m.p. 178–180°, mixed m.p. 180°. Distillation of the filtrate gave triethyl phosphite, 1.25 g. (75%), b.p. 56–57° (21 mm.).

**Pyrolysis of the Adduct.**—The adduct (6.99 g., 0.013 mole) was heated at once at 215°. It melted, and a violent reaction occurred and continued for 1 min. The resulting mixture was distilled *in vacuo*. Triethyl phosphate, 0.88 g., 0.005 mole (34%), b.p. 101–102° (18 mm.) and diphenylacetylene, 1.50 g., 0.0084 mole (69%), b.p. 117–121° (3 mm.) were obtained.

*Anal.* Calcd. for $C_{14}H_{10}$: C, 94.34; H, 5.66. Found: C, 94.10; H, 5.70. Recrystallization of the residue from ethyl acetate gave the dimer of diphenylketene, 2.21 g. (0.0057 mole), m.p. 168–169°.

*Anal.* Calcd. for $C_{28}H_{20}O_2$: C, 86.57; H, 5.19. Found: C, 86.62; H, 5.17.

When a mixture of the adduct (0.01 mole) and triethyl phosphite (0.02 mole) was pyrolzed under same conditions as mentioned above, triethyl phosphate, 0.012 mole (60%), b.p. 105° (31 mm.) and diphenylacetylene, 0.015 mole (70%), b.p. 120–121° (4 mm.) were obtained and 0.014 mole of triethyl phosphite was recovered.

**Pyrolytic Reaction of Diphenylketene with Triethyl Phosphite.**—Triethyl phosphite (7.49 g., 0.045 mole) was added to diphenylketene (5.90 g., 0.030 mole) from a separatory funnel with stirring and the mixture was quickly heated at 215–217° under nitrogen. The reaction mixture soon began to boil and a violent reaction continued for 1 min. The resulting mixture was distilled *in vacuo*. Diphenylacetylene, 3.49 g., 0.020 mole (65%), b.p. 125–127° (6 mm.), and triethyl phosphate, 3.56 g., 0.020 mole (65%), b.p. 111–112° (24 mm.), were obtained, and triethyl phosphite, 3.77 g., 0.023 mole, b.p. 61–62° (29 mm.), was recovered. Recrystallization of the residue from ethyl acetate gave the dimer of diphenylketene, 0.13 g., 0.0004 mole (2%), m.p. 167–168°.

**Acknowledgment.**—The authors wish to express their thanks to Mr. Takanobu Kumamoto for his help during the course of this experiment. Thanks are also due to Mr. Keiji Naruse for the microanalyses.

# Microbiological Transformations. XII. The Substrate Specificity of Hydroxylations by a *Penicillium* sp., A.T.C.C. 12,556

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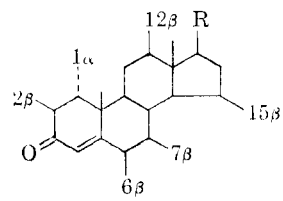
*Received June 1, 1962*

*Penicillium* sp., A.T.C.C. 12,556, which previously has been reported to hydroxylate C-19 steroids at the 1α-position,[2] failed to introduce a hydroxyl group at this carbon atom in the C-21 steroids, progesterone, 16α,17α-epoxyprogesterone, and 17α,21-dihydroxyprogesterone. Instead, hydroxylation occurred largely at the 7β- and 15β-positions. A 1α-hydroxyl group was introduced into testosterone in low yield by this organism, but hydroxylation also occurred at the 6β-, 12β- and 15β-positions. Testololactone was hydroxylated in the 2β-position. 1α-Hydroxylation with this *Penicillium* sp. appears to be limited to normal C-19 steroids, while 7β-hydroxylation is limited to C-21 steroids.

In a previous paper of this series,[2] the hydroxylation of three C-19 steroids in position 1α and 2β by a *Penicillium* sp. A.T.C.C. 12,556 was reported. Here, we would like to report that 1α-hydroxylation with this organism appears to be limited to normal C-19 steroids. C-21-Steroids were hydroxylated largely at the 7β- and 15β-positions; no 1α-hydroxy C-21 steroid was found. The C-19 steroids, testosterone and testololactone, were both hydroxylated in ring A, but no 7β-hydroxy C-19 steroid was found. We do not believe that this degree of substrate specificity has been reported previously for any organism. Also, by variation of substrate, a total of six different positions were hydroxylated by this versatile mold. To our knowledge this is a record for any one organism.

When progesterone was fermented with this species, the two main products were 15β-hydroxyprogesterone and 7β,15β-dihydroxyprogesterone. These two compounds have been reported earlier by other authors.[3–5]

The configuration of a hydroxyl group at C-15 is sometimes difficult to determine, but the n.m.r. spectrum[6] offers a simple solution to this problem.




The 18-methyl peak is shifted from the value of 9.30 p.p.m. for 15α-hydroxyprogesterone and 9.32 p.p.m. for progesterone to 9.06 p.p.m. for 15β-

(1) University of Minnesota, Minneapolis 14, Minnesota.

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

(3) H. L. Herzog, M. J. Gentles, W. Charney, D. Sutter, E. Townley, M. Yudis, D. Kabasakalian, and E. B. Hershberg, *J. Org. Chem.*, **24**, 691 (1959).

(4) J. Fried, R. W. Thoma, D. Perlman, and J. R. Gerke, U. S. Patent 2,753,290, July 3, 1956. *Chem. Abstr.*, **51**, 2071 (1957).

(5) K. Tsuda, T. Asai, E. Ohki, A. Tanaka, and M. Hattori, *Chem. Pharm. Bull.* (Tokyo), **6**, 387 (1958); K. Tsuda, T. Asai, Y. Sato, T. Tanaka, T. Matsuhisa, and H. Hasegawa, *Chem. Pharm. Bull.* (Tokyo), **8**, 626 (1960).

(6) Kindly determined for us by Dr. N. L. McNiven of the Worcester Foundation for Experimental Biology and the Analytical Department of G. D. Searle, & Co. The curves were run at 60 Mc. in deuterochloroform. The shifts are reported as τ values relative to tetramethylsilane as an internal standard.

hydroxyprogesterone. This shift is apparently due to decreased shielding of the methyl group by the nearby pseudo-axial hydroxyl. The differences in the resonance bands due to the hydrogen attached to the 15-carbon atom are not striking (both are multiplets in the 5.65–6.0 region).

Three minor products were also obtained from the fermentation of progesterone. The least polar of all the products was 2β-hydroxyprogesterone, which appeared in very low yield in one run. Although not enough material was available for an analysis, it was identified by comparison of its acetate with an authentic sample.

The second minor product was 2β,15β-dihydroxyprogesterone,[7] and the last of the minor products was identified as 6β-hydroxy-4-pregnene-3,15,20-trione. The structure of this compound is indicated by the ultraviolet spectrum which has a maximum at 236 mμ, characteristic of a 6β-hydroxy compound. The infrared spectrum shows a peak at 5.75 μ, typical of a 5-membered ring ketone. The location of the ketone at C-15 rather than C-16 was suggested by a study of the change of the ultraviolet spectrum in 0.1 *N* methanolic potassium hydroxide with time. Only a slight decrease in intensity resulted, that normally expected for a 6β-hydroxysteroid under these conditions.[8] The enolization of a 16,20-diketone would have been expected to markedly increase the intensity of the ultraviolet absorption.

This compound is interesting since it is the only indication of oxidation of a hydroxyl group which we have seen with this organism.

Fermentation of 17α,21-dihydroxy-4-pregnene-3,20-dione gave a mixture of the 7β- and 15β-hydroxy derivatives in low yield. After most of the 15β-hydroxy material[9] was separated by crystallization, the remaining material was acetylated and then 7β,21-diacetoxy-17α-hydroxy-4-pregnene-3,20-dione[10] could be crystallized.

16α,17α-Epoxyprogesterone was also hydroxylated in the 7β-position by this species of *Penicillium*. The ultraviolet maximum of this material in 0.1 *N* potassium hydroxide in methanol shifted slowly from 241.5 mμ to 283 mμ indicating a 7-hydroxyl group. The hydroxyl group was readily acetylated and the molecular rotatory contribution of the acetoxy group, $\Delta M_D$ (7β OAc −7β H) = +75°, is consistent with the values reported for other 7β-acetoxy steroids.[11]

The fermentation of testosterone gave five products. The two major products were the well known 6β- and 15β-hydroxy compounds. From the intermediate chromatographic fraction between these two materials, 1α-hydroxytestosterone was isolated and shown to be the same as an authentic sample.[2] The fourth compound obtained from a chromatographic column was 12β-hydroxytestosterone, which has been reported recently by Raspe and Kieslich.[12] To establish the structure, we oxidized our material to 4-androstene-3,12,17-trione, which was identical with a sample prepared from 12α-hydroxy-4-androstene-3,17-dione.[13]

The n.m.r. spectrum confirms the assignment of the β-configuration to the 12-hydroxyl group since the signals for the 12α- and 17α-hydrogen atoms are broad multiplets in the 6.11 to 6.59 p.p.m. range.This fact indicates that both hydrogens are axial.[11,14]

The most polar compound from the fermentation was a dihydroxytestosterone with an ultraviolet absorption maximum at 235.5 mμ. A maximum at this wave length suggests that a 6β-hydroxy group is present. When the material was dissolved in methanolic sodium hydroxide solution, a new substance formed. This dehydration product, 6β,17β-dihydroxy-1,4-androstadien-3-one, was independently synthesized from 6β,17β-diacetoxy 4-androsten-3-one by an unambiguous method. The presence of the $\Delta^1$ bond stabilizes the 6β-hydroxy-$\Delta^4$-3-ketone and prevents it from rearranging to a 3,6-diketone.[15] The lost hydroxyl was most probably at position 1α. This assignment is supported by the molecular rotatory contributions of the two hydroxyl groups ($\Delta M_D$ −253°). The calculated value for 1α,6β-dihydroxyl groups is −268°[2,16] while the value for 1β,6β is −459°.[15,16]

Testololactone was hydroxylated by this organism in the 2β-position. The ultraviolet spectrum in base shifted from a single maximum at 239.5 mμ to a strong peak at 231 mμ, ε 16,300 and another low maximum at 350 mμ. This change is characteristic of a 2-hydroxyl group,[8] while the strongly negative molecular rotatory contribution (−706) is typical of a 2β-hydroxyl group.[2]

## Experimental[17]

**Hydroxylation of Progesterone.**—A stainless steel fermentor of 400-l. capacity was charged with medium containing 5000 g. of dextrose, 1000 g. of cotton seed meal

(7) M. Shirasaka, R. Takasaki, R. Hayashi, and M. Tsuruta, *Bull. Agr. Chem. Soc. Japan*, **23**, 245 (1959).

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

(9) S. Bernstein, M. Heller, L. I. Feldman, W. S. Allen, R. H. Blank, and C. E. Linden, *J. Am. Chem. Soc.*, **82**, 3685 (1960).

(10) S. Bernstein, W. S. Allen, M. Heller, R. H. Lenhard, L. I. Feldman, and R. H. Blank, *J. Org. Chem.*, **24**, 286 (1959), incorrectly assigned 7α configuration.[11]

(11) R. C. Tweit, A. H. Goldkamp, and R. M. Dodson, *J. Org. Chem.*, **26**, 2856 (1961).

(12) G. Raspe and K. Kieslich, *Naturwissenschaften*, **48**, 479 (1961).

(13) C. Meystre and A. Wettstein, *Helv. Chim. Acta*, **32**, 1978 (1949).

(14) R. U. Lemieux, R. K. Kullnig, H. J. Bernstein, and W. G. Schneider, *J. Am. Chem. Soc.*, **79**, 1005 (1957).

(15) R. M. Dodson, S. Krayehy, R. T. Nicholson, and S. Mizuba, *J. Org. Chem.*, **27**, 3159 (1962).

(16) S. H. Eppstein, P. D. Meister, H. M. Leigh, D. H. Peterson, H. C. Murray, L. M. Reineke, and A. Weintraub, *J. Am. Chem. Soc.*, **76**, 3174 (1954).

(17) Rotations were measured in chloroform at 25 ± 2° unless otherwise noted.

flour,[18] 600 ml. of corn steep liquor, 50 ml. of concentrated hydrochloric acid, 15 g. of silicone antifoam emulsion[19] and sufficient hot tap water to result in a final volume of approximately 300 l. after sterilization. The vessel and medium were sterilized by direct steam to a temperature of 120° and then were cooled to approximately 25° and maintained at that temperature during incubation.

The medium was inoculated with a culture of *Penicillium* sp. A.T.C.C. 12,556 and, during an initial incubation period of 35 hr., was aerated at the rate of 30 l. of sterile air per minute through a perforated tube type of sparger located near the bottom of the fermentor. Continuous agitation of the culture was maintained by means of a vertically-mounted agitator operating at 180 r.p.m.

One hundred grams of progesterone dissolved in 1200 ml. of acetone was added to the culture and incubation was continued as described above for an additional period of 14 hr. The steroids were extracted from the culture into methylene chloride and recovered by evaporation of the solvent. The residue from the methylene chloride extract was chromatographed on 1700 g. of silica gel. The first 13 l. of 40% ethyl acetate–benzene eluates were concentrated and the residue was recrystallized from methylene chloride–acetone to give 18.75 g. of 15β-hydroxyprogesterone, m.p. 195–197°. A sample was recrystallized to m.p. 198–202°, reported[4] m.p. 204–205°, mixed melting point with authentic material 199–201°.

The next 8 l. of 40% ethyl acetate–benzene eluates were concentrated and the residue recrystallized from methylene chloride–acetone to give 2β,15β-dihydroxyprogesterone, with the following properties: 1.13 g., m.p. 217–219°, $\lambda_{max}^{CH_3OH}$ 243 mμ, ε 14,500, [α]D −51.5° (reported[7] m.p. 206–216°, [α]D −66.7°), and 0.84 g., m.p. 211–214°. The ultraviolet spectrum in base shifted to a maximum at 230 mμ, ε 16,700. The infrared spectrum is the same as that[20] of an authentic sample.

From the last 9 l. of 40% ethyl acetate–benzene eluates, 0.48 g. of 6β-hydroxy-4-pregnene-3,15,20-trione was obtained after several recrystallizations from chloroform–methanol. The compound has the following properties: m.p. 280–286° $\lambda_{max}^{CH_3OH}$ 236 mμ, ε 13,600, [α]D +122±8° (ethanol). The infrared spectrum has peaks at 2.90, 5.75, 5.85, 5.96, and 6.19 μ.

*Anal.* Calcd. for $C_{21}H_{28}O_4$: C, 73.22; H, 8.19. Found: C, 72.96; H, 8.09.

6β-Hydroxy-4-pregnene-3,15,20-trione, 0.1 g., was dissolved in 1 ml. of acetic anhydride and 2 ml. of pyridine. The next day the solution was poured onto ice and sodium carbonate was added. A solid formed slowly and was separated by filtration and recrystallized from acetone–petroleum ether (b.p. 60–70°) to yield 0.07 g. of 6β-acetoxy-4-pregnene-3,15,20-trione with the following properties: m.p. 198–199.5°, $\lambda_{max}^{CH_3OH}$ 234 mμ, ε 13,400, [α]D +117° (ethanol).

*Anal.* Calcd. for $C_{23}H_{30}O_5$: C, 71.48; H, 7.82. Found: C, 71.32; H, 7.94.

The 55% ethyl acetate–benzene eluates were concentrated, and the residue was crystallized from acetone to give 10.3 g. of 7β,15β-dihydroxyprogesterone with the following properties: m.p. 232–234°, $\lambda_{max}^{CH_3OH}$ 240.5 mμ, ε 15,900, [α]D +122°; reported[5] m.p. 231–233°, [α]D +136°.

*Anal.* Calcd. for $C_{21}H_{30}O_4$: C, 72.80; H, 8.73. Found: C, 72.79; H, 8.82.

The monoacetate (7β), prepared by the usual method, has the following properties: m.p. 194–195°, [α]D +132 ±1°, $\lambda_{max}^{CH_3OH}$ 237 mμ, ε 16,300; reported[5] m.p. 194–195°, [α]D +123°.

*Anal.* Calcd. for $C_{23}H_{32}O_5$: C, 71.10; H, 8.30. Found: C, 70.92; H, 8.39.

15β-Hydroxy-4,6-pregnadiene-3,20-dione, prepared by the method of Tsuda[5] from the 7β,15β-dihydroxy compound, has the following properties: m.p. 221–222°, $\lambda_{max}^{CH_3OH}$ 284 mμ, ε 26,300, reported[5] m.p. 220–223°, $\lambda_{max}^{CH_3OH}$ 283.8 mμ, ε 30,000.

*Anal.* Calcd. for $C_{21}H_{28}O_3$: C, 76.79; H, 8.59. Found: C, 76.52; H, 8.67.

A sample prepared by chloranil oxidation[21] of 15β-hydroxyprogesterone was shown to be identical by comparison of infrared spectra.

In another fermentation with the same organism, elution of the chromatographic column with 30% ethyl acetate in benzene gave 65 mg. of crude 2β-hydroxyprogesterone which after crystallization from acetone–cyclohexane, dilute methanol, then acetone–cyclohexane, melted at 184–186°. Because of the very small quantity of pure material obtained, no analysis was attempted on it. The changes in the ultraviolet spectra with time in 0.1*N* methanolic potassium hydroxide were those characteristic of a 2-hydroxy steroid.[8] The sample was acetylated with five drops of pyridine and five drops of acetic anhydride at room temperature for 5 hr. The 2β-acetoxyprogesterone, m.p. 121–123.5°, was recovered by dilution of the reaction mixture with ice and water, then filtration. Crystallization of the 2β-acetoxyprogesterone from dilute acetone raised its melting point to 124–126°. This material was identical (mixed m.p. and infrared spectrum) with a sample of 2β-acetoxyprogesterone, m.p. 125–127°, prepared from 6β-bromoprogesterone by reaction with sodium acetate in acetic acid.[22,23]

**Hydroxylation of 17α,21-Dihydroxy-4-pregnene-3,20-dione.**—A stainless steel fermentation tank of 40-l. capacity was charged with medium containing 120 ml. of corn steep liquor, 5 g. of cotton seed meal flour,[18] 25 g. of dextrose, 40 g. of potassium dihydrogen orthophosphate, 2.5 g. of silicone antifoam emulsion,[19] and sufficient hot tap water to result in a final volume of approximately 40 l. after sterilization. The vessel and medium were sterilized by direct steam to a temperature of 120° and then were cooled to approximately 25° and maintained at that temperature during subsequent incubation.

The fermentor was inoculated with a suspension of spores of *Penicillium* sp., A.T.C.C. 12,556, and was incubated for 28 hr. with aeration through a perforated ring type of sparger at the rate of 5 l. of sterile air per minute. The culture was agitated by means of a vertically mounted, paddle-type stirrer operating at 200 r.p.m.

Ten grams of 17α,21-dihydroxy-4-pregnene-3,20-dione dissolved in 450 ml. of acetone was added and incubation was continued as described above for an additional period of 43 hr. The steroids were extracted from the culture into methylene chloride and were recovered by evaporation of the solvent.

The residue was chromatographed on silica gel. The eluants with 75% ethyl acetate in benzene were concentrated and the residues combined and crystallized twice from acetone–ether to yield 0.21 g. of 15β,17α,21-trihydroxy-4-pregnene-3,20-dione, with the following properties: m.p. 219–222°, [α]D +88.0° (ethanol), reported[9] m.p. 240–242°, [α]D +96° (methanol).

*Anal.* Calcd. for $C_{21}H_{30}O_5$: C, 69.58; H, 8.34. Found: C, 69.50; H, 8.66.

The infrared spectrum in a potassium bromide disk was

(18) Pharmamedia, Traders Oil Mill Co., Fort Worth, Texas.

(19) Antifoam AF Emulsion, Dow Chemical Corp., Midland, Mich.

(20) Kindly supplied by Dr. S. Kraychy of these laboratories. Dr. Kraychy proved the structure by reduction to 15β-hydroxyprogesterone.[7]

(21) E. J. Agnello and G. D. Laubach, *J. Am. Chem. Soc.*, **82**, 4293 (1960).

(22) We are indebted to Mr. R. W. Hamilton of these laboratories for this preparation. 2β-Acetoxyprogesterone (*Anal.* Calcd. for $C_{23}H_{32}O_4$: C, 74.16; H, 8.66. Found: C, 74.04; H, 8.67) was readily converted to the 2α-isomer[23] by prolonged heating with sodium acetate in acetic acid.

(23) F. Sondheimer, S. Kaufmann, J. Romo, H. Martinez, and G. Rosenkranz, *J. Am. Chem. Soc.*, **75**, 4712 (1953).

not identical with that of a sample of authentic material.[24] However, when the material was recrystallized again and seeded with the Lederle material, the m.p. was 233–235° dec. and the infrared spectra were identical.

The filtrate from the original crystallization above was concentrated and dissolved in 3 ml. of acetic anhydride and 3 ml. of pyridine. After standing overnight the solution was poured into water and neutralized with sodium carbonate. The mixture was extracted with ether–methylene chloride and the organic extracts were washed with dilute acid and then concentrated to dryness. When the residue was triturated with ether and rubbed, crystals formed which were crystallized three times from acetone–ligroin to yield 0.13 g. of 7β,21-diacetoxy-17α-hydroxy-4-pregnene-3,20-dione, with the following properties: m.p. 243–244° dec., $[\alpha]_D$ +110°, $\lambda_{max}^{CH_3OH}$ 238 mμ, ε 16,600, shifts in base to 284 mμ, ε 16,600, reported[10] m.p. 246–248°, $[\alpha]_D$ +107.

*Anal.* Calcd. for $C_{25}H_{34}O_7$: C, 67.24; H, 7.68. Found: C, 67.40; H, 7.53.

**Hydroxylation of 16α,17α-Epoxyprogesterone.**—A 40-l. stainless steel fermentor containing 30 l. of sterile cotton seed meal flour–corn steep liquor–dextrose medium was inoculated with spores of *Penicillium* sp., A.T.C.C. 12,556, and was incubated at approximately 25° for 42 hr. with aeration at 15 l. per min. and agitation at 200 r.p.m. Ten grams of 16,17-epoxyprogesterone dissolved in 750 ml. of ethanol was added and incubation was continued 27 hr. Steroids were extracted from the culture into methylene chloride.

The extracts were filtered and concentrated. When ether was added to the residue, a solid formed which was separated and crystallized twice from acetone to yield 1.50 g. of 7β-hydroxy-16α,17α-epoxyprogesterone, with the following properties: m.p. 229–232°, $\lambda_{max}^{CH_3OH}$ 241.5 mμ, ε 15,800, shifts in base to 283 mμ, $[\alpha]_D$ +158.5°.

*Anal.* Calcd. for $C_{21}H_{28}O_4$: C, 73.23; H, 8.19. Found: C, 73.35; H, 7.87.

The remaining material was chromatographed on silica gel.

When the 30% eluates were concentrated and the residue was recrystallized from acetone, 0.46 g. of 7β-hydroxy-16α,17α-epoxyprogesterone, m.p. 237–239°, $[\alpha]_D$ +156 ± 1°, was obtained. An additional 0.11 g., m.p. 232–236°, was obtained from the 40% eluates. The total amount was 2.07 g.

**7β-Acetoxy-16α,17α-epoxyprogesterone.**—7β-Hydroxy-16α,17α-epoxyprogesterone, 0.20 g., was acetylated by the usual method and recrystallized from acetone–ether to yield 0.11 g. of 7β-acetoxy-16α,17α-epoxyprogesterone, with the following properties: m.p. 190–191°; $\lambda_{max}^{CH_3OH}$ 237 mμ, ε 16,200; $[\alpha]_D$ +156 ± 1°.

*Anal.* Calcd. for $C_{23}H_{30}O_5$: C, 71.48; H, 7.82. Found: C, 71.51; H, 7.94.

**Hydroxylation of Testosterone.**—The steroid, 100 g., was fermented with *Penicillium* sp., A.T.C.C. 12,556, as described for the fermentation of progesterone except that the period of steroid conversion was prolonged to 24 hr. The residue was washed with ligroin and the insoluble material was chromatographed on 1700 g. of silica gel. From the early 50% ethyl acetate–benzene eluates, 11.8 g. of 6β-hydroxytestosterone was isolated. A sample was recrystallized from acetone–ether to m.p. 212–214°, $\lambda_{max}^{CH_3OH}$ 236.5 mμ, ε 13,700, $[\alpha]_D$ + 30°. Reported[16] m.p. 216–222°, $\lambda_{max}^{CH_3OH}$ 238 mμ, ε 13,700, $[\alpha]_D$ +32°.

*Anal.* Calcd. for $C_{19}H_{28}O_3$: C, 74.96; H, 9.27. Found: C, 74.60; H, 9.24.

The diacetate prepared in the usual manner had a m.p. of 139–140°, $[\alpha]_D$ + 29 ± 1°, reported[16] m.p. 127–131°, 141–143°, $[\alpha]_D$ −1°. We can not explain the discrepancy in rotations.

*Anal.* Calcd. for $C_{23}H_{32}O_5$: C, 71.10; H, 8.30. Found: C, 71.07; H, 8.35.

From the 60% ethyl acetate–benzene eluates, 4.15 g. of 15β-hydroxytestosterone was obtained. Part of the material was recrystallized from methylene chloride–methanol–acetone to yield material of m.p. 216–218°, $[\alpha]_D$ +67° (ethanol), reported[3] m.p. 220–222°, $[\alpha]_D$ +57° (ethanol).

*Anal.* Found: C, 74.53; H, 9.33.

The infrared spectrum in Nujol was the same in the fingerprint region as that[25] of an authentic sample.

The intermediate fractions between the 6β- and 15β-hydroxytestosterone peaks were rechromatographed and 0.76 g. of 1α-hydroxytestosterone was isolated. Part of the material was recrystallized from methanol to m.p. 239–240°. The infrared spectrum was identical with that of an authentic sample.[2]

From the early 80% ethyl acetate–benzene eluates 12β-hydroxytestosterone was obtained. Some of the material was recrystallized to m.p. 121–125° dec., $[\alpha]_D$ +120°, $\lambda_{max}^{CH_3OH}$ 239.5 mμ, ε 16,500. Reported[12] m.p. 115/117–119°, $\lambda_{max}^{CH_3OH}$ 240 mμ, ε 15,700.

*Anal.* Found: C, 74.88; H, 9.44.

A diacetate prepared in the usual manner melted at 160–161°, $[\alpha]_D$ +89°, $\lambda_{max}^{CH_3OH}$ 239 mμ, ε 17,400.

*Anal.* Found: C, 70.96; H, 8.11.

4-Androstene-3,12,17-trione was prepared by chromium trioxide–pyridine oxidation of 12β-hydroxytestosterone. The triketone melted at 213–215°, $[\alpha]_D$ +297±4°, $\lambda_{max}^{CH_3OH}$ 239 mμ, ε 18,000, mixed m.p. with the triketone synthesized from 12α-hydroxyandrostenedione, 212.5–214.5°. The infrared spectra of the two samples were identical.

*Anal.* Calcd. for $C_{19}H_{24}O_3$: C, 75.97; H, 8.05. Found: C, 76.10; H, 7.98.

From the last of the 80% ethyl acetate–benzene eluates, 1α,6β-dihydroxytestosterone was isolated and crystallized twice from methanol to give 0.19 g. of material, with the following properties: m.p. 256–260° dec., $[\alpha]_D$ +19° (methanol) $\lambda_{max}^{CH_3OH}$ 235.5 mμ, ε 13,800.

*Anal.* Calcd. for $C_{19}H_{28}O_4$: C, 71.22; H, 8.81. Found: C 71.63; H, 8.62.

The steroid, 0.10 g., was partially dissolved in 8 ml. of methanol and 2 ml. of 5% sodium hydroxide solution was added. The mixture was stirred until all the solid was gone and after 4 hr. the solution was diluted to 50 ml. with water and extracted several times with methylene chloride containing a little methanol. The extracts were dried and concentrated to a small volume. When acetone and ligroin were added crystals of 6β,17β-dihydroxy-1,4-androstadien-3-one formed, 43 mg., m.p. 191–192.5°, mixed m.p. with with material prepared below 190–193°. The infrared spectra of the two samples were identical.

**17β-Acetoxy-6β-hydroxy-1,4-androstadien-3-one.**—6β,-17β-Diacetoxy-4-androsten-3-one, 11.6 g., was dissolved in 0.5 l. of benzene and the solution was boiled to remove any water. Then 8.2 g. of 2,3-dichloro-5,6-dicyanobenzoquinone was added. The mixture was refluxed for 2 days, cooled, and filtered. The filtrate was washed with sodium sulfite solution and water, filtered, and concentrated under vacuum. The residue was dissolved in acetone–methanol and sodium hydroxide solution was added. The solution was warmed gently and a solid formed. It was separated by filtration and recrystallized from methylene chloride–methanol to give 4.8 g. of 17β-acetoxy-6β-hydroxy-1,4-androstadien-3-one, with the following properties m.p. 264–266°, $[\alpha]_D$ −10°, $\lambda_{max}^{CH_3OH}$ 245 mμ ε 16,800.

*Anal.* Calcd. for $C_{21}H_{28}O_4$: C, 73.22; H, 8.19. Found: C, 73.06; H, 8.22.

The infrared spectrum indicated the presence of a hydroxyl group (2.92 μ) and an acetoxyl group (5.73 and 8.07 μ). The n.m.r. spectrum suggested a 1,4-bisdehydro-3-oxo moeity by peaks at 2.95, 3.12, 3.80, and 3.95 p.p.m. A broad peak centered at 5.5 p.p.m. was due to the 6α-

(24) Very kindly supplied by Dr. Brian L. Hutchings of Lederle Laboratories.

(25) Very kindly supplied by Dr. H. L. Herzog of the Schering Corp.[3]

hydrogen (6β-hydroxyl) and the 17α-hydrogen (17β-acetoxyl). A peak for a 17α-hydrogen (17β-hydroxyl) which should have appeared at 6.35 was not present.

**6β,17β-Dihydroxy-1,4-androstadien-3-one.**—17β-Acetoxy-6β-hydroxy-1,4-androstadien-3-one, 3.0 g., was mixed with 1/3 l. of methanol and twelve pellets of sodium hydroxide. The mixture was stirred until homogeneous. After 18 hr. the base was neutralized with acetic acid and the solution was concentrated to dryness. Water and methylene chloride were added to the residue and the mixture was filtered. The solid was crystallized from methylene chloride–acetone to give 6β,17β-dihydroxy-1,4-androstadien-3-one, 2.1 g., with the following properties: m.p. 194–195°, $\lambda_{max}^{CH_3OH}$ 246 mμ ε 16,300, [α]D 21 ± 2° (methanol).

*Anal.* Calcd. for $C_{19}H_{26}O_3$: C, 75.46; H, 8.67. Found: C, 75.22; H, 8.56.

**4-Androstene-3,12-,17-trione.**—12α-Hydroxy-4-androstene-3,17-dione,[13] 0.20 g., in acetone was oxidized with chromium trioxide-sulfuric acid solution by the method of Jones and Djerassi.[26] The product was crystallized from acetone–ether to give 4-androstene-3,12,17-trione, 0.10 g., m.p. 212.5–214°.

**Hydroxylation of Testololactone.**—The steroid, 6.5 g., was fermented with *Penicillium* sp., A.T.C.C. 12,556, under conditions similar to those described for the fermentation of 16α,17α-epoxyprogesterone.

The methylene chloride solution was filtered and evaporated. The residue was dissolved in benzene and chromatographed on silica gel.

From the later fractions of 30% ethyl acetate in benzene, 2β-hydroxy-testololactone was obtained. After the material was crystallized twice from acetone-ether it weighed 0.33 g. and had the following properties: m.p. 180–182°, $\lambda_{max}^{CH_3OH}$ 240.5 mμ ε 14,750, [α]D −182 ± 2°, ΔMD (2βOH − 2βH) = −706°.

*Anal.* Calcd. for $C_{19}H_{26}O_4$: C, 71.67; H, 8.23. Found: C, 71.56; H, 8.31.

(26) K. Bowden, I. M. Heilbron, E. R. H. Jones, and B. C. L. Weedon, *J. Chem. Soc.*, 39 (1946); C. Djerassi, R. R. Engle, and A. Bowers, *J. Org. Chem.*, **21**, 1547 (1956).

# Glutarimide Antibiotics. I. The Synthesis of Actiphenol

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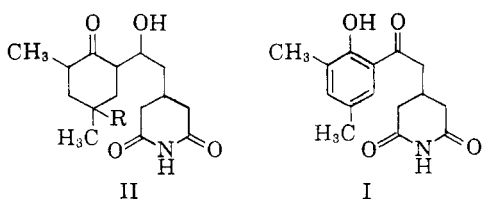
*Received June 4, 1962*

A synthesis of actiphenol is described.

As part of a program aimed at the synthesis of naturally occurring glutarimide compounds we report in this paper a synthesis of actiphenol (I). This substance, which to date is the simplest member of this class of compounds, was first isolated and characterized by Prelog and Highet.[1] They effected its partial synthesis in minute yield by the aromatization of the cyclohexane ring of cycloheximide (II, R=H) with N-bromosuccinimide.

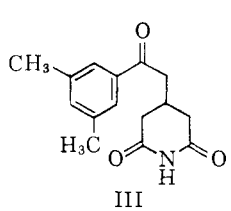
Independently, I was isolated by Rao[2] who gave it the trivial designation C-73. He also came to the same conclusion regarding its structure by relating it to a derivative (III) of E-73 (II, R=OAc). Our synthetic results confirm these findings.



II



I



III

The common intermediate, 3-carboxymethylglutarimide (IV), desired for synthetic approaches to compounds of this glutarimide class has been prepared previously by Phillips, *et al.*,[3] and Lawes.[4] In our hands, however, the former procedure suffered from the capriciousness of the yield in the final cyclization step,[5] while the latter involved the preparation of the somewhat inaccessible methanetriacetic acid.[6,7]

We have found that the desired material can be prepared conveniently using the three step reaction sequence shown in Chart I.

Cope condensation[8] of dimethyl acetonedicarboxylate with cyanoacetic acid in a benzene–acetic acid medium using ammonium acetate as the catalyst afforded dimethyl 3-cyanomethyleneglutarate[9] in 54% yield. Hydrogenation of this material in ethanol over a palladium–charcoal catalyst led to 90% of the expected dimethyl 3-cyanomethylglu-

(1) R. H. Highet and V. Prelog, *Helv. Chim. Acta*, **42**, 1523 (1959).
(2) K. V. Rao, *J. Org. Chem.*, **25**, 661 (1960).
(3) D. D. Phillips, M. A. Acitelli, and J. Meinwald, *J. Am. Chem. Soc.*, **79**, 3517 (1957).
(4) B. C. Lawes, *ibid.*, **82**, 6413 (1960).
(5) M. A. Acitelli, Ph.D. thesis, Cornell University (1957).
(6) E. P. Kohler and G. H. Reid, *J. Am. Chem. Soc.*, **47**, 2803 (1925).
(7) E. C. Kornfeld, R. G. Jones, and T. V. Parke, *ibid.*, **71**, 150 (1949).
(8) A. C. Cope, C. M. Hoffman, C. Wyckoff, and E. Hardenbergh, *ibid.*, **63**, 3452 (1941).
(9) T. Okuda of the Tanabe Saiyaku Co., Tokyo, Japan, has informed us that they also have prepared this material by this method—private communication.