Novel Perfluoroacyl Derivatives of Corticosteroids and Related Substances for Potential Use in Quantitative Gas Chromatography Mass Spectrometry

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We investigated the potential of perfluoroacylation for gas chromatographic mass spectrometric determination of corticosteroids and related substances. Structure elucidation of the reaction products was performed with high and low resolution mass spectrometry and with proton and carbon nuclear magnetic resonance spectrometry. Besides the well known 3-enol ester formation, 17 β -methyl-18-nor-13(14)-ene steroids were formed via loss of the 17- α hydroxyl group followed by a Wagner-Meerwein rearrangement. Compounds that bear an 11 β -hydroxy group formed additionally a 9(11) double bond when acetone was used as solvent, whereas acetonitrile or cyclohexane led to formation of 11 β -perfluoroacyl esters. In particular, perfluoroacylation of cortisol led to a clearly defined product instead of complex mixtures observed before, which thus makes it a valuable alternative to methoxime formation-silylation of cortisol for quantitative gas chromatographic mass spectrometric analyses. (*J Am Soc Mass Spectrom* 1995, 6, 264-276)

During the first gas chromatographic (GC) studies of free corticosteroids, a great variety of decomposition and rearrangement products was observed, in particular for corticosteroids that contain the dihydroxyacetone side chain. These substances predominantly underwent side chain cleavage with formation of the corresponding 17-keto androstanes [1]. Substances with the 17 α -hydroxy-17 β -acyl group underwent D-ring expansion to give D-homosteroids with a 17a-keto group [2]. In addition, by thermal or chemical rearrangement, isomeric D-homosteroids may be formed that bear the keto group on carbon 17 instead of 17a [3]. Furthermore, during GC, elimination of water can occur, and derivatization of the hydroxyl group sometimes even favors the formation of elimination products [4]. Because of these difficulties, a variety of derivatization procedures for corticosteroids was developed to obtain stable products for GC [5]. In addition, chemical degradation of the side chain prior to GC was performed [6]. Today, the

most generally used derivatives for corticosteroids and related substances are the alkyloxime trialkylsilyl derivatives [7, 8]. However, losses due to decomposition of these derivatives still may occur [9, 10] and the detection limits of approximately 200 pg reached with gas chromatography-mass spectrometry (GC/MS) [11-13] are considerably higher than those of other steroid families.

We therefore investigated perfluoroacylation as an alternative derivatization procedure, encouraged by our results with a heptafluorobutyryl derivative of aldosterone [14]. An advantage of perfluoroacylation is the simultaneous derivatization of hydroxyl functions and formation of enol esters with steroids that possess a 3-keto-4-ene substructure [15-17]. Despite the successful application of perfluoroacyl derivatives for derivatization of estrogen- and androgen-type steroids, their use for corticosteroids was very limited [18]. Sometimes it was noticed that attempts at perfluoroacylation gave complex reaction mixtures [19]. Recently, heptafluorobutyrylation of 17 α -hydroxyprogesterone was described, but left open the question about the structure of the product formed [20]. Heptafluorobutyrylation of cortisol was reported after transformation of the dihydroxyacetone side chain into the bis-methylenedioxy derivative [21]. To our knowl-

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edge, only one recent application of perfluoroacylation as the sole derivatization step for corticosteroids that bear the dihydroxyacetone side chain has been described [22]. However, because structure rationalization was only made from mass spectral data, erroneous conclusions about the structure of the obtained products were drawn, as shown by our results that follow.

Here, we describe the mechanism of perfluoroacylation of 17α -hydroxy-20-keto steroids in acetone, which involves the loss of the 17α -hydroxyl group followed by migration of the C-18 methyl group to carbon 17 through a Wagner–Meerwein rearrangement and formation of a 13(14) double bond [23–26]. Structures of the products were confirmed by proton and carbon nuclear magnetic resonance (^1H - and ^{13}C -NMR) spectrometry and by electron impact (EI) mass spectrometry combined with metastable and high resolution measurements. Furthermore, results for corticosteroids with an additional 11β -hydroxy group are presented. The latter substances also underwent the above-described rearrangement via either acetone, acetonitrile, or cyclohexane as the reaction medium. However, derivatization in acetone led additionally to elimination of the 11β -hydroxy group, whereas reaction in acetonitrile [22] and cyclohexane led to 11β -perfluoroacyl esters accompanied by other by-products.

Experimental

Standards and Reagents

Pregn-4-ene-3,20-dione (progesterone, I) was purchased from Sigma Chemical Co. (St. Louis, MO), pregna-4,9(11)-diene-3,20-dione (9(11)-dehydroprogesterone, II) from Steraloids (Wilton, NH), pregna-4,16-diene-3,20-dione (16-dehydroprogesterone, III), 11β -hydroxypregn-4-ene-3,20-dione (11β -hydroxyprogesterone, IV), 17α -hydroxypregn-4-ene-3,20-dione (17α -hydroxyprogesterone, V), 21-hydroxypregn-4-ene-3,20-dione (cortexone, VI), $11\beta,21$ -dihydroxy-

pregn-4-ene-3,20-dione (corticosterone, VII), $17\alpha,21$ -dihydroxypregn-4-ene-3,20-dione (cortexolone, VIII) from Aldrich Chemical Co. (Milwaukee, WI), and $11\beta,17\alpha,21$ -trihydroxypregn-4-ene-3,20-dione (cortisol, IX) Standard Reference Material No. 921 from the National Institute for Standards and Technology (Gaithersburg, MD). The structures of compounds (I)–(IX) are given in Figure 1. All standard solutions were prepared in absolute ethanol from E. Merck (Darmstadt, Germany) in a concentration of approximately 0.1 g/L. All solvents were of pro analysi grade, unless otherwise stated. Acetone was of extra pure quality and also from Merck. The heptafluorobutyric anhydride (HFBA) reagent used for derivatization was from Macherey & Nagel (Düren, Germany) and the pentafluoropropionic anhydride (PFPA) from Alltech (Deerfield, IL). Deuteriochloroform (CDCl_3) was obtained from Aldrich.

Spectrometric Analysis

^{13}C - and ^1H -NMR spectra were recorded by a Bruker AM-360 spectrometer (Karlsruhe, Germany) (^1H at 360 MHz; ^{13}C , proton decoupled, at 90 MHz). Spectra were recorded as 12–20-mM solutions in CDCl_3 at ambient temperature. Chemical shifts were expressed in parts per million relative to external tetramethylsilane. These measurements all were done at the Department of Organic Chemistry (director Professor Dr. F. Borremans) of the University of Ghent (Belgium).

Low resolution mass spectrometry was performed with a Finnigan MAT Incos-XL mass spectrometer (San José, CA), combined with a 5890 Series II GC from Hewlett Packard (HP, Palo Alto, CA) as inlet system. Operation of the GC/MS systems was controlled by a Data General computer system (Westboro, MA) and a 4-MB memory IBM[®] compatible PC workstation as system terminal. GC was done on a 15-m $\times$ 0.18-mm (i.d.) DB-1, that is, methylsilicone, fused silica column with 0.4- μm film thickness from J & W Scientific (Folsom, CA). The capillary 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 working conditions for GC analysis were: injection into the PTV in the "purge on" mode at 60 $^\circ\text{C}$; after 0.3 min the injector was switched to the "purge off" mode for 1 min and simultaneously heated to 280 $^\circ\text{C}$ at a rate of 12 $^\circ\text{C}/\text{s}$; after 1 min at 280 $^\circ\text{C}$, the PTV was cooled down to the starting temperature. The oven temperature was programmed from 190 (hold 1.25 min) to 280 $^\circ\text{C}$ at a rate of 20 $^\circ\text{C}/\text{min}$. The final temperature was held for 10 min. The transfer body and nozzle were set at 280 and 270 $^\circ\text{C}$, respectively. Mass spectrometry was performed under EI (70-eV) ionization, scanning from m/z 50 to 750 in 0.57 s.

For metastable and high resolution ($m/\Delta m = 10\,000$) mass spectrometry, a H-SQ 30 instrument (Fin-

Figure 1. Structures of compounds (I)–(IX).

nigan MAT, Bremen, Germany) was used. The H-SQ 30 is a hybrid instrument equipped with magnetic and electrostatic sectors and two quadrupoles. Metastable decompositions were detected in the "collision" quadrupole. No collision gas was used. The entrance energy was 50 eV, with a resolution of $m/\Delta m = 600$ for precursor scans and unit mass resolution for product scans. Measurements were done in the EI mode at 70 eV and with an accelerating voltage of 3 kV. The ion source temperature was 150 °C. Sample introduction was done via the direct inlet, heated to 80–90 °C. These measurements were performed at the Institute for Organic Chemistry II (director Professor Dr. H. Budzikiewicz), University of Cologne (Germany).

Derivatization Procedure with Perfluoroacyl Anhydrides for Gas Chromatography–Mass Spectrometry Analysis

For structure elucidation of the new products, approximately 5 µg of substance were derivatized, whereas 60 ng were used in preliminary investigations for the applicability of the method for quantification of cortisol in human serum. The appropriate volumes of ethanol standard solution (serum extracts) were evaporated in a 1.5-mL reaction vial at room temperature under nitrogen. The evaporation residues were redissolved in 30 µL acetone or acetonitrile and 30 µL of perfluoroacyl anhydride were added. After closing the vials, derivatization was performed for 1 h at room temperature. Then, the reaction mixtures were evaporated under nitrogen to dryness at room temperature, followed by an additional drying step at 70 °C for 20 s. The residues were redissolved in 100 µL of cyclohexane. Approximately 25–50 ng (2.4 ng for cortisol quantification) of derivatized substance were injected for GC/MS analysis.

Derivatization for NMR analysis was done with HFBA on approximately 3–5 mg of product. To keep the derivatization on the microgram scale as for GC/MS, the sample was dissolved in a maximum of 2 mL of chloroform and 10 portions of 200 µL were pipetted in individual 1.5-mL vials, evaporated, and treated for derivatization as previously described but with 150 µL acetone and 150 µL HFBA. After evaporation of the reaction mixtures to dryness, the residues were dissolved in approximately 70 µL of CDCl₃ and pooled in a NMR tube.

Hydrolysis of the heptafluorobutyl (HFB) derivative of (V) was performed by dissolving the derivative 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.

Derivatization in cyclohexane was performed in a mixture of 30 µL cyclohexane and 50 µL PFPA at 80 °C for 1 h. Evaporation of the reaction mixture and redissolution was performed as described in the foregoing text.

Results and Discussion

Our aim was to develop a one-step derivatization procedure for cortisol with perfluoroacyl anhydrides for potential use in quantitative GC/MS of serum samples. We found reaction conditions that yielded a clearly defined product instead of multiple products as observed before [19]. (Peak shape, detection limits, etc., are discussed in the section Potential Application of the Derivative for Quantification of Cortisol in Human Serum.) Because of the complex nature of cortisol, structure elucidation of the product formed required additional investigations on simpler model substances (see Table 1). (Further in the text, the derivatization products are designated with the extension "a".)

On the basis of our mass spectrometry, ¹H-, and ¹³C-NMR results, we propose the structure (IXa) for the new perfluoroacyl derivative of cortisol (Figure 2, HFB derivative). In the following text, the main elements of structure (IXa) will be rationalized on the basis of the results obtained for cortisol and the model substances. When not mentioned otherwise, derivatization refers to acetone–HFBA.

Proof of the 3,5-Diene-3-perfluoroacyl Ester Substructure in Compounds (Ia), (IIIa), and (Va)–(IXa)

The olefinic resonances at 5.90 (±0.03) (C-4) and 5.54 (±0.03) ppm (C-6) in the ¹H-NMR spectra of the derivatization products (Ia), (IIIa), and (Va)–(IXa) (Table 2) and the upfield shift of the C-19 methyl group [e.g., from 1.20 ppm in (I) to 1.00 ppm in (Ia)] are characteristic for the 3,5-diene-3-perfluoroacyl ester substructure as described before [17]. Perfluoroacylation of the 3-keto-4-ene group also is documented by comparison of the ¹³C-NMR spectra (Table 3) of progesterone (I) and 17α-hydroxyprogesterone (V) with their derivatization products (Ia) and (Va). To our knowledge, no ¹³C-NMR data for steroid enol esters have been published. Therefore, we assigned the signals by comparison with cholesta-3,5-diene [27] and

Table 1. Model compounds used for structure elucidation of the perfluoroacylation product of cortisol

Progesterone (I)
9(11)-Dehydroprogesterone (II)
16-Dehydroprogesterone (III)
11β-Hydroxyprogesterone (IV)
17α-Hydroxyprogesterone (V)
Cortexone (21-hydroxypregn-4-ene-3,20-dione) (VI)
Corticosterone (11β,21-dihydroxypregn-4-ene-3,20-dione) (VII)
Cortexolone (17α,21-dihydroxypregn-4-ene-3,20-dione) (VIII)
Cortisol (IX)

Figure 2. Derivatization products of 17 α -hydroxyprogesterone (Va) and cortisol (IXa) obtained after reaction with HFBA in acetone, and of the latter substance (IXb) after reaction with PFPA in cyclohexane.

data reported for enol acetates [28]. Most affected by the enol ester formation are the carbons C-2 to C-6, which resonate in cholesta-3,5-diene at 23.4, 129.9, 124.6, 141.7, and 123.2 ppm. The introduction of an ester group in this latter substance should cause a strong downfield shift of C-3 and an upfield shift of C-4, whereas C-2 should be nearly unaffected (cf. α -, β -, and β_{gem} -carbon in ref 28). Therefore, we attribute the signals in (Ia) and (Va) at approximately 24, 146, and 118 ppm to C-2, C-3, and C-4, from which it follows that the signals at approximately 138 and 126 ppm can be attributed to C-5 (higher substituted) and C-6. Another interesting feature is the high field shift of the carbons C-8 to C-10 induced by formation of the 3,5-diene structure (compare I/Ia and V/Va), which is similar in the cholestane/cholesta-3,5-diene pair [27]. The preceding findings contradict the assumption of 2,4-diene-3-ol ester formation during derivatization of cortisol with perfluoroacyl anhydrides [22].

Proof of the 9(11) Double Bond Formation upon Derivatization of 11 β -Hydroxyprogesterone (IV), Corticosterone (11 β ,21-dihydroxy-pregn-4-ene-3,20-dione) (VII), and Cortisol (IX)

First, the results for the simpler model compound 11 β -hydroxyprogesterone (IV) will be discussed. Derivatization of (IV) with HFBA led to the product (IVa) with a mass spectrum as shown in Fig. 3a. The highest ion is observed at m/z 508, which indicates heptafluorobutyrylation at C-3, the loss of water, and formation of a double bond. Derivatization of 9(11)-dehydroprogesterone (II) gave a product (IIa) with the same GC retention time and mass spectrum as (IVa) (see Figure 3b), which confirmed the identity of (IIa) and (IVa) and, therefore, the presence of the 9(11) double bond in (IVa).

For the corticosteroid family, no 9(11)-dehydro product was available for comparison. We, therefore, investigated the derivatization products of corticosterone (VII) and cortisol (IX), that is, (VIIa) and (IXa), with GC/MS and $^1\text{H-NMR}$ spectrometry (chemical shifts; see Table 2). The highest ions in the mass spectra of (VIIa) (Figure 4) and (IXa) (Figure 5) are observed at m/z 720 and 718, respectively, which indicates the loss of one molecule of water for (VII) and two molecules of water for (IX) (see also Table 4). In the $^1\text{H-NMR}$ spectra of (VIIa) and (IXa), the 11 α proton at 4.43 and 4.50 ppm present in (VII) and (IX) disappeared and *only one additional vinylic proton* at 5.57 ppm is observed, which confirms the 9(11) double bond in (VIIa) and (IXa). In addition, the C-19 methyl group in (VIIa) and (IXa) is shifted downfield relative to (VIa) and (VIII), which is also in accordance with the presence of a 9(11) double bond [29]. The $^1\text{H-NMR}$ results prove that the loss of water has taken place during the derivatization and not during GC or by mass spectrometric fragmentation. These results contradict the assumption of 11(12) double bond formation during perfluoroacylation of 11 β -hydroxy steroids, as discussed by others [22]. A 9(11) double bond also might have been formed during perfluoroacylation of the bismethylenedioxy derivative of cortisol, because no molecular ion was observed in the mass spectrum of the derivatization product [21].

Proof of the Loss of Water from the 17 α -Hydroxy Compounds (V), (VIII), and (IX)

The mass spectrometry data of the derivatization products (Va) (Figure 6b), (VIIIa) (spectrum not shown), and (IXa) (Figure 5) indicate that all substances that bear a 17 α -hydroxyl (17 α -OH) group underwent the loss of water during perfluoroacylation in acetone (see also Table 4).

The $^1\text{H-NMR}$ data support the loss of the 17 α -OH group, because the signals for the C-16 β protons in (V), (VIII), and (IX) at 2.69, 2.69, and 2.73 ppm disap-

Table 2. Characteristic ^1H -NMR chemical shifts of the substances (I), (III), and (V) to (IX) and their perfluoroacyl derivatives (Ia), (IIIa), and (Va) to (IXa)

Compound	C-4	C-6	C-11 α	C-16 β	C-18 CH ₃	C-19 CH ₃	C-21 CH ₃	C-21 CH ₂	
(I)	5.73	—	—	2.19	0.67	1.20	2.13	—	—
(Ia)	5.87	5.52	—	—	0.67	1.00	2.16	—	—
(III)	5.73	—	—	6.70 ^a	0.95	1.24	2.25	—	—
(IIIa)	5.88	5.51	—	6.78 ^a	0.94	1.04	2.29	—	—
(V)	5.74	—	—	2.69	0.77	1.19	2.28	—	—
(Va)	5.92	5.57	—	—	1.17 ^b	0.99	2.07	—	—
(Vb)	5.75	—	—	—	1.17 ^b	1.17	2.08	—	—
(VI)	5.72	—	—	2.21	0.70	1.19	—	4.17	4.23
(VIa)	5.87	5.51	—	—	0.70	0.99	—	4.81	4.98
(VII)	5.69	—	4.43	—	0.93	1.45	—	4.16	4.23
(VIIa)	5.92	5.52	5.57 ^a	—	0.68	1.17	—	4.83	4.98
(VIII)	5.73	—	—	2.69	0.72	1.19	—	4.31	4.66
(VIIIa)	5.92	5.57	—	—	1.25 ^b	1.00	—	4.95	5.04
(IX)	5.68	—	4.50	2.73	0.96	1.45	—	4.31	4.65
(IXa)	5.93	5.57	5.57 ^a	—	1.28 ^b	1.25	—	4.90	5.05

^a Olefinic protons.^b New 17 β -Methyl group.

peared in (Va), (VIIIa), and (IXa). [The signals for the C-16 β protons in 17-unsubstituted pregnanes appear at approximately 2.2 ppm; therefore, we could not localize them in the normal ^1H -NMR spectra of (Va), (VIIIa), and (IXa).] The absence of the 17 α -OH group is

further supported by comparison of the chemical shifts for the C-21 methylene protons in compounds (VI) to (IX) and their derivatives (Table 2). In the underivatized products, the introduction of a 17 α -OH group increases the *difference in the chemical shift* of the two

Table 3. ^{13}C -NMR data for progesterone (I), HFB derivatization product of progesterone (Ia), 17 α -hydroxyprogesterone (V), 17 α -hydroxyprogesterone derivatization product (Va), hydrolyzed 17 α -hydroxyprogesterone derivatization product (Vb), and Wagner–Meerwein rearrangement product of 17 α -methyltestosterone (X) [42]

Carbon no.	(I)	(Ia)	(V)	(Va)	(Vb)	(X) ^a
1	35.7	33.5 ^b	35.7	33.3 ^b	35.3	35.4
2	33.9	23.9	33.9	24.0	33.6	33.8
3	199.0	146.0	199.1	146.3	199.8	199.2
4	123.8	118.1	123.8	118.0	124.3	124.1
5	170.6	138.2	170.7	138.7	170.9	171.2
6	32.7	126.1	32.8	125.9	33.1	33.3
7	31.9	31.5 ^b	32.0	31.0	31.4	31.1
8	35.5	31.6 ^b	35.5	32.9 ^b	36.7	36.5
9	53.6	47.6	53.3	46.2	51.3	51.6
10	38.5	34.8 ^b	38.5	35.2	38.5	38.5
11	21.0	21.1	20.5	22.7 ^b	23.1 ^b	22.5
12	38.6	38.6	30.1	22.8 ^b	22.4 ^b	22.1
13	43.8	44.1	48.1	141.1	140.7	141.9
14	55.9	56.8	49.9	137.5	137.7	134.6
15	24.3	24.3	23.9	31.3	30.8	29.7
16	22.8	22.8	33.5	35.1	35.0	39.4
17	63.4	63.6	89.7	63.1	63.0	45.3
18	13.3	13.2	15.4	—	—	—
19	17.3	18.6	17.4	18.1	16.8	16.9
20	208.8	210.4	211.1	212.8	212.1	26.6
21	31.4	31.3 ^b	27.8	25.2	25.1	—
17 β -CH ₃	—	—	—	20.9	20.9	26.3

^a Reference 42.^b Assignments may be interchanged.

Figure 3. Low resolution EI mass spectra of the HFB derivative (in acetone) of (a) 11β-hydroxyprogesterone (IVa) and of (b) 9(11)-dehydropregesterone (IIa).

Figure 4. Low resolution EI mass spectrum of the HFB derivative of corticosterone (VIIa).

geminal C-21 protons from approximately 0.06 ppm to 0.34 ppm (compare VI and VII with VIII and IX). Derivatization of products without the 17 α -OH group [(VI) and (VII)] increased the difference slightly to approximately 0.16 ppm. A similar difference (0.09 and 0.15 ppm) is also observed for the derivatives of the compounds with a 17 α -OH group [(VIII) and (IX)], which indicates the absence of the 17 α -OH group in the derivatives (VIIIa) and (IXa). In addition, the *absolute chemical shift* of the geminal C-21 protons is increased by approximately 0.15 and 0.43 ppm through the introduction of a 17 α -OH group (compare VI and VII with VIII and IX). However, compared to (VIa) and (VIIa), the chemical shift increased only by approximately 0.10 and 0.07 ppm in (VIIIa) and (IXa), which further indicates the loss of the 17 α -OH group. In addition, in the ^{13}C -NMR spectrum of (Va), the signal for C-17 is observed at 63.1 ppm (HFB derivative of progesterone: 63.6 ppm) instead of 89.7 ppm in (V), which also is in agreement with the loss of the 17 α -OH group. In summary, the mass spectrometry, ^1H -NMR, and ^{13}C -NMR data all are in accordance with the loss of the 17 α -OH group in (V), (VIII), and (IX) during derivatization.

On the other hand, perfluoroacylation of the hydroxyl group at C-21 in (VI) to (IX) has been achieved, because the ^1H -NMR signals for the C-21 geminal protons are shifted downfield in the derivatives and are similar to those reported for 21-acetates [30].

Proof of the 18-Nor-13(14)-ene Structure that Results from the Loss of the 17 α -OH Group Followed by a Wagner–Meerwein Rearrangement

Initially we assumed that the 17 α -OH loss would result in the formation of a 16 double bond [22], which would have led to (IIIa) upon derivatization of (V), but this was excluded by comparison of the GC/MS data of (IIIa) and (Va) (see Figure 6a and b). The products have different mass spectra and different GC retention times. The formation of a 16 double bond also is excluded by the ^1H -NMR spectra of (Va), (VIIIa), and (IXa), where the signal observed for the C-16 vinylic proton in (IIIa) at 6.78 ppm is absent. These results contradict the assumption of 16 double bond formation as reported recently [22]. Therefore, we searched for other possible structures.

Because 17 α -hydroxypregnanes quite easily yield rearranged products under acidic reaction conditions [3], we investigated their formation during the derivatization step. As a result of rearrangement, two different classes of D-ring homoannulated products (Figure 7, structures A and B) [3] have been described, as well as a Wagner–Meerwein rearranged product in which the C-18 methyl group is migrated to position 17 after loss of the 17 α -OH group (Fig. 2, structure IXa). Because the migration during Wagner–Meerwein rearrangement proceeds suprafacially, the original C-18 methyl group occupies the C-17 β position in the rear-

Figure 5. Low resolution EI mass spectrum of the HFB derivative of cortisol (IXa).

ranged products. This rearrangement quite commonly is observed for 17α -alkyl- 17β -hydroxy substituted androstanes [25, 31-33] and during various approaches for steroid synthesis and transformation [34-36]. For corticosteroids or related substances, Wagner-Meerwein rearrangements have been reported too [26, 37-39].

The formation of homoannulated products under the described reaction conditions can be excluded on the basis of the ^1H -NMR spectra of (Va), (VIIIa), and (IXa). No vinylic proton additional to these from the 3,5-diene substructure or the 9(11) double bond is observed and the C-17 side chain is preserved, as indicated by the ^1H chemical shift for the protons of the C-21 methyl group at 2.07 ppm in (Va). It should

be noted that the shifts for methyl protons for homoannulated products obtained from (V) are all below 1.5 ppm [40, 41]. This led us to the assumption that a Wagner-Meerwein rearrangement had occurred instead. The chemical shifts of the protons of methyl groups in the ^1H -NMR spectra are diagnostic for this process. For the hydrolyzed derivatization product of (V), designated (Vb), where the 3-keto-4-ene structure is restored, two overlapping methyl signals are observed at 1.17 ppm and one at 2.08 ppm; the latter is attributed to the C-21 group. The signal at 1.17 ppm results from the protons of the C-19 methyl group (compare with I, V, VI, and VIII) and from the migrated methyl group. This is in good agreement with the data reported for (Vb) obtained before by reaction of (VI) or (VI) acetate with concentrated sulfuric acid [38, 39]. The signals at 1.17, 1.25, and 1.28 ppm in the products (Va), (VIIIa), and (IXa) are attributed to the protons of the C-17 β methyl group.

Comparison of the ^{13}C -NMR spectra of (Ia) and (Va) allowed us to assign the signals at 137.5 and 141.1 ppm to the newly formed double bond in (Va), which is most probably located between C-13 (141.1 ppm) and C-14 (137.5 ppm). Similar values have been reported for 13(14) double bonds in Wagner-Meerwein rearranged androstane-type steroids [42]. The C-18 methyl rearrangement is indicated further by the absence of a signal below 16 ppm. The signal at 20.9 ppm in (Va) and (Vb) is attributed to the new 17 β -methyl group,

Table 4. Hypothetical molecular ions and highest ions observed in the mass spectra of (IVa)-(IXa) and therefrom calculated numbers of water lost during derivatization with HFBA-acetone

Compound	Hypothetical molecular ion	Highest ion observed	x times loss of water
(IVa)	722	508	1
(Va)	722	508	1
(VIa)	722	722	—
(VIIa)	934	720	1
(VIIIa)	934	720	1
(IXa)	1146	718	2

Figure 6. Low resolution EI mass spectra of the HFB derivative of (a) 16-dehydropregesterone (IIIa) and of (b) 17α-hydroxyprogesterone (Va).

Figure 7. Possible partial structures of D-ring homoannulated steroids obtainable from 17 α -hydroxyprogesterone (V).

because the signals at 25.2 and 25.1 ppm (Va/Vb), due to their low intensity, could be attributed to carbon C-21. A similarly low intensity for C-21 is observed in (I) and (Ia).

Mass Spectral Features of the Rearranged Compounds (Va), (Vb), (VIIIa), and (IXa)

All rearranged compounds showed a small molecular ion and exhibited a strong fragment due to the loss of the original C-17 side chain (see also Figures 5 and 6b; spectra of Vb and VIIIa are not shown), usually considered to be characteristic for a double bond in position 13(14) [36]. On the other hand, we believe that the presence of a tertiary carbon at C-17 is the primary reason for the easy loss of the side chain, because 13(14) unsaturated and saturated products gave nearly identical mass spectra [35] and the mass spectrum of 3 β -hydroxy-18-nor-pregn-5,13(14)-diene-20-one exhib-

ited an intense molecular ion, as recently reported [43]. Therefore, the pronounced C-17 side chain loss seems to be more characteristic for the presence of an additional C-17 methyl group than for the 13(14) double bond. In this view, the mass spectrum alone would not have been a secure indicator for the double bond position. On the contrary, it was only the mass spectrum that made the double bond position questionable because of the observation of an intense fragment at m/z 369 for (Va) and (VIIIa). This could correspond to an A/B-ring fragment that originates from rupture of the C-11/C-12 and C-8/C-14 bonds with transfer of one hydrogen but with an additional (compared to the proposed structure) double bond in ring A or B. In the proposed structure, with the 13(14) double bond, a total of three hydrogens have to be lost to obtain m/z 369. We therefore investigated this unique fragmentation in more detail. First, as in the pentafluoropropionyl ester of (V) the fragment was shifted to m/z 319. Because (Va) and (VIIIa) subsequently showed the fragment at the same mass-to-charge ratio value, the origin from ring A/B could be confirmed. Metastable measurements (precursor and product ion scans) revealed that m/z 369 in (Va) originates from the ion at m/z 465 by loss of 96 u, which corresponds to C_7H_{12} as confirmed by high resolution measurements of both ions (calculated 465.1665, found 465.1641; calculated 369.0726, found 369.0738). Because the corresponding fragment is not present in (IXa), we assume that an

Figure 8. Low resolution EI mass spectrum of the PFP derivative (in cyclohexane) of cortisol (IXb).

allylic fission between C-11 and C-12 with hydrogen transfer initiates its formation. The C-8/C-14 fission could then proceed after double bond isomerization or might be accompanied by a fast H_2 loss (no evidence was found for metastable decomposition involving H_2 loss), which leads to the ion at m/z 369. Loss of H_2 that leads to resonance-stabilized A/B-ring fragments has been reported for other 3,5-diene-3-perfluoroacyl esters to occur presumably from C-1/C-2 [44]. In the hydrolyzed product (Vb), which contains the 3-keto-4-ene moiety, the corresponding fragment (m/z 173) is of only minor importance and is accompanied by m/z 175, which indicates that the 3,5-diene structure in (Va) and related compounds might be the major driving force for its origin and that indeed H_2 loss is involved in its formation.

Products Formed During Perfluoroacylation of Cortisol (IX) in Cyclohexane

By perfluoroacylation of (IX) in cyclohexane, we achieved derivatization of the 11β -hydroxyl group that led to compound (IXb) (see Figure 2). However, forma-

tion of (IXa) still occurred; it was the main product with HFBA, but only a minor product with PFPA. Formation of the 11β and not the 17α derivative was confirmed by the investigation of $9,11,12,12\text{-}^2H_4$ cortisol with a molecular mass increment of 4 u. This is in accordance with results obtained by derivatization of cortisol in acetonitrile [22]. We assume that Wagner–Meerwein rearrangement had occurred as with derivatization in acetone because of the pronounced C-17 side chain loss (m/z 577/677, PFP/HFB-ester) observed in the mass spectrum of (IXb) (PFP-ester; Figure 8). The base peak at m/z 413 (463 for HFB-ester) is formed by additional loss of the respective perfluorinated acid. Products (IXa) and (IXb) also were obtained by using acetonitrile. We therefore conclude that perfluoroacylation of cortisol generally is accompanied by Wagner–Meerwein rearrangement and not by formation of a 16 double bond as described recently [22], independent of the solvent used for derivatization.

It is worth noting that derivatization in cyclohexane led additionally to a product with the highest ion in the mass spectrum at m/z 618/718 (PFP/HFB), which

Figure 9. Total ion chromatogram (mass range m/z 50–750) of cortisol derivatized with HFBA in acetone at room temperature. The derivative (IXa) eluted after 8:02 min. For GC/MS conditions, see Experimental.

had a relative intensity of approximately 50% (mass spectrum not presented). We assume that this product indeed contains a 16 double bond, formed by elimination during injection or gas chromatography [4], supported by the generation of a "foot" in the peak front when the final temperature of the injection system was decreased.

Potential Application of the Derivative for Quantification of Cortisol in Human Serum

Because the new derivative ultimately is intended for quantification of cortisol in human serum by isotope dilution (ID) GC/MS, some characteristics relevant for this application will be addressed shortly. (A full description of this application will be the subject of a forthcoming paper.) First it should be stressed that derivatization was nearly quantitative (compound IXa comprised over 95% of the reaction products; see Figure 9). Furthermore, peak shape was symmetrical (see Figure 10) and elution temperature was moderate (280 °C). The derivative, dissolved in cyclohexane, was stable for several days at room temperature. Quantification by ID-GC/MS was done under selected ion mon-

itoring conditions by using the ion m/z 489, which reached a detection limit of 30 pg (signal-to-noise ratio = 5, determined with a diluted serum extract). Figure 10 represents a typical ion chromatogram of a processed serum sample (1 mL was used, containing approximately 60 ng of cortisol; 2.4 ng were injected). It can be seen that a very good signal-to-noise ratio and separation from other serum constituents is obtained, which allows precise and accurate quantification. Preliminary results on derivatization of other cortisol-like substances (e.g., 6 β -hydroxycortisol, cortisone, 20 α -dihydrocortisol, etc.) under the conditions described herein showed that the reaction is not applicable generally. In particular, cortisone-type compounds and substances with additional (to cortisol) hydroxyl groups tended to form multiple reaction products. However, a detailed description of those results will be given elsewhere.

Conclusion

We were successful in developing a one-step derivatization procedure for cortisol and related compounds by using a reaction with perfluoroacyl anhydrides in

Figure 10. Ion chromatogram at m/z 489 of cortisol-HFB (IXa) (2.4 ng were injected; selected ion monitoring conditions were used). A serum sample with a cortisol concentration of 60 ng/mL was analyzed.

acetone. The derivatization was easy to reproduce and led to stable products with good GC properties. Preliminary experiments show that this procedure might be very useful for quantitative GC/MS.

Note Added in Proof. After the original submission of this manuscript, work from another laboratory came to our attention that describes dehydration of the 11-hydroxyl group of cortisol during reaction of the bis-methylenedioxy-derivative with HFBA in acetone [45].

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