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Mechanisms of Steroid Oxidation by Microorganisms. XIII. C_{22} Acid Intermediates in the Degradation of the Cholesterol Side Chain*

Charles J. Sih, K. C. Wang,† and H. H. Tai

ABSTRACT: Experimental evidence is herein presented to show that C_{22} acids are key intermediates in the microbiological degradation of the cholesterol side chain. Exposure of 19-hydroxycholest-4-en-3-one to microorganisms of the genus *Nocardia* produced four new C_{22} acids besides estrone; the chemical structures of these metabolites have been characterized as 3-hydroxy-19-norbisnorchola-1,3,5(10),17(20)-tetraen-22-oic acid (IIa), 3-hydroxy-19-norbisnorchola-1,3,5-(10)-trien-22-oic acid (IIIa), 3-hydroxy-19-norbisnorchola-1,3,5(10),9(11)-tetraen-22-oic acid (IV), and 3-oxo-19-hydroxybisnorchol-4-en-22-oic acid (Va). From radioactive tracer experiments, it was established that the three-carbon side chain of C_{22} acid can be cleaved under anaerobic conditions to yield propionic acid and 17-keto steroids.

Although the side chain of cholesterol is selectively oxidized, in a stepwise manner, leading to the formation of bile acids and the various classes of steroid hormones, it is believed that relatively little breakdown of the steroid nucelus occurs in mammalian tissues (Talalay, 1957). In contrast, a variety of microorganisms are capable of utilizing sterols, bile acids, and steroid hormones for growth (Turfitt, 1944; Halperin *et al.*, 1954; Schatz *et al.*, 1949). Under suitable conditions, certain microorganisms have the capacity to degrade sterols, such as cholesterol, completely to carbon dioxide and water (Turfitt, 1947). Although there has been considerable progress in elucidating the pathway for the breakdown of the steroid skeleton in recent years (Dodson and Muir, 1961; Sih *et al.*, 1966; Gibson *et al.*, 1966; Lee and Sih, 1967), the mechanism of microbial degradation of the cholesterol side chain is not well understood until now. This paper deals with the identification of C_{22} acids as key intermediates in the breakdown of the cholesterol side chain by microorganisms and the mode of their conversion into 17-keto steroids. A preliminary communication on this subject has already appeared (Sih *et al.*, 1967).

Previous publications have shown that 19-oxygenated derivatives of cholesterol could be efficiently transformed by microorganisms into 17-keto steroids (Sih *et al.*, 1967). By shortening the incubation period to 48 hr, 19-hydroxycholest-4-en-3-one (I) was converted by *Nocardia restrictus* (ATCC 14887) into four acidic products (IIa, IIIa, IV, and Va) (Chart I) besides estrone.

3-Hydroxy-19-norbisnorchola-1,3,5(10),17(20)-tetraen-22-oic Acid (IIa). The first product (mp 241–243°) was initially assigned the structure 3-hydroxy-19-norbisnorchola-1,3,5(10),17(20)-tetraen-22-oic acid (IIa) on the basis of the following data. Molecular weight determination by mass spectrometry showed 326 for the parent compound and 340 for its methyl ester derivative; its ultraviolet absorption peaks ($\lambda_{max}^{CH_3OH}$ 225 mμ (ϵ 19,500) and 280 mμ (ϵ 2300)) are characteristic for a phenol and an α,β,β-trisubstituted α,β-unsaturated carboxylic acid chromophore; its infrared spectrum showed bands at λ_{max}^{Nujol} 2.98, 5.99, 6.21, 6.30, and 6.67 μ; its nuclear magnetic resonance spectrum showed bands at τ 9.21 (3 H, singlet, CH_3 at C-18), 8.11 (3 H, singlet, CH_3 at C-21), 3.57 (singlet), 3.48 (doublet, J = 9 cycles/sec), and 2.97 (doublet, J = 9 cycles/sec) (3 H, aromatic protons).

An analysis of mass spectrum of IIb supports the assigned structure. The spectrum (Figure 1) showed a parent ion peak (*m/e* 340) with peaks at *m/e* 325, 293, and 265, corresponding to the successive loss of CH_3, $HOCH_3$, and CO. The peaks at *m/e* 309 and 281 represent the loss of OCH_3 and $COOCH_3$, respectively, from the parent ion. The *m/e* 253 peak, corre-

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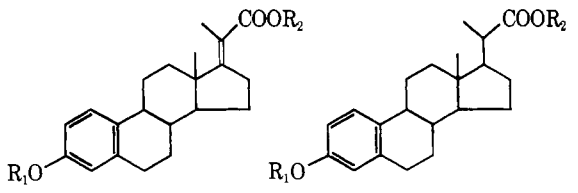
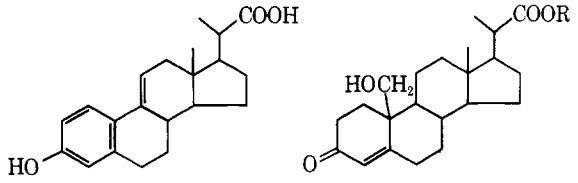
† Present address: Institute of Steroid Chemistry, Syntex Research, Palo Alto, Calif.

CHART I

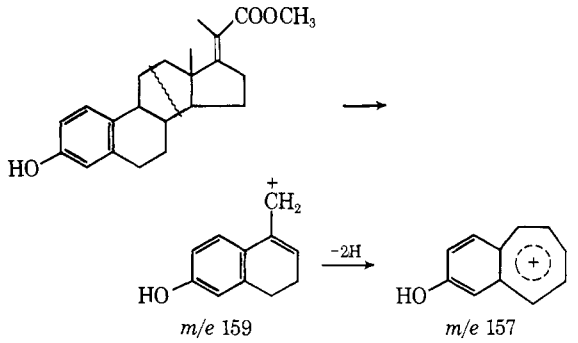


IIa, $R_1 = R_2 = H$ IIIa, $R_1 = R_2 = H$
b, $R_1 = H$; $R_2 = CH_3$ b, $R_1 = H$; $R_2 = CH_3$
c, $R_1 = ACO$; $R_2 = H$ c, $R_1 = ACO$; $R_2 = H$
d, $R_1 = ACO$; $R_2 = CH_3$

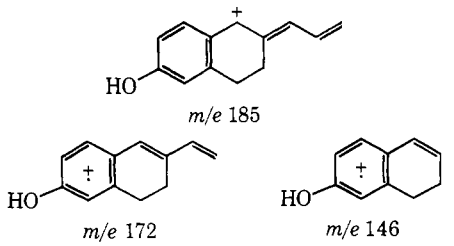


Va, R = H
b, R = CH_3

sponds to the loss of side chain with migration of $\Delta^{17,20}$ double bond to $\Delta^{16,17}$ position prior to loss of the side chain. Subsequent loss of $CH_2{=}CH_2$ leads to the peak at *m/e* 225. Further loss of 42 mass units and the side chain results in the formation of the *m/e* 213 peak, which in turn may give rise, by the loss of a proton, and CH_3, to *m/e* 212 and 199 (Scheme I). The *m/e* 159 peak may be formed *via* rupture of the C-8(14) and C-11(12) bonds, which may lose two protons, resulting in a hydroxybenztropylium ion (*m/e* 157).



The peaks at *m/e* 185, 172, and 146 may correspond to the following fragments.



The structure was further confirmed by partial synthesis. The starting material, 3-acetoxy-19-nor-

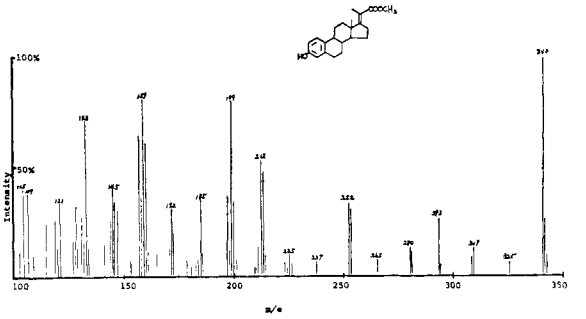


FIGURE 1: The mass spectrum of methyl-3-hydroxy-19-norbisnorchola-1,3,5(10),17(20)-tetraen-22-oate (IIb).

pregna-1,3,5(10)-trien-20-one (VI) was treated with potassium cyanide to give the cyanohydrin VII (Shimizu *et al.*, 1962), which on dehydration with phosphorus oxychloride gave 3-acetoxy-19-norpregna-1,3,5(10),-17(20)-tetraene-20-carbonitrile (VIII) (Kucera and Sorm, 1957). Alkaline hydrolysis of VIII yielded IIa (mp 240.5–242°) identical in all respects (mixture melting point and infrared spectrum) with the sample obtained from fermentation. The addition of cyanide could result in the formation of two stereoisomers. However, the drastic conditions used (at 185–195° for 5 hr) for the hydrolysis of the nitrile (VIII) should result in the thermodynamically favored form. The designation of stereochemistry for IIa in the diagram is preferred since the carboxyl group is larger than the methyl.

3-Hydroxy-19-norbisnorchola-1,3,5(10)-trien-22-oic Acid (IIIa). The second product (mp 216–218°) was initially assigned the structure 3-hydroxy-19-norbisnorchola-1,3,5(10)-trien-22-oic acid (IIIa) from the following data. Molecular weight determination by mass spectrometry showed 328 for the parent compound and 342 for its methyl ester (IIIb); its ultraviolet spectrum exhibited a band at $\lambda_{max}^{CH_3OH}$ 274 mμ (ε 2560); its infrared spectrum showed bands at λ_{max}^{Nujol} 3.04, 5.87, 6.21, and 6.67 μ; its nuclear magnetic resonance spectrum exhibited bands at τ 9.33 (3 H, singlet, CH_3 at C-18), 8.87 (3 H, doublet, J = 7 cycles/sec, CH_3 at C-21), 3.58 (singlet), 3.51 (doublet, J = 9 cycles/sec),

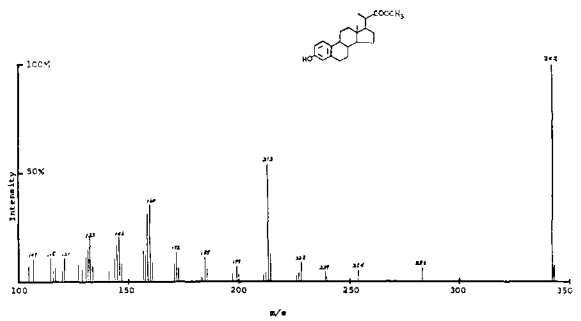
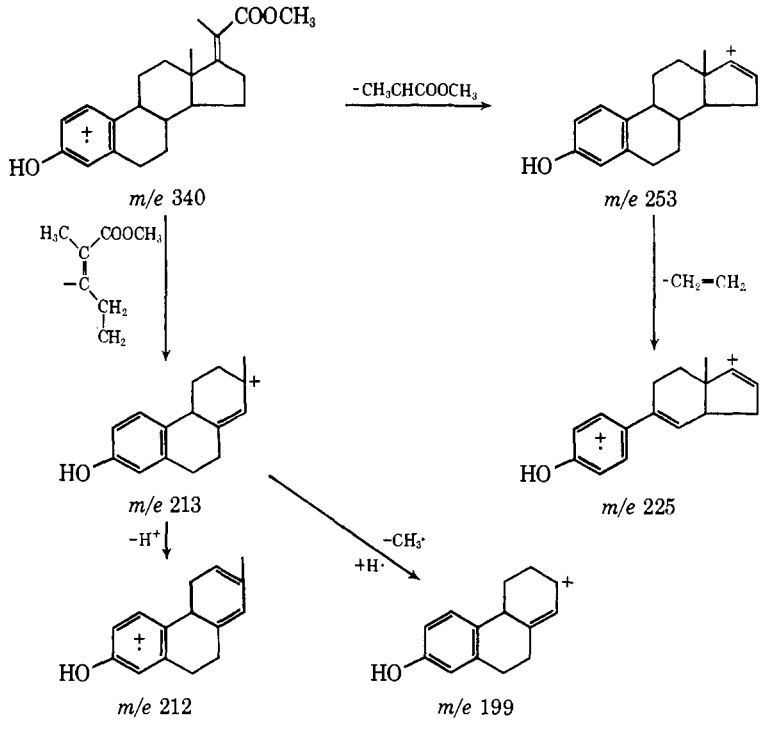


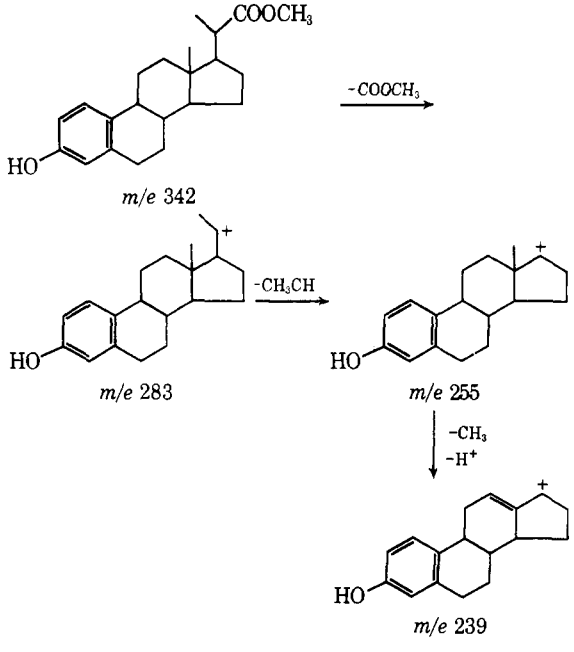
FIGURE 2: The mass spectrum of methyl-3-hydroxy-19-norbisnorchola-1,3,5(10)-trien-22-oate (IIIb).

SCHEME I



and 2.99 (doublet, J = 9 cycles/sec) (3 H, aromatic protons).

An analysis of the mass spectrum of IIIb (Figure 2) supports the assigned structure. The spectrum showed a parent ion peak at *m/e* 342, and peaks at *m/e* 283, 255, and 239, corresponding to the successive loss of $COOCH_3$, CH_3CH, and CH_3 and proton.



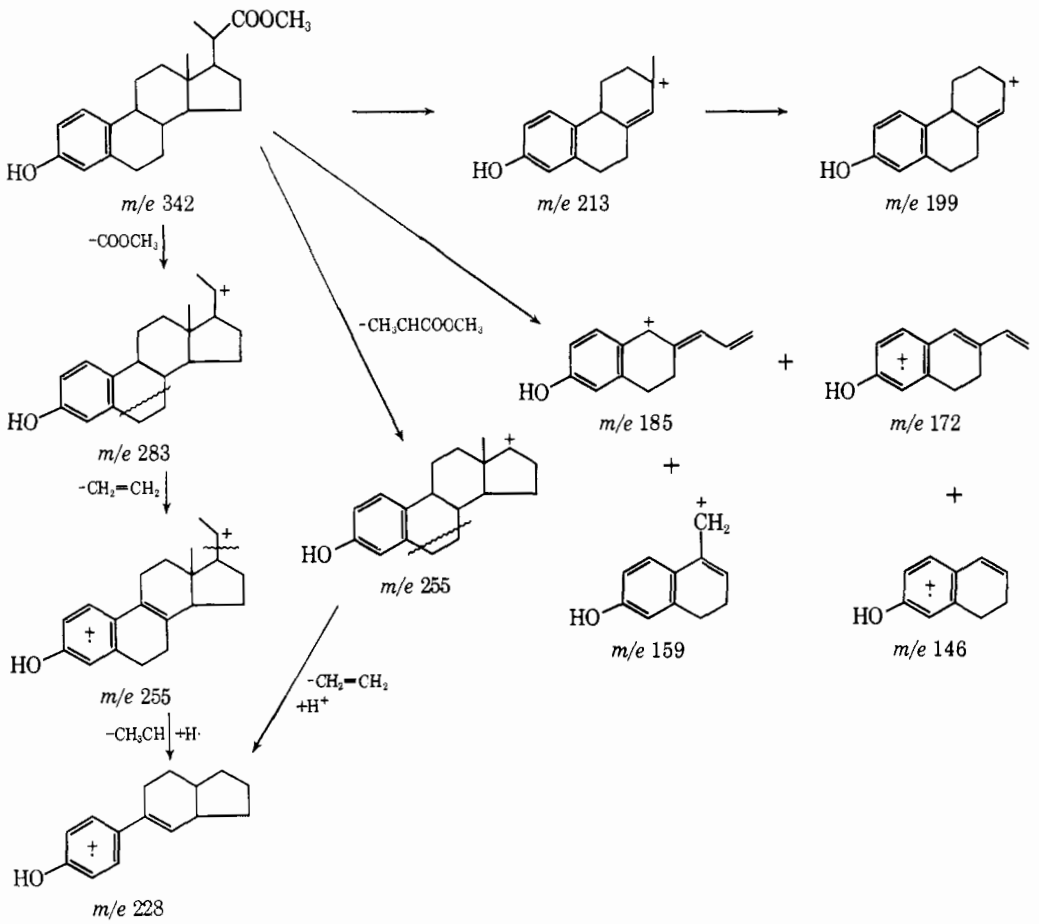
The fragment corresponding to the *m/e* 255 peak may be derived from *m/e* 283 by the loss of $CH_2{=}CH_2$ The *m/e* 228 peak may originate from either of the *m/e* 255 fragments as shown in Scheme II. The peaks at *m/e* 215, 199, 185, 172, 159, and 146 represent the same fragments as shown previously.

The structure of IIIa was confirmed by partial synthesis (Scheme III). The bisnorchola-1,4-diene-3-on-22-oic acid (X), obtained by refluxing IX with DDQ[1] (Burn and Petrow, 1962), was aromatized by treatment with excess of radical anion derived from lithium metal and biphenyl in boiling tetrahydrofuran solution (Dryden *et al.*, 1964). The product (IIIa) (mp 216–218°) was identical in all respects (mixture melting point and infrared spectrum) with a sample obtained from fermentation.

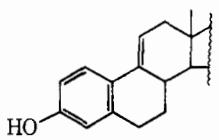
3-Hydroxy-19-norbisnorchola-1,3,5(10),9(11)-tetraen-22-oic Acid (IV). On the basis of the following data, the third product was initially assigned the structure 3-hydroxy-19-norbisnorchola-1,3,5(10),9(11)-tetraen-22-oic acid (IV). Its nuclear magnetic resonance spectrum exhibited bands at τ 9.23 (3 H, singlet, CH_3 at C-18), 8.75 (3 H, doublet, J = 7 cycles/sec, CH_3 at C-21), 4.0 (1 H, vinylic proton at C-11), 3.46 (singlet), 3.39 (doublet, J = 9 cycles/sec), and 2.56 (doublet, J = 9 cycles/sec) (3 H, aromatic protons); its ultraviolet spectrum showed maxima at 264 mμ (ε 13,900) and 300 mμ (ε 3500). These data indicate the presence of one double bond, conjugated to the aromatic ring A.

[1] Abbreviation used: DDQ, 2,3-dichloro-5,6-dicyanobenzoquinone.

SCHEME II



Since the nuclear magnetic resonance spectrum showed the presence of only one vinylic proton, the double bond was assigned to the $\Delta^{9(11)}$ position. The ultraviolet spectrum of XI reported by Dorfman (1953) is also consistent with this assignment. (Owing to the presence of coloring material in our sample, the value of 264 mμ is somewhat lower than expected.)



λ alcohol$_{max}$ 264 mμ (ε 18,000) and 300 mμ (ε 3,000)

Hydrogenation of IV with 10% palladium on carbon as catalyst (Tsuda *et al.*, 1963) resulted in the formation of a product (mp 211–214°) identical in all respects (mixture melting point and infrared spectrum) with a sample of IIIa obtained from fermentation.

3-Oxo-19-hydroxybisnorchol-4-en-22-oic Acid (Va). The fourth product (mp 233–234.5°) was assigned the structure 3-oxo-19-hydroxybisnorchol-4-en-22-oic acid (Va) from the following data. Its ultraviolet spectrum showed a peak at 242 mμ (ε 12,900); its infrared spectrum showed maxima at 2.90, 5.75, 6.00, and 6.07 μ; its nuclear magnetic resonance spectrum exhibited bands at τ 9.35 (3 H, singlet, CH_3 at C-18), 8.93 (3 H, doublet, J = 7 cycles/sec, CH_3 at C-21), 6.26 (2 H, protons on CH_2OH at C-19), and 4.29 (1 H, singlet, vinylic proton at C-4). The hydroxyl group was shown to be acetylable by pyridine–acetic anhydride mixture at room temperature.

Although the mass spectrum of Vb (Figure 3) did not show the expected parent ion peak at *m/e* 374

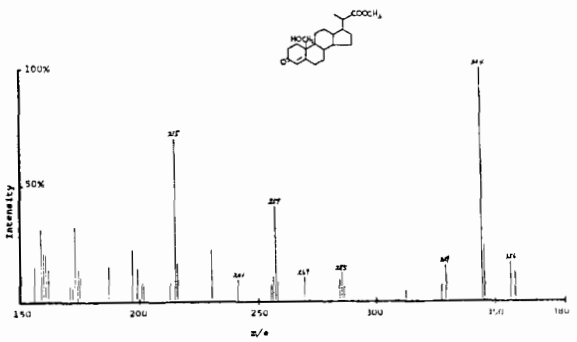
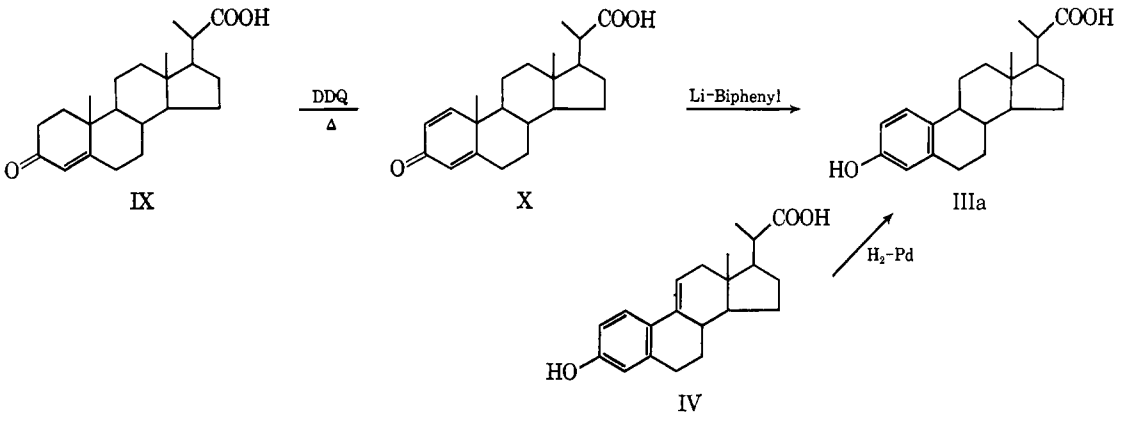


FIGURE 3: The mass spectrum of methyl-3-oxo-19-hydroxybisnorchol-4-en-22-oate (Vb).

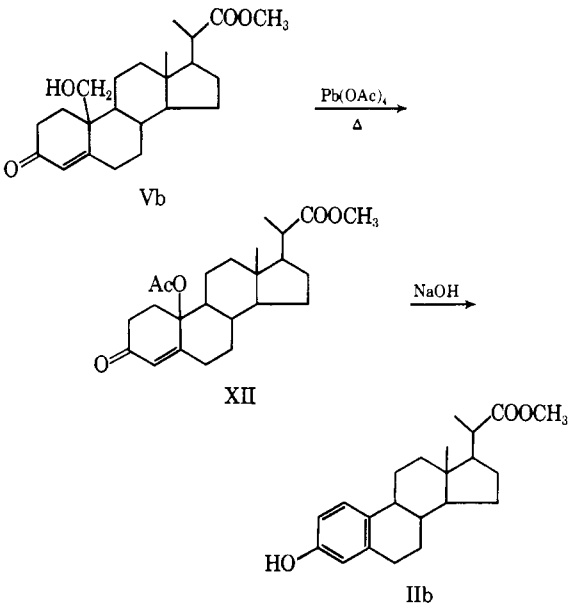
SCHEME III



(Scheme IV), an intense peak was found at *m/e* 344, representing the loss of an HCHO molecule from the parent compound (*m/e* 374). (Since the sample was introduced indirectly at 250°, it is reasonable to assume the loss of HCHO in the inlet system.) The *m/e* 356 peak represents the loss of an H_2O molecule from the parent ion peak; the peak at *m/e* 329 corresponds to the loss of CH_3 from *m/e* 344. The loss of side chain $CH_3CHCOOCH_3$ from *m/e* 344 gives rise to the peak at *m/e* 257; further loss of 42 mass units either from steroid ring D or C-2–C-3 fragment gives the strong peak at *m/e* 215. The loss of CH_3, accompanied with a proton from *m/e* 257, should give rise to the peak at *m/e* 241. The successive loss of $COOCH_3$, followed by CH_3 and a proton from the *m/e* 344 ion, should yield peaks at *m/e* 285 and 269, respectively.

The structure of Vb was confirmed by the following transformations. Compound Vb was refluxed with lead tetraacetate in benzene to give methyl 3-oxo-10β-acetoxy-19-norbisnorchol-4-ene-22-oate (XII) (Alvarez, 1964). Treatment of XII in methanolic sodium hydroxide solution gave IIb (mp 169–171°) identical in all respects (mixture melting point and infrared spectrum) with a sample of IIb obtained by reaction of IIa with diazomethane.

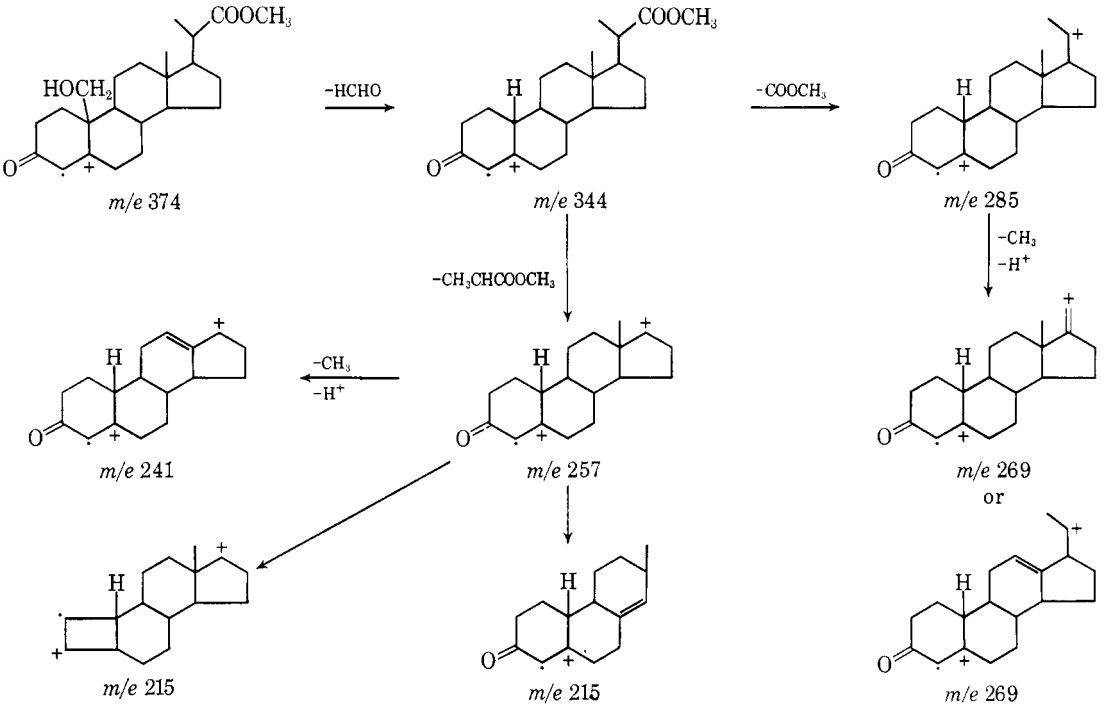
To further verify the intermediacy of these C_{22} acids, IIa was reincubated with both *N. restrictus* and *Nocardia sp.*, but only a trace of estrone was detected after 5 days. Since under these conditions 3-hydroxycholesta-1,3,5(10)-triene was also not metabolized by these organisms, the aromatic A ring appears to interfere with the side-chain cleavage enzymes both at the C_{27} and C_{22} stages. To circumvent this obstacle, 3-oxo-6,19-oxidobisnorchol-4-en-22-oic acid (XVI) was synthesized *via* the conventional hypoiodite reaction (Kalvoda *et al.*, 1961) with minor modifications. The starting material used was methyl-3β-acetoxy-5α-chloro-6β-hydroxybisnorcholan-22-oic acid (XIII) on account of its solubility in apolar solvents. Chromic acid–pyridine was found to be the most suitable reagent for the oxidation of XV; simultaneously, the chlorine atom at C-5 eliminated during the reaction.



The over-all yield of XVI from XIII was about 18%. Conforming to our prediction, it was found that XVI was readily converted into 6,19-oxidoandrost-4-ene-3,17-dione (XVII) by *Nocardia sp.* (ATCC 19170).

In order to determine the metabolic fate of the three-carbon side chain, 3-hydroxybisnorchol-17(20)-en-22-oic acid-22-^{14}C was synthesized *via* a similar sequence of reactions as for IIa, except 3β-acetoxy-5α-pregnan-20-one was used as the starting material. When it was exposed to washed cells of *Nocardia sp.* (ATCC 19170), in the presence of *o*-phenanthroline, a radioactive volatile acid whose chromatographic behavior on a Celite column (Figure 4) was identical with that of propionic acid was obtained. It was isolated as its *S*-benzylisothiuronium salt (mp 151–153°) whose specific activity remained constant after three recrystallizations. Degradation of the propionic acid molecule (Phares, 1951) revealed that all the radioactivity resided in the carboxyl carbon of propionic acid (Sih *et al.*, 1967).

SCHEME IV



Discussion

A considerable amount of effort was expended in the isolation and characterization of metabolites with a view to gaining additional information relative to the degradative sequence of the cholesterol side chain. When 19-hydroxycholest-4-en-3-one was exposed to *N. restrictus* for 48 hr, several phenolic acids accumulated. Unfortunately, all of the products were C_{22} acids, suggesting that their further metabolism is probably rate limiting. Similar results were found using other microorganisms such as *Nocardia sp.* (ATCC 19170) or *Mycobacterium sp.*

Based on general available knowledge on the microbial action on steroids, the mode of formation of IIa, IIIa, and IV from Va could be envisaged as seen in Scheme V. The introduction of a 1,2-double bond into Va results in the formation of a vinylog of a β-hydroxy ketone which may undergo a spontaneous nonenzymic reverse aldolization to yield IIIa and formaldehyde.

Compound IIa can arise via introduction of a $\Delta^{17(20)}$ double bond followed by 1,2-dehydrogenation or *vice versa*. The formation of the $\Delta^{17(20)}$ double bond through hydroxylation at either C-17 or C-20, followed by dehydration, was ruled out on the basis that 3-oxo-bisnorchol-4-en-22-oic acid could be converted into androst-4-ene-3,17-dione by *Nocardia sp.* under *anaerobic* conditions. Although there is no conclusive data available for the assignment of stereochemistry in IIa, it was found that IIa obtained by chemical synthesis was identical in all respects with IIa, obtained by fermentation. The addition of cyanide in the chemical synthesis could result in the formation of two stereoisomers. But under the drastic conditions used for the hydrolysis of the nitrile, the resulting product (IIa) should yield the thermodynamically favored form. Thus, the designation of the stereochemistry of IIa as shown is preferred since the carboxyl group is larger than the methyl.

A plausible pathway leading to the formation of IV may involve: 9α-hydroxylation followed by 1,2-dehydrogenation resulting in the formation of 3,9α-dihydroxybisnorchola-1,3,5(10)-trien-22-oic acid which on dehydration affords IV. The alternative pathway involving 9α-hydroxylation of IIIa followed by dehydration is unlikely in view of the fact that 9α-

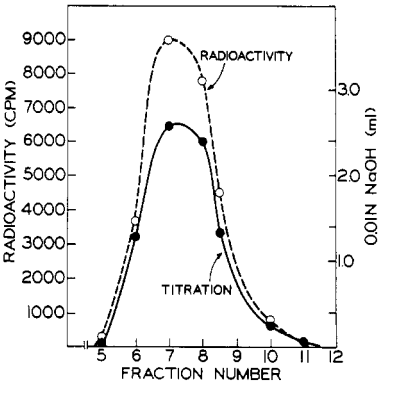
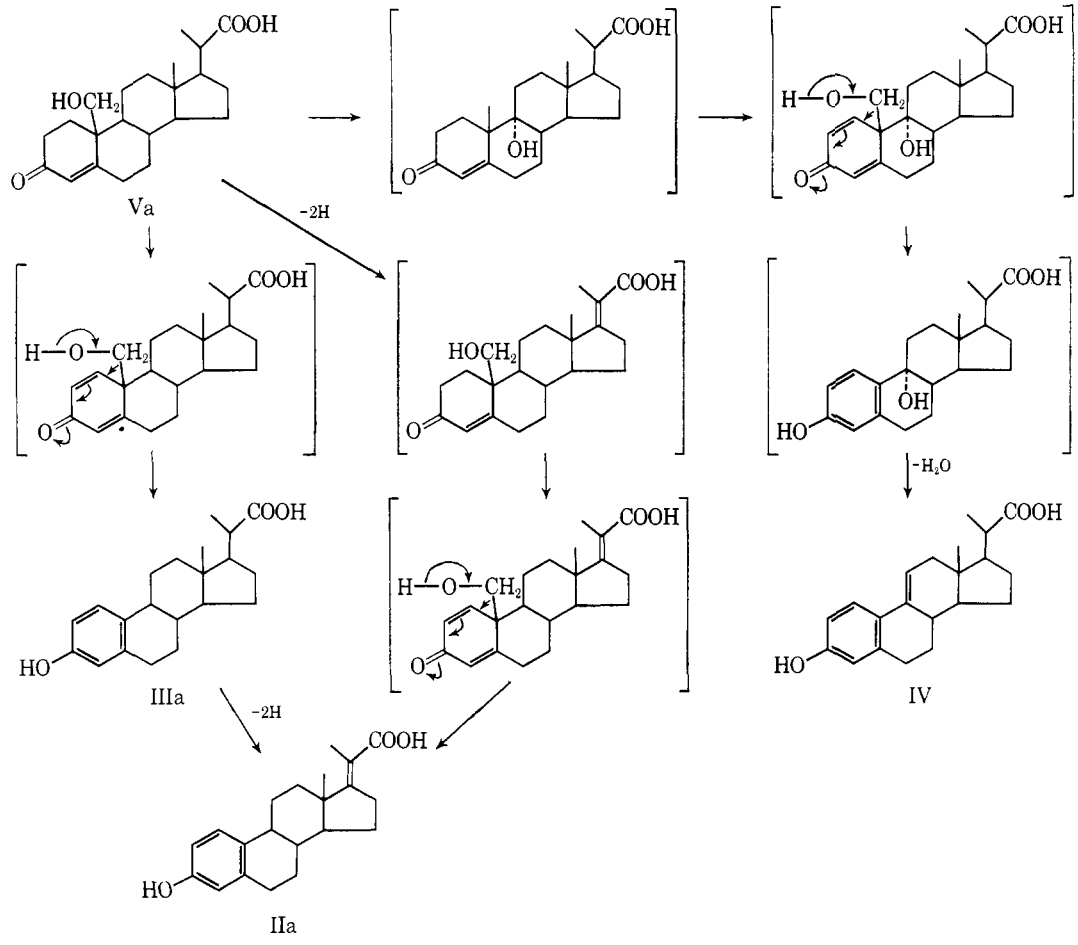


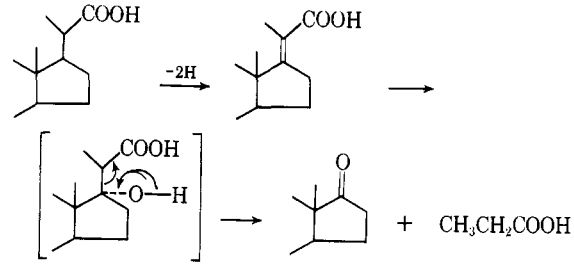
FIGURE 4: Elution profile of propionic acid-^{14}C on a Celite column.

SCHEME V



hydroxylase was inactive on estra-1,3,5(10)-triene-3,17β-diol (Chang and Sih, 1964).

All these results strongly indicate that C_{22} acids are intermediates in the degradative sequence. Although compound IIIa was very poorly metabolized into estrone even after prolonged incubation with *N. restrictus* and *Nocardia sp.* (ATCC 19170), 3-oxobisnorchol-4-en-22-oic acid and 3-oxo-6,19-oxidobisnorchol-4-en-22-oic acid were efficiently converted into androst-4-ene-3,17-dione and 6,19-oxidoandrost-4-ene-3,17-dione (XVII), respectively, by these organisms (Lee, 1966). Furthermore, when radioactive 3-hydroxybisnorchol-17(20)-en-22-oic acid-22-^{14}C was exposed to washed cells of *Nocardia sp.* (ATCC 19170), radioactive propionic acid ($CH_3CH_2{}^{14}COOH$) was isolated as its *S*-benzylisothiuronium salt. These results clearly show that the degradation of the hydrocarbon side chain of cholesterol proceeds *via* C_{22} acid intermediates, which confirms the finding of Whitmarsh. Since 3-oxobisnorchol-4-en-22-oic acid and 3-oxobisnorchol-17(20)-en-22-oic acid could be converted into androst-4-ene-3,17-dione by these microorganisms under *anaerobic* conditions, one may envisage the degradation of the three-carbon side chain involving dehydrogenation, hydration, and aldolytic fission.



Experimental Section

Materials. The conditions of fermentation and maintenance of microorganisms were the same as those described previously (Sih *et al.*, 1967). All of the solvents and inorganic chemicals were reagent grade. Petroleum ether refers to the fraction with a boiling point of 40–70°. Silica gel HF for thin-layer chromatography was purchased from Brinkmann Instruments, Inc. Silicic acid (Mallinckrodt 2847) was used for column chromatography. Standard grade cellulose powder was purchased from H. Reeve Angel and Co. Diazomethane was prepared from Diazald according to the method of Moore and Reed (1961). Diazald (*N*-methyl-*N*-nitro-*p*-toluenesulfonamide) and

2-hydroxydiethanol were products of the Aldrich Chemical Co. Tetrahydrofuran was refluxed with $LiAlH_4$ prior to redistillation. 19-Hydroxycholest-4-en-3-one was prepared according to the method of Kalvoda *et al.* (1961).

Methods. Melting points, determined on a Thomas-Hoover melting point apparatus, are corrected. Infrared spectra were recorded on a Beckman IR5A double-beam infrared recording spectrophotometer. Ultraviolet absorption spectra were determined on a Cary Model 11MS recording spectrophotometer. Mass spectra were determined by Dr. R. Schaffer of Morgan Schaffer Corp. of Montreal, Can. Microanalyses were carried out by Mr. J. Alicino of Metuchen, N. J. Nuclear magnetic resonance spectra were determined on a Varian Associates recording spectrometer (A60) at 60 Mcycles in deuterated chloroform or deuterated dimethyl sulfoxide with tetramethylsilane as an internal standard. Chemical shifts are reported in values (parts per million) (Tier, 1958). Unless otherwise stated, the paper chromatographic system used throughout this work consisted of toluene–propylene glycol (Zaffaroni *et al.*, 1950). Values of $[\alpha]_D$ have been approximated to the nearest degree. 17-Keto function was detected by the Zimmermann reaction (Zimmermann, 1935).

Fermentation of 19-Hydroxycholest-4-en-3-one (I). *N. restrictus* (ATCC 14887) was inoculated into 48.4 l. of Difco nutrient broth (121 2-l. erlenmeyer flasks) at 25° on a rotary shaker (250 rpm, 1-in. stroke). 19-Hydroxycholest-4-en-3-one (24.2 g) (I), dissolved in 605 ml of *N,N*-dimethylformamide, was distributed equally among the flasks and the fermentation was continued for 72 hr. At the end of the fermentation, the culture broth was acidified with glacial acetic acid to a pH of 3.0 and extracted three times with 1.6-l. portions of chloroform. The combined chloroform extract was dried over sodium sulfate and evaporated to dryness *in vacuo* to give 32 g of residue. The residue was chromatographed on a cellulose powder column (5.8 × 60 cm) using propylene glycol as the stationary phase. The column was eluted with a cyclohexane–benzene mixture saturated with propylene glycol. The 20–40% benzene in cyclohexane fractions contained 13.5 g of residue, consisting mainly of starting material. The 50–75% benzene in cyclohexane fractions contained 1.9 g of estrone after work up. The column was further eluted with benzene, and 1.5-l. fractions were collected. Fractions 1–4 contained the 1.8-g mixture of IIa and IIIa. Fractions 5 and 6 contained the 1.2-g mixture of IIIa and IV after work up. Fractions 7 and 8 afforded 2.0 g of a mixture of IIIa and Va. At this point, the column was eluted with 3 l. of chloroform to displace all the material still remaining on the column. The chloroform eluent was washed several times with 1.5-l. portions of water, dried over sodium sulfate, and evaporated to dryness *in vacuo* to give 2.0 g of residue, consisting mainly of IIIa and Va.

Residue (1.8 g) from fractions 1–4 was rechromatographed on a cellulose powder column (4.5 × 50 cm) and was eluted with benzene saturated with propylene glycol, and 20-ml fractions were collected. Fractions 180–255 contained 255 mg of 3-hydroxy-19-norbisnorchola-1,3,5(10),17(20)-tetraen-22-oic acid (IIa) after concentration. Further purification was effected by rechromatography of the residue on a small silicic acid column (1.5 × 7 cm); elution of the column with 0.5% methanol in chloroform afforded 240 mg of IIa. Three recrystallizations from a methanol–acetone–petroleum ether mixture gave analytical sample: mp 241–243°, molecular weight (mass spectrum) 326, $[\alpha]_D^{26}$ +28° (dioxane), $\lambda_{max}^{CH_3OH}$ 280 mμ (ε 2300) and 225 mμ (ε 19,500).

Anal. Calcd for $C_{21}H_{26}O_3 \cdot 0.5CH_3COCH_3$: C, 76.06; H, 8.17. Found: C, 75.78, 75.59; H, 8.76, 8.48.

Fractions 301–440 contained 780 mg of 3-hydroxy-19-norbisnorchola-1,3,5(10)-trien-22-oic acid (IIIa) after evaporation. Further purification was effected by rechromatography of the residue on a small silicic acid column (1.8 × 8 cm); elution of the column with 0.5% methanol in chloroform gave 750 mg (crystals) of compound IIIa. Three recrystallizations from methanol–acetone–petroleum ether mixture gave an analytical sample: mp 216–218°, molecular weight (mass spectrum) 328, $[\alpha]_D^{21}$ +112° (dioxane).

Anal. Calcd for $C_{21}H_{28}O_3 \cdot 0.5CH_3COCH_3$: C, 75.54; H, 8.82. Found: C, 74.96; H, 8.75.

Residue (1.2 g) obtained from fractions 5 and 6 was rechromatographed on a cellulose powder column (4.5 × 50 cm). The column was eluted with benzene, saturated with propylene glycol, and 20-ml fractions were collected. Fractions 160–300 contained 600 mg of 3-hydroxy-19-norbisnorchola-1,3,5(10)-trien-22-oic acid (IIIa) after work-up. Fractions 345–450 yielded 205 mg of an oily brown residue. It was streaked on two 20 × 20 cm silica gel HF thin-layer plates (1 mm thick) and was developed with benzene–dioxane–acetic acid (45:15:1.5, v/v). The ultraviolet light absorbing bands were scratched out and eluted with 60 ml of ethyl acetate. After concentration, 105 mg of an oily yellow residue of 3-hydroxy-19-norbisnorchola-1,3,5(10),9(11)-tetraen-22-oic acid (IV) was obtained: $\lambda_{max}^{alcohol}$ 264 mμ (ε 13,900) and 300 mμ (ε 3500). The residue (2.0 g) from fractions 7 and 8 was chromatographed on a silicic acid–Celite (80:20) column (3 × 50 cm), and the column was eluted with benzene–dioxane–acetic acid mixture. In the benzene–dioxane–acetic acid (80:15:0.5) fraction, 150 mg of 3-hydroxy-19-norbisnorchola-1,3,5(10)-trien-22-oic acid (IIIa) was isolated. In the benzene–dioxane–acetic acid (80:25:1.5) mixture fraction, 225 mg of 3-oxo-19-hydroxybisnorchol-4-en-22-oic acid (Va) was obtained after concentration of the solvent. After repeated crystallization of Va from methanol–acetone–petroleum ether mixture, an analytical sample was obtained: mp 233–234.5°; $\lambda_{max}^{CH_3OH}$ 242 mμ (ε 12,900); $[\alpha]_D^{23}$ +216° (dioxane); λ_{max}^{Nujol} 2.90, 5.75, 6.00, and 6.07 μ.

Anal. Calcd for $C_{22}H_{32}O_4$: C, 73.30; H, 8.95. Found: C, 73.03; H, 8.79.

3-Acetoxy-19-norbisnorchola-1,3,5(10),17(20)-tetraen-22-oic Acid (IIc). 3-Hydroxy-19-norbisnorchola-1,3,5(10),17(20)-tetraen-22-oic acid (40 mg) was dissolved in 6 ml of a mixture of acetic anhydride and pyridine

(1:1) and was left standing at room temperature for 16 hr. The pyridine and excess acetic anhydride were evaporated with a stream of air to yield an oily yellow residue. The residue was dissolved in $CHCl_3$ and was chromatographed on a small silicic acid column (1 × 5 cm), and the column was eluted with $CHCl_3$. Evaporation of the chloroform eluent afforded crystalline residue (37 mg). Several recrystallizations from acetone–petroleum ether mixture gave an analytical sample: mp 234–236° dec; $[\alpha]_D^{25}$ +103° (methanol); $\lambda_{max}^{alcohol}$ 228 mμ (ε 16,600) and 267 mμ (ε 1500); λ_{max}^{Nujol} 5.64, 5.98, 6.20, and 6.70 μ.

Anal. Calcd for $C_{23}H_{28}O_4$: C, 74.97; H, 7.66. Found: C, 74.79; H, 7.71.

Methyl-3-hydroxy-19-norbisnorchola-1,3,5(10),17(20)-tetraen-22-oate (IIb). To a solution of 40 mg of 3-hydroxy 19-norbisnorchola-1,3,5(10),17(20)-tetraen-22-oic acid (IIa) in 5 ml of a chloroform–methanol (1:1) mixture was added excess diazomethane. The mixture was left standing at room temperature for 3 hr; the excess diazomethane and solvent were removed with a stream of air. The oily yellow residue obtained was chromatographed on a small silicic acid column (1 × 5 cm) and the column was eluted with chloroform. The chloroform eluents were further purified by rechromatography on a cellulose powder column (1.7 × 7 cm), and it was eluted with isooctane saturated with propylene glycol. The residue obtained was repeatedly crystallized from ether–petroleum ether mixture to give a sample: mp 195.5–197°; $[\alpha]_D^{24}$ +42° (methanol); $\lambda_{max}^{alcohol}$ 228 mμ (ε 18,900) and 280 mμ (ε 2450); λ_{max}^{Nujol} 2.96, 6.02, 6.20, and 6.65 μ.

Anal. Calcd for $C_{22}H_{28}O_3$: C, 77.61; H, 8.29. Found: C, 77.51; H, 8.57.

3-Acetoxy-20α-hydroxy-19-norpregna-1,3,5(10)-triene-20-carbonitrile (VII). The 3-acetoxy-19-norpregna-1,3,5(10)-triene-20-one (VI) (3.2 g) was dissolved in 130 ml of 95% ethanol, 21 ml of water, and 19 ml of glacial acetic acid, and 20 g of potassium cyanide was then added with stirring. The mixture was stirred for 6 hr at room temperature and left standing at room temperature for another 24 hr. At the end of this period, it was poured into 400 ml of ice water. The resulting precipitate was collected by filtration. The precipitate was dissolved in 500 ml of ethyl acetate and was washed once with 5% sodium carbonate solution, twice with water, dried over sodium sulfate, and evaporated to dryness *in vacuo*. One crystallization from ethyl acetate gave 1.96 g of crystalline VII.

3-Acetoxy-19-norpregna-1,3,5(10),17(20)-tetraene-20-carbonitrile (VIII) (26). The carbonitrile (VII) (1.96 g) from above was dissolved in 25 ml of pyridine and 5 ml of phosphorus oxychloride was then added. The mixture was heated under reflux for 3 hr, cooled to room temperature, and poured into 200 ml of ice water, containing 28 ml of concentrated hydrochloric acid. The mixture was extracted with chloroform, washed with 3% sodium bicarbonate solution and water, dried over sodium sulfate, and evaporated to dryness *in vacuo*. The residue was dissolved in 10 ml of benzene and chromatographed on a silicic acid–Celite (80:20) column (1.5 × 42 cm), and 10-ml fractions were collected. Elution of the column with a benzene–chloroform (1:1) mixture gave 480 mg of VIII in fractions 140–157. Several crystallizations from acetone–petroleum ether mixture gave an analytical sample: mp 180–182°; $[\alpha]_D^{26}$ +30° ($CHCl_3$); $\lambda_{max}^{alcohol}$ 219 mμ (ε 26,000); $\lambda_{max}^{CHCl_3}$ 4.52, 5.72, 6.13, 6.21, and 6.70 μ. Further elution of the column with chloroform gave 205 mg of VI.

Anal. Calcd for $C_{23}H_{27}O_2N$: C, 79.05; H, 7.79; N, 4.01. Found: C, 79.51; H, 8.19; N, 4.38.

3-Hydroxy-19-norbisnorchola-1,3,5(10),17(20)-tetraen-22-oic Acid (IIa) from Hydrolysis of 3-Acetoxy-19-norpregna-1,3,5(10),17(20)-tetraene-20-carbonitrile (VIII). Compound VIII (150 mg) was placed in a 50-ml round-bottom flask with 10 ml of 2-hydroxyethyl ether and 1.2 g of sodium hydroxide in 1.5 ml of water and the flask was placed in an oil bath. The water was removed by raising the temperature to 170° over a 30-min period; it was then heated at 185–195° for 5 hr. The reaction mixture was cooled to room temperature and poured into 300 ml of ice water, acidified with dilute hydrochloric acid to a pH of 3.0, and extracted with chloroform. The chloroform extract was dried over sodium sulfate and evaporated to dryness *in vacuo*. The residue was chromatographed on a cellulose powder column (3 × 35 cm), using propylene glycol as the stationary phase; the column was eluted with benzene, saturated with propylene glycol, and 10-ml fractions were collected. Fractions 120–185 consisted of 53 mg of desired compound IIa after the usual work-up. One crystallization from methanol–acetone–petroleum ether gave mp 240.5–242° identical in all respects (mixture melting point and infrared spectrum) with an authentic sample of IIa obtained from the fermentation of 19-hydroxycholestenone with *N. restrictus*.

3-Acetoxy-19-norbisnorchola-1,3,5(10)-trien-22-oic Acid (IIIc). 3-Hydroxy-19-norbisnorchola-1,3,5(10)-trien-22-oic acid (IIIa) (94 mg) was dissolved in 8 ml of an acetic anhydride–pyridine (1:1) mixture and was left standing at room temperature for 10 hr. The pyridine and excess acetic anhydride were evaporated to dryness with a stream of air to give crystalline residue of IIIc. The residue was recrystallized several times from acetone–petroleum ether to give an analytical sample: mp 202–203.5°; $[\alpha]_D^{23}$ +231° (methanol); $\lambda_{max}^{alcohol}$ 276 mμ (ε 840); λ_{max}^{Nujol} 5.67, 5.88, 6.21, and 6.71 μ.

Anal. Calcd for $C_{23}H_{30}O_4$: C, 74.56; H, 8.16. Found: C, 74.59; H, 8.11.

Methyl-3-acetoxy-19-norbisnorchola-1,3,5(10)-trien-22-oate (IIId). Methyl-3-hydroxy-19-norbisnorchola-1,3,5(10)-trien-20-oate (140 mg) (IIIb) was dissolved in 10 ml of an acetic anhydride–pyridine (1:1) mixture. The mixture was left standing at room temperature for 16 hr and the excess acetic anhydride and pyridine were removed with a stream of air. The oily yellow residue obtained was dissolved in chloroform and was then chromatographed on a silicic acid column (2 × 7.5 cm) for purification. Elution of the column with chloroform gave 138 mg of the desired product after removal of the solvent. Four recrystallizations from

acetone–petroleum ether gave an analytical sample: mp 140.5–142°; $[\alpha]_D^{23}$ +198° ($CHCl_3$); $\lambda_{max}^{alcohol}$ 266 mμ (ε 760); λ_{max}^{Nujol} 5.66, 5.76, 6.20, and 6.68 μ.

Anal. Calcd for $C_{24}H_{32}O_4$: C, 74.97; H, 8.39. Found: C, 74.81; H, 8.04.

3-Oxobisnorchola-1,4-dien-22-oic Acid (X). A mixture of 3-oxobisnorchola-4-en-22-oic acid (IX) (2 g), 2,3-dichloro-5,6-dicyanobenzoquinone (DDQ) (1.8 g), and dry dioxane (25 ml) was heated under reflux for 15 hr. The solution was diluted with ether, and the resulting precipitate was filtered and washed thoroughly with ether. The combined ether solution was concentrated to dryness, dissolved in 10 ml of chloroform, and then chromatographed on a silicic acid column (3 × 14 cm). Elution of the column with a chloroform–acetone (80:20) mixture gave X (1.3 g). Recrystallization from acetone–petroleum ether gave a specimen (mp 234–237°) identical in all respects with an authentic sample (mixture melting point and infrared spectrum) (Lee, 1966).

3-Hydroxy-19-norbisnorchola-1,3,5(10)-trien-22-oic Acid (IIIa) from the Aromatization of 3-Oxobisnorchola-1,4-dien-22-oic Acid (X). A solution of biphenyl (0.8 g) in freshly distilled tetrahydrofuran (8 ml) was heated to reflux under nitrogen with lithium metal (0.05 g) for 1 hr with stirring. The dark green solution was cooled to room temperature and a solution of X (0.5 g in 3 ml of tetrahydrofuran) was added dropwise with stirring. The mixture was stirred at room temperature for 30 min. The excess lithium metal was destroyed by the careful addition of a small amount of water. Finally, the mixture was diluted with water, acidified with dilute hydrochloric acid to a pH of 3.0, and extracted with benzene. The benzene solution was extracted with 6% sodium bicarbonate and the sodium bicarbonate solution was acidified with dilute hydrochloric acid and extracted with chloroform. The chloroform extract was dried over sodium sulfate and evaporated to dryness *in vacuo*. The residue was chromatographed on a cellulose powder column (3.2 × 52 cm) using propylene glycol as stationary phase, and 10-ml fractions were collected. Elution of the column with cyclohexane–benzene (25:75) saturated with propylene glycol gave 305 mg of unreacted X. Further elution of the column with benzene afforded IIIa in fractions 110–180. Crystallization of the residue from methanol–acetone–petroleum ether gave 103 mg of IIIa (mp 213–215°) identical in all respects (mixture melting point and infrared spectrum) with an authentic compound obtained from the fermentation of 19-hydroxycholestenone with *N. restrictus*.

Catalytic Hydrogenation of 3-Hydroxy-19-norbisnorchola-1,3,5(10),9(11)-tetraen-22-oic Acid (IV) to 3-Hydroxy-19-norbisnorchola-1,3,5(10)-trien-22-oic Acid (IIIa). An oily yellow compound (50 mg) (IV) obtained from the fermentation was dissolved in 15 ml of 95% ethanol, 50 mg of 10% palladium on carbon was then added, and the mixture was stirred under hydrogen at atmospheric pressure and 23°. After 3 hr, 1.1 M equiv of hydrogen was consumed. The suspension was filtered and the filtrate was evaporated to dryness. The residue was streaked on one 20 × 20 cm silica gel HF thin-layer plate (1 mm in thickness) and developed with benzene–dioxane–acetic acid (90:25:2). The desired ultraviolet light absorbing band was scratched out and was eluted with 40 ml of a chloroform–methanol (1:1) mixture. The eluent was evaporated to dryness to give a residue. One crystallization of the residue from methanol–acetone–petroleum ether gave a sample of IIIa (mp 211–214°, 25 mg) identical in all respects (mixture melting point and infrared spectrum) with an authentic compound of IIIa obtained from the fermentation of 19-hydroxycholestenone with *N. restrictus*.

Methyl-3-oxo-19-hydroxybisnorchol-4-en-22-oate (Vb). To 110 mg of 3-oxo-19-hydroxychol-4-en-22-oic acid (Va) in 5 ml of a methanol–chloroform (1:1) solution was added excess of diazomethane and the mixture was left standing at room temperature for 3 hr. Excess diazomethane was removed with a stream of air and the solution was evaporated to dryness. Crystallization of the residue from acetone–petroleum ether mixture gave compound Vb of mp 199–200.5°; $[\alpha]_D^{23}$ +238° (methanol); $\lambda_{max}^{alcohol}$ 242 mμ (ε 13,180); λ_{max}^{Nujol} 2.96, 5.77, 6.03, and 6.21 μ.

Anal. Calcd for $C_{23}H_{34}O_4$: C, 73.76; H, 9.15. Found: C, 73.98; H, 8.96.

Methyl-3-oxo-10β-acetoxy-19-norbisnorchol-4-en-22-oate (XII). The methyl ester of V (101 mg) from the above experiment was dissolved in 3 ml of dry benzene and heated under reflux with lead tetraacetate (220 mg) for 2.5 hr. The reaction mixture was cooled to room temperature and the precipitate (lead diacetate) was filtered and washed with benzene. The combined benzene solution was evaporated to dryness and redissolved in ether. The ethereal solution was filtered and the filtrate was evaporated *in vacuo* to dryness. The residue was dissolved in chloroform and chromatographed on a small silicic acid column (1 × 6 cm); elution of the column with chloroform afforded 56 mg of XII. Several crystallizations from acetone–ethyl ether–petroleum ether gave an analytical sample: mp 203–205.5°; $[\alpha]_D^{23}$ +8° ($CHCl_3$); $\lambda_{max}^{alcohol}$ 242 mμ (ε 15,350); λ_{max}^{Nujol} 5.72, 5.75, 5.95, and 6.12 μ.

Anal. Calcd for $C_{24}H_{34}O_5$: C, 71.62; H, 8.51. Found: C, 71.28; H, 8.56.

Methyl-3-hydroxy-19-norbisnorchola-1,3,5(10)-trien-22-oate (IIIb). A. FROM REACTION OF 3-HYDROXY-19-NORBISNORCHOLA-1,3,5(10)-TRIEN-22-OIC ACID (IIIa) WITH DIAZOMETHANE. To 230 mg of IIIa in 10 ml of a methanol–chloroform (1:1) solution was added excess diazomethane. The mixture was left standing at room temperature for 3 hr. Excess diazomethane and solvent were removed with a stream of air. The residue was chromatographed on a silicic acid column (2 × 5 cm); elution of the column with chloroform yielded 211 mg of IIIb. Repeated crystallizations from acetone–petroleum ether gave a sample: mp 172–173°; $[\alpha]_D^{23}$ +214° (methanol); $\lambda_{max}^{alcohol}$ 280 mμ (ε 2570); λ_{max}^{Nujol} 2.92, 5.88, 6.19, 6.32, and 6.70 μ.

Anal. Calcd for $C_{22}H_{30}O_3$: C, 77.15; H, 8.83. Found: C, 77.19; H, 9.28.

B. From aromatization of methyl 3-oxo-10β-acetoxy-19-norbisnorchol-4-en-22-oate (XII). Compound XII (56 mg) was dissolved in 5 ml of 2% sodium hydroxide in methanol and the mixture was left standing at room temperature for 3 hr. At the end of this period, it was poured into 50 ml of water and acidified with dilute hydrochloric acid. The precipitate was filtered, dissolved in chloroform, and streaked on one 20 × 20 cm silica gel HF thin-layer plate (1 mm in thickness). The plate was developed with 5% acetone in chloroform and the desired band was scratched out and eluted with a 60 ml of a methanol–chloroform (1:1) mixture, and the eluent was evaporated to dryness. Crystallization of the residue from acetone–petroleum ether mixture gave 35 mg of IIIb (mp 169–171°) identical in all respects (mixture melting point and infrared spectrum) with a sample of IIIb obtained from A) above.

Methyl-3β-acetoxy-5α-chloro-6β-hydroxybisnorcholan-22-oic Acid (XIII). Methyl-3-acetoxybisnorchol-5-en-22-oic acid (20 g) was dissolved in 400 ml of ethyl ether. After the addition of 33.6 g of bleaching powder and 1260 ml of water, the mixture was stirred vigorously for 5 min. Glacial acetic acid (22 ml) was then added to the reaction mixture and the contents were stirred again at room temperature for 30 min. At the end of this period, it was diluted with a methylene dichloride and ethyl ether mixture (1:1) to dissolve the precipitated product. The organic layer was washed three times with water, dried over anhydrous sodium sulfate, and evaporated to dryness to give 13 g of crude residue (65%). A small sample was recrystallized from acetone–petroleum ether to afford a sample: mp 190–192°; $[\alpha]_D^{26}$ −34.9° (*c* 0.9, in $CHCl_3$); λ_{max}^{Nujol} 2.89, 5.80, 5.88, and 7.93 μ.

Anal. Calcd for $C_{25}H_{39}ClO_5$: C, 66.00; H, 8.60. Found: C, 65.74; H, 8.58.

Methyl-3β-acetoxy-5α-chloro-6,19-oxidobisnorcholan-22-oic Acid (XIV). A suspension consisting of 78 g of lead tetraacetate and 27 g of $CaCO_3$ in 3.5 l. of cyclohexane was heated at 80° for 10 min. The mixture was stirred, irradiated with a 500-w lamp, and refluxed for 60 min. The mixture was then cooled and filtered through Celite. The residue was exhaustively washed with ether and the combined filtrate was washed with 500 ml of 10% sodium thiosulfate solution three times, followed by 1.5 l. of water three times. The organic layer was then dried over sodium sulfate and evaporated to dryness to yield 12.3 g of crude residue. The residue was chromatographed over a silicic acid–Celite (90:10) column (5.6 × 58 cm). The column was washed with benzene and the desired product was eluted with a benzene–chloroform mixture (8:2) to give 8.9 g of crude crystals (71%). Two recrystallizations from acetone–petroleum ether afforded a sample: mp 166–168°, $[\alpha]_D^{26}$ −9.3° (*c* 0.8, in $CHCl_3$), λ_{max}^{Nujol} 5.80 and 8.14 μ.

Anal. Calcd for $C_{25}H_{37}ClO_5$: C, 66.28; H, 8.23. Found: C, 66.30; H, 8.23.

3β-Hydroxy-5α-chloro-6,19-oxidobisnorcholan-22-oic Acid (XV). To 8.5 g of the chloro ester (XIV) from the previous step in 850 ml of methanol was added 8.5 g of potassium carbonate in 42 ml of water. After the reaction mixture had been refluxed for 60 min, it was evaporated to a small volume and acidified with 10% HCl. An oily residue was obtained (4.6 g), which after repeated chromatography on thin-layer plates and column, resisted crystallization. Therefore, this oily residue was used directly for the subsequent step. Its infrared spectrum in Nujol showed bands at 2.94, 3.10, and 5.81 μ.

3-Oxo-6,19-oxidobisnorchol-4-en-22-oic Acid (XVI). The oily residue (4 g) from the previous step was dissolved in 250 ml of pyridine and cooled in an ice bath. Chromic trioxide (25 g) was slowly added with stirring and the reaction mixture was left standing at room temperature for 16 hr. The mixture was dilated with large quantities of water, acidified, and then extracted with 700 ml of ethyl acetate three times. The organic layer was washed with water, dried over sodium sulfate, and the solvent was removed *in vacuo* to yield 3.8 g of residue. The residue was chromatographed over a silicic acid–Celite (9:1) column (3.6 × 38 cm). Elution of the column with chloroform–methanol (99:1) afforded 2.48 g (62%) of the desired product. Recrystallization from acetone–petroleum ether gave an analytical sample: mp 163–164°; $[\alpha]_D^{26}$ −82.3° (*c* 0.9, in $CHCl_3$); λ_{max}^{Nujol} 5.92, 5.98, and 6.2 μ; $\lambda_{max}^{alcohol}$ 241 mμ (ε 11,000).

Anal. Calcd for $C_{22}H_{30}O_4$: C, 73.71; H, 8.44. Found: C, 74.43; H, 8.93.

Transformation of 3-Oxo-6,19-oxidobisnorchol-4-en-22-oic Acid (XVI) by Nocardia sp. (ATCC 19170). Exposure of 200 mg of XVI (300 μg/ml) to *Nocardia sp.* (ATCC 19170) under the usual fermentative conditions (Sih *et al.*, 1967) afforded 22 mg of 6,19-oxidoandrost-4-ene-3,17-dione (XVII) (mp 182–185°) whose infrared spectrum was identical with an authentic specimen and a mixture melting point determination showed no depression.

Synthesis of 3β-Hydroxybisnorchola-5(6),17(20)-dien-22-oic Acid-22-^{14}C. Pregnenolone acetate (60 mg) was dissolved in a mixture consisting of ethanol (2.0 ml), water (0.4 ml), and acetic acid (0.4 ml). To this mixture was added with stirring 360 mg of potassium cyanide, containing 2.8 mg of KCN-^{14}C (1.1053 mc). The reaction mixture was stirred for 6 hr and was then left standing at room temperature for 18 hr. After diluting the system with 6 ml of ice water, it was extracted five times with 20-ml portions of ethyl acetate. The solvent extract was washed twice with water and then evaporated to dryness. The residue was dissolved in 0.6 ml of pyridine. After the addition of $POCl_3$ (0.24 ml), the mixture was refluxed at 100–105° for 3 hr. The excess $POCl_3$ was destroyed by the addition of 0.3 ml of concentrated HCl after the reaction contents had been cooled and diluted with 30 ml of ice water. The aqueous layer was extracted three times each with benzene and chloroform. The combined organic phase (220 ml) was washed twice with water, dried over sodium sulfate, and evaporated to dryness. The residue was dissolved in 5 ml of 2,2′-hydroxy ethyl

ether and 500 mg of NaOH in 0.6 ml of water was then added. The mixture was gradually heated to 175° over a 30-min period and was then refluxed at 190–195° for 5 hr. After the hydrolysis mixture was cooled, it was poured into 60 ml of ice water, acidified, and then extracted three times each with chloroform and ethyl acetate. The combined extract (280 ml) was washed with water and then evaporated to dryness. Thin-layer chromatography of the residue in two systems (cyclohexane–chloroform–acetic acid (65:20:15) and dioxane–benzene–acetic acid (12.5:45:2.5)) revealed that the major radioactive peak had a mobility corresponding to authentic 3-hydroxybisnorchola-5(6),-17(20)-dien-22-oic acid. The product was further purified by chromatography on a silicic acid (3 g) column (1.2 × 8.3 cm). The column was eluted with benzene, benzene–chloroform (1:1), chloroform, and finally chloroform–methanol (99:1). The major radioactive peak resided in the $CHCl_3$–MeOH (99:1) fraction. A total of 43.2 mg of 3-hydroxybisnorchola-5(6),17(20)-dien-22-oic acid, containing 3.132×10^7 cpm, was obtained.

Formation of Propionate-^{14}C from 3β-Hydroxybisnorchola-5(6),17(20)-dien-22-oic Acid-22-^{14}C. Freshly grown *Nocardia sp.* (ATCC 19170) cells (1.2 g) were suspended in 15 ml of 0.05 M phosphate buffer (pH 7.8), containing 22.5 mg of sodium propionate and 8.8×10^5 cpm of 3-hydroxybisnorchola-5(6),17(20)-dien-22-oic acid-22-^{14}C (specific activity 0.16 mc/mmole). *o*-Phenanthroline was added to the reaction mixture to give a final concentration of 1×10^{-3} M. After incubation for 4 days at 25°, the reaction was terminated by acidification and the cells were removed by centrifugation. The supernatant was steam distilled and the distillate was collected, neutralized, and evaporated to 3 ml. To 1.5 ml of the concentrated distillate was added 2.5 mg of sodium propionate. The admixture was acidified and chromatographed on a Celite (15 g) partition column (Swim and Utter, 1957). Fractions containing propionic acid (Figure 4) were pooled. The aqueous phase was neutralized and then evaporated to a small volume (3 ml). An aliquot was admixed with sodium propionate and *S*-benzylisothiuronium chloride to yield a crystalline salt (mp 151–153°) which was recrystallized to constant specific activity. Another aliquot (6500 cpm) was degraded by the method of Phares (1951), and the results have been reported previously (Sih *et al.*, 1967).

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