solution was filtered and the peptide material was precipitated with 200 ml. of absolute, peroxide-free ether and collected immediately by filtration before crystallization of sodium acetate hydrate occurred. The peptide material thus obtained weighed 1.95 g. and had a specific activity of 21 pressor units/mg. For purification this material was placed in the first 8 tubes of the all-glass automatic countercurrent distribution apparatus and distributed in the system 2-butanol–0.1% acetic acid for 1100 transfers. The activity was concentrated in a single peak. The solvent from the tubes containing the active peak was pooled and the solution, which assayed 28,000 pressor units, was concentrated and lyophilized to give 400 mg. of a product which had a specific activity of about 70 units/mg. A portion of this material (195 mg.) was subjected to electrophoresis on a cellulose block at *p*H 4.0 in a pyridine–acetate buffer and yielded, as the most active fraction, 63 mg. of material which assayed 150 units/mg. This material was redistributed in the system 2-butanol–0.1% acetic acid for 2000 transfers to give as the most active fraction 23 mg. of a product which possessed a specific activity of 220 units/mg.

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

Bottromycin. I. A New Sulfur-containing Antibiotic

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A new antibiotic bottromycin[1] has been isolated from fermentation broth by solvent extraction and purified either by chromatography on Florisil or by means of its salts with organic acids. On the basis of analysis of bottromycin and its crystalline derivatives, the antibiotic has been assigned an empirical formula $C_{38}H_{57-61}N_7O_{7-8}S$.

The antibiotic activity present in the fermentation broths of a soil actinomycete *Streptomyces bottropensis*[2] was found to be extractable at neutral or alkaline *p*H by a variety of organic solvents, such as diethyl ether, ethyl acetate and butyl acetate and at acid *p*H by chloroform.

Paper chromatographic methods gave evidence that two antibiotic substances were present in the culture broth, one of which was present in only minor quantities. This one was discarded during the isolation of bottromycin.

The main antibiotic was extracted from the filtered culture broth with butyl acetate. The extracts were concentrated to a small volume by distillation of the azeotropic mixture and thereafter re-extracted with phosphate buffer at *p*H 2. When the aqueous layer was adjusted to *p*H 9 with dilute sodium hydroxide, the crude bottromycin precipitated as an amorphous white product. This crude antibiotic produced a red color with concentrated mineral acids. The crude product was purified by chromatography on Florisil or by precipitation from its solutions in ether or butyl acetate with organic acids, such as salicylic, acetylsalicylic, *p*-aminosalicylic, 3,5-dinitrosalicylic, 3,5-dibromosalicylic, 3,5-dibromo-4-aminosalicylic, benzoic, *p*-aminobenzoic, 3,5-dinitrobenzoic, acetic, phenylacetic, picric, anthranilic acids and benzylpenicillin. Several of the amorphous salts thus obtained could be crystallized from ethyl acetate.

The water-soluble phosphate as well as the relatively insoluble sulfate and hydrochloride also have been prepared. The free bottromycin base could be obtained from its salts by suspending these salts in water–diethyl ether, adjusting the *p*H with dilute sodium hydroxide to 9 followed by repeated extraction of the alkaline water layer with ether. Evaporation of the dried ether extracts yielded the antibiotic as a glittering white amorphous powder The purified product, thus obtained, no longer produces a red color with concentrated mineral acids.

As to whether bottromycin is a chemically pure entity the following comments may be made. When highly purified bottromycin preparations derived from different production batches were compared, identical elementary analyses were always obtained. Also, the biological activities per mg. bottromycin, determined microbiologically, were identical and the value obtained could not be increased by further purification.

On the other hand some of these seemingly pure products gave a definite red coloration when treated with concentrated sulfuric acid whereas others did not. Such batches, after further purification, usually yielded products which no longer gave a positive test.

The molecular weight determined by the isothermic distillation method[3] was found to be about 743 ± 36. On the basis of analyses of bottromycin as well as of its crystalline salts, the antibiotic has been assigned the empirical formula $C_{38}H_{57-61}N_7O_{7-8}S$. The antibiotic is a very weak base (pK'_a = *ca.* 6.5); it is readily soluble in most organic solvents but is virtually insoluble in hexane, cyclohexane and petroleum ether. In ice-water it is more soluble (2.3 mg./ml.) than in water of about 30° (1.3 mg./ml.). The compound is optically active, $[\alpha]^{25}D$ −14.2° (*c* 0.5, in 96% ethanol).

Bottromycin is not adsorbed on alumina or cellulose but is completely adsorbed on activated carbon from which it can be recovered in part (*ca.* 65%) by elution with acetone containing 3% concentrated hydrochloric acid. "Magnesol" also adsorbs the antibiotic. In this case it can be eluted with benzene containing 5% of methyl alcohol.

Amorphous bottromycin or its crystalline salts are fully stable when stored for several months in the refrigerator. Aqueous solutions of the new antibiotic at *p*H 7 show no loss in microbiological activity after 8 days at 0°. However, a solution at *p*H 2 loses 50% of its activity when exposed for 1.5 hours at 100°. Alkaline solutions of *p*H 9 are completely inactivated when kept for the same

(1) Bottromycin is the generic name given to an antibiotic isolated from a culture of *Streptomyces bottropensis* n.sp.

(2) Dutch patent no. 79,749 (16-10-1955) B-mycin.

(3) E. P. Clark, *Ind. Eng. Chem., Anal. Ed.*, **13**, 820 (1941).

period of time at 100°. When dissolved in organic solvents bottromycin is relatively stable even at elevated temperatures.

The ultraviolet absorption spectrum of the free base in 96% ethanol solution shows a strong absorption peak at 203 mμ and a weak shoulder at 240 mμ.

The infrared spectrum (KBr) shows bands at: 3.01 μ (3322 cm.$^{-1}$), 3.23 (3086), 3.31 (3021), 5.73 (1745), 5.90 (1695), 6.05 (1653), 6.48 (1543), 6.68 (1497), 7.32 (1366), 7.65 (1307), 7.94 (1295), 8.53 (1172), 8.74 (1144), 8.93 (1120), 9.23 (1083), 9.45 (1058), 10.01 (999), 10.28 (973), 11.07 (903), 11.75 (851) and 13.17 μ (760 cm.$^{-1}$).

Further studies on the degradation and structure of bottromycin will be reported in two other papers.[4,5]

Experimental

Isolation of Crude Bottromycin.—The filtrate from 3000 l. of bottromycin fermentation broth (9000 γ/l.) was extracted in a Podbielniak extractor with 4,100 l. of butyl acetate. After azeotropic concentration of this extract to 16 l. (1.35 g./l.), the concentrate was washed three times with 5 l. of 5% sodium bicarbonate solution and two times with 5 l. of distilled water. The antibiotic was then re-extracted from the butyl acetate with 5 l. of phosphate buffer of *p*H 2. The aqueous extract was then washed with ether to remove dissolved butyl acetate. After evaporation of dissolved ether the crude bottromycin could be precipitated from the aqueous extract by adjusting the *p*H to 9, with 4 *N* sodium hydroxide. The amorphous white product was filtered and dried *in vacuo* (17.4 g.); yield 64.5%. Microbiological assays were carried out by a plate assay method using *B. subtilis* (A.T.C.C. 6633) as test organism.

Purification of Bottromycin. (a) By Florisil Chromatography.—Commercial Florisil was purified by suspending in 4 *N* hydrochloric acid. After 24 hours the adsorbent was filtered and washed with distilled water until free of acid and then air dried. A solution of crude bottromycin (144 g.) in chloroform (800 ml.) was added to a column previously packed with purified Florisil (1500 g.) in chloroform (column-size: 950/63 mm.). The column was developed by gravity with chloroform containing 7.5% of ethyl alcohol. Fractions of 1.5 l. (1), 1.8 l. (2), 200 ml. (3), 100 ml. (4), 100 ml. (5), 1 l. (6), 60 ml. (7), 660 ml. (8), and finally 3 l. (9) were collected. To each fraction was added an equal volume of 0.1 *N* sulfuric acid after which the organic solvent was removed by evaporation under reduced pressure. Bottromycin was precipitated as an amorphous white product from the various aqueous solutions by adjusting the *p*H to 9 with 4 *N* sodium hydroxide. Fractions 5 and 6 both yielded pure bottromycin (3.6 and 48.5 g.). On addition of concentrated mineral acids no red color appeared. Fractions 4, 7 and 8 and 9 on the other hand, yielded impure products, namely, 2.1, 4.7, 24 and 6.5 g. (red color with mineral acids). However, pure bottromycin could be isolated from these fractions by subjecting them to a second chromatographic procedure.

(b) By Means of its Salts with Organic Acids.—The crude antibiotic (26.8 g.) was dissolved in 1 l. of phosphate buffer *p*H 2. To this solution was added 3 l. of ether whereupon the *p*H was adjusted to 9 with 4 *N* sodium hydroxide. The alkaline aqueous layer was extracted repeatedly with ether. The combined ether extracts (6.7 l.) were dried with anhydrous sodium sulfate and after filtration were concentrated to a volume of 3 l.

Under vigorous stirring a solution of 25 g. of salicylic acid in 150 ml. of ether was added. The salicylate of bottromycin precipitated at once as an amorphous yellowish powder. After storing for three hours in the refrigerator the product was filtered and dried in the desiccator (21.35 g.). This product still gave a red color upon addition of concentrated mineral acids.

A suspension of the crude salicylate was stirred in cold ethyl acetate (100 ml.). After filtration, the washed product was recrystallized from ethyl acetate (150 ml.) to yield 15.05 g. of crystalline bottromycin salicylate; m.p. 161–162° dec. This product gave no red color upon addition of concentrated mineral acids.

Anal. Calcd. for $C_{38}H_{60}N_7O_7S\cdot C_7H_6O_3$: C, 60.27; H, 7.37; N, 10.94; S, 3.57. Found: C, 59.95; H, 7.30; N, 11.17; S, 3.49.

Recovery of Free Bottromycin Base.—The crystalline salicylate (10.05 g.) was dissolved in acetone (220 ml.). To this solution was added successively distilled water (650 ml.), ether (400 ml.) and 1 *N* sodium hydroxide (10 ml.). After shaking, followed by addition of solid sodium chloride in order to break emulsions, the ether layer was separated whereupon the alkaline water layer again was extracted with two 400-ml. portions of ether.

The combined ether extracts were dried over anhydrous sodium sulfate, filtered, concentrated under reduced pressure to a volume of 150 ml., then allowed to evaporate to dryness at room temperature. Glittering white amorphous bottromycin was obtained (8.20 g.); yield 96.5%. The substance melted at 143–147° dec.

Anal. Calcd. for $C_{38}H_{59}N_7O_8S$: C, 58.99; H, 7.63; N, 12.68; S, 4.14. Found: C, 58.94; H, 7.78; N, 12.53; S, 3.88.

Salts of Bottromycin. (1) The *p*-Aminosalicylate.—The *p*-aminosalicylate of bottromycin was prepared by the same method as described for the salicylate. It may be recrystallized from ethyl or butyl acetate. The crystalline salt had a m.p. of 175–180° dec.

Anal. Calcd. for $C_{38}H_{57}N_7O_7S\cdot C_7H_7NO_3$: C, 59.47; H, 7.05; N, 12.33; S, 3.52. Found: C, 59.77; H, 7.11; N, 12.25; S, 3.64.

(2) The Benzylpenicillinate.—Benzylpenicillin sodium salt (712 mg.) was dissolved in a few ml. of distilled water. Butyl acetate (10 ml.) was added followed by 2 *N* phosphoric acid (4 ml.). After shaking, the organic layer was separated, washed twice with water and dried over anhydrous sodium sulfate. After filtration, a solution of purified bottromycin (1.48 g.) in butyl acetate (4 ml.) was added.

The white precipitate formed was filtered off, washed with ether and dried. The crude amorphous penicillinate was dissolved in 50 ml. of cold ethyl acetate, filtered, then concentrated, at 10° under reduced pressure, to a small volume. The penicillinate crystallized after remaining in the refrigerator; 1.57 g. of the pure penicillinate was obtained, m.p. 157–159° dec.

Anal. Calcd. for $C_{38}H_{58}N_7O_8S\cdot C_{16}H_{18}N_2O_4S$: C, 58.59; H, 6.87; N, 11.39; S, 5.79. Found: C, 59.05; H, 6.95; N, 11.26; S, 5.72.

(3) The Sulfate.—Bottromycin (1.48 g.) was dissolved in butyl acetate (4 ml.). To this solution was added ether (40 ml.) and a few drops of concentrated sulfuric acid.

The sulfate, which precipitated at once, was filtered. The hygroscopic salt was dissolved in 20 ml. of alcohol, the solution was filtered and the salt was precipitated by the addition of ether. This purification procedure was repeated and yielded 1.02 g. of amorphous bottromycin sulfate, m.p. 189–196° dec. The product is readily soluble in water.

Anal. Calcd. for $C_{38}H_{60}N_7O_7S\cdot 2H_2O_4S\cdot 3H_2O$: C, 45.23; H, 6.95; N, 9.72; S, 9.53. Found: C, 45.22; H, 6.96; N, 9.60; S, 9.60.

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