Stereospecific Exchange of a β-Methylene Proton in Phenylalanine Preceding Biosynthetic Incorporation into Gliotoxin

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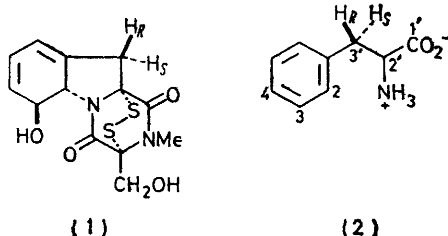
Feeding experiments with the (3'*R*)- and (3'*S*)-forms of [3'-^{2}H]- and [3'-^{3}H]-phenylalanine show that biosynthesis of gliotoxin in *Trichoderma viride* takes place with loss of the *pro-R*- and retention of the *pro-S*-proton from the methylene group of the amino-acid precursor. Gliotoxin derived from [3',3'-$^2H_2$]phenylalanine is mainly monodeuteriated but contains some dideuterio-species; hydrogen (deuterium) loss is not therefore obligatory in gliotoxin biosynthesis. N.m.r. examination of the monodeuteriogliotoxin indicates an *S*-configuration for the new, deuterium-bearing chiral centre corresponding to incorporation of the original 3'-*pro-S*-deuterium with retention of configuration.

Incorporation of [3'-^{3}H]phenylalanine into mycelial protein also involves extensive loss of 3'-*pro-R*-tritium and retention of 3'-*pro-S*-tritium. Hydrolysis of the labelled protein gives [3'-^{3}H]phenylalanine which is shown, by degradation with phenylalanine ammonia-lyase, to have predominantly the 3'*S*-configuration. Protein-phenylalanine and gliotoxin derived from [2'-^{3}H]-phenylalanine contain minimal amounts of tritium.

We conclude that, in *T. viride*, phenylalanine undergoes an unusual exchange reaction involving replacement of the *pro-R*-methylene proton by an external proton with overall retention of configuration. Exchange proceeds substantially faster than incorporation of the amino-acid into protein or gliotoxin but is not an obligatory preliminary step for either process.

Gliotoxin (1) is derived biosynthetically from phenylalanine [1,2] (2) by a process retaining all the aryl protons,[3] in a manner consistent with the involvement of a 2,3(or 3,4)-epoxy-intermediate.[4] We now report in detail, and with an important correction to our preliminary communication [5] on this topic, the fate of the



side-chain protons during the incorporation of phenylalanine into gliotoxin and also into mycelial protein in the fungus *Trichoderma viride*.

DL-[3',3'-$^2H_2$]Phenylalanine [6] (2; $H_R = H_S =$ D) was fed to *T. viride* under conditions [3] which ensured high conversion into gliotoxin and low dilution with endogenous metabolite. The mass spectrum of the resulting gliotoxin showed [*e.g.* from the dominant ($M^+ - 2$S) ion] the presence of monodeuterio- (43%) and dideuterio- (4%) species. Moreover the n.m.r. spectrum showed, within the sensitivity limits of the technique, that the monodeuteriogliotoxin was stereospecifically deuteriated. In gliotoxin, the methylene protons [H_R and H_S in (1)] give a pair of doublets at δ 3·77 and 2·99 (*J* 18 Hz); the former doublet may tentatively be assigned † to H_R and the latter to H_S for the following reasons. Inspection of models and reference to the *X*-ray structure [7] of gliotoxin shows that the C–H_R bond is nearly perpendicular to the plane of the neighbouring C=C double bond, so that allylic coupling between H_R and an olefinic proton might be expected. Indeed the low-field doublet (δ 3·77) shows fine, unresolved coupling whereas the high-field doublet is sharp. Further, the absorption of H_R at lower field than H_S may be explained by the proximity of H_R to the adjacent carbonyl group.‡ In the n.m.r. spectrum of the deuteriated gliotoxin the high-field doublet was of reduced intensity whereas the low-field absorption, which still integrated for one proton, had partly collapsed to a singlet. Thus the predominant biosynthetically monodeuteriated gliotoxin had the stereochemistry (1; H_R = H, H_S = D).

The product (1; H_R = H, H_S = D) could have arisen from the precursor (2; $H_R = H_S$ = D) by replacement by hydrogen, either of the *pro-R*-deuterium with retention, or of the *pro-S*-deuterium with inversion of configuration. The former possibility was shown to apply in the following way. L-(3'*R*)-[3'-^{2}H]- and DL-(3'*S*)-[3'-^{2}H]-Phenylalanine [8] were separately fed, with added DL-[1'-^{14}C]phenylalanine as an internal standard, to *T. viride* cultures. Gliotoxin from the (3'*R*)-form contained little deuterium [6% enhancement of the ($M^+ - 2$S + 1) fragment ion] while that

† Contrast ref. 5.

‡ Our conclusions are not unambiguous but accord well with the findings reported later in this paper. For an opposite assignment in a related structure, based on considerations of the influence of the S–S bridge on chemical shifts, see R. Nagarajan, L. Huckstep, D. H. Lively, D. C. Delong, M. M. Marsh, and N. Neuss, *J. Amer. Chem. Soc.*, 1968, **90**, 2980.

1 R. J. Suhadolnik and R. G. Chenoweth, *J. Amer. Chem. Soc.*, 1958, **80**, 4391.

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3 J. D. Bu'Lock and A. P. Ryles, *Chem. Comm.*, 1970, 1404.

4 N. Johns and G. W. Kirby, *Chem. Comm.*, 1971, 163.

5 J. D. Bu'Lock, A. P. Ryles, N. Johns, and G. W. Kirby, *J.C.S. Chem. Comm.*, 1972, 100.

6 *Cf.* F. Binns, J. A. G. King, A. Percival, N. C. Robinson, and G. A. Swan, *J. Chem. Soc. (C)*, 1970, 1134.

7 J. Fridrichsons and A. McL. Mathieson, *Acta Cryst.*, 1967, **23**, 439.

8 G. W. Kirby and J. Michael, *J.C.S. Perkin I*, 1973, 115.

from the (3'*S*)-form was appreciably enriched (21%) with deuterium.* The n.m.r. spectrum of the latter material confirmed the presence of the monodeuteriogliotoxin (1; $H_R = H$, $H_S = D$). The ^{14}C activity of the gliotoxin specimens showed that the poor incorporation of deuterium from (3'*R*)-[3'-^{2}H]phenylalanine was not merely a consequence of inefficient metabolism in this particular experiment. More precise stereochemical data were sought by using tritiated precursors.

DL-(3'*R*)- and DL-(3'*S*)-[3'-^{3}H]Phenylalanine [8] were mixed with DL-[1'-^{14}C]phenylalanine and converted into cinnamic acid with phenylalanine ammonia-lyase. This enzyme is known [9] to eliminate the elements of ammonia from the substrate with stereospecific removal of the *pro-S*-proton from C-3'. The configurational purities (% *R*) of the amino-acid samples were thus defined (see Table 1) by the $^3H : {}^{14}C$ ratios of the derived cinnamic acids. Feeding experiments with *T. viride* were carried out in the usual way; DL-(3'*RS*)-[3'-^{3}H, 1'-^{14}C]phenylalanine, containing equal amounts of the (3'*R*)- and (3'*S*)-tritiated species, was used as an additional check on the steric course of metabolism. The $^3H : {}^{14}C$ ratios of the various gliotoxin samples were measured and used to calculate (Table 1) the amount of tritium retained during biosynthesis. Clearly (3'*R*)-[3'-^{3}H]phenylalanine (experiment 1) had lost most of its tritium during conversion into gliotoxin, the observed retention (20%) being accounted for largely by the presence of some (3'*S*)-species (17%) in the precursor. Conversely, gliotoxin derived from (3'*S*)-[3'-^{3}H]phenylalanine (experiments 2 and 3) retained nearly all the tritium attributable to the (3'*S*)-labelled components of the precursor mixtures.

TABLE 1

Incorporation of DL-[3'-^{3}H,1'-^{14}C]phenylalanine (2) into gliotoxin (1) and mycelial protein in *T. viride*

Experiment no.	Precursor (2) Configuration	Precursor (2) (*R*)-^{3}H (%) [a]	Gliotoxin (1) Incorpn. (%) [b]	Gliotoxin (1) ^{3}H Retn. (%) [c]	Protein phenylalanine ^{3}H Retn. (%) [c]	Protein phenylalanine (*R*)-^{3}H (%) [a]
1	3'*R*	83	5·7	20	22	34
2	3'*S*	15	8·3	82	82	3
3	3'*S*	13	2·9	85	83	3
4	3'*RS*	48	8·4	49	51	12

[a] Configuration at C-3' by lyase assay. [b] Incorporation based on ^{14}C. [c] Retention of ^{3}H at C-3'.

The formation of detectable amounts of dideuteriogliotoxin from [3',3'-$^2H_2$]phenylalanine (see above) shows that hydrogen loss from the methylene group of the precursor cannot be an obligatory step in gliotoxin biosynthesis, and the small retention of 3'-*pro-R*-tritium in the foregoing experiments points to the same conclusion. This rules out some plausible mechanisms for the biosynthesis of gliotoxin and defines the stereospecific 3'-H loss from phenylalanine as a separate biochemical problem. Important insight into the nature of this *pro-R*-hydrogen loss was gained by examining the tritiated phenylalanine incorporated into mycelial protein. The mycelium from each [3'-^{3}H]phenylalanine feeding was thoroughly washed with hot water then digested with barium hydroxide. The liberated amino-acids were separated by paper chromatography to give crude phenylalanine which was diluted with radio-inactive L-phenylalanine and purified by crystallisation. The tritium content of each sample of protein phenylalanine agreed closely with that of the concurrently produced gliotoxin (Table 1). Tritium loss must therefore be associated with an early stage of metabolism involving either phenylalanine itself or some close metabolic equivalent, for example phenylpyruvic acid. The configuration of tritium in each sample of protein phenylalanine was determined using phenylalanine ammonia-lyase. It was found that the samples were enriched with the (3'*S*)-tritiated form but that loss of tritium from the (3'*R*)-labelled precursor was incomplete. For example, (3'*S*)-[3'-^{3}H]phenylalanine [85% (*S*), 15% (*R*)] (experiment 2) gave protein phenylalanine of high [97% (*S*), 3% (*R*)] configurational purity while (3'*R*)-[3'-^{3}H]phenylalanine [17% (*S*), 83% (*R*)] (experiment 1) gave a product [66% (*S*), 34% (*R*)] still retaining substantial amounts of 3'-*pro-R*-tritium. A time study (Table 2) using DL-(3'*RS*)-[3'-^{3}H,1'-^{14}C]phenylalanine emphasised the rapidity of tritium loss from the precursor relative to incorporation into gliotoxin. After 24 h incubation, when gliotoxin production had only just begun, the metabolite had a high tritium content (72% retention) but this dropped sharply with time reaching the expected final value of *ca.* 50%. Phenylalanine from mycelial protein had, from the outset, a tritium content close to the final value. The mycelium from each experiment was extracted with hot water before hydrolysis of the protein and the small amount of phenylalanine contained in this extract, the 'pool phenylalanine,' was also examined.

TABLE 2

Time study on the metabolism of DL-(3'*RS*)-[3'-^{3}H,1'-^{14}C]-phenylalanine (2) in *T. viride*

Time (h)	Gliotoxin I [a]	Gliotoxin ^{3}H [b]	Pool phenylalanine I [a]	Pool phenylalanine ^{3}H [b]	Pool phenylalanine (*R*) [c]	Protein phenylalanine I [a]	Protein phenylalanine ^{3}H [b]	Protein phenylalanine (*R*) [c]
24	1·3	72	7·5	40	7	20	56	11
48	1·5	54	4·2	44	—	21	54	—
72	6·4	53	2·4	52	—	16	54	—
96	7·0	52	0·7	56	—	17	55	—
120	6·4	53	1·0	46	—	17	52	8

[a] I = incorporation of ^{14}C (%). [b] ^{3}H = ^{3}H retention (%). [c] (*R*) = (3'*R*)-[3'-^{3}H]-content (%) of phenylalanine by lyase assay.

* Our earlier stereochemical conclusions [5] are thus reversed (see footnote in G. W. Kirby and S. Narayanaswami, *J.C.S. Chem. Comm.*, 1973, 323). Later experiments with tritiated phenylalanine showed that deuterium-labelled samples had been accidentally interchanged at some time between preparation of the precursors in Loughborough and final analysis of the products in Manchester. The feedings of (3'*R*)-[3'-^{2}H]- and (3'*S*)-[3'-^{2}H]-phenylalanine were then repeated to confirm the corrected conclusions as reported here; we are grateful to C. Leigh for carrying out the repeat feedings.

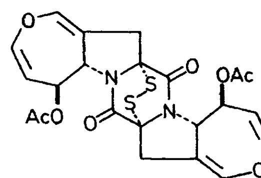
[9] R. Ife and E. Haslam, *J. Chem. Soc.* (*C*), 1971, 2818; R. H. Wightman, J. Staunton, A. R. Battersby, and K. R. Hanson, *J.C.S. Perkin I*, 1972, 2355.

The tritium activities varied with time and were particularly low after 24 and 48 h; the ammonia-lyase assays (see Table 2) again revealed substantial loss of 3′-*pro-R*-tritium from the precursor. There is a small but measurable phenylalanine pool throughout batch culture development but the flux of newly synthesised material through this pool, and the turnover of protein phenylalanine, vary in a more complex manner with time, and a detailed interpretation of the present data is not possible.

The proton which is introduced into the methylene group of phenylalanine during metabolism might come from outside the molecule or, conceivably, from within by a 1,2-migration from C-2′ to C-3′. The latter possibility was tested by feeding DL-[2′-^{3}H,1′-^{14}C]-phenylalanine to *T. viride*. The derived gliotoxin retained little tritium (4·9%) but so did the protein phenylalanine (7·5% retention). The latter observation is consistent with removal of 2′-tritium by, for example, rapid reversible transamination within the organism before incorporation into protein. This is known to occur extensively from studies with [^{14}C,^{15}N]-labelled phenylalanine.[10] The experiment therefore is inconclusive; certainly the proton introduced into the methylene groups of both gliotoxin and mycelial phenylalanine came ultimately from outside the precursor molecule but may possibly have arrived at C-3′ after preliminary attachment to C-2′.

The observed exchange of a methylene proton in phenylalanine was unexpected and its mechanism and metabolic significance remain obscure. It cannot be attributed to phenylalanine ammonia-lyase since this has the opposite stereospecificity. Certainly both removal and replacement of a proton are subject to steric, and so presumably to enzymic, control. Both are appreciably faster than the incorporation of the amino-acid into either protein or gliotoxin. The exchange reaction might involve the same pyridoxal derivative, equilibrating with the pyridoxamine derivative of phenylpyruvic acid, as is involved both in the nett synthesis of phenylalanine and in the known transamination equilibria. There are some precedents: Oshima and Tamija[11] reported exchange of all the protons of L-alanine in ^2H_2O incubations with pyridoxal phosphate and alanine-ketoglutarate aminotransferase. Similarly, Whelan and Long[12] converted L-glutamic acid into the αββ-trideuterio-derivative by using glutamate-oxaloacetate aminotransferase. However, the exchange of a methyl proton in the alanine necessarily provides no stereochemical information while the exchange of both prochiral protons in glutamate demonstrates lack of steric control.

It is unlikely that sterically controlled methylene exchange will be found only for one amino-acid in one organism, and its more general occurrence could lead to the misinterpretation of biosynthetic experiments. Not surprisingly, the incorporation of phenylalanine into acetylaranotin (3) in *Aspergillus*



(3)

terreus also occurs with some loss of methylene deuterium. Brannon *et al.*[13] fed L-[$^2H_8$]phenylalanine to *A. terreus* and isolated acetylaranotin shown, by mass spectrometry, to contain [$^2H_7$]- and [$^2H_{14}$]-species. However, fragment ions attributable to species containing one less deuterium in each half of the molecule were also conspicuous; we have since confirmed substantial loss of one methylene deuteron by using DL-[3′,3′-$^2H_2$,1′-^{14}C]phenylalanine in cultures of an *A. terreus* kindly provided by Dr. Brannon. There is little information available concerning the fate of the methylene proton of aromatic amino-acids in higher plants. No proton loss occurs during the incorporation of tyrosine into the Amaryllidaceae alkaloids[8] except that required by substitution reactions. Phenylalanine loses both methylene protons in forming the C_6–C_1 unit of these alkaloids, but only during and after conversion into cinnamic acid;[14] moreover incorporation of [3′-^{3}H]phenylalanine into the plant protein occurs with complete retention of tritium.[15]

EXPERIMENTAL

Counting Methods.—^{3}H and ^{14}C Activities were measured using a Beckman CPM-100 liquid scintillation spectrometer.

DL-[2′-^{3}H]*Phenylalanine.*—Diethyl 2-acetylamino-2-benzylmalonate (200 mg) was refluxed in tritiated hydrochloric acid, prepared from tritiated water (2 ml) and thionyl chloride (500 mg), for 2 h. The mixture was evaporated and labile tritium removed by addition and re-evaporation of water. The product was liberated by neutralisation and purified by crystallisation from aqueous ethanol. α-Epimerisation[16] of the *N*-benzoyl derivative showed that *ca.* 95% of the tritium was at C-2′.

DL-[3′,3′-$^2H_2$]*Phenylalanine.*—Methyl benzoate (1·72 g) was reduced with $LiALD_4$ (0·35 g) in ether to give [αα-$^2H_2$]-benzyl alcohol which was converted into the benzyl chloride with thionyl chloride–pyridine; reaction with diethyl acetamido(sodio)malonate and hydrolysis[6] gave DL-[3′,3′-$^2H_2$]phenylalanine in 45% overall yield and with less than 5% of mono- or non-deuteriated material (by mass spectroscopy).

(3′R)- *and* (3′S)-[3′-^{2}H]*Phenylalanine.*—These were prepared by the oxazolinone method.[8] The intermediate mixture of *N*-chloroacetyl derivatives was shown by n.m.r.

[10] A. K. Bose, K. S. Khanchandani, R. Tavares, and P. T. Funke, *J. Amer. Chem. Soc.*, 1968, **90**, 3593.
[11] T. Oshima and N. Tamija, *Biochem. J.*, 1961, **78**, 116.
[12] D. J. Whelan and G. J. Long, *Austral. J. Chem.*, 1969, **22**, 1779.
[13] D. R. Brannon, J. A. Mabe, B. B. Molloy, and W. A. Day, *Biochem. Biophys. Res. Comm.*, 1971, **43**, 588.
[14] See ref. 9 and unpublished work by C. Fuganti cited therein.
[15] G. W. Kirby and L. D. Smucker, unpublished work.
[16] H. Matsuo, Y. Fujimoto, and T. Tatsuno, *Tetrahedron Letters*, 1965, 3465.

spectroscopy to contain >90% of a single diastereoisomer (as a racemate).

[3′-^{3}H]*Phenylalanine*.—The (3′*R*)- and (3′*S*)-forms were prepared as for the deuteriated analogues; (3′*RS*)-[3′-^{3}H]-phenylalanine was obtained similarly by omitting the enzymic resolution step.

Phenylalanine Ammonia-lyase Assays.—The enzyme was obtained from potato tubers.[17] Phenylalanine samples were incubated with the enzyme at pH 9·0 and the derived cinnamic acids were diluted with inactive material and purified by sublimation and recrystallisation.

Feeding Experiments with T. viride.—*Trichoderma viride* (strain no. 1828 NRRL, also described as *Gliocladium deliquescens*), obtained from the Commonwealth Mycological Institute (Kew), was subcultured on potato dextrose and grown in shake-culture at pH 3·0—3·5 and 30 °C in a defined sucrose–mineral salt medium [18] or on a glucose–salts–peptone medium.[1,2] Precursors were added in aqueous solutions sterilised by filtration. Incubation times of *ca.* 5 days were suitable for gliotoxin production. The mycelium from each batch was filtered off and the medium extracted with chloroform to afford gliotoxin which was crystallised to constant activity from methanol. The mycelium [typically 6 g (dry weight)] was washed with cold water, dried ($CaCl_2$), then ground with ethanol (20 ml). The solid material was refluxed with water (50 ml) for 30 min to extract the 'pool phenylalanine.' The residual mycelium was digested for 24 h with a refluxing solution of barium hydroxide (10 g) in water (50 ml). The digest was filtered and the filtrate neutralised with sulphuric acid. Filtration through Celite then gave a clear yellow solution containing the protein amino-acids. Phenylalanine was isolated from the 'pool' and protein amino-acid mixture by preparative paper chromatography [Whatman No. 1 sheets developed with n-butanol–acetic acid–water (6 : 2 : 2)]. L-Phenylalanine was added to appropriate fractions and the amino-acid was crystallised to constant activity from aqueous ethanol.

In the time study (see Table 2) ten flasks of medium containing equal amounts of [3′-^{3}H]phenylalanine were inoculated and incubated under the same conditions. Two flasks were worked up after each of the stated periods of incubation.

For minimal dilution of ^{2}H-labelled precursors, the precursor phenylalanine (35—50 mg) was added to each shake culture (150 ml) 20 h after inoculation, and gliotoxin (3—6 mg per flask) was recovered 14 h later. Mass spectra were measured at 190° and 70 eV and the peak heights corrected for natural isotope abundances.

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