^{13}C-NMR and mass spectral data of steroids with a 17,17-dialkyl-18-nor-13(14)-ene substructure

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Abstract We present carbon-13 nuclear magnetic resonance (^{13}C-NMR) and mass spectral data for several androstanes and estranes having a 17,17-dialkyl-18-nor-13(14)-ene substructure. These compounds are formed by a Wagner-Meerwein rearrangement of steroids bearing a tertiary 17-hydroxy group during the derivatization reaction with heptafluorobutyric anhydride. The ^{13}C-NMR assignments are compared with those of natural products having a similar substructure. The mass spectra show characteristic fragment ions for which a fragmentation mechanism is proposed.—**De Brabandere, V. I., L. M. Thienpont, D. Stöckl, and A. P. De Leenheer.** ^{13}C-NMR and mass spectral data of steroids with a 17,17-dialkyl-18-nor-13(14)-ene substructure. *J. Lipid Res.* 1997. **38:** 780–789.

Supplementary key words Wagner-Meerwein rearrangement • mass spectrometry • heptafluorobutyrilation

In the course of our studies for derivatization with heptafluorobutyric anhydride (HFBA) of steroids bearing a tertiary hydroxyl group on carbon-17 (C-17), we observed that this type of derivatization was accompanied by loss of the C-17 hydroxyl group. This dehydration was followed by a Wagner-Meerwein rearrangement, resulting in a 17,17-dialkyl-18-nor-13(14)-ene structure of the derivatization products (1, 2). The Wagner-Meerwein rearrangement is indeed well known for 17-hydroxy steroids. It occurs during acid reaction conditions (3–9) and during the metabolization of some anabolic steroids (10–12). In our previous studies, we published mass and ^{13}C-NMR spectral data of the rearrangement products from pregnane-type steroids (1) and preliminary data from the rearrangement products of androstane and estrane steroids (2).

In this article, we describe the extension of our studies on androstane- and estrane-type steroids. We present mass spectral and ^{13}C-NMR data of 17,17-dialkyl-18-nor-13(14)-ene steroids formed during the heptafluorobutyrilation of a variety of 17β-hydroxy steroids. We discuss the possible mass spectral fragmentation pathway for the formation of a fragment ion that is specific for the 17,17-dialkyl-18-nor-13(14)-ene substructure. In addition, we discuss the ^{13}C-NMR data in light of those found with natural products possessing comparable bicyclo-[4.3.0]-nonene structures.

EXPERIMENTAL

Standards and reagents

The steroids (presented in **Fig. 1**) were purchased from Steraloids (Wilton, NH). Standard solutions were prepared in absolute ethanol from Merck (Darmstadt, Germany) in a concentration of approximately 0.1 g/L. All solvents were analytical grade unless stated otherwise. Acetone was of extra pure quality and also from Merck. The HFBA reagent used for derivatization was from Macherey & Nagel (Düren, Germany). Deuterochloroform ($CDCl_3$) was obtained from Aldrich.

Spectrometric analysis

^{13}C-NMR spectra were recorded proton decoupled at 90 MHz on a Bruker AM-360 spectrometer (Karlsruhe, Germany). Spectra were recorded as 12–20 mmol/L solutions in $CDCl_3$ at ambient temperature.

Abbreviations: EI, electron impact; GC-MS, gas chromatography-mass spectrometry; HFBA, heptafluorobutyric anhydride; NMR, nuclear magnetic resonance (spectroscopy); PTV, programmable temperature vaporizer.

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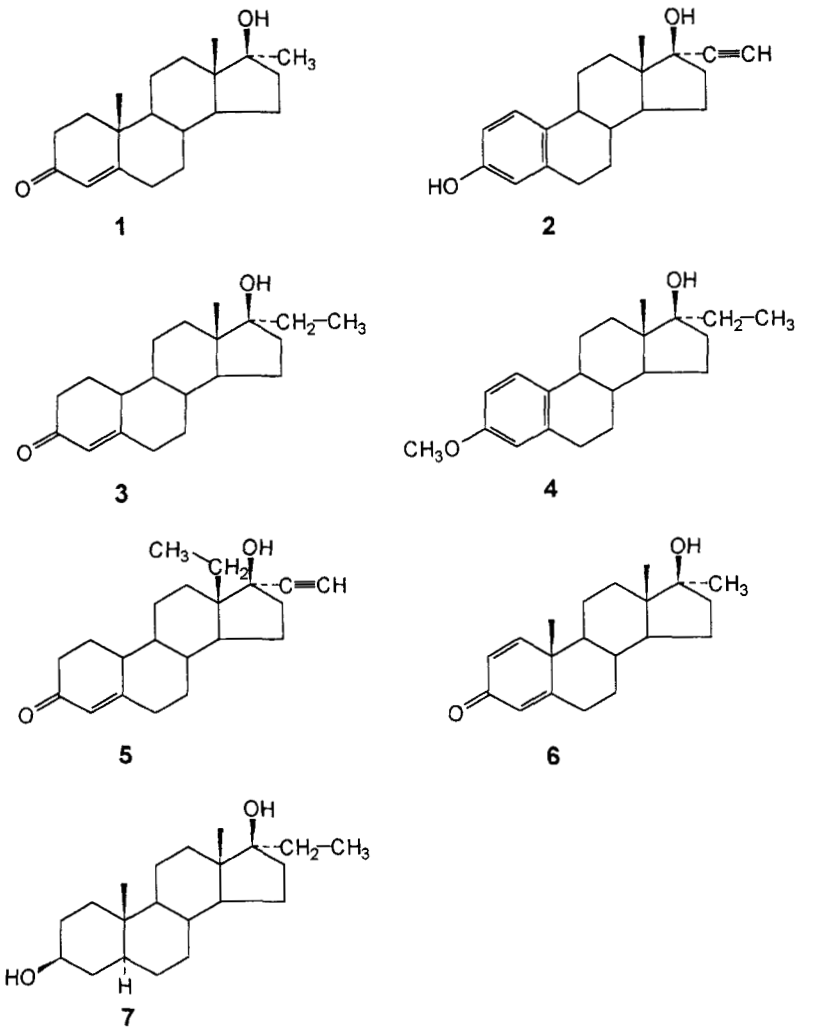


Fig. 1. Chemical structures of the steroids **1** (17α-methyltestosterone), **2** (17α-ethinylestradiol), **3** (norethandrolone), **4** (3-methoxy-17α-ethyl-estradiol), **5** (norgestrel), **6** (methandienone), and **7** (5α,17α-pregnan-3β,17β-diol).

Chemical shifts were expressed in parts per million (ppm) relative to external tetramethylsilane. These measurements were done at the Department of Organic Chemistry of the University of Ghent (Dr. F. Borremans) and by Spectral Service GmbH (Köln Ehrenfeld, Germany).

Mass spectrometry (MS) was performed on a Finnigan MAT Incos-XL mass spectrometer (San José, CA), combined with a 5890 Series II gas chromatograph (GC) from Hewlett Packard (Palo Alto, CA) as inlet system. Operation of the GC-MS system was controlled by a Data General computer system (Westboro, MA) and a 4-MB IBM-compatible PC workstation as the system terminal. MS was operated under electron impact (EI; 70 eV) ionization mode, scanning from m/z 33 to 750 in 0.59 s. GC was done on a 15 m × 0.18 mm (inner diameter) DB-1, that is methylsilicone, fused silica column with 0.4-μm film thickness from J & W Scientific (Folsom, CA). The column was coupled directly to the mass spectrometer. The GC was equipped with a programmable temperature vaporizing (PTV) injection system from Gerstel GmbH (Mülheim a.d., Ruhr, Germany). The injection was performed as follows: injection into the PTV in the purge on mode at 60°C; after 0.3 min the injector was switched to the purge off mode for 1 min and simultaneously heated to 280°C at a rate of 12°C/s; after 1 min at 280°C, the PTV was cooled down to the starting temperature. The oven temperature was programmed from 190 (hold 1.25 min) to 280°C at a rate of 20°C/min. The final temperature was held for 10 min. The transfer body and nozzle were set at 280 and 270°C, respectively.

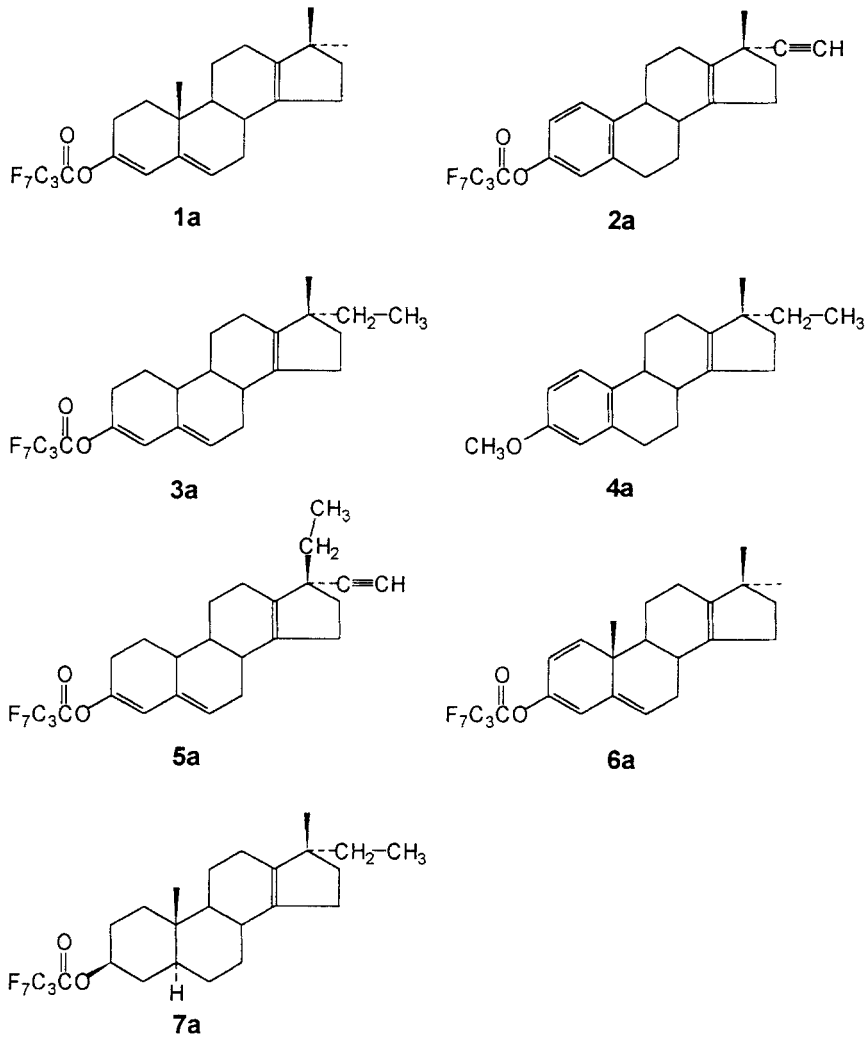


Fig. 2. Derivatization products of steroids **1–7**.

Derivatization with HFBA

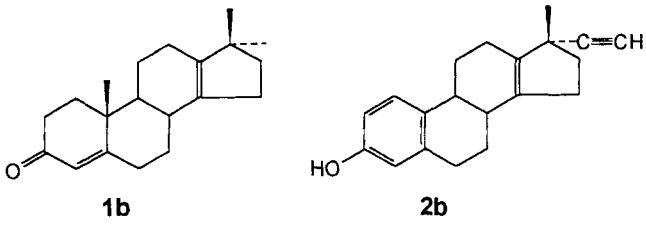
For GC-MS analysis a volume of standard solution containing approximately 5 μg of substance was evaporated to dryness under a stream of nitrogen at room temperature. The evaporation residue was redissolved in 30 μL of acetone and 30 μL of HFBA was added. After closing the vials, derivatization was performed for 1 h at room temperature. Subsequently, the reaction mixture was evaporated to dryness at room temperature under a stream of nitrogen, followed by an additional drying step at 70°C for 20 s. The residues were redissolved in cyclohexane. Approximately 10 ng of derivatized substance were injected for GC-MS analysis.

Derivatization for NMR analysis was done on approximately 5 mg of product. To keep the derivatization on the microgram scale as for GC-MS, the sample was dissolved in 2 mL of chloroform and 10 200-μL portions were pipetted in individual 1.5-mL vials, evaporated, and treated for derivatization as previously described but with 150 μL of acetone and 150 μL of HFBA. After evaporation of the reaction mixtures to dryness, the residues were dissolved in approximately 70 μL of $CDCl_3$ and pooled in a NMR tube.

Hydrolysis of the HFB derivatives of **1** and **2** (see Fig. 1) was performed by dissolving the derivatives in 1 mL of methanol, adding 100 μL of 0.01 M hydrochloric acid, and heating for 3 h at 50°C. Completeness of hydrolysis was assessed by GC-MS.

RESULTS AND DISCUSSION

The HFB-derivatization products of steroids **1–7** are designated with the extension **a** and are presented in



1b 2b

Fig. 3. Hydrolyzation products of the HFB derivatives **1a** and **2a**.

Fig. 2. During the derivatization all steroids lost the tertiary 17-hydroxyl group with formation of the 17,17-dialkyl-18-nor-13(14)-ene substructure. In the following the structures of these compounds will be confirmed by ^{13}C-NMR and/or MS. Because little ^{13}C-NMR data are available on acylated steroids, we also compared with ^{13}C-NMR data obtained on hydrolyzed derivatization products. The structures presented in **Fig. 3** are the hydrolysis products of **1a** and **2a** and are designated with the extension **b**.

^{13}C-NMR spectra

In **Table 1** the ^{13}C-NMR data for **1**, **1a**, **1b**, **2**, **2a**, **2b**, and **3a** are presented. The assignments of **1**, **1b**, and **2** are based on resonances published in the literature (8, 13, 14) and are presented here to facilitate comparison. The acylation of the 3-keto-4-ene function in **1** and **3** to the 3,5-diene-3-heptafluorobutyryl ester in **1a** and **3a** strongly influences the resonances of C-2 to C-6. It causes the disappearance of the carbonyl carbon resonance at 200 ppm (C-3), the introduction of two additional olefinic resonances (C-3 and C-6), and upfield shifts of C-2 (−9.9 ppm), C-4 (−5.7 ppm), and C-5 (−32.6 ppm). Furthermore, the formation of the 3,5-diene structure also causes upfield shifts of C-8 to C-10 (−4.0, −7.3, −3.4 ppm), similar to the cholestane/cholesta-3,5-diene pair (16). For **1a** these resonances of the A/B-ring could be assigned, which may be compared with those we previously reported for the HFB-derivative of progesterone and 17α-hydroxyprogesterone (1) having exactly the same A/B-ring structure. For **3a** the resonances are also very similar but because no C-19 methyl group is present the resonances of C-1, C-2, C-5, and C-8 to C-11 are slightly different. These assignments were confirmed by those of 17α-ethinyl-17β-hydroxy-4-estren-3-one (15), to which the influence of perfluoroacylation of the 3-keto-4-ene moiety (**1a–1**) was complemented. In **2a** the perfluoroacylation of the phenolic hydroxyl group on C-3 causes an upfield shift of C-3 (−5.9 ppm) and downfield shifts of C-2, C-4, and C-10 (+4.6, +5.1, and +7.3 ppm). These chemical shifts were assigned by comparison with those published for estradiol-diacetate (16). For the hydrolysis products **1b** and **2b**, the chemical shifts of the A/B ring are almost identical to those of the original products (**1** and **2**), which confirms the hydrolysis to the 3-keto-4-ene- and the phenolic hydroxyl-function, respectively.

The formation of the 17β-methyl-17α-alkyl-18-nor-13(14)-ene structure is characterized by a strong upfield shift of C-17 to approximately 46 ppm due to the loss

TABLE 1. ^{13}C-NMR Resonances (δ in ppm) of **1**, **1a**, **1b**, **2**, **2a**, **2b**, and **3a**

Carbon	1	1a	1b	2	2a	2b	3a
1	35.6	33.4	35.4	126.5	126.3	126.1	25.2
2	33.9	24.0	33.8	112.6	117.2	112.4	(27.1)
3	199.4	146.1	199.4	153.2	147.3	153.3	147.8
4	123.8	118.1	124.2	115.1	120.2	115.2	119.0
5	171.1	138.5	171.0	138.2	(139.1)	138.4	135.6
6	32.7	126.6	33.3	29.6	29.5	29.7	127.2
7	31.6	31.3	31.0	27.1	(26.4)	26.6	31.2
8	36.4	32.4	36.5	39.3	39.2	39.7	35.4
9	53.7	46.4	51.6	43.4	40.9	40.6	42.2
10	38.6	35.2	38.5	132.5	(139.8)	132.7	38.1
11	20.6	21.7	22.5	26.3	(26.3)	26.6	(27.0)
12	31.3	22.8	22.1	32.6	21.9	22.0	21.9
13	45.2	(141.8)	(141.9)	47.0	(138.6)	(138.4)	(140.3)
14	50.1	(134.8)	(134.6)	49.4	(137.4)	(137.8)	(134.1)
15	23.1	29.6	29.7	22.7	30.0	30.0	30.4
16	38.8	39.5	39.4	38.7	39.6	39.6	40.1
17	81.4	45.3	45.3	79.9	44.2	44.2	49.3
18	13.8			12.6			
19	17.3	18.1	16.8				
20	25.7	(26.8)	(26.5)	87.4	90.6	90.8	32.0
21				74.0	67.9	67.8	9.0
17β-CH_3		(26.2)	(26.3)		25.9	26.0	26.8

The assignments in parentheses are interchangeable.

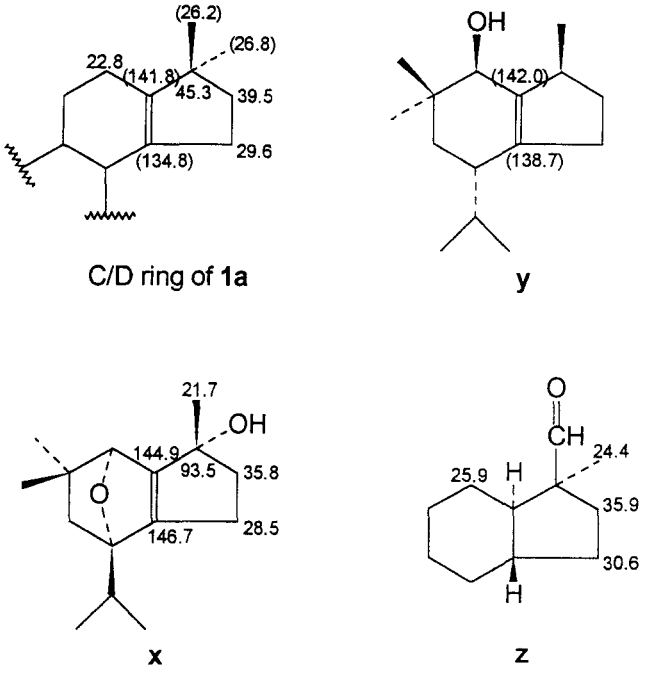


Fig. 4. Comparison of the ^{13}C-NMR resonances in different bicyclo-[4.3.0]-nonene and -nonane structures: the C/D ring of **1a** (see Table 1), 2,5-epoxybrasil-1(6)-en-9-ol (**x**),[18] brasilenol (**y**),[19] and 7,7-formyl,methyl-*trans*-bicyclo-[4.3.0]-nonane (**z**)[20]. The assignments in parentheses are interchangeable.

of the 17-hydroxyl group, and by the presence of the C-13 and C-14 alkene resonances at approximately 140 and 136 ppm. In addition, the rearrangement reaction causes an upfield shift of C-12 from 32.0 ± 0.7 to 22.4 ± 0.5 ppm, and a downfield shift of C-15 from 22.9 ± 0.2 to 29.8 ± 0.2 ppm. The signal of the C-18 methyl group at approximately 13 ppm disappears and is replaced by a signal at 26.1 ± 0.2 ppm, corresponding with the new 17β-methyl group. These findings correspond with those we previously reported for the HFB-derivatization product of 17α-hydroxyprogesterone (1). To further prove this structure and to assure that these assignments are correct, we compared with ^{13}C-NMR data in literature for compounds with similar structures. First of all, the 13(14) double-bond position has only partially been confirmed by other techniques. In ^{1}H-NMR no additional vinylic proton is seen (1, 2, 8) from which it follows that the only possible other positions of the double bond are 8(9) and 8(14). But comparison with ^{13}C-NMR data of 3β-hydroxy-5,8(9)-cholestadiene and 3β-hydroxy-5,8(14)-cholestadiene makes these double-bond positions not probable. In these compounds C-8 resonates at 124.9 ± 1.6 ppm (17), which is at higher field than the resonances we find. Second, we compared with reported ^{13}C-NMR resonances of natural products having a substituted bicyclo-[4.3.0]-nonene (18, 19) and bicyclo-[4.3.0]-nonane (20) structure. In **Fig. 4** these are presented and compared with the C/D ring substructure of **1a**. The agreement of the ^{13}C-NMR resonances of the olefinic carbons in **1a**, **x**, and **y** (all within 141 ± 6 ppm) supports the 13(14) double-bond location. Furthermore, the chemical shifts of C-12, C-15, and C-16 at 22.8, 29.6, and 39.5 ppm are also comparable with those of the corresponding carbons in **x** and **z**.

The upfield shift of C-12 (−9.6 ppm) and the downfield shift of C-15 (+7.0 ppm) in the 17,17-dialkyl-18-nor-13(14)-ene structure relative to the original compounds is probably due to the loss of the C-18 methyl group. In the original steroids the C-18 methyl does indeed have a deshielding effect on C-12 (β-position) and a shielding effect on C-15 (γ-gauche effect). Similar shifts are also observed due to the loss of the angular C-19 methyl group [compare ^{13}C-NMR data of 19-nortestosterone (15) and testosterone (13)], where C-1 (β-position) shifts −8.7 ppm and C-8 (γ-position) shifts +4.3 ppm. Furthermore, we find only a small influence of the 13(14) double bond on the resonances of C-12, C-15, and C-16. In Fig. 4 the chemical shifts of these carbons in the structures with a double bond (**1a** and **x**) and without a double bond (**z**) are comparable.

Mass spectra

GC-MS analysis of the derivatization products of compounds **1–7** always led to nice chromatographic peaks with a recovery of > 85%. The mass spectra have already been published for **1a** and **2a** (2), and are presented in **Figs. 5–9** for **3a–7a**. The molecular ions found in these mass spectra always correspond with the structures proposed in Fig. 2. This confirms the loss of water during the derivatization of all these compounds, and heptafluorobutyrilation in position 3 of compounds **1–3** and **5–7**. Further, all these derivatization products show a characteristic fragmentation pattern with only a small molecular ion and a few abundant fragment ions. This fragmentation is not usual for HFB-derivatives of steroids, which normally show an abundant molecular ion (21). One abundant fragment ion present in all these mass spectra corresponds to the loss of one 17-sidechain. This mass spectral loss of the 17-sidechain has already been reported before for Wagner-Meerwein rearrangement products (1, 2, 22). It is characteristic for the quaternary C-17, which after fragmentation forms a stable tertiary carbonium ion (1). It seems that it is always the largest or more saturated C-17 substituent that is lost, with a methyl or ethinyl group remaining. Another abundant fragment ion corresponds with an A/B-ring fragment formed after cleavages between C-

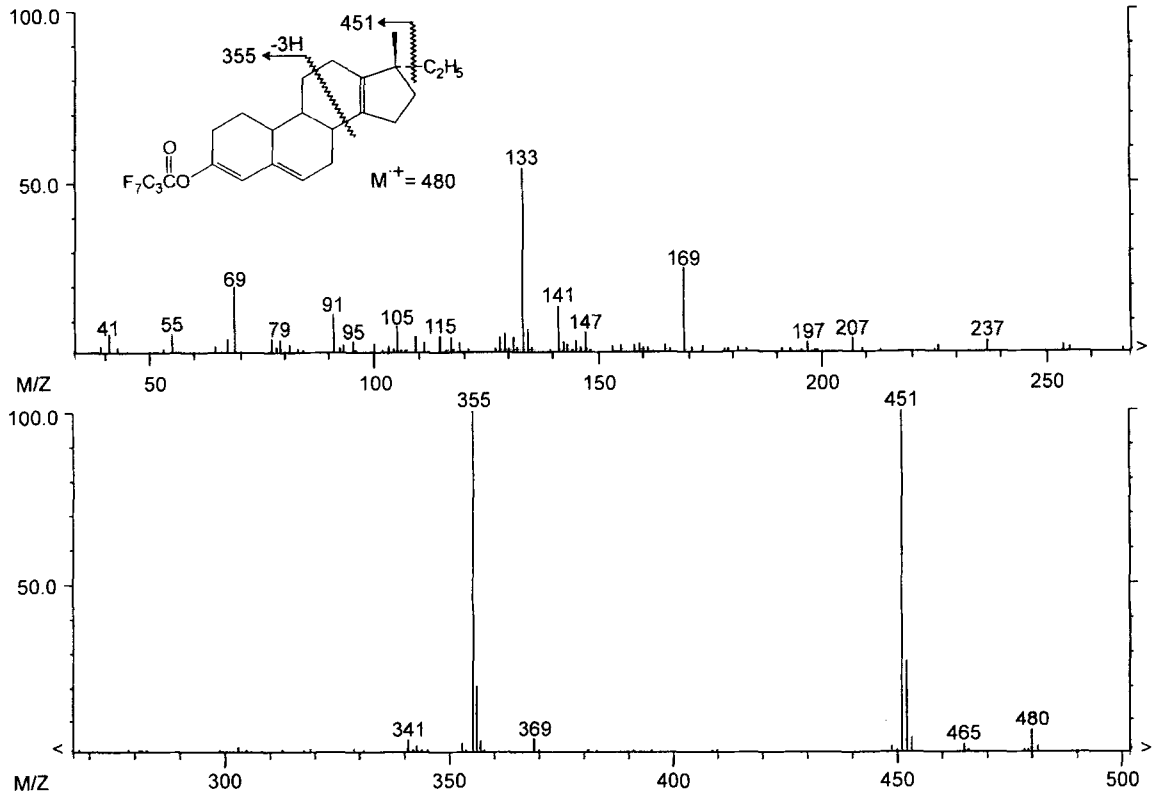


Fig. 5. Mass spectrum of **3a.**

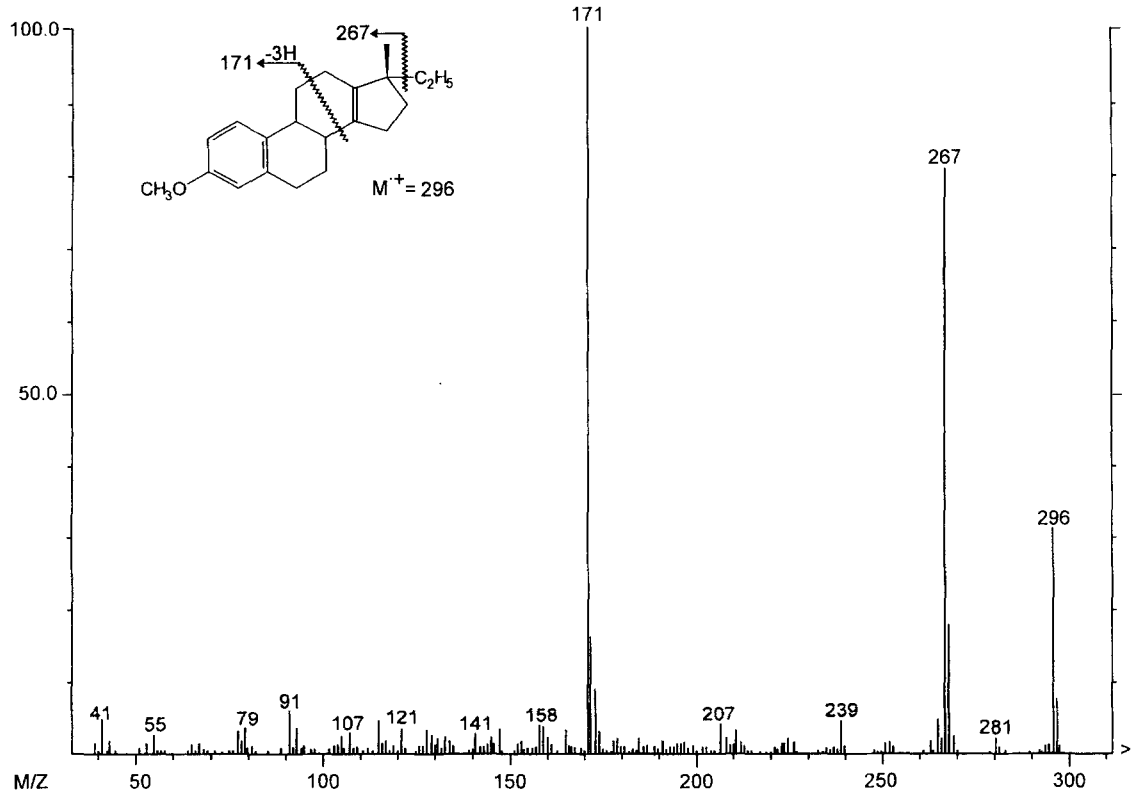


Fig. 6. Mass spectrum of **4a.**

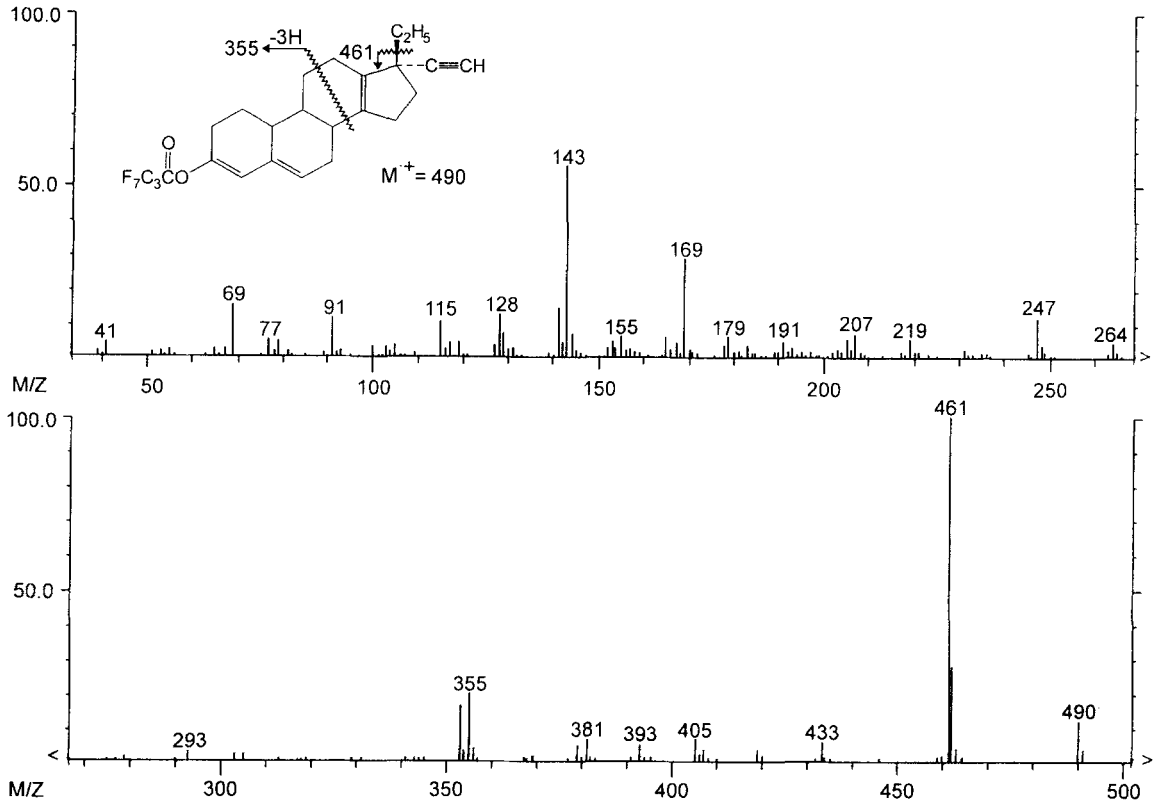


Fig. 7. Mass spectrum of **5a.**

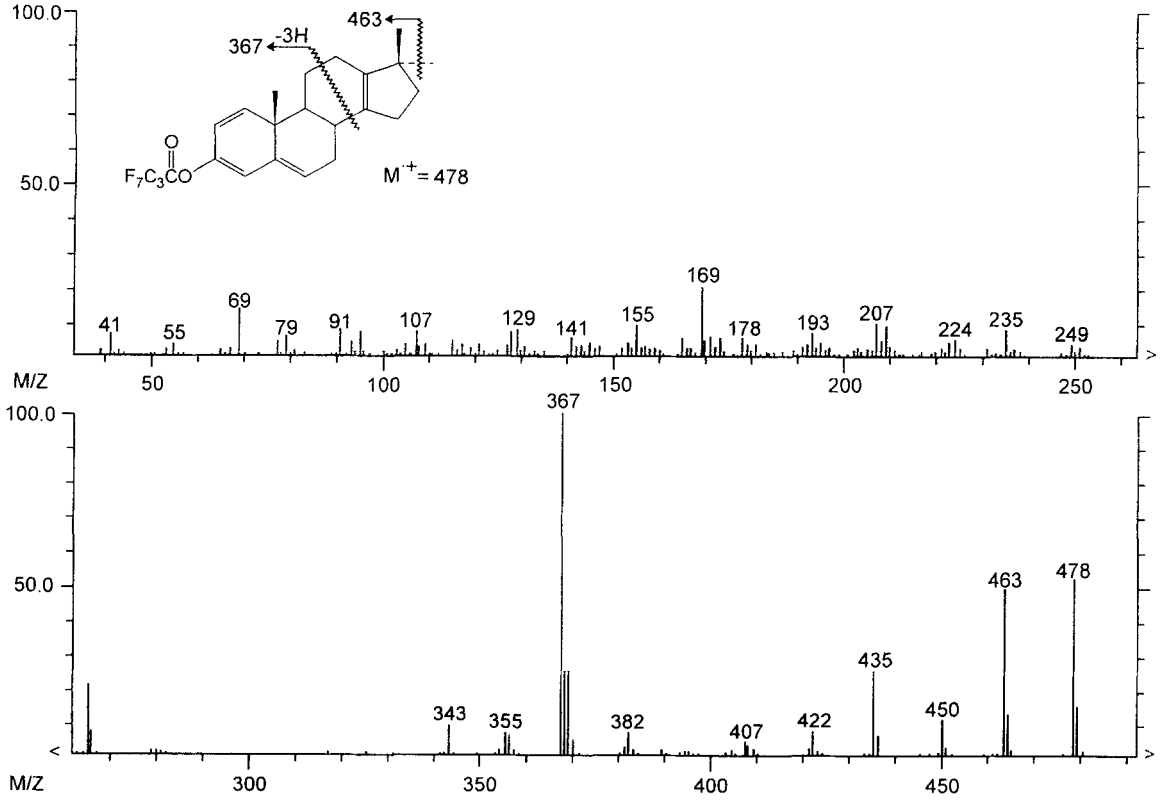


Fig. 8. Mass spectrum of **6a.**

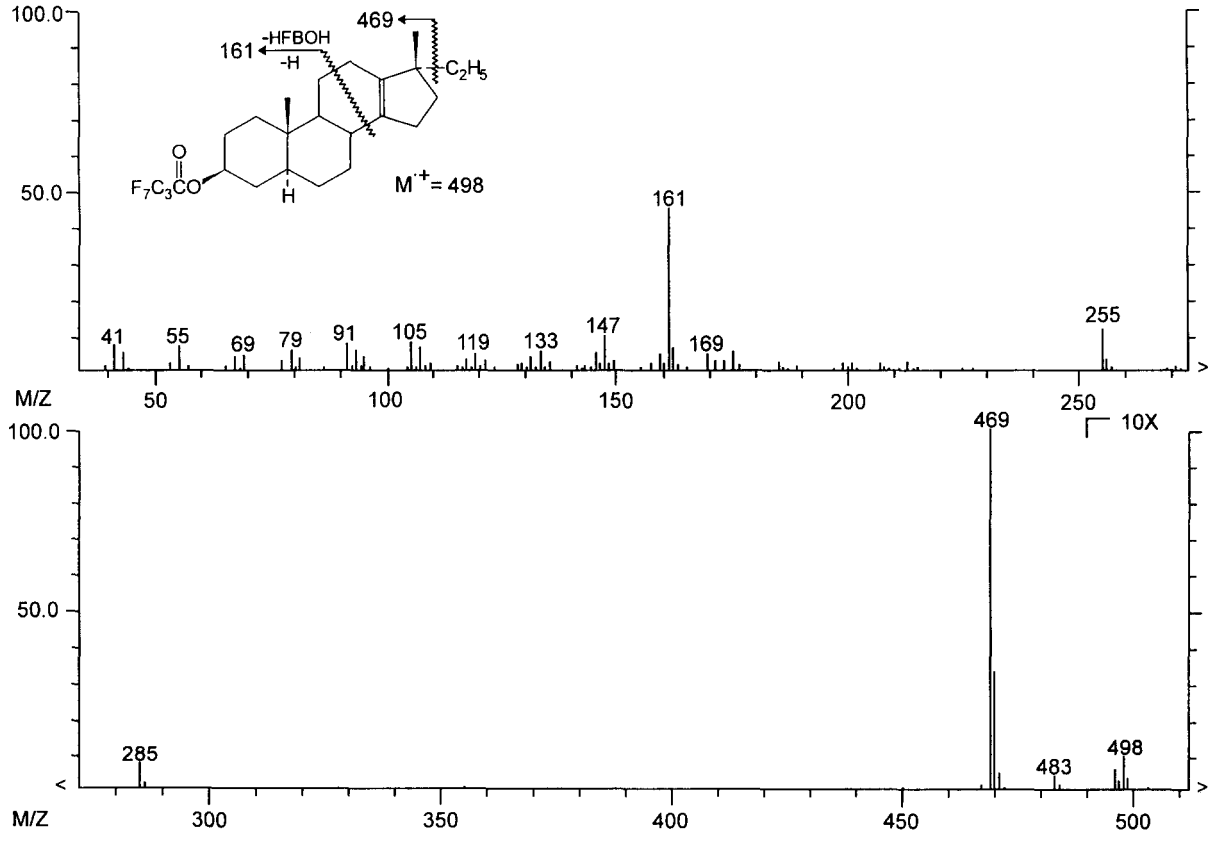


Fig. 9. Mass spectrum of **7a**.

11 and C-12 and between C-8 and C-14. This fragment ion occurs at *m/z* 355 (**3a** and **5a**), *m/z* 171 (**4a**), *m/z* 367 (**6a**), and *m/z* 161 (**7a**), the *m/z* values depending on the A/B-ring substitution and saturation. A corresponding fragment ion was also for **1a** (at *m/z* 369), **2a** (at *m/z* 353), and the HFB- rearrangement products of 17α-hydroxyprogesterone and cortexolone (both at *m/z* 369) an abundant ion (1, 2). It was proven by MS/MS and by metastable ion measurements that this fragment ion originates from the fragment ion produced by the loss of the 17-sidechain (1, 2). It is assumed that the formation of this fragment is initiated by an allylic fission between C-11 and C-12. A possible fragmentation pathway is presented in **Fig. 10**. For all these compounds (except for **7a**), this fragmentation is accompanied by the loss of three hydrogens from the A/B-ring. This is probably caused by one hydrogen transfer to C-14, and an additional loss of two hydrogen atoms during the double bond isomerization in the A/B-ring. This leads to resonance-stabilized fragment ions, which explains their high abundance. In our previous studies, high resolution measurements confirmed the elemental composition of the A/B-ring fragment, and no metastable ions for the H_2 loss were found (1, 2). For **7a** the fragmentation to the A/B-ring is accompanied by the loss of HFBA by a McLafferty rearrangement. This results in only one double bond in the A/B-ring, and here the additional loss of H_2 does not take place. On the other hand, the formation of the A/B-ring fragments is also favored by the formation of resonance-stabilized neutral losses. In the mechanism we propose for compounds **1a**, **3a**, **4a**, **6a**, **7a**, and for the corticosteroids showing this fragmentation, the neutral D-ring fragment shows a thermodynamically stable heteroanular diene structure (see Fig. 10). For compounds **2a** and **5a** a similar structure is possible.

These results support the hypothesis that the characteristic fragmentation seen in the mass spectra from these derivatization products is due to their 17,17-dialkyl-18-nor-13(14)-ene substructure. The mass spectra are indeed very different from those of the usual HFB derivatives. We are therefore convinced that the similarity of the mass spectra of **4a–7a** to those of **1a–3a** proves clearly enough the rearrangement structure so that no additional confirmation of the structures **4a–7a** by ^{13}C-NMR is necessary.

We conclude that the 17,17-dialkyl-18-nor-13(14)-ene substructure gives characteristic ^{13}C-NMR and mass

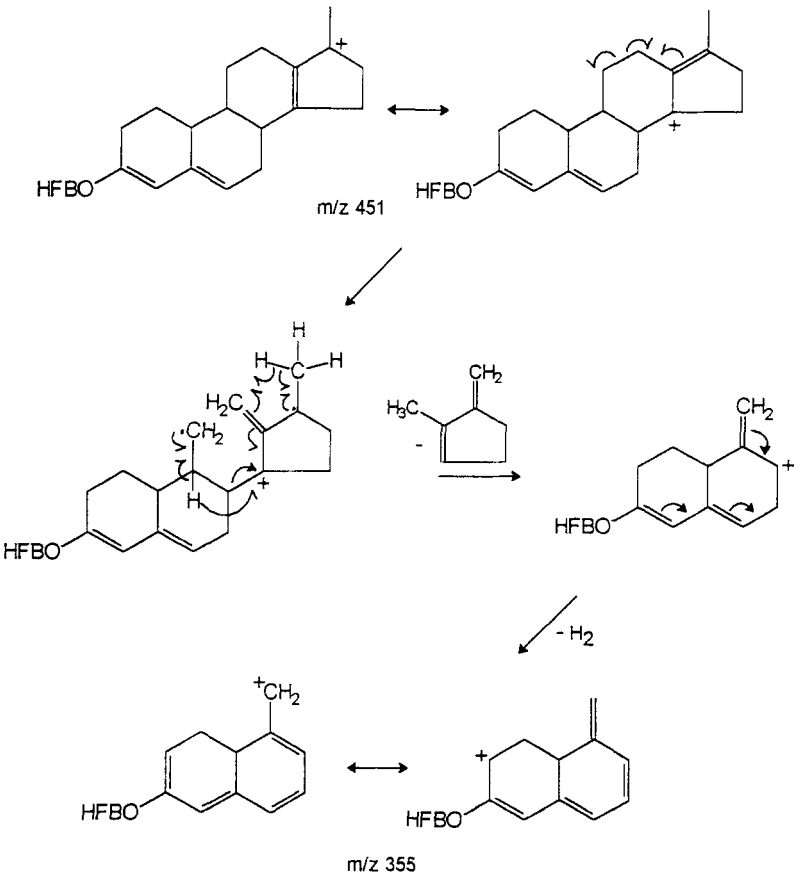


Fig. 10. Possible fragmentation pathway that leads to the A/B-ring fragment, here presented for **3a.**

spectra, and can thus easily be recognized when it occurs in a HFB-derivatization product of a 17-hydroxy-steroid. This is important because the rearrangement during the derivatization reaction implicates that 17-epimeric compounds and 18-nor-13(14)-ene steroids, which are metabolites of anabolic steroids (10–12, 23), will lead to identical derivatives (2). Therefore this perfluoroacylation reaction can only be used for the analysis of 17-hydroxy-steroids when no such metabolites are present.

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