These assay results are in line with the observations of others ([3b,c,12]) on the instability of B_{12b} in the presence of certain reducing agents and assay media constituents such as thiamin and ascorbic acid. The apparent differences in microbiological assay attributed to vitamins B_{12a} and B_{12b}[7] may well be the result of assay methods.

Acknowledgments.—The production and isolation of vitamin B_{12b} is obviously the result of group effort by many individuals. We are grateful to Dr. G. F. Cartland for active direction of the program, to Dr. C. E. Meyer and Messrs. R. A. Delor and H. H. Buskirk for countless microbiological assays, to Dr. George Pish for ultraviolet data, and to Messrs. L. Scholten, W. A. Struck and N. A. Drake for other physical and optical measurements.

Summary

An isolation process for vitamin B_{12b} is described by which it may be obtained as a co-product of neomycin produced by fermentation. The steps include heating the acidified fermentation medium to free the vitamin from the mycelium, adsorbing on carbon, eluting with aqueous acetone, evaporating to a small volume, fractionating between 75 and 95% acetone, extracting into methanol, transferring to water, and deionizing to afford a pink amorphous product of 0.5% purity. The crude product is then extracted from water into phenol, precipitated with acetone, extracted from water into diethylacetic acid, precipitated by petroleum ether, chromatographed on carbon with 50% aqueous acetone, then with 75% aqueous methanol and finally crystallized as dark red needles from aqueous acetone.

Crystalline vitamins B_{12} and B_{12b} are compared on three microbiological assays, and their ultraviolet, visible and infrared absorption curves are presented.

KALAMAZOO, MICHIGAN RECEIVED JUNE 14, 1950

[CONTRIBUTION FROM THE RESEARCH LABORATORIES OF CHAS. PFIZER AND CO., INC.]

Netropsin, a New Antibiotic Produced by a Streptomyces

BY A. C. FINLAY, F. A. HOCHSTEIN, B. A. SOBIN AND F. X. MURPHY

A new antibiotic has been obtained from culture filtrates of a hitherto undescribed Actinomycete, *Streptomyces netropsis*, which was isolated from a soil sample. The antibiotic has been assigned the name Netropsin.[1] Its toxicity appears to be high for parenteral administration; it may, however, have other therapeutic applications.

Netropsin has been prepared as its crystalline sulfate, hydrochloride and picrate. The free base is unstable and has not been isolated as yet. The analysis and molecular weight of netropsin salts indicate it to be a tetraacidic base corresponding to the formula $C_{32}H_{48}N_{18}O_4$. This formula assignment is based primarily upon the analysis of the sulfate; the hydrochloride and picrate are less easily purified and their absolute purity is in doubt. It is relatively stable in acid solution, having a half life of about two hours in 1 *N* sulfuric acid at 100°. When netropsin hydrochloride is dissolved in an equivalent quantity of 0.2 *N* sodium hydroxide, inactivation is complete in less than two hours at 25°.

The ultraviolet absorption spectrum of an aqueous solution of the sulfate at *p*H 5.5 exhibits two peaks, $E^{1\%}_{1\,cm.}$ 429 at 236 mμ, $E^{1\%}_{1\,cm.}$ 436 at 296 mμ (see Fig. 1). This spectrum is virtually unchanged at *p*H 10. The infrared absorption spectrum of a Nujol mull of netropsin sulfate exhibits no sharp peaks. Broad maxima, characteristic of large molecules, occur at the following estimated wave lengths: 3320, 1670, 1550, 1410, 1265, 1210, 1145, 1096, 1057, 961 and 810 cm.$^{-1}$. In Fluorolube-S mulls, additional bands occur at 3100, 1468 and 1434 cm.$^{-1}$. The hydrochloride shows no optical activity in water, methanol or dimethylformamide solutions. The titration of netropsin hydrochloride with sodium hydroxide in aqueous solution indicates all basic groups to be of similar strength.

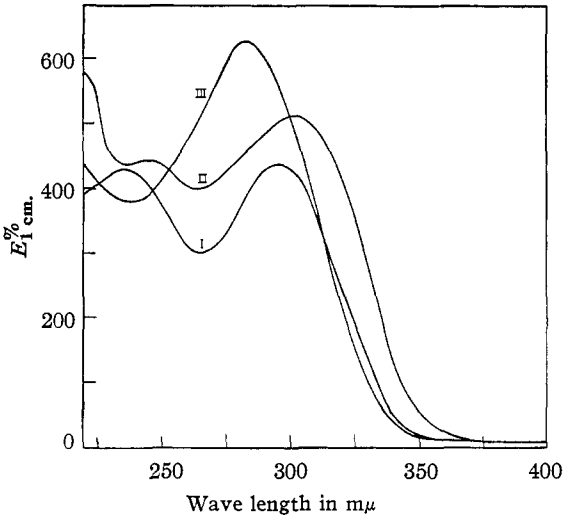

Fig. 1.—Absorption spectra: I, Netropsin sulfate in water, *p*H 5.5; II, degradation product I in 0.1 *N* NaOH; III, degradation product I in 0.1 HCl.

Netropsin gives a positive Sakaguchi test and a positive Ehrlich test. Ninhydrin, Tollens, fuchsin-aldehyde, murexide, ferric chloride, aminoantipyrine (for phenol) and 2,4-dinitrophenylhydrazine tests are all negative. Primary amino nitrogen, as determined by the method of Van Slyke, is absent. It absorbs hydrogen slowly in aqueous solution over Adams platinum catalyst to yield a biologically inactive product. Two crystalline basic hydrolysis products of netropsin have been isolated. The ma-

(1) Netropsin is the trade name of Charles Pfizer and Co. for the antibiotic produced from the fermentation of *Streptomyces netropsis*.

jor product of basic hydrolysis, I, is a monobasic crystalline solid, the analysis of which corresponds to $C_{15}H_{20}N_6O_3$. The ultraviolet absorption spectrum of I in 0.1 *N* hydrochloric acid shows a peak at 282.5 mμ; in 0.1 *N* sodium hydroxide, the peak is shifted to 302.5 mμ (see Fig. 1). It gives a negative Sakaguchi test. A second product, II, gives a positive Sakaguchi test, and may be presumed to contain the guanidine moiety. The analysis indicates the empirical formula to be $C_3H_5N_3O$; it is also monobasic. When netropsin is heated with 0.5 *N* barium hydroxide, ammonia is evolved, and the barium salt of an organic acid is formed.

The limited amount of experimental work does not permit of detailed conclusions regarding the structure of netropsin. However, the following conclusions may be drawn. The strong basicity of netropsin appears to be due to a substituted guanidine group, whose presence is revealed by the positive Sakaguchi test. That this functional group does not account for all of the nitrogen is shown by the isolation of the basic fragment, I, $C_{15}H_{20}N_6O_3$, which gives a negative guanidine test. The negative Van Slyke nitrogen indicates the absence of amino acids. Barium hydroxide hydrolysis provides some evidence for an amide linkage. The ammonia and carbon dioxide are doubtless produced in part from hydrolysis of the guanidine moiety, but the formation of an acidic component in this reaction is improbable unless an amide group is also hydrolyzed.

The *in vitro* activity of crystalline netropsin hydrochloride against a variety of microörganisms was determined by dissolving the antibiotic in nutrient agar, and streaking with the organisms under test. The antibiotic concentrations required to inhibit growth are listed in Table I. Netropsin hydrochloride was found to be active against clothes moth larvae and the black carpet beetle.[2]

TABLE I

CONCENTRATION OF NETROPSIN HYDROCHLORIDE NECESSARY TO INHIBIT GROWTH OF MICROÖRGANISMS ON NUTRIENT AGAR PLATES

Organism	μg./ml.
S. aureus	5
S. albus	3
B. subtilis	5
B. mycoides	20
Salmonella typhosa	10
S. pullorum	8
S. paratyphi A	20
S. paratyphi B	10
K. pneumoniae	10
Sh. paradysenteriae	7
E. coli	7
A. aerogenes	5
Ps. aeruginosa	1000
Proteus vulgaris	40
C. albicans	90
Bodenheimer organism	30

The toxicity of netropsin hydrochloride was determined for mice. The results, expressed as mg./kg. of body weight, are shown in Table II.

TABLE II

MOUSE TOXICITY OF NETROPSIN HYDROCHLORIDE

Route of administration	LD_0, mg./kg.	LD_{50}, mg./kg.
Intravenous injection	10	17
Subcutaneous injection	30	70
Oral	300	>300

There was no significant increase in the survival time of mice infected with *Ps. aeruginosa* and *Salmonella typhosa* when netropsin hydrochloride was administered at non-toxic dosage levels.

Experimental[3]

Assay.—Netropsin was assayed by the agar cup plate method as outlined for the assay of streptomycin.[4] Anhydrous netropsin hydrochloride was used as the standard.

Preparation of Culture Broths.—The organism, *Streptomyces netropsis*,[5] is carried in culture on slants or Roux bottles on Emerson agar.[6] The growth on the surface of such media is used to inoculate shaken Fernbach flasks containing 500 ml. of a fermentation medium, which in turn, at the end of 4 days are used to inoculate submerged fermentations. The fermentation tanks are maintained at 27°, and are aerated by blowing in sterile air through a sparger at the rate of 0.5 to 2 volumes of air per volume of broth per minute.

The fermentation medium consists of soybean meal, 10 g.; dextrose hydrate, 10 g.; distillers solubles, 0.5 g.; sodium chloride, 5 g.; made up to 1 liter in tap water and adjusted to *p*H 7.0 with potassium hydroxide. Calcium carbonate, 1 g., and soybean oil, 1 ml., is added, and the suspension sterilized for 45 minutes at 121°. At the end of 48 hours growth, the above medium shows a potency of about 200 mg. of netropsin per liter.

Isolation of Netropsin Hydrochloride from Culture Filtrates.—Eight and one-half liters of a typical culture broth of the antibiotic, assaying 200 mg. per liter, was filtered with the aid of Super-cel, and the cake washed with 1500 ml. of water. The filtered broth was fed through a 35 × 250 mm. column containing 100 g. of Rohm and Haas IRC-50 cation exchange resin, which had been previously equilibrated to *p*H 7.5 with sodium hydroxide solution. The antibiotic was eluted from the resin column with 2.6 l. of 0.46 *N* HCl. Virtually all the antibiotic was contained in the 700 ml. of eluate emerging at *p*H 7.4 to 0.8. This portion of the eluate was continuously neutralized to *p*H 6.0 as it left the column. Calcium ion was removed by the addition of 1.5 g. of ammonium oxalate in 180 ml. of water.

The calcium free filtrate was concentrated *in vacuo* at 40° to a volume of 70 ml., and was then held at 2° for 16 hours to permit complete crystallization of the antibiotic. The crude antibiotic was dissolved in 10 ml. water at 70° and recrystallized by cooling to 2°. The crystalline netropsin hydrochloride was separated by filtration, washed with acetone, and dried *in vacuo* over calcium chloride. The product weighed 1.2 g.; yield 70%.

Salts of Netropsin.—Netropsin hydrochloride crystallized slowly from aqueous solution in the form of long, thin colorless hydrated prisms which exhibit oblique extinction and are filtered with difficulty. A sample was dried for 3 hours *in vacuo* at 100°. *Anal.* Calcd. for $C_{32}H_{48}N_{18}O_4 \cdot 4HCl$: C, 42.94; H, 5.85; N, 28.18; Cl, 15.85. Found: C, 42.80; H, 5.80; N, 26.57; Cl, 13.76. The anhydrous hygroscopic salt melts with decomposition at 168–172° when placed in a bath at 160° with the temperature rising 2° per minute.

The sulfate proved to be somewhat more satisfactory from a manipulative standpoint. It was prepared by the addition of 0.65 g. of potassium sulfate in 10 ml. water to 1.0 g. of netropsin hydrochloride in 75 ml. of boiling water. On

(2) Private communication, Dr. G. S. Kido, Entomologist, Wisconsin Alumni Research Foundation.

(3) All melting points are corrected.

(4) Federal Register, Section 141.101.

(5) *Streptomyces netropsis* (*sp. nov.*) appears to be related to *Streptomyces reticuli*, Waksman and Henrici, and *Streptomyces rubrireticuli*, Waksman and Henrici, when diagnosed according to Bergey, "Manual of Determinative Bacteriology," Williams and Wilkins, Baltimore, Md., 6th edition, 1948, pp. 929-933.

(6) Gottlieb, Bhattacharyya, Anderson and Carter, *J. Bact.*, 55, 411 (1948).

cooling, the hydrated sulfate was recovered as long colorless needles in virtually quantitative yield. A five-times recrystallized sample, dried at 100°, 0.02 mm. pressure for 6 hours showed no loss in biological activity. *Anal.* Calcd. for $C_{32}H_{49}N_{18}O_4 \cdot 2H_2SO_4$: C, 40.66; H, 5.55; N, 26.69; SO_4, 20.34. Found: C, 40.70; H, 5.57; N, 25.90; SO_4, 20.05. The molecular weight was determined ebullioscopically in water. Calcd. for $C_{32}H_{48}N_{18}O_4 \cdot 2H_2SO_4$: mol. wt., 945 (315 assuming dissociation to three ions in solution). Found: mol. wt., 360 ± 60.

This salt melts at 224–225°, when placed in a bath at 220°, with the temperature rising 2° per minute. It has a solubility of about 30 mg./ml. in water at 80°, less than 0.5 mg./ml. at 25°. It is quite insoluble in the common organic solvents.

Netropsin picrate crystallizes from water, in which it is very slightly soluble, as sheaves of yellow needles. This salt melts at 234° (dec.) when placed in a bath at 225°, with the temperature rising at 2° per minute. The analysis of the picrate dried at room temperature *in vacuo* most nearly conforms to that of a hydrated picrate. *Anal.* Calcd. for $C_{32}H_{48}N_{18}O_4 \cdot 4C_6H_3N_3O_7 \cdot H_2O$: C, 39.92; H, 3.71; N, 24.95. Found: C, 39.94; H, 3.91; N, 23.64.

Hydrogenation of Netropsin.—Hydrogenation of the hydrochloride over Adams platinum catalyst in aqueous acetic acid solutions at atmospheric pressure results in the absorption of 1.6% of hydrogen in 24 hours. The hygroscopic amorphous product is devoid of biological activity.

Alkaline Degradation Products of Netropsin.—One gram of netropsin hydrochloride was suspended in 30 ml. of water and 20 ml. of 0.20 *N* NaOH was added. The resulting solution slowly turned from pale yellow to a deep red color, ammonia was evolved and a colorless crystalline product precipitated. After standing overnight, 0.56 g. of colorless needles was removed by filtration. This material, I, was purified by dissolving in a minimum quantity (7.5 ml.) of 0.20 *N* HCl, warming with Darco G-60 charcoal, and precipitation with an equivalent quantity of sodium hydroxide. The purified product softens at 238°, melts at 245–249° (dec.). *Anal.* Calcd. for $C_{15}H_{20}N_6O_3$: C, 54.20; H, 6.07; N, 25.29. Found: C, 54.20; H, 6.17; N, 25.45.

I is monobasic, forming a sulfate which is very soluble in water. It gives a negative Sakaguchi test, and is readily oxidized by air or ferric chloride to a deep red substance in neutral or acid aqueous solution.

The mother liquor from the basic hydrolysis yielded 60 mg. of a more soluble fraction, II, which crystallizes from water, as a colorless solid, readily soluble in acids, but relatively insoluble in the common organic solvents. *Anal.* Calcd. for $C_3H_5N_3O$: C, 36.36; H, 5.09; N, 42.68. Found: C, 36.84; H, 5.20; N, 42.92. This product decomposes without melting when heated above 300°. It gives a positive Sakaguchi test.

A sample of a sulfate salt of II was analyzed. *Anal.* Calcd. for $C_3H_5N_3O \cdot {}^1/_2H_2SO_4 \cdot H_2O$: $SO_4^=$, 29.40; N, 25.31. Found: $SO_4^=$, 28.42; N, 25.72.

Barium Hydroxide Hydrolysis of Netropsin.—One gram (1.05 mmole) of netropsin hydrochloride was dissolved in 25 ml. of 0.5 *N* barium hydroxide, and heated under reflux for 8 hours. The volatile bases evolved were collected in a known volume of standard hydrochloric acid. This netropsin solution was distilled to a volume of 10 ml., water was added and the concentration repeated to liberate all volatile base. Five millimoles (27% of the total nitrogen) of base was evolved. It was identified as ammonia by analysis of the chloride. The reaction flask contained 0.25 g. (1.24 mmole) of barium carbonate, which was separated by filtration. The excess barium was precipitated by addition of carbon dioxide, and the aqueous phase evaporated to dryness. The amorphous product still contained barium, which could not be precipitated by sulfate ion. This acidic product was not isolated.

Acknowledgment.—We are indebted to the members of the staff of Chas. Pfizer and Co., Inc., and especially to Dr. John B. Routien for isolating and identifying the culture, Dr. S. Y. P'an for determining toxicity, Dr. John A. Means for analytical determinations and absorption spectra, and Mr. K. B. Tate for running the bacterial spectrum and animal protection studies.

Summary

Netropsin is a new antibiotic obtained from culture filtrates of *Streptomyces netropsis*. Analysis of its crystalline salts indicate it to be a tetraacidic base with a formula approximating $C_{32}H_{48}N_{18}O_4$. The physical and biological characteristics of Netropsin are given. Two crystalline degradation products are described.

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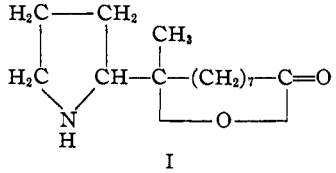
[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY AND CHEMICAL ENGINEERING, UNIVERSITY OF CALIFORNIA]

The Carbon Skeleton of Carpaine

BY HENRY RAPOPORT AND HENRY D. BALDRIDGE, JR.[1]

The alkaloid carpaine[2] was first isolated from the leaves[3] of the papaw tree, *Carica Papaya* L., by Greshoff.[4] Its formula was established as $C_{14}H_{25}NO_2$ by van Rijn[5] and structural investigations, initiated by Barger[6] and continued with Robinson and co-workers,[7,8] culminated in the proposal of I as the structure of carpaine. Although this structure was strongly supported by the reactions of the alkaloid, the only positively identified compounds derived from carpaine were suberic and azelaic acids, obtained by oxidation[7] with potassium permanganate and nitric acid, respectively.



I

A nitrogen-free, hydroxy acid of probable composition $C_{14}H_{28}O_3$, melting indefinitely from 20–25°, was isolated[8] from carpaine by a two-stage exhaustive methylation–Hofmann degradation procedure followed by hydrogenation and hydrolysis. However, this material, called "hydroxyisomyristic acid," was not further identified. This acid obviously provides the key to the carbon skeleton of

(1) U. S. Rubber Company Fellow, 1948-1949.

(2) For references to the pharmacological action of carpaine, see Henry, "The Plant Alkaloids," 4th ed., The Blakiston Company, Philadelphia, Pa., 1949, p. 600.

(3) An interesting account of the use of papaya leaves by natives in the Northern Celebes is found in Fairchild, "Garden Islands of the Great East," Charles Scribner's Sons, New York, N. Y., 1943, pp. 97-99.

(4) Greshoff, *Mededeel. uit's Lands. Plant., Buitenzorg*, **No. 7**, 5 (1890).

(5) van Rijn, *Arch. Pharm.*, **231**, 184 (1893); *ibid.*, **235**, 332 (1897).

(6) Barger, *J. Chem. Soc.*, **97**, 466 (1910).

(7) Barger, Girardet and Robinson, *Helv. Chim. Acta*, **16**, 90 (1933).

(8) Barger, Robinson and Work, *J. Chem. Soc.*, 711 (1937).