SYNTHESIS OF DEUTERIUM LABELLED C_{19}-Δ^{16} STEROIDS

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SUMMARY

Deuterium labelled forms of 5α-androst-16-en-3-one, 3α-hydroxy-5α-androst-16-ene, 3β-hydroxy-5α-androst-16-ene, 3β-hydroxy-5,16-androstadiene and 4,16-androstadien-3-one were prepared to serve as internal standards in quantitative analysis of C_{19}-Δ^{16} steroids by mass spectrometric reverse isotope dilution. In general, the methods used employed deuterium exchange of activated hydrogens adjacent to carbonyl carbons followed by reaction steps which isolated the newly incorporated deuterium. Removal of any remaining exchangeable deuteriums was the final step in each reaction sequence.

Key Words: Androst-16-enes, deuterium, sex odor in pigs.

INTRODUCTION

Gower (1) has reviewed the synthesis of C_{19}-Δ^{16} steroids (androst-16-enes) and discussed their possible physiological role. This group of steroids has been shown to be responsible for the undesirable, permeating "perspiration- or urine-like" sex odor emanating from the tissues of the uncastrated, sexually mature male pig upon heating (2,3,4). The deuterium labelled androst-16-enes were synthesized to permit analysis of tissue extracts by stable isotope dilution.

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Addition of the labelled compounds to pig tissues before analysis compensated for losses incurred during isolation and analysis (5).

MATERIALS

Steroids:

3β-hydroxy-5-androsten-17-one (compound V - Scheme II), obtained from Aldrich Chemical Company and Sigma Chemical Company, was the plentiful and inexpensive starting material for much of this work. Other steroids such as 4,16-androstadien-3-one (compound I - Scheme I) were donated by the Upjohn Company of Kalamazoo, Michigan.

Reagents:

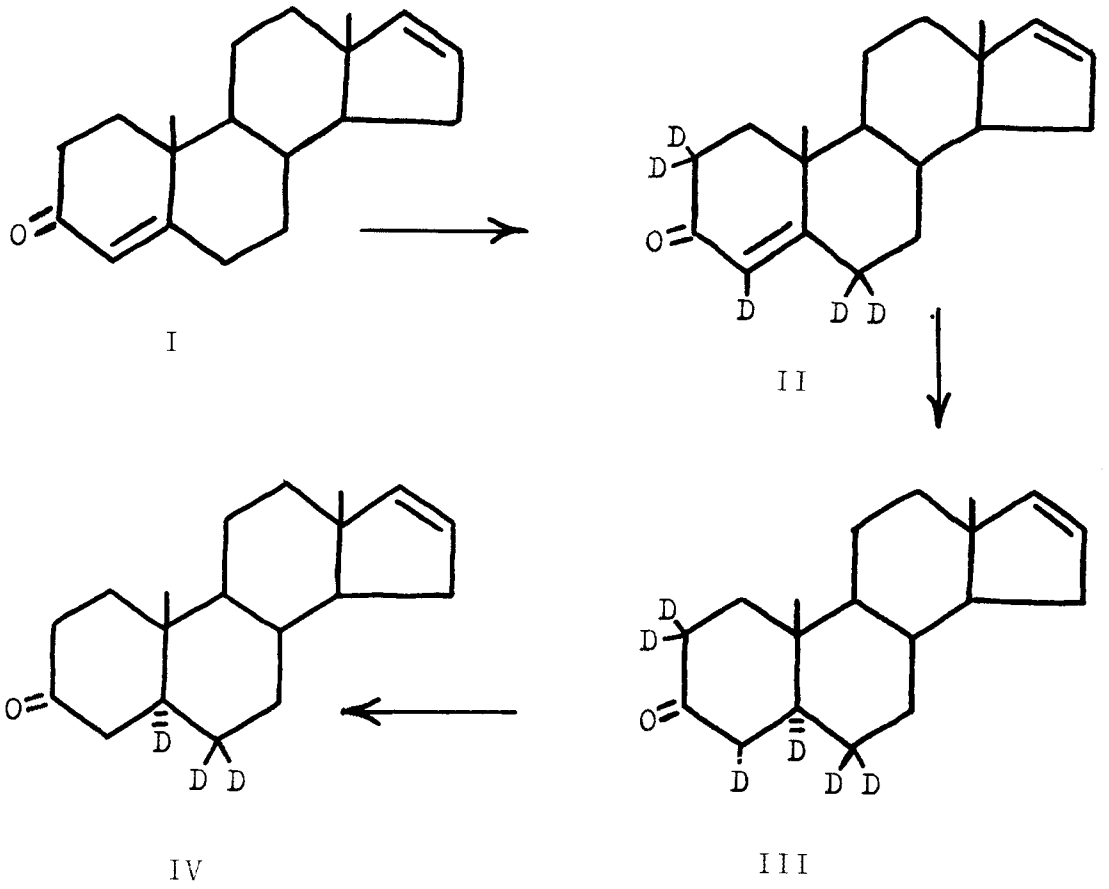
All solvents were analytical grade and were redistilled before use. Electronic grade aluminum isopropoxide, 2M methyllithium in diethyl ether, chloroiridic acid and lithium wire obtained from the Ventron Corporation, Alfa Products, Beverly, Massachusetts. The deuterioammonia (ammonia-d_3), deuterium oxide, lithium aluminum deuteride and deuteriomethanol (CH_3-OD) were obtained from Merck and Company, Inc.

METHODS

The procedures for synthesis and purification of the various C_{19}-Δ^{16} steroids are briefly explained (Schemes I and II). Yields and purity are given whenever available.

5,6,6'-$^2H_3$-5α-androst-16-en-3-one (IV)

The starting material was 4,16-androstadien-3-one (I). The activated hydrogens were exchanged for deuteriums by equilibrating in alkaline deuteriomethanol/deuterium oxide using an adaptation of a procedure described by Djerassi and Tökes (5) for deuteration of 5α-pregn-9-en-12-one. A solution



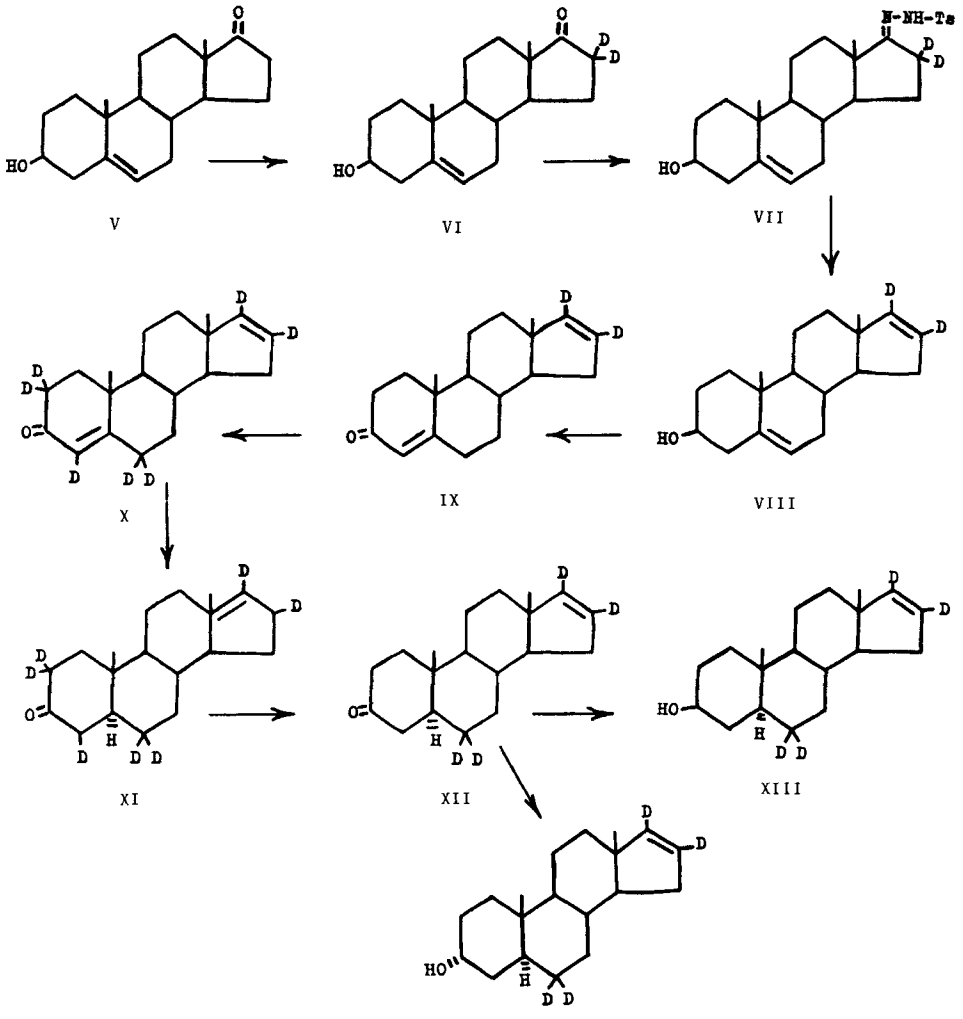
Scheme I. Steps involved in synthesis. Compounds are as follows:
I = 4,16-androstadien-3-one; II = 2,2',4,6,6'-$^{2}H_{5}$-4,16-androstadien-3-one;
III = 2,2',4,5,6,6'-$^{2}H_{6}$-5α-androst-16-en-3-one;and IV = 5,6,6'-$^{2}H_{3}$-5α-androst-16-en-3-one.

of 87 mg of compound I in 15 mL of deuteriomethanol was saturated with 20% sodium deuterioxide in deuterium oxide and refluxed for 3 days. Refluxing was begun before adding the 20% sodium deuterioxide solution to prevent degradation before degassing the solution. The 20% sodium deuterioxide solution was added dropwise after an hour of refluxing until the solution became saturated as indicated by turbidity. Whenever the solution became turbid due to supersaturation, a few drops of deuteriomethanol were added. Refluxing was carried out under a nitrogen atmosphere to protect from air and moisture. The solution was cooled with 10 mL of anhydrous ethyl ether and washed with two successive 5 mL volumes of deuterium oxide. The ether solution was then dried over anhydrous sodium sulfate. The ether was evaporated off using a rotary vacuum evaporator and the dry residue was held in a dessicator over P_2O_5.

The product was a mixture of 36 mg of 5,6,6'-$^2H_3$-5α-androst-16-en-3-one (IV) and of 36 mg of 2,2',4,5,6,6'-$^2H_6$-3β-hydroxy-5α-androst-16-ene. They were separated by preparative thin layer chromatography to give a final yield of 41% for the 3-keto form.

16,17-$^2H_2$-3β-hydroxy-5,16-androstadiene (VIII)

3β-hydroxy-5-androsten-17-one (V, 12.2g) was refluxed in 212 mL of deuteriomethanol for 16 days to exchange protons at the 16 position with deuterium, resulting in the 16,16'-$^2H_2$ analog (VI). This was deliberately done without a base catalyst to avoid interference of the base in subsequent steps. Then p-toluene-sulfonyl-hydrazine (10.2) was added to the solution and the tosylhydrazone derivative (VII) was formed during refluxing for an additional 16 hours in the deuteriomethanol solution. A stream of nitrogen was



Scheme II. Steps in synthesis of steroids. Compounds are identified as follows:
V = 3β-hydroxy-5-androsten-17-one;
VI = 16,16'-$^{2}H_{2}$-3β-hydroxy-5-androsten-17-one;
VII = tosylhydrazone derivative of VI;
VIII =16,17-$^{2}H_{2}$-3β-hydroxy-5,16-androstadiene;
IX = 16,17-$^{2}H_{2}$-4,16-androstadien-3-one;
X = 2,2',4,6,6',16,17-$^{2}H_{7}$-4,16-androstadien-3-one;
XI = 5α-H-3-one derivative of IX;
XII = 6,6',16,17-$^{2}H_{4}$-5α-androst-16-en-3-one.
XIII = 6,6',16,17-$^{2}H_{4}$-3β-hydroxy-5α-androst-16-ene; and
XIV = 6,6',16,17-$^{2}H_{4}$-3α-hydroxy-5α-androst-16-ene.

directed into the warm flask in order to reduce the volume about 20%, and crystallization of the tosylhydrazone derivative occurred during cooling. The crystals were removed by filtration, washed with 40 mL of methanol and dried under vacuum at 65°C for 12 hours. The reaction yielded 16.6 g of steroid-hydrazone.

The next step involved the reduction of the hydrazone with methyllithium using the procedure described by Matthews and Hassner (9). The tosylhydrazone derivative (12.2g) was dissolved in 730 mL of 1,2-dimethoxyethane freshly redistilled from $LiAlH_4$ in a 1-L flask fitted with a 250 mL addition funnel, a drying tube and a magnetic stirring bar. A 2.05 M ether solution of methyl-lithium (30 mL) was added to the hydrazone solution over a 1 hour period through the addition funnel, taking precautions to avoid its explosiveness on exposure to a moist atmosphere and to remove the mineral oil contaminant (10). The highly colored reaction mixture was stirred for 6 hours and then added to 900 mL of ice water with constant stirring. The precipitate was digested on a warm steam bath for 12 hours to aid in filtration. It was filtered, and the residue was washed with water and dried under vacuum at 50°C for 5 hours.

Recrystallization from ethanol-water yielded 7.2 g of compound VIII, a yield of approximately 60%.

16,17-$^2H_2$-4,16-androstadien-3-one (IX)

16,17-$^2H_2$-4,16-androstadien-3-one was prepared by Oppenauer oxidation (10) of compound VIII. A solution of 6 g of VIII in 300 mL of toluene and 47mL of cyclohexanone was distilled for 30 minutes to remove all traces of

water. The distillate (about 10 mL) was removed using a Stark-Dean moisture receiver. A solution containing 3.1 g of aluminum isopropoxide in 30 mL of toluene was added, and the combined solutions were refluxed for 2 hours. 80 mL of water were added, and the volatile components were removed by steam distillation for 4 hours. The residue was extracted with 500 mL of chloroform followed by 300 mL of dichloromethane. The combined extracts were washed with water, dried over anhydrous sodium sulfate and concentrated to 25 mL under vacuum. Then 100 mL of hexane were added, and the solution was further concentrated until crystallization occurred.

The crystals were recovered by filtration and rinsed with a minimum amount of hexane before drying for 6 hours in a vacuum oven at 35°C. The yield of 16,17-$^2H_2$-4,16-androstadien-3-one (IX) was 2.9 g or 48%.

6,6',16,17-$^2H_4$-5α-androst-16-en-3-one (XII)

Compound IX, which was previously synthesized and dried for 24 hours at 50°C over P_2O_5, was converted to compound X by refluxing for 4 days in 110 mL of deuteriomethanol containing 10 drops of a solution of 20% sodium deuterioxide in deutrium oxide. The perdeuteriosteroid was then recovered by crystallization induced by addition of deuterium oxide. It was filtered and washed with a small amount of deuterium oxide. After drying for 3 hours in a vacuum oven at 40°C and then for 8 hours in a dessicator over P_2O_5 at 50°C, the exchange procedure yielded 1.7 g.

The product was converted to its corresponding 5α-H-3-one steroid (XI) by reduction of the Δ^4-double bond in lithium-ammonia solution, as explained

earlier except that protioammonia was used in this instance. A solution of 1.7 g of compound X in 25 mL of tetrahydrofuran was added in a slow stream to the lithium-ammonia solution. The reaction mixture was stirred during refluxing for 1 hour using a Teflon coated stirring bar. The reaction was stopped by adding saturated ammonium chloride in tetrahydrofuran until the blue color disappeared. The ammonia was allowed to evaporate, and the product was extracted with diethyl ether. The ether extract was washed two times with 1 volume of 0.1N HCl, two times with 1 volume of $NaHCO_3$ and three times with 1 volume of water. The solution was dried over anhydrous sodium sulfate, and the remaining solvent was removed under vacuum. The residue was purified by chromatography on a $AgNO_3$-impregnated silica gel column as described by Gower (13) and recrystallized from acetone-hexane. The reaction yielded 67 mg of XI.

The α-deuteriums were removed by equilibrating in methanol-water. The final product was compound XII, and was recrystallized using acetone-hexane as before.

6,6',16,17-$^2H_4$-3α-hydroxy-5α-androst-16-ene (XIV)

The method used was a modification of a procedure for ketone reduction of steroids (11). A solution of 0.63 g of the previously synthesized compound XII, 0.32 g of chloroiridic acid, 3.3 mL of trimethyl phosphite and 6.5 mL of water in 49 mL of 2-propanol was refluxed for 5 days. After cooling, the reaction mixture was transferred to a 250 mL separatory funnel containing 50 mL of ether and 50 mL of water. The product was extracted three times with 50 mL portions of ether. The combined extracts were dried over anhydrous sodium

sulfate and then evaporated to dryness.

The residue was purified by chromatography on a $AgNO_3$-impregnated silica gel column. The product was further purified on a second silica gel column prepared without $AgNO_3$. The appropriate fractions were combined and evaporated to dryness. The product was recrystallized from acetone-hexane to yield 0.1 g.

6,6',16,17-$^2H_4$-3β-hydroxy-5α-androst-16-ene (XIII)

The procedure was adapted from Tokes and Throop (12). A mixture of compound XII (135 mg) and lithium aluminum hydride (66 mg) in dry ether (15 mL freshly distilled from lithium aluminum hydride) was refluxed for 3 hours. The excess hydride was then decomposed by careful dropwise addition of water. The reaction mixture was washed with 0.1N HCl to remove the hydride residue. The solution was then washed with water, dried over anhydrous sodium sulfate and evaporated to dryness.

Gas chromatographic analysis showed that the product consisted of 96% of compound XIII and 4% of the 3α-hydroxy compound (XIV). The 3β-hydroxy compound was further purified by silica gel column chromatography and recrystallized from acetone-hexane. The yield of 6,6',16,17-$^2H_4$-3β-hydroxy-5-α-androst-16-ene was 73 mg or about 50%.

DISCUSSION

The preparation of five deuterium labelled steroids has been described. These compounds were prepared expressly for use as internal standards for quantitative estimation of C_{19}-Δ^{16} steroids in pig tissue as previously

reported (5). To evaluate their suitability for this purpose each labelled steroid was submitted to GC/MS analysis in the selected ion monitoring mode and the abundance of the ion corresponding to the molecular ion of the native form (unlabelled) was compared to the intensity of the most abundant ion among those found for the molecular ion of the labelled form. These results are shown in Table 1.

As can be seen from Table 1, the contamination of the labelled standards by the presence of unlabelled (native) steroid is extremely small in the case of compounds IV, XII, XIII and XIV, and acceptably low in compounds VIII and IX.

The basic requirements for these internal standards, that the label not be lost during sample work-up and that the contamination of the standard by unlabelled compound be low, was met by all five compounds. Compounds IV, XII, XIII and XIV were particularly good with respect to the very low levels of the native molecular ion abundances observed by selected ion monitoring GC/MS analysis, which would facilitate the detection and measurement of the small amount of native compound obtained by isolation from the samples being examined.

A bar graph representation of the mass spectrum for each of the five labelled steroids and their corresponding unlabelled form is shown in Fig. 1. The labelled compounds with varying degrees of isotope incorporation were not subjected to complete mass spectrometric interpretation to determine the relative amounts of each isotopically labelled species. Such examination would require special mass spectrometer settings, i.e., very low ionization energies, as well as tedious measurements and calculations, and was not made a

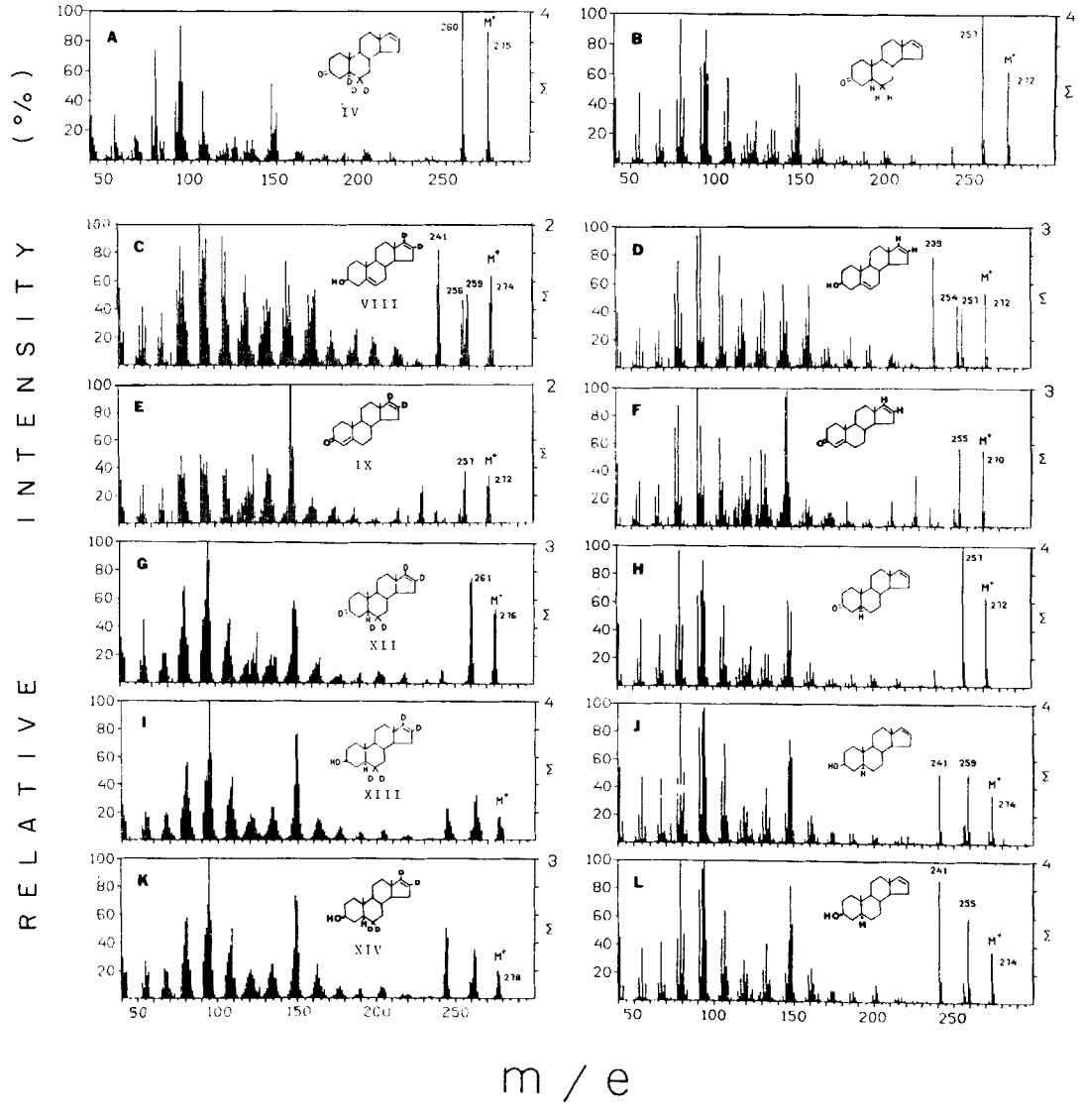


Fig. 1. Mass spectra of the labelled compounds (left) and corresponding unlabelled analogs (right).

part of this work, although it would be interesting and could be the subject of further investigations with these steroid compounds.

Table 1. Selected Ion Monitoring Analysis of Labelled Steroids[a,b]

Compound	M^+ for Unlabelled Steroid (A) (m/z)	M^+ for Labelled Steroid (B) (m/z)	Abundance of A as % of B[c] (%)
IV	272	275	1.6
VIII	272	274	8.1
IX	270	272	8.1
XII	272	276	0.6
XIII	274	278	0.6
XIV	274	278	0.6

a Each value is the mean from six determinations.
b Reference (5) describes mass spectrometry conditions used for selected ion monitoring analysis.
c Abundance of ion A/abundance of ion B X 100.

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