SYNTHESIS OF TRIDEUTERATED TESTOSTERONE LABELED SELECTIVELY AT THE C-19 ANGULAR METHYL GROUP

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SUMMARY

A synthetic procedure for testosterone-19,19,19-d_3 is described. Deuterium labeling of the C-19 position was achieved by the Grignard reaction of 17β-acetoxy-5α,10α-epoxy-estrane-3-cycloethyleneketal with deuterium labeled methyl magnesium bromide (CD_3MgBr). The synthesis resulted in a product with 97.8% d_3 isotopic purity, which is selectively deuterated at C-19 and chemically pure.

Key Words: Testosterone-19,19,19-d_3, Deuterium, Mass fragmentography, Mass spectrum, IR spectrum, NMR spectrum.

INTRODUCTION

A gas chromatograph-mass spectrometer equiped with a multiple ion detector (GC-MS-MID) is being used increasingly in biological assays because of its high sensitivity and high specificity. In this technique carriers which have isotopes already built in the parent compound serve as the ideal internal standard to correct for losses of the compound under study in the initial isolation procedures. This type of internal standard is of particular importance when trace amounts of a substance are analyzed for in a biological sample.

In the study to synthesize deuterium labeled steroids for use in mass fragmentographic assays, special attention was paid to the selective deuterium labeling of the C-19 angular methyl group. The C-19 position was chosen not only because it offered the possibility of introducing three deuterium atoms

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but also because this position seemed not to suffer from the serious problems due to extensive isotope scrambling, loss of labeling atoms, and serious isotope effect. Therefore testosterone-19,19,19-d_3 (testosterone-19-d_3) was synthesized for use as internal standard.

EXPERIMENTAL

IR spectra were recorded with a Shimazu IR-400 spectrometer for KBr tablets. NMR spectra were determined with a JEOL JNM-4H-100 spectrometer for solutions in deuterochloroform with tetramethylsilane as internal standard. Mass spectra were recorded on a Shimazu LKB-9000 spectrometer. The mass spectrometer conditions were: separator temperature, 280°; ionization source temperature, 310°; and ionization energy, 20 eV. GLC was performed with a Shimazu GC-4BPTF. The GLC conditions were: column, 2 m in length and 3 mm i.d. glass column packed with 1.5% SE-30 on chromosorb W (60-80 mesh); injection temperature, 250°; column temperature, 250°; and nitrogen flow rate, 60 ml/min.

17β-Acetoxy-estr-5(10)-ene-3-cycloethyleneketal (3)

To a solution of 4.502 g (1.64 mmole) of 17β-hydroxy-estr-5(10)-en-3-one (1)[1] in 350 ml dry benzene in a 500 ml round flask were added 33 ml (0.59 mole) of ethylene glycol and 0.240 g (1.26 mmole) of p-toluene sulfonic acid. The mixture was then heated under reflux for 5 hr in a flask equipped with a Dean-Stark phase separator. The benzene layer and further benzene extracts were washed with 5% $NaHCO_3$ and H_2O, followed by drying over $MgSO_4$. Evaporation of the benzene furnished 4.768 g (1.50 mmole) of 17β-hydroxy-estr-5(10)-ene-3-cycloethyleneketal (2).

A mixture of 6.643 g (2.09 mmole) of the above product (2), 20 ml of dry pyridine and 10 ml (0.1 mole) of acetic anhydride was kept at room temperature for 4 hr, chloroform and H_2O was added, separated and the aqueous layer was re-extracted. After washing the combined extract with 5% $NaHCO_3$ and H_2O and drying over $MgSO_4$, the chloroform was removed to give rise to 6.939 g (1.93 mmole) of the 17β-acetate (3) which was recrystallized from MeOH-petroleum ether : NMR δ 0.83 (3H, s, 18-CH_3), 2.03 (3H, s, 17-O-CO-CH_3), and 3.94 (4H, s, 3-O-

CH_2-CH_2-O-); IR disappearance of ν_{O-H} 3420 cm^{-1} and appearance of $\nu_{C=O}$ 1740 cm^{-1}.

17β-Hydroxy-5α,10α-epoxy-estrane-3-cycloethyleneketal (5)

To a solution of 6.000 g (1.67 mmole) of 17β-acetate (3) in 90 ml of dimethylformamide and 25 ml of H_2O were added 5.280 g (3.0 mmole) of N-bromosuccinimide gradually and 0.73 g (1.8 mmole) of magnesium oxide. After stirring the reaction mixture (pale yellow suspension) for 2 hr at room temperature, 200 ml of H_2O was added and the precipitate was collected by filtration. The precipitate was then dissolved in dichloromethane to eliminate the excess magnesium oxide. The dichloromethane solution was washed with H_2O and dried over Na_2SO_4. The solvent was evaporated at 25° under reduced pressure, yielding 5.958 g (1.3 mmole) of the pale yellow crude 5α-hydroxy-10β-bromo-17β-acetoxy-estrane-3-cycloethyleneketal (4).

To a stirred suspension of 5.958 g of the above bromohydrin (4) in 60 ml of MeOH was added 8.6 ml of 28% MeONa in MeOH (Wako Pure Chemical Industries, Ltd.) dropwise and the reaction mixture was stirred at 5-10° for 8 hr under N_2 atomosphere. Dilution with H_2O, extraction with dichloromethane, washing with H_2O followed by drying over Na_2SO_4 and evaporation at 25° under reduced pressure gave 4.120 g of the pale yellow residue. After silica gel column chromatography of 3.400 g of the residue using benzene-AcOEt (1:1) as an eluting solvent, the purified product (5) (2.130 g; 0.63 mmole) was obtained as a glasslike solid following evaporation of the solvent under reduced pressure: NMR δ 0.72 (3H, s, 18-CH_3) and 3.88 (4H, s, 3-O-CH_2-CH_2-O-).

17β-Acetoxy-5α,10α-epoxy-estrane-3-cycloethyleneketal (6)

A solution of 1.583 g (0.47 mmole) of the above epoxide (5) in 7 ml of dry pyridine was treated with 3.5 ml (3.7 mmole) of acetic anhydride. After stirring at 5-10° for 5 hr, dichloromethane and H_2O were added, separated and the aqueous layer was re-extracted. After washing the combined extract with 3 N HCl, 5% $NaHCO_3$, and H_2O, the extract was dried over Na_2SO_4. Removal of the dichloromethane by evaporation at 25° under reduced pressure gave 1.379 g (0.36 mmole)

of the 17β-acetoxy epoxide (6): NMR δ 0.78 (3H, s, 18-CH_3), 1.99 (3H, s, 17-O-CO-CH_3), and 3.88 (4H, s, 3-O-CH_2-CH_2-O-); IR disappearance of ν_{O-H} 3450 cm^{-1} and appearance of $\nu_{C=O}$ 1730 cm^{-1}.

5α,17β-Dihydroxy-androstane-3-cycloethyleneketal-19,19,19-d_3 (7)

The deuterium labeled Grignard reagent (1.14 M of CD_3MgBr-tetrahydrofuran solution) was prepared by using methyl bromide-d_3 (CD_3Br, 99 atom% d, Merck). To 35 ml of the above Grignard reagent was added dropwise a solution of 1.000 g (0.26 mmole) of the epoxide (6) in 10 ml of tetrahydrofuran. The solution was refluxed for 2 hr. After cooling, 20 ml of 1.5 M ammonium chloride solution was added dropwise. Dilution with H_2O, extraction with dichloromethane, washing with 3 N HCl, 5% $NaHCO_3$, and H_2O, followed by drying over $MgSO_4$ and evaporation gave 0.821 g of crystalline residue. Purification of the residue by thin layer chromatography (Kieselgel $60F_{254}$ plates, 0.25 mm thickness, Merck) furnished 0.389 g (0.11 mmole) of the product (7) (R_f 0.34, benzene-AcOEt 1:1 as a developing solvent): IR ν_{O-H} 3480 cm^{-1} and ν_{C-D} 2225 cm^{-1}; NMR δ 0.70 (3H, s, 18-CH_3) and 3.94 (4H, s, 3-O-CH_2-CH_2-O-); MS m/e 353 (M^+) and m/e 99 (base peak).

Testosterone-19,19,19-d_3 (9)

A solution of 0.370 g (0.10 mmole) of the above 5α-hydroxy-3-cycloethylene-ketal (7) in 10 ml of MeOH was treated with 1 ml of 1 N H_2SO_4 under reflux for 30 min. After cooling, ether and H_2O were added, separated and the aqueous layer was re-extracted. The combined extract was washed with 5% $NaHCO_3$ and H_2O and dried over $MgSO_4$. Removal of the ether by evaporation resulted in 0.291 g (0.094 mmole) of 5α,17β-dihydroxy-androstan-3-one-19,19,19-d_3 (8) in colorless plates.

The above product (8) was dissolved in 10 ml of MeOH, treated with 0.5 ml of 5% methanolic KOH, heated under reflux for 1 hr, and cooled. After dilution with H_2O and extraction with ether, the combined ether solution was washed with 1 N HCl, 5% $NaHCO_3$, and H_2O, followed by drying over $MgSO_4$. The ether was then removed to give rise to 0.248 g of crystalline residue. The residue was subjected to thin layer chromatography (Kieselgel $60F_{254}$ plates, 0.25 mm

thickness, Merck) and the zone corresponding to testosterone (R_f 0.28, benzene-AcOEt 1:1 as a developing solvent) was scraped off from each plate. Extraction and recrystallization from n-hexane-acetone (7:1) led to 0.172 g (0.059 mmole) of colorless needle crystals of the final product (9): mp 151°; IR ν_{C-D} 2960 cm^{-1} and 2870 cm^{-1}; NMR δ 0.81 (3H, s, 18-CH_3), 3.64 (1H, t, 17-H), and 5.74 (1H, s, 4-H); MS m/e 291 (M^+); Anal. Calcd. for $C_{19}H_{25}D_3O_2$: C, 78.30; H, 9.68. Found: C, 78.33; H, 9.96.

DISCUSSION

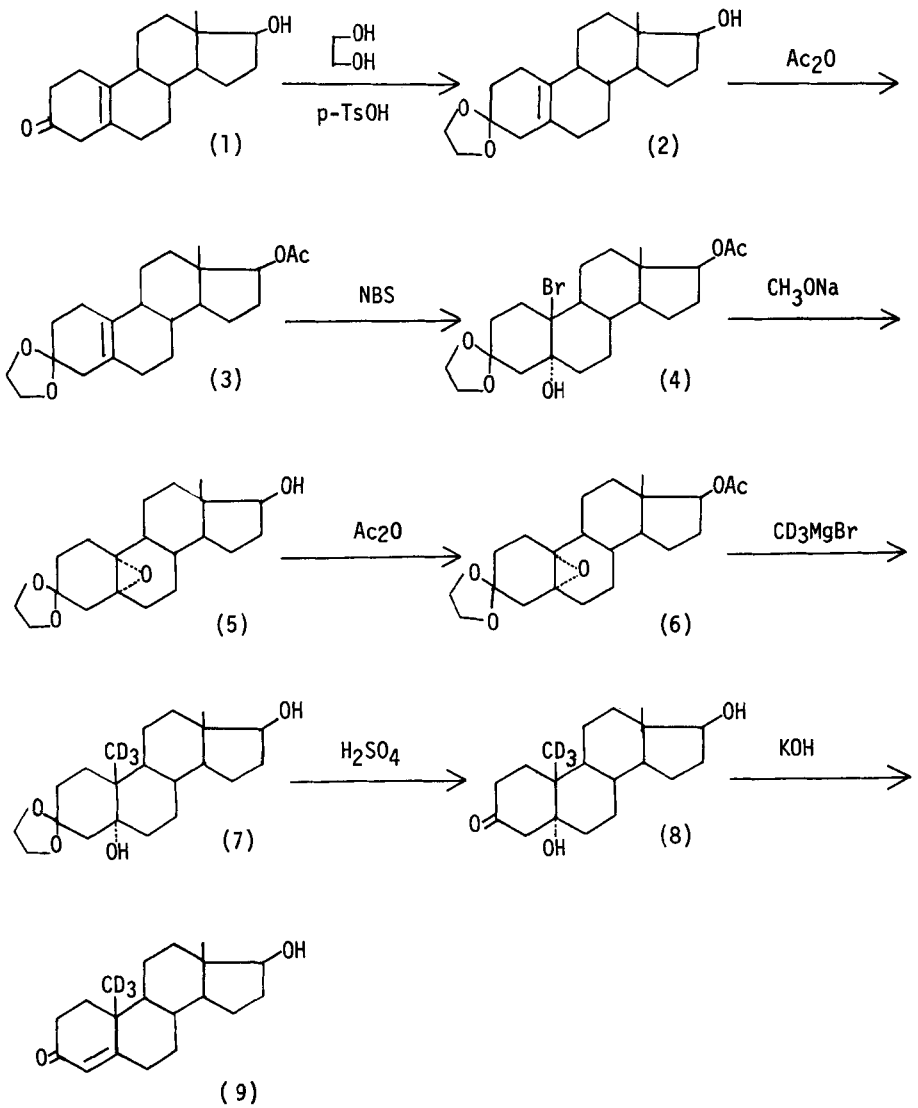
The choice of C-19 position for deuterium labeling was based primarily on a desire to introduce three or more deuterium atoms in the steroid molecule for the accurate, precise, and selective mass spectrometric analysis. Methods for the synthesis of deuterated steroids by catalytic reduction or exchange reaction did not seem promising for the purpose of obtaining the product of high isotopic purity.

The preparation of testosterone-19-d_3 was accomplished by the sequence shown in the scheme. Introduction of three deuterium atoms to the C-19 position of testosterone was achieved by treatment of the 19-nor-5α,10α-epoxy-androstane derivative (6) with methyl Grignard-d_3 followed by hydrolysis and dehydration of the 5α-hydroxy-androstane intermediate (7). The Grignard reaction of the 5α,10α-epoxide (6) with CD_3MgI provided the desired 19-d_3-5α-hydroxy intermediate (7) in very poor yield (~6%). The yield was, however, improved to be more than 40% when CD_3MgBr was used in place of CD_3MgI. This yield was comparable with that reported by Nebelec and Gasc[2] who carried out the above reaction with CH_3MgBr. As has been reported by these authors, the Grignard reaction of (6) in our experiment gave two major products, one of which was led to testosterone-19-d_3 by treatment with 1 N H_2SO_4 followed by 5% KOH and the other to estradiol by treatment with 1 N H_2SO_4.

Djerassi and Kielczewski[3] introduced three deuterium atoms into the C-19 angular methyl group in 5α-androstan-3-one by lithium aluminum deuteride reduction of 3β-acetoxy-androst-5-en-19-oic acid to give 19-d_2-19-ol followed by conversion to the 19-mesylate derivative, displacement with lithium bromide

to afford the 19-bromide intermediate, and reduction with lithium deuteride. This required a tedious sequence of reactions and the isotopic yield was not significantly high. Application of this method for testosterone-19-d_3 seemed, therefore, not to be appropriate.

Formation of the 5α,10α-epoxide (5) of a 5,10-unsaturated 19-nor-3-keto derivative (1) proceeded by way of the trans bromohydrin (4). Contrary to our expectation that alpha attack would be favored, treatment of the 5,10-unsaturated



Scheme

19-nor-steroid (2) with m-chloroperbenzoic acid did not furnish in good yield the desired 5α,10α-epoxide (5). This may be due to the predominant formation of 5β,10β-epoxide. According to Djerassi, et al.,[4] in the absence of the angular methyl group at C-10, the steric factors controlling approach of the reagent are pointed out to be rather subtle. These authors obtained the 5β,10β-epoxide in 65% yield on treatment of 19-nor-17β-hydroxy-androst-5(10)-en-3-one with monoperphthalic acid.

For precise evaluation of the labeling results the deuterium labeled testosterone had to be pure. Conventional purification procedures by thin layer chromatography and recrystallization proved to be quite effective for this purpose. The chemical purity was also confirmed by gas chromatography and the elemental analysis of the product.

The mass spectra of testosterone-19-d_3 and unlabeled reference testosterone

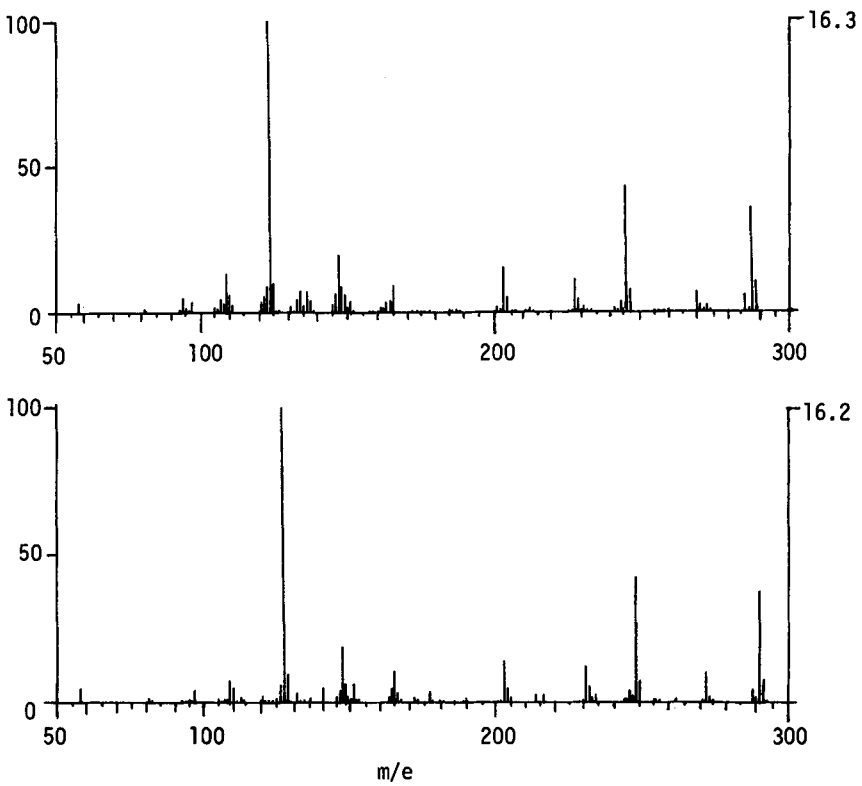


Fig. 1. Mass Spectra of Testosterone (Upper) and Testosterone-19,19,19-d_3 (Lower)

are given in Fig. 1. Mass spectrometric analysis of testosterone-19-d_3 obtained in this experiment demonstrated very high isotopic purity of the product (97.8% d_3, 99.0 atom% d).

In the NMR spectrum of unlabeled testosterone, the signal at 1.21 ppm is typical for the C-19 angular methyl group. The corresponding signal did of course disappear in the spectrum of testosterone-19-d_3.

As is evident from Fig. 2, the IR spectrum of testosterone-19-d_3 clearly shows C-D stretching vibration bands at 2230 cm^{-1} and 2070 cm^{-1}. In unlabeled

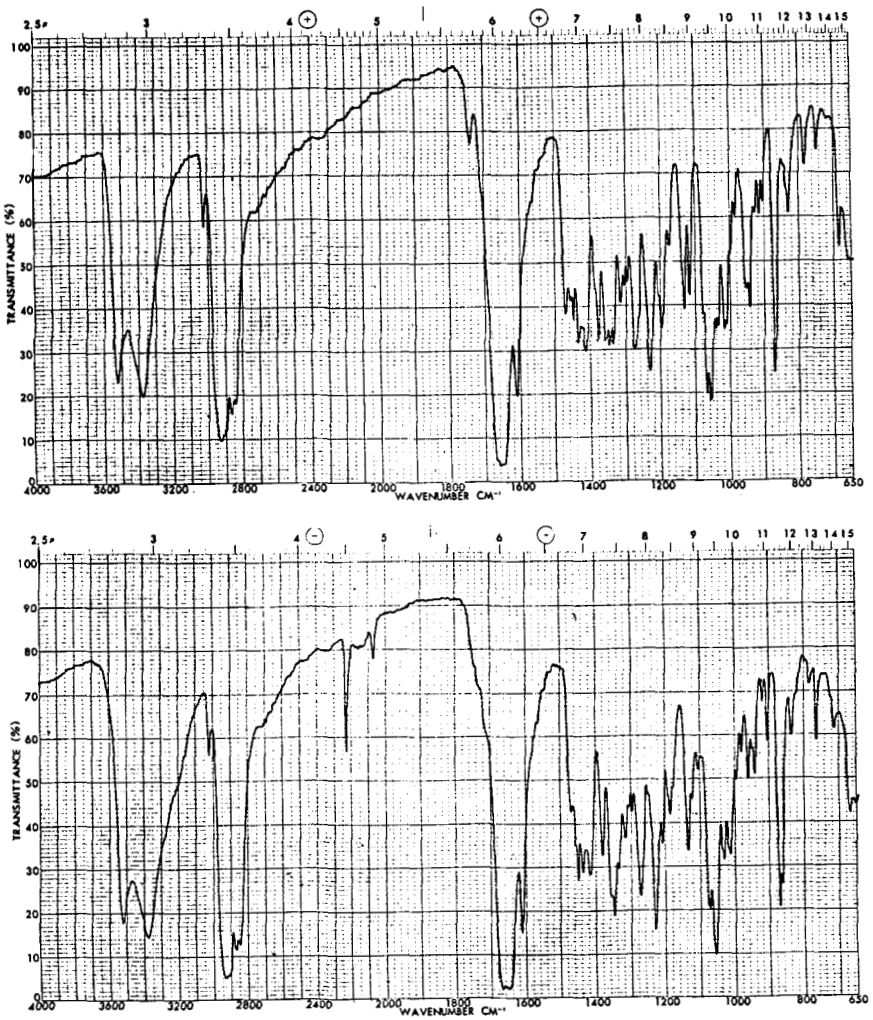


Fig. 2. IR Spectra of Testosterone (Upper) and Testosterone-19,19,19-d_3 (Lower)

testosterone there is a shouldered peak at 1388 cm^{-1} which disappears in the spectrum of testosterone-19-d_3. Introduction of three deuterium atoms into the C-19 position has made it possible to assign the band at 1388 cm^{-1} to a C-H bending vibration of the C-19 angular methyl group, which otherwise could not be appropriately assigned.[5),6)]

In mass spectrometry, an increase of three atomic mass units will enable us to decrease the blank value, leading to a significant increase in sensitivity when testosterone-19-d_3 is used as internal standard. The application of testosterone-19-d_3 obtained in this experiment for the determination of endogenous testosterone in biological fluids by mass fragmentography will be described in the following paper.

The method described in this paper for deuterium labeling of the C-19 position of testosterone will be also applicable for the selective deuterium labeling of the C-19 angular methyl group of other steroids, such as progesterones, corticosteroids, and cholesterol.

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