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An Improved 11 α -Hydroxylation of Progesterone by *Aspergillus ochraceus* TS

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1. The stereospecific hydroxylation of progesterone exclusively to its 11 α -hydroxy derivative by *Aspergillus ochraceus* TS is reported. 2. There is no secondary metabolite (6 β ,11 α -dihydroxyprogesterone) formed even when the transformation was attempted with different concentrations of the substrate (200 μ g/ml–20 mg/ml) for prolonged incubation periods. When transformation was carried out in a medium supplemented with Zn²⁺ at a concentration of 50 μ g/ml identical results were obtained. 3. Similar results were also obtained at different pH values of the culture medium.

The presence of an oxygen function at the 11-position is an obligatory structural requirement for the so-called carbohydrate-regulating hormone activity (Hornberger *et al.*, 1952) of adrenal steroids. Since chemical syntheses (Mackenzie *et al.*, 1948; Chamberlain *et al.*, 1951; Fieser *et al.*, 1951; Stork *et al.*, 1951) of 11-oxygenated steroid are expensive, microbial methods for introduction of an oxygen function at C-11 in the steroid molecule have been developed.

In most of the reactions involving oxygenation at C-11 in the steroid skeleton by different strains of *Aspergillus* species (Fried *et al.*, 1952; Dulaney *et al.*, 1955; Schleg & Knight, 1962; Charney & Herzog, 1967; Sallam *et al.*, 1973; Clegg *et al.*, 1973), a mixture of products including the 11 α -oxygenated derivative is produced. The present paper deals with the transformation of progesterone exclusively into the 11 α -hydroxy derivative by *Aspergillus ochraceus* TS. This is the first report on 11-oxygenation of progesterone where there is no secondary metabolite (6 β ,11 α -dihydroxy derivative) formed under the conditions used. A preliminary account of this work was presented at the Fifth International Fermentation Symposium, Berlin, Germany, 1976.

Materials and Methods

Culture method

A. ochraceus TS was cultivated in flasks (250 ml) containing 100 ml of sterilized medium (composition, % w/v: sucrose, 1; cornsteep liquor, 0.5; K₂HPO₄, 0.05; pH 6.5 before sterilization) inoculated with a spore suspension obtained from a 1-week-old culture maintained on Czapek–Dox agar medium. This volume of inoculum (1 ml), containing 8.4×10^6 spores, was used in all experiments unless otherwise stated.

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The progesterone (200 μ g/ml), either as dry powder or dissolved in aq. ethanol (95%, w/v), was added to a 48 h-grown culture incubated at 30°C on a rotary shaker (220 rev./min) for transformation to continue. A parallel control that received no progesterone was run concurrently.

Extraction and isolation of transformed product

After the transformation both mycelia and filtrate were separately extracted with chloroform (3 $\times$ 50 ml), the chloroform extracts were washed with aq. NaHCO₃ (5%, w/v) and then water, and then dried over anhydrous Na₂SO₄. Complete evaporation of the solvent gave a semi-solid mass from the extract of the filtrate; this was then analysed.

Analysis

The converted product was chromatographed in a packed column (15 cm $\times$ 1.5 cm) of Florisil (Sigma Chemical Co., St. Louis, MO, U.S.A.), eluted with a mixture of ether and benzene (1:1, v/v) and later crystallized from light petroleum and benzene. The purified material was resolved on silica-gel G (M/s; Gouri Chemicals, Calcutta, India) plates along with authentic steroids with ethyl acetate/chloroform/water (60:40:1, by vol.) as solvent system and Liebermann–Burchard spray reagent (Krebs *et al.*, 1969) for development of colour. Its further identity was established by m.p., mixed m.p., optical activity and superimposable i.r. spectra with those of authentic samples of 11 α -hydroxyprogesterone and its corresponding acetate. The converted material was acetylated by the method of Peterson *et al.* (1952). The quantitative determination of the transformation mixture was carried out by the method of Abdel-Fattah & Badawi (1974).

Results and Discussion

The transformed product showed a single spot on t.l.c. with R_f identical with that of an authentic sample of 11α -hydroxyprogesterone; the control experiment without progesterone gave no derivative. The purified material had m.p. 164°C , $[\alpha]_D +172.51$ (c 0.5 in chloroform) [published values: m.p. $166-168^\circ\text{C}$, $[\alpha]_D +174.9$ (Peterson *et al.*, 1952)]. The i.r. spectrum of the material showed an absorption band at 3500cm^{-1} and was superimposable with that of an authentic sample of 11α -hydroxyprogesterone. Further identity of the transformed material was established by preparation of its acetate; m.p. 175°C , $[\alpha]_D +140$ (c 0.5 in acetone) [published values: m.p. $175-177^\circ\text{C}$, $[\alpha]_D +143$]. The i.r. spectrum of the acetate indicated the absence of a band due to a hydroxy group at 3500cm^{-1} and the presence of bands due to acetate at 1730cm^{-1} ($>\text{C}=\text{O}$) and 1255cm^{-1} ($-\text{C}-\text{O}-$) cm^{-1} and was superimposable with that of an authentic sample.

The presence of sucrose and cornsteep liquor in the fermentation media was necessary for maximum yields of the converted material, but medium composition did not modify the exclusive production of the 11α -hydroxyprogesterone product. Similar results were also obtained during the transformation with different progesterone concentrations ranging from $200\mu\text{g/ml}$ to 20mg/ml (Table 1). Shake culture showed better conversion (100%) than still incubation (61%). The pH of this biological hydroxylation is quite broad, ranging from 4 to 8, the maximum conversion being at pH 6.5–7.0. This conversion appears to be quantitative up to a substrate concentration of $400\mu\text{g/ml}$ (95.5% recovery) (Table 1), whereas Abdel-Fattah & Badawi (1974) have reported only partial (25%) conversion of added progesterone even at a lower concentration ($138.8\mu\text{g/ml}$) by a strain of *A. niger* 12Y.

Dulaney *et al.* (1955) reported a similar 11α -

hydroxylation of progesterone by *A. ochraceus* and they suggested that the amount of $6\beta,11\alpha$ -dihydroxyprogesterone formed and the time of its appearance are affected by the concentration of substrate. The dihydroxy derivative appears later and the amount obtained is decreased when the amount of unreacted progesterone remained high. In the present study the hydroxylating enzyme of *A. ochraceus* TS produced only 11α -hydroxyprogesterone at all substrate concentrations ($200\mu\text{g/ml}$ – 20mg/ml ; Table 1) and thus maintained its specificity. Its specificity was not disturbed even when the same transformation was attempted in a medium supplemented with Zn^{2+} at a concentration of $50\mu\text{g/ml}$, contrary to the findings of Dulaney *et al.* (1955), and as such appears to be unique. The specificity of the enzyme also remained unaffected, in spite of variations in pH, as evidenced by the results of transformation at different pH values.

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Table 1. Effect of substrate concentration on transformation of progesterone by *A. ochraceus* TS

Progesterone added to 100ml of medium (mg)	Unconverted progesterone [mg (%)]	11α -Hydroxyprogesterone formed [mg (%)]	$6\beta,11\alpha$ -Dihydroxyprogesterone formed (mg)
20*	0	19† (100)	0
40*	0	37.4§ (100)	0
200†	123 (61.5)	73.98 (36.99)	0
2000†	1132 (56.6)	866 (43.3)	0

* Added at one time.

† Added at intervals.

‡ 95% Recovery.

§ 93.5% Recovery.