Anal. Calcd for $C_{15}H_{22}O_3$: C, 71.97; H, 8.86. Found: C, 71.88; H, 8.79.

Isolation of Encelin (Anhydrofarinosin from *E. farinosa*).—Thin layer chromatograms of the leaves, stems and whole plant of *E. farinosa* showed the presence of, respectively, a single spot of farinosin, a single spot of encelin, and the two spots. The yellow-green stems (including the naked peduncles) were extracted separately and the extract was processed in the usual way.[3] Chromatography of the final crude syrup, isolated by chloroform extraction (5 g from 1 kg of dry plant material), over silica gel (eluent, chloroform–methylene chloride, 1:1) and concentration of the fractions containing encelin (by tlc) yielded 350 mg (0.035%) of the compound. It had mp 195–196°, undepressed on admixture with anhydrofarinosin, and its spectral (uv, ir, nmr) characteristics were identical with those oft he latter.

Anal. Calcd for $C_{15}H_{16}O_3$: C, 73.75; H, 6.60. Found (from plant): C, 73.81; H, 6.84.

Senescent *E. farinosa*.—A collection of *E. farinosa* leaves was made in June, at which time the desert temperatures were in the range of 100°F and the plant had become gray and scarious. Thin layer chromatograms of extracts of the leaves showed the presence of much low-R_f material, but little or no farinosin.

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Microbiological Transformation of Steroids. I. The Synthesis of 19-Nortestololactone[1]

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The major pathway for the oxidative degradation of 19-nortestosterone by the fungus *Aspergillus tamarii* to produce 19-nortestololactone has been found to be analogous to that for testosterone. The consecutive steps to the D-ring δ-lactone involve the initial interaction of the steroid substrates with 17-ketodehydrogenase followed by the action of a lactonase enzyme system. An 11β-hydroxylase enzyme system was also found to be operative on the 19-nortestosterone substrate since 11β-hydroxy-19-nortestosterone, 19-norandrost-4-en-11β-ol-3,17-dione, and 11β-hydroxy-19-nortestololactone were obtained. The latter compounds were also isolated from fermentation of 11β-hydroxy-19-nortestosterone with *A. tamarii*, which apparently represents a contradiction to the published generality that *A. tamarii* lacks the ability to degrade oxidatively the D ring of 11-hydroxylated steroids. This is the first report of successful microbiological synthesis and characterization of a 19-nortestololactone.

Several paper[3-9] and review articles[10-14] have been published concerning the ability of fungi to degrade stereospecifically androstane(ene)- and pregnane(ene)-type steroids to their respective D-ring δ-lactone derivatives. Recent investigations[8,9,15-17] have been oriented toward elucidation of the oxidative mechanism by which δ-lactone formation occurs.

Capek and coworkers[5] have demonstrated the primary pathway in the production of testololactone from progesterone fermentation with *Aspergillus oryzae* to involve the production of testosterone and androst-4-ene-3,17-dione as sequential intermediates. The isolation of testosterone acetate as an intermediate product in the fermentation of progesterone with *Cladosporium resinae*[8] and the isolation of 20β-hydroxy-4-pregnen-3-one in the early hours of incubation of progesterone with *Penicillium lilacinum*[9] indicate still other potential intermediates in the biooxidation of progesterone. It was also reported that 11α-hydroxyprogesterone could be converted into 11α-hydroxytestololactone by *P. lilacinum via* 11α,20β-dihydroxy-4-pregnen-3-one, 11α-hydroxytestosterone, and 11α-hydroxyandrost-4-ene-3,17-dione.[9,19]

More recently, Brannon, *et al.*,[18] found that incubation of progesterone with *Aspergillus tamarii* gave rise not only to the expected testosterone, androst-4-ene-3,17-dione, and testololactone, but also produced 11β-hydroxytestosterone as a terminal by-product; however, formation of 11β-hydroxytestololactone or 11α-hydroxytestololactone by fermentation of 11-hydroxylated pregnenes and androstenes with *A. tamarii* did not occur. Consequently, Brannon, *et al.*,[18] concluded that the fungus *A. tamarii* is unusual in its inability to degrade oxidatively the D ring of 11-hydroxylated steroids.

Our work has been concerned with the interaction of 19-nortestosterone with *A. tamarii* to determine the effect of the absence of the 10β-methyl substituent. Incubation of 19-nortestosterone with *A. tamarii* for 72 hr gave five transformation products. Two of these products, 19-norandrost-4-ene-3,17-dione and 19-nortestololactone, apparently were derived from an oxidative pathway similar to that which has been proved for testosterone.

The D-ring δ-lactone structural assignment for previously unreported 19-nortestotolactone was chiefly derived from its nmr spectrum. Examination of the latter shows the C_{18} methyl signal to be at 82.5 cps. The corresponding signal for the starting material, 19-nortestosterone, has a value of 49.0 cps. These values represent a downfield chemical shift of 33.5 cps. In

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comparison, the downfield chemical shift observed for the C_{18} methyl protons of authentic samples of testosterone and testololactone is 34.5 cps.

Isolation of the three remaining reaction products, namely, 19-norandrost-4-en-11β-ol-3,17-dione, 11β-hydroxy-19-nortestololactone, and 11β-hydroxy-19-nortestosterone, suggested that a second pathway which apparently involved D-ring oxidative degradation of the latter compound was operative. Fermentation of 11β-hydroxy-19-nortestosterone with the same fungus for 72 hr gave 19-norandrost-4-en-11β-ol-3,17-dione and 11β-hydroxy-19-nortestololactone.

The nmr spectrum of the latter compound supported its structural assignment in that comparison of its spectrum with that of 19-nortestololactone indicated that the signal for the C_{18} methyl protons was shifted downfield 11.5 cps. Spectra of authentic samples of 11β-hydroxytestololactone and testololactone indicated a downfield chemical shift of the C_{18} methyl signal to be 12.5 cps. In contrast, the downfield shift expected for an 11α-hydroxylated derivative is 1.5 cps.

It is therefore concluded that the contradiction to the generality that *A. tamarii* cannot degrade an 11-hydroxylated steroid to its D-ring δ-lactone may be explained by the absence of the 10β-methyl substituent group of 19-nor homolog of testosterone.

Since other investigations[20,21] on the fermentation of 19-nor steroids with microorganisms have dealt with either hydroxylation or A-ring dehydrogenation reactions, this investigation represents the first reported microbiological lactonization of a steroid of the 19-norandrostene type.

Experimental Section

The fungus used in this study was *Aspergillus tamarii* Kita 1005 and was obtained from the American Type Culture Collection, Rockville, Md. The 19-nortestosterone was generously supplied by the Upjohn Co., Kalamazoo, Mich. Thin layer chromatograms were run on 250-μ-thick silica gel H coated glass plates, using ethyl acetate as the mobile phase; iodine vapors were used for detection. Steroid mixtures were separated by column chromatography on silica gel H as the adsorbent and ethyl acetate as the eluent. Infrared spectra were recorded with a Baird IR-45 and nmr spectra were obtained with a Varian A-60A spectrometer. Melting points were determined on a Kofler apparatus and are uncorrected. Analyses were performed by Huffman Laboratories, Wheatridge, Colo., and Galbraith Laboratories, Knoxville, Tenn. Optical rotation measurements were obtained with a Zeiss photoelectric polarimeter.

General Methods. Fermentation.—*A. tamarii* Kita spores were inoculated aseptically from Sabouraud dextrose agar slants into 500-ml erlenmeyer flasks which contained 100 ml of sterilized 3% malt extract. After incubation for 48 hr on a rotary shaker at 28°, 50 mg of the steroid, dissolved in 0.4 ml of dimethylformamide (DMF), was added to each flask and incubation was continued for an additional 72 hr at 28° with shaking.

Extraction.—The fermentation was quenched at the end of the incubation period by addition of 40 ml of methylene chloride (CH_2Cl_2) to each flask. The flask contents were transferred to a Waring Blendor, thoroughly mixed with an additional 100 ml of CH_2Cl_2, and filtered. The organic layer was separated and the aqueous layer was extracted with two 30-ml aliquots of CH_2Cl_2. The CH_2Cl_2 layers were then combined, dried over anhydrous magnesium sulfate, and evaporated to a dry residue with a rotary evaporator under vacuum.

Separation of Steroids.—The transformation mixture was dissolved in the minimum amount of CH_2Cl_2 and introduced onto a column containing silica gel H and ethyl acetate. Maximum separation was achieved using a ratio of adsorbent to sample of approximately 100:1. The eluents were collected in 4-ml aliquots and evaporated to a dry residue. The column separation was followed by analysis of the tube residues by thin layer chromatography (tlc).

Transformation of 19-Nortestosterone by *A. tamarii*.—To each of 16 500-ml erlenmeyer flasks, each of which contained a 48-hr growth of *A. tamarii* in 100 ml of the previously described growth medium, was added 50 mg of 19-nortestosterone in 0.4 ml of DMF. After 72 hr of additional incubation, CH_2Cl_2 extraction gave 802.6 mg of crude transformation material. A portion of the crude extract (785.2 mg) was chromatographed on 80 g of silica gel H with ethyl acetate was the eluent. Approximately 180 fractions were collected and evaporated to a dry residue. Analysis by tlc indicated that these fractions could be combined into seven major fractions.

Acetone–hexane recrystallization of the first fraction obtained from the column gave 16.0 mg of 19-norandrost-4-ene-3,17-dione, mp 171–173° (lit.[21] mp 170–173°).

From the second fraction, 16.4 mg of pure starting material, 19-nortestosterone, was obtained: mp 122–123°; ν_{max}^{KBr} 3500, 1675, and 1625 cm^{-1}; nmr ($CDCl_3$) δ 0.83 (3 H), 3.67 (1 H), and 5.82 (1 H).

Fraction three yielded 12.2 mg of 19-norandrost-4-en-11β-ol-3,17-dione: mp 190–194° (lit.[22] mp 193–197°); ν_{max}^{KBr} 3520, 1750, 1670, and 1625 cm^{-1}.

Anal. Calcd for $C_{18}H_{24}O_3$: C, 74.97; H, 8.39. Found: C, 75.00; H, 8.50.

Fraction four gave 552.2 mg (70% yield) of 19-nortestololactone. After recrystallization from acetone–hexane, this compound gave mp 196–199°; ν_{max}^{KBr} 1725, 1670, and 1620 cm^{-1}; nmr ($CDCl_3$) δ 1.37 (3 H), 5.84 (1 H); $[\alpha]^{25}D$ −17.4°.

Anal. Calcd for $C_{18}H_{24}O_3$: C, 74.97; H, 8.39. Found: C, 74.88; H, 8.35.

Recrystallization of fraction five from acetone–hexane gave 26.6 mg of 11β-hydroxy-19-nortestololactone: mp 233–235°; ν_{max}^{KBr} 3500, 1725, 1675, and 1620 cm^{-1}; nmr ($CDCl_3$) δ 1.66 (3 H), 4.32 (1 H), 5.90 (1 H).

Anal. Calcd for $C_{18}H_{24}O_4$: C, 71.03; H, 7.95. Found: C, 71.10; H, 7.99.

The sixth combined fraction yielded 10.0 mg of 11β-hydroxy-19-nortestosterone: mp 214–216° (lit.[23] mp 213–218°); ν_{max}^{KBr} 3480, 1670, and 1625 cm^{-1}. This compound was identical with an authentic sample[24] of 11β-hydroxy-19-nortestosterone.

Fraction seven (147.9 mg) was found to consist of a mixture of the above steroids contaminated with CH_2Cl_2 extractable cellular material.

Transformation of 11β-Hydroxy-19-nortestosterone by *A. tamarii*.—To a 48-hr growth of *A. tamarii* in a 250-ml erlenmeyer flask containing 75 ml of a 3% malt extract medium was added 65.1 mg of 11β-hydroxy-19-nortestosterone. After 72-hr atdditional incubation, the reaction mixture was worked up in the usual manner. In addition, 20 ml of acetone and 20 ml of benzene were used to extract further the aqueous layer. Column chromatography of the solid reaction mixture obtained produced four major fractions after tlc analysis and combination of the tube residues. Fraction one gave 3.3 mg of 19-norandrost-4-en-11β-ol-3,17-dione, which was found to be identical with that previously described. Fraction two yielded 3.0 mg of 11β-hydroxy-19-nortestololactone, whose physical constants were found to be in agreement with those reported above. Fraction three yielded 44.6 mg of starting material, 11β-hydroxy-19-nortestosterone. The fourth fraction (8.3 mg) was found to consist of mixtures of the above steroids and CH_2Cl_2-soluble cellular material.

Registry No.—19-Nortestololactone, 6811-29-6; 19-norandrost-4-ene-11β-ol-3,17-dione, 15313-96-9; 11β-hydroxy-19-nortestololactone, 15313-98-1; 11β-hydroxy-19-nortestosterone, 4075-17-6.

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