THE BIOSYNTHESIS OF GLIOTOXIN

By

JACK ALAN WINSTEAD

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University of Kentucky

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THE BIOSYNTHESIS OF GLIOTOXIN

Thesis Approved:

Robert J. Suhadolnik
Thesis Adviser

Robert J. Sirny

Acheson Hrenoff
Dean of the Graduate School

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CHAPTER I

INTRODUCTION

In 1932, Weindling (1) reported the presence of a highly fungicidal substance in cultures of Trichoderma lignorum that would control damping-off of citrus seedlings caused by Rhizoctonia solani. The fungicidal activity of the Trichoderma was found to be due to the secretion of a lethal principle into the surrounding medium by the young hyphae (2).

The toxic substance was isolated from the medium and crystallized, yielding silky white needles (3). Analysis showed the substance to be $C_{13}H_{14}N_2S_2O_4$ (4). The fungus was reclassified to Gliocladium fimbriatum, thus giving the antibiotic the name gliotoxin (5). Later evidence showed that the fungus Weindling reported as Gliocladium fimbriatum is really a strain of Trichoderma viride (6).

The instability of gliotoxin in alkali and in light, as well as its toxicity, has limited its application. A biosynthetic study of gliotoxin was undertaken to determine the biogenesis of the indole nucleus as well as of the sulfur-containing group. The study of the biosynthesis of antibiotics had been limited until the advent of radioisotopes. It is now possible to determine some of the precursors and intermediates in the biosynthesis of these compounds by using isotopes. Investigations presented in this thesis were conducted using carbon-14 and tritium labeled compounds as possible precursors of gliotoxin.

CHAPTER II

HISTORICAL

Gliotoxin has been isolated and identified from Trichoderma viride (7), Aspergillus fumigatus (8, 9, 10, 11), Aspergillus fumigatus mut. helvola Yuill (12), Penicillium cinerascens (13), Penicillium terlikowski (14), and Penicillium obscurum Biourge (15). A monoacetyl derivative of gliotoxin has been isolated from Penicillium terlikowski Zaleski (16). This is the same organism as Penicillium obscurum Biourge, which has been reported to produce a second unidentified antibiotic (15).

The production of gliotoxin, by the fungus Trichoderma viride in unsterilized acid soil, was demonstrated by the chromatographic separation of an ether extract. The activity was measured by growth-inhibition zones using B. subtilis (17).

Weindling (18) reported that the fungus required an abundance of oxygen and high acidity to produce good yields of gliotoxin. Ammonium salts were found to be the best sources of nitrogen, and glucose and sucrose proved to be good carbon sources.

Johnson et al. (4) used a shaker to furnish the necessary aeration for gliotoxin production. Three grams of gliotoxin was produced from 60 liters of synthetic medium. The medium consisted of sucrose, ammonium sulfate, dipotassium phosphate, magnesium sulfate, ferric chloride, peptone, and water. Brian (7) found that cysteine could

replace the sulfate as the source of sulfur. The pH was adjusted to 3.0-3.8 with concentrated sulfuric or phosphoric acids. The medium was inoculated and the fungus was allowed to grow for 4-5 days at 27-32° with maximum production of gliotoxin occurring during the second day.

The mycelium was filtered from the culture medium and the filtrate was extracted with chloroform (4). The chloroform was evaporated to dryness *in vacuo* and the residue was extracted with petroleum ether and methanol to remove the lipids. The gliotoxin was crystallized from hot methanol or ethanol.

Purified gliotoxin inhibited the growth of human tubercle bacilli *in vitro* and also had strong bacteriostatic action on related organisms (19, 20). It has been found inhibitory against many of the *Bacilli*, *S. aureus*, *P. fischeri*, and the *Clostridia* (21, 22, 23). Gliotoxin has been reported relatively inactive *in vivo* (24) and has also been reported inactive against bacterial viruses (25). Gliotoxin was found toxic to *Pythium* and *Rhizobium*, two genera of the damping-off fungi (26). It exerted marked fungistatic and fungicidal action towards *C. nicotianae* which attacks tobacco in the tropics (27). Gliotoxin was found to have antialgal properties against six cultures of green algae and diatoms (28). However, *T. viride*, the organism producing gliotoxin, is resistant to the toxic effects of the antagonist (7).

Studies of gliotoxin on plants and animals demonstrated a toxic effect at small concentrations. Severe inhibition of root growth of wheat, clover, and mustard seedlings occurred as determined by simple germination tests (29). The lethal dose of gliotoxin for rabbits,

rats, and mice has been reported to be from 45 to 75 mg. per kg. of body weight (4, 30, 31).

At a concentration of 1 gamma per ml., gliotoxin inhibited the subsequent growth of mouse lymphosarcoma cells *in vitro* (32). This inhibitory effect was abolished by previous contact with sulfhydryl compounds. Cysteine was found to inactivate gliotoxin (33). Gliotoxin showed little specificity in its reactions with thiols as compared with other antibiotics such as penicillin (34). The antibacterial activity of gliotoxin, which was reduced by thiol compounds as cysteine, was immediately restored when titrated with iodine solution but more slowly by exposure to air (35).

Gliotoxin can be crystallized from chloroform to yield almost colorless monoclinic crystals which decompose sharply at 221° (capillary m.p. 190-192°) (36). The molecular weight of gliotoxin (from x-ray data) was 328.4 and its density was 1.537 as compared to the formula weight of 326.4 and a determined density of 1.546 (37). The solubility of gliotoxin in a number of solvents has been studied and reported by Johnson *et al.* (4). It is strongly levorotatory and a comparison of its ultraviolet absorption spectrum was similar to that of the spectra of indole and tryptophan (4).

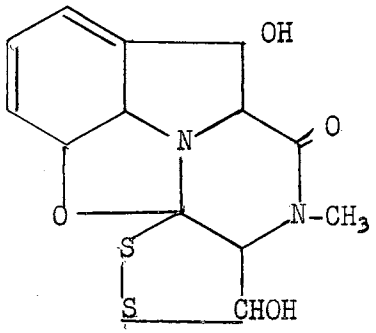
When gliotoxin was refluxed with barium hydroxide, methylamine, hydrogen sulfide, and elementary sulfur were produced (38). Purification of the residue yielded a small amount of crystalline solid identified as indole-2-carboxylic acid. A Zeisel determination showed the absence of methoxyl or ethoxyl groups. The test for a N-methyl group was positive which agreed with the isolation of methylamine. No

reaction occurred with carbonyl reagents. Oxidizing agents rapidly oxidized the sulfur of gliotoxin to sulfate. Reducing agents converted the sulfur in gliotoxin to hydrogen sulfide. Treatment of gliotoxin in pyridine with benzoyl chloride at room temperature produced a crystalline dibenzoate. A Zerewitinoff determination for active hydrogen was somewhat uncertain but indicated two or three active hydrogens. Later work has shown that the two reactive hydrogen atoms were from hydroxyl groups. This interpretation is consistent with the presence of two bands in the infrared absorption spectrum (39). The reaction of gliotoxin with potassium sulfide, potassium thioglycolate, and alkaline plumbite indicated the presence of a sulfide linkage (40).

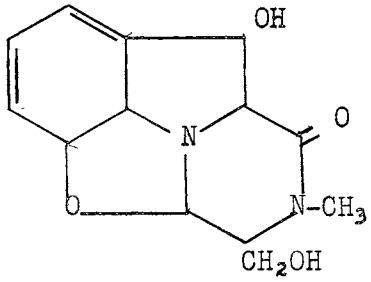
Desulfurization of gliotoxin with aluminum amalgam resulted in the replacement of the sulfur atoms by hydrogen atoms and the liberation of hydrogen sulfide (40, 41, 42). This compound is known as dethiogliotoxin and has been recovered as a colorless crystalline compound in 30 per cent yield.

Dutcher, Johnson, and Bruce (40) have reported that gliotoxin and dethiogliotoxin yielded 0.12 and 0.93 moles of acetic acid, respectively, in the Kuhn-Roth determination. Later work indicated that dethiogliotoxin did not have a $O\text{-}CH_3$ group (42).

The first structures proposed for gliotoxin (I) and dethiogliotoxin (II) were based on a number of degradation products which have been identified (42-50).

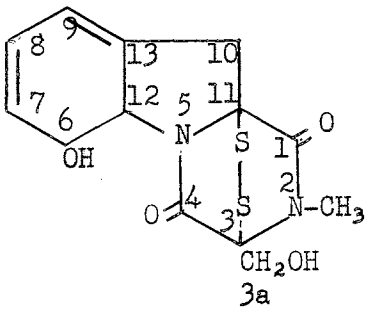


I

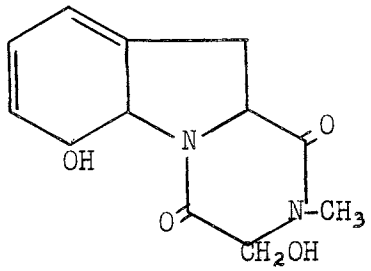


II

On the basis of new experimental evidence, Bell et al. (51) have deduced a new structure for gliotoxin (III). This structure permits the unambiguous explanation for the extensive transformations of gliotoxin and dethiogliotoxin (IV) into degradation products of known structure.

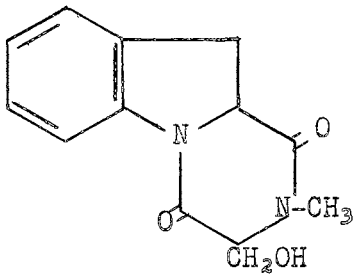


III

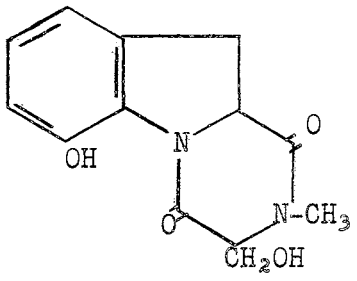


IV

The long-known anhydrodethiogliotoxin was given the structure (V). Dehydrodethiogliotoxin was formed by heating dethiogliotoxin over palladium-charcoal in boiling xylene (52). Its chemical properties and ultraviolet spectrum are consistent with formula (VI), and treatment with acetic anhydride converted the dehydrodethiogliotoxin to an alkali-insoluble, crystalline compound with the formula $C_{15}H_{14}N_2O_4$.



V



VI

Treatment of this derivative with saturated methanolic ammonia at 20° converted it to a sparingly soluble, crystalline compound $(C_7H_7NO_2)_n$, which decomposed at 360°. This product was similar to dimeric substances produced by the action of ammonia on dipeptides containing a serine unit. Chemical and sterochemical considerations excluded the attachment of a disulfide bridge in gliotoxin at any positions except 3 and 11 (51).

A number of schemes involving tryptophan as a precursor have been proposed for indole and indole-like alkaloids (53). Until recently none

of these schemes were studied experimentally. In 1951, Bowden and Marion (54) reported the incorporation of DL-tryptophan-β-C^{14} into the alkaloid, gramine, which is found in barley. Alkaline degradation showed that the radioactivity, in the alkaloid, was entirely in one position. This corresponded to the labeled carbon in the tryptophan administered to the barley plants, thus indicating that tryptophan is a precursor of gramine.

Suhadolnik et al. (55) have studied the incorporation of radioisotopes into the ergot alkaloids cultured on the rye plant. One of the alkaloids of the rye ergot is ergonovine, which contains the lysergic acid moiety. The incorporation of DL-tryptophan, labeled with carbon-14 in the 7a or α-position, was so low as to indicate that tryptophan was not a direct precursor of the lysergic acid moiety. Likewise, the isotopes from carbon-14 labeled phenylalanine and acetate, as well as tritium-labeled tryptophan and anthranilic acid, were not incorporated to a significant extent into the alkaloids. Mothes et al. (56) have reported the incorporation of DL-tryptophan-β-C^{14} into ergometrine, ergonovine, and ergocorine produced on the rye plant. This incorporation was only slightly higher than that reported by Suhadolnik et al. (55). The alkaloids were hydrolyzed and essentially all of the radioactivity was found to be in the lysergic acid moiety. On the basis of these results, a new scheme for lysergic acid biosynthesis has been postulated involving tryptophan.

Suhadolnik and Chenoweth (57) have reported the incorporation of phenylalanine-1-C^{14} and phenylalanine-2-C^{14} into gliotoxin to the extent of 4-12 per cent. The gliotoxin from phenylalanine-1-C^{14} was degraded

to indole-2-carboxylic acid, which retained 82 per cent of the radioactivity, and essentially all of the activity was in the carboxyl carbon. Methionine-CH_3-C^{14} was incorporated to the extent of 0.16-0.27 per cent and was suggested to be a possible source of the N-methyl group. Tryptophan-7a-C^{14} and acetate-2-C^{14} were not incorporated into gliotoxin. It was concluded that phenylalanine is a direct precursor of the indole-type moiety of gliotoxin.

CHAPTER III

EXPERIMENTAL

Tritium and carbon-14 labeled compounds were added to cultures of Trichoderma viride which were growing in an aerated flask. The gliotoxin was isolated and analyzed for radioactivity to determine the extent of incorporation of the labeled compounds. The gliotoxin was then degraded to determine the position of the radioactivity. The labeling pattern indicated whether the added compound was a direct precursor. The methods and procedures are discussed in the following pages.

Isotopes

The DL-serine-3-C^{14}, DL-serine-1-C^{14}, glycine-2-C^{14}, and DL-methionine-CH_3-C^{14} were obtained from the Volk Radiochemical Company. The compounds were dissolved in a known volume of water and an aliquot was added to the culture medium.

The DL-m-tyrosine and DL-phenylalanine were obtained from the California Foundation for Biochemical Research. These were labeled by the tritium exchange method reported by Wilzbach (58). Two hundred-fifty mg. of DL-m-tyrosine was dissolved in 2 ml. of water and transferred to a 10 ml. tritium labeling ampule. The solution of m-tyrosine was taken to dryness in a rotary evaporator to obtain a thin layer of

the compound on the inner wall of the ampule and dried *in vacuo* over phosphorus pentoxide. The tube containing the *m*-tyrosine was evacuated to 0.005 mm. and 5 curies of tritium gas (obtained from Union Carbide Nuclear Company) was pumped into the ampule by means of a Toepler pump. The compound was exposed to the tritium gas for 7 days at room temperature, the gas was removed, and the compound was exchanged 20 times with water to remove any exchangeable tritium. The *m*-tyrosine was crystallized from water. A paper chromatogram of the crystalline *m*-tyrosine using Whatman No. 1 filter paper and pyridine-butanol-water (6:4:3) system gave four radioactive peaks as determined by a windowless chromatogram scanner. One peak remained at the origin and only one peak was ninhydrin positive.

Twenty mg. of the crystalline *m*-tyrosine-H^3 was dissolved in 2 ml. of water and placed on an ion exchange column of Dowex 50-H. The column was developed with 100 ml. of each of the following: water, 0.15 N HCl, 0.3 N HCl, 0.6 N HCl, 1.5 N HCl, and 4 N HCl. The rate of flow was 2 ml. per minute and 4 ml. fractions were collected. The separation is shown in Figure 1.

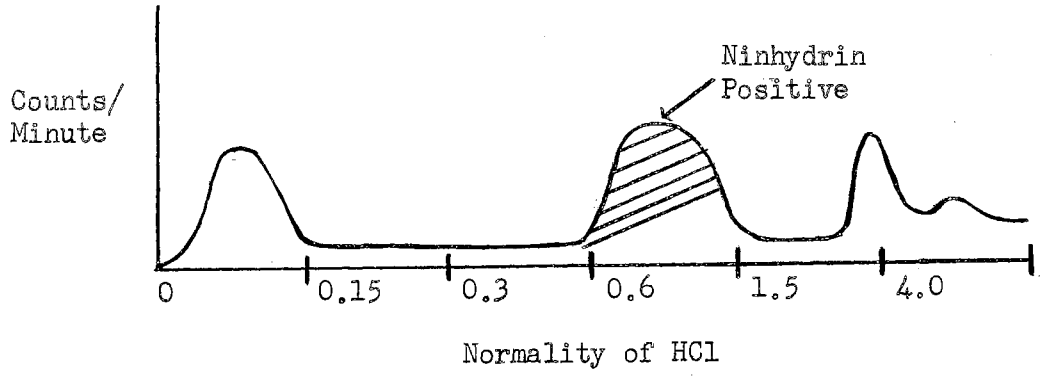


Figure 1. The Separation of Impure *m*-Tyrosine-H^3

The ninhydrin-positive material appeared as one peak when chromatographed and was identified by comparing its R_f with the R_f of authentic m-tyrosine. The radioactivity of the eluted m-tyrosine-H^3 solution from one tube was determined and a known volume was added to the culture medium over a period of 30 hrs. The times of addition are given in Table I.

The ion exchange column was regenerated by converting to the sodium form, washing with water to pH 7-8, converting to the hydrogen form, and then washing with water to pH 5-6.

The DL-phenylalanine was labeled with tritium and purified by the same procedure as the m-tyrosine. A paper chromatogram of the phenylalanine-H^3 indicated four radioactive components. Chromatography of the impure phenylalanine-H^3 on Dowex 50-H resulted in incomplete purification. Repetition of this procedure gave only phenylalanine-H^3 which was eluted with 0.6-1.5 N HCl. It was identified by the ninhydrin test and by comparison of its R_f with the R_f of authentic phenylalanine. A known volume of the tritium labeled phenylalanine solution was added to the culture medium.

Fermentation Procedure

Johnson et al. (4) have reported a shake culture method for the growth of T. viride for maximum production of gliotoxin. The organism grows but fails to produce gliotoxin unless good aeration is maintained. Chenoweth (59) developed an aeration apparatus to draw humidified air through a sintered glass tube into the culture flask by a water aspirator, as diagramed in Figure 2. This procedure provided vigorous

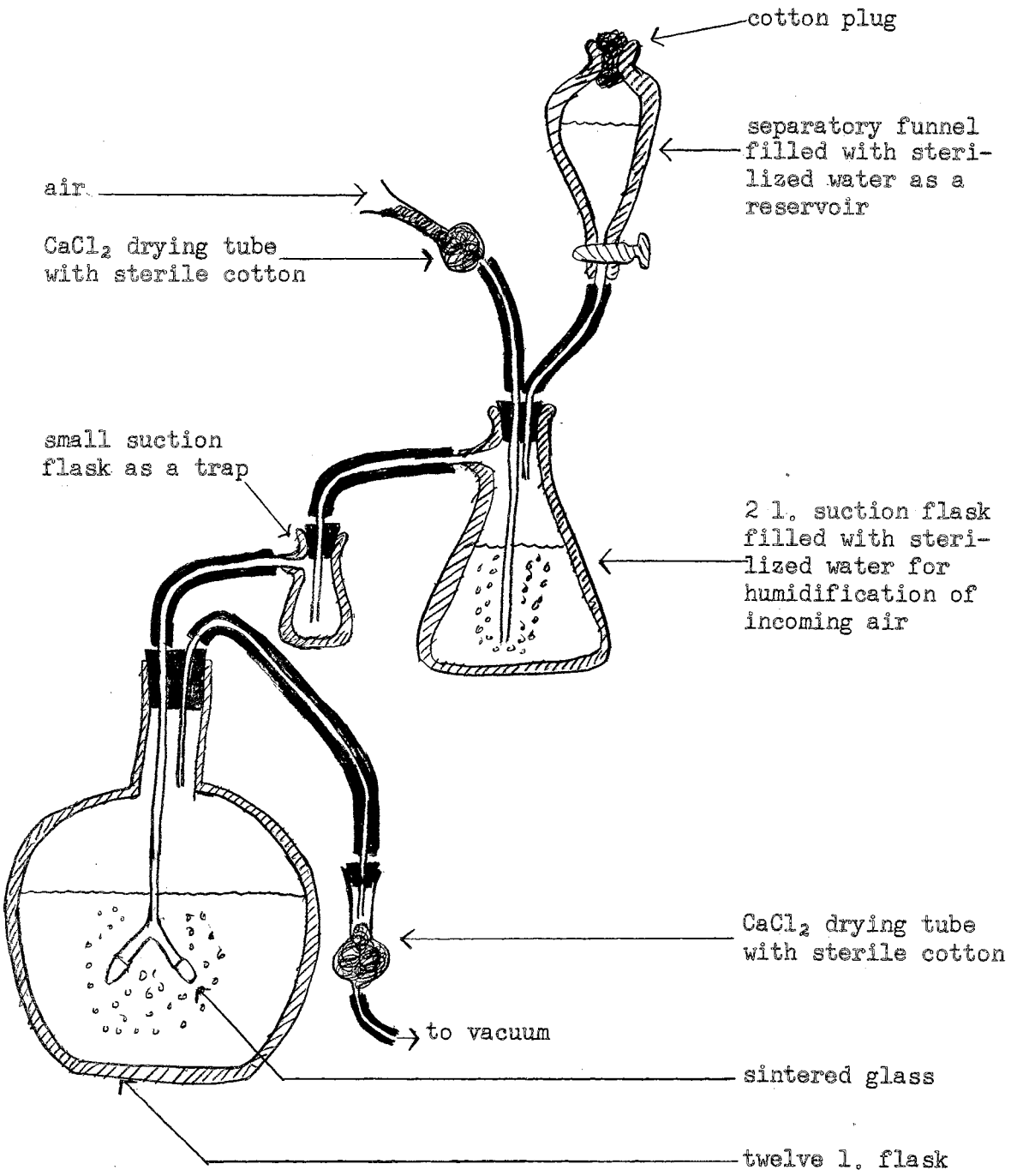
TABLE I

LABELED COMPOUNDS ADDED TO THE FERMENTATION MEDIUM

Flask No.	Substrate	Substrate	Specific Activity of Substrate	Radioactivity added	Times of Isotope Additions	
		mg.	mc./mmole	mμc.	Fraction	Hours
7	DL-Serine-3-C^{14}	0.26	2.02	5,000	1/2	44
					1/2	53
8	DL-Serine-3-C^{14}	0.26	2.02	5,000	Same	
9	DL-Serine-3-C^{14}	0.26	2.02	5,000	1/2	0
					1/4	26
					1/4	49
15	Glycine-2-C^{14}	0.49	1.68	10,960	1/3	23
					1/3	40
					1/3	50
16	Glycine-2-C^{14}	0.49	1.68	10,960	Same	
17	DL-Methionine-CH_3-C^{14}	0.39	1.96	5,170	1/3	42
					1/3	48
					1/3	65
18	DL-Methionine-CH_3-C^{14}	0.39	1.96	5,170	Same	
20	DL-m-Tyrosine-H^3	0.25*	9.6**	2,400	1/4	27
					1/4	41
					1/4	48
					1/4	65
21	DL-m-Tyrosine-H^3	0.25*	9.6**	2,400	Same	
24	DL-Phenylalanine-H^3	2.5*	5.5**	13,700	1/4	27
					1/4	38
					1/2	47
25	DL-Phenylalanine-H^3	2.5*	5.5**	13,700	Same	
26	DL-Serine-1-C^{14}	1.77	1.10	18,500	1/4	31
					1/2	45
					1/4	52

* ml.
** μc./ml.

Figure 2. Diagram of the Apparatus Used to Grow T. viride Cultures



bubbling of air through the medium of the growing T. viride and was used for the major part of these studies. However, some cultures were aerated by passing compressed air through the same apparatus. Both methods were equally successful and the yields compared favorably with those reported by Johnson et al. (4).

The culture medium of Johnson et al. (4), used in these studies, was as follows:

90	g. sucrose
10	g. ammonium sulfate
5	g. anhydrous dipotassium phosphate
2.5	g. anhydrous magnesium sulfate
0.05	g. ferric chloride
0.1	g. peptone
6	l. water

The pH was adjusted to 3.3-3.8 with either concentrated sulfuric or phosphoric acid. The medium was prepared in a twelve liter flask as shown in Figure 2. The aeration apparatus was autoclaved for thirty minutes at fifteen pounds pressure. Essentially no contamination occurred in any of the cultures.

The medium was inoculated with a 50 ml. shake culture or with a potato-dextrose agar slant each of which had been growing for three to six days. The organism used was T. viride, No. 1828 NRRL, which was kindly supplied by Dr. C. W. Hesseltine of the Northern Utilization Research Branch, Peoria, Illinois. The cultures were maintained at 27-32°.

Isolation of Gliotoxin

After five days of growth, the mycelium was removed from the medium by filtering through three sheets of Whatman No. 1 filter paper,

Celite, and four layers of cheesecloth on a Buchner funnel. The filtrate was collected in a four liter suction flask, and the mycelium was dried at 110°. The medium was extracted three times in a four liter separatory funnel with a total volume of chloroform equal to one-fifth the volume of the filtrate. The chloroform was removed by vacuum distillation. The yellow residue was washed twice with a total volume of 50 ml. of petroleum ether (b.p. 90-110°) and absolute methanol (2.3:1). Essentially all of the color and lipids were in the petroleum ether extract. The remaining residue was treated with charcoal, filtered, and the gliotoxin crystallized from hot ethanol or methanol. The mother liquor was separated from the gliotoxin at -10°. The gliotoxin melted at 190-192° in an open capillary which is in agreement with that reported by Johnson et al. (36). More accurate melting points were obtained by packing a small amount of gliotoxin in a commercial melting point tube, flushing the tube with nitrogen, and sealing. A Thiele tube was used for these melting point determinations. All of the isolations of gliotoxin melted with decomposition to form a yellow oil, as described by Weindling (18). The melting points (uncorrected), the yields of gliotoxin, and the yields of mycelium are shown in Table II.

An infrared spectrum[1] of gliotoxin prepared as a potassium bromide pellet was identical with the infrared spectrum reported by Johnson (36). The ultraviolet absorption spectrum had an absorption maximum at 270 mμ. in agreement with that reported by Johnson (36).

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[1]Courtesy of Dr. N. Deno, Department of Chemistry, Pennsylvania State University, University Park, Pennsylvania.

TABLE II

YIELDS AND MELTING POINTS OF GLIOTOXIN AND YIELDS OF MYCELIUM

Flask No.	Total Yield of Gliotoxin	Melting Point	Dry Weight of Mycelium
	mg.		g.
7	350	198-198.5	23.6
8	429	198-199	24.4
9	217	200-202	15.5
15	160	199-200	18.8
16	159	199.5-201	18.8
17	262	197-199	25.0
18	165	198-199	21.3
20	524	201-206.5	26.0
21	418	200-201	26.6
24	310	201-202	26.5
25	320	200-201	28.9
26	485	201-2-2	24.9

Counting Procedures and Results

All of the carbon-14 samples from the isolations described were combusted by the method of Van Slyke *et al.* (60). The CO_2 was collected in an ionization chamber and counted with a vibrating reed electrometer. The tritium-containing samples were counted by the procedure of Wilzbach *et al.* (71).

Samples of the gliotoxin obtained from the various experiments were combusted and counted by the above procedures, and the amount of radioactivity in the isolated gliotoxin was calculated. The incorporation of the isotopes into gliotoxin is shown in Table III.

Degradation Procedures and Results

A method similar to that of Bruce *et al.* (38) was used to degrade gliotoxin and to isolate the N-methyl group as methylamine and was applied to the gliotoxin from flasks 7, 16, 17, and 26. Fifty mg. of carrier gliotoxin was added to 50 mg. of gliotoxin. Ten ml. of 10 per cent NaOH was added to the gliotoxin and refluxed for one hour. The volatile base was collected in 0.1 N HCl. The acidic solution of methylamine hydrochloride was taken to dryness in a rotary evaporator and then dried *in vacuo* over phosphorus pentoxide. A white deliquescent solid melting at 220-222° was obtained. Authentic methylamine hydrochloride melted at 222-224°. Addition of pure methylamine hydrochloride caused no depression of the mixed melting point. The yields were approximately 75 per cent that of theoretical. The isolated methylamine hydrochloride was combusted and counted. The results are shown in Table IV.

TABLE III

INCORPORATION OF RADIOACTIVITY INTO GLIOTOXIN

Flask No.	mμc.	mμc. (Average)	Per Cent**
7	a) 140 b) 134	137	2.73
8	a) 124 b) 118	121	2.42
9	a) 37 b) 35	36	0.72
15	a) 78 b) 116	97	0.88
16	a) 127 b) 130	128	1.17
17	a) 171 b) 181 c) * 169 d) * 180	175	3.39
18	a) 90 b) 89	90	1.74
20	a) 733 b) 762 c) 722	739	30.8
21	a) 1150 b) 973	1062	44.3
24	a) 2260 b) 2550	2405	17.6
25	a) 1970 b) 1930	1950	14.2
26	a) 346 b) 364	355	1.92

* after recrystallization
** based on the quantity of gliotoxin isolated

TABLE IV

CARBON-14 FOUND IN THE METHYLAMINE HYDROCHLORIDE

Flask No.	Substrate Added	Sample Weight	C^{14} in Sample	Specific Activity* of $CH_3NH_2 \cdot HCl$ Average	Specific Activity of Gliotoxin	C^{14} in Methylamine
		mg.	mμc.	mμc./mmole	mμc./mmole	%
7	DL-Serine-3-C^{14}	a) 5.12 b) 5.12	1.21 1.23	32.0	110	29.0
16	Glycine-2-C^{14}	a) 3.52 b) 3.52	1.20 1.43	50.4	268	18.8
17	DL-Methionine-CH_3-C^{14}	a) 6.2 b) 6.2	6.9 7.6	158	219	72.2
26	DL-Serine-1-C^{14}	a) 2.48 b) 2.48	0 0	0	239	0

* Corrected for 2 fold dilution

The radioactive gliotoxin from flasks 9, 21, 24, and 26 was degraded by the method of Suhadolnik and Chenoweth (57) to yield indole-2-carboxylic acid. One hundred-fifty mg. of gliotoxin was refluxed with 15 ml. of 15 per cent $Ba(OH)_2$ for 1.5 hrs. in an atmosphere of nitrogen. At the end of the reflux, 10 per cent H_2SO_4 was added to pH 5. The barium sulfate was removed by filtration. The filtrate was adjusted to pH 1 and taken to dryness in a rotary evaporator. The residue was dried *in vacuo* over phosphorus pentoxide and then sublimed at 110-130° and 10-20 mm. of mercury. The sublimate usually melted at 200-204° (uncorrected). When the melting point was below 200°, the sublimate was recrystallized from hot chloroform. This melting point is similar to that for indole-2-carboxylic acid as reported by Cornforth and Robinson (62) and Dutcher *et al.* (43). Hydrolysis of the ethyl-indole-2-carboxylate[2] with aqueous-methanolic KOH yielded indole-2-carboxylic acid, which after crystallization melted between 202-205° (uncorrected). A mixed melting point of the synthesized indole-2-carboxylic acid with the indole-2-carboxylic acid obtained from the gliotoxin showed no depression in melting point. The indole-2-carboxylic acid samples were combusted and counted for radioactivity. The results are presented in Table V.

Dethiogliotoxin was prepared from the gliotoxin from flasks 7, 8, 15 and 16, and 17 and 18 by the method of Bell (63). Two hundred mg. of labeled gliotoxin with 200 mg. of carrier gliotoxin was added

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[2]Kindly supplied by Dr. H. R. Snyder, Department of Chemistry, University of Illinois, Urbana, Illinois.

TABLE V

DEGRADATION OF GLIOTOXIN TO INDOLE-2-CARBOXYLIC ACID

Flask No.	Substrate Added	Gliotoxin		Indole-2-Carboxylic Acid			
		mμc./mmole	mg.	Melting Point	mμc./mmole	average mμc./mmole	%
9	DL-Serine-3-C^{14}	55	15.0	204-206	a) 9.4 b) 11.6	10.5	19.1
21	DL-m-Tyrosine-H^3	780	15.5	200-202	a) 756 b) 408 c) 1170	778	99.7
24	DL-Phenylalanine-H^3	2340	12.0	201-203	a) 6830 b) 4470	5650	
26	DL-Serine-1-C^{14}	239	2.3	199-200	0	0	0

to 100 ml. of 95 per cent ethanol and dissolved by heating with steam. The solution was cooled to about 25° and treated under nitrogen with 0.8 g. of aluminum foil, which had been cut into one centimeter squares, amalgamated with 5 per cent aqueous mercuric chloride for 30-45 seconds (with constant agitation) and washed several times with water. The amalgamated aluminum was used immediately. During the initial stage of the reaction the temperature increased to about 40°. After slow stirring for three hours, most of the aluminum had reacted. At this time an additional 0.4 g. of amalgamated aluminum was added and the reaction was continued for four hours. The mixture was then heated to boiling and filtered while hot. The residue was treated with 50 ml. of 95 per cent ethanol, boiled for several minutes and filtered. The combined filtrates were evaporated to a volume of 7 ml. After standing at -10° for 24 hrs., the dethiogliotoxin was filtered and dried. Approximately 80 mg. was obtained and melted between 246-250°. A second crop of approximately 40 mg. was obtained by evaporating the mother liquor to 1 ml. The yields were 30-40 per cent. Johnson and Buchanan (42) and Bell (63) reported a melting point range of 246-252°. The infrared[3] and ultraviolet absorption spectra of the dethiogliotoxin were identical with that reported by Johnson (36).

The dethiogliotoxin samples were degraded by the Kuhn-Roth procedure (64), a micro-method for detecting the presence of $C\text{-}CH_3$ groups by oxidation to acetic acid. Two ml. of concentrated H_2SO_4 was added

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[3]Courtesy of Dr. N. Deno, Department of Chemistry, Pennsylvania State University, University Park, Pennsylvania.

to 10-20 mg. of dethiogliotoxin. Eight ml. of 5 N chromic acid was then added. This mixture was refluxed for 1.5 hrs. and the CO_2 evolved was collected in excess NaOH. After 1.5 hrs. the sample was distilled into a known excess of N/100 NaOH and then back titrated with N/100 acetic acid to a phenolphthalein end point. The distillate was not identified. The distillate was taken to dryness, combusted, and counted for radioactivity. The alkaline solution containing the CO_2 collected during reflux was evaporated to a small volume and transferred to a combustion tube. Perchloric acid was then added to the alkaline sample and the CO_2 evolved was passed through a dry ice-acetone trap, collected in an ionization chamber and counted. The results of these degradations are shown in Table VI. In control experiments DL-serine-3-C^{14} and DL-serine-1-C^{14} were degraded by the above procedure. Essentially all of the radioactivity was evolved as CO_2 during reflux. No acetic acid was formed.

TABLE VI

KUHN-ROTH DEGRADATION OF DETHIOGLIOTOXIN

Flask No.	Substrate Added	Dethiogliotoxin Degraded		Distillate		Radioactivity Distillate	Radioactivity CO_2
		mμc./mmole	mg.	mμc.	meq./mmole of dethiogliotoxin	mμc.	mμc.
7	DL-Serine-3-C^{14}	67	12.8	3.23	0.68	0.19	0.78
7	DL-Serine-3-C^{14}	67	16.8	4.23	----	----	1.08
8	DL-Serine-3-C^{14}	49	20.0	3.68	0.76	0.14	----
15 & 16	Glycine-2-C^{14}	110	20.0	8.40	0.32	0.22	3.0
17 & 18	DL-Methionine-CH_3-C^{14}	103	16.4	6.20	0.35	0.70	0.29

CHAPTER IV

DISCUSSION OF RESULTS

A significant incorporation of isotopic labeled compounds into all of the carbons of gliotoxin was observed (Table III). Since Suhadolnik and Chenoweth (57) had reported that the aliphatic side chain of phenylalanine-1-C^{14} and phenylalanine-2-C^{14} was incorporated directly into gliotoxin, it was of interest to determine the origin of the dihydrobenzene moiety. Tritium labeled DL-phenylalanine was used for this study, and was incorporated into gliotoxin to the extent of 14-17 per cent. The fact that incorporation of the phenylalanine-H^3 into gliotoxin was obtained provided additional evidence that all 9 carbons of phenylalanine are incorporated directly into gliotoxin. To show that the tritium was located in the dihydroindoline nucleus, gliotoxin was degraded to indole-2-carboxylic acid. The specific activity, as determined by both the zinc fusion method and the scintillation counting method, of this indole-2-carboxylic acid was greater than that of the gliotoxin (Table V). Thus far no explanation can be proposed for these anomalous results.

The presence of hydroxyl group in the meta position of phenylalanine suggested m-tyrosine as a possible precursor of the 9 carbon fragment of gliotoxin. An experimental test of this hypothesis revealed that DL-m-tyrosine-H^3 was incorporated into gliotoxin to the

extent of 31-44 per cent. When the gliotoxin from the m-tyrosine was degraded to indole-2-carboxylic acid, the specific activity of the indole-2-carboxylic acid was found to be essentially the same as that of the gliotoxin (Table V). These data indicate that m-tyrosine is a precursor of gliotoxin and that the hydroxylation can occur before cyclization of the side chain of phenylalanine.

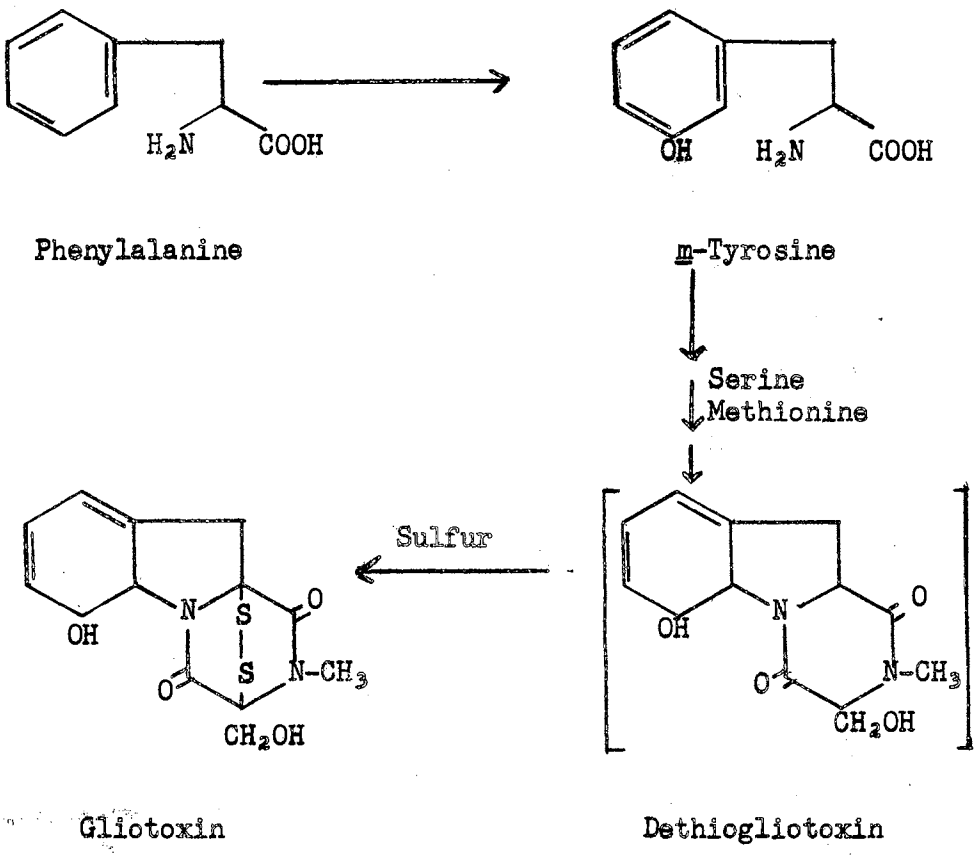
Experiments using DL-methionine-CH_3-C^{14} as an N-methyl donor for gliotoxin was also conducted. DL-methionine-CH_3-C^{14} was incorporated into gliotoxin to the extent of 1.7-3.4 per cent. The alkaline degradation of the labeled gliotoxin to methylamine showed that 72 per cent of the radioactivity was in the N-methyl group (Table IV). Since serine-3-C^{14} and glycine-2-C^{14} were shown to be less efficient as methyl donors (29 and 19 per cent vs. 72 per cent), it is thus indicated that the methyl group of methionine is a more direct methyl donor in gliotoxin biosynthesis.

Since the remaining three carbons of gliotoxin (carbons 3, 3a, and 4) are structurally related to serine, it seemed reasonable that this amino acid might serve as a precursor. The studies conducted showed an incorporation of carbon-14 labeled serine into gliotoxin to the extent of 0.7-2.7 per cent (Table III). The low incorporation of serine (0.7 per cent) can be attributed to the early addition (Table I). In 1945, Dutcher, Johnson and Bruce (40) reported the formation of acetic acid from dethiogliotoxin by the Kuhn-Roth procedure. Bell (63) proposed that the acetic acid formed by this degradation arose from carbons 3 and 3a. The gliotoxin into which the serine-3-C^{14} had been incorporated was degraded by the Kuhn-Roth method. The distillate,

which should have contained carbons 3 and 3a, had essentially no radioactivity, but the CO_2 collected during reflux was radioactive (Table VI). To determine whether or not serine gave rise to acetic acid, as reported by Johnson and Buchanan (42), both serine-3-C^{14} and serine-1-C^{14} were degraded, and essentially all of the radioactivity was evolved as CO_2 during reflux. On the basis of these data, it appears that the Kuhn-Roth degradation of dethiogliotoxin does not specifically yield acetic acid from carbons 3 and 3a. Subsequent degradations of the gliotoxin from the serine-3-C^{14} showed that 19 per cent of the radioactivity was in indole-2-carboxylic acid and 29 per cent was in the N-methyl group. Thus, 52 per cent of the radioactivity is located in carbons 3, 3a, and 4. The specific location of the radioactivity in these three carbons has not been determined. Results of experiments using serine-1-C^{14} showed that no radioactivity was present in the indole-2-carboxylic acid or the N-methyl group of gliotoxin. Thus, all of the radioactivity was in carbons 3, 3a, and 4. The fact that all the radioactivity from serine-1-C^{14} is located in carbons 3, 3a, and 4 strongly suggests that this amino acid (or possibly glycine) is a direct precursor for this portion of gliotoxin.

Since conversion of glycine to serine is known, glycine-2-C^{14} was studied (65). Glycine was incorporated into gliotoxin to a lesser extent than serine. On the basis of the data obtained from the serine and glycine studies, it is postulated that glycine is converted to serine which, in turn, serves as a precursor for carbons 3, 3a, and 4 of gliotoxin.

Since the hydroxylation of phenylalanine can occur before cyclization of the side chain of phenylalanine, reduction of the aromatic ring probably occurs in a subsequent step. On the basis of this assumption and the data presented in this study, the following pathway for the biosynthesis of gliotoxin, which accounts for all of the carbon atoms, is proposed.



CHAPTER V

SUMMARY

1. In this study, the biosynthesis of gliotoxin was investigated. This antibiotic, which contains a dihydroindoline nucleus, is produced by the fungus, Trichoderma viride. These experiments were conducted by using radioactive compounds.

2. The incorporation of radioactivity from the following compounds into gliotoxin was determined: DL-phenylalanine-H^3, DL-m-tyrosine-H^3, DL-methionine-CH_3-C^{14}, DL-serine-3-C^{14}, DL-serine-1-C^{14}, and glycine-2-C^{14}.

3. Degradations of gliotoxin to indole-2-carboxylic acid showed that the radioactivity from phenylalanine and m-tyrosine was located in this moiety.

4. The N-methyl group of the gliotoxin produced from methionine-CH_3-C^{14}, serine-3-C^{14}, glycine-2-C^{14}, and serine-1-C^{14} was found to contain 72, 29, 19, and 0 per cent of the radioactivity, respectively.

5. Radioactive gliotoxin produced from serine-3-C^{14} was degraded to indole-2-carboxylic acid. This product contained 19 per cent of the radioactivity.

6. All of the radioactivity incorporated into the gliotoxin from serine-1-C^{14} and 52 per cent from serine-3-C^{14} was in carbons 3, 3a, and 4.

7. A biosynthetic pathway, which accounts for all 13 carbons of gliotoxin, is proposed.

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VITA

Jack Alan Winstead

Candidate for the Degree of

Master of Science

Thesis: THE BIOSYNTHESIS OF GLIOTOXIN

Major Field: Chemistry

Biographical:

Personal Data: Born near Dixon, Kentucky, June 13, 1932, the son of Roscoe and Venice Winstead.

Education: Attended grade school in Dixon, Kentucky; graduated from Dixon High School in 1950; received the Bachelor of Science degree from the University of Kentucky, with a major in Agriculture, in June, 1954; completed requirements for the Master of Science degree in January, 1959.

Professional experience: Research assistant, University of Kentucky, summer 1954; entered the United States Air Force in 1954, conducted research and analysis by vacuum fusion techniques and emmission spectroscopy, and is now a 1st. Lieutenant.

Member of Phi Lambda Upsilon

Date of Final Examination: January, 1959