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Mass spectrometry of steroid glucuronide conjugates. II — Electron impact fragmentation of 3-keto-4-en- and 3-keto-5α-steroid-17-*O*-β glucuronides and 5α-steroid-3α,17β-diol 3- and 17-glucuronides

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The steroid glucuronide conjugates of 16,16,17-d_3-testosterone, epitestosterone, nandrolone (19-nortestosterone), 16,16,17-d_3-nortestosterone, methyltestosterone, metenolone, mesterolone, 5α-androstane-3α, 17β-diol, 2,2,3,4,4-d_5-5α-androstane-3α,17β-diol, 19-nor-5α-androstane-3α,17β-diol, 2,2,4,4-d_4-19-nor-5α-androstane-3α,17β-diol and 1α-methyl-5α-androstane-3α/β,17β-diol were synthesized by means of the Koenigs–Knorr reaction. Selective 3- or 17-*O*-conjugation of bis-hydroxylated steroids was performed either by glucuronidation of the corresponding steroid ketole and subsequent reduction of the keto group or via a four-step synthesis starting from a mono-hydroxylated steroid including (a) protection of the hydroxy group, (b) reduction of the keto group, (c) conjugation reaction and (d) removal of protecting groups. The mass spectra and fragmentation patterns of all glucuronide conjugates were compared with those of the commercially available testosterone glucuronide and their characterization was performed by gas chromatography/mass spectrometry and nuclear magnetic resonance spectroscopy. For mass spectrometry the substances were derivatized to methyl esters followed by trimethylsilylation of hydroxy groups and to pertrimethylsilylated products using labelled and unlabelled trimethylsilylating agents. The resulting electron ionization mass spectra obtained by GC/MS quadrupole and ion trap instruments, full scan and selected reaction monitoring experiments are discussed, common and individual fragment ions are described and their origins are proposed.

KEYWORDS: anabolic steroids; gas chromatography; mass spectrometry; structure confirmation; mass spectra interpretation; Koenigs–Knorr reaction; selective conjugation

INTRODUCTION

The clinical use of anabolic steroids is indicated in cases of protein and bone wasting, treatment of osteoporosis and during convalescence after chronic debilitating disease.[1] In sports their misuse has been proven many times since the anabolic steroids were banned first by the International Olympic Committee (IOC) at the Olympic Games 1976 in Montreal because of their performance improvement effects. Since 1984, even the use of testosterone has been prohibited[2] and so IOC-accredited laboratories analyse urine samples of high-performance athletes for this class of substances. The determination of anabolic androgenic steroids (AAS) and their metabolites in human urine has become one of the main tasks in doping controls. The common method consists of the isolation of AAS and their metabolites, hydrolysis of conjugates, derivatization of the aglycones with *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) and analysis by means of gas chromatography (GC) and mass spectrometry (MS).[3] The synthesis of phase II metabolites, e.g. glucuronides, of anabolic steroids is of great interest in doping analysis since modern instruments permit the sensitive detection of glucuronic acid conjugates.[4,5] By means of the combination of liquid chromatography (LC) and MS interfaced by electrospray ionization or atmospheric pressure chemical ionization, the metabolites of anabolic steroids can be identified without prior hydrolysis and derivatization. Therefore, reference substances are required to confirm the data obtained by analyses of urine samples. These are not commercially available and their synthesis has to be confirmed by different characterization steps. The structural elucidation includes nuclear magnetic resonance (NMR) spectroscopy and MS, which were presented for 5α-/5β-androstan-3α-ol-17-one glucuronides and 5α-estran-3α-ol-17-one glucuronides in an earlier study.[6] In

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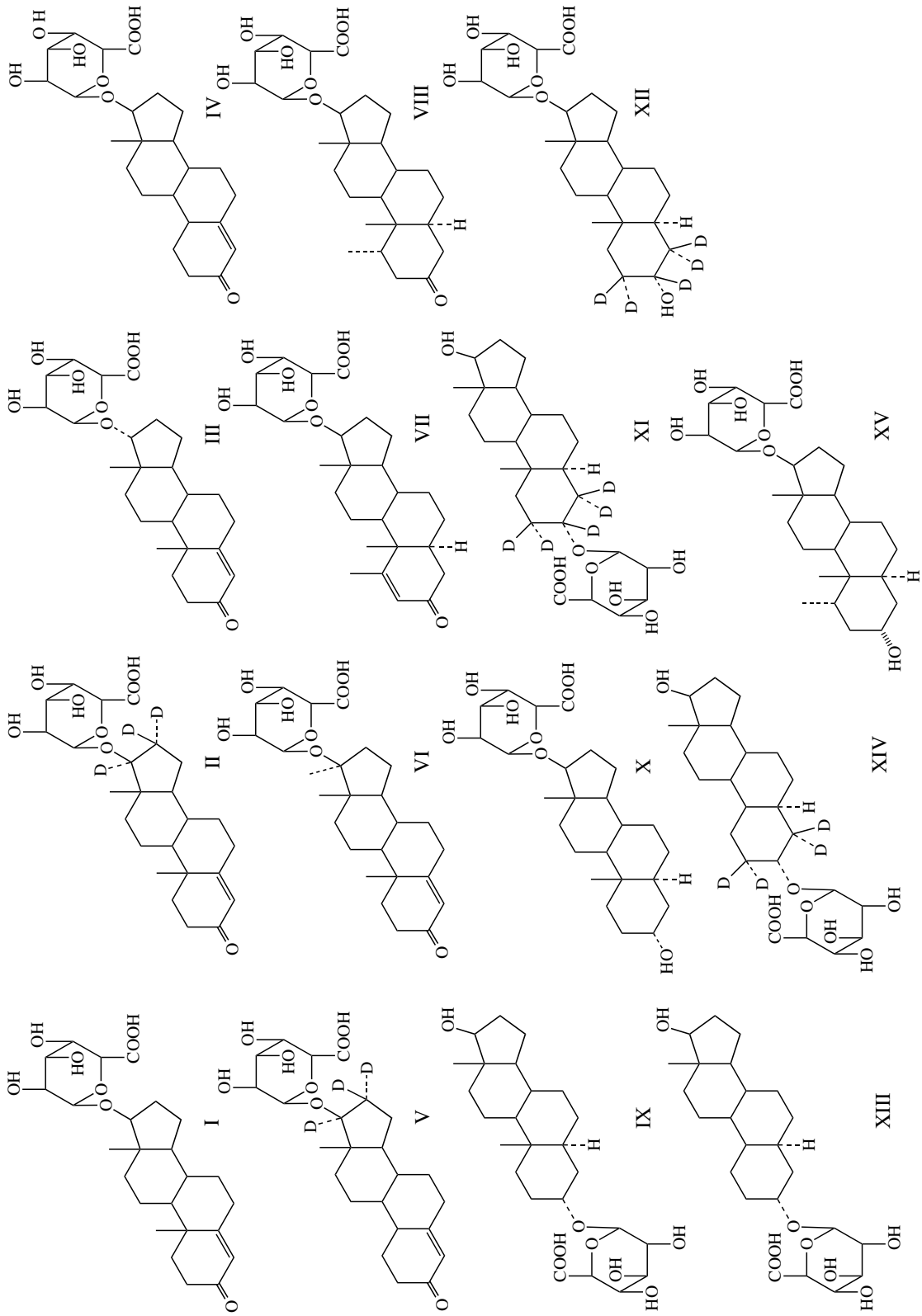

Figure 1. Structure formulae of testosterone glucuronide and synthesized reference compounds: **I**, testosterone glucuronide; **II**, 16,16,17-d_3-testosterone glucuronide; **III**, epitestosterone glucuronide; **IV**, 19-nortestosterone glucuronide; **V**, 16,16,17-d_3-nortestosterone glucuronide; **VI**, methyltestosterone glucuronide; **VII**, metenolone glucuronide; **VIII**, mesterolone glucuronide; **IX**, 5α-androstane-3α,17β-diol-3-*O*-glucuronide; **X**, 5α-androstane-3α,17β-diol-17-*O*-glucuronide; **XI**, 2,2,3,4,4-d_5-5α-androstane-3α,17β-diol-3-*O*-glucuronide; **XII**, 2,2,3,4,4-d_5-5α-androstane-3α,17β-diol-17-*O*-glucuronide; **XIII**, 19-nor-5α-androstane-3α,17β-diol-3-*O*-glucuronide; **XIV**, 2,2,4,4-d_4-19-nor-5α-androstane-3α,17β-diol-3-*O*-glucuronide; **XV**, 1α-methyl-androstane-3α/β,17β-diol-17-*O*-glucuronide.

the present study, synthesized reference material and commercially obtained substances of steroid-17-*O*-glucuronides and androstanediol-3- and 17-*O*-glucuronides (Fig. 1) were characterized by NMR and GC/MS after derivatization to per-TMS derivatives and to the corresponding methyl ester trimethylsilyl ethers. Common and individual fragmentation patterns of the steroidal and glycosidic moiety were obtained and the generation of fragment ions is proposed based on selected reaction monitoring and different deuterium labelling experiments.

EXPERIMENTAL

Chemicals and steroids

N-Methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) was purchased from Chem. Fabrik Karl Bucher (Waldstetten, Germany) and distilled before use, ammonium iodide (purum, p.a.), silver carbonate (purum, p.a.) and toluene (puriss., absolute over molecular sieve) from Fluka (Buchs, Switzerland), sodium hydroxide (p.a.), potassium carbonate (p.a.), glacial acetic acid (p.a.) and iodomethane (for synthesis) from Merck (Darmstadt Germany) and ethanethiol (97%) from Aldrich (Deisendorf, Germany) and methyl 1-bromo-1-desoxy-2,3,4-triacetylglucopyranuronate was synthesized by the procedure of Bollenback *et al*.[7] The reference substances testosterone glucuronide and the unconjugated steroids of androsterone, testosterone, epitestosterone, metenolone, mesterolone, nortestosterone and methyltestosterone were purchased from Sigma (St. Louis, MO, USA). The metabolite of nortestosterone, 19-norandrosterone, and the deuterated counterparts of nortestosterone, testosterone, 19-norandrosterone and androsterone were synthesized in our laboratory.[8,9]

Synthesis of steroid glucuronide conjugates

The monohydroxylated steroids testosterone, d_3-testosterone, nortestosterone, d_3-nortestosterone, mesterolone, metenolone and epitestosterone were conjugated to steroid glucuronides according to the method described earlier.[6] The selectively conjugated bis-hydroxylated steroids were prepared as described below.

Preparation of 3-O-glucuronides of androstane-3α, 17β-diol, 19-norandrostane-3α,17β-diol, deuterated analogues and 17-O-glucuronide of 1α-methyl-5α-androstane-3α/β,17β-diol

Amounts of 20–100 mg of androsterone glucuronide, 19-norandrosterone glucuronide or their deuterated counterparts, respectively, were dissolved in 20 ml of a mixture of ethanol and doubly distilled water (80 : 20, v/v). While stirring, a 1.5 molar excess of sodium borohydride was added and the solution was stirred at ambient temperature for 1 h. The reaction mixture was evaporated to dryness at 50 °C under reduced pressure by means of a rotary evaporator and the residue was dissolved in 20 ml of doubly distilled water. The pH was adjusted to 10–11 (if necessary) by addition of 1 M aqueous NaOH and the solution was extracted twice with 20 ml of *tert* butyl methyl ether to remove unconjugated steroids possibly generated by hydrolysis. Then, the pH of the aqueous layer was adjusted to 2.5 by addition of 3 M HCl and extracted twice with 20 ml of ethyl acetate. The combined organic layers were evaporated to dryness yielding the pure 3-*O*- or 17-*O*-glucuronides in amounts of 71–79% of the theory. In the case of the reduction of 1α-methyl-5α-androstane-17β-ol-3-one-17-O-glucuronide, the 3-keto group yielded the 3α- and 3β-configurations.

Preparation of 17-O-glucuronides of androstane-3α, 17β-diol and d_5-androstane-3α,17β-diol

Protection of the 3-hydroxy group. A 150 mg amount of androsterone or d_5-androsterone, respectively, was dissolved in 25 ml of a mixture of acetic anhydride, acetonitrile and pyridine (10 : 10 : 5, v/v/v) and heated in a water-bath at 60 °C while stirring. After 5 h, complete acetylation of the hydroxy group at C-3 was achieved, and 20 ml of doubly distilled water were added. The pH was adjusted to 7 by carefully adding solid sodium carbonate and the solution was extracted twice with 40 ml of *tert*-butyl methyl ether. The organic layers were combined, evaporated to dryness at 50 °C under reduced pressure and the residue was stored in a desiccator over phosphorus pentoxide and potassium hydroxide under reduced pressure for 10 h.

Reduction of the 17-keto group. According to Rao *et al*.,[10] the reduction of the 17-keto group was performed by solving the crude product in 50 ml of a mixture of ethanol and doubly disstilled water (80 : 20, v/v) and the addition of a 1.5 molar excess of sodium borohydride, obtaining the 17β-OH group.

Conjugation reaction. The glucuronidation and subsequent hydrolysis of the protecting groups of the glycosidic moiety were performed by means of the modified Koenigs–Knorr reaction[11–14] as described earlier.[6]

Hydrolysis of the protecting group at C-3. The obtained 3-acetylated glucuronide conjugate was dissolved in 10 ml of methanol, 1 ml of 2 M aqueous NaOH was added and the solution was stirred for 3 h in a water-bath at 60 °C, yielding complete hydrolysis of the acetyl group at C-3. After cooling to ambient temperature, the solution was evaporated to dryness and the residue was dissolved in 20 ml of doubly distilled water and extracted with *tert*-butyl methyl ether and ethyl acetate as described above. The main product obtained was the 17-*O*-glucuronide of androstane-3α,17β-diol or d_5-androstane-3α,17β-diol, respectively.

Yields

The crude substances were finally purified by HPLC fractionation with overall yields of 22.4 and 18.3% of theory, respectively.

Nuclear magnetic resonance (NMR) analysis

NMR analyses were performed on a Bruker DPX 300 or Bruker DRX 500 instrument. Amounts of 5 mg of the glucuronides of testosterone, d_3-testosterone, epitestosterone, nortestosterone, d_3-nortestosterone, methyltestosterone, metenolone, mesterolone, androstane-3α,17β-diol, d_5-androstane-3α,17β-diol, 19-norandrostane-3α,17β-diol and d_4-19-norandrostane-3α,17β-diol were dissolved in

CD_3OD and ^1H, ^{13}C (DEPT, Distortionless Enhancement by Polarization Transfer), H,H-COSY (Homonuclear Correlated Spectroscopy), HMQC (Heteronuclear Multiple Quantum Correlation) and NOESY (Nuclear Overhauser Effect Spectroscopy) were performed to confirm the structures of the synthesized substances.

Derivatization for GC/MS analysis

The steroid glucuronides were derivatized to per-TMS products (including a TMS-ester group at the glycosidic moiety) by procedure B below, and to methyl ester TMS ether derivatives by procedures A and B, enabling a comparison of the mass spectra and providing information about fragment ion generation. Further, a perdeutero-trimethylsilylation was performed by means of d_{18}-bistrimethylsilylacetamide (d_{18}-BSA) (procedure C).

A. Methylation of the carboxylic group

In a test-tube, 5 μg of the steroid glucuronide are dissolved in 200 μl of acetonitrile, and 20 μl of iodomethane and 10 mg of potassium carbonate are added. The tube is carefully closed and heated for 60 min at 60 °C. After cooling to ambient temperature, the organic layer is transferred to a clean tube, evaporated to dryness by means of a rotary evaporator and the residue is trimethylsilylated as described below.

B. Trimethylsilylation

In a test-tube, 5 μg of the steroid glucuronide are dissolved in 100 μl of a mixture of MSTFA, ammonium iodide and ethanethiol (100 : 0.2 : 0.6, v/w/v) and heated for 20 min at 60 °C.

C. Perdeutero-trimethylsilylation

A 5 μg amount of the steroid glucuronide is dissolved in 100 μl of a mixture of d_{18}-BSA and acetonitrile (1 : 4, v/v) and the sample is heated for 20 min at 60 °C. With this procedure enolization of the 3-keto group is only observed at 3-keto-4-ene structures, and not with mesterolone glucuronide and metenolone glucuronide, in which no double bonds at C-3—C-4 are present. Here, the enolization is performed by evaporation of the d_{18}-BSA solution and additional derivatization as described in step B. A partial exchange of a d_9-TMS group by an unlabelled TMS group was observed with the TMS-ester derivatives whereas the methyl ester derivatives were stable under the conditions of step B.

GC/MS parameters

All spectra presented were generated with a Finnigan GCQ ion trap using the following parameters: injector, ATAS Optic 2, 300 °C; column, HP Ultra1 (Hewlett-Packard), 14 m × 0.2 mm i.d., film thickness 0.11 μm; carrier gas, helium at 1.5 ml min^{-1}; injection volume, 2 μl (100 ng of analyte); split ratio, 1 : 10; oven temperature, initial 180 °C, raised at 20 °C min^{-1} to 320 °C, hold for 5 min; interface temperature, 320 °C; ion source temperature, 225 °C; mass range (full scan), 70–920; ionization, EI (70 eV).

RESULTS AND DISCUSSION

NMR characterization

The compounds synthesized as described above were characterized by different NMR analyses. The β-configuration of the glucuronic acid was proven for all substances by a coupling constant of $^3J = 7.8$ Hz, resulting from the axial–axial positioned hydrogens at C-1′ and C-2′, permitting only an equatorial glycoside bond. The chemical shifts of all carbons including those indicating the position of glucuronidation (3- or 17-*O*-glucuronide) are listed in Table 1.

17-*O*-Glucuronide conjugates of monohydroxylated steroids

The syntheses of the 17-*O*-glucuronides of monohydroxylated steroids were performed according to the methods described earlier yielding the pure substances in amounts of 20.5–41.0% of the theory. After purification by HPLC fractionation, the products were analysed by GC/MS as methyl ester TMS ethers, per-TMS derivatives, methyl ester nonadeutero-TMS ethers and nonadeutero-per-TMS derivatives. The spectra of the glucuronides of testosterone, d_3-testosterone, epitestosterone, nortestosterone, d_3-nortestosterone, methyltestosterone, mesterolone and metenolone as methyl ester TMS ethers and per-TMS derivatives were investigated and those of the conjugates of testosterone and epitestosterone are presented exemplarily in Figs 2 and 3. The main fragmentation scheme will be described with testosterone glucuronide per-TMS and then applied to the other substances.

The fragment ions at m/z 375, 305, 292, 217, 204 and 169 are present in all spectra and originate from the glycosidic moiety, the generation and proposed structures of which were described earlier.[6] In the present study, additional glucuronic acid specific fragments were observed with m/z 233 and 257 as described below.

Proposed fragmentation pattern of testosterone glucuronide, per-TMS (M_r 824)

Like testosterone glucuronide (Fig. 2), all compounds presented here (except mesterolone glucuronide) produce abundant molecular ions due to the α,β-unsaturated 3-keto-steroid structure which forms a stable conjugated electron system after enolization of the ketone to the TMS ether, able to delocalize an induced charge (see Table 3). The consequence is a decreased abundance of secondary ions, e.g. those originating from the glycosidic moiety or from the aglycone.

m/z 502

The fragment at m/z 502 is proposed to be generated from the molecular ion by a cleavage across the glycosidic moiety including a transfer of a TMS group from C-3′ to the ring oxygen [Fig. 4(A)]. Starting with a fission of the O—C-1′ bond of the glucuronic acid, an oxygen radical is formed to which the trimethylsilyl group of C-3′ migrates by a six-center rearrangement. The following neutral loss of 322 u can be described by the cleavage of the C-3′—C-4′ bond and removal of the hydrogen at C-2′. This is comparable to the fragmentation observed with androsterone glucuronide

Table 1. ^{13}C chemical shifts of steroid glucuronide conjugates and ^{1}H chemical shifts of the glycosidic moieties (ppm)[a]

Atom	TG	NTG	MTG	MG	MeG	Adiol-3-G	Adiol-17-G	NorAdiol-3-G	1α-Me-Adiol-17-G
Steroid carbons—									
C-1	36.77	27.64	36.82	176.95	40.31	33.74	33.51	25.16	36.81
C-2	34.71	37.24	34.71	128.95	46.49	26.49[b]	29.58[b]	30.56[b]	36.26
C-3	202.40	202.92	202.39	202.32	215.70	75.73[b]	67.20[b]	75.41	68.15
C-4	124.11	124.73	124.13	42.16	45.42	35.35[b]	36.73[b]	40.37[b]	37.16
C-5	175.32	170.85	175.30	46.20	40.79	40.51	40.32	37.47	33.48
C-6	33.92	36.52	33.97	29.74	29.32	29.53	29.69	34.77	30.01
C-7	32.84	31.94	33.09	31.26	31.77	32.85	32.84	31.80	32.64
C-8	36.77	41.45	37.49	39.40	36.06	36.94	36.73	42.80	36.73
C-9	55.40	51.06	55.41	51.38	49.38	55.81	55.99	49.67	50.15
C-10	40.03	43.79	40.06	44.20	38.61	36.96	37.22	48.43	39.30
C-11	21.78	27.22	21.79	26.55	20.85	21.51	21.50	26.49	20.93
C-12	38.52	38.35	33.09	38.91	37.95	38.15	38.77	38.09	38.66
C-13	44.16	44.37	47.52	44.23	43.97	44.11	44.35	44.21	44.40
C-14	51.72	50.81	50.99	52.70	51.40	52.55	52.27	51.63	52.43
C-15	24.15	24.07	24.44	24.43	23.87	24.30	24.27	24.14	24.30
C-16	29.52[b]	29.77[b]	36.16	29.71	29.35	30.64	29.80	30.64	29.79
C-17	89.11[b]	90.24[b]	89.25	89.97	89.98	82.59	90.48	82.67	90.48
C-18	11.99	11.99	14.88	12.64	12.03	11.69	12.10	11.66	12.17
C-19	17.73		17.70	14.19	14.97	11.91	11.71		14.53
C-20			23.10	25.50	14.64				17.12
Glucuronic acid carbons—									
C-1′	104.46	105.10	100.45	104.90	104.13	103.05	105.10	103.07	105.10
C-2′	75.31	75.06	75.08	75.05	74.17	74.83	75.07	74.86	75.07
C-3′	77.96	77.60	77.69	77.58	76.47	77.67	77.58	77.67	77.58
C-4′	73.80	73.22	73.21	73.20	72.36	73.21	73.24	73.22	73.24
C-5′	76.36	76.63	76.37	76.67	75.72	76.59	76.62	76.58	76.62
C-6′	172.95	172.61	172.74	172.70	172.22	172.66	172.65	172.77	172.88
Glucuronic acid hydrogens—									
H-1′	4.34	4.37	4.48	4.38	4.34	4.36	4.36	4.36	4.36
H-2′	3.19	3.21	3.18	3.20	3.12	3.23	3.19	3.22	3.19
H-3′	3.35	3.34	3.37	3.35	3.32	3.37	3.34	3.37	3.34
H-4′	3.42	3.50	3.52	3.51	3.43	3.52	3.49	3.52	3.49
H-5′	3.49	3.72	3.72	3.73	3.71	3.75	3.71	3.75	3.71

[a] TG = testosterone glucuronide; NTG = 19-nortestosterone glucuronide;
MTG = methyltestosterone glucuronide; MG = metenolone glucuronide; MeG = mesterolone glucuronide;
Adiol-3-G = androstanediol-3-glucuronide; Adiol-17-G = androstanediol-17-glucuronide;
NorAdiol-3-G = norandrostanediol-3-glucuronide;
1α-Me-Adiol-17-G = 1α-methylandrostanediol-17-glucuronide.
[b] Labelled positions in deuterated counterparts.

per-TMS and analogues. The spectrum of the methyl ester tetrakis-TMS derivative [Fig. 2(B)] contains also the ion at m/z 502, proving the loss of the ester group in this fragmentation step. Further, the perdeutero-TMS derivative of testosterone glucuronide shows a corresponding ion with m/z 520 (Table 2), which is evidence for the presence of only two remaining TMS groups in the fragment ion, and the trideuterotestosterone glucuronide per-TMS (Table 2) generates the counterpart to m/z 502 with the ion at m/z 505 indicating that the steroid moiety is present in the ion. The product ion scan of m/z 502 in selected reaction monitoring (SRM) experiments demonstrated the stability of the fragment ion which dissociated mainly to m/z 487 by the loss of 15 u and m/z 474 by the loss of 28 u. These are present in the full-scan spectrum only with abundances of less than 2% of the base peak (Fig. 2).

m/z 388

The generation of m/z 388 is proposed to start from the molecular ion producing the formic acid ester of testosterone mono-TMS by a rearrangement comparable to the retro-Diels–Alder mechanism of the glycosidic ring structure as shown in Fig. 4(B). The fact that the fragment contains one TMS group (at C-3) was proved by the nonadeutero-TMS derivative which generates an ion at m/z 397 corresponding to the unlabelled m/z 388 (Table 2). Further, the spectra of trideuterotestosterone glucuronide per-TMS derivative and

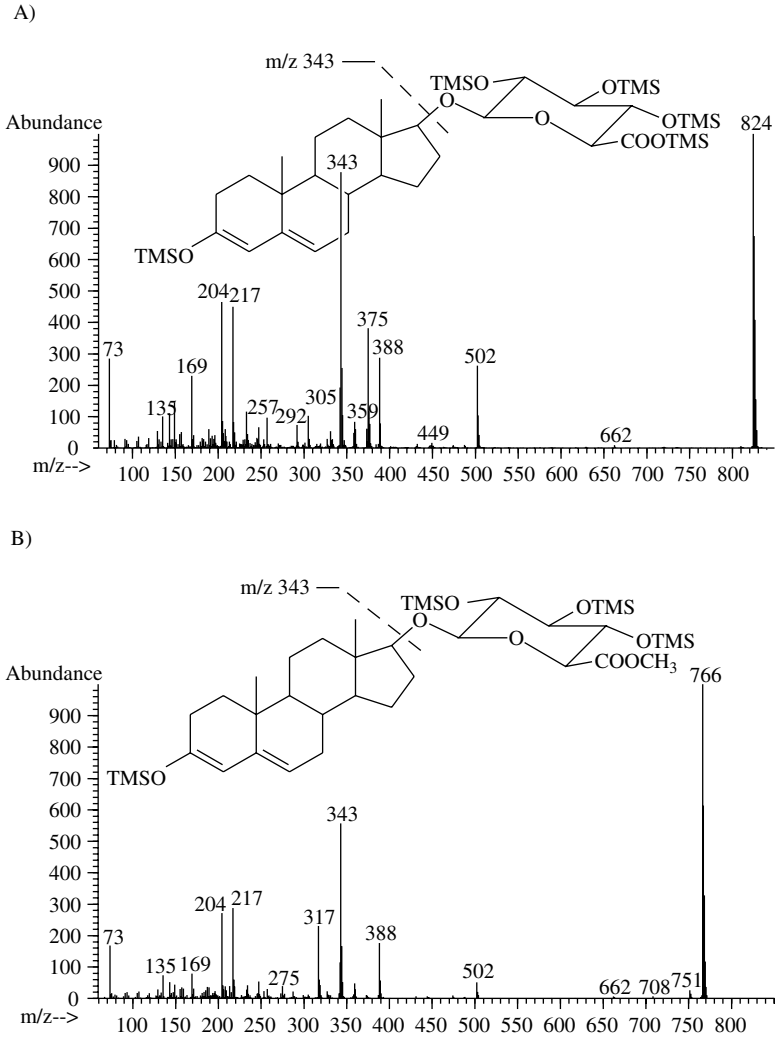

Figure 2. EI mass spectra of testosterone glucuronide: (A) per-TMS derivative (M_r 824); (B) methyl ester tetrakis-TMS derivative (M_r 766).

methyl ester TMS ether (Table 2) show the counterpart to m/z 388 with m/z of 391, and 19-nortestosterone glucuronide (Table 2) contains the ion of m/z 374 (388−14), demonstrating the presence of the steroid nucleus in the fragment of m/z 388. Also here, the SRM experiments demonstrate the stability of the fragment ion which dissociates by the loss of 15 or 28 u mainly to m/z 373 or 360, respectively.

m/z 359, 343 and 342

The fragment ion at m/z 359 is postulated to have two origins since the spectrum of the triply labelled testosterone glucuronide per-TMS contains m/z 359 and 362. The fragment which is not influenced by the deuterium labelling of the steroid moiety is proposed to result from m/z 464 (generated by neutral loss of the steroid moiety) after the losses of a methyl (producing m/z 449, weakly present in some spectra of 17-*O*-glucuronides, per-TMS) and a TMSOH group (−90 u). Evidence for this fragmentation pattern is given by the nonadeutero-TMS derivative of testosterone glucuronide where m/z 359 partly shifts to m/z 383, which comprises two d_9-TMS groups and one hexadeuterodimethylsilyl (d_6-DMS) group which increment the fragment of m/z 359 by 24 u to m/z 383 (Table 2). The ion at m/z 359 originating from the aglycone is proposed to be generated by the fission of the *O*-glycosidic bond between C-1′ and the oxygen while m/z 343 represents the steroid moiety after cleavage of the bond between C-17 and the oxygen. In both cases the loss of a radicalic glycoside is postulated, directly created by the electron impact ionization, producing a positively charged steroid moiety with or without the oxygen at C-17 [Fig. 4(C)]. The spectra of triply labelled testosterone glucuronide and labelled and unlabelled 19-nortestosterone glucuronide support the proposal by the presence of expected shifts by 3 u (in the case of d_3-testosterone glucuronide), −14 u (19-nortestosterone glucuronide) and −11 u (d_3-19-nortestosterone glucuronide) as listed in Table 2. For the origin of m/z 342 a neutral loss of the complete glycoside (482 u) is reasonable with a transfer of a hydrogen partly migrating from C-16 or C-17. This is proved by the shift of this fragment to its counterparts in d_3-testosterone glucuronide or d_3-nortestosterone glucuronide derivatives where the remaining aglycones contain only two deuteria but also all three with relative ratios of 30% (two deuteria remaining) and 70% (three deuteria remaining). For example, m/z 328 of nortestosterone glucuronide shifts to m/z 330 and 331, respectively (Table 2). This indicates that the

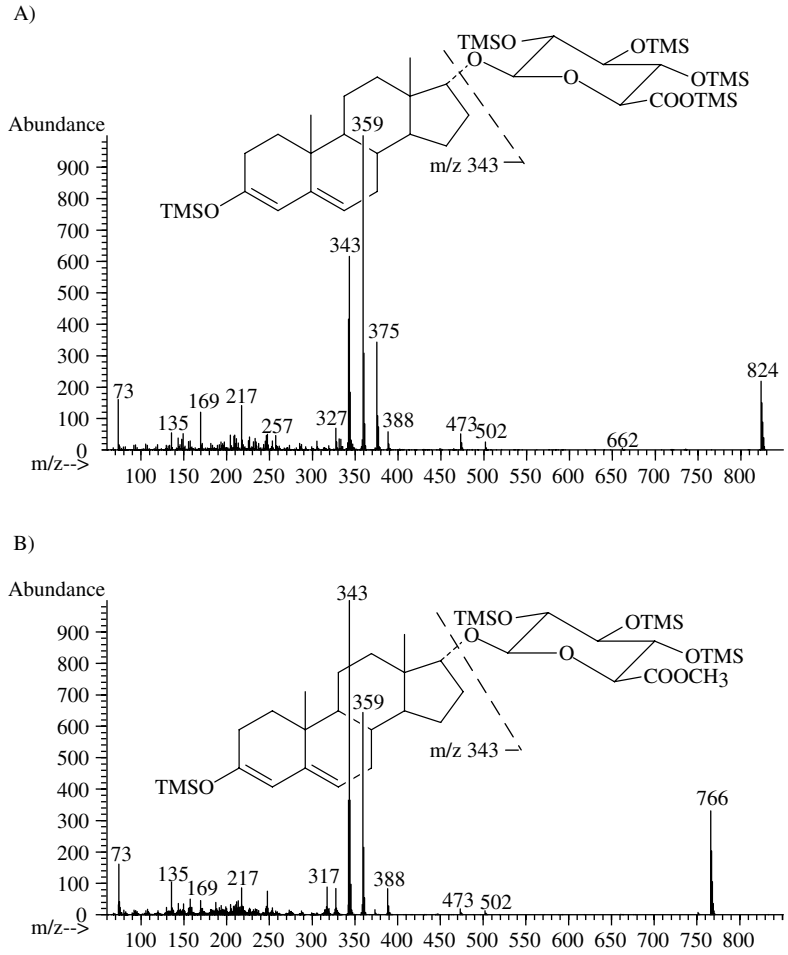

Figure 3. EI mass spectra of epitestosterone glucuronide: (A) per-TMS derivative (M_r 824); (B) methyl ester tetrakis-TMS derivative (M_r 766).

transferred hydrogen with the elimination of the glycosidic moiety can originate from more than one position in the steroid, which was also observed by Budzikiewicz *et al*.[15] in other deuteration experiments with steroid molecules including elimination reactions.

Epitestosterone glucuronide, per-TMS (M_r 824)

In the spectrum of epitestosterone glucuronide per-TMS [Fig. 3(A)], the same fragment ions are present as in the spectrum of testosterone glucuronide per-TMS [Fig. 2(A)] but with significant differences in abundance. Intense signals of the per-TMS derivative are the aglycone fragment without the glycoside bond oxygen (m/z 343) and the ion at m/z 359 which mainly originates from the aglycone retaining the oxygen of the glycosidic bond, as proved by deuteration of the TMS groups. The fragment shifts nearly completely to m/z 368 owing to one TMS group at the steroid moiety and not to m/z 383 which would result from the glucuronic acid moiety.

The spectrum of the methyl ester tetrakis-TMS derivative is comparable with respect to the difference of 58 u (due to the exchange of the TMS group by a methyl group) in the case of the molecular ion (m/z 824 shifts to m/z 766) and the main fragment ion of the glycosidic moiety at m/z 375 which shifts to m/z 317. Further, a switch of abundances is observed with m/z 359 and 343 compared with the per-TMS derivative of epitestosterone glucuronide. The differences in fragment ion abundances compared with testosterone glucuronide was also observed in other studies with testosterone and epitestosterone glucuronides, which were analysed by LC/MS and LC/MS/MS without derivatization.[16,17] The protonated molecule of the conjugate of testosterone is significantly more stable than that of epitestosterone owing to a more stable glycoside bond based on the higher molecular strain of epitestosterone glucuronide.

Nortestosterone glucuronide, per-TMS (M_r 810)

In accordance with Biemann's shift rule, the 19-nortestosterone glucuronide gives rise to mass spectra containing fragment ions corresponding to those of testosterone glucuronide, in compliance with the mass difference of 14 u due to the missing 19-methyl group (Table 3).

Methyltestosterone glucuronide, per-TMS (M_r 838)

The MS behaviour of methyltestosterone glucuronide per-TMS is significantly different from that of testosterone glucuronide per-TMS. Some fragmentation steps are completely missing, e.g. those generating the counterpart to m/z 502 (which should be m/z 516), and present corresponding ions that have clearly different relative abundances (Table 3).

Table 2. Selected fragment ions of testosterone and nortestosterone glucuronide derivatives and their counterparts in deuterium labelling[a–c] experiments (*m/z*)

Fragment ions of TG-per-TMS	A	B	Fragment ions of TG-methyl ester-tetrakis-TMS	C	D	Fragment ions of NTG-per-TMS	E	F	Fragment ions NTG-methyl ester-tetrakis-TMS	G	H
824	827	869	—	—		810	813	855	—	—	—
—	—	—	766	769	802	—	—	—	752	755	788
—	—	—	751	754	—	—	—	—	737	740	—
502	505	520	502	505	520	488	491	506	488	491	506
388	391	397	388	391	397	375	375	402	—	—	—
375	375	402	—	—	—	374	377	383	374	377	383
359	359/362	359/383	359	362	368	345	348	354	345	348	354
343	346	352	343	346	352	329	332	338	329	332	338
342	344/345	351	342	344/345	351	328	330/331	337	328	330/331	337
—	—	—	317	317	335	—	—	—	317	317	335
305	305	332	—	—	—	305	305	332	—	—	—
292	292	319	—	—	—	292	292	319	—	—	—
257	257	275	—	—	—	257	257	275	—	—	—
233	233	251	—	—	—	233	233	251	—	—	—
217	217	235	217	217	235	217	217	235	217	217	235
204	204	222	204	204	222	204	204	222	204	204	222
169	169	178	169	169	178	169	169	178	169	169	178

[a] TG = testosterone glucuronide; NTG = nortestosterone glucuronide.

[b] A = 16,16,17-d_3-testosterone glucuronide per-TMS; B = testosterone glucuronide per-d_9-TMS; C = 16,16,17-d_3-testosterone glucuronide methyl ester tetrakis-TMS; D = testosterone glucuronide methyl ester tetrakis-d_9-TMS; E = 16,16,17-d_3-nortestosterone glucuronide per-TMS; F = nortestosterone glucuronide per-d_9-TMS; G = 16,16,17-d_3-nortestosterone glucuronide methyl ester tetrakis-TMS; H = nortestosterone glucuronide methyl ester tetrakis-d_9-TMS.

[c] Dashes indicate fragment abundance less than 5% of base peak or not detected.

m/z 838

Comparable to epitestosterone glucuronide per-TMS, the molecular ion at *m/z* 838 is not the base peak but *m/z* 357 originating from the aglycone. This may result from a decreased stability of the glycoside bond due to the methyl group additionally located at C-17 in comparison with testosterone glucuronide.

m/z 446

The fragment ion at *m/z* 446 has the same mass/charge value as the unconjugated methyltestosterone per-TMS derivative. Owing to the different retention times of unconjugated methyltestosterone and its glucuronide, it cannot be a contaminant in this spectrum but has to be a fragment ion generated by removal of the glycosidic moiety, accompanied by the transfer of a TMS group from the glucuronic acid to the steroid. This is proved by the nonadeutero-TMS derivative of methyltestosterone glucuronide, where *m/z* 446 shifts to *m/z* 464 owing to two d_9-TMS groups. In the case of the methyl ester TMS ether, the corresponding fragment is not observed.

m/z 402

Analogous to *m/z* 388 of testosterone glucuronide, methyltestosterone glucuronide generates a fragment at *m/z* 402 proposed to be the formic acid ester after a rearrangement of the glycosidic moiety. The abundance of *m/z* 402 is much lower than that of its counterpart in the spectrum of testosterone glucuronide.

m/z 373 and 357

The base peak of the spectrum of methyltestosterone glucuronide per-TMS is at *m/z* 357, originating from the aglycone and containing one TMS group which could be proved with the nonadeutero-TMS derivative producing an ion at *m/z* 366. The glucuronic acid including the oxygen of the glycoside bond is removed as a radical comparable to the generation of *m/z* 343 of testosterone glucuronide [Fig. 4(C)]. In contrast to testosterone glucuronide or epitestosterone glucuronide, only a very weak signal of the aglycone including the oxygen (*m/z* 373) is observed, mainly in the spectrum of the methyl ester-TMS derivative. This may be based on the tertiary carbon C-17 which produces a more stable ion than the terminal oxygen.

The fragments originating from the glucuronic acid (*m/z* 375, 217, 204 and 169) are present with decreased abundances except for *m/z* 375, the abundance of which is higher than 50% of the base peak.

Metenolone glucuronide, per-TMS (M_r 838)

The mass spectrum of metenolone glucuronide per-TMS contains fragment ions the counterparts of which are present in methyltestosterone glucuronide derivatives and testosterone glucuronide derivatives additionally to individually

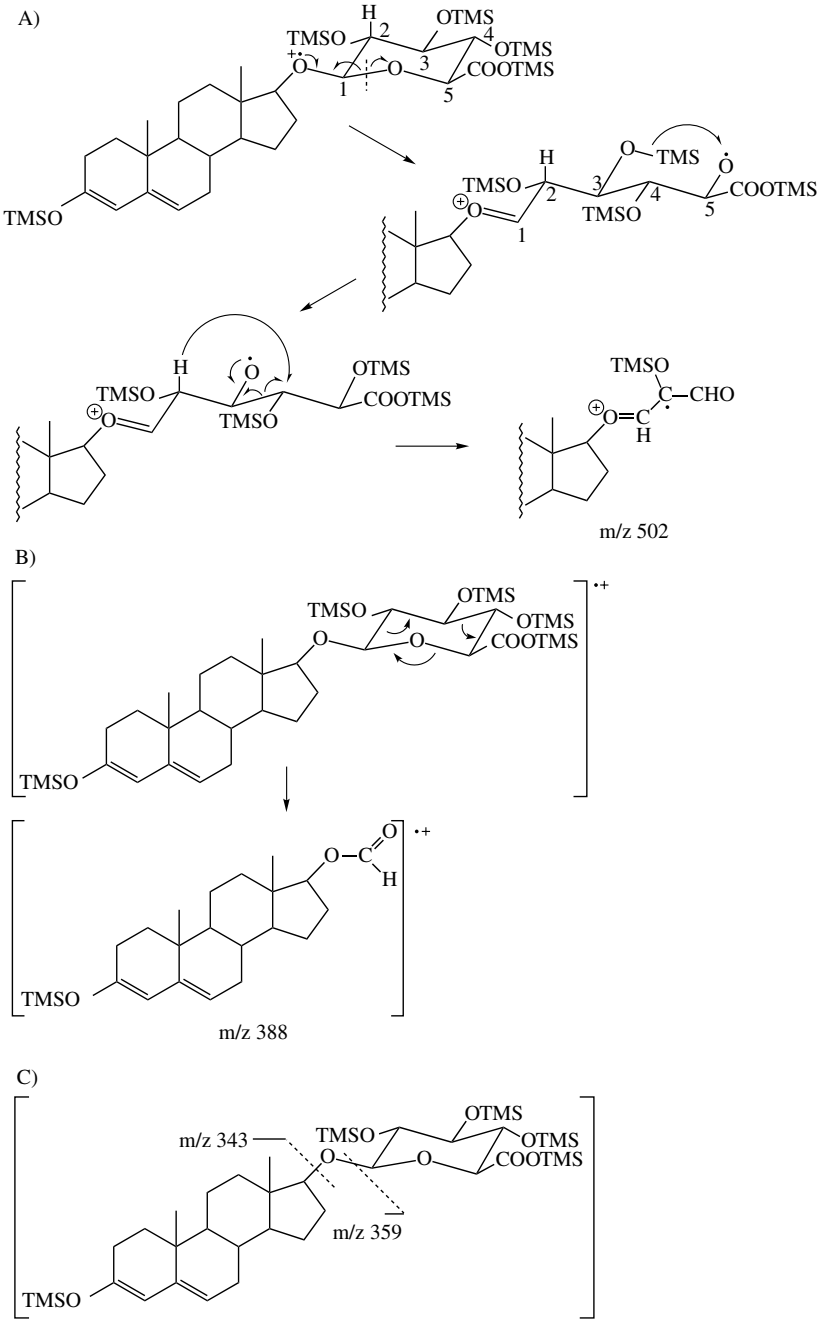

Figure 4. (A) Proposed generation of the fragment ion at *m/z* 502 of testosterone glucuronide per-TMS. After cleavage of the ring structure the TMS group of C-3′ migrates to the oxygen radical originating from the fission of its bond to C-1′. The subsequent fission of the C-3′–C-4′ bond is accompanied by a hydrogen shift resulting in a loss of 322 u, generating *m/z* 502. (B) Postulated fragmentation of testosterone glucuronide per-TMS to the formic acid ester to *m/z* 388 by a mechanism comparable to the retro-Diels–Alder rearrangement. (C) Proposed fissions of testosterone glucuronide per-TMS for the generation of *m/z* 343 and 359.

occurring fragments (Table 3). The enolization of metenolone glucuronide was not achieved with d_{18}-BSA–acetonitrile but with trimethyliodosilane (TMIS) (see step C of derivatization), and therefore one TMS group (located at the oxygen of C-3) of the analyte was not labelled.

m/z 838 and 823

The molecular ion at m/z 838 is present in the spectrum of the per-TMS derivative with an abundance of 25% of the base peak. The loss of a methyl group (−15 u) leads to m/z 823 with an abundance of 10% of the base peak whose counterparts in the spectra of, e.g., testosterone glucuronide were observed only in very weak abundances. The origin of the leaving radical is not clear but deuterium-labelled TMS groups did not increment the loss from 15 to 18 u, indicating that the methyl group is expelled from the steroid.

m/z 516

The ion at m/z 516 corresponding to m/z 502 of testosterone glucuronide is present in nearly the same abundance as

Table 3. Selected fragment ions of steroid-17-*O*-glucuronides per TMS (*m/z*, with abundances (%) relative to the base peak in parentheses)[a,b]

Ion	TG	EpiTG	NTG	MTG	MG	MeG
M^+	824 (100)	824 (22)	810 (100)	838 (16)	838 (19)	—
$M^+ - 15$	—	—	—	—	823 (5)	825 (13)
$M^+ - 15 - 90$	—	—	—	—	—	735 (3)
$M^+ - 249$	—	—	—	—	—	591 (6)
$M^+ - 322$	502 (26)	502 (3)	488 (21)	—	516 (21)	518 (39)
$M^+ - 322 - 15$	—	—	—	—	—	503 (12)
	—	—	—	—	—	428 (20)
	—	—	—	446 (13)	—	—
$M^+ - 436$	388 (29)	388 (6)	374(20)	402 (4)	402 (4)	—
$M^+ - 465$	359 (9)	359 (100)	345 (8)	373 (6)	373 (9)	375 (66)
$M^+ - 481$	343 (88)	343 (62)	329 (71)	357 (100)	357 (66)	359 (100)
	—	—	—	—	—	269 (15)
	—	—	—	—	208 (63)	—
	—	—	—	—	195 (46)	—

[a] TG = testosterone glucuronide; EpiTG = epitestosterone glucuronide; NTG = 19-nortestosterone glucuronide; MTG = methyltestosterone glucuronide; MG = metenolone glucuronide; MeG = mesterolone glucuronide.

[b] Dashes indicate fragment abundance less than 3% of base peak or not detected.

m/z 502 in Fig. 2(A). It is incremented by 9 u in the case of trimethylsilylation with d_{18}-BSA–acetonitrile followed by enolization with TMIS, indicating the same composition of the fragment as proposed for testosterone glucuronide. With steroid glucuronides containing the 3-keto-4-ene structure, the enolization was achieved using only d_{18}-BSA–acetonitrile where the corresponding fragment ions are shifted by 18 u.

m/z 402, 373 and 357

According to Biemann's shift rule, the fragments at *m/z* 402, 373 and 357 are the counterparts to *m/z* 388, 359 and 343 of testosterone glucuronide (Table 3). Owing to the enolization performed with unlabelled TMIS, these ions are not influenced by nonadeutero-TMS derivatization. This proves the location of the TMS group at C-3 in the fragments, which is selectively not labelled.

m/z 208 and 195

The ions at *m/z* 208 and 195 occur individually with metenolone glucuronide and originate from the A-ring of the steroid after derivatization to the TMS-enol ether. According to studies by Powell *et al*.[18] and Shapiro and Djerassi,[19] the postulated fission of the bond between C-9 and C-10 is followed by the migration of a hydrogen from C-8 and subsequent McLafferty rearrangement forming the fragment at *m/z* 208 which can be stabilized by an isomerization to the aromatic ring structure as shown in Fig. 5 (path 1). A second route (path 2) to the fragment at *m/z* 208 is described by the initial cleavage of the C-6—C-7 bond also followed by the transfer of the C-8 hydrogen and a McLafferty rearrangement directly leading to the aromatic ring, which may be the reason for the high abundance of the fragment.[20] The generation of the ion at *m/z* 195 is probably based on a comparable beginning of route 1 followed by the cleavage of the bond between C-5 and C-6 forming a crossed-conjugated π-electron system which may be responsible for the high abundance of the fragment ion (Fig. 5).

Mesterolone glucuronide, per-TMS (M_r 840)

The per-TMS derivative of mesterolone glucuronide is the only conjugate in this study which does not have a C—C double bond in the A-ring of the steroid except that produced by enolization. The consequence is a missing molecular ion in the spectrum of the per-TMS derivative and an increased degree of dissociation. This is comparable to the spectra of steroid diol monoglucuronides, which will be discussed later.

m/z 825 and 735

The fragments at *m/z* 825 and 735 are generated by the common losses of a methyl group from the molecular ion and a subsequent neutral loss of TMSOH (−90 u).

m/z 591

The origin of the ion at *m/z* 591 is proposed to be a loss of 249 u from the molecular ion by the mechanism postulated in Fig. 6. The initial ionization of the glycoside bond oxygen is identical with that proposed for the generation of *m/z* 502 [Fig. 4(A)] and results in a radicalic oxygen of the glucuronic acid ring structure. A hydrogen transfer from C-2′ to the oxygen radical is followed by the fission of the bond between C-3′ and C-4′ producing the fragment at *m/z* 591 (840–249). This ion formation is also known from androsterone glucuronide conjugates per-TMS derivatives and analogues.[6]

m/z 518 → 503, m/z 428

Corresponding to *m/z* 502 of testosterone glucuronide, a fragment at *m/z* 518 is generated in the case of mesterolone glucuronide. It shifts to *m/z* 527 with nonadeutero-TMS

JMS

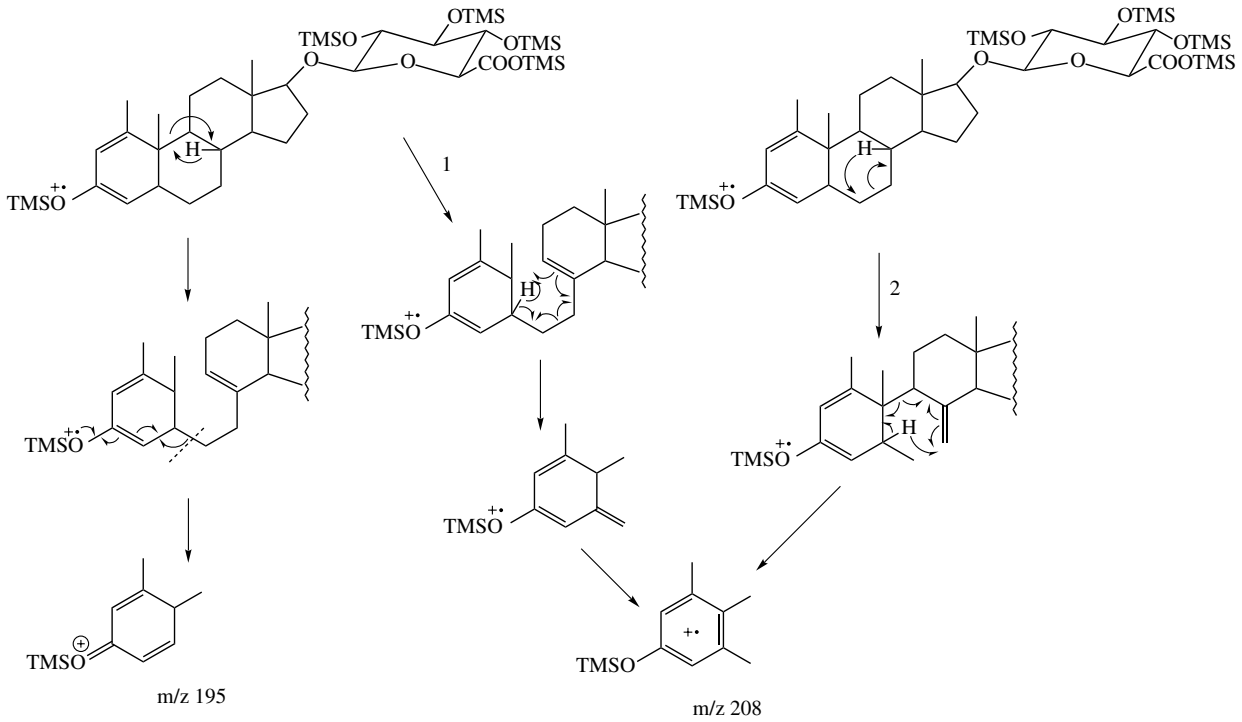

Figure 5. Proposed fragmentation scheme for the generation of *m/z* 208 and 195 of metenolone glucuronide. The ion at *m/z* 208 may result from two paths which both shift the same hydrogens and use a McLafferty rearrangement, yielding a fragment stabilized by an aromatic ring structure. The migration of the hydrogen positioned at C-8 to the resulting fragment was proved by deuteration experiments and the importance of the hydrogen of C-5 was shown by its substitution by a methyl group which made the fragment generation impossible.[18] The ion at *m/z* 195 is proposed to result from the same start as *m/z* 208 producing a crossed-conjugated π-electron system.

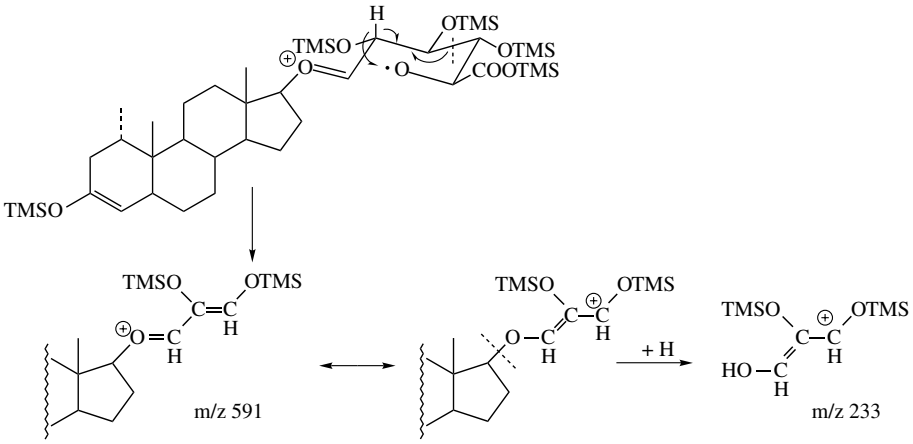

Figure 6. Proposed generation of the M^+-249 fragment. The ion is observed in all spectra of steroid 3,17-diol monoglucuronides and mesterolone glucuronide per-TMS as shown with *m/z* 591. The subsequent elimination of the steroid nucleus generates *m/z* 233 that is also found in many spectra of pertrimethylsilylated steroid glucuronides.

derivatization and subsequent enolization of the 3-keto group with unlabelled TMIS. The ion at m/z 503 originates from m/z 518 by the loss of a methyl group, which could be proved by a product ion scan of m/z 518, and this phenomenon was also observed with androsterone glucuronide and its 19-nor-analogue, where the lost methyl group was identified as C-18 of the steroid moiety. The counterpart to m/z 503 is detected in the spectrum of the d_9-TMS derivative with m/z 512 indicating that here the lost methyl group is not located at the deuterated TMS group. Further evidence for its origin (e.g. C-18, C-19, C-20) has not yet been found. The loss of TMSOH (−90 u) from m/z 518 generating m/z 428 is assigned to the TMS group of the glycosidic moiety due to the presence of m/z 428 in the spectrum of the deuterium-labelled TMS derivative where m/z 527 eliminates 99 u (d_9-TMSOH).

m/z 375, m/z 359 → 269

The fragment at m/z 375 of mesterolone glucuronide per-TMS originates from two sources: (a) the glycosidic moiety

as observed in all other glucuronic acid conjugates presented in this study and (b) the aglycone including the oxygen of the glycoside bond, as demonstrated with the nonadeutero-TMS derivative. Here, we find an ion at m/z 402 corresponding to m/z 375 whose origin is the glucuronic acid, and a fragment at m/z 375 due to the unlabelled part of the steroid being the counterpart to m/z 359 of testosterone glucuronide per-TMS. The aglycone generated by fission of the glycoside bond without retaining the oxygen at the steroid produces m/z 359, corresponding to m/z 343 of testosterone glucuronide per-TMS. A subsequent loss of TMSOH from m/z 359 generates m/z 269, the counterparts of which are not observed in any spectrum of α,β-unsaturated 3-keto-steroid glucuronides, but in high abundances in spectra of steroid diol glucuronides. Additionally, the fragments originating solely from the glucuronic acid moiety are present in the spectrum of mesterolone glucuronide per-TMS with increased abundances.

Glucuronide conjugates of bishydroxylated steroids

The selectively 3- or 17-conjugated monoglucuronides of androstanediol and d_5-androstanediol were prepared as described above. Their fragmentation schemes were investigated and compared with the spectra of monohydroxylated steroid glucuronide conjugates and with norandrostanediol-3-*O*-glucuronide, d_4-norandrostanediol-3-*O*-glucuronide and 1α-methyl-5α-androstanediol-17-*O*-glucuronide.

Androstane-3α,17β-diol-3-*O*-β-glucuronide/ androstane-3α,17β-diol-17-*O*-β-glucuronide, per-TMS (M_r 828)

In contrast to the spectra derived from α,β-unsaturated 3-keto-steroid-17-*O*-glucuronides, the spectra of androstanediol-3-glucuronide and -17-glucuronide (Figs 7 and 8) do not contain the molecular ion but show an intense fragmentation generating ions mainly originating from the glycosidic moiety. A few fragment ions indicating the steroid structure are present, commonly produced with all glucuronide conjugates of 3,17-bishydroxylated steroids, e.g. the methyl ester-TMS derivatives generate a weak signal of $M^+ - 15$ and all spectra of the per-TMS derivatives contain an ion of $M^+ - 249$, the aglycone fragment ($M^+ - 481$) and its secondary ion after a neutral loss of TMSOH (−90 u) (Table 5).

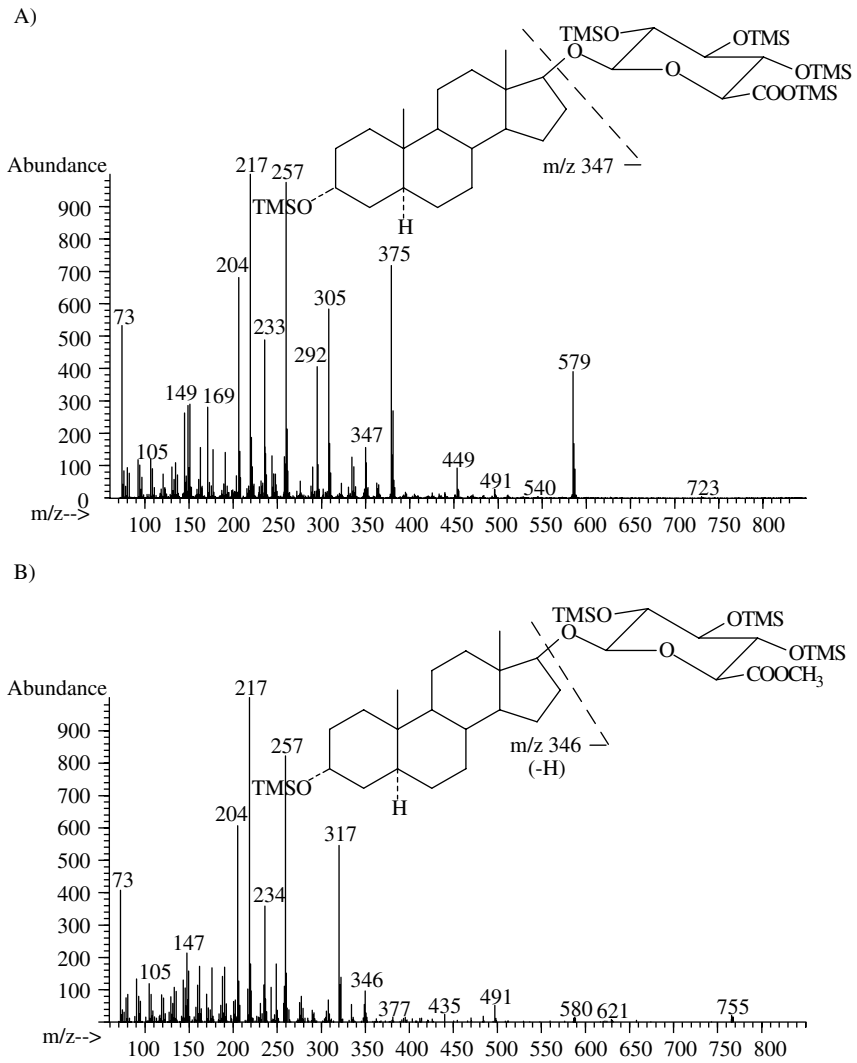

Figure 7. EI mass spectra of androstane-3α,17β-diol-17-*O*-glucuronide: (A) per-TMS derivative (M_r 828); (B) methyl ester tetrakis-TMS derivative (M_r 770).

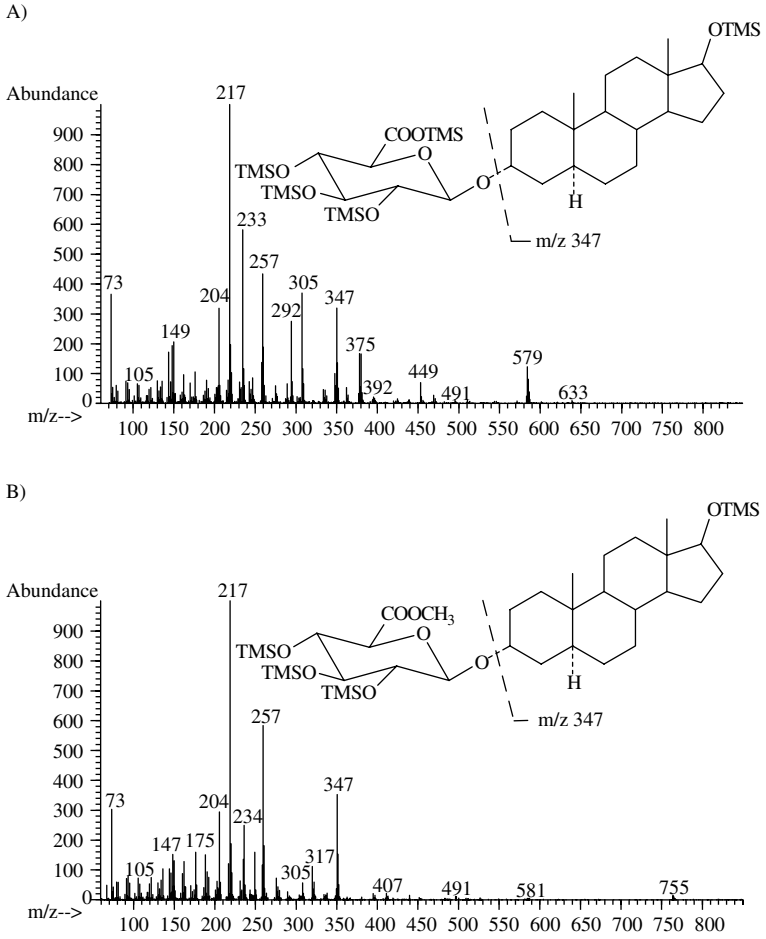

Figure 8. EI mass spectra of androstane-3α,17β-diol-3-*O*-glucuronide: (A) per-TMS derivative (M_r 828); (B) methyl ester tetrakis-TMS derivative (M_r 770).

m/z 579

The ion at *m/z* 579 corresponds to the fragment at *m/z* 591 of mesterolone glucuronide per-TMS. The proposed mechanism of the loss of 249 u is presented in Fig. 6 and evidence for the composition of the generated fragment is given by deuterium-labelled trimethylsilylation where the counterpart of *m/z* 579 is present with *m/z* 606 (Table 4). The increment of 27 u is due to three nonadeutero-TMS groups. Further, its corresponding ion in the spectra of d_5-androstanediol includes all five deuteria showing the fragment at *m/z* 584.

Androstane-3α,17β-diol-3-O-β-glucuronide, m/z 347 → 257

The ion at *m/z* 347 is proposed to originate from the molecular ion after loss of the glycosidic moiety including the glycoside bond oxygen (−481 u). The remaining steroid nucleus contains one TMS group as its fragment at *m/z* 347 shifts to *m/z* 356 in the case of nonadeutero-TMS derivatization (Table 4) and generates *m/z* 257 by the loss of TMSOH or d_9-TMSOH. The spectrum of d_5-androstanediol-3-*O*-glucuronide per-TMS contains the counterparts to *m/z* 347 and 257 with *m/z* 352 and 262, but still a weak signal of *m/z* 257 is present, suggesting a second origin for this fragment. The presence of *m/z* 257 in most of the spectra of per-TMS steroid glucuronides and in product ion spectra of *m/z* 375 indicates its glycosidic generation by elimination of HCOOTMS (−118 u). The fact that the ion shifts to *m/z* 275 with d_9-TMS derivatization (Table 4) supports its postulated origin and composition.

Androstane-3α,17β-diol-17-O-β-glucuronide, m/z 346

The methyl ester-TMS derivative of androstanediol-3-glucuronide also generates the aglycone fragment at *m/z* 347 as well as partly the per-TMS derivative of androstanediol-17-glucuronide [Fig. 7(A)], but the methyl ester-TMS derivative of the latter gives rise to a spectrum missing this fragment and containing an ion at *m/z* 346. This phenomenon is also observed with the d_5-androstanediol-17-glucuronide compared with its 3-*O*-glucuronide. The latter generates a fragment of *m/z* 352 (Table 4) whereas the aglycone of the 17-*O*-conjugated steroid is decremented by 1 u to *m/z* 351.

m/z 233

The fragment ion at *m/z* 233 is present in all spectra derived from steroid-3,17-diol-monoglucuronides and in several spectra of monohydroxylated steroid conjugates. Its origin is the fragment M^+-249 (in the case of androstanediol glucuronide *m/z* 579), as demonstrated with product ion experiments and its proposed structure is shown in Fig. 6 after loss of the steroid nucleus. The hydrogen that is transferred to the ion cannot origin from the α-positioned carbons

Table 4. Selected fragment ions of androstanediol-3-glucuronide derivatives and their counterparts in deuterium labelling experiments (m/z)[a–c]

Fragment ions of ADG-per-TMS	A	B	Fragment ions of ADG-methyl ester-tetrakis-TMS	C	D
—	—	—	755	760	788
579	584	606	—	584	606
449	449	482	—	—	—
—	—	—	407	407	434
377	377	—	—	—	—
375	375	402	—	—	—
347	352	356	347	352	356
—	—	—	317	317	335
305	305	332	305	305	332
292	292	319	—	—	—
257	257 / 261 / 262	257 / 275	257	261 / 262	257
—	—	—	247	247	265
—	—	—	234	234	252
233	233	251	—	—	—
217	217	235	217	217	235
204	204	222	204	204	222

[a] ADG = androstanediol-3-glucuronide.

[b] A = 2, 2, 3, 4, 4-d_5-androstanediol-3-glucuronide per-TMS; B = androstanediol-3-glucuronide per-d_9-TMS; C = 2, 2, 3, 4, 4-d_5-androstanediol-3-glucuronide methyl ester tetrakis-TMS; D = androstanediol-3-glucuronide methyl ester tetrakis-d_9-TMS.

[c] Dashes indicate fragment abundance less than 5% of base peak or not detected.

Table 5. Selected fragment ions of steroid diol monoglucuronide per-TMS compounds (m/z, with abundances (%) relative to base peak in parentheses)[a]

Ion	Adiol-3-G	Adiol-17-G	NorAdiol	1α-MeAdiol
$M^+ - 249$	579 (13)	579 (40)	565 (15)	593 (33)
$M^+ - 481$	347 (32)	347 (16)	333 (26)	361 (7)
$M^+ - 481 - 90$	257 (43)	257 (96)	243 (52)	271 (68)
	233 (58)	233 (49)	233 (57)	233 (58)

[a] Adiol-3-G = androstanediol-3-glucuronide; Adiol-17-G = androstanediol-17-glucuronide; NorAdiol = 19-norandrostanediol-3-glucuronide; 1α-MeAdiol = 1α-methylandrostanediol-17-glucuronide.

C-2, C-3 or C-4 because in the case of d_5-androstanediol-3-glucuronide the fragment at m/z 233 is not incremented to m/z 234 (Table 4). Further, in the spectra of d_3-testosterone glucuronide and d_3-nortestosterone glucuronide per-TMS the hydrogen migration also does not originate from the α-positioned carbon C-16, proved by the presence of m/z 233.

The fragment ions originating solely from the glycosidic moiety (m/z 449, 375, 305, 292, 217, 204 and 169) are also present with high abundances.

Norandrostane-3α,17β-diol-3-*O*-β-glucuronide, per-TMS (M_r 814) and d_4-norandrostane-3α,17β-diol-3-*O*-β-glucuronide, per-TMS (M_r 818)

The 19-nor-analogues of androstanediol-3-*O*-glucuronide give rise to spectra which contain all corresponding fragments in compliance with a mass difference of 14 u (in the case of norandrosterone glucuronide per-TMS) or 10 u (in the case of the d_4-labelled compound) for those ions comprising the steroid nucleus (Table 5).

1α-Methyl-5α-androstane-3α/β,17β-diol-17-*O*-glucuronide, per-TMS (M_r 842)

The fragment ions present in the spectrum of 1α-methylandrostane-3,17-diol-17-glucuronide-per-TMS (Table 5) also correspond to those observed with androstane-3,17-diol-17-glucuronide-per-TMS.

m/z 593 → 233

The ion at m/z 593 generated by the neutral loss of 249 u from the molecular ion is the counterpart to m/z 579 of androstanediol glucuronide per-TMS in compliance with a difference of +14 u according to the Biemann rule and the additional methyl group at C-1. The resulting secondary ion at m/z 233 is also present in comparable abundance.

m/z 361, m/z 360 → 271

The fragments at m/z 361, 360 and 271 originating from the aglycone correspond to m/z 347 and 257 of androstanediol glucuronide per-TMS, also incremented by 14 u. In the spectrum of the methyl ester-TMS derivative is an aglycone fragment observed with m/z 360, which is decremented by 1 u compared with the per-TMS derivative. This was detected only with the 17-*O*-glucuronide of androstanediol and not with its 3-conjugated analogue, thus allowing the differentiation between 3-*O*- and 17-*O*-androstanediol glucuronides.

CONCLUSION

The mass spectra of 17-*O*-glucuronides of α,β-unsaturated 3-keto steroids, 3-keto or 3α,17β-diol steroids derivatized to the trimethylsilylated compounds present significantly different fragmentation schemes depending on their structural properties. The steroid glucuronides with a 3-keto-1- or -4-ene structure generate stable molecular ions with high abundances. Testosterone glucuronide per-TMS shows a decreased degree of dissociation owing to the stability of the M^+ and its analogues all contain the molecular ion with various abundances. Additionally, a fragment proposed to be the formic acid ester of the steroid is generated by all α,β-unsaturated 3-keto steroid glucuronides. The saturation of the steroid A-ring results in a less stable molecular ion rapidly generating $M^+ - 15$, which is found in the case of mesterolone glucuronide per-TMS. A further consequence is the general rise in fragmentation generating more and different fragment ions which are comparable to those known from 17-keto-3-*O*-glucuronide conjugates such as androsterone glucuronide. The monoglucuronides of 3,17-bishydroxylated steroids derivatized to per-TMS products do not generate the molecular ion or $M^+ - 15$. Owing to the absence of any conjugated π-electron systems, the induced charge cannot be delocalized as in steroids containing keto functions and/or double bond structures, which results in a high degree of decomposition generating only a few diagnostic fragments.

All spectra contain the aglycone ion independent of the steroid structure and, except for the androstanediol glucuronides and related compounds, a fragment proposed to be the aglycone including the glycoside oxygen. Changes in the configuration or substitution at carbon C-17 show a great influence on the fragment abundance, e.g. epitestosterone glucuronide per-TMS has significantly different ion abundances to testosterone glucuronide per-TMS. Further, the spectrum of 17-methyltesosterone glucuronide per-TMS contains a very abundant aglycone fragment and very few other ions with lower abundance except m/z 375 resulting from the glycosidic moiety. The spectra of bishydroxylated monoglucuronide per-TMS compounds all show an $M^+ - 249$ ion generated by the loss of a part of the glycosidic moiety. Besides this fragment, only the aglycone and its secondary ion resulting from a neutral loss of TMSOH can be considered as diagnostic for the steroid nucleus. High abundances of ions originating from the glucuronic acid are also usually present. The differentiation between a 3- or 17-conjugation of a 3,17-bishydroxylated steroid is hardly possible from the ion abundances but can be seen by the decrement of the aglycone fragment. Especially with the methylation of the carboxylic acid group and subsequent trimethylsilylation of the hydroxy groups, the aglycone fragment is decreased by 1 u in the case of 17-*O*-conjugation compared with 3-*O*-conjugation.

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