[CONTRIBUTION FROM THE DEPARTMENTS OF CHEMISTRY AND BIOCHEMISTRY, UNIVERSITY OF WISCONSIN]

The Chemistry of Antimycin A. III. The Structure of Antimycic Acid[1,2]

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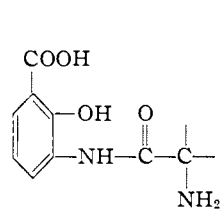
Antimycic acid, a degradation product of antimycin A, has been shown to be N-(3-aminosalicyloyl)-L-threonine by hydrolysis to L-threonine and 3-aminosalicylic acid.

Mild alkaline degradation of the antibiotic antimycin A has afforded, among other products, a substance $C_{11}H_{14}N_2O_5$, which has been termed antimycic acid (I).[3] The degradations outlined in this communication along with initial observations reported previously, are uniquely accommodated by the structure N-(3-aminosalicyloyl)-L-threonine for I.

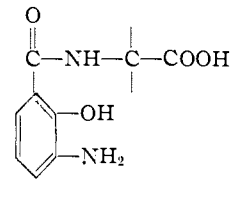
Fusion of antimycic acid with solid potassium hydroxide led to a mixture of water-soluble amino acids which could be separated by suitable extraction techniques. One of these was a ninhydrin-positive substance which was shown to be glycine by its neutral equivalent, R_f value on paper chromatograms, and activity in microbiological assays based on the growth of *Leuconostoc mesenteroides*. A second, ether-soluble acid II possessed the formula $C_7H_7NO_3$ and contained a basic nitrogen as well as a phenolic function and a carboxyl group. Electrometric titration showed pK_a values of 2.3 and 4.8 and no other inflections up to *p*H 11.5. The analytical data suggested an aminohydroxybenzoic acid structure; conversion of II to salicylic acid by means of diazotization and replacement of the diazo function by hydrogen supported this surmise. Assignment of the amino group in II to the 3-position on the aromatic ring was accomplished by comparison of II with authentic 3-aminosalicylic acid. The infrared spectra of the two acids were identical, and the mixed melting point of their methyl esters was undepressed.

There remained in antimycic acid two carbon atoms unaccounted for. Saponification of I by concentrated aqueous sodium hydroxide afforded a better yield of 3-aminosalicylic acid but only small amounts of ninhydrin-positive material; this result suggested the attachment of the glycine portion in I to the undetected two-carbon fragment. This possibility was realized in the elaboration of a suitable structure for I by utilizing data previously reported.[3]

Because antimycic acid possesses but one carboxyl group, either the carboxyl group of glycine or that of 3-aminosalicylic acid must be masked by the existence in I of an amide link between the two degradation products. Of the two possibilities (III and IV), III is improbable, because it predicts one mole of amino nitrogen by the Van Slyke technique, whereas antimycic acid yields no nitrogen under the conditions of this method. Actually, a substance



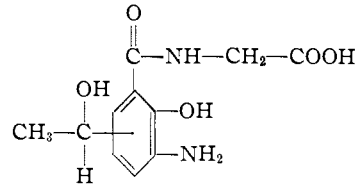
III



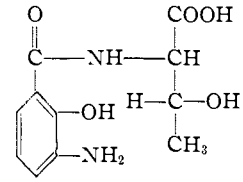
IV

possessing partial structure IV would be expected to form a fairly stable diazonium salt, as does its parent, *o*-aminophenol,[4] and to yield little or no nitrogen in the Van Slyke test. Also a substance of type III should show a positive ninhydrin reaction, whereas antimycic acid and 3-aminosalicylic acid do not. The presence of a weakly basic nitrogen and a second (phenolic) acidic function in I are in keeping with structure IV, but can hardly be said to exclude III.

Moreover, the optical activity of antimycic acid and the presence of one C-methyl group and an additional hydroxylic oxygen (as demanded by the molecular formula) limit to V and VI the possible structures which account also for the formation of



V



VI

glycine and 3-aminosalicylic acid in the basic fusion. This transformation may be viewed in each case as a retrograde–aldol type of cleavage. Structure V is comparable to 6-carboxy-3,3-dimethylphthalide, which, upon drastic treatment with alkali, is converted (probably *via* the anion of the hydroxy acid) to acetone and isophthalic acid.[5] Structure VI, on the other hand, is an N-acyl threonine. Wieland and Wirth[6] found that saturated barium hydroxide converted both threonine and N-acyl threonines into glycine and presumably acetaldehyde, which would hardly be expected to remain, under

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(2) Presented in part at the 44th annual meeting of the American Society of Biological Chemists, Chicago, April, 1953.

(3) G. M. Tener, F. M. Bumpus, B. R. Dunshee and F. M. Strong, THIS JOURNAL, **75**, 1100 (1953).

(4) A. Hantzsch and W. B. Davidson, *Ber.*, **29**, 1522 (1896).

(5) T. B. Wood, W. T. N. Spivey and T. H. Easterfield, *J. Chem. Soc.*, **75**, 20 (1899).

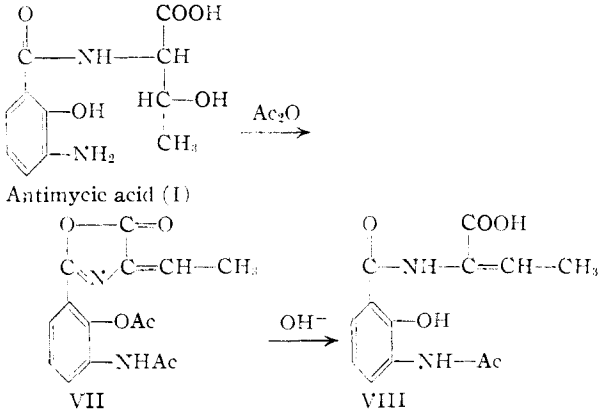
(6) T. Wieland and L. Wirth, *Chem. Ber.*, **82**, 468 (1949).

the strongly basic conditions, in any recognizable form.

A selection between the two possibilities can be made, however, by taking into account the relatively easy thermal decarboxylation of antimycic acid.[3] A substance possessing structure V would appear to offer no ready route for such elimination, whereas VI would be expected to dehydrate initially with the formation of N-(3-aminosalicyloyl)-2-aminobuten-1-oic acid (VIa), which, like most α,β-unsaturated acids, should be susceptible to ready decarboxylation.[7]

Confirmation of structure VI was attained by identification of the products of drastic acid hydrolysis. Autoclaving a solution of antimycic acid in 3 *N* hydrochloric acid for 15 hours resulted in the formation of II and a different ninhydrin-positive product, which was shown by means of paper chromatographic studies and microbiological assay to be L-threonine. We therefore consider antimycic acid (I) to be N-(3-aminosalicyloyl)-L-threonine (VI).

Several additional observations on the chemical behavior of antimycic acid can now be clarified on the basis of this structure. Acetylation[3] afforded a neutral "bisanhydrodiacetate," $C_{15}H_{14}N_2O_5$, (VII), which could be selectively hydrolyzed to an "anhydromonoacetate," $C_{13}H_{14}N_2O_5$ (VIII); the latter product was phenolic and possessed a carboxyl group. This sequence of reactions can be best explained by assuming (i) acetylation of the phenolic hydroxyl and the free amino groups of I with concurrent conversion of the peptide linkage into an azlactone and dehydration of the β-hydroxy carbonyl system to give VII, and (ii) preferential hydrolysis of the O-acetyl group in VII and opening of the azlactone ring to yield VIII, the anhydromonoacetyl derivative. A consequence of the proposed course of reaction is the absence of an



asymmetric center in either of the two transformation products. By way of confirmation, VIII was tested for possible rotation and was found to be optically inactive.

The formation of an ester–ether by treating antimycic acid successively with acidic methanol and diazomethane is readily interpretable on the basis of VI.

(7) Structure VIa closely resembles the presumed intermediate in the pyruvic acid decarboxylation catalyzed by organic bases (R. E. Schachat, E. I. Becker and A. D. McLaren, *J. Org. Chem.*, **16**, 1349 (1951); *J. Phys. Chem.*, **56**, 722 (1952)).

Experimental[8]

Alkaline Fusion of Antimycic Acid.—To 375 mg. of antimycic acid contained in a Pyrex glass test-tube was added 840 mg. of powdered potassium hydroxide and the mixture was heated slowly in a nitrogen atmosphere to 300°. After 15 minutes at 300°, the reaction mixture was cooled to room temperature, and carefully neutralized with dilute sulfuric acid. An amorphous inorganic precipitate (probably silica) was filtered off and washed twice with water. The combined filtrate and washings were adjusted to *p*H 3.5 with dilute sulfuric acid and the resulting solution (25 ml.) extracted repeatedly with ether (6 × 25 ml.). The dried ether extract yielded 238 mg. of a solid residue after evaporation. This material, compound II, was purified by sublimation at 200° and 10 mm. pressure, followed by recrystallization from methanol. Fine white needles resulted that decomposed between 231–235°.

Anal. Calcd. for $C_7H_7O_3N$: C, 54.9; H, 4.61; N, 9.15; neut. equiv., 153. Found: C, 55.10; H, 4.96; N, 9.38; neut. equiv., 159, determined by titration with alcoholic sodium hydroxide to a thymolphthalein end point. The ultraviolet spectrum of this compound showed peaks at 224 and 326 mμ with specific extinctions of 129 and 19.3, respectively.

Isolation of Glycine.—To the aqueous layer left after the ether extraction above was added six volumes of absolute ethanol. The precipitate that formed was filtered off and extracted three times with 50-ml. portions of boiling 95% ethanol. The combined ethanol extracts were concentrated to dryness *in vacuo*, the resulting yellow gum dissolved in 3 ml. of boiling water, and sufficient alcohol added to induce a slight turbidity. The hot solution was filtered, and the clear filtrate on cooling deposited 14 mg. of glistening white plates which decomposed at 210–215°. No ash was left after combustion. The compound gave strong ninhydrin and qualitative nitrogen tests, but only a very slight test for sulfur and none for phenols.

Anal. Calcd. for $C_2H_5O_2N$: neut. equiv., 75.1. Found: neut. equiv., 75.7, determined by titration with alcoholic sodium hydroxide to a thymolphthalein end-point. The R_f value on paper chromatograms developed with a phenol–water–formic acid (40.4:20.5:1) system[9] was 0.51 whereas that of known glycine was 0.52. Microbiological assay using *L. mesenteroides*[10] also indicated that at least 90% of the isolated product consisted of glycine.

Deamination of Compound II.—Compound II (36.8 mg.) was deaminated by the procedure of Hodgson and Turner.[11] Addition of the diazotized amine to the alcohol–cuprous oxide mixture resulted in rapid evolution of gas. After the initial reaction period, the mixture was heated for five minutes, cooled and poured into three volumes of water. This aqueous solution was extracted with ether (6 × 35 ml.), the ether layer concentrated to dryness, and the residue boiled for 20 minutes in 5 ml. of 10% sodium hydroxide. The cooled alkaline solution was extracted three times with ether, made acidic to congo red with 5% sulfuric acid, and again extracted with ether (3 × 30 ml.). This second ether extract was dried over anhydrous sodium sulfate and concentrated to dryness at room temperature in a stream of dry air. The residue was sublimed at 15 mm. and 130° to yield 1.3 mg. of long colorless needles, m.p. 154.5–156.5°. A mixed melting point with authentic salicylic acid (m.p. 157.5–158.0°) was 155–157°. Confirmation of the identity of this product was obtained by comparison of its infrared spectrum with that of an authentic sample.

Esterification of Compound II.—To 44 mg. of compound II suspended in 20 ml. of ether and 1 ml. of methanol was added 40 ml. of ethereal diazomethane (from 1 g. of nitrosomethylurea). After standing 10 minutes, the solution was concentrated to dryness *in vacuo* and the residue recrystal-

(8) Melting and boiling points uncorrected. Microanalyses by Micro-Tech Laboratories, Skokie, Illinois. Infrared spectra were determined with the Baird spectrophotometer by D. R. Johnson and R. Meiklejohn, the potentiometric titration was carried out by Dr. R. M. Bock, and the microbiological assays by J. C. Alexander and A. E. Denton. The authors wish to express their sincere thanks for this assistance.

(9) J. S. Cohen, M.S. thesis, University of Wisconsin, 1952.

(10) J. C. Alexander and C. A. Elvehjem (unpublished procedure).

(11) H. H. Hodgson and H. S. Turner, *J. Chem. Soc.*, 748 (1942).

lized from methanol–water and then from petroleum ether. A good yield of very light tan crystals resulted, m.p. 85.0–85.5°.

Anal. Calcd. for $C_8H_9O_3N$: C, 57.5; H, 5.43. Found: Cl, 58.0; H, 5.60.

The methyl ester of 3-aminosalicylic acid prepared in the above manner was found to melt at 85–86° and the melting point of a mixture of the known ester and that from compound II was 85.5–86.5°. Confirmation of the identity of compound II as 3-aminosalicylic acid was obtained by comparison of the infrared spectra of known and isolated samples of the free acid.

Saponification of Antimycic Acid.—Antimycic acid (436 mg.) was dissolved in 4 ml. of aqueous sodium hydroxide (cold saturated) and heated under reflux in a nitrogen atmosphere for two hours. After cooling, the *p*H was adjusted to 2.5 with 5% sulfuric acid, and the mixture was extracted 10 times with ether (20 ml. each). After drying the ether extract over anhydrous sodium sulfate, the solvent was removed *in vacuo* and the resulting residue purified by sublimation at 0.01 mm. and 200° to yield 212 mg. (81%) of 3-aminosalicylic acid (compound II). The aqueous mother liquors contained only a very small amount of ninhydrin-positive material as judged by the feeble color produced after spotting on filter paper.

Acid Hydrolysis of Antimycic Acid.—Antimycic acid (96.5 mg.) was hydrolyzed for 15 hours in 5 ml. of 3 *N* hydrochloric acid at 15 lb. pressure of steam. The resulting solution was concentrated to dryness *in vacuo*, two ml. of water added and the solution again concentrated to dryness. The residue was dissolved in 1 ml. of water and the resulting solution adjusted to *p*H 3 with 5% sodium bicarbonate. A small amount of precipitate (15.2 mg.) was filtered off and the filtrate was extracted with ether (4 × 6 ml.). The ether layer contained 3-aminosalicylic acid. The aqueous phase was adjusted to *p*H 6 and a portion chromatographed by the procedure used for glycine. Its R_f value (0.59) was the same as that of known threonine chromatographed simultaneously. The single spot produced by the unknown was readily distinguishable from that of glycine. Confirmation of the presence of L-threonine was obtained by microbiological assay using *S. faecalis*.[12] By this assay a recovery of more than 85% of the theoretical amount of threonine was obtained on acid hydrolysis. Since this test organism does not respond to D-threonine or to D- or L-allothreonine, the hydrolytic product must have possessed the common L-configuration.

(12) A. M. Violante, R. J. Sirny and C. A. Elvehjem, *J. Nutrition*, **47**, 307 (1952).

MADISON, WISCONSIN

[CONTRIBUTION FROM THE RESEARCH LABORATORIES OF THE UPJOHN COMPANY]

Synthesis of Pteroic and Pteroylglutamic Acids.[1] I.

BY D. I. WEISBLAT, B. J. MAGERLEIN, A. R. HANZE, D. R. MYERS AND S. T. ROLFSON

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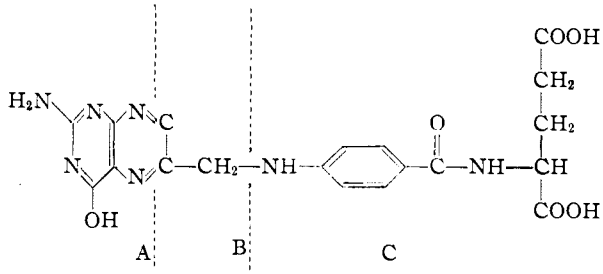
A new general synthesis of pteroic and pteroylglutamic acids is reported wherein an N-tosyl-*p*-aminobenzoate or N-tosyl-*p*-aminobenzoylglutamate is alkylated with a substituted propylene oxide molecule, the product oxidized to a ketone, and condensed with 2,4,5-triamino-6-hydroxypyrimidine. Pteroic and pteroylglutamic acids are then formed from the N^{10}-tosyl compounds by the use of an improved detosylation procedure.

Since the initial publication of the structure and synthesis of pteroylglutamic acid,[2] many papers and patents have appeared describing syntheses of this member of the vitamin B complex.[3] Since the pteroylglutamic acid molecule consists of a 2,4,5-triamino-6-hydroxypyrimidine portion (A), a three carbon system (B), and a *p*-aminobenzoylglutamic acid portion (C), these syntheses of



pteroylglutamic acid may be divided into three general classes. The first and most widely investigated type of synthesis employs the simultaneous reaction of the three components, A + B + C, the earliest type of which employed 2,3-dibromopropanal as the B portion.[3a] Other three carbon molecules are 2,2,3-tribromopropanal,[3g] 1,1,3-tribromopropanone-2,[3g,h] glyceraldehyde ditosylate,[3e] dihydroxyacetone[3e] and dichloroacetone.[4] The second type of synthesis, represented as AB + C, involves condensation of the pyrimidine portion (A) with a trifunctional three carbon component (B) to form a product (AB), which in turn is condensed with the *p*-aminobenzoylglutamic acid portion. Representative AB molecules which have been used are N-[(2-amino-4-hydroxy-6-pteridyl)-methyl]-pyridinium iodide (I),[3b] the corresponding bromo derivative II,[3d] the hydroxy derivative III[3e] and the pteridyl aldehyde IV.[3f] In the third

(1) Presented in part before the Division of Biological Chemistry at the XIIth International Congress of Pure and Applied Chemistry, New York, September 10 to 13, 1951.

(2) R. B. Angier, J. H. Boothe, B. L. Hutchings, J. H. Mowat, J. Semb, E. L. R. Stokstad, Y. SubbaRow, C. W. Waller, D. B. Cosulich, M. J. Fahrenbach, M. E. Hultquist, E. Kuh, E. H. Northey, D. R. Seeger, J. P. Sickels and J. M. Smith, Jr., *Science*, **103**, 667 (1946).

(3) (a) C. W. Waller, B. L. Hutchings, J. H. Mowat, E. L. R. Stokstad, J. H. Boothe, R. B. Angier, J. Semb, Y. SubbaRow, D. B. Cosulich, M. J. Fahrenbach, M. E. Hultquist, E. Kuh, E. H. Northey, D. R. Seeger, J. P. Sickels, J. M. Smith, Jr., THIS JOURNAL, **70**, 19 (1948); (b) M. E. Hultquist, E. Kuh, D. B. Cosulich, M. J. Fahrenbach, E. H. Northey, D. R. Seeger, J. P. Sickels, J. M. Smith, Jr., R. B. Angier, J. H. Boothe, B. L. Hutchings, J. H. Mowat, J. Semb, E. L. R. Stokstad, Y. SubbaRow and C. W. Waller, *ibid.*, **70**, 23 (1948); (c) R. B. Angier, E. L. R. Stokstad, J. H. Mowat, B. L. Hutchings, J. H. Boothe, C. W. Waller, J. Semb, Y. SubbaRow, D. B. Cosulich, M. J. Fahrenbach, M. E. Hultquist, E. Kuh, E. H. Northey, D. R. Seeger, J. P. Sickels and J. M. Smith, Jr., *ibid.*, **70**, 25 (1948); (d) J. H. Boothe, C. W. Waller, E. L. R. Stokstad, B. L. Hutchings, J. H. Mowat, R. B. Angier, J. Semb, Y. SubbaRow, D. B. Cosulich, M. J. Fahrenbach, M. E. Hultquist, E. Kuh, E. H. Northey, D. R. Seeger, J. P. Sickels and J. M. Smith, Jr., *ibid.*, **70**, 27 (1948); (e) P. Karrer and R. Schwyzer, *Helv. Chim. Acta*, **31**, 777 (1948); (f) F. Weygand, A. Wacher and V. Schmied-Kowarzik, *Chem. Ber.*, **82**, 25 (1949); (g) F. Weygand and V. Schmied-Kowarzik, *ibid.*, **82**, 333 (1949); (h) F. E. King and P. C. Spensley, *Nature*, **164**, 574 (1949); (i) H. S. Forrest and J. Walker, *J. Chem. Soc.*, 2002 (1949).

(4) D. I. Weisblat and A. R. Hanze, U. S. Patent 2,560,616 (1951).