The Biosynthetic Incorporation of [*phenyl*-^{3}H]Phenylalanine into Gliotoxin

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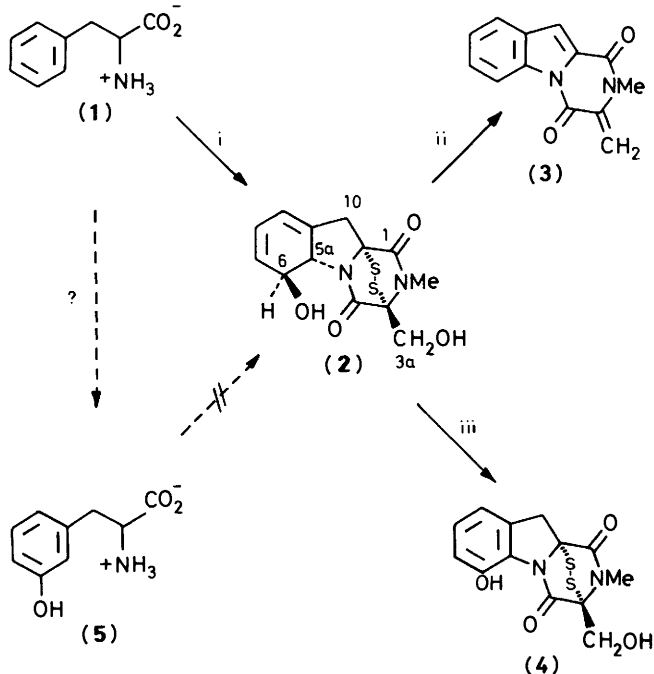
When mixtures of DL-[2′,4′,6′-$^3H_3$]-*m*-tyrosine and DL-[1-^{14}C]phenylalanine were fed to cultures of *Gliocladium deliquescens* good incorporation (4.9 and 1.6% in separate experiments) of ^{14}C and negligible incorporation (*ca.* 0.04%) of ^{3}H into the derived gliotoxin (**2**) was observed. Incorporation of DL-[1-^{14}C, 3′-^{3}H]-, DL-[1-^{14}C, 2′-^{3}H]-, and L-[1-^{14}C, 4′-^{3}H]-phenylalanine into gliotoxin occurred without loss of tritium. Degradation of the [^{14}C, ^{3}H]gliotoxin gave, in each case, bis(anhydrodethio)gliotoxin (**3**) and dehydrogliotoxin (**4**) having a tritium content indicating that the incorporation of phenylalanine had occurred without migration of tritium. These results, taken together, show that neither *m*-tyrosine (3′-hydroxyphenylalanine) nor any other hydroxybenzene derivative can be an obligatory intermediate on the biosynthetic pathway from phenylalanine to gliotoxin. The possible involvement of arene oxides in gliotoxin biosynthesis is discussed.

Suhadolnik and Chenoweth [1] showed that the reduced indole nucleus of gliotoxin (2) is derived biosynthetically from phenylalanine (1) rather than tryptophan (Scheme 1). Both



Scheme 1. *Reagents:* i, *G. deliquescens*; ii, Al_2O_3; iii, *o*-chloranil

DL-[1-^{14}C]- and DL-[2-^{14}C]phenylalanine were incorporated efficiently (4—12%) into gliotoxin in cultures of the fungus *Trichoderma viride* (more recently described as *Gliocladium deliquescens*). Degradation of gliotoxin derived from DL-[1-^{14}C]phenylalanine showed that radioactivity resided largely at position 1, indicating that the carbon skeleton of the amino acid had been incorporated intact; this conclusion has been amply confirmed by later work.[2] Winstead and Suhadolnik [3] reported a high incorporation (30—44%) of tritium into gliotoxin when generally tritiated DL-*m*-tyrosine (DL-3′-hydroxyphenylalanine) (5) was fed to the fungus, and proposed the biosynthetic sequence, (1) ⟶ (5) ⟶ (2). However, subsequent attempts by three independent research groups to substantiate this finding have failed. Bu'Lock and Ryles [4] fed DL-[1-^{14}C]phenylalanine and DL-[1-^{14}C]-*m*-tyrosine in parallel to *G. deliquescens* and observed good incorporation (5.4%) of the former and negligible incorporation (0.053%) of the latter into gliotoxin. A similar, negative result was obtained with *o*-tyrosine and 2′,3′-dihydroxyphenylalanine. More compellingly, incorporation of DL-[2′,3′,4′,5′,6′-$^2H_5$]phenylalanine was shown by mass spectrometry to give pentadeuteriogliotoxin as the major deuteriated species. This clearly excludes any hydroxybenzene derivative from consideration as an obligatory intermediate in gliotoxin biosynthesis. Our own experiments,[5] using tritium rather than deuterium but leading to the same conclusion, will be described in the sequel. Finally, Brannon *et al.*[6] showed that DL-[3-^{14}C]phenylalanine was incorporated much more efficiently than DL-[2-^{14}C]-*m*-tyrosine into gliotoxin in both *G. deliquescens* and *Penicillium terlikowskii*.

Radiolabelling of DL-*m*-tyrosine (5) was effected in tritiated 5M-hydrogen chloride at 100 °C for 1 h. A control experiment in DCl showed (^{1}H n.m.r. spectroscopy) that exchange had occurred, as expected,[7] *ortho* and *para* to the phenolic hydroxy group and not to any detectable extent in the side chain. DL-[2′,4′6′-$^3H_3$]-*m*-Tyrosine was fed to *G. deliquescens* [strain NRRL-1828] under the standard conditions.[1,3] Only a small incorporation (0.0095%) of tritium into gliotoxin was observed (Table 1) although it was shown, in a control experiment, that no significant loss of tritium from *m*-tyrosine occurred under the same conditions in the absence of the organism. Furthermore, when a mixture of DL-[2′,4′,6′-$^3H_3$]-*m*-tyrosine and DL-[1-^{14}C]phenylalanine (^{3}H:^{14}C ratio, 11.5) was fed, the derived gliotoxin contained barely detectable amounts of tritium (^{3}H:^{14}C ratio, *ca.* 0.1) although good incorporation (4.9%) of ^{14}C was observed. A similar result was obtained when the relative weights of the amino acids was changed *ca.* 300-fold. These results contrasted markedly with those of Winstead and Suhadolnik [3] and cast doubt on the status of *m*-tyrosine as a precursor for gliotoxin. However, it was conceivable that the low incorporation of *m*-tyrosine was a consequence of poor uptake of the amino acid by the fungus under our particular culture conditions. The following experiments using phenylalanine tritiated in the phenyl group were therefore carried out.

Hydroxylation of phenylalanine to give *m*-tyrosine must involve either loss or migration, by the familiar NIH shift, of hydrogen from the site of attack. To test this possibility, DL-[3′-^{3}H]phenylalanine [8] was mixed with DL-[1-^{14}C]phenylalanine and fed to *G. deliquescens*. No loss of tritium (Table 1) occurred during incorporation into gliotoxin. Dehydration

Table 1. Incorporation of phenylalanine (**1**) and *m*-tyrosine (**5**) into gliotoxin (**2**) in *Gliocladium deliquescens*

Precursor; wt. (mg)		$^3H:^{14}C$	% Incorp. (^{14}C)	Wt. of (**2**) (mg)	$^3H:^{14}C$ in (**2**)
DL-[2′,4′,6′-$^3H_3$]-*m*-Tyrosine	20	11.5	4.9	233	*ca.* 0.1
DL-[1-^{14}C]Phenylalanine	0.07				
DL-[2′,4′,6′-$^3H_3$]-*m*-Tyrosine	9.6	11.9	1.6	159	*ca.* 0.1
DL-[1-^{14}C]Phenylalanine	9.3				
DL-[2′,4′,6′-$^3H_3$]-*m*-Tyrosine	8.6		0.0095*	215	
DL-[1-^{14}C, 3′-^3H]Phenylalanine	13	7.1	3.3	189	7.6
DL-[1-^{14}C, 3′-^3H]Phenylalanine	17	6.5	2.3	215	6.7
DL-[1-^{14}C, 2′-^3H]Phenylalanine	13	38.2	7.5	277	36.2
L-[1-^{14}C, 4′-^3H]Phenylalanine	0.01	4.7	7.5	333	4.9

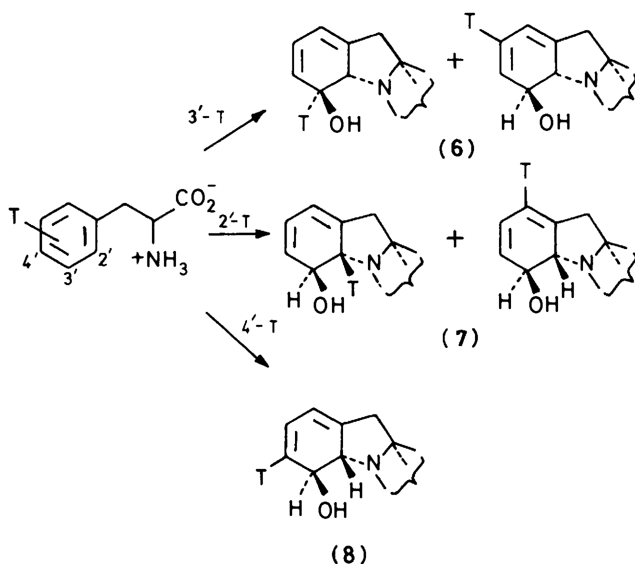
* % Incorporation of ^3H.

Table 2. Degradation of gliotoxin (**2**) derived from phenylalanine (**1**) to give bis(anhydrodethio)gliotoxin (**3**) and dehydrogliotoxin (**4**)

	Gliotoxin (**2**)		(**3**)		(**4**)	
Precursor (**1**)	^{14}C*	$^3H:^{14}C$	^{14}C*	$^3H:^{14}C$	^{14}C*	$^3H:^{14}C$
DL-[1-^{14}C, 3′-^3H]	0.58	7.5	0.52	7.5	0.57	3.9
DL-[1-^{14}C, 3′-^3H]	0.41	6.7	0.39	6.8	0.40	3.6
DL-[1-^{14}C, 2′-^3H]	0.21	36.2	0.19	17.2	0.20	17.6
L-[1-^{14}C, 4′-^3H]	0.62	4.9	0.56	4.7	0.61	4.6

* Specific activity; μCi/mmol.

and desulphurisation gave[2a] bis(anhydrodethio)gliotoxin (**3**)* (Scheme 1) without loss of tritium (Table 2), whereas dehydrogenation gave[9] dehydrogliotoxin (**4**) with loss of 48% of the tritium. Repetition of the entire experiment gave essentially the same results which are consistent with the labelling pattern (**6**) (Scheme 2). Clearly, neither loss nor overall migration of tritium



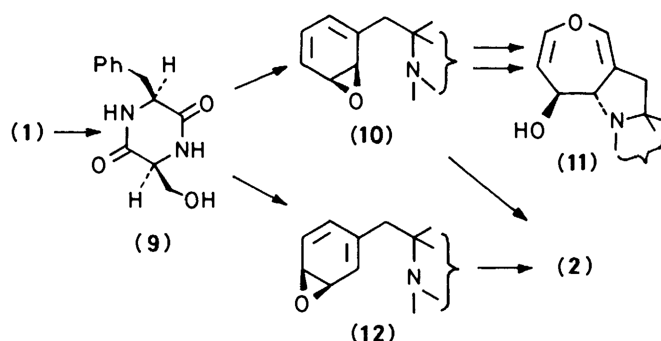
Scheme 2.

occurs during the incorporation of [3′-^3H]phenylalanine into gliotoxin. The observed location of only half the tritium at position 6 merely reflects the symmetry of the phenyl group in the precursor. However, this result alone does not rigorously exclude *m*-tyrosine from consideration as a precursor of gliotoxin. It could be argued that hydroxylation of phenylalanine occurs with migration, and subsequent complete retention, of tritium to a neighbouring carbon and that incorporation of *m*-tyrosine into gliotoxin then occurs with migration of tritium in the reverse sense. This possibility was discounted in the following way. A mixture of DL-[2′-^3H]-phenylalanine and DL-[1-^{14}C]phenylalanine gave gliotoxin without significant loss of tritium. Degradation of the gliotoxin, as before, gave results (Table 2) consistent with the labelling pattern (**7**). Clearly, tritium does not migrate from position 2′ in the precursor to position 6 in the gliotoxin. Finally, L-[4′-^3H]phenylalanine with L-[1-^{14}C]phenylalanine gave gliotoxin free from tritium at positions 5a and 6 [labelling pattern (**8**)]. These three experiments (Scheme 2), taken together, show that no hydroxylated benzene derivative lies on the biosynthetic pathway between phenylalanine (**1**) and gliotoxin (**2**). Further, not only are the three distinct phenyl hydrogens of the amino acid retained in gliotoxin but they remain at their original positions in the molecule.

The tritiated *m*-tyrosine used by Winstead and Suhadolnik[3] was prepared by the Wilzbach method and may well have been contaminated with highly radioactive phenylalanine, formed radiolytically. This could account for the observed incorporation of tritium into gliotoxin. The high value of this incorporation is still surprising but might have arisen from a synergistic effect of *m*-tyrosine on the secondary metabolism of phenylalanine.† Ali *et al.* reported[10] the incorporation of dehydrogliotoxin (**4**) into gliotoxin (**2**). Again, it now appears that this transformation can, at the best, be of only minor importance for gliotoxin biosynthesis and that dehydrogliotoxin cannot be an obligatory intermediate on the pathway. The most attractive biosynthetic proposal, fully consistent with the foregoing results, is that involving an arene oxide (Scheme 3). Neuss *et al.*[11] pointed out that the oxepin ring (**11**), characteristic of the aranotin group of metabolites, and the cyclohexadienol ring of gliotoxin (**2**) could be derived from a common arene oxide (**10**). Alternatively,[5] the isomeric epoxide

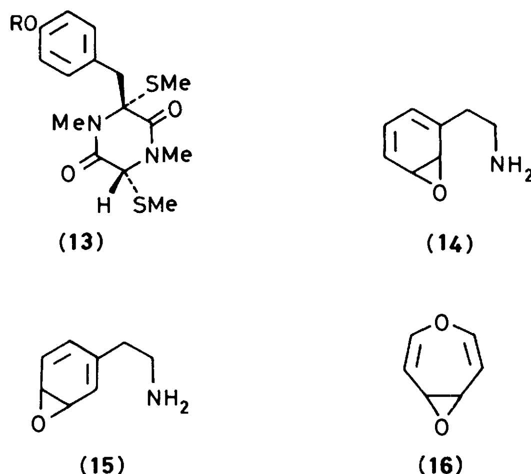
* 2-Methyl-3-methylenepyrazino[1,2-*a*]indole-1,4(2*H*,3*H*)-dione.

† Personal communication from Dr. J. D. Bu'Lock; see also ref. 4.



Scheme 3.

(**12**) could cyclise to give gliotoxin. Later studies make it clear that epoxidation must occur after formation of the dioxopiperazine[12] (**9**) and possibly[13] after introduction of sulphur. Recently, Hanson and O'Leary[14] isolated two new metabolites (**13**; R = H) and (**13**; R = 3,3-dimethylallyl) from *G. deliquescens* and suggested that the 4′-hydroxy group might have arisen by rearrangement of an epoxide of the type (**12**). However, it is not certain that the biosynthesis of gliotoxin is related to that of (**13**). Attempts to reproduce the putative cyclisations, (**10**) ⟶ (**2**) and (**12**) ⟶ (**2**), using simple chemical models have so far been unsuccessful. Rastetter and Nummy[15] found that the amino epoxides (**14**) and (**15**)



rearranged readily, to give the corresponding *ortho* and *para* phenols, respectively, but did not cyclise under a variety of conditions. However, intermolecular ring-opening of both benzene oxide and the oxepin oxide (**16**) with amines was achieved,[16] thus providing some analogy for the biosynthetic proposals. The biosynthetic incorporation of phenylalanine (**1**) into gliotoxin (**2**) has by now been studied in great detail[2] with the fate of all atoms, other than the carboxylic oxygens, determined by isotopic labelling. Nevertheless, direct evidence for the involvement of an arene oxide remains elusive and may, perhaps, only come with the synthesis and feeding of appropriate epoxy-precursors.

Experimental

General.—M.p.s. were measured with a Kofler hot-stage apparatus. ^{1}H N.m.r. spectra were recorded at 90 MHz.

Radioactive Materials and Counting Methods.—DL-[1-^{14}C]-, L-[1-^{14}C]-, and L-[4′-^{3}H]-Phenylalanine were obtained from the Radiochemical Centre, Amersham. ^{3}H and ^{14}C Activities were measured with a Beckmann Scintillation Counter Type CPM-100. Gliotoxin (**2**) and its degradation products (**3**) and (**4**) (typically 1 mg) were counted in *N,N*-dimethylformamide (0.1 ml) and scintillation fluid (5 ml) prepared from 2,5-diphenyloxazole (3.8 g), 1,4-bis(4-methyl-5-phenyloxazol-2-yl)-benzene (0.2 g), and toluene (1 l). m-Tyrosine (*ca.* 1 mg) was kept at room temperature for 20 min in saturated ethanolic hydrogen chloride (1.0 ml). The solution was evaporated at room temperature under reduced pressure over sodium hydroxide pellets and the residue was dissolved in *N,N*-dimethylformamide and scintillation fluid, as before. Phenylalanine (*ca.* 1 mg) was heated in saturated ethanolic hydrogen chloride at *ca.* 70 °C for 10 min. The solution was evaporated over sodium hydroxide, as before, and the residue counted in *N,N*-dimethylformamide (1 ml) and scintillation fluid (10 ml).

DL-[2′,4′,6′-$^3H_3$]-m-*Tyrosine* (**5**).—The identity of DL-*m*-tyrosine (Koch-Light Ltd.) was checked by permethylation with an excess of dimethyl sulphate in 10% aqueous sodium hydroxide. The resulting alkaline solution of the quaternary ammonium salt when heated gave *m*-methoxycinnamic acid having physical properties identical with those of an authentic sample. DL-*m*-Tyrosine (100 mg) was heated in 5M-DCl at 100 °C and the exchange[7] of hydrogen monitored by ^{1}H n.m.r. spectroscopy. The 3-proton multiplet, δ 6.91, diminished in intensity with time and the 1-proton multiplet, δ 7.33, sharpened to give a singlet without change in intensity. Exchange of the 2′, 4′, and 6′ protons was complete in 15 min. No exchange of the side-chain protons was observed even after 16 h of heating. Thionyl chloride (300 mg) was added dropwise with cooling to tritiated water (1.0 ml, 200 mCi) to form tritiated 5M-hydrochloric acid. DL-*m*-Tyrosine (100 mg) was dissolved in this solution, which was then freed from sulphur dioxide with a stream of nitrogen. The mixture was heated in a sealed tube at 100 °C for 1 h and then evaporated to dryness. Readily exchangeable tritium was removed by repeated (× 4) addition and evaporation of water. The residue was dissolved in water and the solution neutralised and then evaporated to small volume and diluted with ethanol (2 ml) to induce crystallisation of DL-[2′,4′,6′-$^3H_3$]-*m*-tyrosine (54%, 2.3 mCi/mmol).

Stability of Tritium Labels in m-*Tyrosine.*—A solution of DL-[2′,4′,6′-$^3H_3$]-*m*-tyrosine (2 mg, 100 μCi) and DL-[1-^{14}C]-phenylalanine (10 μCi) in water (50 ml) containing phenylmercury nitrate (2 mg) was adjusted to pH 3 with hydrochloric acid, sterilised by filtration, and sealed in 5 × 10 ml sample bottles. The bottles were maintained at 30 °C and opened at 2-day intervals. The solutions were neutralised and evaporated and the residues counted for ^{3}H and ^{14}C. No significant change in ^{3}H:^{14}C ratio was observed during 10 days.

DL-[2′-^{3}H]- *and* DL-[3′-^{3}H]-*Phenylalanine.*—2-Bromotoluene was converted *via* [2-^{3}H]toluene into [2-^{3}H]benzyl bromide following the published method.[8] This was converted[17] *via* 5-[2-^{3}H]benzyl-3-phenylhydantoin into DL-[2′-^{3}H]phenylalanine. DL-[3′-^{3}H]Phenylalanine was prepared similarly from 3-bromotoluene.

Fermentation Conditions and Isolation of Gliotoxin (**2**).—*Gliocladium deliquescens* (strain no. 1828 NRRL) was maintained on potato dextrose agar and grown in shake-culture at pH 3.0—3.5 in a defined[18] medium at 25—30 °C. Precursors were added to cultures immediately after inoculation and incubation was continued for 5 days. Gliotoxin was isolated as described before[12c] and crystallised from methanol to constant specific activity. The yields of gliotoxin in Table 1 were obtained from 4 l of culture medium for each feeding experiment.

Bis(anhydrodethio)gliotoxin (**3**).—Gliotoxin (50 mg) was stirred at room temperature with grade II, neutral alumina (2 g) in dry benzene (10 ml) for 12 h. The mixture was filtered and the alumina extracted with hot benzene. The combined benzene solutions were evaporated and the residue (24 mg) was chromatographed on grade II, neutral alumina (50 g) in benzene to give *bis(anhydrodethio)gliotoxin* (**3**) (16 mg), m.p. 160—161 °C (from methanol) (Found: C, 69.3; H, 4.6; N, 12.4. $C_{13}H_{10}N_2O_2$ requires C, 69.0; H, 4.4; N, 12.4%); $\nu_{max.}$($CHCl_3$) 1 715 and 1 675 cm^{-1}; δ($CDCl_3$) 3.40 (s, NMe), 5.27 (1 H, d, *J* 1.5 Hz, 3a-H), 6.15 (1 H, d, *J* 1.5 Hz, 3a-H), 7.2—7.9 (4 H, m, ArH and 10-H), and 8.50 (dd, *J* 8 and 2 Hz, 6-H). The mass spectrum accorded well with that reported by Bose *et al.*[2a] who did not record any other properties of the derivative (**3**).

Dehydrogliotoxin (**4**).—Gliotoxin (52 mg) was heated under reflux in benzene (3.5 ml) containing tetrachloro-1,2-benzoquinone (38 mg) for 3 h. Chromatography[9] of the mixture gave dehydrogliotoxin (37 mg), m.p. 181—185 °C (lit.,[9] 185—186 °C); δ($CDCl_3$) 3.22 (s, NMe), 3.25 and 4.28 (ABq, *J* 19 Hz, 10-CH_2), 4.28 and 4.44 (ABq, *J* 12 Hz, 3a-CH_2), 6.7—7.2 (m, ArH), and 10.42 (s, 6-OH).

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