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SYNTHESIS OF DEUTERIUM-LABELLED 17α-HYDROXYPREGNENOLONE FOR USE AS INTERNAL STANDARD IN STABLE ISOTOPE DILUTION/MASS SPECTROMETRY

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Summary

Using a 4-step scheme, we have synthesized deuterium-labelled 17α-hydroxypregnenolone for use as internal standard for an isotope dilution/mass spectrometry plasma assay. 17α-Hydroxyprogesterone served as starting material. Its 17α-hydroxygroup was protected by tetrahydropyranylation. Introduction of deuterium by base-catalyzed enolization and protection of the 3-hydroxygroup by acetate formation yielded [2,2,4,21,21,21- $^{2}H_{6}$]3ß,17α-dihydroxypregna-3,5-diene-20-one 3-acetate 17-tetrahydro-pyranyl ether. After reductive deuteration of the 3-ene and removal of the protecting groups [2,2,4,4,21,21,21 - $^{2}H_{7}$] 17α-hydroxypregnenolone was obtained.

Keywords: synthesis, steroid, deuterium, 17α-hydroxypregnenolone, gas chromatography, mass spectrometry

Introduction

Nonclassic 3β-hydroxysteroid dehydrogenase deficiency, an adrenal enzyme defect, is an important cause of female hyperandrogenism, a frequent disorder in women [1]. Its diagnosis is solely based on hormonal analysis, because molecular genetic studies have shown no abnormalities [2]. The plasma steroid 17α-hydroxypregnenolone (3ß,17α-dihydroxy-pregn-5-en-20-one) represents the marker hormone of this enzyme deficiency. So far, the determination of 17α-hydroxypregnenolone has been

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exclusively based on immunoassays. However, their reliability can suffer severely from phenomena like cross reactivity or matrix effects [3]. Isotope dilution/mass spectrometry (ID/MS) represents the most specific method for the determination of steroid hormones [4]. In our attempt to develop an ID/MS assay for the determination of 17α-hydroxypregnenolone, we decided to synthesize a deuterium labelled analog in a 4-step scheme (Figure 1), because a suitable stable isotope labelled standard was commercially unavailable, and no synthetic scheme has been described so far [5].

Experimental

17α-Hydroxyprogesterone was obtained from Makor Chemicals. Methylene chloride (Merck) was treated with sicapent (Merck) and distilled prior to use. Methanol (MeOH; Merck), acetonitril (AcCN; Riedel de Haen) and water (Fluka) used for HPLC separation were of gradient grade for chromatography. For gas chromatography/mass spectrometry (GC/MS), steroids were derivatized with methoxyamin hydrochloride (Eastman Kodak) and trimethylsilylimidazol (TMS; Pierce) [6], or heptafluorobutyric anhydride (Pierce) [3]. All other chemicals and reagents were of analytical reagent grade and purchased from Aldrich or Fluka. They were used without further purification.

Mass spectra (EI 70 eV) were recorded on a Finnigan SSQ 7000 mass spectrometer (direct inlet) and a GC/MS unit (Dani 6500 GC directly interfaced to a Hewlett Packard 5970B mass selective detector). Infrared spectra were recorded on a Perkin-Elmer 883 IR-spectrometer using KBr pellets. HPLC analyses were carried out on a Supelcosil PLC-18 column (250 x 21,2 mm, 18μm, Supelco) using a Sykam S 1021 pump and a Gynkotek UV detector. Analytical HPLC separation was performed on a Nucleosil 300-C_{18} column (250 x 8 mm, 4μm, Macherey-Nagel, Germany). ^{1}H-NMR spectra were determined on a Bruker AMX-500 500 MHz spectrometer with tetramethylsilane as internal standard in pyridine-d_5.

17α-Hydroxypregn-4-ene-3,20-dione 17-tetrahydropyranyl ether (**2**). A solution of 17α-hydroxyprogesterone (**1**; 1.0 g, 3.0 mmol) and dihydropyran (1.1 ml, 12.0 mmol) in dry methylene

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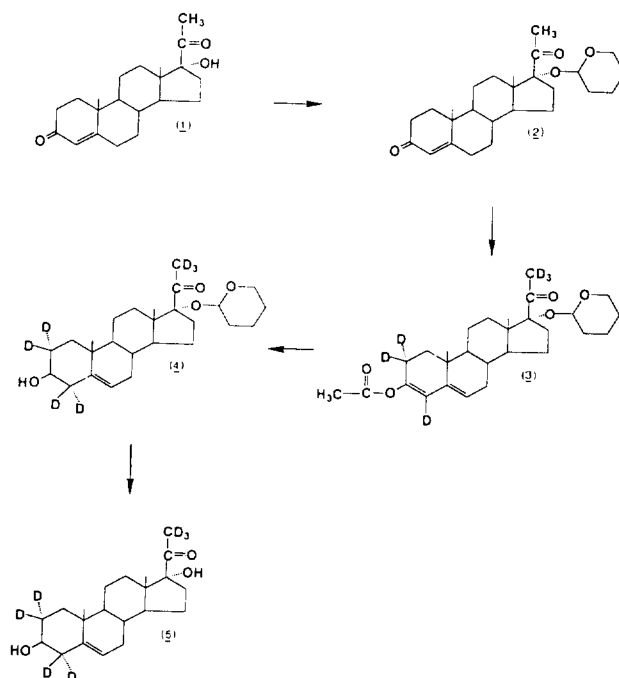

Figure1: Synthetic procedure

chloride containing pyridinium p-toluenesulfonate (PPTS; 0.3 g, 1.2 mmol) was stirred at room temperature for 25 h. Crystalline PPTS was prepared from pyridine and p-toluenesulfonic acid [7]. The reaction mixture was diluted with ether (80 ml) and washed with half-saturated brine (50 ml) to remove the catalyst. The organic phase was dried over Na_2SO_4 and evaporated to dryness. Purification was done by a preparative HPLC separation using $MeOH/H_2O$ (95.5 : 4.5) to give 168 mg of 2. IR (KBr): 2940, 2860, 1710, 1670,1610, 1030 cm^{-1}. MS: m/z 371 (M^+ - CH_3CO), m/z 287 (M^+ - CH_3CO - C_5H_8O, base peak), m/z 85 (C_5H_8O).

[2,2,4,21,21,21- $^2H_6$] 3,17α-Dihydroxypregna-3,5-diene-20-one 3-acetate 17-tetrahydropyranyl ether (3). A suspension of 2 (154.8 mg, 0.4 mmol) and potassium tert-butoxide (393.4 mg, 3.5 mmol) in tert-butanol-d_1 (5 ml) was stirred for 24 h under a nitrogen atmosphere. Then, acetic anhydride (0.8 ml) was added to the reaction mixture and stirring was continued for 1 h. The mixture

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was poured into ice-water and the aqueous solution was extracted with ether (100 ml). The etheral extract was washed twice with cold water, aqueous sodium bicarbonate and again with cold water. The organic phase was dried over Na_2SO_4 and evaporated to afford 3 as an oil which gave orange crystals. The crude product was chromatographed on a preparative HPLC column with MeOH/H_2O (95.5 : 4.5). 36 mg of 3 were obtained. IR (KBr): 2940-2870, 1750, 1700, 1220, 1030 cm $^{-1}$. MS: m/z 462 (M^+), m/z 420 (M^+ - CH_2CO), m/z 374 (M^+ - CH_2CO - CD_3CO), m/z 289 (M^+ - 173, D-ring fragmentation: C_{13}-C_{17}, C_{15}-C_{16}), m/z 85 (C_5H_8O, base peak).

[2,2,4,4,21,21,21- $^2H_7$] 3β,17α-Dihydroxypregn-5-ene-20-one 17-tetrahydropyranyl ether (4). Sodium borohydride (118 mg, 3.1 mmol) was placed in methanol-d_1 (10 ml) and deuterium oxide (1 ml). A solution of 3 (40 mg, 0.1 mmol) in methanol-d_1 (10 ml) was added dropwise to the suspension at 0°C with stirring. Afterwards the mixture was allowed to stand over night at room temperature. Concentrated hydrochloric acid was added dropwise to the reaction mixture until no more hydrogen was generated. To obtain complete separation of the layers, water (5 ml) was added to the mixture before the product was extracted repeatedly with ether (5 x 10 ml). The combined extracts were washed with water (2 x 10 ml) and dried over Na_2SO_4. Evaporation of the solvent gave 37 mg of a crude oily product. The product was not purified further. GC/MS (MO-TMS derivative): m/z 524 (M^+).

[2,2,4,4,21,21,21-$^2H_7$]3β,17α-Dihydroxypregn-5-ene-20-one (d_7 -17α-hydroxypregnenolone) (5). A solution of 4 (36.6 mg, 0.1 mmol) and PPTS (4.9 mg, 0.02 mmol) in ethanol (8 ml) was stirred at 55°C (bath temperature) for 3.5 h. After evaporation of the solvent, the product was purified by HPLC using a preparative C_{18}-column and MeOH/H_2O (85 : 15). A further analytical HPLC separation with AcCN/H_2O/trifluoroacetic anhydride (48:52:0.05) was required, to yield 1.2 mg of [2,2,4,4,21,21,21- $^2H_7$] 17α-hydroxypregnenolone (5) in high purity. MS: m/z 339 (M^+), m/z 293 (M^+ - CD_3CO), m/z 275 (M^+ - CD_3CO - H_2O), m/z 46 (CD_3CO, base peak).

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Results and Discussion

Compared with ^{13}C-labelling techniques, the incorporation of deuterium in the steroid molecule is generally less difficult. Regarding our synthesis, we aimed at introducing deuterium into chemically stable sites by exchange and reductive deuteration reactions. Furthermore, labelling of the steroid nucleus was required, because loss of the side chain occurs in case perfluoroacylation is used for derivatization in GC/MS.

Commercially available and cheap 17α-hydroxyprogesterone (1) served as starting material. First, its 17α-hydroxygroup was protected by an acid-catalyzed tetrahydropyranylation using the mild catalyst pyridinium p-toluenesulfonate (PPTS) in methylene chloride [7] to give 17α-hydroxypregn-4-ene-3,20-dione 17-tetrahydropyranyl ether (2) in 13.4% yield. Compared with other protecting groups, THP-ethers are more stable, particularly with respect to strong bases and metalorganic compounds [8]. In contrast to Miyashita et al. [7], who obtained excellent yields in steroids with secondary hydroxygroups, in our case the low yield is explained by the sterically hindered tertiary 17-hydroxyl function. Neither increasing the excess of dihydropyran nor prolonging the reaction time could improve the yield. Replacing PPTS by the stronger p-toluene sulfonic acid according to Dehennin [9] was less satisfactory because decomposition of the product occured.

Incorporation of deuterium was achieved using conditions similar to those by Ockels and Budzikiewicz for the synthesis of deuterated analogs of 3β-hydroxyandrost-5-ene [10, 11]. Using potassium tert-butoxide and tert-butanol-d_1, deuterium was introduced by a base-catalyzed enolization in positions 2, 2, 4, 21, 21, 21. Protection of the 3-hydroxygroup was achieved by acetate formation thus obtaining [2,2,4,21,21,21-$^2H_6$]3,17α-dihydroxypregna-3,5-diene-20-one 3-acetate 17-tetrahydropyranyl ether (3) in 20.9% yield. Reduction of the 3-ene was achieved by sodium borohydride reduction in methanol-d_1 and deuterium oxide [10]. Treatment with

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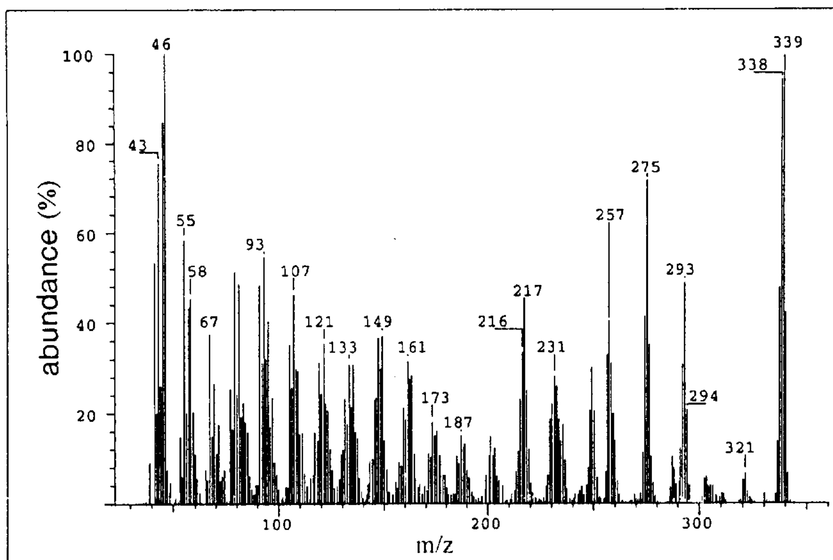

Figure 2: Mass spectrum (EI 70 eV; direct inlet) of native [2,2,4,4,21,21,21-$^{2}H_{7}$] 17α-hydroxypregnenolone.

concentrated hydrochloric acid led to the cleavage of the 3-acetate group yielding [2,2,4,4,21,21,21-$^{2}H_{7}$] 3ß,17α-dihydroxypregn-5-ene-20-one 17-tetrahydropyranyl ether (4).

In the final step, the protecting tetrahydropyranyl group was removed by an acid-catalyzed reaction in ethanol using PPTS [7] to give the end product [2,2,4,4,21,21,21-$^{2}H_{7}$] 17α-hydroxypregnenolone (5) in 4.1% yield. Figure 2 shows the mass spectrum (EI, direct inlet) of our labelled 17α-hydroxypregnenolone with sevenfold deuterated 17α-hydroxypregnenolone (m/z 339) representing the main product of our synthesis. The isotope composition after correction of natural abundances was: d_0: 0%, d_1: 0%, d_2: 0%, d_3: 0%, d_4: 3%, d_5: 15%, d_6: 34%, d_7: 38%, d_8: 10%.

NMR spectroscopy confirmed the labelled positions at C-2, C-4 and C-21. Due to the low amount of synthesized standard, only recording of a ^{1}H-NMR spectrum in pyridine-d_5 was possible. To get a

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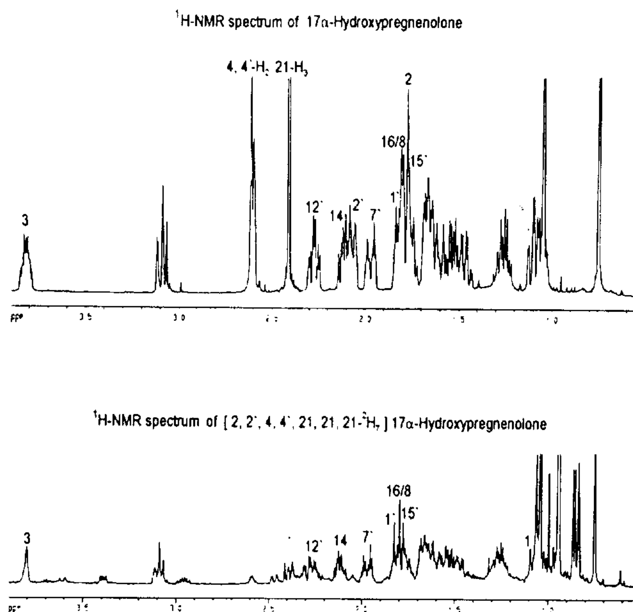

Figure 3: ^{1}H-NMR spectra of 17α-hydroxypregnenolone (above) and [2,2,4,4,21,21,21-$^2H_7$] 17α-hydroxypregnenolone (below) in pyridine-d_5

complete assignment for all protons and carbons of the steroid, the nondeuterated 17α-hydroxypregnenolone was recorded. By comparison of the ^{1}H-NMR spectra of the deuterated and nondeuterated compound (Figure 3), we could determine the positions of deuterium: the C-21 methyl signals (δ=2.41 ppm) and the proton resonance signals for H-4 and H-4` (δ=2.60/2.61 ppm) disappeared; the area of the overlapping signals from H-2`and H-14 (δ=2.02-2.18 ppm) decreased by twofold in the spectrum of the deuterated compound, because of the missing couplings from H-2`; the H-2 proton resonance signal (δ=1.76 ppm) disappeared, too; the multiplet proton signals from H-3 (δ=3.83 ppm) and H-1 (δ=1.11 ppm) of unlabelled 17α-hydroxypregnenolone became singlets in the labelled compound. The major difficulty in characterizing the ^{1}H-NMR spectrum was assigning the narrow band of signals (δ=1.72-1.85 ppm) from H-1`, H-2, H-8, H-15` and H-16. The assignments of chemical shifts for these protons, as well as for H-2` and H-14 (δ=2.02-2.18 ppm)

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were further complicated by overlapping multiplets in these signals. In order to establish the correct chemical shift values, two dimensional NMR techniques were used in addition to ^{1}H-, ^{13}C- and DEPT 135 spectra. The following experiments were performed: HMQC, HMBC and H,H Cosy. From the resulting data we obtained the chemical shifts of all protons and carbons.

To our knowledge, our procedure presents the first attempt to synthesizing stable isotope labelled 17α-hydroxypregnenolone, a diagnostically most important steroid hormone. We aimed at using reactions relatively simple to perform, and finally obtained an extremely pure deuterated 17α-hydroxypregnenolone with high isotopic content. No unlabelled 17α-hydroxypregnenolone was present. Although the overall yield of our synthesis was low, the synthesized amount will suffice by far, because only 1 ng of internal standard will be required per plasma sample.

We have succeeded in synthesizing deuterated 17α-hydroxypregnenolone labelled at the steroid nucleus and the side chain. The positions are chemically stable [9, 10]. We have already tested our product by determining its isotopic composition before and after plasma work up of our GC-MS plasma method [12]. Heptafluorobutyrate derivatives were prepared. Heptafluorobutyrate formation leads to cleavage of the 17-hydroxygroup and thus to loss of water [13]. The isotope composition was determined on the dominant ion of the spectrum representing the steroid nucleus after loss of the side chain (M-43). The isotopic distributions were practically identical before and after work up (*before:* m/z 467: 0%, m/z 468: 0,6%, m/z 469: 4,7%, m/z 470: 22,3%, m/z 471: 43,6%, 472: 23,1%, 473: 5,7%, m/z 474: 0%; *after:* m/z 467: 0,2%, m/z 468: 0,7%, m/z 469: 4,8%, m/z 470: 21,6%, m/z 471: 43,0%, 472: 23,3%, 473: 5,6%, m/z 474: 0,8%). This experiment proved the stability of the deuterium labels during our work up procedure and thus demonstrated the suitability of our compound as internal standard for ID/GC-MS analysis of 17α-hydroxypregnenolone.

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