SYNTHESIS OF C_{19} STEROID MONOSULPHATES LABELLED WITH DEUTERIUM AT SPECIFIC POSITIONS

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SUMMARY

Synthesis of C_{19} steroid monosulphates labelled with deuterium is reported. Deuterium was introduced either at hydroxylated carbons or in methylene groups, in order to permit use of the steroids in studies of intermolecular hydrogen transfer *in vivo*. Methylene groups were deuterated by equilibration of carbonyl α-hydrogens with deuterium oxide. Deuterium at hydroxylated carbons was introduced by metal deuteride reduction. The sulphate group was introduced in the final step of a reaction sequence or, in some cases, in the step preceding the final one followed by removal of an acetate group.

Key Words: Androstanediols, androstanolones, sulphate esters, deuterium labelling, gas chromatography-mass spectrometry

INTRODUCTION

Steroid sulphates are subject to direct metabolism *in vivo* without prior hydrolysis. Thus, hydroxyl groups may be oxidized and carbonyl groups may be reduced by hydroxysteroid oxidoreductases. When two steroid sulphates are metabolized simultaneously hydrogen may be transferred *via* a coenzyme from the steroid that is being oxidized to the steroid that is being reduced (1,2). Intermolecular hydrogen transfer may also take place during the reversible oxidoreduction of a hydroxysteroid. These processes may be studied by gas chromatography-mass spectrometry of the metabolites formed from a mixture of steroid molecules deuterated at the hydroxylated carbon and in metabolically stable positions. Labelled C_{19} steroid sulphates, suitable for these studies, have now been prepared. Such deuterated steroids are also useful as internal standards in quantitative analyses by selected ion monitoring gas chromatography-mass spectrometry.

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EXPERIMENTAL

GENERAL

Infrared spectra were recorded using either solutions in carbon tetrachloride or discs of potassium bromide in a Perkin-Elmer 254 Infrared Spectrophotometer.

Liquid-gel chromatography was carried out using Lipidex 1000 and Lipidex 5000 (Packard International, S.A., Zürich, Switzerland) as stationary phases in reversed- or straight-phase systems (3).

Thin-layer chromatography was carried out on glass plates with 0.25 mm layers of silica gel 60 (Merck, Darmstadt, Germany). Reactions were monitored using 2 x 5 cm plates. The mobile phase was ethyl acetate/cyclohexane (1:1) when unconjugated steroids were analyzed and chloroform/methanol/water (65:25:4) when steroid sulphates were analyzed. Spots were visualized by spraying the plate with vanillin/sulphuric acid solution and subsequent heating.

Gas-liquid chromatography was carried out using a Pye 104 instrument equipped with either a packed 1.5% SE-30 column or a 25 m x 0.34 mm open tubular glass capillary column coated with SE-30 (4). In the latter case solid injection in an all-glass system was used (5) with nitrogen as carrier gas at an inlet pressure of 60 kPa. Androstanediols were analyzed as the trimethylsilyl (TMS) ether derivatives (6) and androstanolones as the O-methyloxime TMS ethers (7).

Gas chromatography-mass spectrometry was carried out on a modified LKB 9000 instrument equipped with a 1.5% SE-30 packed column and with helium as carrier gas. Magnetic scan spectra were taken for identification purposes. Repetitive accelerating voltage scanning was used with continous recording on magnetic tape for determination of the deuterium content. The values were bunched to give 20 values per mass spectrometric peak (8). The isotope excess was calculated using the intensities of the molecular ions by comparison with unlabelled compounds.

Reagents. Deuterium oxide (99.8%) was obtained from Norsk Hydro (Oslo, Norway). Monodeuteroethanol (99%), lithium aluminium deuteride (98%), and

sodium borodeuteride (98%) were from Merck AG (Darmstadt, Germany). All steroids were purchased from Sigma Chemical Co (St Louis, Mo, USA). When necessary the steroids were purified by liquid-gel chromatography before use.

COMMON EXPERIMENTAL PROCEDURES

Deuterium exchange of carbonyl α-hydrogens. Sodium (2 mmol) in small pieces was added to a stirred mixture of monodeuteroethanol (50 ml) and deuterium oxide (5 ml). After complete reaction the 3- or 17-oxosteroid (1.0-1.4 mmol) was added and the solution was heated under reflux for 48 h under an argon atmosphere. The reaction mixture was cooled and neutralized with 2 M HCl. Ethyl acetate (50 ml) was added and the solution was washed with water and taken to dryness. The residue was purified by chromatography on a 80-120 g column of Lipidex 5000 in hexane/chloroform (4:1).

Preparation of tetrahydropyranyl (THP) ethers. The hydroxysteroid (0.9-1.2 mmol) was dissolved in freshly distilled 3,4-dihydro-2-H-pyran (3 ml). Phosphorous oxychloride (3 drops) was added under stirring. After 2 h at room temperature the solution was diluted with diethyl ether (50 ml), washed with 1 M $NaHCO_3$ and water, and taken to dryness. The residue was purified by chromatography on a 70 g column of Lipidex 1000 in hexane/chloroform (4:1). This procedure is a modification of the method described by Greenhalgh *et al.* (9).

Acetylation. The hydroxysteroid (0.6-0.9 mmol) was dissolved in a mixture of anhydrous pyridine and acetic anhydride (3 ml, 2:1). The solution was heated under reflux for 30 minutes, ethyl acetate (50 ml) was added and the solution was repetitively washed with 1 M $NaHCO_3$, 2 M HCl and water before it was taken to dryness.

Lithium aluminium hydride (deuteride) reduction. The 3- or 17-oxosteroid (0.6-0.9 mmol) was dissolved in anhydrous diethyl ether (30 ml). The hydride (deuteride) (2 mmol) was added under stirring. The solution was kept at room temperature (3-oxosteroids) or was heated under reflux (17-oxosteroids) until the reduction was complete. The reagent excess was destroyed

by dropwise addition of water and the solution was neutralized with 1 M HCl. The organic phase was washed with water and taken to dryness.

Sodium borohydride (borodeuteride) reduction. The oxosteroid (0.7-1.2 mmol) was dissolved in abs. ethanol (15 ml). The hydride (deuteride) (1.5 mmol) was added and the mixture was stirred at room temperature for 3 h. After evaporation of the solvent, the residue was partitioned between ethyl acetate and water. The organic phase was washed with water and taken to dryness.

Hydrolysis of acetates. The steroid acetate (0.2-0.5 mmol) was dissolved in 0.6 M KOH in methanol (10 ml). The solution was heated under reflux for 30 minutes, cooled, neutralized with 2 M HCl, and taken to dryness. Unconjugated steroids were isolated by extracting the residue with ethyl acetate, washing the solution with water, and evaporating the solvent. Steroid sulphates were isolated by extraction chromatography on a column (130-170 ml) of Amberlite XAD-2 in water (10). The sulphates were eluted with methanol.

Cleavage of THP ethers. The THP ether (0.4-0.8 mmol) was heated under reflux with p-toluenesulphonyl chloride (0.2 mmol) in abs. ethanol (15 ml) for 10 minutes. After cooling, ethyl acetate (50 ml) was added and the solution was washed with 1 M $NaHCO_3$ and water and was taken to dryness. The residue was purified by chromatography on a 60-100 g column of Lipidex 5000 in hexane/chloroform (7:3).

Jones' oxidation. The hydroxysteroid (0.4-1.1 mmol) was dissolved in acetone (10 ml). Jones' reagent (11) was added dropwise under stirring. Excess reagent was destroyed by adding 2-propanol. After evaporation of the solvent, the residue was extracted with ethyl acetate and the solution was washed with water and taken to dryness.

Sulphation. The hydroxysteroid (0.2-0.4 mmol) was dissolved in a mixture of anhydrous dimethylformamid and molten freshly distilled N,N'-dicyclohexylcarbodiimide (3-6 ml, 6:1, v/v) and the solution was cooled to -10° in an ice/ethanol cold bath. A precooled mixture of conc. sulphuric acid and anhydrous dimethylformamide (0.2-0.4 ml, 1:10) was added under stirring and the mixture was kept at -10° for about 30 minutes. By this time thin layer chromatography indicated absence of unconjugated steroid.

The carbodiimide excess was destroyed by adding 5 ml of 70% aqueous ethanol and the mixture was made slightly alkaline with 1 M KOH and was centrifuged. The supernatant was taken to dryness and the steroid sulphate was purified by chromatography on Sephadex LH-20 and Amberlite XAD-2 as described elsewhere (2). This procedure is a modified version of the method described by Mumma et al. (12).

Crystallization and analysis of chemical purity. The final products were recrystallized from abs. ethanol or methanol/ethyl acetate mixtures. The crystals were analyzed by thin layer chromatography and infrared spectrometry, in some cases by comparison with authentic standards. The purity of the steroid moiety was determined by gas-liquid chromatography of the TMS ethers or O-methyloxime TMS ethers of the steroids liberated by solvolysis (13).

SYNTHESIS OF INDIVIDUAL COMPOUNDS

Potassium 5α-[17α-D]androstane-3ß,17ß-diol 3-sulphate (I:E)

3ß-Hydroxy-5α-androstan-17-one (1.03 mmol) was converted into the THP ether, which was reduced with lithium aluminium deuteride. The resulting mixture of isomers was separated on a 120 g column of Lipidex 5000 in hexane/chloroform (4:1). The isolated compound I:A was acetylated to I:B, which was subjected to ether cleavage resulting in I:C. Esterification with sulphuric acid afforded I:D, the acetate group of which was finally removed and the sulphate I:E (0.21 mmol) was isolated.

Potassium 5α-[2,2,4,4-D_4]androstane-3ß,17ß-diol 3-sulphate (II:C)

17ß-Hydroxy-5α-androstan-3-one (1.03 mmol) was subjected to deuterium exchange. The deuterated androstanolone was acetylated and reduced with sodium borohydride. The resulting 3-hydroxysteroid epimers were separated on a 75 g column of Lipidex 1000 in methanol/water/chloroform (7:2:1). The isolated compound II:A was esterified with sulphuric acid to II:B, which was hydrolyzed to the sulphate II:C (0.30 mmol).

Potassium 5α-[17α-D]androstane-3α,17ß-diol 3-sulphate (I:K)

3α-Hydroxy-5α-androstan-17-one (1.03 mmol) was converted into the THP

ether, which was reduced with lithium aluminium deuteride. Product separation on a 115 g column of Lipidex 5000 in hexane/chloroform (7:3) afforded compound I:F, which was acetylated to I:G. THP ether cleavage resulted in I:H, which was sulphated to I:J. Alkaline hydrolysis of the acetate group yielded I:K (0.22 mmol).

Potassium 3ß-hydroxy-5α-[2,2,4,4-D_4]androstan-17-one sulphate (III:C)

17ß-Hydroxy-5α-androstan-3-one (1.37 mmol) was labelled adjacent to the carbonyl group by deuterium exchange and was converted into the THP ether. Reduction with lithium aluminium hydride afforded II:D, which was isolated by chromatography of the products on a 120 g column of Lipidex 5000 in hexane/chloroform (7:3). Acetylation gave II:E, which underwent THP ether cleavage to II:F. Oxidation to the androstanolone acetate III:A was followed by hydrolysis to III:B, which was conjugated with sulphuric acid to III:C (0.27 mmol). When lithium aluminium deuteride was used instead of the hydride the pentadeuterated sulphate III:D was obtained.

Potassium 3α-hydroxy-5α-[2,2,4,4-D_4]androstan-17-one sulphate (III:G)

17ß-Hydroxy-5α-androstan-3-one (1.37 mmol) was equilibrated with deuterium oxide and oxidized with Jones' reagent. The dione underwent partial reduction with sodium borohydride in aqueous pyridine (14). The reaction products were separated on a 120 g column of Lipidex 5000 in hexane/chloroform (8:3). A small amount of 3α-hydroxysteroid III:E was isolated together with the predominant 3ß-epimer III:B. The latter compound was converted into the mesylate III:F with methanesulphonyl chloride (0.5 ml) in pyridine (1.0 ml). The solution was stirred at room temperature for 15 minutes, diluted with ethyl acetate, washed with 1 M HCl, 1 M $NaHCO_3$ and water, and taken to dryness. The crude product was purified on a 50 g column of Lipidex 5000 in hexane/chloroform (4:1). The mesylate was epimerized in collidine/water (15) and compound III:E was isolated by chromatography in the same system. This material was pooled with the minor reduction product and was sulphated to III:G (0.18 mmol).

Potassium 5α-[3α-D]androstane-3ß,17ß-diol 17-sulphate (IV:E)

17ß-Hydroxy-5α-androstan-3-one (1.37 mmol) was converted into the THP ether, which was reduced with sodium borodeuteride. The hydroxysteroid IV:A

was isolated by chromatography on a 120 g column of Lipidex 5000 in hexane/chloroform (4:1). Acetylation afforded IV:B, which underwent ether cleavage to IV:C. Esterification with sulphuric acid gave IV:D, and hydrolysis of the acetate group yielded IV:E (0.28 mmol).

Potassium 5α-[3ß-D]androstane-3α,17ß-diol 17-sulphate (IV:L)

17ß-THP-5α-androstan-3-one (1.15 mmol) was reduced with sodium borodeuteride and the products were separated on a 120 g column of Lipidex 5000 in hexane/chloroform (4:1). A small amount of the 3α-hydroxysteroid IV:F was isolated together with the 3ß-epimer IV:A. The latter compound was epimerized *via* the mesylate in the same way as in the preparation of compound III:G. The product was pooled with the minor fraction from the reduction and converted into the acetate IV:H, which underwent ether cleavage to IV:J. Esterification with sulphuric acid gave the mixed ester IV:K, and alkaline hydrolysis yielded the sulphate IV:L (0.16 mmol).

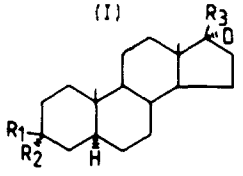
Potassium 17ß-hydroxy-5α-[16,16,17α-D_3]androstan-3-one sulphate (VI:C)

3α-Hydroxy-5α-androstan-17-one (1.03 mmol) was equilibrated with deuterium oxide. The dideuterated steroid was converted into the THP derivative, which was reduced with lithium aluminium deuteride. The 17ß-hydroxysteroid V:A was isolated by chromatography on a 115 g column of Lipidex 5000 in hexane/chloroform (7:3). Acetylation to V:B was followed by ether cleavage resulting in compound V:C. The hydroxysteroid was oxidized to VI:A, which was hydrolyzed to VI:B. Finally, sulphation gave VI:C (0.18 mmol).

RESULTS AND DISCUSSION

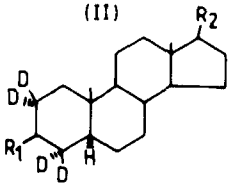
Intermediates and products of the reaction pathways are summarized in Figure 1. The labelled compounds were prepared from steroids carrying oxygen at C-3 and C-17. The acid labile THP group and the base labile acetate group were used to protect hydroxyls. The THP group was shown to be stable towards the conditions of metal hydride reduction and acetylation, thus confirming

(I)



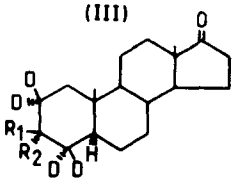
	R_1	R_2	R_3
A	THP[a]	H	OH
B	THP	H	OAc
C	OH	H	OAc
D	OSO_3^-	H	OAc
E	OSO_3^-	H	OH
F	H	THP	OH
G	H	THP	OAc
H	H	OH	OAc
J	H	OSO_3^-	OAc
K	H	OSO_3^-	OH

(II)



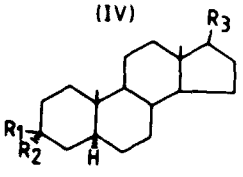
	R_2	R_2
A	OH	OAc
B	OSO_3^-	OAc
C	OSO_3^-	OH
D	OH	THP
E	OAc	THP
F	OAc	OH

(III)



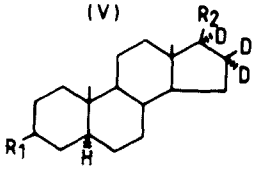
	R_1	R_2
A	OAc	H
B	OH	H
C	OSO_3^-	H
D	OSO_3^-	D
E	H	OH
F	Ms[b]	H
G	H	OSO_3^-

(IV)



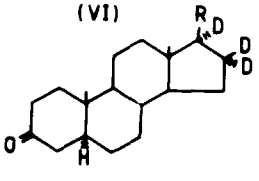
	R_1	R_2	R_3
A	OH	D	THP
B	OAc	D	THP
C	OAc	D	OH
D	OAc	D	OSO_3^-
E	OH	D	OSO_3^-
F	D	OH	THP
G	Ms	D	THP
H	D	OAc	THP
J	D	OAc	OH
K	D	OAc	OSO_3^-
L	D	OH	OSO_3^-

(V)



	R_1	R_2
A	THP	OH
B	THP	OAc
C	OH	OAc

(VI)



	R
A	OAc
B	OH
C	OSO_3^-

[a] Tetrahydropyran-2'-yloxy-

[b] Methanesulphonyloxy-

Figure 1. Intermediates and products.

earlier results (16). The acetate group was essentially stable to the conditions of THP ether cleavage provided that the reaction time was limited.

The difficulty of preparative separation of steroid monosulphates (17) necessitated the reactions following sulphation to lead to homogenous products in a quantitative yield. This, together with the acid lability of these compounds, restricted the possible reactions of the sulphates. Thus, the only reaction that followed sulphation was removal of acetate protecting groups under alkaline conditions.

Metal hydride reductions of 3- and 17-oxosteroids result in mixtures of hydroxysteroids where one epimer is predominant. Thus, 17ß-hydroxysteroid is nearly exclusively obtained from 17-oxosteroid, and in the 5α-series the major reduction product of a 3-oxosteroid is a 3ß-hydroxysteroid (18). By the use of epimerization reactions it was possible to convert the 3ß-hydroxysteroid into the 3α-epimer *via* the mesylate. It was shown that the THP group was stable to the conditions of these reactions. The 3- and 17-hydroxysteroid epimers were effectively separated by chromatography using Lipidex 1000 and Lipidex 5000 as stationary phases.

The isotopic purity of the androstanolone sulphates with deuterium in the A ring was lower than that of the other compounds (Table 1). It was shown that the former compounds lost deuterium in the reaction steps following equilibration of the 3-oxosteroid with deuterium oxide, i.e. THP ether formation and chromic acid oxidation. In contrast, the androstanolone sulphate VI:C with deuterium at C-16, resulting from deuterium exchange of the 17-oxosteroid, retained its labelling under similar conditions. These two reactions are catalyzed by Lewis acids, phosphorous oxychloride and chromic acid respectively, which are potential catalysts of enolization and thus may promote an exchange of the α-hydrogens of the steroid ketone. These results are contrary to the findings of Wheeler *et al.* that steroid 17-ketones are more easily enolized than 5α-steroid 3-ketones under acid catalysis (19).

Table 1. Isotope composition of steroid sulphates, determined from the intensities of the molecular ion of the TMS ether (androstanediols) and the O-methyloxime TMS ether (anrostanolones) derivatives of the steroids liberated by solvolysis.

Compound (see Fig. 1)	Deuterium excess (atoms%)					
	D_0	D_1	D_2	D_3	D_4	D_5
I:E	8.0	92.0	-	-	-	-
II:C	-	-	1.8	12.2	86.0	-
I:K	4.6	95.4	-	-	-	-
III:C	-	1.0	4.4	25.7	68.8	0.1
III:D	-	-	2.4	8.7	28.6	60.3
III:G	-	1.0	4.5	23.6	70.8	0.1
IV:E	7.3	92.7	-	-	-	-
IV:L	7.7	92.3	-	-	-	-
VI:C	-	1.5	5.2	93.3	-	-

No loss of deuterium label took place at C-3 during epimerization of 3ß-hydroxysteroids in agreement with earlier findings (15). It was also shown that the neighbouring hydrogens were stable to exchange during the reaction.

The mass spectra of the TMS ethers of unlabelled 5α-androstane-3,17ß-diols contain base peaks at m/z 129 (20). This fragment has been suggested to consist of a trimethylsiloxy group and three carbons and four hydrogens, which could arise from fission of either ring A or ring D (20,21). The absence of any significant shift of the m/z 129 peak in the mass spectra of any of the ring A deuterated androstane-3,17ß-diols compared with an almost complete shift to m/z 130 in the mass spectra of the 17α-deuterated androstanediols showed that this fragment originated from the D ring (Fig. 2).

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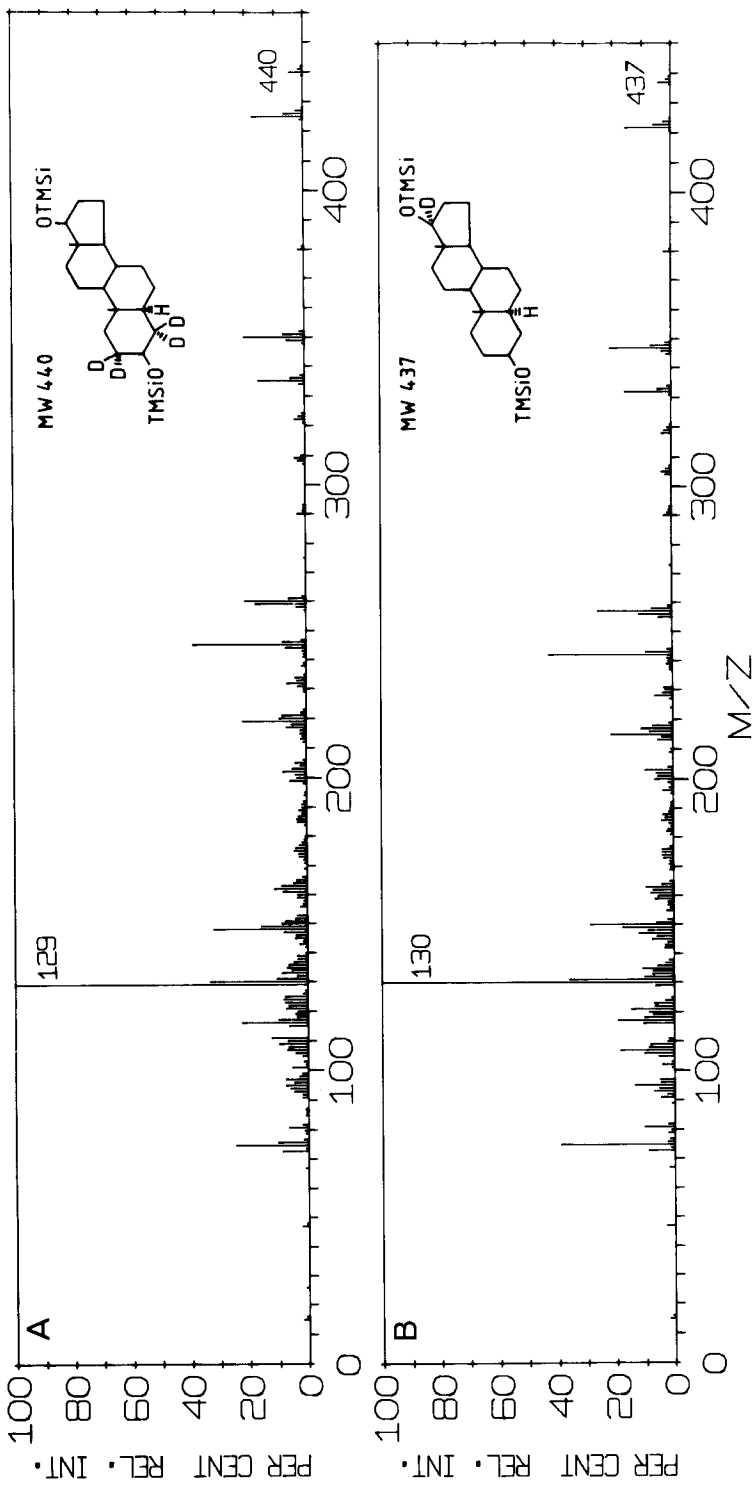

Figure 2. Mass spectra of androstanediol TMS ethers, labelled in the A ring (A) and in the D ring (B).

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