177°. Three recrystallizations of this material from benzene–petroleum ether (b.p. 60–70°) yielded 3.5 g. (30%) of phenothiazine, melting and mixture melting points 184–186°.

N-Lithio-2-chlorophenothiazine.—To 11.7 g. (0.05 mole) of 2-chlorophenothiazine in 50 ml. of tetrahydrofuran was added slowly 0.054 mole of phenyllithium in 40 ml. of diethyl ether. The 40 ml. of diethyl ether was then distilled and an additional 50 ml. of tetrahydrofuran added to the reaction flask along with 1 g. (0.15 g. atom) of lithium. Stirring was then continued for 45 min. at 25°. Termination of the reaction was by carbonation. Basic extraction of the carbonation mixture failed to yield any acidic material. The neutral layer was evaporated and the residue recrystallized once from ethanol and twice from benzene–petroleum ether (b.p. 60–70°) to produce 1.3 g. (11%) of phenothiazine, identified as in the preceding experiment.

N-Ethyl-3-chlorophenothiazine.—A mixture of 13.1 g. (0.05 mole) of N-ethyl-3-chlorophenothiazine, 2 g. (0.3 g. atom) of lithium and 50 ml. of tetrahydrofuran was stirred for 1 hr. at 50°. Color Test I[2] was positive; therefore the reaction was terminated by carbonation. Extraction of the reaction mixture with dilute potassium hydroxide resulted in the isolation of 10 g. of acidic material which contained no thiophenolic compounds. This acidic material was a brown semi-solid which decomposed to a black oil when heated in ethanol or benzene. It was not purified. No neutral compounds were found when the solvent was evaporated from the neutral layer.

N-Ethylphenothiazine. Run I.—A mixture of 11.3 g. (0.05 mole) of N-ethylphenothiazine, 1 g. (0.15 g. atom) of lithium and 50 ml. of tetrahydrofuran was stirred at 25° for 45 min. Carbonation was accomplished in the usual manner. From the basic extract was isolated 9.5 g. of crude material melting 104–113°. Two recrystallizations of this material from petroleum ether (b.p. 60–70°)–benzene produced 7 g. (51%) of 2-carboxy-2′-mercaptoethyldiphenylamine, m.p. 114–116°. Its infrared spectrum has bands characteristic of the S–H and the carboxyl functions. The neutral layer contained 1 g. (9%) of starting material.

Anal. Calcd. for $C_{15}H_{15}O_2NS$: S, 11.76. Found: S, 11.73, 11.54.

Run II.—A mixture of 11.3 g. (0.05 mole) of N-ethylphenothiazine, 2 g. (0.3 g. atom) of lithium and 50 ml. of tetrahydrofuran was stirred at 50° for 40 min. At that time the reaction became exothermic and the color changed from brown to green. An ice-bath was used to keep the reaction temperature at 50°. Stirring was continued for an additional 10 min. before carbonation was effected. Only a trace of acidic material was found in the basic extract. The residue after evaporation of the solvent from the neutral layer was dissolved in benzene and chromatographed on alumina. Evaporation of the eluate followed by two recrystallizations of the residue from methanol–water produced 2.6 g. (27%) of pure N-ethylcarbazole identified by the method of mixture melting point and by its infrared spectrum.

N-Phenylphenothiazine.—A mixture of 6.3 g. (0.025 mole) of N-phenylphenothiazine, 0.5 g. (0.075 g. atom) of lithium and 60 ml. of tetrahydrofuran was stirred at 25° for 1 hr. and then carbonated in the usual manner. The basic extract contained 3.1 g. of material which was purified by vacuum sublimation and two recrystallizations from methanol–water to yield 1.5 g. (19%) of 2-carboxy-2′-mercaptotriphenylamine. The infrared spectrum contained the bands characteristic of S–H and carboxyl. The neutral layer contained a small amount of unidentified oil. Three other runs with reaction periods of up to 12 hr. at reflux failed to produce any identifiable material other than the aforementioned acid.

Anal. Calcd. for $C_{19}H_{15}O_2NS$: S, 9.96. Found: S, 9.89, 10.06.

N-Benzylphenothiazine.—A mixture of 7.2 g. (0.025 mole) of N-benzylphenothiazine, 0.5 g. (0.075 g. atom) of lithium and 50 ml. of tetrahydrofuran was stirred at 25° for 45 min. and then carbonated. An unresolvable mixture resulted and no compounds could be identified from either the neutral layer or the basic extract which contained 1.4 g. of acidic material.

A second run was refluxed for 8 hr. and then carbonated. This time there was obtained a neutral and a thiophenolic material, but neither could be purified or identified.

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

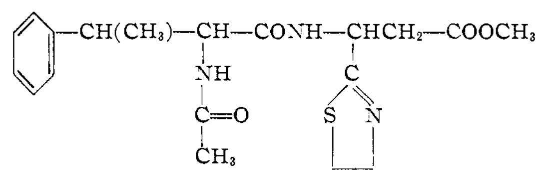
The Chemistry and Partial Structure of Bottromycin. IV.

BY J. M. WAISVISZ AND M. G. VAN DER HOEVEN

RECEIVED JULY 23, 1957

The antibiotic bottromycin ($C_{38}H_{57-61}N_7O_{7-8}S$) yields upon mild alkaline hydrolysis a crystalline compound ($C_{37}H_{57}N_7O_7S$) which is biologically inactive. Upon esterification of this compound with methanolic hydrochloric acid a biologically active compound is produced which is identical with the original bottromycin. Also other biologically active esters of above-mentioned crystalline compound could be prepared.

Recently, Waisvisz and co-workers in a series of publications[1–3] reported on the isolation, purification and partial structure-elucidation of the antibiotic bottromycin, produced by a new species *Streptomyces bottropensis*. Upon acetylation of bottromycin, *e.g.*, with acetic anhydride two crystalline decomposition products were obtained. One of these compounds was identified as the methyl ester of a N-acetyl dipeptide of the structure



These results suggested that it might be worthwhile to study whether the molecule of bottromy-

(1) J. M. Waisvisz, *et al.*, THIS JOURNAL, **79**, 4520 (1957).
(2) J. M. Waisvisz, *et al.*, *ibid.*, **79**, 4522 (1957).
(3) J. M. Waisvisz, *et al.*, *ibid.*, **79**, 4524 (1957).

cin also might contain this methyl ester grouping.

Indeed, Kunz hydrolysis[4] of bottromycin showed a consumption of one mole of sodium hydroxide for each mole of bottromycin decomposed.

From the reaction mixture we were able to isolate a biologically inactive white, amorphous compound (referred to as Kunz bottromycin) which, contrary to bottromycin, was very poorly soluble in most organic solvents such as acetone, diisobutyl ketone, ethyl acetate, benzene, dibutyl ether, chloroform, carbon tetrachloride, dichloroethane, cyclohexane, cyclohexanone and dioxane. The compound was found to be moderately soluble in several alcohols and was quite soluble in N,N-dimethylformamide and methylpyrrolidone.

Amorphous white Kunz bottromycin can be crystallized from acetone, methanol or ethanol which yields either an anhydrous crystalline product (m.p. 185–186°) or a crystalline monohydrate (m.p. 195–196°).

Anhydrous crystalline Kunz bottromycin has the empirical formula $C_{37}H_{57}N_7O_7S$. The infrared spectrum of Kunz bottromycin failed to show the absorption maximum of 5.73 μ (1745 cm.$^{-1}$), typical of a carbonyl grouping of an ester and therefore present in the infrared spectrum of bottromycin itself.

Strong hydrolysis of Kunz bottromycin (for instance, with concentrated hydrochloric acid) yields the same seven ninhydrin-positive compounds as were obtained by direct strong hydrolysis of bottromycin. This could be demonstrated by paper chromatographic technique.

It was possible to re-esterify the inactive Kunz bottromycin by means of a methanolic solution of hydrochloric acid. This yielded a biologically active, amorphous compound, which was according to the infrared spectrum and autobiograms identical with bottromycin.

A crystalline salt of "synthetic" bottromycin and salicylic acid could be prepared which was biologically active and which was in every respect identical with the salicylic acid salt of bottromycin. Both have the empirical formula $C_{45}H_{64}N_7O_{10}S$.

Esterification of Kunz bottromycin with other alcohols such as ethanol and *n*-butyl alcohol yielded compounds which also were biologically active, though the butyl ester was less active than the methyl ester (bottromycin). From these unnatural esters we were also able to prepare active crystalline salicylates.

Experimental

Kunz Hydrolysis of Bottromycin.—Four hundred and three mg. of bottromycin was dissolved in 25 ml. of acetone; 25 ml. of 0.1 *N* sodium hydroxide was added whereupon the mixture was kept in the refrigerator for 2.5 hours. Subsequent titration with hydrochloric acid showed that 23.55 ml. of hydrochloric acid (0.0854 *N*) was required for neutralization compared with 29.05 ml. of the same acid for the control test. Assuming a molecular weight of 759 for bottromycin one may calculate a consumption of 0.89 mg. equivalent sodium hydroxide for each mmole of bottromycin.

Isolation of Kunz Bottromycin. Method 1.—Four grams of bottromycin was dissolved in 250 ml. of acetone. Two hundred and fifty ml. of 0.1 *N* sodium hydroxide was added and the mixture was allowed to remain in the refrigerator for 2.5 hours. The *p*H then was adjusted carefully to 6.0 with 4 *N* hydrochloric acid. The clear solution was evaporated to dryness *in vacuo* and the residue was dried in a vacuum desiccator above phosphorus pentoxide.

6.41 g. of a cream-colored compound was obtained which was then subsequently extracted with chloroform for 16 hours in a Soxhlet apparatus. Chloroform extracts were dried over sodium sulfate, filtered, then evaporated to dryness. The slightly cream colored residue (4.11 g.) had a melting point of 173–176° and was biologically inactive (microbiological assays were carried out by a plate assay method using *B. subtilis* (A.T.C.C. 6633 as test organism).

Method 2.—Forty grams of bottromycin was dissolved in 2500 ml. of acetone; 2500 ml. of 0.1 *N* sodium hydroxide was added and the mixture was left for 2.5 hours in the refrigerator. The *p*H of the slightly opaque solution was adjusted to 6.2–6.3 with concentrated hydrochloric acid. The clear solution thus obtained was concentrated *in vacuo* to 2.5 l. A precipitate formed which was filtered off, washed and dried *in vacuo* over phosphorus pentoxide. Thirty-two and seven tenths grams of product was obtained and yields could be increased with another 3.6 g. by further concentration of the mother liquors.

Purification of Crude Kunz Bottromycin.—Three and eight tenths g. of crude Kunz bottromycin was dissolved in 750 ml. of boiling ethyl acetate. After filtration and cooling an amorphous precipitate formed that was collected, washed with ethyl acetate and dried; yield 2.25 g. (m.p. 183–185°).

After a second "crystallization" from 500 ml. of ethyl acetate 1.72 g. of amorphous purified Kunz bottromycin was obtained (m.p. 185.5–187°).

Anal. Calcd. for $C_{37}H_{57}N_7O_7S$: C, 59.76; H, 7.67; N, 13.19; S, 4.31. Found: C, 59.90; H, 7.62; N, 12.98; S, 4.29.

A second portion of 2.3 g. of crude Kunz bottromycin was crystallized from 350 ml. of acetone. Well formed, cubic crystals were obtained (1.46 g.) which after washing with acetone and ether had a melting point of 185–186°.

Anal. Calcd. for $C_{37}H_{57}N_7O_7S$: C, 59.76; H, 7.67; N, 13.19; S, 4.31. Found: C, 59.51; H, 7.58; N, 13.05; S, 4.07.

Upon slow evaporation of the acetone mother liquor a small amount (188 mg.) of a second crystalline product was obtained. These crystals were double refractive and had a melting point of 195–196°. They appeared to be the hydrate of Kunz bottromycin.

Anal. Calcd. for $C_{37}H_{57}N_7O_7S \cdot H_2O$: C, 58.35; H, 7.75; N, 12.88; S, 4.22. Found: C, 58.06; H, 7.78; N, 12.87; S, 3.85.

Hydrolysis of Kunz Bottromycin.—Three mg. of Kunz bottromycin was heated for 72 hours with 1 ml. of concentrated hydrochloric acid in a Carius tube at 110°. The hydrolyzate obtained was compared paper chromatographically with a similar hydrolyzate prepared from bottromycin. The descending method was applied using Whatman no. 1 filter paper and the system 1-butanol–acetic acid–water (100:12:100) as eluent.

It could be demonstrated that all seven ninhydrin-positive degradation products (compound I–VII) present in the bottromycin hydrolyzate[2] also were present in the Kunz bottromycin hydrolyzate.

Esterifications of Kunz Bottromycin. I. Preparation of the Methyl Ester.—Five g. of Kunz bottromycin was dissolved in 1500 ml. of 0.1 *N* non-aqueous methanol solution of hydrochloric acid. After 41 hours at room temperature this solution was evaporated *in vacuo*. The residue obtained was dissolved in non-aqueous methanol and again evaporated to dryness. Finally, it was dissolved in a minimum amount of methanol and diluted with 250 ml. of water. After further addition of 4 ml. of 30% sodium hydroxide solution and 200 ml. of ether the mixture was thoroughly shaken in a separatory funnel and the resulting aqueous layer re-extracted four times with a total amount of 800 ml. of ether. The combined ether extracts were dried over sodium sulfate, then evaporated to dryness; obtained, 2.57 g. of a white amorphous compound with a biological activity of 70% of that of bottromycin.

The infrared spectrum showed again clearly an absorption at 5.72 μ (1745 cm.$^{-1}$) typical for the carbonyl group of an ester and also present in bottromycin, but absent in Kunz bottromycin.

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

The "synthetic" bottromycin thus obtained was compared by means of paper chromatography with authentic bottromycin. The following eluent systems were used: water saturated 1-butanol–*p*-toluenesulfonic acid–piperidine (300:6:2.4); *t*-butyl alcohol–formic acid–water (70:15:15), and 1-butanol–acetic acid–water (1:2:4). All three autobiograms showed only a single spot of biologically active compound on the agar test plates. The R_f values of corresponding spots, as obtained with the authentic and "synthetic" bottromycin, were also identical.

Preparation of the Salicylic Acid Salt of "Synthetic" Bottromycin (Methyl Ester of Kunz Bottromycin).—One g. of "synthetic" bottromycin was dissolved in 50 ml. of ether. After filtration 200 mg. of salicylic acid, dissolved in 10 ml. of ether, was added. A spontaneous precipitate of the amorphous salt was formed which, after storage in the refrigerator and isolation, yielded 1.02 g. of a white product. It could be obtained in crystalline form by crystallization from 20 ml. of ethyl acetate; yield 734 mg., decomposition point 158–160°. Biological activity of this salicylate was identical with that of the salicylate of authentic bottromycin.

Anal. Calcd. for $C_{38}H_{58}N_7O_7S \cdot C_7H_6O_3$: C, 60.40; H, 7.16; N, 10.96; S, 3.58. Found: C, 60.26; H, 7.17; N, 11.28; S, 3.66.

II. Preparation of the Ethyl Ester and its Salicylic Acid Salt.—Two and a half g. of Kunz bottromycin was dissolved in 750 ml. of 0.1 *N* ethanolic hydrochloric acid solution (water-free) and kept for 72 hours at room temperature. The ethyl ester was isolated by the same technique as described above for the methyl ester. One and twenty-five hundredths g. of a white amorphous compound was obtained with a biological activity of the same order as that of bottromycin. Its infrared spectrum also showed again absorption at 5.72 μ (1745 cm.$^{-1}$). One g. of this ethyl ester dissolved in 50 ml. of ether was treated with 200 mg. of salicylic acid dissolved in 10 ml. of ether. One and four hundredths g. of salicylic acid salt was obtained and after crystallization from 20 ml. of ethyl acetate 742 mg. of crystalline salt was obtained with a decomposition point of 152–156°. Biological activity of the salicylic acid salt of the ethyl ester of Kunz bottromycin also appeared to be of the same order as obtained for bottromycin itself.

Anal. Calcd. for $C_{39}H_{61}N_7O_7S \cdot C_7H_6O_3$: C, 60.73; H, 7.37; N, 10.78; S, 3.52. Found: C, 60.35; H, 7.32; N, 11.08; S, 3.84.

III. Preparation of the *n*-Butyl Ester and its Salicylic Acid Salt.—By the same procedure as described for the methyl- and ethyl ester 3.02 g. of amorphous white butyl ester was prepared from 3.3 g. of Kunz bottromycin. However, its biological activity was only 30% of that of bottromycin.

In the same way a salicylate of this ester was prepared. One g. of butyl ester was dissolved in 60 ml. of ether, 200 mg. of salicylic acid dissolved in 10 ml. ether was added. Nine hundred and seventy-five mg. of amorphous salicylate was obtained which after crystallization from 20 ml. of ethyl acetate yielded 810 mg. of an acicular crystalline compound. Its decomposition range was 150–160°. Microbiological activity of this compound was only 45% of the activity of bottromycin.

Paper chromatographic studies on the *n*-butyl ester of Kunz bottromycin proved that this compound was not identical with bottromycin since two distinct spots were obtained on autobiograms when both were mixed. The following elution system was used: 1-butanol–acetic acid–water (1:2:4) in combination with Whatman paper no. 20.

Anal. Calcd. for $C_{41}H_{63}N_7O_7S \cdot C_7H_6O_3$: C, 61.60; H, 7.38; N, 10.48; S, 3.43. Found: C, 61.43; H, 7.33; N, 10.92; S, 3.52.

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[CONTRIBUTION FROM THE DEPARTMENTS OF MICROBIOLOGY, BIOCHEMISTRY AND PEDIATRICS, SCHOOL OF MEDICINE, UNIVERSITY OF PENNSYLVANIA, AND THE SLOAN KETTERING INSTITUTE FOR CANCER RESEARCH]

A New Antioxidant from Yeast. Isolation and Chemical Studies[1]

BY MARTIN FORBES, FRIEDRICH ZILLIKEN, GLYN ROBERTS AND PAUL GYÖRGY

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A new antioxidant of the empirical formula $C_{16}H_{12}O_6$ has been isolated in crystalline form from different types of yeast. The method of isolation of the substance, its biological activity and its physical and chemical properties as well as those of a crystalline dimethoxy derivative ($C_{17}H_{14}O_6$) and a crystalline degradation product are described.

Introduction

Dietary supplements of ethanolic extracts of British baker's yeast, of Fleischmann's baker's yeast, as well as the ether extracts of the ethanol soluble fractions of these, were found to prevent the occurrence of hemorrhagic liver necrosis in rats, kept on a diet which regularly induces this fatal liver injury.[2] Like vitamin E, these extracts prevented also the characteristic susceptibility[3] to hemolysis by dialuric acid of the red blood cells of vitamin E deficient rats. Paper chromatographic analysis of these ether extracts of yeast, however, showed no spots corresponding to α-, β-, γ- or δ-tocopherol but indicated the presence of a substance giving a positive reaction in the Emmerie–Engle test and exhibiting a strong blue fluorescence when exposed to long wave ultraviolet light. An eluate of the section (R_f = 0.84) of the paper chromatogram incorporating the blue fluorescent material, in contrast to all other sections of the chromatogram, when admixed *in vitro* to red blood cells of vitamin E depleted rats, was found to prevent their hemolysis by dialuric acid.[2] This communication deals with the isolation and crystallization of the blue fluorescent compound from the ether soluble fraction of yeast and its chemical and physical properties.

Results and Discussion

Isolation.—A typical experiment showing the isolation of the hemolysis preventing principle is presented in Tables I and II. The methanol step does not substantially enrich the activity but is

(1) Supported in part by the Subcommittee on Liver of the Advisory Committee on Metabolism, through Army Contract No. DA-49-007-MD-78, Office of the Surgeon General of the United States Army, and by the American Cancer Society Research Grant (C-2367) from the National Cancer Institute of National Institutes of Health, United States Public Health Service.

(2) M. Forbes and P. György, *J. Nutr.*, in press (1957).

(3) C. S. Rose and P. György, *Blood*, **5**, 1062 (1950).