Anal. Calcd. for $C_{12}H_{13}PO_4$: C, 57.20; H, 5.16. Found: C, 57.30; H, 5.59.

Sodium β-Naphthyl Methyl Phosphate.—Finely powdered sodium β-naphthyl monohydrogen phosphate,[2] 0.3 g., was slowly added to a cold ethereal solution of diazomethane obtained from 2.0 g. of N-nitrosomethylurea. The addition caused a vigorous reaction with evolution of gas. After one hour at room temperature the mixture was filtered. The residue was triturated with boiling ethyl acetate to which a small amount of methanol was added, leaving a small amount of high-melting residue. On concentration the filtrate gave a white crystalline precipitate, 0.2 g., m.p. 222–223°.

Anal. Calcd. for $C_{11}H_{10}PO_4Na$: C, 50.70; H, 3.87. Found: C, 51.02; H, 3.70.

β-Naphthyl Dimethyl Phosphate (a) Methylation of β-Naphthyl Dihydrogen Phosphate.—The dihydro acid, 2.3 g., was added slowly to a cold solution of diazomethane distilled with 100 cc. of ether from 10 g. of N-nitrosomethylurea. When the diazomethane and excess solvent were distilled there remained a pale yellow oil, 2.5 g., b.p. 160–165°, 0.5 mm., $n^{25}D$ 1.5612.

Anal. Calcd. for $C_{12}H_{13}PO_4$: C, 57.20; H, 5.16. Found: C, 57.71; H, 5.60.

(b) **From β-Naphthyl Phosphoryl Dichloride.**—The phosphoryl dichloride,[2] 12.1 g., was refluxed in 100 cc. of reagent methanol with 10 g. of potassium carbonate for one hour. After filtration the solution was distilled to remove the solvent. There was obtained an almost colorless oil, 11.58 g., b.p. 160–165°, 0.5 mm., $n^{25}D$ 1.5655.

Anal. Calcd. for $C_{12}H_{13}PO_4$: C, 57.20; H, 5.16. Found: C, 57.20; H, 5.33.

Sodium Half-Salt of α-Naphthyl Dihydrogen Phosphate.—A precipitate was obtained by the addition of aqueous sodium chloride to a solution of α-naphthyl dihydrogen phosphate.[2] Concentrated solutions were required because of the solubility of the product, a factor which also made the removal of occluded sodium chloride difficult. When titrated potentiometrically, however, the product gave the same characteristic curve as was obtained with the β-isomer. The first equivalence point at *p*H 3.5 was reached after addition of 3.55 cc. and the second at *p*H 9 after addition of 10.4 cc. of 0.1000 *N* alkali. The discrepancy in the neutral equivalence is a result of the occluded sodium chloride.

6-Bromo-2-naphthyl Dihydrogen Phosphate.—6-Bromo-2-naphthol[10] was converted to the phosphoryl dichloride with phosphorus oxychloride and pyridine and then hydrolyzed to the dihydrogen phosphate by exposure to moisture by the method previously described.[2] A nicely crystalline product was obtained by recrystallization from acetic acid, m.p. 207–209°.

Anal. Calcd. for $C_{10}H_8PO_4Br$: C, 39.58; H, 2.66. Found: C, 39.74; H, 2.93.

Sodium Half-Salt of 6-Bromo-2-naphthyl Dihydrogen Phosphate.—The half-salt was obtained by addition of aqueous sodium chloride to an aqueous solution of the phosphate. Potentiometric titration of the product had to be carried out in the presence of methanol because of the limited solubility of the half-salt in water. The first equivalence point at *p*H 4 was reached after addition of 1.6 cc. and the second at *p*H 9 after the addition of 4.6 cc. of standard alkali.

(10) C. F. Koelsch, *Org. Syntheses*, **20**, 18 (1940).

CAMBRIDGE 38, MASS. RECEIVED MAY 5, 1951

[CONTRIBUTION FROM THE RESEARCH LABORATORIES OF HOFFMANN-LA ROCHE INC.]

The Isolation of Three New Crystalline Antibiotics from Streptomyces[1]

BY JULIUS BERGER, A. I. RACHLIN, W. E. SCOTT, L. H. STERNBACH AND M. W. GOLDBERG

Three new crystalline antibiotics were isolated from cultures of three unidentified streptomyces. Their most likely empirical formulas are: $C_{46-47}H_{80-82}O_{13}$ (X-206), $C_{25}H_{40}O_7$ (X-464) and $C_{34}H_{52}O_8$ (X-537A). The rather toxic antibiotics are active *in vitro* against certain gram-positive bacteria and mycobacteria, and inactive *in vivo* against bacterial and protozoan infections, when tested at tolerated dose levels.

In the course of our search for new antibiotics, three Streptomyces were isolated from soil samples of Montclair, N. J., Salem, Va., and Hyde Park, Mass. These unidentified organisms were referred to as X-206, X-464 and X-537A, respectively. When grown on a variety of media in aerated submerged culture, all three produced antimicrobially active substances. Three antibiotics were obtained in crystalline form, one from each of the organisms, and in each case from the cells of the cultures, which contained 5–10 times as much active substance as the filtered broth. Although the new antibiotics are chemically different, their biological activities and certain chemical properties are so similar that they are being reported here as a group.

All three antibiotics are colorless, optically active, organic acids, containing C, H and O. Antibiotics X-206 and X-464 were isolated by alcohol extraction of the cells and purified by chromatography of their alkali salts. Antibiotic X-537A was obtained in form of the sodium salt by extraction of the cells with butyl acetate. The salt was soluble in benzene and hot petroleum ether, and insoluble in water, which facilitated its separation from other products. Similar unusual solubility properties were also observed for the alkali salts of the X-206 and X-464 antibiotics, possibly indicating the presence of some common structural feature in all three of them.

The analytical results so far obtained can best be correlated with the following empirical formulas for the three antibiotics: X-206, $C_{46-47}H_{80-82}O_{13}$; X-464, $C_{25}H_{40}O_7$ and X-537A, $C_{34}H_{52}O_8$. Since all three compounds, and particularly antibiotic X-206, are of rather high molecular weight, related empirical formulas are, of course, not excluded.

Only antibiotic X-537A has a characteristic ultraviolet absorption spectrum, with maxima at 317 mμ (ε 3700) and 249 mμ (ε 6400) for the free acid in isopropyl alcohol. It is also the only one of the three compounds to give a positive ferric chloride reaction. An absorption spectrum of this type, together with the observed ferric chloride reaction, would be consistent with the presence in the X-537A antibiotic of an aromatic ring, substituted by a hydroxyl and a carboxyl group. However, this is not yet supported by any other experimental evidence.

All three antibiotics are active *in vitro* against certain gram-positive bacteria and mycobacteria, but they are inactive against gram-negative

(1) Presented before the Division of Agricultural and Food Chemistry (Fermentation Subdivision) of the American Chemical Society, 119th Meeting, Cleveland, Ohio, April, 1951.

bacteria and fungi. Table I summarizes our observations.

TABLE I

ANTIMICROBIAL SPECTRUM OF ANTIBIOTICS X-206, X-464, X-537A

Test organism	Plate units per mg. X-206	X-464	X-537A
Bacillus E	800	2000	800
Sarcina lutea	900	2000	500
Staphylococcus aureus	40	40	20
Mycobacterium phlei	200	200	20
Corynebacterium simplex	0.2	2	20
Bacillus cereus	20	60	120
Bacillus simplex	20	20	20
Bacillus mesentericus	70	180	50
Saccharomyces cerevisiae	15	0.5	0
Scopulariopsis brevicaulis	1	5	0
Gram-negatives[a]	0	0	0

[a] These were *Pseudomonas aeruginosa, Aerobacillus polymyxa and Escherichia coli.*

The three antibiotics were also tested in our Chemotherapy Department by Dr. R. J. Schnitzer and his associates. All three were found to be rather toxic, the LD_{50} in mice being 11 mg./kg. (subc.) for X-206, 2.5 mg./kg. (intraperit.) for X-464 and 40 mg./kg. (intraperit.) for X-537A. Tolerated dose levels showed no significant activity *in vivo* against a variety of bacterial and protozoan infections.

Experimental[2]

Culture and Assay Methods (With the assistance of Robert G. Pollock).—The organisms were grown in aerated submerged culture, both in shaken flasks and in 50 to 1000 gallon stainless steel and iron fermentation tanks. All broths were adjusted to *p*H 6.5–7.5 before sterilization, and were incubated at 28° after inoculation with spore suspensions or vegetative growth. Antibiotic activity was followed by the familiar cup-plate agar diffusion assay, in which the test organism Bacillus E was employed. This is the same culture that was used in the University of Wisconsin for differential penicillin assays.[3] In general, the concentration of antibiotic required to produce an inhibition of 20–22 mm. in diameter with any of the test organisms was considered to be 1 unit per ml. Whole broths containing the cells were diluted for assay in 1% phosphate buffer, *p*H 6.0.

Following the usual procedures, each of the organisms was tested on over 20 different media, to determine which would support the highest antibiotic production. A variety of media containing complex nitrogen sources such as soyflour, fishmeal, distillers residues, cottonseed meal, were found to support good production of all three antibiotics. Typical flask yields with such media ranged from 20–120 units per ml. for X-206, 10–300 units for X-464, and 30–400 units for X-537A. So-called synthetic media generally gave very low yields.

From the degree of variability observed between results of different flask experiments, it was suspected that there might be a wide variation in antibiotic yields with different strains of each of the three streptomyces. This indeed proved to be the case. With cultures X-206 and X-464, the antibiotic production depended not only on the strain, but also on the medium on which the particular strain was grown. On a medium containing 1% corn distillers dried grains with solubles, 1% glucose and 0.1% U.S.P. salts mixture No. 1, eight out of fifteen strains of culture X-464 gave an average of 300 Bacillus E plate units per ml., while 6 out of 15 gave less than 40 units per ml.

With culture X-537A, the variation in antibiotic yield between 15 strains was from 20 to 1500 Bacillus E units per ml. crude broth.

(2) All melting points are corrected.

(3) H. Higuchi and W. H. Peterson, *Ind. Eng. Chem., Anal. Ed.*, **19**, 68 (1947).

For tank fermentations, 5–10% inoculum consisting generally of 3 day old submerged growth from an aerated bottle was used per tank. The medium usually contained 2% soybean flour, 2% brown sugar, 0.5% cornsteep liquor and 0.1% K_2HPO_4. Tank fermentations were carried on at 28°, under positive air pressure, with air-flows of 5–10 cu. ft. of air per minute per 40 to 80 gallon liquid charge. Broths were usually harvested after 4 to 6 days fermentation, filtered with Hyflo filter-aid, and subjected to extraction as described below.

Isolation of Antibiotic X-206 (With the assistance of S. Kaiser). **Mixture of the Sodium and Potassium Salts.**—Crude broth containing the antibiotic was filtered and crystalline active material was isolated from both the filtered broth (A) and the separated mycelium (B) as described below.

(A).—To 2700 liters of filtered broth (*p*H 6.2), showing in the cup plate test a potency of 10–20 Bacillus E units per ml. (total 27–54 million E units), were added 200 kg. of sodium chloride and 1300 liters of butyl acetate. The mixture was stirred for one-half hour, the emulsion which formed was broken by the addition of a detergent and the butyl acetate solution separated. It contained 25–50 million E units and was concentrated *in vacuo* to a volume of 70 liters. The solution was then extracted with ice-cold 0.2 molar dipotassium phosphate buffer (*p*H 8.9), adding 3 *N* potassium hydroxide to maintain the *p*H at 8.9, to remove impurities. It was then concentrated *in vacuo* to 4 liters (concentrate A). All the activity was present in this concentrate, which contained 300 g. of solids.

(B).—The separated mycelium (110 kg. wet weight, including 45 kg. filter-aid), containing about 200 million E units, was extracted at room temperature with 160 liters of ethanol and then with 1000 liters of methanol. The combined filtered ethanol and methanol solutions were concentrated *in vacuo* to a volume of 20 liters and mixed with 50 liters of butyl acetate. The butyl acetate solution was extracted with dipotassium phosphate buffer and alkali as described above, yielding concentrate B, containing 1050 g. solids and 90–100 million E units.

A crystalline mixture of the sodium and potassium salts of antibiotic X-206 was obtained by chromatographic purification of both concentrates. The purification of concentrate A is described below.

The extract was concentrated *in vacuo* with the addition of xylene, in order to remove most of the butyl acetate. The dark residual oil (300 g.) was dissolved in 1 liter of benzene and adsorbed on a column (80 mm. width) containing 2.5 kg. of activated alumina (Harshaw, chromatographic grade). The chromatogram was developed with benzene, followed by elution with ether, containing increasing amounts of ethanol as shown in Table II.

TABLE II

Solvent	Liters	Weight, g.	Activity, u./mg.	Total activity, million E units
Benzene	7	130	0	0
Ether + 3.5% ethanol	5	20	0	0
Ether + 3.5% ethanol	7	2.5	100	0.25
Ether + 8.3% ethanol	6	16.5	800	13.2
Ether + 13% ethanol	9	14.5	800	11.4
Ether + 23% ethanol	12	6.5	800	5.2
Total		190.0		30.05

In the above described, as well as in other chromatograms, benzene removed a large amount of inactive material. Ether, containing ethanol, eluted the active material. The total eluted activity was generally in good agreement with the activity of the crude concentrates. About 30–40% of the initial total weight remained on the alumina.

The fractions assaying 800 E units per mg. (in other experiments sometimes only 500–600 units per mg.) were purified as follows: The crude brown oil (37.5 g.) was dissolved in the smallest possible amount of ether. About 500 ml. of petroleum ether (b.p. 30–60°) was added and the mixture was evaporated at atmospheric pressure until most of the ether was removed. The mixture was then cooled to 0° for 24 hours and the insoluble impurities were filtered off. The filtrate was further concentrated *in vacuo* and the precipitation of impurities with petroleum ether was repeated.

The filtered solution was then evaporated and the oily residue dissolved in a minimum amount of ether. A large amount of petroleum ether was added, and the mixture concentrated on the steam-bath, until crystallization began. If the oil did not crystallize, it was dissolved in petroleum ether and heated on the steam-bath; sometimes this procedure had to be repeated several times until crystallization occurred. The crystalline compound was filtered off and washed with petroleum ether, containing very small amounts of ether. The mother liquors were concentrated, yielding more crystalline material. However, it was not possible to convert more than 50–65% of the amorphous 800 E units per mg. material into the crystalline product.

Thus, from concentrate A containing 25–50 million E units of activity, there was isolated 22 g. of white crystalline material, assaying 800 E units per mg. (total 18 million E units) and 11 g. of a non-crystallizable orange colored oil, also assaying 800 E units per mg. (total 9 million E units). Both fractions had the same antibacterial spectrum. The crystalline material, prisms melting at 201–203°, proved to be a mixture of the sodium and potassium salts of the antibiotic. It is insoluble in water, but soluble in ethanol and in ether.

Free Antibiotic X-206.—To a cooled solution of 10 g. of the above described crystalline salt mixture in 100 ml. of methanol were added 20 ml. of 3 *N* ice-cold sulfuric acid. The mixture was immediately extracted with petroleum ether, the extract washed with water, dried and concentrated *in vacuo*. The dry residue was recrystallized from methanol with the addition of water, giving 7.6 g. colorless prisms melting at 126–128°. The melting point changed after 2 years (desiccator at room temperature) to 112–116°. The activity (800 E units per mg.) however remained unchanged. The product is optically active, $[\alpha]^{29}D$ +15.0° (methanol, *c* 2). It has no characteristic absorption in the ultraviolet. It is soluble in alcohols, esters, acetone, ether and petroleum ether, and practically insoluble in water and in aqueous alkali.

The antibiotic gives no coloration with ferric chloride or tetranitromethane. The anthrone test for carbohydrates is negative. It decolorizes bromine in carbon tetrachloride solution, yielding a dark resinous mass. It reacts with a solution of lead tetraacetate in acetic acid, consuming about 2–3 atoms of oxygen per equivalent weight.

The antibiotic is unstable in mineral acid or strongly alkaline solutions. A solution in a mixture of 70% ethanol and 30% of 3 *N* sulfuric acid lost after 48 hours (20°) 90% of its biological activity. Treatment with alkali (70 ml. of methanol, 30 ml. of 3 *N* potassium hydroxide) reduced the activity to 10% after 72 hours at 20°. Samples treated for 4 days at room temperature with glacial acetic acid, or a mixture of 60 ml. of methanol and 40 ml. of 25% ammonia, showed full activity.

Hydrogenation experiments carried out at room temperature and atmospheric pressure, with prehydrogenated platinum oxide as catalyst, resulted in the absorption of 1.2 moles of hydrogen in methanol and 3 moles of hydrogen in acetic acid. The products were amorphous and biologically inactive.

Saponification experiments (0.2 *N* barium hydroxide solution, 140°, 15 hours; 1.5 *N* sulfuric acid, 70°, 30 hours) gave inactive amorphous products, soluble in organic solvents, and showing the same high oxygen content as the original starting material.

The comparison of the analyses of the crystalline antibiotic with the results obtained from its salts shows the presence of two molecules of water of crystallization in the free acid. The solvent of crystallization is not methanol, since no methoxyl was found analytically. Amorphous dry samples of the antibiotic, prepared by vacuum concentration of solutions of crystalline analytically pure material in petroleum ether, show a higher content of carbon, approaching that calculated for the anhydrous material.

Crystalline Material.—Calcd. for $C_{46}H_{80}O_{13}\cdot 2H_2O$: C, 62.99; H, 9.66; O, 27.35; equiv. wt., 877. Calcd. for $C_{47}H_{82}O_{13}\cdot 2H_2O$: C, 63.33; H, 9.73; O, 26.94; equiv. wt., 891. Found: C, 63.10, 63.26, 63.60; H, 9.40, 9.73, 9.80; O, 26.90 (direct determination); equiv. wt., 883, 870, 874 (electrometric titration in aqueous methanol); methoxyl = 0.

Amorphous Product.—Calcd. for $C_{46}H_{80}O_{13}$: C, 65.68; H, 9.59. Calcd. for $C_{47}H_{82}O_{13}$: C, 66.01; H, 9.67. Found: C, 65.08, 65.21; H, 9.21, 9.95.

Sodium Salt.—To a solution of antibiotic X-206 in methanol was added an equivalent amount of 1 *N* aqueous sodium hydroxide. The solution was concentrated *in vacuo*, and the residue recrystallized repeatedly from petroleum ether containing a very small amount of ether; prisms or needles melting at 185–187°. The salt shows full antibiotic activity.

Anal. Calcd. for $C_{46}H_{79}O_{13}Na$: C, 64.01; H, 9.23; Na, 2.67. Calcd. for $C_{47}H_{81}O_{13}Na$: C, 64.35; H, 9.31; Na, 2.62. Found: C, 63.61, 64.57; H, 9.04, 9.57; Na, 2.62, 2.68.

Potassium Salt.—Prepared as was the sodium salt; needles melting at 211–213°. Has full antibiotic activity.

Anal. Calcd. for $C_{46}H_{79}O_{13}K$: C, 62.84; H, 9.06; K, 4.44. Calcd. for $C_{47}H_{81}O_{13}K$: C, 63.19; H, 9.14; K, 4.38. Found: C, 63.09; H, 9.19; K, 4.17, 4.33.

A mixture of the sodium and potassium salts was prepared by recrystallizing together equal amounts of the sodium and potassium salts from petroleum ether containing a small amount of ether; prisms melting at 201–203°. This mixture shows the same properties as that obtained in the chromatographic purification of the antibiotic.

Silver Salt.—Four hundred and forty mg. of antibiotic X-206 and 86 mg. of silver nitrate were dissolved in ether. The filtered solution was concentrated *in vacuo*. The residue was dissolved in a few drops of ether. Petroleum ether was added and the solution was filtered again. The filtrate was concentrated and the residue recrystallized 4 times by dissolving in a few drops of ether, adding petroleum ether (b.p. 30–60°) and evaporating most of the ether; needles or prisms, m.p. 153–156° (dec.).

Anal. Calcd. for $C_{46}H_{79}O_{13}Ag$: C, 58.28; H, 8.41; Ag, 11.38. Calcd. for $C_{47}H_{81}O_{13}Ag$: C, 58.68; H, 8.49; Ag, 11.21. Found: C, 58.58, 58.58; H, 8.24, 8.08; Ag, 11.55.

Barium Salt.—Two samples were prepared in the same manner as the sodium and potassium salts. Sample A was recrystallized from a mixture of ether and petroleum ether, forming prisms melting at 154–156°. Sample B was purified by dissolving in a small amount of methanol, evaporating the alcohol and crystallizing the residue from petroleum ether; prisms melting at 149.5–151.5°. Both samples showed full antibiotic activity. However, for some unknown reason, the analysis of this salt does not fully agree with the empirical formulas calculated for the other salts and the free acid.

Anal. Calcd. for $(C_{46}H_{79}O_{13})_2Ba$: C, 60.79; H, 8.76; Ba, 7.56. Found (A): C, 61.67; H, 9.22; Ba, 6.61, 6.73. Found (B): C, 61.87; H, 9.17; Ba, 6.90.

Methyl Ester.—An amorphous, biologically inactive ester was obtained by treating the free X-206 antibiotic in ether with diazomethane, and evaporating the solution *in vacuo* to dryness.

Anal. Calcd. for $C_{47}H_{82}O_{13}$: CH_3O, 4.80. Found: CH_3O, 5.31.

Isolation of Antibiotic X-464.—The active substance was extractable from the whole broth with either butyl alcohol or butyl acetate over the *p*H range of 2.5 to 9.0. Most of it was present in the cells.

The contents of one kettle of broth (550 liters) were filtered with the aid of Hyflo Super-Cel. The damp filter cake was leached at 25° with three successive portions of methanol (total volume 250 liters), the solids were removed by filtration, and the combined aqueous methanol extracts, containing 130 million Bacillus E units, were concentrated by vacuum flash distillation to 45 liters. This concentrate was extracted with an equal volume of butyl acetate. Twenty-seven liters of this butyl acetate extract were washed first with 0.2 molar dipotassium phosphate buffer (*p*H 8.9), then with water, and finally concentrated *in vacuo* to a semisolid mass. This residue, on extraction with 4.5 liters of petroleum ether (b.p. 30–60°), left behind 69 g. of an inactive amorphous product. The petroleum ether extract, on evaporation *in vacuo* yielded 1 kg. of an oil which contained 20 million Bacillus E units of activity.

This oil was partitioned between 2 liters of 90% aqueous methanol and 3 liters of petroleum ether (b.p. 30–60°). The petroleum ether layer was extracted with two successive portions of 90% aqueous methanol which had previously been saturated with petroleum ether. The combined aqueous methanol extracts were concentrated *in vacuo*, giving 179 g. of an oil which contained all of the activity.

The 179 g. of active oil was dissolved in 1 liter of benzene and chromatographed by the flow method through a column

(80 mm. width) containing 1800 g. of activated alumina (Harshaw, chromatographic grade). The column was eluted with the following succession of solvents: benzene, benzene + 10% ether, ether, ether + 10% ethanol. These eluates contained 85% of the total weight of the material passed through the column and all of the activity. The residues obtained from the benzene + 10% ether and the ether eluates, on trituration with petroleum ether (b.p. 30–60°), yielded 8 g. of crystalline material, m.p. 225–235°. This product had an activity of 2000 Bacillus E units per mg.; yield 13% based on the activity found in the original methanol extracts. On ignition, an alkaline residue remained which, on the basis of flame tests, appeared to contain sodium and potassium.

Free Antibiotic X-464.—The free acid was liberated from the salt by partition between ether and an excess of dilute hydrochloric acid. The white crystalline acid melted at 170–172° (dec.); assay, 2000 Bacillus E units per mg.

For analytical purposes 0.5 g. of the acid was dissolved in 25 ml. of warm ethyl acetate plus 3 drops of water. The solution was evaporated *in vacuo* and the amorphous residue was dissolved in 4 ml. of dry ethyl acetate at room temperature. The resulting solution slowly deposited white crystals, m.p. 172–174° (dec.). The melting point did not change after two more recrystallizations. Two preparations were analyzed.

Anal. Calcd. for $C_{25}H_{40}O_7$: C, 66.34; H, 8.91; O, 24.75; mol. wt., 452.6. Found: C, 66.43, 66.00, 66.11; H, 8.69, 9.08, 9.14; O, 25.21, 24.75, 25.20 (direct determination); mol. wt. (Rast, camphor) 450, 440, 447; $[\alpha]^{27}D$ +65.9° (methanol, *c* 2).

The antibiotic showed no characteristic absorption in the ultraviolet.

Isolation of Antibiotic X-537A (With the assistance of B. R. Meltsner).—Although some active material was found in the filtered broth whence it could be extracted with *n*-butanol or butyl acetate at a *p*H range of 2–10, the bulk was in the cells and could be obtained in solution by leaching with methanol or butyl acetate.

Accordingly, a batch of 204 liters of broth was filtered with the aid of Hyflo Super-Cel. The wet filter cake was suspended in 100 liters of butyl acetate and the mixture was stirred overnight at room temperature. After filtration, the water layer was separated and discarded. The butyl acetate solution, assaying 30 million Bacillus E units, was concentrated *in vacuo* to 3 liters, washed with 10% sodium carbonate solution, and dried with anhydrous sodium sulfate.

On further concentration to 300 ml. and dilution with 350 ml. of petroleum ether (b.p. 50–60°), 41 g. of solid material (A), assaying 25 million Bacillus E units, separated.

This solid material (A) was then extracted in a Soxhlet apparatus with 4 liters petroleum ether (b.p. 50–60°) for 40 hours. The extract was taken to dryness *in vacuo*, the crystalline residue suspended in 100 ml. of petroleum ether and filtered, yielding 24.49 g. assaying 500 E units per mg.: total 12.25 million E units. The mother liquor of the solid (A) yielded an additional 5.73 g. of the crystalline antibiotic.

After recrystallization from ether–petroleum ether, this material, which contained sodium, was dissolved in ether and washed with dilute sulfuric acid to convert it to the free acid. Removal of the ether left an oily residue which crystallized from ethanol. Several recrystallizations from ethanol did not change the melting point, which remained unsharp at 100–109°; $[\alpha]^{26}D$ −7.2° (methanol, *c* 1); assay 800 E units per mg. The ultraviolet absorption spectrum of the free acid showed maxima at 317 mμ (ε 3700) and 249 mμ (ε 6400) in isopropyl alcohol. The acid was soluble in organic solvents and insoluble in water, but could be obtained in crystalline condition only from alcohols, from which it appeared to crystallize with one or more molecules of solvent. With alcoholic ferric chloride a green-blue coloration was obtained. The anthrone test for carbohydrate was negative. The antibiotic reacted with ethereal diazomethane, but no crystalline product could be isolated. It contained no nitrogen or halogen, phosphorus or sulfur, and on combustion left no ash.

Anal. Calcd. for $C_{34}H_{52}O_8$: C, 69.36; H, 8.90. Calcd. for $C_{34}H_{52}O_8 \cdot C_2H_5OH$: C, 68.11; H, 9.22. Calcd. for $C_{34}H_{52}O_8 \cdot 2C_2H_5OH$: C, 67.03; H, 9.48. Found (dried at 40°): C, 68.04, 67.72; H, 9.30, 9.66.

A sample recrystallized twice from isopropyl alcohol also melted at 100–109° and was analyzed.

Anal. Calcd. for $C_{34}H_{52}O_8$: C, 69.36; H, 8.90. Calcd. for $C_{34}H_{52}O_8 \cdot C_3H_7OH$: C, 68.48; H, 9.32. Calcd. for $C_{34}H_{52}O_8 \cdot 2C_3H_7OH$: C, 67.77; H, 9.68. Found (dried at 50°): C, 67.60; H, 9.32. Found (dried at 60°): C, 68.24, 68.24; H, 9.35, 9.26.

Barium Salt.—The barium salt was prepared by shaking an ether solution of the free acid with aqueous barium hydroxide. The salt, which remained in the ether, was recrystallized twice from benzene–ligroin; m.p. 156–160°.

Anal. Calcd. for $(C_{34}H_{51}O_8)_2Ba$: C, 62.27; H, 7.82; Ba, 10.46. Found (dried at 100°): C, 62.13, 62.04; H, 7.75, 8.05; Ba, 10.47, 10.60; (dried at 120°): C, 62.02; H, 8.20.

Sodium Salt.—The sodium salt was prepared by shaking an ether solution of the free acid with aqueous sodium carbonate. As above, the salt remained in the ether. Various samples of the sodium salt were crystallized from ethanol, ether–petroleum ether and benzene–ligroin. However, it was not possible to obtain consistent analytical results, in view of the difficulties encountered in drying these samples. The melting points ranged from 168–179°, $[\alpha]^{26}D$ −30° (methanol, *c* 1). A sample recrystallized from benzene–ligroin and dried at 100° *in vacuo*, melting at 168–171°, was electrometrically titrated. 0.6204 g. of sodium salt used 10.1 ml. of 0.1 *N* HCl.

Anal. Calcd. for $C_{34}H_{51}O_8Na$: equiv. wt., 610.7. Found: equiv. wt., 604.

The ultraviolet absorption spectrum of the sodium salt in isopropyl alcohol showed a maximum at 308 mμ (ε 4100) and an inflection at about 245 mμ.

Potassium Salt.—A solution of the free acid in ether was shaken with aqueous potassium carbonate, dried, and the ether removed *in vacuo*. The crystalline residue was recrystallized twice from petroleum ether and dried *in vacuo* at 100°; m.p. 177–178°. It probably still contained solvent of crystallization.

Anal. Calcd. for $C_{34}H_{51}O_8K$: C, 65.13; H, 8.20; K, 6.24. Found: C, 65.23, 65.55; H, 9.17, 8.94; K, 6.17, 6.24.

Drying at 120° resulted in material on which no repeatable analysis could be obtained.

Acknowledgment.—We wish to express our thanks to Dr. R. J. Schnitzer, Dr. E. Grunberg and their associates for the chemotherapeutic data, to Mr. L. A. Dolan for assistance in the large scale extraction operations, to Dr. Al Steyermark and his staff for the microanalyses and to Dr. A. Motchane for the ultraviolet spectra.

NUTLEY, NEW JERSEY RECEIVED MAY 14, 1951