Microbiological Hydroxylation of Steroids of Unnatural Configuration[1a]

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Abstract: The microbiological attack of *Aspergillus ochraceus* on racemic 19-nortestosterone, racemic 13β-ethyl-17β-hydroxygon-4-en-3-one, and racemic 17β-hydroxy-13β-propylgon-4-en-3-one resulted in monohydroxylation of both the *d* and *l* antipodes at several positions. From racemic 19-nortestosterone were obtained *l*-1β-hydroxy, 6β-hydroxy, *l*-10β-hydroxy, and *d*-11α-hydroxy products; from the 13β-ethyl analog were obtained *l*-1β-hydroxy, *d*-6β-hydroxy, *d*-10β-hydroxy, and *dl*-11α-hydroxy products; from the 13β-propyl analog was obtained the *l*-1β-hydroxylated product.

As part of an over-all study of the microbiological alteration of racemic 13β-alkylgonane derivatives we undertook the microbiological resolution of select totally synthetic 13β-alkylgon-4-en-3-ones[2] for biological evaluation.

Microbiological resolution of racemic steroids has been reported in relatively few instances,[3] and only the natural *d* antipodes[4] have been transformed. Microbiological reductions[5a] and hydroxylations[5b] of both antipodes of simpler bicyclic systems have been reported however. Very recently, additional examples of microbial metabolism of but one antipode of racemic steroid-like molecules have been published.[6]

Our resolution work involves three types of reactions: (a) nuclear hydroxylation of Δ^4-3-ketones, (b) C-1,2 dehydrogenation of Δ^4-3-ketones, and (c) C-17 ketone–alcohol oxidation–reduction reactions on several substrates. In this regard our work parallels that of Vischer, *et al.*,[3a,b] with the important distinction that in select cases we find both the *d* and *l* enantiomers may be hydroxylated and dehydrogenated microbiologically.

The present report deals with the first of these three reaction types, namely nuclear hydroxylation, as a means of resolution. Steroids of unnatural *l* configuration result. A second paper will detail our experience with microbiological dehydrogenations leading to resolved steroids in the *d* series.

In seeking 11-hydroxylation of select 13β-alkylgon-4-en-3-one derivatives it was noted that *Aspergillus ochraceus*[7] gave a smooth conversion of *dl*-13β-ethyl-17β-hydroxygon-4-en-3-one (Ib)[2] into a major, more polar product, together with a series of minor polar components. Isolation of the major component was readily accomplished by direct crystallization from the concentrated solvent extract, and yields averaging 52% of a resolved monohydroxylated product (IIb) were obtained. Chromatography of the mother liquors afforded two other optically active monohydroxylated derivatives, Vb (2.9%) and VIIb (3.9%), and traces (0.12%) of an optically inactive monohydroxylated derivative (VIIIb). Several more polar trace components were not examined further.

Repetition of the fermentation with the racemic 13β-propyl derivative (Ic) gave a poorer transformation, but a 10% yield of a single resolved monohydroxylated product (IIc) was obtained. The chromatographic pattern of transformation products included minor components of mobilities comparable to those of the minor components (Vb, VIIb, and VIIIb) obtained from racemic Ib (see Figure 1).

The structures of the products IIb and IIc followed from their spectral behavior in a variety of solvents. In alcohol both derivatives showed minor bathochromic shifts of *ca.* 2 mμ relative to their respective parents Ib and Ic. In concentrated sulfuric acid, spectra typical of a Δ^4-3-ketone were obtained. In 0.066 *N* alkaline ethanol, spectra typical of the phenoxide ion were obtained immediately on solution, which on acidification had features of spectra of steroidal phenols.

(1) (a) Part VIII of the series Totally Synthetic Steroid Hormones. For part VII, see G. C. Buzby, Jr., E. Capaldi, G. H. Douglas, D. Hartley, D. Herbst, G. A. Hughes, K. Ledig, J. McMenamin, T. Pattison, H. Smith, C. R. Walk, and G. R. Wendt, *J. Med. Chem.*, **9**, 338 (1966). (b) Author to whom inquiries should be addressed at the University of Texas Medical Branch, Galveston, Texas 77550.

(2) (a) H. Smith, Belgian Patents 608,369 and 608,370 (March 16, 1962); (b) H. Smith, *et al.*, *Experientia*, **19**, 394 (1963); (c) H. Smith, *et al.*, *J. Chem. Soc.*, 4472 (1964).

(3) (a) E. Vischer, J. Schmidlin, and A. Wettstein, *Experientia*, **12**, 50 (1956); (b) A. Wettstein, E. Vischer, and C. Meystre, U. S. Patent 2,844,513 (July 22, 1958); (c) W. S. Johnson, W. A. Vredenburgh, and J. E. Pike, *J. Am. Chem. Soc.*, **82**, 3409 (1960); (d) K. V. Yorka, W. L. Truett, and W. S. Johnson, *J. Org. Chem.*, **27**, 4580 (1962).

(4) The nomenclature convention of L. F. Fieser and M. Fieser, "Steroids," Reinhold Publishing Corp., New York, N. Y., 1959, p 336, is used throughout. All structural formulas are drawn in the usual manner for the *d* enantiomers even where racemic modifications and *l* enantiomers are involved.

(5) (a) V. Prelog in "Steric Course of Microbiological Reactions," Ciba Foundation Study Group No. 2, Little, Brown and Co., Boston, Mass., 1959, pp 79–90; (b) A. Schubert, A. Rieche, G. Hilgetag, R. Siebert, and S. Schwarz, *Naturwissenschaften*, **45**, 623 (1958).

(6) (a) Y. Kurosawa, H. Shimojima, and Y. Osawa, *Steroids Suppl.*, **1**, 185 (1965); **6**, x (1965); (b) P. J. Curtis, *Biochem. J.*, **97**, 148 (1965).

(7) *A. ochraceus* is a well-known 11α-hydroxylating microorganism[8] and is particularly able to 11α-hydroxylate steroids whose C-18 methyl group is functionalized[8l] or is part of the E ring of 18,20-cyclopregnanes[8i] or of conessine.[8o]

(8) (a) H. C. Murray and D. H. Peterson, U. S. Patent 2,649,402 (Aug 18, 1953); (b) E. L. Dulaney, E. O. Stapley, and C. Hlavac, *Mycologia*, **47**, 464 (1955); (c) E. L. Dulaney, W. J. McAleer, M. Koslowski, E. O. Stapley, and J. Jaglom, *Appl. Microbiol.*, **3**, 336 (1955); (d) E. O. Karow and D. M. Petsiavas, *Ind. Eng. Chem.*, **48**, 2213 (1956); (e) E. A. Weaver, H. E. Kenney, and M. E. Wall, *Appl. Microbiol.*, **8**, 345 (1960); (f) A. G. Timofeeva, E. G. Gusakova, and A. A. Shpangis, *Izv. Akad. Nauk SSSR, Ser. Biol.*, **4**, 574 (1961); (g) R. I. Mateles and G. J. Fuld, *Antonie van Leeuwenhook J. Microbiol. Serol.*, **27**, 33 (1961); (h) E. A. Weaver and H. E. Kenney, U. S. Patent 2,989,439 (June 20, 1961); (i) H. Wehrli, M. Cereghetti, K. Schaffner, J. Urech, and E. Vischer, *Helv. Chim. Acta*, **44**, 1927 (1961); (j) S. G. Knight, U. S. Patent 3,031,379 (April 24, 1962); (k) M. C. Schleg and S. G. Knight, *Mycologia*, **54**, 317 (1962); (l) A. Wettstein, E. Vischer, J. Urech, and O. Jeger, U. S. Patent 3,055,806 (Sept 25, 1962); (m) J. de Flines, W. F. van der Waard, W. J. Mijs, and S. A. Szpilfogel, *Rec. Trav. Chim.*, **82**, 129 (1963); (n) C. Vézina, S. N. Sehgal, and K. Singh, *Appl. Microbiol.*, **11**, 50 (1963); (o) S. M. Kupchan, C. J. Sih, S. Kubota, and A. M. Rahim, *Tetrahedron Letters*, **No. 26**, 1767 (1963).

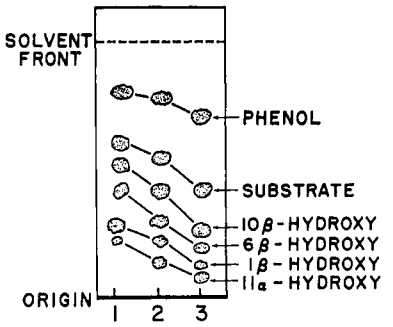


Figure 1. Thin layer chromatographic behavior of fermentation products from 1, *dl*-Ic; 2, *dl*-Ib; and 3, *dl*-Ia (solvent system ethyl acetate).

These spectral characteristics are sufficiently distinctive to support the assignment of a 1-hydroxy-Δ^4-3-ketone structure to both IIb and IIc.[9]

The diol IIb gave a diacetate IId, and the facile dehydration of IIb and IIc indicated by spectra was substantiated by preparation of the phenols IIIb and IIIc. Chromic acid oxidation of IIIb gave the 17-ketone IVa. Methylation of the phenols IIIb and IIIc with dimethyl sulfate and alkali gave the respective 3-methyl ethers IIId and IIIe, which on reduction with lithium metal and liquid ammonia, followed by acid hydrolysis, gave the optically active Δ^4-3-ketones Ib and Ic.

The specific rotations of the derivatives IIIb, IIIc, IIId, IIIe, IVa, IVb, Ib, and Ic prepared from the 1-hydroxy Δ^4-3-ketones IIb and IIc were all negative, where positive specific rotations are exhibited by similar derivatives of natural *d* steroids.[10] On this basis we assign the unnatural *l* configuration to these steroids, and necessarily also to the 1-hydroxy Δ^4-3-ketones IIb and IIc. To our best knowledge the transformation of racemic Ib and Ic to IIb and IIc, respectively, represents the first such case of a microbiological hydroxylation of the unnatural *l* antipode of a complex polycyclic system.

The *l* assignment of the optically active Ib obtained from IIb is further established by comparison of its optical rotatory dispersion with that of natural *d*-19-nortestosterone and of *d*-Ib, where a mirror image relationship was obtained (Figure 2).

The *l* derivatives, 13β,17α-diethyl-17β-hydroxygon-4-en-3-one and 13β-ethyl-17α-ethynyl-17β-hydroxygon-4-en-3-one, prepared in the course of this investigation have been reported previously.[2c]

The orientation of the 1-hydroxyl group in IIb and IIc may be assigned on the basis of molecular rotational contributions in the usual manner. The ΔM_D^{OH} values of +360 and +387° for IIb and IIc, respectively, must be reversed in sign to −360 and −387° in order to compare the increments with data for *d* steroids. These corrected ΔM_D values more nearly correspond with those of 1β-hydroxy Δ^4-3-ketones than with those of 1α-hydroxy Δ^4-3-ketones, which have much less negative ΔM_D values.[9] As has been pointed out by Dodson, *et al.*,[12] molecular rotational increments should be obtained on the acetate derivatives of 1-hydroxy Δ^4-3-ketones for confident assignment of configuration. A ΔM_D^{OAc} value of −410° (corrected to the *d* series)[13] is obtained for the 1-acetoxy derivative IId *vs.* the 17β-acetate of *l*-Ib. This high negative

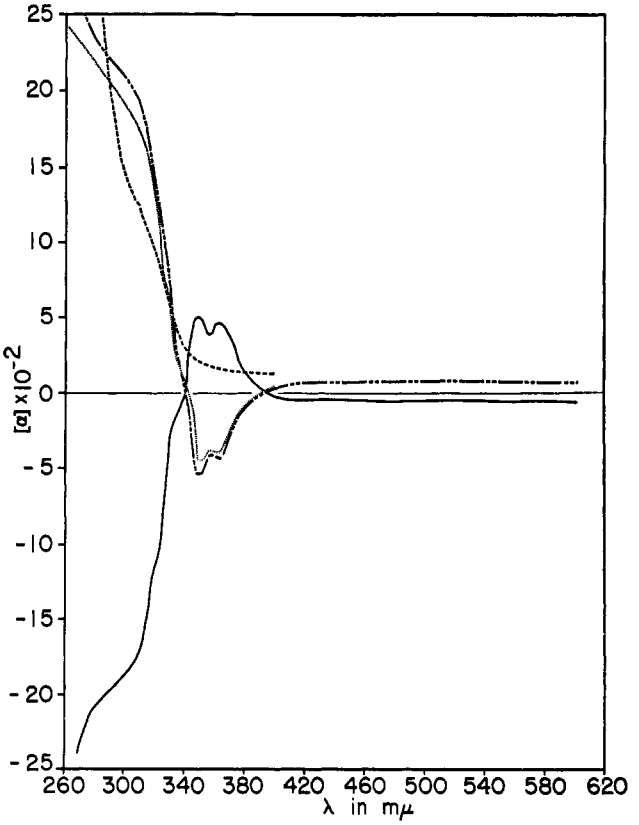


Figure 2. Optical rotatory dispersion of ———, *l*-Ib; – – – –, *l*-IIb; –·–·–·, *d*-19-nortestosterone (*d*-Ia); and ···, *d*-Ib.

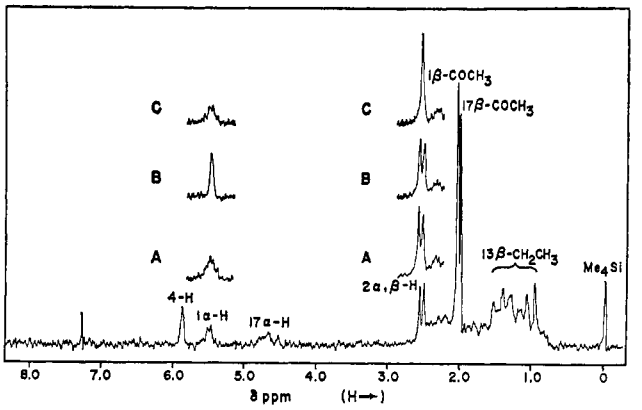


Figure 3. Proton nuclear magnetic resonance spectra of *l*-1β,17β-diacetoxy-13β-ethylgon-4-en-3-one (*l*-IId) at 60 Mc; inserts A, B, and C are at 100 Mc.

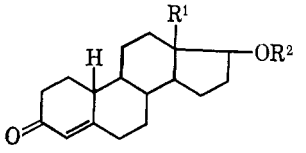
(9) L. L. Smith, *Steroids*, **1**, 570 (1963).

(10) (a) The *d* enantiomer of IIIc is known as a methylene chloride solvate of positive specific rotation.[11a] (b) The known *d* enantiomer of Ic has a positive specific rotation.[11b] (c) In addition we have prepared the *d* enantiomers of Ib, Ic, IIIb, IIIc, IVa, and IVb;[2c] see also L. L. Smith, G. Greenspan, and T. J. Foell, U. S. Patent 3,189,528 (June 15, 1965). Their specific rotations are all positive and agree in magnitude with the values reported herein. A further description of the synthesis and characterization of these *d* steroids is in preparation.

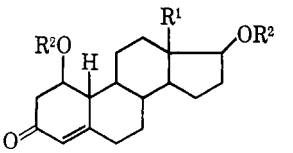
(11) (a) L. Velluz, G. Nominé, R. Bucourt, A. Pierdet, and P. Dufay, *Tetrahedron Letters*, No. 3, 127 (1961); G. Nominé, R. Bucourt, and A. Pierdet, Belgian Patent 599,343 (July 20, 1961); (b) G. Nominé and R. Bucourt, U. S. Patents 3,061,617 (Oct 30, 1962), 3,074,978 (Jan 22, 1963).

(12) R. M. Dodson, S. Kraychy, R. T. Nicholson, and S. Mizuba' *J. Org. Chem.*, 27, 3159 (1962).

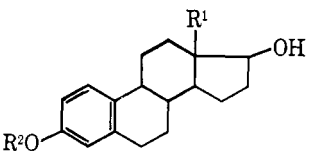
(13) The $\Delta[M]_D$ value of −420° previously reported[9] for 1β-acetoxy Δ^4-3-ketones in the 19-nor series should read −410°.



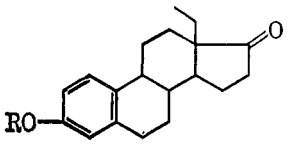
Ia, $R^1 = CH_3$; $R^2 = H$
b, $R^1 = C_2H_5$; $R^2 = H$
c, $R^1 = n\text{-}C_3H_7$; $R^2 = H$
d, $R^1 = C_2H_5$; $R^2 = COCH_3$



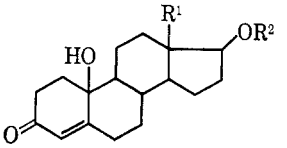
IIa, $R^1 = CH_3$; $R^2 = H$
b, $R^1 = C_2H_5$; $R^2 = H$
c, $R^1 = n\text{-}C_3H_7$; $R^2 = H$
d, $R^1 = C_2H_5$; $R^2 = COCH_3$



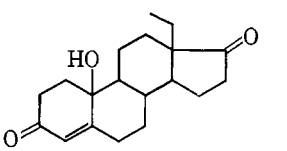
IIIa, $R^1 = CH_3$; $R^2 = H$
b, $R^1 = C_2H_5$; $R^2 = H$
c, $R^1 = n\text{-}C_3H_7$; $R^2 = H$
d, $R^1 = C_2H_5$; $R^2 = CH_3$
e, $R^1 = n\text{-}C_3H_7$; $R^2 = CH_3$



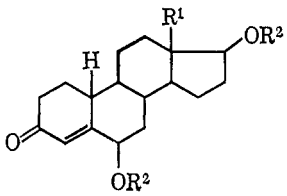
IVa, R = H
b, R = CH_3



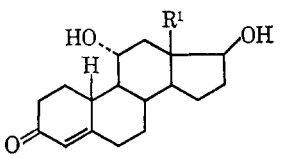
Va, $R^1 = CH_3$; $R^2 = H$
b, $R^1 = C_2H_5$; $R^2 = H$
c, $R^1 = C_2H_5$; $R^2 = COCH_3$



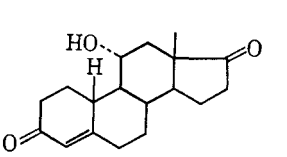
VI



VIIa, $R^1 = CH_3$; $R^2 = H$
b, $R^1 = C_2H_5$; $R^2 = H$
c, $R^1 = C_2H_5$; $R^2 = COCH_3$



VIIIa, $R^1 = CH_3$
b, $R^1 = C_2H_5$



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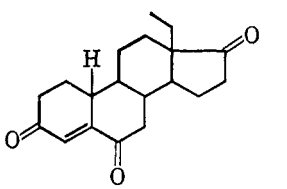
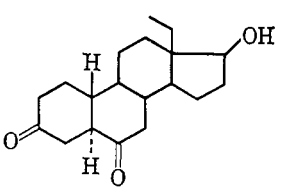


X



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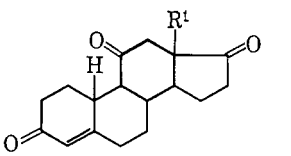

XIIa, $R^1 = CH_3$
b, $R^1 = C_2H_5$

rotational increment is consistent with the 1β assignment and is at variance with the low positive increment associated with 1α-acetoxy Δ^4-3-ketones.[14]

Further evidence for the 1β assignment in IId obtains from its proton nuclear magnetic resonance spectra (Figure 3). Bands between 0.9 and 1.6 ppm are associated with the 13β-ethyl group, the methyl triplet centered about 0.94 ppm (J = 7 cps), the methylene quartet centered about 1.33 ppm (J = 7 cps). The bands at 2.00 and at 2.02 ppm are assigned to the 1β- and 17β-acetoxyl methyl protons.[15] The triplet centered about 4.67 ppm is assigned to the 17α proton,[17] and the 5.88-ppm resonance to the C-4 vinyl proton. The remaining resonances of interest are the multiplet centered about 5.50 ppm, assigned to the 1α proton, and a sharp doublet at 2.54 ppm associated with the two C-2 protons adjacent to carbonyl.

The 5.50-ppm multiplet is near the region associated with equatorial protons on carbon bearing axial acetoxyl groups[19] and most closely resembles the signal pattern of a 1β-acetoxy Δ^4-3-ketone reported by Tori and Kondo,[24] while differing in appearance from their published signal pattern for 1α-hydroxy and 1α-acetoxy Δ^4-3-ketones.

The quasi-equatorial nature of the 1α proton in *l*-IId is further demonstrated by analysis of the spin–spin coupling pattern in 60-Mc spectra and by spin-decoupling spectra at 100 Mc. The (first-order) coupling constant for the 5.50-ppm multiplet is 4 cps, which is the separation of the 2.54-ppm doublet. The coupling of the 1α proton with both C-2 protons is

(14) R. M. Dodson, A. H. Goldkamp, and R. D. Muir, *J. Am. Chem. Soc.*, **82**, 4026 (1960).

(15) 17β-Acetoxyl methyl protons for a variety of 17β-acetoxy steroids have chemical shifts in the region 2.0–2.2.[16]

(16) N. S. Bhacca and D. H. Williams, "Applications of NMR Spectroscopy in Organic Chemistry, Illustrations from the Steroid Field," Holden-Day, Inc., San Francisco, Calif., 1964, p 33.

(17) The 17α-proton triplet of 17β-acetoxy steroids has been detected within the range 4.46–5.11 ppm in a variety of steroids.[18]

(18) L. L. Smith, *Steroids*, **4**, 395 (1964).

(19) Equatorial protons of 3α-acetoxy (axial) steroids,[20a,b] 7α-acetoxy (axial) steroids,[20c,f] 11β-acetoxy (axial) steroids,[20f] 15β-acetoxy (quasi-axial) steroids,[20f] and 16α-acetoxy (axial) D-homosteroids[20d] have been found in the range 4.93–5.45 ppm, while axial protons of 3β-acetoxy (equatorial) steroids,[20a,b] 7β-acetoxy (equatorial) steroids,[20c,f] 11α-acetoxy (equatorial) steroids,[20f] 15α-acetoxy (quasi-equatorial) steroids,[20f] and 16α-acetoxy (equatorial) D-homosteroids[20e] are within the range 4.46–5.15 ppm. No distinction in chemical shift is made for the 6α and 6β protons of 6β- and 6α-acetoxy Δ^4-3-ketones, however,[20f,21] and the generalization may not hold in all cases.[22,23]

(20) (a) J. N. Shoolery and M. T. Rogers, *J. Am. Chem. Soc.*, **80**, 5121 (1958); (b) J. N. Shoolery, in "NMR and EPR Spectroscopy," Pergamon Press, New York, N. Y., 1960, pp 100–121; (c) R. C. Tweit, A. H. Goldkamp, and R. M. Dodson, *J. Org. Chem.*, **26**, 2856 (1961); (d) L. L. Smith, M. Marx, J. J. Garbarini, T. Foell, V. E. Origoni, and J. J. Goodman, *J. Am. Chem. Soc.*, **82**, 4616 (1960); (e) M. Heller, S. M. Stolar, and S. Bernstein, *J. Org. Chem.*, **26**, 5036 (1961); (f) Y. Kawazoe, Y. Sato, T. Okamoto, and K. Tsuda, *Chem. Pharm. Bull.* (Tokyo), **11**, 328 (1963).

(21) D. J. Collins, J. J. Hobbs, and S. Sternhell, *Tetrahedron Letters*, **No. 4**, 197 (1963).

(22) K. L. Williamson and W. S. Johnson, *J. Am. Chem. Soc.*, **83**, 4623 (1961).

(23) See ref 16, pp 77–85.

(24) K. Tori and E. Kondo, *Steroids*, **4**, 713 (1964).

established by a double irradiation experiment at 100 Mc, where the 5.50-ppm multiplet collapsed to a singlet when the 2.54-ppm doublet was irradiated (Figure 3, curve B), and where the 2.54-ppm doublet became a sharp singlet on irradiation of the 5.50-ppm multiplet (Figure 3, curve C). These decoupling experiments confirm that the two C-2 protons have the same chemical shifts and are coupled to the 1α proton with a coupling constant of $J = 4$ cps. Since the 1α proton appears as a singlet (half-width *ca.* 2 cps) when decoupled from the C-2 protons, the residual coupling between the 1α and 10β protons must be very small.

By assuming that the Karplus correlation[25] can apply in this system, the dihedral angles $H_{1\alpha}$–C–C–$H_{2\alpha}$ and $H_{1\alpha}$–C–C–$H_{2\beta}$ must be equal, and their magnitude should be about 40°. The dihedral angle $H_{1\alpha}$–C–C–$H_{10\beta}$ is then a right angle. These dihedral angles can be accommodated in Dreiding models of IId with a "twist" A-ring conformation, making the 1β-acetoxyl group quasi-axial and the 1α proton quasi-equatorial.

The secondary products, Vb, VIIb, and VIIIb, from *A. ochraceus* attack on Ib were more readily identified. Data obtained from systematic spectral examination[9] of the least polar Vb suggested a 10β-hydroxy Δ^4-3-ketone structure; particularly was this true of the hypsochromic shift in the ultraviolet absorption spectra in ethanol and the characteristic absorption spectra in concentrated sulfuric acid, where spectra typical of the $\Delta^{4,9}$-3-ketone system were obtained.[9]

Acetylation of Vb produced a 17β-monoacetate (Vc), and chromic acid oxidation of Vb gave a monohydroxy diketone (VI), the spectral properties in each case supporting the 10β-hydroxy Δ^4-3-ketone feature.

Acid dehydration of VI gave the phenol IVa,[26] which from its positive specific rotation is assigned a *d* configuration. The positive specific rotation of Vb, Vc, and VI also support the *d* assignment.[27] Molecular rotational increments of +51 and +42° for Vb and its 17β-monoacetate Vc[28] are in agreement with the *d*-10β-hydroxy Δ^4-3-ketone assignment.

Proof of structure of Vb was completed by synthesis of *dl*-13β-ethyl-10β,17β-dihydroxygon-4-en-3-one *via* monoperphthalic acid oxidation of *dl*-13β-ethyl-17β-hydroxygon-5(10)-en-3-one[2] followed by alkaline hydrolysis of the intermediate 5ξ,10ξ-epoxide. The racemic product and its 17β monoacetate were identical in infrared spectral (solution) and chromatographic behavior with *d*-Vb and *d*-Vc from the fermentation source.

The secondary product VIIb was recognized as a 6β-hydroxy derivative by the hypsochromic shift in its ultraviolet absorption spectra in ethanol and by its behavior in 0.066 *N* alkaline ethanol. A diacetate VIIc was formed, and chromic acid oxidation of VIIb gave a trione (X) with spectral properties characteristic of the Δ^4-3,6-dione system. Rearrangement in alkali of VIIb gave a product (XI) having properties consistent with those anticipated in a 5α-3,6-dione derived from a 6-hydroxy Δ^4-3-ketone system. Reductive cleavage of the 6-acetoxyl group of VIIc with zinc and acetic acid followed by saponification of the 17β-acetate ester yielded *d*-Ib, identified by comparison with the authentic *d* enantiomer prepared in other work. Molecular rotational increments of −379° for VIIb and −240° for its diacetate VIIc are consistent with the *d*-6β-hydroxy assignment.[9]

Identity was completed by synthesis of *dl*-VIIb from *dl*-Ib *via* enol ether formation and monoperphthalic acid oxidation in the usual fashion. Racemic VIIb and its diacetate (VIIc) had infrared spectral (solution) and chromatographic properties identical with those of *d*-VIIb and *d*-VIIc, respectively. These data establish that VIIb is *d*-13β-ethyl-6β,17β-dihydroxygon-4-en-3-one.

The trace product VIIIb, shown to be a monohydroxylated derivative of Ib by elemental analysis, was optically inactive, as was the trione XIIb obtained therefrom by chromic acid oxidation. Systematic spectral examination of VIIIb in ethanol, in 0.066 *N* alkaline ethanol, and in concentrated sulfuric acid ruled against A- or B-ring hydroxylic substitution. Infrared spectra of the trione XIIb included bands at 5.75 (17 ketone) and 5.84 μ, the latter band being interpreted as evidence for a six-membered cyclic ketone. Only 11- and 12-hydroxylated derivatives of Ib could lead to such ketonic absorption.[29]

The racemic VIIIb is assigned the 11α-hydroxy formulation after exclusion of 12 hydroxylation the trione XIIb lacks any evidence in its infrared spectra of the 12,17-diketone interactions described by de Flines, *et al.*[30] Comparison of VIIIb and XIIb with *dl*-13β-ethyl-11α,17β-dihydroxygon-4-en-3-one and *dl*-13β-ethylgon-4-ene-3,11,17-trione, respectively, prepared by total synthesis, established their identity and confirmed the assigned 11α-hydroxy structure for VIIIb.

The lack of optical activity in VIIIb implies that *A. ochraceus* attacks both *d* and *l* enantiomers in this instance. Thus, in one fermentation, racemic Ib is transformed into *d*, *l*, and *dl* products.

Fermentation of *l*-Ib with *A. ochraceus* gave a single product transformation as evidenced by thin layer chromatography, the product being recognized as the 1β-hydroxy derivative (IIb). No other products could be detected by ultraviolet light absorption or by phosphomolybdic acid on thin layer chromatoplates. Fermentation of *d*-Ib, on the other hand, gave a thin layer chromatographic pattern essentially the same as that obtained with racemic Ib except for spot intensity. The 6β- and 10β-hydroxylated products (VIIb and Vb) were predominant, with minor amounts of the 11α-hydroxy derivative (VIIIb) and of a component having chromatographic behavior identical with that of the 1β-hydroxy derivative (*l*-IIb). Although the identity of this fourth transformation product of the *d* substrate could not be established, it probably is a *d*-1β-hydroxy derivative (IIb); thus 1β hydroxylation as well as

(25) M. Karplus, *J. Chem. Phys.*, **30**, 11 (1959).

(26) 10β-Hydroxy Δ^4-3-ketones dehydrate readily in acid to form the related phenol: see (a) R. L. Pederson, J. A. Campbell, J. C. Babcock, S. H. Eppstein, H. C. Murray, A. Weintraub, R. C. Meeks, P. D. Meister, L. M. Reineke, and D. H. Peterson, *J. Am. Chem. Soc.*, **78**, 1512 (1956); (b) J. Perez Ruelas, J. Iriarte, F. Kincl, and C. Djerassi, *J. Org. Chem.*, **23**, 1744 (1958); (c) A. von Wartburg, J. Binkert, and E. Angliker, *Helv. Chim. Acta*, **45**, 2139 (1962).

(27) 10β-Hydroxy Δ^4-3-ketones derived from steroids of natural *d* configuration all have positive specific rotations.[26a-c]

(28) The ΔM_D values for Vc and VIIc were calculated using the M_D value of −147° for *l*-Ib 17β-monoacetate (Id), reversed in sign to +147° for the *d* series.

(29) A possible though unprecedented alternative, the hydroxylation of the methylene portion of the 13β-ethyl group, was considered unlikely.

(30) J. de Flines, W. F. dan der Waard, W. J. Mijs, L. A. van Dijck, and S. A. Szpilfogel, *Rec. Trav. Chim.*, **82**, 139 (1963).

11α-hydroxylation may obtain with both *d* and *l* enantiomers.

This remarkable product distribution of Ib could not be demonstrated to the same extent with the 13β-propyl derivative (Ic). The fermentation was less satisfactory, the racemic substrate being recovered in several cases along with the major transformation product (IIc). However, the presence of minor steroidal components having a chromatographic mobility suggesting their identity as 6β-, 10β-, and 11α-hydroxy derivatives of Ic did establish the complexity of the attack of *A. ochraceus* on Ic.

An extraneous sterol recognized as ergosterol was isolated from these *A. ochraceus* fermentations with *dl*-Ic.[31]

A. ochraceus fermentation of racemic 19-nortestosterone (Ia) yielded 37% of the expected 11α-hydroxy derivative (*d*-VIIIa), together with minor amounts of other monohydroxylated products. A secondary product (Va) was identified as the 10β-hydroxy derivative by systematic spectral examination[9] in the same manner used for Vb. Infrared spectral (solution) and chromatographic behavior of Va showed properties identical with those of authentic *d*-10β-hydroxy-19-nortestosterone.[26a,b] The 10β-hydroxy product Va in this case had a negative specific rotation and was thus assigned the *l* configuration.

A minor product IIa of the fermentation was recognized to be a 1β-hydroxylated derivative of Ia by chromatographic and systematic spectral examination where properties analogous with those of the 1β-hydroxy steroids IIb and IIc were obtained. Dehydration of IIa gave a single phenolic product having spectral and chromatographic properties corresponding to those of natural estradiol-17β. Insufficient material was available for full characterization or for preparative-scale dehydration to estradiol for confident assignment of configuration, but an optical rotatory dispersion curve for IIa was essentially superimposable on that from the known *l*-IIb (Figure 2), thereby establishing that IIa belongs to the *l* series.

After this work was completed, it was possible to make a comparison between *l*-IIa and *d*-IIa obtained by microbial hydroxylation of natural Ia with *Botryodiplodia malorum*.[32] Identical spectral and chromatographic properties confirmed our assignment of structure to IIa.

A trace product (VIIa) was recognized as 6β-hydroxy-19-nortestosterone on the basis of chromatographic comparisons and systematic spectral examination. It was not possible to establish its optical properties.

Fermentation of *d*-19-nortestosterone with *A. ochraceus* led to a different product picture, a 72% yield of *d*-11α-hydroxy-19-nortestosterone and a small amount of *d*-11α-hydroxyestr-4-ene-3,17-dione (IX) being obtained. Conversion of both products by chromic acid oxidation to the common *d*-estr-4-ene-3,11,17-trione (XIIa) served to establish their relationship to one another. Since these studies were completed, de Flines, *et al.*, have published essentially identical results with *A. ochraceus* on *d*-19-nortestosterone.[8m]

These transformations thus involve both *d* and *l* enantiomers as well as different positions of hydroxylative attack. Four sites of hydroxylation (1β, 6β, 10β, and 11α) are common to racemic 19-nortestosterone (Ia) and to racemic 13β-ethyl-17β-hydroxygon-4-en-3-one (Ib), and there is chromatographic evidence for the same hydroxylation products obtained with the 13β-propyl derivative (Ic, Figure 1).

The three racemic substrates studied, Ia, Ib, and Ic, differ from one another only in the nature of the angular alkyl group at the 13β position of the steroid nucleus. The progression from the 13β-methyl group of Ia to the 13β-ethyl group of Ib is accompanied (in fermentation product distribution) by: (a) a reversal of the configuration of the major product isolated, the major product from racemic Ia being a *d* steroid and from racemic Ib being an *l* steroid; (b) a reversal of the proportions of the 11α- and 1β-hydroxylated products, 11α hydroxylation being the major transformation and 1β hydroxylation being a very minor product with *dl*-Ia, but 1β hydroxylation being a major transformation and 11α hydroxylation being the minor transformation with *dl*-Ib; and (c) a reversal of the configuration of the major secondary 10β-hydroxylated product obtained, an *l*-10β-hydroxy product being obtained from *dl*-Ia and a *d*-10β-hydroxy product being obtained from *dl*-Ib.

Since spore studies of Vézina, *et al.*,[8n] have shown that the 11α-hydroxylation activity of *A. ochraceus* is not dependent on an adaptive enzyme system, it was hoped that use of spores would favor 11α hydroxylation of racemic Ib were the 1β-, 6β-, and 10β-hydroxylases adaptive. However, spores of *A. ochraceus* NRRL 405 in nitrogen-free medium afforded complete transformation of *dl*-Ib to the 1β-, 6β-, 10β-, and 11α-hydroxylated products in about the same distribution found in vegetative cell fermentations. The several hydroxylating systems thus do not appear to be adaptive and are assumed to be constitutive in nature.

While these results could be regarded as a series of highly stereospecific hydroxylations, involving in each instance a separate enzyme system for each hydroxylated product formed, it may be more appropriate to consider these transformations as the result of a low order of stereospecificity, with hydroxylation occurring at the 1β, 10β, or 11α position depending on the extent of interaction between substrate and enzyme. This concept is supported by an earlier report of 1β or 11α hydroxylation of 17α,21-dihydroxypregn-4-ene-3,20-dione by *Absidia orchidis*.[33]

Experimental Section[34]

19-Nortestosterone Fermentations. Vegetative growth of *A. ochraceus* NRRL 405 was obtained in 1-l. flasks containing 200 ml of a medium consisting of 10 g/l. of yeast extract and 10 g/l. of

(31) Ergosterol has been isolated from other species of *Aspergillus* as the palmitate: *cf.* M. W. Miller, "The Pfizer Handbook of Microbial Metabolites," McGraw-Hill Book Co., Inc., New York, N. Y., 1961, p 167; H. Otomasu, E. Watanabe, and S. Nakajima, *J. Pharm. Soc. Japan*, **83**, 107 (1963).

(32) C. C. Bolt, W. J. Mijs, F. J. Zeelen, S. A. Szpilfogel, J. de Flines, and W. F. van der Waard, *Rec. Trav. Chim.*, **84**, 626 (1965).

(33) V. Schwarz, M. Ulrich, and K. Syhora, *Steroids*, **4**, 645 (1964).

(34) All melting points were taken on a Kofler block under microscopic magnification. Ultraviolet absorption spectra were recorded on solutions in 95% ethanol, in 0.066 *N* alkaline ethanol according to Meyer,[35] and in concentrated sulfuric acid. Infrared spectra were obtained on potassium bromide disks and on chloroform solutions using a Perkin-Elmer Model 21 spectrophotometer. Optical rotatory dispersion data were obtained on dioxane solutions using a Rudolph spectropolarimeter. Specific rotations were obtained on 0.5–1.0% solutions in chloroform except as noted to the contrary. Proton

glucose in distilled water. Incubation was carried out at 28° on a rotary shaker (250 rpm) for 65.5 hr. Mycelial transfers (9%) were made to 1- and 2-l. flasks containing 200 and 400 ml of the same medium, and after 24.5 hr a 50-mg/ml ethanolic solution of crystalline *dl*-19-nortestosterone[2c,37] was added to give a final steroid concentration of 0.25 g/l. The course of the transformation was followed by taking methyl isobutyl ketone extracts at appropriate times and chromatographing the extracts in petroleum ether (bp 36–58°)–benzene–methanol–water (3:7:5:5) paper chromatographic system; the steroidal products were detected by ultraviolet light absorption. After 25–27 hr the flasks were harvested.

l-10β,17β-Dihydroxyestr-4-en-3-one (Va). Harvested fermentation broth (6 l.) obtained by the action of *A. ochraceus* NRRL 405 on 1.5 g of crystalline *dl*-19-nortestosterone was extracted once with an equal volume of ethyl acetate, then three times with half-volumes of the same solvent. The combined extracts were concentrated under vacuum to *ca.* 50 ml, at which time solids appeared. The solids (275 mg) were filtered and shown not to contain steroids by thin layer chromatography. The mother liquor was chromatographed on 100 g of silica gel. Benzene–ethyl acetate (7:3) eluted 100 mg of crude Va, which was recrystallized from acetone, giving the analytical sample: mp 214–218° (sweating from 200°); $[\alpha]_D$ −71.5° (2%, methanol);[38] λ_{max} 236 mμ (ε 14,545); λ_{max}^{KBr} 3.05, 6.02, 6.17 μ, etc.

Anal. Calcd for $C_{18}H_{26}O_3$: C, 74.44; H, 9.03. Found: C, 73.44; H, 8.72.

The melting point was not depressed by an authentic sample of *d*-10β,17β-dihydroxyestr-4-en-3-one[26b] (mp 208–213°, with sweating from 195°).

Spectra of Va in concentrated sulfuric acid after 2 hr showed λ_{max} ($E_{1cm}^{1\%}$) 283 (316), 398 (436), 456 (396), 500 (143), 544 mμ (23); λ_{min} ($E_{1cm}^{1\%}$) 251 (241), 335 (116), 429 (287), 536 mμ (40).[39]

The ultraviolet and infrared spectra and the thin layer chromatographic behavior of Va were identical with those of authentic *d*-Va. In addition, the infrared spectra of *l*-Va were identical with the published spectra of *d*-Va.[40]

6β,17β-Dihydroxyestr-4-en-3-one (VIIa). Continued elution of the silica gel column from which *l*-Va was isolated afforded mixed fractions of IIa and VIIa. These fractions were chromatographed with ethyl acetate–methanol (17:3) on silica gel chromatoplates (250 μm thick). The more mobile ultraviolet light absorbing zone was eluted, yielding 0.5 mg of VIIa: mp 211–219°; λ_{max} 238 mμ (ε 13,200); $\lambda_{max}^{0.066\ N\ NaOH}$ (after 24 hr) 244 mμ (ε 8750), 276 (4300), inflection, 365 (900). Thin layer chromatographic behavior was identical with that of authentic 6β-hydroxy-19-nortestosterone.

l-1β,17β-Dihydroxyestr-4-en-3-one (IIa). The more polar ultraviolet light absorbing zone from the thin layer chromatoplate from which VIIa was isolated was eluted and the product was recrystallized twice from acetone, yielding 2.5 mg: mp 192–198°; λ_{max} 243 mμ (ε 16,695); $\lambda_{max}^{0.066\ N\ NaOH}$ (immediately) 243 mμ (ε 10,600), 302 mμ (ε 2750) (acidified, λ_{max} 280 mμ (ε 2770), 288 mμ (ε 2440)); λ_{max}^{KBr} 2.90, 3.00, 6.00, 6.13 μ, etc. Insufficient material was available for elemental analysis.

Optical rotatory dispersion of IIa in dioxane (*c* 0.02456) was obtained over the range 265–400 mμ: $[\alpha]_{400}$ +163°, $[\alpha]_{348}$ +269°, $[\alpha]_{335}$ +554°, $[\alpha]_{320}$ +1025°, $[\alpha]_{265}$ +3540°.

Infrared spectral and thin layer chromatographic behavior of *l*-IIa was identical with that of an authentic sample of *d*-IIa, mp 201–204° with sweating from 194° (lit.[32] mp 212–215°).

d-11α,17β-Dihydroxyestr-4-en-3-one (VIIIa). A. From *dl*-Ia. Continued elution of the column (from which Va, IIa, and VIIa had been eluted) with benzene–ethyl acetate (3:2) and with pure ethyl acetate yielded 280 mg of *d*-VIIIa, which was recrystallized three times from ethyl acetate and twice from acetone, affording a sample: mp 179–181°, $[\alpha]_D$ −42.5°, λ_{max} 242 mμ (ε 16,320), identical with an authentic sample.[41]

B. From *d*-Ia. To 9.6 l. of mycelial growth of *A. ochraceus* WLR 182 (a strain whose transformation pattern is very similar to that of NRRL 405) prepared in yeast extract–glucose medium was added 4.8 g of *d*-19-nortestosterone as a 40 mg/ml ethanolic solution. Incubation was carried out at 26 and 28°. Flasks were harvested after 28 or 45 hr; the broth was extracted with ethyl acetate, and the extracts were processed in the usual manner, yielding 3.1 g of product, mp 145–160°, which was recrystallized several times from ethyl acetate to yield the pure *d*-VIIIa: mp 167–170°, $[\alpha]_D$ −44.0°, λ_{max} 242.5 mμ (ε 16,323). A second crystalline form, mp 182–184°,[26a] was also encountered.

d-11α-Hydroxyestr-4-ene-3,17-dione (IX). Mother liquor from which *d*-VIIIa has been removed under B above was chromatographed on Florisil. Elution with chloroform–methanol (49:1) gave 430 mg of crude *d*-IX, which was recrystallized several times from ethyl acetate, yielding the analytical sample: mp 200–219°; $[\alpha]_D$ ±0°, −18.4° (methanol); λ_{max} 240 mμ (ε 15,816); λ_{max}^{KBr} 2.99, 5.75, 6.08, 6.23 μ, etc.;[42] mauve color by Zimmermann test.

Anal. Calcd for $C_{18}H_{24}O_3$: C, 74.97; H, 8.39. Found: C, 74.78; H, 8.35.

d-Estr-4-ene-3,11,17-trione (XIIa). A solution of 142 mg of *d*-VIIIa in 10 ml of acetone was cooled to 0° and 0.25 ml of 8 *N* chromic acid was added. After 5 min an additional 0.07 ml of chromic acid was added. Ten minutes later 1 ml of methanol was added and the mixture was poured onto ice. After extraction with chloroform–ether (1:4), washing, drying, evaporation, and crystallization of the residue from acetone, there was obtained 80 mg of XIIa, mp 205–209°. Several recrystallizations from acetone and from acetone–ether afforded the analytical sample: mp 207–211°; $[\alpha]_D$ +313.5°, +286° (methanol); λ_{max} 240 mμ (ε 16,100); λ_{max}^{KBr} 5.74, 5.84, 5.98, 6.18 μ, etc.[43]

Similar oxidation of the 11α-hydroxy-3,17-dione IX gave a trione, mp 200–206°, λ_{max} 240 mμ (ε 14,443), identical with that obtained from *d*-VIIIa as evidenced by thin layer chromatography and infrared spectra.

13β-Ethyl-17β-hydroxygon-4-en-3-one Fermentations. A spore suspension of *A. ochraceus* WLR 182 was transferred to 500-ml flasks containing 100 ml of a medium composed of 50 g/l. of glucose, 20 g/l. of peptone, and 5g/l. of corn steep liquor in distilled water, plus 2 ml per flask of a salts solution composed of 14.6 g/l. of calcium nitrate tetrahydrate, 5 g/l. of potassium dihydrogen phosphate, 10 g/l. of potassium chloride, 10 g/l. of magnesium sulfate heptahydrate, 1 g/l. of ferrous sulfate, and 3 g/l. of manganous sulfate hydrate in distilled water.[44] Incubation was carried out on a rotary shaker (250 rpm) at 28° for 64 hr, and 10% mycelial transfers were made to 2-l. flasks with 400 ml of the above medium. After 24 hr 3 g of *dl*-Ib[2] was added as a 30-mg/ml solution in ethanol. After 30 hr the broth was harvested.

l-13β-Ethyl-1β,17β-dihydroxygon-4-en-3-one (IIb). Six liters of harvested broth was extracted three times with 1.5 l. of ethyl acetate. The residue obtained on evaporation of the extracts was slurried in 75 ml of acetone and filtered after 1 hr. The solids weighed 550 mg. Concentration of the acetone filtrate yielded 326 mg of product, both fractions being homogeneous by thin layer chromatography. An additional 50 mg of product could be recovered on further working of the mother liquors. Recrystallization of first crop material from methanol several times gave the analytical sample: mp 198–200°; $[\alpha]_D$ +67.9° (50% methanol in chloroform); λ_{max} 243 mμ (ε 15,500); $\lambda_{max}^{0.066\ N\ NaOH}$ 243 mμ (ε 9630), 302 mμ (ε 3140) (acidified, λ_{max} 222 mμ (ε 12,350), 281 (3600), 288 (3140)); λ_{max}^{KBr} 2.90, 3.03, 6.03, 6.14 μ, etc.

Anal. Calcd for $C_{19}H_{28}O_3$: C, 74.96; H, 9.27. Found: C, 74.54; H, 8.95.

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spectra were obtained with a Varian Associates A-60 spectrometer on 10–15% solutions of steroid in deuteriochloroform, measuring chemical shifts downfield from tetramethylsilane as the internal standard. Thin layer chromatography was conducted on silica gel chromatoplates prepared with rice starch as binder.[36] Compounds were detected *via* ultraviolet light absorption and with acidified phosphomolybdic acid reagent.[35] All characterized compounds were chromatographically homogeneous by thin layer chromatographic standards (steroidal impurities estimated less than 1%). Wherever possible, infrared spectra and thin layer chromatographic behavior were compared between resolved compounds and their respective racemic modifications.[2]

(35) A. S. Meyer, *J. Org. Chem.*, **20**, 1240 (1955).

(36) L. L. Smith and T. Foell, *J. Chromatog.*, **9**, 339 (1962).

(37) S. N. Ananchenko and I. V. Torgov, *Tetrahedron Letters*, **No. 23**, 1553 (1963); K. K. Koshoev, S. N. Ananchenko, A. V. Platonova, and I. V. Torgov, *Izv. Akad. Nauk SSSR, Ser. Khim.*, **11**, 2058 (1963).

(38) The *d* enantiomer is known: mp 199–205°, $[\alpha]_D$ +76°, λ_{max} 237 mμ (ε 15,025);[26a] mp 208–210°, $[\alpha]_D$ +80° (methanol), λ_{max} 234–236 mμ (ε 13,200);[26b] mp 205–210°, $[\alpha]_D$ +70°.[8m]

(39) Complete data on Va, VIIa, VIIIa, IX, IIb, IId, Vb, VIIb, IIc, etc., are included in a manuscript in preparation dealing with spectra of 19-nor Δ^4-3-ketones in sulfuric acid.

(40) A. von Wartburg, *Helv. Chim. Acta*, **46**, 591 (1963).

(41) Reported constants:[26a] mp 167–178°, also 185–187°; $[\alpha]_D$ −46°; λ_{max} 242 mμ (ε 15,475); ν_{max}^{Nujol} 3345, 1650, and 1610 cm^{-1}.

(42) Reported constants:[26a] mp 212–214°; $[\alpha]_D$ +5°; ν_{max}^{Nujol} 3590, 1742, 1672, and 1618 cm^{-1}.

(43) Reported constants: mp 213.5–215°, $[\alpha]_D$ +145°, λ_{max} 240 mμ (ε 14,600);[26a] mp 208–210°, $[\alpha]_D$ +296°.[8m]

(44) German Patent 1,009,627 (1957).

Optical rotatory dispersion in dioxane (*c* 0.0462): $[\alpha]_{400}$ +187°, $[\alpha]_{348}$ +195°, $[\alpha]_{335}$ +490°, $[\alpha]_{320}$ +928°, $[\alpha]_{310}$ +1270°, $[\alpha]_{265}$ +3250°.

Proton spectra showed C-2 protons at 2.46 ppm (doublet, J = 3.5 cps), 17α proton at 3.60 ppm (multiplet), 1α proton at 4.35 ppm (singlet), C-4 vinyl proton at 5.78 ppm.

***l*-1β,17β-Diacetoxy-13β-ethylgon-4-en-3-one (IId).** Acetylation of 348 mg of *l*-IIb with 0.95 ml of acetic anhydride in 3 ml of pyridine yielded a crude diacetate which was recrystallized from ether, giving 276 mg. Further recrystallization from ether–hexane afforded the analytical sample: mp 149–152°, resolidifying and remelting 230–232°; $[\alpha]_D$ +67.9°; λ_{max} 240 mμ (ϵ 17,000); λ_{max}^{KBr} 5.77, 5.98, 6.15 μ, etc.

Anal. Calcd for $C_{23}H_{32}O_5$: C, 71.10; H, 8.30. Found: C, 71.18; H, 8.39.

Proton spectra of IId are presented in Figure 3.

***d*-13β-Ethyl-10β,17β-dihydroxygon-4-en-3-one (Vb).** The mother liquors from which *l*-IIb had been removed by crystallization (representing a total of 41 g of racemic Ib as intial substrate) were combined and chromatographed on 800 g of silica gel. Benzene–ethyl acetate (9:1) eluted nonsteroidal components and eventually 420 mg of *l*-IIIb, $[\alpha]_D$ −55.8°. Benzene–ethyl acetate (4:1) eluted 610 mg of *d*-Vb: mp 224–227°; $[\alpha]_D$ +66.5°; λ_{max} 236 mμ (ϵ 14,850); λ_{max}^{KBr} 3.05, 6.03, 6.17 μ, etc.

Anal. Calcd for $C_{19}H_{28}O_3$: C, 74.96; H, 9.27. Found: C, 74.90; H, 9.21.

Spectra in concentrated sulfuric acid after 2 hr showed λ_{max} ($E_{1\,cm}^{1\%}$) 236 (395, inflection), 275 (395, inflection), 285 (402), 315 (296, inflection), 400 (282, inflection), 452 (384), 498 (204), 538 mμ (35).

***d*-17β-Acetoxy-13β-ethyl-10β-hydroxygon-4-en-3-one (Vc).** A solution of 45 mg of *d*-Vb in 0.5 ml of pyridine and 0.4 ml of acetic anhydride was held at room temperature for 15 hr, after which time the solvents were removed under vacuum. The residue was dissolved in ether and the solution was extracted with 3 *N* hydrochloric acid and 0.1 *N* sodium hydroxide solution and evaporated. The product was crystallized from acetone–hexane yielding 44 mg, mp 180–183°. After recrystallization from acetone–hexane and drying under vacuum there was obtained 40 mg: mp 182–184°; $[\alpha]_D$ +54.7°; λ_{max} 237 mμ (ϵ 13,850); λ_{max}^{KBr} 3.02, 5.72, 6.05, 6.15, 8.05, 9.60 μ, etc.; $\lambda_{max}^{CHCl_3}$ 2.95, 5.80, 5.99, 8.0 μ, etc.

Anal. Calcd for $C_{21}H_{30}O_4$: C, 72.80; H, 8.73. Found: C, 72.61; H, 8.57.

***d*-13β-Ethyl-10β-hydroxygon-4-ene-3,17-dione (VI).** A solution of 120 mg of *d*-Vb in 15 ml of acetone at 0° was treated with 0.125 ml of 8 *N* chromic acid reagent. After 10 min the solution was poured onto ice and worked up in the usual manner. The residue was crystallized from ethyl acetate, yielding 90 mg of VI, mp 202–206°; $[\alpha]_D$ +124.6° (50% methanol in chloroform); λ_{max} 235 mμ (ϵ 14,735); λ_{max}^{KBr} 2.85, 5.80, 5.99, 6.17 μ, etc.

***d*-13β-Ethyl-3-hydroxygona-1,3,5(10)-trien-17-one (IVa).** Dry hydrogen chloride was passed through a solution of 85 mg of VI in 5 ml of glacial acetic acid at 5° for 1.5 hr. The solution was then poured into 50 ml of ice water and the solids filtered (70 mg) and recrystallized from ethyl acetate and from methanol–chloroform, yielding the phenol IVa: mp 246–249°; mmp 252–253° with authentic *d*-IVa, mp 252–253°; $[\alpha]_D$ +108.1° (50% methanol in chloroform); infrared spectra and chromatographic behavior identical with that of an authentic sample of *d*-IVa.

***dl*-13β-Ethyl-10β,17β-dihydroxygon-4-en-3-one (Vb).** A solution of 500 mg of *dl*-13β-ethyl-17β-hydroxygon-5(10)-en-3-one[2] and 1.5 equiv of monoperphthalic acid in chloroform was held at 5° for 18 hr, then diluted with ether, washed with sodium bicarbonate solution and with water, and evaporated under vacuum. The residue was crystallized from ethyl acetate, yielding 102 mg of *dl*-5ξ,10ξ-epoxy-13β-ethyl-17β-hydroxygonan-3-one, λ_{max}^{KBr} 2.90, 5.80 μ, etc. From the mother liquor 55 mg of *dl*-Vb (see below) was recovered. A slurry of 80 mg of the 5ξ,10ξ-epoxide in 12 ml of 5% methanolic potassium hydroxide solution was refluxed for 1 hr, poured onto ice, and extracted with chloroform. The chloroform extract was washed with water, dried, and evaporated, yielding 44 mg of *dl*-Vb. This crude product was combined with the 55 mg of Vb obtained directly from the peracid oxidation step and recrystallized twice from methanol, thus affording the analytical sample: mp 250–253°; λ_{max} 236 mμ (ϵ 15,315); λ_{max}^{KBr} 3.04, 6.03, 6.19 μ, etc.

Anal. Calcd for $C_{19}H_{28}O_3$: C, 74.96; H, 9.27. Found: C, 75.03; H, 9.10.

***dl*-17β-Acetoxy-13β-ethyl-10β-hydroxygon-4-en-3-one (Vc).** Thirty milligrams of *dl*-13β-ethyl-10β,17β-dihydroxygon-4-en-3-one was acetylated with 0.3 ml of pyridine and 0.25 ml of acetic anhydride in the usual manner. The crude product was recrystallized twice from acetone, yielding 26 mg; mp 143–144°; λ_{max} 238.5 mμ (ϵ 14,560); $\lambda_{max}^{CHCl_3}$ 2.95, 5.82, 6.00, 6.15, 8.0 μ, etc.

Anal. Calcd for $C_{21}H_{30}O_4$: C, 72.80; H, 8.73. Found: C, 72.59; H, 8.63.

***d*-13β-Ethyl-6β,17β-dihydroxygon-4-en-3-one (VIIb).** Continued elution of the column (from which *d*-Vb had been eluted) with benzene–ethyl acetate (4:1) yielded small amounts of mixed fractions. Elution with benzene–ethyl acetate (3:2) afforded 810 mg of the 6β-hydroxylated product. After several recrystallizations from ethyl acetate there was obtained 630 mg; mp 191–195°; $[\alpha]_D$ −75.0° (1% methanol in chloroform); λ_{max} 238 mμ (ϵ 14,270); λ_{max}^{KBr} 3.02, 6.03, 6.16 μ, etc.

Anal. Calcd for $C_{19}H_{28}O_3$: C, 74.96; H, 9.27. Found: C, 74.69; H, 9.09.

***d*-6β,17β-Diacetoxy-13β-ethylgon-4-en-3-one (VIIc).** Fifty milligrams of *d*-VIIb was dissolved in 0.5 ml of pyridine and 0.4 ml of acetic anhydride. After 30 hr the solvents were removed under vacuum and the residue was recrystallized from acetone–hexane, yielding 42 mg: mp 102–104°; $[\alpha]_D$ −23.9°; λ_{max} 235 mμ (ϵ 14,290); λ_{max}^{KBr} 5.76, 5.95, 6.15, 8.15, 9.65 μ, etc.

Anal. Calcd for $C_{23}H_{32}O_5$: C, 71.10; H, 8.30. Found: C, 71.25; H, 8.32.

Proton spectra of *d*-VIIc showed 6β- and 17β-acetoxyl methyl protons at 2.01 and 2.02 ppm, 17α proton at 4.60 ppm (triplet, J = 8 cps), 6α proton at 5.37 ppm (multiplet), C-4 vinyl proton at 5.87 ppm.[45]

***d*-13β-Ethylgon-4-ene-3,6,17-trione (X).** Thirty milligrams of *d*-VIIb was dissolved in 0.4 ml of pyridine and a solution of 30 mg of chromium trioxide in 0.4 ml of pyridine was added. After 20 hr the solution was poured into water and extracted with ether–chloroform (4:1). The extracts were washed with 3 *N* hydrochloric acid, dilute sodium hydroxide solution, and water, and were dried and evaporated under vacuum. The residue was crystallized from acetone–ether, yielding 18 mg of product which by thin layer chromatography was not completely oxidized. The material in acetone solution was reoxidized with 8 *N* chromic acid reagent in the usual manner and the product crystallized from acetone–ether and recrystallized from aqueous methanol, yielding 12 mg of the trione: mp 217–220°; λ_{max} 254 mμ (ϵ 9740); λ_{max}^{KBr} 5.77, 5.90, 5.95, 6.25 μ, etc.

Anal. Calcd for $C_{19}H_{24}O_3$: C, 75.97; H, 8.05. Found: C, 75.74; H, 7.94.

***d*-13β-Ethyl-17β-hydroxy-5α-gonane-3,6-dione (XI).** A solution of 20 mg of *d*-VIIb in 1 ml of methanol containing 25 mg of potassium hydroxide and 0.1 ml of water was heated under nitrogen for 30 min. The solution was poured into water, extracted with chloroform–ether (1:3), washed with water, then evaporated. The residue was crystallized from acetone–hexane, yielding 13 mg: mp 178–188°; λ_{max} none; $\lambda_{max}^{0.066\ N\ KOH}$ 246 mμ (ϵ 4820), 270 (3700, shoulder), 370 (2190); λ_{max}^{KBr} 2.95, 5.85 μ, etc. The product gave an instant yellow color on papergrams when sprayed with 10% alcoholic alkali, which also fluoresced intensely yellow under ultraviolet light. Insufficient material was available for elemental analysis.

***d*-13β-Ethyl-17β-hydroxygon-4-en-3-one (Ib).** A mixture of 125 mg of *d*-VIIc, 120 mg of zinc dust, and 15 ml of glacial acetic acid was refluxed for 3.5 hr. The cooled mixture was filtered and the acetic acid was removed under vacuum, thus affording 70 mg of crude product which could not be crystallized. The material was dissolved in 10 ml of 95% methanol and 0.5 ml of 5% potassium hydroxide solution was added. After refluxing for 40 min the saponification was complete as judged by thin layer chromatography, and the solution was neutralized and evaporated. The product (50 mg) was chromatographed on thin layer chromatoplates using ethyl acetate–hexane (1:1) and the appropriate zones were removed and eluted with ethyl acetate, yielding 10 mg of crystals, mp 151–158°; $[\alpha]_D$ +56.6°. Thin layer chromatographic, infrared spectral, and mixture melting point comparisons of this sample with authentic *d*-Ib established the identity of the two preparations.

***dl*-13β-Ethyl-6β,17β-dihydroxygon-4-en-3-one (VIIb).** Six grams of *dl*-Ib in 60 ml of dioxane, 0.06 ml of ethanol, and 6.0 ml of triethyl orthoformate was treated with 6 drops of 72% perchloric acid. The solution was mixed with 1.2 ml of pyridine, diluted with water, and extracted with ether. The washed ether extracts were dried, diluted with ether to 100 ml, and cooled in Dry Ice, and 51

(45) The 6α proton of 6β-acetoxy Δ^4-3-ketones has been found at 5.3–5.43 ppm, not greatly displaced from the 6β proton of 6α-acetoxy Δ^4-3-ketones (5.4–5.46 ppm).[20f,21]

ml of an ether solution containing 5.1 g of monoperphthalic acid was added (−70°). The ether solution was allowed to warm up to room temperature overnight and was washed with sodium bicarbonate solution and water, dried, and evaporated. The residue, 450 mg, was recrystallized several times from methanol–ether and from chloroform-ether, yielding a product: mp 158–160° and 196–199°; λ_{max} 238 mμ (ε 12,345); λ_{max}^{KBr} 2.94, 3.03, 6.01, 6.17 μ, etc.

Anal. Calcd for $C_{19}H_{28}O_3 \cdot H_2O$: C, 70.77; H, 9.38. Found: C, 70.62; H, 8.48.

***dl*-6β,17β-Diacetoxy-13β-ethylgon-4-en-3-one (VIIc).** A solution of 35 mg of *dl*-VIIb in 0.35 ml of pyridine and 0.30 ml of acetic anhydride was worked up in the usual manner after 15 hr, yielding 33 mg of VIIc: mp 171–175°; λ_{max} 235 mμ (ε 13,450); $\lambda_{max}^{CHCl_3}$ 5.78, 6.10, 8.0 μ, etc.

Anal. Calcd for $C_{23}H_{32}O_5$: C, 71.10; H, 8.30. Found: C, 70.89; H, 8.04.

***dl*-13β-Ethyl-11α,17β-dihydroxygon-4-en-3-one (VIIIb).** Still further elution of the column (from which Vb and VIIb had been eluted) with benzene–ethyl acetate (3:2 and 2:3) afforded 420 mg of *l*-IIb, and finally elution with pure ethyl acetate gave *dl*-VIIIb. Recrystallization from ethyl acetate gave 70 mg; mp 215–217°; $[\alpha]_D$ ±0° (50% methanol in chloroform); λ_{max} 242 mμ (ε 15,235); λ_{max}^{KBr} 3.00, 6.01, 6.17 μ, etc.

Anal. Calcd for $C_{19}H_{28}O_3$: C, 74.96; H, 9.27. Found: C, 74.43; H, 9.34.

A mixture melting point between VIIIb and *dl*-13β-ethyl-11α,17β-dihydroxygon-4-en-3-one prepared by total synthesis (mp 211.5–214.5°) was undepressed (212–215°). Identity of the two samples was also established by thin layer chromatography and infrared spectral data.

***dl*-13β-Ethylgon-4-ene-3,11,17-trione (XIIb).** To a solution of 13 mg of *dl*-VIIIb in 3 ml of acetone at 0° was added 30 μl of 8 *N* chromic acid. After 10 min the solution was poured onto ice, extracted with chloroform, and recovered in the usual manner. The residue was crystallized from acetone–hexane yielding 8 mg; mp 158–159°; $[\alpha]_D$ ±0° (50% methanol in chloroform); λ_{max} 240 mμ (ε 15,395); λ_{max}^{KBr} 5.75, 5.84, 6.02, 6.18 μ, etc. Insufficient material was available for elemental analysis.

Comparison of XIIb with *dl*-13β-ethylgon-4-ene-3,11,17-trione obtained by total synthesis (mp 163.5–165°) established the identity of the two samples.

***l*-13β-Ethylgona-1,3,5(10)-triene-3,17β-diol (IIIb).** A solution of 965 mg of IIb in 400 ml of 0.1 *N* sodium hydroxide solution and 30 ml of methanol was heated to 55° and filtered. The insolubles were dissolved in methanol and filtered, and the filtrate was combined with the initial filtrate. After 30 min 10% hydrochloric acid was added to give pH 6.2, the mixture was cooled to 20–25°, and the product was filtered, yielding 847 mg. Recrystallization from aqueous methanol, from acetone–ether, from ether, and from benzene gave the pure phenol as a benzene solvate, mp 189–190°.

Anal. Calcd for $C_{19}H_{26}O_2 \cdot C_6H_6$: C, 82.37; H, 8.85. Found: C, 81.49; H, 8.75.

Recrystallization from benzene–petroleum ether twice, with vacuum drying at 100° for 16 hr, gave the unsolvated phenol: mp 189.5–190.0°; $[\alpha]_D$ −53.4°; λ_{max} 223 mμ (ε 7600), 283 (2100), 288.5 (1950); λ_{max}^{KBr} 2.95, 6.20, 6.31, 6.67 μ, etc.

Anal. Calcd for $C_{19}H_{26}O_2$: C, 79.68; H, 9.15. Found: C, 79.73; H, 9.13.

Infrared spectral and thin layer chromatographic comparison of *l*-IIIb with racemic IIIb prepared *via* total synthesis[2] established the identity of the two samples.

***l*-13β-Ethyl-3-hydroxygona-1,3,5(10)-trien-17-one (IVa).** A solution of 602 mg of *l*-IIIb in 20 ml of acetone was treated with 660 μl of 8 *N* chromic acid reagent. The mixture was stirred vigorously for 1 min and poured into ice water, and the product was extracted with chloroform. The chloroform extracts were washed with sodium bicarbonate solution and with water, dried, and evaporated under vacuum. The residue was dissolved in benzene and chromatographed on silica gel. Elution with 10% ethyl acetate in benzene afforded the product, which was recrystallized from petroleum ether–benzene, yielding 275 mg, homogeneous by thin layer chromatography. Additional material (83 mg) contaminated with small amounts of unoxidized diol (IIIb) was recovered from the mother liquor. Rechromatographing the major fraction on silica gel (elution with 5% ethyl acetate in benzene) gave the pure product, mp 245–251°; $[\alpha]_D$ −103.0° (ethyl acetate); λ_{max} 223 mμ (ε 7310), 282 (2110), 288 (1980); λ_{max}^{KBr} 2.96, 5.83, 6.21, 6.65 μ, etc.

Anal. Calcd for $C_{19}H_{24}O_2$: C, 80.24; H, 8.51. Found: C, 80.18; H, 8.59.

***l*-13β-Ethyl-3-methoxygona-1,3,5(10)-trien-17β-ol (IIId).** A solution of 1.266 g of *l*-IIIb in 40 ml of boiling ethanol was treated four times with 7.2 ml of 60% sodium hydroxide and 10 ml of dimethyl sulfate. After the final addition of dimethyl sulfate the solution was cooled, 100 ml of water added, and the mixture cooled in ice and filtered. The solids (1.354 g) were chromatographed on silica gel (elution with 2% ethyl acetate in benzene) and recrystallized from methanol, yielding the analytical sample: mp 107.5–109.5°; $[\alpha]_D$ −50.8°; λ_{max} 222 mμ (ε 8460), 282 (2100), 288 (1965); λ_{max}^{KBr} 3.06, 6.20, 6.35, 6.66 μ, etc.

Anal. Calcd for $C_{20}H_{28}O_2$: C, 79.95; H, 9.39. Found: C, 79.74; H, 9.13.

***l*-13β-Ethyl-17β-hydroxygon-4-en-3-one (Ib).** A solution of 909 mg of *l*-IIId in 22.5 ml of redistilled 1-methoxy-2-propanol (Dowanol) was added to 135 ml of redistilled liquid ammonia and 34 ml of 1-methoxy-2-propanol. Lithium metal (0.9 g) was added in small pieces. After stirring for 30 min the ammonia was distilled from the reaction vessel (2 hr) and 113 ml of water was added. The precipitated product was filtered and washed twice with 34 ml of water. The dried $\Delta^{2,5(10)}$-enol methyl ether weighed 777 mg; mp 120–125°; λ_{max}^{KBr} 2.92, 5.89, 5.99, 8.18, 12.65 μ, etc. Without further purification this enol ether was dissolved in 15 ml of methanol and 0.65 ml of water, and 1 ml of concentrated hydrochloric acid was added. The reaction mixture was stirred under nitrogen for 2 hr, neutralized with solid sodium bicarbonate, and filtered. The filtrate was evaporated under vacuum and chromatographed on silica gel. Elution with 10% ethyl acetate in benzene afforded 400.5 mg of *l*-Ib, mp 156–158°, homogeneous on thin layer chromatoplates. Recrystallization from ether–hexane gave the analytical sample: mp 158–160°; $[\alpha]_D$ −54.5°; λ_{max} 241 mμ (ε 17,500); λ_{max}^{KBr} 2.97, 6.00, 6.16 μ, etc.

Anal. Calcd for $C_{19}H_{28}O_2$: C, 79.12; H, 9.79. Found: C, 79.38; H, 9.66.

Optical rotatory dispersion of *l*-Ib in dioxane over the range 270–600 mμ: $[\alpha]_{600}$ −58.7°, $[\alpha]_{363}$ +455°, $[\alpha]_{357}$ +412°, $[\alpha]_{350}$ +515°, $[\alpha]_{335}$ −131°, $[\alpha]_{320}$ −1190°, $[\alpha]_{310}$ −1704°, $[\alpha]_{295}$ −2015°, $[\alpha]_{270}$ −2380°.

***l*-17β-Acetoxy-13β-ethylgon-4-en-3-one (Id).** One hundred milligrams of *l*-Ib was dissolved in 1 ml of dry pyridine and 0.8 ml of acetic anhydride. After 12 hr the solvents were removed under vacuum and the residue (90 mg), mp 78–80°, was recrystallized from aqueous acetone, yielding the pure monoacetate: mp 83–85°; $[\alpha]_D$ −44.5°; λ_{max} 240 mμ (ε 16,845); λ_{max}^{KBr} 5.78, 5.95, 6.19, 8.02, 9.63 μ, etc.

Anal. Calcd for $C_{21}H_{30}O_3$: C, 76.32; H, 9.15. Found: C, 76.06; H, 8.82.

***l*-13β-Ethyl-3-methoxygona-1,3,5(10)-trien-17-one (IVb).** A solution of 3.94 g of *l*-IIId less in 300 ml of acetone (under nitrogen) containing 2.5 g of anhydrous magnesium sulfate was treated with 5 ml of 8 *N* chromic acid reagent (2.67 g of chromium trioxide and 2.3 ml of concentrated sulfuric acid, diluted to 10 ml with water) added dropwise over 15 min. The mixture was stirred for 15 min longer and 20 ml of 2-propanol was added, along with excess sodium bicarbonate. After 5 min of further stirring, the mixture was filtered. The solids were washed with methylene chloride and the washes were combined with the initial filtrate. The combined filtrates were evaporated under vacuum to a slurry, 150 ml of ether was added, and the solution was washed three times with 15 ml of water, dried over anhydrous magnesium sulfate, and evaporated under vacuum. The residue was dissolved in 150 ml of boiling methanol, filtered, cooled overnight, and filtered. The crystalline product (2.7035 g) (an additional 282.3 mg was recovered from the mother liquors) was chromatographed on silica gel (elution with benzene), yielding the pure methyl ether: mp 146–148°; $[\alpha]_D$ −102.5° (50% methanol in chloroform); λ_{max} 222 mμ (ε 7300), 278.5 (1910), 286 (1810); λ_{max}^{KBr} 3.05, 5.78, 6.22, 6.37, 6.70 μ, etc.

Anal. Calcd for $C_{20}H_{26}O_2$: C, 80.49; H, 8.78. Found: C, 80.20; H, 8.54.

17β-Hydroxy-13β-propylgon-4-en-3-one Fermentations. One- and two-liter shake flasks with mycelial growth of *A. ochraceus* WLR 182 in the corn steep medium already described were supplemented with 6 g of *dl*-Ic[2] as a 50-mg/ml ethanolic solution. Incubations were carried out at 26 and 28°. Fermentation broth was harvested over a 4-day period after 3–7 days of fermentation.

***l*-1β,17β-Dihydroxy-13β-propylgon-4-en-3-one (IIc).** Ten liters of harvested broth was extracted twice with 8 l. of ethyl acetate. The combined extracts were washed with water and evaporated under vacuum. The residue was dissolved in ethyl acetate and ether was added. The solids were filtered after standing overnight, yielding 700 mg of IIc: mp 216–220°; $[\alpha]_D$ +47.3° (50% methanol

in chloroform); λ_{max} 243.5 mμ (ε 15,150); $\lambda_{max}^{0.66\ N\ NaOH}$ 243 mμ (ε 8560), 302.5 mμ (ε 2670) (acidified, λ_{max} 281 mμ (ε 2355), 288 mμ (ε 2198)); λ_{max}^{KBr} 2.78, 3.01, 6.00, 6.14 μ, etc.

Anal. Calcd for $C_{20}H_{30}O_3$: C, 75.43; H, 9.50. Found: C, 75.16; H, 9.65.

Ergosterol. From the mother liquors from which pure *l*-IIc had crystallized there was obtained 260 mg of crude ergosterol. Several recrystallizations from methanol gave 185 mg of a purified product, homogeneous on chromatoplates but still not of high purity, mp 147–155°, [α]D −110° (50% methanol in chloroform). The material was identified as ergosterol by thin layer chromatography (detection with the Liebermann–Burchard reagent) and by comparison of infrared, ultraviolet, and proton spectra with those of an authentic sample.

***dl*-17β-Hydroxy-13β-propylgon-4-en-3-one (Ic).** From a partially complete fermentation of 900 mg of *dl*-Ic with *A. ochraceus*, a product was isolated (370 mg) in the usual manner which analyzed as a mixture of unaltered substrate Ic and the 1β-hydroxy product IIc by thin layer chromatography. Chromatography on silica gel (elution with 50% chloroform in benzene and with chloroform) gave 45 mg of Ic, mp 151–152°, [α]D ±0° (50% methanol in chloroform). The product was identified as *dl*-Ic by infrared spectra.

Further elution of the column with chloroform–methanol (99:1) gave 40 mg of *l*-IIc, mp 211–214°; [α]D +52.9° (50% methanol in chloroform).

***l*-13β-Propylgona-1,3,5(10)-triene-3,17β-diol (IIIc).** A solution of 250 mg of *l*-IIc in 10 ml of methanol was treated with 50 ml of 0.1 *N* sodium hydroxide solution. The solution was warmed at 50° for 10 min and cooled to room temperature, and 2.3 ml of 3.5% hydrochloric acid was added (pH 6.0). The precipitated solids were filtered, washed with water, and dried at 58° under vacuum, yielding 200 mg, mp 91–96°. Several recrystallizations from benzene gave a benzene solvate; mp 105–108°, resolidifying by 130° and remelting at 167°; [α]D −51.3° (ethanol); λ_{max} 223 mμ (ε 6870), 282 (1990), 288 (1810); λ_{max}^{KBr} 2.73, 2.80, 3.02, 3.42, 6.23, 6.70 μ, etc.

Anal. Calcd for $C_{20}H_{28}O_2 \cdot 0.5C_6H_6$: C, 81.43; H, 9.20. Found: C, 81.86; H, 9.45.

The benzene solvate was chromatographed on silica gel and sublimed under vacuum, thus affording the unsolvated pure product, mp 105 and 181°; [α]D −59.2° (ethanol);[46] λ_{max} 282 mμ (ε 2080), 287.5 mμ (ε 1930).

Anal. Calcd for $C_{20}H_{28}O_2$: C, 79.95; H, 9.39. Found: C, 79.87; H, 9.31.

***l*-3-Methoxy-13β-propylgona-1,3,5(10)-trien-17β-ol (IIIe).** A solution of 2.10 g of *l*-IIIc in 65 ml of boiling ethanol was treated with 12 ml of 60% sodium hydroxide solution and then with 16.2 ml of dimethyl sulfate. The additions of base and dimethyl sulfate were repeated in order three more times over 1 hr. After being cooled the solution was poured into 200 ml of water and the precipitate was extracted into chloroform. The chloroform extract was washed with water, dried, and evaporated under vacuum, and the product (2.5 g) was chromatographed on 60 g of Florisil. Elution with petroleum ether–benzene (2:3) gave 1.676 g of crystalline product, homogeneous on thin layer chromatoplates. Several recrystallizations from hexane gave the analytical sample: mp 101–103°; [α]D −58.7°; λ_{max} 278 mμ (ε 2015), 287 mμ (ε 1965); $\lambda_{max}^{CHCl_3}$ 2.95, 6.22, 6.35, 6.70 μ, etc.

Anal. Calcd for $C_{21}H_{30}O_2$: C, 80.21; H, 9.62. Found: C, 80.12; H, 9.50.

***l*-17β-Hydroxy-13β-propylgon-4-en-3-one (Ic).** To a solution of 220 ml of redistilled liquid ammonia and 55 ml of redistilled 1-methoxy-2-propanol under reflux was added a solution of 1.37 g of *l*-IIIe in 37 ml of 1-methoxy-2-propanol (distilled over calcium hydride). Lithium ribbon (1.46 g) was added in small pieces, the precipitated solids were filtered and shown to contain substantial amounts of unreduced IIIe by ultraviolet and infrared spectroscopy. An additional reduction under the same conditions gave 1.10 g of product, mp 115–125°, still contaminated by about 15% of an aromatic impurity as judged by ultraviolet absorption spectra. Without further purification the crude $\Delta^{2,5(10)}$-enol ether was dissolved in 25 ml of methanol and 1 ml of water, and 1.35 ml of concentrated hydrochloric acid was added. After being stirred for 30 min under nitrogen, the solution was poured into water and extracted with ether, and the ether extracts were washed, dried, and evaporated under vacuum, yielding 970 mg of product. The material was chromatographed on 30 g of Florisil. Fractions eluted with benzene were not worked further but probably contained over-reduced products. Material eluted with benzene–chloroform (1:1) weighed 324 mg and after recrystallization from ethyl acetate–hexane afforded 152 mg of pure Δ^4-3-ketone: mp 155–157°; [α]D −74.9°; λ_{max} 241 mμ (ε 15,140); λ_{max}^{KBr} 2.93, 6.03, 6.20 μ, etc.[47]

Anal. Calcd for $C_{20}H_{30}O_2$: C, 79.37; H, 9.94. Found: C, 79.42; H, 10.00.

Proton spectra showed 17α-proton at 3.69 ppm (triplet, J = 7 cps), C-4 vinyl proton at 5.78 ppm (singlet).

Spore Experiment. Conidiospores of *A. ochraceus* NRRL 405 were produced by incubating the culture for 5 days on Sabouraud dextrose agar in Kolle flasks at 28°. After 5 weeks of refrigeration the spores were harvested with distilled water and filtered through glass wool, and the suspension was centrifuged for 15 min at 3600 rpm. After the supernatant was decanted the spores were washed three times with distilled water and resuspended in water to give a count of 1×10^8 spores/ml. To 30 ml of the suspension in a 250-ml flask was added 125 mg of glucose and 7.5 mg of *dl*-Ib dissolved in 0.5 ml of ethanol. Incubation was carried out at 28° on a rotary shaker at 250 rpm. Transformation of the substrate was complete at 92 hr, as evidenced by paper chromatography of a methyl isobutyl ketone extract of the broth. Four ultraviolet light absorbing zones with the same mobility and intensity as obtained with vegetative cells were observed. Microscopic examination of the broth at this point showed virtually no germination of spores.

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(46) The *d* enantiomer is known as a methylene chloride solvate, mp 110°, [α]D +57° (0.2% in ethanol).[11a]

(47) The *d* enantiomer is known: mp 163°, [α]D +64° (methanol), λ_{max} 240–241 mμ (ε 16,100).[11b]