Kuhn–Roth Oxidation of Digitoxigenin. Digitoxigenin (77 mg.) was added to a solution of chromium trioxide (5 g.) in 2 *N* sulfuric acid (10 ml.) and the mixture was distilled. Water was added to the distillation flask to maintain the volume at about 10 ml. When about 80 ml. of distillate had been collected it was titrated with 0.1 *N* sodium hydroxide (2.2 ml.) and then evaporated to dryness. The residue was crystallized from ethanol–ether affording sodium acetate (18 mg.).

1-Acetamidonaphthalene. Sodium acetate (2 mg.) and 1-aminonaphthalene hydrochloride (4 mg.) were dissolved in water (1 ml.). 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride[19] (20 mg.) was added. After stirring for a few minutes with a glass rod, the oily precipitate which was first formed became solid and was filtered off after 10 min. The pink precipitate (3–4 mg.) was dried at 60° and then sublimed *in vacuo* (140°, 0.01 mm.). The white sublimate was dissolved in a little boiling benzene. On addition of petroleum ether 1-acetamidonaphthalene separated out as colorless needles (2–3 mg.), m.p. 159–160°.

Acknowledgments. We thank Robert McLeester (Botany Department) and Professor Herbert Jonas (School of Pharmacy) for the cultivation of the *Digitalis* plants. We also thank Professor Ole Gisvold (School of Pharmacy) for providing us with a detailed method for the isolation of digitoxin prior to publication.[6]

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Biosynthesis of Plant Steroids. II. The Distribution of Activity in Digitoxigenin Derived from Mevalonic Acid-2-C^{14} [1]

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The steroid moiety of digitoxigenin, the aglycone of the cardiac glycoside digitoxin, has been degraded systematically. It has been established that digitoxigenin, derived from mevalonic acid-2-C^{14}, is labeled only at C-1, C-7, and C-15, and the activity is equally divided between these positions. This result strongly suggests that the plant steroids are produced from squalene by the same series of metabolic reactions as those which lead to animal steroids such as cholesterol.

In the previous paper[4] evidence was obtained which strongly favored the formation of digitoxigenin from a pregnane derivative and one molecule of acetic acid. It was assumed as a working hypothesis that the pregnane was formed in *Digitalis* plants from mevalonic acid, *via* squalene, by the same route as that which leads to the formation of animal steroids. There is now considerable evidence, circumstantial[5] and experimental,[6] which supports this general hypothesis. However, in none of these experiments involving radioactive precursors was the steroid nucleus degraded to determine the complete pattern of labeling. In mammalian tissues the cyclization of squalene derived from mevalonic acid-2-C^{14} leads to a pregnane derivative labeled at C-1, C-7, and C-15. Our degradation of digitoxigenin, illustrated in Figure 1, was thus designed to determine directly the activity as these three positions.

Activity at C-1 was obtained by the following route. Digitoxigenin (VI) was dehydrated with dilute sulfuric acid and the resultant diene was hydrogenated over platinum. The 3-hydroxy group was then oxidized with chromium trioxide yielding 3-oxo-5β-cardanolide (I).[7] This ketone was brominated in acetic acid affording 2,4-dibromo-3-oxo-5β-cardanolide which was dehydrobrominated to 3-oxo-card-1,4-dienolide (II) by refluxing in collidine. This dienone was hydroxylated with 2 moles of osmium tetroxide and the resultant 1,2,4,5-tetrahydroxy-3-oxo-cardanolide oxidized with lead tetraacetate yielding 1,5-dioxo-1,5-seco-A-trisnorcardanolide (III). This β-ketoaldehyde was cleaved on refluxing with ethanolic sodium hydroxide to formic acid and the ketone IV in which the methyl group at C-10 can be expected to have the more stable (equatorial) α-configuration.[8] The formic acid which originated from C-1 of digitoxigenin was collected as its *p*-bromophenacyl ester.

To obtain information on the activity at C-7, digitoxigenin was converted to 7β-hydroxydigitoxigenin (V) by means of the microorganism *Rhizopus nigricans*.[9]

(1) A preliminary account of part of this work has appeared as a communication: E. G. Gros and E. Leete, *Chem. Ind.* (London), 698 (1963), and was also presented at a lecture at the 148th National Meeting of the American Chemical Society, Chicago, Ill., Aug. 30–Sept. 4, 1964, Abstracts, p. 26C. This investigation was supported by a research grant (GM-13246) from the U. S. Public Health Service.

(2) Fellow of the Consejo Nacional de Investigaciones Científicas y Técnicas, Argentina.

(3) Alfred P. Sloan Foundation Fellow.

(4) E. Leete, H. Gregory, and E. G. Gros, *J. Am. Chem. Soc.*, **87**, 3475 (1965).

(5) C. Djerassi in "Biochemistry of Steroids," E. Mosettig, Ed., Pergamon Press, Inc., New York, N. Y., 1958, p. 1.

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(7) H. M. E. Cardwell and S. Smith, *J. Chem. Soc.*, 2012 (1954).

(8) J. Castells, E. R. H. Jones, G. D. Meakins, and R. W. J. Williams, *ibid.*, 1159 (1959).

(9) We are indebted to Sra. Elba T. Gros for carrying out this microbiological oxidation.

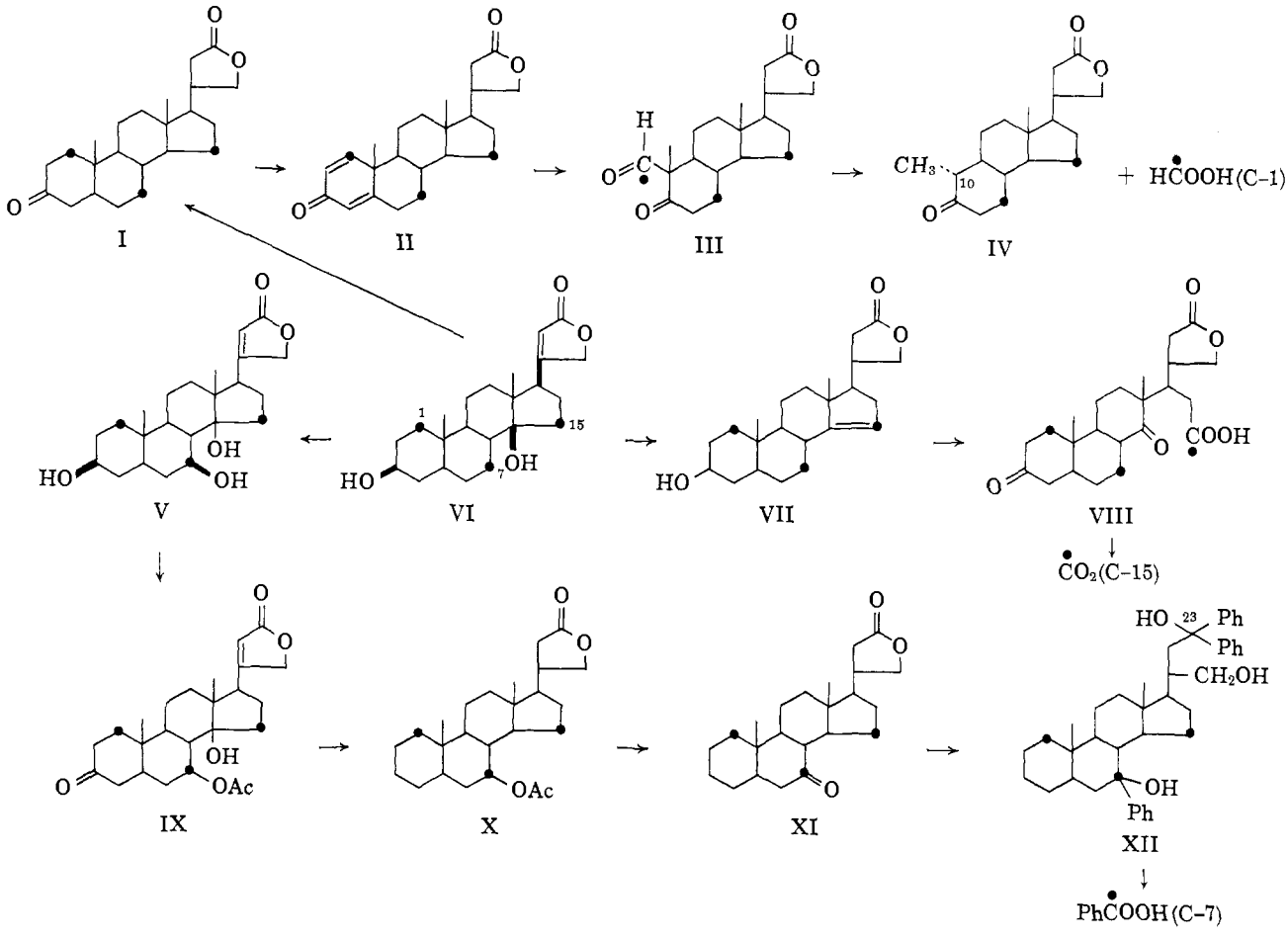

Figure 1. Degradation of digitoxigenin derived from mevalonic acid-2-C^{14}.

In this microbiological oxidation, discovered independently by Nozaki[10] and Tamm,[11] the *R. nigricans* is incubated first with a relatively large amount of progesterone. The mycelium is then filtered off, washed with water, and then incubated with digitoxigenin. In the work-up of the 7β-hydroxydigitoxigenin some 6β,11α-dihydroxyprogesterone[12] was also isolated, presumably remaining with the mycelium during the transfer from the first to the second incubation. Having obtained 7β-hydroxydigitoxigenin, the general plan was to remove the 3β-hydroxy group and then convert the 7β-hydroxy group to a ketone. Reaction with phenyllithium would then yield a 7-phenyl derivative which on oxidation would yield benzoic acid representing the activity at C-7. To conserve the precious 7β-hydroxydigitoxigenin, exploratory procedures for the removal of the 3β-hydroxy group were carried out on digitoxigenin. Oxidation with chromium trioxide afforded digitoxigenone[13] which on treatment with ethanedithiol in the presence of boron trifluoride etherate yielded 3-ethylenedithioketal-5β-card-8(14)- or -14(15),20(22)-dienolide. On refluxing this compound with Raney nickel in ethanol, 5β-cardanolide[14] was obtained. The 7β-hydroxydigitoxigenin was catalytically oxidized to 7β,14-dihydroxy-3-oxo-5β-card-20(22)-enolide with oxygen in the presence of platinum.[15] After acetylation of the 7-hydroxy group, the 3-keto was removed by the same procedure used on digitoxigenone resulting in the formation of 7β-acetoxy-5β-cardanolide (X). Methanolic potassium hydroxide removed the acetyl group and opened the lactone ring which relactonized on acidification. Oxidation with Kiliani's reagent yielded 7-oxo-5β-cardanolide (XI) which was allowed to react with excess phenyllithium in boiling benzene. The crude reaction product, presumably XII, was oxidized with chromic acid affording a mixture of benzoic acid and benzophenone. We were concerned that some benzoic acid might arise by oxidation at C-23. However, the oxidation of a similar compound, 3α,7β-diacetoxy-24,24-diphenyl-5β-cholan-24-ol,[16] under the same conditions yielded only benzophenone and no benzoic acid.

Activity at C-15 was determined by making use of a reaction sequence first carried out by Jacobs and Elderfield.[17] 3β,14-Dihydroxy-5β-card-14(15)-enolide (VII) was oxidized with alkaline potassium permanganate to 3,14,15-trihydroxy-5β-cardanolide which was further oxidized with chromium trioxide in sulfuric acid to 3,14-dioxo-14,15-seco-5β-cardanolid-15-oic acid (VIII). A

(10) Y. Nozaki, *Agr. Biol. Chem.* (Tokyo), **25**, 559 (1961); Y. Nozaki, E. Masuo, and D. Satoh, *ibid.*, **26**, 399 (1962).
(11) G. Juhasz and Ch. Tamm, *Helv. Chim. Acta*, **44**, 1063 (1961).
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(13) K. Meyer and T. Reichstein, *Helv. Chim. Acta*, **30**, 1508 (1947).
(14) W. A. Jacobs and N. M. Bigelow, *J. Biol. Chem.*, **101**, 697 (1933); J. L. Breton, J. Delgado, and A. G. Gonzalez, *Chem. Ind.* (London), 513 (1959).
(15) H. Ishii, Y. Nozaki, T. Okumura, and D. Satoh, *Yakugaku Zasshi*, **80**, 1150 (1960); **81**, 805 (1961).
(16) This compound was obtained by the acetylation of the reaction product of 3α,7β-dihydroxy-5β-cholanic acid methyl ester with phenylmagnesium bromide.
(17) W. A. Jacobs and R. C. Elderfield, *J. Biol. Chem.*, **99**, 693 (1932).

Schmidt reaction on this diketo acid yielded carbon dioxide which arose from C-15, since no carbon dioxide was produced when 20,22-dihydrodigitoxigenin was subjected to the same Schmidt reaction conditions.

The activities of the degradation products of digitoxigenin derived from mevalonic acid-2-C^{14} are recorded in Table I. Within experimental error it was found that one-third of the radioactivity was located at each of the positions C-1, C-7, and C-15. We have also previously established[4] that mevalonic acid-3'-C^{14} yields digitoxigenin labeled at C-18, C-19, and C-21. Our experiments have thus provided convincing evidence that plant and animal steroids are produced by the same biosynthetic route.

Table I. Degradation Products of Digitoxigenin

	Specific activity, d.p.m./mmoles	Relative activity
(a) Determination of activity at C-1		
3-Oxo-5β-cardanolide (I)	1.42×10^4	100
3-Oxocard-1,4-dienolide (II)	1.42×10^4	100
1,5-Dioxo-1,5-seco-A-trisnorcardanolide (III)	1.39×10^4	98
5-Oxode-A-10β-cardanolide (IV)	0.97×10^4	68
p-Bromophenacyl formate (C-1)	0.49×10^4	34
(b) Determination of activity at C-7		
Digitoxigenin (VI)	1.75×10^5	100
7β-Hydroxydigitoxigenin (V)	1.75×10^5	100
7β-Acetoxy-3-oxo-5β-card-20(22)-enolide (IX)	1.80×10^5	103
7β-Acetoxy-5β-cardanolide (X)	1.76×10^5	100
7-Oxo-5β-cardanolide (XI)	1.76×10^5	100
Benzoic acid[a] (C-7)	0.61×10^5	35
Benzophenone oxime[a]	0	0
(c) Determination of activity at C-15		
3β-Hydroxy-5β-card-14(15)-enolide (VII)	4.39×10^5	100
3,14-Dioxo-14,15-seco-5β-cardanolid-15-oic acid (VIII)	4.42×10^5	101
Barium carbonate[b] (C-15)	1.38×10^5	31

[a] Obtained by the oxidation of the phenylation product XII. [b] Obtained from a Schmidt reaction on the acid VIII.

Experimental[18]

Production of Radioactive Digitoxin from DL-*Mevalonic Acid-2-*C^{14}. Details of the feeding experiments and the yields of digitoxin are given in part I.[4] The degradations carried out to determine activity at C-1 and C-7 utilized digitoxin obtained by spraying the leaves of *Digitalis purpurea* plants with an aqueous solution of the tracer (experiments 5, 6, and 7). The activity at C-15 was determined on digitoxin obtained from excised leaves of *D. purpurea* which had been placed in a solution of the tracer (experiment 4).

Determination of Activity at C-1. 3-Oxo-5β-cardanolide (I).[7] Digitoxigenin (5.0 g.) was dissolved in a mixture of ethanol (140 ml.) and 10% sulfuric acid (140 ml.) and refluxed for 2 hr. Water (140 ml.) was then added and the ethanol was removed *in vacuo*. The resultant 3β-hydroxy-5β-card-14(15),20(22)-dienolide (β-anhydrodigitoxigenin) was extracted from the aqueous suspension with chloroform, which was then washed with water. The residue obtained on evaporation of the dried (magnesium sulfate) chloroform extract was dissolved in ethanol and hydrogenated in the presence of platinum oxide (0.6 g.) at atmospheric pressure. The theoretical amount of hydrogen was absorbed after 2 hr. The catalyst was filtered off and the filtrate was evaporated to dryness yielding a crystalline residue (3.5 g.) which was dissolved in 90% acetic acid (80 ml.) and treated dropwise with a solution of chromium trioxide (2.7 g.) in a mixture of concentrated sulfuric acid (4 ml.) and water (16 ml.). After stirring for 30 min. the mixture was poured onto ice–water. The precipitate obtained was filtered off, washed with water, and dried *in vacuo*. Crystallization of the product from methanol afforded colorless needles of 3-oxo-5β-cardanolide (2.4 g.), m.p. 233–235°. The infrared spectrum had absorptions at 1770 (lactone) and 1710 $cm.^{-1}$ (ketone).

2,4-Dibromo-3-oxo-5β-cardanolide. A solution of bromine (2.13 g.) in acetic acid (5 ml.) was added slowly to a stirred solution of 3-oxo-5β-cardanolide (2.3 g.) in acetic acid (50 ml.) at room temperature. After 20 hr. the reaction mixture was added to ice–water and the resultant precipitate was filtered off and dried *in vacuo*. Crystallization from acetone–ethanol and then ethyl acetate yielded colorless needles of 2,4-dibromo-3-oxo-5β-cardanolide (2.12 g.), m.p. 213–214° dec. The infrared spectrum had absorptions at 1768 and 1740 $cm.^{-1}$.

Anal. Calcd. for $C_{23}H_{32}Br_2O_3$: C, 53.50; H, 6.25; Br, 30.95. Found: C, 53.45; H, 6.23; Br, 30.81.

3-Oxocard-1,4-dienolide (II). The 2,4-dibromo-3-oxo-5β-cardanolide (2.05 g.) was dissolved in 2,4,6-collidine (10 ml.) and refluxed for 1.5 hr. The mixture was then cooled and collidine hydrobromide was filtered off and washed with ether. The combined filtrates and washings were evaporated to dryness *in vacuo*. The residue was dissolved in chloroform and washed with 2 *N* hydrochloric acid, saturated sodium bicarbonate, and water, and finally dried over magnesium sulfate. The dark residue obtained on evaporation of the chloroform was boiled with methanol and filtered, and the filtrate was evaporated. The residue was dissolved in benzene and chromatographed on neutral Woelm alumina (activity I). Fractions obtained on elution of the column with benzene containing 15–20% chloroform yielded, on evaporation, crystals of the dienone. Recrystallization from methanol yielded colorless prismatic needles of 3-oxocard-1,4-dienolide (325 mg.), m.p. 243–246°. The ultraviolet spectrum had λ_{max} 245.5 mμ (log ε 4.14). The infrared spectrum had absorptions at 1770 (lactone), 1665, 1626, and 1605 $cm.^{-1}$ (1,4-dien-3-one).

Anal. Calcd. for $C_{23}H_{30}O_3$: C, 77.93; H, 8.53. Found: C, 77.49; H, 8.66.

1,5-Dioxo-1,5-seco-A-trisnorcardanolide (III). A solution of osmium tetroxide (500 mg.) in benzene (14 ml.) was added to the dienone II (321 mg.) dissolved in dry pyridine. After standing in the dark at room temperature for 6 days, the reaction mixture was cooled to 4° and hydrogen sulfide was passed in for 3 hr. After standing overnight the mixture was filtered and the black residue was washed with dioxane. The residue obtained on evaporation of the combined filtrates was

(18) Melting points are uncorrected. Radioactivity measurements were all carried out in a Nuclear Chicago liquid scintillation spectrometer, Model 724, using as solvents either toluene or dioxane–water with the usual scintillators (*cf.* A. R. Friedman and E. Leete, *J. Am. Chem. Soc.*, **85**, 2141 (1963)). Microanalyses were carried out by Mrs. Olga Hamerston, Miss Kathleen Nelson, and Mr. T. S. Prokopov of the University of Minnesota, and by the Clark Microanalytical Laboratories, Urbana, Ill. Infrared spectra, unless otherwise stated, were determined in KBr pellets on a Perkin-Elmer 421 spectrophotometer. Ultraviolet spectra were determined in 95% ethanol.

extracted with boiling methanol. The residue obtained on evaporation of the filtered methanol extract was dissolved in chloroform (40 ml.) and added to a solution of lead tetraacetate (3.2 g.) in a mixture of acetic acid (80 ml.) and chloroform (40 ml.). After standing in the dark at room temperature for 1 day, water and ether were added. The ether layer was separated and the aqueous layer was extracted with more ether. The combined ether extracts were washed successively with water, 10% sodium bicarbonate, and finally water. The residue, obtained on evaporation of the dried (magnesium sulfate) ether extract, was dissolved in chloroform and chromatographed on neutral Woelm alumina (activity III). Fractions obtained on elution with chloroform–methanol (1:1) yielded on evaporation the ketoaldehyde III. Crystallization from methanol–ether afforded colorless, cubic prisms (90.6 mg.), m.p. 218–220°, which gave positive reactions with aldehyde reagents (Tollens, triphenyltetrazolium chloride). It had no strong absorptions in the ultraviolet and its infrared spectrum in chloroform had absorptions at 2875, 2740 (aldehyde C–H), 1775 (lactone), 1726 (aldehyde), and 1700 cm.$^{-1}$ (ketone).

Anal. Calcd. for $C_{20}H_{28}O_4$: C, 72.26; H, 8.49. Found: C, 72.19; H, 8.43.

5-Oxode-A-10β-cardanolide (IV). The ketoaldehyde III (78 mg.) was dissolved in a 1% solution of sodium hydroxide in ethanol (8 ml.) and the mixture was refluxed for 30 min. under nitrogen. Water (40 ml.) was then added and the solution was acidified with 1 *N* sulfuric acid. After standing for several hours at 5°, the solution deposited crystals of the ketone IV. Recrystallization from a mixture of ethanol and ether yielded colorless crystals of 5-oxode-A-10β-cardanolide (12 mg.), m.p. 160–162°. The infrared spectrum had absorptions at 1772 (lactone) and 1712 cm.$^{-1}$ (ketone).

Anal. Calcd. for $C_{19}H_{28}O_3$; C, 74.96; H, 9.27. Found: C, 75.16; H, 9.06.

The aqueous filtrate from which the ketone had been removed was made faintly alkaline with sodium hydroxide and evaporated to dryness. The residue was dissolved in a little water, made acidic with 1 *N* sulfuric acid, and then distilled almost to dryness. The distillate was titrated with 0.2 *N* sodium hydroxide (phenolphthalein indicator) and concentrated to 1 ml. A few drops of hydrochloric acid was added and the solution was added to *p*-bromophenacyl bromide (40 mg.) dissolved in ethanol (15 ml.) and the mixture was refluxed for 1 hr. On cooling the solution and adding a little water, crystals separated. Recrystallization from ethanol afforded colorless needles of *p*-bromophenacyl formate (6 mg.), m.p. 101–102°, not depressed on admixture with an authentic specimen.

Determination of the Activity at C-7. 7β-Hydroxydigitoxigenin (V). This microbiological oxidation was carried out according to the procedure of Nozaki and co-workers.[10] We thank Drs. Ishii and Nozaki of the Shionogi Co., Amagasaki, Hyogo-ken, Osaka, Japan, for a culture of *Rhizopus nigricans* (ATCC 6227b). The mold was grown in a conical flask containing water (500 ml.), dextrose (17.5 g.), peptone (15 g.), and corn steep liquor (5 g.) for 24 hr. Progesterone (125 mg.) was then added and the mixture was shaken for an additional 24 hr. The mycelium was then filtered off, washed with water, and then added to a flask containing digitoxigenin (10 mg.) in water (500 ml.). After incubating for 2 days the mixture was filtered, the mycelium being washed with water, chloroform, and ethanol. The filtrates from ten such fermentations were concentrated *in vacuo* to remove organic solvents. The aqueous solution was then extracted with chloroform and then with a mixture of chloroform and acetone (3:1). The combined organic extract was washed with water and then dried over magnesium sulfate. The residue obtained on evaporation was dissolved in ethyl acetate. On standing at room temperature, a crystalline solid separated which gave a negative Kedde reaction. Recrystallization of this material from methanol afforded colorless prisms of 6β,11α-dihydroxyprogesterone,[12] m.p. 252–256°, having an absorption in the ultraviolet at λ_{max} 234.5 mμ (log ε 4.12) and in the infrared at 3420, 3360, 1697, 1665, and 1615 cm.$^{-1}$. It was also characterized as its diacetyl derivative, m.p. 156–157°. The filtrate obtained after removal of the progesterone derivative was evaporated and the residue was chromatographed on silicic acid.[19] The eluents from the column were monitored by paper chromatography. The paper used was Whatman No. 1 which had been impregnated with a mixture of formamide and acetone (1:3). The developing solvent was benzene–chloroform (7:5) which had been saturated with formamide. The cardenolides were detected with Kedde's reagent (3,5-dinitrobenzoic acid (1 g.) and potassium hydroxide (2.8 g.) dissolved in 50% aqueous methanol (100 ml.)); R_D = distance traveled by compound/distance traveled by digitoxigenin. Fractions containing 7β-hydroxydigitoxigenin[20] were combined and rechromatographed on neutral Woelm alumina (activity I)[11] yielding (from 103 mg. of radioactive digitoxigenin) 7β-hydroxydigitoxigenin (46 mg.), m.p. 276–282°, R_D 0.43.

7β,14-Dihydroxy-3-oxo-5β-card-20(22)-enolide.[15] 7β-Hydroxydigitoxigenin (60 mg.), dissolved in acetone (7 ml.), was added to a suspension of Adams' catalyst (60 mg., previously reduced with hydrogen) in water (3 ml.). The mixture was stirred in the presence of oxygen for 20 hr. After removal of the catalyst the solution was evaporated and the residue was crystallized from acetone–ether, affording colorless needles of 7β,14-dihydroxy-3-oxo-5β-card-20(22)-enolide (44 mg.), m.p. 258–264°, R_D 0.86. It had absorptions in the infrared at 3370, 1800, 1725, 1705, and 1652 cm.$^{-1}$.

7β-Acetoxy-14-hydroxy-3-oxo-5β-card-20-(22)-enolide (IX).[15] The previous compound (220 mg.) was dissolved in a mixture of pyridine (2.5 ml.) and acetic anhydride (2 ml.) and allowed to stand at room temperature for 2 days. The mixture was then added to ice–water, and extracted with chloroform which was washed with 2 *N* hydrochloric acid, 10% sodium carbonate, and finally water. The residue obtained on evaporation of the dried chloroform solution was crystallized from acetone–ether yielding colorless plates of the acetyl derivative IX (141 mg.), m.p. 206–208°.

7β-Acetoxy-5β-cardanolide (X). The previous compound IX (310 mg.) and ethanedithiol (0.3 ml.) were dissolved in acetic acid (6 ml.) and the solution was heated on a steam bath while hot boron trifluoride

(19) E. Titus, A. Murray, and H. Spiegel, *J. Biol. Chem.*, **235**, 3399 (1960).

(20) We thank Dr. Ch. Tamm of the University of Basel for an authentic specimen of this compound.

etherate (0.3 ml.) was added. The mixture was then allowed to cool to room temperature. After 2 hr. chloroform (25 ml.) and ether (25 ml.) were added, and the solution was washed with water, 5% sodium hydroxide (until the odor of the thiol disappeared), and water, and finally dried over magnesium sulfate. The residue (320 mg.), obtained on evaporation of the chloroform, was crystallized from chloroform–methanol affording fine fluffy needles of the 3-ethylene dithioketal (238 mg.), m.p. 194–195°. This product was dissolved in absolute ethanol (60 ml.) and refluxed with Raney nickel (W-2, 4.0 g.) for 20 hr. The reaction mixture was then filtered through Celite. The residue was extracted with boiling ethanol for 2 hr. and filtered again. The combined filtrates were evaporated and the residue was crystallized from methanol affording colorless plates of 7β-acetoxy-5β-cardanolide (165 mg.), m.p. 212–214°. It had absorptions in the infrared at 1785, 1730, and 1248 cm.$^{-1}$.

Anal. Calcd. for $C_{25}H_{38}O_4$: C, 74.58; H, 9.52. Found: C, 74.56; H, 9.22.

7-Oxo-5β-cardanolide (XI). The 7β-acetoxy-5β-cardanolide (160 mg.) and potassium hydroxide (4 g.) were dissolved in methanol (40 ml.) and the mixture was refluxed for 1.5 hr. Water (40 ml.) was then added and the methanol was removed *in vacuo*. On acidification of the aqueous solution with 2 *N* hydrochloric acid, a precipitate was formed which was extracted with chloroform. The chloroform solution was washed with sodium carbonate and water, and then dried over sodium sulfate. On evaporation of the chloroform, colorless plates of 7β-hydroxy-5β-cardanolide (102 mg.), m.p. 260–268°, were obtained. It had absorptions in the infrared at 3500 (hydroxyl) and 1775 cm.$^{-1}$ (lactone). This compound was dissolved in 90% acetic acid (20 ml.), and 3 ml. of Kiliani's reagent (400 ml. water, 80 g. of concentrated sulfuric acid, 53 g. chromium trioxide) was added. After 20 min. at room temperature the mixture was added to ice–water and extracted with chloroform which was then washed with sodium bicarbonate and water. The residue, obtained on evaporation of the dried (magnesium sulfate) chloroform extract, was crystallized from methanol affording colorless needles of 7-oxo-5β-cardanolide (55 mg.), m.p. 238–240°. It had absorptions in the infrared at 1776 (lactone) and 1708 cm.$^{-1}$ (ketone).

Phenylation of 7-oxo-5β-cardanolide and Oxidation of the Product. An ether solution of phenyllithium (1 *M*, 5 ml.) was added to a solution of 7-oxo-5β-cardanolide (40 mg.) in benzene (5 ml.). The ether was distilled off and the benzene solution was refluxed for 4 hr. Water (10 ml.) and 2 *N* hydrochloric acid were then added to the cooled reaction mixture until it was acidic. The mixture was then extracted with ether which was then washed successively with water, sodium carbonate, and water. The infrared spectrum of the residue obtained on evaporation of the dried ether extract had no absorptions in the carbonyl region (1600–1800 cm.$^{-1}$), but hydroxyl absorptions were observed at 3540 and 3340 cm.$^{-1}$. This crude phenylation product was dissolved in 10% sulfuric acid (2 ml.) and added to a solution of chromium trioxide (3 g.) in 10% sulfuric acid (8 ml.). The mixture was immediately distilled in a stream of nitrogen into a cooled flask containing 10% sodium hydroxide (5 ml.). Water was added to the reaction flask to maintain the volume at about 10 ml. Distillation was continued for 3 hr. The alkaline distillate was extracted with ether which was then dried over magnesium sulfate. The residue (48 mg.), obtained on evaporation of the ether extract, was found to be a mixture of benzophenone and biphenyl (formed in the original preparation of phenyllithium from bromobenzene and lithium). This residue was dissolved in ethanol (4 ml.) and water (3 ml.) was added when biphenyl (29 mg.), m.p. 68°, separated out. Hydroxylamine hydrochloride (30 mg.) and potassium hydroxide (180 mg.) were added to the filtrate and the mixture was heated on a steam bath for 10 min. Dilute hydrochloric acid was then added to the cooled solution and on standing, colorless crystals of benzophenone oxime (11 mg.) separated, m.p. 144°, not depressed on admixture with an authentic specimen. The aqueous alkaline solution from which the benzophenone had been extracted was acidified with hydrochloric acid and extracted with ether. The dried ether extract was evaporated and the residue was sublimed affording benzoic acid (10 mg.), m.p. 121–122°.

3α,7β-Diacetoxy-24,24-diphenyl-5β-cholan-24-ol. 3α,7β-Dihydroxy-5β-cholanic acid (purchased from Steraloids Inc., Flushing, N. Y.) was converted to its methyl ester, m.p. 118–120°, with diazomethane,[21] and then reacted with phenylmagnesium bromide in boiling benzene for 20 hr. The resultant phenylation product was acetylated in pyridine with acetic anhydride at 50° for 20 hr. Crystallization from methanol afforded colorless plates of 3α,7β-diacetoxy-24,24-diphenyl-5β-cholan-24-ol, m.p. 181–182°.

Anal. Calcd. for $C_{40}H_{54}O_5$: C, 78.13; H, 8.85. Found: C, 77.82; H, 8.56.

Oxidation of this compound (45 mg.) using the same procedure as that used on compound XII yielded only benzophenone (8 mg.) and no trace of benzoic acid.

5β-Cardanolide from Digitoxigenone. Digitoxigenone[13] (300 mg.) was added to a mixture of acetic acid (4 ml.) and ethanedithiol (0.2 ml.) and the solution was heated to 90°. Boron trifluoride etherate (0.2 ml.) was added and the solution was allowed to cool when 3-ethylenedithioketal-5β-card-8(14)- or -14(15),20-(22)-dienolide separated (116 mg.). Crystallization from chloroform–methanol afforded colorless needles, m.p. 226–228°.

Anal. Calcd. for $C_{25}H_{34}O_2S_2$: C, 69.72; H, 7.96; S, 14.89. Found: C, 69.50; H, 7.62; S, 15.23.

The dithioketal (50 mg.) was refluxed in absolute ethanol (20 ml.) with Raney nickel (W-2, 1 g.) for 20 hr. The product was isolated by the same procedure as that used for compound X. 5β-Cardanolide was obtained as colorless plates from ethanol, m.p. 167–169°. It had an absorption in the infrared at 1780 cm.$^{-1}$ (lactone).

Anal. Calcd. for $C_{23}H_{36}O_2$: C, 80.17; H, 10.53. Found: C, 80.14; H, 10.36.

Determination of the Activity at C-15.[17] Digitoxigenin was hydrogenated over platinum and the resultant 3β,14-dihydroxy-5β-cardanolide was dehydrated by refluxing in dilute sulfuric acid[7] affording 3β-hydroxy-5β-card-14(15)-enolide, m.p. 184–186°. This compound

(21) T. Kanazawa and T. Sato, *Nippon Kagaku Zasshi*, **76**, 463 (1955).

was oxidized first with alkaline potassium permanganate in pyridine solution. The 14,15-diol thus obtained was then dissolved in acetic acid and oxidized with Kiliani's reagent yielding the diketo acid VIII, m.p. 195–199°. Sodium azide (40 mg.) was added to a cooled solution of this diketo acid (28 mg.) in a mixture of chloroform (0.4 ml.) and concentrated sulfuric acid (0.5 ml.). The mixture was warmed to 45–50° and the evolved carbon dioxide was collected as barium carbonate (8.1 mg.).

The Structures of Mitorubrin and Mitorubrinol

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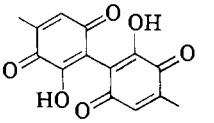
Two new metabolites, for which the names mitorubrin and mitorubrinol are suggested, have been isolated from Penicillium rubrum cultures. Spectral evidence was used to derive their structures.

The isolation and characterization of mold and fungal metabolites has generally been impelled by some conspicuous feature of the microorganism, such as its pigmentation or its ability to elaborate a toxic or therapeutic agent. *Penicillium rubrum* is notable both for the coloration which its mycelium develops during growth and for its ability to synthesize a substance of high toxicity. The latter feature has attracted attention in association with the poisoning of livestock through feedstuff which was shown to be infected with *P. rubrum*.[2,3] During the course of the present work it was found that the substances responsible for pigmentation of the fungus are distinct from the agent(s) causing toxicity and in the present paper we shall discuss the isolation and structure determination of two pigments.

When *P. rubrum* is grown on a natural medium such as wheat or corn its mycelium develops a brilliant orange coloration interspersed with flecks of darker red. Extraction of the culture with ethyl acetate after 2 to 3 weeks of growth affords a dark red extract which contains, in addition to pigments, a large amount of lipid and fat-soluble material. Since the pigments are relatively insoluble in nonpolar solvents, they can be concentrated by extracting with solvents of increasing polarity. The lipid material is removed in the initial extraction with petroleum ether and the final residue after extraction with ethyl ether contains, according to thin layer chromatography, three principal pigments together with some colorless, polar material. Chromatography of this residue on silicic acid yields two pure pigments which we have named mitorubrin and mitorubrinol, respectively.

A search among the known mold products disclosed only one metabolite of *P. rubrum* which has been previously isolated and identified.[4] This substance, phoenicin, has been assigned the bisquinonoid structure **1**, and its synthesis has been reported.[5] The obvious dissimilarity between the physical and spectral properties of phoenicin and those of mitorubrin (and mitorubrinol) precluded the possibility of close structural resemblance.



1

Mitorubrin was obtained as orange-yellow prisms, m.p. 218°, and is optically active. Bioassays with mice showed that it possesses none of the physiological properties associated with the toxic metabolite which is elaborated by the same fungus. The infrared spectrum of the pigment shows broad absorption attributable to hydroxyl groups, a carbonyl band at 1715, and broad, complex absorption in the region of 1600–1660 $cm.^{-1}$. The ultraviolet spectrum of mitorubrin shows maxima, in ethanol, at 216 mμ (ε 18,200), 266 (18,200), 292 (10,100), and 346 (16,100). Solutions of this substance change from yellow to orange on addition of sodium hydroxide and this is accompanied by a large shift in the absorption spectrum, with maxima occurring, in base, at 246 mμ (ε 20,200), 320 (23,600), 346 (28,600), and 485 (5600). The original spectrum is regenerated upon immediate acidification, but prolonged contact with base results in degradation of the molecule. Attempts to match the rather complex ultraviolet spectrum of mitorubrin with known chromophores were not immediately successful and led to the suggestion that the spectrum might be the result of a superposition of two separate chromophores present in the molecule.

The appearance of a clearly defined molecular ion peak in the mass spectrum indicated that the molecular weight of mitorubrin is 382 and a high-resolution mass spectrum established the composition as $C_{21}H_{18}O_7$.[6]

We now wish to consider the spectroscopic and chemical properties of mitorubrin in terms of structure **2** which we feel is uniquely compatible with the evidence available.

(1) National Institutes of Health Predoctoral Fellow, 1963–1965.

(2) J. E. Burnside, *et al.*, *Am. J. Vet. Res.*, **18**, 817 (1957); J. Forgacs, H. Koch, W. T. Carll, and R. H. White-Stevens, *ibid.*, **19**, 744 (1958).

(3) B. J. Wilson and C. H. Wilson, *J. Bacteriol.*, **83**, 693 (1962); **84**, 293 (1962).

(4) E. A. Friedheim, *Helv. Chim. Acta*, **21**, 1464 (1938); T. Curtin, G. Fitzgerald, and J. Reilly, *Biochem. J.*, **34**, 1605 (1940).

(5) T. Posternak, H. W. Ruelius, and J. Tcherniak, *Helv. Chim. Acta*, **26**, 2031 (1943).

(6) The high resolution mass spectrum was obtained through the courtesy of Professor K. Biemann, Massachusetts Institute of Technology.