Biosynthetic Studies on Gliotoxin Using Stable Isotopes and Mass Spectral Methods[1]

Ajay K. Bose,[2a,b] K. G. Das,[2b] P. T. Funke,[2b] Irene Kugajevsky,[2b] O. P. Shukla,[2b] K. S. Khanchandani,[2b] and R. J. Suhadolnik[2c]

Contribution from the Department of Chemistry and Chemical Engineering, Stevens Institute of Technology, Hoboken, New Jersey 07030, and Research Laboratories, Albert Einstein Medical Center, Philadelphia, Pennsylvania 19141. *Received May 6, 1967*

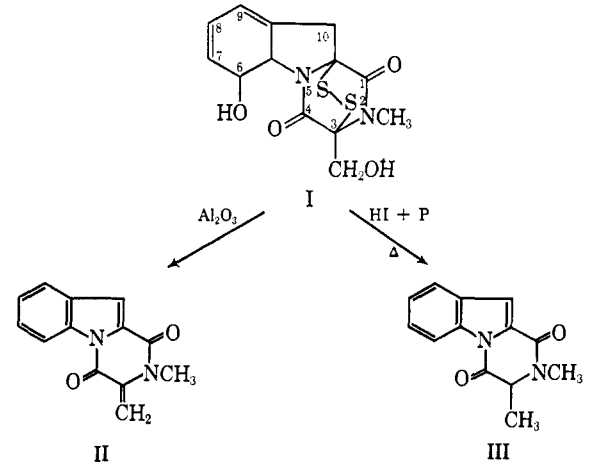
Abstract: The biosynthesis of gliotoxin has been studied using ^{15}N- and ^{13}C-labeled compounds and the organism *Trichoderma viride* grown under aerobic conditions. Mass spectrometry was employed for determining the extent and the location of isotope labeling. The desthio derivative II, obtained by passing I through a column of alumina, was found suitable for spectral measurements. With milligram size samples of I or II and an analytical type of mass spectrometer, the isotope enrichment level could be determined rapidly. From a study of the fragmentation pattern of labeled and unlabeled II it was concluded that phenylalanine can be incorporated into I by at least two pathways: (a) intact and (b) *via* a nitrogen-free intermediate that undergoes reamination. Glycine can serve as the source of both nitrogen atoms in I.

In recent years, extensive use has been made of radioactive precursors, especially ^{14}C-labeled compounds, for studies on biosynthesis. Nonradioactive isotopes, such as ^{13}C and ^{15}N, have received much less attention partly due to the difficulty of detection of incorporation and the scarcity of labeled starting materials. Many of the starting materials have now become available commercially. Since the art of mass spectrometry has advanced to the stage that the details of fragmentation can be determined, a study of the fragmentation pattern with and without incorporation of isotopes should allow one to deduce the position(s) of the labeled atom(s) in most molecules. If the compound under such mass spectral examination be isolated from a biological system to which substrates labeled with nonradioactive isotopes had been added, valuable information on biosynthesis would become available without the need for chemical degradation associated with studies involving ^{14}C-labeled precursors.

For examining the scope and limitations of the mass spectral approach to studies on biosynthesis, we have initiated an investigation on gliotoxin produced by the organism *Trichoderma viride*. Some aspects of the biosynthesis of this antibiotic have been established by the use of ^{14}C-labeled compounds.[3] Since incorporation levels have been reported as quite high, exceeding 10% in some cases,[4] this system represents a convenient test case. Although the biosynthetic origin of the carbon skeleton has been shown to arise from phenylalanine and glycine or serine,[4] no information is available as to the source of the nitrogen atoms in gliotoxin.

An essential requirement for obtaining a satisfactory mass spectrum is that the compound to be studied must be thermally stable at the temperatures necessary to attain a pressure of $\sim 10^{-3}$ mm in the heated inlet of the mass spectrometer used. It must also give a reasonably simple fragmentation pattern. If one is interested only in the total incorporation of isotopes, direct insertion in the source of a mass spectrometer will be sufficient and the compound need only be stable at the temperature necessary to volatilize it to generate a pressure of $\sim 10^{-5}$ mm. Gliotoxin was found to decompose thermally in the heated inlet. In the direct inlet, the molecular ion was very weak or nonexistent; however, the $(M - S_2)^+$ peak was obtained; this proved satisfactory for the determination of the total isotope enrichment level[5] greater than 0.5%.

Since gliotoxin was not found suitable for quantitative mass spectral studies it was necessary to obtain a derivative which could fulfill the conditions set above. After some experimentation, the aromatic desthio compound II was found satisfactory. This derivative is readily obtained by passing a solution of gliotoxin through an activated alumina (Alcoa, grade F-2) column.[6] Some of the earlier studies were carried out on another derivative, III, which is prepared by the treatment of I with red phosphorus and hydriodic acid,[7]



(1) Biosynthetic Studies. I. Presented at the Fourth International Symposium on the Chemistry of Natural Products, Stockholm, June 1966, Abstract 150.

(2) (a) To whom inquiries should be directed; (b) Stevens Institute of Technology; (c) Albert Einstein Medical Center.

(3) R. J. Suhadolnik and R. G. Chenoweth, *J. Amer. Chem. Soc.*, **80**, 4391 (1958).

(4) J. A. Winstead and R. J. Suhadolnik, *ibid.*, **82**, 1644 (1960).

(5) The enrichment level is calculated by taking the intensity of a fragment P as 100 in both the labeled and the unlabeled species and subtracting the intensity of the P + 1 peak of the unlabeled compound from that of the P + 1 peak of the labeled compound.

(6) J. S. Warner, Ph.D. Thesis, Cornell University, 1953.

(7) J. D. Dutcher and J. R. Johnson, *J. Amer. Chem. Soc.*, **66**, 617 (1944).

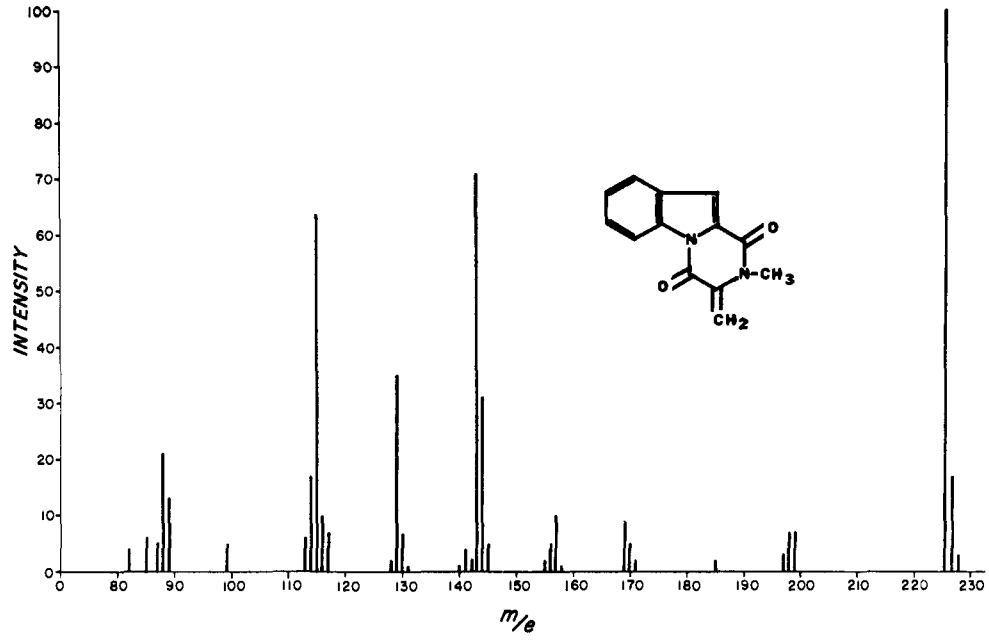


Figure 1.

but difficulties were encountered in the conversion of I into III in a reproducible fashion and the use of III was discontinued in favor of II.

Experimental Section

A Consolidated Electrodynamics Corporation Model 21-103C mass spectrometer equipped with a thermostated, all-glass heated inlet was employed. The ion source was maintained at 250° and a 10-μA ionizing current of electrons of 70-eV energy was used for ionizing the sample. All spectra were recorded under nonfocused conditions. Compounds II and III were volatalized into a 3-l. bulb maintained at 150°. Standard analytical procedures were followed in the analysis. The magnet current was maintained at 484 mA and spectra were scanned starting at 3000 V.

It was found that the instrument had to be "conditioned" to the sample for a period of 2 hr at which time spectra were reproducible to 2%. This "conditioning" was not very important if the peak P corresponds to the molecular ion so that the P + 1 peak is the contribution of naturally occurring isotopes only. However, if this P + 1 peak is a fragment ion, then the previous criteria must be followed including careful control of the pressure of the sample. The pressure was maintained in the range sufficient to give accuracy but at the same time to minimize changes in fragmentation due to possible ion–molecule reactions.

The organism *Trichoderma viride* was grown under aerobic conditions in a chemically defined medium to which a trace of peptone had been added.[3] The following labeled compounds were introduced in the medium after the rapid growth of mycelia had ceased (about 60 hr): ^{15}N-glycine, ^{15}N-phenylalanine, ^{13}C-2-glycine, ^{13}C-formate (the isotope level was 50–60% for ^{13}C compounds and 90–95% for ^{15}N compounds). The gliotoxin that was harvested a few days later was converted to the derivative II or III. In each experiment about 50 mg of the labeled compound was added to a medium of about 1.5 l. The amount of recrystallized gliotoxin obtained ranged between 30 and 100 mg.

Results

Table I shows the total per cent isotope enrichment of gliotoxin as measured from the spectra of compounds II and III. In order to determine the location of the isotope incorporated, it is necessary to identify the composition of the ions which have relatively large intensities. A detailed examination was made of the spectrum of II since most of our experiments involved this compound. The molecular ion is the base peak for II (Figure 1) and there are several peaks with intensity exceeding 20% of that of the base peak. A high-resolution mass spectrum[8] of II established the composition of various fragments, some of which are shown in Table II. Some of the peaks are doublets, for example, the one at nominal mass 143; some fragments (*viz.*, *m/e* 129) must involve skeletal rearrangement. Obviously such peaks are not suitable for the exact calculation of isotope enrichment unless care is taken to ensure that the spectra are recorded under uniform conditions and are reproducible. It is our experience, however, that by using an analytical type mass spectrometer and exercising reasonable care, even such peaks can provide at least a qualitative indication of isotope incorporation.

Table I

Substrate	Expt	Isotope enrichment,[a] %
^{15}N-Glycine	1	8.9[b]
	2	2.8
	3	8.3
	4	8.3
^{15}N-Phenylalanine	1	4.4[b,c]
	2	1.3
	3	0.6
	4	3.9
	5	4.0
2-^{13}C-Glycine	1	1.4
	2	2.5
1-^{13}C-Glycine	1	2.4
^{13}C-Formate	1	1.0
	2	2.5

[a] Based on compound II unless mentioned otherwise. [b] Based on compound III. [c] Due to the smallness of the quantity of material available, the error is probably quite large. In other experiments the error is about ±0.2.

By analogy with the mass spectra of numerous indole alkaloids,[9] it can be assumed that the fragment of *m/e*

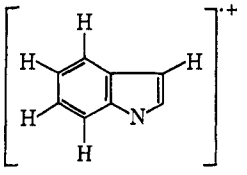
(8) Recorded on a double-focusing mass spectrometer (CEC 21-110) at Hoffman-LaRoche, Inc., Nutley, N. J.

(9) K. Bieman, "Mass Spectrometry," McGraw-Hill Book Co., Inc., New York, N. Y., 1962; H. Budzikiewicz, C. Djerassi, and D. H.

Table II. High-Resolution Mass Spectrum of Compound II; Selected Peaks

m/e	Compn	Fragment
226	$C_{13}H_{10}N_2O_2$	$[M]^+$
199	$C_{12}H_9NO_2$	$[M - HCN]^+$
198	$C_{12}H_{10}N_2O$	$[M - CO]^+$
197	$C_{12}H_7NO_2$	$[M - NCH_3]^+$
	$C_{12}H_9N_2O$	$[M - CHO]^+$
170	$C_{11}H_{10}N_2$	$[M - 2CO]^+$
144	C_9H_6NO	
143	C_9H_5NO	
	$C_{10}H_9N$	
130	C_9H_8N	
129	C_9H_7N	
116	C_8H_6N	
115	C_8H_5N	

115 corresponds to IV (or its high-energy equivalents). The fragments of nominal mass 143 also must contain the indole moiety. It is very likely that in these ions, the nitrogen is derived from the indole system (*i.e.*, N_5) although some contribution from the other nitrogen (*i.e.*, N_2) cannot be ruled out without further investigation.[10] The fragmentation of III also leads to ions of *m/e* 115 and 143. Although their composition has not been verified by high-resolution mass spectrometry, it appears reasonable to assume that these ions have the same composition and genesis whether they are derived from II or III.



IV
m/e 115

In an early incorporation experiment using ^{15}N-glycine, a very high enrichment level (8.9%) was reached (see Table I). For compound III derived from this sample, the isotope level data are shown in Table III. The data based on II are reported in Table IV. In these tables, the increment Δ is defined by eq 1 and 2 where *P* refers to the height of the peak at *m/e* = P.

$$I_{rel} = [(P + 1)/P]\,(100\%) \quad (1)$$

$$\Delta = \text{labeled } I_{rel} - \text{unlabeled } I_{rel} \quad (2)$$

For the molecular ion, Δ represents the isotope enrichment level. For other ions this is not entirely true unless corrections[11] are made for the enhanced isotope content of the adjoining fragments P + 1, P − 1, etc.; in general these Δ values represent the upper limit of the isotope enrichment. However, they do serve to give a qualitative picture of the isotope distribution.[11] From Tables III and IV it becomes apparent that when ^{15}N-glycine is added to the medium, both N_2 and N_5 in gliotoxin are labeled. The carbon atoms of glycine, however, do not seem to become incorporated in the indole moiety.

Table III. Incorporation Data on Compound III

Fragment P, *m/e*	Fragment	Compound III from ^{15}N-glycine Δ(±0.2), %
228	M^+	8.9
213	$[M - CH_3]^+$	8.9
143		2.3
115		2.3

Table IV. Incorporation Data on Compound III

Fragment P, *m/e*	Compound II from substrate shown: ^{15}N-Phenylalanine	^{15}N-Glycine	2-^{13}C-Glycine	1-^{13}C-Glycine
	Δ			
226	1.3	2.8, 10.4	1.4	2.4
143	1.0	0.8, 3.5	0.3	0.0
115	0.8	0.6, 3.2	0.0	0.7

Discussion

From these studies, it has been found that a moderately pure sample of gliotoxin of about 0.1-mg size is adequate for determining whether significant incorporation (>0.5% isotope enrichment) has occurred. Using the direct inlet it is necessary to scan only the upper part of the spectrum; the usual impurities are of much lower molecular weight than gliotoxin and therefore do not affect the region near the $(M - S_2)^+$ peak for gliotoxin. Repeated runs with unlabeled I have shown that the relative intensity of the peaks at *m/e* 262 and 263 is 100:15.5. Any significant increase in this relative intensity indicates incorporation of a stable isotope. The isotope enrichment data provided by this approach are not as accurate as those from the mass spectra of II recorded under standardized conditions, but they can be obtained quickly and with no chemical manipulation. The convenience and the saving in time in using this technique for routine incorporation studies are obvious.

We have found that ^{15}N-phenylalanine, 2-^{13}C-glycine, ^{15}N-glycine, 1-^{13}C-glycine, and ^{13}C-formate are incorporated in gliotoxin. This is in agreement with the observations of Winstead and Suhadolnik[4] who used ^{14}C-labeled compounds. The data in Table I show that the level of incorporation with the same substrate varied considerably from experiment to experiment. Work is in progress for defining the conditions for optimal incorporation.

Winstead and Suhadolnik[4] have demonstrated that glycine is a precursor for gliotoxin, but since they used ^{14}C label, they did not obtain any information on the fate of the nitrogen. The randomization of the nitrogen of glycine observed by us may well be attributed to the metabolism of glycine to the "active formate"[12] which releases the amino group into a pool in the cell. This amino nitrogen pool appears to be used for the formation of a substantial amount of phenylalanine,

Williams, "Structure Elucidation of Natural Products by Mass Spectrometry," Vol. 1, Holden-Day, Inc., San Francisco, Calif., 1964.

(10) We have plans for the synthesis of II with ^{15}N label at a specific site to determine whether any scrambling of nitrogen takes place under electron impact.

(11) An efficacious calculation (with the aid of a computer program) which includes such corrections is under study. Preliminary calculations based on a set of simultaneous equations for the cluster of peaks around *m/e* 143 and 115 are compatible with the qualitative conclusions regarding isotope distribution based on uncorrected Δ values.

(12) B. D. Davis, *Advan. Enzymol.*, **16**, 247 (1955).

probably by the well-known transamination process.[13]

Winstead and Suhadolnik have demonstrated that 1-^{14}C-phenylalanine is incorporated in gliotoxin. In view of the present finding that ^{15}N-phenylalanine also undergoes incorporation, this indicates that intact phenylalanine can provide the indolecarboxylic acid moiety in II. Thus, phenylalanine incorporation in I can proceed along at least two pathways: (a) intact, (b) *via* a nitrogen-free intermediate which undergoes transamination. Work is in progress for obtaining further information about these pathways.

Conclusion

The mass spectral technique appears quite feasible for determination of isotope labeling from biosynthetic studies. However, about 20 mg of unlabeled samples must be available for purposes of establishing optimum operating conditions for the mass spectrometer. Furthermore, in order to determine the exact location of the labeled atom great care must be taken in stabilizing the conditions in the mass spectrometer and the peaks used for calculation chosen with due consideration to several factors. For achieving adequate accuracy in the measurements on a fragment of *m/e P*, the P + 1 peak should be small and preferably due only to naturally occurring isotopes. The P − 1 peak also should be quite small; its intensity should not exceed 10% of that of the P ion. It is very desirable to study the high-resolution mass spectrum of the compound to avoid errors caused by molecular rearrangement and unusual modes of fragmentation. Not only are proton transfers quite common under electron impact, but many cases of skeletal rearrangement also are now well authenticated.[9,14] Extra caution has to be exercized in using deuterium-labeled precursors.

For most compounds, it should be possible to prepare some simple derivative which would satisfy the above requirements. Hence, after some preliminary mass spectral studies and chemical work, a routine can be set up for extended biogenetic studies involving much less human labor than in the usual ^{14}C experiments. Since nitrogen has no radioactive isotope, the use of ^{15}N labeling and mass spectroscopy assumes considerable importance for biosynthetic studies on the numerous polynitrogen compounds found in nature. There is every indication that in coming years, mass spectrometers will have much higher sensitivity than now and therefore detection of isotopes at enrichment levels of 0.01% or even less will be reliable. The use of stable isotopes and mass spectral methods can be expected to become increasingly important for studies on biosynthesis and metabolism.[15]

Acknowledgment. Partial support for this investigation by a U. S. Public Health Research Grant (GM 12122) to Dr. A. K. Bose and National Science Foundation Grant (GB-4487) to Dr. R. J. Suhadolnik is gratefully acknowledged. Thanks are extended to Dr. F. Vane and Dr. L. Jampolsky of Hoffman-LaRoche, Inc., for the high-resolution spectrum of compound II.

(13) The chemically defined medium used for these experiments is free of amino acids except for a very small quantity represented by the peptone added.

(14) H. Budzikiewicz, C. Djerassi, and D. H. Williams, "Interpretation of Mass Spectra of Organic Compounds," Holden-Day, Inc., San Francisco, Calif., 1964; P. Brown and C. Djerassi, *Angew. Chem. Intern. Ed. Engl.*, **6**, 477 (1967).

(15) For recent reports on the use of mass spectroscopy in conjunction with ^{15}N- and deuterium-labeled substrates, see G. R. Waller, R. Ryhage, and S. Meyerson, *Anal. Biochem.*, **16**, 277 (1966); E. S. Hall, F. McCapra, T. Money, K. Fujumoto, J. R. Hausou, B. S. Mootoo, G. T. Phillips, and A. I. Scott, *Chem. Commun.*, 348 (1966); M. Lengant, E. Zissman, and E. Lederer, *Tetrahedron Letters*, 1049 (1967).