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DHT FORMATION AND DEGRADATION IN CULTURED HUMAN SKIN FIBROBLASTS: DHT ACCUMULATION IN THE GENITAL SKIN

ISAO MORIMOTO,[1]* SUMIYA ETO,[1] SHUNJI INOUE,[2] MOTOMORI IZUMI,[2] SHIGENOBU NAGATAKI,[2] YUTAKA SAITO[3] and TANETOSHI HARA[4]

[1]The First Department of Internal Medicine, School of Medicine, University of Occupational and Environmental Health, 1-1, Iseigaoka, Yahatanishi-Ku, Kitakyushu; [2]The First Department of Internal Medicine and [3]Department of Urology, Nagasaki University School of Medicine, Nagasaki and [4]Department of Urology, Nagasaki City Hospital, Nagasaki, 852 Japan

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Summary—The conversion of testosterone to dihydrotestosterone (DHT) by 5α-reductase and the interconversion between DHT and 5α-androstane-3α,17β-diol (3α-diol) by 3α-hydroxysteroid oxidoreductase (3α-HSOR) were studied in fibroblasts derived from the genital skin of 22 males and 6 females, and from the nongenital skin of 19 males and 9 females with normal gonadal function. The formation of DHT from testosterone (5α-reduction) was significantly greater in fibroblasts from genital skin than in those from nongenital skin in both males (2.15 ± 1.43 vs 0.81 ± 0.46 pmol/mg protein/h, mean ± SD, $P < 0.001$) and females (2.52 ± 1.99 vs 0.69 ± 0.18, $P < 0.01$). Furthermore, DHT formation from 3α-diol (3α-HSOR oxidation) was also significantly greater in genital skin fibroblasts than in nongenital skin fibroblasts of males (5.47 ± 3.37 vs 2.52 ± 1.74 pmol/mg protein/h, $P < 0.01$). However, the degradation of DHT to 3α- and/or 3β-diol (3α- and/or 3β-HSOR reductions) was not different between genital and nongenital skin fibroblasts of either males or females. Respective ratios of DHT formation to DHT degradation (5α-reduction/3α-HSOR reduction, 3α-HSOR oxidation/3α-HSOR reduction) were also significantly greater ($P < 0.002$) in genital skin fibroblasts than in nongenital skin fibroblasts of males. On the other hand, both DHT formation and degradation were not different between male and female genital skin fibroblasts. These results suggest that the increased production of DHT in genital compared to nongenital skin results from increased 5α-reduction and 3α-HSOR oxidation.

INTRODUCTION

Secreted testosterone is peripherally converted into dihydrotestosterone (DHT) by 5α-reductase and then acts on the nucleus. It is then metabolized in nongonadal tissues to 5α-androstane-3α,17β-diol (3α-diol) by 3α-hydroxysteroid oxidoreductase (3α-HSOR) [1]. DHT plays a central role in male phenotypic differentiation during fetal life and in the development of male sexual characteristic appearance in certain tissues postnatally [2]. 3α-Diol appears to be a potent androgen and can induce benign prostatic hypertrophy [3]. In these experiments, it was suggested that the prostatic hypertrophy resulted from accumulation of DHT backconverted from 3α-diol in the prostate. Our previous study of *in vivo* androgen kinetics [4] indicated that the conversion of DHT to 3α-diol is reversible and a large amount of 3α-diol backconverts to DHT. These reactions (testosterone→DHT⇄3α-diol) occurred in extrasplanchnic target tissues [5, 6].

Cultured skin fibroblasts have been used to study androgen metabolism [7–14]. Testosterone is converted to DHT by 5α-reductase in skin [15, 16]. Wilson [10] demonstrated reduced 5α-reductase activity in skin fibroblasts obtained from male patients with 5α-reductase deficiency. Other studies had demonstrated that greater activity of 5α-reductase was observed in genital skin fibroblasts than in nongenital skin fibroblasts [10, 11, 17], and a similar result was observed in androgen metabolism by skin slices [16]. Thus, skin fibroblasts retain the metabolic characteristics of the skin from which they are derived.

This is the first report on consecutive studies of the conversion of testosterone to DHT (5α-reduction) and the interconversion between DHT and 3α-diol (3α-HSOR) using genital and nongenital skin fibroblasts. Our results indicate

*To whom correspondence should be addressed.

that DHT accumulation in genital skin fibroblasts is not only the result of increased 5α-reduction but also increased 3α-HSOR oxidation.

MATERIALS AND METHODS

Materials

[1,2-^{3}H]testosterone (SA, 57 Ci/mmol), [4-^{14}C]DHT (SA, 57.7 mCi/mmol), [1.2-^{3}H]3α-diol (SA, 24 Ci/mmol) and [4-^{14}C]DHT (SA, 57.7 mCi/mmol) were purchased from New England Nuclear Corporation (Boston, MA). These radioactive steroids were purified by Sephadex LH-20 microcolumn (7 × 100 mm) using a benzene–methanol (85:15 v/v) solvent system. [4-^{14}C]3α-diol was prepared by sodium borohydride reduction of [4-^{14}C]DHT [3] and was then purified by aluminum oxide thin layer chromatography (TLC) with benzene–methanol (96:4). Nonradioactive steroids were obtained from Sigma Chemical Company (St Louis, MO). Precoated silica gel F_{254} plates and aluminum oxide F_{254} plates for TLC were purchased from Merk (Darmstadt, F.R.G.). Eagle's minimal essential medium (MEM) and fetal calf serum (FCS) were purchased from Gibco (Chagrin Falls, OH), and trypsin and EDTA solutions were from Nakarai Chemical (Tokyo, Japan). Tissue culture plates were obtained from Falcon (Lincoln Park, NJ).

Cell culture and assays

The cell strains of fibroblasts used in this study were derived from genital or nongenital skin of patients undergoing various types of surgery. Genital skin specimens were obtained from the foreskin or scrotum of 22 male adult patients (age 36 ± 13 yr, mean ± SD) undergoing various urological surgical procedures (phimosis, retentio testis, benign prostatic hypertrophy, prostatic cancer and tumor of the kidney and urinary bladder), and from the intact region of the labia majora of 6 females (age 56 ± 10 yr) with vulvar dystrophy (lichen sclerosis and leukoplakia). Nongenital skin specimens were obtained from the skin of the abdomen, neck and upper arm of 19 males (age 41 ± 14 yr, mean ± SD) and 9 females (age 35 ± 16 yr) at surgery for various conditions (cholelithiasis, tumor of the stomach and large intestine, hyperthyroidism, thyroid tumor and fracture of the upper arm). Gonadal function of the above patients was clinically normal.

The culture of fibroblasts was done as described by Wilson [10] and our previous report [17]. Fibroblasts were maintained in a humidified CO_2 incubator at 37°C in 75 cm flasks containing 10 ml Eagle's MEM supplemented with 10% (v/v) FCS, 100 units/ml penicillin, 100 μg/ml streptomycin and 24 mM NaHCO, at a pH of 7.4. One week after the initial cell growth, the cells were subcultured with 0.05% trypsin and 0.05% EDTA dissociation. Assays of androgen metabolism in this study were performed using fibroblasts of the 3rd and 4th subculture stages.

For assays, fibroblasts were seeded at a concentration of approx. 1×10^5 cells per 5 ml of medium in 60 mm Petri dishes. On the third culture day, the medium was changed and on the sixth culture day, the cells were incubated with medium minus FCS. The cells were confluent (average cell density approx. 1×10^6 cells/dish) on the seventh day, and the monolayers were used for the assays. The cells were washed with MEM without FCS twice and then incubated with 1 ml of MEM with respective tritiated steroids for substrate. After incubation for 2 h at 37°C in a humidified CO_2 incubator, the medium was removed for purification. The cells were dissolved in 1 ml of 0.1 N NaOH and the protein content determined by the Bio Rad Protein Assay Kit (Richmond, CA). Blanks were run for each assay using medium containing tritiated steroids incubated in culture dishes without cells.

Purification of steroids

Each medium containing radioactive steroids was extracted three times with 5 ml of ethyl ether after the addition of a known quantity of ^{14}C steroids (3000–6000 cpm) to monitor recovery. Extracts were evaporated to dryness and the residues redissolved in 30 μl of ethanol containing 10 μg of authentic nonradioactive steroids, and these were purified by the following procedures [18]: the purification of DHT in the study of 5α-reduction was performed by two-step silica gel TLC using chloroform–methanol (98:1.75) followed successively using chloroform–ethyl acetate–methanol (85:15:3). In the study of the conversion of 3α-diol to DHT (3α-HSOR oxidation), steroids were chromatographed on thin layer silica gel using a chloroform–methanol (98:1.75) solvent system, and then rechromatographed to separate DHT from androsterone using aluminum oxide plates (type F_{254}) with a solvent system of methylene

chloride–diethyl ether (9:1) [19]. In the study of the conversion of DHT to 3α- and/or 3β-diol (3α- and/or 3β-HSOR reductions), the purification of 3α- and 3β-diol was performed by the two-step TLC procedure used for the purification of DHT in the study of 5α-reduction. After the 2nd chromatograph, the areas corresponding to the 3α- and 3β-diols were scraped off and eluted by ethyl acetate. One-third of the elutions were counted for the formation of (3α + 3β)-diol and two thirds were rechromatographed for the separation between 3α- and 3β-diol on aluminum oxide plates using a benzene–methanol (96:3.5) solvent system. After the final chromatograph, the areas corresponding to DHT, 3α- and 3β-diol were eluted into vials with ethyl acetate. The samples were dried, scintillation fluid added and these radioactivities counted in a scintillation counter (Aloka LSC-903, Tokyo, Japan). The amount of each steroid produced, corrected for recovery losses, were expressed as pmol/mg protein/h. The amount of 3β-diol produced was obtained by subtraction of 3α-diol formation from (3α + 3β)-diol formation. The scintillation counter had a ^{3}H counting efficiency of 43% and a ^{14}C efficiency of 66%. The ^{14}C-to-^{3}H feedthrough was 14.8% and the ^{3}H-to-^{14}C feedthrough was less than 0.01%. The radiochemical purity of each steroid produced was confirmed by a constant ^{3}H-to-^{14}C ratio after crystalization and acetylation [5, 17, 18].

Statistical analyses

All data are expressed as the mean ± SD or the mean ± SEM. Statistical analysis of significance was done by Student's t-test.

RESULTS

Evaluation of methods

Initial studies were designed to establish satisfactory assay conditions in cultured fibroblasts for effective measurement of DHT formation and degradation (5α-reduction, 3α- and/or 3β-HSOR reductions and 3α-HSOR oxidation). The relationships between the amount of substrate added and the formation of metabolic products by genital skin fibroblasts are shown in Fig. 1. When the amount of substrate in the three assays was increased from 0.005 to 2 μM, the rates of metabolite formation rose linearly until a plateau was attained. DHT formation from testosterone (5α-reduction) or 3α-diol (3α-HSOR oxidation) approached a plateau at a substrate concentration of about 0.25 μM, and either 3α- or (3α + 3β)-diol formation from DHT [3α- or (3α + 3β)-HSOR reduction] was at a substrate concentration of about 0.50 μM. The K_m was 0.08 μM for the 5α-reduction, 0.125 μM for the 3α-HSOR oxidation and 0.20 μM for the 3α- or (3α + 3β)-HSOR reduction.

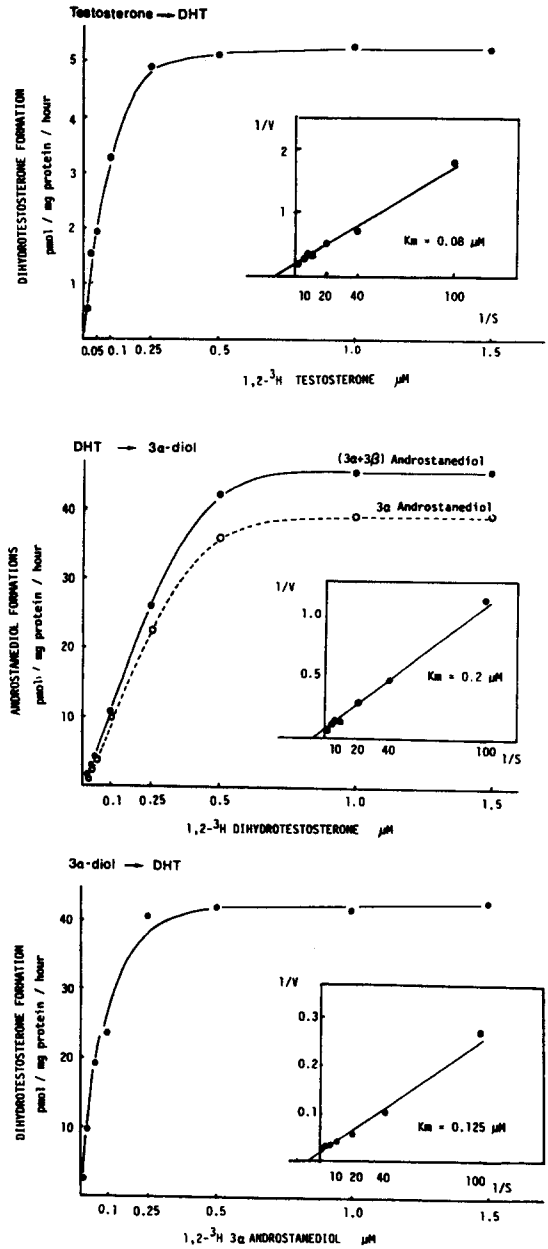

Fig. 1. Steroid formation in genital skin fibroblasts (No. 48, 2-yr-old male) incubated with various concentrations of substrate. The formation of DHT from testosterone (upper), 3α- or (3α + 3β)-diols from DHT (middle), and DHT from 3α-diol (lower) are shown. Each point is the mean of duplicate determinations.

When fibroblasts were incubated with 0.1 μM of substrate, steroid metabolite formation in the

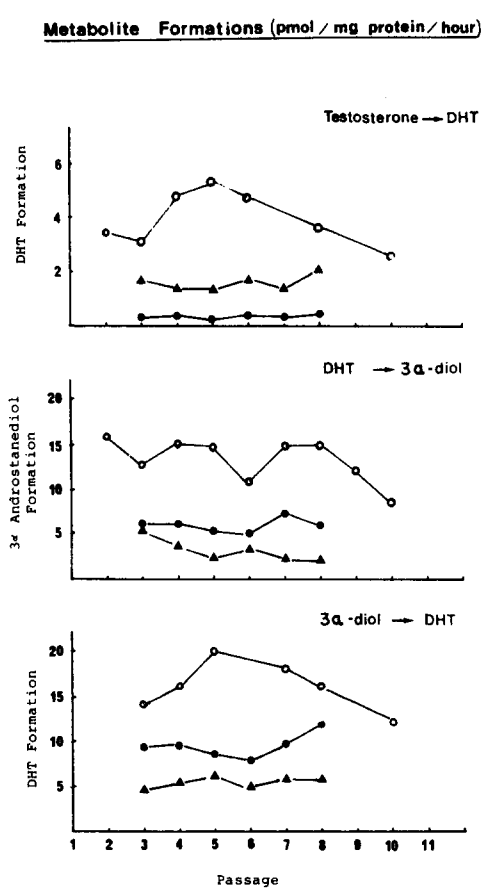

Fig. 2. Metabolite formation over serial subcultures. Fibroblasts were incubated with 0.1 μM of each substrate for 2 h. The formation of DHT from testosterone (upper), 3α-diol from DHT (middle) and DHT from 3α-diol (lower) are shown. Each point is the mean of duplicate determinations. ○ = Genital skin fibroblasts (No. 48, 2-yr-old male); ▲ = genital skin fibroblasts (No. 46, 6-yr-old male); and ● = nongenital skin fibroblasts (No. 15, 33-yr-old male).

three systems increased in a time dependent manner for 4 h (results not shown). In studies done on the relationship between the growth phase of fibroblasts and the formation of steroid metabolites, the formation of these metabolites in three assays increased linearly during the growth phase (results not shown) [17]. To determine the effect of serial subcultures of fibroblasts on the formation of steroid metabolites, assays of the three enzyme systems were performed at various subculture stages. Formation of respective metabolites did not change significantly between the 3rd and 8th subculture stages (Fig. 2). Similar results were observed in nongenital skin fibroblasts. Standard assays for the three enzyme systems were performed 7 days after seeding fibroblasts at the 3rd and 4th subcultures. The substrate concentration was 0.1 μM and the incubation for 2 h in each assay.

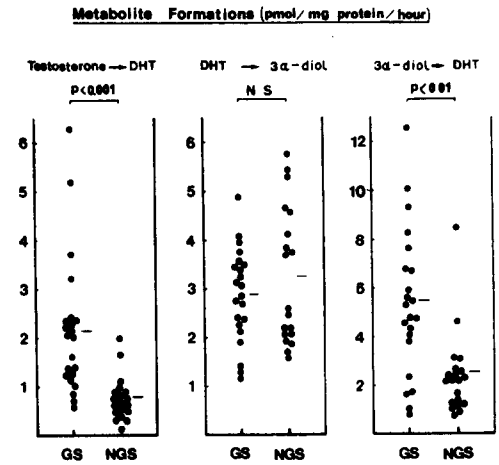

Fig. 3. DHT formation and degradation (testosterone→DHT⇄3α-diol) in 22 male genital skins (GS) and 19 male nongenital skins (NGS) incubated with 0.1 μM of steroids for substrate. Each point represents the mean value obtained from respective assays using the 3rd and 4th subcultured fibroblasts. The horizontal lines indicate the mean value in each group.

Androgen metabolism of cultured skin fibroblasts

The quantitative rates of androgen metabolism in skin fibroblasts obtained from different subjects are shown in Table 1 and Fig. 3. The results are divided into the activities which produced DHT and those which removed it. In the study of DHT formation from testosterone (5α-reduction), DHT formation of the genital skin fibroblasts from 22 males was 2.15 ± 1.43 pmol/mg protein/h (mean $\pm$ SD) and was significantly greater than that of the nongenital skin fibroblasts from 19 males, 0.81 ± 0.46 pmol/mg protein/h ($P < 0.001$). In

Table 1. DHT formation and degradation in cultured genital and nongenital skin fibroblasts derived from either males or females incubated with 0.1 μM of substrates (mean $\pm$ SD)

		Forming DHT		Removing DHT		
	No.	T→DHT (pmol/mg protein/h)	3α-diol→DHT	DHT→3α-diol	DHT→3β-diol (pmol/mg protein/h)	DHT→(3α + 3β)-diol
Males						
Genital skin	22	2.15 ± 1.43**	5.47 ± 3.37*	2.87 ± 0.94	1.05 ± 0.54	3.94 ± 1.26
Nongenital skin	19	0.81 ± 0.46	2.52 ± 1.74	3.28 ± 2.17	1.17 ± 0.63	4.46 ± 1.62
Females						
Genital skin	6	2.52 ± 1.99*	4.60 ± 4.10	2.64 ± 0.31	1.16 ± 0.42	3.75 ± 0.59
Nongenital skin	9	0.69 ± 0.18	4.46 ± 3.41	3.52 ± 2.71	0.84 ± 0.43	4.75 ± 2.87

**$P < 0.001$, *$P < 0.01$.

skin fibroblasts from females, DHT formation from testosterone in the genital skin was also significantly greater than that found in the nongenital skin (2.52 ± 1.99 vs 0.69 ± 0.18, $P < 0.01$).

DHT formation from 3α-diol (3α-HSOR oxidation) was also significantly higher in male genital skin fibroblasts than in male nongenital skin fibroblasts (5.47 ± 3.37 vs 2.52 ± 1.74 pmol/mg protein/h, mean ± SD, $P < 0.01$). However, no difference of DHT formation from 3α-diol was noted between the genital and nongenital skin fibroblasts of females.

In the study of DHT degradation, 3α- and/or 3β-diol formation from DHT did not differ significantly between fibroblasts derived from the genital skin and nongenital skin of either males or females (Table 1).

No statistical differences of DHT formation and DHT degradation were noted between the genital skin fibroblasts derived from males and females, and the similar results were found in nongenital skin fibroblasts.

To evaluate a difference in individual androgen metabolism (testosterone→DHT⇄3α-diol) between fibroblasts derived from genital and nongenital skin, the ratios of enzyme activities forming DHT to those removing DHT were determined (index of net formation of DHT in fibroblasts) (Fig. 4). The ratio of 5α-reduction to 3α-HSOR reduction (testosterone→DHT/DHT→3α-diol) was significantly greater in genital skin fibroblasts than that found in nongenital skin fibroblasts of either males (0.88 ± 0.76 vs 0.30 ± 0.24, mean ± SD, $P < 0.002$) or females (0.93 ± 0.68 vs 0.29 ± 0.14, $P < 0.05$). The ratio of 3α-HSOR oxidation to 3α-HSOR reduction (3α-diol→DHT/DHT→3α-diol) was also significantly greater in the genital skin fibroblasts than in the nongenital skin fibroblasts of male (2.16 ± 1.16 vs 0.75 ± 0.34, mean ± SD, $P < 0.001$), but not of females (1.83 ± 1.40 vs 1.75 ± 1.66).

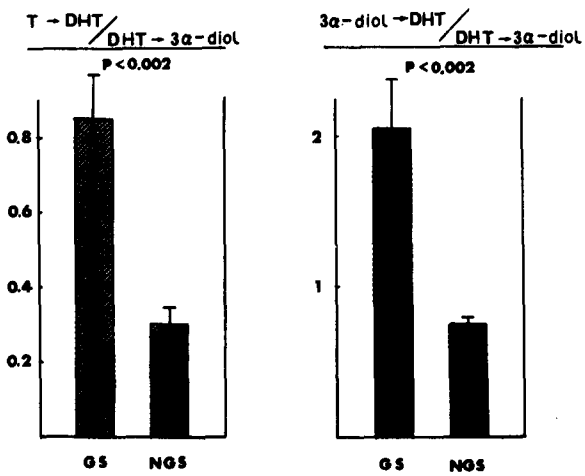

Fig. 4. The ratio of DHT formation to DHT degradation in cultured 22 male genital (GS) and 19 nongenital skins (NGS) fibroblasts. DHT formation is the conversion of testosterone to DHT (5α-reduction) or that of 3α-diol to DHT (3α-HSOR oxidation) and DHT degradation is the conversion of DHT to 3α-diol (3α-HSOR reduction). The vertical bars represent the mean ± SEM.

DISCUSSION

Lamberigt *et al.* [20] and Mowszowicz *et al.* [21] had previously reported that 5α-reductase activity in fibroblasts increased during serial subcultures. In this study, however, no significant changes in DHT formation and degradation were found during the early subculture stages. Despite this, assays for 5α-reduction, 3α-oxidation and 3α- and/or 3β-reductions were done simultaneously using fibroblasts of the same subculture stage, to ensure constancy of the data. Rates of metabolite formation in respective assays were increased linearly from 0.01 to 0.25 μM of substrate, and the three assays in this study were done at 0.1 μM of each steroid for substrate.

In this study of androgen metabolism in cultured skin fibroblasts, DHT formation from testosterone (5α-reduction) was significantly greater in genital skin fibroblasts than in nongenital skin fibroblasts of either males or females as previously described by several investigators [10, 17, 21] and consistent with the concept that DHT plays a central role in the development of the external genitalia and urogenital sinus [2]. These studies indicate that skin is the tissue where DHT is converted from testosterone and that skin fibroblasts retain the original function of skin from which they are derived. In our experiments, interesting results were observed in studies of the interconversion of DHT and 3α-diol in male skin fibroblasts. Formation of DHT from 3α-diol (3α-HSOR oxidation) in genital skin fibroblasts was significantly greater than that in nongenital skin fibroblasts. On the other hand, degradation of DHT to 3α- and/or 3β-diol was not different between genital and nongenital skin fibroblasts of either males or females.

Ratios of DHT formation and DHT degradation (5α-reduction/3α-HSOR reduction, 3α-HSOR oxidation/3α-HSOR reduction) were

significantly greater in genital skin fibroblasts than in nongenital skin fibroblasts. In female genital skin fibroblasts, DHT formation from 3α-diol was not statistically different from that in nongenital skin. This may result from a small number of female samples and a difference of age distribution in female samples as compared with male samples.

DHT formation and degradation in this study did not differ between male and female genital skin fibroblasts. Our previous study [17] demonstrated that 5α-reductase activity did not differ significantly between genital skin of males and females, and 5α-reductase activity of skin fibroblasts from hypogonadal males was similar to that from normal males. Therefore, enzyme activities in skin are not affected by the sex or hormonal status of donors.

In conclusion, this *in vitro* study indicates that the increased production of DHT in genital compared to nongenital skin results from a combination of increased 5α-reduction of testosterone and increased 3α-HSOR oxidation of 3α-diol.

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