[CONTRIBUTION FROM THE BAKER LABORATORY OF CHEMISTRY AT CORNELL UNIVERSITY]

Gliotoxin, The Antibiotic Principle of *Gliocladium fimbriatum*. III. The Structure of Gliotoxin: Degradation by Hydriodic Acid[1]

BY JAMES D. DUTCHER,[2] JOHN R. JOHNSON AND WILLIAM F. BRUCE

The isolation and general physical properties of gliotoxin, as well as a preliminary study of the nature of the functional groups have been previously described.[1,3] In an effort to elucidate the chemical structure of gliotoxin, it was early observed that the sulfur atoms possess marked lability; this lability made the degradation of gliotoxin to a discrete product very difficult. A reaction was therefore sought which would eliminate the sulfur atoms and leave a more tractable compound with which to work. The reactions which were known to remove sulfur were accordingly examined more closely in order to determine the nature of the organic residue. While the majority of these procedures gave organic products which could not be crystallized, they gave some indication of the nature of the combination of sulfur in gliotoxin and will therefore be described in a later paper. By reduction with hydriodic acid and red phosphorus in glacial acetic acid, a crystalline, neutral, optically inactive organic product (I) was secured. By analysis its empirical formula was found to be $C_{13}H_{12}N_2O_2$. This substance was therefore derived from gliotoxin simply by the apparent loss of two molecules of water and two of hydrogen sulfide, as equation 1 shows

(1) $C_{13}H_{14}N_2O_4S_2 + 6H$ (HI and P) $\longrightarrow$ $C_{13}H_{12}N_2O_2 + 2H_2S + 2H_2O$

It may be seen from this formulation that both of the sulfur atoms and two of the four oxygen atoms in gliotoxin were eliminated without the loss of any carbon or nitrogen.

The determination of the chemical nature of this reduction product was achieved in the following manner: Treatment with 0.5 *N methanolic* potassium hydroxide at room temperature resulted in reaction with one mole of alkali to yield the salt of a 13-carbon acid of formula $C_{13}H_{14}N_2O_3$, which was characterized by the preparation of the ethyl ester. The equation for this hydrolysis may be written

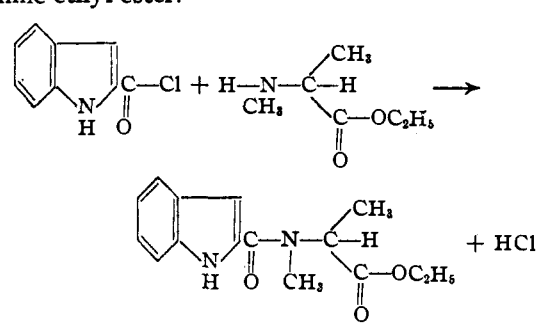
(2) $C_{13}H_{12}N_2O_2 + H_2O \xrightarrow[\text{KOH}]{0.5\ N\ \text{methanolic}} C_{12}H_{13}N_2OCO_2H$

The formation from the original neutral product of an acid with the same number of carbon atoms is indicative of the presence of a lactam or lactone linkage. Further cleavage of this acid was accomplished by refluxing with 20% *aqueous* potassium hydroxide solution. One mole of alkali was consumed beyond that required for neutralization and from this hydrolysis mixture was isolated in good yield an acid of the composition $C_9H_7NO_2$. The equation for this hydrolysis may be written

(3) $C_{13}H_{14}N_2O_3 + H_2O \xrightarrow[\text{KOH}]{20\%\ \text{aqueous}} C_8H_6NCO_2H + C_4H_9NO_2$

This acid was identified as indole-2-carboxylic acid by comparison with an authentic specimen of the free acid. The methyl and ethyl esters of indole-2-carboxylic acid also proved identical with the corresponding derivatives of the hydrolysis product. The isolation of this acid confirmed the presence in gliotoxin of an indole nucleus substituted in the 2-(or α) position.[1]

An examination of the equation 3 shows that a 4-carbon fragment and a nitrogen atom were split off. It was known that one of the nitrogen atoms of gliotoxin bore a methyl group[1] (Herzig–Meyer determination of N-methyl groups) and since indole-2-carboxylic acid which was isolated has an unsubstituted nitrogen atom it could be assumed as a working hypothesis that the four carbon fragment represented an α- or β-methyl-aminopropionic acid residue. Because of the small amount of material available it was not possible to isolate this product from the hydrolysis mixture but verification of the hypothesis was obtained by the synthesis of the ethyl ester of the 13-carbon acid previously mentioned (equation 2) from indole-2-carboxylic acid chloride and *dl*-α-N-methylalanine ethyl ester.

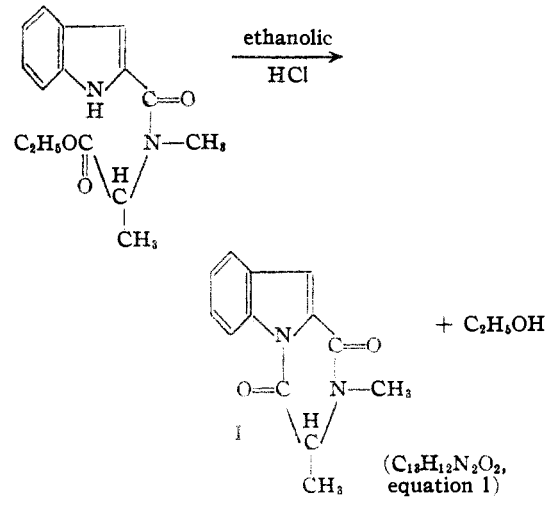
(4) 

Furthermore, it was observed that under suitable conditions this ester of the 13-carbon acid could be cyclized by the elimination of a mole of ethyl alcohol to yield a product identical with I, the neutral product obtained by the hydriodic acid reduction of gliotoxin. In view of both the ready hydrolysis and ease of cyclization of this product there seems little doubt that this cyclization takes place through the imide hydrogen of the indole nitrogen atom.

(1) Second paper, THIS JOURNAL, 66, 614 (1944).

(2) Du Pont post-doctorate fellow; present address, Squibb Institute for Medical Research, New Brunswick, N. J.

(3) Johnson, Bruce and Dutcher, THIS JOURNAL, 65, 2005 (1943).

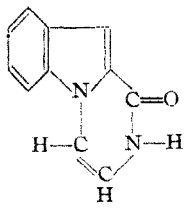
(5) 

($C_{13}H_{12}N_2O_2$, equation 1)

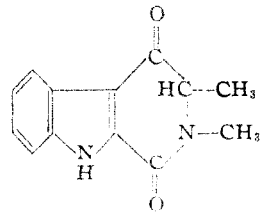
A compound with a similar ring system has been prepared by Kermack, Perkin and Robinson,[4] who found that indole-2-carboxyacetalyl amide cyclized readily to give a compound of formula II.

According to the Ring Index such a system is called pyrazino-[1.2-a]-indole (No. 1630). Employing the preferred numbering of the Ring Index, the compound obtained from gliotoxin is named 2,3-dimethyl-1,4-diketotetrahydropyrazino-[1.2-a]-indole (equation 5). In a later report dealing with the synthesis of various homologs of this neutral degradation product it will be shown more conclusively that the alternative formulation involving cyclization through the 3-(or β)-carbon atom of the indole nucleus to form a carboline derivative corresponding to formula III does not represent the structure of the product (I) derived from gliotoxin.

The elucidation by this means of the complete carbon and nitrogen skeleton of gliotoxin leaves undetermined only the position and function of two oxygen and two sulfur atoms. These matters will be the subject of subsequent reports.



II



III

We wish to thank the Cornell Research Foundation and E. I. du Pont de Nemours and Co. for generous support of this work.

Experimental

Hydriodic Acid Reduction of Gliotoxin.—One gram of gliotoxin was refluxed in 25 ml. of glacial acetic acid with 1.0 g. of red phosphorus and 1.0 ml. of hydriodic acid, sp. gr. 1.7, until no more hydrogen sulfide was evolved. This required approximately four hours. The hydrogen sulfide which was formed was trapped in alkaline plumbite solution. An amount of lead sulfide equivalent to 96% of the sulfur in the gliotoxin was obtained. The warm acetic acid solution was filtered from excess phosphorus and the filtrate and washings poured into 200 ml. of cold water containing 2.0 g. of sodium bisulfite. The solution was thoroughly extracted with ether and the ether washed free of acetic acid with dilute sodium carbonate solution. After drying over anhydrous sodium sulfate the ether solution was evaporated in vacuum and yielded a pale yellow sirup which weighed 380 mg. After addition of a few drops of ethanol and storage in the ice-chest for several days the sirup crystallized. The crystalline material was washed free of pigmented mother liquor and then recrystallized from ethanol. It was obtained as large, dense, rhombic crystals which melted at 122°.[5] It was optically inactive. This compound was slightly soluble in hot water but insoluble in cold water or dilute acid or alkali solution. It was moderately soluble in most organic solvents. It was readily oxidized by potassium permanganate solution, gave a negative Ehrlich reaction (an alcoholic solution treated with *p*-dimethylaminobenzaldehyde and concentrated hydrochloric acid) and coupled immediately in 1% sodium carbonate solution with diazotized sulfanilic acid to yield a deep orange-red solution.

Anal. Calcd. for $C_{13}H_{12}N_2O_2$: C, 68.40; H, 5.26; N, 12.30. Found: C, 68.25; H, 5.24; N, 12.69.

Hydrolysis of Hydriodic Acid Reduction Product.—A methanol solution of 480 mg. of the compound m. p. 122° was treated with 5.0 ml. of 0.5 *N* methanolic potassium hydroxide at room temperature for four hours. Titration with standard acid showed the consumption of 4.2 ml. of the 0.5 *N* alkali, corresponding to a saponification equivalent of 228. With the assumption that one mole of alkali per mole of compound was consumed, the formula of the substance was $C_{13}H_{12}N_2O_2$, molecular weight 228. The methanolic solution was strongly acidified and concentrated in vacuu to a small volume from which a crystalline precipitate separated. This precipitate, which weighed 320 mg., was recrystallized from acetic acid and was obtained as rosets of colorless needles, m. p. 187°. This compound was shown to be an acid by titration with standard alkali using phenolphthalein as indicator. Neutral equivalents of 247, 250 and 245 were obtained. The formula $C_{12}H_{13}N_2O\cdot CO_2H$ requires a molecular weight of 246.

Anal. Calcd. for $C_{13}H_{14}N_2O_3$: C, 63.40; H, 5.70; N, 11.40. Found: C, 63.23; H, 5.98; N, 11.32, 11.63.

The *ethyl ester* was prepared by refluxing the acid in absolute alcohol with a trace of sulfuric acid. Addition of water to the hot solution caused the precipitation, on cooling, of long shining needles. The ester was recrystallized from aqueous alcohol and from benzene–petroleum ether mixture. It melted at 127°.

Anal. Calcd. for $C_{15}H_{18}N_2O_3$: C, 65.70; H, 6.57; N, 10.22. Found: C, 66.10; H, 6.85; N, 10.51.

The yield of ester was good and there was no evidence of any cyclization under these conditions to yield the neutral product $C_{13}H_{12}O_2N_2$, m. p. 122°. This product was obtained from the same ester, as shown below, under other reaction conditions.

Further Hydrolysis of the Acid $C_{13}H_{14}N_2O_3$.—Two hundred and thirty-three mg. of this acid was dissolved in 5.0 ml. of 20% aqueous potassium hydroxide and refluxed for four hours. A stream of nitrogen was passed through the flask during refluxing and then passed into standard acid solution. It was found that *no* volatile amine was produced during hydrolysis. The cooled solution was made up to 50 ml. in a volumetric flask and 5.0-ml. aliquots titrated. A blank on the alkali was run in the same manner. By subtracting one equivalent of alkali for the neutralization of the original carboxyl group, a saponification equivalent of 233 was obtained, in good agreement with the molecular weight of the acid, 246. After acidification of the entire solution, the material was concentrated in

(4) Kermack, Perkin and Robinson, *J. Chem. Soc.*, **119**, 1602 (1921).

(5) All melting points are uncorrected.

vacuum. At a small volume a colorless product crystallized out. This product was filtered off, washed with water and recrystallized from acetic acid. The melting point was 204° with sublimation and decomposition; 150 mg. was obtained.

Anal. Calcd. for $C_9H_7NO_2$: N, 8.70; neut. equiv., 161. Found: N, 8.67; neut. equiv., 160, 165.

The **methyl ester** was prepared by refluxing for one hour with methanol and a trace of sulfuric acid. It crystallized from aqueous acetone as platelets; m. p. 151°.

Anal. Calcd. for $C_{10}H_9NO_2$: N, 8.00. Found: N, 8.02.

The **ethyl ester** was prepared in a similar way with ethanol and sulfuric acid. Needles were obtained on recrystallization from aqueous ethanol or benzene–petroleum ether; m. p. 121°.

Anal. Calcd. for $C_{11}H_{11}NO_2$: N, 7.40. Found: N, 7.36.

These compounds were proved to be identical with indole-2-carboxylic acid, m. p. 204°, indole-2-carboxylic acid methyl ester, m. p. 151°, and indole-2-carboxylic acid ethyl ester, m. p. 122°, by mixed melting point determinations with synthetic samples.

Synthesis of the Hydriodic Acid Reduction Product of Gliotoxin.—After the isolation of indole-2-carboxylic acid from the strong aqueous alkaline hydrolysis of the 13-carbon acid it was found impossible to obtain any further crystalline material. In view of the possibility that the remainder of the molecule might be either α- or β-N-methylalanine the following synthesis was carried out. Indole-2-carboxylic acid was synthesized by the method of Reissert[6] and converted to the acid chloride by the action of thionyl chloride. To 25 ml. of dry ether containing 1.0 g. of thionyl chloride, 175 mg. of finely powdered indole-2-carboxylic acid was slowly added. Solution occurred readily and after all the acid had been added the mixture was allowed to stand at room temperature for forty minutes before the ether and excess thionyl chloride were removed in vacuum. The slightly pigmented semi-crystalline residue was redissolved in dry ether and used without further purification since it decomposed readily. Since the 13-carbon acid obtained from gliotoxin possessed no optical activity, inactive α-N-methyl alanine ethyl ester, prepared by the method of Zelinsky, *et al.*,[7] was used. An ethereal solution of 175 mg. of this ester was added to the solution of indole-2-carboxylic acid chloride. The mixture warmed slightly and became cloudy. It was allowed to stand at room temperature for one hour after which the ether and excess amino acid ester were removed in vacuum. The crystalline residue was taken up in absolute alcohol and recrystallized by the addition of water to the warm solution. Colorless needles melting at 126° were obtained. The yield was 148 mg. and represented 55% of the theoretical yield on the basis of the indole-2-carboxylic acid used.

This synthetic ester agreed exactly in all properties with the ester of the 13-carbon acid and showed no depression in a mixed melting point determination. On saponification it yielded the identical 13-carbon acid; m. p. 187°.

It was observed in a second preparation of this ester that if the alcoholic solution of the initial condensation product of the indole-2-carboxylic acid chloride and *dl*-α-N-methyl alanine ester was allowed to stand for thirty-five hours, instead of obtaining the open chain ester of the 13-carbon acid, a product melting at 122° and identical with the hydriodic acid reduction product of gliotoxin was obtained. The presence of a small amount of hydrochloric acid was apparently responsible for this cyclization since the addition of solid sodium carbonate to the ethereal solution, after condensation, to remove all traces of hydrochloric acid prevented the cyclization. The neutral, cyclized product was then obtained from the ester by allowing an alcoholic solution containing 1% hydrochloric acid to stand for forty-eight hours. From 750 mg. of indole-2-carboxylic acid, 525 mg. of the cyclized product was obtained; this represents 50% of the theoretical yield.

(6) A. Reissert, *Ber.*, **30**, 1036 (1897).

(7) Zelinsky, Annenkoff and Kulikoff, *Z. physiol. Chem.*, **73**, 468 (1911).

Summary

Treatment of gliotoxin with phosphorus and hydriodic acid in acetic acid gave a crystalline product in which analysis showed that no sulfur and two less oxygen atoms were present; but the same number of carbons as in gliotoxin were found. This product was degraded stepwise to N-(indole-2-carboxoyl)-N-methyl-α-alanine and to indole-2-carboxylic acid. The crystalline reduction product, 2,3-dimethyl-1,4-diketotetrahydropyrazino-[1,2-a]-indole (I) and the several degradation products have been identified by synthesis.

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Gliotoxin, the Antibiotic Principle of *Gliocladium fimbriatum*. IV. The Structure of Gliotoxin: The Action of Selenium

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In seeking to establish the structure of gliotoxin, the antibiotic substance produced by the fungus *Gliocladium fimbriatum*,[2] a distillation with selenium was carried out. A curious but informative reaction occurred which yielded a product that shed considerable light upon the structure of the gliotoxin molecule and served to verify the observations previously reported for the hydriodic acid reduction of gliotoxin.[3]

When an intimate mixture of selenium and gliotoxin was heated at 230 to 250° a crystalline sublimate was obtained which analysis showed to have the formula $C_{12}H_8N_2O_3$. Sulfur, hydrogen sulfide and water were the only other products of the reaction which were observed, but under the conditions of the reaction carbon dioxide formaldehyde or methane might have been overlooked. Expressed in the form of an equation the reaction was

$$C_{13}H_{14}N_2O_4S_2 \xrightarrow[250^\circ]{Se} C_{12}H_8N_2O_3\ (II) + 2\,H_2S + [C] + H_2O$$

(1) Du Pont post-doctorate fellow; present address, Squibb Institute for Medical Research, New Brunswick, N. J.

(2) Johnson, Bruce and Dutcher, THIS JOURNAL, **65**, 2005 (1943).

(3) Dutcher, Johnson and Bruce, *ibid.*, **66**, 617 (1944).