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MICROBIAL HYDROXYLATIONS—IX:* ON THE DIHYDROXYLATION OF 19-NORSTEROIDS BY *CURVULARIA LUNATA*

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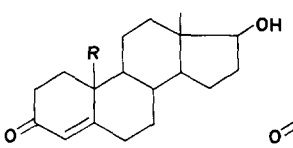
SUMMARY

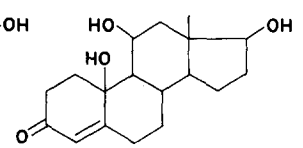
10β-Hydroperoxy-17β-hydroxy-4-estren-3-one was transformed by vegetative cell cultures of *Curvularia lunata* NRRL 2380 to 10β,17β-dihydroxy-4-estren-3-one which was transformed in turn on prolonged fermentation to 10β-hydroxy-4-estrene-3,17-dione and 10β,11β,17β-trihydroxy-4-estren-3-one. These results support sequential hydroxylation as the means by which 10β,11β,17β-trihydroxy-4-estren-3-one be formed from 17β-hydroxy-4-estren-3-one.

INTRODUCTION

The formation of small amounts of 10β,11β- and 6β,10β-dihydroxylated derivatives of 19-nor-4-ene-3-ketosteroids by *Curvularia lunata* NRRL 2380 [1, 2] was not readily reconciled with sequential monohydroxylations, and we have suggested the possibility of an alternative mechanism involving a microbial dioxygenase [2]. We have recently established the action of dioxygenases from higher plants on sterols to form hydroperoxides [3] and have offered evidence supporting the intermediacy of cholesterol 20α-hydroperoxide in the cleavage of the sterol side-chain by mammalian adrenal cortex mitochondrial enzymes [4–6]. The present study considers whether a putative dioxygenase of *C. lunata* NRRL 2380 transform 17β-hydroxy-4-estren-3-one (I) via hydroperoxide or epidioxide intermediates to the *cis*-1,3-dihydroxylated product 10β,11β,17β-trihydroxy-4-estren-3-one (IV).



(I) R = H
(II) R = OOH
(III) R = OH



(IV)

Were the 10β,11β,17β-triol IV derived from substrate I by rearrangement of a hydroperoxide intermediate in a manner analogous to formation of the vicinal diols 5-cholestene-3β,20α,21-triol and 5-cholestene-3β,20α,22R-triol from cholesterol 20α-hydroperoxide [4], then 10β-hydroperoxy-17β-hydroxy-4-estren-3-one (II) would be a good prospective intermediate. To test the hypothesis we examined the metabolism of the 10β-hydroperoxide II and of its corresponding 10β-alcohol III in vegetative cell cultures of *C. lunata* NRRL 2380, and our results are reported herein.

EXPERIMENTAL

Melting points were taken on a calibrated Kofler block under microscopic magnification. Infrared absorption spectra were obtained on a Perkin–Elmer Model 337 spectrophotometer equipped with a beam condensing lens, using 1·5 mm dia potassium bromide disks incorporating the sample. Thin-layer chromatography was conducted on 0·25 mm thick 20 × 20 cm chromatoplates of Silica Gel HF_{254} (E. Merck, GmbH., Darmstadt) irrigated several times in ascending fashion with benzene–ethyl acetate (1:1, v/v or 1:3, v/v) mixtures. Mobility data are given as R_F values. Steroids were detected by their ultraviolet light absorption properties and by their characteristic colors with 50% aqueous sulfuric acid. The hydroperoxide II was additionally detected with the N,N-dimethyl-*p*-phenylenediamine reagent [7]. Preparative t.l.c. was conducted similarly except that 0·5 mm thick chromatoplates were used.

Gas chromatography was conducted on 0·83 m long 4 mm dia silanized glass U-tubes packed with 3% SP-2401 on 100–120 mesh Supelcoport (Supelco Co., Bellafonte, Pa.) using nitrogen as carrier gas at 20

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ml/min flow rate. Injection port temperature was 250°C, column temperature was 230°C, detector oven temperature was 250°C. Retention time data (t_R) are given in terms of the 10β-alcohol III as unit retention time. The absolute retention time for III on 3% SP-2401 was 10·9 min.

Vegetative cell cultures of *Curvularia lunata* NRRL 2380 were grown for 24 h at 28°C in 200 ml of a 1% sucrose–1% Difco tryptone medium as previously described in detail [2]. Steroid substrates II and III were added as dimethylformamide solutions so as to give final steroid concentrations of 250 μg/ml with final dimethylformamide concentrations less than 0·25% (v/v). Aliquots of fermentation broth were removed at intervals, extracted with methyl isobutyl ketone, and analyzed by t.l.c. and by gas chromatography. Fermentations were terminated by addition of 100 ml of methyl isobutyl ketone. Products were thereby extracted, and the aqueous layer was re-extracted twice more with 100 ml portions of methyl isobutyl ketone. The combined solvent extracts were evaporated under vacuum to give the steroid product mixtures.

In one experiment an induction of steroid hydroxylases was insured by addition of 40 mg of I (final concentration 200 μg/ml) to a flourishing culture of *C. lunata*. After 5 h aeration of the flask, during which time hydroxylated products of I were seen to form, the fermentation broth was filtered, the mycelium washed thoroughly six times with distilled water, and resuspended in the 1% sucrose–1% Difco tryptone medium. Addition of substrate II then followed in the usual manner.

10β-Hydroperoxy-17β-hydroxy-4-estren-3-one (II)

Oxygen was slowly bubbled through a solution of 450 mg of 17β-hydroxy-5(10)-estren-3-one in 25 ml of chloroform held in a glass flask exposed to fluorescent (ceiling) lighting. After 22 h approximately half of the starting material had been consumed. The solution was applied to 0·5 mm thick 20 × 20 cm chromatoplates of Silica Gel HF_{254} and the steroid mixture was resolved by irrigation twice with benzene–ethyl acetate (1:1, v/v). Starting material (R_F 0·66), I (R_F 0·47), and product II (R_F 0·42) were resolved. The hydroperoxide (U.V. light absorbing, positive reaction to N,N-dimethyl-*p*-phenylenediamine, brown color with 50% aqueous sulfuric acid) was eluted with acetone, yielding 113·1 mg (28·2%) of product homogeneous by thin-layer chromatography. Recrystallization from benzene gave pure II, m.p. 179–184°C (literature m.p. 181–185°C [8]); $\tilde{\nu}_{max}^{KBr}$ 3350, 3200, 1660, 1055, 1020, 940 cm^{-1}; R_F 0·42 in benzene–ethyl acetate (1:1, v/v). The pyrolysis pattern obtained from II by gas chromatography on 3% SP-2401 included five major components at t_R 0·24, 0·37, 0·53, 0·72 and 1·00 (Fig. 1A) and several minor components including one at t_R 2·48 (Fig. 1B).

The pyrolysis products of II were collected in glass capillaries, rinsed therefrom with acetone, the solvent evaporated, and each product examined by t.l.c. and gas chromatography and by I.R. absorption spectra. The identity of only one major product could be determined. The t_R 1·00 component identified as the 10β-alcohol III was characterized by m.p. 198–202°C (literature m.p. 199–205°C [9], 208–210°C [10], 206–

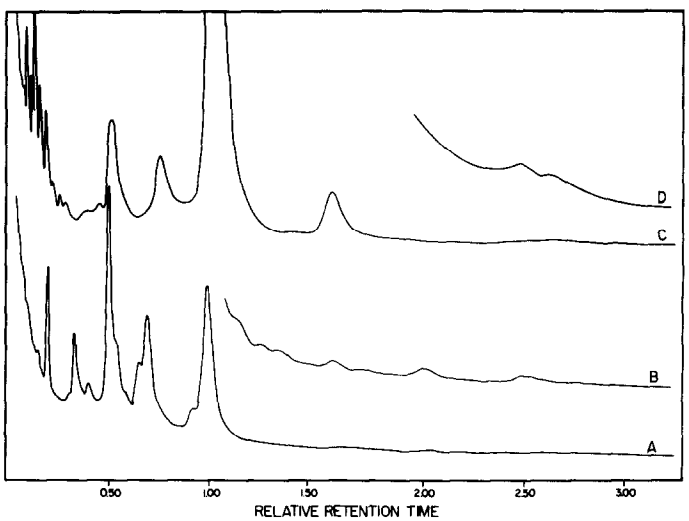


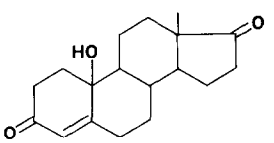
Fig. 1. Gas chromatographic elution curve (3% SP-2401) of pyrolysis and fermentation products of 10β-hydroperoxy-17β-hydroxy-4-estren-3-one (II). Curve A, pyrolysis of II; curve B, polar trace pyrolysis products of II; curve C, fermentation (2 h duration) products of II; curve D, polar trace fermentation products of II.

209°C and 205–210°C [1], 196–206°C [8], 207–210°C [11], 210–215°C [2]); R_F 0·54 in benzene–ethyl acetate (1:3, v/v); yellow–orange color with 50% aqueous sulfuric acid spray; t_R 1·00 on 3% SP-2401; $\tilde{\nu}_{max}^{KBr}$ 3350, 1640, 1060, 945 cm^{-1}; identical in these properties with those of an authentic reference sample of III.

The long retained trace pyrolysis product at t_R 2·48 (Fig. 1B) was shown not to be the 10β,11β,17β-triol IV by gas chromatography. The 10β,11β,17β-triol IV with t_R 2·63 was definitively resolved from the t_R 2·48 component whose identity remains unknown.

Transformations of 10β-hydroperoxy-17β-hydroxy-4-estrene-3-one (II)

After addition of 51·6 mg of II to 200 ml of a flourishing vegetative cell culture of *C. lunata* aliquots of broth were withdrawn for chromatographic analysis at 15, 30 and 60 min. In these samples the 10β-alcohol III was the major product, with traces of the 17-ketone V



(V)

but with no trace of the 10β,11β,17β-triol IV. After 90 min the fermentation was terminated and the products were recovered from the methyl isobutyl ketone extracts. Substrate II was not detected chromatographically in the 60 min sample or among the isolated products. The solid product, 32·4 mg (66·4%), m.p. 193–198°C, was identified as the 10β-alcohol III by but with no trace of the 10β,11β,17β-triol IV. After comparison of t.l.c. and gas chromatographic behavior and of I.R. absorption spectra with those of an authentic reference sample of III.

A companion experiment in which prior induction of steroid hydroxylases had been arranged was conducted at the same time, using 51·6 mg of II as substrate in otherwise identical conditions. In the 15, 30 and 60 min samples the 10β-alcohol III was the major product, with traces of the 17-ketone V present, but with no 10β,11β,17β-triol IV detected at any time. Substrate II was not detected in the 60 min sample nor in the products harvested after 90 min. The product, 30·7 mg (62·4%), m.p. 195–200°C, was identified as the 10β-alcohol III by comparison of chromatographic and I.R. spectral properties with those of an authentic reference sample of III.

A third experiment using 52·0 mg of substrate II was conducted and assayed at 15, 30 and 60 min and harvested after 120 min. In the first three samples the major product III and the minor product V were detected but no IV was found. During the period substrate II was totally consumed. After 120 min the broth was harvested and products recovered. Gas chromatographic analysis showed one major product at t_R 1·00 identified as III, a minor component at t_R 1·56 identified as V, and two rapidly eluted minor components not identified. Very weak recorder responses near t_R 2·5 (Fig. 1C) suggested the possible presence of the 10β,11β,17β-triol IV, which an expanded elution curve (Fig. 1D) confirmed.

The yellow, oily product from the 2 h fermentation was chromatographed on a 0·5 mm thick chromatoplate irrigated with benzene–ethyl acetate (1:3, v/v). The most polar zone (R_F 0·21) yielded 0·2 mg (0·4%) of IV. The mid-zone (R_F 0·54) afforded 30·1 mg (61·6%) of III, and the most mobile zone (R_F 0·67) gave 1·6 mg (3·3%) of V. Product identities were made in each case by comparison of spectral and chromatographic properties with those of the appropriate authentic reference steroid.

A solution of 13·4 mg of II in 0·5 ml of dimethylformamide was added to the clear filtrate obtained by removal of mycelium (through cheese cloth) from a 200 ml flourishing vegetative cell culture of *C. lunata*. The preparation was incubated in the usual manner with samples withdrawn at intervals for analysis. Sample composition after 30 min was 90% II, 10% III; after 60 min, 75% II, 25% III; after 120 min, 60% II, 40% III. Small amounts (1–2%) of II remained after prolonged (18 h) incubation.

Transformations of 10β,17β-dihydroxy-4-estren-3-one (III)

Fermentation of 27·8 mg of III for 14 h gave a crude product shown by chromatography to contain III, IV and V as the major product. Preparative t.l.c. using benzene–ethyl acetate (1:3, v/v) afforded from the mobile zone (R_F 0·67) 13·4 mg (51·4%) of the 17-ketone V, m.p. 194–203°C (literature m.p. 198–201°C [9], 195–197°C [1]); λ_{max}^{EtOH} 235 nm (ε 13,000) (literature λ_{max}^{EtOH} 235·5 nm (ε 14,025) [9]); $\tilde{\nu}_{max}^{KBr}$ 3420, 1720, 1665, 1625 cm^{-1}; R_F 0·67 in benzene–ethyl acetate (1:3, v/v); orange–tan color with 50% aqueous sulfuric acid spray; t_R 1·56 on 3% SP-2401; identical in these properties with those of an authentic reference sample of V.

From the more polar zone there was recovered 0·8 mg (2·8%) of IV which was recrystallized from acetone to give pure IV, m.p. 232–238°C dec. (literature m.p. 230–240°C dec. [2]); $\tilde{\nu}_{max}^{KBr}$ 3300, 1675, 1640 cm^{-1}; R_F 0·21 in benzene–ethyl acetate (1:3, v/v); lemon yellow color with 50% aqueous sulfuric acid spray; t_R 2·64 on 3% SP-2401; identical in these

properties with those of an authentic reference sample of IV.

From the mid-zone of the chromatoplate (R_F 0·54) there was recovered 6·1 mg of unaltered substrate III, identified as such by spectral and chromatographic data.

Fermentation of 52·5 mg of III for a shorter period of 60 min also showed formation of the products III, IV and V. The crude product mixture obtained by solvent extraction of the 60 min fermentation was chromatographed on a 0·5 mm thick chromatoplate using benzene–ethyl acetate (1:3, v/v) and the more mobile zone (R_F 0·67) eluted with acetone yielded 1·4 mg (2·7%) of the 17-ketone V, m.p. 195–200°C, identified by I.R. spectral and chromatographic properties. From the more polar zone (R_F 0·21) 0·1 mg (0·2%) of IV was obtained, identified as such by I.R. spectral and chromatographic data. From the mid-zone (R_F 0·54) there was recovered 47·1 mg of unaltered substrate III, m.p. 197–202°C, identified as such by I.R. spectral and chromatographic properties.

RESULTS

Our results establish that both the 10β-hydroperoxide II and the 10β-alcohol III serve as substrates for *C. lunata* vegetative cell cultures and that both II and III are metabolized along similar courses to the 17-ketone V and 10β,11β,17β-triol IV products. The major mode of transformation of the 10β-hydroperoxide II is by reduction to the corresponding 10β-alcohol III, which is then subject to further metabolism by two separate pathways to the products IV and V.

Reduction of the 10β-hydroperoxide II and dehydrogenation of the product 10β-alcohol III to V occur rapidly and can be detected within 15 min of fermentation. Reduction of II is complete within 60 min. Preinduction of steroid hydroxylases by substrate I appeared to diminish the initial rate of reduction of II, but the yield of III after 90 min was the same whether the culture had been preinduced with I or not. The hydroperoxide II was more slowly reduced in the absence of mycelium, over half the substrate charge remaining after 120 min. Unreduced substrate II could still be detected after 18 h of incubation.

The 17-ketone V detected throughout fermentations of II increased in amount with time and was formed from III rather than from II. A yield of 3·3% of V from III was obtained after 120 min, of 51·4% after 14 h. Formation of the 17-ketone is another example of 17β-hydroxysteroid dehydrogenase action previously demonstrated in *C. lunata* fermentations of 19-norsteroids. The dehydrogenase action in vegetative cell cultures may not be readily demonstrated, but the enzyme is quite active in cell-free preparations of steroid hydroxylases [12, 13].

The trace product IV could not be detected in fermentations of II of less than 90 min duration nor during that period in which substrate II could still be detected. Only after II had been consumed (to III and V) was IV detected at all. The 10β,11β,17β-triol IV was formed directly from the 10β-alcohol III as substrate after 60 min of fermentation, and the presence of IV among products from substrate II surely derives from initial reduction of II to III. Yields of IV obtained were 0·2% from III after 60 min, 0·4% from II after 120 min, and 2·8% from III after 14 h.

The presence of very small amounts of IV in these fermentations of II or III cannot be attributed to thermal decomposition of either steroid or to other artifactuous process. The 10β,11β,17β-triol IV was not formed on pyrolysis of the 10β-hydroperoxide II although an unidentified component of similar retention time (t_R 2·48) was formed (Fig. 1B). This t_R 2·48 component may also have been formed in fermentations of II (Fig. 1C and 1D), but the point is unsettled, and we have not pursued the matter of the identity of the t_R 2·48 component or of the other pyrolysis or fermentation components indicated in the gas chromatographic elution curves of Fig. 1.

DISCUSSION

Our results definitively rule out the 10β-hydroperoxide II as a substrate from which the 10β,11β,17β-triol IV is derived in *C. lunata* fermentations. Furthermore, our data strongly suggest that the 10β-hydroperoxide II or a formal equivalent is not a likely intermediate in the transformation of substrate I to the 10β,11β,17β-triol IV by a hydroperoxide rearrangement reaction. Rather, our present studies support in clear fashion that sequential monohydroxylation of I leads to the 10β,11β,17β-triol IV and that microbial dioxygenases are not indicated. Our results and these conclusions stand without regard to whether steroid hydroxylases had been induced prior to addition of II as substrate.

Our results suggest that the triol IV be formed from I by initial 10β-hydroxylation followed by 11β-hydroxylation of III to yield IV. However, this obvious pathway still does not explain formation of racemic 10β,11β,17β-triol IV from racemic I where the 10β-alcohol III of natural configuration is also formed. Rather, formation of racemic IV from racemic I may be possible with the reverse order of monohydroxylations. Racemic I yielded racemic 11β,17β-dihydroxy-4-estren-3-one [2], and this product may be acted upon by a 10β-hydroxylase to give racemic IV. We have not examined this possibility however. Our prior reported

failure to observe 10β-hydroxylation of racemic 11β,17β-dihydroxy-4-estren-3-one or of 11β-hydroxylation of III [2] may be understood in terms of the sensitivity used in our prior t.l.c. analyses. Detection of IV in our present work using 50 mg charges of substrates II or III has succeeded largely through our careful use of gas chromatography as a necessary adjunct to routine thin-layer chromatography.

Our specific failure to detect the 10β,11β,17β-triol IV at early times of *C. lunata* fermentations of II is in distinction to the previously demonstrated rearrangement reaction of cholesterol 20α-hydroperoxide by adrenal cortex mitochondria to give 5-cholestene-3β,20α,21-triol and 5-cholestene-3β,20α,22R-triol, which rearrangement occurred rapidly in a matter of minutes [4, 14] and which was uncompromised by reduction of the 20α-hydroperoxide to the corresponding alcohol 5-cholestene-3β,20α-diol.

The rapid peroxidase action of flourishing vegetative cells of *C. lunata* in destroying the 10β-hydroperoxide II prior to 11β-hydroxylation finds precedent in a similar reduction of 17α-hydroperoxy-4-pregnene-3,20-dione by *Aspergillus ochraceus* NRRL 405 prior to 11α-hydroxylation [15, 16]. Additional studies using vegetative cell cultures of microorganisms for steroid hydroperoxide metabolism in which the hydroperoxide group must escape immediate reduction do not appear warranted.

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