The Biosynthesis of the Fungal Toxin Gliotoxin; the Origin of the "Extra" Hydrogens as Established by Heavy-isotype Labelling and Mass Spectrometry

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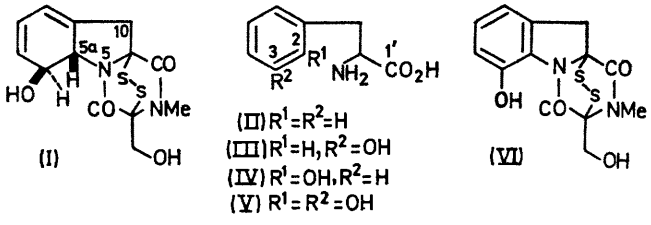
Summary Phenylalanine, but not *o*- or *m*-tyrosine or 2,3-dopa, is incorporated into gliotoxin with very high efficiency, allowing the use of heavy-isotope labelling methods: all five aromatic hydrogens are retained in the incorporation of phenylalanine, and biosynthesis by way of a 2,3-epoxide now appears plausible.

KEY steps in the biosynthesis of the important fungal toxin gliotoxin (I) remain obscure despite a variety of experimentation[1–3] showing that the molecule is assembled from phenylalanine, an aliphatic amino-acid such as serine, methionine (Me donor), and a sulphur donor. For some time, evidence that the phenylalanine (II) is incorporated by way of *m*-tyrosine (III)[2] has been accepted. Our original aim was to study this unusual oxidation, but in the event we demonstrated that (III) is not involved at all. Nor are *o*-tyrosine (IV) nor 2,3-dopa (V), substances which might conceivably, like (III), afford an *o*-quinone from which the indole skeleton of (I) could be formed. We



report evidence for quite a different pathway which is also more acceptable on chemical, stereochemical, and comparative grounds.

In the earlier work,[2] "[^{3}H]-*m*-tyrosine" was obtained by the Wilzbach method, and its apparent incorporation might be ascribed to contamination with radiochemically-formed [^{3}H]phenylalanines. Authentic [1′-^{14}C]-DL-(III) was prepared from *m*-methoxybenzaldehyde and benzoyl[1-^{14}C]-glycine. Also, unlabelled (III) gave at least 85% of the theoretical [2,4,6-$^2H_3$]-(III) by treatment with DCl in D_2O at 100° followed by exchange of labile deuterium in cold dilute HCl, the product being verified by m.s. and n.m.r. A sample of [4,5,6-$^2H_3$]-(V) was similarly prepared and authenticated, and then using the same method, products presumed (but not directly verified) to be [3,5-^{3}H]-DL-(IV) and [4,5,7-^{3}H]-DL-(V) were obtained. The results obtained when these labelled species were added to gliotoxin-producing *Trichoderma viride* (strain NRRL-75, submerged cultures with optimal aeration in glucose–ammonia–peptone–salts medium) are given in Table 1 together with data for [1′-^{14}C]-DL-(II), the only amino-acid showing significant incorporation. Since we have found that both the yield of (I) and the endogenous turnover of amino-acids in *T. viride* are somewhat sensitive to exogenous aromatic amino-acids,[4] we took care to investigate the incorporations of (II) and (III) under comparable conditions; by any criterion the incorporation of (III) was insignificant. This was not due to failure of (III) to enter the cells since it was actively removed from the medium and metabolised by other routes.

The stereochemistry of the amino-alcohol system in (I)[5] could arise by the interaction of an epoxide with the phenylalanine-derived N atom. Other fungal dioxopiperazines (*e.g.* the brevianamide series) and epidithia-dioxopiperazines (in the aranotin and sporidesmin series) contain similar amino-alcohol systems in equivalent locations, and, moreover, the aranotins contain oxepin rings which could also arise by rearrangements of similar epoxides.[6] The plausibility of such a pathway is impaired only by the reported transformation of labelled dehydrogliotoxin (VI) into (I).[7] The origin of the "extra" H atoms in (I), at C-5a and C-6, was therefore of interest; if the 2,3-epoxide of a phenylalanine derivative is a precursor, these H atoms should come directly from (II), but if (VI) is an intermediate they must be introduced by a subsequent hydrogenation step. To minimise interference by selective isotope effects we decided to add a fully-deuteriated precursor under conditions of minimal biosynthetic dilution. As shown in Table 2, conditions giving incorporation of (II) as high as 6·5% and dilutions as low as ×2·7 were established using [1′-^{14}C]-DL-(II). Repeated exchange of DL-(II) in 85 : 15 D_2SO_4–D_2O at 50° (8·5 days for each equilibration) followed by chemical purification gave [2,3,4,5,6-$^2H_5$]-DL-(II) showing < 15% Ar–H by n.m.r. and giving in the mass spectrometer molecular ion peaks at 165—170 in the ratio 2 : 3 : 5 : 11 : 50 : 100 (unlabelled phenylalanine shows a similarly large M − H peak). This material was fed at 1·4 mM at 20 h, and 0·07 mM (I) was recovered at 34 h; the labelled product was examined in the mass spectrometer. The peak groups for the molecular ion and for the base peak, M − 2S, both showed quite clearly that a [$^2H_5$]-(I) was the only major deuteriated species (*ca.* 25% of the corresponding ^{1}H peak). In the mass spectrum of unlabelled (I) there are important peaks at *m/e* 226 and 227 corresponding to losses of $2H_2O$ and H_2O + HO, respectively, from the (M − 2S) ion; in the spectrum from the labelled (I) it was necessary to resolve the corresponding isotope peaks from adjacent unlabelled peaks (M − 2S − CH_4O, M − 2S − CH_3O, *etc.*). When this was done it could be seen that the major deuteriated species in this region were the $^2H_4$-ions, *m/e* 230 and 231, again at *ca.* 25% of the corresponding ^{1}H-peaks (at *m/e* 226 and 227). Hence just one of the five ^{2}H atoms in (I) is involved in this dehydration process, as expected. By reaction with *o*-chloranil[8] the labelled (I) was then converted into dehydrogliotoxin (VI); in the mass spectrum of this product the molecular ion is inconveniently small, but at the base peak, M − 2S, the principal deuteriated species was now the $^2H_3$-ion at *m/e* 263 (about 8% of the ^{1}H-ion at *m/e* 260; the isotope effect in the dehydrogenation reaction is obviously considerable).

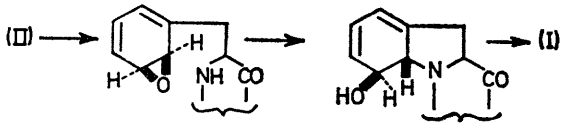
TABLE 1

Precursor DL-amino-acid	Label	Amount added µM	Amount added µCi	Incorporation period (h from inoculation)	Recovered (I) (µM)	Incorporation in (I) (%)	Dilution in in (I)
m-Tyrosine (III)	2,4,6-$^2H_3$	910	—	30—72	100	not detectable	—
	1′-^{14}C	110	37·3			0·0024	×4×10^3
m-Tyrosine (III)	1′-^{14}C	5·3	1·73	30—72	280	0·0043	×1·2×10^6
m-Tyrosine (III)	1′-^{14}C	52	17·3	36—72	210	0·053	×7·7×10^3
Phenylalanine (II)	1′-^{14}C	58	10·1		110	5·4	×33
Phenylalanine (II)	1′-^{14}C	6·3	1·06		140	11·0	×200
2,3-Dihydroxyphenylalanine (V)	4,5,6-^{3}H	4·2	77·7	20—36	160	0·04	×9·3×10^4
o-Tyrosine (IV)	3,5-^{3}H	5·6	93·8		120	0·003	×6·1×10^6

TABLE 2

Optimisation of [1′-^{14}C]-DL-*phenylalanine incorporation*

Amount fed µM	Amount fed µCi	Incubation period (h from inoculation)	Incorporation (%)	Dilution
1330	2·59	20—34	3·4	×2·7
133	2·27		3·7	×28
13·3	2·27		6·5	×107



SCHEME

We are satisfied that in the biosynthesis of gliotoxin (I) neither *m*-tyrosine (III) nor dehydrogliotoxin (VI) can be intermediates, and that it is formed from phenylalanine by a route in which all the aromatic hydrogens of the latter are retained. Of the various pathways so far put forward, one involving the intermediate formation of a 2,3-epoxide from a phenylalanine derivative, as shown in the Scheme, seems the most likely on present evidence; other steps in the biosynthesis are now open to further investigation.

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