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Streptolin. Preliminary Investigation and Separation of Acid Hydrolysis Products

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Streptolin A, a pure component derived from, and exhibiting the same elemental analysis as the mixture streptolin AB, has been tentatively assigned the minimum formula $C_{17}H_{31-33}N_5O_8$. Acid hydrolysis of streptolin AB followed by separation on Dowex-50 affords five major fractions, among which are: (1) a reducing fragment, (2) ammonia, (3) a basic substance possessing the empirical formula $C_4H_{8-10}N_2O_3$ and (4) an isomer of lysine previously described by Carter, *et al.*, and Haskell, *et al.*[6]

Streptolin, an antibiotic active against both gram-negative and gram-positive bacteria, was first isolated by Rivett and Peterson[1a] from the deep culture fermentation of an actinomycetes designated as *Streptomyces* No. 11. In this paper we wish to report results obtained in the initial phases of our research on the chemistry of streptolin, particularly the chromatographic separation of acid hydrolysis products.

Crude streptolin was initially recrystallized as the reineckate until a turbidometric assay[1] of 19,000 units/milligram against *E. coli* was attained. Conversion of the reineckate to the hydrochloride (assay 44,000 u./mg.) represented the final stage of purification accomplished during the time this research was carried out, and the latter material was used in this investigation. Subsequently Larson, Sternberg and Peterson[1b] were able to show that streptolin at this stage is actually a mixture of isomers, designated, respectively, as "streptolin A" and "streptolin B"; the mixture used herein was therefore termed "streptolin AB." Analysis of streptolin AB helianthate,[1b] hydrochloride and reineckate[1b] indicated either the minimum formula $C_{17}H_{31-33}N_5O_8$ or $C_{24}H_{45-47}N_7O_{11}$ for the free base. Since isomer A gives the same analytical values as streptolin AB, we tentatively consider the isomers to be best represented by either of these formulas.

Streptolin AB gave positive qualitative results in the biuret and ninhydrin reactions. Reducing properties were demonstrated through the use of the Shaffer–Somogyi copper reagent[2a] and the Park and Johnson ferroferricyanide method.[2b] A quantitative Sakaguchi determination, when run on either streptolin AB sulfate or its acid hydrolysate, indicated less than 1% of arginine. Alkoxyl, alkimide and C-methyl determinations gave negligible values. Streptolin AB gave no reaction with phenylhydrazine or 2,4-dinitrophenylhydrazine; the antibiotic activity was not diminished after treatment with either of these reagents. Neither the antibiotic nor its acid hydrolysis product absorbed hydrogen over platinum oxide in acetic acid at room temperature and atmospheric pressure. The ultimate analytical data indicate that streptolin, on the basis of the C_{17}-formula, has three basic centers; in addition, streptolin hydrochloride which has been dried *in vacuo* contains one less protonated basic center than either the reineckate or the helianthate.

The preponderant portion of research effort was expended on the hydrolysis of streptolin AB and identification of the derived fragments. Of the various routes attempted, hydrolysis with hydrochloric–formic acid[3] followed by chromatography on Dowex-50 ion-exchange resin[4] proved to be the most fruitful. Hydrolysis with constant-boiling hydrochloric acid alone resulted in considerable humin formation; partition or absorption chromatography was ineffectual. For comparison purposes, a small, analytical Dowex-50 column was first calibrated, using the Moore and Stein photometric ninhydrin procedure,[5] with known amino acids. Elutions were carried out successively with 1.5, 2.5 and 4.0 *N* hydrochloric acid, 1-ml. fractions

(1) (a) R. W. Rivett and W. H. Peterson, THIS JOURNAL, **69**, 3006 (1947); (b) L. M. Larson, H. Sternberg and W. H. Peterson, *ibid.*, **75**, 2036 (1953).

(2) (a) P. A. Shaffer and M. Somogyi, *J. Biol. Chem.*, **100**, 695 (1933); (b) J. T. Park and M. J. Johnson, *ibid.*, **181**, 149 (1949).

(3) G. L. Miller and V. du Vigneaud, *ibid.*, **118**, 101 (1937).

(4) W. H. Stein and S. Moore, *Cold Spring Harbor Symp. Quant. Biol.*, **14**, 179 (1950).

(5) S. Moore and W. H. Stein, *J. Biol. Chem.*, **176**, 367 (1948).

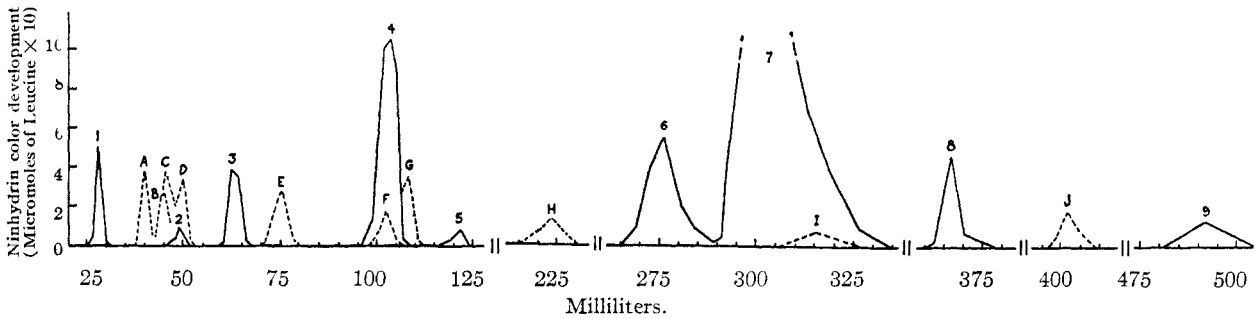

Fig. 1.—Analytical fractionation of streptolin AB hydrolysate on Dowex-50: — —, streptolin hydrolysate; - - -, known amino acids: A, aspartic acid; B, serine; C, threonine: D, glutamic acid; E, glycine; F, ammonia; G, valine; H, α,γ-diaminobutyric acid; I, lysine; J, arginine.

being collected. Figure 1 shows the plot of tube number against the photometric ninhydrin value in terms of micromoles of an arbitrary standard, leucine. When the acid hydrolysate of streptolin AB was subjected to chromatographic separation on the analytical column, well-defined fractions were obtained, all of which gave positive ninhydrin tests. Not one of the major peaks, however, appeared in the same position as any one of the known amino acids used in the calibration (Fig. 1).

In order to obtain greater amounts of these fractions, a larger, preparative column was employed, and 10-ml. fractions were collected. Only 2.5 and 4.0 *N* hydrochloric acid were used as eluants. Four of the peaks, No. 2, 5, 8 and 9, represent only a small percentage of the total hydrolysate and are assumed to be due to impurities or artifacts. Fraction no. 1 appears to be derived from the reducing moiety of streptolin and is under investigation in these Laboratories. Fraction no. 4 was identified as ammonia by comparison and by characterization as benzamide. On the larger column, the degree of resolution between peak no. 6 and 7 was not as high as on the analytical column. However, by evaporating the combined richer fractions corresponding to peak no. 6, a crystalline hydrochloride (m.p. 215–216°) was readily obtained. Analysis of the *p*-hydroxyazobenzene-*p'*-sulfonate (dec. 258–260°) of the free base indicated the empirical formula $C_4H_{8-10}N_2O_3$. Only oily material was obtained on evaporating the fractions corresponding to peak no. 7. The substance formed, on the other hand, a crystalline dipicrate (m.p. 199–200°) and a di-(*p*-hydroxyazobenzene-*p'*-sulfonate) (dec. 243.5–244.0°); these salts gave analyses corresponding to a $C_6H_{14}O_2N_2$ free base. Because of its proximity to lysine on the calibrated column, the base is regarded as a position isomer of this amino acid.[6]

Experimental[7]

Streptolin AB Hydrochloride.—Four grams of streptolin reineckate (turbidometric assay 19,000 u./mg. against *E. coli*[1]) was placed in 8 ml. of methanol and was stirred until in solution; 10–20 ml. of excess methanol was added. Three milliliters of concentrated hydrochloric acid in 35–40 ml. of methanol was added rapidly and stirring continued for 15 minutes. The solution was then divided into four parts, and each part was added, with stirring, to 130 ml. of acetone contained in a 250-ml. centrifuge bottle. A flocculent precipitate formed immediately, and the mixture was centrifuged. The acetone was decanted, and the precipitate was washed with acetone by centrifugation and decantation until the red color disappeared. The material was then dried over phosphorus pentoxide in a vacuum desiccator. The yield was 1.40 g. Turbidometric assay against *E. coli*[1] gave the value 44,000 u./mg. After drying at 1 mm. at room temperature, the hydrochloride gave an analysis which, on the basis of the $C_{17}H_{31-35}N_5O_8$ formula, indicates that only two basic centers are involved in salt formation; the helianthate and reineckate, on the other hand, are derived from a tribasic molecule.[2]

Anal. Calcd. for $C_{17}H_{31}N_5O_8(HCl)_2$: C, 40.31; H, 6.57; N, 13.83. Calcd. for $C_{17}H_{33}N_5O_8(HCl)_2$: C, 40.16; H, 6.94; N, 13.78. Found: C, 39.95; H, 6.66; N, 13.65.

Acid Hydrolysis of Streptolin AB.—Streptolin AB hydrochloride (500 mg., 43,000 u./mg.) was hydrolyzed by refluxing for 20 hours in a solution of 20 ml. of 20% hydrochloric acid and 20 ml. of 50% formic acid. Both acids had been redistilled before use in order to eliminate ammonia. After the reflux period the solvents were removed *in vacuo*. The addition of water followed by removal *in vacuo* was repeated several times, and finally the residue was dried at 100° under 0.2 mm. pressure for 5 hours. The dark brown residue was then taken up in water. The resulting solution was filtered through a sintered glass funnel and then diluted to 25 ml. with water which had been distilled from sulfuric acid.

Fractionation of Streptolin AB Hydrolysate. Standards, Solvents and Reagents.—Aqueous 2.0 *M* solutions of the amino acids listed in Fig. 1 were prepared. These solutions were preserved by the addition of a few drops of chloroform and of toluene to each solution and storage at 3°. To calibrate the column, 0.5 ml. of each of these solutions was used as described below. In the ninhydrin analysis, 0.1 ml. of the 2 *M* leucine solution was used as a standard.

Constant-boiling hydrochloric acid was used to prepare 1.5, 2.5 and 4.0 *N* acids. Dilutions were made on a weight basis with water distilled from sulfuric acid to prevent contamination by ammonia.

The ninhydrin color reagent was prepared as needed by a modification of the Moore and Stein method.[5] A 0.2 *M* citrate buffer was diluted for use with an equal volume of water, and 320 mg. of stannous chloride dihydrate was added. This solution was so distributed in four 50-ml. glass-stoppered bottles that they were filled completely. When stored at 3°, each portion maintained its potency for at least a month. The reagent solution was prepared for immediate use by dissolving 20 mg. of ninhydrin in 1 ml. of equal parts of methyl cellosolve and citrate buffer. The ninhydrin was first dissolved in the methyl cellosolve and the buffer added to give a homogeneous solution.

A diluent solution was prepared from equal volumes of pure water and *n*-propyl alcohol.

Analytical Fractionation.—To prepare the column, 32 g. of Dowex-50 (250–300 mesh) was first washed successively with three portions of 2 *N* sodium hydroxide, one portion of water and finally one of 2 *N* hydrochloric acid. The wash was removed each time by centrifugation and decantation.

(6) Our fraction no. 7 appears to be identical with an antibiotic hydrolytic fragment isolated by H. E. Carter, *et al.*, Abstracts of Papers, 119th Meeting, American Chemical Society, Cleveland, Ohio, April, 1951, p. 25A; and by Haskell, *et al.*, THIS JOURNAL, **74**, 599 (1952).

(7) All melting points except those noted were taken on a micro hot-stage and are corrected.

The resin was then slurried with 1.5 *N* hydrochloric acid and poured into a Pyrex column 50 cm. long with a 9 mm. inside diameter. After settling by gravity, the resin was conditioned by the percolation of 1.5 *N* acid for 48 hours at the rate of 4 ml./hour.

To prepare the charge, 0.6 ml. of the stock solution of streptolin AB hydrolysate (12 mg. of streptolin hydrochloride/ml.) was evaporated to dryness under a stream of nitrogen at 100°. The residue was taken up in 0.6 ml. of 1.5 *N* hydrochloric acid and the resulting solution placed on the column. As the charge reached the resin bed level, the column was filled with 1.5 *N* acid; then a 125-ml. dropping funnel of acid was fitted onto the top. Collection of the eluate in 1.0 ml. fractions was started just as the charge disappeared below the resin bed. The rate of flow was controlled by slight air pressure so as to yield four 1-ml. fractions per hour. After 75 tubes had been collected, the remaining acid was removed from the column and replaced with 2.5 *N* acid. After 325 fractions had been collected, the 2.5 *N* acid was replaced with 4.0 *N* acid, and fractions were collected to tube 500.

The fractions were grouped in batches of 25 consecutive tubes, and the batches were evaporated at 15–20 mm. in a vacuum desiccator immersed in a boiling water-bath. This operation required about four hours, after which time they were allowed to stand eight to ten hours at 0.2 mm. over a mixture of solid potassium hydroxide and calcium chloride. Each batch of collected fractions was then analyzed by the photometric ninhydrin method of Moore and Stein.[5] Since traces of ammonia can be detected by this procedure, it is important that the blanks and analytical samples be handled under strictly parallel conditions. Two leucine standards and two blanks were run with each batch, and the color yield was determined directly in terms of optical density with a Coleman 6A Junior Spectrophotometer. One milliliter of ninhydrin reagent was pipetted into a tube containing the sample. The tube was shaken, capped with a 10-ml. beaker and placed in a vigorously boiling water-bath for 20 minutes. The tube was removed, 5 ml. of diluent added and the tube shaken. The sample was allowed to stand 15 minutes, and then readings were taken at 570 mμ. Readings were essentially constant for one hour after the reaction was completed. The optical density readings were converted to micromoles of leucine by reference to a previously prepared standard curve and the results plotted as a function of tube number (Fig. 1).

Preparative Fractionation.—A glass chromatographic tube 60 cm. long and having a 28-mm. inside diameter was filled with 300 g. of Dowex-50 resin which had been washed with 4.0 *N* hydrochloric acid six times and centrifuged each time. The column was placed on the fraction cutter, and 2.5 *N* acid was used to equilibrate the column as described above. No pressure was used, and the column ran at the rate of 27 ml. per hour. Three hundred milligrams of dry, hydrolyzed streptolin AB was dissolved in 1 ml. of 2.5 *N* acid and the solution placed on the column. The tube was filled with 2.5 *N* acid and a one-liter reservoir mounted on top. Ten-ml. fractions were collected. After the 200th fraction had drained, the 2.5 *N* acid was replaced with 4.0 *N* acid and 165 five-ml. fractions collected. The unit volume of fractions 365 to 450 was raised to 10 ml. Every third fraction was analyzed photometrically as described for the analytical separation. In preparative runs, the ninhydrin procedure was omitted, and the material was isolated by grouping the tubes in accordance with the initially obtained ninhydrin data. The combined tube contents were reduced to dryness *in vacuo*, taken up in distilled water and finally evaporated to dryness in a jet of nitrogen.

Fraction No. 4.—The dry residue was identified as ammonium chloride by conversion to benzamide with benzoyl chloride by the Schotten–Baumann method.

Fraction No. 6.—Evaporation of the combined tubes afforded, on standing, a crystalline hydrochloride, m.p. 215–216°. The *p*-hydroxyazobenzene-*p'*-sulfonate, after repeated recrystallization from water, decomposed sharply at 258–260°.

Anal. Calcd. for $C_4H_8N_2O_3(C_{12}H_{10}N_2O_4S)$: C, 46.82; H, 4.42; N, 13.65. Calcd. for $C_4H_{10}N_2O_3(C_{12}H_{10}N_2O_4S)$: C, 46.60; H, 4.89; N, 13.59. Found: C, 46.62; H, 4.72; N, 13.62.

Fraction No. 7.—About 50 mg. of crude fraction no. 7 was dissolved in 2 ml. of water. Two hundred milligrams of *p*-hydroxyazobenzene-*p'*-sulfonic acid was dissolved in 2 ml. of hot water, and the two solutions were mixed. The fine, golden needles of the di-(*p*-hydroxyazobenzene-*p'*-sulfonate) which formed on cooling were recrystallized repeatedly from water until the dec. point 243.5–244°[8] was reached.

Anal. Calcd. for $C_6H_{14}N_2O_2 \cdot 2C_{12}H_{10}N_2O_4S$: C, 51.28; H, 4.87; N, 11.95. Found: C, 50.91; H, 5.08; N, 11.56.

The dipicrate of fraction no. 7 was prepared by adding 4 ml. of saturated, aqueous picric acid solution to a solution of 50 mg. of crude fraction no. 7 in 4 ml. of 95% ethanol. The yellow precipitate which formed was recrystallized from ethanol until the melting point 200–201° was attained.

Anal. Calcd. for $C_6H_{14}N_2O_2(C_6H_3N_3O_7)_2$: C, 35.78; H, 3.34; N, 18.55. Found: C, 36.38; H, 3.63; N, 18.06.

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(8) Taken in a melting point bath and corrected.

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[CONTRIBUTION FROM THE LABORATORY OF ORGANIC CHEMISTRY OF THE UNIVERSITY OF WISCONSIN]

Streptolin. The Structure and Synthesis of Isolysine

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Isolysine ($C_6H_{14}N_2O_2$), an acid-hydrolysis product derived from streptolin AB,[1] viomycin[3] and streptothricin,[4] possesses a carboxyl group and two primary amino groups, but no C-methyl groups, exhibits optical activity, but gives no copper or cobalt complex. It is oxidized to succinic acid by potassium permanganate, but is not attacked by potassium periodate. Of the structures consistent with these characteristics (β,ϵ-diaminocaproic acid, γ,ϵ-diaminocaproic acid and α-aminomethyl-δ-aminovaleric acid), the first was confirmed as that of isolysine by a synthesis involving the Arndt–Eistert homologation sequence starting with L-ornithine. Syntheses of the two remaining structures are also presented.

In a recent communication[1] we described in detail the chromatographic separation of the hydrolysis products derived from streptolin AB, an antibiotic produced by a strain of Streptomyces.[2] The last major fraction (no. 7) to be eluted by acid on a Dowex-50 column was a base, $C_6H_{14}N_2O_2$, which we regarded as a position isomer of lysine. This substance, which we designate as "isolysine," has also been obtained from viomycin by Haskell, *et al.*,[3] and from streptothricin by Carter, *et al.*[4] We now wish to record pertinent chemical characteristics

(1) E. E. Smissman, R. W. Sharpe, B. F. Aycock, E. E. van Tamelen and W. H. Peterson, THIS JOURNAL, **75**, 2029 (1953).

(2) R. W. Rivett and W. H. Peterson, *ibid.*, **69**, 3006 (1947).

(3) T. H. Haskell, S. A. Fusari, R. P. Frohardt and Q. R. Bartz, *ibid.*, **74**, 599 (1952).

(4) H. E. Carter, W. R. Hearn and W. R. Taylor, "Abstracts of Papers," 119th Meeting, American Chemical Society, Cleveland, Ohio, April, 1951, p 25A.