Actinomycin J_2, a By-product from a Strain of Actinomyces.

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An antibiotic (activity; 1:10,000,000 against *staphylococcus aureus Terajima*) has been obtained from *actinomyces flavus*,[1] which seems to be related to actinomycin A of Waksman[2], but considering the fact that the position of the absorption maximum in 95 % alcohol is 435 mμ, compared with the values 450 mμ and 230~250 mμ obtained by Waksman. and that there is a difference in the percentage of carbon (author: 55. 45 %, Waksman: 59.01 %), it is suggested that this substance is not quite identical which actinomycin A. Thus we shall call this substance actinomycin J_1. Meanwhile, another antibiotic, with a minor activity (1:160,000) seemed to have been extracted from the same strain, corresponding to actinomycin B of Waksman. We have studied its chemical structure and finally come to the conclusion of its being the dodecyl ester of 5-ketostearic acid:

$$CH_3(CH_2)_{10}CH_2OCO(CH_2)_3CO(CH_2)_{12}CH_3$$

by means of synthesis. Though this synthesized sample was identical with the natural specimen, it demonstrated no activity. Therefore, it must be concluded that the activity must have been due to a minute amount of the former actinomycin J_1 existing as an impurity in the second substance.

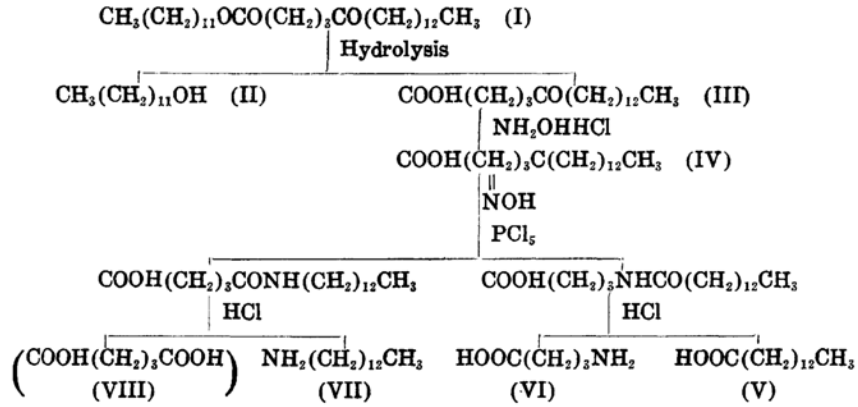
This second substance may be named actinomycin J_2 for convenience, notwithstanding its inactivity, and the experimental data for the determination of its chemical structure and synthesis are here reported.

Actinomyces flavus was cultivated on a Czapeck-Dox solution, extracted after two weeks and crystallized from methanol (m. p. 59–61°C).

It possesses a waxlike nature and odor, dissolving in all the wax-dissolving solvents. The fact that the ultra-violet absorption spectra showed no selective absorption suggested that it was probably a simple aliphatic compound containing none of the specific groups. The tetranitromethane and bromine tests were negative, thus excluding the presence of a double bond. Analysis gave the formula $C_{30}H_{58}O_3$(I), and by the action of ethanolic potassium hydroxide, the original substance was hydrolysed into two fractions, $C_{12}H_{26}O$ (II) and $C_{18}A_{36}O_3$ (III). (Scheme I)

(1) Y. Hirata and K. Nakanashi, *J. Penicillin*, 2 (1949), 180.

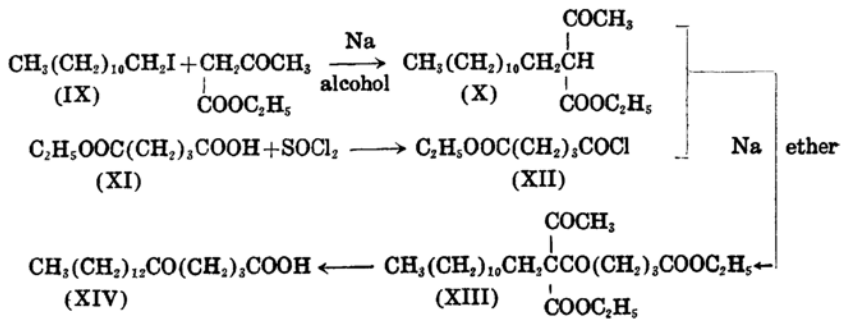
(2) S. A. Waksman and H. B. Woodruff, *J. Bact.*, 40 (1940), 583; *J. Biol. Chem.*, 142 (1942), 519.



Bubbles which were generated by the action of sodium bicarbonate showed (III) to be an acid, while the negative results obtained from an effort to esterify the substance by means of *p*-nitrobenzoyl chloride and to dehydroxylize it by reduction with hydroiodic acid in a sealed tube, indicated that (III) was not a hydroxy acid. On the other hand, such derivatives as the 2,4-dinitrophenyl hydrazone and the oxime (IV) showed it to be a keto acid. The oxime was submitted to the Beckmann rearrangement and after hydrolysis with hydrochloric acid, (V) (VI) and a minute amount of (VII) were obtained, thus suggesting its structure to be 5-keto-stearic acid. (VII) could not be identified.

The melting point of (II) (22°) and the *α*-naphthyl urethane (80°) corresponded with that of dodecyl alcohol. Therefore, the sturcture of J_2 was established to be the dodecyl ester of 5-keto-stearic acid and this was further proved by synthesizing the substance and mixing it with the natural specimen, upon which no depression of melting point was observed. The synthesis was carried out by two methods, of which the latter gave a considerable yield (42 %).

The first method, analogous to that employed by Robinson and Robinson[3] (scheme II) is concluded by gradual hydrolysis under three steps of Scheme II. First Method.



(3) G. M. Robinson and R. Robinson, *J. Chem. Soc.*, 127 (1925), 175.

Second Method.

$$\underset{\text{(XV)}}{Br(CH_2)_2COOC_2H_5} + \underset{\text{(XV)}}{\begin{matrix}COCH_3\\|\\HCH\\|\\COOC_2H_5\end{matrix}} \xrightarrow[\text{alc.}]{Na} \underset{\text{(XVI)}}{\begin{matrix}COCH_3\\|\\HC(CH_2)_2COOC_2H_5\\|\\COOC_2H_5\end{matrix}} + \underset{\text{(XVII)}}{CH_3(CH_2)_{12}COCl}$$

$$\xrightarrow[\text{ether}]{Na} \underset{\text{(XVIII)}}{\begin{matrix}COCH_3\\|\\CH_3(CH_2)_{12}COC(CH_2)_2COOC_2H_5\\|\\COOC_2H_5\end{matrix}} \longrightarrow \underset{\text{(XIX)}}{CH_3(CH_2)_{12}CO(CH_2)_3COOH}$$

the condensation products of the form :

$$CH_3(CH_2)_m\overset{\overset{COCH_3}{|}}{\underset{\underset{COOC_2H_5}{|}}{C}}-CO(CH_2)_{12}COOC_2H_5$$

The process resulted in a very poor yield of the keto acid, owing to the fact that a considerable amount of the dibasic acid, $COOH(CH_2)_nCOOH$, and a ketone, $CH_3(CH_2)_{m+1}COCH_3$, were produced ; furthermore, it is rather tedious to prepare monoethyl glutarate (XI) in good yield. Bradley and Robinson[4] suggest that the hydrolysis of a series of substituted dibenzoyl methanes, $RCOCH_2COR'$ occurred with the production of the stronger of the acids, RCOOH and R'COOH in preponderating amounts. Thus in the diketo acids of the type shown above (XIII), owing to the fact that the dibasic acid, $COOH(CH_2)_nCOOH$ is stronger than acetic acid, the linkage marked with dots is degraded mainly, resulting in the undesired by-products, thd dibasic acid and a ketone.

In the second method (scheme II)[5] the synthesis was carried out by way of the diketo acid possessing the following general formula :

$$CH_3(CH_2)_{m+1}\overset{\overset{COCH_3}{|}}{\underset{\underset{COOC_2H_5}{|}}{C}}OC(CH_2)_{n-1}COOC_2H_5$$

In this case, acetic acid is stronger than the acid, $CH_3(CH_2)_{m+1}COOH$, and this would make the hydrolysis proceed so as to give the desired keto acid, $CH_3(CH_2)_{m+1}CO(CH_2)_nCOOH$, as the main product.

Finally the synthesis of J_2, namely, the dodecyl ester of 5-ketostearic acid chloride was carried out by the reaction of dodecyl alcohol and the keto acid chloride in pyridine.

Experimental.

Cultivation. Actinomyces flavus (received from Dr. Umezawa)[6] was grown on a

(4) W. Bradley and R. Robinson, *J. Chem. Soc.*, **1926**, 2356.

(5) G. M. Robinson, *J. Chem. Soc.*, **1920**, 747.

(6) H. Umezawa, S. Hayano, T. Takeuchi and Y. Hara, *J. Penicillin.* **1** (1947), 129.

modified Czapeck-Dox medium ($NaNO_3$ 6 g., K_2HPO_4 2 g., KCl 1 g., $MgSO_4 7H_2O$ 1 g., $FeSO_4 7H_2O$ 0.02 g., glucose 60 g., peptone 10 g., distilled water 1000 c.c.) at 27°C for two weeks and shaken occasionally.

The culture liquid became brown-black and the floating bodies gradually acquired a yellowish color.

Extraction. The body and culture liquid were separated and the former extracted with acetone. The latter was shaken with clay and adsorbed, extracted with acetone, and the combined acetone solutions evaporated by submitting to a flash evaporator. The resulting orange yellowish turbid aqueous solution was extracted with ether, and red plates (actinomycin J_1) were obtained by concentrating the ether solution. After removing the crystals by decantation the concentration was repeated, upon which a second crop of J_1 precipitated. J_2 was obtained from the residue as white wax-like plates using methanol or ethanol as recrystallization solvent. Yields were 150 mg./l. and 80 mg./l. for J_1 and J_2 respectively. Melting point of J_2: 59-61°.

(Found: C, 76.36; H, 12.44. Calculated for $C_{30}H_{38}O_8$: C, 77.2; H, 12.5%). Molecular weight determined by the Rast method gave 455. Calculated: 463.

Ultra-violet absorption spectra. The 0.1%, 0.01%, and 0.001% solutions in 96.5% alcohol showed no selective absorption, the absorption of a 100 mm. solution beginning from 310 mμ.

Hydrolysis. Hydrolysis of J_2 was carried out by the action of 4% alcoholic KOH on the sample (185 mg.) at room temperature for three days.

The alcohol was evaporated on a steam-bath and after addition of water to the residue, submitted to steam distillation, upon which (II) came over into the distillate as white crystals, possessing an odor of higher alcohol. The residue was acidified, extracted with ether, the ether evaporated and the residue (III) recrystallized from ethyl alcohol. m. p.: 81°. Found: C, 71.82; H, 13.05. Calculated for $C_{18}H_{36}O_3$: C, 72.0; H, 12.0%.

Dodecyl alcohol (II). M. p. 22°, alone or mixed with dodecyl alcohol. α-Naphthyl urethane: m. p. 79° (ligroin).

***p*-Bromophenacyl ester of 5-keto-stearic acid.** 1N NaOH was titrated to an alcoholic solution of the sample (25 mg.), using phenolphthalein as indicator. The solution should be kept slightly acidic and the concentration of alcohol kept above 70%. One to two per cent. less than the equivalent amount of *p*-Bromophenacyl bromide is added to this solution, boiled on the bath under reflux for 1 hour, the flask cooled, water added and the separated solid recrystallized from dilute alcohol. m. p. 105° (uncorr.).

2,4-Dinitrophenyl hydrazone of 5-ketostearic acid. The alcohol and sulfuric acid solution of 2,4-dinitrophenyl hydrazine was added to the alcoholic solution of the keto acid and left overnight in the ice-box. After removing the red crystals of 2,4-dinitrophenyl hydrazine sulfate which may occur, the remaining red oil crystallized after 1-2 days. M. p. 110° (uncorr.).

5-Ketostearic acid oxime (IV). A mixture of the keto acid (70 mg.), anhydrous pyridine (1.3 c.c.) and hydroxyl amine hydrochloride (30 mg.) (molecular ratio; 1:1:3) was gently boiled for 3 hours under reflux, poured into water and acidified with HCl. The resulting oxime was extracted with ether, and crystallized from alcohol. This method using pyridine enabled the oxime to be prepared more easily and completely than the usual method with alcoholic KOH. m. p. 64°. Found: N, 4.90. Calculated for $C_{18}H_{37}O_2N$: N, 4.4%.

Beckmann rearrangement and hydrolysis of the oxime (IV). PCl_5 was added under cooling with an ice-mixture to the dilute ether solution of the oxime obtained above and kept overnight ot 0°, A sufficient amount of PCl_5 should be added to retain a precipitate of PCl_5. The mixture is shaken with ice-water, the ether evaporated after drying over K_2CO_3 and the residue heated eight hours with conc. HCl at 160° in a sealed tube.

Myristic acid (V). Water is poured into the reaction mixture and the white crystals

extracted with ether. M. p. 53° (alcohol), alone or mixed with myristic acid. Found: C, 72.58; H, 12.01. Calculated for $C_{14}H_{28}O_2$: C, 73,7; H, 12.3 %.

n-Tridecyl amine (VII). The acidic solution was made alkaline, extracted with ether and dry HCl gas passed through, upon which a small amount of fine white needles were obtained. Hygroscopic, decomposed at 100°. Pt-salt ($2C_{13}H_{29}N+2HCl+PtCl_4$): 230° (decomp.).

Glutaric acid (VIII). This was unobtainable owing to the minute amount of sample used.

γ-Aminobutyric acid (VI). The alkaline residue was acidified with HCl, evaporated on the water-bath, separated from NaCl by washing with absolute alcohol and the alcohol evaporated. The remaining syrup crystallized into hygroscopic needles when kept in an ice-box. The Pt-salt was obtained and analysed. Found: Pt, 31.02; Calculated for $2NH_2(CH_2)_3COOH+2HCl+PtCl_4$: Pt. 31.88 %.

Dodecyl-acetoacetic acid ethyl ester (X). Dodecyl iodide (IX) was prepared by passing HI gas (2.5 mol) through dodecanol (55 g., 0.3 mol) at room temperature, and after washing with water and dilute KOH, distilled; the portions 165-170°/20 mm. collected (40 g.).

16 g. of an oil, b. p. 205°/255 mm., were obtained according to the usual method, employing Na (2.1 g.), ethyl acetoacetate (17.7 g.), absolute alcohol (30 c c.), n-dodecyl iodide (28 g.): the time of reaction was 5 hours.

4-Carbethoxybutyryl chloride (XII). Monoethyl glutarate (XI) was prepared by a method analogous to that employed by Grün and Wirth[7] in the semihydrolysis of diethyl sebacate; namely, diethyl glutarate (8 g.) was dissolved in half its volume of absolute alcohol and to this boiling solution a calculated amount of 2 N KOH alcoholic solution was added under vigorous stirring. The resulting white mass was poured into a considerable amount of ice-water, and the alcohol removed by distillation under reduced pressure. After extracting the unchanged diester completely with ether, the residue was acidified and extracted with ether. A crude product of the monoester was obtained by evaporating the ether. The ether extract was dried over Na_2SO_4 and distilled. 3 g. of an oil, 143°/7 mm. was obtained. Monoethyl glutarate (1.8 g.) was heated gently on the water bath with thionyl chloride (6 g.) for 3 hours, the excess thionyl chloride removed completely by distillation under reduced pressure and by keeping the residue in vacuum. A high temperature should be avoided throughout all the processes, owing to the unstability of the monoester, The chloride (XII) thus obtained was directly submitted to further procedures.

5-Ketostearic acid (XVI) (First method). Na (0.28 g.) was granulated under toluene, washed with ether, suspended in ether (20 c.c.) and (X) gradually added, the clear solution of the sodio derivative being completed by heating on the bath for several minutes. To this solution, 4-carbethoxy-butyryl chloride (XIII) dissolved in ether (3 c.c.) was added at 0°; after remaining 30 minutes at room temperature, the mixture was boiled under reflux for 15 minutes, cooled, washed with water and the ether evaporated. The residue was agitated for 8 hours with 5 % NaOH (75 c.c.), acidified with acetic acid and collected by means of ether. The product was next boiled with 5 % H_2SO_4 (100 c c.) for 8 hours and steam-distilled in order to remove the methyl tridecyl ketone. The hydrolysis was completed by boiling with 5 % NaOH (400 c.c.) for 5 hours. The product crystallized from light petroleum ether (b. p. 40-80°) and methyl alcohol, but the yield was poor, m. p. 81.5°, alone or mixed with the natural keto acid.

Diethyl α-acetoglutarate (XVI). Ethyl 3-bromopropionate (XV) (11.4 g.) ("Organic

(7) A Grün and T. Wirth, *Ber.*, 55 (1922), 2207.

Synthesis" Coll. Vol. I, p. 263) Na (2.6 g.), absolute alcohol (57 c.c.) and ethylacetoacetate (14.8 g.) were submitted to the usual method, the time of reaction being 5 hours. 2.8 g. of an oil, 158-162°/15 mm. were obtained.

5-Ketostearic acid (Second method) (XIX). Na (0.3 g.) was granulated under toluene, washed with ether, suspended in ether (30 c.c.), and diethyl α-acetoglutarate (XVI) (2.8 g.) gradually added (30 minutes) upon which the sodio-derivative was produced as a white powder. After the addition of myristic acid chloride (XVII) (3 g.), the mixture was left overnight to complete the precipitation of NaCl, boiled under reflux for 20 minutes, washed with water, the ethere evaporated and the residue agitated with 3% NaCH (170 c.c.) for 9 hours. The solution was concentrated for one hour on the bath, upon which the crude Na-salt of the keto acid formed a gelatinous mass. The free acid, prepared by the treatment with HCl and extraction with ether, was crystallized from light petroleum ether (b. p. 40–80°) and methyl alcohol. M. p. 81°, alone or mixed with the natural specimen. Yield: 1.5 g. or 42%.

Actinomycin J_2. 5-Ketostearic acid (350 mg.) was converted into its chloride by the action of thionyl chloride at 50°, the excess reagent being removed by reduced pressure. A solution of dodecyl alcohol (250 mg.) in pyridine (3 c.c.) was added to the chloride, heated at 50° for 5 minutes, left overnight at room temperature, poured into water and extracted with ether. The ethereal solution was washed with dil. H_2SO_4 and water, the ether evaporated and the residue dissolved in hot alcohol. The plates which appeared on cooling seemed to be a mixture of dodecyl alcohol and the keto acid. After removing this portion, the ester was obtained by concentrating the remaining alcoholic solution. M. p. 60°, alone or mixed with the natural substance.

Summary.

The constitution of actiomycin J_2 obtained from *actinomyces flavus* has been studied and idenified to be the dodecyl ester of 5-ketostearic acid by means of hydrolysis of the original substance, followed by the Beckmann rearrangement and hydrolysis of the oxime of 5-ketostearic acid into myristic acid, γ-aminobutyric acid and a small amount of *n*-tridecyl-amine. Next, the 5-ketostearic acid was synthesized according to two methods, which were concluded respectively by the hydrolysis of condensation products of the type: $CH_3(CH_2)_mC(COCH_3)(COOC_2H_5)CO(CH_2)_nCOOC_2H_5$ and $CH_3(CH_2)_{n+1}CO(COCH_3)(COOC_2H_5)(CH_2)_{n-1}COOC_2H_5$, of which the latter gave a considerable yield, while that of the former was very poor owing to the undesired direction of hydrolysis. The condensarion products of 5-ketostearic acid chloride and dodecyl alcohol showed no depression of meltihg point when mixed with actinomycin J_2, thus proving the constitution of actinomycin J_2 to be the dodecyl ester of 5-ketostearic acid.

These experiments were begun since it seemed that actinomycin J_2 was an antibiotic, but considering the fact that the synthesized specimen demonstrated no such activity, it must be conculded that the original activity was probably due to a minute amount of actinomycin J_1 existing as an impurity, and that actinomycin J_2 is a mere by-product.

In conclusion, the authors wish to acknowledge their indebtedness to

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