Microbial Transformation of 19-Hydroxy pregnanes

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_Nocardia_ species aromatized 19-hydroxyprogesterone, 3β,19-dihydroxy-pregn-5-en-20-one 3-acetate and pregn-5-ene-3β,19,20β-triol 3-acetate, without cleavage of the side chain, into 3-hydroxy-19-norpregna-1,3,5(10)-tri-en-20-one. _Septomyxa affinis_ aromatized the ring A and cleaved the side chain of 19-hydroxyprogesterone to yield estrone. With 19-hydroxy pregna-4,7-diene-3,20-dione as substrate, the transformation was more complex and many products were formed.

Microbial transformation of 19-hydroxylated steroids resulting in the formation of aromatic ring A steroids was described for the first time by Dodson and Muir (4). They showed that 19-hydroxyandrost-4-en-3,17-dione was readily converted into estrone by _Pseudomonas_ sp. Similar transformations of 19-hydroxyandrostanes with _Nocardia restrictus_ were reported by Sih and Rahim (7). Sih and Wang (8) later reported that aromatization occurred with cleavage of the sterol side chain when 19-hydroxycholest-4-en-3-one was incubated with _N. restrictus_. An organism isolated from soil (CSD-10), which used cholesterol as the sole carbon source, gave higher yields of estrone from the same substrate. Similarly, 19-hydroxyisotest-4-en-3-one was converted to estrone.

Studies by Sih and his collaborators and earlier work done in our laboratories on the transformations of 19-hydroxylated androstanes (1) and cholestanes (2) led us to investigate the conversion of 19-hydroxy pregnanes. Synthesis and microbial transformation of a few 19-hydroxypregnanes are described in this paper.

**MATERIALS AND METHODS**

Microorganisms. A large number of bacteria and fungi were screened for their ability to aromatize steroids. Of these, an organism of the genus _Nocardia_, which in preliminary studies was found to aromatize cholest-5-ene-3β,19-diol 3-acetate with degradation of the side chain, and _Septomyxa affinis_ ATCC 6737, an organism known to cleave the side chain of C-17 steroids during the course of 1-dehydrogenation (9), were selected for the present study.

_Transformation of steroids_. _Nocardia_ species was cultured in a mineral salt medium containing (per liter): NH₄NO₃, 1.0 g; KH₂PO₄, 1.0 g; MgSO₄·7H₂O, 0.2 g; CaCl₂, 0.02 g; FeCl₃, 0.05 g; pH 7.0 (medium A). To this medium, 0.3 g of cholesterol per liter was added. The organism was incubated in stationary culture at 25°C for 7 to 10 days. Submerged cultures were started by transferring 5 ml of the growth from the above stationary culture to 250-ml Erlenmeyer flasks containing 50 ml of medium A. To each flask, 15 mg of an inducer steroid, such as progesterone or 1,4-androstadiene-3,17-dione, was added and the flasks were incubated at 25°C on a rotary shaker (240 rev/min) for 3 to 4 days. For steroid transformations, 50 ml of the vegetative growth from the above shaken culture were transferred to a 2-liter Erlenmeyer flask containing 450 ml of Nutrient Broth (Difco); the steroid, dissolved in dimethylformamide, was added to yield a final concentration of 0.3 g per liter. All flasks were incubated at 28°C on a rotary shaker (240 rev/min) for 3 to 4 days.

Spores of _S. affinis_ were prepared as described previously (10). Spores were suspended in 0.05 M phosphate (5 × 10⁴ spores per ml; pH 6.0), the steroid was added to give a concentration of 0.3 to 1.0 mg/ml, and the flasks were incubated at 28°C on a rotary shaker.

For transformations with the vegetative growth of _S. affinis_, the organism was grown on a medium containing 1% cereolose, 2% cornsteep liquor, and 25 to 50 μg/ml of an inducer steroid such as 1,4-androstadiene-3,17-dione or 3-keto-bisnor-4-cholen-22-al (pH 4.9) for 24 hr; the steroid was added and incubation was continued for 24 to 96 hr at 28°C on a rotary shaker.

At the end of the incubation period, the reaction mixtures were acidified to pH 4.0 and extracted with methylene chloride. The solvent extracts were successively washed with 1% NaHCO₃ and water and evaporated to dryness. The residues were chromatographed on silica gel plates [thin-layer chromatography (TLC)]. The following solvent systems were used for development: 10% collidine in CCl₄ (v/v) and toluene-triethylamine-isopropyl ether (77:5: 15:7.5, v/v/v). The plates were sprayed with 1% p-nitrobenzenediazonium fluoroborate in 50% acetic acid to develop the phenolic spots and oversprayed with 50% p-toluenesulfonic acid in ethanol. The reaction products were also analyzed by gas-liquid chromatography (GLC) by using a Perkin Elmer model 800 apparatus (6 ft length), 2.5 mm (internal diameter) glass column packed with 5% DC-710 on
RESULTS AND DISCUSSION

Synthesis of 19-hydroxylated pregnanes. Pregnan-5-ene-3β,19,20β-triol 3-acetate was prepared by adding 250 mg of sodium borohydride to a stirred suspension of 500 mg of 3β,19-dihydroxyprogren-5-ene-20-one 3-acetate in 10 ml of methanol with ice cooling. After stirring for 1 hr, water was added and the precipitated gum was crystallized from methanol-water and then from acetone-hexane (Tm 181 to 183 C, [α]DHexane -64°).

Analysis: C23H36O4; calculated: C, 73.37; H, 9.64 found: C, 73.14; H, 9.43

To prepare 19-hydroxyprogren-4,7-diene-3,20-dione, 1.88 g of sodium methoxide was added to a solution of 2.6 g of 19-hydroxyprogren-4,6-diene-3,20-dione (A. Wettstein, G. Anner, K. Heusler, and J. Kalvoda, U.S. Patent 3, 211, 761, 1965) in 12 ml of dimethylsulfoxide. Thirty seconds later, 50 ml of hexane previously cooled to -70 C was added. After an additional 30 sec, 5.0 ml of cold concentrated hydrochloric acid was added and the mixture was vigorously stirred for 2 min. To the mixture, 20 ml of water was added, and the gummy solid was filtered and taken up in ethyl acetate; the solution was washed with sodium bicarbonate solution and water. After drying and evaporation of solvent, the residue was suspended in ether and filtered, yielding 1.6 g of crude product (Tm 165 to 174 C). Crystallization from acetone-hexane and then from methanol-water gave the pure product (Tm 177 to 179 C, [α]DHexane + 90°), and maximal ultraviolet absorption at 238 nm (ε 16,350).

Analysis: C17H20O5; calculated: C, 76.79; H, 8.59 found: C, 76.67; H, 8.69

Microbial transformation. Transformation of 360 mg of 3β,19-dihydroxyprogren-5-en-20-one 3-acetate by Nocardia sp. yielded 165 mg of dry residue which was chromatographed on 5 g of silica gel. The fractions eluted with benzene containing 5% (v/v) ether were evaporated to dryness and crystallized from acetone-hexane (Tm 249 to 251.5 C; infrared maxima (CHCl3) at 3,630, 3,400 (OH), 1,690 (20-ketone), 1,610, 1,590 cm⁻¹ (aromatic ring A). Acetylation with acetic anhydride-pyridine yielded a compound with infrared maxima (CHCl3) at 1,740 (phenolic acetate) and 1,690 cm⁻¹ (20-ketone). The product was assigned the structure: 3-hydroxy-19-nor-pregn-1,3,5(10)-trien-20-one (Tm 247 to 249 C, reference 3).

By using the routine procedure, 35 mg of pregn-5-ene-3β,19,20β-triol-3-acetate was transformed to yield 25 mg of dry residue identified as 3-hydroxy-19-nor-pregn-1,3,5(10)-trien-20-one by TLC and infrared spectrum. 19-Hydroxyprogesterone (6) was also transformed into the above phenol.

19-Hydroxyprogren-4,7-diene-3,20-dione was not transformed by Nocardia sp. or by the few other bacteria examined. Spores and vegetative mycelium of S. affinis rapidly transformed 19-hydroxyprogesterone into estrone. To 400 ml of spore suspension, 400 mg of 19-hydroxyprogesterone was added. After 22 hr of incubation, the reaction mixture was extracted and the usual work up yielded 300 mg of slightly yellow residue which, when analyzed by TLC, showed a main spot for estrone. The residue was washed with a small amount of ether and crystallized from ether to yield 229 mg of a product identical to estrone (Tm, infrared spectrum, and GLC).

When 3β,19-dihydroxyprogren-5-en-20-one 3-acetate was incubated with S. affinis, small amounts of estrone and 19-hydroxyprogesterone were formed.

By the procedure described above, 20 mg of 19-hydroxyprogren-4,7-diene-3,20-dione was incubated with the vegetative growth of S. affinis. After 48 hr of incubation, the reaction mixture was extracted and worked up as usual to yield 14 mg of dry residue. Analysis (GLC) indicated the presence of 5.5% equilin, 3.5% equilenin, traces of 7a,13,5(10),7-tetraene-3,17β-diol and estr-1,3,5(10),6,8-pentaene-3,17β-diol, and one or two unidentified peaks. Attempts to improve the yield of equilin by changing the composition of the culture medium were unsuccessful.

In our preliminary studies, Nocardia sp. was found to transform cholest-5-ene-3β,19-diol
3-acetate into estrone. However, this organism aromatized ring A of 3β,19-dihydroxy-pregn-5-en-20-one 3-acetate, 19-hydroxyprogesterone and pregn-5-ene-3β,19,20β-triol 3-acetate without cleaving the side chain (Fig. 1). Apparently, the cholestane and pregnane side chains are degraded through different enzymatic pathways and Nocardia sp. lacks an active enzyme system for the degradation of the C-17 side chain of 19-hydroxy pregnanes. S. affinis has previously (9) been shown to 1-dehydrogenate various pregnanes with concomitant cleavage of the side chain. It was therefore reasonable to expect that 1-dehydrogenation of 19-hydroxy pregnanes accompanied by cleavage of the side chain would lead to the formation of estrogens. Indeed S. affinis spores and mycelial growth transformed 19-hydroxy progesterone quantitatively into estrone (Fig. 1). With the objective of obtaining equilin, we subjected 19-hydroxypregn-4,7-diene-3,20-dione to the action of S. affinis. The reaction was more complex, and small amounts of equilin and equilenin, traces of estra-1,3,5(10),7-tetraene-3,17β-diol and estra-1,3,5(10),6,8-pentaene-3,17β-diol, and one or two unidentified products were formed.

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