[CONTRIBUTION FROM THE RESEARCH LABORATORIES OF THE KON. NED. GIST- EN SPIRITUSFABRIEK]

Bottromycin. II. Preliminary Degradation Studies

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Bottromycin, $C_{38}H_{57-61}N_7O_{7-8}S$, is cleaved by vigorous acid hydrolysis into six ninhydrin-positive substances, two of which have been identified as glycine and valine. Two of the four unidentified fragments have been isolated and shown to have the formulas $C_6H_8N_2O_2S$ and $C_{10}H_{13}NO_2$. Acetylation of the antibiotic yielded two crystalline ninhydrin-negative compounds $C_{19}H_{23}N_3O_4S$ and probably $C_{21}H_{34}N_4O_4$.

In a previous publication Waisvisz and coworkers[1] have reported the isolation, purification and some of the properties of a new sulfur-containing antibiotic, bottromycin.

Analyses and molecular weight determinations[1] indicated a probable molecular formula for bottromycin as $C_{38}H_{57-61}N_7O_{7-8}S$.

Although bottromycin gives a negative ninhydrin test, a Van Slyke nitrogen test indicates the presence of one primary amino group.

Ready decolorization of bromine suggests the unsaturated character of the antibiotic. A Friederich N-alkyl determination and an acetyl determination on the intact antibiotic proved that no such groups are present. The reaction of Kuhn-Roth revealed the presence of two or three C-methyl groups. A Zeisel determination indicated the presence of one methoxyl group.

Desulfurization of the antibiotic with Raney nickel and precipitation of the reaction product with salicylic acid yielded an amorphous sulfur-free degradation product.

Refluxing a solution of bottromycin in 20% aqueous or alcoholic potassium hydroxide resulted in extensive degradation and only tarry or oily products could be isolated.

Hydrolysis of the antibiotic with concentrated hydrochloric acid at 110° for 72 hours yielded a hydrolyzate containing several compounds which gave a positive ninhydrin reaction.

By paper chromatographic methods we were able to demonstrate the presence of seven ninhydrin-positive compounds in this hydrolyzate (compounds no. I/VII, numbers according to the position of the fragments on paper chromatograms).

CHART I

Bottromycin, $C_{38}H_{57-61}N_7O_{7-8}S$

Vigorous acid hydrolysis

7 ninhydrin-positive reacting compd.		$C_{19}H_{23}N_3O_4S$ (VIII)	$C_{21}H_{34}N_4O_4$? (IX)
		↓ vigorous acid hydrolysis	↓ vigorous acid hydrolysis
Glycine	(I)		
Degradation product of III	(II)		
$C_6H_8N_2O_2S$	(III)	$C_6H_8N_2O_2S$ (III)	Glycine (I)
Unidentified	(IV)	$C_{10}H_{13}NO_2$ (VII)	Unidentified (IV)
Valine	(V)		Valine (V)
Unidentified	(VI)		Unidentified (VI)
$C_{10}H_{13}NO_2$	(VII)		

"Acetylation"

No. I, V, VI and VII developed a purple color on spraying with ninhydrin, II and IV produced faint yellow colors, whereas III showed a brown spot turning purple on exposure to daylight. Up to the present two compounds (I and V) have been identified as glycine (I) and valine (V).

Fractionation of the acid hydrolyzate on an Amberlite IR-4B resin column resulted in the isolation of VII as a pure crystalline compound (m.p. 176–177°) and the isolation of an unidentified ninhydrin-negative substance (m.p. 218° after sublimation). Compound VII crystallizes from 96% alcohol as a monohydrate. Molecular weight determination by the method of Rast indicated that this compound has a molecular weight of about 187. It is optically active. On the basis of analysis of VII and its dinitrophenyl, acetyl- and tosyl-derivatives we have assigned to it the empirical formula $C_{10}H_{13}NO_2$.

Acetylation of bottromycin with boiling acetic anhydride yielded, in addition to tarry and amorphous products, two crystalline degradation products VIII and IX. Compound VIII, recrystallized from ethyl acetate, diisopropyl ether or benzene, contains sulfur and melts at 215–216° (after sublimation). The molecular weight of VIII, determined in camphor, is approximately 408 ± 20. Analysis disclosed that this ninhydrin-negative product has the molecular formula $C_{19}H_{23}N_3O_4S$. According to group analyses, compound VIII contains one acetyl-, one methoxyl- and also one C-methyl group. Kunz-hydrolysis[2] of VIII showed that one molecule utilizes one molecule of sodium hydroxide under the conditions of the reaction, which means that one group of the molecule is subjected to saponification.

By paper chromatographic methods it was shown that mild hydrolysis of compound VIII produced three ninhydrin-positive compounds, namely, the above-mentioned III and VII, and in addition a product with a rather high R_f-value (VIIIa). VIIIa which could be isolated from the hydrolyzate and recrystallized from alcohol or water. The melting point of this sulfur-containing degradation product was 203–206°. Analyses indicated a probable molecular formula $C_{16}H_{19}N_3O_3S \cdot {}^1/_2H_2O$. The same product (VIIIa) could be isolated from a mild acid hydrolyzate of the antibiotic.

Vigorous hydrolysis (72 hours with concentrated hydrochloric acid at 110°) of VIII or VIIIa yielded mainly the two products III and VII. Both products were isolated from these hydrolyzates either by fractional crystallization of their hydrochlorides or by

(1) J. M. Waisvisz, *et al.*, THIS JOURNAL, **79**, 4520 (1957).

(2) A. Kunz and C. S. Hudson, *ibid.*, **48**, 1982 (1926); M. L. Wolfrom, M. Konigsberg and S. Stolzberg, *ibid.*, **58**, 490 (1936).

column chromatography on Amberlite IR-4B.

III, recrystallized from alcohol, melts at 197.5–201.5° dec. and has the molecular formula $C_6H_8N_2O_2S$. A mono-dinitrophenyl derivative of III was prepared.

A third unidentified product (IIIa), which showed a salmon-pink spot on the paper chromatograms, was isolated from the above-mentioned hydrolyzates as its crystalline hydrochloride. Continued vigorous acid hydrolysis of IIIa yielded III. The other crystalline product IX obtained by acetylation of the antibiotic with boiling acetic anhydride could be recrystallized from ethyl acetate and melted at 165–170°. This ninhydrin-negative compound contained no sulfur. Its composition is best represented by the formula $C_{21}H_{34}N_4O_4$.

A Friederich determination on IX disclosed the presence of one or two N-alkyl groups. However, the same determination on bottromycin failed to demonstrate the presence of N-alkyl groups in the intact antibiotic.

A Kuhn-Roth determination showed the presence of two C-methyl groups. Vigorous hydrolysis of IX with concentrated hydrochloric acid yielded a hydrolyzate which contained the ninhydrin-positive reacting compounds I (= glycine), IV, V (= valine) and VI. A crystalline mixture of the four substances was isolated. Further work on the purification and structure of these compounds will be reported later.

It has been found that alkaline hydrolysis or oxidation of III results in the formation of II, in other words, II is a degradation product of III.

Taking into account the above-mentioned facts, it seems likely that bottromycin has a peptide-like structure.

Experimental

Acid Hydrolysis of Bottromycin.—Purified bottromycin (2 g.) was heated for 72 hr. in a Carius tube with concentrated hydrochloric acid (10 ml.) at 100–110°. Paper chromatograms (run in butanol–acetic acid–water 100:12:100) showed the presence of seven ninhydrin-positive compounds in this hydrolyzate.

Fractionation of the Acid Hydrolyzate of Bottromycin on an Amberlite IR-4B Resin Column (Isolation of VII).—The acid hydrolyzate was evaporated *in vacuo* and redissolved in water (50 ml.). This solution was added to an Amberlite IR-4B (OH-form) resin column (310 × 28 mm.). The column was at first developed by gravity with distilled water (205 fractions of 12.5 ml. were collected) followed by 0.5 *N* hydrochloric acid (65 fractions of 12.5 ml. and one of 600 ml.). Although the paper chromatograms (run in butanol–acetic acid–water 100:12:100) disclosed that a poor elution and separation had been achieved, nevertheless we could isolate by freeze-drying (1) from the water fractions (no. 1/10) an amorphous ninhydrin-negative compound. This product was crystallized from ethyl acetate–acetone and melted at 218° (after sublimation in needles at 184°).

(2) The water fractions (no. 33/60) contained, according to the paper chromatograms, a single ninhydrin-positive compound VII. After freeze-drying and several recrystallizations from alcohol we obtained 18 mg. of pure VII melting at 176–177°. The mother liquors yielded another 11 mg. (m.p. 173°) of the same compound. The hydrochloric acid fractions contained the impure III together with VI.

Anal. Calcd. for $C_{10}H_{13}NO_2 \cdot H_2O$: C, 60.91; H, 7.61; N, 7.11. Found: C, 60.91; H, 7.64; N, 7.17.

Acetylation of Bottromycin (Isolation of VIII and IX).—A solution of bottromycin (50 g.) in acetic anhydride (500 ml.) was heated for 3 hours on a steam-bath. The solution was then concentrated *in vacuo* to a small volume (100 ml.) and cooled in the refrigerator, whereupon spontaneous crystallization took place. The snow-white crystals were filtered off, washed with acetic anhydride (20 ml.) and dried. The crude product (14.2 g.) was recrystallized from ethyl acetate (800 ml.) and yielded VIII (11.4 g.) melting at 215–216° (after sublimation in needles).

From the recrystallization mother liquor we isolated another crop of VIII (2.3 g.) melting at 209–211°.

Anal. Calcd. for $C_{19}H_{23}N_3O_4S$: C, 58.61; H, 5.91; N, 10.80; S, 8.23; CH_3CO, 11.05; OCH_3, 7.97; mol. wt., 392. Found: C, 58.52; H, 6.05; N, 10.62; S, 8.29; CH_3CO, 11.57; OCH_3, 8.14; mol. wt. (Rast), 408.

The reaction mother liquor was evaporated to dryness and the tarry residue dissolved in acetone (20 ml.). Crystallization occurred very slowly. After one week the crystals were collected, washed with a small quantity of cold acetone and dried.

Recrystallization from ethyl acetate (100 ml.) yielded 3.8 g. of IX melting at 165–170°.

Anal. Calcd. for $C_{12}H_{34}N_4O_4$: C, 62.04; H, 8.43; N, 13.78. Found: C, 61.86; H, 8.35; N, 13.67.

Hydrolysis of VIII with 6 *N* Hydrochloric Acid. a. Isolation of VIIIa.—Compound VIII (120 mg.) was dissolved in 6 *N* (11 ml.) hydrochloric acid and heated on a steam-bath for 4.5 hours. The hydrolyzate was evaporated to dryness, redissolved in water and concentrated. Finally the concentrate was dissolved in water (2 ml.) and extracted several times with a 5% solution of methyldioctylamine in chloroform until the *p*H of the water layer was about 6. The remaining water layer was evaporated to dryness and the solid residue recrystallized from ethanol (1 ml.). VIIIa (21 mg.), m.p. at 203–206°, was obtained.

Anal. Calcd. for $C_{16}H_{19}N_3O_3S \cdot {}^1/_2H_2O$: C, 56.11; H, 5.85; N, 12.28; S, 9.38. Found: C, 55.89; H, 5.87; N, 12.03; S, 9.72.

Hydrolysis of VIII with Concentrated Hydrochloric Acid. a. Isolation of III and VII by Fractionation on an Amberlite IR-4B Resin Column.—Compound VIII (3.21 g.) was heated with concentrated hydrochloric acid (16 ml.) in a Carius tube at 110° for 72 hours. Paper chromatograms (run in butanol–acetic acid–water 100:12:100) showed that mainly two ninhydrin-positive compounds III and VII were present in this hydrolyzate. The acid solution was evaporated to dryness, dissolved in water (50 ml.) and added to a previously packed IR-4B (OH-form) resin column (430 × 42 mm.). The column was successively developed with water (135 fractions of 50 ml. and one of 300 ml.) and 0.5 *N* hydrochloric acid (17 fractions of 50 ml., one of 850 ml. and 3 fractions of 100 ml.). According to the paper chromatograms the water fractions 1–24 contained mainly compound III, the fractions 25–110 contained substance VII, whereas the hydrochloric acid fractions contained both III and VII. After evaporation and several recrystallizations from alcohol, the water fractions 1–24 yielded 68 mg. of III melting at 197–201.5°. From the water fractions 25–100 we obtained by evaporation and recrystallization from alcohol 500 mg. of VII melting at 176–177°.

Anal. Calcd. for $C_6H_8N_2O_2S$: C, 41.86; H, 4.66; N, 16.28; S, 18.60. Found: C, 41.86; H, 4.71; N, 15.91; S, 17.95.

b. Isolation of the Hydrochlorides of III and VII by Fractional Crystallization.—Compound VIII (10 g.) was heated with concentrated hydrochloric acid (50 ml.) in a Carius tube at 110° for 72 hr. The hydrolyzate crystallized when stored in the refrigerator. The white crystals were collected, washed with two small portions of cold concentrated hydrochloric acid, and dried to yield 2.88 g. of solid K_1. The mother and wash liquors were combined and concentrated *in vacuo* to half their volume. A second crop of crystals (1.15 g.) K_2 was isolated. After concentration of the filtrate to a still smaller volume another 0.5 g. of solid product K_3 was obtained.

The remaining mother liquor was evaporated to dryness and the resulting brown gum was dissolved in a small quantity of cold alcohol. Spontaneous crystallization set in. After storing in the ice-box for a few hours, the yellowish product was collected, washed with two small portions of cold alcohol and dried to give K_4 (4.1 g.), as a crystalline solid.

Paper chromatographic methods showed that K_1 and K_2 were both the pure hydrochloride of VII, $[\alpha]^{25}D$ −10.6 (*c*

5%, water). K_3 was a mixture of III and VII, whereas K_4 proved to be the pure hydrochloride of III.

Preparation of Derivatives of III and VII. a. The 2,4-Dinitrophenyl Derivative of III.—According to the method of Sanger[3] the dinitrophenyl derivative of III was prepared by treating the hydrochloride of III (239 mg.) with 2,4-dinitrofluorobenzene (0.56 ml.). After three recrystallizations from 60% methyl alcohol, the yellow, acicular crystalline derivative (223 mg.) was obtained, m.p. 148–151°.

Anal. Calcd. for $C_{12}H_{10}N_4O_6S$: C, 42.61; H, 2.98; N, 16.57; S, 9.46. Found: C, 42.47; H, 2.91; N, 16.49; S, 9.34.

b. The 2,4-dinitrophenyl derivative of VII was prepared from the hydrochloride (283 mg.) and 2,4-dinitrofluorobenzene (0.28 ml.). After three recrystallizations from 60% methanol we obtained the yellow derivative (254 mg.), m.p. 175–179°.

Anal. Calcd. for $C_{16}H_{15}N_3O_6$: C, 55.65; H, 4.38; N, 12.17. Found: C, 55.62; H, 4.37; N, 12.27.

c. The Tosyl Derivative of VII.—The hydrochloride of VII (500 mg.) was dissolved in 1 *N* sodium hydroxide (10 ml.). A solution of *p*-toluenesulfonyl chloride (1 g.) in ether (12.5 ml.) was added and the mixture was shaken mechanically for 4 hr. The ether layer was discarded and the water layer acidified with 4 *N* hydrochloric acid. A white precipitate formed, which was recrystallized several times from 60% alcohol. The acicular, white crystals (414 mg.) melted at 169–171°.

Anal. Calcd. for $C_{17}H_{19}NO_4S$: C, 61.25; H, 5.75; N, 4.20; S, 9.60. Found: C, 61.23; H, 5.79; N, 4.25; S, 9.73.

d. The Acetyl Derivative of VII.—A solution of the hydrochloride of VII (5 g.) in 1 *N* sodium hydroxide (50 ml.) was cooled in an ice-bath. Acetic anhydride (3 ml.) and 1 *N* sodium hydroxide (25 ml.) were added simultaneously and gradually under stirring over a period of 15 minutes. The mixture was stirred for an additional 30 minutes, whereupon another portion of acetic anhydride (3 ml.) and sodium hydroxide (65 ml.) were added in 13 minutes. After another 30 minutes the solution was filtered, acidified with concentrated hydrochloric acid and cooled; the precipitated solid was filtered off, washed with water and dried.

The product was recrystallized from 20% alcohol. A white, acetyl derivative (3.4 g.), m.p. 177–185°, $[\alpha]^{25}D$ +35.0° (*c* 2%, 96% ethyl alcohol), was obtained.

(3) F. Sanger, *Biochem. J.*, **39**, 507 (1945).

Anal. Calcd. for $C_{12}H_{15}NO_3$: C, 65.14; H, 6.79; N, 6.33. Found: C, 64.84; H, 6.79; N, 6.42.

Kunz-hydrolysis of VIII.—Compound VIII (197.9 mg.) was dissolved in purified acetone (25 ml.). To this solution was added 0.1011 *N* sodium hydroxide (25 ml.). After 2.5 hours in the refrigerator, titration with 0.0906 *N* hydrochloric acid showed that 0.98 milliequivalent of alkali had been consumed per milliequivalent of VIII.

Identification of Valine and Glycine in the Acid Hydrolyzate of Bottromycin by Means of Paper Chromatography.—A hydrolyzate of bottromycin with concentrated hydrochloric acid (72 hr. hydrolysis) was compared with known amino acids by means of paper chromatographic methods. The one-dimensional descending method was used (Whatman no. 1 paper). The following eluent systems were used: *n*-propanol–water (70:30), *n*-propanol–water–diethylamine (85:15:3), *n*-butanol–acetic acid–water (100:12:100), isobutyl alcohol–formic acid–water (70:15:13), phenol–water–NH_3–NaCN, pyridine–isoamyl alcohol–water (35:35:30). In all six systems spots I and V coincided with the spots of glycine and valine, respectively. Identity was demonstrated also as follows. The hydrolyzate was evaporated to dryness under vacuum and treated with 2,4-dinitrofluorobenzene, according to the method of Levy.[4] This yielded a mixture of dinitrophenyl derivatives. By paper chromatographic technique this mixture was compared with the dinitrophenyl derivatives of glycine and valine. The following systems were used: 1, the Blackburn system[5] (phthalate buffered paper strips of *p*H 6.0 and as eluent: (a) phthalate buffer–saturated *n*-propanol–petroleum ether 3:7); (b) phthalate buffer saturated amyl alcohol; 2, the Biserte system[6] (toluene–pyridine–ethylene chlorhydrin–0.8 *N* ammonia, 5:1:3:3). In all cases the spots of dinitrophenylvaline and dinitrophenylglycine coincided with two spots of dinitrophenyl derivatives obtained with bottromycin hydrolyzate.

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(4) A. L. Levy, *Nature*, **174**, 126 (1954).
(5) S. Blackburn and A. G. Lowther, *Biochem. J.*, **48**, 126 (1951).
(6) G. Biserte and R. Osteux, *Bull. soc. chim. biol.*, **33**, 50 (1951).

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[CONTRIBUTION FROM THE RESEARCH LABORATORIES OF THE KON. NED. GIST-EN SPIRITUSFABRIEK]

The Structure of the Sulfur-containing Moiety of Bottromycin

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The degradation of the antibiotic bottromycin by means of boiling acetic anhydride yielded two crystalline acetyl products $C_{19}H_{23}N_3O_4S$ and probably $C_{21}H_{34}N_4O_4$. The sulfur-containing moiety proved to be the methyl ester of a N-acetyl dipeptide. The two components of this peptide are two new naturally occurring amino acids, α-amino-β-phenylbutyric acid and β-(2-thiazole)-β-alanine.

Previously[1] it was reported that treatment of bottromycin with boiling acetic anhydride yields two crystalline acetyl-degradation products: $C_{19}H_{23}N_3O_4S$ (VIII) and a compound with the probable formula $C_{21}H_{34}N_4O_4$ (IX).

This paper deals with the elucidation of the structure of the sulfur-containing moiety. It has already[1] been reported that VIII contains one acetyl, one methoxyl and, in addition, one C-methyl group. Furthermore on Kunz hydrolysis one group of the molecule is split off, whereas mild acid hydrolysis yields a crystalline substance $C_{16}H_{19}N_3O_3S$ (VIIIa). Finally, vigorous hydrolysis of VIII or VIIIa yields two ninhydrin-positive compounds $C_{10}H_{13}NO_2$ (VII) and $C_6H_8N_2O_2S$ (III).

The ultraviolet spectrum of VII showed the typical absorption maxima of the phenyl nucleus (252.5–258 and 264 mμ).

By paper electrophoretic methods it could be demonstrated that VII has basic properties whereas the hydroxamic acid test[2] proved that, in addition, a carboxyl group was present in the molecule.

(1) J. M. Waisvisz, *et al.*, THIS JOURNAL, **79**, 4522 (1957).

(2) F. Feigl, "Qualitative Analysis by Spot Tests," 2nd ed., Elsevier Press, London, 1939, p. 294.