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STEREOSELECTIVE REDUCTION OF 17α-METHYLTESTOSTERONE DOUBLE BOND *IN VIVO*

TADASHI WATABE, SACHIKO YAGISHITA and SHOJI HARA

Division of Organic Chemistry, Tokyo College of Pharmacy
Ueno-sakuragi, Taito-ku, Tokyo 110, Japan

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Abstract—Urinary tetrahydro metabolites of 17α-methyltestosterone in rabbits have been examined by GLC and TLC for studies on the reduction ratio of its double bond *in vivo* and found that a 5β-steroid was formed preferentially to the corresponding 5α-isomer in the ratio of 4·6 to 1. The tetrahydro metabolites were isolated and identified as 17α-methyl-5β-androstane-3α,17β-diol and 17α-methyl-5α-androstane-3β,17β-diol. None of the other two possible diol isomers, dihydro-3-ketols, and the unchanged steroid were found in any detectable amount in the urinary extract.

17α-METHYLTESTOSTERONE has been shown in our previous paper to be converted to polar 16-oxygenated steroids in rabbits and excreted as glucuronides in significant amounts in their urine.[1] It is interesting that all the polar metabolites are 5β-steroids, of which the major one is a 16β-hydroxy derivative, while testosterone has been known to give an approximately 1:1 mixture of 5α- and 5β-steroids[2] and to be hydroxylated at the 16α-position *in vivo*[3, 4] and *in vitro*.[5, 6] The remarkable difference in the *in vivo* metabolism of 17α-methyltestosterone from that of testosterone seems to be attributable to a steric effect of the 17α-methyl group. Predominant formation of a tetra-hydro 5β-steroid has already been demonstrated by Rongone and Segaloff in a woman with ovarian carcinoma who was given a large dose of 17α-methyltestosterone.[7] Their finding, however, does not necessarily provide direct information about the *in vivo* production ratio of 5α- and 5β-steroid diols which have a slight difference in chromatographic mobilities on adsorbents, since it is based on the quantities of the diols isolated and purified as crystals after repeated adsorption column chromatography. The above mentioned facts have attracted our attention in view of the role of the 17α-methyl group in the *in vivo* reduction of 17α-methyltestosterone double bond and have encouraged us to undertake further investigation on urinary excretion ratio of its tetrahydro metabolites.

MATERIALS AND METHODS

Extraction of urinary metabolites. Five male albino rabbits, weighing 2·5–3·0 kg, were dosed intragastrically with 0·5 g each of 17α-methyltestosterone (Roussel-Uclaf, Paris) suspended in 10% gum arabic solution. The pooled 48-hr urine was adjusted at pH 5·0 with acetic acid, incubated at 38° for 48 hr with a calf liver β-glucuronidase preparation (800 Fishman units/ml urine) and extracted continuously with ether for 20 hr. The ethereal solution was washed successively with 0·5 N HCl, 1 N NaOH and water, dried over anhydrous Na_2SO_4 and evaporated to give 0·94 g of a gum.

Chromatography. GLC was carried out with a Shimadzu model GC-1C gas chromatograph, equipped with a hydrogen flame ionization detector and a glass column, 180 cm long and 4 mm internal diameter, packed with 1·5% SE-30 on Chromosorb W (60–80 mesh). The temperature of the detector bath was 250°, and the flow rate of nitrogen as a carrier gas was 38 ml/min. Samples were dissolved in acetone and injected directly onto the column. Standard calibration curves for the quantitative determination of tetrahydro metabolities were prepared by estimating peak areas corresponding to 0·005–0·06 μmoles of the authentic samples. TLC was carried out using glass plates, 20 × 5 cm^2, coated with silica gel (Wakogel B-5) and solvent mixtures as indicated in Table 1. When the plate was twice developed, the chromatogram obtained after the first development was dried well by blowing of warm air and then developed again by the same solvent system up to the first solvent front. All the chromatograms were visualized by spraying with concentrated sulfuric acid followed by heating. Silica gel (Wakogel C-200) was also used for column chromatography. The gel column was prepared using benzene in such a size that the ratio of its diameter and height was 1:15.

Preparation of steroid samples. 17α-Methylandrostane-3,17-diols and 17β-hydroxy-17α-methylandrostan-3-ones described below are all known compounds and the recorded melting points are not in disagreement with previously published values.

The four diols were prepared by the reaction of the corresponding 3-hydroxy-androstan-17-ones with 2·5 molar equivalent methylmagnesium iodide in absolute ether in the usual manner. Their i.r. spectral data which are not described in the literature and their melting points recorded are as follows: 17α-methyl-5α-androstane-3α,17β-diol (I),[7] m.p. 188–190°, λ_{max}^{KBr} 3367, 1174, 1149, 1085, 1072, 1032, 1014, 1005, 971, 953, 935 cm^{-1}; 17α-methyl-5α-androstane-3β,17β-diol (II),[8] m.p. 212–214°, λ_{max}^{KBr} 3448, 3226, 1166, 1135, 1074, 952, 936 cm^{-1}; 17α-methyl-5β-androstane-3α,17β-diol (III),[9] m.p. 164–165°, λ_{max}^{KBr} 3330, 1163, 1110, 1092, 1089, 1042, 980, 952, 934 cm^{-1}; and 17α-methyl-5β-androstane-3β,17β-diol (IV),[9] m.p. 177–178°, λ_{max}^{KBr} 3436, 1156, 1083, 1036, 981, 943 cm^{-1}.

17β-Hydroxy-17α-methyl-5α-androstan-3-one[10] and 17β-hydroxy-17α-methyl-5β-androstan-3-one[11] were prepared by the previous methods. Homogeneity of all the synthetic steroids was confirmed by TLC and GLC.

RESULTS

Determination of the ratio of 5α- and 5β-methylandrostanediols in the urinary extract

The neutral extract, obtained on administration of 17α-methyltestosterone to male rabbits, followed by treatment of the 48-hr urine with β-glucuronidase, showed three peaks (A, B, and C in Fig. 1) due to its metabolites in the gas chromatogram. The other peaks appearing at smaller retention times were due to naturally occurring materials in the urine. GLC of four authentic 17α-methylandrostanediols, two 3-keto-17α-methylandrostanols, and previously isolated 16-oxygenated steroids[1, 12] under the same conditions indicated that major metabolite peak A corresponded to 17α-methyl-5β-androstane-3α,17β-diol (III) or/and 17α-methyl-5β-androstane-3β,17β-diol (IV), peak B 17α-methyl-5α-androstane-3α,17β-diol (I) or/and 17α-methyl-5α-androstane-3β,17β-diol (II), and peak C the 16-ketosteroid (Table 1). Furthermore, it suggested that neither 17β-hydroxy-17α-methylandrostan-3-ones nor the unchanged steroid were present in any detectable amount in the urinary extract, since these three steroids were distinguishable as isolated peaks from those of the diols when a mixture of all the

authentic specimens were injected. Quantification of the peaks A and B in the chromatogram illustrated in Fig. 1 indicated that the 5β- and 5α-diols exists in the ratio of 4·6:1 and in the quantities of 301 and 65 mg, respectively.

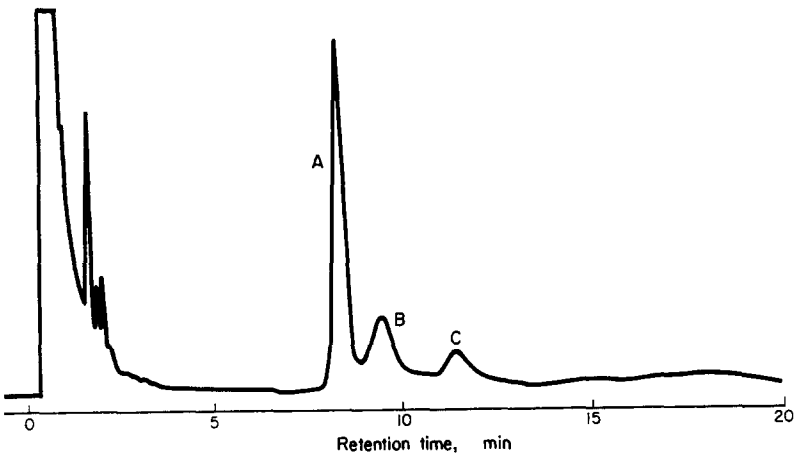

FIG. 1. GLC of the crude neutral extract from rabbit urine after administration of 17α-methyltestosterone. Conditions—column: 1·5% SE–30 on Chromosorb W (60–80 mesh), column temperature: 210°, carrier gas: N_2 (38 ml/min), and detector: FID.

A few other stationary phases, commonly used for the separation of steroids, such as QF-1, could not also differentiate the 3α-diols of both 5α- and 5β-methyl-androstanes from their 3β-epimers.

Identification of the methylandrostanediols

Absence of the unchanged steroid and both of 3-ketomethylandrostanols in the extract was also confirmed by TLC: in the chromatogram, obtained in the System 1 (Table 1), both steroids added to the extract appeared as isolated spots exhibiting characteristic colouration on visualization of the chromatogram.

Attempts to resolve the isomer pairs by silica gel TLC was successful only when each of the four authentic diols was spotted at a different origin and co-chromatographed, but when they were applied as a mixture to the plate, they appeared as an unresolved long single spot. Twice development of the plate, however, increased the difference in their mobilities and was found to give well isolated spots in the chromatogram even when they were applied as a mixture (System 3 in Table 1).

For careful identification of the urinary diols, an aliquot (200 mg) of the extract was subjected to silica gel (10 g) column chromatography and they were separated by eluting the column with benzene containing 4 per cent acetone. After recombination of all the column chromatographic fractions containing the diols, the combined fraction (76 mg) was examined by twice developing TLC (System 3), showing two spots at areas corresponding to diols II and III. But none of the other two isomeric diols, I and IV, were detected. From the results of experiments carried out using synthetic mixtures consisting of various amounts of the diols I and IV and a known amount of

TABLE 1. R_f AND RETENTION TIME (R_t) VALUES OF 17α-METHYLTESTOSTERONE AND ITS DERIVATIVES

Steroid*	R_f on silica gel†			R_t on 1·5% SE-30‡ (min)
	System 1	System 2	System 3	
17α-Me-5α-A-3α,17β-diol (I)	0·50	0·51	0·65	9·6
17α-Me-5α-A-3β,17β-diol (II)	0·48	0·46	0·58	9·6
17α-Me-5β-A-3α,17β-diol (III)	0·45	0·40	0·48	8·4
17α-Me-5β-A-3β,17β-diol (IV)	0·52	0·62	0·75	8·4
17α-Methyltestosterone	0·55	—	—	13·0
17β-Hydroxy-17α-Me-5α-A-3-one	0·70	—	—	10·6
17β-Hydroxy-17α-Me-5β-A-3-one	0·70	—	—	10·0
3α,17β-Dihydroxy-5β-A-16-one	0·37	—	—	11·3
17α-Me-5β-A-3α,16β,17β-triol	0·27	—	—	Not eluted
17α-Me-5β-A-3α,16α,17β-triol	0·12	—	—	Not eluted

* Abbreviations used are A: androstane and Me: methyl.
† System 1: Benzene–Acetone (4:1);
System 2: Benzene–Ethyl acetate (2:1);
System 3: twice developed by the same system as 2.
‡ Conditions used are same as indicated in Fig. 1.

1:4·6 ratio mixture of the diols II and III, it was found that the diols I and IV could not be present in the urinary diol fraction in larger ratios than 0·25 and 0·4 to 10 of the diol III, respectively.

Taking 60 mg of the urinary diol mixture and subjecting it to a large scale of column chromatography (50 g of silica gel), using benzene and increasing ratios of ethyl acetate, the diols II and III were obtained as pure states in yields of 8 mg and 22 mg, respectively, and a larger part of the mixture remained unresolved. Identity of the purely isolated diols with the corresponding authentic specimens was confirmed by the mixed melting point test and by superimposability of their i.r. spectra.

DISCUSSION

In addition to our previous work concerning polar 16-oxygenated 5β-metabolites of 17α-methyltestosterone,[1] the present studies establish that *in vivo* reduction of the double bond produces 5β-methylandrostanediol preferentially. From these studies, it is evident that all representative urinary metabolites, as far as detected by TLC and identified, have 5β-configuration. Prior to our investigation, Rongone and Segaloff have also obtained a similar result with urinary diol metabolites of this steroid, administered to a woman with ovarian carcinoma.[7] However, we would like to point out that their investigation might be incomplete by following reasons: first, on the basis of our experience in silica gel column chromatography of 17α-methylandrostane-3,17β-diols, even when a much larger adsorbent column was used, quantitative resolution of their two diols under the conditions described by them was found to be very difficult and incidentally, any extent of contamination of a 5α- or 5β-diol is almost impossible to be removed from its 3-epimer by fractional crystallization. Secondly, although as mentioned in the present studies, only careful TLC results in successful resolution and identification of the four steroid diols simultaneously, they did not try to search the urinary extract for any possibility of presence or absence of the other two isomeric diols. And thirdly, GLC which is the best tool, as far as widely accepted, for

obtaining the information about the exact ratio of 5α- and 5β-steroid isomers, was not used in their investigation.

The preferential formation of the 5β-diol may imply a steric effect of the methyl group on the *in vivo* reduction of the double bond since testosterone is well known to give an equal ratio mixture of 5α- and 5β-steroids *in vivo*.[2] In neither biological nor chemical reduction of steroid 4-double bond has been known such long range steric hindrance of an alkyl substituent in ring D, although as only one biological instance, modification of 19-nortestosterone by substitution of methyl group for 1α-hydrogen atom has been demonstrated to result in a marked change in the ratio of 5α- and 5β-reduction *in vivo*: in contrast with the unsubstituted norsteroid whose double bond is reduced in the equal ratio,[13] the modified one is converted preferentially to the corresponding 5β-derivative.[14]

Considering the above mentioned facts, it might be predictable that steroid Δ^4-5α-reductase[15] is susceptible to steric hindrance of such small substituent as methyl group located not only in ring A, but also in ring D. In other words, the reductase might require the unsubstituted α-side of the steroid molecule, at least with rings A and D, for the maximal interaction with the substrate *in vivo*.

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