[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY AND CHEMICAL ENGINEERING, UNIVERSITY OF ILLINOIS]

Biosynthesis of Gliotoxin. I.[1] Incorporation of Phenylalanine-1- and -2-C^{14}

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Trichoderma viride incorporated the isotope from phenylalanine-1-C^{14} and phenylalanine-2-C^{14} into the gliotoxin, which it secretes into the culture medium, to the extent of 4–12%. Alkaline degradation of gliotoxin from phenylalanine-1-C^{14} resulted in indole-2-carboxylic acid which retained 82% of the radioactivity, and essentially all of the C^{14} was in the carboxyl carbon. Thus, phenylalanine is a direct precursor of the indole moiety of gliotoxin. Tryptophan-7a-C^{14} and acetate-2-C^{14} were not incorporated. Methionine-CH_3-C^{14} was incorporated to a slight extent and may serve as the source of the N-methyl group.

Falck[4] reported in 1931 that *Trichoderma viride* prevented the decay of wood by *Coniophora cerebella*. He suggested that "enzymes" of *T. viride* were toxic to the wood destroying fungus. In 1932 Weindling[5] observed an antagonism between *T. viride* and the soil fungus *Rhizoctonia solani* and concluded that this antagonism was due to toxic substances produced by the *Trichoderma*. This toxic substance was later isolated in crystalline form by Weindling and Emerson[6] and was named gliotoxin[7] since Weindling had referred to the fungus which produces it as *Gliocladium fimbriatum*.[8] Brian[9] later reported that the organism from which gliotoxin was isolated was actually *T. viride*.

As a result of a series of investigations, Johnson and his collaborators[10,11] proposed that gliotoxin has a pentacyclic structure. More recently Bell, *et al.*,[12] have proposed a revised structure. Since gliotoxin has a reduced indole nucleus, tracer experiments were undertaken to determine the precursors of this system. This communication presents evidence that neither tryptophan nor acetate are direct precursors but phenylalanine apparently contributes nine carbons to the gliotoxin structure. The methyl-C^{14} of methionine was incorporated slightly.

Experimental

Fermentation Procedure.—The organism used in these experiments was *T. viride*, No. 1828 NRRL which was kindly supplied to us by Dr. C. W. Hesseltine of the Northern Utilization Research Branch, Peoria, Illinois. It was subcultured on potato-dextrose agar and a suspension of spores from one culture tube was used for inoculating six liters of the medium of Johnson, Bruce and Dutcher.[10] The fermentation was done in subdued light and the temperature was maintained at 25° since little gliotoxin was produced when the temperature rose above 30°. The period required for maximum production of gliotoxin was five days and the yield of isolated compound was 25 to 95 mg./l., which compared favorably to the yield reported by Johnson, *et al.*[10]

Administration of Isotopes.—The C^{14} isotopically labeled precursors used in this study were added to the medium at one time or gradually starting at the end of the first day and continuing to the end of the fourth day. The compounds studied are shown in Table I.

TABLE I

LABELED COMPOUNDS ADDED TO THE FERMENTATION MEDIUM

Exp. no.	Isotopes	Specific activity, μc./m*M*	Amount of compd. added Mg.	Amount of compd. added mμc.	Time of addition, hr.
I-4	DL-Tryptophan-7a-C^{14}	153	2.7	2030	24–96
I-3	Sodium acetate-2-C^{14}	1000	3.2	39000	24–96
II-2	Sodium acetate-2-C^{14}	1000	3.8	46300	45
I-1	DL-Methionine-CH_3-C^{14}	420	0.5	1410	72
II-5	DL-Methionine-CH_3-C^{14}	420	1.0	2820	24–35
II-4	DL-Phenylalanine-1-C^{14}	422	1.91	4880	24–35
III-1	DL-Phenylalanine-1-C^{14}	422	2.0	5120	24–59
I-5	DL-Phenylalanine-2-C^{14}	840	0.57	2900	47–69
II-3	DL-Phenylalanine-2-C^{14}	840	1.08	5500	45–47

Isolation of Gliotoxin, Mycelium, Lipids and CO_2.—At the end of five days, the mycelium was separated by filtration and dried at 110°. The clear filtrate was extracted three times with 10% of its volume of chloroform. The chloroform was removed by vacuum distillation. The lipids were then removed from the chloroform residue by extraction three times with a total volume of 50 ml. of petroleum ether (b.p. 90–100°). The gliotoxin was dissolved in a minimum volume of methanol. This solution was treated with charcoal, crystallized at 2° overnight and purified to a constant melting point by recrystallization from methanol. The crystalline products from all experiments melted between 196–202° with decomposition on the Kofler block. The addition of authentic gliotoxin caused no depression of melting points.[13] The CO_2 released from the fermentation flask was collected in 10% sodium hydroxide from a stream of CO_2-free air.

Alkaline Degradation of Gliotoxin.—The gliotoxin was degraded according to the procedure of Dutcher, *et al.*[14] One hundred and ninety-six mg. of gliotoxin (100 mμc., Table II, exp. III-1) was refluxed with 10% barium hydroxide for 1.5 hr. Concentrated sulfuric acid was added to *p*H 2 and the insoluble barium sulfate was removed by filtration. The indole-2-carboxylic acid was sublimed at 110–115° *in vacuo* and was recrystallized from chloroform (yield 14 mg.; theoretical yield 97 mg.). The indole-2-carboxylic acid melted at 203–204° with no depression on mixing with authentic compound.[15] Combustion of 1.6 and 0.81 mg. of the isolated indole-2-carboxylic acid resulted in 1.4 and 0.66 mμc. Converted to a theoretical yield, this amounts to 85.0 and 79 mμc. or 85 and 79% (average 82%) of the C^{14} from the 100 mμc. of gliotoxin degraded.

Decarboxylation of Indole-2-carboxylic Acid.—5.36 mg. (4.5 mμc.) of the indole-2-carboxylic acid was mixed with

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(3) Taken in part from a thesis submitted by R. G. Chenoweth in partial fulfillment of the requirements for the Bachelor of Science degree in Chemistry, August, 1957.

(4) R. Falck, *Mitt. Forstwirt. Forstwiss.*, 480 (1931).

(5) R. Weindling, *Phytopathology*, **22**, 837 (1932).

(6) R. Weindling and O. H. Emerson, *ibid.*, **26**, 1068 (1936).

(7) R. Weiudling, *ibid.*, **31**, 991 (1941).

(8) R. Weiudling, *ibid.*, **27**, 1175 (1937).

(9) P. W. Brian, *Nature*, **154**, 667 (1944).

(10) J. R. Johnson, W. F. Bruce and J. D. Dutcher, THIS JOURNAL, **65**, 2005 (1943).

(11) J. R. Johnson and J. B. Buchanan, *ibid.*, **75**, 2103 (1953).

(12) M. R. Bell, J. R. Johnson, B. S. Wildi and R. B. Woodward, *ibid.*, **80**, 1001 (1958).

(13) Kindly supplied by Dr. James D. Dutcher, The Squibb Institute, New Brunswick, New Jersey.

(14) J. D. Dutcher, J. R. Johnson and W. F. Bruce, THIS JOURNAL, **66**, 614, 617 (1944).

(15) Kindly supplied by Dr. H. R. Snyder, Department of Chemistry, University of Illinois.

TABLE II

INCORPORATION OF RADIOACTIVITY INTO GLIOTOXIN[a]

Exp. no.	Substrate	Gliotoxin yield, mg.	C^{14} Incorporated mμc.	%
I-4	DL-Tryptophan-7a-C^{14}	568	0	0
I-3	Sodium acetate-2-C^{14}	390	6.8	.02
II-2	Sodium acetate-2-C^{14}	533	2.4	.005
I-1	DL-Methionine-CH_3-C^{14}	568	2.3	.16
II-5	DL-Methonine-CH_3-C^{14}	258	7.5	.27
II-4	DL-Phenylalanine-1-C^{14}	195	411	8.4
III-1	DL-Phenylalanine-1-C^{14}	418	213	4.2
I-5	DL-Phenylalanine-2-C^{14}	140	359	12.4
II-3	DL-Phenylalanine-2-C^{14}	300	382	6.9

[a] See Table I for mg. and mμc. of substrate added.

probably reflects its conversion to more direct precursors. The slight incorporation of the methyl-C^{14} of methionine into gliotoxin suggests this compound as a possible methyl donor for the N-methyl group. To demonstrate that the lack of incorporation of tryptophan and acetate into gliotoxin was not due to a lack of permeability, the mycelium, lipids and carbon dioxide were analyzed for radioactivity and the results are shown in Table III.

The high incorporation of tryptophan and acetate into the mycelium and lipids provides evidence that these compounds are utilized for purposes other than gliotoxin synthesis.

The extensive incorporation of phenylalanine into gliotoxin suggested that this amino acid forms the

TABLE III

YIELDS OF C^{14} IN PRODUCTS FROM *T. viride*

Exp. no.	Substrate	Mycelium mμc.	%	Lipids mμc.	%	CO_2 mμc.	%	Total %
I-4	DL-Tryptophan-7a-C^{14}	870	43.0					43.0
I-3	Sodium acetate-2-C^{14}	15000	38.5	226	0.6			39.1
II-4	DL-Phenylalanine-1-C^{14}	240	4.9	320	6.6			11.5
III-1	DL-Phenylalanine-1-C^{14}	811	15.8	126	2.4	1280	25.0	43.2

TABLE IV

Compd.	Degraded Mg.	Degraded mμc.	Degradation product formed: Indole-2-carboxylic acid Mg.	Indole-2-carboxylic acid mμc.	Indole-2-carboxylic acid %	CO_2 Mg.	CO_2 mμc.	CO_2 %
Gliotoxin	196	100	97[a]	82	82	...	..	..
Indole-2-carboxylic acid	12.88[b]	4.5	..	..	..	2.73[c]	3.7	82

[a] Theoretical yield. See Experimental: Alkaline Degradation of Gliotoxin. [b] This figure represents 5.36 mg. of radioactive indole-2-carboxylic acid plus 7.52 mg. of carrier indole-2-carboxylic acid. [c] Represents 77.5% decarboxylation of the indole-2-carboxylic acid. If the decarboxylation were 100%, essentially all of the isotope would be in the CO_2.

7.52 mg. of carrier and decarboxylated to indole and CO_2 by heating a break-seal tube evacuated to 10^{-4} mm. in an electric oven at 225–230° for 1 hr. 0.062 mmole or 2.73 mg. of CO_2 was released (77.5% yield. Theoretical decarboxylation of the 12.88 mg. of indole-2-carboxylic acid is 0.080 mmoles or 3.5 mg.). The CO_2 was counted and 3.7 mμc. was present.

C^{14} Counting Procedure.—An aliquot of the 10% NaOH used for trapping the CO_2 was added to a combustion tube. Perchloric acid (35%) was added, and the CO_2 released was collected in an ionization chamber in the same manner. The samples of mycelium, lipid, gliotoxin and indole-2-carboxylic acid were combusted according to the procedure of Van Slyke, Steele and Plazin,[16] and the C^{14} was measured with the vibrating reed electrometer.

Results and Discussion

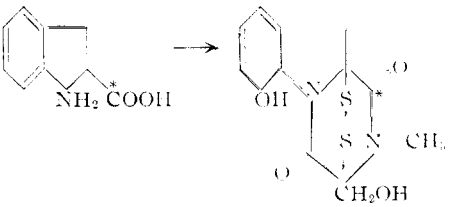
By using a chemically defined fermentation medium, it has been possible to study the incorporation of C^{14}-labeled isotopes into gliotoxin. The yields of C^{14} in the isolated gliotoxin from the substrates used are shown in Table II.

It is clear from the data shown in Table II that tryptophan, although structurally similar to the reduced indole moiety of gliotoxin, is not a direct precursor. The slight incorporation of acetate

(16) D. D. Van Slyke, R. Steele and J. Plazin, *J. Biol. Chem.*, **192**, 769 (1951).

reduced indole structure by closure of the side chain on the 6-membered ring through the α-amino group. The results of the degradation shown in Table IV indicate that essentially all of the C^{14} from phenylalanine-1-C^{14} was located in the carboxyl carbon of indole-2-carboxylic acid.

The evidence obtained to date indicates that the function of phenylalanine, in the production of gliotoxin by *T. viride*, is concerned with the formation of a nine carbon fragment which can be isolated as indole-2-carboxylic acid by alkaline degradation. The incorporation of phenylalanine-1-C^{14} into gliotoxin can be shown as



Phenylalanine Gliotoxin

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