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

Gliotoxin. XI. A Related Antibiotic from *Penicillium terlikowski*: Gliotoxin Monoacetate[1]

BY JOHN R. JOHNSON, AKLAQ R. KIDWAI AND JOHN S. WARNER

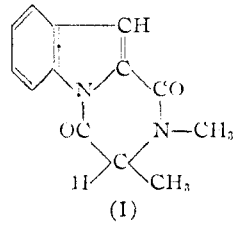
RECEIVED AUGUST 5, 1952

A crystalline antibiotic substance of the molecular formula $C_{15}H_{16}N_2O_5S_2$ has been obtained, together with gliotoxin, from cultures of *Penicillium terlikowski* Zaleski. The new substance has been shown to be a monoacetate of gliotoxin and has been converted by methanolysis to gliotoxin and methyl acetate. Efforts to obtain a crystalline acetate by acetylation of gliotoxin have been unsuccessful.

Several years ago Mull, Townley and Scholz[2] reported the isolation of gliotoxin and a related new antibiotic substance, which they designated as "Isolate 2," from the culture medium of a mold considered to be *Penicillum obscurum* Biourge. More recently Dr. Kenneth B. Raper of the Northern Regional Research Laboratory, who had made the identification of the original culture in 1945, has re-examined the organism and now designates it as *Pencillium terlikowski* Zaleski.[3] Brian[4] also has reported the isolation of gliotoxin from cultures of *Penicillium terlikowski* but did not observe the new antibiotic in his culture medium.

Isolate 2 formed large, pale yellow, rhombic crystals, m.p. 159–160°, and was optically active, $[\alpha]^{19}D$ −197° (*c* 0.600 in chloroform). The crystals are quite different from those of gliotoxin and can be distinguished from the latter quite readily under the microscope. The ultraviolet absorption spectrum of Isolate 2 is almost identical with that of gliotoxin—both have a characteristic maximum at 268 mμ. Mull, Townley and Scholz observed that Isolate 2, on hydrolysis with barium hydroxide and on reduction with hydriodic acid, gave products identical with those obtained from gliotoxin itself.[5] These experimental observations indicate clearly that the fundamental carbon–nitrogen skeleton of Isolate 2 is the same as that of gliotoxin and suggest that the sulfur and oxygen functions are similarly disposed in both compounds.

The analytical data of Mull, Townley and Scholz and their determinations of molecular weight (by the Rast method) led to the formula $C_7H_8NO_2S$ but that must be doubled to account for the formation of the hydriodic acid reduction product (I), which has thirteen carbons and two nitrogen atoms. The doubled formula, $C_{14}H_{16}N_2O_4S_2$, differs from that of gliotoxin, $C_{13}H_{14}N_2O_4S_2$, by a CH_2 increment. This methylene group cannot represent methylation of an hydroxyl group since methoxyl determination on Isolate 2 demonstrated the absence of such a group. Another possibility would involve conversion of the disulfide functions of gliotoxin to a methylene derivative of the type R–S–CH_2–S–R, analogous to the transformation of cystine into djenkolic acid.[6] Attempts in our laboratory to convert gliotoxin to a methylene derivative were unsuccessful.



(I)

To obtain a quantity of Isolate 2 for further chemical study we grew cultures of *Penicillium terlikowski* Zaleski on Weindling medium, at first on a small scale in the laboratory. In a typical preparation ten liters of the culture medium, eight days after inoculation with the mold spores, furnished by benzene extraction 800–1100 mg. of a mixture of gliotoxin and Isolate 2. The purified Isolate 2 formed pale yellow rhombic crystals, m.p. 162–163°. This material had the same crystallographic properties as the original Isolate 2, m.p. 159–160°, described by Mull, Townley and Scholz. Although the analytical data for the two samples are not in close agreement (see Table I), we believe that both materials are essentially the same substance.[7]

TABLE I

ELEMENTARY ANALYSIS OF ISOLATE 2 FROM *P. terlikowski* ZALESKI

	C	H	N	S
Mull, Townley, Scholz	49.43	4.68	8.14	18.84
Cornell sample	49.05	4.33	7.51	17.65
	49.00	4.35	7.37	17.62
Calcd. for:				
$C_{15}H_{16}N_2O_5S_2$	48.90	4.38	7.61	17.40
$C_{14}H_{16}N_2O_4S_2$	49.41	4.71	8.24	18.82
$C_{13}H_{14}N_2O_4S_2$ (gliotoxin)	47.85	4.32	8.59	19.65

Our analytical data clearly indicate a formula

(1) Preceding paper, THIS JOURNAL, **75**, 2103 (1953).

(2) R. P. Mull, R. W. Townley and C. R. Scholz, *ibid.*, **67**, 1626 (1945).

(3) We are grateful to Dr. C. R. Scholz of the Research Department of Ciba Pharmaceutical Products, Inc., for his friendly coöperation in making available to us unpublished notes of chemical studies of Isolate 2, and to Dr. Philip C. Eisman for supplying us with an active culture of *Penicillium terlikowski* Zaleski (NRRL A-573).

(4) P. W. Brian, *Trans. Brit. Mycol. Soc.*, **29**, 173, 211 (1946).

(5) J. D. Dutcher, J. R. Johnson and W. F. Bruce, THIS JOURNAL, **66**, 617 (1944).

(6) M. D. Armstrong and V. du Vigneaud, *J. Biol. Chem.*, **168**, 373 (1947).

(7) The appreciable deviation of the published analytical values of Mull, Townley and Scholz from those required for the C_{15} formula may have arisen from elimination of the elements of acetic acid during the seven crystallizations to which the material was subjected. Dr. C. R. Scholz has informed us that their sample of Isolate 2 which had been crystallized only once, following the chromatographic purification on alumina, had a composition in good agreement with our results and with the values for the C_{15} formula: C, 48.82; H, 4.52; N, 7.60; S, 17.48. The observation that the Rast molecular weight values are only half of the value required for the C_{15} formula would be consistent with the view that Isolate 2 undergoes elimination of the elements of acetic acid under these conditions.

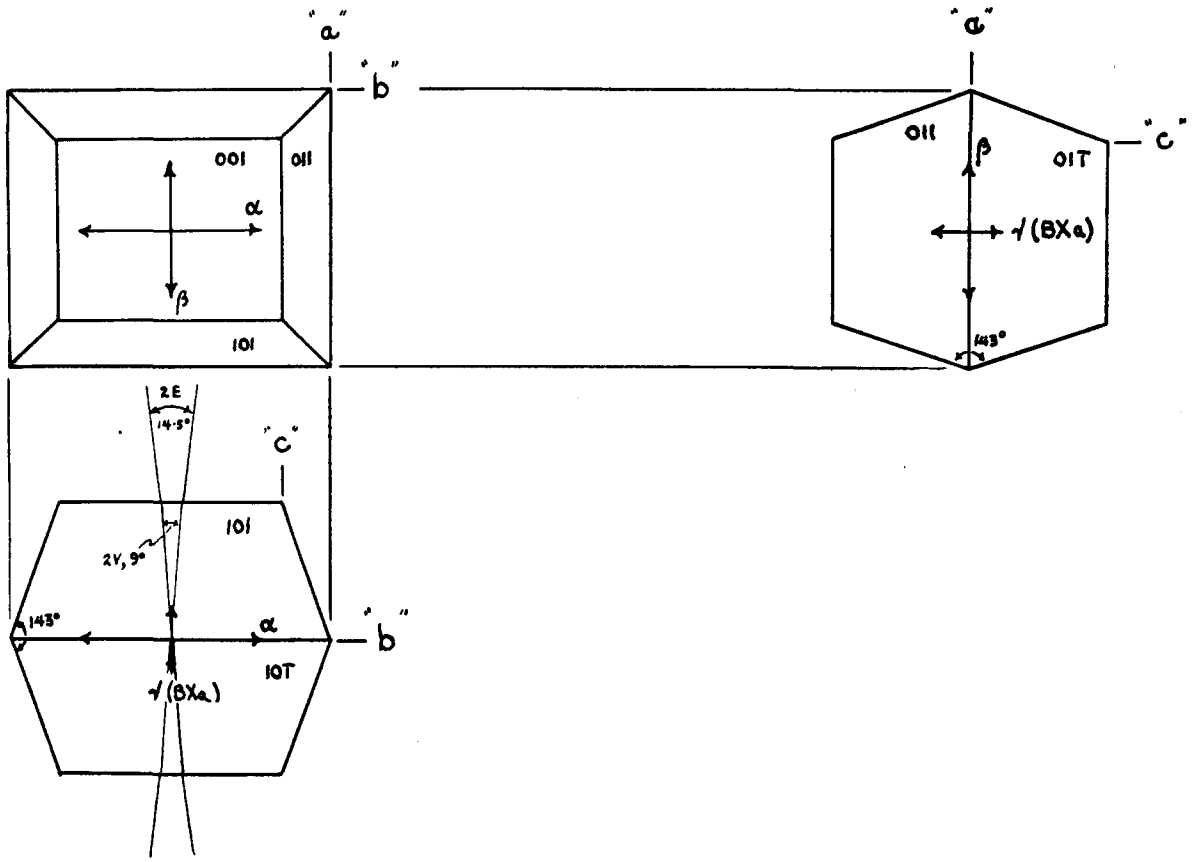


Fig. 1.—Crystalline form of gliotoxin monoacetate.

$C_{15}H_{16}N_2O_5S_2$ for Isolate 2; the difference between it and gliotoxin now becomes C_2H_2O, which is an addition of CH_2 *and* CO such as would correspond to the acetylation of an hydroxyl group: –O–H to –O–CO–CH_3.

Zerewitinow determination of active hydrogen showed that Isolate 2 (in anisole, 95°) contains two active hydrogen atoms per mole, as against three for gliotoxin. Carbon-methyl determination gave 0.81 mole of acetic acid per mole of Isolate 2, while gliotoxin gave a negligible amount (0.13–0.20 mole). An acetyl determination[8] gave a value corresponding to one acetyl group per mole of Isolate 2.

The view that Isolate 2 is an acetyl derivative of gliotoxin was established unequivocally by means of an ester exchange reaction between Isolate 2 and methanol, in the presence of an acid catalyst. From the reaction mixture there were isolated gliotoxin and methyl acetate. The gliotoxin was identified by comparison with an authentic specimen, and the methyl acetate by saponification and conversion of the acetate to the solid S-benzylthiouronium salt. There is no experimental evidence indicating which of the hydroxyl groups of gliotoxin has been acetylated in the formation of Isolate 2.

Attempts to convert gliotoxin into Isolate 2 have been entirely unsuccessful. Acetylating agents such as acetyl chloride and pyridine, acetic anhydride, isopropenyl acetate and ketene, failed to furnish a crystalline mono- or diacetate of gliotoxin. Quantitative experiments reported in an earlier paper[9] indicate that gliotoxin reacts with one mole of acetyl chloride or acetic anhydride in the presence of pyridine but no crystalline acetyl derivative could be isolated. Under similar conditions benzoyl chloride gives a crystalline dibenzoate.

It was hoped that the monoacetate might be converted by further acetylation into a crystalline diacetate but none of the usual methods of acetylation led to the desired product.

Experimental

Isolation of Antibiotics from Cultures of *Penicillium terlikowski* Zaleski.—The mold was grown on Weindling culture medium, which had the following composition: 250 g. of glucose, 20 g. of ammonium tartrate, 1 g. of potassium dihydrogen phosphate, 5 g. of anhydrous magnesium sulfate, 10 ml. of "minor elements concentrate," 1 g. of yeast extract (dried powder) and 10 l. of distilled water. The "minor elements concentrate" consisted of: 100 mg. of ferrous sulfate hydrate, 15 mg. of copper sulfate hydrate, 100 mg. of zinc sulfate hydrate, 10 mg. of manganese sulfate hydrate, 10 mg. of potassium molybdate hydrate, in 100 ml. of distilled water. We wish to express our thanks to Professor Georges Knaysi of the Bacteriology Department, Cornell University, for his very helpful advice and for assistance in growing the mold cultures under favorable conditions.

The organism was grown by introducing a freshly prepared suspension of the mold spores into 1-l. portions of the sterilized medium, contained in 3-l. erlenmeyer flasks, and incubating at 25° for eight days. On the second day after inoculation, small floating colonies 1–2 mm. in diameter were observed and on the third day there were numerous

(8) For this determination we wish to thank Mr. J. F. Alicino of the Squibb Institute for Medical Research, New Brunswick, N. J.

(9) W. F. Bruce, J. D. Dutcher, J. R. Johnson and L. L. Miller, THIS JOURNAL, **66**, 616 (1944).

floating colonies with a white aerial mycelium, almost forming a pellicle. A similar layer of growth covered the bottom of the flask. On the fifth day a heavy wrinkled white mat of mycelium had formed and on the eighth day there were some submerged areas containing spores.

Ten liters of culture medium, after eight days incubation, was freed of mycelium by decantation and filtered through several layers of cheese cloth. The resulting clear greenish-yellow solution was divided into three portions and extracted thoroughly by repeatedly shaking with benzene, using a total of about 5-l. of solvent. The benzene extracts were washed with water, combined, and concentrated on a steam-bath to a volume of about 300 ml. The last of the benzene was distilled off under reduced pressure in an all-glass apparatus. The slightly yellow crystalline residue was triturated with two 25-ml. portions of petroleum ether to wash out fatty material. The crystalline mixture of antibiotics, after washing, weighed 1.1 g.

The mixture was dissolved in 100 ml. of benzene, warmed with decolorizing carbon and filtered. The filtrate was concentrated to a volume of about 40 ml., diluted with petroleum ether (b.p. 30–60°) until a turbidity developed, and cooled in a refrigerator. This procedure effected precipitation of most of the gliotoxin, which was separated by filtration. The crystalline precipitate was taken up in 40 ml. of benzene and the gliotoxin fraction reprecipitated by dilution with petroleum ether and cooling, as before. The crude gliotoxin after the second precipitation melted at 180–190°; after several crystallizations from ethanol it was obtained in substantially pure condition, m.p. 190–192°.

The mother liquors from the isolation of gliotoxin were evaporated to dryness under reduced pressure and the residue was recrystallized twice from ethanol. This gave 300 mg. of a pure product, m.p. 162–163°, for which analytical data are shown in Table I. Acetyl determination gave the value 11.1%, *vs.* 11.7% calculated for one acetyl group. Methoxyl analysis showed 2.0 and 1.8%, which is much lower than the value 8.3% required for one methoxyl group.

The crystals of gliotoxin monoacetate are quite different from those of gliotoxin[10] and are readily identified under the microscope. Dr. John H. Andreen has kindly prepared for us a crystallographic description of gliotoxin monoacetate, crystallized from benzene (Fig. 1). The crystals are orthorhombic and in their most common habit possess the forms: basal pinacoid, 001; macrodome, 101; and brachydome, 011. In some crystals the basal pinacoid, 001, is very small or entirely absent. The interfacial angles $011:01\bar{1}$ and $101:10\bar{1}$ are 143 ± 1°. The optic axial plane is 100, with γ, the acute bisectrix, parallel to "c". The sign of double refraction is positive. In sodium light the optic axial angles are $2V = 9 \pm 1°$, and $2E = 14.5 \pm 1°$. For sodium light the refractive indices are: α, 1.6110; β, 1.6120; and γ (calcd.), 1.80 ± 0.05.

Methanolysis of Gliotoxin Monoacetate.—In a small flask 500 mg. of the new antibiotic, m.p. 162–163°, was placed with 100 mg. of *p*-toluenesulfonic acid and 30 ml. of methanol. The mixture was heated and methanol was distilled off slowly at the rate of about 15 ml. per hour. The distillate was collected in a receiver cooled in ice. More methanol was added from time to time to maintain a volume of 20–30 ml. of liquid in the flask.

After ten hours heating, the reaction mixture was reduced to a volume of 15 ml., 50 ml. of chloroform added, and the organic solvent washed with 100 ml. of water. The aqueous layer was extracted again with 50 ml. of chloroform. The combined chloroform extracts were washed with 50 ml. of water and then evaporated to dryness. Benzene (50 ml.) was added and evaporated to dryness to remove all of the chloroform. After two crystallizations from methanol the residue formed pale yellow crystals, m.p. 188–189°; weight 150 mg. A mixture with authentic gliotoxin showed no depression of the melting point.

The distillate (150 ml.) obtained during the ten hours heating with methanol was treated with 5 ml. of *N* sodium hydroxide solution and refluxed for two hours to saponify methyl acetate. Titration of an aliquot indicated that 76.4% of the theoretical amount of acetic acid had been formed. The neutralized solution was evaporated to dryness and the resulting sodium acetate was identified as the S-benzylthiouronium salt, according to the procedure of Shriner and Fuson.[11] The derivative melted at 130–132° and showed no depression when mixed with an authentic specimen of the acetate derivative.

(10) J. R. Johnson, W. F. Bruce and J. D. Dutcher, THIS JOURNAL, **65**, 2008 (1943).

(11) Shriner and Fuson, "The Systematic Identification of Organic Compounds," John Wiley and Sons, Inc., New York, N. Y.

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[CONTRIBUTION FROM THE RESEARCH LABORATORIES OF MERCK & CO., INC.]

Approaches to the Total Synthesis of Adrenal Steroids. VII. A New Method for the Attachment of Ring D. Part A

BY L. H. SARETT, W. F. JOHNS, R. E. BEYLER, R. M. LUKES, G. I. POOS AND G. E. ARTH

RECEIVED DECEMBER 22, 1952

The first phase of a new method for attaching ring D to 1-ketopolyhydrophenanthrenes is described. A three-carbon system corresponding to C-17, C-20 and C-21 of the pregnane skeleton is added. Thus the condensation of methallyl iodide with 2α,4b-dimethyl-7-ethylenedioxy-1,2,3,4,4aα,4b,5,6,7,8,10,10aβ-dodecahydrophenanthrene-4β-ol-1-one (I) gives the 2β-methyl-2-methallyl derivative II. Application of the same reaction to the 2-methyl-1,4-diketone IV gives chiefly the epimeric 2α-methyl-2-methallyl compound V. In partial ozonolyses and partial hydroxylations with osmium tetroxide the side chain double bond is affected preferentially. The structures of dilactols related to these 1-keto-2-methallyl compounds are discussed.

The gain of five carbon atoms is required to convert the tricyclic methyl hydroxyketone[1] I into a pregnane derivative. Three of these, which represent C-17, C-20 and C-21 of the pregnane skeleton, may be conveniently introduced by a condensation with an allylic halide. It is with this condensation and attendant stereochemistry that the present paper is concerned.

The reaction of 2α,4b-dimethyl-7-ethylenedioxy-1,2,3,4,4aα,4b,5,6,7,8,10,10aβ-dodecahydrophenanthrene-4β-ol-1-one (I) with methallyl iodide in the presence of potassium *t*-butoxide gave a good yield of a single crystalline 2-methyl-2-methallyl derivative II.[2] The corresponding diketone III resulted from oxidation of this alcohol with the chromium trioxide–pyridine complex.[3] When the methyl diketone IV was methallylated, the major product consisted of an isomeric methyl meth-

(1) R. M. Lukes, G. I. Poos, R. E. Beyler, W. F. Johns and L. H. Sarett, THIS JOURNAL, **75**, 1707 (1953).

(2) The results of structure assignments given below are anticipated here for the sake of clarity.

(3) G. I. Poos, G. E. Arth, R. E. Beyler and L. H. Sarett, THIS JOURNAL, **75**, 422 (1953).