cyclo-(L-Phenylalanyl-L-seryl) as an Intermediate in the Biosynthesis of Gliotoxin

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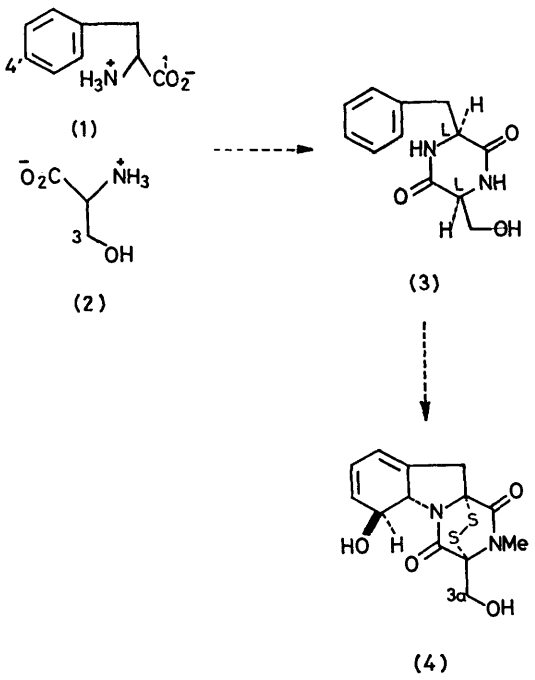
Feeding experiments with the four stereoisomers of *cyclo*-(phenylalanylseryl) have shown that only the LL-isomer is incorporated efficiently (48%) into gliotoxin in '*Trichoderma viride*' (*Gliocladium deliquescens*). *cyclo*-(L-[4'-^{3}H]Phenylalanyl-L-[3-^{14}C]seryl) gave gliotoxin with essentially unchanged ^{3}H : ^{14}C ratio. Successive feedings of non-radioactive *cyclo*-(L-phenylalanyl-L-seryl) and L-[U-^{14}C]phenylalanine to cultures of *T. viride* led to the recovery of *cyclo*-(L-phenylalanyl-L-seryl) containing 1.3% of the radioactivity of the amino-acid. It is concluded that the *cyclo*-dipeptide is a natural metabolite of *T. viride* and is either an intermediate, or is interconvertible with an intermediate, on the biosynthetic pathway from phenylalanine to gliotoxin.

GLIOTOXIN (4) is derived biosynthetically [1] from phenylalanine (1) and serine (2). Only recently, however, has the nature of intermediates lying on the pathway been explored experimentally.[2] MacDonald and Slater [2a] fed *cyclo*-(L-[1-^{14}C]phenylalanyl-L-seryl) (3) to cultures of



Penicillium terlikowski but observed little incorporation into gliotoxin (4), although they demonstrated transport of the *cyclo*-dipeptide into the mycelia. In contrast, Bu'Lock and Leigh have reported [2b] efficient (21%) incorporation of a mixture of *cyclo*-(L-phenylalanyl-L-seryl) and *cyclo*-(L-phenylalanyl-D-seryl) into gliotoxin in '*Trichoderma viride*' (*Gliocladium deliquescens*). Their precursor mixture was prepared from L-[*Ar*-^{3}H]phenylalanine and DL-[1-^{14}C]serine and, significantly, the isotope ratios in the precursor and the metabolite were identical within experimental error. Bu'Lock and Leigh give cogent mycological reasons for the discrepancy between their results and those of MacDonald and Slater. Moreover, our observation [2c] of efficient conversion of *cyclo*-(L-alanyl-L-phenylalanyl) into the 'unnatural' metabolite, 3a-deoxygliotoxin, in *T. viride*, lends circumstantial support to Bu'Lock and Leigh's findings. Nevertheless, further experiments appeared desirable. We decided (*a*) to test whether *cyclo*-(L-Phe-L-Ser) rather than *cyclo*-(L-Phe-D-Ser) or another stereoisomer serves as a precursor for gliotoxin, and (*b*) to seek direct evidence for the formation of *cyclo*-(L-Phe-L-Ser) in *T. viride*.

Non-radioactive samples of the four stereoisomers of *cyclo*-(phenylalanylseryl) were prepared from the appropriate enantiomers of phenylalanine methyl ester hydrochloride and *N*-benzyloxycarbonylserine by a standard method.[2a] The products were readily obtained stereochemically pure by crystallisation. *cyclo*-(L-[U-^{14}C]Phenylalanyl-L-seryl), *cyclo*-(L-[4'-^{3}H]phenylalanyl-L-[3-^{14}C]seryl), and *cyclo*-(L-[U-^{14}C]phenylalanyl-D-seryl) were prepared separately by this method. Radiochemical and stereochemical purities were checked by autoradiography and radioscanning of thin-layer chromatograms, and by radiodilution analysis using the pure, inactive stereoisomers. Similarly, a mixture of the remaining two isomers was obtained from D-phenylalanine and DL-[3-^{14}C]serine. Dilution analysis of this mixture with, in turn, *cyclo*-(D-Phe-L-Ser) and *cyclo*-(D-Phe-D-Ser) established the composition: *cyclo*-(D-phenylalanyl-L-[3-^{14}C]seryl), 68% and *cyclo*-(D-phenylalanyl-D-[3-^{14}C]seryl), 28%. The radiolabelled *cyclo*-dipeptides (typically 32 mg, 6 μCi) were added in dimethyl sulphoxide (2 ml) to 1-day old, shake cultures (2 l) of '*Trichoderma viride*' (*Gliocladium deliquescens* NRRL 1828). Incubations were continued for 4 days and gliotoxin was extracted from the culture filtrates with chloroform. The results are summarised in the Table.

[1] (*a*) R. J. Suhadolnik and R. G. Chenoweth, *J. Amer. Chem. Soc.*, 1958, **80**, 4391; (*b*) J. A. Winstead and R. J. Suhadolnik, *ibid.*, 1960, **82**, 1644; (*c*) A. K. Bose, K. G. Das, P. T. Funke, I. Kugajevsky, O. P. Shukla, K. S. Khanchandani, and R. J. Suhadolnik, *ibid.*, 1968, **90**, 1038; (*d*) A. K. Bose, K. S. Khanchandani, R. Tavares, and P. T. Funke, *ibid.*, p. 3593; (*e*) J. D. Bu'Lock and A. P. Ryles, *Chem. Comm.*, 1970, 1404; (*f*) N. Johns and G. W. Kirby, *ibid.*, 1971, 163; (*g*) D. R. Brannon, J. A. Mabe, B. B. Molloy, and W. A. Day, *Biochem. Biophys. Res. Comm.*, 1971, **43**, 588; (*h*) N. Johns, G. W. Kirby, J. D. Bu'Lock, and A. P. Ryles, *J.C.S. Perkin I*, 1975, 383.

[2] (*a*) J. C. MacDonald and G. P. Slater, *Canad. J. Biochem.*, 1975, **53**, 475; (*b*) J. D. Bu'Lock and C. Leigh, *J.C.S. Chem. Comm.*, 1975, 628; (*c*) G. W. Kirby and D. J. Robins, *ibid.*, 1976, 354.

The incorporation of activity into the chloroform extracts was strikingly dependent upon the stereochemistry of the precursor. Extracts from incubations of the LL-isomer (3) (experiments 1, 3, 5, and 7) contained consistently *ca.* 50% of the precursor's activity; this was shown, by autoradiography and radioscanning of chromatograms, to reside largely in gliotoxin. In contrast, the other three stereoisomers gave very little radioactive material extractable into chloroform. The gliotoxin (*ca.* 200 mg) derived from the feeding of *cyclo*-(L-[4′-^{3}H]phenylalanyl-L-[3-^{14}C]seryl) (experiment 7) was filtrate was extracted with chloroform, to remove gliotoxin, and then continuously with ethyl acetate to yield the *cyclo*-dipeptide (3). This was diluted with pure, inactive (3) and the mixture was crystallised to constant activity. The residual activity in (3) corresponding to 1.3% of that administered as L-[U-^{14}C]phenylalanine. The *cyclo*-dipeptide, had, therefore, been formed from phenylalanine and was presumably present in the organism, though possibly in only small amounts, under normal conditions of growth.

cyclo-(L-Phenylalanyl-L-seryl) (3) satisfies all the usual

Metabolism of *cyclo*-(phenylalanylseryl) in *T. viride*

Experiment no.[a]	Precursor	Composition[b] (%) by radiodilution analysis	Incorporation (%) of ^{14}C into $CHCl_3$ extract
1 }	*cyclo*-(L-[U-^{14}C]Phe-L-Ser)	109 LL, 0.3 DL, 0.3 LD	40
2 }	*cyclo*-(L-[U-^{14}C]Phe-D-Ser)	109 LD, 0.5 LL	0.43
3 }	*cyclo*-(L-[U-^{14}C]Phe-L-Ser)	109 LL, 0.3 DL, 0.3 LD	58
4 }	*cyclo*-(L-[U-^{14}C]Phe-D-Ser)	109 LD, 0.5 LL	0.43
5 }	*cyclo*-(L-[U-^{14}C]Phe-L-Ser)	109 LL, 0.3 DL, 0.3 LD	49
6 }	*cyclo*-(D-Phe-DL-[3-^{14}C]Ser)	68 DL, 28 DD, 0.1 LL	1.1
7	*cyclo*-(L-[4′-^{3}H]Phe-L-[3-^{14}C]Ser)	100 LL, 0.2 DL, 0.2 LD	59

[a] Incubations conducted in parallel are bracketed. [b] The configurations (L or D) of components are given in the order, Phe-Ser.

crystallised repeatedly from methanol; the specific activity rose initially and soon reached a constant value corresponding to a 48% incorporation of the precursor. Radiochemical purity was checked by conversion of the metabolite into anhydrodesthiogliotoxin. Further, the ^{3}H : ^{14}C ratios in the precursor (3) (10.9) and the derived gliotoxin (4) (11.1) were essentially the same. This constant isotope ratio provides strong evidence for the intact incorporation of a dipeptide, cyclic or acylic, into gliotoxin. Independent experimental support for this view was readily obtained. If the *cyclo*-dipeptide had at first been cleaved into its constituent amino-acids then the resulting phenylalanine would have been transformed, with an intact carbon skeleton, into gliotoxin in the usual way. However, the serine would have suffered a different fate. [3-^{14}C]Serine is incorporated [1b] into gliotoxin with partial scrambling of the radiolabel, 25% passing, presumably *via* the one-carbon pool, into the *N*-methyl group. We therefore hydrolysed our biosynthetically labelled gliotoxin with alkali and collected the liberated methylamine as the hydrochloride. This, and the derived *N*-methyl-*N*′-phenylthiourea, contained essentially no (<0.1%) ^{14}C or ^{3}H activity. Thus there seems little doubt that the precursor (3) is converted into gliotoxin without prior degradation into its constituent amino-acids.

It remained possible that the *cyclo*-dipeptide (3) was not a normal biosynthetic intermediate but underwent some structural modification in the organism before passing on to the natural pathway. The status of (3) as a natural intermediate was therefore tested by an 'intermediate-trapping' experiment. Non-radioactive (3) was incubated with *T. viride* and, 2 h later, L-[U-^{14}C]phenylalanine was added to the medium. After a further 2 h the organism was harvested and the culture

criteria for a biosynthetic precursor of gliotoxin (4), at least in *T. viride*. It is formed from phenylalanine and is converted efficiently into gliotoxin and must, therefore, be either an intermediate, or be converted reversibly into an intermediate, on the direct biosynthetic pathway.

EXPERIMENTAL

General.—M.p.s were measured with a Kofler, hot-stage apparatus. I.r. spectra were recorded for Nujol mulls and ^{1}H n.m.r. spectra for $(CD_3)_2SO$ solutions at 90 MHz. Analytical t.l.c. separations were carried out on precoated, silica GF_{254} plates and preparative separations on GF_{254} (type 60) plates. Optical rotations were measured with a Perkin-Elmer 141 polarimeter.

Radiochemical Methods.—^{14}C and ^{3}H activities were measured with a Philips liquid scintillation analyser using toluene–methanol solutions. A Panax Thin Layer Scanner RTLS-1A was used for the radioscanning of t.l.c. plates and Ilford Red Seal 100 FW X-ray film for autoradiograms.

Fermentation Conditions.—'*Trichoderma viride*' (*Gliocladium deliquescans*, strain no. 1828 NRRL) was maintained on potato dextrose agar and grown in shake-culture at pH 3.0—3.5 in a defined [3] medium at 24 °C. Precursors (typically 32 mg, 6 μCi) in dimethyl sulphoxide (2 ml) were added to 1 day old cultures (20 × 100 ml) of *T. viride* through a sterile syringe. After 4 days, the mycelium was filtered off and treated with methanol. The filtrate was neutralised with sodium hydroxide, saturated with sodium chloride, and extracted (×6) with chloroform (10% by volume). The extracts were dried ($MgSO_4$) and evaporated. Gliotoxin was crystallised from methanol.

cyclo-(L-*Phenylalanyl*-L-*seryl*) *and its Enantiomer.*—*N*-Benzyloxycarbonyl-L-serine (2.39 g, 10 mmol) and L-phenylalanine methyl ester hydrochloride (2.16 g, 10 mmol) were condensed using dicyclohexylcarbodi-imide (11 mmol) in dichloromethane (125 ml) containing triethylamine (12.5

[3] J. R. Johnson, W. F. Bruce, and J. D. Dutcher, *J. Amer. Chem. Soc.*, 1943, **65**, 2005.

mmol) at room temperature for 5 h. The mixture was filtered to remove dicyclohexylurea and the filtrate was washed successively with dilute hydrochloric acid, water, saturated aqueous sodium hydrogen carbonate, and water, then dried ($MgSO_4$), and evaporated. The residue was suspended in acetone and the suspension filtered to remove more urea. The crude product was hydrogenolysed at ambient temperature and pressure using 10% palladium–carbon catalyst (340 mg) in methanol (125 ml) containing acetic acid (0.2 ml). The resulting dipeptide ester was cyclised with saturated ammoniacal methanol (25 ml) at room temperature for 24 h. The product which separated was washed with ether and crystallised from methanol to give cyclo-(L-*phenylalanyl*-L-*seryl*) (655 mg), m.p. 235—240 °C (decomp.) (lit.,[2a] 244—246 °C) $[\alpha]_D^{20}$ −105° (*c* 0.99 in Me_2NCHO) (lit.,[2a] −20.1°) (Found: C, 61.3; H, 6.1; N, 11.8. $C_{12}H_{14}N_2O_3$ requires C, 61.5; H, 6.0; N, 12.0%), $\nu_{max.}$ 3 395, 3 190, and 1 675 cm^{-1}; δ 7.95br (s, 1 H), 7.85br (s, 1 H), 7.25 (m, 5 H), 4.85 (t, *J* 5 Hz, 1 H), 4.1 (m, 1 H), 3.7 (m, 1 H), and 2.8—3.5 (m, 4 H).

cyclo-(D-Phenylalanyl-D-seryl) was prepared similarly from the appropriate D-amino-acids. Its physical properties, apart from optical rotation, $[\alpha]_D^{20}$ +104° (*c* 0.99 in Me_2NCHO), were identical with those of the enantiomer.

cyclo-(L-*Phenylalanyl*-D-*seryl*) *and its Enantiomer*.—L-Phenylalanine and D-serine were converted by the preceding method into cyclo-(L-*phenylalanyl*-D-*seryl*) (25% yield based on phenylalanine), m.p. 258—268 °C (decomp.) (methanol) (Found: C, 61.7; H, 5.9; N, 12.0. $C_{12}H_{14}N_2O_3$ requires C, 61.5; H, 6.0; N, 12.0%), $[\alpha]_D^{20}$ +11.2° (*c* 0.99 in Me_2NCHO), $\nu_{max.}$ 3 470, 3 200, and 1 675 cm^{-1}; δ 8.05br (s, 1 H), 7.75br (s, 1 H), 7.25 (m, 5 H), 4.93 (t, *J* 4.5 Hz, 1 H), 4.2 (m, 1 H), and 2.8—3.85 (m, 5 H). *cyclo*-(D-Phenylalanyl-L-seryl) had identical physical properties apart from optical rotation, $[\alpha]_D^{20}$ −10.2° (*c* 1.01 in Me_2NCHO).

Synthesis and Radiodilution Analysis of Labelled Precursors.—Radiolabelled (see Table) samples of *cyclo*-(L-Phe-L-Ser) and (*cyclo*-(L-Phe-D-Ser) were prepared from the appropriately labelled amino-acids by the foregoing method. A mixture of *cyclo*-(D-Phe-D-[3-^{14}C]Ser) and *cyclo*-(D-Phe-L-[3-^{14}C]Ser) was similarly obtained from D-phenylalanine and DL-[3-^{14}C]serine. The labelled compounds (*ca.* 1 mg) were diluted with various (see Table) unlabelled stereoisomers of *cyclo*-(Phe-Ser) (50 mg) and the mixtures crystallised from methanol to constant specific activity.

Degradation of Labelled Gliotoxin.—As a check on radiochemical purity, [^{3}H, ^{14}C]gliotoxin (from experiment 7 in the Table) was converted [1c] into anhydrodesthiogliotoxin by stirring in benzene with grade II, neutral alumina overnight. The ^{3}H and ^{14}C activities of this derivative were, respectively, 108 and 109% of those of the gliotoxin.

[^{3}H, ^{14}C]Gliotoxin (23 mg) was heated with 10% aqueous sodium hydroxide and the liberated methylamine collected as the hydrochloride.[1b] This, in the minimum quantity of water, was treated with concentrated aqueous sodium hydroxide (1 drop) and phenyl isothiocyanate (0.02 ml). The mixture was agitated for 2 min, diluted with methanol, and applied to t.l.c. plates. *N*-Methyl-*N*′-phenylthiourea (6 mg), m.p. 109—113 °C, was obtained having a specific molar activity <0.1% that of the gliotoxin.

Detection of cyclo-(L-*Phe*-L-*Ser*) *in* T. viride.—Non-radioactive *cyclo*-(L-Phe-L-Ser) (40 mg) in dimethyl sulphoxide (2 ml) was fed to a 1-day old culture (10 × 100 ml) of *T. viride*. After 2 h, L-[U-^{14}C]phenylalanine (9 μg, 25 μCi) was added and 2 h later the organism was harvested. The culture filtrate was extracted first with chloroform then continuously with ethyl acetate for 6 days. One third of the latter extract was diluted with non-radioactive *cyclo*-(L-Phe-L-Ser) (50 mg) which was recovered and crystallised from methanol to constant specific activity. The final activity corresponded to a 1.3% incorporation of phenylalanine into *cyclo*-(L-Phe-L-Ser).

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