Biosynthesis of Hyalodendrin and Didethiobis(methylthio)hyalodendrin, Sulphur-containing 2,5-Dioxopiperazines of the 3*S*,6*S* Series

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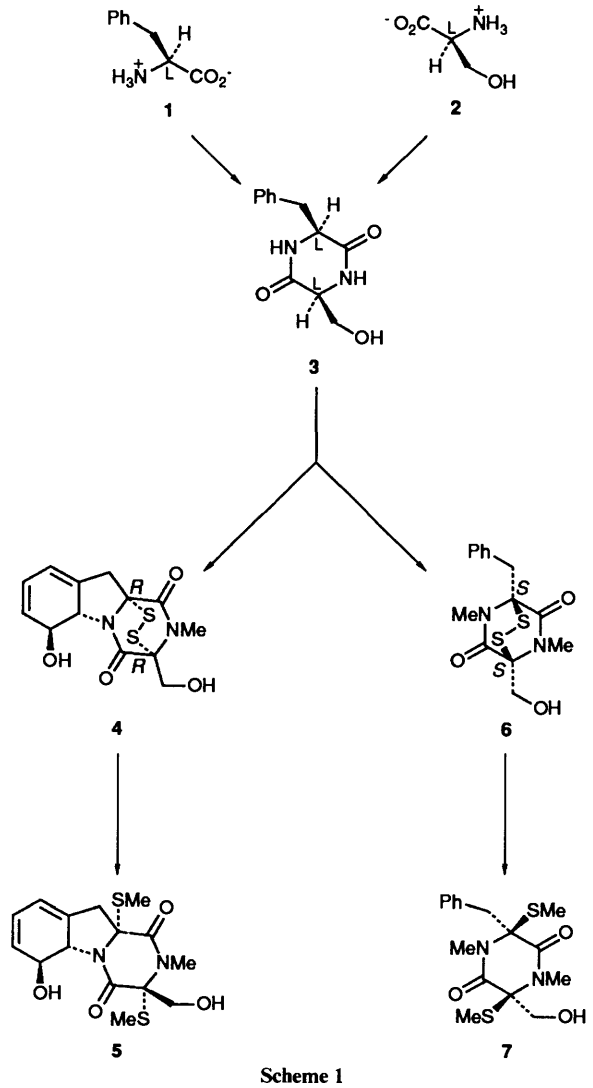
cyclo-(L-[4′-^{3}H]Phe-L-[3-^{14}C]Ser) **8**, a known biosynthetic precursor of the (3*R*,6*R*)-epidithiodioxopiperazine gliotoxin **4**, was efficiently incorporated (42%), in *Hyalodendron* sp. (FSC-601) cultures, into the 3*S*,6*S* metabolite didethiobis(methylthio)hyalodendrin (DBH) **7** without significant change in the ^{3}H:^{14}C ratio. None of the three diastereoisomers of *cyclo*-(L-Phe-L-Ser) was incorporated into DBH to any significant extent. The ^{13}C label from *cyclo*-(L-Phe-L-[3-^{13}C]Ser) was located, in the expected site, in DBH by ^{13}C NMR spectroscopy. Gliotoxin derived in *Gliocladium virens* from the doubly labelled precursor **8** was degraded to locate, in similar manner, the ^{14}C label. The *N*-methyl derivative **16** of *cyclo*-(L-Phe-L-Ser) was not incorporated detectably into either gliotoxin or DBH. Radioactivity from the doubly labelled, linear dipeptides **17** and **18**, possible precursors for the cyclodipeptide **8**, was incorporated with moderate efficiency into gliotoxin. However, the ^{3}H:^{14}C ratios for the dipeptides and the derived gliotoxin differed substantially, indicating that the peptides had undergone cleavage in the fungus before incorporation.

Gliotoxin **4**, the first known member of a large group of epidithiodioxopiperazine derivatives produced by fungi, is derived biosynthetically from phenylalanine **1** and serine **2** *via* *cyclo*-(L-phenylalanyl-L-seryl) **3** (Scheme 1). The closely related metabolite **5**,[2] recently shown [3] to act as an inhibitor of platelet-activating factor, is formed from gliotoxin in *Gliocladium virens* by reduction and methylation, a transformation that is readily effected chemically. We showed,[4] using ^{14}C- and ^{3}H-labelled substrates, that only the LL-diastereoisomer **3** was incorporated efficiently (up to 50%) into gliotoxin **4** in *G. virens*; incorporation of the other three stereoisomers was insignificant. Hyalodendrin **6** and its co-metabolite **7** have the less common 3*S*,6*S* configuration for the dioxopiperazine rings.[5e] We were interested therefore to discover whether these metabolites would be formed from the LL-precursor **3**, like gliotoxin, or from any other stereoisomer. In particular, *cyclo*(D-Phe-D-Ser) would be the appropriate precursor if introduction of sulphur occurred stereospecifically with retention of configuration. *Hyalodendron* sp. (FSC-601) produces both hyalodendrin **6** and didethiobis(methylthio)hyalodendrin (DBH) **7**,[5d] the latter predominating with higher incubation temperatures and longer incubation times. We selected DBH **7** for study since it was the major metabolite under our culture conditions and was more easily crystallised than was hyalodendrin **6**. DBH was characterised further as its crystalline *O*-acetate. The biosynthetic transformation **6** ⟶ **7** has not formally been demonstrated (but see ref. 2), although the corresponding chemical conversion has been reported.[5d]

cyclo-(L-[U-^{14}C]Phe-L-Ser)[4] was fed, in dimethyl sulphoxide (DMSO), to cultures of *Hyalodendron* sp. (FSC-601), the original strain reported[5b] to produce the hyalodendrin metabolites. After 9 days, the culture medium was extracted with chloroform, and DBH **7** was then isolated and purified to constant specific radioactivity by TLC and recrystallisation. Good incorporation (10%, see Table 1, experiment 1) of radioactivity into the purified product was observed, accompanied by only a moderate, 28-fold isotopic dilution (specific activity of the precursor/specific activity of the product) of the radiolabel. To demonstrate intact incorporation of the LL-isomer **3**, *cyclo*-(L-[4′-^{3}H]Phe-L-[3-^{14}C]Ser) **8**, was fed to cultures in the same way (Table 1, experiment 2). On this occasion, exceptionally high (42%) incorporation and low dilution (12-fold) were observed. Importantly, the ^{3}H:^{14}C ratios in the precursor **8** (10.9) and the derived DBH (11.1) were the same, within experimental error. This indicated that biosynthetic

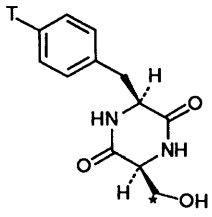


Scheme 1

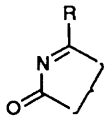
Table 1 Incorporation of cyclodipeptides into didethiobis(methylthio)hyalodendrin (DBH) **7** in *Hyalodendron* cultures

Experiment	Precursor	^{14}C Specific activity of DBH ($\mu Ci\ mmol^{-1}$)	^{14}C Incorporation into DBH[c] (%)	Dilution
1	*cyclo*-(L-[U-^{14}C]Phe-L-Ser)	3.00	10	28
2	*cyclo*-(L-[4′-^{3}H]Phe-L-[3-^{14}C]Ser) **8**	7.60	42	12
3	[a] *cyclo*-(D-Phe-DL-[3-^{14}C]-Ser)	0.05	0.36	1250
4	[a] *cyclo*-(L-[U-^{14}C]Phe-L-Ser)	7.95	28	8.3
5	[a] *cyclo*-(L-[U-^{14}C]Phe-D-Ser)	0.06	0.48	526
6	[a] *cyclo*-(L-[U-^{14}C]Phe-L-Ser)	1.97	15	29
7	[b] *cyclo*-(L-Phe-DL-[3-^{13}C]-Ser) + *cyclo*-(L-[U-^{14}C]Phe-L-Ser) [b]	0.59	9.7	40[c] 37
8	[a] *cyclo*-(L-[U-^{14}C]Phe-L-[*N*-Me]Ser)	6×10^{-4}	0.003	1.4×10^{5}
9	[a] *cyclo*-(L-[U-^{14}C]Phe-L-Ser)	3.30	33	26

[a] Precursors fed separately but in parallel. [b] Precursors fed as a mixture. [c] Calculated from the weight of DBH isolated and the specific activity after repeated crystallization. [d] ^{13}C Dilution measured by ^{13}C NMR spectroscopy.



8 * = ^{14}C



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incorporation had occurred without prior hydrolysis of the cyclodipeptide and subsequent incorporation of phenylalanine and serine after independent dilution with endogeneous amino acids. In another experiment, the same, doubly labelled precursor **8** was fed to the fungus in the usual way, but incubation was terminated after only 2 days. The quantities of metabolites were then quite small, but radioscanning of a TLC plate showed there to be approximately equal ^{14}C activity in the hyalodendrin **6** and DBH **7**. Both products were isolated by TLC and were found to have ^{3}H:^{14}C ratios essentially identical with those of the precursor, *viz.* hyalodendrin **6**, ratio 11.2; DBH **7**, 10.6; and the precursor **8**, 10.9. The specific ^{14}C activities of the hyalodendrin (16.3 $\mu Ci\ mmol^{-1}$) and DBH (16.1) were essentially the same and, as expected, the isotopic dilution (5.6) was lower in this short-term experiment. Unfortunately, there were insufficient amounts of the metabolites to allow recrystallisation to constant specific radioactivity.

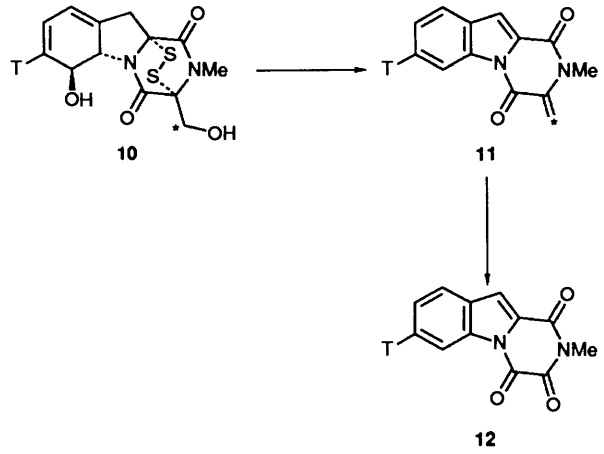
Feeding experiments with the other diastereoisomers of *cyclo*-(Phe-Ser) were next carried out to test the stereospecificity of DBH **7** biosynthesis. A mixture of *cyclo*-(D-Phe-L-[3-^{14}C]Ser) and *cyclo*-(D-Phe-D-[3-^{14}C]Ser) (7:3) prepared, as before,[4] from D-phenylalanine and DL-[3-^{14}C]serine, was fed to *Hyalodendron* cultures in the usual way (experiment 3). For comparison, *cyclo*-(L-[U-^{14}C]Phe-L-Ser) was fed separately to the organism in a parallel experiment (experiment 4). The incorporation of ^{14}C into DBH from the mixture of DL- and DD-cyclodipeptides (0.36%) was much lower than that from the LL-cyclodipeptide (28%). A correspondingly large difference in isotopic dilutions (1250 *versus* 8.3) emphasised the inefficiency of both DL- and DD-*cyclo*-(Phe-Ser) as precursors for DBH **7**. Moreover, the small, though experimentally significant, ^{14}C activity in DBH derived from the mixed precursors may have arisen from a correspondingly small amount of *cyclo*-(L-Phe-L-[3-^{14}C]Ser), present as a contaminant in the synthetic mixture or formed by partial epimerisation in the fungus. Finally (experiments 5 and 6), *cyclo*-(L-[U-^{14}C]Phe-D-Ser) was fed in parallel with *cyclo*-(L-[U-^{14}C]Phe-L-Ser). Once more, the LD-cyclodipeptide was incorporated into DBH much less efficiently (0.48%), and with a greater isotopic dilution (526), than was the LL-cyclodipeptide **3** (15%, dilution 29).

The foregoing experiments, taken with those reported[4] on the biosynthesis of gliotoxin **4**, clearly indicate that *cyclo*-(L-Phe-L-Ser) **3** is the common precursor for these epidithiodioxopiperazines of the antipodal 3*R*,6*R* and 3*S*,6*S* series. That is, the introduction of sulphur does not proceed with stereospecific retention of configuration, as is commonly observed for hydroxylation at saturated carbon. Instead, imino intermediates **9** may be involved, with sulphur being added, perhaps as the nucleophilic thiol group of cysteine, from opposite faces of the molecule in different organisms. It is interesting that the enantiomer of DBH **7**, gliovictin, has been isolated, from *Helminthosporium victoriae*[6] and *Penicillium turbatum*.[5c]

Since no earlier studies had been reported on the biosynthesis of hyalodendrin, it was thought judicious to demonstrate that incorporation of the labelled precursor **8** had taken place without 'scrambling' of one or other of the isotopically labelled atoms. We elected to monitor the fate of C-3 of the serine unit, using *cyclo*-(L-Phe-L-[3-^{13}C]Ser) in conjunction with *cyclo*-(L-[U-^{14}C]Phe-L-Ser). This 'doubly labelled' precursor provides a second test of intact incorporation, with the advantage that the position of ^{13}C in DBH **7** can be determined by ^{13}C NMR spectroscopy. For convenience in synthesis, *cyclo*-(L-Phe-L-[3-^{13}C]Ser) was prepared as a mixture with the LD-diastereoisomer, using commercially available DL-[3-^{13}C]serine. The last compound was converted into the *N*-benzyloxycarbonyl derivative, which was coupled with L-phenylalanine methyl ester by dicyclohexylcarbodiimide (DCC). The resulting dipeptide was hydrogenolysed, then cyclised with methanolic ammonia. Crystallisation of the product gave several crops of material having different ratios of the LL- and LD-cyclodipeptides, as determined by ^{13}C NMR spectroscopy. A crop with the LL:LD ratio 4.2:1 was used in the biosynthetic experiment (experiment 7). This was mixed with *cyclo*-(L-[U-^{14}C]Phe-L-Ser) and fed to cultures of *Hyalodendron*, as before. The derived DBH **7** was isolated and purified in the usual way. The incorporation of ^{14}C was 9.7% and the corresponding, isotopic dilution was 37 (in calculating dilution, it was assumed that the LD-[^{13}C]cyclodipeptide was not incorporated). The proton-decoupled ^{13}C NMR spectrum of the DBH showed an enhanced signal at δ_C 64.3, arising from the hydroxymethylene carbon, and other signals all equal in intensity to those from DBH having ^{13}C at natural abundance. The intensity of the signal δ_C 64.3 was 3.0-times that at natural abundance. This value, taken with the ^{13}C isotopic

abundance (90%) of the precursor (measured by mass spectrometry), gave a calculated dilution [(90 − 1.1)/(3 × 1.1) − 1.1] of 40, in good agreement with the more precise figure (37) from the ^{14}C incorporation. Thus, it appears again that the precursor **3** is incorporated intact. Also, C-3 of the seryl unit is incorporated without scrambling. The former conclusion is especially important because enzymic hydrolysis of the LL-cyclodipeptide **3** would give L-phenylalanine and L-serine, both almost certainly the prime precursors[7] of the metabolites **6** and **7**. Further, the lack of incorporation of the LD-,DL-, and DD-isomers might then simply reflect their resistance to enzymic hydrolysis. However, the site-specific incorporation of ^{13}C provides additional evidence for intact incorporation of the cyclodipeptide **3**. Early studies[8a] on gliotoxin **4** biosynthesis showed that [3-^{14}C]serine was incorporated with partial scrambling of the radiolabel, 25% passing, presumably *via* the one-carbon pool, into the *N*-methyl group. In the present study, there was no detectable enhancement of any of the 4 methyl signals of the ^{13}C-labelled DBH **7**. To date, it appears that LL-cyclodipeptides are irreversibly committed as biosynthetic precursors for sulphur-containing dioxopiperazines. As a further example, *cyclo*-(L-Phe-L-Phe) was shown,[9] by ^{13}C–^{15}N labelling, to be converted in *Aspergillus terreus* into didethiobis(methylthio)acetylaranotin with not more than 5% dissociation and subsequent recombination of the phenylalanine moieties.

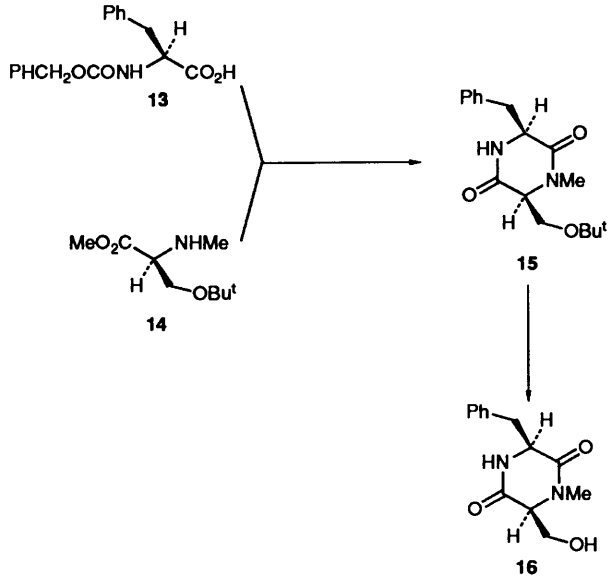
In the course of this investigation, related experiments, some already reported,[4] were carried out on the biosynthesis of gliotoxin **4** in *Gliocladium virens*. For example, the doubly labelled precursor **8** was incorporated efficiently into gliotoxin without significant change in the ^{3}H:^{14}C ratio. The derived gliotoxin **10** was shown[4] to contain no ^{3}H or ^{14}C in the *N*-methyl group, but otherwise the site of neither label was defined. This has now been done by the following degradation (Scheme 2). Treatment of gliotoxin **10** (^{3}H:^{14}C ratio 11.1; ^{14}C activity 1.10 μCi mmol^{-1}) with alumina gave the anhydrodethiogliotoxin **11** (^{3}H:^{14}C 11.2; ^{14}C activity 1.21 μCi mmol^{-1}). Ozonolysis of the latter gave formaldehyde, isolated as its dimedone derivative, containing ^{14}C but no ^{3}H, as expected. However, in several experiments, the specific ^{14}C activity of the formaldehyde was somewhat less than half the expected value. Possibly, this low activity arose from dilution of the [^{14}C]formaldehyde with the unlabelled aldehyde derived from the *N*-methyl group. Therefore, gliotoxin **10** (^{3}H:^{14}C ratio 13.8; ^{3}H activity 14.8 μCi mmol^{-1}) was converted, as before, into the derivative **11** (^{3}H:^{14}C 13.6; ^{3}H activity 13.7 μCi mmol^{-1}). Oxidation with chromium trioxide then gave the trioxopiperazine **12**,[8b] which was devoid of ^{14}C and had a ^{3}H activity (15.3 μCi mmol^{-1}) in good agreement with that of the gliotoxin **10**. As explained before, for hyalodendrin, the absence



Scheme 2

of scrambling of the serine-derived, ^{14}C label is additional evidence for the intact incorporation of the precursor **8**.

No precursor following *cyclo*-(L-Phe-L-Ser) **3** on the biosynthetic pathways to gliotoxin **4** and hyalodendrin **6**, has so far been identified. There is evidence[10] that the introduction of sulphur into the dioxopiperazine ring can occur next in the biosynthesis of gliotoxin and other epidithiodioxopiperazines. However, alternative pathways may also exist as part of a 'metabolic grid.' The *N*-methyldioxopiperazine **16** might therefore serve as a precursor for both gliotoxin and hyalodendrin. This possibility was tested, with negative conclusions, as follows. The protected derivatives of L-phenylalanine **13** and *N*-methyl-L-serine **14** were coupled with DCC and the resulting dipeptide was hydrogenolysed and cyclised to give the t-butyl ether **15**. This was cleaved with hydrobromic acid to afford the dioxopiperazine **16** (Scheme 3) Similarly, L-[U-^{14}C]phenylalanine gave *cyclo*-(L-[U-^{14}C]-Phe–*N*-methyl-L-Ser). When this was fed to *G. virens*, in parallel with *cyclo*-(L-[U-^{14}C]Phe-L-Ser), the resulting gliotoxin was essentially inactive (1.8 × 10^{-3} μCi mmol^{-1}), whereas the



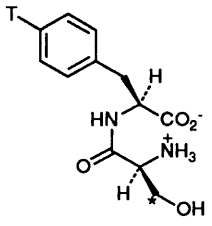
Scheme 3

gliotoxin derived from the *cyclo*-(L-[U-^{14}C]Phe-L-Ser) was, as usual, highly radioactive (2.91 μCi mmol^{-1}, incorporation *ca.* 55%). Thus, the specific activities of the two samples of gliotoxin differed by a factor of >1500. Although negative experiments must be treated with caution, this result provides substantial evidence against *N*-methylation as the step following the formation of *cyclo*-(L-Phe-L-Ser) in gliotoxin biosynthesis, for the following reasons. The two labelled dioxopiperazines were fed concurrently to separate flasks of the same batch of *G. virens* cultures. Further, it is likely that the *N*-methyl derivative **16**, like the NH parent compound **3**, will penetrate the cell walls of the fungus: the derivative **16** is more soluble than its parent **3** in both lipophilic solvents and water; in addition, the penetration of the efficient precursor **3** is unlikely to result from some specific, active transport, since compound **3** is presumably formed *within* the cell during biosynthesis.

Similarly, [U-^{14}C]Phe-labelled samples of the dioxopiperazines **16** and **3** were fed in parallel to *Hyalodendron* cultures (Table 1, experiments 8 and 9). Again, there was a striking difference in the incorporations and specific activities for the DBH **7** resulting from the *N*-methyl **16** (0.003%; 6 × 10^{-4} μCi mmol^{-1}) and NH **3** (33%; 3.30 μCi mmol^{-1}) compounds. This provides evidence, with the foregoing caveat, that the *N*-methyl derivative **16** is not a precursor for hyalodendrin and DBH,

although the isomeric *N*-methyl compound would need to be tested also, for completeness.

The biosynthetic conversion of L-phenylalanine **1** and L-serine **2** into the cyclic dipeptide **3** necessarily requires the formation, presumably sequential, of two peptide linkages. Either or both of the corresponding linear dipeptides might therefore be a free intermediate, like the cyclodipeptide, in the biosynthesis of gliotoxin **4** or hyalodendrin **6**. To test this possibility in *Gliocladium virens*, the doubly labelled linear dipeptides **17** and **18** were synthesized by standard methods. In



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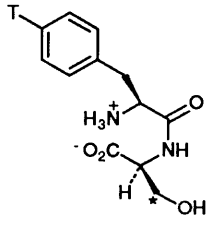

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the first set of experiments, H-L-[3-^{14}C]Ser-L-[4′-^{3}H]Phe-OH **17** was fed, in aqueous ethanol, to 1-day-old cultures of *G. virens*. After incubation for 3 days the resulting gliotoxin **4** was isolated in crystalline form, weighed to provide an estimate of incorporation (see Table 2, footnote *d*), then recrystallized to constant specific activity of ^{14}C and ^{3}H. The results of duplicate experiments are shown in Table 2. Intact incorporation of the peptide **17** would have resulted in equal % incorporations of ^{3}H and ^{14}C. In fact, the incorporation of ^{3}H, from phenylalanine, was *ca.* 5-times greater than that of ^{14}C, from serine. Clearly, the linear dipeptide **17**, unlike the cyclodipeptide **8**, must have been extensively cleaved and the two constituent amino acids then separately incorporated into gliotoxin. A substantially greater (*ca.* 6×) incorporation of [^{3}H]phenylalanine than of [^{14}C]serine into gliotoxin has been observed earlier by Winstead and Suhadolnik.[8a] Similar results were obtained when H-L-[4′-^{3}H]Phe-L-[3-^{14}C]Ser-OH **18** was fed to 1-day-old cultures for 3 days. Again, a *ca.* 5-fold difference in ^{3}H and ^{14}C incorporations was observed. However, with successively shorter incubation times, 2 days and 1 day, in more mature cultures, 2- and 3-day old, the incorporation of ^{14}C into gliotoxin approached that (*ca.* 5%) of ^{3}H. Indeed, in the last experiment the ^{14}C and ^{3}H incorporations barely differed by experimental error. It remains possible that, under these conditions, intact incorporation of the dipeptide **18** competes effectively with enzymic hydrolysis to the amino acids. However, the clear evidence that the linear peptides **17** and **18** are readily cleaved means that no firm conclusion can be drawn about their status as free intermediates in gliotoxin biosynthesis. Plans to conduct comparable studies in *Hyalodendron* were consequently abandoned.

In conclusion, *cyclo*-(L-Phe-L-Ser) **3** is an efficient, irreversibly committed intermediate in the secondary metabolic pathways leading to both the 3*S*,6*S* and 3*R*,6*R* epidiothiodioxopiperazines hyalodendrin **6** and gliotoxin **4**. Unlike the corresponding linear dipeptides **17** and **18**, this cyclopeptide resists enzymic cleavage in fungal cultures. The lack of incorporation of the *N*-methyl derivative **16** is consistent with the idea[10] that introduction of sulphur may immediately follow cyclodipeptide formation on biosynthetic pathways to the sulphur-containing dioxopiperazines.

Experimental

General.—M.p.s were measured with a Kofler hot-stage apparatus. Optical rotations were measured with a Perkin-Elmer 141 polarimeter. Mass spectra were obtained by electron impact at 70 eV. NMR spectra were obtained, at the frequencies indicated, with Varian T60 and XL100, and Perkin-Elmer R32 spectrometers. Light petroleum refers to the fraction boiling in the range 40–60 °C.

Radiochemical Methods.—^{14}C and ^{3}H Activities were measured with a Philips liquid scintillation analyser, generally using toluene–methanol solutions. Exceptionally, the linear dipeptides **17** and **18** were dissolved in methanol–triton X 100–toluene (0.06:1.0:2.0). Radioactive compounds were detected on TLC plates with a Panax Thin Layer Scanner.

Chromatographic Methods.—Analytical and preparative TLC was carried out with Merck Kieselgel GF_{254} plates. Linear dipeptides were detected with ninhydrin. Cyclic dipeptides were detected as follows. Plates were dried at 100 °C for 5 min, then cooled and placed for 5 min in a tank containing chlorine (alternatively, the plates were sprayed with aq.-sodium hypochlorite). The plates were kept in air for *ca.* 5 min before being sprayed with a mixture of potassium iodide (1 g) in water (500 cm^3) and *o*-tolidine (3,3′-dimethylbenzidine) (160 mg) in acetic acid (30 cm^3).[11] Later, the less toxic 2,2′,6,6′-tetramethylbenzidine (200 mg) was used in place of *o*-tolidine. Cyclodipeptides gave blue or yellow spots, depending upon their concentration. Sulphur-containing metabolites were detected by spraying with silver nitrate in aq. acetone. Epidisulphides immediately gave yellow spots, which soon turned dark brown. Bis(methylthio) derivatives very slowly gave white spots on a grey background.

Isolation, Purification and Characterisation of Didethiobis(methylthio)hyalodendrin (DBH) **7**.—A culture of *Hyalodendron* sp. (FSC 601) was kindly provided by Dr A. Taylor (NRC, Halifax, Canada). Specimens were deposited with the CAB International Mycological Institute (Kew) (IMI 238244). The fungus was grown on 2% malt–agar slants, then transferred to the recommended[5b] medium (100 cm^3 portions in 500 cm^3 conical flasks) at pH 3.5. The organism was thereafter grown at 25 °C in shake culture (orbital shaker at 160 rpm) for 14 days. The mycelia were filtered off through Celite and washed with methanol. The filtrate and washings were extracted repeatedly with chloroform and the combined extracts were washed with water, dried ($MgSO_4$), and evaporated. The residue was chromatographed on silica plates, developed with benzene–ethyl acetate (4:1), and the band with R_f 0.12–0.24, detected with UV light, was eluted with chloroform and then with ethyl acetate. Evaporation of the eluates gave didethiobis(methylthio)hyalodendrin **7** (20–50 mg per dm^3 culture medium), m.p. 141.5–142.5 °C (from dichloromethane–cyclohexane) (lit.,[5d] 140–140.5 °C); $[\alpha]_D^{24}$ +63.5° (*c* 2.5 in $CHCl_3$) (lit.,[5d] +64°); δ_H(60 MHz; $CDCl_3$) 3.27 and 3.00 (2 × s, 2 × NMe), 2.26 and 2.10 (2 × s, 2 × SMe), and multiplets in agreement with literature[5d] data; δ_C(25.2 MHz; $CDCl_3$) 13.4 and 14.3 (2 × SMe), 29.4 and 30.9 (2 × NMe), 42.3 (Ph*C*H$_2$), 64.3 (*C*H$_2$OH), 71.6 and 73.5 (C-3 and -6), 127.7 (*p*-Phe-C), 128.6 (2 × *m*-Phe-C), 130.0 (2 × *o*-Phe-C), 134.1 (*ipso*-Phe-C) and 165.4 and 165.3 (2 × C=O).

For further characterization, DBH **7** (12.1 mg) was treated for 12 h at room temperature with pyridine (0.1 cm^3) and acetic anhydride (0.1 cm^3). Methanol (0.5 cm^3) was then added and the mixture was evaporated. Crystallization of the residue from acetone–diethyl ether gave *O-acetyldidethiobis(methylthio)hyalodendrin* (10.7 mg, 79%), m.p. 137–139 °C (Found: C, 54.7; H, 6.2; N, 6.9. $C_{18}H_{24}N_2O_4S_2$ requires C, 54.6; H, 6.1; N, 7.1%); δ(60 MHz; $CDCl_3$) 7.22 (br s, Ph), 4.37 (s, CH_2O), 3.87 and 2.70 (ABq, *J* 14, CH_2Ph), 3.28 and 3.05 (2 × s, 2 × NMe), 2.33 and 2.23 (2 × s, 2 × SMe) and 1.66 (s, Ac).

Table 2 Incorporation of H-L-[3-^{14}C]Ser-L-[4′-^{3}H]Phe-OH **17** and H-L-[4′-^{3}H]Phe-L-[3-^{14}C]Ser-OH **18** into gliotoxin **4** in *Gliocladium virens*

	Precursor				
	17		**18**		
Age of culture (days)[a]	1	1	1	2	3
Incubation time (days)[b]	3	3	3	2	1
Precursor conc.[n] (mg dm^{-3})[c]	11	6	14	14	14
Incorporation of ^{14}C (%)[d]	0.86	1.20	0.60	2.90	4.40
Incorporation of ^{3}H (%)[d]	4.45	6.40	3.10	3.90	4.90

[a] Age at time of feeding. [b] Incubation time after feeding. [c] Concentration of precursor (^{3}H:^{14}C ratio typically 12:1) in culture medium. [d] Incorporations were calculated from the weight of crystalline gliotoxin and the specific activities of ^{14}C and ^{3}H after repeated recrystallization.

Feeding Experiments with Hyalodendron *sp.*—Precursors, in DMSO, were fed to cultures in *Hyalodendron* (8–15 mg dm^{-3}) on the fifth day of growth. DBH **7** was isolated 9 days later, as described above. The crude product mixture contained dimethyl sulphone, formed microbially from DMSO which had similar chromatographic properties to DBH. The mixture was, therefore, dissolved in dichloromethane (10 cm^{3}) and washed with water (3 × 5 cm^{3}). The solution was dried ($MgSO_4$) and evaporated and the residue of DBH was crystallized to constant specific radioactivity. The following details of the experiment (Table 1, experiment 7) with the ^{13}C-labelled precursor **3** illustrate general procedures and method of calculation. *cyclo*-(L-[U-^{14}C]Phe-L-Ser) (2.7 mg, 1.05 μCi) was mixed with *cyclo*-(L-Phe-DL[3-^{13}C]Ser) (10.6 mg, 80.8% LL-isomer, 90% ^{13}C at C-3) and fed, in DMSO, to *Hyalodendron* (8 × 100 cm^{3} culture medium). The derived DBH **7** (61 mg, 0.173 mmol) was recrystallised to a constant specific activity, 0.59 μCi mmol^{-1}, corresponding to a 9.7% ^{14}C incorporation and 37-fold ^{14}C dilution. Calculation of the ^{13}C dilution (40-fold) is given in the main text.

Isolation and Purification of Gliotoxin.—*Gliocladium virens* (NRRL 1828), obtained from the CAB International Mycological Institute (Kew) (IMI 101525, listed as *G. deliquescens*; the same strain was referred to earlier[4] as *Trichoderma viride*), was maintained on potato dextrose–agar and grown in shake-culture at pH 3.0–3.5 in a defined medium[12] at 24 °C. After a suitable period of grown (see the main text, and below), the mycelium was filtered off and washed with methanol. The filtrate and washings were neutralized with aq. sodium hydroxide, saturated with sodium chloride, and repeatedly extracted with chloroform. The extracts were dried ($MgSO_4$) and evaporated. The semicrystalline residue was triturated with methanol and the crystalline gliotoxin (typically 100 mg per dm^{3} medium) was collected, then recrystallized from methanol.

Feeding Experiments with Gliocladium virens.—Cyclodipeptides (typically 16 mg, 3 μCi per dm^{3} culture medium) were fed in DMSO (1 cm^{3}) to 1-day-old shake-cultures of *G. virens*. Cultures were extracted 3 days after feeding. The radiolabelled *N*-methyl derivative **16** (11 mg, 2 μCi per dm^{3}) was incubated for 4 days after feeding, as was the radiolabelled derivative **3** in the control experiment. The linear dipeptides **17** and **18** were fed in aq. ethanol to cultures of *G. virens* (6–14 mg dm^{-3}). Table 2 lists the culture ages (1–3 days) and incubation times (3–1 days) for each experiment.

Synthesis of Radiolabelled Cyclodipeptides.—The radiolabelled forms of *cyclo*-(L-Phe-L-Ser) **3** and its diastereoisomers, listed in Table 1, were prepared from the appropriately labelled *N*-benzyloxycarbonylserine and phenylalanine methyl ester hydrochloride, as described before.[4] The stereochemical purity of the diastereoisomers was checked, as before,[4] by radiodilution analysis.

cyclo-(L-*Phenylalanyl*-DL-[3-^{13}C]*seryl*).—DL-[3-^{13}C]Serine (90 atom% 3-^{13}C), obtained from Prochem-BOC Ltd., was found to contain *ca.* 23% [^{12}C]glycine. The mixture (0.516 g) was separated by column chromatography on a Biorad, analytical-grade cation-exchange resin, AG 50W-X8 (200–400 mesh, H^+-form). Elution with 1 mol dm^{-3} hydrochloric acid gave successively DL-[3-^{13}C]serine and [^{12}C]glycine. Fractions containing serine were evaporated, the residue was dissolved in water, and the solution was adjusted to pH 6.8 with 1 mol dm^{-3} lithium hydroxide. Addition of ethanol and cooling to 0 °C gave the serine as plates (0.340 g). Glycine (0.103 g) was obtained similarly from later fractions. Treatment of the serine in aq. sodium hydrogen carbonate with benzyl chloroformate in the usual way[13] gave *N*-benzyloxycarbonyl-DL-[3-^{13}C]serine (83%), m.p. 125–126 °C (lit.,[14] 125 °C for the ^{12}C derivative).

Condensation of this derivative (480 mg, 2 mmol) and L-phenylalanine methyl ester hydrochloride (431 mg, 2 mmol) with DCC and triethylamine in dichloromethane, in the usual way, gave *N*-benzyloxycarbonyl-DL-[3-^{13}C]seryl-L-phenylalanine methyl ester. Hydrogenolysis with 10% palladium–carbon catalyst and subsequent cyclisation in methanolic ammonia gave *cyclo*-(L-Phe-DL[3-^{13}C]Ser) as a crystalline mixture of diastereoisomers (94 mg) (crop A), m.p. 244–255 °C and 262–264 °C (decomp.). Concentration of the ammoniacal, methanolic mother liquors gave more cyclodipeptide (20 mg) (crop B), m.p. 235–240 °C. Further concentration gave a final batch of product (51 mg) (crop C), m.p. 230–235 °C [*cyclo*-(L-Phe-L-Ser) and *cyclo*-(L-Phe-D-Ser) have m.p.s 244–246 °C and 258–268 °C (decomp.), respectively]. The composition of each of the 3 crops of crystalline mixtures was determined by ^{13}C NMR spectroscopy. Spectra of reference samples gave, for *cyclo*-(L-Phe-L-Ser) **3**, δ_C[25.2 Hz; $(CD_3)_2SO$] 39.8 (Ph*C*H$_2$), 55.5 and 57.1 (C-3 and -6), 63.1 (CH_2O), 126.4 (*p*-Phe-C), 128.1 (2 × *m*-Phe-C), 129.9 (2 × *o*-Phe-C), 136.6 (*ipso*-Phe-C) and 165.8 and 166.6 (2 × C=O); and for *cyclo*-(L-Phe-L-Ser), δ_C 37.8 (Ph*C*H$_2$), 55.0 and 56.4 (C-3 and -6), 62.4 (CH_2O), 126.5 (*p*-Phe-C), 127.9 (2 × *m*-Phe-C), 130.1 (2 × *o*-Phe-C), 136.2 (*ipso*-Phe-C) and 166.8 and 167.4 (2 × C=O). The following compositions were determined from the relative heights of the ^{13}C signals at δ_C 63.1 and 62.4: crop A, LL:LD 1:3.0; crop B, 3.8:1; crop C, 4.2:1. The last crop (80.8% LL) was used in the feeding experiment (Table 1, experiment 7). In the ^{1}H spectra [100 MHz; $(CD_3)_2SO$] of the cyclodipeptides the NH signals for the 2 diastereoisomers were distinct, δ 7.94 and 8.06 for the LL- isomer and δ 7.85 and 8.16 for the LD-isomer. However, partial overlap of the broad signals limited the accuracy of ^{1}H NMR spectroscopy for analysis of the mixture.

Degradation of the Doubly Labelled Gliotoxin **10** (*Scheme* 2).—The doubly labelled gliotoxin **10** was converted[7] into the bis(anhydrodethio)gliotoxin **11** by treatment with alumina in benzene, without significant loss of ^{3}H or ^{14}C (see the main text for ^{3}H and ^{14}C data). The product **11** was ozonolysed in ethyl acetate at −25 °C. The mixture was allowed to warm to room

temperature and the excess of ozone and the solvent were evaporated in a stream of nitrogen. The residue was steam distilled in the presence of zinc dust and the distillate was collected in saturated, aq. dimedone. The crystalline dimedone derivative of formaldehyde which formed was collected and recrystallized from aq. ethanol, m.p. 190–191.5 °C (lit.,[15] 191.4 °C). This derivative was devoid of ^3H, but had a specific molar ^{14}C activity only 48% of that of the derivative **11**. The experiment was repeated with a similar result (44%). In another experiment, after ozonolysis in chloroethane at −25 °C, the ozonide was decomposed by addition of dimethyl sulphide at −25 °C. The resulting formaldehyde dimedone again had a low ^{14}C activity (37%). Alternatively, the derivative **11** was oxidized[8b] in acetic acid with aq. chromium trioxide at room temperature overnight to yield the trioxo derivative **12**, which was crystallized repeatedly from methanol–chloroform (1:1) to give pure material, m.p. 262–264 °C (lit.,[8b] 262–263 °C); ν_{max}(KBr)/cm^{-1} 1735 and 1690; *m/z* 228 (M^+, 39%), 143 (100), 115 (65) and 88 (22), *m** 92.5 (143 ⟶ 115) and 67.3 (115 ⟶ 88) (see the main text for ^3H and ^{14}C data).

N-*Benzyloxycarbonyl*-O-*t*-*butyl*-L-*serine*.—*N*-Benzyloxycarbonyl-*O*-t-butyl-L-serine methyl ester[16] (10.64 g, 34.4 mmol) was treated with 2 mol dm^{-3} sodium hydroxide (34.4 cm^3) in ethanol (34.4 cm^3) for 10 min at room temperature. The mixture was neutralized with acetic acid, then evaporated. The residue was dissolved in dichloromethane–water and the water layer was extracted with dichloromethane. The combined dichloromethane solutions were dried ($MgSO_4$) and evaporated. The residue was dissolved in 10% aq. sodium hydrogen carbonate and the solution was washed with diethyl ether, then acidified, with cooling, with conc. hydrochloric acid. The mixture was extracted repeatedly with diethyl ether and the extracts were dried ($MgSO_4$) and evaporated to yield *N*-benzyloxycarbonyl-*O*-t-butyl-L-serine (8.65 g, 85%), m.p. 81–86 °C (from cyclohexane) (lit.,[16] 87–87.5 °C);$[\alpha]_D^{18}$ +18.0° (*c* 2.4 in EtOH) (lit.,[16] +22.7 °). The wide m.p. range and low optical rotation were consequences of partial racemisation.

N-*Benzyloxycarbonyl*-O-*t*-*butyl*-N-*methyl*-L-*serine*.—Iodomethane (2.5 cm^3) and sodium hydride (660 mg, 27.5 mmol) were added to a stirred, ice-cold solution of *N*-benzyloxycarbonyl-*O*-t-butyl-L-serine (1.475 g, 5 mmol) in dry tetrahydrofuran, according to the general procedure of Cheung and Benoiton.[17] The mixture was stirred at 0 °C for 3 days, then was filtered, and the filtrate was evaporated. The residue was dissolved in water (100 cm^3)–diethyl ether (30 cm^3). The ether layer was extracted with aq. sodium hydrogen carbonate and the extracts were combined with the aq. layer. The cooled, combined aq. solutions were acidified to pH 3–4 with citric acid, and were then extracted with ethyl acetate. The extracts were washed with water, dried ($MgSO_4$), and evaporated to give *N*-benzyloxycarbonyl-*O*-t-butyl-*N*-methyl-L-serine[18] as an oil (1.51 g, 98%) (Found: C, 62.25; H, 7.2; N, 4.5. Calc. for $C_{16}H_{23}NO_5$: C, 62.1; H, 7.4; N, 4.5%); $[\alpha]_D^{15}$ +4.8° (*c* 1.5 in $CHCl_3$); ν_{max}($CHCl_3$)/cm^{-1} 3300–2800, 1770w, 1700, 1395 and 1365; δ(60 MHz; $CDCl_3$) 9.65–9.25 (br s, CO_2H, exch. with D_2O), 7.35 (s, Ph), 5.15 (s, $PhCH_2$), 5.00–4.55 (m, 2-H), 3.95–3.60 (m, CH_2O), 3.03 (s, NMe) and 1.15 (s, Bu^t); *m/z* 309 (M^+, weak).

N-*Benzyloxycarbonyl*-O-*t*-*butyl*-N-*methyl*-L-*serine Methyl Ester*.—The foregoing serine derivative was treated with diazomethane in diethyl ether, in the usual way, to give the *methyl ester* as an oil (Found: C, 63.0; H, 8.0; N, 4.3. $C_{17}H_{25}NO_5$ requires C, 63.2, H, 7.7; N, 4.3%); $[\alpha]_D^{21}$ −7.6° (*c* 2.7 in $CHCl_3$); ν_{max}(liq. film)/cm^{-1} 3700–3100, 1740 and 1695; δ(60 MHz; $CDCl_3$) 7.40 (s, Ph), 5.20 (s, $PhCH_2$), 4.95–4.50 (m, 2-H), 3.90–3.60 (m, CH_2OBu^t), 3.75 (br s, OMe), 3.05 (s, NMe) and 1.15 (s, Bu^t); *m/z* 323 (M^+, weak).

O-t-*Butyl*-N-*methyl*-L-*serine Methyl Ester* **14**.—The foregoing *N*-benzyloxycarbonyl derivative (3.45 g, 10.7 mmol) in methanol (100 cm^3) was hydrogenated at ambient temperature and pressure with 10% palladium–carbon to give the oily *methylamino ester* **14** (1.90 g, 94%) (Found: C, 57.1; H, 10.2; N, 7.3. $C_9H_{19}NO_3$ requires C, 57.1; H, 10.1; N, 7.4%); ν_{max}(liq. film)/cm^{-1} 3600–3200, 2800 and 1740; δ(60 MHz; $CDCl_3$) 3.95 (s, OMe), 3.75-3.20 (3 H, m, 2- and 3-H), 2.45 (s, NMe), 2.05 (br s, NH, exch. with D_2O) and 1.15 (s, Bu^t).

N-*Benzyloxycarbonyl*-L-*phenylalanyl*-O-*t*-*butyl*-N-*methyl*-L-*serine Methyl Ester*.—DCC (228 mg, 1.1 mmol) was added to an ice-cold solution of *N*-benzyloxycarbonyl-L-phenylalanine **13** (299 mg, 1 mmol) and the methylamino ester **14** (189 mg, 1 mmol) in dichloromethane (10 cm^3). The mixture was kept at room temperature overnight, then filtered to remove dicyclohexylurea. The filtrate was washed successively with dil. hydrochloric acid, water, aq. sodium hydrogen carbonate, and water, then dried ($MgSO_4$) and evaporated. The residual oil was dissolved in the minimum amount of acetone and the solution was set aside to allow separation of more dicyclohexylurea, which was filtered off. The filtrate was evaporated to yield the required product as an oil (414 mg, 88%), sufficiently pure for subsequent use (TLC showed the presence of a small amount of the urea). A sample was purified by TLC [R_f 0.60 on silica plates developed with light petroleum–ethyl acetate–acetic acid (12:6:1)] to give the oily *dipeptide ester* (Found: C, 66.2; H, 7.1; N, 5.8. $C_{26}H_{34}N_2O_6$ requires C, 66.4; H, 7.2; N, 6.0%); $[\alpha]_D^{15}$ +6.8° (*c* 1.14 in $CHCl_3$); ν_{max}($CHCl_3$)/cm^{-1} 3420, 1740, 1720 and 1650; δ(60 MHz; $CDCl_3$) 7.35 (s, Ph), 7.25 (s, Ph), 5.85–5.55 (m, NH), 5.10 (br s, $PhCH_2O$), 5.20–4.60 (m, 2 × $CHCH_2$), 3.85–3.55 (m, Bu$^tOCH_2$), 3.70 (s, OMe), 3.05 (s, NMe), 3.20–2.80 (m, $PhCH_2CH$) and 1.15 (s, Bu^t).

cyclo-(L-*Phenylalanyl*-O-*t*-*butyl*-N-*methyl*-L-*seryl*) **15**.—The foregoing benzyloxycarbonyl derivative (2.185 g, 4.65 mmol) in methanol (100 cm^3) containing acetic acid (6 cm^3) was hydrogenated with 10% palladium–carbon (220 mg). The mixture was filtered and the filtrate was evaporated. Methanol was added to the residue and evaporated to remove traces of acetic acid. The residue was dissolved in hot acetone and light petroleum (b.p. range 40–60 °C), and the solution was set aside to allow crystallization of a by-product, which was recrystallized from ethyl acetate to afford cyclo-(L-*phenylalanyl*-O-*t*-*butyl*-N-*methyl*-D-*seryl*) as cubes (120 mg, 8.5%), m.p. 213–215 °C (Found: C, 67.3; H, 8.05; N, 9.4. $C_{17}H_{24}N_2O_3$ requires C, 67.0; H, 7.9; N, 9.2%); $[\alpha]_D^{20}$ −124° (*c* 1.0 in $CHCl_3$); ν_{max}(KBr)/cm^{-1} 3270, 1688 and 1645; δ(60 MHz; $CDCl_3$) 7.25 (m, Ph), 5.88 (br s, NH), 4.36 (dd, *J*10 and 4, $CHCH_2Ph$), 3.90–3.47 (m, 2 × CH_2), 2.96 (s, NMe), 2.95–2.62 (m, $CHCH_2OBu^t$) and 1.12 (s, Bu^t); *m/z* 304 (M^+, weak).

The mother liquors were evaporated and the residue was chromatographed on an alumina column (60 g). Elution with ethyl acetate–light petroleum (1:1) gave the required LL-*cyclodipeptide* **15** (442 mg, 31%), m.p. 133–138 °C (from ethyl acetate–light petroleum) (Found: C, 67.3; H, 8.1; N, 9.3%); $[\alpha]_D^{20}$ −150° (*c* 1.4 in $CHCl_3$); ν_{max}(KBr)/cm^{-1} 3350, 3235, 1680 and 1650; δ(60 MHz; $CDCl_3$) 7.41–7.14 (m, Ph), 5.85–5.75 (br s, NH), 4.20–3.88 (2 H, m, 2 × CH), 3.80–3.70 (m, $PhCH_2$), 3.55–2.98 (m, Bu$^tOCH_2$), 2.99 (s, NMe) and 1.20 (s, Bu^t); δ(CD_3OD) 7.29 (m, Ph), 4.14 (br t, *J* 7, $CHCH_2Ph$), 4.02 (t, *J* 3, $CHCH_2OBu^t$), 3.81–3.31 (m, CH_2OBu^t), 3.24 (br d, *J* 7, CH_2Ph), 3.01 (s, NMe) and 1.22 (s, Bu^t); *m/z* 304 (M^+, weak).

cyclo-(L-*Phenylalanyl*-N-*methyl*-L-*seryl*) **16**.—The cyclodi-

peptide **15** (99 mg, 0.33 mmol) was stirred with 45% hydrogen bromide in acetic acid (2 cm^3) at room temperature for 10 min. The mixture was diluted with dry diethyl ether (50 cm^3) and kept at *ca.* 5 °C overnight to precipitate a white solid. The solid, which was filtered off, collapsed to an oil when exposed to the air. PLC gave the *cyclodipeptide* **16** (30 mg, 37%), m.p. 164.5–165 °C (from ethyl acetate) (Found: C, 62.6; H, 6.5; N, 11.5. $C_{13}H_{16}N_2O_3$ requires C, 62.9; H, 6.45; N, 11.3%); $[\alpha]_D^{21}$ −128° (*c* 1.0 in MeOH); ν_{max}(KBr)/cm^{-1} 3300, 1665 and 1650; δ[90 MHz; $(CD_3)_2SO$] 7.92 (br s, NH, exch. with D_2O), 7.50–7.05 (m, Ph), 5.18 (br t, *J* 6, OH, exch. with D_2O), 7.50–7.05 (m, Ph), 5.18 (br t, *J* 6, OH, exch. with D_2O), 4.04 (m, changing after D_2O exchange to br t, *J* 6.5, C*H*CH_2Ph), 3.81 (m, changing after D_2O exchange to br t, *J* 2.5, C*H*CH_2OH), 3.62 and 3.26 (2 × m, changing after D_2O exchange to 2 × dd, J_{AB} 12, J_{vic} *ca.* 3, CH_2OH), 3.07 (br d, *J* 6, CH_2Ph) and 2.86 (s, NMe); *m/z* 248 (M^+, 6%), and 218 (M − CH_2O, 100).

When the cleavage of the t-butyl ether **15** was carried out in hydrogen bromide in acetic acid for 30 min instead of 10 min the product **16** was mixed with the corresponding O-*acetyl derivative*, obtained by TLC, m.p. 172–177 °C (from acetone) (Found: C, 61.75; H, 6.0; N, 9.5. $C_{15}H_{18}N_2O_4$ requires C, 62.1; H, 6.2; N, 9.65%); $[\alpha]_D^{24}$ −142° (*c* 0.6 in MeOH); ν_{max}(KBr)/cm^{-1} 3250, 1750, 1685 and 1640; δ(90 MHz; CD_3OD) 7.35 (br s, Ph), 4.40–3.95 (3 H, m, CH_2OAc and C*H*CH_2Ph), 3.52 (dd, *J* 12 and 6, C*H*CH_2OAc), 3.25–3.05 (m, CH_2Ph), 3.05 (s, NMe) and 2.05 (s, Ac); *m/z* 290 (M^+).

cyclo-(L-[U-^{14}C]*Phenylalanyl*-N-*methyl*-L-*seryl*.—L-[U-^{14}C]Phenylalanine, purchased from Amersham International plc, was converted in the usual way into the *N*-benzyloxycarbonyl derivative (84 μCi $mmol^{-1}$), which was then coupled with the serine derivative **14**. Deprotection, cyclisation, and cleavage of the t-butyl ether group gave the radiolabelled derivative **16** (83 μCi $mmol^{-1}$). Radioscanning of TLC plates run in 3 different solvent systems showed no significant, radiolabelled impurities. Dilution analysis with unlabelled **16** gave a radiochemical purity of 98%.

L-[3-^{14}C]*Seryl*-L-[4′-^{3}H]*phenylalanine* **17**.—L-[3-^{14}C]Serine and L-[4′-^{3}H]phenylalanine (Amersham International plc) were converted by standard methods into the corresponding *N*-benzyloxycarbonyl and benzyl ester hydrochloride derivatives, respectively. These were coupled with DCC to give *N*-benzyloxycarbonyl-L-[3-^{14}C]seryl-L-[4′-^{3}H]phenylalanine benzyl ester. This derivative was hydrogenolysed, in aq. ethanol containing acetic acid, with 10% palladium–carbon to give the dipeptide **17**. Radiodilution analysis with H-L-Ser-L-Phe-OH, H-L-Ser-D-Phe-OH and H-D-Ser-L-Phe-OH showed this product to be ≥96% radiochemically pure and to contain ≤0.2% of either of the LD- and DL-diastereoisomer.

L-[4′-^{3}H]*Phenylalanyl*-L-[3-^{14}C]*serine* **18**.—This dipeptide was prepared by variants of literature methods which were found to be more suitable for small-scale syntheses. Details are given below, for unlabelled material. Radiodilution analysis of the doubly labelled dipeptide **18** with H-L-Phe-D-Ser-OH and H-D-Phe-D-Ser-OH showed the presence of ≤0.3 and ≤0.2%, respectively, of these potential impurities.

L-*Serine Benzyl Ester Toluene*-p-*sulphonate*.—L-Serine (105 mg, 1 mmol) and toluene-*p*-sulphonic acid (189 mg, 1.1 mmol) were stirred and heated at 100 °C in benzyl alcohol (4 cm^3) until a clear solution was obtained. Tetrachloromethane (7 cm^3) was added and the mixture was heated under reflux in a Soxhlet apparatus charged with molecular sieves. Heating was continued for 6 h, more tetrachloromethane (5 cm^3) being added as soon as an initial cloudiness had disappeared. The mixture was cooled, then diluted with dry diethyl ether to precipitate the required toluene-*p*-sulphonate salt as an oil (289 mg) of sufficient purity for the following preparation.

N-*Benzyloxycarbonyl*-L-*phenylalanyl*-L-*serine Benzyl Ester*.—The foregoing, oily L-serine benzyl ester toluene-*p*-sulphonate was coupled with *N*-benzyloxycarbonyl-L-phenylalanine, in dichloromethane, with DCC and triethylamine, in the usual way. The crude product slowly crystallised from diethyl ether as needles (43%). Recrystallization from ethyl acetate–light petroleum (3:1) gave needles, m.p. 115–118 °C (Found: C, 67.9; H, 5.9; N, 5.7. $C_{27}H_{28}N_2O_6$ requires C, 68.1; H, 5.9; N, 5.9%); $[\alpha]_G^{20}$ +17° (*c* 1.2 in $CHCl_3$); ν_{max}(KBr)/cm^{-1} 3600–3150, 3310, 1737 and 1695; δ(90 MHz; $CDCl_3$) 7.28, 7.23 and 7.14 (3 × br s, 3 × Ph), 7.10–6.78 (m, NH, exch. with D_2O), 5.50 (br d, *J* 8.0, NH, exch. with D_2O), 5.14 and 4.99 (2 × s, 2 × OCH_2Ph), 4.75–4.30 (m, 2 × CH), 4.00–3.78 (m, CH_2OH), 3.15–2.95 (m, CHCH_2Ph) and 2.80 (m, OH, exch. with D_2O); *m/z* 458 (M − H_2O, weak).

L-*Phenylalanyl*-L-*serine*.—Water (28 cm^3) and acetic acid (0.8 cm^3) were added dropwise to a stirred mixture of the foregoing, protected dipeptide (400 mg, 0.84 mmol) in ethanol (56 cm^3). The solution was hydrogenated with 10% palladium–carbon catalyst (40 mg), then filtered. The filtrate was evaporated and the residual oil was dissolved in ethanol. The solution was evaporated and the residue was triturated with chloroform to yield L-phenylalanyl-L-serine hydrate (233 mg, 102%), m.p. 121–125 °C (lit.,[19] 116–125 °C) (Found: C, 53.9; H, 6.8; N, 10.25. Calc. for $C_{12}H_{16}N_2O_4 \cdot H_2O$: C, 53.3; H, 6.7; N, 10.4%); $[\alpha]_D^{20}$ +35° (*c* 2.3 in water) (lit.,[19] +30°); ν_{max}(KBr)/cm^{-1} 3700–2300, 1675 and 1605; δ(90 MHz; CF_3CO_2H) 8.35 (br d, *J* 7, CONH), 7.37 (8 H, br s, Ph and $\overset{+}{N}H_3$), 5.60–4.60 (m, 2 × CH), 4.60–4.10 (m, CH_2OH) and 3.70–3.15 (m, CH_2Ph); *m/z* 234 (*M* − H_2O, weak).

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References

1 G. W. Kirby and D. J. Robins, *The Biosynthesis of Mycotoxins*, ed. P. S. Steyn, Academic, New York, 1980, ch. 9.
2 G. W. Kirby, D. J. Robins, M. A. Sefton and R. R. Talekar, *J. Chem. Soc., Perkin Trans. 1*, 1980, 119.
3 M. Okamoto, K. Yoshida, I. Uchida, M. Nishikawa, M. Kohsaka and H. Aoki, *Chem. Pharm. Bull.*, 1986, **34**, 340.
4 G. W. Kirby, G. L. Patrick and D. J. Robins, *J. Chem. Soc., Perkin Trans. 1*, 1978, 1336.
5 (*a*) G. M. Strunz, M. Kakushima, M. A. Stillwell and C. J. Heissner, *J. Chem. Soc., Perkin Trans. 1*, 1973, 2600; (*b*) M. A. Stillwell, L. P. Magasi and G. M. Strunz, *Can. J. Microbiol.*, 1974, **20**, 759; (*c*) K. H. Michel, M. O. Chaney, N. D. Jones, M. M. Hoehn and R. Nagarajan, *J. Antibiot.*, 1974, **27**, 57; (*d*) G. M. Strunz, C. J. Heissner, M. Kakushima and M. A. Stillwell, *Can. J. Chem.*, 1974, **52**, 325; (*e*) G. M. Strunz, M. Kakushima and M. A. Stillwell, *Can. J. Chem.*, 1975, **53**, 295.
6 F. Dorn and D. Arigoni, *Experientia*, 1974, **30**, 134.
7 N. Johns and G. W. Kirby, *J. Chem. Soc., Perkin Trans. 1*, 1985, 1487 and citations therein.
8 (*a*) J. A. Winstead and R. J. Suhadolnik, *J. Am. Chem. Soc.*, 1960, **82**, 1644; (*b*) M. S. Ali, J. S. Shannon and A. Taylor, *J. Chem. Soc. C*, 1968, 2044.

9 M. I. Pita Poente, G. W. Kirby and D. J. Robins, *J. Chem. Soc., Chem. Commun.*, 1981, 619.
10 G. W. Kirby, W. Lösel, P. S. Rao, D. J. Robins, M. A. Sefton and R. R. Talekar, *J. Chem. Soc., Chem. Commun.*, 1983, 810; G. W. Kirby, D. J. Robins and W. M. Stark, *J. Chem. Soc., Chem. Commun.*, 1983, 812; G. W. Kirby, D. J. Robins and W. M. Stark, *J. Chem. Res. (S)*, 1986, 302.
11 H. J. Issaq and E. W. Barr, *J. Chromatogr.*, 1977, **132**, 121.
12 J. R. Johnson, W. F. Bruce and J. D. Dutcher, *J. Am. Chem. Soc.*, 1943, **65**, 2005.
13 St. Guttmann and R. A. Boissonnas, *Helv. Chim. Acta*, 1958, **41**, 1852.
14 J. P. Greenstein and M. Winitz, *Chemistry of the Amino Acids*, Wiley, New York, 1961, vol. 2, p. 894.
15 *Handbook of Tables for Organic Compound Identification*, ed. Z. Rappoport, CRC Press, Boca Raton, Florida, 3rd edn., 1967.
16 F. M. Callahan, G. E. Anderson, R. Paul and J. E. Zimmerman, *J. Am. Chem. Soc.*, 1963, **85**, 201.
17 S. T. Cheung and N. L. Benoiton, *Can. J. Chem.*, 1977, **55**, 906.
18 I. J. Galpin, A. K. A. Mohammed and A. Patel, *Tetrahedron*, 1988, **44**, 1773.
19 K. Suzuki, T. Abiko and N. Endo, *Chem. Pharm. Bull.*, 1969, **17**, 1671.

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