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BIOTRANSFORMATION OF TESTOSTERONE AND OTHER ANDROGENS BY SUSPENSION CULTURES OF *NICOTIANA TABACUM* "BRIGHT YELLOW"*

MASAO HIROTANI and TSUTOMU FURUYA

School of Pharmaceutical Sciences, Kitasato University, Minato-ku, Tokyo, Japan

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Key Word Index—*Nicotiana tabacum* "Bright Yellow"; Solanaceae; tissue culture; biotransformation; esterification; glucosylation; steroid; testosterone; Δ^4-androstene-3,17-dione; 5α-androstane-3β,17β-diol; epiandrosterone; 5α-androstan-17β-ol-3-one; epiandrosterone palmitate; 5α-androstane-3β,17β-diol dipalmitate; epiandrosterone glucoside; 5α-androstane-3β,17β-diol 3- and 17-monoglucosides; testosterone-glucoside.

Abstract—It has been shown that the cultured cells of *Nicotiana tabacum* "Bright Yellow" are capable of transforming testosterone to Δ^4-androstene-3,17-dione, 5α-androstan-17β-ol-3-one, 5α-androstane-3β,17β-diol, its dipalmitate and 3- and 17-monoglucosides, epiandrosterone, its palmitate and glucoside, testosterone glucoside. 5α-Androstane-3β,17β-diol dipalmitate and 3- and 17-monoglucosides, epiandrosterone palmitate and glucoside, and testosterone glucoside have been found for the first time as metabolites of testosterone in plant systems. Δ^4-Androstene-3,17-dione was converted to testosterone. 5α-Androstan-17β-ol-3-one, which has been recognized as an active form of testosterone in mammals, was also detected. It has also been demonstrated that [4-^{14}C]testosterone is actively incorporated in these transformations.

INTRODUCTION

THERE is little information on the biotransformation of foreign steroids by cultured plant cells. However, tobacco, *Sophora* and *Digitalis* cells can convert the C_{21} steroids progesterone[1,2] and pregnenolone[1] and the cardiac glycoside digitoxin,[3] while these cells cannot transform the C_{27} sterol cholesterol, the C_{28} ergosterol and the C_{29} sitosterol.[4]

We now wish to report the biotransformation by cultured tobacco cells of the C_{19} steroid testosterone (**1**)[5] to Δ^4-androstene-3,17-dione (**2**), 5α-androstan-17β-ol-3-one (**3**), 5α-androstane-3β,17β-diol (**5**), its dipalmitate (**7**) and 3- and 17-monoglucosides (**8a,b**), epiandrosterone (**6**), its palmitate (**9**) and glucoside (**10**) and testosterone glucoside (**11**). The time

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* Part XXII in the series "Studies on Plant Tissue Cultures". For Part XXI see SYŌNO, K. and FURUYA, T. (1974) *Plant & Cell Physiol.* **15**, 7.

[1] FURUYA, T., HIROTANI, M. and KAWAGUCHI, K. (1971) *Phytochemistry* **10**, 1013.

[2] FURUYA, T., KAWAGUCHI, K. and HIROTANI, M. (1973) *Phytochemistry* **12**, 1621.

[3] FURUYA, T., HIROTANI, M. and SHINOHARA, T. (1970) *Chem. Pharm. Bull.* (*Tokyo*) **18**, 1080.

[4] Unpublished data.

[5] FURUYA, T., SYŌNO, K., KOJIMA, H., HIROTANI, M., IKUTA, A., HIKICHI, M., KAWAGUCHI, K. and MATSUMOTO, K. (1972) Proc. IV. IFS: *Ferment. Technol. Today* 705.

course of the metabolic products using [4-^{14}C]testosterone has been observed and a pathway of testosterone metabolism proposed.

RESULTS

Testosterone (**1**) (total 960 mg) was administered to tobacco suspension callus culture and after 23 days growth with continuous shaking the callus was harvested. The $CHCl_3$-soluble fraction of the callus contained several transformation products and was chromatographed on silica gel (see Experimental). Fraction B gave (**7**), $C_{51}H_{92}O_4$, m.p. 79–80°, the IR showed ester absorption bands at 1740 and 1177 cm^{-1}. On alkaline hydrolysis, 5α-androstane-3β,17β-diol (**5**) and palmitic acid were obtained. Therefore, (**7**) is an ester of 5α-androstane-3β,17β-diol with palmitic acid and this was confirmed by comparison (m.m.p., IR, MS and NMR) with synthetic 5α-androstane-3β,17β-diol dipalmitate.

Fraction D gave (**9**), $C_{35}H_{60}O_3$, m.p. 78–79°. The IR showed absorption bands at 1735 and 1180 (COO) cm^{-1} and the MS gave the main fragment peaks at *m/e* 528 [M^+] and 272 [$M^+–C_{15}H_{31}COOH$], thus indicating that (**9**) is fatty acid ester of sterol. Alkaline hydrolysis gave epiandrosterone (**6**) and palmitic acid. Structure (**9**) was confirmed by comparison (m.m.p., IR, MS and NMR) with synthetic epiandrosterone palmitate. The presence of several transformation products such as Δ^4-androstene-3,17-dione (**2**; R_f 0·53), 5α-androstan-17β-ol-3-one (**3**; R_f 0·44), 5α-androstane-3β,17β-diol (**5**; R_f 0·26), epiandrosterone (**6**; R_f 0·39) was detected in fractions F, G and I by TLC and GLC; (**5**), m.p. 161–162° and (**6**), prisms, m.p. 166–167° were isolated.

After administration of Δ^4-androstene-3,17-dione (**2**) and epiandrosterone (**6**) respectively, $CHCl_3$ extracts were prepared, as described above for testosterone, and the transformation products detected (Table 1).

TABLE 1. BIOTRANSFORMATION OF TESTOSTERONE AND OTHER ANDROGENS BY TOBACCO SUSPENSION CULTURES

	Product										
Substrates	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
Testosterone (**1**)		+*	+		+	+	+	+	+	+	+
Δ^4-Androstene-3,17-dione (**2**)	+		+		+	+	+	+	+	+	
Epiandrosterone (**6**)									+	+	

* Present.

Fraction K (86·1 mg), after acetylation, was rechromatographed on silica gel, and gave the acetates of (**8**), (**10**) and (**11**). MS and GLC data suggest that (**8**) acetate, m.p. 202–203° is a mixture of 5α-androstane-3β,17β-diol 3-β-D-glucoside (**8**a) pentaacetate and 5α-androstane-3β,17β-diol 17-β-D-glucoside (**8**b) pentaacetate. The IR spectrum of (**10**) acetate, $C_{33}H_{48}O_{11}$, m.p. 193–194°, had absorption bands at 1750 (COO), 1735 (CO), 1240 and 1040 (COO) cm^{-1}. The main mass fragment peaks were observed at *m/e* 331 [$C_{14}H_{19}O_9$] and 272 [$M^+–C_{14}H_{20}O_{10}$]. The peak at *m/e* 331 suggested that (**10**) acetate is a tetraacetyl-β-D-glucopyranoside.[6] The NMR analysis of (**10**) acetate showed an anomeric proton doublet $\delta = 4{\cdot}58$ (1H) with a coupling constant of 7·0 Hz, indicating that sugar has the β-configuration.[7] On acid hydrolysis of (**10**) acetate, epiandrosterone (**6**) and glucose were obtained.

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[6] BIEMANN, K., DEJOHGH, D. C. and SCHNOES, H. K. (1963) *J. Chem. Soc.* **85**, 1763.

[7] LEMIEUX, R. V. and STEVENS, J. D. (1965) *Can. J. Chem.* **43**, 2059.

Therefore, the acetate is epiandrosterone tetraacetylglucopyranoside and this was confirmed by m.p. and spectral comparison with synthetic epiandrosterone-β-D-glucoside (**10**) tetraacetate. The UV spectrum of (**11**) acetate, $C_{33}H_{46}O_{11}$, m.p. 166–167°, showed absorption at λ_{max}^{EtOH} 241. IR absorption peaks were found at 1750 (COO) and 1675 (C = C–CO) cm^{-1}. The main mass fragment peaks were observed such as *m/e* 618 [M^+], 331 [$C_{14}H_{19}O_9$] and 271 [$M^+-C_{14}H_{19}O_{10}$]. The peak at *m/e* 331 suggested that (**11**) acetate is a tetraacetyl-β-D-glycopyranoside. The NMR analysis of (**11**) acetate showed an anomeric proton doublet $\delta = 4{\cdot}50$ (1H) with a coupling constant of 7·5 Hz, indicating that sugar has the β-configuration. Acid hydrolysis of (**11**) acetate afforded testosterone (**1**) and glucose. Therefore, this acetate is testosterone tetraacetylglucopyranoside and this was confirmed by direct comparison with synthetic material. After incubation of [4-^{14}C]testosterone with tobacco suspension culture the transformation products such as 5α-androstane-3β,17β-diol (**5**) and epiandrosterone (**6**) reached their maximum on the 5th day and thereafter steadily decreased (Fig. 1). In contrast, the palmitates and glucosides reached the maximum on about the 8th day. The incorporation of [4-^{14}C]testosterone into palmitates and glucosides remained level until the 14th day (Fig. 1).

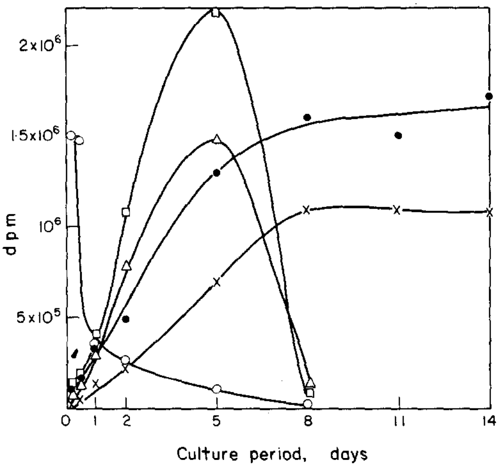
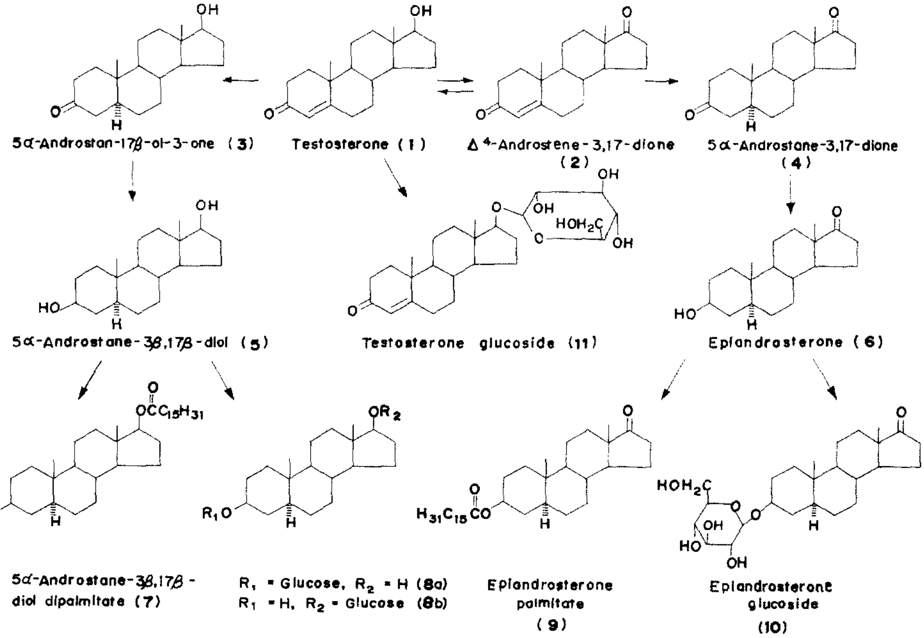


FIG. 1. TIME COURSE OF THE METABOLIC PRODUCTS OF TESTOSTERONE: ○——○, testosteron fraction; □——□, 5α-androstane-3β,17β-diol fraction; △——△, epiandrosterone fraction; ×——×, palmitate fraction; ●——●, glucoside fraction.

DISCUSSION

In our laboratory, we have hitherto studied the biotransformation of progesterone,[1,2] pregnenolone,[1] digitoxin,[3] cholesterol, sitosterol and ergosterol in suspension culture of several species of higher plants. Progesterone, pregnenolone and digitoxin gave some transformation products, but we have been unable to detect metabolic products from cholesterol, sitosterol and ergosterol.[4] We have now found that testosterone (**1**), Δ^4-androstene-3,17-dione (**2**) and epiandrosterone (**6**) are metabolized by tobacco suspension culture effectively. These results suggest that suspension callus cultures have the ability to metabolize steroids lacking a long side chain at C-17 more effectively than those having such a long side chain. These results agree with data reported by Schneider[8] that glucosides were not formed from cholesterol and cholestanol. Biosynthesis of palmitates of 5α-androstane-3β, 17β-diol (**5**) and epiandrosterone (**6**) from testosterone (**1**) seem to be carried out in the same way as the biosynthesis of 5α-pregnanolone palmitate described earlier.[1] Glucosides of 5α-androstane-3β,17β-diol (**5**), epiandrosterone (**6**) and testosterone (**1**) seem to be formed by the same biosynthetic pathway as that of glucosides from progesterone[2] and digitoxin[3] by suspension culture of *Digitalis purpurea*. Stohs and El-Olemy[9] suggested that epiandrosterone (**6**) and 5α-androstane-3β,17β-diol (**5**) as metabolites of Δ⁴-androstene-3,17-dione (**2**) are present as conjugates. It appears that these conjugates are identical with the glucosides now isolated and identified. The possible biotransformation pathway of testosterone is shown in Scheme 1. (**1**) was first converted to Δ⁴-androstene-3,17-dione (**2**) by a 17β-hydroxysteroid dehydrogenase in the callus tissue. Then, (**2**) was metabolized to epiandrosterone (**6**) via 5α-androstane-3,17-dione (**4**). In addition, the formation of 5α-androstane-3β,17β-diol (**5**) from (**1**) can be explained by stereospecific reduction of an α,β-unsaturated ketone, similar to that which progesterone undergoes. 5α-Androstan-17β-ol-3-one (**3**) which was also detected as an intermediate in the callus tissue has been considered as an active form of the mammalian sex hormone.[10] Stoh and El-Olemy[9] reported that (**3**) was not present in the suspension cultures of *Dioscorea deltoidea* after administration of [4-^{14}C]Δ⁴-androstene-3,17-dione (**2**). The pathway by which 5α-androstane-3β,17β-diol (**5**) is biosynthesized via (**3**) from (**1**) is not fully understood in animals.[11] Our present results with tobacco suspension culture suggest the presence of a similar pathway to that proposed in animals.



SCHEME 1. BIOTRANSFORMATION OF TESTOSTERONE AND OTHER ANDROGENS BY TISSUE CULTURE OF *Nicotiana tabacum*.

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[8] SCHNEIDER, J. J. (1970) *J. Biol. Chem.* **245**, 5505.

[9] STOHS, S. J. and EL-OLEMY, M. M. (1972) *Lloydia* **35**, 81.

[10] BRUCHOUSKY, N. and WILSON, T. D. (1968) *J. Biol. Chem.* **243**, 2012.

[11] HEFTMANN, E. (1970) in *Steroid Biochemistry*, Academic Press, New York.

By administration of Δ^4-androstene-3,17-dione (**2**), the same eight transformation products (Table 1) as those from testosterone (**1**) were found; (**1**) was also detected by TLC and GLC. Recently, Krehula *et al.*[12] reported the co-occurrence of testosterone (**1**), epitestosterone and Δ^4-androstene-3,17-dione (**2**) in the pollen of *Pinus silvestris*. Thus the interconversion of (**1**) and (**2**) is reversible in tobacco callus as it is in mammals. Nevertheless, neither the biotransformation of 5α-androstane-3β,17β-diol (**5**) from epiandrosterone (**6**) nor the formation of (**6**) from (**5**) was observed. Thus, the dehydrogenase for the reaction (**1**) $\rightleftarrows$ (**2**) is less active in the case of the comparable transformation (**5**) $\rightleftarrows$ (**6**).

In the tobacco callus system, the results of the time course experiments from using [4-^{14}C]testosterone show more glucosylation than esterification. Palmitates and glucosides reached their maximal level on 14th day and thus appear to be end products. The time course of (**5**) and (**6**) formation was identical with that observed in non-radioactive experiments. To conclude, these are probably two metabolic pathways for testosterone (**1**); one is (**1**) $\rightarrow$ (**3**) $\rightarrow$ (**5**) $\rightarrow$ (**7**) and (**8a,b**), and the other is (**1**) $\rightarrow$ (**2**) $\rightarrow$ (**4**) $\rightarrow$ (**6**) $\rightarrow$ (**9**) and (**10**).

EXPERIMENTAL

Determination of m.p.s was by means of a Kofler hot plate and are uncorrected. IR spectra were taken in KBr. NMR spectra were determined in $CDCl_3$ using tetramethylsilane as internal reference. MS were run, using a direct insertion probe, on an JMS–OIS instrument.

Tissue culture and administration of testosterone (**1**) *and Δ^4-androstene-3,17-dione* (**2**) *and epiandrosterone* (**6**). The tissue culture were derived from the stem of tobacco (*Nicotiana tabacum* "Bright Yellow") and have been subcultured for about 5 yr. Plant tissue suspension cultures were grown on modified Murashige and Skoog's tobacco medium containing 0·5 ppm 2,4-D (2,4-dichlorophenoxyacetic acid), 0·1 ppm kinetin and 3% sucrose. The medium (250 ml) was dispensed in 1 liter flask containing 20–30 mg of crystalline testosterone, Δ^4-androstene-3,20-dione and epiandrosterone, respectively. The callus (30–40 g) from 3–4 weeks static cultures was incubated at 29° in a shaker for about 3 wks. [4-^{14}C]testosterone $6{\cdot}6 \times 10^6$ dpm (0·529 mCi/mmol, Daiichi Pure Chemicals Co., Ltd.) was administered to the tobacco callus similarly. Samples of callus (30–40 g) from 4 weeks static culture were incubated at 29° in a shaker for 5, 12, 24 hr, 2, 5, 8, 11 and 14 days, respectively. After incubation, the calluses were harvested at each time interval, three flasks being used for each experiment.

Extraction procedure. The calluses were harvested with Nylon cloth and homogenized with cold acetone in a Waring blender and allowed to stand for 10 days at room temp. Each homogenate was filtered and the residue extracted twice with fresh solvent. The residue was reextracted with MeOH. The filtrates were combined and the organic solvent was removed under red. press. The residue was extracted with $CHCl_3$ and the aqueous solution was extracted with H_2O-saturated BuOH. The $CHCl_3$ solution was washed with H_2O, dried and evaporated to dryness. The H_2O-saturated BuOH solution was evaporated to dryness.

Separation of transformation products of [4-^{14}*C*]*testosterone.* The calluses and media, from three flasks, were separated with Nylon cloth. The callus was extracted under reflux with acetone and filtered. The filter cake was refluxed with MeOH and filtered. The filtrates were combined, and then the organic solvent was evaporated off under reduced pressure. The residue redissolved in H_2O, was extracted with $CHCl_3$ ($\times$ 4–9) and the aq. soln was extracted with H_2O-saturated BuOH ($\times$ 4–7). Each fraction was evaporated to dryness. Each $CHCl_3$ extract was chromatographed on silica-gel (35 g) and eluted as follows: fraction No. 1 (fr. No. 1), 30% *n*-hexane in C_6H_6 430 ml; fr. No. 2, C_6H_6 1000 ml; fr. No. 3–22, 5% Me_2CO in C_6H_6; fr. No. 23–32, 10% Me_2CO in C_6H_6; each fraction 50 ml, fr. No. 33, 5% MeOH in $CHCl_3$ 500 ml and 10% MeOH in $CHCl_3$ 500 ml. Fr. No. 1 and 2 were combined. Each fr. No. 1–32 was counted with 10 ml of the solution containing 3·0 g PPO (2,5-diphenyloxazole) and 0·3 g dimethyl POPOP {1,4-Bis[2-(5-phenyloxazolyl)]-benzene} per 1 liter of toluene in Packard Tri-Carb liquid scintillation counter. Fr. No. 33 combined with BuOH fraction was counted with 10 ml of the solution containing 100 g naphthalene, 5·0 g PPO and 0·2 g POPOP per 1 liter of dioxane.

Separation of transformation products of testosterone (**1**). After administration of testosterone (total 980 mg) to the tobacco culture, the callus (total fr. wt 2329 g) was harvested and extracted using the same method as that

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[12] KREHULA, M. S., TAJIC, M. and KOLBAH, D. (1971) *Experientia* **27**, 108.

decribed above. The $CHCl_3$ extract (total 1·5657 g) was chromatographed on silica gel (180 g) and eluted as follows: fraction A (Fr. A), 30% *n*-hexane in C_6H_6 600 ml and C_6H_6 880 ml; Fr. B, C_6H_6 760 ml; Fr. C, C_6H_6 250 ml and 3% Et_2O in C_6H_6 660 ml; Fr. D, 3% Et_2O in C_6H_6 420 ml and 5% Et_2O in C_6H_6 460 ml; Fr. E, 5% Et_2O in C_6H_6 540 ml and 10% Et_2O in C_6H_6 1000 ml; Fr. F, 10% Me_2CO in C_6H_6 200 ml; Fr. G, 10% Me_2CO in C_6H_6 600 ml; Fr. H, 20% Me_2CO in C_6H_6 220 ml; Fr. I, 20% Me_2CO in C_6H_6 500 ml; Fr. J. $CHCl_3$ 500 ml and 10% EtOH in $CHCl_3$ 1280 ml and Fr. K, 10% EtOH in $CHCl_3$ 1090 ml and 20% EtOH in $CHCl_3$ 840 ml.

Isolation and identification of 5α-androstane-3β,17β-diol dipalmitate (**7**) *and epiandrosterone palmitate* (**9**). Fr. B after evaporation of solvent was recrystallized from MeOH to yield leaflets (**7**) (4·5 mg), m.p. 79–80° (Found: C, 79·83; H, 12·16. $C_{51}H_{92}O_4$ requires: C, 79·70; H, 11·98%). ν_{max}: 2920, 2850 (CH), 1740, 1177 (COO) cm^{-1}. NMR: δ 0·77 (C-18, *s*, 3H), 0·83 (C-19, *s*, 3H), 1·27 (CH_2, *s*, 48H) ppm. MS: *m/e* 512, $[M^+{-}C_{15}H_{31}COOH]$, 256 $[M^+{-}C_{15}H_{31}COOH{-}C_{15}H_{31}COOH]$ and 241 $[M^+{-}2C_{15}H_{31}COOH{-}Me]$. (**7**) was identified by IR, NMR, MS and m.m.p. comparison with synthetic material.

The colourless amorphous material obtained from Fr. D. was rechromatographed on silica gel (70 g) and eluted as follows: fraction 1 (Fr. 1) C_6H_6 100 ml; Fr. 2, C_6H_6 300 ml and 1% Et_2O in C_6H_6 700 ml and Fr. 3, 1% Et_2O in C_6H_6 520 ml. Fr. 3, after evaporation of solvent was recrystallized from MeOH to yield leaflets (**9**) 11·53 mg, m.p. 78–79°, $C_{35}H_{60}O_3$ (requires: 528·454) 528·455 by high resolution mass spectrometry. ν_{max} 2920, 2850 (CH) 1735 (CO) 1180 (COO) cm^{-1}. NMR: δ 0·86 (C-18, *s*, C-19, *s*, 6H), 1·26 (CH_2, *s*, 24H), 2·16 (CH_2·COO, *t*, 2H, *J* 10 Hz) ppm. MS: *m/e* 528 $[M^+]$, 272 $[M^+{-}C_{15}H_{31}COOH]$ and 257 $[M^+{-}C_{15}H_{31}COOH{-}Me]$ (**9**) was identified by IR, NMR, MS and m.m.p. comparison with synthetic material.

Detection of Δ^4-*androstene-3,20-dione* (**2**), *5α-androstan-17β-ol-3-one* (**3**), *5α-androstane-3β,17β-diol* (**5**) *and epiandrosterone* (**6**). Fr. F, Fr. G and Fr. I were investigated by TLC with authentic compounds. Several transformation products [(**2**) and (**3**) from Fr. F, (**5**) from Fr. I and (**6**) from Fr. G] were identified by GLC and TLC. R_t and R_f are as follows: R_t (min) systems 1 and 2. R_f C_6H_6–Me_2CO (4:1); $CHCl_3$–MeOH (19:1). (**2**), R_t 20·1, 45·4; R_f 0·53, 0·66. (**3**), 17·1, 17·4; 0·44, 0·56. (**5**) 17·2, 8·9; 0·26, 0·39. (**6**), 16·6, 14·7; 0·39, 0·54.

Isolation and identification of 5α-androstane-3β,17β-diol (**5**) *and epiandrosterone* (**6**). The amorphous material (160·0 mg) from Fr. I was purified on preparative TLC (R_f 0·39, $CHCl_3$–MeOH, 19:1) several times. (**5**) was recrystallized from MeOH to yield needles (33 mg), m.p. 161–162°, $C_{19}H_{32}O_2$ (requires: 292·240) 292·235 by high resolution mass spectrometry. ν_{max} 3470 (OH), 2930, 2850 (CH) cm^{-1}. MS: *m/e* 292 $[M^+]$, 277 $[M^+{-}Me]$, 274 $[M^+{-}H_2O]$, 259 $[M^+{-}Me{-}H_2O]$. (**5**) was identified by GLC, IR, MS and m.m.p. comparison with authentic 5α-androstane-3β,17β-diol.

Fr. G (166·5 mg) evaporated to a small volume, was subjected to rechromatography on silica gel (50 g) as follows: fraction 1, C_6H_6 200 ml, 5% Me_2CO in C_6H_6 500 ml, and 8% Me_2CO in C_6H_6 350 ml; fraction 2, 8% Me_2CO in C_6H_6 60 ml. Fraction 2, after evaporation of solvent was recrystallized from MeOH to yield columnar prisms (9·1 mg), m.p. 166–167°, $C_{19}H_{30}O_2$ (requires: 290·225) 290·229 by high resolution mass spectrometry. ν_{max} 3470 (OH), 2930, 2850 (CH), 1730 (CO) cm^{-1}. MS: *m/e* 290 $[M^+]$, 275 $[M^+{-}Me]$, 272 $[M^+{-}H_2O]$, 257 $[M^+{-}Me{-}H_2O]$, (**6**) was identified by GLC, IR, MS and m.m.p. comparison with authentic epiandrosterone.

Isolation and identification of epiandrosterone glucoside (**10**) *acetate, 5α-androstane-3β,17β-diol 3- and 17-monoglucosides* (**8*a,b***) *acetates and testosterone glucoside* (**11**) *acetate*. The amorphous material were obtained from Fr. K, was acetylated (86·1 mg) by reacting with Ac_2O–pyridine and allowing to stand at room temperature overnight. The acetates were separated and purified on silica gel (70 g) column chromatography and eluted as follows: fraction a (fr. a), C_6H_6 200 ml and 2% Me_2CO in C_6H_6 1000 ml; fr. b, 2% Me_2CO in C_6H_6 220 ml; fr. c, 5% Me_2CO in C_6H_6 250 ml; fr. d, 5% Me_2CO in C_6H_6 270 ml; fr. e, 5% Me_2CO in C_6H_6 900 ml. The solvent was distilled off from fr. b the residue recrystallized from MeOH to yield needles (**8**) acetate (3·35 mg), m.p. 202–203°. MS: *m/e* 604 $[M^+{-}MeCOOH]$, 544 $[M^+{-}MeCOOH{-}MeCOOH]$, 331 $[C_{14}H_{19}O_9]$ 316 $[M^+{-}C_{14}H_{20}O_{10}]$ and 257 $[M^+{-}C_{14}H_{20}O_{10}{-}MeCOO]$. GLC: R_t 20·7 and 21·4 (min) in system 3. (**8**) acetate was shown to be a mixture of 5α-androstane-3β,17β-diol 3-monoglucoside (**8**a) acetate and 5α-androstane-3β,17β-diol 17-monoglucoside (**8**b) acetate by MS and GLC data.

Fr. d was evapotrated *in vacuo* to give a residue which was recrystallized from MeOH to yield needles (**10**) acetate (3·5 mg), m.p. 193–194°, (Found: C, 63·90, H, 7·77. $C_{33}H_{48}O_{11}$ requires: C, 63·97, H, 7·74%). ν_{max} 2920, 2850 (CH), 1750 (COO), 1735 (CO), 1240 and 1040 (COO). NMR: δ 0·83 (C-18, *s*, 3H), 0·87 (C-19, *s*, 3H), 2·02, 2·03, 2·05, 20·8 (MeCO, *s*, 4 × 3H), 4·58 (1′-H, *d*, 1H, *J* 7·0 Hz) ppm. MS: *m/e* 560 $[M^+{-}MeCOOH]$, 331 $[C_{14}H_{19}O_9]$, 272 $[M^+{-}C_{14}H_{20}O_{10}]$. GLC: R_t 16·7 (min) in system 3. (**10**) acetate was identified by IR, MS, NMR, GLC and m.m.p. comparison with synthetic material.

Fr. e after removal of the solvent was recrystallized from MeOH to yield needles (**11**) acetate (4·5 mg), m.p. 166–167°, $C_{33}H_{46}O_{11}$ (requires: 618·304) 618·302 by high resolution mass spectrometry. λ_{max}^{EtOH} 241 nm. ν_{max} 2930, 2850 (CH), 1750 (COO), 1675 (C=C–CO), 1230, 1035 (COO). NMR: δ 0·76 (C-18, *s*, 3H), 1·20 (C-19, *s*, 3H), 2·0, 2·02, 2·04, 2·08 (MeCO, *s*, 4 × 3H), 4·50 (1′-H, *d*, 1H, *J* 7·5 Hz) ppm. MS: *m/e* 618 $[M^+]$, 331 $[C_{14}H_{19}O_9]$, 271 $[M^+{-}C_{14}H_{19}O_{10}]$. GLC: R_t 20·0 (min) in system 3. (**11**) acetate was identified by UV, IR, MS, NMR, GLC and m.m.p. comparison with synthetic material.

Detection of testosterone (**1**), *5α-androstan-17β-ol-3-one* (**3**), *5α-androstane-3β,17β-diol* (**5**), *its dipalmitate* (**7**) *and glucoside* (**8**), *epiandrosterone* (**6**), *its palmitate* (**9**) *and glucoside* (**10**) *as transformation produtcs of* Δ^4-*androstene-3,17-dione* (**2**) *by TLC and GLC*. The $CHCl_3$ extract (23·3 mg) was obtained by the administration of Δ^4-androstene-3,17-dione (**2**) (40 mg) to tobacco callus. Testosterone (**1**), 5α-androstan-17β-ol-3-one (**3**), 5α-androstane-3β,17β-diol (**5**) and epiandrosterone (**6**) were detected by GLC and TLC. R_t and R_f are as follows:

	(1)	(3)	(5)	(6)
R_t (min) system 1	—	17·1	17·2	16·6
R_t (min) system 2	25·5	17·4	8·9	14·7
R_f C_6H_6–Me_2CO (4:1)	0·33	0·44	0·26	0·39
R_f $CHCl_3$–MeOH (19:1)	0·51	0·56	0·39	0·54

(**7**), (**8**), (**9**) and (**10**) were detected by TLC, respectively. These data are as follows:

	(7)	(8)	(9)	(10)
n-hexane–$CHCl_3$ (4:1)	0·62	—	0·35	—
n-hexane–Me_2CO (3:1)	0·86	—	0·69	—
$CHCl_3$–MeOH (7:3)	—	0·35	—	0·42

Detection of epiandrosterone palmitate (**9**) *and glucoside* (**10**) *converted from epiandrosterone* (**6**) *by TLC*. The $CHCl_3$ extract (336 mg) was obtained by the administration of epiandrosterone (**6**) (total 100 mg) to tobacco callus (total 731·6 g). Epiandrosterone palmitate (**9**) and glucoside (**10**) were detected by TLC. R_f values are as follows: (**9**) R_f 0·35, *n*-hexane–$CHCl_3$ (4:1); 0·69, *n*-hexane–Me_2CO (3:1). (**10**) R_f 0·42, $CHCl_3$–MeOH (7:3).

Hydrolysis of 5α-*androstane*-3β,17β-*diol dipalmitate* (**7**) *and epiandrosterone palmitate* (**9**). Ten mg (**7**) obtained by tobacco callus was refluxed with 5% alcoholic KOH for 1 hr. After dilution with 30 ml H_2O, EtOH was removed under red. press. The residue was extracted with ether and the evaporation of ether afforded the crystalline residue which was identified by TLC (R_f 0·25, C_6H_6–Me_2CO (4:1); 0·45, $CHCl_3$–MeOH (7:1), respectively) and GLC (R_t 17·2 (min), system 1). The alkaline solution after removal of 5α-androstane-3β,17β-diol (**5**) was acidified with dil. HCl and extracted with ether. After removal of ether, the white powder obtained was identified with palmitic acid by TLC (R_f 0·48, $CHCl_3$–MeOH, 7:1). By the same method, 5 mg (**9**) gave epiandrosterone (**6**) by TLC [R_f 0·39, C_6H_6–Me_2CO (4:1); 0·54, $CHCl_3$–MeOH (19:1)] and GLC [R_t 16·6 (min), system 1] and palmitic acid was identified by TLC (R_f 0·48, $CHCl_3$–MeOH, 7:1).

Synthesis of 5α-*androstane*-3β,17β-*diol dipalmitate* (**7**) *and epiandrosterone palmitate* (**9**).[13] 5α-Androstane-3β,17β-diol (**5**) (300 mg), m.p. 162–163°, prepared by reduction ($NaBH_4$) of epiandrosterone (**6**), was dissolved in pyridine (18 ml) and palmitoyl chloride (1·5 g) was added to this solution, then the reaction mixture was warmed in water bath for few min allowed to stand overnight. The crude product was chromatographed on silica-gel (145 g) and eluted as follows: fraction Y, 30% *n*-hexane in C_6H_6 1000 ml; fraction Z, 30% *n*-hexane in C_6H_6 1000 ml and C_6H_6 300 ml. Fraction Z gave leaflets (**7**) from MeOH, m.p. 79–80° (460 mg, yield 58%) (Found: C, 79·83; H, 12·16. $C_{51}H_{92}O_4$ requires: C, 79·70; H, 11·98%). R_f 0·62 (*n*-hexane–$CHCl_3$, 4:1). The IR, NMR and MS were identical with those of the metabolic product (**7**). Epiandrosterone palmitate (**9**) was synthesized in 82% yield by the same method as used for (**7**). Synthetic epiandrosterone palmitate gave leaflets (**9**) from MeOH, m.p. 81–82° (Found: C, 79·37; H, 11·55. $C_{35}H_{60}O_3$ requires: C, 79·49; H, 11·44%). The IR, NMR and MS were identical with those of the metabolic product (**9**).

Hydrolysis of mixture of epiandrosterone glucoside (**10**) *acetate and* 5α-*androstan*-3β,17β-*diol* 3- *and* 17-*monoglucosides* (**8a,b**) *acetates, and testosterone glucoside* (**11**) *acetate*. The mixture of (**8a,b**) and (**10**) acetates (26·9 mg) obtained from fr. c was refluxed with 10 ml of 10% alcoholic HCl for 1 hr. The product was obtained in amorphous form which was identified by TLC (R_f 0·26 and 0·38, C_6H_6–Me_2CO, 4:1) and GLC [R_t 8·9 and 14·7 (min) in system 2]. The acidic solution after removal of 5α-androstane-3β,17β-diol (**5**) and epiandrosterone (**6**) was neutralized with dil. KOH and concentrated under reduced pressure. The sugar was the same as that from the hydrolysate of authentic β-D-glucose pentaacetate by TLC (R_f 0·47 and 0·34; BuOH:AcOH:H_2O = 5:1:1). Testosterone glucoside (**11**) acetate from fr. e gave testosterone (**1**) by TLC (R_f 0·33, C_6H_6–Me_2CO, 4:1) and GLC (R_t 25·5, system 2), and the same hydrolysate of β-D-glucose pentaacetate (R_f 0·47 and 0·34, BuOH:AcOH:H_2O = 5:1:1).

Synthesis of epiandrosterone β-D-*glucoside* (**10**) *tetraacetate and testosterone* β-D-*glucoside* (**11**) *tetraacetate*.[14] Epiandrosterone (**6**) (1 g) was treated with acetobromglucose (5 g) in the usual way and the product was obtained as needles (834 mg, yield 39%) from MeOH, m.p. 193–194°. (Found: C, 63·90; H, 7·77. $C_{33}H_{48}O_{11}$ requires: C, 63·87; H, 7·74%). The IR, NMR, MS and GLC were identical with those of the transformation product (**10**) acetate. Testosterone glucoside (**11**) tetraacetate was synthesized in 47% yield by the same method. Synthetic testosterone glucoside (**11**) tetraacetate gave needles from MeOH, m.p. 166–167° (Found: C, 63·62; H, 7·54. $C_{33}H_{46}O_{11}$ requires: C, 64·04; H, 7·44%). The UV, IR, NMR, MS and GLC were identical with those of the transformation product (**11**) acetate.

Synthesis of 5α-*androstane*-3β,17β-*diol* 3-β-D-*glucoside* (**8a**) *pentaacetate*. Epiandrosterone glucoside (**10**) tetraacetate (300 mg), prepared as described above, was dissolved in MeOH (80 ml) and reduced with $NaBH_4$ (100 mg).

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The product was crystallized from MeOH to give 94·5 mg (31%) of needles, m.p. 188–190° (Found: C, 63·19, H, 7·85. $C_{35}H_{52}O_{12}$ requires: C, 63·02, H, 7·93%). GLC: R_t 21·4 (min) in system 3.

Synthesis of 5α-androstane-3β,17β-diol 17β-D-glucoside (**8b**) *pentaacetate*. Testosterone glucoside tetraacetate was reduced with $NaBH_4$ and then acetylated with Ac_2O–pyridine. The product (yield 16%) was crystallized from MeOH to give needles, m.p. 212–214° (Found: C, 63·07; H, 7·79. $C_{35}H_{52}O_{12}$ requires: C, 63·02; H, 7·93%). GLC: R_t 20·7 (min) in system 3.

Condition of GLC. Gas chromatographic analysis was run on a Shimadzu gas chromatograph GC-4BMF instrument fitted with a hydrogen flame ionization detector. The condition of GLC are as follows:

System	Column	Liquid phase	Column temp. (°C)	Detector temp. (°C)	Carrier gas N_2 (ml/min)
1	2 m × 3 mm	1% OV 1 on Chromosorb W (80 ~ 100 mesh)	200	250	38
2	2 m × 3 mm	1·5% QF 1 on Chromosorb W (60 ~ 80 mesh)	215	250	50
3	2 m × 3 mm	1% OV 1 on Chromosorb W (80 ~ 100 mesh)	275	300	47

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