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A THESIS PRESENTED BY

ROBERT ORR CAIN

entitled

"Studies related to the biogenesis of
Bicyclomycin"

in partial fulfilment of the regulations
governing the degree of Ph.D of the
University of Stirling

Chemistry Department
University of Stirling
Scotland
March 1980

Dedication

To my in-laws and out-laws who, together with Aer Lingus, helped immensely in the production of this thesis, but principally to my wife, Jean, and daughter, Kirsty, for their silence, when necessary, and infinite patience.

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SECTION 3

EXPERIMENTAL

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ABSTRACT

The literature concerning the biogenesis of naturally-occurring dioxopiperazines is reviewed.

Initially, it was hoped to probe the biogenesis of Bicyclomycin using substrates labelled with stable isotopes. A working hypothesis for the biogenesis of Bicyclomycin is proposed and seemed to indicate that leucine and isoleucine were the principal precursors.

To test the validity of this theory [$1-^{13}\text{C}$] leucine and [$1-^{13}\text{C}$] isoleucine were synthesised by conventional methods.

Small scale shake culture fermentations did produce Bicyclomycin but the yield in the case of both *Streptomyces S. aizunensis* and *S. sapporonensis* was very low. The efficiency of Bicyclomycin isolation was shown to be ca. 10%.

The production of Bicyclomycin was stimulated by nutrient deprivation (carbohydrate and nitrogen sources) and by the introduction of a thermal shock during fermentation.

In the case of *S. sapporonensis*, thermal shock increased the yield of the crude antibiotic by a factor of five. In general *S. aizunensis* showed a greater production of the crude antibiotic under shake culture conditions thus reflecting its lower oxygen requirement during fermentation. Large scale fermentation experiments (20L) using *S. aizunensis* produced a broth with modest antibiotic activity. This was purified by several methods but the final yield of Bicyclomycin was disappointing. *S. sapporonensis* proved no more efficient than *S. aizunensis* in large scale fermentations.

However, phenylacetamide was isolated as an antibiotic from S.sapporonensis.

Limited mutation studies produced one mutant with an enhanced antimicrobial spectrum.

The key features of the postulated biogenesis of Bicyclomycin were tested by the synthesis of a target compound 3,6-di-(2'-hydroxybenzyl)-2,5-dihydroxypyrazine. All reactions were tested first on a series of parallel model compounds derived from 3,6-dibenzyl-2,5-dioxypyrazine.

A new method for the removal of alkyl groups from ethers of aromatic 1,4-diazines was discovered. These ethers are particularly resistant to cleavage by normal methods but will produce the dealkylated product in high yield when treated with iodotrimethylsilane.

The target compound failed to undergo the key photo-oxygenation step, required by biosynthetic proposals, due to its inherent insolubility in most polar solvents.

A second route designed to produce the target compound by way of a substituted 2,5-dichloropyrazine derivative proved only partly successful.

The utility and preparation of 2,5-dichloropyrazines was investigated.

Several N-oxides of these diazines were produced and the reaction of these compounds with sodium allyloxide was investigated.

Thermolysis of one derivative gave a small amount of material whose structure seems to be consistent with a Claisen rearrangement. This is a novel process for 1,4-diazines.

Preparation of substrates, for Claisen rearrangement, based on 2,5-diethoxypyrazine met with only limited success.

The ^{13}C spectra of most synthetically produced dioxopiperazine and pyrazine derivatives are reported, together * with structural assignments for Bicyclomycin itself. It is hoped that these data may be useful in future biosynthetic work.

SECTION 1

"The biogenesis of natural dioxopiperazines"

A review of current literature
ending in August 1979

Introduction

2,5-dioxopiperazines may be conveniently regarded as anhydro-dimers of amino acids.

The natural occurrence of these peptide derivatives is ubiquitous throughout nature but is most common among the lichens, yeasts, bacteria and fungi. They may also be found in protein hydrolysates where they are thought to be present as reaction artefacts.

The parent compound cyclo-glycyl-glycine was first synthesised by Curtius in 1888 and the more complex derivatives have represented a synthetic challenge ever since.

The purpose of this review is to consider aspects of importance which contribute to an understanding of the biosynthesis of this class of compounds.

Consequently synthetic studies will only be considered if they are biomimetic or act as a model system for a proposed biosynthetic process.

The bulk of this review will concentrate on hard facts obtained from feeding experiments and speculative routes will be kept to a minimum.

Naturally occurring dioxopiperazines have been recently¹ reviewed but consideration of detailed aspects of biosynthetic studies was minimal. Similarly the chemistry of cyclic hydroxamic acids has also been reviewed² with total omission of biosynthetic considerations.

1.0 The Simple Dioxopiperazines

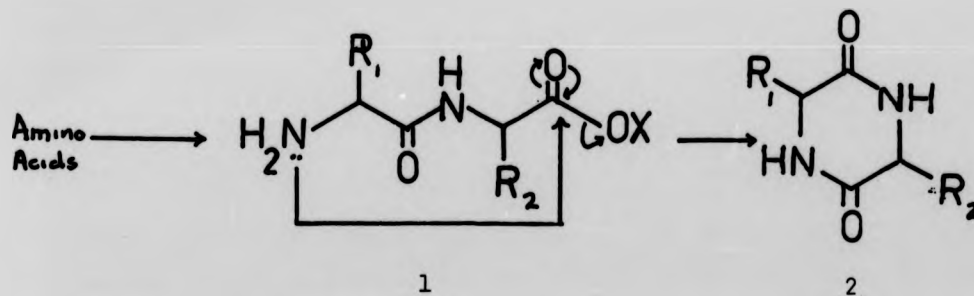
The occurrence of simple dioxopiperazines is widespread in nature and they are usually secondary metabolites produced by fungi, yeasts, algae and higher organisms.

The structural diversity of these simple cyclic dipeptides is as spectacular as the variation in the structure of the amino acids from which they are formed.

The biogenesis of these compounds is not as simple as it would appear at first sight.

There is still controversy concerning their origin although the fact that they are derivatives of two amino acids is beyond reasonable doubt.

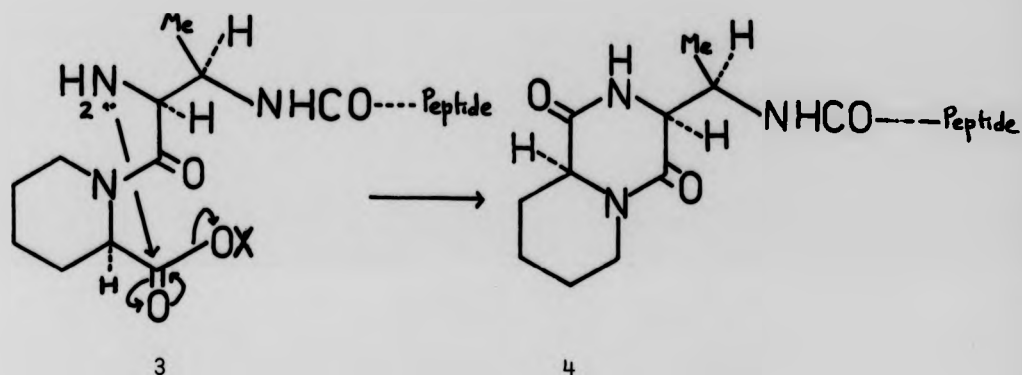
In some cases it is assumed¹ that a dipeptide is formed and cyclisation of an activated ester (1) leads to the formation of a dioxopiperazine system (2) i.e. (Scheme 1)



Scheme 1

In a few other cases a different mechanism has been proposed which involves the formation of a dioxopiperazine at the terminus of a linear polypeptide e.g. Amphomycin³.

Cleavage of the dioxopiperazine moiety may then follow or the system may remain intact as in Amphotycin (4) (Scheme 2).

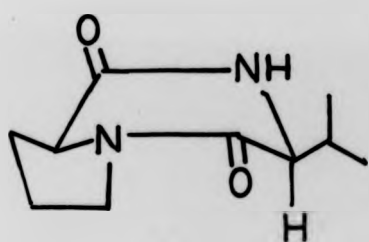


Scheme 2

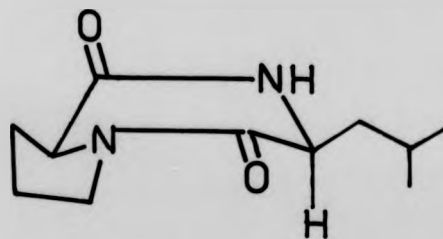
No definitive statement can be made concerning the biogenesis of any cyclic dipeptide since one case may hold for one class of metabolites and alternative mechanisms operate for a different group.

Many of the simpler examples of dioxopiperazines such as the Streptomyces lavendulae dipeptides belong to this group e.g. cyclo-L-valyl-L-proline (5) and other dipeptides derived from leucine, phenylalanine, isoleucine and proline⁴. These derivatives (5-8) appear to be derived by a cyclisation involving the activated ester closure, but the alternative mechanism cannot be ruled out.

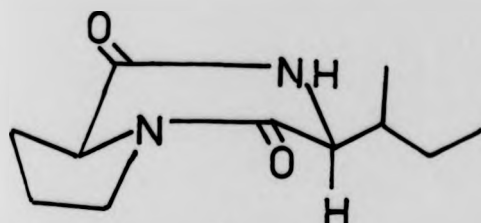
Similarly, the natural product cyclo-L-valyl-L-proline (5) occurs in many other organisms⁵ e.g. Streptomyces K-73,



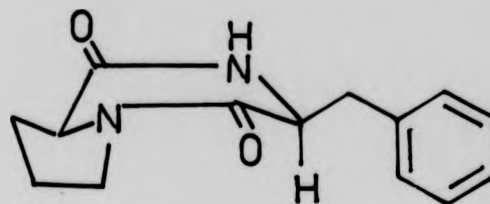
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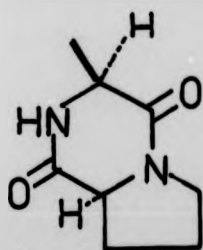
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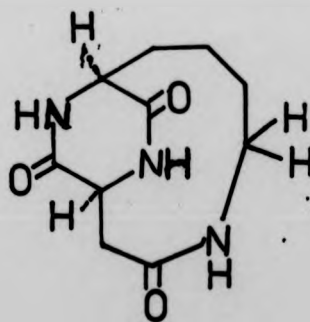
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Rosellina necatrix, Aspergillus ochraceus and Oospora destructor illustrating the ubiquitous nature of these compounds.

It is of interest to note that related compounds e.g. cyclo-L-prolyl-L-alanine⁶ (9) in some cases possess marked biological activity e.g. (9) is active against lymphocyte leukemia. The occurrence of a large number of cyclic dipeptides containing the proline residue may be related to the known propensity of proline derivatives to undergo cyclhydration.



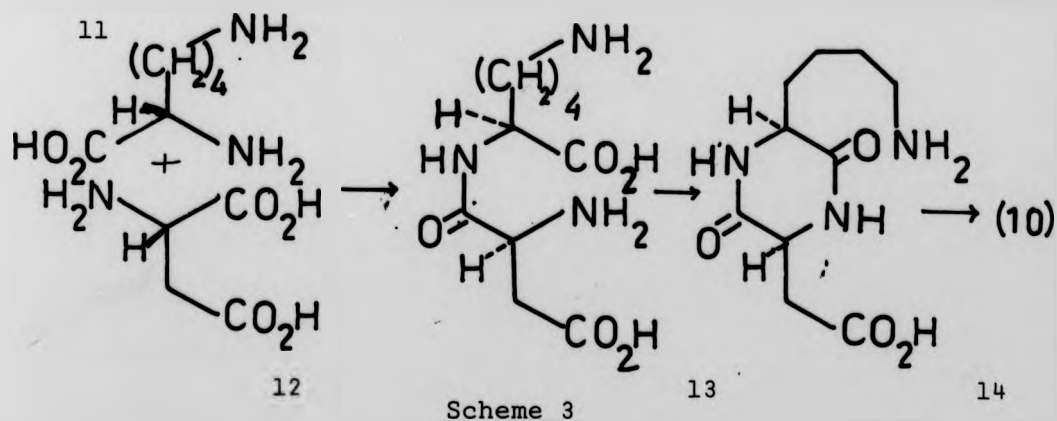
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Another product of great interest is Cairomycin B (10), a broad spectrum gram negative antibiotic. Cairomycin B⁷ has been isolated from Streptomyces As-C-19 and on acid hydrolysis yields aspartic acid and lysine⁷.

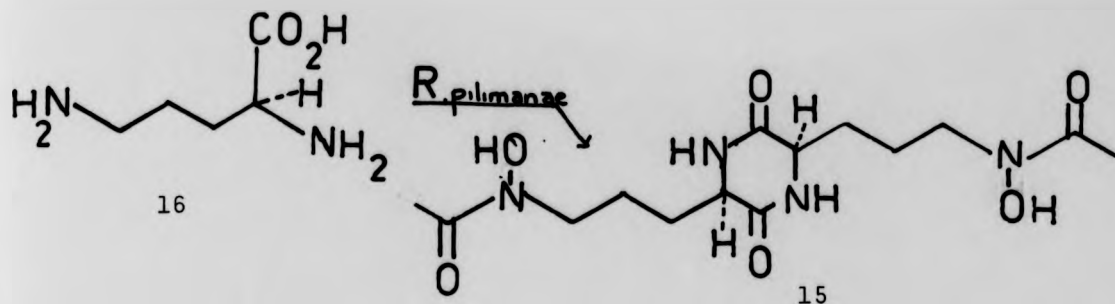
It is therefore reasonable to assume that the biogenesis of this antibiotic may involve these two amino acids (Scheme 3).



Neither of the intermediates (13) or (14) have been reported from cultures of Streptomyces As-C-19 and the above scheme is, at best, speculative.

More complex dioxopiperazines such as rhodotorulic acid⁸ (15), a metabolite of the lichen Rhodotorula pilimanae (CBS4478), has been the subject of biosynthetic investigation.

Ornithine (16) was thought to be the major precursor of rhodotorulic acid and was fed to an actively growing culture of R. pilimanae⁹ (Scheme 4).



Scheme 4

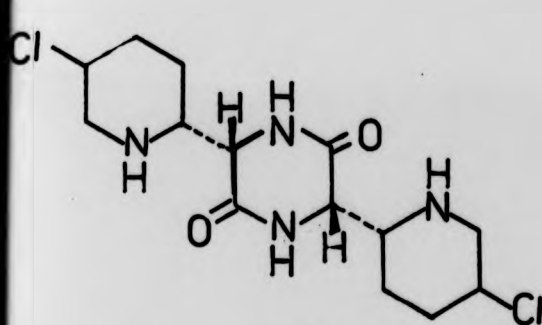
resulting in incorporation⁹ of labelled ornithine into the acid (15).

Similarly R. pilimanae was grown in cultures containing 99.8% D₂O and in degradation no deuterium incorporation was observed in the N-hydroxyl functionality of ornithine, suggesting that hydroxylation occurred via molecular oxygen rather than by hydration processes.

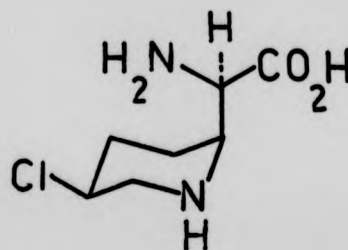
Evidence for acetylation and dimerisation being sequential to N-hydroxylation was also obtained⁹.

One of the more unusual compounds in this class of metabolites is the anti-tumour compound 593A (17), a metabolite of Streptomyces griseoluteus¹⁰⁻¹³.

The most unusual feature of this metabolite is the β -chloramine functionality, most commonly present in the synthetic nitrogen mustards. The biosynthetic proposals¹⁴ invoke dimerisation of streptolutine (18) as yet an unknown amino acid.



17



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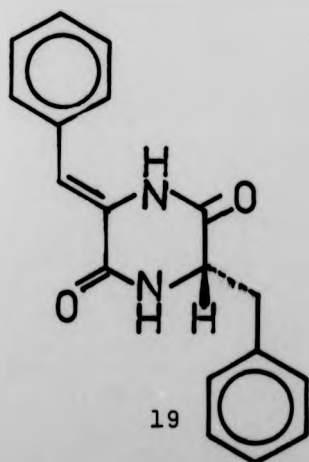
1.1 The Natural Arylidene Dioxopiperazines

The arylidene dioxopiperazines can be regarded as products of dehydrogenation of the corresponding saturated analogues and occur naturally with the saturated analogues.

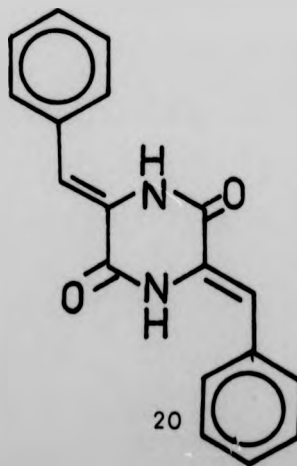
This would seem to suggest an in vivo dehydration of a hydroxylated intermediate or a dehydrogenation process.

Most of the natural metabolites have been shown to possess the (Z) configuration¹⁵ as one might expect.

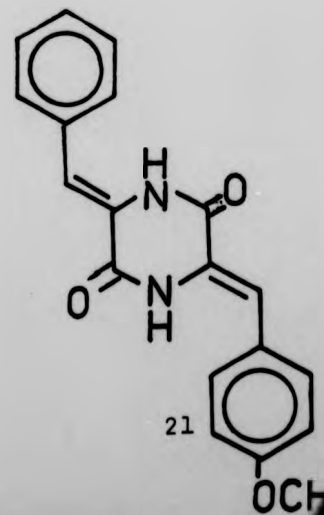
The structural variation of this class of compounds can be demonstrated in compounds (19)¹⁶ and (20)¹⁷ from Streptomyces noursei and (21)¹⁸ from Streptomyces thioluteus.



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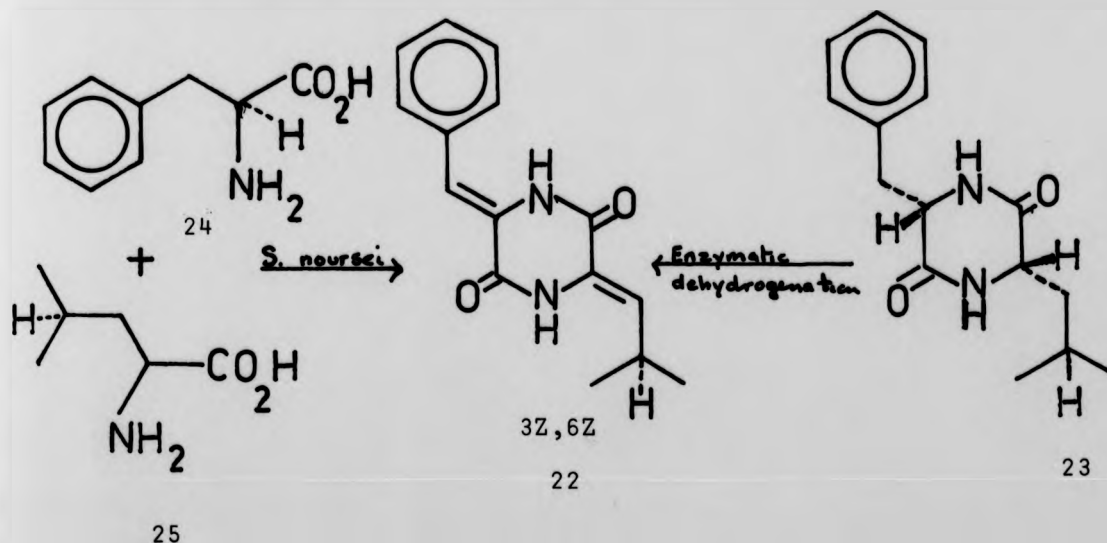


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Most biosynthetic studies have been carried out on albonoursin (22), a metabolite of *S.noursei*¹⁹, which possesses the expected (3Z,6Z) configuration²⁰.

Phenylalanine (24) and leucine (25) have been shown to be effective precursors of albonoursin (22) in cultures of *S.noursei*.²¹

Other workers²⁰ have inferred the existence of the intermediate (23) on the basis that the dehydrogenation must be enzyme mediated to generate the Z-isomer preferentially in this and other related compounds (Scheme 5).



Scheme 5

However no feeding experiments using (3R) or (3S) tritiated phenylalanine have been conducted and the stereochemical course of dehydrogenation remains unsolved.

In a similar fashion, phenylalanine has been shown to be incorporated effectively into the bis-arylidene compound (20)²¹.

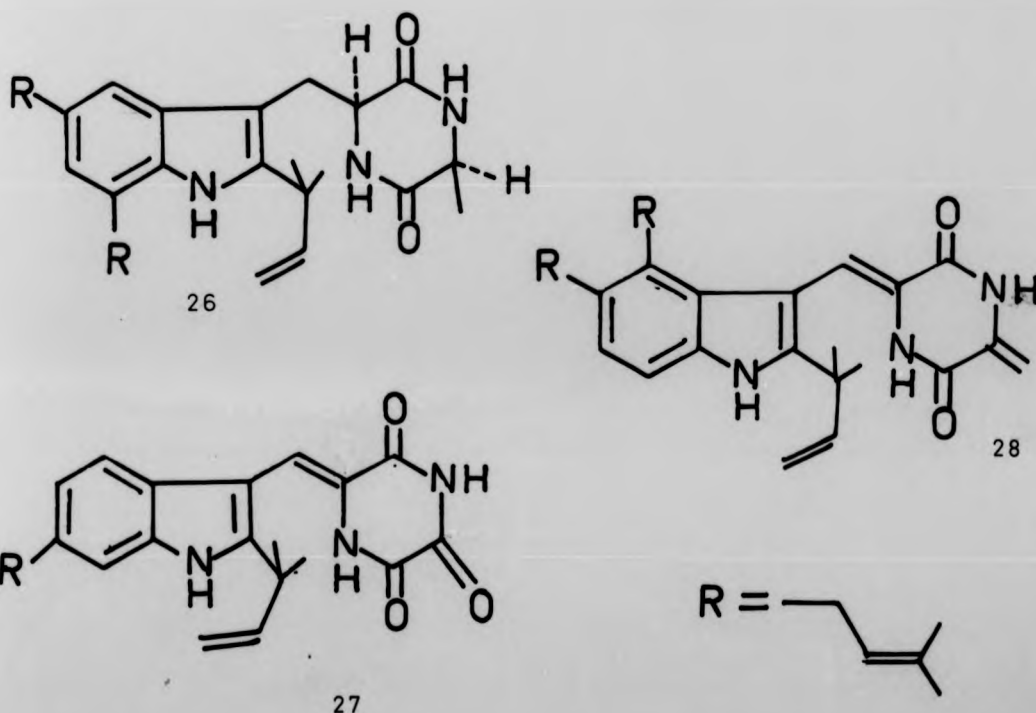
1.2 The Biogenesis of Dioxopiperazines via Amide-amide Interaction

The type of closure is observed almost exclusively in the ergot alkaloid series and has been the subject of a recent review by Floss²².

The reader is recommended to read this fully comprehensive review on the biogenesis of ergot alkaloids as it will not be further examined in this review.

2.0 The Echinulins

The compounds of the echinulin type represent a rather unusual class of natural products in that, in the same molecule both normal and reversed prenylated moieties occur. Most occur as fungal products and the range of structural types is illustrated with reference to echinulin (26), neoechinulin (27) and the arylechinulin (28).

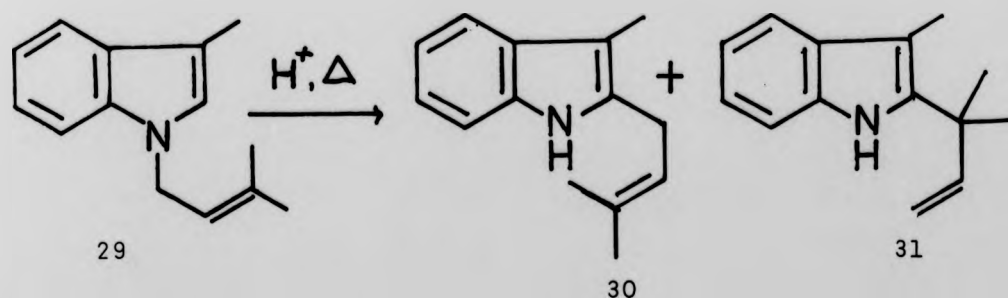


As can be seen, the prenylation pattern can vary widely although the reversed isoprene unit is always situated at the 2-position of the indole ring.

2.1 Model Studies

The first step in the biogenesis of these metabolites is considered to be prenylation of the tryptophan unit prior to closure of the dioxopiperazine ring.

Only two authors, Casnati and Pochini^{23,24} propose the possible intermediacy of an N-prenylated indole and rearrangement to the 2-substituted indole (Scheme 6)^{23,24}



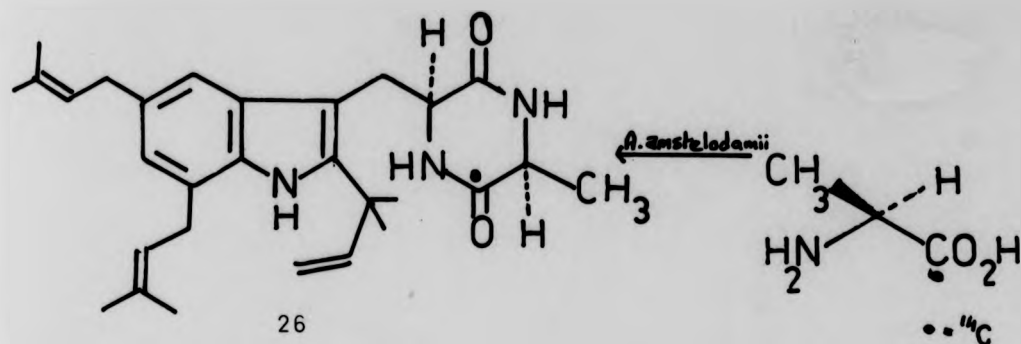
Scheme 6

Most workers now consider this theory too unlikely²⁵.

2.2 Tracer Studies

Biosynthetic theory would predict that echinulin (26) is probably derived from alanine, tryptophan and mevalonate and this was later shown to be the case (Scheme 7).

Feeding experiments²⁶ with (1-¹⁴C) alanine (32) established that alanine was incorporated into echinulin (26) in cultures of Aspergillus amstelodamii (0.12% incorporation).



Scheme 7

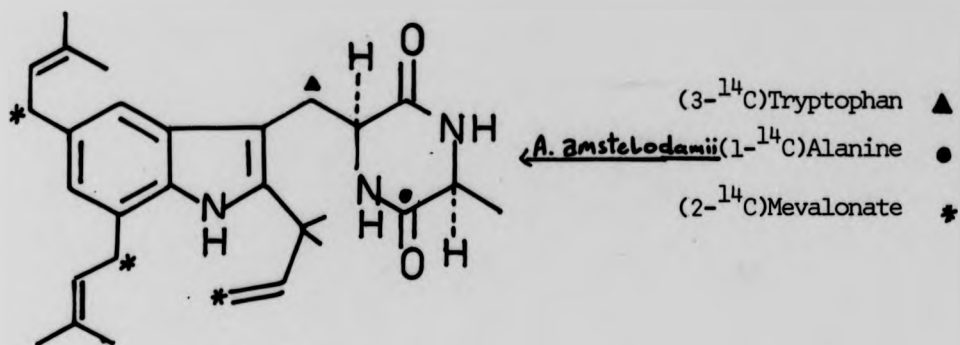
(2-¹⁴C) mevalonate was also found to be an efficient precursor (4.25% incorporation) and degradation gave radioactive acetone from the isoprene moieties and α,α -dimethylvaleric acid from the reversed prenyl moiety²⁶.

Both D,L-(3-¹⁴C)-tryptophan and L-(3-¹⁴C)-tryptophan were efficient precursors for echinulin (26)²⁷ and incorporations were 1.36% and 2.76% respectively. On degradation the alanyl unit was found to be radiochemically inactive.

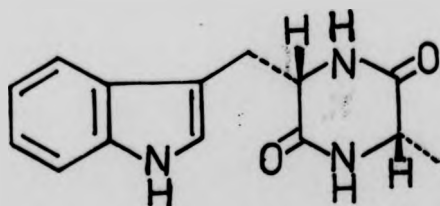
Further studies²⁸ showed that tryptophan labelled in the 1-, 2- or 3-position and D,L-(3-¹⁴C)-tryptophan were incorporated without label randomisation. The biosynthetic results related to echinulin are summarised in Scheme 8.

Subsequent feeding experiments with cyclo-L-alanyl-L-(3-¹⁴C)-tryptophan (33) showed that this too was an efficient precursor of echinulin (26) i.e. (9.9 + 16.2% incorporation)²⁹.

Competition experiments showed that the precursor was incorporated intact.



Scheme 8

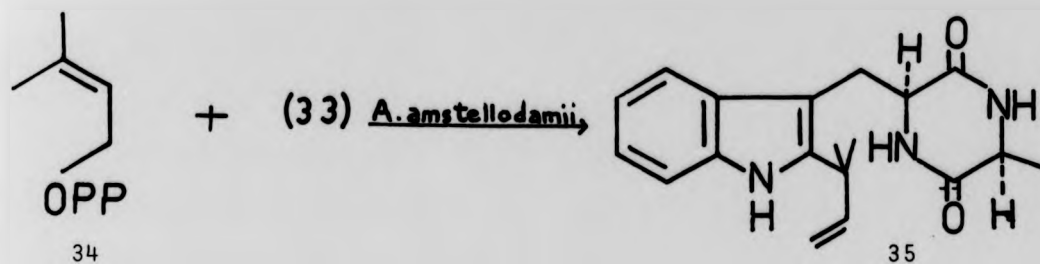


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These results seem to indicate that prenylation probably occurs after dioxopiperazine formation.

Investigations using cell free extracts of *A. amstelodamii* proved that it was possible to transfer the 3,3-dimethylallyl moiety from 3,3-dimethyl allyl pyrophosphate (34) to cyclo-L-alanyl-L-tryptophan (33) to give the mono-prenylated product (35)³⁰ (Scheme 9).

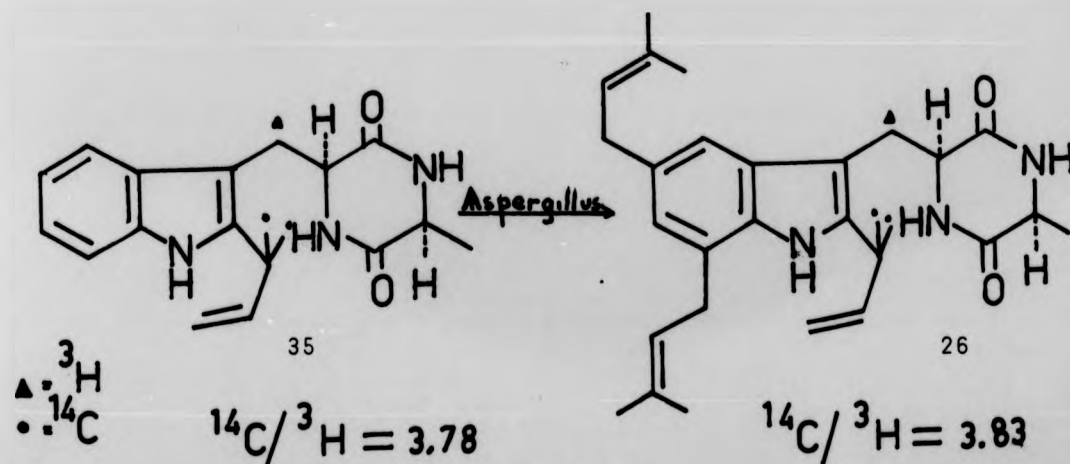
The same reaction was shown to occur in whole active cultures³¹.



Scheme 9

No incorporation of the prenyl group occurred when tryptophan, L-alanyl-L-tryptophan, or L-tryptophan-L-alanine were used as substrates.

It was, therefore, no surprise to many workers when the mono-prenylated product (35) was shown to be a highly efficient precursor of echinulin (26), using the doubly labelled compound (35)³² (Scheme 10).

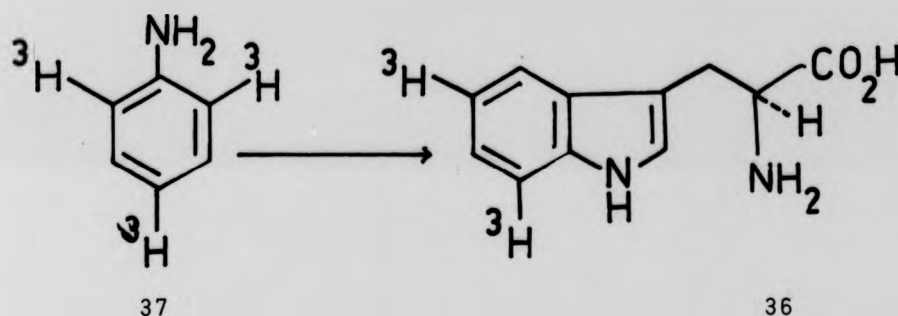


Scheme 10

The isotopic ratios were unchanged in the recrystallised echinulin³².

At the same time, some light was shed on the mechanism of alkylation subsequent to the incorporation of the monoprenylated product (35).

Tritiated tryptophan (36) was synthesised from 2,4,6-tri-tritioaniline (37).



Feeding (36) to A.amstellodomi resulted in inactive echinulin. It would appear that ring prenylation occurs with removal of the tritium from the alkylation site. Tryptophan synthesised from 3,5-ditritio aniline gave active echinulin since alkylation does not occur at these sites³³.

Similar results were demonstrated in feeding experiments with cyclo-L-(U-¹⁴C)-alanyl-L-(5,7-³H)-tryptophan (35) in A.amstellodomi.

Incorporation of this substrate into neoechinulin (27) and neoechinulins A (38), B (39), C (40) and D (41) was demonstrated and the incorporation values are as shown in Figure 1.³⁴

The ¹⁴C/³H ratio was constant in all cases and equal to that of the precursor. Therefore the substrate (35) was

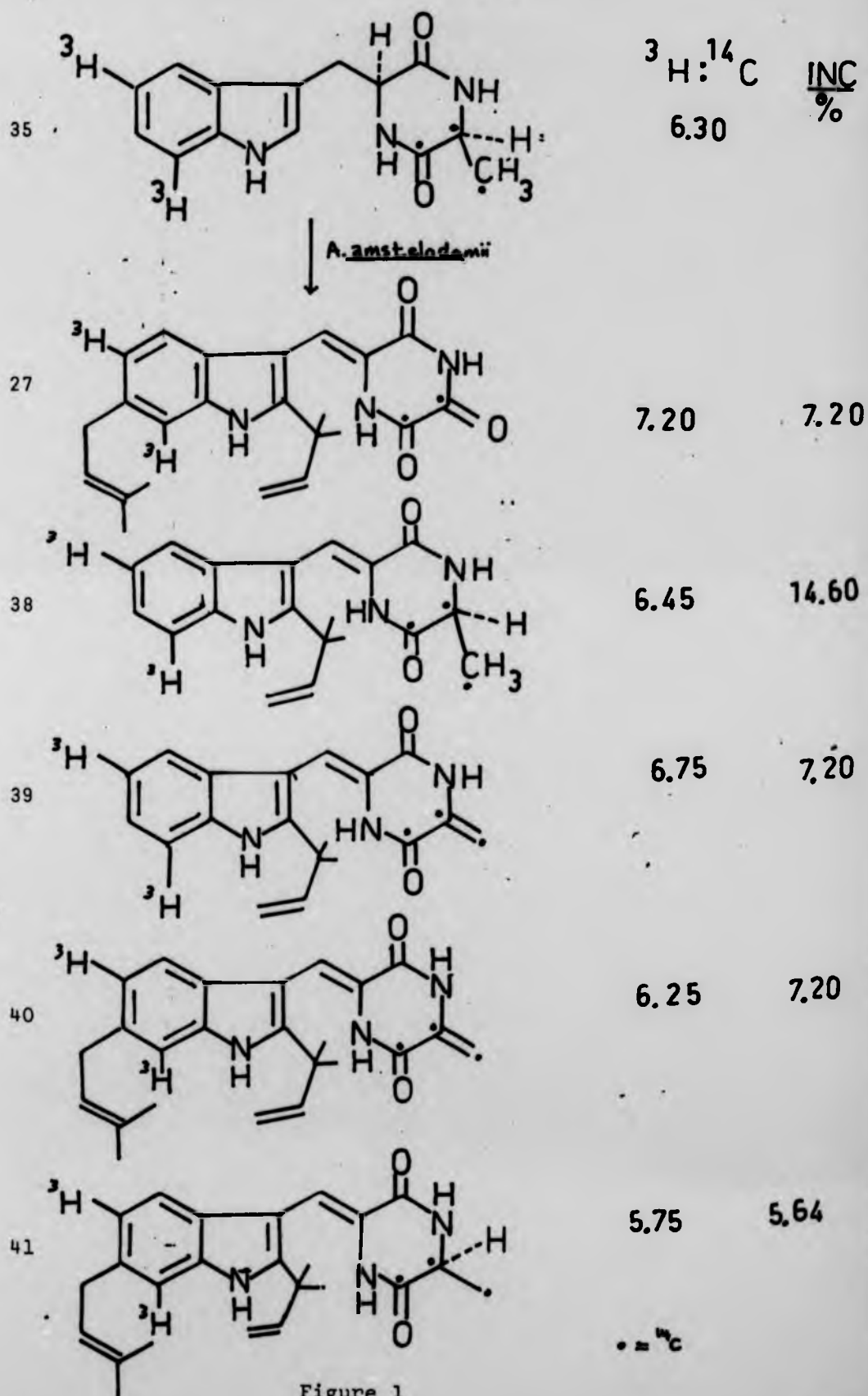
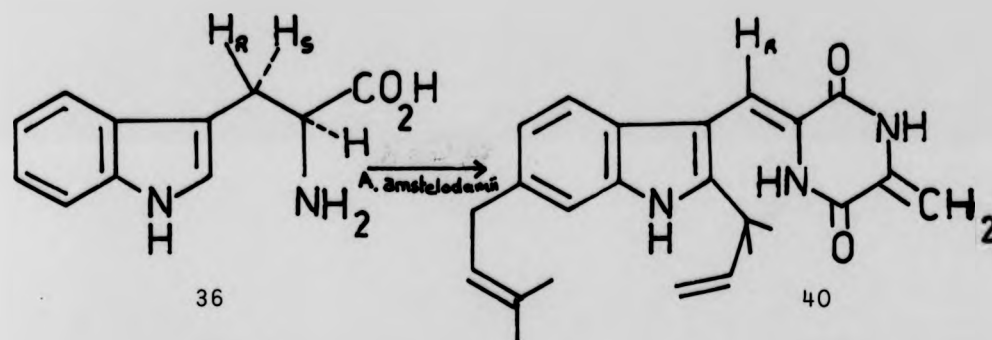


Figure 1

incorporated intact.

More recent work has demonstrated that the structural assignments for neoechinulins A-D (38-41) were, in fact, correct.³⁵

The stereochemical course of the dehydrogenation of the L-tryptophan unit has been shown to involve stereospecific removal of the pro-S hydrogen in the biogenesis of cryptoechinulin A³⁶ (40) (Scheme 11).



Scheme 11

Feeding (3R) ($3'$ - ^3H , $3'$ - ^{14}C) tryptophan gave cryptoechinulin (40) with 98% tritium retention and incorporations of 1-6%.

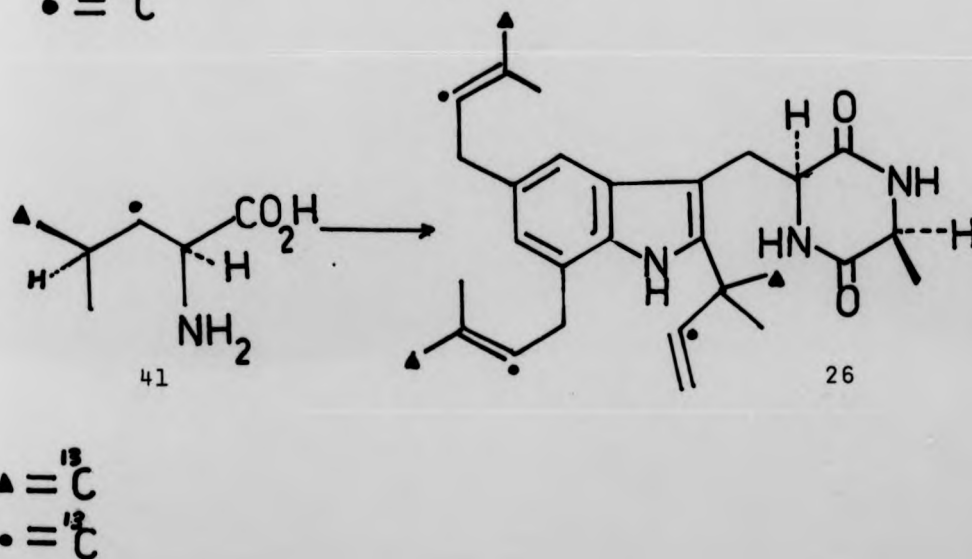
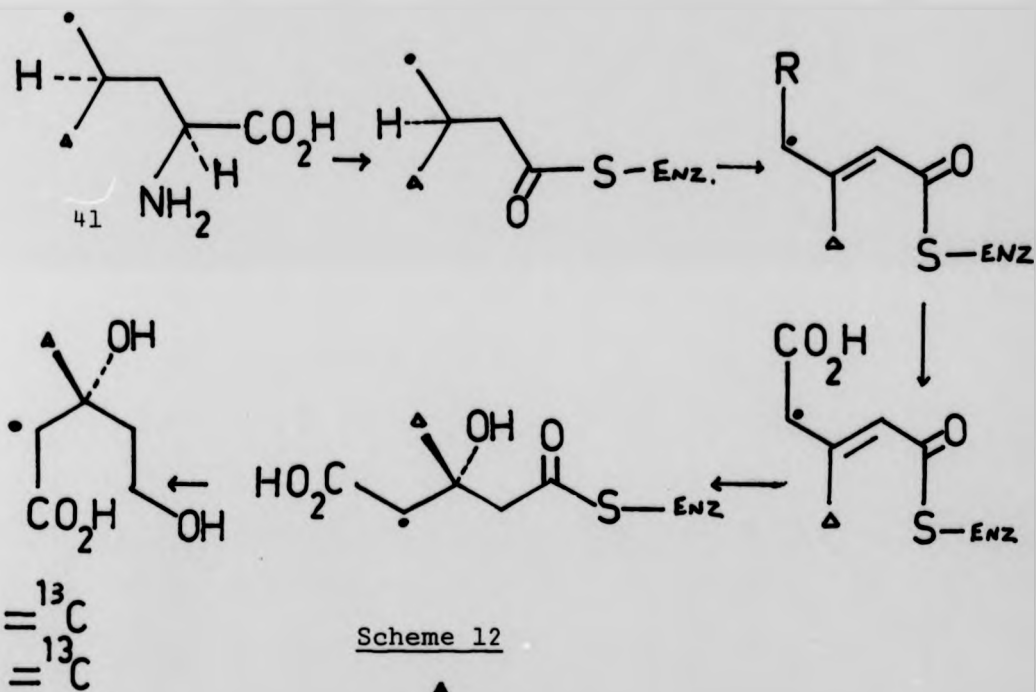
The (3S), ($3'$ - ^3H , $3'$ - ^{14}C) substrate gave cryptoechinulin (40) with only 5% tritium retention.

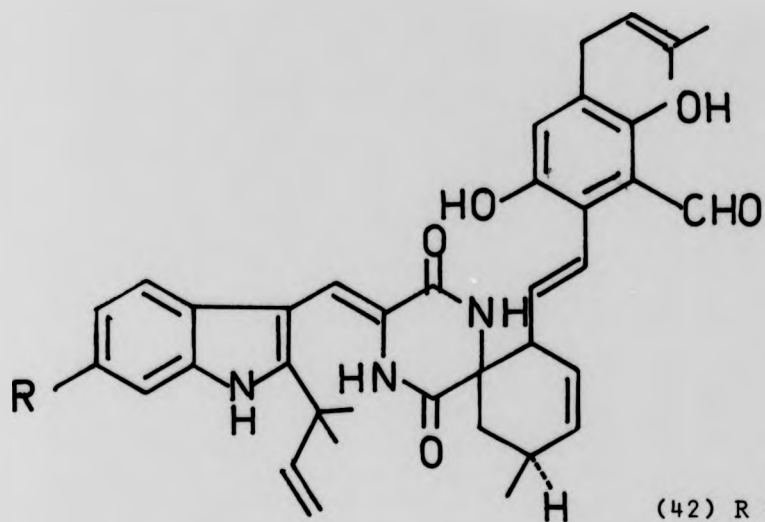
It is known that leucine (41) may act as precursor for mevalonate after carboxylation and subsequent hydration (Scheme 12).

With this proposal (Scheme 12) (4S) (5 - ^{13}C) leucine (41) was fed to cultures of A.amstelodamii and was incorporated

into echinulin (26) giving enhancement of the signals designated³⁷ (▲) (Scheme 13).

The future trend in research in this area will probably be the investigation of the biogenesis of more complex structures such as (42) and (43) from *A.amstelodamii*.³⁸





(42) R = H

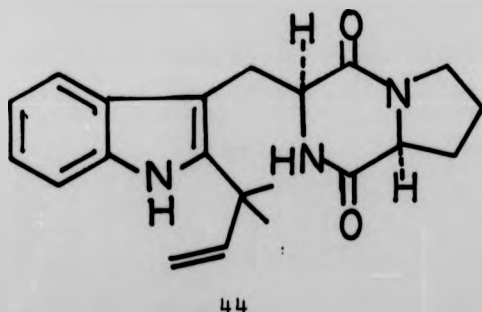
(43) R =

The general theories relating intermediates in the biogenesis of the echinulins with other metabolites will be discussed later.

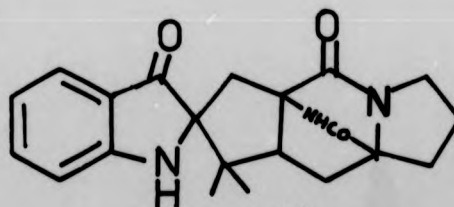
3.0 The Brevianamides and Related Compounds

The brevianamides bear a close structural similarity to the echinulins in that they too contain a reverse isoprene moiety at position 2 of the indole ring. However, proline rather than alanine is utilised in dioxopiperazine ring formation.

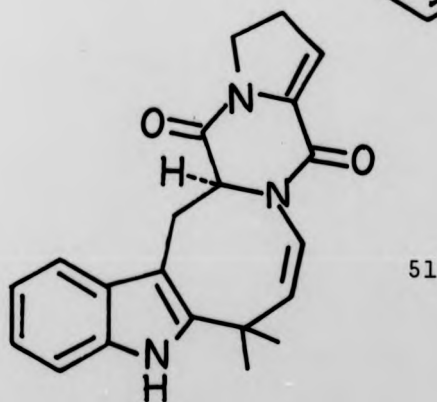
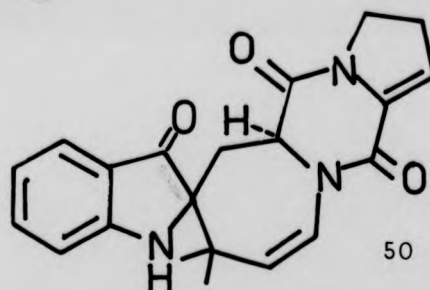
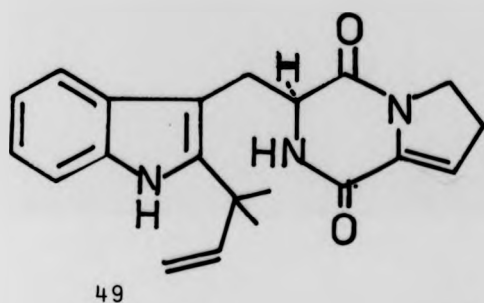
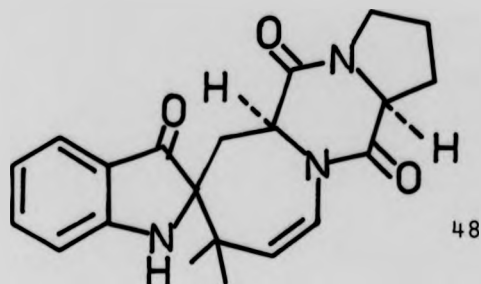
Most of these metabolites occur in species belonging to the genus Aspergillus e.g. (44) and Penicillium e.g. (45).



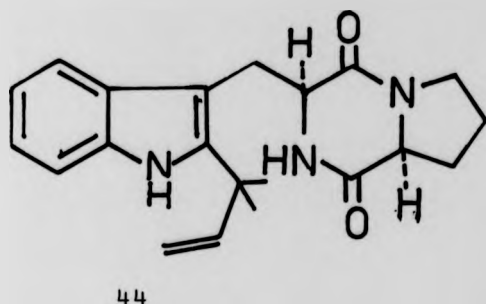
44



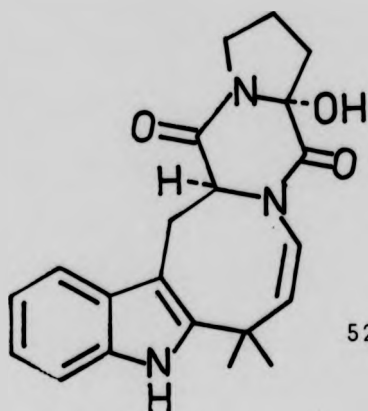
45



Further proof of the co-occurrence of austamides and echinulin derivatives in the same microbial species was apparent when the hydroxylated austamide (52) was found to co-occur with the mono-prenylated structure (44) in *A.ustus* cultures⁴¹.



44

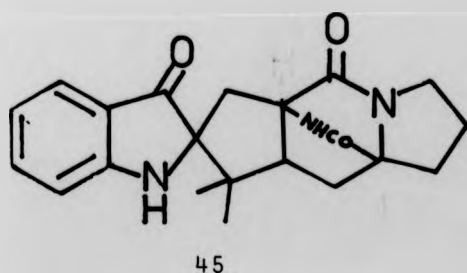


52

3.2 Tracer Studies

Various workers have probed the biogenesis of brevipinamide A (45).^{42,43} Initial studies showed the expected precursors - mevalonate, tryptophan, proline and acetate were incorporated but incorporation values were low.

Later studies by the same authors gave much improved incorporation figures⁴³ (Scheme 14).



45

← L-(5-³H)-Proline (0.4%)
 L-(3-¹⁴C)-Tryptophan (17.6%)
 D,L-(2-¹⁴C)-Mevalonate (0.2%)

Scheme 14

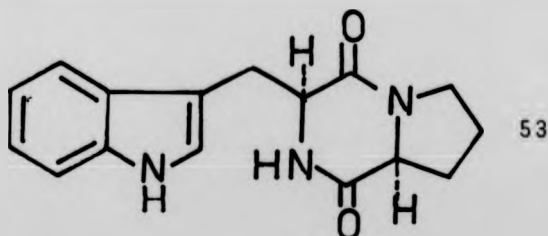
Proline was found to be incorporated into mycelial protein and this explains the low incorporation into brevianamide A.

It is interesting to note that the dioxopiperazine (53) is incorporated intact into (45) (Table 1).

TABLE 1

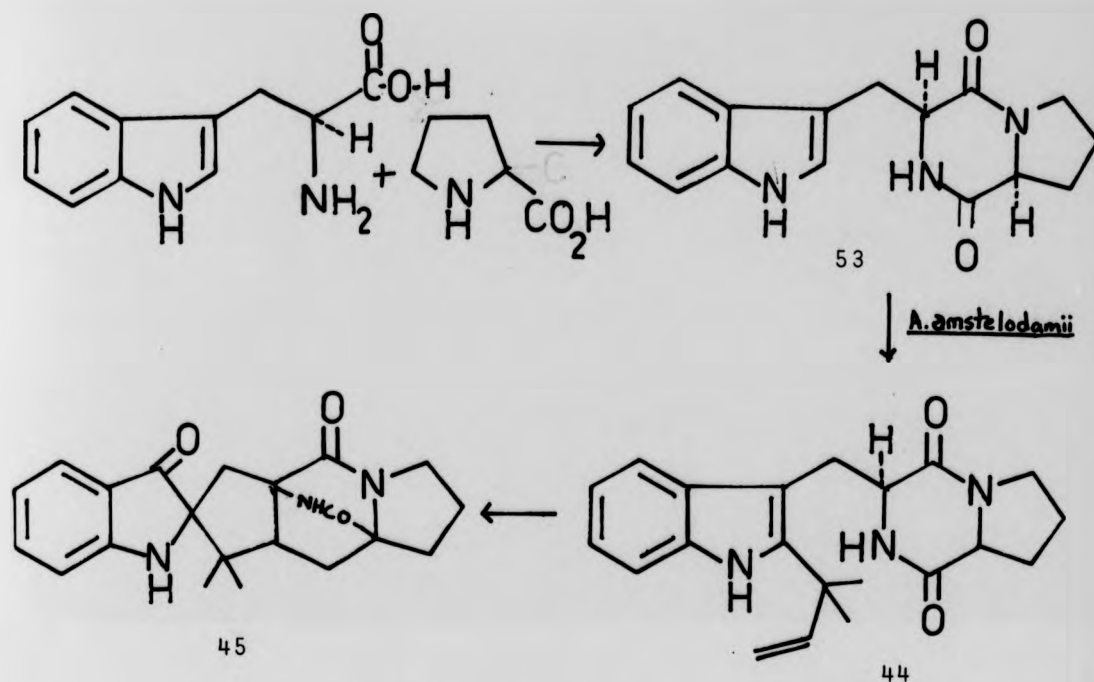
Incorporation of Dioxopiperazine (53) into Brevianamide A (45).

Precursor	Incorporation into (45)	Isotopic Ratio $^{14}\text{C} : ^3\text{H}$
cyclo-L-(3- ^{14}C)-tryptophanyl-L-proline (53)	1.8%	-
cyclo-L-(3- ^{14}C)-tryptophanyl-L-(5- ^3H)-proline	3.2%	3.82 : 1
($^{14}\text{C}/^3\text{H} = 3.67 : 1$)	3.6%	3.74 : 1



Birch and co-workers⁴³ then viewed the biogenesis of the brevianamides A (45) and B (45)-epimers at the spiro centre as depicted in Scheme 15.

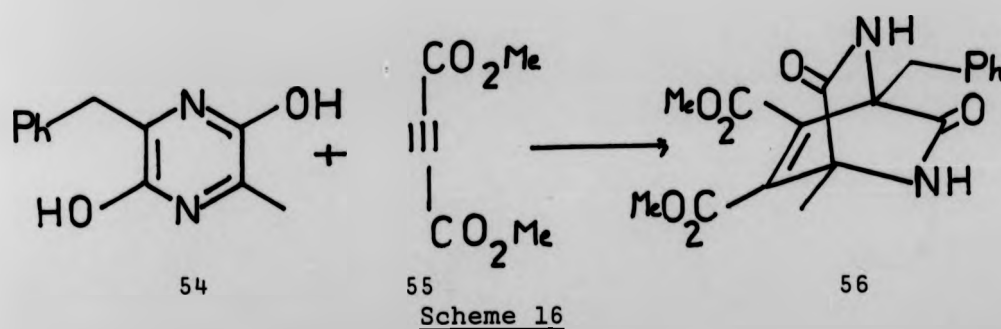
Further evidence for this theory came with the discovery that the dioxopiperazine (53) functions as an excellent isoprene acceptor in cell-free systems from A.amstelodamii³¹ using dimethylallyl pyrophosphate as an isoprene source.



Scheme 15

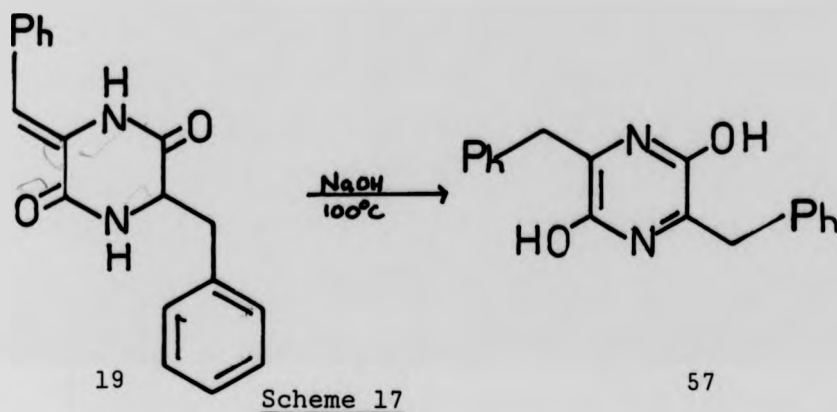
Shortly before this time, brevianamides C (46) and D (47) were dismissed as true metabolites in *P. brevicompactum* since they could not be isolated from cultures grown under low light conditions. Most workers now regard them as photochemical artefacts³⁹.

Further biosynthetic possibilities were raised when it was shown that the cycloaddition reaction of the hydroxypyrazine (54) and dimethylacetylene dicarboxylate (55) gave the adduct⁴⁴ (56) (Scheme 16).



Scheme 16

Some previous work had shown that arylidene dioxo-piperazines could be converted to the corresponding hydroxypyrazines by base treatment (Scheme 17).⁴⁵

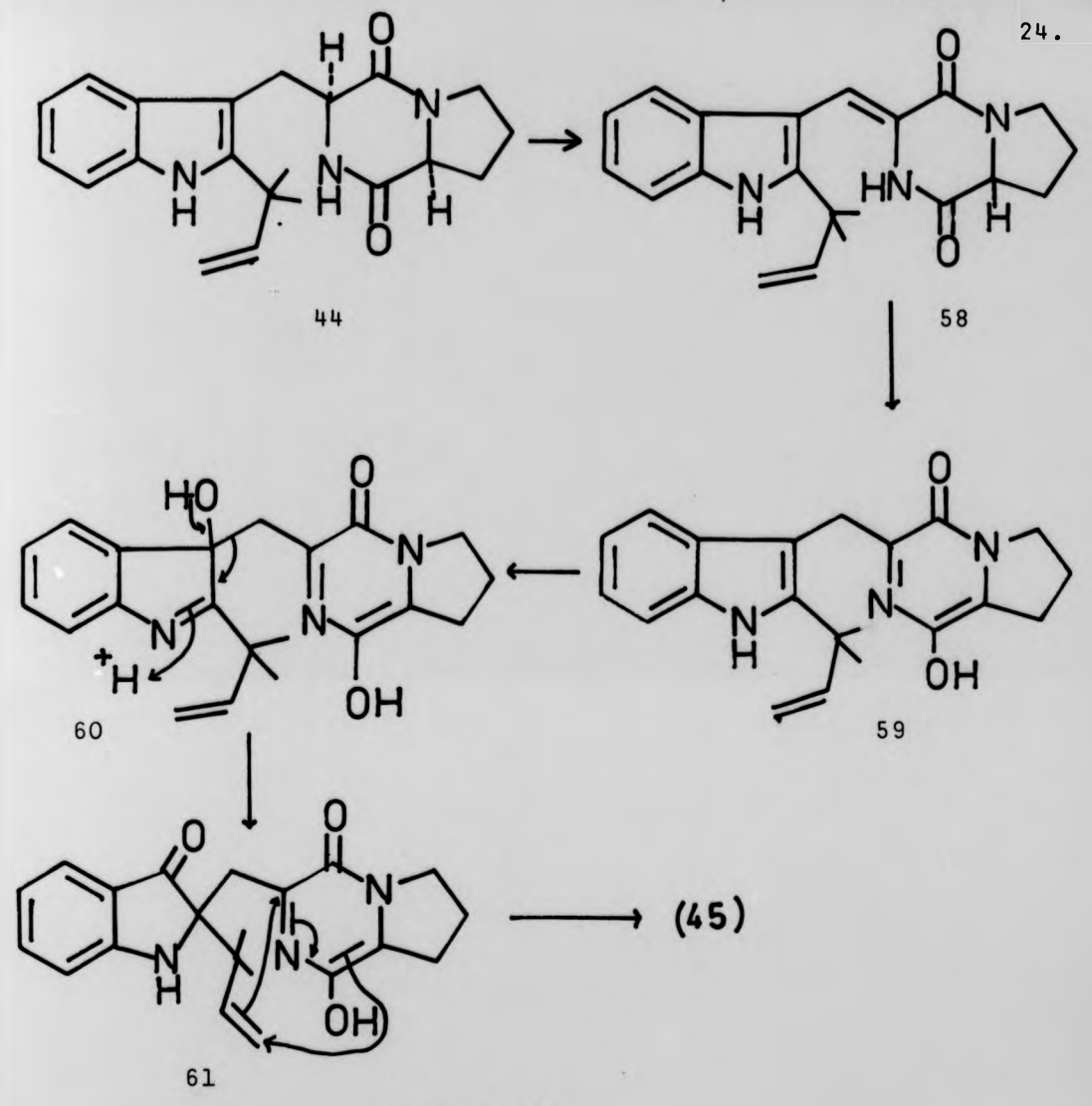


These workers⁴⁶ suggested that these facts could be applied to the biogenesis of brevianamides A and B (Scheme 18).

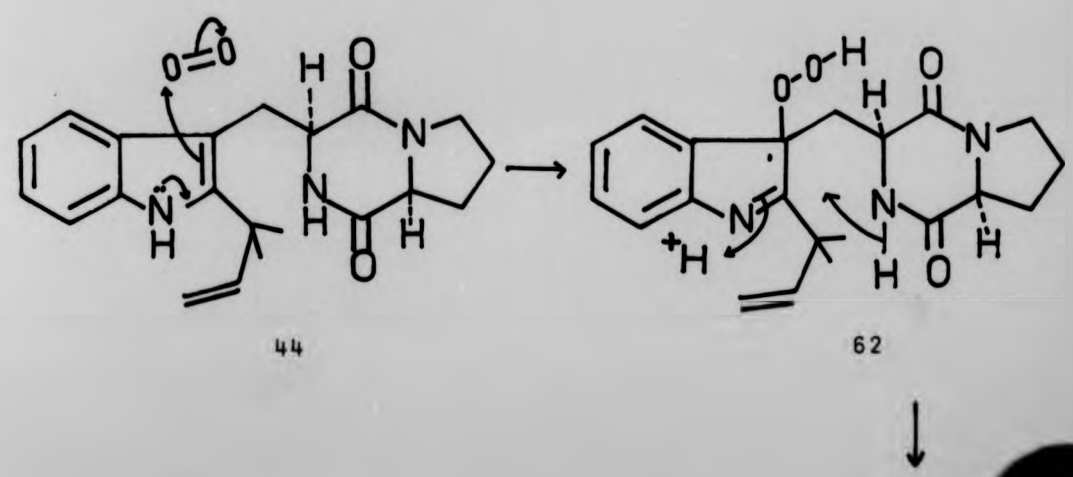
If analogous reactions in the echinulin series hold here, conversion of (44) to (58) should be accompanied by loss of the pro-S-proton to give the arylidene system (58). Reactions analogous to the base catalysed isomerisation of (58) to (59) are known to be facile processes⁴⁷. Hydroxylation of the indole-3-position (60) and rearrangement should generate the indoxyl intermediate (61). Cycloaddition will then generate the known brevianamide (45).

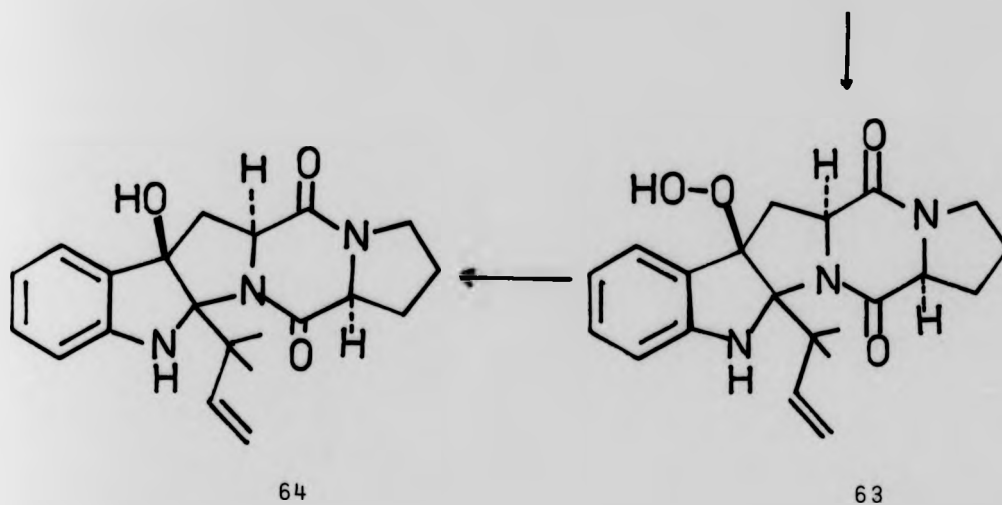
These workers suggested that (44) is a key intermediate in the biogenesis of brevianamide E (64)⁴⁶ (Scheme 19).

Again, it was postulated that autoxidation of the indole would give the 3-hydroperoxyindolenine which should close to a derivative of brevianamide E.



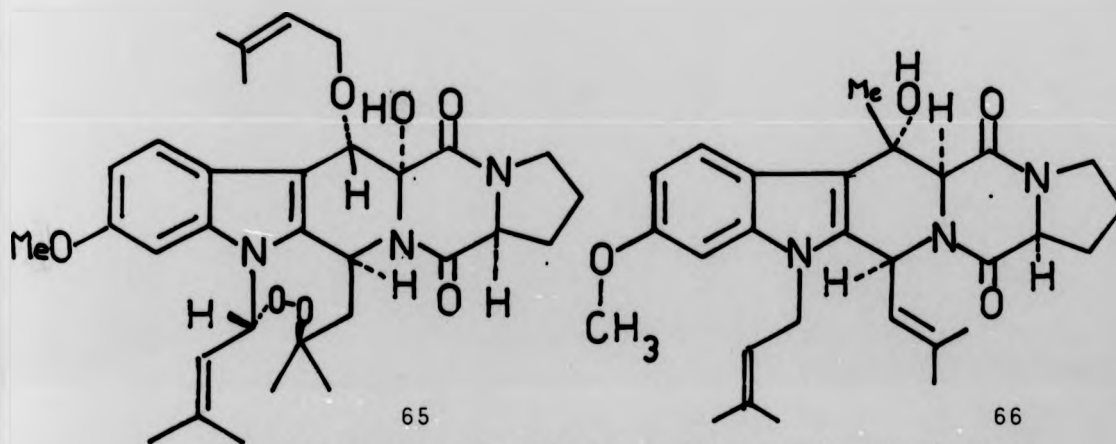
Scheme 18





Scheme 19

Studies on the more complex brevianamides e.g. Fumitremorgin A (65)⁴⁸ and B (66)⁴⁹ showed that tryptophan, proline and mevalonate were all efficient precursors.⁵⁰



Both were isolated from cultures of *A.fumigatus*.

The related structure verrucologen (67) has been isolated from *P.verruculosum*.⁵¹

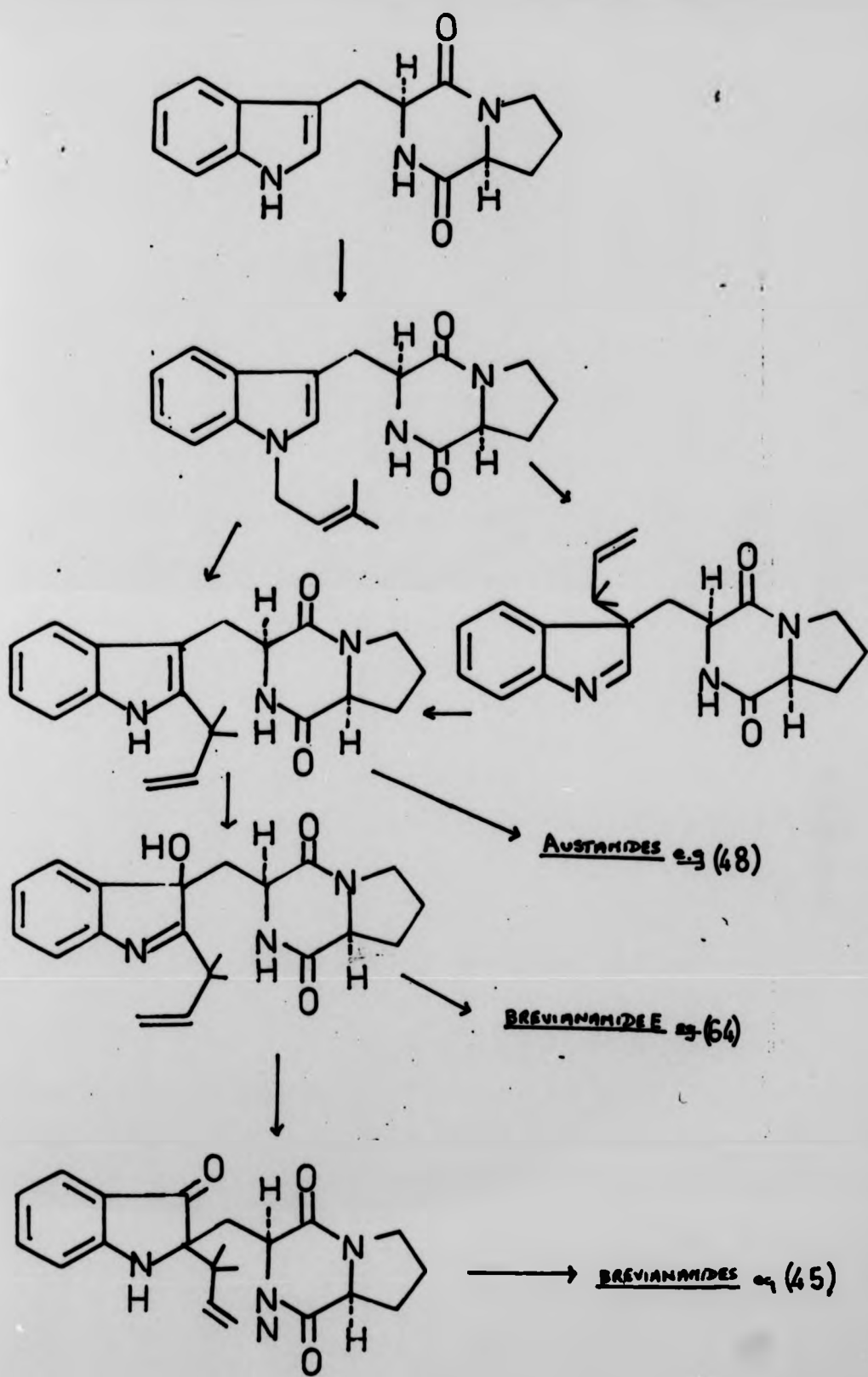
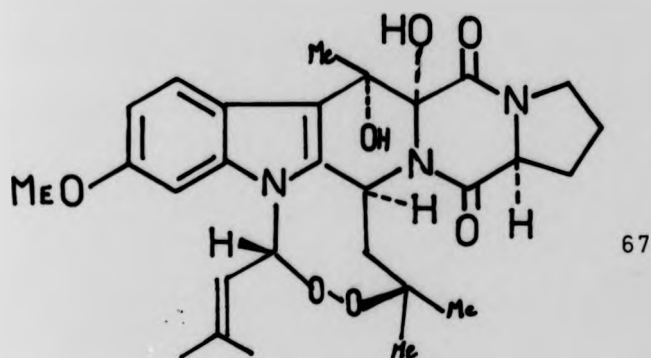


Figure 2



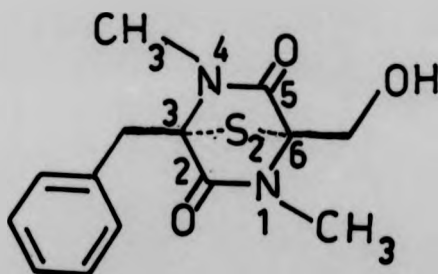
Finally Grundon, in a recent review of hemiterpene biogenesis, has proposed the biogenetic scheme (Figure 2) to account for the common biogenesis of the austamides and brevianamides⁵².

4.0 The Simple Polythiodioxopiperazines

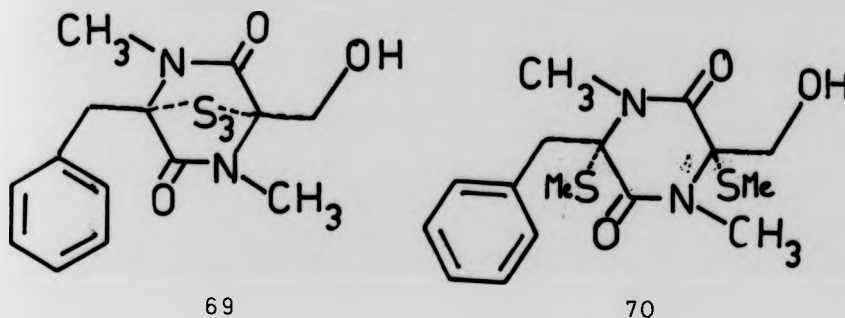
The simple sulphur bridged dioxopiperazines represent a rather small and limited group of fungal metabolites.

Structurally there is little variation within this group but small variations in structure may lead to dramatic increases or total loss of biological activity.

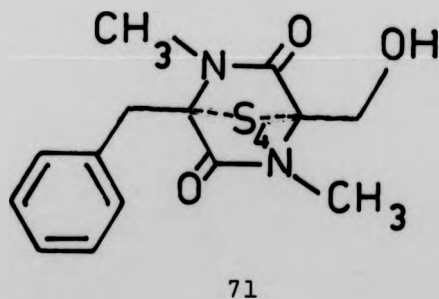
The first member of this group to be isolated and characterised was from *Hylodendrin* sp^{53,54} and is probably derived via serine and phenylalanine.



Other workers found hyalodendrin (68), the trithio analogue (69) and the opened analogue (70) in cultures of Helminthosporium victoriae⁵⁵ and another unidentified species⁵⁶.



Similarly, hyalodendrin (68), the open form (70) and the unusual tetrathio analogue (71) were isolated as gram negative antibiotics from Penicillium turbatum⁵⁷. Antiviral activity was also noted in (68) and (71).



4.1 Anomalies in Biosynthesis

One important feature to consider here is the species variation in biogenesis⁵⁸.

Hyalodendrin (68) produced by Hylodendrin sp. has the (3S,6S) configuration while that isolated from P.turbatum has the (3R,6R) stereochemistry yet both possess biological activity.

Similarly the other metabolites (71) and (70) from P.turbatum share the (3R,6R) stereochemistry and the associated activity.

It is interesting to reflect that the opened analogue (70) from H.victoriae⁵⁵ is inactive, yet possesses the (3S,6S) stereochemistry⁵⁸.

The bridged structure seems to be the essential for antiviral activity and probably antibiotic activity also.

4.2 Tracer Studies

Since no labelling studies have been reported, the general view of biogenesis is that the amino acids combine to form dioxopiperazines prior to the introduction of sulphur.

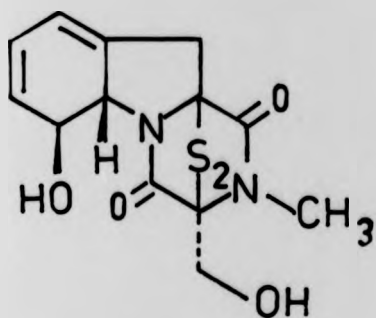
The mechanism of sulphur addition and the form in which it reacts, remain speculative.

5.0 The Complex Polythiodioxopiperazines

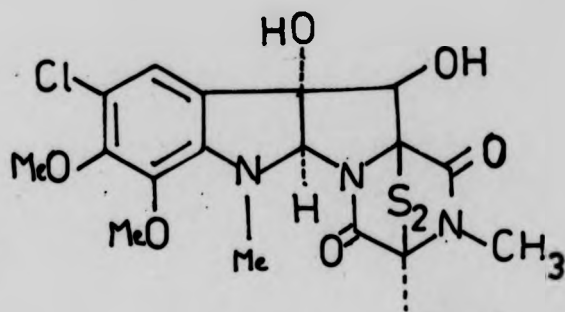
The complex sulphur bridged dioxopiperazines represent a highly divergent class of natural products.

The novel structure of gliotoxin^{59,60} (72) and its high antiviral activity led other workers to look for similar compounds, and the related compound sporidesmin A⁶¹ (73) was subsequently discovered in Pithomyces chartarum.

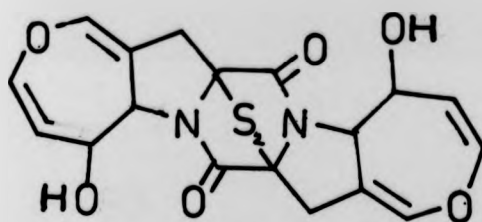
Ring expanded analogues (74), open sulphur analogues (75) and dihydroaromatic analogues (76) have been isolated, mainly from Arachniotus aureus⁶².



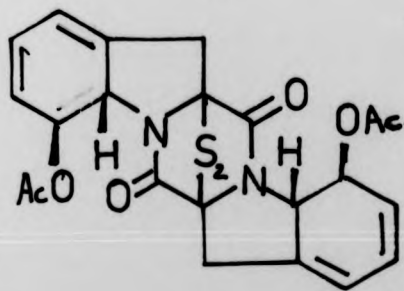
72



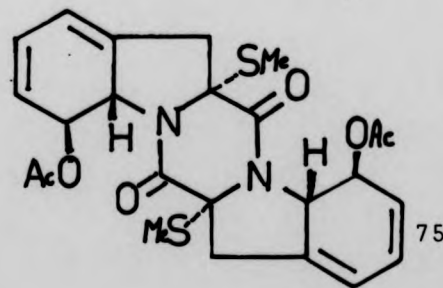
73



74



76

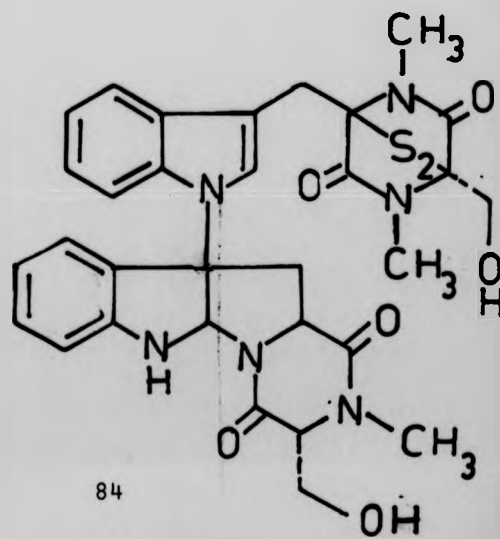
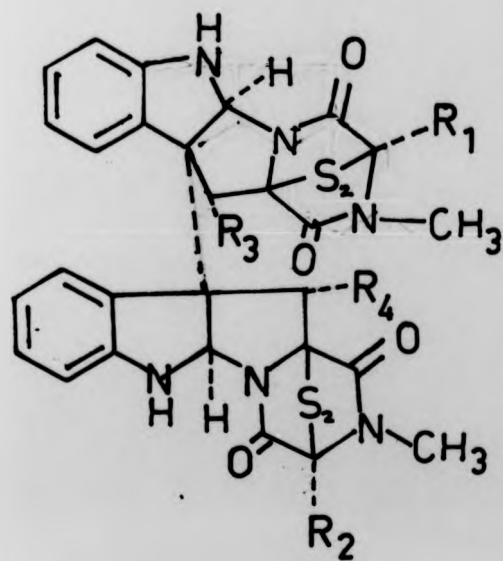
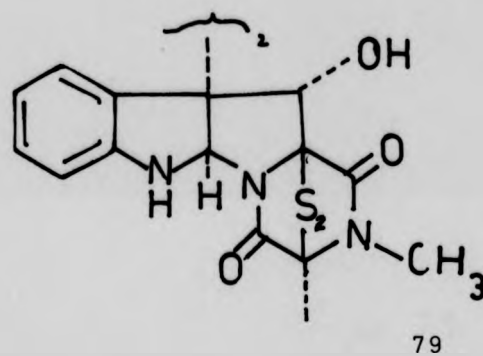
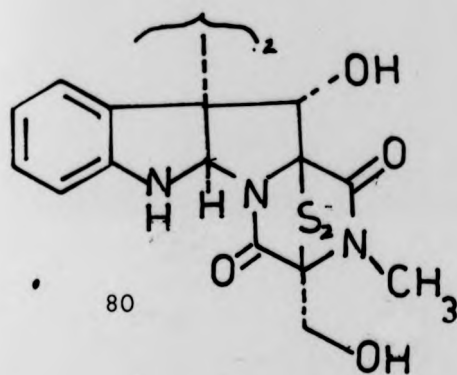
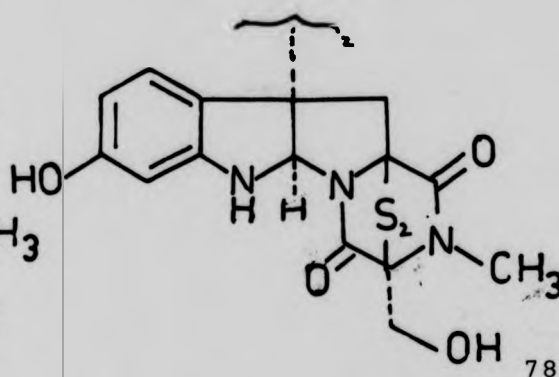
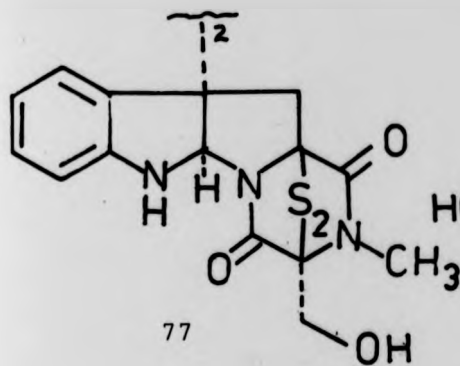


75

Further investigation showed that several elaborate dimeric structures were also possible e.g. chaetocin⁶³ (77), 11,11-dihydroxychaetocin⁶⁴ (75) and verticillins A (79) and B (80).⁶⁵

A similar group of related antiviral compounds, the melinacidins⁶⁶ (81-83), have also been isolated.

Other related compounds include metabolites reported from *Chaetomium* sp (84)⁶⁷.



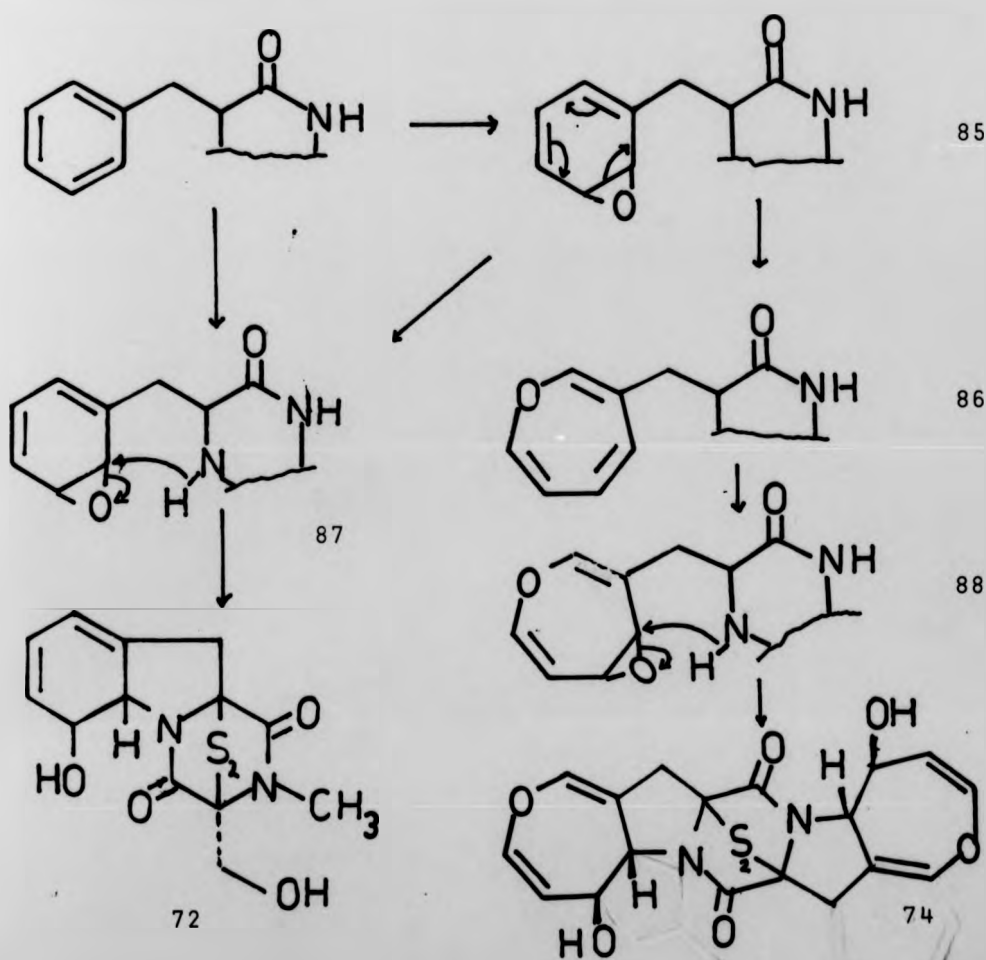
R_1	R_2	R_3	R_4	
$-CH_3$	$-CH_2OH$	$-H$	$-OH$	81
$-CH_2OH$	"	$-OH$	$-H$	82
		$-OH$	$-OH$	83

Considering the diversity of the structural types involved in this series of natural products, the biosynthetic results would suggest a simple and fairly common biogenetic rationale for their occurrence in nature.

5.1 Formation of the Dihydroaromatic Ring System

One of the most unusual features of gliotoxin (72) and the apo-aranotin type of metabolites is the existence of a hydroxylated dihydroaromatic ring system.

The initial theory for the biosynthesis of this functionality is as shown in Scheme 20.



Scheme 20

In this postulate⁶², the authors envisage formation of the 2,3-arene oxide (85) to form a common intermediate between the aranotin and gliotoxin metabolites.

Ring expansion to the oxepine (86), followed by subsequent 2,3-epoxide formation (88) and closure should generate the aranotin skeleton (74).

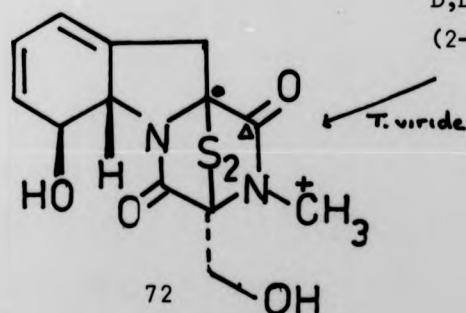
Conversely, direct closure of the adjacent amino functionality onto the 2,3-arene oxide generates gliotoxin (72).

5.2 Tracer Studies

(a) Gliotoxin

Workers⁶⁸ who first studied the biogenesis of gliotoxin in Trichoderma viride found that phenylalanine labelled at positions 1- and 2-, methionine and acetate were all efficient precursors of gliotoxin (Scheme 21).

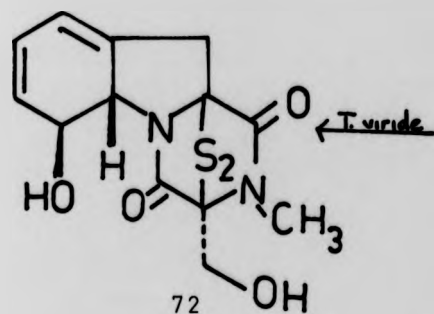
	% incorporation
D,L- (1- ¹⁴ C)-phenylalanine ▲	8.4
D,L- (2- ¹⁴ C)-phenylalanine ●	12.4
D,L- (1- ¹⁴ C)-methionine +	0.27
(2- ¹⁴ C) acetate	0.02



Scheme 21

The same authors found that incorporation of D,L-tyrosine was not detectable.

Stable isotope studies were also conducted using T.viride^{69,70} (Scheme 22).



	% isotope enrichment
^{15}N -glycine	8.9
^{15}N -phenylalanine	4.4
(2- ^{13}C)-glycine	2.5
(1- ^{13}C)-glycine	2.4
(1- ^{13}C)-formate	2.5

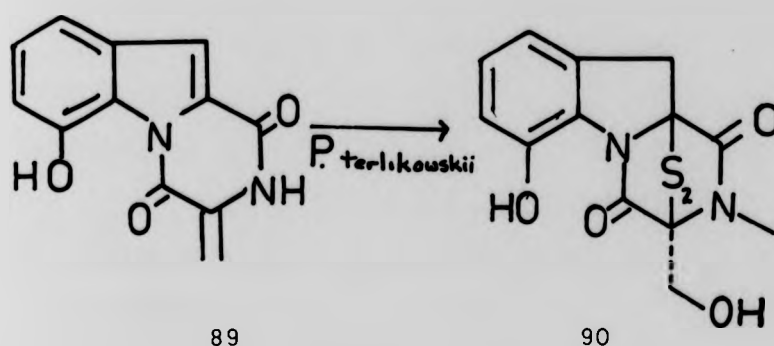
Scheme 22

Thus the efficiency of phenylalanine, glycine and formate were established. Using a mixture of $^{14}\text{C}/^{13}\text{C}$ and $^{15}\text{N}/^{14}\text{C}$ compounds the same incorporations were also established⁷⁰.

However refinement of the techniques during further studies showed the ^{15}N -phenylalanine was specifically incorporated, but ^{15}N -glycine was scrambled over both nitrogen atoms thus indicating substantial transamination activity. In the same study m-tyrosine⁷⁰ was claimed as precursor but this was subsequently disproved by Bu'Lock⁷¹.

Bu'Lock⁷¹ showed that (3,5- ^3H) o-tyrosine, (2,4,6- ^3H) m-tyrosine and (4,5,6- ^3H) 2,3-dihydroxy phenylalanine were at best poorly incorporated. [0.04 + 0.02%]. This was later to lead Bu'Lock to investigate the arene oxide theory in more detail.

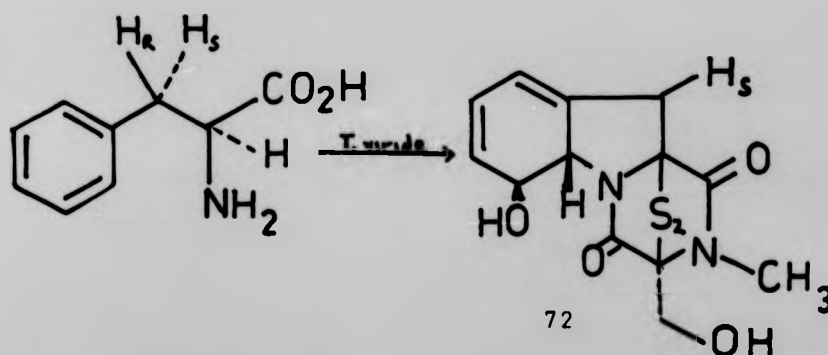
At this time, efforts were enhanced by the isolation of the plausible intermediate (89) from P.terlikowskii - a gliotoxin producing strain⁷² (Scheme 22).



Scheme 22

This intermediate (89) was shown to be incorporated into dehydrogliotoxin (90).

Further evidence for the involvement of this intermediate in gliotoxin biogenesis was shown by the fact that the pro-R-proton⁷³ is lost from (3-²H) phenylalanine during incorporation into gliotoxin (72). The pro-S-proton and configuration at the carbon atom in the 3-position are retained, i.e. removal and reprotonation must occur from the same face of the molecule by an enzyme mediated process⁷⁴ (Scheme 23).

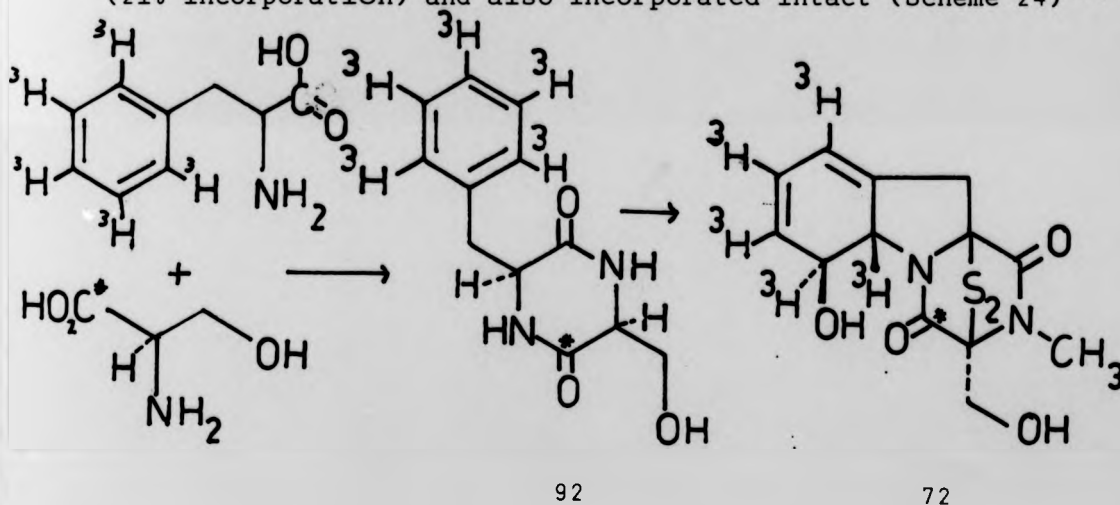


Scheme 23

In the most recent studies, feeding study cyclo-L-phenylalanyl-L-serine (92) has produced conflicting results.

Slater and co-workers⁷⁵ found low incorporation figures (0.3%) for incorporation into gliotoxin (72) in P.terlikowskii.

Bu'Lock, however, showed that the same substrate was incorporated into gliotoxin in T.viride with high efficiency (21% incorporation) and also incorporated intact (Scheme 24)^{76,77}.



Scheme 24

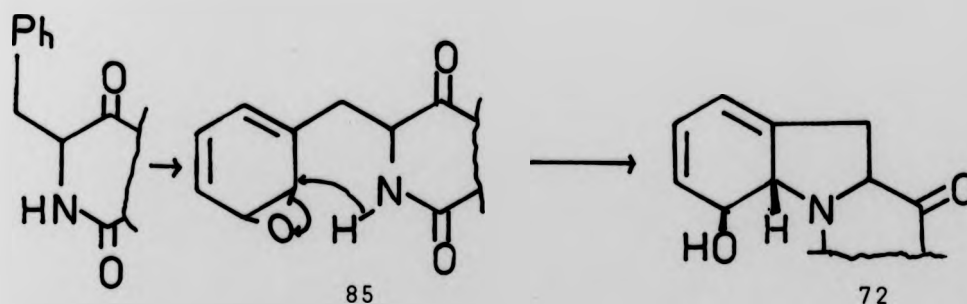
Evidence for intact incorporation came from the $^{14}\text{C}/^3\text{H}$ ratio in the precursor (1:13) and the isolated gliotoxin (1:12.5).

Bu'Lock stated that Slater's results were poor because of the high levels of exogenous precursor applied. It is possible that this alone might be sufficient to perturb normal metabolic processes by inhibition of the equilibrium of the exogenous sample with other in vivo biosynthetic intermediates.

This may be caused by the massive quantities fed actually blocking endogenous synthesis of these substrates⁷⁶.

Since the postulated formation of the dehydroaromatic ring existed, some workers set out to investigate this facet of gliotoxin biogenesis.

Elegant work by Kirby, using specifically tritiated phenylalanines has provided more evidence for the arene oxide theory and largely supersedes the now dated work of Winstead and Suhadolnik⁷⁸. The general route postulated in Kirby's work⁷⁹ is shown in Scheme 25.

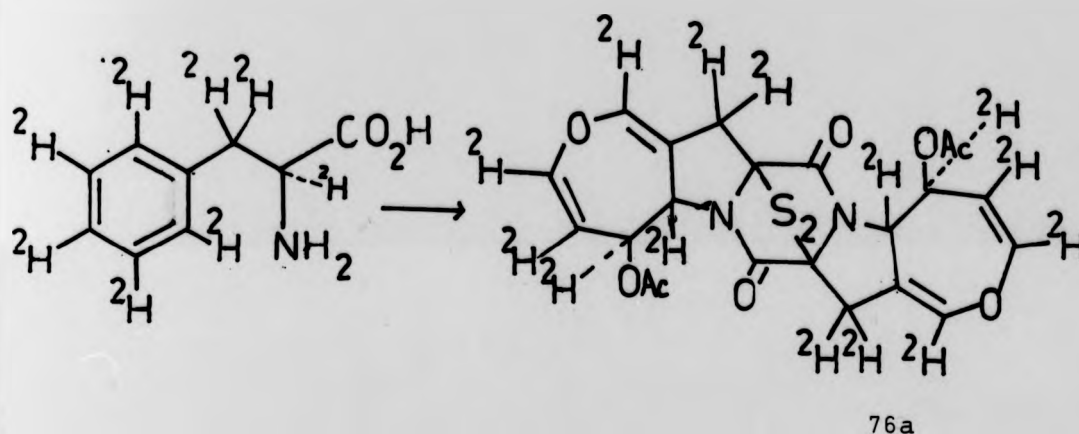


Scheme 25

Feeding D,L-(2,4,6-³H)-m-tyrosine gave an incorporation of 0.001% into gliotoxin (72) and D,L-(2,4,6-³H)-m-tyrosine and D,L-(1-¹⁴C)-phenylalanine gave an incorporation of 4.9% for ¹⁴C but tritium incorporation was barely detectable⁷⁹. Thus the role of m-tyrosine is in dispute.

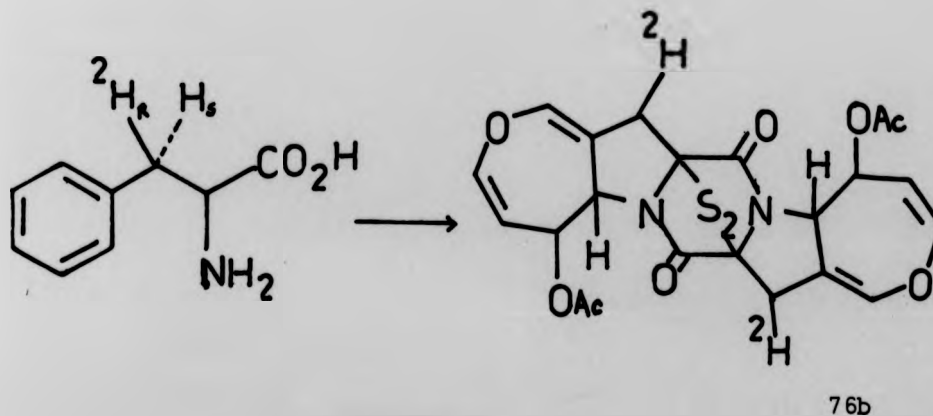
Similarly, experiments showed that L-phenylalanine

labelled with deuterium in the aromatic ring and methylene group) was incorporated into *Aranotius* sp to give acetylaranotin (76a) which contained tetradeca- and heptadecadeutero-fragments. (Scheme 26).



Scheme 26

Other workers showed that D,L-(R,3'-²H,1-¹⁴C) phenylalanine was incorporated acetyl aranotin (76b) with substantial loss of the pro-R-deuteron⁷⁴ (Scheme 27).



Scheme 27

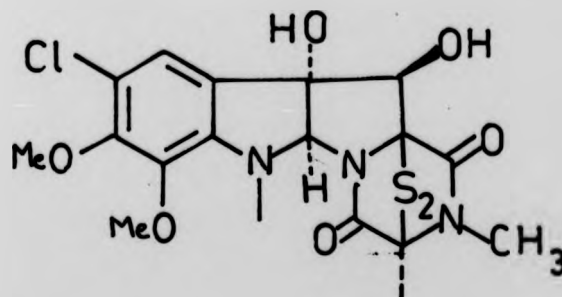
Thus the divergent nature of the biogenesis of this group of natural products, depicted in Scheme 20, now seems to accommodate the feeding results thus far documented.

(b) Sporidesmins

The metabolites of Pithomyces chartarum are also fungal toxins responsible for facial eczema of sheep.

These metabolites may be regarded as tryptophan analogues of the gliotoxins, since the phenylalanine residue has been replaced by tryptophan.

The major metabolite is Sporidesmin A⁴¹ (73).

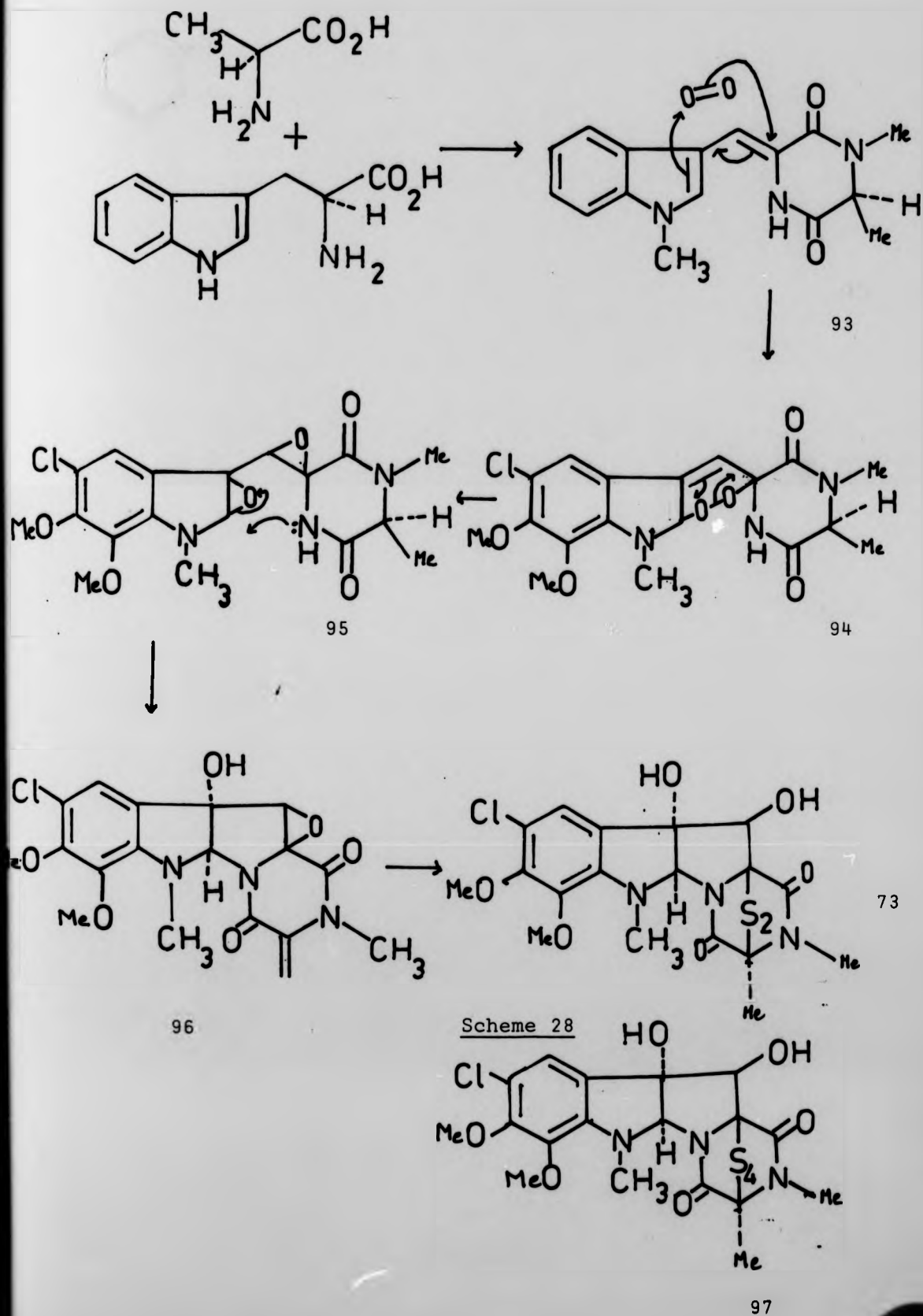


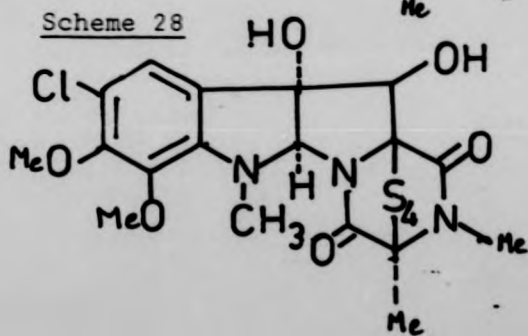
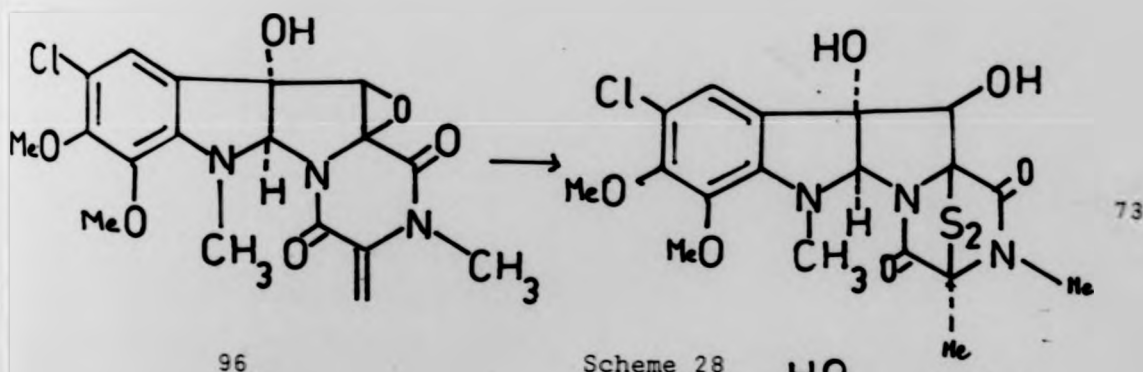
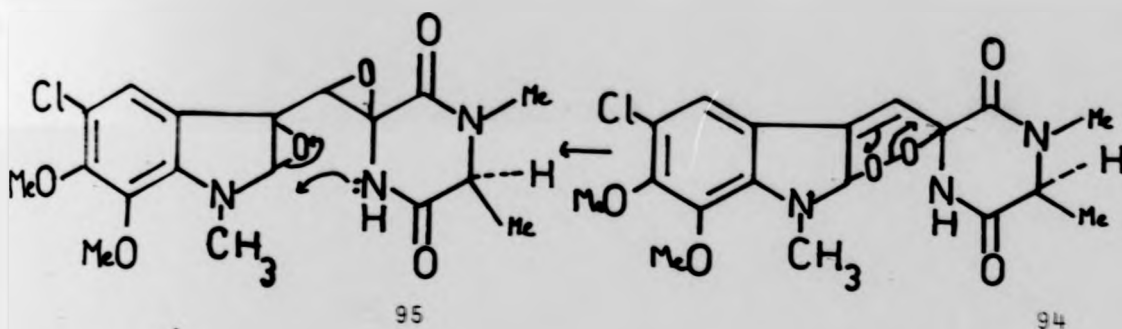
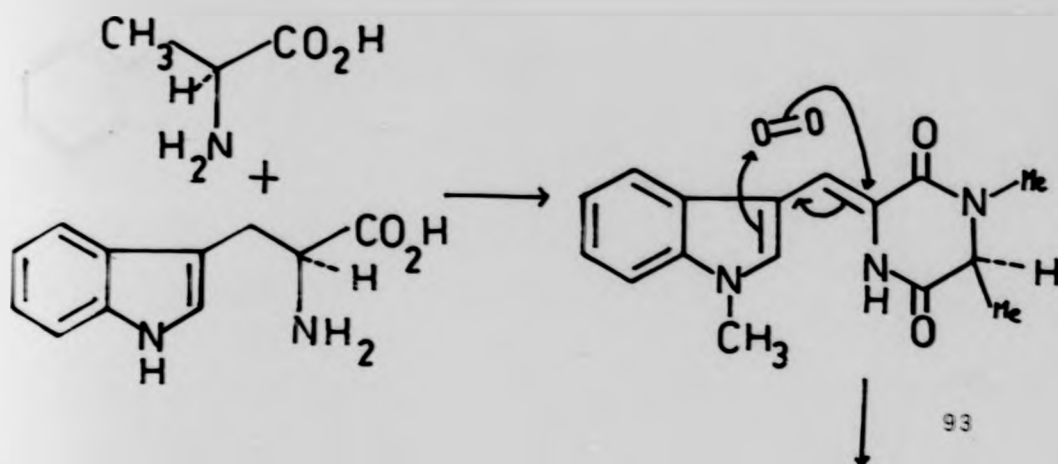
73

The biogenesis of the sporidesmins has been postulated, by Sammes¹, to occur as depicted in Scheme 28.

Precedent for the singlet oxygen addition⁸⁰ and structural evidence for intermediates, is available but the postulate, in general, must be regarded as speculative until feeding experiments produce some factual data.

The tetrathio-derivative (97) has also been isolated from cultures of P. chartarum⁸¹.



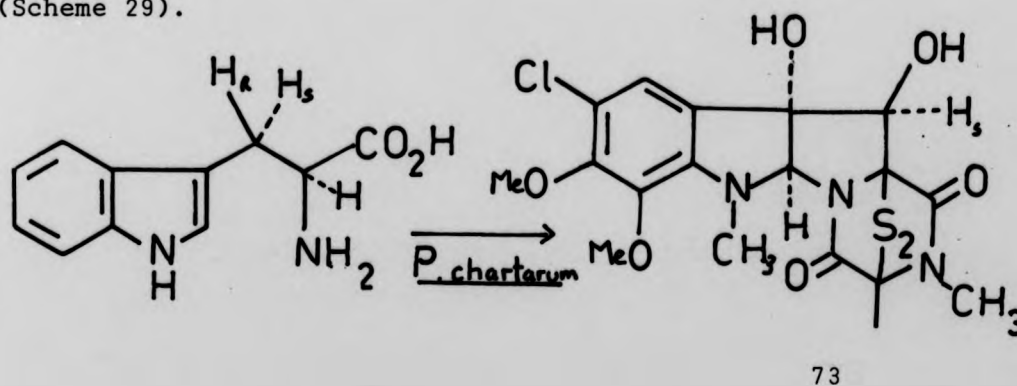


No evidence for the stereospecific removal of the 3-proton from tryptophan to give the dehydroamino-acid exists, and the only analogy occurs in the echinulin series.

The stereochemical course of hydroxylation at the C-3 position has been established⁸² e.g. in the formation of (73).

In cultures of *P.chartarum*, the pro-R proton is removed and hydroxylation occurs from the same face of the molecule thus preserving the existing stereochemistry at this site.

The incorporation levels for ¹⁴C were normally 0.4-0.5% and tritium retention of the pro-S tritium was 90-95% (Scheme 29).

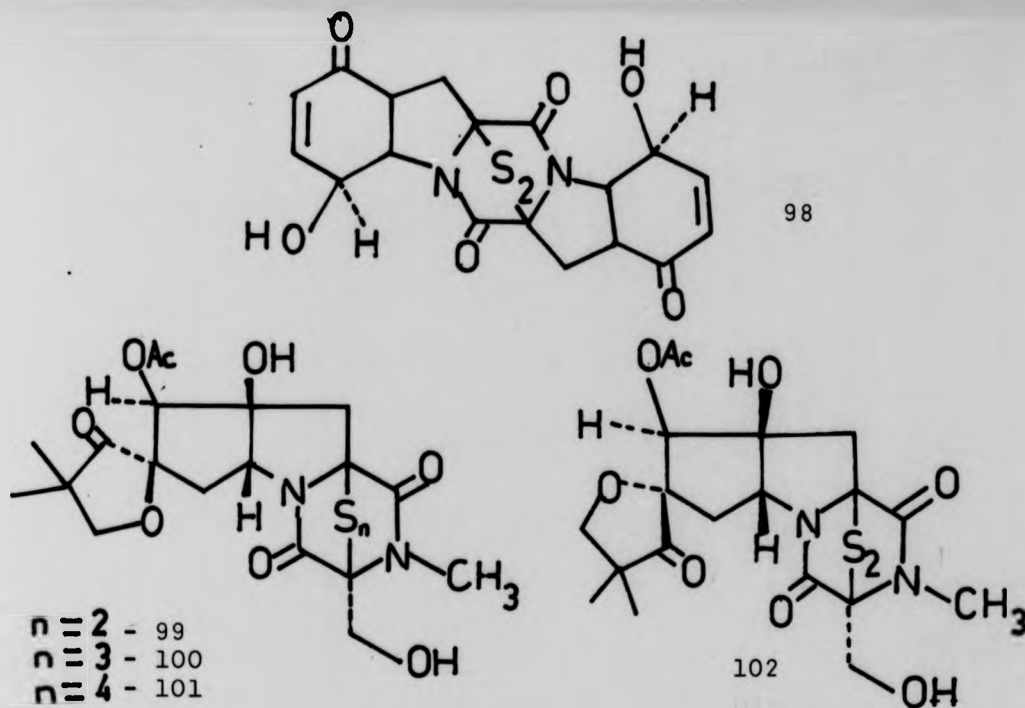


Scheme 29

More complex metabolites such as metabolite (98)⁸³ and sirodesmins A (99),⁸¹ B (100),⁸¹ C (101)⁸¹ and G (102)⁸⁴ probably arise by similar biosynthetic routes.

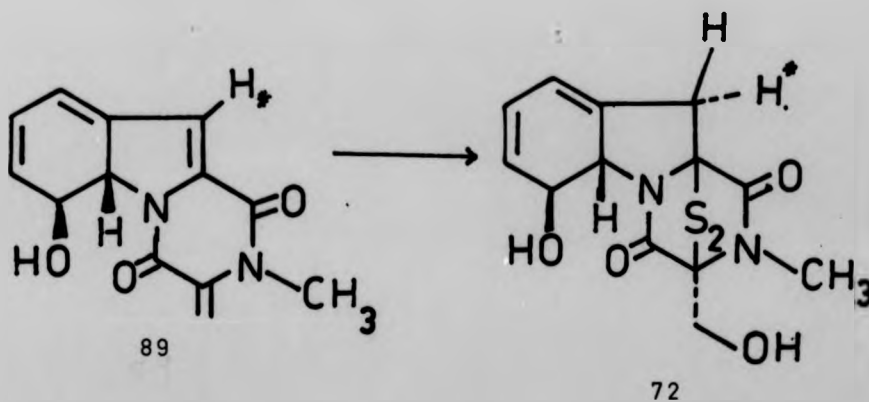
A biosynthetic route for the origin of sirodesmins has been suggested but is, as yet, unproven⁸⁴.

The method for the insertion of sulphur is not known although several in vitro methods for the formation of polythio-dioxopiperazines exist^{85,86,87}.



Addition of sulphur to dehydropeptides such as (89) have been ruled out since the benzylic protons are not exchanged during biogenesis⁸⁸ and the overall stereochemistry at C-3 of phenylalanine is retained.

This view does not account for insertion of RSH by an enzyme mediated process which could give rise to gliotoxin (72) where the C-3 stereochemistry in the phenylalanine derivative is still preserved (Scheme 30).



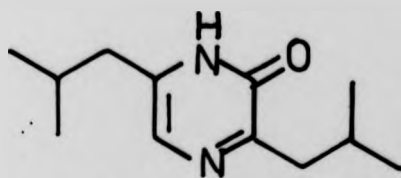
Scheme 30

6.0 The Aspergillic Acids

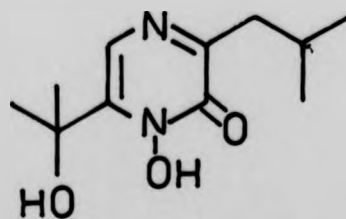
The diverse group of cyclic hydroxamic acids has been the subject of a past review.²

Structurally, the cyclic hydroxamic acids are not as diverse as some of the naturally occurring dioxopiperazines, and their biological activity is also highly variable.

This is typified by the fact that flavacol⁸⁹ (103) has no activity yet muta-aspergillic acid has pronounced effect⁹⁰ (104) in terms of biological activity.



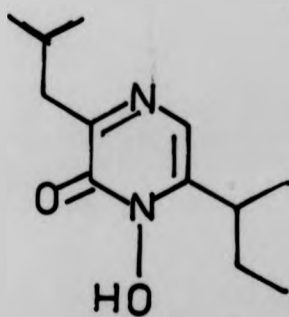
103



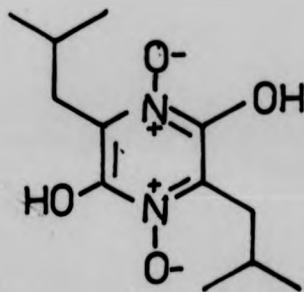
104

It should be noted that flavacol, although included in this group, is not a hydroxamic acid but a pyrazinone.

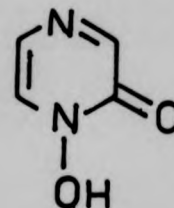
Other members of this class are aspergillic acid⁹¹ (105), pulcheriminic acid⁹² (106) and enimycin (107).⁹³



105



106



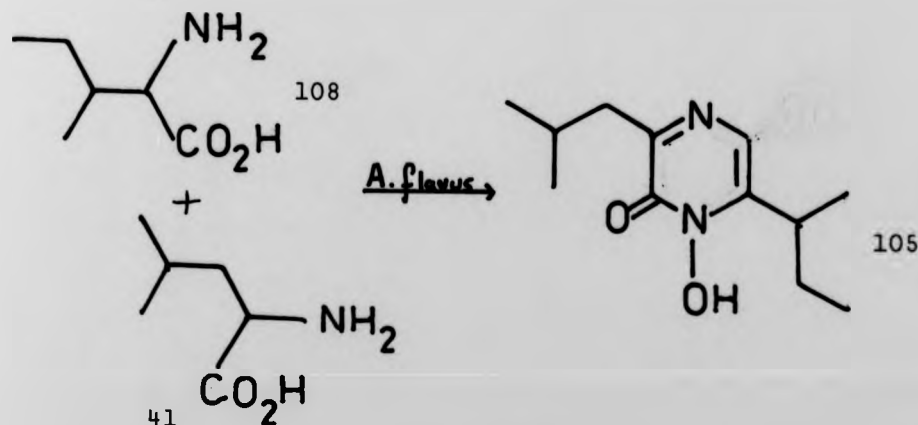
107

6.1 Tracer Studies

A considerable number of biosynthetic postulates and studies have been proposed in this area.

The metabolites consist of combinations of amino acids - mainly leucine, isoleucine and valine, and it is here that biosynthetic studies started.

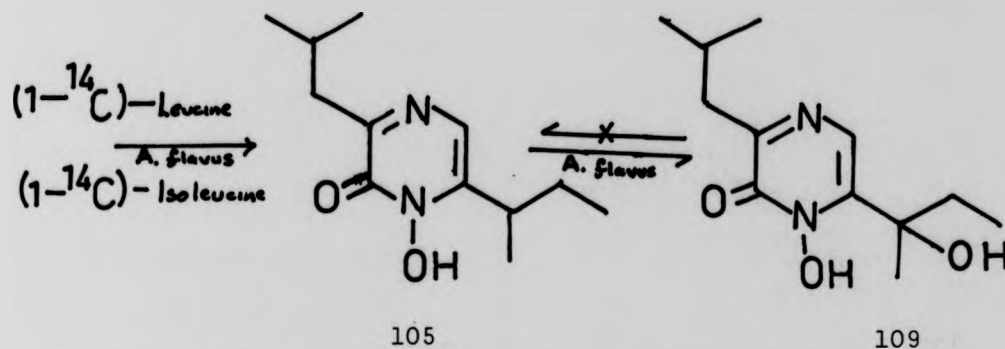
In the case of aspergillic acid (105), the amino acids leucine (41) and isoleucine (108) were shown to be efficient precursors, being incorporated into both aspergillic acid (105) and mycelial protein in A.flavus⁹⁴ (Scheme 31).



Scheme 31

The L-amino acid was consistently found to give better incorporation values in cultures of A.flavus.

A similar study showed that leucine and isoleucine were specifically incorporated into the appropriate parts of aspergillic acid (105). The aspergillic acid, isolated and labelled with ¹⁴C, was shown to be an efficient precursor of hydroxyaspergillic acid⁹⁵ (109) (Scheme 32).



Scheme 32

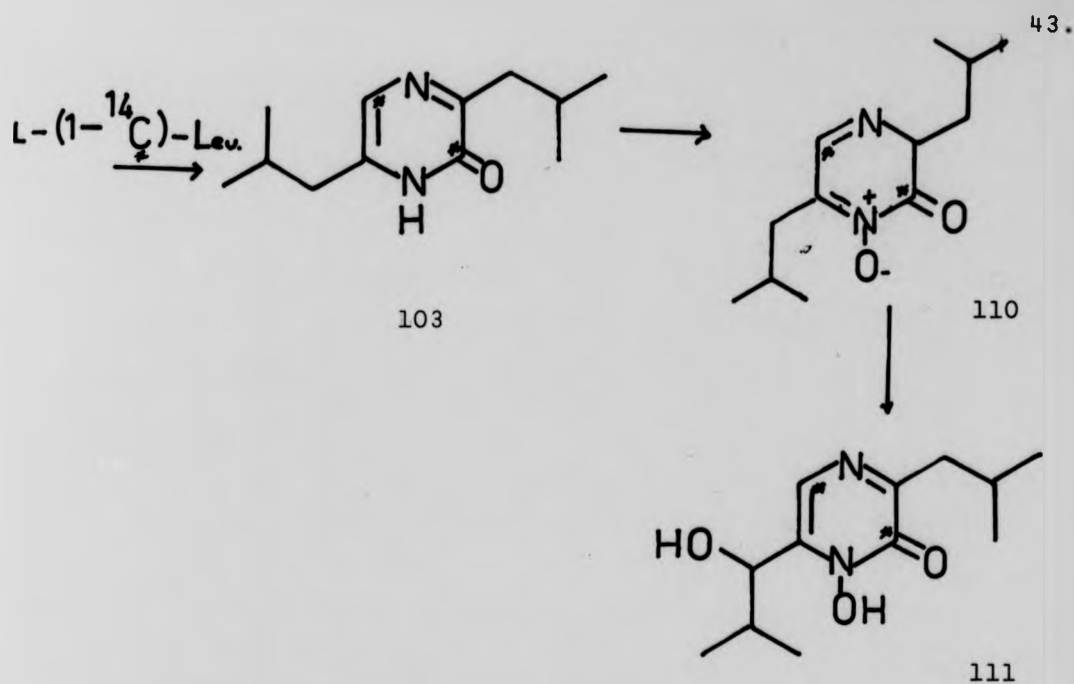
This was supported by time course studies i.e. early in fermentation, the concentration of aspergillic acid (105) was far in excess of hydroxyaspergillic acid (109). The converse was true later in the fermentation. In a similar fashion, feeding hydroxyaspergillic acid (109) did not lead to incorporation into aspergillic acid (105).

In the neoaspergillic acid series, it has been shown that L-leucine is incorporated into flavacol (103) which was shown to be a precursor of both neoaspergillic acid (110) and neohydroxyaspergillic acid (111)⁹⁶ (Scheme 33).

Feeding labelled neohydroxyaspergillic acid (111) did not lead to incorporation in either neoaspergillic acid (110) or flavacol⁹⁶ (103).

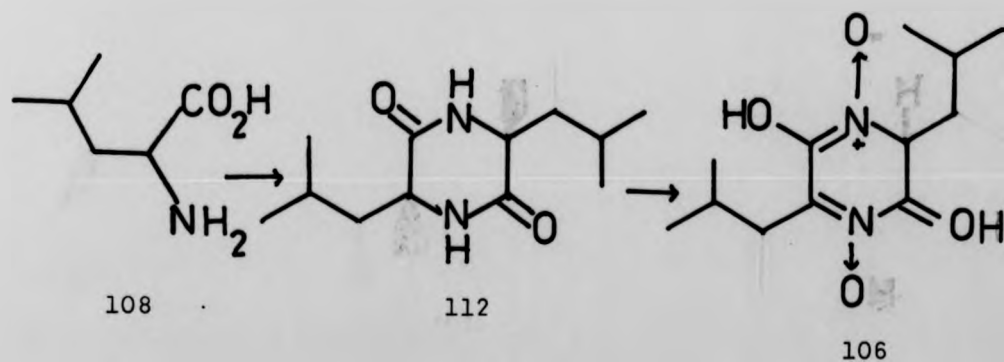
L-(1-¹⁴C)-leucine has also been shown to be incorporated into pulcheriminic acid (106)⁹⁷.

Trapping experiments showed this occurred by way of



Scheme 33

cyclo-L-leucyl-L-leucine (112) and that the cyclic dipeptide was also incorporated intact into pulcheriminic acid⁹⁷ (106) (Scheme 34).



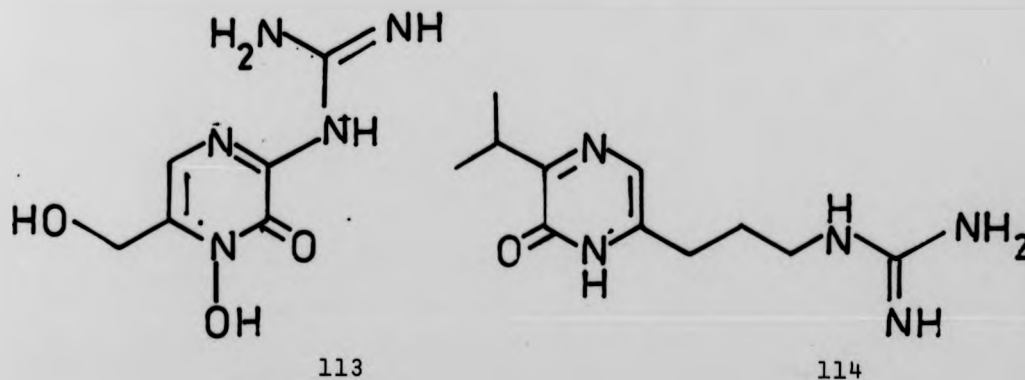
Scheme 34

It has also been shown that pulcheriminic acid (106) may arise by direct aerial oxidation of cyclo-L-leucyl-L-leucine⁹⁹ (112).

Similar studies have not been so successful e.g. cyclo-L-leucyl-L-leucine was only a poor precursor for neoaspergillic acid (110) and was in fact hydrolysed and incorporated into mycelial protein at a rate far in excess of actual incorporation into the cyclic hydroxamic acids (103), (110) and (111)⁹⁶.

In general dioxopiperazines are, at best, only poor precursors for the aspergillic acids and the relationship between the two is by no means certain.

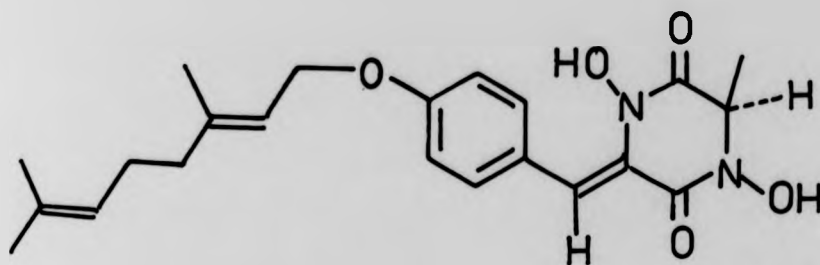
More recently, derivatives, such as stizolamine (113)⁹⁹ and argualin (114),¹⁰⁰ have been isolated but their biogenesis has not, as yet, been investigated, although both are probably derived from arginine and some other amino acid.



7.0 Miscellaneous Metabolites

7.1 Mycelianamide

The metabolite, mycelianamide (115), was first isolated from Penicillium griseofulvum (Dierckx)¹⁰¹ and its structure was determined by Birch and co-workers¹⁰².

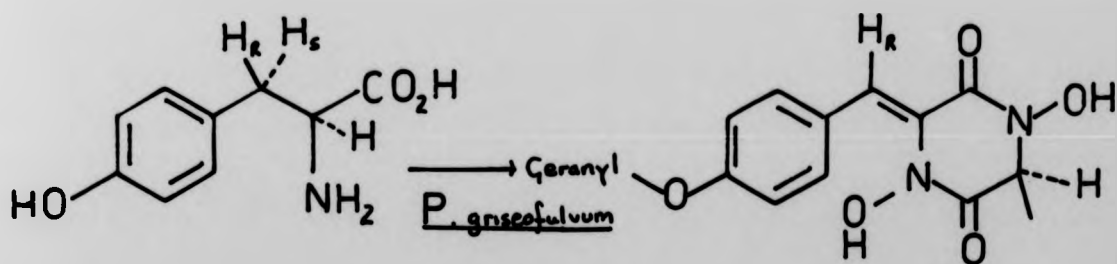


115

It is formally derived from mevalonate, alanine and tyrosine and this has been proven by feeding studies¹⁰².

The stereochemical nature of the proton removal to generate the arylidene derivative has been investigated.

Formation of the α,β -dehydroamino acid unit in mycelianamide (115) proceeds with high retention of the pro-R proton and virtually complete loss of the pro-S methylene hydrogen atom of the precursor¹⁰³ (Scheme 35).



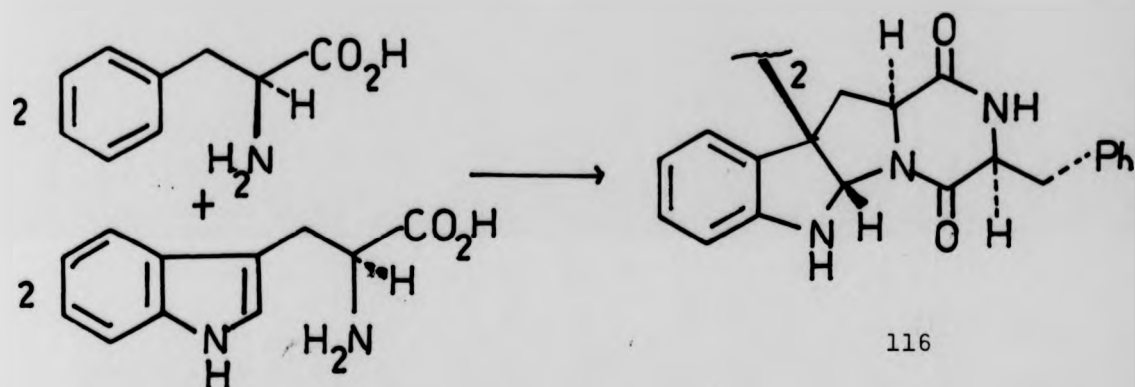
115

Scheme 35

Doubly labelled tyrosine ($3\text{-}^3\text{H}, 2\text{-}^{14}\text{C}$) was incorporated with moderate efficiency (^{14}C incorporation was 3%, retention of pro-R tritium 77%).

7.2 Ditryptophenaline¹⁰⁴ (116)

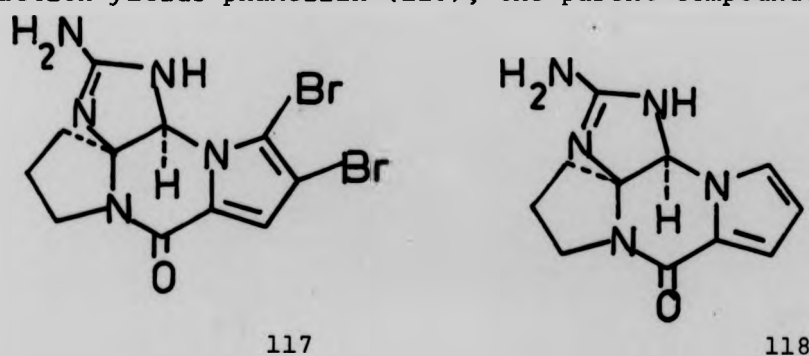
This was a new metabolite of Aspergillus flavus and is unique for two reasons. Unlike any other A.flavus metabolite, it contains no sulphur and secondly it is formally derived from two aromatic amino acids and exists as a dimeric structure (Scheme 36).



Scheme 36

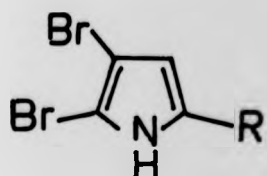
7.3 Phakellins

Dibromophakellin (117) is a novel natural product from the marine sponge Phakellia flabellata¹⁰⁵. Catalytic reduction yields phakellin (118), the parent compound.

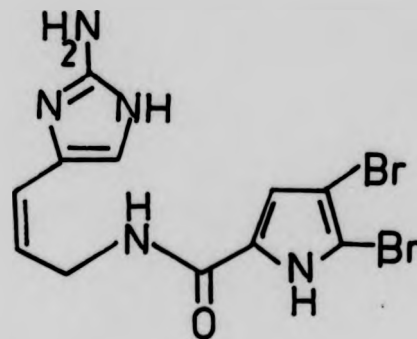


It may be derived by way of cyclo-L-prolyl-L-proline. The related bromopyrroles (119-122)^{106,107} are suggested to be

biogenetically related to phakellin (118).



- 119 - $R = \text{CO}_2\text{H}$
 120 - $R = \text{CN}$
 121 - $R = \text{CONH}_2$

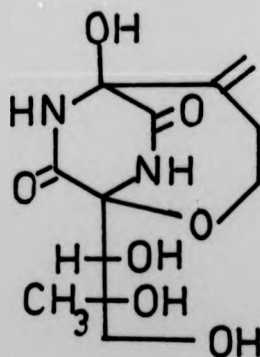


122

7.4 Bicyclomycin

This metabolite (123) has promoted much biosynthetic speculation in these and other laboratories.

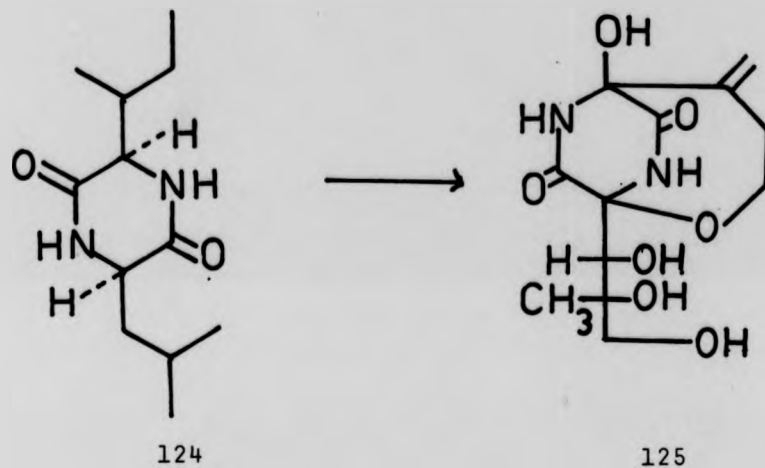
Bicyclomycin (123)¹⁰⁸ is a metabolite of several Streptomyces strains and is an excellent gram negative antibiotic¹⁰⁹



123

Present ideas concerning the biogenesis of this metabolite (123) centre around the metabolite bearing a good

relationship to cyclo-isoleucyl-leucine (124). The molecule is, of course, a highly functionalised derivative of (124) and work is continuing in these laboratories to probe the biogenesis of this antibiotic (Scheme 37).



Scheme 37

The work on this problem is dealt with fully in the following section of this thesis.

REFERENCES

1. P. G. Sammes, Fortsh.der Org.Chem., 1975, 32, 51
2. J. B. Bapat, D. C. Black and R. F. Brown, Adv.Heterocyclic Chem., 1969, 10, 199
3. M. Bodansky, G. F. Singler and A. Bodansky, J.Amer.Chem.Soc., 1973, 95, 2352
4. K. Kubo, T. Arai and K. Takahashi, Experientia, 1977, 33, 12
5. H. Ogura, K. Furuhata and K. Furuhata, Chem.Pharm.Bull.(Japan), 1975, 21, 1397
6. G. R. Pettit, R. B. van Dreele, G. Bollinger, P. M. Troxler and P. Brown, Experientia, 1973, 29, 521
7. R. I. Shimi, N. Abedallah and S. Fatha, Antimicrob.Agents and Chemotherapy, 1977, 13, 373
8. G. L. Atkins and J. B. Neilands, Biochemistry, 1968, 7, 3734
9. H. A. Akers, M. Llinas and J. B. Neilands, Biochemistry, 1972, 11, 2283
10. F. M. Shabel, M. W. Trader, W. R. Laster, S. C. Shaddix and R. W. Brockman, Cancer Treat.Rep., 1976, 60, 1325
11. R. W. Brockman, S. C. Shaddix, M. Williams and R. F. Struck, Cancer Treat.Rep., 1976, 60, 1317
12. G. P. Wheeler, V. H. Bono, B. J. Bowden, D. S. Adamson and R. W. Brockman, Cancer Treat.Rep., 1976, 60, 1307
13. G. R. Pettit, R. B. van Dreele, D. L. Herald, D. T. Edgar and H. B. Wood, J.Amer.Chem.Soc., 1976, 98, 6742
14. G. O. Gutterman, E. L. Ricks, D. E. Wolf and J. Madas, J.Antibiotics, 1976, 23, 305
15. K. W. Blake and P. G. Sammes, J.Chem.Soc.,(C), 1970, 980
16. R. Brown, C. Kelly and S. E. Wibberley, J.Org.Chem., 1965, 30, 277
17. B. Heinman, M. A. Kaplan, R. D. Muir and I. R. Hooper, Antibiotics and Chemotherapy, 1953, 3, 1239
18. N. N. Gerber, J.Org.Chem., 1967, 32, 4055
19. J. Rao and A. Cullen, J.Amer.Chem.Soc., 1960, 82, 1127
20. C-G. Shin, M. Hayabuaka, K. Mikami and J. Yoshima, Tet.Letts., 1977, 863
21. L. Dolezilova, I. Malek, L. Pokoma, E. Paleckova, Z. Vanek and M. Vandracek, Folia Mikrobiol.(Prague), 1966, 11, 47

22. H. G. Floss, Tetrahedron Report, No. 14, 1976, 32, 404
23. G. Casnati and A. Pochini, J.C.S.Chem.Comm., 1970, 1328
24. G. Casnati, R. Marchelli and A. Pochini, J.C.S.Perkin I, 1974, 759
25. E. H. Jackson and E. A. Smith, Tetrahedron, 1965, 21, 989
26. A. J. Birch, G. E. Blance, S. David and H. Smith, J.Chem.Soc., 1961, 3128
27. A. J. Birch and K. R. Farrar, J.Chem.Soc., 1963, 4277
28. J. C. MacDonald and G. P. Slater, Can.J.Microbiol., 1965, 12, 455
29. G. P. Slater, J. C. MacDonald and T. Nakashimi, Biochemistry, 1970, 9, 2886.
30. C. M. Allen, Biochemistry, 1972, 11, 2154
31. C. M. Allen and C. L. Deyrup, Phytochem., 1975, 14, 971
32. C. M. Allen, J.Amer.Chem.Soc., 1973, 95, 2386
33. G. Casnati, R. Marchelli and A. Dossena, J.C.S.Chem.Comm., 1975, 779.
34. G. Casnati, G. P. Gardini, G. Palla and C. Fuganti, J.C.S.Perkin I, 1974, 2397
35. R. Marchelli, A. Dossena, A. Pochini and E. Drada, J.C.S.Perkin I, 1977, 713.
36. R. Candillo, C. Fuganti, D. Ghiringhelli and P. Grasselli, J.C.S.Chem.Comm., 1975, 713
37. R. Candillo, C. Fuganti, D. Ghiringhelli and P. Grasselli, J.C.S.Chem.Comm., 1977, 474
38. R. Candillo, C. Fuganti, D. Ghiringhelli and G. Gratti, J.C.S.Chem.Comm., 1976, 435
39. A. J. Birch and R. A. Russell, Tetrahedron, 1972, 28, 299
40. P. Steyn, Tetrahedron, 1973, 29, 107
41. P. Steyn and R. Vleggar, Phytochem., 1976, 15, 355
42. A. J. Birch and J. J. Wright, Tetrahedron, 1970, 26, 2329
43. A. J. Birch, J. Baldas and R. A. Russell, J.C.S.Perkin I, 1974, 50
44. A.E.A. Porter and P. G. Sammes, J.C.S.Chem.Comm., 1970, 1103
45. A.E.A. Porter and P. G. Sammes, J.Chem.Soc., 1970, 2350.

46. P. J. Machin, A.E.A. Porter and P. G. Sammes, J.C.S.Perkin I, 1973, 405
47. K. W. Blake and P. G. Sammes, J.Chem.Soc., (C), 1970, 980
48. N. Eickman, J. Gluardy, R. J. Cole and J. N. Kirksey, Tet.Letts., 1975, 1052
49. M. Yamazaki, K. Sasago and K. Miyaka, J.C.S.Chem.Comm., 1974, 408
50. M. Yamazaki, H. Fujimoto and T. Kawasaki, Tet.Letts., 1975, 124
51. J. Fayes, D. Lakensguard, R. J. Cole and J. N. Kirksey, J.Amer.Chem.Soc., 1974, 96, 6785
52. M. F. Grundon, Tetrahedron Report, No. 49, 1978
53. G. M. Strunz, M. Kakushima and M. A. Stillwell, J.C.S.Perkin I, 1973, 260
54. M. A. Stillwell, Can.J.Microbiology, 1974, 26, 759
55. R. L. Devault and R. Rosenbrook, J.Antibiotics, 1973, 26, 532
56. P. Michel, J.Antibiotics, 1974, 27, 57
57. F. Dorn and D. Arigoni, Experientia, 1974, 30, 135
58. G. M. Strunz, M. Kakushima and M. A. Stillwell, Can.J.Chem., 1975, 53, 295
59. M. R. Bell, J. R. Johnston, B. S. Wildii and R. B. Woodward, J.Amer.Chem.Soc., 1958, 80, 1001
60. A. F. Beecham, F. Fridricksons and A. Mathieson, Tet.Letts., 1966, 3131
61. S. Safe and A. Taylor, J.C.S.Perkin I, 1972, 472
62. N. Neus, R. Nagarajan, B. Molloy and L. Huckstep, Tet.Letts., 1968, 4467
63. D. Hauser, H. P. Weber and H. P. Sigg, Helv.Chim.Acta., 1970, 53, 1061
64. D. Hauser, F. Loosli and K. Nicklaus, Helv.Chim.Acta., 1972, 55, 2182
65. H. Hinato, M. Matsumoto and T. Katayama, J.C.S.Perkin I, 1973, 1819
66. D. Argondelis and Y. Mizak, J.Antibiotics, 1977, 30, 468
67. A. G. McInnes, A. Taylor and J. A. Walter, J.Amer.Chem.Soc., 1976, 98, 6741

68. R. L. Suhadolnik and R. G. Chenoveth, J.Amer.Chem.Soc., 1958, 80, 439
69. A. K. Bose, K. G. Das, P. T. Funke, I. Kugajevski, O. P. Shulka and R. L. Suhadolnik, J.Amer.Chem.Soc., 1968, 90, 1038
70. A. K. Bose, K. S. Khanchandani, P. Funke and R. Tavares, J.Amer.Chem.Soc., 1968, 90, 3593
71. J. D. Bu'Lock and A. P. Ryles, J.C.S.Chem.Comm., 1970, 1404
72. M. S. Ali, J. S. Shannon and A. Taylor, J.Chem.Soc.(C), 1968, 2044
73. M. S. Ali, R. Rahman and A. Taylor, 5th I.U.P.A.C.Symp. Nat.Prod.London (1968) Abstract C18 p94.
74. N. Johns, G. W. Kirby, J. D. Bu'Lock and A. P. Ryles, J.C.S.Perkin I, 1975, 383
75. J. C. MacDonald and G. A. Slater, Can.J.Biochem., 1975, 53, 475
76. J. D. Bu'Lock and C. Leigh, J.C.S.Chem.Comm., 1975, 628
77. G. W. Kirby, G. L. Patrick and D. J. Robins, J.C.S.Perkin I, 1978, 1336
78. J. A. Winstead and R. L. Suhadolnik, J.Amer.Chem.Soc., 1960, 82, 1644
79. N. Johns and G. W. Kirby, J.C.S.Chem.Comm., 1971, 163
80. C.S.M. Foote, S. Mazur, P. A. Burns and D. Lerdal, J.Amer.Chem.Soc., 1973, 95, 586
81. E. Francis, R. Rahman, S. Safe and A. Taylor, J.C.S.Perkin I, 1972, 470
82. G. W. Kirby and M. T. Varley, J.C.S.Chem.Comm., 1974, 833
83. P. G. Deffieux, Acta Cryst., 1977, B33, 1475
84. P. J. Curtis, D. Greatbanks, B. Hesp, A. Forbes-Cameron and A. A. Freer, J.C.S.Perkin I, 1975, 383
85. T. Fukayama and Y. Kishi, J.Amer.Chem.Soc., 1976, 98, 6723
86. D. L. Coffen, D. A. Katonak, N. R. Nelson and F. Sancilio, J.Org.Chem., 1977, 42, 948
87. T. Hino and T. Sako, Tet.Letts., 1971, 3127
88. D. R. Brannan, J. A. Mabe, B. Molloy and W. Day, Biochem.Biophys.Res.Comm., 1971, 43, 588

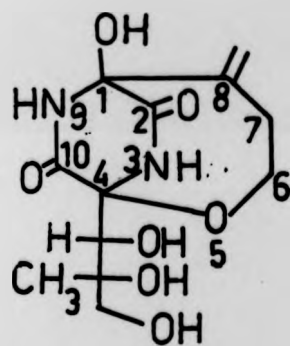
89. G. Dunn, G. T. Newbold and F. S. Spring, J.Chem.Soc., 1949, 2586
90. S. Nakamura, Agric.Biol.Chem.(Tokyo), 1961, 25, 74
91. G. T. Newbold, W. Sharp and F. S. Spring, J.Chem.Soc., 1951, 2679
92. A. H. Cook and C. A. Slater, J.Chem.Soc., 1956, 4133
93. M. Tenao, J.Antibiotics, 1963, 16, 182
94. J. C. MacDonald, J.Biol.Chem., 1961, 512
95. J. C. MacDonald, J.Biol.Chem., 1962, 1977
96. J. C. MacDonald and R. G. Micetich, J.Biol.Chem., 1965, 1692
97. J. C. MacDonald, Biochem.J., 1965, 96, 533
98. E. Canale-Parola and P. L. Offen, J.Bacteriology, 1972, 111, 86
99. T. Yoshida, Phytochem., 1976, 15, 1723
100. K. Tatsuka, K. Fujimoto, M. Yamashita, T. Tsuchiya and S. Umegawa, J.Antibiotics, 1973, 26, 606
101. A. E. Oxford and H. Raistrick, Biochem.J., 1948, 43, 323
102. A. J. Birch, I. A. Massey-Westropp and R. W. Rickards, J.Chem.Soc., 1956, 3717
103. G. W. Kirby and S. Narayanaswami, J.C.S.Perkin I, 1976, 1564
104. J. P. Springer, G. Buchi, B. Kobbe, A. Demain and J. Clardy, Tet.Letts., 1977, 2403
105. G. M. Sharma and P. R. Burkeholde, J.C.S.Chem.Comm., 1971, 51
106. S. Forenza, L. Minale and R. Ricco, J.C.S.Chem.Comm., 1971, 1129
107. E. E. Gracia, L. Benjamin and R. Fryer, J.C.S.Chem.Comm., 1973, 78
108. T. Kamiya, S. Maeno, H. Aobi, M. Kohsaka, H. Sikai and H. Iminaka, J.Antibiotics, 1972, 25, 569
109. M. Nishida, Y. Miwi and T. Mutsbarsara, J.Antibiotics, 1972, 25, 582

SECTION 2

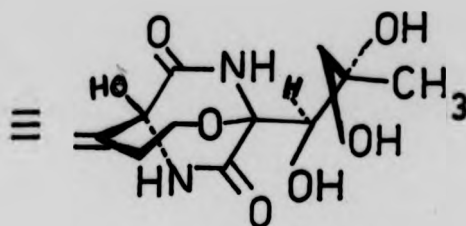
Part 1

1.0 Introduction

Bicyclomycin (1) has been cited^{as} one of the most promising antibacterial agents discovered in recent years.¹ The high water solubility and low toxicity of this compound make it ideal for the production of various pharmaceutical preparations.



1



2

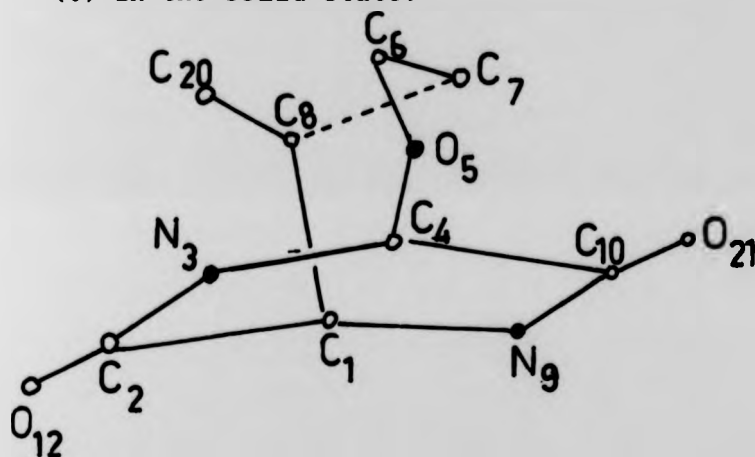
In general, the in vivo activity of the antibiotic is better than that expected from the original in vitro assays.²

Bicyclomycin (1) was discovered simultaneously by two Japanese groups and was shown to occur in culture filtrates of Streptomyces sapporonensis,³ S. aizunensis^{4,5} and S. griseoflavus var. bicyclometricus⁶.

Chemical and spectroscopic investigation⁷ led to the conclusion that amide groups, a terminal olefin and a glycol moiety were the salient features of the structure.

X-ray diffraction studies⁸ showed that the two amide groups are part of a dioxopiperazine ring which is in the boat conformation and that the glycol substituents are all 'gauche'. The relative stereochemistry of the system is depicted as structure (2).

The molecule can be seen to possess a caged structure (3) in the solid state.



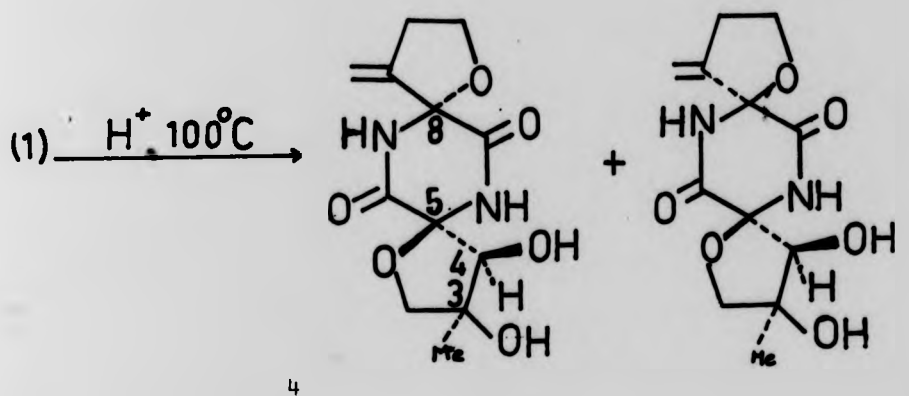
3

More recent work has helped to establish the absolute configuration of bicyclomycin (1)⁹.

Treatment of bicyclomycin (1) with perchloric acid at 100°C leads to the rapid conversion of bicyclomycin (1) into a 1:1 mixture of diastereoisomers (4) and (5) (Scheme 1), epimeric at C-8.

X-ray diffraction studies of the p-bromobenzoate⁹ derivative of (4) lead to the assignment of the 3S, 4S, 5S, 8R absolute stereochemistry for diastereoisomer (4). Similarly diffraction studies gave the absolute stereochemistry of the p-bromobenzoate derivative of (5) as 3S, 4S, 5S, 8S.

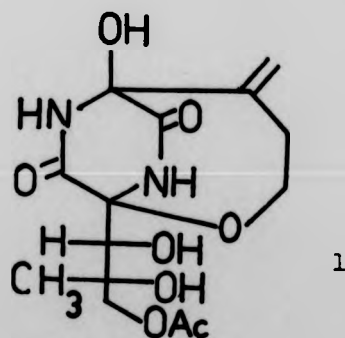
These results establish the absolute configuration of



Scheme 1

bicyclomycin (1) as 1S, 6R, 1'S, 2'S (using the original numbering system).

Various mono-acetylated derivatives (1) R = Ac have been prepared and patented^{7,10,11,12} as antibacterial agents.



The acylated derivatives bearing cyclohexyl, nicotinyl and 2-thienyl substituents, are more readily absorbed into mammalian subjects due to the increased lipophilicity of these analogues. These compounds have been patented¹³ for orally administered preparations.

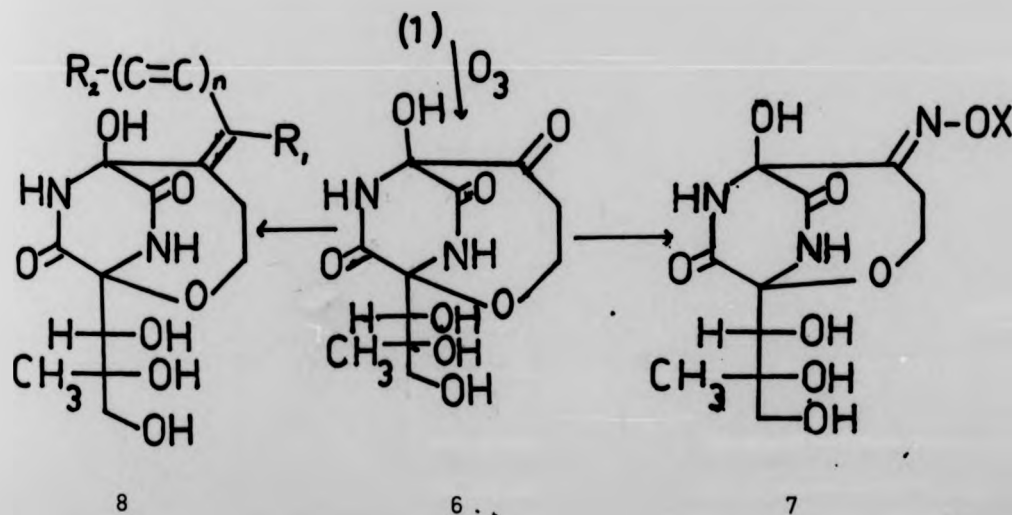
The absorption, excretion and tissue distribution of the parent antibiotic (1) and the various acylated derivatives already mentioned, have already been studied¹⁴ and, interestingly, the antibiotic is excreted without metabolic transformation¹⁴.

The therapeutic value of bicyclomycin (1) has been demonstrated in primate shigellosis¹⁵, and the compound has been shown not to cross react with benzylpenicillin in plasma¹⁶.

One outstanding application of bicyclomycin (1) is that it may be used to treat diseases in which the causal organism is known to be resistant to conventional β -lactam antibiotic therapy.

In the recent literature, workers at Ciba-Geigy A.G. have patented^{17,18,19} new analogues of bicyclomycin (1) as antibacterial agents.

In these patents, 5-norbicyclomycin-5-one (6), the ozonolysis product of bicyclomycin (1), has been used as the starting material for two groups of potent derivatives (7)^{17,18} and (8)¹⁹. Scheme 2.



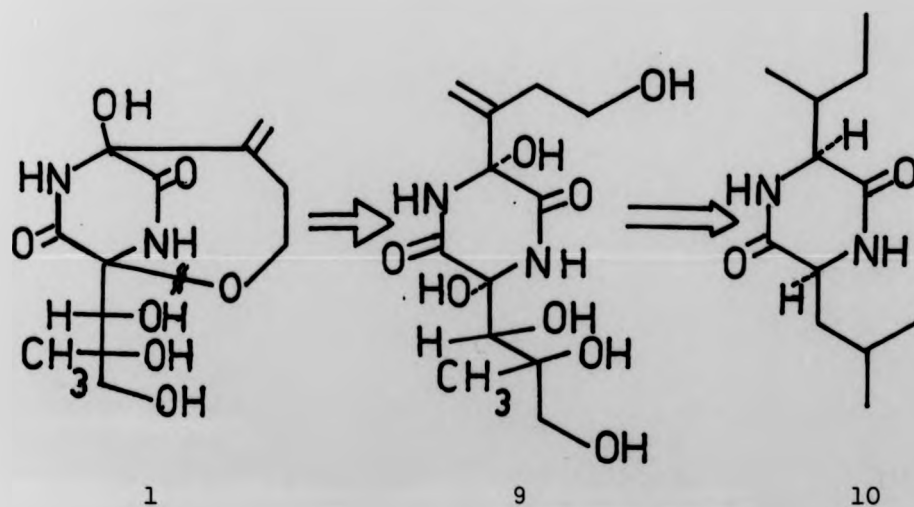
Scheme 2

1.1 A postulated biosynthetic scheme for bicyclomycin

The bicyclic ring system of bicyclomycin (1) is unique amongst natural products although the fact that it is formally a bridged dioxopiperazine suggests that it is probably an amino acid derivative.

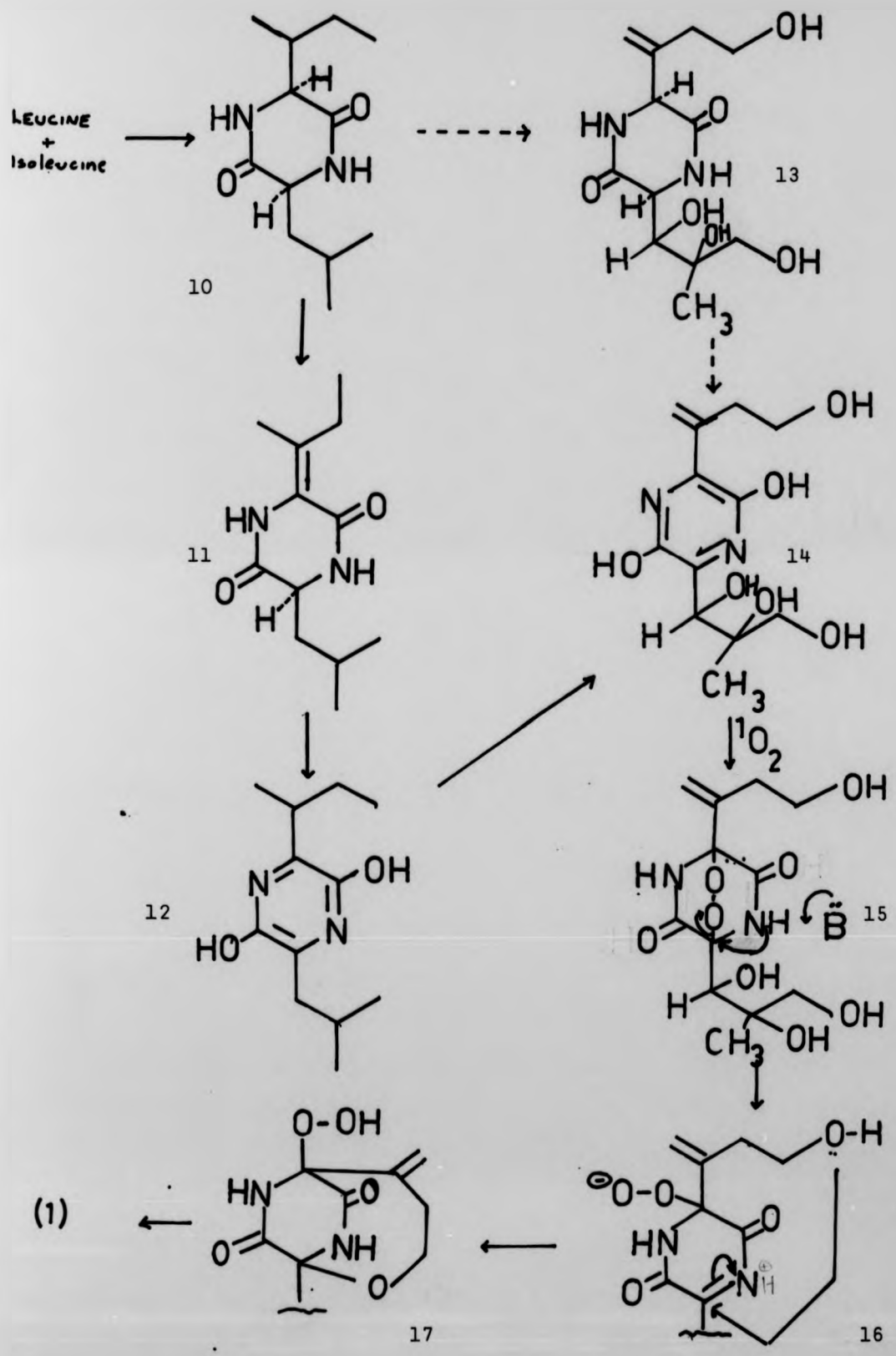
If one applies antithetic analysis²⁰ some light may be thrown on the possible mode of biogenesis in vivo.

Cleavage of the ether linkage (Scheme 3) to generate a monocyclic system, generates a highly functionalised dioxopiperazine (9), which on removal of all oxygen atoms save the two amide carbonyl groups, results in the cyclic dipeptide, cyclo-isoleucyl-leucine (10), which is formally derived from leucine and isoleucine.



Scheme 3

Conversion of cyclo-isoleucyl-leucine (10) into bicyclomycin (1) clearly proceeds through several oxidative steps,



Scheme 4

consistent with the high oxygen requirement during the growth of S. sapporonensis and S. aizunensis, although the precise sequence of these steps must remain a topic for conjecture.

Conversion of (10) into the dihydroxypyrazine (14) may proceed by way of an alkylidene dioxopiperazine (eg. 11), followed by isomerisation of the double bond into the ring, or by direct oxidation of the piperazine ring. Both reactions have excellent precedent in the literature^{21,22,23,24}.

Our original assertion that leucine and isoleucine may be involved has recently been supported by the findings of a Japanese group, who have shown that feeding [1-¹⁴C]leucine and [1-¹⁴C]isoleucine to S. sapporonensis²⁵ results in labelled biocyclomycin *i.e.* presumably (2-¹⁴C), (10-¹⁴C) bicyclomycin. The role of cyclo-isoleucyl-leucine is still speculative as yet.

In line with our hypothesis [1-¹³C]leucine and [1-¹³C]isoleucine were synthesised from K¹³CN by classical Strecker synthesis prior to the publication of the work on the ¹⁴C amino acids²⁵.

Hydroxylation and dehydrogenation of the side chains probably occur through several steps, corresponding to oxidations of the benzylic positions and hydroxylation at the saturated carbon. Again both of these oxidations have precedent in the biosynthetic literature.

An alternative scheme might involve prior oxidation of the side chains of cyclo-isoleucyl-leucine (10) followed by oxidation of the dioxopiperazine ring, although this seems less likely.

Reaction of the functionalised dihydroxypyrazine

with singlet oxygen would be expected to result in the formation of a bridged dioxopiperazine (eg. 15). This is well precedented, in that 2,5-dihydroxypyrazines are known to undergo [4 + 2] cycloadditions with singlet oxygen and olefins, and the resulting cycloaddition products have been fully characterised^{26,27}.

Reductive cleavage of the oxygen-oxygen bond (Scheme 4) to furnish the 3,6 dihydroxydioxopiperazine, followed by dehydrative cyclisation, may be ruled out since this is expected to give rise to the cis arrangement of the oxygen substituents at C-3 and C-6 of the dioxopiperazine ring, whereas the trans stereochemistry is observed in bicyclomycin.

It, therefore, seems probable that cleavage of the ring oxygen bond of the bicyclic peroxide (15), is assisted by nitrogen participation and is followed by recyclisation to form the hydroperoxide of bicyclomycin (17) by an n-endo trig type closure²⁸.

Fermentation Studies

1.2 Introduction

As previously reported, bicyclomycin (1) is produced by several strains of Streptomyces sp. during aerobic fermentation conditions^{29,30}.

Our studies were confined to S. sapporonensis and S. aizunensis since these particular species were reported to produce bicyclomycin in reasonable quantity.

Both species were obtained as lyophilised samples from the American Type Culture Collection (ATCC) and were grown after rehydration on a range of solid agar based media.

Two methods exist for the isolation of the bicyclomycin (1) from culture filtrates and both are the subject of patent applications^{29,30}.

The most practical of the two methods involves adjustment of the culture filtrate to pH 7.0, followed by adsorption of the organic fraction of the extract onto activated carbon.

Selective desorption using ethyl acetate, followed by acetone induced precipitation of impurities furnished an oil with moderate antibiotic activity. Column chromatography on silica and combination of all antibiotic fractions produced bicyclomycin at the level of 50 mg. l⁻¹.

The second method involves solvent reduction of the filtrate followed by several solvent induced precipitations to produce a white powder after lyophilisation.

Extraction of the lyophilised powder produced bicyclomycin (1) at the same concentration as the previous method.

The whole isolation process was monitored using E.coli IAM 1159 or ATCC 27166 - a hypersensitive mutant which can detect bicyclomycin at very low concentrations^{3,25}.

1.3 Fermentation studies related to bicyclomycin production

(a) Shake culture studies

Initially, fermentation studies using S. sapporonensis (ATCC 21532) were confined to shake culture techniques.

The decision to employ this cultural technique was based on the reported isolation figures and the quantity of material necessary for isotopic feeding experiments.

S. sapporonensis (ATCC 21532) was grown initially on malt agar³ slopes and plates to produce stock cultures whose growth was limited by storage at 4°C.

Similarly, stock cultures of S. aizunensis (ATCC 21775) were maintained on glucose-asparagine agar⁴.

The colonies produced by both organisms were morphologically and culturally identical to those reported by the original Japanese workers²⁹.

The stock cultures were used to prepare actively growing vegetative cultures in liquid media, which was used as the inoculant for the shake culture experiments.

After inoculation, shake culture fermentations were continued for six days at 30°C. Since both organisms were reported to have high oxygen requirement the agitation rate was high ca 200 r.p.m. and the invaginations in the culture flasks seemed to aerate the broth to an adequate degree.

The filtrates were adjusted to pH 7.0 and the organic fraction adsorbed onto activated carbon.

Liberation of the organic fraction with ethyl acetate: methanol (5:1) and subsequent lyophilisation gave an off-white powder.

P.l.c. of the crude powder and comparison with some authentic material showed that bicyclomycin was probably present in the extract, as evidenced by a spot of similar R.f. when visualised with potassium periodocuprate spray³¹.

Using this spray reagent, bicyclomycin (1) appears as a white spot on a brown background.

The quantity of bicyclomycin present in the initial

S. sapporonensis fermentation was ca 20 mg and was quantified by using quantitative t.l.c. and several known bicyclomycin standards.

A second S. sapporonensis shake culture experiment, which was continued for 60 hours, produced ca 2.5 mg of bicyclomycin as estimated by quantitative t.l.c.

This may be indicative that, during long periods of fermentation, when the nutrient supply is limiting, some degradation of bicyclomycin may occur.

The two isolated materials were combined and purified by p.l.c. in methanol:chloroform (5:1) to yield 3 mg of bicyclomycin as a single spot.

The material had a small positive rotation i.e. $+ 0.03^{\circ}$. This corresponds to a molecular rotation of $+ 50.0^{\circ}$ ($c = 6 \times 10^{-4}$, MeOH). The reported rotation for bicyclomycin is $+ 63.5^{\circ}$ ($c = 1$, MeOH) which corresponds to an optical purity of 78.7%.

Mass spectroscopy of this material showed prominent peaks at 114 a.m.u. and the usual -NHCO extrusion peak for dioxopiperazines³² at 43 a.m.u. The molecular ion region was too ill-defined to be diagnostic of the compound's identity.

The parent compound, bicyclomycin (1), run under similar conditions, gave a rather poor molecular ion at 320 a.m.u. but was otherwise comparable to the data reported for its mass spectrum and that of the isolated compound. The isolated material had a melting point of 181-188.5°C (Literature m.p. bicyclomycin = 188-189°C).²

A third shake culture fermentation using S. sapporonensis was carried out for 60 hours but in this case the filtrate was divided into two equal parts. One part was spiked with authentic

bicyclomycin (10 mg) and both were worked up as usual.

The unspiked extract produced ca 1 mg bicyclomycin and the spiked extract ca 2 mg (quantified by t.l.c. against bicyclomycin standards).

It would seem therefore, that the extraction process was either only about 10% efficient or that bicyclomycin was decomposing under the isolation conditions³³.

Two possible explanations may account for the low bicyclomycin yields from these cultures.

Firstly, the medium used, i.e. liquid malt-yeast medium, may be limiting bicyclomycin production since it has only a modest content of nitrogenous material present in it, and secondly, the aeration rate may not be adequate for bicyclomycin production since in the literature^{4,5}, aeration rates of 20-30 l.min⁻¹ were cited.

Initially, the parameter of medium composition was thought to be of prime importance, and as such was investigated prior to the study of increased aeration rates.

A series of experiments utilising different medium formulations was undertaken and the primary aim of this series of experiments was to examine whether the antibiotic (1) could be produced by cultures of S. sapporonensis and S. aizunensis in the media cited²⁹ for commercial production viz Pharamedia^R/starch under shake culture conditions.

The results of these experiments, in terms of bicyclomycin production, is shown in Table 1.

TABLE 1

Crude Bicyclomycin production in Pharmamedia:
starch mixtures using S. sapporonensis and S. aizunensis
(60 hours, 30°C, 180 r.p.m.)

Organism	Media	Isolated yield crude bicyclomycin* (mg/l)
<u>S. sapporonensis</u> (ATCC 21532)	Pharmamedia/starch	6.6
	Pharmamedia alone	4.9
	Nitrogen limited media ^a	2.4
	Carbohydrate limited media ^b	4.4
<u>S. aizunensis</u> (ATCC 21775)	Starch/Pharmamedia	21.8

* material had chromatographic properties identical to the previously isolated bicyclomycin [$\alpha_D^{25} = +51.6^\circ - +52^\circ$] in most cases

- a. The main nitrogen source - pharmamedia reduced by 60%
b. Carbohydrate content reduced by 60%

As can be seen in Table 1, reduction in carbohydrate and nitrogen sources substantially reduce the levels of bicyclomycin and this is indicative that at least high levels of nitrogenous material, and to a lesser extent carbohydrate, are necessary for bicyclomycin production.

Similarly it may be seen from Table 1 that S. aizunensis is far superior to S. sapporonensis in terms of bicyclomycin production, under these conditions.

These results are probably a reflection of the lower

oxygen demand in this species i.e. ca 10L air min⁻¹ compared with that of S. sapporonensis i.e. ca 30L air min⁻¹.

In all cases the material had identical spectroscopic characteristics to that previously isolated by shake culture techniques.

A second parameter investigated was that of sudden temperature reduction during active fermentation. This phenomenon creates stress on the organism and other workers³⁴ have suggested that this may lead to the production of high concentrations of the antibiotic.

The technique involved cooling the active cultures to 0°C for 1 hour during a 60 hour fermentation and then processing the culture filtrate as normal.

The results are shown in Table 2.

The effect of reducing available nitrogen sources is most dramatic and again, to a lesser effect, the reduction of carbohydrate, also decreases the level of bicyclomycin production significantly.

The general trend, however, shows that thermal stress leads to a two fold increase in bicyclomycin production. The commercial formulation for the culture of S. sapporonensis however increases five fold.

The S. aizunensis culture had a higher concentration of bicyclomycin than S. sapporonensis, although in the thermally shocked cultures, the difference between the two species was far less than in the control situation. This probably reflects the lower oxygen demand of S. aizunensis.

Several morphological differences between the species were noted during the fermentations in Pharmamedia-starch

formulations.

TABLE 2

The production of crude bicyclomycin in thermally stressed cultures of S. sapporonensis and S. aizunensis using various media formulations (30°C, 60 hours, 190 r.p.m. - 1 hour at 0°C)

Organism	Media	Isolated yield of crude bicyclomycin* (mg/l)
<u>S. sapporonensis</u> (ATCC 21532)	Starch/Pharmamedia	30
	Pharmamedia alone	7.2
	Nitrogen deficient media ^a	4.6
	Carbohydrate deficient media ^b	9.2
<u>S. aizunensis</u> (ATCC 21775)	Starch/Pharmamedia	39.4

* identical to that in Table 1

a. " " " " "

b. " " " " "

The first noticeable variation was that S. aizunensis produces very small colonies and no discrete colonies could be discerned during active fermentation. In the case of this organism, it was also noticed that the medium consistency decreased more rapidly with time, than in fermentations utilising S. sapporonensis. This result may reflect an increase in the production or activity of extracellular hydrolytic enzymes in S. aizunensis.

Similarly soluble pigment production in S. aizunensis was much more visible than that in S. sapporonensis.

In the control situation, the production of bicyclomycin in S. sapporonensis is only 13% of that quoted in the patent

application. S. aizunensis in the control situation yields 43% of the cited value.

The effect of thermal stress raises these values to 60% and 79% respectively.

It should be remembered however, that from rotation measurement, these samples are only 78% optically pure.

On completion of these experiments, all the fractions were collected, concentrated and examined by t.l.c.

The material was no longer homogeneous under t.l.c. conditions and ^{13}C studies confirmed this.

After chromatographic separation and identification, it was found that only 20% (26 mg) remained homogeneous. This was crystallised from acetone and the m.p., mass spectrum, and n.m.r. confirmed this as pure bicyclomycin.

It is possible, in the light of these findings, that bicyclomycin is unstable to storage over a protracted period, if not previously crystallised. However, with the levels shown in Table 1, crystallisation of individual isolated aliquots was not possible.

Later results are also in agreement with this view.

During the isolation of bicyclomycin from S. sapporonensis shake cultures, a white solid crystallised out of the aqueous methanolic solution used for isolation purposes. The crude material was filtered off and shown to be homogeneous in three different t.l.c. solvent systems.

The melting point of the compound was sharp i.e. 126-128.5°C and no significant rotation was observed on the sodium D line.

The material was crystallised from toluene to give colourless needles (m.p. 133-134.5°C) and the purified material was unreactive towards ninhydrin or potassium periodocuprate. Lassaigne's test³⁵ gave positive results for nitrogen and sulphur.

In methanolic solution the ultraviolet absorbance reached a maximum at 273 nm, but this shifted to 306 nm on the addition of a few drops of aqueous sodium hydroxide solution.

Similarly the proton resonances e.g. τ 2.3(S)1H, 2.7(d)2H and 2.9(dd)1H shifted in the presence of NaOD to resonances at τ 1.9(s)1H, 2.6(d)2H, 3.09(dd)1H.

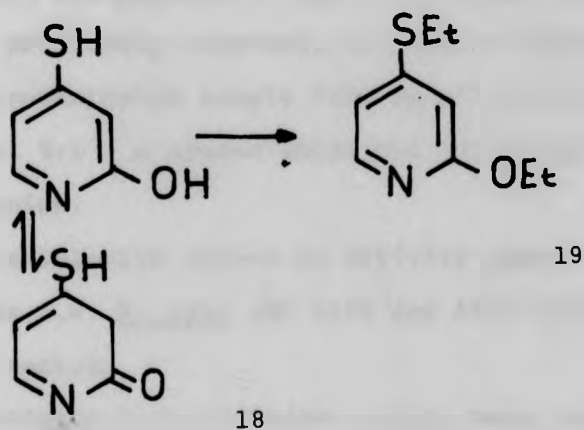
The ultraviolet and n.m.r. data coupled with a positive ferric chloride test, would indicate a phenolic type function.

Mass spectral examination of the compound showed the molecular ion at 127.0092 a.m.u. which gives an elemental analysis of C_5H_5NOS .

Microanalysis gave similar results.

Carbon resonances for the compound, discussed in Section 3, suggest a pyridone type structure (18) and reaction of the compound with triethyloxonium tetrafluoroborate in dry dichloromethane gave the alkylated product (19).

Further examination of the material was discontinued when assays showed the material had no biological activity against clinical isolates of E. coli (ATCC 27166 and MRE 600).



1.4 Large scale fermentation studies

After consideration of the poor yields obtained by shake culture experiments, it was decided to scale the process up, in the hope that a potentially useful quantity of the antibiotic would be produced.

A large scale fermentation unit (LHE 1000, 20L) was purchased and installed.

The first batch fermentation was carried out precisely as cited in the literature⁴, using *S. aizunensis* grown in Soyflour/glucose medium⁴ with an aeration rate of 10L min⁻¹.

The exact fermentation parameters were followed, the only addition being the injection of a 10 second sample of an antifoaming agent (ca 5 ml) every 20 mins.

After a fermentation of 72 hours, the broth was centrifuged in a continuous centrifuge to remove mycelial debris and insoluble matter in the medium.

This produced 20L of a clear brown solution. The pH

of the solution was adjusted to pH 7.0 and the solution then worked up as previously reported, to yield a brown oil.

A concentrated sample (100 mg/ml) was prepared in water:methanol 9:1 - a system which did not inhibit growth of the test organism.

This solution showed no activity against the cited test organisms i.e. E. coli IAM 1159 and ATCC 27166 when plated out in Oxoid medium.

Initially disc diffusion assays were used but these were unsuccessful and no inhibition of growth was observed.

A recent publication has stressed³⁷ that the assay results vary with the thickness and quality of the paper used.

In our experiments, 1 cm circles of Whatman filter paper were used, with a loading of 100 μ l (10 mg crude material) and substantial inhibition zones should have resulted.

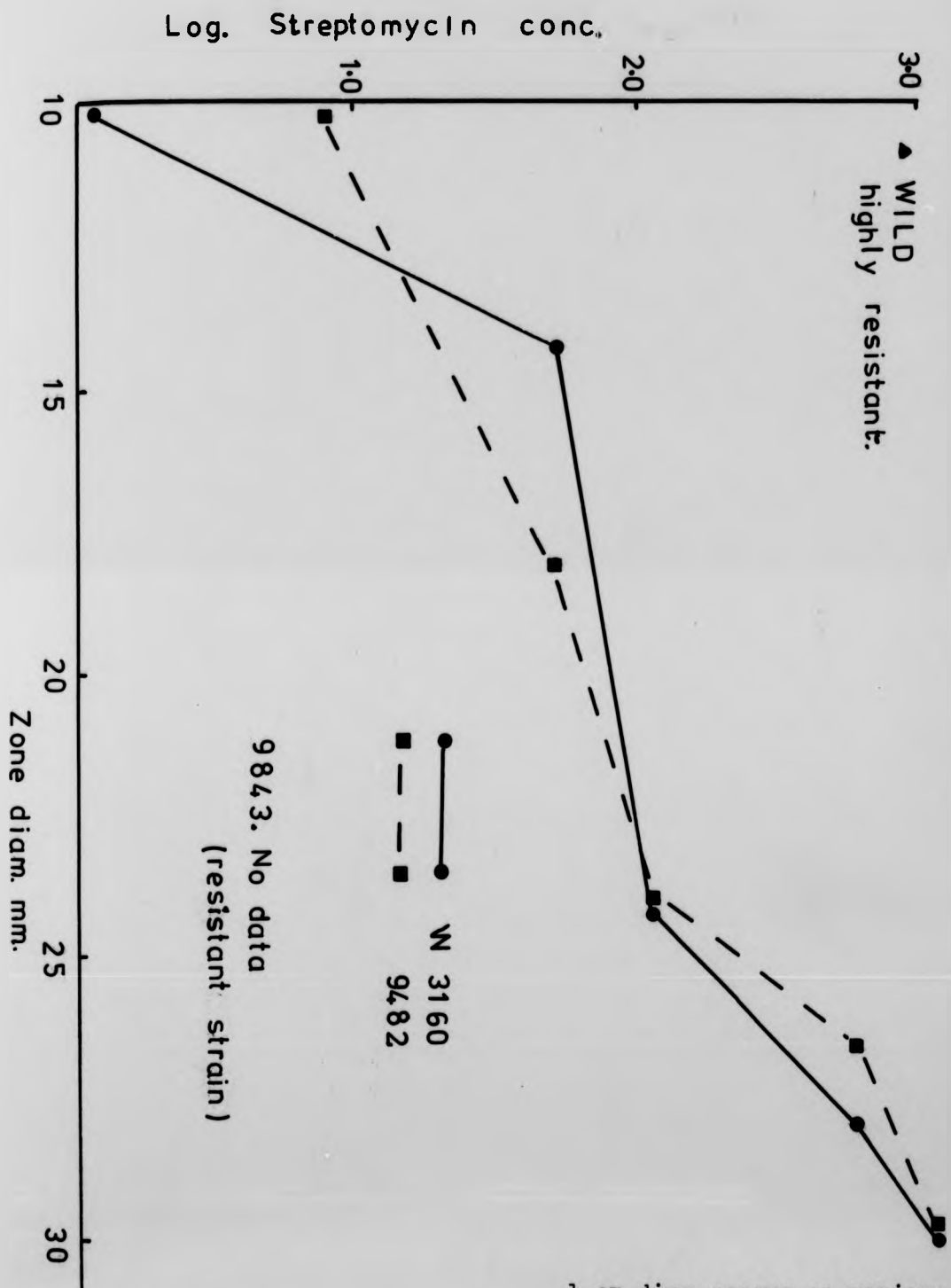
One may speculate that the antibiotic may have been adsorbed onto the paper and thus only the carrier solvent diffused into the agar on which the test organisms were growing, but this seems unlikely.

The method was then assessed using a range of industrial microorganisms e.g. E. coli W 3160, Wild 1, MRE 600, 9843 and 9482.³⁶ (Fig.1).

Only E. coli 9482 (a streptomycin resistant strain) gave a satisfactory zone of inhibition.

A concentration series obtained by serial dilution of a known standard (5×10^5 - 5×10^3 μ g ml⁻¹) was assayed using E. coli 9482 as the test organism.

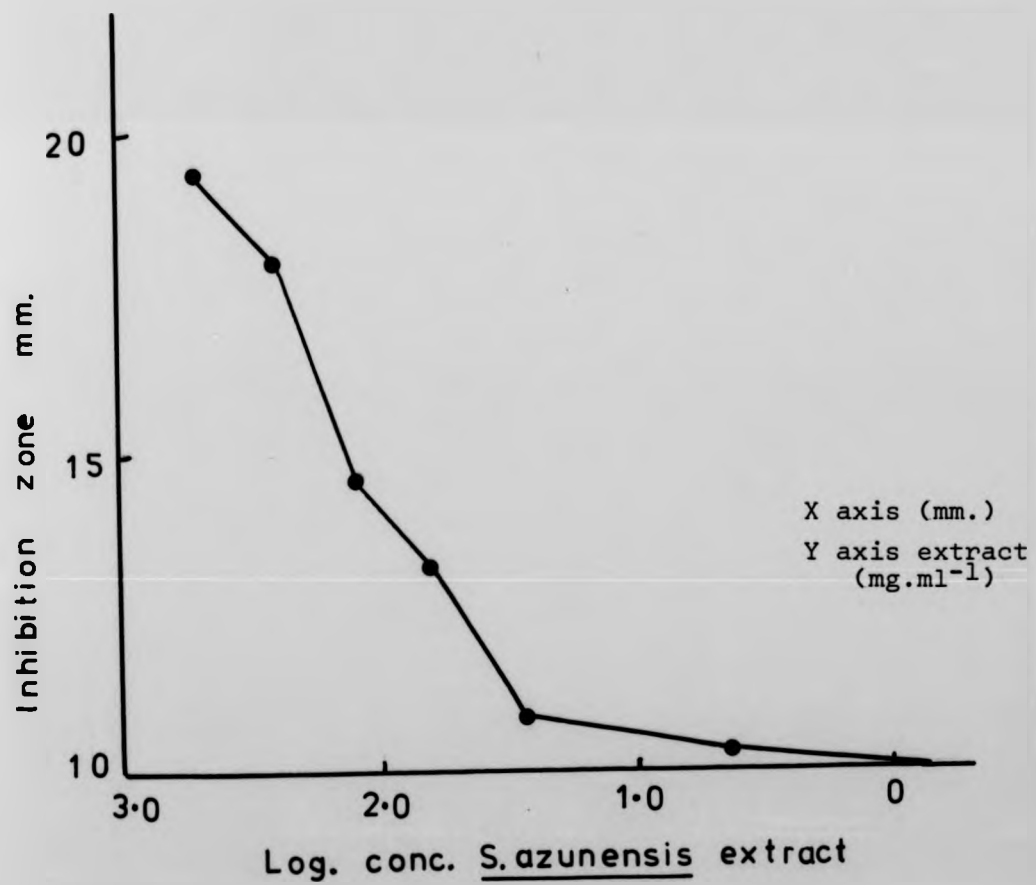
The assay used in this case was the cylinder cup



1 cm disc assays on various E.coli strains using streptomycin sulphate

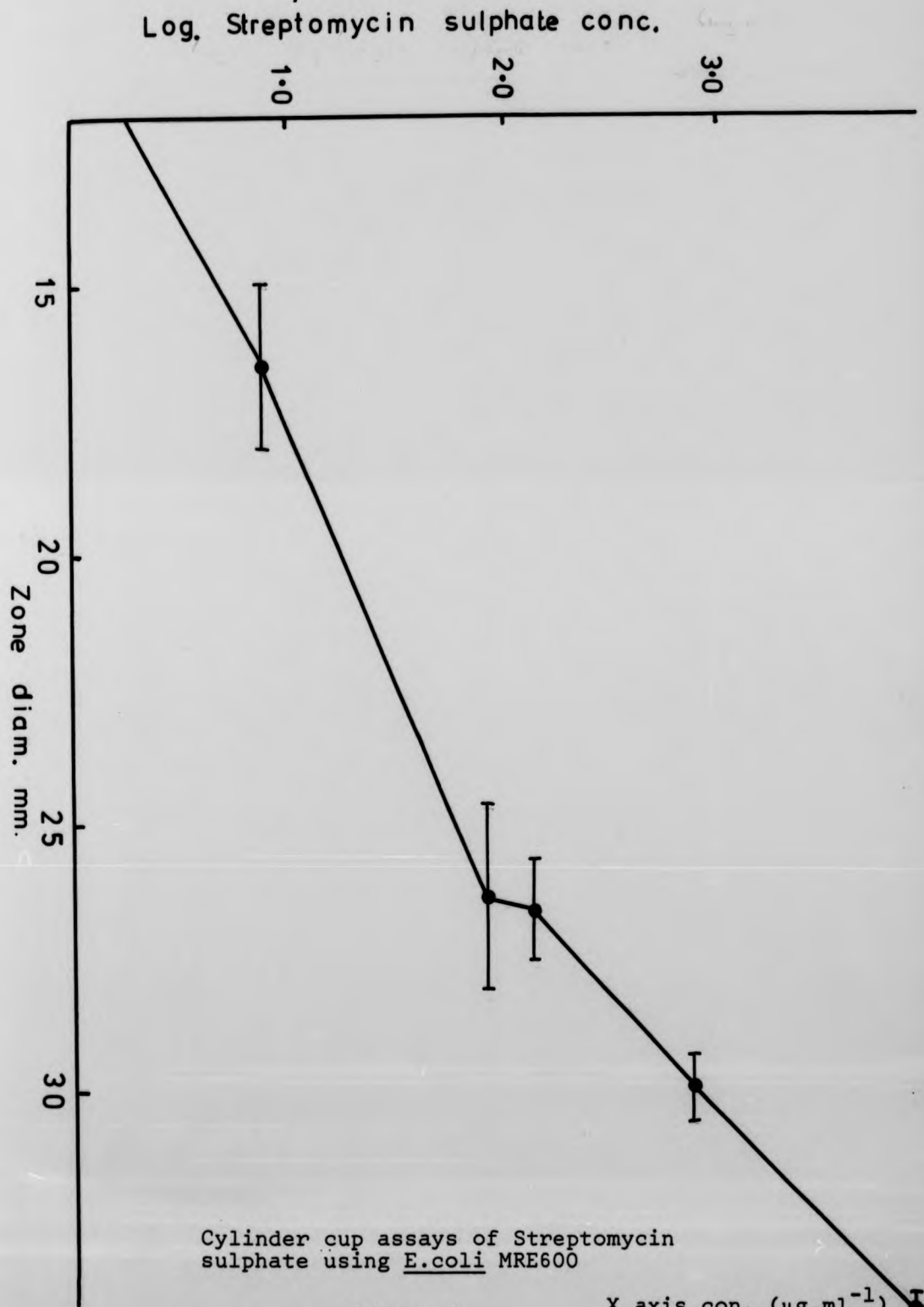
P.S Log Strep.conc. add ($\mu\text{g}.\text{ml}^{-1}$) also Zone diameter axis (mm.).

Figure 1



Cylinder cup assays of S. aizunensis extract using E. coli 9843

Figure 2



Cylinder cup assays of Streptomycin sulphate using *E.coli* MRE600

Figure 3

X axis con. ($\mu\text{g} \cdot \text{ml}^{-1}$)
Y axis (mm.)

method. A central well was cut in the centre of a media-filled petri dish in an actively growing culture of the test organism, usually grown in a petri dish. All assays were performed in duplicate and the petri dishes were randomised in the incubator to remove temperature bias.

After 48 hours at 30°C, the size of the inhibition zone was measured and the results are displayed graphically in Fig. 2.

The cylinder cup assay³⁸ method worked well and showed reasonable reproducibility.

The system was then calibrated using streptomycin sulphate (Fig.3).

A sample of the crude material (1.2g) was subjected to column chromatography on Kieselgel H using methanol:chloroform (1:5) as an eluant. Twenty-nine fractions (5 ml each) were collected from the column and each fraction was assayed using E. coli 9482 grown in Sensitest agar.

Using this technique, the fractionated extract was shown to contain activity in fractions 9, 15 and 19 (Table 3).

TABLE 3

Cylinder cup assays³⁸ of column effluent from a sample of S. aizunensis broth using E. coli 9482 as the test organism^a

Fraction	No. of u.v. spots (t.l.c.).	Inhibition Zone	Average
9	5	16.5, 18.0 mm	17.25 mm
15	4	12.0, 12.4 mm	12.20 mm
19	3	11.2, 11.1 mm	11.15 mm

a. E. coli 9482 grown on Sensitest agar, 30°C, 40 hours, using 10 mm wells. All fractions were evaporated and dissolved in MeOH:H₂O 9:1 (1 ml).

Fractions 5-10 and 11-18 were combined as two discrete fractions, and assays showed that fractions 5-10 contained the greatest bacteriostatic activity.

The combined fractions (5-10) were further separated into 80 fractions by slow passage through a silica gel column (80-100 mesh) using CHCl_3 :methanol 5:1 as an eluant.

The resultant activity was assayed using the system previously mentioned and the results are displayed in Table 4.

The conditions for growth of the test organism and assay are identical to those stated for the assays in Table 3.

TABLE 4

Biological assay of refractionated S. aizunensis extract

Fraction	5	10	15	20	25	30	35	40	45
Inhibition Zone	10.1	10.2	12.0	13.5	13.5	12.3	11.0	12.1	12.1
Fraction	50	55	60	65	70	75	solvent blank		
Inhibition Zone	11.4	11.2	12.3	11.4	11.1	10.0	10.0		

Since the activity of each fraction was small, the 80 fractions were combined on either side of peak activity i.e. 14-40, 45-50, 60-65 and were then concentrated in order to facilitate separation of the active components at a later stage.

The combined fractions were assayed as before and the results are displayed in Table 5.

TABLE 5

Activity of 100 μ l aliquots of combined fractions of S. aizunensis broth (cylinder cup assay^a, 10 mm Wells, E. coli 9482 on sensitest agar, 30°C, 40 hours).

Fraction Nos.	15 - 40	45 - 50	60 - 65
Inhibition zone (mm) ^b	17.25	12.01	13.04

- a. Conditions as previously reported in Table 3.
 b. Mean of three assays.

Combined fractions 15-40 were separated on Whatman No. 1 paper (5 x 20 cm) using chloroform:methanol (3:1) as an eluant. Three discrete spots were visible and the solution was chromatographed again and each spot subjected to bioautographic assay after thorough drying.

Each spot was removed by cutting it out and then placing it in the centre of a growing culture of E. coli 9482 grown on Sensitest agar.

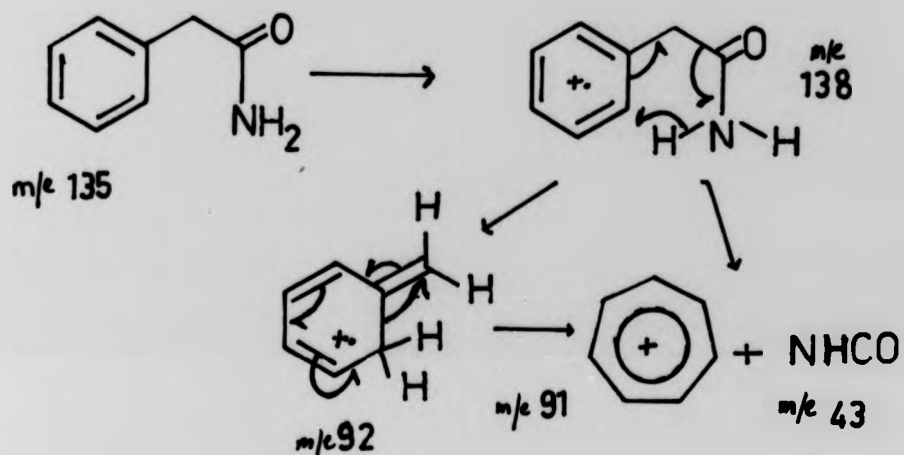
All the spots gave rise to inhibition zones. The best inhibition zone arose from the spot with the highest Rf value which was fluorescent under u.v. light.

In subsequent experiments, this material was isolated and was shown to have a melting point of 148-149.5°C. The mass spectral data showed the molecular ion was prominent at 135 a.m.u. with other prominent peaks at 91 and 92 a.m.u. A small peak at 43 a.m.u. was also observed. Recrystallised material melted at 157.0-159.0°C.

The infra-red showed peaks at 3509, 3396 and 1674 cm^{-1} (amide 1 band).

Most of these results are in close agreement with the data reported for phenyl acetamide - a known antibiotic previously isolated from S. sapporonensis cultures³⁹.

The mass spectral evidence may be rationalised as shown in Scheme 5.



Scheme 5

The smaller, medium R_f spot was not u.v. detectable but gave a positive reaction with potassium periodocuprate. The R_f was similar to that of bicyclomycin.

Even when the whole extract was processed, the amount of this material was less than 10 milligrams, and the activity against E. coli IAM 1159, ATCC 27166 and 9482 was negligible (inhibition zone = 10.1 mm).

Identical results were obtained from a second fermentation using a freshly acquired sample of S. aizunensis, so the low yield was not related to long storage times for the stock cultures.

Similar results were obtained from large scale

fermentations of S. sapporonensis, although the initial bioassays of the crude extract looked very promising (Table 6).

TABLE 6

Bioassays of crude S. sapporonensis culture filtrates using E. coli strains (Sensitest agar, 30°C, 40 hours) in a cylinder cup assay.

<u>E. coli</u> strain	Inhibition Zone	Mean
W 3160	21.5, 23.0, 20.0	21.5
Wild 1	20.0, 20.0, 20.0	20.0
9482	18.5, 19.5, 19.0	19.0
9843	21.0, 21.0, 19.0	20.3
ATCC 27166	12.5, 12.5, 13.0	12.6
Solvent blank	10.0, 10.0, 10.0	10.0

These results indicate that the extract from S. sapporonensis (in the crude form) has a wider range of biological activity than that from S. aizunensis which only showed inhibition of E. coli 9482.

The sample was fractionated using methods identical to those used for S. aizunensis.

In the final fractionation step and subsequent bioautography¹⁴, the results were similar to those obtained for S. aizunensis and are displayed in Table 7.

TABLE 7

Bioassays of combined fractions of S. extracts, using cylinder cup assays (10 mm Wells, E. coli 9482, Sensitest agar, 30°C, 40 hours).

Fraction number	15 - 35	40 - 50	55 - 65
Inhibition zone* (mm)	16.27	11.80	13.01

* mean of four results

Bioautography of this extract failed to show any of the component thought to be phenylacetamide (20).

Similarly, the amount of material corresponding to the chromatographic data for bicyclomycin was small (< 15 mg). This material gave a positive reaction with potassium periodocuprate.

The inhibition zone of this material did not increase when using the medium and test organism cited by the original workers i.e. heart infusion agar/E. coli ATCC 27166.

Our experiments, which have duplicated the reported experimental conditions and patented methods for bicyclomycin production, have not produced bicyclomycin in sufficient quantity for feeding experiments.

Even in large scale fermentation experiments i.e. 20L, the concentration of bicyclomycin in the fermentation broth has been lower than the values cited in the literature.

Two possible explanations may account for these findings.

It is possible that the organisms submitted to the American Type Culture Collection are low yielding strains of these organisms, or that somehow the culture integrity has declined. In view of the excellent record of the ATCC, this latter explanation seems unlikely.

Similarly puzzling is the low biological activity of authentic bicyclomycin under the conditions normally used to assess its activity.

The sample gave proton and ^{13}C n.m.r. consistent with its structure when fresh. However when the compound was left for an eight month period and reassayed, both the activity and

chromatographic homogeneity of the sample had declined, even in the crystalline state.

The results were emphasised by the rapid degradation of isolated samples prior to crystallisation.

In the original references to the antibiotic, it was noted that the antibiotic, in solution, is both acid and base labile but no deterioration of the solid was mentioned.

The inactivity and deterioration of authentic material may indicate that the antibiotic has a limited storage life in the crystalline state.

It is not possible to determine whether the formulated drug is more stable on the available information.

1.5 Mutation studies

Since it is possible that a mutant strain is used for the production of bicyclomycin, some small scale experiments were attempted using S. aizunensis.

A spore suspension of S. aizunensis in water was irradiated with ultraviolet light for 30 mins. The survival rate was calculated from samples cultured in the dark and was ca 3%.

The suspension was diluted and plated under red light conditions and incubated in the dark for 14 days.

However, due to technical difficulties, most of the spores reverted to the parental genotype by the photo-reactivation⁴⁰ process in which light cancels the effect of ultraviolet light.

The purpose of the ultraviolet irradiation is to split the bases in the DNA apart, so that recombination can occur

thus altering the genotype of the microorganism.

However, one morphological mutant was isolated, purified and cultured.

When grown on glucose-asparagine agar⁴, the mutant did not produce the yellow soluble pigment which is a characteristic of the parental genotype.

Colony shape and colour of the aerial mycelium was not consistent with the parent strain.

The mutant strain showed antibiosis against E. coli 9482 and MRE 600, while the parent showed activity against E. coli 9482 but not against MRE 600.

These results would seem to indicate marginal biochemical divergency from the parental strain but further work will have to be carried out in this area to produce a high yielding strain before biosynthetic studies can be initiated.

A recent paper by the original workers supports our view of bicyclomycin biogenesis in that (1-¹⁴C)leucine and (1-¹⁴C)isoleucine gave labelled bicyclomycin in S. sapporonensis cultures.²⁵ Isolation, purification and incorporation values were omitted from this report and, as such, must be regarded with caution until full details are released.

Introduction

The total synthesis of bicyclopiperylin has been reported in the chemical literature. However, the total synthesis of the bicyclopiperylin derivatives (4) and (5) from bicyclopiperylin (1), has been recently accomplished (Scheme 1).

N,N-diacetylbicyclopiperylin was treated with the Grignard reagent (2) to give the allylicene bicyclopiperylin (3). The allylicene derivative in dimethyl ether, however, retains the remaining N-acetyl moiety.

Hydrolysis with p-toluenesulfonic acid in dimethyl ether leads to the bicyclopiperylin (23).

The bicyclopiperylin (23) was converted into the N,N-diacetylbicyclopiperylin (24) in two steps, to give a satisfactory yield of diacetylated bicyclopiperylin.

SECTION 2

Part 2

The amine was then formed in the 4-position with LDA or -70°C . The ketone was prepared and the reaction mixture was treated with glacial acetic acid.

The final N-acetyl moiety was removed by hydrolysis in ethanol.

On treatment of bicyclopiperylin (25) with the Grignard reagent (2) and subsequent hydrolysis, the bicyclopiperylin (26) was obtained.

Hydrolysis of the ester gave the bicyclopiperylin (27) which generates the tetrahydrofuran ring bearing the allylicene moiety.

It should be noted that the bicyclopiperylin (28) is

2.0 Introduction

No total synthesis of bicyclomycin has been reported in the chemical literature. However, the total synthesis of the acid catalysed rearrangement products (4) and (5) from bicyclomycin (1), has been recently accomplished⁹ (Scheme 6).

N,N-diacetyldioxopiperazine is condensed with the aldehyde (A) to give the alkylidene dioxopiperazine (21). Hydrazinolysis of the alkylidene derivative in d.m.f. removes the remaining N-acetyl moiety.

Transketalisation with p-toluene sulphonic acid in ethylene glycol leads to the spirodioxopiperazine (23).

The dioxopiperazine is converted into the N,N-diacetyl compound by acetylation in two steps, to give a satisfactory yield of diacetylated product (24).

The anion was then formed in the 5-position with LDA at -76°C . The ketone was then introduced and the reaction mixture warmed to -40°C and quenched with glacial acetic acid.

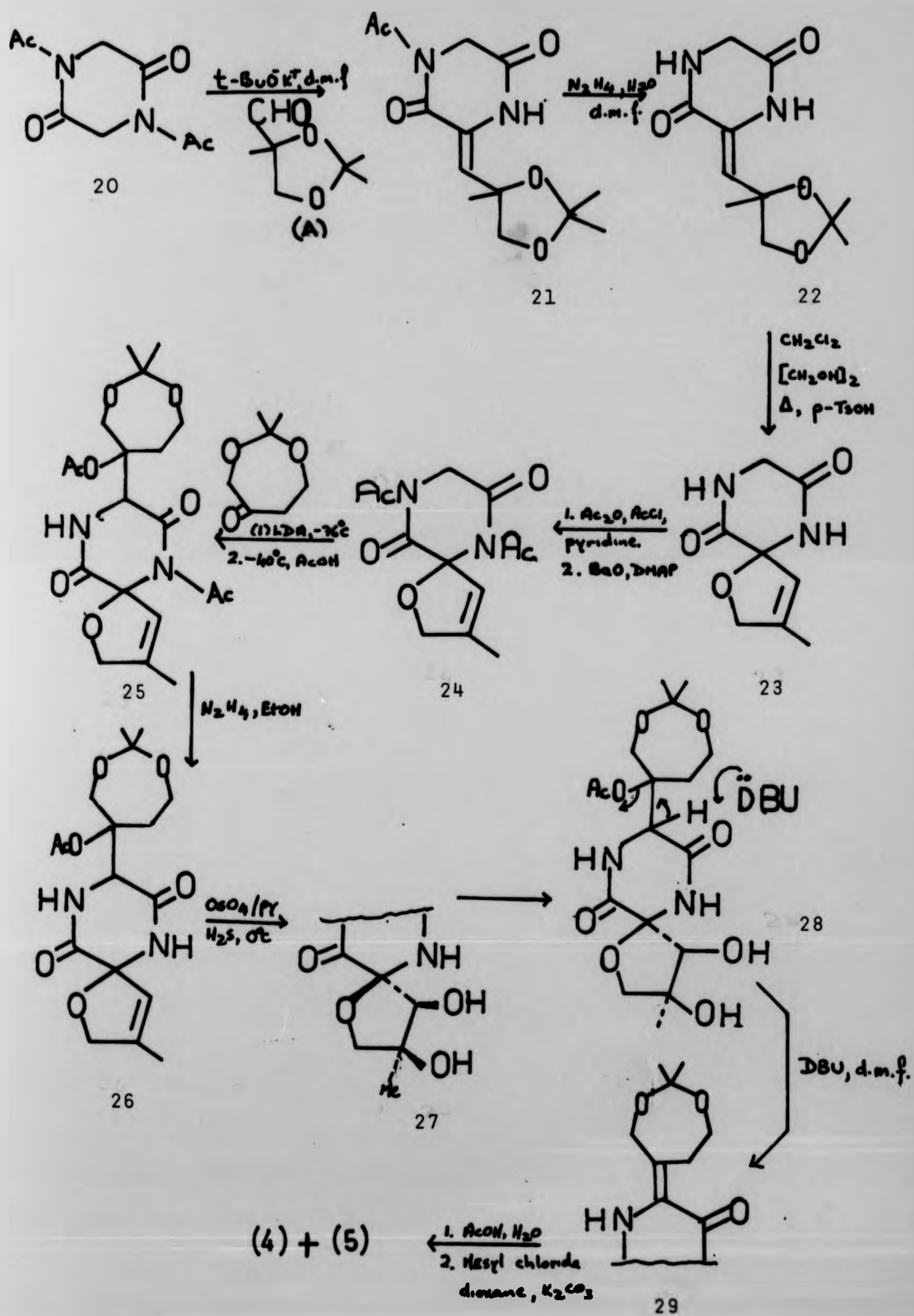
The final N-acetyl function was then removed by hydrazinolysis in ethanol.

Osmium tetroxide in pyridine/ H_2S generated the cis-diol (27) and removal of the 5 proton with DBU and subsequent elimination of the acetyl group generated the alkylidene compound (29).

Hydrolysis of the ketal gave the tetraol which then generates the tetrahydrofuranoid ring bearing the exomethylene moiety.

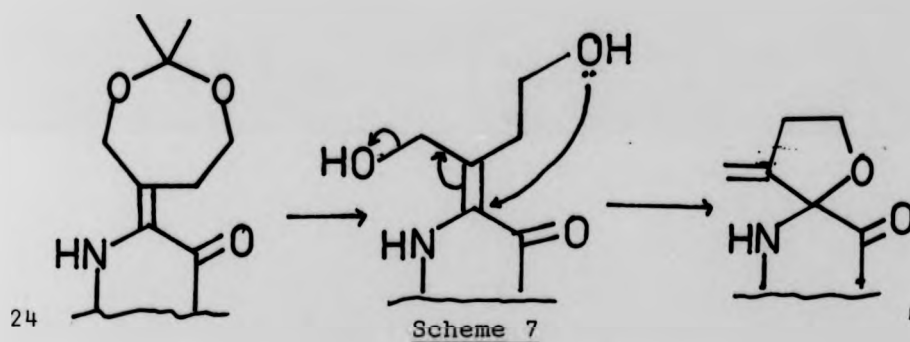
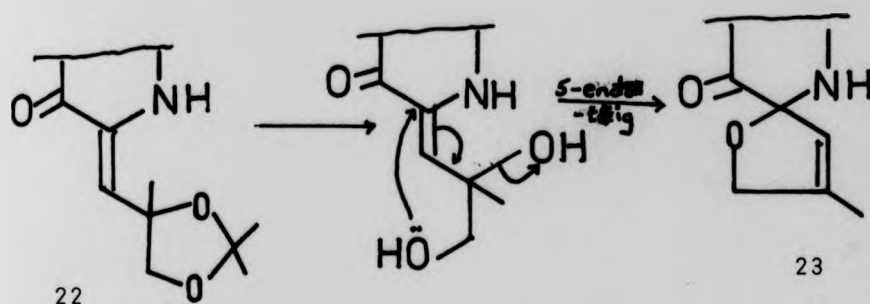
would seem to

It should be noted that both these reactions¹⁰ occur via a 5-endo-trig process (Scheme 7) which Baldwin²⁸ states is



Scheme 6

an unfavourable process.



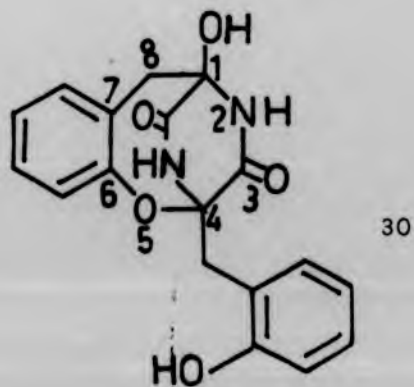
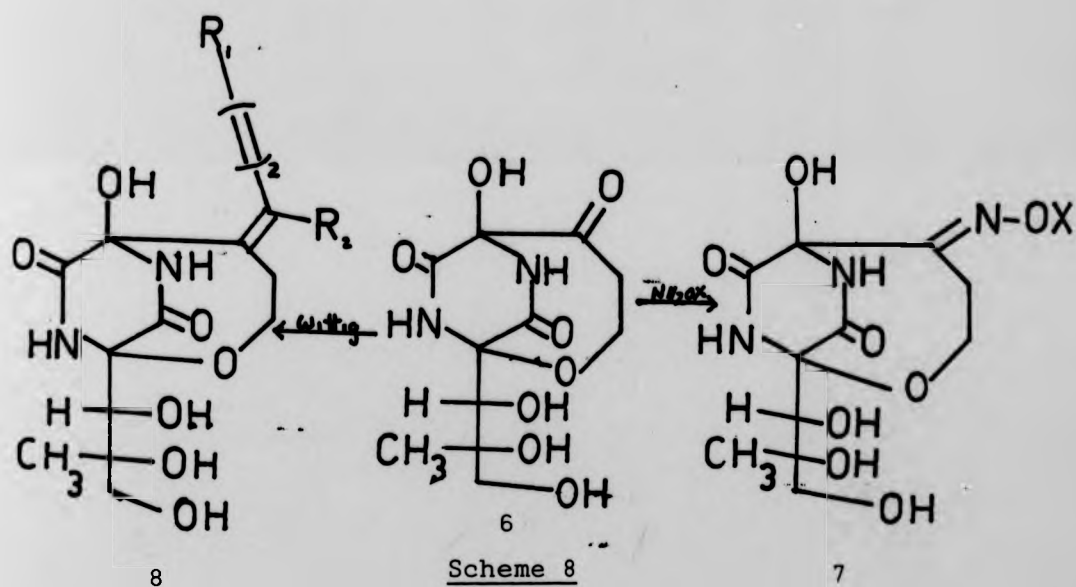
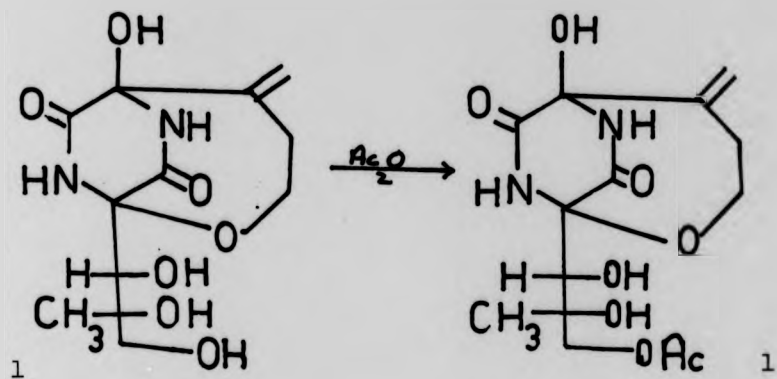
As such, these ring closures represent two examples of closures which do not obey Baldwin's rules²⁸.

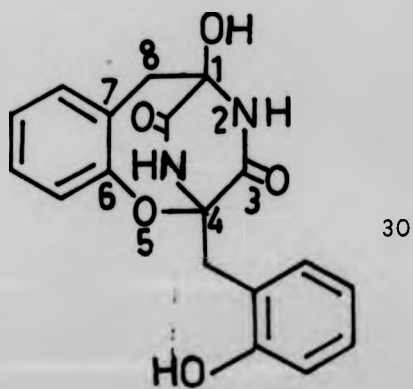
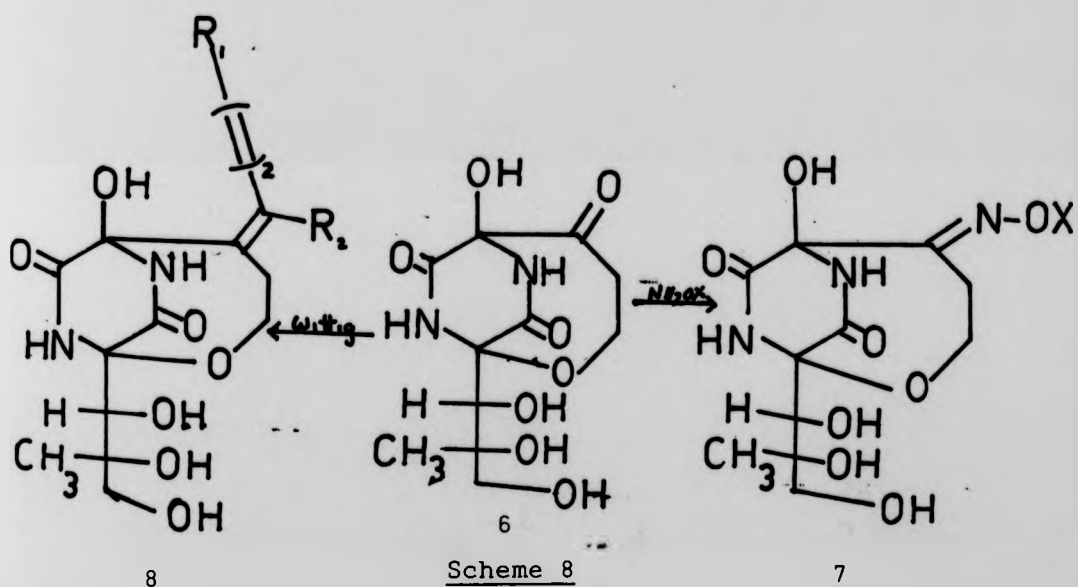
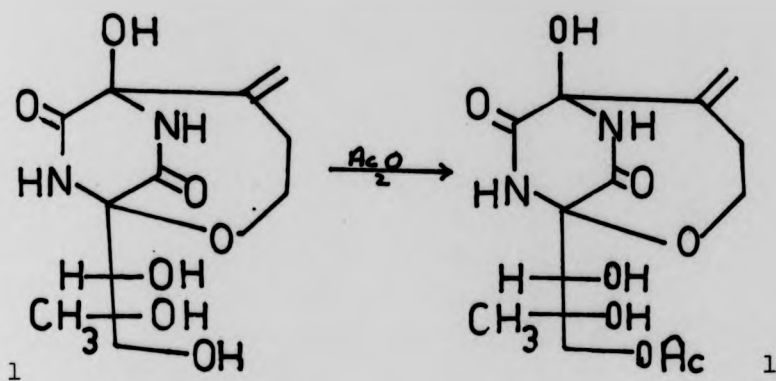
The compounds were then converted to their acetonides and separated by chromatographic means.

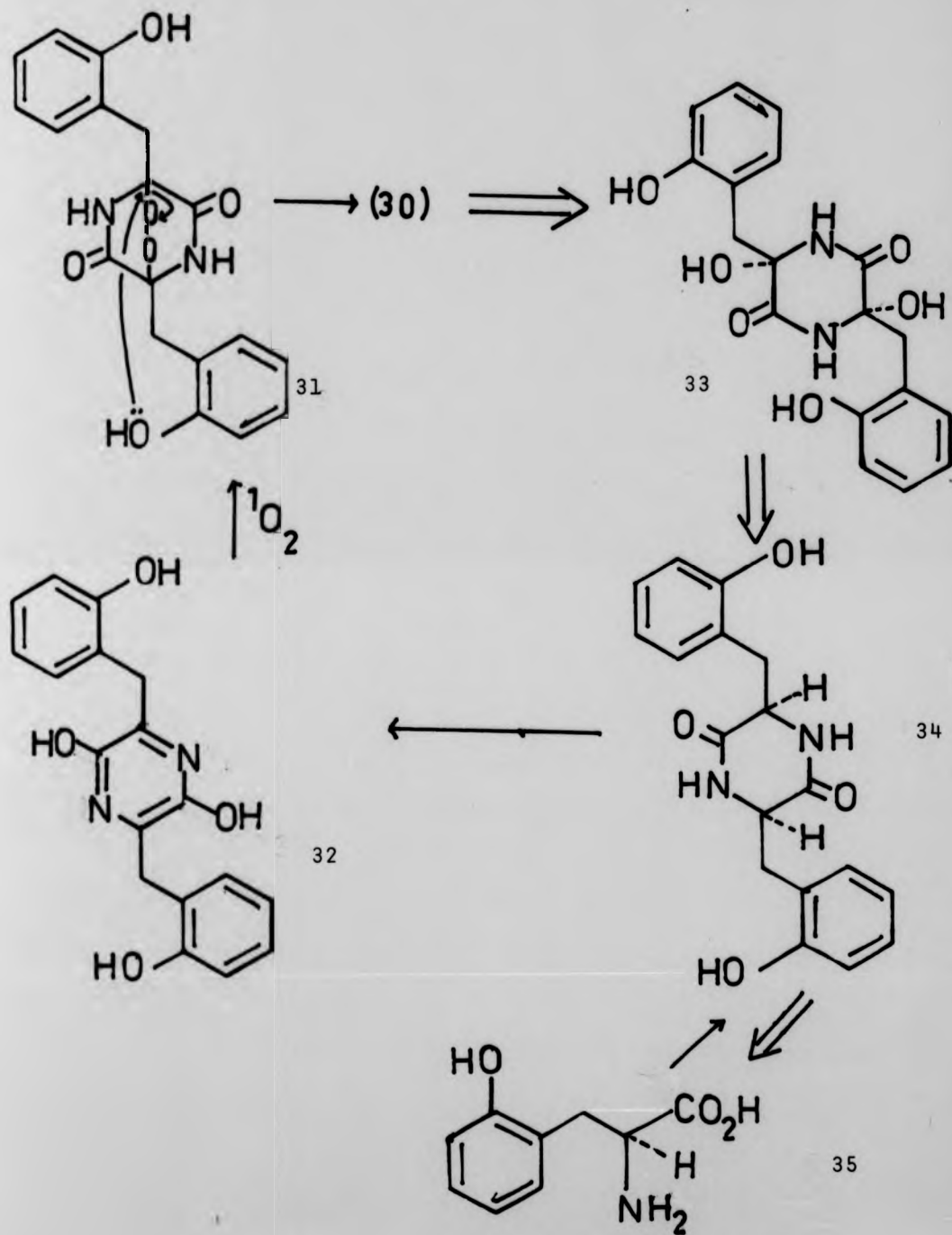
Conversion of bicyclomycin (1) to the rearrangement products (4) and (5) was accomplished by heating at 100°C for 15 mins in the presence of 0.1N perchloric acid⁹.

Some authors consider the acid stability of bicyclomycin to be unusual, since it possesses an inherently unstable hemiaminal ring system⁴¹.

If acidic material was present in the bicyclomycin fractions then this might possibly explain the degradation of the samples during storage.







Scheme 9

The same authors suggest that synthetic routes to bicyclomycin should be constructed so that the energy minimum represented by (4) and (5) may successfully be avoided⁹.

Partial syntheses of bicyclomycin are also absent from the chemical literature and this is not surprising, for the reason outlined previously.

As previously reported, bicyclomycin can be used to produce many biologically active derivatives¹⁰, and more recently 5-norbicyclomycinone (6) shows promise of being a suitable nucleus for analogue production^{17,18,19}.

Some representative reactions are shown in Scheme 8.

In our synthetic strategy, it was decided that a compound of type (30) should be our ultimate synthetic goal.

The rationale behind this strategy may become clearer to the reader when we consider the antithetic analysis²⁰ of the compound.

This involves initial disruption of the C(4)-O(5) bond. The rough outline for our synthetic strategy is depicted with the antithetic analysis in Scheme 9.

The synthetic scheme was based on the biosynthetic reactions proposed earlier in this section and is designed to be biomimetic in nature.

The target compound in this strategy is the tetraol (32) which on photo-oxygenation and ring closure using an alkoxide anion as proposed in the biogenetic scheme 4, should result in the formation of (30).

The route chosen for the synthesis of the model (30) again reflects a biomimetic approach in that synthetic intermediates were sought, which were analogous to the proposed

biosynthetic intermediates on the route leading to bicyclomycin.

A highly functionalised dioxopiperazine (34) is formed by the condensation of an amino acid which is subsequently aromatised to the corresponding 2,5-dihydroxypyrazine (32). It was hoped that this derivative would then react with singlet oxygen to form the cyclic-epi-dioxopiperazine (31), and the final stage, as in the biosynthesis (Scheme 4) involves the opening of the peroxide via attack by an oxyanion anion.

The choice of a symmetrical system was reasoned to be of prime importance since it would mean the dimerisation of a single amino acid in the first reaction. If two differing amino acids had been used, formation of the dioxopiperazine would have required two steps i.e. formation of a dipeptide from amine-protected and carboxylic acid protected amino acids and subsequent removal of the amino protecting group with concomitant attack on the protected acid moiety.

Similarly, a symmetrical intermediate increases the possibility of the ring closure reaction in the final step since either of the phenolic groups may participate in the final cyclisation step.

The symmetrical system also facilitates the protection of sensitive functionalities while transformations are being carried out on other parts of the molecule.

The main reasons for choosing the model (32) for synthesis are that it should provide an excellent system for probing the possible biosynthetic reactions which have been proposed for bicyclomycin, as well as providing a reasonably simple route to bicyclomycin-type compounds for biological evaluation.

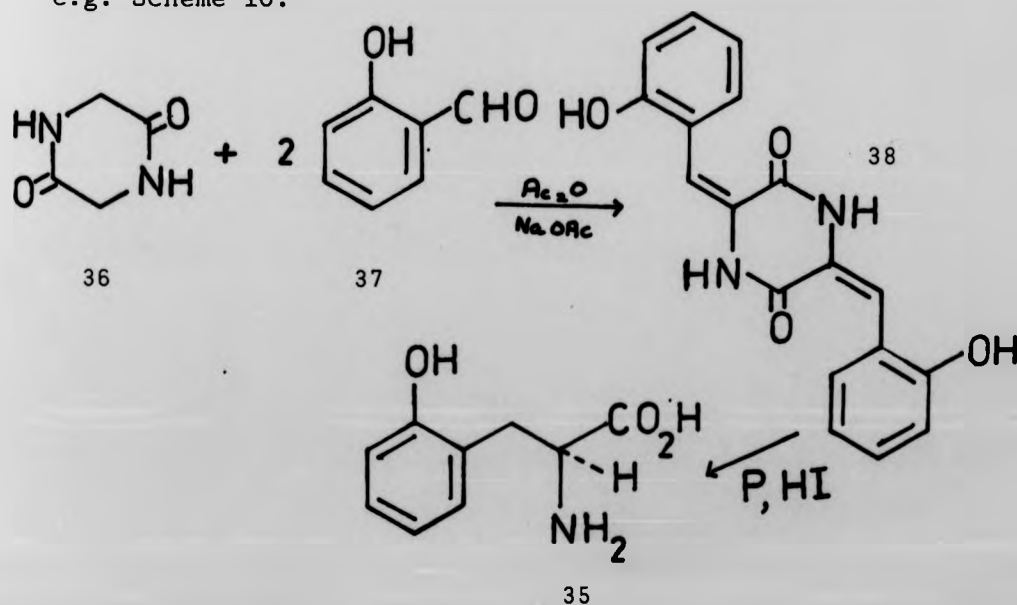
The synthetic scheme should also provide information on the chemistry of highly functionalised 2,5-dihydropyrazines and their cyclic peroxides.

2.1 Synthetic routes to the target intermediate
i.e. 3,6-di-(2'-hydroxybenzyl)-2,5-dihydropyrazine (32)

(a) From ortho-tyrosine

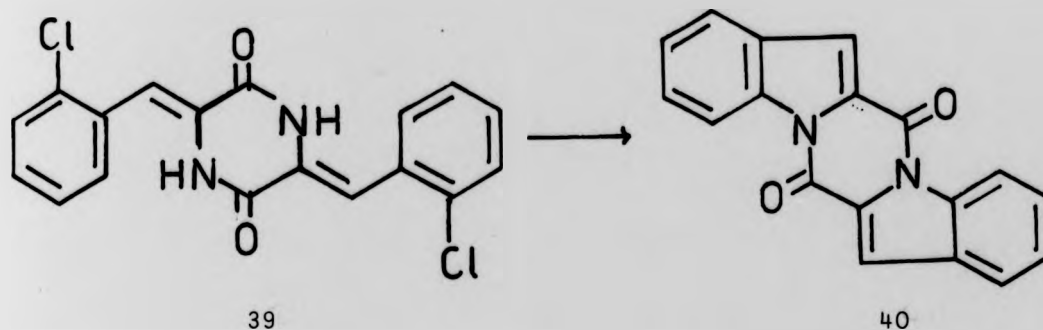
In the antithetic approach shown in Scheme 9, ortho-tyrosine is the key starting material and, as such, it was necessary to utilise a series of chemical transformations, which would lead to ortho-tyrosine in high yield.

Most synthetic routes to o-tyrosine have made use of the acidity of activated methylene groups adjacent to amide groups, and exploited this⁴² feature by condensing aromatic aldehydes with amide containing systems in a basic medium, e.g. Scheme 10.



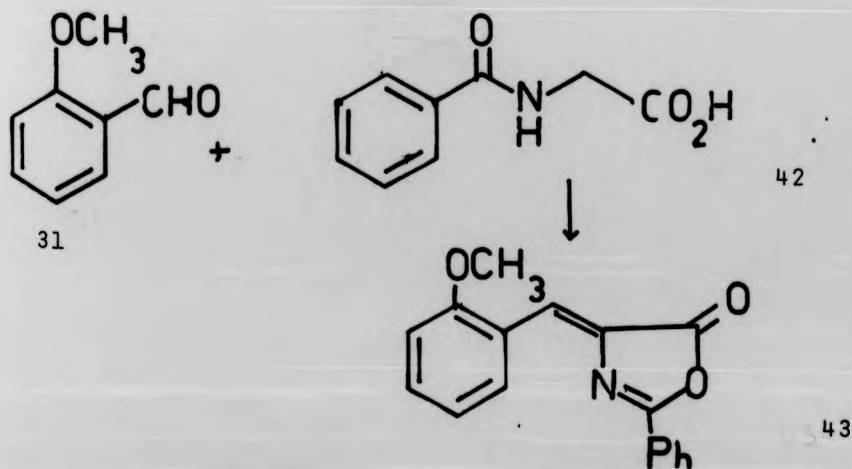
Scheme 10

This type of approach has been used to produce compounds resembling metabolites of *Aranotinus* sp. eg. (40) and other naturally-occurring dioxopiperazines⁴³. Scheme 11.



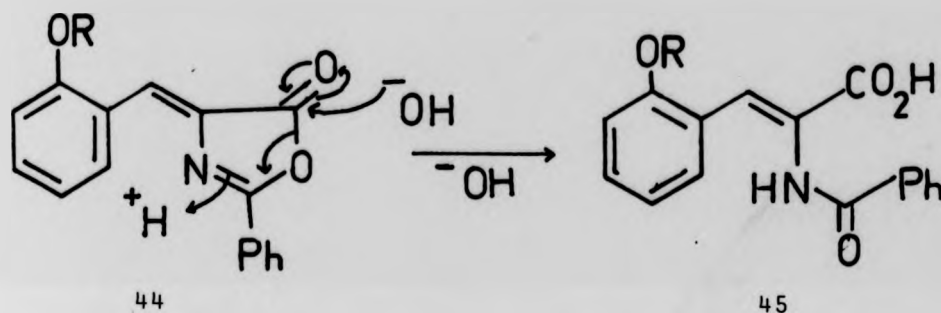
Scheme 11

A more common, if somewhat older^{44,45}, approach is to react an aromatic aldehyde such as anisaldehyde with an N-protected amino acid such as benzoyl glycine under basic conditions to form the azlactone intermediate (43). Scheme 12.



Scheme 12

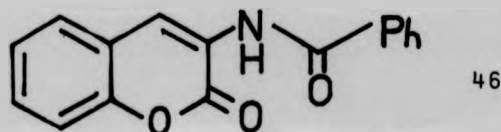
The treatment of an azlactone with a mildly basic solution usually results in the formation of an α,β -dehydro-amino acid⁴⁶ derivative e.g. (45), Scheme 13.



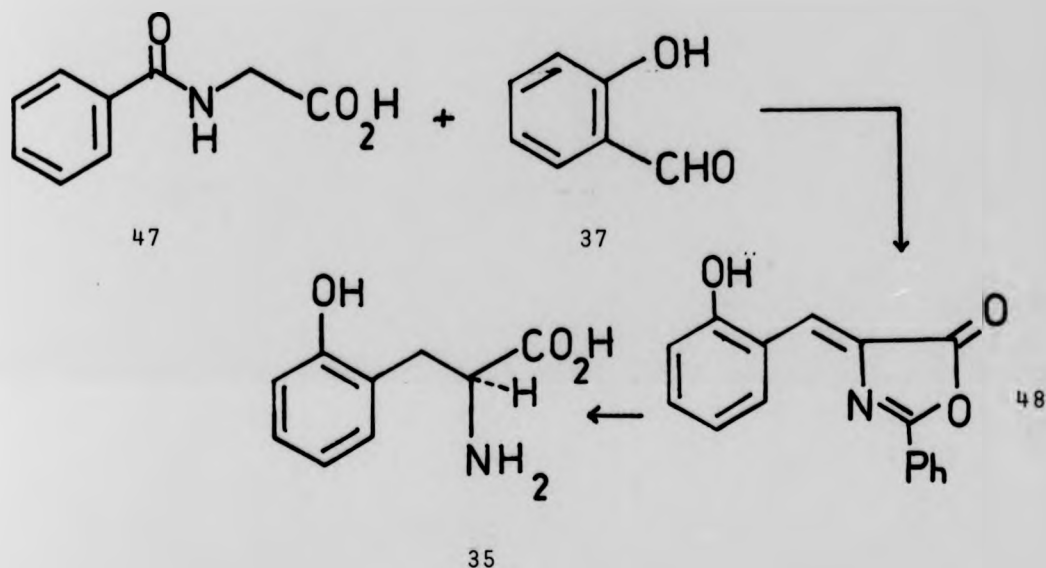
Scheme 13

The parent amino acid is then formed by reduction and hydrolysis of the α,β -dehydro analogue. This reduces the double bond and deprotects the amino functionality in one simultaneous process.

One disadvantage is evident in the latter series of transformations, in that coumarins⁴⁵ may be produced as a minor by-product e.g. (44) when $R' = OAc$ generates (46).



Our synthetic approach to *o*-tyrosine is a composite route utilising the best aspects of both approaches while maintaining high yields, Scheme 14.



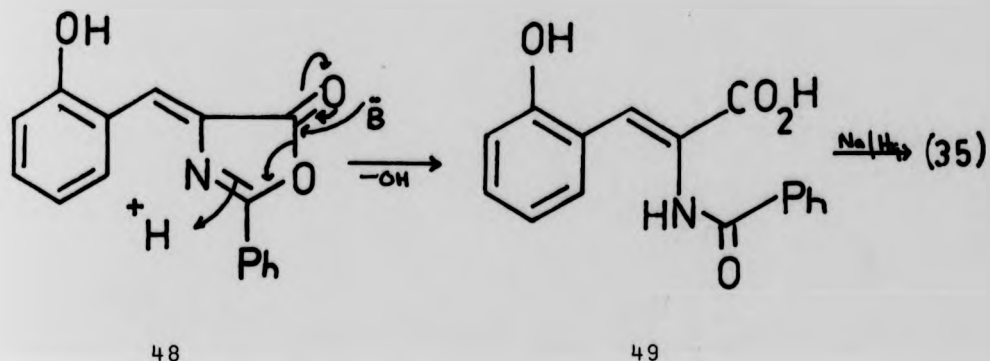
Scheme 14

The overall yield from glycine is 38.0%. Condensation of salicylaldehyde with benzoyl glycine⁴⁷ was accomplished using fused sodium acetate and acetic anhydride. The intermediate aromatic azlactone (48) is reduced in good yield to racemic *o*-tyrosine (35) using red phosphorus in hydroiodic acid, containing some acetic anhydride.

T.l.c. of the crystalline amino acid, in butanol:acetic acid:water and subsequent visualisation with ninhydrin spray reagents, showed that the compound was homogeneous.

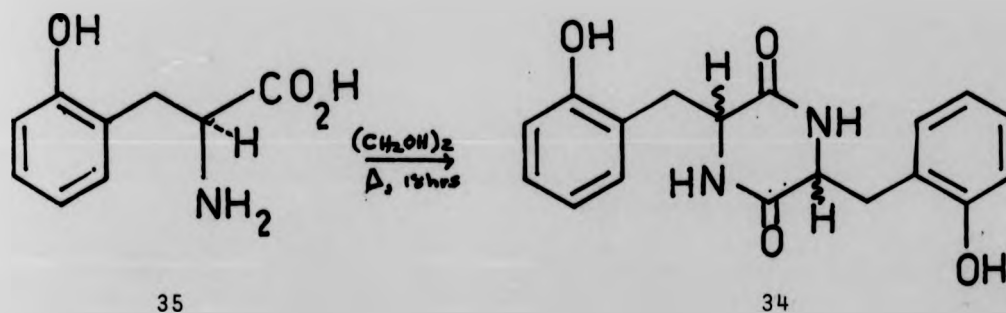
A parallel route in which the azlactone was taken to the α,β -dehydro amino acid⁴⁷ (49) and finally reduced by sodium

amalgam⁴⁸, also gave the desired product (35) but in low yield (17%), Scheme 15.



Scheme 15

The next product in the antithetic strategy is the dioxopiperazine (34) formed by the condensation of *o*-tyrosine with itself. This compound was synthesised by cyclodehydration of *o*-tyrosine in refluxing ethylene glycol. The dioxopiperazine was obtained in 20% yield after recrystallisation from glacial acetic acid, Scheme 16.

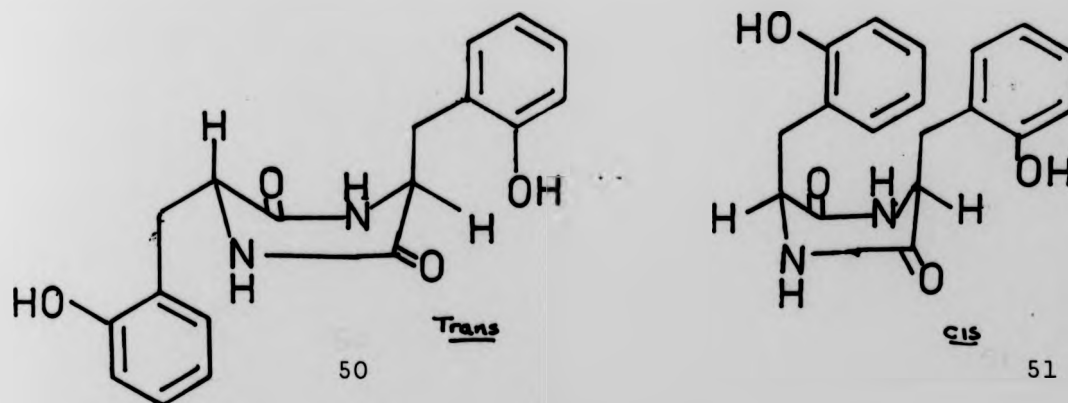


Scheme 16

The low yield at this step is thought to be due to polymer formation where amide bonds have been formed between different molecules.

The recrystallised material gave satisfactory micro-analytical and spectroscopic data consistent with the structure (34).

T.l.c. confirmed the presence of two diastereoisomeric products in the recrystallised material i.e. the cis and trans diastereoisomers (50) and (51).



The diastereoisomers did not vary in intensity or ratio in four different t.l.c. systems using ninhydrin as a visualisation reagent.

Since the antithetic analysis would suggest that a 2,5-dihydroxypyrazine system would be an acceptable intermediate, the formation of cis and trans diastereoisomers does not create a problem.

The next two transformations on the dioxopiperazine ring convert it into an aromatic system where the stereochemical features of the ring are common for the cis and trans isomers.

Since a subsequent step involved alkylation of the amide carbonyl, it was necessary to protect the phenolic groups pendant from the aromatic ring.

Selective alkylation of the two phenolic groups proved difficult in the initial studies since acetylation was either incomplete or also produced N-acetylated species. This may be a reflection of the stereochemical constraints in the cis isomer where the phenolic moiety may be shielded within the 'boat' configuration of the dioxopiperazine ring.

Selective acetylation was achieved using dry acetic anhydride in dry dimethyl sulphoxide with a catalytic quantity of iminazole.

Again t.l.c. analysis of the recrystallised product showed the presence of two, equal intensity, diastereoisomeric spots.

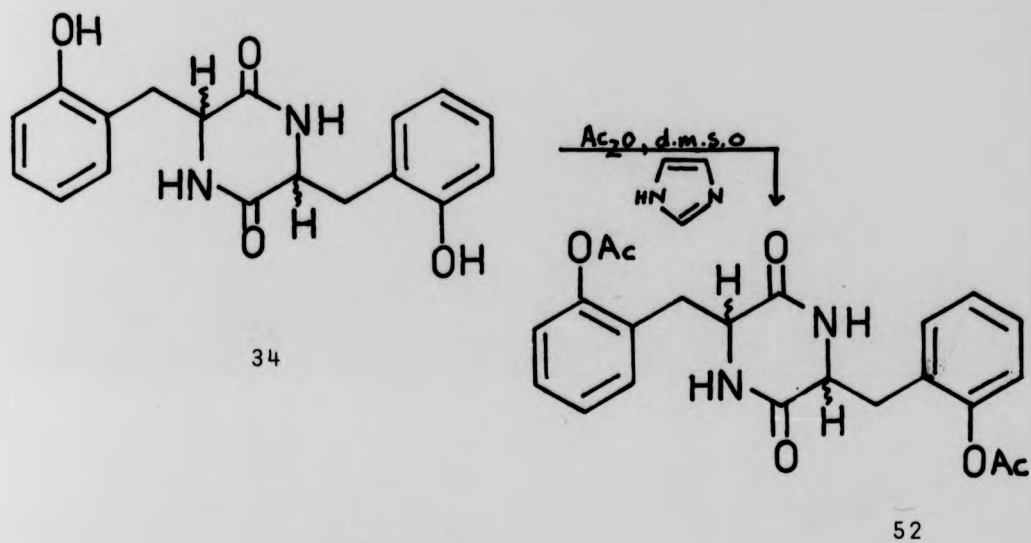
However, the acetylation reaction (Scheme 17) gave, in the best instance, only a 60% yield. Again this may be due to stereochemical constraints within the systems.

Spectroscopic data and microanalytical results were consistent with the structure (52).

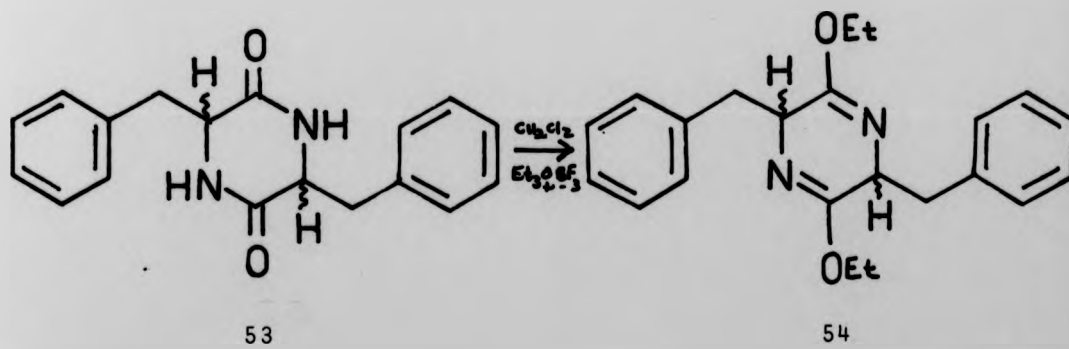
Model studies had shown that 3,6-dibenzyl-2,5-dioxopiperazine (53) prepared from D,L-phenylalanine by an analogous route also existed as a diastereoisomeric mixture⁴⁹.

Similarly it had been shown that this mixture could be converted smoothly to the bis-imidate (54) by reaction with four

equivalents of Meerweins salt⁴⁹⁻⁵¹ (triethyloxonium tetrafluoroborate) in dichloromethane (Scheme 18).

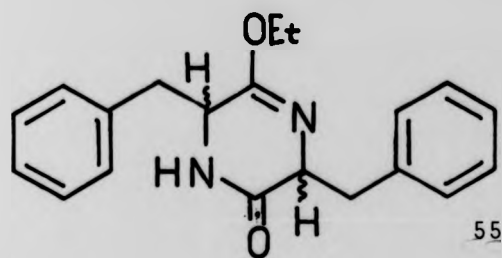


Scheme 17

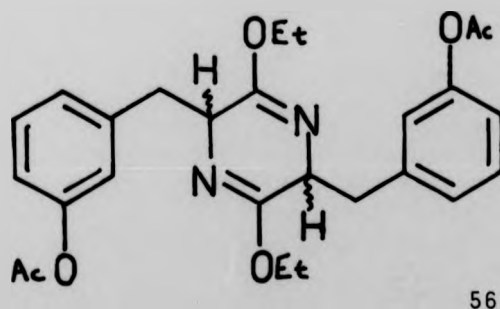


Scheme 18

The mono-imidate (55) was formed⁴⁹ as a minor product and was removed by fractional crystallisation.



Reaction of the acetylated intermediate (52) with Meerweins salt under identical conditions gave the corresponding bis-imidate (56) in 89% yield.

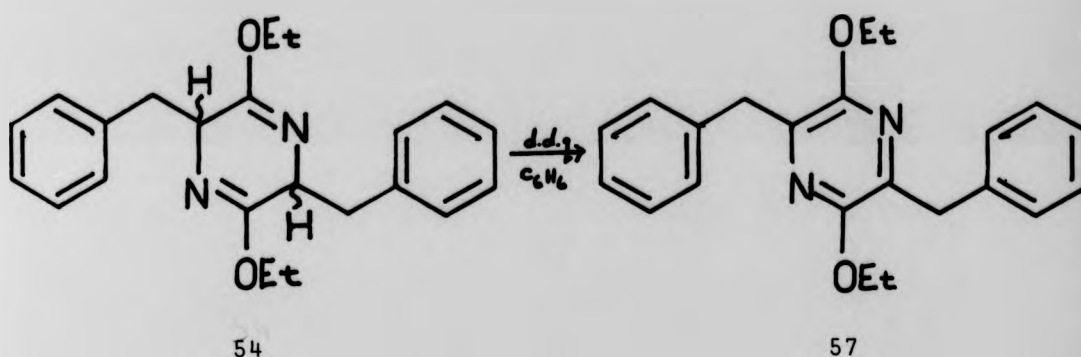


Microanalysis and spectroscopic data were consistent with the structure proposed i.e. (56).

Model studies with 3,6-dibenzyl-2,5-diethoxy-3,6-dihydropyrazine (54) had shown that aromatisation of the ring could be achieved using d.d.q. (dicyano-dichloro-benzoquinone)

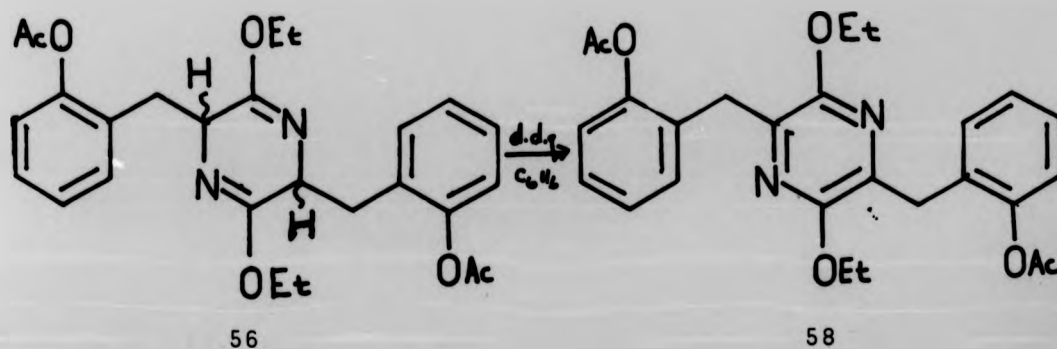
or σ -chloroanil (tetrachlorobenzoquinone) in a neutral aromatic solvent⁵². Yields of the pyrazine (57) were 60-75% and 40% respectively (Scheme 19).

Aromatisation of the ring was also demonstrated by the loss of the multiplet at τ 7.3-8.4 (2H) for the dihydropyrazine protons.



Scheme 19

Conversion of the more highly functionalised bis-imidate (56) to the corresponding pyrazine (58) was achieved smoothly in 76% yield using d.d.q. in dry benzene (Scheme 20).

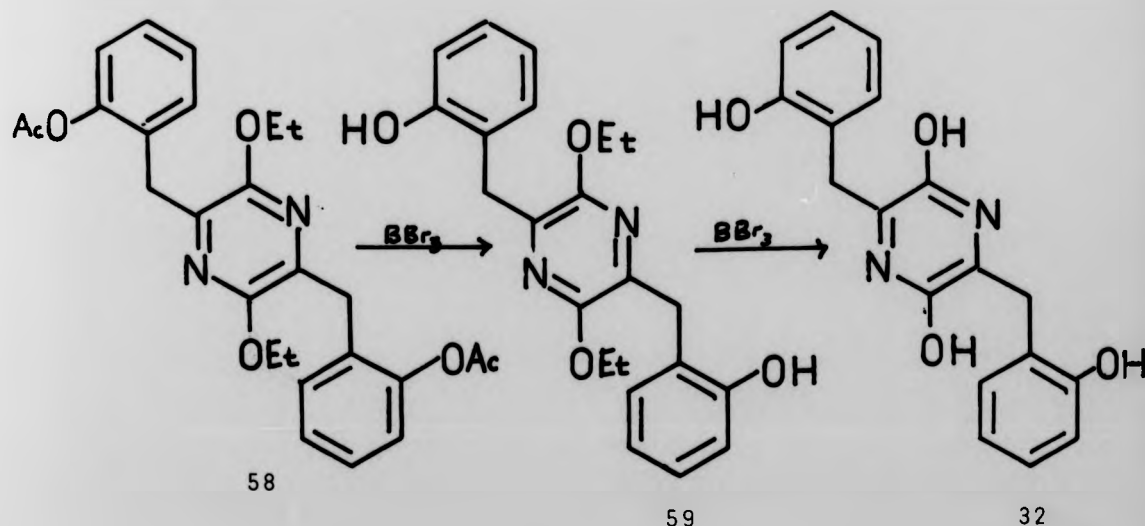


Scheme 20

In compound (56), the benzylic protons and dihydropyrazine protons form a 6H multiplet in the n.m.r. at τ 6.1 but on conversion to the fully aromatic pyrazine (58), this multiplet collapses to a 4H singlet at τ 6.2 which corresponds to the benzylic protons alone.

Other spectroscopic and micro analytic data were consistent with the proposed structure (58).

Initially the synthetic strategy involved simultaneous dealkylation of the two aryl-alkyl ether functionalities and deacetylation of the substituted phenols in the intermediate (58). (Scheme 21).

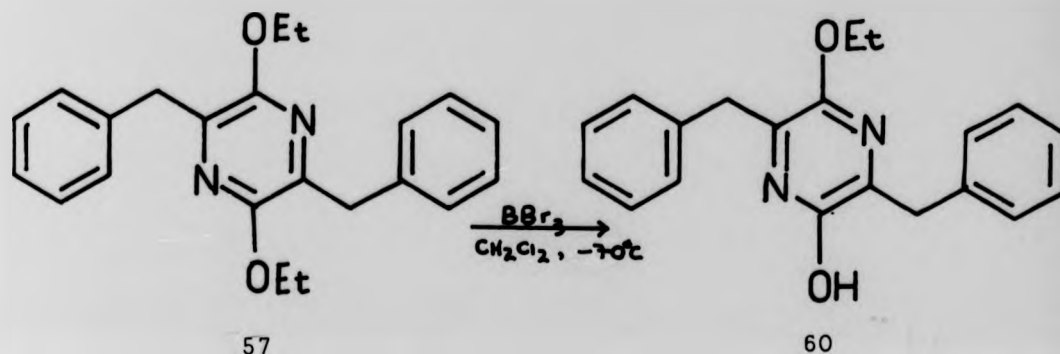


Scheme 21

The target compound (32) would be generated in one step thus increasing the direct yield from (58) since only one reaction is used and the number of transfers will be reduced.

The reagent selected for this transformation was boron tribromide - a standard ether cleavage reagent. When used in an excess it was considered that this reagent might also deacetylate the intermediate.

Previous work using the model system i.e. 3,6-dibenzyl-2,5-diethoxypyrazine (57) had shown however, that even in excess and at low temperature, complete dealkylation was not accomplished (Scheme 22).



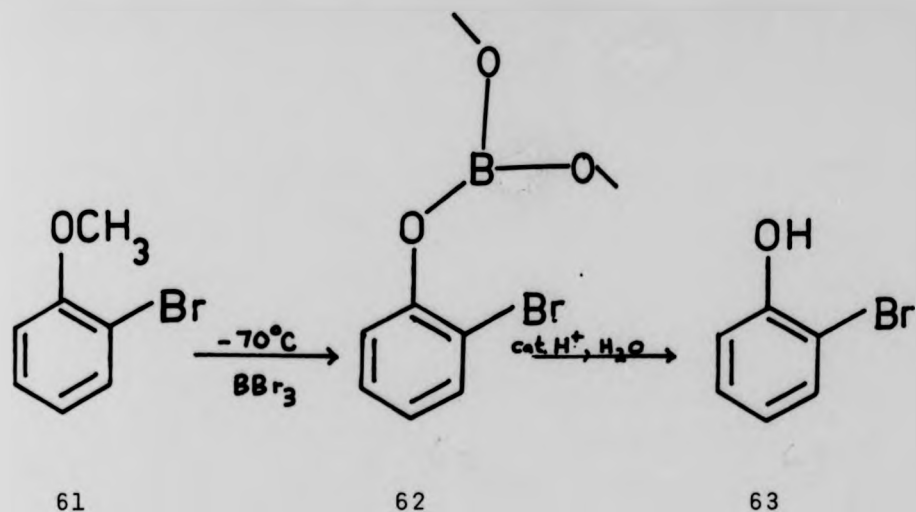
Scheme 22

The major product was identified as 3,6-dibenzyl-2-ethoxy-5-hydroxypyrazine (60) on the basis of the decreased integral for the ethyl portions of the n.m.r. spectrum.

Additional information was obtained by measurement of the accurate mass of the molecular ion i.e. 320.1525 (elemental fit C₂₀H₂₀N₂O₂), and also by the appearance of an absorption at 3500-3200 cm⁻¹ in the infrared spectrum.

Under normal conditions, the cleavage of an aryl-alkyl ether by boron tribromide requires 3 equivalents of the ether for each molecule of boron tribromide⁵³ and subsequent formation of a boron complex. The complex is quenched by water and produces the free phenolic derivative (Scheme 23).

Similar reactions of 3,6-dibenzyl-2,5-diethoxypyrazine with boron tribromide at -40°C and room temperature also gave the mono-dealkylated compound (60) as the major product.



Scheme 23

However, further treatment with a tenfold excess of boron tribromide in dry dichloromethane gave a canary yellow solid which was insoluble in chloroform, carbon tetrachloride and dimethyl sulphoxide.

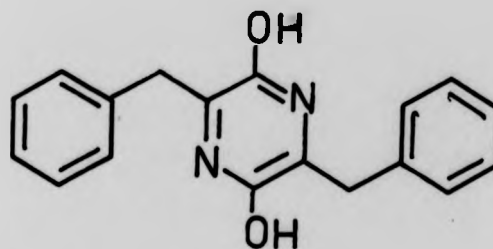
The n.m.r. spectrum showed the absence of the ethyl resonances and the infrared spectrum showed increased absorption in the $3400\text{--}3200\text{ cm}^{-1}$ region.

Mass spectral results were ambiguous since the molecular ion was of very low intensity.

Reaction of this material with four equivalents of Meerweins salt in dry dichloromethane gave 3,6-dibenzyl-2,5-diethoxypyrazine (57) as the major product.

On the basis of this finding and the spectroscopic data, the structure was tentatively assigned as 3,6-dibenzyl-2,5-dihydroxypyrazine (64).

The only contradictory evidence is that the melting point is lower than that reported for the compound, *i.e.* 165°C [Lit. 260°C] ^{54,52}.



64

One possible explanation may be that dimethylformamide has been occluded into the crystalline structure of the material during recrystallisation from this solvent.

The assignment of structure for this compound will be dealt with later in this section, in the light of subsequent work.

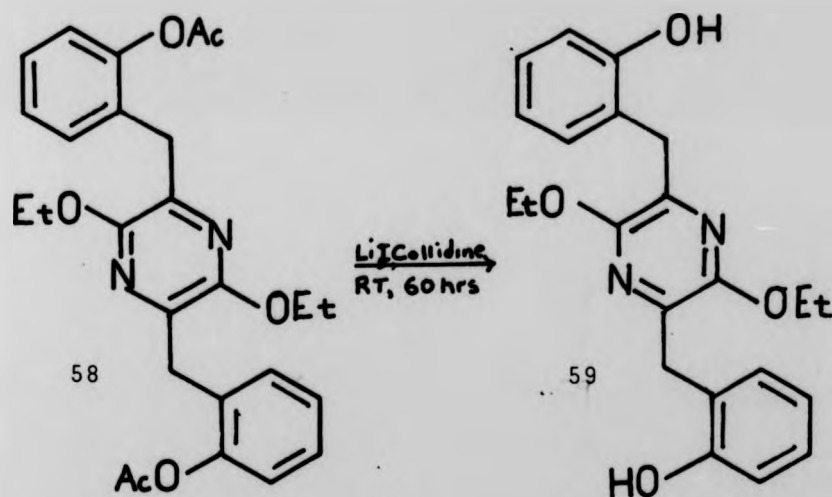
Since there was now some evidence for complete dealkylation of the model diethoxypyrazine system (57), it was decided to evaluate the use of boron tribromide with the parent compound (58).

The parent compound, 3,6-di-(2'-acetoxybenzy-)-2,5-diethoxypyrazine (58), when reacted with boron tribromide, under a variety of different temperatures, gave starting material as the major product along with four unidentified products, all in low yield.

On reviewing the literature, it was noted that lithium iodide, under various conditions, will facilitate ether and ester cleavage reactions.

Accordingly, a small aliquot of the parent compound (58) was reacted with anhydrous lithium iodide in collidine⁵⁵ at room temperature.

Spectroscopic data confirmed the structure of the major product as starting material and the other reaction product (ca. 14%) as the bis-phenol derivative (59). N.m.r. spectroscopy confirmed the absence of the two acetyl groups (Scheme 24).



Scheme 24

Since neither of these reagents provided the desired simultaneous dealkylation and deacetylation reaction, it was decided to produce the target compound (32) by two discrete steps.

Since deacetylation of the compound seemed, at first sight, to be the easier of the two transformations, work was concentrated on this area initially.

Several reagents which have literature precedent for deacetylation reactions were investigated, and the results are shown in Table 8.

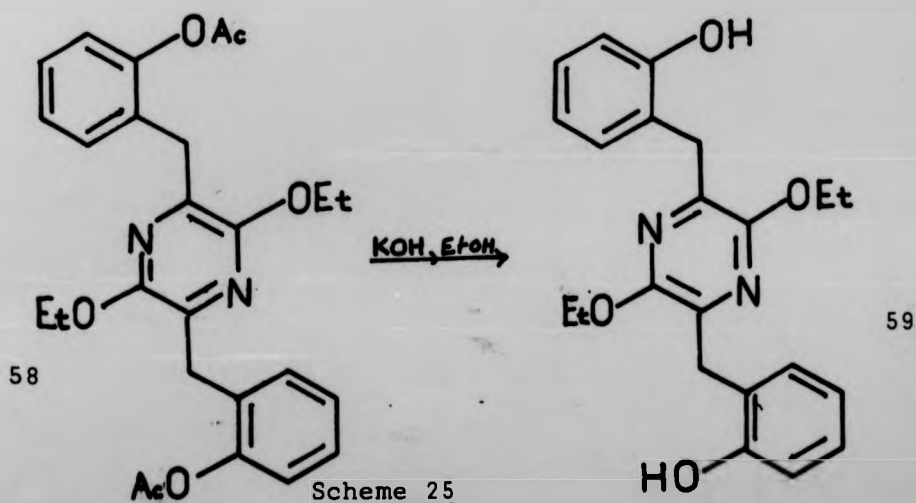
TABLE 8

Deacetylation of 3,6-di-(2'-acetoxybenzyl)-2,5-diethoxy pyrazine (58)

Reagent	Reaction Time	Temperature	% deacetylated product
Lithium iodide/ collidine ⁵⁵	60 hours	25°C	14%
Hydrazine hydrate ⁵⁶	36 hours	"	30%
ethanolic KOH 2 eq.	24 hours	"	86%
ethanolic KOH 4 eq.	60 hours	"	72-90%

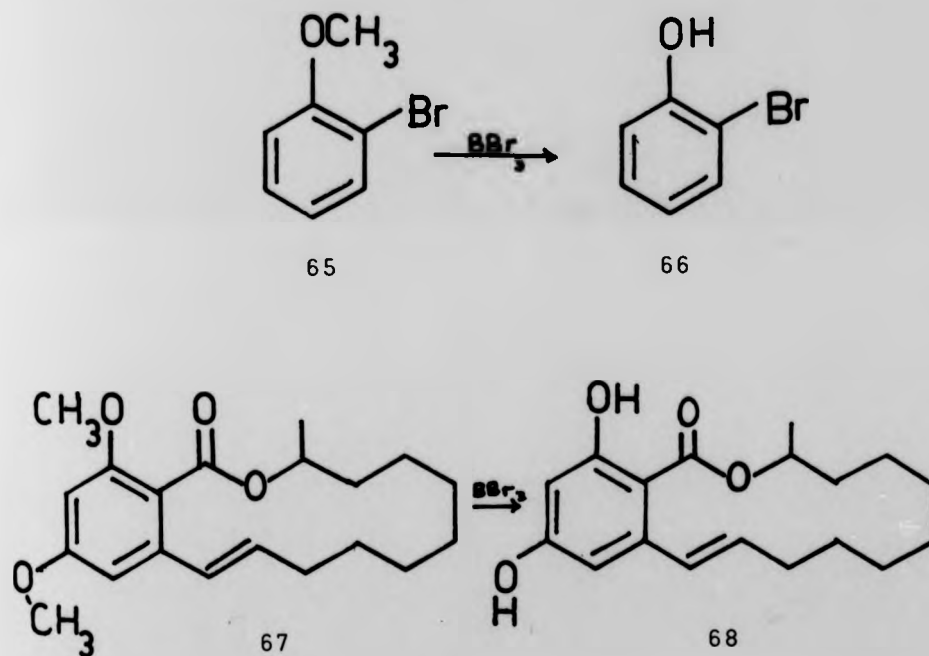
As can be seen from Table 8, ethanolic potassium hydroxide seems to be the reagent of choice on the basis of yield and on absence of other reaction products.

In a large scale reaction, the parent compound (58) was stirred with four equivalents of ethanolic potassium hydroxide for two hours. T.l.c. analysis showed the absence of starting material. This reaction provided the desired deacetylated product (59) in 93% isolated yield (Scheme 25).



Our attention now focussed on the cleavage of aryl-alkyl ether moiety, to complete the synthesis of the target intermediate (32).

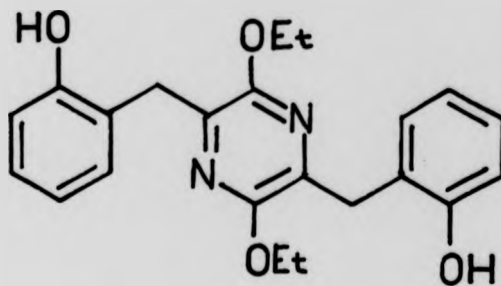
Boron tribromide was selected initially as the reagent of choice since it is well documented as a reagent for the dealkylation of alkyl-aryl ethers^{57,58} e.g. Scheme 26.



Scheme 26

Treatment of 3,6-dia(2'-hydroxybenzyl)-2,5-diethoxypyrazine (58) pyrazine (59) with tenfold excess of boron tribromide at -78°C , warming to room temperature and quenching the intermediate in water gave only starting material.

The search for a suitable dealkylation reagent was continued using 2,5-diethoxy-3,6-dibenzyl pyrazine (57) as a model for the more complex system (59).



59

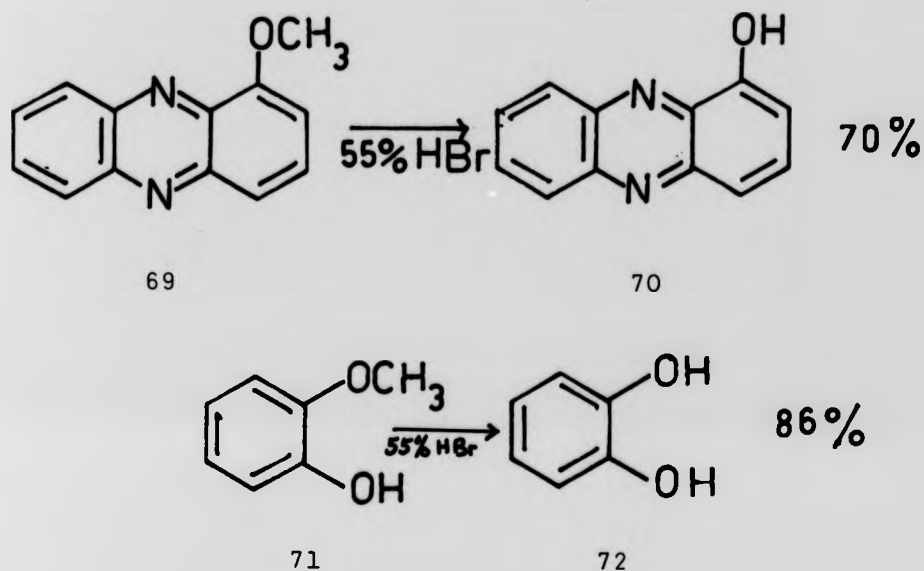
Prolonged heating in the presence of dilute mineral acid did not result in the production of the desired 2,5-dihydroxypyrazine derivative (64).

Similarly hydrolysis of the remaining ether moiety in the previously isolated monohydroxypyrazine (64) in the mineral acid did not occur, and starting material was recovered in high yield.

Treatment of alkyl-aryl ethers^{59,60} e.g. (69) and (71) with 55% HBr has been shown to result in good yields of the corresponding phenol derivatives and these offer a good analogy for the case under investigation (Scheme 27).

However treatment of 2,5-diethoxy-3,6-dibenzylpyrazine (57) with 55% HBr again resulted in the isolation of starting material as the major product, together with several minor products which remain unidentified.

Reaction of 2,5-diethoxy-3,6-dibenzylpyrazine (57) with HBr in glacial acetic⁶¹ acid gave an identical result, and starting material was recovered in high yield.



Scheme 27

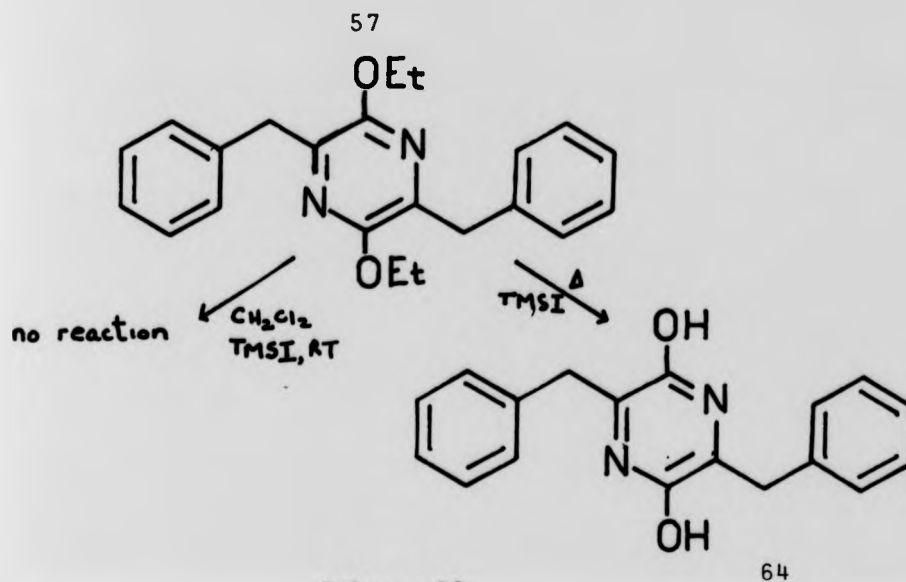
The target intermediate (32) seemed to be inaccessible by conventional chemical methods.

However, at about this time, Jung and Lyster reported^{62,63,64} a convenient synthesis of iodotrimethylsilane, and had shown that this reagent could be used in ester hydrolysis reactions⁶⁵⁻⁷⁰ to give quantitative yields of the corresponding acids.

This reagent had also been used by these authors to effect quantitative alkylation of simple alkyl-aryl ethers⁶³.

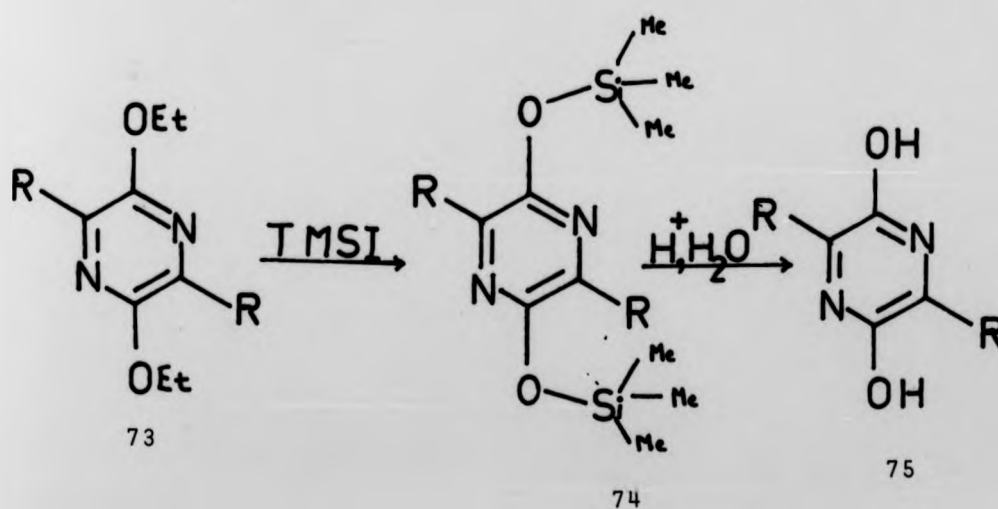
More recently a mild method for *in situ* generation of iodotrimethyl silane has also been reported⁷¹.

Reaction of 2,5-diethoxy-3,6-dibenzylpyrazine (57) with iodotrimethylsilane in dry dichloromethane (1.5 equiv.) produced only starting material, but when the pyrazine (57) was refluxed in neat iodotrimethylsilane, the desired 2,5-dihydroxypyrazine (64) was obtained in 95% yield (Scheme 28).



Scheme 28

The reaction probably proceeds⁶² via the TMS-ether which is decomposed using a proton source (*i.e.* water plus a trace of HCl) (Scheme 29).



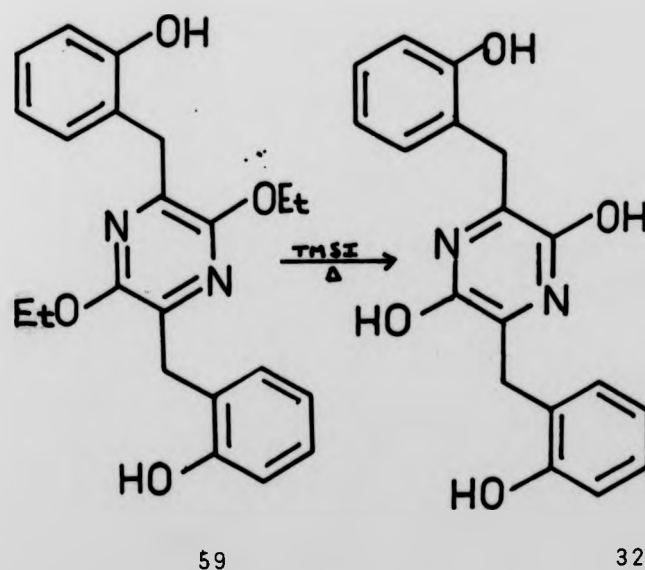
Scheme 29

Identification of the reaction product as 2,5-dihydroxy-3,6-dibenzylpyrazine (64) was accomplished by conventional means.

The molecular ion in the mass spectrum of (64) is the base peak and gave an accurate mass of 292.1212 ($C_{18}H_{16}N_2O_2$ requires 292.1212). Microanalysis, proton and carbon n.m.r. confirmed the structure as (64).

The melting point of the compound was 256.5-259.0°C and is close to the reported literature value i.e. 258-260°C.

Reaction of iodotrimethylsilane (TMSI) with the compound in our model series i.e. 3,6-di-(2'-hydroxybenzyl)-2,5-diethoxypyrazine (59) generated our synthetic target - the intermediate (32) in good yield (80%) (Scheme 30).



Scheme 30

The structure was confirmed by n.m.r. and mass spectroscopy in the first instance and satisfactory micro-analytical data was subsequently obtained.

The mass spectrum shows the molecular ion at 324 a.m.u. (53% abundance) and gave an accurate mass at 324.1111 a.m.u. for the molecular ion. Due to the high insolubility of the compound

in all solvents, except T.F.A. in which it was sparingly soluble, no ^{13}C data were available for structural confirmation.

The successful route leading to the key intermediate (32) is shown in Fig. 4.

2.2 via 2,5-dichloropyrazines

While work was being carried out on the synthesis of the target molecule (32) by the previous route, a complimentary synthesis was also being investigated which was based on the preparation of functionalised 2,5-dichloropyrazine derivatives.

The aim of this work was to provide an alternative route to the key intermediate (32) should the first route fail or prove impractical.

The synthetic scheme envisaged, based on known chloropyrazine chemistry, is shown in Fig. 5.

The route omits several steps which appear in the other strategy and the salient points of this route are as follows:-

- (a) aromatise the ring in one step instead of two.
- (b) nucleophilic displacement of chloride by benzyloxy anion - no ether cleavage.
- (c) production of a system containing only one protecting group. This facilitates unmasking of all the protected hydroxylic functionalities simultaneously, thereby increasing the yield.

Initial studies were based on the chlorination of 3,6-dibenzylpiperazine-2,5-dione (53).

Direct chlorination of this dioxopiperazine using phosphorus oxychloride⁷² gave the desired 2,5-dichloro-3,6-dibenzylpyrazine as the major product but in variable yield

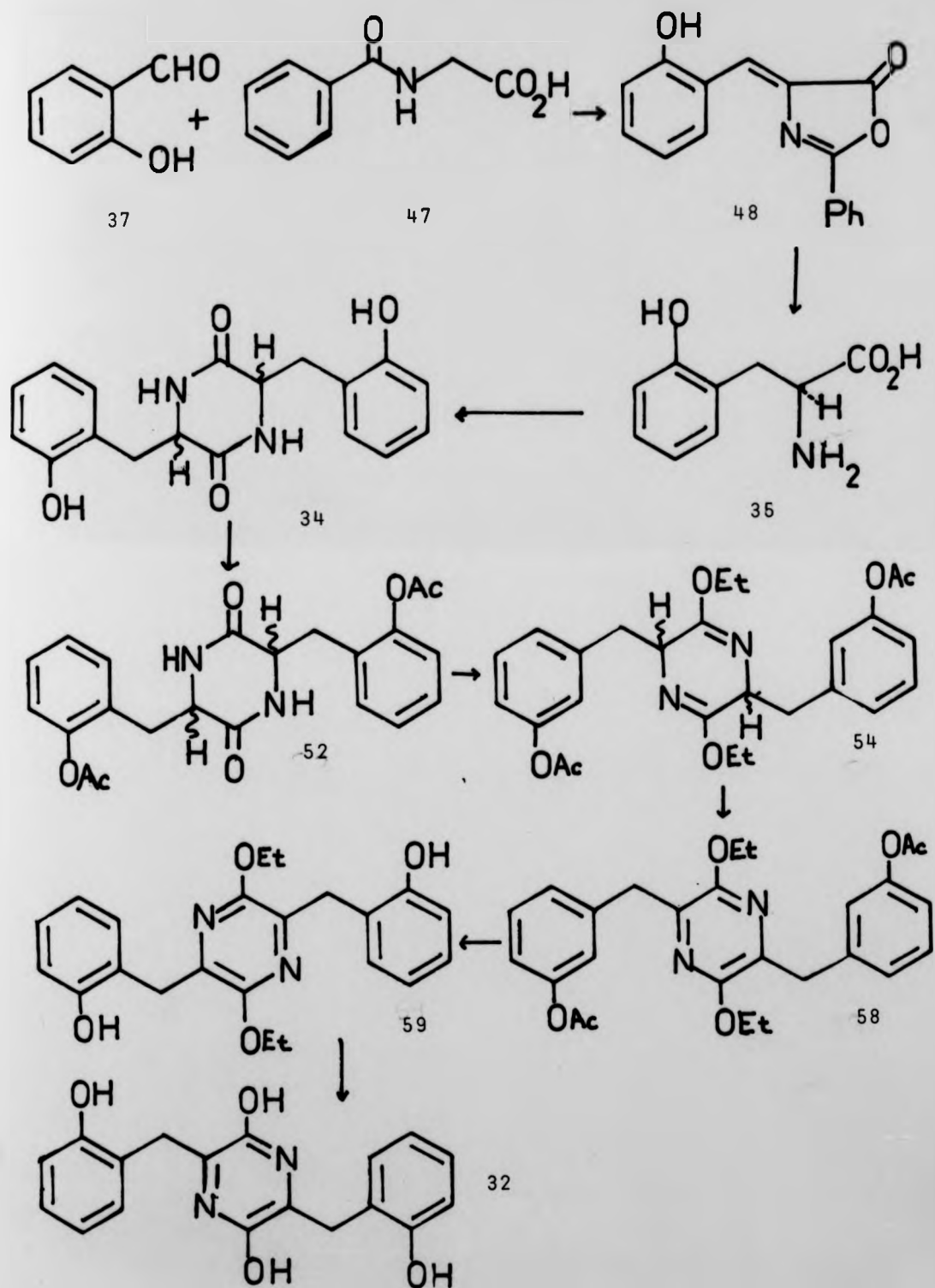


Figure 4

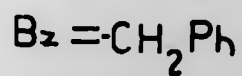
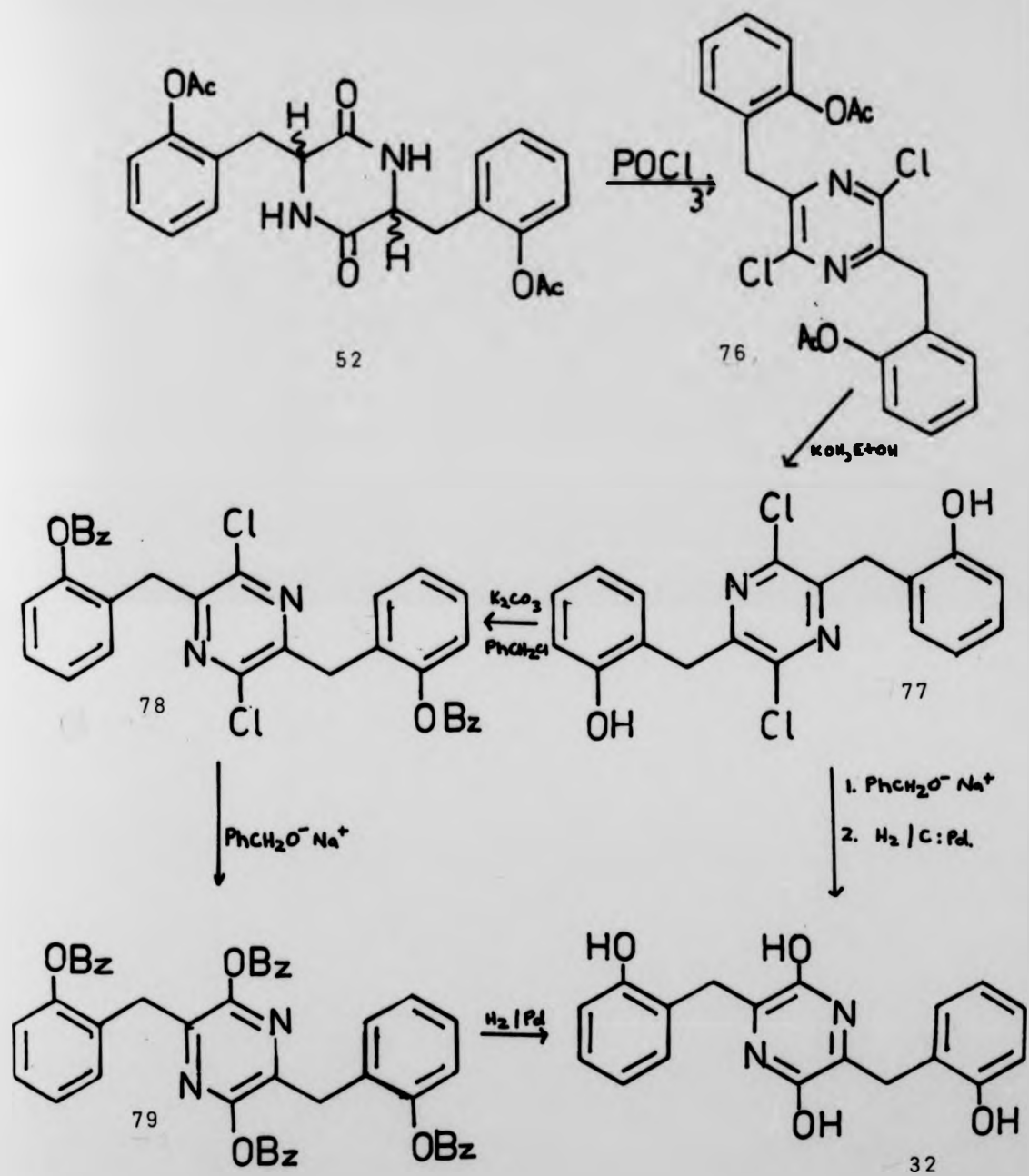
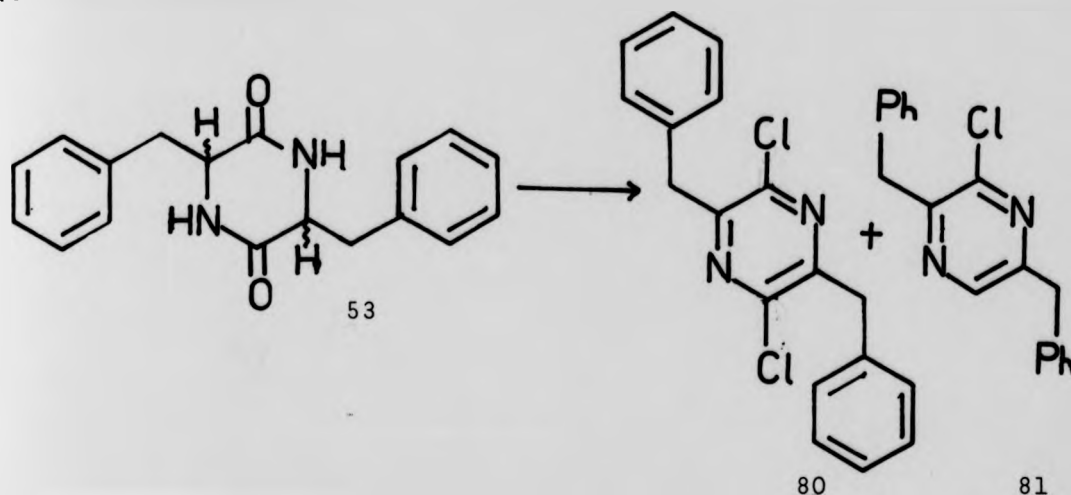


Figure 5

(3 - 30%) (Scheme 31).



Scheme 31

The mechanism of this reaction presumably follows initial nucleophilic attack at the amide carbonyl by chloride, followed by dehydration and subsequent double bond rearrangement to the pyrazine.

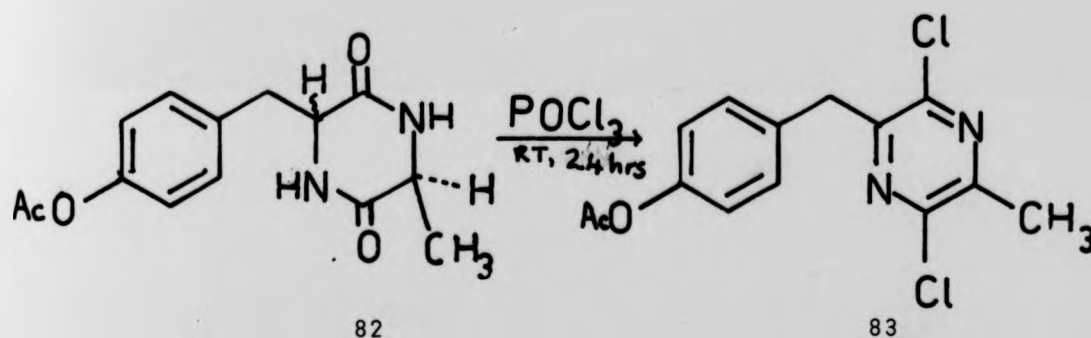
The quantity of monochloropyrazine (81) produced in this reaction is small (ca 7%) and this material was obtained by p.l.c. of the reaction mixture.

The structure of the dichloropyrazine (80) was confirmed by a clear molecular ion in the mass spectrum at 328 a.m.u. with the associated $m+2$ and $m+4$ peaks in the ratio $m+ : m+2 : m+4 = 9 : 6 : 1$. This is diagnostic of a compound bearing two chlorine substituents.

Similarly, the monochloropyrazine (81) exhibits an $M+$ to $m+2$ ratio of 3 : 1 in the mass spectrum. Proton and carbon n.m.r. were consistent with the structures proposed.

Both chlorinated pyrazines i.e. (80) and (81) were obtained as crystalline solids.

Of more direct relevance to our intended strategy was the reported reaction of an acetylated dioxopiperazine with phosphorus oxychloride, which involved retention of the acetate moiety⁷³ (Scheme 32).

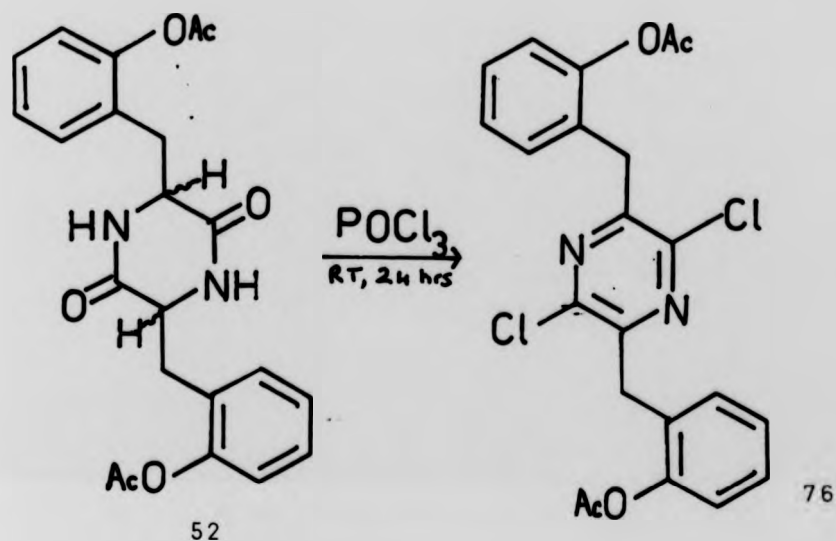


Scheme 32

Reaction of the acetylated dioxopiperazine derived from σ -tyrosine (52) with phosphorus oxychloride was conducted under identical conditions to the above and gave the desired product (76) in 30% yield (Scheme 33).

The structure was confirmed by n.m.r., ¹³C.m.r. and mass spectroscopy.

Retention of the acetyl groups was evident by the occurrence of a 6 proton singlet at τ 7.7 in the proton n.m.r. spectrum. The material was recrystallised from hexane to give a pale yellow solid (m.p. 205-207°C).



Scheme 33

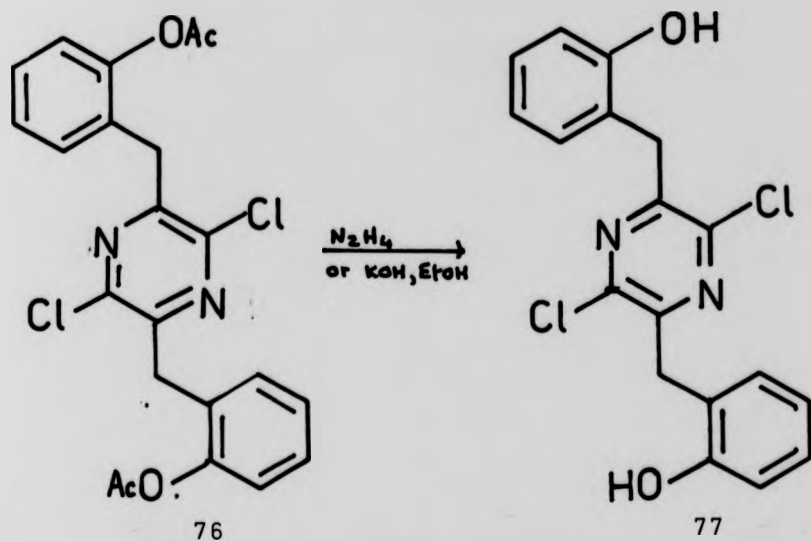
The second transformation in this route *i.e.* deacetylation of the intermediate (76) proved troublesome.

Reaction of the acetylated dichloropyrazine (76) with ethanolic potassium hydroxide solution gave the free phenolic derivative (77) which gave a positive reaction with ferric chloride. The yield of the phenol (77) was low.

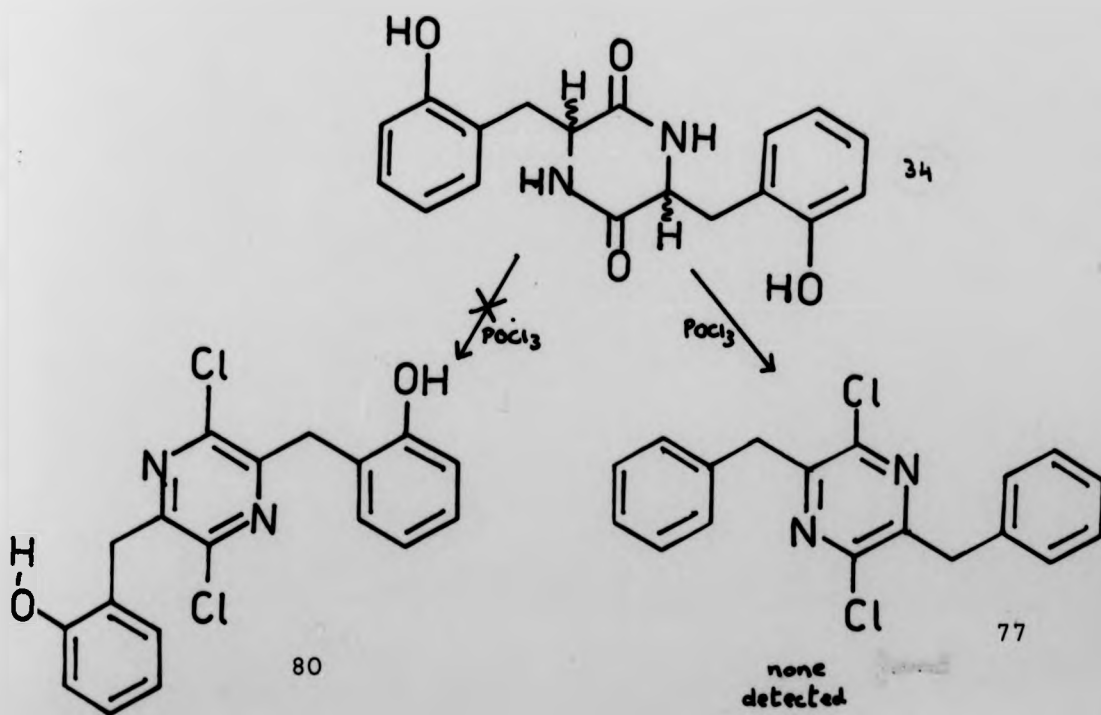
Similarly, reaction with hydrazine hydrate⁵⁶ produced starting material as the major product with only a trace of the phenolic derivative (77) (Scheme 34).

When faced with this unexpected failure, it was decided to circumvent this problem by attempting direct chlorination of cyclo- σ -tyrosinyl- σ -tyrosine (34) *i.e.* Scheme 35.

Treatment of the dioxopiperazine (34) with POCl₃ at room temperature for five days gave a clear yellow solution which showed one major spot by t.l.c., along with a fluorescent spot and one at high R_f.



Scheme 34

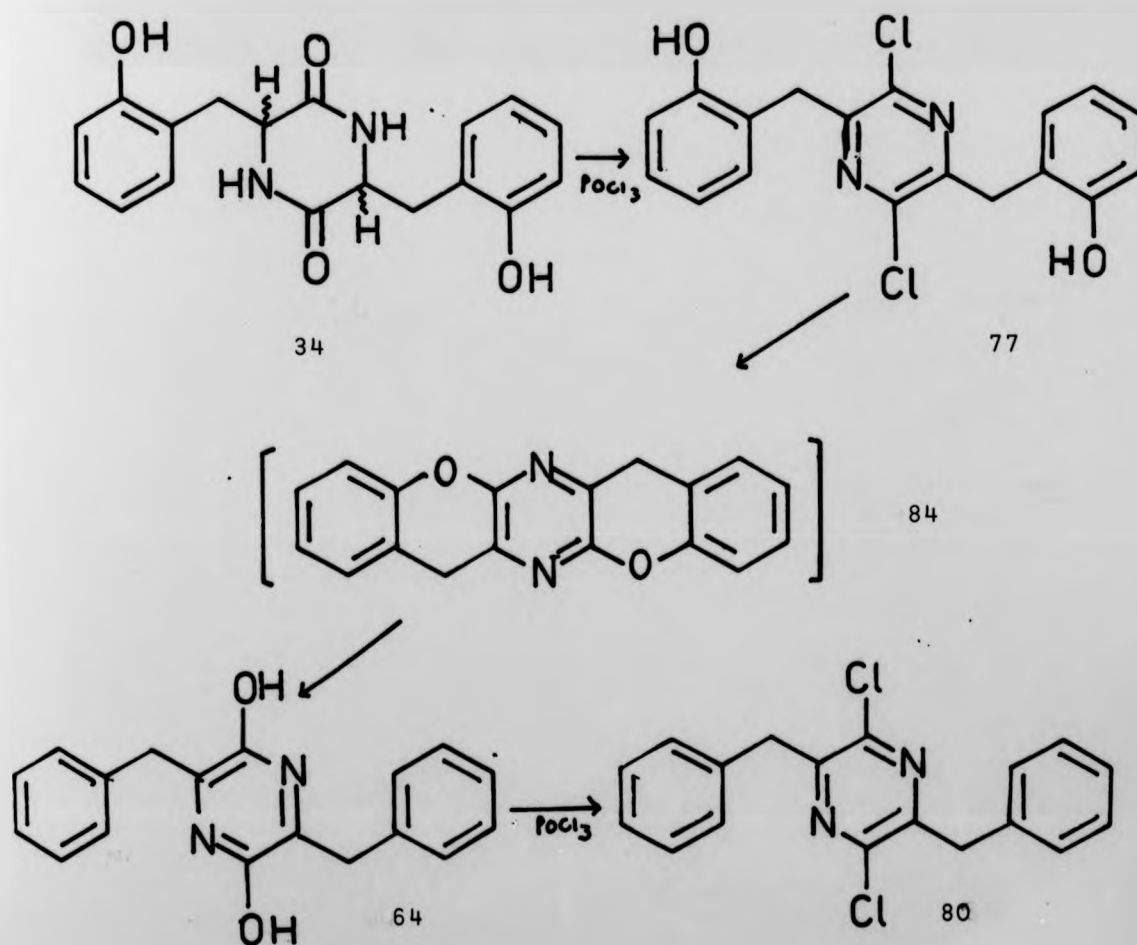


Scheme 35

Analysis showed the high R_F spot to be a chloropyrazine (80) $m+293$ a.m.u. ($m+ : m+2 = 3 : 1$). Further analysis showed the compound to be 3,6-dibenzyl-2-chloropyrazine (81) since the spectroscopic data was identical to our authentic sample.

The major spot had identical physical and spectroscopic properties to 3,6-dibenzyl-2,5-dichloropyrazine (80) shown by comparison with an authentic sample.

One possible method of conversion which would account for loss of the phenolic hydroxyl groups is given below (Scheme 36).

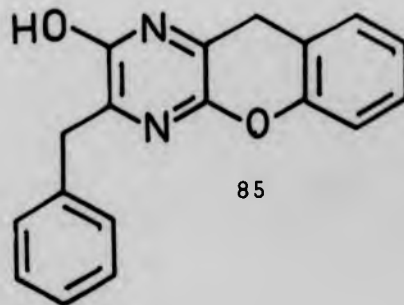
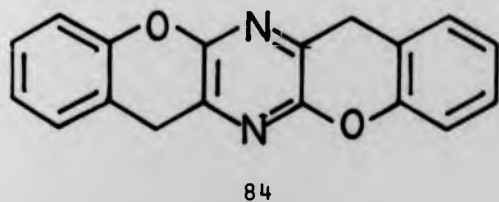


Scheme 36

The scheme is not intended to be a mechanistic rationale but merely as a suggestion of a possible route from the substituted dioxopiperazine (34) to 2,5-dichloro-3,6-dibenzylpyrazine (80).

The pentacyclic intermediate (84) is suggested since it may fit the n.m.r. data for the fluorescent compound. The fluorescence may arise by conjugation of the aromatic ring with the diazine ring through the p-orbitals of the oxygen bridges. This is only a speculation however since no analogies could be found.

The fluorescent material was isolated and gave a clean n.m.r. spectrum i.e. τ 5.7(s) 4H, 2.8(s) 8H and the infrared spectrum shows a strong C-O-C stretch at 1410 cm^{-1} which is quite high for an ether absorbance. The symmetry of the n.m.r. favours (84) as a tentative structure while (85) remains an alternative but less likely possibility.



Failure to produce the desired 2,5-dichloro-3,6-di-(2'-hydroxy-benzyl)pyrazine was disappointing since this intermediate was of key importance in the early part of this route.

However, the last three pyrazine ring transformations were successfully demonstrated using 2,5-dichloro-3,6-dibenzylpyrazine (80).

2,5-dichloro-3,6-dibenzylpyrazine was reacted with sodium benzyloxide prepared in situ in dry benzyl alcohol and gave the desired dibenzyl ether in 68% yield.

The n.m.r. of the compound was diagnostic having only two resonances i.e. τ 2.9(s) and 6.0(s), in the ratio 5 : 2. For the proposed structure (86) the ratio would be 20 : 8 or 5 : 2.

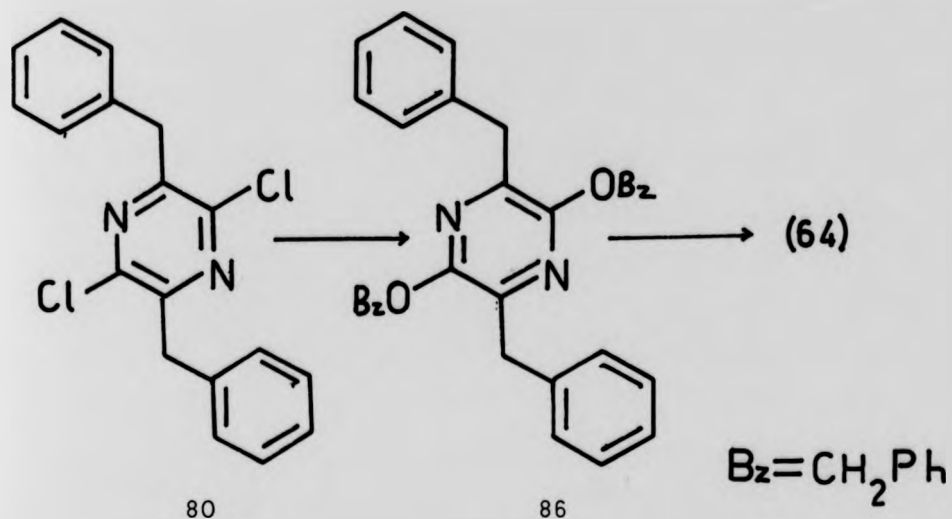
The structure was assigned as the dibenzyl ether (86) on the basis of other spectroscopic data.

Removal of the benzyl-ether protecting groups by cleavage with 1M HCl generated the dihydroxypyrazine (64) previously reported in Scheme 37 in 72% yield.

Spectroscopic properties were identical to those reported previously.

The nucleophilic displacement of the chlorine atoms in (80) was not always facile e.g. when using methoxide, hydroxide under phase transfer conditions, or sodium benzyloxide generated in dry benzene, starting material was recovered.

With the exception of the deacetylation step, this route would appear to hold some practical value in the synthesis of functionalised 2,5-dihydroxypyrazines.

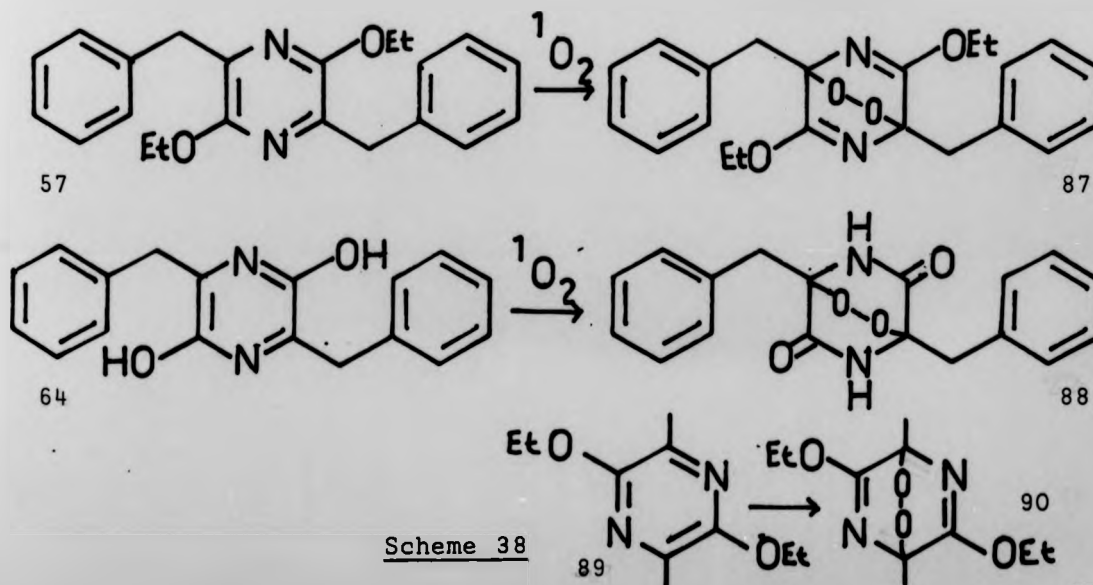


Scheme 37

However, when compared with the previous route to the key intermediate (32), it seemed less likely to succeed in the short period of time available to assess its merits.

2.3 Photo-oxygenation studies on the intermediate (32)

Fundamental work in the photo-oxygenation of dihydroxypyrazine has been conducted by Sammes and Markham^{26,27} and the examples shown in Scheme 38 have been reported.



Scheme 38

It has been demonstrated that pyrazines such as (57) and (64) give the corresponding endoperoxide^{26,27} on reaction with singlet oxygen and, clearly, the key step in the proposed biogenesis of bicyclomycin has adequate precedent in these examples.

To establish experimental conditions for our system, the photo-oxygenation of 2,5-diethoxy-3,6-dibenzylpyrazine (57) was further investigated and found to be dependent on solvent and sensitiser used (Table 9).

TABLE 9

Photo-oxygenation of 2,5-diethoxy-3,6-dibenzylpyrazine (57)
(300W spot lamp)

Experiment	Reaction Time ^a	Sensitiser ^b	Solvent ^c	Isolated Peroxide ^d Yield %
A	17 hours	Methylene blue	CH ₂ Cl ₂	70
B	6 hours	" "	MeOH	85
C	12 hours	Rose Bengal	CH ₂ Cl ₂	74
D	5 hours	" "	MeOH	92

- a. Time interval after which no starting material remained (t.l.c.)
- b. 10mg in each case
- c. All solvents were scrupulously dried
- d. Isolated yield from p.l.c. plates.

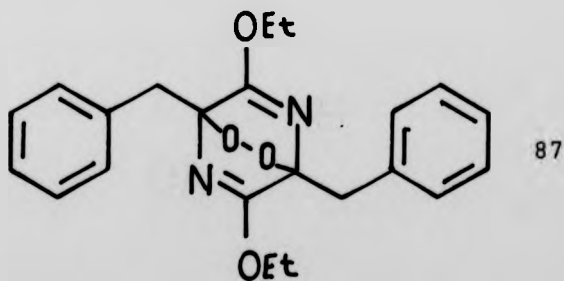
As far as can be ascertained from the literature these reaction times and yields in dichloromethane broadly concur with those cited by Sammes^{26,27,74}.

In our experiments, variation of solvent polarity produced shorter reaction times and increased yields. The reason for the increased rate may be that the more polar solvent induces a slight polarisation in the pyrazine prior to singlet oxygen addition or facilitate charge distribution over the ring at the point of incipient peroxide formation.

One point that should be mentioned however is that in methanol Rose Bengal is fully soluble and is therefore in more intimate contact with the solvent, substrate and oxygen gas, thus increasing the "sensitisation" effect and increasing the rate of singlet oxygen production.

The reaction is a truly photochemical one since the process will not occur in the absence of light.

The structure of the peroxide formed was confirmed by conventional spectroscopic methods, including ^{13}C n.m.r. and is best represented by (87).



Irradiation of our small model system i.e. 2,5-dihydroxy-3,6-dibenzylpyrazine (64) did not proceed as reported in the literature^{26,74}.

Photooxygenation of the dihydroxypyrazine (64) in d.m.s.o./ CH_2Cl_2 (1 : 10) in the presence of oxygen and sensitiser for 36 hours, did not produce the reported product (88) nor did the cited product precipitation occur when the sample was concentrated.

A second attempt, using warm d.m.s.o./methanol (1 : 9), to dissolve the solid and sensitiser, did produce some insoluble material after irradiation in the presence of oxygen for 48 hours.

A small amount of the material was isolated and the molecular ion in the mass spectrum was identical to that for starting material and was again the base peak 292 a.m.u.

This result, however, does not preclude formation of the endoperoxide since the first process to occur in the mass spectrometer is a retro-Diels-Alder reaction as occurs with 2,5-diethoxy-3,6-epidioxy-3,6-dibenzyl-3,6-dihydropyrazine (87). No ion for the endoperoxide is observed (Scheme 39).

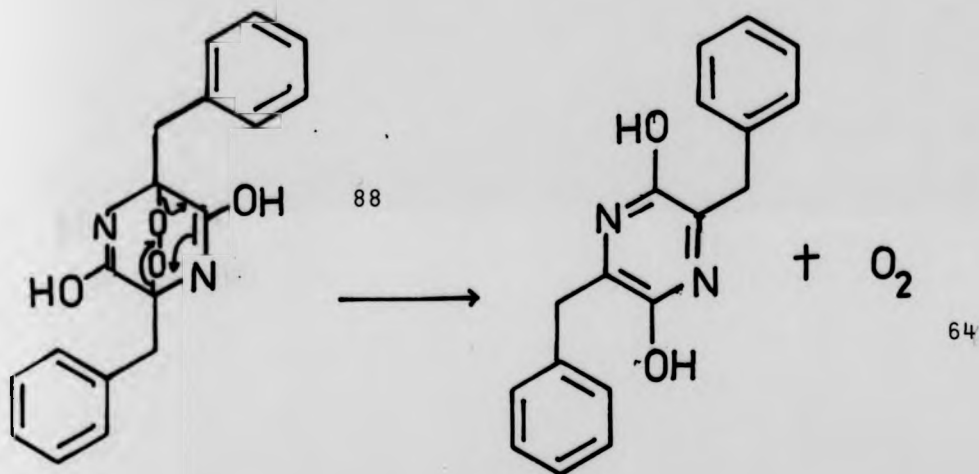
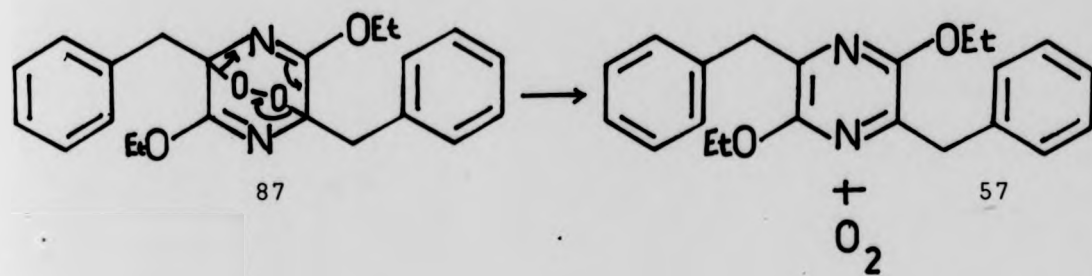
The reaction product was too insoluble for an n.m.r. spectrum to be obtained, but the infrared spectrum did show small hydroxylic absorption and amide absorption at 1690 cm^{-1} .

The peroxide would be expected to exist predominantly in the amide form (88).

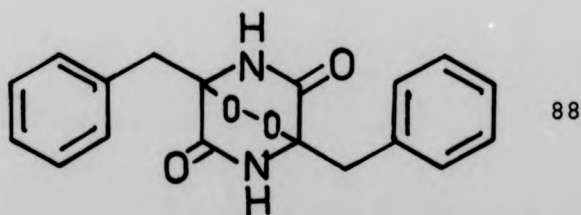
Initially 2,5-diethoxy-3,6-di(2'-hydroxy-benzyl)-pyrazine (59) was irradiated in preference to the model (32) on solubility grounds.

After 10 hours irradiation in the presence of oxygen and methylene blue in dichloromethane, a second reaction product was observed and isolated by p.l.c.

The quantity of material isolated was low (10 mg) and the n.m.r. indicated that peroxide formation had occurred.



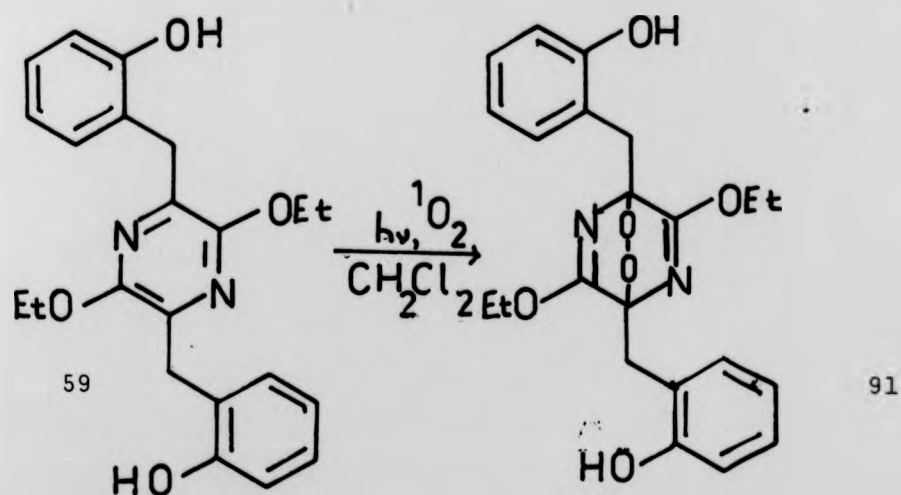
Scheme 39



Evidence for this was provided by the n.m.r. spectrum since there was a shift to high field of the benzylic protons from $\tau 6.0$ to $\tau 6.6$. An identical shift in the proton resonances was observed between 2,5-diethoxy-3,6-dibenzylpyrazine (57) and its endo-peroxide (87).

No mass measurements could be usefully used for structural elucidation since the compound decomposed in the probe of the mass spectrometer at 150°C .

The structure of the insoluble material was tentatively assigned as the endo-peroxide (91) (Scheme 40).



Scheme 40

Photooxygenation of the synthesised model (32) with either methylene blue or Rose Bengal did not produce the desired product (31) since it was insoluble (even in high dilution) in methanol, methanol/d.m.s.o. and methanol/d.m.s.o./HMPA.

Clearly the use of (32) as a model system, although a potentially useful system for study, would appear to be of no

real value due to its limited solubility in any suitable solvent. In hindsight, the low solubility of the model is not unexpected since many dihydroxypyrazines are known to be extremely insoluble in all solvents and two structural features appear to be involved.

Firstly the highly polar "imidic acid" groups tend to be soluble in aqueous and hydroxylic solvents. As would be expected the phenyl moieties have added solubility in organic solvents and clearly, in the model chosen, the worst of both solubility profiles seems to result in almost complete insolubility in all common solvents. It may prove possible to photo-oxygenate the model in alkaline solution i.e. as the tetraphenoxide, but this has not been investigated due to lack of time.

Consideration was, therefore, given to a simpler model, in which the total molecular weight would be substantially reduced, thus increasing solubility in hydroxylic solvents. Under these conditions it is possible that a successful photo-oxygenation might be achieved.

OTHER MODEL STUDIES

2.4 Route C - Routes via direct chlorination of glycine anhydride

In this section we are primarily interested in synthetic routes which generate 2,5-dichloropyrazine (93) in high yield from piperazine-2,5-dione (glycine anhydride) (92) (Scheme 41).

It was proposed to use 2,5-dichloropyrazine as the starting material for a synthetic route to a second model system, Fig.6.

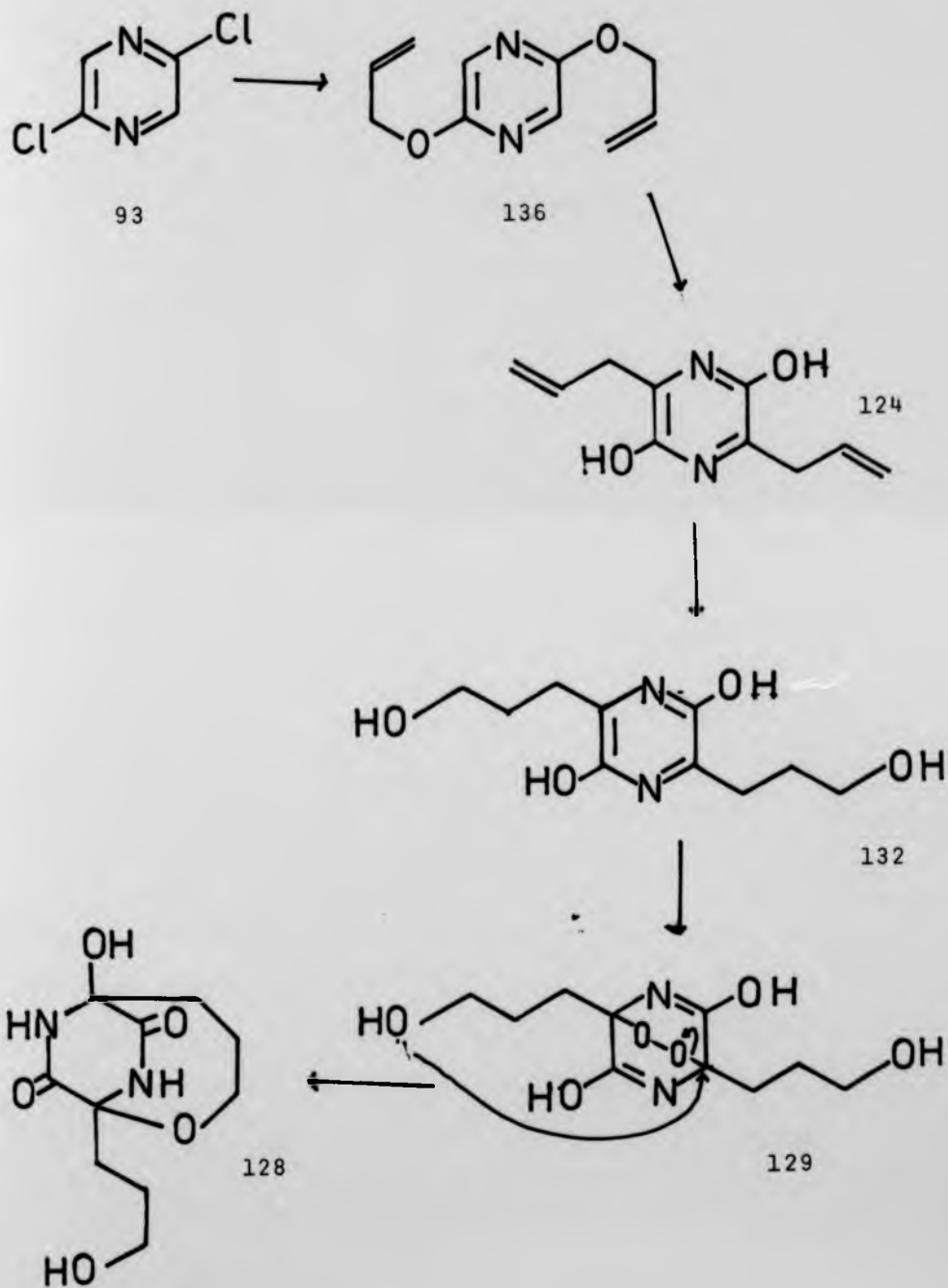
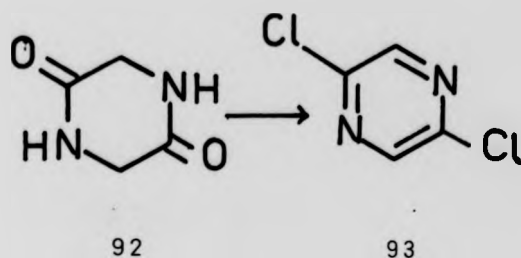


Figure 6

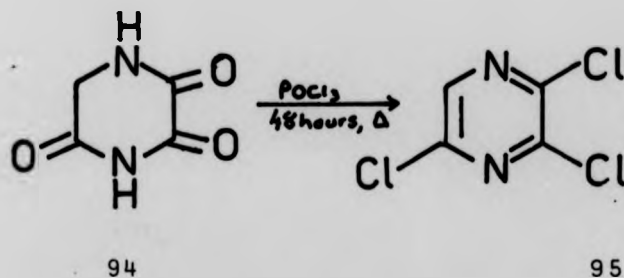


Scheme 41

The target compound (126), although similar to the intermediate (32) in the two other routes, was chosen to try and optimise the solubility characteristics of the compound.

Both the long chain alcohol substituents and the "imidic acid" moiety should be soluble in hydroxylic solvents, thus enhancing the probability of a successful photo-oxygenation.

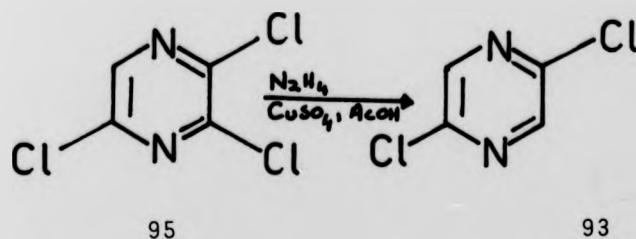
Our problems with this route were immediately apparent since no direct method for the chlorination of glycine anhydride could be found in the literature. However, chlorination reactions in analogous systems are known⁷⁷ (Scheme 42).



Scheme 42

Although no explanation is offered in this paper, the remaining portion of 2,3,5-trioxopiperazine (94) will probably be in the form of various lower halopyrazines.

A synthetic route from 2,3,5-trichloropyrazine (95) to 2,5-dichloropyrazine (93) has been reported⁷⁵ but the yield of 2,5-dichloropyrazine (93) was low (ca 40%) due to the simultaneous formation of the 2,3- and 2,6-dichloropyrazine isomers (Scheme 43).

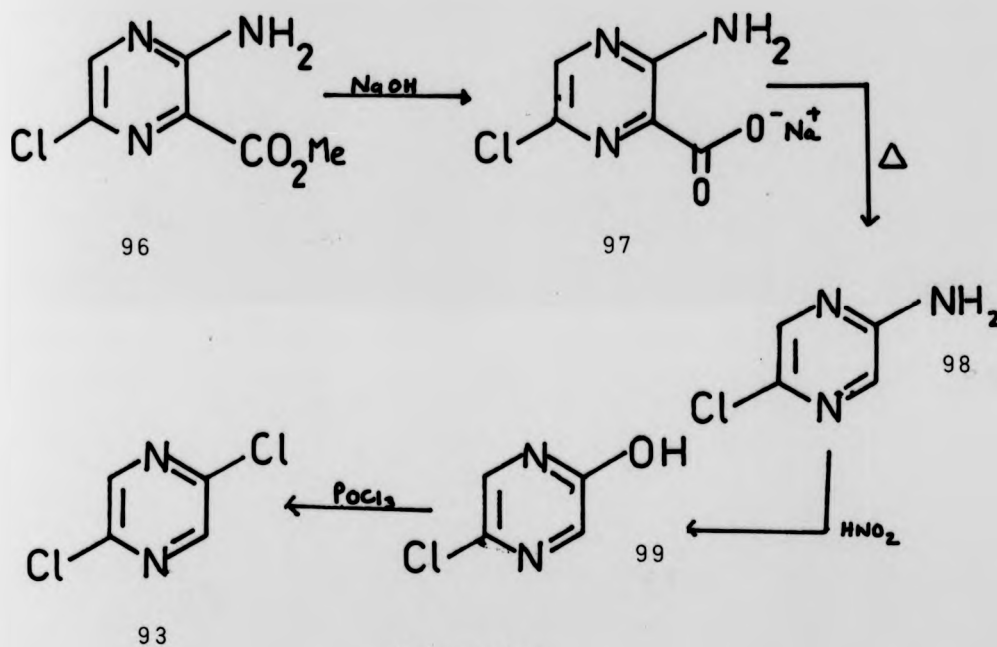


Scheme 43

An alternative synthetic route to 2,5-dichloropyrazine (93) has been reported⁷⁶ but it is long and the cost of starting material (96) would probably be prohibitive (Scheme 44).

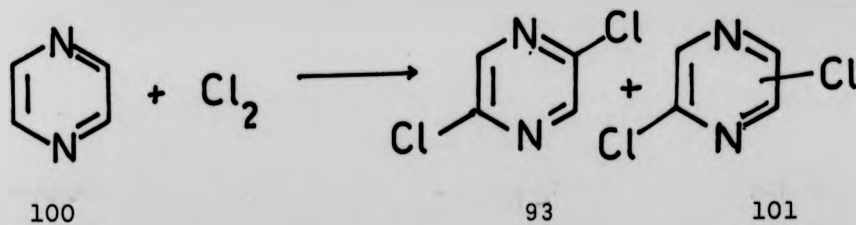
The scheme involves saponification of 2-amino-3-carboxymethyl-5-chloropyrazine (96) in sodium hydroxide to produce the free acid (97) which is then decarboxylated to 2-amino-5-chloropyrazine (98). Reaction of the amine functionality with nitrous acid produces the anticipated

2-hydroxy-5-chloropyrazine (99) which undergoes normal chlorination with phosphorus oxychloride to generate 2,5-dichloropyrazine (93).



Scheme 44

Direct chlorination of pyrazine (100) will also yield 2,5-dichloropyrazine (93) when reacted under high temperature and pressure, but all possible isomers are also formed⁷⁷ (Scheme 45).



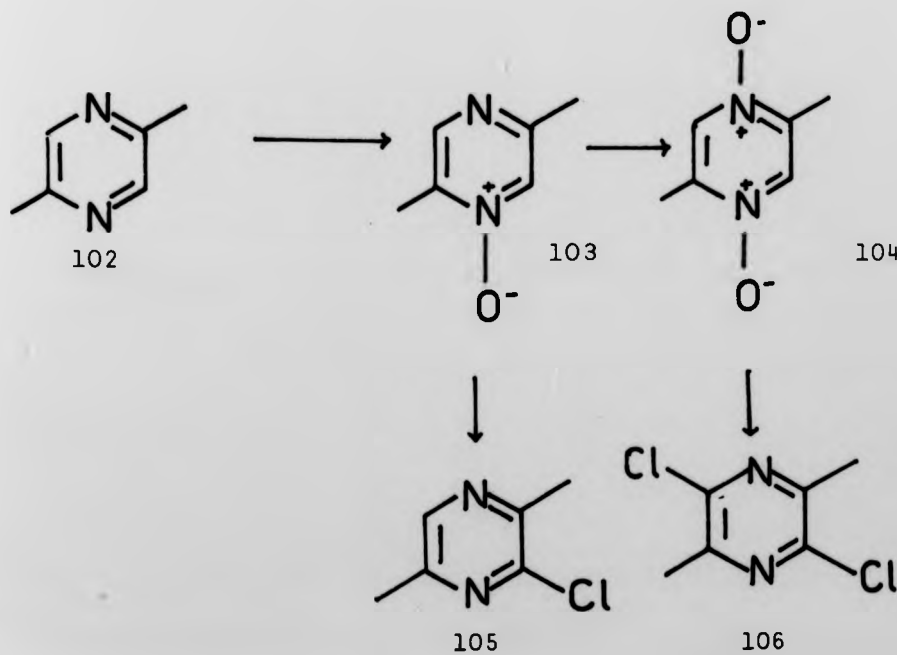
Scheme 45

These chlorination reactions, specified previously, produce 2,5-dichloropyrazine (93), in fair to modest yield, from an unactivated 1,4-diazine ring system.

The most common method of chlorine introduction in substituted 1,4-diazines is by activating the ring towards nucleophilic attack by forming an N-oxide⁷⁸⁻⁸¹.

In most cases the N-oxide function will activate the unsubstituted carbon adjacent to it towards nucleophilic attack by a slight withdrawal of electron density towards the N-oxide functionality.

An illustration of this point which also bears relevance to our route in general is shown in Scheme 46.



Scheme 46

In the case of 2,5-dimethylpyrazine (102), a mixture of mono- (103) and di-N-oxides (104) is formed. Chlorination of these under conventional conditions produced the corresponding mono- and dichloro-2,5-dimethyl pyrazines (105) and (106)⁷⁸.

Of prime importance in this synthetic route was the production of 2,5-dichloropyrazine (93) in good yield from 2,5-dioxopiperazine (92).

Direct chlorination was attempted using phosphorus oxychloride as the solvent and chlorination reagent.

When 2,5-dioxopiperazine (92) was stirred overnight with excess phosphorus oxychloride, a red solution resulted. The solution was heated to 100°C over 30 minutes, cooled and quenched by slowly pouring the reaction mixture into phosphate buffer (0°C, pH 7.0). Chloroform extraction of this material and subsequent concentration yielded a red semi-solid (11%).

G.l.c. analysis of the reaction mixture showed that the major products were 2-chloropyrazine (107) and 2,5-dichloropyrazine (93) which were present as 78% and 10% of the semi-solid respectively. Two minor impurities were also noted (Fig.7).

P.l.c. of the solid in neat chloroform gave the two compounds as single g.c. peaks and these were fully characterised.

2-Chloropyrazine (107) was shown to have identical spectroscopic data with commercial 2-chloropyrazine and the structure was confirmed by g.c./m.s. and ¹³C.m.r.⁸²

2,5-dichloropyrazine (93) was characterised by n.m.r. i.e. τ 1.75(s) and g.c./m.s. i.e. m^+ = 149 a.m.u. Isotopic peaks in the mass spectrum showed the presence of two chlorine atoms

Figure 7

G.c. trace of chlorinated pyrazines -
the reaction mixture of DKP and POCl₃ analysed
on Apiezon L (5%) at 115°C

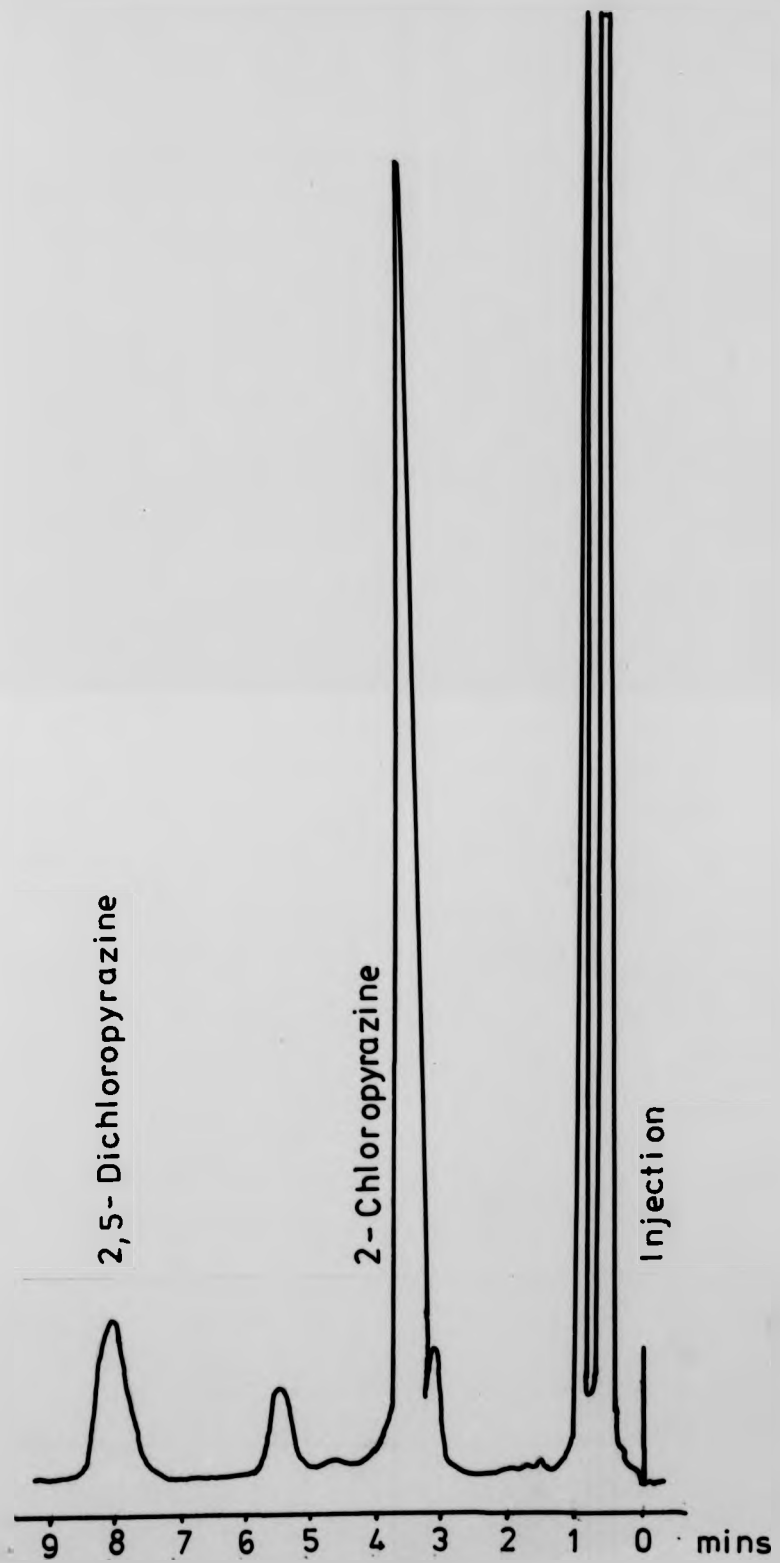


Figure 7

in the compound. Accurate mass measurement and ^{13}C .m.r. confirmed the structure as 2,5-dichloropyrazine.

When authentic samples were used to spike g.c. samples of the reaction mixture, only the peak with the correct assignment was enhanced.

This work tends to confirm the unacceptable yield of 2,5-dichloropyrazine (93) from dioxopiperazine using phosphorus oxychloride as the sole chlorination reagent.

One minor peak was tentatively assigned as 2,3,5-trichloropyrazine on the basis of g.c./m.s. data which showed three chlorine atoms in the isotopic distribution peaks, i.e. $m^+ = 183$ a.m.u. The direct yield of 2,5-dichloropyrazine (93) by this method was 5 - 7%.

Most of the starting material seemed to be consumed in the production of black tarry material associated with this reaction.

The alternative to this reaction was to produce 2,5-dichloropyrazine (93) from 2,5-dioxopiperazine (92) by a mixed chlorination system.

Stirring 2,5-dioxopiperazine (92) overnight with phosphorus oxychloride and phosphorus pentachloride in a 1 : 1 molar ratio and quenching in phosphate buffer produced, after extraction, an oily red semi-solid in 43% yield.

G.l.c. analysis of the mixture, using the previously mentioned systems showed that this reaction produced 2,5-dichloropyrazine (93) as the major product (88%) and the only other product in significant amount was 2-chloropyrazine (107) (11%). The percentage composition is based on the calculation of peak areas (Fig.8.).

Figure 8

G.c. trace of chlorinated pyrazines -
the reaction mixture of POCl₃ and
3-chloropyrazine-1-oxide analysed on
Apiezon L (5%) 120°C

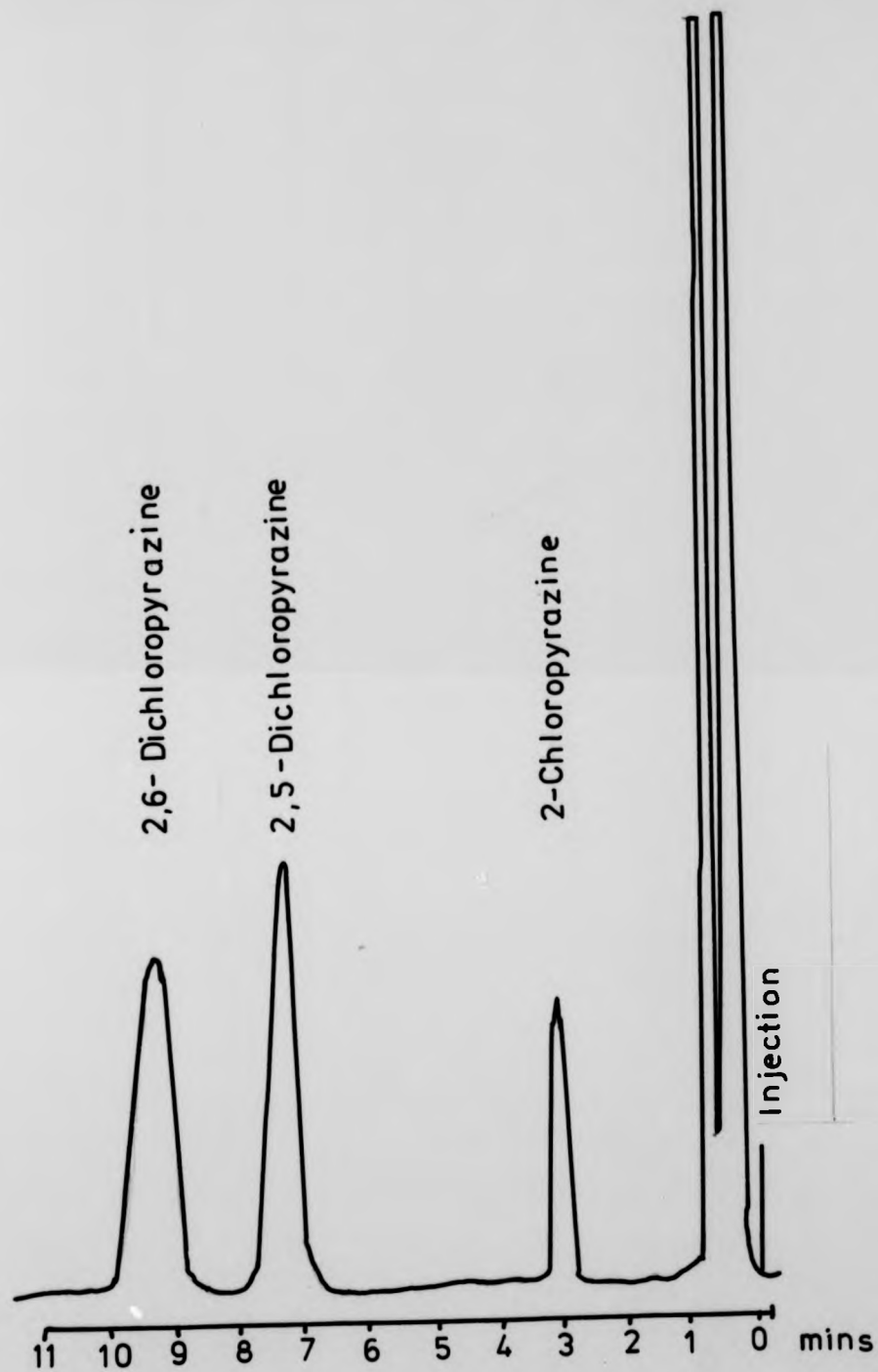


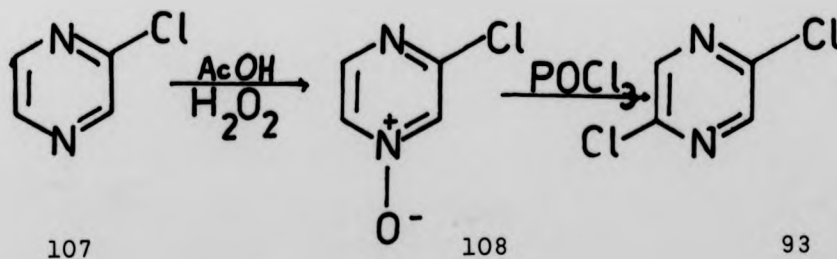
Figure 8

Both materials were confirmed structurally by physical and spectroscopic analysis, as well as by co-injection with authentic samples.

Large scale reactions increased the direct yield of 2,5-dichloropyrazine (93) from 2,5-dioxopiperazine (92) to 54%. Thus mixed chlorination systems appear to be superior in the direct chlorination and dehydration of 2,5-dioxopiperazine.

A second possible route to 2,5-dichloropyrazine appeared to be worthy of examination.

The reaction of mono-halopyrazine-N-oxides with phosphorus oxychloride to produce dichloropyrazines has been known for some time. This reaction involves the loss of the oxygen atom to the chlorination agent during the reaction (Scheme 47).



Scheme 47

The general type of reaction shown above should produce 2,5-dichloropyrazine (93) in modest yield and thus the scope of the reaction was briefly investigated as an

alternative to direct chlorination of the piperazine nucleus.

The treatment of commercial 2-chloropyrazine (107) with peroxyacetic acid gave the 4-N-oxide (108) in 58% yield. The N-oxide (108) was characterised and the data are in agreement with those published.

Reaction of the N-oxide (108) with a slight excess of phosphorus oxychloride was shown to produce two major products which were separated by p.l.c.

Product analysis (g.l.c.) showed that one major product was 2,5-dichloropyrazine (93). P.l.c. and isolation of the second product gave a cubic crystalline solid which, on the basis of the n.m.r. spectrum, was tentatively identified as 2,6-dichloropyrazine (109). This was later confirmed by comparison of spectroscopic data and m.p. with an authentic sample.

An excellent analytical method for the determination of the ratio of 2,5- and 2,6-dichloro-isomers is to monitor the height of the resonances in the proton n.m.r. spectrum at τ 1.75 and τ 1.45. This comparison of peak heights of 2,5- and 2,6-dichloropyrazine is in agreement with the data obtained by comparison of g.c. peak areas.

The reaction of the N-oxide (108) with phosphorus oxychloride produces 2,5-dichloropyrazine (93) and its 2,6-isomer (109) in roughly equal proportions (45%:42%). A small quantity (12%) of 2-chloropyrazine (107) is also present in the reaction mixture (Fig.9.).

2,5-Dichloropyrazine (93) was produced in 20% yield by this reaction, based on starting material.

Using a known literature preparation⁸¹, 2-chloropyrazine (107) was reacted with potassium persulphate in sulphuric

acid to generate 2-chloropyrazine-1-oxide (110) in 70% yield.

Reaction of this N-oxide (110) was less satisfactory than the previous reactions since some starting material i.e. the 1-N-oxide (110) was observed by t.l.c. and g.l.c.

The predominant product, as expected, was 2,6-dichloropyrazine (50%). The insertion of chlorine adjacent to a 2-halopyrazine-1-N-oxide has literature precedent.

The presence of the 2-chloropyrazine-1-oxide (25%) is probably indicative of the reaction not having gone to completion and the occurrence of 2-chloropyrazine as in the case of the 4-N-oxide (168) probably represents removal of the oxygen by PCl_3 produced in the medium or some other degradative mechanism.

The g.l.c. trace for this experiment is shown in Fig.10 and the summarised product analysis for all the chlorination reactions in Fig.11.

The direct yield of the reaction products calculated from starting material is shown in Table 10.

TABLE 10

Overall yields of chlorinated pyrazines from dichloropyrazine (92) and various chlorination reagents

Starting material	Reagent	1-chloro isomer	2,6-dichloro isomer	2,5-dichloro isomer
(92)	POCl_3 / PCl_5	3.0%	-	23% 54%
(92)	POCl_3	28.0%	-	4%
(107)	POCl_3	5.0%	18.0%	19%
(107)	POCl_3	8.0%	27.0%	-

Figure 9

G.c. trace of chlorinated pyrazines -
the reaction mixture of $\text{POCl}_3:\text{PCl}_5$ (1:1)
with DKP. Analysed on Apiezon L (5%) 118°C

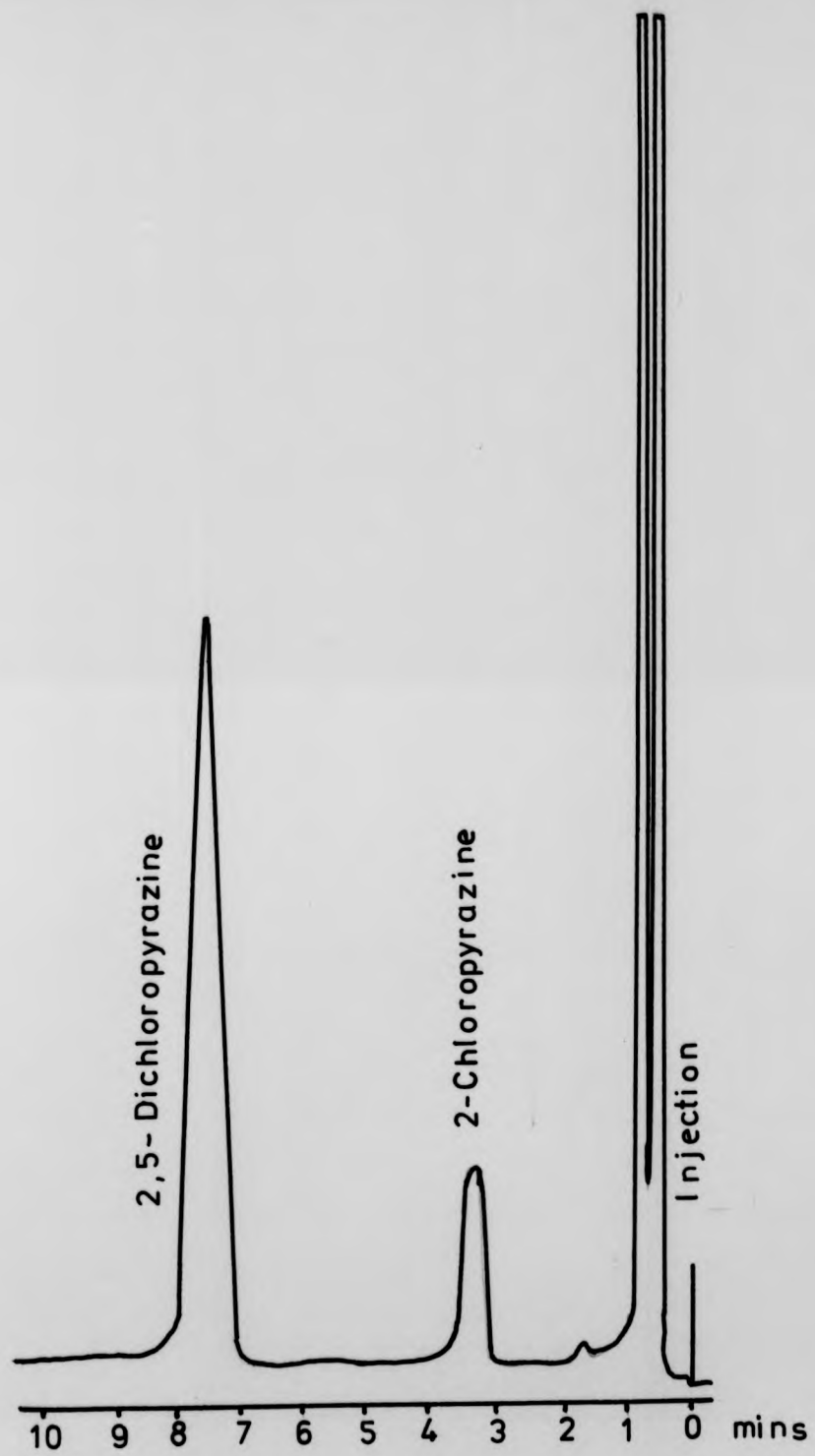
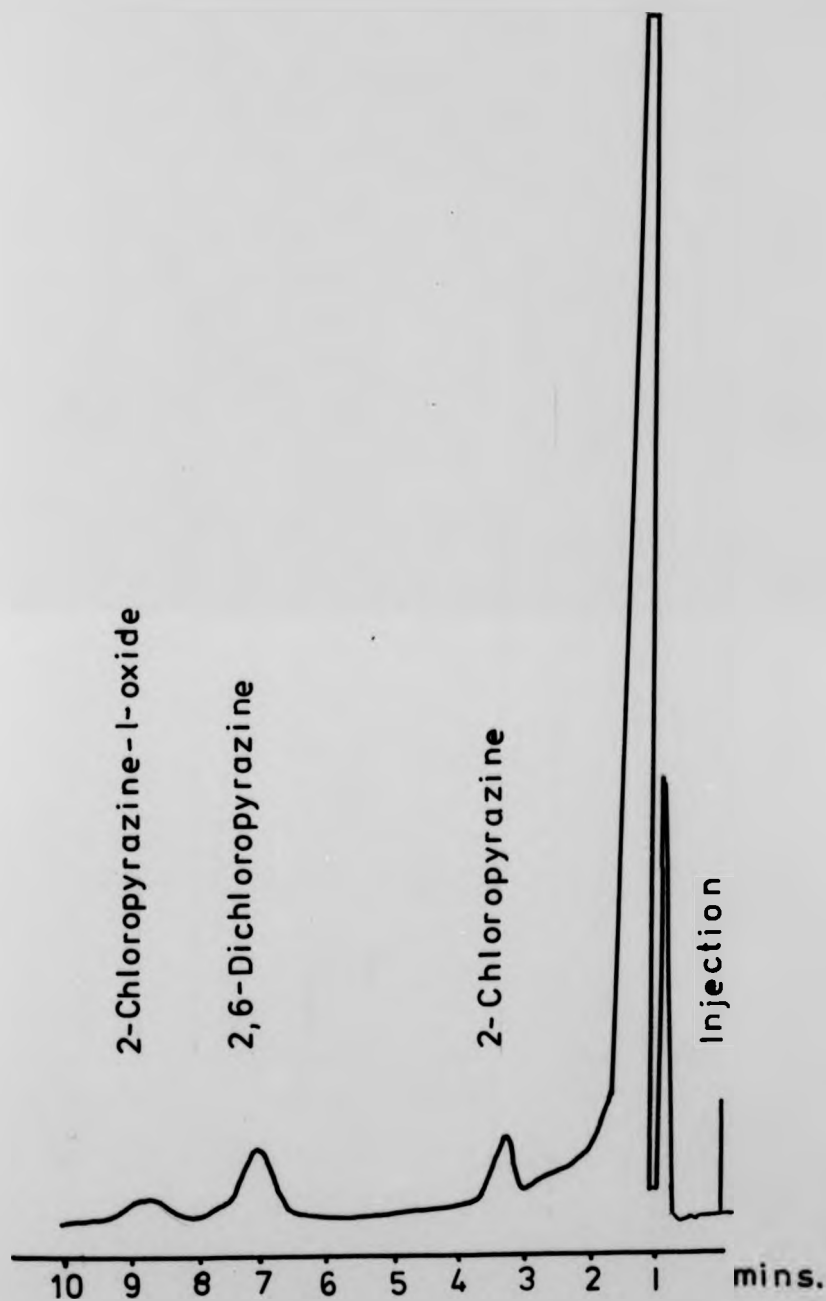


Figure 9



G.c. trace of chlorinated pyrazines -
the reaction mixture of POCl₃ and
2-chloropyrazine-1-oxide analysed at
122°C on 5% Apiezon L.

Figure 10

Figure 11

Summarised product analysis on chlorinated
pyrazines from chlorination of DKP and
N-oxides (calculated on areas only)

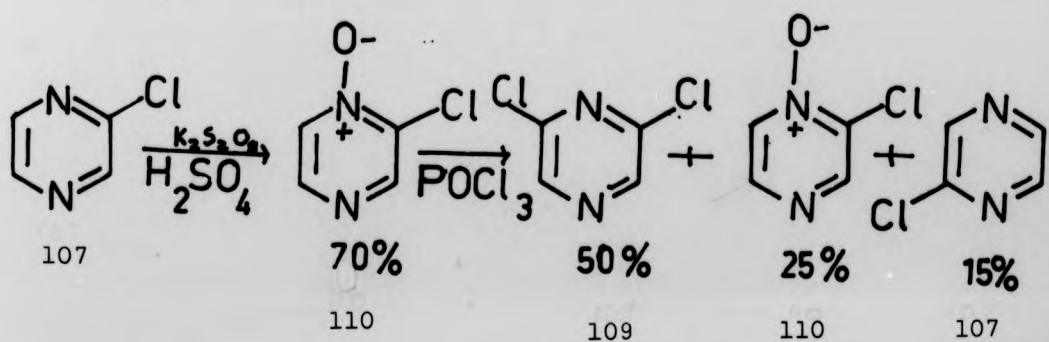
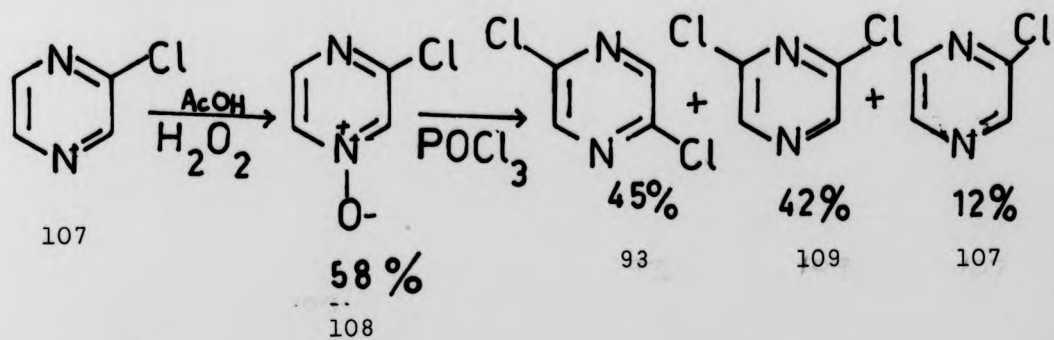
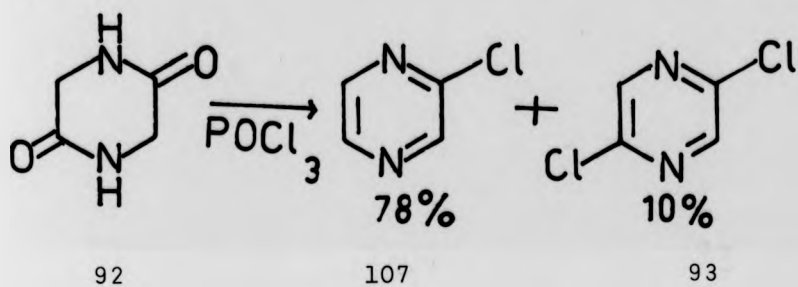
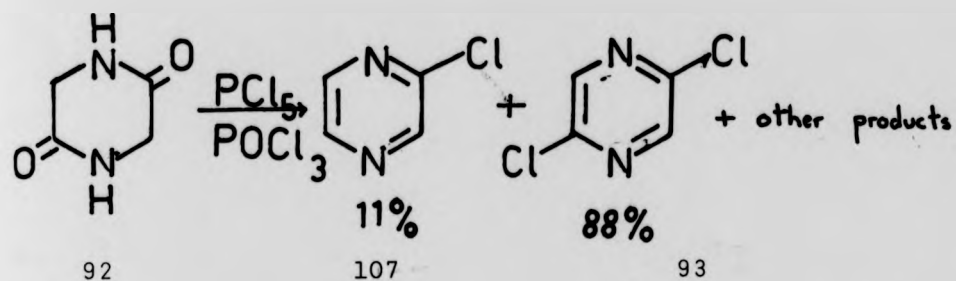
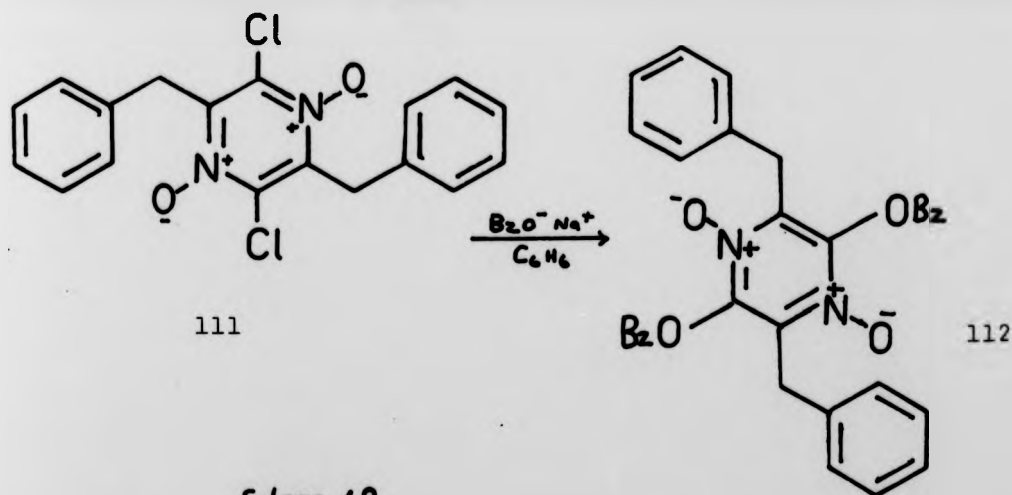


Figure 11

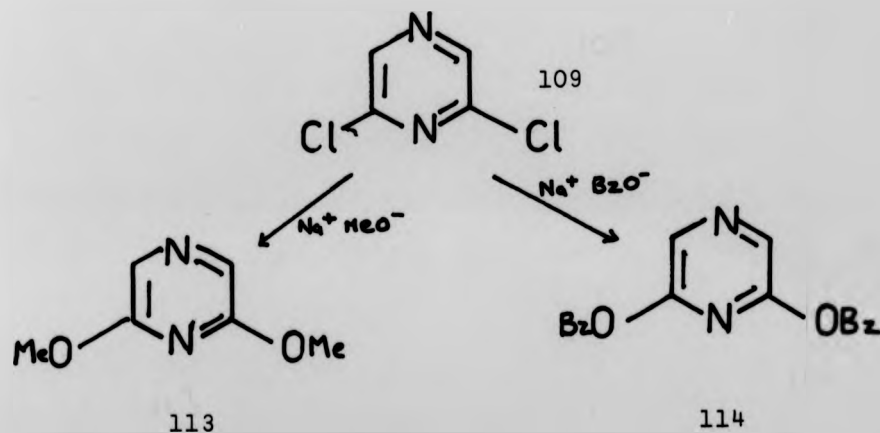
The method chosen to produce 2,5-dichloropyrazine for further synthetic transformations was the mixed chlorination method, since isolation was simpler and the yield overall was better in large scale experiments.

Referring back to Fig.6, it can be seen that the next step in this route is the formation of a 2,5-O-allylpyrazine (136).

Precedent for the displacement of a halogen from a halopyrazine-N-oxide⁴⁹ and an unactivated halopyrazine nucleus⁸³ exist in the literature e.g. Schemes 48 and 49.

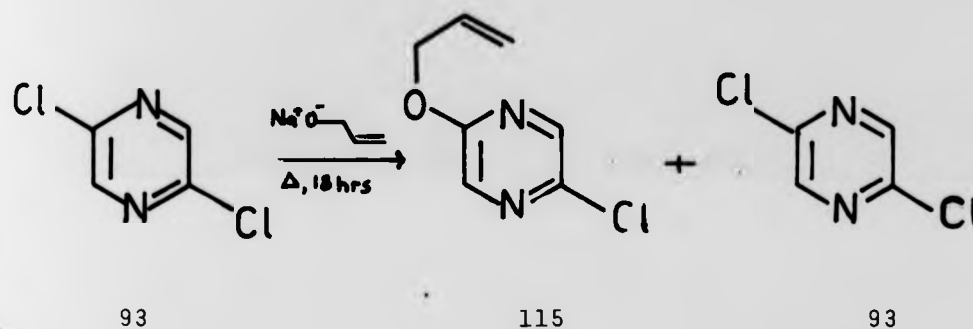


Scheme 48



Scheme 49

However, the reaction of 2,5-dichloropyrazine (93) with sodium allyloxide generated in allyl alcohol gave only low yields of monoalkylated product (115). Even after 18 hours reflux, none of the dialkylated product was found in the reaction mixture (Scheme 50).



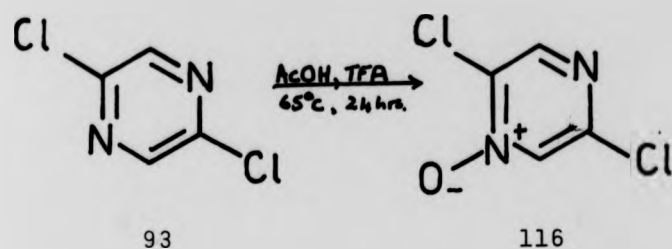
Scheme 50

Since the above reaction was unsatisfactory, it was decided to attempt alkylation using 2,5-dichloropyrazine-1-oxide (116) as the starting material.

Treatment of 2,5-dichloropyrazine (93) with peroxyacetic or peroxytrifluoroacetic acid at 65-70°C for 24 hours gave yields (ca 60%) of the mono-N-oxide (116) (Scheme 51).

In the mono-N-oxide (116), the molecular ion was also the base peak of the mass spectrum and elemental analysis derived from an accurate mass measurement was satisfactory.

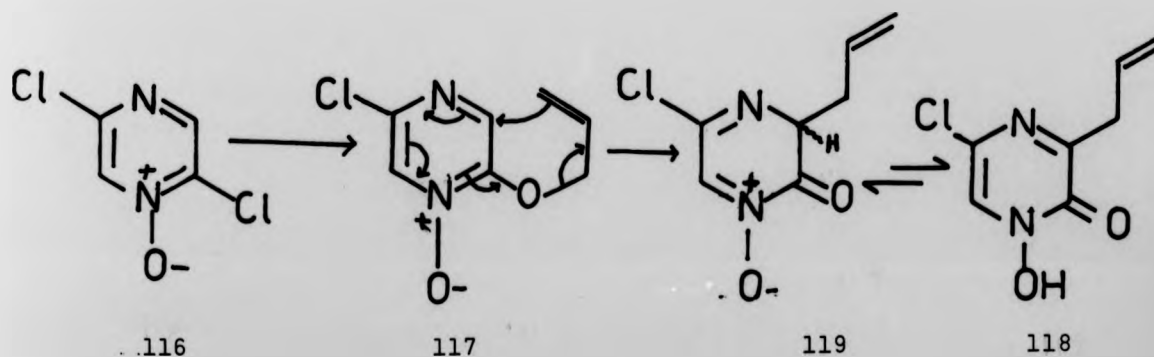
The original intention was to form the bis-N-oxide to permit displacement of both chlorine atoms.



Scheme 51

Various literature references^{49,52} cite the use of > 90% H₂O₂ to perform this transformation but this grade of hydrogen peroxide is not commercially available and a reference sample did not arrive from La Porte Industries until the end of the present period of study.

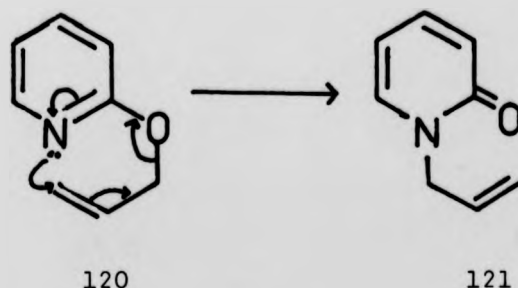
In the interim, it was decided to use 2,5-dichloropyrazine-1-oxide (116) as a model for the Claisen rearrangement envisaged in the reaction scheme (Fig.6). This is depicted in Scheme 52.



Scheme 52

Displacement of the 2-chlorine atom is more likely activated by the N-oxide⁸⁷.

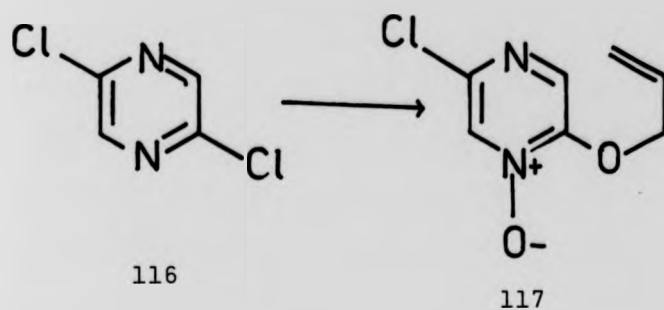
The Claisen rearrangement has no precedent in the 1,4-diazine series although it has precedent in both 1,2- and 1,3-diazines⁸⁴. Rearrangement from O → N is an example of the Chapman rearrangement. It has numerous precedents in the chemical literature. Similarly, this has been observed for 2-alkylated pyridines⁸⁵ (Scheme 53) which undergo Claisen rearrangement to the N-allyl derivatives e.g. (121).



Scheme 53

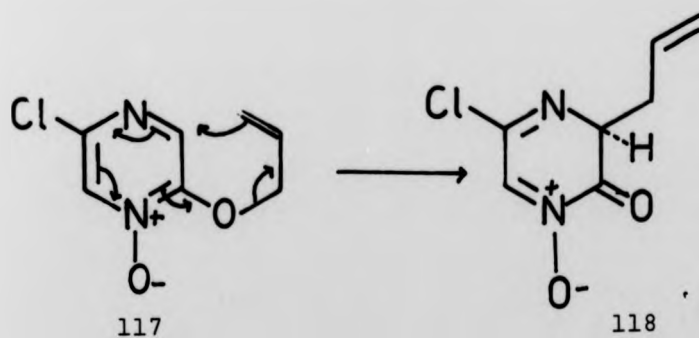
Reaction of 2,5-dichloropyrazine-1-oxide (116) with sodium hydride and dry allyl alcohol in dry benzene produced the mono-alkylated product (117) in 67% yield after 18 hours reflux. Spectral data was consistent with the structure (117) (Scheme 57).

The mono-alkylated product (117) was heated under reflux in toluene for 48 hours. T.l.c. showed the presence of some starting material but also a new spot of lower R_f . P.l.c. of the reaction mixture yielded starting material and a viscous oil whose n.m.r. and i.r. were consistent with the rearrangement product (119). The yield of this product was only 15%.

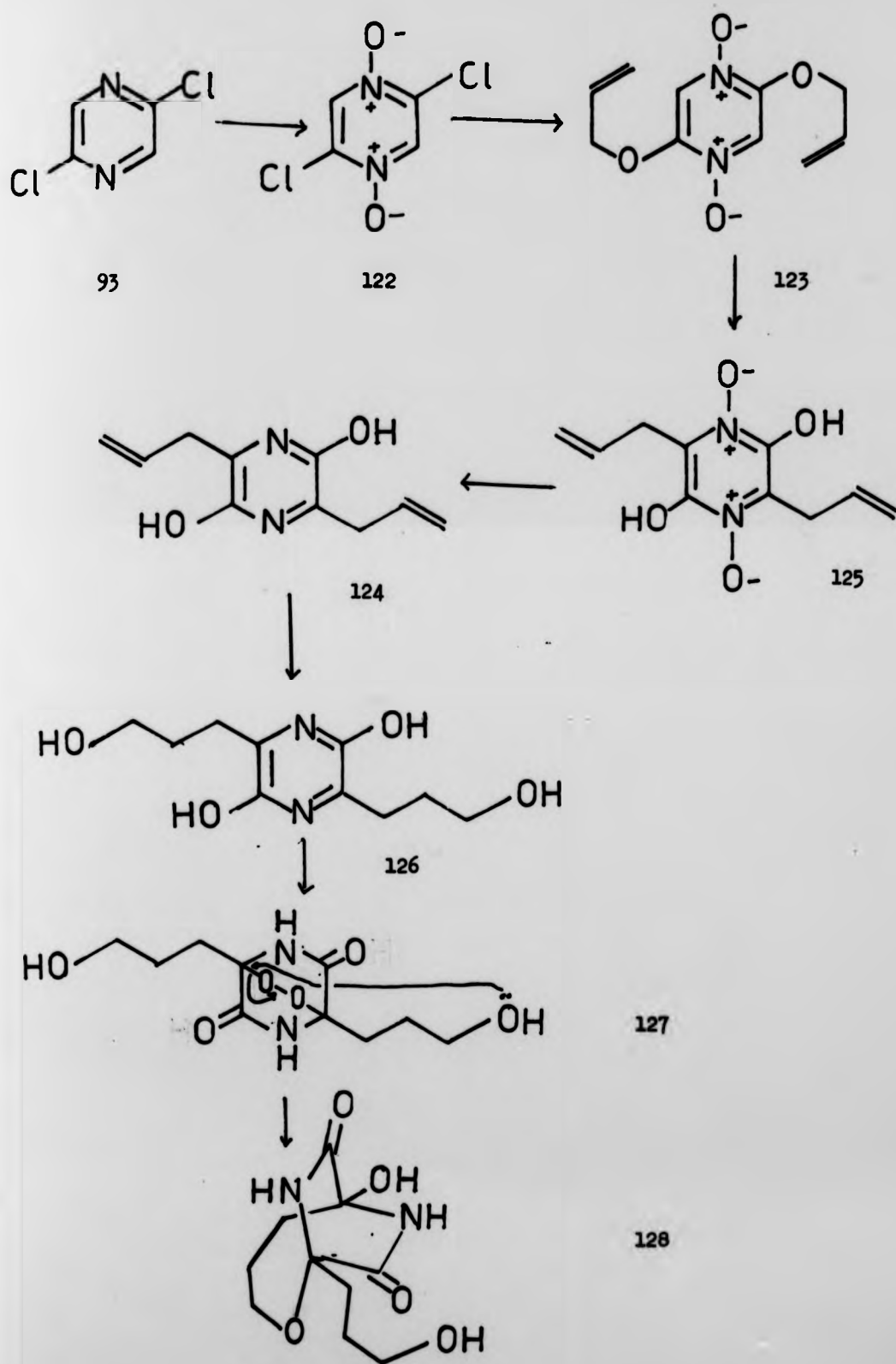


Scheme 54

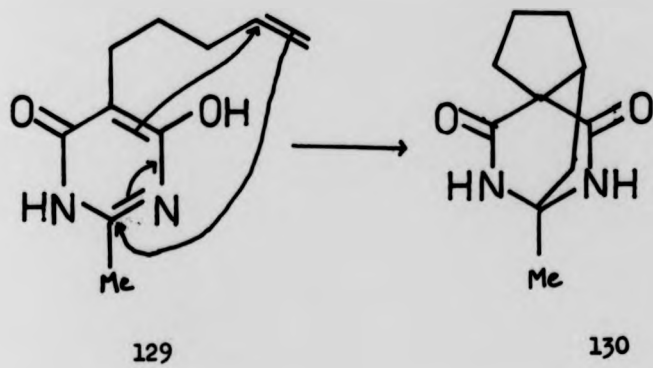
The proton n.m.r. showed the usual allylic proton resonances at τ 4.0(m) 1H and τ 5.9(pd) 2H. A small change in the chemical shift of the methylene triplet from τ 5.9 to τ 6.2 was also observed (Scheme 55).



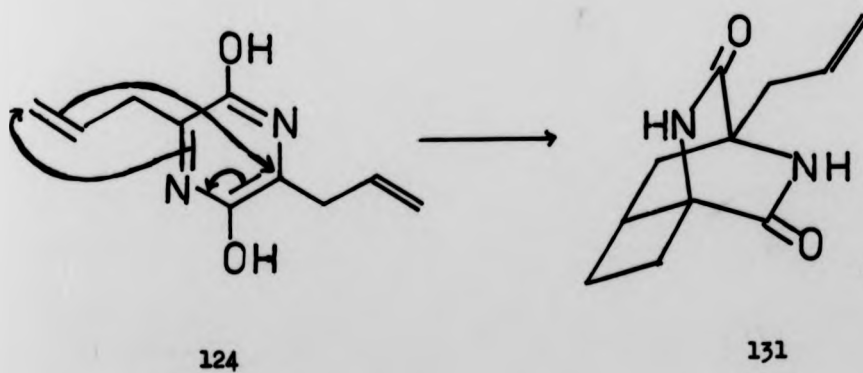
Scheme 55



Scheme 56

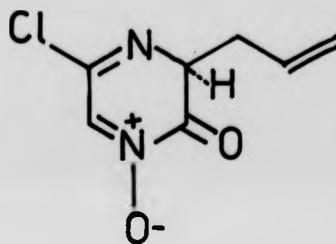


Scheme 57



Scheme 58

However, amide absorption was noted in the i.r. Mass spectral data, however, was ambiguous since the anticipated molecular ion was of low intensity. The product has been tentatively assigned as structure (119).



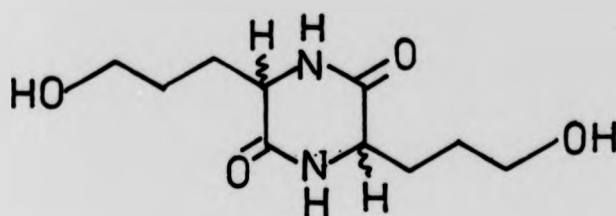
119

The Claisen rearrangement product may therefore constitute a possible route into intermediates which will lead to model systems not dissimilar from bicyclomycin, e.g. Scheme 56.

The pyrazine ring did not act as a diene in this reaction as it does in the previously discussed photooxygenation. This would have led to products such as (131) and analogous reactions are known in the 1,3-diazine series. Hydroxypyrimides, preferring the tautomeric amide form (129), act as true dienes in this case⁸⁶ (Scheme 57).

Similarly the intermediate (132) could be derived via the dimer of a homologated serine-type compound (132) using the normal methods outlined in Scheme 56.

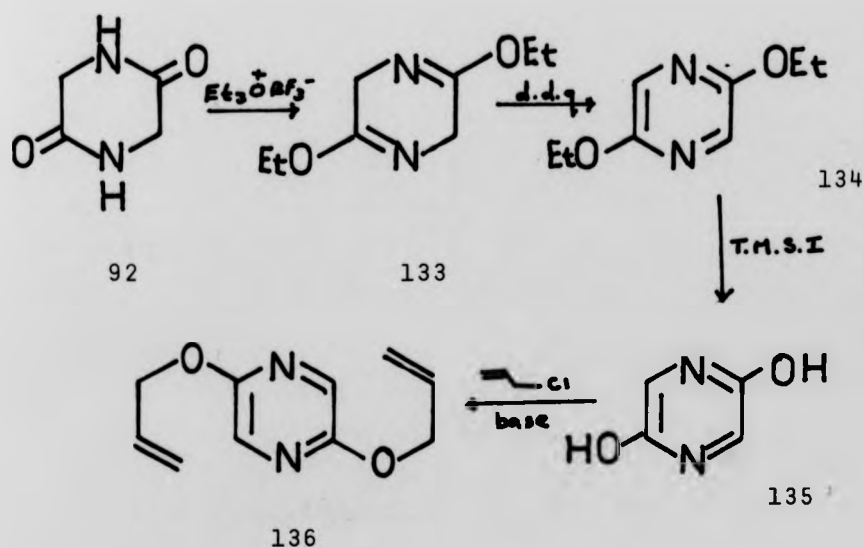
In either case the intermediate (132) should be soluble in hydroxylic solvents and a successful photooxygenation should result.



132

A final route for the preparation of Claisen rearrangement substrates was investigated.

Since the reaction of allyl alcohols with dichloropyrazines had proved problematic, it was decided to prepare a dihydroxypyrazine and react it with allyl halides (Scheme 62).



Scheme 59

2,5-diethoxy-3,6-dihydropyridazine⁸⁷ (133) was prepared in 88% yield by stirring as a suspension of dioxopiperazine (92) and triethyloxonium tetrafluoroborate in dry dichloromethane for

24 hours.

Spectroscopic data confirmed the structure as shown i.e. (133). Aromatisation of the dihydropyrazine (133) was carried out using d.d.q. in dry benzene and subsequent chromatographic separation, yielded 2,5-diethoxypyrazine (134) in 60% yield⁸⁷. Structure (134) was confirmed by spectroscopic means including ¹³C n.m.r.

The conversion of the diethoxypyrazine (134) to the dihydroxy pyrazine (135) with trimethylsilyl iodide was unsuccessful principally due to the fact that the pyrazine (134) is a volatile liquid with a lower boiling point than T.M.S.I. which is also the bulk solvent.

Similarly, stirring 2,5-diethoxypyrazine (134) with T.M.S.I. or a dichloromethane solution of T.M.S.I. did not produce the desired product.

However, this route is probably worth further investigation since so little literature exists on the Claisen rearrangements of the 1,4-diazines.

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SECTION 2

Part 3

3.0 Introduction

In accordance with the projected interest in this field of research, the ^{13}C n.m.r. spectra of synthetic and naturally-occurring intermediates, were recorded and interpreted.

The rationale behind this exercise was two fold. Initially we could use the derived data as a tool for the structural elucidation of natural products and secondly, by inference, we could derive data on particular resonances in pyrazines, dioxopiperazines and bicyclomycin analogues for use in projected biosynthetic studies.

3.1 Authentic bicyclomycin

Authentic bicyclomycin (ex Fujisawa Pharmaceutical Co.) was recorded at the SRC ^{13}C Centre, University of Edinburgh. The anticipated twelve carbon resonances consistent with this structure were observed.

The important resonances in biogenetic studies are those for the carbonyl groups designated C-6 and C-8. Both of these resonances were low in intensity due to the lack of Nuclear Overhauser effect (n.o.e.) in the carbonyl groups.

Although these resonances cannot be unequivocally assigned the resonance at 172.88 ppm may be C-8 which is taken to lower field by the effect of the adjacent exocyclic double bond. The chemical shift increment due to this double bond would be small due to the distance from the carbon involved (C-8).

However this may be sufficient to explain the small difference between the C-6 (169.32 ppm) and C-8 (172.8 ppm) resonances.

The bridgehead carbons C-5 and C-7 are observed as singlets in uncoupled spectra and resonate at 82.67 ppm and 88.43 ppm respectively.

The hydroxylated side chain also yielded the anticipated resonances and were confirmed by the multiplicity of the resonances in the uncoupled spectra.

The resonances determined were C-1 at 66.94 ppm (t), C-2 at 77.89 ppm (s) and C-4 at 71.38 ppm (d). The methyl resonance (C-3) occurred at 23.52 ppm (q).

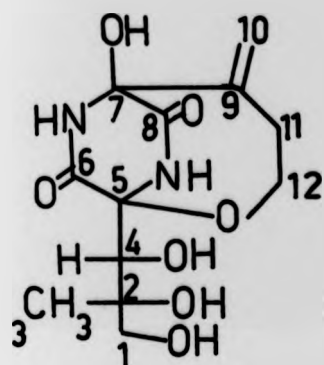
The resonances of the olefinic carbons fall in the expected area, C-9 at 147.67 ppm and C-10 at 118.19 ppm which occurred as a triplet in the uncoupled spectra.

The adjacent carbons C-11 and C-12 fall at 35.53 ppm (t) and 67.81 (t) respectively.

The variation in chemical shift of C-11 and C-12 occurs because of the deshielding effect of the oxygen atom adjacent to C-12.

The structural data and assigned resonances for bicyclomycin (1) are given in Table 11.

TABLE 11

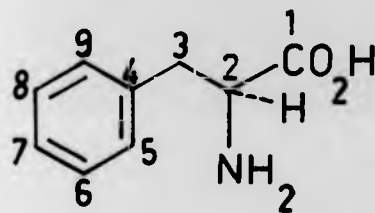


<u>Carbon No.</u>	<u>Shift</u>	<u>Multiplicity</u>
1	66.94	t
2	77.89	s
3	23.52	q
4	71.38	d
5	82.67	s
6	169.32	s
7	88.43	s
8	172.88	s
9	147.67	s
10	118.19	t
11	35.53	t
12	67.81	t

3.2 ^{13}C data for substituted pyrazines

The resonances for the pyrazine systems now discussed have been unequivocally assigned on the basis of the resonances for pyrazine (145.6 ppm) and the standard aromatic resonances for phenyl alanine (Table 12).

TABLE 12

	<u>Carbon No.</u>	<u>Shift</u>	<u>Multiplicity</u>
	1	174.31	s
	2	56.86	d
	3	37.17	t
	4	135.92	s
	5,9	129.94	d
	6,8	130.19	d
	7	128.52	d
	136		

In general there is little variation on the effect of substituents on the C-1 atom of the pyrazine nucleus since all lie between 151 and 152 ppm i.e. 6 - 7 ppm higher than pyrazine itself.

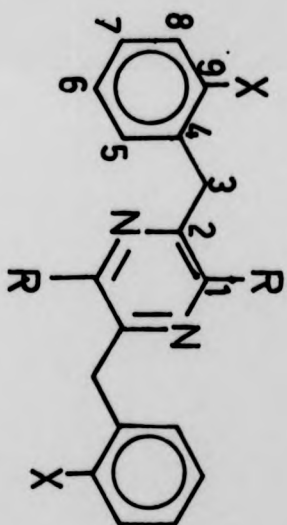
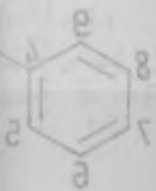


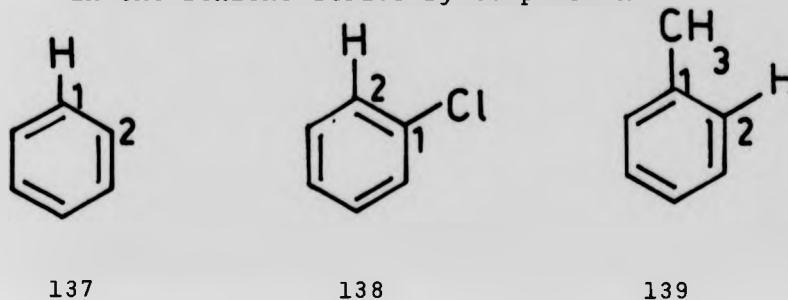
TABLE 13 ^{13}C resonances for substituted pyrazines

Substituents	X	R	Carbon resonances (ppm)									R	
			1	2	3	4	5	6	7	8	9		
(64)	H	OH	151.25	139.09	38.54	136.19	128.31	128.70	126.70	128.70	128.31	-	-
(80)	H	Cl	152.09	145.59	40.39	136.68	128.61	128.84	126.96	128.84	128.61	-	-
(57)	H	OEt	152.10	139.23	37.93	137.68	128.12	129.22	126.02	129.22	128.69	14.74	62.00
(59)	OH	OEt	151.13	138.41	33.15	124.39	130.76	117.58	128.71	120.60	155.69	14.66	63.21
(93)	2,5-di-chloro-	pyrazine	147.64	143.62	-	-	-	-	-	-	-	-	-



The same is true for the C-2 of the pyrazine nucleus except in the case of (80) where the electronegative substituent at C-1 has the effect of raising the C-2 resonance by 6 ppm above its listed counterparts.

In the benzene series by comparison



the parent resonance is 128.7 ppm. Chlorine substitution at C-1 raises the C-1 resonance to 135 ppm i.e. 6 ppm almost identical to the chloropyrazine C-2 shift. The example of toluene only shifts the C-1 resonance by 9 ppm.

Most of the other resonances (in the benzyl substituent) follow the same trend as phenylalanine (136) throughout the cited examples.

The substituent effects for carbon atoms in the phenyl ring follow the same trends as those known to occur in substituted benzenes bearing the same substitution pattern.

A prime example of this is shown by compound (59) bearing a hydroxyl group in the phenyl ring.

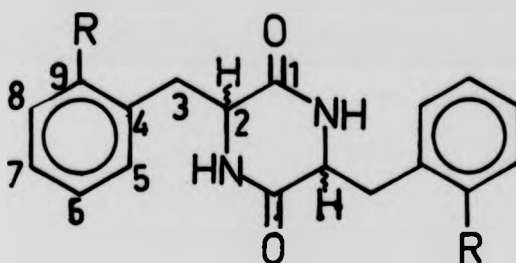
In the benzene series this has the effect of raising the resonance of the adjacent carbon atom by 26.9 ppm. In compound (59) the chemical shift of this carbon is raised by 27.29 ppm.

3.3 ^{13}C data for some dioxopiperazines

Some dioxopiperazines (52) to (53) were subjected to ^{13}C analysis. All are diastereoisomeric mixtures but this will not alter carbon resonances significantly since it is only the carbon resonances we are interested in. The results are depicted in Table 14.

TABLE 14

^{13}C .m.r resonances for a series of dioxopiperazines



Substituent	Carbon Resonance								
	1	2	3	4	5	6	7	8	9
(52) OAc	167.25	54.19	32.42	127.88	131.54	122.56	128.85	125.62	149.53
(34) OH	167.91	54.69	32.23	123.08	131.41	115.59	128.03	119.15	155.81
(53) H	167.21	54.56	38.83	136.05	130.11	126.82	128.19	126.82	130.11

Again the effect of electronegative substituent on C-9 is evident in (52) and (34) and the basic phenyl resonance is raised by 20.83 and 27.11 ppm respectively. The resonances expected in a mono-substituted benzene would be 151.7 and 155.6 ppm and bear a good correlation to those found. Compound (53) C-9 resonance is typical of a mono-substituted benzene bearing a

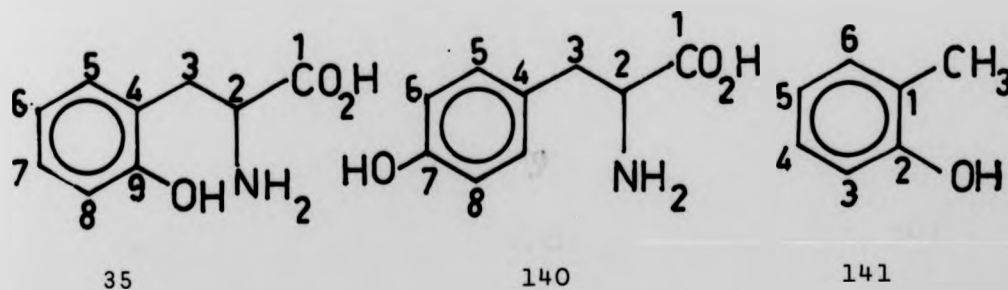
methyl group. In this case the carbon resonance ortho to the substituent would be 129.0 ppm.

3.4 ^{13}C resonances for amino acids

Of prime importance to our model studies and subsequent work was ortho-tyrosine (35), tyrosine (140) and ortho-hydroxy-toluene (141) were all compared by ^{13}C .m.r. (Table 15).

TABLE 15

Compound	Carbon resonance								
	1	2	3	4	5	6	7	8	9
(140)	169.10	57.34	39.82	127.01	132.54	116.52	157.89	116.52	132.54
(35)	174.40	56.22	32.25	127.75	130.62	121.13	127.03	115.71	154.70
(141)	124.71	150.13	115.7	127.30	121.13	130.73			



A good correlation can be seen to exist between all carbon bearing hydroxyl groups e.g. (35) 154.7 ppm, (140) 157.89 ppm and (141) 150.13 ppm.

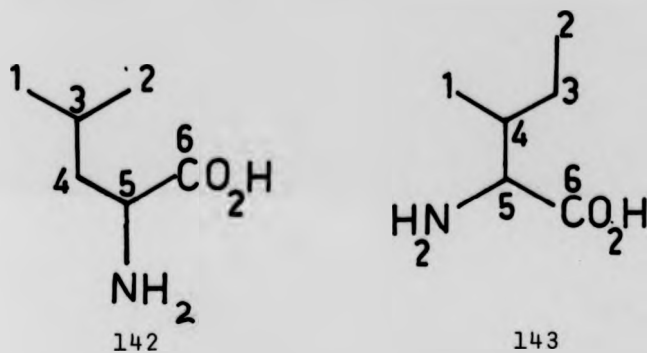
A common trend is also evident between all three when one looks at the phenyl resonances.

The amino acids leucine and isoleucine were of paramount importance to our biogenetic work. These compounds

were subjected to ^{13}C analysis and the results are displayed in Table 16.

TABLE 16

Carbon	1	2	3	4	5	6
Leucine (142)	22.9	21.85	24.84	44.57	54.98	184.48
isoleucine (143)	8.4	13.23	36.23	21.63	58.63	180.55



The observed results are consistent with those reported in the literature for leucine.

If these values had not already been reported it would have been necessary to use an additivity rule for the branched system under study.

Normally one would use Grant's rule Eq-1⁹¹

$$\delta(k) = c + \sum_i n_{ik} \cdot A_i \quad \text{Eq.1}$$

where the shift of the saturated carbon k is derived from Eq.1 and the terms are defined as

c = constant of reference standard ($\text{CH}_4 = 2.1$ ppm)

A_i = shift increment associated with the i^{th} substituent,

and n_{ik} represents the number of substituents at i relative to k .

Typical values for A_1 (α substituents) is +9.1 ppm, A_2 (β substituents) is +9.4 ppm and A_3 (γ substituents) is -2.5 ppm.

Similarly it would be possible to use the Lindemann-Adam rule⁹¹ (Eq.2) but this is not so flexible in application since it ignores α -methyl groups and their effects.

$$\delta_c(k) = A_n + \sum_{m=0}^2 N_m^\alpha \cdot \alpha_{nm} + N_{\gamma n}^\gamma + N_{\delta n}^\delta \quad \text{Eq.2}$$

The terms are defined as follows:

n = no hydrogens at atom k

m = no " at carbon

N_m^α = no of CH_m groups (α methyl groups ignored)

N_γ = no of carbons γ to k

N^δ = no of carbons δ to k .

Values for A_n , α_{nm} , γ_n and δ_n are taken from computed tables.

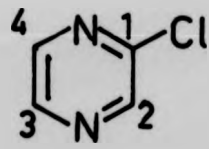
In the case of leucine (142) and isoleucine (143) the Grant equation (Eq.1) would have been more appropriate.

3.5 Miscellaneous structures

The following miscellaneous structures were also subjected to ^{13}C analysis.

(a) 2-chloropyrazine (107) Table 17.

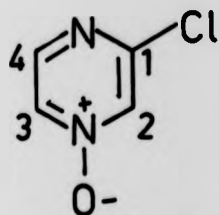
TABLE 17

	<u>Carbon No.</u>	<u>Shift</u>	<u>Multiplicity</u>
	1	149.31	s
	2	143.58	d
	3	142.23	d
	4	144.71	d

Again the same trend in chemical shifts is evident compared with chlorobenzene where δ meta > δ ortho > δ para.

(b) 3-chloropyrazine-1-oxide (108) Table 18.

TABLE 18

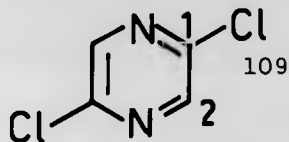


108

<u>Carbon No.</u>	<u>Shift</u>	<u>Multiplicity</u>
1	151.66	s
2	133.09	d
3	133.39	d
4	145.84	d

(c) 2,5-dichloropyrazine (93) Table 19.

TABLE 19



109

<u>Carbon No.</u>	<u>Shift</u>	<u>Multiplicity</u>
1	147.64	s
2	143.82	d

(d) 3,6-dibenzyl-2,5-diethoxy-3,6-epidioxo-3,6-dihydropyrazine

This product shows identical phenyl resonances when compared with the parent compound 3,6-dibenzyl-2,5-diethoxy-pyrazine (57). An increase in the C-2 resonance compared with 3,6-dibenzyl-2,5-dioxopiperazine is created due to the electro-negative dioxo-bridge adjacent to this carbon (Table 20).

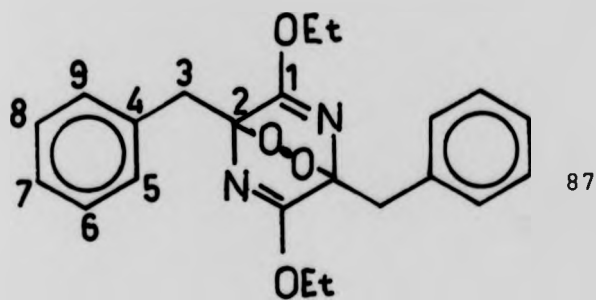


TABLE 20

<u>Carbon No.</u>	<u>Shift (ppm)</u>	<u>Multiplicity</u>
1	174.86	s
2	88.60	s
3	36.66	t
4	133.87	s
5	130.52	d
6	126.79	d
7	127.77	d
8	126.50	d
9	130.93	d

REFERENCES

1. Y. Yagisawa, Japan Med.Gaz., 1973, 10, 13
2. M. Nishida, Y. Mine and T. Matsubara, J.Antibiotics, 1972, 25, 532
3. T. Miyoshi, N. Miyairi, H. Aoki, M. Kohsaka, H. Sakai and H. Imanaka, J.Antibiotics, 1972, 25, 569
4. S. Miyamura, N. Ogasawara, H. Otsuka, S. Niwayama, H. Tanaka, T. Take, T. Uchiyama and H. Ochai, J.Antibiotics, 1973, 26, 479
5. S. Miyamura, N. Ogasawara, H. Otsuka, S. Niwayama, H. Tanaka, T. Take, T. Uchiyama, H. Ochai, K. Abe, K. Koizuma, K. Asao, K. Matsuki and T. Hoshino, J.Antibiotics, 1972, 25, 610
6. H. Saki, T. Miyoshi, N. Miyari, H. Aoki, M. Kohsaka and H. Imanaka, Jap.Kokai, 1974, 74, 94, 898
7. T. Kamiya, S. Maeno, M. Hashimoto and Y. Mine, J.Antibiotics, 1972, 25, 576
8. Y. Tokuma, S. Koda, T. Miyoshi and Y. Morimoto, Bull.Chim.Soc.Japan, 1974, 47, 18
9. H. Maay, J. F. Blount, D. L. Coffen, T. S. Steppe and F. Wong, J.Amer.Chem.Soc., 1978, 100, 6786
10. T. Kamiya and S. Maeno (Fujisawa Pharm.Co.), S.African Pat. 7,462,795 (1976)
11. T. Kamiya and S. Maeno (Fujisawa Pharm.Co.), Austrian Pat. 335,607 (1977)
12. T. Kamiya, S. Maeno and Y. Kit, Ger.Offen. 2,647,322 1977
13. T. Kamiya, S. Maeno and Y. Kit, Belgian Pat. 815, 530 1974

14. M. Nishida, Y. Mine and T. Matsubara, J.Antibiotics, 1972, 25, 594
15. M. Nishida, Y. Mine, S. Nonoyama, T. Kamimura and S. Fukada, J.Antibiotics, 1974, 27, 456
16. M. Nishida, Y. Mine, S. Nonoyama, T. Kamimura and S. Fukada, J.Antibiotics, 1974, 27, 976
17. B. Muller, N. Kump and B. Wacker (Ciba-Geigy A.G.) Ger.Offen 2,722,164 (1977)
18. B. Muller, N. Kump and B. Wacker (Ciba-Geigy A.G.) Belgian Pat. 854,841 (1977)
19. B. Muller, N. Kump and B. Wacker (Ciba-Geigy A.G.) Ger.Offen 2,734,579 (1977)
20. E. J. Corey and W.L. Jorgensen, J.Amer.Chem.Soc., 1976, 98, 203
21. A.E.A. Porter and P. G. Sammes, Chem.Comm., 1970, 1103
22. P. J. Machin, A.E.A. Porter and P. G. Sammes, J.C.S. Perkin I, 1973, 405
23. A. J. Birch and J. J. Wright, Tetrahedron, 1970, 26, 2329
24. K. W. Blake and P. G. Sammes, J.Chem.Soc.(C) 1970, 980
25. A. Somieya, M. Iseki and N. Tanaka, J.Antibiotics, 1978, 31, 712
26. P. G. Sammes and J. L. Markham, J.C.S.Perkin I, 1979, 1885
27. P. G. Sammes and J. L. Markham, Chem.Comm., 1976, 417
28. J. E. Baldwin, Chem.Comm., 1976, 734
29. T. Kamiya and S. Maeno (Fujisawa Pharm.Co.), Ger.Offen. 2,334,927 (1974)
30. T. Kamiya and S. Maeno (Fujisawa Pharm.Co.), British Patent 1,334,179 (1974)
31. Personal communication from Roche Products Ltd.

32. H. J. Svec and G. A. June, J.Amer.Chem.Soc., 1964, 86, 2278
33. This work was conducted by Dr. J. J. Usher during a short postdoctoral visit to this University.
34. Personal communication from Bacteriology Department, M.R.C. Unit, Edinburgh University.
35. A. I. Vogel 'Textbook of Quantitative Organic Analysis', p.613.
36. I am indebted to Dr. M. Horne, Biology Department, Stirling University, for pure isolates of these industrial strains.
37. R. A. Rippere, J.Pharm.Sci., 1978, 67, 367
38. D. Collins, 'Microbiological Methods', p.296, 1974, Wiley.
39. Personal communication from Mr. G. A. Smith.
40. D. Collins, 'Microbiological Methods', 1974, Wiley.
41. E. J. Corey, D. J. Brunelle and K. C. Nicolaou, J.Amer.Chem.Soc., 1977, 99, 7359
42. W. P. Dickson and R. G. Marshall, J.C.S., 1929, 1495
43. D. L. Coffen, D. A. Katonak, N. R. Nelson and F. Sancilio, J.O.C., 1977, 42, 948
44. E. Erlenmeyer and W. Stadlin, Annalen, 1904, 337, 283
45. G. W. Kirby, J. Michael and S. Narayana swami, J.C.S. Perkin I, 1972, 202
46. E. Erlenmeyer and J. T. Halsey, Annalen, 1899, 307, 138
47. A. I. Vogel, 'Practical Organic Chem', p.584 and 910.
48. E. Erlenmeyer and J. T. Halsey, Chem.Ber., 1897, 30, 2981
49. K. W. Blake, Ph.D. Thesis, London University, 1970, 181
50. H. Meerwein, E. Battenberg, H. Gold, E. Pfiel and G. Willfend, J.Prakt.(2), 1940, 154, 83
51. H. Meerwein, Org.Syn., 1966, 46, 113

52. A.E.A. Porter, Ph.D. Thesis, London University, 1971
53. F.M.W. McOmie and M. L. Watts, Chem.Ind., 1963, 1658
54. P. J. Machin, A.E.A. Porter and P. G. Sammes,
J.C.S. Perkin I, 1973, 405
55. G. W. Kakover, J. B. Brown and J. Fried, J.Org.Chem.,
1962, 27, 4710
56. Fieser and Fieser, 'Reagents for Organic Synthesis',
I, p.434.
57. F. L. Benton and T. E. Dillon, J.Amer.Chem.Soc.,
1942, 64, 1128.
58. I. Vlattas, I. T. Harrison, L. Tokes, J. H. Fried and
A. D. Cross, J.Org.Chem., 1968, 33, 4176
59. A. R. Surry, Org.Syn.Coll.Vol.3, 1955, 753
60. H. T. Clarke and E. R. Taylor, ibid. I, 1941, 150
61. G. B. Bennet, Synthesis, 1977, 589
62. M. E. Jung and M. A. Lyster, J.Amer.Chem.Soc., 1977, 99, 968
63. M. E. Jung and M. A. Lyster, J.Org.Chem., 1977, 42, 3761
64. M. E. Jung and M. A. Lyster, Chem.Comm., 1978, 315
65. M. E. Jung, W. A. Andrus and P. L. Ornstein, Tett.Letts.,
1977, 4178
66. M. E. Jung and P. L. Ornstein, Tett.Letts, 1977, 4178
67. G. A. Olah, B.I.B. Gupta and S. C. Nara, Synthesis,
1977, 583
68. B. I. Rosen and W. P. Weber, J.Org.Chem., 1977, 42, 3463
69. T-L. Ho and G. A. Olah, Synthesis, 1977, 417
70. T-L. Ho and G. A. Olah, Proc.Natl.Acad.Sci., 1978, 75, 4
71. M. E. Jung and T. A. Blumenkopf, Tett.Letts., 1978, 3657
72. K. W. Blake and P. G. Sammes, J.Chem.Soc.C., 1970, 1070
73. Personal communication from Professor P. G. Sammes,
City University, London, 1972

74. J. L. Markham, Ph.D. Thesis, London University, 1977
75. L. Cere, A. Marchese and P. F. Rossi, Rass.Chim., 1975, 27, 294
76. G. Palamidessi and L. Bernardi, J.Org.Chem., 1964, 29, 2491
77. Ger.Offen. 1,911,023 (1971), Chem.Abs., 1971, 74, 3169 (d)
78. G. T. Newbold and F. S. Spring, J.Chem.Soc., 1947, 1183
79. B. Klein, N. E. Hetman and M. E. O'Donnell, J.Org.Chem., 1963, 28, 1682
80. R. A. Baxter and F. S. Spring, J.Chem.Soc., 1948, 1858
81. G. E. Mixan and R. G. Pews, J.Org.Chem., 1954, 29, 2491
82. Aldrich 'N.m.r. catalogue' a. 71
83. G.W.H. Cheeseman and E.S.G. Törzs, J.C.S., 1965, 6681
84. B. S. Thyagarajan, Adv.Heterocyclic Chem., 1967, 8, 143
85. E. Ochai, J.Org.Chem., 1953, 18, 534
86. B. L. Davies, O. A. Leci and P. G. Sammes, J.C.S. Perkin I, 1978, 1293
87. K. W. Blake, Ph.D. Thesis, Imperial College, London, 1970, 177
88. Dr. I. Sadler, SRC Fourier Transform NMR Unit, Edinburgh University.
89. Levy and Nelson, '¹³C n.m.r. for Organic Chemists', 1973, Wiley Interscience.
90. Johnston and Jankowski, 'Carbon-13 Spectra', 1972, Wiley Interscience.
91. Wehrli and Wirthlin, 'Interpretation of ¹³C n.m.r. spectra', Hayden 1978
92. D. M. Grant and E. G. Paul, J.Amer.Chem.Soc., 1964, 86, 2484
93. J. Oehldrich and J. M. Cook, J.Org.Chem., 1977, 42, 891

SECTION 3

EXPERIMENTAL

PART I

General Methods

All stock microorganisms were grown on sterile media and stored at 4°C to ensure slow even growth. Normal microbiological techniques were used throughout. All media and instruments were routinely autoclaved at 15 p.s.i. (110°C) for 20 minutes.

All E.coli strains were cultured for 18 hours at 100 r.p.m., 36°C in 10% nutrient broth (Oxoid).

All assays were carried out on nutrient broth (Oxoid) agar (10mm thick) and all plates were kept uniform, since variation in the thickness of the agar can lead to variability in the size of inhibition zone produced.

Cylinder cup assays were carried out in the standard method - a well (10mm diameter) was cut in the agar with a sterile cork-borer and the base was sealed with a few drops of the appropriate agar and allowed to cool. The surface was then seeded with the E.coli strain (ca. 10^5 bacteria ml^{-1}) and the central well filled with a known volume of the preparation to be tested. The plate was then incubated for 36 hours at 36°C and the size of the zone measured (external diameter).

Disc assays were made up as above - the only difference being that there was no well and that the preparation was added to a filter paper disc (Whatman No. 1, 1 cm) and this was placed on the seeded agar surface.

Fermentation Methods

Shake culture

This implies the use of one litre invaginated flasks containing 200-300ml of medium (previously autoclaved in the flask). These flasks were seeded with stock cultures of the Streptomyces strain and placed in an orbital incubator.

Fermentations were conducted at 30°C, 180 r.p.m., for 144 hours.

Large scale fermentations were conducted in an LHE 1000 (20L) fermentation using a silicone based anti-foaming agent.

In this case the medium was placed in the 20L vessel and the two autoclaved at 15 p.s.i. (110°C) for 60 minutes.

Revival of the Organism

A freeze-dried sample of S. sapporonensis, (ATCC 21532) was obtained in a glass vial in the form of freeze-dried mycelium.

The vial was opened in a sterile Airflow cabinet and 1.0 ml of sterile distilled water was added to the contents and the solid material was then suspended by agitation of the suspension with a sterile wire loop.

Preparation of Malt Agar

Malt extract agar (50g) and yeast extract powder (3g) were added to water (1.0 litres) and the solution was then boiled for 5 minutes to dissolve most of the solids present. The resulting solution was then autoclaved at 15 p.s.i. (110°C) for 15 minutes, and when it had cooled to blood heat, was poured onto

30 sterile Petri dishes and into 3 conical flasks (250 ml). The plates were left to cool in the cabinet and the flasks were set aside in the cold room to be used in the establishment of stock cultures.

Plating out the Organism

A small amount of the suspended revived organism was plated out onto each of the malt agar plates using a sterile wire loop.

After 14 days the cultures had grown well and distinct white colonies could be easily observed. The plates were then stored in the cold room (4°C) to slow down growth and to minimise cross contamination.

Streptomyces aizunensis (ATCC 21775) was cultured in an identical manner.

Liquid culture fermentation techniques

Preparation of vegetative cultures

Six small portions of an actively growing S. sapporoensis culture were added, in sterile fashion, to previously autoclaved malt extract liquid media (300 ml). After 48 hours in an orbital incubator at 180 r.p.m. and at 30°C, a vigorously growing vegetative (anaerobic) culture was produced.

Preparation of malt extract liquid media

Into a two litre conical flask was placed the following mixture: peptone (6.0g); yeast extract powder (3.6g); malt extract powder (36g) and water (1.2 litres). The medium was then boiled for 5 minutes and was added, in portions of 300 ml, to each of four invaginated 2L culture vessels. The vessels were plugged with cotton wool, covered with foil caps and autoclaved for 15 minutes at 15 p.s.i. (110°C).

The vegetative culture was then added in equal portions to the four fermentation vessels and these were cultured at 30°C for six days at a shake speed of 180 r.p.m.

Isolation Method

Fermentation No. 1.

The contents of the flask were first cooled and then filtered through a Celite plug in a sintered glass funnel, under vacuum.

The culture broth (1500 ml) was then adjusted to pH 7.0 using aqueous sodium hydroxide solution (20%). To this solution was added active carbon (40g) and the solution was then stirred for 20 minutes. The active carbon was then filtered off and the cake sucked dry under vacuum.

Removal of the cake and extraction with a mixture of ethyl acetate:MeOH (5:1) (2 x 300 ml) yielded a brown gum (1.35g). After evaporation of the solvent, lyophilisation of the gum yielded a crude product (800 mg) which showed some activity after t.l.c. (benzene:MeOH:CHCl₃-1:1:1) and subsequent visualisation with potassium iodo-cuprate spray (KIP). Rf Authentic Bicyclomycin 0.56 in above solvent mixture. Rf of KIP positive spots 0.07; 0.23; 0.33; 0.55; 0.69; 0.71; 0.88; 0.93.

P.l.c. of the crude product using CHCl₃:MeOH (5:1) gave a band with the same Rf as the authentic antibiotic, i.e. Bicyclomycin has an Rf value of 0.21 in CHCl₃:MeOH (5:1). The crude product when eluted with the same solvents gave bands at Rf 0.59, 0.40, 0.21 and 0.15.

Lyophilisation of the eluent from the band with Rf 0.21 gave a crude colourless gum (140 mg). The crude material was then boiled with hot acetone and filtered. The filtrate was then concentrated, and the acetone insoluble material was dissolved in methanol (1.0 ml).

T.l.c. of the various fractions in benzene:chloroform:methanol (1:1:1) showed that only the acetone extract had any comparable Rf values with that of bicyclomycin.

Authentic Bicyclomycin Rf 0.62.

Acetone extract Rf 0.61.

Methanol extract Rf 0.59.

All of the acetone soluble fraction was loaded onto a 20 x 20 cm plate and eluted with benzene:chloroform:methanol (1:1:1). After elution of the material corresponding to the bicyclomycin marker, it was found that the amount of sample obtained was disappointingly small (ca. 1.0mg) (quantitative t.l.c. using 5 and 1 mg loading of standards on precoated Merck SiO₂ plates GF₂₅₄ 20 x 20 cm.).

Fermentation No. 2.

The fermentation was carried out under the conditions mentioned previously except that the culture time was only 60 hours.

After treatment with active carbon, a brown gum (1.5g) was again obtained. The gum was then extracted with acetone (30 ml) and stirred for 30 minutes. Subsequent filtration and concentration yielded a white solid (110 mg).

P.l.c. of this solid in benzene:chloroform:methanol (1:1:1) and then t.l.c. on an analytical plate (20 x 20 cm.) using the same solvent system and a repeated elution method, eventually yielded only two products which reacted positively

with KIP spray. One of these was only present in trace quantities.

Bicyclomycin Rf 0.46 KIP positive.

Spot No. 1 Rf 0.47 KIP positive. Rotation +0.014

Spot No. 2 Rf 0.41 KIP negative. Rotation -0.002

Mass spectral data showed that the compound with the positive rotation was very similar to bicyclomycin; m/e 114 normal for a DKP ring and m/e 43 extrusion of -NHCO . It is possible, therefore, that this compound may represent some related material or, possibly, a precursor of bicyclomycin.

Fermentation No. 3.

Culturing of the organism was as previously described in Fermentation No. 2.

The filtrate (3L) was adjusted to pH 7.0 and then charcoal (100g) was added and the solution stirred for 40 minutes. The filter cake was then sucked dry and extracted with a mixture of methanol and ethyl acetate as previously reported, and when concentrated, gave a gum (4.2g).

The gum was then dissolved in an equimolar solution of benzene:methanol and chloroform and passed through a short silica plug to remove the highly polar base line material. The resulting solution was then concentrated and dissolved in methanol (10 ml), then passed onto a silica gel column (80-100 mesh) and then eluted with toluene:chloroform:methanol (1:1:1). Six fractions (110 ml) were collected but only fractions 2, 3 and

4 showed a spot with the same Rf as authentic bicyclomycin on t.l.c.

Fractions 2, 3 and 4 were pooled and subjected to p.l.c. in CHCl_3 :MeOH (5:1) and a broad band around Rf 0.45-0.6 was removed and re-eluted by p.l.c. with CHCl_3 :MeOH (2:1) and the band corresponding to bicyclomycin Rf 0.41 was removed. As in the first two fermentations the amount of material left was far too small for further examination.

A portion of the original filtrate (300 ml) was given to Dr. J. J. Usher and to this was added 10 mg of authentic bicyclomycin. After working through the work up procedure, the process was shown to be 10% efficient (based on bicyclomycin recovery). A second experiment gave a similar result.

At this time, it was shown that methanol extraction of the mycelium of S. sapporonensis from actively growing cultures had no traces of the antibiotic present in them.

Preparation of Soyflour medium

The composition for the soyflour medium is given in Table 1.

The resulting broth was then divided between two fermentation vessels. Culturing and isolation were as previously described in this section but again, little material was obtained.

Since the culture of the organism was showing more progress with media based on Pharmamedia, the initial work using these media was continued.

TABLE 1

Composition for soyflour medium¹

<u>Material</u>	<u>Quantity</u>
Glucose	10g
Soybean meal	10g
Peptone	1.0g
NaNO ₃	1.0g
KH ₂ PO ₄	0.5g
MgSO ₄ .7H ₂ O	0.25g
FeSO ₄ .7H ₂ O	5 mg
Distilled Water	500 ml

Preparation of Pharmamedia media

The composition of the medium (per 300 ml) was as given in Table 2.

TABLE 2

Composition of Pharmamedia medium²

<u>Material</u>	<u>Quantity</u>
Potato starch	15g
Pharmamedia	9g
MgSO ₄ .7H ₂ O	3g
NaHPO ₄	0.75g
KH ₂ PO ₄	3.27g
Distilled Water	300 ml

Six two litre fermentation flasks were filled with the medium described and autoclaved at 15 p.s.i. (110°C) for 20 minutes, then inoculated with a portion of a vegetative culture of S. sapporonensis and cultured at 30°C, 180 r.p.m. for three

days. Three flasks were then immersed in ice for one hour and then all six flasks were incubated for a further three days at 30°C, 180 r.p.m. The two treatments were then worked up separately.

Work up procedure

After 6 days the cultures were removed and the mycelium was removed by filtration through Celite beds. The culture fluid was then adjusted to pH 7 in each treatment with 1M NaOH and this usually gave rise to cloudiness in the culture fluid. The filtrate (ca. 1200 ml) was then stirred with active carbon (35g) and after about 30 minutes the carbon was removed by vacuum filtration.

The carbon was then stirred with methanol:ethyl acetate (5:1, 600 ml) for 45 minutes. The carbon was removed by filtration and the solution concentrated to a brown gum.

This gum was suspended in methanol (10 ml) and applied to two p.l.c. plates (20 x 40 cm/SiO₂), eluted with toluene:chloroform:methanol (1:1:1) and the band (Rf 0.46-0.84) corresponding to bicyclomycin (Rf 0.57) was removed and eluted with methanol. On t.l.c. in the same solvent system, this gave a spot with a positive KIP reaction at Rf 0.58 in the mixture, compared with Rf 0.59 for authentic bicyclomycin.

The methanolic solution was then loaded onto a second pair of p.l.c. plates (20 x 20 cm/SiO₂) and eluted with chloroform:methanol (5:1) and the band corresponding to bicyclomycin (Rf 0.21-0.34) was removed and eluted again with methanol. (Rf of bicyclomycin in this solvent system was 0.30).

The resulting methanolic solution was concentrated to an almost colourless oil which was subjected to t.l.c. (20 x 20 cm/SiO₂) in toluene:chloroform:methanol (1:1:1) and the band corresponding to bicyclomycin was removed and eluted with methanol (10 ml).

On concentration, this yielded 6.6 mg of a colourless oil which ran as a single spot on t.l.c. in toluene:chloroform:methanol (1:1:1) Rf 0.42 and the Rf of authentic bicyclomycin was 0.42 also. The material solidified during storage.

In a similar fashion the temperature shocked was shown to yield ca. 30 mg of the antibiotic. Rf in the same solvent system was 0.42. No crystallisation of the material was attempted.

Preparation of Carbohydrate deficient medium

The composition of the medium (per 300 ml) was as given in Table 3.

TABLE 3

Composition of carbohydrate deficient media

<u>Material</u>	<u>Quantity</u>
Potato starch	2.83g
Pharmamedia	9g
MgSO ₄ ·7H ₂ O	3g
Na ₂ HPO ₄	0.75g
KH ₂ PO ₄	3.27g
distilled water	300 ml

This gave a 60% reduction in the amount of available carbohydrate. Sterilisation and culturing in the media were identical to the method previously described in general methods.

Purification

Work up was as described previously i.e. charcoal adsorption; organic solvent extraction; p.l.c. in the two solvent systems and finally t.l.c. in toluene:chloroform:methanol (1:1:1).

The thermal shock treatment showed a spot with Rf 0.42 and the control showed two spots of Rf 0.42 and 0.39 (authentic bicyclomycin Rf 0.42).

The control sample was then run in the above solvent and the band with Rf 0.42 removed to yield 4.4 mg of the crude antibiotic. The thermally shocked treatment yielded 9.2 mg.

Preparation of nitrogen deficient medium

The composition of the nitrogen deficient medium was as given in Table 4.

TABLE 4

The composition of nitrogen deficient medium (per 300 ml)

<u>Material</u>	<u>Quantity</u>
Potato starch	14g
Pharmamedia	2.9g
MgSO ₄ .7H ₂ O	3.0g
Na ₂ HPO ₄	0.75g
KH ₂ PO ₄	3.27g
distilled water	300 ml

The sterilisation and incubation conditions were as previously described.

Purification

The same methods were employed in purification of the crude antibiotic. After chromatography, t.l.c. showed that

the antibiotic was present in both the control and thermally shocked extracts. The Rf in each case was 0.31 (in chloroform:methanol 5:1) and the Rf of authentic antibiotic was 0.30.

This yielded 4.6 mg of the crude antibiotic from the shocked treatment and 2.9 mg from the control.

Culturing *S. aizunensis* on 'Pharmamedia'

S. aizunensis (ATCC 21775) was grown in a vegetative culture and used to inoculate six flasks containing Pharmamedia medium. Culturing and isolation were as previously described.

Several morphological differences were observed

- (a) Colony size was reduced and, in some cases, colonies were not observed.
- (b) The broth thinned very quickly.
- (c) The resulting mycelium free broth was highly coloured.
- (d) The mycelium from submerged culture was brown (in the other strain it is grey).

After isolation, however, the yields of crude antibiotic were 7.2 mg from the shocked culture and 4.9 mg from the control. Both extracts ran as single spots on t.l.c. (toluene:chloroform:methanol 1:1:1) Rf 0.42.

Mass culturing of shock treatments on Pharmamedia

In this case all six flasks were thermally shocked using *S. sapporonensis* as the culturing organism. Incubation and work up were as before except that a white solid (3.82g) crystallised out of the extract prior to chromatography.

The combined filtrates yielded, after chromatography,

5.9 mg of a gum which ran as a single spot on t.l.c. (benzene:chloroform:methanol 1:1:1) Rf 0.47. The Rf of authentic bicyclomycin was 0.47.

Characterisation of the white solid from *S. sapporonensis* culture fluid

The crude material had the following characteristics.

m.p. 126-129°C.

Rf (chloroform:methanol 5:1) 0.63 (KIP positive)

Rf (n-BuOH:AcOH:MeOH 3:1:1) 0.07 (negative ninhydrin test)

n.m.r. (CD₃OD) τ 2.3(s) 1H, 2.4 and 2.6 both (d) 2H, 2.9 (dd) 1H.

$\nu_{\text{CHCl}_3}^{\text{max}}$ 3550; 3200 (broad); 2820; 1630- 1410; 1350; 1300; 1200; 1040; 920; 850 cm⁻¹.

The material was then crystallised from toluene (three times) and gave white needles.

m.p. 133-134°C.

n.m.r. (CD₃OD) τ 2.3(s) 1H, 2.5 and 2.7(d) 2H, 2.9(dd) 1H.

Treatment with NaOD gave a shift in the chemical shifts of some of the aromatic protons i.e.

n.m.r.* (after NaOD) τ 1.9(s) 1H, 2.6(d) 2H, 3.09(dd) 1H.

$\nu_{\text{KBr}}^{\text{max}}$ 3200-2800 (weak); 1610; 1420; 1350; 1310; 1240; 1210; 1050; 950; 880; 820 cm⁻¹.

These data may be indicative of a phenolic group (the solid also gave a positive ferric chloride test).

Sodium fusion with molten sodium (Lassaigne's³ test) gave positive results for nitrogen (production of Prussian blue with ferrous sulphate and a trace of acid) and sulphur (positive tests with ferrous sulphate and sodium nitroprusside).

Mass spectral information was also sketchy i.e. there is no evidence that we can observe the molecular ion but the base peak is 127. Accurate mass measurement gave this as 127.0092 (best fit was C_5H_5ONS - at the moment this is not given a precise structure).

General methods for assays in large scale fermentations

All E.coli strains were cultured for 18 hours at 100 r.p.m., 30°C in 10% nutrient broth (Oxoid).

All assays were carried out on nutrient broth (Oxoid) agar (10 mm thick) and all plates were kept uniform since variation in the thickness of the agar can lead to variability in the size of the inhibition zone produced.

Cylinder cup assays⁴ were carried out using the standard method - a well (10 mm diameter) was cut in the agar with a sterile cork-borer and the base was sealed with a few drops of the appropriate agar and allowed to cool. The surface was then seeded with the E.coli strain (ca. 10^5 bacteria. ml^{-1}) and the central well filled with a known volume (100 μl) of the preparation to be tested. The plate was then incubated for 36 hours at 36°C and the size of the zone measured (external diameter).

Disc assays⁴ were made up as above - the only difference being that there was no well and that the preparation was added to a filter paper disc (Whatman No. 1, 1 cm) and this was placed on the seeded agar surface.

Large scale fermentation of S. aizunensis

A vegetative culture of the organism was produced as previously reported. Into a twenty litre vessel was placed

fifteen litres of azumycin medium, the composition of which is given in Table 5.

TABLE 5

Composition of azumycin medium⁵

<u>Material</u>	<u>Quantity</u>
Glucose	280g
Soybean meal	280g
Peptone	28g
NaNO ₃	28g
KH ₂ PO ₄	14g
MgSO ₄ .7H ₂ O	7g
KCl	7g
FeSO ₄ .7H ₂ O (pH 7)	14 mg
distilled water	1400 ml

The medium was then autoclaved for 60 minutes at 15 p.s.i. (110°C). The fermenter was then assembled and fermentation was conducted at 30°C, 300 r.p.m. and 10L air min⁻¹ for 60 hours.

The broth was then centrifuged (Sorvall SS-34) to remove debris (10,000 r.p.m., 10°C) and the resulting solution was then adjusted to pH 7 and stirred with active carbon for a period of one hour.

After removing the carbon by filtration, it was extracted with ethyl acetate:methanol (5:1), concentrated and suspended in a little methanol.

A portion (1g) was taken to dryness and used to make up a range of dilutions (500 to 5 mg/ml) in a solvent composed of

0.1 ml methanol, 0.9 ml water and 4 drops of acetonitrile (HPLC grade). The solvent did not inhibit the growth of any of the E.coli strains used.

The extract was applied at the rate of 100 μ l/well. The mean results are given below. Assays were carried out in triplicate.

Conc.(mg/ml)	500	250	125	62.5	50	30	5
Zone (mm)	19.4	18.05	14.6	13.2	11.5	10.5	10.3

M.I.C. approx 5 mg.ml⁻¹.

It should be noted that the test organism was E.coli var. 9482 and that the strains Wild I, W3160++, 9843 and MRE 600 were not inhibited by the crude broth.

Fractionation of the crude broth

1g of crude material was separated by Kieselgel H column chromatography using chloroform:methanol (5:1) and 29 x 5 ml fractions were collected. When tested by cylinder cup assay using E.coli 9482, only three fractions showed pronounced activity i.e. fractions 9, 15 and 19. The details of the activity of these fractions are given in the Discussion.

Fractions 5 to 10 were pooled, as were fractions 11 to 18.

Fractionation on a silica gel column using chloroform:methanol (5:1) gave 80 x 0.5 ml fractions.

These were also assayed by cylinder cup assay using the test organism E.coli 9482 and every fifth fraction as a test.

Fraction	5	10	15	20	25	30	35	40	45	50	55
Zone (mm)	10	10	11	12	13.5	13.5	12	11	12	12	11
Fraction	60	65	70	75	solvent						
Zone	12	11.5	11	10	10						

The activity of each fraction was so small that it was decided to pool the active fractions into three groups i.e. 15-40, 45-50, and 60-65. The activity of the pooled fractions is given in Table 7, Section 2.

Paper chromatography

Fractions 15 to 40 were pooled and taken to dryness and applied to a strip of chromatography paper (Whatmans 5 x 20 cm) and eluted with chloroform:methanol (3:1). Three spots were observed and tested against E.coli 9482 by the "bio-autographic method" which involved cutting out each of the spots and placing them on a pre-seeded bed of agar. All three were slightly active but the highest and lowest spot showed the greater activities. The highest R_f spot was identified as phenylacetamide.

m.p. = 148-149.5°C Lit. = 157.0-158.0°C ⁷

m.s. = M+ = 135 (100%), 92, 91 and 43

I.R. 3509, 3396 and 1674 were prominent.

n.m.r. (CDCl₃) τ2.72 (5H,m,Ar-H), 6.45 (3H,s,PhCH₂)

m.p. (ex MeOH) = 157.0°-159.0°C.

Mutation experiments with S. aizunensis

A suspension of spores from an S. aizunensis

culture was irradiated with u.v. light (254 nm) for 30 minutes. Survival rate was 3% (shown by plating out known volumes). The suspension was then plated out onto glucose-asparagine agar, the composition of which is given in Table 6.

TABLE 6

Composition of glucose-asparagine agar⁶

<u>Material</u>	<u>Quantity</u>
Glucose	10g
Agar	15g
Asparagine	0.5g
K ₂ HPO ₄	0.5g
distilled water	1 litre
adjust pH to pH 7.0	

Most of the viable spores reverted to the parental genotype under the influence of white light but one mutant, designated BC - M - 1, remained different. It was compared with the parent strain.

The characteristics of this mutant were as follows:-

Soluble pigment on Glucose-Asparagine Agar

Parent strain gave a strong yellow pigment whereas the mutant produced no pigment at all.

Colony morphology

The colonies of the parent strain were round, spherical and had a small amount of segmentation. The mutant existed as a single, fused unit with much more segmentation.

Colour

Sub aerial mycelium was grey in both cases but aerial

mycelium was more compact in the mutant and showed definite colour segmentation.

Antibiosis

Parent. negative MRE 600, Wild I, W3160++ and 9843
positive 9482

Mutant. negative Wild I, W3160++, 9843
positive 9482 and MRE 600 (slight).

The above investigation would seem to point at the mutant being at least biochemically divergent,⁸ in that it produces no pigment and may have different antibiotic properties.

Insufficient time remained to pursue a further investigation of mutant strains.

GENERAL ASSAY RESULTS

Assays using various *E.coli* strains

All organisms were obtained as homogeneous preparations from Dr. Horne (Biology) and grown on nutrient agar for 6 days at 37°C. The test material was taken up in sterile water and transferred on to discs or into wells using a sterile hypodermic (Hamilton, 500 µl).

(a) Assay of streptomycin sulphate by disc assay

E.coli strains Wild 1, W3160, 9482 and 9843 were cultivated on petri dishes. A series of serial dilutions of streptomycin sulphate (Glaxo) were made up in sterile water.

The bacteria were spread onto the surface of a fresh nutrient agar petri dish using a glass spreader. A 1 cm Whatman No. 1 disc was placed in the centre of the plate and 100 µl of the antibiotic solution added to the disc. The cultures were then incubated for 36 hours at 36°C and the inhibition zone measured.

The results are given in Table 7 below and in Figure 1 in the discussion section.

TABLE 7 Streptomycin assay

Concn. µg/ml H ₂ O	Inhibition zone diameter (mm)			
	Wild 1	W3160	9482	9843
10 ³	10.4;10.6	30.0;30.2	30.2;30.1	No inhibition
5 x 10 ²	No inhibition	28.3;28.0	25.5;28.0	resistant strain
10 ²	" "	24.1;24.3	24.4;24.1	
7.5 x 10 ¹	" "	16.3;16.3	18.1;18.0	
7.5	" "	No inhibition	10.2;10.2	

(b) Assay of crude *S.aizunensis* extract

Crude extract material (500 mg) from a *S.aizunensis* fermentation was made into a series of serial dilutions 500-5 mg.ml. in glass vials, using 90% H₂O, 10% MeOH and 4 drops acetonitrile (HPLC grade) as solvent (Total volume = 1 ml per dilution).

Several nutrient agar plates were prepared, well cut in them and seeded with 0.1 ml stock solutions of *E.coli* 9482 and 9843 (ca 10⁴ bacteria ml⁻¹).

The crude extract was applied to each well at the rate of 100 µl/well. Incubated at 36°C for 40 hours. The results are given in Table 8 and Figure 2 in the discussion section.

TABLE 8 Cylinder cup assay of crude *S.aizunensis* extract

Concn. (mg/ml)	9843 Inhibition Zone (mm)		
	Replicate 1	Replicate 2	Mean
5 x 10 ²	19.5	19.3	19.4
2.5 x 10 ²	18.2	17.9	18.1
1.25 x 10 ²	14.5	14.7	14.6
6.3 x 10	13.2	13.2	13.2
5.0 x 10	12.5	11.5	12.0
3.0 x 10	11.2	10.5	10.9
0.5 x 10	10.2	10.3	10.3

(c) Assay of streptomycin sulphate using *E.coli* MRE 600

Several plates were prepared using nutrient agar and had well cut in them (10 mm). The plates were then seeded with MRE 600 in nutrient broth (Oxoid No.2) at ca 10⁵ bacteria ml⁻¹.

Each well was dosed with 100 μ l of a known antibiotic dilution then incubated for 40 hours at 35°C. The results are given in Table 9 and Figure 3 in the discussion section.

TABLE 9 Streptomycin assay by cylinder cup method

Conc. (μ g.ml ⁻¹)	Zone diameter (mm.)				Mean	$\Sigma(X-\bar{X})^2$	S.D
	1	2	3	4	\bar{X}		
6×10^3	34.5	33.0	34.0	35.0	34.12	5.00	+ 1.85
6×10^2	33.0	29.0	28.0	28.0	29.50	21.51	+ 2.31
1.5×10^2	26.0	28.0	25.5	27.0	26.62	3.67	+ 0.95
7.5×10	29.0	24.0	25.5	23.5	26.37	17	+ 2.66
7.5	18.0	15.0	17.0	16.0	16.5	2.2	+ 0.73

(d) Assay of unknown metabolite from S.sapporonensis fermentation

The metabolite was made up as an 50% aqueous acetone solution and applied at the rate of 100 μ l per well. The assay was conducted using several strains and incubating at 36°C for 40 hours. Results are depicted in Table 10.

TABLE 10 Sapporonensis metabolite assay

Concn. μ g.ml ⁻¹ aqueous acetone	Strain and inhibition zone (mm)			
	Wild 1	W3160	9482	9843
3×10^3	10.2	-	-	-
10^3	No inhibition	No inhibition	No inhibition	No inhibition
2×10^2	-	-	-	-
H ₂ O:Acetone (1:1) blank	No inhibition	No inhibition	No inhibition	No inhibition

The material appears to be inactive E.coli.

(e) Cylinder cup assays using *E.coli* 27166 - a sensitive mutant

Several cylinder cup assays were prepared to test bicyclomycin, streptomycin and the unknown metabolite (BCH-Z) against the hypersensitive mutant strain, *E.coli* ATCC 27166⁵. The bacterium was prepared as a stock culture on agar slopes.

The two suggested assay agars⁵ were used and serial dilutions of the antibiotics used at the dosage of 100 μ l well.

TABLE 11 Cylinder cup assay of Bicyclomycin and Streptomycin sulphate against *E.coli* 27166 Media

Sensitest agar and Heart Infusion agar

100 μ l each 10 mm well, 36^o 60 hours.

Concn.	Zone Size (mm)							
	Heart Infusion				Sensitest			
	1	2	3	\bar{X}	1	2	3	\bar{X}
BCM 10 ³								
5x10 ²	11.5	10.5	10.0	10.66	11.0	10.5	10.0	10.50
10 ²	11.0	11.0	11.0	11.0	11.5	10.5	10.0	10.66
10	10.5	10.0	10.5	10.33	-	-	-	
Strep 10 ³	28.0	28.0	-	28.0	27.5	-	-	27.5
10 ²	23.0	24.0	-	23.5	23.0	-	-	23.0
BCM-2 10 ³ *	14.0	14.0	-	14.0	18.5*	-	-	

*acetone solution

* sparse *E.coli* growth in zone

No significant difference was found between the agar media. Similarly the supposed hypersensitive mutant ATCC 27166 was no better at detecting the antibiotics (by zone area) than some of the less costly bacteria utilised previously, e.g. MRE600.

(a) Qy

physiologically

against the

The bacteria

dilutions

TABLE II

Control

BCM 10³

2x10³

10²

10

Step 10³

10²

BCM-2 10³

acetone
solution

No significant

Similarity

no better

some of the

e.g. MRE50

PART II

General Experimental Techniques

Infrared (i.r.) spectra were recorded on a Perkin Elmer 577 grating spectrometer as carbon tetrachloride or chloroform solutions in 0.1 mm NaCl cells. In some cases neat liquids were used and in other cases KBr discs were used depending on the solubility properties of the samples. In all cases a polystyrene standard film (0.05 mm) was used to calibrate the spectrometer.

Proton magnetic resonance spectra (n.m.r.) were recorded at 60 MHz on a Hitachi-Perkin Elmer R24 spectrometer and at 90 MHz on a H-P.E. R32 spectrometer.

All spectra used tetramethylsilane (T.M.S.) as an internal standard, and solvents used included CDCl_3 , DMSO-d_6 , CCl_4 and in some cases T.F.A. and D_2O . The following notation is used in presenting n.m.r. data for both carbon and proton spectra. s = singlet; d = doublet; m = multiplet; t = triplet; dt = double triplet; dd = double doublet; q = quartet; p-q = pseudo-quartet. Assignments for proton nmr were made on the τ scale.

^{13}C spectra were run by Dr. Ian Sadler, SRC Fourier Transform Unit, Edinburgh University.

Mass spectra were recorded on a Jeol D100 spectrometer using direct probe insertion or GC/MS in combination using an O.V.1 column.

All melting points were determined on a Kohfler hot stage apparatus and are uncorrected.

Chromatography

Gas liquid chromatography (g.l.c.) was carried out using a Perkin Elmer F-11 chromatograph fitted with a Vitatron

recorder. Columns used were O.V.1 and 5% Apiezon L at ca. 120°C.

T.l.c. refers to the use of 5 x 20 cm plates precoated with Kieselgel 60 GF₂₅₄ (0.25 mm).

Preparative plates were prepared using silica gel GF₂₅₄ type 60 and were prepared in 5 x 20, 20 x 20 and 20 x 40 cm sizes all of which were 2 mm thick. All plates were baked at 100°C for 2 hours to standardise activity.

Column chromatography was carried out using columns packed with silica gel 100-200 mesh, Kieselgel HF₂₅₄ or aluminium oxide of varying activity. Fractions were collected on a Microcol fraction collector at 200 drop intervals.

Compounds were visualised by the quenching of fluorescence at 366 or 254 nm, or by the use of iodide or ceric spray reagents.

High pressure liquid chromatography (H.P.L.C.) was carried out using a Waters Associates 6000A chromatograph using a Cecil u.v. detector as the detection system. All H.P.L.C. solvents were redistilled and degassed before use.

General methods

All compounds which were not crystalline were evaporated to dryness under reduced pressure and stored at 0 - 4°C.

Small amounts of material were crystallised in Craig tubes and the mother liquors removed by centrifugation (2,000 r.p.m., 15 minutes).

All solvents were normally dried and usually redistilled before use and are G.P. grade unless otherwise stated. Solvents were dried as follows.

Dimethylformamide (d.m.f.) was dried by distillation from calcium hydride under reduced pressure.

Methanol was dried by the iodine/magnesium method⁹ and then distilled.

Chloroform was initially extracted with water to remove ethanol and then distilled from P_2O_5 .

Dichloromethane was distilled from P_2O_5 .

Pyridine was dried by standing over KOH pellets prior to distillation.

Ether, benzene and toluene were dried by insertion of sodium wire.

Petrol normally refers to the 60-80° fraction unless otherwise stated.

Solutions were normally dried using previous baked magnesium sulphate unless otherwise stated.

Nitrogen (white spot) was further purified by passage through Fiesers solution, concn. H_2SO_4 and a KOH tower.

Stirring normally implies magnetic stirring, and heating implies the use of Electrothermal mantles unless otherwise stated.

Microdistillation was carried out using a Kugelrohr apparatus.

Preparation of 3,6-dibenzyl-2,5-dioxopiperazine¹⁰ (53)

D,L-phenylalanine (6.0g, 36.36 mmol⁻¹) was suspended in ethylene glycol (12 ml) and refluxed for a period of 24 hours. The crystalline product was removed by vacuum filtration, washed with ethanol (5 ml), ether (2 x 10 ml) and finally dried in a vacuum oven at 60°C (0.1 mm). The yield of crystalline product was 4.07g (76%).

m.p. 300-303°C [Lit. = 301-302°C]¹⁰

n.m.r. (T.F.A.)- τ 2.4 (2H,bs), amide protons, 3.2 (10H, m) aromatic protons. 5.9 (4H,bm) benzylic protons, 7.3 to 8.4 (2H,m) methine protons.

T.l.c. (Butanol, acetic acid, water 3:1:1) showed the presence of two diastereoisomers. ¹³C spectra were consistent with the structure of the product (53) (see section 3 of this thesis).

Preparation of 3,6-dibenzyl-2,5-diethoxy-3,6-dihydropyrazine¹⁰ (54)

3,6-dibenzyl-2,5-dioxopiperazine (4.0g, 13.8 mmol⁻¹) was suspended in freshly dried dichloromethane (25 ml) and stirred at ambient temperature with the exclusion of atmospheric moisture.

To the stirred suspension was added freshly prepared triethyloxonium tetrafluoroborate (5.24g, 27.6 mmol⁻¹) as a dichloromethane solution (10 ml), over a 30 minute period. The mixture was then stirred at ambient temperature for 18 hours.

After this period, a further quantity of triethyl-oxonium tetrafluoroborate (5.24g, 27.6 mmol⁻¹) was added and the solution stirred for a further 36 hours.

The solution was then poured onto a saturated solution of sodium bicarbonate (50 ml) and gently shaken until effervescence

ceased. The aqueous solution was still very slightly alkaline at this point.

Separation of the organic layer, followed by washing with saturated aqueous sodium bicarbonate (50 ml), drying (MgSO_4) and concentration under reduced pressure yielded a white product (4.7g, 97%).

The crude product (4.7g) was dissolved in 60-80° petroleum ether (ca. 100 ml) and placed in an ice-bath.

During this treatment, the mono-alkylated product i.e. 3,6-dibenzyl-2-ethoxy-5-oxo-3,6-dihydropyrazine crystallised out as a white solid (0.95g).

m.p. = 88-89.5°C [Lit. = 88.5-90°C]¹¹

¹³C spectra were consistent with the structure and other spectral data were in accord with the literature values.

The remaining organic solution was concentrated under reduced pressure to give a diastereoisomeric mixture of the two 3,6-dibenzyl-2,5-diethoxy-3,6-dihydropyrazines (54) in 75% yield (3.61g).

The product was oxidised without further purification.

Chloranil oxidation of 3,6-dibenzyl-2,5-diethoxy-3,6-dihydropyrazine (54)

3,6-dibenzyl-2,5-diethoxy-3,6-dihydropyrazine (2.63g, 7.55 mmol⁻¹) was dissolved in dry benzene (30 ml) and to the stirred solution was added chloranil (2.78g, 11.3 mmol⁻¹).

The mixture was refluxed for 2.5 hours and the reaction was followed by t.l.c. ($\text{C}_6\text{H}_6/\text{SiO}_2$).

After cooling, the reaction mixture was filtered through a bed of Celite (10g) which was then carefully washed

with benzene (15 ml).

The combined organic extracts were concentrated to a small volume and loaded onto an alumina column (Grade III, 15 x 2 cm). Elution of the desired compound was achieved using benzene.

Fractions (100 ml) were collected and analysed by t.l.c. (neat benzene) since the product desired had a higher R_f value than the starting material.

Microdistillation of the appropriate fractions yielded the desired product, 3,6-dibenzyl-2,5-diethoxypyrazine in 48% yield (1.26g).

b.p. = 125-130°C (0.15 mm)

m.p. (sealed tube) = 47.5-49°C [Lit. = 45-46.5°C]¹⁰

n.m.r. (CDCl₃) τ2.8 (10h,s Ar-H), 5.7 (4H,q,-CH₂CH₃)

6.1 (4H,s,PhCH₂-) and 8.7 (6H,t,-CH₂CH₃).

$\nu_{\text{CHCl}_3}^{\text{max}}$ 2980, 1690, 1410, 1340, 1110, 1040 and 910 cm⁻¹

m⁺ = 348 a.m.u.

Accurate mass measurement = 348.1838 (C₂₂H₂₄N₂O₂ requires 348.1839 a.m.u.)

¹³C spectra were consistent with the structure proposed (57).

Oxidation of 3,6-dibenzyl-2,5-diethoxy-3,6-dihydropyrazine (54) with D.D.Q.¹⁰

3,6-dibenzyl-2,5-diethoxy-3,6-dihydropyrazine (2.63g, 7.55 mmol⁻¹) was dissolved in dry benzene (30 ml) and to this was added a solution of D.D.Q. (Dichloro-dicyanobenzoquinone) (2.56g, 11.29 mmol⁻¹) in dry benzene (10 ml). The solution was stirred at ambient temperature for 18 hours.

T.l.c. (neat benzene) showed the complete absence of any starting material.

Filtration through Celite removed any of the quinol by-product and the solution was then reduced in volume and loaded onto a grade III alumina column (2 x 15 cm).

Elution with benzene and monitoring of the fractions collected, showed that this reaction was cleaner than the previous chloranil oxidation.

Fractions containing the desired pyrazine were combined and microdistilled to give the product in 75% yield (1.97g). A second batch was purified by p.l.c. (SiO_2 GF₂₅₄ (Merck), neat benzene) to give an identical product in 70% yield (1.83g).

b.p. = 127-131°C (0.13 Torr)

m.p. (sealed tube) = 46-47.5°C [Lit. = 45-46.5°C]¹⁰

n.m.r. (CDCl_3) τ 2.8 (10H,s,Ar-H), 6.1 (4H,s, PhCH_2 -),

5.7 (4H,q, CH_2CH_3) and 8.7 (6H,t,- CH_2CH_3)

$\nu_{\text{max}}^{\text{CHCl}_3}$ 2980, 1690, 1410, 1350, 1110, 1040 and 910 cm^{-1}

m^+ = 348 a.m.u. Accurate mass = 348.1838 ($\text{C}_{22}\text{H}_{24}\text{N}_2\text{O}_2$ requires 348.1839 a.m.u.)

Reaction of 3,6-dibenzyl-2,5-diethoxypyrazine (57) with 2M HCl

3,6-dibenzyl-2,5-diethoxypyrazine (170 mg, 0.5 mmol^{-1}) was added to 2M hydrochloric acid (1.5 ml) and heated, with stirring, at 60-65°C on an oil bath for 2 hours.

T.l.c. (SiO_2 , C_6H_6) showed only one major spot ($R_f = 0.89$) corresponding to unchanged starting material.

After a further seventeen hours at 60-65°C, no change was observed.

A further portion of 2M HCl (1 ml) was added and the

solution refluxed for four hours, during which a red, oily deposit collected in the base of the apparatus.

The reaction mixture was poured into saturated sodium bicarbonate solution (20 ml). Extraction of the aqueous layer with ether (2 x 10 ml), drying the extract (MgSO_4) and concentration under reduced pressure yielded a yellow oil (140 mg) probably 3,6-dibenzyl-2,5-dihoxypyrazine (64) n.m.r. (CDCl_3) τ 2.8 (10h,s,Ar-H), 6.0 (4H,s,- CH_2Ph),

6.5 (2H,bs) - signal disappears with D_2O introduction.

Mass spectral results were inconclusive.

The reaction was not reproducible, e.g. refluxing with a tenfold excess of 2M HCl for 48 hours gave a quantitative recovery of starting (m.p. $44-46^\circ\text{C}$, starting material = $46-47.5^\circ\text{C}$) with identical spectral properties.

Similarly, refluxing 3,6-dibenzyl-2,5-diethoxypyrazine (57) for four days in a tenfold excess of 2M HCl gave starting material as the only product. T.l.c. (toluene) showed only one major spot of R_f (0.91).

m.p. $45.5-47.5^\circ\text{C}$ [Lit. = $45-46.5^\circ\text{C}$]¹⁰

n.m.r. (CDCl_3) τ 2.8 (10h,s,Ar-H), 6.1 (4H,s, PhCH_2-),

5.7 (4H,q,- CH_2CH_3), 8.7 (6H,6,- CH_2CH_3)

$\nu_{\text{CHCl}_3}^{\text{max}}$ 2980, 1690, 1500 (weak), 1420, 1350, 1250 (weak),

1100, 1040 and 910 cm^{-1}

$m^+ = 348\text{ a.m.u.}$

Reaction of 3,6-dibenzyl-2,5-diethoxypyrazine (57) with HBr/Acetic acid

3,6-dibenzyl-2,5-diethoxypyrazine (1.0g, 2.87 mmol^{-1}) was refluxed in a mixture of HBr (47%, 50 ml) and glacial acetic acid (50 ml) for one hour.

The acids were removed by concentration under reduced pressure and the residue taken up in 1% sodium hydroxide solution. The solution was slowly neutralised by the passage of carbon dioxide through the solution. No precipitation of the corresponding dihydroxypyrazine occurred.

The solution was extracted with chloroform (2 x 30 ml) and concentration of this extract gave an 80% recovery of starting material (0.8g).

Spectral characteristics of this product were identical to those previously reported.

Reaction of 3,6-dibenzyl-2,5-diethoxypyrazine with boron tribromide

3,6-dibenzyl-2,5-diethoxypyrazine (160 mg, 0.45 mmol^{-1}) was dissolved in dry dichloromethane (5 ml). The solution was then cooled to 0°C in an ice salt bath.

To this solution was added an excess of boron tribromide (4 drops, ca. 300 mg, 1.2 mmol^{-1}) and the solution stirred at 0°C for four hours. The solution was then allowed to warm to ambient temperature.

A small aliquot (0.5 ml) was removed from the cloudy red solution and quenched in water/ether (1:1). T.l.c. (SiO_2 , neat toluene) showed that no starting material was present.

The reaction mixture was then quenched with water (1.5 ml) and the colour of the solution changed from red to deep

yellow. The organic phase was removed and concentrated to yield a bright yellow solid (120 mg).

The n.m.r. was similar to that of the starting material with the exception of the benzylic region which was slightly split into two singlets, τ 6.1(s) and τ 6.2(s) 4H total. Similarly, in the i.r. spectrum all the peaks are identical to starting material with the exception of weak to moderate absorption at $3550-3310\text{ cm}^{-1}$.

The solid was recrystallised from $60-80^\circ$ petroleum ether to give a pale yellow crystalline solid (57 mg).

m.p. = $146-148^\circ\text{C}$ (starting material $46-48^\circ\text{C}$).

n.m.r. τ 2.8 (10H,s,Ar-H), 6.1 (4H,s,PhCH₂⁻ including shoulder at 6.2(s)), 5.7 (2H,q,-CH₂CH₃) and 8.7 (3H,t,-CH₂CH₃)

$\nu_{\text{CHCl}_3}^{\text{max}}$ 3550-3310 (weak), 2980, 1740, 1620, 1390, 1350, 1250 (weak), 1100 and 910 cm^{-1}

m^+ = 320 a.m.u. Accurate mass = 320.1526 (C₂₀H₂₀N₂O₂ requires 320.1525 a.m.u.)

The spectral data suggests an ethyl group has been removed and probably replaced by a proton. It is tentatively suggested that this compound is 3,6-dibenzyl-2-ethoxy-5-hydroxypyrazine.

More recently, workers in other laboratories have produced similar finding concerning this type of reaction.¹⁷

Reaction of dibenzyl-2-ethoxy-5-hydroxypyrazine with 1M HCl

3,6-dibenzyl-2-hydroxy-5-ethoxypyrazine (34 mg, 0.11 mmol⁻¹) was refluxed in 1M HCl (2.5 ml) for 60 hours.

The solution was then quenched with saturated sodium

bicarbonate solution (10 ml) and the aqueous layer extracted with ether (2 x 10 ml). Concentration of the solution yielded a solid (29 mg).

T.l.c. (SiO_2 , C_6H_6) of the isolated solid was identical to that of starting material i.e. a major spot of Rf 0.46 and a barely visible spot at Rf 0.86. Several other minor spots were noted in the isolated solid at Rf 0.32 and 0.02 (baseline)

m.p. = 150-152°C (starting material = 146-148°C)

n.m.r. (CDCl_3) τ 2.8 (10H,bs,Ar-H), 5.8 (2H,q,- CH_2CH_3) and 8.75 (3H,t,- CH_2CH_3), 6.1 (4H,s,Ph- CH_2 - slight non integral shoulder at 6.2). No definite hydroxyl resonance could be observed.

$\nu_{\text{CHCl}_3}^{\text{max}}$ 3550-3310 (weak), 2980, 1690, 1420, 1390, 1350, 1250 (weak), 1100, 910 cm^{-1}

m^+ = 320 a.m.u.

No reaction between HCl and the hydroxypyrazine is evident from the above data.

Reaction of 3,6-dibenzyl-2-ethoxy-5-hydroxypyrazine with Meerweins salt

3,6-dibenzyl-2-ethoxy-5-hydroxypyrazine (50 mg, 0.15 mmol^{-1}) was dissolved in dry dichloromethane (10 ml). To the stirring solution was added triethyloxonium tetrafluoroborate (55 mg, 0.32 mmol^{-1}) as a dichloromethane solution (5 ml). The mixture was stirred at ambient temperature.

The mixture was run into saturated sodium bicarbonate solution (5 ml) and extracted with ether (2 x 10 ml).

Concentration, followed by p.l.c. (SiO_2 , toluene) gave 3,6-dibenzyl-2,5-diethoxypyrazine as the major product in 81% yield (42.28 mg).

m.p. = 46.5-48°C [Lit. = 45-46.5°C]¹⁸

Spectral characteristics of the major product were identical to those previously reported.

The reaction of 3,6-dibenzyl-2-hydroxy-5-ethoxypyrazine (60) with boron tribromide

3,6-dibenzyl-2-hydroxy-5-ethoxypyrazine (150 mg, 0.46 mmol^{-1}) was dissolved in dry dichloromethane (5 ml). To this was added boron tribromide (15 drops, ca 1.0g, 4.48 mmol^{-1}) in tenfold excess at 0°C . The reaction was warmed up to room temperature and a yellow solid came out of solution after ca 24 hours. When the reaction was complete (t.l.c.), the reaction was quenched in water (2 ml) and stirred for 1 hour to yield, on concentration, a canary yellow solid (109 mg). m.p. = $163-165^\circ\text{C}$. A portion was removed and crystallised from dimethylformamide to give 57 mg of a crystalline yellow solid which was insoluble in chloroform, carbon tetrachloride and dimethylsulphoxide.

m.p. = $158-160.5^\circ\text{C}$ (sealed tube-decomposition evident at m.p.)

n.m.r. (T.F.A) τ 3.3 (10H,s,Ar-H), 6.4 (4H,s,-CH₂Ph), signals at 7.3(s) and 7.4(s) traces of d.m.f. No hydroxylic resonances could be found.

$\nu_{\text{KBr}}^{\text{max}}$ 3200-2400, 1660, 1620, 1440-1370, all weak to moderate absorptions.

The compound proved to be light sensitive and turned brown after 2 days. No molecular ion could be determined in the mass spectrum although the tropylium cation m/e 91 and peaks at m/e 92 and m/e 110 were the most prominent.

Initially it was thought that this might be 3,6-dibenzyl-2,5-dihydroxypyrazine but the melting point was far too low even allowing for the possible incorporation of dimethyl-

formamide into the growing crystals.

In the light of later findings, this compound is certainly not 3,6-dibenzyl-2,5-dihydroxypyrazine.

Reaction of 3,6-dibenzyl-2,5-diethoxypyrazine with iodotrimethylsilane

3,6-dibenzyl-2,5-diethoxypyrazine (200 mg, 0.56 mmol⁻¹) and freshly prepared iodotrimethylsilane (1 ml, excess) were refluxed for 10 hours. T.l.c. of a quenched aliquot showed that no starting material remained. The addition of chloroform and acetone (1 ml of each) lead to the precipitation of a yellow solid which was insoluble in ether, chloroform, acetone, benzene, methanol and hexane. The solid was dried at 50°C in vacuo to give 160 mg (95%) of the desired 3,6-dibenzyl-2,5-dihydroxypyrazine.

m.p. = 256.5-259.5°C [Lit. = 258.5-261°C]⁸

n.m.r. (T.F.A.) τ3.2 (10h,bs,Ar-H), 6.2 (4H,s,-CH₂Ph),

-1.5 (2H,bs,hydroxylic protons) - only visible
in DMSO-d₆

$\nu_{\text{KBr}}^{\text{max}}$ 3600-2650, 1690, 1640, 1370, 1290, 1135, 1020, 1000,
760 cm⁻¹

m⁺ = 292 a.m.u. Accurate mass = 292.1212 (C₁₈H₁₆N₂O₂
requires 292.1214)

¹³C spectra added structural confirmation to the existing spectral data.

Preparation of Benzoyl glycine (47)¹²

Glycine (250g, 3.3M) was dissolved in 10% aqueous sodium hydroxide (2.5L) and to this was added, with stirring, benzoyl chloride (600g, 4.26M). The mixture was stirred for 2 hours. The solution was then made acidic to Congo red indicator paper and the resulting white precipitate removed by gentle suction.

The solid was then boiled in carbon tetrachloride (1.5L) for 10 minutes to remove benzoic acid.

A portion of the material was recrystallised from boiling water to give colourless crystals.

m.p. = 186.5-187.5°C [Lit. = 187°C]¹²

n.m.r. (CDCl₃) τ1.4 (1H,t,NH), 2.6 (5H,m,Ar-H), 6.3 (2H,d,CH₂)

m⁺ = 179 a.m.u.

Total yield was 462g (77.5%).

Preparation of the azlactone (48)

Into each of two round bottom flasks (2L) was placed an intimate mixture of benzoyl glycine (230g, 1.28M), fused sodium acetate (105g, 1.28M) and salicaldehyde (264g, 2.16M). To these was added acetic anhydride (350 ml/flask) and the flasks were heated on a steam bath for ca 40 minutes. This resulted in a deep yellow solution which cooled to a solid mass of yellow crystals.

Recrystallisation from ethanol gave the azlactone as a white crystalline solid. Yield = 474g (70%).

m.p. = 170-171°C

n.m.r. (CDCl₃) τ1.2 (1H,s,PhOH) exchanges in D₂O, 2.2 (1H,s, Ph-CH=), and 2.6 (9H,m,Ar-H).

$\nu_{\text{CHCl}_3}^{\text{max}}$ 3390, 3000, 1710, 1600, 1500, 1480, 1365, 1250, 1160, 940, and 850 cm⁻¹

m⁺ = 265 a.m.u.

Yield 474.2g (70%).

Reduction of the azlactone (48)¹³

The azlactone (66g, 0.25M) was added to a suspension of acetic anhydride (330 ml) and purified red phosphorus (52.3g,

1.69M) stirring in a nitrogen atmosphere. To this mixture was added hydroiodic acid (330 ml) over a period of 90 minutes. The mixture frequently formed a gel which was disrupted by an increase in stirring rate. After the addition of the acid was complete, the mixture was refluxed for 3 hours.

After cooling, the mixture was filtered to remove the unreacted red phosphorus and the remaining solution was concentrated under reduced pressure.

To the residue, water (100 ml) was added and the slurry taken to dryness under reduced pressure.

The solid was then partitioned between water (200 ml) and ether (200 ml). The ether layer was discarded.

To the aqueous solution, active carbon (3.5g) was added and a trace (10 mg) of sodium sulphide. The solution was boiled to remove traces of ether, filtered hot and made neutral to Congo red by the addition of concentrated ammonia solution. The amino acid was filtered off and dried in a vacuum oven at 100°C.

Yield = 30-35g (~ 71%)

m.p. = 247-249°C [Lit. = 249-250°C]¹⁴

T.l.c. (BuOH, AcOH, H₂O 3:1:1) showed the material to be

identical to an authentic sample

¹³C n.m.r. proved satisfactory.

Preparation of cyclo-o-tryosyl l-o-tyrosine

o-Tyrosine (210g, 1.16M) was suspended in ethylene glycol (500 ml) and refluxed for 18 hours. The suspension was then filtered and dried in a vacuum oven for 5 hours at 100°C.

Recrystallisation from boiling glacial acetic acid gave the material as a white crystalline solid 41.7g (20%) which was

dried at 100°C (0.1 mm) for two days to remove traces of acetic acid. The product did, however, still have an acetic odour.

m.p. 250-251°C (decomposition)

n.m.r. (d.m.s.o.-d₆) τ 3.2 (8H,m,Ar-H), 6.3 (2H,pt, CH₂-CH < $\begin{matrix} \text{CO}_2\text{H} \\ \text{NH}_2 \end{matrix}$),
7.1 (4H,ds,Ph-CH₂-CH-), 5.9(s) HOD.

T.l.c. in the following solvent systems showed spots of equal intensity at the following R_f values and are assigned to the diastereoisomers of the dioxopiperazine.

System	R _f	
CHCl ₃ :MeOH (19:1)	0.04	0.10
CHCl ₃ :MeOH (9:1)	0.51	0.64
CHCl ₃ :MeOH (2:1)	0.63	0.70
Butanol:acetic acid:water (3:1:1)	0.70	0.77

Acetylation of cyclo-o- tryosyl -o-tyrosine (34)

Cyclo-o- tryosyl -o-tyrosine (1.51g, 5.0 mmol) and iminazole (50 mg, 0.73 mmol) were suspended in dry DMSO (5 ml) and dry acetic anhydride (10 ml) and the mixture was then stirred for 18 hours. The reaction mixture cleared after 2.5 hours and then precipitation of the product occurred. After 18 hours, dry diethyl ether (200 ml) was added dropwise to the stirred mixture and the flask was then stored at -15°C for 6 hours. The resulting white precipitate was filtered, washed twice with dry ether (50 ml) and then air dried. Most of the solid was dissolved in refluxing methanol (100 ml) then the solution was filtered and the clear filtrate kept at -15°C for 3 days.

The resulting white crystalline solid was filtered off, washed with a little methanol and ether, and dried overnight at 100°C over KOH pellets at 1.0 mm, to yield only 1.21g of acetylated product (60%).

m.p. (sealed tube) 211-217°C

n.m.r. (DMSO) τ 2.9 (8H,m,Ar-H), 6.5 (2H,m, methine ring protons),
7.2 (4H,d,CH₂Ph protons), 7.8 (6H,s,- $\overset{\text{O}}{\text{C}}\text{-CH}_3$),
6.3 HOD

Found C = 64.2%, H = 5.3%, 6.8%N, C₂₂H₂₂N₂O₄ requires
C = 64.4%, H = 5.4%, 6.8%N.

m⁺ = 410 a.m.u. but a weak molecular ion at best.

¹³C.m.r. was consistent with the proposed structure (52).

T.l.c. in a mixture of CHCl₃:MeOH (95:5) showed spots of equal intensity at Rf 0.2 and 0.3 and these were assigned as the expected diastereoisomeric products. A small impurity was also observed at Rf 0.12.

The filtrate from recrystallisations was taken to dryness and yielded 0.12g of a white solid, with the same spectral characteristics.

Preparation of bis-(2-acetoxybenzyl)-2,5-diethoxy-3,6-dihydropyrazine

The acetylated dioxopiperazine (0.386g, 1.0 mmol) was suspended in dry dichloromethane (5 ml) and stirred mechanically. Triethyl-oxonium fluoroborate (0.38g, 2 mmol) was added in dry dichloromethane (5 ml) and the mixture was stirred overnight at ambient temperature.

The next morning, a further equivalent of triethyl oxonium tetrafluoroborate was added and the mixture was stirred

for a further 24 hours.

The resulting suspension was then poured onto saturated sodium bicarbonate solution (10 ml) and washed in with dry dichloromethane (5 ml) and shaken until effervescence ceased. The organic layer was then separated and the aqueous layer was washed with dichloromethane (5 ml). The combined organic layers were dried (MgSO_4) and then evaporated to give a white solid.

The solid was dried under vacuum (10 mm) to give 0.405g (87%) of a white solid.

T.l.c. in CHCl_3 -MeOH (95:5) gave a single large spot (Rf 0.66) with a very small impurity spot at Rf 0.21.

Yield = 0.18g.

n.m.r. (CDCl_3) τ 2.8 (8H,m,Ar-H), 5.8 (6H,m, CH_2CH_3 + ring protons)
 O
 7.0 (4H,m,Ph CH_2 -), 7.6 (6H,s, CH_3C)
 8.6 (6H,t,- CH_2CH_3)

$\nu_{\text{CHCl}_3}^{\text{max}}$ = 2990, 1750, 1690, 1490, 1370, 1240, 1180, 1040 and
 930 cm^{-1} .

The product was initially crystallised from methanol.

m.p. = 150-152°C. Found C = 66.0%, H = 6.5%, N = 5.5%.

$\text{C}_{26}\text{H}_{30}\text{N}_2\text{O}_6$ requires C = 66.9%, H = 6.5%, N = 6.0%. This is consistent with $\text{C}_{26}\text{H}_{30}\text{N}_2\text{O}_6 \cdot \frac{1}{2}\text{MeOH}$ i.e. methanol inclusion into the crystals.

Here satisfactory material was obtained from ethyl acetate.

m.p. = 144-145°C.

Found C = 66.8%, H = 6.3%, N = 6.1% $\text{C}_{26}\text{H}_{30}\text{N}_2\text{O}_6$ requires

C = 66.9%, H = 6.5%, N = 6.0%

M^+ = 466 a.m.u. the molecular ion, however, was of low intensity.

Preparation of the acetylated pyrazine analogue (58)

The bis-imidate (1.52g: 3.22 mmol) and DDQ (0.88g: 3.86 mmol) were mixed in benzene (50 ml) and stirred overnight at ambient temperature.

T.l.c. in CHCl_3 -MeOH (95:5) showed that all of the starting material had disappeared. The solution was then loaded onto a short alumina column and eluted with benzene (300 ml) then CHCl_3 (70 ml).

The main fraction yielded 0.66g (44%) of the pyrazine.
 n.m.r. (CDCl_3) τ 2.9 (4H,m,Ph-H), 5.8 (4H,q,- CH_2CH_3),
 6.1 (4H,s,Ph CH_2 -), 7.7 (6H,s, CH_3CO -),
 8.7 (6H,6,- CH_2CH_3)

$\nu_{\text{CHCl}_3}^{\text{max}}$ = 2980, 1780, 1430, 1380, 1350, 1220, 1050 and 920 cm^{-1} .

m.p. 86-87°C (hexane), 83-84.5°C (ethyl acetate)

m^+ = 464 a.m.u.

Found C = 67.1% H = 5.9%, N = 5.8% $\text{C}_{26}\text{H}_{28}\text{N}_2\text{O}_6$ requires

C = 67.2%, H = 6.1%, N = 6.0%

^{13}C .m.r. data were consistent with the proposed structure.

Deacetylation reactions on bis-acetoxy,benzyl pyrazines

1. Reactions with Hydrazine hydrate¹⁵

The bis-acetoxy pyrazine (58) (100 mg: 0.215 mmol) was dissolved in dry dichloromethane (5 ml) and stirred at room temperature. To this was added, in one portion, hydrazine hydrate (21.5 mg: 2 equiv. 0.431 mmol) as a dichloromethane solution (1 ml). The reaction was followed by t.l.c. (10% MeOH in toluene).

After two hours, no change was noted. A further equivalent of hydrazine was added; and after 3 hours, a brown

oil began to come out of solution but even after 24 hours, starting material was still present.

The dichloromethane solution was evaporated and the residue was redissolved in dry ethanol (10 ml) and stirred overnight. In the morning no starting material could be seen. The solution was taken to dryness and applied to a p.l.c. plate (20 x 20 cm) as a methanol solution (1 ml) and eluted with 5% methanol in toluene.

The main band (Rf 0.82) was eluted with chloroform and degassed to yield the bis-phenol (24.9 mg) (59) (30%).

m.p. 126-127°C

n.m.r. (CDCl₃) 1.4 (2H,s,-OH), 2.9 (4H,m,Ar-H), 3.1 (4H,m,Ar-H),
5.7 (4H,s,Ph-CH₂-), 6.6 (4H,q,-OCH₂CH₃),
8.5 (6H,6,-OCH₂CH₃)

m⁺ = 380

$\nu_{\text{CHCl}_3}^{\text{max}}$ = 3500-3100, 2990, 1590, 1490, 1440, 1420, 1380, 1350,
1320, 1220 and 1030 cm⁻¹

Accurate mass = 380.1736 (C₂₂H₂₄N₂O₄ requires 380.1840)

¹³C.m.r. was consistent with the structure proposed using all data together.

2. Reaction with two equivalents of ethanolic KOH

The pyrazine (100 mg: 0.215 mmol) was dissolved in ethanolic KOH (24 mg KOH in 3 ml EtOH) and stirred for 24 hours. The reaction was followed by t.l.c. (10% MeOH in toluene). No starting material remained after this time. P.l.c. of the reaction mixture, as before, yielded the bis-phenol as the major product (68.1 mg, 85.7%).

m.p. 118-120°C

n.m.r. (CDCl₃) τ1.3 (2H,s,-OH), 3.09 (8H,m,Ar-H),
5.7 (4H,q,-OCH₂CH₃), 6.1 (4H,s,CH₂Ph),
8.6 (6H,t,-OCH₂CH₃)

m⁺ = 380

v_{max}
CHCl₃ 3500-3100, 2990, 1590, 1490, 1440, 1420, 1380, 1350,
1320, 1220 and 1030 cm⁻¹

Accurate mass = 380.1736 (C₂₂H₂₄N₂O₄ requires 380.1840)

T.l.c. comparison with the previous sample (5% MeOH in CHCl₃)
showed that they were identical.

3. Reaction with four equivalents ethanolic KOH

The pyrazine (110 mg, 0.220 mmol) was stirred
with KOH (48.24 mg, 4 equiv. 0.88 mmol) in ethanol (5 ml).

The solution was stirred for 60 hours, then made
neutral by the addition of 2 drops of 5M HCl, and then
concentrated under reduced pressure. Again t.l.c. showed
that all of the starting material had gone. P.l.c. of the
mixture, as before, yielded the bis-phenol as the main product
(57.29 mg, 71.6%).

m.pt. = 127-128°C

n.m.r. (CDCl₃) τ1.5 (2H,bs,PhOH), 3.1 (8H,m,Ar-H),
5.8 (4H,q,-OCH₂CH₃), 6.15 (4H,s,-CH₂Ph),
8.7 (6H,t,-OCH₂CH₃)

Spectral data were as previously reported.

4. Reaction with lithium Iodide in collidine¹⁶

The pyrazine (100 mg, 0.22 mmol) was added to freshly
distilled dry collidine (10 ml) and anhydrous lithium iodide

(57.54 mg, 2 equi. 0.42 mmol). The mixture was then refluxed for 60 hours.

T.l.c. of the mixture showed a variety of products including a trace of starting material ($R_f = 0.71$).

P.l.c., as before, enabled the bis-phenol to be isolated in small yield (11.0 mg, 14.4%).

m.p. = 125-127°C

n.m.r. τ 1.5 (2H,s,PhOH), 3.1 (8H,m,Ar-H), 5.8 (4H,q,OCH₂CH₃),
6.15 (4H,s,-CH₂Ph), 8.7 (6H,t,-OCH₂CH₃)

$\nu_{\text{max}}^{\text{CHCl}_3}$ 3500-3100, 2980, 1590, 1490, 1435, 1420, 1380, 1350,
1320, 1220 and 1030 cm^{-1}

T.l.c. in 5% methanol in CHCl₃ showed this product to be identical with those isolated previously.

Large scale conversion of the bis-acetoxypyrazine to the bis-phenol (59)

The pyrazine (900 mg, 1.93 mmol) was added to a solution of ethanolic KOH (10 ml EtOH, 0.433g KOH, 4 equiv. 7.72 mmol) and stirred for 2 hours and then t.l.c. showed that all the starting material had been consumed. The solution was then taken to dryness and stored in the fridge overnight. The material was then applied to three p.l.c. plates (SiO₂: 20 x 20 cm) and eluted with 5% MeOH in toluene. The band corresponding to the bis-phenol was removed and eluted with chloroform to yield the bis-phenol as a yellow solid (683 mg, 93%).

The material was then crystallised from cyclohexane.

m.p. = 127-128°C

n.m.r. (CDCl₃) τ 1.5 (2H,bs, phenolics) 3.1 (8H,m,Ar-H),
5.8 (4H,q) ethyl CH₂, 6.15 (4H,s) benzylic CH₂,
8.7 (6H,t) ethyl CH₃

$\nu_{\text{CHCl}_3}^{\text{max}}$ 3500-3100(OH), 2990, 1590, 1490, 1440, 1420, 1380,
1350, 1320, 1220(s) and 1030 cm^{-1}

m^+ = 380 a.m.u.

Accurate mass = 380.1736 ($\text{C}_{22}\text{H}_{24}\text{N}_2\text{O}_2$ requires 380.1840)

Calculated: C = 75.8, H = 6.9, N = 8.0.

Found C = 75.6, H = 6.6, N = 7.9

^{13}C was consistent with the structure proposed.

Reaction of the bis-phenol (59) with BBr_3

The bis-phenol (59) (50 mg, 0.13 mmol^{-1}) was dissolved in dry dichloromethane (8 ml) and stirred at room temperature. To this was added an excess of BBr_3 (10 drops, 750 mg, 2.99 mmol) and immediately a white precipitate appeared but which disappeared during the subsequent 60 hours of stirring.

The dichloromethane was evaporated and the resulting solid was suspended in sodium bicarbonate solution. A solid formed and was removed by centrifugation. The bicarbonate layer was made slightly acid and extracted into ether, then dried and concentrated to give starting material (27 mg, 54%).

The rest of the material was present only in small amounts and could not be positively identified.

Reaction of TMSI with bis-3,6-(2-hydroxybenzyl)-2,5-diethoxypyrazine (59)

The bis-phenol (100 mg, 0.26 mmol) was refluxed with iodotrimethylsilane (1 ml) for 4.5 hours and allowed to cool. Addition of water (0.1 ml) and acetone (0.3 ml) led to the precipitation of a yellow solid 66.3 mg (79%) identified as bis-3,6-(2-hydroxybenzyl)-2,5-dihydroxypyrazine.

m.p. = $210.5\text{-}213^\circ\text{C}$

m^+ = 324 (53.2%)

Accurate mass = 324.1111 - elemental fit $\text{C}_{18}\text{H}_{16}\text{N}_2\text{O}_4$

n.m.r. (DMSO-d_6) τ 3.1 (8H,m), 6.2(4H,s), 1.3(2H,bs), 1.5(2H,bs).

Found C = 66.5, H = 4.7, N = 8.5, $\text{C}_{18}\text{H}_{16}\text{N}_2\text{O}_2$ requires

C = 66.6%, H = 4.9, N = 8.6%.

Chlorination of 3,6-dibenzyl-piperazine-2,5-dione (53)

The dioxopiperazine (2.49g, 10 mmol) was stirred with neat POCl_3 (17.4 ml) for 20 hours at ambient temperature. Aliquots of the orange suspension were removed after stirring overnight and t.l.c. (benzene: SiO_2) showed that some starting material was still present.

However, after 48 hours none of the starting material remained.

The reaction mixture was run into phosphate buffer (pH 7, 0°C) (100 ml). A buff-coloured solid separated out and was removed by filtration and then vacuum dried. Yield of the crude solid was 2.81g.

The material was thoroughly extracted with boiling petrol (5 x 20 ml) and concentrated to yield a crystalline residue (200 mg). Fractionation of this on Kieselgel H gave 183 mg (5.5%) of 2,5-chloro-3,6-dibenzylpyrazine.

m.p. = $106.5-108.5^\circ\text{C}$

n.m.r. (CDCl_3) 2.75 (10H,s,Ar-H), 5.8 (4H,s,- CH_2Ph)

m^+ = 328 a.m.u. and the associated isotopic peaks were also evident

The remaining 2.6g was, in fact, starting material confirmed by m.s. and n.m.r. This was probably in the form of a weak complex with POCl_3 during t.l.c. since the aliquots were not subjected to work up prior to t.l.c.

A second experiment yielded 10% of the mono-chloropyrazine and 6% of the dichloropyrazine.

m.p. = $106.5-108^\circ\text{C}$

n.m.r. = $\tau 2.75$ (10H,s,Ar-H), 5.8 (4H,s,- CH_2Ph)

m^+ = 328 m^+ : $m+2$: $m+4$ = 9:6:1 .*. two chlorine substituents.

Large scale preparation of 3,6-dibenzyl-2,5-dichloropyrazine (80)

3,6-dibenzyl-2,5-dichloropyrazine (6.00g, 20.4 mmol) was refluxed for 2 hours with POCl_3 (60 ml). The excess POCl_3 was removed by vacuum distillation and the residue (ca 6-7 ml) was carefully decomposed by running into it, phosphate buffer (pH 7, 100 ml). The buff solid which separated out was filtered and continuously extracted with boiling petrol. This extract was concentrated and yielded a red solid which was fractionated by column chromatography ($\text{SiO}_2:\text{CHCl}_3$) to give the dichloropyrazine as a yellow solid. Yield 1.91g (28.48%).

m.p. = 107-108.5°C

m^+ = 328 a.m.u. $m^+ : m+2 : m+4 = 9 : 6 : 1$ ∴ two chlorine substituents

n.m.r. = 2.75 (10H,s,Ar-H), 5.8 (4H,s,- CH_2Ph).

Benzylation of 2,5-dichloro-3,6-dibenzylpyrazine (80)

The dichloropyrazine (365 mg, 1.11 mmol) was made up to 10 ml as a benzene solution. Benzyl alcohol (dried over CaH_2 , 455 mg) was added and sodium hydride (200 mg, 8.3 mmol from 58% dispersion). After one hour, stirring under nitrogen, hydrogen evolution appeared to have ceased and the solution of the dichloride in benzene was slowly added. No colour change occurred.

After 2 hours stirring, t.l.c. (CHCl_3) showed that there was no change in the starting material, and this was also the case when stirring was continued overnight.

The reaction mixture was then poured into 0.5M NaOH (50 ml) and the yellow aqueous layer was removed and further extracted with chloroform (2 x 5 ml). The combined organic layers were dried and concentrated to a yellow solid. m.s. data

gave the m^+ as 328 a.m.u. and was, therefore, starting material. One small spot, separated out on Kieselgel H (CHCl_3), yielded 20 mg of a white solid.

m.p. = 132-135°C

n.m.r. = τ 2.8 (15H,s,Ar-H), 5.8 and 5.9 (6H,d,- CH_2 -Ph)

m^+ = 400 $m^+ : m+2$ = 3:1 therefore one chlorine substituent.

Probably a trace of the mono-chloro-mono-benzyl ether.

Direct Benzylation of the 3,6-dibenzyl-2,5-dichloropyrazine (80)

Sodium (30 mg, 22 mmol) was dissolved in dry benzene (4 ml) and dry benzyl alcohol (0.15 ml, 1.56 mg, 1.22 mmol). When the alkoxide solution had formed, the dichloropyrazine (200 mg, 0.61 mmol) was added dropwise as a benzene solution and stirred at room temperature for 4 days.

The solution looked like a suspension due to the formation of sodium chloride (insoluble in benzene) but this disappeared when the solution was quenched with 0.5M NaOH (5 ml). The alkaline extract was removed and worked up as before. Combination of the organic layers and degassing, yielded an oily semi-solid (320 mg, 68%).

n.m.r. (CDCl_3) 2.9 (20H,s,Ar-H), 6.0 (8H,s,- CH_2 Ph)

m^+ = 472. The characteristic pattern for chlorine was noticeably absent.

$\nu_{\text{CHCl}_3}^{\text{max}}$ 3000-2800, 1410, 1370, 1340, 1320, 1240, 1200, 1160
1080, 1040, 900 and 860 cm^{-1}

Chlorination of cyclo-(o-tyrosine) $_2$ (34)

The dioxopiperazine (185 mg, 0.612 mmol) was stirred with POCl_3 (1.2 ml) at room temperature for 5 days. After stirring for this period of time, the mixture was dark yellow

in colour, and t.l.c. showed the presence of a fluorescent component at Rf 0.34 and some material of high Rf.

The solution was run into phosphate buffer as previously described and then extracted from the aqueous layer with chloroform (50 ml). Fractionation on SiO₂ (CHCl₃) gave 17 fractions (5 ml each). Fractions 3-10 gave a dichloro compound and fractions 11-13 gave the fluorescent compound.

Analysis

(a) Fractions 1-3

High Rf material in the form of a white solid.

m^+ = 326 i.e. starting material (confirmed by mixed melting point and n.m.r.)

(b) Fractions 3-10

This was the major product m.pt. = 106.5-108°C

m^+ = 328 - thought to be the 3,6-dibenzyl-2,5-dichloropyrazine

n.m.r. (TFA) τ 2.75 (10H,s,Ar-H), 6.1 (4H,s,-CH₂Ph),

no OH resonances were observed

m.s. data was compared with an authentic sample;

both 93-95% FSD.

Sample m/e	330	329	328	327	295	294	293
Ratio with authentic 3,6-dibenzyl-2,5- dichloropyrazine	0.95	0.96	1.0	0.95	0.99	0.87	0.97

(c) Fractions 11-13

This sample contained most of the fluorescent compound, which was in very low concentration (\sim 10 mg).

n.m.r. τ 5.7 (4H,s,PhCH₂) τ 2.8 (8-9H,-non integral,s,Ar-H).

The infra-red spectrum run in CHCl₃ showed the presence of C-O-C stretch at 1410 cm⁻¹.

the reaction.

m.p. = 107-109°C

n.m.r. (CDCl₃) τ2.75 (10H,s,Ar-H), 5.8 (4H,s,-CH₂Ph)

m⁺ = 328 m⁺:m+2:m+4 = 9:6:1 ∴ 2 chlorines present.

Reaction of 3,6-dibenzyl-2,5-dichloropyrazine (80) with sodium methoxide

The pyrazine (100 mg; 0.3 mmol) was added to freshly prepared sodium methoxide (20 mg Na in 5 ml dry methanol) and refluxed for 24 hours. Again only starting material (91 mg; 91%) was recovered.

m.p. = 106.5-108.5°C

n.m.r. (CDCl₃) = τ2.75 (10H,s,Ar-H), 5.8 (4H,s,-CH₂Ph)

m⁺ = 328

Reaction of 3,6-dibenzyl-2,5-dichloropyrazine (80) with hydroxide under phase transfer conditions

The pyrazine (100 mg; 0.3 mmol) was dissolved in CHCl₃ (10 ml). To this was added an aqueous solution of KOH (10 ml) containing 70 mg (1.24 mmol) KOH. To the two phase mixture was added tri-t-butyl ammonium iodide (10 mg) and the solution stirred rapidly for 48 hours.

During this time, the reaction was followed by t.l.c. (CHCl₃) but no product formation could be observed. Concentration of the organic layer, followed by drying with MgSO₄, gave a pale yellow solid (90 mg).

m.p. = 105.5-107.5°C

m⁺ = 328 a.m.u. m⁺:m+2:m+4 = 9:6:1 ∴ 2 chlorines present.

Spectroscopic properties and m.p. confirmed that this was unchanged starting material.

Photo-oxygenation of 3,6-dibenzyl-2,5-diethoxypyrazine (57)¹⁷

The pyrazine (200 mg, 0.57 mmol) was dissolved in dichloromethane (40 ml) containing methylene blue (2 mg). Oxygen was bubbled through the solution and then irradiation (250W tungsten lamp) was continued for 18 hours.

T.l.c. (CHCl₃) confirmed that all starting material had gone, and p.l.c. and subsequent elution of the major band (Rf 0.53) with the same solvent, gave the endoperoxide as a colourless oil (150 mg, 70%).

m.s. m/e = 91 a.m.u. was base peak, m/e = 348 a.m.u. = m⁺ - O₂

n.m.r. (CDCl₃) τ2.7 (10H,m,Ar-H), 5.8 (4H,q,OCH₂CH₃),

6.6 (4H,s,CH₂Ph), 8.7 (6H,t,OCH₂CH₃).

$\nu_{\text{CHCl}_3}^{\text{max}}$ = 3100-2800, 1740, 1635, 1500, 1450, 1375, 1330, 1220
1080, 1040, 1020 and 925 cm⁻¹

Found C = 69.3%, H = 6.1%, N = 7.2 C₂₂H₂₄N₂O₄ requires

C = 69.5%, H = 6.3%, N = 7.4%.

¹³C.m.r. was consistent with the proposed structure

i.e. 3,6-dibenzyl-2,5-diethoxy-3,6-epidioxo-3,6-dihydropyrazine.

Photo-oxygenation of 3,6-dibenzyl-2,5-dihydroxy-pyrazine (64)¹⁷

The dihydroxypyrazine (100 mg; 0.34 mmol) was dissolved in DMSO/CH₂Cl₂ (1:10) in a total volume of 50 ml. Irradiation was continued for 36 hours using methylene blue as a sensitiser.

The reaction mixture was concentrated to a small volume (2 ml) but no precipitation of the endoperoxide occurred as reported. The only material isolated was starting material.

Photo-oxygenation of 3,6-di(α -hydroxybenzyl)-2,5-diethoxypyrazine (59)

The pyrazine (100 mg; 0.26 mmol) was dissolved in dry methanol (50 ml) to give a pale yellow solution. Addition of methylene blue (2 mg) turned the solution a deep emerald green, and the solution was then irradiated for 10 hours after which the solution was entirely blue.

T.l.c. in 10% methanol in toluene, showed starting material as the major spot. P.l.c. using the same solvents, showed a thin band below starting material and this was eluted (CHCl_3) to give an off-white solid (10 mg).

m.p. = 135-139°C m^+ = 380 a.m.u.

n.m.r. (CDCl_3) τ 1.5 (2H,bs,PhOH-exchange when D_2O added)

3.1 (8H,m,Ar-H), 5.8 (4H,q, OCH_2CH_3)

6.1 (4H,s,- CH_2Ph), 8.7 (6H,t, OCH_2CH_3)

$\nu_{\text{max}}^{\text{CHCl}_3}$ 3500-3100, 2990, 1490, 1440, 1380, 1350, 1320, 1220, 1140, 1120 and 1030 cm^{-1} .

Photo-oxygenation of 3,6-dibenzyl-2,5-dichloropyrazine (57)

The pyrazine (150 mg; 0.46 mmol) was irradiated as a dichloromethane solution using methylene blue as a sensitiser and followed by t.l.c. (CHCl_3). Even after 48 hours, no product formation could be noted. The solution was concentrated down and an almost quantitative recovery of starting material was achieved.

The previous material was concentrated down and resuspended in methanol/dichloromethane (1:1) on the basis that the chloro-imine might be displaced by methanol to give the 3,6-dibenzyl-2,5-dimethoxypyrazine but after 48 hours of irradiation, only starting material was recovered.

Chlorination of piperazine-2,5-dionePreparation of dioxopiperazine¹⁸ (92)

Glycine ethyl ester hydrochloride (250g; 1.79M) was suspended in water (200 ml) at 0-5°C. Triethylamine (250 ml) was added dropwise over 1.5 hours, keeping the temperature below 5°C at all times. The solution was then stirred until it had reached room temperature and was left to stand for 2 days. The solution was then chilled for 4 hours and the crystalline mass removed by filtration. The crystals were then washed with water (250 ml) and then methanol (250 ml) and finally dried in a vacuum oven (60°C).

Yield 53.1g (52%)

m.p. greater than 300°C

n.m.r. (TFA) τ 2.3 (2H,bs,-NH-CO-) and 6.1 (4H,s,-CH₂-NH-CO-).

Chlorination of DKP with POCl₃

Dioxopiperazine (8.0g; 70.17 mmol) was suspended in POCl₃ and stirred overnight. During this period the solution turned from yellow to a deep red brown.

After 24 hours the mixture was placed on an oil bath and the temperature was raised to 100°C over 1 hour, and then maintained at that temperature for 30 minutes.

The solution was then quenched in ice cold phosphate buffer (pH 7, 800 ml) and the brown aqueous solution was then extracted with chloroform (3 x 500 ml) and dried (MgSO₄) to give an oily semi-solid (3.75g).

During the reaction, a large amount of insoluble black polymeric material was produced as had been reported in previous reactions.

A small aliquot of the product was made up to a 5% solution in ether prior to g.l.c. analysis.

G.C. conditions

Column 15 ft 5% Apiezon L. Gases Air = H₂ = N₂ = 20 p.s.i.

Injection temperature - 3 Oven = 114°C

Attenuation = 16X Range = 10

Chart 10mm/min

The best results were obtained by using a 1 μ l loading of a 5% solution and this gave four distinct peaks.

- (1) R.T. 3.5 mins. (78.8%)
- (2) R.T. 5.4 mins. (4.7%)
- (3) R.T. 7.9 mins. (10.4%)
- (4) a small peak at R.T. 3.0 mins. (3.2%)

Authentic 2,5-dichloropyrazine co-injected with peak 3.

A similar aliquot was then subjected to G.C./M.S. analysis on the same column at 120°C. In this analysis some resolution of peaks was lost and peak 4 was swamped out by peak 1.

Analyses are as follows.

Peak 1. m^+ = 114 a.m.u. peaks at 87,86,79,75,51

m^+ ; $m+2$ = 3:1 2-chloropyrazine

Peak 2. m^+ = 107 a.m.u. peaks at 81,79,72 (100%), 61,59,57,

45,44,43 - unknown

Peak 3. m^+ = 149 a.m.u. peaks at 148 (100%) 121,113,88,86,

85,62,60 2,5-dichloropyrazine.

Chlorination of DKP with POCl₃/PCl₅

Dioxopiperazine (1.6g; 14 mmol) was added to a mixture of POCl₃ (5 ml) and PCl₅ (5.34g; 25.6 mmol) and gently warmed to create a straw coloured solution. The solution was then stirred at ambient temperature for 72 hours during which time it turned deep red in colour.

The solution was then quenched in ice cold phosphate buffer (pH 7, 200 ml) and then allowed to warm up to room temperature. Extraction of the brown aqueous layer with chloroform (2 x 500 ml) gave a yellow organic phase which was dried (MgSO₄) and concentrated to give an oily yellow semi-solid (0.528g; 26%).

Again a small aliquot was made up to a 5% solution and introduced onto the same g.l.c. system described (Oven = 118°C).

Three peaks were observed and the average values for three runs are given below.

- (1) RT 1.7 mins (0.35%)
- (2) RT 3.4 mins (10.99%)
- (3) RT 5.7 mins (88.45%)

Authentic 2,5-dichloropyrazine co-injects with peak 3 and has the same spectral characteristics (after p.l.c.) and 2-chloropyrazine has the same spectral characteristics and g.l.c. properties as peak 2.

T.l.c. of the crude mixture (10% MeOH/toluene) agreed with these findings and the n.m.r. of the crude oil showed the major peak as an intense singlet at τ 1.75 - again confirming the major product as 2,5-dichloropyrazine.

Scale up of the previous reaction

Dioxopiperazine (5.0g; 43.5 mmol) was added to a mixture of POCl_3 (15.6 ml) and PCl_5 (16.68g; 80 mmol) and warmed to effect solution and stirred for 48 hours. The solution was then worked up as previously described to yield a red mobile oil (4.01g; 61.2%).

N.m.r. showed only one major signal - a singlet at $\tau 1.7$ - the 2,5-dichloro isomer and g.l.c. analysis on Apiezon L (120°C) showed the 2,5-dichloro isomer to be dominant with only a small amount of the monochloropyrazine (< 5%) as a contaminant.

N-oxide preparations

Preparation of 3-chloropyrazine-1-oxide¹⁹ (108)

To a stirred solution of 2-chloropyrazine (6.0g; 52.4 mmol) in glacial acetic acid (15.5 ml), 30% hydrogen peroxide (10 ml) was added and the solution heated at $65-75^\circ\text{C}$ for 17 hours.

The clear solution was then reduced to a third of its volume and then an equal amount of water was added. The solution was concentrated again.

Extraction of the aqueous phase with chloroform (2 x 100 ml) and after adjusting the pH of the aqueous phase to pH 9 and then extracting again with chloroform, yielded, on concentration, the N-oxide as a white solid (4.0g; 50%).

The material was then crystallised from methanol.

m.p. $95-96.5^\circ\text{C}$ [Lit. $95-96^\circ\text{C}$]¹⁹

m.s. $m^+ = 130$ a.m.u. (100%) 79, 75, 52, 51 and $m^+ : m+2 = 3:1$
one chlorine.

$\nu_{\text{max}}^{\text{CHCl}_3}$ 3000-2870, 1590, 1440, 1410, 1340, 1110, 1100, 1050,
1010, 950 and 850 cm^{-1}

Starting material had the following characteristics.

Clear liquid B.p . 63°C (29 mm)

n.m.r. (CDCl₃) τ1.5 (1H,s); 1.65 (1H,d) and 1.77 (1H,s).

m⁺ = 114 (100%) other peaks at 87, 86, 79, 60, 52, 51 a.m.u.

$\nu_{\text{max}}^{\text{CHCl}_3}$ 3000, 1510, 1460, 1400, 1390, 1060, 1010, 850 cm⁻¹

Preparation of 2-chloropyrazine-1-oxide (110)²⁰

2-chloropyrazine (0.6g; 5.2 mmol) was added to concentrated sulphuric acid at 10°C. To the stirring solution was added potassium persulphate (1.54g; 5.72 mmol; 1.1 eq.) and the solution was then stirred at ambient temperature for 24 hours.

The mixture was then quenched in ice (30g) and extracted with chloroform (2 x 30 ml). The organic phase was then dried (MgSO₄) after washing with bicarbonate (50 ml) and saturated brine (50 ml). Concentration of the solution yielded a white solid (475 mg; 70%) which was recrystallised from ethanol.

m.p. 133-134.5°C [Lit. 133-134°C]²⁰

n.m.r. (CDCl₃) τ1.4 (1H,s), 1.6-1.7 (1H,m)

m⁺ = 130 (100%) other peaks at 79, 75, 52, 51 and 40 a.m.u.

m⁺:m+2 = 3:1

$\nu_{\text{max}}^{\text{CHCl}_3}$ 3000-2890, 1590, 1460, 1400, 1310, 1180, 1115, 1050,
1010, 880 cm⁻¹

Spectral data is identical to that reported in the literature.¹⁶

Chlorination of 3-chloropyrazine-1-oxide (108)²⁰

The N-oxide (4.0g; 30.76 mmol) was added in portions to POCl₃ and refluxed for 1 hour, after which it was quenched

in phosphate buffer (150 ml/150g ice). Extraction with chloroform (2 x 75 ml) gave a red solution which was back extracted with water (50 ml), 5% bicarbonate (50 ml) and then dried (CaCl_2). Concentration of the solution gave 3.4g of a red mobile oil.

n.m.r. (CDCl_3) τ 1.45 (2H,s) and τ 1.75 (2H,s).

integral ratio 1:1 i.e. a mixture of the 2,5- and 2,6-dichloropyrazine isomers.

T.l.c. could not resolve the major spot using the normal solvent systems (10% MeOH in benzene). However, the isomers were separated on silica GF₂₅₄ by p.l.c. in 40-60 petrol with 2% methanol.

The highest Rf band yielded a colourless crystalline solid.

m.p. 51-54°C (Lit. for 2,6 isomer 52-53.5°C)²⁰

m^+ = 149 a.m.u. peaks at 148 (100%), 115, 89, 87, 86, 62, 60 and 51 a.m.u.

$\nu_{\text{max}}^{\text{CHCl}_3}$ 3000, 1580, 1450, 1320, 1110, 1056, 910, 880 and 810 cm^{-1}

n.m.r. (CDCl_3) τ 1.45 (2H,s)

The lower Rf band had the appearance of a yellow oil.

n.m.r. (CDCl_3) showed two peaks τ 1.75(s) and a very small peak at τ 1.45(s).

m.s. and i.r. data are consistent with those published for the 2,5-isomer.

G.l.c. analysis is fully discussed in the text in the discussion section of this thesis.

Chlorination of 2-chloropyrazine-1-oxide (110)²⁰

2-chloropyrazine-1-oxide (100 mg; 0.77 mmol) was stirred at room temperature with POCl_3 (2 ml) for 1 hour. Some of the solid still had not dissolved but gentle warming with an air blower brought this about. The mixture was then stirred for an overnight period.

The solution was then quenched in ice (30g) and extracted with chloroform (2 x 25 ml) and the organic phase back extracted with 5% bicarbonate (50 ml) and saturated brine (50 ml) and then dried (CaCl_2). Concentration yielded a semi-solid (87 mg; 76%).

G.l.c. analysis on Apiezon L showed that the 2,6-isomer was the major product with the N-oxide and 2-chloropyrazine as minor contaminants. P.l.c. separation (GF_{254}) in petrol/2% methanol gave the 2,6-isomer as a crystalline solid.

m.p. 52-54°C [Lit. 52-53.5°C]²⁰

n.m.r. (CDCl_3) τ 1.45 (2H,s)

m^+ = 149 a.m.u. peaks at 148 (100%), 115, 113, 89, 87, 86, 62, 51 and 50 a.m.u.

$\nu_{\text{max}}^{\text{CHCl}_3}$ 3000, 1580, 1450, 1400, 1320, 1180, 1050, 910, 88 and 810 cm^{-1}

G.l.c. analysis appears in the discussion section.

Preparation of 2,5-diethoxy-3,6-dihydropyrazine (133)²¹

Dioxopiperazine (1.0g; 8.7 mmol) was suspended in methylene chloride (5L ml) and Meerweins salt (6.0g; 31.6 mmol) was added with stirring. The solid anhydride took ca. 3 hours to go into solution and after a further 3 hours, a gelatinous precipitate formed.

The precipitate was carefully decomposed by the addition of a saturated solution of sodium bicarbonate (100 ml) and the solution was stirred until effervescence ceased and the aqueous layer was still alkaline (pH 7.5). The organic layer was separated, washed with water, dried (MgSO_4) and concentrated under reduced pressure.

On standing for a few minutes, the product solidified in the form of long needle crystals.

The crystalline mass was then extracted with petrol (60-80) (2 x 50 ml) and the filtrate was cooled in a fridge.

A precipitate of the 3,6-dihydro-5-ethoxypyrazinone was removed and characterised. wt. of monoimidate = 0.61g.

m.p. 156-157°C [Lit. 158-159°C]²¹

n.m.r. (CDCl_3) τ 8.7 (3H,t), 6.1-5.7 (6H,m),

2.3 (1H,s) goes in D_2O .

The remaining filtrate was cooled after being concentrated, and yielded the title compound as long colourless needles.

Yield 0.89g (60%)

Loss of yield may be attributed to the volatile nature of the compound.

m.p. 76-78°C [Lit. 77-78°C]²¹

n.m.r. (CDCl_3) τ 8.25 (6H,t), 6.0 (4H,s) and 5.85 (4H,q)

m^+ = 170 m.wt. = 170 peaks corresponding to sequential loss of ethoxy groups also present.

$\nu_{\text{max}}^{\text{CHCl}_3}$ = 3000-2800, 1690, 1480, 1440, 1370, 1350, 1250-1200,
1140, 1080, 1040, 920 cm^{-1}

A second synthesis using 8 equivalents of Meerweins salt in 2 x 4 eq. batches, 24 hours apart, and identical work up, gave an 88% yield of the desired bis-imidate.

and other attempts the main product was starting material (50-70%).

Attempted dealkylation of 2,5-diethoxypyrazine (134) with BBr_3

The diethoxypyrazine (100 mg; 0.6 mmol) was suspended in dry dichloromethane (5 ml) and an excess of BBr_3 (6 drops) added at 0°C (ice/salt bath). The pale, clear solution turned a cloudy yellow colour and a white solid separated out.

On quenching with water, a white solid came out of solution and when the solution was taken to dryness, a pale brown solid 2,5-dihydroxypyrazine (soluble in DMSO but not in TFA, MeOH, CHCl_3 etc.) was obtained. (weight = 41 mg).

m.p. $122-124^\circ\text{C}$.

n.m.r. $\tau 1.9$ (2H,s) does not exchange with D_2O

contains a trace of starting material.

Preparation of 2,5-dichloropyrazine-1-oxide

Trifluoroacetic acid (10 ml) was chilled to 0°C and to this was added hydrogen peroxide (80%, 9 ml) and stirred for 30 minutes to effect peroxytrifluoroacetic acid formation.

To the stirring solution at 5°C was added 2,5-dichloropyrazine (1.50g, 10.06 mmol) and the mixture stirred at ambient temperature for 30 minutes.

After this period, a vigorous reaction occurred and the vessel had to be cooled to control the reaction. The reaction was stirred for a further hour at ambient temperature after the reaction subsided.

The solution was then taken to dryness and the semi-solid produced taken up in chloroform.

T.l.c. (2% methanol in CHCl_3) showed that no starting material was present.

P.l.c. (2% MeOH: CHCl_3) gave three distinct products.

(a) 2,5-dichloropyrazine-1-oxide (0.84g, 51%)

m.p. = 103-105.0°C

n.m.r. (CDCl_3) τ 1.65 (1H,s), τ 1.85 (1H,s)

$\nu_{\text{CHCl}_3}^{\text{max}}$ 3100, 3005, 2940, 2860, 1710, 1560, 1480, 1410,
1370, 1290, 1120, 950, 910, and 840 cm^{-1}

m^+ = 164 (100%), 109, 101, 76, 74, 60, 52 and 51

$m^+ : m+2 : m+4$ = 9:6:1 \therefore 2 chlorine substituents

Found C = 28.8, H = 1.1, N = 16.7 $\text{C}_4\text{H}_2\text{N}_2\text{Cl}_2\text{O}$ requires

C = 29.09, H = 1.21, N = 16.96.

(b) m.p. = 87-90°C Yield = 247 mg (15%)

m^+ = 179 (61%), 164, 125, 111, 97, 85, 83, 82,

71, 69 and 57 (100%)

$m^+ : m+2 : m+4$ = 9:6:1 \therefore 2 chlorine substituents

n.m.r. (CDCl_3) τ 1.83 (2H,s)

$\nu_{\text{CHCl}_3}^{\text{max}}$ 3005, 2920, 2860, 1560, 1430, 1370, 1360, 1260,
1200, 1120, 1020 and 950

This may be a trace of 2,5-dichloropyrazine-1,4-dioxide.

(c) The third fraction was not identified.

Preparation of 2-O-allyl-5-chloropyrazine-1-oxide (117)

Dry benzene (2 ml) was placed in a 5 ml pear-shaped flask. To this was added sodium hydride (60 mg, 2.5 mmol) and some hydrogen evolution occurred. When hydrogen evolution had ceased freshly distilled allyl alcohol (118 μ l, 100 mg 1.72 mmol) was added. Further hydrogen evolution was observed. The

solution was stirred at room temperature for 2 hours.

To the solution was added 2,5-dichloropyrazine-1-oxide (75.8 mg, 0.5 mmol) and the solution refluxed for 18 hours. T.l.c. showed that slight trace of starting material was still present. However a large iodine positive spot was observed at high Rf. After a further two hours the reaction was quenched with water (20 ml) and the organic phases extracted with 0.5N NaOH (20 ml) and CHCl_3 (50 ml). The aqueous layers were further extracted with chloroform (50 ml) and the combined organic extracts subjected to a further base extraction (0.5N NaOH, 50 ml), dried and concentrated to an orange oil. P.l.c. (2% MeOH in CHCl_3) gave starting material as a minor product (15%) and an orange oil as the major product (125 mg, 67%).

n.m.r. (CDCl_3) τ 1.5 (s) 1H, 4.0 (m) 1H, 4.8 and 4.7 (bs) 1H each, 5.9 (t) 2H.

$\nu_{\text{max}}^{\text{CHCl}_3}$ 3100, 3005, 2940, 2860, 1710, 1600, 1510, 1480, 1410, 1370, 1290, 1120, 950 and 840

m^+ = 186 but low intensity ion

Found C = 44.8%, H = 3.5%, N = 14.9%, $\text{C}_7\text{H}_7\text{N}_2\text{O}_2\text{Cl}$ requires 45.04%C, 3.75%H, 15.01%N.

Preparation of 5-chloro-3-allyl-pyrazin-2-one (119)

2-O-allyl-5-chloropyrazine-1-oxide (100 mg, 0.54 mmol) was refluxed in toluene (20 ml) for 48 hours.

T.l.c. 2% MeOH in CHCl_3 showed the presence of some starting material, and a new spot at low Rf.

The mixture was evaporated under reduced pressure and applied to 40 x 20 p.l.c. plate (SiO_2 GF₂₅₄, 2 mm) and eluted with 2% methanol in chloroform.

The high Rf spot yielded starting material (75 mg, 75%) and had the same spectral characteristics.

The lower Rf spot was present in low concentration and gave 15 mg (15%) of a mobile yellowish oil.

n.m.r. τ 1.65 (1H,s), 4.2 (1H,m), 6.2 (2H,t), 4.5 and 4.6 (1H,m)

$\nu_{\text{max}}^{\text{CHCl}_3}$ 3100, 3005, 2940, 2860, 1710, 1675, 1500, 1460, 1410, 1370, 1290, 950 and 840 cm^{-1}

m^+ = 186 a.m.u. but again of low intensity.

Tentatively assigned this product as the rearranged compound due to the presence of the amide I band absorption at 1675 cm^{-1} .

Preparation of (1- ^{13}C) Leucine

KCN (1- ^{13}C ; 91.8% enriched) (500 mg; 7.58 mmol) was dissolved in water (0.8 ml) in a Quickfit test tube and cooled in ice. Ammonium chloride (411 mg; 7.6 mmol) was then added along with an aqueous solution of ammonia (1.03 ml; 21.2 mmol) and

3-methyl-but-1-al (660 mg; 7.67 mmol) in 95% ethanol (2.3 ml) was added.

The solution was stirred and then cooled in the test tube. After warming to room temperature the tube was heated to 60°C with stirring and then stirred for 18 hours at this temperature.

The solution was then poured onto 9 ml of ice cold HCl and was washed in with 1.0 ml water. The mixture was then refluxed vigorously in an oil bath at 120°C for 2.5 hours and then evaporated to give the crude leucine hydrochloride, contaminated with some KCl and NH_4Cl .

The brownish solid was taken up in water (20 ml) and applied to a column of Dowex 50 x 8 (H^+ form) on a 30 x 1.5 cm

column (prewashed with distilled water) over a period of 5 minutes. The column was then washed with distilled water (50 ml) until the effluent was neutral. The amino acid was then eluted with a molar solution of pyridine (1.5%) and fractions (50 ml) were collected at various stages. Fractions 2-20 inclusive contained most of the ninhydrin positive material and were subsequently pooled and taken to dryness to yield an off-white solid. This solid was dissolved in hot distilled water (15 ml), filtered and the residues also washed in. The combined hot filtrates were then treated with ethanol (36 ml) and stored at 4°C for 3 days. The resulting white plates were filtered off, washed twice with ethanol, the ether and then dried at 60°C in a vacuum oven to give enriched leucine (0.24g; 24%). m.p. (sealed tube) 271-273°C (Lit. 293-295°C).

T.l.c. in a n-BuOH:AcOH:H₂O (1:1:1) with ninhydrin spray showed the amino acid to be homogeneous.

¹³C.m.r. showed incorporation only into the carboxylic acid moiety.

Preparation of (1-¹³C) Isoleucine

The method of preparation was identical to that given for leucine only using 2-methyl-but-1-al instead of 3-methyl-but-1-al

Yield of isoleucine = 0.03g

m.p. (sealed tube) 247-250°C (Lit. 275°C)

T.l.c. in the same solvent system as for leucine with ninhydrin visualisation, again showed the amino acid to be homogeneous.

¹³C.m.r. showed incorporation only into the carbon of the carboxylic acid moiety.

REFERENCES

1. M. Nishida, Y. Mino, T. Matsubura, J.Antibiotics 1972 25 532
2. T. Kamiya, S. Maeno and Y. Kit, Ger.Offen 2,647,322 (1977)
3. A. I. Vogel, "Textbook of Quantitative Organic Analysis", p.613
4. D. Collins, "Microbiological methods", 1974, Wiley, p.296
5. S. Miyamura, N. Ogasawara, H. Otsuka, S. Niwayama, H. Tanaka, T. Take, T. Uchiyama and H. Octai, J.Antibiotics 1973 27 479
6. ATCC Handbook
7. Dictionary of Organic Compounds
8. W. Wakesman, "The Genus Steptomyces", Wiley Interscience 1976
9. A. Vogel, "Practical Organic Chemistry", p.169, Longmans (1966)
10. A.E.A. Porter, Ph.D. thesis, London University 1971.
11. K. W. Blake, Ph.D. thesis, London University 1970
12. A. Vogel, "Practical Organic Chemistry", p.584
13. *ibid.* p.910
14. W. P. Dickson and R. G. Marshall, J.C.S. 1929, 1495
15. L. Fieser and M. Fieser, Reagents for Organic Synthesis I 434
16. G. W. Kakover, J. B. Brown and J. Fried, J.Org.Chem. 1962 27 4710
17. P. G. Sammes and J. Markham, J.C.S.Chem.Comm. 1976 417
18. J. P. Greenstein and M. Winitz, "Chemistry of the Amino Acids" 1961, 2 p.73 Wiley Interscience
19. B. Klien, N. E. Hetman and M. E. O'Donnell, J.Org.Chem. 1963 28 1682
20. C. E. Mixan and R. G. Pews, J.Org.Chem. 1977 42 1769
21. K. W. Blake, P. G. Sammes, A.E.A. Porter, J.C.S.Perkin I 1972, 2494

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