Reduction of the 20-Carbonyl Group of C-21 Steroids by Spores of Fusarium solani and Other Microorganisms

I. Side-Chain Degradation, Epoxide Cleavage, and Substrate Specificity

ROSAIRE PLOURDE, OSSAMA M. EL-TAYEB, AND HAMDALLAH HAFEZ-ZEDAN

Faculté de Pharmacie, Université de Montréal, Montréal, Québec, Canada

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The spores of Fusarium solani reduced the C₂₀-carbonyl group, 1-dehydrogenated ring "A" and cleaved the side chain of 16α,17α-oxidopregnen-4-ene-3,20-dione (16α,17α-oxidopregnen-4-ene-3,20-dione(I)) to give the following products: 20α-hydroxy-16α,17α-oxidopregnen-4-ene-3,20-dione(II); 20α-hydroxy-16α,17α-oxidopregna-1,4-dien-3-one(III); 16α-hydroxy-17α-oxa-androst-1,4-diene-3,17-dione (16α-hydroxy-1-dehydrotestololactone)(IV); and 16α,17β-dihydroxy-androst-1,4-diene-3,17-dione (16α-hydroxy-1-dehydrotestosterone)(V). When II was used as a substrate, it was metabolized into III, IV, and V at a slower rate than I. Furthermore, 16α-hydroxy-androst-4-ene-3,17-dione (16α-hydroxyandrostenedione)(X) was transformed into IV and V. Pregn-4-ene-3,20-dione (progesterone)(XII) was transformed into androsta-1,4-diene-3,17-dione (androstanedione)(VIII) and 17α-oxa-androst-1,4-diene-3,17-dione (1-dehydrotestololactone)(IX), while 17α-hydroxy-pregn-4-ene-3,20-dione (17α-hydroxyprogesterone)(VI) was converted into its 1-dehydro analogue (VII) without accumulation of any 20-dihydro compounds. Substrate specificity in the 20-reductase system of F. solani, Cylindrocarpon radicicola, Septomyxa affinis, Bacillus lentus, and three strains of B. sphaericus are demonstrated. The 20-reductase is active only on steroids having the 16α,17α-oxido, and Δ⁴-3-keto functions. Evidence of competition between side-chain degrading enzymes and the 20-reductase for the steroid molecule and evidence of side-chain degradation followed by epoxide cleavage (and not the reverse) are presented. A mechanism for the epoxide opening by nongerminating spores of F. solani is postulated.

Several microorganisms are known to cleave epoxides or to degrade the 17β-acetyl side chain of progesterone and related steroids, or both, to give ring "D" lactones as final products. Microbiological epoxide cleavage has been reported by Camerino and Vercellone (4) and Camerino and Sciaky (3), Prochážka, et al. (18), Wix and Albrecht (34), El-Tayeb et al. (6), and Tömörkény et al. (29) using yeast cells, Rhizopus nigricans, Fusarium caucasicum, Cylindrocarpon radicicola, and Mycobacterium phlei, respectively. Wix and Albrecht (34) reported the conversion of 16α,17α-oxido-progesterone with F. caucasicum into androstenedione, probably via epoxide opening, forming a 17α-hydroxy intermediate, followed by removal of side chain. On the other hand, El-Tayeb et al. (6), using C. radicicola, demonstrated the eventual conversion of the same substrate into 16α-hydroxy-1-dehydrotestosterone and 16α-hydroxy-1-dehydrotestololactone, probably via side-chain cleavage followed by epoxide opening (Fig. 1).

Alternative mechanisms for steroid side-chain cleavage by various organisms involve: (i) direct oxygenation at C₂₁-C₂₂, (ii) reduction at C₂₅; or (iii) hydroxylation at C₁₇. Peterson et al. (17) suggested that C. radicicola degrades
Fig. 1. Conversion of 16α,17α-oxidoprogesterone into 16α-hydroxy-1-dehydrotestololactone and 16α-hydroxy-1-dehydrotestosterone and 16α-hydroxy-1-dehydrotestololactone. Conversion, probably via side-chain cleavage followed by epoxide opening, demonstrated by El-Tayeb et al. (6) in C. radicicola.

progesterone side chain via mechanism i, and the first intermediate of the sequence, 17β-acetoxy-androst-4-en-3-one (testosterone acetate), was first isolated by Fonken et al. (8) after exposure of progesterone to Cladosporium resinae. Recently, Rahim and Sih (19) separated two enzymes from C. radicicola: an oxygenase that catalyzes the conversion of progesterone into testosterone acetate, which was then hydrolyzed by an esterase to yield 17β-hydroxy-androst-4-en-3-one (testosterone). Sebek et al. (22) reported the possibility of 20β-hydroxy derivatives (mechanism ii) as intermediates in side-chain degradation of progesterone and 11α-hydroxyprogesterone by Penicillium lilacinum. On the other hand, the possibility of a 17α-hydroxy intermediate (mechanism iii) in the oxidative cleavage of the side chain of C21 steroids has precedence in higher organisms (5).

In previous communications (10, 12, 21, 24, 25, 30), it was established that nongerminating spores of fungi and actinomyces can affect a wide range of conversions of steroid molecules. The nongerminating spores of certain microorganisms showed identical (31) or modified (10, 11; H. H. Zedan, M.S. thesis, Univ. of Cairo, Cairo, Egypt, 1969) activities on steroids as compared to corresponding growing cultures. Whereas the mechanisms of steroid transformation by growing cells have been extensively studied, Singh and Rakhit (23) appear to have been the first to have elucidated the mechanism of side-chain degradation of C-21 steroids with the nongerminating spores of Septomyxa affinis; the mechanism was suggested for steroids that do not have a 16α,17α-epoxide group.

A comparative study of the activities of nongerminating spores and growing cells of F. solani and other microorganisms on different steroids has been made to determine the role of 20-reductase in microbial side-chain degradation and the side-chain removal of 16α,17α-epoxysteroids.

MATERIALS AND METHODS

Microorganisms and chemicals. Stock cultures of C. radicicola ATCC 11011, F. solani (Faculty of Pharmacy, Univ. of Cairo), S. affinis ATCC 6737, and P. lilacinum ATCC 10114 were maintained on nutrient dextrose agar (Difco) supplemented with 1% yeast extract. Bacillus lentus ATCC 13805, and the strains of B. sphaericus ATCC 245, 7055, and 7055 were maintained on nutrient agar. They were stored at 4 C and subcultured monthly.

Analytically pure 20α-hydroxy-16α,17α-oxidopregn-4-en-3-one; 20α-hydroxy-16α,17α-oxidopregna-1,4-dien-3-one; 16α-hydroxy-1-dehydrotestosterone, and 16α-hydroxy-1-dehydrotestololactone were prepared from 16α,17α-oxidoprogesterone, while 1-dehydrotestololactone was prepared from progesterone by incubation with C. radicicola as previously described by El-Tayeb et al. (6, 7). Other chemicals were reagent grade.

Sporeulation. Spores of F. solani and C. radicicola were produced on Sabouraud dextrose agar, and S. affinis was sporeulated on a medium consisting of 1% glucose, 0.5% yeast extract, 3% malt extract, and 2.5% agar (Difco). To all the sporation media, progesterone (100 µg/ml) was added as an inducer. Abundant sporation was attained after incubation for 5 to 7 days at 26 to 28 C. Spores were harvested in 1% phosphate buffer (pH 6.0), and spore suspensions were filtered through sterile cheesecloth, washed five times by centrifugation, resuspended in the buffer solution, and stored at 4 C.

Steroid conversion by spores. The spores, suspended in the phosphate buffer, were counted in a hemocytometer, and the suspension was diluted to give 1.5 x 10⁶ spores per ml. Transformations were carried out in 125-ml Erlenmeyer flasks, each containing 25 ml of the spore suspension. Sterile glucose solution was added to each flask to give a final concentration of 0.5 mg/ml, and a 50 mg/ml solution of
the steroid substrate dissolved in N,N-dimethylformamide (DMF) was added to give a concentration of 0.5 mg per ml of medium. The flasks were incubated on a reciprocating shaker (4-cm stroke, 150 cycles/min) at 26 to 28 C for 48 to 72 hr (unless otherwise specified).

**Steroid conversion by growing cells.** Transformation of steroids by growing fungal mycelia was carried out in a medium consisting of 1% glucose, 2% peptone, 1% yeast extract, and 0.25% calcium carbonate. For transformation by bacilli, nutrient broth supplemented with 0.5% yeast extract was used.

A fresh stock culture was used to inoculate 50 ml of the sterile medium in 250-ml Erlenmeyer flasks; the inoculated flasks were incubated on a shaker for 48 hr. A 5% inoculum from the resulting growth was used to inoculate a fresh medium, which was further incubated for 24 hr. A fresh flask was incubated with a 5% inoculum of the resulting growth and again was incubated for 24 hr; the substrate was then added, and the culture medium was incubated as for spore-mediated transformation.

**Steroid determination.** All melting points were determined on a Renco capillary melting point apparatus. Infrared (IR) spectra were recorded from KBr discs on a Perkin Elmer model 257 double-beam IR spectrophotometer. Ultraviolet (UV) absorption spectra were recorded in 95% ethanol on a Unicam recording spectrophotometer (model 800). MN-Silica Gel G/UV254 (Macherey, Nagel & Co., Germany) was used for thin-layer chromatography (TLC) analyses.

At the end of the incubation period, the whole reaction mixture was extracted with chloroform and the extract was dried over anhydrous sodium sulfate and evaporated to dryness under vacuum.

The course of steroid transformation was followed by TLC on Silica Gel G plates developed in benzene-isopropanol (6:1). The chromatoplates were viewed under a short-wave UV lamp, sprayed with 50% aqueous sulfuric acid, and heated at 110 C for 10 min.

When particularly specified, \( \Delta^1 \)-3-ketosteroids were differentiated from \( \Delta^4 \)-3-ketosteroids on the plates by spraying with the strong methanolic isonicotinic acid hydrazide (INH) solution of Smith and Foell (26); \( \Delta^4 \)-3-ketosteroids give a yellow color within 5 to 10 min, whereas 1 to 2 hr are required for the same color to appear in the case of \( \Delta^1 \)-3-ketosteroids (12). Similarly, the presence of steroids bearing a 17\( \beta \)-methyl ketone side chain was revealed on TLC plates by the nitroprusside reaction of Lisbon (13) for methyl ketones.

The transformation products were separated from crude extract by preparative TLC, recrystallized, and identified by comparison with authentic samples [melting point (MP), mixed MP, IR spectra, and chromatographic properties].

For quantitative estimation of steroids, samples were spotted on TLC plates, developed, and air-dried. The UV absorbing spots, along with blank spots of the same size, were scraped off and eluted with 95% ethanol. The absorption of the eluates was measured spectrophotometrically at 240 nm. Results were expressed as percentage of total steroids.

**RESULTS**

**Transformation of 16\( \alpha \),17\( \alpha \)-oxidopregesterone by spores of F. solani.** Spores of *F. solani* converted 16\( \alpha \),17\( \alpha \)-oxidopregesterone(I) to 20\( \alpha \)-hydroxy-16\( \alpha \),17\( \alpha \)-oxido-pregn-4-en-3-one(II), 20\( \alpha \)-hydroxy-16\( \alpha \),17\( \alpha \)-oxido-pregna-1,4-dien-3-one (III), 16\( \alpha \)-hydroxy-1-dehydrotestolactone(IV), and 16\( \alpha \)-hydroxy-1-dehydrotestosterone(V).

After a 36-hr incubation period of 50 mg of I with spores and the usual work-up of extraction, separation, and recrystallization from acetone-petroleum ether, the following products were obtained: 10 mg of II, 10 mg of III, 6 mg of IV, and 3 mg of V. The properties of the isolated compounds were identical with those of corresponding authentic standards (Table 1).

The relative proportions of II, III, IV, and V varied with the period of incubation. Figure 2 illustrates the course of transformation of I by spores. Spore I disappeared gradually, and the concentration reached a plateau at 28 hr. The four metabolites (II, III, IV, and V) showed different transformation rates. As II and III exhibited an increase in their concentrations followed by gradual disappearance, compounds IV and V accumulated. 16-Hydroxyandrosteno-1,4-diene-3,17-dione, which might be expected as an intermediate between compounds V and IV, was not detected.

When compound II was used as a substrate, it was metabolized at a slower rate than I but with a similar conversion pattern, whereas 16\( \alpha \)-hydroxyandrenedione(X) was converted into IV and V. Figure 3 represents the conversion behavior of compound II and its transformation products on incubation with spores.

**Transformation of compound I by mycelium of F. solani.** Compound I was quantitatively converted with growing *F. solani* into II, III, IV, and V within 12 hr (Fig. 4). Although the transformation patterns of I by the spores and the mycelium were similar, the substrate was transformed at a slightly higher rate by the mycelium. Furthermore, lower yields of the 20\( \alpha \)-hydroxy metabolites (II and III) and higher yields of the side-chain degradation products (IV and V) were obtained in the mycelial fermentation (Fig. 5).

**Transformation of 17\( \alpha \)-hydroxyprogesterone by spores of F. solani.** Spores of *F. solani* converted 17\( \alpha \)-hydroxyprogesterone (VI) into 17\( \alpha \)-hydroxyprogna-1,4-diene-3,20-dione (VII), which was accumulated in the medium after a lag period of 6 hr and reached a plateau (36%) after 72 hr, as illustrated in
Table 1. Physicochemical properties of the isolated steroids

| Compound | Name | Melting point | INH | H₂SO₄ |
|----------|------|---------------|-----|-------|
| II       | 20α-Hydroxy-16α,17α-oxidopregn-4-en-3-one | 245–247 C | +++ | Greenish blue |
| III      | 20α-Hydroxy-16α,17α-oxidopregna-1,4-dien-3-one | 225–227 C | + | Red |
| IV       | 16α-Hydroxy-17α-oxa-androsta-1,4-diene-207-209 C | 207–209 C | + | Brown |
| V        | 16α,17β-Dihydroxy-androsta-1,4-dien-3-one | 199–202 C | + | Orange brown |
| VII      | 17α-Hydroxy-pregna-1,4-diene-3,20-dione | 249–250.5 C | + | Brown |
| VIII     | Androsta-1,4-diene-3,17-dione | 139–141 C | + | Orange red |
| IX       | 17α-Oxa-androsta-1,4-diene-3,17-dione | 219–221 C | + | Brown |

*These properties as well as the infrared spectra were identical with those of authentic standards and there was no depression in mixed melting points. See references 6 and 7 for more comprehensive identification of these and related steroids.

* Symbols: (+++), yellow color within a few minutes; (+), yellow color after 1 hr or more.

Fig. 2. Time course of the metabolism of 16α,17α-oxidoprogesterone (O) by the spores of Fusarium solani. Spores, 1.5 x 10⁸/ml; substrate, 0.5 mg/ml; glucose, 0.5 mg/ml; pH 6.0. Symbols: O, 20α-hydroxy-16α,17α-oxidopregnen-4-en-3-one; Δ, 20α-hydroxy-16α,17α-oxidopregna-1,4-dien-3-one; ▲, 16α-hydroxy-1-dehydrotestosterone; □, 16α-hydroxy-1-dehydrotestosterone.

Fig. 6.
When 200 mg of VI was incubated for 48 hr with spores, 63 mg of VII was obtained. Compound VII was purified by repeated crystallization from acetone; MP found was 249 to 250.5 C [reported 245 to 262 C by J. N. Gardner, U.S. Patent 3,356,696, 1967 (to Schering Corp., Bloomfield, N.J.)], IR spectra were 2.92 μm (hydroxyl), 5.88 μm (20-ketone), 6.06, 6.2, 6.28 μm (Δ¹ʻ4-3-ketone). The identification of VII was also confirmed by a combination of microbiological and chemical analytical methods. Through the action of P. lilacinum ATCC 10114, which brings about side-chain degradation of many steroids (16), the isolated compound VII was transformed into compound VIII, identical in all respects (Table 1) with an authentic sample of androstadienedione; another compound was obtained which was tentatively identified as 1-dehydrotestolactone (IX). This indicates the presence of a Δ¹ʻ4-3-ketone group in the original structure of VII. The
Fig. 3. Time course of the metabolism of 20α-hydroxy-16α,17α-oxidopregn-4-en-3-one (○) by the spores of Fusarium solani. Spores, 1.5 x 10^8/ml; substrate, 0.5 mg/ml; glucose, 0.5 mg/ml; pH 6.0. Symbols: △, 20α-hydroxy-16α,17α-oxidopregna-1,4-dien-3-one; ▲, 16α-hydroxy-1-dehydrotestololactone; □, 16α-hydroxy-1-dehydrotestosterone.

Fig. 4. Time course of the metabolism of 16α,17α-oxidoprogesterone (○) by the mycelium of Fusarium solani. The conditions are explained in the text. Symbols: ●, 20α-hydroxy-16α,17α-oxidopregn-4-en-3-one; △, 20α-hydroxy-16α,17α-oxidopregna-1,4-dien-3-one; ▲, 16α-hydroxy-1-dehydrotestololactone; □, 16α-hydroxy-1-dehydrotestosterone.
Fig. 5. Formation of 20α-hydroxy metabolites (20α-hydroxy-16α,17α-oxidopregnen-4-en-3-one and its 1-dehydro analogue) and side-chain degradation products (16α-hydroxy-1-dehydrotestolactone and 16α-hydroxy-1-dehydrotestosterone) from 16α,17α-oxidoprogesterone on incubation with the spores and the mycelium of Fusarium solani. The conditions are explained in Fig. 2 and 4. Symbols: 20α-hydroxymetabolites (△) and side-chain degradation (○) products by spores; 20α-hydroxy metabolites (▲) and side-chain degradation (●) products by mycelium.

Fig. 6. Time course of the metabolism of 17α-hydroxyprogesterone (○) into 17α-hydroxy-pregna-1,4-diene-3,20-dione (●) by the spores of Fusarium solani. Spores, 1.5 x 10⁸/ml; substrate, 0.5 mg/ml; glucose, 0.5 mg/ml; pH 6.0.
presence of this group was confirmed by its behavior toward isonicotinic acid hydrazide reagent (26); compound VII gave a yellow spot on TLC plates after 1 to 2 hr. As a final proof, VII was not transformed by strains of B. sphaericus ATCC 7055, 7054, and 245 or by B. lentus ATCC 13805, all known to 1-dehydrogenate various steroids (1, 9, 20, 33), which indicates that the Δ1-double bond is already present in compound VII. The presence of an intact 17β-methyl-ketone side chain was revealed by a positive nitroprusside reaction (13).

The 6-hr lag period suggests the presence of an inducible system. However, the addition of 0.05 mg of either peptone, yeast extract, or ammonium sulfate per ml to the fermentation medium as well as the reuse of spores in two successive fermentations did not eliminate the lag period or enhance the yield. Furthermore, the same reaction course was observed when the process was conducted in the presence of tetracycline hydrochloride (100 μg/ml of medium), an antibiotic which inhibits protein synthesis.

Transformation of VI by mycelium of F. solani. F. solani mycelium transformed VI into androstadienedione(VIII) and 1-dehydrotestololactone(IX), identified by comparison with authentic standards as shown in Table 1.

Transformation of 3β-hydroxy-16α, 17α-oxido-pregn-5-en-20-one (17α-hydroxy-pregnenolone) by spores and mycelium of F. solani. 3β-Hydroxy-16α, 17α-oxido-pregn-5-en-20-one (17α-hydroxypregnenolone)(XI) was not metabolized by either the spores or the mycelium.

Transformation of compound I and other steroids by spores or growing cells, or both, of various microorganisms. The activity of the spores and mycelia of C. radicicola and S. affinis, as well as the cells of three strains of B. sphaericus and one of B. lentus, on I, VI, and XI and on progesterone(XII) is presented in Table 2.

The reaction products listed in Table 2 were identified on the basis of comparisons of their Rf values and color reactions (isonicotinic acid hydrazide, H2SO4, and nitroprusside tests) with those of authentic samples of the respective steroids.

DISCUSSION

The spores of F. solani are capable of bringing about a variety of conversions of the steroid molecule: introduction of Δ1-double bond, reduction of the C-20 carbonyl group into a 20α-hydroxy group, cleavage of the methyl ketone side chain, oxygenation of ring D into a six-membered heterocyclic lactone, and cleavage of the 16α, 17α-epoxide group.

In a previous communication, El-Tayeb et al. (6) suggested that the 20-reductase of C. radicicola (mycelium) is probably competing with the side-chain-degrading enzymes for C-21 steroids. Thus, substrates which are metabolized rapidly by side-chain-degrading enzymes would be transformed very little, if any, into 20-hydroxy compounds, while substrates which are slowly metabolized by side-chain-degrading enzymes would be converted into 20-hydroxy derivatives. Accordingly, one may assume that the spores of F. solani would accumulate larger quantities of 20-hydroxy steroids than the vegetative mycelium, since it has been demonstrated that the former are much less active in side-chain degradation than the corresponding growing mycelium (11). From Fig. 2, 4, and 5, it is evident that higher yields of II and III accumulated within the first 24 hr, when the spores, instead of the growing cells, of F. solani were incubated with I; furthermore, the amount of II produced by the growing mycelium disappeared rapidly (after 24 hr) from the fermentation medium, while the side-chain degradation products (IV and V) accumulated very early. There was also higher rate of side-chain degradation and higher yields of C-19 steroids obtained after 24 hr with growing mycelium. This suggests competition between side-chain-degrading enzymes and the 20-reductase. It is also interesting to note that the amount of 20α-hydroxy derivatives produced by the mycelium from compound I is much higher in F. solani than in C. radicicola (6). This is significant since we know that F. solani is a weaker side-chain-degrading fungus than C. radicicola (11).

From the results obtained in this study (Fig. 7), it is clear that the course of 20-keto reduction or side-chain degradation, or both, by F. solani (spores or mycelium) was significantly modified by minor changes in the functional groups on the steroid molecule. Thus, while both the spores and the mycelium metabolized progesterone quantitatively into androstadiene-dione and 1-dehydrotestololactone (11) without accumulation of any 20-hydroxy compounds, the mycelium converted 17α-hydroxy-progesterone into the same products and the spores converted that same steroid into its 1-dehydro analogue without side-chain degradation. Thus, even though 17α-hydroxyprogesterone was not affected by the side-chain-degrading enzymes of the spores, the C-20 keto group was not reduced. This inability of the spores to reduce the 20-carbonyl function does not result
from impermeability, since the substrate could be transformed into its 1-dehydro derivative. Alternative explanations present themselves for the cleavage of the side chain of 17α-hydroxyprogesterone by mycelium and not by spores. (i) The spores may contain only 1-dehydrogenase and lack the side-chain-degrading enzymes. (ii) The main reaction in both mycelium and spore processes may be 1-dehydrogenation; further incubation may induce de novo protein synthesis of the side-chain-cleaving enzymes in the mycelium. This induction would not take place in spores due to absence of adequate nitrogen supplement. (iii) Both mycelium and spores contain both 1-dehydrogenase and side-chain-degrading enzymes. In spore-mediated reactions, the side-chain-degrading enzymes may be inactive due to a lack of certain cofactors which may be present in the mycelium-mediated reactions. The preparation of a cell-free extract from spores may explain the difference of activity. Vischer and Wettstein (32) already found that the presence of a 17α-hydroxy group on the steroid molecule blocks side-chain degradation by the F. solani mycelium; however, our obser-

| Organism and steroid substrate | Products | Reactions |
|-------------------------------|----------|-----------|
| Cylindrocarpon radicicola ATCC 11011; Septomyxa affinis ATCC 6737 (spores, mycelia): Progesterone, 17α-hydroxyprogesterone | Androstadienedione | 1-Dehydrogenation, side-chain degradation |
| 16α, 17α-Oxidopregnenolone 16α, 17α-Oxidoprogesterone | 1-Dehydrotestolactone | 1-Dehydrogenation, side-chain degradation, ring D lactonization |
| Bacillus sphaericus ATCC 7054, ATCC 7055 (vegetative cells): Progesterone 17α-Hydroxyprogesterone 16α, 17α-Oxidopregnenolone 16α, 17α-Oxidoprogesterone | None | Nil |
| B. sphaericus ATCC 245, B. lentus ATCC 13805 (vegetative cells): Progesterone 17α-Hydroxyprogesterone 16α, 17α-Oxidopregnenolone 16α, 17α-Oxidoprogesterone | 1-Dehydro derivative | 1-Dehydrogenation |
| 20α-Hydroxy derivative | 1-Dehydro derivative | 1-Dehydrogenation |
| 16α-Hydroxy-1-dehydro-testosterone 16α-Hydroxy-1-dehydro-testolactone | None | Reduction of the C-20 ketone |
| 20α-Hydroxy derivative | 1-Dehydro derivative | Reduction of the C-20 ketone, 1-dehydrogenation |
| 20α-Hydroxy-1-dehydro derivative | 1-Dehydro derivative | 1-Dehydrogenation, side-chain degradation, epoxide cleavage |
| Androstenedione | 1-Dehydrogenation, side-chain degradation, epoxide cleavage, ring D lactonization |
| Oxidation of the 17β-hydroxy group |

* Spores, 1.5 × 10⁴/ml; substrate, 0.5 mg/ml; glucose, 0.5 mg/ml; incubation, 24 to 72 hr.
* Incubation period, 24 hr.
Fig. 7. Transformation of steroids by the spores and the mycelium of Fusarium solani. S, spores; M, mycelium.

vation is the first example of such a difference between two different forms (spores and mycelium) of a given strain.

The question as to whether a nonspecific 17β-hydroxy steroid dehydrogenase might be responsible for catalyzing the reduction of the keto function at C₂₀ has been investigated. Although all examined strains of B. sphaericus and B. lentus (Table 2) oxidized the 17β-hydroxy group of testosterone, only two strains of B. sphaericus (ATCC 7054, 7055) were able to reduce the C₂₀ ketone of 16α,17α-oxidoprogesterone. This observation attenuates the possible participation of a 17β-hydroxy steroid dehydrogenase.

To examine the need for a 16α,17α-oxido group as the only requirement for the activity of the 20-reductase system, 16α,17α-oxidopregnolone was used as a substrate. Neither the mycelium nor the spores of F. solani could transform it. However, the permeability phenomena are not excluded.

The ability of several microbial cultures to reduce the 20-carbonyl group of 16α,17α-oxidoprogesterone to the 20α-hydroxy group and the influence of structure alterations in the
molecule on their activity are illustrated in Table 2. It is probable that the C₂₀ ketone reduction or the side-chain degradation, or both, could be hindered through alteration of the spatial relationship in the steroid molecule in such a way that the points of reactivity between the enzyme and the substrate may be blocked.

The possibility of a 20-hydroxy intermediate in steroid side-chain degradation was reported by Sweat and co-workers (27), who proposed that 17α, 20α-dihydroxyprogren-4-en-3-one can serve as an intermediate precursor of androstenedione in bovine and human ovaries, while Sebek et al. (22) suggested the possibility of participation of these 20-dihydro compounds as intermediates in microbiological side-chain degradation. Our results showed that 20α-hydroxy-16α, 17α-oxidopregnen-3-one was metabolized at a slower rate than 16α, 17α-oxidoprogesterone and the 20-reductase of other microorganisms which do not carry out side-chain cleavage (Table 2). It is therefore unlikely that 20α-hydroxy compound is an obligatory intermediate in the side-chain degradation by F. solani spores. This has also been shown to be true with C. radicicola mycelium (6).

Rahim and Sih reported that 20α-hydroxy-16α, 17α-oxidopregen-4-en-3-one was not metabolized by the side-chain-degrading enzymes isolated from mycelium-free extracts of C. radicicola (19). Similar observations were reported for other biological systems; Lynn and Brown (14) have demonstrated that testicular microsomes failed to cleave the side chains of 20α- and 20β-hydroxysteroids, and Ball and Kadis (2) reported that the side chain of progesterone was not cleaved with sow ovary, although a 20α-hydroxy compound (17α, 20α-dihydroxyprogren4-en-3-one) was formed. Therefore, it appears that these 20α-hydroxy steroids have no biological role in side-chain degradation and that they are unusual metabolites of 16α, 17α-oxidoprogesterone.

Figure 8 represents three alternate mechanisms for the opening of the 16α, 17α-oxido group. Mechanism A involves the opening of the oxido group into a 17α-hydroxy group (the presence of a 17β-side chain prevents the at-

![Fig. 8. Possible mechanisms for the cleavage of the 16α, 17α-oxido group of 16α, 17α-oxidoprogesterone.](http://aem.asm.org/)
tack of the hydride ion from the back side of the molecule, thereby preventing the formation of a 16α-hydroxy derivative) via a reductive type of opening in a manner similar to that by metal hydrides. Side-chain degradation of the resulting 17α-hydroxy compound would yield the corresponding 17-ketosteroid which on reduction gives the 17β-hydroxy derivative and on oxidation gives the respective steroid lactone. The presence of a 16α-hydroxy group on the molecules of the side-chain degradation metabolites (IV and V) and the inability of the spores to cleave the side chain of 17α-hydroxyprogesterone rule out this mechanism. Mechanism B could proceed via the opening of the 16α,17α-oxido group into a 16α, 17α-dihydroxy derivative which parallels the acid-catalyzed opening of epoxides. Side-chain degradation followed by reduction or oxygenation would give rise to 16α,17β-dihydroxy- and 16α-hydroxy ring D lactone-steroids, respectively. This mechanism is also ruled out by the fact that the presence of a 17α-hydroxy function in the steroid structure inhibited the side-chain degradation activity of the spores. Mechanism C could involve side-chain degradation of the 16α,17α-oxido compound followed by a nonenzymic rearrangement to the 16α-hydroxy-17-ketone. Reduction of the 17-keto group would give the 16α,17β-dihydroxy metabolite (V), while its oxidation through a Baeyer-Villiger-type reaction could yield the corresponding lactone (IV). Our results are in complete agreement with mechanism C, which involves a nonenzymic epoxide opening.

The resistance of 16α-hydroxy-1-dehydrotestosterone (V) to further oxidation is consistent with the observation of Talalay and Marcus (28) that 17β-hydroxysteroid dehydrogenase from Pseudomonas testosteroni was unable to oxidize 17β-hydroxy steroids bearing oxygen functions at C₁₄, and with the observation of El-Tayeab et al. (6), who reported that V is a final product in the fermentation of 16α,17α-oxidospregesterone with C. radicicola (mycelium). However, the reverse reaction can obviously take place, since 16α-hydroxy-androstenedione was readily transformed to V by nongerminating spores and mycelium of F. solani as indicated by X → V on Fig. 7.

We conclude that the metabolism of 16α,17α-oxidospregesterone by nongerminating spores of F. solani can tentatively be represented by Fig. 9, which is consistent with previous findings (6) with C. radicicola mycelium.

The 6-hr lag period that was apparent in the bioconversion of 17α-hydroxyprogesterone by spores of F. solani (Fig. 6) suggested that enzyme adaptation had occurred. As described by Mandel and Vitolis (15) for the induced trehalose mechanism in spores of the fungus Myrothecium verrucaria, the de novo protein synthesis could occur at the expense of the endogenous amino acid pool of the spores. We examined this possibility by three independent methods: (i) reuse of one batch of spores in successive fermentations of the same substrate; (ii) addition of very low concentration of an exogenous protein supplement (0.05 mg of peptone, yeast extract, or ammonium sulfate per ml of medium) to the reaction medium before incubation; and (iii) biotransformation in the presence of a protein synthesis inhibitor (100 μg of tetracycline HCl per ml of medium).

In all cases, the 6-hr lag period and the same pattern of transformation were obtained, thus eliminating the possibility of enzymatic adap-
tation during the process (although one has to bear in mind the question of cell permeability to the antibiotic used). This observation, which is consistent with the report of Vézina et al. (30) on hydroxylation of steroids by the conidia of Aspergillus ochraceus, is being investigated.

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