MICROBIOLOGICAL 1α-HYDROXYLATION OF NORETHISTERONE

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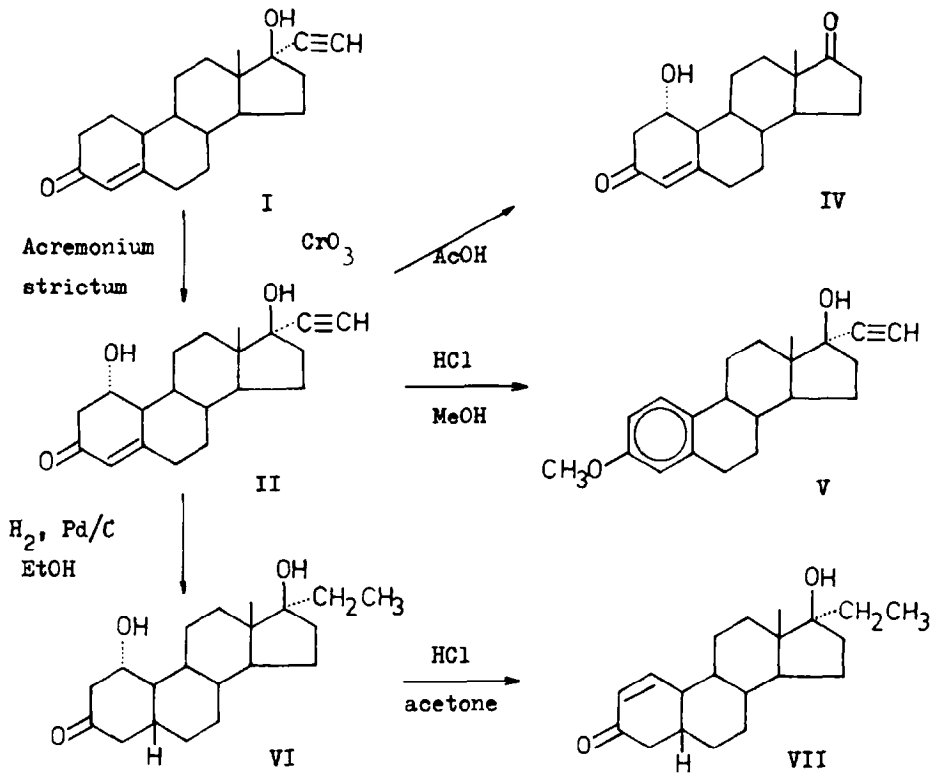
ABSTRACT

Acremonium strictum and Acremonium kiliense converted norethisterone predominantly into its 1α-hydroxyl derivative. Chemical and spectroscopic /UV, IR, PMR, MS, ORD, CD/ methods were used in establishing the structure and stereochemistry of the product. The 1α-hydroxyl group is shown to be axially oriented in the preferred, normal half-chair ring A conformation.

As part of an over-all study of the structure-activity correlation for microbiologically obtained norethisterone derivatives, in previous communications we described the microbiological hydroxylation of norethisterone /17-ethynyl-17β-hydroxy-4-estren-3-one, I/ with various fungi at positions 6β, 10β, 15α, and 15β/1,2/. In this paper we report the conversion of norethisterone by Acremonium strictum and Acremonium kiliense into its 1α-hydroxyl derivative /1α,17β-dihydroxy-17-ethynyl-4-estren-3-one, II/ which was found to possess contraceptive activity connected with its inhibiting the implantation of fertilized ova /3/.

Acremonium strictum and Acremonium kiliense isolated by us from soil produced compound II under various aerobic conditions. The best yields were obtained when the microorganisms were grown at 24°C in media containing malt extract, peptone and soybean meal. With Acremonium kiliense, formation of 10β-hydroxy-norethisterone /10β,17β-dihydroxy-17-ethynyl-4--estren-3-one, III/ was also noted as a minor product.



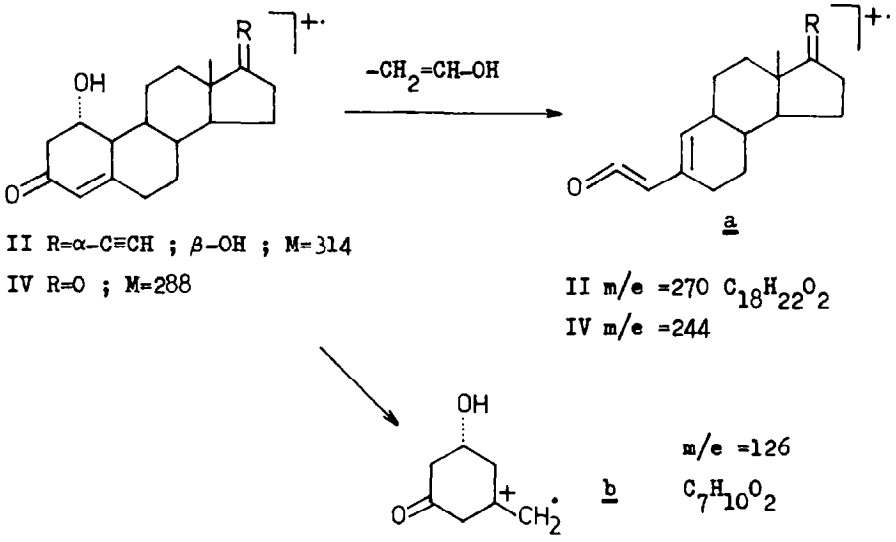
The structure of 1α-hydroxy-norethisterone was deduced as follows. The ultraviolet /UV/ and infrared /IR/ spectra showed that the Δ^4-3-ketone structure of the parent molecule remained unchanged during transformation. In the mass spectrum /MS/ of II the molecular ion /$M^{+\cdot}$/ appeared at m/e 314 and its elemental composition $C_{20}H_{26}O_3$ indicated monohydroxylation of norethisterone.

According to the proton magnetic resonance /PMR/ spectrum of II, the microbiologically introduced hydroxyl group is of secondary nature. Its adjacent /geminal/ proton, resonating at 4.46 ppm /$CDCl_3$/, exhibited a multiplicity /incompletely resolved octet/ corresponding to interaction with three vicinal protons.

Treatment of II with CrO_3 in acetic acid afforded a monohydroxylated 4-estrene-3,17-dione /IV/, indicating that the microbiologically introduced secondary alcohol function was not converted into ketone.

The formation of ion b /Scheme I/ in the mass spectra of II and IV can be explained by the well-known fragmentation process of Δ^4-3-keto-

steroids /4,5/. Accurate mass measurement gave an elemental composition of $C_7H_{10}O_2$ for this ion, suggesting thereby positions 1, 2 or 6 as the possible sites of microbiological hydroxylation. Since the PMR spectrum necessitates the presence of three hydrogens vicinal to the hydroxyl group, positions 2 and 6 must be ruled out and thus the mass spectral fragmentation pattern may be depicted as in Scheme I. It is interesting to note that loss of CH_2=CHOH, not observed with several other hydroxylated norethisterones at our disposal, seems to be unique for 1-hydroxy-Δ^4-3-ketosteroids. The above structural conclusion was in accordance with the observation that treatment of II with methanolic HCl afforded, via elimination of hydroxyl group, the aromatic 17-ethynyl--3-methoxy-1,3,5/10/-estratrien-17β-ol /V/.



Scheme I. Mass spectral fragmentation pattern of 1α-hydroxy-norethisterone /II/ and 1α-hydroxy-4-estrene-3,17-dione /IV/.

The configuration at C-1 was derived from the following chemical and spectral observations. The molecular rotational contribution of the alcohol function, ΔM_D^{OH}, was found in both II and IV to be considerably smaller /-52 and -41, respectively/ than the values usually encountered with 1β-hydroxy-19-nor-Δ^4-3-ketones /-360 to -390/ /6/. A straightforward evi-

dence for the correctness of structure II was furnished by the finding that hydrogenation of compound II gave 1α,17β-dihydroxy-17-ethyl-5β--estran-3-one /VI/ as the major product. In its PMR spectrum the resonance of C-1H appeared at 4.06 ppm /$CDCl_3$/ suggesting equatorial hydroxyl group. Furthermore, double resonance experiments showed that the sum of the vicinal couplings of the same proton, 21.4 Hz, consists of the terms $J_{1,2a} + J_{1,2e}$ = 17.6 Hz and $J_{1,10}$ = 3.8 Hz. From the magnitude of these couplings it can be confidently stated that the proton geminal to the hydroxyl function is, indeed, axially oriented /large vicinal couplings with C-2 hydrogens/ and the angular proton at C-10 is equatorial with respect to ring A, i.e. the hydrogenation product is a 5β,10β-steroid. The ORD curve with a negative Cotton-effect /a= -19.3/ centered at 286 nm provided corroboration for this conclusion.

Elimination of the 1α-hydroxyl group from VI afforded 17-ethyl-17β--hydroxy-5β-estr-1-en-3-one /VII/ exhibiting PMR coupling parameters /$J_{1,10}$ = 5.5 Hz and negligibly small allylic $J_{2,10}$/ typical of 5β-Δ^1-3-keto-19-norsteroids /7/.

Conclusions regarding the preferred ring A conformation in II were derived from ORD, CD, and PMR evidences. In a study of epimeric 1-methyl-19-norprogesterones, Djerassi et al. /8/ have shown that, because of the steric compression arising between an equatorial C-1 methyl group and the C-11αH, ring A will assume such conformations in which the C-1 methyl group is axially oriented in both α- and β-configurations. The same conformational situation is expected to occur with the epimeric 1-hydroxyl-19-norsteroids, too. In fact, the ORD spectrum of II exhibited a negative Cotton effect /a = -140/ centered at 320 nm, whereas the rather strong positive background rotation, along with the growing positive tendency of the ORD curve at lower wavelengths, indicated a positive Cotton effect in the K-band. Both the negative and the strong positive Cotton effects associated, respectively, with the $n \rightarrow \pi^*$ /R-band/ and the lowest $\pi \rightarrow \pi^*$ /K-band/ transitions of the enone chromophore appeared in the CD spectrum of II. According to empirical rules /9/ and theoretical calculations /10/, the signs of the Cotton effects reflect positive chirality of the enone chromophore which, in turn, requires axial orientation for the 1α-hydroxyl group, that can be readily accomodated in a normal /positive/ half-chair conformation of

ring A.

In complete agreement with this conclusion, the PMR spectrum of II showed that J values of C-1H /$J_{1,2e}$ = 3.8 Hz, $J_{1,2a}$ = 3.0 Hz, and $J_{1,10}$ = 3.0 Hz/ correspond to a spacial arrangement in which this proton is placed between the geminal hydrogens at C-2 and has an equatorial-axial relationship with C-1OH. The relative order of the $J_{1,2}$ couplings /i.e. $J_{ee} > J_{ea}$/ follows the general trend established for the gauche rotamers in ideal or moderately distorted $-XCH_2CH_2Y-$ fragments /11/, while their magnitudes are reasonably close to the expectation values for X = OH, Y = -C=OCH=CH-. Although no specific conclusion as to the ring A geometry seems to be warranted in terms of dihedral angles, the coupling constants suggest an undistorted, or slightly distorted half-chair form as the preferred conformation. Since acetylation of the hydroxyl group resulted in no detectable changes in the coupling parameters, the conformational "purity" of this form must be fairly high.

While this paper was in preparation, Greenspan et al. /12/ reported the microbiological 1β-hydroxylation of norethisterone. To our best knowledge, the preparation of II described in this paper, represents the first case of 1α-hydroxylation by microorganisms of a 19-norsteroid.

EXPERIMENTAL

Microorganisms

Acremonium strictum and Acremonium kiliense have been deposited at the National Institute for Public Health, Budapest, Hungary, under NN 106 and 107, respectively.

Maintenance

The strains were maintained at 20°C on potato-glucose agar slants.

Growing conditions

The cultures were grown in 500 ml Erlenmeyer flasks containing 100 ml of the following medium: 50 g of malt extract /60 % dry wt./, 20 g of peptone, 10 g of soybean meal, 5 g of KH_2PO_4 and 5 g of $MgSO_4 \cdot 7\ H_2O$ in 1 l of tap water.

The incubations were carried out at 24°C on a rotary shaker /300 r.p.m., 20 mm amplitude/ for 48 hours.

Preparation of 1α-hydroxy-norethisterone /II/ by Acremonium strictum

After distributing 5 g of norethisterone dissolved in 100 ml of dimethylformamide among 50 Erlenmeyer flasks containing Acremonium strictum culture, the incubation was continued for 24 hours as described above. The pooled broths were then filtered to remove mycelium, and the filtrate was extracted three times with one third volume of ethyl acetate. The combined extracts were evaporated in vacuo. The residue, containing 1α-hydroxy-norethisterone as the major product, was chromatographed on preparative silica gel thin layer plates in chloroform: ethanol /9:1/. 1.48 g of pure 1α-hydroxy-norethisterone was obtained. This was recrystallized twice from acetone giving the analytical sample:

m.p. 208-211°C; $[\alpha]_D$ -46° /c=1, methanol/; λ_{max} 241 nm /ε=16100/;
IR /KBr/: νOH 3420, ν/≡CH/ 3240, νC=O 1650, νC=C 1615 /shoulder/ cm^{-1};
PMR /100,1 MHz, $CDCl_3$/: δC-4H 5.9 /1 H, s/, δC-1H 4.46 /1 H, m, $J_{1,2e}$ = 3.8, $J_{1,2a}$ = 3.0, $J_{1,10}$ = 3.0/, δC-2He 2.72 /1 H, q, $J_{2e,2a}$ = -16.5/, δC-2Ha 2.52 /1 H, q/, δC-21H 2.56 /1 H,s/, δC-18H 0.92 /3 H, s/ ppm;
ORD /c=0.1, ethanol/: $[M]_{344}$ -4400 /trough/, $[M]_{324}$ 0, $[M]_{290}$ + 10000 /infl./, $[M]_{270}$ + 13000!
CD /c=0.1, ethanol/:$[\theta]_{322}$ -9800 /trough/,$[\theta]_{245}$ +28800 /peak/;
MS: m/e /%/: 314 /100/, 296 /19/, 270 /39/, 247 /47/, 215 /17/, 213 /28/, 187 /20/, 160 /15/.

Preparation of 1α-hydroxy-norethisterone /II/ and 10β-hydroxy-norethisterone /III/ by Acremonium kiliense

The incubation was performed similarly as described for transformation with Acremonium strictum, except that, instead of 5 g, only 1 g of norethisterone was distributed among 50 Erlenmeyer flasks containing Acremonium kiliense culture. After 16 hours of incubation the conversion of norethisterone was complete and II, as well as smaller amounts of the somewhat less polar III, were accumulated in the broth. The transformation products were extracted from the broth and separated on preparative silica gel thin layer plates in ethyl acetate: n-heptane /7:3/. 210 mg of II and 63 mg of III were obtained. Compound III /m.p.263-265°C,$[\alpha]_D$ -13.5° /c=1, dioxane// proved to be identical with an authentic sample of 10β-hydroxy-norethisterone /1, 13, 14/.

1α-Hydroxy-4-estrene-3,17-dione /IV/

To a solution of 400 mg of II in 50 ml of acetic acid a solution of 400 mg CrO_3 in 5 ml of water was added. The reaction mixture was kept at room temperature for 1 hour and then diluted with 100 ml of water and extracted twice with equal volume of ethyl acetate. The combined extracts

were washed with water, dried over Na_2SO_4 and evaporated in vacuo. The residue containing IV was chromatographed on preparative silica gel thin layer plates in ethyl acetate : n-heptane /8:2/. 145 mg of 1α-hydroxy-4-estrene-3,17-dione was obtained. Recrystallization from acetone gave the analytical sample:

m.p. 224-227°C; $[\alpha]_D$ + 115° /c=1, chloroform/; λ_{max} 241 nm /ε= 16100/;
IR /KBr/: νOH 3480, νC=O 1735, 1655 νC=C 1610 cm^{-1};
PMR /60 MHz, $CDCl_3$/; δC-4H 5.89 /1 H, s/, δC-1H 4.45 /1 H, m/,
δC-18H 0.95 /3 H, s/ ppm;
MS: m/e /%/: 288 /100/, 273 /33/, 244 /75/, 126 /21/.

Preparation of 17-ethynyl-3-methoxy-1,3,5/10/-estratrien-17β-ol /V/ from II

150 mg of II was dissolved in 5 ml of 4 % methanolic HCl. After 30 minutes at room temperature the solution was diluted with 50 ml of water and extracted twice with half volume of chloroform. The combined extracts were evaporated in vacuo. The residue was recrystallized from methanol yielding 115 mg of V: m.p. 150-152°C.
IR spectrum of V was identical with that of an authentic sample.

1α,17β-Dihydroxy-17-ethyl-5β-estran-3-one /VI/

800 mg of II dissolved in 40 ml of ethanol was hydrogenated with 160 mg of 5 % Pd on carbon as the catalyst. After an uptake of 3 moles of hydrogen the catalyst was removed by filtration and the filtrate evaporated in vacuo. The residue containing 1α,17β-dihydroxy-17-ethyl-5β-estran-3-one as the major product was chromatographed on preparative silica gel thin layer plates in ethyl acetate : n-heptane /6:4/. 450 mg of pure 1α,17β-dihydroxy-17-ethyl-5β-estran-3-one was obtained. This was recrystallized twice from acetone giving the analytical sample:

m.p. 195-198°C; $[\alpha]_D$ -21° /c=1, methanol/;
IR /KBr/: νOH 3400, νC=O 1710 cm^{-1};
PMR /100,1 MHz,$CDCl_3$/: δC-1H 4.06 /1 H, t, d, $J_{1,2}$ = 8.8, $J_{1,10}$ = 3.8/,
δC-2H 2.54 /2 H, d/, δC-21H 0.97 /3 H, t, J=7/, δC-18H 0.92 /3 H, s/ppm;
ORD /c=0.1, ethanol/: $[M]_{306}$ -1190 /trough/, $[M]_{284}$ 0, $[M]_{267}$ + 740 /peak/;
MS: m/e /%/: 320 /16/, 302 /21/, 291 /18/, 273 /37/, 248 /68/, 246 /28/, 231 /100/, 230 /43/, 160 /40/, 85 /32/.

17-Ethyl-17β-hydroxy-5β-estr-1-en-3-one /VII/

200 mg of 1α,17β-dihydroxy-17-ethyl-5β-estran-3-one was dissolved in 5 ml of acetone and one drop of concentrated hydrochloric acid was added. The reaction mixture was refluxed for 10 minutes, then diluted with 50 ml

of water and extracted twice with 25 ml of ethyl acetate. The combined extracts were washed with water, dried over Na_2SO_4 and evaporated in vacuo. The residue was chromatographed on preparative silica gel thin layer plates in ethyl acetate : n-heptane /1:1/. 145 mg of 17-ethyl-17β-hydroxy-5β-estr-1-en-3-one was obtained. Recrystallization from acetone gave the analytical sample:

m.p. 139-142°C; $[\alpha]_D$ + 228° /c=1, methanol/; λ_{max} 231 nm /ε= 9300/;
IR /KBr/: νOH 3530, 3460, νC=O 1665, νC=C 1605 cm^{-1};
PMR /60 MHz, $CDCl_3$/: δC-1H 7.18 /1 H, dd, $J_{1,2}$ = 10, $J_{1,10}$ = 5.5/, δC-2H 6.0 /1 H, d, $J_{1,2}$ = 10/, δC-21H 0.98 /3 H, t, J=7/, δC-18H 0.96 /3 H, s/ ppm;
MS: m/e /%/: 302 /100/, 273 /38/, 231 /62/, 108 /44/, 95 /22/, 85 /21/.

Equipments: M.p.s were measured with a Kofler hot-stage apparatus and are uncorrected. UV spectra were recorded on a Unicam model SP 500 spectrophotometer and IR spectra with a Perkin-Elmer 457 instrument. Varian A60-D and XL-100-15 spectrometers were employed for recording PMR spectra. Mass spectra were taken on a Varian MAT SM-1 instrument. ORD and CD spectra were measured on a ZEISS recording electric spectropolarimeter /REPM-12/ and on a JASCO J-40C dichrograph, respectively, in quartz cells of 1 and 10 mm lengths at room temperature.

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