BIOSYNTHESIS OF GLIOTOXIN

AND RELATED COMPOUNDS

A Thesis presented

in part fulfilment of the requirements for the

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in the

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by

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SUMMARY.

The open chain dipeptides, L-phenylalanyl-L-serine and L-seryl-L-phenylalanine were synthesised, radioactively labelled with ^{14}C and ^{3}H. Both precursors were poorly incorporated into gliotoxin, produced by Trichoderma viride. The isotope ratio measured in gliotoxin was different from those of the precursors which suggested that the precursors were cleaved by the fungus to their constituent amino-acids. It is believed that the amino-acids were then incorporated separately into gliotoxin.

The stereoisomers of cyclo-(phenylalanyl-seryl) were synthesised, labelled with ^{14}C. A high incorporation of the LL-stereoisomer into gliotoxin was observed but the other stereoisomers were poorly incorporated. cyclo-(L-Phenylalanyl-L-seryl), doubly labelled with ^{14}C and ^{3}H, was incorporated into gliotoxin with essentially no change in the isotopic ratio, which suggested that the precursor was incorporated intact. Degradation studies, carried out on doubly labelled gliotoxin from this experiment, demonstrated that the ^{14}C label was in the expected position in the gliotoxin skeleton. Doubly labelled gliotoxin was converted to anhydro-desthiogliotoxin with no change in either the specific activity or the isotopic ratio, which substantiated the radiochemical purity of the sample. Methylamine, derived from the N-methyl group of gliotoxin, was shown to be non-radioactive.

L-Phenylalanine, labelled with ^{14}C, was fed to Trichoderma viride and ^{14}C labelled cyclo-(L-phenylalanyl-L-seryl) was isolated in an intermediate trapping experiment. The fungus was

shown, thus, to be capable of synthesising cyclo-(L-phenylalanyl-L-seryl).

cyclo-(L-Phenylalanyl-N-methyl-L-seryl) was synthesised, labelled with ^{14}C. The precursor was poorly incorporated into gliotoxin.

The cyclic dipeptides mentioned above were fed to a Hyalodendron species. Only cyclo-(L-phenylalanyl-L-seryl) was incorporated significantly into hyalodendrin and bisdethiodi-(methylthio)hyalodendrin. cyclo-(L-Phenylalanyl-L-seryl), doubly labelled with ^{14}C and ^{3}H, was incorporated into the metabolites with essentially no change in the isotopic ratio.

PUBLICATION.

Some of the work described in this thesis has been published in the following paper:

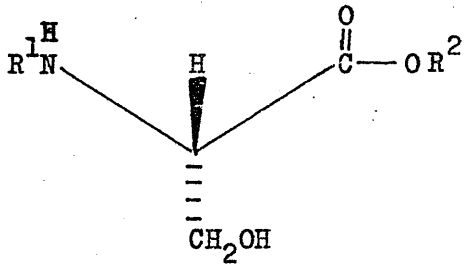
G.W. Kirby, D.J. Robins, and G.L. Patrick, "cyclo-(L-Phenylalanyl-L-seryl) as an Intermediate in the Biosynthesis of Gliotoxin", J. Chem. Soc. Perkin I, 1978, 1336.

NOTE ON NOMENCLATURE.

The following abbreviations are used in the text:

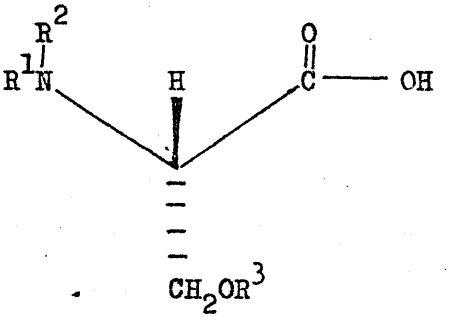
Z	Benzyloxycarbonyl	Phe	Phenylalanine
Bu^t	t-Butyl	Ser	Serine
Boc	Butyloxycarbonyl	Ala	Alanine
DCC	Dicyclohexylcarbodiimide	Trp	Tryptophan
DCU	Dicyclohexylurea	Pro	Proline
NBS	N-Bromosuccinimide	Leu	Leucine
Bz	Benzyl	Ile	Isoleucine
p-TsOH	p-Toluenesulphonic acid		
pts	p-Toluenesulphonate		

Amino-acids which are mono-substituted on the amino and/or the acidic group are represented as shown;



R^1-L-Ser-OR^2

Amino-acids which are disubstituted on the amino group and/or substituted on a side group are represented as shown;

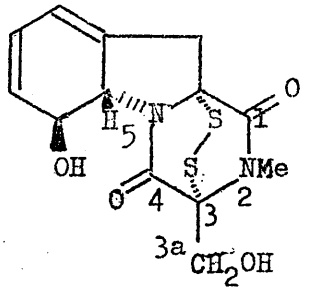


R^1-L-Ser(NR^2,OR^3)-OH

CHAPTER 1 GLIOTOXIN

1.1 Introduction

The first indication that a biologically active compound was produced by the fungus *Trichoderma viride* appeared in 1931, when *T.viride* was found to prevent the decay of wood by *Coniophora cerebella*.[1] In 1932 Weindling[2] observed an antagonism of *T.viride* against the soil fungus *Rhizoctonia solani*, and in 1936[3] he isolated an active compound, unstable to light or basic solution, which was later named gliotoxin (1). The compound was subsequently isolated from cultures of *Gliocladium fimbriatum*[4] (later identified as *T.viride*),[5] *Aspergillus fumigatus*[6], *Penicillium terlikowskii*[7], *Penicillium obscurum*[8] (later identified as *P.terlikowskii*[7]), *Penicillium cinerascens*[9], *Aspergillus chevalieri*[10], *Aspergillus terreus*[11], and *Aspergillus* MO-10.[12]



(1)

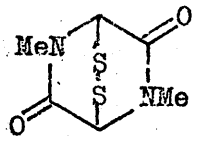
1.2 Biological Activity.

Gliotoxin was found to inhibit the growth of a wide range of organisms. It was found[13] to have bacteriostatic properties which were stronger than those of penicillin, gramicidin, actinomycin, streptothricin, and pyocyanase, and fungicidal properties which were comparable to those of actinomycin. The growth of a range of pathogenic organisms was found to be halted by a gliotoxin level of 10 $\mu g\ ml^{-1}$ and some organisms were inhibited by as little as 0.2-0.3 $\mu g\ ml^{-1}$.[4] Antiviral properties were also observed[14] against RNA viruses such as poliovirus, herpes and Asian influenza viruses. Gliotoxin is toxic to aphids, rabbits, rats, mice, and other mammals.

The mode of gliotoxin's biological action is unclear. Certainly the disulphide bridge is crucial to its activity since all analogues and degradation products of gliotoxin without the disulphide bridge are inactive. Furthermore, the simple epidithiodioxopiperazine (2) has itself been found to have some biological activity[15]. The discovery[16] of small quantities of thiopurines and thiopyrimidines in ribonucleic acids and their ready reaction with disulphides has led to the suggestion that gliotoxin reacts at these centres to play some biological role as yet unknown.

1.3 Structure and Stereochemistry of Gliotoxin.

The structure of gliotoxin was elucidated by chemical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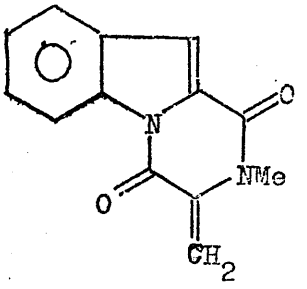
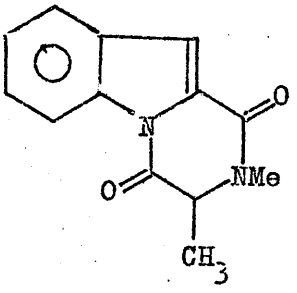
S
S
NMe
O
O

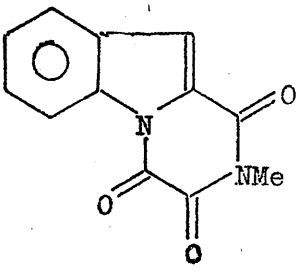
(2)


N
O
O
NMe
CH2

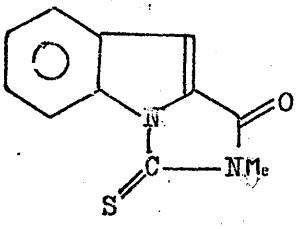
(3)


N
O
O
NMe
CH3

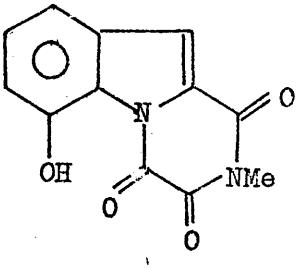
(4)


N
O
O
NMe
O

(5)


N
O
C
NMe
S

(6)


N
O
OH
NMe
O
O

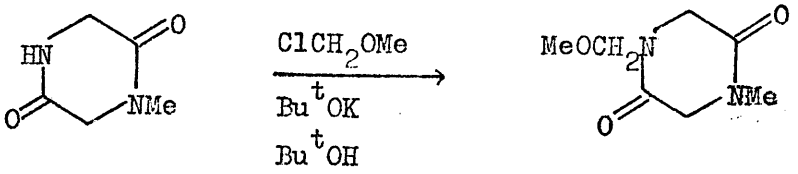
(7)

methods and the study of degradation products. This work has been covered in several reviews[17-20] and is merely summarised here. The basic skeleton of the molecule was established by degradation of glictoxin to three separate products (3)-(5) whose structures were proven by chemical synthesis. The synthesis of the thiohydantoin (6) from gliotoxin suggested that the sulphur atoms were linked to the noncarbonyl carbon atoms of the dioxopiperazine ring. The position of the secondary hydroxyl group in gliotoxin was established by degradation of gliotoxin to (7), whilst the primary hydroxyl group was located by the u.v. and n.m.r. spectroscopic data of other degradation products.

X-ray crystallographic analysis[21] confirmed the structure, established the absolute configuration and showed that the strained disulphide bridge has a twisted conformation, with a dihedral angle about C-S-S-C of 12°. The sulphur atoms lie closer to the carbonyl groups of the dioxopiperazine ring than to the nitrogen atoms.[22]

1.4 Synthesis of Gliotoxin.

A total synthesis of gliotoxin was achieved in 1976 by Kishi and co-workers[23] (Figure 1). Starting from glycine sarcosine anhydride (8), the dithiol (9) was synthesised in four steps. Formation of the thioacetal (10) from (9) gave a mixture of diastereomers. Kishi and co-workers[24] had already shown that the thioacetal was a useful protected precursor for the disulphide bond of the epidithiodioxopiperazine structure and had already



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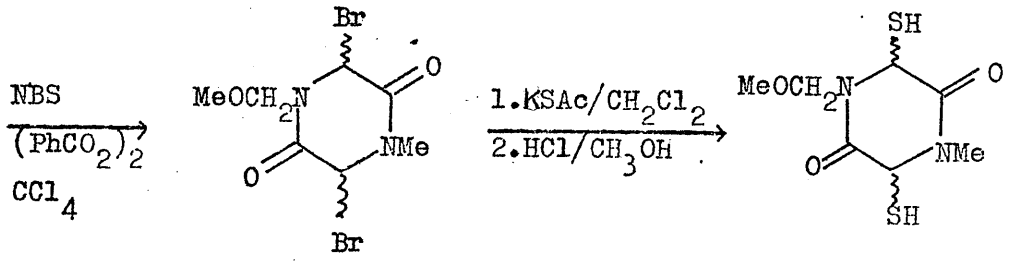

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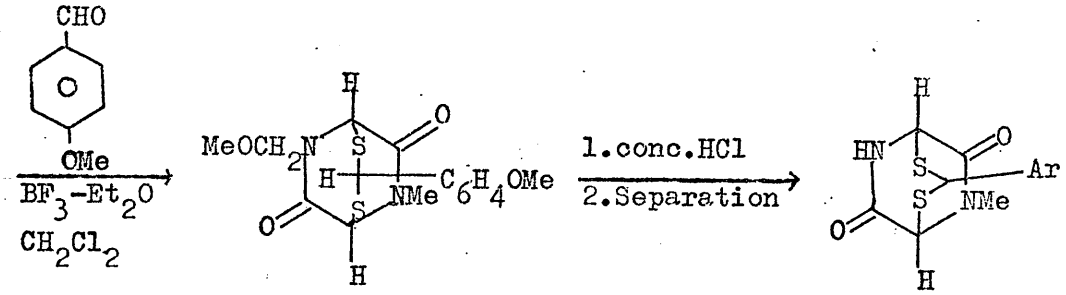
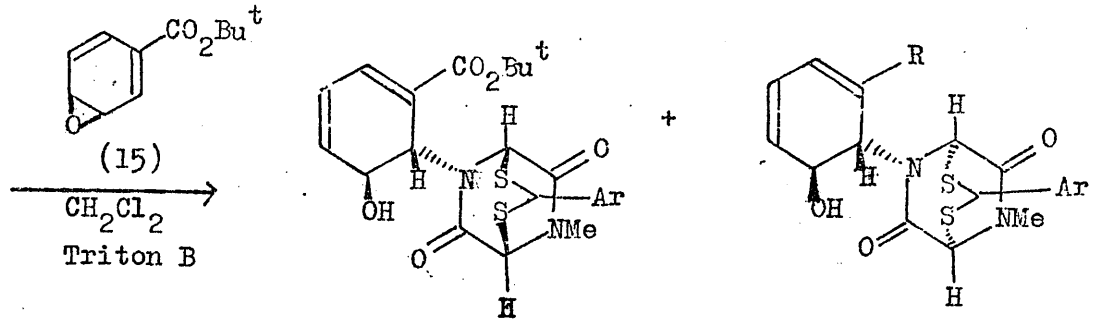
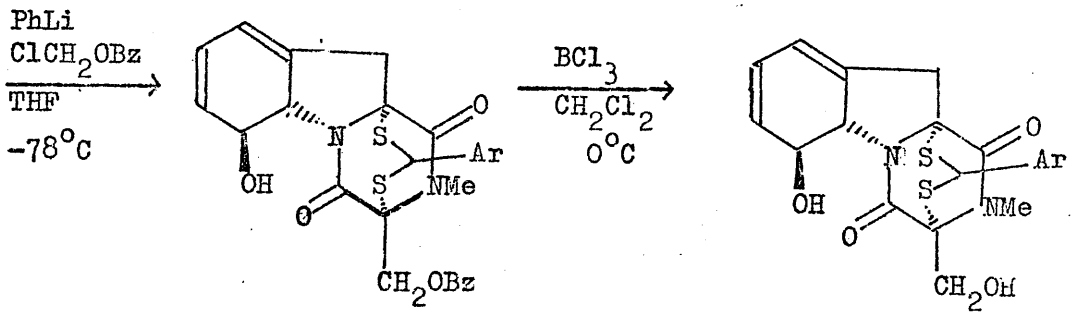


(10) (14)

Figure 1. Synthesis of Gliotoxin.





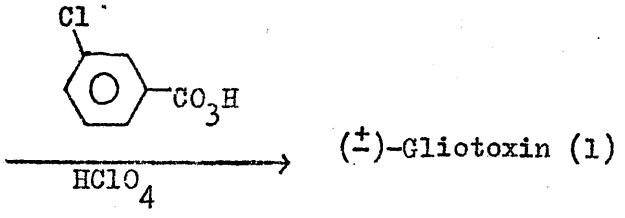
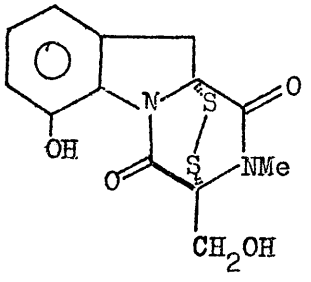
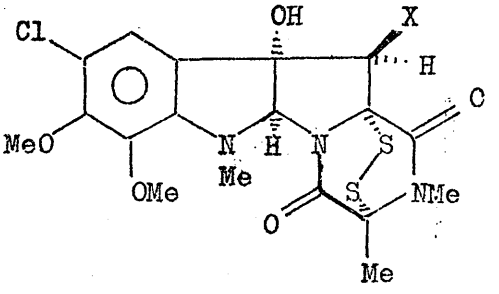


Figure 1. Synthesis of Gliotoxin.

devised syntheses for dehydrogliotoxin (11)[25], sporidesmin A (12)[26], and sporidesmin B (13)[27] from related intermediates.



(11)



(12) X= OH

(13) X= H

Removal of the methoxymethyl protecting group of (10) gave a mixture of diastereomers (14) which was separated by chromatography of the N-benzoyl derivatives. The thioacetal (14) was obtained after aminolysis in 30% overall yield from (8) with the aromatic residue anti to the NH group. Coupling of (14) with the benzene oxide (15) was achieved by a novel, solvent dependent Michael reaction to yield a 1:1 mixture of two products (16) and (17) of which the latter had the required stereochemistry. The alcohol (17) was converted into the chloride (18) in seven steps.[23] The benzylgliotoxin adduct (19) was obtained in 45% yield by alkylation of (18) (Figure 1). The benzyl protecting group was removed to yield the gliotoxin anisaldehyde adduct (20) in 50% yield. Finally, the disulphide bridge was formed by oxidation to the sulphoxide followed by perchloric acid treatment which resulted in a facile carbon-sulphur bond fission. The resulting

carbonium ion experienced resonance stabilisation from the p-methoxybenzene ring. With the loss of anisaldehyde, the disulphide bridge formed spontaneously to yield a,l-gliotoxin.

1.5 Biosynthesis of Gliotoxin.

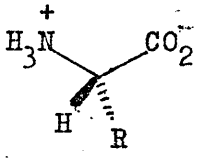
The biosynthesis of gliotoxin and related metabolites has been reviewed recently.[18,28] Only a brief summary will be given here to place the author's own studies in context.

Labelling studies[29-32] revealed that gliotoxin is derived from the amino-acids L-phenylalanine (21) and L-serine (22) and that the N-methyl carbon is provided by L-methionine (23).

A high incorporation of m-tyrosine into gliotoxin reported by Winstead and Suhadolnik[30] led to the proposal that m-tyrosine is an intermediate in the biosynthetic pathway to gliotoxin. This result, however, could not be confirmed by other workers[33-35] and rigorous labelling experiments showed that m-tyrosine could not be an obligatory intermediate. The most likely explanation for the incorporation observed by Winstead and Suhadolnik is that their m-tyrosine had been contaminated with L-phenylalanine.

The dioxopiperazine, cyclo-(L-phenylalanyl-L-seryl) (24), was studied by MacDonald and Slater[36] as a possible biosynthetic intermediate for gliotoxin. They observed only a low incorporation of cyclo-(L-[1-^{14}C]phenylalanyl-L-seryl) into gliotoxin produced by Penicillium terlikowskii, even though the precursor was taken up into the mycelium, and concluded that the cyclic dipeptide was not a free intermediate. Bu'Lock and Leigh[37], on the other hand, observed a high incorporation (21%) of a mixture of cyclo-

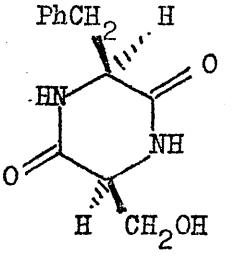
(L-[Ar-^{3}H]phenylalanyl-L-[1-^{14}C]seryl) and cyclo-(L-[Ar-^{3}H] phenylalanyl-D-[1-^{14}C]seryl) into gliotoxin produced by Trichoderma viride. Furthermore the isotope ratios $^{14}C:^{3}H$ of the precursor mixture and gliotoxin were the same within experimental error, implying that the precursor had been incorporated intact. It was suggested[37] that the high levels of (24) fed to the cultures by MacDonald and Slater had resulted in misleading results.



(21) R= CH_2Ph

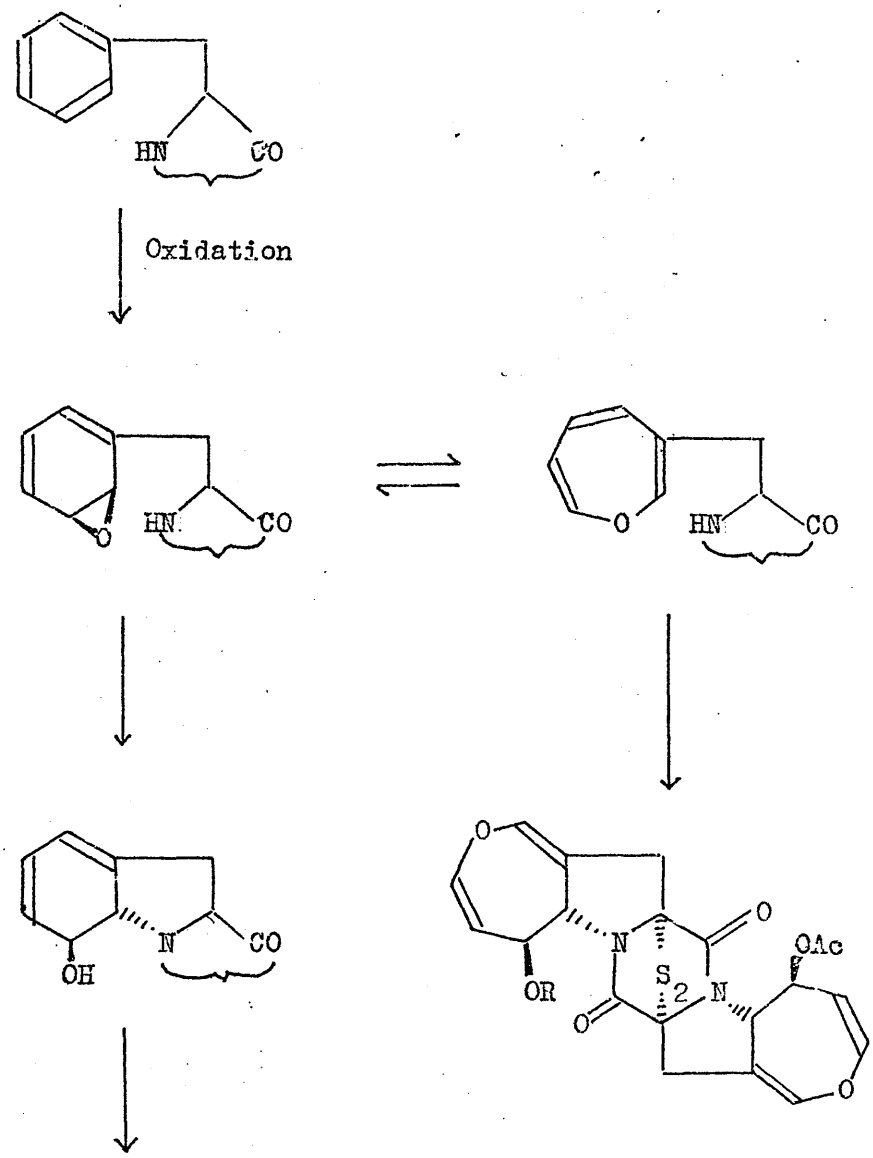
(22) R= CH_2OH

(23) R= CH_2CH_2SMe



(24)

Little research has been carried out into the later stages of the biosynthesis. At least three further steps are required; N-methylation, oxidative cyclisation on to the aromatic ring, and incorporation of the disulphide bridge. N-Methylation has been shown already to involve methionine but it is not known at what stage it occurs. Oxidative ring closure is thought to involve a benzene oxide-oxepin intermediate which may also be involved in the biosynthesis of aranotin[38,39] (25) (Figure2).



Gliotoxin

(25) R= H

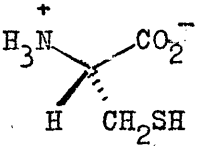
(26) R= Ac

Figure 2. Postulated Mechanism for Oxidative Ring Closure.

It may be significant that both gliotoxin and acetylaranotin (26) were isolated[11] from cultures of the same organism, Aspergillus terreus.

L-[^{35}S]Methionine was incorporated into gliotoxin but when inactive L-cysteine was added, the incorporation dropped and the amount of gliotoxin obtained increased. This suggested that L-cysteine was a better sulphur source for gliotoxin than L-methionine.[3]

Olefinic derivatives such as (28) were shown not to be obligatory intermediates for gliotoxin by Johns et al.[40] Stereoselectively deuteriated and tritiated (3R)- and (3S)-phenylalanines were fed to T.viride and, although stereospecific loss of the (3R)-proton was observed in gliotoxin, the loss was not quantitative signifying that the observed loss was associated with extensive transamination of L-phenylalanine.



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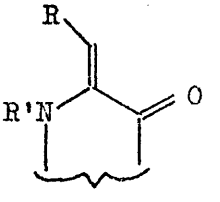
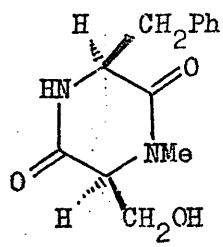


(28)

To conclude, at the outset of this work, phenylalanine and serine had been established as the basic biosynthetic precursors for gliotoxin but the status of cyclo-(L-phenylalanyl-L-seryl) was confused by conflicting results. It was proposed, therefore, to study the various stereoisomers of cyclo-(phenylalanyl-seryl) as possible intermediates for gliotoxin and this work is described

in Chapter 4. No experiments using open chain dipeptides of phenylalanine and serine as precursors of gliotoxin have been published. The synthesis and results of feeding these compounds is the subject of Chapter 3. A study of cyclo-(L-phenylalanyl-N-methyl-L-seryl) (29) as a possible biosynthetic intermediate for gliotoxin is recorded in Chapter 6.

Finally, a biosynthetic study of hyalodendrin (30), a fungal metabolite closely related to gliotoxin, is described in Chapter 7. This metabolite differs from gliotoxin in that the disulphide bridge has the opposite absolute configuration and both nitrogen atoms are methylated. It was interesting therefore to investigate whether gliotoxin and hyalodendrin share common biosynthetic intermediates.



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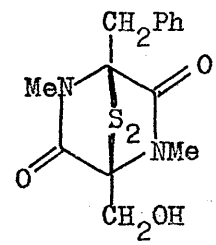

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CHAPTER 2 DIOXOPIPERAZINES AS BIOSYNTHETIC INTERMEDIATES.

Since cyclo-(L-Phe-L-Ser) (24) has been proposed as a key intermediate in gliotoxin biosynthesis, it is relevant to review cases where other simple dioxopiperazines have been identified or proposed as biosynthetic intermediates for natural products.

2.1 Echinulin and Related Compounds.

Simple dioxopiperazines have been established as biosynthetic intermediates for such metabolites as echinulin (31), neoechinulin A (32), and brevianamide A (33)[48].

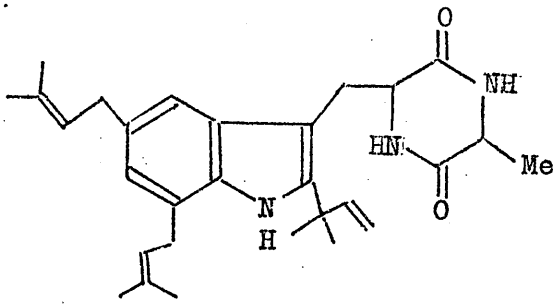
2.1.1 Echinulin and Neoechinulin. Echinulin (31), isolated from Aspergillus amstelodami, was shown to be derived from tryptophan[41], alanine[42], and mevalonic acid.[43] Slater et al.[44] subsequently fed, to this organism, cyclo-(L-alanyl-L-[3'-^{14}C]tryptophyl) (34) which was incorporated into echinulin, thus demonstrating that isoprenylation is a late step in the biosynthetic pathway. Further evidence supporting (34) as an intermediate was provided by Allen[45] who was able to isolate a partially purified enzyme from A.amstelodami, capable of transferring the isoprene unit from 3-methyl-2-butenyl-1-pyrophosphate (35) to the cyclic dipeptide (34). No transfer was observed with tryptophan or either of the open chain dipeptides of tryptophan and alanine. The product of the enzyme mediated isoprenylation of (34) was identified as preechinulin (36), a metabolite isolated from an echinulin-producing culture of A.chevalieri.[46] A sample of

preechinulin, prepared as above and doubly labelled with ^{14}C and ^{3}H, was incorporated into echinulin without any change in the isotope ratio, thus establishing it and the dioxopiperazine precursor (34) as intermediates.[47]

Neoechinulin (32) is a metabolite closely related to echinulin. Marchelli *et al.*[49] have shown that *cyclo*-(L-[U-^{14}C] Ala-L-[5,7-$^{3}H_2$]Trp) was incorporated, without change in the ^{14}C:^{3}H ratio, into neoechinulin A (32) and related metabolites.

2.1.2 Brevianamides and Austamides. A large range of metabolites, shown to be derived from proline, tryptophan, and mevalonic acid,[50] have been isolated from *Penicillium brevicompactum* and *Aspergillus ustus*. These metabolites, the brevianamides and austamides, all contain an isoprene unit at position 2 of the indole nucleus. The simple dioxopiperazine, *cyclo*-(L-[5-^{3}H]prolyl-L-[3-^{14}C]tryptophyl) (37) was incorporated intact into brevianamide A (33) and was also identified as a metabolite of *P.brevicompactum*.[51]

2.1.3 Fumitremorgen and Verrucologen. Several metabolites related to brevianamide have been isolated from *Aspergillus fumigatus*, *Penicillium verruculosum*, and *Aspergillus caespitocus*[55], for example fumitremorgen B[53] (38) (also known as lanosulin[52]) and verruculogen TR_1[54] (39). These compounds are interesting in that the indole nitrogen is isoprenylated since N-isoprenylated intermediates have been proposed for the brevianamides.[49] *cyclo*-(L-Pro-L-Trp) (37) is presumably an intermediate to these compounds but this has still to be proved.



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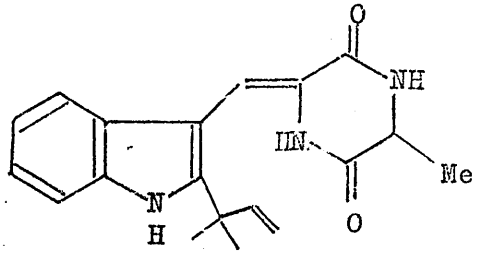

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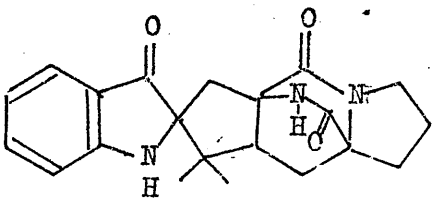

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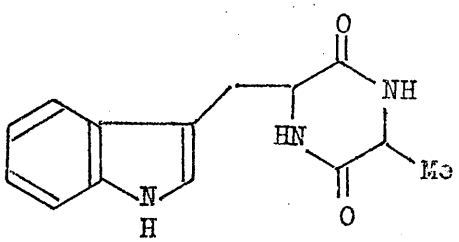

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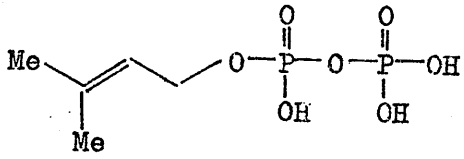

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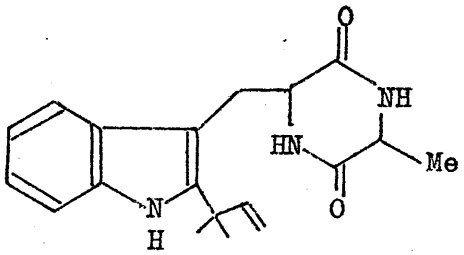

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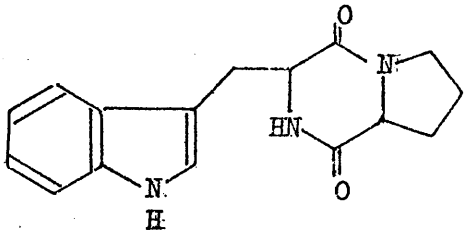

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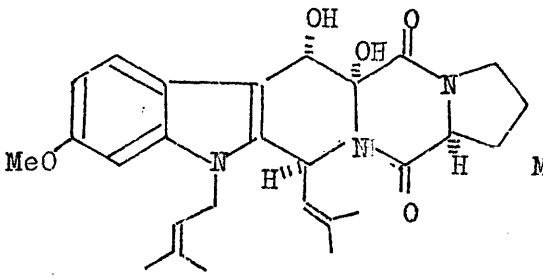

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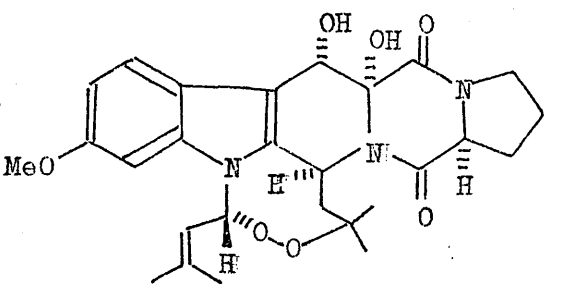


(39)

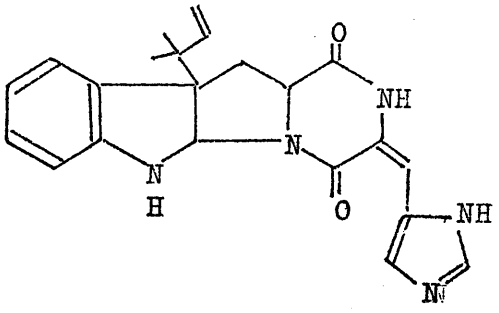
2.1.4 Roquefortine and Oxaline. Roquefortine (40), isolated from Penicillium roqueforti[56], has been found to have neurotoxic properties[57] and is related to oxaline (41), an unusual alkaloid from Penicillium oxalium.[58] Mevalonic acid, tryptophan, and histidine were incorporated into roquefortine[59] but cyclo-(L-histidyl-L-tryptophyl) (42) remains to be tested.

2.2 Bicyclomycin.

Bicyclomycin (43), a metabolite of Streptomyces sapporonensis, is an antibiotic active against Gram-negative bacteria. It has been suggested[19] that it might arise from cyclo-(L-leucyl-L-isoleucyl) (44) but biosynthetic studies are still to be carried out.

2.3 Dibromophakellin.

It has been suggested that cyclo-(L-prolyl-L-prolyl) (45) might be an intermediate for dibromophakellin (46) isolated from the marine sponge, Phakellia flabellata but research into the biosynthesis remains to be carried out.[19]



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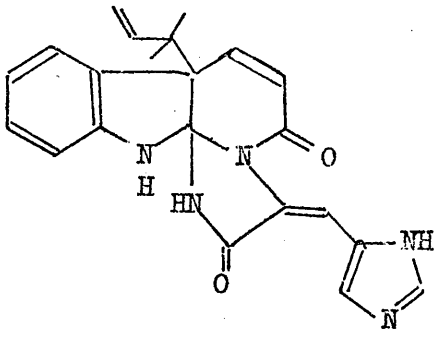

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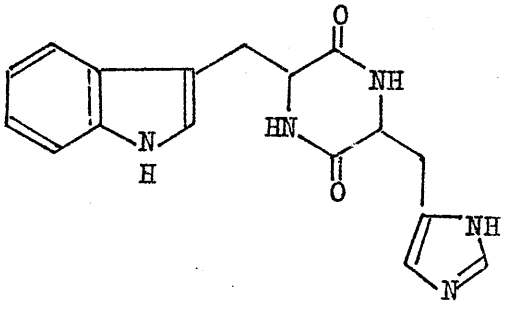

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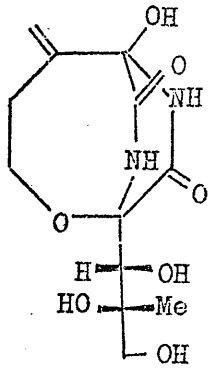

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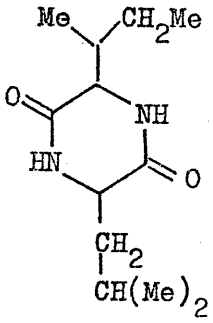

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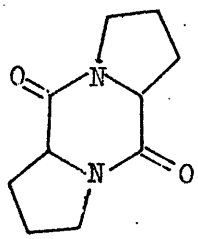

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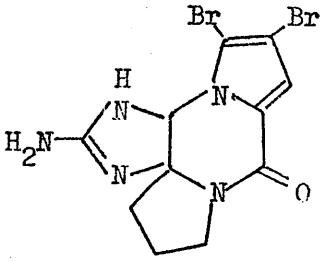


(46)

2.4 Hydroxypyrazine Derivatives.

2.4.1 Aspergillic Acid and Related Compounds. Biosynthetic studies on these compounds have been carried out by MacDonald and his collaborators. Experiments[60] with Aspergillus sclerotiorum revealed that L-[1-^{14}C]leucine and [^{14}C]flavacol (49) were incorporated into neoaspergillic acid (48). Similar experiments showed that leucine and isoleucine were incorporated into aspergillic acid (47) isolated from Aspergillus flavus.[61] Deoxyaspergillic acid (50) was incorporated into aspergillic acid when fed to A.sclerotiorum.[62] Labelling experiments with cyclo-(L-[^{14}C]Leu-L-Leu) (51) and cyclo-(L-Leu-L-[^{14}C]Ile) (44) were not so successful however, and low incorporations into neoaspergillic acid and aspergillic acid respectively were observed.[62] MacDonald and Slater[36] suggested that the simple dioxopiperazine was not an intermediate and that a linear dipeptide was formed on an enzyme complex then modified before cyclisation. However the amount of substrate fed was similar to the amount of metabolite isolated. This may have resulted in the suppression of equilibration with exogenous material as suggested by Bu'Lock and Leigh[37] in the case of the cyclic dipeptide precursor of gliotoxin.

2.4.2 Pulcherriminic Acid. Pulcherriminic acid (52) is an effective chelating agent and may have a function in nature as an iron chelate. It was isolated from Candida pulcherrima and was shown to be derived from two molecules of L-leucine.[63]

Radioactively labelled cyclo-(L-Leu-L-Leu) (51) was also isolated from this experiment and a high incorporation of cyclo-(L-[^{14}C] Leu-L-Leu) into pulcherriminic acid was observed.[63]

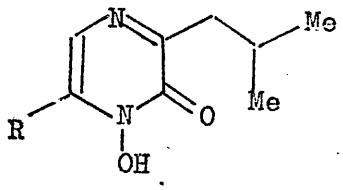
2.4.3 Mycelianamide. Mycelianamide (53) is a metabolite of Penicillium griseofulvin. Labelling studies have established mevalonic acid[64,65] and tyrosine[66] as precursors. cyclo-(L-Alanyl-L-[^{14}C]tyrosyl) (54) and cyclo-(L-alanyl-D-[^{14}C]tyrosyl) were not incorporated very well into mycelianamide[36] but the dose levels were high with respect to the amount of metabolite isolated and the arguments put forward by Bu'Lock and Leigh[37] may apply as discussed previously.

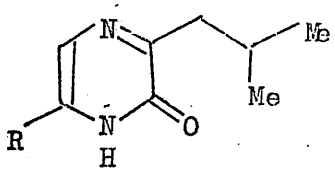
2.5 Epidithiodioxopiperazines

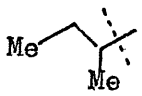
2.5.1 Gliotoxin and Hyalodendrin. The biosynthesis of these metabolites has been discussed in Chapter 1.

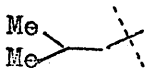
2.5.2 Aranotin and Epicorazine. cyclo-(L-Phenylalanyl-L-phenylalanyl) (55) has been suggested as the intermediate for aranotin (25) and epicorazine A (56). The dioxopiperazine (55) has been isolated from epicorazine-producing cultures[67] of Epicoccum nigrum but biosynthetic studies remain to be carried out.

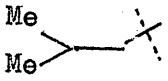
2.5.3 Sporidesmins. Sporidesmin (57) and related metabolites are thought to be derived from cyclo-(L-alanyl-L-tryptophyl) (34).

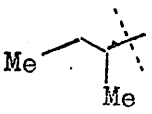


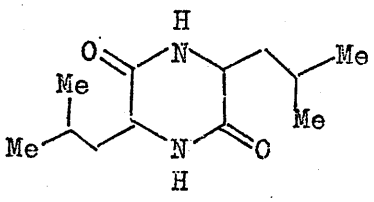


(47) R= 

(49) R= 

(48) R= 

(50) R= 



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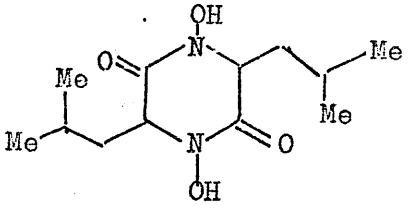

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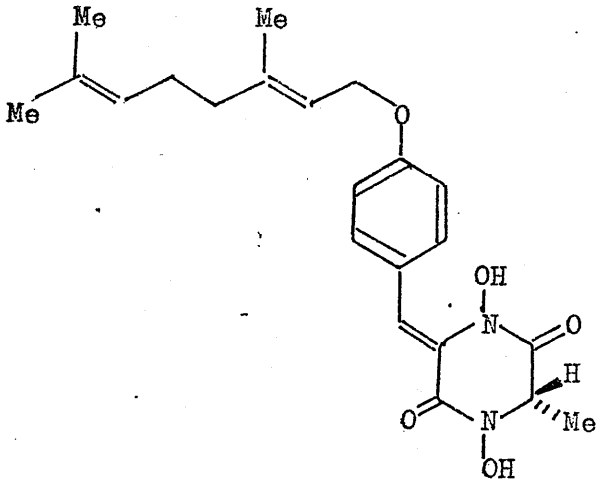

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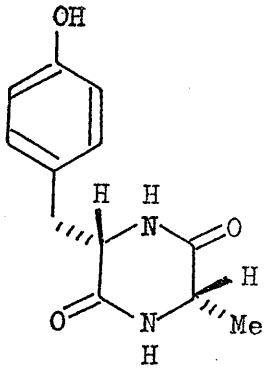
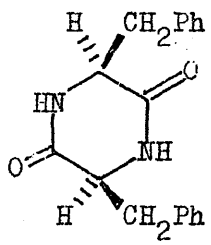


(54)

Kirby and Varley[68] fed cyclo-(L-[3-^{3}H]Ala-L-[3-^{14}C]Trp) to Pithomyces chartarum and observed a good incorporation (2.05% based on ^{14}C) into sporidesmin. The ^{3}H:^{14}C ratio was 51% of that of the precursor however, which suggested that either the precursor was being degraded to its constituent amino acids or that a dehydroalanyl derivative was involved in the biosynthetic pathway.



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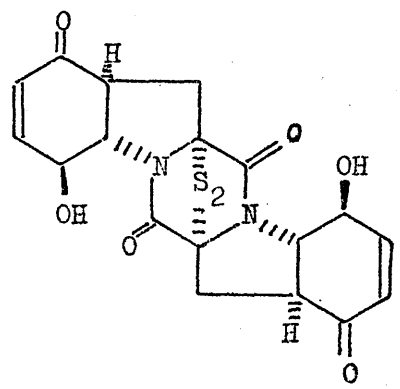

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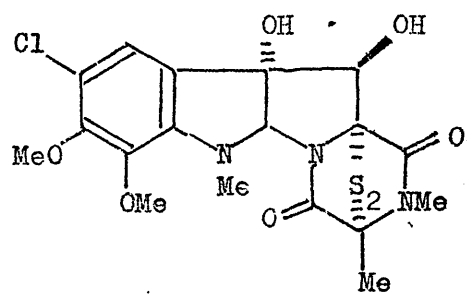
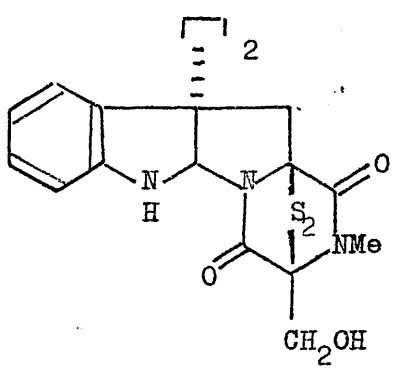


(57)

2.5.4 Other Epidithiodioxopiperazines. Chaetocin (58) and chetomin (59) may be derived from cyclo-(L-seryl-L-tryptophyl) (60) as might the verticillins e.g. verticillin B (61). The newly discovered sirodesmins, e.g. sirodesmin A (62) may be derived from cyclo-(L-seryl-L-tyrosyl) (63).*

2.6 Conclusion.

Simple dioxopiperazines have been shown to be biosynthetic intermediates for a variety of natural products containing a dioxopiperazine nucleus as well as being natural products in their own right. However, much research still requires to be done on a number of natural products for which simple dioxopiperazines have been proposed as likely intermediates.



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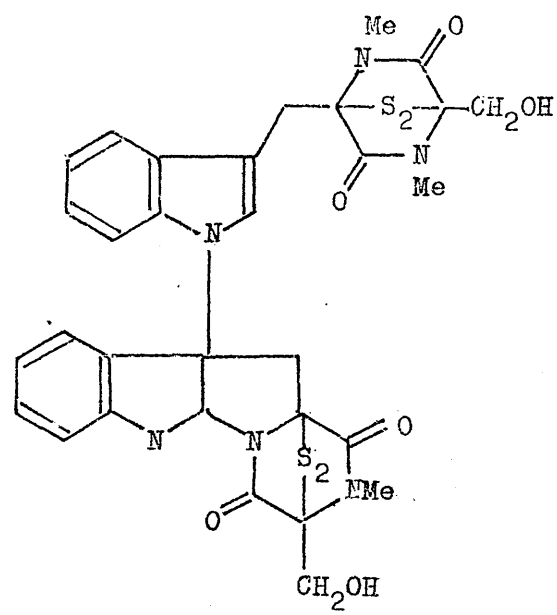
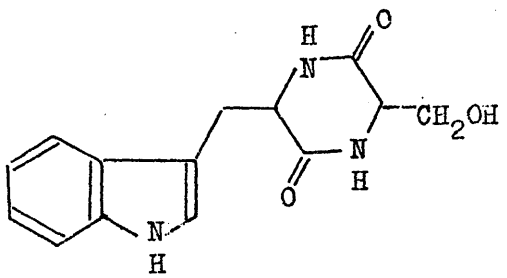


(59)

* Tyrosine has recently been shown to be a precursor for the sirodesmins, J.D.Bu'Lock, personal communication to G.W.Kirby.



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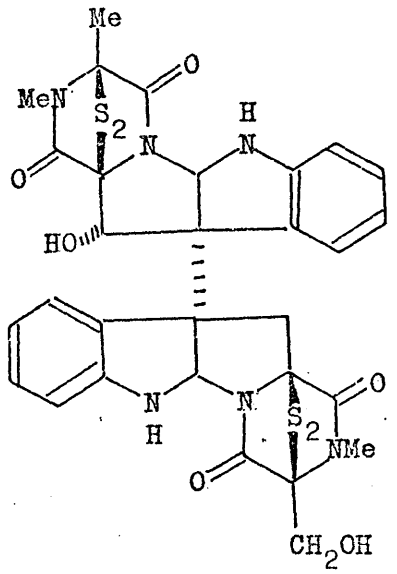

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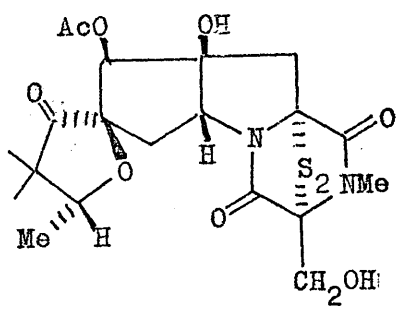

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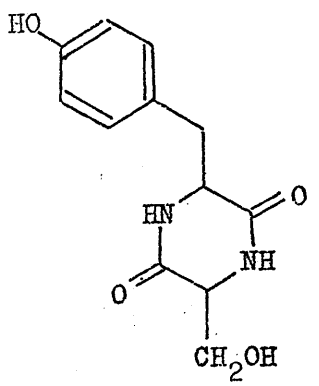


(63)

CHAPTER 3 L-PHENYLALANYL-L-SERINE AND L-SERYL-L-PHENYLALANINE AS POSSIBLE BIOSYNTHETIC PRECURSORS FOR GLIOTOXIN.

Introduction.

L-Phenylalanine (21) and L-serine (22) have been established as biosynthetic precursors for gliotoxin (1).[29-32] It seemed feasible that an open chain dipeptide derived from these two amino-acids might be a biosynthetic intermediate and therefore the synthesis of the two possible combinations, L-phenylalanyl-L-serine (64) and L-seryl-L-phenylalanine (65), doubly radioactively labelled with ^{14}C and ^{3}H, was planned. Feeding experiments and comparison of the incorporations of each precursor into gliotoxin would then be carried out.

3.1 Synthesis of L-Seryl-L-phenylalanine (65).

H-L-Ser-L-Phe-OH (65) was synthesised by Miyoshi *et al.*[69] by three different routes. The most convenient route is shown in Figure 3.

3.1.1 L-Phenylalanine Benzyl Ester Hydrochloride (66). The ester hydrochloride (66) was synthesised by the method of Patel and Price[70], by heating phenylalanine in benzyl alcohol in the presence of thionyl chloride. High yields (90%) were obtained of the crude product (66). However, the recovery from

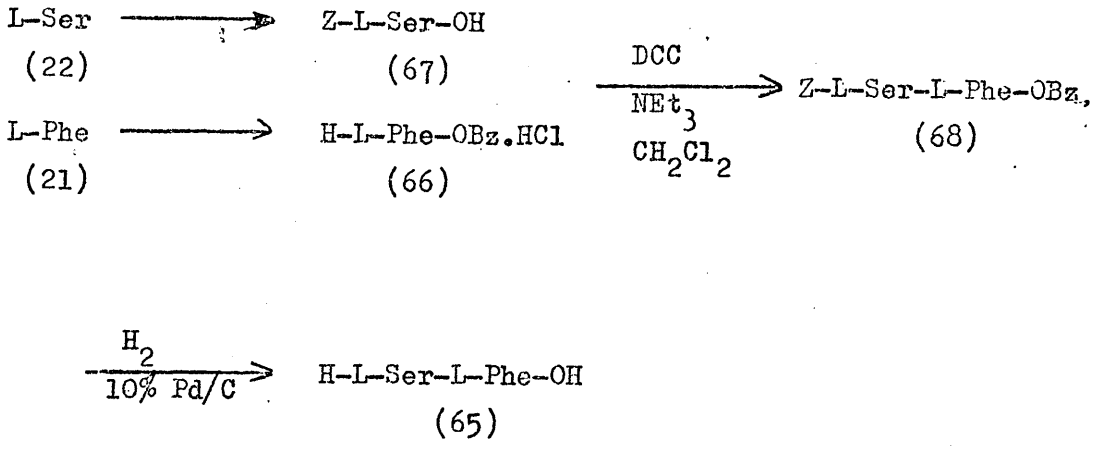
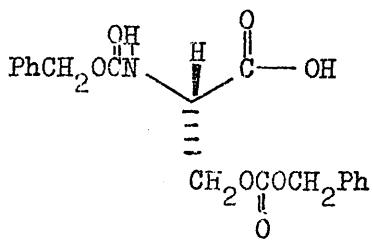


Figure 3 Synthesis of H-L-Ser-L-Phe-OH (65).

crystallisation, carried out in ethanol rather than ethanol-ether[70], was low (ca. 60%) and crystallisation of the mother liquors was usually necessary. Although the melting point was consistently low, the i.r. spectrum was in accord with the literature[70] and the n.m.r. spectrum was consistent with the structure (66).

3.1.2 N-Benzyloxycarbonyl-L-serine (67). Z-L-Ser-OH (67) was synthesised by the method of Guttman and Boissonas[71] by stirring a solution of L-serine (22) in aqueous sodium bicarbonate with benzyloxycarbonyl chloride (Figure 3). The Schotten-Baumann conditions[72] involving sodium hydroxide as base could not be used in this case since a mixture of (67) and N-benzyloxycarbonyl-O-benzyloxycarbonyl-L-serine (69) was obtained.[73] High yields were obtained as long as the solution was vigorously stirred. The optical rotation and melting point agreed with literature values. I.r. and n.m.r. spectra were in accord with the structure (67).

3.1.3 Coupling Procedure. The two protected amino-acids (66) and (67) were coupled by the DCC method (Figure 3) to give the protected dipeptide (68). The major contaminant was DCU (70).



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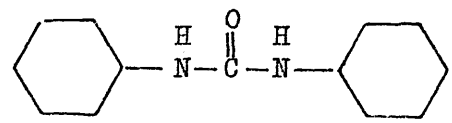


(70)

This was removed by precipitation from a solution of (68) in acetone, by crystallisation, and by ether trituration of solid (68). The melting point and optical rotation of the protected dipeptide (68) agreed with literature values.[69] The i.r. spectrum was consistent with the proposed structure. The n.m.r. spectrum was complicated, but several signals sharpened after addition of D_2O to the solution thus allowing the assignments shown in Figure 4 to be made. Complication of the signals arises from the

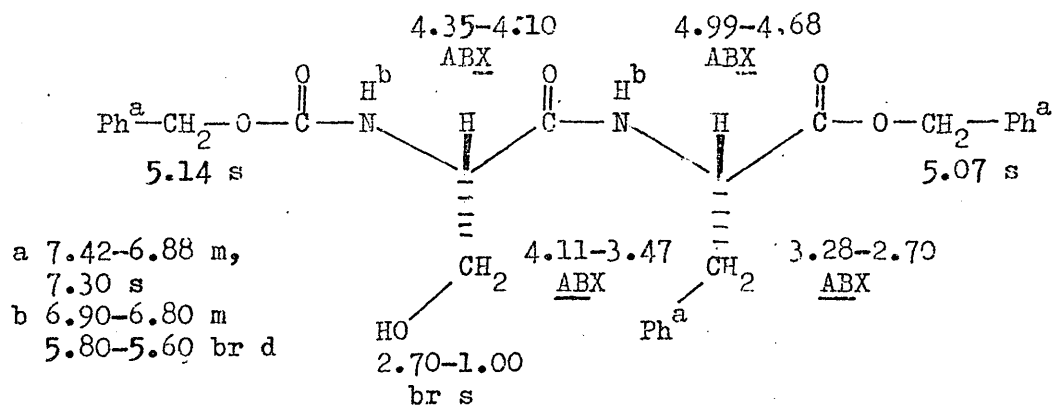


Figure 4 N.m.r. Spectrum of (68) in $CDCl_3$ (δ values).

fact that the methylene protons adjacent to a chiral centre are not in identical environments and are therefore diastereotopic. Of the two AB methylene multiplets, the one at lower field is most likely to arise from the methylene group adjacent to the hydroxyl group. Confirmation of this was provided by the sharpening of this multiplet when the hydroxyl proton was exchanged for deuterium. The methine signals were assigned on the basis of decoupling experiments and comparison of vicinal coupling constants measured after the sample was shaken with D_2O.

The mass spectrum of (68) was dominated by fragmentation of the benzyloxycarbonyl group (Figure 5). The parent ion was weak and the base peak was due to the tropylium ion at m/e 91.

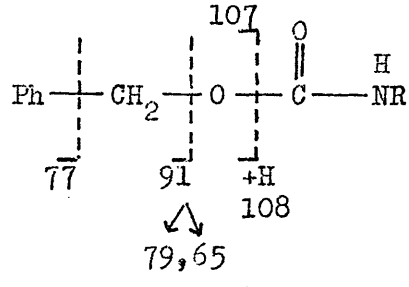
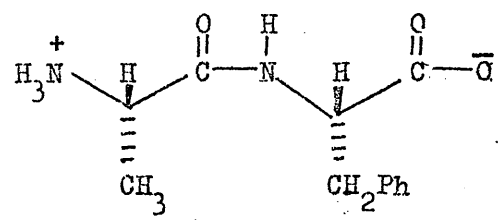


Figure 5 Fragmentation of the Benzyloxycarbonyl Group.

3.1.4 Formation of Linear Dipeptide (65). Both protecting groups were efficiently removed by hydrogenolysis (Figure 3) as long as the solvent ratio of ethanol to water was 2:1. With any other composition, either starting material or product was found to crystallise out and coat the catalyst, thus stopping the reaction. The triturated linear dipeptide (65) was identical

with a sample crystallised from methanol-water (1:1). T.l.c. gave one spot when developed in three different solvent systems. The optical rotation, $[\alpha]_D$ +51.0°, was comparable with the literature value of +53.8°.[71] The i.r.spectrum was characteristic of a zwitterion and closely resembled a literature spectrum of L-alanyl-L-phenylalanine (71). Fragmentations observed in the mass spectrum are outlined in Figure 6. The base peak at m/e 60 resulted from a well known amine fragmentation route. A MacLafferty rearrangement to give a peak at m/e 222 was observed but this was weak as was that of the parent ion. The n.m.r. spectrum of (65) was assigned as shown in Figure 7. Assignments were based on the sharpening of signals observed when D_2O was added to the sample and on decoupling experiments.



(71)

3.2 Synthesis of H-L-[3-^{14}C]Ser-L-[4'-^{3}H]Phe-OH.

It was decided to label both halves of the dipeptide (65), using ^{14}C and ^{3}H, so that any hydrolysis of the dipeptide by the fungus would be detected by a change in the isotopic ratio measured in gliotoxin. The synthesis, starting from L-[3-^{14}C] serine and L-[4'-^{3}H] phenylalanine, was carried out on a 1 mmol

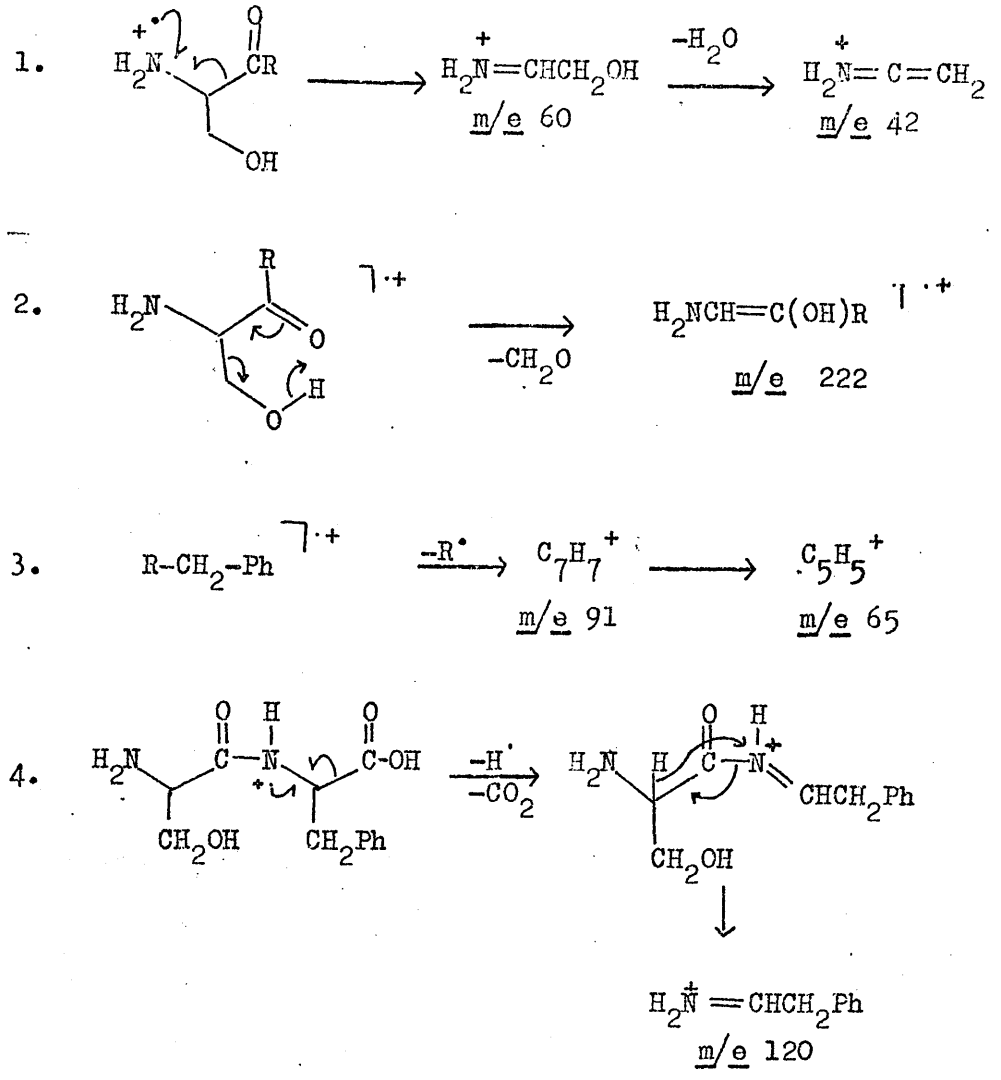


Figure 6 Mass Spectrum of H-L-Ser-L-Phe-OH (65).

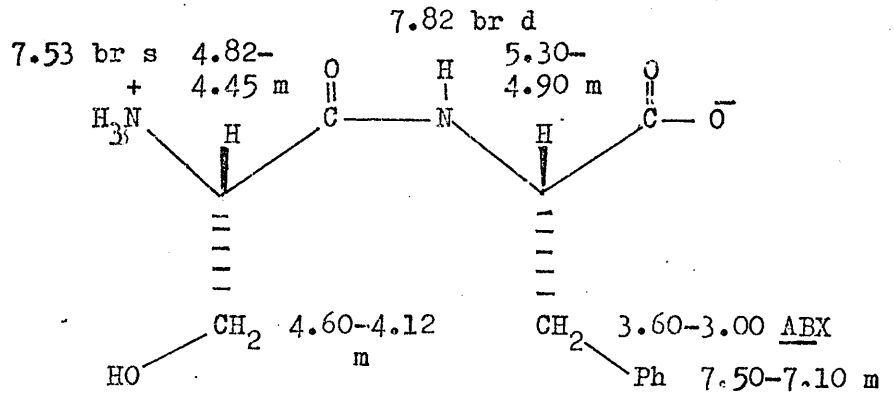


Figure 7 N.m.r. Spectrum of H-L-Ser-L-Phe-OH (65) in CF_3CO_2H. (δ values).

scale. H-L-[4'^{3}H]-Phe-OBz.HCl and Z-L-[3-^{14}C]Ser-OH were synthesised in high yield as previously described (Figure 3) and coupled to give the protected dipeptide (68) in 56% yield with ^{14}C and ^{3}H specific activities essentially unchanged. Only one band was observed on Panax scanning and autoradiography of an analytical t.l.c. plate. Hydrogenolysis of doubly labelled (68) gave the linear dipeptide (65) in 86% yield with unchanged specific activity. Panax scanning and autoradiography of t.l.c. plates run in five different solvent systems established that neither cyclo-(L-Phe-L-Ser) (24) nor any other contaminant was present. However, this did not reveal whether epimerisation had occurred since H-L-Ser-D-Phe-OH (73) was found to have the same R_f value as the LL-isomer in all solvent systems tried. Dilution analysis was carried out to test for possible epimerisation by diluting a small quantity of labelled dipeptide (65) (usually 1mg) with unlabelled carrier (usually 50mg) and crystallising to constant activity. The results (Table 1) show that the radiochemical purity was not less than 96% and that not more than 0.2% H-L-Ser-D-Phe-OH (73) or H-D-Ser-L-Phe-OH (74) was present.

3.3 Synthesis of L-Seryl-D-phenylalanine (73) and D-Seryl-L-phenylalanine (74).

The linear dipeptides (73) and (74), required for dilution analysis (see above), were synthesised as described for the LL-isomer (65) (Figure 3).

3.3.1 N-Benzyloxycarbonyl-D-seryl-L-phenylalanine Benzyl Ester (75) and its Enantiomer (76). Z-D-Ser-OH (77), synthesised from D-serine (78) in 90% yield, was coupled with H-L-Phe-OBz.HCl (66) to form Z-D-Ser-L-Phe-OBz (75) in 54% yield. The i.r. and mass spectra were very similar to those of the LL-isomer (68) and the n.m.r. spectrum of the benzyl ester was consistent with the structure (75).

The LD-isomer (76) was similarly synthesised from Z-L-Ser-OH (67) and H-D-Phe-OBz.HCl (79) in 49% yield and had identical physical properties to (75) with the exception of optical rotation.

3.3.2 Formation of Linear Dipeptides. Hydrogenolysis of Z-L-Ser-D-Phe-OBz (76) gave H-L-Ser-D-Phe-OH (73) in 44% yield after recrystallisation. The i.r. and mass spectra were virtually identical to those of the LL-diastereomer (65). The n.m.r. spectrum of (73) was similar to that of (65). Multiplets due to methine protons were again assigned by decoupling experiments.

H-D-Ser-L-Phe-OH (74) was synthesised in the same fashion in better yield (61%) and had identical physical properties to those of (73) except for optical rotation, $[\alpha]_D$ $+25.8^\circ$ compared with -29.3° for the enantiomer (73).

3.4 Synthesis of L-Phenylalanyl-L-serine:- Literature Routes.

The only reported synthesis of H-L-Phe-L-Ser-OH (64) was carried out by Suzuki et al.[75] and is shown in Figure 8. The

acid (80) (Figure 8) was also synthesised by Bodanzky and coworkers[76,77] as shown in Figure 9. Both routes were carried out to compare yields and applicability to small scale synthesis.

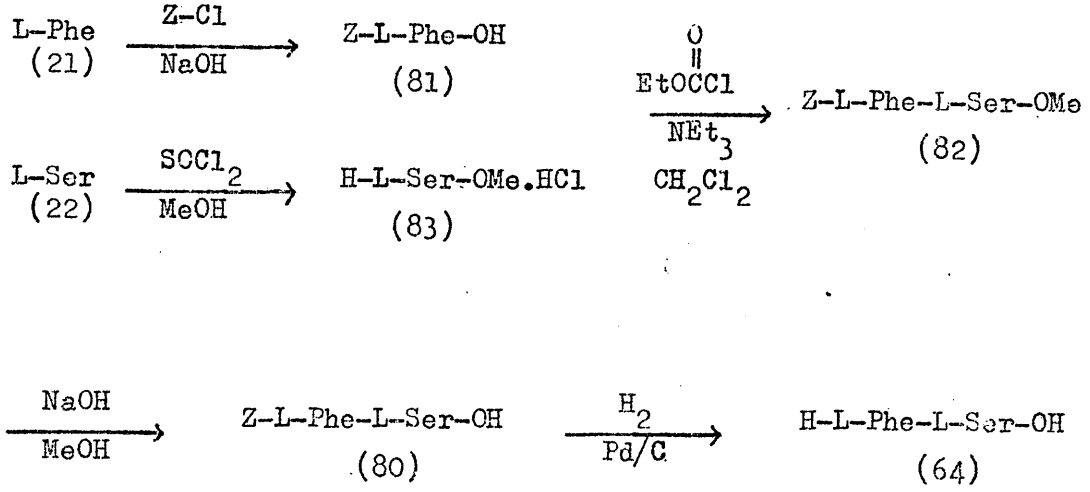


Figure 8 Synthesis of H-L-Phe-L-Ser-OH (64).[75]

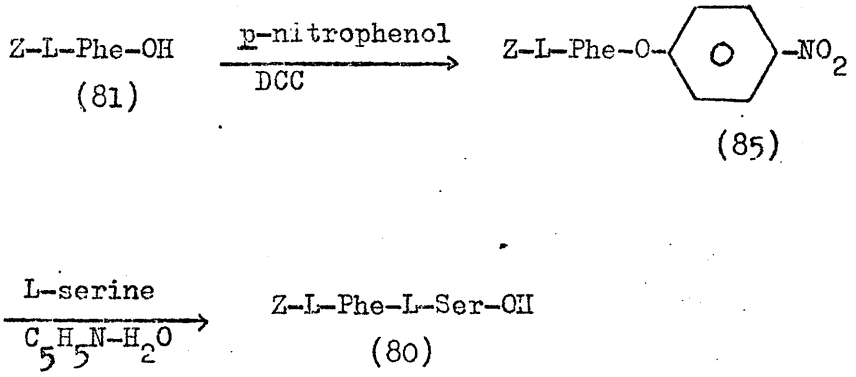


Figure 9 Synthesis of Z-L-Phe-L-Ser-OH (23).[76,77]

3.4.1 Benzyloxycarbonyl-L-phenylalanine (81) Z-L-Phe-OH (81) was synthesised by the Schotten-Baumann procedure[72] (Figure 8) with certain modifications. It was found that better yields were

obtained if the reagents were added dropwise over the required time period rather than in portions. In addition, the solution was acidified to pH 1 since acidification to Congo Red[72] had resulted in precipitation of (81) contaminated with its sodium salt.[78] With vigorous stirring yields of 90% or more were achieved.

3.4.2 Benzyloxycarbonyl-L-phenylalanyl-L-serine Methyl Ester (82). (82). L-Serine (22) was converted into H-L-Ser-OMe.HCl (83) in high yield by the method of Brenner and Huber[79] (Figure 8). The ester (83) was coupled with Z-L-Phe-OH (81) by the mixed anhydride method described by Nicolaides and De Wald[81] (Figure 8) to give the protected dipeptide (82) in 70% yield. The major absorptions in the i.r. spectrum were due to the primary hydroxyl group with bands at 3310 and 1060 cm^{-1}; the monosubstituted aromatic rings with bands at 750 and 700 cm^{-1}; and amide, urethane and ester carbonyl stretching absorptions. The n.m.r.spectrum was consistent with the structure (82). The mass spectrum was again dominated by fragmentation of the benzyloxycarbonyl group.

3.4.3 Removal of the Ester Group. Suzuki et al.[75] saponified the methyl ester (82) with 1.1 equivalents of base in aqueous methanol and achieved a 62% yield of the acid (80) (Figure 8). Since the release of an acidic group during the reaction should account for 1 equivalent of base, the reaction was tried with 2 equivalents of base. The crude yield was high (91.4%) but the product was found on t.l.c. to consist of 2 compounds, presumably

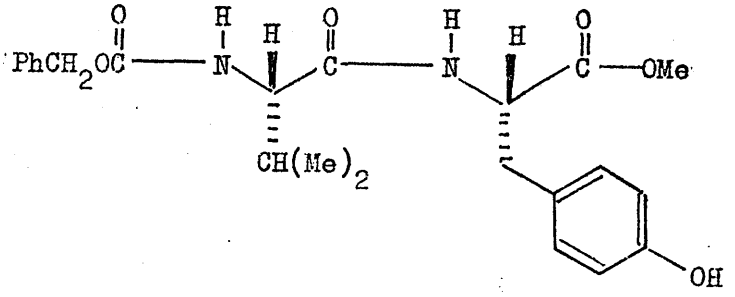
resulting from epimerisation. The recovery from crystallisation was poor and the optical rotation of the product (80) was very low compared with the literature value.[75] However, the melting point and spectroscopic data were in accord with the structure (80). The possibility exists that the reported[75] optical rotation was a mistake since Bodanzky et al.[76] reported a low optical rotation for the same compound closer to our observed figures.

A variety of reaction conditions, including those followed by Suzuki et.al.[75], were tried but epimerisation still occurred to some extent and crystallisation was inefficient.

Some attempts were made to cleave the ester enzymatically. Walton et al.[82] had succeeded in hydrolysing the ester of benzyloxycarbonyl-L-valine-L-tyrosine methyl ester (84) using chymotrypsin, without cleaving the amide bond. Unfortunately the procedure failed with Z-L-Phe-L-Ser-OMe (82) since chymotrypsin is selective with repect to the substrate and prefers amino-acids with an aromatic side chain. In the case of (82) the aromatic residue is too far away from the ester group. The same procedure was tried with a pig liver esterase but no reaction was observed.

An attempt to remove the benzyloxycarbonyl group of (82) in methanolic hydrogen chloride, such that the resulting amine salt could be treated with the enzyme, was unsuccessful. Several spots were observed on t.l.c. after hydrogenolysis of (82) and it was presumed that side reactions, involving the chloride anion and the seryl moiety of the starting material (82), were occurring. Hydrogenolysis in methanol could not be used since some

dioxopiperazine was formed.



(84)

3.4.4 Synthesis of Z-L-Phe-L-Ser-OH (80).[76] Attention was switched to the procedure employed by Bodanzky et al.[76], to synthesise the acid (80) (Figure 9). The p-nitrophenyl ester (85) of Z-L-Phe-OH was synthesised as described by Bodanzky and Vigneaud[77] in 71% yield, then coupled with L-serine by the literature method.[76] The success of the coupling reaction depended crucially on maintaining the pH at 8.7 by the controlled addition of sodium hydroxide. It was found that the addition was best done automatically but this presented practical difficulties when the reaction was carried out on a small scale. The synthesis was attempted on 3.75 mmol of starting material but the reaction mixture failed to go clear after 16h as described[76], and remained cloudy after 40h. A final yield of 42% was obtained, 20% below the literature yield.[76] The product was impure, failing to give a satisfactory analysis or well resolved spectra.

3.5 Synthesis of L-Phenylalanyl-L-serine (64)

Neither of the literature routes[75,76] were considered suitable for a small scale radioactive synthesis due to epimerisation in one case and the need for rigid pH controls in the other. It was decided therefore, to synthesise the dipeptide (64) in a similar fashion to that adopted for H-L-Ser-L-Phe-OH (65) (Figure 3), namely via Z-L-Phe-L-Ser-OBz (86) (Figure 10).

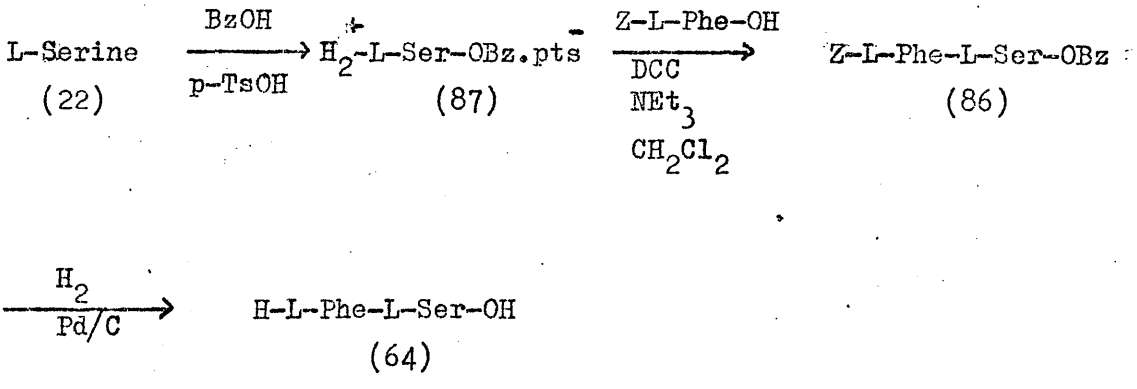
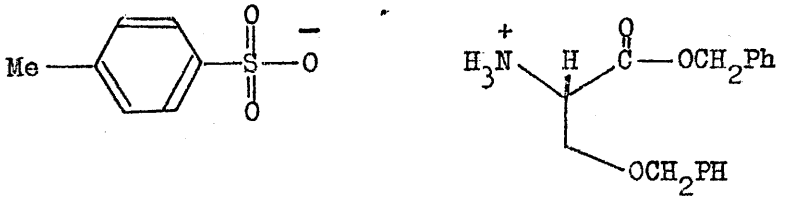


Figure 10 Synthesis of L-Phenylalanyl-L-serine (64).

3.5.1 L-Serine Benzyl Ester p-Toluenesulphonate (87). The ester salt (87) (Figure 10) was synthesised by the method of Foelsch[83], modified to a 1 mmol scale. The procedure involved heating a solution of the amino-acid and p-toluenesulphonic acid in benzyl alcohol and azeotroping off the water formed with carbon tetrachloride. p-Toluenesulphonic acid was required both as a catalyst and as a means of dissolving the amino-acid. The reaction

depended crucially on dissolving the reagents completely and keeping them dissolved during the addition of carbon tetrachloride. More solvent was required than reported (for benzenesulphonic acid)[83] for total dissolution. The amount of carbon tetrachloride initially added was also crucial. If too much was added the starting materials were precipitated but if too little was added then the water was not azeotroped off efficiently. It was discovered that refluxing the carbon tetrachloride through a Soxhlet apparatus containing molecular sieves was a far more efficient procedure on small scale than continuous distillation. After several minutes reflux, more carbon tetrachloride could be added without risk of precipitation. Applying these conditions on a 1 mmol scale, the ester (87) was obtained in 79% yield with a satisfactory n.m.r. spectrum. It failed to crystallise from the recommended solvent due to the presence of small quantities of dibenzylated serine derivative (88).[83]

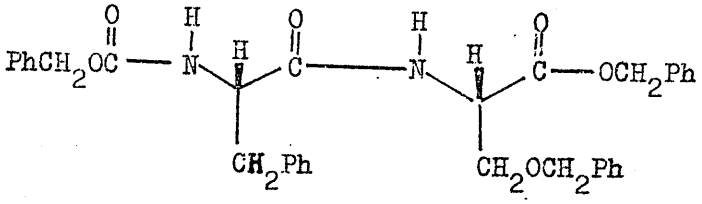


(88)

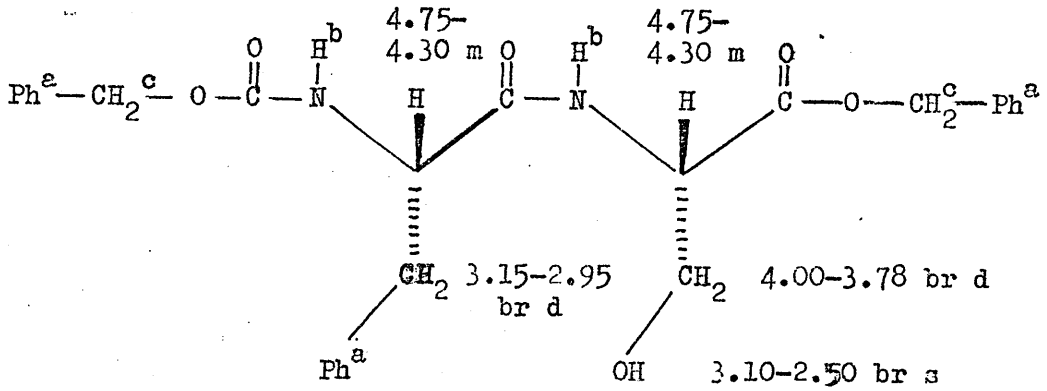
3.5.2 Coupling Procedure The protected amino-acids Z-L-Phe-OH (81) and H-L-Ser-OBz.pts (87) were coupled by the DCC procedure (Figure 10) to give a crude product contaminated with DCU and

one other impurity. This impurity, purified by preparative t.l.c., was believed to be benzyloxycarbonyl-L-phenylalanyl-O-benzyl-L-serine benzyl ester (89) on the basis of the n.m.r. spectrum. Both impurities were efficiently removed in solution by filtration after cooling a suspension of the crude product in ether for several days. The final yield (43%) was low compared with the crude yield (89%) but further product could be obtained by preparative t.l.c. of the ether filtrate. A sample of Z-L-Phe-L-Ser-OBz (86) was crystallised and fully characterised. The i.r. spectrum, outwith the fingerprint region, was similar to that of Z-L-Ser-L-Phe-OBz (68) with characteristic hydroxyl, carbonyl and aromatic absorptions. The n.m.r. spectrum (Figure 11) contained a complicated multiplet due to both methine protons. Doublets due to the methylene protons were observed rather than the ABX patterns observed in spectra of similar compounds (68), (75), and (76). These patterns were altered when D_2O was added but no conclusions could be made. No parent ion was observed in the mass spectrum of (86) due to rapid dehydration and loss of the benzyloxycarbonyl group.

The product (86) showed unusual melting properties. When the crystals were warmed quickly the melting point was 116-119°C. However on slow heating a mixture of needles, m.p. 136-138°C, and prisms, m.p. 210°C, were formed. Possibly decomposition of (86) occurred on slow heating before the m.p. was reached.



(89)



a 7.28 s, 7.23 s, 7.14 s b 7.10-6.78 m, 5.60-5.40 br d
c 5.14 s, 4.99 s

Figure 11 N.m.r. Spectrum of Z-L-Phe-L-Ser-OBz (86) in $CDCl_3$ (δ values).

3.5.3. Formation of Linear Dipeptide Hydrogenolysis (Figure 10) rapidly cleaved the protecting groups of Z-L-Phe-L-Ser-OBz (86) and H-L-Phe-L-Ser-OH (64) was obtained as a white solid (t.l.c. showed a single spot) which failed to crystallise. The melting point and optical rotation of (64) agreed with those of the literature.[75] The solid did not analyse correctly but the results which were obtained were more in line with a monohydrate, in

agreement with the results obtained by Suzuki *et al.*[75] The i.r. spectrum showed characteristic absorptions of a zwitterion and the n.m.r. spectrum was consistent with the structure (64). No parent ion was observed in the mass spectrum due to rapid dehydration. The amine fragmentation described previously (Figure 6) was observed again, giving a fragment ion peak at m/e 120 which was also the base peak.

3.6 Synthesis of H-L-[4'-^{3}H]Phe-L-[3-^{14}C]Ser-OH.H_2O.

H-L-[3-^{14}C]Ser-OBz.pts was synthesised in 66% yield and coupled with an equivalent of Z-L-[4'-^{3}H]Phe-OH which had been synthesised in 90 % yield (Figure 10). Z-L-[4'-^{3}H]Phe-L-[3-^{14}C]-Ser-OBz was obtained in good yield (82%). Panax scans and autoradiography of an analytical t.l.c. plate revealed the presence of a small quantity of dibenzylated derivative (89). Hydrogenolysis (Figure 10) gave H-L-[4'-^{3}H]Phe-L-[3-^{14}C]Ser-OH. H_2O in 97% yield. The overall yield from L-serine was 52% compared with an overall yield of 72% from L-phenylalanine. The ^{14}C specific activity was 88% of the original value when dilutions were taken into account, whilst the specific activity of ^{3}H was 89% of the original value. The radiochemical purity was checked by scanning and autoradiography of t.l.c. plates which had a single radioactive band corresponding to (64). The extent of epimerisation was estimated by dilution analysis since H-L-Phe-D-Ser-OH (30) (see below) had the same R_f value as that of the product in all solvent systems tried. The results of the

dilution analysis (Table 2) showed that contamination by H-L-Phe-D-Ser-OH (90) and H-D-Phe-L-Ser-OH (91) was not more than 0.3 and 0.2% respectively.

3.7 Synthesis of L-Phenylalanyl-D-serine and its Enantiomer.

3.7.1. Benzyloxycarbonyl-D-phenylalanyl-L-serine Benzyl Ester (92) and its Enantiomer (93) The protected dipeptides (92) and (93) were synthesised from their respective protected amino-acids as described for Z-L-Phe-L-Ser-OBz (86) (Figure 10). Yields of 41-47% were obtained and samples were purified by preparative t.l.c. and crystallisation for analysis. Bands, characteristic of the primary hydroxyl group, the ester, amide, and urethane carbonyls, and the aromatic ring, were observed in the i.r. spectrum. The n.m.r. spectrum of (92) is shown in Figure 12. Both amide protons exchanged very slowly with deuterium when the sample was shaken with D_2O. The multiplets due to the methine protons overlapped and could not be distinguished. The multiplets due to the methylene groups adjacent to a methine group were more complicated than the simple ABX system and could not be analysed. Fragmentation of the benzyloxycarbonyl protecting group again dominated the mass spectra of (92) and (93).

3.7.2 Formation of Linear Dipeptides (90) and (91). Due to the insolubility of the protected dipeptides (92) and (93) in ethanol-water, hydrogenolysis was carried out overnight on a suspension of the reactant. H-L-Phe-D-Ser-OH (90) and H-D-Phe-

Carrier	LL**		LD		DL	
Isotope	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H
S.A* Initial	0.91	11.21	0.76	9.34	0.78	9,55
S.A. 1st xtll	0.91	11.59	5.25×10^{-3}	0.11	1.98×10^{-2}	0.27
S.A. 2nd xtll	0.93	11.53	undetected		4.00×10^{-5}	0.02

Table 1 Dilution Analysis Results for H-L-[3-^{14}C]Ser-L-[4'-^{3}H]Phe-OH.

Carrier	LD***		DL	
Isotope	^{14}C	^{3}H	^{14}C	^{3}H
S.A* Initial	0.71	9.84	0.56	7.79
S.A. 1st Xtll	0.02	0.26	0.03	0.56
S.A. 2nd xtll	1.13×10^{-3}	0.08	1.23×10^{-3}	0.06
S.A. 3rd xtll	1.9×10^{-3}	0.06	undetected	

Table 2 Dilution Analysis Results for H-L-[4'-^{3}H]Phe-L-[3-^{14}C]Ser-OH.H_2O.

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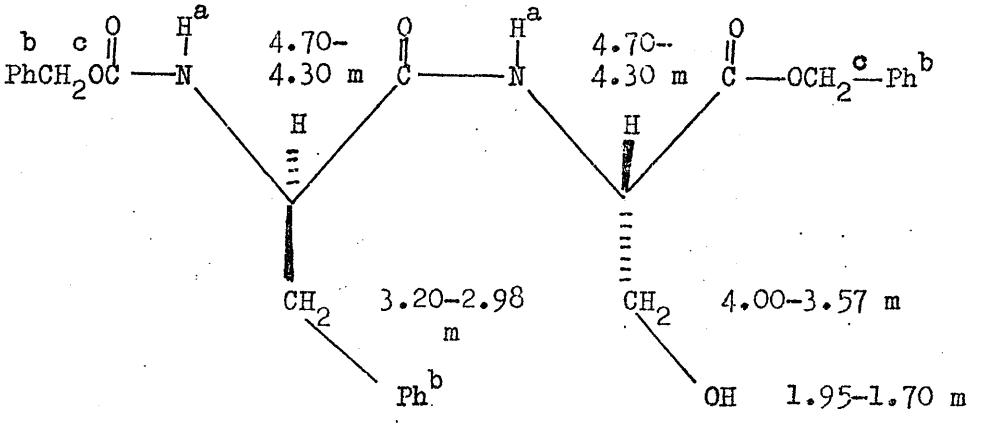
* Specific activity (μCi mmol^{-1}).

** The configurations(L or D) of components are given in the order, Ser-Phe.

*** The configurations (L or D) of components are given in the order, Phe-Ser.

L-Ser-OH (91) were obtained in ca. 75% yield after crystallisation. The melting points of (90) and (91) were broad and, as melting took place, needles were formed which in turn decomposed over a large temperature range. Possibly the dipeptides were cyclising in the molten state to the dioxopiperazine which decomposes at 258-268°C (page 136). The i.r. spectra of (90) and (91) showed bands



a. 6.78-6.49 m, 5.50-5.30 m. b. 7.29 s, 7.21 s.
c. 5.15 s, 5.05 s

Figure 12 N.m.r. Spectrum of Z-D-Phe-L-Ser-OBz (92) in $CDCl_3$ (δ values).

characteristic for a zwitterion. The n.m.r. spectra of (90)and (91) were consistent with the structures and the mass spectra were closely similar to that of the LL-isomer (64).

3.8 Feeding Experiments.

The doubly labelled dipeptides, L-phenylalanyl-L-serine (64) and L-seryl-L-phenylalanine (65), were fed in aqueous ethanol to one day old cultures of T.viride at a concentration of 6-14 mg l^{-1}. The cultures were grown for a further three days then worked up as described in the Experimental Section. Incorporations of radioactivity into the total chloroform extract and the crude, crystalline gliotoxin obtained from the extracts by trituration with methanol, are recorded in Tables 3 and 4. The latter figures are more accurate as a measure of precursor incorporations into gliotoxin since the specific activity of the crude crystalline gliotoxin remained reasonably constant after several crystallisations.

Neither of the labelled dipeptides were incorporated very well into gliotoxin and the isotopic ratio (^{3}H:^{14}C) in gliotoxin was quite different from that of the dipeptide precursor in all the experiments carried out. These results strongly suggest that the dipeptide precursors were cleaved by the fungus into their constituent amino-acids. It would be reasonable to assume that the pool sizes of L-phenylalanine and L-serine would be different. Therefore, the efficiency of uptake of the two amino-acids into gliotoxin would be different. This would result in a different isotope ratio in gliotoxin compared with that of the precursor, which would account for the results obtained.

H-L-[4'-^{3}H]Phe-L-[3-^{14}C]Ser-OH (64) was also fed to two day and three day old cultures. The cultures were grown for four days in all as above, then worked up in the normal manner. The results

	Incorporation (%)			
Age of Culture when fed	1 day		1 day	
Concentration (mg l^{-1})	11		6	
Isotope	^{14}C	^{3}H	^{14}C	^{3}H
Chloroform Extract	2.23	5.40	5.80	15.8
Triturated Extract	0.86	4.45	1.20	6.4
Aqueous (Extracted)	12.6	35.2	16.0	30.0
Mycelium (Calculated)	85.2	59.4	78.2	54.2

Table 3 Feeding of H-L-[3-^{14}C]Ser-L-[4'-^{3}H]Phe-OH

	Incorporation (%)					
Age of Culture when fed	1 day		2 days		3 days	
Concentration (mg l^{-1})	14		14		14	
Isotope	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H
Chloroform Extract	3.40	8.60	6.90	6.50	8.80	7.10
Triturated Extract	0.60	3.10	2.90	3.90	4.40	4.90
Aqueous (Extracted)	0.00	42.0	0.00	38.0	9.30	40.8
Mycelium (Calculated)	96.6	48.4	93.1	55.5	81.2	52.1

Table 4 Feeding of H-L-[4'-^{3}H]Phe-L-[3-^{14}C]Ser-OH.H_2O.

(Table 4) indicate that cleavage of the precursor was occurring as explained above. However, it is interesting that the isotopic ratio in gliotoxin was almost the same as that of the precursor, and the incorporation value for ^{14}C was greater, when the dipeptide was fed to a three day old culture. These results could be accounted for by variation of the pool sizes of L-phenylalanine and L-serine with the age of the culture. Another possibility is that some incorporation of the linear dipeptide into gliotoxin is taking place, and that this incorporation occurs more readily with increasing age of the culture.

3.9 Conclusion.

Clear evidence for the involvement of linear dipeptides in the biosynthesis of gliotoxin could not be obtained. In fact, the results obtained suggest that the dipeptides were cleaved by the fungus to their constituent amino-acids, which were then incorporated separately into gliotoxin. It is possible linear dipeptides are enzyme bound biosynthetic intermediates for gliotoxin. Therefore, equilibration between these intermediates and added linear dipeptides may be prevented.

CHAPTER 4 THE ROLE OF CYCLIC DIPEPTIDES IN GLIOTOXIN BIOSYNTHESIS.

Introduction.

As described in Chapter 1, the status of cyclo-(L-Phe-L-Ser) (24) as a biosynthetic intermediate for gliotoxin had been placed in doubt by the results of MacDonald and Slater.[36] The incorporation into gliotoxin obtained by Bu'Lock and Leigh[37] on feeding cyclo-(L-[Ar-^{3}H]Phe-DL-[3-^{14}C]Ser) to Trichoderma viride could have been partly the result of incorporation of cyclo-(L-Phe-D-Ser) (94). It was decided to synthesise and feed the four possible stereoisomers of cyclo-(Phe-Ser) to settle the issue. The labelled structures selected were cyclo-(L-[U-^{14}C]Phe-L-Ser), cyclo-(L-[U-^{14}C]Phe-D-Ser), cyclo-(L-[4'-^{3}H]Phe-L-[3-^{14}C]Ser), and cyclo-(D-Phe-DL-[3-^{14}C]Ser). The last compound was a mixture of two diastereomers, one of which was the DD-isomer. This isomer could not be synthesised separately since neither radioactively labelled D-phenylalanine nor D-serine was commercially available and so it was planned to synthesise the mixture from DL-[3-^{14}C]serine and to separate the two diastereomers if necessary.

4.1 Synthesis of cyclo-(L-Phenylalanyl-L-seryl) (24) and cyclo-(D-Phenylalanyl-D-seryl) (95).

The synthesis of cyclo-(L-Phe-L-Ser) (24) and the DD-enantiomer (95) was carried out as shown in Figure 13 and was basically the same route used by MacDonald and Slater.[36] The only deviation from their procedure was the final step where ammoniacal methanol was used to encourage cyclisation.

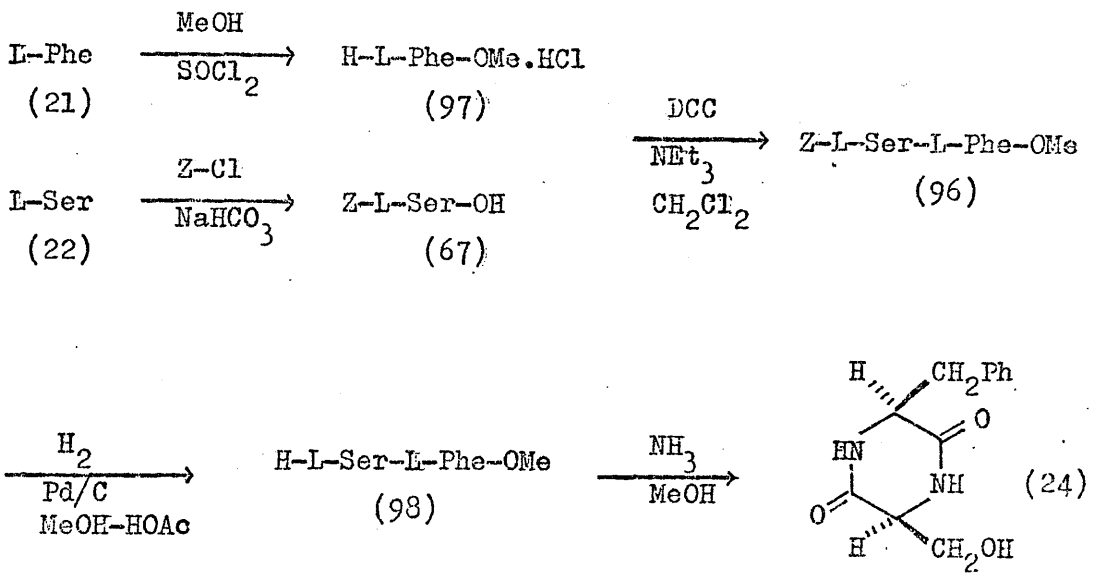


Figure 13 Synthesis of cyclo-(L-Phe-L-Ser) (24).

4.1.1 N-Benzyloxycarbonyl-L-seryl-L-phenylalanine Methyl Ester (96).

H-L-Phe-OMe.HCl (97) was synthesised in good yield by the method of Brenner and Huber[79] and was coupled with Z-L-Ser-OH (67) as described by MacDonald and Slater[36b] (Figure 13) to give the protected dipeptide (96) as an oil contaminated with DCU. A sample was purified for analysis by preparative t.l.c. and crystallisation. The i.r. spectrum was closely similar to that of Z-L-Phe-L-Ser-OBz (86). It was not possible to analyse completely the n.m.r. spectrum run at 60 MHz. However signals due to the aromatic, methyl ester and benzyloxycarbonyl groups were clear. Fragmentation

of the benzyloxycarbonyl group was the dominant feature of the mass spectrum.

Z-D-Ser-D-Phe-OMe (97) was similarly synthesised.

4.1.2 Deprotection and Cyclisation Procedure. Normally the crude protected dipeptide (96)was used for the next step in the synthesis, namely hydrogenolysis of the benzyloxycarbonyl group (Figure 13). Hydrogenolysis was superior to treatment with 45% HBr/HOAc since it was found that side reactions involving acetylation and elimination of the hydroxyl function occurred by the latter method. This behaviour of serine derivatives has been recorded in the literature.[85] An oil was obtained after hydrogenolysis and was shown by t.l.c. and n.m.r. spectroscopy to be a mixture of (24) and the open chain methyl ester (98). Acetic acid was also present in the product and it was concluded that the acid had protonated the amino group of (98) thus halting cyclisation which should have occurred readily in the absence of acid.[36b] Several methods were tried to induce cyclisation, the best being treatment of the crude product with ammoniacal methanol. There was an added advantage in that the cyclised product crystallised from this solution. The crystals were contaminated with DCU but this could be removed by thoroughly washing the crystals with ether. Crystallisation from methanol usually resulted in a recovery of 65% but more product could be obtained from the mother liquors. The overall yield from L-phenylalanine was in the order of 30-40%. Coupling was the stage with the lowest yield in the synthesis.

cyclo-(L-Phe-L-Ser) (24) did not show up on t.l.c. under u.v. light or with iodine and required a multiple spray procedure, (see Experimental Section) for detection. The optical rotation ($[\alpha]_D$ -105^o) was high, five times the reported value[36b], and melting was observed to occur with decomposition at a lower temperature than that recorded.[36b] Nevertheless the product analysed correctly and the spectral results confirmed that the correct product had been obtained.

The i.r. spectrum contained absorptions characteristic of the dioxopiperazine ring. Amide N-H stretching was at 3200 cm^{-1}, shifted to lower frequency compared with the normal trans value of 3300 cm^{-1}.[86] Carbonyl stretching gave a band at 1670 cm^{-1} which was at higher frequency than the normal absorption of ca 1650 cm^{-1} for trans amides.[86] The amide II absorption at 1550 cm^{-1}, characteristic of trans amides[86], was absent in the spectrum of (24).

Due to the complicated multiplets observed in the n.m.r. spectrum of (24), spectra were run in three different solvent systems. Cyclic dipeptides containing an aromatic side chain generally prefer the dioxopiperazine ring in a boat conformation with the aromatic ring hanging over the boat.[19] This conformation is thought to be the result of dipole induced dipole interactions between the aromatic ring and the amide groups of the dioxopiperazine ring. One result of this conformation is that protons in the axial position at the opposite end of the boat from the aromatic residue experience a shielding effect from the aromatic ring current. Therefore in cyclo-(L-Phe-L-Ser) (24)

the methylene protons adjacent to the hydroxyl group might be expected to be at higher field in the n.m.r. spectrum than normal. This was found to be the case in $(CD_3)_2SO$ where a widely spread ABX multiplet was observed for these protons (Figure 14). When the sample was shaken with D_2O this multiplet sharpened appreciably due to the exchange of the hydroxyl proton with deuterium. The methine protons were assigned on the basis of decoupling experiments.

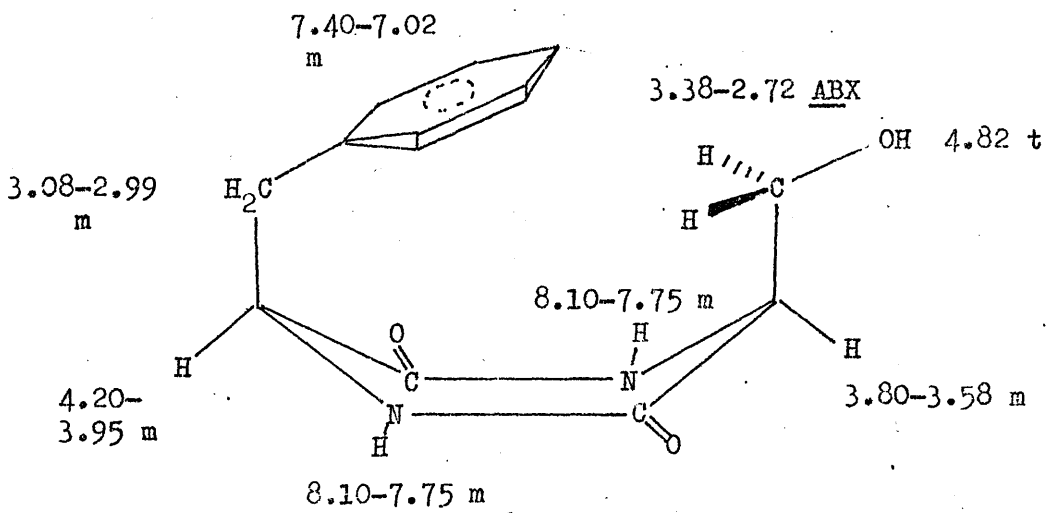


Figure 14 N.m.r. Spectrum of cyclo-(L-Phe-L-Ser) (24) in $(CD_3)_2SO$ (δ values).

When the spectrum was run in CD_3OD, hyperfine splitting (J= 1Hz) of the multiplets due to the methine protons was observed. This splitting was the result of long range coupling between the

methine protons analagous to homoallylic coupling.[88] The fact that this splitting was observed suggests that there is significant double bond character in the amide bonds and that the molecule exists more as a flattened boat in order to satisfy the stereochemical requirements for homoallylic coupling.[88]

Three characteristic fragmentations have been observed for simple dioxopiperazines in their mass spectra.[19] One of these was observed in the mass spectrum of cyclo-(L-Phe-L-Ser) (24) (Pathway 1, Figure 15).

cyclo-(D-Phe-D-Ser) (95) was synthesised in a similar fashion and had identical physical properties to (24) except for an optical rotation of $[\alpha]_D$ + 104° compared with $[\alpha]_D$ -105° for (24).

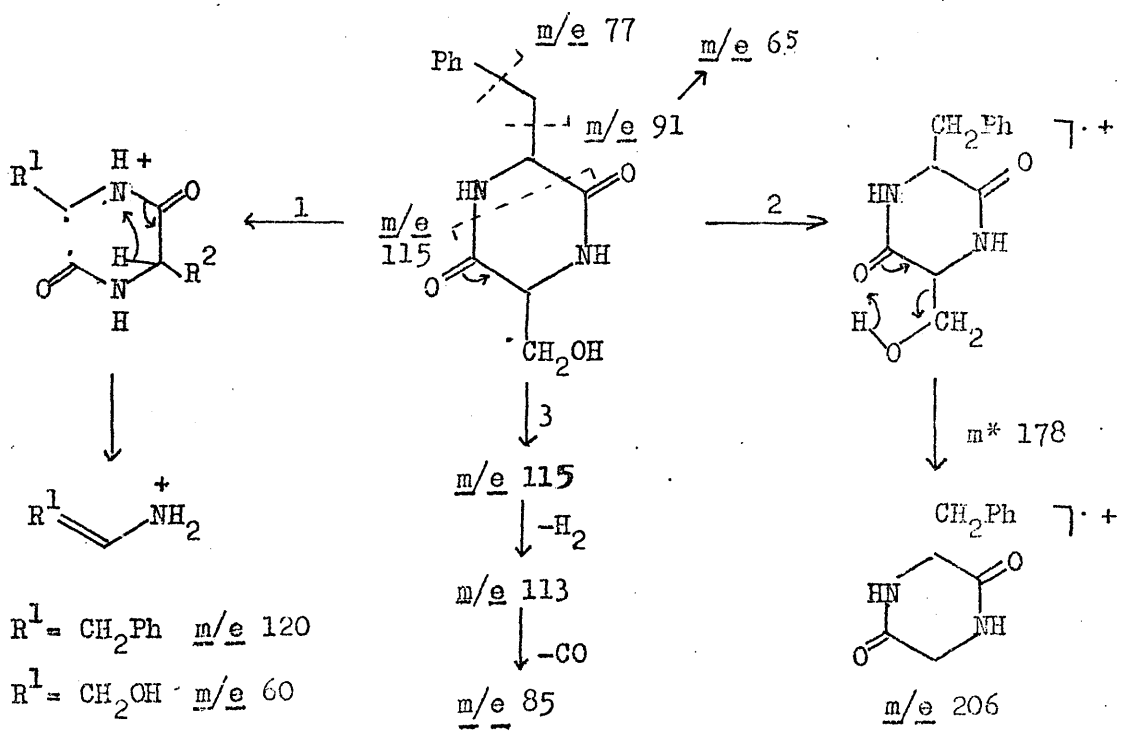


Figure 15 Mass Spectrum of cyclo-(L-Phe-L-Ser) (24).

4.2 Synthesis of cyclo-(L-Phenylalanyl-D-seryl) (94) and its Enantiomer (99).

The procedure described earlier was used to synthesise Z-L-Ser-D-Phe-OMe (100) and its enantiomer (101) as oils. A sample of (100) was purified by preparative t.l.c. for analysis.

Hydrogenolysis and cyclisation of the protected dipeptides was successfully carried out as described for the LL-stereoisomer (Figure 13). Samples obtained by trituration of the crude product and by recrystallisation were compared but no difference in purity was observed. The cyclic dipeptides (94) and (99) had the same Rf value on t.l.c. as that of the LL-stereoisomer (24) in all the solvent systems tried. This is probably not too suprising since (24) and (94) both exist in a boat conformation with the aromatic ring over the dioxopiperazine ring and a hydroxyl group presumably spending most of its time exo to the ring. The i.r. spectrum was characteristic of the dioxopiperazine ring. The n.m.r. spectrum of (94) run in CF_3CO_2H is shown in Figure 16. The multiplet due to the methine proton derived from serine is shifted upfield by 1.1 p.p.m. compared with the same signal for the LL-stereoisomer. Assignments were made on the basis of decoupling experiments and comparison with spectra run in different solvents. The mass spectrum was virtually identical to that of the LL-stereoisomer.

The DL-isomer (99) showed the same spectral characteristics as those of (94) except for an optical rotation of $[\alpha]_D$ -10.2^o compared with $+11.2^o$ for (94).

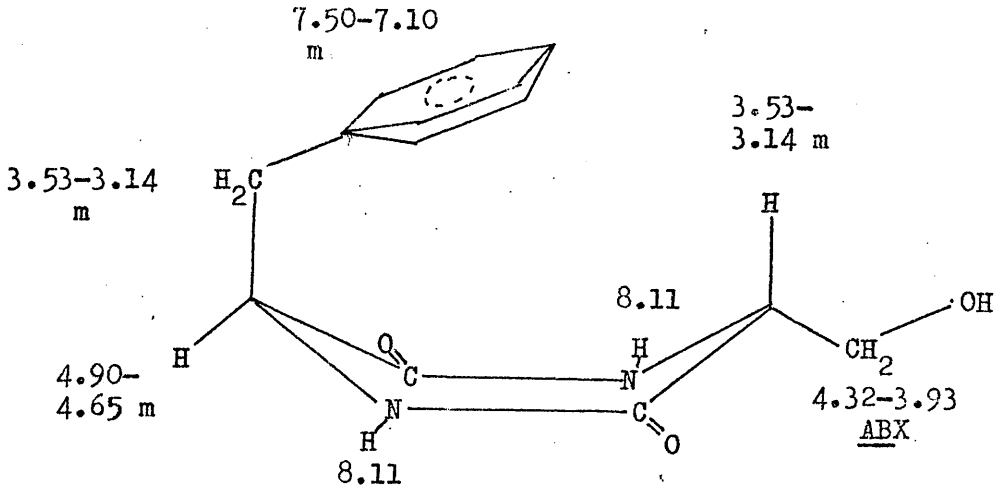


Figure 16 N.m.r. Spectrum of cyclo-(L-Phe-D-Ser) (94) in CF_3CO_2H (δ values).

4.3 Synthesis of Radioactively Labelled Precursors.

cyclo-(L-[U-^{14}C]Phe-L-Ser) was synthesised by the route described above (Figure 13) with one exception. H-L-[U-^{14}C]Phe-OMe.HCl (97) was synthesised by the method of Rachel[89] by concentrated hydrochloric acid. This method was more convenient for small scale synthesis. The cyclised product was obtained in 33% overall yield based on L-phenylalanine with a specific activity 85% of the original specific activity. One radioactive band was observed by Panax scanning and autoradiography of t.l.c. plates run in two different solvent systems. Dilution analysis showed that the LD- and DL-isomers were present in not more than 0.3% (Table 5).

cyclo-(L-[U-^{14}C]Phe-D-Ser) was synthesised in 30% overall yield with a specific activity of 90% of the original value and found to contain not more than 0.5% of the LL-stereoisomer (Table 5). cyclo-(D-Phe-DL-[3-^{14}C]Ser) was synthesised in 15% overall yield from DL-[3-^{14}C]serine. The mixture was found to have a DL:DD ratio of 68:28 and to contain less than 0.1% of the LL-isomer (Table 5). The specific activity was 87% of the original.

Z-L-[3-^{14}C]Ser-OH, synthesised from L-[3-^{14}C]serine in 91% yield, with a specific activity 97% of the theoretical value, was coupled with H-L-[4-^{3}H]Phe-OMe.HCl and converted into the cyclic dipeptide in 28.6% overall yield with the ^{14}C specific activity 98% and the ^{3}H specific activity 97% of the expected value. Dilution analysis (Table 5) showed the presence of not more than 0.2% of the LD- and DL-isomers.

All the radiolabelled samples were shown to contain a single radioactive compound by Panax scanning and autoradiography of t.l.c. plates.

4.4 Feeding Experiments with Cyclic Dipeptides.

The radiolabelled cyclic dipeptides, normally 16 mg (3 μCi) l^{-1}, were added in dimethyl sulphoxide (1ml) to 1 day old, shake cultures of T.viride. The cultures were incubated for a further three days, then worked up as described in the Experimental Section. cyclo-(L-[4-^{3}H]Phe-L-[3-^{14}C]Ser) was fed as a separate experiment but all the other precursors were fed in parallel with cyclo-(L-[U-^{14}C]Phe-L-Ser) as reference. The results (Table 6)

Precursor	Composition (%) by Radiodilution Analysis.
cyclo-(L-[U-^{14}C]Phe-L-Ser)	109 LL*, 0.3 DL, 0.3 LD
cyclo-(L-[U-^{14}C]Phe-D-Ser)	109 LD, 0.5 LL
cyclo-(D-Phe-DL-[3-^{14}C]Ser)	68 DL, 28 DD, 0.1 LL
cyclo-(L-[4'-^{3}H]Phe-L-[3-^{14}C]Ser)	100 LL, 0.2 DL, 0.2 LD

* The configurations (L or D) of components are given in the order, Phe-Ser.

Table 5 Dilution Analysis on Radiolabelled Cyclic Dipeptides.

Exp. No.[a]	Precursor	Incorporation ^{14}C (%) in Chloroform Extract	S.A. ^{14}C Gliotoxin[c]	S.I.[b]
1	cyclo-(L-[U-^{14}C]Phe-L-Ser)	40	6.19	1.5×10^{-1}
2	cyclo-(L-[U-^{14}C]Phe-D-Ser)	0.43	0.06	1.3×10^{-3}
3	cyclo-(L-[U-^{14}C]Phe-L-Ser)	58	7.17	3.4×10^{-1}
4	cyclo-(L-[U-^{14}C]Phe-D-Ser)	0.43	0.03	4.3×10^{-5}
5	cyclo-(L-[U-^{14}C]Phe-L-Ser)	49	11.7	2.6×10^{-1}
6	cyclo-(D-Phe-DL-[3-^{14}C]Ser)	1.1	0.06	1.2×10^{-3}
7	cyclo-(L-[4'-^{3}H]Phe-L-[3-^{14}C]Ser)	59	3.13	1.5×10^{-1}

a Incubations bracketed were conducted in parallel.

b $\frac{\text{Specific}}{\text{Incorporation}}$ = Specific Activity (Gliotoxin)/Specific Activity (Precursor)

c S.A.= Specific Activity (μCi mmol^{-1})

Table 6 Feeding Results for the Cyclic Dipeptides.

showed that only cyclo-(L-Phe-L-Ser) (24) was incorporated into gliotoxin significantly. Incorporations of 40-50% of the radioactivity fed in the form of the LL-cyclic dipeptide were observed in the crude chloroform extract, double the incorporation observed by Bu'Lock and Leigh.[37] Radioscanning and autoradiography of t.l.c. plates run in four different solvent systems revealed that the vast majority of the activity was present in gliotoxin. Gliotoxin, after crystallisation to constant activity, normally had a specific activity of 6-12 μCi mmol^{-1}. In contrast, poor incorporations (0.4-1.1%) of the other isomers were observed into the crude chloroform extract and the specific activity of the crystallised gliotoxin was very low, 0.03-0.06 μCi mmol^{-1}; less than 1% of the specific activity of crystallised gliotoxin from a parallel LL-cyclic dipeptide feeding.

A more accurate incorporation figure for an LL-cyclic dipeptide feeding was obtained in the following manner. A sample of the mother liquors from the crystallisation of gliotoxin was diluted with inactive gliotoxin by a factor of fourteen and the mixture crystallised to constant activity. From this figure it was possible to calculate how much labelled gliotoxin was present in the sample taken and hence in the total sample. This procedure was also carried out for the mother liquors obtained from the trituration of crude gliotoxin. In this way it was shown that at least 43% of cyclo-(L-[U-^{14}C]Phe-L-Ser) had been incorporated into gliotoxin in the experiment tested, and that very little gliotoxin was lost during trituration.

Having established that cyclo-(L-Phe-L-Ser) (24) was incorporated efficiently into gliotoxin, we needed to show that the incorporation observed was not due to cleavage of the cyclic dipeptide by the fungus into its constituent amino-acids, which would then be incorporated separately. To surmount this problem, the LL-cyclic dipeptide was fed, doubly labelled, with ^{3}H in the phenylalanyl moiety and ^{14}C in the seryl moiety, and the derived gliotoxin was crystallised to constant activity as before. It was found that the $^{3}H:^{14}C$ ratio in gliotoxin (11.1) was essentially the same as in the precursor (10.9). This is good evidence that the precursor was not cleaved to its constituent amino-acids, since it is unlikely that both amino-acids would then be taken up into gliotoxin with the same efficiency. The pool sizes of L-phenylalanine and L-serine would be expected to be quite different. This assumption was supported by the results obtained from feeding doubly labelled open chain dipeptides, where cleavage did appear to occur and the $^{14}C:^{3}H$ ratio did change (Chapter 3).

This experiment did not disprove the possibility that the cyclic dipeptide was an unnatural precursor which was converted by the fungus into the true intermediate. This possibility was tested by means of an 'intermediate trapping' experiment. Non radioactive LL-cyclic dipeptide was incubated with T.viride and, 2 hours later, L-[U-^{14}C]phenylalanine was added to the medium. After a further 2 hours, the organism was harvested and the culture filtrate was extracted with chloroform to remove gliotoxin, and then was extracted continuously with ethyl acetate to give the cyclic

dipeptide (24). This was diluted with unlabelled (24) and the mixture was crystallised to constant activity. The residual activity in (24) corresponded to 1.3% of that administered as L-[U-^{14}C]phenylalanine. This demonstrated that the fungus had indeed converted some of the fed phenylalanine into (24) under normal conditions of growth.

A further check was carried out whereby the doubly labelled gliotoxin obtained above was degraded to prove that the ^{14}C label was in the expected position. Details of this and other degradation experiments are given in Chapter 5.

4.5 Conclusion.

cyclo-(L-Phenylalanyl-L-seryl) (24) was shown to be a biosynthetic intermediate for gliotoxin (1). Incorporations of labelled cyclo-(L-Phe-L-Ser) into gliotoxin were high. Doubly labelled cyclo-(L-Phe-L-Ser) was incorporated into gliotoxin with the isotopic ratio (^{14}C:^{3}H) unchanged. It was shown by an 'intermediate trapping' experiment that the fungus was capable of synthesising the cyclic dipeptide (24) from L-phenylalanine. The other stereoisomers of cyclo-(phenylalanyl-seryl) were poorly incorporated into gliotoxin.

CHAPTER 5 DEGRADATION STUDIES ON GLIOTOXIN.

Introduction.

The incorporation of cyclo-(L-[4'-^{3}H]Phe-L-[3-^{14}C]Ser) into gliotoxin with an essentially unchanged $^{3}H:^{14}C$ ratio, backed by the intermediate trapping experiment described in the previous Chapter, provided strong evidence that the LL-cyclic dipeptide is a true intermediate for gliotoxin. It was decided to strengthen the case further by degrading the gliotoxin derived biosynthetically from cyclo-(L-[4'-^{3}H]Phe-L-[3-^{14}C]Ser). The degradations were aimed at finding out a) whether the N-methyl group was labelled with ^{14}C and b) whether the ^{14}C label was completely located as expected in the hydroxymethylene unit.

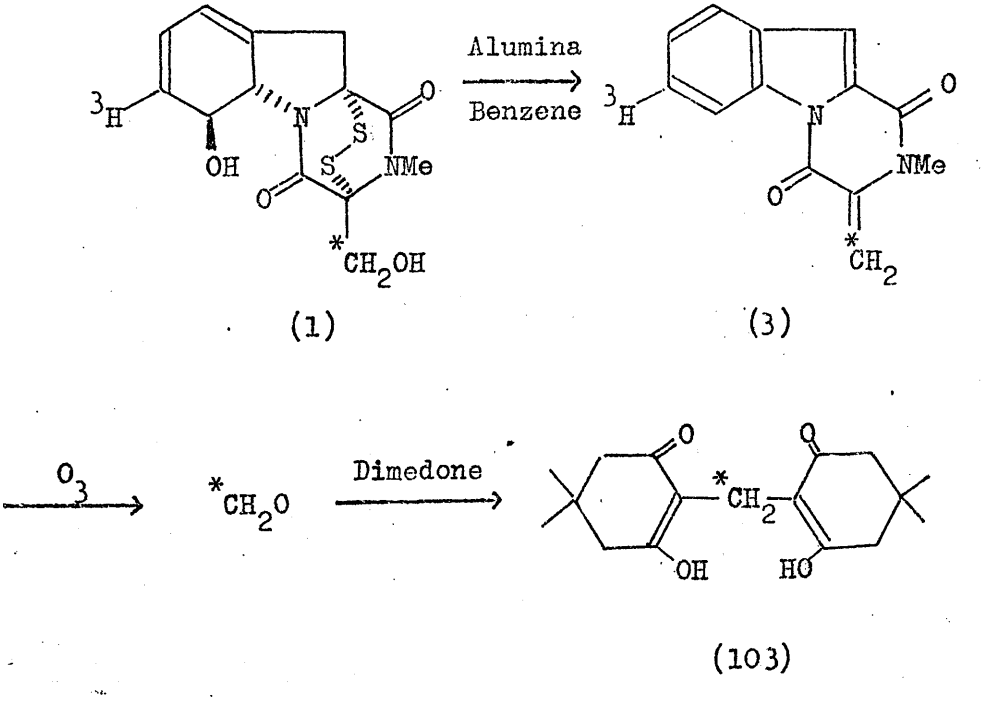
5.1 Degradation Studies to Isolate the N-Methyl Group.

The possibility that the LL-cyclic dipeptide (24) was degraded by the organism to its constituent amino-acids, followed by uptake of the latter into gliotoxin, had already been shown to be unlikely since the isotope ratios in precursor and metabolite were identical. However there remained the outside possibility that both amino-acids were incorporated with exactly the same efficiency. This possibility could be ruled out if the N-methyl unit in gliotoxin was shown to be free from ^{14}C, for the following reasons. It is well known that serine and glycine

are biosynthetically interconvertable and that, in this process, the C-3 of serine (the position labelled in the cyclic dipeptide), enters the one-carbon pool. This same pool supplies the S-methyl group of L-methionine which in turn has been shown[30] to be a good precursor for the N-methyl group in gliotoxin. Evidence of this biosynthetic process was supplied by Winstead and Suhadolnik[30] who, by feeding [3-^{14}C]serine to Trichoderma viride, demonstrated that 25% of the radioactivity incorporated was contained in the N-methyl group. They hydrolysed gliotoxin with sodium hydroxide thus generating methylamine, derived from the N-methyl unit, which was trapped in hydrochloric acid and counted as the salt. This procedure was modified for the degradation of doubly labelled gliotoxin on account of the small scale employed. A sample of the labelled gliotoxin was diluted with inactive gliotoxin and methylamine was generated and trapped as described.[30] The basic solution was then steam distilled to ensure that all generated methylamine had passed into the traps. The methylamine hydrochloride obtained (ca 3.5mg) contained only 1.16% of the original radioactivity. The hydrochloride salt was converted into the phenylisothiourea derivative of methylamine by treatment with phenylisothiocyanate under basic conditions. The derivative was purified by preparative t.l.c. Very little radioactivity was observed in the derivative, and it was calculated that the specific activity was not more than 0.1% of the original specific activity of the diluted gliotoxin. Thus it was shown conclusively that degradation of the precursor (24) to its constituent amino-acids was not occurring.

5.2 The Location of the ^{14}C Label in Doubly Labelled Gliotoxin - part 1.

Having shown that the ^{14}C label was not in the N-methyl group we needed still to prove its location. The expected position for the label was the hydroxymethylene group of gliotoxin (Figure 17) which, it was planned, could be isolated by conversion of gliotoxin into anhydrodesthiogliotoxin (3) followed by ozonolysis of (3) to produce formaldehyde which could be trapped and counted as its dimedone derivative (103).



(1) (3) (103)

Figure 17 Degration Scheme.

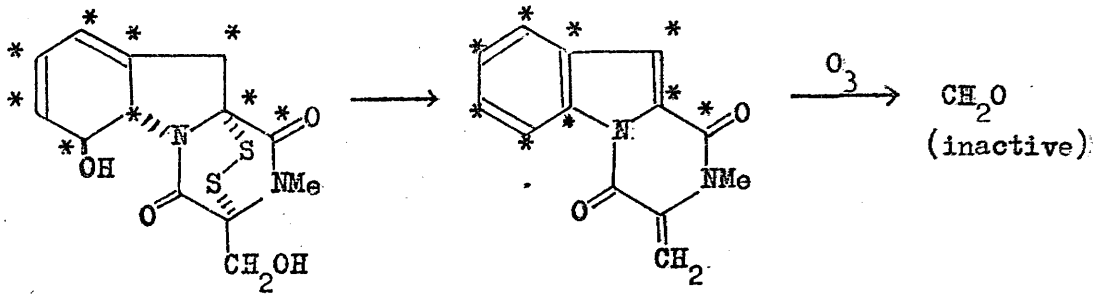
5.2.1 Synthesis and Ozonolysis of Anhydrodesthiogliotoxin (3). Anhydrodesthiogliotoxin was synthesised by a method of Johns[90] using a modified work up procedure. The yields obtained were not very good and varied considerably (10-25%). The melting point and i.r. and mass spectra agreed with the literature.[90] The n.m.r. spectrum was closely similar to that of the literature[90] except for the N-methyl singlet which was at δ 3.45 (lit.,[90] δ 4.00).

5.2.2 Degradation of Gliotoxin-1. Samples of doubly labelled gliotoxin were converted into anhydrodesthiogliotoxin (3) as described above, with essentially unchanged specific activity (105-109%) and isotope ratio (10.7-11.5) as expected. This result further confirms the radiochemical purity of the gliotoxin isolated. The olefin (3) was ozonised and the product worked up with zinc to produce formaldehyde, trapped as its dimedone derivative, which was crystallised to constant activity. The results were suprising since the specific activity was less than half of the expected value. Variation of the temperature and time of ozonolysis made little difference to this result. A different work up procedure involving dimethyl sulphide resulted in lower specific activities since it was found that formaldehyde was being generated from dimethyl sulphide itself.

Two explanations for the results obtained were possible; 1) the label had been scrambled within the molecule or 2) formaldehyde was being generated from another part of (3), diluting the labelled formaldehyde and thus lowering the specific activity.

The latter explanation seemed more likely and of all the carbon atoms present only that in the N-methyl group was at the correct oxidation level to generate formaldehyde.

5.2.3 Degradation of Gliotoxin-2. ^{14}C-Labelled gliotoxin derived biosynthetically from cyclo-(L-[U-^{14}C]Phe-L-Ser) was converted into anhydrodesthiogliotoxin (3). The product was ozonised and the reaction mixture treated with dimethyl sulphide to give unlabelled formaldehyde (Figure 18). This is in accord with the above theory since none of the carbons in the upper half of gliotoxin should be capable of generating formaldehyde. However, the yield of dimedone derivative obtained from this experiment was poor and could in fact have been derived from dimethyl sulphide.



(1) (3)

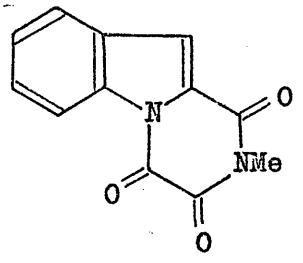
Figure 18 Degradation Scheme.

5.3 The Location of the ^{14}C Label in Gliotoxin-part 2.

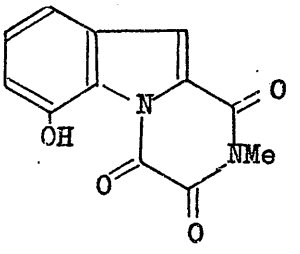
It would have been extremely informative if the trioxopiperazin

(5) could have been isolated from the ozonolysis described above, since it should have contained ^{3}H but no ^{14}C if this latter label was confined to 3a-carbon. However, due to the scale of the reaction and the suspected break up of the molecule, this isolation was not attempted.

A search of the literature revealed that (5) and another trioxopiperazine (7) had been synthesised from gliotoxin. These syntheses were studied to see whether they were suitable for small scale degradation of gliotoxin.



(5)



(7)

5.3.1 Synthesis of the Trioxopiperazine (7). Compound (7) had been synthesised by Taylor and co-workers[91] by the route shown in Figure 19. Gliotoxin was dehydrogenated to give dehydrogliotoxin (11) in 69% yield. Acetylation of (11) gave an uncharacterised acetate in 55% yield, which was converted into the phenol (104) in 85% yield. Finally oxidation of (104) gave (7) via the glycol (105) in 23% yield. This route was impracticable due to the low yields obtained during acetylation and oxidation. A modified route was planned (Figure 20) whereby it was hoped that treatment of

dehydrogliotoxin (11) with HOAc/Ac_2O would give the phenol (104) directly in better yield and that chromic acid oxidation of (104) would give the trioxopiperazine (7) in an analagous fashion to the chromic acid oxidation of anhydrodesthiogliotoxin (3) described later.

5.3.2 Dehydrogliotoxin (11). Gliotoxin was dehydrogenated to dehydrogliotoxin (11) with 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (106) (Figure 20) in an improved yield of 81%. Unreacted gliotoxin (7%) was recovered by chromatography. The i.r. spectrum contained absorptions characteristic of the dioxopiperazine ring and of the aromatic and hydroxyl groups. The parent ion peak in the mass spectrum of (11) was weak due to the rapid loss of the disulphide bridge and subsequent dehydration to an ion of m/e 242 which gave the base peak.

5.3.3 The Phenol (104). Dehydrogliotoxin was converted into the phenol (104) (Figure 20) in 60% yield after preparative t.l.c. and crystallisation, an improvement on the 47% yield from the original two step process.[91] The i.r. spectrum corresponded closely with that reported[91] showing the absence of the hydroxyl group and the presence of the cis-amide carbonyls in conjugation with double bonds. Since the base peak in the mass spectrum of dehydrogliotoxin corresponded with the composition of (104) it was expected that the spectra of both compounds would be closely similar and this was found to be the case. The n.m.r. spectrum of (104) agreed exactly with that of the literature.[91]

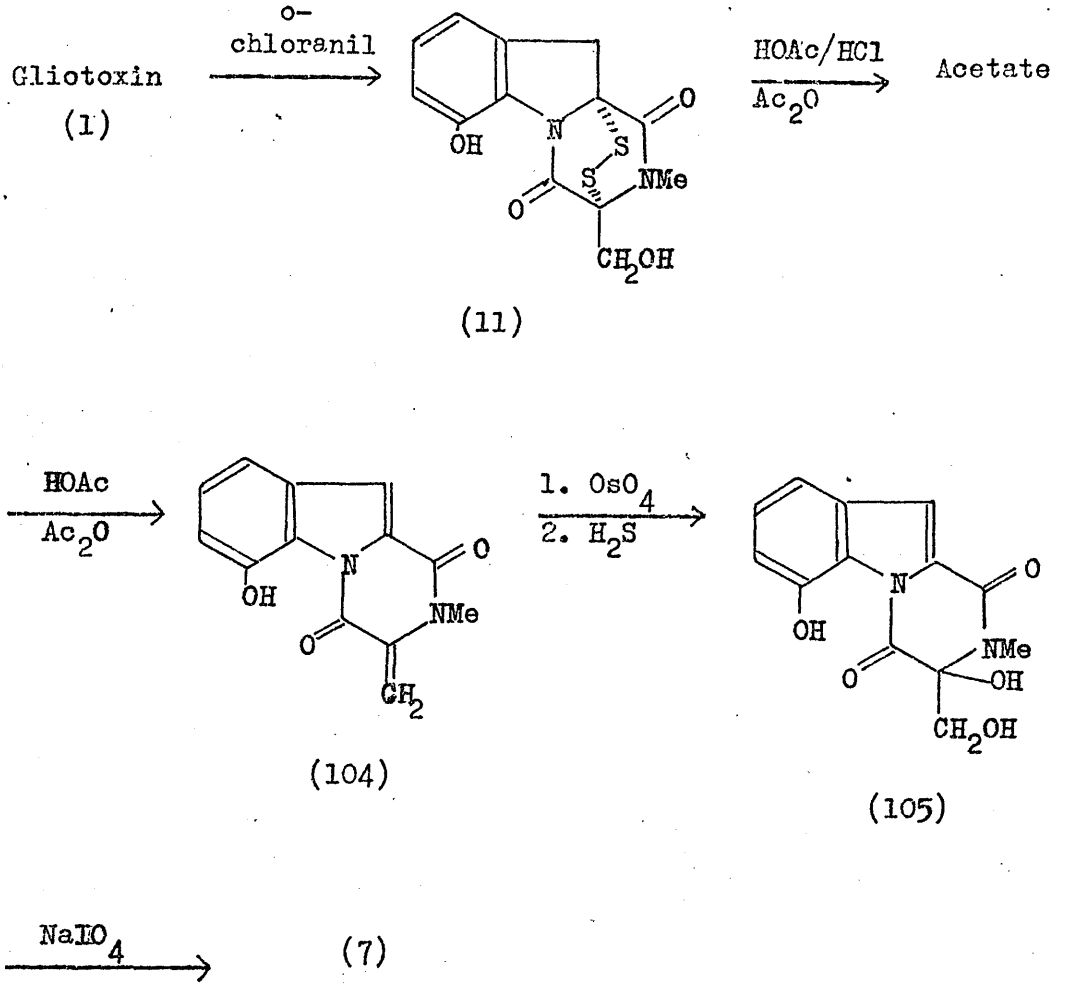


Figure 19 Synthesis of Trioxopiperazine (7).[91]

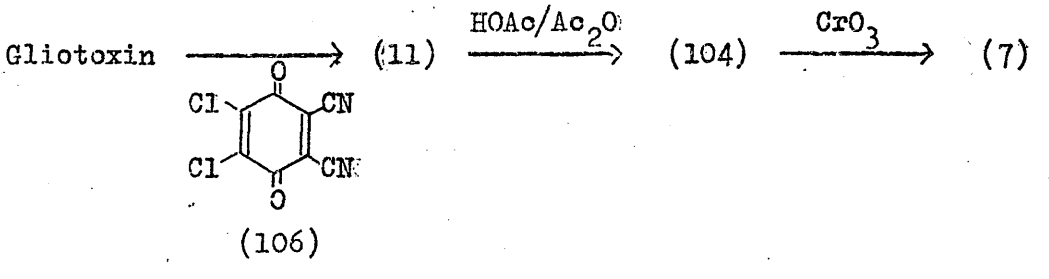
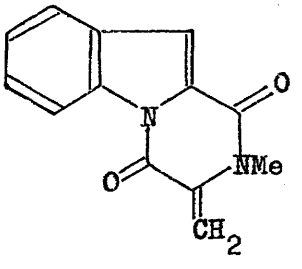


Figure 20 Planned Synthesis of Trioxopiperazine (7).

5.3.4 Oxidation of the Phenol (104). The phenol (104) was oxidised with chromic acid by the method of Ali et al.[92] However, the product obtained was a black solid which could not be characterised and it was concluded that the phenol had been too reactive and that it had been degraded.

In an attempt to reduce this reactivity a sample was acetylated as described by Ali et al.[92] and treated with chromic acid as above but again no simple product could be detected and so this approach was discontinued.

5.3.5 Synthesis of the Trioxopiperazine (5). The trioxopiperazine (5) had originally been obtained by treating gliotoxin with selenium.[93] However, the yield was very low. More recently (5) was synthesised from anhydrodesthiogliotoxin (3) by several routes (Figure 21), the best of which involved oxidation with chromic acid.[92]



(3)

		Yield
1. OsO_4 2. H_2S 3. PTSA	(5)	13.4%
1. OsO_4 2. H_2S 3. $NaIO_4$	(5)	<40.0%
CrO_3	(5)	85.0%

Figure 21. Syntheses of Trioxopiperazine (5).

This procedure was carried out on a sample of doubly labelled anhydrodesthiogliotoxin, synthesised from doubly labelled gliotoxin, as before with unchanged $^{3}H:^{14}C$ ratio and specific activity. The isolated trioxopiperazine was crystallised to constant specific activity and found to be identical (103%) in ^{3}H content to the original, diluted gliotoxin. No ^{14}C activity could be detected at all proving that it must have been totally in the 3a-position

The melting point and the i.r. and mass spectra of the crystallised product corresponded closely with those of the literature.[92] In the mass spectrum, a peak at m/e 88 with an accompanying metastable ion at m/e 67.3 was observed, whereas the peak at m/e 89, the literature value, was relatively weak. A metastable ion at m/e 92.5 was also observed rather than at m/e 91.5 [92] but the former value fits the fragmentation m/e 143 to m/e 115 much better and the literature value was considered to be a misprint.

5.4 Analogue Synthesis - 1.

The results obtained above demonstrated that the anomalous ozonolysis must have involved generation of formaldehyde from another part of (3) which, as already stated, was most likely to be the N-methyl group. It was decided to synthesise an analogue of (3) having the structure shown in Figure 22, with the N-methyl group labelled in order to detect if this group could form formaldehyde on ozonolysis.

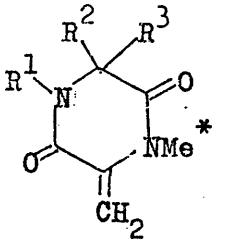


Figure 22 Analogue Structure.

An attempt to synthesise the analogue (107) was made following the route shown in Figure 23. Glycine anhydride (108) was

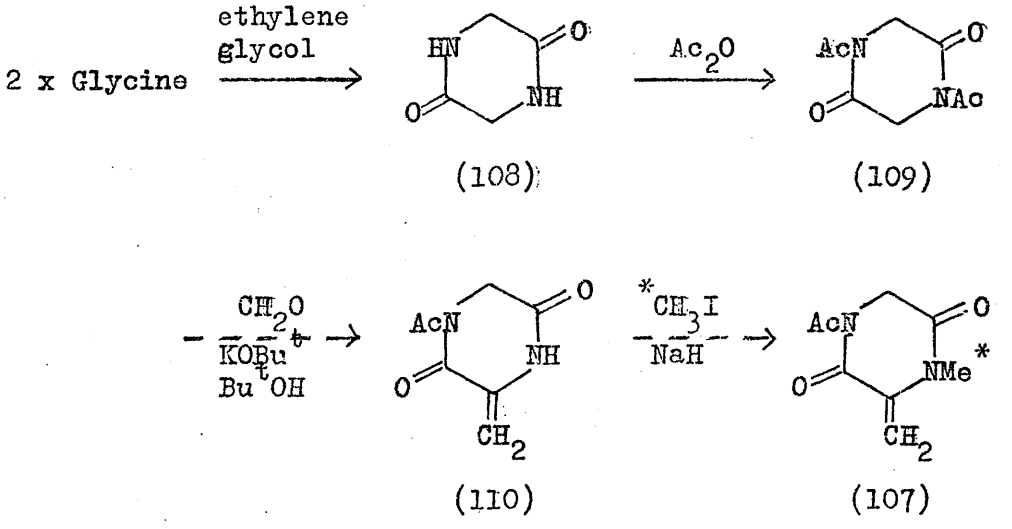


Figure 23 Planned Synthesis of Analogue (107).

synthesised in 74% yield by the method of Schott et al.[94] I.r. and mass spectra were characteristic of the dioxopiperazine structure. Acetylation of the anhydride yielded the di-acetylated product (109) in 84% yield. It was planned to condense (109) with formaldehyde to give (110) which could then be methylated with radioactively labelled iodomethane. It had been shown that (109)

could be dialkylated with aromatic aldehydes and monoalkylated with aliphatic aldehydes.[95] The mechanism (Figure 24) frees a lone pair on nitrogen which reduces the electron withdrawing power of the neighbouring carbonyl group making dialkylation possible only with reactive aldehydes. If formaldehyde could be condensed with (109), it would then be possible to label the desired amide nitrogen as shown (Figure 23). Unfortunately, all attempts to synthesise (110) following the literature procedure[95] were unsuccessful, the only product being a plastic like film showing several spots on t.l.c. It was decided therefore to abandon this approach.

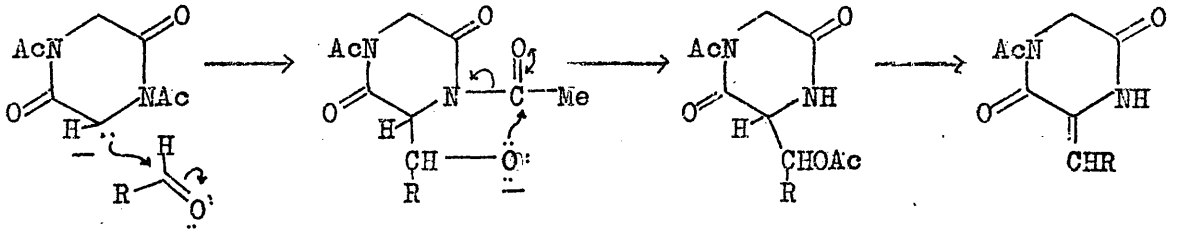


Figure 24 Mechanism of Alkylation.[95]

5.5 Analogue Synthesis— 2.

Following the failure of the above synthesis it was decided to synthesise the literature compounds (111) and (112), ozonise both, and compare the yields of formaldehyde generated. If the N-methyl group was being converted into formaldehyde then a greater yield might be expected from (111). It was also proposed to label (111) radioactively in the N-methyl group. The literature synthesis[96] was employed for both compounds with slight variations (Figure 25).

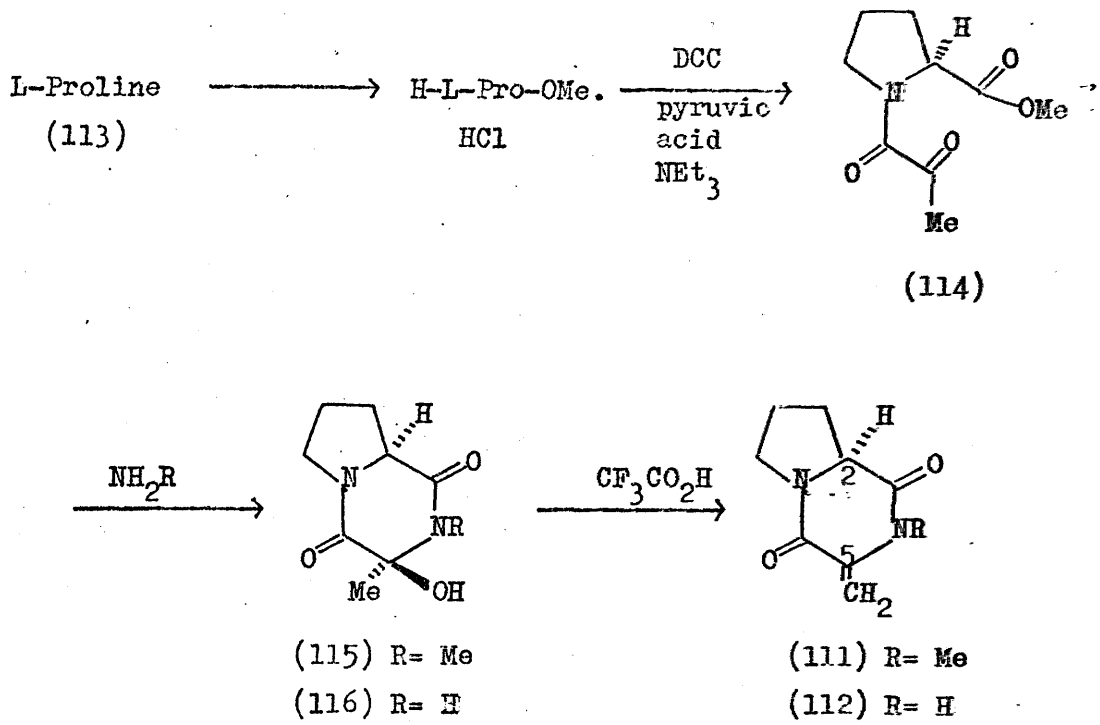


Figure 25 Analogue Synthesis - 2.

5.5.1 N-Pyruvoyl-L-proline Methyl Ester (114). L-Proline was methylated as its hydrochloride salt by the method of Brenner and Huber.[79] The product was an oil which failed to crystallise and was coupled to pyruvic acid by the DCC method to give an oil, heavily contaminated with DCU. Fractional distillation gave (114) but the recovery was low due to decomposition or polymerisation of the product in the distillation flask. The optical rotation of (114) was suprisingly high, almost twenty degrees higher than the literature value.[96b] However, the i.r. and n.m.r spectra were identical with reported spectra.[96b] The n.m.r. spectrum was interesting in that two singlets were observed for each methyl group due to hindered rotation about the amide bond.

The mass spectrum, which was not reported[96b], showed a very weak parent ion peak, the base peak being at m/e 128 with other strong peaks at m/e 70 and m/e 43. A fragmentation scheme is given in Figure 26.

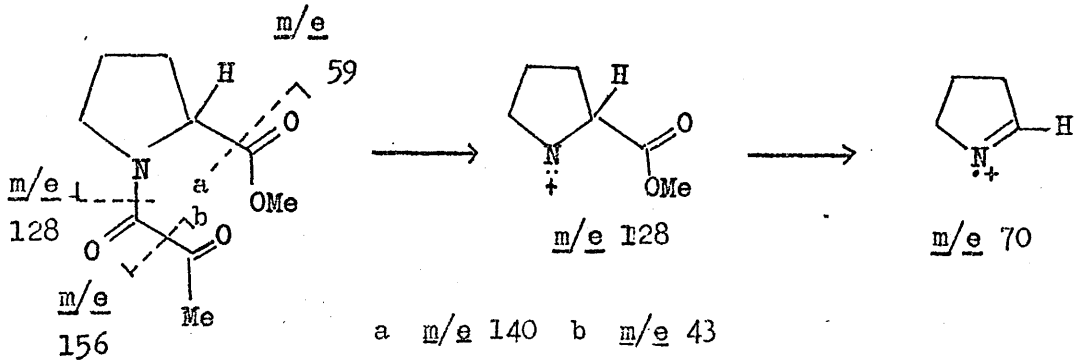


Figure 26 Mass Spectrum of N-Pyruvoyl-L-proline Methyl Ester (63).

5.5.2 Cyclisation with Methylamine. Cyclisation of (114) with methylamine was carried out as described by Lee [96b] to give the dioxopiperazine (115). The yield was half of that expected and the melting point was low. The elemental analysis was correct however and the optical rotation and n.m.r. spectral data were identical to literature values.[96b] The i.r. and mass spectra, which were not reported, were also consistent with the structure (115). The hydroxyl group was revealed in the i.r.spectrum by O-H stretching and C-O stretching bands. Two carbonyl absorptions were present. The parent ion peak in the mass spectrum was weak due to rapid dehydration. Fragmentations, characteristic of the dioxopiperazine and proline rings, were present (Figure 27).

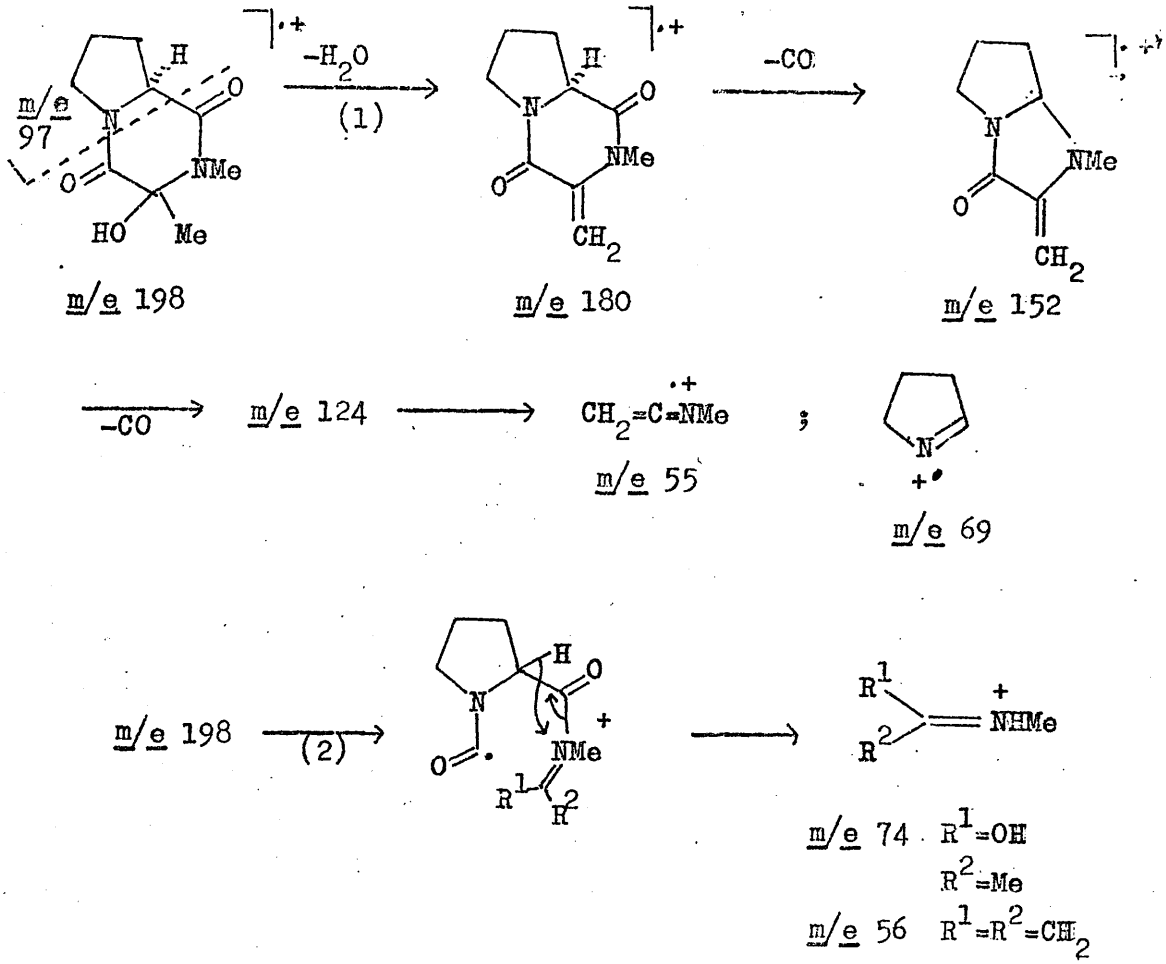


Figure 27 Mass Spectrum of Dioxopiperazine (115).

5.5.3 Dehydration Procedure. Dehydration of the dioxopiperazine (115) in trifluoroacetic acid was followed by n.m.r. spectroscopy and found to be complete within a few minutes. The olefin (111) obtained failed to crystallise and decomposed after a few hours.

5.5.4 Cyclisation with Ammonia. The dioxopiperazine (116) (Figure 25) was synthesised by treating (114) with ammonia in dimethoxyethane

as described by Lee[96b] to give prisms with a decomposition point, 20°C higher than the reported melting point.[96b] However, the elemental analysis was correct and the optical rotation was identical to the reported value.[96b] The n.m.r. spectrum was similar to that reported[96b] and the i.r.spectrum showed the characteristic absorptions for a dioxopiperazine ring. Fragmentation pathways, analagous to those for the methylated compound (115) were also present in the mass spectrum of (116).

5.5.4 Dehydration Procedure. Dehydration of the dioxopiperazine (116) was carried out as described by Lee[96b] to give the olefin (112) which analysed correctly and gave i.r. and mass spectra which were consistent with the structure (112). The mass spectrum was closely similar to that of the reactant as expected, since dehydration of the latter was an important process.

5.6 Ozonolysis Experiments.

5.6.1 Ozonolysis of methylated dioxopiperazine (111). Due to the instability of the dehydrated dioxopiperazine (111), ozonolysis was carried out as soon as the product (111) had been freed of trifluoroacetic acid. The same reaction conditions were used as for the ozonolysis of anhydrodesthiogliotoxin (3). A dimethyl sulphide work up was used to generate formaldehyde which was trapped in dimedone solution. The yield varied from 45-54%.

5.6.2 Ozonolysis of Dioxopiperazine (112). The same conditions were applied to the unmethylated dioxopiperazine (112) using ethyl acetate as solvent since (112) was insoluble in ethyl chloride. The yield of dimedone derivative obtained was 48%.

The yields were essentially the same but this does not disprove the theory that the N-methyl group was contributing to the recovered formaldehyde to some extent.

5.7 Radiolabelled Synthesis of the Analogue (111).

More detailed studies were pursued on the cyclisation reaction to produce the methylated dioxopiperazine (115) with methylamine, since it was proposed to introduce the radioactive label at this stage with [^{14}C]methylamine. This, in turn, had to be generated from [^{14}C]methylamine hydrochloride. In order to get as high an incorporation of radioactivity into the product as possible it was necessary, ideally, to treat the reactant with one equivalent of methylamine. The reaction was studied to see if this was possible with unlabelled methylamine hydrochloride. It was found that methylamine could be generated by refluxing the salt in sodium hydroxide solution and could then be passed in a slow stream of nitrogen into the reaction flask which was cooled to -40 to -50°C to ensure efficient trapping of the gas. Usually 5-8h was required for the process.

The amount of methylamine hydrochloride used and the reaction times were varied. Reaction solutions were evaporated to dryness and studied by n.m.r. spectroscopy in CD_3OD.

Using one equivalent of methylamine, the reaction failed to go to completion since some methyl ester was still in evidence in the n.m.r. spectrum. It was calculated that the yield was 66%. With two equivalents of methylamine the reaction went to completion but, as with the previous experiment, two products were present on t.l.c. and two N-methyl singlets were present in the n.m.r.spectrum. The singlet of lower intensity was due to the desired product and the other, presumably, was due to the 2S, 5S-diastereomer. It was estimated that the 2S,5R:2S,5S ratio was 1:3. When more than two equivalents of methylamine hydrochloride were used a solid was obtained with the 2S,5R:2S,5S ratio nearer 1:1. These results implied that the 2S,5S-diastereomer was formed kinetically and was then equilibrated in the presence of excess base to a 1:1 mixture of the two forms. This was at odds with the results obtained by Bycroft and Lee[96] and it must be concluded that the methylamine was not sufficiently dried by the potassium hydroxide and soda lime columns included in the apparatus. Bycroft and Lee[96] reported that 1:1 mixtures of the two diastereomers were obtained in protic solvents. Bigger drying columns were impracticable and so it was decided to carry out the radiolabelled synthesis as follows. N-Pyruvoyl-L-proline methyl ester (114) would be treated with one equivalent of [^{14}C] methylamine at 0°C for two days to produce a non-quantitative mixture of the two diastereomers, mostly 2S,5S. Ten equivalents of unlabelled methylamine would then be added to equilibrate the mixture. Before this procedure was attempted it was necessary to show that equilibration did not release methylamine. This

was demonstrated by treating a solution of a 1:1 mixture of the diastereomers with ammonia, generated by refluxing an ammonia solution. If equilibration required the loss of methylamine then ammonia would take its place resulting in the loss of the N-methyl singlets in the n.m.r. spectrum. This was not observed.

The radiolabelled synthesis was carried through by the method described to give a diastereomeric mixture of 2S,5S:2S,5R (ratio 5:3) containing 68.5% of the radiolabelled methylamine added. Crystallisation of the mixture yielded 36mg (18%) of the 2S,5R-diastereomer and a further 9% was obtained by diluting the mother liquors with unlabelled product (115) and recrystallising.

Panax scanning and autoradiography of t.l.c. plates of (115) run in three different solvent systems showed the presence of a small quantity of the 2S,5S-diastereomer. Dilution analysis of a sample of the labelled product, with unlabelled product as carrier, showed that the product was 94.4% pure and it was decided not to purify further since both diastereomers would yield the same product on dehydration.

Dehydration of labelled (115) was followed by n.m.r. spectroscopy and the crude product (111) was ozonised. The ozonide was worked up as before. Formaldehyde dimedone was obtained in 51% yield with a negligible specific activity corresponding to 0.2 - 0.4% of the original value.

It appears that the N-methyl group in this particular molecule (111) does not form formaldehyde on ozonolysis. This result cannot, however, rule out the possibility of this occurring

with anhydrodesthiogliotoxin (3) and it may be that the indole double bond has some important function to play.

The obvious experiment to try is an ozonolysis of anhydrodesthiogliotoxin labelled specifically in the N-methyl group. However, the synthesis of this derivative would not be straightforward.

5.8 Conclusion.

The position of the ^{14}C label in doubly labelled gliotoxin, derived biosynthetically from cyclo-(L-[4'-^{3}H]Phe-L-[3-^{14}C]Ser), was shown to be at carbon-3a by degradation of gliotoxin to the trioxopiperazine (5). The N-methyl group of gliotoxin was shown to be inactive. These results demonstrate that the ^{14}C label was not scrambled within the molecule during incorporation of the cyclic dipeptide.

Doubly labelled gliotoxin was converted to anhydrodesthiogliotoxin with essentially the same isotope ratio and specific activity. Ozonolysis of the latter compound gave formaldehyde with less than half of the expected specific activity in ^{14}C. Since the label has not been scrambled, formaldehyde is being formed from another part of anhydrodesthiogliotoxin apart from the exo methylene group. The N-methyl group is the most likely contributer. However, this has still to be proved since experiments with model compounds did not show that formaldehyde is generated from N-methyl groups of the model compounds on ozonolysis.

CHAPTER 6 THE ROLE OF CYCLO-(L-PHENYLALANYL-N-METHYL-L-SERYL) AS A POSSIBLE BIOSYNTHETIC INTERMEDIATE FOR GLIOTOXIN.

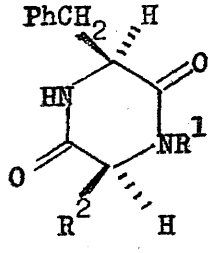
Introduction.

Following the confirmation of cyclo -(L-phenylalanyl-L-seryl) (24) as a biosynthetic intermediate for gliotoxin (Chapter 4), attention was turned to other possible intermediates. At least three further stages are required to convert cyclo-(L-Phe-L-Ser) into gliotoxin: N-methylation, incorporation of the disulphide bridge, and ring closure to form the dihydroaromatic system. The order of these steps is unknown but several possible intermediates can be formulated. The most accessible intermediate in terms of synthesis is cyclo-(L-phenylalanyl-N-methyl-L-seryl) (29), which could be formed in the biosynthetic pathway by N-methylation of cyclo-(L-Phe-L-Ser).

Specific methylation of cyclo-(L-Phe-L-Ser) did not seem feasible since experiments with cyclo-(L-Phe-L-Ala) (117) had given the NN-dimethylated cyclic dipeptide with no evidence of the mono-substituted product, even when less than one equivalent of methylating agent was used. Racemisation, O-methylation, and elimination might also occur on the L-seryl portion of cyclo-(L-Phe-L-Ser) and so it was decided to synthesise (29) by coupling an ester derivative of N-methyl-L-serine with Z-L-Phe-OH (81) such that hydrogenolysis of the protecting group would release a primary amino group necessary for subsequent cyclisation.

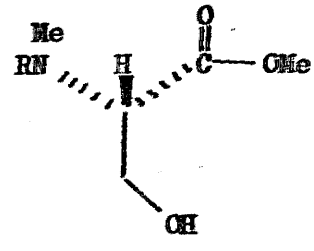
Earlier work had shown that secondary amino groups did not cyclise readily.[97]

Attempts to methylate Z-L-Ser-OH (67) to give N-benzyloxycarbonyl-N-methyl-L-serine methyl ester (118), which could then be hydrogenolysed to the desired N-methyl-L-serine methyl ester (119), were unsuccessful. Using iodomethane and silver oxide in dimethylformamide[98] at room temperature, a mixture of the unsaturated derivative (120) and the serine derivative (121) was obtained in a ratio of 1:2 in agreement with the literature.[99] The same result was obtained at 0°C.



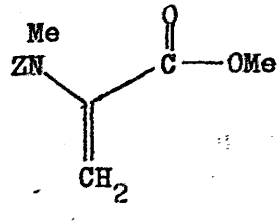
(29) R^1= Me R^2= CH_2OH

(117) R^1= H R^2= Me

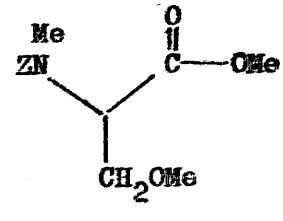


(118) R= Z

(119) R= H

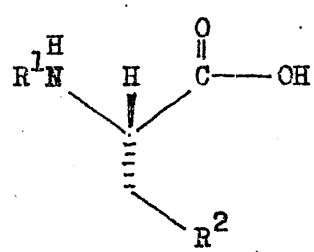


(120)



(121)

A recent paper by Cheung and Benoiton[100] reviewed several methods of N-methylating amino-acid derivatives. The method involving least racemisation was the treatment of N-benzyloxycarbonyl or N-t-butyloxycarbonyl derivatives of amino-acids with eight equivalents of iodomethane and three equivalents of sodium hydride in tetrahydrofuran with or without dimethylformamide. In the absence of dimethylformamide the acid group was not methylated. These workers also reported[100] that the serine derivatives (122) and (123) underwent elimination under these conditions at room temperature, but by reducing the temperature to 5°C and increasing the reaction time to three days, the N-methylated products were obtained in high yield.



(122) R^1= Z, R^2= OBu^t
(123) R^1= Boc, R^2= OBz

It was decided to synthesise N-benzyloxycarbonyl-O-t-butyl-L-serine (122) rather than the O-benzylated compound (123) since the t-butyl group could be retained until the final stage of the synthetic route to (29). The benzyl protecting group, which was difficult to introduce, would be lost during hydrogenolysis. The planned synthesis is shown in Figure 28, including the known literature route to (122).

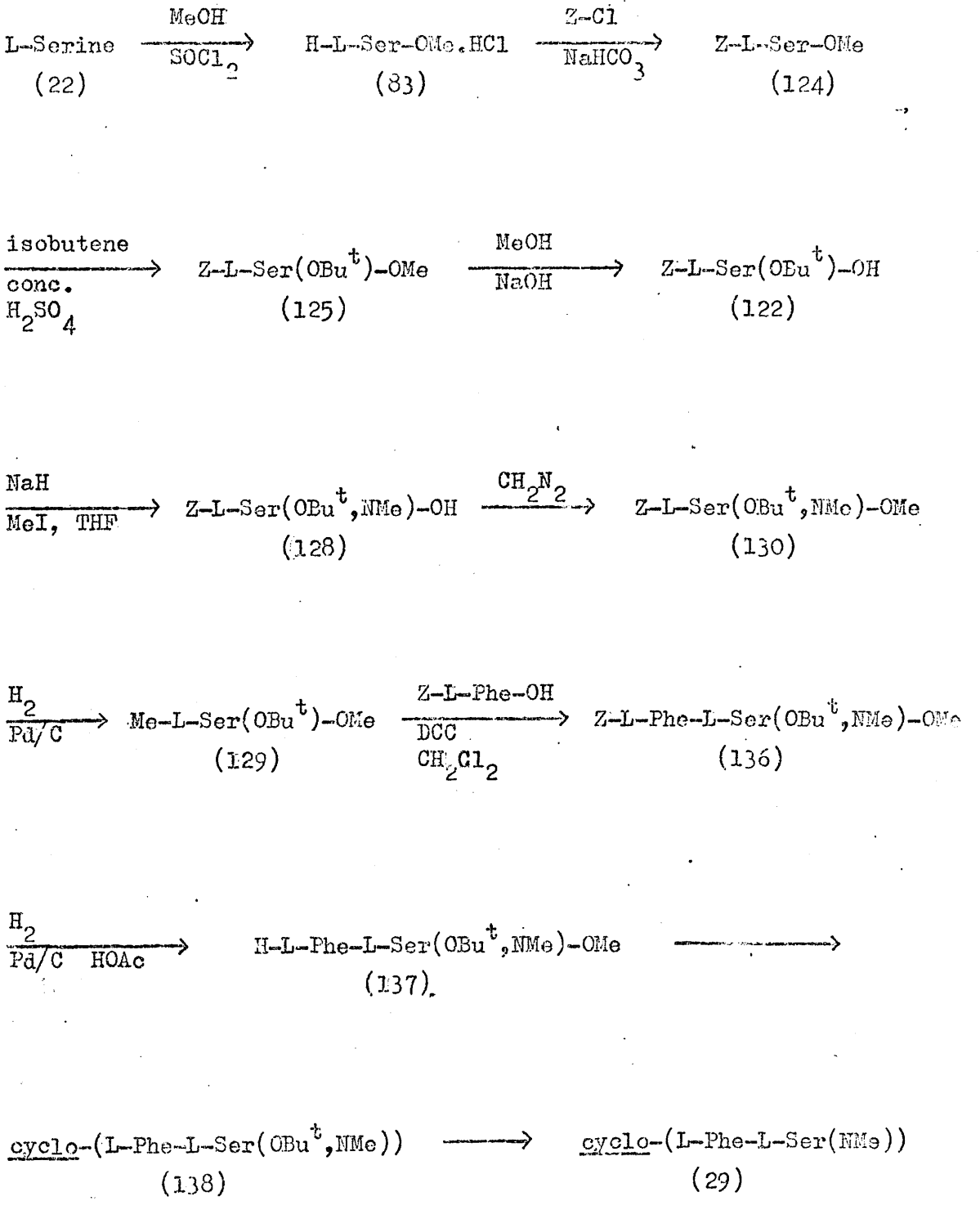


Figure28 Synthesis of cyclo-(L-Phenylalanyl-N-methyl-L-seryl).

6.1 Synthesis and N-Methylation of N-Benzyloxycarbonyl-O-t-butyl L-serine (122).

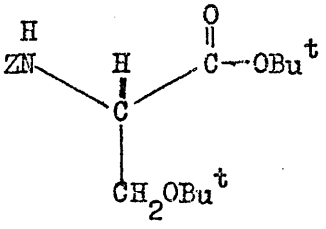
6.1.1 N-Benzyloxycarbonyl-L-serine Methyl Ester (124).

H-L-Ser-OMe.HCl (83) was converted into Z-L-Ser-OMe (124) in good yield (80%) by the method of Guttman and Boissonas[71] (Figure 28). The product failed to crystallise due to the presence of benzyl chloride as an impurity. The success of the reaction depended on neutralisation of the hydrochloride salt with ice cold sodium bicarbonate solution and vigorous stirring throughout the reaction. A sample of the oil was purified by vacuum distillation for analysis. The i.r.spectrum showed absorptions, characteristic of the urethane, ester, aromatic, and hydroxyl groups. The presence of the benzyloxycarbonyl group was clearly indicated by the n.m.r. and mass spectra of (124).

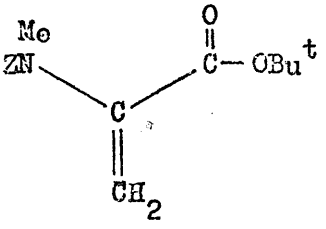
6.1.2 N-Methylation of L-Serine Ester Derivatives. The substrates used by Cheung and Benoiton[100] for N-methylation were free acids and therefore it would be necessary to hydrolyse the methyl ester of (125) to obtain the same starting material. Since, however, the methyl ester group would have to be reformed afterwards, attempts were made to N-methylate the methyl ester (125) directly, using the reaction conditions of Cheung and Benoiton.[100] N-Methylation did take place but so did elimination of t-butanol and hydrolysis of the ester. At -20°C, N-methylation failed to proceed but hydrolysis was still found to occur. Similar experiments with N-benzyloxycarbonyl-O-t-butyl-L-serine t-butyl

ester (126), prepared by the method of Callahan et al.[101], were equally unsuccessful, and a yield of 57% was obtained of the unsaturated product (127) at 5°C.

These results showed that the methine proton of the serine ester derivatives was too acidic to allow selective N-methylation and that the free acid had to be synthesised.



(126)



(127)

6.1.4 N-Benzyloxycarbonyl-O-t-butyl-L-serine (122). Hydrolysis of the methyl ester (125) was carried out by the method of Callahan et al.[101] (Figure 28) to give the acid (122) as an oil, which was purified by preparative t.l.c. The optical rotation was less than half of the reported value[101], implying that racemisation had occurred. Racemisation was reduced by replacing methanol as solvent with ethanol, by increasing the water:ethanol ratio as much as possible within the limits of solubility, and by decreasing the reaction time. The acid (122) was obtained as a solid in better yield (85%) with no more than 10% racemisation. Only 0.8% of the starting material was recovered showing that hydrolysis was virtually complete. The melting point was broad due to partial

racemisation. The i.r. spectrum showed the characteristic broad acid O-H stretching absorption as well as absorptions due to the acid and urethane carbonyl and C-O stretching vibrations. The n.m.r. spectrum was noticeable for the ABX multiplet due to the seryl methylene group. Benzyloxycarbonyl and t-butyl fragmentations dominated the mass spectrum.

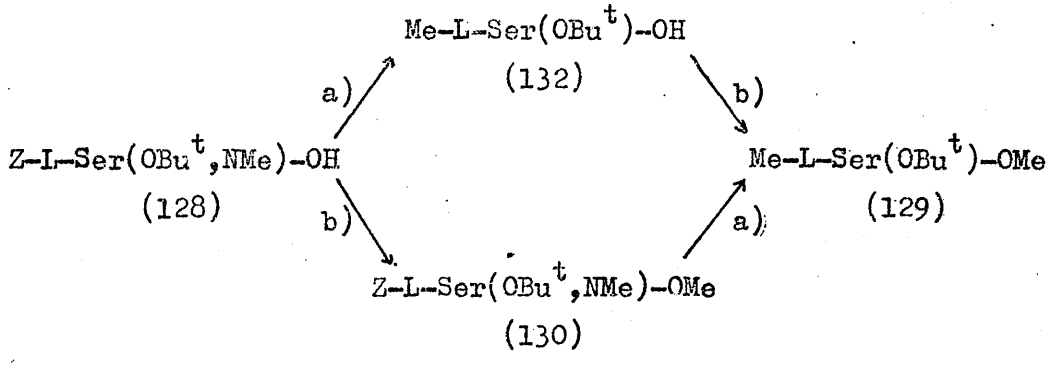
Unsuccessful attempts were made to remove the methyl ester of (125) using other procedures e.g. lithium iodide in dimethylformamide[103], and sodium thiomethoxide in hexamethylphosphoramide.[104] A different approach using the t-butyl ester (126) was tried whereby selective removal of the t-butyl ester was attempted with benzene and p-toluenesulphonic acid. However, both ester and ether t-butyl groups were removed.

6.1.3 N-Methylation Procedure. N-Methylation of the free acid (122) was carried out as described by Cheung and Benoiton[100] (Figure 28). However, it was observed that a certain amount of elimination (11-15%) was still taking place and that the reaction proceeded better at 0°C with no elimination. Furthermore it was discovered that the reaction failed to go to completion if more than five mmol of starting material was used. Under the revised conditions the N-methylated compound (128) was obtained in high yield (94-98%), as an oil which showed one spot on t.l.c. and analysed correctly. Since the urethane group was tertiary, no urethane II absorption was observed in the i.r. spectrum. Both monomer and hydrogen bonded dimer, acid carbonyl stretching

absorptions were seen. The multiplet due to the methine proton in the n.m.r. spectrum was shifted downfield by ca. 0.25 p.p.m. compared with that of the reactant. Benzyloxycarbonyl and t-butyl fragmentations were again the dominant features of the mass spectrum.

6.2 Synthesis of O-t-Butyl-N-methyl-L-serine Methyl Ester (129).

Two steps were required to convert the acid (128) into the desired methyl ester (129), namely, formation of the methyl ester and hydrogenolysis of the benzyloxycarbonyl group. Since these steps could be carried out in either order (Figure 29), the two alternatives were tried and compared with respect to yield and ease of synthesis.



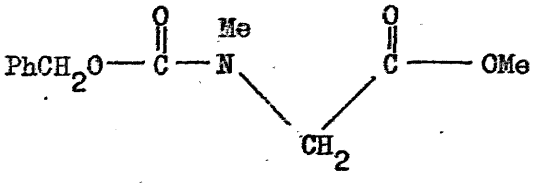
a) H_2, Pd/C b) CH_2N_2

Figure 29 Synthesis of Methyl Ester (129).

6.2.1 N-Benzyloxycarbonyl-O-t-butyl-N-methyl-L-serine Methyl Ester (130). The methyl ester (130) was formed quantitatively

by treating the acid (128) with a slight excess of diazomethane. The ester was a slightly hygroscopic oil which showed a single spot on t.l.c. and analysed correctly. Evidence for the methyl ester group was clearly seen in the i.r. and n.m.r.spectra.

One interesting feature in the n.m.r. spectrum (Figure 30) was the extremely broad singlet observed due to the ester methyl group, caused by hindered rotation about the urethane bond. The same situation was found with N-benzyloxycarbonyl-N-methyl-glycine methyl ester (131), which at +35°C showed broad doublets in the n.m.r. spectrum due to the methylene and ester methyl groups and broad singlets for the phenyl and adjacent methylene groups.[105] At +60°C, rotation was rapid and every signal was a sharp singlet whilst at -30°C the methyl ester, phenyl, and glycyl methylene groups showed doublets due to hindered rotation.[105]



(131)

The mass spectrum was dominated by benzyloxycarbonyl and t-butyl fragmentations as expected. Other peaks of significance were at m/e 162, 146, and 102 which might be produced as shown in Figure 31.

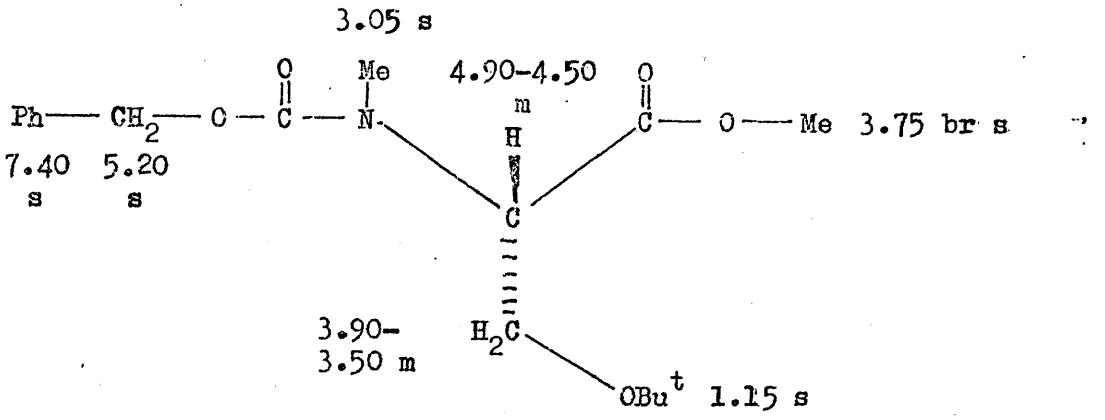
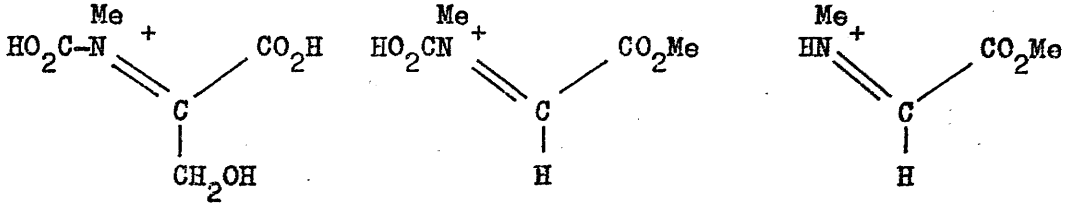


Figure 30 N.m.r. Spectrum of Z-L-Ser(OBu^t,NMe)-OMe (130) in $CDCl_3$ (δ values).



m/e 162 m/e 146 m/e 102

Figure 31 Possible Ions in the Mass Spectrum of the Ester (130).

6.2.2 O-t-Butyl-N-methyl-L-serine Methyl Ester (129).

Z-L-Ser(OBu^t,NMe)-OMe (130) was easily converted into Me-L-Ser(OBu^t)-OMe (129) by hydrogenolysis (Figure 29). An oil, giving a single spot on t.l.c, was obtained in high yield. After several days at room temperature, the compound was found to have decomposed slightly and was therefore best stored at 0°C. Absorptions due to the methyl ester and t-butyl ether groups were clearly seen in the

i.r. spectrum. A sharp singlet was observed in the n.m.r. spectrum (Figure 32) due to the ester methyl group. The multiplet due to the methine proton and the singlet due to the N-methyl group had shifted significantly upfield compared with the equivalent signals in the n.m.r. spectrum of the reactant (130).

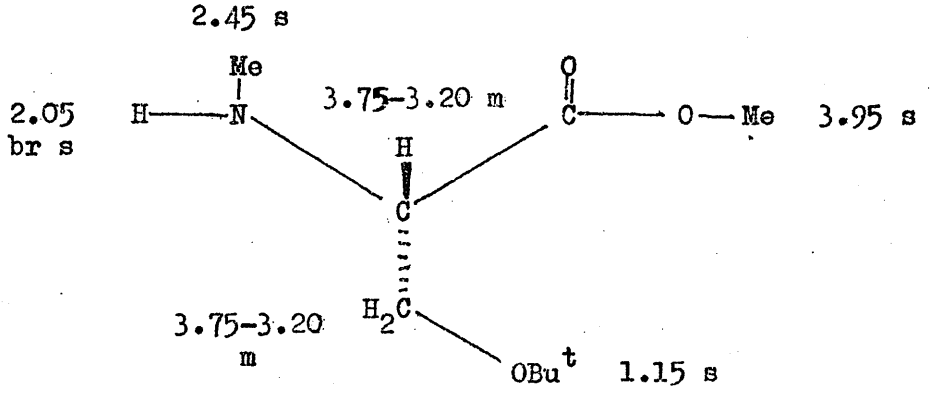


Figure 32 N.m.r. Spectrum of Me-L-Ser(OBu^t)-OMe (129) in $CDCl_3$ (δ values).

6.2.3 O-t-Butyl-N-methyl-DL-serine (132). The alternative route was attempted whereby the acid (128) was hydrogenolysed (Figure 29). The amino-acid (132) was obtained as a solid in 85% yield. The i.r. spectrum revealed that the product existed as a zwitterion. The t-butyl group was obvious in the n.m.r. and mass spectra.

6.2.4 Methylation of the Amino-acid (132). The amino acid (132) was treated with diazomethane (Figure 29) but the reaction failed to go to completion. The previous route where esterification preceded hydrogenolysis (Figure 29) was therefore favoured.

6.3 Synthesis of cyclo-(L-Phenylalanyl-N-methyl-L-seryl) (29).

6.3.1 Coupling Procedure. Me-L-Ser(OBu^t)-OMe (128) and Z-L-Phe-OH (81) were coupled by the DCC method (Figure 28) for twenty four hours rather than five hours since coupling was slower. This was presumably due to steric hindrance from the N-methyl group. Triethylamine was not required since the free base (129) was used. In fact, the presence of triethylamine resulted in the formation of an impurity, identified by n.m.r. spectroscopy (Figure 33), as the N-acyl urea (133) of Z-L-Phe-OH present at a ratio of 3:4 with respect to the desired product. N-Hydroxysuccinimide (134), known to inhibit the formation of N-acyl derivatives, was found to be very effective in eliminating formation of (133). However, the active ester (135) which it formed with Z-L-Phe-OH was not sufficiently reactive to react completely with Me-L-Ser(OBu^t)-OMe (129). After twenty four hours a mixture of the product to active ester of 3:7 was obtained.

In the absence of triethylamine and N-hydroxysuccinimide,, a high yield (88%) of the crude product, benzyloxycarbonyl-L-phenylalanyl-O-t-butyl-N-methyl-L-serine methyl ester (136). was obtained, contaminated slightly with DCU. A sample was purified by preparative t.l.c. and analysed. The LD-isomer, Z-L-Phe-D-Ser(OBu^t,NMe)-OMe, had the same Rf value as that of the LL-isomer and the only indication of its presence was a small shoulder on the singlet due to the t-butyl group in the n.m.r. spectrum.

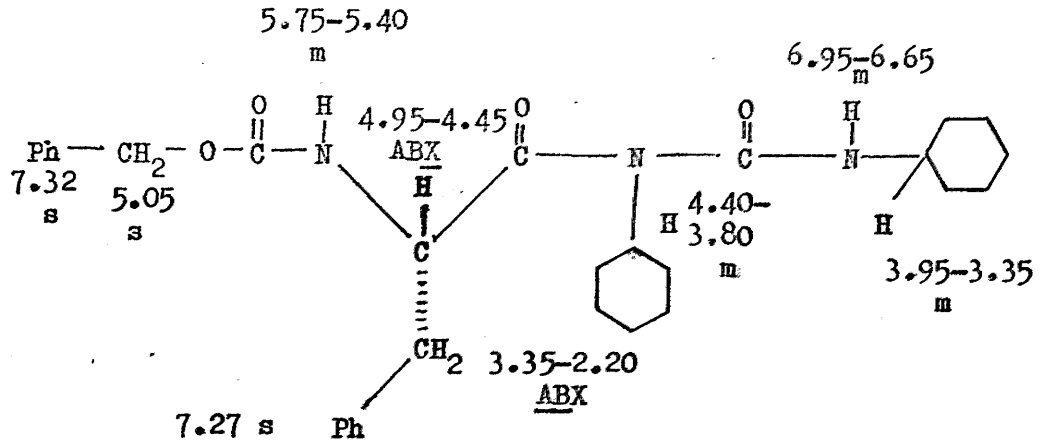
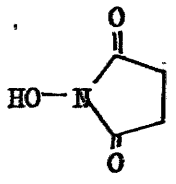
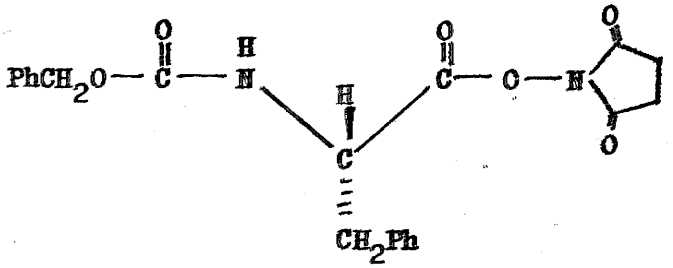


Figure 33 N.m.r. Spectrum of N-acyl urea derivative (133) in $CDCl_3$ (δ values).



(134)



(135)

6.3.2 Hydrogenolysis of the Protected Dipeptide (136). The benzyloxycarbonyl group of the protected dipeptide (136) was removed quantitatively by hydrogenolysis (Figure 28) to give L-phenylalanyl-O-t-butyl-N-methyl-L-serine methyl ester (137), which cyclised spontaneously once residual acetic acid had been removed completely. Two impurities were observed on t.l.c., one of which was DCU. Purification by crystallisation,

preparative t.l.c., and column chromatography gave cyclo-(L-phenylalanyl-O-t-butyl-N-methyl-L-seryl) (138) and the impurity, cyclo-(L-phenylalanyl-O-t-butyl-N-methyl-D-seryl) (139). Both compounds were recrystallised and analysed.

The optical rotations for both compounds were large and negative. The i.r. spectra of both compounds showed absorptions characteristic of the dioxopiperazine structure. The amide II absorption was absent and the absorptions due to the secondary amide C=O stretching modes were at high frequency (ca. 1680 cm^{-1}) compared with the normal trans-value (ca. 1650 cm^{-1}). An absorption due to the tertiary amide C=O stretching mode was present at ca. 1650 cm^{-1} in both spectra. The n.m.r. spectrum of the LL-isomer (138) run in CD_3OD (Figure 34) was compared with that of cyclo-(L-Phe-L-Ser) (Figure 14). The ABX multiplet due to the methylene group derived from serine was again widely spread and shifted upfield due to the influence of the aromatic ring. However, both the shift and the spread of the multiplet was not so great as observed with cyclo-(L-Phe-L-Ser) which implies that the boat might be flatter thus decreasing the influence of the aromatic ring on the methylene group. The n.m.r. spectrum of the LL-isomer (138) was also run in $CDCl_3$ and compared with the n.m.r. spectrum of the LD-isomer (139) (Figure 35). The multiplet due to the methine proton derived from serine was shifted ca. 1.2 p.p.m. upfield in the n.m.r.spectrum of the LD-isomer (139) compared with the equivalent signal in the spectrum of the LL-isomer (138). An extremely large combined vicinal

splitting was also observed in the former spectrum for this multiplet. The fragmentations observed in the mass spectra of (138) and (139) are shown in Figure 36. Fragmentations whereby the charge resided in the dioxopiperazine ring were favoured for the LL-isomer (138), whereas the strongest ions resulting from the LD-isomer (139) contained the charge on the side groups.

6.3.3 Deprotection Procedure. Several methods of removing the t-butyl protecting group from cyclo-(L-Phe-L-Ser(OBu^t,NMe)) (138) were attempted. Refluxing (138) in benzene with p-toluenesulphonic acid, heating with trifluoroacetic acid or chloroform-hydrochloric acid had no effect, and starting material was recovered in every case. Refluxing (138) in benzene-concentrated sulphuric acid resulted in degradation, leading to many spots on t.l.c. The only method which worked was treatment with 45% HBr-HOAc. The acetyl derivative (140) of cyclo-(L-Phe-L-Ser(NMe)) was also formed as a by-product but the extent of this reaction was reduced by short reaction times. Deprotection was virtually complete but unfortunately, the crude product required to be purified by preparative t.l.c. and the recovery of cyclo-(L-Phe-L-Ser(NMe)) (29) was not good. The overall yield after crystallisation was 37%. The acetyl derivative was obtained in 6% yield when the reaction mixture was allowed to stand for thirty minutes.

The i.r. spectrum of (29) contained absorptions, characteristic of the hydroxyl group, the dioxopiperazine ring, and the tertiary amide group. The base peak (m/e 218) in the mass spectrum of (29)

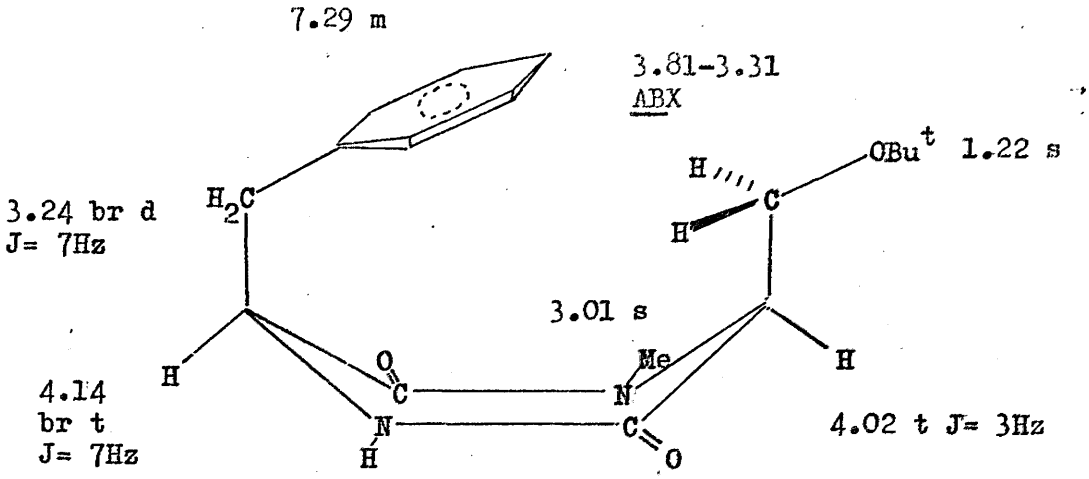


Figure 34 N.m.r. Spectrum of cyclo-(L-Phe-L-Ser(OBu^t,NMe)) in CD_3OD (δ values).

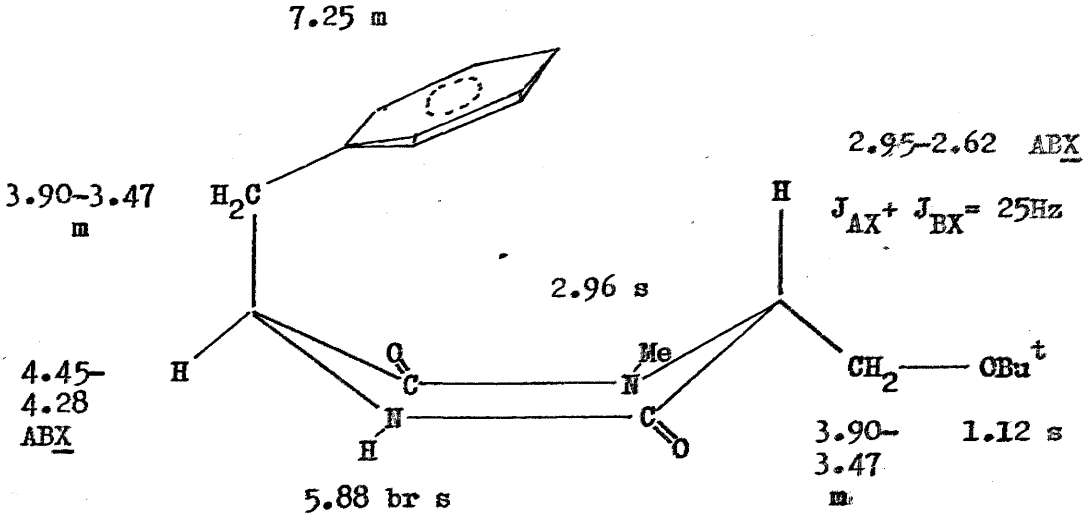
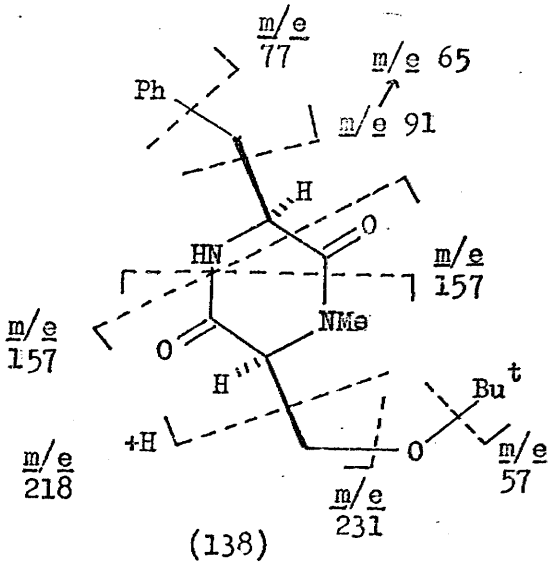
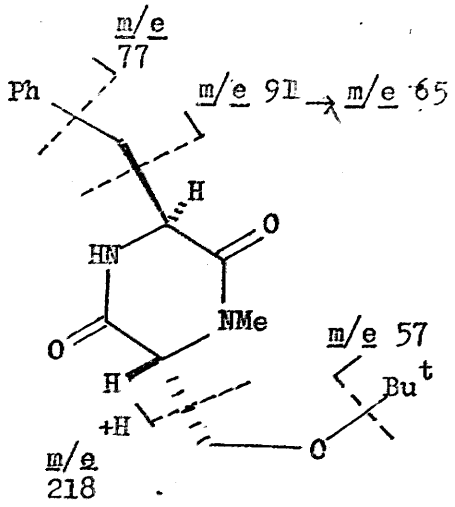


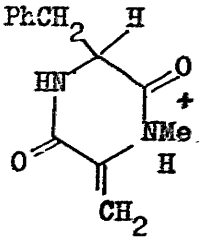
Figure 35 N.m.r. Spectrum of cyclo-(L-Phe-D-Ser(OBu^t,NMe)) in $CDCl_3$ (δ values).



(138)

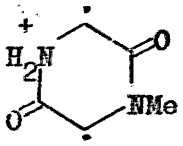


(139)

1) m/e 304 ⟶  ⟶ $CH_2=C=\overset{+}{N}HMe$ m/e 56

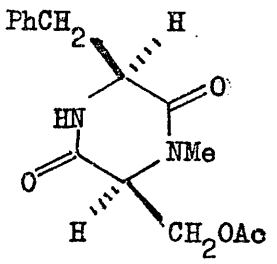
m/e 231

2) m/e 304 ⟶ $PhCH_2\overset{+}{C}H=NH_2$ m/e 120

3) m/e 304 ⟶  —CO⟶ m/e 99

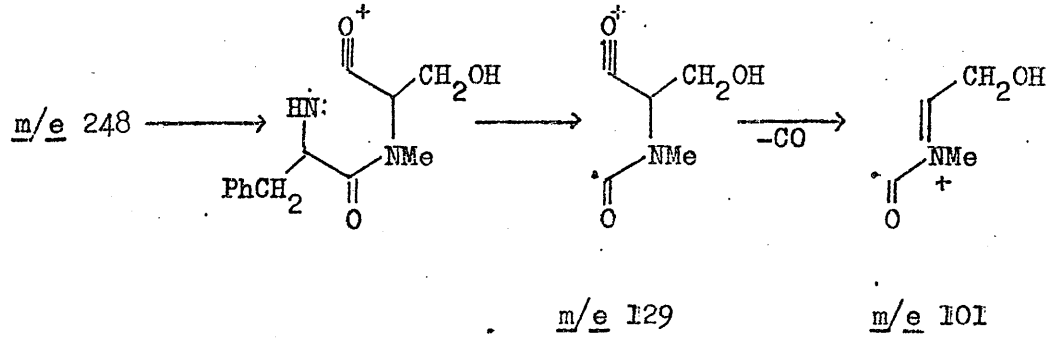
m/e 127

Figure 36 Mass Spectra of cyclo-(L-Phe-L-Ser(OBu^t,NMe)) (138) and cyclo-(L-Phe-D-Ser(OBu^t,NMe)) (139).



(140)

corresponded to a McLafferty rearrangement and an associated metastable ion peak at m/e 191.5 was observed. The remainder of the spectrum was similar to that of the reactant (138). Two new peaks were observed and these were thought to be due to the ions shown in Figure 37. The n.m.r. spectrum run in $(CD_3)_2SO$ was



m/e 129 m/e 101

Figure 37 Possible Derivation of Ions in the Mass Spectrum of (L-Phe-L-Ser(NMe)) (29).

complicated but several multiplets sharpened after the solution was shaken with D_2O and assignments were made as shown in Figure 38. The ABX multiplet due to H^c and H^d was again widely spread and shifted upfield although not so much as the equivalent

multiplet in the n.m.r. spectrum of cyclo-(L-Phe-L-Ser) (24).

The acetyl derivative (140) had a large negative optical rotation. Strong absorptions due to the ester group were present in the i.r. spectrum and there was no O-H stretching absorption. The mass spectrum contained many of the ion peaks discussed already. A peak at m/e 230 was either the result of elimination or a McLafferty rearrangement. The n.m.r. spectrum is shown in Figure 39. The multiplet due to H^c and H^d was at lower field due to the increased electron withdrawing influence of the acetyl group. An ABX multiplet, thought to be due to H^a was suprisingly shifted upfield and experienced large vicinal coupling.

6.3.4 cyclo-(L-Phenylalanyl-N-methyl-D-seryl) (141). cyclo-(L-Phe-D-Ser(NMe)) (141) was synthesised from the protected cyclic dipeptide (139) as above. The reaction proceeded cleanly with little evidence of the acetyl impurity. The recovery from preparative t.l.c. was again poor and the final yield was 9.5%. It was found that the melting points of the LL- and LD-isomers, (29) and (141), were virtually identical and that both compounds had the same Rf value on t.l.c. The mass spectrum of (141) was very similar to that of (29) but there were differences in the i.r. spectra. The n.m.r. spectrum of (141) was confused by solvent peaks but it was clear that the methine proton of the seryl moiety had been shifted upfield as expected.

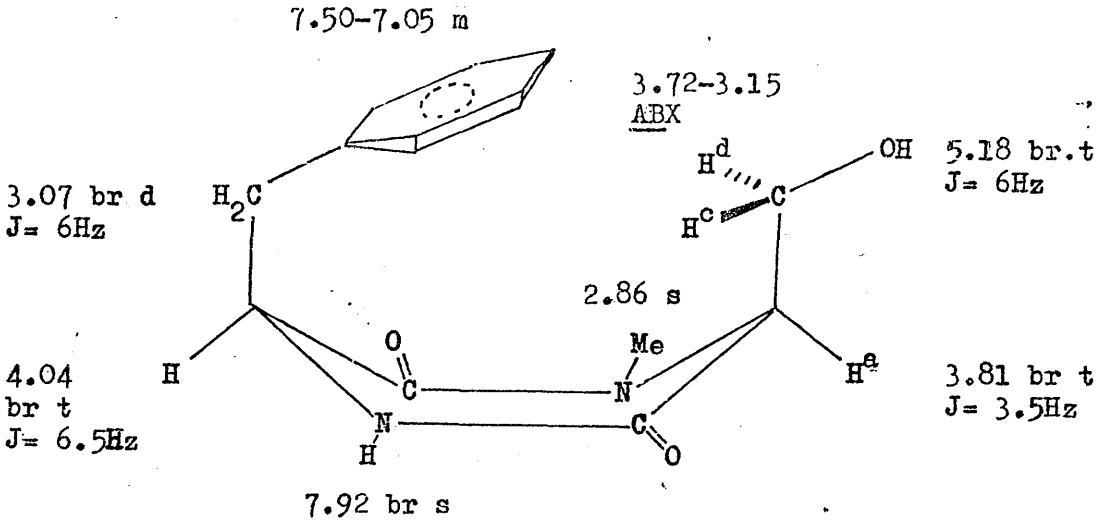


Figure 38 N.m.r. Spectrum of cyclo-(L-Phe-L-Ser(NMe)) (29) in $(CD_3)_2SO$ (δ values).

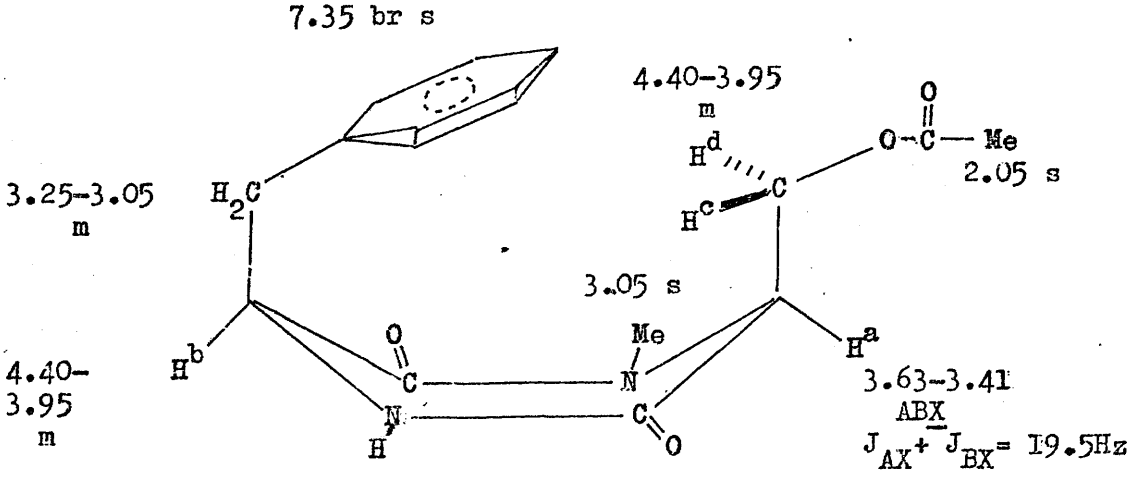


Figure 39 N.m.r. Spectrum of cyclo-(L-Phenylalanyl-O-acetyl-N-methyl-L-seryl) in CD_3OD (δ values).

6.4 Synthesis of cyclo-(L-[U-^{14}C]Phe-L-Ser(NMe)).

The discovery that the diastereomers (29) and (141) had similar R_f values on t.l.c. presented a problem in the radiolabelled synthesis. Since recovery of the protected and free cyclic dipeptides from preparative t.l.c. had been poor, it had been hoped that the LD-diastereomer (141) could have been separated from the product by preparative t.l.c. after the final step. Separation of the diastereomer had been achieved to some extent by eluting four times with ethyl acetate-methanol; 10:1 but the separation was still quite small. It was decided, therefore, to carry out a total synthesis on a one mmol scale in order to see whether crystallisation of the final product was sufficient to remove the LD-diastereomer.

6.4.1 Trial Synthesis. Coupling of Me-L-Ser(OBu^t)-OMe (129) with Z-L-Phe-OH (81) was achieved in 81% yield (Figure 28) and hydrogenolysis gave a crude oil which was estimated by n.m.r. spectroscopy to consist of a 9:1 ratio of LL : LD diastereomers. The crude product took longer to dissolve in 45% HBr/HOAc than the pure compound but the rest of the deprotection procedure was carried out as normal to give the recrystallised product in 24% overall yield from Z-L-Phe-OH (81). There was no evidence of the LD-diastereomer from t.l.c. and it was concluded, therefore, that one crystallisation had been effective in removing the impurity.

6.4.2 Radiolabelled Synthesis. The radiolabelled synthesis was carried out as above. L-[U-^{14}C]Phenylalanine was converted into Z-L-[U-^{14}C]Phe-OH in 97% yield with 87% of the total radioactivity retained. The product was coupled with Me-L-Ser (OBu^t)-OMe (129), then hydrogenclysis and deprotection gave cyclo-(L-[U-^{14}C]Phe-L-Ser(NMe)) in 16% overall yield with a specific activity 98.8% of the expected value. Only one radioactive band was observed on t.l.c., carried out in three different solvent systems. Multiple elution revealed the absence of the LD-diastereomer (141) as did dilution analysis, carried out with (29) as diluent. Dilution analysis revealed a radiochemical purity of 98.2%.

6.5 Feeding of cyclo-(L-[U-^{14}C]Phenylalanyl-N-methyl-L-seryl) to Trichoderma Viride.

cyclo-(L-[U-^{14}C]Phe-L-Ser(NMe)) was fed, in dimethyl sulphoxide, in parallel with cyclo-(L-[U-^{14}C]Phe-L-Ser) at a level of 11 mg l^{-1}, 2μCi l^{-1}, to a one day old culture of T.viride. The culture was grown a further four days and worked up as described in the Experimental Section.

A significant quantity of radioactivity was incorporated into the crude chloroform extracts from both precursors but it was shown by radioscanning and autoradiography of t.l.c. plates of the crude extracts that, whereas most of the radioactivity incorporated from feeding cyclo-(L-Phe-L-Ser) resided in gliotoxin,

Precursor	^{14}C Incorporation (%) into		^{14}C S.A.[a] of
	Chloroform Extract	Triturated Extract	Pure Gliotoxin
cyclo-(L-[U-^{14}C]Phe-L-Ser)	84	55	2.91
cyclo-(L-[U-^{14}C]Phe-L-Ser(NMe))	22	3.7	1.8×10^{-3}

a S.A. = Specific activity (μCi mmol^{-1}).

Table 7 Feeding of cyclo-(L-[U-^{14}C]Phe-L-Ser(NMe)).

the vast majority of the radioactivity present from feeding cyclo-(L-Phe-L-Ser(NMe)) was in recovered precursor. Autoradiography showed that a very small amount of radioactivity, too small to be detected by radioscanning, had been incorporated into gliotoxin and other metabolites.

After trituration of the residue from the chloroform extract with methanol, the radioactive incorporation dropped dramatically to 3.7% due to the loss of most of the N-methylated precursor. Similar treatment carried out on the extract from the reference feeding resulted in a drop of radioactive incorporation to 55% (Table 7). Furthermore, after repeated crystallisations, the specific activity of gliotoxin isolated from the N-methylated precursor feeding dropped sharply to level off at a very low value of 0.002 μCi mmol^{-1}. In contrast, the specific activity of gliotoxin from the reference feeding rose slightly to a constant value of 2.91 μCi mmol^{-1}. The ratio of specific activities was ca. 1500. Thus, the N-methylated precursor had been incorporated into gliotoxin with less than 0.1% the efficiency of cyclo-(L-Phe-L-Ser).

These results, however, cannot be taken to prove that the N-methylated precursor is not a biosynthetic intermediate since other factors such as cell wall permeability or presence of the N-methylated cyclic dipeptide as an enzyme-bound intermediate may have prevented incorporation.

CHAPTER 7 HYALODENDRIN AND BISDETHIODI(METHYLTHIO)HYALODENDRIN.

Introduction.

Hyalodendrin[106] (30) and the related fungal metabolite[107] (142) are closely similar to gliotoxin. They appear to be derived from phenylalanine and serine and both nitrogen atoms of the epidithiodioxopiperazine ring are methylated. Ring closure to the dihydroaromatic system has not occurred but it is not known whether this is due to the lack of the necessary enzyme required for this step or whether both nitrogen atoms are methylated before ring closure has a chance to occur.

It seemed likely that cyclo-(Phe-Ser) would prove to be an intermediate for these metabolites and it was decided to study hyalodendrin specifically, since the disulphide bridge was of the opposite stereochemistry to that of gliotoxin. There existed, therefore the possibility that cyclo-(D-Phe-D-Ser) was the intermediate rather than cyclo-(L-Phe-L-Ser).

7.1 Bisdethiodi(methylthio)hyalodendrin (143).

The conditions of growth used by Strunz et al.[106] were followed but it was not possible, with the facilities then available, to grow the culture at the required 20-21°C, the lowest possible temperature being 25°C. At this temperature bisdethiodi(methylthio)-

hyalodendrin[108] (143) was produced in greater quantities than hyalodendrin. This compound (143) is, most likely, a degradation product of hyalodendrin, analogous to bisdethiodi(methylthio)-gliotoxin (144) which has been shown[109] to be derived from gliotoxin. The fact that (143) also crystallised much more easily than hyalodendrin made it a more attractive compound to study. Chemical correlation with hyalodendrin had been achieved by Strunz et al.[108] by treating hyalodendrin with sodium borohydride in the presence of methyl iodide and pyridine at 0°C to produce (144). Therefore, any results obtained from studying (144) should also hold true for hyalodendrin.

7.2 Feeding of cyclo-(Phenylalanyl-seryl) Diastereomers.

Growth conditions for the Hyalodendron species and the work up procedure are described in the Experimental Section. The precursors listed in Table 8, dissolved in dimethylsulphoxide, were fed at a level of 8-10 mg l^{-1}, ^{14}C activity 2.8-3.0 μCi l^{-1} of medium. The results are summarised in Table 8.

7.2.1 Feeding of cyclo-(L-[U-^{14}C]Phe-L-Ser). On feeding the radiolabelled LL-cyclic dipeptide (24), 20% of the radioactivity fed was incorporated into the chloroform extract. Radioscanning and autoradiography of t.l.c. plates run in three different solvent systems showed that the majority of this activity was present in (143), whilst a smaller quantity was present in hyalodendrin.

Exp.No.[a]	Precursor	^{14}C Incorporation (%) into Chloroform Extract	Metabolite[b] (143)	S.A.[c] (143)	S.I.[d]
1	cyclo-(L-[U-^{14}C]Phe-L-Ser)	20	10	3.00	3.6×10^{-2}
2	cyclo-(L-[U-^{14}C]Phe-L-Ser)	63	28	7.95	1.2×10^{-1}
	cyclo-(D-Phe-DL-[3-^{14}C]Ser)	2.2	0.36	0.05	8.0×10^{-4}
3	cyclo-(L-[4'-^{3}H]Phe-L-[3-^{14}C]Ser)	63	42	7.60	8.3×10^{-2}
4	cyclo-(L-[U-^{14}C]Phe-L-Ser)	44	33	3.30	3.8×10^{-2}
	cyclo-(L-[U-^{14}C]Phe-L-Ser(NMe))	12	3.0×10^{-3}	6×10^{-4}	7.1×10^{-6}

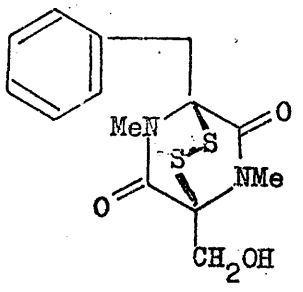
a Incubations bracketed were conducted in parallel.

b The metabolite was bisdethiodi(methylthio)hyalodendrin.

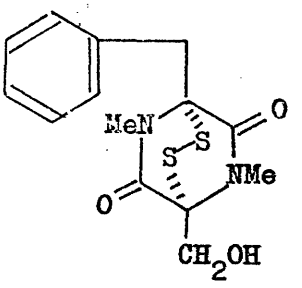
c Specific activity (μCi mmol^{-1}) of purified metabolite (143).

d $\frac{\text{Specific}}{\text{Incorporation}}$ = Specific Activity(Metabolite[b])/ Specific Activity (Precursor)

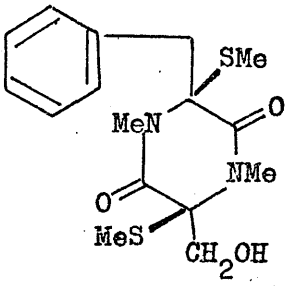
Table 8 Feeding Results of Cyclic Dipeptides to Hyalodendron Species (FSC-601).



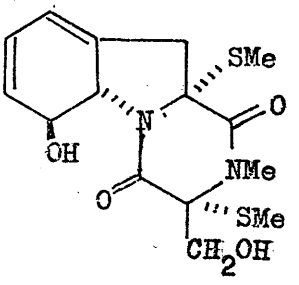
(30)



(142)



(143)



(144)

The extract was purified by preparative t.l.c. and the S-methyl derivative was found to contain 10% of the radioactivity fed. On repeated crystallisation the specific activity of the metabolite soon reached a constant value of 3.0 μCi mmol^{-1} and it was shown by radioscanning and autoradiography of t.l.c. plates run in different solvent systems, that the metabolite was radiochemically pure. The physical properties of (143) were identical to the literature values.

7.2.2 Feeding of cyclo-(D-Phe-DL-[3-^{14}C]Ser). The radiolabelled mixture of DD- and DL-diastereomers were fed in parallel with

cyclo-(L-[U-^{14}C]Phe-L-Ser). The latter was incorporated efficiently whereas the former mixture was poorly incorporated into the S-methyl metabolite. The specific activity of the pure metabolite was 0.6% of that value measured for the reference feeding. Measurement of the radioactivity of the aqueous broth demonstrated that ca. 20% of the radioactivity fed as the diastereomeric mixture had probably been incorporated into the mycelium.

It was concluded that cyclo-(L-Phe-L-Ser) and not cyclo-(D-Phe-D-Ser) or cyclo-(D-Phe-L-Ser) was the biosynthetic intermediate for bisdethiodi(methylthio)hyalodendrin and therefore also for hyalodendrin.

7.2.3 Feeding with Doubly Labelled cyclo-(L-Phe-L-Ser).

cyclo-(L-[4'-^{3}H]Phe-L-[3-^{14}C]Ser) was incorporated extremely well (ca. 42%) into the S-methyl metabolite (143) and the isotope ratio of the crystallised metabolite (11.1) was essentially unchanged from that of the precursor (10.9). This result, as explained in Chapter 4, demonstrates that the precursor was not degraded into its constituent amino-acids which might then have been incorporated into the metabolite.

An attempt was made to isolate hyalodendrin itself by feeding the precursor to a five day old culture and working up two days later. The chloroform extract contained 28% of the radioactivity fed with the isotopic ratio (11.2) relatively unchanged from that of the precursor (10.9). Radioscanning showed that the activity was almost evenly divided between hyalodendrin and the dimethylthio metabolite (143). Preparative t.l.c. gave a small

quantity of both compounds as solids. Hyalodendrin had a ^{14}C activity of 16.3 μCi mmol^{-1} and a ^{3}H:^{14}C ratio of 11.2 while the corresponding figures for bisdethiodi(methylthio)hyalodendrin were 16.1 μCi mmol^{-1} and 10.6. In both cases the isotope ratio was essentially unchanged and the ^{14}C activities were virtually identical, as expected. Unfortunately, there was insufficient metabolite for further crystallisations to constant activity.

7.3 Feeding of cyclo-(L-[U-^{14}C]Phenylalanyl-N-methyl-L-seryl).

cyclo-(L-[U-^{14}C]Phe-L-Ser(NMe)) was fed in parallel with labelled cyclo-(L-Phe-L-Ser) on the same scale described above. Incorporation of the former precursor into the dimethylthio metabolite proved to be extremely small, and the radioactivity measured in the chloroform extract was due to unchanged precursor. Only 0.003% of the activity fed was present in bisdethiodi(methylthio)-hyalodendrin after preparative t.l.c. From the radioactivity of the aqueous broth after chloroform extraction it was concluded that ca. 16% of the radioactivity fed was probably incorporated into the mycelium.

7.4 Biosynthetic Studies with ^{13}C.

Degradative studies require to be carried out on doubly labelled bisdethiodi(methylthio)hyalodendrin, derived from cyclo-(L-[4'-^{3}H]Phe-L-[3-^{14}C]Ser), to prove that the labels are in the expected positions. As an alternative to chemical

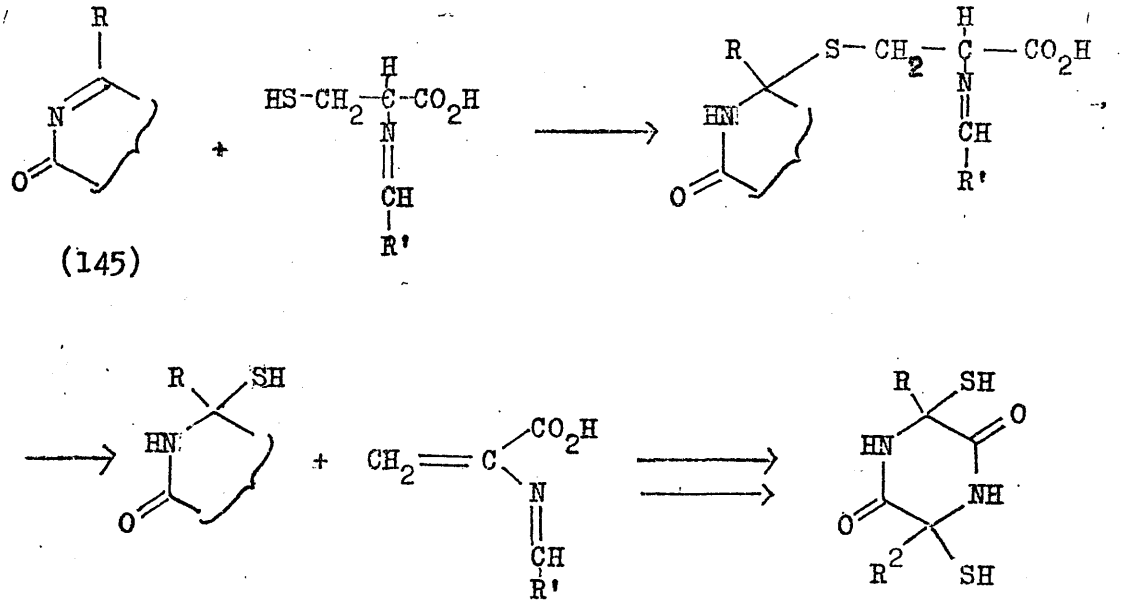
degradation it was proposed to synthesise and feed cyclo-(L-Phe-DL-[3-^{13}C]Ser). The dimethylthio metabolite isolated from such a feeding would then be studied by ^{13}C n.m.r. spectroscopy which should demonstrate whether the label was in the expected position.

cyclo-(L-Phe-DL-[3-^{13}C]Ser) was synthesised from DL-[3-^{13}C] serine by the method described in Chapter 4. However, t.l.c. showed two compounds in the final product and it was concluded that the impurity was cyclo-(glycyl-L-phenylalanyl) on the basis of ^{1}H n.m.r.spectroscopy and t.l.c.. A sample of DL-[3-^{13}C]serine was checked for purity by ^{1}H n.m.r. spectroscopy and found to contain glycine. Several unexplained peaks were also present in the n.m.r. spectrum. Due to the difficulties involved in purifying the final mixture of products this research was terminated.

7.5 Conclusion.

Since cyclo-(L-Phe-L-Ser) appears to be the biosynthetic intermediate for bisdethiodi(methylthio)hyalodendrin rather than cyclo-(D-Phe-D-Ser), inversion of the chiral centres of the cyclic dipeptide must occur during biosynthesis. This is contrary to the situation in gliotoxin biosynthesis where the chirality of the LL-cyclic dipeptide is retained in gliotoxin.

Inversion would most likely take place during the incorporation of the disulphide bridge. A reaction scheme (Figure 40) for the incorporation of sulphur was suggested by Kirby and Robins[28]



(145)

Figure 40 Postulated Reaction Scheme[28]

in their discussion concerning gliotoxin biosynthesis. Structure (145) might be common to both gliotoxin and hyalodendrin biosynthesis. The opposing stereochemistry of the disulphide bridge in these metabolites would then arise from the sulphur source attacking opposite sides of the structure (145).

CHAPTER 8 EXPERIMENTAL

8.1 General Notes

Melting points are corrected and were measured with a Kofler, hot stage apparatus. Infra red spectra were recorded for KBr discs, unless otherwise stated, with Perkin-Elmer 197, 257 or 580 Infra red Spectrophotometers or a Perkin-Elmer 225 Grating Infra red Spectrophotometer. Nuclear Magnetic Resonance Spectra were recorded for $CDCl_3$ solutions, unless otherwise stated, with Varian T-60, Perkin-Elmer R32, or Varian XL-100 spectrometers. Tetramethylsilane was used as internal standard. Mass spectra were obtained with an AEI MS 12 spectrometer. Analytical t.l.c. separations were carried out on precoated, silica GF_{254} plates and preparative separations on GF_{254} (type 60) plates. Open chain dipeptides were detected with a ninhydrin spray. Cyclic dipeptides were detected by the following procedure. The plate was sprayed with bleach then allowed to stand for thirty minutes before spraying with absolute ethanol. After ten minutes, the plate was sprayed with a 1:1 mixture of 1% potassium iodide solution and a 2% solution of o-toluidine in 10% acetic acid in water.[117] All concentrations were carried out by rotary evaporation under reduced pressure. Organic solutions were dried over magnesium sulphate or sodium sulphate and solid samples were dried over phosphorus pentoxide in vacuo.

The following t.l.c. systems are referred to in the text and are quoted as superscripts (R_f^A): A) n-butanol-acetic acid-

water; 4:1:1 B) n-butanol-acetic acid-pyridine-water; 15:10:3:2 C) methanol-water; 7:3 D) toluene-acetone; 2:1 E) ethyl acetate-chloroform-diethylamine; 1:1:0.1 F) ethyl acetate-petrol ether (40-60°C)-acetic acid; 1:1:0.1 G) di-isopropyl ether-chloroform-acetic acid; 6:3:1 H) chloroform-acetic acid; 3:1 I) methanol-ethyl acetate; 1:1 J) toluene-acetone; 5:2 K) ethyl acetate-methanol; 10:1 L) chloroform-methanol; 15:1 M) chloroform-methanol; 15:2 N) ethyl acetate-methanol; 6:1 O) toluene-acetone; 94:6.

The following abbreviations are used in the text: pet. ether (petrol ether 40-60°C); br (broad); δ (delta value in p.p.m. for n.m.r.); exch. (exchanged).

8.2 Radiochemical Methods.

^{14}C and ^{3}H activities were measured with a Philips liquid scintillation analyser using toluene-methanol solutions. Open chain dipeptides were measured using methanol-triton X100-toluene (0.06:1.0:2.0) 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 was used for autoradiography.

8.3 Fermentation Conditions

'Trichoderma viride' (Gliocladium deliquescens, strain no.

1828 NRRL) was maintained on potato dextrose agar and grown in shake-culture at pH 3.0-3.5 in a defined medium[4] at 24°C. Precursors were added to 1 day old cultures 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 (x6) with chloroform (10% by volume). The extracts were dried and evaporated. Gliotoxin (ca. 100mg l^{-1} of medium) was crystallised from methanol.

Hyalodendron sp. (FSC-601) was grown as a shake culture at 25°C as described by Stillwell et al.[106] Radiolabelled precursors (ca. 8-10 mg l^{-1}, ^{14}C activity 2.8-3.0 μCi l^{-1}) dissolved in dimethylsulphoxide (1ml) were fed to 5 day old cultures and growth continued for a further 9 days. Mycelia were filtered and killed with methanol. The aqueous filtrate was extracted (x6) with chloroform (10% by volume) and the organic extracts were dried and evaporated. The residue was taken up in chloroform and washed twice with water then dried, filtered and concentrated to a solid. Bisdethiodi(methylthio)hyalodendrin was purified by preparative t.l.c. (toluene-acetone; 94:6) and crystallised from methylene chloride-cyclohexane to give needles, usually 50 mg l^{-1} of medium, m.p. 139-140°C (lit.,[108] 140-140.5°C), $[\alpha]_D^{24}$ + 63.5° (c, 2.5, $CHCl_3$) (lit.,[108] + 64°). Mass, n.m.r. and i.r. spectra were in agreement with the literature.[108]

8.4 Experimental for Chapter 3.

L-Phenylalanine Benzyl Ester Hydrochloride (66) - Thionyl chloride (20 ml, 278 mmol) was added dropwise over 20 min to a stirred suspension of L-phenylalanine (3.3 g, 20 mmol) in benzyl alcohol (125 ml) at 5 °C. After heating on a steam bath for 5 h, the solution was dried and filtered before dry ether was added to turbidity. On cooling overnight a solid crystallised which was filtered and dried, (5.22 g, 89.5%), m.p. 188-191 °C. Recrystallisation from ethanol gave (66) (3.07 g) m.p. 195-196.5 °C (lit.,[70] 202 °C); $\nu_{max.}$ 3 300 - 2 500, 1 754, 1 490, 1 230, 750 - 740, and 697 cm^{-1}; δ [$(CD_3)_2SO$] (60 MHz) 9.30 - 8.55 (3H, m, $\overset{+}{N}H_3$), 7.35 (5H, s, Ph), 7.25 (5H, s, Ph), 5.15 (2H, s, OCH_2Ph), 4.50 - 4.10 (1H, m, $CHCH_2$), and 3.40 - 3.00 (2H, m, $CHCH_2$). Recrystallisation of the mother liquor yielded more (66) (1.08 g), m.p. 191-192 °C.

N-Benzyloxycarbonyl-L-seryl-L-phenylalanine Benzyl Ester (68) - L-Phenylalanine benzyl ester hydrochloride (66) (2.91 g, 10 mmol) was dissolved in methylene chloride (50 ml) at 0 °C by the addition of triethylamine (1.8 ml, 12.5 mmol). N-Benzyloxycarbonyl-L-serine (67) (2.39 g, 10 mmol) was added followed by DCC (2.38 g, 11 mmol) and the mixture allowed to stand in the dark for 5 h. DCU was removed by filtration through a Celite pad and washed with methylene chloride. The filtrate was washed with 1M hydrochloric acid, water, saturated sodium hydrogen carbonate solution and water, then dried, filtered and concentrated to an oil.

The residue was dissolved in a small volume of acetone and the precipitate removed by filtration. The filtrate was concentrated to give (68) (3.384 g, 71.1%). Crystallisation from ethyl acetate - petroleum ether (3:1) and further washing with ether gave needles (2.36 g, 50%), m.p. 128 - 130 °C (lit.,[69] 129-130 °C), $[\alpha]_D^{25}$ - 7.31° (c 2.34, Me_2NCHO) (lit.,[69] - 8.2°), $[\alpha]_D^{19}$ - 13.9° (c 1.8, $CHCl_3$), R_F^F 0.47; $\nu_{max.}$($CHCl_3$) 1 750 - 1 700, 1 680, and 1 500 cm^{-1}; δ 7.42 - 6.88, 7.30 (15H, m, 3 x Ph), 6.90 - 6.80 (1H, m, D_2O exch., NH), 5.80 - 5.60 (1H, br d, J 7 Hz, D_2O exch., NH), 5.14 (2H, br s, $OC\underline{H}_2Ph$), 5.07 (2H, s, $OC\underline{H}_2Ph$), 4.99 - 4.68 (1H, m, alters on D_2O shake to br t, J 6 Hz, $C\underline{H}CH_2Ph$), 4.35 - 4.10 (1H, m, sharpens on D_2O shake to AB$\underline{X}$, $J_{AX} + J_{BX}$ 9.5 Hz, $C\underline{H}CH_2OH$), 4.11 - 3.47 (2H, $\underline{ABX}$, J_{AB} 11.5 Hz, J_{AX} 4.0 Hz, J_{BX} 6.0 Hz, sharpens on D_2O shake, $C\underline{H}_2OH$), 3.28 - 2.87 (2H, $\underline{ABX}$, J_{AB} 14 Hz, $J_{AX} = J_{BX}$ 6 Hz, $C\underline{H}_2Ph$), 2.70 - 1.00 (1H, br m, D_2O exch., OH); m/e 476 (weak, M), 120 (15%, $PhCH_2CH{=}NH_2$), 108 (26, $PhCH_2OH$), 107 (17, $PhCH_2O$), 91 (100), 79 (38), 77 (25), 65 (16).

N-Benzyloxycarbonyl-L-serine (67) - N-Benzyloxycarbonyl-L-serine (67) was synthesised from L-serine by the method of Guttman and Boissonas as crystals (86%), m.p. 116-118 °C (lit.,[71] 119.5 °C), $[\alpha]_D^{20}$ + 5.2° (c, 1.04, AcOH) (lit.,[71] + 5.6°), R_F 0.13 (ethyl acetate - pet. ether - acetic acid; 20:20:1); $\nu_{max.}$(Nujol) 3 550 - 2 400, 3 340 - 3 320, 1 690, 1 520, 745 - 725, and 695 cm^{-1};

δ [$(CD_3)_2SO$] (60 MHz) 7.45 (5H, s, Ph), 5.10 (2H, s, $OC\underline{H}_2Ph$), 4.30 - 3.95 (1H, m, $C\underline{H}CH_2$), 3.90 - 3.60 (2H, m, $CHC\underline{H}_2$).

L-Seryl-L-phenylalanine (65) - Water (16 ml), followed by acetic acid (0.4 ml), was added dropwise at room temperature to a stirred solution of N-benzyloxycarbonyl-L-seryl-L-phenylalanine benzyl ester (68) (269.5 mg, 0.57 mmol) in ethanol (32 ml). The solution was hydrogenated for 3 h over 10% palladium on charcoal catalyst (30 mg). The catalyst was removed by filtering through a Celite pad which was then washed with water (3 x). The combined filtrates were concentrated to yield a solid (149 mg) which was triturated with chloroform and ether to give (65) (125.4 mg, 88%), m.p. 218-225 °C (lit.,[69] 226-227 °C), $[\alpha]_D^{20}$ + 51.04° (c 2.4, AcOH) (lit.,[69] + 53.8°), R_F^A 0.40, R_F^B 0.56, R_F^C 0.78; (Found: C, 57.00; H, 6.66; N, 10.90. $C_{12}H_{16}N_2O_4$ requires C, 57.14; H, 6.35; N, 11.11%); $\nu_{max.}$ 3 600 - 2 200, 3 200, 3 070, 1 680, 1 570, 1 500, 1 405, 1 075, 745, and 715 - 700 cm^{-1}; δ (CF_3COOH) 7.82 (1H, br d, J 7.5 Hz, D_2O exch., amide NH), 7.53 (3H, br s, D_2O exch., $\overset{+}{N}H_3$), 7.50 - 7.10 (5H, m, Ph), 5.30 - 4.90 (1H, m, $C\underline{H}CH_2Ph$), 4.82 - 4.45 (1H, m, $C\underline{H}CH_2OH$), 4.60 - 4.12 (2H, m, sharpens on D_2O exchange, $C\underline{H}_2OH$), 3.60 - 3.00 (2H, ABX, J_{AX} 6 Hz, J_{BX} 8 Hz, J_{AB} 15 Hz, $C\underline{H}_2Ph$); m/e 252 (weak, M), 222 (5%, $M-CH_2O$), 120 (13, $PhCH_2CH{=}NH_2$), 91 (30), 77 (6), 65 (7), and 60 (100, $H_2N{=}CHCH_2OH$).

L-[4′-^{3}H]Phenylalanine Benzyl Ester Hydrochloride - An aqueous solution of L-[4′-^{3}H]phenylalanine (500μCi) was added to L-phenylalanine (165.5 mg, 1 mmol). The resultant solution was concentrated slowly and dried in vacuo overnight. The solid was suspended in benzyl alcohol (7 ml), cooled to 5 °C and thionyl chloride (1 ml) was added over 20 min. The reaction mixture was maintained at 95 °C for 5 h. Subsequent work-up was as described for (66) yielding crystals (264 mg, 91%), ^{3}H activity 448.2 μCi mmol^{-1}.

N-Benzyloxycarbonyl-L-[3-^{14}C]serine - L-[3-^{14}C]Serine (50 μCi) was diluted with L-serine (105 mg, 1 mmol) and converted as described earlier to Z-L-[3-^{14}C]Ser, 196 mg,(82%), ^{14}C activity 50.1 μCi mmol^{-1}.

N-Benzyloxycarbonyl-L-[3-^{14}C]seryl-L-[4′-^{3}H]phenylalanine Benzyl Ester - N-Benzyloxycarbonyl-L-[3-^{14}C]serine (239 mg, 1 mmol, 41.1 μCi mmol^{-1}) was coupled with L-[4′-^{3}H]phenylalanine benzyl ester hydrochloride (291.5 mg, 1 mmol, 405.9 μCi mmol^{-1}) in the presence of DCC (228 mg, 1.1 mmol) and triethylamine (0.18 ml) as described above to give, after crystallisation and washing, a white solid (268.3 mg, 56.6%), ^{14}C activity 39.3 μCi mmol^{-1}, ^{3}H:^{14}C 12.1, showing one radioactive band on tlc, R_F 0.27 (EtOAc - $CHCl_3$ - Et_2NH; 7.5:7.5:0.6) by Panax scanning and autoradiography.

L-[3-^{14}C]Seryl-L-[4'-^{3}H]phenylalanine - N-Benzyloxycarbonyl-L-[3-^{14}C]seryl-L-[4'-^{3}H]phenylalanine benzyl ester (268.3 mg, 0.56 mmol, 39.3 μCi mmol^{-1}) was hydrogenated and worked up as described earlier to yield a solid, (121 mg, 85.7%), ^{14}C activity 38.72 μCi mmol^{-1}, ^{3}H:^{14}C 12.3; R_F^A 0.40, R_F^B 0.56, R_F^C 0.78, R_F^H 0.00. A single radioactive product was observed by Panax scanning and autoradiography of tlc plates. Dilution analysis with H-L-Ser-L-Phe-OH (65), H-L-Ser-D-Phe-OH (73) and H-D-Ser-L-Phe-OH (74) showed that the radiochemical purity was not less than 96% and that not more than 0.2% H-L-Ser-D-Phe-OH (73) or H-D-Ser-L-Phe-OH (74) was present.

N-Benzyloxycarbonyl-D-serine (77) - N-Benzyloxycarbonyl-D-serine (77) was synthesised from D-serine (78) in 90% yield following the same procedure described for the L-enatiomer. Physical properties were identical to Z-L-Ser-OH except for optical rotation, $[\alpha]_D^{20}$ - 4.3° (c 1.0° AcOH) (lit.,[74] - 5.6°)

D-Phenylalanine Benzyl Ester Hydrochloride (79) - D-Phenylalanine benzyl ester hydrochloride was synthesised in a similar fashion to the L-enantiomer (66) yielding light silvery crystals (95%) which were recrystallised from ethanol (47%) m.p. 191 - 194 °C.

N-Benzyloxycarbonyl-L-seryl-D-phenylalanine Benzyl Ester (76) - The protected dipeptide (76) was synthesised on an 18

mmol scale in the same manner described for the enantiomer (75) to give, after recrystallisation, a solid (4.17 g, 48.7%), having identical physical properties to its enantiomer with the exception of optical rotation, $[\alpha]_D^{20}$ - 2.82° (c, 4.6, $CHCl_3$).

N-Benzyloxycarbonyl-D-seryl-L-phenylalanine Benzyl Ester (75) - N-Benzyloxycarbonyl-D-serine (77) (4.302 g, 18 mmol) was coupled with L-phenylalanine benzyl ester hydrochloride (66) (5.247 g, 18 mmol) by the DCC procedure described above to give the protected dipeptide (75) (4.663 g, 54.4%), m.p. 116-119 °C, R_F^E 0.44, $[\alpha]_D^{19}$ + 1.9° (c, 4.8, $CHCl_3$); (Found: C, 67.82; H, 5.80; N, 5.88. $C_{27}H_{28}N_2O_6$ requires C, 68.07; H, 5.88; N, 5.88%); $\nu_{max.}$ 3 600 - 3 150, 1 740, 1 695, 1 655, 1 550 - 1 530, 1 020, 760 - 730, and 700 cm^{-1}; δ 7.50 - 6.70, 7.30 (16H, m, 3 x Ph and NH), 5.90 - 5.60 (1H, br d, J 8 Hz, D_2O exch., NH), 5.13 (2H, d, J 2 Hz, $OC\underline{H}_2Ph$), 5.08 (2H, s, $OC\underline{H}_2Ph$), 4.97 - 4.75 (1H, m, sharpens on D_2O shake, $C\underline{H}CH_2Ph$), 4.33 - 4.08 (1H, m, sharpens on D_2O shake, $C\underline{H}CH_2OH$), 4.05 - 3.48 (2H, ABX, sharpens on D_2O shake, J_{AX} 5.5 Hz, J_{BX} 3.4 Hz, J_{AB} 12 Hz, $C\underline{H}_2OH$), 3.07 (2H, br d, $C\underline{H}_2Ph$), and 2.75 - 2.25 (1H, br m, D_2O exch., OH); m/e 476 (weak, M), 120 (16%, $PhCH_2CH{=}NH_2$), 108 (55), 107 (43), 92 (22), 91 (100), 79 (77), 77 (55), 65 (25).

L-Seryl-D-phenylalanine (73) - N-Benzyloxycarbonyl-L-seryl-D-phenylalanine benzyl ester (76) (2.8 g, 5.88 mmol) was

hydrogenated for 3 h and worked up as described earlier for the LL dipeptide (65) to giwe a slightly yellow solid, (1.068 g, 72%). Crystallisation from methanol - water (2:1) gave the dipeptide (73) as prisms, (652 mg, 44%), m.p. 249 - 252 °C, $[\alpha]_D^{20}$ - 29.34° (c 2.9, AcOH), R_F^A 0.40, R_F^B 0.56, R_F^C 0.78, R_F^H 0.00 (Found: C, 57.01; H, 6.58; N, 10.7. $C_{12}H_{10}N_2O_4$ requires C, 57.14; H, 6.35; N, 11.1%); $\nu_{max.}$ 3 500 - 2 250, 3 330, 3 090, 2 130, 1 685, 1 570 - 1 500, 1 410, 1 050, 760 and 710 cm^{-1}; δ (CF_3COOH) 7.84 (1H, br d, NH), 7.32 (m, $\overset{+}{N}H_3$, Ph), 5.40 - 5.02 (1H, m, sharpens on D_2O shake to ABX, J_{AX} + J_{BX} 14 Hz, C<u>H</u>CH_2Ph), 4.77 - 4.40 (1H, m, C<u>H</u>CH_2OH), 4.30 - 3.72 (2H, ABX, J_{AB} 12.5 Hz, J_{AX} 4.0 Hz, J_{BX} 6.5 Hz, sharpens on D_2O shake, C<u>H</u>$_2$OH), 3.70 - 2.92 (2H, ABX, J_{AB} 14.5 Hz, J_{AX} 9.0 Hz, J_{BX} 5.0 Hz, sharpens on D_2O shake, C<u>H</u>$_2$Ph); m/e 252 (weak, M), 222 (5%, M-CH_2O), 120 (13), 91 (30), 77 (6), 65 (7), 60 (100).

D-Seryl-L-phenylalanine (74) - D-Seryl-L-phenylalanine was synthesised as above on a 2.12 mmol scale to give a triturated yield of 75% and a recrystallised yield of 61%, m.p. 251-253 °C, $[\alpha]_D^{20}$ + 25.8° (c, 2.2, AcOH) having identical spectroscopic properties to its enantiomer (73).

Benzyloxycarbonyl-L-phenylalanine (81) - A solution of L-phenylalanine (8.275 g, 50 mmol) in freshly prepared 2M sodium hydroxide (32.5 ml, 65 mmol) was cooled to 0 °C and stirred

vigorously. Benzyloxycarbonyl chloride (90%; 7.5 ml) and 4M sodium hydroxide solution (12.5 ml, 50 mmol) were added dropwise over a period of 30 min and stirring continued for a further 10 min. Excess benzyloxycarbonyl chloride was removed by ether extraction (3 x). The aqueous solution was cooled and acidified to pH 1 with concentrated hydrochloric acid. A white solid was precipitated which was filtered, washed and dried (14.2 g, 95%). Recrystallisation from methylene chloride - pet. ether gave (81) as needles (13.4 g, 90%) m.p. 85-87 °C (lit.,[78] 87-89 °C), $[\alpha]_D^{21}$ + 6.23° (c 2.6, EtOH) (lit.,[78] + 5.1°); $\nu_{max.}$ 3 460 - 2 400, 3 317, 1 705, 1 695, 1 530, 1 270, 740, and 700 cm^{-1}; δ 9.55 - 9.10 (1H, m, D_2O exch., COOH), 7.40 - 7.00 (5H, m, $CHCH_2\underline{Ph}$), 7.28 (5H, s, $OCH_2\underline{Ph}$), 5.35 - 5.10 (1H, m, D_2O exch., NH), 5.06 (2H, s, $OC\underline{H}_2Ph$), 4.80 - 4.50 (1H, m, $C\underline{H}CH_2$), 3.20 - 2.90 (2H, m, $C\underline{H}_2Ph$).

Benzyloxycarbonyl-D-phenylalanine was similarly prepared in 90% yield.

L-Serine Methyl Ester Hydrochloride (83) - L-Serine (5.25 g, 50 mmol) was converted by the method of Brenner and Huber[79] to the methyl ester hydrochloride (83), (7.05 g, 91%) m.p. 161-165 °C (lit.,[80] 165-166 °C); $\nu_{max.}$ (Nujol) 3 350, 1 740, 1 590, 1 505, 1 250, 1 040, cm^{-1}; δ (CF_3COOH) (60 MHz) 7.70 (3H, m, $\overset{+}{N}H_3$), 4.70 - 4.50 (4H, m, $C\underline{H}C\underline{H}_2$), and 4.00 (3H, s, OCH_3).

Benzyloxycarbonyl-L-phenylalanyl-L-serine Methyl Ester (82) - Benzyloxycarbonyl-L-phenylalanyl-L-serine methyl ester (82) was synthesised as described by Nicolaides[81] in 70% yield, m.p. 120-122 °C (lit.,[81] 122-123 °C) (Found: C, 63.28; H, 6.20; N, 6.88. $C_{21}H_{24}N_2O_6$ requires C, 63.00; H, 6.00; N, 7.00%); $\nu_{max.}$ 3 600 - 3 150, 3 310, 1 745, 1 700, 1 660, 1 540, 1 060, 750 and 700 cm^{-1}; δ 7.50 - 7.00 (1H, m, NH), 7.22 (5H, s, Ph), 7.17 (5H, s, Ph), 5.86 (1H, br d, J 8.0 Hz, NH), 5.13 - 4.82 (2H, AB, J_{AB} 12.5 - 14.5 Hz, $OC\underline{H}_2Ph$), 4.75 - 4.40 (2H, m, 2 x CH), 3.95 - 3.75 (2H, m, $C\underline{H}_2OH$) 3.67 (3H, s, OCH_3), 2.50 - 2.25 (1H, br s, D_2O exch., OH), 3.30 - 2.80 (2H, ABX, J_{AX} 6.0 Hz, J_{BX} 8.0 Hz, J_{AB} 14 Hz, $C\underline{H}_2Ph$); m/e 400 (weak, M), 120 (10%, $PhCH_2CH{=}NH_2$), 92 (16), 91 (100), 79,(10), 77 (12), and 65 (16).

Benzyloxycarbonyl-L-phenylalanyl-L-serine (80) by Saponification - Benzyloxycarbonyl-L-phenylalanyl-L-serine methyl ester (82) (1 g, 2.5 mmol) was shaken with 2M sodium hydroxide (2.52 ml, 5.0 mmol) in methanol (20 ml) for 15 min then acidified with acetic acid. The solvent was removed by evaporation and the residue was dissolved in sodium hydrogen carbonate solution and washed with ether to remove starting material. The aqueous solution was cooled, acidified with concentrated hydrochloric acid and extracted with methylene chloride. The combined organic extracts were dried, filtered and concentrated to give (80) (882 mg, 91.4%). Crystallisation from ethyl acetate - ether gave a stone coloured solid (80) (88 mg, 9%), m.p. 147-151.5 °C

(lit.,[75] 147-149 °C and[76] 155-156 °C), $[\alpha]_D^{22}$ + 0.2° (c 2.1, Me_2NCHO) (lit.,[75] + 44.8° and[76] - 2,6°); $\nu_{max.}$ 3 700 - 2 400, 1 710, 1 665, 1 535, 1 055, 745 and 700 cm^{-1}; δ[$(CD_3)_2CO$] (60 MHz) 7.30 (10 H, s, Ar), 6.70 - 6.20 (1H, m, D_2O exch., NH), 5.50 - 4.80 (3H, D_2O exch., COOH,OH,NH), 5.00 (2H, s, OCH_2Ph), 4.90 - 4.40 (2H, m,2CH), 4.20 - 3.60 (2H, m, CH_2OH), 3.15 - 2.85 (2H, m, CH_2Ph); m/e 386 (weak, M), 148, 120 ($PhCH_2CH{=}NH_2$), 92, 91 (100%), 79, 77, and 65.

Recrystallisation of the mother liquor yielded a further crop (239 mg, 24.4%), m.p. 142-148 °C, $[\alpha]_D^{22}$ -1.1° (c 2.3, Me_2NCHO). The mother liquor was concentrated to a sticky solid (555 mg), m.p. 59-61 °C, $[\alpha]_D^{22}$ - 15.8° (c 2.0, Me_2NCHO).

Benzyloxycarbonyl-L-phenylalanine p-Nitrophenyl Ester (85) - Benzyloxycarbonyl-L-phenylalanine-p-nitrophenyl ester (85) was synthesised as described by Bodanzky and Vigneaud[77] in 71 % yield, m.p. 124.5-126.5 °C (lit.,[77] 126-126.5 °C), $\nu_{max.}$ 3 360, 1 760, 1 697, 1 617, 1 595, 1 528, 1 490, 1 350, and 700 cm^{-1}; δ (60 MHz) 8.30, 8.15, 7.80 - 6.90 (14H, m, Ar), 5.50 - 5.10 (1H, m, NH), 5.15 (2H, s, OCH_2Ph), 5.00 - 4.70 (1H, m, $CHCH_2Ph$), 3.15 (2H, br d, J 6.5 Hz, CH_2Ph).

Benzyloxycarbonyl-L-phenylalanyl-L-serine (80) - Benzyloxycarbonyl-L-phenylananine p-nitrophenyl ester (85)(1.575 g, 3.75 mmol) was coupled with L-serine (392.5 mg, 3.74 mmol) as described by

Bodanzky et al.[76], reacting for 40 h rather than 16 h. An off white solid was obtained, (610 mg, 42%), m.p. 150 -154 °C (lit.,[76] 155-156 °C, 154 °C sint.), $[\alpha]_D^{20}$ - 3.3° (c 2.5, Me_2NCHO) (lit.,[76] - 2.6°).

L-Serine Benzyl Ester p-Toluenesulphonate (87) - L-Serine (165 mg, 1 mmol) and p-toluenesulphonic acid (209 mg, 1.1 mmol) were heated at 100 °C with stirring in benzyl alcohol (4 ml) until dissolved. Dry carbon tetrachloride (7 ml) was added dropwise and the reaction apparatus set up for reflux through a Soxhlet apparatus containing molecular sieves. A slight cloudiness developed which disappeared after a few minutes boiling. The solution was refluxed for 6 h during which more CCl_4 (5 ml) was added, after cooling dry ether was added. Cooling for 16 h gave an oil (289 mg, 79%); δ (CD_3OD) (60 MHz) 7.75, 7.60, 7.25, 7.10 (Aromatic), 7.32 (s, Ph), 5.20 (s, $C\underline{H}_2Ph$), 4.25 - 3.80 (m, $C\underline{H}C\underline{H}_2$), 2.35 (s, Me). Crystallisation from i-propanol - ether was unsuccessful.

D-Serine benzyl ester p-toluenesulphonate was similarly prepared.

Benzyloxycarbonyl-L-phenylalanyl-L-serine Benzyl Ester (86) - Crude L-serine benzyl ester p-toluenesulphonate (87) (1.768 g, 4.82 mmol) was coupled with benzyloxycarbonyl-L-phenylalanine (1.441 g, 4.82 mmol) in the presence of DCC (1.099 g, 5.3 mmol) and triethylamine (0.915 ml) by the procedure described

previously for (65). The product was treated with acetone to give a yellow oil (2.044 g, 89%) which was taken up in ether and cooled for several days to give the protected dipeptide (86) as needles, (986 mg, 43%), R_F^F 0.40. A sample was recrystallised from ethyl acetate - pet. ether (3:1) to give needles, m.p. 115-118 °C, $[\alpha]_D^{20}$ + 17.3° (c 1.16, $CHCl_3$) (Found: C, 67.90; H, 5.92; N, 5.67. $C_{27}H_{28}N_2O_6$ requires C, 68.07; H, 5.88; N, 5.88%); $\nu_{max.}$ 3 600 - 3 150, 3 310, 1 737, 1 695, 1 645, 1 540, 1 045, 745, and 699 cm^{-1}; δ 7.28, 7.23, 7.14 (15H, s, Ar), 7.10 - 6.78 (1H, m, D_2O exch., NH), 5.60 - 5.40 (1H, br d, J 8.0 Hz, D_2O exch., NH), 5.14 (2H, s, OCH_2Ph), 4.99 (2H, s, OCH_2Ph), 4.75 - 4.30 (2H, m, 2 x CH), 4.00 - 3.78 (2H, br d, CH_2OH), 3.15 - 2.95 (2H, m, $CHCH_2Ph$), 3.10 - 2.50 (1H, br m, D_2O exch., OH); m/e 458 (weak, $M-H_2O$), 120 (10%), 108 (11), 107 (9), 92 (19), 91 (100), 79 (23), 77 (23), and 65 (27).

L-Phenylalanyl-L-serine (64) - Water (28 ml) and acetic acid (0.8 ml) were added dropwise to a stirred solution of benzyloxycarbonyl-L-phenylalanyl-L-serine benzyl ester (86) (400 mg, 0.84 mmol) in ethanol (56 ml). The solution was hydrogenated over 10% palladium on charcoal (40 mg) for 30 min. The catalyst was removed by filtration, then washed and the filtrate concentrated to an oil at 30 - 40 °C. Residual acetic acid was removed by azeotroping with ethanol and the residue was triturated with chloroform to yield (64) as a solid (233 mg,

102%), m.p. 121-125 °C (lit.,[75] 116-125 °C), $[\alpha]_D^{20}$ + 34.8° (c 2.3, water) (lit.,[75] + 30.1°), R_F^A 0.38, R_F^B 0.65, R_F^C 0.62, R_F^H 0.00, (Found: C, 53.86; H, 6.79; N, 10.25. $C_{12}H_{16}N_2O_4 \cdot H_2O$ requires C, 53.33; H, 6.67; N, 10.37%); $\nu_{max.}$ 3 700 - 2 300, 1 675, 1 605, 1 525, 1 390, 750, and 700 cm^{-1}; δ (CF_3COOH) 8.35 (1H, br d, J 7 Hz, amide NH), 7.37 (8H, br s, Ph, $\overset{+}{N}H_3$), 5.60 - 4.60 (2H, m, 2 x CH), 4.60 - 4.10 (2H, m, CH_2OH), 3.70 - 3.15 (2H, m, CH_2Ph); m/e 234 (weak, M-H_2O), 120 (100%), 103 (25), 91 (73), 77 (16), 73 (45), 65 (16), and 60 (14).

Benzyloxycarbonyl-L-[4′-^{3}H]phenylalanine - L-[4′-^{3}H]Phenylalanine (165 mg, 1.0 mmol, 500 μCi $mmol^{-1}$) was reacted with benzyloxycarbonyl chloride as described earlier to give (81), (270.0 mg, 90.3%, ^{3}H activity 554 μCi $mmol^{-1}$).

L-[3-^{14}C]Serine Benzyl Ester p-Toluenesulphonate - L-[3-^{14}C]Serine (131.2 mg, 1.25 mmol, 40 μCi $mmol^{-1}$) was dissolved with p-toluenesulphonic acid (261.5 mg) in benzyl alcohol (5 ml) at 100 °C. The procedure described earlier was followed to give (87) as an oil, (302 mg, 66%).

Benzyloxycarbonyl-L-[4′-^{3}H]phenylalanyl-L-[3-^{14}C]serine Benzyl Ester - L-[3-^{14}C]Serine benzyl ester p-toluenesulphonate (330 mg) was coupled with benzyloxycarbonyl-L-[4′-^{3}H]phenylalanine (270.0 mg, 0.9 mmol, 554 μCi $mmol^{-1}$) as described previously to give, after crystallisation from ether, the protected

dipeptide (86) (350.5 mg, 82%), R_F^F 0.40. Panax scanning and autoradiography was carried out on the above tlc plate revealing one major radioactive signal at R_F 0.40 and one minor band at R_F 0.60.

L-[4'-^{3}H]Phenylalanyl-L-[3-^{14}C]serine Monohydrate - Hydrogenation of the protected dipeptide, benzyloxycarbonyl-L-[4'-^{3}H]phenylalanyl-L-[3-^{14}C]serine benzyl ester (350 mg) was carried out as described earlier to give the product (64) after trituration (192 mg, 96.7%, ^{14}C activity 32.19 μCi mmol^{-1}, 88%, ^{3}H:^{14}C, 13.92), R_F^A 0.38, R_F^B 0.65, R_F^C 0.62, R_F^H 0.00. A single radioactive band was observed on tlc by Panax scanning and autoradiography. Stereochemical purity was confirmed by dilution analysis with L-phenylalanyl-D-serine (90) and D-phenylalanyl-L-serine (91). The results showed maximum impurities of 0.3% and 0.2% of the LD- and DL-dipeptides respectively.

Benzyloxycarbonyl-D-phenylalanyl-L-serine Benzyl Ester (92) - L-Serine benzyl ester p-toluenesulphonate (87) (1.712 g, 4.66 mmol) was coupled with benzyloxycarbonyl-D-phenylalanine (1.393 mg, 4.66 mmol) by the DCC method described earlier. A solid product was obtained which was triturated thouroughly with ether leaving the protected dipeptide (92) (1.04 g, 47%) R_F^F 0.45 containing a minor impurity at R_F^F 0.65. A sample was purified for analysis by preparative tlc and crystallisation from ethyl acetate - pet.

ether (1:1), m.p. 139-141 °C, $[\alpha]_D^{15}$ + 20.7° (c 0.80, $CHCl_3$) (Found: C, 68.10; H, 5.92; N, 6.12. $C_{27}H_{28}N_2O_6$ requires C, 68.10; H, 5.88; N, 5.88%); $\nu_{max.}$ 3 470, 3 300, 1 735, 1 715, 1 695, 1 650, 1 540, 1 046, 750, 732, and 695 cm^{-1}; δ 7.29, 7.21 (15H, s, Ar), 6.78 - 6.49 (1H, m, NH), 5.50 - 5.30 (1H, br d, J 7 Hz, NH), 5.15 (2H, s, OCH_2Ph), 5.05 (2H, s, OCH_2Ph), 4.70 - 4.30 (2H, m, 2 x CH), 4.00 - 3.57 (2H, m, CH_2OH), 3.20 - 2.98 (2H, m, CH_2Ph), 1.95 - 1.70 (1H, m, D_2O exch., OH); m/e 476 (weak, M), 210 (10%), 92 (15), 91 (100), 79 (8), 77 (8), 65 (13), 39 (10).

Benzyloxycarbonyl-L-phenylalanyl-D-serine Benzyl Ester (93) - The synthesis of benzyloxycarbonyl-L-phenylalanyl-D-serine benzyl ester (93) was carried out as described for the DL-enantiomer to yield a crude product in 41% yield. Purification of a sample by tlc and crystallisation, as described earlier for (92), gave solid (93), m.p. 136.5-140 °C, $[\alpha]_D^{15}$ - 17.9° (c 0.34, $CHCl_3$). The i.r., n.m.r., and mass spectra were identical to (92), (Found: C, 67.82; H, 6.02; N, 6.02. $C_{27}H_{28}N_2O_6$ requires C, 68.10; H, 5.88; N, 5.88%).

L-Phenylalanyl-D-serine (90) - Acetic acid (0.6 ml) and 10% palladium on charcoal (50 mg) were added to a suspension of benzyloxycarbonyl-L-phenylalanyl-D-serine benzyl ester (93) (504 mg, 1.059 mmol) in ethanol - water (2:1; 100 ml) and

hydrogenated overnight. The catalyst was removed by filtration, then washed with water. The filtrate was concentrated to give a solid (279 mg), which was triturated with chloroform and crystallised from water - acetone to give the dipeptide (90) (200 mg, 75%), m.p. 215 - 225 °C (with formation of needles, m.p. 230 - 252 °C, dec.), $[\alpha]_D^{20}$ + 49.80° (c 1.96, CF_3COOH), R_F^A 0.38, R_F^B 0.65, R_F^C 0.62, R_F^H 0.00, (Found: C, 57.29; H, 6.46; N, 11.03. $C_{12}H_{16}N_2O_4$ requires C, 57.14; H, 6.35; N, 11.11%); $\nu_{max.}$ 3 700 - 2 400, 3 270, 3 070, 1 688, 1 625, 1 545, 1 522, 1 422, 1 382, 1 048, 1 032, 750 and 700 cm^{-1}; δ (CF_3COOH) 8.32 (br d, J 7 Hz), 7.94 (br d, J 7 Hz), 7.70 - 7.20 (8H, m, Ph, $\overset{+}{N}H_3$), 5.05 - 4.55 (2H, m, 2 x CH), 4.50 - 3.91 (2H, ABX, J_{AB} 12 Hz, J_{AX} 4 Hz, J_{BX} 3 Hz, CH_2OH), 3.41 (2H, br d, J 8 Hz, CH_2Ph); m/e 254 (weak, $M-H_2O$), 143 (44%, $M-H_2O-C_7H_7$), 120 (76), 91 (100), 83 (22), and 77 (20).

D-Phenylalanyl-L-serine (91) - D-Phenylalanyl-L-serine (91) was synthesised in the same manner as its LD-enantiomer (90) having identical physical properties with the exception of optical rotation.

Attempted Enzymic Hydrolsis of the Ester (80) - The ester (80) was treated with chymotrypsin as described by Walton et al.[82] The starting material failed to dissolve and was recovered. The same procedure was followed with pig liver esterase but again starting material was recovered.

Attempted Hydrogenation of the Ester (80) - The ester (80) (100 mg) was dissolved in methanolic hydrochloric acid and hydrogenated as described previously. The product showed several spots on t.l.c.

8.5 Experimental for Chapter 4.

L-Phenylalanine Methyl Ester Hydrochloride (97) - L-Phenylalanine methyl ester hydrochloride was synthesised from L-phenylalanine in 90% yield by the method of Brenner and Huber[79] as needles, m.p. 152-154 °C (lit.,[84] 159-161 °C), $[\alpha]_D^{19}$ + 34.4° (c 4.6, CF_3COOH), + 35.6° (c 4.0, EtOH); $\nu_{max.}$ (nujol) 1 750, 1 585, 1495, 1 232, 730, and 690 cm^{-1}; δ (CF_3COOH) (60 MHz) 7.40 (8H, m, Ph, $\overset{+}{N}H_3$), 4.65 (1H, m, CH), 4.05 (3H, s, OMe), and 3.55 (2H, m, CH_2Ph).

D-Phenylalanine Methyl Ester Hydrochloride - D-Phenylalanine methyl ester hydrochloride was synthesised as above in 93% yield with identical physical properties with the exception of optical rotation.

N-Benzyloxycarbonyl-L-seryl-L-phenylalanine Methyl Ester (96) - N-Benzyloxycarbonyl-L-serine (67) (239 mg, 1 mmol) was coupled with L-phenylalanine methyl ester hydrochloride (215.5 mg, 1 mmol) by the DCC method described by MacDonald and Slater[36b] to give an oil (353 mg, 88%). A sample was purified for analysis by preparative tlc, R_F 0.26 (EtOAc - Pet. ether - HOAc; 20:20:1) followed by crystallisation from acetone - pet. ether to give (96) as needles, m.p. 75-77 °C, $[\alpha]_D^{15}$ + 15.4° (c 1.18, MeOH) (Found: C, 63,23; H, 5.99; N, 6.90. $C_{21}H_{24}N_2O_6$ requires C, 63.00; H, 6.00; N, 7.00%) $\nu_{max.}$ 3 600 - 3 150, 1 745, 1 690, 1 660, 1 550, 1 060, 760 - 745, and 705 cm^{-1}; δ (60 MHz) 7.50 - 6.80, 7.35 (10H, m,

Ar), 5.90 (1H, br d, J 7 Hz, D_2O exch., NH), 5.10 (2H, s, OCH_2Ph), 5.10 - 4.65 (1H, m, CH), 4.50 - 3.35 (3H, m, CH, CH_2OH), 3.70 (3H, s, OCH_3) 3.50 - 3.20 (1H, m, D_2O exch., OH), 3.20 - 2.80 (2H, m, CH_2Ph); m/e 400 (weak, M), 162 (12%, PhCH=CHCOOMe), 131 (5, PhCH=CHCO), 120 (5, $PhCH_2CH=NH_2$), 92 (10),91 (100), 79 (6), 77 (8) and 65 (14).

N-Benzyloxycarbonyl-D-seryl-D-phenylalanine Methyl Ester (98) - The dipeptide (98) was synthesised as described above as a crude oil which was not characterised.

cyclo-(L-Phenylalanyl-L-seryl) (24) - L-Phenylalanine methyl ester hydrochloride (2.155 g, 10 mmol) was coupled with N-benzyloxycarbonyl-L-serine (2.390 g; 10 mmol) by the DCC method previously described to give an oil (2.685 g). The oil, dissolved in methanol (90 ml), was hydrogenated for 5h in the presence of 10% palladium on charcoal (240 mg) and acetic acid (0.1 ml). The catalyst was removed by filtering through a Celite pad which was washed with methanol. The filtrate and washings were combined and concentrated to an oil which was dissolved in a small volume of methanol and saturated with ammonia at 0 °C. On standing at room temperature overnight, a solid separated which was filtered and washed thouroughly with ether to give (24), (814 mg, 35% based on H-L-Phe-OMe.HCl), m.p. 237-240 °C, $[\alpha]_D^{20}$ - 95.6° (c, 1.0, DMF),

showing one spot on tlc. Recrystallisation from methanol gave (24) (527 mg), m.p. 235-240 °C (decomp.) (lit.,[36b] 244-246 °C) $[\alpha]_D^{20}$ - 105° (c 1.0, DMF) (lit.,[36b] - 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%) R_F^H 0.30, R_F^I 0.58; $\nu_{max.}$ 3 405, 3 200, 1 670, 1 460, 1 345, 1 075, 761, and 705 cm^{-1}; δ [$(CD_3)_2SO$] 8.10 - 7.75 (2H, m, D_2O exch., 2 x NH), 7.40 - 7.02 (5H, m, Ph), 4.82 (1H, t, J 6 Hz, D_2O exch., OH), 4.20 - 3.95 (1H, m, sharpens on D_2O shake to broad t, $J_{AX} + J_{BX}$ 11 Hz, C<u>H</u>CH_2Ph), 3.80 - 3.58 (1H, m, C<u>H</u>CH_2OH), 3.38 - 2.72 (2H, <u>ABX</u>, J_{AB} 11 Hz, J_{AX} 3.5 Hz, J_{BX} 6.0 Hz, sharpens on D_2O shake, C<u>H</u>$_2$OH), 3.08 - 2.99 (2H, q, J_{AX} 6 Hz, J_{BX} 5 Hz, C<u>H</u>$_2$Ph); δ (CF_3COOH) 8.45 - 8.00 (2H, m, 2 x NH), 7.50 - 7.10 (5H, m, Ph), 4.90 - 4.60 (1H, m, C<u>H</u>CH_2Ph), 4.56 - 4.32 (1H, m, C<u>H</u>CH_2OH), 3.81 - 3.05 (2H, <u>ABX</u>, J_{AB} 12 Hz, J_{AX} 4 Hz, J_{BX} 6 Hz, C<u>H</u>$_2$OH), 3.34 (2H, d, J 5.5 Hz, C<u>H</u>$_2$Ph); δ (CD_3OD) 7.29 (5H, m, Ph), 4.32 - 4.20 (1H, m, $J_{AX} + J_{BX}$ 11 Hz, J_{CX} 1 Hz, C<u>H</u>CH_2Ph), 3.94 - 3.82 (1H, dq, $J_{AX} + J_{BX}$ 9.5 Hz, J_{CX} 1 Hz, C<u>H</u>CH_2OH), 3.57 - 2.82 (2H, <u>ABX</u>, J_{AB} 11.0 Hz, J_{AX} 3.5 Hz, J_{BX} 6.0 Hz, C<u>H</u>$_2$OH), 3.26 - 3.16 (2H, q, J_{AX} 6.5 Hz, J_{BX} 5.0 Hz, C<u>H</u>$_2$Ph); <u>m/e</u> 234 (17%, M), 204 (21, M-CH_2O), 143 (9, M-C_7H_7), 120 (9, $PhCH_2CH{=}NH_2$), 115 (14), 113 (17), 92 (22), 91 (100), 85 (22), 77 (7), 65 (12), and 60 (6). Crystallisation of the mother liquors gave a further crop (100 mg), m.p. 233-240 °C (decomp.).

cyclo-(D-Phenylalanyl-D-seryl) (95) - cyclo-(D-Phe-D-Ser) (95) was synthesised as described above on a 10 mmol scale to give a solid (666 mg, 28.5% from H-D-Phe-OMe.HCl) after trituration with ether. Recrystallisation from methanol and recrystallisation of the mother liquors from methanol gave (95) (454 mg), m.p. 235 - 241 °C, $[\alpha]_D^{20}$ + 104° (c 1.00, DMF), having identical spectroscopic and chromatographic properties to (24).

N-Benzyloxycarbonyl-L-seryl-D-phenylalanine Methyl Ester (101) - N-Benzyloxycarbonyl-L-serine (67) (2.39 g, 10 mmol) was coupled with D-phenylalanine methyl ester (2.155 g, 10 mmol) by the normal DCC procedure to give a sticky solid, (2.56 g, 64%) a sample of which was purified by preparative tlc, R_F 0.30 (EtOAc - Pet ether - AcOH; 20:20:1), to give the protected dipeptide, m.p. 108 - 112 °C, $[\alpha]_D^{15}$ - 35.4° (c 1.0, $CHCl_3$); δ (60 MHz) 7.35 (5H, s, $PhCH_2O$), 7.55 - 6.80 (6H, m, NH, $PhCH_2CH$), 5.90 (1H, br d, J 7 Hz, NH), 5.10 (2H, s, $PhCH_2O$), 5.10 - 4.65 (1H, m, $CHCH_2$), 4.50 - 3.40 (4H, m, CH, CH_2OH), 3.70 (3H, s, OMe), 3.25 - 2.90 (2H, m, CH_2Ph); m/e 400 (M), 162 (49%, PhCH=CHCOOMe), 131 (20, PhCH=CHCO), 108 (51), 107 (41), 91 (100), 79 (74), 77 (68), 65 (26).

cyclo-(L-Phenylalanyl-D-seryl) (94) - N-Benzyloxycarbonyl-D-serine (2.39 g, 10 mmol) and L-phenylalanine methyl ester hydrochloride (97) (2.155 g, 10 mmol) were coupled in the normal

fashion to give the crude protected dipeptide (2.558 g, 64%) which was hydrogenated and cyclised as for the LL-diastereomer. The crude solid product was washed thoroughly with ether to give (94) (721.6 mg, 31% from H-L-Phe-OMe.HCl) which was recrystallised from methanol to give cyclo-(L-phenylalanyl-D-seryl) (94) as needles (529 mg, 23%), m.p. 258-268 °C, $[\alpha]_D^{20}$ + 11.2° (c 1.0, DMF), R_F^H 0.39, R_F^I 0.62; (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%); $\nu_{max.}$ 3 480, 3 200, 1 685, 1 670, 1 338, 1 108, 1 093, 769, 722, and 707 cm^{-1}; δ [$(CD_3)_2SO$] 8.04 (1H, br s, D_2O exch., NH), 7.75 (1H, br s, D_2O exch., NH), 7.21 (5H, s, Ph), 4.92 (1H, t, J 5.5 Hz, D_2O exch., OH), 4.26-4.09 (1H, m, $J_{AX}+J_{BX}$ 9 Hz, C<u>H</u>CH_2Ph), 3.80-3.35 (2H, m, D_2O gives <u>ABX</u>, J_{AB} 11.5, J_{AX} 4, J_{BX} 3 Hz, C<u>H</u>$_2$OH), 3.30-2.80 (3H, m, C<u>H</u>$_2$Ph, C<u>H</u>CH_2OH); δ (CF_3COOH) 8.11 (2H, br s, 2 x NH), 7.50 - 7.10 (5H, m, Ph), 4.90 - 4.65 (1H, m, C<u>H</u>CH_2Ph), 4.32 - 3.93 (2H, <u>ABX</u>, J_{AB} 12 Hz, J_{AX} 3.5 Hz, J_{BX} 3.5 Hz, C<u>H</u>$_2$OH), 3.53 - 3.14 (3H, m, C<u>H</u>CH_2OH and C<u>H</u>$_2$Ph); <u>m/e</u> 234 (11%, M), 204 (20, M-CH_2O), 143 (6, M-C_7H_7), 115 (8), 113 (9), 92 (15), 91 (100), 85 (22), 77 (9), 65 (18). Crystallisation of the mother liquor gave a further crop (83 mg) m.p. 257-264 °C.

cyclo-(D-Phenylananyl-L-seryl) (100) - cyclo-(D-Phenylalanyl-L-seryl) (100) was synthesised as above and the product had identical physical properties with the exception of optical rotation, $[\alpha]_D^{20}$ - 10.2° (c 1.0, DMF).

cyclo-(L-[U-^{14}C]Phenylalanyl-L-seryl) - L-[U-^{14}C]Phenylalanine (165 mg, 1 mmol, 100 μCi mmol^{-1}) was suspended in freshly distilled 2,2-dimethoxypropane (10 ml). Concentrated hydrochloric acid (1.0 ml) was added dropwise with swirling and the solution was stored at room temperature for 24 h. The brown solution was concentrated at 30-40 °C to give a brown residue. The residue was triturated with ether to yield the methyl ester hydrochloride (97) (214.1 mg, 99%), m.p. 155-157 °C (lit.,[84] 159-161 °C), which was coupled in the previously described manner with N-benzyloxycarbonyl-L-serine (239 mg, 1 mmol) to give a sticky solid (320 mg). Hydrogenation and cyclisation gave an off white solid which was washed thoroughly with ether to give the product (78.3 mg, 33.5% from L-phenylalanine, ^{14}C activity, 84.74 μCi mmol^{-1}) R_F 0.10 ($CHCl_3$-AcOH; 9:1), R_F^I 0.65; Panax scanning and autoradiography of tlc plates and dilution analysis with inactive stereomers showed that the product contained no more than 0.3% of either the LD- or DL-cyclic dipeptides (94) and (100).

cyclo-(L-[U-^{14}C]Phenylalanyl-D-seryl) - cyclo-(L-[U-^{14}C]Phenylalanyl-D-seryl) was synthesised as above to give after trituration, a solid, (71 mg, 30% from L-phenylalanine) ^{14}C activity, 45.01 μCi mmol^{-1}; R_F^H 0.33, R_F^I 0.60. Radioscanning and autoradiography of tlc plates showed that there was only one radioactive band. Dilution analysis revealed that not more than

0.5% of the LL-diasteriomer (24) was present.

cyclo-(D-Phenylalanyl-D,L-[3-^{14}C]seryl) - D,L-[3-^{14}C]Serine (116 mg, 1.1 mmol, 100 μCi mmol^{-1}) was converted as previously described to N-benzyloxycarbonyl-D,L-[3-^{14}C]serine (190 mg, 72%). The sample was diluted to 1 mmol with inactive material and converted in the usual manner to the product (49.1 mg, 15% from D,L-serine, ^{14}C activity, 62.64 μCi mmol^{-1}. Radioscanning and autoradiography of tlc plates was carried out as above, dilution analysis showed that the DL:DD ratio was 68:28 and that not more than 0.1% of the LL-isomer was present in the mixture.

cyclo-(L-[4'-^{3}H]Phenylalanyl-L-[3-^{14}C]seryl) - L-[3-^{14}C] Serine (110.6 mg, 1.05 mmol, 100 μCi mmol^{-1}) was converted to N-benzyloxycarbonyl-L-[3-^{14}C]serine as before (230 mg, 91.4%, ^{14}C activity, 92.27 μCi mmol^{-1}, 97.2% of original specific activity).

L-[4'-^{3}H]Phenylalanine (166.0 mg, 1 mmol, 1 mCi mmol^{-1}) was converted to L-[4'-^{3}H]phenylalanyl methyl ester hydrochloride as before (228 mg, 100%).

Z-L-[3-^{14}C]Ser-OH was diluted to 1 mmol with inactive compound and coupled with H-L-[4'-^{3}H]Phe-OMe.HCl as before to give a yellow solid (311 mg) which was hydrogenated and cyclised to give an off white solid after trituration, (66.8 mg, 28.6% based on H-L-Phe-OH, ^{14}C activity, 90.405 μCi mmol^{-1}, 98% of

reactant's specific activity), Radiochemical purity was checked by the methods already described. Not more than 0.2% of the DL- or LD-isomers, (94) and (100) were present.

Intermediate Trapping Experiment - cyclo-(L-Phenylalanyl-L-seryl) (24) (80 mg) was added, in dimethyl sulphoxide (4 ml) to a one day old shake culture (1 l) of Trichoderma viride. L-[U-^{14}C]Phenylalanine (25 μCi) was fed 2 h later. After a further 2 h, the normal work up procedure was carried out. The aqueous solution, after extraction with chloroform, was extracted continuously with ethyl acetate for 3 days. The organic extract was dried, filtered, and concentrated to a brown solid (34 mg, 0.763 μCi). Unlabelled cyclo-(L-Phe-L-Ser) (48.0 mg) was added to a sample of the residue (11 mg). The mixture was triturated with chloroform then crystallised to constant activity (0.33 μCi mmol^{-1}) with methanol. The activity present in the total ethyl acetate extract as cyclo-(L-[U-^{14}C]Phe-L-Ser) was calculated to be 0.202 μCi (0.80%).

The aqueous solution was extracted with ethyl acetate for a further 3 days and the procedure described above was repeated. cyclo-(L-[U-^{14}C]Phe-L-Ser) (0.12 μCi) (0.5%) was present in the extract.

8.6 Experimental for Chapter 5.

Degradation of Glictoxin by Basic Hydrolysis - Gliotoxin (23.2 mg, ^{14}C activity 0.762 μCi $mmol^{-1}$) was heated at reflux in 10% sodium hydroxide (7 ml) for 1 h under a stream of nitrogen, which, after passage through the apparatus, was bubbled through 2M hydrochloric acid. Steam distillation was carried out for 10 min, and the distillate was collected in hydrochloric acid. The combined acid solutions were concentrated to a yellow film which was solidified by precipitation from methanol- ethyl acetate, to give a yellow solid (3.5 mg) R_F 0.55 (MeOH - Pet. ether - AcOH; 1:1:0.1), ^{14}C activity 0.012 μCi $mmol^{-1}$ (1.16%)

The hydrochloride salt was dissolved in a minimum of water and phenylisothiocyanate (0.02 ml) was added, followed by concentrated sodium hydroxide solution (1 drop). The mixture was cooled for 3 days by which time crystals had formed. The solvent was removed by evaporation and the residue dissolved in methanol - chloroform and applied to preparative tlc which was eluted with EtOAc - Pet. ether (2:5). The product was obtained as an oil which was solidified by scratching, (6 mg, 51% from gliotoxin), m.p. 109 - 113 °C (lit.,[110] 113 °C), R_F 0.20 (EtOAc - pet. ether; 2:5), ^{14}C activity 2.131 nCi $mmol^{-1}$ (0.07%); δ (60 MHz) 7.35 (m, Ph) and 3.1 (s, Me).

Anhydrodesthiogliotoxin (3) - Gliotoxin (46.6 mg, 0.143 mmol) was suspended in dry benzene (10 ml) and stirred

overnight at room temperature with grade II neutral alumina (2.03 g). The alumina was removed by filtration and washed with hot chloroform (6 x). The combined filtrates were concentrated to give a light yellow solid (26 mg) which was purified by preparative tlc and crystallisation from methanol to give (3) as slightly yellow crystals (8 mg, 23%) m.p. 158-160 °C (lit.,[90] 160-161 °C) R_F 0.5 (toluene - acetone; 9:1); $\nu_{max.}$ ($CHCl_3$) 1 710, 1 675, 1 605, 1 395, 1 355 cm^{-1}; δ (60 MHz) 8.60 (1H, d, J 7,7 Hz, indole CH), 7.80 - 7.25 (4H, m, Ar), 6.15 (1H, d, J 1.5 Hz, olefinic H), 5.25 (1H, d, J 1.5 Hz, olefinic H), 3.45 (3H, s, NMe); m/e 227, 226, 199, 198, 197, 170, 169, 144, 143, 130, 129, 116, 115, 114, 89, 88, 55, and 54.

Degradation of Gliotoxin to Anhydrodesthiogliotoxin (3) and subsequent Ozonolysis (a) - Gliotoxin (52.4 mg, ^{14}C activity, 1.1 μCi $mmol^{-1}$, $^{3}H:^{14}C$ 11.1) was converted to (3) as described previously, (10 mg, 27.5%, ^{14}C acivity, 1.21 μCi $mmol^{-1}$, 108%, $^{3}H:^{14}C$ 11.2.

The product, dissolved in ethyl acetate, was ozonised for 30 min at - 25 °C. The solvent was evaporated slowly at room temperature to give the ozonide which was broken down by steam distillation in the presence of zinc. The aqueous distillate was collected in dimedone solution and allowed to stand overnight resulting in the crystallisation of the dimedone derivative of

formaldehyde (11.32 mg, 88%) m.p. 189-190 °C (lit.,[111] 190-191 °C) ^{14}C activity 0.53 μCi mmol^{-1}. The derivative was recrystallised from ethanol - water to give (5.9 mg, 43%) m.p. 190 - 191.5 °C, R_F 0.72 (EtOAc - Pet. ether; 1:1), ^{14}C activity 0.580 μCi mmol^{-1} (48%), ^{3}H activity 0.0 μCi mmol^{-1}.

(b) - The degradation of gliotoxin was repeated to give anhydrodesthiogliotoxin (10%), ^{14}C activity 0.955 μCi mmol^{-1} (105%), ^{3}H:^{14}C 10.7, which was ozonised for 2 min and worked up as above to yield the dimedone derivative (44%), m.p. 190-191 °C, ^{14}C activity 0.408 μCi mmol^{-1} (43%).

(c) - Anhydrodesthiogliotoxin (4.9 mg, ^{14}C activity 0.49 μCi mmol^{-1}, ^{3}H:^{14}C 11.5) dissolved in ethyl chloride was ozonised for 10 min at - 25 °C. After the solution had been flushed with nitrogen, dimethyl sulphide (0.02 ml) was added and the solution was allowed to stand at - 25 °C for 1 h. The temperature was allowed to rise to 0 °C whereupon water was added and the flask was allowed to stand at this temperature for 45 min before standing overnight at room temperature. Steam distillation was carried out and the dimedone derivative was obtained as before (5.5 mg, 87%) and crystallised to constant activity (2.7 mg) m.p. 192-193 °C, ^{14}C activity 0.18 μCi mmol^{-1} (37%).

Degradation of Gliotoxin derived from cyclo-(L-[U-^{14}C]Phe-L-Ser) - Gliotoxin (50.72 mg, ^{14}C activity 0.57 μCi mmol^{-1})

derived from the feeding of cyclo-(L-[U-^{14}C]Phe-L-Ser), was converted as above to anhydrodesthiogliotoxin (3.8 mg, 11%, ^{14}C activity 0.570 μCi mmol^{-1}, 100%).

Ozonolysis was carried out at - 25 °C for 5 min and dimethyl sulphide used for the work up to give the dimedone derivative of formaldehyde (0.5 mg, 10%, ^{14}C activity 5.0 x 10^{-4} μCi mmol^{-1}) which was recrystallised (0.15 mg, ^{14}C activity 1.2 x 10^{-4} μCi mmol^{-1}).

Dehydrogliotoxin (11) - A solution of gliotoxin (207 mg, 0.635 mmol) in chloroform was heated at reflux in the presence of 2,3-dichloro-1,4-benzoquinone (DDQ) for 6 h. The resulting phenolic precipitate was filtered and the filtrate concentrated to give a residue which was crystallised from chloroform - methanol giving (11) as brown needles (148 mg, 72%) m.p. 184-185.5 °C (lit.,[91] 185-186 °C); $\nu_{max.}$ 3 490, 1 670, 1 610, 1 400, 1 060, 725, and 700 cm^{-1}; m/e 324 (weak, M) 260 (60%, M-S_2), 243 (35), 242 (100, M-S_2-H_2O), 230 (30, M-S_2-CH_2O), 214 (15, M-S_2-H_2O-CO), 185 (13, M-S_2-H_2O-CH_3NCO), 160 (30), 159 (100), 131 (36), 103 (43), 76 (40, C_6H_4), 64 (45), 56 (35, CH_2=C=$NHCH_3$), with metastable ions at m/e 225, 189.5, 108, 81, and 56.

1,2,3,4-Tetrahydro-6-hydroxy-2-methyl-3-methylene-1,4-dioxopyperazino[1,2-a]indole (104) - A solution of dehydrogliotoxin (11) (52.5 mg, 0.162 mmol) in acetic acid (5 ml) and acetic

anhydride (0.164 ml) was heated at reflux for 3 h. The solvent was removed by evaporation and the residue applied to preparative chromatography to give the product (104) (42 mg) which was crystallised from ethanol – chloroform giving yellow needles (24 mg, 61.1%), m.p. 217-219 °C (lit.,[91] 218-219 °C); $\nu_{max.}$ 1 680, 1 595, 1 580, 1 417 – 1 410, 1 380 – 1 350, 1 185, 775, and 730 cm^{-1}; m/e 242 (100%, M), 214 (11, M-CO), 185 (18, M-CH_3NCO), 159 (100), 145 (25), 131 (50), 103 (70), 102 (15), 76 (80), 75 (20), 56 (80, CH_2=C=NHCH_3), 55 (35, CH_2=C=NCH_3), 54 (35, CH_2=C=C=O), with metastable ions at m/e 189, 108, 81, and 56; δ (60 MHz) 10.7 (1H, s, OH), 7.54 (1H, s, indole H), 7.36 – 6.95 (3H, m, Ar), 6.25 (1H, d, J 1.5 Hz, CH), 5.35 (1H, d, J 1.5 Hz, CH), and 3.42 (3H, s, NMe).

[7-^{3}H]1,2,3,4-Tetrahydro-2-methyl-1,3,4-trioxopyrazino-[1,2a]-indole (5) – Gliotoxin (102.1 mg, ^{14}C activity 1.07 μCi $mmol^{-1}$, ^{3}H:^{14}C 13.8) was converted to anhydrodesthiogliotoxin (3) as described earlier to give yellow needles (11.2 mg, 16%), m.p. 153-156 °C, ^{14}C activity 0.982 μCi $mmol^{-1}$ (91.5%), ^{3}H:^{14}C 13.6.

The product (3), in acetic acid (15 ml), was stirred at room temperature overnight with chromic anhydride solution (0.44 ml) from a stock solution of chromic anhydride (260 mg) in water (10 ml). The reaction was worked up as described by

Ali et al.[92] to yield a crude residue which was triturated with methanol then crystallised (3 x) from methanol - chloroform (1:1) to give (5) as an off white solid (2 mg, 18%), m.p. 262-264 °C (lit.,[92] 262-263 °C), ^{14}C activity 0.0 μCi mmol^{-1}, ^{3}H activity 15.3 μCi mmol^{-1} (103%); $\nu_{max.}$ 1 735, 1 690, 1 400, 1 350, 740 cm^{-1}; m/e 228 (39%, M), 143 (100), 115 (65), 88 (22), with metastable ions at 92.5 and 67.3.

Glycine Anhydride (108) - Glycine (50 g, 670 mmol) was converted by the method of Schott et al.[94] to glycine anhydride (27.81 g, 74%), m.p. 285-300 °C (decomp.) (lit.,[112] 311-312 °C, decomp., rapid heating); $\nu_{max.}$ 3 195 - 3 165, 3 050, 1 707 - 1 680, 1 470, 1 445, 1 340; cm^{-1}; m/e 114 (62%, M), 86 (10, M-CO), 71 (43, M-HNCO), 56 (10, M-CH_2NCO), 43 (42, HNCO).

NN-Diacetyl Glycine Anhydride (109) - Glycine anhydride (1.018 g, 8.93 mmol) was heated at 100 °C in acetic anhydride (5 ml) for 3 h then cooled to 0 °C and poured onto ice (250 ml). An oil separated which solidified on stirring and scratching. The ice-solid mixture was filtered, collected and the ice melted to form a solution which was neutralised with sodium hydroxide solution. The solution was extracted with chloroform several times and the combined organic extracts were dried, filtered and concentrated to give a solid which was crystallised from ether - pet. ether (60-80 °C) to give the product (109)

(1.48 g, 84%) m.p. 101-102 °C (lit.,[113] 102 °C); $\nu_{max.}$ (nujol) 1 730 - 1 700, cm^{-1}; δ (60 MHz) 4.65 (4H, s, 2 x CH_2), 2.65 (6H, s, 2xMe); m/e 198 (18%, M), 170 (33, M -CO), 156 (21, M+H-CH_3CO), 114 (26, M+H_2-2CH_3CO), and 43 (100, CH_3CO).

L-Proline Methyl Ester Hydrochloride - L-Proline (14.4 g, 0.125 mol) was converted into its methyl ester hydrochloride by the method of Brenner and Huber[79] to give an oil (19.6 g, 95%), δ (CD_3OD) 4.65 - 4.15 (1H, m, CH), 3.85 (3H, s, OCH_3), 3.60 - 3.05 (2H, m, NCH_2), and 2.70 - 1.75 (4H, m, 2 x CH_2).

N-Pyruvoyl-L-proline Methyl Ester (114) - Triethylamine (2.4 ml) was added to a suspension of L-proline methyl ester hydrochloride (3.3 g, 20 mmol) in methylene chloride (80 ml) at 0 °C. Pyruvic acid (1.76 g, 20 mmol) and DCC (4.12 g, 20 mmol) were added and the mixture stood at room temperature over-overnight. The DCU was filtered off through a Celite pad which was washed with 1M hydrochloric acid (2 x), water, saturated sodium hydrogen carbonate solution (2 x) and water, then dried, filtered and concentrated to give a yellow oil (3.397 g, 84.5%). Fractional distillation gave a clear oil (729 mg, 18%). b.p. 72 °C/0.06 mm Hg (lit.,[96b] 121 °C/0.1 mm Hg), $[\alpha]_D^{15}$ - 87.3° (c 1.1, chloroform) (lit.,[96b] - 69°); $\nu_{max.}$ (thin film) 1 744, 1 725, 1 640, 1 350, cm^{-1}; δ (60 MHz) 5.10 - 4.35 (1H, m, CH), 4.05 - 3.50 (2H, m, NCH_2), 3.80, 3.78 (3H, two s, OCH_3), 2.45

and 2.43 (3H, two s, CH_3CO), 2.60 - 1.65 (4H, m, $CHCH_2CH_2$); m/e 199 (weak, M), 156 (38%, M-CH_3CO), 140 (25, M-CH_3OCO), 128 (100, M-CH_3COCO), and 70 (91).

(2S,5R)-4,5-Dimethyl-3,6-dioxo-5-hydroxy-1,2-trimethylene-piperazine (115) - A solution of distilled N-pyruvoyl-L-proline methyl ester (114) (209 mg, 1.05 mmol) was cyclised with methylamine as described by Lee[96b] to give needles (894 mg, 38%), m.p. 135-137 °C (lit.,[96b] 145-148 °C), $[\alpha]_D^{20}$ - 147.6° (c 0.5, $CHCl_3$) (lit.,[96b] - 146°) (Found: C, 54.67; H, 6.91; N, 13.98. $C_9H_{14}N_2O_3$ requires C, 54.54; H, 7.07; N, 14.14%); $\nu_{max.}$ 3 280, 1 672 - 1 660, 1 640, 1 100 cm^{-1}; δ 5.01 (1H, br s, D_2O exch., OH), 4.45 - 4.18 (1H, m, CH), 3.70 - 3.34 (2H, m, NCH_2), 3.01 (3H, s, NMe), 2.55 - 1.60 (4H, m, $CHCH_2CH_2$), 1.73 (3H, s, Me); m/e 198 (weak, M), 180 (24, M-H_2O), 152 (11, M-H_2O -CO), 151 (11), 124 (62, M-H_2O-2CO), 95 (13), 74 (11, CH_3(OH) C=NMeH), 70(65), 69 (19), 68 (20), 58 (14), 56 (31), 55 (100), and 54 (46).

(2S,5R)-[4-^{14}C]Methyl-3,6-dioxo-5-hydroxy-5-methyl-1,2-trimethylenepiperazine - Sodium hydroxide (40 mg, 1 mmol) was added to an aqueous solution of [^{14}C] methylamine hydrochloride (67.5 mg, 1 mmol, 250 μCi $mmol^{-1}$ in 2.5 ml water). The solution was heated at reflux for 8 h, and the methylamine generated was carried in a stream of nitrogen into a flask

containing a solution of N-pyruvoyl-L-proline methyl ester (114) (199 mg, 1 mmol) in dry dimethoxyethane (10 ml), cooled to -50 °C. The reaction flask was stored at 0 °C for 48 h. Methylamine (10 mmol) was passed into the reaction flask by the same procedure and the mixture was left at 0 °C for 5 days. Excess methylamine was removed by passing a stream of nitrogen through the reaction mixture and into a trap of hydrochloric acid. Concentration of the reaction mixture gave a solid residue which was crystallised from ethanol - pet. ether to give needles (36.3 mg, 18%), ^{14}C activity 171.2 μCi $mmol^{-1}$ (68.5%), R_F 0.46 (Acetone - toluene; 2:1), R_F 0.37 (EtOAc - MeOH; 6:1), R_f 0.47 (acetone).

Unlabelled product (115) (40 mg) was added to the mother liquor and its crystallisation was repeated to yield a further crop of (115) (35 mg, ^{14}C activity 95.3 μCi $mmol^{-1}$. Radiochemical purity of the first crop was tested by Panax scanning, autoradiography and dilution analysis and found to be 94.4%. The S,S-diastereomer was the only impurity, R_F 0.43 (acetone - toluene; 2:1), 0.26 (EtOAc - MeOH; 6:1), 0.38 (acetone).

(2S)-3,6-Dioxo-4-methyl-5-methylene-1,2-trimethylenepiperazine (111) - The hydroxydioxopiperazine (115) (50 mg) was treated with anhydrous trifluoroacetic acid (0.5 ml) for 1 min resulting in quantitative conversion to the dehydrated product (111);

δ (CF_3COOH) (60 MHz) 5.90 (1H, d, J 2 Hz, exo methylene) 5.15 (1H, d, J 2 Hz, eco methylene), 4.65-4.15 (1H, m, CH), 4.05-3.65 (2H, m, NCH_2), 3.25 (3H, s, NCH_3), and 2.75-1.90 (4H, m, $CHC\underline{H}_2C\underline{H}_2$).

(2S,5R)-3,6-Dioxo-5-hydroxy-5-methyl-1,2-trimethylene-piperazine (116) - N-Pyruvoyl-L-proline methyl ester (114) (500 mg, 2.5 mmol) was cyclised in the presence of ammonia as described by Lee[96b] to give plates (234 mg, 51%), m.p. 194 - 198 °C (decomp.) (lit.,[96b] 177 °C), $[\alpha]_D^{20}$ - 147.7° (c 0.10, $CHCl_3$) (lit.,[96b] - 144°) (Found: C, 52.15; H, 6.70; N, 14.98. $C_8H_{12}N_2O_3$ requires C, 52,17; H, 6.52; N, 15.22%) $\nu_{max.}$ (Nujol) 3 200, 3 070, 1 670 - 1 645, cm^{-1}; δ [$(CD_3)_2SO$] 8.77 (1H, br s, D_2O exch.), 6.43 (1H, br s, D_2O exch.), 4.40 - 4.05 (1H, m, CH), 3.60 - 3.10 (2H, m, NCH_2), 2.30 - 1.60 (4H, m, $CHC\underline{H}_2C\underline{H}_2$), and 1.4 (3H, s, Me); m/e 184 (3%, M), 166 (30, M-H_2O), 138 (18, M-H_2O -CO), 110 (55, M-H_2O-2CO), 97 (18), 70 (100), 69 (32), 68 (30), and 60 (28).

(2S)-3,6-Dioxo-5-methylene-1,2-trimethylenepiperazine (112) - The preceding compound (116) (250 mg, 1.36 mmol) was treated with anhydrous trifluoroacetic acid as described[96b] to yield (112) (87 mg, 38.5%), m.p. slow decomposition (lit.,[96b] 240 °C) (Found: C, 57.66; H, 6.20; N, 16.71. $C_8H_{10}N_2O_2$ C, 57.83; H, 6.02; N, 16.87%) $\nu_{max.}$ (Nujol) 3 200, 3 080, 1 675, 1 630 cm^{-1}; m/e 166 (80%, M), 138 (36, M-CO), 137 (17), 110 (100, M-2CO), 82 (16), 70 (29), 69 (12) and 68 (17).

Ozonolysis of (2S)-3,6-Dioxo-5-methylene-1,2-trimethylene-piperazine (112) - A solution of (112) (11.8 mg, 0.071 mmol) in ethyl acetate (5 ml) was ozonised and worked up with dimethyl sulphide as described previously to give the dimedone of formaldehyde (10.0 mg, 48%), m.p. 191-192 °C.

Ozonolysis of (2S)-3,6-Dioxo-[4-^{14}C]methyl-5-methylene-1,2-trimethylenepiperazine (111) - (2S,5R)-[4-^{14}C]Methyl-3,6-dioxo-5-hydroxy-5-methyl-1,2-trimethylenepiperazine (24.25 mg, 2.79 μ Ci mmol^{-1}) was dissolved in trifluoroacetic acid and shaken for 5 min. The solvent was removed under vacuum. The residue (111) was dissolved in ethyl chloride, ozonised, and worked up with dimethyl sulphide as described previously to give the dimedone derivative of formaldehyde (19.3 mg, 54%, 0.02 μCi mmol^{-1}). Recrystallisation from methanol-ether gave needles (11.5 mg), m.p. 191-191.5°C, ^{14}C activity 0.01 μCi mmol^{-1}.

Attempted Oxidation of the Phenol (104) - The phenol (104) (10 mg) was treated with chromic acid as described by Ali et al.[92] A black solid was obtained which could not be characterised.

The phenol (10 mg) was acetylated as described by Ali et al.[92] and treated with chromic acid as above. No simple product could be detected.

Attempted Alkylation of NN-Diacetyl Glycine Anhydride (109)- The diacetylated anhydride (109) was treated with formaldehyde by the method of Gallina and Liberatori[95]. A plastic film was obtained which showed several spots on t.l.c.

8.7 Experimental for Chapter 6.

N-Benzyloxycarbonyl-L-serine Methyl Ester (124) - L-Serine methyl ester hydrochloride was converted into N-benzyloxycarbonyl-L-serine methyl ester by the method of Guttman and Boissonas[71] in 80% yield as an oil. A sample was purified by vacuum distillation (155 °C, 0.1 mm Hg), $[\alpha]_D^{15}$ + 5.75° (c 2.0, chloroform), $[\alpha]_D^{15}$ - 10.9° (c 1.6, methanol) (lit.,[114] - 13.2°); $\nu_{max.}$ (thin film) 3 680 - 3 140, 1 760 - 1 680, 1 560 - 1 490, 1 065, 745, and 705 cm^{-1}; δ (60 MHz) 7.40 (5H, s, Ph), 6.20 - 5.65 (1H, m, D_2O exch., NH), 5.15 (2H, s, $PhCH_2O$), 4.70 - 4.30 (1H, m, $CHCH_2$), 4.05 - 3.80 (2H, br d, J 4 Hz, CH_2OH), 3.75 (3H, s, OCH_3), 2.75 (1H, br s, D_2O exch., OH); m/e 253 (weak, M), 162 ($M-C_7H_7$), 150, 108, 107, 92, 91, 79, 77, and 65.

N-Benzyloxycarbonyl-DL-serine methyl ester was similarly prepared in 87% yield.

N-Benzyloxycarbonyl-O-t-butyl-L-serine Methyl Ester (125) - The t-butyl ester (125) was synthesised from N-benzyloxycarbonyl-L-serine methyl ester (124) by the method of Callahan et al.[101] to give an oil which was taken up in pet. ether (80-100 °C) with slight warming and decanted from an insoluble oil. The solvent was removed by evaporation and the residual oil was taken up again in a minimum of pet. ether (80-100 °C) at room temperature. The solution was cooled overnight to give crystals which were recrystallised as needles (125) (53%), m.p. 41-43 °C (lit.,[115] 42-44 °C), $[\alpha]_D^{11}$ + 4.6° (c 1.4, EtOH) (lit.,[101]

+ 6.0°), $[\alpha]_D^{11}$ + 4.0° (c 1.2, MeOH) (lit.,[115] + 4.0°); $\nu_{max.}$ 3 480,- 3 300, 1 750, 1 730, 1 510, 1 395, 1 365, 1 205, 740, and 700 cm^{-1}; δ (60 MHz) 7.35 (5H, s, Ph), 5.65 - 5,40 (1H, br d, NH), 5.15 (2H, s, $PhCH_2O$), 4.60 - 4.25 (1H, m, $CHCH_2$), 3.95 - 3.40 (2H, ABX, J_{AX} 4.0 Hz, J_{BX} 3.0 Hz, J_{AB} 9.5 Hz, $CHCH_2$), and 3.75 (3H, s, OMe), 1.10 (9H, s, Bu^t); m/e 309 (weak, M), 223 (22%), 162 (28), 108 (10), 107 (10), 92 (11), 91 (100), 79 (15), 77 (10), 65 (12), 57 (37).

N-Benzyloxycarbonyl-O-t-butyl-DL-serine methyl ester was similarly prepared in 74% yield, m.p. 77-79 °C (lit.,[114] 78.5-79 °C).

N-Benzyloxycarbonyl-O-t-butyl-L-serine (122) - A solution of N-benzyloxycarbonyl-O-t-butyl-L-serine methyl ester (10.64 g, 34.4 mmol) in 2M sodium hydroxide (34.4 ml) and ethanol (34.4 ml) was shaken for 10 min. Acetic acid was added to neutrality and after removal of ethanol by evaporation, the residue was taken up into a mixture of methylene chloride and water. The aqueous layer was separated and extracted with methylene chloride (2 x). The combined extracts were dried, filtered and concentrated. The residue was then taken up in 10% sodium hydrogen carbonate solution (150 ml). After washing twice with ether (20 ml), the aqueous solution was cooled and acidified with concentrated hydrochloric acid, then extracted with ether (4 x). The organic extracts were dried, filtered and concentrated to an oil which

solidified after several hours under vacuum (9.472 g, 93%). Crystallisation from cyclohexane gave needles (8.65 g, 85.2%), m.p. 81-86 °C (lit.,[101] 87-87.5 °C); R_F 0.5 (pet. ether - EtOAc-AcOH; 2:1:0.1), $[\alpha]_D^{18}$ + 18.0° (c 2.4, EtOH) (lit.,[101] + 22.7°); $\nu_{max.}$ 3 435, 3 300 - 2 800, 1 750, 1 730, 1 685, 1 530, 1 385, 1 365, 1 210, 1 080, 730, and 695 cm^{-1}; δ 11.40 - 11.20 (1H, br s, D_2O exch., COOH), 7.37 (5H, s, Ph), 5.90 - 5.55 (1H, m, D_2O exch., NH), 5.15 (2H, s, $PhC\underline{H}_2O$), 4.70 - 4.30 (1H, m, $C\underline{H}CH_2$), 4.05 - 3.45 (2H, ABX, J_{AB} 9.5 Hz, J_{AX} 4.0 Hz, J_{BX} 3.0 Hz, $C\underline{H}_2OBu^t$), 1.15 (9H, s, Bu^t); m/e 295 (weak, M), 209 (6%, $M+H-CH_2OBu^t$), 148 (7), 108 (5), 107 (9), 92 (11), 91 (100), 90 (11), 79 (9), 77 (13), 65 (25), 59 (15) and 57 (50).

N-Benzyloxycarbonyl-O-t-butyl-DL-serine was also synthesised by the above procedure yielding an oil (86%).

N-Benzyloxycarbonyl-O-t-butyl-N-methyl-L-serine (128) - Methyl iodide (2.5 ml) and sodium hydride (660 mg, 27.5 mmol) were added to a stirred, ice cold solution of N-benzyloxycarbonyl-O-t-butyl-L-serine (122) (1.475 g, 5 mmol) in dry tetrahydrofuran (20 ml). Stirring was continued for 3 days at 0 °C. The solution was decanted from excess sodium hydride and then ethyl acetate followed by a few drops of water, were added to the solution to destroy suspended sodium hydroxide and hydride. The solvent was removed by evaporation and the residue was taken up in a mixture of water (100 ml) and ether (30 ml). The organic phase was

separated and extracted with sodium hydrogen carbonate solution. The combined aqueous extracts were cooled, acidified to pH 3-4 with citric acid and extracted with ethyl acetate. The organic extracts were washed with water, dried, filtered and concentrated to give (128) as an oil (1.51 g, 98%) which showed one spot on tlc, R_F 0.3 (pet. ether - EtOAc - AcOH; 2:1:0.1), $[\alpha]_D^{15}$ + 4.8° (c 1.5, $CHCl_3$), (Found: C, 62.25; H, 7.20; N, 4.54. $C_{16}H_{23}NO_5$ requires C, 62.13; H, 7.44; N, 4.53%); $\nu_{max.}$ ($CHCl_3$) 3 300 - 2 800, 1 770, 1 730 - 1 690, 1 395, 1 365, cm^{-1}; δ (60 MHz) 9.65 - 9.25 (1H, br s, D_2O exch., COOH), 7.35 (5H, s, Ph), 5.15 (2H, s, $PhCH_2O$), 5.00 - 4.55 (1H, m, $CHCH_2$), 3.95 - 3.60 (2H, m, CH_2OBu^t), 3.03 (3H, s, NMe), and 1.15 (9H, s, Bu^t); m/e 309 (weak, M), 223 (9%, $M+H-CH_2OBu^t$), 148 (8), 92 (15), 91 (100), 77 (9), 65 (19), and 57 (36).

N-Benzyloxycarbonyl-O-t-butyl-N-methyl-DL-serine was similarly prepared as an oil (87%).

N-Benzyloxycarbonyl-O-t-butyl-N-methyl-L-serine Methyl Ester (130) - N-Benzyloxycarbonyl-O-t-butyl-N-methyl-L-serine (128) (3.68 g, 11.9 mmol) was dissolved in ether and a solution of diazomethane[116] in ether was added until a yellow solution was observed. The solution was stood overnight and washed with sodium hydrogen carbonate solution, dried, filtered, and concentrated to a yellow oil, (3.624 g, 94%), R_F 0.45 (pet. ether - EtOAc; 4:1). A sample was purified by preparative tlc to give the methyl ester

(130) as a clear oil, $[\alpha]_D^{21}$ - 7.6° (c 2.7, $CHCl_3$), (Found: C, 63.00; H, 8.01; N, 4.31. $C_{17}H_{25}NO_5$ requires C, 63.16; H, 7.74; N, 4.33%); $\nu_{max.}$ (thin film) 3 700 - 3 100, 1 740, 1 695, 1 395, 1 360, 735 and 695 cm^{-1}; δ (60 MHz) 7.40 (5H, s, Ph), 5.20 (2H, s, $PhCH_2O$), 4.95 - 4.50 (1H, m, $CHCH_2$), 3.90 - 3.50 (2H, m, CH_2OBu^t), 3.75 (3H, br s, COOMe), 3.05 (3H, s, NMe), and 1.15 (9H, s, Bu^t); m/e 323 (weak, M), 237 (20%, M+H-CH_2OBu^t), 162 (16), 146 (20), 102 (25), 92 (14), 91 (100), 65 (10), 59 (10), 57 (35).

O-t-Butyl-N-methyl-L-serine Methyl Ester (129) - A solution of N-benzyloxycarbonyl-O-t-butyl-N-methyl-L-serine methyl ester (130) (3.454 g, 10.7 mmol) in methanol (100 ml) was hydrogenated over 10% palladium on charcoal for 40 min. The catalyst was removed by filtration through a Celite pad. The filtrate was concentrated to give the amino ester (129) (1.9 g, 94%), R_F 0.6 ($CHCl_3$ - MeOH - Et_2NH; 10:1:0.01) (Found: C, 57.1; H, 10.23; N, 7.29. $C_9H_{19}NO_3$ requires C, 57.14; H, 10.05; N, 7.41%); $\nu_{max.}$ (thin film) 3 600 - 3 200, 2 800, 1 740, 1 390, 1 360, and 1 085 cm^{-1}; δ (60 MHz) 3.95 (3H, s, OMe), 3.75 - 3.20 (3H, m, $CHCH_2$), 2.45 (3H, s, NMe), 2.05 (1H, br s, D_2O exch., NH), and 1.15 (9H, s, Bu^t).

Benzyloxycarbonyl-L-phenylalanyl-O-t-butyl-N-methyl-L-serine Methyl Ester (136) - DCC (228 mg, 1.1 mmol) was added

to an ice cold solution of benzyloxycarbonyl-L-phenylalanine (81) (299 mg, 1 mmol) and the methyl ester (129) (189 mg, 1 mmol) in methylene chloride (10 ml), then the reaction was allowed to stand overnight at room temperature. DCU was removed by filtration through a Celite pad and the latter was washed with methylene chloride. The filtrate was washed with 1M hydrochloric acid, water, sodium hydrogen carbonate solution and water, then dried, filtered and concentrated to an oil. The oil was taken up in a minimum of acetone and a further precipitate of DCU was removed by filtration. Evaporation of the solvent left an oil (414 mg, 88%) showing one major spot on tlc corresponding to the product (136), R_F 0.60 (pet. ether - EtOAc - AcOH; 12:6:1) and one minor spot corresponding to DCU, R_F 0.95).

A sample of the oil was purified by preparative tlc for analysis to give the protected dipeptide (136), $[\alpha]_D^{15}$ + 6.75° (c 1.14, $CHCl_3$) (Found: C, 66.22; H, 7.08; N, 5.80. $C_{26}H_{34}N_2O_6$ requires C, 66.38; H, 7.23; N, 5.96%); $\nu_{max.}$ ($CHCl_3$) 3 430, 1 740, 1 720, 1 650, 1 500, 1 395, and 1 365 cm^{-1}; δ (60 MHz) 7.35 (5H, s, Ph), 7.25 (5H, s, Ph), 5.85 - 5.55 (1H, m, NH), 5.10 (2H, br s, $PhCH_2O$), 5.20 - 4.60 (2H, m, 2 x CH), 3.85 - 3.55 (2H, m, CH_2OBu^t), 3.70 (3H, s, COOMe), 3.05 (3H, s, NMe), 3.20 - 2.80 (2H, m, CH_2Ph), 1.15 (9H, s, Bu^t).

cyclo-(L-Phenylalanyl-O-t-butyl-N-methyl-L-seryl) (138) - A solution of benzyloxycarbonyl-L-phenylalanyl-O-t-butyl-N-

methyl-L-serine methyl ester (128) (2.185 g, 4.65 mmol) in methanol (100 ml) was hydrogenated for 5 h in the presence of 10% palladium on charcoal (220 mg) and acetic acid (6 ml). The catalyst was removed by filtration through a Celite pad, which was washed with hot methanol and the filtrate was concentrated to an oil. Residual acetic acid was removed by azeotroping with methanol and the resulting oil was taken up in acetone - pet. ether and cooled. Crystals were filtered off and washed with ethyl acetate then ether. Recrystallisation from ethyl acetate yielded cyclo-(L-phenylalanyl-O-t-butyl-N-methyl-D-seryl) (139) as cubes (120.2 mg, 8.5%), m.p. 213-215 °C, R_F^F 0.45 (Found: C, 67.30; H, 8.05; N, 9.39. $C_{17}H_{24}N_2O_3$ requires C, 67.10; H, 7.89; N, 9.21%), $[\alpha]_D^{20}$ - 123.8° (c 1.0, $CHCl_3$); $\nu_{max.}$ 3 270, 1 688, 1 645, 1 100, 760 - 750, and 705 cm^{-1}; δ 7.25 (5H, m, Ph), 5.88 (1H, br s, NH), 4.45 - 4.28 (1H, ABX, $J_{AX} + J_{BX}$ 13.5 Hz, $CHCH_2Ph$), 3.90 - 3.47 (4H, m, 2 x CH_2), 2.96 (3H, s, NMe), 2.95 - 2.62 (1H, ABX, $J_{AX} + J_{BX}$ 25 Hz, $CHCH_2OBu^t$), and 1.12 (9H, s, Bu^t); m/e 304 (weak, M), 218 (35%, M+H-CH_2OBu^t), 127 (50), 99 (16, $CH{=}CHOBu^t$), 91 (100), 65 (20), 57 (100), 56 (25).

The mother liquor, taken up in ethyl acetate - pet. ether (1:1), was separated by column chromatography on alumina o (60 g). Elution with ethyl acetate - pet. ether (1:1) gave fractions containing a solid which was crystallised from acetone - pet. ether to give the cyclo-dipeptide (138), (442 mg, 31.3%), m.p.

133-138 °C, $[\alpha]_D^{20}$ - 149.7° (c 1.4, $CHCl_3$), R_F^F 0.35, (Found: C, 67.32; H, 8.09; N, 9.32. $C_{17}H_{24}N_2O_3$ requires C, 67.10; H, 7.89; N, 9.21%); $\nu_{max.}$ 3 350, 3 235, 1 680, 1 650, 1 390, 1 365, 1 095, 750, and 700 cm^{-1}; 7.41 - 7.14 (5H, m, Ph), 5.85 - 5.75 (1H, br s, NH), 4.20 - 3.88 (2H, m, 2 x CH), 3.80 - 3.70 (2H, m, $C\underline{H}_2Ph$), 3.55 - 2.98 (2H, ABX, J_{AX} 11 Hz, J_{BX} 3.5 Hz, J_{AB} 13 Hz, $C\underline{H}_2OBu^t$), 2.99 (3H, s, NMe), and 1.20 (9H, s, Bu^t); δ (CD_3OD) 7.29 (5H, m, Ph), 4.14 (1H, br t, J 7 Hz, $C\underline{H}CH_2Ph$), 4.02 (1H, t, J 3 Hz, $C\underline{H}CH_2OH$), 3.81 - 3.31 (2H, ABX, J_{AX} 3 Hz, J_{BX} 3 Hz, J_{AB} 10 Hz, $C\underline{H}_2OBu^t$), 3.24 (2H, br d, J 7 Hz, $C\underline{H}_2Ph$), 3.01 (3H, s, NMe), and 1.22 (9H, s, Bu^t); m/e 304 (weak, M), 218 (90%, $M+H-CH_2OBu^t$), 127 (100), 99 (20), 91 (32), 57 (57), and 56 (7).

cyclo-(L-Phenylalanyl-N-methyl-L-seryl) (29) - cyclo-(L-Phe-L-(NMe,OBu^t)Ser) (138) (99 mg, 0.33 mmol) was dissolved in 45% HBr - AcOH (1-2 ml) over a period of 10 min. Dry ether (ca. 50 ml) was added and the contents were mixed and cooled overnight to precipitate a white solid which was triturated with ether. On exposure to air an oil (160 mg) was formed which was applied to preparative tlc. The product (29) was obtained as an oil which was solidified by slow evaporation from ethyl acetate, then recrystallised from ethyl acetate to give fan-like crystals (30 mg, 37%), m.p. 164.5-165 °C, $[\alpha]_D^{21}$

-127.6° (c,1.0, MeOH); R_F 0.15 (chloroform - methanol - diethylamine; 15:1:0.1); (Found: C, 62.60; H, 6.49; N, 11.50. $C_{13}H_{16}N_2O_3$ requires C, 62.90; H, 6.45, N, 11.29%); $\nu_{max.}$ 3 300, 1 665, 1 650, 1 090, 750, and 705 cm^{-1}; δ [$(CD_3)_2SO$] 7.92 (1H, br s, D_2O exch., NH), 7.50 - 7.05 (5H, m, Ph), 5.18 (1H, br t, J 6 Hz, D_2O exch., OH), 4.04 (1H, m, sharpens on D_2O shake to br t, J 6.5 Hz, $C\underline{H}CH_2Ph$), 3.81 (1H, m, sharpens on D_2O shake to br t, J 3.5 Hz, $C\underline{H}CH_2OH$), 3.72 - 3.15 (2H, m, sharpens on D_2O shake to ABX, $J_{AX} = J_{BX}$ 3.5 Hz, J_{AB} 12 Hz, $C\underline{H}_2OH$), 3.07 (2H, br d, J 6 Hz, $C\underline{H}_2Ph$), 2.86 (3H, s, NMe); m/e 248 (6%, M), 218 (100, $M-CH_2O$), 189 (20), 157 (13, $M-C_7H_7$), 129 (23, $M-PhCH_2CH=NH$), 127 (63, $M-C_7H_7-CH_2O$), 120 (12), 99 (25), 91 (64), and 65 (14) with metastable ion at 191.5.

cyclo-(L-Phenylalanyl-O-acetyl-N-methyl-L-seryl) (140) - The previous experiment was allowed to stand for 30 min, then worked up as usual. The acetyl derivative was obtained from preparative tlc as an oil which was solidified by slow evaporation from acetone. The solid was triturated then crystallised from acetone, to give the acetyl product (140), (5 mg, 6.0%), m.p. 172 -177 °C, $[\alpha]_D^{24}$ -141.7° (c 0.6, MeOH), R_F 0.35 (chloroform - methanol - diethylamine; 15:1: .1), (Found: C, 61.75; H, 6.03; N, 9.46. $C_{15}H_{18}N_2O_4$ requires C, 62.07; H, 6.21; N, 9.65%); $\nu_{max.}$ 3 250, 1 750, 1 685, 1 640, 1 230, 755, and 710 cm^{-1}; δ (CD_3OD)

7.35 (5H, br s, Ph), 4.40 - 3.95 (3H, m, $C\underline{H}_2OAc$, $C\underline{H}CH_2Ph$), 3.63-3.41 (1H, ABX, $J_{AX} + J_{BX}$ 19.5 Hz, $C\underline{H}CH_2OAc$), 3.25-3.05 (2H, m, $C\underline{H}_2Ph$), 3.05 (3H, s, NMe), 2.05 (3H, s, OMe); m/e 290 (8%, M), 230 (64, M-CH_3COOH), 157 (37), 127 (24, M-C_7H_7-CH_2OAc), 111 (29), 91 (100), and 65 (30).

cyclo-(L-Phenylalanyl-N-methyl-D-seryl) (141) - cyclo-(L-Phenylalanyl-O-t-butyl-N-methyl-D-seryl)(139) (80 mg, 0.263 mmol) was converted as described for (138) into the cyclic dipeptide (141), (15 mg), m.p. 159-166.5 °C and recrystallised from methanol - ether - pet. ether (6.3 mg, 9.5%) m.p. 163.5-165 °C, $[\alpha]_D^{22}$ - 47.8° (c 2.8, MeOH), (Found: C, 62.63; H, 6.35; N, 11.14. $C_{13}H_{16}N_2O_3$ requires C, 62.90; H, 6.45; N, 11.29%); $\nu_{max.}$ 3 380, 3 260, 1 690, 1 645 - 1 630, cm^{-1}; δ (CD_3OD) 7.20 5H, s, Ph), 4.38 (1H, br t, J 5 Hz, $C\underline{H}CH_2Ph$), 3.85 (2H, d, J 2.5 Hz, $C\underline{H}_2OH$), 3.50 - 3.08 (m, $C\underline{H}CH_2OH$, CH_2Ph), 2.83 (3H, s, NMe); m/e 248 (16%, M), 218 (96, M-CH_2O), 188 (28), 157 (20, M-C_7H_7), 129 (30, M-$PhCH_2CH{=}NH$), 127 (76, M-C_7H_7- CH_2O), 120 (18), 99 (25), 91 (100).

cyclo-(L-[U-^{14}C]Phenylalanyl-N-methyl-L-seryl) - L-[U-^{14}C] Phenylalanine (165.5 mg, 100 μCi $mmol^{-1}$) was converted as previously described into benzyloxycarbonyl-L-[U-^{14}C]phenylalanine (97%, ^{14}C activity, 83.8 μCi $mmol^{-1}$), then coupled with O-t-butyl-N-methyl-L-serine methyl ester (129) (1 mmol) to give an oil (87%). Hydrogenation was carried out as normal to give an oil

The oil was dissolved in 45% HBr/AcOH over a 30 min period and worked up as usual to give, after preparative tlc and crystallisation the product (29) as cubes (39 mg, 16% from Z-L-Phe-OH), m.p. 164 - 165 °C, ^{14}C activity 82.78 μCi $mmol^{-1}$ (98.8%). The presence of only one radioactive product was established by radioscanning and autoradiography of tlc plates rum in 3 solvent systems and by dilution analysis with unlabelled cyclo-(L-Phe-L-(NMe)Ser) as diluent.

N-Benzyloxycarbonyl-O-t-butyl-L-serine t-Butyl Ester (126) - N-Benzyloxycarbonyl-L-serine (4,78 g, 20 mmol) was converted to (126) by the method of Callahan et al.[101] to give an oil (5.23 g, 75%); $\nu_{max.}$ (film) 3 490 - 3 250, 1 760 - 1 690, 1 500, 1 390, 1 360, 735, and 695 cm^{-1}; δ (60 MHz) 7.35 (5H, s, Ph), 5.80 - 5.40 (1H, m, NH), 5.15 (2H, s, $PhCH_2$), 4.55 - 4.15 (1H, m, CH), 4.00-3.40 (2H, m, CH_2OBu^t), 1.65 (9H, s, $COOBu^t$) and 1.10 (9H, s, CH_2OBu^t); m/e 351 (weak, M), 209 (35%), 148 (25), 91 (100), 57 (68).

O-t-Butyl-N-methyl-DL-serine (132) - A solution of N-benzyl-oxycarbonyl-O-t-butyl-N-methyl-DL-serine (535 mg, 1.79 mmol) in methanol (20 ml) and acetic acid (0.1 ml) was hydrogenated for 2 h over 10% palladium on charcoal (50 mg). The solution was filtered and evaporated to give a solid which was crystallised from methanol - ether to yield the amino-acid (132) (271 mg,

86%), m.p. 191-198 °C (sublimation), (Found: C, 54.80; H, 9.56; N, 7.93. $C_{18}H_{17}NO_3$ requires C, 54,86; H, 9.71; N, 8.00%); $\nu_{max.}$ (Nujol) 3 600 - 2 200, 1 650 - 1 590,- cm^{-1}; δ (CD_3OD) (60 MHz) 4.20 - 3.25 (3H, m, $CHCH_2$), 2.75 (3H, s, NMe), 1.25 (9H, s, Bu^t); m/e 176 (weak, M+1), 175 (weak, M), 102 (45%, M-OBu^t), 89 (100, M+H-CH_2OBu^t), 88 (45, M-CH_2OBu^t), 74 (64), 57 (100), and 56 (44).

Attempted Hydrolysis of the Ester (125) - The ester (125) was treated with lithium iodide in dimethylformamide[103] and with sodium thiomethoxide in hexamethylphosphoramide.[104] Starting material was recovered in both experiments and there was no evidence for hydrolysis.

Attempted Hydrolysis of the Ester (126) - The ester (126) was treated with benzene and p-toluenesulphonic acid. The product was studied by t.l.c. and n.m.r. spectroscopy and found to be a mixture of the starting material and two other products of which Z-L-Ser-OH was predominant.

Attempted Deprotection of the Butyl Ether (138)- The butyl ether (138) was treated, in turn, with benzene and p-toluene-sulphonic acid under reflux, trifluoroacetic acid, and chloroform-hydrochloric acid. Starting material was recovered in all these experiments. The ether (138) was treated with benzene-concentrated sulphuric acid under reflux. The product showed several spots on t.l.c.

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