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MICROBIOLOGICAL TRANSFORMATION OF STEROIDS—XI

THE ACTION OF *CORYNEBACTERIUM SIMPLEX* ON NON-CORTICOID STEROID SUBSTRATES

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Abstract—*Corynebacterium simplex* (A.T.C.C. 6946) causes the following reactions to occur with appropriate steroid substrates: (1) oxidation of Δ^5-3β-hydroxyl to Δ^4-3-ketone, (2) oxidation of Δ^5-3β-hydroxyl to $\Delta^{1,4}$-diene-3-ketone, (3) oxidation of secondary 17β-hydroxyl to 17-ketone, (4) oxidation of 20β-hydroxyl to 20-ketone, (5) oxidation of 19-nor-Δ^4-3-ketone to $\Delta^{1,3,5\,(10)}$-triene-3-hydroxyl and (6) hydrolysis of 3- and 21-acetate groups.

THE preceding paper of this series described the transformation of Δ^3-3-ketosteroids of the cortical series into the corresponding $\Delta^{1,4}$-diene-3-ketosteroids and the reduction of the 20-carbonyl group to the 20β-hydroxyl group in some of these compounds by the action of *C. simplex* (A.T.C.C. 6946). Application of this organism to a variety of non-corticoid steroid substrates has revealed the presence of enzyme systems capable of effecting several other reactions.

Bacterial species were known from the work of the Mamoli school[1] to be useful in oxidation of Δ^5-3-hydroxy- to Δ^4-3-ketosteroids, in oxidation of 17β-hydroxy- to 17-ketosteroids, and in the hydrolysis of 21-acetates. In particular the activities of *Corynebacterium mediolanum*,[2] *Micrococcus dehydrogenans*[3] and *Flavobacterium dehydrogenans*[4] have been reported.

In 1953 related reactions with a variety of fungal species were described. *Cylindrocarpon radicicola*[5] and *Fusarium* sp.[6] were shown to possess powerful oxidizing systems capable of transforming progesterone into 1-dehydrotestololactone and 1,4-androstadiene-3,17-dione respectively. *Fusarium* sp. were also employed to convert pregnenolone and dehydro-epiandrosterone into 1,4-androstadiene-3,17-dione.[6] At the same time a member of the actinomycete family, *Streptomyces lavendulae*[5] was shown to display similar oxidizing properties.

As a part of our studies with *Corynebacterium simplex*[7] we have examined the

[1] For reviews of the work of this period see F. G. Fischer's chapter in *Newer Methods of Preparative Organic Chemistry* p. 182 Interscience, New York (1948); C. Arnaudi, *Zentr. Parasitenk* **105**, 352 (1942).

[2] L. Mamoli, *Ber. Dtsch. Chem. Ges.* **72**, 1863 (1939).

[3] A. Ercoli, *Boll. Sci. Facolta Chem. Ind. Bologna* 279 (1940).

[4] A. Ercoli, *Z. physiol. Chem.* **270**, 266 (1941).

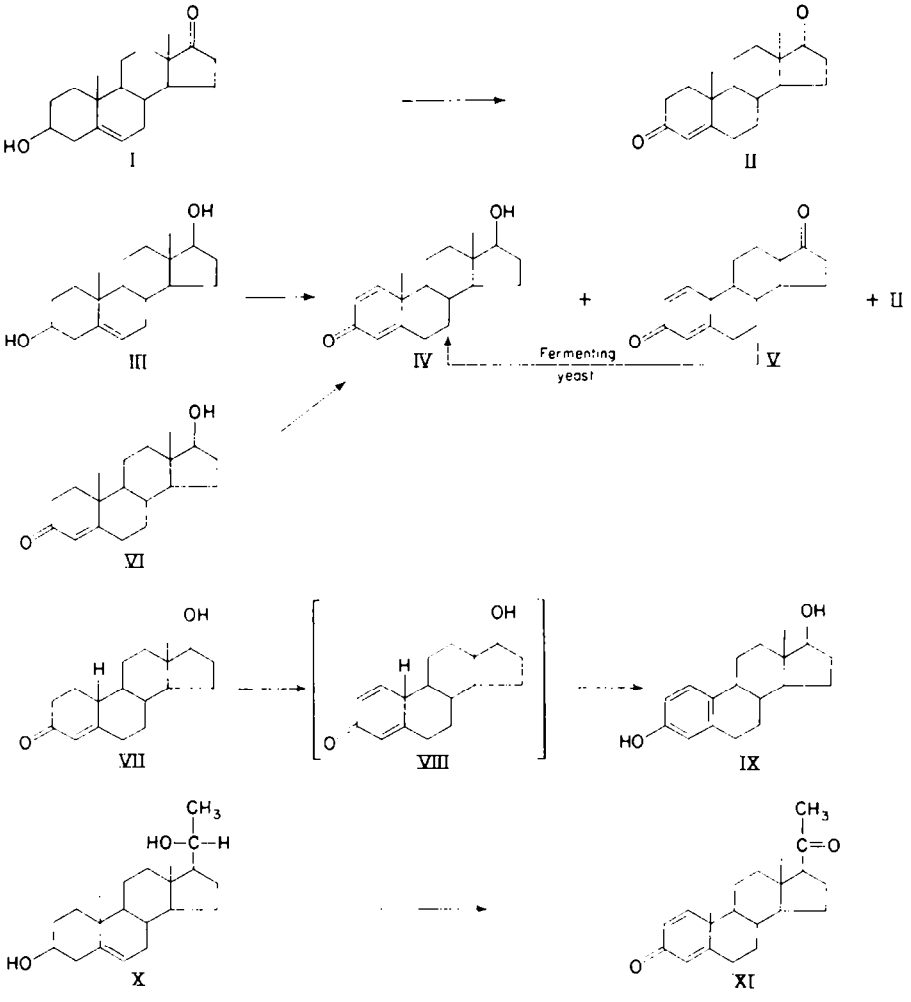
[5] J. Fried, R. W. Thoma and A. Klingsberg, *J. Amer. Chem. Soc.* **75**, 5764 (1953).

[6] E. Vischer and A. Wettstein, *Experientia* **9**, 371 (1953).

[7] H. L. Herzog, C. C. Payne, M. T. Hughes, M. J. Gentles, E. B. Hershberg, A. Nobile, W. Charney, C. Federbush, D. Sutter and P. L. Perlman, *Tetrahedron* **18**, 581 (1962).

action of this species on a variety of non-corticoid steroid substrates. The transformations noted with this organism, other than 1-dehydrogenation,[7] parallel in principle the earlier observations of Mamoli[3] on *C. mediolanum*.

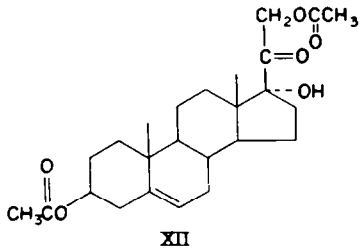
For example, incubation of dehydroepiandrosterone (I) with *C. simplex* afforded 4-androstene-3,17-dione (II) in good yield, illustrating that the organism is capable of oxidizing the 3-hydroxyl group and isomerizing the 5,6-double bond, presumably after the oxidation. With 5-androstene-3β,17β-diol (III) the reaction did not yield testosterone as the major product. Instead it proceeded further with the introduction of Δ^1-unsaturation, together with some oxidation of the 17-hydroxyl group to yield a mixture of 1,4-androstadiene-17β-ol-3-one (IV),[8] 1,4-androstadiene-3,17-dione (V)[9] and II in the proportion of about 3:1:1 respectively, as estimated by infrared, rotational and polarographic evidence. With testosterone (VI) as the starting



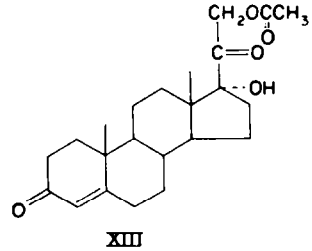
[8] H. H. Inhoffen and G. Zuhlsdorf, *Ber. Dtsch. Chem. Ges.* **74**, 1911 (1941).
[9] H. H. Inhoffen, G. Zuhlsdorf and Huang-Minlon, *Ber. Dtsch. Chem. Ges.* **73**, 451 (1940).

material the reaction followed about the same course as with 5-androstene-3β,-17β-diol except that the ratio of 17β-ol to 17-one products was increased. Compound IV was also prepared by reduction of V with a fermenting yeast. From the experiments with *C. simplex* one may conclude that the rates of the three processes promoted by the organism bear the following relationship: Δ^4-3-ketone-to $\Delta^{1,4}$-diene-3-ketone > Δ^5-3-hydroxyl to Δ^4-3-ketone > 17β-hydroxyl to 17-ketone.[10] It is interesting that dehydroepiandrosterone acetate, testosterone propionate and 4-androstene-3,17-dione did not react appreciably *under the conditions of the experiment.*

When 19-nortestosterone (VII)[11] was used as the substrate β-estradiol (IX) was formed in good yield,[12] the latter being the enol form of 19-nor-1,4-androstadiene-17β-ol-3-one (VIII). In this instance the rate of introduction of the Δ^1-unsaturation is very much more rapid than the rate of oxidation of the hydroxyl at C-17 since there was no evidence for the presence of either estrone or 19-nor-4-androstene-3.17-dione.[13]

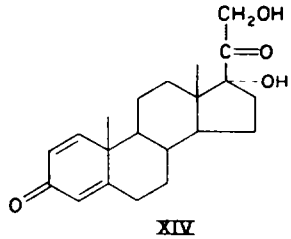


XII

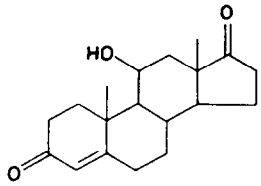


XIII

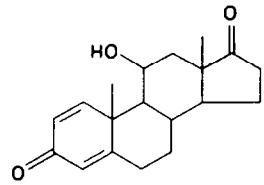
+



XIV



XV



XVI

[10] Relationships of this kind may be substrate specific and should be used with caution.

[11] A. J. Birch and S. M. Mukherji, *J. Chem. Soc.* 2531 (1949).

[12] cf. H. L. Herzog, U.S. 2,928,805.

[13] cf. S. Kushinsky, *J. Biol. Chem.* **230**, 31 (1958); A. Bowles, C. Casas-Campillo and C. Djerassi, *Tetrahedron* **2**, 165 (1958). Under the experimental conditions of Kushinsky, which involved forced aeration during the transformation, both estrone and 19-nor-4-androstene-3,17-dione were form in addition to estradiol. In our experiment, in shake flasks, with much less oxygen available to the culture, oxidation at 17- was apparently so slow as to be unimportant.

4-Androstene-11β-ol-3,17-dione[14] (XV) was transformed into 1,4-androstadiene-11β-ol-3,17-dione (XVI) with the aid of *C. simplex*. The product so produced was identical with a sample of XVI obtained from the degradation of 1,4-pregnadiene-11β,17α,21-triol-3,20-dione by sodium bismuthate.[15]

Examination of the reactions of several non-corticoid C-21 steroids revealed other interesting transformations. 5-Pregnene-3β,20β-diol (X) was converted into 1,4-pregnadiene-3,20-dione (XI) in moderate yield. In addition to the previously observed oxidation of the 3-hydroxyl group and the introduction of Δ^1-unsaturation, the 20-hydroxyl group is also oxidized. The same compound (XI) has been prepared by Vischer *et al.*[16] by the action of the filamentous fungus, *Calonectria decora* on progesterone. These authors indicate that from this reaction XI is accompanied by V and more polar products.

The action of *C. simplex* on 5-pregnene-3β,17α,21-triol-20-one 3,21-diacetate (XII)[17] afforded, in low yield, Reichstein's Compound S acetate (XIII) and 1,4-pregnadiene-17α,21-diol-3,20-dione.[7] In addition to the previously noted oxidations in the A-ring the 3-acetate and 21-acetate groups are hydrolyzed.

Among the C-21 steroids which did not react to an appreciable extent *under the conditions employed* were progesterone, pregnane-3α,17α,21-triol-11,20-dione and pregnane-17α,21-diol-3,11,20-trione.

Certain tentative conclusions about the structural requirements for reaction with *C. simplex* may be drawn from the data which has been presented, within the framework of the reaction conditions which have been employed. The introduction of C-1 unsaturation in the A-ring does not occur in the absence of the Δ^4-3-ketone system (or a system capable of generating a Δ^4-3-ketone by oxidation of a 3-hydroxyl group, such as Δ^5-3β-hydroxyl). Reaction of any kind appears to be favoured by the presence of a free hydroxyl group in the molecule. The examples wherein reaction proceeded to an important extent in the absence of a free hydroxyl group were the reactions of desoxycorticosterone acetate noted in the preceding paper of this series[7] and the reaction of 19-nortestosterone acetate of Kushinsky.[13]

EXPERIMENTAL[18]

General procedure for the microbiological transformations. The procedure described in the preceding paper of this series[7] was employed throughout.

4-Androstene-3,17-dione (II). The crude chloroform extracts from the transformation of 1·5 g dehydroepiandrosterone (I) were concentrated to a residue which was then crystallized from ether–hexane. There resulted 0·83 g II in two crops, m.p. 155–165° and 147–152° respectively. The combined crystalline products were recrystallized again from ether–hexane affording 0·54 g m.p. 165–168°. Admixture with an authentic sample of II did not alter the m.p. The infrared spectrum of II was identical with that of an authentic sample.

Reaction of 5-androstene-3β,17β-diol (III). The crude chloroform extracts from the transformation of 3·0 g III were concentrated to a residue and crystallized from a small vol ether. There resulted 1·2 g

[14] M. E. Herr and F. W. Heyl, *J. Amer. Chem. Soc.* **75**, 5927 (1953).

[15] H. L. Herzog, C. C. Payne, M. A. Jevnik, D. Gould, E. L. Shapiro, E. P. Oliveto and E. B. Hershberg, *J. Amer. Chem. Soc.* **77**, 4781 (1955).

[16] E. Vischer, C. Meystre and A. Wettstein, *Helv. Chim. Acta* **38**, 835 (1955).

[17] J. Heer and K. Miescher, *Helv. Chim. Acta.* **34**, 359 (1951).

[18] All m.p.s. are corrected. Analyses and optical data were obtained by the Microanalytical and Physical Chemistry Departments of these laboratories and by the Galbraith Laboratories, Knoxville, Tenn.

of a crystalline mixture m.p. 128–135°, $[\alpha]_D^{25}$ +68·7° (chloroform), λ_{max}^{Nujol} 2·89 μ (OH), 5·72 μ (17-carbonyl), 6·01, 6·16 and 6·23 μ ($\Delta^{1,4}$-diene-3-one.) Examination of the carbonyl band indicated that about 40% (±10%) of the mixture possessed this functional group. The fingerprint region of the spectrum corresponded fairly well to that of authentic 1,4-androstadiene-17β-ol-3-one(IV), indicating that IV is the predominent component. Polarographic assay of the $\Delta^{1,4}$-diene-3-ketone system, using 1,4-androstadiene-3,17-dione as a reference, indicated that about 80% (±10%) of the mixture contained this functional group system and the remainder of the mixture contained the Δ^4-3-ketone system. From the evidence presented it is possible to construct an approximate composition for the mixture. The infrared and polarographic exidence are consistent with a mixture of IV, V and II in the proportion 3:1:1 respectively. This composition is confirmed by calculating the rotation of such a mixture according to the relation 0·6 (+22·5°) + 0·2 (116°) + 0·2 (185°) = predicted rotation.[19] In this way $[\alpha]_D^{25}$ calculated = 73·7° which is in good agreement with the observed value.

1,4-*Androstadiene*-17β-*ol*-3-*one* (IV) *from testosterone* VI. The chloroform extract from the transformation of 1·35 g VI was concentrated to a small vol and chromatographed on 30 g Florisil, which had been prepared with hexane. Testosterone (0·1 g) was recovered from the 20% ether–hexane fraction and IV was obtained from the 30% ether–hexane through 100% ether fractions. Crystallization of these fractions from methylene chloride–hexane gave 0·42 g IV, m.p. 157–160°, $[\alpha]_D^{25}$ +34° (chloroform). Further crystallization raised the m.p. to 165–167°. The infrared spectrum was in good agreement with that of authentic IV, but had a weak 17-carbonyl band in addition. From the rotation the purity of the chromatographed and once-crystallized product is estimated to be 85–90%.

1,4-*Androstadiene*-17β-*ol*-3-*one* (IV) *from* 1,4-*androstadiene*-3,17-*dione* (V). A culture of *Saccharomyces cerevisiae* (A.T.C.C. 4125) was grown for 48 hr on an agar medium of the following composition: yeast extract (Difco), 10 g; cerelose, 60 g; potassium dihydrogen phosphate, 4·49 g; disodium hydrogen phosphate, 8·83 g; agar, 20 g and tap water to make 1 l. The cell material from one agar slant was suspended in 5 ml saline and 1 ml of this suspension was added to 100 ml of the aforedescribed medium (without agar) in a 300 ml erlenmeyer flask. The resulting mixture was incubated at 30° on a shaker for 24 hr.

A fermenter containing 2 l. of the agar-free medium was inoculated with the 100 ml incubated mixture prepared previously and aerated at a rate of 1½ volumes of air per volume of medium per minute. After 6 hr of growth 2 g V dissolved in 50 ml ethanol was added to the fermenter and the reaction was allowed to proceed for 96 hr. The pH of the broth was then adjusted to 3·5 with dilute hydrochloric acid and the reaction mixture was extracted throughly with chloroform. The chloroform extracts were concentrated and there resulted 4·7 g of a dark brown, oily residue. The residue was taken up in hexane and extracted 3 times with 90% aqueous ethanol. The ethanol extracts were combined and taken to dryness, affording 2·0 g crude crystalline product. Chromatography on Florisil (70 g) and elution with 25% ether-in-hexane afforded a series of cyrstalline fractions which when pooled and crystallized from aqueous methanol gave 1·83 g IV, m.p. 161–165°. An additional crystallization from the same solvents yielded 1·64 g IV, m.p. 163–167°, $[\alpha]_D^{25}$ +16·7° ($CHCl_3$), $\lambda_{max}^{methanol}$ 245 mμ (ε = 15,000), λ_{max}^{Nujol} 2·89 μ (OH), 6·02, 6·16 and 6·24 μ ($\Delta^{1,4}$-diene-3-one). Lit m.p.[9] 169°, $[\alpha]_D^{25}$ +22·5°.

Estradiol (IX) *from* 19-*nortestosterone* (VII). The crude chloroform extract from the transformation of 0·100 g VII was crystallized from acetone–hexane affording 0·075 g IX, m.p. 171–173°. The product was soluble in dil aqueous potassium hydroxide, did not depress the m.p. of estradiol on admixture and possessed the identical infra-red spectrum.

1,4-*Pregnadiene*-3,20-*dione* (XI) *from* 5-*pregnene*-3β,20β-*diol* (X). The crude chloroform extract from the transformation of 1·0 g X was crystallized from a small vol ether. There resulted 0·35 g, m.p. 135–138°. Further crystallization from methylene chloride–hexane gave XI, m.p. 152–153°, $[\alpha]_D^{25}$ +122° (chloroform), $\lambda_{max}^{methanol}$ 246 mμ (ε = 15,900) λ_{max}^{Nujol} 5·88 μ (20-carbonyl), 6·01, 6·15 and 6·22 μ ($\Delta^{1,4}$-diene-3-one). No hydroxyl band was present. (Found: C, 80·38; H, 8·91; Calc. for $C_{21}H_{28}O_2$; C, 80·73; H, 9·03%).

1,4-*Pregnadiene*-17α,21-*diol*-3,20-*dione* (XIV) *and* 4-*pregnene*-17α,21-*diol*-3,20-*dione* 21-*acetate* (XIII) *from* 5-*pregnene*-3β,17α,21-*triol*-20-*one* 3,21-*diacetate* (XII). The crude chloroform extract from the transformation of 1·0 g XII was chromatographed on 20 g Florisil. Elution with methylene chloride afforded first about 30 mg XII, m.p. 165–170°, with an infra-red spectrum identical with that

[19] Rotations are in chloroform.

of starting material. In subsequent methylene chloride fractions XIII appeared. A pool of all methylene chloride eluates with m.p. over 200° followed by crystallization from methylene chloride–hexane afforded 25 mg XIII, m.p. 213–215°. The infra-red spectrum was in good agreement with that of Reichstein's Compound S acetate. Elution of the column with 0·5% methanol-in-methylene chloride gave XIV. Collection of all fractions melting over 225°, and crystallization from acetone gave 80 mg XIV, m.p. 238–240°, identical with the product identified previously.[7]

1,4-Androstadiene-11β-ol-3,17-dione (XVI) *from 4-androstene-11β-ol-3,17-dione* (XV). The crude chloroform extract from the transformation of 0·4 g XV was crystallized from methylene chloride–hexane affording 0·35 g crystalline solid, m.p. 167–172°. Recrystallization from the same solvent mixture afforded XVI, m.p. 176–179°, with an infra-red spectrum identical with that of authentic material.[16] It was noted that crystallization of XV in the presence of small amounts of acetic acid afforded an acetic acid solvate, the m.p. of which went through a phase change between 100 and 120°, final melting occuring as noted previously.

The rotation of the solvate $[\alpha]_D^{25}$ +111° (acetone), was in good agreement with that calculated from the unsolvated value[15] by making allowance for the increase in molecular weight.

(Found: C, 70·10; H, 7·59; Calc. for $C_{19}H_{24}O_3$. $C_2H_4O_2$: C, 69·97; H, 7·83%).