Mechanism of Hydroxylation of Steroids.
Hydroxylation of 16α-Methyl-4-pregnene-21-ol-3,20-dione Acetate with *Mucor Griseo-Cyanus*

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Mucor griseo-cyanus, which introduces an hydroxyl group in the 14-position of several steroids, converts 16α-methyl-4-pregnene--21-ol-3,20-dione acetate (I) into a monohydroxyderivative for which the structure 16α-methyl-4-pregnene-7α,21-diol-3,20-dione (III) has been determined.

16α-Methyl-4-pregnene-21-ol-3,20-dione acetate (I) and 16α--methyl-4-pregnene-7α,21-diol-3,20-dione (III) are transformed by *Mucor griseo-cyanus*, with a long time of incubation, into a dihydroxyderivative for which the structure 16α-methyl-4-pregnene--7α,12β,21-triol-3,20-dione triacetate (VI) has been determined.

The 16α-methyl group in compound I inhibits the 14α-hydroxylase of *Mucor griseo-cyanus* by steric hindrance.

The mechanism of hydroxylation of steroids by microorganisms or by mammals (for instance in the conversion of the 5-pregnene-3β-ol-20-one into the adrenocortical hormones) is not known[1]. Some information about the mechanism of hydroxylation of steroids must be deduced by the steric requirements of such hydroxylases.

Mucor griseo-cyanus introduces quite specifically an hydroxyl group in the 14α-position of many steroids[2]. Previously[3] it has been found that *Mucor griseo-cyanus* ATCC 1207 a (+) converts 4-pregnene-21-ol-3,20-dione into a 14α-monohydroxyderivative and, with a long time of incubation, into a 7α,14α--dihydroxyderivative: the introduction of a 7α-hydroxyl group takes place only after the introduction of the 14α-hydroxyl group.

It seems that *Mucor griseo-cyanus* produces either one dihydroxylase, which in the steroids introduces a hydroxyl group first in the 14α-position and then in the 7α-position, or two different hydroxylases. In the second case the 7α-hydroxylase works at a slower rate or works well only after the introduction of the 14α-hydroxyl group. In order to determine the steric requirements of the 14α-hydroxylase of *Mucor griseo-cyanus*, it seemed interesting to incubate a steroid having a substituent near the 14α-position with such an organism.

In the course of our recent studies 16α-methyl-4-pregnene-21-ol-3,20-dione acetate (I) was incubated in the presence of *Mucor griseo-cyanus* ATCC 1207 a (+). This treatment gave rise to the introduction of one hydroxyl group into the steroid molecule, and two new products were isolated. On the basis

of the experiments which will be described later in this paper, the following structures are ascribed to the products of biotransformation of I: 16α-methyl--4-pregnene-7α,21-diol-3,20-dione 21-acetate (II) and 16α-methyl-4-pregnene--7α,21-diol-3,20-dione (III) [see Scheme].
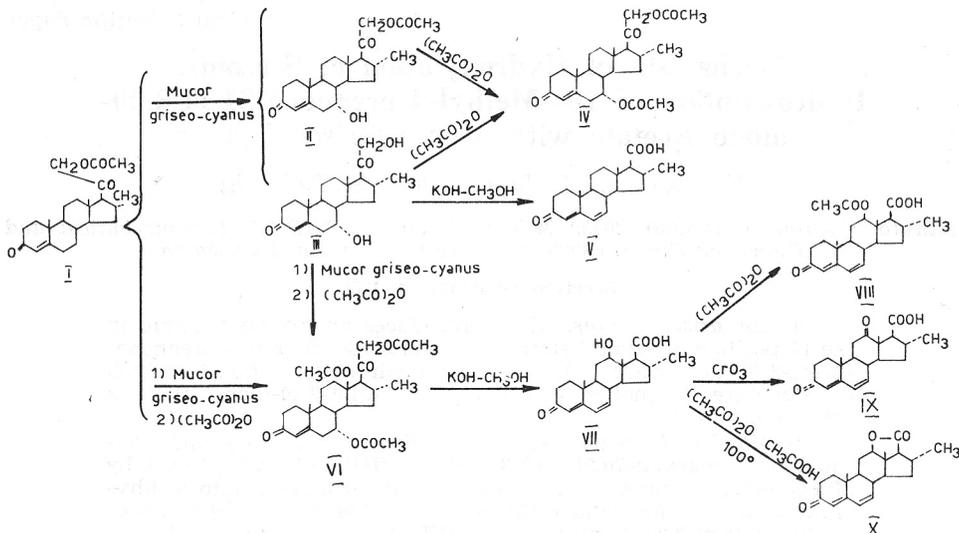
It should be noted that during the incubation, some of the starting material I remained unchanged, 13% being recovered. However, the compound which would have been formed through the hydrolysis of the acetyl group at the 21-position in I, has never been detected. Therefore, it might be assumed that the hydrolysis of the acetyl group in compound III is preceded by hydroxylation at position 7α.

Compounds II and III when treated with acetic anhydride give the same diacetate IV. Reaction of III with potassium hydroxide in methanol, in the presence of air, afforded a new compound to which the structure 16α-methyl--3-keto-4,6-etiadienoic acid (V) has been attributed. The spectral data for compound V confirm its structure; maximum at 285 nm in ultraviolet is characteristic of the 3-keto-4,6-dienic group[4], as well as the absorptions at 1630 and 1610 cm^{-1} in infrared[3]. The fact that V shows the absorption in ultraviolet establishes the presence of the 6,7-double bond, which might be formed through the dehydration of the hydroxyl group in position 7[3-5]; a hydroxyl group in position 6 in an analogous reaction would have produced a 5α-3,6--diketosteroid with no absorption[6].

The configuration of the hydroxyl group in position 7 in compounds II and III has been deduced by the difference in molecular rotation. It is known[7,8] that a 7α-hydroxyl group in steroids contributes to a molecular rotation of — 60° to — 70° while a 7α-acetoxyl group makes a variation of about — 300°; a 7β-hydroxyl group gives a contribution of + 95 to + 110°, a 7β--acetoxyl group makes a variation of + 208°. The observed difference in molecular rotation between compounds II and I (hydroxyl group) is of — 65°, and the difference between IV and I (acetoxyl group) is of — 343°. Both values are in good agreement with the data for the contribution of a 7α-group,

confirming thus that the first hydroxyl group, introduced by *Mucor griseo-cyanus* into a steroid molecule having the 16α-methyl group, entered position 7α.

In order to know whether the 14α-hydroxylase of *Mucor griseo-cyanus* is completely inhibited or only deactivated by the 16α-methyl group in compound I, incubation of I was prolonged from 4 to 12 hours. This resulted in the introduction of two hydroxyl groups; after acetylation with acetic anhydride in pyridine at room temperature, a new product was isolated to which the structure 16α-methyl-4-pregnene-7α,12β,21-triol-3,20-dione triacetate (VI) has been attributed. The identical product was obtained from compound III through incubation with *Mucor* followed by acetylation, indicating that one of the hydroxyl groups in VI (as acetate) is in position 7α.

Attention was now turned to the determination of the position of the second hydroxyl group introduced by *Mucor griseo-cyanus*. The fact that the product was easily acetylated with acetic anhydride to yield VI eliminates all structures having the hydroxyl group in a tertiary position, *e. g.* in 8β, 9α, 16, 17α, or even in the most generally attacked position 14α.
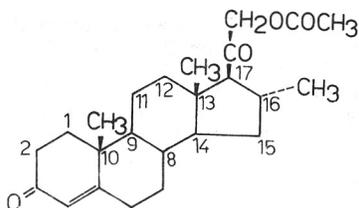
Compound VI undergoes a dehydration reaction with potassium hydroxide in methanol giving the new 16α-methyl-3-keto-12β-hydroxy-4,6-etiadienoic acid (VII). Its structure has been determined on the basis of its ultraviolet and infrared spectral data, which are characteristic of the 3-keto-4,6-dienic group[3,4] (maximum at 286 nm and absorptions at 1640 and 1610 cm^{-1}). The formation of the acid VII makes the existence of the second hydroxyl group in VI in position 1 impossible. Such a derivative upon treatment with potassium hydroxide in methanol would yield a 3-keto-$\Delta^{1,4}$-steroid[9,10]. On the other hand, had the second hydroxyl group entered at position 6, the analogous treatment with potassium hydroxide in methanol would produce a diketoderivative showing an ultraviolet maximum[11] different from the value found for compound VII.

The second hydroxyl group introduced by *Mucor* cannot be in position 2 because compound VII does not reduce 2,3,5-triphenyltetrazolium[10].

For the second hydroxyl group introduced by *Mucor* only positions 11α, 12α, 12β, 15α, and 15β remained as possibilities.

Hydroxy acid VII was converted into a ketoacid IX; this ketoacid has no infrared band of a cyclopentanonic ring near 1750 cm^{-1}; so positions 15α and 15β were excluded. The inspection of the contribution to the molecular optical rotation shows that the difference between the rotations of compounds IV and VI is + 53°. The published[7] data for the contribution to molecular optical rotation are as follows:

11α-acetoxyl-group	— 178°
12α-acetoxyl group	+ 280°
12β-acetoxyl group	+ 76°

Thus, it could be deduced that the second hydroxyl group introduced by *Mucor griseo-cyanus* is in position 12β. In order to confirm it, hydroxy acid VII was treated with acetic anhydride in acetic acid at 100 °C, and lactone X was isolated; this lactone has an infrared band at 1810 cm⁻¹, in the region characteristic of steroid γ-lactones[12]. A hydroxyl group in position 11α or 12α cannot give a lactone with a carboxyl group in the 17β-position.

The NMR spectrum of compound VI shows the presence of three methyl groups in the same regions where they appear in compounds I and IV (positions 10, 13, and 16). A multiplet at $\tau = 6.11$ is ascribed, according to Tweit[13], to the proton in the 12α position in steroids having an hydroxyl group in 12β-position.

In conclusion, a 16α-methyl group in compound I completely inhibits the 14α-hydroxylation by *Mucor griseo-cyanus* ATCC 1207 a (+), and such a microorganism introduces a hydroxyl group first in the 7α-position and then in the 12β-position.

<div align="center">EXPERIMENTAL</div>

Melting points are uncorrected, UV spectra were determined in methanol, IR spectra were recorded on a Perkin-Elmer 137 infrared spectrophotometer. The NMR spectra were recorded on a Varian 100 MC spectrometer with tetramethylsilane as internal standard. Deuterochloroform was used as solvent.

The biotransformations with *Mucor griseo-cyanus* ATCC a (+) were carried out under the same conditions as described by Canonica *et al.*[3]

Biotransformation of I with Mucor griseo-cyanus

Compound I[14] (12 g) in acetone (1 l) was added to micelium suspended in water (22 l) and the incubation was carried out at 28 °C for 4 hours. The micelium was filtered on celite, the broth was extracted with chloroform (24 l) three times, the chloroform was washed with 5% aqueous sodium bicarbonate, then with water, and evaporated.

The residue was chromatographed on a column of Florosil (500 g) eluted with hexane-ethyl acetate (4 : 6) collected in 22-ml fractions.

The residue of the first 5 fractions was crystallized from benzene-petroleum ether to give pure I (1.6 g); m. p. UV, and IR spectra were indistinguishable from those of an authentic sample[14]. NMR spectrum (in CDCl₃) : τ 9.3 (s, C₁₃—CH₃), 9.08 (d, C₁₆—CH₃), 8.86 (s, C₁₀—CH₃), 7.91 (s, OAc), 7.74 (m, —CH₂-positions 2 and 6), 7.3 (d, >CH—, position 17), 5.52 (d, —CH₂—, position 21), 4.38 (s, —CH=, position 4).

The fractions from 6 to 11 gave (after evaporation and crystallization from methanol-ether) pure *16α-methyl-4-pregnene-7α,21-diol-3,20-dione 21 acetate* (II) (0.65 g) m. p. 198—200 °C, $[\alpha]_D^{20}$ 132.5° (c 1.0, CHCl₃) UV max. 241—2 nm (»ε« near 16100): IR bands at 3440, 1750, 1730, 1670, 1620, and 1235 cm⁻¹.

<div align="center">

Anal. C₂₄H₃₄O₅ (402.51) calc'd.: C 71.61; H 8.51%
found: C 71.42; H 8.39%

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The fractions from 12 to 22 gave (after evaporation and crystallization from methanol-ether) pure *16α-methyl-4-pregnene-7 ,21-diol-3,20-dione* (III) (.1 g) m. p. 209—211 °C, $[\alpha]^{20}$ 117.7 (c 1.0 CHCl₃) UV max. 241—2 nm (»ε« near 15200); IR bands at 3400, 1715, 1660, and 1610 cm⁻¹.

<div align="center">

Anal. C₂₂H₃₂O₄ (360.49) calc'd.: C 73.29; H 8.90%
found: C 72.91; H 9.20%

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16α-Methyl-4-pregnene-7α,21-diol-3,20-dione diacetate (IV)

A. — A solution of II (0.3 g) in pyridine (1.5 ml) was treated with acetic acid (0.6 ml) for 40 hours at 20 °C. After dilution with water, the separated crystals were collected and crystallized from methanol-water to give pure IV (0.2 g) m. p. 184——186 °C, $[\alpha]_D^{20}$ 56.9 (c 2.0, CHCl₃); UV max. 238—9 nm (»ε« near 15400); IR bands at

1745, 1730, 1680, 1630, and 1240 cm^{-1}; NMR spectrum (in $CDCl_3$): τ 9.3 (s, C_{13}—CH_3), 9.09 (d, C_{16}—CH_3), 8.83 (s, C_{10}—CH_3), 8.04 (s, OAc, position 7), 7.9 (s, OAc, position 21), 7.7 (m, —CH_2—, position 2), 7.55 (t, —CH_2—, position 6), 5.52 (d, —CH_2—, position 21), 5.11 (m, —CH<, position 7), 4.41 (m, —CH=, position 4).

Anal. $C_{26}H_{36}O_6$ (444.65) calc'd.: C 70.24; H 8.16%
found: C 70.11; H 8.30%

B. — A solution of III (0,7 g) in pyridine (3.5 ml) was treated with acetic anhydride (1.4 ml) for 40 hours at 20 °C. After dilution with water, the separated crystals were collected and crystallized from methanol-water to give pure IV (0.5 g) m. p. 184—6 °C. The product had identical m. p. $[\alpha]_{20}^{D}$, UV max., IR and NMR spectrum with the product obtained as described under *A* and the mixed m. p. was without depression.

16α-Methyl-3-keto-4,6-etiadienoic acid (V)

A solution of III (0.7 g) in methanol (160 ml) was treated with KOH (2.8 g) for 40 hours at 20 °C. After evaporation of the solvent *in vacuo* dilution with water and acidification with HCl, the solid product was filtered, dissolved in 10% aqueous sodium hydroxide, filtered and again precipitated with HCl. The separated product was crystallized from methanol-water (9 : 1) to give pure V (0.5 g) m. p. 327—330 °C, $[\alpha]_{20}^{D}$ 91.4°; UV max 285 nm (»ε« near 26000), IR bands at 1710, 1630 and 1610 cm^{-1}, NMR spectrum (in pyridine-d_5): τ 0.08 (s, C_{13}—CH_3), 6.51 (s, CH_3OH from crystallization), 4.14 (m, —CH=, positions 4,6,7).

Anal. $C_{21}H_{29}O_3$ 1/2 CH_3OH (354.95) calc'd.: C 74.91, H 8,77%
found: C 75.32, H 8.88%

16α-Methyl-4-pregnene-7α,12β,21-triol-3,20-dione triacetate (VI)

A. — I (2 g) was incubated as indicated above with *Mucor griseo-cyanus* ATCC 1207 a (+) for 12 hours, the product was extracted with chloroform and the solvent was evaporated and the residue was treated with hexane and filtered. The separated crude product (1 g) was dissolved in pyridine (20 ml) and treated with acetic anhydride (4 ml) for 40 hours at room temperature. After dilution with water, filtration and crystallization from ether, compound VI was obtained (0.2 g): m. p. 168—170 °C, $[\alpha]_D^{20}$ 60.9° (c 2.0, $CHCl_3$), UV max 236—237 nm (»ε« near 16100), IR bands at 1740, 1720, 1670, 1620, and 1240 cm^{-1}, NMR spectrum (in $CDCl_3$. τ 9.2 (s, C_{13}—CH_3, 9.09 (d, C_{16}—CH_3), 8.83 (s, C_{10}—CH_3), 8.75 (s, OAc), position 12 β), 8.04 (s, OAc, position 7α), 7.9 (s, OAc, position 21), 7.55 (t, —CH_2—, position 6), 6.11 (m, —CH<, position 12 α), 5.54 (s, —CH_2—, position 21), 4.41 (s, —CH=, position 4).

Anal. $C_{28}H_{38}O_8$ (502.58) calc'd.: C 66.20; H 7.52%
found: C 66.31; H 7.62%

B. — III (0.5 g) was incubated as indicated above with *Mucor griseo-cyanus* ATCC 1207 a (+) for 10 hours. The product was isolated as described under A to give pure VI (0.1 g) m. p. 168—170 °C. The product has identical m. p., $[\alpha]_{20}^{D}$, UV max., IR and NMR spectrum with the product obtained as described under *A*, and the mixed m. p. was without depression.

16α-Methyl-3-keto-12β-hydroxy-4,6-etiadienoic acid (VII)

A solution of VI (0.26 g) in methanol was treated with potassium hydroxide (1.04 g) for 40 hours at room temperature. After dilution with water, acidification with HCl, filtration and crystallization from methanol-water (9 : 1), pure VII was obtained (0.16 g): m. p. 251—257 °C; UV max 286—287 nm (»ε« near 24900); IR bands at 3420, 1730, 1640, 1610 cm^{-1}; NMR spectrum (in pyridine-d_5): τ 9.05 (s, C_{13}—CH_3) 8.85 (d, C_{16}—CH_3), 8.77 (s, C_{10}—CH_3), 4.14 (m, —CH=, positions 4,6,7), 3.55 (broad signal, 12 β—OH, —COOH)

Anal. $C_{21}H_{27}O_4$ ·1/2 H_2O calc'd.: C 71.10; H 8.28%
found: C 71.00; H 7.93%

16α-Methyl-3-keto-12β-acetoxy-4,6-etiadienoic acid (VIII)

Compound VII (20 mg) was heated with acetic anhydride (0.05 ml) in pyridine (0.5 ml) for 40 hours at room temperature to give monoacetate VIII (15 mg), m. p. 204—206 °C; UV max. 286 nm (»ε« near 22500), IR bands at 1720, 1640, 1610, and 1250 cm^{-1}.

Anal. $C_{23}H_{30}O_5$ (386.47) calc'd.: C 77.47; H 7.82%
found: C 77.23; H 7.95%

16α-Methyl-3,12β-diketo-4,6-ethiadienoic acid (IX)

A solution of VII (40 mg) in acetic acid (2.0 ml) and water (0.1 ml) was treated with CrO_3 (13 mg) for 20 hours at room temperature to give compound IX (15 mg) m. p. 275—178 °C, UV max 286 nm (»ε« near 23200); IR bands at 1730, 1710, 1640, and 1610 cm^{-1}.

Anal. $C_{21}H_{26}O_3$ (342.42) calc'd.: C 73.65; H 7.65%
found: C 73.41; H 7.81%

16α-Methyl-3-keto-12β-hydroxy-4,6-ethiadienoic acid lactone (X)

A solution of VII (100 mg) in acetic acid (3.7 ml) was treated with acetic anhydride (2.5 ml) for 2 hours at 100 °C. After dilution with water (20 ml) at 60 °C, cooling, filtration and crystallization from aqueous methanol, compound X was obtained (10 mg): m. p. 180—185 °C; UV max 286 nm (»ε« near 23.600); IR bands at 1810, 1640, and 1610 cm^{-1}.

Anal. $C_{21}H_{26}O_3$ (326.42) calc'd.: C 77.26; H 8.02%
found: C 77.12; H 8.15%

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IZVLEČEK

Mehanizem hidroksiliranja steroidov. Hidroksiliranje 16-metil-4-pregnen-7α-21-diol--3,20-dion acetata z *Mucor griseo-cyanus*-om

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Mucor griseo-cyanus, ki hidroksilira številne steroide na mestu 14α, biotransformira 16α-metil-4-pregnen-21-ol-3,20-dion acetat (I) v njefov monohidroksi derivat, ki je 16-metil-4-pregnen-7α,21-diol-3,20-dion (III).

16α-metil-4-pregnen-21-ol-3,20-dion acetat (I) in 16α-metil-4-pregnen-7α,21-diol--3,20-dion (III) transformira *Mucor griseo-cyanus* z dolgotrajno inkubacijsko dobo v dihidroksi derivat, katerega strukturo smo določili in je 16α-metil-4-pregnen-7α, 12β,21-triol-3,20-dion (VI).

Metilna skupina na mestu 16 inhibira zaradi steričnih ovir 14α-hidroksilazo *Mucor griseo-cyanusa.*

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