amido - 2′ - deoxy - 3′,4′,6′ - tri - *O* - acetyl - β - D - glucopyranoside, 21588-60-3; cyclohexyl 2′-acetamido-2′-deoxy-α-D-glucopyranoside, 21559-73-9; cyclohexyl 2′-acetamido-2′-deoxy-3′,4′,6′-tri-*O*-acetyl-α-D-glucopyranoside, 21559-74-0.

Acknowledgments.—The authors gratefully acknowledge the interest and support of Dr. T. F. Gallagher throughout this investigation. We also thank Beatrice S. Gallagher for the determination and interpretation of the infrared spectra.

Microbial Hydroxylations. IV. Differential Metabolism of 19-Nor Steroid Antipodes by *Curvularia lunata*[1]

YONG YENG LIN,[2] MASAYOSHI SHIBAHARA,[2] AND LELAND L. SMITH

Department of Biochemistry, University of Texas Medical Branch, Galveston, Texas 77550

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In order to explore the generality of prior findings that microbial systems hydroxylate both the natural *d* and the unnatural *l* enantiomers of racemic 19-norsteroids, the metabolic disposition of *d*- and *dl*-19-nortestosterone and its *dl*-13β-ethyl homolog by vegetative cell cultures of *Curvularia lunata* NRRL 2380 was examined. From *d*-19-nortestosterone there was obtained the *d*-10β-hydroxy, *d*-11β-hydroxy, *d*-14α-hydroxy, *d*-6β-hydroxy, and *d*-10β,11β-dihydroxy derivatives. From *dl*-19-nortestosterone there wss obtained the *d*-10β-hydroxy, *d*-14α-hydroxy, *dl*-11β-hydroxy, *dl*-6β-hydroxy, *l*-12α-hydroxy, and *dl*-10β,11β-dihydroxy derivatives together with *l*-10β,11β,17β-trihydroxy-5α-estran-3-one. From *dl*-13β-ethyl-17β-hydroxygon-4-en-3-one there was obtained the *dl*-10β-hydroxy, *l*-12α-hydroxy, *d*-14α-hydroxy, *dl*-6β-hydroxy, and *d*-6β,10β-dihydroxy derivatives. Structures of each product were established by elemental analysis, spectral behavior in ethanol, alkaline ethanol, and concentrated sulfuric acid, proton spectra, derivitization, and optical rotatory dispersion. These results support and expand on our prior similar results using *Aspergillus ochraceus* NRRL 405 on these same substrates.

The previously held notion that microbial systems metabolize only the natural *d* antipode[3] of racemic steroid preparations and reject the unnatural *l* antipode[4] is compromised by our prior findings,[5] wherein microbial hydroxylase and dehydrogenase systems were demonstrate to metabolize both the *d* and the *l* enantiomers of racemic 19-nortestosterone (*dl*-17β-hydroxyestr-4-en-3-one) (Ia) and its 13β-alkyl homologs. In order to establish the generality of the differential hydroxylation of the antipodes of racemic steroids in other microbial systems, we have examined the metbolism of natural and racemic Ia and of its racemic homolog *dl*-13β-ethyl-17β-hydroxygon-4-en-3-one (IIa) by vegetative cell cultures of *Curvularia lunata* NRRL 2380, a well-known microorganism used broadly to introduce the 11β-hydroxyl group into a wide variety of steroids.[6]

We have carefully reexamined the transformation of *d*-Ia by *C. lunata* NRRL 2380 and confirmed that the 10β-hydroxy derivative *d*-IIIa is the major product, with diminished amounts of the 11β-hydroxy, 14α-hydroxy, and 10β,11β-dihydroxy products *d*-IVa, *d*-Va, and *d*-VIa.[7] In addition to these products, we isolated the 6β-hydroxysteroid *d*-VIIa, not previously noted in these fermentations.[7] Estimation of the composition of the product mixture by gas chromatography[8] gave *d*-IIIa, 55.5% (52.0%); *d*-IVa, 17.5% (2.6%); *d*-Va, 13.3% (9.5%); *d*-VIa, 0.8% (0.5%); *d*-VIIa, 4.4% (1.4%); recovered *d*-Ia, 5.9%.

Fermentation of racemic Ia with *C. lunata* NRRL 2380 under the same conditions yielded a similar thin-layer and gas chromatographic pattern of hydroxylated products. However, fractionation of the product mixture afforded two additional minor products, VIIIa and IXa, not found in the *d*-Ia fermentations. The fortuitous superposition on thin layer chromatograms of the trace product IXa with Va and of VIIIa with IVa and VIa gave no hint of the formation of these components during fermentation, and their presence in the product mixture was noted only after isolation. Gas chromatographic analysis[8] of the product mixture from *dl*-Ia fermentation (with optical configurations given) gave *d*-IIIa, 50.3% (18.9%); *d*-Va, 7.3% (0.5%); *dl*-VIa, 10.3% (1.3%); *dl*-VIIa, 4.8% (4.3%); *l*-IXa, 4.8% (1.3%); *dl*-IVa and *l*-VIIIa, 19.7%[9] (6.1% and 1.4%); and recovered substrate, 2.8%.

The structures for the known steroids IIIa, IVa, Va, and VIIa were assigned on the basis of their spectral behavior in ethanol, in alkaline ethanol, and in concentrated sulfuric acid by systematic comparisons,[10]

(1) (a) Paper III of this series: L. Tan and L. L. Smith, *Biochim. Biophys. Acta*, **164**, 389 (1968); (b) presented in part before the 6th International Symposium on the Chemistry of Natural Products, Mexico City, April 21–25, 1969; (c) supported by funds from the Robert A. Welch Foundation, Houston, Texas.

(2) Robert A. Welch Foundation Postdoctoral Fellow, 1968–1969.

(3) The nomenclature of L. F. Fieser and M. Fieser, *Steroids*, Reinhold Publishing Corp., New York, N. Y., 1959, p 336, for the enantiomers of racemic steroids is used throughout. Structural formulas are drawn in the usual manner for the *d* enantiomer even where the racemic modification and *l* enantiomer may be involved.

(4) (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. Amer. Chem. Soc.*, **82**, 3409 (1960); (d) K. V. Yorka, W. L. Truett, and W. S. Johnson, *J. Org. Chem.*, **27**, 4580 (1962).

(5) (a) L. L. Smith, G. Greenspan, R. Rees, and T. Foell, *J. Amer. Chem. Soc.*, **88**, 3120 (1966); (b) G. Greenspan, L. L. Smith, R. Rees, T. Foell, and H. E. Alburn, *J. Org. Chem.*, **31**, 2512 (1966).

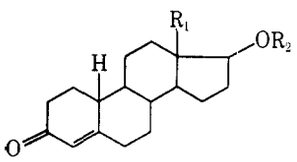
(6) (a) W. Charney and H. L. Herzog, "Microbial Transformations of Steroids, A Handbook," Academic Press, Inc., New York, N. Y., 1967, pp 383–389; (b) A. A. Akhrem and Yu. A. Titov, "Mikrobiologicheskie Transformatsii Steroidov," U. S. S. R. Academy of Sciences, Moscow, 1965, pp 215–386; (c) A. Čapek, O. Hanč, and J. Tadra, "Microbial Transformations of Steroids," Czech Academy of Sciences, Prague, 1966, p 135.

(7) J. de Flines, W. F. van der Waard, W. J. Mijs, and S. A. Szpilfogel, *Rec. Trav. Chim.*, **82**, 129 (1963).

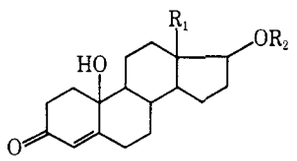
(8) The composition figures are not absolute yields of product based on substrate but are the relative proportions for each component in the crude product mixture. The yield figures in parenthesis following each gas chromatographic proportion are actual isolated yields for each component, based on substrate.

(9) Resolution of *dl*-IVa and *l*-VIIIa was not achieved on 3% QF-1 columns. Accordingly, the value of 19.7% represents both components together.

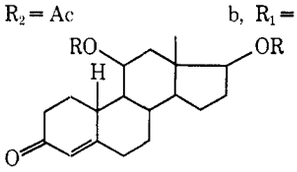
(10) (a) L. L. Smith, *Steroids*, **1**, 570 (1963); (b) L. L. Smith, *Texas Rep. Biol. Med.*, **24**, 674 (1966).



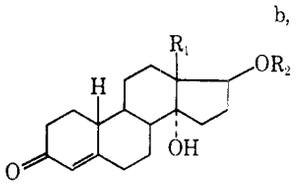
Ia, R_1 = Me, R_2 = H
b, R_1 = Me, R_2 = Ac
IIa, R_1 = Et, R_2 = H
b, R_1 = Et, R_2 = Ac



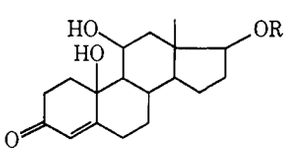
IIIa, R_1 = Me, R_2 = H
b, R_1 = Me, R_2 = Ac
XIVa, R_1 = Et, R_2 = H
b, R_1 = Et, R_2 = Ac



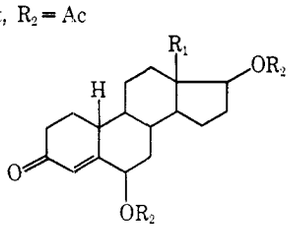
IVa, R = H
b, R = Ac



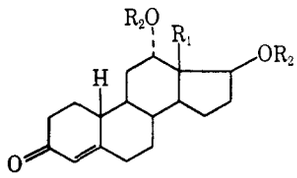
Va, R_1 = Me, R_2 = H
b, R_1 = Me, R_2 = Ac
XVa, R_1 = Et, R_2 = H
b, R_1 = Et, R_2 = Ac



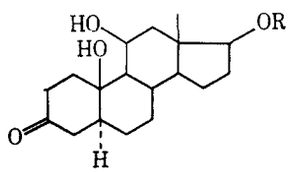
VIa, R = H
b, R = Ac



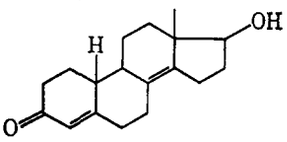
VIIa, R_1 = Me, R_2 = H
b, R_1 = Me, R_2 = Ac
XVIa, R_1 = Et, R_2 = H
b, R_1 = Et, R_2 = Ac



VIIIa, R_1 = Me, R_2 = H
b, R_1 = Me, R_2 = Ac
XVIIa, R_1 = Et, R_2 = H
b, R_1 = Et, R_2 = Ac



IXa, R = H
b, R = Ac



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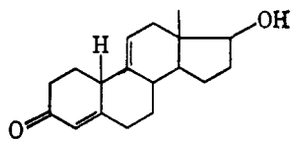

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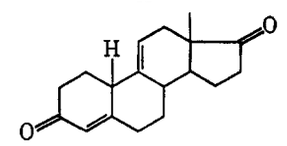

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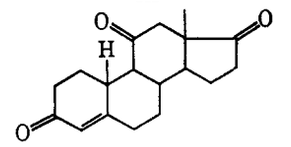
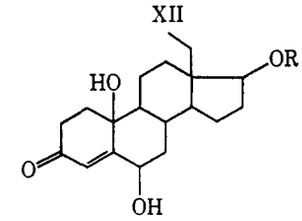
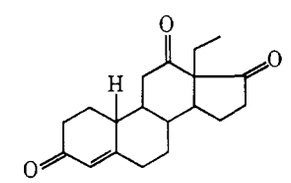


XIII



XVIIIa, R = H
b, R = Ac



XIX

and by comparison with authentic samples where possible. Each hydroxysteroid possessed unique characteristics of spectra such that an unambiguous assignment of structure was obtained.

The 14α-hydroxysteroid structure of Va assigned by de Flines, *et al.*,[11] was supported by proton spectra. A moderate paramagnetic shift of 0.1 ppm in the position of the C_{18}-methyl proton signal and of 0.6 ppm in the chemical shift of the 17α proton (relative to *d*-Ib), attributed to the microbially introduced tertiary hydroxyl group, can be reconciled only with a 14α-hydroxysteroid formulation.[12] Furthermore, dehydration of *d*-Va with acid gave a dienone X to which the structure *d*-17β-hydroxyestra-4,8(14)-dien-3-one was assigned, based on comparison with a racemic reference sample.[13]

The structures of products IVa as 11β-hydroxylated derivatives of Ia follow by analogy with the work of de Flines, *et al.*,[11] and from formation of a trione XIII and of the diacetates IVb, whose spectral properties are consistent with the 11β-hydroxy formulation.[14]

Additionally, proton spectra of IVb exhibited the C_{18}-methyl proton signal shifted downfield by 0.08 ppm; this deshielding effect approached that of 11β-acetoxyl-substituted steroids.[12,15]

The optical rotatory dispersion curves of IIIa and Va derived from *dl*-Ia were essentially superimposable on that of *d*-Ia, thus demonstrating the *d* configuration of these two products, whereas the dispersion curves for IVa and VIIa derived from *dl*-Ia were flat and featureless, thus establishing the racemic nature of these two products.

The 10β,11β-dihydroxysteroid structure for VIa was assigned without evidence by de Flines, *et al.*,[7] to an uncharacterized triol obtained from *d*-Ia in *C. lunata* NRRL 2380 fermentations. We have obtained the same triol from *d*-Ia fermentations and a racemic modification, as evinced by a flat and featureless optical rotatory dispersion curve, from *dl*-Ia fermentations. Both products VIa were obtained in low yields and a fully satisfying structural assignment cannot be offered. The triol *dl*-VIa formed a 17β-monoacetate, *dl*-VIb, thus indicating the tertiary or hindered secondary nature of the two microbially introduced hydroxyl groups, one of which must be at the 10β position in view of the hypsochromic shift in ultraviolet light absorption of 4 mμ[10a] and spectral behavior in concentrated sulfuric acid charactristic of the $\Delta^{4,9}$-3-ketone system.[10b] Thus, three ditertiary (8β,10β, 9α,10β, and 10β,14α) combinations and one tertiary-hindered secondary

(11) J. de Flines, W. F. van der Waard, W. J. Mijs, L. A. van Dijck, and S. A. Szpilfogel, *Rec. Trav. Chim. Pays-Bas*, **82**, 149 (1963).
(12) L. L. Smith, *Steroids*, **4**, 395 (1964).
(13) W. F. Johns, *J. Org. Chem.*, **31**, 3780 (1966).
(14) S. M. Fox, V. E. Origoni, and L. L. Smith, *J. Amer. Chem. Soc.*, **82**, 2580 (1960), and references cited therein.
(15) Y. Kawazoe, Y. Sato, M. Natsume, H. Hasegawa, T. Okamoto, and K. Tsuda, *Chem. Pharm. Bull.*, **10**, 338 (1962).

(10β,11β) possibility need to be considered for VIa structures.[16]

Microbial 8β hydroxylation is of rare occurrence[17] and totally undescribed for *C. lunata* strains.[6] Although this sort of argument is of dubious merit in these matters, this improbability, together with a lack of spectral evidence for 8β hydroxylation,[18] leads us to discount this possibility. The strong infrared interactions shown by *d*-VIa and *dl*-VIa in the hydroxyl region would not be expected of *trans*-diols such as 9α,10β- and 10β,14α-diols, and we reject these possibilities accordingly. We therefore suggest the 10β,11β-dihydroxy structure for *d*-VIa and *dl*-VIa.

The minor product VIIIa could not be obtained in crystalline form, but its diacetate VIIIb was fully characterized. Location of the newly introduced hydroxyl group at a secondary C- or D-ring site was established by formation of the diacetate VIIIb and by consideration of spectra in ethanol, alkaline ethanol, and sulfuric acid.[10] Optical rotatory dispersion spectra of VIIIa appeared as a mirror image of dispersion spectra of *d*-Ia; therefore, the optical configuration of VIIIa is the unnatural *l* configuration. Nonidentity of the diacetate *l*-VIIIb with the previously described 11α,17β-diacetoxy,[20a] 12β,17β-diacetoxy,[20b] 16α,17β-diacetoxy,[20c] and 16β,17β-diacetoxy[20c] derivatives of I left as possibilities the 12α,17β-, 15α,17β-, and 15β,17β-diacetoxy derivatives.

Proton spectra of *l*-VIIIb, though inadequate for complete structure assignment, suggest only the 12α,17β- and 15α,17β-diacetoxy structures. The C_{18}-methyl proton signal at 0.94 ppm is only slightly shifted downfield (0.06 ppm) from that of *d*-Ib,[21] thus eliminating 12β-, 15β-, and 16β-acetoxy structures.[12] The signals from the proton on carbon geminal to the newly introduced acetoxyl group and the 17α proton both appear as multiplets, unresolved from one another at 4.9 ppm. The indicated deshielding of the 17α proton by about 0.3 ppm[22] is characteristic of the anisotropic effect of an acetoxyl group located *cis*-1,3 diaxially from the 17α proton, thus at the 12α or 15α position.[23] The proton on carbon bearing the unassigned (12α- or 15α-) acetoxyl group gave a poorly recognized signal unresolved from background and from the signal of the 17α proton, with an indicated chemical shift in the 4.9–5.0-ppm vicinity. Although the geminal proton in 15α-acetoxysteroids is found in this range (4.94–5.13 ppm)[12] and the geminal proton for 12α-acetoxysteroids is at lower field (5.37[12] and 5.25 ppm for *l*-XVIIb herein), we do not regard the indicated chemical shift for the said proton as reliable for the present structural assignment. A diminishing sample obviated further structure studies of *l*-VIIIb.

The structure of *l*-VIIIb as a 12α-acetoxyl derivative of *l*-Ib is reasonably formulated on the basis of analogy with the major 12α-hydroxy product *l*-XVIIa and its diacetate *l*-XVIIb formed in *C. lunata* fermentations on *dl*-IIa. Thin layer gas chromatographic mobilities of the two products *l*-VIIIb and *l*-XVIIb and of their parent alcohols *l*-VIIIa and *l*-XVIIa were sufficiently closely related such that a homolog relationship could be surmised. Thus, although 12α hydroxylation by *C. lunata* strains has not been previously demonstrated and 15α hydroxylation has been,[25] we presently regard *l*-VIIIa as a 12α-hydroxylated derivative of *l*-Ia.[26]

The minor product, IXa, was recognized to be a saturated 3-ketosteroid from its absorption at 1700 cm^{-1} with no selective absorption in the 240-mμ region. Under mild acetylation conditions, IXa formed a dihydro-17β-monoacetate, IXb, which retained strong absorption at 3450 and 3350 cm^{-1}, thus demonstrating the presence of two unacylable hydroxyl groups. Spectra of IXa in concentrated sulfuric acid resembled in detail spectra obtained from $\Delta^{4,9}$-3-ketones.[10b]

Prior experience with 9α-, 10β-, and 11α-hydroxy-19-nor-Δ^4-3-ketones in concentrated sulfuric acid suggests the ready formation of the $\Delta^{4,9}$-3-ketone system and the ready isomerization of the $\Delta^{5(10)}$ double bond in $\Delta^{5(10)}$-3-ketones to the conjugated Δ^4-3-ketone system.[10b] The present spectral data on 11β-, 12α-, and 14α-hydroxy-19-nor-Δ^4-3-ketones likewise suggests that these hydroxylated steroids be dehydrated in acid with the formation of a double bond which isomerizes ultimately to afford the $\Delta^{4,9}$-3-ketone system. Thus, 9α-, 10β-, 11α-, 11β-, 12α-, and 14α-hydroxy derivatives of 19-nor-Δ^4-3 ketones appear to give spectra in concentrated sulfuric acid characteristic of $\Delta^{4,9}$-3-ketones, whereas 1β-, 2α, 2β-, and 6β-hydroxy-Δ^4-3-ketones do not.[10b] Such behavior offers a rapid differentiation between the two groups of hydroxy-Δ^4-3-ketones.

Spectra in concentrated sulfuric acid characteristic of the $\Delta^{4,9}$-3-ketone system for the saturated 3-ketone IXa may be visualized as arising through removal in acid of one of the microbially introduced hydroxyl groups, located at the A/B ring juncture, with possible isomerization of the unsaturation thereby produced, to

(16) The 10β,11β-dihydroxy formulation recognizes the interaction between the 10β- and 11β-hydroxyl groups, as evinced by infrared absorption data, and also the steric hindrance of the 10β-hydroxyl on the 11β-hydroxyl group, the 10β-hydroxyl taking the place of the 19-angular methyl group in the androstanes and pregnanes where the 11β-hydroxyl is sufficiently hindered so as not to react with acetic anhydride–pyridine.[14] It is also assumed that these microbial hydroxylations do not involve inversion of configuration at the sites hydroxylated.

(17) (a) E. Kondo, K. Morihara, Y. Nozaki, and E. Masuo, *J. Agr. Chem. Soc. Jap.*, **34**, 844 (1960); (b) K. Tori and E. Kondo, *Tetrahedron Lett.*, 645 (1963).

(18) 8β-Hydroxy-Δ^4-3-ketones were not included in the prior systematic treatment of spectra.[10a] More recent publication of data for this previously undescribed steroid system established that a bathochromic shift of 2–3 mμ is associated with introduction of the 8β-hydroxyl group into a Δ^4-3-ketone system.[17,19]

(19) T. Kubota and M. Ehrenstein, *J. Org. Chem.*, **29**, 345, 351 (1964).

(20) (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. Amer. Chem. Soc.*, **78**, 1512 (1956); (b) J. de Flines, W. F. van der Waard, W. J. Mijs, L. A. van Dijck, and S. A. Szpilfogel, *Rec. Trav. Chim. Pays-Bas*, **82**, 139 (1963); (c) J. de Flines, W. F. van der Waard, W. J. Mijs, and S. A. Szpilfogel, *ibid.*, **82**, 121 (1963).

(21) Signals for *d*-Ib recorded under the same conditions as used for *l*-VIIIb include 0.88 (C_{18}-methyl protons), 2.02 (acetate protons), 4.59 (m, 17α proton), and 5.77 ppm (C_4-vinyl proton).

(22) The deshielding effect was calculated using the 4.59-ppm value for the 17α-proton signal in *d*-Ib spectra, recorded at the same time as spectra of *l*-VIIIb. The 17α-proton signal for 17β-acetoxysteroids appears variously between 4.46 and 5.11 ppm.[12]

(23) A deshielding effect by the 15α-acetoxyl group of *ca.* 0.6 ppm on the 17α proton and of *ca.* 0.08 ppm on the C_{18}-methyl protons of estra-1,3,5(10)-triene-3,15α,16α,17β-tetraol tetracetate[24a] *vs.* estra-1,3,5(10)-triene-3,16α,-17β-triol triacetate[24b] is indicated. Similar deshielding of the 17α proton by the 12α-acetoxyl group does not appear to be recorded in the literature.

(24) (a) J. Fishman and H. Guzik, *J. Org. Chem.*, **33**, 3133 (1968); (b) M. Neeman and Y. Hashimoto, *J. Amer. Chem. Soc.*, **84**, 2972 (1962).

(25) L. Canonica, U. Valcavi, and C. Scolastico, *Gazz. Chim. Ital.*, **93**, 368 (1963).

(26) Although *d*-15α,17β-dihydroxyestr-4-en-3-one has been described [*cf.* J. de Flines, W. F. van der Waard, W. J. Mijs, and S. A. Szpilfogel, *Rec. Trav. Chim. Pays-Bas*, **82**, 143 (1963)], the 15α,17β-diacetate is undescribed and no direct comparison of samples has been made.

afford the conjugated Δ^4-3-ketone system, whereas the other microbially introduced hydroxyl group is similarly eliminated, with concomitant isomerization and conjugation of the second unsaturation to give the $\Delta^{4,9}$-3-ketone system. This spectral and chemical behavior of IXa thus implied that the microbially introduced unacylable hydroxyl groups were at tertiary or hindered secondary sites along the steroid "backbone" or immediately adjacent to it. For formation in sulfuric acid of a $\Delta^{5(10)}$-3-ketone system which would isomerize to the Δ^4-3-ketone, a hydroxyl at the 10β position is indicated. By arguments similar to those adduced for the structure of *d*-VIa and *dl*-VIa, the most probable structure for IXa becomes a 10β,14α- or a 10β,11β-dihydroxy-4,5-dihydro derivative of Ia.

Dehydration of IXa with hydrogen chloride in acetic acid gave an unsaturated ketone XI, recognized as a Δ^4-3-ketone by its absorption at 241 mμ. Chromic acid oxidation of XI yielded the unsaturated dione XII. The absence of either microbially introduced hydroxyl group in XII was indicated by its infrared absorption spectrum, and thin layer and gas chromatographic properties of XI were very similar, though not identical, with those of the 4,8(14)-dien-3-one *dl*-X.[13] Since the 14α-hydroxyl group in *d*-Va is eliminated under these conditions to form *d*-X, and XI is not identical with X, 14α-hydroxyl substitution in IXa is not indicated. The dehydration product XI is probably the 4,9(11)-dien-3-one-17β-hydroxyestra-4,9(11)-dien-3-one, formed by elimination of the 10β-hydroxyl group and migration of the double bond, thereby formed into the conjugated Δ^4-3-ketone feature, with concomitant elimination of the 11β-hydroxyl group to form the 4,9(11)-diene XI, not isomerized under these conditions to the $\Delta^{4,9}$-3-ketone system. From these several evidences, we reject structures other than the 4,5-dihydro-10β,11β,-17β-triol structure of IXa.

The dihydroxyketone IXa was correlated with the 10β,11β-dihydroxy product VIa by catalytic hydrogenation of *dl*-VIa, reduction of which gave a mixture of 5-epimeric 3-ketones together with nonketonic (overreduced) material. Gas chromatography of the mixed epimers resolved two well-separated components recognized as the 5 epimers. The more rapidly eluted component was assigned the 5β-3-ketone structure, in keeping with prior experience with the order of elution of the 5-epimeric 3-ketones of the androstane and pregnane series.[27] The more slowly eluted component was therefore the 5α epimer, and the coincidence of the retention times of this 5α component with that of IXa from the *C. lunata* fermentation demonstrated the identity of these two components and thereby the 5α configuration of IXa.

A strong negative Cotton effect of amplitude 1557° was present in the optical rotatory dispersion curve of IXa. The shape of the dispersion curve appeared to be a mirror image of the dispersion curves of *d*-5α-3-keto steroids widely published in the literature,[28] where strong positive Cotton effects of amplitude *ca.* 1600–1700° are characteristic. Epimeric 5β-3-keto steroids regularly exhibit less pronounced negative Cotton effects of diminished amplitude (*ca.* 650–800°).[28,29] Since the dispersion curves of 19-nor-3-ketones differ from those of related androstane-, pregnane-, and cholestane-3-ketones only in regard to slightly increased amplitudes,[30] the structure of IXa as the unnatural *l*-5α-3-ketone was determined.

The origin of the saturated ketone *l*-IXa as a true metabolite of *dl*-Ia is suggested by the relatively high proportion (4.8%) of IXa in the tranformation product mixture and by the isolation yield of 1.3%, in excess of the 1% level of contamination of the racemic substrate allowed by extensive gas, thin layer chromatographic, and spectral analyses. The possibility of overreduced material from the lithium–liquid ammonia reduction step in the total synthesis of *dl*-Ia being carried over into our microbial fermentations may be discounted, and the presence of an *l*-steroid Δ^4-reductase in *C. lunata* NRRL 2380 accordingly is demonstrated. Whether the saturated ketone *l*-IXa be derived from its Δ^4-3-ketone analog *dl*-VIa formed in greater amounts in the fermentation of *dl*-Ia or whether any metabolic relationship obtains between *l*-IXa and *dl*-VIa remains unsettled.

All of the optically active hydroxy-Δ^4-3-ketones derived from the attack of *C. lunata* NRRL 2380 on *dl*-Ia possessed dispersion curves of the same shape as *d*-Ia, with the noted exception of *l*-VIIIa, where a mirror-image shape obtained. Although the structures assigned to the minor products *l*-VIIIa and *l*-IXa must be considered as tentative, their unnatural *l* configurations are secure, and the differential metabolism of the *d* and *l* antipodes in these studies is accordingly established.

The 13β-ethyl homolog *dl*-IIa was transformed by *C. lunata* into a spate of metabolites very similar to that obtained with *dl*-Ia. The gas chromatographic pattern of transformation products from *dl*-IIa was almost exactly like that from *dl*-Ia except that the homologous products XIVa, XVa, XVIa, and XVIIa were retained on column slightly longer than the estrane derivatives IIIa, Va, VIIa, and VIIIa. However, it was apparent that no homolog of the 11β-hydroxysteroid *dl*-IVa was formed from *dl*-IIa, and, although a dihydroxylated product XVIIIa was present, a 5α-dihydro transformation product therefrom derived was not. From gas chromatographic analysis of the transformation product mixture, there was obtained[8] *dl*-XIVa, 30.2% (16.7%); *d*-XVa, 14.2% (4.8%); *dl*-XVIa, 6.5% (0.8%); *l*-XVIIa, 38.5% (14.3%); *d*-XVIIIa, 1.7% (0.3%); recovered *dl*-IIa, 8.8% (3.5%).

The structure of XIVa as the 10β-hydroxylated product followed from its formation of a 17β-monoacetate XIVb and from spectral properties of the two derivatives in ethanol and in concentrated sulfuric acid. The 10β-hydroxysteroids XIVa and XIVb matched in their chromatographic properties those of the homologous 10β-hydroxysteroids *d*-IIIa and *d*-IIIb and in their other physical properties with *d*-XIVa and *d*-XIVb previously described.[5a] Optical rotatory dispersion

(27) The 5β epimer in a number of androstane and pregnane 3 ketones is eluted before the 5α epimer on QF-1 columns; *cf.* (a) I. S. Hartman in "Gas Chromatography in the Analysis of Steroid Hormones," H. H. Wotiz and S. J. Clark, Ed., Plenum Press, New York, N. Y., 1966, pp 108–112; (b) G. H. Thomas in "The Gas–Liquid Chromatography of Steroids," J. K. Grant, Ed., Cambridge University Press, Cambridge, 1967, pp 134–136.

(28) (a) C. Djerassi, "Optical Rotatory Dispersion, Applications to Organic Chemistry," McGraw-Hill Book Co., Inc., New York, N. Y., 1960, pp 49–51; (b) C. Djerassi, *Rec. Chem. Progr.*, **20**, No. 3, 101 (1959); (c) C. Djerassi, *Proc. Chem. Soc.*, 314 (1964).

(29) D. C. DeJongh, J. D. Hribar, P. Littleton, K. Fotherby, R. W. A Rees, T. J. Foell, and H. Smith, *Steroids*, **11**, 649 (1968).

(30) C. Djerassi, O. Halpern, V. Halpern, and B. Riniker, *J. Amer. Chem. Soc.*, **80**, 4001 (1958).

established that the product was racemic. In this respect, formation of the racemic *dl*-XIVa differs from our present results with *C. lunata* on *dl*-Ia where only the *d*-10β-hydroxy product *d*-IIIa was obtained and with our prior work with both *dl*-Ia and *dl*-IIa with *Aspergillus ochraceus* NRRL 405 where *l*-IIIa and *d*-XIVa were obtained.[5a]

Although obtained only as a minor product, the *d*-14α-hydroxylated product XVa was the major steroid product of *d* configuration observed in these studies. The structure of *d*-XVa as the 14α-hydroxy homolog of Va was assigned on the basis of thin-layer and gas chromatographic and spectral comparisons with *d*-Va and on the basis of formation of a monoacetate XVb. As in the case of *d*-Vb, proton spectra of XVb showed the 17α-proton to be deshielded by 0.53 ppm. Optical rotatory dispersion of XVb showed a negative Cotton effect typical of steroid Δ^4-3-ketones of the natural *d* configuration.

Although major amounts of a transformation product of unnatural *l* configuration were not recovered from *dl*-Ia fermentations, a major *l* product (*l*-XVIIa) was obtained in fermentation of *dl*-IIa, the *l* configurational assignment being made on the basis of the positive cotton effect and mirror image appearance of the optical rotatory dispersion spectrum. The product formed a diacetate, *l*-XVIIb, absorbed light a 241 mμ, and gave a spectrum in concentrated sulfuric acid which was different from those of 10β-, 11α-, 11β-, or 14α-hydroxy analogs. After 24 hr, the spectrum resembled that of the $\Delta^{4,9}$-3-ketone system. These points indicated hydroxyl substitution in *l*-XVIIa at a secondary position remote from the Δ^4-3-ketone chromophore, with the 12 and 15 positions being most probable. Chromic acid oxidation of *l*-XVIIa gave a trione, *l*-XIX, whose characteristic absorption at 1760, 1700, 1670, and 1610 cm^{-1} clearly suggested that the newly introduced hydroxyl group in XVIIa was located in a six-membered ring, thus at the 12 position. The 1700-cm^{-1} absorption of *l*-XIX was of diminished intensity, considerably weakened by interaction with the 17-ketone group. These effects have been noted in 3,12,17-triones of the estrane and androstane series[20b] and are suitable for an assignment of the 12,17-diketone structure to *l*-XIX. Thus, a 12-hydroxyl was indicated for *l*-XVIIa. The 12α configuration was assigned from proton spectra of the diacetate *l*-XVIIb, where the 17α-proton was deshielded by 0.3 ppm in comparison with the 17α proton of *dl*-IIb. This paramagnetic deshielding may be attributed to the anisotropic effect of the 12α-acetoxyl group on the nearby *cis*-1,3-diaxial 17α proton. The 12-proton signal in *l*-XVIIb at 5.25 ppm, while not well resolved into a triplet as required of 12α-acetoxysteroids,[31] had a half-width of *ca.* 7 Hz, which signal shape and frequency[12] define an equatorial 12β-proton and thus an axial 12α-acetoxyl group in *l*-XVIIb.

Trace products from the fermenation of *dl*-IIa included the 6β-hydroxysteroid XVIa, recognized as such on the basis of formation of a diacetate *dl*-XVIb, by which means the product was recovered from the fermentation. Spectral behavior of *dl*-XVIb in ethanol, in alkaline ethanol, and in concentrated sulfuric acid defined the 6β-acetoxy-Δ^4-3-ketone system suitably, and comparison of chromatographic properties between *dl*-XVIb and *dl*-VIIb suggested the homolog nature of their relationship. Comparison of the properties of *dl*-XVIb with those of *dl*-XVIb previously described[5a] also supported the assigned structure. Proton spectra of *dl*-XVIb showed the C_4-vinyl proton at a deshielded position, at 0.18 ppm lower field than the C_4-proton of *dl*-IIb. Optical rotatory dispersion spectra established the racemic nature of *dl*-XVIb and therefore of *dl*-XVIa.

The other trace product, *d*-XVIIIa, recovered from the fermentation of *dl*-IIa as the 17β-monoacetate *d*-XVIIIb, was recognized as a dihydroxylated product on the basis of chromatographic behavior. The chromatographic properties of *d*-XVIIIb and *dl*-VIb suggested that they were not homologs, although the chromatographic properties of the parent alcohols *d*-XVIIIa and *dl*-VIa indicated their very close structural relationship. On the basis of a strong hypsochromic shift in the ultraviolet-light absorption to 233 mμ, spectra in concentrated sulfuric acid characteristic of a $\Delta^{4,9}$-3-ketone and strong interactions in the hydroxyl absorptions in the solution infrared spectrum, it is suggested that *d*-XVIIIb contain a 10β-hydroxyl group and a second hydroxyl group *cis* to it and positioned so as to add strain to the A-ring Δ^4-3-ketone chromophore. Of the possibilities (1β,10β, 2β,10β, 6β,10β, 7β,10β, 8β,10β), only the 6β,10β-dihydroxy feature is consistent with the 233-mμ absorption.[32] Furthermore, spectra in alkaline ethanol were consistent with the presence of a 6-hydroxy-Δ^4-3-ketone system in *d*-XVIIIb. Optical rotatory dispersion of the trace product XVIIIb established the natural configuration. On these bases, we assign to XVIIIa the *d*-6β,10β-dihydroxy structure.

The resistance to acetylation under the usual mild conditions with acetic anhydride and pyridine, as evinced by the behavior of the 10α,11β-dihydroxysteroids *dl*-VIa and *l*-IXa and the 6β,10β-dihydroxysteroid *d*-XVIIIa, must be attributed to the strong interactions between the *cis*-1,3-diaxial hydroxyl groups. Although the mere presence of the 10β-hydroxyl group as a steric factor simulating the steric hindrance of the C_{19}-methyl group of the androstane and pregnane series may account for the failure to acetylate the 11β-hydroxyl group in *dl*-VIa and *l*-IXa, the 6β-hydroxyl group in androstanes and pregnanes is generally readily acetylated despite any hindrance or interaction between it and the angular methyl group *cis*-1,3 diaxial to it in the B ring.

These studies with *C. lunata* constitute another example of the differential metabolism of 19-nor steroids by microorganisms to afford *d*-, *l*-, and *dl*-hydroxylated products.[33] As in our prior example of differential

(31) K. Tori and E. Kondo, *Steroids*, **4**, 713 (1964); *Nippon Kagaku Zasshi*, **87**, 1117 (1966).

(32) Hypsochromic effects associated with introduction of hydroxyl groups into 19-nor-Δ^4-3-ketones include 10β, *ca.* 4 mμ; 6β, *ca.* 3 mμ; 11β, −1 mμ (based on derivatives described herein and in our prior report).[5a] The 233-mμ maximum of *d*-XVIIIb may be viewed as a consequence of the additive effects of the two hydroxyls, which situation also obtains for the 10β,11β-diol *dl*-VIb.

(33) A trivial instance of 21 hydroxylation of both *d* and *l* antipodes of a racemic progesterone derivative by *Aspergillus niger* NRRL 599 had been reported; *cf.* B. Gadsby, M. R. G. Leeming, G. Greenspan, and H. Smith, *J. Chem. Soc., C*, 2647 (1968). Other recent microbial hydroxylation to form racemic hydroxylated products includes instances of steroidlike tricyclic ketones[34a] and azabicycloalkanes.[34b]

(34) (a) S. J. Daum, M. M. Riano, P. E. Shaw, and R. L. Clarke, *J. Org. Chem.*, **32**, 1435 (1967); (b) R. A. Johnson, M. E. Herr, H. C. Murray, L. M. Reineke, and G. S. Fonken, *ibid.*, **33**, 3195 (1968).

metabolism of racemic 19-nor steroids with *A. ochraceus*, where hydroxylations at the 1β, 6β, 10β, and 11α positions were obtained,[5a] hydroxylations at the 6β, 10β, 11β, 12α, and 14α positions were obtained with *C. lunata*. In both instances, fermentation of the *d* substrate gave a different product pattern than obtained with the racemic substrate *dl*-Ia. With *A. ochraceus*, a 17β-hydroxysteroid alcohol dehydrogenase activity was obtained in *d*-Ia fermentations[5a,7] but not in *dl*-Ia fermentations, whereas with *C. lunata*, a steroid Δ^4-reductase activity was exhibited with the racemic substrate *dl*-Ia but not with the natural *d*-substrate *d*-Ia.

As in the case of *A. ochraceus* fermentations, the *l*-estrane antipode of the *dl*-Ia substrate proved to be a relatively poor substrate in *C. lunata* fermentations. Whereas over 50% of the *d* enantiomer of *dl*-Ia was recovered as hydroxylated products (represented by *d*-IIIa, *d*-Va, *dl*-IVa, *dl*-VIa, and *dl*-VIIa), only 17% of the *l* enantiomer was so recovered (as *l*-VIIIa, *l*-IXa, *dl*-IVa, *dl*-VIa, and *dl*-VIIa). However, in the case of the 13β-ethyl homolog IIa, the *l* enantiomer became the better substrate, with 46% being recovered as the products *l*-XVIIa, *dl*-XIVa, and *dl*-XVIb, whereas the *d* enantiomer of *dl*-IIa was recovered in 27% yield as *d*-XVa, *dl*-XIVa, *dl*-XVIb, and *d*-XVIIIb, The unmetabolized substrate recovered from fermentation of the 13β-ethylgonane *dl*-IIa was racemic, as was the case in *A. ochraceus* metabolism of *dl*-IIa reported previously.[5a]

Still other comparisons between our prior results with *A. ochraceus* and our present results with *C. lunata* are warranted. Although the major *d* product from *A. ochraceus* metabolism of *dl*-Ia was the 11α-hydroxy product *d*-11α,17β-dihydroxyestr-4-en-3-one, only trace amounts of 11α hydroxylation were obtained with the 13β-ethylgonane homolog *dl*-IIa as substrate. With *C. lunata*, the major *d* product pathway of 10β hydroxylation obtained with *dl*-Ia as substrate was retained when *dl*-IIa was employed as substrate, albeit with racemic product *dl*-XIVa resulting. Again in exact analogy with our prior work, a trace *l* product (*l*-1β-hydroxy with *A. ochraceus*, *l*-12α-hydroxy with *C. lunata*) obtained from the racemic estrane substrate *dl*-Ia became a major *l* product with *dl*-IIa as substrate.

The question whether multiple monohydroxylations of a given steroid substrate by a microorganism result from multiple, individual, highly specific hydroxylases acting independently from one another to give a unique spate of hydroxysteroid products or whether a singe hydroxylase of diminished specificity is involved has been put forth previously.[5a,35] Instances of distinct multiple hydroxylase systems acting independently of one another have been demonstrated,[36] but the many subtle differences in product nature and distribution in the present *C. lunata* and in prior *A. ochraceus*[5a] studies of *d* and *dl* substrates of the estrane and 13β-ethylgonane series support the provocative concept of aberrant binding of the *l* enantiomer on the hydroxylase in a geometry not identical with that assumed by the *d* enantiomer, and of the 13β-ethylgonane homolog in a position not precisely that taken by the estrane substrate, with resultant differential hydroxylation obtaining. Consistent discrimination of the hydroxylase system of *C. lunata* to provide 14α-hydroxy products only of the *d* configuration (*d*-Va, *d*-XVa) and 12α-hydroxy products solely of the *l* configuration (*l*-VIIIa, *l*-XVIIa) suggests that these hydroxylations arise from separate hydroxylase systems from those involved in 6β, 10β, and 11β hydroxylations, where mixed optical configurations of hydroxysteroid products obtain.

Some success has been achieved by others in attempting to map the binding or active sites of microbial reductases and dehydrogenases as they act on steroids and steroidlike molecules.[37] Indeed, some appeal obtains in use of the composite *d*- and *l*-steroid structure devised by Ringold, *et al.*,[37b] to demonstrate the equivalence of certain positions in *d* and *l* antipodes. In this matter, the *d*-14α and *l*-12α positions appear to be equivalent, so that aberrant binding of the *l* antipode on the *d* antipode's nominal binding site for 14α hydroxylation might result in 12α hydroxylation. However, work on cell-free microbial hydroxylase systems, necessary to a rigorous treatment of these matters, has not progressed to the point where such studies may be satisfactorily carried out. In the few instances where cell-free microbial hydroxylases have been described,[38,39] it appears that a separation of hydroxylase activities has not been achieved, the 10β- and 11β-hydroxylase activities of *C. lunata* NRRL 2380 on 19-nor steroids and the 11β- and 14α-hydroxylase activities on androstanes and pregnanes co-occurring in the cell-free systems.[38b] Such limitations notwithstanding, and on the assumption that a single enzyme hydroxylates a substrate in all positions, several concepts of the steric requirements of substrate–enzyme interaction have been set forth and disputation given for microbial hydroxylation of steroids,[40a] monocyclic alcohols,[40b] and a variety of cyclic nitrogenous compounds.[40c]

Formation of a racemic 10β,11β-dihydroxylated product *dl*-VIa from *dl*-Ia, in the face of formation of a *d*-10β-monohydroxylated product *d*-IIIa from the same racemic substrate, and formation of a *d*-6β,10β-dihydroxylated product *d*-XVIIIa from *dl*-IIa at the

(35) A similar suggestion was made in regard to formation of 1β and 11α hydroxylations in certain *Absidia orchidis* fermentations; *cf.* V. Schwarz, M. Ulrich, and K. Syhora, *Steroids*, **4**, 645 (1964).

(36) Strain selection and medium differences afforded a separation between 2β- and 16α-hydroxylase activity in *Streptomyces roseochromogenus* ATCC 3347; *cf.* (a) L. L. Smith, H. Mendelsohn, T. Foell, and J. J. Goodman, *J. Org. Chem.*, **26**, 2859 (1961); (b) J. J. Goodman and L. L. Smith, *Appl. Microbiol.*, **9**, 372 (1961); (c) L. L. Smith, H. Mendelsohn, and J. J. Goodman, U. S. Patent 3,063,989 (Nov 13, 1962). Furthermore, nutritional differences may be demonstrated for the 6β- and 11α-hydroxylases of *A. ochraceus* NRRL 405; *cf.* (d) E. L. Dulaney, E. O. Stapley, and C. Hlavac, *Mycologia*, **47**, 464 (1955); and, for the 1β- and 7β-hydroxylases of *Absidia orchidis*, *cf.* (e) Y. Nazaki, *Agr. Biol. Chem.* (Tokyo), **25**, 884 (1961). Other examples also obtain.

(37) (a) V. Prelog, *Pure Appl. Chem.*, **9**, 119 (1964); (b) H. J. Ringold, J. M. H. Graves, A. Clark, and T. Bellas, *Recent Progr. Hormone Res.*, **23**, 349 (1967); "Proceedings of the Second International Congress on Hormonal Steroids," Milan, May 23–28, 1966; L. Martini, F. Fraschini, and M. Motta, Ed., "Excerpta Medica Foundation," Amsterdam, 1967, pp 219–226.

(38) (a) M. H. J. Zuidweg, W. F. van der Waard, and J. de Flines, *Biochim. Biophys. Acta*, **58**, 131 (1962); (b) M. H. J. Zuidweg, *ibid.*, **152**, 144 (1968); (c) J. E. Wilson and C. S. Vestling, *Arch. Biochim. Biophys.*, **110**, 401 (1965); (d) F. N. Chang and C. J. Sih, *Biochemistry*, **3**, 1551 (1964); (e) L. M. Kogan and E. A. Yelin, Abstracts, 2nd International Congress on Hormonal Steroids, Milan, **3**, 233 (1966); E. A. Elin and L. M. Kogan, *Dokl. Akad. Nauk* SSSR, **167**, 1175 (1966).

(39) We have recently prepared cell-free systems from *A. ochraceus* NRRL 405 which 11α hydroxylates progesterone and 19-nortestosterone; M. Shibahara, J. A. Moody, and L. L. Smith, unpublished observations.

(40) D. R. Brannon, F. W. Parrish, B. J. Wiley, and L. Long, *J. Org. Chem.*, **32**, 1521 (1967); (b) G. S. Fonken, M. E. Herr, H. C. Murray, and L. M. Reineke, *J. Amer. Chem. Soc.*, **89**, 672 (1967); (c) R. A. Johnson, M. E. Herr, H. C. Murray, and G. S. Fonken, *J. Org. Chem.*, **33**, 3217 (1968).

same time that racemic 6β- and 10β-monohydroxy products (*dl*-XVIa and *dl*-XIVa) are formed, suggests that these minor steroid triols be formed by other enzymes or by other hydroxylation mechanisms.

The 10β,11β,17β-triol *d*-VIa could not be demonstrated when *d*-IIIa was subjected to the action of *C. lunata* under standard conditions, nor could *dl*-VIa be demonstrated as deriving from *dl*-IVa. Chromatographic examination of *C. lunata* fermentations of *dl*-Ia did not give evidence for the sequential formation of the triol *dl*-VIa from either 10β-or 11β-monohydroxy product IIIa or IVa. Although the argument may be put forth that neither *d*-IIIa nor *dl*-IVa were suitable inducers of the requisite hydroxylases leading to VIa, or that neither *d*-IIIa nor *dl*-IVa could diffuse into the microbial cell for metabolic disposition, we currently view these results as indicating that the 10β,11β-dihydroxy products do not form from Ia by sequential hydroxylation. As a possible alternate means of derivation of the 10β,11β-dihydroxysteroid *dl*-VIa from *dl*-Ia and also the 6β,10β-dihydroxysteroid *d*-XVIIIa from *dl*-IIa (and the 7α,14α,17α,21-tetrahydroxypregn-4-ene-3,20-dione from 17α,21-dihydroxypregn-4-ene-3,20-dione), all metabolites being *cis*-1,3-diaxial diols, initial formation of a cyclic *cis*-1,3-peroxide followed by reduction of the peroxide to a *cis*-1,3-diol might be postulated.[41]

Experimental Section[42]

***dl*-17β-Hydroxyestr-4-en-3-one** (*dl*-**Ia**).—Racemic Ia was obtained by condensation of 6-methoxytetralone with 2-methylcyclopentane-1,3-dione, stereospecific reduction to racemic estradiol methyl ether, and lithium–liquid ammonia reduction, all by published means.[45] Racemic Ia used in these studies was characterized: mp 116–118° (from isopropyl alcohol–diethyl ether); uv λ_{max} 241 mμ (ε 16,670); ir $\tilde{\nu}_{max}^{KBr}$ 1660 and 1610 cm^{-1}. Thin layer and gas chromatography showed the preparation to be greater than 99% Ia, with no detectable traces of other components.

***dl*-13β-Ethyl-17β-hydroxygon-4-en-3-one** (*dl*-**IIa**).—Racemic IIa was prepared by acid hydrolysis of *dl*-13β-ethyl-3-methoxygona-2,5(10)-dien-17β-ol. Racemic IIa used in these studies was characterized: mp 145–147°; uv λ_{max} 241 mμ (ε 16,000); ir $\tilde{\nu}_{max}^{KBr}$ 3410, 1660, and 1610 cm^{-1}. Thin layer and gas chromatography of *dl*-IIa showed no other detectable component in the preparations used.

Fermentation Conditions with *Curvularia lunata* NRRL 2380.—Vegetative cell cultures of *C. lunata* were grown in 1% sucrose–1% Difco tryptone medium (also containing sodium nitrate, 0.2%; dipotassium hydrogen phosphate, 0.1%; magnesium sulfate heptahydrate, 0.05%; potassium chloride, 0.05%; ferrous sulfate heptahydrate, 0.001%, adjusted to pH 7 with sulfuric acid, with 0.25% calcium carbonate added prior to sterilization)[46] inoculated with surface growth of the organism from agar slants. Inoculation of flourishing vegetative cultures were made into 500-ml erlenmeyer flasks containing 200 ml of the 1% sucrose–1% Difco tryptone medium, and incubations were carried out on a New Brunswick rotary shaker (250 rpm) at 28° for 4 days, at which point mycelial transfers were made into a 14-l. New Brunswick stirred fermentor containing 8 l. of the 1% sucrose–1% Difco tryptone medium. Aerated stirring was conducted for 24 hr, and the selected steroid substrate was added as a solution in dimethylformamide (1 g/5 ml) so as to give a final steroid concentration of 250 μg/ml of fermentation broth. Aeration on the rotary shaker was continued until thin layer chromatography of methyl isobutyl ketone extracts of broth samples taken at different times indicated that the transformation of substrate was complete. At this time, the fermentation broth was extracted twice with equal volumes of methyl isobutyl ketone. The solvent extracts were washed with water, dried over anhydrous sodium sulfate, and concentrated under diminished pressure to yield the crude mixed steroid product.

Fermentation of *d*-17β-Hydroxyestr-4-en-3-one (*d*-**Ia**).—Two grams of crystalline *d*-Ia in 10 ml of dimethylformamide was added in the fluorishing vegetative cell culture as described. After 12 hr of incubation, the products were recovered, and the total crude product was recrystallized twice from ethyl acetate, yielding 340 mg of crude *d*-IIIa and a mother liquor fraction which was worked up separately. The mother liquor material was chromatographed on silica gel using ethyl acetate as eluting solvent, and four major fractions were taken, based on thin layer chromatographic analysis of the effluent.

Fermentation of *dl*-17β-Hydroxyestr-4-en-3-one (*dl*-**Ia**).—Two grams of *dl*-Ia were added to 8 l. of fluorishing *C. lunata* NRRL 2380 vegetative cell culture in the manner previously described. After 12 hr, thin layer chromatography indicated that the transformation of substrate was almost complete. The products were recovered by solvent extraction and fractionated on silica gel irrigated with ethyl acetate. Five major fractions were taken, based on thin layer chromatographic monitoring of the effluent from the column.

***d*-10β,17β-Dihydroxyestr-4-en-3-one** (*d*-**IIIa**). **A. From *d*-Ia.**—The 340 mg of crude IIIa was combined with the first fraction eluted from the silica gel column chromatogram of the combined mother liquor material and recrystallized from ethyl acetate to give 1.1 g of *d*-IIIa, mp 210–215° (lit. mp 199–205°,[20a] 208–210°,[47a] 205–210°,[47b] and 206–209°[7]); uv λ_{max} 237 mμ (ε 13,900) [lit. λ_{max} 237 mμ (ε 15,025),[20a] 234–236 mμ (ε 13,200)[47a]; $\lambda_{max}^{H_2SO_4}$ ($E_{1\,cm}^{1\%}$) 279 (354), 397 (409), and 457 mμ (367); ir $\tilde{\nu}_{max}^{KBr}$ 3300–3400, 1660, and 1615 cm^{-1}; ORD $[\alpha]_{400}$ +49°, $[\alpha]_{373}$ −347°, $[\alpha]_{367}$ −327°, $[\alpha]_{360}$ −396°, $[\alpha]_{330}$ +1326°. Identity of the sample with authentic *d*-IIIa was estabished by direct comparison of chromatographic and infrared spectral properties.

B. From *dl*-Ia.—Evaporation of the solvent from the first, most mobile column fraction from the *dl*-Ia fermentation prod-

(41) A cyclic five-membered peroxide intermediate is suggested to account for introduction of the 9- and 11-oxygen atoms of prostagalndin E_1 wherein only one molecule of oxygen is involved. *cf.* B. Samuelsson, *J. Amer. Chem. Soc.*, **87**, 3011 (1965); D. H. Nugteren, R. K. Beerthuis, and D. A. van Dorp, *Rec. Trav. Chim. Pays-Bas*, **85**, 405 (1966).

(42) Melting points were taken on a calibrated Kofler block under microscopic magnification. Ultraviolet light absorption spectra were recorded with a Cary Model 14 spectrophotometer, using 95% ethanol, 0.066 *N* alkaline ethanol according to Meyer,[43] and concentrated sulfuric acid as solvents for steroids. Spectra in alkaline ethanol were recorded after 24 hr, and in concentrated sulfuric acid after 2 hr, except where noted otherwise. Infrared absorption spectra were recorded on 0.5-mm, diameter potassium bromide disks incorporating the sample and on 0.001 *M* solutions of samples in chloroform or carbon tetrachloride, by means of a Perkin-Elmer Model 337 spectrophotometer equipped with a beam condenser. Optical rotatory dispersion spectra were recorded using a Rudolph self-recording instrument over the range 250–400 mμ, using dioxane solutions of steroids. Proton spectra were recorded on a Varian Model A60A 60-MHz spectrometer, using 15% solutions of steroids in deuteriochloroform containing tetramethylsilane as an internal reference. Chemical shifts are expressed as δ values in parts per millions downfield from the internal reference. Abbreviations used include the following: t, triplet; m, multiplet.

Thin layer chromatography was conducted on 5 × 20 and 20 × 20 cm chromatoplates, 0.25 mm thick, of silica gel HF_{254} (E. Merck GmbH., Darmstadt), using ethyl acetate as irrigating solvent for steroid alcohols and ethyl acetate–chloroform (1:1) for steroid acetates. Resolved steroids were detected under ultraviolet light (254 and 366 mμ) after which the chromatoplate was sprayed with 50% aqueous sulfuric acid and warmed until color development was complete. Preparative thin layer chromatography was conducted on 20 × 20 cm chromatoplates, 1 and 2 mm thick, of silica gel PF_{254} irrigated with the same solvents. Samples were applied to the chromatoplate as a fine line by means of a Rodder Streaker (Rodder Instrument Co., Los Altos, Calif.). All substrates, each microbial fermentation, and isolation and purification procedures were carefully monitored by thin layer chromatography as a routine.

Gas chromatography was conducted on 1.83-m-long silanized glass U-tubes, 6-mm o.d., filled with 3% QF-1 (trifluoropropylmethyl silicone) on 100–120 mesh Gas-Chrom Q (Applied Science Laboratories, State College, Pa.) at 230° by techniques previously described.[44] Areas under elution peaks were estimated and used to obtain relative composition of hydroxy steroid product mixtures.

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

(44) J. E. van Lier and L. L. Smith, *Biochemistry*, **6**, 3269 (1967); (b) J. E. van Lier and L. L. Smith, *Anal. Biochem.*, **24**, 419 (1968).

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

(46) G. M. Shull, D. A. Kita, and J. W. Davisson, U. S. Patent 2,658,023 (Nov 3, 1953).

(47) (a) J. Perez Ruelas, J. Iriarte, F. Kincl, and C. Djerassi, *J. Org. Chem.*, **23**, 1744 (1958); (b) A. von Wartburg, J. Binkert, and E. Angliker, *Helv. Chim. Acta*, **45**, 2139 (1962).

ucts, followed by recrystallization from ethyl acetate, give 400 mg of *d*-IIIa, mp 206–212°, identical in infrared spectra, thin layer chromatography, and optical rotatory dispersion with *d*-Ia obtained from fermentation of *d*-Ia with *C. lunata* NRRL 2380.

d-**17β-Acetoxy-10β-hydroxyestr-4-en-3-one** (*d*-**IIIb**).—Acetylation of 100 mg of *d*-IIa (from A above) was accomplished with 0.5 ml of acetic anhydride and 1 ml of pyridine in the usual manner. After 24 hr, the solution was poured onto ice, the product was extracted with diethyl ether, the solvent was removed under vacuum, and the residue was crystallized from ethyl acetate, yielding 80 mg of *d*-IIIb, mp 174–176° (lit. mp 184–185°,[20a] 182–183° [47a]); uv λ_{max} 236 mμ (ε 14,300); ir $\tilde{\nu}_{max}^{KBr}$ 3370, 1710, 1680, and 1625 cm^{-1}; $\tilde{\nu}_{max}^{CCl_4}$ 3 630 cm^{-1} (sharp); nmr δ 0.88 (3 H, C_{18} protons), 2.02 (3 H, acetate methyl protons), 4.58 (m, 1 H, 17α-proton), 5.70 ppm (1 H, C_4 vinyl proton).

d-**6β17β-Dihydroxyestr-4-en-3-one** (*d*-**VIIa**).—The second silica gel column chromatogram fraction containing *d*-VIIa was evaporated under vacuum to give 30 mg of *d*-VIIa, mp 215–220° (lit. mp 217–219°,[20a] 209–213°,[11] 211–219° [5a]), with infrared absorption spectrum identical with that of *dl*-VIIa.

dl-**6β,17β-Dihydroxyestr-4-en-3-one** (*dl*-**VIIa**).—The second fraction eluted from the silica gel column chromatogram of crude products from fermentation of *dl*-Ia contained *dl*-VIIa. Recrystallization of the crude material from ethyl acetate gave 90 mg of *dl*-VIIa, mp 220–226°; uv λ_{max} 238 mμ (ε 13,500); $\lambda_{max}^{0.066NNaOH}$ (ε) 242 (3840), 266 (1840), 313 (1510), and 367 mμ (1850); $\lambda_{max}^{H_2SO_4}$ ($E_{1\,cm}^{1\%}$) 297 (650), 377 (117), 397 (122), and 459 mμ (90); ir $\tilde{\nu}_{max}^{KBr}$ 3350, 3290, and 1660 cm^{-1}; no optical activity over the range 250–400 mμ.

Anal. Calcd for $C_{18}H_{26}O_3$ (mol wt 290.39): C, 74.44; H, 9.03. Found: C, 74.54; H, 9.03.

d-**6β,17β-Diacetoxyestr-4-en-3-one** (*d*-**VIIb**).—Thirty milligrams of *d*-VIIa was acetylated with 0.5 ml of acetic anhydride and 1 ml of pyridine in the usual manner, yielding, after recrystallization from hexane, 18 mg of *d*-VIIb, mp 130–131° (lit. mp 137–138°[20a], 132–133° [11]); uv λ_{max} 234 mμ (ε 12,330) [lit. λ_{max} 236 mμ (ε 13,500)[20a]]; ir $\tilde{\nu}_{max}^{KBr}$ 1735, 1680, and 1625 cm^{-1}.

Anal. Calcd for $C_{22}H_{30}O_5$ (mol wt 374.46): C, 70.56; H 8.08. Found: C, 70.43; H, 8.08.

d-**14α,17β-Dihydroxyestr-4-en-3-one** (*d*-**Va**). **A. From** *d*-**Ia.**—Recrystallization of the third silica gel column chromatogram fraction of the crude products obtained from *d*-Ia from ethyl acetate gave 200 mg of *d*-Va, mp 191–192° (lit.[11] mp 192–194°); uv λ_{max} 241 mμ (ε 16,200); $\lambda_{max}^{H_2SO_4}$ ($E_{1\,cm}^{1\%}$) 282 (227), 392 (277), and 458 mμ (257); ir $\tilde{\nu}_{max}^{KBr}$ 3400, 1660, and 1610 cm^{-1}.

Anal. Calcd for $C_{18}H_{26}O_3$ (mol wt 290.39): C, 74.44; H, 9.03. Found: C, 74.35; H, 8.94.

B. From *dl*-**Ia.**—From the ethyl acetate–hexane mother liquors from which *l*-XIa had been isolated, crude crystals of *d*-Va were recovered. Recrystallization from ethyl acetate gave 10 mg of *d*-Va, identical in chromatographic properties, infrared spectra, and optical rotatory dispersion with *d*-Va isolated from fermentation of *d*-Ia.

d-**17β-Acetoxy-14α-hydroxyestr-4-en-3-one** (*d*-**Vb**).—A 100-mg sample of *d*-Va (from A above) was acetylated in the usual manner with 0.5 ml of acetic anhydride and 1 ml of pyridine. The crude acetate was recrystallized from diethyl ether to yield 90 mg of *d*-Vb, mp 191–196° (lit. mp 183–186°[11]); uv λ_{max} 241 mμ (ε 15,000); ir $\tilde{\nu}_{max}^{KBr}$ 3460, 1720, 1660, and 1610 cm^{-1}; $\tilde{\nu}_{max}^{CCl_4}$ 3630 cm^{-1} (sharp); nmr δ 0.97 (3 H, C_{18} protons), 2.02 (3 H, acetate methyl protons), 5.18 (m, 1 H, 17α-proton), and 5.79 ppm (1 H, C_4-vinyl proton); ORD $[\alpha]_{430}$ +22°, $[\alpha]_{368}$ −297°, $[\alpha]_{364}$ −286°, $[\alpha]_{356}$ −330°, $[\alpha]_{330}$ +605°.

Anal. Calcd for $C_{20}H_{28}O_4$ (mol wt 332.42): C, 72.26; H, 8.49. Found: C, 72.14; H, 8.41.

d-**17β-Hydroxyestra-4,8(14)-dien-3-one** (*d*-**X**).—Dry hydrogen chloride was passed through a solution of 5 mg of *d*-Va in 1 ml of glacial acetic acid. After 2 hr, the mixture was poured into ice-water and extracted with ethyl acetate. Removal of solvent under vacuum gave *d*-X, identified by thin layer chromatographic behavior and by retention time (1.8 min on 3% QF-1) identical with that of authentic *dl*-X.[13]

d-**11β,17β-Dihydroxyestr-4-en-3-one** (*d*-**IVa**).—The fourth fraction from the silica gel column of crude steroid products from *d*-Ia contained two dihydroxyketones. Rechromatography on silica gel using slow elution with ethyl acetate and careful attention to the composition of eluted material afforded the more mobile component in a pure state. Recrystallization of the more mobile steroid from ethyl acetate gave 60 mg of *d*-IVa, mp 221–224° (lit. mp 224–227°,[48] 214–219° [7]); uv λ_{max} 242 mμ (ε 15,600) [lit. λ_{max} 243 mμ (ε 16,100)[48]]; $\lambda_{max}^{H_2SO_4}$ ($E_{1\,cm}^{1\%}$) 280 (345), 399 (384), and 455 mμ (435); ir $\tilde{\nu}_{max}^{KBr}$ 3390–3450, 1660, and 1620 cm^{-1}.

Anal. Calcd for $C_{18}H_{26}O_3$ (mol wt 290.39): C, 74.44; H, 9.03. Found: C, 74.23; H, 8.91.

d-**11β,17β-Diacetoxyestr-4-en-3-one** (*d*-**IVb**).—A 40-mg sample of *d*-IVa was acetylated in the usual manner, yielding, after recrystallization of the crude product from ethyl acetate, 25 mg of *d*-IVb, mp 171–173°; uv λ_{max} 239 mμ (ε 16,500); ir $\tilde{\nu}_{max}^{KBr}$ 1740, 1735, 1670, and 1615 cm^{-1}; nmr δ 0.96 (3 H, C_{18} protons), 1.99 (3 H, 17β-acetate methyl protons), 2.06 (3 H, 11β-acetate methyl protons), 4.52 (m, 1 H, 17α proton), 5.16 (m, 1 H, 11α proton), and 5.84 (1 H, C_4-vinyl proton).

Anal. Calcd for $C_{22}H_{30}O_5$ (mol wt 374.46): C, 70.56; H, 8.08. Found: C, 70.74; H, 8.13.

dl-**11β,17β-Dihydroxyestr-4-en-3-one** (*dl*-**IVa**).—Evaporation of the eluting solvent from the fourth column fraction obtained from silica gel chromatography of the crude products from fermentation of *dl*-Ia afforded crude *dl*-IVa. Recrystallization from ethyl acetate–methanol gave 130 mg of *dl*-IVa, mp 226–229°; uv λ_{max} 242 mμ (ε 14,900); ir $\tilde{\nu}_{max}^{KBr}$ 3350–3400, 1660, and 1620 cm^{-1} (slightly different from *d*-IVa in the fingerprint region); no optical activity over the region 250–400 mμ.

Anal. Calcd for $C_{18}H_{26}O_3$ (mol wt 290.39): C, 74.44; H, 9.03. Found: C, 74.34; H, 9.02.

dl-**11β,17β-Diacetoxyestr-4-en-3-one** (*dl*-**IVb**).—Acetylation of 50 mg of *dl*-IVa in the usual manner gave a crude diacetate which was recrystallized from diethyl ether to give crystalline *dl*-IVb, mp 174–176°, identical in thin layer and gas chromatographic behavior and in infrared spectra with *d*-IVb.

dl-**Estr-4-ene-3,11,17-trione** (*dl*-**XIII**).—A solution of 40 mg of *dl*-IVa in acetone was oxidized in the usual manner with Jones reagent, to yield 25 mg of *dl*-XIII, recrystallized from acetone: mp 185–189°; uv λ_{max} 239 mμ (ε 14,300); ir $\tilde{\nu}_{max}^{KBr}$ 1740, 1720, 1660, and 1620 cm^{-1}; $\tilde{\nu}_{max}^{CHCl_3}$ 1740, 1710, 1660, and 1615 cm^{-1}. An authentic sample of *d*-estr-4-ene-3,11,17-trione gave identical spectra in chloroform and identical frequencies for the major functional groups in KBr; however, in KBr the fingerprint region of the spectrum of *d*-XIII differed from that of *dl*-XIII.

d-**10β,11β,17β-Trihydroxyestr-4-en-3-one** (*d*-**VIa**).—The more polar component from the silica gel chromatogram from which *d*-IVa had been eluted as a more mobile component was isolated on evaporation of the eluting solvent, yielding 10 mg of *d*-VIa. Recrystallization from ethyl acetate gave 5 mg of *d*-VIa, mp 230–240° dec; uv λ_{max} 237 mμ (ε 12,800); $\lambda_{max}^{H_2SO_4}$ ($E_{1\,cm}^{1\%}$) 280 (296), 333 (128), 405 (287), and 454 mμ (455); ir $\tilde{\nu}_{max}^{KBr}$ 3350, 3270, 1680, and 1630 cm^{-1}; ORD $[\alpha]_{450}$ −682°, $[\alpha]_{410}$ −724°, $[\alpha]_{375}$ −1363°, $[\alpha]_{368}$ −1278°, $[\alpha]_{360}$ −1363°, $[\alpha]_{330}$ +639°. Direct comparison by thin layer chromatography and infrared spectra of *d*-VIa and an authentic sample[7] established their identity.

dl-**10β,11β,17β-Trihydroxyestr-4-en-3-one** (*dl*-**VIa**).—Repeated recrystallization of the solids obtained from the mother liquor from which *dl*-IVa had been obtained gave 30 mg of *dl*-VIa, mp 233–234° dec; identical in chromatographic behavior and in infrared spectra with *d*-VIa. Optical rotatory dispersion spectra of the sample showed a flat featureless curve between 250 and 400 mμ. Insufficient pure material was available for elemental analyses.

dl-**17β-Acetoxy-10β,11β-dihydroxyestr-4-en-3-one** (*dl*-**VIb**).—A 20-mg sample of *dl*-VIa was acetylated in the usual fashion to give an oily product. Preparative thin layer chromatography of the material, using chloroform–ethyl acetate (1:1) as irrigating solvent, on a 20 × 20 cm silica gel PF_{254} chromatoplate 1 mm thick, afforded, on recovery from the chromatoplate, 8 mg of *dl*-VIb, mp 182–184°; uv λ_{max} 237 mμ (ε 13,500); ir $\tilde{\nu}_{max}^{KBr}$ 3400, 3300, 1735, 1660, and 1610 cm^{-1}; $\tilde{\nu}_{max}^{CCl_4}$ 3630, 3480, 1740, 1670, and 1620 cm^{-1}.

Anal. Calcd for $C_{20}H_{28}O_5$ (mol wt 348.42): C, 68.94; H, 8.10. Found: C, 69.52; H, 8.17.

l-**10β,11β,17β-Trihydroxy-5α-estran-3-one** (*l*-**IXa**).—Evaporation of the third column fraction obtained from silica gel chromatography of the crude products from *dl*-Ia gave crude *l*-IXa. Recrystallization from ethyl acetate–hexane gave 30 mg of *l*-IXa, mp 267–270°; uv $\lambda_{max}^{H_2SO_4}$ ($E_{1\,cm}^{1\%}$) 279 (286), 400 (339), and 455 mμ (318); ir $\tilde{\nu}_{max}^{KBr}$ 3330–3410 and 1700 cm^{-1}; ORD $[\alpha]_{400}$ −72°, $[\alpha]_{316}$ −882°, $[\alpha]_{272}$ +675°, $[\alpha]_{260}$ +567°.

(48) J. W. Ralls, U. S. Patent 2,778,841 (Jan 22, 1957); *Chem. Abstr.*, **51**, 8824*i* (1957).

l-**17β-Acetoxy-10β,11β-dihydroxy-5α-estran-3-one** (*l*-**IXb**).—Twenty milligrams of *l*-IXa was acetylated in the usual fashion. The crude acetate was crystallized from ethyl acetate–hexane to yield 13 mg of *l*-IXb, mp 188–191°; ir $\tilde{\nu}_{max}^{KBr}$ 3450, 3350, 1735, 1720, and 1700 cm^{-1}; $\tilde{\nu}_{max}^{CCl}$ 3630, 3510, 1740, 1720, and 1690 cm^{-1}; nmr δ 1.06 (3 H, C_{18}-methyl protons) and 2.04 ppm (3 H, acetate methyl protons).

Anal. Calcd for $C_{20}H_{30}O_5$ (mol wt 350.44): C, 68.54; H, 8.63. Found: C, 68.77; H, 8.67.

l-**17β-Hydroxyestra-4,9(11)-dien-3-one** (*l*-**XI**).—Dry hydrogen chloride was passed through a solution of 6 mg of *l*-IXa in 1 ml of glacial acetic acid at 22° for 1 hr, after which time the mixture was poured into ice–water, extracted with ethyl acetate, and the extracts evaporated under reduced pressure, yielding 4 mg of crystalline *l*-XI, mp 120–130°; uv λ_{max} 241 mμ (ε 15,400); $\lambda_{max}^{H_2SO_4}$ ($E_{1\,cm}^{1\%}$) 285 (607), 400 (680), and 460 mμ (96); ir $\tilde{\nu}_{max}^{KBr}$ 3420, 1670, and 1615 cm^{-1}. Infrared spectra and gas chromatographic behavior of *l*-XI were different from those of *d*-X. An insufficient sample of *l*-XI was available for elemental analysis.

l-**Estra-4,9(11)-diene-3,17-dione** (*l*-**XII**).—Three milligrams of *l*-XI dissolved in 2 ml of acetone was treated with 0.2 ml of the Jones reagent for 30 min. Excess reagent and acetone were removed and the residue was taken up in diethyl ether. Evaporation of the ether gave *l*-XII, mp 140–150°; uv λ_{max} 240 mμ (ε 15,000); ir $\tilde{\nu}_{max}^{KBr}$ 1735, 1670, and 1615 cm^{-1}. A 3 *N* methanolic hydrochloric acid solution of *l*-XII was refluxed and the spectrum was recorded: uv $\lambda_{max}^{3\ N\ MeOH\text{-}HCl}$ 243 (ε 14,000) and 313 mμ (ε 2800).

dl-**10β,11β,17β-Trihydroxy-5α-estran-3-one** (*dl*-**IXa**).—Two milligrams of *dl*-VIa in ethanol was hydrogenated at room temperature for 2 hr over 5 mg of 5% palladium on calcium carbonate. Removal of catalyst and evaporation of solvent gave an oil which contained three major components by gas chromatographic analysis (on 3% QF-1). The most mobile components were recognized to be overreduced materials, probably isomeric estrane-3ξ,10β,17β-tetraols. The second and third peaks eluted from the gas chromatogram were recognized as *dl*-10β,11β,17β-trihydroxy-5β-estran-3-one and *dl*-10β,11β,17β-trihydroxy-5α-estran-3-one (*dl*-IXa), respectively, based on the usual order of elution of 5 epimers on gas chromatography. The retention time (19 min) of *dl*-IXa obtained *via* catalytic reduction was identical with that of *l*-IXa recovered from the *C. lunata* fermentation of *dl*-Ia.

l-**12α,17β-Diacetoxyestr-4-en-3-one** (*l*-**VIIIb**).—Evaporation of the fifth most polar fraction from the silica gel column chromatogram of the crude products from *dl*-Ia fermentation gave 30 mg of an oil containing *l*-VIIIa, ir $\tilde{\nu}_{max}^{KBr}$ 3350–3450, 1660, and 1610 cm^{-1}, which could not be crystallized. Acetylation of the oil with acetic anhydride–pyridine in the usual manner gave 20 mg of *l*-VIIIb, mp 187–190°; uv λ_{max} 240 mμ (ε 16,400); ir $\tilde{\nu}_{max}^{KBr}$ 1735, 1660, and 1610 cm^{-1}; nmr δ 0.94 (3 H, C_{18}-methyl protons), 2.00 (3 H, 17β-acetate methyl protons), 2.06 (3 H, 12α-acetate methyl protons), 4.92 (m, 2 H, 12β and 17α protons, unresolved), and 5.83 ppm (1 H, C_4-vinyl proton); ORD $[\alpha]_{430}$ −33°, $[\alpha]_{364}$ +225°, $[\alpha]_{362}$ +218°, $[\alpha]_{358}$ +225°, $[\alpha]_{343}$ −119°.

Anal. Calcd for $C_{22}H_{30}O_5$ (mol wt 374.46): C, 70.56; H, 8.08. Found: C, 70.53; H, 8.11.

Fermentation of *dl*-**13β-Ethyl-17β-hydroxygon-4-en-3-one** (*dl*-**IIa**).—Two grams of *dl*-IIa was added to 8 l. of a fluorishing vegetative cell culture of *C. lunata* NRRL 2380 in the manner previously described. After 24 hr of aeration, thin layer chromatograms indicated that the transformation was almost complete. The products were recovered by extraction of the broth with methyl isobutyl ketone, and the solvent extract was evaporated under vacuum. The product residue was dissolved in ethyl acetate and adsorbed onto a column of silica gel which was further irrigated with ethyl acetate.

dl-**17β-Acetoxy-13β-ethylgon-4-en-3-one** (*dl*-**IIb**).—The recovered substrate *dl*-IIa indicated by thin layer chromatography in very early fractions from the silica gel column chromatogram of fermentation products of *dl*-IIa was obtained by evaporation of the ethyl acetate under vacuum and crystallization of the residue from diisopropyl ether to give 70 mg of *dl*-IIa which was acetylated with acetic anhydride–pyridine in the usual manner. After recrystallization of the crude acetate from hexane, there was obtained pure *dl*-IIb, mp 113–116°; uv λ_{max} 241 mμ (ε 16,500); ir $\tilde{\nu}_{max}^{KBr}$ 1730, 1670, and 1620 cm^{-1}; nmr δ 2.05 (3 H, acetoxyl protons), 4.73 (m, 1 H, 17α proton), and 5.83 ppm (1 H, C_4-vinyl proton); no optical activity over the range 250–400 mμ; identified with an authentic sample of *dl*-IIa by chromatographic and infrared spectral comparisons.

dl-**13β-Ethyl-10β,17β-dihydroxygon-4-en-3-one** (*dl*-**XIVa**).—The initial major fraction eluted from the silica gel column by ethyl acetate contained XIVa by thin layer chromatography. After removal of ethyl acetate under vacuum, the residue was recrystallized from ethyl acetate–hexane–methanol to give 350 mg of *dl*-XIVa, mp 230–232° (with sweating from 225°) (lit.[5a] mp 224–227°[5a]); uv λ_{max} 236 mμ (ε 13,800) [lit. λ_{max} 236 mμ (ε 14,850); $\lambda_{max}^{H_2SO_4}$ ($E_{1\,cm}^{1\%}$) 281 (372), 397 (363), and 456 mμ (347); ir $\tilde{\nu}_{max}^{KBr}$ 3370, 1660, and 1610 cm^{-1}; optically inactive over the range 250–400 mμ.

Anal. Calcd for $C_{19}H_{28}O_3$ (mol wt 304.42): C, 74.96; H, 9.27. Found: C, 75.07; H, 9.28.

dl-**17β-Acetoxy-13β-ethyl-10β-hydroxygon-4-en-3-one** (*dl*-**XIVb**).—A sample of 80 mg of *dl*-XIVa was acetylated with acetic anhydride and pyridine in the usual manner, yielding 80 mg of oily monoacetate *dl*-XIVb, ir $\tilde{\nu}_{max}^{KBr}$ 3450, 1730, 1670, and 1620 cm^{-1}, which could not be crystallized (lit. *d*-XIVb mp 182–184°[5a]).

d-**13β-Ethyl-14α,17β-dihydroxygon-4-en-3-one** (*d*-**XVa**).—The second major fraction eluted by ethyl acetate from the silica gel column contained XVa by thin layer chromatography. After removal of ethyl acetate under vacuum, the residue was recrystallized from ethyl acetate–hexane to give 100 mg of *d*-XVa, mp 205–207°; uv λ_{max} 241 mμ (ε 16,300); $\lambda_{max}^{H_2SO_4}$ ($E_{1\,cm}^{1\%}$) 283 (323), 392 (254), and 459 mμ (285); ir $\tilde{\nu}_{max}^{KBr}$ 3550, 3470, 1660, and 1610 cm^{-1}. The sample could not be brought to satisfactory analytical purity for elemental analysis, but was fully characterized as the monoacetate *d*-XVb.

Anal. Calcd for $C_{19}H_{28}O_3$ (mol wt 304.42): C, 74.96; H, 9.27. Found: C, 73.78; H, 8.97.

d-**17β-Acetoxy-13β-ethyl-14α-hydroxygon-4-en-3-one** (*d*-**XVb**).—Acetylation of 60 mg of *d*-XVa in the usual manner yielded, after recrystallization from ethyl acetate, 40 mg of *d*-XVb, mp 202–205°; uv λ_{max} 241 mμ (ε 16,300); ir $\tilde{\nu}_{max}^{KBr}$ 3480, 1730, 1670, and 1630 cm^{-1}; ORD $[\alpha]_{430}$ +107°, $[\alpha]_{366}$ −245°, $[\alpha]_{362}$ −240°, $[\alpha]_{356}$ −277°, $[\alpha]_{332}$ +552°; nmr δ 2.05 (3 H, acetate protons), 5.26 (1 H, 17α proton), and 5.83 ppm (1 H, C_4-vinyl proton).

Anal. Calcd for $C_{21}H_{30}O_4$ (mol wt 346.45): C, 72.80; H, 8.73. Found: C, 72.71; H, 8.68.

l-**13β-Ethyl-12α,17β-dihydroxygon-4-en-3-one** (*l*-**XVIIa**).—The third major fraction from the silica gel column was evaporated under vacuum and the residue was recrystallized from ethyl acetate–hexane, yielding 300 mg of *l*-XVIIa, mp 158–160°; uv λ_{max} 241 mμ (ε 17,600); $\lambda_{max}^{H_2SO_4}$ ($E_{1\,cm}^{1\%}$) 282 (530), 343 (289), and 455 mμ (119); after 24 hr, 277 (463), 410 (289), and 450 mμ (354); ir $\tilde{\nu}_{max}^{KBr}$ 3450, 1670, and 1620 cm^{-1}; ORD $[\alpha]_{430}$ −24°, $[\alpha]_{364}$ +362°, $[\alpha]_{361}$ +358°, $[\alpha]_{355}$ +382°, $[\alpha]_{328}$ −1015°.

Anal. Calcd for $C_{19}H_{28}O_3$ (mol wt 304.42): C, 74.96; H, 9.27. Found: C, 74.73; H, 9.27.

l-**12α,17β-Diacetoxy-13β-ethylgon-4-en-3-one** (*l*-**XVIIb**).—Acetylation of 100 mg of *l*-XVIIa with acetic anhydride and pyridine in the usual fashion gave, after recrystallization from ethyl acetate, 80 mg of *l*-XVIIb, mp 129–130°; uv λ_{max} 239 mμ (ε 17,700); ir $\tilde{\nu}_{max}^{KBr}$ 1740, 1670, and 1620 cm^{-1}; nmr δ 2.02 (3 H, 17β-acetoxyl protons), 2.08 (3 H, 12α-acetoxyl protons), 5.02 (t, 1 H, 17α proton), 5.25 (m, 1 H, 12β proton), and 5.81 ppm (1 H, C_4-vinyl proton).

Anal. Calcd for $C_{23}H_{32}O_5$ (mol wt 388.49): C, 71.10; H, 8.30. Found: C, 71.51; H, 8.26.

l-**13β-Ethylgon-4-ene-3,12,17-trione** (*l*-**XIX**).—One hundred milligrams of *l*-XVIIa was oxidized with Jones reagent at room temperature for 30 min, after which time the preparation was worked up in the usual fashion. The crude product was crystallized from acetone–hexane to yield 70 mg of the 3,12,17-trione *l*-XIX, mp 211–214°; uv λ_{max}^{MeOH} 238 mμ (ε 17,500); $\lambda_{max}^{0.04\ N\ NaOH}$ 238 mμ (ε 17,000); ir $\tilde{\nu}_{max}^{CHCl_3}$ 1760, 1700 (intensity *ca.* 30% of the 1760-cm^{-1} band), 1670, and 1620 cm^{-1}; nmr δ 0.89 (t, 3 H, J = 7 Hz, C_{18a}-methyl protons) and 5.85 ppm (1 H, C_4-vinyl proton).

Anal. Calcd for $C_{19}H_{24}O_3$ (mol wt 300.38): C, 75.97; H, 8.05. Found: C, 75.99; H, 8.00.

dl-**6β,17β-Diacetoxy-13β-ethylgon-4-en-3-one** (*dl*-**XVIb**).—The eluates from the silica gel column chromatogram containing XVIa as indicated by thin layer chromatography were evaporated under vacuum and acetylated with acetic anhydride–pyridine in the usual fashion. After recrystallization from hexane, there was obtained 17 mg of *dl*-XVIb, mp 163–165° (lit. mp 171–175°[5a]); uv λ_{max} 236 mμ (ε 12,700) [lit. λ_{max} 235 mμ (ε 13,450)[5a]]; ir $\tilde{\nu}_{max}^{KBr}$ 1740, 1680, and 1630 cm^{-1}; nmr δ 2.05 (3 H, 17β-acetoxyl protons), 2.08 (3 H, 6β-acetoxyl protons), 4.68 (m, 1 H, 17α

proton), 5.47 (m, 1 H, $W_{1/2}$ = 7 Hz, 6α proton), and 6.01 ppm (1 H, C_4-vinyl proton; no optical activity over the range 250–450 mμ.

d-**17β-Acetoxy-13β-ethyl-6β,10β-dihydroxygon-4-en-3-one** (*d*-**XVIIIb**).—The fraction eluted from the silica gel column shown to contain the dihydroxylated transformation product XVIIIa by thin layer chromatography was evaporated under vacuum and acetylated with acetic anhydride–pyridine in the usual manner. After recrystallization from hexane–diethyl ether, there was obtained 7 mg of the 17β-monoacetate *d*-XVIIIb, mp 174–176°; uv λ_{max} 233 mμ (ε 13,800); $\lambda_{max}^{0.066\ N\ NaOH}$ 238 (ε 5600) and 275 mμ (ε 2300); ir $\tilde{\nu}_{max}^{KBr}$ 3320 (br), 1730, 1680, and 1630 cm^{-1}; $\tilde{\nu}_{max}^{CCl_4}$ 3630, 3480 (br), 1730, and 1670 cm^{-1}; ORD $[\alpha]_{430}$ +65°, $[\alpha]_{375}$ −308°, $[\alpha]_{362}$ −356°, $[\alpha]_{336}$ +600°. An insufficient sample was available for combustion analyses.

Chromatographic Relationships.—Chromatographic data showing homolog relationships between certain products are given in order as follows: gas chromatographic relative retention times on 3% QF-1, followed by thin layer chromatographic relative mobilities using ethyl acetate for irrigation with hydroxy steroids and ethyl acetate–chloroform (1:1) for irrigation with steroid acetates. Mobility data are expressed in terms of *dl*-Ia as unity for estranes and in terms of *dl*-IIa as unity for the 13β-ethylgonane homologs, except for the thin layer mobility data on the acetates, where *dl*-Ib served as unit marker for the estranes and *dl*-IIb for the 13β-ethylgonanes. A homologous relationship is demonstrated for those pairs showing the same retention data and thin layer mobility data, both as the free alcohols and as the acetates. Mobility data follow: *dl*-Ia, 1.00, 1.00; *dl*-IIa, 1.00; 1.00; *d*-IIIa, 1.30, 0.84; *dl*-XIVa, 1.33, 0.86; *d*-Va, 1.77, 0.52; *d*-XVa, 1.83, 0.49; *dl*-VIIa, 1.46, 0.65; *dl*-XVIa, 1.52, 0.69; *l*-VIIIa, 1.93, 0.32; *l*-XVIIa, 2.06, 0.31; *dl*-VIa, 3.21, 0.36; *d*-XVIIIa, 3.21, 0.31; *dl*-IVa, 1.93, 0.36; *l*-IXa, 2.22, 0.52; *dl*-Ib, 1.63, 1.00; *dl*-IIb, 1.71, 1.00; *d*-IIIb, 2.13, 0.64; *dl*-XIVb, 2.14, 0.63; *d*-Vb, 3.27, 0.61; *d*-XVb, 3.58, 0.56; *dl*-VIIb, 3.44, 0.97; *dl*-XVIb, 3.44, 0.97; *l*-VIIIb, 3.08, 0.75; *l*-XVIIb, 3.08, 0.75; *dl*-VIb, 2.98, 0.39; *d*-XVIIIb, 4.89, 0.34; *dl*-IVb, 4.87, 0.78; *l*-IXa, 3.67, 0.54.

Registry No.—*dl*-Ia, 5972-58-7; *dl*-IIa, 793-54-4; *d*-IIIb, 21317-53-3; *d*-IVa, 4075-17-6; *dl*-IVa, 21317-55-5; *d*-IVb, 21317-56-6; *dl*-IVb, 21317-57-7; *d*-Va, 2162-37-0; *d*-Vb, 21317-59-9; *d*-VIa, 21317-60-2; *dl*-VIa, 21317-61-3; *dl*-VIb, 21317-62-4; *dl*-VIIa, 21317-63-5; *d*-VIIb, 21317-64-6; *l*-VIIIb, 21317-65-7; *dl*-IIb, 21317-66-8; *l*-IXa, 21317-67-9; *dl*-IXa, 21317-68-0; *l*-IXb, 21317-69-1; *l*-XI, 21317-70-4; *l*-XII, 21317-71-5; *dl*-XIII, 21317-72-6; *dl*-XIVa, 6615-05-0; *dl*-XIVb, 6615-06-1; *d*-XVa, 21317-75-9; *d*-XVb, 21317-76-0; *dl*-XVIb, 6615-11-8; *l*-XVIIa, 21321-83-5; *l*-XVIIb, 21321-84-6; *d*-XVIIIb, 21321-85-7; *l*-XIX, 21321-86-8.

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Synthesis of Racemic Phytosphingosine and the *lyxo* Isomer

KEIITI SISIDO, NORIYUKI HIROWATARI, AND TYÛZÔ ISIDA

Department of Industrial Chemistry, Kyôto University, Kyôto, Japan

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dl-erythro-2-Benzylamino-3-hydroxy-4-ethylenedioxyoctadecanoic acid (**15**), prepared from *dl-trans*-2,3-epoxy-4-ethylenedioxyoctadecanoic acid (**14**) and benzylamine, was converted into methyl *dl-erythro*-2-benzylamino-3-hydroxy-4-oxooctadecanoate hydrochloride (**19**). The ester hydrochloride **19** was reduced with lithium aluminum hydride to yield *dl*-2-benzylamino-1,3,4-trihydroxyoctadecane (**20a,b**), and, from the *dl-ribo* isomer (**20a**), racemic phytosphingosine (**25a**) was obtained by catalytic hydrogenolysis. The same compound **25a** and the diastereoisomeric *dl-lyxo* compound (**25b**) were prepared from *dl*-2-benzylamino-3,4-dihydroxyoctadecanoic acids (**23a,b**) and their lactones (**24a,b**) by reduction with lithium aluminum hydride followed by hydrogenolysis.

In 1963, Carter and Hendrickson[1] established by degradative studies that phytosphingosine was D-*ribo*-2-amino-1,3,4-trihydroxyoctadecane,[2] and the syntheses of this optically active aminotriol from sphingosine[3] and sugars[4] have been published. Also, a total synthesis to prepare the stereoisomers of racemic 2-amino-1,3,4-trihydroxyoctadecane has been reported,[5] but the configurations of the products were not defined. The present paper describes the syntheses of racemic phytosphingosine and its *lyxo* isomer.

The process is based on the method described previously for a synthesis of racemic dihydrosphingosine,[6] *i.e.*, on the stereospecific reaction of *dl-trans* glycidic acid with benzylamine to yield *dl-erythro*-2-benzylamino-3-hydroxy acid.

1-Bromo-2-hexadecanone (**3**) was prepared from *n*-pentadecanoyl chloride (**1**) by treatment with diazomethane followed by gaseous hydrogen bromide. The reaction of the α-bromo ketone **3** with 2 mol equiv of carbomethoxymethylenetriphenylphosphorane in boiling benzene[7] gave methyl 4-oxo-*trans*-2-octadecenoate (**4**). That the keto ester **4** has the *trans* configuration was proved by the infrared absorption spectrum and by an independent synthesis from 4-ethylenedioxy-*trans*-2-octadecenoic acid (**8**). This ketal acid **8** was prepared

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