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Synthesis and GC/MS-Characterization of Glucuronide Conjugates of Anabolic Steroids (2)

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Introduction

The mass spectrometric behaviour of steroid glucuronide conjugates derivatized to the per-TMS and methyl ester TMS-ether products was described for the glucuronides of androsterone, 19-norandrosterone, testosterone, 19-nortestosterone, metenolone and methyltestosterone showing common and individual fragmentation pattern[1-2]. Those ions generated from the glycosidic moiety were present in all spectra while fragments originating from the steroid nucleus varied within the different spectra, depending on the structure of the steroid nucleus, e.g. presence of C–C-double bonds and position of keto functions. In the present study the individual fragmentation of the phase-II-metabolite of metenolone is described demonstrating the influence of functional groups on the dissociation of a charged molecule and thus enabling the identification of steroids bearing this structure. In addition, two possibilities of the preparation of selectively conjugated bishydroxylated steroids are presented and characteristic fragmentations are found indicating the conjugation site[3].

Experimental

Chemicals and Steroids

All chemicals used were obtained as described in the literature[2,4] and sodium borohydride (for synthesis) and ethanol (p.a.) were obtained from Merck (Darmstadt, Germany). The reference standards or authentic standards of mesterolone and androsterone were purchased from Sigma (St. Louis, USA), the phase-I-metabolite of metenolone and the deuterated analogues of androsterone and norandrosterone were synthesized in our laboratory[5,6].

Synthesis of selectively conjugated bishydroxylated Steroids

For the preparation of monoglucuronides of androstanediol one way is described in the literature[7] starting with androsterone or 5α-dihydrotestosterone, followed by the protection of the hydroxy group by acetylation, reduction of the keto group with sodium borohydride and subsequent conjugation by means of the Koenigs-Knorr reaction[8,9] as shown in figure 1. The removal of the protecting groups of the glycosidic moiety was easily performed as described earlier[2,10] but the acetyl group of the steroid was resistant under these conditions. The complete hydrolysis of the ester with conservation of the glucuronide was achieved by dissolving the glucuronic acid conjugate in 10ml of methanol, adding 1 ml of 2M aqueous NaOH and stirring at 60°C for 3 hours.

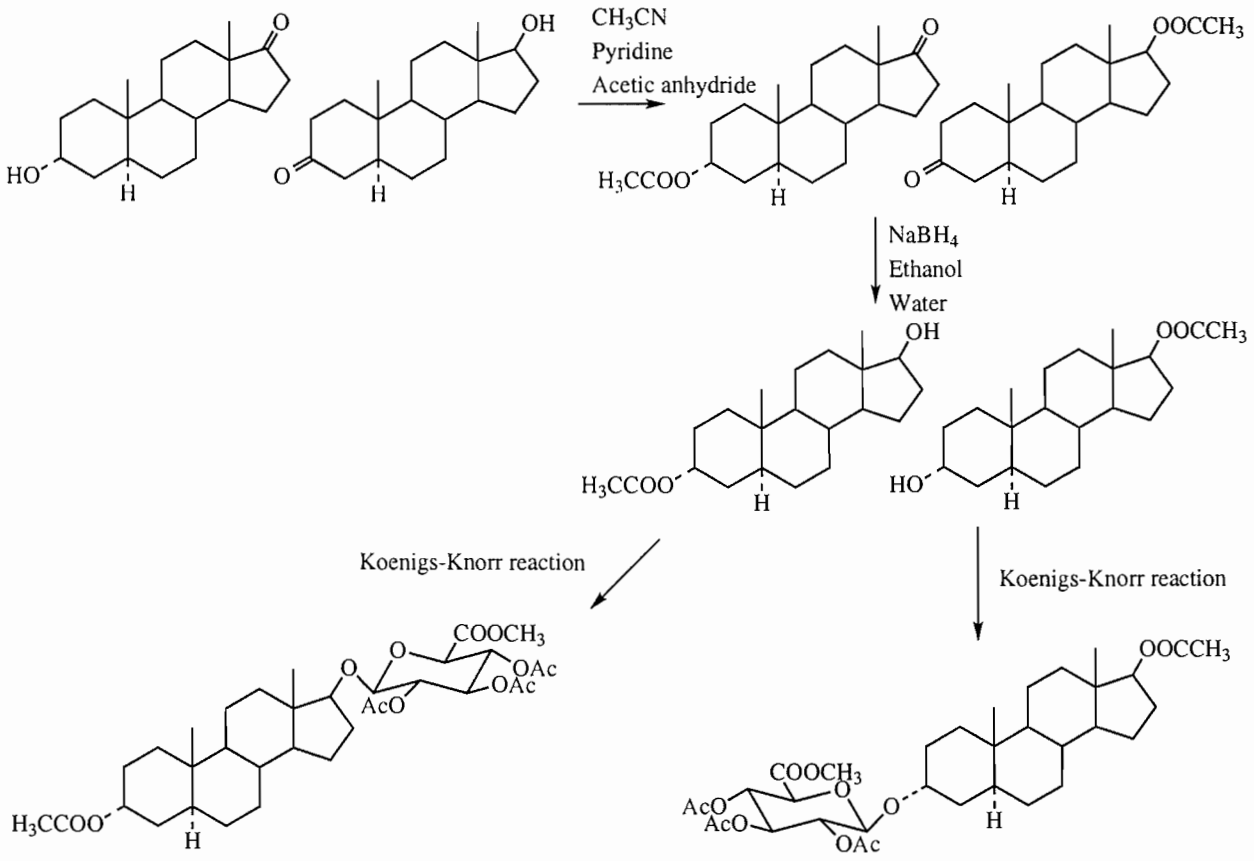


Figure 1: Approach to the synthesis of selectively conjugated androstanediol

A second possibility for the synthesis of 3α,17β-hydroxylated steroid monoglucuronides is the reduction of a remaining 17-keto group after glucuronidation of a 3-hydroxy group, e.g. of norandrosterone. In order to do this, the steroid conjugates were treated in 20 ml of a mixture of ethanol and bidestilled water (80:20, v:v) with sodium borohydride in a 1.5 molar excess. After one hour the reaction mixture was evaporated to dryness and the residue was redissolved in 20 ml of bidistilled water. The pH was adjusted to 10-11 (if necessary) by the addition of 1M aqueous NaOH and the solution was extracted twice with 20 ml of *tert*-butylmethyl ether to remove the unconjugated steroids possibly generated by the hydrolysis. The pH of the aqueous layer was then adjusted 2.5 with aqueous HCl (3M) and extracted twice with ethyl acetate. The combined organic layers were evaporated to dryness giving the pure 3-O-glucuronides in yields of 71-79%.

Derivatization for the GC/MS analysis

A) Trimethylsilylation

The obtained steroid glucuronide conjugates (5 μg) were derivatized to the per-TMS products with 100 μl of a mixture of MSTFA/ethanethiol/NH_4I (100:0.6:0.2, v:v:w) at 60°C for 20 minutes.

B) Methylation of the carboxylic group

The methyl ester per-TMS derivatives of the glucuronides (5 μg) were obtained by esterification of the glucuronic acid with methyl iodide (20 μl) in a volume of 200 μl of acetonitrile and K_2CO_3 (ca. 50 mg) as catalyst. The mixture was heated for 1 h at 60°C and the supernatant was transferred into a reagent tube. The sample was evaporated to dryness and solved in 100 μl of the derivatization mixture mentioned above (part A).

C) Perdeutero-trimethylsilylation

5 μg of the steroid glucuronide are dissolved into 100 μl of a mixture of d_{18}-bis-trimethylsilyl-acetamide (d_{18}-BSA) and acetonitrile (1:4, v:v) and the mixture is heated for 20 minutes at 60°C. Under these conditions there is no enolization observed of the 17-keto group.

GC/MS parameters

The analyses were performed on a Finnigan GCQ ion trap GC/MS system equipped with an ATAS Optic 2 injector system. The conditions were as follows:

column:	HP Ultra 1, 14m x 0.2mm i.d., film thickness 0.11 µm
carrier gas:	helium, 1.5 ml/min, split 1:10
injector temp.:	325°C
temp. programm:	0 min 140°C, +20°C/min, 5 min 320°C
interface temp.:	325°C
ionization:	EI (70 eV), mass range 70-920 amu.

Results and discussion

Figure 2 shows the mass spectrum of the phase-II-metabolite of metenolone, 1-methylen-5α-androstan-3-ol-17-one-3-O-glucuronide, derivatized to the per-TMS product. The main difference to other mass spectra of androstan-3-ol-17-one-3-O-glucuronides is the intense fragment m/z 553 the origin of which was studied by different derivatization techniques. The tetrakis-TMS derivative of 1-methylen-5α-androstan-3α-ol-17-one-3-O-glucuronide without the enol-TMS group at position 17 gives rise to a spectrum containing the fragment at m/z 481 as listed in table 1. The shift of 72 mass units from the ions at m/z 553 to m/z 481 proves the participation of this TMS group in the investigated ion. Further, the methyl ester-TMS ether derivative generates a fragment at m/z 495 corresponding to m/z 553, and therefore we conclude that the ester group is present in the ion m/z 553.

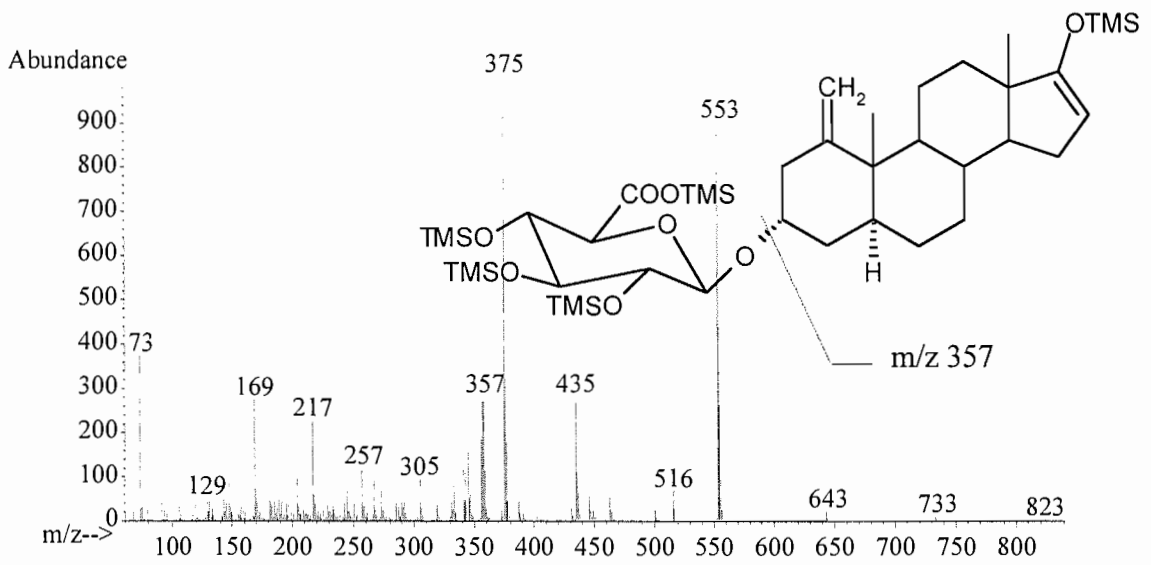
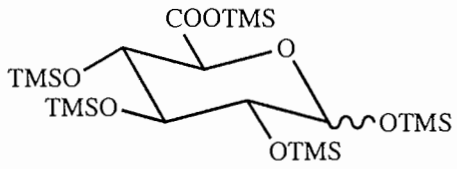


Figure 2: EI-mass spectrum of 1-methylen-5α-androstan-3-ol-17-one-3-O-glucuronide per-TMS, mol wt = 838.

The trimethylsilylation with d_{18}-BSA without enolization of the 17-keto group produces a fragment ion at m/z 517 which is incremented from m/z 481 by 36 mass units, proving the presence of four TMS groups containing 9 deuterium atoms each. As a final conclusion we observe a fragment ion at m/z 553 consisting of the glycosidic moiety including the TMS group of position 17 as shown in figure 3. Therefore, a long-range transfer is proposed which was observed in other cases with different positions and compounds in earlier studies[11,12,13].

Derivative	Fragment Ion (m/z)	Shift (amu)
per-TMS	553	-
Me-TMS	495	58
tetrakis-TMS	481	72
tetrakis-d_9-TMS	517	36

Table 1: Shifts of m/z 553 in different derivatives of 1-methylen-5α-androstan-3-ol-17-one-3-O-glucuronide. Per-TMS = pentakis-TMS, Me-TMS = Methyl ester-TMS ether



m/z 553

Figure 3: Proposed structure of the ion at m/z 553 of 1-methylen-5α-androstan-3-ol-17-one-3-O-glucuronide per-TMS

The selectively conjugated steroids of androstanediol, d_5-androstanediol, norandrostanediol, d_4-norandrostanediol and 17α-methyl-5α-androstane-3α,17β-diol were prepared as described above and their mass spectra were recorded after derivatization to the per-TMS and methyl ester-TMS ether products. In figure 4 the mass spectra of androstanediol 3- and 17-O-glucuronide per-TMS are presented and their fragmentation patterns differ only in the intensities of fragment ions relative to the base peak at m/z 217. Compared to that, the methyl ester-TMS ether derivatives, as shown in figure 5, have a different fragmentation concerning the aglycone fragment. In case of a 3-O-glucuronide the aglycone is represented by m/z 347 but in case of the 17-O-glucuronide the corresponding fragment is decremented by one amu to m/z 346. This phenomenon is reproducible even for the deuterated counterparts of the 3- and 17-O-glucuronide where the ion at m/z 352 is present in the spectrum of the 3-O-conjugated steroid and m/z 351 is found in the 17-O-conjugated d_5-androstanediol. Even here, a proton is removed but not a deuterium. The other glucuronidated diols like 19-norandrostanediol, its d_4-labelled counterpart and also the diols of 17-methyl steroids show the same mass spectrometric behaviour concerning their conjugation site. The 19-norandrostanediol-3-O-glucuronide per-TMS and methyl ester-TMS ether generate both a aglycone fragment at m/z 333 and the d_4-labelled analogue shows the corresponding ion at m/z 337 which do not show a decrement of one mass unit, due to their conjugation at position 3 (fig. 6).

The methyl ester-TMS ether derivative of the 3-O-glucuronide of 17α-methyl-5α-androstane-3α,17β-diol also produces the expected aglycone fragment at m/z 361 while its 17-O-conjugate generates an ion at m/z 360, as shown with their compared mass spectra in figure 7. In addition to the aglycone fragment another ion indicates the position of conjugation with the 17-methyl steroiddiols, namely the ion at m/z 143, which is also known from unconjugated steroids containing a 17-methyl-17-hydroxy-structure. The ion at m/z 143 is abundant only in the 3-O-glucuronide derivatives.

Conclusion

Individual functional groups of steroids can influence the mass spectrometric behaviour intensively as demonstrated with the phase-II-metabolite of metenolone, which contains a methylene unit at C-1 of the steroidal A-ring. The consequence of its presence is a long-range transfer of a TMS group from C-17 which was not observed in any other steroid glucuronide derivatized to its per-TMS product.

The selective conjugation of diols of steroids to monoglucuronides is possible by two different approaches and the evidence for the position of conjugation is given by the different fragmentation patterns exhibited by the 3- or 17-O-glucuronides, especially as the methyl ester-TMS ether derivatives. All 17-O-glucuronides generate an aglycone fragment ion which is decremented by one amu when compared to their 3-O-conjugated counterparts. Further, the 17-methyl-17-O-glucuronides do not produce the characteristic fragment at m/z 143 which usually occurs in all spectra of TMS derivatives of 17-methyl-17-hydroxylated steroids.

Acknowledgements

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