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THE BIOSYNTHESIS OF
SULPHUR-CONTAINING DIOXOPIPERAZINES

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

by

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(Of Scotland)

"That knuckle-end of England—that land of Calvin, oat-cakes,
and sulphur."

Rev. Sydney Smith

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SUMMARY

This thesis records an investigation into the biosynthesis of the aranotins, a group of sulphur-containing dioxopiperazine metabolites produced by the fungi Aspergillus terreus and Amauroascus aureus.

A series of analogues of the known aranotin precursor, cyclo-(L-phenylalanyl-L-phenylalanyl), fluorinated on one or both aromatic rings, was prepared, both unlabelled and labelled with ^{14}C . These compounds were fed to Aspergillus terreus, as were o-, m-, and p-fluorophenylalanines, and their metabolism was followed by ^{19}F n.m.r. spectroscopy of the extracts. A fluorinated bisdethiodi(methylthio)-acetylpoaranotin was isolated from cultures which had been fed DL-p-fluorophenylalanine or cyclo-(L-phenylalanyl-L-p-fluorophenylalanyl).

A new natural metabolite of A. terreus, cis- α,α' -di(methylthio)-cyclo-(phenylalanyl-phenylalanyl), of unknown absolute configuration, was isolated and characterised. Several fluorinated analogues of this compound were detected spectroscopically in extracts which had been fed fluorinated precursors.

cyclo-(L-Phenylalanyl-L-p-fluorophenylalanyl), stereospecifically monodeuteriated at the methylene group of the p-fluorophenylalanyl residue, was fed to A. terreus, and it was shown that neither the 3R - nor the 3S -deuterium was lost obligatorily during the conversion of this compound into cis- α,α' -di(methylthio)-cyclo-(phenylalanyl-p-fluorophenylalanyl), although there was a partial loss from each position.

The natural product, cis- α,α' -di(methylthio)-cyclo-(phenylalanyl-phenylalanyl), was synthesised as a racemate. The compound, labelled with ^{35}S , was fed to A. terreus, and was not significantly incorporated into bisdethiodi(methylthio)acetylaranotin. The racemic epidisulphide,

α, α' -epidithio-cyclo-(phenylalanyl-phenylalanyl), was synthesised. This compound, doubly labelled with ^3H and ^{35}S , was fed to A. terreus, and was incorporated into acetylaranotin and bisdethiodi(methylthio)-acetylaranotin, with some changes in the isotopic ratios. An "intermediate trapping" experiment confirmed the ability of the fungus to biosynthesise α, α' -epidithio-cyclo-(phenylalanyl-phenylalanyl), of unknown absolute configuration, from L-phenylalanine.

Abbreviations used in the text

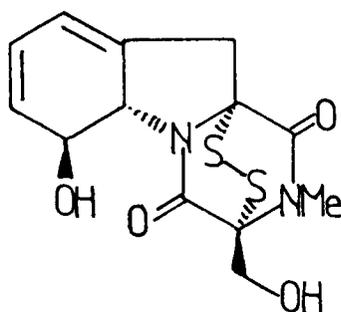
n.m.r.	=	nuclear magnetic resonance
p.p.m.	=	parts per million
t.l.c.	=	thin layer chromatography
u.v.	=	ultraviolet
i.r.	=	infrared
BDA	=	bisdethiodi(methylthio)acetylaranotin (40)
BDAA	=	bisdethiodi(methylthio)acetylapanotin (41)
DMF	=	<u>N,N</u> -dimethylformamide
DMSO	=	dimethylsulphoxide
LDA	=	lithium di-isopropylamide
THF	=	tetrahydrofuran
TFA	=	trifluoroacetic acid
Phe	=	phenylalanyl
Ala	=	alanyl
Gly	=	glycyl
Ser	=	seryl
Trp	=	tryptophyl
Tyr	=	tyrosyl
Pro	=	prolyl

Deuterium is represented by D.

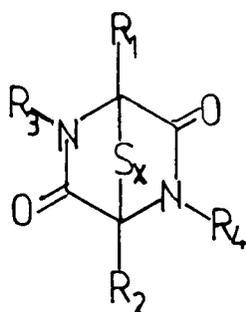
CHAPTER 1

INTRODUCTION1.1 General

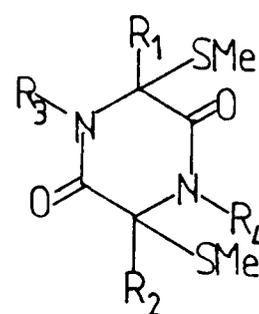
In 1936, Weindling and Emerson¹ isolated a toxic compound from Gliocladium fimbriatum (Trichoderma viride) which they named gliotoxin. Its structure, finally determined in 1958 by Bell et al.,² contains the sulphur-bridged dioxopiperazine moiety (2), which is a characteristic of a number of fungal metabolites discovered since. There are now ca. 40 members of the family, containing either the sulphur-bridged ring (2) or the closely related cis-di(methylthio)-dioxopiperazine ring system (3). Some other metabolites appear to be the products of rearrangement of epidithiodioxopiperazines.^{3,4,5}



(1)



(2); x = 2, 3, or 4



(3)

Several reviews have been published concerning the structures, chemistry, biosynthesis, and biological activity of these compounds.^{6,7,8} The biosynthetic studies up to 1980 have been reviewed by Kirby and Robins,⁹ and since this review, three new sulphur-containing dioxopiperazines have been reported.^{3,4,10}

The toxicology of this class of compounds has been the subject of much interest, and has been reviewed by Taylor.¹¹ The epidi-, tri-, and tetra-sulphides show pronounced antiviral activity, and are also toxic to fungi, bacteria, and mammals. The di(methylthio)- compounds show

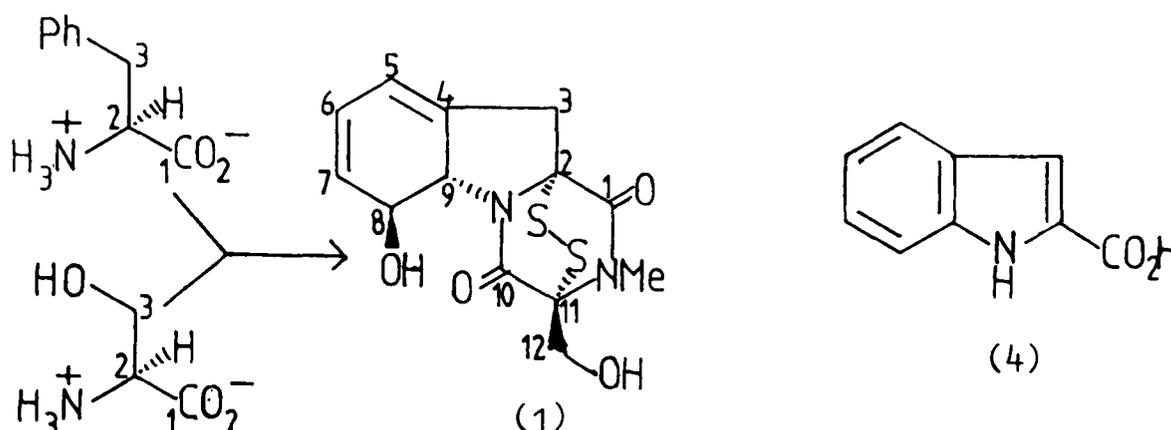
no toxicity. The mechanism of action of the epipolysulphides is not known, but they are believed to interfere with DNA transcription.

All the sulphur-containing dioxopiperazines isolated to date from natural sources have been found in fungi, and their biosynthetic procedures are discussed below (Section 1.2). There is another producer of these compounds, however, *i.e.* Homo sapiens⁸, and his methods are detailed in Section 1.3.

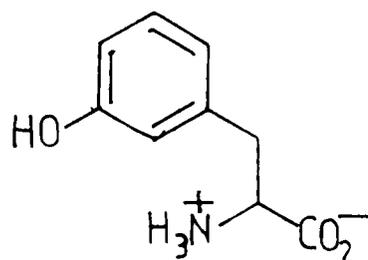
1.2 Biosynthesis of sulphur-containing dioxopiperazines

1.2.1 Biosynthesis of gliotoxin (1)

Because gliotoxin was the first epidithiodioxopiperazine to be discovered, most of the earlier biosynthetic investigations were concerned with it. Examination of the structure of gliotoxin (1) suggests a derivation from two amino-acids, the "lower half" (C-10 - C-12) resembling serine, and the "upper half" (C-1 - C-9) a modified aromatic amino-acid such as phenylalanine or *m*-tyrosine. Accordingly, in 1958 Suhadolnik and Chenoweth¹² fed DL-[1-¹⁴C] phenylalanine and DL-[2-¹⁴C] phenylalanine to T. viride and observed significant incorporations into gliotoxin (8.4 and 12.4% respectively). Degradation of the metabolite obtained from feeding DL-[1-¹⁴C] phenylalanine located the label at the C-1 carbonyl group (82% of the total activity), confirming that the amino-acid was incorporated intact and without rearrangement. Similarly,¹³ DL-[1-¹⁴C] serine was incorporated (1.9%)



into gliotoxin, degradation showing that the label resided in neither the N-methyl group nor the indole-2-carboxylic acid (4) derived from the phenylalanine portion. However, when DL-[3-¹⁴C]serine was fed, the gliotoxin isolated contained activity (25% of the total) in the N-methyl group. This is consistent with transfer of the serine methylene group to the C₁ pool of the organism, and in agreement with this result, DL-[methyl-¹⁴C]methionine was found to be a more efficient methyl source. This group also reported¹³ a large incorporation (44.3%) of DL-[U-³H]-m-tyrosine (5) into gliotoxin. Several attempts to repeat this observation have failed,^{14,15,16} and m-tyrosine is not now considered to be a precursor.



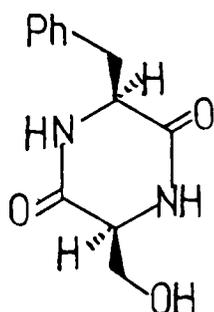
(5)

Bose et al.^{17,18} investigated the origin of the nitrogen atoms in the dioxopiperazine nucleus. It was demonstrated that [¹⁵N]glycine, [¹⁵N]aspartic acid, and [¹⁵N]glutamic acid could all act as sources of the nitrogen atoms. Although DL-[¹⁵N]phenylalanine gave gliotoxin with a large proportion of the label in the expected position, when doubly labelled DL-[1-¹⁴C, ¹⁵N]phenylalanine was used the ¹⁵N was diluted to a greater extent than the ¹⁴C label. These results suggest that deamination and re-amination of the amino-acid occur readily. When optically active phenylalanines were fed,¹⁸ it was found that D-[1-¹⁴C]phenylalanine was incorporated almost as efficiently as L-[1-¹⁴C]phenylalanine. Thus it appears that inversion of the chiral centre of phenylalanine can take place easily, possibly by the deamination-reamination process

mentioned above.

In 1975, MacDonald and Slater¹⁹ investigated the possibility that the cyclic dipeptide cyclo-(L-Phe-L-Ser) (6) was an intermediate on the biosynthetic pathway. However, on observing only a low incorporation of cyclo-(L-[1-¹⁴C]Phe-L-Ser) into gliotoxin by Penicillium terlikowskii, they concluded that the cyclic dipeptide (6) was not a free intermediate. Their results were not in accord with those of Bu'Lock and Leigh,²⁰ who reported a high incorporation (21%) of activity from a mixture of cyclo-(L-[Ar-³H]Phe-L-[1-¹⁴C]Ser) and cyclo-(L-[Ar-³H]Phe-D-[1-¹⁴C]Ser) into gliotoxin by Trichoderma viride. Bu'Lock and Leigh proposed that the poor incorporation by P. terlikowskii could be explained by the large amount of cyclo-(L-Phe-L-Ser) fed to the organism.

Kirby et al.²¹ fed all four stereoisomers of [¹⁴C]-cyclo-(Phe-Ser) separately to T. viride (Gliocladium deliquescens), and found that only the LL- isomer was efficiently incorporated (48%). Feeding of cyclo-(L-[4'-³H]Phe-L-[3-¹⁴C]Ser) gave gliotoxin with conservation of the ³H:¹⁴C isotopic ratio, and hydrolysis gave inactive methylamine. These results suggest that cyclo-(L-Phe-L-Ser) (6) is metabolised to gliotoxin without separation of the two amino-acid residues, and that the methylene group of the seryl residue is not exchanged with the C₁ pool (as it was with the free amino-acid). Also, an "intermediate trapping" experiment demonstrated the presence of cyclo-(L-Phe-L-Ser) in the organism; cyclo-(L-Phe-L-Ser) was added to the cultures, followed 2 h later by L-[U-¹⁴C]phenylalanine. The re-isolated cyclo-(L-Phe-L-Ser)



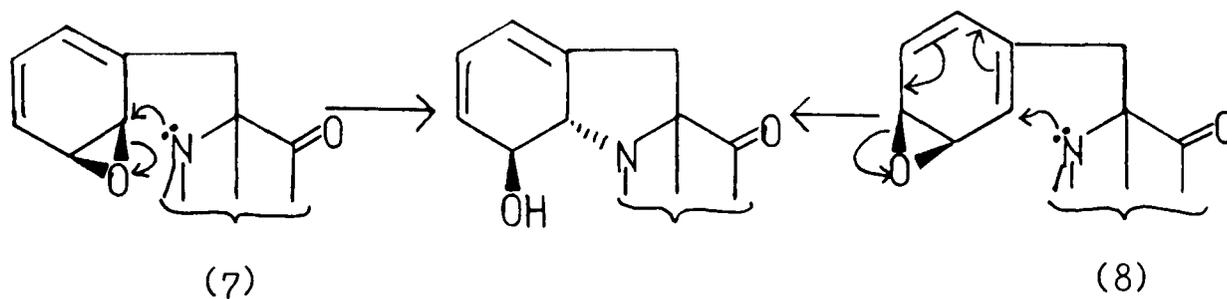
(6)

contained 1.3% of the activity fed, showing that cyclo-(L-Phe-L-Ser) is synthesised in the organism from L-phenylalanine.

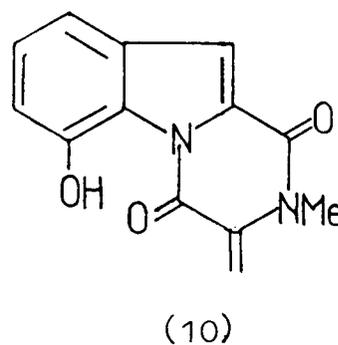
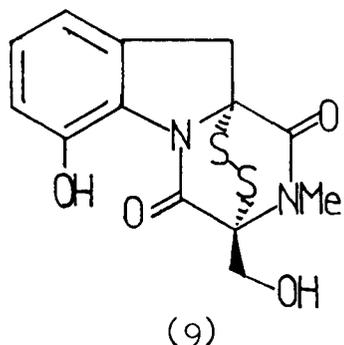
Following cyclic dipeptide formation, there remain at least three transformations necessary to produce gliotoxin, i.e. oxidative cyclisation of nitrogen with the phenyl group, N-methylation, and introduction of the disulphide bridge. The relative order of these steps is unknown. The S-methyl group of methionine has been shown to be incorporated efficiently into gliotoxin,¹² suggesting a relationship with other biological methylations.

It is now fairly well established that m-tyrosine (5) is not a precursor for gliotoxin. Bu'Lock and Ryles¹⁴ fed DL-[2',3',4',5',6',-D₅]phenylalanine to T. viride and found no significant loss of deuterium. Similarly, Johns and Kirby¹⁵ found that DL-[1-¹⁴C, 3'-³H]-phenylalanine was converted into gliotoxin by T. viride with no loss of tritium. The label was shown to be located equally at C-6 and C-8 of gliotoxin by degradations. Also, the possibility that migration to a neighbouring carbon atom was taking place, followed by return to the original site with loss of hydrogen, was discounted by feeding DL-[1-¹⁴C, 2'-³H]phenylalanine and DL-[1-¹⁴C, 4'-³H]phenylalanine; these were incorporated into gliotoxin with no loss of tritium.

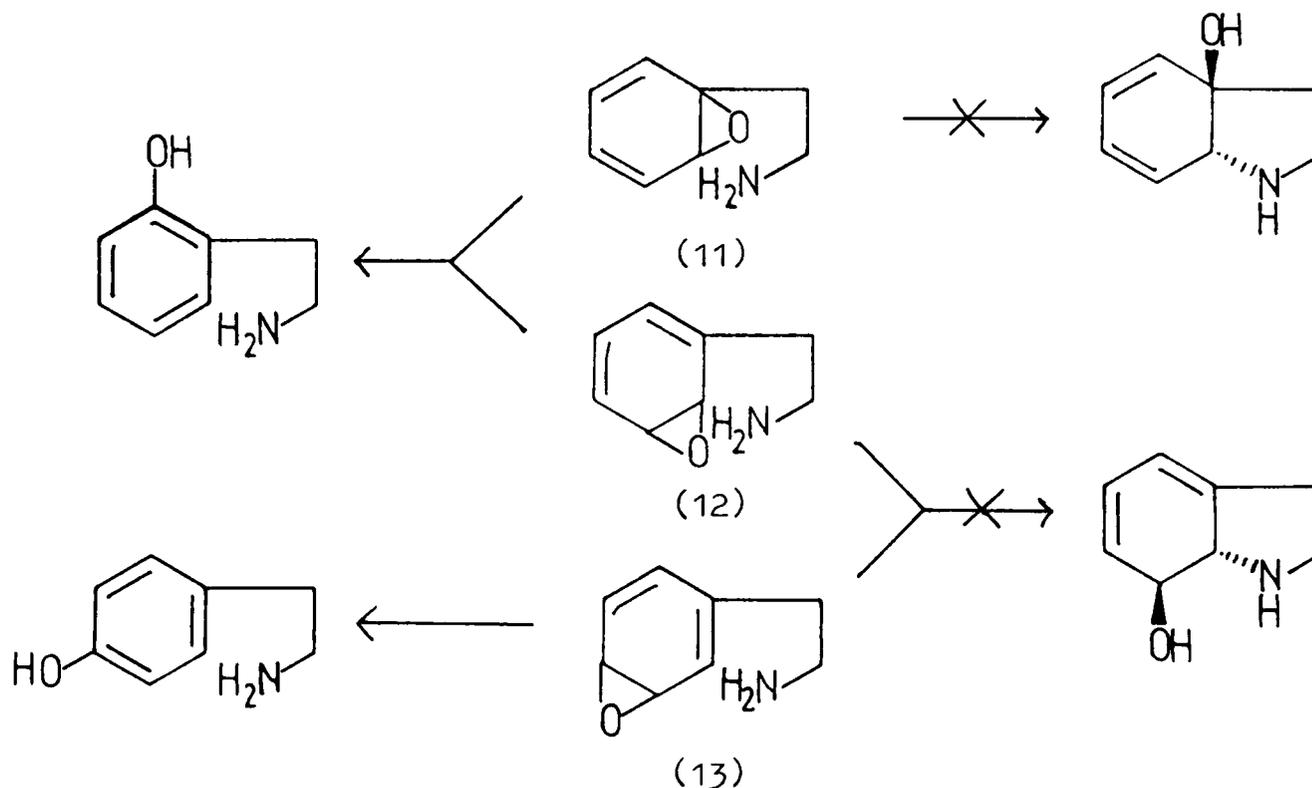
At present, the most popular explanation of the formation of the cyclohexadienol ring system involves the intermediacy of an arene oxide which is opened by nucleophilic attack of nitrogen. Both 2',3'-



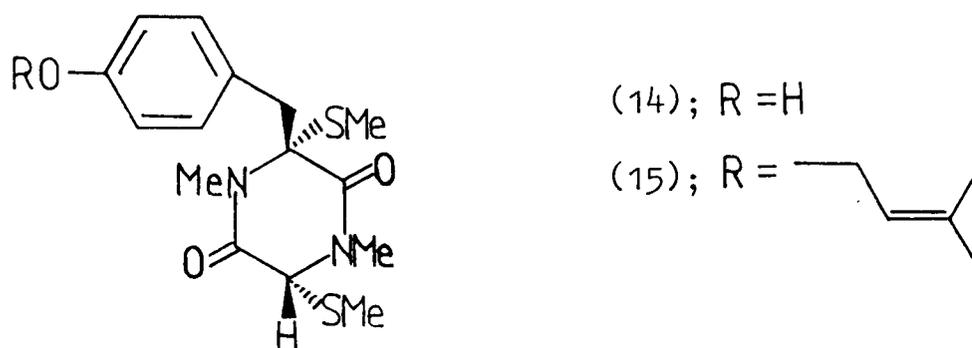
and 3',4'-epoxides (7) and (8) are possible intermediates, although the 3',4'-arene oxide is not a suitable intermediate in arantoin biosynthesis (see below) and attack by nitrogen on this compound would be disfavoured by Baldwin's rules (5-endo-trig).²² This mechanism accounts for all the experimental results except those where phenolic precursors have been incorporated, *i.e.* *m*-tyrosine (5)¹³ and the gliotoxin derivatives (9) and (10).²³ The weight of evidence in favour of an arene oxide intermediate must bring the validity of these results into question.



Rastetter *et al.* demonstrated the feasibility of nucleophilic attack on arene oxides by amines *in vitro*,²⁴ although the model compounds (11), (12), and (13) did not cyclise under their conditions,²² rearrangement to the phenols taking place.

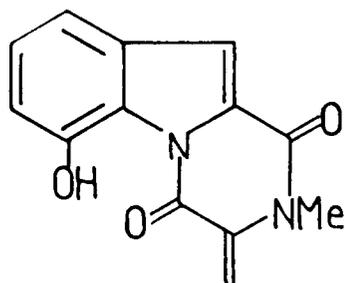


In connection with this proposed mechanism, Hanson and O'Leary²⁵ isolated two new metabolites (14) and (15), from Gliocladium deliquescens. They pointed out that the para-oxygen function could have arisen from a 3',4'-arene oxide, but not from a 2',3'-oxide. However, oxidation of the aromatic ring in these compounds may be unrelated to the oxidation in gliotoxin biosynthesis.



Little is known to date as to the mechanism of sulphur introduction. Neuss et al.²⁶ found that L-[³⁵S]methionine gave a good incorporation of sulphur into gliotoxin by P. terlikowsii (10.2%) and T. viride (3.3%). When unlabelled L-cysteine was added in 1000-fold excess to cultures of T. viride fed L-[³⁵S]methionine, gliotoxin production was more than doubled, and the incorporation of ³⁵S was reduced to 0.1%, suggesting that cysteine can also act as a sulphur source for gliotoxin production.

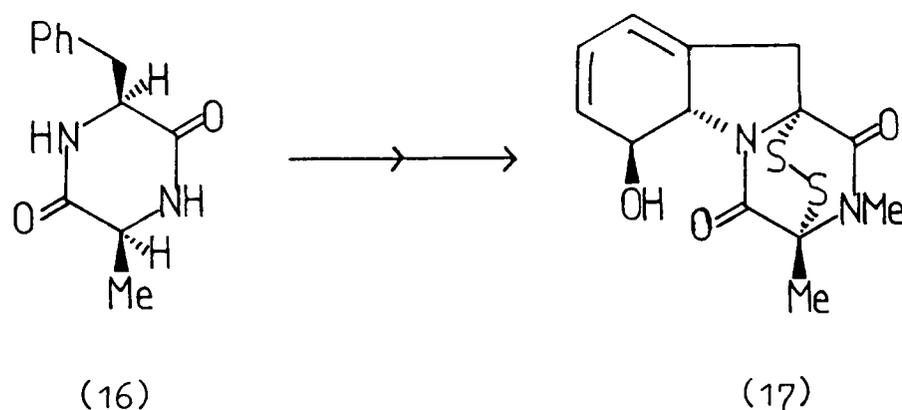
Unsaturated derivatives of cyclo-(Phe-Ser) such as (10) have been proposed as possible precursors to gliotoxin.⁸ When phenylalanine, tritiated or deuteriated at the methylene group, was fed²⁷ to T. viride, (G. deliquescens) and incorporated into gliotoxin, there was a stereo-



(10)

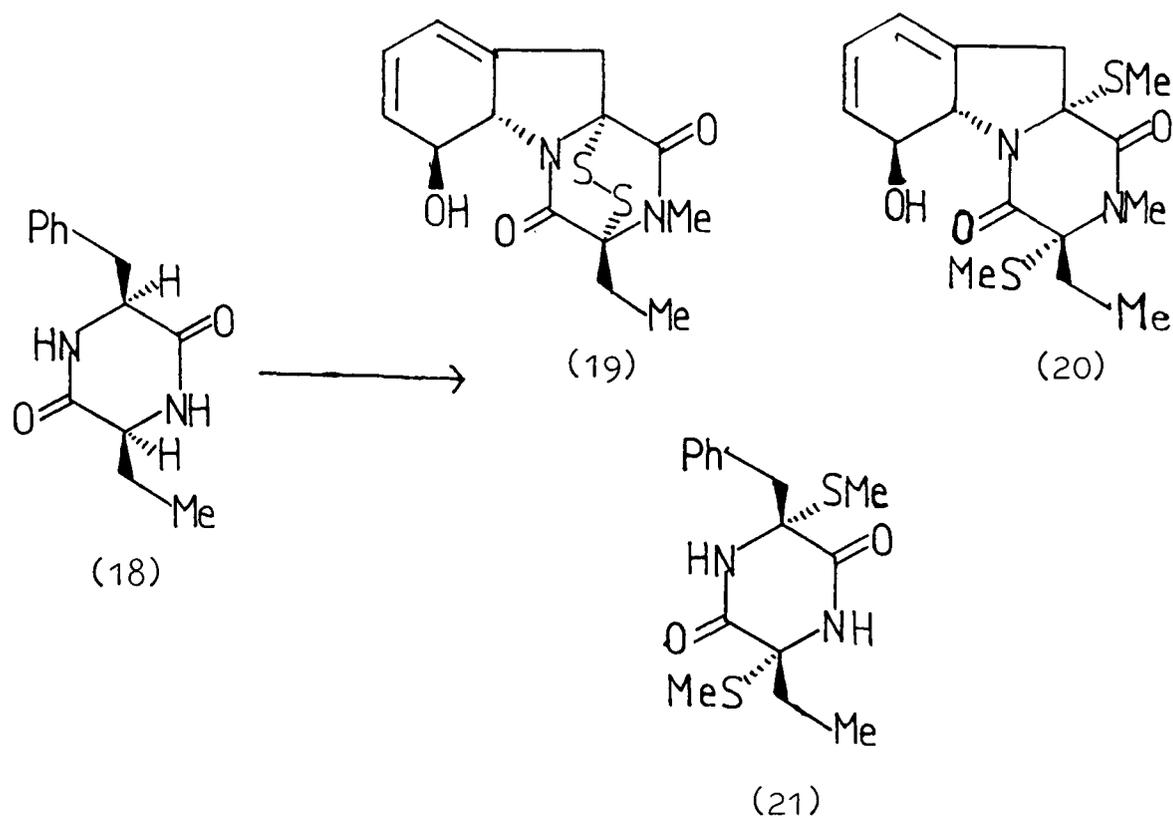
selective loss of label, e.g. tritium was lost from [$3R$ - 3H]phenylalanine but retained in [$3S$ - 3H]phenylalanine. When [3 - D_2]phenylalanine was fed, the gliotoxin isolated contained, besides unlabelled material, mainly monodeuteriated compound. However, small amounts of dideuterio-gliotoxin were present, eliminating olefinic derivatives (in the phenylalanine moiety) as obligatory intermediates. The predominance of the monodeuteriated species could be attributed to a stereospecific exchange of the $3R$ -hydrogen in L-phenylalanine, which was found to have taken place in the amino-acid recovered from the mycelial proteins.²⁷ Thus loss of the label could have occurred before formation of the cyclic dipeptide.

Structural analogues of the natural precursors have been fed to G. deliquescens. cyclo-(L-Phe-L-Ala) (16) was incorporated with high efficiency (ca. 40%) into the gliotoxin analogue (17).²⁸ This indicates that the C-12 hydroxyl group is not involved in an essential part of the biosynthetic route.



Another analogue of cyclo-(Phe-Ser), cyclo-(L-Phe-L- α -amino-buteryl) (18) was fed to G. deliquescens, and incorporation into three metabolites was observed.²⁹ The structures of two of these compounds were tentatively assigned as the gliotoxin analogue (19), and the bisdethiodi(methylthio)-analogue (20). The third compound was assigned

the structure (21), and this has been confirmed by synthesis of the racemate.³⁰ The isolation of this metabolite suggests that

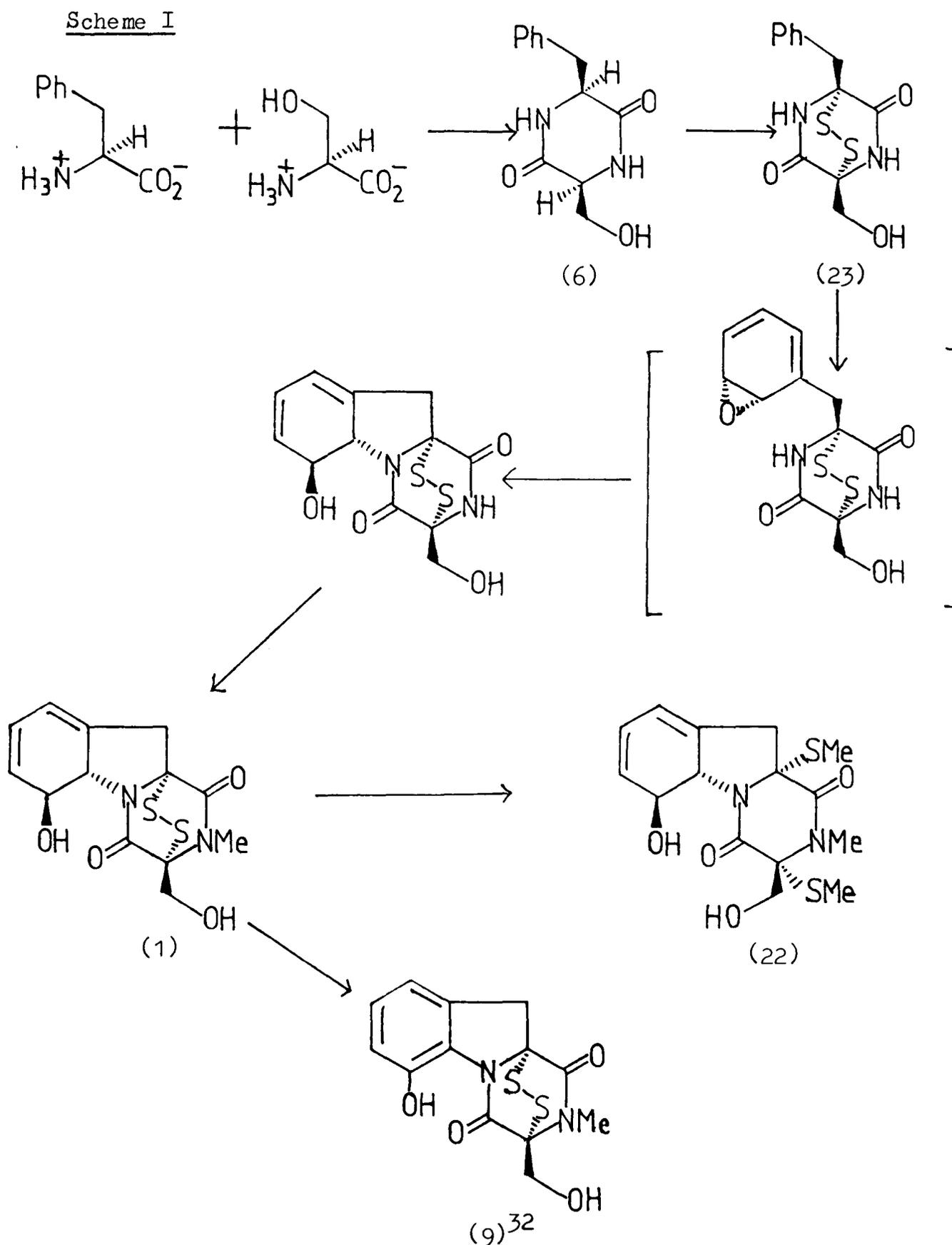


biosynthetic introduction of sulphur occurs immediately after formation of the cyclic dipeptide, and before N-methylation or oxidative cyclisation.

In G. deliquescens, as in other dioxopiperazine-producing fungi, the bisdethiodi(methylthio)- derivative of gliotoxin (22) co-occurs with the epidisulphide.³¹ The relationship between the two was studied by Kirby et al.,³¹ who fed [¹⁴C]gliotoxin (derived biosynthetically from [U-¹⁴C]phenylalanine) to G. deliquescens, and observed a significant incorporation (8.6%) into bisdethiodi(methylthio)gliotoxin (22). A further 27.4% of the activity was recovered as gliotoxin. When [¹⁴C]bisdethiodi(methylthio)gliotoxin was fed, the incorporation into gliotoxin was negligible (0.2%), and 58% of the activity was recovered as unchanged (22). It was thus demonstrated that, in G. deliquescens, bisdethiodi(methylthio)gliotoxin (22) is formed by irreversible

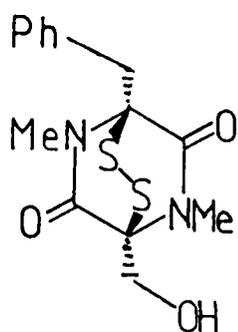
reductive methylation of gliotoxin; this result might also apply to the analogous metabolites in other fungi.

A biosynthesis for gliotoxin that agrees with most of the evidence presented to date is given in Scheme I. N-Methylation could occur either before or after cyclisation of the aromatic ring with the amide nitrogen.

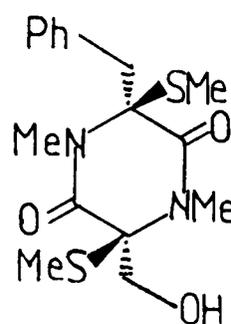


1.2.2 Biosynthesis of hyalodendrin

Hyalodendrin (24) was first isolated by Strunz et al.³³ from a species of Hyalodendron. Interestingly, a comparison of its circular dichroism curve with those of other epidithiodioxopiperazines³³ indicated that the configuration of the disulphide bridge in hyalodendrin is antipodal to that of gliotoxin, the aranotins, and the sporidesmins. Strunz et al. also isolated the related bisdethiodi(methylthio)hyalodendrin (25).³⁴

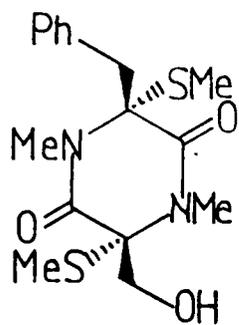


(24)

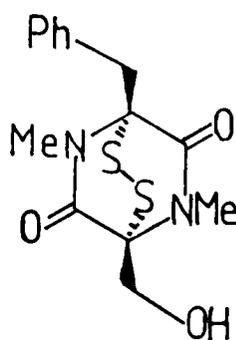


(25)

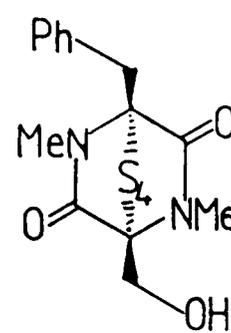
Related metabolites have been found in several other fungi. Dorn and Arigoni³⁵ isolated gliovictin (26) from Helminthosporium victoriae, differing from bisdethiodi(methylthio)hyalodendrin (25) only in its absolute configuration. The same metabolite was isolated from Penicillium turbatum by Michel et al.,³⁶ along with the hyalodendrin antipode (27) and the tetrasulphide (28). The enantiomer of this tetrasulphide has been isolated from Hyalodendron by Strunz et al..³⁷



(26)

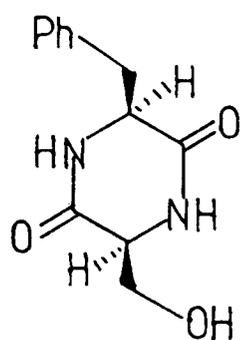


(27)

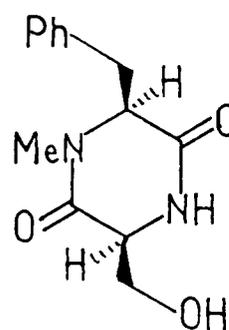


(28)

Hyalodendrin bears a close resemblance to gliotoxin, the only differences being the absolute configuration, the lack of oxidative cyclisation, and the methylation of the second nitrogen. Accordingly, Patrick³⁸ and Pita³⁹ investigated whether cyclo-(Phe-Ser) (6) is a precursor for this group of metabolites. Because of the growth conditions used, bisdethiodi(methylthio)hyalodendrin (25) was isolated from the cultures of Hyalodendron rather than the epidisulphide (24), but their conclusions should also hold for hyalodendrin. All four isomers of cyclo-(Phe-Ser) were fed (labelled with ¹⁴C),³⁸ and only cyclo-(L-Phe-L-Ser) (6a) was incorporated to a significant degree (28%). Doubly labelled cyclo-(L-[4'-³H]Phe-L-[3-¹⁴C]Ser) was incorporated (42%) without significant change in the isotopic ratio, indicating that the cyclic dipeptide is transformed into (25) without return to its constituent amino-acids. Patrick also showed³⁸ that the monomethylated cyclic dipeptide (29) was not incorporated into the hyalodendrin derivative (25). The intermediacy of cyclo-(L-Phe-L-Ser) (6a) is therefore



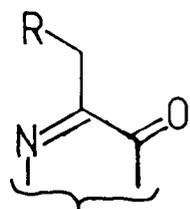
(6a)



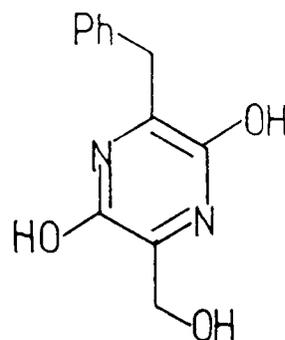
(29)

well established, and it may be that sulphur is introduced before N-methylation, as was suggested for gliotoxin. The fact that sulphur is inserted with retention of configuration in gliotoxin biosynthesis, but with inversion in the case of hyalodendrin, is unexplained, but might indicate a non-chiral intermediate, for example an imine such

as (A), or the pyrazinediol (30).



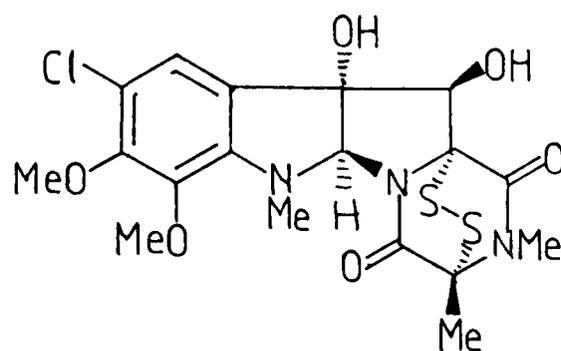
(A)



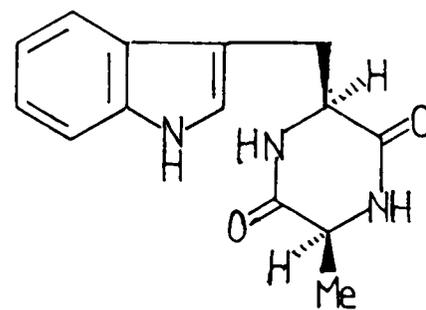
(30)

1.2.3 Biosynthesis of the sporidesmins

Pithomyces chartarum produces a series of related metabolites,⁹ of which sporidesmin (31) is the most abundant. These compounds are implicated in facial eczema, a disease of sheep. Tryptophan and alanine seem strong candidates for progenitors of the carbon skeleton,



(31)



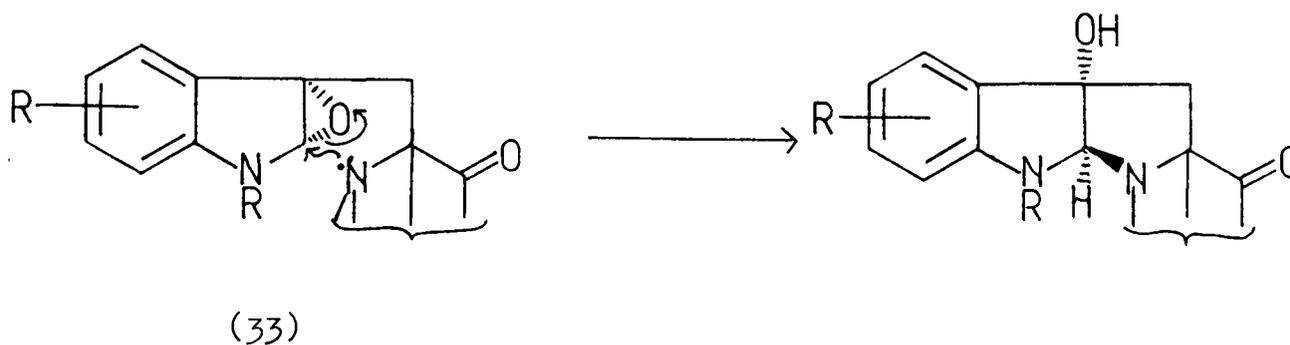
(32)

and both have been shown to be incorporated into sporidesmin by P. chartarum.⁴⁰ Activity from [¹⁴C]serine, [1-¹⁴C]glycine, and L-[methyl-¹⁴C]methionine was also incorporated to a lesser extent, but DL-[3-¹⁴C]cysteine and L-[U-¹⁴C]phenylalanine did not serve as precursors.

Following the analogy of gliotoxin biosynthesis, Kirby and Varley⁴¹ tested cyclo-(L-Ala-L-Trp) (32) as a possible precursor. cyclo-(L-[3-³H]Ala-L-[3-¹⁴C]Trp) was incorporated (2% of the ¹⁴C activity), but the ³H:¹⁴C isotopic ratio was reduced to 51% of that of the precursor. This could be explained, either by breakdown of the cyclic dipeptides into the amino-acids and re-incorporation, or by loss of tritium from the methyl group of the alanyl residue.

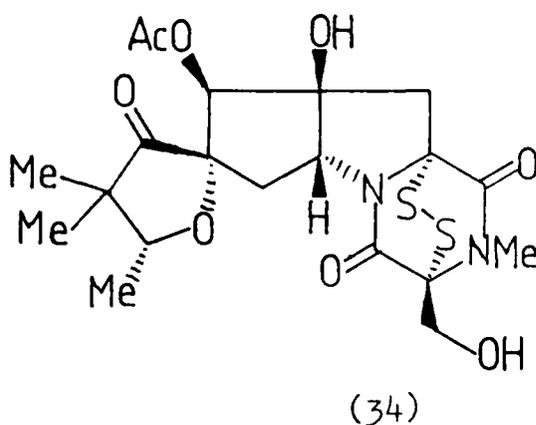
Therefore the status of cyclo-(L-Ala-L-Trp) (32) as an intermediate is not yet clear.

The cyclisation of the dioxopiperazine ring to the indolyl system in sporidesmin biosynthesis could take place by attack of nitrogen on an epoxide (33), in an analogous way to the proposed cyclisation mechanism in gliotoxin, but no work has been done on this possibility to date.



1.2.4 Biosynthesis of the sirodesmins

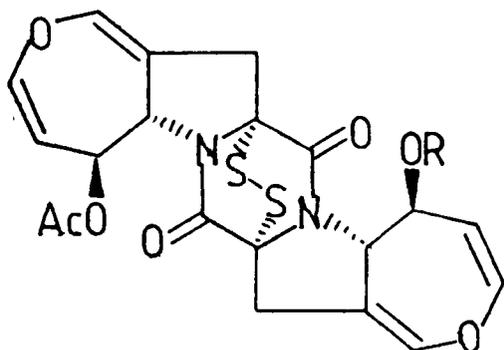
The sirodesmins constitute a family of epipolythiodioxopiperazines containing an unusual spiro-fused tetrahydrofuranone system, sirodesmin PL (34) being a typical example. The other members of the group have



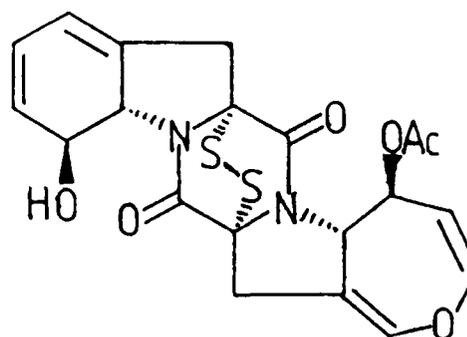
different stereochemistry in the side-group and/or different numbers of sulphur atoms in the bridge (2, 3, or 4). Ferezou et al.⁴² have investigated the biosynthesis of sirodesmin PL (34) in Phoma lingam. L-[U-¹⁴C]Serine and L-[U-¹⁴C]tyrosine were both incorporated into

1.2.5 Biosynthesis of the aranotins

In 1968, Neuss *et al.*^{43,44} reported the isolation of five new metabolites from cultures of Arachniotus aureus (Eidam) Shroeter. Three of these compounds, aranotin (37), acetylaranotin (38), and apo-aranotin (39) are epidisulphides; the other two are bisdethiodi(methylthio)acetylaranotin (BDA) (40) and bisdethiodi(methylthio)acetylapo-aranotin (BDAA) (41). In the same year, Miller *et al.* reported the

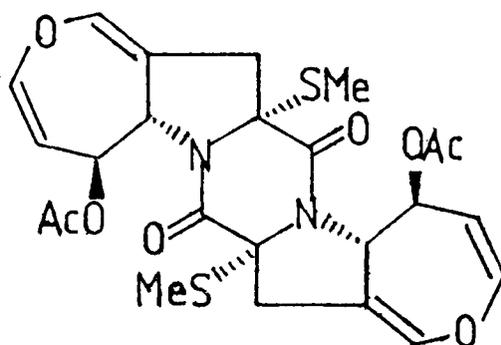


(37); R = H

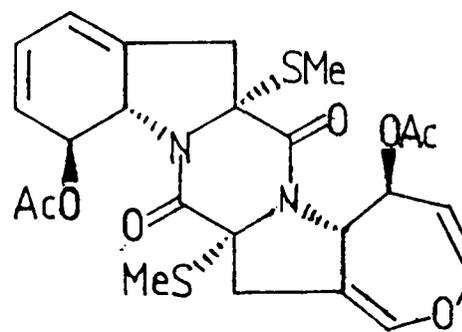


(38); R = Ac

(39)



(40)



(41)

isolation of two metabolites from Aspergillus terreus cultures. These two compounds, which they named LL-S88 α and LL-S88 β , proved to be identical to acetylaranotin (38) and BDA (40) respectively. X-Ray and circular dichroism studies^{46,47} confirmed that the aranotins had the same configuration as gliotoxin (1) at the disulphide bridge.

The first biosynthetic experiments on the aranotins were carried out by Neuss and coworkers,²⁶ who fed a range of amino-acids to A. aureus. Both D- and L-phenylalanines were incorporated into BDA

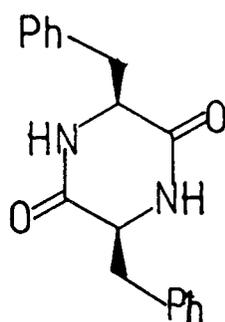
(2.42 and 3.15% respectively), as was L-[methyl- ^{14}C]methionine (6.46%). Isolation of the methyl iodide produced by Zeisel degradation of BDA from the last experiment showed that 96% of the activity from the labelled methionine was in the S-methyl groups of BDA. When a mixture of L-[^{35}S]methionine and L-[methyl- ^3H]methionine was fed to A. aureus, the tritium label was incorporated with greater efficiency (11.3%) than the ^{35}S (2.77%), indicating that the methylthio- group of methionine was not transferred intact to BDA. DL-[3- ^{14}C]Serine and DL-[3- ^{14}C]tryptophan were also incorporated into BDA, though at a much lower level (0.76 and 0.67% respectively). Here, Zeisel degradation again located much of the label in the S-methyl groups (73 and 84% respectively). Unfortunately, BDA from the phenylalanine feeding experiments was not similarly degraded.

Feeding experiments by Brannon et al.¹⁶ established that DL-[3- ^{14}C]phenylalanine was incorporated into BDA (40) much more efficiently (6.64%) than DL-[2- ^{14}C]-m-tyrosine (0.46%) or DL-3',5'-[2- ^{14}C]-dihydroxyphenylalanine (0.18%) by A. aureus. When a mixture of DL-[3- ^{14}C]phenylalanine and DL-[2'- ^3H]phenylalanine was fed, both isotopes were incorporated (7.04 and 5.67% respectively) into BDA, showing that there was little loss of tritium from the ortho- position of phenylalanine in its transformation to the arantoin ring system.

This research group also investigated the fate of the other hydrogen atoms in phenylalanine.¹⁶ When fully C-deuteriated (D_8) DL-phenylalanine was fed to A. terreus, mass spectrometry of the isolated acetylarantoin (38) revealed large amounts of [D_7] and [D_{14}] species. This result suggested that [D_8]phenylalanine contributed to both halves of the arantoin molecule, and that only one deuterium atom was lost as an obligatory step, probably from the α - position of the

phenylalanine fed. Some further loss of deuterium had evidently occurred, however, since there were substantial abundances of $[D_6]$ and $[D_{13}]$ ions. Johns et al.²⁷ showed that this loss was from the methylene group of phenylalanine by feeding DL-[3,3-D₂, 1-¹⁴C]phenylalanine to Aspergillus terreus; mass spectrometry of the isolated acetylaranotin showed substantial loss of one deuterium atom. This loss could be explained by the stereospecific exchange of the methylene hydrogens in phenylalanine which was shown to take place in Gliocladium deliquescens, and thus might be unconnected with dioxopiperazine biosynthesis.²⁷

The comparison of the aranotins and gliotoxin (1) suggested the cyclic dipeptide cyclo-(L-Phe-L-Phe) (42) as a possible aranotin precursor. Accordingly, Kirby et al.⁴⁸ fed cyclo-(L-[U-¹⁴C]Phe-L-Phe) to cultures of A. terreus and observed a high incorporation (19.9%) in into BDA (40) (which was produced in preference to acetylaranotin (38) under their growth conditions). A mixture of cyclo-(L-[1-¹⁴C]Phe-D-Phe)



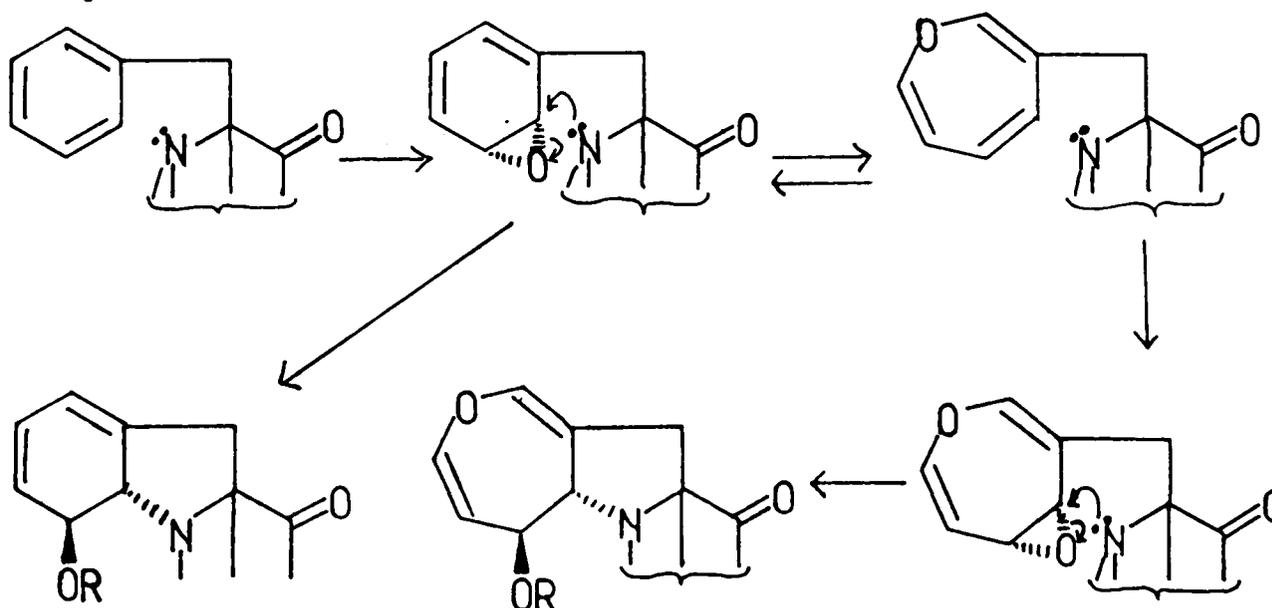
(42)

and cyclo-(D-[1-¹⁴C]Phe-D-Phe) was not significantly incorporated (0.5%). To establish that the cyclic dipeptide (42) was incorporated without dissociation into its constituent amino-acids and recombination, the doubly labelled cyclo-(L-[¹⁵N]Phe-DL-[1-¹³C]Phe) was synthesised and fed. The one-bond coupling between ¹⁵N and ¹³C creates a symmetrical doublet in the ¹³C n.m.r. signal of the amide carbonyl, and comparison of the intensities of the ¹³C n.m.r. amide carbonyl signals

in the isolated BDA with those of the precursor demonstrated that a minimum of 90% of the LL-precursor was transformed intact into BDA.

It was also of interest to determine the fate of the methylene hydrogen atoms in cyclo-(L-Phe-L-Phe) (42).³⁹ A mixture of cyclo-(DL-[3,3-D₂]Phe-DL-[3,3-D₂]Phe) and cyclo-(L-[U-¹⁴C]Phe-L-Phe) was fed to A. terreus, and the deuterium content of the BDA produced was determined by mass spectrometry. Some [D₄] species were detected, indicating, as did the experiments of Brannon et al.,¹⁶ that deuterium loss from the methylene group is not obligatory; but overall the results suggested a loss of ca. 20% of the total deuterium label.

After cyclic dipeptide formation, several transformations are required to obtain the aranotin metabolites. The mechanism of sulphur introduction has not been investigated; it is probable that this takes place in a similar manner to the analogous conversion in gliotoxin biosynthesis, and that the di(methylthio)- compounds (BDA and BDAA) are derived from the appropriate epidisulphides. Neuss et al.⁴³ have advanced a plausible explanation for the formation of the unusual oxepin rings (Scheme III); the existence of members of the aranotin family having a cyclohexadienol ring system (acetylpoaranotin and BDAA) suggests that this mechanism might also operate in gliotoxin biosynthesis.



Scheme III

Further work concerning aranotin biosynthesis is described in this thesis (Discussion).

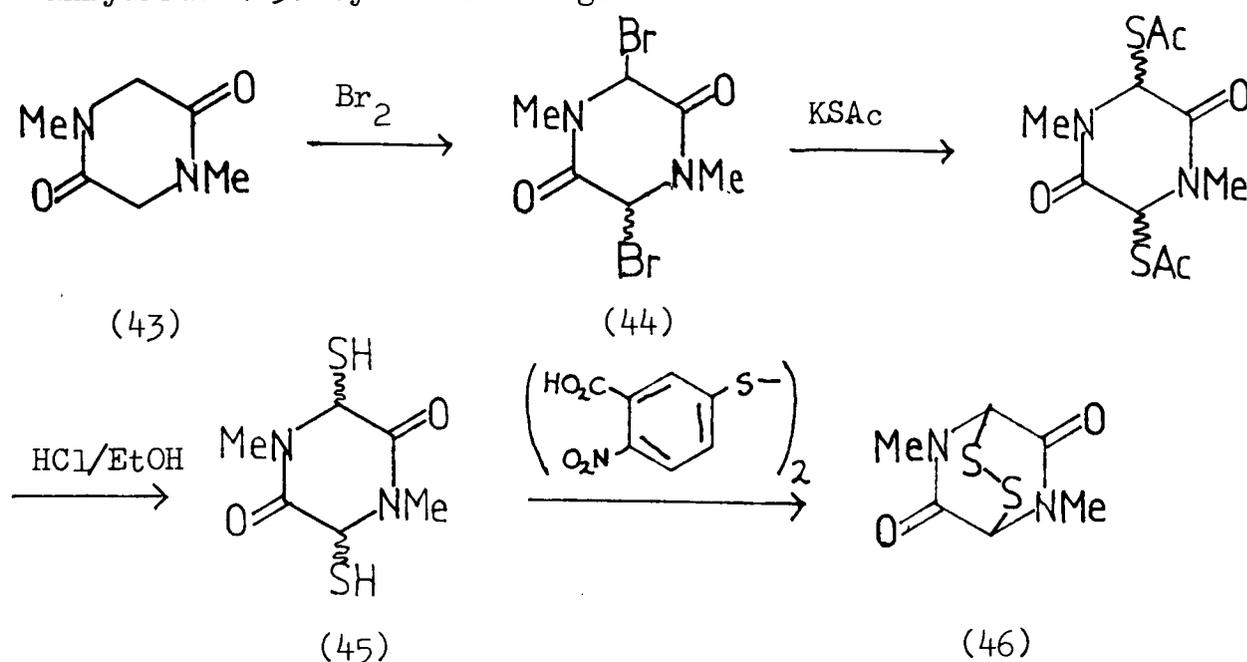
1.2.6 Summary

All the metabolites investigated to date have amino-acid biogeneses, and all appear to have simple cyclic dipeptides as intermediates in the biosyntheses. Interestingly, all the known sulphur-containing dioxopiperazines have at least one moiety which appears to be derived from one of the aromatic amino-acids (phenylalanine, tryptophan or tyrosine). The other amino-acid residue in the dioxopiperazine ring can be derived from phenylalanine, serine, alanine, or glycine.

The origin of sulphur and the mechanism of its introduction are not fully determined for any metabolite. The presence in some fungal extracts of epitrisulphides and epitetrasulphides is intriguing, and although there is some suggestion that these might be artefacts,⁴⁹ their wide occurrence⁹ and the mildness of the isolation procedures argue against this possibility. The finding that, in gliotoxin biosynthesis, the bisdethiodi(methylthio)- derivative is formed irreversibly from the epidisulphide, may apply to other sets of metabolites in different organisms. Although the mechanism of cyclisation of nitrogen to the amino-acid side-chains has not been determined, the intervention of arene oxides is an attractive possibility in several cases.

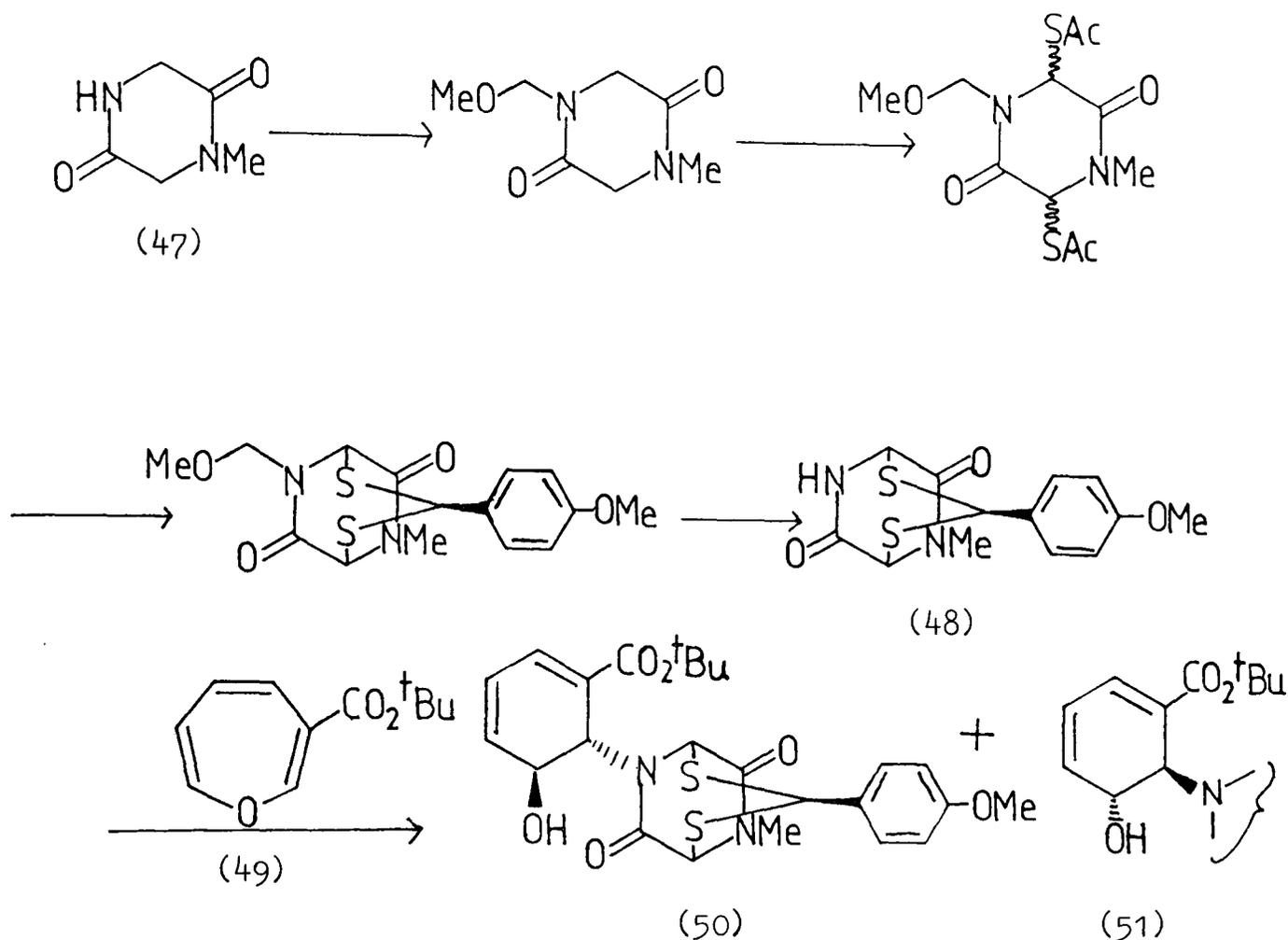
1.3 Synthesis of sulphur-containing dioxopiperazines

More than 30 years elapsed between the isolation of gliotoxin and the first synthesis of an epidithiodioxopiperazine. This was achieved in 1968 by Trown,⁵⁰ who prepared the epidisulphide (46) from sarcosine anhydride (43) by the route given below.



Most syntheses of sulphur-containing dioxopiperazines have introduced sulphur into the preformed dioxopiperazine ring by the above method, *i.e.* by displacement of bromide with a sulphur nucleophile. In particular, Kishi and coworkers⁵¹ have used this approach for the syntheses of gliotoxin (1),^{51,52} dehydrogliotoxin (9),^{51,53} spori-desmin A (31),⁵⁴ and hyalodendrin (24).⁵¹ Kishi's strategy was to introduce sulphur at an early stage, and elaborate the protected sulphur-containing dioxopiperazine before removal of the sulphur protecting groups to give the desired product.

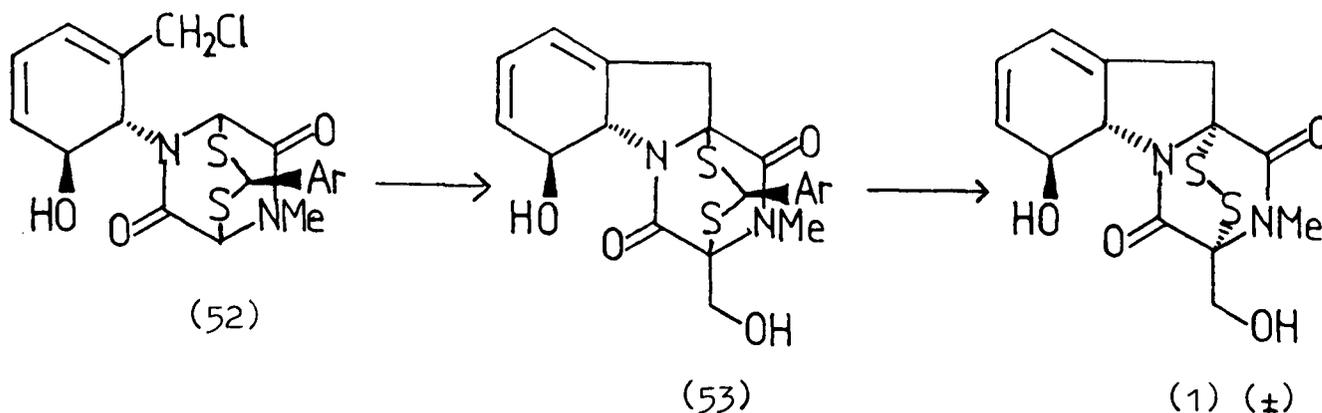
In the synthesis of gliotoxin,^{51,52} the key intermediate was the dithioacetal (50), which was prepared by an extension of Trown's method, as shown below.



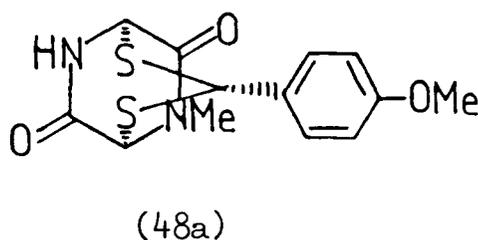
Glycine sarcosine anhydride (47) was methoxymethylated with chloromethyl methyl ether and potassium *t*-butoxide. Sulphur was introduced by radical bromination with *N*-bromosuccinimide followed by displacement with potassium thioacetate, giving a mixture of *cis*- and *trans*- isomers of the di(acetylthio)- derivative. After methanolysis, the dithiol was protected with *p*-anisaldehyde and boron trifluoride etherate, and the methoxymethyl group was removed by acid hydrolysis. This gave a mixture of dithioacetals which was separated by selective *N*-benzoylation and ammonolysis. The diastereomer (48) reacted with the arene oxide-oxepin (49) in basic conditions, giving a mixture of isomers (50) and (51).

The ester (50) was converted to the alkyl chloride (52) by a standard route. This compound, on treatment with phenyl-lithium (3.2

equivalents) cyclised to give a gliotoxin type pyrrolidine ring, and the hydroxymethyl function was introduced by treating the anion with benzyl chloromethyl ether, followed by cleavage of the benzyl ether with boron trichloride. This gave the protected racemic gliotoxin (53) which was deprotected by treatment with *m*-chloroperbenzoic acid and perchloric acid.

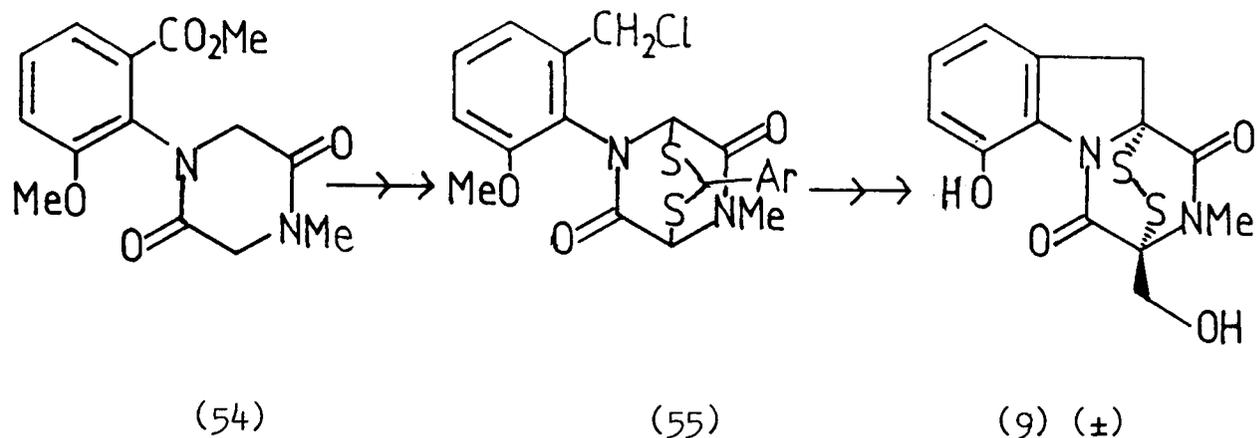


Optically active gliotoxin was obtained by conducting the same series of reactions on the resolved dithioacetal (48a), and its properties were identical to those of the natural product (1).

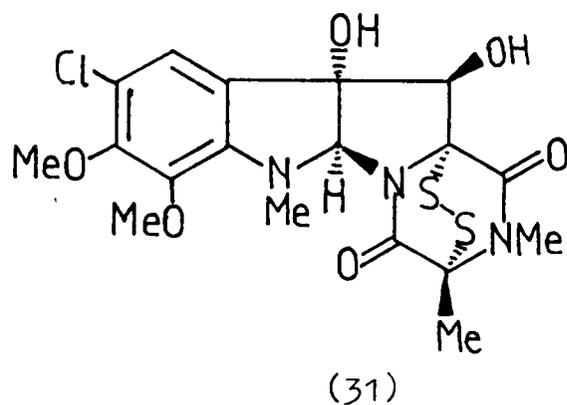


(±)-Dehydrogliotoxin (9) was synthesised by a similar route. The *N*-aryl cyclic dipeptide (54) was prepared by an Uhlmann-type reaction between glycine sarcosine anhydride (47) and 2-iodo-3-methoxybenzoic acid followed by esterification of the carboxyl group. After introduction of the dithioacetal as before, both diastereoisomers were converted into the chloromethyl derivatives (55), which were treated with phenyl-lithium, cyclised, alkylated, and deprotected as in the

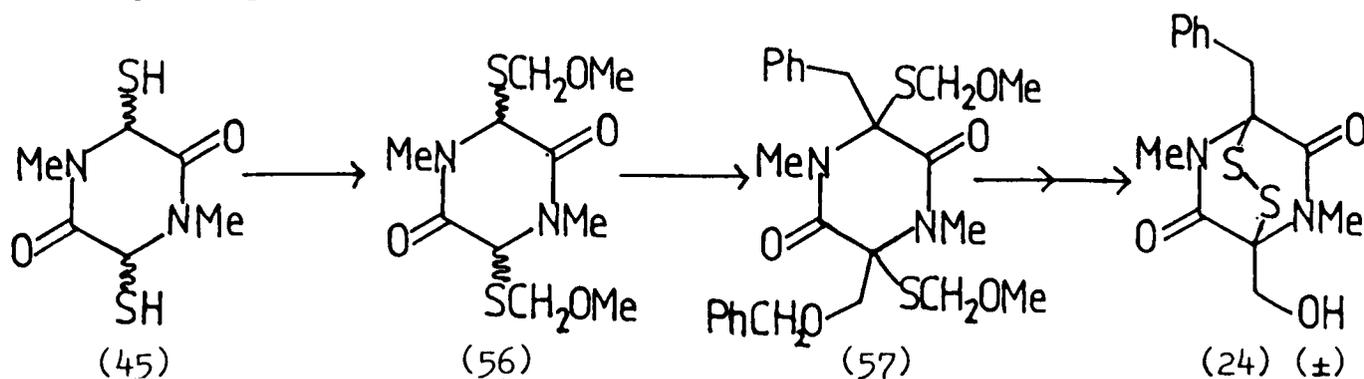
gliotoxin synthesis (see above).



(±)-Sporidesmin A (31) was also synthesised by Kishi using this method.

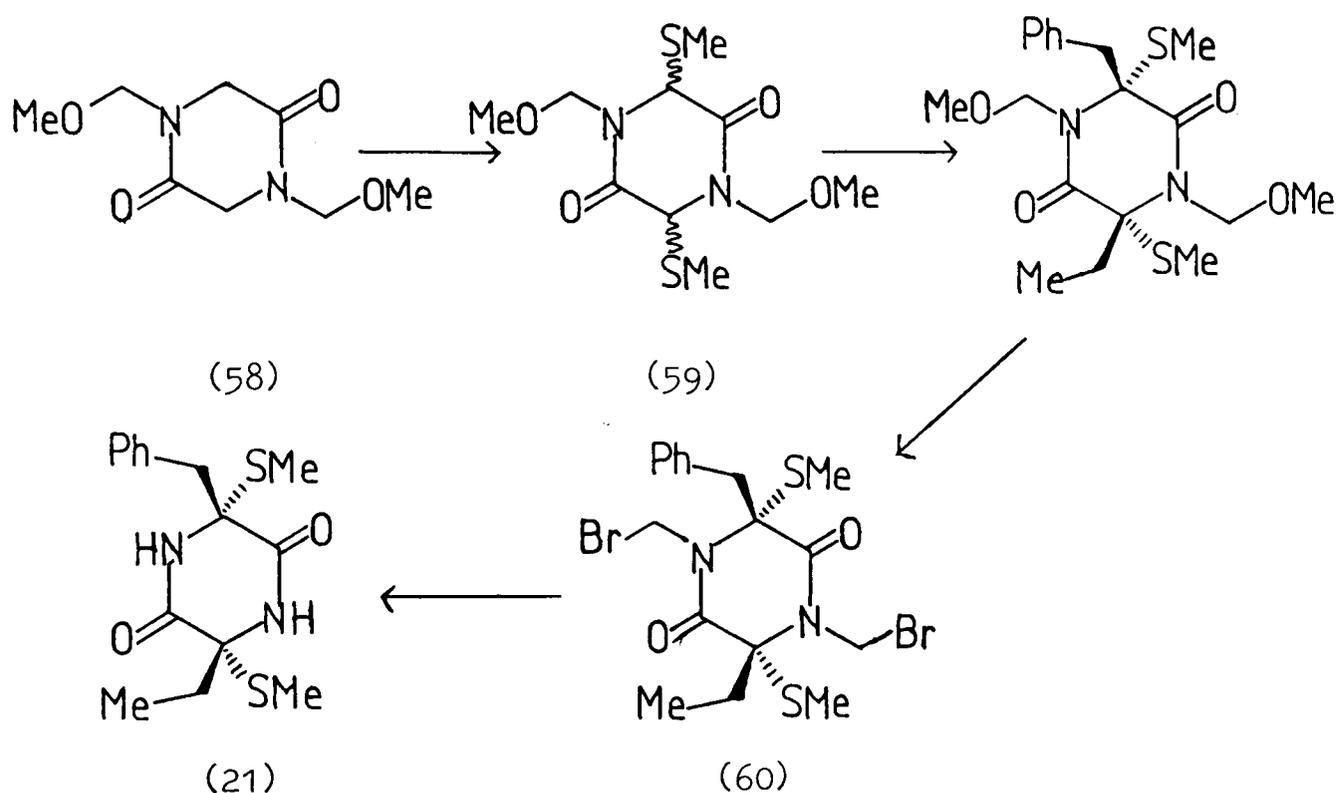


(±)-Hyalodendrin (24) was prepared by a slightly different method;⁵¹ the protected derivative (56) was formed from the dithiol (45) (prepared by the procedure described above) on treatment with chloromethyl methyl ether and base. Alkylation of this compound using lithium di-isopropylamide (2.3 equivalents) followed by benzyl bromide and bromomethyl benzyl ether, then deprotection with boron trichloride and oxidation of the dithiol to the disulphide, gave racemic hyalodendrin (21). Alkylation was highly stereoselective, giving mainly the cis-dialkyl compound (57).



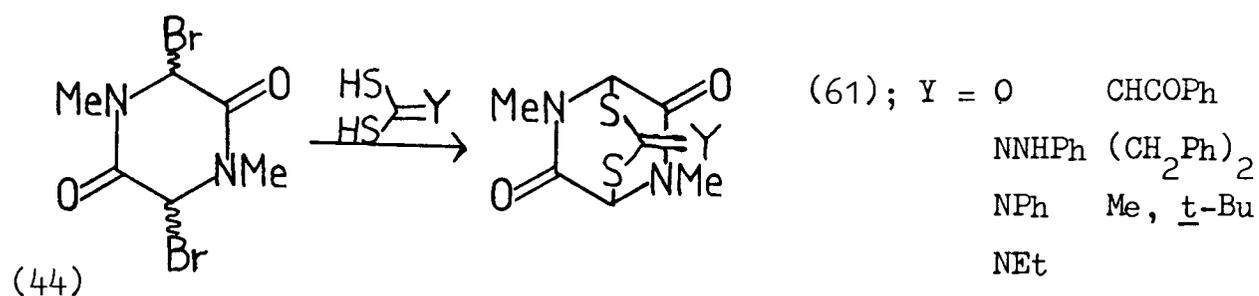
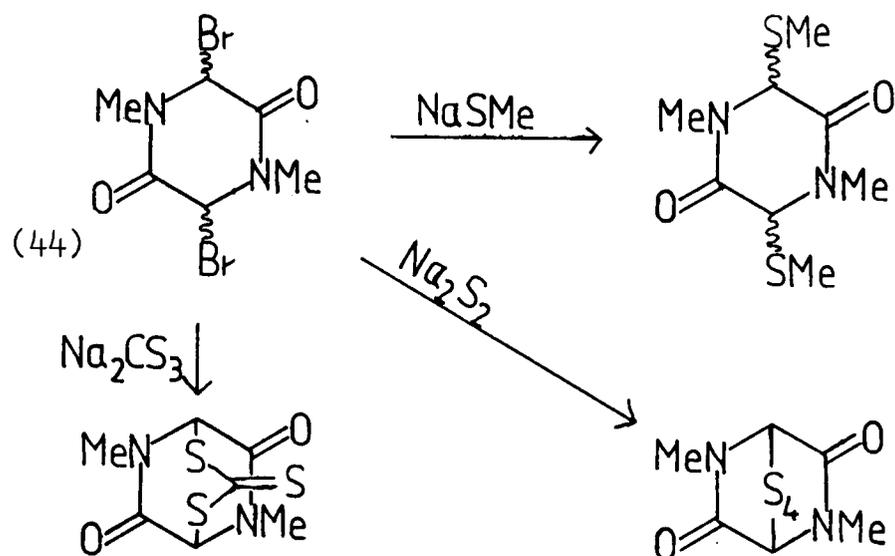
Hyalodendrin was also synthesised by Strunz and Kakushima⁵⁵ by an adaptation of Kishi's route using the anisaldehyde dithioacetal protecting group.

A modification of this method was used by Kirby *et al.*³⁰ to synthesise the unnatural metabolite (21). Sulphur was introduced into di(methoxymethyl) glycine anhydride (58) by the usual method, and the dithiol was methylated to give (59). This compound was alkylated with benzyl bromide and ethyl iodide; treatment of the product with boron tribromide gave a mixture containing the di(bromomethyl)-compound (60) which was deprotected with aqueous sodium bicarbonate to give (21).

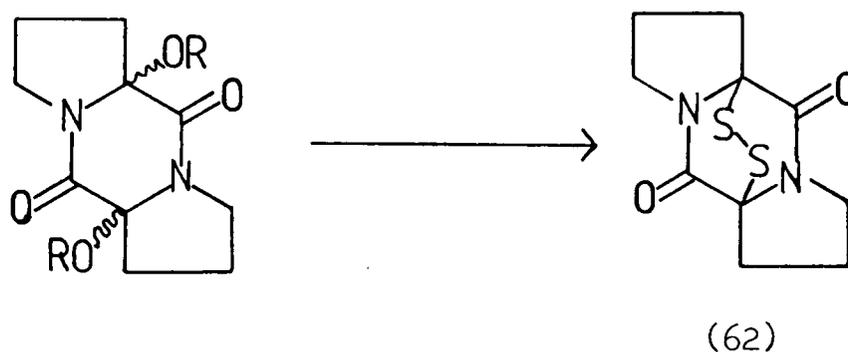


Several other groups have used nucleophilic substitution of bromine as their method for sulphur introduction. Poisel and Schmidt⁵⁶ treated sarcosine anhydride dibromide (44) with a variety of sulphur nucleophiles and obtained the expected sulphenylated products. Olsen *et al.*⁵⁷ treated the same compound (44) with several geminal dithiols and obtained the sulphur-containing substitution products (61), but were

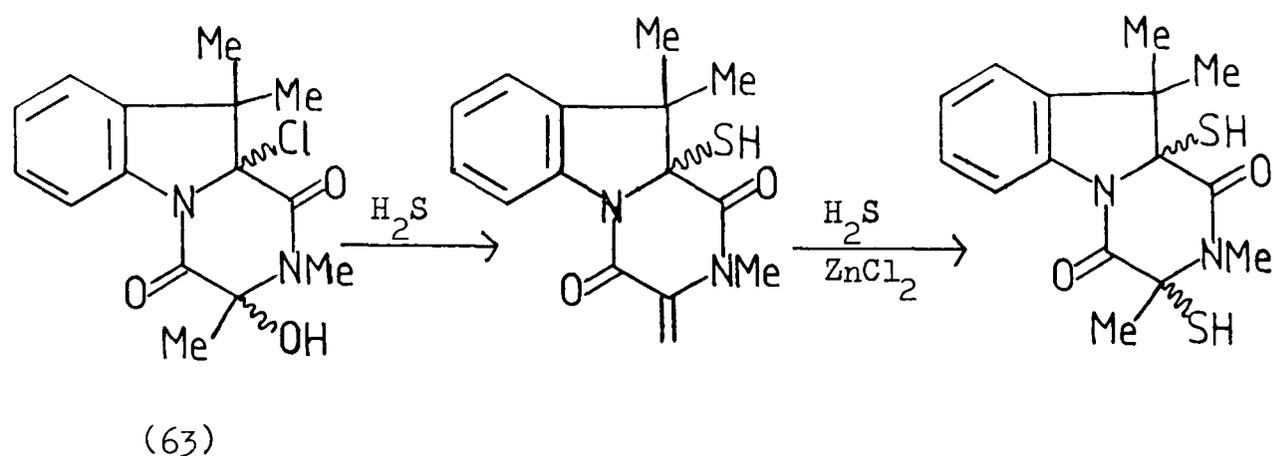
not able to form epidisulphides from them.



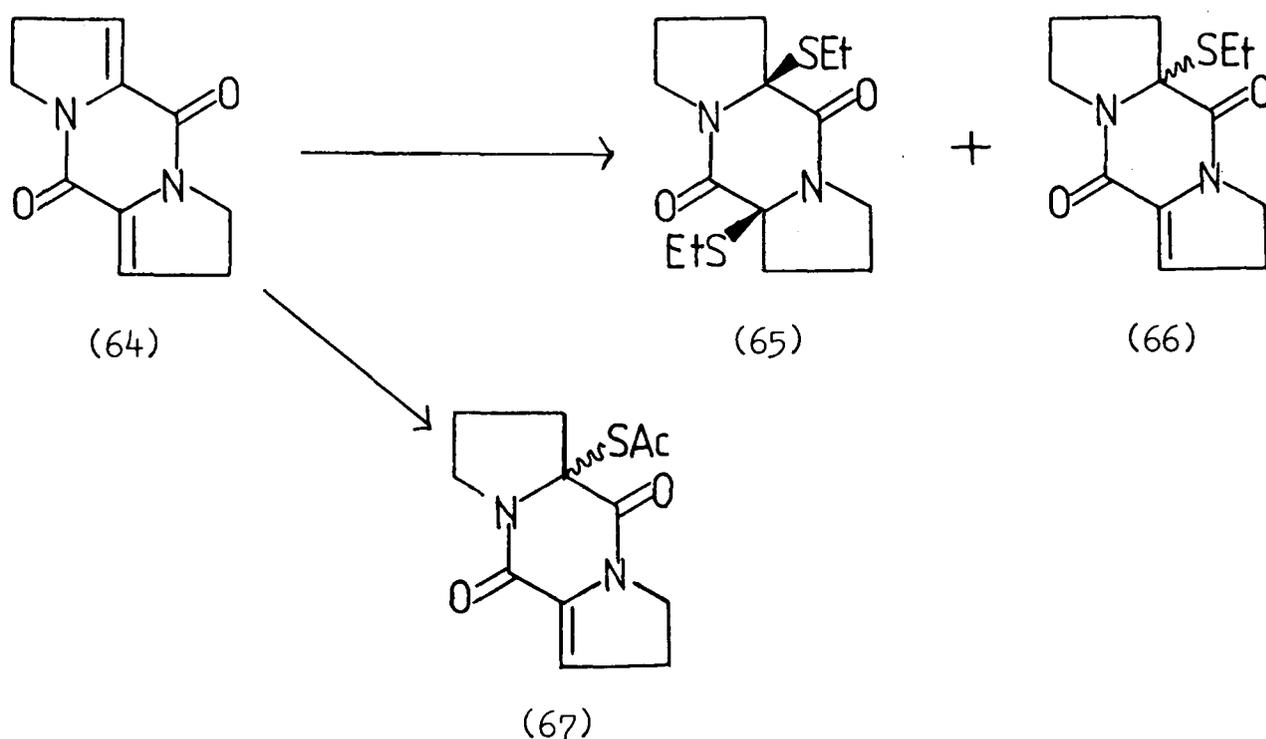
Similar nucleophilic displacements of leaving groups other than bromide have been successful; Schmidt *et al.*⁵⁸ replaced hydroxyl and methoxyl groups with thiols using zinc chloride catalysis.



This method was also used by Yoshimura *et al.*⁵⁹ with methoxide as the leaving group. Ottenheijm and coworkers⁶⁰ carried out a similar reaction on the dioxopiperazine (63).

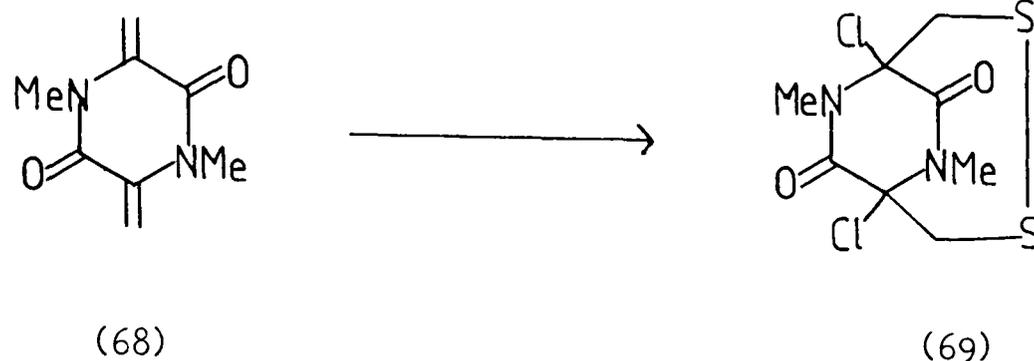


The second reaction above represents yet another means of sulphur introduction, *i.e.* addition of a thiol (or hydrogen sulphide) across a double bond. Machin and Sammes⁶¹ found that, under strongly acidic conditions, thiols added to the didehydro- derivative of proline anhydride (64) in the desired sense; *cis*-di(ethylthio)-*cyclo*-(Pro-Pro) (65) was obtained using ethanethiol and hydrogen chloride in methylene chloride, along with the monoaddition product (66). Thiolacetic acid gave only mono-adduct (67).

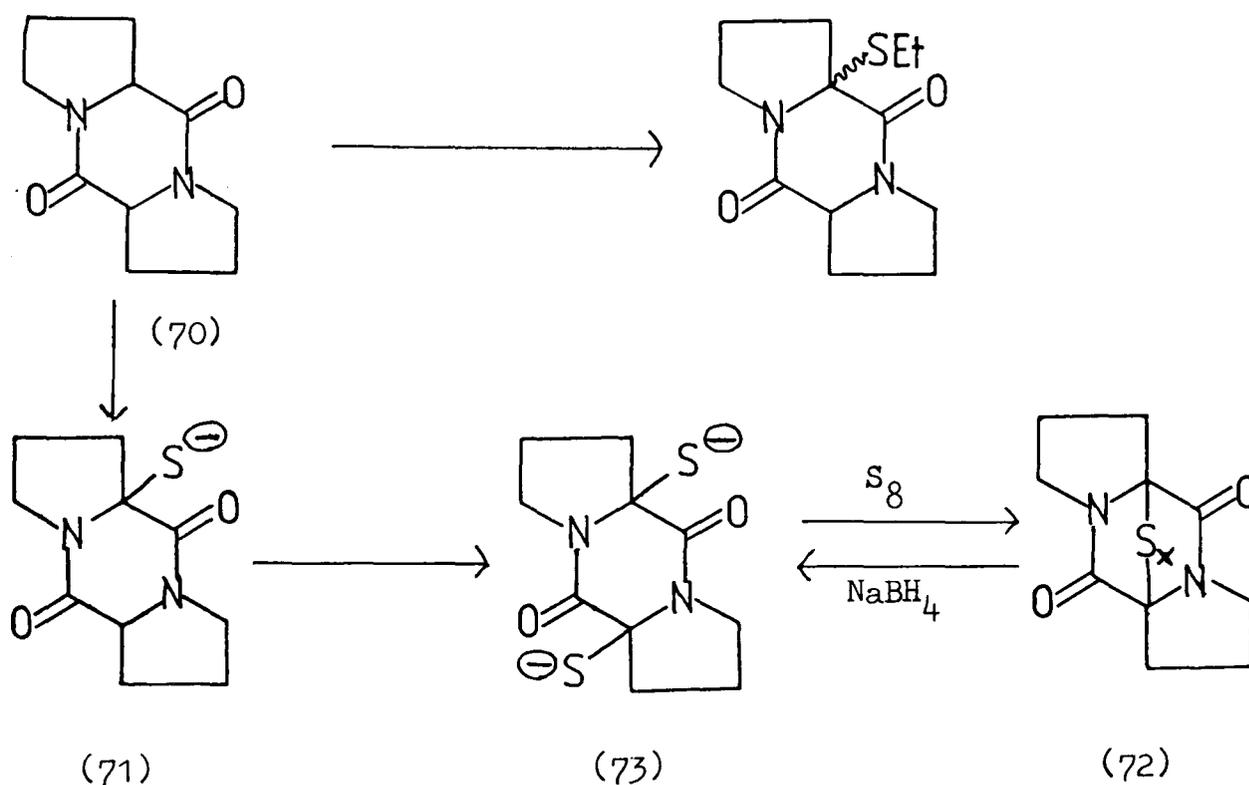


Yoshimura *et al.*⁶² found that addition of methanesulphenyl chloride and sulphur monochloride to dialkylidene dioxopiperazines took place in the opposite sense; *e.g.* dimethylene sarcosine anhydride (68) gave the

derivative (69) with sulphur monochloride.

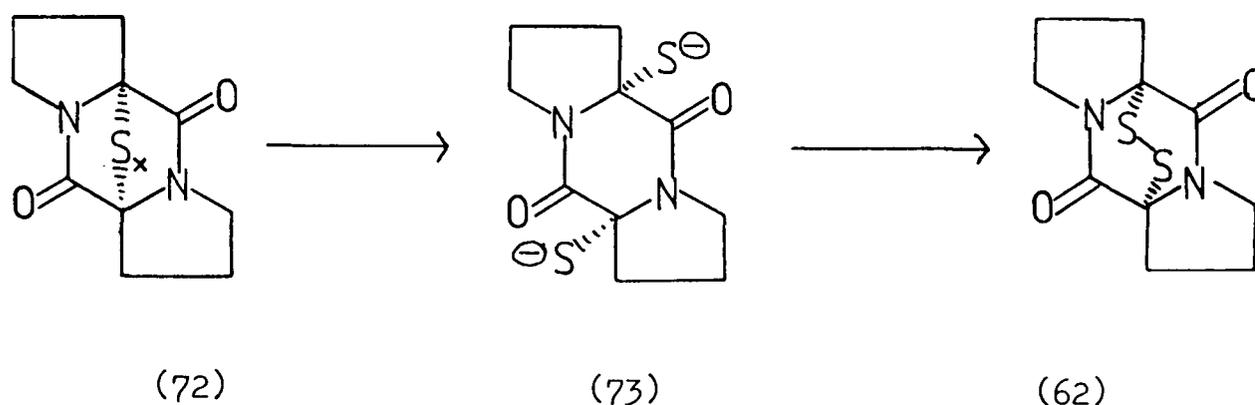


The use of electrophilic sources of sulphur has been less frequent. Poisel and Schmidt^{63,64} treated proline anhydride (70) with lithium di-isopropylamide or sodamide in liquid ammonia, to give the anion which reacted with sulphur electrophiles yielding thiolate or alkylthio- derivatives. The thiolate derivative (71) could be treated

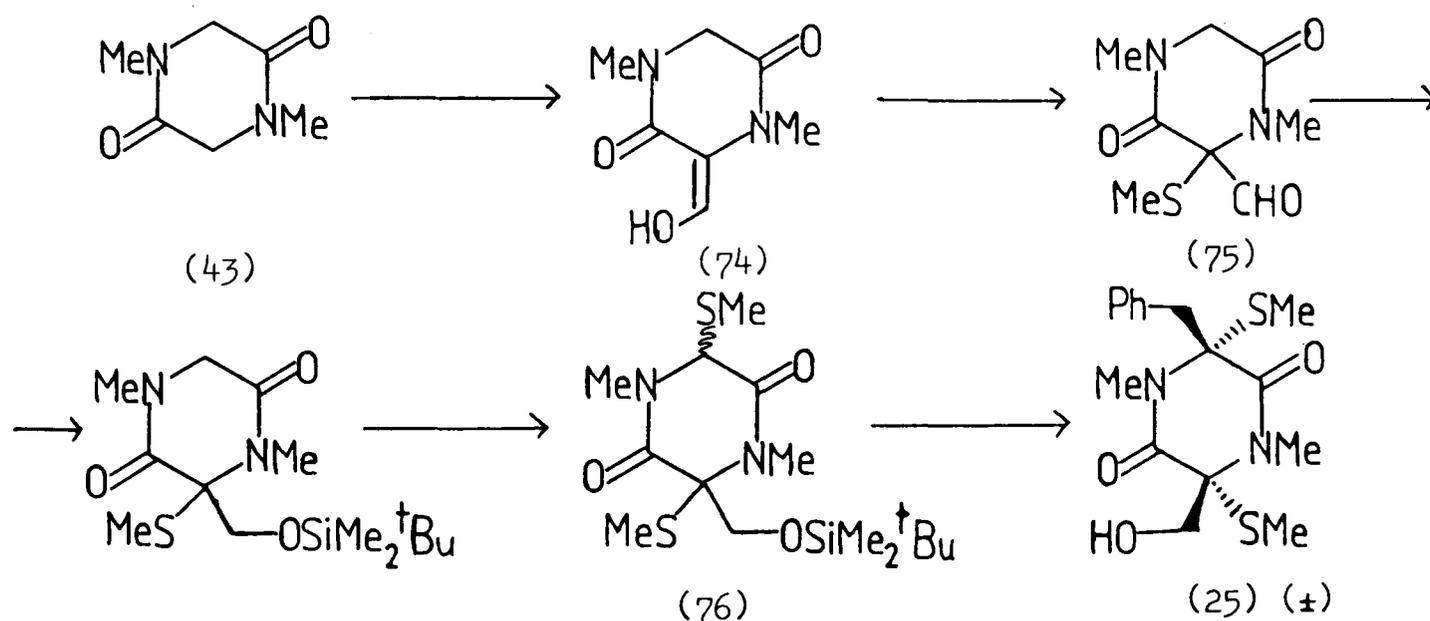


with more base, then sulphur, giving an amorphous oligosulphide (72). Treatment of this compound with sodium borohydride in methanol gave a solution of the cis-dithiolate (73) which could be alkylated with

alkyl halides to give the cis-di(alkylthio)- derivatives, or acidified and the resultant dithiol oxidised with potassium triiodide to the epidisulphide (62). When cyclo-(L-Pro-L-Pro) was used as the starting material, optically active disulphide (62) was obtained.

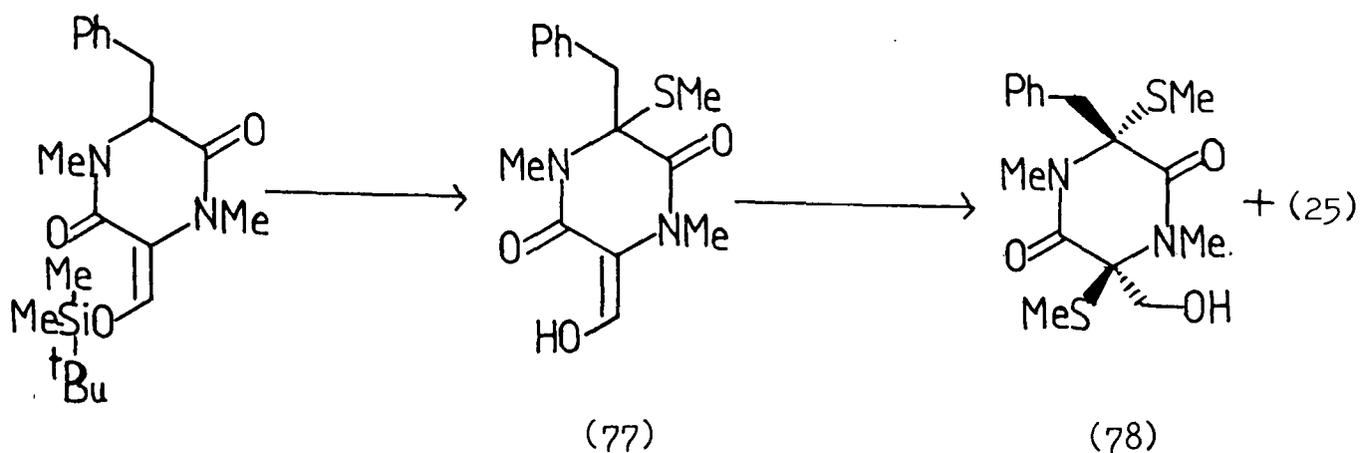


Williams and Rastetter^{65,66} used several sulphur electrophiles in their syntheses of (±)-hyalodendrin (24) and (±)-bisdethiodi(methylthio)hyalodendrin (25). Formylation of sarcosine anhydride (43) gave the enol (74) which was sulphenylated with methanesulphenyl chloride in THF, giving the formyl methylthio- compound (75). Reduction to the alcohol and protection with t-butyldimethylsilyl chloride was followed by a second sulphenylation with dimethyldisulphide and lithium di-isopropylamide at -78°C. This gave a mixture of diastereoisomers (76) which were not separated. Benzylation of this mixture took place in a highly stereoselective manner to give (±)-bisdethiodi(methylthio)-

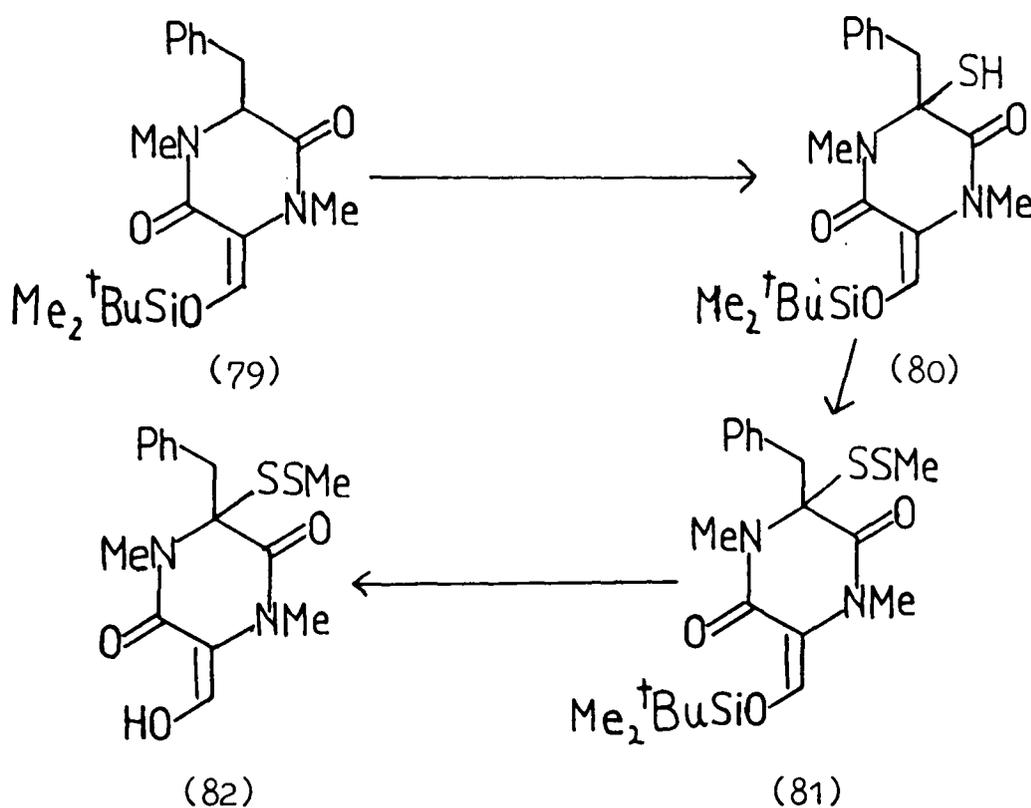


hyalodendrin (25) in 38% yield from sarcosine anhydride.

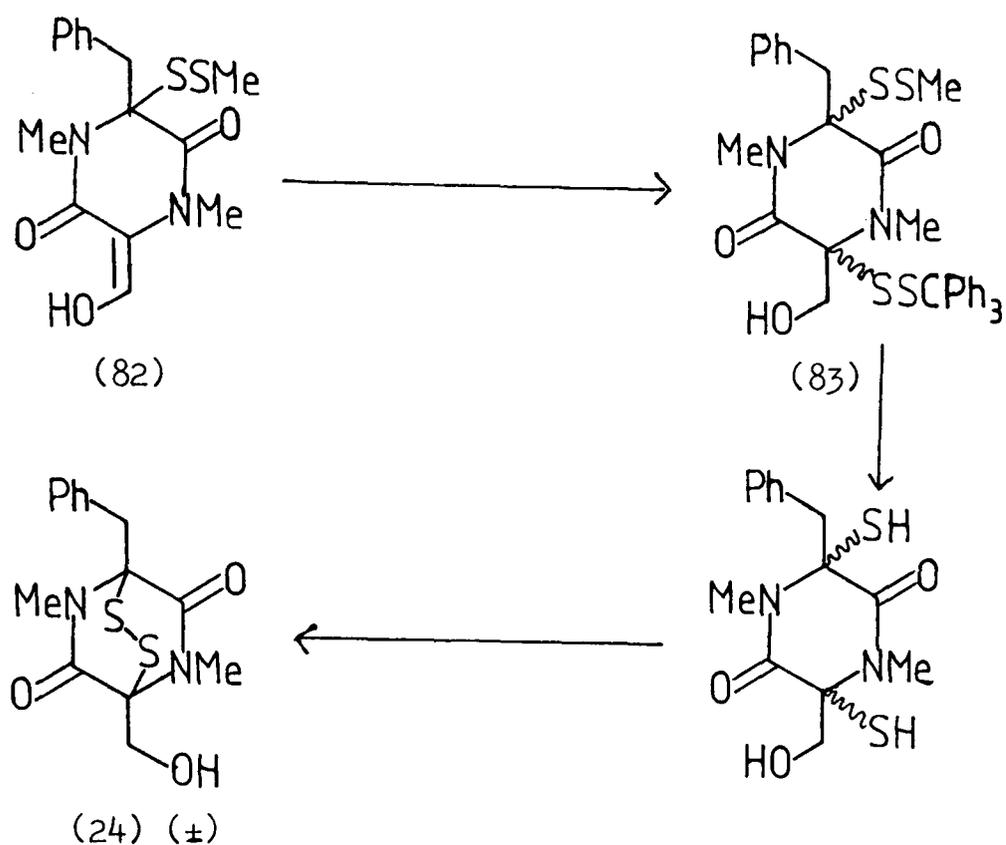
On the other hand, sulphenylation of the benzyl compound (77) gave a mixture of cis- and trans- isomers of (25) in the ratio 2:5.



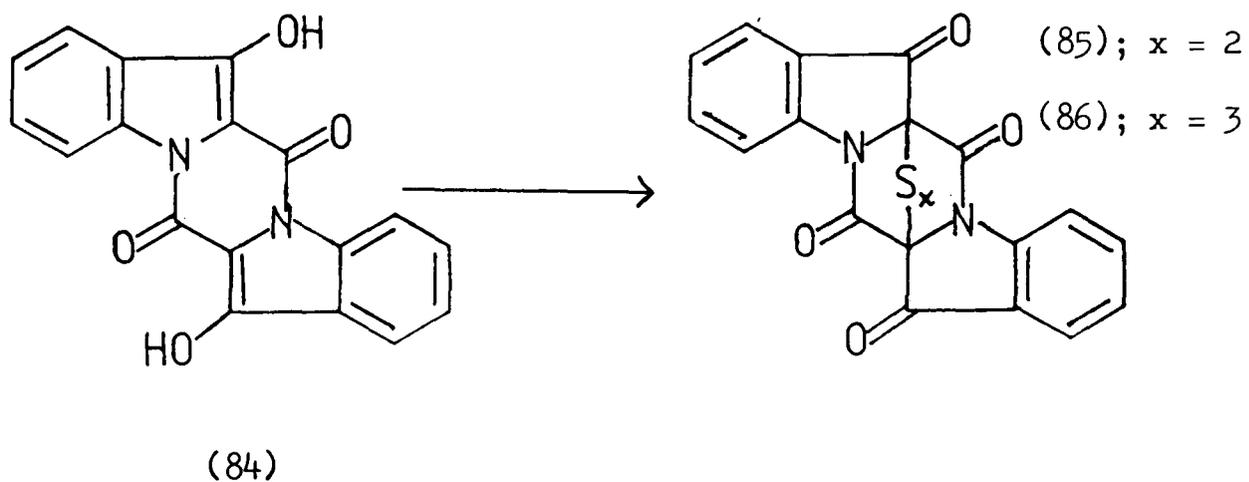
(±)-Hyalodendrin was synthesised by a similar method. The protected compound (79) was converted into the thiol (80) by adding the enolate in THF to a solution of monoclinic sulphur, followed by a reductive work-up (sodium borohydride). The thiol was protected as the methylidisulphide (81); hydrolysis of the silyl protecting group gave the enol (82), and treatment of this compound with triphenylmethyl chlorodisulphide led to the doubly sulphenylated compound (83), as a mixture of cis- and trans- isomers in the ratio 1:2. Reductive



deprotection with sodium borohydride followed by oxidation of the dithiol with potassium triiodide gave (\pm)-hyalodendrin in 12% overall yield.

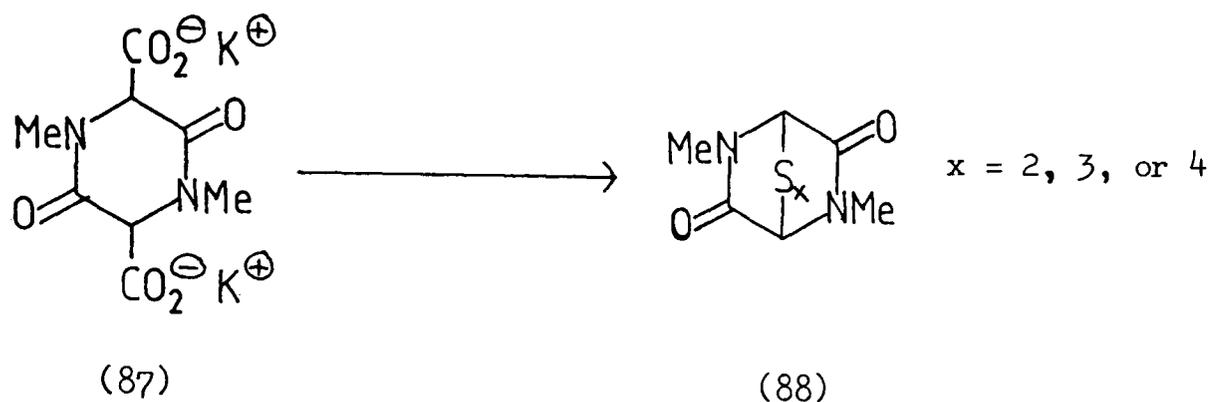


Coffen and coworkers⁶⁷ used a related reaction in their synthesis of aromatic analogues of the aranotins. Treatment of the dienol (84) with sulphur monochloride in methylene chloride with pyridine as a base, gave the disulphide (85), or when excess sulphur monochloride was used, the trisulphide (86).

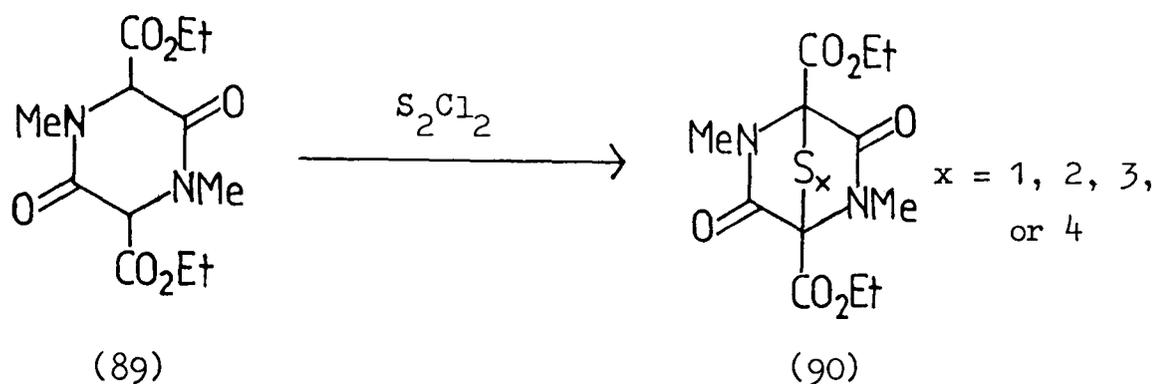


Sato and Hino⁶⁸ introduced sulphur by decarboxylation of the

dipotassium salt (87) in the presence of sulphur monochloride, obtaining a mixture of di-, tri-, and tetra-sulphides (88).



Sato and Hino also formed ethylthio- and phenylthio- derivatives of sarcosine anhydride by the above method with sulphenyl chlorides as electrophiles. In a previous paper⁶⁹ they had reported the formation of the mono-, di-, tri-, and tetra-sulphides (90) from diethoxycarbonyl sarcosine anhydride (89).



Summary

Syntheses of sulphur-containing dioxopiperazines have been performed using both nucleophilic and electrophilic sources of sulphur. In general, substitution with sulphur nucleophiles has been preferred for the syntheses of the naturally occurring compounds; the usual strategy has been to elaborate a relatively simple sulphur-containing dioxopiperazine nucleus by alkylation of anions at the 3- and 6-positions. The use of sulphur electrophiles has been less widespread, but they seem to be more suitable for the sulphenylation of dioxopip-

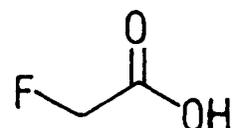
erazines already substituted at the 3- and 6- positions. Many of the naturally occurring sulphur-containing dioxopiperazines have been synthesised, the major exceptions being the aranotins; the difficulties in constructing the oxepin ring system have probably deterred anyone from attempting this synthetic challenge.

1.4 The use of fluorinated analogues in biosynthesis

The use of analogues in biosynthetic studies can be of assistance in elucidating pathways. The ability (or inability) of living systems to convert chemically modified precursors into analogues of their normal metabolites can throw some light on the enzymic transformations occurring. There is also the possibility that analogue substrates will be metabolised in an abnormal way, giving rise to new structural types.

Fluorine has attracted attention as a replacement for hydrogen in precursors. Its properties are especially suited to this purpose; it forms a strong bond with carbon (ca. 460 kJ/mol), and thus is not readily displaced; its Van der Waals radius (1.35 Å) is comparable with that of hydrogen (1.20 Å), and therefore its steric interactions with enzymes will be similar; many fluorinated compounds are readily available; the element is uncommon in living organisms, so is easy to observe in isolation; and the transformations of fluorinated analogues can be traced using the sensitive technique of ^{19}F n.m.r. spectroscopy.

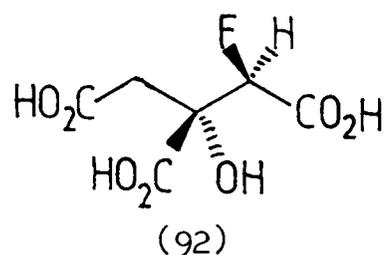
The first work on fluorinated compounds in biological systems was concerned with fluoroacetic acid (91). This simple compound was



(91)

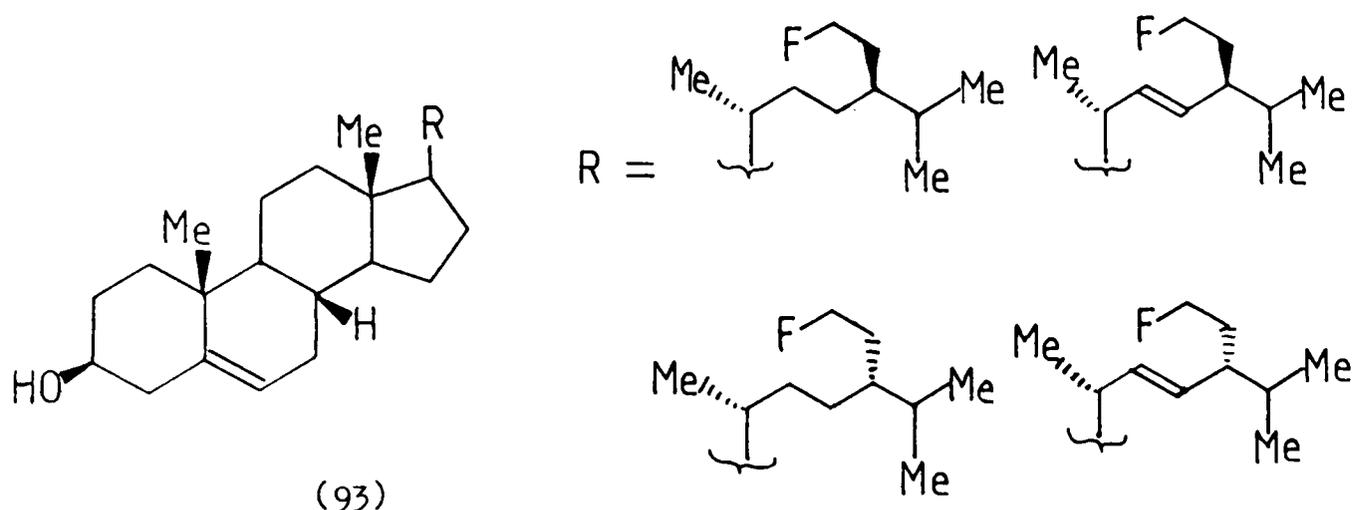
isolated from Dichapetalum cymosum (Gifblaar), a plant responsible for cattle poisoning in South Africa. Animals which were given fluoroacetic acid were found⁷⁰ to have a markedly increased concentration of citric

acid in their organs, and this suggested some interference in the tricarboxylic acid pathway. Administration of fluoroacetic acid to kidney homogenates resulted in the production of (+)-erythro-fluoro-citric acid (92). This compound was found to be a competitive



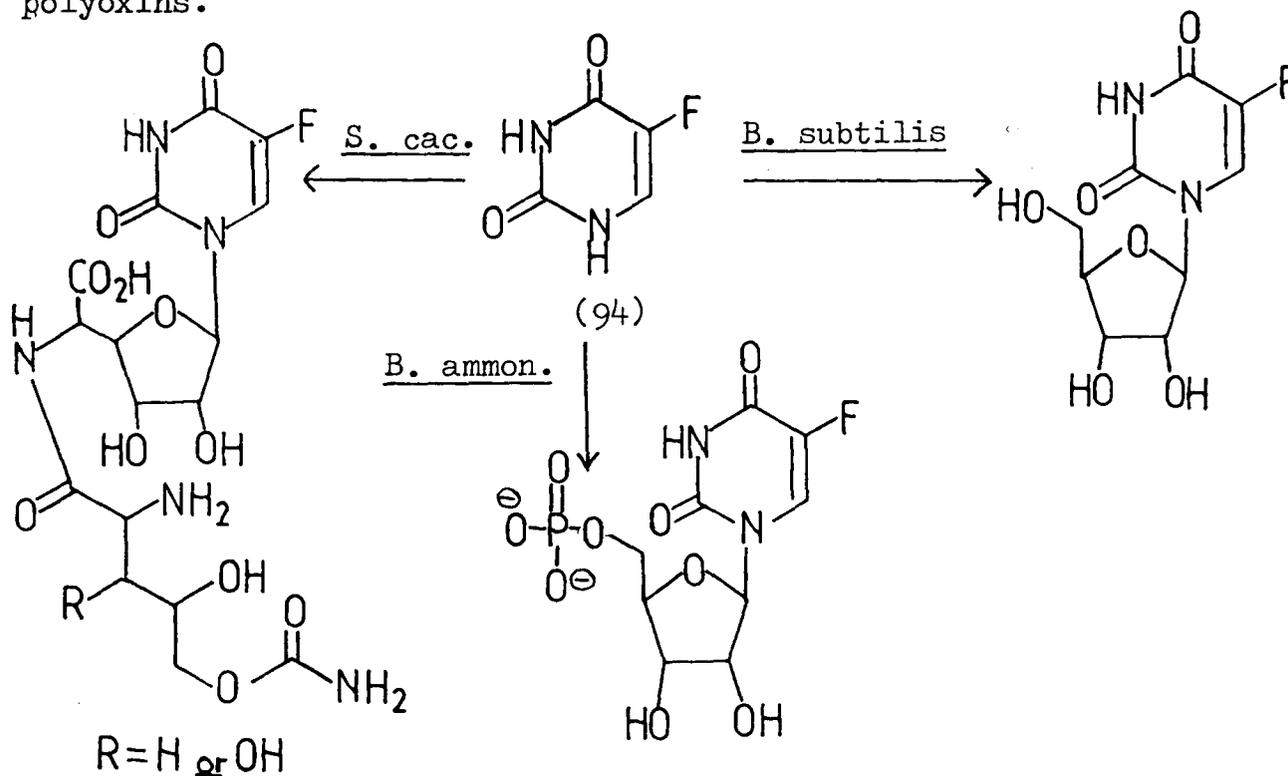
inhibitor of the enzyme aconitase, but the inhibition was not sufficient to account for all the biological effects. Later work⁷¹ showed that the site of fluorocitrate action is at the mitochondrial membrane; the compound is a potent inhibitor of citrate transport across this barrier. Evidence for the formation of two fluorocitrate-protein adducts was found, these being believed to be thioesters. Fluorinated fatty acids and esters containing an even number of carbon atoms in the fatty acid chain were shown to be toxic,⁷⁰ but those with odd numbers of carbon atoms were relatively non-toxic. A possible explanation for this observation is that the even-numbered chains are degraded to acetate and fluoroacetate, this being the ultimate toxic principle.

Recently, Prestwich and Phirwa⁷² prepared the fluorinated phyto-sterol derivatives (93). These compounds were very toxic to insects,

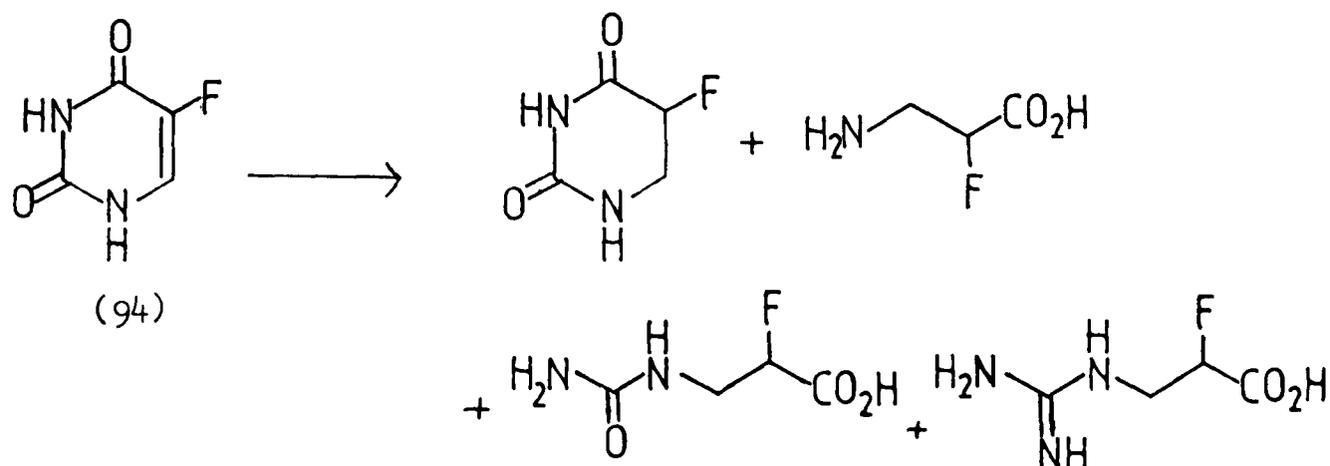


and their toxicity was attributed to degradation of the steroid side-chain, giving fluoroacetate. Insects are particularly susceptible to these compounds because they obtain their steroids by degradation of those in their food.

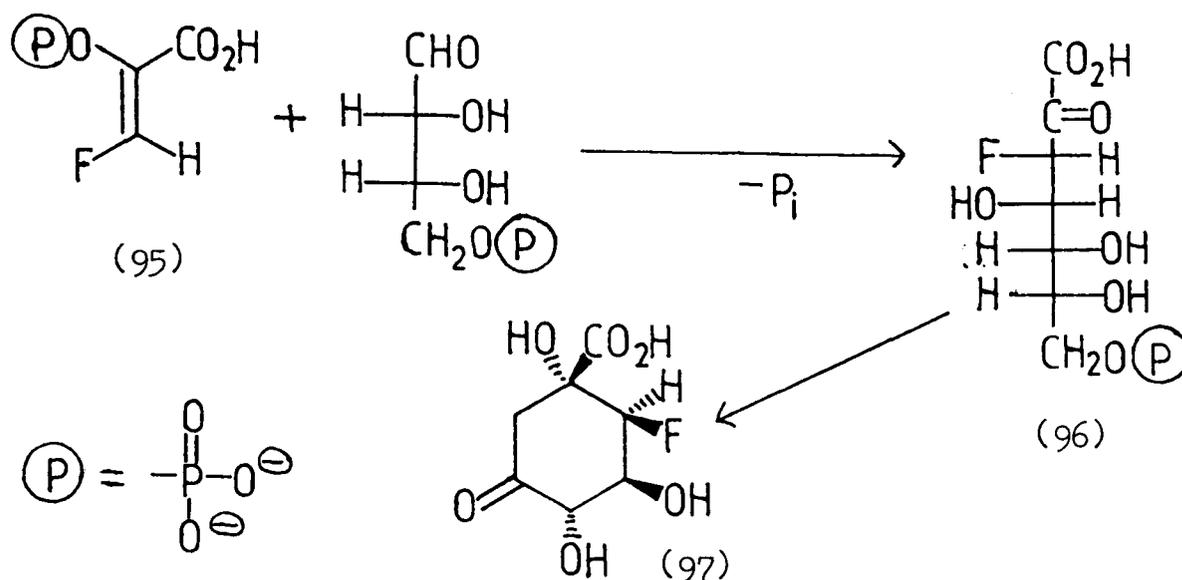
The metabolism of 5-fluorouracil (94), an antitumour drug, has been studied in various systems. It is converted by Bacillus subtilis to 5-fluorouridine, by Brevibacterium ammoniagenes to 5-fluorouridylic acid, and by Streptomyces cacaoi (var. ascoensis) to 5-fluoropolyoxins.⁷³



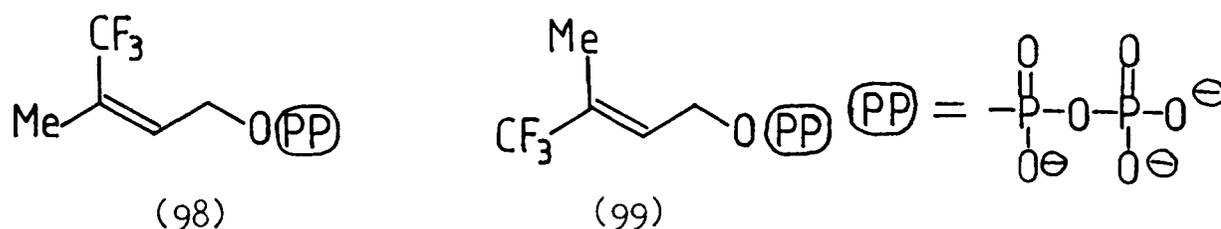
The degradation of 5-fluorouracil (94) in animals has also been studied, and various products have been isolated.⁷³



Fluorinated analogues have been used in investigations of the shikimate pathway. (Z)-3-Fluoro-2-phosphoenolpyruvic acid (95) was prepared and was shown to be a substrate for several enzymes.⁷⁴ It was converted in the presence of erythrose-4-phosphate to (3S)-3-deoxy-3-fluoro-arabino-heptulosonic acid-7-phosphate (96) by an enzyme from *Escherichia coli*, and this compound was further converted to (6S)-fluoro-3-dehydroquinic acid (97) by dehydroquinate synthase.

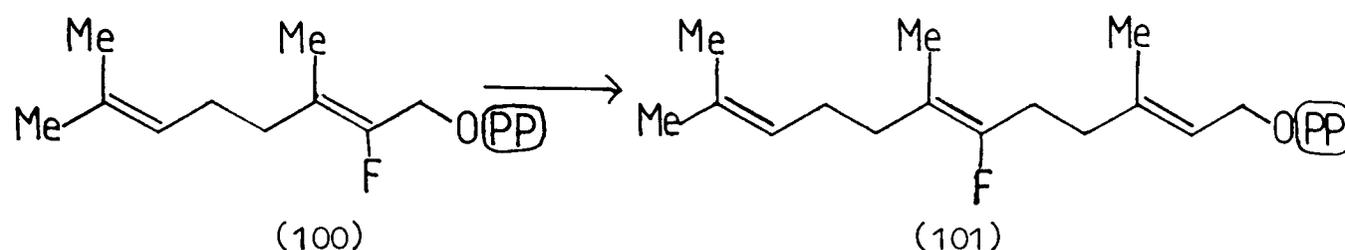


An interesting study of squalene biosynthesis used fluorinated analogues. The rates of condensation of the E- and Z- trifluoromethyl analogues (98) and (99) of dimethylallyl pyrophosphate with isopentenyl pyrophosphate, mediated by pig-liver synthetase, were substantially lower than those of the normal substrates.⁷⁵ These decreases correlate

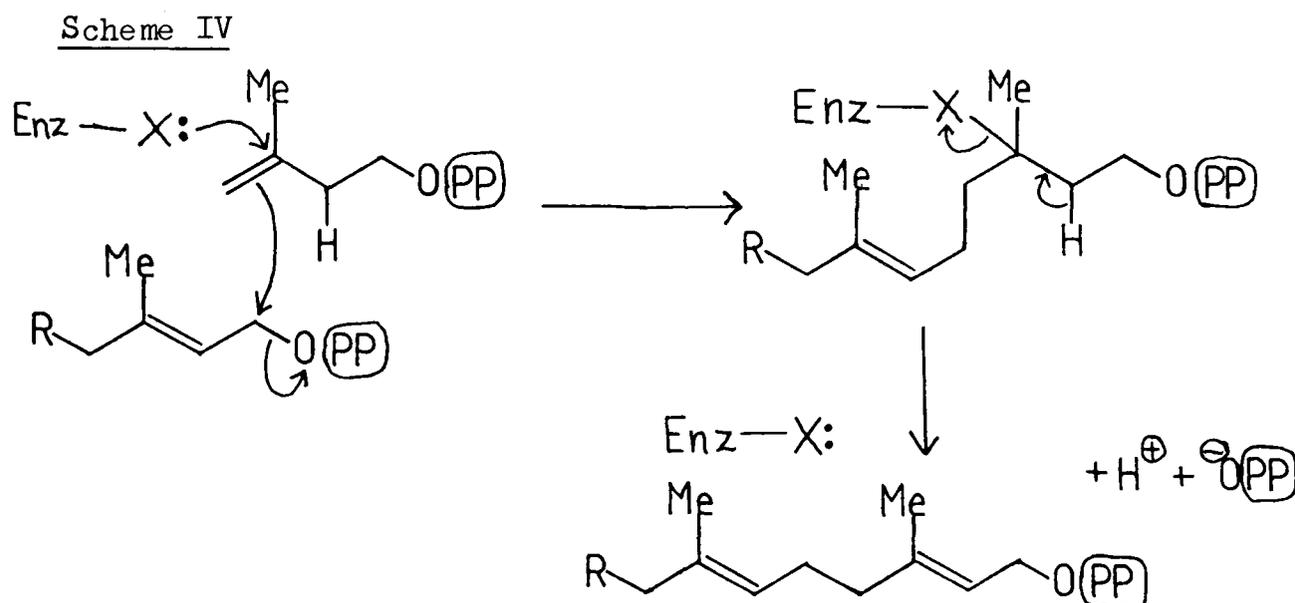


well with the decreases in solvolysis rates of the corresponding methanesulphonate derivatives under S_N1 conditions, but not under S_N2 conditions, when the fluorine substitution produced slight enhancement of the solvolysis rates. 2-Fluorogeranyl pyrophosphate (100) also

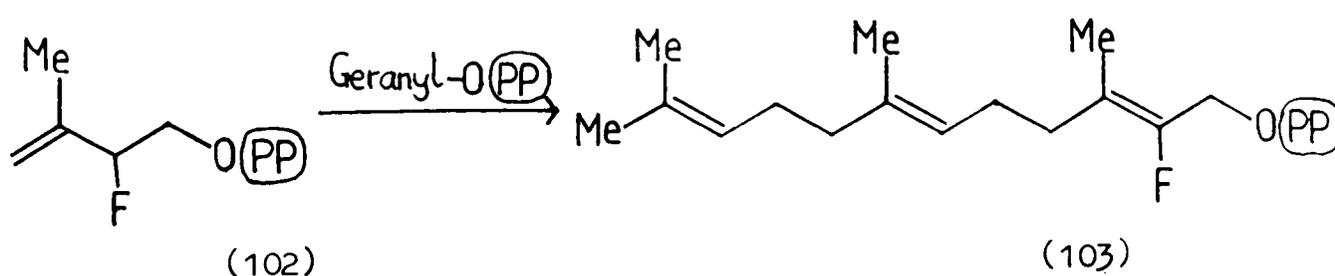
acted as a substrate for the enzyme, giving 6-fluorofarnesyl pyrophosphate (101); again, the rate decrease was comparable to the decrease in the S_N1 solvolysis rate of the analogue methanesulphonate from that of the geranyl derivative. These results suggested that the



condensation-elimination mechanism which has been proposed for this reaction (Scheme IV) was incorrect, since it involves S_N2 displacement of pyrophosphate.

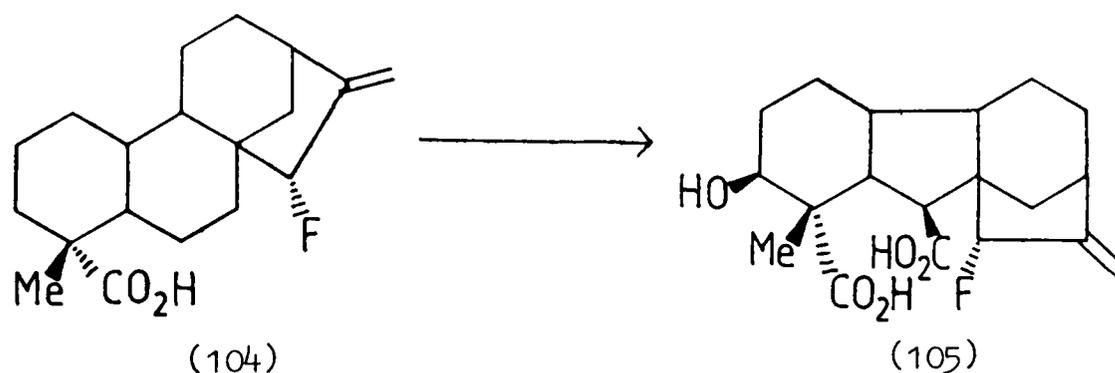


This group also showed that, in the presence of pig-liver synthetase, racemic 2-fluoroisopentenyl pyrophosphate (102) was converted into 2-fluorofarnesyl pyrophosphate (103). In the normal biosynthesis,



there is stereospecific loss of the pro-R hydrogen atom from C-2 of isopentenyl pyrophosphate; therefore, if (2R)-2-fluoroisopentenyl pyrophosphate were attacked by an enzymic nucleophile, as in Scheme IV, the covalent intermediate might be incapable of eliminating HX, and thus the enzyme might be deactivated. No significant deactivation took place, and this gave further support to the authors' arguments against an S_N2 mechanism involving a covalently enzyme-bound intermediate; however, the possibilities were not excluded that the enzymic nucleophilic attack is reversible, or that the R- isomer of (102) is not a substrate for the enzyme.

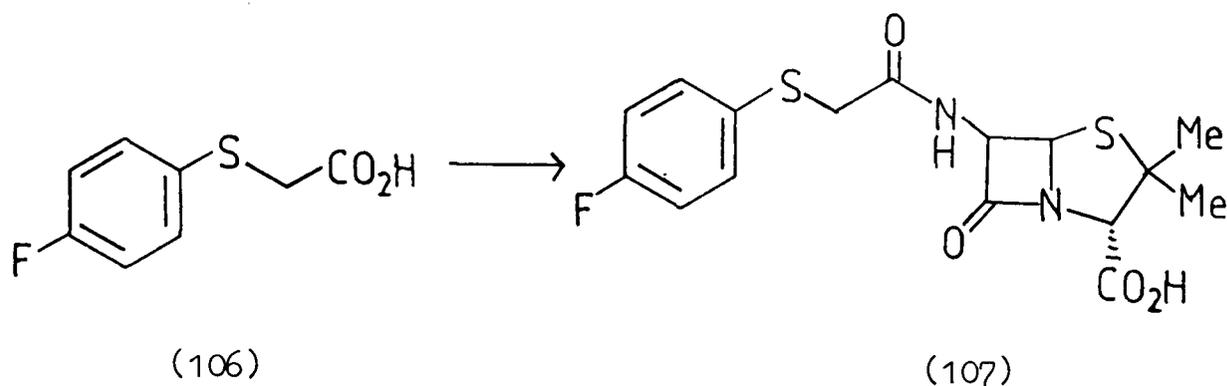
The metabolism of fluorinated precursor analogues by Gibberella fujikuroi has produced a number of fluorinated gibberellins and related compounds,⁷⁶ e.g. the acid (104) was converted by G. fujikuroi into the gibberellin A_4 analogue (105). An interesting feature of this work is the use of a suppressant of terpenoid biosynthesis, so that the only substances metabolised are the more advanced precursors such as (104).



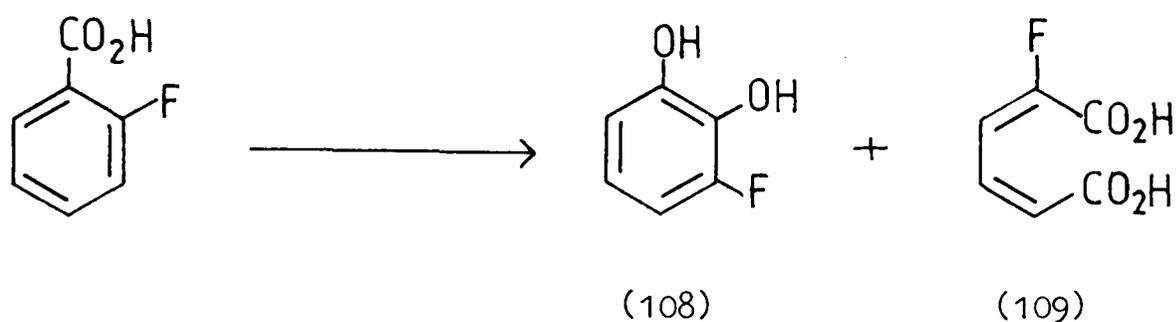
Several simple fluorine-containing aromatic compounds have been incorporated into analogues of natural products, and some examples of these conversions are given below:

(4-Fluorophenylthio)acetic acid (106) was used as the side-chain for a

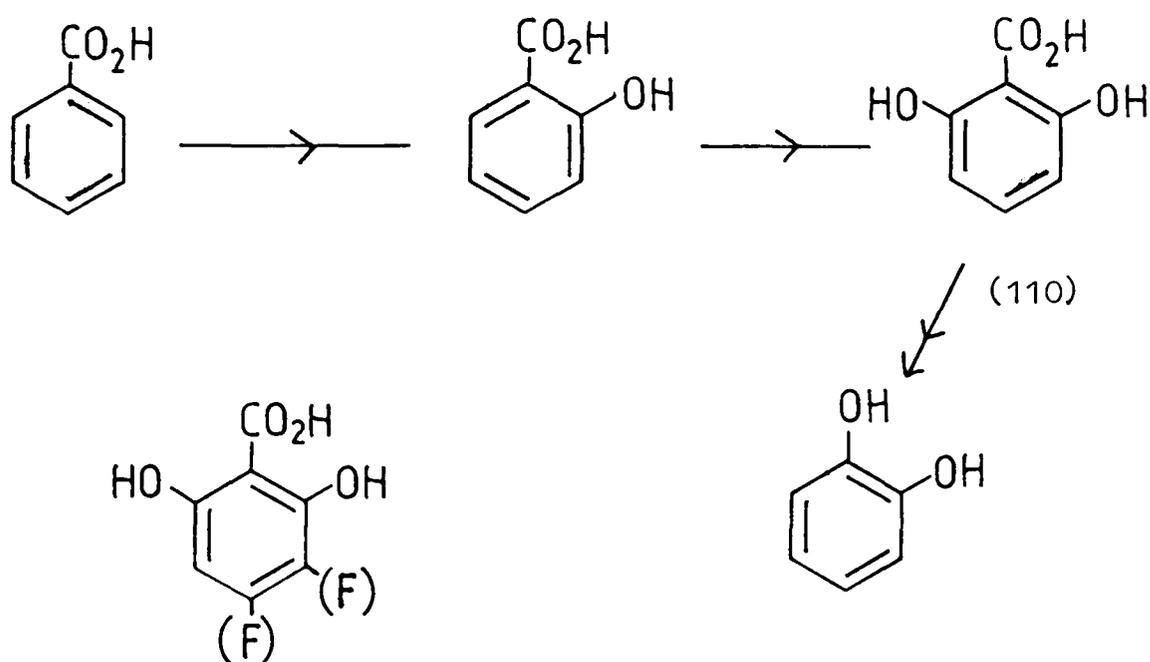
penicillin (107).⁷⁷



o-Fluorobenzoic acid was converted by a Pseudomonas species into 3-fluorocatechol (108) and 2-fluoromuconic acid (109).⁷⁸

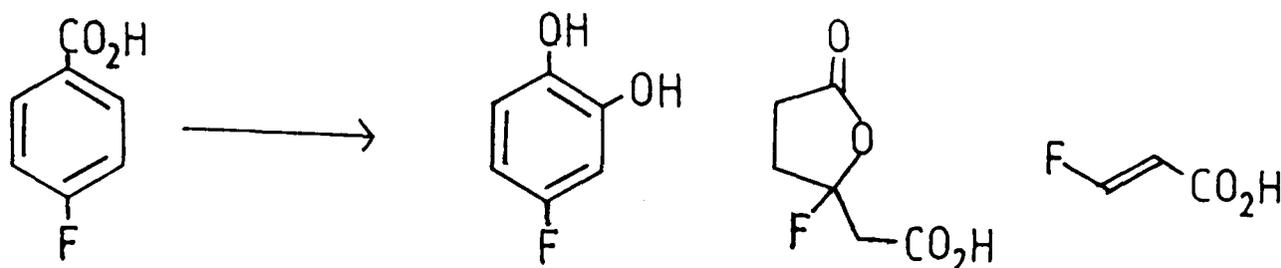


Acetobacter vinelandii transforms benzoic acid into catechol via intermediate hydroxylated compounds, as shown below.⁷⁹ m-Fluoro- and p-fluoro-benzoic acids were also converted into the intermediate (110) analogues.⁷⁹

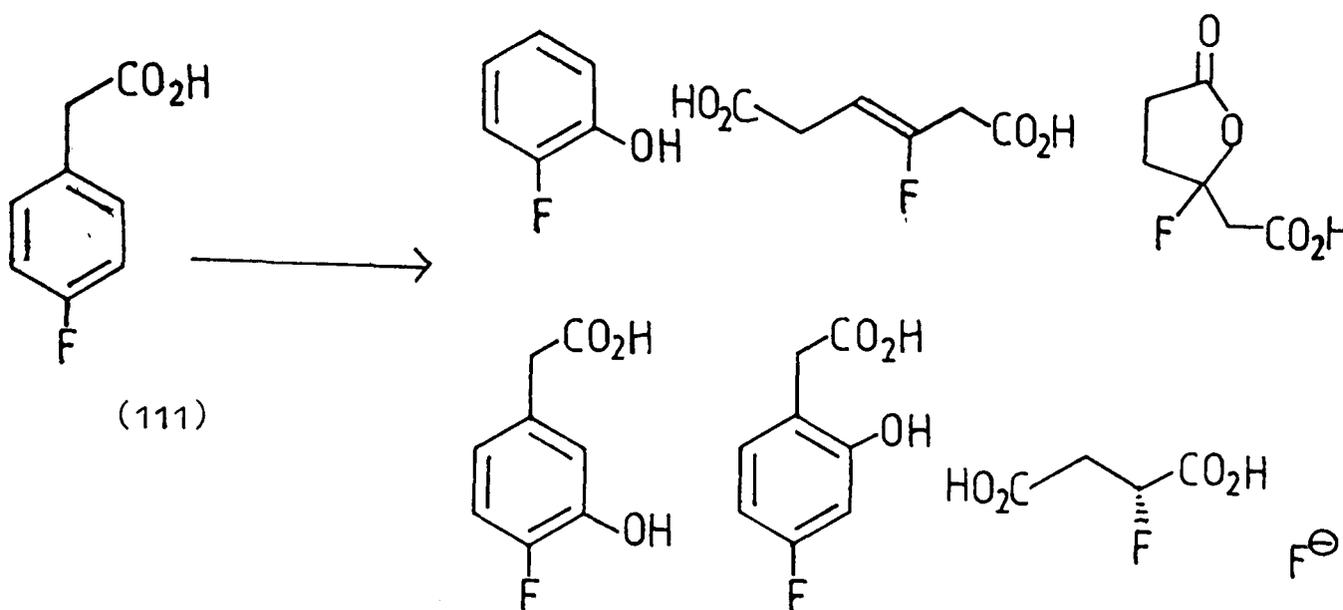


Similarly, a Pseudomonas species, given p-fluorobenzoic acid as its sole carbon source, produces 4-fluorocatechol, 4-carboxymethyl-4-fluoro-

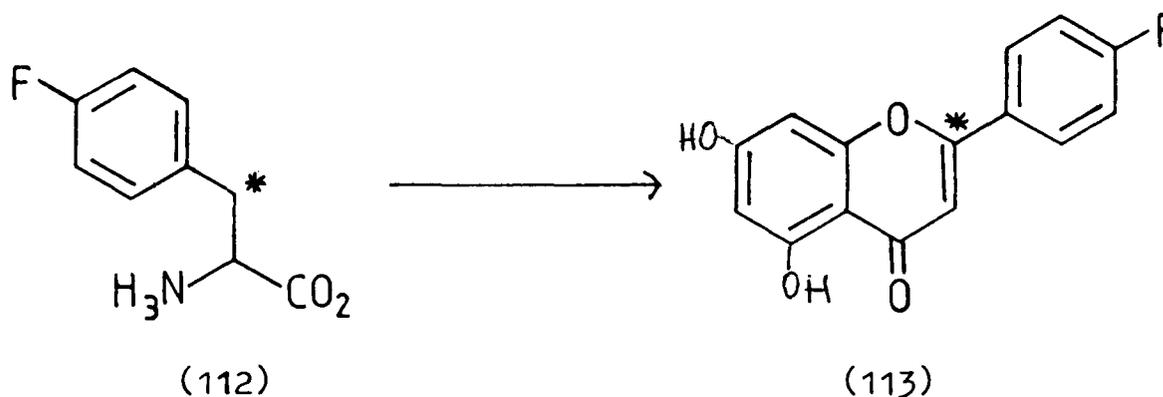
but-2-enolide, and β -fluoroacrylic acid.⁸⁰ Ultimately all the fluorine was released as F^- ion.



Also, 4-fluorophenylacetic acid (111) was oxidised by a *Pseudomonas* species to the derivatives shown below.⁸¹

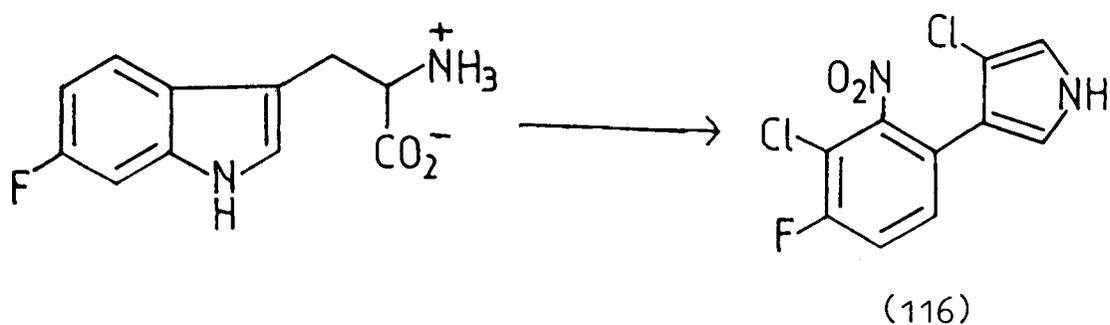
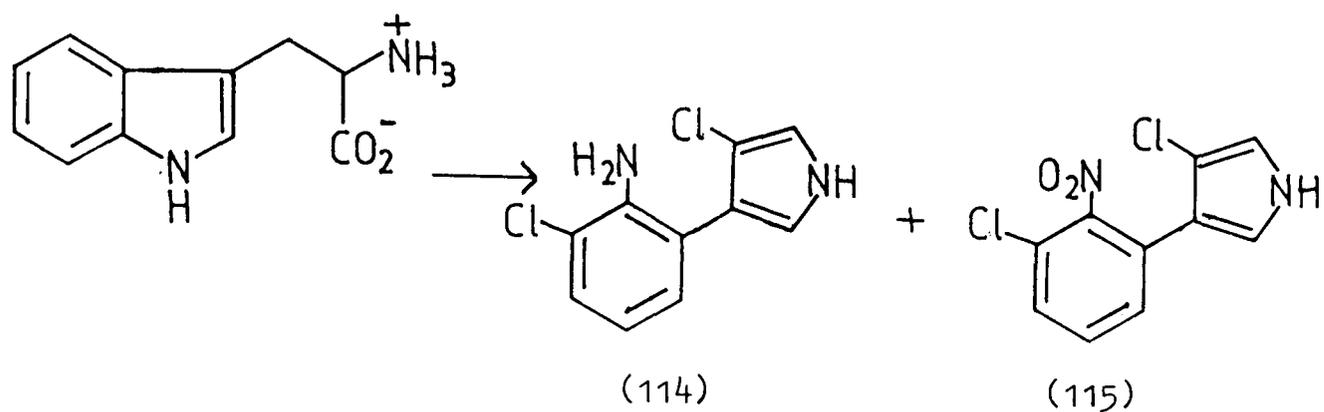


$[3-^{14}C]$ -*p*-Fluorophenylalanine (112) was incorporated by *Scutellaria galericulata* into $[2-^{14}C]$ -4'-fluorochrysin (113).⁸²

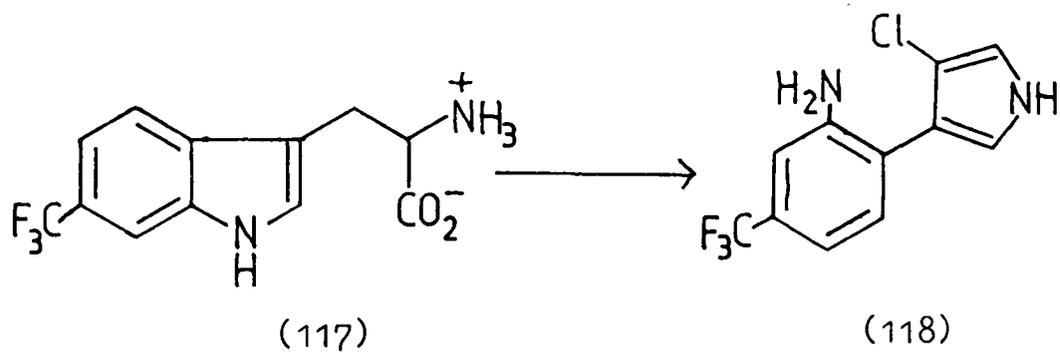


Tryptophan is metabolised by *Pseudomonas aureofaciens* to pyrrol-nitrins (114) and (115),⁸³ and the analogue 6-fluorotryptophan was

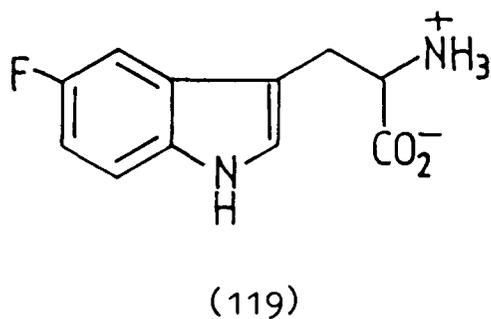
converted to the fluoropyrrolnitrin (116).⁸³ Similarly, 6-trifluoro-



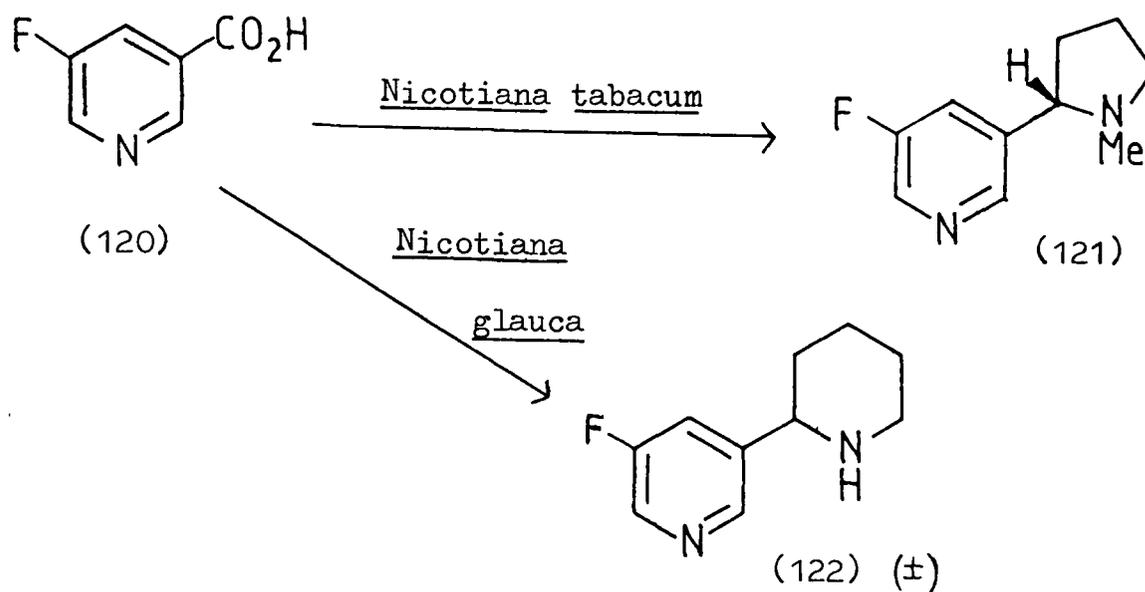
methyltryptophan (117) was converted into the analogue (118), which has not been chlorinated on the benzene ring.⁸³



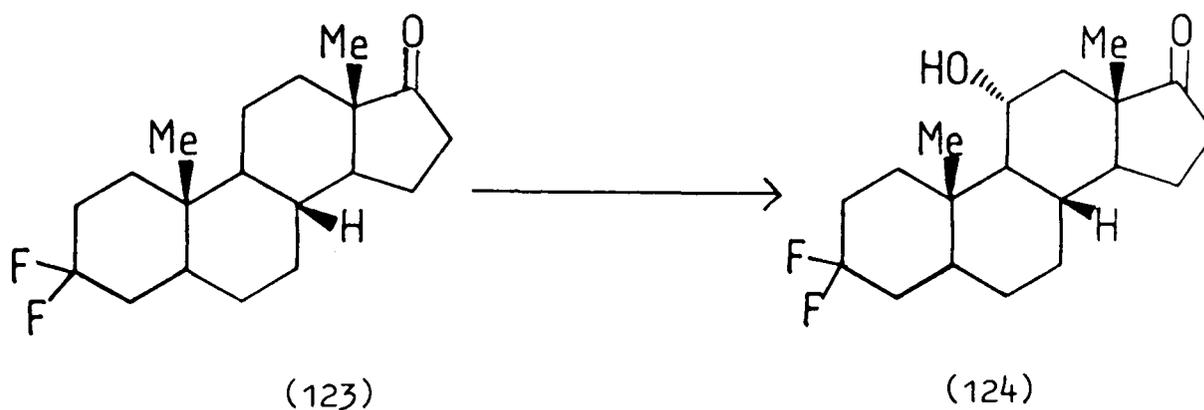
In contrast, 5-fluorotryptophan (119) was not incorporated into ergot alkaloids by *Claviceps purpurea*.⁸⁴



Leete et al. studied the conversion of 5-fluoronicotinic acid (120) into the 5-fluoro- analogues of the alkaloids nicotine (121)⁸⁵ and anabasine (122).⁸⁶

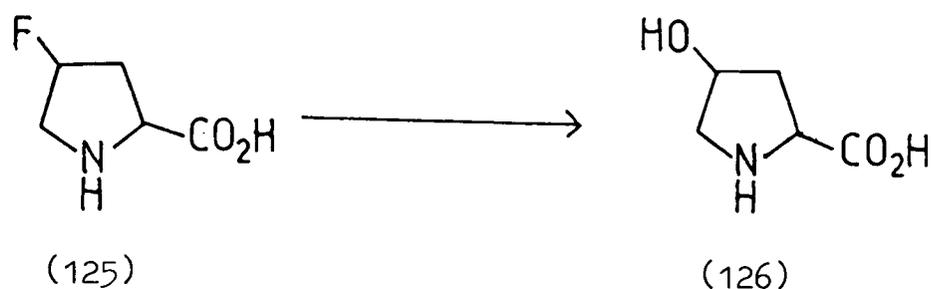


Fluorinated compounds have been used in investigations of enzymic hydroxylations. Bird et al.⁸⁷ prepared a series of fluorinated 5α -androstanones and compared their metabolism to that of the unfluorinated compounds by Calonectria decora, Rhizopus nigricans, and Aspergillus ochraceus. These fungi hydroxylate the steroids in characteristic positions, and it was found that substitution of fluorine for hydrogen did not alter these positions if the fluorine was distant from the site of hydroxylation, e.g. 3,3-difluoro- 5α -androstan-17-one (123) was converted into the 11α -hydroxy derivative (124) by A. ochraceus in 45% yield.

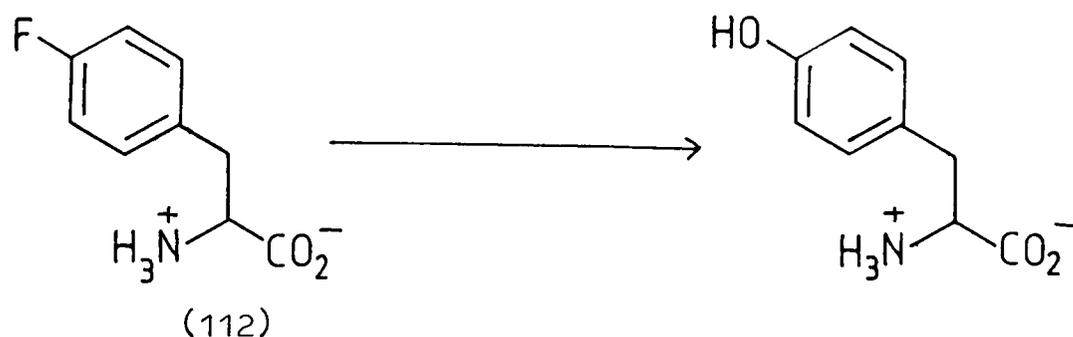


However, hydroxylation was inhibited at or adjacent to carbons bearing fluorine.

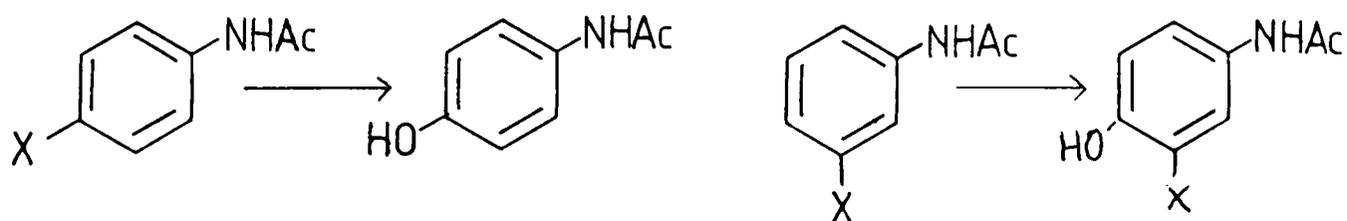
Several organisms can replace fluorine with a hydroxyl group, e.g. an enzyme preparation from Streptomyces antibioticus transforms 4-fluoroproline (125) into 4-hydroxyproline (126).⁸⁸



Liver microsomes from several species of animals can convert *p*-fluorophenylalanine (112) into tyrosine.⁸⁹



Halide-substituted acetanilides have been used for studies of hydroxylation by liver microsome preparations.^{90,91} 4-Fluoro- and 4-chloro-acetanilides were hydroxylated with loss of halogen to give 4-hydroxyacetanilide. The 3-halo- compounds were hydroxylated in the 4- position without loss of the halide substituent.



X = F or Cl

In summary, fluorinated analogues of natural substrates have been metabolised by a wide variety of biological systems. In most cases, the fluorinated precursor analogues were transformed into analogues of the "normal" metabolites; this was nearly always true when the fluorine was remote from the sites of the transformations. Interestingly, some organisms have the peculiar ability to replace fluorine with a hydroxyl group, but apart from this the fluorine substituent is not normally lost in biological transformations. There are few examples of the utilisation of the chemical properties of fluorine substituents to alter the course of biosynthetic processes; the investigation of squalene biosynthesis using fluorinated analogues of the normal pyrophosphate precursors⁷⁵ was an interesting demonstration of the deliberate use of fluorine's electronegativity to elucidate a biological mechanism.

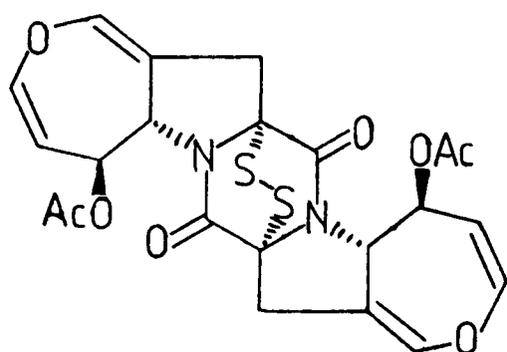
CHAPTER 2DISCUSSION2.1 Review of biosynthesis

The research described in the introduction (Section 1.3) has elucidated the earlier stages of the biosynthesis of sulphur-containing dioxopiperazines to a considerable degree. For many of these metabolites, the amino-acid precursors have been determined, and in several cases it has been shown that cyclic dipeptides are intermediates on the biosynthetic pathways. Our knowledge of the transformations taking place after cyclic dipeptide formation is, however, rather limited. The feature common to all members of this metabolite family, apart from the dioxopiperazine ring, is the presence of sulphur, either as an epipolythio- bridge or as methylthio-groups; but when this project was begun, the timing and mechanism of sulphur introduction remained obscure. The cyclisation of the aromatic rings of the precursors onto nitrogen of the dioxopiperazine ring, with concomitant oxygenation, is a characteristic of many of the metabolites, e.g. gliotoxin and the aranotins, but little is known of the biosynthetic pathways leading to these systems. The only relevant observations are that in gliotoxin and aranotin biosynthesis, no hydrogens are lost obligatorily from the aromatic ring of phenylalanine. This, coupled with the observed stereochemistry of the natural products, allowed Neuss to propose the intermediacy of arene oxides in the biosynthesis of these metabolites (Introduction, Section 1.3), but it is unlikely that any such compounds would be stable enough to be tested as precursors by normal methods. There remains, however, the possibility of obtaining information on these biosynthetic transformations indirectly, by the use of analogue

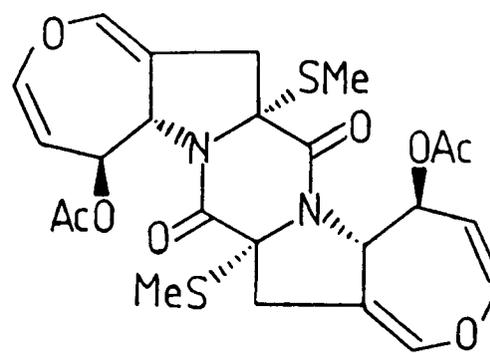
precursors. These might be accepted by the fungi as normal precursors and be metabolised to give analogues of the natural products, or they might be metabolised abnormally to give new metabolite types, whose structures might reveal something of the nature of the processes required to form them. Furthermore, a late step in the biosynthesis might be inhibited by the structural change in the precursor, and thus, the analogue of a normally transient intermediate might accumulate and be identified.

2.2 Aspergillus terreus

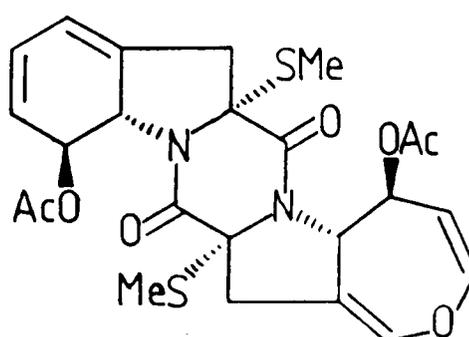
In all our feeding experiments, the fungus used was Aspergillus terreus, strain NRRL 3319. This fungus has been reported⁹² to produce acetylaranotin (38), but under our conditions the main metabolites were BDA (40) and BDAA (41), although a small amount of acetylaranotin was usually present. The change could be related to the higher temperature in our growth room, as compared with the literature conditions (28°C rather than 25°C). Other than this, the growth conditions of Miller and Trown⁹² were followed.



(38)



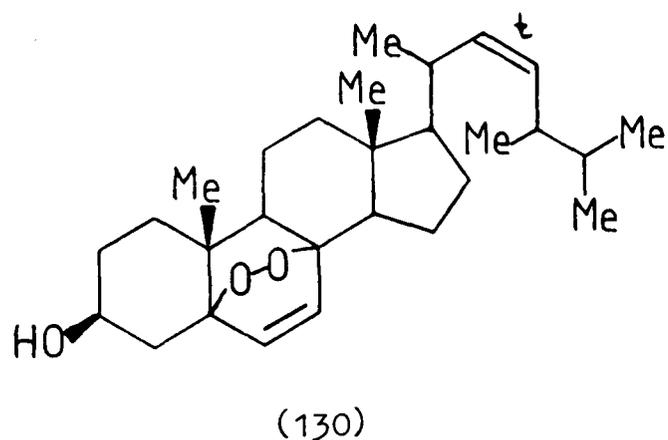
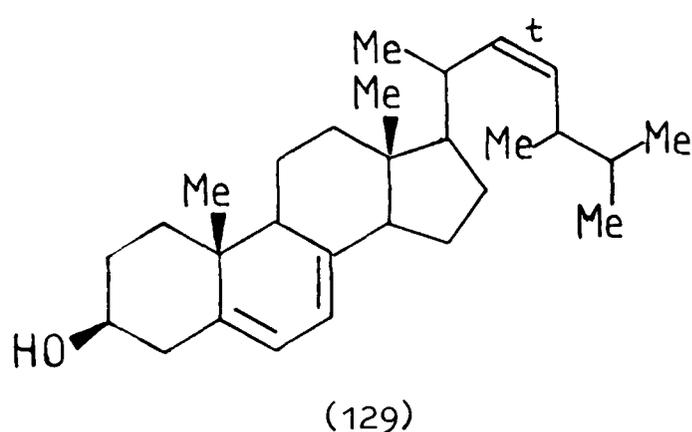
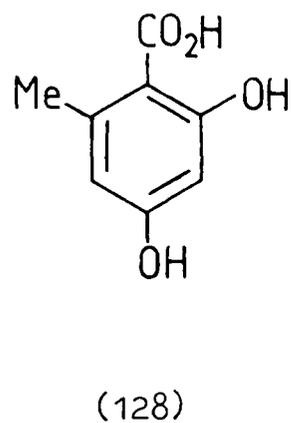
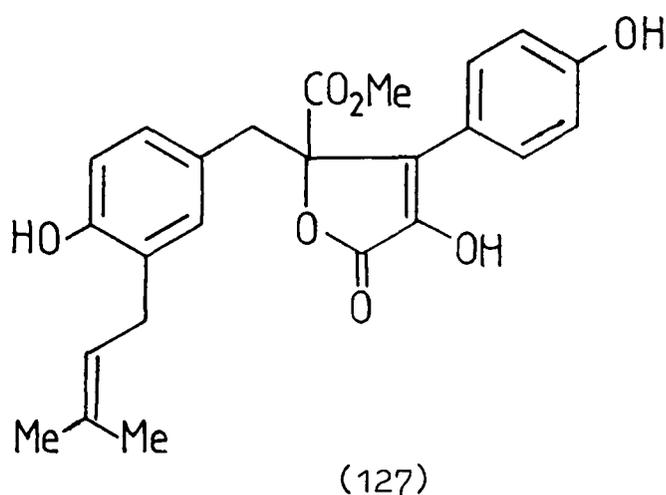
(40)



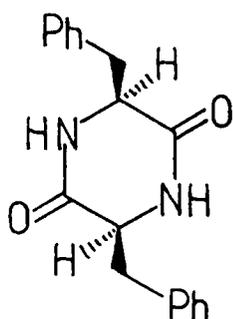
(41)

Few problems were encountered with the growth of this fungus, but one significant requirement for the production of aranotin metabolites was normal daylight illumination. Continuous artificial lighting stopped the production of aranotins, and total darkness also had an adverse effect, although in this case some aranotin production continued.

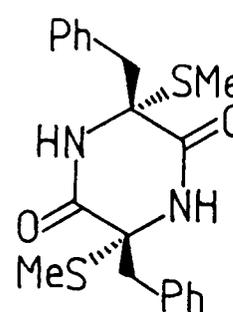
In the course of this work, a number of metabolites were isolated which had not been previously reported from this strain of *A. terreus*. The metabolite (127), previously found by Kiriya *et al.*⁹³ in another strain of *A. terreus* (IFO 8835), was isolated from several of our extracts by preparative t.l.c.. Also, small amounts of orsellinic acid (128) and ergosterol (129) were found. A compound identified as ergosterol *endo*-peroxide (130) was also obtained from some extracts, although it is not certain that this was not an artefact.



cyclo-(L-Phe-L-Phe) (42) was isolated for the first time from extracts of this fungus grown without added precursor, although it has been shown to be produced by A. terreus in an intermediate-trapping experiment.⁴⁸ The isolation of cis-di(methylthio)-cyclo-(Phe-Phe) (131)⁹⁴ is discussed in Section 2.6.



(42)



(131)

2.3 Preparation and feeding of fluorinated analogues

2.3.1 Rationale

It had already been demonstrated⁴⁸ that cyclo-(L-Phe-L-Phe) (42) is an intermediate in the biosynthesis of arantins by Aspergillus terreus. Therefore, we thought that this compound would be a suitable subject for mimicking with analogues. The analogues which we chose to prepare were fluorinated on the aromatic rings of cyclo-(L-Phe-L-Phe); these were thought to be suitable for several reasons.

i) The fluorine substituent, being of small size (Van der Waals radius ca. 1.3 Å) should not change the molecule sterically so much that it would no longer be accepted by the fungal enzymes.

ii) A fluorine substituent on the aromatic ring should have a significant effect on the formation of arene oxides, but should be sufficiently removed from the site of sulphur introduction to have little effect on this process. Thus it was thought that there might

be produced metabolites containing sulphur, but with abnormally oxygenated (or unoxygenated) fluorinated aromatic rings.

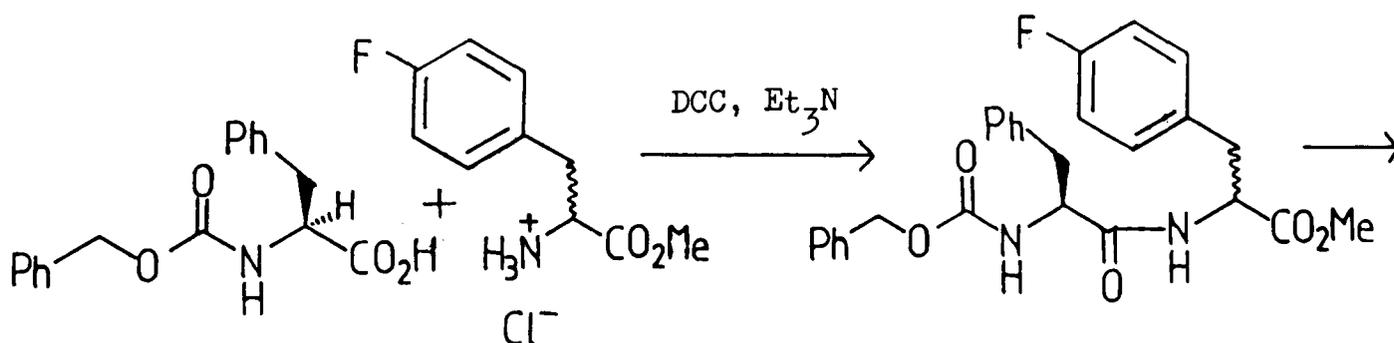
iii) Since the only fluorinated compounds in the cultures would be derived from the materials fed, the unnatural metabolites would be easily detectable by ^{19}F n.m.r. spectroscopy of fungal extracts or substances derived therefrom.

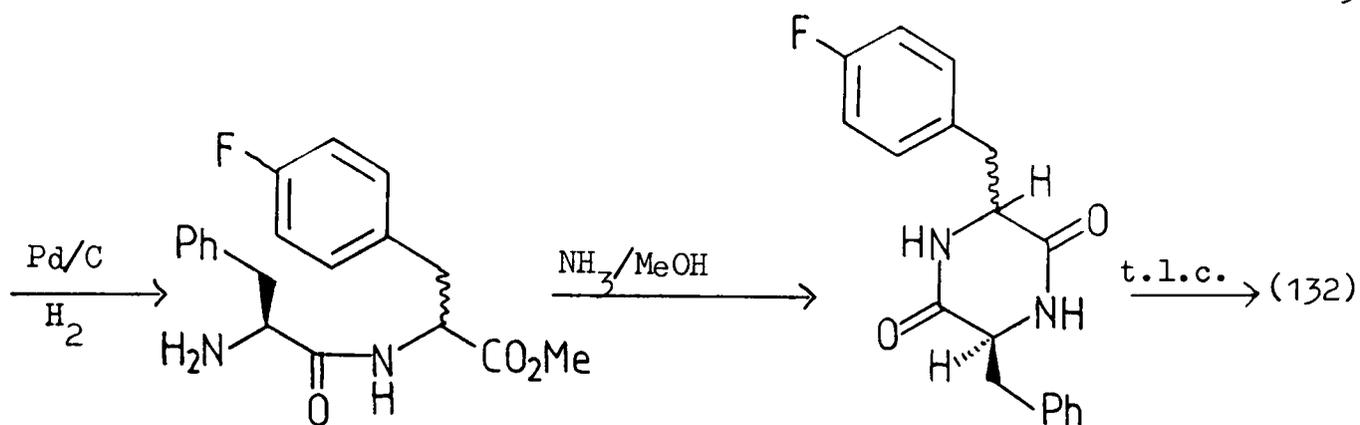
Also, fluorinated analogues had been used successfully before in biological systems, as detailed in the Introduction (Section 1.4).

2.3.2 Preparation of fluorinated analogues of cyclo-(L-Phe-L-Phe)

We therefore set out to prepare a range of fluorinated analogues of arantoin precursors. The only such compound available commercially in radioactive form was DL-p-fluorophenylalanine, although DL-o- and m-fluorophenylalanines were available unlabelled.

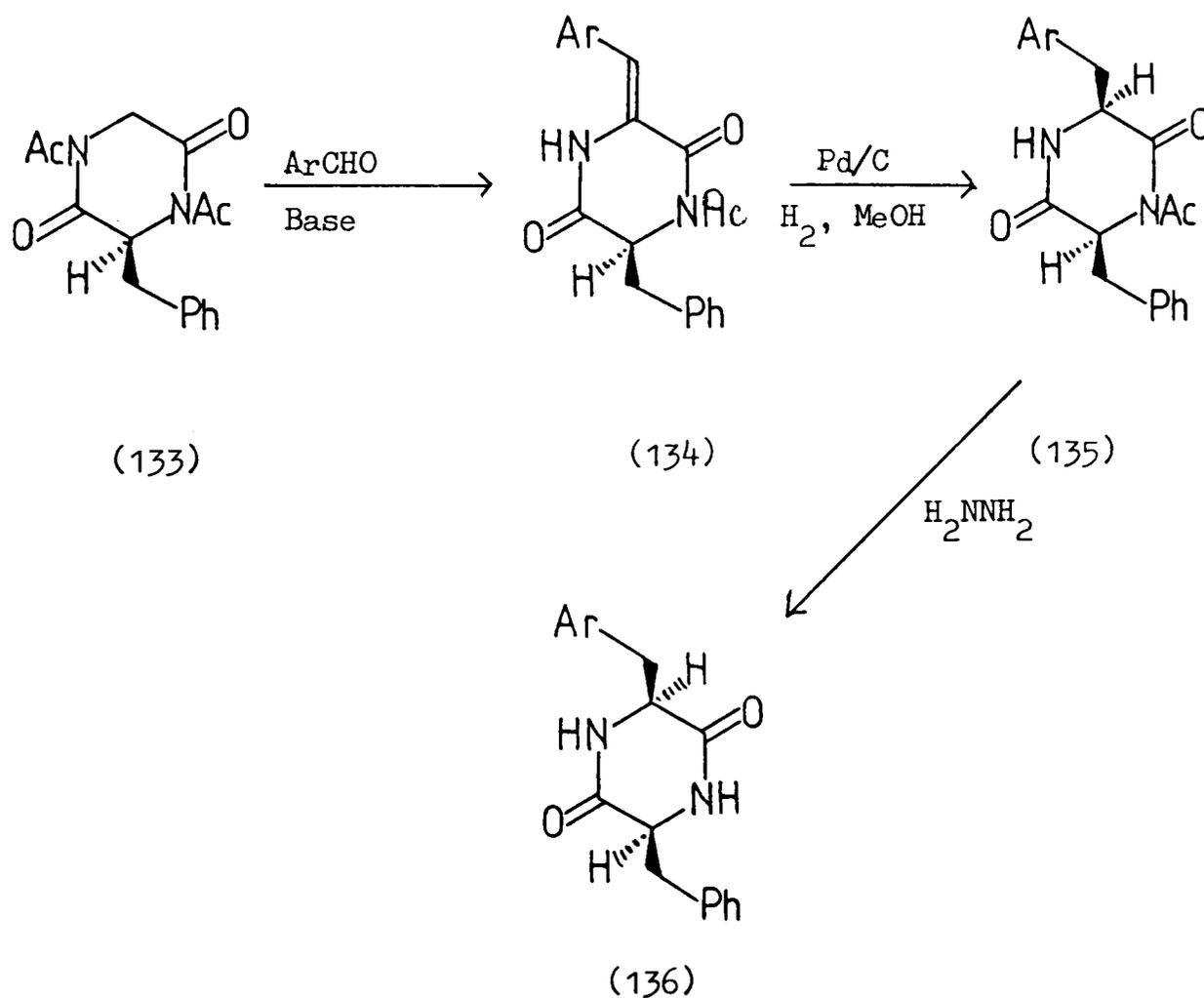
Our first objective was the preparation of cyclo-(L-Phe-L-p-Fluorophenylalanine) (132). This was achieved by a standard peptide-type synthesis⁹⁵ from L-phenylalanine and DL-p-fluorophenylalanine, followed by separation of the 1:1 mixture of LL- and LD- isomers by preparative t.l.c. (solvent system; chloroform: methanol: acetic acid, 9:0.5:0.5), but rather poor yields were obtained since only half of the product mixture was of use, and the t.l.c. separation was difficult; more than 20 mg of mixture per 20 x 20 cm, 1 mm-thick plate caused streaking. We therefore thought it desirable to devise a synthesis of the single, LL- isomer.





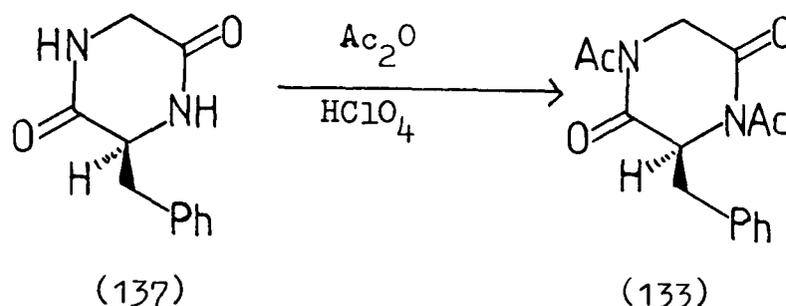
DCC = dicyclohexylcarbodiimide

It was known that aldehydes could be condensed with N,N'-diacetyl cyclic dipeptides containing a glycylic residue (cf. the Perkin condensation) giving alkylidene or arylidene derivatives.⁹⁶⁻⁹⁸ It was reasonable to suppose that this reaction could be carried out with substituted benzaldehydes and N,N'-diacetyl-cyclo-(Gly-L-Phe) (133), and that the arylidene derivatives so produced (134) would be catalytically hydrogenated to give predominantly the cis- (LL) compounds (135);⁹⁹ deacetylation would then give the desired analogues (136) of cyclo-(L-Phe-L-Phe).

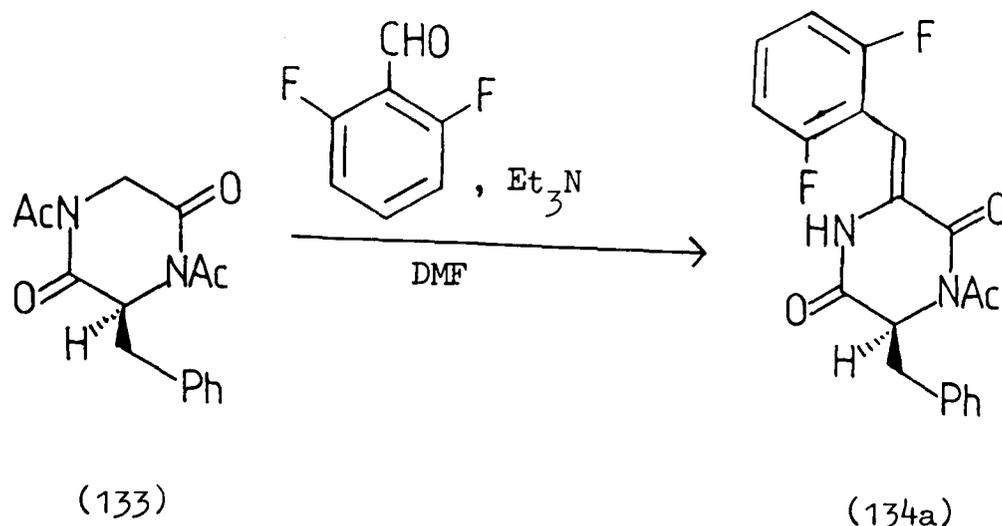


Accordingly, we embarked on this route.

cyclo-(Gly-L-Phe) (137)¹⁰⁰ was prepared by the general method of Blaha.⁹⁵ It was necessary to find a method of acetylation which would not cause racemisation of the cyclic dipeptide. Treatment with acetic anhydride under neutral or basic (pyridine or sodium acetate) conditions gave the diacetyl derivative (133),¹⁰¹ but with substantial racemisation. Interestingly, the L- enantiomer had a higher melting point (103-104°C) than the racemate (85-87°C), and crystallised separately from solutions of the partially racemised compound (133). Acid-catalysed acetylation (HClO₄) proceeded without racemisation to give the desired L-enantiomer in good yield.



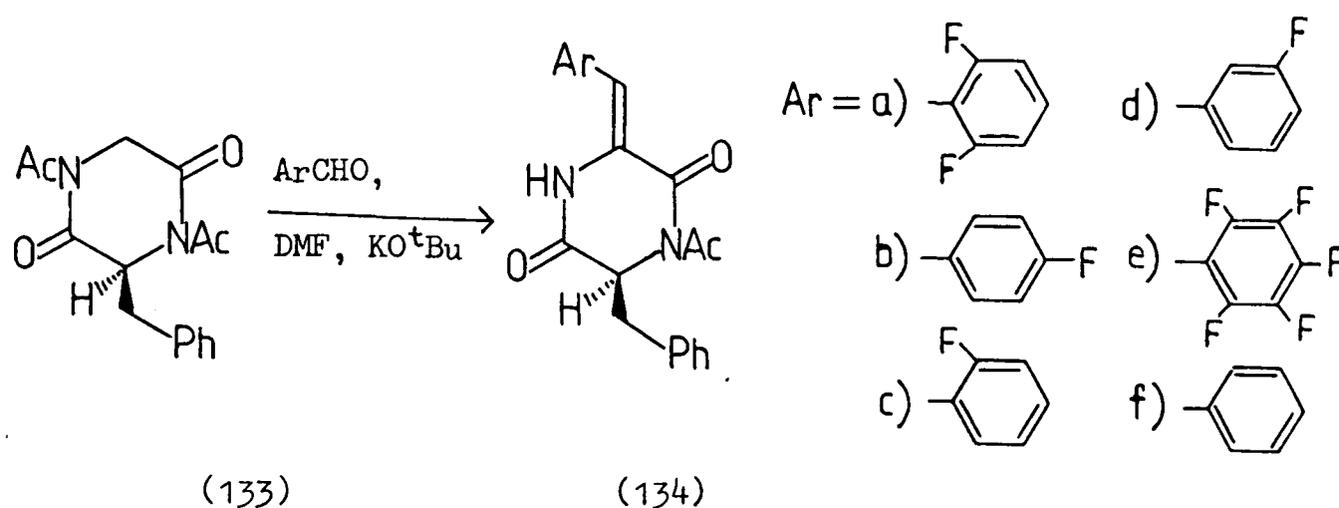
Our first attempts at condensation of the diacetyl cyclic dipeptide (133) with aromatic aldehydes were conducted using 2,6-difluorobenzaldehyde. Gallina and Liberatori⁹⁷ had found that activated aldehydes e.g. p-nitrobenzaldehyde, would condense with N,N'-diacetyl-cyclo-(Gly-Gly) in DMF in the presence of triethylamine, but that more unreactive aldehydes required a stronger base (potassium t-butoxide). Because of the necessity to avoid racemisation, we tried the weaker base, triethylamine, first. Reaction of N,N'-diacetyl-cyclo-(Gly-L-Phe) (133) in DMF with 2,6-difluorobenzaldehyde and triethylamine took place over ca. 3 days at room temperature, giving the N-acetyl arylidene compound (134a) in reasonable yield. The acetyl group of



the glycyI residue was lost, in accordance with the mechanism proposed by Gallina and Liberatori,⁹⁷ and Z- geometry of the double bond was inferred by comparison of the chemical shift of the vinylic proton in the n.m.r. spectrum to those of known examples.⁹⁷ However, the considerable variation in optical activity of samples from different runs suggested that some racemisation was taking place, and this was confirmed by n.m.r. spectroscopy with the use of a chiral europium shift reagent; the ratio of L- to D- isomer in the sample of highest optical activity was ca. 2:1. An alternative method of preparation of (134), which would not cause racemisation, was therefore sought.

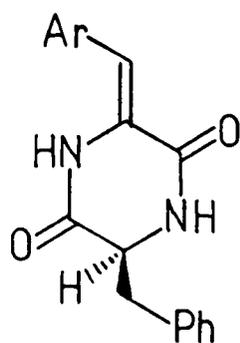
Kanmera et al.⁹⁸ had used a very similar route to our proposed method, in their asymmetric syntheses of α -amino-acids. They condensed N,N'-diacetyl-cyclo-(L-Ala-Gly) with aldehydes, and after deacetylation of the product, catalytic hydrogenation gave predominantly the LL-cyclic dipeptide, which was hydrolysed to its constituent amino-acids. The condensation was carried out using potassium t-butoxide at room temperature, and no racemisation was observed. We therefore tried potassium t-butoxide as the base in our reaction. Using one equivalent of base at 0°C, the condensation of 2,6-difluorobenzaldehyde with (133) was virtually complete after 1h, and n.m.r. spectroscopy of the

product (134a) using the chiral europium shift reagent did not detect any D- isomer. This method was therefore used to prepare the series of compounds (134a-f); all of these were obtained as oils.



The nature of the aldehyde did not appear to have a great effect on the rate of the reaction. The reasons for the conservation of optical activity when potassium t-butoxide was used rather than triethylamine, are not clear, but one factor might be that the starting material is consumed much faster than in the reactions with triethylamine; once the acetyl group is removed from the glycylyl residue, the α -hydrogen of the phenylalanyl residue is likely to become much less acidic.

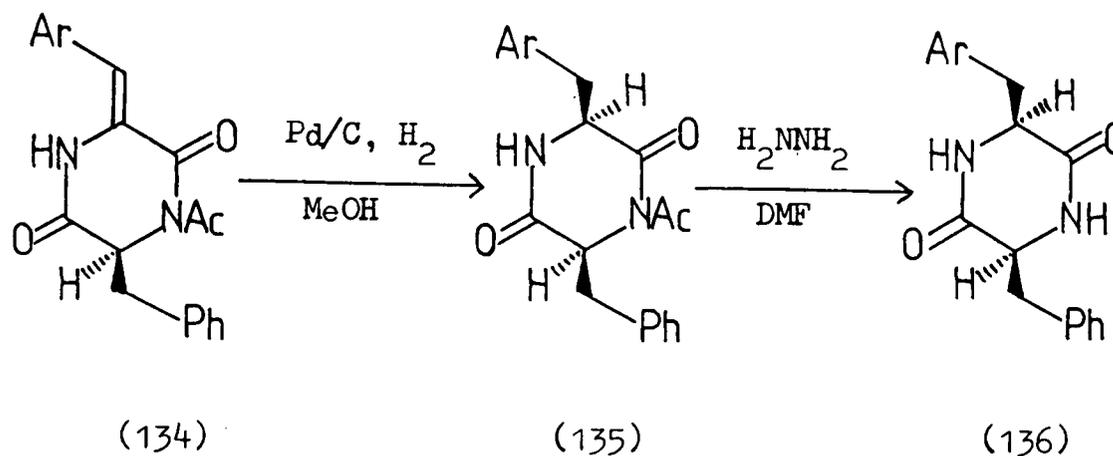
Preliminary experiments showed that the products of deacetylation (138) of the arylidene compounds were very insoluble in most solvents,



(138)

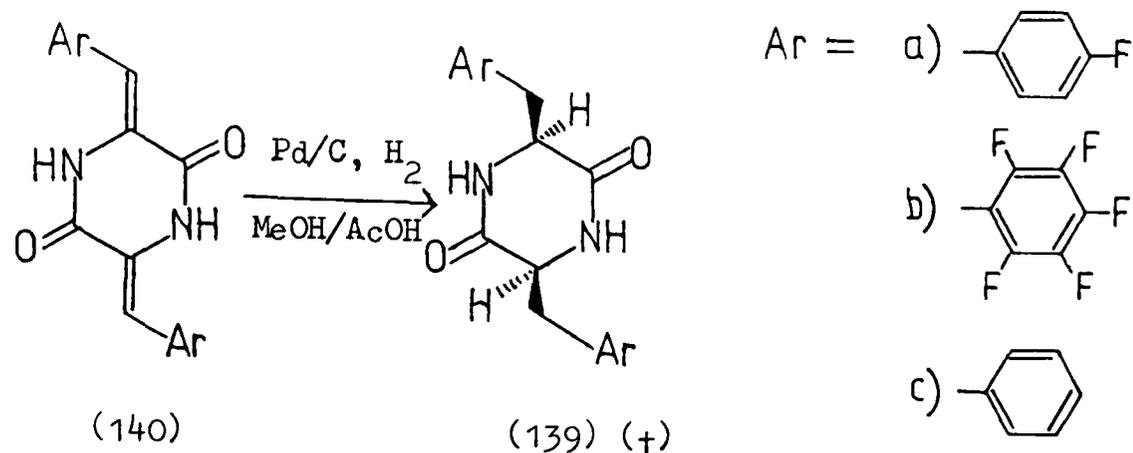
and this discouraged the use of the method of Kanmera et al.⁹⁸ for the hydrogenation of the double bond. The N-acetyl arylidene compounds

(134) were much more soluble; catalytic hydrogenation of these, in methanol with a trace of acetic acid, was usually complete after 24 h. The N-acetyl cyclic dipeptides (135) were not isolated; hydrazinolysis of the crude products of hydrogenation gave the cyclic dipeptides (136a-f), which were easier to purify.

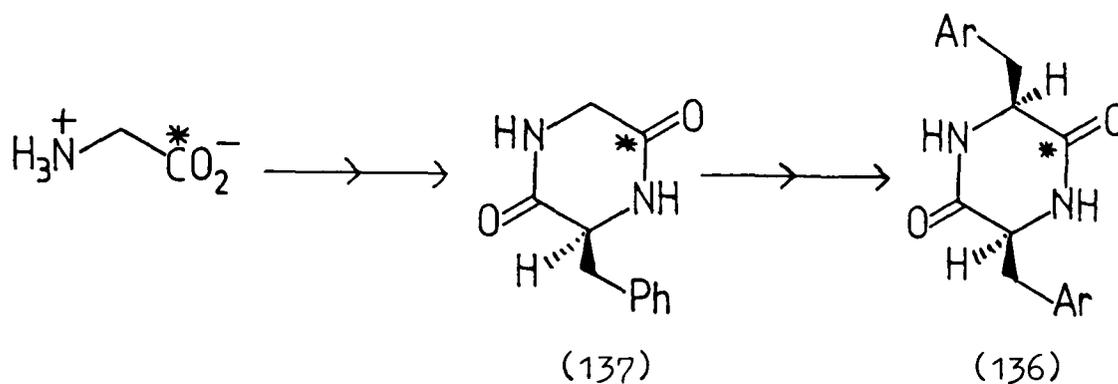


T.l.c. and n.m.r. spectroscopy gave no indication of the presence of any trans- isomers in the crystallised cyclic dipeptides, thus verifying that hydrogenation had taken place with virtually complete stereospecificity on the opposite face of the arylidene compound to the phenylalanyl side-chain. As a further demonstration that racemisation did not occur at any stage, cyclo-(L-Phe-L-Phe) (42 = 136f) was prepared by the above method, and its optical rotation was comparable to that of material prepared by other methods or isolated from natural sources.^{102,103}

The symmetrical fluorinated analogues of cyclo-(Phe-Phe) (139a and 139b) were prepared by a similar method. Di-(p-fluorobenzylidene)-piperazinedione (140a) was prepared by Sasaki's general method,⁹⁶ and the preparation of di-(pentafluorobenzylidene) piperazinedione (140b) followed the method of Gallina and Liberatori.⁹⁷ Both compounds were catalytically hydrogenated in methanol-acetic acid to give exclusively the cis- racemic cyclic dipeptides (139a and b).

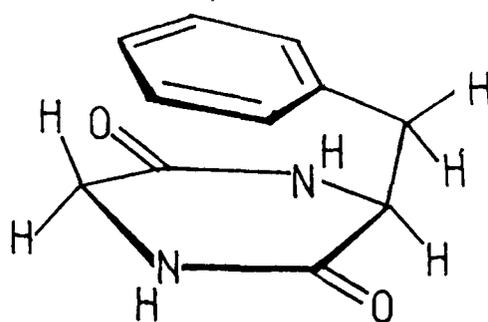


The radio-labelled compounds were prepared by the foregoing methods, using $[1-^{14}\text{C}]$ glycine to prepare the intermediate cyclo-($[1-^{14}\text{C}]$ Gly-L-Phe); hence all the radioactive cyclic dipeptides used in the feeding experiments were labelled at the carbonyl of the fluorinated phenylalanyl residue.



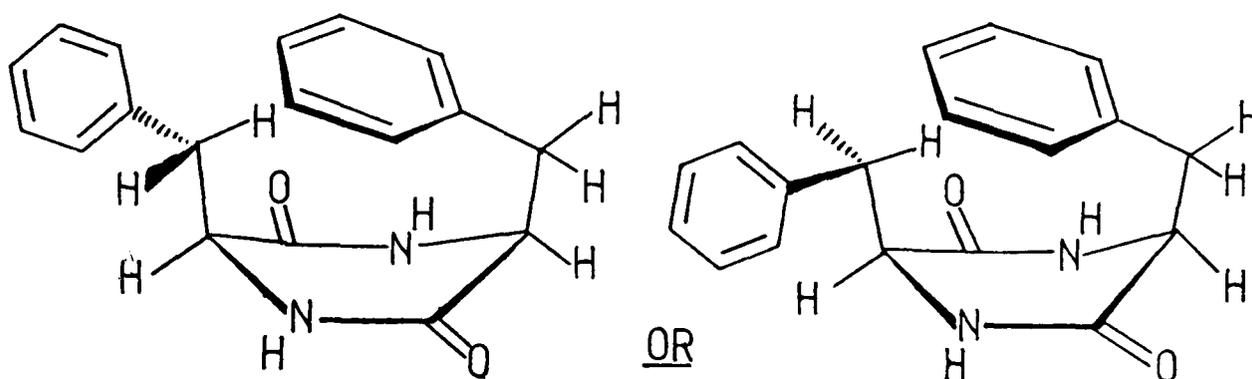
2.3.3 N.m.r. spectra of cyclo-(Phe-Phe) analogues

There has been considerable interest shown in the n.m.r. spectra of cyclic dipeptides with aromatic side-chains.¹⁰⁴⁻¹⁰⁶ In compounds such as cyclo-(Gly-Phe), the n.m.r. signal of one proton of the glycyll methylene group is shifted substantially upfield (by ca. 1 p.p.m. from the expected position at δ ca. 4.0), and this is attributed to a tendency for the molecule to adopt a conformation where the phenyl group is lying over the dioxopiperazine ring; the aromatic ring then shields the cis- glycyll proton. In cyclo-(L-Phe-L-Phe) (42), the



cyclo-(Gly-Phe)

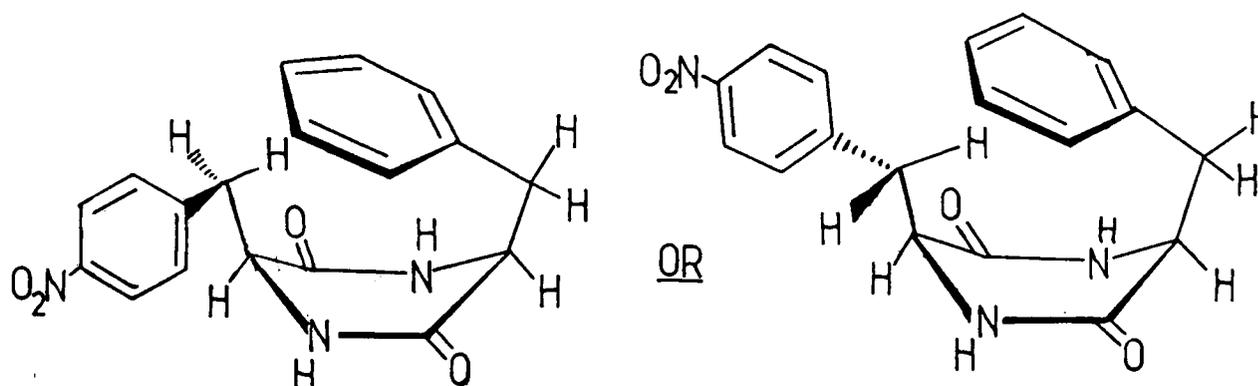
signals due to the protons of the methylene group are split into an ABX system; one of the methylene proton signals is to considerably higher field (δ 2.34) than expected from comparison with other phenylalanine derivatives. (methylene signals at δ 3.1). This shift is again attributed to shielding by the aromatic ring of a benzyl methylene proton at the other end of the molecule;¹⁰⁴ the side-chains might prefer to occupy positions such that one of the methylene protons is in the shielding region of the phenyl group of the other side-chain.



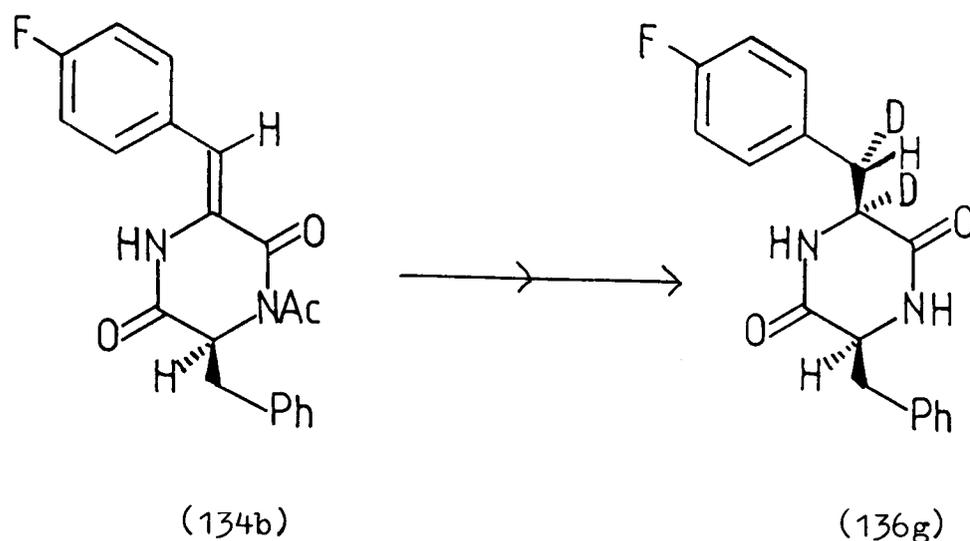
The larger coupling constant to the α -proton associated with the upfield methylene proton signal is consistent with a trans- vicinal relationship, in agreement with the postulate that this proton is preferentially located nearest to the distal benzyl group.

Because of time averaging, the signals from both methylene groups in cyclo-(L-Phe-L-Phe) (42) are identical. It was noted by Liberek et al.¹⁰⁴ that in unsymmetrical dipeptides of this type, the methylene proton signals of the group with the more electron-rich aromatic ring were shifted less than those of the electron-poor ring. They inferred

that this was due to the electron-rich ring spending a greater fraction of the time over the dioxopiperazine, so that the dominant configuration was that shown below (for the *p*-nitrophenyl analogue).

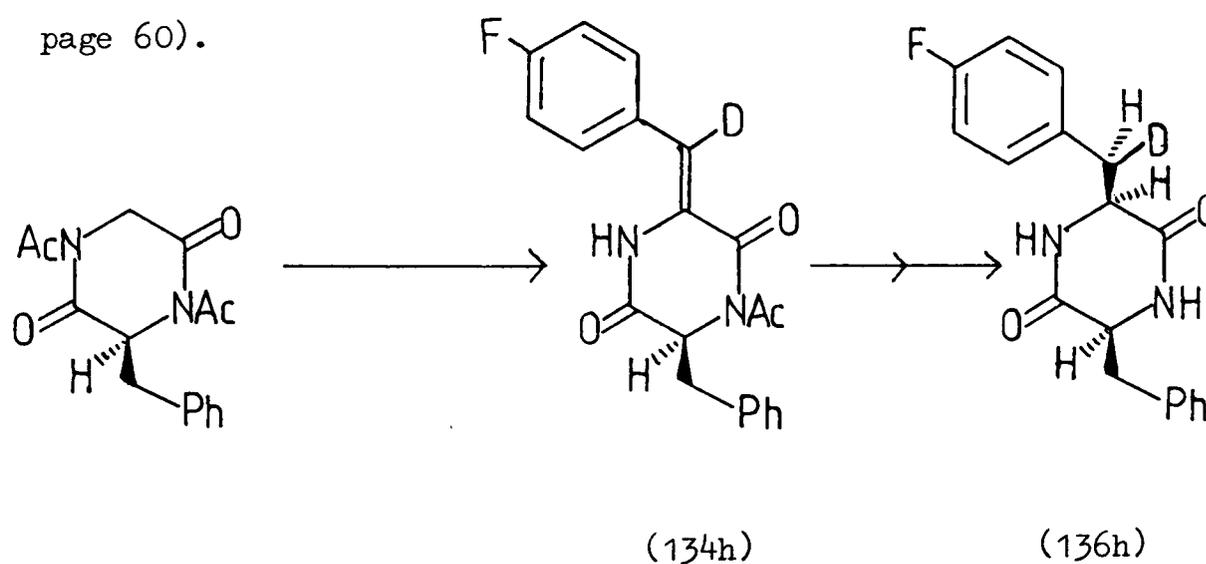


These workers¹⁰⁴ located the signals for each residue by non-stereospecific deuterium labelling of phenylalanine in the methylene group. Our synthesis of cyclo-(Phe-Phe) analogues, as described in Section 2.4, had potential for stereospecific labelling with deuterium; we could thus determine which proton was abnormally shielded. cyclo-(L-Phe-L-*p*-Fluorophe) (136b) was chosen for this study, since one of the methylene protons was at exceptionally high field in the n.m.r. spectrum in TFA. (δ 2.07). The arylidene compound (134b) was reduced with deuterium gas instead of hydrogen, and the product was deacetylated as before. The isolated cyclic dipeptide (136g) was stereospecifically deuteriated at the α -position and one of the methylene positions in the *p*-fluorophenylalanyl residue; the n.m.r. spectrum showed a collapse of the high-field doublet of doublets at δ 2.07 to a broad singlet, and the disappearance of a one-proton signal at lower field (δ 3.01). Assuming Z-geometry for the double bond in the arylidene compound, the pro-S hydrogen of the cyclic dipeptide (136g) should be replaced by deuterium; thus, the high-field n.m.r. signal is from the pro-R hydrogen of the fluorinated aromatic residue.



Similarly, when [formyl-D]-p-fluorobenzaldehyde was used to prepare the arylidene compound (134h), hydrogenation and deacetylation gave the cyclic dipeptide (136h); in this case the high-field, pro-R proton signal was not observed.

The proton n.m.r. spectra of these deuteriated compounds, together with that of the undeuteriated (136b), are illustrated in Figure 1. (on page 60).



These data suggest a predominant configuration for (136b) as shown below, with the pro-R hydrogen of the fluorophenylalanyl residue closest to the phenyl ring.

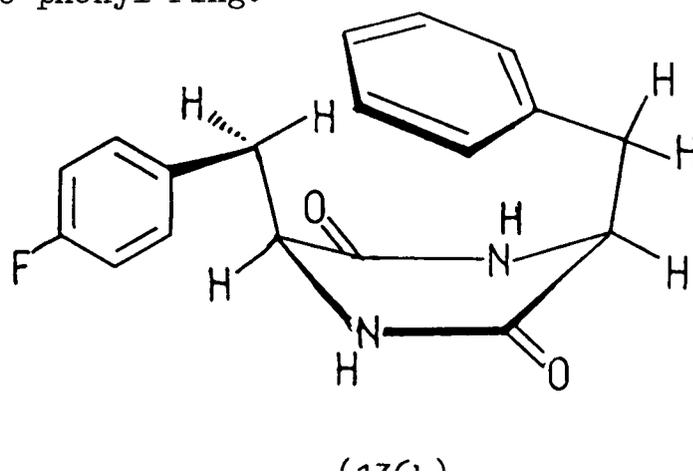
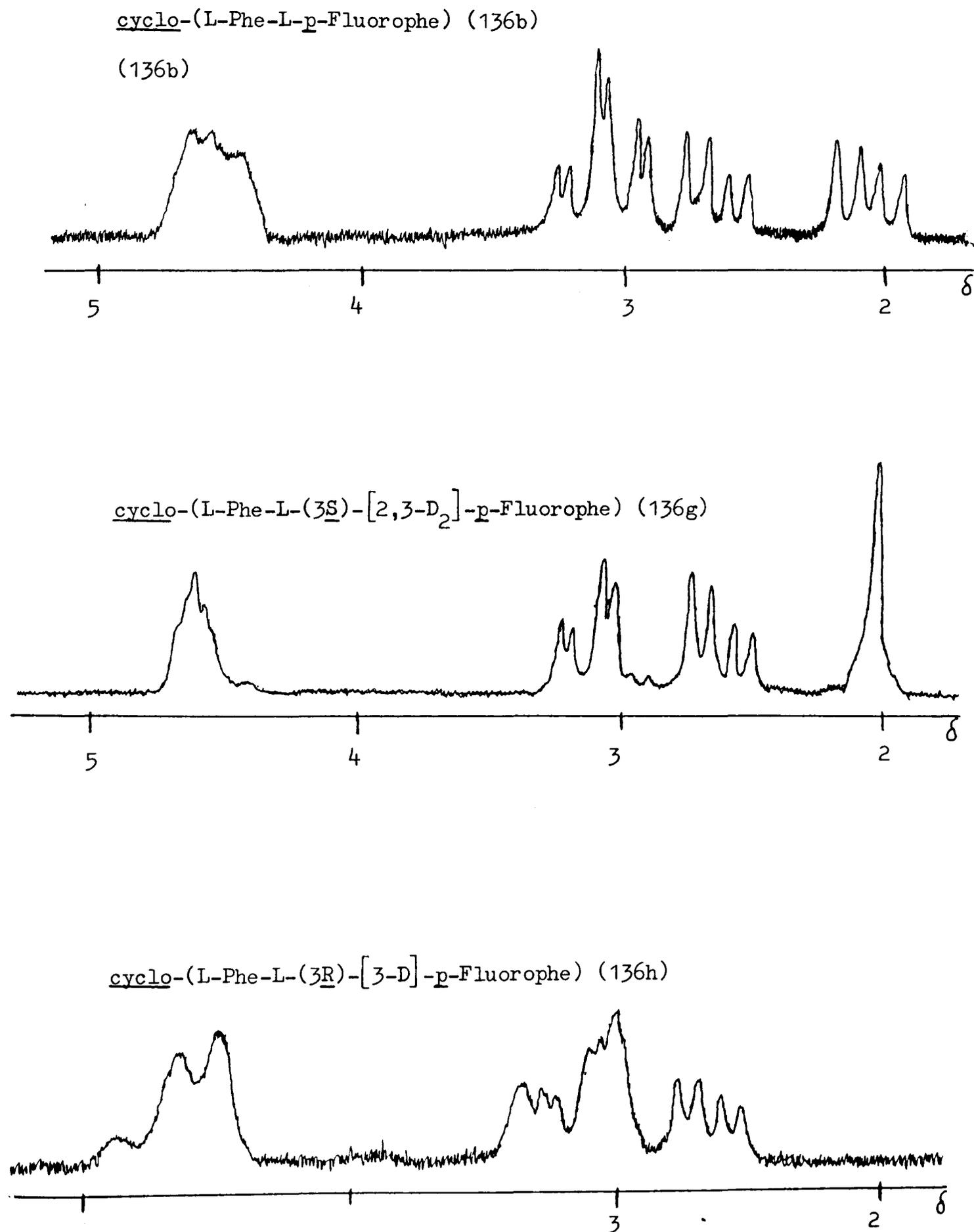
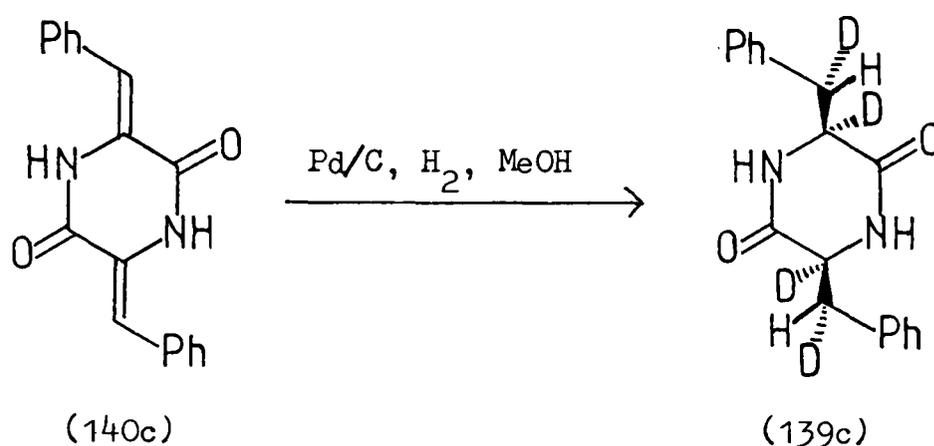


Figure 1 ^1H n.m.r. spectra of cyclo-(L-Phe-L-p-Fluorophe) and its deuteriated analogues (136g) and (136h) (methylene and methine signals) (in TFA).

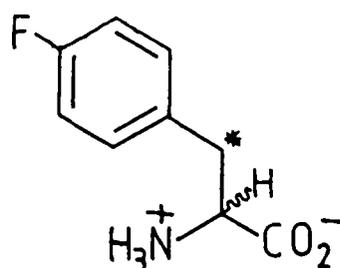


Because the geometry of the double bond of the arylidene compound (134b) was not absolutely certain, further confirmation of this result was obtained by reducing dibenzylidenepiperazinedione (140c)⁹⁶ with deuterium; the configuration of (140c) is well established.¹⁰⁷ Again, the proton n.m.r. signals from the methylene group of cyclo-(L-Phe-L-Phe) (42) were reduced to a broad singlet at δ 2.30 in (\pm)-tetra-deuterio-cis-cyclo-(Phe-Phe) (139c). Therefore the upfield methylene proton is once again pro-R in cyclo-(L-Phe-L-Phe) .



2.3.4 Feeding experiments with fluorinated analogues

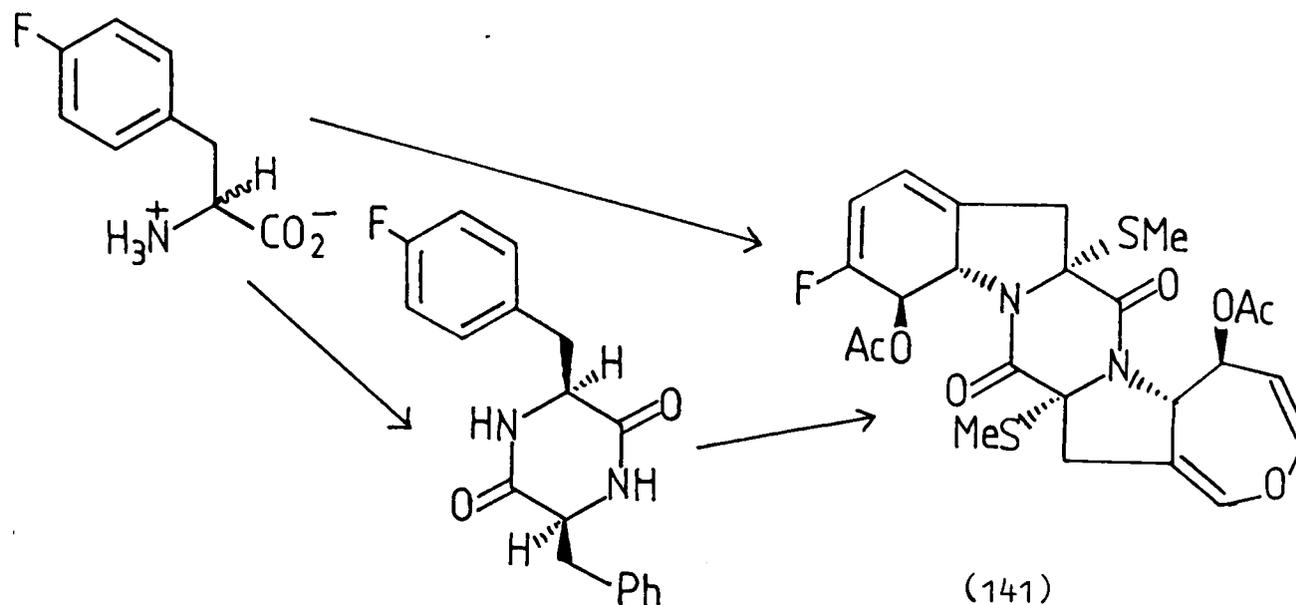
Our first feeding experiments were conducted using DL-p-fluorophenylalanine (112), an analogue of the natural precursor, L-phenylalanine. When fed as a tracer to Aspergillus terreus, DL-[3-¹⁴C]-p-fluorophenylalanine was incorporated at a very high level (ca. 40%) into the total chloroform extract, and t.l.c. followed by radio-scanning and autoradiograms of the t.l.c. plates showed that nearly all



(112)

of this activity was in a compound whose t.l.c. properties were very like those of BDAA (41). Unlabelled DL-p-fluorophenylalanine was then fed at a sub-lethal dose (it was found to kill the fungus at 5 mM, but to be relatively innocuous at 0.5 mM), and the broth extract was subjected to n.m.r. spectroscopy. The proton-decoupled spectrum contained a strong signal at δ -123, and several other weaker signals clustered around δ -115; the latter shift is characteristic of p-fluorobenzyl compounds, e.g. cyclo-(L-Phe-L-p-Fluorophe) (136b). The signal at δ -123 was also present in the spectra of extracts from cultures fed cyclo-(L-Phe-L-p-Fluorophe) (136b), and radioactivity from this compound was also observed to be incorporated into the BDAA-like metabolite (by autoradiography of chromatograms). Isolation of this compound proved difficult because of its similarity to BDAA, but it was eventually purified by repeated t.l.c. separation. The mass spectrum of this compound had a molecular ion at m/z 536, and the accurate mass of this ion gave the molecular formula as $C_{24}H_{25}FN_2O_7S_2$, corresponding to a fluorinated BDAA. The next highest significant ion in the mass spectrum was at m/z 489, produced by loss of [SMe] from the molecular ion; this loss occurs in the mass spectra of BDA, BDAA, etc. The proton n.m.r. spectrum contained a set of signals attributable to an aranotin ring system, the chemical shifts and couplings being almost identical to those of the spectrum of BDAA. There were four other proton signals in the olefinic region; by selective decoupling experiments the signals were shown to be consistent with a fluorocyclohexadienol acetate system, with fluorine on the carbon adjacent to the carbon bearing the acetate group. All of the protons in the cyclohexadienol ring were coupled to fluorine; these couplings were correlated by selective proton decoupling of the proton-coupled ^{19}F n.m.r.

spectrum. The experimental data thus established the structure of the analogue metabolite as the fluoro-BDAA (141).

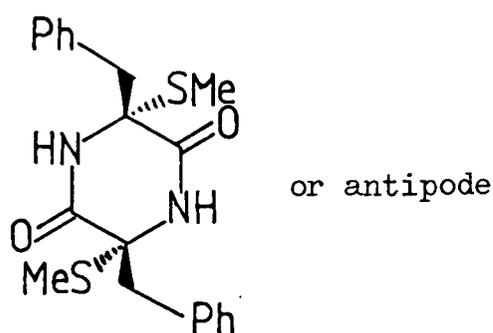


The incorporation of cyclo-(L-Phe-L-p-Fluorophe) into the analogue was not measured directly, because of losses of material on isolation, but ^{19}F n.m.r. of the fungal extract using p-fluorobenzoic acid as a standard gave an approximate incorporation of 1.8% into fluoro-BDAA, and a total incorporation of fluorine into the extract of 6.2%. This value is in fairly good agreement with the incorporation of ^{14}C label into the total chloroform extract of 8.0%.

It was of interest to determine the nature of the other fluorinated metabolites present in the extract. On autoradiograms of extracts from cultures fed DL-p-fluorophenylalanine, spots were observed which corresponded to cyclo-(L-Phe-L-p-Fluorophe) and cis-cyclo-(p-Fluorophe-p-Fluorophe) standards. Chromatographic separation of the extract and crystallisation of the material extracted from near the baseline, gave cyclo-(L-Phe-L-Phe) (42) with smaller amounts of cyclo-(L-Phe-L-p-Fluorophe) (136b) and cis-cyclo-(p-Fluorophe-p-Fluorophe) (139a) as determined by ^{19}F n.m.r. spectroscopy and mass spectrometry.

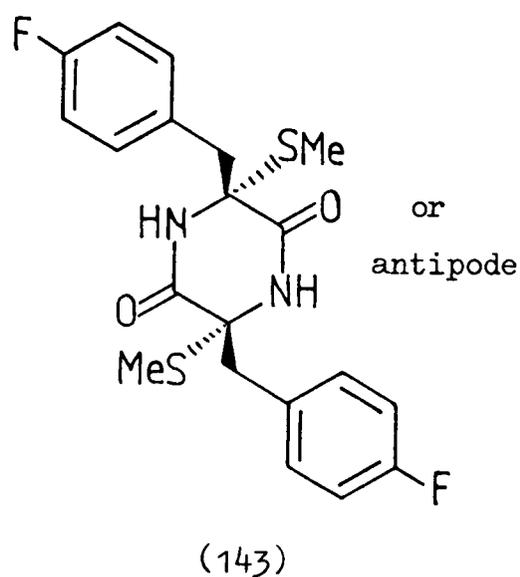
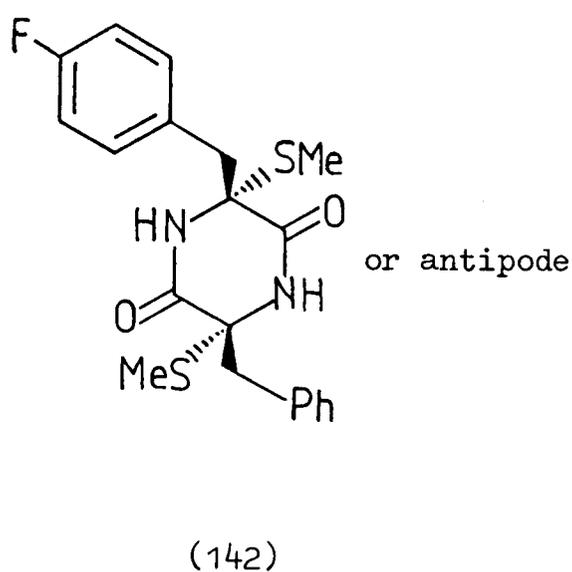
Activity on autoradiograms was also observed at a spot on t.l.c.

plates running with higher R_f than BDAA and the other arantoin metabolites. Preparative t.l.c. allowed the isolation of a crystalline substance from this part of the extract; the compound could be obtained from both fed and unfed extracts. The structure (131) was assigned on the basis of its spectral properties. The highest significant ion in the mass spectrum was at m/z 339; this corresponds to loss of SMe from a di(methylthio)-cyclo-(Phe-Phe). The accurate mass measurement of this ion gave a formula of $C_{19}H_{19}N_2O_2S$, and a molecular ion of formula $C_{20}H_{22}N_2O_2S_2$ was also observed. The 1H n.m.r. spectrum confirmed the presence of SMe groups, benzyl groups and NH protons. Since the metabolite was optically active, the relative configuration must be cis-. This compound is the first reported sulphur-containing dioxopiperazine natural product without substitution on nitrogen; its synthesis and its relationship with the biosynthesis of the arantoin are discussed in Section 2.5.

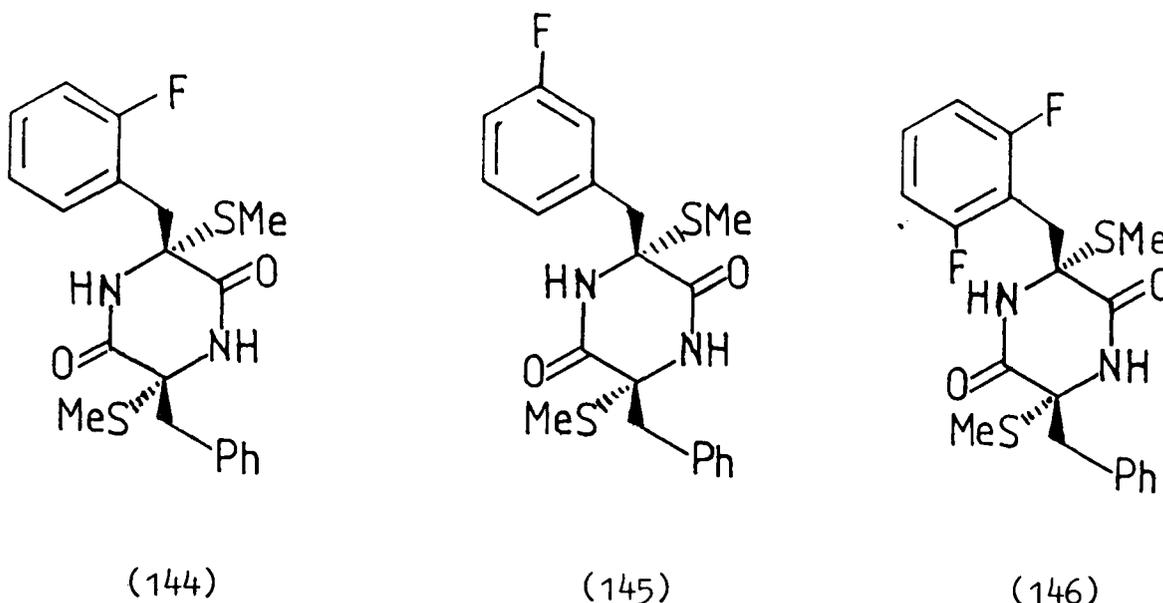


When the above compound (131) was isolated from extracts which had been fed DL-p-fluorophenylalanine, mass spectrometry and ^{19}F n.m.r. showed it to contain small amounts of the analogues containing either one or two fluorophenyl rings (142) and (143). Similar analogues were obtained from feedings of cyclo-(L-Phe-L-o-Fluorophe) (136c), cyclo-(L-Phe-L-m-Fluorophe) (136d), cyclo-(L-Phe-L-p-Fluorophe) (136b), cyclo-(L-Phe-L-2',6'-Difluorophe) (136a), and (\pm)-cyclo-(p-Fluorophe-

p-Fluorophe) (139a). The di(methylthio)- analogues were all isolated by co-crystallisation with the natural product (131), and were characterised by ^{19}F n.m.r. spectroscopy and mass spectrometry of the mixtures. Of course, only the analogues containing one fluorinated ring were observed, except for the metabolite from (139a), where the precursor has two fluorinated aromatic rings. Spots corresponding to the analogue metabolites were also seen on autoradiograms when the radioactive precursors were fed.



Apart from the detection of these analogues, the feeding experiments with fluorinated precursors have not been very fruitful. (\pm)-cis-cyclo-(p-Fluorophe-p-Fluorophe) (139a) was fed to A. terreus, but the ^{19}F n.m.r. spectrum of the extract had only three prominent signals, all of which were in the "aromatic" region (δ ca. -115). Two of these signals may be assigned to the starting material (139a) and the di(methylthio)- analogue (143). A similar pattern was observed with cyclo-(L-Phe-L-o-Fluorophe) (136c) and cyclo-(L-Phe-L-m-Fluorophe) (136d); three signals were present in the ^{19}F n.m.r. spectrum in each case, and two of these were accounted for by the starting materials and the di(methylthio)- analogues (144) and (145). The amino-acids, DL-o-fluorophenylalanine and DL-m-fluorophenylalanine, gave a wider range of fluorinated metabolites, but because the amino-acids were not



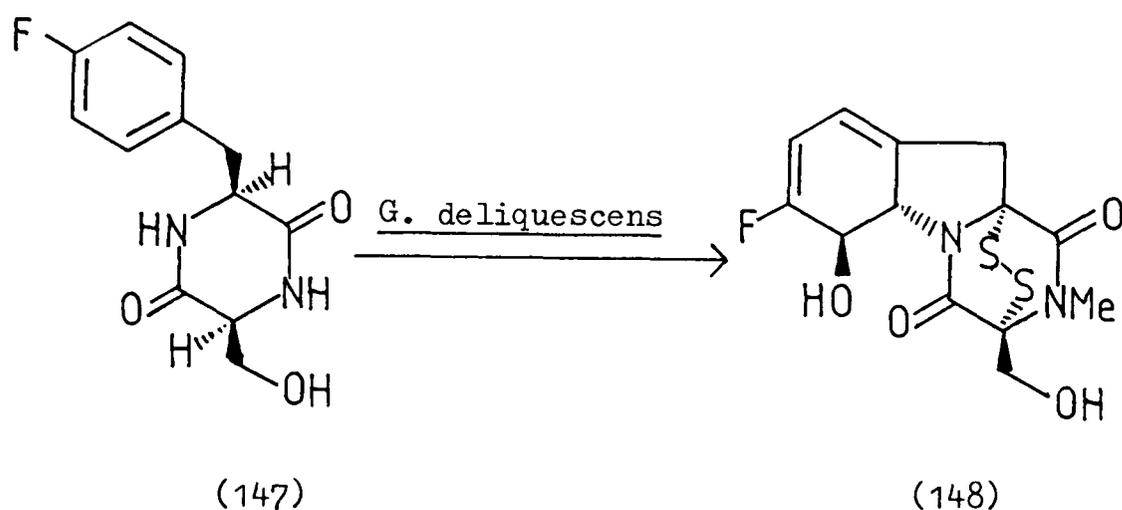
radioactive we were unable to isolate and characterise any of these.

More interesting was cyclo-(L-Phe-L-2',6'-Difluorophe) (136a), which was metabolised to give a number of compounds, including some whose behaviour on t.l.c. was close to that of the aranotins. Unfortunately, because of losses on successive t.l.c. separations, only the starting material and the di(methylthio)-cyclo-(Phe-Phe) analogue (146) were detected with certainty. Similar problems were encountered with cyclo-(L-Phe-L-Pentafluorophe) (136e). The ^{19}F n.m.r. spectrum of the fungal extract indicated the presence of several compounds, all of which possessed pentafluorophenyl rings as shown by the characteristic ^{19}F splitting pattern. However, none of these was isolated in a pure state, because of insufficient quantities.

Finally, the "analogue" (\pm)-cis-cyclo-(Pentafluorophe-Pentafluorophe) (139b) was fed to A. terreus. Not surprisingly, this compound was not metabolised, and the signals in the ^{19}F n.m.r. spectrum of the extract were those of the starting material only.

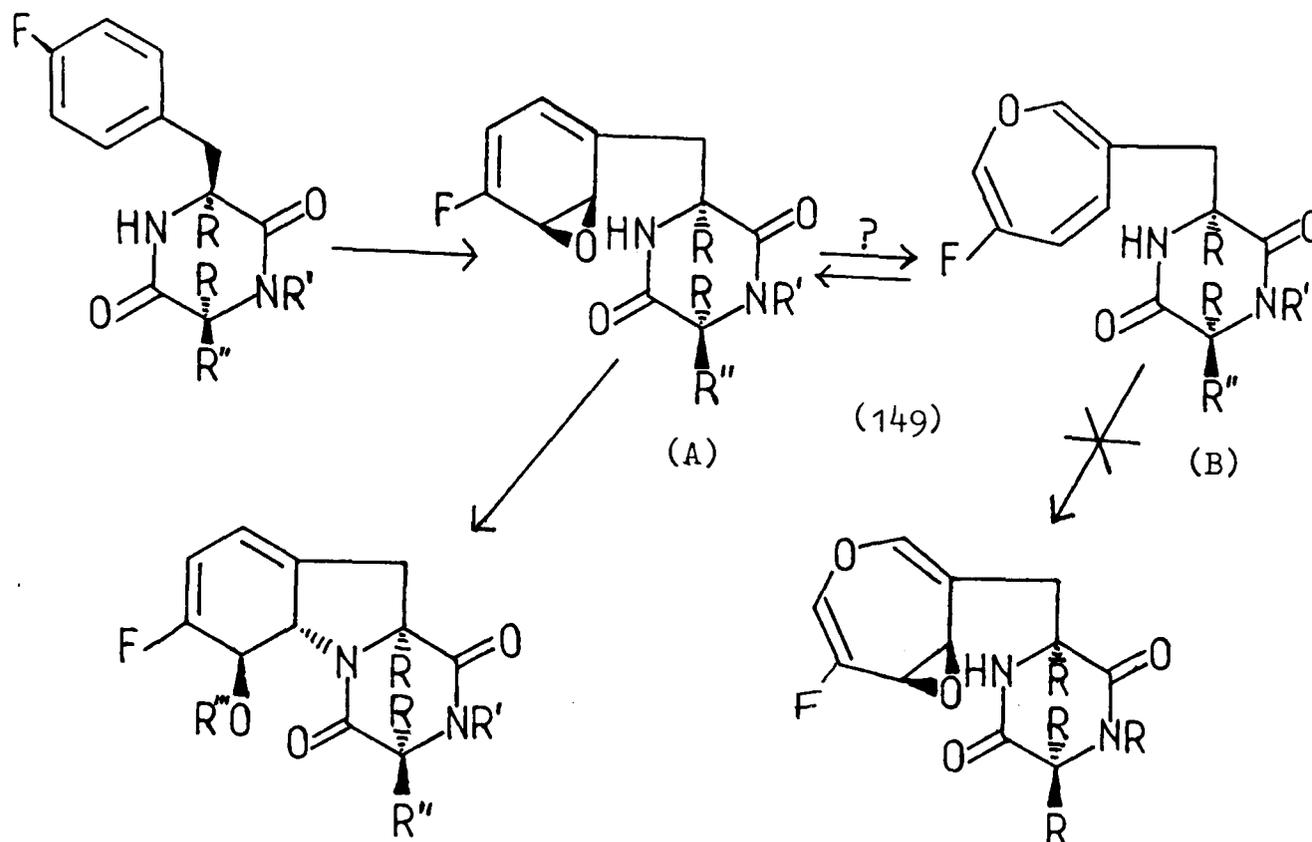
The most interesting result was therefore the incorporation of DL-p-fluorophenylalanine (112) and cyclo-(L-Phe-L-p-Fluorophe) (136b) into fluoro-BDAA (141). In this case, the modified precursor has evidently been accepted as a substrate by the aranotin-producing

enzymes, and has been oxygenated on the aromatic ring bearing fluorine. However, no compounds having fluorine in an aranotin (oxepin) ring were detected, so fluorine appears to prevent a second oxygenation of the aromatic ring from taking place. Also, the non-incorporation of cis-cyclo-(p-Fluorophe-p-Fluorophe) into ring-oxygenated metabolites may be significant; it might be necessary to oxidise one ring to an aranotin system before operations at the other end can commence. An interesting experiment related to this discussion was the observation of the incorporation of cyclo-(L-p-Fluorophe-L-Ser) (147) into the fluorogliotoxin (148).¹⁰⁸



There are several possible explanations for the failure of cyclo-(L-Phe-L-p-Fluorophe) (136b) to give compounds fluorinated on an aranotin ring. Firstly, there might be two separate enzymes or enzyme active sites, one of which forms aranotin rings, and the other cyclohexadienol rings. The former enzyme might then not accept the fluorinated aromatic ring as a substrate. Alternatively, the fluorine substituent might affect the behaviour of the monooxygenated intermediate, e.g. (149) (A) \rightleftharpoons (B) in such a way that it is not oxygenated a second time. For example, the fluorinated oxepin (B) might not be an acceptable substrate for the enzyme performing the second oxidation; or, the fluorine substituent might alter the position of the equilib-

rium between arene oxide and oxepin, so that oxygenation cannot take



place to any detectable extent because the oxepin is not present in sufficient amount; or, the fluorine substituent might increase the reactivity of the arene oxide (A), so that nucleophilic attack by nitrogen takes place before a second oxygenation can occur. In principle, a distinction between these alternatives might plausibly be made on the grounds of chemical reactivity. Unfortunately, though not surprisingly, there appear to be no examples in the literature of fluorinated arene oxides or, of course, the corresponding oxepins.

After our first result, the failure of the o- and m-fluoro- analogues (136c) and (136d) to be incorporated into aranotin-like compounds was somewhat unexpected. That the di(methylthio)-cyclo-(Phe-Phe) analogues [(144) and (145)] were detected is an indication .

that the cyclic dipeptides were absorbed into the fungal cells, so cell wall impermeability was probably not the cause of their non-incorporation. Moreover, introduction of sulphur into the molecules was not prevented by fluorine substitution, so it appears that fluorination had now inhibited even the first oxidation step. The 2',6'-difluoro- and pentafluoro- analogues (136a) and (136e) were converted into unnatural metabolites (detected by ^{19}F n.m.r. and autoradiography) which might be related to aranotin biosynthesis, but further work is necessary to determine the structures of these compounds.

2.3.5 Conclusions

In at least some cases, fluorine substitution on aranotin precursors had the predicted effect, i.e. it inhibited oxygenation of the aromatic rings. Several fluorinated analogues of the natural metabolites were detected, but isolation and characterisation was hindered by the low conversions of the precursors to metabolite analogues. The use of similar analogues in related biosynthetic systems, e.g. the gliotoxin-producing fungus Gliocladium deliquescens,¹⁰⁸ might prove to be enlightening.

2.3.6 Feeding of deuteriated fluorinated analogues

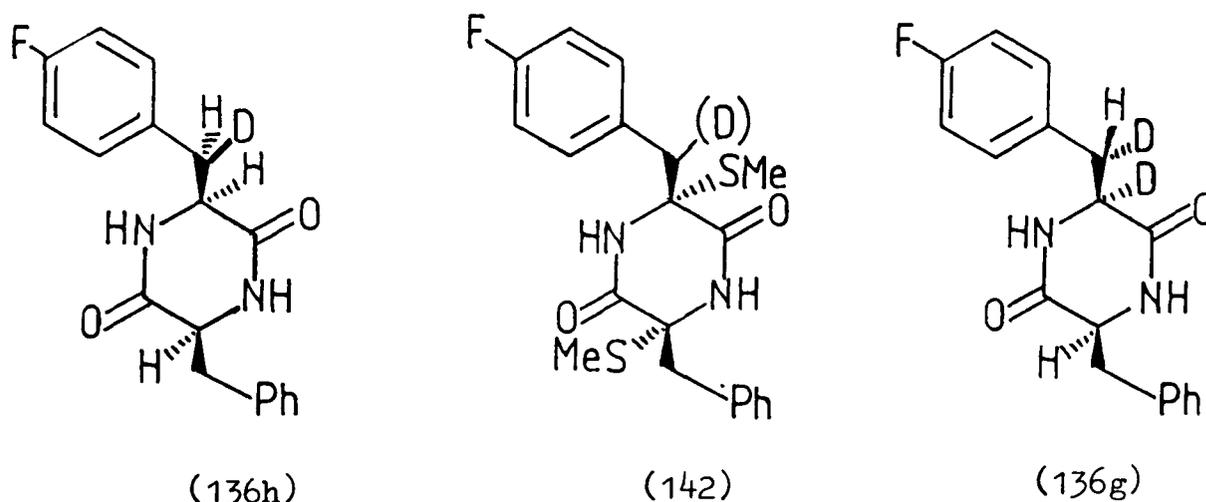
Several attempts have been made to establish whether the methylene hydrogens in precursors of sulphur-containing dioxopiperazines are lost during the biosynthesis (see Introduction). This is important since it is possible to envisage sulphur introduction by addition of thiols to unsaturated precursors such as (140c). This research has been hampered by various problems; enzymic exchange of the methylene protons of phenylalanine was noted by Johns and Kirby,²⁷ in their

investigations with gliotoxin, and Pita et al.,³⁹ in their research on aranotin biosynthesis, had difficulty in interpretation of mass spectral data because of overlap of isotope (^{13}C , ^{34}S) peaks from natural, non-deuteriated BDA with the peaks arising from deuteriated BDA. However, the evidence gathered to date, as discussed in the Introduction, tends to support the view that there is no obligatory loss of hydrogen from the methylene groups of gliotoxin and aranotin precursors, although some experimenters have observed a small loss of deuterium and tritium label from these positions.^{16,39}

Our isolation of a new, sulphur-containing metabolite, di(methylthio)-cyclo-(Phe-Phe) (131), along with its fluorinated analogues, gave us the opportunity to tackle this question in an unusual way. We had already prepared cyclo-(L-Phe-L-p-Fluorophe) stereospecifically labelled with deuterium at both the pro-R and pro-S positions of the fluorobenzyl methylene group [(136g) and (136h)]. Feeding these compounds to A. terreus, isolation of the mixture of di(methylthio)-cyclo-(Phe-Phe) and the p-fluoro- analogue (142), and mass spectrometry, should give a fairly accurate indication of how much deuterium is lost from each position during sulphur introduction. The advantage of this approach lies in the fact that the mass spectral peaks for the fluorinated products are, of course, shifted to higher mass, well clear of the isotope peaks of the natural metabolites.

Firstly, the deuterium content at the methylene group of the precursor analogues had to be quantified. The cyclo-(L-Phe-L-(3R)-[3-D]-p-Fluorophe) (136h) was unfortunately slightly contaminated with the dehydro-cyclic dipeptide (138h), because of incomplete hydrogenation; however, the signals in the mass spectrum due to this compound could easily be subtracted, and a minimum deuterium content at the

methylene group of 95% was calculated, in accord with the proton n.m.r. spectrum which gave a similar value. The other analogue, cyclo-(L-Phe-L-(3S)-[2,3-D₂]-p-Fluorophe) (136g) was similarly calculated to have a minimum deuterium content at the methylene group of 93%. Di(methylthio)-cyclo-(Phe-Phe) (131) and the p-fluoro- analogue (142) were isolated from cultures fed each deuteriated compound, and the intensities of the ions in the mass spectrum corresponding to loss of a methylthio- group from the fluorinated analogues (i.e. the highest significant ions) were compared to the intensities in the undeuteriated analogue (142). In both cases, the most abundant ion was at m/z 358, corresponding to the retention of one deuterium atom. By comparison of the intensities of the ions at m/z 357 to that of the ion at m/z 356 in the undeuteriated analogue (142), and taking into account the percentage deuteriation of each precursor, the loss of deuterium from each methylene group was calculated as 6% from the (3R)-[3-D]- compound (136h), and 11% from the (3S)-[3-D]- analogue (136g). The only factor



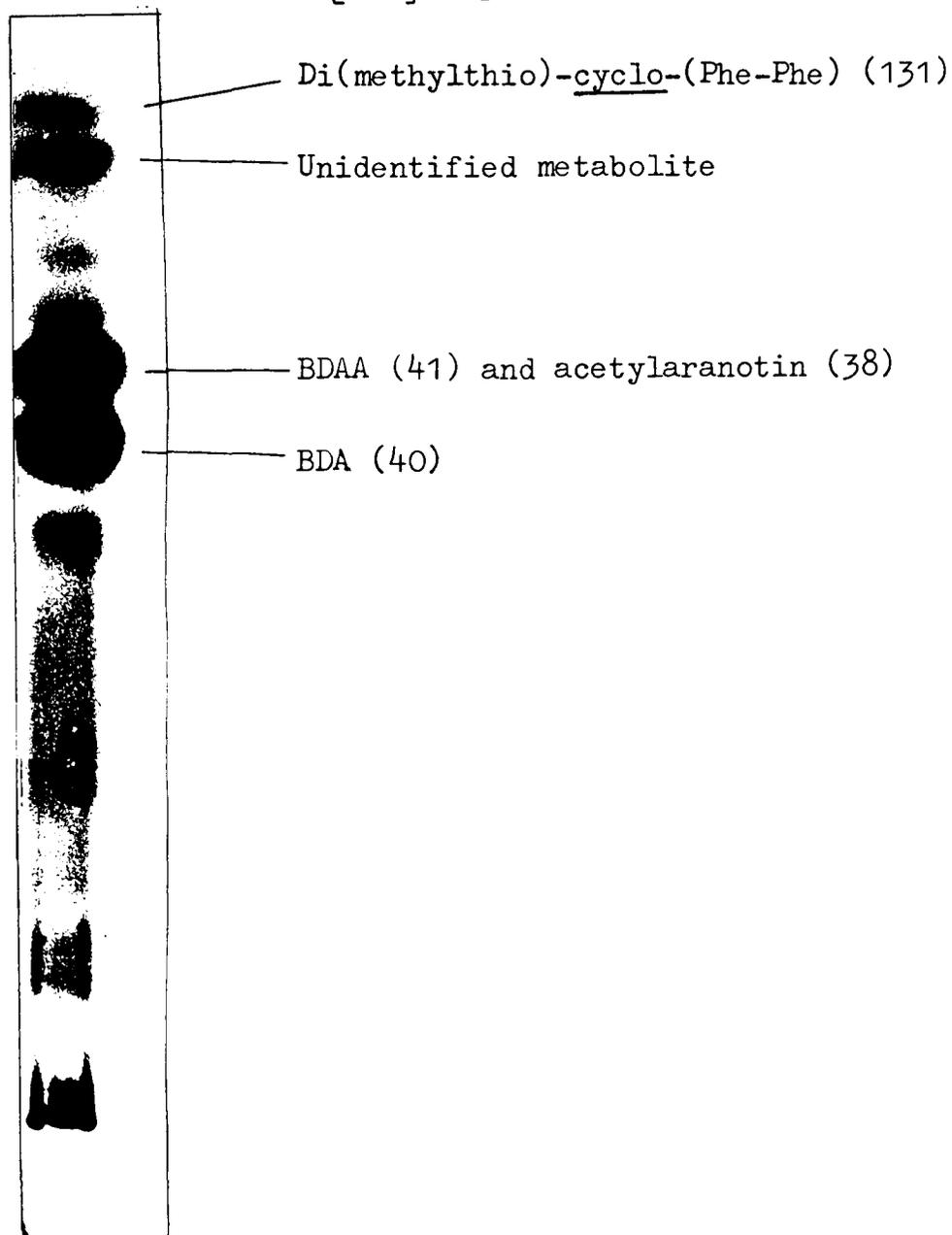
which could invalidate these results would be a shift of the α -deuterium in (136g) to the β -position in the sulphur-containing product, but this would appear to be unlikely. There is, therefore, a small but significant loss of deuterium label from both the R- and S-positions of the fluorophenylalanyl group, but clearly, obligatory loss of

a hydrogen atom during biosynthesis can be discounted.

2.4 Feeding of $^{35}\text{SO}_4^{2-}$

Radioactive sodium [^{35}S] sulphate was fed to cultures of A. terreus so that it would be possible to observe all sulphur-containing metabolites by autoradiography and radioscanning of chromatograms. As expected, most of the label was located in spots corresponding to BDA and BDAA, but other metabolites were present, and there was significant activity in the di(methylthio)-cyclo-(Phe-Phe) spot (Figure 2).

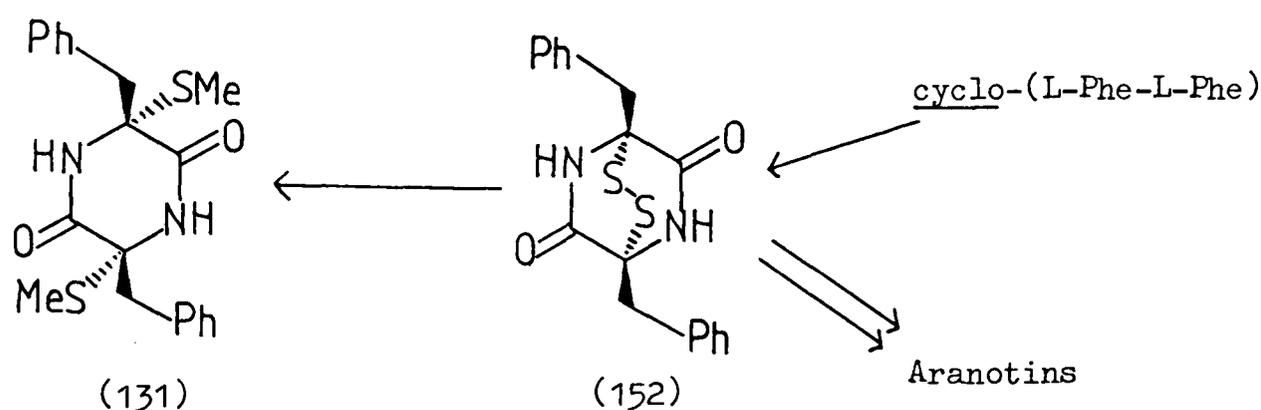
Figure 2 T.l.c. autoradiogram (solvent system A) of total extract fed sodium [^{35}S] sulphate



2.5 Synthesis of sulphur-containing dioxopiperazines

2.5.1 General background

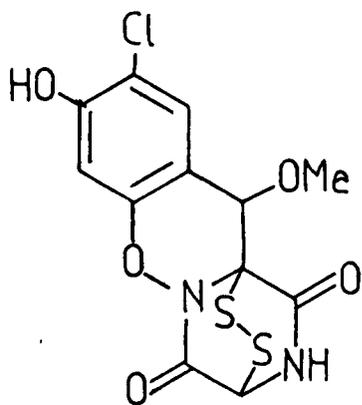
The isolation of di(methylthio)-cyclo-(Phe-Phe) (131) from extracts of Aspergillus terreus (see Section 2.1.6) gave a hint as to how the biosynthesis of the aranotins might proceed from cyclo-(L-Phe-L-Phe) (42). The fact that this compound did not have any structural changes from cyclo-(Phe-Phe) other than the replacement of the α -hydrogens with methylthio- groups suggested that introduction of sulphur could be the step immediately following cyclic dipeptide formation in aranotin biosynthesis. We did not believe that di(methylthio)-cyclo-(Phe-Phe) was an intermediate on the pathway to the aranotins, because S-methylation was expected to take place after formation of the aranotin skeleton, analogously to gliotoxin biosynthesis; rather, the new metabolite was thought to be a product of reductive methylation of the epidisulphide (152), which might be the true intermediate. Of



course, it was desirable to check these predictions by experiment; we therefore set out to synthesise the natural product (131) and the epidisulphide (152).

There were no natural products recorded in the literature containing a secondary amide and an epidisulphide in the dioxopiperazine ring, except antibiotic A30641, whose structure (153)¹⁰⁹ is in some doubt.⁵

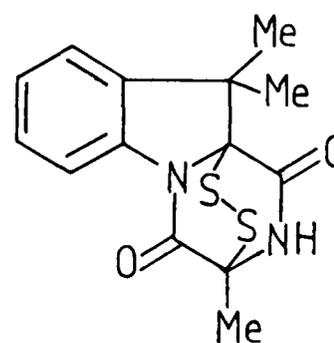
The only other example from natural sources of a dioxopiperazine with a sulphur-bearing carbon α - to a secondary amide nitrogen is the unnatural metabolite (21) isolated by Kirby *et al.* from *Gliocladium deliquescens*.³⁰ Neither have any epidisulphides with secondary amides



(153)

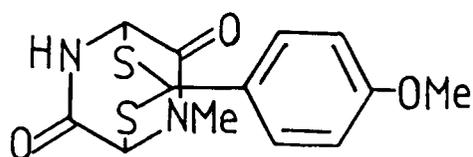


(21)

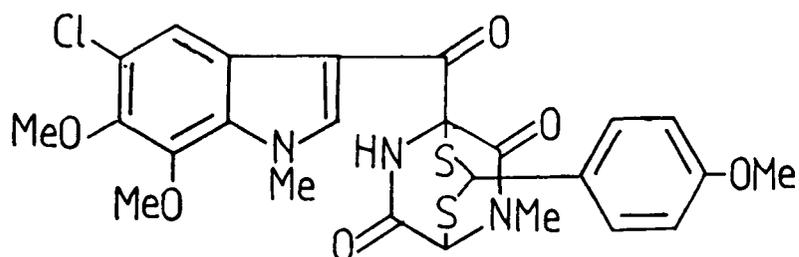


(154)

been synthesised. Ottenheijm *et al.*¹¹⁰ claimed that they had prepared the gliotoxin analogue (154), but that it was unstable. Unfortunately, no data were reported for this compound. Kishi's group, in their syntheses of gliotoxin (1) and sporidesmin (31) made use of the dithioacetals (48) and (155), which were prepared by removal of an N-protective methoxymethyl group with concentrated hydrochloric acid in ethanol or trifluoroacetic acid.



(48)



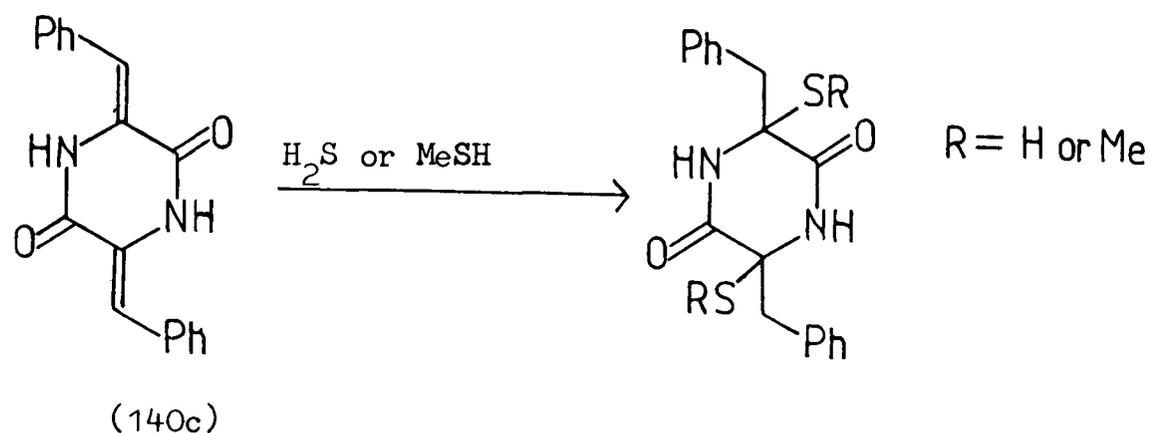
(155)

At this time, in our laboratories, work was in progress to synthesise the unnatural metabolite (21) by an adaptation of Kishi's method.³⁰ The synthetic targets (131) and (152), however, seemed to

lend themselves, by their symmetry and unreactive side-chains, to a shorter synthesis.

2.5.2 Synthetic procedures

One of the simplest methods that could be envisaged would be the addition of methanethiol or hydrogen sulphide across the double bonds of dibenzylidenepiperazinedione (140c), giving the di(methylthio)-compound in one step, or the epidisulphide (152) in two, after oxidation of the dithiol. Machin and Sammes⁶¹ had shown that under acidic



conditions, addition of thiols to double bonds in N-alkylated dioxo-piperazines took place in the desired sense, with sulphur on the carbon α - to the nitrogen (see Introduction, Section 1.3). Also, Ottenheijm *et al.*⁶⁰ had performed a similar reaction with hydrogen sulphide using zinc chloride catalysis.

We therefore attempted the addition of methanethiol to dibenzylidenepiperazinedione (140c) under similar conditions. Zinc chloride was prepared in situ by saturation of a suspension of zinc dust in methylene chloride with hydrogen chloride gas. Dibenzylidenepiperazinedione (140c) was added, then methanethiol. The products isolated were found to be predominantly starting material, along with a very small amount of a compound tentatively identified as the mono-adduct

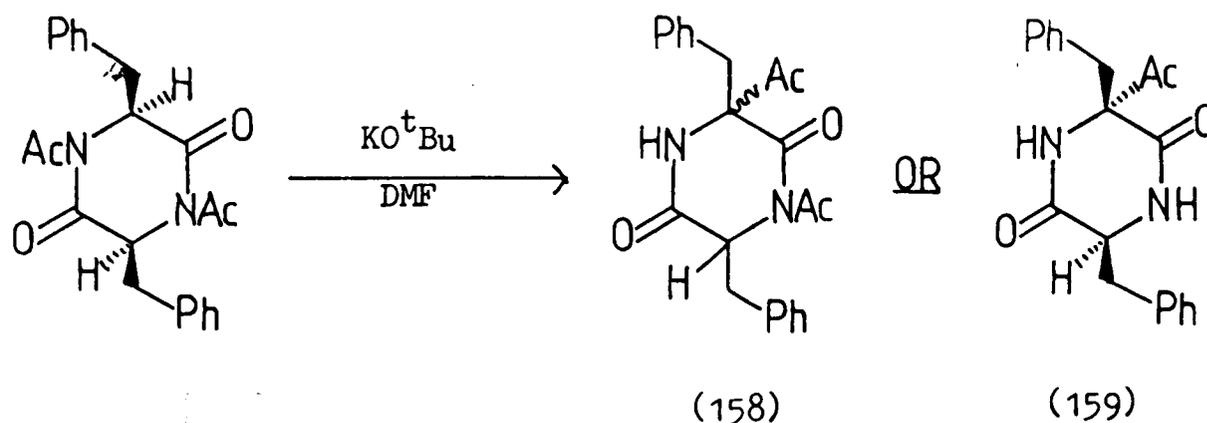
(156). No di(methylthio)- compound (131) was detected. A repeat of this reaction produced only starting material, so although it appears



(156)

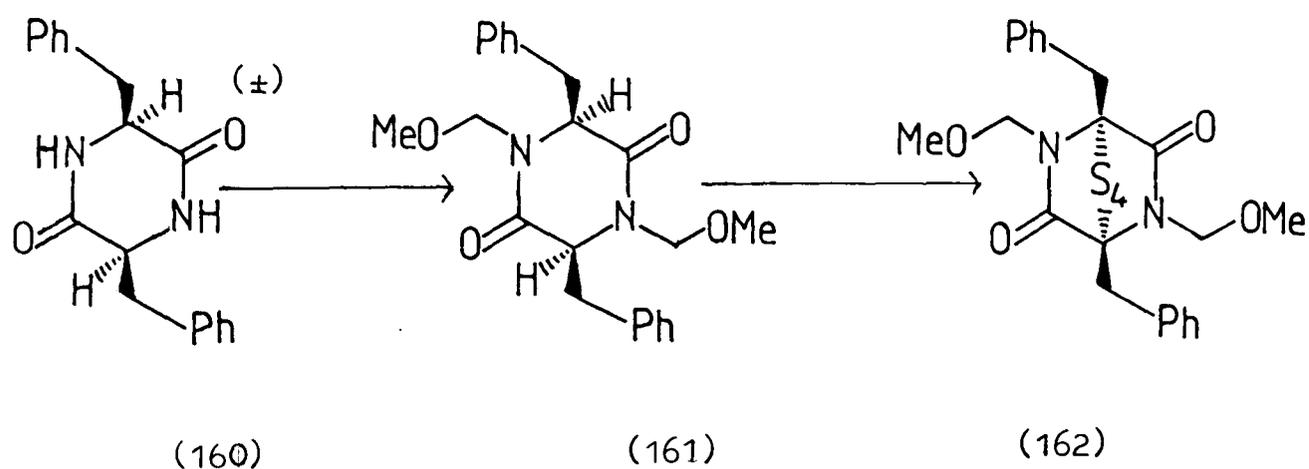
to be feasible to introduce sulphur in this way, our attempts were abandoned because of the uncertainty of achieving the target compounds (131) and (152).

Several research groups had reported the introduction of sulphur by generation of anions at C-3 and C-6 of the dioxopiperazine ring, and reaction of these with sulphur electrophiles (Introduction, Section 1.3). For these reactions it was necessary to protect the secondary amide functions. cyclo-(L-Phe-L-Phe) (42) was treated with acetic anhydride under acidic conditions to give the diacetyl compound (157). Attempted sulphenylation of this compound in DMF with dimethyl disulphide, methanesulphenyl chloride, or sulphur, using triethylamine or potassium t-butoxide as base, failed. Interestingly however, when potassium t-butoxide was used as the base, the unusual C-acetyl compounds (158) and (159) were obtained. It is not known whether the



acetyl group is transferred intramolecularly, but inspection of molecular models suggests that this would be difficult.

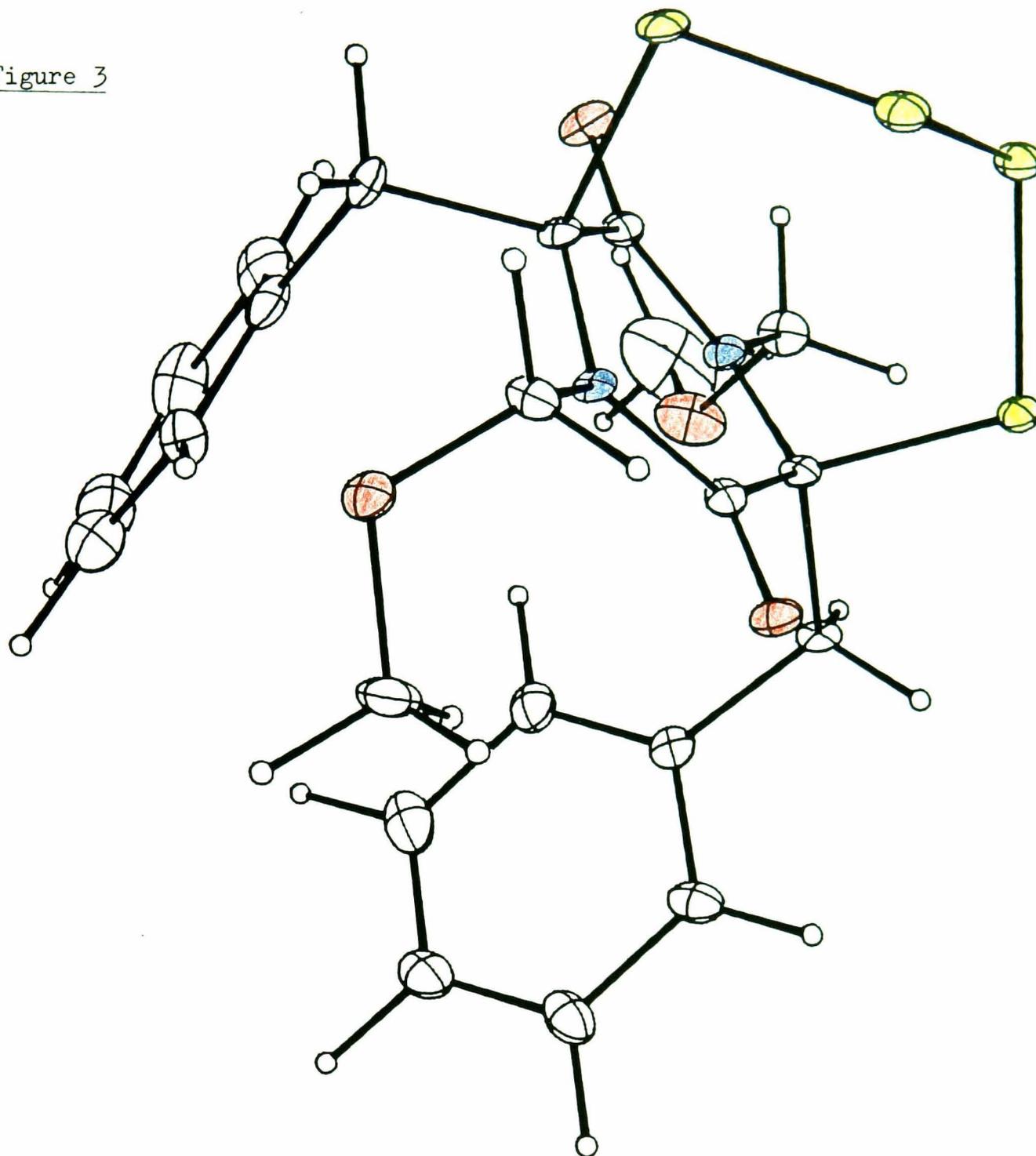
The formation of these rearrangement products precluded the use of acetyl protecting groups in such reactions. An alternative was the methoxymethyl group, although if it were used, a much stronger base would be required to remove the methine protons. (\pm)-Di(methoxymethyl)-cis-cyclo-(Phe-Phe) (161) was prepared in good yield by treatment of the racemic cis-cyclic dipeptide (160)¹¹¹ with potassium t-butoxide and chloromethyl methyl ether in DMSO. Treatment of this compound with LDA in THF, in the presence of excess sulphur and a trace of DMF, gave a moderate yield of epitetrasulphide (162) which was readily obtained by crystallisation from the viscous reaction products.



Initially, elemental analysis suggested that this compound contained five sulphur atoms, but by t.l.c. purification a sample was obtained which gave an analysis corresponding to the tetrasulphide (162). An X-ray crystal analysis was carried out by Dr. A.A. Freer, and this confirmed that the compound was indeed the tetrasulphide (162). A representation of the three-dimensional structure is given below (Figure 3, page 78).

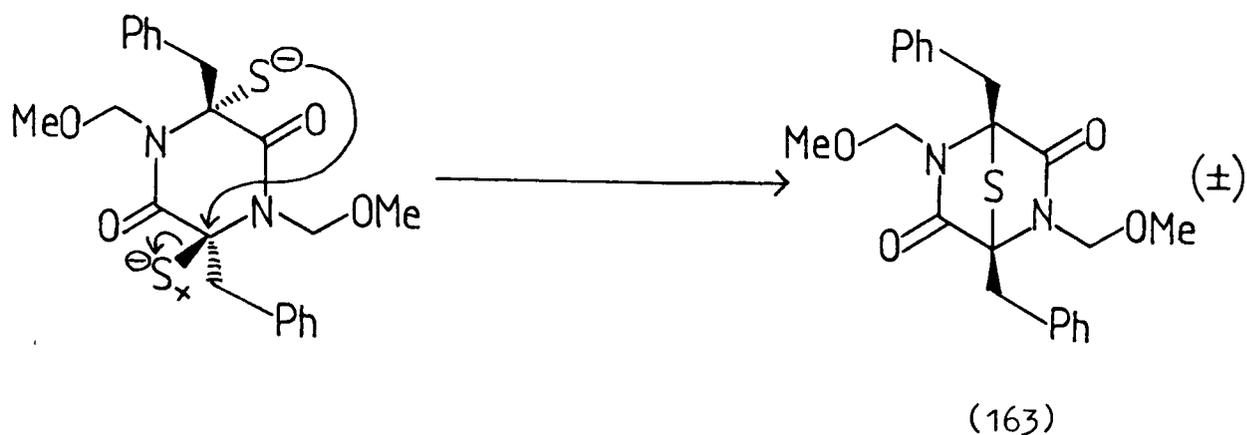
The necessity to add a small amount of DMF to the reaction mixture is difficult to explain. The original (\pm)-di(methoxymethyl)-cyclo-

Figure 3

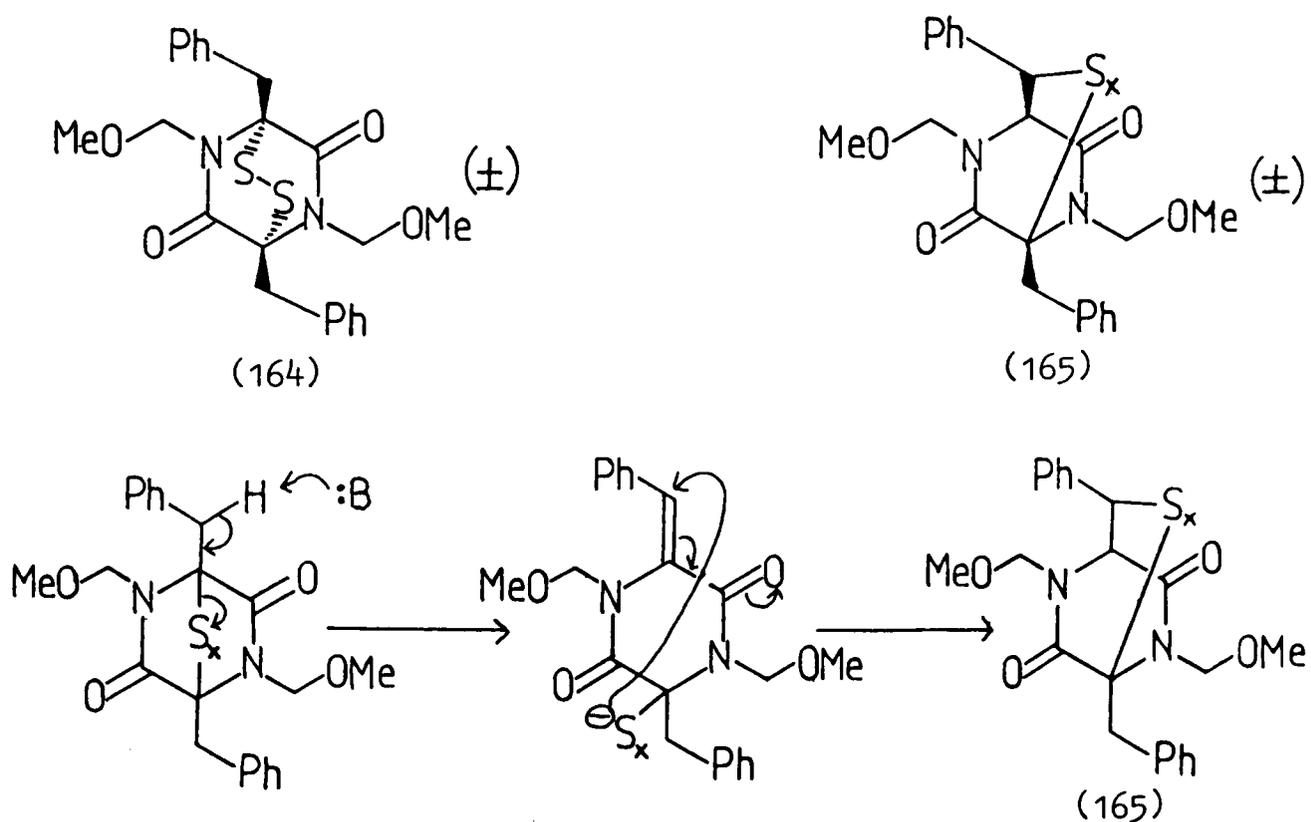


(Phe-Phe) used for these experiments had been prepared in DMF, and very small amounts of the solvent remained in the product. When rigorously pure di(methoxymethyl) compound (161) was used without added DMF, the yield of the sulphuration reaction dropped to zero, the only product being a viscous, polymer-like material. Other amide solvents, such as hexamethylphosphortriamide or *N,N'*-dimethylimidazolidin-2-one had a similar beneficial effect to DMF. When larger than trace amounts of these compounds (e.g. 1 mole equivalent) were added to the reaction mixture, the yield of tetrasulphide was once more adversely affected.

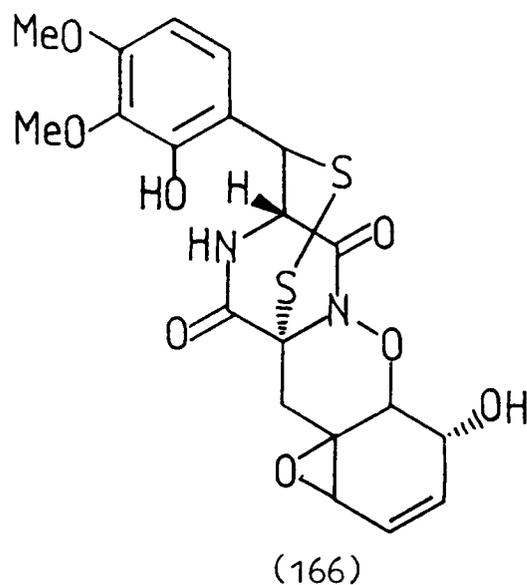
The reaction mixture was normally quenched with acetic acid at -10°C . If it was allowed to warm to room temperature, the epimono-sulphide (163) was obtained. This compound could be derived from a trans-dithiolate species by intramolecular attack:



Other compounds were obtained from the reaction mixture after quenching at -10°C ; the epidisulphide (164) was isolated, along with an unsymmetrical sulphur-containing compound, believed to have the structure (165), the number of sulphur atoms in the compound being unknown. This compound might have arisen by elimination at one end of an oligosulphide, and Michael-type addition to the unsaturated derivative formed. If $x = 2$, this compound is related to the system found

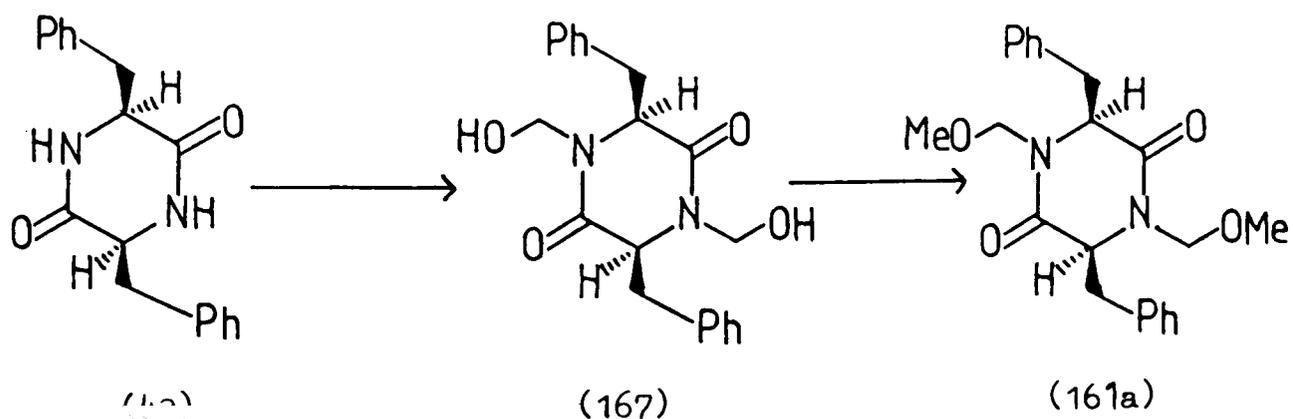


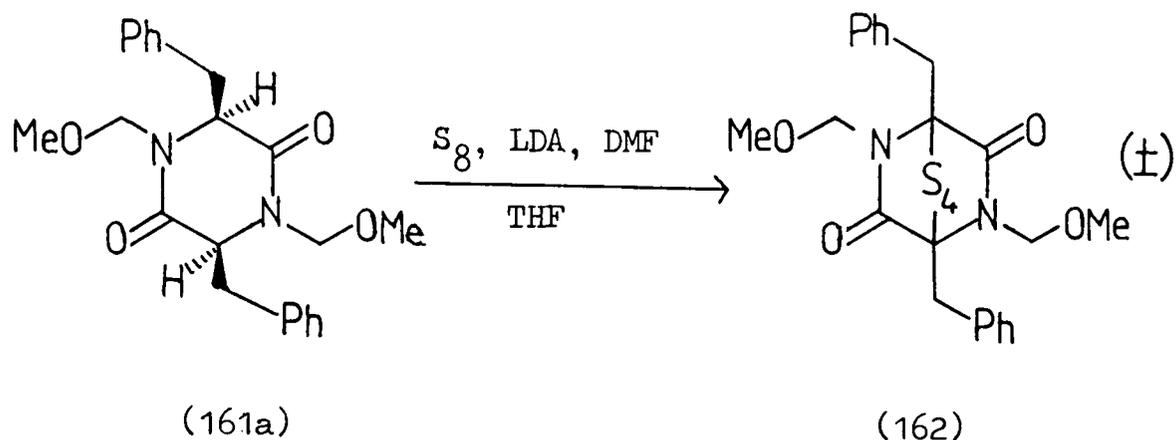
in gliovirin (166), a metabolite of Gliocladium virens.⁴



The rest of the starting material was converted in the sulphuration reaction into a gummy substance with a very broad n.m.r. spectrum, and this was thought to be a polymer made up from mainly trans-sulphenylated units.

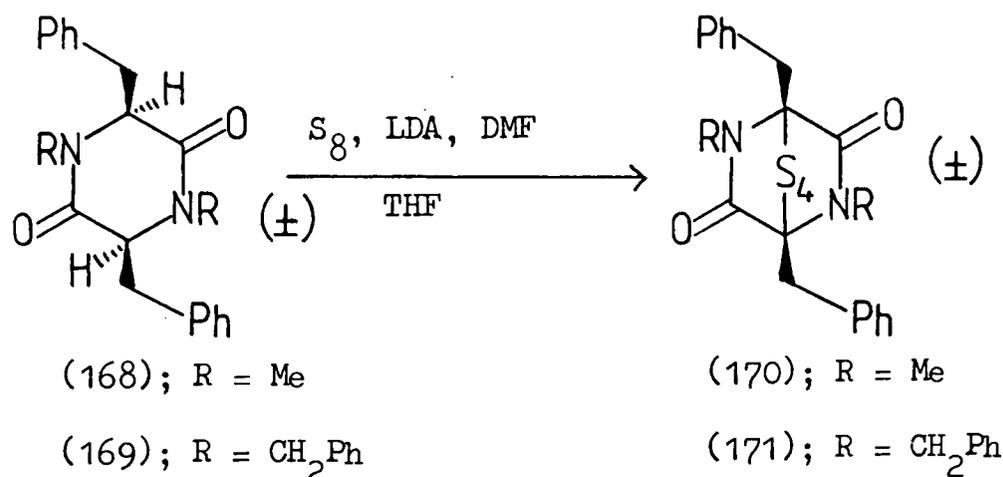
Schmidt⁶⁴ had obtained optically active epipolysulphide when cyclo-(L-Pro-L-Pro) (70) was treated with sulphur and sodamide in liquid ammonia (see Introduction, Section 1.3). To test whether retention of configuration occurred in our system, the optically active protected compound di(methoxymethyl)-cyclo-(L-Phe-L-Phe) (161a) was prepared by hydroxymethylation of cyclo-(L-Phe-L-Phe) (42) with formalin, and replacement of the hydroxy groups by methoxyls in methanoltionyl chloride. This alternative route was used to avoid epimerisation of the chiral centres. However, on sulphuration, the tetrasulphide (162) was optically inactive, and its melting point properties





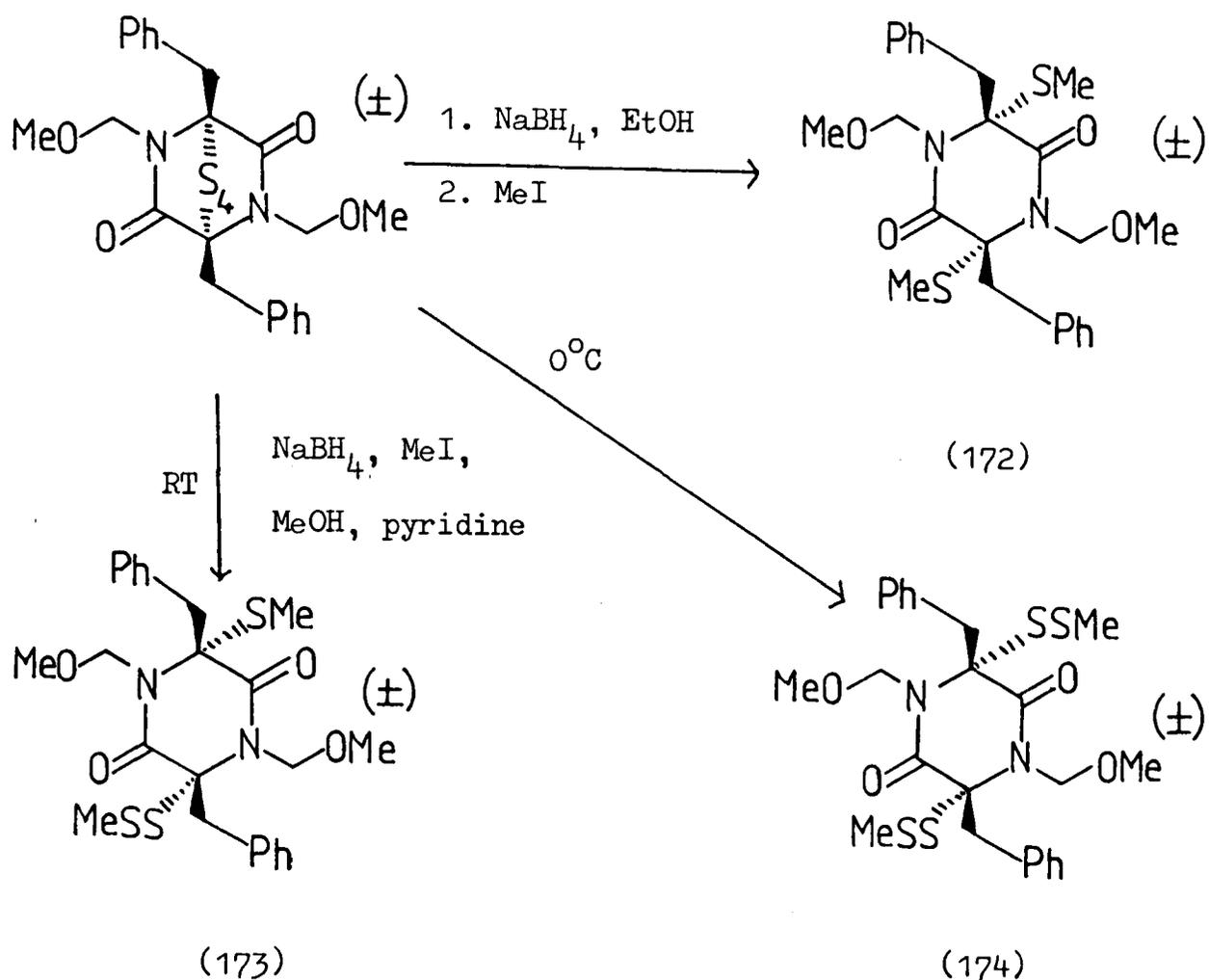
were identical to those of the racemate.

To test the general applicability of the sulphuration reaction, the N,N'-dimethyl and N,N'-dibenzyl derivatives of (\pm)-cis-cyclo-(Phe-Phe) (168) and (169), were prepared, and reaction of these compounds under the same conditions as before gave the corresponding epitetrasulphides (170) and (171). The dimethyl epitetrasulphide (170) was formed in somewhat better yield (41%) than the di(methoxymethyl) compound.

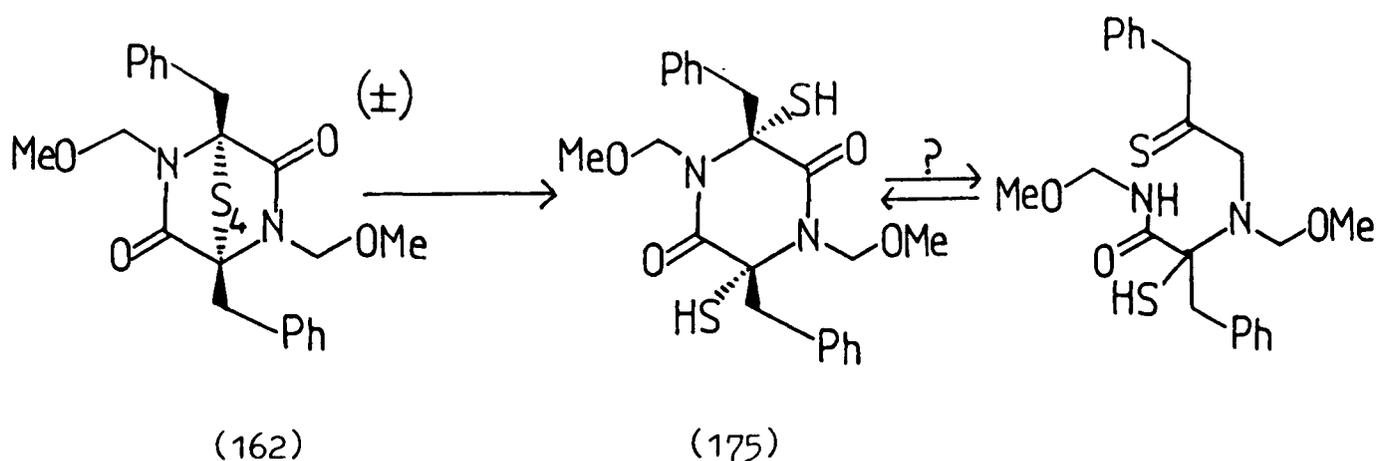


The di(methoxymethyl) epitetrasulphide (162) was converted in high yield to the cis-di(methylthio)-dioxopiperazine (172) by reduction with sodium borohydride and methylation of the resultant cis-dithiolate with methyl iodide. Initially, attempts were made to reduce the epitetrasulphide (162) in the presence of methyl iodide,¹¹² but these resulted in the compounds (173) and (174), containing methylthio-

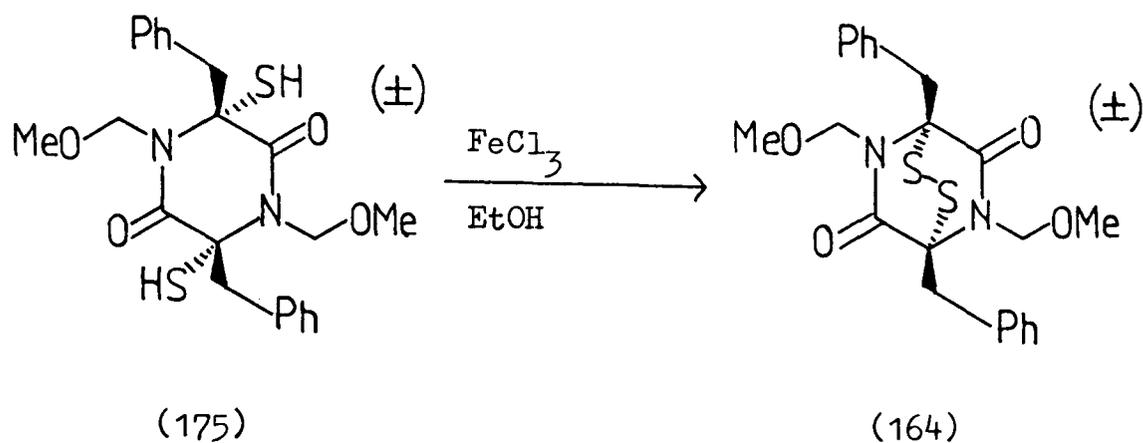
groups.



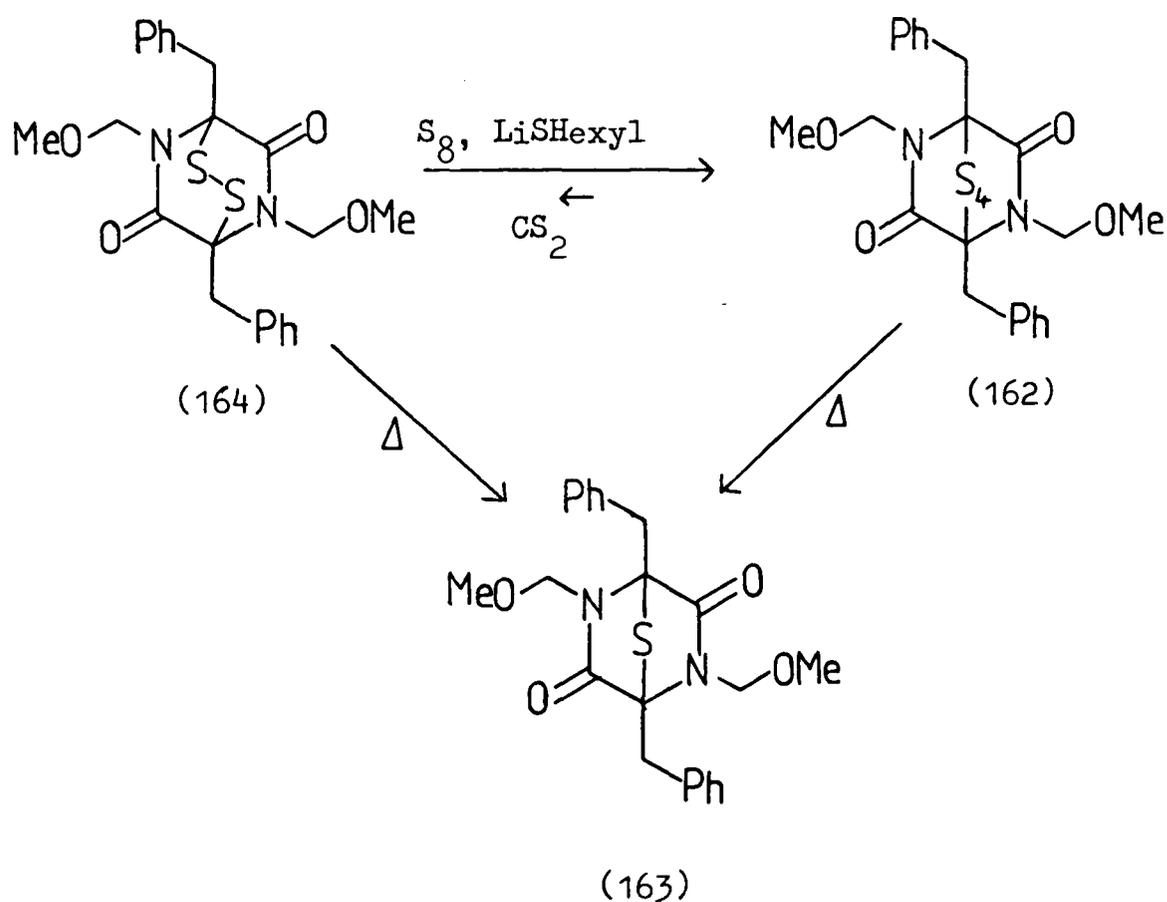
To prepare the epidisulphide (164), the epitetrasulphide was reduced with sodium borohydride to the dithiol (175), which was not purified because of its instability. This compound was interesting in that on heating a solution in methanol, a beautiful purple-red colour was produced, which disappeared on recooling. This might be due to transient formation of a thioketone, as was suggested by Ottenheijm for a related compound.¹¹³



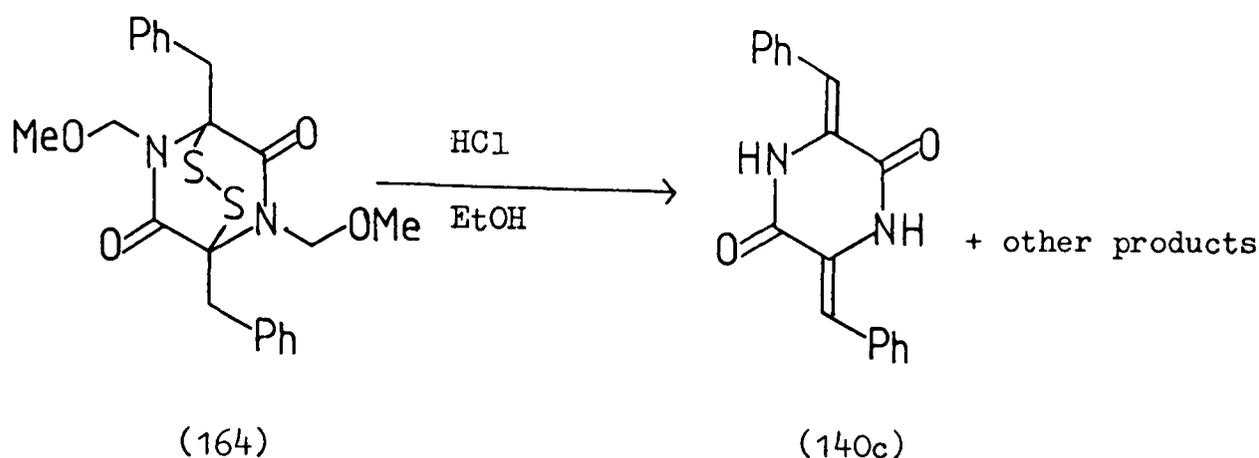
The dithiol (175) was readily oxidised to the epidisulphide (164). Ferric chloride solution in ethanol gave better yields in this reaction than iodine in pyridine.⁶⁰



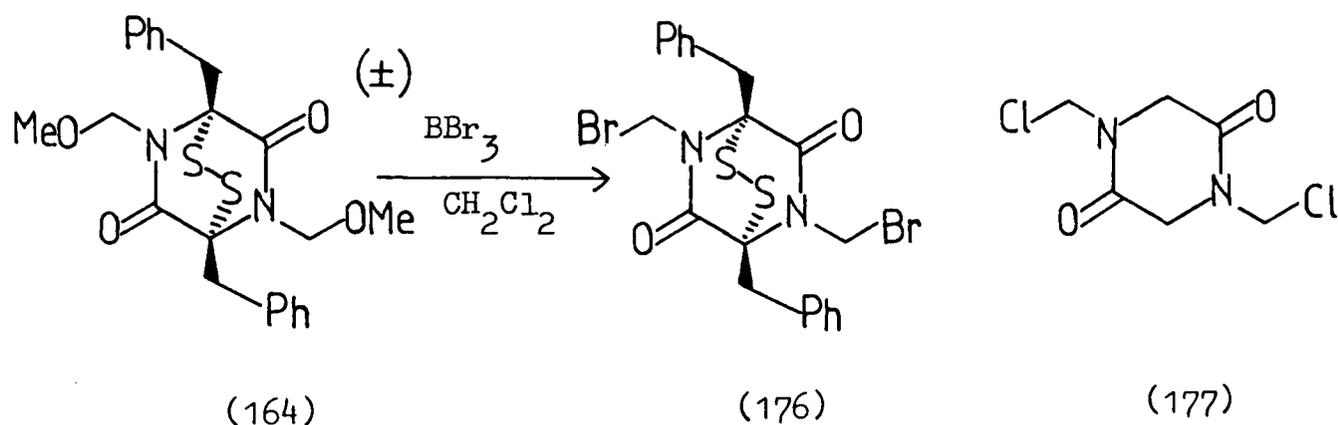
The disulphide (164) could be reconverted into the epitetrasulphide (162) by treatment with sulphur in basic conditions. Even when a deficiency of sulphur was used, no epitrisulphide was observed, contrary to what is found in similar systems.⁸ Heating the epidisulphide gave, besides sulphur, the epimonosulphide (163) and traces of epitetrasulphide (162). Similarly, heating the epitetrasulphide afforded epimonosulphide (163).



Attempts to remove the methoxymethyl protecting groups from the epidisulphide (164) with hydrochloric acid, in ethanol or trifluoroacetic acid,^{51,54} were not successful; extensive decomposition occurred, and dibenzylideneepiperazinedione (140c) was one of the main products.

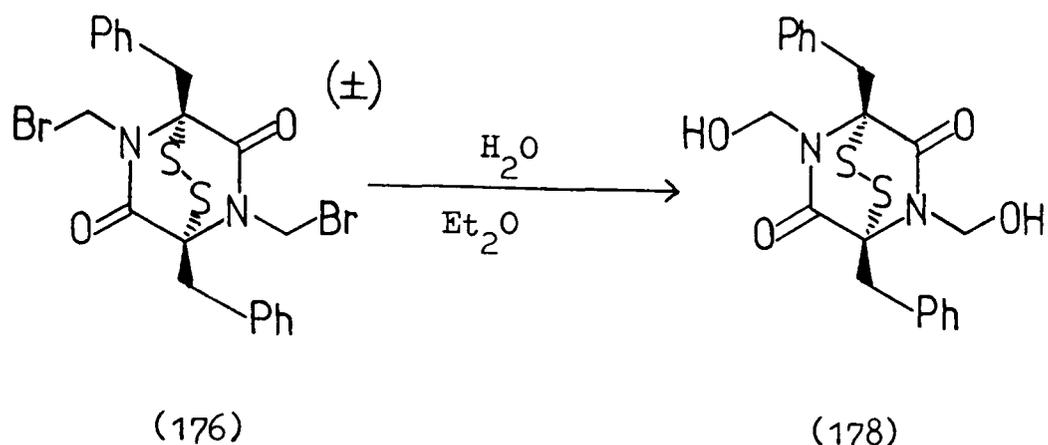


Treatment of the di(methoxymethyl)-epidisulphide (164) with boron tribromide at -40°C gave, rather unexpectedly, the di(bromomethyl) compound (176) in very high yield. This parallels the research of Cherbuliez and Feer,¹¹⁴ who found that in derivatives of glycine anhydride e.g. the N,N'-di(chloromethyl) compound (177), the methylene position was especially susceptible to nucleophilic attack.

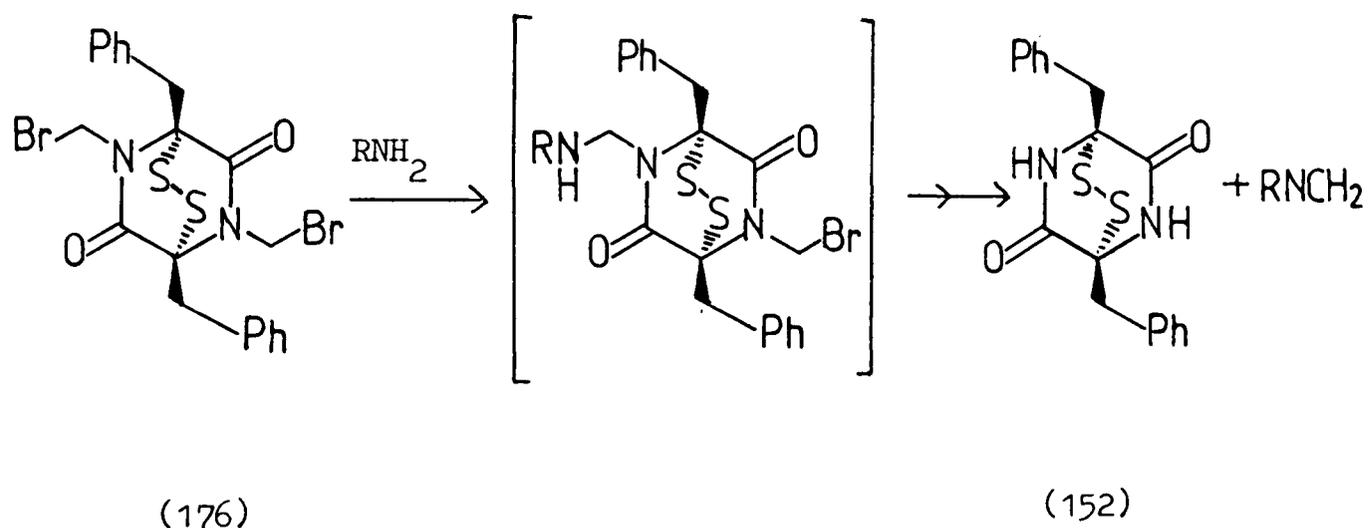


The di(bromomethyl) compound (176), when stirred with water, was hydrolysed to the di(hydroxymethyl) epidisulphide (178). This compound was much more stable than expected, and neither heat nor basic conditions (sodium bicarbonate) effected the removal of the hydroxymethyl groups. With more strongly basic conditions, decomposition

occurred with the evolution of hydrogen sulphide.



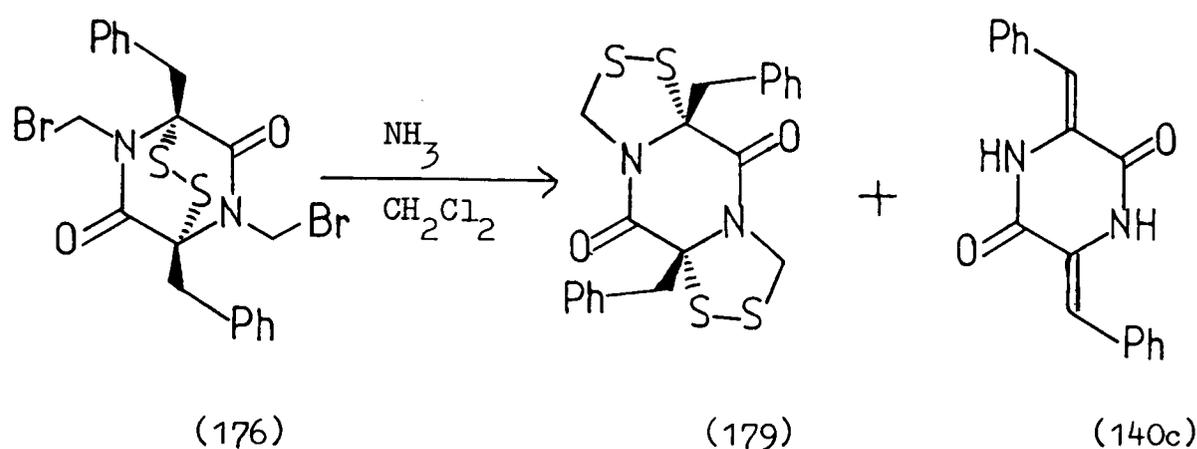
Another possible means of deprotection was to displace bromine with ammonia or a primary amine, and then the methylene group might be lost as an imine. Thus, treatment of a solution of the di(bromomethyl) compound (176) with ammonia in methylene chloride (ca. 10% saturated) for ca. 30 min gave the deprotected disulphide (152) on crystallisation from benzene, in moderate yield (ca. 50%), along with hexamethylenetetramine and hexamethylenetetramine hydrobromide (detected by n.m.r. spectroscopy only). The epidisulphide (152) was surprisingly soluble in relatively non-polar solvents (chloroform, carbon tetrachloride,



ether), whereas di(methylthio)-cyclo-(Phe-Phe) (131) was relatively insoluble in most solvents. It crystallised from benzene or toluene as a solvate, which by n.m.r. spectroscopy and elemental analysis

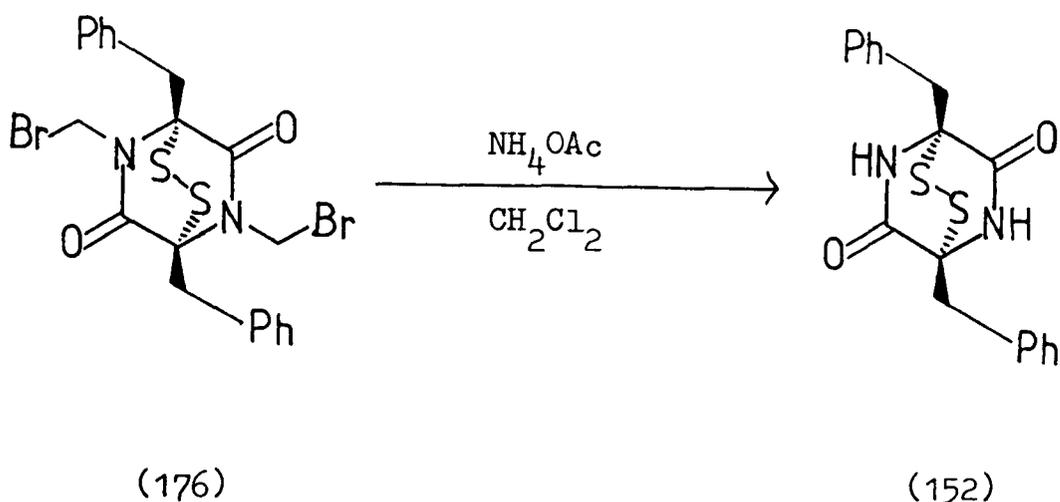
contained 0.75 benzene or toluene molecules for each epidisulphide molecule. Crystallisation also occurred from concentrated solutions in chloroform, but this material would not give a consistent analysis; this was attributed to residual traces of chloroform in the crystals. The compound was stable under neutral or slightly acidic conditions, but not under basic (potassium carbonate, morpholine) conditions.

When the di(bromomethyl) compound (176) was treated with a saturated solution of ammonia in methylene chloride, no deprotected epidisulphide was obtained; instead, the unusual bis(dithio)- compound (179) was isolated as a gum, along with dibenzylidenepiperazinedione (140c)

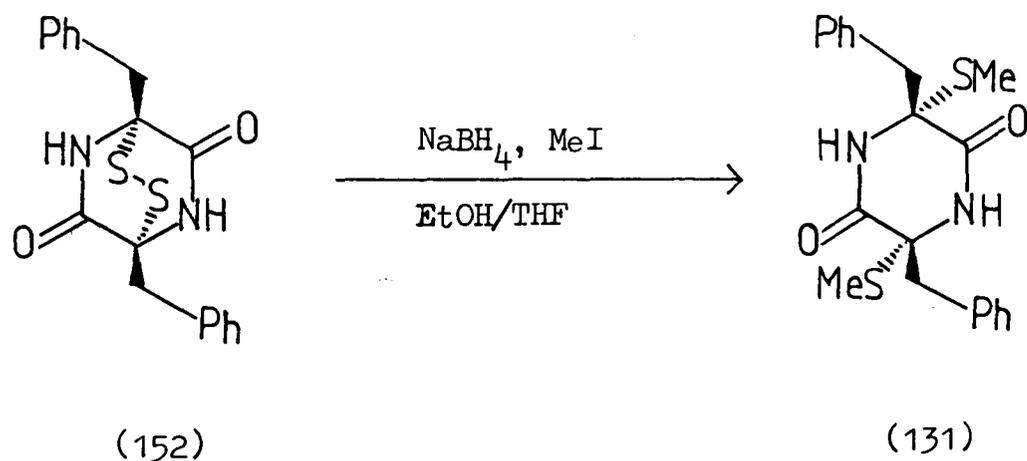


(140c). The structure of (179) was assigned on the basis of its spectroscopic properties only. The mechanism of its formation is not known.

The yield of epidisulphide (152) from treatment with ammonia was probably diminished by decomposition because of the basic conditions. We therefore tried using ammonium acetate as a suspension in methylene chloride, as a weakly basic deprotection reagent. The salt should be partially dissociated to free ammonia and acetic acid and, since it is used in excess as a suspension, it should provide a constant, reproducible concentration of ammonia. Indeed, deprotection proceeded almost quantitatively in 24h, with very little decomposition.

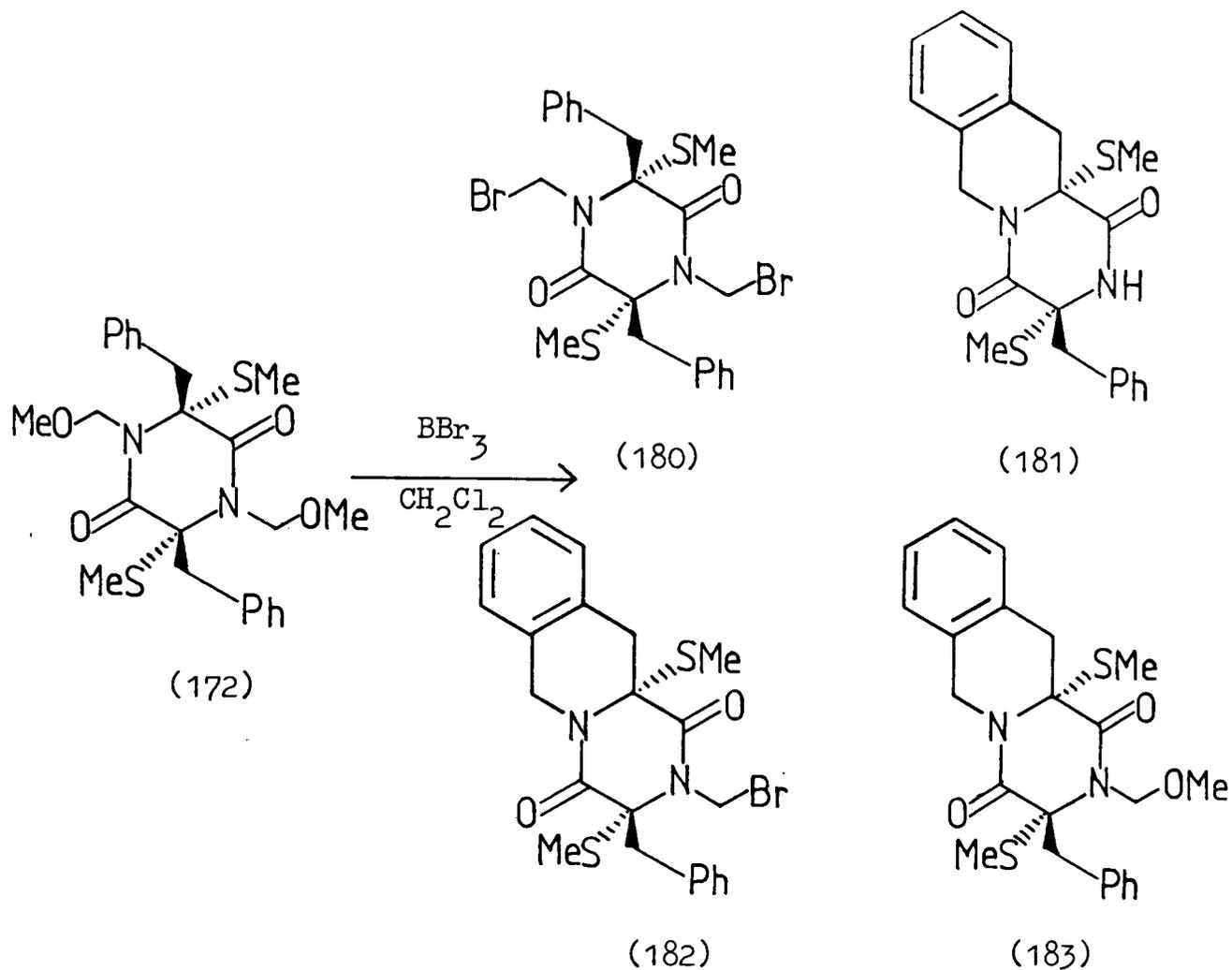


The epidisulphide, on treatment with sodium borohydride in ethanol and methyl iodide at 0°C , gave a reasonable yield (49%) of cis-di(methylthio)-cyclo-(Phe-Phe) (131). Its spectral properties were identical to those of the natural product obtained from Aspergillus terreus.



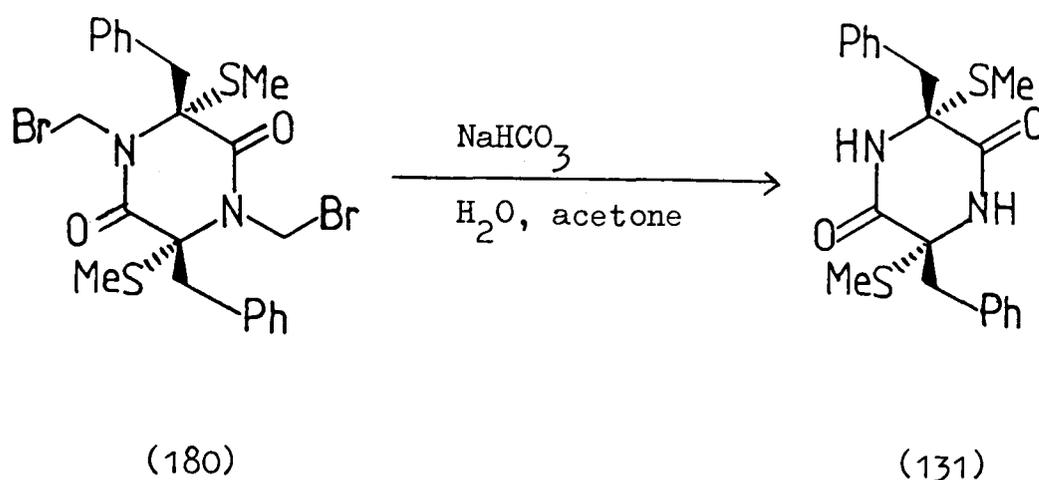
(±)-cis-Di(methylthio)-cyclo-(Phe-Phe) (131) was also prepared from the di(methoxymethyl) compound (172). When this compound was treated with boron tribromide at -60°C , a mixture of products was obtained, with the desired di(bromomethyl) derivative (180) as a minor component (ca. 30% of the total material). The remainder of the mixture consisted of three compounds [(181), (182), and (183)], all of which had undergone intramolecular cyclisation onto the phenyl ring. Compound (181) was fully characterised; the structures of (182) and

(183) were deduced from spectral data.



It is interesting that this aromatic substitution occurs with the di(methylthio)- compound (172) but not with the epidisulphide (164). It could be that the epidisulphide has an unsuitable conformation for the reaction to take place.

After boron tribromide treatment of (172), the total reaction products were stirred with aqueous sodium bicarbonate solution, and this gave, on crystallisation from acetone, the racemic natural product (131) in 31% yield from the di(methoxymethyl) compound (172). The mother liquors from the crystallisation contained the cyclised compounds (181) and (183). Thus the natural product (131) was synthesised both by the above method, and by reductive methylation of the deprotected epidisulphide (152).



2.5.3 Radio-labelled sulphur-containing dioxopiperazine synthesis and feeding experiments

Having made the proposed biosynthetic intermediate (152), and the natural product (131), we then had to prepare them in radioactive form for feeding experiments. Two labels (^3H and ^{35}S) were required to determine whether the precursors were incorporated intact.

The [^3H]- and [^{35}S]-di(bromomethyl)epidissulphides (176) were prepared separately, and combined in the final, deprotection step to give epidissulphide (152) with a suitable $^3\text{H}:^{35}\text{S}$ isotopic ratio. cyclo-(DL-[4'- ^3H]Phe-DL-Phe) was prepared by heating L-[4'- ^3H]phenylalanine in ethylene glycol under reflux. Strangely, this reaction did not behave in the same way as with unlabelled L-phenylalanine. The mixture became very dark after a short time, so the reaction was stopped earlier than was usual in case all the product was destroyed. The reaction did, however, yield a reasonable amount of cyclic dipeptide. The reaction sequence to the di(bromomethyl) compound (176) was carried out as before, giving a 2.3% overall yield from L-phenylalanine.

Similarly, ^{35}S -labelled elemental sulphur was used to label the disulphide bridge, and the di(bromomethyl)-[^{35}S]epidissulphide (176)

was obtained in 15% yield from di(methoxymethyl)-cyclo-(Phe-Phe) (161). For the final step, the ^3H - and ^{35}S -labelled compounds were combined in suitable proportions, and deprotected with ammonium acetate in 89% yield. This material was then fed to Aspergillus terreus.

In the first feeding experiment, after administration of the disulphide (152) (45 mg; ^3H , 16.8 μCi ; ^{35}S , 3.3 μCi), the fungus was given its normal growth period (4 days) before extraction. The extract was separated by t.l.c. to give BDA (40) (10.0 mg), di(methylthio)-cyclo-(Phe-Phe) (131) (0.6 mg) and cyclo-(L-Phe-L-Phe) (42) (8 mg). The incorporations into BDA were reasonable (^3H , 2.7%; ^{35}S , 4.4%), and the dilution by endogenous material was low (^3H , 5.41; ^{35}S , 3.40), but the $^3\text{H}:^{35}\text{S}$ isotopic ratio had not remained constant (5.16 \rightarrow 3.24). Substantially more sulphur activity than tritium had been incorporated. Since no precursor was recovered in the extract, both optical isomers of the racemic compound fed had been consumed, and thus the discrepancy in the ratio could have been caused by breakdown of the "wrong" (SS) optical isomer and incorporation of the fragments at different rates. Nevertheless, the incorporation of both labels at significant levels, with the low dilution of the precursor in the isolated BDA, did suggest that the epidisulphide (152) is a precursor of the arantins. It was desirable, however, to demonstrate more fully that the precursor is incorporated intact, at least to some extent.

The di(methylthio)-cyclo-(Phe-Phe) (131) isolated from this experiment was almost entirely derived from the disulphide fed, judging by the exceptionally low dilution figures (^3H , 1.20; ^{35}S , 1.06), and accounted for ca. 1% of the activity fed. In this compound, the isotopic ratio (4.53) was much closer to that of the precursor (5.16).

The cyclo-(L-Phe-L-Phe) isolated contained 0.3% of the tritium

activity of the precursor, with a dilution of 72.7, and this shows that there is some biological mechanism whereby the epidisulphide (152) can be reduced back to the cyclic dipeptide (42).

In a separate experiment, [^{35}S]-cis-di(methylthio)-cyclo-(Phe-Phe) (131) (5.5 mg, 0.36 μCi), prepared by reductive methylation of the deprotected epidisulphide (152) was fed to A. terreus. The incorporation of activity into BDA was low (0.26%), and the dilution was high (306); this agrees with our proposed biosynthesis where this compound (131) does not lie on the main biosynthetic route.

In an attempt to ascertain the causes of the alterations in isotopic ratio between the epidisulphide (152) and the metabolites, a second sample of doubly labelled precursor was synthesised, having higher specific activities than in the first run. This material was used for two feeding experiments run in parallel. In the first experiment, the culture was harvested $5\frac{1}{2}$ h after feeding the epidisulphide ((4.8 mg; ^3H , 2.06 μCi ; ^{35}S , 0.42 μCi ; ratio, 4.86). The isolated BDA (3.2 mg) had incorporated tritium (1.2%) and ^{35}S (2.7%), but the $^3\text{H}:$ ^{35}S ratio (2.63) had changed even more than in the long-period experiment. Once again, there was no detectable epidisulphide remaining in the extract (by t.l.c., radioscanning, and autoradiography). Because the culture was harvested at an early stage, there was a significant amount of acetylaranotin (38) in the extract (1.2 mg), and the precursor had been incorporated into this compound at a higher level (^3H , 3.1%; ^{35}S , 3.4%), lower dilution (^3H , 6.74; ^{35}S , 5.91), and less distortion of the $^3\text{H}:$ ^{35}S isotopic ratio (4.86 \rightarrow 4.41) than in the case of BDA. The low dilution in acetylaranotin can be rationalised by proposing that, because it is being converted into BDA, a large proportion of the acetylaranotin present had been formed during the $5\frac{1}{2}$ h between feeding the radioactive epidisulphide and harvesting, whereas most of the BDA

was already present when feeding took place. It is difficult to explain why the $^3\text{H}:^{35}\text{S}$ isotopic ratio was lower in BDA than in acetylaranotin. Assuming that all the BDA is derived from acetylaranotin, the activity in the isolated BDA should have been incorporated, in general, sooner after feeding than that in the acetylaranotin. Thus it is possible that a sulphur-containing product of degradation of the epidisulphide was absorbed into the cells more quickly than the precursor itself, and was therefore metabolised first, so that the acetylaranotin produced at this stage was high in sulphur activity; later, as the intact precursor was being metabolised, the isotopic ratio in acetylaranotin would approach that of the epidisulphide, but the BDA ratio would take longer to reach a higher value.

Also isolated from this extract was cis-di(methylthio)-cyclo-(Phe-Phe) (131) (ca. 0.2 mg). In this material, the $^3\text{H}:^{35}\text{S}$ isotopic ratio was almost exactly that of the precursor (4.86→4.85). The good incorporation (ca. 1.3%), and low dilution (3.54) strongly suggest that A. terreus can make this compound from the epidisulphide (152) without breakdown.

In the second feeding experiment, after feeding the disulphide (3.2 mg; ^3H , 1.37 μCi ; ^{35}S , 0.28 μCi ; ratio, 4.86), the culture was grown for 24h before harvesting. BDA (3.9 mg) isolated from this culture had a $^3\text{H}:^{35}\text{S}$ isotopic ratio of 3.18, higher than in the previous experiment but still substantially lower in tritium than the precursor.

A further experiment was designed to determine whether the epidisulphide (152) is produced by A. terreus. Autoradiography of chromatograms from extracts of cultures fed radioactive aranotin precursors (e.g. $^{35}\text{SO}_4^{2-}$ or L-[U- ^{14}C] phenylalanine) did not show any activity corresponding to this compound, but this might be due to its transient

nature and low concentration. Therefore, to test whether the epidisulphide was present in the cultures, the compounds on the arantoin biosynthetic pathway were labelled by administration of L-[U- ^{14}C]-phenylalanine (50 μCi) to 2-day old cultures. After incubation for 3 h, (\pm)-epidithio-cyclo-(Phe-Phe) (152) (40 mg) was added, and 30 min later the culture was harvested and the broth extracted. The epidisulphide was recovered by t.l.c., although there was very little of it left, (ca. 1 mg) and this was diluted with more epidisulphide (2 mg) to give enough material for crystallisation to constant activity. The specific activity ($1.08 \mu\text{Ci mmol}^{-1}$) represents an incorporation from L-phenylalanine into the recovered material of 0.015%. This activity demonstrates that the epidisulphide (152) can be biosynthesised from L-phenylalanine by Aspergillus terreus.

BDA and acetylarantoin were also isolated from this extract. The BDA contained 1.5% of the activity fed, and the acetylarantoin 3.0%. These incorporations after $3\frac{1}{2}$ h parallel quite closely the incorporation of tritium from the epidisulphide (152) into the same metabolites (1.2 and 3.1%, respectively) after $5\frac{1}{2}$ h.

2.5.4 Conclusions

In conducting this investigation we asked ourselves the question: Is epidithio-cyclo-(Phe-Phe) (152) an intermediate in the biosynthesis of the arantoin? Our experiments have provided strong evidence in favour of the affirmative; from doubly labelled (\pm)-[^{35}S]epidithio-cyclo-([4'- ^3H]Phe-Phe) (152), both isotopes were incorporated into arantoin with reasonable efficiency, even after a very short time, and with very low dilution by endogenous material. It was also shown that the epidisulphide can be produced by A. terreus from L-[U- ^{14}C]-phenylalanine. The changes in isotopic ratio from the precursor to

the products lessens the certainty of our conclusions, but this change can be explained by breakdown of some of the precursor (for example the "wrong", SS-isomer) to simpler compounds, and re-incorporation of the sulphur label with greater efficiency than the tritium. In this respect it might be advantageous to resolve the epidisulphide (152) so that only one optical isomer was being fed.

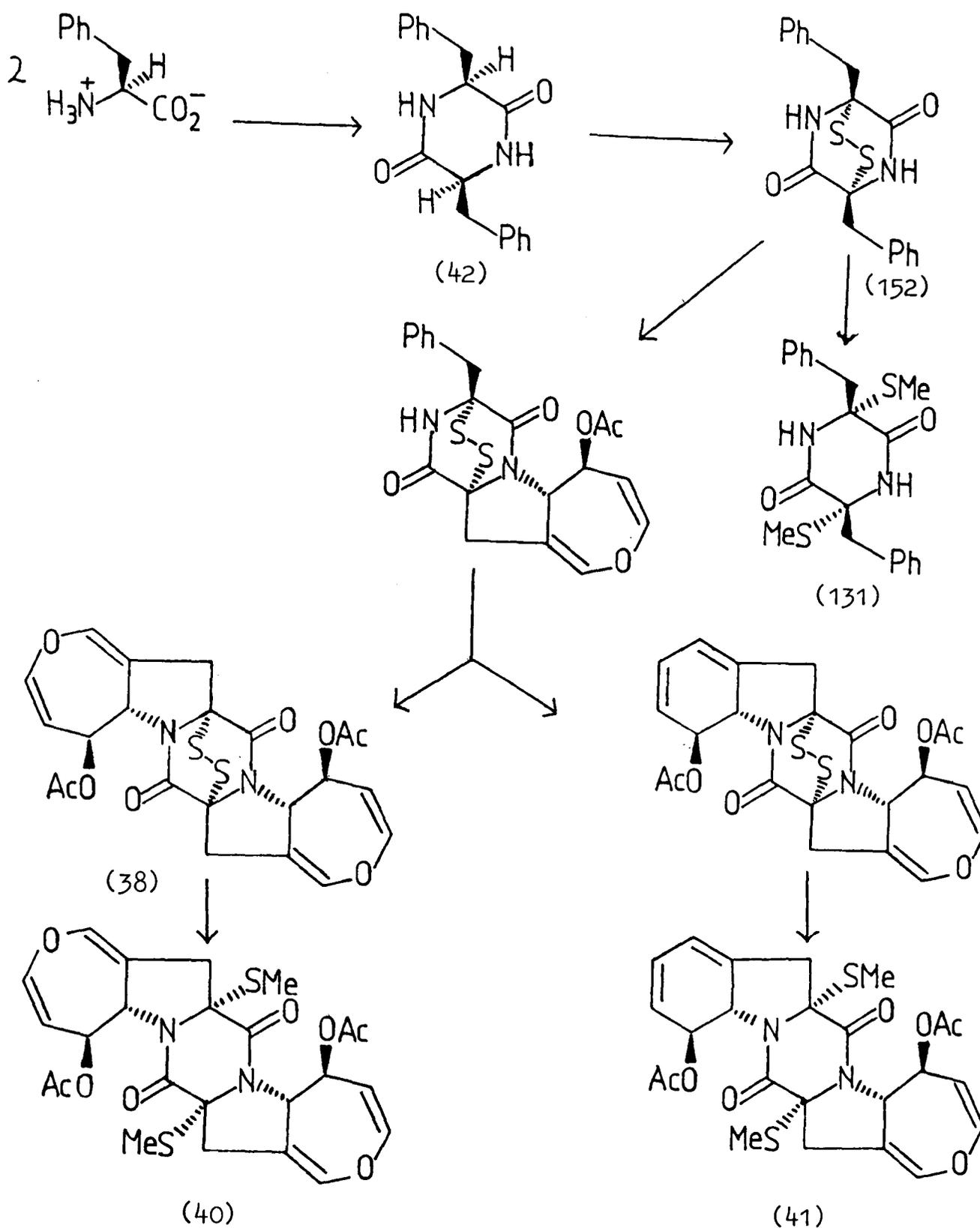
The situation is much clearer for di(methylthio)-cyclo-(Phe-Phe) (131). The epidisulphide (152) was converted into this compound with very low dilution by endogenous metabolite, and with no change in the $^3\text{H}:^{35}\text{S}$ isotopic ratio (in the short term experiment). It can therefore be concluded with some certainty that this metabolite can be derived from the intact epidisulphide precursor (152). The low incorporation of the di(methylthio)- compound into BDA suggests that it is not an intermediate in aranotin biosynthesis.

If the biosynthesis of the aranotins is analogous to that of gliotoxin, the methylthio- groups in BDA should be formed by reductive methylation of the disulphide bridge in acetylaranotin. The data from the above experiments tend to support this hypothesis, since in the short-term feeding experiments much of the activity from precursors is found in acetylaranotin, with low dilution by endogenous metabolite, whereas incubation of the fungus for several days results in most of the activity reaching BDA.

Although no apoaranotin metabolites e.g. BDAA (41) were isolated during this study, the results for acetylaranotin and BDA should also apply to these compounds.

Combining the results acquired in this project, including the experiments with fluorinated precursors, with previous research, a probable pathway for the biosynthesis of the aranotin metabolites can

be outlined, as shown below.



Many aspects of the pathway remain unclear, however. It has not yet been demonstrated conclusively that the di(methylthio)- compounds originate from the corresponding epidisulphides [except for the new metabolite (131)], although there is much evidence in support of this

hypothesis. The detailed mechanism of sulphur introduction, and the source of the sulphur atoms, remain unknown. Also, the mechanism of oxygenation of the aromatic rings of the precursors to the aranotin and gliotoxin-type ring systems, and the order in which these oxidations occur, are still unclear. Further experiments with this fungus might include feeding of other possible sulphur-containing precursors, such as dithiol and monothiol derivatives of cyclo-(L-Phe-L-Phe). Also, it would be interesting to feed the resolved epidisulphide (152) and thus perhaps establish conclusively that this compound is incorporated intact into the aranotins. A further search of the broth extracts for new metabolites might be enlightening, since their structures might reveal the order of some of the biosynthetic steps.

CHAPTER 3EXPERIMENTAL SECTION3.1 General procedures

Melting points were recorded on a Reichert hot-stage apparatus, and are uncorrected.

Optical rotations were measured at 589 nm (unless otherwise stated) using an Optical Activity Ltd AA-100 polarimeter.

Elemental analyses were carried out by Mrs. W.Harkness and her staff.

Ultraviolet spectra were recorded on a Pye-Unicam SP-800 spectrometer.

Infrared spectra were recorded on a Perkin-Elmer 580 spectrometer by Mrs. F.Lawrie and her staff. Unless otherwise stated, the samples were prepared by dispersion in potassium bromide discs.

Proton n.m.r. spectra were recorded on a Perkin-Elmer R 32 (90 MHz) spectrometer, unless otherwise stated. 100 MHz spectra were obtained from a Varian XL-100 spectrometer in the pulsed Fourier Transform (FT) mode. 360 MHz spectra were recorded on a Bruker WH-360 instrument, by Dr. I.Sadler and his staff (University of Edinburgh). Unless otherwise stated, deuteriochloroform (CDCl_3) was used as the solvent, with tetramethylsilane (TMS) as internal reference. All proton spectra are quoted to the nearest 0.1 p.p.m.

Carbon-13 n.m.r. spectra were recorded on a Varian XL-100 spectrometer, in CDCl_3 , with TMS as internal reference, at 25.2 MHz, unless otherwise stated.

Fluorine n.m.r. spectra were also recorded on the Varian XL-100 machine, in CDCl_3 unless otherwise stated, at 94.1 MHz. Signals were

recorded in p.p.m. from trichlorofluoromethane, using a deuterium locking system. The offsets of CFCl_3 from the deuterium signals were, for CDCl_3 , 49 661 Hz; for d_6 -DMSO, 50 019 Hz; and for CD_3OD , 49 827 Hz. The spectra from the Varian XL-100 were recorded by Dr. D.S.Rycroft, Mr. J.Gall, and Mr. R.Sharp.

Radioactive samples were counted on a Philips liquid scintillation counter, model PW 4510 D649, in Packard scintillation vials. The samples were dissolved in scintillator solution (10 ml); this was AnalaR toluene containing 2,5-diphenyloxazole (4 g/l) and 1,4-bis-2-(4-methyl-5-phenyloxazolyl) benzene (0.1 g/l). Materials containing ^{35}S were counted using the same programme as for ^{14}C . The results were found to be consistent if the quenching value was not significantly altered. Activities of ^{35}S ($t_{\frac{1}{2}} = 88$ days) have been adjusted to account for decay, so that incorporations, purities etc. can be evaluated directly from the quoted results. The scintillation counter was operated by Mrs. M.Tait and her staff. Autoradiograms were prepared using Ilford Red Seal 100FW film. A Panax Thin Layer Scanner, model RTLS-1A, was also used for detection of activity on t.l.c. plates.

Analytical t.l.c. was carried out on precoated Kieselgel GF₂₅₄ plates of thickness 0.25 mm, available from Merck. Spots were located initially using u.v. light (254 nm). Cyclic dipeptides were detected by the method of Issaq and Barr;¹¹⁵ after treatment of the dried, developed t.l.c. plates with chlorine gas for 5 min., the plates were sprayed with an aqueous solution of o-tolidine. Cyclic dipeptides with a secondary amide function gave blue spots, or yellow spots at higher concentrations. Tertiary amides gave cream-coloured spots. Later, a solution of tetramethylbenzidine was substituted to avoid the

use of the carcinogenic o-tolidine. The solution was made by dissolving 2,2',6,6'-tetramethylbenzidine (200 mg) in acetic acid (30 ml), then adding water (500 ml) and potassium iodide (1 g). The colours produced were the same. Sulphur-containing compounds were detected by spraying the plates with a 2% silver nitrate solution in aqueous acetone; compounds containing S-S bonds gave brown spots after ca. 1 min. Preparative t.l.c. separations were carried out on 20 x 20 cm plates coated with a 1 mm-thick layer of silica gel GF₂₅₄ (Fluka). The solvent systems used for t.l.c. were:

A; di-isopropyl ether: chloroform: acetic acid, 6:3:1

B; chloroform: ethyl acetate, 1:1

C; toluene: ethyl acetate, 3:1

D; benzene: acetic acid, 9:1

Organic solvents were removed on a Buchi rotary film evaporator at water-pump pressure.

3.2 Preparation of fluorinated cyclic dipeptides

cyclo-(Gly-L-Phe) (137)^{95,100} To an ice-cooled suspension of L-phenylalanine methyl ester hydrochloride (5.39 g, 25 mmol) in dry methylene chloride (50 ml) was added triethylamine (2.78 g, 27.5 mmol), followed by N-benzyloxycarbonylglycine (5.22 g, 25 mmol), and dicyclohexylcarbodiimide (5.67 g, 27.5 mmol). The mixture was left in the dark at room temperature for 12 h, with occasional swirling, and was then filtered through celite. The filtrate was washed with 1 M hydrochloric acid (2 x 10 ml), water (2 x 10 ml), saturated aqueous sodium bicarbonate solution (2 x 10 ml), water (1 x 10 ml), and brine (1 x 10 ml), and evaporated to dryness. The resultant oil was dissolved in acetone (25 ml), filtered through celite, and the solvent was evaporated. The oil was then dissolved in methanol (50 ml) and acetic acid (5 ml); 10% Pd/C catalyst (200 mg) was added, and the mixture was hydrogenated at atmospheric pressure for 14 h. After the catalyst had been filtered off, the solution was concentrated to a yellow oil which was dissolved in methanol (30 ml). To the solution was added saturated ammoniacal methanol (50 ml). After standing overnight at room temperature, the solid was filtered off, washed with methanol and ether, and dried under vacuum, to give cyclo-(Gly-L-Phe) (137) (4.50 g, 88%), m.p. 268-270°C (lit.,¹⁰⁰ 265.5°C), $[\alpha]_D^{20}$ +100.0° (c 2.7 in AcOH) (lit.,¹⁰⁰ +100.5°); ν_{\max} . 3 200 (NH), and 1 675 cm⁻¹ (CO); δ (TFA) 3.07 (1 H, d, J 18 Hz, H_{pro-S} of CH₂N), 3.35 (2 H, m, CH₂Ph), 3.99 (1 H, d, J 18 Hz, H_{pro-R} of CH₂N), 4.78 (1 H, m, CHCH₂Ph), 7.1-7.5 (5 H, m, Ph), 8.00 (1 H, br s, NH), and 8.33 (1 H, br s, NH); m/z 204 (M^+ , 52%), 161, 91, (100), 86, and 43.

N,N'-Diacetyl-cyclo-(Gly-L-Phe) (133).¹⁰¹ - cyclo-(Gly-L-Phe) (137) (700 mg, 3.43 mmol) was stirred with acetic anhydride (6 ml) and perchloric acid (2 drops) for 40 min. at 75°C. The mixture was cooled, poured into water (70 ml), and extracted with chloroform (3 x 15 ml). The solvents were removed and the residual oil was crystallised from ethanol-water to give diacetyl-cyclo-(Gly-L-Phe) (133) (830 mg, 83%), m.p. 103-104°C, $[\alpha]_D^{20} + 76.4$ (c 1.0 in EtOH) (Found: C, 62.4; H, 5.5; N, 9.5. C₁₅H₁₆N₂O₄ requires C, 62.5; H, 5.6; N, 9.7%); ν_{\max} . 1 720 (2 COMe) and 1 700 cm⁻¹ (2 ring CO); δ 2.52 (3 H, s, Ac), 2.54 (3 H, s, Ac), 2.50 (1 H, d, J 19 Hz, H_{pro-S} of CH₂N), 3.28 (2 H, m, CH₂Ph), 4.48 (1 H, d, J 19 Hz, H_{pro-R} of CH₂N), 5.42 (1 H, m, CHCH₂Ph), and 7.0 - 7.4 (5 H, m, Ph); m/z 288 (M⁺, 100%), 203, 91, and 43.

General method for the preparation of the cyclic dipeptides (136a-f)

To an ice-cooled solution of diacetyl-cyclo-(Gly-L-Phe) (133) (288 mg, 1 mmol) and the relevant aromatic aldehyde (1.1 mmol) in DMF (2 ml) was added slowly with stirring a 0.5 M solution of potassium t-butoxide in t-butanol (2 ml). The mixture was stirred at 0°C for 1 h, when t.l.c. indicated that the reaction was completed. Acetic acid (0.2 ml) was added, the mixture was poured into water (20 ml), and the products were extracted with ethyl acetate (3 x 10 ml). After removal of the solvent, the residual oil containing the monoacetyl-arylidene derivative (134a - f) was dissolved in methanol (10 ml) and acetic acid (2 drops), 10% Pd/C catalyst (50 mg) was added, and the mixture was hydrogenated at atmospheric pressure for 24 h. The catalyst was filtered off, the solvents were evaporated, and the residue containing the N-acetyl cyclic dipeptide was dissolved in DMF (2 ml). Hydrazine hydrate (30 μ l) was added, and the mixture was left at room

temperature for 1 h, then acidified with acetic acid (0.1 ml). The mixture was added to water (10 ml), the product was filtered off, washed with methanol and ether, and crystallised from acetic acid-water.

cyclo-(L-Phe-L-2',6'-Difluorophe) (136a), (215 mg, 65%), m.p. 287-289°C, $[\alpha]_D^{21} -29.6^\circ$ (c 0.5 in AcOH) (Found: C, 65.55; H, 4.95; N, 8.5; F, 11.2. $C_{18}H_{16}F_2N_2O_2$ requires C, 65.45; H, 4.9; N, 8.5; F, 11.5%); ν_{max} . 3 200 (NH), 1 680 (CO), and 1 670 cm^{-1} (CO); δ (TFA) 2.5 - 2.9 (2 H, m, 2 $CH_{\text{pro-R}}^{\text{HAr}}$), 3.0 - 3.4 (2 H, m, 2 $CH_{\text{pro-S}}^{\text{HAr}}$), 4.61 (2 H, m, 2 $CHCH_2Ar$), 6.8 - 7.5 (8 H, m, Ar), 8.16 (1 H, br s, NH), and 8.35 (1 H, br s, NH); $\delta_F\{^1H\}$ (CD_3OD) -113.2 (s); m/z 330 (M^+ , 100%), 239, 203, and 91.

cyclo-(L-Phe-L-p-Fluorophe) (136b), (110 mg, 35%), m.p. 307 - 308°C, $[\alpha]_D^{20} -95.8^\circ$ (c 0.5 in AcOH) (Found: C, 69.4; H, 5.7; N, 9.0; F, 6.2. $C_{18}H_{17}FN_2O_2$ requires C, 69.4; H, 5.5; N, 8.95; F, 6.1%); ν_{max} . 3 210 (NH), 1 680 (CO), and 1 660 cm^{-1} (CO); δ (TFA) 2.07 (1 H, dd, J 15 and 8 Hz, $CH_{\text{pro-R}}^{\text{HAr}}$), 2.66 (1 H, dd, J 15 and 7 Hz, $CH_{\text{pro-R}}^{\text{HPh}}$), 3.01 (1 H, dd, J 15 and 3 Hz, $CH_{\text{pro-S}}^{\text{HAr}}$), 3.17 (1 H, dd, J 15 and 4 Hz, $CH_{\text{pro-S}}^{\text{HPh}}$), 4.40 - 4.75 (2 H, m, 2 $CHCH_2Ar$), 7.0 - 7.6 (9 H, m, Ar), 8.02 (1 H, br s, NH), and 8.14 (1 H, br s, NH); $\delta_F\{^1H\}$ -116.0; m/z 312 (M^+ , 100%), 221, 203, 109, and 91.

cyclo-(L-Phe-L-o-Fluorophe) (136c), (200 mg, 64%), m.p. 300 - 302°C, $[\alpha]_D^{21} -74.6^\circ$ (c 0.5 in AcOH) (Found: C, 69.35; H, 5.4; N, 8.8; F, 6.4. $C_{18}H_{17}FN_2O_2$ requires C, 69.2; H, 5.5; N, 8.95; F, 6.1%); ν_{max} . 3 310 (NH), 3 200 (NH), 1 675 (CO), and 1 665 cm^{-1} (CO); δ (TFA)

2.35 (1 H, dd, J 15 and 7 Hz, $\text{CH}_{\text{pro-R}}^{\text{HAr}}$), 2.49 (1 H, dd, J 14 and 8 Hz, $\text{CH}_{\text{pro-R}}^{\text{HPh}}$), 3.04 - 3.38 (2 H, m, 2 $\text{CH}_{\text{pro-S}}^{\text{HAr}}$), 4.62 (2 H, m, 2 CHCH_2Ar), 7.0 - 7.5 (9 H, m, Ar), 8.14 (1 H, br s, NH), and 8.24 (1 H, br s, NH); $\delta_{\text{F}}\{^1\text{H}\}$ (d_6 -DMSO) -112.7; m/z 312 (M^+ , 20%), 221, 203, 109, and 91 (100).

cyclo-(L-Phe-L-m-Fluorophe) (136d), (170 mg, 54%), m.p. 300 - 303°C, $[\alpha]_{\text{D}}^{21}$ -74.4° (c 0.5 in AcOH) (Found: C, 69.2; H, 5.5; N, 9.0; F, 6.3. $\text{C}_{18}\text{H}_{17}\text{FN}_2\text{O}_2$ requires C, 69.2; H, 5.5; N, 8.95; F, 6.1%); ν_{max} . 3 200 (NH), 1 680 (CO), and 1 670 cm^{-1} (CO); δ (TFA) 2.04 (1 H, dd, J 15 and 8 Hz, $\text{CH}_{\text{pro-R}}^{\text{HAr}}$), 2.62 (1 H, dd, J 15 and 8 Hz, $\text{CH}_{\text{pro-R}}^{\text{HPh}}$), 2.99 (1 H, dd, J 15 and 3 Hz, $\text{CH}_{\text{pro-S}}^{\text{HAr}}$), 3.12 (1 H, dd, J 15 and 4 Hz, $\text{CH}_{\text{pro-S}}^{\text{HAr}}$), 4.38 - 4.72 (2 H, m, 2 CHCH_2Ar), 6.7 - 7.5 (9 H, m, Ar), 8.01 (1 H, br s, NH), and 8.11 (1 H, br s, NH); $\delta_{\text{F}}\{^1\text{H}\}$ (d_6 -DMSO) -116.7; m/z 312 (M^+ , 16%), 109, and 91 (100).

cyclo-(L-Phe-L-Pentafluorophe) (136e), (crystallised from acetic acid-ether), (290 mg, 76%), m.p. 309 - 311°C, $[\alpha]_{\text{D}}^{21}$ -39.6° (c 0.5 in AcOH) (Found: C, 56.0; H, 3.2; N, 7.0; F, 24.6. $\text{C}_{18}\text{H}_{13}\text{F}_5\text{N}_2\text{O}_2$ requires C, 56.25; H, 3.4; N, 7.3; F, 24.7%); ν_{max} . 3 200 (NH), and 1 680 cm^{-1} (CO); δ (TFA) 2.17 (1 H, dd, J 15 and 9 Hz, $\text{CH}_{\text{pro-R}}^{\text{HC}_6\text{F}_5}$), 2.92 (1 H, dd, J 15 and 4 Hz, $\text{CH}_{\text{pro-S}}^{\text{HC}_6\text{F}_5}$), 3.22 - 3.41 (2 H, m, CH_2Ph), 4.40 (1 H, m, CHCH_2Ph), 4.78 (1 H, m, $\text{CHCH}_2\text{C}_6\text{F}_5$), 7.1 - 7.6 (5 H, m, Ph), 8.30 (1 H, br s, NH), and 8.39 (1 H, br s, NH); $\delta_{\text{F}}\{^1\text{H}\}$ (d_6 -DMSO) -141.0 (2 F, m), -157.6 (1 F, t), and -163.5 (2 F, m); m/z 384 (M^+ , 6%), 265, 203, 181, and 91 (100).

cyclo-(L-Phe-L-Phe) (136f) = (42), (185 mg, 63%), m.p. 307 -

310°C, (lit.,¹⁰² 308 - 310°C), $[\alpha]_D^{21}$ -123.6° (c 0.5 in AcOH); $[\alpha]_D^{21}$ -237° (c 0.06 in pyridine) lit.,¹⁰³ $[\alpha]_D^{25}$ -242° (c 0.059 in pyridine); ν_{\max} . 3 210 (NH), 1675 (CO), and 1 665 cm^{-1} (CO); δ (TFA) 2.34 (2 H, dd, J 15 and 8 Hz, $\text{CH}_{\text{pro-R}}^{\text{HPh}}$), 3.09 (2 H, dd, J 15 and 4 Hz, $\text{CH}_{\text{pro-S}}^{\text{HPh}}$), 4.58 (2 H, m, CHCH_2Ph), 7.0 - 7.6 (10 H, m, Ph), and 8.07 (2 H, br s, NH); m/z 294 (M^+ , 33%), 203, 175, and 91 (100).

Although the intermediate arylidene compounds (134a - f) were not isolated in pure form, their n.m.r. spectra were recorded, and are given in Table 1.

Determination of the optical purity of (134a)

After carrying out the condensation of diacetyl-cyclo-(Gly-L-Phe) (133) with 2,6-difluorobenzaldehyde as described above, a sample of the oil obtained was purified by preparative t.l.c. (solvent system B) to give the pure arylidene compound (134a) as an oil, λ_{\max} . (EtOH) 307 nm (ϵ 16 000); ν_{\max} . 3 400 (NH), 1 710 (CO), and 1 695 cm^{-1} (CO); δ 2.59 (3 H, s, Ac), 3.22 (2 H, d, J 5 Hz, CH_2Ph), 5.33 (1 H, t, J 5 Hz, CHCH_2Ph), 6.52 (1 H, s, CHAr), 6.8 - 7.5 (8 H, m, Ar), and 7.81 (1 H, br s, NH); m/z 370 (M^+ , 77%), 328, 237, (100), 209, 91, and 43. On addition of the chiral shift reagent tris[3-(trifluoromethylhydroxymethylene)-d-camphorato]europium(III) [$\text{Eu}(\text{tfc})_3$] (0.2 mol eq) to the n.m.r. sample, the spectrum became; δ 2.82 (3 H, s, Ac), 3.84 (1 H, dd, J 14 and 6 Hz, CHHPh), 4.18 (1 H, dd, J 14 and 3 Hz, CHHPh), 6.6 - 7.8 (10 H, m, Ar, CHAr , and CHCH_2Ph), and 8.90 (1 H, br s, NH). The spectrum of a partially racemic sample (prepared using triethylamine as base - see Discussion) contained an additional ABX system ca. 0.1 p.p.m. upfield, and an additional NH signal 0.2 p.p.m. upfield of the

COMPOUND	CH_3CO (s)	CH_2 (d, J 5 Hz)	CHCH_2 (t, J 5 Hz)	C=CHAr (br s)	Ar (m)	NH (br s)
(134a); Ar = $\text{C}_6\text{H}_3\text{F}_2$	2.58	3.22	5.35	6.52	6.8 - 7.5	7.74
(134b); Ar = p-F-Ph	2.56	3.19	5.30	6.55	6.8 - 7.35	8.60
(134c); Ar = o-F-Ph	2.58	3.23	5.34	6.62	6.95 - 7.5	7.95
(134d); Ar = m-F-Ph	2.58	3.19	5.27	6.53	6.7 - 7.5	8.56
(134e); Ar = C_6F_5	2.60	3.20	5.25	6.40	6.9 - 7.4	8.83
(134f); Ar = Ph	2.57	3.24	5.36	6.62	7.0 - 7.7	8.4 (br)

TABLE 1 N.m.r. data for arylidene compounds (134a - f)

corresponding signals from the S-enantiomer. These signals were not detectable in the first sample.

cyclo-(L-Phe-L-(3R)-[3-D]-p-Fluorophe) (136h). - This compound was prepared by the general method, using [formyl-D]-p-fluorobenzaldehyde (preparation given below). Diacetyl-cyclo-(Gly-L-Phe) (133) (200 mg, 0.69 mmol) gave the deuteriated cyclic dipeptide (136h) (130 mg, 60%), m.p. 306 - 309°C, ν_{\max} . 3 210 (NH), 1 680 (CO), and 1 660 cm^{-1} (CO); δ (TFA) 2.63 (1 H, dd, J 15 and 7 Hz, $\text{CH}_{\text{pro-R}}^{\text{HPh}}$), 2.98 (1 H, br d, J 4 Hz, $\text{CH}_{\text{pro-S}}^{\text{DAr}}$), 3.12 (1 H, dd, J 15 and 4 Hz, $\text{CH}_{\text{pro-S}}^{\text{HPh}}$), 4.46 (1 H, m, CHCHDAr), 4.62 (1 H, m, CHCH_2Ph), 7.0 - 7.5 (9 H, m, Ar), 7.97 (1 H, br s, NH of p-Fluorophe), and 8.09 (1 H, br s, NH of Phe); also small signals at 3.33 (m), 4.82 (m), 7.25 (m), and 8.42 (br s), due to a small amount (ca. 15%) of the arylidene compound (138h); m/z 313 (M^+ , 70%), 312 (11), 311 (28), 203 (41), 110 (76), and 91 (100).

α -Morpholino- α -(p-fluorophenyl)acetonitrile (184). - This compound was prepared by the method of Bennett et al.,¹¹⁶ substituting p-fluorobenzaldehyde for benzaldehyde, on a 6 millimolar scale, giving (184) (1.07 g, 81%), m.p. 67 - 68°C, (Found: C, 65.25; H, 5.8; N, 12.75. $\text{C}_{12}\text{H}_{13}\text{FN}_2\text{O}$ requires C, 65.45; H, 5.95; N, 12.7%); ν_{\max} . 2 870, 2 830, 2 230 (CN), 1 610, 1 600, 1 505, 1 110, and 860 cm^{-1} ; δ 2.57 (4 H, m, NCH_2), 3.71 (4 H, m, OCH_2), 4.80 (1 H, m, CH), 7.09 (2 H, t, J 9 Hz, o- to F), and 7.52 (2 H, dd, J 9 and 6 Hz, m- to F); m/z 220 (M^+ , 43%), 189, 134 (100), 109, and 86.

[Formyl-D]-p-fluorobenzaldehyde. - α -Morpholino- α -p-fluorophenyl)-acetonitrile (184) (1.0 g, 4.55 mmol) was dissolved in dry ether (5 ml). The solution was cooled to 0°C in a flask equipped with a rubber septum, and a 1.5 M solution of n-butyllithium in hexane (4 ml) was added with

stirring. After 10 min., D_2O (0.2 ml) was added dropwise, then thionyl chloride was added until the mixture gave an acid reaction on wet litmus paper. The mixture was then added to water (50 ml) and extracted with ether (3 x 10 ml). The brown oil so obtained was heated under reflux with 2 M hydrochloric acid (50 ml) for 1 h, the crude deuterio-fluorobenzaldehyde was extracted with ether (3 x 10 ml), and was purified using a Kugelrohr ball-to-ball distillation apparatus. Pure [formyl-D]-p-fluorobenzaldehyde was thus obtained (200 mg, 35%), δ 7.13 (2 H, t, J 8 Hz, \underline{o} -H to F), 7.82 (2 H, dd, J 8 and 5 Hz, \underline{o} -H to CDO), and 9.84 (residual CHO, s, ca. 3% of 1 H).

cyclo-(L-Phe-L-(3S)-[2,3-D₂]-p-Fluorophe) (136g). - This compound was prepared by the general method, except that the reduction step was carried out using deuterium gas, with methanol-OD as the solvent. The deuterium was generated by addition of D_2O via a pressure-compensated dropping funnel to lithium metal contained in a three-necked flask attached to the evacuated hydrogenation apparatus. When atmospheric pressure was reached (indicated by inflation of a rubber teat attached to one arm of the three-necked flask), the reservoir was allowed to fill, and the three-necked flask was detached. The diacetyl compound (133) (144 mg, 0.5 mmol) gave the deuteriated cyclic dipeptide (136g) (60 mg, 38%), m.p. 304 - 307°C; ν_{\max} . 3 210 (NH), 1 680 (CO), and 1 660 cm^{-1} (CO); δ (TFA) 2.06 (1 H, br s, $\underline{CH}_{\underline{pro-R}}^{DAr}$), 2.63 (1 H, dd, J 15 and 7 Hz, $\underline{CH}_{\underline{pro-R}}^{HPh}$), 3.13 (1 H, dd, J 15 and 4 Hz, $\underline{CH}_{\underline{pro-S}}^{HPh}$), 4.43 (ca. 0.1 H, m, residual $\underline{CHCHD}Ar$), 4.63 (1 H, m, \underline{CHCH}_2Ph), 7.0 - 7.6 (9 H, m, Ar), 8.00 (1 H, br s, NH of p-Fluorophe), and 8.12 (1 H, br s, NH of Phe); m/z 314 (\underline{M}^+ , 46%), 313 (12), 223 (7), 204 (36), 203 (6), 110 (91), 109 (8), and 91 (100).

2,6-Difluorobenzaldehyde. - 2,6-Difluorobenzoic acid (6.5 g, 41 mmol) was added to thionyl chloride (10 ml) containing pyridine (1 drop), and the mixture was heated under reflux for 3 h. Excess thionyl chloride was removed under reduced pressure to give crude 2,6-difluorobenzoyl chloride as an oily liquid, which was used without further purification. $\nu_{\max.}$ (thin film) 1 780 (CO) and 1 740 cm^{-1} (CO).

To a solution of the crude acid chloride (7.2 g, ca. 40 mmol) and triphenylphosphine (21.6 g, 82 mmol) in acetone (100 ml) was added bis-(triphenylphosphine) copper(I) borohydride (24.8 g, 40 mmol).¹¹⁷ The mixture was stirred for 45 min., and the solids were removed by filtration. The filter cake was washed with ether, and the solvents were evaporated from the combined filtrate and washings. The residue was treated with ether (100 ml) and filtered; the filtrate was evaporated, and the residue was dissolved in chloroform (100 ml). The solution was stirred with finely powdered copper(I) chloride (10 g) for 2 h, the mixture was filtered, and the filtrate was evaporated. The residue was treated with methanol (50 ml) and filtered. After evaporation of the methanol from the filtrate, the remaining oil was distilled at water-pump pressure to give 2,6-difluorobenzaldehyde (3.8 g, 65%), b.p. 104 - 105°C/40 Torr (lit.,¹¹⁸ 85 - 87°C/20 Torr); $\nu_{\max.}$ (thin film) 2 870, 2 770 (CHO), and 1 700 cm^{-1} (CO); δ 6.8 - 7.8 (3 H, m, Ar), and 10.33 (1 H, s, CHO); $\delta_{\text{F}}\{^1\text{H}\}$ -115.2 (s); m/z 141 (M^+ - H).

3,6-Di-(p-fluorobenzylidene)piperazine-2,5-dione (140a).⁹⁶ - Glycine anhydride (300 mg, 2.63 mmol), p-fluorobenzaldehyde (820 mg, 6.61 mmol), sodium acetate (870 mg, 10.61 mmol), and acetic anhydride (3 ml) were heated together at 120°C for 4 h. The mixture was then

shaken with water (25 ml), the precipitate was filtered off, and was washed with methanol and ether to give (140a) (530 mg, 62%), m.p. $> 328^{\circ}\text{C}$, ν_{max} . 3 220 (NH), 1 685 (CO), 1 630 (C=C), 1 510, 1 410, and 1 230 cm^{-1} ; δ (TFA) 7.1 - 7.6 (m); m/z 326 (M^+ , 100%), 136, 135, 134, 109, 108, and 107 (Found: m/z 326.0878. $\text{C}_{18}\text{H}_{12}\text{F}_2\text{N}_2\text{O}_2$ requires 326.0867).

(±)-3,6-Di-(p-fluorobenzyl)piperazine-2,5-dione (139a). - The diarylidene compound (140a) (60 mg, 0.18 mmol) was suspended in methanol (60 ml) and acetic acid (0.5 ml), 10% Pd/C catalyst (30 mg) was added, and the mixture was hydrogenated for 70 h at atmospheric pressure. The catalyst was filtered off and washed with acetic acid (10 ml), and the filtrates were evaporated to dryness. The residue was crystallised from acetic acid-water to give (139a) (35 mg, 58%), m.p. $291 - 293^{\circ}\text{C}$ (Found: C, 65.25; H, 4.95; N, 8.55; F, 11.6. $\text{C}_{18}\text{H}_{16}\text{F}_2\text{N}_2\text{O}_2$ requires C, 65.45; H, 4.9; N, 8.5; F, 11.5%); ν_{max} . 3 210 (NH), and 1 670 cm^{-1} (CO); δ (TFA) 2.44 (2 H, dd, J 15 and 8 Hz, $\text{CH}_{\text{pro-R}}^{\text{HAr}}$ in SS-enantiomer), 3.13 (2 H, dd, J 15 and 4 Hz, $\text{CH}_{\text{pro-S}}^{\text{HAr}}$ in SS-enantiomer), 4.60 (2 H, m, CHCH_2Ar), 7.12 (8 H, d, J 7 Hz, Ar), and 8.1 (2 H, br s, NH); $\delta_{\text{F}}\{^1\text{H}\}$ -116.3; m/z 330 (M^+ , 7%), 221, and 109 (100).

3,6-Di-(pentafluorobenzylidene)piperazine-2,5-dione (139b).⁹⁷ - Triethylamine (220 mg, 2.18 mmol) was added to a solution of 1,4-diacetylpiperazine-2,5-dione (200 mg, 1.01 mmol) and pentafluorobenzaldehyde (400 mg, 2.04 mmol) in DMF (2 ml), and the mixture was stirred at 100°C for 2 h. The dark-coloured mixture was then shaken with water (10 ml), the solid was filtered off, and was washed with methanol

and ether to give (140b) (170 mg, 36%), m.p. > 322°C (Found: C, 45.9; H, 0.75; N, 6.0; F, 40.4. $C_{18}H_4F_{10}N_2O_2$ requires C, 46.0; H, 0.85; N, 5.95; F, 40.4%); ν_{\max} . 3 200 (NH), 1 690 (CO), 1 660, and 1 645 cm^{-1} (C=C); m/z 470 (M^+ , 96%), 208 (100), 207, and 180.

(±)-cis-3,6-Di-(pentafluorobenzyl)piperazine-2,5-dione (139b). -

The diarylidene compound (140b) (140 mg, 0.30 mmol) was suspended in methanol (100 ml) and acetic acid (10 ml), with 10% Pd/C catalyst (100 mg), and the mixture was hydrogenated at atmospheric pressure for 50 h. The catalyst was filtered off, the filtrate was evaporated to dryness, and the residue was crystallised from methanol-water to give (139b) (130 mg, 92%), m.p. 292 - 294°C (Found: C, 45.7; H, 1.4; N, 5.65; F, 39.85. $C_{18}H_8F_{10}N_2O_2$ requires C, 45.6; H, 1.7; N, 5.9; F, 40.05%); ν_{\max} . 3 200 (NH), 1 680 (CO), 1520, and 1 510 cm^{-1} ; δ (TFA) 3.01 - 3.72 (4 H, m, CH_2Ar), 4.71 (2 H, m, $CHCH_2Ar$), and 8.48 (2 H, br s, NH); $\delta_F\{^1H\}$ (d_6 -DMSO) -141.6 (2 F, m), -156.9 (1 F, t), and -163.2 (2 F, m); m/z 474 (M^+ , 7%), 293, 265 (100), 181, and 163.

(±)-cis-cyclo-([2,3-D₂]-Phe-[2,3-D₂]-Phe) (139c). - 3,6-Dibenzylidenepiperazine-2,5-dione (140c)⁹⁶ (97 mg, 0.33 mmol) was suspended in methanol-OD (8 ml) with 10% Pd/C catalyst (30 mg), and the mixture was treated with deuterium gas (preparation given above) at atmospheric pressure for 20 h. The catalyst was filtered off and washed with acetic acid (10 ml). The combined filtrates were evaporated to dryness, and the residue was crystallised from acetic acid-water to give tetradeuterio-cis-cyclo-(Phe-Phe) (139c) (38 mg, 39%), m.p. 325°C (sublimed), ν_{\max} . 3 210 (NH), 1 675 (CO), and 1 450 cm^{-1} ; δ (TFA) 2.30 (2 H, br s, $CHDPh$), 7.0 - 7.5 (10 H, m, Ph), and 8.06 (2 H, br s, NH);

m/z 299 (5.7%), 298 (M^+ , 18), 297 (7), 294 (15), 206, 202, 178, 174, 116, 93, 92 (100), and 91.

Preparation of ^{14}C -labelled cyclic dipeptides

N-Benzoyloxycarbonyl-[1- ^{14}C]-glycine. - This compound was prepared by the method of Greenstein and Winitz,¹¹⁹ using [1- ^{14}C]glycine (250 μCi , 51.2 mCi/mmol) which was diluted by the addition of unlabelled glycine (225 mg, 3 mmol) to the aqueous solution. This procedure gave the N-protected derivative (562 mg, 90%), m.p. 122 - 123°C (lit.,¹¹⁹ 120°C), specific activity 83.4 $\mu\text{Ci}/\text{mmol}$.

cyclo-([1- ^{14}C]Gly-L-Phe) (137). - The cyclic dipeptide was prepared by the method described previously for unlabelled material. N-Benzoyloxycarbonyl-[1- ^{14}C]-glycine (510 mg, 2.44 mmol), gave the cyclic dipeptide (137) (250 mg, 50%), m.p. 270 - 272°C.

N,N'-Diacetyl-cyclo-([1- ^{14}C]Gly-L-Phe) (133). - This compound was prepared as described earlier for unlabelled material. cyclo-([1- ^{14}C]-Gly-L-Phe) (137) (250 mg, 1.23 mmol) gave the diacetyl derivative (133) (240 mg, 68%), m.p. 102 - 103°C, specific activity 84.4 $\mu\text{Ci}/\text{mmol}$. This material was used to prepare the labelled fluorinated cyclic dipeptides (136a - e) (data given in Table 2).

COMPOUND	From (133) (mg)	Yield (mg)	Yield (%)	m.p. (°C)	Specific activity ($\mu\text{Ci}/\text{mmol}$)
(136a); Ar = $\text{C}_6\text{H}_3\text{F}_2$	50	28.5	50	280 - 282	83.2
(136b); Ar = <u>p</u> -F-Ph	40	20.9	48	312 - 314	83.6
(136c); Ar = <u>o</u> -F-Ph	40	20.3	47	299 - 302	83.5
(136d); Ar = <u>m</u> -F-Ph	45	24.3	50	300 - 302	83.0
(136e); Ar = C_6F_5	45	36.5	61	300 - 303	82.7

TABLE 2 Experimental data for radio-labelled fluorinated dipeptides

3.3 Synthesis of sulphur-containing dioxopiperazines

N,N'-Diacetyl-cyclo-(L-Phe-L-Phe) (157).¹⁰¹ - A mixture of cyclo-(L-Phe-L-Phe) (294 mg, 1 mmol), acetic anhydride (2 ml), and perchloric acid (1 drop) was stirred at 90°C for 1 h. The acetic anhydride was evaporated under reduced pressure, and the residue was crystallised from acetone-water, giving the diacetyl derivative (157) (240 mg, 64%), m.p. 176 - 177°C, $[\alpha]_D^{21} +177.5^\circ$ (c 1.0 in CHCl₃); ν_{\max} . 1 715 (CO), 1 705 (CO), and 1 220 cm⁻¹; δ 2.47 (6 H, s, Ac), 2.5 - 2.91 (4 H, m, CH₂Ph), 5.30 (2 H, dd, J 7 and 5 Hz, CHCH₂Ph), and 7.02 - 7.45 (10 H, m, Ph); m/z 378 (M⁺, 20%), 336, 245, 203, 131, 91 (100), and 43.

Reaction of (157) with potassium t-butoxide

i) To diacetyl-cyclo-(L-Phe-L-Phe) (157) (126 mg, 0.33 mmol) in DMF (1 ml) was added dimethyl disulphide (0.15 ml). The mixture was cooled to 0°C, and a 0.5 M solution of potassium t-butoxide in t-butanol (0.67 ml) was added with stirring. After 25 min., acetic acid (0.2 ml) was added, the mixture was poured into water, (25 ml), and the products were extracted with chloroform (3 x 10 ml). The extract was washed with water (2 x 10 ml) and evaporated to dryness. The residual gum was a 1:1 mixture of cis- and trans- isomers of the rearranged compound (158); ν_{\max} . (CHCl₃) 3 440 (NH), 3 005, 1 780, 1 740 (C-Ac), 1 710 (N-Ac), 1 675 (CONR₂), 1 495, and 1 265 cm⁻¹; δ 1.20 (1 H, dd, J 16 and 7 Hz, cis-CHHPh), 1.82 (3 H, s, trans-CCOMe), 2.01 (3 H, s, cis-CCOMe), 2.41 (3 H, s, N-Ac), 2.63 (3 H, s, N-Ac), 2.7 - 3.3 (7 H, m, cis- and trans- CH₂Ph), 3.67 (1 H, dd, J 10 and 6 Hz, trans-CHCH₂Ph), 4.95 (1 H, dd, J 7 and 3 Hz, cis-CHCH₂Ph), 6.28 (1 H, br s, NH), 6.8 - 7.5 (20 H, m, Ph), and 7.57 (1 H, br s, NH);

m/z 378 (M^+ , 24%), 277, 245, 203, 186, 131, 120, 91 (100), and 43.

ii) To an ice-cooled solution of diacetyl-cyclo-(L-Phe-L-Phe) (90 mg, 0.24 mmol), in DMF (1 ml) was added a 0.5 M solution of potassium t-butoxide in t-butanol (1 ml) with stirring. After 1 h, acetic acid (0.1 ml) was added, the mixture was added to water (15 ml), and the products were extracted with ethyl acetate (3 x 5 ml). The solvent was evaporated and the residue was crystallised from ethyl acetate to give (159) (31 mg, 39%), m.p. 243 - 244°C, ν_{\max} 3 280 (NH), 1 770; 1 705 (CO), 1 670 (CONH), 1 660 (CONH), and 700 cm^{-1} ; δ 0.86 (1 H, dd, J 14 and 11 Hz, CHCHHP_H), 1.92 (3 H, s, COMe), 2.63 (1 H, dd, J 14 and 4 Hz, CHCHHP_H), 2.93 and 3.23 (2 H, ABq, J 13 Hz, CAcCH₂Ph), 4.32 (1 H, dd, J 11 and 4 Hz, CHCH₂Ph), 6.35 (1 H, br s, exchangeable with D₂O, NH), 6.8 (2 H, m, Ar), 7.0 - 7.4 (8 H, m, Ar), and 7.45 (1 H, br s, exchangeable with D₂O, NH); m/z 336 (M^+ , 27%), 319, 245, 203, 186, 120, 119, 118, 91 (100), and 43.

Addition of methanethiol to 3,6-dibenzylidenepiperazine-2,5-dione.

Zinc metal (25 mg) was suspended in dry methylene chloride (30 ml), and dry hydrogen chloride was bubbled through the suspension until saturation was reached. After stirring for a further 2 h, most of the zinc had dissolved, and dibenzylidenepiperazine-2,5-dione (140c) (580 mg, 2.0 mmol) was added, followed by methanethiol (0.6 ml; cooled to ca. 0°C). The mixture was stirred at ca. 10°C for 24 h. After removal of the volatile components by evaporation under reduced pressure, the residual solid was treated with chloroform (50 ml), and the insoluble material was filtered off (this was nearly all starting material). Evaporation of the chloroform gave a solid (45 mg) which was purified by t.l.c. (solvent system B) to give a white solid (156) (ca. 10 mg),

m.p. 300 - 303°C (decomp.); δ (100MHz) 2.26 (3 H, s, SMe), 3.19 and 3.60 (2 H, ABq, CH_2Ph), 6.35 (1 H, br s, exchangeable with D_2O , NH), 6.87 (1 H, s, CHPh), 7.0 - 7.6 (ca. 18 H, m, Ar), and 7.89 (1 H, br s, exchangeable with D_2O , NH); m/z 292 (24%), 291 ($\text{M}^+ - \text{SMe}$, 87), 290 (100), 118 (73), 117 (53), 91 (58), 90 (31), 48 (MeSH^+ , 10), and 47 (MeS^+ , 13).

Sulphur. - The sulphur used in the reactions given below was heated to increase its solubility; technical grade flowers of sulphur was heated in an oven at 110°C overnight.

Lithium di-isopropylamide (LDA). - LDA was prepared by the addition of a 1.5 M solution of *n*-butyllithium in hexane (1.0 equivalents) to a stirred solution of di-isopropylamine in THF, at 0°C.

(±)-1,4-Di(methoxymethyl)-3,6-cis-dibenzylpiperazine-2,5-dione (161). - Potassium *t*-butoxide (7.6 g, 68 mmol) was added slowly to a stirred suspension of (±)-3,6-cis-dibenzylpiperazine-2,5-dione (160)¹¹¹ (9.1 g, 31 mmol) in DMSO (50 ml). When all the solids had dissolved (ca. 30 min.), chloromethyl methyl ether (5.0 ml, 66 mmol) was added dropwise over 20 min. After stirring for a further 10 min., the mixture was poured into water (300 ml), the products were extracted with methylene chloride (3 x 50 ml), and the extract was washed with water (2 x 50 ml). The solvent was evaporated and the residual oil was purified by passing it through a short column of silica gel H, with ether as the eluent. This gave reasonably pure protected compound (161) as an oil (8.7 g, 74%), $\nu_{\text{max.}}$ (CHCl_3) 1 675 (CO), 1 460, 1 090, and 710 cm^{-1} ; δ 2.47 (2 H, dd, \underline{J} 14 and 6 Hz, CHHPh), 2.85 (2 H, dd, \underline{J} 14 and 4 Hz, CHHPh), 3.23 (6 H, s, Me), 4.14 and 5.13 (4 H, ABq,

\underline{J} 10 Hz, $\underline{\text{CH}}_2\text{OMe}$), 4.42 (2 H, dd, \underline{J} 6 and 4 Hz, $\underline{\text{CH}}\text{CH}_2\text{Ph}$), and 7.0 - 7.4 (10 H, m, Ar); $\underline{m/z}$ 382 ($\underline{\text{M}}^+$, 0.6%), 350, 91, and 45 (100); (Found: $\underline{m/z}$ 382.1905. $\text{C}_{22}\text{H}_{26}\text{N}_2\text{O}_4$ requires 382.1893).

(±)-Di(methoxymethyl)-3,6-epitetrahydro-3,6-dibenzylpiperazine-2,5-dione (162). - A solution of the protected dipeptide (161) (2.10 g, 5.50 mmol), and sulphur (1.72 g, 5.50 mmol of S_8) in THF (60 ml) and DMF (0.02 ml) was cooled to -78°C . LDA (12.1 mmol) in THF (15 ml) was added by syringe via a rubber septum, whereupon the mixture became orange-red. The mixture was stirred at -78°C for 30 min., then allowed to warm up to -10°C over ca. 90 min. At this point the colour was a deep red, and there were no suspended solids remaining. Acetic acid (2 ml) was added, the mixture was poured into water (300 ml), and the products were extracted with methylene chloride (3 x 50 ml). The solvent was evaporated off, and excess sulphur was removed by treating the residue with ethyl acetate (25 ml) and filtering off the insoluble solids. After evaporation of the solvent, the syrupy residue was crystallised from acetone to give the tetrasulphide (162) (1.04 g, 35%; calculated for $\text{C}_{22}\text{H}_{24}\text{N}_2\text{O}_4\text{S}_5$ - see Discussion), m.p. $160 - 162^\circ\text{C}$. An analytical sample, purified by t.l.c. (solvent system C), followed by crystallisation from acetone, had m.p. $165 - 167^\circ\text{C}$ (Found: C, 51.7; H, 4.5; N, 5.7; S, 25.3. $\text{C}_{22}\text{H}_{24}\text{N}_2\text{O}_4\text{S}_4$ requires C, 51.95; H, 4.75; N, 5.5; S, 25.2%); ν_{max} 1 680 cm^{-1} (CO); δ 3.10 (6 H, s, Me), 3.45 and 3.63 (4 H, ABq, \underline{J} 15 Hz, $\underline{\text{CH}}_2\text{Ph}$), 4.89 and 5.45 (4 H, ABq, \underline{J} 10 Hz, $\underline{\text{CH}}_2\text{OMe}$), and 7.10 (10 H, br s, Ph); δ_{C} 41.0 (t), 57.8 (q), 75.8 (t), 79.2 (s), 127.0 (d), 128.2 (d), 130.5 (d), 134.1 (s), and 169.3 (s); $\underline{m/z}$ 380 ($\underline{\text{M}}^+ - \text{S}_4$, %), 325, 91, 64, and 45 (100).

Separation of the crude tetrasulphide (162) (200 mg) by t.l.c.

(solvent system C) afforded as a minor product, the bridged sulphide (165) (5 mg), m.p. 140 - 142°C (from acetone); ν_{\max} . 1 685 (CO), 1 455, 1 365, 745, and 700 cm^{-1} ; δ 3.02 (3 H, s, Me), 3.36 (3 H, s, Me), 3.45 and 3.67 (2 H, ABq, \underline{J} 15 Hz, $\underline{\text{CH}}_2\text{Ph}$), 4.47 and 4.71 (2 H, ABq, \underline{J} 6 Hz, $\underline{\text{CH}}\underline{\text{CH}}\text{Ph}$), 4.90 and 5.43 (2 H, ABq, \underline{J} 10 Hz, $\underline{\text{CH}}_2\text{OMe}$), 4.95 and 5.65 (2 H, ABq, \underline{J} 10 Hz, $\underline{\text{CH}}_2\text{OMe}$), and 7.0 - 7.2 (10 H, br s, 2 Ph); $\underline{m/z}$ 410, 380, 378, 325, 117, 116, 91, 64, and 45 (100%).

The mother liquors from crystallisation of the tetrasulphide (162) were evaporated to a viscous oil. This was separated by column chromatography using silica gel H and toluene-ethyl acetate as eluent, and crystallisation of one of the fractions from methanol gave the epidithio-sulphide (164) (90 mg, 3.7%), m.p. 110 - 112°C. Spectral data were identical to those given below.

(±)-1,4-Di(methoxymethyl)-3,6-epidithio-3,6-dibenzylpiperazine-2,5-dione (164). - To a stirred, ice-cooled solution of tetrasulphide (162) (540 mg, 1 mmol) in THF (4 ml) and ethanol (4 ml) was added slowly a suspension of sodium borohydride (200 mg, 5.26 mmol) in ethanol (3 ml). After 20 min. the solution was carefully acidified with acetic acid and added to water (30 ml). On extraction with methylene chloride (3 x 5 ml) and evaporation, the crude dithiol (175) was obtained as a foul-smelling oil, δ 3.30 (6 H, s, Me), 3.55 and 3.93 (4 H, ABq, \underline{J} 14 Hz, $\underline{\text{CH}}_2\text{Ph}$), 3.74 (2 H, s, exchangeable with D_2O , SH), 5.03 and 5.32 (4 H, ABq, \underline{J} 10 Hz, $\underline{\text{CH}}_2\text{OMe}$), and 6.8 - 7.5 (10 H, m, Ph). A solution of the dithiol in ethanol gave a purple-red colour on heating which disappeared on cooling. The oil was dissolved in ethanol (10 ml), and a solution of ferric chloride (800 mg, 4.03 mmol) in ethanol (5 ml) was added. The mixture was stirred for 1 h, water

(60 ml) was added, and the product was extracted with methylene chloride (3 x 10 ml). After removal of the solvent, the epidisulphide (164) was crystallised from methanol; (400 mg, 90%), m.p. 108 - 110°C (Found: C, 59.6; H, 5.55; N, 6.1; S, 14.6. $C_{22}H_{24}N_2O_4S_2$ requires C, 59.45; H, 5.45; N, 6.3; S, 14.45%); ν_{\max} . 1 695 cm^{-1} (CO); δ 3.40 (6 H, s, Me), 3.96 and 4.13 (4 H, ABq, J 14 Hz, \underline{CH}_2Ph), 4.82 and 5.42 (4 H, ABq, J 10 Hz, \underline{CH}_2OMe), and 7.2 - 7.5 (10 H, m, Ph); δ_C 35.8 (t), 56.9 (q), 73.8 (s), 74.1 (t), 127.6 (d), 128.4 (d), 130.4 (d), 134.3 (s), and 166.3 (s); m/z 380 ($M^+ - S_2$, 5%), 325, 91, 64, and 45 (100).

(±)-1,4-Di(methoxymethyl)-3,6-epithio-3,6-dibenzylpiperazine-2,5-dione (163). - A solution of protected dipeptide (161) (0.38 g, 1.0 mmol) and sulphur (0.26 g, 1 mmol of S_8) in THF (10 ml) and DMF (5 μ l) was cooled to -78°C, and LDA (2.2 mmol) in THF (5 ml) was added by syringe via a rubber septum. The stirred mixture was allowed to warm to room temperature over 1 h, then acetic acid (0.5 ml) was added and the mixture was poured into water (50 ml). Extraction with methylene chloride (3 x 10 ml), followed by evaporation of the solvent, gave a viscous oil, which was dissolved in ethyl acetate (5 ml) and filtered. The ethyl acetate was evaporated off, and the residue was separated by column chromatography (silica gel H, with toluene-ethyl acetate as the eluent). Crystallisation of one of the fractions from ethyl acetate-hexane gave the epimonosulphide (163) (110 mg, 27%), m.p. 129 - 131°C (Found: C, 64.15; H, 6.1; N, 6.75; S, 7.9. $C_{22}H_{24}N_2O_4S$ requires C, 64.05; H, 5.85; N, 6.8; S, 7.75%); ν_{\max} . 1 725 (CO), and 1 090 cm^{-1} ; δ 3.23 (6 H, s, Me), 3.31 and 3.86 (4 H, ABq, J 14 Hz, \underline{CH}_2Ph), 4.70 and 4.85 (4 H, ABq, J 11 Hz, \underline{CH}_2OMe), and 7.25 (10 H, s, Ph); δ_C 31.8 (t), 56.3 (q), 72.8 (t), 76.9 (s), 127.7 (d),

128.6 (d), 130.0 (d), 134.3 (s), and 173.8 (s); m/z 412 (M^+ , 0.3%), 380, 91, and 45 (100).

(±)-1,4-Di(bromomethyl)-3,6-epidithio-3,6-dibenzylpiperazine-2,5-dione (176). - The epidisulphide (164) (222 mg, 0.5 mmol) was dissolved in methylene chloride (5 ml), and the solution was cooled to -78°C . A solution of boron tribromide (260 mg, 1.04 mmol) in methylene chloride (1 ml) was added with stirring. The mixture was allowed to warm to -40°C over 40 min., at which point wet ether (10 ml) and 5% aqueous sodium bicarbonate (5 ml) were added. The mixture was poured into water (30 ml), extracted with ether (3 x 10 ml), and the ether extract was washed with 20% glucose solution (10 ml) and water (2 x 10 ml). After evaporation of the solvent, the residue was crystallised from carbon tetrachloride to give the di(bromomethyl) derivative (176), (260 mg, 96%), m.p. $167 - 168^\circ\text{C}$ (decomp.). A satisfactory analysis was not obtained. ν_{max} 1710 cm^{-1} (CO); δ 4.03 and 4.17 (4 H, ABq, J 15 Hz, CH_2Ph), 5.04 and 5.81 (4 H, ABq, J 11 Hz, CH_2Br), and 7.38 (10 H, br s, Ph); m/z 480, 478, and 476 ($M^+ - \text{S}_2$, 4, 9, and 4%), 91 (100), 82, 81, 80, 79, and 64; (Found: 541.9190. $\text{C}_{20}\text{H}_{18}^{79}\text{Br}^{81}\text{BrN}_2\text{O}_2\text{S}_2$ requires 541.9156. Found: 479.9673. $\text{C}_{20}\text{H}_{18}^{81}\text{Br}_2\text{N}_2\text{O}_2$ requires 479.9650).

(±)-1,4-Di(hydroxymethyl)-3,6-epidithio-3,6-dibenzylpiperazine-2,5-dione (178). - A solution of the di(bromomethyl) compound (176) (80 mg, 0.15 mmol) in ether (10 ml) was stirred vigorously with water (30 ml) for 18 h. The water was decanted and replaced with a further 30 ml, and the mixture was stirred again for 20 h. The ether layer was separated and evaporated to dryness, and the residue was crystall-

ised from acetone to give (178) (50 mg, 81%), m.p. 263 - 280°C (decomp.); ν_{\max} . 3 420 (OH), 1 680 (CO), and 1 030 cm^{-1} ; δ (100 MHz) (d_6 -DMSO) 3.88 and 4.11 (4 H, ABq, J 15 Hz, CH_2Ph), 4.83 (2 H, dd, J 12 and 8 Hz, CHHOH), 5.30 (2 H, dd, J 12 and 8 Hz, CHHOH), 6.49 (2 H, t, J 8 Hz, exchangeable with D_2O , OH), and 7.2 - 7.5 (10 H, m, Ph); m/z 292 ($\text{M}^+ - \text{S}_2 - (\text{CH}_2\text{O})_2$, 35%), 290 (100), 201, 118, 117, 116, and 91.

(±)-3,6-Epidithio-3,6-dibenzylpiperazine-2,5-dione (152). - The di(bromomethyl) epidisulphide (176) (27 mg, 0.05 mmol) was stirred with powdered ammonium acetate (100 mg, 1.30 mmol) in methylene chloride (3 ml) for 20 h. The mixture was washed with water (3 x 1 ml), and evaporated to dryness. The residue crystallised from benzene to give the deprotected disulphide (152) (19.5 mg, 94%), m.p. 246 - 249°C (decomp.) (Found: C, 65.0; H, 4.9; N, 6.85; S, 16.4. $\text{C}_{18}\text{H}_{16}\text{N}_2\text{O}_2\text{S}_2 \cdot 0.75 \text{C}_6\text{H}_6$ requires C, 65.1; H, 5.0; N, 6.75; S, 15.45%); [A sample was also crystallised from toluene; m.p. 251 - 253°C (decomp.) (Found: C, 65.9; H, 5.25; N, 6.55; S, 14.9. $\text{C}_{18}\text{H}_{16}\text{N}_2\text{O}_2\text{S}_2 \cdot 0.75 \text{C}_7\text{H}_8$ requires C, 65.6; H, 5.2; N, 6.6; S, 15.05%);]; ν_{\max} . (CHCl_3) 3 360 (NH), 1 710 (CO), 1 455, 1 335, and 705 cm^{-1} ; ν_{\max} . (KBr) 3 360 (NH), 3 200 (NH), 1 690 (CO), 1 425, 1 340, and 700 cm^{-1} ; δ 3.42 and 3.70 (4 H, ABq, J 16 Hz, CH_2Ph), 6.55 (2 H, br s, exchangeable with D_2O , NH), 7.36 (4.5 H, s, benzene), and 7.40 (10 H, s, Ph); δ_C 37.7 (t), 71.1 (s), 128.5 (d), 129.0 (d, benzene), 129.3 (d), 130.9 (d), 132.4 (s), and 165.0 (s); m/z 292 ($\text{M}^+ - \text{S}_2$, 28%), 290, 201, 173, 91 (100), 65, and 64.

Bis(dithio)- compound (179). - To a solution of di(bromomethyl) epidisulphide (176) (14 mg, 0.026 mmol) in methylene chloride (1 ml)

was added a saturated solution of ammonia in methylene chloride (1 ml). After 10 min., the mixture was evaporated to dryness, and to the residue was added methylene chloride (2 ml). The mixture was filtered, and the filtrate was evaporated to dryness. Separation of the residue by t.l.c. (solvent system B) gave the bis(dithio)- compound (179) as an oil (4 mg, 35%); $\nu_{\max.}$ (CHCl_3) 1 720 (CO), 1 665, and 1 365 cm^{-1} ; δ 2.63 and 3.34 (4 H, ABq, \underline{J} 14 Hz, $\underline{\text{CH}}_2\text{Ph}$), 4.43 and 5.65 (4 H, ABq, \underline{J} 11 Hz, $\underline{\text{CH}}_2\text{S}$), and 7.0 - 7.5 (10 H, m, Ph); m/z 446 (\underline{M}^+ , 3.5%), 382, 117, 116, 91 (100), and 64; (Found: m/z 446.0249. $\text{C}_{20}\text{H}_{18}\text{N}_2\text{O}_2\text{S}_4$ requires 446.0251).

The material collected on the filter was washed with water and methanol to give 3,6-dibenzylidenepiperazinedione (140c) (2.4 mg, 32%), m.p. 302 - 306°C (lit.,⁹⁶ 298 - 300°C); $\nu_{\max.}$ 3 200 (NH), 1 680 (CO), 1 630 (C=C), 1 400, and 690 cm^{-1} ; m/z 290 (\underline{M}^+ , 21%), 118, 117, 116, 90 (100), and 89.

(±)-Di(methoxymethyl)-cis-3,6-di(methylthio)-3,6-dibenzylpiperazine-2,5-dione (172). - The tetrasulphide (162) (27 mg, 0.05 mmol) was dissolved in THF (0.5 ml) and ethanol (0.5 ml), cooled to 0°C, and sodium borohydride (15 mg, 0.39 mmol) in ethanol (1 ml) was added slowly with stirring. After 5 min., methyl iodide (1 ml) was added. The reaction mixture was stirred for 30 min., then allowed to warm to room temperature, and acetic acid (0.1 ml) was added. The mixture was poured into water (20 ml) and the products were extracted with methylene chloride (3 x 5 ml). After evaporation of the solvent, the residue was crystallised from methanol to give (172) (21 mg, 89%), m.p. 117 - 119°C (Found: C, 60.75; H, 6.5; N, 5.75; S, 13.7. $\text{C}_{24}\text{H}_{30}\text{N}_2\text{O}_4\text{S}_2$ requires C, 60.75; H, 6.35; N, 5.9; S, 13.5%); $\nu_{\max.}$ 1 675 (CO), 1 090, and 700 cm^{-1} ; δ 2.14 (6 H, s, SMe), 2.96 (4 H, s, $\underline{\text{CH}}_2\text{Ph}$), 3.38 (6 H,

s, OMe), 4.72 and 5.14 (4 H, ABq, \underline{J} 10 Hz, $\underline{\text{CH}}_2\text{OMe}$), and 7.0 - 7.3 (10 H, m, Ph); $\underline{\text{C}}$ 13.8 (q), 44.6 (t), 57.8 (q), 74.5 (s), 76.6 (t), 127.5 (d), 128.4 (d), 131.0 (d), 134.2 (s), and 166.8 (s); $\underline{\text{m/z}}$ 474 ($\underline{\text{M}}^+$, 2%), 427, 390, and 91 (100).

(±)-Di(methoxymethyl)-cis-3,6-di(methyldithio)-3,6-dibenzylpiperazine-2,5-dione (174). - To an ice-cooled solution of epitetrasulphide (162) (100 mg, 0.19 mmol), in pyridine (1 ml) and methyl iodide (5 ml) was added a suspension of sodium borohydride (100 mg) in methanol (2 ml). The mixture was stirred for 40 min., then carefully acidified with acetic acid and evaporated to dryness. The residue was extracted with ether (3 x 5 ml), and after evaporation of the solvent the extract was separated by t.l.c. (solvent system C), giving the di(methyldithio)- compound (174) (50 mg, 50%), which was crystallised from methanol; m.p. 88 - 89°C (Found: C, 53.75; H, 5.55; N, 5.15; S, 23.4. $\text{C}_{24}\text{H}_{30}\text{N}_2\text{O}_4\text{S}_4$ requires C, 53.5; H, 5.6; N, 5.2; S, 23.8%); ν_{max} . 1 680 (CO), 1 360, and 1 290 cm^{-1} ; δ 2.48 (6 H, s, SMe), 3.20 (6 H, s, OMe), 3.20 and 3.92 (4 H, ABq, \underline{J} 16 Hz, $\underline{\text{CH}}_2\text{Ph}$), 5.02 (4 H, s, $\underline{\text{CH}}_2\text{OMe}$), and 7.10 (10 H, br s, Ph); $\underline{\text{m/z}}$ 459 ($\underline{\text{M}}^+$ -SSMe, 0.44%), 380, 130, 91, 79, 64, 47, and 45 (100).

(±)-1,4-Di(methoxymethyl)-cis-3-methyldithio-6-methylthio-3,6-dibenzylpiperazine-2,5-dione (173). - To an ice-cooled solution of epitetrasulphide (162) (55 mg, 0.1 mmol) in methyl iodide (2 ml) and pyridine (0.5 ml) was added a suspension of sodium borohydride (50 mg) in methanol (1 ml). The mixture was allowed to warm up to room temperature, and it was stirred for a further 1 h, then evaporated to dryness under reduced pressure. The residue was extracted with ether

(3 x 5 ml), and on evaporation the extract gave the compound (173) as a gum; δ 2.22 (3 H, s, SMe), 2.42 (3 H, s, SSMe), 2.97 (2 H, s, CH_2Ph), 3.12 and 3.63 (2 H, ABq, \underline{J} 15 Hz, CH_2Ph), 3.25 (3 H, s, OMe), 3.39 (3 H, s, OMe), 4.62 and 5.02 (2 H, ABq, \underline{J} 9 Hz, CH_2OMe), 5.07 (2 H, s, CH_2OMe), and 6.80 - 7.35 (10 H, m, Ph); m/z 458 ($\underline{M}^+ - \text{MeSH}$, 0.1%), 427 ($\underline{M}^+ - \text{MeSS}$, 14), 381, 380, 379 (100), 303, 130, 91, and 45.

(±)-cis-3,6-Di(methylthio)-3,6-dibenzylpiperazine-2,5-dione (131).

- A solution of the di(methoxymethyl) compound (172) (24 mg, 0.05 mmol) in methylene chloride (1 ml) was cooled to -78°C , then boron tribromide (25 mg, 0.1 mmol) in methylene chloride (0.1 ml) was added with stirring. The mixture was allowed to warm to -40°C over 30 min., then wet ether was added (2 ml), and the mixture was poured into 5% aqueous sodium bicarbonate (5 ml). After extraction with ether (3 x 3 ml), the extract was washed with 20% glucose solution (3 ml) and water (2 x 5 ml). The oil obtained on evaporation of the solvent was a mixture of the desired di(bromomethyl) derivative (180) and various by-products (see Discussion). The oil was dissolved in acetone (5 ml) and water (3 ml), 5% aqueous sodium bicarbonate (1 ml) was added, and the mixture was stirred for 18 h. The mixture was then added to water (20 ml) and extracted with chloroform (3 x 5 ml). The solvent was evaporated and the residue crystallised from chloroform to give the deprotected compound (131) (6 mg, 31%), m.p. $302 - 304^\circ\text{C}$ (decomp.). A satisfactory analysis was not obtained. ν_{max} 3 180 (NH), 1 670 (CO), 1 410, 765, 700, and 485 cm^{-1} ; δ (100 MHz) 2.25 (6 H, s, Me), 2.67 and 3.02 (4 H, ABq, \underline{J} 14 Hz, CH_2Ph), 5.83 (2 H, br s, exchangeable with D_2O , NH), and 7.04 - 7.5 (10 H, m, Ph); m/z 339 ($\underline{M}^+ - \text{SMe}$, 1.8%), 292, 291, 290 (100), 212, 118, 117, 91, 90, 89, and 47; (Found:

m/z 386.1142 (0.16%). $C_{20}H_{22}N_2O_2S_2$ requires 386.1123. Found: 339.1166 (13%). $C_{19}H_{19}N_2O_2S$ requires 339.1168).

The supernatants from the crystallisation were evaporated to dryness, and the residue was crystallised from methanol, giving the cyclised compound (181) (6.5 mg, 32%), m.p. 193 - 197°C (decomp.) (Found: C, 63.3; H, 5.7; N, 7.1; S, 16.25. $C_{21}H_{22}N_2O_2S_2$ requires C, 63.3; H, 5.55; N, 7.05; S, 16.1%); ν_{max} . 3 200 (NH), 1 670 (CO), 1 660 (CO), and 1 390 cm^{-1} ; δ 1.93 and 3.08 (2 H, ABq, J 17 Hz, CCH_2 Ar), 2.09 (3 H, s, Me), 2.42 (3 H, s, Me), 3.06 and 3.73 (2 H, ABq, J 15 Hz, CH_2 Ph), 4.46 and 5.22 (2 H, ABq, J 18 Hz, NCH_2 Ar), and 6.8 - 7.3 (10 H, m, 1 H exchangeable with D_2O , Ph, Ar, and NH); δ_C (50.3 MHz) 14.0 (q), 36.6 (t), 43.2 (t), 47.0 (t), 66.1 (s), 67.6 (s), 125.5 (d), 126.7 (d), 126.9 (d), 127.6 (d), 128.3 (d), 128.5 (d), 130.5 (d), 133.6 (d), 164.1 (s), and 166.5 (s); m/z 351 ($M^+ - SMe$, 60%), 304, 303, 130, 129, 128, 91 (100), and 47.

A sample of the di(bromomethyl) compound (180) was obtained by preparative t.l.c. (solvent system C) of the products of boron tribromide treatment; m.p. 151 - 153°C (decomp.); ν_{max} . 1 680 (CO), 1 350, and 1 240 cm^{-1} ; δ 2.24 (6 H, s, Me), 2.63 and 2.93 (4 H, ABq, J 15 Hz, CH_2 Ph), 4.99 and 5.62 (4 H, ABq, J 8 Hz, CH_2 Br), and 6.9 - 7.4 (10 H, m, Ar); m/z 445 and 443 ($M^+ - SMe - HBr$, 0.8%), 411, 91, 90, 77, 61 (100), and 47.

Alternative synthesis of (131). - The deprotected epidisulphide (152) (20.7 mg, 0.05 mmol) was dissolved in methyl iodide (2 ml), and sodium borohydride (15 mg, 0.39 mmol) in ethanol (1 ml) was added with stirring to the ice-cooled solution. After 20 min., acetic acid (0.1 ml) was added, and the mixture was evaporated to dryness. The

residue was dissolved in acetone (1 ml), water (10 ml) was added, and the precipitate was filtered off. Crystallisation from acetone gave the deprotected di(methylthio)- compound (131) (9.8 mg, 52%), m.p. 301 - 303°C (decomp.); spectral data identical to those of the sample prepared by the first method.

N,N'-Di(methoxymethyl)-cyclo-(L-Phe-L-Phe) (161a). - cyclo-(L-Phe-L-Phe) (42)³⁹ (441 mg, 1.5 mmol), DMSO (5 ml), 40% formalin (10 ml), and sodium bicarbonate (50 mg) were heated together under reflux for 90 min. The mixture was then poured into water (50 ml) and extracted with methylene chloride (3 x 10 ml). Evaporation of the solvent gave crude di(hydroxymethyl)-cyclo-(L-Phe-L-Phe) (167) (480 mg); δ 2.35 (2 H, dd, J 14 and 7 Hz, CHHPH), 2.79 (2 H, dd, J 14 and 4 Hz, CHHPH), 4.13 and 5.03 (4 H, ABq, J 10 Hz, CH_2OH), 4.39 (2 H, m, CHCH_2Ph), 4.45 (2 H, br s, OH), and 6.9 - 7.4 (10 H, m, Ph). This oil was dissolved in methanol (5 ml) and thionyl chloride (0.5 ml) was added. After 1 h, the solvents were evaporated off, and the residue was purified by precipitation of the impurities from ethyl acetate-pet. ether (60 - 80°C). The supernatants were evaporated to give the di(methoxymethyl) derivative (161a) as a colourless oil (220 mg, 38%), having spectral data identical with those of the racemic material (161); $[\alpha]_D^{20} +26.6^\circ$ (c 2.2 in CHCl_3).

Formation of the tetrasulphide (162) from (161a). - The reaction of the protected cyclic dipeptide (161a) with sulphur was carried out as before: (161a) (220 mg, 0.58 mmol) and sulphur (148 mg, 0.58 mmol of S_8) in THF (10 ml) and DMF (2 μ l), were treated with LDA (1.3 mmol) in THF (4 ml). After work-up, crystallisation from acetone gave the

tetrasulphide (162) (28 mg, 9%), m.p. 161 - 163°C, which was purified by t.l.c. (solvent system C) to give pure epitetrasulphide (162), m.p. 164 - 167°C, $[\alpha]_D^{22} 0^\circ$ (c 0.5 in CHCl_3).

Equilibration of the disulphide (164) and tetrasulphide (162) in basic conditions. - To a solution of epidisulphide (164) (22 mg, 0.05 mmol) in carbon disulphide (0.5 ml) was added sulphur (3.2 mg, 0.1 mmol of S) in carbon disulphide (32 μl). A catalytic amount of lithium hexane-1-thiolate in carbon disulphide [prepared by the addition of *n*-butyllithium (1 equivalent) to a solution of hexane-1-thiol in carbon disulphide] was added. N.m.r. spectroscopy of the solution after 30 min. indicated that ca. 70% of the epidisulphide had been converted to the epitetrasulphide, and no signals attributable to other oligosulphides were observed. After 24 h, the n.m.r. spectrum showed that ca. 95% of the disulphide (164) had been converted into the tetrasulphide (162), and once again no evidence for other oligosulphides was observed. Addition of more sulphur increased further the proportion of tetrasulphide, but no other changes in the spectrum were observed.

Action of heat on the disulphide (164) and tetrasulphide (162). - The tetrasulphide (162) (27 mg, 0.5 mmol calculated from $\text{C}_{22}\text{H}_{24}\text{N}_2\text{O}_4\text{S}_5$) was heated at 130°C for 2 h, at 0.1 Torr. The products, examined by t.l.c. and n.m.r. spectroscopy, along with elemental sulphur (sublimed), were the epimonosulphide (163), epidisulphide (164), and epitetrasulphide (162), in the ratio 2 : trace : 3.

The disulphide (164) (22 mg, 0.05 mmol) was heated for 2 h at 0.06 Torr, up to 120°C. The product, apart from sublimed sulphur, was

the epimonosulphide (163), together with a small amount of epitetrasulphide (162) (by t.l.c. and n.m.r. spectroscopy).

(±)-1,4-Dimethyl-3,6-cis-dibenzylpiperazine-2,5-dione (168). -

To a stirred suspension of (±)-cis-3,6-dibenzylpiperazine-2,5-dione (160)¹¹¹ (2.50 g, 8.5 mmol) in DMSO (40 ml) was added potassium t-butoxide (2.10 g, 18.7 mmol). When all the solids had dissolved (ca. 30 min.), methyl iodide (2 ml, 32 mmol) was added slowly, and the mixture was stirred for a further 1 h, then added to water (200 ml). Extraction with methylene chloride (3 x 20 ml), followed by evaporation of the solvent, gave an oil which was crystallised from ethyl acetate to give the dimethyl derivative (168) (2.22 g, 81%), m.p. 138 - 141°C (Found: C, 74.45; H, 6.65; N, 8.6. C₂₀H₂₂N₂O₂ requires C, 74.5; H, 6.9; N, 8.7%); ν_{\max} . 1 650 cm⁻¹ (CO); δ 2.16 (2 H, dd, J 15 and 7 Hz, CHHPh), 2.68 (6 H, s, Me), 2.78 (2 H, dd, J 15 and 4 Hz, CHHPh), 4.00 (2 H, dd, J 7 and 4 Hz, CHCH₂Ph), and 6.95 - 7.40 (10 H, m, Ph); m/z 322 (M⁺, 19%), 231, 203, and 91 (100).

(±)-1,4-Dimethyl-3,6-epitetrathio-3,6-dibenzylpiperazine-2,5-dione (170). - A solution of the dimethyl cyclic dipeptide (168) (644 mg, 2.0 mmol) and sulphur (512 mg, 2.0 mmol of S₈) in THF (30 ml) and DMF (10 μ l) was cooled to -78°C in a round-bottomed flask sealed with a rubber septum. LDA (4.4 mmol) in THF (10 ml) was added by syringe, and the stirred mixture was allowed to warm to -10°C over 90 min. Acetic acid (0.5 ml) was added, the mixture was poured into water (250 ml), and the products were extracted with methylene chloride (3 x 30 ml). After evaporation of the solvent, the residue was treated with ethyl acetate (10 ml) and the mixture was filtered. The solvent was evaporated and the viscous residue was crystallised from acetone

giving crude epitetrasulphide (170) (370 mg, 41%), m.p. 184 - 186°C. An analytical sample was purified by t.l.c. and crystallised from acetone; m.p. 192 - 194°C (Found: C, 53.6; H, 4.4; N, 6.2; S, 28.25. $C_{20}H_{20}N_2O_2S_4$ requires C, 53.55; H, 4.5; N, 6.25; S, 28.6%); ν_{\max} . 1 675 cm^{-1} (CO); δ 3.15 and 3.85 (4 H, ABq, J 16 Hz, CH_2Ph), 3.15 (6 H, s, Me), and 6.73 - 7.25 (10 H, m, Ph); m/z 320 ($M^+ - S_4$, 33%), 229, 201, 116, 91, and 64 (100).

(±)-1,4-Dibenzyl-3,6-cis-dibenzylpiperazine-2,5-dione (169). - To a stirred suspension of (±)-cis-3,6-dibenzylpiperazine-2,5-dione (160)¹¹¹ (1.18 g, 4.0 mmol) in DMSO (15 ml) was added potassium t-butoxide (0.95 g, 8.5 mmol). After 1 h, a clear orange solution resulted, to which was added benzyl bromide (1.5 ml, 12 mmol). After a further 1 h, the mixture was acidified with acetic acid, added to water (300 ml), and extracted with methylene chloride (3 x 50 ml). The solvent was evaporated, the residue was dissolved in ethyl acetate (10 ml), and the solution was filtered. After evaporation of the solvent, the residual oil was crystallised from ethyl acetate-pet. ether (60 - 80°C) to give the dibenzyl derivative (169) (1.40 g, 74%), m.p. 142 - 144°C (Found: C, 81.0; H, 6.65; N, 5.7. $C_{32}H_{30}N_2O_2$ requires C, 81.0; H, 6.35; N, 5.9%); ν_{\max} . 1 660 (CO), 1 450, and 700 cm^{-1} ; δ 2.36 (2 H, dd, J 15 and 8 Hz, $CHCHHPh$), 2.91 (2 H, dd, J 15 and 5 Hz, $CHCHHPh$), 3.43 and 5.34 (4 H, ABq, J 16 Hz, NCH_2Ph), 4.12 (2 H, dd, J 8 and 5 Hz, $CHCH_2Ph$), and 6.8 - 7.4 (20 H, m, Ph); m/z 383 ($M^+ - C_7H_7$, 69%), 355, and 91 (100).

(±)-1,4-Dibenzyl-3,6-epitetra-thio-3,6-dibenzylpiperazine-2,5-dione (171). - A solution of the dibenzyl derivative (169) (474 mg,

1.0 mmol) and sulphur (384 mg, 1.5 mmol of S_8) in THF (15 ml) with DMF (20 μ l) was cooled to -78°C in a flask sealed with a rubber septum. LDA (2.2 mmol) in THF (5 ml) was added by syringe, and the stirred mixture was allowed to warm up to 0°C over 90 min. Acetic acid (0.2 ml) was added, the mixture was poured into water (200 ml), and the products were extracted with methylene chloride (3 x 20 ml). The solvent was evaporated off, the residue was treated with ethyl acetate (5 ml), and the excess sulphur was filtered off. The solvent was evaporated, and the viscous residue was crystallised from acetone, to give tetrasulphide (171) (120 mg, 20%), m.p. $192 - 194^\circ\text{C}$ (decomp.) (Found: C, 63.95; H, 4.65; N, 4.55; S, 21.3. $C_{32}H_{28}N_2O_2S_4$ requires C, 63.95; H, 4.7; N, 4.65; S, 21.35%); ν_{max} 1670 (CO), and 700 cm^{-1} ; δ 3.46 and 3.64 (4 H, ABq, J 16 Hz, CCH_2Ph), 4.46 and 5.43 (4 H, ABq, J 15 Hz, NCH_2Ph), 6.50 (4 H, m, $o\text{-H}$ of 2 Ph), and 6.8 - 7.4 (16 H, m, other Ph); m/z 472 ($M^+ - S_4$, 2.5%), 470, 381, 199, 91 (100), 65, and 64.

Synthesis of radio-labelled sulphur-containing compounds

A) i) (\pm)-1,4-Di(bromomethyl)-3,6-epidithio-3,6-di [$4 - ^3\text{H}$]benzylpiperazine-2,5-dione (176). - L-[$4' - ^3\text{H}$]Phenylalanine (1 mCi) in water (1 ml) was added to unlabelled L-phenylalanine (660 mg, 4 mmol), and the water was evaporated under reduced pressure. To the solid was added ethylene glycol (technical grade; 3 ml), and the mixture was heated under reflux for 4 h. The reaction mixture had grown very dark-coloured, so heating was not continued (the normal reaction time was 24 h). After cooling, the mixture was filtered, and the crystals were washed with methanol and ether, giving a mixture of cis- and trans- isomers of cyclo-([$4' - ^3\text{H}$]Phe-Phe) (160) (320 mg, 54%), m.p. $305 - 308^\circ\text{C}$. The cyclic dipeptide was suspended in DMSO (3 ml), and potassium t-butoxide (270 mg, 2.41 mmol) was added with stirring.

When the solids had dissolved, chloromethyl methyl ether (0.2 ml, 2.5 mmol) was added dropwise. After 20 min., the mixture was poured into 5% aqueous sodium bicarbonate (20 ml) and the products were extracted with methylene chloride (3 x 5 ml). The solvent was evaporated, and the residue was purified by passing it through silica gel H (ca. 2 g), using ether as the eluent, to give the di(methoxymethyl) derivative (161) (300 mg, 72%). The protected cyclic dipeptide was converted into the tetrasulphide as described earlier. Crystallisation from acetone gave 65 mg (15%), and a further crop was obtained by adding non-radioactive tetrasulphide (162) (40 mg) to the mother liquors and recrystallising, giving a combined total of 110 mg, m.p. (for first crop) 158 - 161°C (decomp.). The tetrasulphide was converted into the epidisulphide (164), and thence to the di(bromomethyl) derivative (176) as described previously, giving (176) (33 mg, 29% from epitetrasulphide), m.p. 164 - 166°C (decomp.); specific activity 368 $\mu\text{Ci}/\text{mmol}$.

ii) (±)-1,4-Di(bromomethyl)-3,6-[³⁵S]epidithio-3,6-dibenzylpiperazine-2,5-dione (176). - N.B. All reactions involving ³⁵S-labelled compounds were performed in a well-ventilated fume cupboard.

The protected cyclic dipeptide (161) (1.00 g, 2.62 mmol) was sulphurated, converted to the epidisulphide, and cleaved with boron tribromide, as described for the unlabelled compounds, using sulphur (675 mg, 2.6 mmol) to which was added [³⁵S]sulphur (rhombic; nominal 2 mCi).

Tetrasulphide (162) (357 mg, 25%), m.p. 161 - 163°C (decomp.)

Disulphide (164) (215 mg, 73%), m.p. 108 - 110°C

Di(bromomethyl) disulphide (176) (219 mg, 83%), m.p. 167 - 168°C (decomp.); specific activity 41.6 $\mu\text{Ci}/\text{mmol}$.

iii) (±)-3,6-[³⁵S]Epidithio-3,6-[4-³H]dibenzylpiperazine-2,5-dione (152). - The [³H]di(bromomethyl) epidisulphide (176) (33 mg, 0.061 mmol) and [³⁵S]di(bromomethyl) epidisulphide (54 mg, 0.1 mmol) were combined, dissolved in methylene chloride, and deprotected with ammonium acetate as described for the unlabelled compound. The deprotected epidisulphide (152) was crystallised from chloroform-hexane to constant activity (51 mg, 89%), m.p. 249 - 252°C (decomp.); one spot by t.l.c.; specific activity (³H) 133 μCi/mmol, (³⁵S) 25.8 μCi/mmol.

cis-3,6-Di([³⁵S]methylthio)-3,6-dibenzylpiperazine-2,5-dione (131). - The [³⁵S]di(bromomethyl) disulphide (176) (60 mg, 0.11 mmol) was converted to the deprotected disulphide (152) as before. Without crystallisation, the disulphide was dissolved in methyl iodide (1 ml) and ethanol (0.5 ml), and reductively methylated with sodium borohydride as before. The product was crystallised from chloroform and acetone. At this point the di(methylthio)- derivative was found to contain some unreacted disulphide (152), so the mixture was separated by t.l.c. (solvent system A), and the [³⁵S]di(methylthio)-cyclic dipeptide (131) was crystallised from acetone (6 mg, 14%), m.p. 292 - 295°C, specific activity 25.1 μCi/mmol.

B) i) (±)-1,4-Di(bromomethyl)-3,6-epidithio-3,6-di[4-³H]benzylpiperazine-2,5-dione (176). - The preparation of cyclo-(Phe-Phe) (cis- and trans- mixture) (160) was carried out as in A) above, starting with L-[4-³H]phenylalanine (250 μCi) diluted with unlabelled L-phenylalanine (330 mg, 2.0 mmol). This gave the cyclic dipeptide (160) (220 mg, 75%), m.p. 304 - 306°C. The di(methoxymethyl) derivative (161) was prepared by the method used in the preparation of N,N'-di(methoxy-

methyl)-cyclo-(L-Phe-L-Phe) (161a). This procedure gave (161) (170 mg, 60%). The protected cyclic dipeptide was carried through to the di(bromomethyl) epidisulphide (176) as before.

Epitetrasulphide (162) (27 mg, 11%), m.p. 160 - 163°C (decomp.)

Epidisulphide (164) (18 mg, 81%), m.p. 107 - 110°C

Di(bromomethyl) epidisulphide (176) (18 mg, 82%), m.p. 164 - 166°C (decomp.), specific activity 238 $\mu\text{Ci}/\text{mmol}$.

ii) (\pm)-1,4-Di(bromomethyl)-3,6-[^{35}S]epidithio-3,6-dibenzylpiperazine-2,5-dione (176). - The reaction sequence was carried out as in A) above, using protected cyclic dipeptide (161) (0.28 g, 0.73 mmol) and sulphur (180 mg, 0.7 mmol) containing [^{35}S] sulphur (nominal activity 2.2 mCi), giving;

Epitetrasulphide (162) (25 mg, 6%), m.p. 158 - 161°C (decomp.)

Epidisulphide (164) (19 mg, 92%), m.p. 109 - 111°C

Di(bromomethyl) epidisulphide (176) (19.5 mg, 84%), m.p. 166 - 168°C (decomp.), specific activity 343 $\mu\text{Ci}/\text{mmol}$.

iii) (\pm)-3,6-[^{35}S]Epidithio-3,6-di[4- ^3H]benzylpiperazine-2,5-dione (152). - The [4- ^3H]di(bromomethyl) disulphide (176) (18 mg, 0.033 mmol) and [^{35}S]di(bromomethyl) disulphide (3 mg, 0.006 mmol) were combined, and deprotected as for the unlabelled material. Crystallisation from benzene gave epidisulphide (152) (9 mg, 56%), m.p. 245 - 249°C (decomp.); one spot on t.l.c.; specific activity (^3H) 178 $\mu\text{Ci}/\text{mmol}$, (^{35}S) 36.6 $\mu\text{Ci}/\text{mmol}$.

3.4 Biosynthetic experiments

Fermentation conditions

Aspergillus terreus (NRRL 3319) was obtained from Lilly Research Laboratories, Indiana, U.S.A. Cultures were started on Czapek-Dox agar slants, made from:

Glucose	50 g	Magnesium sulphate (anhydrous)	1 g
Agar	20 g	Dipotassium hydrogen phosphate	0.5 g
Sodium nitrate	2 g	Ferrous sulphate (anhydrous)	0.01 g
Potassium chloride	1 g	Deionised water	1 litre

After incubation for 7 days, the slants were used to inoculate 500 ml wide-necked conical flasks containing 100 ml each of the nutrient medium:

Glucose	15 g	Magnesium sulphate heptahydrate	0.5 g
Sucrose	15 g	Potassium dihydrogen phosphate	1 g
Sodium nitrate	2 g	Ferrous sulphate heptahydrate	0.01 g
Potassium chloride	0.5 g	Beef extract (Oxoid "Lab-Lemco")	0.5 g
Yeast extract	0.125 g	Deionised water	1 litre

The medium was sterilised by autoclaving at 121°C for 20 min.

The seed flasks were grown at 28°C for 2 days, when aliquots (10 ml) from them were used to inoculate similar flasks containing the same medium (100ml). These flasks were normally grown for 7 days at 28°C; feeding was carried out after 48 h. Both the seed flasks and the main culture flasks were agitated on an orbital shaker at 160 r.p.m. Natural daylight provided the only illumination.

All operations concerning the growth and propagation of the fungus were carried out by Mrs. M.Tait and her staff.

Extraction procedure

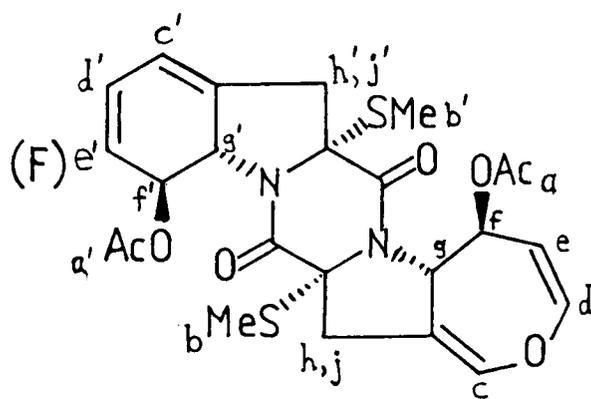
The cultures were harvested by filtration and washing of the mycelium with methanol. The combined broth and washings were adjusted to pH 2.5 with concentrated hydrochloric acid, and extracted with methylene chloride or chloroform (5 x 10% of the broth volume). The extracts were concentrated to ca. 100 ml, washed with water (3 x 10 ml) and evaporated to dryness.

Isolation of metabolites

The extracts were separated by preparative t.l.c. (solvent system A).

i) BDA (40). - The t.l.c. separation gave two u.v.-active bands at R_f ca. 0.5, the lower of which contained BDA, which was crystallised from methanol, (ca. 20 mg per litre of broth), m.p. 200 - 203°C (decomp.) [(lit.,⁴³ 213 - 217°C (decomp.)]; δ (360 MHz) (see Figure 1 for assignment) 2.06 (6 H, s, H_a), 2.25 (6 H, s, H_b), 2.9 - 3.1 (4 H,

Figure 1



Assignments for BDA and acetylaranotin refer to the aranotin ring only.

complex, H_h, H_j), 4.68 (2 H, dd, J 8 and 2 Hz, H_e), 5.17 (2 H, br d, J 8 Hz, H_g), 5.80 (2 H, ddd, J 8, 2, and 2 Hz, H_f), 6.28 (2 H, dd, J 8 and 2 Hz, H_d), and 6.56 (2 H, br s, H_c).

ii) BDAA (41). - The upper band of the two at R_f ca. 0.5 in the t.l.c. separation described above contained BDAA. This was purified further by t.l.c. (solvent system B) and crystallised from ether-hexane (ca. 15 mg per litre of broth), m.p. 131 - 134°C (decomp.) (lit.,⁴³ 105 - 107°C (decomp.); δ (360 MHz) 2.07 (3 H, s, H_a), 2.10 (3 H, s, H_a), 2.22 (3 H, s, H_b), 2.30 (3 H, s, H_b), 2.86 (1 H, br d, J 16 Hz, H_h), 2.9 - 3.1 (3 H, complex, H_h , H_j , H_j), 4.68 (1 H, dd, J 8 and 2 Hz, H_e), 5.16 (1 H, m, H_g), 5.19 (1 H, br d, J 14 Hz, H_e), 5.58 (1 H, br d, J 8 Hz, H_e), 5.79 (1 H, ddd, J 8, 2, and 2 Hz, H_f), 5.94 (1 H, m, H_c), 5.96 (1 H, m, H_d), 6.15 (1 H, br d, J 14 Hz, H_f), 6.28 (1 H, dd, J 8 and 2 Hz, H_d), and 6.55 (1 H, br s, H_c).

iii) Acetylaranotin (38). - This compound was occasionally found in extracts, especially those where the fungus was harvested at an early stage. The band containing BDAA (41) from the t.l.c. separation, when further separated (solvent system B) gave, besides BDAA, a band at higher R_f . This was extracted and crystallised from methanol to give acetylaranotin (ca. 2 mg per litre of broth harvested after 3 days of growth), m.p. 212 - 215°C (decomp.) [lit., 210 - 215°C (decomp.)]; δ (100 MHz) 2.03 (6 H, s, H_a), 3.00 (2 H, ddd, J 18, 2, and 2 Hz, H_h), 4.06 (2 H, ddd, J 18, 2, and 2 Hz, H_j), 4.64 (2 H, dd, J 8 and 2 Hz, H_e), 5.14 (2 H, dd, J 8 and 2 Hz, H_g), 5.72 (2 H, ddd, J 8, 2, and 2 Hz, H_f), 6.35 (2 H, dd, J 8 and 2 Hz, H_d), and 6.66 (2 H, dd, J 3 and 2 Hz, H_c); m/z 440 ($M^+ - S_2$, 1.0%), 291 (3), 115 (4), 106 (4), 64 (5), 60 (5), 45 (10), 44 (12), 43 (28), and 32 (100).

iv) cis-3,6-Di(methylthio)-3,6-dibenzylpiperazine-2,5-dione (131).
- The t.l.c. separation of the total extract gave a u.v.-active band at R_f ca. 0.75. Extraction of this band afforded a gum containing

several compounds, which was crystallised from methanol and recrystallised from acetone to give the di(methylthio)-cyclic dipeptide (131) (ca. 0.5 mg per litre of broth), m.p. 291 - 293°C (decomp.), $[\alpha]_{\text{Hg}}^{21} -122^\circ$ (c 0.019 in CHCl_3), $[\alpha]_{\text{D}}^{21} -52.1^\circ$ (c 0.28 in AcOH); $\nu_{\text{max.}}$ 3 180 (NH), 1 670 (CO), 1 420, 700, and 485 cm^{-1} ; δ (100 MHz) 2.25 (6 H, s, Me), 2.67 and 3.01 (4 H, ABq, J 14 Hz, CH_2Ph), 5.77 (2 H, br s, exchangeable with D_2O , NH), and 7.04 - 7.5 (10 H, m, Ph); m/z 339 ($\text{M}^+ - \text{SMe}$, 96%), 292 (61), 291 (49), 119 (30), 118 (39), 117 (18), 91 (100), 90 (10), 48 (8), and 47 (13).

v) cyclo-(L-Phe-L-Phe) (42). - The bands near the baseline in the t.l.c. separation of the total extract (solvent system A) (R_f ca. 0.2) were cut off and extracted with methanol. Crystallisation of the extracted material from methanol gave cyclo-(L-Phe-L-Phe) (ca. 5 mg per litre of broth), m.p. 312 - 315°C (lit.,¹⁰² 308 - 310°C), $[\alpha]_{\text{D}}^{20} -125^\circ$ (c 0.5 in AcOH), having spectral data as for the synthesised material (136f).

Non-dioxopiperazine metabolites isolated from extracts

i) Orsellinic acid. - This metabolite was isolated on one occasion from an extract of cultures fed DL-p-fluorophenylalanine. After t.l.c. separation of the total extract (solvent system A), the band at R_f 0.75 (containing di(methylthio)-cyclo-(Phe-Phe)) was subjected to a second t.l.c. separation (solvent system B). A band at R_f ca. 0.5 was cut off and extracted, giving crude orsellinic acid (3 mg), identified by its mass spectrum and proton n.m.r. spectrum; δ (100 MHz) (CD_3OD) 2.48 (3 H, br s, Me), 6.18 (2 H, ABq, J 2 Hz, Ar), and 7.2 (1 H, br s, OH);

m/z 168 (M^+ , 44%), 150, 122 (100), 94, 69, and 66.

ii) Ergosterol (129). - This compound was occasionally isolated from extracts; t.l.c. (solvent system A) gave a u.v.-active band at R_f ca. 0.8, which was extracted and crystallised from acetone to give ergosterol (129) (ca. 3 mg per litre of broth), m.p. 162 - 164°C (lit., ¹²⁰ 163°C), δ (100 MHz) 0.63 (3 H, s, Me), 0.84 (3 H, d, J 7 Hz, Me), 0.85 (3 H, d, J 7 Hz, Me), 0.92 (3 H, d, J 7 Hz, Me), 0.96 (3 H, s, Me), 1.05 (3 H, d, J 6 Hz, Me), 1.05 - 2.6 (ca. 20 H, complex), 3.63 (1 H, m, $\underline{C}HOH$), 5.24 (2 H, m, side-chain olefinic CH), and 5.34 - 5.65 (2 H, m, ring olefinic CH); m/z 396 (M^+ , 14%), 394, 378, 363, 337, 267, 251, and 69.

iii) Ergosterol endo-peroxide (130). - This compound was isolated from an extract of 1.5 litres of culture which had been fed cyclo-(p-Fluorophe-p-Fluorophe) (139a). Separation by t.l.c. (solvent system A) and extraction of a band at high R_f (ca. 0.8) gave a gum which was tentatively identified as the peroxide (130) from its proton n.m.r. spectrum and mass spectrum; δ 0.8 - 2.3 (ca. 40 H, complex), 3.99 (1 H, m, $\underline{C}HOH$), 5.23 (2 H, m, side-chain olefinic CH), 6.39 and 6.56 (2 H, ABq, J 9 Hz, ring olefinic CH); m/z 428 (M^+ , 3%), 396, 266, 249, 109, 107, 105, 95, 69, and 55.

iv) Phenolic metabolite (127).⁹³ - The supernatants from crystallisation of di(methylthio)-cyclo-(Phe-Phe) (131) were evaporated to dryness, and separated further by t.l.c. (solvent system A or B), giving the metabolite (127) as an oil (ca. 3 mg per litre of broth); $\nu_{max.}$ ($CHCl_3$) 3 590, 3 500, 3 600 - 3 150 (br), 3 030 (CH), 1 755 (CO),

1 740 (CO), 1 610 (C=C), 1 520, 1 390, 1 265, and 1 180 cm^{-1} ; δ (100 MHz) 1.70 (3 H, br s, C-Me), 1.74 (3 H, br s, C-Me), 3.18 (2 H, br d, \underline{J} 7 Hz, CH_2 of dimethylallyl), 3.55 (2 H, br s, CH_2Ar), 3.80 (3 H, s, OMe), 5.1 (2 H, br s, 2 OH), 5.16 (1 H, br t, \underline{J} 7 Hz, CH of dimethylallyl), 5.8 (1 H, br s, OH), 6.52 - 6.65 (3 H, m, CH of trisubstituted aromatic ring), 6.96 and 7.65 (4 H, ABq, \underline{J} 9 Hz, CH of disubstituted aromatic ring); m/z 396 ($\underline{M}^+ - \text{CO}$, 6%), 394, 380, 378, 175 (100), 131, 91, and 78.

Feeding of fluorinated compounds

The fluorinated dipeptides were fed to A. terreus after 48 h of growth, as solutions in DMSO; the total volume of DMSO was <0.5% of the volume of the medium. The concentration of dipeptide in the medium was ca. 0.1 mM. Fluorinated amino-acids were fed as solutions in DMSO-water mixtures; the concentration of amino-acid in the medium was normally ca. 0.05 mM.

After extraction, the ^{19}F n.m.r. spectrum (proton-noise decoupled) of a representative sample of the total extract was recorded (in CDCl_3), and the most significant signals in these spectra are given below (s = strong signal, m = medium, w = weak).

Total extract from cultures fed:

DL-o-Fluorophenylalanine; $\delta_{\text{F}}\{^1\text{H}\}$ -106.9 (m), -115.7 (s), -117.3 (m), -118.0 (m), -130.1 (w).

DL-m-Fluorophenylalanine; $\delta_{\text{F}}\{^1\text{H}\}$ -113.7 (s), -114.1 (s), -123.1 (w), -137.3 (m).

DL-p-Fluorophenylalanine; $\delta_{\text{F}}\{^1\text{H}\}$ -115.1 (m), -115.4 (m), -115.5 (m), -116.5 (m), -122.1 (w), -122.2 (s).

cyclo-(L-Phe-L-o-Fluorophe) (136c); $\delta_F\{^1H\}$ -115.6 (s), -117.0 (w), -117.3 (w).

cyclo-(L-Phe-L-m-Fluorophe) (136d); $\delta_F\{^1H\}$ -113.2 (s), -114.1 (w), -114.3 (w).

cyclo-(L-Phe-L-p-Fluorophe) (136b); $\delta_F\{^1H\}$ -115.3 (m), -115.6 (m), -116.8 (m), -122.3 (s).

cyclo-(L-Phe-L-2', 6'-Difluorophe) (136a); $\delta_F\{^1H\}$ -108.0 (m), -108.1 (m), -110.2 (m), -111.1 (s), -113.8 (s); -114.5 (m), -115.1 (w), -116.7 (s).

cyclo-(L-Phe-L-Pentafluorophe) (136e); $\delta_F\{^1H\}$ -140.0 - -142.8 (several signals, s), -144.5 (s), -156.0 (s), -156.5 (s), -157.4 (s), -158.6 (w), -162.4 - -164.3 (several signals, s).

(±)-cis-cyclo-(p-Fluorophe-p-Fluorophe) (139a); $\delta_F\{^1H\}$ -115.3 (s), -115.6 (s), -115.8 (s).

(±)-cis-cyclo-(Pentafluorophe-Pentafluorophe) (139b); $\delta_F\{^1H\}$ -142.6 (m), -156.6 (m), -163.0 (m).

The extracts were then separated by t.l.c. or column chromatography (solvent system A). When cyclo-(L-Phe-L-Phe) (42) and cis-3,6-di(methylthio)-3,6-dibenzylpiperazine-2,5-dione (131) were isolated as described above, the mono- and di-fluoro analogues tended to co-crystallise. These fluorinated analogues were detected using mass spectrometry and ^{19}F n.m.r. spectroscopy on the mixtures.

i) From cultures fed DL-p-fluorophenylalanine (112):

Mixture of cyclo-(L-Phe-L-Phe) (42), cyclo-(L-Phe-L-p-Fluorophe) (136b), and cis-cyclo-(p-Fluorophe-p-Fluorophe) (139a); $\delta_F\{^1H\}$ -115.3 (136b), and -115.6 (139a); m/z 330 [(139a), 2%], 312 [(136b), 6], 294 [(42), 17], 109 [C_7H_6F , 35], and 91 [C_7H_7 , 100]; (Found: 330.1187. Calc. for $C_{18}H_{16}N_2O_2F_2$ 330.1197; Found: 312.1281. Calc. for

$C_{18}H_{17}N_2O_2F$ 312.1291).

ii) Di(methylthio)-cyclo-(Phe-Phe) analogues

a) From cultures fed DL-p-fluorophenylalanine (112);

Mixture of (131) with p-fluoro- and p,p'-difluoro-analogues (142) and (143); $\delta_F\{^1H\}$ -114.3; m/z 375 [(143), M^+ -SMe, 1.5%], 357 [(142), M^+ -SMe, 11], 339 [(131), M^+ -SMe, 100], 309 [(142), M^+ -SMe-MeSH, 14], 291 [(131), M^+ -MeSH-SMe, 94], 109 [$C_7H_6F^+$, 7] and 91 [$C_7H_7^+$, 50]; (Found: m/z 357.1077. $C_{19}H_{18}FN_2O_2S$ requires 357.1073. Found: 309.1035. $C_{18}H_{14}FN_2O_2$ requires 309.1039.

b) From cultures fed cyclo-(L-Phe-L-p-Fluorophe) (136b);

Mixture of (131) with p-fluoro-analogue (142); $\delta_F\{^1H\}$ -114.3; m/z 357 [(142), M^+ -SMe, 7%], 339 [78], 309 [(142), M^+ -MeSH-SMe, 6], 292 [72], 291 [100], 109 [$C_7H_6F^+$, 6], and 91 [94].

c) From cultures fed cyclo-(L-Phe-L-o-Fluorophe) (136c);

Mixture of (131) with o-fluoro-analogue (144); $\delta_F\{^1H\}$ -115.4; m/z 357 [(144), M^+ -SMe, 7%], 339 [78], 310 [(144), M^+ -2 SMe, 10], 309 [(144), M^+ -MeSH-SMe, 10], 292 [57], 291 [62], 109 [8], and 91 [100].

d) From cultures fed cyclo-(L-Phe-L-m-Fluorophe) (136d);

Mixture of (131) with m-fluoro-analogue (145); $\delta_F\{^1H\}$ -112.5; m/z 357 [(145), M^+ -SMe, 3%], 339 [37], 291 [27], 290 [24], 109 [13], and 91 [100].

e) From cultures fed cyclo-(L-Phe-L-2',6'-Difluorophe) (136a);

Mixture of (131) with 2',6'-difluoro-analogue (146); $\delta_F\{^1H\}$ -110.6; m/z 375 [(146), M^+ -SMe, 7%], 339 [42], 328 [4], 327 [4], 292 [27], 291 [29], 127 [$C_7H_5F_2^+$, 4], 91 [71], and 57 [100].

f) From cultures fed (+)-cis-cyclo-(p-Fluorophe-p-Fluorophe)

(139a);

Mixture of (131) with 4,4'-difluoro-analogue (143); m/z 375 [(143), $M^+ - SMe$, 1.5%], 339 (36), 292 (22), 291 (16), 109 (30), and 91 (100).

Fluoro-BDAA (141).

DL-p-Fluorophenylalanine (112) (13.7 mg) was fed to A. terreus (1.5 litres of medium) and extracted as described above, after 5 days. The extract was separated by preparative t.l.c. (solvent system B), and a band at R_f ca. 0.5, containing a mixture of arantoin metabolites, was cut off and extracted. This material was further separated (solvent system A), and the band containing BDAA (and fluoro-BDAA) was cut off and extracted. A third separation (solvent system D) on analytical t.l.c. plates (25 mg of mixture per 20 x 20 cm plate) gave fluoro-BDAA (141) as a narrow band just above the strong BDAA band. This was further purified by another t.l.c. separation on analytical plates (solvent system B) to give fluoro-BDAA as an oil, which was crystallised from ether-hexane (2.4 mg after crystallisation), m.p. 131 - 134°C (decomp.), $[\alpha]_D^{20}$ -123° (c 0.03 in $CHCl_3$); λ_{max} . (EtOH) 266 nm (ϵ 3350); ν_{max} . 3 080 (olefinic CH), 2 930 (saturated CH), 1 745 ($COCH_3$), 1 675 (CON), 1 375, 1 230, and 1 040 cm^{-1} ; δ (360 MHz) (see Fig. 1 for assignments) 2.07 (3 H, s, H_a), 2.15 (3 H, s, H_a), 2.20 (3 H, s, H_b), 2.29 (3 H, s, H_b), 2.83 (1 H, br d, J 15 Hz, H_h), 2.97 - 3.06 (3 H, complex, H_h , H_j , H_j), 4.68 (1 H, dd, J 8 and 2 Hz, H_e), 5.14 (1 H, m, H_g), 5.38 (1 H, very br d, J ca. 15 Hz, H_g), 5.61 (1 H, br ddd, J 11, 5.5, and 2 Hz, H_d), 5.79 (1 H, ddd, J 8, 2, and 2 Hz, H_f), 5.85 (1 H, m, H_c), 6.28 (1 H, dd, J 8 and 2 Hz, H_d), ca. 6.32 (1 H, very br, H_f), and 6.55 (1 H, br s, H_c); δ_F (55°C) -121.9 (m); from selective proton decoupling $J_{H_c,F} = 5$ Hz, $J_{H_d,F} = 11$ Hz, $J_{H_f,F} = 2$ Hz, and $J_{H_g,F} = 4$ Hz; m/z 536 (M^+ , 0.8%), 489, 429,

381, 321, 162, 160, 134, 132, and 43 (100); (Found: m/z 536.1089.

$C_{24}H_{25}FN_2O_7S_2$ requires 536.1087.

Feeding experiments with radio-labelled fluorinated compounds

The results of feeding the ^{14}C -labelled fluorinated cyclic dipeptides (136a - e) are summarised in Table 3.

The incorporation of cyclo-(L-Phe-L-[1- ^{14}C]-p-Fluorophe) (136b) into fluoro-BDAA was measured by n.m.r. spectroscopy: To the total extract was added p-fluorobenzoic acid (1.5480 mg), and the proton-decoupled ^{19}F n.m.r. spectrum integral was used to give an approximate incorporation. Integral heights; fluoro-BDAA (δ -123) 4.8 cm, p-fluorobenzoic acid (δ -107) 51.1 cm, other fluorinated compounds (δ -114 - -117) 12.0 cm. Incorporation into fluoro-BDAA = 1.8%. Total recovery of fluorinated compounds = 6.2% (cf. incorporation of ^{14}C into the total extract = 8.0%).

Feeding of the deuteriated cyclic dipeptides (136g) and (136h)

cyclo-(L-Phe-L-(3R)-[3-D]-p-Fluorophe) (136h) (85 mg, 0.27 mmol) and cyclo-(L-Phe-L-(3S)-[2,3-D₂]-p-Fluorophe) (136g) (85 mg, 0.27 mmol) were each fed to 1.4 litres of A. terreus culture medium. The cultures were harvested 5 days later, and the mixtures of di(methylthio)-cyclo-(Phe-Phe) (131) and its fluorinated, deuteriated analogues (142) were isolated as described previously. The two samples were analysed by mass spectrometry.

i) From the culture fed cyclo-(L-Phe-L-(3R)-[3-D]-p-Fluorophe) (136h); m/z 358 [(142), M^+ -SMe, 3.6%], 357 [0.47], 339 [(131), M^+ -SMe, 34], and 91 [100].

ii) From the culture fed cyclo-(L-Phe-L-(3S)-[2,3-D₂]-p-Fluorophe)

COMPOUND FED	Activity fed (μCi)	Weight fed (mg)	Medium vol. (l)	Activity in extract (μCi)	Radioactive compounds in extract		
					Corresp. cyclic dip.	Di(SMe) cpd.	Other compounds
DL- $[\beta\text{-}^{14}\text{C}]$ - <u>p</u> -Fluorophenyl- alanine (112)	50	0.46	0.5	22.0	(<u>p</u> -F) + (<u>p</u> ₂) +	+	Fluoro-BDAA +++
DL- $[\beta\text{-}^{14}\text{C}]$ - <u>p</u> -Fluorophenyl- alanine (112)	10	32.4	1.4	0.93	(<u>p</u> -F) + (<u>p</u> ₂) +	+	Fluoro-BDAA ++
(136b); Ar = <u>p</u> -F-Ph	5.00	18.7	0.6	0.40	+	+	Fluoro-BDAA ++
(136c); Ar = <u>o</u> -F-Ph	5.08	19.0	0.8	0.66	++	+	
(136d); Ar = <u>m</u> -F-Ph	6.36	23.9	1.0	0.78	++	+	
(136a); Ar = 2,6-di-F-Ph	6.83	27.1	0.8	1.38	++	++	Several unidentified compounds
(136e); Ar = penta-F-Ph	4.32	16.3	0.5	1.12	++	?	Several unidentified compounds

TABLE 3 Experimental data for feeding experiments
with radio-labelled fluorinated compounds

+ = small amount present ++ = moderate amount
+++ = substantial amount

(136g); m/z 358 [(142), M^+ -SMe, 0.93%], 357 [0.17], 339 [(131), M^+ -SMe, 6.3], and 91 [100].

Feeding of sodium [^{35}S]sulphate

A solution of sodium [^{35}S]sulphate (298 μCi , <1 mg) in sterile water (5 ml) was fed to 0.8 litres of A. terreus culture. On extraction 4 days later, the total extract contained 75 μCi , an incorporation of ^{35}S into organic metabolites of 25%. Radioscanning and autoradiograms of t.l.c. plates showed that most of the activity was in BDA and BDAA, with lesser amounts in di(methylthio)-cyclo-(Phe-Phe) (131) and some other, unidentified metabolites.

Feeding of 3,6- ^{35}S epidithio-3,6-[4- ^3H]dibenzylpiperazine-2,5-dione (152)

A) The doubly labelled deprotected epidisulphide (152) (45 mg, 0.126 mmol; specific activity (^3H) 133 $\mu\text{Ci}/\text{mmol}$, (^{35}S) 25.8 $\mu\text{Ci}/\text{mmol}$; total activity (^3H) 16.8 μCi , (^{35}S) 3.3 μCi ; $^3\text{H}:^{35}\text{S}$ ratio 5.16) was dissolved in DMSO (1.5 ml) and fed to A. terreus (0.6 litres of culture). After 4 days, the cultures were harvested, extracted, and the extract separated by t.l.c. to give:

BDA (40) (10.0 mg), crystallised from methanol to constant activity; specific activity (^3H) 24.6 $\mu\text{Ci}/\text{mmol}$, (^{35}S) 7.6 $\mu\text{Ci}/\text{mmol}$; $^3\text{H}:^{35}\text{S}$ ratio 3.24; incorporation (^3H) 2.7%, (^{35}S) 4.4%; dilution (^3H) 5.41, (^{35}S) 3.40.

Di(methylthio)-cyclo-(Phe-Phe) (131) (0.6 mg), crystallised from chloroform and recrystallised from acetone; specific activity (^3H)

110.5 $\mu\text{Ci}/\text{mmol}$, (^{35}S) 24.4 $\mu\text{Ci}/\text{mmol}$; $^3\text{H}:^{35}\text{S}$ ratio 4.53; incorporation (^3H) 1.0%, (^{35}S) 1.1%; dilution (^3H) 1.20, (^{35}S) 1.06.

cyclo-(L-Phe-L-Phe) (42) (8 mg), crystallised twice from acetic acid-water, specific activity (^3H) 1.83 $\mu\text{Ci}/\text{mmol}$; incorporation 0.3%; dilution 72.7.

B) The disulphide (152) (4.8 mg, 0.012 mmol; specific activity (^3H) 178 $\mu\text{Ci}/\text{mmol}$, (^{35}S) 36.6 $\mu\text{Ci}/\text{mmol}$; total activity (^3H) 2.06 μCi , (^{35}S) 0.42 μCi ; $^3\text{H}:^{35}\text{S}$ ratio 4.86) was fed to 0.6 litres of culture, and the cultures were harvested after $5\frac{1}{2}$ h. Preparative t.l.c. of the extract gave:

BDA (40) (3.2 mg), crystallised twice from methanol; specific activity (^3H) 4.21 $\mu\text{Ci}/\text{mmol}$, (^{35}S) 1.93 $\mu\text{Ci}/\text{mmol}$; incorporation (^3H) 1.2%, (^{35}S) 2.7%; dilution (^3H) 42.3, (^{35}S) 19.0. To obtain a constant ratio on successive crystallisations, it was necessary to add unlabelled BDA (5 mg) (due to lack of material); $^3\text{H}:^{35}\text{S}$ ratio 2.63.

Acetylaranotin (38) (1.2 mg), crystallised twice from methanol; specific activity (^3H) 26.4 $\mu\text{Ci}/\text{mmol}$, (^{35}S) 6.20 $\mu\text{Ci}/\text{mmol}$; $^3\text{H}:^{35}\text{S}$ ratio 4.26; incorporation (^3H) 3.1%, (^{35}S) 3.4%; dilution (^3H) 6.74, (^{35}S) 5.91. A sample was diluted with unlabelled acetylaranotin and crystallised to constant ratio; $^3\text{H}:^{35}\text{S}$ ratio 4.41.

Di(methylthio)-cyclo-(Phe-Phe) (131) (ca. 0.2 mg), crystallised from chloroform and recrystallised from acetone; specific activity (^3H) 50.3 $\mu\text{Ci}/\text{mmol}$, (^{35}S) 10.4 $\mu\text{Ci}/\text{mmol}$; $^3\text{H}:^{35}\text{S}$ ratio 4.85; incorporation (^3H and ^{35}S) 1.3%; dilution (^3H and ^{35}S) 3.54.

C) The disulphide (152) (3.2 mg, 0.008 mmol, specific activities etc. as in B), total activity (^3H) 1.37 μCi , (^{35}S) 0.28 μCi) was fed

to 0.4 litres of culture, and harvested after 24 h. T.l.c. gave BDA (3.9 mg), crystallised to constant activity from methanol; specific activity (^3H) 3.28 $\mu\text{Ci}/\text{mmol}$, (^{35}S) 1.03 $\mu\text{Ci}/\text{mmol}$; $^3\text{H}:^{35}\text{S}$ ratio 3.18; incorporation (^3H) 1.7%, (^{35}S) 2.6%; dilution (^3H) 54.3, (^{35}S) 35.5.

Feeding of [^{35}S]di(methylthio)-cyclo-(Phe-Phe) (131)

The above compound, prepared from the [^{35}S]epididysulphide (152), (5.5 mg, 0.014 mmol; specific activity 25.1 $\mu\text{Ci}/\text{mmol}$; total activity 0.36 μCi) was dissolved in DMSO (1 ml) and fed to 0.3 litres of culture. The culture was harvested 5 days later, BDA (6.0 mg) was isolated by t.l.c., and crystallised to constant activity from methanol; specific activity 0.082 $\mu\text{Ci}/\text{mmol}$; incorporation 0.26%; dilution 306.

Intermediate trapping experiment

To 0.5 litres of culture was fed L-[U- ^{14}C]phenylalanine (50 μCi ; specific activity 10 mCi/mmol) in water (1 ml). After 3 h, epididysulphide (152) (40 mg, 0.097 mmol) in DMSO (1 ml) was added, and after a further 30 min., the culture was harvested and the broth extracted. T.l.c. (solvent systems A and C) recovered some of the disulphide (152) (ca. 1 mg). This was diluted with unlabelled disulphide (2 mg), and crystallised from toluene to constant activity; specific activity 1.08 $\mu\text{Ci}/\text{mmol}$; incorporation into recovered (152) 0.015%. BDA (40) and acetylaranotin (38) were also isolated:

BDA (40) (4.5 mg), crystallised to constant activity from methanol; specific activity 89.6 $\mu\text{Ci}/\text{mmol}$; incorporation 1.5%.

Acetylaranotin (38) (1.8 mg), crystallised to constant activity from methanol; specific activity 422 $\mu\text{Ci}/\text{mmol}$; incorporation 3.0%.

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