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BIOTRANSFORMATION OF PROGESTERONE AND PREGNENOLONE BY PLANT SUSPENSION CULTURES*

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Abstract—Progesterone was converted to 5α-pregnanolone palmitate in high yield by tobacco (*Nicotiana tabacum* var. Bright Yellow) and *Sophora angustifolia* callus tissues. In *Sophora angustifolia* callus, 5α-pregnanolone was detected at the same time. The results showed that progesterone undergoes stereospecific reduction of the α,β-unsaturated keto group and esterification. Pregnenolone was also converted to pregnenolone palmitate and 5α-pregnanolone palmitate by tobacco and *Sophora angustifolia* callus tissues.

INTRODUCTION

MICROBIAL transformations[1,2] have extensively been studied and are of great significance for the synthesis of steroids and other organic compounds. Recently, the secondary metabolites produced by plant tissue cultures have been studied,[3] but very few investigations have hitherto been reported on the biotransformation of organic compounds by plant tissue cultures.[4–6] We have studied the biotransformation of steroids in plant suspension cultures and wish to report the biotransformation of progesterone (I) to 5α-pregnanolone (III) and 5α-pregnanolone palmitate (IV), and pregnenolone (II) to pregnenolone palmitate (V) and 5α-pregnanolone palmitate (IV) by tobacco (*Nicotiana tabacum* var. Bright Yellow) or *Sophora augustifolia*[7] ("Kurara", in Japanese) callus tissues (Chart 1).

RESULTS

Progesterone (total 190 mg) was administered to tobacco suspension callus culture. After shaking culture for 4 weeks, the callus was harvested. The neutral fraction was separated with $CHCl_3$ and the $CHCl_3$ extract was chromatographed on silica gel to give waxy colorless crystals, which were recrystallized from MeOH to give needles, m.p. 108–110°, its formula as $C_{37}H_{64}O_3$ (IV) being determined by high resolution mass spectrometry.The i.r. spectrum (KBr, cm^{-1}) had absorption bands at 1740 (—C(=O)—O—) and 1180 (—C(=O)—O—), and the NMR spectrum (δ, ppm) showed signals at 0·88 (CH_3—, t, $J = 6$ c/s, 3H), 1·24 (—CH_2—, s, 24H), and 2·24 (—CH_2—C(=O)—O—, t, $J = 7$ c/s, 2H).
These spectral data suggested that (IV) was a fatty acid ester of sterol.

* Part III in the series "Studies on Plant Tissue Cultures": for Part II, see *Plant & Cell Physiol.*, in press (1971).

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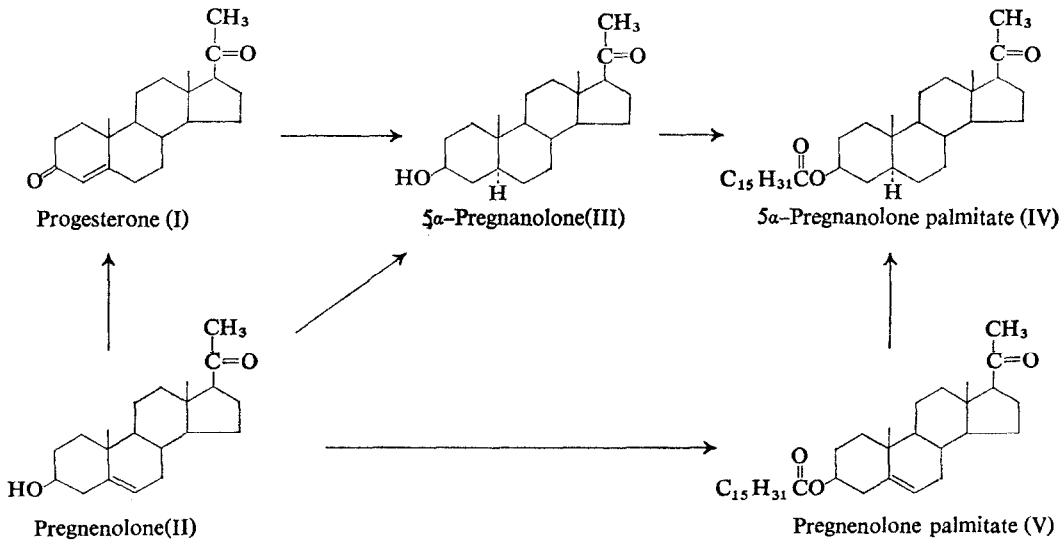

CHART 1. SCHEME FOR THE BIOTRANSFORMATION OF PROGESTERONE AND PREGNENOLONE BY PLANT TISSUE CULTURES.

Alkaline hydrolysis of (IV) gave the alcohol 5α-pregnanolone (III), m.p. 189° and an esterifying acid, characterized as palmitic acid by TLC and mass spectrometry. Compound (IV), therefore seemed to be an ester of 5α-pregnanolone and palmitic acid and this was confirmed by a mixed m.p. with authentic 5α-pregnanolone palmitate.

Progesterone was also converted into 5α-pregnanolone and its palmitate by *Sophora angustifolia* callus. The presence of 5α-pregnanolone was detected by TLC and GLC (Table 1).

TABLE 1. BIOTRANSFORMATION OF PROGESTERONE AND PREGNENOLONE BY PLANT TISSUE CULTURES

Callus	Substrate	Metabolic product (III)	(IV)	(V)
Nicotiana tabacum var. Bright Yellow	Progesterone		+	
Sophora angustifolia	Progesterone	+	+	
Nicotiana tabacum var. Bright Yellow	Pregnenolone		+	+
Sophora angustifolia	Pregnenolone		+	+

+ Indicates the presence of metabolic products.

Pregnenolone (II) was administered to the suspension callus cultures of tobacco and *Sophora angustifolia*. The ester was separated by the same method as progesterone. Alkaline hydrolysis of the ester gave an alcohol portion containing a mixture of pregnenolone and 5α-pregnanolone. The esterifying acid was identified as palmitic acid by TLC and mass spectrometry. The results showed that pregnenolone is converted to pregnenolone palmitate (V) and 5α-pregnanolone palmitate (IV) by suspension cultures of tobacco and *Sophora angustifolia* (Table 1). It was not possible to detect 5α-pregnanolone in the cultures.

DISCUSSION

It is of much interest that esterification with palmitic acid which was recently detected in tobacco callus tissue,[8] is carried out effectively by tobacco and *Sophora angustifolia* callus tissues at the same time as the stereospecific reduction of the double bond of progesterone and pregnenolone (Graves and Smith).[5] It is well known that some steroids[9–11] and triterpenoids[12] are esterified with fatty acids in higher plants, but esterification of exogeneous steroids by plant tissue cultures was unknown. The occurrence of palmitates of pregnenolone and 5α-pregnanolone has been demonstrated for the first time.

As shown in Chart 1, the formation of 5α-pregnanolone palmitate (IV) from progesterone (I) could be explained by reduction of an α,β-unsaturated ketone to 5α-pregnanolone (III) followed by esterification. The conversion of pregnenolone (II) into 5α-pregnanolone palmitate (IV) could be also explained by assuming that either the reduction of pregnenolone palmitate (V), or the esterification of 5α-pregnanolone (III) formed via progesterone (I) by Oppenauer oxidation of pregnenolone as Caspi and Lewis's report,[13] or the esterification of 5α-pregnanolone (III) by the direct reduction of Δ^5 double bond of pregnenolone could occur. Thus plant suspension callus cultures appear to be useful for studying the biotransformation of steroids.

EXPERIMENTAL

NMR spectra were determined in $CDCl_3$ using tetramethylsilane as internal reference.

Tissue Culture and Administration of Progesterone (I) and Pregnenolone (II)

The tissue cultures used were derived from the stem of tobacco (*Nicotiana tabacum* var. Bright Yellow) and the seedling of *Sophora angustifolia*. They were subcultured for about 3 yr and 4 yr, respectively. 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 l. flask containing 10 mg progesterone or 10 mg pregnenolone suspended with Tween 80. The callus (10–15 g) from 4 (tobacco) or 5 (*Sophora angustifolia*) weeks static cultures was incubated at 29° in a shaker for about 4 or 5 weeks.

Extraction Procedure

The calluses were harvested with Nylon cloths and homogenized with cold acetone in a Waring blender and allowed to stand for a week at room temperature. Each homogenate was filtered and the residue extracted with fresh solvent three times. The filtrates were combined and acetone was removed under reduced pressure. The residue after acidification was extracted with $CHCl_3$ and the $CHCl_3$ solution was washed with 0·25N NaOH. The $CHCl_3$ solution was washed with H_2O, dried and evaporated to dryness.

Isolation and Identification of 5α-Pregnanolone Palmitate (IV)

The $CHCl_3$ extract (483·2 mg) obtained by the administration of progesterone (total 190 mg) to tobacco callus was chromatographed on silica gel (250 g) and eluted as follows: fraction A, 50% benzene in petroleum 300 ml; B, 70% benzene in petroleum 1820 ml and C, benzene 2500 ml. From fraction C the colorless powder (162 mg) was obtained and rechromatographed on silica gel (100 g) as follows: fraction 1, *n*-hexane 1000 ml; 2, *n*-hexane containing 10, 20, 30 and 50% benzene, respectively, total 5000 ml; and 3, 60% benzene in *n*-hexane, 2000 ml. Fraction 3 after evaporation of solvent was recrystallized from MeOH to yield colorless

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needles (IV) (57 mg), m.p. 108–110°, $C_{37}H_{64}O_3$, (required; 556·486) 556·483 by high resolution mass spectrometry. No characteristic u.v. spectrum was observed. The i.r., ν_{max}^{KBr} cm^{-1} 2930, 2870 (CH), 1740 (—C(=O)—O—), 1710 (>C=O) and 1180 (—C(=O)—O—). The NMR spectrum (δ, ppm), 0·60 (C-18, s, 3H), 0·82 (C-19, s, 3H), 0·88 (CH_3—, t, $J = 6$ c/s, 3H), 1·24 (—CH_2—, s, 24H), 2.10 (CH_3CO—, s, 3H), 2·24 (—CH_2·COO, t, $J = 7$ c/s, 2H). The main fragment peaks were observed such as *m/e* 556 [M^+], 300 [$M^+ - C_{15}H_{31}COOH$] and 285 [$M^+ - C_{15}H_{31}COOH - CH_3$]. (IV) was identified by i.r., NMR, mass spectra and mixed m.p. comparison with authentic material.

By the same method as that described in the tobacco callus, 5α-pregnanolone palmitate was separated from the $CHCl_3$ extract (162 mg) obtained by the administration of progesterone (5 mg) to *Sophora angustifolia* callus. Then the presence of 5α-pregnanolone palmitate was detected by TLC (R_f 0·67, benzene–ether, 4:1).

Hydrolysis of 5α-Pregnanolone Palmitate (IV)

(IV) obtained (13·0 mg) by the tobacco callus was refluxed with 10 ml of 5% alcoholic KOH for one hour. After diluting with 40 ml H_2O, EtOH was removed under reduced pressure. The solution was extracted with ether and the evaporation of ether afforded the crystalline residue which was recrystallized from MeOH (0·5 mg), m.p. 189°, identified by mass fragment peaks such as *m/e* 318 [M^+], 300 [$M^+ - H_2O$], 285 [$M^+ - H_2O - CH_3$] and R_f 0·32 (TLC, benzene–ether, 2:1). The alkaline solution after removing 5α-pregnanolone 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 0·44, $CHCl_3$ – MeOH, 5:1) and mass spectrometry, *m/e* 256 [M^+].

Detection of 5α-Pregnanolone

The $CHCl_3$ extract (62 mg) obtained by the administration of progesterone (20 mg) to *Sophora angustifolia* callus was chromatographed on silica gel (50 g). From the $CHCl_3$ eluate, 5α-Pregnanolone was detected by TLC (R_f 0·35, $CHCl_3$–AcOEt, 4:1; R_f 0·26, benzene–AcOEt, 6:1) and GLC (t_R 11·8 min).

Isolation and Identification of the Mixture Containing 5α-Pregnanolone Palmitate (IV) and Pregnenolone Palmitate (V)

The isolation of each ester (53 mg from tobacco callus, 99·8 mg from *Sophora angustifolia*) obtained by the administration of pregnenolone to tobacco (140 mg) and *Sophora angustifolia* (320 mg) callus tissues and the alkaline hydrolysis of each ester were performed by the same method as that described for 5α-pregnanolone palmitate. In the crystalline hydrolysates, 5α-pregnanolone and pregnenolone were detected by TLC (R_f 0·32, 0·27, respectively, benzene–ether, 2:1) and GLC (t_R 11·9, 10·8 min, respectively). The esterifying acid was identical with palmitic acid by TLC (R_f 0·44, $CHCl_3$:MeOH, 5:1) and mass spectrometry, *m/e* 256 [M^+].

Synthesis of 5α-Pregnanolone Palmitate [14] *(IV)*

5α-Pregnanolone (500 mg), m.p. 190°, prepared by catalytic reduction (Pd-C) of pregnenolone was dissolved in pyridine (60 ml) and palmitoyl chloride (2 g) was added to this solution, then the reaction mixture was warmed in water bath for few minutes and allowed to stand overnight. The solution was poured into the ice water and filtrated. The precipitation was dissolved in $CHCl_3$. The $CHCl_3$ solution was washed with $NaHCO_3$ solution and H_2O. The $CHCl_3$ solution was dried over anhydrous Na_2SO_4 and evaporated to dryness. The residue gave to colorless needles from MeOH, m.p. 110° (549·6 mg, yield 63%), $C_{37}H_{64}O_3$ (required; 556·486) 556·483 by high resolution mass spectrometry. R_f 0·67 (TLC, benzene–ether, 4:1). The i.r., NMR and mass spectra were identical with those of the metabolic product (IV).

Synthesis of Pregnenolone Palmitate (V)

Pregnenolone palmitate was synthesized by the same method as used for 5α-pregnanolone palmitate, yield 58%. Synthetic pregnenolone palmitate gave colorless needles from MeOH, m.p. 96–97°, $C_{37}H_{62}O_3$ (required; 554·470) 554·464 by high resolution mass spectrometry. The i.r., ν_{max}^{KBr} cm^{-1} 2930, 2870 (CH), 1740 (—C(=O)—O—), 1710 (>C=O) and 1180 (—C(=O)—O—). The NMR spectrum (δ, ppm), 0·62 (C-18, s, 3H), 1·02 (C-19, s, 3H), 1·25 (—$(CH_2)_{12}$—, s, 24H), 2·10 (CH_3CO—, s, 3H).

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Conditions of GLC

Gas chromatographic analysis was run on a Shimadzu gas chromatograph GC-1C instrument fitted with a hydrogen flame ionization detector. A glass U-column (1·8 m × 4 mm i.d.) packed with 1% QF-1 on Gaschrom Q (80–100 mesh) was operated under the following conditions. The temperatures of the detector block and column oven were 250° and 195°, respectively. The carrier gas was N_2 with a flow rate of 72·7 ml/min.

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