REFERENCE COMPOUNDS FOR THE STUDY OF MOXESTROL METABOLISM

J. SALMON, D. COUSSEDIERE, C. COUSTY,
V. DELAROFF and J.P. RAYNAUD

Centre de Recherches Roussel Uclaf,
93230 Romainville, France

Received 3/19/79

SUMMARY

Reference compounds for the subsequent identification of the metabolites of the potent estrogen, moxestrol (R 2858), in various species were isolated from the bile of phenobarbital pretreated rats or obtained via enzymatic hydroxylation by microorganisms. A few of them were prepared by chemical synthesis. The structures of all these compounds were determined by physical and chemical methods.

INTRODUCTION

Moxestrol (R 2858 or 11ß-methoxy-17-ethynyl-1,3,5(10)-estratriene-3,17ß-diol) is a highly potent estrogen (1-6), active in menopausal women at the extremely low once-weekly dose of 50 to 100 µg (7-12). This dose is so low that moxestrol metabolites cannot be isolated and purified from human urine in order to determine their structure; they have to be identified by thin-layer chromatography and GC mass spectrography using standards synthesized chemically or preferably prepared biologically in vitro or in vivo. The present study describes the preparation of these reference molecules and the determination of their structure.

On incubation of ethynyl estradiol with rat liver homogenates, a high degree of metabolism was observed confirming available results (13), but moxestrol only yielded a

small amount of just two metabolites. Attempts to obtain larger amounts of metabolites were then based on extraction from the bile of treated animals, in particular of guinea-pigs, since liver steroid metabolism in guinea-pig often resembles that in human (14-15), but the only product found was conjugated moxestrol. It was only when using phenobarbital-pretreated rats that sufficient quantities of biotransformed products were obtained from bile enabling the determination of the structure of twelve metabolites.

Since certain structural modifications undergone by ethynyl estradiol in man were not encountered in moxestrol-treated rats, products of moxestrol hydroxylation were obtained in vitro by the use of microorganisms or by chemical synthesis.

MATERIALS AND METHODS

Materials

Spectra were measured with a Cary 14 spectrophotometer (Ultraviolet (UV) spectra), a WH 90 Bruker FF spectrometer (Proton nuclear magnetic resonance (NMR) spectra) and a CEC 110C, 70 eV or Finnigan 3300 quadrupolar (Mass spectra (MS)). Thin-layer chromatograms were scanned with a "Dünnschicht-scanner Berthold LB2721 + LB2560".

Radioactivity was measured in a Packard Tricarb 3375 liquid scintillation spectrometer. A solution of butyl-PBD* (0.4%, w/v) in toluene or, for aqueous solutions, of butyl-PBD* (0.4%, w/v) and naphthalene (8%, w/v) in methoxy ethanol : toluene (4:6) was added to the samples. Quenching was corrected by the automatic external standard method.

All solvents and reagents were of analytical grade.

---

*butyl-PBD = 2-(4'-t-butylphenyl)-5-(4"-biphenyl)-1,3,4-oxadiazole

Chromatography

The chromatographic procedures are detailed in Table 1.

TABLE 1. Chromatographic procedures

Adsorption on silicagel

Silicagel 60 F254 precoated plates (Migration: 15 cm) and Silicagel H packed columns (E. Merck, Darmstadt)

Solvents:

A	Chloroform:methanol	(95:5)
B	Chloroform:methanol	(9:1)
C	Chloroform:ethanol	(9:1)
D	Methylene chloride:methanol	(9:1)
E	Ethyl acetate:cyclohexane	(2:8)
F	Ethyl acetate:cyclohexane	(5:5)
G	Ethyl acetate:cyclohexane	(9:1)
H	Ethyl acetate:benzene	(2:8)
I	Ethyl acetate:benzene	(5:5)
J	Ethyl acetate	
K	Chloroform	

Elution from plates by:
Methylene chloride:acetone (5:5, v/v)

Use of Sephadex columns

Sephadex LH20 (Pharmacia, Uppsala)

Eluants:

L	Methylene chloride:methanol	(95:5)
M	Heptane:chloroform:ethanol	(5:5:1) (water-saturated)

Partition chromatography

Silicagel 60 F254 precoated plates (E. Merck, Darmstadt) saturated in vapour of stationary phase for 15 hr before and 1.5 hr after spotting.
Migration (15 cm) in mobile phase:

N	Benzene:ethyl acetate:methanol:water	(8:2:7:3)
O	Hexane:methanol:water	(10:7:3)
P	Benzene:methanol:water	(10:7:3)

Chemical reactions for metabolite identification

Silver salt formation: Silicagel 60 F 254 precoated plates (E. Merck, Darmstadt) were dipped to a depth of 5 cm into an acetonitrile solution of silver nitrate (10%, w/v), dried 1 hr at 50°C and then left at room temperature for 1 hr. If the metabolite migrated on the treated plate, there was no unsubstituted ethynyl group present.

Borohydride reduction: Sodium borohydride (10 mg) was added to the metabolite dissolved in 0.2 ml of methanol. After 15 min at 15°C and addition of 2 ml of water, the product formed was extracted with ethyl acetate. If, on chromatography, it was more polar than the metabolite, one or more carbonyl groups were present in the initial product.

Acetylation: The metabolite, dissolved in pyridine: acetic anhydride (2:1), was left for 16 hr at 20°C and then acidified with N HCl. The acetylated compound was extracted with methylene chloride. If, on chromatography, it was less polar than the metabolite, one or more hydroxyl groups (phenol or alcohol) were present in the initial product.

Acetonide formation: The metabolite, dissolved in 0.1 ml of a 70% perchloric acid acetone solution (1%, v/v), was left 30 min at 20°C, then neutralized with triethylamine. If, on chromatography, a product less polar than the metabolite was detected, an α-diol was present. Furthermore, under these conditions, if the acetonide was on the D ring, owing to the rigidity of this ring, the diol was, in all likelihood, in a cis configuration.

Isolation of metabolites from rat bile

Radiolabelling: 6,7[^{3}H]-moxestrol (6,7[^{3}H]-11ß-methoxy-17-ethynyl-1,3,5(10)-estratriene-3,17ß-diol) (47.5 Ci/mmole) was synthesized at the Roussel Uclaf Research Centre. Before use, its purity was checked by TLC (>98% pure).

Animals: Thirty Sprague-Dawley rats, weighing 400 g, were pretreated for 3 days with phenobarbital (100 mg/kg/day orally). On the 4th day, they were anaesthetized with urethane; a catheter was introduced into the bile duct and 5 mg of [^{3}H]-moxestrol (16 µCi/mg) in 0.2 ml of 92% acetylmethylamine were injected intravenously. Bile was collected for 24 hr.

Enzymatic lysis of conjugates (Fig. 1): 25 ml of 0.2 M sodium acetate, acetic acid to pH 5.2 and 5 ml of Helix pomatia juice (Industrie Biologique Française, 100 000 UF ß-glucuronidase and 800 000 UR sulfatase per ml) were added to the 490 ml of bile collected and left under argon in a sealed container for 24 hr at 37°C. After checking the pH, a further 5 ml of Helix pomatia juice were added and the mixture was again left under argon for a further 24 hr at 37°C. Following extraction with 3x1 litre of ethyl acetate, each extract was washed with 3x100 ml of water. The organic phase was concentrated (<40°C, in vacuo) to a residue containing all glucuroconjugated steroids and some sulfoconjugated steroids. The aqueous phase and washings were acidified to pH 1 with pure sulfuric acid. After saturation with ammonium sulfate (750 g/l), addition of 0.45 volume of 30% sulfuric acid (v/v), and extraction with 3x1 vol of ethyl acetate, the organic phases thus obtained were pooled without washing or drying; para-toluene sulfonic acid (0.2 g/100 ml) was added and the mixture was stored under argon in a sealed container at 37°C for 24 hr. The ethyl acetate solution was neutralized with saturated bicarbonate solution and washed with 3x0.5 vol of water. The organic phase was concentrated (<40°C, in vacuo) to a residue containing the sulfoconjugated steroids.

Separation and purification of metabolites: The residue obtained on enzymatic lysis was chromatographed on silicagel H (Merck) (100 parts by weight). On elution with system I, system J, then methanol (5 v/w of silica), eleven fractions were isolated, each containing 1 to 3 products. These were analysed and purified by TLC. The residue obtained after solvolysis was also chromatographed on silicagel H (Merck) (100 parts by weight). After elution with system D, three fractions were isolated (Table 2).

Hydroxylation by microorganisms

The microorganism, Gibberella zeae, was grown in medium containing corn steep (1%, w/v), soja flour (1%, w/v), malt extract (0.5%, w/v), calcium carbonate (0.1%, w/v), sodium chloride (0.5%, w/v) and glucose (1%, w/v). Mycelia were produced in two steps at 28°C on a rotative shaker (250 rpm). One gram of moxestrol was added to 2 litres of the final medium and left for 96 hr (28°C - 250 rpm). After filtration, the solution was extracted with 3x1 litre of ethyl acetate. The extract was evaporated (40°C, in vacuo) and the residue was chromatographed in system B. After acetylation, 7ß-acetoxy moxestrol (Ib), 15α-acetoxy moxestrol (IIb) and 6ß-acetoxy moxestrol (IIIb) were separated and purified by chromatography in system E.

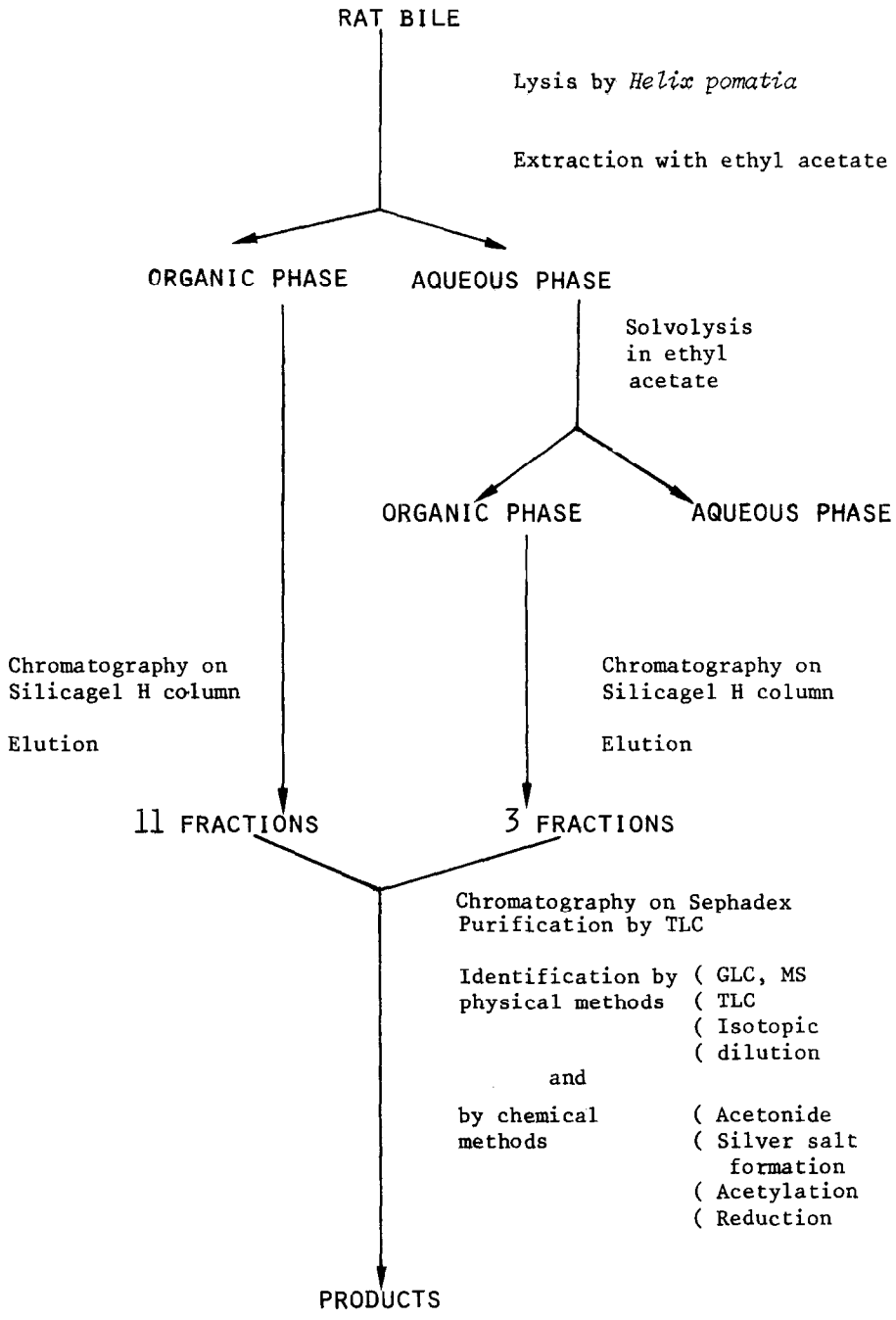


Fig. 1. *Procedure for the separation and identification of metabolites*

TABLE 2. TLC analysis of conjugates (after lysis)

	Mixture of glucuroconjugates and some sulfoconjugates (enzymatic lysis)						
	System D - Chloroform:methanol (9:1)						
Fraction n°	1	2	3	4	5	6	7
Number of peaks	1	3	2	1	2	2	2
Rx (moxestrol)* of peaks	1.57	1.60 1.38 1.10	1.10 1.00	0.82 0.82	1.00 0.78	1.12 0.58	0.59 0.41
% radioactivity in peaks	97	33 55 14	27 72	90	10 80	30 47	31 44
	System G - ethyl acetate:cyclohexane (9:1)						
Fraction n°	8	9	10	11			
Number of peaks	1	1	1	2			
Rx (moxestrol)* of peaks	0.69	0.65	0.58	0.65 0.0			
% radioactivity in peaks	95	95	95	40 40			
	Sulfoconjugates (solvolysis)						
	System D - Chloroform:methanol (9:1)						
Fraction n°	1	2	3				
Number of peaks	2	1	1				
Rx (moxestrol)* of peaks	2.05 1.43	0.78	0.58				
% radioactivity in peaks	40 40	90	85				

*Rx(moxestrol) = R_F of the product/R_F of moxestrol on the same plate.

Chemical syntheses

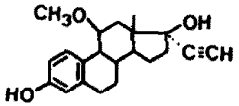
6α-Acetoxy moxestrol (IVb): 12 mg of 6ß-hydroxy moxestrol (obtained by microbiological hydroxylation as described above) were dissolved in 1 ml of acetone at 0°C and 14 µl of an aqueous solution containing CrO_3 (0.27, w/v) and sulfuric acid (0.23, v/v) were added. After 15 min at 0°C and dilution with water, the 6-oxo derivative was extracted with ethyl acetate, purified (6.2 mg) by chromatography in system B, and reduced with sodium borohydride. Chromatography in system G gave 2.3 mg of 6α-hydroxy moxestrol (IVa) which, after acetylation, gave 2.3 mg of 6α-acetoxy moxestrol (IVb).

2-Hydroxy moxestrol (Va) and 4-hydroxy moxestrol (VI): 1.5 g of moxestrol was dissolved in 500 ml of acetone, 720 ml of water and 80 ml of acetic acid. After addition of 5 g of Fremy salt (16) (potassium nitrosodisulfonate) and stirring for 15 min at 20°C, the mixture of the two orthoquinones formed was extracted with 1x500 ml and 2x250 ml of chloroform. The extract was washed with N HCl, then with water. Acetic acid (100 ml) and potassium iodide (3 g) were added; the solution was stirred for 5 min and 0.1 N sodium thiosulfate was added until decolorization. After addition of 200 ml of water, the aqueous phase was separated and extracted with 200 ml of methylene chloride:ethyl acetate (1:1). The organic phase (1st and 2nd extractions) was washed with N HCl, then with water, and concentrated (40°C, in vacuo). The residue was a mixture of moxestrol, 2-hydroxy moxestrol (Va) and 4-hydroxy moxestrol (VI), which were separated by column chromatography in system B, then in system L.

Methylation of 2-hydroxy moxestrol (Va): 33 mg of 2-hydroxy moxestrol (Va) were dissolved in 0.5 ml of methanol. 0.5 ml of diazomethane in chloroform (12 g/l) were added twice at 15 mins interval. After 15 min at room temperature, the solvent was evaporated in vacuo. Chromatography in system K gave 13 mg of dimethoxy moxestrol (Vd) and a mixture which, after chromatography in system M, was separated into the 2-methyl (Vb) and 3-methyl (Vc) ethers of 2-hydroxy moxestrol (Va).

RESULTS

The reference number, structure and chemical name of all the compounds identified in this study are given in Fig. 2.



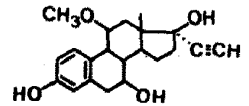
R 2858

Name : 11β-methoxy-19-nor-17α-pregna-1,3,5(10)-trien-20-yne-3,17β-diol

MS : 326(100), 249(49), 226(45), 211(55), 200(39), 189(25), 172(36), 159(30), 157(38), 146(62), 145(43).

NMR : (C_5D_5N) δ1.42(3,S,18-CH_3) 3.19(3,S,11β-OCH_3) 3.44(1,S,-C≡CH) 4.16(1,m,11α-H).

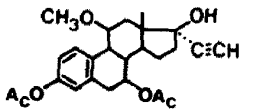
Ia



Ia name: 11β-methoxy-19-nor-17α-pregna-1,3,5(10)-trien-20-yne-3,7β,17β-triol

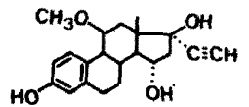
Ib MS : 426(2), 366(49), 334(70), 292(86), 289(50), 224(100), 209(39), 43(51)

Ib



Ib NMR : ($CDCl_3$) δ1.12(3,S,18-CH_3) 1.52(1,S,-OH) 2.04(3,S,7β-OAc) 2.24(3,S,7-OAc) 2.58(1,S,-C≡CH) 2.96(2,m,6-CH_2) 3.28(3,S,11β-OCH_3) 4.21(1,m,1α-H) 4.99(1,m,$J_{7\alpha-6\beta} \sim J_{7\alpha-8\beta} = 10Hz$ $J_{7\alpha-6\alpha}=6Hz$, 7α-H) 6.72(1,J∿2Hz,4-H) 6.84(1,J∿9Hz J∿2Hz,2-H) 7.10(1,d J∿9Hz,1-H).

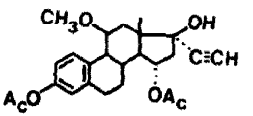
IIa



IIa name: 11β-methoxy-19-nor-17α-pregna-1,3,5(10)-trien-20-yne-3,15α,17β-triol

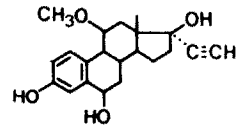
IIb MS : 426(18), 384(16), 366(29), 348(5), 334(4), 324(12), 308(42), 266(71), 224(12), 213(12), 211(15), 159(14), 157(19), 147(20), 146(55), 145(27), 43(100).

IIb



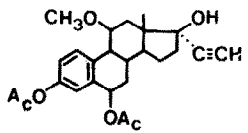
IIb NMR : ($CDCl_3$) δ1.14(3,S,18-CH_3) 1.52(1,S,-OH) 2.05(3,S,15α-OAc). 2.26(3,S,3-OAc) 2.67(1,S,-C≡CH) 3.24(3,S,11β-OCH_3) 4.20(1,m,11α-H) 5.04(1,m,15β-H) 6.74(1,S,4-H) 6.79(1,d d J=9Hz J=2Hz,2-H) 7.11(1d J=9Hz,1-H).

Fig. 2. *Reference number, structure, chemical name and specifications of the compounds identified in this study*

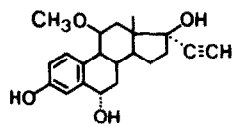
IIIa 

IIIa name: 11β-methoxy-19-nor-17α-pregna-1,3,5(10)-trien-20-yne-3,6β,17β-triol.

IIIb MS : 426(4), 384(5), 366(21), 324(11), 282(15), 279(32), 224(27), 198(37), 157(22), 145(21), 144(28), 43(100).

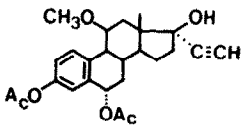
IIIb 

IIIb NMR : ($CDCl_3$) δ1.12(3,S,18-CH_3) 2.04(3,S,6β-OAc) 2.27(3,S,3-OAc) 2.61(1,S,-C≡CH) 3.3(3,S,11β-OCH_3) 4.22(1,m,11α-H) 5.94(1,m,width at half-height 7.5 Hz, 6α-H).

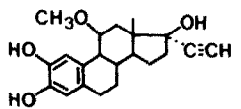
IVa 

IVa name : 11β-methoxy-19-nor-17α-pregna-1,3,5(10)-trien-20-yne-3,6α,17β-triol

IVa MS : 384(26), 366(86), 352(14), 324(24), 282(19), 279(18), 224(45), 198(43), 211(18), 209(21), 198(43), 157(29), 145(23), 144(37), 43(100).

IVb 

IVb NMR : ($CDCl_3$) δ1.07(3,S,18-CH_3) 2.14(3,S,6α-OAc) 2.27(3,S,3-OAc) 2.61(1,S,-C≡CH) 3.27(3,S,11β-OCH_3) 4.22(1,m,11α-H) 6.00(1,m,width at half-height 20 Hz, 6β-H).

Va 

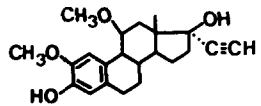
Va name : 11β-methoxy-19-nor-17α-pregna-1,3,5(10)-trien-20-yne-2,3,17β-triol

Va MS : 342(100), 265(26), 242(25), 227(32), 216(21), 205(13), 201(13), 173(21), 162(31), 161(30).

Va NMR : (C_5D_5N) δ1.44(3,S,18-CH_3) 3.17(3,S,11β-OCH_3) 3.49(1,S,-C≡CH) 4.16(1,m,11α-H) 7.00(1,S,4-H) 7.24(1,S,1-H).

Fig. 2. (Contd.)

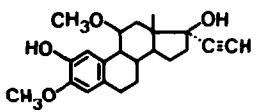
Vb



Vb MS : 356(100), 279(16), 256(16), 241(24), 230(21), 187(24), 176(31), 175(28), 161(21).

Vb NMR : ($CDCl_3$) δ1.09(3,S,18-CH_3) 2.60(1,S,-C≡CH) 3.32(3,S,11β-OCH_3) 3.85(3,S,2-OCH_3) 3.84(3,S,2 or 3-OCH_3) 6.47(1,S,4-H) 6.58(1,S,-1-H).

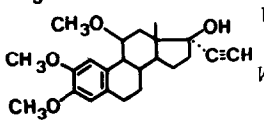
Vc



Vc MS : same as Vb

Vc NMR : ($CDCl_3$) δ1.09(3,S,18-CH_3) 2.60(1,S,-C≡CH) 3.31(3,S,11β-OCH_3) 3.83(3,S,3-OCH_3) 6.56(1,S,4-H) 6.77(1,S,1-H).

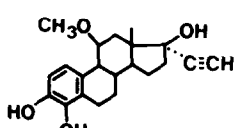
Vd



Vd MS : 370(100), 293(9), 270(8), 255(14), 244(9), 229(14), 190(24), 189(21).

Vd NMR : ($CDCl_3$) δ1.08(3,S,18-CH_3) 2.59(1,S,-C≡CH) 3.31(3,S,11β-OCH_3) 3.83(3,S,2 or 3-OCH_3) 3.84(3,S,2 or 3-OCH_3) 6.47(1,S,4-H) 6.58 (1,S,1-H).

VI

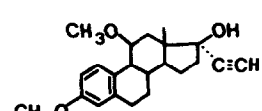


VI name : 11β-methoxy-19-nor-17α-pregna-1,3,5(10)-trien-20-yne-3,4,17β-triol

VI MS : 342(100), 287(11), 265(29), 244(25), 242(25), 227(23), 216(24), 215(34), 173(55), 162(27).

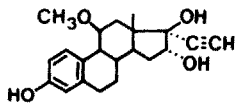
VI NMR : ($CDCl_3$ + C_5D_5N) δ1.14(3,S,18-CH_3) 2.59(1,S,-C≡CH) 3.25(3,S,11β-OCH_3) 4.17(1,m,11α-H) 6.59 and 6.74(2,AB system J=8Hz,1-H and 2-H).

VII

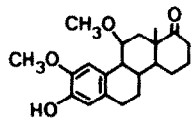


VII name : 3,11β-dimethoxy-19-nor-17α-pregna-1,3,5(10)-trien-20-yne-17β-ol

Fig. 2. (Contd.)

VIII 

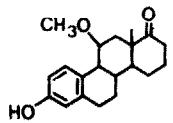
VIII name : 11β-methoxy-19-nor-17α-pregn-1,3,5(10)-trien-20-yne-3,16α, 17β-triol

IX 

IX name : 2,11β-dimethoxy-3-hydroxy-D-homo-estra-1,3,5(10)-trien-17a-one

IX MS : 344(100), 284(10), 280(63), 255(75), 215(58).

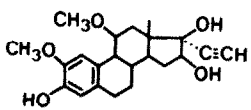
IX NMR : ($CDCl_3$) δ1.34(3,S,18-CH_3) 3.27(3,S,11β-OCH_3) 3.84(3,S,2-OCH_3) 4.14(1,m,11α-H) 6.63(1,S,1-H or 4-H) 6.67(1,S,1-H or 4-H).

X 

X name : 3-hydroxy-11β-methoxy-D-homo-estra-1,3,5(10)-trien-17a-one

X MS : 314(100), 296(8), 282(20), 185(2), 146(35).

X NMR : ($CDCl_3$) δ1.35(3,S,18-CH_3) 3.25(3,S,11β-OCH_3) 4.17(1,m,11α-H) 6.56(1,S,4-H) 6.63(1,d J=10Hz,2-H) 7.06(1,d J=10Hz,1-H).

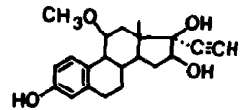
XI 

XI name : 2,11β-dimethoxy-19-nor-17α-pregna-1,3,5(10)-trien-20-yne-3,16β,17β-triol

XI MS : 372(31), 295(12), 219(100), 189(56), 176(89).

XI NMR : ($CDCl_3$) δ1.10(3,S,18-CH_3) 2.61(1,S,-C≡CH) 3.32(1,S,11β-OCH_3) 3.86(3,S,2-OCH_3) 4.18(1,m,11α-H and 16α-H) 6.64(2,S,1-H and 4-H).

Fig. 2. (Contd.)

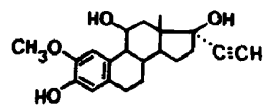


XII

XII name: 11β-methoxy-19-nor-17α-pregna-1,3,5(10)-trien-20-yne-3,16β, 17β-triol).

XII MS : 342(100), 324(32), 292(14), 278(46), 211(43), 189(79),146(75).

XII NMR : ($CDCl_3$ + C_5D_5N) δ1.12(3,S,18-CH_3) 2.54(1,S,-C≡CH) 3.27(3,S,11β-OCH_3) 4.23(2,m,11α-H and 16α-H) 6.62(1,S,4-H). 6.72(1,d J=9Hz,2-H) 7.01(1,d J=9Hz,1-H).

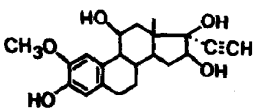


XIII

XIII name : 2-methoxy-19-nor-17α-pregna-1,3,5(10)-trien-20-yne-3,11β, 17β-triol

XIII MS : 342(100), 274(12), 256(14).

XIII NMR : ($CDCl_3$) δ1.15(3,S,18-CH_3) 2.6(1,S,-C≡CH) 3.86(2,S,2-OCH_3) 4.78(1,m,11α-H) 6.69(1,S,1-H or 4-H) 6.77(1,S,1-H or 4-H).

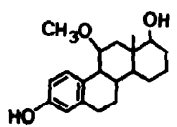


XIV

XIV name : 2-methoxy-19-nor-17α-pregna-1,3,5(10)-trien-20-yne-3,11β, 16β,17β-tetraol.

XIV MS : 358

XIV NMR : ($CDCl_3$ + C_5D_5N) δ1.20(3,S,18-CH_3) 2.56(1,S,-C≡CH) 3.81(3,S,2-OCH_3) 4.28(1,m,16α-H) 4.70(1,m,11α-H).



XV

XV name : 3,17aβ-dihydroxy-11β-methoxy-D-homo-estra-1,3,5(10)-triene

Fig. 2. (Contd.)

Metabolites extracted from rat bile

Seventy percent of the radioactivity administered to rats was recovered in the bile within 24 hr.

Steroids isolated after enzymatic lysis : The crude bile was separated into 11 fractions by chromatography; they accounted for 78% of bile radioactivity (Table 2).

Fraction 1, the least polar, contained only one product. This product had an ethynyl group (TLC on silver nitrate coated plate) and was purified by TLC in systems N, O and P successively. In these systems, as in system D, it had the same R_F as the 3-methyl ether of moxestrol (VII) prepared by chemical synthesis (17). The quantity of purified material was, however, insufficient for physical structure determination.

Fraction 2 contained three products, of which only one, according to chromatography on silver nitrate coated plates, had an ethynyl group. This product was obtained in minute amounts and was identical to 2-methoxy moxestrol (Vb) from Fraction 3. Of the remaining two products, one (IX) also methoxylated in 2, was purified in systems O, P, then F, the other (X) in system P. According to chromatography data, the products behaved like 11ß-methoxy estrone, but this was not confirmed by other data, i.e. by the GC MS fragmentation pattern, by the NMR data (δ 1.07 (3S, 18-CH_3) for 11ß-methoxy estrone but δ 1.35 (3S, 18-CH_3) for IX and X) and by the IR data (C=O 1703 cm^{-1}, C=O of cyclohexanone $\sim$ 1700 cm^{-1}, C=O of cyclopentanone 1739 cm^{-1}). Reduction of X gave XV; the 17-OH group was ß-oriented in view of the selectivity of the reagent (18).

Fraction 3 contained two ethynylated products. Data on the mass and NMR spectra and R_F of one product purified in system P, then F, indicated that it was identical to 2-methoxy moxestrol (Vb) prepared by chemical synthesis. The other product, after chromatography in

system L, had the same NMR and mass spectra and R_F in different systems as authentic moxestrol.

Fraction 4 contained only one product which was purified in system L and had the same mass and NMR spectra and R_F in different systems as 4-hydroxy moxestrol (VI) prepared by chemical synthesis.

Fraction 5 contained two products which were separated by chromatography in system L. Each had an ethynyl group and, as they were able to form an acetonide, a 16ß-hydroxyl group. One of them was purified in system F and had NMR and mass spectra which agreed with the structure of 2-methoxy-16ß-hydroxy moxestrol (XI). The other was purified in system D and had NMR and mass spectra which agreed with the structure of 16ß-hydroxy moxestrol (XII).

Fraction 6 contained two products which were separated by chromatography in system L. One of them did not have an ethynyl group and was not present in amounts sufficient for physical analysis. The other, after purification in systems N and G, had the same NMR and mass spectra and R_F in different systems as 2-hydroxy moxestrol (Va) prepared by chemical synthesis.

Fraction 7 contained two products. The very small quantities obtained on chromatography in systems G and D (two migrations) only enabled identification of one of them, namely, 2-hydroxy moxestrol (Va), on the basis of its mass spectrum and R_F.

Fraction 8 contained only one product which was chromatographed in systems G and N and which was identified on the basis of NMR and mass spectra as the 2-methoxy derivative of moxestrol demethylated in 11 (XIII). The hydroxy and methoxy groups are in positions 2 and 3 as the protons in 1 and 4 appear as singlets, but since there is no reference compound with an 11ß-hydroxy substitution, there is no NMR proof that the methoxy group is in position 3 rather than in position 2. However, as no

compounds methylated in position 3 were detected in rat bile, we assumed that this methoxy group was in position 2.

It was not possible to isolate a pure product from Fraction 9.

Fraction 10 yielded a pure product after chromatography in systems N and G. Since it formed an acetonide, it had a 16ß-hydroxyl group. As above, the methoxy group is assumed to be in position 2 (XIV).

Fraction 11 contained numerous polar products none of which has been obtained in pure form.

Steroids isolated after solvolysis : The three fractions obtained on chromatography accounted for only 4.5% of bile radioactivity. One product, isolated from the Fraction 2 was identified as 16ß-hydroxy moxestrol (XII) previously obtained by enzymatic lysis (same R_F of the product and of the acetonide). Another product, isolated from Fraction 3 after chromatography in systems N and D, was identified as 15α-hydroxy moxestrol (IIa) obtained by microbiological hydroxylation (same R_F in systems B and G and same mass spectrum of the acetylated derivative IIb).

Products obtained by incubation with microorganisms

Incubation of moxestrol with Gibberella zeae introduced a hydroxyl group into positions 7ß, 15α or 6ß, as evidenced by the mass and NMR spectra of the acetylated derivatives (Ib, IIb and IIIb) isolated as described in Materials and Methods.

Products obtained by chemical synthesis

Oxydation followed by borohydride reduction of 6ß-hydroxy moxestrol (IIIa) gave the 6α-hydroxy derivative

(IVa). A comparison of the NMR spectra of their acetates (IIIb and IVb respectively) confirmed the equatorial or axial position of the hydroxyl group.

In the case of moxestrol, as for estrone (19), oxydation by Fremy salt introduced a hydroxyl group into position 2 (Va) or 4 (VI). The NMR spectra indicated the presence of two protons in positions 1 and 4 for Va and ortho coupling of the protons in positions 1 and 2 for VI.

Methylation of 2-hydroxy moxestrol (Va) by diazomethane gave the fully methylated compound, Vd, and the two partially methylated compounds, Vb and Vc. On the basis of the frequency of protons 1 and 4 of compounds Va, b, c and d, when compared with published data (20), a 2-methoxy-3-hydroxy structure can be attributed to compound Vb and a 2-hydroxy-3-methoxy structure to compound Vc. Furthermore, as for estrone (21) and ethynyl estradiol (22) derivatives, the 2-methylated isomer is less polar than the 3-methylated isomer.

The syntheses of the 3-methyl ether of moxestrol (VII) and of 16α-hydroxy moxestrol (VIII) have been described (17, 23).

DISCUSSION

The reference compounds, listed in Fig.2, were prepared by the above-described chemical and biochemical methods and identified by classic physical and chemical methods.

A proposed scheme for the metabolic fate of moxestrol in the rat bile has been set out in Fig. 3 showing the following biotransformations: Hydroxylation in 2, in 4, in 15α and in 16β; methylation of moxestrol and of the hydroxylated derivatives in some of these positions

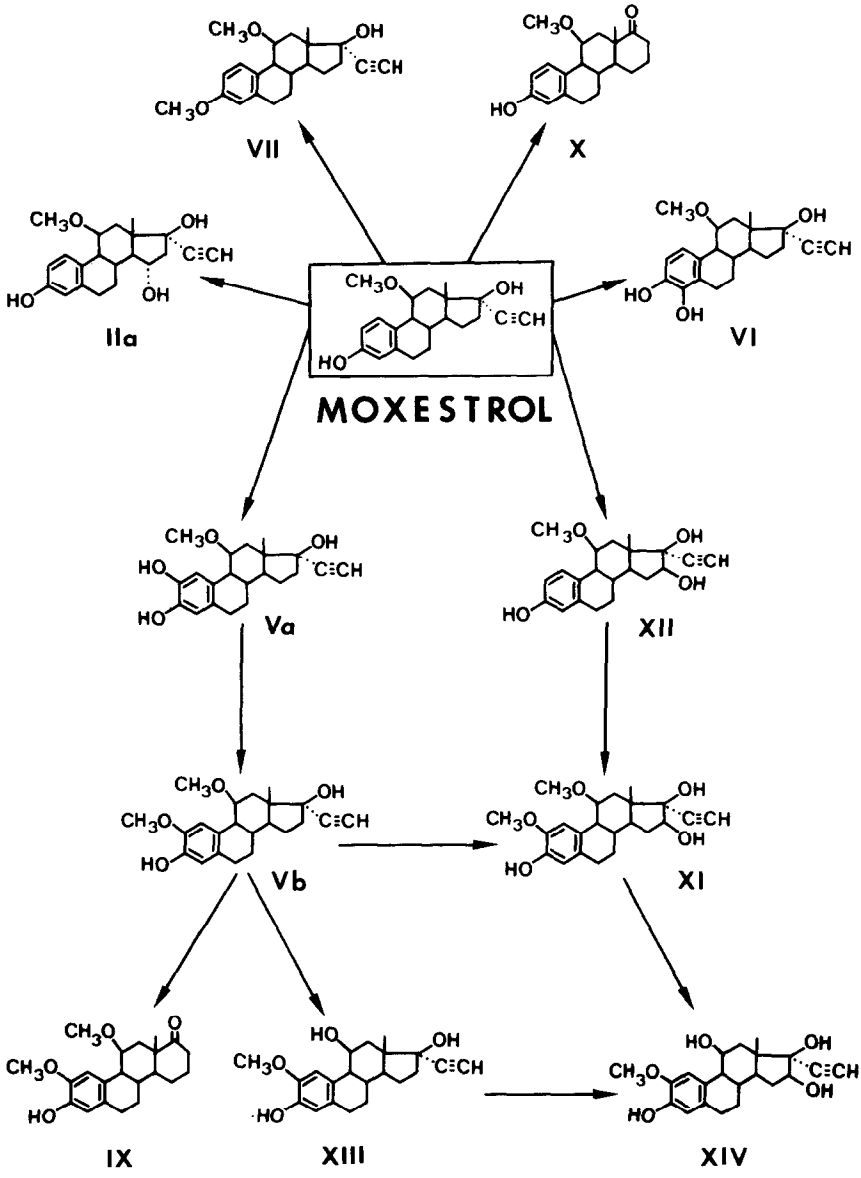


Fig. 3. *The metabolic fate of moxestrol in rat bile*

(e.g. in 2 and 3); demethylation in 11; conversion of the D-ring into a D-homo structure with a keto function.

Microbiological hydroxylation of moxestrol has given the 7β and 15α hydroxy derivatives as well as the 6-hydroxy derivative, a possible metabolite in man by analogy with ethynyl estradiol metabolism. The availability of all these compounds will enable us to identify metabolites in other species (dog, monkey and man) and establish comparative metabolic pathways.

ACKNOWLEDGEMENTS

The expert technical assistance of A. Rullier of the Metabolism Department and of D. Jovanovic and R. Smolik of the Physics Department is gratefully acknowledged.

REFERENCES

1. RAYNAUD, J.P. BOUTON, M.M., GALLET-BOURQUIN, D., PHILIBERT, D., TOURNEMINE, C. and AZADIAN-BOULANGER, G. Mol. Pharmacol., 9, 520-533 (1973)
2. AZADIAN-BOULANGER, G. and BERTIN D. Chimie Thérapeutique, 8, 451-454 (1973)
3. BOUTON, M.M. and RAYNAUD, J.P. Endocrinology, in press (1979)
4. DOUGHTY, C., BOOTH, J.E., McDONALD, P.G. and PARROTT, R.F. J. Endocrinol., 67, 419-424 (1975)
5. McEWEN, B.S., PLAPINGER, L., CHAPTAL, C., GERLACH, J. and WALLACH, G. Brain Res., 96, 400-406 (1975)
6. DEGOS, F., BENHAMOU, J.P. MENARD, J., SALMON, J. and RAYNAUD, J.P. Eur. J. Clin. Invest., 8, 243-248 (1978)
7. SOUTOUL, J.H., MAILLET, M. and ANTHONIOZ, P.H. C.R. Soc. Franc. Gynec., 39, 695-704 (1969)
8. ROBYN, C. VEKEMANS, M., ROZENCWEIG, M., CHIGOT, D. and RAYNAUD, J.P. J. Clin. Pharmacol., 18, 29-34 (1978)
9. FRANCHIMONT, P., LEGROS, J.J. and MEURICE, J. Horm. Metab. Res., 4, 288-292 (1972)
10. BONHOMME, J. Rev. Franç. Gynéc., 70, 519-523 (1975)
11. DELACROIX, P. Gazette Médicale de France, 81, 5463-5467 (1974)

12. GAUTIER, P.J. Gazette Médicale de France, 83, 1346-1351 (1976)
13. BALL, P., GELBKE, H.P., HAUPT, O. and KNUPPEN, R. Hoppe-Seyl. Z. Physiol. Chem., 354, 1567-1575 (1973)
14. BURSTEIN, S., UNGAR, F., GUT, M. and DORFMAN, R.I. Endocrinology, 56, 267-270 (1955)
15. WYNGAARDEN, J.B., PETERSON, R.E. and WOLFF, A.R. J. Biol. Chem., 212, 963-972 (1955)
16. MOSER, W. and HOWIE, R.A. J. Chem. Soc., A, 3039-3047 (1968)
17. BERTIN, D. and PIERDET, A. United States Patent 3 579 545 (1971)
18. CLINTON, R.O., CHRISTIANSEN, R.G., NEUMANN, H.C. and LASKOWSKI, S.C. J. Am. Chem. Soc., 79, 6475-6480 (1957)
19. GELBKE, H.P., HAUPT, O. and KNUPPEN, R. Steroids, 21, 205-218 (1973)
20. FISHMAN, J. and LIANG, J.S. Tetrahedron, 24, 2199-2204 (1968)
21. MARKS, F. and HECKER, E. Biochim. Biophys. Acta, 187, 250-265 (1969)
22. TALAAT-ABDEL-AZIZ, M. and WILLIAMS, K.I.H. Steroids, 15, 695-710 (1970)
23. GASC, J.C. and PIERDET, A. French Patent 2 152 392 (1971)