BIOSYNTHETIC STUDIES IN HETEROCYCLIC FUNGAL METABOLITES

A thesis presented in part fulfilment of the requirement for the Degree of Doctor of Philosophy

by

Maria Isabel Pita Boente

Department of Chemistry

University of Glasgow

January 1981.

CONTENTS

ACKNOWLEDGEMENT

Three years usually represent a short period in someone's life. Yet, when the three years are spent doing something enjoyable, in pleasant surroundings and with interesting people, they acquire a very special significance. Unfortunately, it is not possible to acknowledge all the people that somehow contributed to such an experience.

Professor G.W.Kirby and Dr. D.J.Robins continuously provided the support, the encouragement and the ideas that made possible the execution and the writing-up of this thesis. They also introduced me to the fascinating world of British cryptic crosswords, and were as patient with my often clumsy attempts at them, as they were with my frequent mishaps at the bench.

The Staff of the Mycology Unit, although usually dreading what I would ask for next, were always ready to help and, with an endurance worthy of Job, put up with my almost continuous upsetting of their culture growth schedules.

My fellow lab-mates contributed to creating an atmosphere in which, in spite of all its trials and tribulations, Chemistry can become a most satisfying and absorbing occupation. Among them Harry McGuigan and David McLean were very special sources of inspiration and consolation when sprits were low. Dr. Robert Hill and Marshall Stark undertook the tedious job of, respectively, typing and proof-reading this script. Dr. David Rycroft and Tony Ritchie often went beyond the call and time of duty to produce and discuss innumerable n.m.r. and mass spectra.

Three very special ladies had many a "wee blether" with me throughout these three years: Liz Breslin, Helen Prentice and Pearl

Tait. They taught me a lot about Glasgow, its people and their ways and made this foreigner feel very much at home.

My companion, Jose, although intrigued by my anthropomorphic relationship with my fungi, patiently listened to my problems and tried very hard to teach me not to make a drama out of a crisis.

The Venezuelan National Council for Scientific and Technological Research (CONICIT) provided the essential economic support.

To all mentioned above, I can only say: "Thank-you very much!"

Marie

SUMMARY

The tryptophan analogues, β-(2-azaindol-3-yl)-, β-(7-azaindol-3-yl)- and β-(1-benzothien-3-yl)-alanine, were synthesised and fed to Penicillium cyclopium cultures. The three compounds caused inhibition of normal fungal growth and metabolite production. 3-Acetyl-5-(indol-3-ylmethyl)-tetramic acid, a known intermediate in cyclopiazonic acid biosynthesis, labelled with ^{14}C, was synthesised. After administration of this compound to cultures of P. cyclopium, no radioactivity was detected in the metabolites. It is presumed that cell-wall permeability problems were responsible for this result. When grown on a replacement medium containing no exogenous nitrogen source, P. cyclopium produced α- and β-cyclopiazonic acids only when small amounts of 3-acetyl-5-(indol-3-ylmethyl)tetramic acid were added. However, when 3-[$^{2}H_3$]acetyl-5-(indol-3-ylmethyl)-[5-^{2}H]tetramic acid was administered, the α- and β-cyclopiazonic acids isolated contained no deuterium. These results suggest that the tetramic acid is first degraded to tryptophan, which can then lose the ^{2}H-atom in a transamination reaction, prior to incorporation into the metabolites.

cyclo-(L-Phenylalanyl-D-seryl), labelled with ^{14}C, was synthesised and administered to cultures of Hyalodendron sp. and found to be poorly incorporated into bisdethiobis(methylthio)hyalodendrin. This finding complemented previous results obtained in this group and indicated that of the four cyclo-(phenylalanyl-seryl) stereoisomers, only the LL-isomer is an efficient precursor for hyalodendrin biosynthesis. Moreover, experiments involving the feeding of cyclo-(L-phenylalanyl-L-[3-^{13}C]seryl) indicated that the precursor is incorporated intact into the metabolite. Exploratory studies on the status of cyclo-

(L-N-Me-Phe-L-Ser) as a precursor for bisdethiobis(methylthio)hyalodendrin are also described.

The stereoisomers of cyclo-(phenylalanyl-phenylalanyl), labelled with ^{14}C, were synthesised and fed to cultures of Apergillus terreus. Only the LL-isomer was incorporated efficiently into bisdethiobis(methylthio)acetylaranotin. L-[U-^{14}C]Phenylalanine was fed to A. terreus and ^{14}C-labelled cyclo-(L-phenylalanyl-L-phenylalanyl) was isolated in an "intermediate-trapping" experiment. This indicated that the fungus was capable of producing cyclo-(L-phenylalanyl-L-phenylalanyl). Furthermore, feeding experiments using cyclo-(L-[^{15}N]phenylalanyl-L-[1-^{13}C]phenylalanyl) and cyclo-(L-[3,3-$^{2}H_2$]phenylalanyl-L-[3,3-$^{2}H_2$]phenylalanyl) indicated that, in its transformation into bisdethiobis(methylthio)acetylaranotin, cyclo-(L-phenylalanyl-L-phenylalanyl) is incorporated intact but with partial, i.e. non-obligatory, exchange of methylene hydrogen.

GENERAL INTRODUCTION

In its earliest days, organic chemistry was confined to the study of those materials which are available from natural sources. Following the isolation of these materials, chemists were then concerned with the investigation of their structures, which eventually led to attempts at their synthesis. The next logical question that chemists asked themselves was how Nature went about making such a variety of structurally different compounds. Wohler's synthesis of urea from ammonium cyanate had already disproved the old theory of a "vital force" at work. The study of the biosynthesis of natural products was thus originated and it received its greatest push forward with the advent of nuclear technology in the early forties, which led to the availability of radioactively labelled compounds, chiefly ^{14}C and ^{3}H.

Living organisms synthesise and degrade chemical compounds by means of a series of enzyme-mediated chemical reactions, collectively known as metabolism. The products of these metabolic processes have been classified as primary and secondary metabolites. Primary metabolites are derived from metabolic pathways common to all organisms and are essential to their survival and well being. Examples of primary metabolites are amino acids, sugars, nucleic acids, common fatty acids and polymers derived from them. Secondary metabolites are products biosynthesised from primary metabolites by specialised pathways and are species, and often strain, specific. Secondary metabolism is well developed in plants and microorganisms and leads to compounds such as phenols, alkaloids and terpenes.

The exact purpose of this wide range of secondary metabolites has been the subject of much speculation. It has been suggested that these metabolites may have the dual role of being a means of food storage and of influencing the growth, health, behaviour and population biology of other living organisms sharing the same environment. We will be concerned here with some secondary metabolites produced by fungi.

Together with the algae and bacteria, fungi have been traditionally classified as members of the Thallophyta, a division of the plant kingdom which comprises organisms with no true roots, stems or leaves. The individual reproductive bodies of fungi are the spores and these are borne by sporophores, which are the visible fruiting bodies commonly known as mushrooms and toadstools. Under favourable conditions, spores become detached from the sporophores and, if they reach a suitable environment, they grow and produce the vegetative phase of the fungus. This is known as the mycelium and consists of a network of fine, branched filaments, individually known as hypha.

Fungi lack photosynthetic pigments and are therefore unable to manufacture organic compounds from carbon dioxide and water. They require oxidisable organic compounds as energy sources. In their natural habitat, these can be obtained from dead or living plants, animals or other microorganisms. In cultures, they are grown on artificial media containing a carbon source (usually a sugar), a nitrogen source (usually ammonia, nitrate or an amino acid) and a variety of other vital ions and trace elements.

Fungi can be induced to utilise most organic compounds and this enables the investigation of their secondary metabolism. Administration of isotopically labelled precursors, followed by the isolation and investigation of the metabolic products, has made possible the

elucidation of the pathways leading to a variety of compounds of biological interest. In establishing biosynthetic routes by use of isotopically labelled precursors, the investigator must overcome several experimental difficulties. The first and most obvious one is that the organism under study must be producing the metabolites of interest in amounts sufficient to permit their isolation and purification. The added precursor must then be able to enter the system. Once inside, it must become metabolically equivalent to the endogenous substrate it is intended to label. The labelled metabolite obtained in the end will be diluted with endogenous metabolite and the extent of this dilution will affect the sensitivity of the detection system. Therefore, the experiment must be designed so as to allow a detectable amount of labelled precursor to be incorporated, without this amount causing perturbations to the normal state of the organism. The sensitivity of the detection system depends on the nature of the label used. Radioactive tracers offer the advantage of accurate and sensitive detection, but do not yield information as to the precise location of the label in the final metabolite. This has to be established by long and often complicated procedures involving the degradation of the metabolite to simple products, usually containing just one or two carbons, through which the exact position of the label can be ascertained. Frequently, the amounts of metabolite available, its impure condition or the lack of suitable chemical reactions, make complete degradation studies impossible. The use of stable isotopes, detectable by mass spectrometry and/or nuclear magnetic resonance spectroscopy has allowed the determination of the exact location of the introduced label(s) without having to resort to chemical degradations. However, stable isotopes also have their drawbacks, the main one being the need to

introduce larger amounts of the precursor to permit reasonable detection.

Some of the techniques briefly outlined above have been used throughout this study. In the first part of this work, some aspects of the biosynthesis of a fungal toxin, cyclopiazonic acid, will be investigated. The second part deals with a variety of fungal metabolites with antibiotic properties which possess as a common feature an epidithiodioxopiperazine moiety.

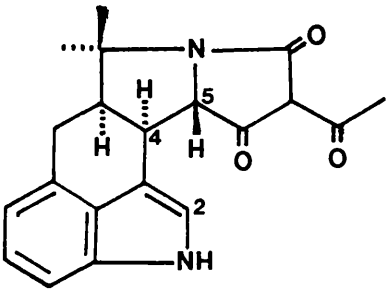
PART I

STUDIES RELATED TO THE BIOSYNTHESIS OF CYCLOPIAZONIC ACID

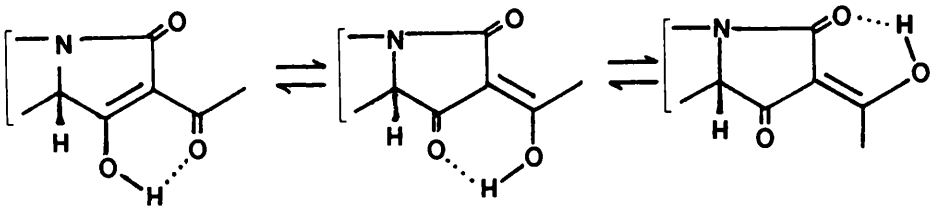
CHAPTER 1

INTRODUCTION

Penicillium cyclopium Westling is a saprophytic fungus frequently found growing on stored grain and cereal products. Outbreaks of cattle poisoning in the United States in 1964 were attributed by Albright *et al.*[1] to the presence of *P.cyclopium* in corn used as feedstuff. The fungus has also been isolated on two other occasions from contaminated feedstuffs which had caused diseases among farm animals.[2] In 1968, Holzapfel investigated a strain (1082) of *P. cyclopium*, obtained from ground nuts and grown on maize meal. He isolated the main toxic principle, cyclopiazonic acid, and, on the basis of chemical and spectroscopic evidence, assigned to it the structure and relative stereochemistry shown in (1).[3]



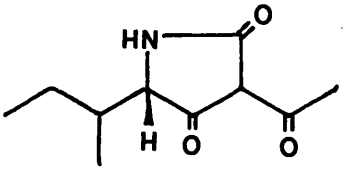
(1)



(2) (3) (4)

Holzapfel[3] initially formulated cyclopiazonic acid as the enol tautomer (2) of the acetyltetramic acid. Ten years later, ^{13}C nuclear magnetic resonance (n.m.r.) studies by Steyn and Wessels[4] indicated that cyclopiazonic acid exists, in deuteriochloroform, as a mixture of three tautomers (2), (3) and (4), of which (4) is the preferred tautomer.

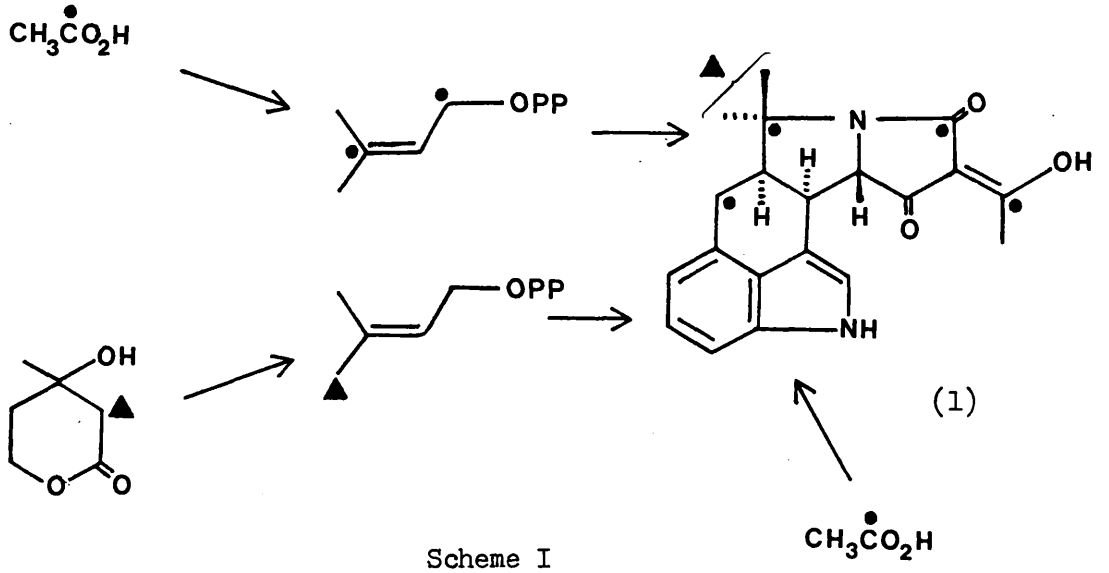
In 1971, from analysis of its structure, Holzapfel and Wilkins[5] suggested that cyclopiazonic acid is biosynthesised from tryptophan and either a C_5 unit derived from mevalonic acid and two acetic acid molecules, or two C_5 units derived from mevalonic acid. The first possibility is analogous to the formation of tenuazonic acid (5) in Alternaria tenuis Auct. from one molecule of L-isoleucine and two molecules of acetic acid.[6]



(5)

In order to establish the major precursors used by the fungus to make cyclopiazonic acid (1), Holzapfel and Wilkins[5] fed [1-^{14}C]acetate, [2-^{14}C]mevalonic acid and DL-[U-^{14}C]tryptophan to cultures of P. cyclopium grown on a synthetic medium.[7] The cyclopiazonic acid isolated from the tryptophan-fed culture contained 24.7% of the radioactivity, thus confirming that tryptophan is a direct precursor of cyclopiazonic acid. Acetic and mevalonic acids gave incorporations of 3.5 and 7.0% respectively.

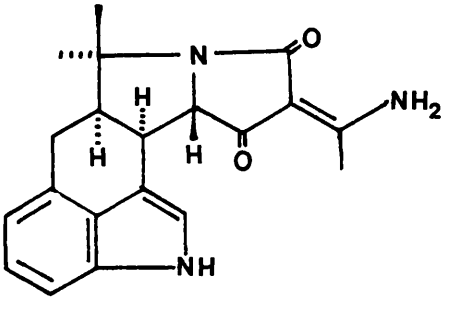
Degradation of the cyclopiazonic acid (1) derived from these two feeding experiments showed that, as with the biosynthesis of tenuazonic acid (5), the other atoms of (1) are derived from two molecules of acetic acid and an isoprene unit formed from mevalonic acid, as shown in Scheme 1.



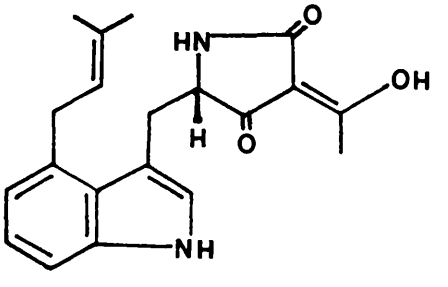
(1)

Scheme I

Two other compounds were isolated from P. cyclopium (1082) by Holzapfel and coworkers: cyclopiazonic acid imine and bissecodehydropiazonic acid, and their structures were shown to be (6) and (7) respectively.[8] The latter is usually referred to in the literature as β-cyclopiazonic acid (7), whilst cyclopiazonic acid itself is known as α-cyclopiazonic acid (1). Subsequently, α-cyclopiazonic acid,



(6)



(7)

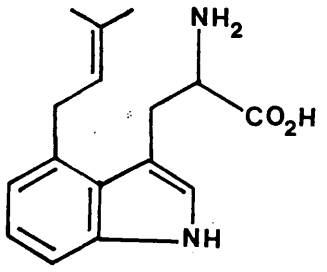
cyclopiazonic acid imine and β-cyclopiazonic acid were also isolated from cultures of Aspergillus versicolor (Vuill.) Tiraboschi.[9] α-Cyclopiazonic acid is also present in Aspergillus flavus Link.[10]

After studies of the reaction of α-cyclopiazonic acid with aqueous ammonia, Holzapfel et al.[8] suggested that cyclopiazonic acid imine (6) might not be the product of an enzymic reaction, but rather that it might result from the direct reaction of α-cyclopiazonic acid and ammonia from the ammonium pool of the fungus. With regard to β-cyclopiazonic acid (7), these workers found that its concentration increased rapidly during the early stages of the fermentation and then decreased rapidly as soon as α-cyclopiazonic acid production accelerated. However, if the fungus was grown on a zinc- or iron-deficient medium, β-cyclopiazonic acid continued to accumulate. Shortly after, five isoenzymes, collectively designated as β-cyclopiazonate oxidocyclase, were isolated from P. cyclopium (1082) and found to be capable of quantitatively converting in vitro β-cyclopiazonic acid into α-cyclopiazonic acid in the presence of air.[11]

These observations suggested that β-cyclopiazonic acid (7) might be a precursor of α-cyclopiazonic acid (1). To test this hypothesis, Holzapfel and Wilkins[5] prepared labelled β-cyclopiazonic acid by growing P. cyclopium on a synthetic medium containing a low zinc concentration and sodium [1-^{14}C]acetate. The labelled β-cyclopiazonic acid thus obtained was then fed to cultures of the fungus grown on the full synthetic medium and the α-cyclopiazonic acid produced was found to contain 67% of the added label. This is compelling evidence that β-cyclopiazonic acid (7) is a direct precursor of (1).

Attention was then turned towards the order in which the simple

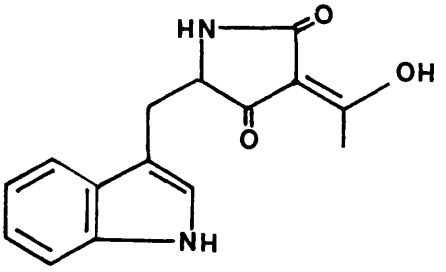
precursors of β- and α-cyclopiazonic acids are assembled. It was suggested, in keeping with the biosynthetic route to the clavine alkaloids,[12] that dimethylallyl tryptophan (8) is an early precursor.



(8)

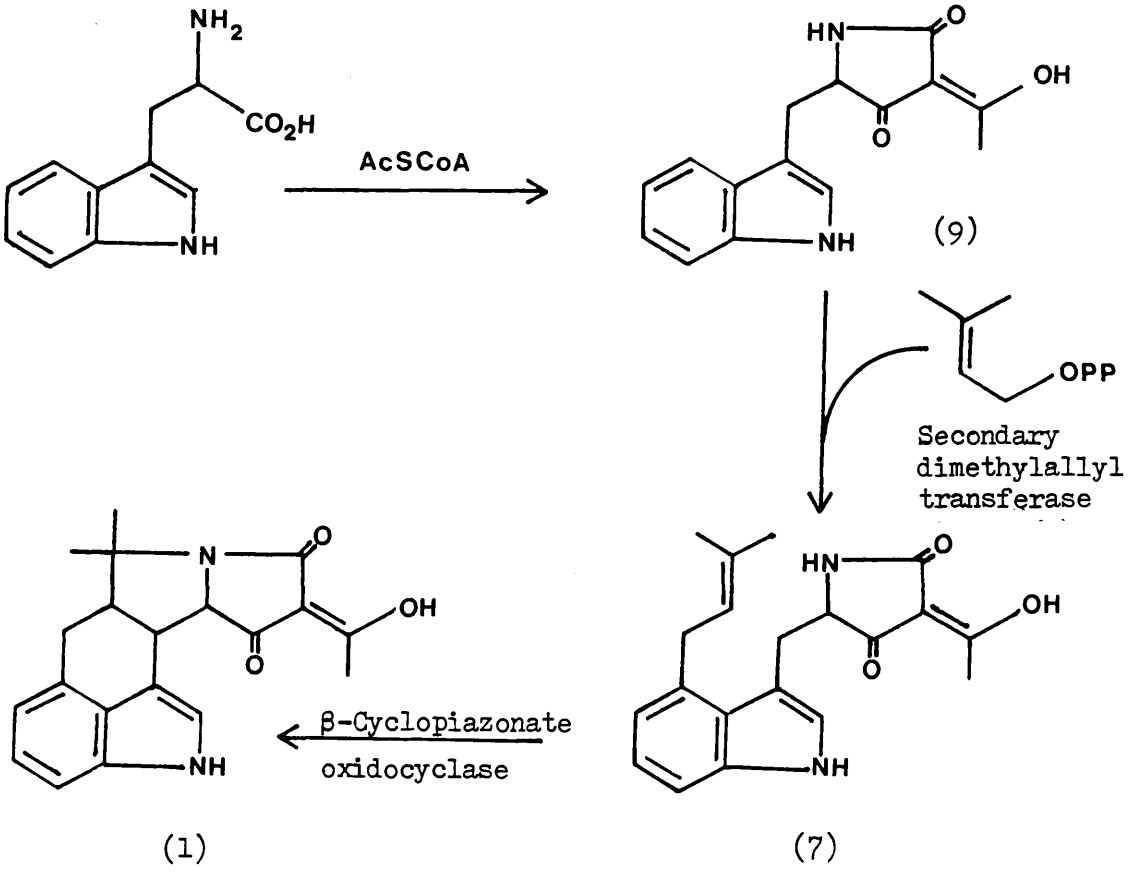
However, this compound has never been isolated from P. cyclopium cultures. McGrath and coworkers[13] fed 4-dimethylallyl[G-^{3}H]tryptophan, mixed with L-[$^{14}CH_2$]tryptophan, to cultures of P. cyclopium and isolated α-cyclopiazonic acid containing 8.4% of the ^{14}C label but no tritium, thus excluding the possible intermediacy of (8).

A second possibility in the sequence of events would involve incorporation of the acetoacetate portion of cyclopiazonic acid prior to incorporation of dimethylallyl pyrophosphate. This possibility was investigated by McGrath and coworkers.[13,14] A mixture of L-[G-^{3}H]-tryptophan and [1-^{14}C]dimethylallyl pyrophosphate was incubated for 1h at 30°C with a cell-free extract prepared from P. cyclopium cultures. The α-cyclopiazonic acid (1) isolated from the reaction mixture contained no ^{3}H or ^{14}C, while the β-cyclopiazonic acid (7) produced contained no ^{3}H but 19% of the added ^{14}C. This indicated that the cell-free extract contained both an enzyme and a substrate which, along with dimethylallyl pyrophosphate, produces β-cyclopiazonic acid. This cosubstrate was isolated and identified as 3-acetyl-5-(indol-3-ylmethyl)-tetramic acid (9, cyclo-acetoacetyl-L-tryptophyl).



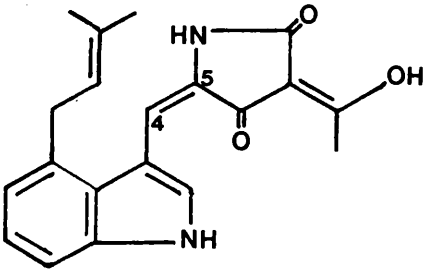
(9)

Incubation of cyclo-acetoacetyl-L-[G-^{3}H]tryptophyl and [1-^{14}C]dimethylallyl pyrophosphate with a cell-free extract produced β-cyclopiazonic acid (7) with a ^{3}H:^{14}C ratio which indicated a 1:1 reaction of the substrates. The enzyme responsible for this transformation, secondary dimethylallyl transferase, was isolated and found to be present only in the mycelium. These results lead to a more detailed picture of the pathway followed in α-cyclopiazonic acid biosynthesis, shown in Scheme II.



Scheme II

Having established the sequence of events in the biosynthesis of α-cyclopiazonic acid, examination of individual steps was possible. The first step, involving the formation of 3-acetyl-5-(indol-3-ylmethyl)-tetramic acid (9), is analogous to the biosynthesis of tenuazonic acid (vide supra). The isoprenylation of tryptophan or a tryptophan derivative is not novel either. Apart from the already mentioned analogy with the biosynthesis of clavine alkaloids,[12] dimethylallyl pyrophosphate has also been found to be transferred to the 2-position of the indole nucleus of cyclo(alanyltryptophyl) in Aspergillus amstelodami.[15] The conversion of β-cyclopiazonic acid (7) into α-cyclopiazonic acid (1) may be regarded as a didehydrogenation followed by cyclization. Schabort et al.[11] suggested that 4,5-didehydro-β-cyclopiazonic acid (10) is an intermediate in this cyclization.



(10)

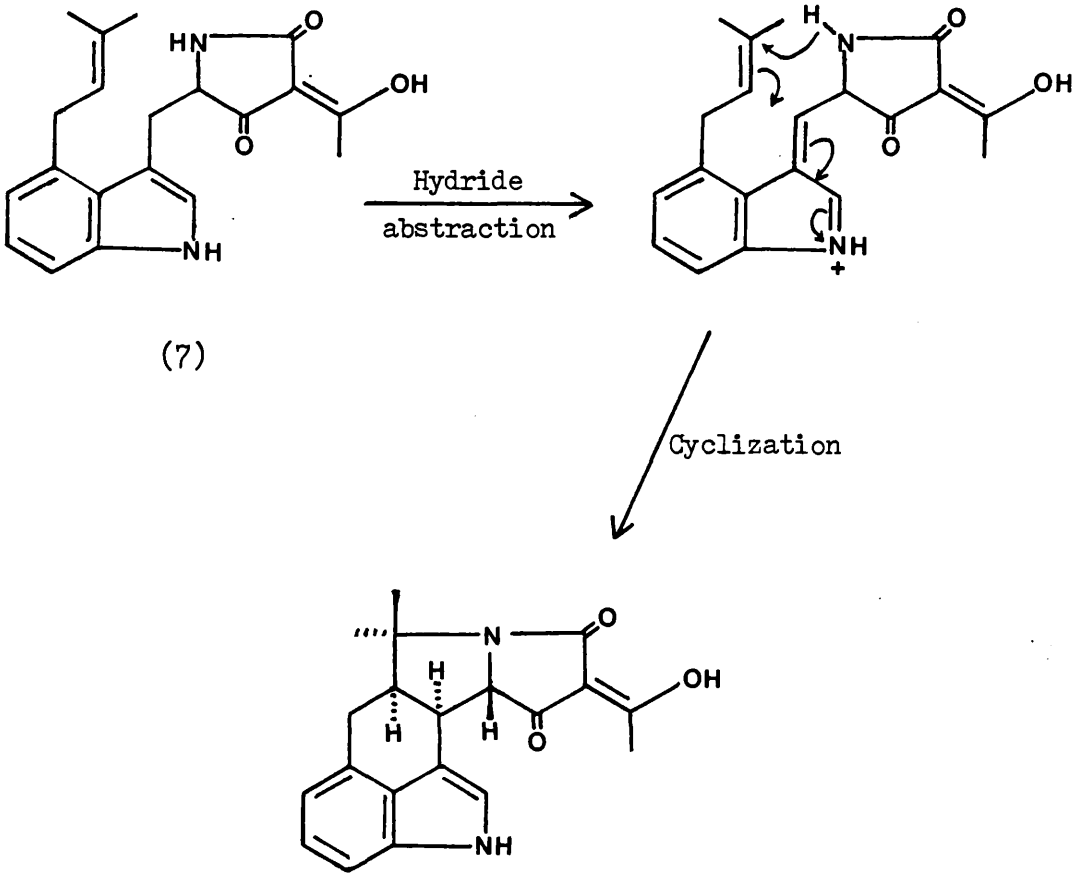
This possibility would require the loss of a hydrogen atom at C-5 of β-cyclopiazonic acid and was tested by Holzapfel and Schabort.[16] They synthesised DL-[5-^{3}H]-β-cyclopiazonic acid and DL-[4-^{14}C]-β-cyclopiazonic acid following literature procedures.[17] These two compounds were then incubated together with purified β-cyclopiazonate oxidocyclase in the presence of 2,6-dichlorophenolindophenol, a teminal electron acceptor. The α-cyclopiazonic acid (1) formed in

the reaction contained 44% of the initial radioactivity and the $^{3}H/^{14}C$ ratio was essentially unchanged. This result demonstrates that retention of tritium at C-5 of β-cyclopiazonic acid (7) is essentially complete during its biochemical conversion into α-cyclopiazonic acid and thus excludes the possibility of a 4,5-didehydro-derivative such as (10).

This retention of the label at C-5 also indicates that α-cyclopiazonic acid has the same configuration at that position as β-cyclopiazonic acid. Circular dichroism studies[3,8] of both α- and β-cyclopiazonic acid suggest that they both have the (S)-configuration at C-5. This conclusion was supported[16] by feeding suitable precursors to *P. cyclopium* cultures grown on a ferrous ion deficient medium, which results in the supression of α-cyclopiazonic acid production and accumulation of β-cyclopiazonic acid. In this way, separate feedings of L-[3-^{14}C]tryptophan and D-[3-^{14}C]tryptophan produced β-cyclopiazonic acid (7) that contained 22.1% of the activity of the labelled L-tryptophan and 5.6% of the activity of the labelled D-tryptophan. This result suggests that β-cyclopiazonic acid and thus α-cyclopiazonic acid have the (S)-configuration at C-5.

Steyn and coworkers[18] suggested that a 1,4-didehydro derivative of β-cyclopiazonic acid could be the precursor for the final oxidative cyclization reaction. Formation of this intermediate would involve loss of one of the C-4 methylene protons in β-cyclopiazonic acid. To test their hypothesis, the authors fed stereospecifically labelled (3R)- and (3S)-[3-^{3}H,3-^{14}C]tryptophan separately to cultures of *P. cyclopium*. Both stereoisomers were incorporated into β-cyclopiazonic acid (7) with high retention of tritium. The α-cyclopiazonic acid isolated from cultures fed with (3R)-[3-^{3}H,3-^{14}C]tryptophan showed essentially complete retention of tritium. Conversely, in its

transformation to α-cyclopiazonic acid, (3S)-[3-^{3}H,3-^{14}C]tryptophan lost almost all its tritium. These results showed that the methylene group of tryptophan remains intact during the early stages of the biosynthesis and that C-C bond formation at C-4 occurs from the side of the molecule opposite to proton removal. Furthermore, together with the previous findings that the proton at C-5 in β-cyclopiazonic acid (7) is not involved in the oxidative cyclization step, these results are consistent with the cyclization proceeding <u>via</u> a 1,4-didehydroderivative of (7) as shown in Scheme III.



Scheme III

The biosynthesis of cyclopiazonic acid and related tetramic acids has been reviewed recently by Holzapfel.[19]

The present study had two main objectives. Firstly, to establish, by the use of stable isotopic labels, that the C-5 proton of β-cyclopiazonic acid (7) is not involved in the final oxidative cyclization step which leads to α-cyclopiazonic acid (1). This study was initiated before the work of Holzapfel and Schabort[16] had appeared. Second, to determine if structurally modified versions of the natural precursors and intermediates can be converted by P. cyclopium into the corresponding analogues of α-cyclopiazonic acid.

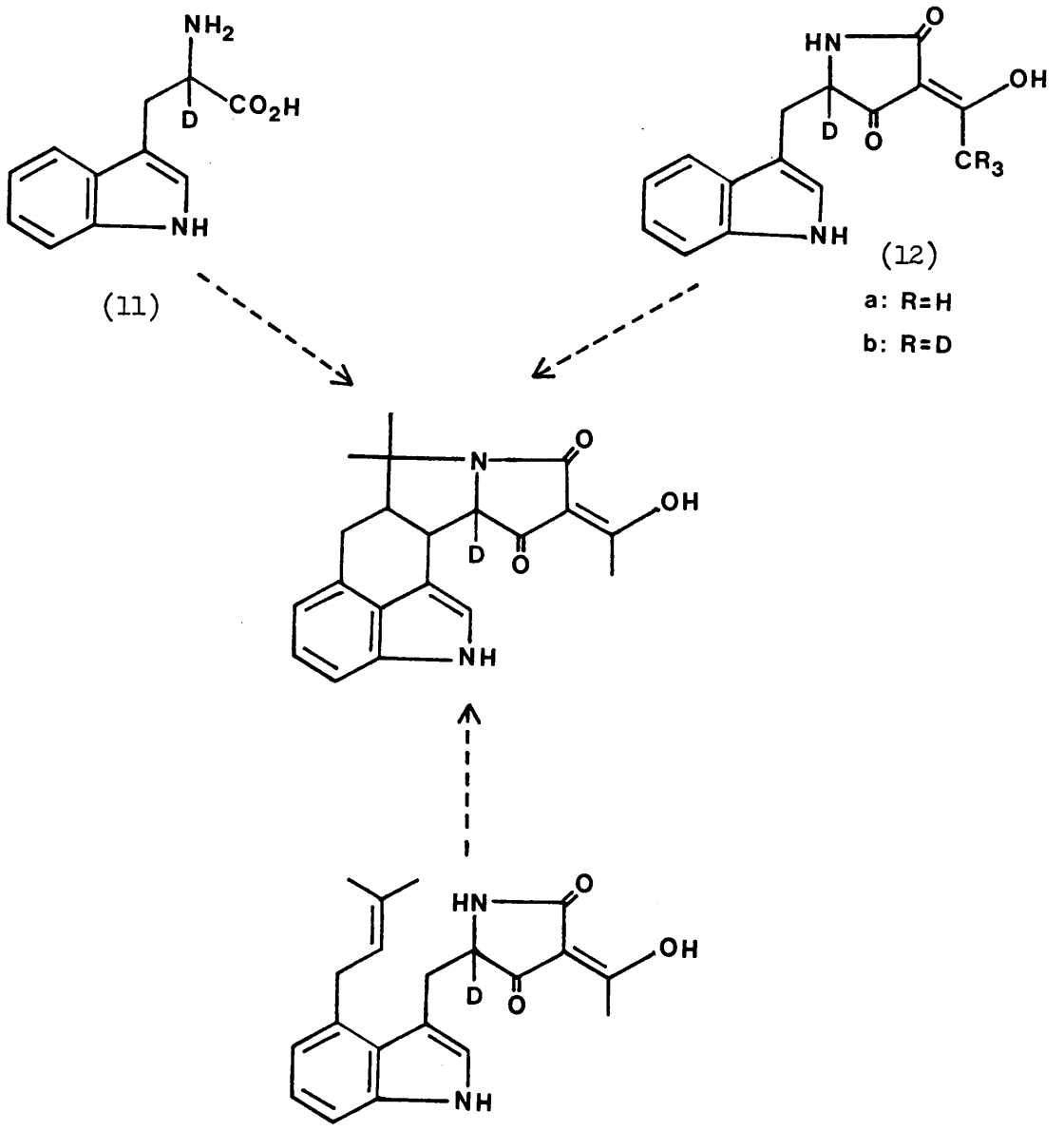
CHAPTER 2

DISCUSSION

The first objective in any biosynthetic investigation is to establish that the organism under study is growing well and producing the desired metabolite(s). The *P. cyclopium* culture used in this study was kindly supplied by Dr P. S. Steyn, of the National Chemical Research Laboratory, Pretoria, South Africa. It took some time, however, before it could be grown in a reproducible and satisfactory manner. Consequently, one of the feeding experiments was carried out by Dr. Steyn in his laboratories with material synthesised by us.

2.1 Synthesis of deuterium labelled precursors.- Our initial efforts were directed towards the synthesis of a suitable precursor with a deuterium label in what would become the C-5 position of α-cyclopiazonic acid (1). The three possible deuterium labelled precursors are shown in Scheme IV.

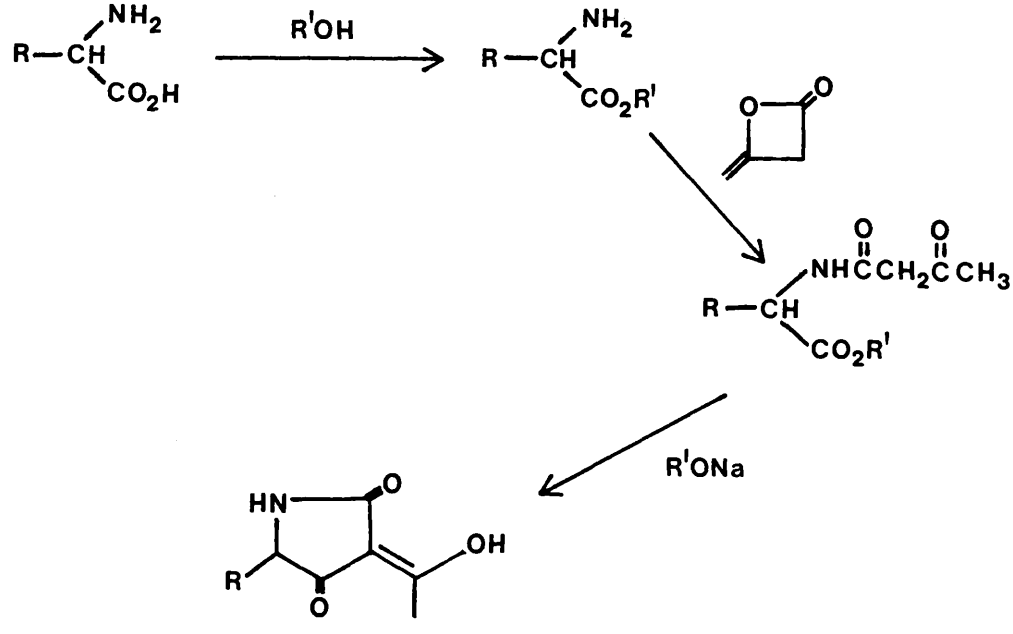
The precursor was chosen according to the following considerations. Of the three possibilities, [α-^{2}H]tryptophan (11) is obviously the easiest compound to synthesise with a high ^{2}H content.[20] However, because stable isotope experiments require the administration of the precursor at concentrations higher than those normally required when using radioisotopes, the effect of adding large amounts of tryptophan to the mould culture had to be considered. Neethling and McGrath[7] have reported that tryptophan does not form a pool in *P. cyclopium* and that exogenous tryptophan depresses both mycelium weight and



Scheme IV

α-cyclopiazonic acid yield, possibly by inhibition of tryptophan synthetase. This clearly excluded [α-^{2}H]tryptophan (11) as the precursor of choice. The [5-^{2}H]tetramic acid derivative (12a) was chosen instead since the synthesis appeared relatively straightforward and it was a known precursor of the cyclopiazonic acids.

The discovery of the antiviral and antitumor properties of tenuazonic acid (2) led to the synthesis of a large number of 3-acetyl-5-substituted tetramic acids by Yuki et al.,[21] Harris, Fisher and Folkers,[22] and Mulholland's group at I.C.I.[23] among many others. All these syntheses essentially follow Lacey's original method,[24] outlined in Scheme V, starting from the amino acid, which is esterified, aceto-acetylated, and finally cyclized with a sodium alkoxide.

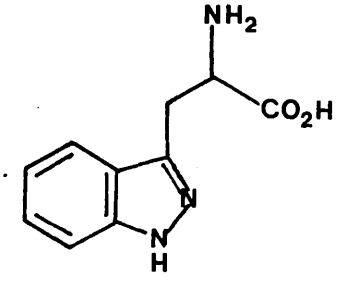


Scheme V

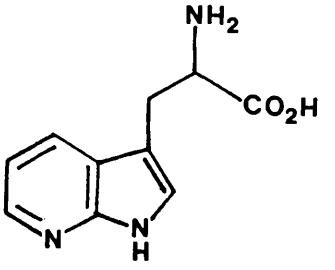
The initial plan was therefore to synthesise [α-^{2}H]tryptophan (11) and submit it to the reaction conditions described above, using CD_3ONa as the base for the cyclization. First, however, the cyclization of unlabelled N-acetoacetyltryptophan methyl ester using CD_3ONa in refluxing deuteriated methanol and benzene was investigated. The ^{1}H-n.m.r. spectrum of the tetramic acid produced in this reaction showed that the cyclization had proceeded with partial exchange of the C-5

and acetyl protons. Thus, if these conditions were to be used for [α-^{2}H]-tryptophan (11), the product would be only partially tetradeuteriated tetramic acid. Milder conditions were then tried to effect the desired cyclization of the N-acetoacetyltryptophan methyl ester, namely, heating it under reflux in (a) mixtures of piperidine and methanol, (b) NN-dimethyl-aniline and methanol, and (c) anhydrous potassium carbonate in acetone. Uncyclized N-acetoacetyltryptophan methyl ester was recovered from all these reactions. The behaviour of N-acetoacetyltryptophan methyl ester in sodium deuteroxide in deuterium oxide was then followed by ^{1}H-n.m.r. spectroscopy at room temperature. It was observed that both α- and acetyl-protons exchanged with deuterium at very much the same rate; partial hydrolysis of the methyl ester was also evident. Unlabelled 3-acetyl-5-(indol-3-ylmethyl)-tetramic acid (9) was synthesised in 77% yield by slight modifications of the literature procedure.[21] Its behaviour in the presence of sodium deuteroxide in deuterium oxide was also followed by ^{1}H-n.m.r. spectroscopy at 35°C. Almost immediately, integration of the spectrum showed that the C-5 and methyl protons were being exchanged, whilst the pattern corresponding to the methylene protons became simpler. After 1.5 h, approximately 50% of the methyl protons had exchanged and the ABX pattern due to the C-5 and methylene protons was no longer clear. The signals due to the C-5 and methyl protons had virtually disappeared after 4.5 h and the pattern due to the methylene protons had simplified to an AB quartet. After 21.5 h at 35°C, the exchange was complete. It was therefore decided to settle for the tetradeuteriated tetramic acid (12b) as the precursor of choice. The 3-acetyl-5-(indol-3-ylmethyl)tetramic acid (9) was heated overnight under reflux in sodium deuteroxide solution to yield 3-[$^2H_3$]acetyl-5-(indol-3-yl-methyl)-[5-^{2}H]tetramic acid, containing 82% [$^2H_4$]species, in 92% yield. A sample of this compound was sent to Dr. Steyn for feeding to P.cyclopium cultures. The results obtained will be discussed later.

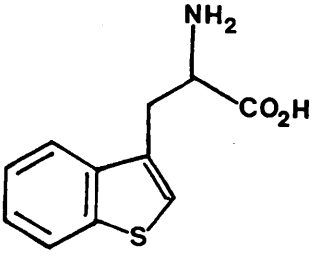
2.2 Synthesis of tryptophan analogues. The synthesis of tryptophan analogues was then undertaken. Tryptophan analogues have been synthesised to be used in pharmacological, biological and biosynthetic studies. Three analogues were chosen as the most likely to mimic tryptophan behaviour: β-(2-azaindol-3-yl)alanine (14), β-(7-azaindol-3-yl)alanine (15) and β-(1-benzothien-3-yl)alanine (16).



(14)



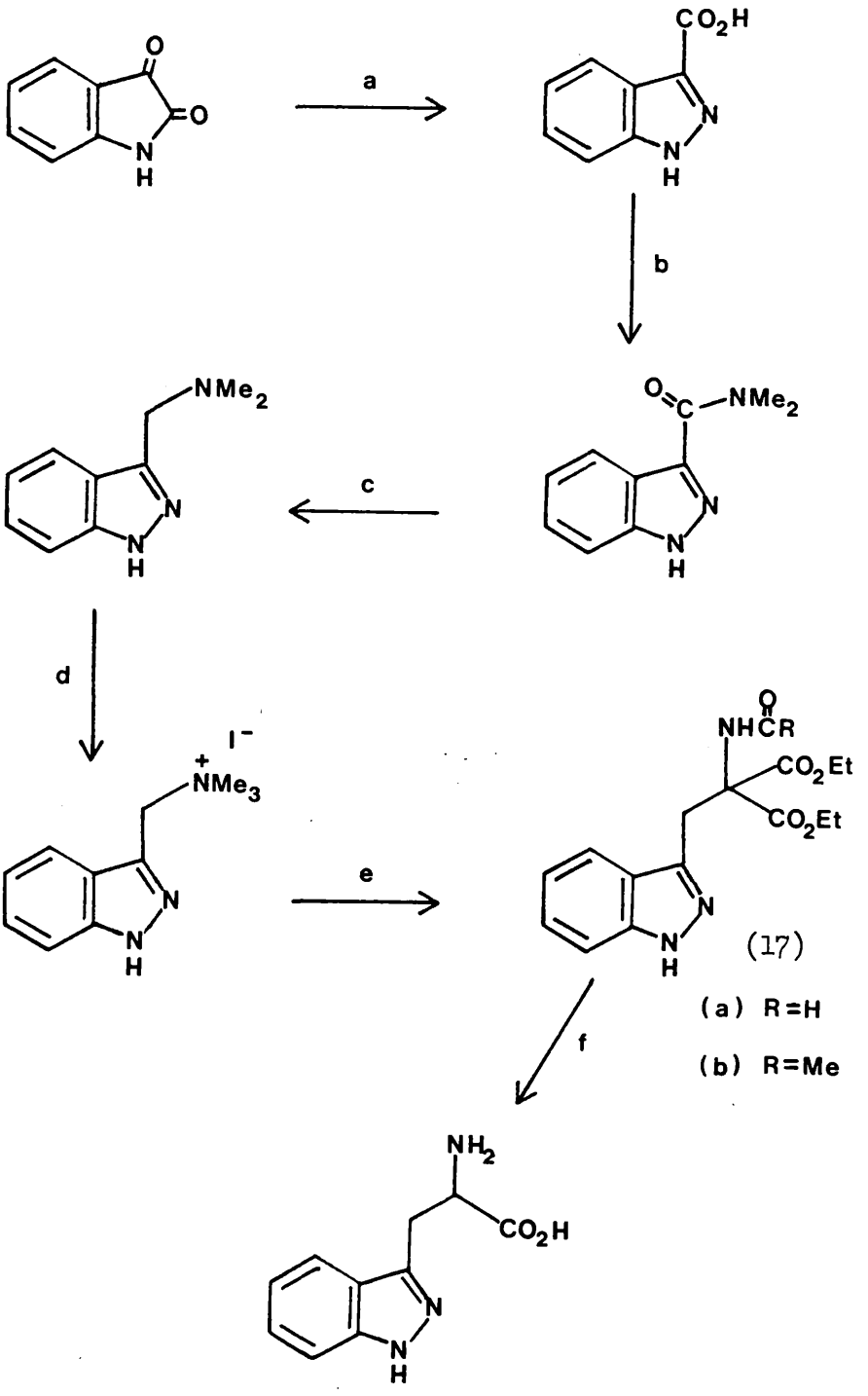
(15)



(16)

The synthesis of all three compounds have been reported in the literature and those procedures were followed with only slight modifications.

The reaction sequence used to synthesise the 2-azaindole analogue (14), starting from isatin, is shown in Scheme VI.[25]



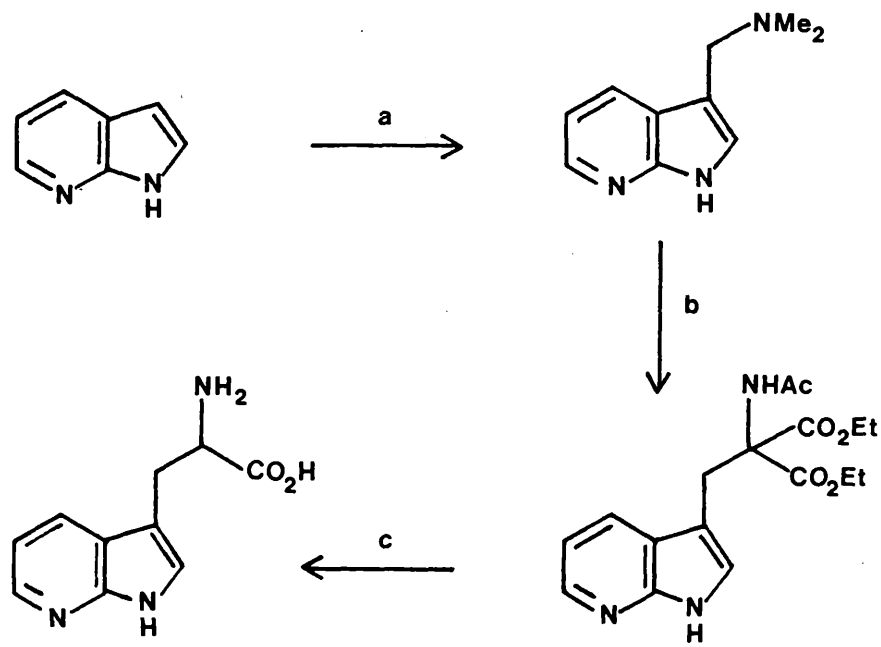
(14)

Reagents: (a) aq. NaOH; $H_2SO_4/NaNO_2$; $HCl/SnCl_2$; Δ ; (b) $SOCl_2$; Me_2NH; (c) $LiAlH_4$; (d) MeI; (e) $NaOEt/RCONHCH(CO_2Et)_2$; (f) conc. HCl

Scheme VI

In the original literature procedure,[25] the acetamido diester (17b) was saponified to the malonic acid derivative, which was then decarboxylated to the substituted propionic acid and transformed to the amino acid (14) by hydrolysis of the acetyl group in barium hydroxide solution. We found that the amino acid (14) could be more easily obtained by heating the formamido diester (17a) in refluxing concentrated hydrochloric acid for 7h. The amino acid (14) was also similarly prepared, albeit in lower yield, from the crude acetamido diester (17b).

β-(7-Azaindol-3-yl)alanine (15) was prepared by the method described by Robison and Robison,[26] as shown in Scheme VII.

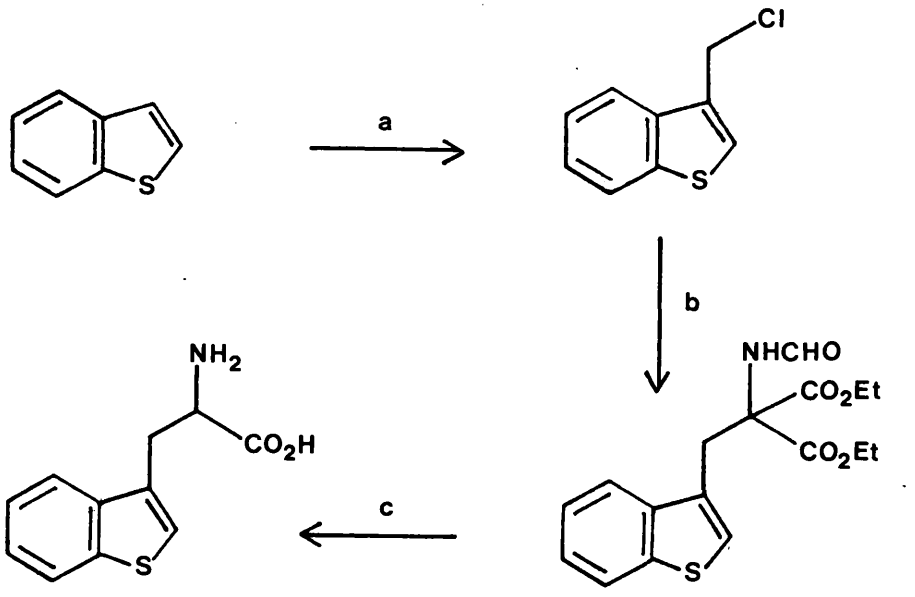


(15)

Reagents: (a) $(CH_2O)_n/Me_2NH/^nBuOH$; (b) $NaOEt/AcNHCH(CO_2Et)_2$; (c) conc. HCl

Scheme VII

The thiotryptophan analogue (16) was synthesised from benzothiophene according to the procedure described by Avakian et al.[27] and depicted in Scheme VIII.



(16)

Reagents: (a) 1,3,5-Trioxane/HCl/HOAc; (b) NaOEt/$HCONHCH(CO_2Et)_2$; (c) conc. HCl

Scheme VIII

2.3 Feeding experiments.- Having synthesised the required tryptophan analogues, attention was turned to the evaluation of P. cyclopium cultures which were by then being grown in our laboratories. Once the correct growth conditions for metabolite production had been obtained, efforts were concentrated on the isolation of the two main metabolites, α- and β-cyclopiazonic acids. This turned out to be quite a problem. A considerable number of time-consuming extractions and chromatographies were required to isolate the crude metabolites.

In general, mycelial extracts were cleaner than broth extracts. The weight of the mycelium obtained (ca. 14.3 g/l of culture) compared favourably with the literature value (15 g/l of culture[7]). In several instances, a third major compound was isolated from the cultures. ^{1}H-N.m.r. spectroscopy and mixed melting point determination with an authentic sample, obtained from Prof. C.J.W.Brooks in our Department, showed this compound to be 6-methylsalicylic acid. Although not previously reported for P. cyclopium, 6-methylsalicylic acid is a well known and widespread fungal metabolite.[28] On one occasion, another metabolite was isolated from the mycelial extract. Melting point and optical rotation determinations, together with ^{1}H n.m.r. spectroscopic evidence, indicated that this compound was ergosterol. Preparation of its Diels-Alder adduct with Cookson's dienophile,[29] followed by determination of its mixed melting point with that of the same adduct prepared from commercial ergosterol, confirmed their identity. Ergosterol has been previously isolated from the mycelium, but not the broth, of a strain of P. cyclopium (NRRL A-15-465) and qualitative assays have indicated its presence in several other strains.[30]

In order to evaluate the efficiency of our cultures in producing α-cyclopiazonic acid (1), two separate feedings of DL-[$^{14}CH_2$]tryptophan (25μCi) were carried out. On average, 208 mg of α-cyclopiazonic acid were obtained with a specific activity of 1.94 μCi/mmol, corresponding to an incorporation of 9.6% for the L-isomer. This value was considerably lower than the 24.7% reported by Holzapfel and Wilkins,[5] but was considered as sufficient evidence that our culture was producing α-cyclopiazonic acid at an acceptable level.

The tryptophan analogues were now fed to our cultures of P. cyclopium. The results of these feeding experiments are summarized in Table 1.

TABLE 1

FEEDING OF TRYPTOPHAN ANALOGUES TO *P. cyclopium*

Experiment	Amino acid fed		Concentration of amino acid (mmol/l of culture)	Mycelial Weight (g/100 ml of culture)	pH of broth at harvesting*
1	None	(a)	-	1.032	7.0 (5.1)
		(b)	-	0.968	7.3
2	DL-Tryptophan	(a)	0.98	0.849	6.2 (3.0)
		(b)	2.00	0.316	6.6
3	DL-β-(2-Azaindol-3-yl)alanine (14)	(a)	1.06	0.260	5.5 (3.6)
		(b)	1.96	0.055	3.0
4	DL-β-(7-Azaindol-3-yl)alanine (15)	(a)	1.04	<0.001	3.0 (3.0)
		(b)	1.96	0.003	2.4
5	DL-β-(1-Benzothien-3-yl)alanine (16)	(a)	0.84	0.013	3.0 (3.0)
		(b)	1.92	0.003	2.6

*Figures in parentheses refer to pH values for the broth two days after addition of the amino acid.

The first observation that can be made from the table above is that, as already noted by Neethling and McGrath,[7] doubling the concentration of tryptophan causes a very significant decrease in the mycelial weight. This, however, does not seem to be accompanied by any significant decrease in the final pH of the broth. Of the three analogues fed, only β-(2-azaindol-3-yl)alanine (Experiment 3) shows the same dependence of mycelial weight on amount of amino acid administered. Yet, this decrease is, in this case, accompanied by a significant lowering of the final pH value. The other two analogues, at both concentration levels, show a large suppression on mycelial weight. Attention must be turned now to the pH values obtained. On the fourth day after inoculation, the non-fed control (Experiment 1a) has a broth pH of 5.1. This increases with time, until it reaches a neutral pH on the seventh day, when harvesting is done. Administration of tryptophan (Experiment 2) decreases the pH of the broth more than in the control, but once again, towards the end of growth the pH approaches neutrality. β-(2-Azaindol-3-yl)alanine (14), when fed at the lower concentration (Experiment 3a), causes roughly the same effect as observed on addition of tryptophan. However, at the higher concentration (Experiment 3b), there is no increase of pH with age. This same effect is observed with the two other analogues (Experiments 4 and 5). It is worth noting that McGrath *et al.*[31] have determined that the optimum pH value for the various enzymes involved in α-cyclopiazonic acid (1) biosynthesis lies in the 6.5 - 8.0 range. Thence, it seems that the initial low pH in the early stages of growth must increase before the metabolites can be produced. In other words, if the organism is unable to overcome that initial low pH, there can be no production of α-cyclopiazonic acid. However, one must keep in mind that, because of their

poor solubility in water, the amino acids were fed in fairly acidic solutions (see Experimental Section) and this fact, coupled with the presence of an unnatural amino acid, could be responsible for the results obtained.

The cultures from Experiments 1, 2 and 3a were extracted as usual. Analytical thin layer chromatography (t.l.c.) showed that α- and β-cyclopiazonic acids had only been produced in Experiments 1 and 2 (the control and tryptophan-fed experiments). It seems, therefore, that although (14) does not have as drastic an effect as the other analogues on the growth of the organism, it does affect its normal behaviour.

At this point, it was decided to investigate the use of analogues of the tetramic acid (9). These could be synthesised from the previously prepared tryptophan analogues by following the route described in Scheme V. Prior to undertaking these syntheses, however, it was necessary to investigate the response of P. cyclopium to the administration of 3-acetyl-5-(indol-3-ylmethyl)tetramic acid (9). As described in the introduction, earlier studies involving this intermediate were restricted to cell-free extracts of the organism. It was therefore necessary to synthesise the radioactively labelled tetramic acid (9). This was obtained by the usual procedure,[20] starting from DL-[$^{14}CH_2$]tryptophan, in 62% yield and with a specific activity of 391.7 μCi/mmol.

In the first feeding experiment involving this precursor, an ethanolic solution (1.5 ml) of the labelled tetramic acid (0.086 mmol., 33.7 μCi) was distributed between 22 flasks of P. cyclopium culture (each containing 100 ml of medium), on the seventh day after inoculation, when growth was well established. After 24 h, the

culture was harvested and extracted as described in the Experimental Section. The mycelium extract contained only 2% of the added radioactivity, whilst the broth contained 64%. Analytical t.l.c. plates of both extracts and of the labelled precursor, as a reference, were scanned for radioactivity and autoradiographed. The scans and autoradiograms showed that the radioactivity in the broth extract was concentrated mainly in the band corresponding to the fed precursor, whilst that of the mycelium extract, barely detectable, was concentrated in the baseline. Preparative layer chromatography of both extracts yielded unlabelled α- and β-cyclopiazonic acids. No efforts were made to recover the labelled precursor, although there were indications that it had undergone some degradation, since the baselines of the preparative t.l.c. plates showed considerable activity.

Neethling and McGrath[7] have reported that the highest rate of β-cyclopiazonic acid biosynthesis occurs on the third day after inoculation. It seemed appropriate, therefore, to introduce the tetramic acid (9) at an earlier time. In the second feeding experiment, the labelled precursor (0.060 mmol., 23.5 μCi) in ethanolic solution (1.5 ml) was distributed between 2.5 l of culture, on the second day after inoculation. Cultures were harvested 120 h later. The mycelium extract contained approximately 3% of the added radioactivity; the broth extract contained 77%. Once again, scans and autoradiograms showed no detectable radioactivity in the mycelial extract, whilst most of the radioactivity of the broth extract was accounted for in the band corresponding to the precursor administered, with a smaller amount present in the baseline. Unlabelled tetramic acid was added to the crude broth extract. Great difficulties were experienced in recovering any tetramic acid from this complex mixture. Eventually,

some material, highly contaminated with assorted impurities, was isolated and found to be radioactive. No reliable figure for its specific activity could be obtained. The only conclusions that could be drawn from these experiments were: i) there is no tetramic acid present in the mycelial extracts; ii) most of the radioactivity present in the broth extracts is due to the tetramic acid, which also seems to suffer a certain amount of degradation to highly polar material. It seems, therefore, that 3-acetyl-5-(indol-3-ylmethyl)tetramic acid (9) is not incorporated, under the conditions used, by whole cultures of P. cyclopium. These results could be rationalized by an inability of the precursor (9) to penetrate the cell wall of the organism. McGrath et al.[13] have reported that secondary dimethylallyltransferase, the enzyme mediating the conversion of (9) into β-cyclopiazonic acid (7), is found only in the mycelium. Hence, if the tetramic acid cannot pass through the cell membrane, it cannot be transformed into β- and eventually, α-cyclopiazonic acid. The idea of feeding analogues of (9) was therefore abandoned, partly because it was probable that the same membrane permeability problems would be encountered, partly because no suitable control experiment would be available.

It was about this time that Dr. Steyn's results on the conversion of 3-acetyl-5-(indol-3-ylmethyl)tetramic acid (9) into α-cyclopiazonic acid (1) were obtained.[32] A system was developed to study this conversion by means of a replacement culture technique in which the replacement medium contained no $NaNO_3$, thus depriving the organism of any exogenous nitrogen source. Under these conditions, no α-cyclopiazonic acid was produced. Addition of small levels of ^3H-labelled tetramic acid (9), led to a highly efficient conversion of

(9) into α-cyclopiazonic acid. When higher levels of 3-[$^{2}H_{3}$]acetyl-5-(indol-3-ylmethyl)-[5-^{2}H]tetramic acid (12b) were fed to the replacement cultures, α- and β-cyclopiazonic acids, together with some recovered acid (9), were isolated. ^{1}H N.m.r. spectroscopic analysis of the acid (9) revealed that, although the deuterium atoms at the acetyl methyl group had exchanged completely, the deuterium atom at the C-5 position was retained. Both α- and β-cyclopiazonic acids produced contained no deuterium. If 3-[$^{2}H_{3}$]acetyl-5-(indol-3-ylmethyl)-[5-^{2}H]tetramic acid had been a direct substrate for the isoprenylation reaction, α-cyclopiazonic acid should have retained the deuterium atom at the C-5 position. Therefore, it seems that the tetramic acid (9) is not incorporated under these conditions, but rather that it is first degraded to the amino acid and then subjected to a transamination reaction, which would account for the observed loss of deuterium.

2.3 Conclusions.- The results that had been obtained to this date were by no means encouraging. The objectives that had been set at the beginning of the project could not be realised. Moreover, considerable difficulties were being experienced in keeping the cultures at an appropriate production level. Work with this organism was therefore brought to an end.

CHAPTER 3

EXPERIMENTAL

3.1 General Procedures

Melting points were measured on a Reichert hot-stage melting point apparatus and are uncorrected. Microanalyses were carried out by Mrs. W. Harkness and her staff. Infrared spectra were obtained from Perkin-Elmer 257 or 580 infrared spectrometers by Mrs. F. Lawrie and her staff and only significant absorptions are quoted. Ultraviolet spectra were obtained on a Pye-Unicam SP 800 spectrometer. Mass spectra were recorded on a GEC-AEI MS12 spectrometer by Mr. A. Ritchie and his staff.

Proton nuclear magnetic resonance spectra were recorded on Varian T-60A (60 MHz), Perkin-Elmer R32 (90 MHz) and Varian XL-100 (100 MHz) spectrometers, using tetramethylsilane as the internal standard. The following abbreviations are used: s - singlet, d - doublet, t - triplet, q - quartet, m - multiplet, dd - double doublet, ddd - doublet of double doublets, and b - broad. Spectral parameters are derived from first order analyses unless otherwise stated.

Analytical thin layer chromatography (t.l.c.) was carried out on commercially prepared plates coated with Kieselgel GF_{254} (Merck) 0.25 mm thick. For preparative t.l.c., plates (20 x 20 cm) coated with Kieselgel GF_{254} (Fluka) 1 mm thick were used. Plates precoated with oxalic acid were prepared by either dipping Kieselgel GF_{254} (Merck) precoated plates in 8% methanolic solution of oxalic acid and air-drying for 24 h, or by coating glass plates with a slurry prepared with Kieselgel GF_{254} (Fluka) with 0.2 M aqueous oxalic acid in a 1:2 ratio to a 1 mm thickness.

3.2 Radioactive Methods

Radioactive tryptophan was obtained from The Radiochemical Centre, Amersham. Radioactive samples were counted on a Philips liquid scintillation counter, model PW4510 D649. Liquid samples were measured using an Oxford micropipette. Solid samples were weighed on metal foil boats in a Mettler UM-6 microbalance. Samples were placed in Packard scintillation vials and dissolved in scintillation solution (10 ml). The scintillation solution contained 2,5-diphenyloxazole (4 g) and 1,4-cis-2-(4-methyl-5-phenyloxalyl)benzene (0.1 g) per litre of Analar grade toluene. Solid samples were crystallised to constant activity. The radioactivity measurements are subject to a statistical error of 2%. A Panax Thin Layer Scanner, model RTLS-1A, was used for radioscanning of t.l.c. plates. Autoradiography was carried out using Ilford Red Seal 100FW and Kodak XR-P1 X-ray films.

3.3 Fermentation Conditions

Penicillium cyclopium Westling strain 1082 from the culture collection of the Microbiological Research Group, C.S.I.R., Pretoria, was used in this investigation. The fungus was grown at 25°C in shake culture (200 rpm) in a synthetic medium (100 ml) contained in 500 ml Erlenmeyer flasks. The synthetic medium had the following composition:[7]

Glucose	60 g
$NaNO_3$	4.5 g
$MgSO_4.7H_2O$	0.5 g
KCl	0.5 g
K_2HPO_4	1 g

$Na_2B_4O_7.10H_2O$	0.7 mg
$(NH_4)_6Mo_7O_{24}.4H_2O$	0.5 mg
$CuSO_4.5H_2O$	0.3 mg
$MnSO_4.4H_2O$	0.11 mg
$ZnSO_4.7H_2O$	17.6 mg
$FeSO_4.7H_2O$	10 mg
Deionised water	1 litre

The pH of the medium was adjusted to 5.5 with hydrochloric acid and the solution was sterilized by autoclaving at 120°C and 15 psi for 15 min. The flasks were inoculated with 5 ml of inoculum from a two-day-old culture of P. cyclopium grown on the above synthetic medium. The mould was grown on slants of 2% malt agar for 7 - 10 days. These were shaken with a sterile 0.1% w/v solution of Tween-80 and the spore suspension thus obtained was used for setting up the seed flasks.

3.4 Feeding Experiments

DL-[β-^{14}C]Tryptophan (25 μ Ci, 0.5 ml of aqueous solution containing 2% ethanol) was fed to cultures (ca. 2.7 l) six to seven days after inoculation and cultures were harvested 24 h later.

Tryptophan analogues were fed in parallel with unlabelled tryptophan to cultures (0.5 l) on the second day after inoculation, in aqueous solutions (ca. 15 ml) containing 10% ethanol and 4 - 5 drops of concentrated hydrochloric acid. The solutions were sterilized by passage through a millipore filter. Two different feeding concentrations were used (1 and 2 mM) and in both cases harvesting was done seven days after inoculation.

The labelled tetramic acid derivative was fed under two different conditions. Firstly, an ethanolic solution (1.5 ml) of the compound was fed to cultures (ca. 2.2 l) on the seventh day after inoculation, in 0.04 mM concentration, and harvesting was done 24 h later. In the second experiment, the compound was fed in ethanolic solution (1.5 ml) at a concentration of 0.024 mM to two-day-old cultures (ca. 2.5 l), which were harvested seven days after inoculation.

3.5 Metabolite Isolation[13]

The cultures were subjected to suction filtration. The mycelium was washed with methanol, dried in a desiccator over P_2O_5 at reduced pressure for 48 h, and then was continuously extracted with chloroform in a Soxhlet extractor for 96 h. The broth was acidified to pH 2 with hydrochloric acid, mixed with the mycelial methanolic washings and continuously extracted with chloroform for 96 h. The chloroform extract was concentrated under reduced pressure. The residue was dissolved in chloroform (ca. 100 ml) and extracted with saturated sodium bicarbonate solution (5 x 20 ml). The aqueous solution was acidified to pH 2 with hydrochloric acid and then re-extracted with chloroform (6 x 20 ml). The extracts were washed with water, dried over $MgSO_4$ and concentrated to dryness under vacuum and weighed. Each extract was purified by successive preparative t.l.c. In the first, the crude extract was chromatographed on silica gel plates developed with ethyl acetate: methanol: conc. aqueous ammonia (85:15:10). The ultraviolet active bands, corresponding to α-cyclopiazonic ($R_f \sim 0.5$) and β-cyclopiazonic ($R_f \sim 0.4$) acids, were scraped off and eluted with methanol. The methanolic solutions of α- and β-cyclopiazonic acid were concentrated and separately applied to plates precoated

with oxalic acid and developed using chloroform: methylisobutylketone (4:1). The α-cyclopiazonic acid (R_f ~0.7) and β-cyclopiazonic acid (R_f ~0.5) bands were eluted with chloroform. The chloroform extracts were concentrated under reduced pressure and afforded the crude metabolites. Typical total crude yields were ca. 120 mg/l for α-cyclopiazonic acid and ca. 100 mg/l for β-cyclopiazonic acid.

3.6 Synthetic Procedures

N-Acetyl-DL-tryptophan.- Treatment of a solution of DL-tryptophan in 1M sodium hydroxide with acetic anhydride, according to the method of du Vigneaud and Sealock,[33] gave plates (82% yield) after crystallisation from water, m.p. 204 - 207°C (lit.,[33] 205 - 206°C) (Found: C, 63.2; H, 5.6; N, 11.0. Calc. for $C_{13}H_{14}O_3N_2$: C, 63.4; H, 5.7; N, 11.4%); δ (d_6-Me_2CO) (90 MHz) 7.25 (6H, m, ArH and NH), 4.77 (1H, m, α-CH), 3.25 (2H, m, β-CH_2), and 1.88 (3H, s, COMe); m/e 246 (M^+), 187 (M - NHCOMe - H) and 130 (C_9H_8N).

N-Acetyl-L-[α-^{2}H]tryptophan.-[20] N-Acetyl-DL-tryptophan (1.75 g, 7 mmoles) was heated for ten min in refluxing anhydrous 1,4-dioxan (35 ml) containing acetic anhydride (1.5 ml). Pyridine (0.5 ml) was added to the cooled solution, which was stirred for five min, and then deuterium oxide (8 ml) was added. The mixture was evaporated to dryness under reduced pressure. The brown oil obtained was resubmitted to the reaction conditions and was then dissolved in hot water (ca. 10 ml), treated twice with charcoal and, on cooling, gave white crystals (0.84 g, 48% yield, 0.90 atom deuterium), m.p. 204 - 206°C (lit.,[33] 205 - 206°C for non-deuteriated compound); δ (d_6-Me_2CO/D_2O)

(90 MHz) 7.44 (5H, m, ArH), 3.44 (2H, AB q, J_{AB} 15 Hz, β-CH_2), and 2.15 (3H, s, COMe); m/e 247 (M^+) and 130 (C_9H_8N).

DL-[α-^{2}H]Tryptophan (11).- N-Acetyl-DL-[α-^{2}H]tryptophan (0.79 g, 3 mmoles) was heated in refluxing 2M hydrochloric acid (20 ml) for three h. The pink solution was cooled, taken to pH 5.9 with dilute ammonia solution, treated twice with charcoal and kept at 0°C overnight. This afforded white crystals (0.52 g, 85% yield, 0.90 atom deuterium), m.p. 258 - 260°C (dec.) [lit.,[34] 265°C (dec.) for nondeuteriated compound]; ν_{max}(KBr) 3 400, 3 040, 1 660, 1 610, and 1 580 cm^{-1}; δ(d_6-Me_2CO/D_2O) (90 MHz) 7.70 (5H, m, ArH) and 3.44 (2H, AB q, J_{AB} 15 Hz, β-CH_2); m/e 205 (M^+), 160 (M - CO_2H), 130 (C_9H_8N), and 117 (C_8H_7N).

DL-Tryptophan methyl ester hydrochloride.- This compound was prepared by the method of Brenner and Huber[35] in 80% yield, m.p. 225 - 226°C (dec.) lit.,[36] 225°C (dec.,) ; δ($CDCl_3$) (90 MHz) For the free base, 8.90 (1H, bs, NH, exchangeable with D_2O), 7.16 (5H, m, ArH), 3.76 (1H, X part of ABX system, J_{AX} 8, J_{BX} 5 Hz, α-CH), 3.62 (3H, s, CO_2Me), 3.11 and 3.04 (2H, AB part of ABX system, J_{AX} 8, J_{BX} 5, J_{AB} 15 Hz, β-CH_2), and 1.57 (2H, bs, NH_2 exchangeable with D_2O); m/e 218 (M - HCl), 159 (M - HCl - CO_2Me) and 130 (C_9H_8N).

DL-3-Acetyl-5-(indol-3-ylmethyl)tetramic acid (9).-[21] DL-Tryptophan methyl ester hydrochloride (0.90 g, 3.5 mmoles) was dissolved in water (10 ml) and an equivalent amount of triethylamine was added. The free amino acid methyl ester was extracted into diethyl ether.

The ether extracts were dried and evaporated to give a yellow oil (98% recovery) which was dissolved in anhydrous methanol (6 ml) and cooled to 0°C. Redistilled diketene (0.5 ml, 6.3 mmoles) was added dropwise, with stirring, and the solution was stirred at 0°C for 10 min and then at room temperature for 2 h. The solvent was evaporated leaving a viscous yellow oil, the crude N-acetoacetyl-tryptophan methyl ester. This ester gave only one spot on t.l.c. (R_f = 0.33, EtOAc) which turned purple on spraying with methanolic $FeCl_3$ solution; $\delta(CDCl_3)$ (90 MHz) 8.55 (1H, bs, NH, exchangeable with D_2O), 7.22 (5H, m, ArH), 4.89 (1H, m, α-CH), 3.60 (3H, s, CO_2Me), 3.25 (2H, m, β-CH_2), 3.19 (2H, s, $COCH_2CO$), 2.16 (1H, s, NH), and 2.00 (3H, s, COMe).

The crude oil from above was dissolved in anhydrous benzene (2 ml) and to this was added a solution of sodium methoxide (4.4 mmoles) in methanol and the mixture was refluxed for 3h. The mixture was left at room temperature overnight, and then extracted with water (5 x 30 ml). The water extracts were acidified to pH 2 with dilute hydrochloric acid and extracted with ether (5 x 20 ml). The combined ether extracts were dried and concentrated. The residue was recrystallised from ethanol to give pale yellow crystals (0.73 g, 77% yield) and gave one spot on t.l.c. (R_f = 0.61, $CHCl_3$: MeOH: HOAc - 80:20:2), which turned brownish orange when sprayed with methanolic $FeCl_3$ solution; m.p. 173 - 174°C (lit.,[21] 171 - 173°C) (Found: C, 66.6; H, 5.0; N, 10.2. Calc. for $C_{15}H_{14}N_2O_3$: C, 66.7; H, 5.2, and N, 10.4%); $\lambda_{max}(CHCl_3)$ 226 (112 x 10^3) and 280 (57 x 10^3) nm; $\nu_{max}(CHCl_3)$ 3 480, 3 440, 1 715, 1 660, and 1 620 cm^{-1}; $\delta(CDCl_3)$ (90 MHz) 10.70 (1H, bs, OH, exchangeable with D_2O), 8.15 (1H, bs, NH, exchangeable

with D_2O), 7.35 (5H, m, ArH), 6.05 (1H, s, NH exchangeable with D_2O), 4.10 (1H, X part of ABX system, J_{AX} 10, J_{BX} 4 Hz, 5-H), 3.45 and 2.83 (2H, AB part of ABX system, J_{AX} 10, J_{BX} 4, J_{AB} 15 Hz, β-CH_2), and 2.45 (3H, s, COMe); m/e 270 (M^+) and 130 (C_9H_8N).

DL-3-[$^2H_3$]-Acetyl-5-(indol-3-ylmethyl)-5-[^2H]-tetramic acid (12b).- DL-3-Acetyl-5-(indol-3-ylmethyl)tetramic acid (0.50 g, 1.9 mmoles) was heated overnight in a refluxing 0.4 M sodium deuteroxide solution (40 ml). The cold solution was acidified to pH 2 with dilute DCl solution and was extracted with chloroform (10 x 5 ml). The combined chloroform extracts were dried, concentrated and the residue was recrystallised from ethanol to give pale yellow crystals (0.47 g, 92% yield, 82% [$^2H_4$]species) m.p. 171 - 173°C (lit.,[21] 171 - 173°C for non-deuteriated compound); δ ($CDCl_3$) (90 MHz) 11.20 (1H, bs, OH, exchangeable with D_2O), 8.15 (1H, bs, NH, exchangeable with D_2O), 7.33 (5H, m, ArH), 6.05 (1H, bs, NH, exchangeable with D_2O), 3.42 (1H, d, J_{AB} 15 Hz, β-CH), and 2.80 (1H, d, J_{AB} 15 Hz, β-CH); m/e 274 (M^+) and 130 (C_9H_8N).

DL-3-Acetyl-5-(indol-3-yl-[^{14}C]methyl)tetramic acid.- DL-[β-^{14}C]-Tryptophan (250 μCi) diluted with unlabelled DL-tryptophan(126 mg, 0.62 mmoles) was suspended in anhydrous methanol (1 ml), cooled to 0°C and thionyl chloride (100 μl) was added to give a clear yellow solution which was kept at 0°C for 45 h, followed by 1 h at room temperature. The solvent was removed under dry nitrogen and anhydrous methanol (1.5 ml) was added to the residue, followed by 3.67 M sodium methoxide solution (200 μl, 0.73 mmole), which liberated the amino ester. The reaction mixture was cooled to -40°C and redistilled

diketene (100 µl, 1.27 mmoles) was added dropwise with stirring. The reaction was stirred for 24 h at room temperature. The solvent was removed under dry nitrogen and the residue was purified by preparative t.l.c. developing with ethyl acetate. The radioactive band (R_f ~0.3 - 0.4) was eluted with 20% methanol in ethyl acetate. The N-acetoacetyl methyl ester was heated for 1 h in a refluxing solution of benzene (1 ml) and 3.67 M sodium methoxide solution (170 µ l, 0.62 mmole), and then left at room temperature for 48 h. The product was isolated as described previously for the unlabelled analogue (9) and was crystallised from ethanol to give pale yellow crystals (103 mg, 62%, 391.7 µ Ci/mmol), m.p. 173°C (lit.,[20] 171 - 173°C).

2-Azaindole-3-carboxylic acid.- 2-Azaindole-3-carboxylic acid was prepared from isatin by the method described by Snyder et al.[25] The crude product was crystallised from water, giving pale yellow needles (33% yield), m.p. 268 - 268.5°C (block preheated to 220°C) (lit.,[25] 268 - 268.5°C) (Found: C, 59.2; H, 3.9; N, 17.4. Calc. for $C_8H_6N_2O_2$: C, 59.3; H, 3.7; N, 17.3%); δ (CD_3OD) (90 MHz) 8.08 (1H, d, J 8 Hz, H-7) and 7.30 (3H, m, ArH); m/e 162 (M^+), 145 (M - OH), and 118 (M - CO_2H + H).

NN-Dimethyl-2-azaindole-3-carboxylic acid amide.- This was prepared from 2-azaindole-3-carboxylic acid, dimethylamine and thionyl chloride according to the procedure of Snyder et al.[25] The crude product gave pale beige crystals (78% yield) from nitromethane, m.p. 191 - 192°C (lit.,[25] 187 - 188.5°C) (Found: C, 63.4; H, 6.1; N, 22.1. Calc. for

$C_{10}H_{11}N_3O$: C, 63.5; H, 5.9; 22.2%); ν_{max}(KBr) 3 440, 3 160, and 1 600 cm^{-1}; $\delta(CDCl_3)$ (90 MHz) 8.08 (1H, d, J 8 Hz, H-7), 7.31 (3H, m, ArH), and 3.30 (6H, s, 2 x Me); m/e 189 (M^+), 145 (M - NMe_2), and 118 (M - $CONMe_2$ + H).

3-Dimethylaminomethyl-2-azaindole.- NN-Dimethyl-2-azaindole-3-carboxylic acid amide was reduced with lithium aluminium hydride in refluxing anhydrous tetrahydrofuran, as described by Snyder et al.[25] Crystallisation from diethyl ether afforded white crystals (77% yield), m.p. 126 - 127°C (lit.,[25] 125 - 126°C) (Found: C, 68.6; H, 7.7; N, 24.0. Calc. for $C_{10}H_{13}N_3$: C, 68.5; H, 7.5; N, 24.1%); ν_{max}(KBr) 3 120, 3 080, 3 040, 2 990, 2 960, 2 870, 2 830, 2 810, and 1 620 cm^{-1} δ ($CDCl_3$) (90 MHz) 7.82 (1H, d, J 8 Hz, H-7), 7.20 (3H, m, ArH), 3.88 (2H, s, CH_2), and 2.32 (6H, s, 2 x Me); m/e 175 (M^+), 160 (M - Me), 145 (M - 2 x Me), and 132 (M - NMe_2 + H).

Methiodide of 3-dimethylaminomethyl-2-azaindole.- A mixture of 3-dimethylaminomethyl-2-azaindole and methyl iodide in ethanol was heated to give a clear solution, which was then left at 0°C overnight. The crude solid formed was filtered, dried (84% yield) and recrystallised (94% recovery) from ethanol, m.p. 192 - 194°C (lit.,[25] 192 - 193°C). No attempts were made to further characterise this compound due to its instability.

Ethyl α-formamido-α-ethoxycarbonyl-β-(2-azaindol-3-yl)propionate (17a).- Treatment of diethyl formamidomalonate with sodium ethoxide and the methiodide of 3-dimethylaminomethyl-2-azaindole, according

to the published procedure,[25] gave white crystals (53% yield) from ethanol, m.p. 140 - 142°C (lit.,[25] 140 - 140.5°C) (Found: C, 57.9; H, 5.8; N, 12.6. Calc. for $C_{16}H_{19}N_3O_5$: C, 57.7; H, 5.7; N, 12.6%); ν_{max}(KBr) 3 390, 3 260, 2 990, 1 760, 1 735, and 1 645 cm^{-1}; δ ($CDCl_3$) (60 MHz) 8.10 (1H, s, CHO), 7.33 (6H, m, ArH and 2 x NH, which exchange with D_2O), 4.43 (4H, q, $C\underline{H}_2Me$) and 4.10 (2H, s, CH_2-C), and 1.26 (6H, t, 2 x $CH_2\underline{Me}$); m/e 333 ($\underline{M}^+$), 289 (M - NHCHO), 288 (M - OEt), 260 (M - CO_2Et), 243 (M - 2 x OEt), 232 (M - NHCHO - 2 x Et + H), 214, 198, 186 (M - 2 x CO_2Et - H), and 131 ($C_8H_7N_2$).

β-(2-Azaindol-3-yl)alanine (14).- Ethyl α-formamido-α-ethoxycarbonyl-β-(2-azaindol-3-yl)propionate (1.61 g, 5 mmoles) was heated in refluxing concentrated hydrochloric acid for 7 h. The resulting clear solution was evaporated under reduced pressure to give a white foam, which was dissolved in water (ca. 5 ml) and neutralized with dilute ammonia solution. The solid obtained on cooling was filtered and crystallised from water to give a white crystalline solid (85% yield) which gave a positive ninhydrin test, m.p. 248 - 251°C (dec.) [lit.,[25] 249 - 250°C (dec.)]; ν_{max}(KBr) 3 420, 3 200 - 2 900, 1 620, 1 500, 1 400, and 1 350 cm^{-1}; δ(d_6-DMSO/D_2O) (100 MHz)[37] 7.82 (1H, d, J_{45} 7.9 Hz, H-4), 7.54 (1H, d, J_{76} 8.3 Hz, H-7), 7.38 (1H, ddd, J_{64} 0.9, J_{65} 6.5, J_{67} 8.4 Hz, H-6), 7.13 (1H, ddd, J_{54} 8.0, J_{56} 6.5, J_{57} 1.4 Hz, H-5), 3.79 (1H, X part of ABX system, J_{AX} 2.8, J_{BX} 9.4 Hz, α-CH), 3.55 and 3.29 (2H, AB part of ABX system, J_{AB} 8.0, J_{AX} 2.8, J_{BX} 9.4 Hz, β-CH_2); m/e 205 ($\underline{M}^+$), 187 (M - H_2O), 160 (M - CO_2H), 132 ($C_8H_8N_2$), and 118 ($C_7H_6N_2$).

The amino acid was also obtained by reacting the methiodide of 3-dimethylaminomethyl-2-azaindole with dimethyl acetamidomalonate and sodium ethoxide in ethanol, followed by hydrolysis in

refluxing concentrated hydrochloric acid, without prior purification of the acetamido diester (17b). Evaporation of the hydrolysis mixture to dryness, followed by neutralization to pH 7 with dilute ammonia solution and cooling, gave a solid which was treated with charcoal and twice recrystallised from water, giving white crystals (16% yield from the methiodide) with the same characteristics described before.

3-Dimethylaminomethyl-7-azaindole.- This compound was prepared as described by Robison and Robison[26] from 7-azaindole, dimethylamine hydrochloride and paraformaldehyde (75% crude yield), m.p. 147 - 152°C (lit.,[26] 144 - 152°C); δ($CDCl_3$) (90 MHz) 8.24 (1H, d, ArH), 8.00 (1H, d, ArH), 7.22 (1H, bs, ArH), 7.03 (1H, m, ArH), 3.56 (2H, s, CH_2), 2.21 (6H, s, NMe_2), and 1.82 (1H, bs, NH, exchangeable with D_2O); m/e 175 (M^+) and 131 (M - NMe_2).

Ethyl α-acetamido-α-ethoxycarbonyl-β-(7-azaindol-3-yl)propionate.- The unrecrystallised 3-dimethylaminomethyl-7-azaindole was heated in refluxing xylene with powdered sodium hydroxide and diethyl acetamidomalonate as described in the literature,[26] giving the crude title compound in 59% yield. A sample recrystallised from benzene had m.p. 172 - 173.5°C (lit.,[26] 170 - 170.5°C) (Found: C, 58.9; H, 5.8; N, 12.3. Calc. for $C_{17}H_{21}N_3O_5$: C, 58.8; H, 6.1; N, 12.1%); ν_{max}(KBr) 3 420, 3 300, 1 740, and 1 645 cm^{-1}; δ($CDCl_3$) (90 MHz) 8.37 (1H, bs, NH, exchangeable with D_2O), 7.97 (1H, d, ArH), 7.22 (2H, m, ArH), 6.80 (1H, s, ArH), 4.37 (4H, q, CH_2Me), 3.97 (2H, s, CH_2-C), 2.07 (3H, s, COMe), 1.35 (6H, t, CH_2Me), and 1.05 (1H, s, NH, exchangeable with D_2O); m/e 347 (M^+), 302 (M - OEt), 289 (M - NHAc), 216 [$C(CO_2Et)_2NHAc$], and 131 ($C_8H_7N_2$).

β-(7-Azaindol-3-yl)alanine (15).- Ethyl α-acetamido-α- ethoxycarbonyl-β-(7-azaindol-3-yl)propionate was hydrolysed in concentrated hydrochloric acid by the method described.[26] The crude amino acid was treated with charcoal and recrystallised from water to give white crystals (84% yield) which gave a positive ninhydrin test, m.p. 254 - 256°C (dec.) [lit.,[26] 257 - 259°C (dec.)]; ν_{max}(KBr) 3 420, 3 240 - 2 900, 1 630, 1 585, 1 500, 1 450, 1 350, and 1 330 cm^{-1}; δ (d_6-DMSO/D_2O) (100 MHz)[38] 8.19 (1H, dd, J_{64} 1.5, J_{65} 4.6 Hz, H-6), 8.11 (1H, dd, J_{46} 1.2, J_{45} 8.0 Hz, H-4), 7.32 (1H, s, H-2), 7.08 (1H, dd, J_{54} 7.9, J_{56} 4.7 Hz, H-5), 3.59 (1H, X part of ABX system, J_{AX} 3.9, J_{BX} 8.7 Hz, α-CH), 3.31 and 3.08 (2H, AB part of ABX system, J_{AX} 3.9, J_{BX} 8.7, J_{AB} 8.4 Hz, β-CH_2); m/e 205 (M^+), 160 (M - CO_2H), 131 ($C_8H_7N_2$), and 118 ($C_7H_6N_2$).

3-Choromethylbenzothiophene.- 3-Chloromethylbenzothiophene was prepared from benzothiophene and trioxymethylene in acid solution as described by King and Nord.[39] The crude product was purified by bulb-to-bulb distillation (95 - 100°C/0.05 Torr) to give a colourless oil that solidified on standing. The solid was recrystallised from petroleum ether (40 - 60°C) to give white plates (56%), which decomposed on standing, m.p. 37 - 39°C (lit.,[39] 39 - 40°C) (Found: C, 59.0; H, 3.6; S, 17.7. Calc. for C_9H_7ClS: C, 59.2; H, 3.8; S, 17.5%); δ (CCl_4) (60 MHz) 7.9 - 7.7 (2H, m, ArH), 7.4 - 7.3 (3H, m, ArH), and 4.8 (2H, s, CH_2); m/e 184 (M^+ with ^{37}Cl), 182 (M^+ with ^{35}Cl), 149 [M(^{34}S) - Cl], 147 [M(^{32}S) - Cl], 134 (C_8H_6S), and 108 (C_6H_4S).

Ethyl α-formamido-α-ethoxycarbonyl-β-(1-benzothien-3-yl)propionate.- 3-Chloromethylbenzothiophene was reacted with diethyl formamidomalonate

in the presence of sodium ethoxide in ethanol, according to the procedure described by Avakian *et al.*,[27] to give white crystals from benzene - petroleum ether (60 - 80°C), m.p. 103 - 105°C (lit.,[27] 106 - 107°C); δ($CDCl_3$) (90 MHz) 7.93 (1H, s, CHO), 7.53 (4H, m, ArH), 7.08 (1H, s, H-2), 4.26 (4H, q, CH_2Me), 3.97 (2H, s, CH_2-C), 1.52 (1H, bs, NH, exchangeable with D_2O), and 1.23 (6H, t, CH_2Me); *m/e* 349 (M^+), 304 (M - OEt), 276 (M - CO_2Et), 259 (M - 2 x OEt), 248 (M - NHCHO - 2 x Et), 230 (M - OEt - CO_2Et + H), and 202 (M - 2 x CO_2Et - H).

β-(1-Benzothien-3-yl)alanine (16).- Ethyl α-formamido-α-ethoxycarbonyl-β-(1-benzothien-3-yl)propionate (1.94 mmoles) was heated in refluxing concentrated hydrochloric acid (10 ml) for six hours. The solution was evaporated to dryness and the residue was dissolved in aqueous ethanol (1:1, 8 ml) and neutralized to pH 7 with dilute ammonia. On cooling, a solid was obtained which was recrystallised from aqueous ethanol (1:1) to give white crystals (74% yield) which gave a positive ninhydrin test, m.p. 230 - 232°C (dec.)[lit.,[40] 242 - 244°C (dec.)] (Found: C, 59.9; H, 4.8; N, 6.2; S, 14.5. Calc. for $C_{11}H_{11}NO_2S$: C, 59.7; H, 5.0; N, 6.3; S, 14.5%); ν_{max}(KBr) 3 430, 3 060, 3 030, 1 630, 1 500, and 1 400 cm^{-1}; δ(d_6-DMSO/D_2O) (100 MHz)[41] 7.97 (2H, m, H-4 and H-7), 7.52 (1H, s, H-2), 7.43 (2H, m, H-5 and H H-6), 3.70 (1H, X part of ABX system, J_{AX} 3.9, J_{BX} 9.3 Hz, α-CH), 3.50 and 3.16 (2H, AB part of ABX system, J_{AX} 3.9, J_{BX} 9.3, J_{AB} 9.3 Hz, β-CH_2); *m/e* 221 (M^+), 176 (M - CO_2H), 160 (M - CO_2H - NH_2), 147 (C_9H_7S), and 134 (C_8H_6S).

PART I

REFERENCES

1. J.L.Albright, S.D.Aust, J.H.Byers, T.E.Fritz, B.O.Brodie, R.E. Olsen, R.P.Link, J.Simon, H.E.Rhoades, and R.L.Brewer, J. Am. Vet. Med. Assoc.,1964, 144, 1013.
2. B.J.Wilson, C.H.Wilson, and A.W.Hayes, Nature (London), 1968, 220, 77.
3. C.W.Holzapfel, Tetrahedron, 1968, 24, 2101.
4. P.S.Steyn and P.L.Wessels, Tetrahedron Lett., 1978, 4707.
5. C.W.Holzapfel and D.C.Wilkins, Phytochemistry, 1971, 10, 351.
6. C.E.Stickings and R.J.Townsend, Biochem. J.,1961, 78, 412.
7. D.C.Neethling and R.M.McGrath, Can. J. Microbiol., 1977, 23, 856.
8. C.W.Holzapfel, R.D.Hutchison, and D.C.Wilkins, Tetrahedron, 1970, 26, 5239.
9. S.Ohmomo, M.Sugita, and M.Abe, J. Agric. Chem. Soc. (Japan), 1973, 47, 57.
10. K.C.Luk, B.Kobbe, and J.M.Townsend, Appl. Environ. Microbiol., 1977, 33, 211.
11. J.C.Schabort, D.C.Wilkins, C.W.Holzapfel, D.J.J.Potgieter, and A.W.Neitz, Biochim. Biophys. Acta, 1971, 250, 311.
12. P.F.Heinstein, S.L.Lee, and H.G.Floss, Biochem. Biophys. Res. Commun.,1971, 44, 1244.
13. R.M.McGrath, P.S.Steyn, N.P.Ferreira, and D.C.Neethling, Bioorg. Chem., 1976, 4, 11.
14. R.M.McGrath, P.S.Steyn, and N.P.Ferreira, J. Chem. Soc., Chem. Commun., 1973, 812.

15. C.M.Allen, Biochemistry, 1972, 11, 2154.

16. C.W.Holzapfel and J.Schabort, S. Afr. J. Chem.,1977, 30, 233.

17. C.W.Holzapfel and P.J.Gildenhuys, S. Afr. J. Chem., 1977, 30, 125.

18. P.S.Steyn, R.Vleggaar, N.P.Ferreira, G.W.Kirby, and M.J.Varley, J. Chem. Soc., Chem. Commun., 1975, 465.

19. C.W.Holzapfel, in "The Biosynthesis of Mycotoxins - A Study in Secondary Metabolism", ed. P.S.Steyn, Academic Press, New York, 1980, p. 327.

20. H.Matsuo, Y.Fujimoto, and T.Tatsuno, Tetrahedron Lett.,1965, 3465.

21. H.Yuki, K.Kariya, and Y.Hashimoto, Chem. Pharm. Bull., 1967, 15, 727.

22. S.A.Harris, L.V.Fisher, and K.Folkers, J. Med. Chem.,1965, 8, 478.

23. T.C.P.Mulholland, R.Foster, and D.B.Haydock, J. Chem. Soc., Perkin Trans. 1, 1972, 2121.

24. R.N.Lacey, J. Chem. Soc., 1954, 850.

25. H.R.Snyder, C.B.Thompson, and R.L.Hinman, J. Am. Chem. Soc., 1952, 74, 2009.

26. M.L.Robison and B.L.Robison, J. Am. Chem. Soc., 1955, 77, 457.

27. S.Avakian, J.Moss, and G.J.Martin, J. Am. Chem. Soc., 1948, 70, 3075.

28. W.B.Turner, "Fungal Metabolites", Academic Press, London, 1971, p. 87.

29. S.S.H.Gilani and D.J.Triggle, J. Org. Chem.,1966, 31, 2397.

30. D.T.C.Yang and B.J.Wilson, J. Chin. Chem. Soc. (Taipei), 1970, 17, 185.

31. R.M.McGrath, P.N.Nourse, D.C.Neethling, and N.P.Ferreira, Bioorg. Chem., 1977, 6, 53.

32. P.S.Steyn, personal communication.

33. V. du Vigneaud and R.R.Sealock. J. Biol. Chem., 1932, 96, 511.

34. D.I.Weisblat and D.A.Lyttle, J. Am. Chem. Soc., 1949, 71, 3079.

35. M.Brenner and W.Huber, Helv. Chim. Acta, 1953, 36, 1109.

36. D.O.Holland and J.H.C.Nayler, J. Chem. Soc., 1953, 285.

37. cf. E.B.Dennler, C.R.Portal, and A.R.Frasca, Spectrochim. Acta, Part A, 1967, 23, 2243.

38. cf. P.J.Black and M.L.Heffernan, Aust. J. Chem., 1965, 18, 353.

39. W.J.King and F.F.Nord, J. Org. Chem., 1948, 13, 635.

40. N.B.Chapman, R.M.Scrowston, and R.Westwood, J. Chem. Soc., (C), 1969, 1855.

41. cf. N.B.Chapman, D.F.Ewing, R.M.Scrowston, and R.Westwood, J. Chem. Soc., (C), 1968, 764.

PART II

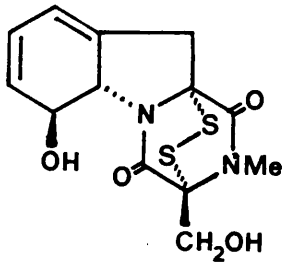
BIOSYNTHETIC STUDIES ON SOME METABOLITES CONTAINING THE DITHIODIOXOPIPERAZINE MOIETY.

CHAPTER 1

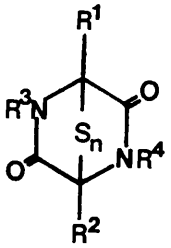
The role of cyclic dipeptides in epipolythiodioxopiperazine biosynthesis

1.1 General

The isolation of gliotoxin (1) in 1936 by Weindling and Emerson,[1] and the subsequent discovery and isolation of other fungal metabolites with related structural features has established the epipolythiodioxopiperazine family of metabolites. The forty-odd metabolites in this family possess as their common feature either the sulphur-bridged dioxopiperazine moiety indicated in (2) or its closely related bisdethiobis(methylthio) variant (3).



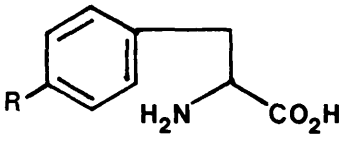
(1)



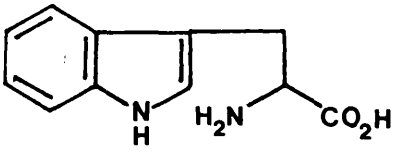
(2)

(3)

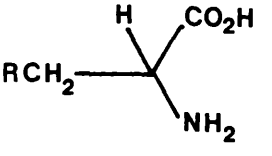
Structural analysis of the compounds belonging to this family indicated that one or both of the residues R^1 and R^2 are derived from the aromatic amino acids phenylalanine (4, R = H), tryptophan (5) or tyrosine (4, R = OH). The other residue may be derived from alanine (6, R = H), serine (6, R = OH) or glycine. In a number of cases, feeding experiments with the appropriate amino acids have supported this theory.



(4)



(5)

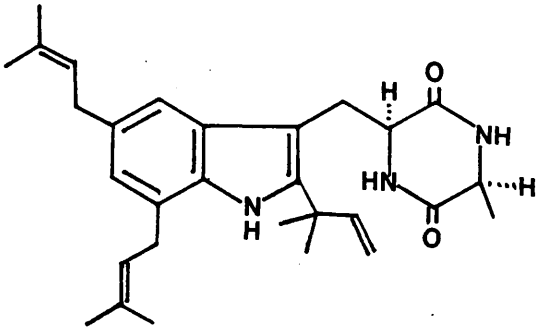


(6)

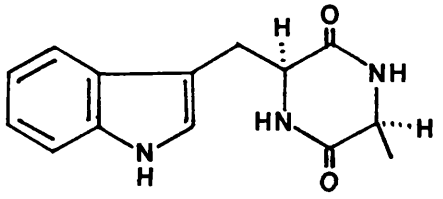
Several reviews on the structure, chemistry, biosynthesis, and biological activity of these metabolites have appeared in the literature during the last ten years.[2-5] Recently, Kirby and Robins have provided a comprehensive review on the biosynthesis of gliotoxin and related epipolythiodioxopiperazines.[6]

Simple cyclic dipeptides have been shown to be precursors to more complex dioxopiperazines.[3] Two examples are echinulin (7), shown to be derived from cyclo-(L-alanyl-L-tryptophyl) [8, cyclo-(L-Ala-L-Trp)] and brevianamide A (9), which is biosynthesised from cyclo-(L-prolyl-

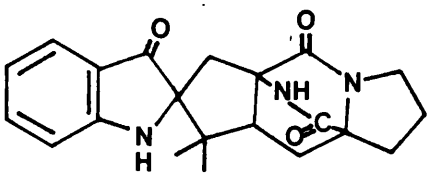
L-tryptophyl) [10, cyclo-(L-Pro-L-Trp)]. The purpose of this chapter is to survey briefly the role of cyclic dipeptides (dioxopiperazines) as intermediates in the biosynthesis of epipolythiodioxopiperazines.



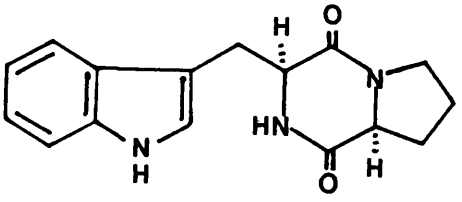
(7)



(8)



(9)

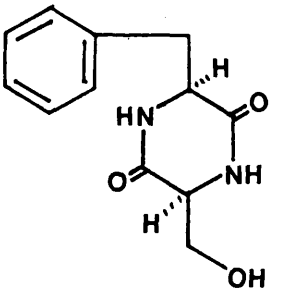


(10)

1.2 Gliotoxin

MacDonald and Slater[7] were the first to suggest that cyclo-(L-phenylalanyl-L-seryl) [11, cyclo-(L-Phe-L-Ser)] might be an intermediate in the biosynthesis of gliotoxin (1). However, when these authors fed cyclo-(L-[1-^{14}C]Phe-L-Ser) to cultures of Penicillium terlikowskii, a known gliotoxin producer, they observed only a low

incorporation of the cyclic dipeptide into gliotoxin, despite its efficient uptake into the mycelium. They concluded that the cyclic dipeptide (11) could not be a free intermediate. Shortly after,



(11)

Bu'Lock and Leigh[8] fed a mixture of cyclo-(L-[Ar-^{3}H]Phe-D-[1-^{14}C]Ser) and cyclo-(L-[Ar-^{3}H]Phe-L-[1-^{14}C]Ser) to Trichoderma viride (probably a Gliocladium sp.) and observed a high incorporation (21%) of the radioactivity into gliotoxin (1). Moreover, the ^{14}C:^{3}H isotope ratios of the precursor mixture and the isolated gliotoxin were effectively unchanged, indicating that the cyclic dipeptide was incorporated intact into gliotoxin. Bu'Lock and Leigh[8] suggested that the misleading results obtained by MacDonald and Slater[7] were due to the administration of precursor in such high levels that equilibration of exogenous material with biosynthetic intermediates had been blocked.

The observations of Bu'Lock and Leigh were later confirmed and extended by Kirby et al.[9] ^{14}C-Labelled samples of all four possible stereoisomers of cyclo-(phenylalanylseryl) were separately fed to cultures of T. viride (Gliocladium deliquescens). The LL-isomer was efficiently incorporated (up to 50%) into gliotoxin, whereas none of

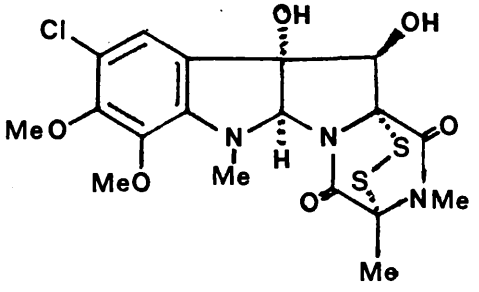
the other stereoisomers was incorporated to any significant extent. Administration of cyclo-(L-[4-^{3}H]Phe-L-[3-^{14}C]Ser) to the fungus gave gliotoxin (1) with essentially the same isotope ratio as the precursor, and hydrolysis of the isolated metabolite produced inactive methylamine. These results[9] supported the hypothesis that (11) is incorporated intact into gliotoxin. Breakdown of the cyclic dipeptide to [3-^{14}C]serine, and equilibration with the C_1 metabolite pool would have resulted in ^{14}C-activity appearing in the methylamine.

The status of the cyclic dipeptide (11) as a free intermediate in T. viride was also investigated by the same group[9] by means of an "intermediate-trapping" experiment. Non-radioactive (11) was fed to the mould culture and, 2 hours later, L-[U-^{14}C]Phe 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 was then extracted with ethyl acetate to remove the cyclodipeptide (11). This was diluted with unlabelled (11) and the mixture was crystallised to constant activity. The residual activity corresponded to 1.3% of that administered as L-[U-^{14}C]Phe.

The presence of cyclo-(phenylalanylseryl) as a free biosynthetic intermediate has also been demonstrated recently in G. virens, another gliotoxin producing fungus . Behling and Fischer[10] incubated DL-[3-^{14}C]serine with protoplasts of G. virens and isolated from this mixture radioactive cyclo-(phenylalanylseryl). The experimental conditions used by these authors did not lead to gliotoxin biosynthesis. Nevertheless, this observation supports the status of (11) as an intermediate in gliotoxin biosynthesis.

1.3 Sporidesmins

Sporidesmin A (12) and related metabolites are thought to be derived from cyclo-(L-Ala-L-Trp) (8). The metabolism of (8) in Pithomyces chartarum was studied by Varley.[11] Administration of cyclo-(L-[3-^{3}H]Ala-L-[3-^{14}C]Trp) resulted in a 2.05% ^{14}C incorporation into sporidesmin A, but the ^{3}H:^{14}C ratio of the product was only 51%



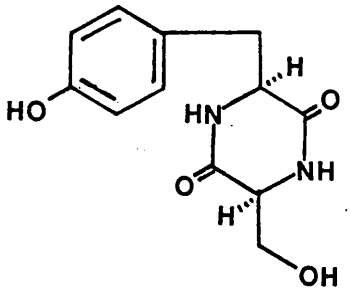
(12)

that of the precursor. The author proposed that this result could have arisen by either: (a) partial or complete cleavage of (8) into its constituent amino-acids, followed by their separate incorporation or (b) loss of ^{3}H from the methyl group of the alanine unit via reversible formation of a dehydroalanyl derivative at some point in the biosynthetic pathway. Further studies are needed to settle the role of the cyclodipeptide (8) in sporidesmin biosynthesis.

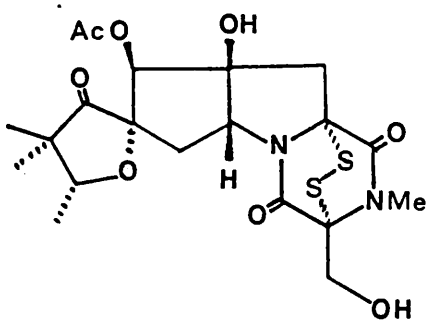
1.4 Sirodesmins

Curtis et al.[12] were the first to suggest that a cyclo-(seryl-tyrosyl) derivative might be a precursor to the sirodesmins.

Recently, Ferezou et al.[13] fed cyclo-(L-Ser-L-[U-^{14}C]Tyr) (14) to Phoma lingam cultures at two different concentration levels. The isolated metabolite, sirodesmin A (14) contained 12.5% and 4.35% of the precursor radioactivity from the low- and high-level feedings



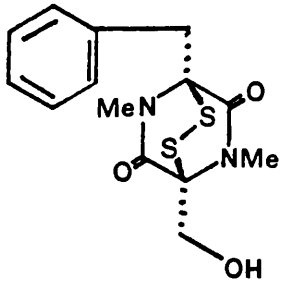
(13)



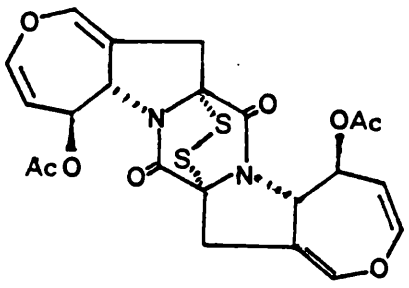
(14)

respectively. These results are in agreement with Bu'Lock and Leigh's previous observations[8] regarding levels of administered precursors in biosynthetic experiments.

1.5 Other epipolythicdicxopiperazines



(15)



(16)

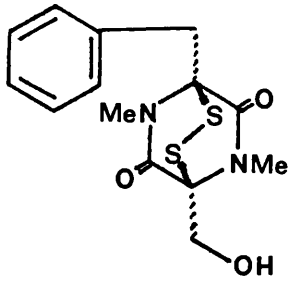
Hyalodendrin (15) and acetylaranotin (16) are two members of the epipolythiodioxopiperazine family that show an interesting resemblance to gliotoxin (1). The study of their biosynthesis, and the role played in it by cyclic dipeptides, is the object of this work and is discussed in the following chapters.

CHAPTER 2

Hyalodendrin

2.1 Isolation, biological activity and structural determination

In 1973, Strunz et al.[14] found potent antifungal properties associated with a species of Hyalodendron, an imperfect fungus. The search for the active principle responsible for this activity led to the isolation of a new metabolite, designated hyalodendrin and assigned the structure (15) on spectroscopic and chemical evidence. Comparison



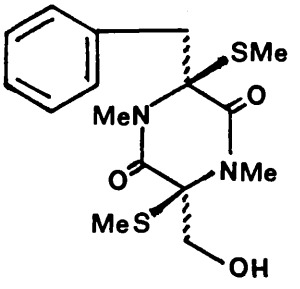
(15)

of the circular dichroism curve of hyalodendrin with those obtained for other epidithiodioxopiperazines showed[14] that the disulphide bridge in hyalodendrin is antipodal to that of gliotoxin (1), the sporidesmins and the aranotins.

The biological activity of hyalodendrin (15) has been studied by Stillwell's group.[15] They found that the antimicrobial activity of hyalodendrin against fungi associated with decay and deterioration in

trees and wood products was, with few exceptions, considerably greater than that of nystatin, cryptosporiopsin or scytalidin. Hyalodendrin also compared favourably with nystatin and benomyl in its ability to inhibit the growth of Ceratocystis ulmi, the causal organism of Dutch elm disease. Its potential use as a control agent of this devastating tree disease was suggested by the authors.[15] Hyalodendrin also showed antibacterial and antifungal activity against several human pathogens. However, hyalodendrin is also acutely toxic to mice, the LD_{50} having been established at 75 mg/kg.

A second metabolite was isolated from Hyalodendron sp. by Strunz et al.[16] Comparison of its spectroscopic properties with those of hyalodendrin (15), showed that this new metabolite was bisdethio-bis(methylthio)hyalodendrin (17), in which the disulphide bridge has



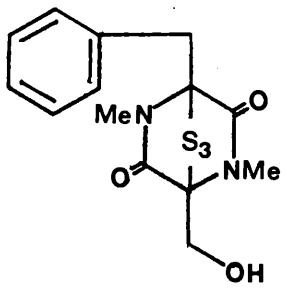
(17)

been replaced by two methylthio groups. Isolation of bisdethiobis(methylthio) modifications of epidithiodioxopiperazine metabolites are well precedented in the literature.[6] In all these cases, (17) included, the absence of a sulphur bridge results in a total lack of biological activity.

Strunz et al. treated hyalodendrin (15) with sodium borohydride

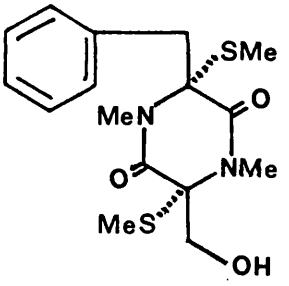
in the presence of methyl iodide and pyridine at 0°C.[16] Comparison of the chromatographic behaviour and spectra of the product of this reaction, together with a mixed melting point determination, established its identity with the natural metabolite (17). Regrettably, the optical rotations of the synthetic product and of natural bisdethiobis(methylthio)-hyalodendrin were not compared.

Also in 1973, De Vault and Rosenbrook[17] reported the isolation of three metabolites from an unidentified fungus, NRRL 3888. On the basis of ^{1}H n.m.r. and mass spectroscopic evidence, they assigned to two of these metabolites the same structures, (15) and (17), independently given to hyalodendrin and its congener, but no efforts were made to determine the absolute configuration of the sulphur substituents in either metabolite. The third metabolite was found to possess a bridge containing three sulphur atoms, as shown in (18).



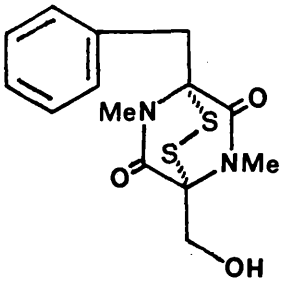
(18)

Two other groups have isolated another metabolite closely related to bisdethiobis(methylthio)hyalodendrin (17). Helminthosporium victoriae was found by Dorn and Arigoni[18] to produce gliovictin (19), a metabolite with the same gross structure as (17), but with the (R)-configuration at positions 3 and 6. Independently, Michel at al.[19]

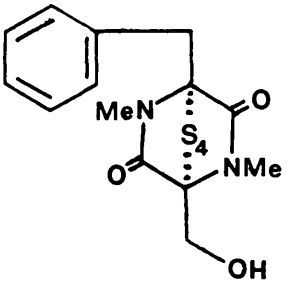


(19)

isolated from Penicillium turbatum a compound, antibiotic A26771E, to which they assigned the structure (19). Thus antibiotic A26771E and gliovictin (19) are identical. P. turbatum was also found to produce two more metabolites with the (R)-configuration at positions 3 and 6: the hyalodendrin antipode (20), whose structure, excluding absolute configuration, was established by X-ray diffraction studies, and the epitetrathiodioxopiperazine (21). Both compounds possess antiviral and antibacterial activity.[19]



(20)



(21)

Strunz et al.[20] have also isolated an epitetrathiodioxopiperazine from cultures of Hyalodendron sp. Its gross structure was found, by spectroscopic methods, to be identical to that of (21), isolated from

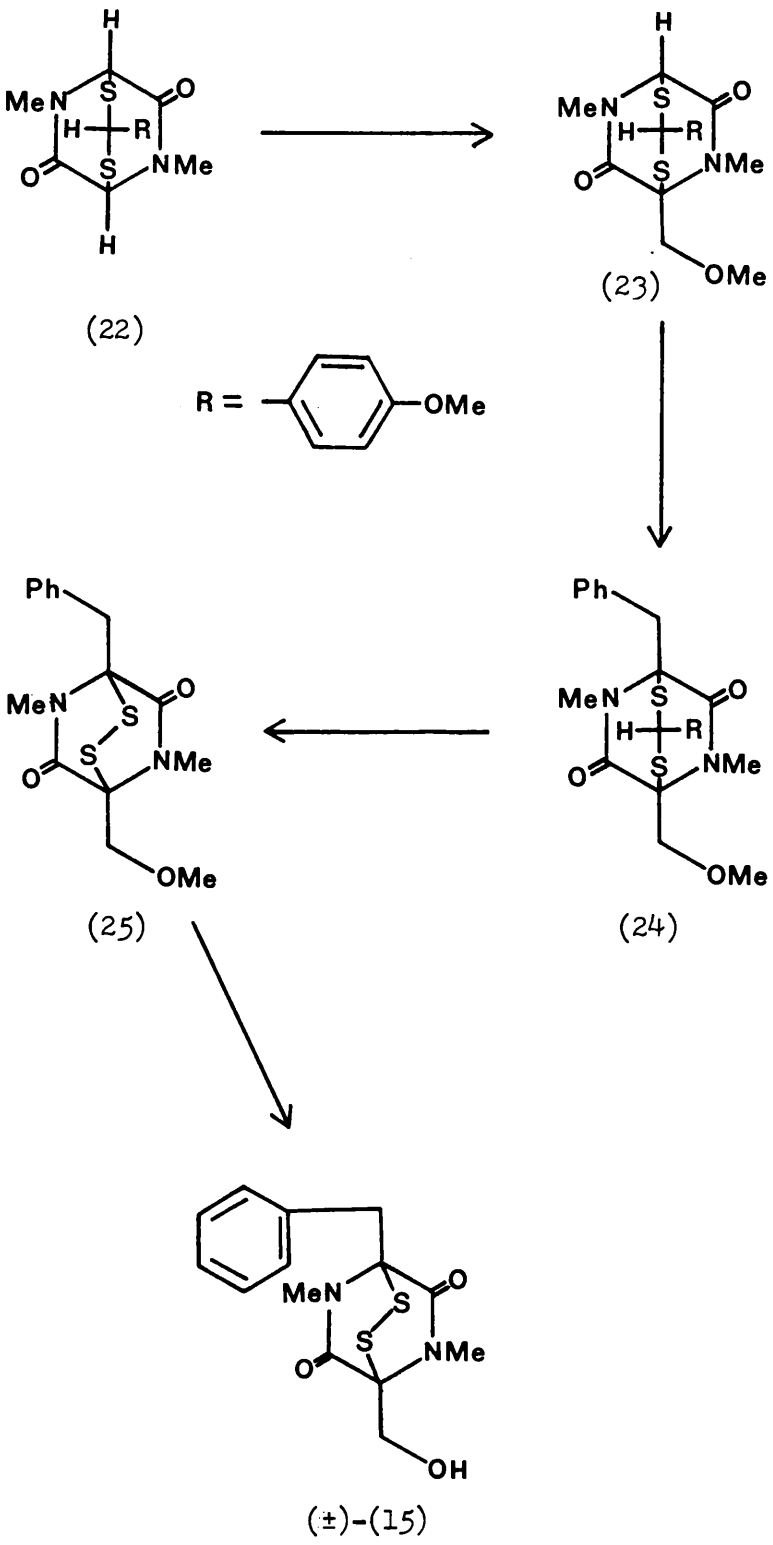
P. turbatum. However, comparison of the circular dichroism curve of the Hyalodendron tetrasulphide with that of (21), established that these compounds are enantiomers.[20] The structure and relative, but not absolute, configuration of the (S)-tetrasulphide was confirmed by X-ray studies.[21]

Summarizing, P. turbatum and Hyalodendron are each able to produce a family of polythiodioxopiperazine metabolites enantiomeric with each other. The reasons for this enantiomeric selectivity in nature are not yet understood.

2.2 Synthesis

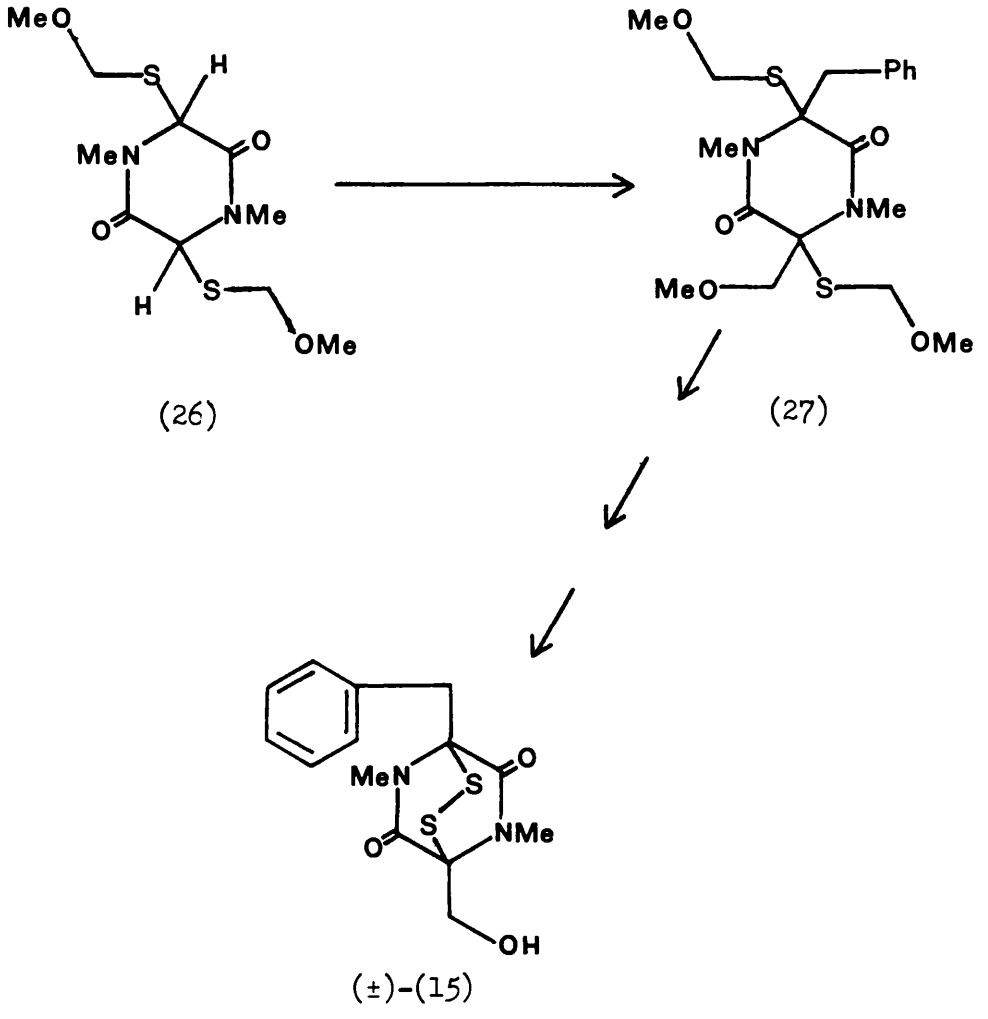
Hyalodendrin was first synthesised by Strunz and Kakushima,[22] using a slight modification of the method developed by Kishi *et al.* for the total synthesis of dehydrogliotoxin[23] and sporidesmin A.[24] In this method, the thioacetal (22), prepared from the *cis*-dithiol and *p*-anisaldehyde, serves as a protected precursor of the epidithiomoiety and can be metalated and alkylated selectively stepwise at the bridgehead position as shown in Scheme I. Treatment of (22) with one equivalent of n-butyl lithium, followed by alkylation with chloromethyl methyl ether afforded the methyl ether (23). This was again metalated and alkylated with benzyl bromide to give the protected hyalodendrin precursor (24). Oxidation of (24) with *m*-chloroperbenzoic acid, followed by treatment with perchloric acid in tetrahydrofuran according to Kishi's procedure,[23,24] gave, after chromatography, the racemic hyalodendrin methyl ether (25). The methyl ether was cleaved using boron trichloride and, after chromatography of the reaction mixture, racemic hyalodendrin (15) was obtained in 3% overall yield from (22).

A couple of years later Kishi and coworkers[25] reported another, shorter, synthesis of hyalodendrin starting from 1,4-dimethyl-3,6-di(methoxymethylthio)piperazine-2,5-dione (26) (Scheme II; all compounds were racemic). The dianion generated from treatment of (26) with 2.3 equivalents of lithium di-isopropylamide was alkylated first with benzyl bromide and then with bromomethyl methyl ether to give, in a one-pot reaction, the unsymmetrically disubstituted, protected dithiopiperazinedione (27). Removal of the sulphur protecting groups



Scheme I

was achieved using boron trichloride. Iodine oxidation of the resulting dithiol, to give the desired disulphide bridge, was followed by further treatment with boron trichloride to liberate the primary alcohol group of hyalodendrin (15). The overall yield of (15) from (26) was 16%. The authors[25] report a melting point of 101 - 102°C for their product. This is close to that obtained by Strunz et al.[14] for natural hyalodendrin (100.5 - 101.5°C) but not to that reported by Strunz and Kakushima[22] for their synthetic racemic hyalodendrin (131 - 134°C). Whether this discrepancy is due to a printing error or to the omission of reporting a resolution step is not clear.

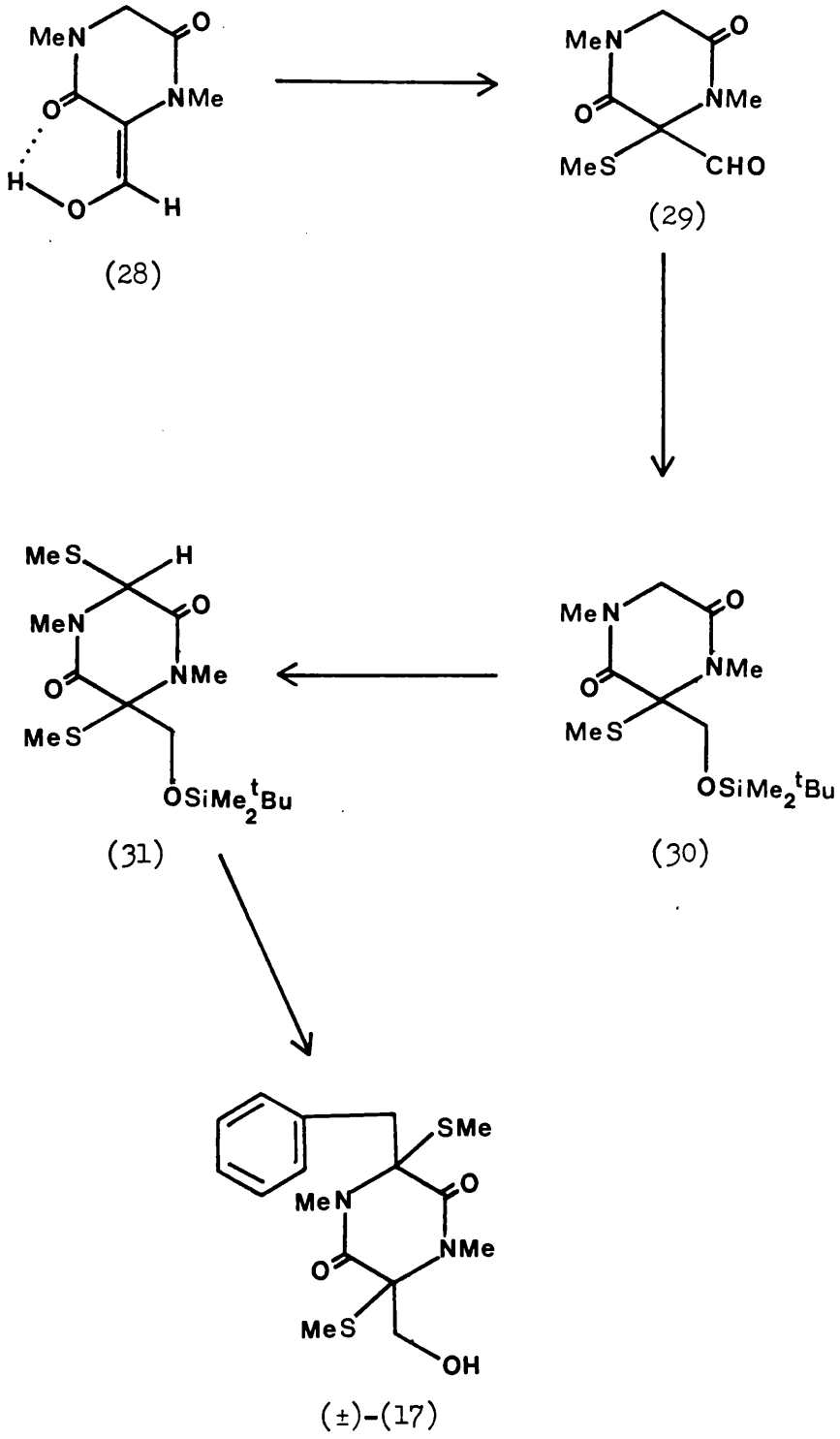


Scheme II

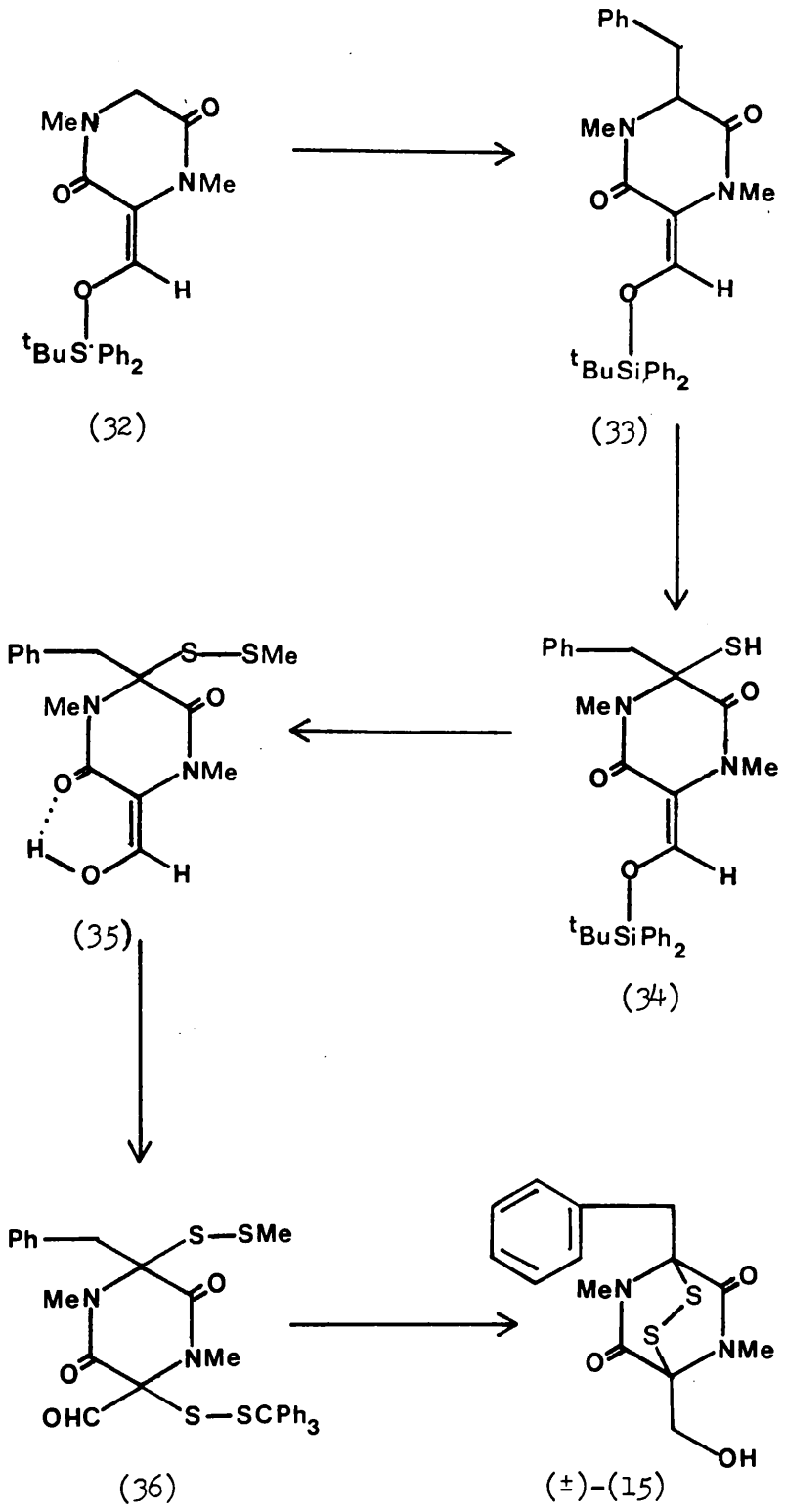
Williams and Rastetter[26] have recently synthesised racemic bisdethiobis(methylthio)hyalodendrin (17) (Scheme III) and hyalodendrin (15) (Scheme IV). Their method is based on the stereoselective alkylation and sulphenylation of enolates derived from dioxopiperazines. The authors found that reactions of enolates of dioxopiperazines already containing a sulphur substituent, with a carbon or sulphur electrophile, occur preferentially, although to varying degrees, on the face opposite to the existing sulphur substituent (vide infra).

Addition of a slight excess of methanesulphenyl chloride, in the presence of one equivalent of triethylamine, to the enol (28) (Scheme III), obtained from monoformylation of sarcosine anhydride (1,4-dimethyl-piperazine-2,5-dione), gave the product (29). Its aldehyde group was reduced with tri-t-butoxyaluminium hydride to give a primary alcohol, which was protected with t-butyldimethylsilyl chloride to give (30). The enolate derived from treatment of this compound with lithium di-isopropylamide was sulphenylated with dimethyl disulphide to give a mixture of diastereomers (31), which was not separated. Benzylation of the enolate of (31), followed by removal of the silyl protecting group, afforded racemic bisdethiobis(methylthio)hyalodendrin (17) in 38% overall yield from sarcosine anhydride.

Hyalodendrin was synthesised along similar lines (Scheme IV), albeit in lower yield. Benzylation of the enolate of (32), prepared from the potassium salt of (28) and t-butyldiphenylsilyl chloride, afforded the silyl ether (33). Sulphenylation of the enolate of this compound, using monoclinic sulphur, followed by reductive work up, gave the thiol (34). This was converted into the enolic methyl disulphide (35) by reaction with methanesulphenyl


MeN
O
NMe
O
H
O
H
(28)
MeN
O
NMe
O
MeS
CHO
(29)
MeS
H
MeN
O
NMe
O
MeS
OSiMe2tBu
(31)
MeN
O
NMe
O
MeS
OSiMe2tBu
(30)
SMe
MeN
O
NMe
O
MeS
OH
(±)-(17)

Scheme III


MeN
O
NMe
O
H
O
tBuSiPh2
(32)
Ph
(33)
SH
(34)
S—SMe
(35)
OHC
S—SCPh3
(36)
S
OH
(±)-(15)

Scheme IV

chloride in the presence of triethylamine, followed by acid hydrolysis of the silyl protecting group. The enolate of (35) was generated with triethylamine and sulphenylated with triphenylmethyl chloro-disulphide to give (36) as a mixture of diastereomers, favouring the undesired anti-isomer. Reduction of (36) with sodium borohydride, followed by oxidation with potassium tri-iodide in pyridine and chromatography of the product, gave racemic hyalodendrin (15) in 12% overall yield from sarcosine anhydride.

2.3 Biosynthesis

As mentioned in Chapter 1, hyalodendrin (15) and other metabolites of the same group (*vide supra*), although structurally simpler, bear a close resemblance to gliotoxin (1). They too appear to be derived from phenylalanine and serine. Both nitrogen atoms in the dioxopiperazine nucleus are methylated and ring closure to the dihydroaromatic system has not occurred. Whether the absence of this late-stage oxidation is due to the lack of the necessary enzymes or whether oxidation cannot take place after the nitrogen atoms are methylated, is yet to be established. Moreover, in the group of metabolites produced by *Hyalodendron* sp., the sulphur-bridged system is of a configuration opposite to that of gliotoxin. In the light of the results obtained by Kirby *et al.*[9] on the biosynthesis of gliotoxin, it seemed likely that *cyclo*-(phenylalanylseryl) might be an intermediate in the biosynthetic route leading to the *Hyalodendron* metabolites. The antipodal character of the disulphide bridge of hyalodendrin as compared to that of gliotoxin, further suggested comparative experiments with the LL- and DD-forms of *cyclo*-(phenylalanylseryl).

These investigations were started by Patrick[27] in our laboratories. The temperature (21°C) used by Strunz *et al.*[14] for growing *Hyalodendron* sp. was not available in our growth room; instead, the cultures were grown at 25°C. As a result, bisdethiobis(methylthio)-hyalodendrin (17), and not hyalodendrin (15), was found to be the major metabolite produced. Since (17) and (15) have the same configuration of interest at the sulphur bridge and, moreover, since the former

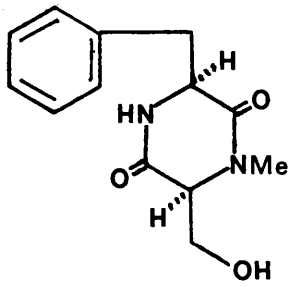
is easier to crystallise than the latter, our studies were all carried out with this compound. Results obtained with bisdethiobis(methylthio)-hyalodendrin (17) should also hold true for hyalodendrin (15).

Patrick synthesised [9,27] cyclo-(L-[U-^{14}C]Phe-L-Ser) and cyclo-(D-Phe-DL-[3-^{14}C]Ser) from the appropriate enantiomers of phenylalanine methyl ester hydrochloride and N-benzyloxycarbonylserine by standard methods. Administration of cyclo-(L-[U-^{14}C]Phe-L-Ser) to cultures of Hyalodendron sp. resulted in a total incorporation of 10% of the precursor radioactivity into bisdethiobis(methylthio)hyalodendrin (17). The radiolabelled mixture of DD- and DL-diastereomers was fed in parallel with cyclo-(L-[U-^{14}C]Phe-L-Ser). Whereas the latter was efficiently (28%) incorporated into the metabolite (17), no significant incorporation was obtained from the former mixture. The specific activity of the pure bisdethiobis(methylthio)hyalodendrin isolated from the administration of the diastereomeric mixture was only 0.6% of the value obtained for the pure compound (17) isolated from the parallel reference feeding.

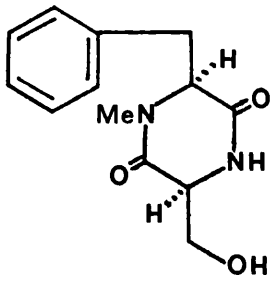
Doubly labelled cyclo-(L-[4'-^{3}H]Phe-L-[3-^{14}C]Ser) was also synthesised and administered to Hyalodendron sp. cultures by Patrick.[27] Incorporation into the metabolite (17) was high (42%) and the $^{3}H/^{14}C$ ratio of the pure crystalline metabolite (11.1) was essentially unchanged from that of the precursor (10.9), thus indicating, as discussed in Chapter 1 for gliotoxin, that the precursor is incorporated intact into the metabolite.

Having demonstrated the involvement of cyclo-(L-Phe-L-Ser) in hyalodendrin biosynthesis, attention was turned to possible later intermediates. Several further stages are required to convert cyclo-

(L-Phe-L-Ser) into hyalodendrin: N-methylation of both nitrogen atoms and incorporation of the disulphide bridge. The order of these steps is unknown, but several intermediates can be postulated. The most accessible in terms of synthesis are cyclo-(L-Phe-L-N-Me-Ser) (37) and cyclo-(L-N-Me-Phe-L-Ser) (38), which might be formed in the biosynthetic pathway by selective N-methylation at either nitrogen



(37)

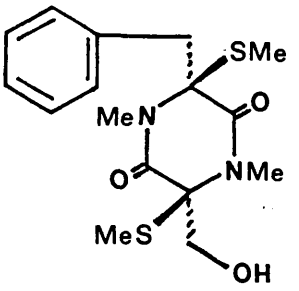


(38)

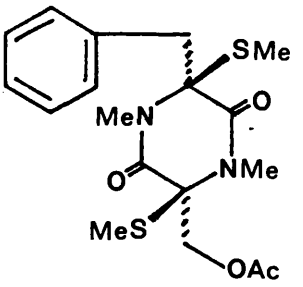
of cyclo-(L-Phe-L-Ser). Patrick synthesised[27] the previously unreported dioxopiperazine (37) by dicyclohexylcarbodiimide coupling of the methyl ester of N-methyl-L-serine t-butyl ether and N-benzyloxycarbonyl-L-phenylalanine, followed by hydrogenolysis of the benzyloxycarbonyl group to give spontaneously the t-butyl protected cyclized dipeptide, which was finally deprotected with 45% hydrobromic acid in glacial acetic acid. The cyclic dipeptide (37) was fed[27] in parallel with cyclo-(L-[U-^{14}C]Phe-L-Ser) to cultures of Hyalodendron sp. Only 0.003% of the total radioactivity administered as (37) was found in the isolated bisdethiobis(methylthio)hyalodendrin, whereas the reference feeding proceeded with 33% total incorporation.

Patrick's results[27] indicated that cyclo-(L-Phe-L-Ser), but not the related DD- or DL-stereoisomer is a precursor to hyalodendrin. Furthermore, there was some indication that the LL-stereoisomer is incorporated intact. Finally, there was no support to the hypothesis that cyclo-(L-Phe-L-N-Me-Ser) (37) could be an intermediate in hyalodendrin biosynthesis. Several points, however, remained to be clarified. Firstly, cyclo-(L-Phe-D-Ser) remained to be investigated as a possible biosynthetic precursor. Second, since no degradation studies had been carried out on the metabolite isolated from any feeding experiment, it was desirable to feed a ^{13}C-labelled precursor to show that incorporation had occurred without "scrambling" of the label. Finally, the possibility that the other N-methylated cyclodipeptide (38) might be incorporated also had to be investigated. These experiments were carried out by the author and are described below.

As discussed before, Hyalodendron sp. was grown at 25°C, thus producing bisdethiobis(methylthio)hyalodendrin (17) as the major metabolite. In a typical run, 20 - 25 mg of pure crystalline (17) were obtained from a litre of mould culture. The melting point, optical rotation, mass and ^1H-n.m.r. spectra of the isolated metabolite (17) agreed with the data reported in the literature.[16] It was found that there was some solvent effect on the chemical shift of one of the N-methyl signals. Thus, in $CDCl_3$, the two N-methyl signals appear at δ 3.03 and 3.30 ppm, whilst in CCl_4 they are observed at 2.97 and 3.13 ppm. For further ^1H-n.m.r. comparison, the acetate (39) of bisdethiobis(methylthio)hyalodendrin was synthesised by treatment of (17) with pyridine and acetic anhydride. The previously unreported acetate (39) gave ^1H-n.m.r. spectral signals for the N-methyl protons



(17)



(39)

at δ 3.28 and 3.05 ppm, approximately the same chemical shifts as those of (17) in $CDCl_3$. However, the S-methyl signals shifted closer to each other, appearing at 2.33 and 2.23 ppm, as opposed to 2.30 and 2.13 ppm for bisdethiobis(methylthio)hyalodendrin. The acetyl protons of (39) appeared at 1.66 ppm, at considerably higher field than usual for simple acetyl protons.

All the feeding experiments to be described were carried out by administering the precursor of choice in dimethyl sulphoxide to 5-day old cultures of the fungus. Extraction of the fed cultures afforded, besides the natural metabolites, crystallisable amounts of a white solid, soluble in most organic solvents and in water. Mixed melting point determination and comparison of the ^1H-n.m.r. spectra showed this material to be dimethyl sulphone. Since this compound was only isolated from cultures that had been fed, it seems that Hyalodendron sp. is capable of oxidising the administered dimethyl sulphoxide to the corresponding sulphone. Because dimethyl sulphone and bisdethiobis(methylthio)hyalodendrin have the same R_f in the chromatographic system used to purify the desired metabolite, it was necessary to find some method to separate these two compounds. Washing a methylene chloride solution of the mixture with several

portions of water afforded pure metabolite (17), devoid of any sulphone impurities, which could then be crystallised from methylene chloride - cyclohexane.

cyclo-(L-[U-^{14}C]Phe-D-Ser) was synthesised by dicyclohexylcarbodiimide coupling of N-benzyloxycarbonyl-D-serine and L-[U-^{14}C]phenylalanine methyl ester hydrochloride in the presence of triethylamine, followed by hydrogenolysis of the N-protecting group and cyclisation to the dioxopiperazine with saturated ammoniacal methanol. The product was obtained in 38% yield from the protected amino acids, with a specific activity of 31.5 μCi/mmol. A solution of this compound (12.1 mg, 1.63 μCi) in dimethyl sulphoxide (1 ml) was distributed among 9 flasks of Hyalodendron culture (total volume, 900 ml). In a parallel feeding, cyclo-(L-[U-^{14}C]Phe-L-Ser) (12.3 mg, 2.99 μCi), in dimethyl sulphoxide (1 ml), was administered to 8 flasks (total volume, 800 ml) of the culture growing under the same conditions. Both cultures were cropped off and extracted as described in the Experimental section. Radioscanning and autoradiography of analytical t.l.c. plates of both crude extracts showed that the radioactivity was only detectable in the extract derived from feeding the LL-isomer. Moreover, in this extract most of the radioactivity was present in bisdethiobis-(methylthio)hyalodendrin (17), whilst a smaller quantity was present in hyalodendrin (15). The bisdethiobis(methylthio)hyalodendrin from both experiments was isolated, purified and crystallised to constant activity. It was found that whilst the metabolite (17) isolated from the culture fed the LL-isomer contained 15% of the administered radioactivity, that isolated from the culture fed the LD-isomer contained only 0.48% of the radioactivity of the precursor. These

results indicate that cyclo-(L-Phe-D-Ser) is not a precursor in hyalodendrin biosynthesis.

As an alternative to carrying out chemical degradation studies on doubly labelled bisdethiobis(methylthio)hyalodendrin derived from cyclo-(L-[4'-^{3}H]Phe-L-[3-^{14}C]Ser), it was decided to synthesise and feed cyclo-(L-Phe-DL-[3-^{13}C]Ser). The metabolite isolated from such an experiment could then be studied by ^{13}C-n.m.r. spectroscopy which should show whether the label was in the expected position or whether it had been "scrambled" throughout the molecule. Patrick[27] had synthesised cyclo-(L-Phe-DL-[3-^{13}C]Ser) from DL-[3-^{13}C]serine by the method described above for the LD-isomer. However, t.l.c. and ^{1}H-n.m.r. spectroscopy of the product showed the presence of two compounds: the desired cyclo-(L-Phe-DL-[3-^{13}C]Ser) and the unexpected cyclo-(glycyl-L-phenylalanyl) [cyclo-(Gly-L-Phe)]. A sample of the commercial DL-[3-^{13}C]serine, supplied by Prochem, was checked for purity by ^{1}H-n.m.r. spectroscopy and found to contain glycine. Patrick did not pursue this line of work any further.[27]

Initially, our attempts were directed to separating unlabelled mixtures of cyclo-(L-Phe-L-Ser) and cyclo-(Gly-L-Phe) by preparative chromatography on silica gel. Analytical t.l.c. showed that a mixture of chloroform, methanol and acetic acid (90:5:5) separated the two dioxopiperazines, cyclo-(L-Phe-L-Ser) and cyclo-(Gly-L-Phe) having R_f values of 0.22 and 0.40 respectively. Preparative chromatography using the same solvent mixture was then attempted. Again, separation was good but recovery of either dioxopiperazine from the silica gel required several elutions with methanol. This in turn resulted in the extraction of undesirable organic and inorganic impurities off the silica gel. Nevertheless, successive crystallisations gave the

desired dioxopiperazine reasonably pure, albeit with a very low recovery. This chromatographic separation was then attempted on the mixture of cyclic dipeptides synthesised by Patrick[27] from DL-[3-^{13}C]-serine contaminated with glycine. Recovery was again difficult and low yielding. More important, however, was the fact that ^{13}C-n.m.r. spectroscopy of the mixture of diastereomers of cyclo-(phenylalanylseryl) showed that the LD- to LL-isomer ratio had changed from 1:1.5 to 6.5:1.1 after the chromatographic separation. It seemed that the cyclodipeptide was epimerising to the more stable trans-LD-isomer during preparative chromatography on silica gel. Since we had already established that only the LL-isomer is a precursor in hyalodendrin biosynthesis and, since we could not afford to lose 86% of our labelled precursor, it was apparent that this separation method would not be very fruitful.

It was therefore decided to effect the purification at an earlier stage. Thus, efforts were directed towards the separation of the two amino acids, glycine and DL-[3-^{13}C]serine. After several attempts using various different conditions on mixtures of the unlabelled amino acids, it was found that the best method was chromatography using an acidic ion-exchange resin of very small bead size eluted at a very slow flow rate with 1M hydrochloric acid. Very good resolution was obtained under these conditions and the recovered separated amino acids were crystallised after neutralisation and evaporation of the appropriate fractions, from aqueous ethanol. The recovered glycine accounted for 23% of the total weight of the commercial DL-[3-^{13}C]-serine sample.

The pure DL-[3-^{13}C]serine was converted to its benzyloxycarbonyl

derivative and then into cyclo-(L-Phe-DL-[3-^{13}C]Ser) by the procedure previously described for the synthesis of cyclo-(L-[U-^{14}C]Phe-D-Ser). Crystallisation of the product from methanol afforded three crops. The first crop of crystals was found, by ^{13}C-n.m.r. spectroscopy, to have an LL:LD-isomer ratio of 1:3. In the second and third crops the LL:LD-isomer ratio was 3.8:1 and 4.1:1, respectively.

We were now ready to feed our Hyalodendron culture with the ^{13}C-labelled dioxopiperazine. However, our culture was starting to show signs of deterioration. Metabolite production was low and great differences in pH and physical appearance (colour, amount of growth, etc.) were evident among flasks of the same culture batch. However, the feeding experiment with the ^{13}C-labelled precursor was carried out by administering a mixture of the LL- and LD-isomers "spiked" with cyclo-(L-[U-^{14}C]Phe-L-Ser) to the mould culture. The crude extract of the culture broth showed great amounts of impurities and dimethyl sulphone, and very little bisdethiobis(methylthio)hyalodendrin. Indeed, after three preparative t.l.c. separations only 4.3 mg of the desired metabolite were obtained, far too little to crystallise to constant activity, but just enough for ^{13}C-n.m.r. spectroscopy. Inspection of the ^{13}C-n.m.r. spectrum (2.6 x 10^5 scans at 90.6 MHz - Edinburgh n.m.r. service) of the metabolite showed a three-fold enhancement of the signal corresponding to the hydroxyl-bearing carbon, with no significant enhancement of any of the other signals. The corresponding ^{13}C-enrichment, together with the ^{13}C-enrichment for the administered cyclo-(L-Phe-DL-[3-^{13}C]Ser), was used to calculate the dilution of the precursor in the biosynthetic transformation (see Appendix). The calculations showed that in its transformation into bisdethiobis-

(methylthio)hyalodendrin, cyclo-(L-Phe-L-Ser) suffered a dilution of 41.

Although these results were very encouraging, the small quantity of pure metabolite isolated did not permit a comparison between the incorporation and dilution values observed from radioactivity measurements with those calculated from the ^{13}C-n.m.r. spectrum. Therefore, when a new Hyalodendron culture was available, this experiment was repeated. Larger quantities of pure crystalline bisdethiobis(methylthio)hyalodendrin were obtained this second time, thus enabling crystallisation to constant radioactivity and obtention of a better ^{13}C-n.m.r. spectrum. The ^{13}C-n.m.r. spectra of natural abundance and ^{13}C-enriched metabolite are shown in Figures 1a and 1b, respectively. Again, only the signal corresponding to the hydroxyl-bearing carbon showed an enhancement. The measured enhancement was 3.0 and the dilution calculated therefrom was 40.4. The bisdethiobis(methylthio)hyalodendrin isolated from this experiment was recrystallised to constant radioactivity and found to have a specific activity of 0.59 μCi/mmol, whereas the precursor had a specific activity of 21.82 μCi/mmol. Based on these values and on the amount of pure material isolated, the total incorporation of the labelled precursor was calculated to be 9.7% (minimum value). Moreover, the dilution of the radioactive precursor was found to be 37, in close agreement with that measured from the ^{13}C-enrichment. These results showed that cyclo-(L-Phe-L-Ser) is incorporated intact into hyalodendrin.

The next objective in our work was to synthesise cyclo-(L-N-Me-Phe-L-Ser) (38), a compound previously unreported in the literature. Specific monomethylation of cyclo-(L-Phe-L-Ser) did not seem feasible. Even when less than one equivalent of methylating agent was used

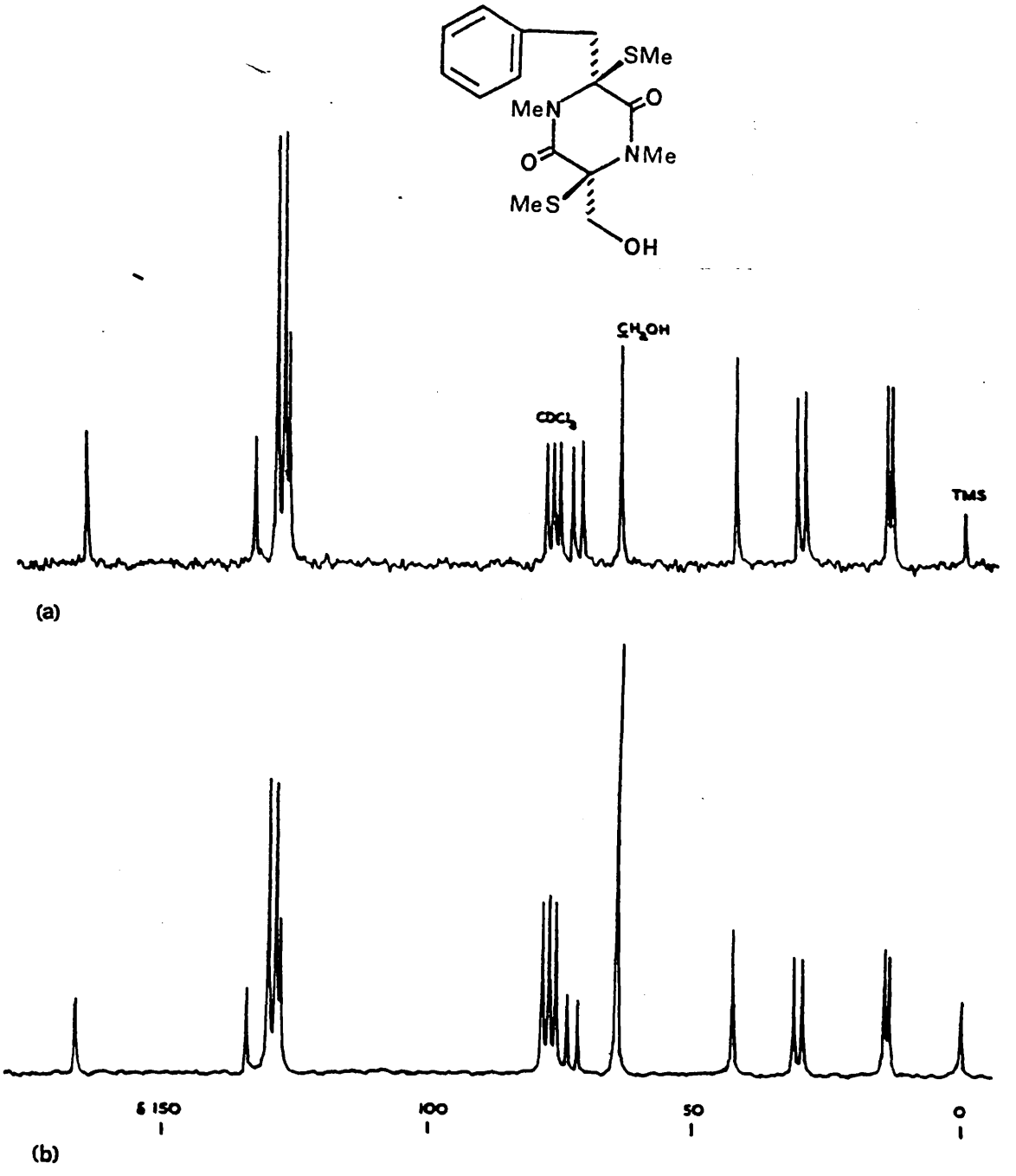


FIGURE 1.- ^{13}C-n.m.r. spectra of bisdethiobis(methylthio)hyalodendrin

(a) Natural abundance

(b) From feeding of cyclo-(L-Phe-DL-[3-^{13}C]Ser) to Hyalodendron sp.

on cyclo-(L-Phe-L-Ala), the NN-dimethylated cyclic dipeptide was obtained with no evidence of the monomethylated product.[28] Racemisation, O-methylation and elimination might also occur on the seryl portion of cyclo-(L-Phe-L-Ser). Therefore, it was decided to synthesise the N-methyl dioxopiperazine (38) by the coupling of benzyloxycarbonyl-L-serine with N-methyl-L-phenylalanine methyl ester hydrobromide, so that hydrogenolysis of the N-protecting group would release a primary amino group, necessary for the subsequent cyclization. It was believed at the time that cyclization of a secondary amine to the desired dioxopiperazine would not proceed readily.[29]

N-Methyl-L-phenylalanine methyl ester hydrobromide was synthesised by methylation of benzyloxycarbonyl-L-phenylalanine with an excess of sodium hydride and methyl iodide in a tetrahydrofuran - dimethylformamide solvent mixture, followed by acid hydrolysis of the benzyloxycarbonyl protecting group, according to the method of Coggins and Benoiton.[30] The dicyclohexylcarbodiimide coupling of this N-methyl methyl ester with benzyloxycarbonyl-L-serine in the presence of triethylamine was attempted twice. In both instances, t.l.c. of the crude reaction mixture, after 4 and 24 h, showed very little dipeptide formation, but several other unidentified side products were observed along with the unreacted amino acid derivatives. Since it was apparent that this route would not easily lead to the desired product, a different route was attempted.

The new route involved coupling of N-benzyloxycarbonyl-N-methyl-L-phenylalanine with L-serine methyl ester hydrochloride, followed by hydrogenolysis of the N-protecting group to give a secondary amine which then had to be cyclized to the desired dioxopiperazine (38).

N-Benzyloxycarbonyl-N-methyl-L-phenylalanine was synthesised by the method of McDermott and Benoiton[31] using an excess of sodium hydride and methyl iodide on the N-protected amino-acid in tetrahydrofuran. Dicyclohexylcarbodiimide coupling of this compound with the L-serine derivative proceeded cleanly to give the desired N-benzyloxycarbonyl-N-methyl-L-phenylalanyl-L-serine methyl ester. The N-protecting group of this dipeptide was removed by palladium on charcoal hydrogenolysis in a methanol-acetic acid (85:15) solvent mixture. Upon complete removal of the acetic acid, the unprotected dipeptide cyclized spontaneously to give the desired cyclo-(L-N-Me-Phe-L-Ser) (38).

This synthetic route was repeated using L-[3-^{14}C]serine methyl ester hydrochloride. Again, coupling proceeded smoothly. Analytical t.l.c. of the crude protected dipeptide in two different solvent systems, followed by radioscanning of the plates, showed only one radioactive band. Removal of the N-protecting group was achieved by hydrogenolysis in the same conditions used for the unlabelled compound. However, upon removal of the solvent, there was no evidence of dioxopiperazine formation. Inspection of the crude hydrogenolysis reaction mixture by analytical t.l.c. showed the presence of three spots, one of them corresponding to the desired N-methyl dioxopiperazine (38). A methanol solution of this mixture was treated with ammoniacal methanol to induce cyclization. T.l.c. of the product of this treatment, together with radioscanning of the chromatogram, once again revealed the presence of two compounds, one corresponding to the desired product (38), the other being the same as one of those observed previous to treatment with ammonia. This unknown compound gave a colour reaction to o-tolidine spray (see Experimental) typical of dioxopiperazines. However, the ratio of the total radioactivity

present in this compound to that present in the N-methyl dioxopiperazine (38) had nearly doubled upon treatment with ammonia, as shown by radio-scanning. The two dioxopiperazines were separated by preparative t.l.c. on silica gel and both were recovered, but no attempts were made to identify the undesired one. Attempts to crystallise the crude recovered cyclo-(L-N-Me-Phe-L-[3-^{14}C]Ser) were unsuccessful. This compound can only be recrystallised from methanol - diethyl ether mixtures and, in this instance, the presence of traces of silica gel carried through from the chromatography, resulted in gelling and inhibition of crystallisation. The crude N-methyl compound (38) was dissolved in a known amount of methanol. An aliquot of this solution was taken for counting; another aliquot, after evaporation of the solvent and redissolution in dimethyl sulphoxide was fed to Hyalodendron cultures. A third aliquot was diluted with a known amount of unlabelled (38) and crystallised to constant specific radioactivity . This permitted calculation of the radiochemical purity of the precursor fed which was found to be 59.7%. The fact that the N-Me dioxopiperazine fed to the mould culture contained a high level of other unidentified radioactive impurities must be borne in mind for the discussion that follows.

The bisdethiobis(methylthio)hyalodendrin (17) isolated from cultures that had been administered impure cyclo-(L-N-Me-Phe-L-[3-^{14}C]-Ser) was crystallised to a constant specific activity of 1.00 μCi/mmol. The metabolite contained 2.5% of the total radioactivity administered and the cyclo-(L-N-Me-Phe-L-[3-^{14}C]Ser) present in the feeding mixture had suffered a dilution of 5.5 in its transformation into (17). However, these results are highly questionable on account of the

TABLE 1

Results of incorporations of dioxopiperazines into bisdethiobis(methylthio)hyalodendrin (17) by cultures of Hyalodendron sp.

Experiment No.		Precursor	S.A. of (17) (μCi/mmol)	Incorporation (%) of ^{14}C into (17)	Dilution
1.		cyclo-(L-[U-^{14}C]Phe-L-Ser)	3.00	28	27.7
2.	a	cyclo-(L-[U-^{14}C]Phe-L-Ser)	7.95	28	8.3
3.	a	cyclo-(D-Phe-DL-[3-^{14}C]Ser)	0.05	0.36	12.5
4.	a	cyclo-(L-[U-^{14}C]Phe-L-Ser)	1.97	15	28.9
5.	a	cyclo-(L-[U-^{14}C]Phe-D-Ser)	0.06	0.48	53.5
6.		cyclo-(L-[4'-^{3}H]Phe-L-[3-^{14}C]Ser)	7.60	42	12.1
7.	a	cyclo-(L-[U-^{14}C]Phe-L-Ser)	3.30	33	26.3
8.	a	cyclo-(L-[U-^{14}C]Phe-L-N-Me-Ser)	0.0006	0.003	1.5×10^{5}
9.	a	cyclo-(L-[U-^{14}C]Phe-L-Ser)	1.97	15	28.9
10.	a	cyclo-(L-N-Me-Phe-L-[3-^{14}C]Ser)	1.00	2.5	5.5
11.	b	cyclo-(L-[U-^{14}C]Phe-L-Ser)	0.59	9.7	37.0
	b	cyclo-(L-Phe-DL-[3-^{13}C]Ser)	-	--	40.4

[a] Precursors administered separately but in parallel

[b] Precursors administered as a mixture

uncertainties regarding the real composition of the administered precursor. Clearly, the synthetic route to cyclo-(L-N-Me-Phe-L-Ser) must be studied closer and maybe modified for the radiolabelled synthesis. In retrospect, it also seems that it would be better to have the ^{14}C-label at a different position. If the precursor were to be dissociated to its constituent amino-acids in the course of the biosynthetic experiment, the hydroxyl-bearing carbon (C-3) of serine would enter the C_1 pool and might still be incorporated into the metabolite, giving what would be very misleading results. As it stands, the information obtained from this feeding experiment does not allow any conclusions to be drawn on the possible intermediacy of cyclo-(L-N-Me-Phe-L-Ser) (38) in hyalodendrin biosynthesis.

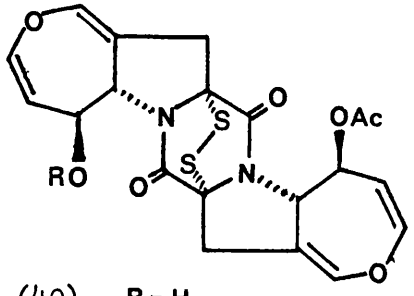
The results of the feeding experiments carried out by Patrick[27] and the author on Hyalodendron are summarized in Table 1. It is clear that cyclo-(L-Phe-L-Ser) is an efficient precursor for both gliotoxin and bisdethiobis(methylthio)hyalodendrin despite the antipodal configuration of the sulphur in these two metabolites. It is possible that biosynthetic introduction of sulphur is preceded by a step which destroys the chiral centre correspondingto the α-carbon of the amino acid, perhaps by the formation of an imino-group.[6] Nucleophilic addition of a thiol (e.g. cysteine) could then occur from either face of the imino-group depending upon the organism.

CHAPTER 3

Acetylaranotin

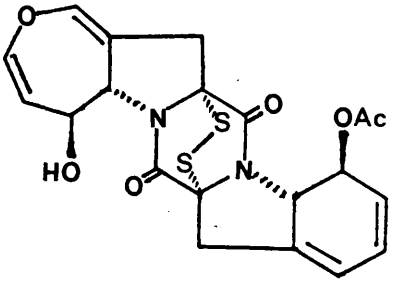
3.1 Isolation, biological activity and initial biosynthetic studies

Whilst investigating fungi as a possible source of antiviral compounds, workers at Lilly Research Laboratories isolated five different metabolites from cultures of Arachniotus aureus (Eidam) Schroeter.[32,33] These metabolites comprise what is now known as the aranotin family of the epithiodioxopiperazines. Three of these possess the familiar disulphide bridge: aranotin (40), acetylaranotin (16) and

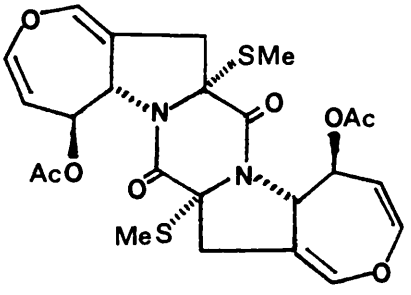


(40) R = H

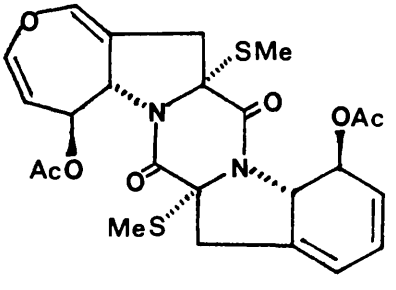
(16) R = Ac



(41)



(42)



(43)

apoaranotin (41). The other two metabolites are the related bis(methylthio)-variants: bisdethiobis(methylthio)acetylaranotin (BDA) (42) and bisdethiobis(methylthio)acetylapoaranotin (BDAA) (43).

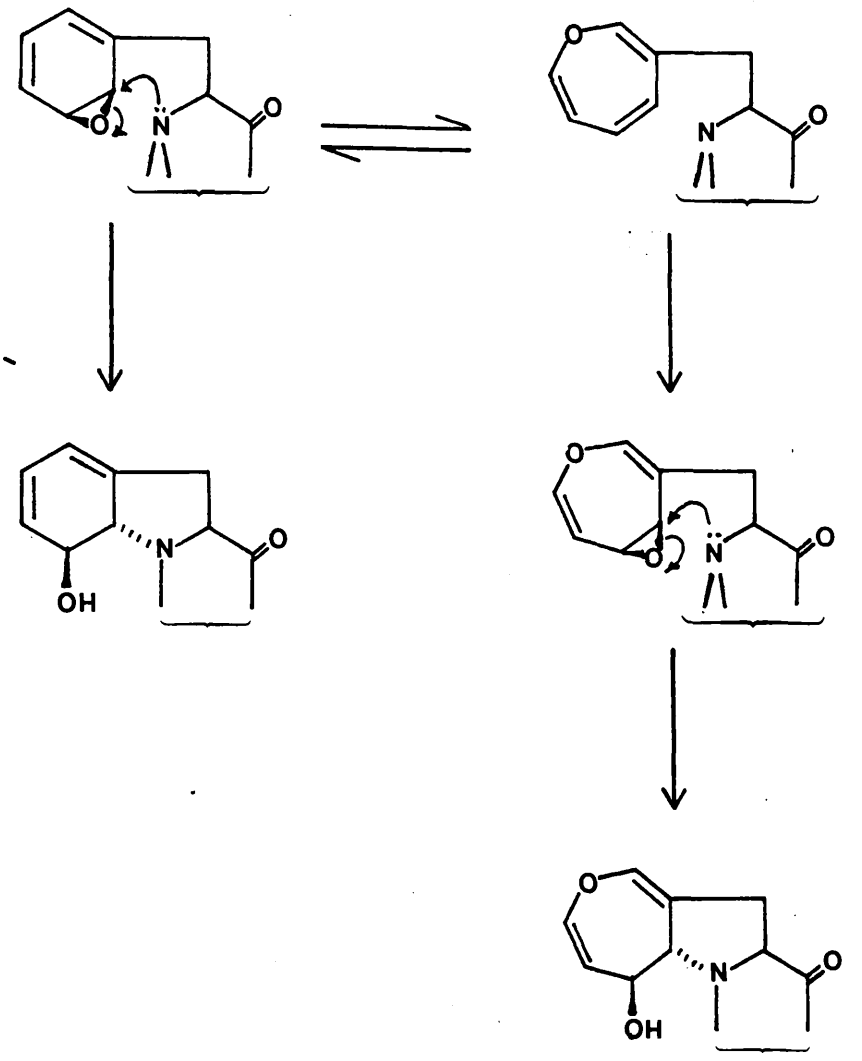
At about the same time, workers at Lederle Laboratories, A Division of American Cyanamid, reported the isolation, from culture filtrates of Aspergillus terreus, of two compounds which they designated as LL-S88α and LL-S88β.[34] Comparison of the chemical and physical properties, including X-ray analysis,[35] of LL-S88α and acetylaranotin (16) proved these two substances to be identical. Similarly, LL-S88β was found to be identical with BDA (42).

As already noted briefly for other members of the polythiodioxopiperazine family of metabolites, the presence of the disulphide bridge is essential for biological activity. Indeed, neither BDA (42) nor BDAA (43) display any antibiotic properties. The other three members of the aranotin family [(40), (16) and (41)] were effective in controlling Coxsackie virus infections in mice when administered intraperitoneally at levels of 5 - 8 mg/kg.[36] The LD_{50} for mice was determined to lie at 35 - 50 mg/kg.[36] The three epidisulphide aranotins were also active, to varying degrees, against several types of polio, Rhino-, measles, influenza and parainfluenza virus.[34,36-38] These three metabolites are devoid of any antibacterial or antifungal activity. It is believed[34,38] that the antiviral properties of these compounds result from their ability to block completely viral RNA synthesis at levels which do not affect cellular RNA synthesis.

The gross structures of the five aranotin metabolites were assigned on the basis of chemical and spectroscopic evidence. The structure and relative configuration of acetylaranotin (16) was also confirmed

by X-ray studies.[35] Comparison of the circular dichroism curves of acetylaranotin (16) and apoaranotin (41) with that of gliotoxin (1), showed that the configurations of the corresponding asymmetric centres of the three compounds are identical.[39] These results were confirmed from the X-ray studies[40] done on BDA (42), which revealed that the absolute configuration of the sulphur-bearing centres, and thus those of aranotin (40) and acetylaranotin (16), were identical with those of gliotoxin. Moreover, in apoaranotin (41), both the oxepin moiety characteristic of the aranotin family and the cyclohexadienol characteristic of gliotoxin are present in the same molecule. This suggests a close biosynthetic relationship between the aranotins and gliotoxin which has been explained[33,38] by the intervention of an arene oxide capable of rapid valence tautomerism to an oxepin ring (Scheme V).

Initial biosynthetic studies on the production of BDA (42) by Arachniotus aureus were carried out by Neuss and coworkers.[38] They found that L-[$^{14}CH_3$]methionine, DL-[3-^{14}C]serine and DL-[2-^{14}C]-tryptophan were incorporated quite efficiently into BDA. Zeisel degradation of the metabolite (42) isolated from each experiment produced radioactive methyl iodide, derived from the S-methyl groups, accounting for most of the recovered radioactivity. Moreover, the most efficient (6.46% incorporation) and specific (96% in the S-methyl group) source of the S-methyl carbon was found to be methionine. The effectiveness of methionine as a sulphur donor was demonstrated by the authors[38] by feeding a mixture of L-[^{35}S]methionine and L-[Me-^{3}H]methionine to cultures of A. aureus. The BDA derived from this experiment contained higher amounts of ^{3}H (11.3% incorporation)



Scheme V

than of ^{35}S (2.77%), thus indicating that an intact S-methyl group was probably not transferred to the dioxopiperazine ring system. Both L- and D-[1-^{14}C]phenylalanines were incorporated into BDA with similar efficiency (3.15 and 2.42% respectively), but no attempts were made to locate the position of the label.

Investigations carried out later by another group,[41] showed that DL-[3-^{14}C]phenylalanine is a more efficient precursor for BDA in A. aureus than DL-[2-^{14}C]-m-tyrosine and DL-3',5'-dihydroxy-[2-^{14}C]-

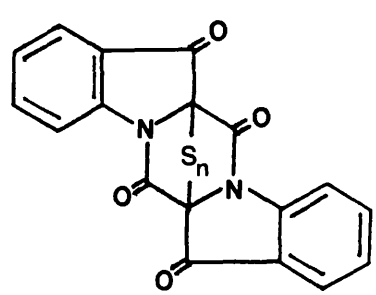
phenylalanine. These results agree with the findings of Bu'Lock and Ryles[42] that phenylalanine, but not o- or m-tyrosine or 2',3'-dihydroxyphenylalanine, is incorporated into gliotoxin (1) by T. viride. Feeding a mixture of DL-[3-^{14}C]phenylalanine and DL-[2'-^{3}H]phenylalanine to A. aureus gave BDA (42) which contained 7.04 and 5.67% of the radioactivity administered as ^{14}C and ^{3}H, respectively.[41] This demonstrated that the tritium in the ortho position of the phenyl ring is largely retained in BDA. These results were confirmed and expanded by feeding[41] fully C-deuteriated ($^{2}H_{8}$) DL-phenylalanine to Aspergillus terreus, which is a good source of acetylaranotin (16). The deuterium content of the isolated acetylaranotin was determined by mass spectrometric comparison of the proportions of the dominant (M^{+}- 2S) ions in natural abundance and deuterium-labelled acetylaranotin. The latter was found to contain large amounts of [$^{2}H_{14}$] and [$^{2}H_{7}$] species. It was, therefore, concluded that [$^{2}H_{8}$]phenylalanine had contributed to both halves of the symmetrical acetylaranotin molecule, with loss of only one deuterium atom, presumably from the α-position. The [$^{2}H_{7}$]acetylaranotin formed could be accounted for by concurrent incorporation of endogenous, undeuteriated, phenylalanine and deuteriated precursor. In connection with similar work being carried out in gliotoxin biosynthesis, Johns et al.[43] pointed out that the mass spectrum of the acetylaranotin isolated from the DL-[$^{2}H_{8}$]phenylalanine feeding also showed conspicuous fragment ions attributable to the loss of one deuterium in each half of the molecule. These authors[43] confirmed their observations by administering DL-[3',3'-$^{2}H_{2}$, 1'-^{14}C]phenylalanine to cultures of A. terreus and isolating acetylaranotin that showed, by mass spectrometry, substantial loss of one

methylene deuteron. It seems, therefore, that the biosynthesis of acetylaranotin proceeds without obligatory loss of hydrogen from either the methylene or the phenyl group of phenylalanine.

The results obtained to date on the biosynthesis of the aranotins, when taken together with results obtained in studies of gliotoxin biosynthesis, strongly suggest that cyclo-(L-Phe-L-Phe) is a precursor for all the members of the aranotin group. This hypothesis has been tested by the author and is discussed in Section 3.3.

3.2 Synthetic attempts

Although the syntheses of several complex sulphur-containing dioxopiperazines have been reported in the literature, none of the members of the aranotin family have been synthesised, probably owing to the added difficulty introduced by the presence of the oxepin ring. Coffen and coworkers,[44] at Hoffman-La Roche, synthesised the aromatic analogues (44) and (45) in the hope that the dihydro-oxepin ring might not be crucial to the biological activity of the aranotin series. However, when tested in mice, both compounds failed to show any antiviral activity.



(44) n = 2

(45) n = 3

3.3 Biosynthesis

Our studies on the biosynthesis of acetylaranotin were carried out on Aspergillus terreus. The growth conditions used led to the production of BDA (42) as the major metabolite. BDAA (43) was the next most abundant metabolite isolated. As discussed previously for hyalodendrin, results obtained with BDA (42) should also hold true for acetylaranotin (16). BDA and BDAA were isolated from culture broth extracts and separated by means of two successive preparative thin layer chromatographies (see Experimental section). Generally, BDA was obtained in a highly pure crystalline state, but the BDAA isolated always contained some BDA, since the two could not be well resolved in any chromatographic system tested. In a typical run, ca. 10 mg of pure crystalline BDA were obtained per litre of mould culture. The melting point, mass and ^{1}H-n.m.r. spectra of the isolated BDA agreed with the data reported in the literature.[32,34]

The various cyclo-(Phe-Phe) stereoisomers used in our work were synthesised by dicyclohexylcarbodiimide coupling of the relevant benzyloxycarbonyl and methyl ester hydrochloride derivatives of phenylalanine, followed by hydrogenolysis of the N-protecting group and cyclisation of the resultant dipeptide with saturated ammoniacal methanol. Since the methyl ester hydrochloride derivative of phenylalanine can be obtained in higher chemical yields than the benzyloxycarbonyl derivative, the labelled phenylalanine or the more expensive of the two labelled phenylalanines, was converted into its methyl ester hydrochloride derivative.

Feeding experiments were carried out by administering the cyclic

dipeptides dissolved in dimethyl sulphoxide to three-day old cultures of the mould. Due to the low solubility of the cyclic dipeptides administered, relatively large volumes of dimethyl sulphoxide (up to 1 ml/100 ml culture) had to be used. Nevertheless, the organism did not seem to be affected by the amounts of solvent administered. The final pH value of the culture broth and the yield of metabolite isolated from cultures that had been fed dimethyl sulphoxide (1 ml/100 ml culture) were comparable to those obtained from unfed cultures. Moreover, in contrast to what was observed in Hyalodendron sp., A. terreus seems to reduce most of the administered dimethyl sulphoxide to dimethyl sulphide. Since the mould is grown at 28 - 30°C, most of this compound evaporated as it formed. Nevertheless, the organic extract of the culture broth was washed with water to remove residual dimethyl sulphoxide.

After the feeding experiments involving radioactive precursors had been concluded, the A. terreus cultures started to show signs of deterioration, as demonstrated by a decrease in the yield of metabolite and the appearance of a yellow pigment in the culture broth. Upon thorough investigation, we discovered that there were two major factors leading to this deterioration. Firstly, following suggestions[45] of the suppliers of our mould culture, tomato paste-oatmeal agar slants were being used for the continuous propagation of the mould culture. Since the production of the yellow pigment was already observable at this stage, other agar systems routinely used for mycological purposes were investigated, namely malt, potato dextrose and Czapek-Dox agar. Of these, Czapek-Dox agar slants gave the best growth and the minimum production of the undesired yellow pigment. Successive subculturing of the fungus on these slants resulted in the total disappearance of the pigment. The second factor was that, for

several months, the fermentation stage of the fungal growth was carried out under 24 h of artificial illumination, because cultures of other fungi being grown concurrently had these light requirements. Since the timing of this additional illumination seemed to coincide with the deterioration of A. terreus, the amount and kind of illumination was restricted to normal daylight hours. We had already observed that total exclusion of light, by wrapping the culture flasks in black plastic, led to a decrease in mycelial growth. Thus, the use of Czapek-Dox agar slants and of normal daylight hours illumination during the fermentation stage, returned the culture to its previous normal, clean, productive behaviour.

In the intervening time, in case our A. terreus cultures did not recover, we tried to get cultures of Amauroascus aureus (previously known as Arachniotus aureus) into production. The growth conditions first used were those described in the literature[36] for Arachniotus aureus, in which the main carbon source in the fermentation medium is molasses. In spite of two successive sterilizations of the culture medium at 24 h intervals, the fungal culture was contaminated with large amounts of bacteria, mostly cocci, and mycelial growth was very poor. No sulphur-containing metabolites could be detected with silver nitrate spray reagent (see Experimental section) on chromatograms of culture broth extracts. Next, the growth conditions used for A. terreus were tried with Amauroascus aureus. No bacterial contamination was observed this time, but mycelial growth was still poor and no sulphur-containing metabolites were produced. This growth medium is a much "poorer" medium than the one suggested for Arachniotus aureus, but since the latter is so "rich" that bacterial contamination is almost inevitable, we settled on a compromise between the two. Growth was

initiated on the A. terreus medium and on the fifth day, when mycelial growth was well on its way, each culture flask was supplemented with some "rich" medium and grown for a further three days. This resulted in some production of BDA (42) and BDAA (43), as shown by ^{1}H-n.m.r. spectra of the crude broth extract. The first feeding experiments involving stable isotopes (vide infra) were carried out in a culture of Amauroascus aureus grown in this manner. Unfortunately and for reasons unknown, although mycelial growth was good, there was no metabolite production. It was at around this time that the A. terreus cultures started to show signs of recovery and, therefore, all work on Amauroascus aureus was abandoned. Thus, in the end, all feeding experiments were carried out using A. terreus cultures.

The efficiency of A. terreus in producing BDA (42) was evaluated by feeding L-[U-^{14}C]phenylalanine to the mould culture. Figure 2 shows an autoradiogram obtained from a t.l.c. plate of the crude culture broth extract from this feeding; most of the radioactivity is distributed between the major metabolites, BDA and BDAA. The isolated pure BDA (42) accounted for 10.1% of the radioactivity administered. Since no feeding of [^{14}C]phenylalanine to A. terreus has been reported, this result cannot be compared to any literature value. However, feedings of L-[1-^{14}C]phenylalanine[38] and DL-[3-^{14}C]phenylanine[41] to the closely related organism Arachniotus aureus gave comparable, albeit lower, incorporation values.

We were now ready to test whether cyclo-(L-Phe-L-Phe), rather than cyclo-(D-Phe-D-Phe) or cyclo-(L-Phe-D-Phe), served as a precursor for BDA in A. terreus. A solution of cyclo-(L-[U-^{14}C]Phe-L-Phe) (32.9 mg, 4.87 μCi) in dimethyl sulphoxide (19 ml) was distributed among 24 flasks of A. terreus culture (total volume, 2.4 l). After three days

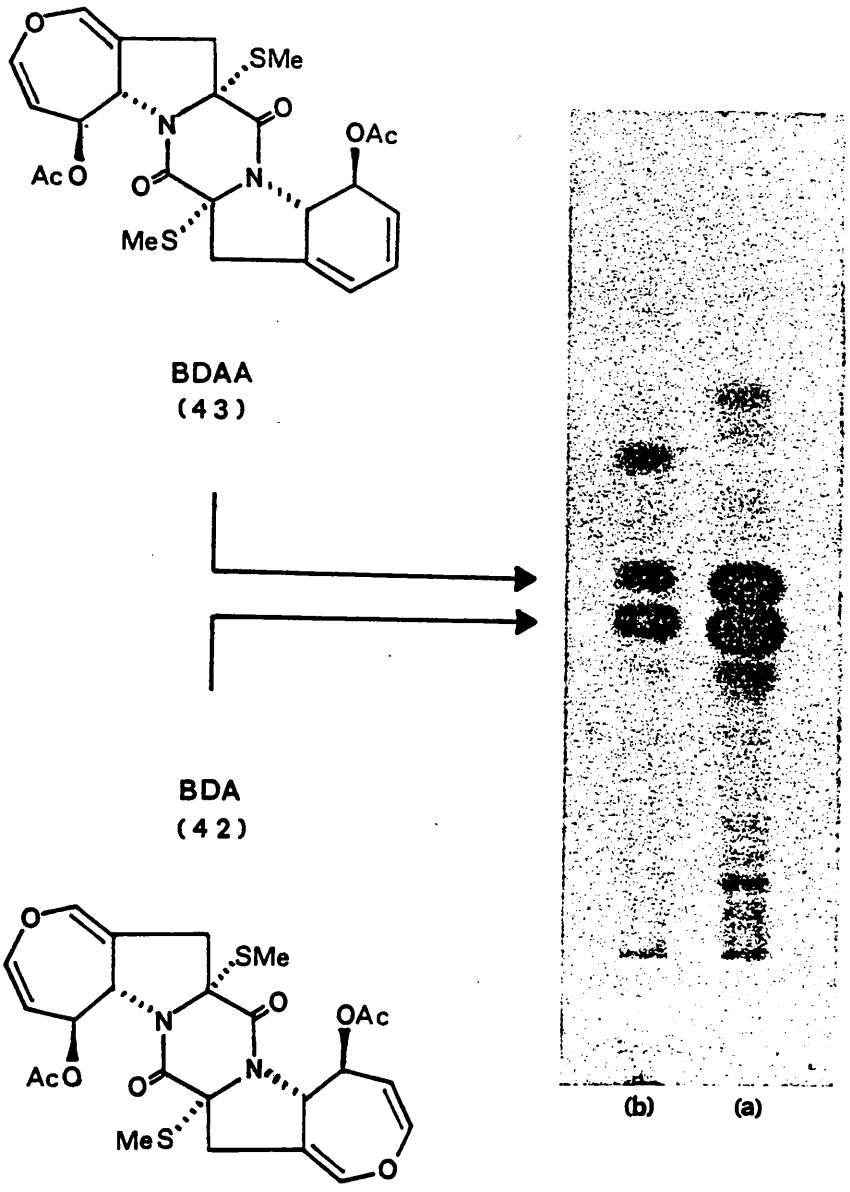


FIGURE 2.- Feeding of L-[U-^{14}C]phenylalanine to Aspergillus terreus. Autoradiogram of t.l.c. plate (silica gel; isopropyl ether - chloroform - acetic acid, 6:3:1): (a) Total crude extract of culture broth at end pH (7.2); (b) Total crude extract of culture broth after acidification to pH 3.

growth, analytical t.l.c. plates of the crude broth culture extracts in several solvent systems, were scanned for radioactivity and submitted to autoradiography. Only the bands corresponding to BDA, BDAA and cyclo-(L-Phe-L-Phe) were found to be radioactive. The crude extracts were chromatographed and the BDA isolated was recrystallised to constant radioactivity. Attempts to recover the cyclic dipeptide from the crude culture broth extracts were unsuccessful due partly to the strong adsorption of the compound on to the silica gel used for chromatography and partly to its low solubility in most organic solvents. Attempts to recrystallise the isolated BDAA were also unsuccessful, owing to the small quantities of compound available and to its contamination with BDA. The pure BDA was found to have a specific activity of 9.54 μCi/mmol, whereas the precursor had had a specific activity of 43.5 μCi/mmol. Based on these values and on the amounts of material isolated, the minimum value for the total incorporation of ^{14}C- labelled precursor was calculated to be 19.9% and the dilution of cyclo-(L-[U-^{14}C]Phe-L-Phe) in its transformation into BDA was 4.6.

Owing to the commercial unavailability of ^{14}C-labelled D-phenylalanine, the two other diastereomers of cyclo-(Phe-Phe) were synthesised together from D-phenylalanine and DL-[1-^{14}C]phenylalanine as a 4:6 mixture (determined by ^{1}H-n.m.r. spectroscopy) of cyclo-(D-Phe-D-[1-^{14}C]Phe) and cyclo-(D-Phe-L-[1-^{14}C]Phe). A solution of this mixture in dimethyl sulphoxide was administered to A. terreus cultures. Measurement of the radioactivity of the culture broth extract and of the aqueous residue accounted for 61% of the administered radioactivity, thus indicating that the remainder must be in the mycelium and hence, that there had been no cell-wall permeability problems. Radioactive scanning of t.l.c. plates of the total culture broth extract showed

that most of the radioactivity was associated with the bands corresponding to the two diastereomeric cyclic dipeptides. The BDA isolated from this extract was crystallised to a constant specific activity of 0.22 μCi/mmol. Using the total weight of pure metabolite isolated and the specific activity of the precursor (20.73 μCi/mmol), the minimum incorporation of the cyclo-(Phe-Phe) diastereomers into BDA was calculated to be 0.5% and the dilution value was 94.

From the results discussed above (Table 2, Experiments 2 and 3), the incorporation of activity into BDA (42) is strikingly dependent upon the stereochemistry of the precursor. Of the three possible stereomers of cyclo-(Phe-Phe) only the LL-isomer is incorporated efficiently. This finding closely resembles those obtained for gliotoxin[9] and hyalodendrin (Chapter 2). The small incorporation of radioactivity from the (DD + DL)-mixture might have arisen from cleavage of the cyclodipeptide followed by incorporation of [^{14}C]-phenylalanine.

The next point to be tested was the possibility that cyclo-(L-Phe-L-Phe) might not be a normal biosynthetic intermediate, but rather that it underwent some structural modification in the organism prior to entry into the normal pathway. Therefore, the status of cyclo-(L-Phe-L-Phe) as a natural intermediate was tested by means of an "intermediate-trapping" experiment. Non-radioactive cyclo-(L-Phe-L-Phe) was incubated with A. terreus and, 2 h later, L-[U-^{14}C]phenylalanine was added to the medium. After a further 2 h, the organism was harvested and the culture extracted with chloroform. The extract was concentrated and cooled to 0°C, and the solid that separated was found to be cyclo-(L-Phe-L-Phe) by comparison with an authentic sample (melting point, ^{1}H-n.m.r. spectroscopy). The cyclic dipeptide was recrystallised to constant radioactivity and found to contain 10.4%

TABLE 2

Results of incorporations of radioactive precursors into bisdethiobis(methylthio)acetylaranotin (BDA) (42) by cultures of Aspergillus terreus

Experiment No.	Precursor	S.A. of (42) (μCi/mmol)	Incorporation (%) of ^{14}C into (42)	Dilution
1.	L-[U-^{14}C]phenylalanine	62.99	10.1	8.1×10^3
2.	cyclo-(L-[U-^{14}C]Phe-L-Phe)	9.54	19.9	4.6
3.	cyclo-(D-Phe-DL-[1-^{14}C]Phe)	0.22	0.5	94
4.	cyclo-(L-[4'-^{3}H]Phe-L-[3-^{14}C]Ser)	-	1.5[a]	-

[a] Refers to the mixture of BDA (42) and BDAA (43).

of the total radioactivity administered as L-[U-^{14}C]phenylalanine. cyclo-(L-Phe-L-Phe) had, therefore, been formed from phenylalanine and was presumably present in the organism under normal conditions of growth.

Our attention was then directed to investigating the claims[34,37] that A. terreus is also a gliotoxin (1) producer. Although we had never detected any gliotoxin in our culture broth extracts, it was possible that the compound was there, but in amounts too low to permit chromatographic detection. Therefore, cyclo-(L-[4'-^{3}H]Phe-L-[3-^{14}C]Ser) (^{3}H/^{14}C, 13.1), a known gliotoxin precursor,[9] was administered to our cultures of A. terreus to increase the sensitivity of detection of any gliotoxin present. However, radioactive scanning of chromatograms of the total crude extract obtained from this experiment did not show any radioactivity corresponding to gliotoxin. In fact, the only radioactivity detected was present in the bands corresponding to BDA and BDAA, and in the baseline of the chromatogram. After chromatographic purification, the mixture of BDA and BDAA was recrystallised to constant radioactivity. Attempts to obtain gliotoxin from the extract by chromatography were fruitless. The baseline of the chromatograms was found to contain a complex mixture of polar material and was not investigated further. The pure crystalline material, corresponding to a mixture of BDA and BDAA was found to have a ^{3}H:^{14}C ratio of 50.8, corresponding to total incorporation values for ^{3}H and ^{14}C of 5.5 and 1.5%, respectively. This seemed to indicate that the cyclo-(L-[4'-^{3}H]Phe-L-[3-^{14}C]Ser) had been cleaved by the organism into L-[4'-^{3}H]phenylalanine and L-[3-^{14}C]serine. The phenylalanine, after dilution with endogenous material, was then

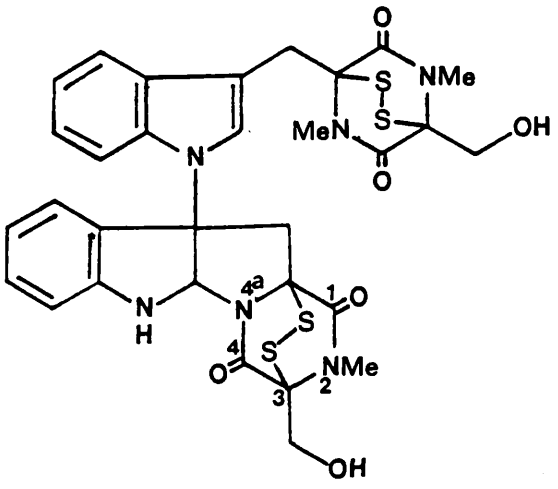
incorporated, with an intact carbon skeleton, into BDA in the usual way. The serine, however, having no direct role in BDA biosynthesis, passed into the metabolic C_1-pool, later to appear in the S-methyl groups and acetate portions of BDA and BDAA. No attempts were made to chemically degrade the isolated metabolites to confirm this hypothesis, but no other plausible explanation could be found to account for the results obtained. The purpose of this experiment was to investigate the alleged production of gliotoxin by A. terreus and the only conclusion that may be drawn is that our cultures of A. terreus are not gliotoxin producers.

A more important outcome of this experiment is that it poses the question: is cyclo-(L-Phe-L-Phe) also being cleaved by A. terreus into two phenylalanine units which are then reincorporated into the metabolite? In other words, is cyclo-(L-Phe-L-Phe) incorporated intact in its biosynthetic transformation into BDA? Because of the symmetry of the precursor, the usual means of testing for this possibility, namely, using a $^{3}H,^{14}C$-labelled precursor, is not feasible. Cleavage of the cyclic dipeptide would lead to ^{3}H- and ^{14}C-labelled phenylalanine, indistinguishable to the organism, which could then mix with endogenous unlabelled phenylalanine, to give ultimately a mixture of ^{3}H- and ^{14}C-labelled BDA in a ratio identical to the initial $^{3}H:^{14}C$ ratio of the precursor. Therefore, it was necessary to find a way of labelling the precursor that would reveal any breakdown. Cyclo-(L-[^{15}N]-Phe-[1-^{13}C]Phe) was decided upon since it was hoped that cleavage of ^{13}C-^{15}N bond would be evident from loss of $^{13}C^{15}N$-coupling in the ^{13}C- ^{1}H n.m.r. spectrum of the derived metabolite.

Once again, the commercial unavailability of L-[1-^{13}C]phenylalanine

meant that the pure LL-isomer could not be made. However, since it had already been established (vide supra) that the LD-isomer is not incorporated significantly into BDA, its presence should not present any problems. Thus, a 53:47 mixture of cyclo-(L-[^{15}N]Phe-L-[1-^{13}C]Phe) and cyclo-(L-[^{15}N]Phe-D-[1-^{13}C]Phe) was synthesised and fed, mixed with cyclo-(L-[U-^{14}C]Phe-L-Phe) in an amount equivalent to the ^{13}C,^{15}N-labelled LL-isomer, to A. terreus cultures. The BDA isolated from this experiment was recrystallised to constant specific activity. The amount of total radioactivity incorporated into the pure crystalline BDA was calculated to be 14.4% and the total cyclo-(L-Phe-L-Phe) administered had undergone a dilution of 6.0 in its transformation into BDA.

Figure 3 shows the ^{13}C-^{1}H n.m.r. carbonyl signals of natural abundance and ^{13}C,^{15}N-enriched BDA. The lower field signal corresponds to the acetate carbonyl, whilst that at δ 164.4 ppm corresponds to the amide carbonyl in the dioxopiperazine moiety. The amide carbonyl signal of ^{13}C,^{15}N-enriched BDA shows two satellites due to a ^{13}C-^{15}N one-bond coupling, J_{CN} 13 Hz. No other differences were discernible



(46)

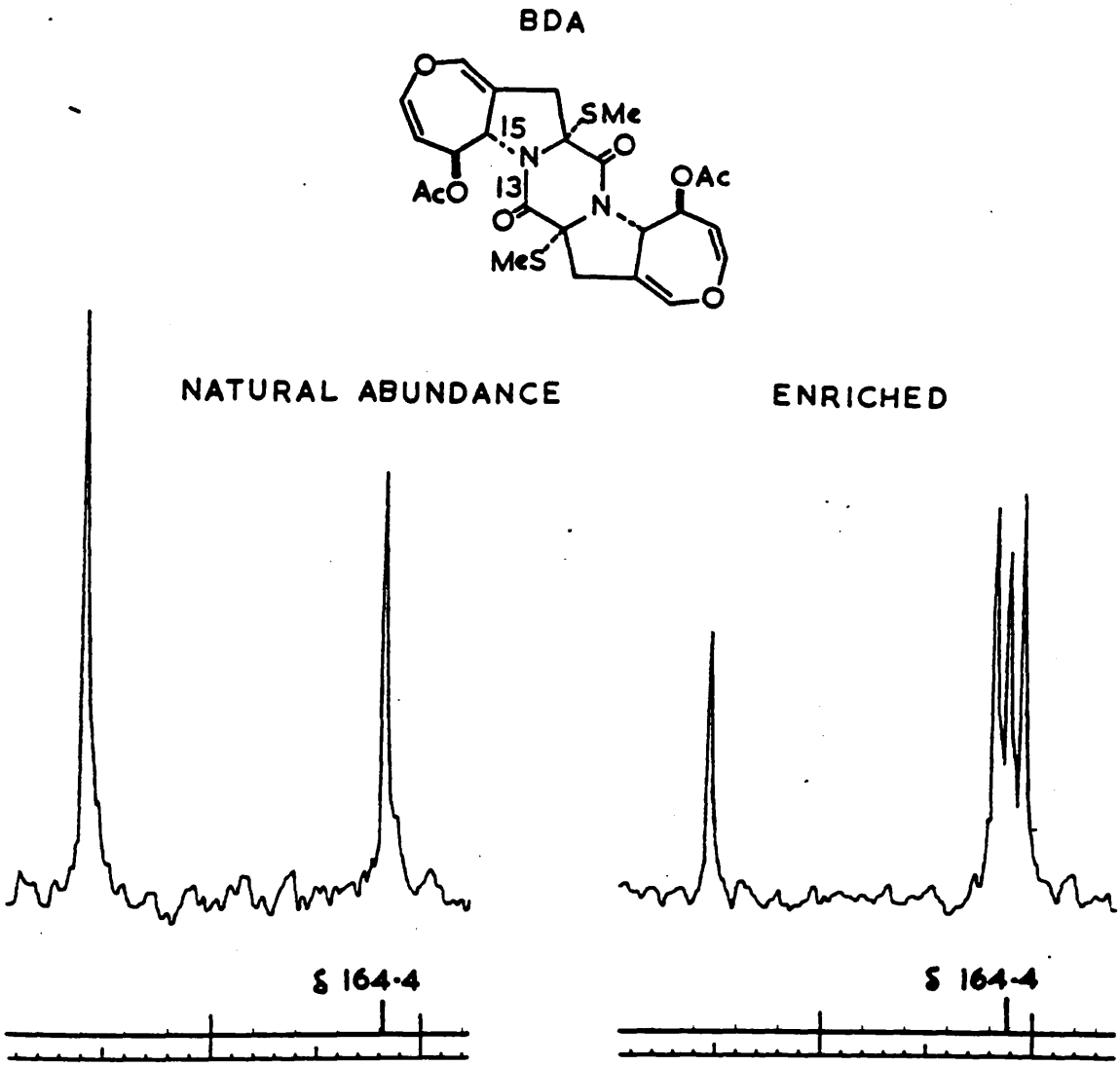


FIGURE 3.- ^{13}C-n.m.r. spectra (carbonyl region) of bisdethiobis(methylthio)acetylaranotin.

between the spectra of natural abundance and enriched BDA. Brewer et al.[46] determined the structure of chetomin (46), another epidithiodioxopiperazine, by ^{13}C- and ^{15}N-spectroscopy. They reported one-bond coupling constants of 14.6 and 13.7 Hz for $J_{4,4a}$ and $J_{1,2}$, respectively, and a two-bond ^{15}N-^{13}C coupling constant of 5 Hz for $J_{4a,3}$. Thus, the J_{CN} value obtained for BDA is in good agreement with the only close enough example that could be found in the literature. No efforts were made to pick out two-bond couplings in our spectrum, since they would probably be very small and would not give any new additional information.

In the discussion that follows, we shall refer to the intensity of only the central peak of the triplet of Figure 3. No quantitative comparison involving the two outer satellite peaks can be made, since the nuclear Overhauser effects and spin-lattice relaxation times for ^{13}C are different when bonded to ^{15}N and ^{14}N. All that may be said about them is that they arise from a BDA molecule that has a ^{13}C-labelled carbonyl group directly bonded to a ^{15}N atom. The central peak, in turn, arises from a ^{13}C-labelled carbonyl group directly bonded to a ^{14}N atom. Whether the ^{13}C-content in this carbonyl group is derived partly from [1-^{13}C]phenylalanine resulting from cleavage of the administered cyclodipeptide is the crux of the experiment.

In the natural abundance spectrum of BDA the ratio of the heights of the acetate and amide peaks was found to be 1.37. This value was corroborated with similar measurements on other ^{13}C-n.m.r. spectra of natural abundance BDA, recorded at different times, but using the same experimental conditions. Inspection of the relative heights of the acetate and amide peaks in the spectrum of the enriched BDA, reveals that the situation here is very different, the central amide

peak being larger than the acetate peak. Using the height of the acetate peak in this spectrum (6.45 cm) and the 1.37 value previously calculated, the height of the peak expected from exclusively a natural abundance ^{13}C-contribution can be calculated to be 4.71 cm. Yet, the measured height of the amide peak was 8.40 cm. How can this discrepancy of 3.69 cm be accounted for? First, there will be a contribution to the total measured height arising from overlap with the flanking peaks resulting from ^{13}C-^{15}N coupling. The contribution due to this overlap to the total measured height of the central peak can be estimated to be 1.30 cm. Second, the administered precursor contained a certain amount of species having ^{13}C-labelled carbonyl groups not directly bonded to ^{15}N-atom. These arise from several sources. Importantly, the 'doubly-labelled' precursor was synthesised from [^{15}N]phenylalanine and [1-^{13}C]phenylalanine containing only 96.5% and 91.0%, respectively, of labelled molecules and, clearly, some [^{13}C,^{14}N]cyclodipeptide must be formed in the synthesis. Further, account must be taken of the ^{13}C present at natural abundance both in the [^{15}N]phenylanine used in the synthesis and in the [^{14}C]cyclo-dipeptide added to the precursor mixture to provide an independent measure of dilution.

It was therefore necessary to calculate the abundance of each species in the cyclo-(L-Phe-L-Phe) fed to A. terreus. Table 3 lists the ten possible isotopic combinations in the cyclodipeptide mixture administered and the percentage abundances calculated (see Appendix) for each species. It is necessary now to consider which of these ten species will contribute to the central amide carbonyl peak in the spectrum of ^{13}C,^{15}N-enriched BDA, that is, those species not containing a ^{13}C atom directly bonded to a ^{15}N atom.

TABLE 3

Comparison of the mixture of cyclo-(L-[^{15}N]Phe-L-[1-^{13}C]Phe) and cyclo-(L-[U-^{14}C]Phe-L-Phe):

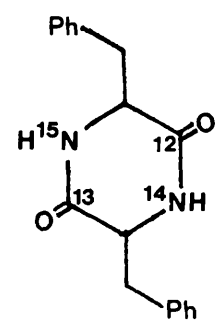
(a) As fed to Aspergillus terreus

(b) As calculated for random recombination

	SPECIES	PERCENTAGE ABUNDANCE (a)	(b)
A	15— 13—	46.87	13.04
B	15— —	4.97	25.25
C	— 13—	2.69	24.32
D	15— 13 13—	0.52	0.19
E	15— 13— 15	0.17	0.14
F	— —	44.66	23.54
G	— 13— 15	0.06	0.36
H	— 13 13—	0.02	6.28
I	15— — 15	0.02	6.77
J	15— 13 13— 15	0.002	0.001

Notation used: (i) a dash stands for phenylalanine, (ii) two dashes stand for the cyclodipeptide; (iii)numbers indicate ^{13}C or ^{15}N; where no numbers are given ^{12}C or ^{14}N is implied. Thus,

15—
13— stands for



Inspection of the Table shows that only species C, D, G, and H fulfil this requirement. However, the ^{13}C-n.m.r. signal that would be obtained from species D and G will be split by a two-bond $^{13}C^{15}N$-coupling and will not lie on top of the central amide peak, therefore these two species may be discarded. From the sum of the relative abundances of species C and H, and with the help of the dilution value (6.0), obtained independently by means of the ^{14}C-tracer (vide supra), the expected height for the amide peak including the ^{13}C-contribution due to precursor can be calculated (see Appendix) to be 5.89 cm. If we add to this calculated value the correction due to overlap (1.30 cm) of the satellite peaks, we obtain a height of 7.19 cm, which still leaves a discrepancy of 1.21 cm on comparison with the peak height measured directly from the spectrum (8.40 cm).

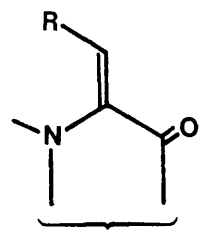
This residual discrepancy may lie within the limits of error of the various measurements. However, dissociation of the doubly-labelled precursor would also lead to enhancement of the central peak. This possibility must be considered quantitatively since it is central to the purpose of the experiment. In the following calculation it is assumed that the precursor molecules dissociate and recombine without any dilution by endogenous [^{14}N]-species. That is, we shall calculate the maximum dissociation needed to account for the additional height of the central amide peak. Any additional recombination with, for example, endogenous [^{14}N]phenyl-alanine would increase the amount of $^{13}C,^{14}N$-labelled product and decrease the percentage dissociation needed to explain the discrepancy.

Dissociation of the precursor, having the composition given in Table 3, and recombination of the separate halves at random would

recreate the same ten species initially present, but this time the percentage abundance of each species would be altered. The calculated outcome is shown in Table 3. However, a brief inspection of ^{13}C-n.m.r. spectrum of the enriched BDA (Figure 3) indicates that there could be only partial dissociation of the cyclodipeptide. Had there been complete dissociation, the heights of the $^{13}C^{15}N$-satellites would be very small relative to the height of the central peak since the singly-labelled [^{13}C,^{14}N]-precursor would be a major species. It is now possible to calculate the maximum extent of dissociation needed to account for the extra height of the central amide peak. Using the same arguments as before, only species C and H need be considered. In the cyclodipeptide mixture resulting from complete dissociation and random recombination, these two species have increased considerably in abundance. The abundances of the species C and H, together with dilution value (6.0) and the height of the central peak measured from the spectrum (7.10 cm, after correction for satellite overlap), can be used to calculate (see Appendix) the maximum extent to which the dissociation-recombination process might have occurred. As a result, it can be said that the residual discrepancy of 1.21 cm in the height of the central amide peak may be accounted for by 5.0% dissociation of the initial LL-precursor fed. Therefore, the answer to the initial question: "Is cyclo-(L-Phe-L-Phe) incorporated intact in its biosynthetic transformation into BDA?", is: "Yes, a minimum of 95% of the LL-precursor fed is incorporated intact."

The next point to be investigated concerned the fate of the methylene protons of cyclo-(L-Phe-L-Phe) in its transformation into BDA (42). There was evidence in the literature[43] suggesting that there

might be some non-obligatory loss of a methylene deuteron during incorporation of DL-[3,3-$^{2}H_{2}$,1-^{14}C]phenylalanine into BDA. However, it was not clear whether the deuterium atom was lost from the amino-acid itself or from some other intermediate along the biosynthetic pathway leading to BDA. Since structures such as (47) can be suggested for intermediates involved in the introduction of sulphur, it was



(47)

desirable to confirm, or disprove, the earlier conclusions using a different precursor. Accordingly, we decided to feed cyclo-(L-[3,3-$^{2}H_{2}$]-Phe-L-[3,3-$^{2}H_{2}$]Phe) to A. terreus and to examine, by mass spectrometry, the deuterium content in the isolated BDA.

The tetradeuteriated LL-cyclodipeptide may be synthesised, by the general method discussed previously, from L-[3,3-$^{2}H_{2}$]phenylalanine. However, since it is easier to synthesise racemic [3,3-$^{2}H_{2}$]phenyl-alanine and since we already knew that the DD- and DL-cyclodipeptides were not incorporated efficiently into BDA, it was decided to synthesise the desired tetradeuteriated cyclodipeptide as a mixture of the three possible stereoisomers. This was achieved by refluxing a solution of DL-[3,3-$^{2}H_{2}$]phenylalanine in ethylene glycol, which gave a statistical mixture (1:2:1) of the LL-, LD- and DD-cyclodipeptide isomers.

DL-[3,3-$^{2}H_{2}$]Phenylalanine was synthesised, by slight modifications

of literature procedures from [formyl-^{2}H]benzaldehyde. The synthetic route involved a Cannizzaro reaction of the [^{2}H]benzaldehyde to give [methylene-$^2H_2$]benzyl alcohol, which was then converted into [methylene-$^2H_2$]benzyl chloride and thence into the correspondingly dideuteriated acetamidomalonate derivative. Hydrolysis of the latter yielded the desired amino-acid. Mass spectrometry analysis of the dideuteriated diethyl acetamidomalonate showed that the percentage abundances of [$^2H_1$]- and [$^2H_2$]-species were, respectively, 51.0 and 34.9%. The results arise from the fact that the deuterium content in the [^{2}H]benzaldehyde was less than 100%. Accordingly, mass spectrometry of the tetradeuteriated cyclodipeptide revealed, as shown in Table 4, that the most abundant species was that containing three deuterium atoms. In the ideal experiment, an abundance greater than 90% for the [$^2H_4$]-species would simplify considerably the analysis of the results obtained from feeding experiments. Nevertheless, the [$^2H_4$]-content was adequate for our purpose, as will be demonstrated later.

A solution of cyclo-(DL-[3,3-$^2H_2$]Phe-DL-[3,3-$^2H_2$]Phe), mixed with cyclo-(L-[U-^{14}C]Phe-L-Phe) in an amount equivalent to the deuteriated LL-cyclodipeptide, in dimethyl sulphoxide, was fed to A. terreus cultures. Radioactivity measurements of the isolated BDA, after it had been recrystallised to a constant specific activity of 3.00 μCi/mmol, revealed that 18.5% of the ^{14}C-label had been incorporated and that the total LL-cyclodipeptide administered had suffered a dilution of 7.4 in its transformation into BDA. Analysis of the deuterium composition of the [M - SCH_3]$^{+\cdot}$ fragment ions observed in the mass spectra of the isolated BDA, gave the distribution shown in Table 5. The fact that [$^2H_4$]BDA was present, in an amount

TABLE 4

Composition of synthetic cyclo-(DL-[3,3-$^2H_2$]Phe-DL-[3,3-$^2H_2$]Phe)

SPECIES	PERCENTAGE ABUNDANCE
$^2H_0$	4.68 ± 0.40
$^2H_1$	10.68 ± 0.24
$^2H_3$	28.04 ± 0.20
$^2H_3$	38.45 ± 1.46
$^2H_4$	18.14 ± 1.94

TABLE 5

Composition of $[M - SMe]^{+\cdot}$ fragment ion of BDA:

(a) isolated from feeding experiment

(b) calculated from precursor composition

SPECIES	PERCENTAGE ABUNDANCES		
	(a)	(b)	(a) - (b)
$^2H_0$	91.04 ± 0.29	86.74 ± 2.84	+4.30 ± 2.85
$^2H_1$	1.55 ± 0.23	1.80 ± 0.04	-0.25 ± 0.23
$^2H_2$	1.51 ± 0.16	3.80 ± 0.03	-2.29 ± 0.16
$^2H_3$	4.43 ± 0.18	5.21 ± 0.02	-0.78 ± 0.27
$^2H_4$	1.47 ± 0.11	2.46 ± 0.26	-0.99 ± 0.28

greater than the calculated (see Appendix) experimental error, indicates that deuterium loss is not an obligatory process for the biosynthesis of the metabolite. If this had been the case, no $[^2H_4]$-species would have been detected.

Of course, no direct comparison may be made between the abundance of each species in precursor and in metabolite. In its transformation into BDA, the cyclodipeptide undergoes a dilution by endogenous material present in the organism and thus the relative abundance of $[^2H_0]$BDA is increased. An allowance must also be made for the small (0.016%) natural abundance deuterium content in the endogenous metabolite and its contribution to the abundance of $[^2H_1]$-species must be considered. With this in mind, and using the dilution value (7.4) obtained from the ^{14}C-tracer, the percentage abundances of the synthetic precursor listed in Table 4 can be used to calculate (see Appendix) the percentage abundances predicted for each species in the metabolite, assuming a biosynthetic process in which (a) all four deuterium atoms are retained, and (b) no dissociation and recombination of the precursor occurs. The calculated values and the discrepancies between the observed and calculated values are listed in Table 5.

The discrepancies [(a) - (b) in Table 5] between the observed and calculated relative abundances of $[^2H_0]$- and $[^2H_1]$-BDA are of the same order as the errors associated with their measurement and, therefore, no significant quantitative conclusion may be drawn from them. Conversely, the errors associated with the measurements of the three other species are considerably smaller than the corresponding discrepancies. The total percentage of deuterium (5.94%) present in

the BDA isolated from the feeding experiment was calculated (see Appendix) from the percentage abundances of the species in Table 5 [column (b)] taking account of the number of deuterium atoms present in each species. Similarly, the predicted total percentage of deuterium (7.42%) was calculated using the values in Table 5 [column (b)]. Thus, ca. 20% of deuterium had been lost during the experiment.

The partial loss of deuterium can be accounted for by an exchange process occurring either during the biosynthesis or in the mass spectrometer. This latter possibility is considered unlikely. The mass spectral data were obtained by repetitive scanning of the mass spectrum of the isolated BDA for 6.5 min in an MS 9 mass spectrometer. Comparison of the percentage abundance of each species in each scan did not reveal any trend indicating an exchange process. Also, essentially the same spectrum was obtained with an MS 12 spectrometer. Therefore, the loss of deuterium most probably occurs during the biosynthetic conversion of the precursor into the metabolite. However, as pointed out before, the observed presence of [$^{2}H_{4}$]BDA excludes the possibility that this loss of deuterium is an obligatory step in BDA biosynthesis. The mechanism that would account for such a loss is not evident, but it is clear that this point ought to be investigated further.

In conclusion, we have established that cyclo-(L-Phe-L-Phe), rather than cyclo-(D-Phe-D-Phe) or cyclo-(D-Phe-L-Phe), is incorporated virtually intact and efficiently into bisdethiobis-(methylthio)acetylaranotin by A. terreus. Moreover, cyclo-(L-Phe-L-Phe) has been identified as a natural metabolite of A. terreus and is either an intermediate, or is interconvertible with an intermediate, on the biosynthetic pathway from phenylalanine to BDA.

Furthermore, this pathway appears to proceed with partial (i.e. non-obligatory) loss of the hydrogen originally in the methylene groups of cyclo-(L-Phe-L-Phe).

CHAPTER 4

EXPERIMENTAL

4.1 General procedures

Only procedures not mentioned in Part I, Chapter 3, will be dicussed here. Optical rotations were measured at 589 nm (D line of sodium) in a Perkin Elmer 141 polarimeter. The mass spectra of BDA (42), discussed in Section 3.3, were obtained in a VG updated MS-902S, coupled to a VG data system, with a dynamic resolving power of 3,000. Samples were introduced into the probe at 170°C and an ionization potential of 70 eV was used.

Proton noise decoupled ^{13}C-nuclear magnetic resonance spectra were run at 25.2 MHz in the pulsed Fourier transform mode on a Varian XL-100 n.m.r. spectrometer with VFT-100 accessory, at ambient temperature (ca. 25°C) and using tetramethylsilane as internal standard. Standard conditions for data acquisition were: spectral width, 5000 Hz; acquisition time, 0.4 s; pulse width, 10 μs (pulse angle, ca. 20°) and digital resolution, 1.25 Hz/data point. When measuring enhancements, in order that the peak heights were not affected by the digital resolution, a severe exponential weighting factor (sensitivity enhancement 0.05 s) was used to multiply the free induction decay. This resulted in the line widths being about 10 Hz at half height.

After t.l.c., sulphur containing compounds were detected by spraying the plates with silver nitrate reagent.[47] This was prepared by adding a saturated solution of silver nitrate (1 ml) to acetone (20 ml); the precipitated silver nitrate was then redissolved by

dropwise addition of water (ca. 0.5 ml). Compounds containing S-S linkages initially gave yellow spots which, in time, first turned brown and finally black. Compounds containing MeS-groups gave, after ca. 12 h, white spots on a grey background. Cyclic dipeptides were detected as follows.[48] After development, the t.l.c. plates were dried at 100°C for 5 min, cooled to room temperature, and then placed for 5 min in a tank saturated with chlorine gas. Chlorine gas was generated by adding concentrated hydrochloric acid to a potassium permanganate solution in a small beaker placed at the bottom of the tank. Excess chlorine was removed by allowing the plate to stand in the fume-cupboard for ca. 5 min. The plate was then sprayed, in the fume-cupboard, with o-tolidine reagent until the spots became visible. The reagent was prepared by dissolving o-tolidine (160 mg) in glacial acetic acid (30 ml), diluting the solution with water (500 ml) and adding potassium iodide (1 g). Cyclic dipeptides, depending on their concentration, gave blue or yellow spots.

4.2 Fermentation conditions

Hyalodendron sp. (FSC-601) was obtained from Dr. A.Taylor of the Atlantic Regional Laboratory, National Research Council, Canada. The fungus was grown for 9 days on 2% malt-agar slants. Material from these slants was used directly to inoculate 500 ml Erlenmeyer flasks each containing synthetic medium (100 ml) of the following composition:

Glucose	10 g
KH_2PO_4	1.5 g
$MgSO_4.7H_2O$	0.5 g

NH_4NO_3	0.7 g
KCl	1 g
Thiamine.HCl	1 mg
$Na_2B_4O_7.10H_2O$	0.88 mg
$CaCl_2.2H_2O$	0.037 mg
$FeSO_4.7H_2O$	0.25 mg
$MnSO_4.4H_2O$	0.04 mg
$Na_2MoO_4.2H_2O$	0.025 mg
$ZnSO_4.7H_2O$	0.031 mg
Deionised water	1 litre

The pH of the medium was adjusted to 3.5 with dilute hydrochloric acid and the solution was sterilized by autoclaving at 121°C and 15 psi for 15 min. The culture was grown at 25°C on an orbital shaker at 160 rpm for 14 days.

Aspergillus terreus (NRRL 3319) was obtained from Lilly Research Laboratories, Indiana, USA. Initially, the mould was grown on slants having the following composition:

Tomato paste	20 g
Oatmeal flour	20 g
Agar	20 g
Deionised water	1 l

It was found later (see Section 3.3) that better growth was obtained with Czapek-Dox slants, which consist of:

Glucose	50 g
$NaNO_3$	2 g
KCl	1 g

$MgSO_4$	1	g
K_2HPO_4	0.5	g
$FeSO_4$	0.01	g
Agar	20	g
Deionised water	1	litre

Slants were incubated for one week and washings therefrom were used to inoculate 500 ml Erlenmeyer flasks containing the synthetic medium (100 ml) described below. These seed flasks were grown at 28°C for 4 days and aliquots (10 ml) from them were used to inoculate 500 ml Erlenmeyer flasks containing synthetic medium (100 or 120 ml) of the following composition:

Glucose	15	g
Sucrose	15	g
$NaNO_3$	2	g
KH_2PO_4	1	g
$MgSO_4.7H_2O$	0.5	g
KCl	0.5	g
$FeSO_4.7H_2O$	0.01	g
Beef extract	0.5	g
Yeast extract	0.125	g
Deionised water	1	litre

The medium was sterilized by autoclaving at 121°C, and 15 psi for 20 min. The fungus was grown at 28°C for one week on an orbital shaker at 160 rpm.

4.3 Feeding experiments

Precursors, dissolved in dimethyl sulphoxide (DMSO), were fed to Hyalodendron sp. on the fifth day of growth, at the levels shown in Table 6. Cultures were harvested 9 days later. Precursors, dissolved in DMSO, were fed to A. terreus 48 h after inoculation, at the levels shown in Table 7. Cultures were harvested 5 days later.

The "intermediate-trapping" experiment was carried out by administering cyclo-(L-Phe-L-Phe) (100 mg) in DMSO (10 ml) to two-day-old cultures of A. terreus (1 l), followed, 2 h later, by the addition of L-phenylalanine (25 μCi). After a further 2 h, the culture was harvested.

4.4 Metabolite isolation

Hyalodendron sp. cultures were harvested by suction filtration through a pad of Celite 545 filter-aid. The mycelium was washed with methanol and the mycelial washings and filtrate were combined and extracted with chloroform (6 x 10% total volume). The combined extracts were concentrated to ca. 100 ml, washed with water (3 x 10 ml), dried over $MgSO_4$, and evaporated to dryness. The residue was chromatographed on silica gel t.l.c. plates developed with benzene-ethyl acetate (4:1). The band between $R_f \sim 0.12 - 0.24$, detected with ultraviolet light (254 nm), was eluted with chloroform (2 x 30 ml) and ethyl acetate (2 x 30 ml). The solvents were evaporated, yielding crude bisdethiobis(methylthio)hyalodendrin (17). When the cultures had been fed with precursors dissolved in DMSO, this residue was dissolved in methylene chloride (10 ml), which was washed with water

PRECURSOR	PRECURSOR WEIGHT (mg)	TOTAL RADIOACTIVITY (μCi)	VOLUME OF CULTURE (l)	PRECURSOR CONCENTRATION (mg/l)	S.A. PRECURSOR (μCi/mmol)
cyclo-(L-[U-^{14}C]Phe-L-Ser)	12.3	3.00	0.9	13.7	56.98
cyclo-(L-[U-^{14}C]Phe-D-Ser)	12.1	1.63	0.9	13.4	31.54
cyclo-(L-N-Me-Phe-L-[3-^{14}C]Ser)	13.8	2.40	0.9	15.3	5.44
cyclo-(L-[U-^{14}C]Phe-L-Ser)	2.7	1.05	0.8	14.1[b]	21.82[b]
cyclo-(L-Phe-DL-[3-^{13}C]Ser)	10.6[a]	-			

[a] 80.8% LL-isomer

[b] Refers exclusively to LL-isomer

TABLE 6 Feeding conditions for Hyalodendron sp.

PRECURSOR	PRECURSOR WEIGHT (mg)	TOTAL RADIOACTIVITY (μCi)	VOLUME OF CULTURE (l)	PRECURSOR CONCENTRATION (mg/l)	S.A. PRECURSOR (μCi/mmol)
L-Phenylalanine	-	25.0	0.8	-	513×10^3
cyclo-(L-[U-^{14}C]Phe-L-Phe)	32.9	4.87	2.4	13.7	43.50
cyclo-(D-Phe-DL-[1-^{14}C]Phe)	31.4	2.21	1.5	20.9	20.73
cyclo-(L-[4'-^{3}H]Phe-L-[3-^{14}C]Ser)	27.0	7.45 (^{14}C) 99.31 (^{3}H)	2.0	13.5	64.58 860.70
cyclo-(L-[U-^{14}C]Phe-L-Phe)	5.5	0.81	1.2	10.0[c]	19.94[c]
cyclo-(L-[^{15}N]Phe-DL-[1-^{13}C]Phe)	12.3[a]	-			
cyclo-(L-[U-^{14}C]Phe-L-Phe)	5.4	0.80	1.2	8.8[c]	22.16[c]
cyclo-(DL-[3,3-$^{2}H_2$]Phe-DL-[3,3-$^{2}H_2$]Ser)	20.8[b]	-			

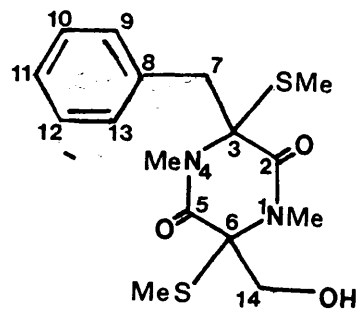
[a] 52.7% LL-isomer

[b] 25.0% LL-isomer

[c] Refers exclusively to LL-isomer

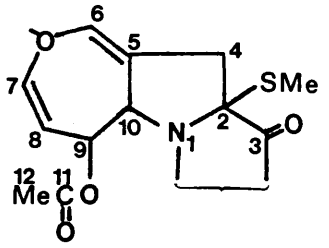
TABLE 7 Feeding conditions for A. terreus

(3 x 5 ml) to remove dimethyl sulphone, dried and evaporated to dryness. Bisdethiobis(methylthio)hyalodendrin (ca. 20 - 25 mg per litre of mould culture) was crystallised from methylene chloride - cyclohexane, m.p. 141.5 - 142.5°C (lit.,[16] 140 - 140.5°C), $[\alpha]_D^{31}$ + 62° (c 1.01. $CHCl_3$) [lit.,[16] $[\alpha]_D^{23}$ + 64° (c 1.071, $CHCl_3$)], + 48° (c 0.13, CH_3OH); ^{13}C-n.m.r. δ ($CDCl_3$) 13.4 (MeN), 14.3 (MeN), 29.4 (MeS), 30.9 (MeS), 42.3 (C-7), 64.3 (C-14), 71.6 (C-3 or C-6), 73.5 (C-3 or C-6), 127.7 (C-11), 128.6 (C-10 and C-12), 130.0 (C-9 and C-13), 134.1 (C-8) and 165.4 ppm (C-2 and C-5).



A. terreus cultures were harvested by suction filtration and the mycelium was washed with methanol. The combined culture broth and mycelial washings were acidified to pH 3 with concentrated hydrochloric acid and were extracted with $CHCl_3$ (6 x 10% total volume). The combined extracts were concentrated (ca. 100 ml), washed with water (3 x 10 ml), dried over $MgSO_4$ and evaporated to dryness. The residue was chromatographed on silica gel t.l.c. plates developed with chloroform - ethyl acetate (1:1). The band at R_f ∿ 0.5 0.7, detected with ultraviolet light (254 nm), was eluted with chloroform (3 x 30 ml) and ethyl acetate (3 x 30 ml). The combined eluates were evaporated to dryness, leaving a residue which contained BDA (42) and BDAA (43). This mixture was rechromatographed on silica gel t.l.c. plates developed with isopropyl ether - chloroform - acetic acid (6:3:1). The closely running bands at R_f 0.21 - 0.33 and 0.33 - 0.45, detected with ultraviolet light (360 nm), were eluted with chloroform (3 x 30 ml) and ethyl acetate (3 x 30 ml). The material from the faster-running band consisted mostly of BDAA,

contaminated with varying amounts of BDA, and, generally, could not be crystallised. The material from the slower-running band gave, after recrystallisation from methanol, pale yellow crystals of pure BDA (ca. 10 mg per litre of mould culture) m.p. 201 - 203°C (dec.) [lit., 213-217° (dec.)[32], 215 - 236° (dec.)[34], 230° (dec.)[37]]; ^{13}C-n.m.r. δ ($CDCl_3$) 14.7 (MeS), 21.0 (C-12), 40.5 (C-4), 60.3 (C-5), 70.5 (C-2), 71.7 (C-9), 105.8 (C-8), 109.5 (C-10), 137.7 (C-6 or C-7), 139.7 (C-6 or C-7), 164.4 (C-3) and 169.9 ppm (C-11).



4.5 Labelled amino acids

L-[U-^{14}C]Phenylalanine, L-[4'-^{3}H]phenylalanine, DL-[1-^{14}C]phenylalanine and L-[3-^{14}C]serine were obtained from The Radiochemical Centre, Amersham. DL-[3-^{13}C]Serine (containing ca. 23% glycine; 0.90 atom ^{13}C), DL-[1-^{13}C]phenylalanine (0.91 atom ^{13}C) and L-[^{15}N]phenylalanine (0.965 atom ^{15}N) were obtained from Prochem-B.O.C. Limited.

The commercial DL-[3-^{13}C]serine was freed from the glycine it contained by ion-exchange chromatography using a BIO-RAD analytical grade cation exchange resin, AG 50W-X8 (H^+, 200 - 400 mesh). The commercial sample (0.516 g) in deionised water (10 ml), was applied to the column (1 m long, 1 cm inner diameter) which was then washed with deionised water (40 ml). The amino-acids were eluted with 1M hydrochloric acid (flow rate: 0.4 ml/min) and fractions (10 ml) were collected with an automatic fraction collector. Each fraction was analysed by t.l.c. on Merck aluminium sheets precoated with Cellulose

F_{254} (0.10 mm thick) developed with a mixture of n-butanol - acetone - diethylamine - water (10:10:2:5).[49] The spots were revealed by spraying the plates with a solution of ninhydrin in n-butanol. In this solvent system, serine had R_f = 0.40, whereas glycine had R_f = 0.21. Fractions 37 - 43 were found to contain the desired DL-[3-^{13}C]-serine. These were combined and evaporated to dryness under reduced pressure. The residue was dissolved in water (5 ml) and taken to pH 6.8 with 1M lithium hydroxide. Ethanol was added until a slight cloudiness appeared and the solution was cooled to 0°C. The crystalline amino-acid was filtered off, washed with water and dried under vacuum over P_2O_5. The white plates of pure DL-[3-^{13}C]serine (0.340 g) thus obtained had m.p. 238°C (dec.) [lit.,[50] 246°C (dec.)]. Glycine (0.103 g) was recovered in a similar manner from fractions 51 - 57. Since previous chromatographies of mixtures of the two amino-acids had shown that the average recovery for serine was ca. 89%, the commercial sample contained ca. 23% of glycine.

4.6 Synthetic procedures

Cyclo-(L-[U-^{14}C]Phe-L-Ser) and cyclo-(L-[4'-^{3}H]Phe-L-[3-^{14}C]Ser) were prepared by Patrick[9,27] and cyclo-(L-[U-^{14}C]Phe-L-Phe) was prepared by Talekar[29] by methods similar to those described below for the same compounds having different isotopic labelling patterns.

Acetate of bisdethiobis(methylthio)hyalodendrin (39).- A solution of bisdethiobis(methylthio)hyalodendrin (12.1 mg), pyridine (2 drops) and acetic anhydride (2 drops) was kept at room temperature for 12 h. Methanol (0.5 ml) was added and the solution evaporated to dryness.

The residue was recrystallised from acetone - diethyl ether to give white needles of the acetate (39) (10.7 mg, 79.1% yield), m.p. 137 - 139°C (Found: C, 54.7; H, 6.2; N, 6.9. $C_{18}H_{24}N_2S_2O_4$ requires C, 54.6; H, 6.1; N, 7.1%); δ ($CDCl_3$) (60 MHz) 7.22 (5H, s, ArH), 4.37 (2H, s, -CH_2-Ph), 3.30 (2H, ABq, J_{AB} 14 Hz, -CH_2O-), 3.28 (3H, s, NMe), 3.05 (3H, s, NMe), 2.33 (3H, s, SMe), 2.23 (3H, s, SMe) and 1.66 (3H, s, SMe).

N-Benzyloxycarbonyl-D-serine.- Benzyl chloroformate (1 ml, 96% pure) was added, with vigorous stirring, to a solution of D-serine (0.53 g, 5 mmol) in aqueous saturated sodium bicarbonate (20 ml). Stirring was continued for a further 4 h, and then the reaction mixture was extracted with ether (3 x 10 ml) and cooled to 0°C. The cold solution was acidified to pH 2 with concentrated hydrochloric acid and then extracted with ethyl acetate (5 x 10 ml). The combined ethyl acetate extracts were dried over anhydrous Na_2SO_4 and concentrated to a solid. This was triturated with ether (3 x 10 ml) and then dried under vacuum to give N-benzyloxycarbonyl-D-serine (1.01 g, 84.5% yield), m.p. 117.5 - 118°C (lit.,[51] 119.5°C); $[\alpha]_D^{22}$ - 5.2° (c 1.07, HOAc) [lit.,[52] $[\alpha]_D^{20}$ - 5.6° (c 1, HOAc)].

N-Benzyloxycarbonyl-DL-[3-^{13}C]serine.- This racemate was synthesised from DL-[3-^{13}C]serine (335.8 mg, 3.2 mmol), by the method described above for the D-enantiomer, in 82.7% yield, m.p. 125 - 126°C (lit.,[53] 125°C).

L-Serine methyl ester hydrochloride.- Using the method of Guttman and Boisonnas,[51] L-serine (5.25 g, 50 mmol) was converted, by

the action of thionyl chloride and methanol, into its methyl ester hydrochloride (6.92 g, 89% yield), m.p. 164 - 166°C (lit.,[51] 168°C)

DL-Phenylalanine methyl ester hydrochloride.- A solution of DL-phenylalanine (164.4 mg, 0.99 mmol), 2,2-dimethoxypropane (10 ml) and concentrated hydrochloric acid (1 ml) was kept in the dark for 18 h, as described by Rachele.[54] Evaporation of the solvents left a dark brown residue which was dissolved in anhydrous methanol (1 ml). Cold anhydrous ether was added to this solution until crystals started to appear. Crystallisation was complete after cooling to 0°C for 24 h. The crystals were filtered, washed with anhydrous ether and dried under vacuum. The white plates of the hydrochloride (193.8 mg, 91% yield) had m.p. 157 - 158.5°C (lit.,[55] 158°C); m/e 179 (M^+, free amino ester), 120 (M - CO_2Me), 103 (M - C_7H_7 + H), 91 (C_7H_7) and 88 [$CHNH_2(CO_2Me)$].

DL-[1-^{14}C]Phenylalanine methyl ester hydrochloride.- DL-[1-^{14}C]-Phenylalanine methyl ester hydrochloride was synthesised, as described above, from DL-[1-^{14}C]phenylalanine(50 μCi), diluted with DL-phenylalanine (382.7 mg, 2.3 mmol), in 89% yield, with a specific activity of 21.07 μCi/mmol and m.p. 157 - 158.5°C (lit.,[55] 158°C).

DL-[1-^{13}C]Phenylalanine methyl ester hydrochloride.- DL-[1-^{13}C]-Phenylalanine methyl ester hydrochoride was synthesised, as described above, from DL-[1-^{13}C]phenylalanine (255.8 mg, 1.54 mmol) in 75% yield and with m.p. 158 - 158.5°C (lit.,[55] 158°C).

L-Phenylalanine methyl ester hydrochloride.- L-Phenylalanine (330 mg, 2 mmol) was submitted to the reaction conditions described above for racemic phenylalanine, to give the L-methyl ester hydrochloride (409.4 mg, 95% yield), m.p. 157 - 159°C (lit.,[56] 158.5 - 159.5°C); $[\alpha]_D^{26}$ + 36° (c 2, EtOH) [lit.,[56] $[\alpha]_D^{25}$ + 40° (c 2, EtOH)].

L-[U-^{14}C]Phenylalanine methyl ester hydrochloride.- The radioactive methyl ester hydrochloride was synthesised as described for the unlabelled racemate, from L-[U-^{14}C]phenylalanine (50 μCi) diluted with L-phenylalanine (188 mg, 1.1 mmol), in 94% yield with a specific activity of 41.28 μCi/mmol and m.p. 153 - 155°C (lit.,[56] 158.5 - 159.5°C).

N-Benzyloxycarbonyl-L-phenylalanine.- This compound was obtained using L-phenylalanine, benzyl chloroformate and aqueous sodium hydroxide, according to the method of Grassman.[57] On recrystallisation from methylene chloride - petroleum ether (40 - 60°C), the pure amino-acid derivative was obtained (71% yield), m.p. 85 - 87°C (lit.,[57] 88 - 89°C); $[\alpha]_D^{22}$ + 5.7° (c 1.96, EtOH) [lit.,[57] $[\alpha]_D^{20}$ + 5.1° (c 2, EtOH)].

N-Benzyloxycarbonyl-L-[^{15}N]phenylalanine.- N-Benzyloxycarbonyl-L-[^{15}N]phenylalanine was synthesised, as described above, from L-[^{15}N]-phenylalanine (303.1 mg, 1.8 mmol) in 84.5% yield and with m.p. 86 - 87°C (lit.,[57] 88 - 89°C).

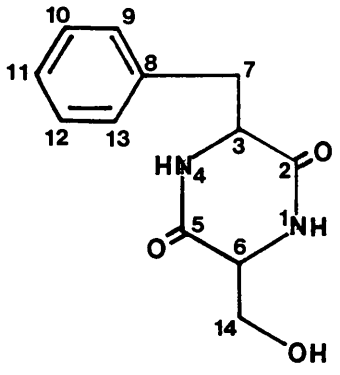
N-Benzyloxycarbonyl-D-phenylalanine.- N-Benzyloxycarbonyl-D-phenylalanine was synthesised as described above for the L-enantiomer, in 80% yield with identical properties except for opposite optical rotation.

N-Benzyloxycarbonyl-N-methyl-L-phenylalanine.- This compound was synthesised, according to the method of McDermott and Benoiton,[31] from N-benzyloxycarbonyl-L-phenylalanine and excess methyl iodide and sodium hydride in tetrahydrofuran. The derivative, previously reported as an oil,[31] was crystallised from methylene chloride - petrolem ether (40-60°C) to give white crystals (66% yield), m.p. 62 - 64.5°C, $[\alpha]_D^{22}$ - 74° (c 2.05, MeOH) (Found: C, 69.3; H, 6.2; N, 4.2. $C_{18}H_{19}NO_4$ requires C, 69.0; H, 6.1; N, 4.5%); δ ($CDCl_3$) (90 MHz) 10.79 (1H, bs, CO_2H, exchangeable with D_2O), 7.33 (5H, d - rotamers, ArH), 5.07 (2H, d - rotamers, $-CH_2-O-$), 4.92 (1H, m, α-CH), 3.22 (2H, m, β-CH_2) and 2.80 (3H, d - rotamers, NMe); m/e 313(M^+), 178 (M - C_7H_7OCO), 165 ($C_7H_7OCONHMe$) and 91 (C_7H_7).

cyclo-(L-Phe-DL-[3-^{13}C]Ser).- L-Phenylalanine methyl ester hydrochloride (435 mg, 2 mmol) was suspended in anhydrous methylene chloride (25 ml), cooled to 0°C and anhydrous, freshly distilled triethylamine (0.39 ml, 2.8 mmol) was added to give a clear solution. N-Benzyloxycarbonyl-DL-[3-^{13}C]serine (478 mg, 2 mmol) was added to this solution followed by dicyclohexylcarbodiimide (460 mg, 2.2 mmol). The solution was kept in the dark at room temperature for 6 h. The precipitated dicyclohexylurea was removed by filtration. The filtrate was washed with 1M HCl (2 x 5 ml), water (2 x 5 ml), saturated aqueous sodium bicarbonate (2 x 5 ml) and water (2 x 5 ml). The methylene chloride layer was dried over $MgSO_4$ and evaporated. The residue was suspended in anhydrous acetone and filtered to remove more urea. the acetone was evaporated and the residue was dissolved in anhydrous methanol (20 ml) containing glacial acetic acid (4 drops). This solution

was hydrogenolysed at room temperature and atmospheric pressure for 4 h using 10% palladium - carbon catalyst (70 mg). The catalyst was filtered off, the solvents were evaporated, and the residue was dissolved in anhydrous methanol (1 ml). This solution was then treated with saturated ammoniacal methanol (10 ml) at room temperature for 24 h. The product which separated was filtered off, washed with cold anhydrous methanol (2 x 2 ml) and anhydrous ether (3 x 2 ml) and finally dried under vacuum. The crystalline cyclodipeptide (94.4 mg) (crop A) showed two m.p.'s at 244 - 255°C and 262 - 264°C (dec.). Concentration of the ammoniacal mother liquors, followed by cooling and washing of the resulting crystals as described above, afforded more cyclodipeptide (20.1 mg) (crop B), m.p. 235 - 240°C. Further concentration of the mother liquors as above gave crop C (51.1 mg), m.p. 230 - 235°C.

The 25.2 MHz natural abundance ^{13}C-n.m.r. spectra of cyclo-(L-Phe-L-Ser) and cyclo-(L-Phe-D-Ser) were obtained using samples of the cyclic dipeptides that had been prepared by Patrick.[9,27] For the LL-isomer: δ (d_6-DMSO) 166.6 (C-2 or C-5), 165.8 (C-2 or C-5), 136.6 (C-8), 129.9 (C-9 and C-13), 128.1 (C-10 and C-12), 126.4 (C-11), 63.1 (C-14), 57.1 (C-3 or C-6), 55.5 (C-3 or C-6) and 39.8 ppm (C-7). For the LD-isomer: δ (d_6-DMSO) 167.4 (C-2 or C-5), 166.8 (C-2 or C-5), 136.2 (C-8), 130.1 (C-9 and C-13), 127.9 (C-10 and C-12), 126.5 (C-11), 62.4 (C-14), 56.4 (C-3 or C-6), 55.0 (C-3 or C-6) and 38.6 (C-7).



The ^{13}C-n.m.r. spectra of crops A, B and C were obtained and compared with those previously obtained for the pure LL- and LD-

isomers. The relative abundances of LL- and LD-isomers present in the ^{13}C-labelled mixture were calculated from the heights of the signals at δ 63.1 and 62.4. Thus the LL:LD ratios were found to be 1:3.0 for crop A, 3.8:1 for crop B and 4.2:1 for crop C. These results were confirmed by ^{1}H-n.m.r. spectroscopy. The NH protons in the LL-isomer gave signals at δ 7.94 and 8.06 ppm, whereas the corresponding signals for the LD-isomer appeared at δ 7.85 and 8.16 ppm. However, no quantitative information could be obtained from the heights of these peaks since, owing to partial overlap, their integration was not very accurate. Nevertheless, for crop A, four distinct signals were observed in that region, the two inner ones being much weaker (x $\frac{1}{4}$) than the outer ones, whereas for both crops B and C, the inner peaks were higher, the outer ones being barely visible. Therefore, overall 165 mg of cyclo-(L-Phe-DL-[3-^{13}C]Ser) (35% yield from the amino-acid derivatives) were obtained, of which ca. 85 mg corresponded to the LD-isomer and ca. 80 mg corresonded to the LL-isomer.

cyclo-(L-[U-^{14}C]Phe-D-Ser).- This cyclic dipeptide was synthesised by the preceding method, from L-[U-^{14}C]phenylalanine methyl ester hydrochloride (224 mg, 1.04 mmol, 43.0 μCi), N-benzyloxycarbonyl-D-serine (248.5 mg, 1.04 mol), dicyclohexylcarbodiimide (272.3 mg, 1.32 mmol) and triethylamine (160 μl) in methylene chloride (10 ml). The product which separated after treatment with saturated ammoniacal methanol was filtered off, washed with anhydrous diethyl ether (3 x 2 ml) and crystallised from methanol to give cyclo-(L-[U-^{14}C]Phe-D-Ser) (92 mg, 38% yield from the amino-acid derivatives), m.p. 258 - 265°C (dec.) [lit.,[9] 258 - 268°C (dec.)], with a specific activity of 31.54 μCi/mmol; R_f 0.62 (methanol - ethyl acetate, 1:1); δ (d_6-DMSO) (90 MHz)

8.00 (1H, bs, NH, exchangeable with D_2O), 7.70 (1H, bs, NH, exchangeable with D_2O), 7.24 (5H, m, ArH), 4.90 (1H, t, J 5.5 Hz, OH, exchangeable with D_2O), 4.20 (1H, m, C<u>H</u>CH_2Ph) and 3.80 - 2.85 (5H, m, C<u>H</u>CH_2OH, CH_2OH and C<u>H</u>$_2$Ph).

<u>cyclo</u>-(L-<u>N</u>-Me-Phe-L-Ser).- L-Serine methyl ester hydrochloride (162.mg; 1.04 mmol) was suspended in anhydrous methylene chloride (12 ml), cooled to 0°C and anhydrous, freshly distilled triethylamine (140 µl) was added to give a clear solution. <u>N</u>-Benzyloxycarbonyl-<u>N</u>-methylphenylalanine (333 mg, 1.06 mmol) was added to this solution followed by dicyclohexylcarbodiimide (223 mg, 1.08 mmol). The reaction mixture was kept in the dark at room temperature for 4 h, then at 0°C overnight and finally at room temperature for a further 1 h. The reaction mixture was worked up as described for the ^{13}C-labelled cyclodipeptide, namely, by removal of the urea, washing of the filtrate (with 1 <u>M</u> hydrochloric acid, water, saturated aqueous sodium bicarbonate and water), drying of the organic layer, removal of more urea and finally dissolution of the residue in a methanol - acetic acid mixture (85:15, 10 ml). This was hydrogenolysed for 8 h at room temperature and atmospheric pressure using 10% palladium - carbon catalyst (71 mg). The catalyst was filtered off and the solvents were evaporated. The unprotected dipeptide cyclised spontaneously upon complete removal of the acetic acid. The crude cyclodipeptide was recrystallised from methanol - diethyl ether to give white needles of pure <u>cyclo</u>-(L-<u>N</u>-Me-Phe-L-Ser) (<u>ca</u>. 35 mg, 13.6% yield), R_f 0.50 (methanol - ethyl acetate, 1:1), m.p. 199 - 201°C, $[\alpha]_D^{22}$ + 2° ± 1° (<u>c</u> 0.165, methanol) (Found: C, 52.5; H, 7.0; N, 9.1. $C_{13}H_{16}N_2O_3.2\frac{1}{2}H_2O$ requires C, 53.2; H, 7.2; N, 9.6%)

(m/e 249.1235. Calc. for $C_{13}H_{17}N_2O_3$: M, 249.1239); δ (d_6-DMSO) (90 MHz) 9.13 (1H, d, J 8 Hz, NH, exchangeable with D_2O), 7.33 (5H, s, ArH), 5.26 (1H, t, J 5 Hz, OH, exchangeable with D_2O), 4.38 (1H, dt, J 8 and 4 Hz, $CHCH_2OH$), 4.17 (1H, t, J 7 Hz, $CHCH_2Ph$), 3.74 (2H, d, J 4 Hz, CH_2OH), 3.64 (3H, s, NMe), and 3.15 (2H, d, J 7 Hz, CH_2Ph). Irradiation of the signal at 9.13 ppm caused the signal at 5.26 ppm to disappear[58] and that at 4.38 ppm to collapse to a triplet. Irradiation of the signal at 4.38 ppm caused: a) collapse of the signal at 9.13 ppm to a singlet, b) collapse of a signal at 5.26 ppm to a very broad singlet, and c) collapse of the signal at 3.74 ppm to a broad singlet. Irradiation of the signal at 4.17 ppm caused the signal at 3.15 ppm to collapse to a singlet. m/e 249 (M^+ + H), 221 (M + 1 - CO), 189 (M + 1 - CO - CH_3OH), 134 ($C_7H_7CH{=}NHMe$), 119 ($C_7H_7CH{=}NH$) and 91 (C_7H_7).

cyclo-(L-N-Me-Phe-L-[3-^{14}C]Ser).- Thionyl chloride (160 µl) was added to a solution of L-[3-^{14}C]serine in anhydrous methanol (2 ml) at 0°C. L-Serine (106 mg, 1 mmol) was then added and the solution was kept at room temperature for 2 h and then at 0°C overnight. The solvents were removed under a stream of nitrogen, leaving white needles of the methyl ester hydrochloride. These were dissolved in anhydrous methylene chloride (6 ml) and anhydrous, frshly distilled triethylamine (140 µl) was added to free the amino ester. N-Benzyl-oxycarbonyl-N-methyl-L-phenylalanine (315 mg, 1 mmol) was then added to the methylene chloride solution followed by dicyclohexylcarbodiimide (250 mg, 1.2 mmol). The reaction mixture was kept in the dark at room temperature for 4 h, then at 0°C overnight, and then worked up

as described above. The protected dipeptide gave only one spot, R_f 0.58 [ethyl acetate - petroleum ether (40 - 60°C) - acetic acid, 4:4:1]. The protecting group was removed by hydrogenolysis for 13 h at room temperature and atmospheric pressure using 10% palladium - carbon catalyst (80 mg) in methanol (8.5 ml) containing acetic acid (1.5 ml). Filtration of the mixture and evaporation of the filtrate afforded a mixture of three compounds, only one of which had the R_f expected for the cyclodipeptide. On the assumption that complete cyclisation had not taken place, the mixture was dissolved in methanol (2 ml) and treated with saturated ammoniacal methanol (10 ml) at room temperature for 24 h. Evaporation of the solvent and inspection by t.l.c. showed a mixture of two products, one of which (R_f 0.54; methanol - ethyl acetate, 1:1) was the desired cyclo-(L-N-Me-Phe-L-L-Ser). The two compounds were separated by preparative t.l.c. using chloroform - methanol - acetic acid (90:5:5). The material recovered from the band at $R_f \sim 0.11$, could not be crystallised (see Section 2.3). The crude product (46.2 mg) was dissolved in methanol (5 ml). Half of this solution was used in a feeding experiment (Table 1, experiment No. 10); the other half was used for an isotope dilution analysis. This revealed that the specific activity of the cyclo-(L-N-Me-Phe-L-[3-^{14}C]Ser) was 5.45 μCi/mmol, and that it was contaminated with 40.3% of radioactive im impurities which could not be identified.

cyclo-(D-Phe-DL-[1-^{14}C]Phe).- To a solution of DL-[1-^{14}C]-phenylalanine methyl ester hydrochloride (433 mg, 2 mmol, 42.3 μCi) in anhydrous methylene chloride (20 ml) was added anhydrous, freshly distilled triethylamine (0.3 ml), N-benzyloxycarbonyl-D-phenylalanine (598 mg, 2 mmol) and dicyclohexylcarbodiimide (413 mg, 2 mmol), in

that order. The reaction mixture was kept in the dark at room temperature for 4 h, and then at 0°C overnight. The precipitated dicyclohexylurea was removed by filtration. The filtrate was washed with 1 M hydrochloric acid (2 x 5 ml), water (2 x 5 ml), saturated aqueous bicarbonate (2 x 5 ml) and water (2 x 5 ml). The organic layer was dried over $MgSO_4$ and evaporated to dryness. The residue was suspended in anhydrous acetone (10 ml) and filtered to remove more urea. To the acetone solution was added 10% palladium - carbon catalyst (71 mg) and the mixture hydrogenolysed at room temperature and atmospheri atmospheric pressure for 3 h. The catalyst was removed by filtration and the filtrate was evaporated to dryness. The residue was dissolved in anhydrous methanol (2 ml), cooled to 0°C and treated with saturated ammoniacal methanol (10 ml). The solution was left at room temperature overnight. The solid that separated was filtered, washed with ether 2 x 5 ml) and recrystallised from methanol (large excess). The mixture of cis(DD)- and trans(DL)-cyclodipeptides crystallised as very fine white needles (63.3 mg, 11% yield from amino acid derivative), m.p. 295 - 297°C, specific activity 20.73 μCi/mmol, R_f 0.25 (DD-isomer) and 0.51 (DL-isomer) (isopropyl ether - chloroform - acetic acid, 6:3:1); δ (CF_3CO_2H) (90 MHz) 8.11 (bs, NH), 7.84 (bs, NH), 7.36 and 7.12 (m, ArH), 4.58 (bm, α-CH), 3.96 (m, α-CH), 3.11 (m, β-CH), and 2.27 (m, β-CH). The signals at δ 8.11 and 4.58 ppm corresponded to the DD-isomer, whereas those at δ 7.84 and 3.96 ppm corresponded to the DL-isomer (by comparison with the ^{1}H-n.m.r. spectra of the two separate isomeric cyclodipeptides). Integration of these signals showed that the DL:DD ratio was 4:6.

cyclo-(L-[^{15}N]Phe-DL-[1-^{13}C]Phe).- Using the procedure described above, DL-[1-^{13}C]phenylalanine methyl ester hydrochloride (240 mg, 1.1 mmol) and N-benzyloxycarbonyl-L-[^{15}N]phenylalanine (330 mg, 1.1 mmol) were coupled to give the protected dipeptide. This compound was deprotected by hydrogenolysis and cyclised, as before, to give cyclo-(L-[^{15}N]Phe-DL-[1-^{13}C]Phe) in two crops: the first (40.8 mg) had m.p. 295 - 297°C and the second (54.8 mg) had m.p. 281 - 291°C (overall 29% yield from the amino-acid derivative). In the first crop the LL:LD ratio was 52.7:47.3, whereas in the second it was 59:41, as calculated from integration of the ^{1}H-n.m.r. spectrum. ^{13}C-n.m.r. δ (CF_3CO_2H) (25.2 MHz) 173.3 (carbonyl group of LD-isomer) and 172.6 ppm (carbonyl group of LL-isomer), J_{CN} 18 Hz (assignments made on comparison with published data[59]).

Morpholine perchlorate.- A solution of morpholine (50 g, 0.6 mol) in methanol (200 ml) was cooled to 0°C and perchloric acid (50 ml of 72% aqueous solution, d 1.67 g/ml, 0.6 mol) was added dropwise and with stirring, to keep the exothermic reaction under control. The resulting pale yellow solution was allowed to cool to room temperature, whereupon the perchlorate crystallised as small white needles. These were filtered off, washed with cold methanol (2 x 25 ml), and dried first by suction and then under vacuum, over P_2O_5, for 24 h. No attempts were made to characterise the product (72.6 g, 64.7%) due to its potentially explosive character.

α-Phenyl-α-morpholinoacetonitrile.- α-Phenyl-α-morpholinoacetonitile was synthesised by the method of Bennett et al.[60] The crude

product was recrystallised from petroleum ether (60 - 80°C) to give white needles (87% yield), m.p. 69.5 - 70°C (lit.,[60] 69 - 70°C) (Found: C, 71.2; H, 7.2; N, 14.0. Calc. for $C_{12}H_{14}N_2O$:C, 71.3; H, 6.9; N, 13.9%); $\nu_{max.}$ (KBr) 2 980, 2 945, 2 910, 2 825, 2 230 and 1 455 cm^{-1}; δ (CCl_4) (90 MHz) 7.44 (5H, m, ArH), 4.74 (1H, s, α-H), 3.67 (4H, t, $-CH_2O-$) and 2.53 (4H, t, $-CH_2-N-$); m/e 202 (M^+), 171 (M - CH_3O) and 116 (M - C_4H_8NO).

α-Phenyl-α-[α-^{2}H]morpholinoacetonitrile.- α-Phenyl-α-morpholinoacetonitrile (50.5 g, 0.25 mol) in anhydrous NN-dimethylformamide (200 ml), under dry, oxygen-free nitrogen, was treated with sodium hydride (mineral oil suspension; 0.76 mol) at room temperature for 1 h to generate the green benzylic anion. Addition of D_2O (26 ml) was followed by acidification of the solution at 0°C to pH ∿ 2 with a slight excess of thionyl chloride. The brown mixture was diluted with water (ca. 20 ml) and extracted with chloroform (4 x 30 ml). The combined chloroform extracts were washed with water (3 x 10 ml), dried over $MgSO_4$ and evaporated to give a syrupy residue. This was crystallised from petroleum ether (60 - 80°C) giving white needles (45.6 g, 90% yield, 0.94 atom deuterium), m.p. 68 - 70°C (lit.,[60] 69 - 70°C for nondeuteriated compound) (Found: C, 71.1; H, 7.6; N, 13.8. Calc. for $C_{12}H_{13}DN_2O$ C, 70.9; H, 7.4; N, 13.8%); $\nu_{max.}$ (KBr) 2 980, 2 940, 2 910, 2 820, 2 255 and 1 455; δ (CCl_4) (90 MHz) 7.40 (5H, m, ArH), 4.68 (0.06H, s, residual α-CH), 3.62 (4H, t,$-CH_2O-$) and 2.49 (4H, t, $-CH_2N-$); m/e 203 (M^+), 172 (M - CH_3O) and 117 (M - C C_4H_8NO).

[formyl-^{2}H]Benzaldehyde.- α-Phenyl-α-[α-^{2}H]morpholinoacetonitrile (47.5 g, 0.23 mol) was heated for 1 h in refluxing 2 M hydrochloric acid (250 ml). The solution was cooled to room temperature and extracted with methylene chloride (8 x 30 ml). The combined methylene chloride extracts were dried over $MgSO_4$ and the solvent was distilled off through a short fractionating column at atmospheric pressure. [formyl-^{2}H]-Benzaldehyde (21.7 g, 88% yield, 0.95 atom deuterium) was obtained by distillation of the residue, b.p. 170 - 178°C (lit.,[50] 178°C for nondeuteriated compound); $\nu_{max.}$(film) 3 060, 2 100, 2 060, 2 040 and 1 685 cm^{-1}; δ (CCl_4) (90 MHz) 9.97 (0.05H, s, residual CHO) and 7.86 and 7.57 (5H, m, ArH); m/e 107 (M^+) and 79 (M - CO).

[methylene-$^2H_2$]Benzyl alcohol.- [formyl-^{2}H]Benzaldehyde (30 g) was heated for 2.5 h in refluxing 25% aqueous potassium hydroxide solution (80 ml) and then left at room temperature overnight. Water was added, to dissolve the precipitated potassium benzoate, and the solution was extracted with diethyl ether (4 x 20 ml). The combined ether extracts were concentrated (to ca. 30 ml) and washed with saturated aqueous sodium metabisulphite (2 x 5 ml), 10% aqueous sodium carbonate (2 x 5 ml) and water (2 x 5 ml). The organic layer was dried over $MgSO_4$ and evaporated under reduced pressure. Upon distillation, the yellow residue afforded the pure alcohol (11.8 g, 76.5% yield, ca. 1.60 atom deuterium), b.p. 65 - 75°C/30 Torr (lit.,[50] 205°C, for nondeuteriated compound); δ (CCl_4) (60 MHz) 7.00 (5H, s, ArH), 4.33 (0.44H, bs, residual CH_2) and 3.53 (1H, bs, OH, exchangeable with D_2O).

[methylene-$^2H_2$]Benzyl chloride.- To a solution of [methylene-$^2H_2$]-benzyl alcohol (6.5 g, 60 mmol) and pyridine (0.7 ml) in anhydrous diethyl ether (50 ml) was added, dropwise and with stirring, a solution of thionyl chloride (9.8 g, 82 mmol) in anhydrous diethyl ether (35 ml). The mixture was stirred overnight and was the washed with water (2 x 10 ml) and dried over $MgSO_4$. The ether was evaporated and bulb-to-bulb distillation of the residue afforded the pure chloride (5.9 g, 78% yield, 1.72 atom deuterium), b.p. 110 - 115°C/30 Torr (lit.,[50] 179°C for nondeuteriated compound); δ (CCl_4) (60 MHz) 7.33 (5H, s, ArH) and 4.53 (0.29H, bs, residual CH_2).

Ethyl 2-acetamido-2-ethoxycarbonyl-3-phenyl-[3,3-$^2H_2$]propionate.- [methylene-$^2H_2$]Benzyl chloride was used to prepare this compound a according to the procedure described by Snyder et al.[61] for the nondeuteriated analogue. The crude solid was recrystallised from water to give white crystals (62% yield, 1.42 atom deuterium), m.p. 105 - 106°C (lit.,[61] 106°C for nondeuteriated compound) (Found: C, 62.0; H, 7.5; N, 4.3. Calc. for $C_{16}H_{19}D_2O_5N$: C, 62.1; H, 7.4; N, 4.5%); $\nu_{max.}$ (KBr) 3 280, 3 010, 2 995, 1 755 and 1 650 cm^{-1}; δ (CCl_4) (90 MHz) 7.16 (5H, m, ArH), 6.57 (1H, bs, NH), 4.28 (4H, q, $-OCH_2-$), 3.60 (0.60H, bs, residual CH_2Ph), 1.97 (3H, s, $COCH_3$) and 1.30 (6H, t, CH_2CH_3); m/e 309 (M), 279, 174 and 119.

DL-[3,3-$^2H_2$]Phenylalanine.- The [$^2H_2$]-acetamidomalonate derivative (5.3 g, 17 mmol) was heated in refluxing concentrated hydrochloric acid for 18 h. The solution was evaporated to dryness under reduced pressure, the residue dissolved in water and the solution

was taken to pH 6.5 with 5 M sodium hydroxide. Cooling of this solution afforded a white solid which was crystallised first from ethanol - water (2:1) to give a mixture (2.2 g), positive to ninhydrin, consisting of the amino-acid and sodium chloride. This mixture was recrystallised three times from water to give the pure amino-acid (1.6 g, 56% yield) as white plates, m.p. 215 - 217°C (dec.) [lit.,[50] 284 - 288°C (dec.), for the nondeuteriated compound] (Found: C, 64.9; H, 7.6; N, 8.4. Calc. for $C_9H_9D_2NO_2$: C, 64.7; H, 7.8; N, 8.4%); $\nu_{max.}$(KBr) 3 450, 3 200 - 2 500, 1 630, 1 595 and 1 500 cm^{-1}; δ (CF_3CO_2H) (90 MHz) 7.36 (5H, m, ArH) and 4.62 (1H, bs, α-CH).

cyclo-(DL-[3,3-$^2H_2$]Phe-DL-[3,3-$^2H_2$]Phe).- DL-[3,3-$^2H_2$]Phenylalanine (0.563 g, 3.4 mmol) was heated, under a nitrogen atmosphere, in refluxing ethylene glycol for 4 h, according to the method of Sannié.[62] On cooling to room temperature the solution deposited white crystals. They were filtered off, washed with cold methanol (2 x 5 ml) and diethyl ether (2 x 5 ml), and dried under vacuum. The cyclic dipeptide (319.5 mg, 63.1% yield) had m.p. 293 - 295°C (dec.); δ (CF_3CO_2H) (90 MHz) 8.07 (2H, bs, 2 x NH of cis-isomers), 7.78 (2H, bs, 2 x NH of trans-isomer), 7.33 and 7.11 (10H, m, ArH), 4.56 (2H, bs, α-CH of cis-isomers), 3.93 (2H, bs, α-CH of trans-isomer), 3.11 (1.6H, bm, residual β-CH) and 2.29 (0.3H, bm, residual β-CH); m/e 298 (M^+), 205 (M - $C_7H_5D_2$), 177 (M - $C_6H_5CD_2CHNH$), 122 ($H_2\overset{+}{N}$=CH-$CD_2C_6H_5$) and 93 ($C_7H_5D_2$).

APPENDIX

A. Studies involving radioactive isotopes

In the ^{14}C- and ^{3}H-biosynthetic studies described in this work, the incorporation (% I) of the radiolabelled precursor is given by the equation:

$$\% I = \frac{S_m}{S_p} \times \frac{M_m}{M_p} \times 100 \qquad (1)$$

where,

S_m: molar specific activity of metabolite

S_p: molar specific activity of precursor

M_m: number of moles of isolated metabolite

M_p: number of moles of administered precursor

The dilution (D) of the radiolabel in the biosynthetic transformation of precursor into metabolite is given by the equation:

$$D = \frac{S_p}{S_m} \qquad (2)$$

B. Studies involving ^{13}C-labelled precursors

(i) Terminology

In ^{13}C-biosynthetic experiments, information on the incorporation and dilution of the precursor is obtained from the enhancement (P) of

the ^{13}C-n.m.r. signals corresponding to the labelled sites. In single-site labelling experiments, the enhancement (P) of the site is given by the ratio:

$$P = \frac{i_{lab.}}{i_{nat.\ abund.}} \qquad (3)$$

where the $\underline{i}$'s denote peak heights, with $i_{lab.}$ standing for the height of the signal of the labelled centre in the spectrum of the enriched metabolite and $i_{nat.\ abund.}$ for the height of the same signal in the natural abundance spectrum. The value of $i_{nat.\ abund.}$ used in this calculation is obtained as follows. Excluding the signal of the labelled site, the intensity of each peak in the spectrum of the enriched metabolite is divided by the intensity of the corresponding peak in the natural abundance spectrum. The average of these ratios is then calculated and used to estimate what the peak intensity of the labelled site would have been, had it not been enriched above natural abundance. The value thus obtained is $i_{nat.\ abund.}$; $i_{lab.}$ is then measured directly from the spectrum of the enriched metabolite.

Results are commonly expressed as percentage ^{13}C-enrichments, that is, P.r, where r represents the natural abundance ^{13}C-content (1.1%). The dilution may, in turn, be obtained from the formula:

$$D = \frac{e - r}{Pr - r} \qquad (4)$$

where e is the percentage ^{13}C-enrichment of the precursor at the specific site of labelling. The value of e is usually obtained either from the supplier of the labelled precursor or by independent calculation

from n.m.r. or mass spectral data.

The validity of equation (4) can be demonstrated by examination of what goes on during a biosynthetic experiment. Let us assume that we administer A moles of a labelled precursor, having a percentage ^{13}C-enrichment value of e at a specific site, to an organism. In response to this, B moles of labelled metabolite, also having a percentage ^{13}C-enrichment value of e, will be produced by the organism, but they will be diluted with E moles of unlabelled, endogenous metabolite. The dilution (D) of the precursor is the ratio of the total moles of metabolite produced over the moles of metabolite derived exclusively from precursor, that is,

$$D = \frac{B + E}{B} \qquad (5)$$

The percentage ^{13}C-enrichment of the metabolite (P.r) will have contributions from the fraction of moles of metabolite possessing the same percentage ^{13}C-enrichment as the precursor and the fraction of moles of metabolite only having the ^{13}C-isotopic natural abundance. Hence,

$$P.r = \frac{B}{B + E} e + \frac{E}{B + E} r$$

$$= \frac{Be + Er}{B + E}$$

If we subtract r from both sides of this identity, we obtain:

$$P.r - r = \frac{Be + Er}{B + E} - r$$

$$= \frac{B(e - r)}{B + E}$$

Rearrangement of this last equation leads to equation (4).

(ii) ^{13}C-Experiment on Hyalodendron sp.

In the feeding of cyclo-(L-Phe-DL-[3-^{13}C]Ser) to Hyalodendron sp., the percentage ^{13}C-enrichment for this precursor at C-3 was determined to be ~90%, from mass spectral data. The percentage ^{13}C-enrichment of the isolated metabolite was found, upon measurement of P as described before, to be 3.0 x 1.1%. Thus, the dilution value for this experiment can be calculated by substituting the numerical values in equation (4).

$$D = \frac{90\% - 1.1\%}{3 \times 1.1\% - 1.1\%} = 40.4$$

(iii) $^{13}C^{15}N$-Experiment on A. terreus

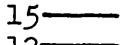
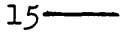
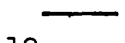
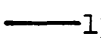
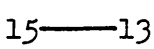
The composition of the precursor fed, listed in Table 3 [column (a)] was calculated as follows:

1. The isotopic compositions of the commercial [^{15}N]phenylalanine and [1-^{13}C]phenylalanine listed below were calculated based on the ^{15}N- and ^{13}C-enrichments of 96.5 and 91%, respectively, quoted by the suppliers. We shall designate n as the ^{15}N-enrichment and r as the ^{13}C-enrichment. Thus, for the commercial [^{15}N]phenylalanine, n = 0.965 and r = 0.011, and for commercial [1-^{13}C]phenylalanine, n = 0.0037 and r = 0.91. By the same token, for unlabelled phenylalanine, n = 0.0037 and r = 0.011.

SPECIES	FORMULA	RELATIVE ABUNDANCE in [^{15}N]Phe	in [1-^{13}C]Ph	in Phe
15——	n(1 - r)	0.9544	0.0003	0.0037
——	(1 - n)(1 - r)	0.0346	0.0897	0.9853
15——13	nr	0.0106	0.0034	0.00004
——13	r(1 - n)	0.0004	0.9066	0.0110

2. The composition of a dioxopiperazine synthesised by coupling two amino acids can be obtained by construction of a 4 x 4 matrix using the sets of values listed above. Because the matrix will be symmetrical about its main diagonal, 12 of the 16 combinations obtained will really be 6 sets of mirror images. Pictorially, $\overset{15——}{13——}$ represents the same dioxopiperazine as $\overset{——15}{——13}$. Thus, to obtain the composition of the $^{13}C,^{15}N$-labelled dioxopiperazine, we use the values calculated for [^{15}N]phenylalanine and [1-^{13}C]phenylalanine, whereas for cyclo-(L-[U-^{14}C]Phe-L-Phe) we use the set of values obtained for unlabelled phenylalanine. However, the two dioxopiperazines were present in different amounts in the total mixture fed, 6.5 mg corresponding to the doubly labelled LL-dioxopiperazine and 5.5 mg corresponding to the ^{14}C-labelled dioxopiperazine. Therefore, the percentage abundance of each species in the $^{13}C^{15}N$- and ^{14}C-labelled dioxopiperazines must be multiplied by 6.5/12 and 5.5/12, respectively, before they are added to give the final composition of the LL-cyclo-dipeptide administered.

As discussed in Section 3.3, only species C and H will contribute to the central amide peak in the ^{13}C-n.m.r. spectrum of BDA, when the

		$^{13}C,^{15}N$-LABELLED DIOXOPIPERAZINE	^{14}C-LABELLED DIOXOPIPERAZINE	ADMINISTERED DIOXOPIPERAZINE MIXTURE
A		86.52	0.008	46.87
B		8.56	0.73	4.97
C		3.14	2.17	2.69
D		0.96	-[a]	0.52
E		0.32	-[a]	0.17
F		0.31	97.08	44.66
G		0.11	0.008	0.06
H		0.04	0.001	0.02
I		0.03	0.004	0.02
J		0.004	-[a]	0.002

[a] less than 0.0001

latter is obtained after a 6.0 dilution of the administered precursor. Moreover, species H will contribute twice, since it possesses two ^{13}C-^{14}N units. Rearrangement of equation (4), gives:

$$P = \frac{e - r}{rD} + 1$$

Therefore, since:

$$e = 2.69\% + 2(0.002\%) = 2.73\%$$

$$D = 6.0$$

$$P = 1.25$$

Using equation (3) and the height of amide peak (4.71 cm) expected from exclusively a natural abundance ^{13}C-contribution, we obtain:

$$i_{lab.} = P \times i_{nat.\ abund.}$$

$$= 5.89 \text{ cm}$$

If we now assume that there has been complete dissociation to give phenylalanine, we discover that the relative abundances of each amino-acid species have become:

SPECIES	RELATIVE ABUNDANCE
15——	0.2603
——	0.4852
15—— 13	0.0038
—— 13	0.2506

Combination of these values, as previously explained, in a 4 x 4 matrix, yields a new set of percentage abundances for the dioxopiperazine that would result from random recombination as discussed in Section 3.3, and listed in Table 3 [column (b)].

	SPECIES	PERCENTAGE ABUNDANCE		SPECIES	PERCENTAGE ABUNDANCE
A	15—— 13——	13.04	F	—— ——	23.54
B	15—— ——	25.25	G	—— 13——15	0.36
C	—— 13——	24.32	H	——13 13——	6.28
D	15——13 13——	0.19	I	15—— ——15	6.77
E	15—— 13——15	0.14	J	15——13 13——15	0.001

Again, only species C and H will contribute to the central amide peak, this time with e = 36.88%. However, dissociation has only occurred to a certain fractional extent, x. The value of x may be calculated as follows.

$$e = 2.73(1 - x) + 36.88x$$
$$= 2.73 + 34.15x$$

and $D = 6.0$

$$\therefore P = 1.25 + 5.17x$$

And, consequently,

$$i_{lab.} = 4.71(1.25 + 5.17x)$$
$$= 5.89 + 24.35x$$

Recalling that the value for $i_{lab.}$ measured directly from the ^{13}C-n.m.r. spectrum (Figure 3) is, after correction for overlap, 7.10 cm, the identity:

$$i_{lab.} = 7.10 = 5.89 + 24.35x$$

can be solved for x,

$$x = 4.97\%$$

Hence, as discussed in Section 3.3, at least 95% of the LL-cyclodipeptide is incorporated intact.

C. Studies involving ^{2}H-labelled precursors

The percentage abundances and errors for the various deuteriated species listed in Table 4 were calculated as follows. Three scans

of the spectrum of cyclo-(DL-[3,3-$^2H_2$]Phe-DL-[3,3-$^2H_2$]Phe) were obtained in an MS-12 mass spectrometer. In each scan, the heights of the peaks (for $M^{+\cdot}$) at m/e = 294, 295, 296, 297 and 298 (corresponding to the $^2H_0$-, $^2H_1$-, $^2H_2$-, $^2H_3$- and $^2H_4$-dioxopiperazines) were measured (with a ruler) and the heights obtained were normalized to a total height of unity. The mean ($\bar{x}$) of the three values obtained for each species was calculated, along with the standard error (standard deviation from the mean), according to the equations:

$$\text{MEAN} = \bar{x} = \frac{x_1 + x_2 + \ldots + x_n}{n}$$

$$\text{STANDARD ERROR} = \sqrt{\frac{\sum |\bar{x} - x|^2}{n(n-1)}}$$

The spectrum of a sample of unlabelled cyclo-(DL-Phe-DL-Phe) was obtained under the same conditions and, from the heights measured, the M + 1/M and M + 2/M corrections were calculated to be 0.22 and 0.04 respectively. These values agreed with those reported in mass spectral tables. The mean and standard error for each species in the deuteriated cyclodipeptide were adjusted, taking account of these corrections, and then renormalized to give the percentage abundances and standard errors listed in Table 4.

The values listed in Table 5 [column (a)] were obtained in a similar way, with the following modifications: (i) thirty scans of the MS-9 spectrum of the isolated deuteriated BDA were used to calculate the mean intensity and standard error for the fragments of m/e = 487, 488, 489, 490 and 491, corresponding to the five ($^2H_0$ to $^2H_4$) species of the $[M - SMe]^{+\cdot}$ fragment ion; (ii) thirty

scans of the MS-9 spectrum of unlabelled BDA were used to calculate the P + 1/P and P + 2/P corrections, which were 0.27 and 0.10, respectively. No tabulated values were available for comparison.

The values in Table 5 [column (b)] were calculated using Table 4 and the dilution value of 7.4 ± 0.21 obtained by means of the ^{14}C-tracer. Since deuterium has a natural abundance of 0.016%, both the $^{2}H_0$- and $^{2}H_1$-species suffer the influence of the dilution by endogenous material. Thus, 6.36 ± 0.21 and 0.027 ± 0.0009 were added, respectively, to the percentage abundances of the $^{2}H_0$- and $^{2}H_1$-species and then the abundances of all the species and their errors were normalized to a total of 100.

The total percentages of deuterium in the isolated and predicted BDA were calculated by adding the products of multiplying the percentage abundance of each species by the number of deuterium atoms it contains, and normalizing the sum to a percentage. Thus,

	PREDICTED BDA	ISOLATED BDA
$^{2}H_0$	0	0
$^{2}H_1$	1.80 ± 0.04	1.55 ± 0.23
$^{2}H_2$	7.60 ± 0.06	3.02 ± 0.32
$^{2}H_3$	10.42 ± 0.04	13.29 ± 0.54
$^{2}H_4$	9.84 ± 1.04	5.88 ± 0.44
Total ^{2}H atoms	29.66 ± 1.04	23.74 ± 0.80
Total % ^{2}H	7.42 ± 0.26	5.94 ± 0.20

The loss of deuterium can be calculated from these values:

$$\text{Loss of deuterium} = \frac{7.42 - 5.94}{7.42} \times 100 = 19.95 \pm 0.23\%$$

PART II

REFERENCES

1. R.Weindling and O.Emerson, Phytopath., 1936, 26, 1068.
2. A.Taylor, in "Microbial Toxins", eds. S. Kadis and A.Ciegler, Academic Press, London and New York, 1971, vol VII, p. 337.
3. P.G.Sammes, in "Progress in the Chemistry of Organic Natural Products", eds. W.Herz, H.Grisebach and G.W.Kirby, Springer-Verlag, Vienna, 1975, vol XXXII, p. 51.
4. C.Leigh and A.Taylor, in "Advances in Chemistry Series", ed. J.V. Rodricks,American Chemical Society, Washington D.C., 1976, no. 149, p. 228.
5. G.W.Kirby, Pure Appl. Chem., 1979, 51, 705.
6. G.W.Kirby and D.J.Robins, in "The Biosynthesis of Mycotoxins - A Study in Secondary Metabolism", ed. P.S.Steyn, Academic Press, New York, 1980, p. 301.
7. J.C.MacDonald and G.P.Slater, Can. J. Biochem., 1975, 53, 475.
8. J.D.Bu'Lock and C.Leigh, J. Chem. Soc., Chem. Commun., 1975, 628.
9. G.W.Kirby, G.L.Patrick and D.J.Robins, J.Chem. Soc.,Perkin Trans. 1, 1978, 1336.
10. R.A.Behling and A.G.Fischer, Int. J. Biochem., 1980, 11, 457.
11. M.J.Varley, Ph.D. Thesis, Loughborough University of Technology, 1975.
12. P.J.Curtis, D.Greatbanks, B.Hesp, A.Forbes-Cameron and A.A.Freer, J. Chem. Soc. Perkin Trans. 1, 1977, 180.
13. J.P.Ferezou, A.Quesneau-Thierry, C.Servy, E.Zissman and M.Barbier, J. Chem. Soc. Perkin Trans. 1, 1980, 1739.
14. G.M.Strunz, M.Kakushima, M.A.Stillwell and C.J.Heissner, J. Chem. Soc. Perkin Trans. 1, 1973, 2600.

15. M.A.Stillwell, L.P.Magasi and G.M.Strunz, Can. J. Microbiol., 1974, 20, 759.

16. G.M.Strunz, C.J.Heissner, M.Kakushima and M.A.Stillwell, Can. J. Chem., 1974, 52, 325.

17. R.L.De Vault and W.Rosenbrook, J. Antibiotics, 1973, 26, 532.

18. F.Dorn and D.Arigoni, Experientia, 1974, 30, 134.

19. K.H.Michel, M.O.Chaney, N.D.Jones, M.M.Hoehn and R.Nagarajan, J. Antibiotics, 1974, 27, 57.

20. G.M.Strunz, M.Kakushima and M.A.Stillwell, Can. J. Chem., 1975, 53 295.

21. F.R.Ahmed and M.Przybylska, Acta Crystallogr., 1977, 33B, 168.

22. G.M.Strunz and M.Kakushima, Experientia, 1974, 30, 719.

23. Y.Kishi, T.Fukuyama and S.Nakatsuka, J. Am. Chem. Soc., 1973, 95, 6492.

24. Y.Kishi, S.Nakatsuka, T.Fukuyama and M.Havel, J. Am. Chem. Soc., 1973, 95, 6493.

25. T.Fukuyama, S. Nakatsuka and Y.Kishi, Tetrahedron Lett., 1976, 3393.

26. R.M.Williams and W.H.Rastetter, J. Org. Chem., 1980, 45, 2625.

27. G.L.Patrick, PhD. Thesis, University of Glasgow, 1979.

28. G.L.Patrick, Honours B.Sc. Thesis, University of Glasgow, 1976.

29. R.R.Talekar, unpublished results.

30. J.R.Coggins and N.L.Benoiton, Can. J. Chem., 1971, 49, 1968.

31. J.R.McDermott and N.L.Benoiton, Can. J. Chem., 1973, 51, 1915.

32. R.Nagarajan, L.L.Huckstep, D.H.Lively, D.C.De Long, M.M.Marsh and N.Neuss, J. Am. Chem. Soc., 1968, 90, 2980.

33. N.Neuss, R.Nagarajan, B.B.Molloy and L.L.Huckstep, Tetrahedron Lett., 1968, 4467.

34. P.A.Miller, P.W.Trown, W.Fulmor, G.O.Morton and J.Karliner, Biochem. Biophys. Res. Commun., 1968, 33, 219.

35. D.B.Cosulich, N.R.Nelson and J.H. van den Hende, J. Am. Chem. Soc., 1968, 90, 6519.

36. D.C.De Long, D.H.Lively and N.Neuss, U.S.P. 3,745,158/1973; Chem. Abs., 1970, 73, P108254g.

37. P.A.Miller and P.W.Trown, U.S.P. 3,701,774/1972; Chem. Abs., 1973, 78, P56432v.

38. N.Neuss, L.D.Boeck, D.R.Brannon, J.C.Cline, D.C.De Long, M.Gorman, L.L.Huckstep, D.H.Lively, J.Mabe, M.M.Marsh, B.B.Molloy, R.Nagarajan, J.D.Nelson and W.M.Stark, Antimicrob. Agents Chemother., 1968, 213.

39. R.Nagarajan, N.Neuss and M.M.Marsh, J. Am. Chem. Soc., 1968, 90, 6518.

40. J.W.Moncrief, J. Am. Chem. Soc., 1968, 90, 6517.

41. D.R.Brannon, J.A.Mabe, B.B.Molloy and W.A.Day, Biochem. Biophys. Res. Commun., 1971, 43, 588.

42. J.D.Bu'Lock,and A.P.Ryles, Chem. Commun., 1970, 1404.

43. N.Johns, G.W.Kirby, J.D.Bu'Lock and A.P.Ryles, J. Chem. Soc., Perkin Trans. 1, 1975, 383.

44. D.L.Coffen, D.A.Katonak, N.R.Nelson and F.D.Sancilio, J. Org. Chem., 1977, 42, 948.

45. K.F.Koch, personal cummunication.

46. D.Brewer, A.G.McInnes, D.G.Smith, A.Taylor, J.A.Walter, H.R.Loosli and Z.L.Kis, J. Chem. Soc., Perkin Trans. 1, 1978, 1248.

47. R.Rahman, S.Safe and A.Taylor, J. Chromatogr., 1970, 53, 592.

48. H.J.Issaq and E.W.Barr, J. Chromatogr., 1977, 132, 121.

49. E. von Arx and R.Neher, J. Chromatogr., 1963, 12, 329.

50. Handbook of Chemistry and Physics, ed. R.C.Weast, C.R.C. Press, Cleveland, Ohio, 1974, 55th edition.

51. S.Guttman and R.A.Boisonnas, Helv, Chim. Acta, 1958, 41, 1852.

52. E.Boer and J.Muurukas, J. Biol. Chem., 1955, 212, 25.

53. J.P.Greenstein and M.Winitz, "Chemistry of the Amino Acids", John Wiley and Sons Inc., New York, 1961, vol. II, p. 894.

54. J.R.Rachele, J. Org. Chem., 1963, 28, 2898.

55. J.P.Greestein and M.Winitz, "Chemistry of the Amino Acids", John Wiley and Sons Inc., New York, 1961, vol. II, p. 930.

56. M.Goodman and W.J.McGahren, Tetrahedron, 1967, 23, 2031.

57. W.Grassman and E.Wünsch, Chem. Ber., 1958, 91, 462.

58. Cf. J.Feeney and A.Heinrich, Chem Commun., 1966, 295.

59. H.R.Kricheldorf, Org. Magn. Reson., 1980, 13, 52.

60. D.J.Bennett, G.W.Kirby and V.A.Moss, J. Chem. Soc (C), 1970, 2049.

61. H.R.Snyder, J.F.Shekleton and C.D.Lewis, J. Am. Chem. Soc., 1945, 67, 310.

62. M.C.Sannié, Bull. Chim. Soc., 1942, 9, 487.