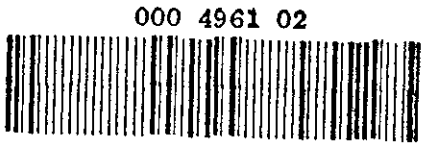
Biosynthesis of some nitrogenous fungal metabolites



BIOSYNTHESIS OF SOME NITROGENOUS FUNGAL METABOLITES

by

NICHOLAS JOHNS B.Sc.

A Doctoral Thesis

Submitted in partial fulfilment of the requirements

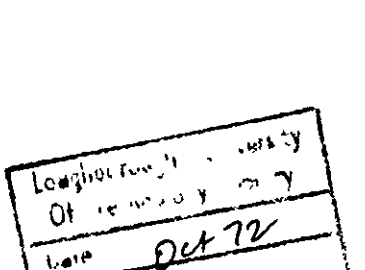
for the award of

Doctor of Philosophy of the Loughborough University of Technology

August 1972

Supervisor Professor G.W. Kirby, M.A., Ph.D., Sc.D., F.R.I.C.

Department of Chemistry



Foreword

Coming to Loughborough University to work for Professor G.W. Kirby was largely a matter of chance; of a general desire to return to chemistry after stifling years in a chemical sales organisation. It was a lucky choice. Throughout the past three years Professor Kirby has shown such great consideration and kindness, to my family as well as myself, that although it is true that the work described in this book could not have been accomplished without his great help, it is not sufficient merely to acknowledge him in the usual way.

N.J.

Acknowledgements

Apart from Professor G.W. Kirby, his supervisor, the author wishes to thank the following who have contributed to this work.

The Science Research Council, for a generous maintenance grant.

Dr. A. Hofmann (Sandoz A.G., Basle, Switzerland) and Dr. J. Woolley (The Polytechnic, Leicester) for gifts of *Claviceps purpurea* cultures.

Dr. R.J. Stretton and Mr. J. Watson Walker for microbiological advice and facilities.

Drs. J.D. Bu'Lock (Manchester) and B.W. Bycroft (Nottingham) for collaborative work on gliotoxin and clavine alkaloids, respectively.

Dr. S. Narayanaswami for gifts of chemicals as indicated, and for much helpful discussion and proof-reading.

Dr. A.P. Ryles, Mr. R. Pinchin and Mr. W.R. Gretton for private communications as indicated, and for helpful discussion.

Mrs. Arlene Wright for typing and cups of tea.

His wife Helen, and children, for three years' sympathy and patience.

Contents

Biosynthesis of Some Nitrogenous Fungal Metabolites

Summary

A. Feedings of Trichoderma viride with ring-tritiated aromatic amino-acids have shown that m-tyrosine is not an obligatory precursor of gliotoxin and that during conversion of phenylalanine into gliotoxin, neither loss nor migration of the ring protons occurs. A phenylalanine epoxide intermediate is proposed.

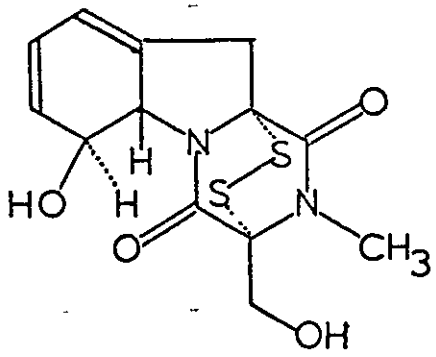
B. Gliotoxin derived from side-chain tritiated phenylalanines shows complete loss of 2'-label and partial loss of (3'S) and (3'R) label, the latter predominating. Similar losses are encountered in protein phenylalanine from the same feedings. Assay of the mycelial phenylalanine samples with phenylalanine ammonia-lyase shows predominant retention of the (3'-pro S)-proton. Gliotoxin biosynthesis therefore involves loss of the 2'-proton and retention of the 3'-protons. Loss of the (3'-pro R)-proton is an independent process. The relationship between 3'-label loss and incubation time is investigated.

C. DL [2-^{3}H]Tryptophan retains all of its label on conversion into agroclavine and elymoclavine by Claviceps purpurea (pennisetum). A biosynthetic hypothesis involving an indole-2-thioether intermediate is therefore unlikely, but no syntnesis has been found to conclusively eliminate the possibility of migration.

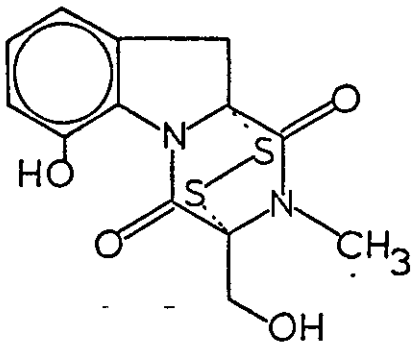
Introduction

The Epidithiadiketopiperazine Antibiotics

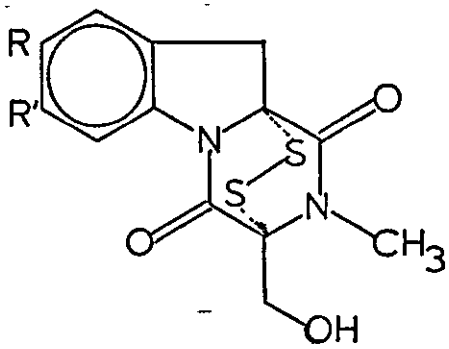
Gliotoxin (1), discovered in the 1930's by Weindling[1], was the first known member of a group of antibiotics which have since come to light. All are characterised by containing the 2,5-diketopiperazine-3,6-epidisulphide grouping. Gliotoxin[2] is a powerful toxin to animal, bacterial and fungal cells. More recently it has been shown to have antiviral properties as well[3]. Several moulds, including Gliocladium fimbriatum[2], Aspergillus species, particularly A. fumigatus[4,5], Trichoderma viride[6] and Penicillium terlikowskii[7], have been shown to produce gliotoxin. A number of other related metabolites have been obtained from the last organism, particularly dehydrogliotoxin (2)[7] and its isomer (3) or (4)[8,9]. The polyketopiperazine derivatives (5), (6) and (7) of indole-2-carboxylic acid and of 7-hydroxyindole-2-carboxylic acid have also been reported from cultures of P. terlikowskii[10]. The structure of gliotoxin for many years remained a mystery. Much chemical evidence, but very little spectral evidence, has been put forward to support its structure. Indeed although several references have been made in the literature to the n.m.r. spectrum of gliotoxin it has not yet been published[7]; neither have the i.r. or mass spectra. The u.v. spectrum has been reported by Johnson[2]. Much work has been dedicated to establishing the structure of gliotoxin by chemical degradation, the degradation products being identified by synthesis. An ample review of this work already exists[8]. Scheme I shows the degradations and appropriate references. From this evidence, early workers[15] deduced the complete structure (8) for gliotoxin. In 1953 the existence of a



(1) GLIOTOXIN

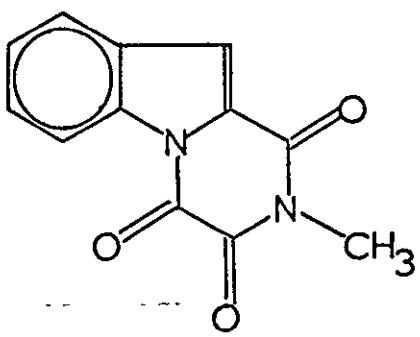


(2) DEHYDROGLIOTOXIN

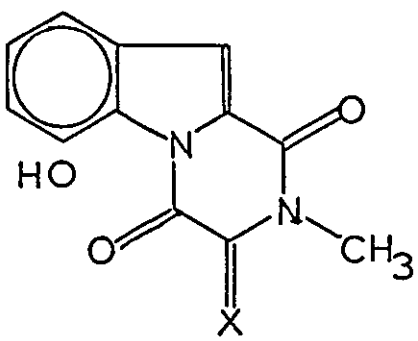


(3) R=OH, R'=H

(4) R=H , R'=OH

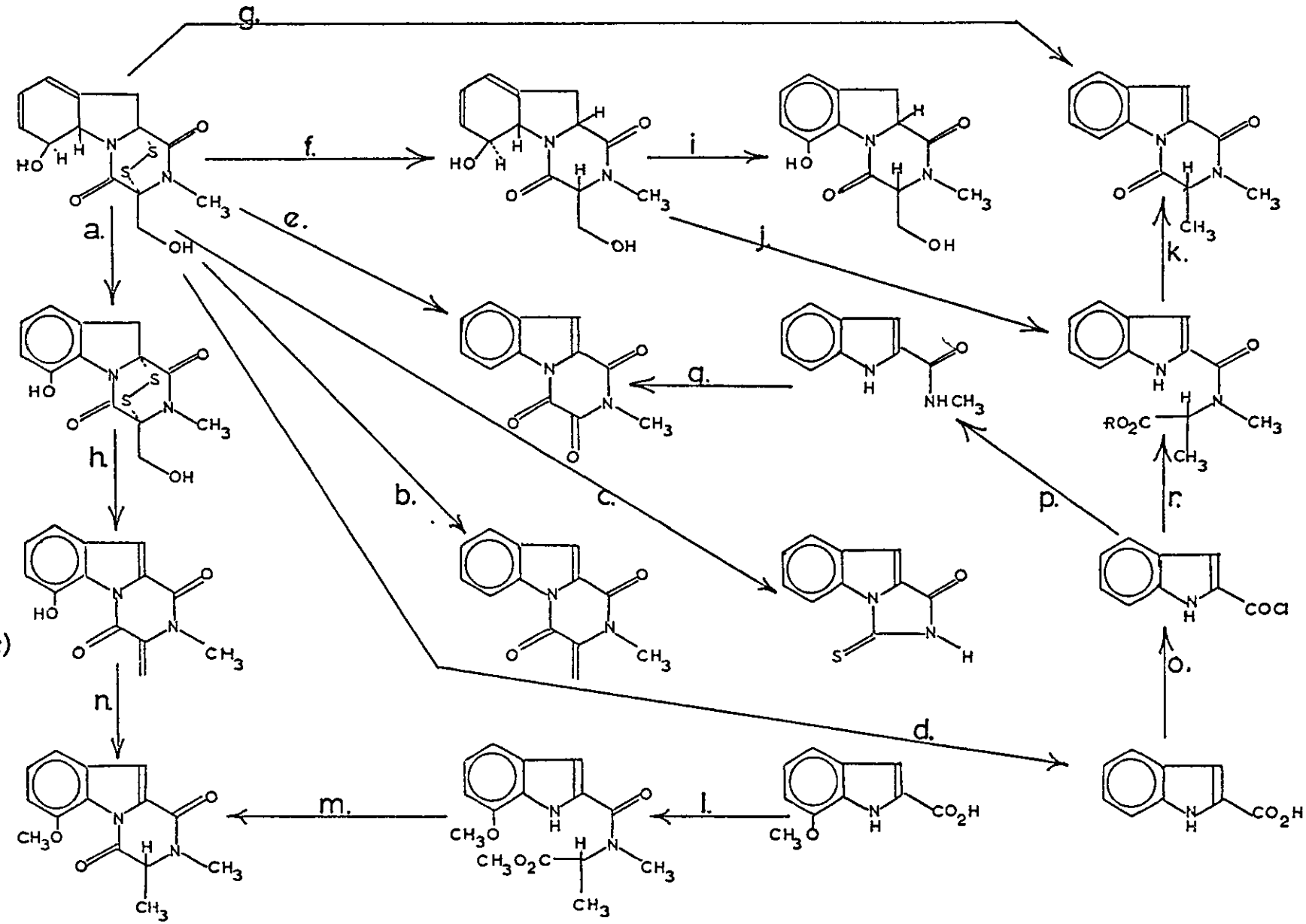


(5)



(6) X=O

(7) $X=CH_2$



Scheme I

(notes on following page)

Notes to Scheme I

a. *o*-Chloranil in refluxing benzene[7].

b. Grade II neutral alumina column[11,12].

c. Alcoholic sodium methoxide[13,14,15,16].

d. Aqueous barium hydroxide[17,18].

e. Heating with selenium[19].

f. Aluminium amalgam in ethanol[13,16]. Raney nickel[20].

g. Red phosphorus and hydriodic acid[19].

h. Hydrogen chloride in acetic acid[7].

i. [21]

j. R=H, Reflux in 2*N*-methanolic KOH[13].

k. R=Me, Trimethylamine or methanolic sodium methoxide[22].

l. (i) Thionyl chloride. (ii) N-Methylalanine methylester[7].

m. Base in methanol, *cf.*(k)[7].

n. (i) Dimethylsulphate in base. (ii) Zinc in acetic acid/ acetic anhydride[7,10].

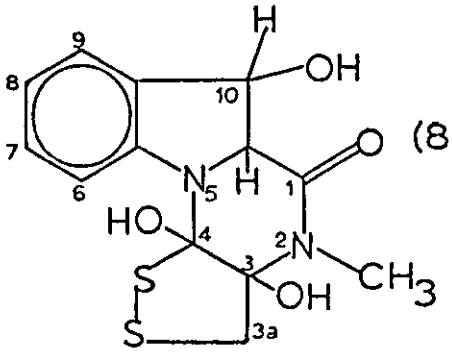
o. Thionyl chloride[23].

p. Methylamine[23].

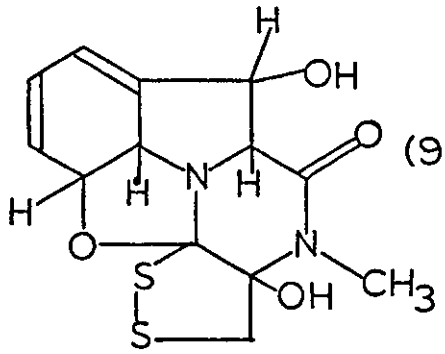
q. Oxalyl chloride[19].

r. *N*-methylalanine methyl ester[23], R = Me.

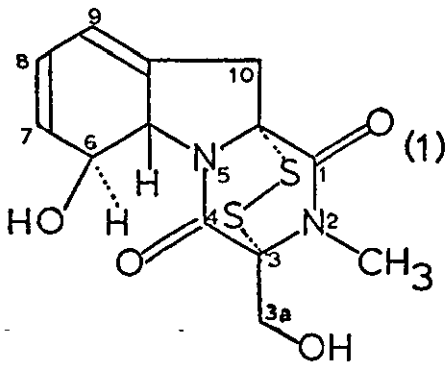
STRUCTURES of GLIOTOXIN



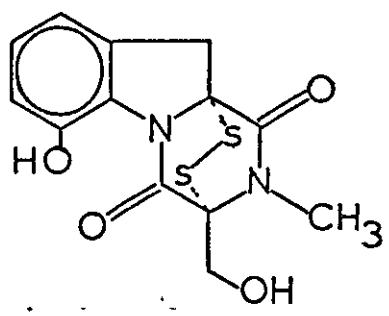
(8) after Elvidge (1949)



(9) after Johnson (1953)



(1) after Woodward (1958)

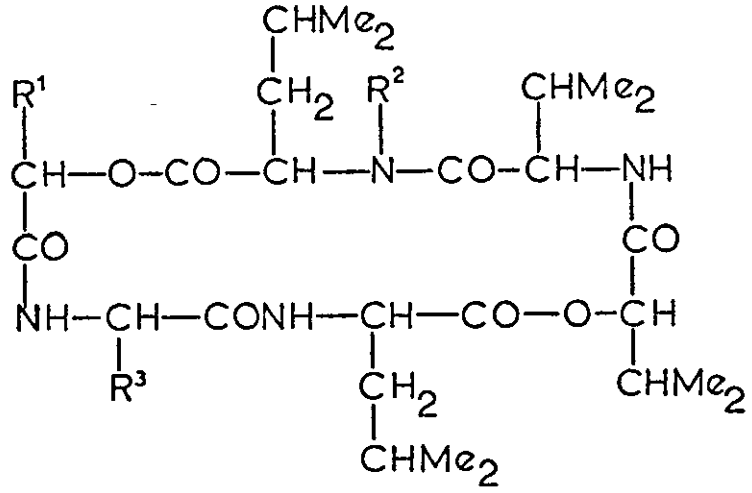


(2)

cyclohexadiene unit within the molecule was recognised[16] and the structure was modified to (9). The structure (1) which is now accepted as correct was put forward in 1958[24]. The absolute configuration has since been established by X-ray crystallography[76].

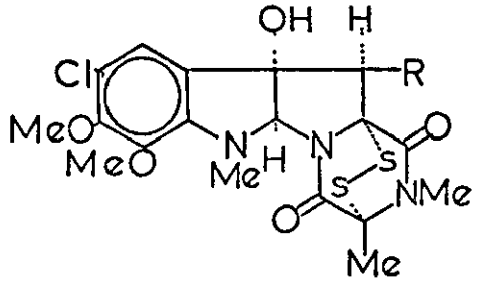
Although Turner[32] implies that the absolute configuration of dehydrogliotoxin (2) from P. terlikowskii is not known, it seems reasonably certain on the basis of published evidence[7,9] that the natural material is identical to the product obtained when gliotoxin is refluxed with o-chloranil in benzene. Since it is not likely that the disulphide bridge can undergo inversion under these conditions dehydrogliotoxin (2) should have the same configuration as gliotoxin (1).

The sporidesmins, another group of epidithiadiketopiperazine antibiotics have been isolated, together with the sporidesmolide depsipeptides (10), from the fungus Sporidesmium bakeri, which has since been renamed Pithomyces chartarum. Screening of the organism was carried out after its presence in pasture had been correlated with occurrence of facial eczema in sheep. That facial eczema is associated with an extract termed "sporidesmin" was first reported in 1959[25,26,27]. Four years later, the first compounds of this group, sporidesmin (11) and sporidesmin B (12) were purified and characterised chemically and spectroscopically[28]. The absolute configurations shown in these structures was established by X-ray crystallography[29]. Configuration at the disulphide bridge is the same as in gliotoxin. Subsequently, a number of closely related metabolites have been isolated and characterised. Sporidesmin C (13) has a



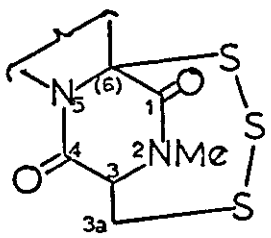
(10)

Sporidesmolide	1	2	3	4
R^1=	Me_2CH,	Me_2CH,	Me_2CH,	Me_2CHCH_2
R^2=	Me	Me	H	Me
R^3=	Me_2CH,	Me_2CHCH_2,	Me_2CH,	Me_2CH

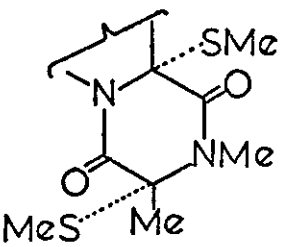


(11), R=OH, Sporidesmin

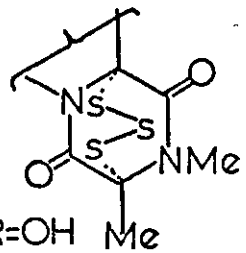
(12), R=H, Sporidesmin B



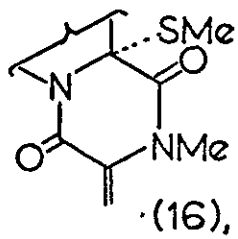
(13), R=OH, Sporidesmin C



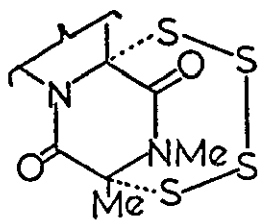
(14), R=OH, Sporidesmin D



(15), R=OH Sporidesmin E



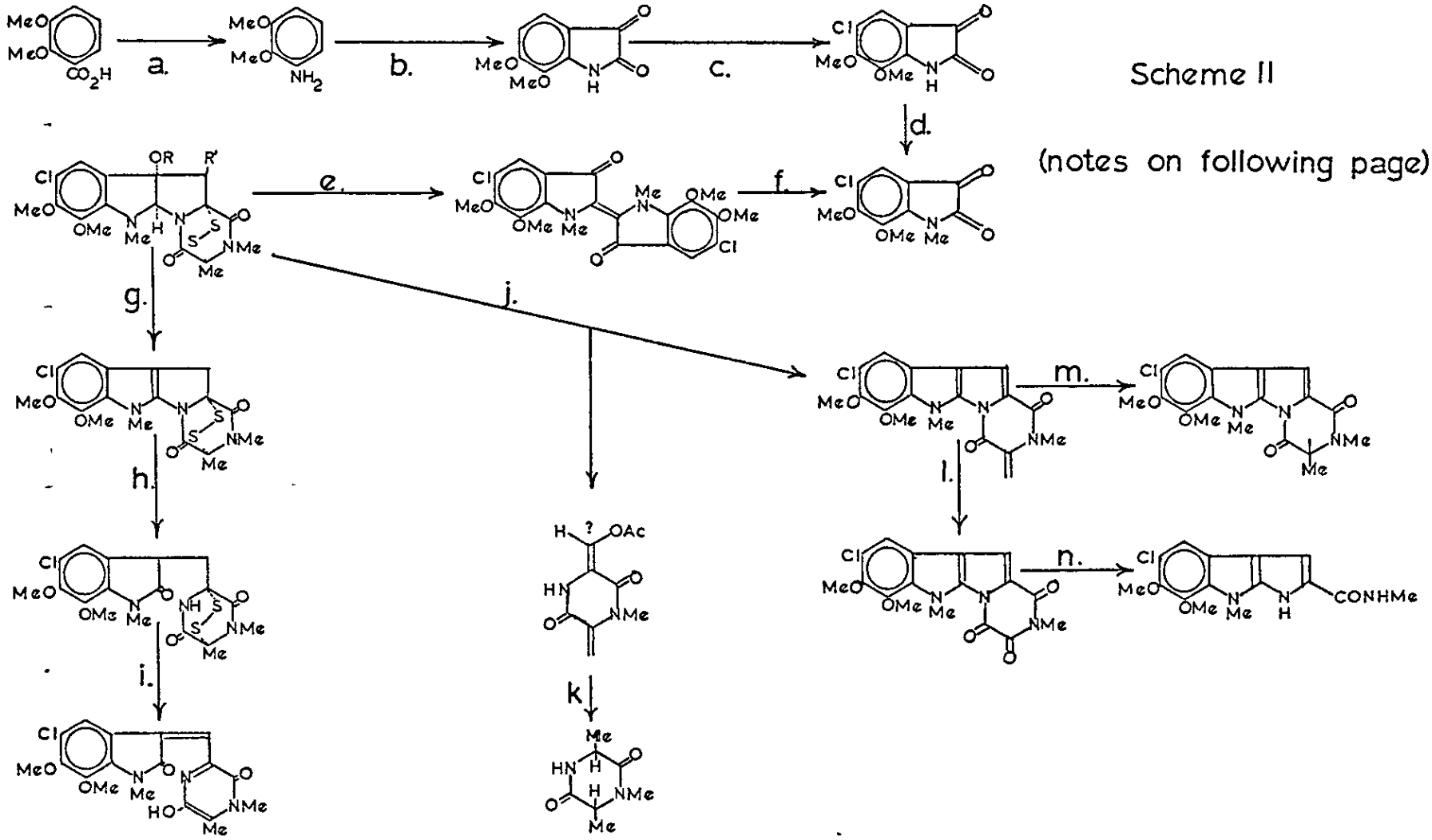
(16), R=OH Sporidesmin F



(17), R=OH, Sporidesmin G

trisulphide, instead of a disulphide bridge, connecting C-6 of the diketopiperazine ring and the methylene (C-3a) attached to C-3 of the diketopiperazine ring. Mass spectral and n.m.r. data have been published[30], but no information on the configuration of the molecule is available. Sporidesmins D[31] (14) and F (16) have been assigned their structures[77] on the basis of simple degradations from sporidesmin itself and almost certainly have the configurations shown. Another epitrisulphide, sporidesmin E[33] (15) has sulphur bound only to the carbon atoms of the diketopiperazine grouping[34]. Its structure has been correlated with that of sporidesmin by both synthesis[35] and degradation[36]. Of particular interest is that there are two possible conformers for the central sulphur atom, and the n.m.r. spectrum undergoes simplification when the sample is warmed to 55°C. This phenomenon has also been observed with model compounds[37]. An attempt to separate the two conformers of sporidesmin E at low temperature was unsuccessful[36]. Recently a further anaolgue of sporidesmin having a tetrasulphide bridge[38] was isolated and has been named sporidesmin G (17). The free energy of interconversion of its conformers is much lower than in sporidesmin E[38].

As in the case of gliotoxin, much of the chemistry of the sporidesmins has consisted of degrading the molecule in order to produce fragments which may be identified by synthesis. The work is well reviewed[8] and so will not be presented in detail here. An outline of the degradations carried out on sporidesmin with appropriate references is shown in Scheme II. Generally the sporidesmin and gliotoxin



Scheme II

(notes on following page)

Notes to Scheme II

a. Sodium azide in dilute sulphuric acid[39].

b. (i) Chloral hydrate. (ii) Hydroxylamine[39].

c. t-Butyl hypochlorite[39].

d. (i) Isomerise in sodium hydroxide. (ii) Dimethyl-sulphate[39].

e. R=H, R'=OH, Mercury; shake at room temperature[39].

f. Methanolic sodium hydroxide[39].

g. R=Ac, R'=H, Boron trifluoride etherate[40].

h. Boron trifluoride etherate[41].

i. Triethylamine in benzene[41].

j. R=Ac, R'=OAc, Zinc in acetic acid/acetic anhydride[42].

k. Hydrogenation palladium/charcoal in methanol[42].

l. Potassium permanganate[42].

m. Zinc in acetic acid[42].

n. Dilute sodium hydroxide[42].

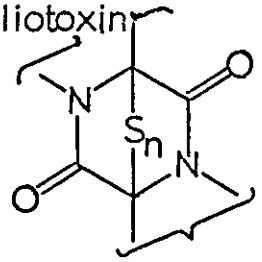
degradation products are analagous, although frequently arrived at by different procedures.

More recently, work on the chemistry of the sporidesmins and of dehydrogliotoxin has been directed at the bridged diketopiperazine disulphide moiety. Safe and Taylor[35] have reported that the homologous series (18) n=2,3,4 etc. may be ascended stepwise by reaction of a parent compound ($n \geq 2$) with one molar equivalent at a time of dihydrogen disulphide. In this way, sporidesmin E having all the properties of the natural compound, and a tri- and tetra-thio analogue of dehydrogliotoxin may be obtained[35]. The same authors[36] have since reported that the homologous series (18) may be descended down to n=1 without loss of configuration about the C-S bonds. In this case the reagent used was triphenylphosphine. When the compounds (19) and (20), (X=S) were oxidised with m-chloroperbenzoic acid, the sulphoxide bridged species (19) and (20) (X=SO) were produced. Loss of configuration in the sulphoxide was explained by the sequence shown in Scheme III.

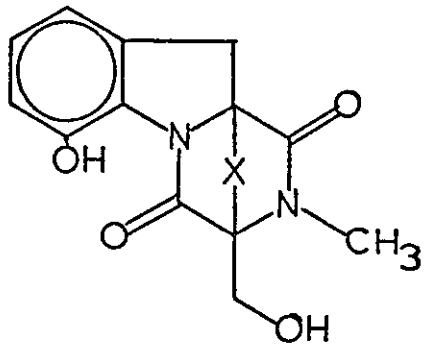
Since it is the epidithiadiketopiperazine structure which is known to confer antibiotic properties on these metabolites[43], numerous attempts have been made to synthesise this type of structure. For example Corey[44], Lautenschlaeger[45] and Weil[46] have all independently shown that disulphur dichloride can add across unconjugated cyclic dienes. 2-Mercaptoalanine, produced by a modification of Erlenmeyer's azlactone synthesis and its methyl ester, can be coupled, using ferric ion to give a bis (2,2-alanino)-disulphide[47]. No indication is given of the stereochemistry, and presumably

sporidesmin or
dehydrogliotoxin

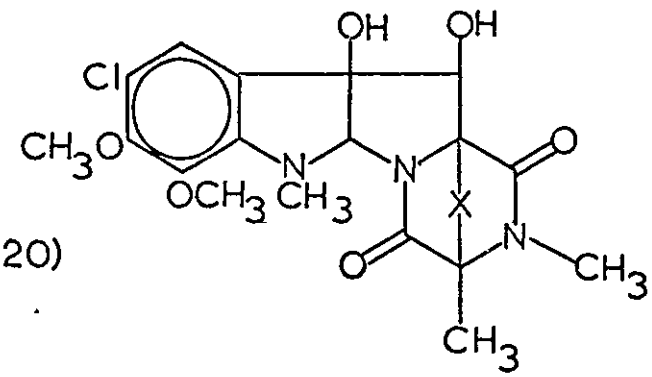
(18)



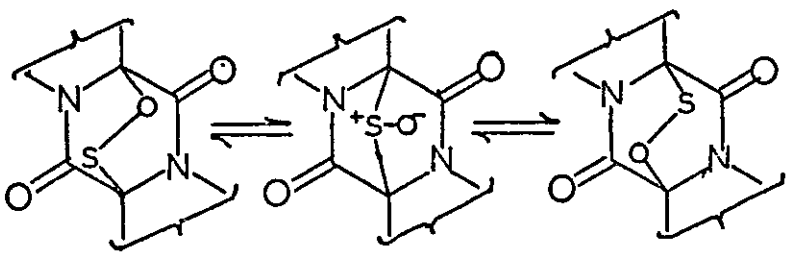
(19)



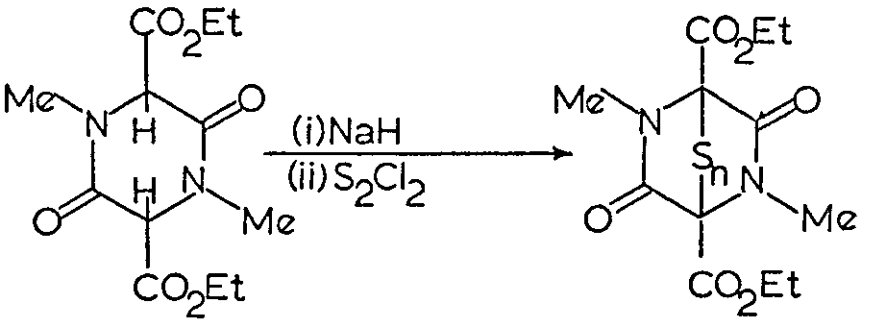
(20)



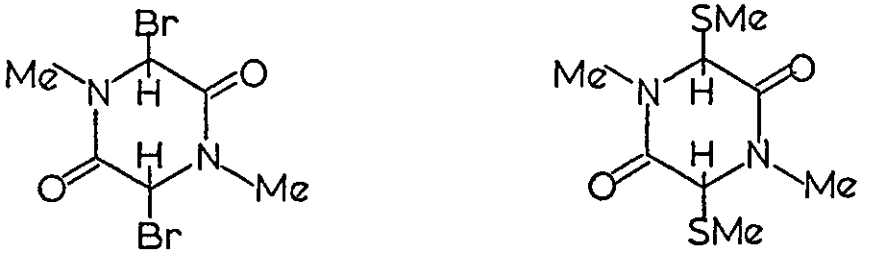
Scheme III



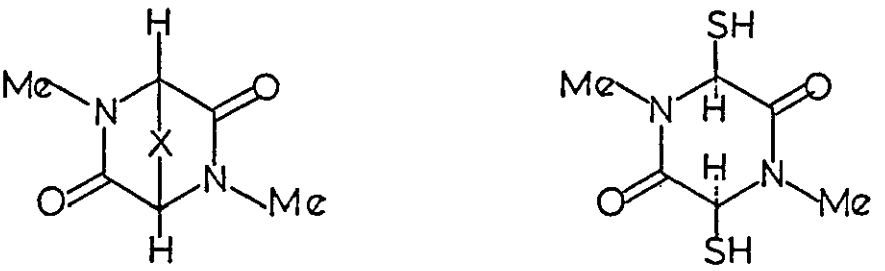
racemates are used throughout. Other workers[48] have prepared 3,6-diethoxycarbonyl-1,4-dimethylpiperazine-2,5-dione (21). The 3,6-dicarbanion of this compound, prepared by heating (21) with sodium hydride in a suitable solvent readily gives a range of products (22), (n=0,1,2,3,4) with disulphur dichloride. Yields were very low[48]. Poisel and Schmidt[49] have since carefully studied the addition of sulphur across various diketopiperazines. Treatment of 2,5-dibromo-1,4-dimethylpiperazine-3,6-dione (23) with methanolic methyl mercaptide, gave a mixture of cis- and trans-2,5-dimethylthiopiperazinediones (24). When this material was treated with base it isomerised to one form. Bridged species (25) (X=S(CS)S, X=S_4) were formed by treating the dibromo compound with thiocarbonate or tetrasulphide respectively. Reduction of (25) (X=S_4) with borohydride gave the cis-dithiol (26) which on treatment with phosgene yielded (25) (X=S(CO)S). This latter compound was also obtained from (25) (X=(CS)S) by treatment with mercuric acetate[49]. The dithiol (26) could be oxidised to the disulphide-bridged species (25) (X=S_2) by iodine/potassium iodide at a water/chloroform interface or by ferric ion and oxygen[50]. The same workers have metallated L-prolyl-L-proline anhydride (27) with butyllithium. It is claimed that the 3-carbanion can be reprotonated to give (27) without change of stereochemistry. However the 3,6-dicarbanion reprotonates or alkylates to give a trans-diketopiperazine derivative. Stepwise metallation followed by treatment successively with sulphur and ethyl bromide gave (28) (R=SEt)[51]. Treatment of (27) with sulphur in the



(21) (22)



(23) (24)



(25) (26)

(27) (28)

(29) (30)

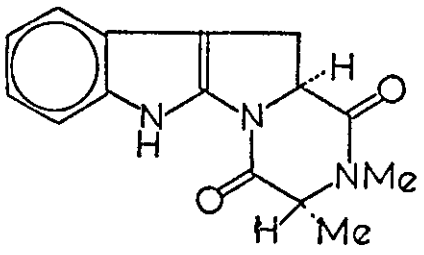
presence of sodamide gave a mixture (28) (R=SH) and (29) ($X=S_n$)[52]. Reduction of the oligosulphide (29) ($X=S_n$) with borohydride to the mixture of cis-enantiomers (30) (R=SH) followed by oxidation with iodine/iodide yielded (29) ($X=S_2$).

Trown[53] reported the preparation of di(acetylthio)-N,N-dimethylpiperazine-2,6-dione from the dibromo compound (23). This was hydrolysed to the dithiol (presumably (26)) and the sulphur atoms linked by treatment with 5,5'-dithiobis-(2-nitrobenzoic acid). No mention is made of the stereochemistry. A yield of 70% is quoted for the last reaction.

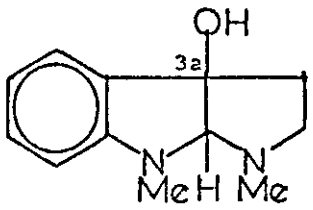
Although no attempt at the synthesis of gliotoxin or dehydrogliotoxin has yet been reported, Witkop[54] has synthesised the parent diketopiperazine (31) of sporidesmin in good yield, and other workers have prepared a [2,3-b]-pyrroloindole with a hydroxyl function at position 3a (32)[55]. In a recent paper [56], the indole derivative (33) was reported, which has the same oxidation state as sporidesmin, and which could furnish a convenient starting material for total synthesis.

When the work on which this thesis is based was begun in 1969, biosynthetic studies on gliotoxin and sporidesmin had been carried out which merited further investigation. These are reviewed below. Appropriate references to biosynthetic work published since 1969 will be presented as the author's work is described.

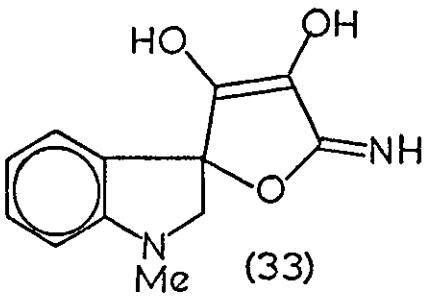
Suhadolnik[18] showed in 1958 that gliotoxin was derived from phenylalanine rather than from tryptophan and acetate. In particular the 1' and 2' carbon atoms of phenylalanine were retained in gliotoxin as shown by carbon-14 labelling



(31)

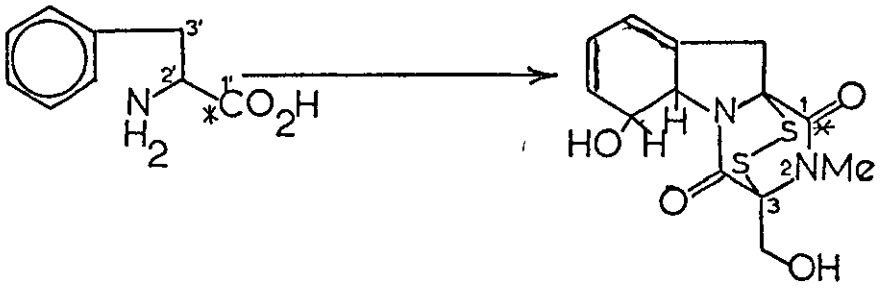


(32)

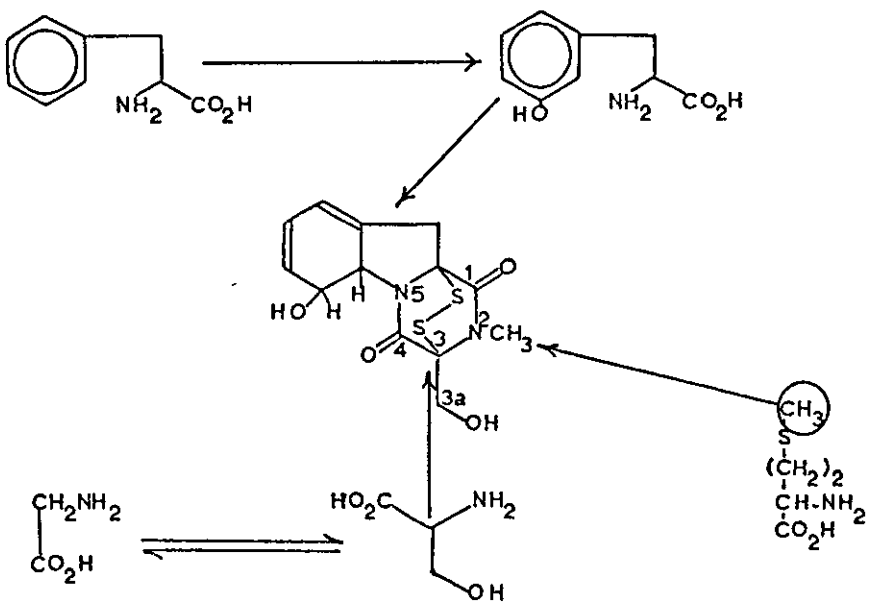


(33)

Scheme IV

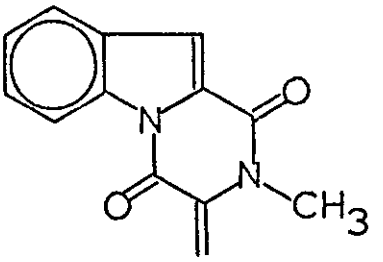


Scheme V

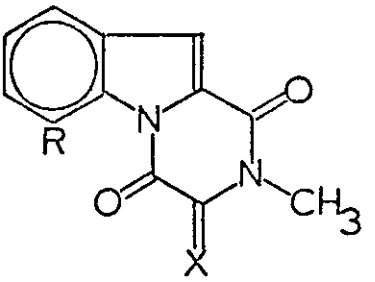


studies, implying that all nine carbons of phenylalanine were incorporated, since all activity in gliotoxin derived from [1'-^{14}C]phenylalanine was located at the 1-position in gliotoxin (Scheme IV). A slight incorporation of [methyl-^{14}C] methionine was interpreted in terms of methionine being the source of the N-methyl group in gliotoxin. In a second paper[57] the biosynthetic relationship between phenylalanine, in this case tritiated, and gliotoxin was again demonstrated, and ^{3}H-m-tyrosine was incorporated even more readily. The [methyl-^{14}C] methionine experiment was repeated, and in this case the label was found by degradation to be located entirely in the N-methyl group of gliotoxin. When [1-^{14}C] serine was fed the label was entirely located at C-4 in gliotoxin. The label from [3-^{14}C] serine appeared at C-3a and N-CH_3 in the metabolite. [2-^{14}C] Glycine was also incorporated to a slight extent into gliotoxin, the label being located at C-3 and N-CH_3. The findings of Suhādolnik's group are summarised in Scheme V.

Bose et al.[12] discovered that sufficiently good incorporation of precursors into gliotoxin occurred to make heavy-isotope labelling practicable. He used mass spectrometry both to measure the incorporation levels and to locate the label. All gliotoxin was degraded to the anhydrodesthio-derivative (34) before analysis. [^{15}N]-Phenylalanine was found to be incorporated into the N-5 of gliotoxin. The label from [^{15}N] glycine was found in both N-2 and N-5. Label from [1-^{13}C]- and [2-^{13}C] glycine and from [^{13}C] formate were incorporated but the position of incorporation was not conclusively shown. Bose et al.



(34)

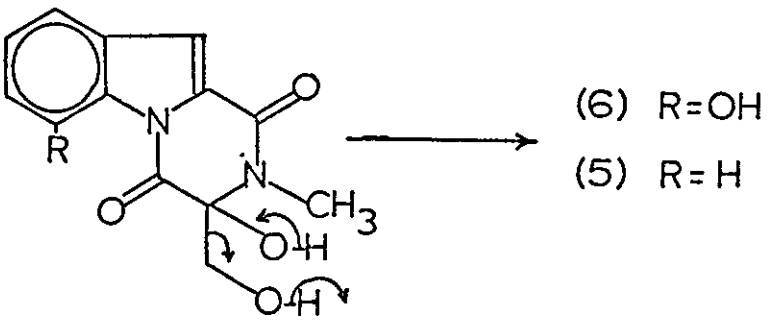


(7) X=CH_2, R=OH

(6) X=O , R=OH

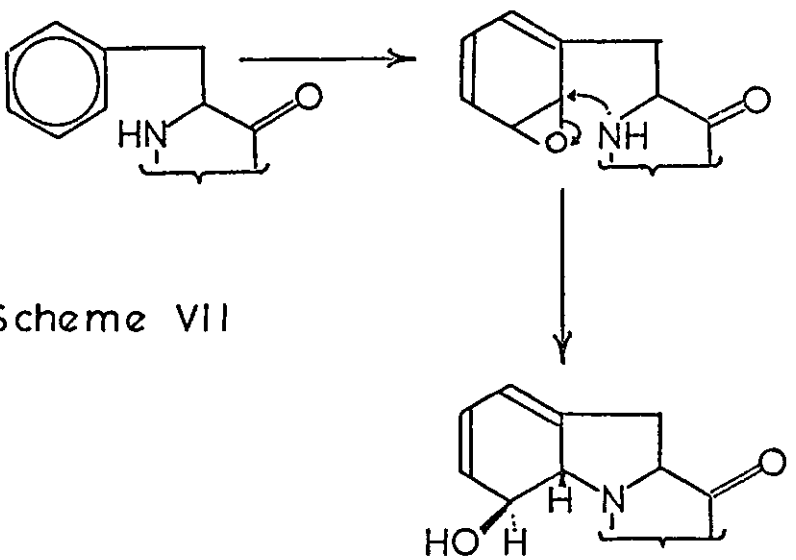
(5) X=O , R=H

Scheme VI



(6) R=OH

(5) R=H



Scheme VII

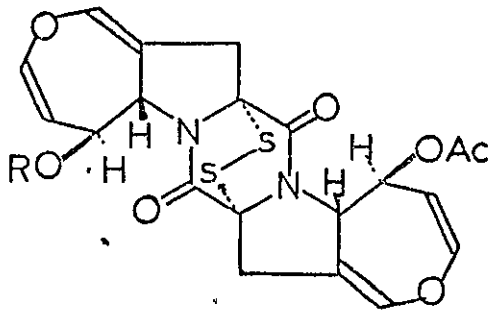
deduced that phenylalanine may be incorporated either intact or *via* a nitrogen-free intermediate of some sort. In a later communication[50] the same workers found that whereas there was no appreciable difference between the dilution factors of ^{13}C and ^{14}C in gliotoxins derived from [1'-^{13}C,1'-^{14}C]-, and [1'-^{13}C,3'-^{14}C]phenylalanines, the dilution factor of ^{15}N was about three times that of ^{14}C when [1'-^{14}C,^{15}N]phenylalanine was fed, whether D-[1'-^{14}C]- or L-[1'-^{14}C]phenylalanine was fed together with DL-[^{15}N]phenylalanine. Although no label from [1-^{14}C]aspartate was incorporated, ^{15}N from labelled aspartate could be detected at both N-2 and N-5 of gliotoxin. Likewise ^{15}N enrichment due to labelled glutamate could be detected at both positions. When Taylor *et al.*[10] discovered diketopiperazines of indole-2-carboxylic acid and of 7-hydroxyindole-2-carboxylic acid in cultures of *P. terlikowskii*, which also produces gliotoxin and dehydrogliotoxin, it seemed likely that these were intermediates in the biosynthetic route. Indeed Taylor postulated (7) as the precursor of gliotoxin, accounting for (5) and (6) by the postulated secondary routes shown in Scheme VI. Later Taylor's group claimed[59] that (7), labelled with ^{14}C in the benzenoid ring was incorporated into dehydrogliotoxin and gliotoxin by *P. terlikowskii*. The route from phenylalanine was taken to be *via* *m*-tyrosine, (7) and dehydrogliotoxin to gliotoxin itself. However in the same year as this report the discovery of aranotin (see below) prompted the suggestion[60,61] that gliotoxin might be formed by attack of the amino-acid nitrogen upon an arene oxide species. (Scheme VII). This route and that proposed by

Suhadolnik and Taylor are clearly mutually exclusive. The author's work has partly been to establish which is the actual biosynthetic route.

Work on sporidesmin has been oriented towards preparing the labelled toxin for tracer studies of its metabolism by sheep. Brook and Matthews[62] have reported that ^{35}S from labelled sulphate is readily incorporated into sporidesmin (the crude preparation). Towers and Wright[63] have reported incorporation of [3'-^{14}C] tryptophan, of uniformally labelled [^{14}C] serine, alanine and glycine, of L-[methyl-^{14}C] methionine and of [^{35}S] cysteine(L), methionine(L) and sulphate but not of [U-^{14}C] cysteine or phenylalanine. Although the results indicate that tryptophan and alanine are the likely precursors, rather than the 5-chloro-6,7-dimethoxyindole derivatives postulated by Taylor[39], no values for the incorporations are presented, and no fast conclusions can be drawn.

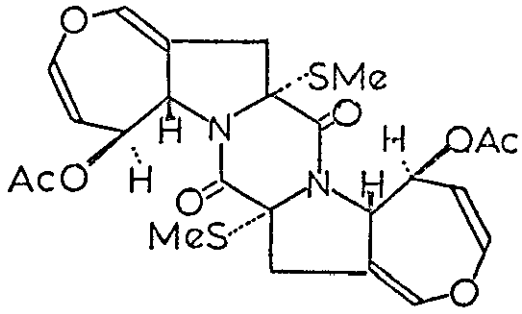
The aranotins are another class of dithiadiketopiperazine antibiotics, which possess an oxepin ring as part of their general structure. Compounds of this type are derived from Arachniotus aureus (Eidam) Schroeter[60] and from Aspergillus terreus[64].

Aranotin (35) (R=H), acetylaranotin (35) (R=Ac) and bisdethiodi-(methylthio)-acetylaranotin(BDA)(36) were discovered to be the main antiviral metabolites of Arachniotus aureus[65]. Relationships between the structures were established by acetylation of aranotin to acetylaranotin and partial hydrogenation of the latter and of BDA to a common diketopiperazine derivative using Raney nickel[20].

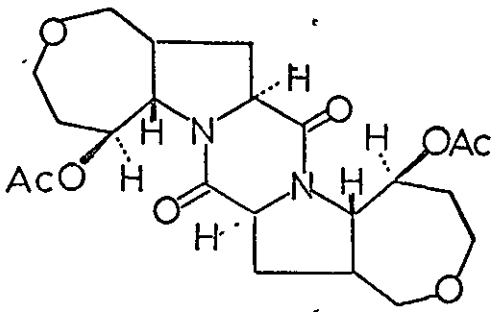


(35) R=H Aranotin

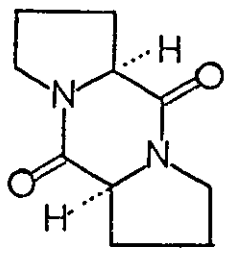
R=Ac Acetylaranotin



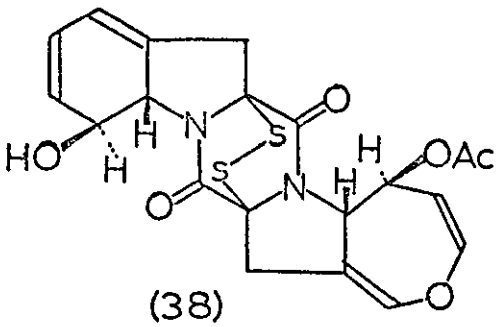
(36) B.D.A.



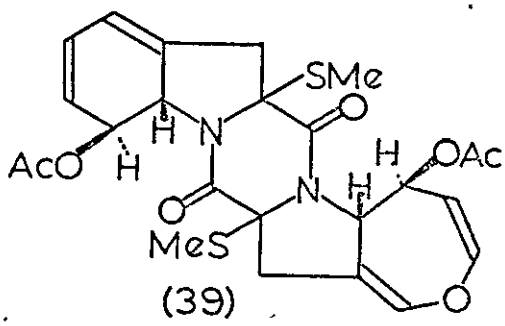
(37)



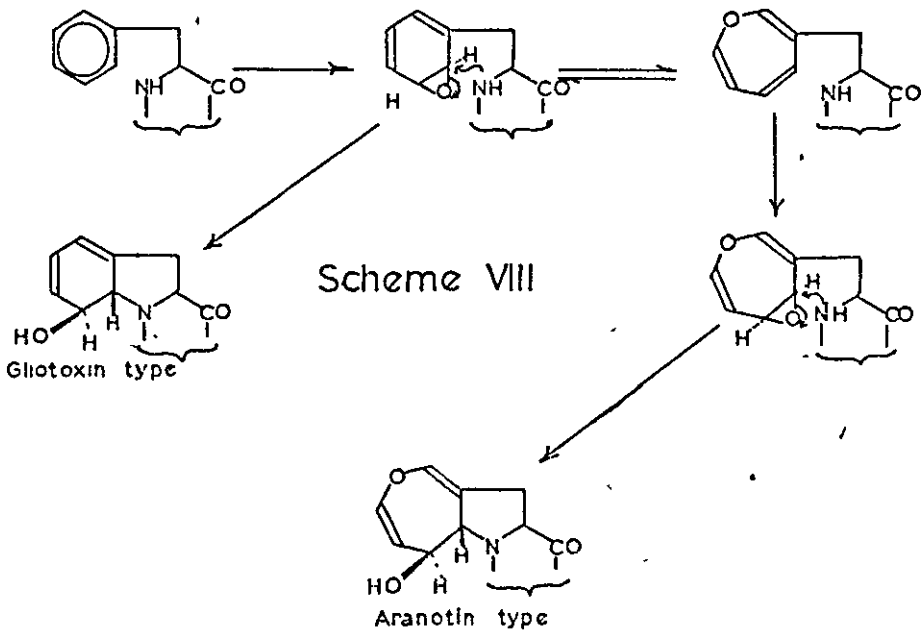
(27)



(38)



(39)



Scheme VIII

This latter reaction is of particular interest in that from the aranotin series a diketopiperazine having the same configuration as the parent is obtained, whereas that from gliotoxin is partially racemised[20]. An explanation for this phenomenon is suggested by the work of Poisel and Schmidt[52].

The existence of an oxepin ring in aranotin was deduced from studies of the n.m.r. and mass spectra of the compound itself and of the cyclic ether produced by complete hydrogenation over Raney nickel.

Metabolites designated LL-S88α and LL-S88β with antiviral properties have been isolated from Aspergillus terreus and their spectral properties recorded.[66] In a later paper[64] LL-S88α was reported to be identical with acetylaranotin. It is likely from the evidence[66] that LL-S88β is BDA (36).

Comparison of circular dichroism curves of acetylaranotin and gliotoxin shows good correlation between the two[20], as would be expected from the work of Taylor[40]. Good correlation is also observed between c.d. curves of perhydrodesthio acetylaranotin (37) and L-prolyl-L-proline anhydride (27).

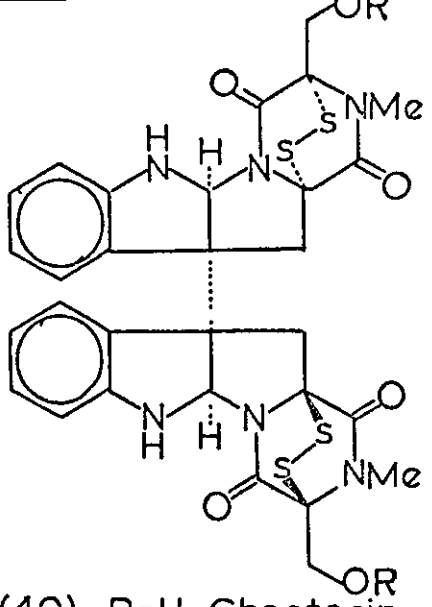
The absolute configurations of the acetylaranotin[67] and BDA molecules[68] have been assigned as (35) and (36) on the basis of X-ray crystallographic data.

Two other metabolites related to both the aranotin and the gliotoxin series have been isolated from Arachniotus aureus cultures. These are named apoaranotin (38) and bisdethio-di(methylthio)-acetylapoaranotin (BDAA) (39)[69]. The stereochemistry of their carbon skeleton has been correlated with that of gliotoxin and aranotin but no attempt has been made to show the configuration of the

disulphide bridge. Nevertheless it is almost certain to be R,R as in the other aranotins.

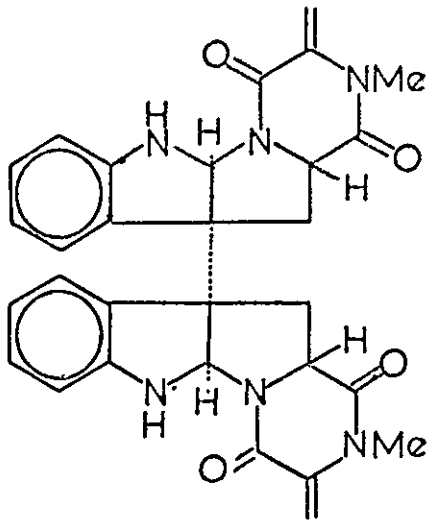
The discovery of the aranotins and particularly of the apoaranotins represents a landmark in the biosynthetic work on gliotoxin. Since arene oxides are known to undergo tautomerism to oxepins[70], a biosynthetic pathway can be put forward[69] which will account both for the hydroxylated cyclo-hexadienyl unit of gliotoxin and for the oxepin grouping in the aranotins (Scheme VIII). In both groupings the stereochemistry of the C-OH and C-N bonds suggest a biosynthetic route involving rearside attack by nitrogen of an epoxide function.

In 1968[60] preliminary feeding experiments with cultures of Arachniotus aureus showed that D- and L- [1'-^{14}C]phenylalanine were readily incorporated into BDA, as was L methyl-^{14}C methionine. Only low incorporations of variously carbon-14 labelled tyrosine, proline, leucine, serine and tryptophan, were observed, and it was demonstrated by Zeisel degradation that most of the activity incorporated from L- methyl-^{14}C methionine, DL-[3-^{14}C]serine and DL [2'-^{14}C]tryptophan was present in the S-methyl groups. When equimolar amounts of L- [^{35}S] - and L-[methyl-^{3}H] methionine were fed, the incorporation of tritium was higher than that of sulphur-35, indicating that the S-methyl group of methionine is not transferred in one step to the diketopiperazine grouping of BDA. Incubation with [$^{35}S_2$] BDA demonstrated that this compound was not the precursor of acetylaranotin. When equimolar quantities of L-[^{35}S]-and L-[methyl-^{3}H] methionine were fed to P. terlikowskii, incorporation was of the same order for both isotopes. By feeding L-[^{35}S]methionine to P. terlikowskii in the presence of a large excess of various sulphur-containing compounds, it

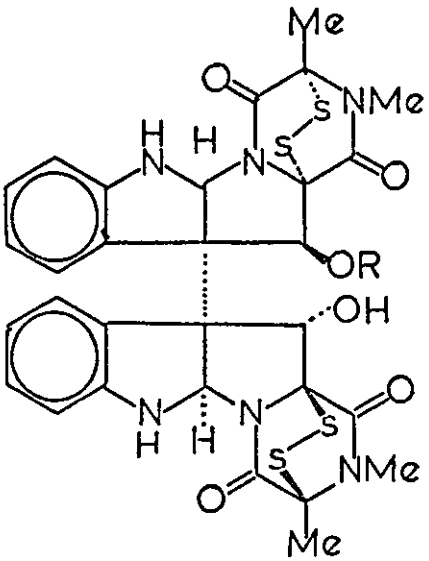


(40) R=H Chaetocin

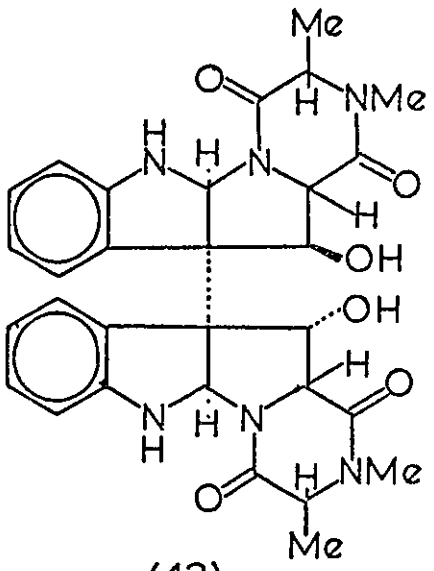
R=Ac Diacetylchaetocin



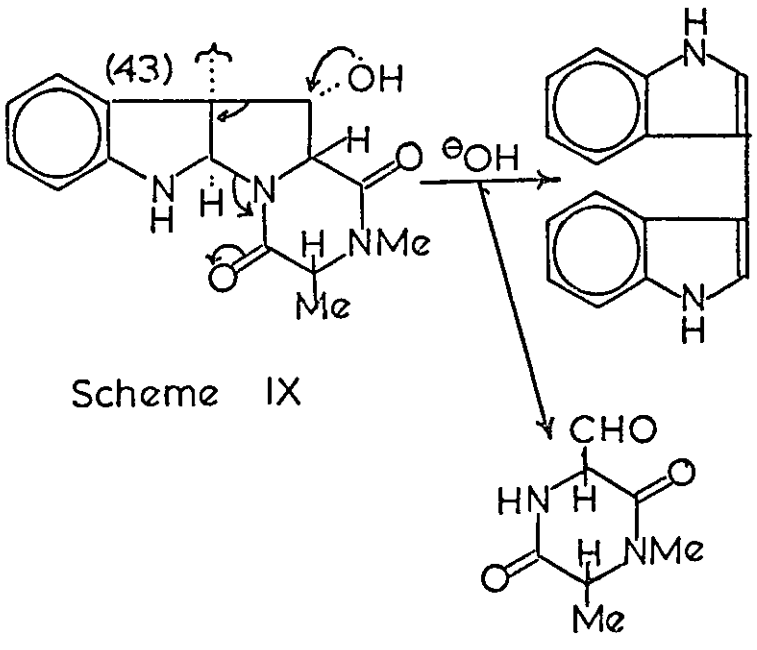
(41)



(42) R=H Verticillin A



(43)



Scheme IX

was possible to show that DL-homocysteine and L-cysteine both stimulate gliotoxin production, but homocysteine increases ^{35}S incorporation from methionine, while cysteine greatly reduces it. DL-Cystathionine also reduced ^{35}S incorporation but did not affect gliotoxin production. Sulphite and thiosulphate drastically reduced both the production of gliotoxin and the incorporation of label. The authors concluded that cysteine was the likely donor of sulphur in gliotoxin biosynthesis by P. terlikowskii.

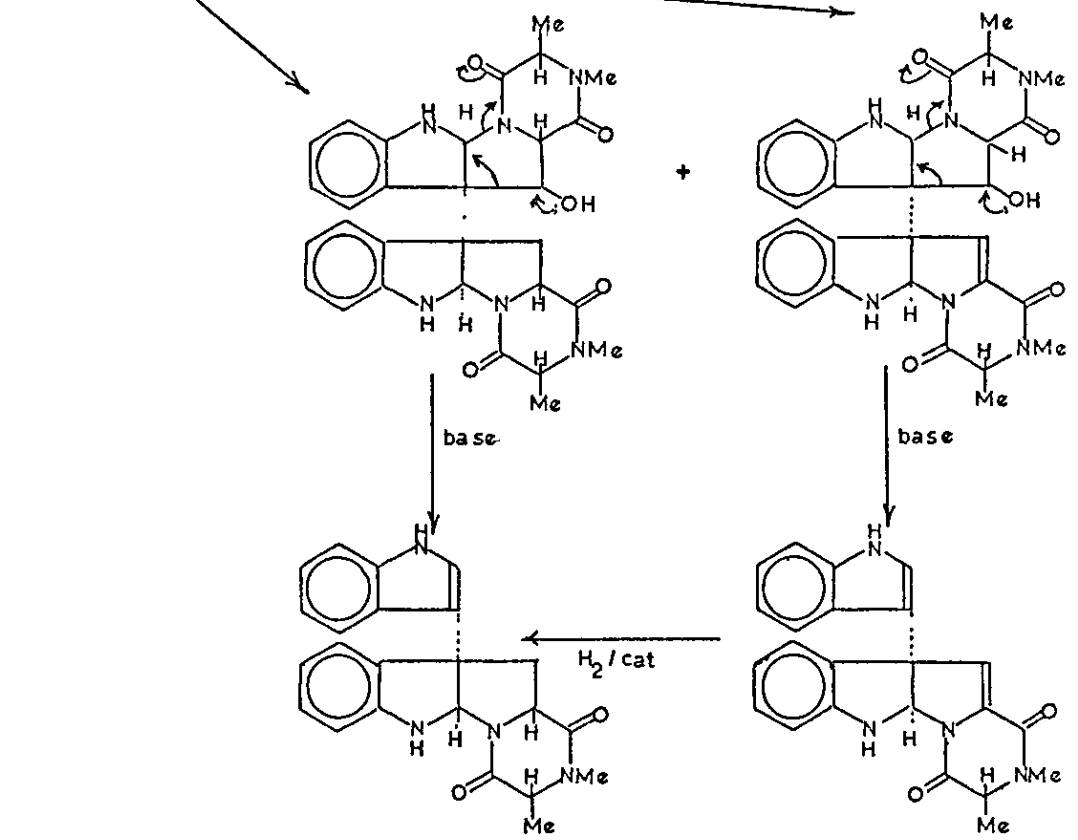
Recent investigation of antibiotic fungal extracts has led to the discovery of three new dithiadiketopiperazine metabolites. From Chaetomium minutum has been isolated the bridged diindole compound chaetocin (40) (R=H)[71]. The gross structure has been elucidated by spectroscopic studies. Treatment with pyridine and acetic anhydride gave the diacetate (40) (R=Ac) which yields (41) on desulphurisation with aluminium amalgam. Compounds analogous to (41) occur in the gliotoxin and sporidesmin series. By means of X-ray diffraction the absolute configuration (40) has been assigned. Configuration about the disulphide bridge is S,S whereas all such metabolites previously described had the R,R stereochemistry.

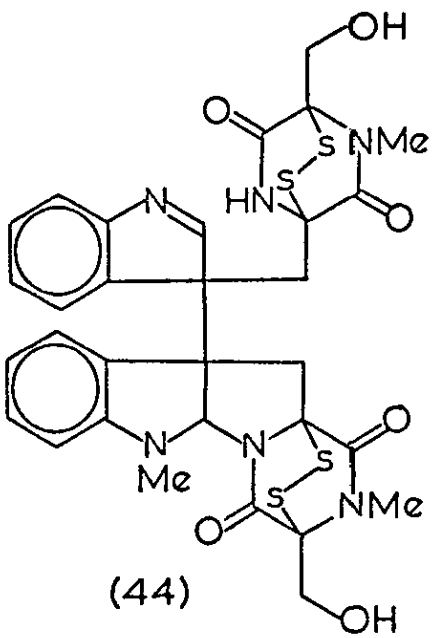
Circular dichroism maxima occur at the same wavelengths as those of gliotoxin, sporidesmin B and acetylaranotin. The extinction coefficients are comparable in magnitude, but in the opposite sense in the two series. DL-[6-^{3}H,3'-^{14}C]-Tryptophan was incorporated (2%) into chaetocin; the ^{3}H:^{14}C ratio altered from 9.2:1 in the precursor to 11.4:1 in purified diacetylchaetocin (40) (R=Ac).

A similar metabolite, verticillin A, (42) (R=H) has been obtained from Verticillium mould, strain No. TM-759[72]. The acetyl and benzoyl derivatives (42) (R=Ac, Bz) have been reported. Desulphurisation with aluminium amalgam gives the compound (43) which readily breaks down on treatment with base to 3,3'-biindolyl and 3-formyl-1,6-dimethylpiperazine-2,5-dione (Scheme IX). Treatment of acetylverticillin A (42), (R=Ac) with aluminium amalgam followed by base gave the sequence shown in Scheme X. From these reactions it was established that verticillin A has a symmetrical bis-indolyl type structure and hence that it is an isomer of chaetocin. The configuration of the disulphide bridge was established as S,S due to the similarity of circular dichroism curves of chaetocin and verticillin A. Assignment of configuration to the hydroxyl groups was based on i.r. studies of hydrogen bonding, and on the Cotton effect shown by the benzoyl derivative (42), (R=Bz).

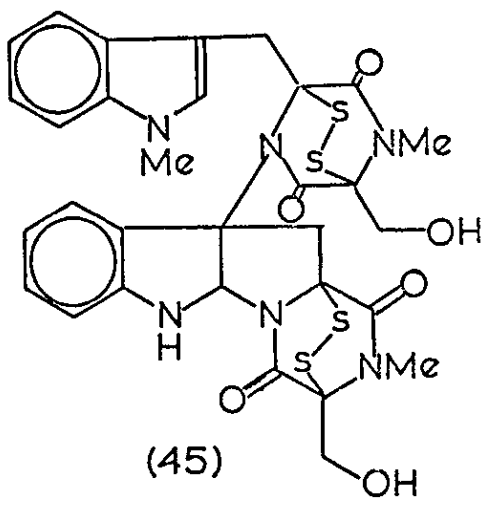
An antibiotic extract was obtained from the organism Chaetomium cochliodes as long ago as 1949[73]. On the basis of its sulphur content and high lability, Taylor[8] suggested it might be an epidithiadiketopiperazine derivative, and in 1967 demonstrated a correlation between bacterial resistance to gliotoxin, sporidesmin and chaetomin, as the Chaetomium metabolite came to be called[74]. Recently interest in chaetomin has revived as a result of a possible correlation between meadow-fungus and ruminant disease (cf. sporidesmin) and publication of the structures of chaetocin and verticillin A has prompted Safe and Taylor[75] to assign the

Scheme X





(44)



(45)

Chaetomin?

structure (44), or (45) to chaetomin. They report a chaetomin diacetate, an N-methylchaetomin diacetate and a chaetomin bistrimethylsilyl ether, prepared by treatment with, respectively acetic anhydride, dimethylsulphate and trimethylsilyl chloride in pyridine. Chaetomin loses two atoms of sulphur per molecule on treatment with triphenylphosphine. Methanolic methyliodide in the presence of pyridine and sodium borohydride gives a tetra-S-methyl compound from which a formyl group has been lost. Treatment of chaetomin diacetate under the same conditions gives a tetra-S-methyl compound from which acetoxymethanol has apparently been lost. Prolonged heating with acetic acid and acetic anhydride gave a compound described as anhydrodesthiochaetomin. Spectral data are given, but no structures assigned for these derivatives. Assignment of the structure of the parent compound is very largely based on spectral properties. The circular dichroism spectrum is closely similar to that of chaetocin, indicating the S,S configuration (opposite to gliotoxin) about the disulphide bridge.

Section A

Biosynthesis of the Tetrahydroindole Skeleton of Gliotoxin

A.1 Isolation and Chemistry of Gliotoxin

Before feeding experiments with the organism Trichoderma viride were carried out it was cultured on Johnson's medium[2] by the technique described by Suhadolnik[18] in the absence of radio-labelled precursors. Gliotoxin was obtained by chloroform extraction of the medium after 5 days. It was easily obtained crystalline by washing the residue from the chloroform extract with a little methanol. Recrystallisation from methanol gave material in which no impurities are detectable by t.l.c. (benzene 2, acetone 1 ; silica) or by n.m.r. This product was generally still greenish in colour. A further recrystallisation yielded almost white crystals. No improvement in melting point was noted after the second recrystallisation. However it was alarming that the properties of this material did not quite agree with any of those published by Johnson[2]. The melting range was 180-195°C. with decomposition whereas Johnson[2] reports a "decomposition point" of 221°C. The specific rotation was only -255° whereas Johnson[2] reports $[\alpha]_D^{22}$ -270°±10. An ultra-violet spectrum had the outline reported by Johnson, but the λ_{max} was at 263 nm with a shoulder at about 250 nm; Johnson reported 2700Å.[2]

An i.r. spectrum (fig. 1) showed a broad band about 3,400 cm^{-1} with a shoulder at 3,200 cm^{-1}, indicating a very strongly hydrogen-bonded hydroxyl group, together with a less strongly H-bonded group. The presence of small sharp peaks between 3050 cm^{-1} and 2800 cm^{-1} indicated the stretching of olefinic, aliphatic and N-methyl C-H bonds. Carbonyl stretching absorption occurred as a single sharp

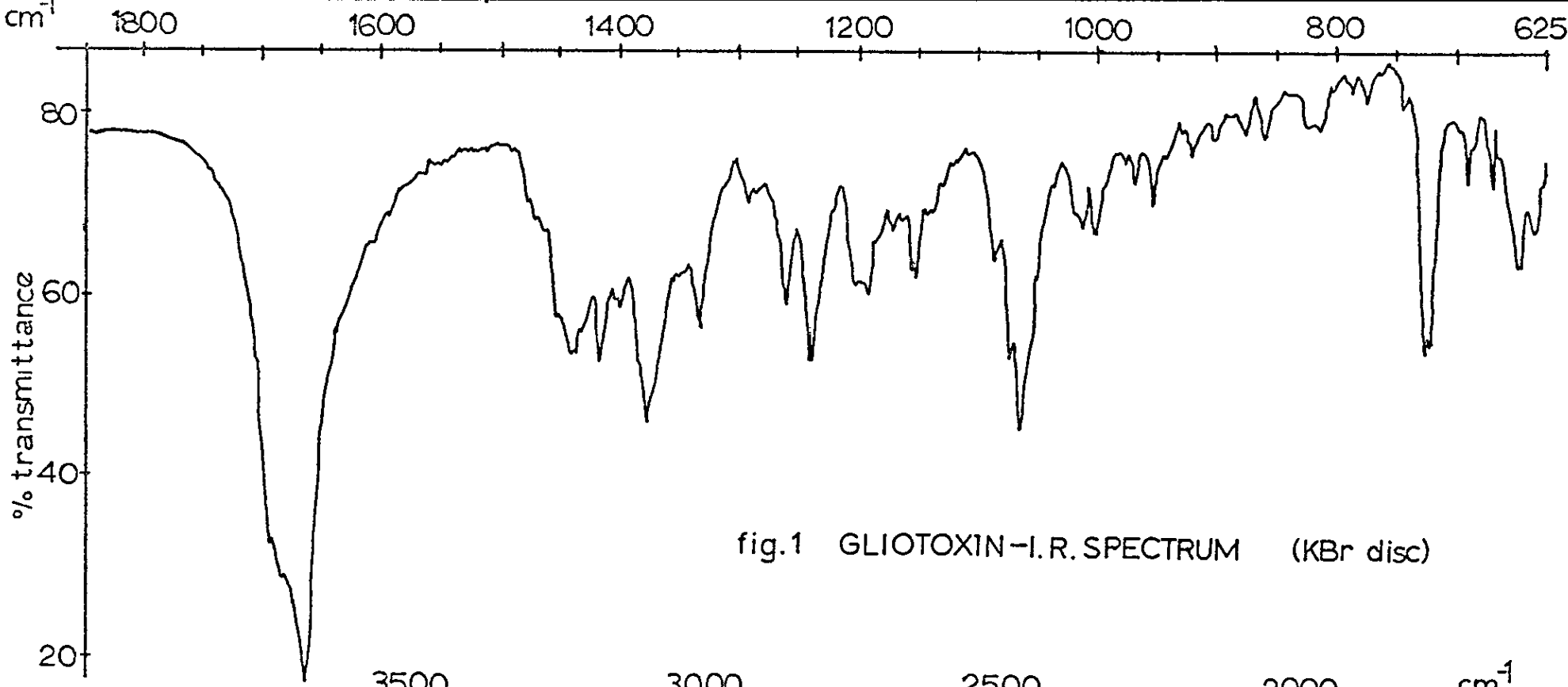
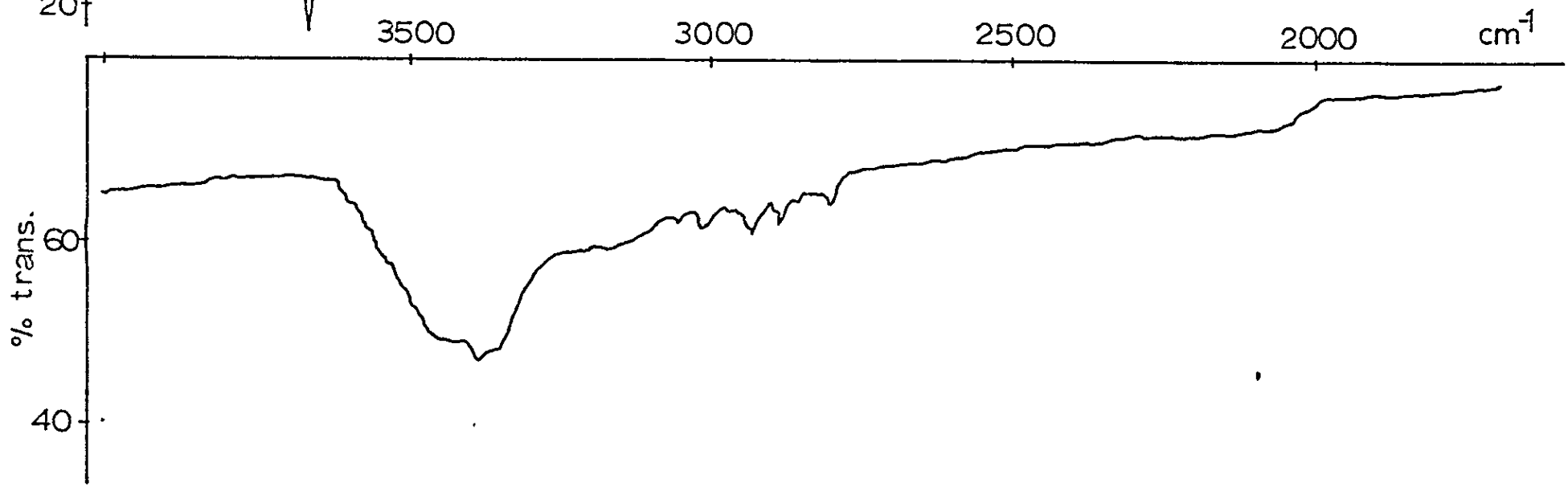
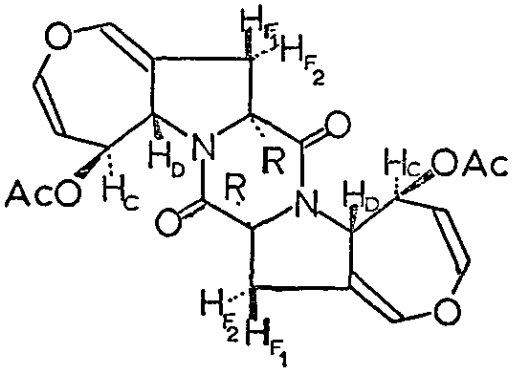


fig.1 GLIOTOXIN – I.R. SPECTRUM (KBr disc)



band at 1665 cm^{-1}, which was expected for a tertiary amide in a 6-membered ring.

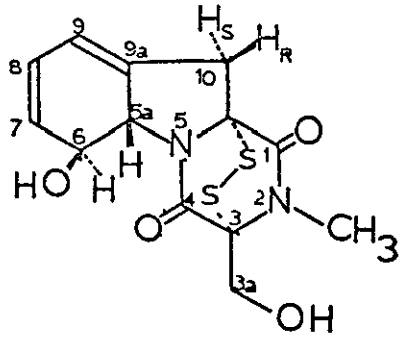
The n.m.r. spectrum of gliotoxin, (figs. 2 and 3) showed a 4-proton multiplet τ4·0-4·4, the integral of which became 3 on addition of D_2O. The protons at positions 5a and 6 resonated together at τ5·2. An AB quartet centred at τ5.68 accounted for the prochiral methylene group at position 3a. Addition of D_2O sharpened this signal considerably. There was another perturbed doublet of doublets at τ6·28 and τ7·08 which was attributed to the methylene group at C-10. Assignment of these protons is somewhat controversial. Nagarajan et al.[65] have assigned H_R to the upfield signal and H_S to the downfield proton in aranotin following the observation that the lower signal moved upfield upon desulphurisation with Raney nickel. The actual changes in chemical shift reported[20] are as follows.



	R,R=S-S	R=H	Change
	τ	τ	Hz
H_{F-1}	7·30	7·205	- 9·5
H_{F-2}	5·88	7·025	+114·5
H_D	4·91	4.80	- 11
H_C	4·17	4·44	+ 27
H=R	-	5·64	-

fig.2 – GLIOTOXIN (1)

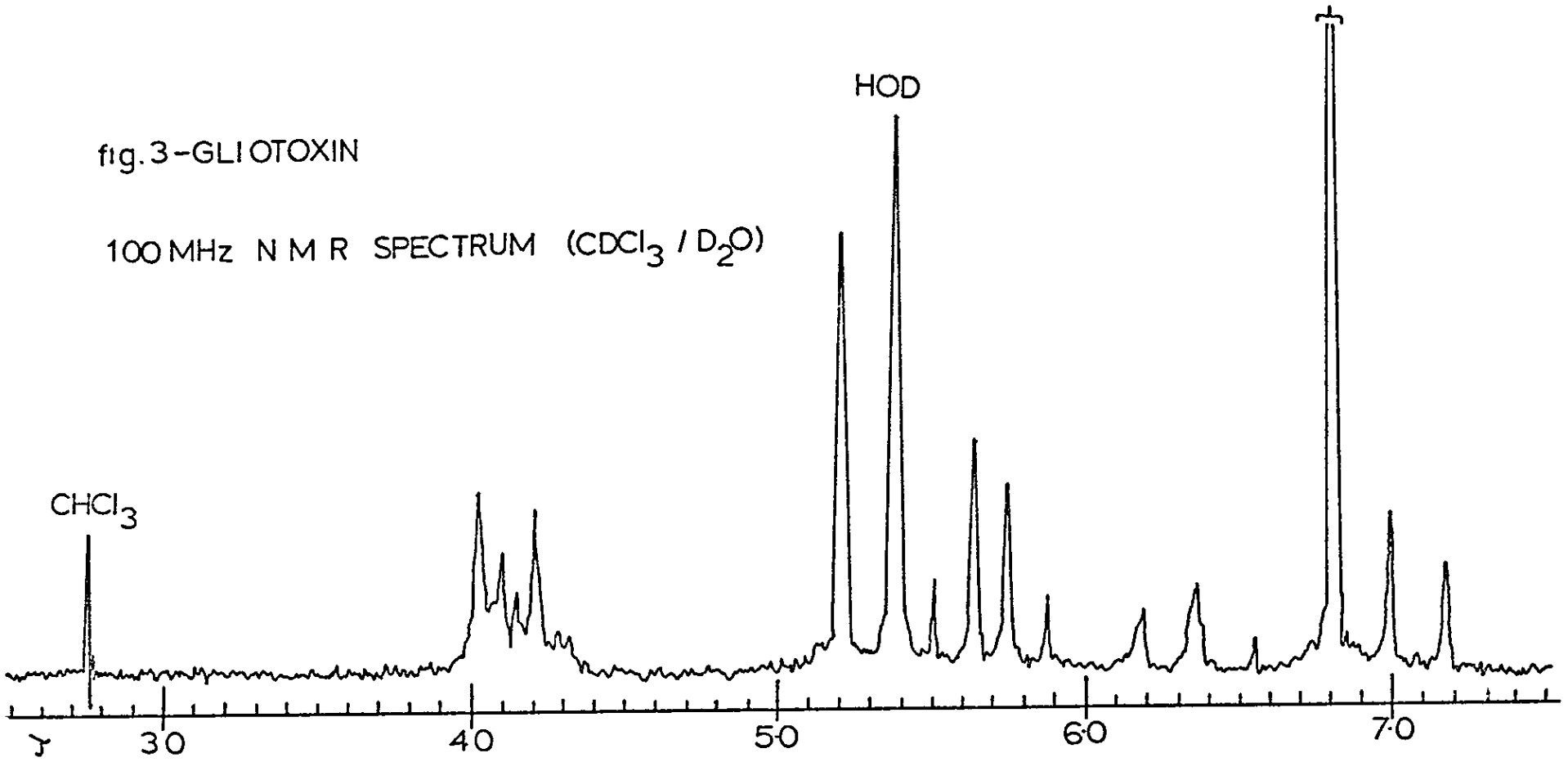
100MHz. N.M.R. SPECTRUM ($CDCl_3$)



(1)

fig. 3 - GLIOTOXIN

100 MHz N M R SPECTRUM ($CDCl_3$ / D_2O)

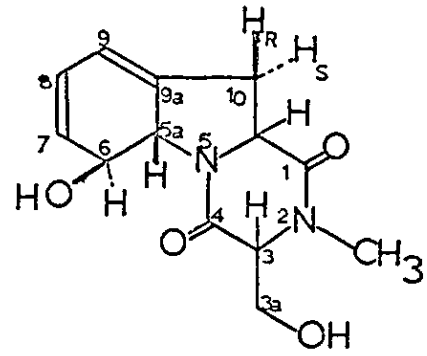


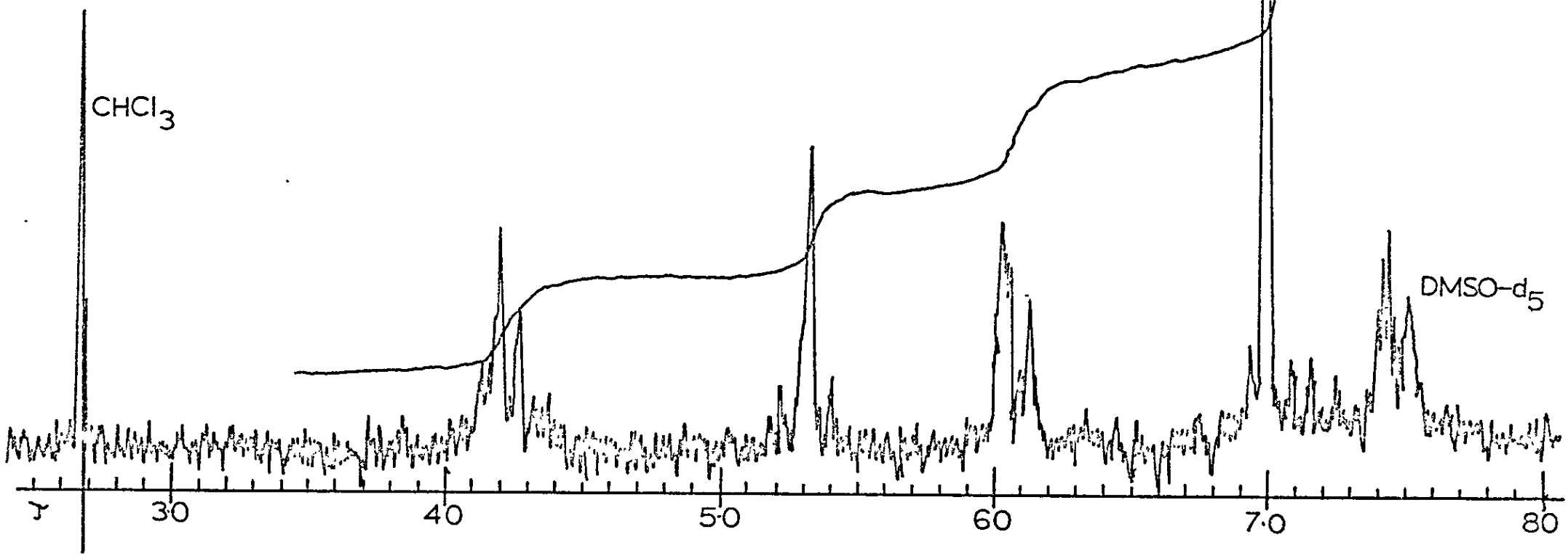
Since protons located on the same side of the molecule as the disulphide bridge move upfield on desulphurisation, while those on the opposite side move downfield, Nagarajan, Neuss & Marsh[20] have postulated that the d-orbitals of sulphur are responsible for shifting the H_{F_2} signal downfield. On the basis of this argument Bu'Lock[78] has assigned the downfield signal to the (S)-proton at position 10 in gliotoxin, and used the assignment to draw conclusions about the stereochemistry of deuterium label incorporated at this position. However, the author's own results (see Section B) conflict with Bu'Lock's argument, and it has become necessary to reassign the (10R)- and (10S)-protons of gliotoxin. From a model it can be seen that the (10R)-proton is well situated for deshielding by the carbonyl group of position-1; the same is true in aranotin.' Removal of the disulphide bridge releases the diketopiperazine from a rather strained boat form and it becomes more planar. The (10R)-proton is then twisted out of the proximity of the deshielding carbonyl group. Slight coupling of the downfield doublet is probably due to allylic coupling with the C-9 proton. In gliotoxin the (10R)-proton is in a plane at right angles to that of the 9-9a unsaturation, and hence is ideally situated for allylic coupling with H-9. It is also possible to explain the changes in shift of H_C, H_D and H_{F_2} reported by Nagarajan *et al.*[20] in terms of motion into or out of the fringe of carbonyl deshielding, but these changes are so small relative to that of H_{F_1}, that a detailed explanation is unnecessary.

An n.m.r. spectrum of desthiodihydro-gliotoxin (46),

fig.4 - DESTHIODIHYDROGLIOTOXIN (46)

100 MHz. N.M.R SPECTRUM ($CDCl_3$/DMSO-d_6/D_2O)



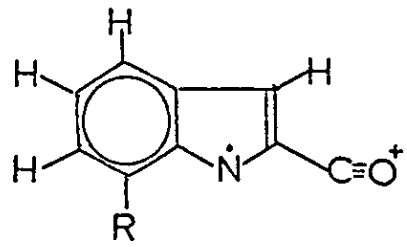


produced by the action of amalgamated aluminium on gliotoxin is presented (fig. 4). Although the region around τ7 is obscured somewhat by the N-methyl group, an AB quartet is visible, centred at τ7·12. If it is taken to be a doublet of overlapping doublets (J=19Hz) then these latter are situated at τ7·08 and τ7·18. A full assignment is given in the experimental section.

The mass spectrum obtained for gliotoxin is also shown (fig. 5). Loss of sulphur is a typical fragmentation for bridged compounds of this type[30,65,71]. Since the molecular ion is in such low abundance, no pattern due to sulphur isotopes was observed. Ions derived from 7-hydroxyindole e.g. (47) at m/e 159 and from indole itself e.g. (48) at m/e 143 indicate derivation from a structure which can aromatise either by loss of hydrogen or by loss of water.

Dehydrogenation of gliotoxin with o-chloranil by the procedure of Lowe, Taylor and Vining[7] gave a good yield of dehydrogliotoxin. The n.m.r. spectrum of this compound is shown (fig. 6). It is closely similar to that of gliotoxin except that aromatic signals about τ3·0 have replaced the olefin multiplet of the gliotoxin spectrum. Since position 10 is now benzylic, the doublet of doublets due to that methylene group has moved downfield and the lower-field doublet overlaps the AB quartet due to the 3a methylene protons.

The mass spectrum of dehydrogliotoxin contains only ions which may be assigned 6-hydroxy indole structures. No derivatives of indole itself are observed. As with gliotoxin the base peak is $(M-S_2)^+$, in this case at m/e 260.

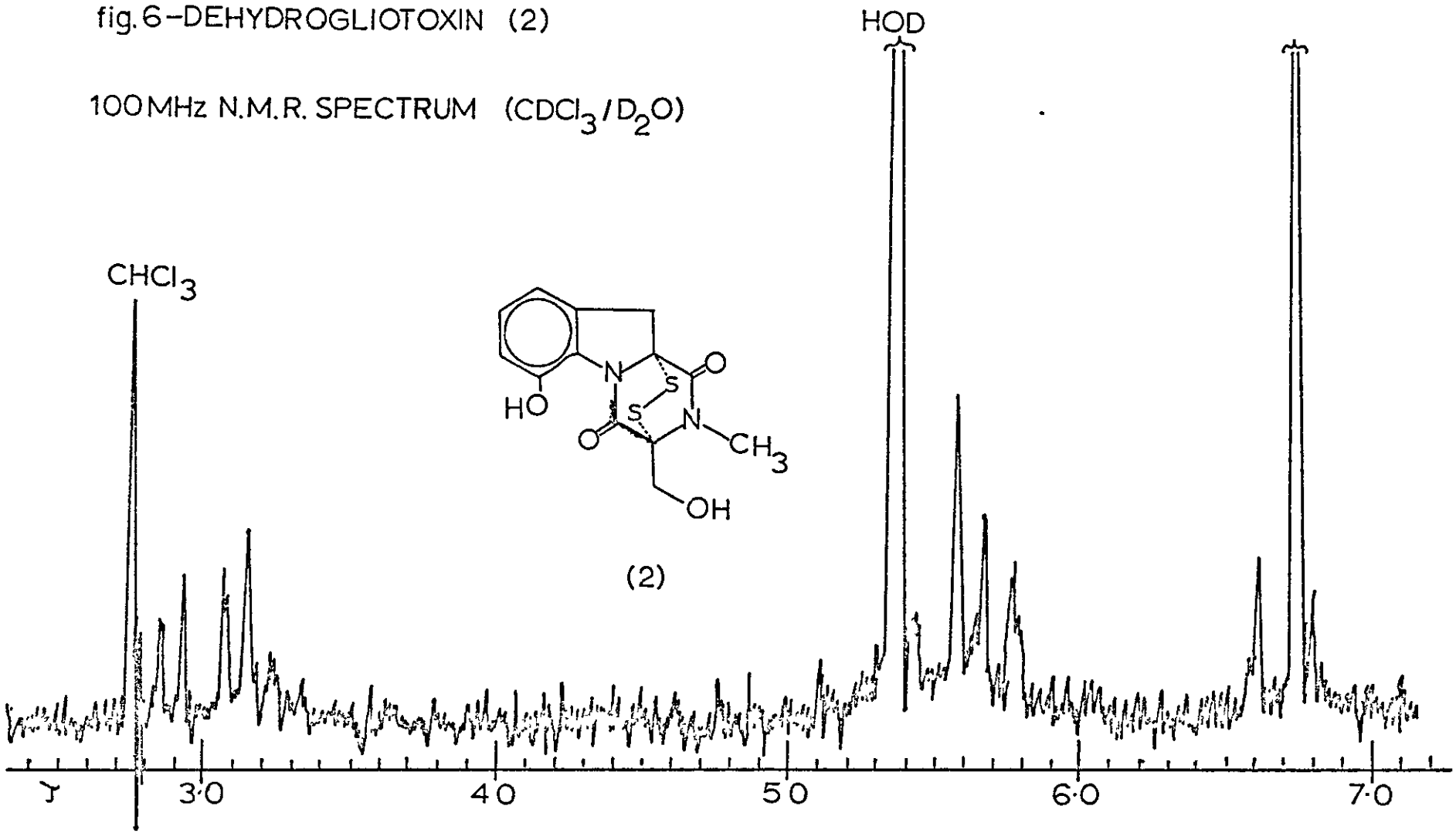


(47) R = Ȯ m/e 159
(48) R = · m/e 143

fig. 5 – GLIOTOXIN, MASS SPECTRUM

fig. 6 – DEHYDROGLIOTOXIN (2)

100 MHz N.M.R. SPECTRUM ($CDCl_3$ / D_2O)



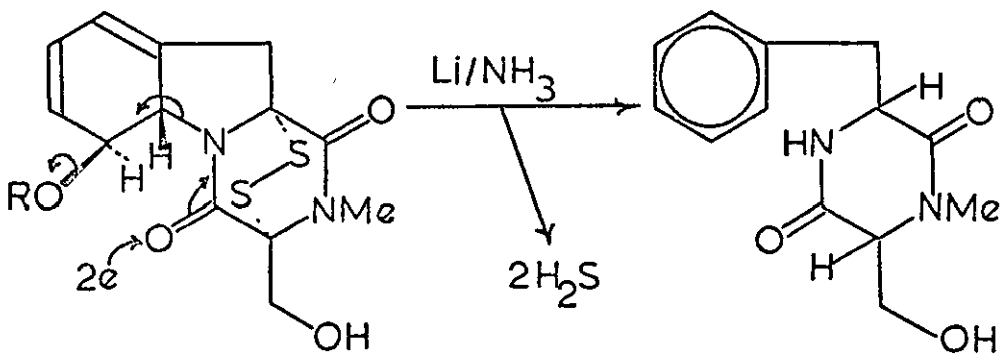
(2)

The infra-red spectrum is also similar to that of gliotoxin except that it has a single sharp peak at 3,500 cm^{-1} instead of the broad band at 3,400 cm^{-1} in the gliotoxin spectrum. On treating a solution with base *in situ* in the u.v. spectrometer cell, a bathochromic shift was observed in the spectrum. Thus the compound is phenolic.

Anhydrodesthiogliotoxin (34) was prepared by treating with gliotoxin with grade II neutral alumina[12] in benzene. Since the compound has only been tenuously described in the literature[11], it was characterised by microanalysis as well as by spectroscopy, and was found to have the structure assigned by Bose *et al*.[12]

As a result of one of the biosynthetic problems encountered, it became desirable to assay the stereospecificity of tritium incorporation at position 10 of gliotoxin. In theory this could be achieved by converting gliotoxin into a peptide of phenylalanine by cleavage with lithium in liquid ammonia[99], (Scheme XI R=H) but in practice no aromatic product was isolated. An *OO*-diacyl derivative was therefore sought, since an acyloxy group is a better leaving group and might facilitate cleavage (Scheme XI, R=acyl). Although dibenzoyl gliotoxin has been reported[79,15] no attempt was made to prepare this material because these compounds had not been well characterised and because a tribenzoate[79] had also been claimed. Acetylation therefore, with acetic anhydride or with acetyl chloride in the presence of pyridine, gave the same product (by t.l.c; benzene; silica). This material would not crystallise but was extensively purified and characterised as the 3a-*O*-monoacetyl derivative of

Scheme XI

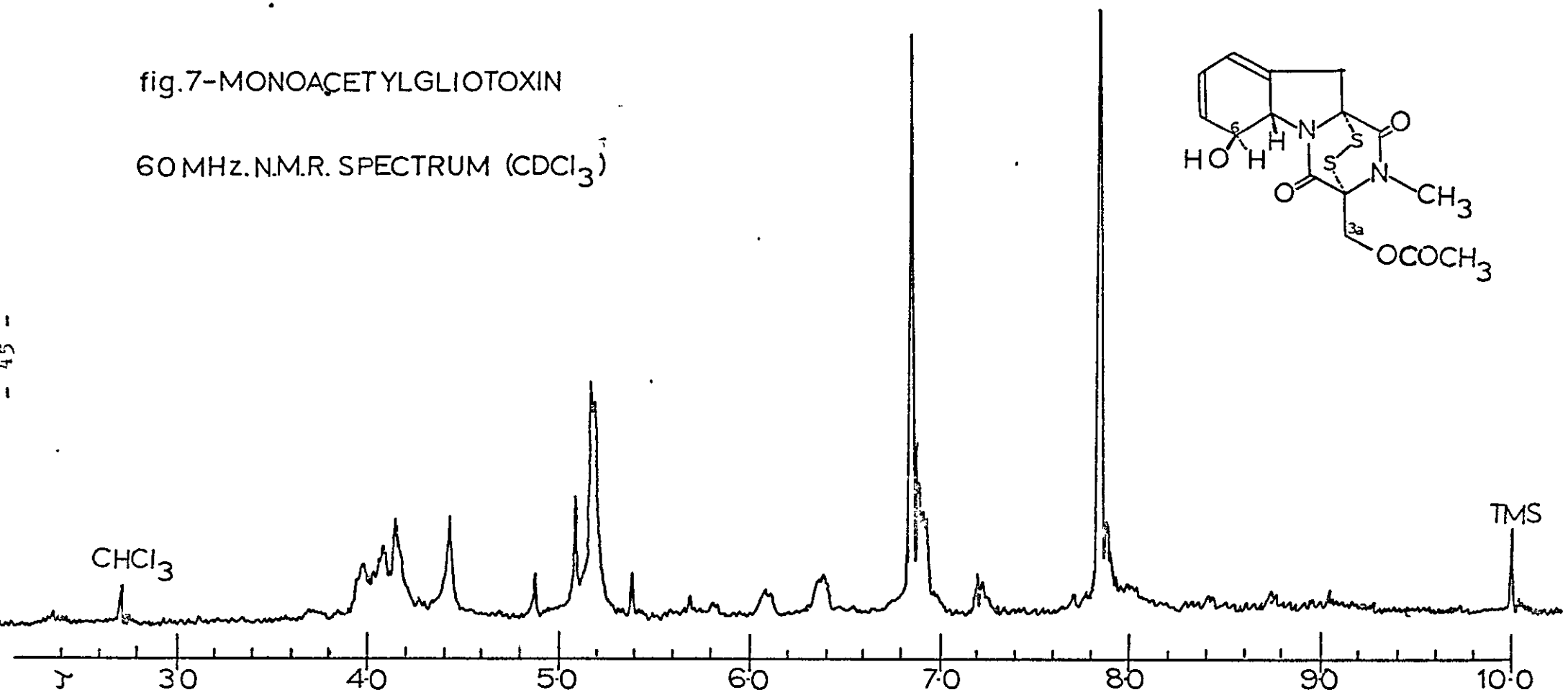


gliotoxin. The n.m.r. spectrum (fig. 7) was identical to that of gliotoxin except for the singlet at τ7·86 due to an acetyl group, and the fact that the AB quartet due to the 3a methylene protons was centred at τ5·15 instead of τ5.68 as in gliotoxin. This was clearly due to deshielding by an acetyl group. Interestingly, the proton from the 6-hydroxy group was visible in this spectrum as a sharp peak at τ4·30, which disappeared on addition of D_2O to the sample.

No matter what acetylation conditions were used, this monoacetyl gliotoxin was the only product isolated. The approach was eventually abandoned since both gliotoxin and time were in short supply.

fig.7-MONOACETYLGLIOTOXIN

60 MHz. N.M.R. SPECTRUM ($CDCl_3$)



A.2 Labelling and Feeding of m-Tyrosine

The ring protons of phenolic amino-acids are known to be readily exchanged on heating with deuteriated or tritiated acid[80]. In general only the protons ortho and para to the phenolic hydroxyl are exchanged, but it was not certain in the case of m-tyrosine that the presence of two activating groups in the ring would not enable exchange of the proton meta to the hydroxyl group as well. m-Tyrosine was therefore heated with 5N-deuterium chloride in a sealed n.m.r. tube, and the spectrum run at intervals. Changes in the integrals of the aromatic signals at τ2·67 and 3·09 were as tabulated below.

Table 1

Time	o min.	5 min.	10 min.	15 min.	4 hr.	16 hr.
τ 2·67	1·08(t)	0.96(t)	1·15(s)	1·06(s)	1·15(s)	0·86(s)
τ 3·09	2·96(d)	1·15(d)	0·75(d)	0·49(-)	0·51(-)	0·49(-)

The aromatic portions of the spectra, with assignments, are shown (fig. 8).

Since three of the ring protons had been exchanged it seemed reasonable to assume that these were at the positions ortho and para to the hydroxyl group (fig. 8-H_B) and that the remaining proton was located at the meta position (H_A). Exchange treatment of m-tyrosine in 5N-tritiated hydrochloric acid thus afforded DL-[2,4,6-$^3H_3$]-m-tyrosine of high specific activity.

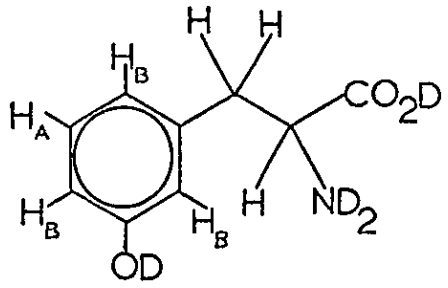
In order to conclusively test Suhadolnik's result[57] that m-tyrosine is a better precursor of gliotoxin than phenylalanine, the above tritiated m-tyrosine was mixed with DL-[1'-^{14}C]phenylalanine (from a commercial source),

fig 8–m-TYROSINE 60MHz N.M.R. SPECTRA

AROMATIC PROTON SIGNALS

AFTER.

	hours.	min.
a.	0	0
b.	0	5
c.	0	10
d.	0	15
e.	4	0
f.	16	0



a known precursor of gliotoxin[18,12] and fed to cultures of Trichoderma viride (strain NRRL 1828). Culture technique and work-up of the gliotoxin were exactly as described by Suhadolnik[18]. Three feedings were carried out. In the first, m-tyrosine labelled by exchange, and high-activity DL-[1'-^{14}C]phenylalanine from a commercial source was used, as described. In the second feeding, unlabelled phenylalanine was added to the precursor to make the quantities fed equimolar. Finally DL-[2,4,6-$^{3}H_3$]-m-tyrosine alone was fed, as a control experiment. Results of these three feedings are shown in table 2, below.

Table 2

Precursor	Wt.(mg)	^{3}H:^{14}C	gliotoxin Wt.(mg)	% incorp.	^{3}H:^{14}C
DL-[2,4,6-$^{3}H_3$]-m-Tyrosine DL-[1'-^{14}C]-Phenylalanine	20 0·07	11·5	233	4·9(^{14}C)	9x10^{-3}
DL-[2,4,6-$^{3}H_3$]-m-Tyrosine DL-[1'-^{14}C]-Phenylalanine	9.6 9·3	11·9	159	1·6(^{14}C)	0·13
DL-[2,4,6-$^{3}H_3$]-m-Tyrosine	8·6		215	9·5x10^{-3} (^{3}H)	

The variability in these gliotoxin yields is attributable to the fact that on hot days it was impossible to keep the incubation temperature below 30°C.[18,6]

From the above results it appeared unlikely that m-tyrosine is a better precursor of gliotoxin than is

phenylalanine. However the possibility remained that label might be exchanged out of DL-[2,4,6-$^{3}H_3$]-m-tyrosine in the culture medium before biosynthesis could occur. However, when a mixture of this tritiated precursor and DL-[1'-^{14}C]phenylalanine was maintained under sterile conditions at pH3 and 30°C. no change in ^{3}H:^{14}C ratio occurred. Decarboxylation of phenylalanine does not occur under these conditions.

Another possibility might have been that the commercial m-tyrosine was in fact some other isomer, e.g. o- or p-tyrosine. To test this, material from the same batch as was taken for exchange labelling was degraded to the corresponding methoxycinnamic acid by exhaustive methylation followed by a Hofmann elimination in hot 2N-sodium hydroxide. This methoxycinnamic acid was compared with p-methoxycinnamic acid (prepared by Hofmann elimination of authentic L-tyrosine) and with o-methoxycinnamic acid (prepared by Perkin condensation of O-methylsalicylaldehyde and malonic acid in the presence of pyridine). N.m.r. spectra and melting points were taken into account. The properties of the derivative from the m-tyrosine were in good agreement with expected and published results, and were different from the properties of either of the other two isomers. This was taken as conclusive proof that the commercial material was genuine m-tyrosine. It should be pointed out that "alkyl" isomers of m-tyrosine such as 3-amino-3-(m-hydroxyphenyl)-propionic acid were excluded on the basis of the alkyl portion of the n.m.r. spectrum of m-tyrosine, which closely resembled that of phenylalanine[81]. The conclusion drawn from this preliminary

work, therefore, was that Suhadolnik's result for m-tyrosine as a precursor of gliotoxin was in error, and it seemed worthwhile to study in detail the biosynthesis of the tetrahydroindole skeleton.

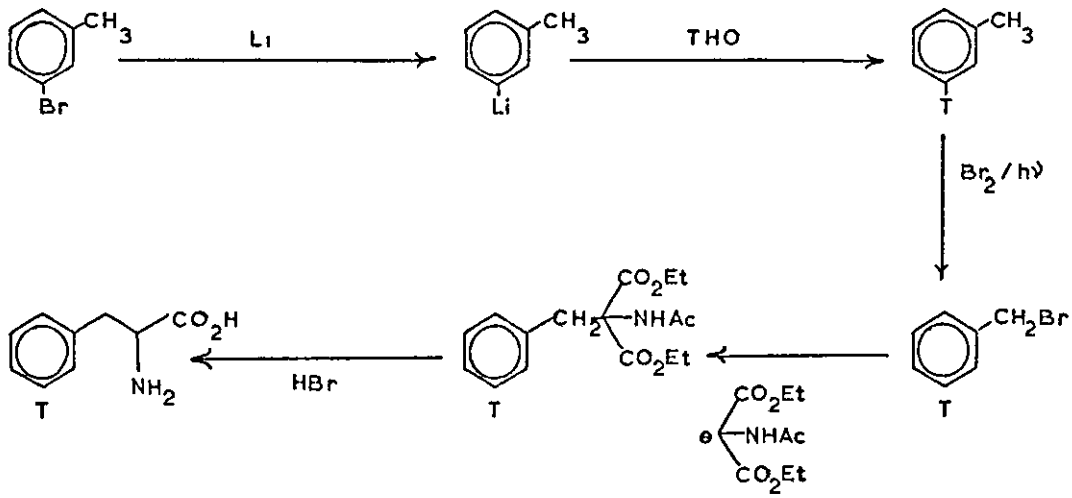
A.3 Preparation and Feeding of Ring-Labelled Phenylalanines

In order to observe any loss or migration of the ring hydrogens, phenylalanines labelled at the 2, 3 and 4 positions of the benzene ring were fed to T. viride cultures and the derived gliotoxins degraded to establish the position of the label.

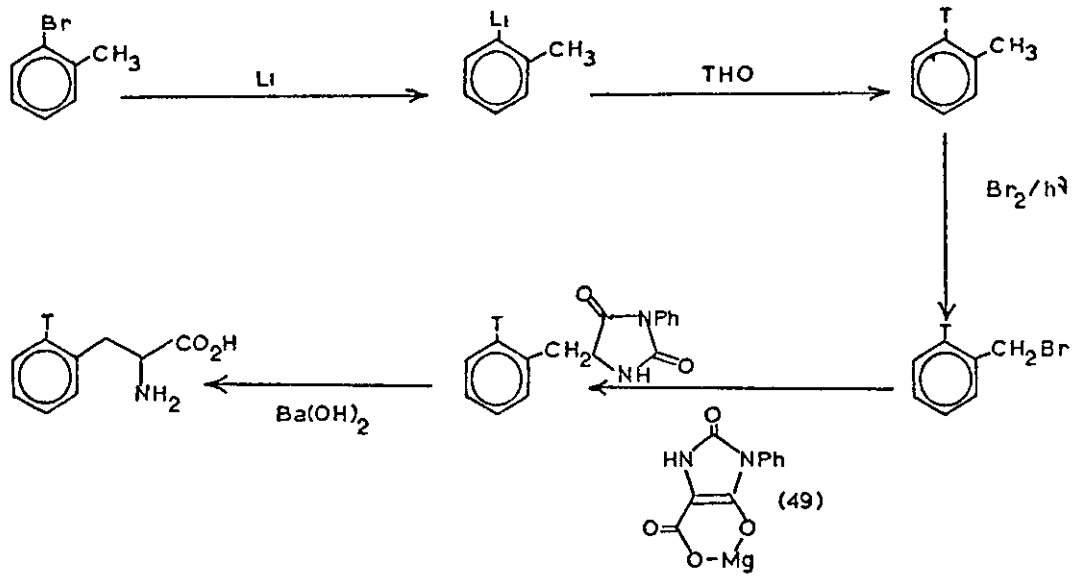
DL-[3-^{3}H]Phenylalanine was the first ring-labelled precursor prepared, the route being that shown in Scheme XIIa. The individual steps are all well established reactions and similar synthetic sequences have been used previously for the preparation of precursors by members of Professor G.W. Kirby's research group[82,83]. The starting material for synthesis of DL-[3-^{3}H]phenylalanine was m-bromotoluene. From this was prepared 3-methylphenyllithium by direct reaction with lithium metal in ether. Treatment of the ethereal solution of aryllithium with excess of tritiated water gave [3-^{3}H]toluene. When the 3-methylphenyllithium was treated with deuterium oxide, comparison of the integrals of the two singlets in the n.m.r. spectrum of the product indicated loss of 0·7 proton from the phenyl ring.

Treatment of the labelled toluene with bromine under strong irradiation with visible light gave crude benzyl bromide. Reaction of the latter with the carbanion derived from diethyl 2-acetamidomalonate, yielded diethyl 2-[3-^{3}H]benzyl-2-acetamidomalonate in rather poor yield. This compound was carefully purified, and converted into DL-[3-^{3}H]phenylalanine by treatment with refluxing mineral acid. The author did not degrade the precursor to establish the position of the label; instead the position

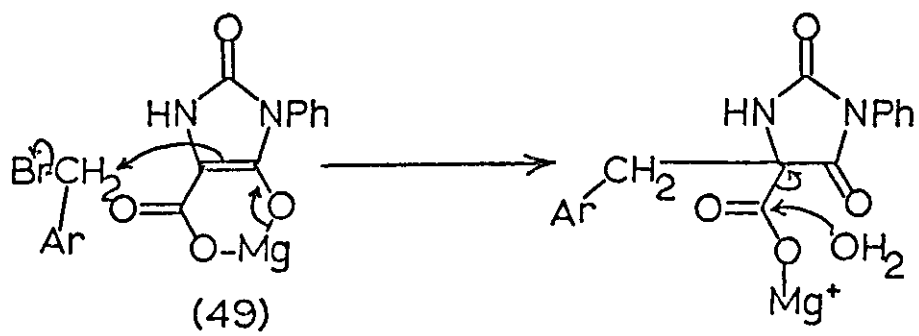
Scheme XIIa - DL-[3-^{3}H]Phenylalanine



Scheme XIIb - DL-[2-^{3}H]Phenylalanine



Scheme XIIc



of the label in the derived gliotoxin was established by degradation. BDH Chemicals Ltd., the supplier of the m-bromotoluene was contacted and claimed that material of the batch in question contained not less than 99.5% of the m-isomer, as shown by gas/liquid chromatography. Another worker has since found by degradation that not less than 99% of the tritium in this sample of precursor is meta to the alaninyl group, i.e. in the 3 or 3,5 positions[84].

Synthesis of DL-[2-^{3}H]phenylalanine was carried out by the sequence shown in Scheme XIIb. Metallation tritiation and bromination were carried out as in the [3-^{3}H]benzyl bromide reaction sequence. The [2-^{3}H]benzyl bromide was then distilled, and treated with a solution of compound (49) in dimethylformamide[85]. Work-up yielded 5-[2-^{3}H]benzyl-3-phenylhydantoin, which was recrystallised and hydrolysed in saturated aqueous barium hydroxide/dioxan[86] in a sealed tube at 165°C. Solutions of (49) are prepared in situ by dissolving 3-phenylhydantoin in a solution of magnesium methoxycarbonate, $CH_3O\ Mg\ O.(CO).OCH_3$. The latter is obtained by passing carbon dioxide through magnesium methoxide dissolved in dimethylformamide[87]. The mechanism of the reaction is shown in Scheme XIIc[85]. Its facility apparently depends on a.) the ability of magnesium to form a cation and b.) the relative stability of carbanions at the hydantoin 5-position[88].

No degradation was employed to locate the label in DL-[2-^{3}H]phenylalanine, instead the derived gliotoxin was degraded. Commercial o-bromotoluene from the same source as the m-bromotoluene was the starting material. The

manufacturer again claimed 99% purity and the gliotoxin degradations bear this claim out.

L-[4-^{3}H]Phenylalanine was obtained from a commercial source, and also used for feeding experiments. It was not degraded to locate the label, but again degradations carried out on the derived gliotoxin have demonstrated that it is predominantly the [4-^{3}H]-compound.

DL-[2-^{3}H]Phenylalanine and DL-[3-^{3}H]phenylalanine were mixed with commercial DL-[1'-^{14}C]phenylalanine and L-[4-^{3}H]phenylalanine was mixed with commercial L-[1'-^{14}C]-phenylalanine. T. viride was cultured in the presence of each doubly-labelled precursor; the results are shown in table 3, below.

Table 3

Precursor Phenylalanine	Wt.(mg)	^{3}H:^{14}C	Gliotoxin Wt.(mg)	% incorp. ^{14}C	^{3}H:^{14}C
DL-[1'-^{14}C,3-^{3}H]-	13	7·1	189	3·3	7·6
DL-[1'-^{14}C,3-^{3}H]-	17	6·5	215	2·3	6·7
DL-[1'-^{14}C,2-^{3}H]-	13	38·2	277	7·5	36·2
L-[1'-^{14}C,4-^{3}H]-	ca.10^{-2}	4·7	333	7·5	4·9

This work demonstrated that no loss of tritium relative to carbon-14 was occurring during the biosynthesis. No loss of carbon-14 from the 1'-position was expected[18,12], so complete retention of tritium was apparently occurring. Further investigation of the mechanism of biosynthesis of the tetrahydroindole skeleton is only possible if the position of the label in the metabolite can be unambiguously shown by degradation.

A.4. Degradation of Gliotoxin.

Two degradative procedures only were used in this stage of the work, each being chosen to remove a certain atom of hydrogen (tritium) from the gliotoxin molecule. Gross breakdown of the molecular structure and drastic conditions were avoided in order to minimise possible loss of tritium by hydrogen exchange or by scrambling throughout the molecule. Gliotoxin (1) should lose the hydrogen (tritium) atom at position 6 on conversion into dehydrogliotoxin (2), whereas this hydrogen will be retained on conversion into anhydrodesthiogliotoxin (34). The hydrogen at position 5a will be lost in both cases, whereas that at position 7 will be retained in both cases, provided the elimination of hydrogen and of water respectively, proceeds in a straightforward manner.

Because the biological system cannot detect asymmetry in the phenyl ring of DL-[2-^{3}H]- and DL-[3-^{3}H]phenylalanine the label in gliotoxin derived from these precursors should be evenly divided between positions 5a and 9 (in the case of DL-[2-^{3}H]phenylalanine) and positions 6 and 8 (in the case of DL-[3-^{3}H]phenylalanine). All the label in gliotoxin derived from L-[4-^{3}H]phenylalanine would be expected to be at the 7-position. Loss of label from the 5a or 6 position of gliotoxin, due to degradation should therefore be observed as loss of 50% tritium relative to ^{14}C; see Scheme XIII.

When the degradations shown in Scheme XIII were carried out on gliotoxins derived from the ring-labelled phenylalanines, the figures shown in table 4 were obtained.

Scheme XIII -(retention of T, %)

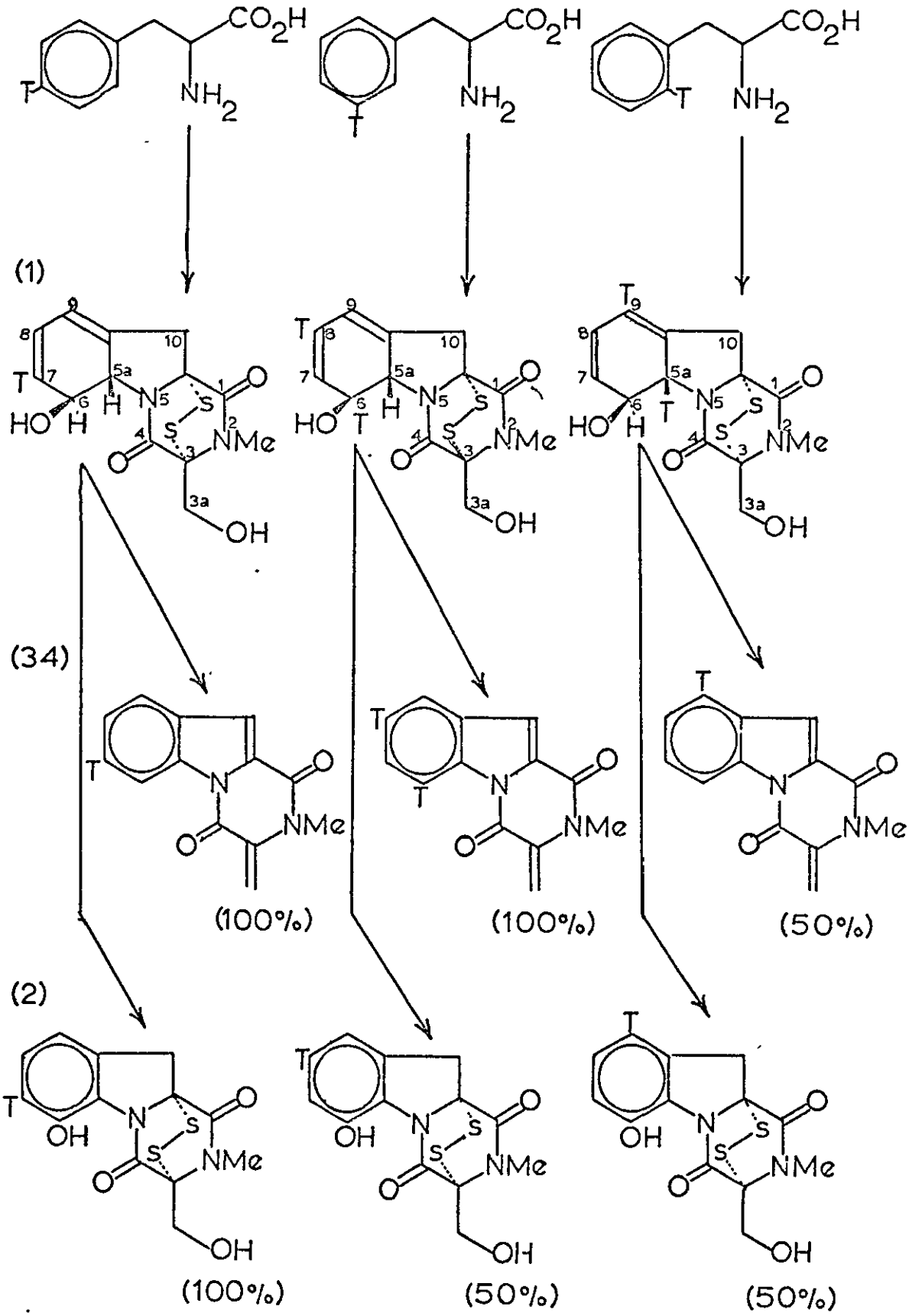


Table 4

Precursor Phenylalanine	$^{3}H:^{14}C$	Gliotoxin(1)		(34)		(2)	
		^{14}C*	$^{3}H:^{14}C$	^{14}C*	$^{3}H:^{14}C$	^{14}C*	$^{3}H:^{14}C$
DL-[1'-^{14}C,3-^{3}H]-	7·1	0·58	7·5	0·52	7·5	0·57	3·9
DL-[1'-^{14}C,3-^{3}H]-	6·5	0·41	6·7	0·39	6·8	0·40	3·6
DL-[1'-^{14}C,2-^{3}H]-	38·2	0·21	36·2	0·19	17·2	0·20	17·8
L-[1'-^{14}C,4-^{3}H]-	4·7	0·62	4·9	0·56	4·7	0·61	4·6

* ^{14}C specific activity: μCi/mmole

It can be seen that whereas the ^{14}C specific activity of the gliotoxins and their degradation products are approximately the same, the $^{3}H:^{14}C$ ratio in both (34) and (2) from the DL-[2-^{3}H]phenylalanine-derived gliotoxin and in the (2) from DL-[3-^{3}H]phenylalanine-derived gliotoxin has fallen by about 50%.

All the work on ring-tritiated phenylalanine feedings may be conveniently summarised in terms of %-retention of tritium relative to carbon-14, as is shown in table 5 below.

Table 5

Precursor Phenylalanine	$^{3}H:^{14}C$	Gliotoxin		(34)		(2)	
		$^{3}H:^{14}C$	%	$^{3}H:^{14}C$	%*	$^{3}H:^{14}C$	%*
DL-[1'-^{14}C,3-^{3}H]-	7·1	7·5	106	7·5	100	3·9	52
DL-[1'-^{14}C,3-^{3}H]-	6·5	6·7	103	6·8	101	3·6	54
DL-[1'-^{14}C,2-^{3}H]-	38·2	36·2	94	17·2	48	17·8	49
L-[1'-^{14}C,4-^{3}H]-	4·7	4·9	102	4·7	96	4·6	94

* Tritium incorporation relative to ^{14}C; based on gliotoxin rather than precursor.

Thus the degradations used apparently proceed by elimination of hydrogen or water in a straightforward manner. No migration or loss of ring-hydrogen occurs in the biosynthesis of gliotoxin from phenylalanine.

A.5. Discussion of Results.

The evidence detailed in table 2 indicates that m-tyrosine is probably not a direct precursor of gliotoxin. In the first competitive experiment phenylalanine is incorporated 1000 times more efficiently than is m-tyrosine. Even when stoichiometrically equivalent quantities of the two amino-acids are used, phenylalanine is still 100 times more readily incorporated. When no phenylalanine is present m-tyrosine incorporations are still comparably low.

The identity of the m-tyrosine fed cannot be questioned. Likewise the possibility that label has been lost by exchange in the acid medium has been excluded. Other workers have since established that labelled m-tyrosine is gradually removed from the medium by T. viride[89], and that label from m-tyrosine accumulates inside the cells of P. terlikowskii[90], showing that no problem of permeability exists. It is thus difficult to explain the results of Suhadolnik[57], who reports 40% incorporation of m-tyrosine as opposed to only 17% incorporation of phenylalanine. His incorporations are larger than those encountered in the above work because his feedings were carried out when gliotoxin production was maximal (3 days after inoculation) and because more active precursors were used. However 40% is higher than any of his other reported incorporations. Perhaps m-tyrosine was broken down to phenylalanine or some other precursor during the Wilzbach labelling process which was used.

Further evidence that m-tyrosine is not the precursor of gliotoxin is indirectly supplied by the observation that o-m- and p- ring-tritiated phenylalanines are incorporated

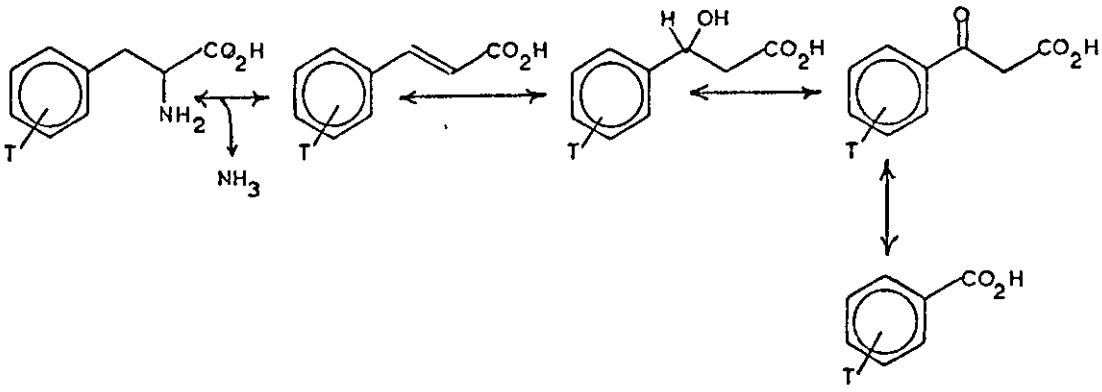
without loss or migration of the label. If m-tyrosine were formed by hydroxylation of phenylalanine prior to gliotoxin biosynthesis, the hydrogen at position 3 of the phenyl ring would be expected either to be lost, or to migrate to the adjacent position in the ring (N.I.H. Shift). No such loss or migration of this or the other ring hydrogens was detected, so m-tyrosine is fully excluded as an obligatory precursor of gliotoxin.

An explanation for the high tritium incorporations relative to carbon-14 in the DL-[3-^{3}H]- and [4-^{3}H]phenylalanine feedings (106, 103 and 102% respectively) may be that a small fraction of the precursor has undergone decarboxylation, by some process such as -oxidation to tritiated benzoic acid, of which a small proportion may have been reconverted to tritiated phenylalanine. The envisaged pathway is shown in Scheme XIV. In this way a small quantity of phenylalanine labelled with tritium but not with carbon-14 could arise. Breakdown of α,β-unsaturated fatty acids to acetate is a well documented process[91] as is the deamination of phenylalanine to pyruvate[92]. The reversibility of the processes is known.

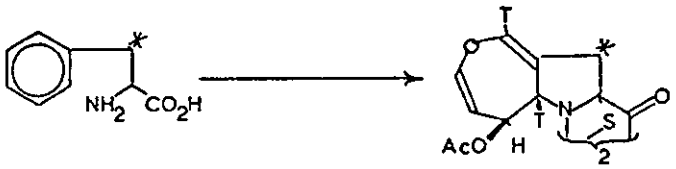
It is more difficult to account for the rather low relative incorporation of tritium from DL-[2-^{3}H]phenylalanine (94%). Brannon et al.[90] found a similar anomalously low incorporation (80%) of tritium relative to carbon-14, from this [2-^{3}H]- precursor into acetylaranotin[90] (see Scheme XV).

However, despite the slight anomaly of the gliotoxin result, all the facts support a pathway of the type suggested originally by Brannon, involving a phenylalanine epoxide[60]

Scheme XIV

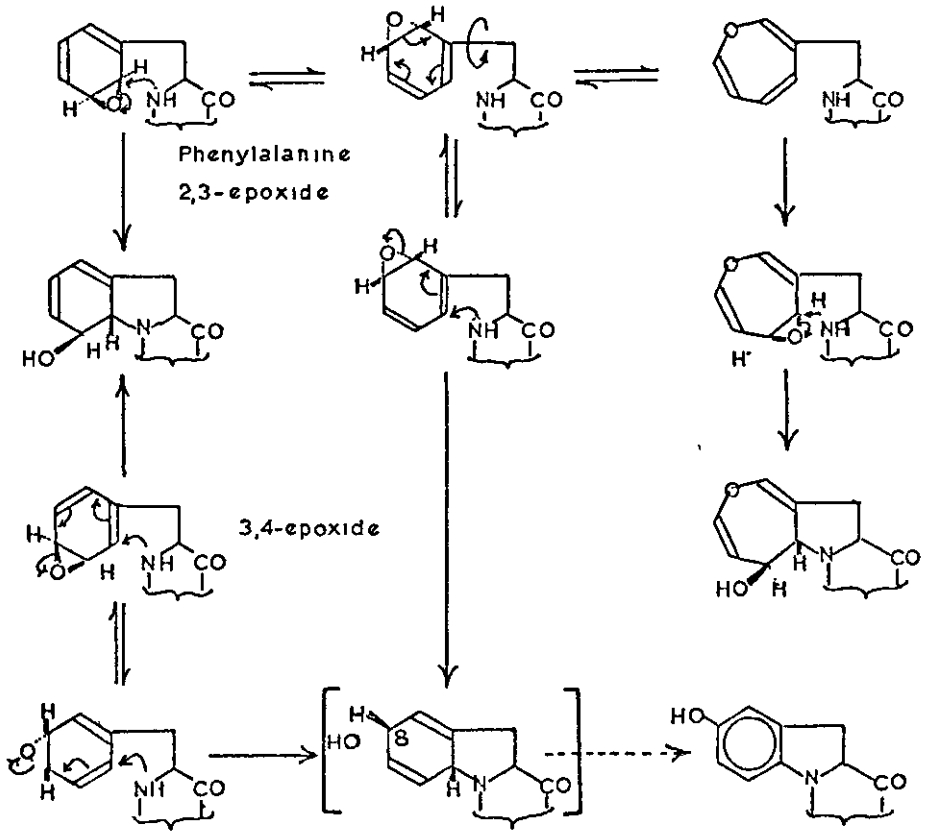


Scheme XV



80% relative
T-retention

Scheme XVI



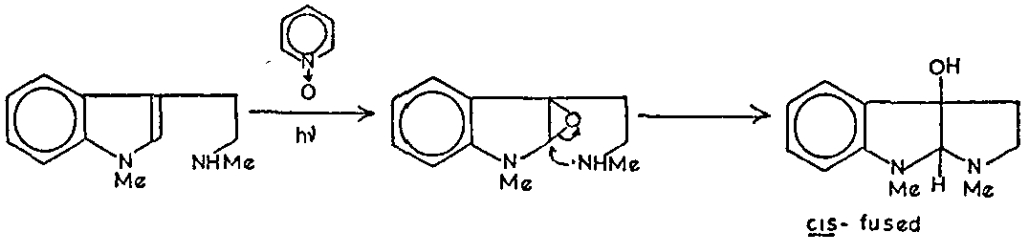
(Scheme XVI). The same scheme shows that a phenylalanine-3,4-epoxide, while not accounting for the oxepin ring of the aranotins, can account adequately for the cyclohexadienyl moiety found in gliotoxin and apoaranotin. In fact if it is assumed that both the 3,4- and the 2,3-epoxides are formed, the latter preferentially, then the apoaranotin series may be cases in which the phenyl groups of the same dipeptide are epoxidised differently; one to give a 2,3-epoxide and the other a 3,4-epoxide.

It is interesting that both epoxides could give rise to an isomer of gliotoxin bearing a hydroxyl group at position 8 (Scheme XVI). No such compound has so far been isolated, but Taylor reported an isomer of dehydrogliotoxin[8] which has since been confirmed by Ryles[9] to bear a hydroxyl group at position 7 or 8. The 7-hydroxy isomer is ruled out if biosynthesis occurs in the manner shown in Scheme XVI.

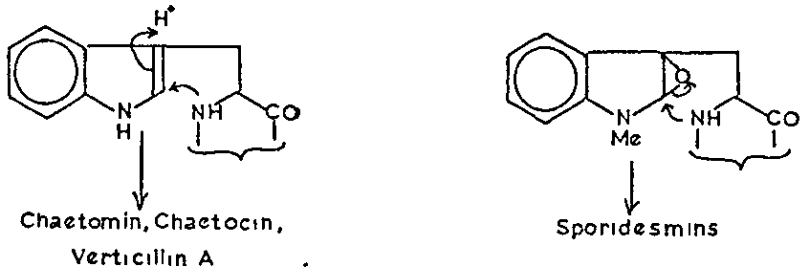
It has been claimed[55] that N-methyltryptamine-(indole-2,3)-epoxide cyclises spontaneously in good yield to a 3-hydroxypyrrolo-[2,3b] indole (Scheme XVII). As a possible biosynthetic route to the sporidesmins, analogous to gliotoxin biosynthesis, this type of reaction deserves mention, but it fails to account for non-hydroxylated sporidesmin analogues, chaetomin, verticillin A and chaetocin. However a possible reaction sequence is shown in Scheme XVIII.

It is well known that aromatic nuclei can be hydroxylated by living systems to form phenols. The enzymes responsible are termed mixed-function oxidases since only one atom per molecule of oxygen used in the process enters the product. The other is reduced to water by $NADPH_2$[93]. Migration of

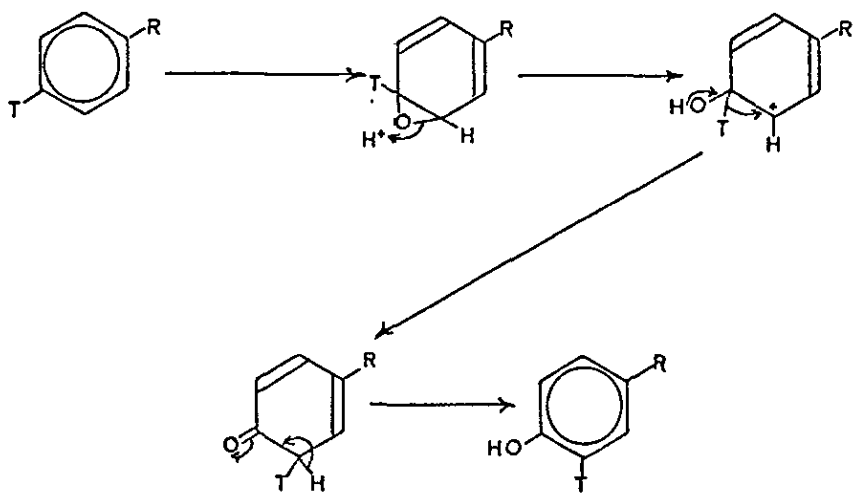
Scheme XVII



Scheme XVIII



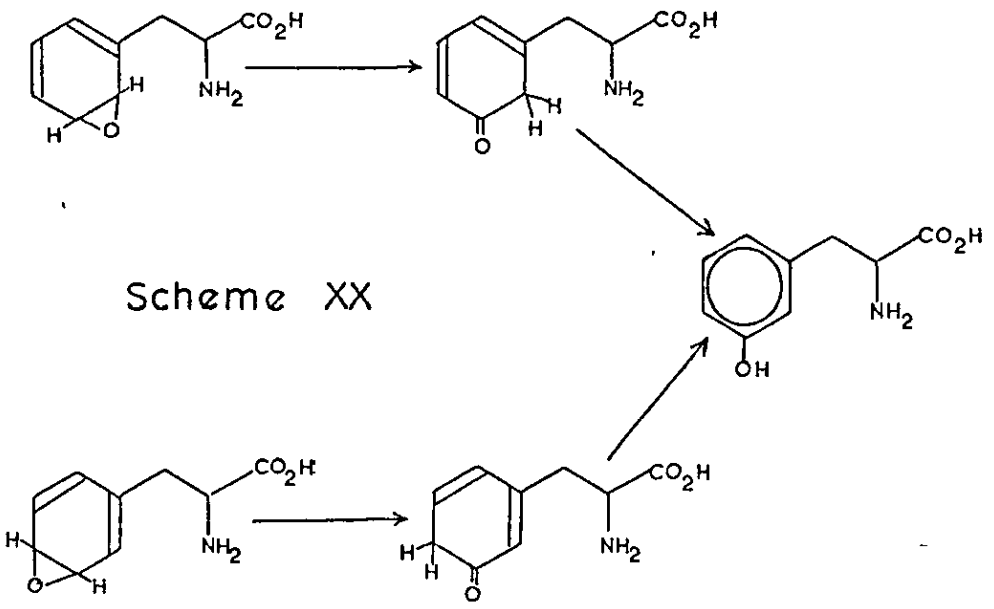
Scheme XIX - NIH Shift



hydrogen from the site of hydroxylation to the neighbouring carbon of the aromatic ring has been established by labelling techniques and is known as the "N.I.H. Shift"[94]. Extensive studies of the phenomenon have led to its explanation in terms of arene-oxide formation, followed by isomerisation (Scheme XIX). As has been mentioned, if m-tyrosine were a precursor of gliotoxin migration of tritium from the meta-position of precursor phenylalanine, on N.I.H. Shift principles would be expected. From Scheme XX it can be seen that the 2,3- and 3,4-epoxides of phenylalanine put forward (Scheme XVI) as precursors of gliotoxin, are also the theoretical precursors of m-tyrosine. Most of the hydroxylations studied have been para-directed, or more recently ortho-[95]. Only one documented example of an N.I.H. Shift of label from the meta position is known[96] and this is disappointing in that migration is only observed of 35% of the tritium (the remainder is lost) compared with 95% migration generally observed in the case of para-hydroxylations[94].

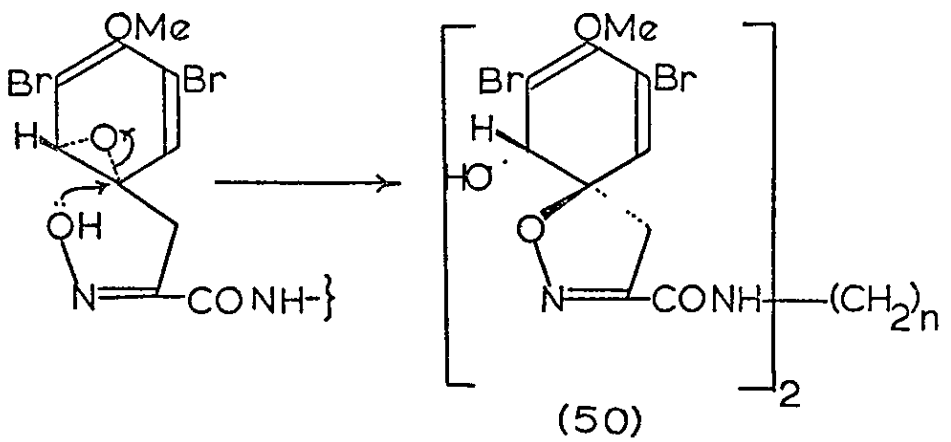
Biosynthetic mechanisms involving arene oxide formation have been put forward for the metabolites aerothionin (50),(n=4), homoaerothionin (50)(n=5)[97] and for aeroplysinin (51)[98] obtained from Verongia sponges. In the case of (50) attack of the epoxide by the oxygen atom of an oxime function is envisaged. In the case of aeroplysinin (51), attack is by a carboxylate anion.

Other workers have obtained results which are also compatible with the proposed mechanism for gliotoxin biosynthesis. Bu'Lock and Ryles[89] have fed both

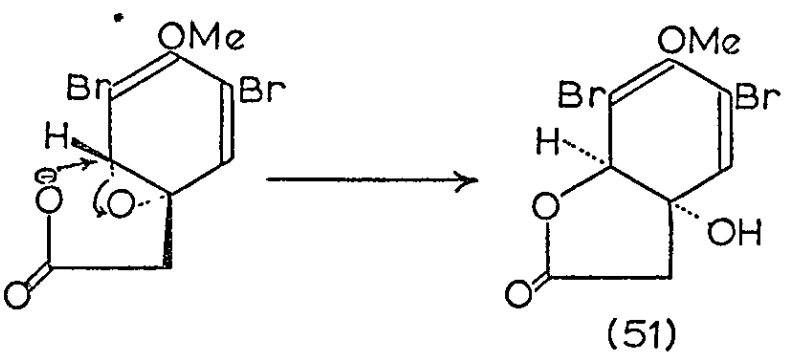


Scheme XX

Verongia sponge metabolites



(50)



(51)

DL-[2,4,6-$^{3}H_{3}$]- and DL-[1'-^{14}C] m-tyrosine to T. viride, and report no incorporation of either into gliotoxin. Likewise DL-[3,5-$^{3}H_{2}$]-o-tyrosine and DL-2,3-dihydroxy-[4,5,6-$^{3}H_{3}$] phenylalanine were shown not to be precursors[89]. On the other hand DL-[1'-^{14}C]phenylalanine gave incorporations as high as 11%. DL-[2,3,4,5,6-$^{2}H_{5}$]Phenylalanine was fed under conditions designed to give maximum incorporation, and the only significant deuteriated species in the gliotoxin produced was found to be the pentadeuterio compound by mass spectrometry. Bu'Lock and Ryles therefore concluded that m-tyrosine could not be an intermediate, since its formation would imply loss of at least one atom of deuterium. Their pentadeuteriated result is also compatible with the idea of an arene oxide intermediate.

Brannon et al.[90] have demonstrated that while DL-[2'-^{14}C]-m-tyrosine is readily taken up by the cells of Penicillium terlikowskii, it is not incorporated into gliotoxin. Under the same conditions DL-[3'-^{14}C]phenylalanine is both readily taken up and converted into labelled gliotoxin. The organism Arachniotus aureus readily admitted label into its cells in the form of DL-[2'-^{14}C]-m-tyrosine, and DL-[3'-^{14}C]phenylalanine, and to a lesser extent DL-3,5-dihydroxy-[2'-^{14}C]phenylalanine, but incorporations of the hydroxylated amino-acids into acetylaranotin were extremely low (0.17-0.46%), whereas up to 6.6% of the labelled phenylalanine was incorporated. An incorporation of 80% tritium relative to carbon-14 was observed in acetylaranotin when DL-[3'-^{14}C,2-^{3}H]phenylalanine was fed to cultures of Aspergillus terreus. When

the latter organism was fed DL-[$^2H_8$]phenylalanine the acetylaranotin produced showed only $^2H_{14}$, $^2H_7$ and ^2H_o enrichment ions in its mass spectrum, indicating that all the ring hydrogens and both of the methylene hydrogens are retained during the biosynthesis of aranotin. Brannon and his coworkers concluded that the biosynthesis of acetylaranotin in both A. aurues and A. terreus closely parallels that of gliotoxin in the formation of the carbon-nitrogen skeleton, and that disulphide bridge formation does not involve addition of electrophilic sulphur to a double bond as claimed by Taylor[59].

The work of Bu'Lock and Ryles and of Brannon, inter-relates the biosyntheses of gliotoxin and aranotin showing, as one would expect, that the route is similar whatever the producing organism.

Section B

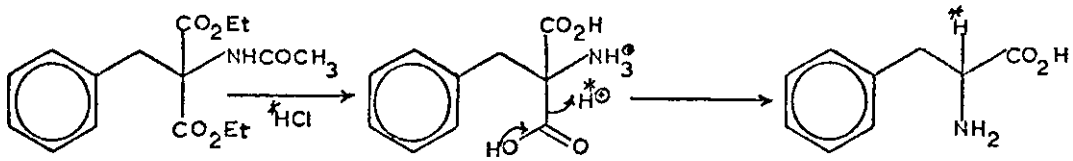
The Fate of the Side-Chain in Gliotoxin Biosynthesis

B.1 Preparation of Precursors

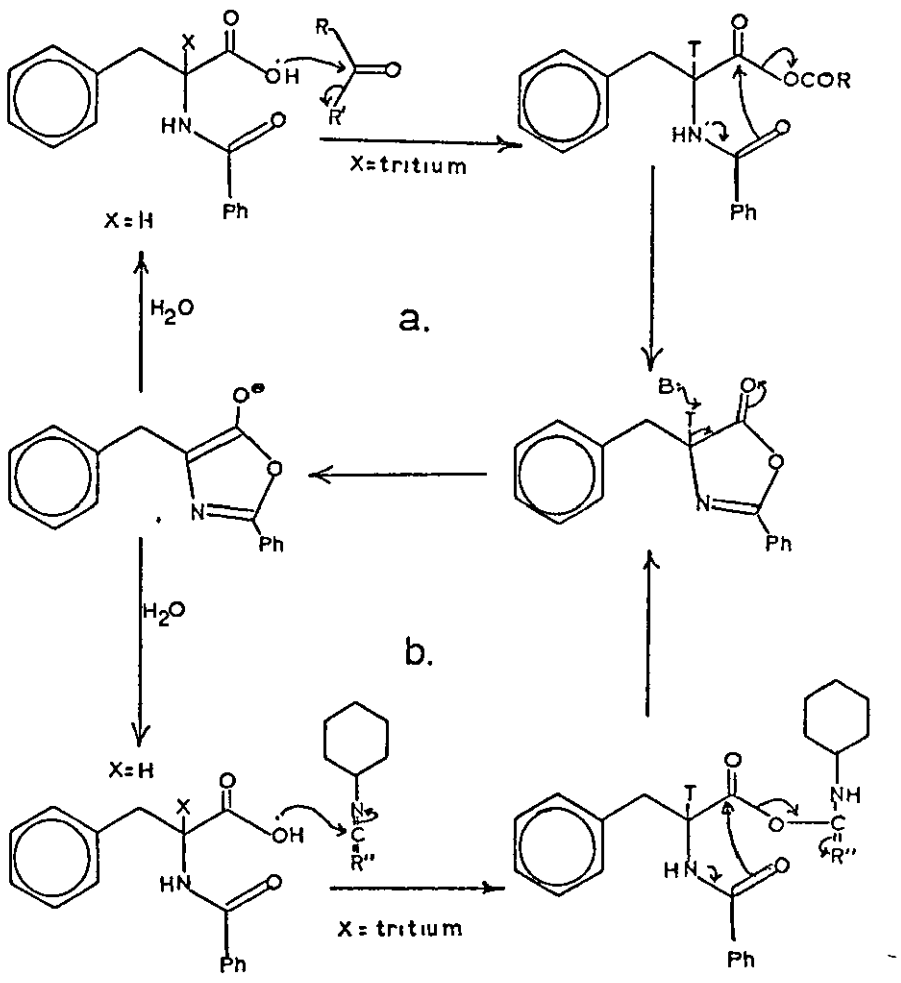
DL-[2'-^{3}H]Phenylalanine was prepared (Scheme XXI) from unlabelled diethyl 2-benzyl-2-acetamidomalonate by hydrolysis and decarboxylation in tritiated hydrochloric acid. The starting material had been prepared from reagent grade benzyl bromide and diethyl acetamidomalonate while the synthetic route to DL-[3-^{3}H]phenylalanine was being investigated.

The position of the label in the precursor was ascertained by mixing a portion with DL-[1'-^{14}C]phenylalanine, diluting with the unlabelled amino-acid and converting the diluted material to the N-benzoyl derivative. It is known[113] that N-acyl derivatives of optically active amino-acids are racemised in the presence of an acid anhydride and a trace of base. Formation of an oxazolinone occurs when the elements of water are removed by an acid anhydride or by dicyclohexylcarbodiimide[105]. Treatment with base readily forms the aromatic enol anion, removing the 2'-hydrogen atom of the original amino-acid. The sequence is shown in detail in Scheme XXII. Regeneration of the racemic N-acyl amino-acid is accomplished by adding water. If tritiated or deuteriated water is used, the 2'-hydrogen of the recovered acyl amino-acid is labelled[105].

The initial DL-[1'-^{14}C,2'-^{3}H]phenylalanine had a ^{3}H:^{14}C ratio of 5:1. After N-benzoylating by a Schotten-Baumann technique the ratio had fallen to 1·9:1. Thus some loss of label had evidently occurred by the route shown in Scheme XXIIa (R = Ph, R' = Cl). Under these conditions it was highly unlikely that label from elsewhere



Scheme XXI



Scheme XXII

in the molecule could have been lost since all readily-exchangeable tritium had been removed from the labelled phenylalanine during work-up.

When the DL-*N*-benzoyl-[1'-^{14}C,2'-^{3}H]phenylalanine was heated with dicyclohexylcarbodiimide, basified with pyridine and regenerated with water, according to the sequence shown in Scheme XXIIb, the ^{3}H:^{14}C ratio in the product was 0·27:1. Thus about 95% of the precursor label must have been in the 2'-position. The remainder may have been introduced into the phenyl ring, *o* and *p* to the alanine grouping, by proton exchange in the strong acid used to hydrolyse the acetamido function. The benzoylated amino-acid was also heated with acetic anhydride, basified and regenerated according to Scheme XXIIa (R = CH_3, R' = $O.CO.CH_3$) but in this case the ^{3}H:^{14}C ratio only fell to 1·1:1, indicating incomplete conversion to the oxazolinone.

Synthesis of stereoselectively labelled 3'-deuterio phenylalanines was carried out by the procedure of Kirby and Michael as shown in Scheme XXIII (R = ^{2}H)[126]. The procedure used to label the formyl group of benzaldehyde consisted first of converting the aldehyde into a Strecker base with cyanide and morpholine. The aldehyde proton is thus activated, and can be removed with base (sodium hydride) and the resulting anion deuteriated or tritiated by treatment with D_2O or tritiated water[106]. Hydrolysis with dilute mineral acid yielded labelled benzaldehyde, which was converted into the labelled, unsaturated oxazolinone (52). In recent years there has been some controversy as to whether this compound has the (*Z*)-

configuration shown (52)[127], or the opposite (E)-configuration about the exocyclic double bond[128]. However it has now been established[129] that the more stable (Z)-isomer only, is formed by the condensation of benzaldehyde with hippuric acid. It is also reported[130], that no change in stereochemistry occurs on hydrolysis of the oxazolinone (52) to the 2'-benzoylaminocinnamic acid (53). Kirby, Michael and Narayanaswami[131] have shown that hydrogenation of both the (E)- and the (Z)-2'-benzoylaminocinnamic acid proceeds cis with high stereoselectivity, to give the mixture of enantiomeric amino-acid derivatives (54), (R = ^{2}H; R' = COPh). Hydrolysis of this mixture from the (Z)-cinnamic acid to (54), (R = ^{2}H, R' = H) followed by chloroacetylation under Schotten-Baumann conditions[146] gave (54), (R = ^{2}H; R' = $COCH_2Cl$). This latter mixture was resolved enzymatically by the technique of Greenstein[132] using carboxypeptidase A. Only the (2'S)-enantiomer is dechloroacetylated by the enzyme. (2'R,3'S)-N-Chloroacetyl-[3'-^{2}H]phenylalanine can be recovered from the reaction mixture by solvent extraction and (2'S,3'R)-[3'-^{2}H]phenylalanine by ion exchange chromatography.

The n.m.r. spectrum of (54) (R = ^{2}H; R' = $COCH_2Cl$) is shown (fig. 9). An enlargement of the 2' (ca. τ = 5·5) and 3' (ca. τ = 6·9) proton signals is given in fig. 10, together with a comparable spectrum of unlabelled material and the patterns obtained by Kirby and Michael in their synthesis of stereoselectively labelled tyrosine[126], A being the spectrum of unlabelled N-acetyl-4-methoxyphenylalanine, B that of a mixture of enantiomeric N-acetyl-4-methoxy-

Scheme XXIII

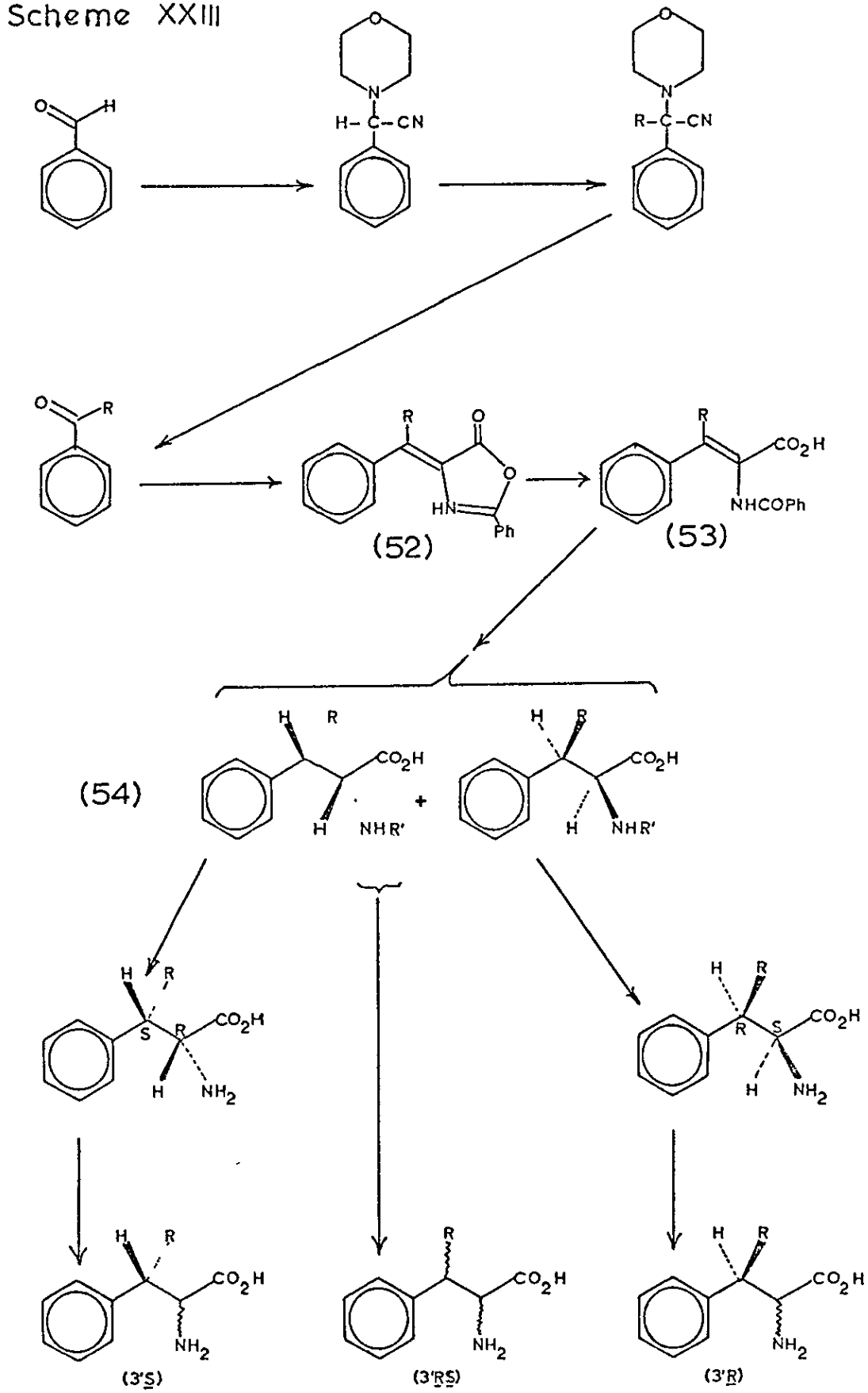


fig. 9- N-CHLOROACETYL-[3'-^{2}H]PHENYLALANINES (54),(R=$COCH_2Cl$)

60MHz. N.M.R. SPECTRUM (DMSO-d_6)

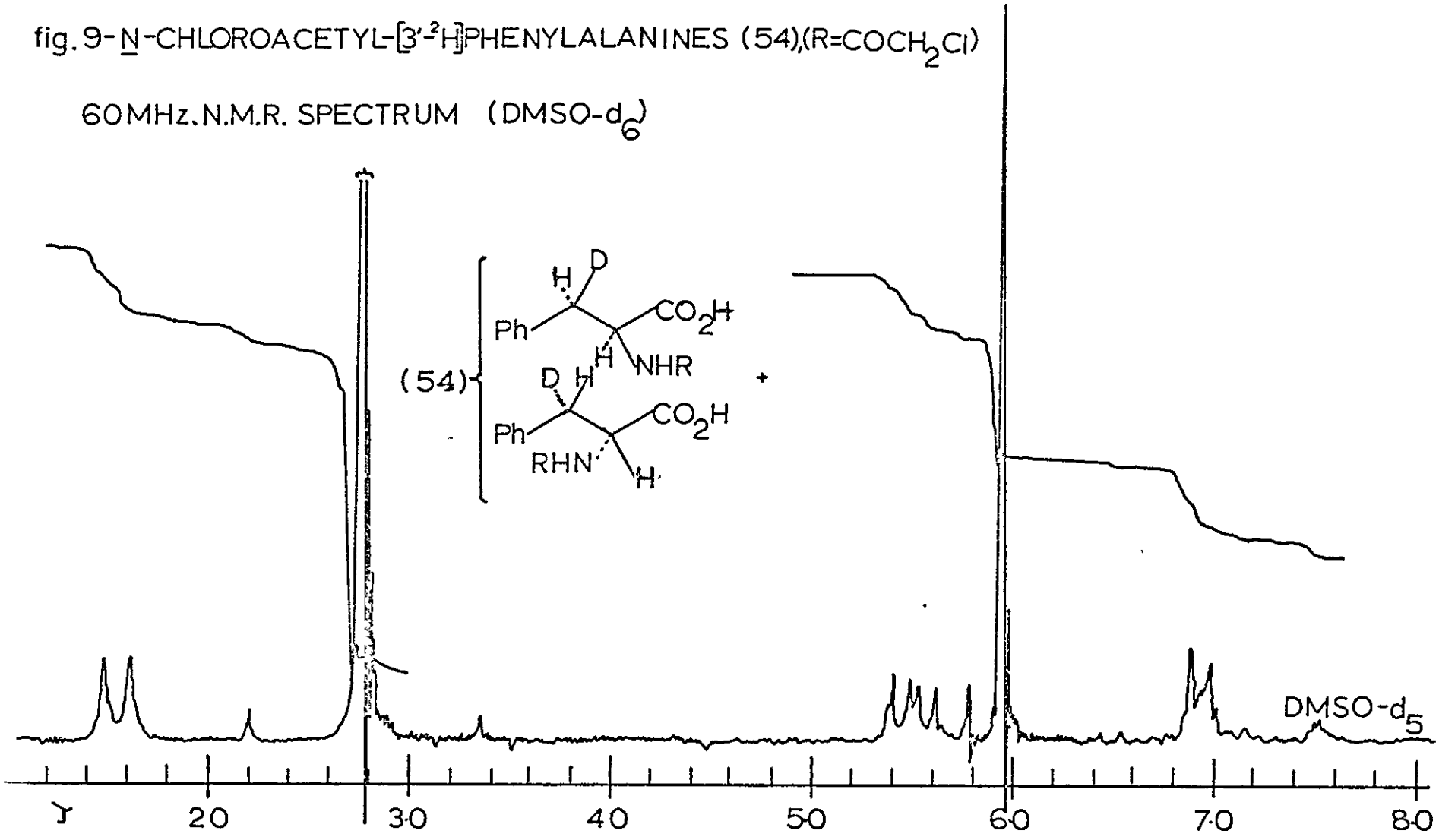
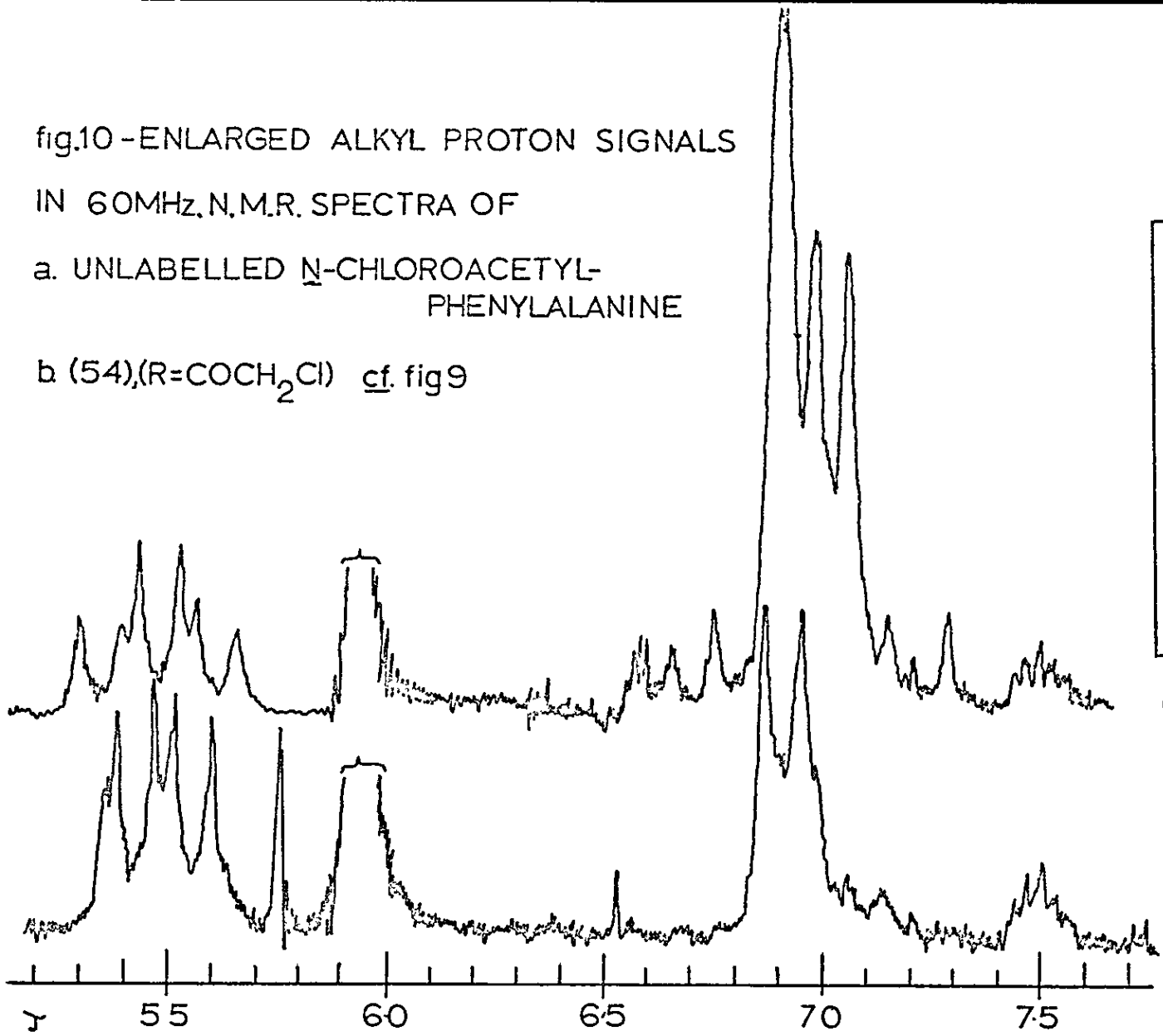


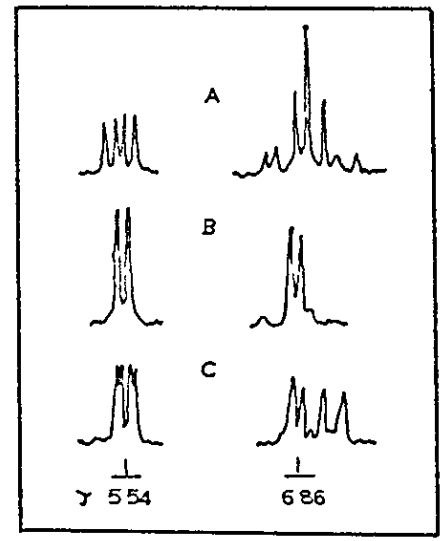
fig.10 - ENLARGED ALKYL PROTON SIGNALS IN 60MHz. N.M.R. SPECTRA OF

a. UNLABELLED N-CHLOROACETYL-PHENYLALANINE

b (54), (R=$COCH_2Cl$) cf. fig 9



c.



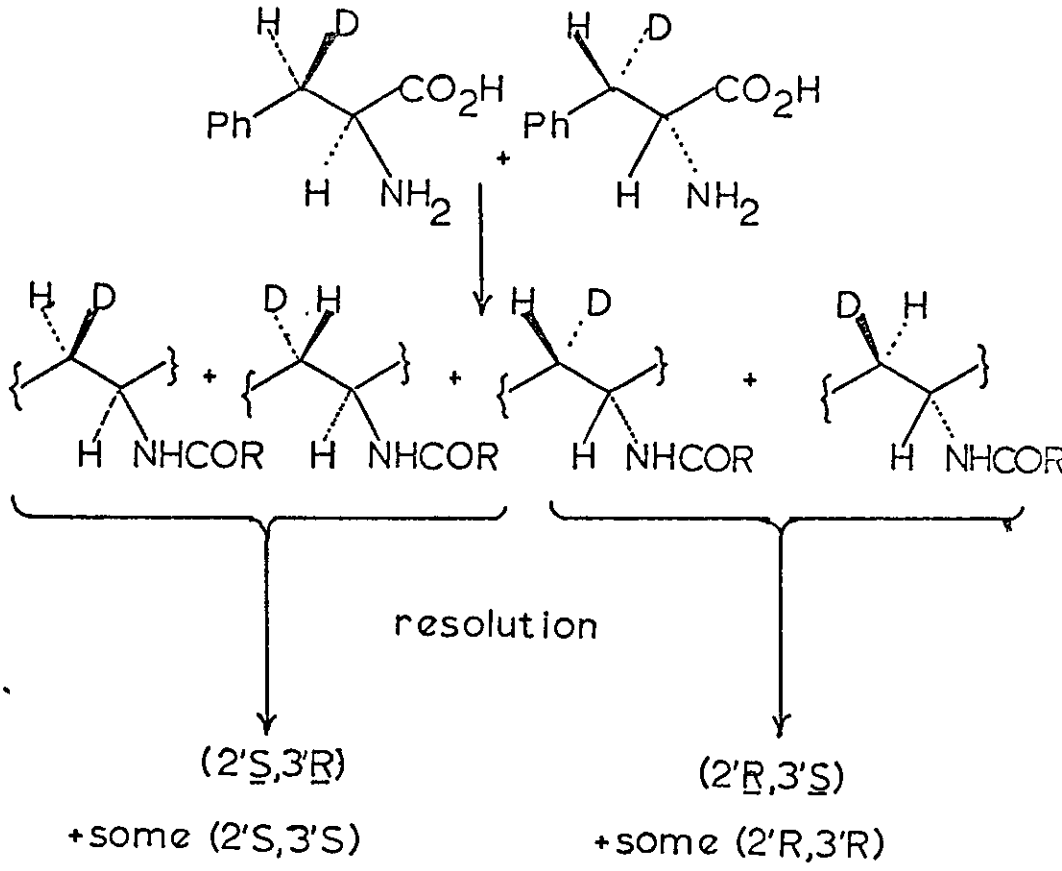
c. N-ACETYL-4-METHOXY-PHENYLALANINE

N.M.R. SPECTRA AFTER KIRBY & MICHAEL[126]

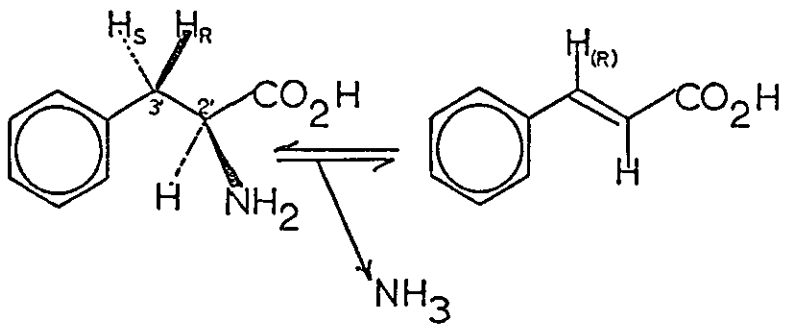
[3'-^{2}H]phenylalanines, and C, that of the N-acetyl-4-methoxy-[3'-^{2}H]phenylalanine after epimerisation at the 2' position. The enlargements of the spectra for the N-chloroacetyl compounds correspond well with spectra A and B of Kirby and Michael, indicating high stereoselectivity of labelling. Due to coupling with the amide proton, the 2'-H signal is more complicated in the chloroacetyl spectrum. Simplification by exchanging the amide proton with D_2O was impracticable as the signal due to the 2' proton was then obscured by the HOD singlet.

The only stage in the synthesis at which loss of the relative configuration at the 2' and 3' centres is likely to occur, is the chloroacetylation of the enantiomeric mixture of labelled phenylalanines to produce (54), (R = $COCH_2Cl$). As has been shown with DL-[2'-^{3}H]phenylalanine, epimerisation can easily take place under these conditions, due to loss of the 2' proton. In the labelling sequence (Scheme XXIII) the effect would be to produce phenylalanine in which the relative stereochemistry at the 2' and 3' positions was reversed, i.e. threo to erythro, and each sample of phenylalanine would contain some of the enantiomerically 3'-labelled compound after 2' centre had been completely resolved. (Scheme XXIV)

However, since the n.m.r. spectrum (fig. 9 and 10) after chloroacetylation showed only the threo labelled compounds and some unlabelled phenylalanine, it was concluded that little, if any epimerisation at the 2' position had occurred during chloroacetylation. In the deuteriated series (Scheme XXIII, R = ^{2}H) the resolved (2'R,3'S)- but



Scheme XXIV -partial epimerisation prior to resolution



Scheme XXV

not the (2'S,3'R)-[3'-^{2}H]phenylalanine was epimerised to the (2'R,S) form without loss of configuration at the 3' centre, by heating with acid in a sealed tube[126]. (2'S,3'R)- and (2'R,S,3'S)-[3'-^{2}H]phenylalanines were used by Bu'Lock and Ryles in feeding experiments with T. viride.

Stereoselectively tritiated phenylalanines were prepared by an analogous sequence (Scheme XXIII, R = ^{3}H). The author's starting material in this case was the mixture of enantiomeric labelled phenylalanines (54), (R = ^{3}H, R'= H), kindly provided by Dr. S. Narayanaswami, who also donated a small quantity of resolved (2'R,3'S)-N-chloroacetyl-[3'-^{3}H]phenylalanine, which was added to the author's material at the appropriate stage. Both enantiomers after resolution were epimerised at the 2'-centre to give (2'R,S,3'R)- and (2'R,S,3'S)-[3'-^{3}H]phenylalanine. The position of the label was known from n.m.r. and mass spectrometry studies on the deuteriated series. In order to assay its stereochemical purity, a portion of each tritiated precursor, diluted and mixed with (2'R,S)-[1'-^{14}C]phenylalanine was incubated with a solution of L-phenylalanine ammonia-lyase of known enzymic activity[111] obtained from potato tubers by the method of Havir and Hanson[92]. It has recently been shown[133,110] that this enzyme stereospecifically removes the pro-S hydrogen from the 3' position of phenylalanine (Scheme XXV). After incubation the cinnamic acid produced was extracted, diluted with inactive material and purified by sublimation. Results from the assays are shown below in table 6. Two portions of the (3S')-[3'-^{3}H]precursor were assayed. As

a control experiment, (2'R,S,3'R,S)-[3'-^{3}H]phenylalanine, prepared by epimerising a mixture of enantiomeric labelled phenylalanines (54), (R=^{3}H, R'=H) in acid in a sealed tube was also assayed.

Table 6

	Cinnamic acid		
Precursor Phenylalanine	^{3}H:^{14}C	^{3}H:^{14}C	Assay % R
(2'R,S,3'R)-[1'-^{14}C,3'-^{3}H]-	5·0	4·2	83·1
(2'R,S,3'S)-[1'-^{14}C,3'-^{3}H]-	4·6	0·69	15·0
(2'R,S,3'S)-[1'-^{14}C,3'-^{3}H]-	12·6	1·7	13·3
(2'R,S,3'R,S)-[1'-^{14}C,3'-^{3}H]-	6·4	3·1	47·5

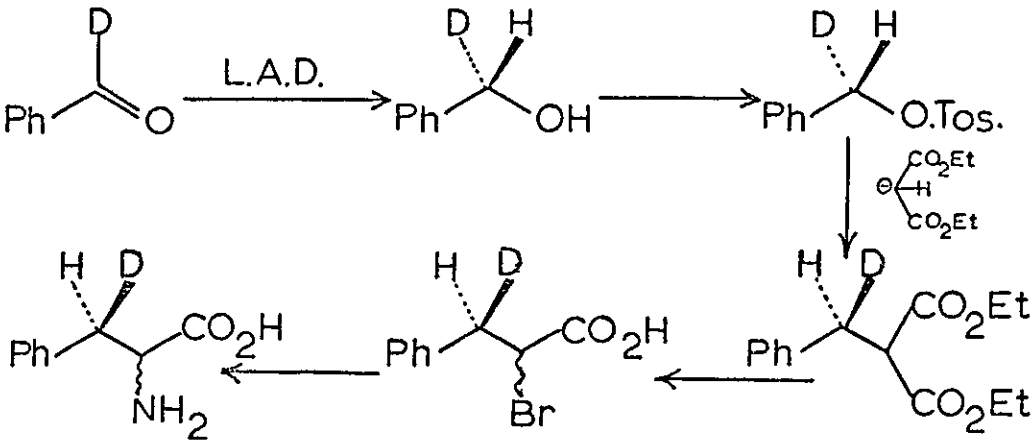
The stereoselectivity achieved in the synthesis is thus comparable to the 85% and 15% (R)-form (respectively) estimated by Kirby and Michael[126] for their stereoselective tyrosine synthesis, and subsequently confirmed by an assay using L-tyrosine ammonia-lyase. It will be noticed that the assay figures for (3'R)- and (3'S)-labelled phenylalanine do not add up to 100%. This is not strictly necessary in fact, since the two precursors came, in part, from two different batches which may have been chloroacetylated (with consequent risk of epimerisation) under different conditions.

It might, alternatively, be possible to explain the discrepancy between the observed proportions of (3'-R)-label in the stereoselectively-labelled phenylalanines (table 6) in terms of a secondary isotope effect. Such an explanation would account for the low retention of

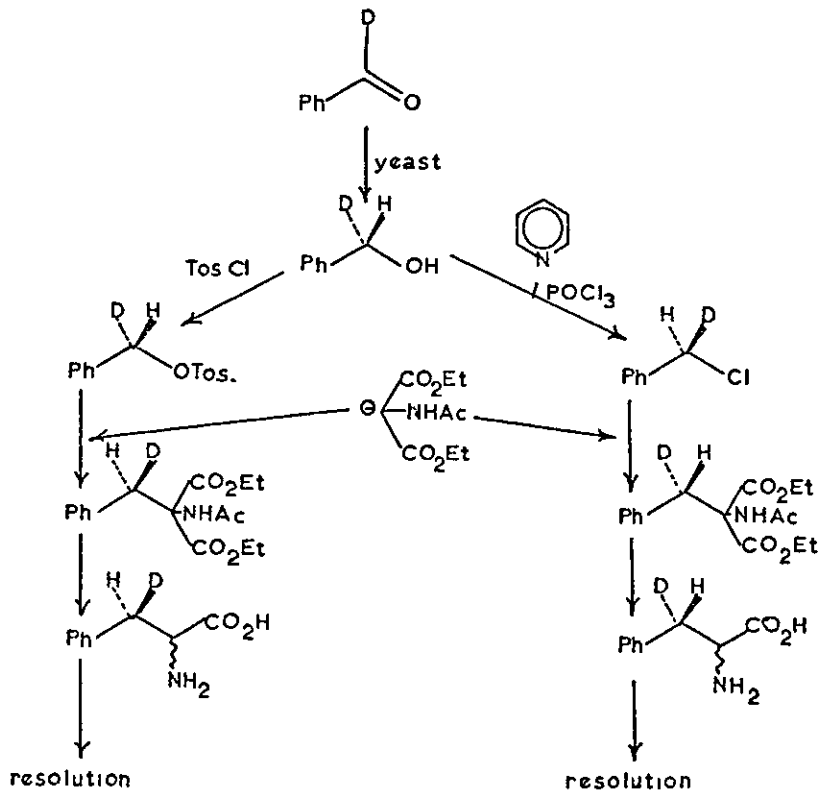
tritium in the control assay of racemically (3')-tritiated phenylalanine (47·5%). Since the rate-determining step in the L-phenylalanine ammonia-lyase reaction probably involves the breaking of the C-H_S bond, the primary isotope effect should not affect the ^3H:^{14}C ratio of the cinnamic acid, as both (2'S,3'R)-[3'-^{3}H]phenylalanine and (2'S)-[1'-^{14}C]-phenylalanine are converted into product at the same rate. (2'S,3'S)-[3'-^{3}H]Phenylalanine will lose ammonia more slowly but will, of course, give inactive cinnamic acid. The point is discussed in detail by Battersby[134], who has demonstrated that during the reaction (2'S,3'S)-[3'-^{3}H]-phenylalanine accumulates, since it is less readily converted to cinnamic acid than the species which have protium in the (3'S)- configuration.

Although the primary isotope effect is not observed in the conversion to cinnamic acid, a secondary isotope effect whereby the presence of (3'R) tritium slightly strengthens the 3'-carbon - 3'S-protium bond could conceivably account for the results in table 6. However the only results of enzymic assays of complementary stereoselectively labelled phenylalanines as yet published are those of Battersby[134], who quotes assays of 88% (R) and 11% (R) for two samples of phenylalanine. The labelling procedure used is not reported in this article. If the samples were prepared by a route involving alcohol dehydrogenase, followed by tosylation and attack of the tosylate by malonate carbanion[133] (Scheme XXVI) or (Scheme XXVII), then it cannot be expected that they should have complementary proportions of (3'R) label. On

Scheme XXVI[133]



Scheme XXVII[110]



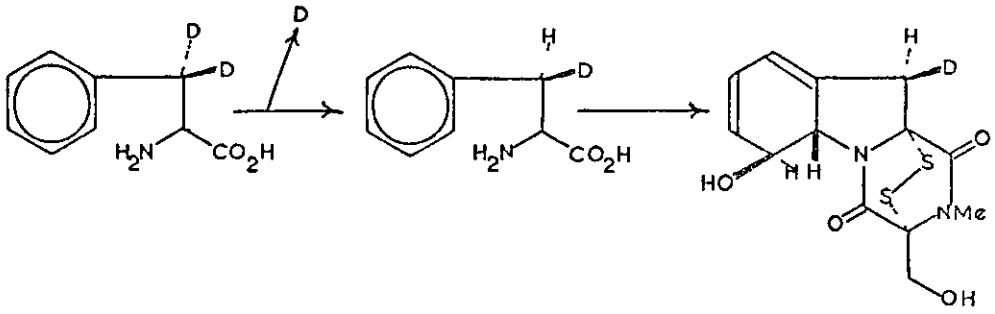
the other hand, if they were produced by a sequence analogous to that of Kirby and Michael[126], the samples should have complementary proportions of (3'R) label and therefore Battersby's results would indicate no secondary isotope effect. The possibility of a secondary isotope effect is an interesting one, and has apparently not yet been noted in the literature.

Apart from Battersby et al.[133] who have stereoselectively labelled phenylalanine both by a method analogous to that of Kirby and Michael and by the independent method shown in Scheme XXVI, which uses liver alcohol dehydrogenase to generate the asymmetric centre, only one other group has synthesised stereoselectively deuteriated phenylalanine. Haslam and Ife[110] investigated the stereochemical course of the L-phenylalanine ammonia-lyase reaction using (2'S,3'R)- and (2'S,3'S)-[3'-^{2}H]phenylalanine. These labelled amino-acids were prepared by the route shown in Scheme XXVII. The initial asymmetric step was carried out by an actively fermenting culture of baker's yeast. Resolution to give the (2'S) forms was carried out via the chloroacetyl derivatives using Acylase I. Despite the fact that they rely on a total Walden inversion at one, or even two stages, high stereoselectivities, up to 90%, have been claimed.

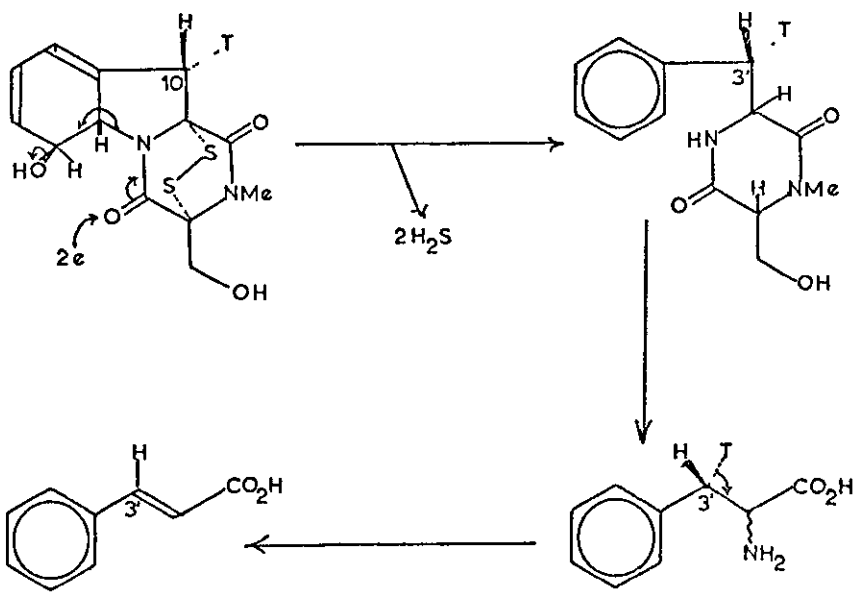
B.2 Feeding of 2'- and 3'- Labelled Phenylalanines

It has been mentioned in Section A that Brannon et al. had concluded, from feedings carried out on A. aureus using $^{2}H_{8}$ phenylalanine, that no loss of label occurs from the methylene position on conversion of phenylalanine into aranotin[90]. However, Bu'Lock and Ryles kindly communicated their discovery that about 50% of the deuterium at the 2'-position in phenylalanine is lost on conversion into gliotoxin by T. viride. Moreover they found by n.m.r. spectroscopy that the remaining label was apparently stereospecifically located in the gliotoxin. It seemed unlikely that biosynthesis of the disulphide bridge proceeds differently in gliotoxin and aranotin, and therefore Ryles[9] fed (2'R,S)-[2'-^{2}H]phenylalanine in the hope of observing a 2'-3' shift during biosynthesis which could have accounted for the anomaly between his and Brannon's results. No such migration occurred. By feeding (2'S,3'R)- and (2'R,S,3'S)-[3'-^{2}H]phenylalanines prepared by the author as described above, Bu'Lock and Ryles[78] obtained the dilutions (^{14}C: x 5·1; ^{2}H: x 5·8) and (^{14}C: x 3·3; ^{2}H: x 19). The difference between the n.m.r. spectra of gliotoxin samples from the two feedings are shown (fig. 12). From these spectra, and from the above dilution figures, Bu'Lock and Ryles have concluded that the incorporation of phenylalanine into gliotoxin proceeds in the manner shown in Scheme XXVIII. Corroborating evidence of a metabolic pathway which removes one of the 3'-protons from phenylalanine was obtained by extracting phenylalanine from the amino-acid pool of T. viride incubated with (2'R,S,3'R,S)-[3'-^{3}H]-phenylalanine.

Scheme XXVIII



Scheme XXIX



The workers found[78] that 25% loss of tritium had occurred from the pool-phenylalanine, and 35% from the extracted mycelium.

In the meantime we began parallel studies with tritiated phenylalanines to confirm these findings and provide more accurate values for hydrogen (tritium) loss than can be achieved with the deuterium-labelling technique, despite the latter's usefulness. Moreover it was a point of interest whether the gliotoxin and mycelial phenylalanine would show the same extent of tritium loss, and at what rate during the organism's period of growth the apparently stereospecific loss of tritium occurred. Accordingly (2'R,S)-[1'-^{14}C,2'-^{3}H]Phenylalanine and (2'R,S, 3'R,S)-, (2'R,S,3'R)- and (2'R,S,3'S)-[1'-^{14}C,3'-^{3}H]phenylalanine were fed to T. viride as already described. The results of the feedings are shown in table 7 below.

Table 7

Precursor Phenylalanine	Gliotoxin				
	^{3}H:^{14}C	yield (mg)	*% incorp.	^{3}H:^{14}C	^{3}H ret. %
(2'R,S)-[1'-^{14}C,2'-^{3}H]-	5·01	277	7·6	0·24	4·9
(2'R,S,3'R,S)-[1'-^{14}C,3'-^{3}H]-	6·45	317	8·4	3·17	49·1
(2'R,S,3'R)-[1'-^{14}C,3'-^{3}H]-	5·01	133	5·7	1·01	20·2
(2'R,S,3'S)-[1'-^{14}C,3'-^{3}H]-	4·60	168	8·3	3·78	82·2
(2'R,S,3'S)-[1'-^{14}C,3'-^{3}H]-	12·61	211	2·9	10·69	84·8

* based on ^{14}C only

The second feeding of (2'R,S,3'S)-[1'-^{14}C,3'-^{3}H]-phenylalanine was carried out in order to establish for certain

that the previous (3'R) and (3'S) feedings had not been accidentally interchanged. Although particular care had been taken to avoid this, it seemed the only explanation at the time for the discrepancy between these and Ryles' results.

The next stage was to extract phenylalanine from the mycelium for examination. Cultures used for gliotoxin production were five days old, and hardly any detectable amino-acid could be extracted with boiling water[135]. However considerable hydrolysis of the mycelial protein occurred on refluxing for 24 hours in 20% barium hydroxide solution. This treatment was employed rather than the usual 6N-hydrochloric acid hydrolysis in order to avoid the extensive charring of the cell-wall polysaccharides which occurs in the presence of acid. Initially it was hoped to separate the phenylalanine chromatographically on ion exchange resin as reported by Chandra and Vining[136], this method having been recommended by Dr. A.P. Ryles. However in practice only very poor separations of the amino-acids from hydrolysed mycelial protein were possible on this system. Following the observation that radioscans of paper chromatograms of mycelial extracts showed only single peaks, located at the Rf (0·6) of phenylalanine, preparative paper chromatography was used to isolate the phenylalanine. The mycelial phenylalanine was obtained by (1/100) dilution with unlabelled L-phenylalanine and purified by recrystallisation from aqueous ethanol. Incorporation and relative tritium retention figures for mycelial phenylalanines from the five feedings previously

described, are shown in table 8.

Table 8

Precursor Phenylalanine	$^{3}H:^{14}C$	Mycelial Phenylalanine			Gliotoxin ^{3}H ret. %
		*% incorp.	$^{3}H:^{14}C$	^{3}H ret. %	
(2'R,S)-[1'-^{14}C,2'-^{3}H]-	5·01	4·2	0·38	7·5	4·9
(2'R,S,3'R,S)-[1'-^{14}C,3'-^{3}H]-	6·45	3·5	3·26	50·5	49·2
(2'R,S,3'R)-[1'-^{14}C,3'-^{3}H]-	5·01	5·0	1·08	21·5	20·2
(2'R,S,3'S)-[1'-^{14}C,3'-^{3}H]-	4·60	5·6	3·75	81·6	82·2
(2'R,S,3'S)-[1'-^{14}C,3'-^{3}H]-	12·61	4·3	10·47	83·0	84·8

* based on ^{14}C.

It was heartening to see such a close similarity between tritium retention in the mycelial phenylalanine and incorporation into gliotoxin. The configuration of tritium in the mycelial phenylalanine was next investigated. Mycelial phenylalanine samples were isolated by preparative paper chromatography, as described above and were incubated, without dilution, with L-phenylalanine ammonia-lyase solution. The labelled cinnamic acid was extracted, diluted with unlabelled compound and purified by sublimation. Assay results are shown in table 9 below.

Table 9

Original Precursor Phenylalanine	Mycelial Phenylalanine $^{3}H:^{14}C$	Cinnamic acid	
		$^{3}H:^{14}C$	^{3}H ret. %
(2'R,S,3'R,S)-[1'-^{14}C,3'-^{3}H]-	3·26	0·38	11·6
(2'R,S,3'R)-[1'-^{14}C,3'-^{3}H]-	1·08	0·36	33·7
(2'R,S,3'S)-[1'-^{14}C,3'-^{3}H]-	3·75	0·13	3·4
(2'R,S,3'S)-[1'-^{14}C,3'-^{3}H]-	10·47	0·28	2·6

Having established the labelling pattern in the mycelial phenylalanine a technique was sought for establishing the configuration of the label at position 10 in gliotoxin. As has already been mentioned, it was hoped to cleave the gliotoxin molecule with lithium in liquid ammonia (Scheme XXIX) to give a cyclic peptide of phenylalanine which could in principle be hydrolysed, yielding phenylalanine. No inversion at position 3' (of phenylalanine) would be expected to occur, and assay with L-phenylalanine ammonia-lyase should show the configuration of tritium in the original gliotoxin. This work, however was unsuccessful and was eventually abandoned.

An experiment was next designed which would give an idea of the rate of tritium loss from the 3'-position of phenylalanine. T. viride was grown in five litres of culture medium in the presence of racemic [1'-^{14}C,3'-^{3}H]-phenylalanine. A sample of one litre was worked up every twenty-four hours for five days. Each sample was extracted for gliotoxin and if necessary unlabelled metabolite was added to the extract to render isolation possible. The extract medium was reduced to 1/100 its volume and examined for radioactivity and amino-acids by paper chromatography and scanning. Washed, dried mycelium, which had been filtered off prior to gliotoxin extraction, was extracted first with boiling water to obtain phenylalanine from the amino-acid pool, and then with barium hydroxide to hydrolyse the protein. Phenylalanine from these extracts was isolated as above by preparative paper chromatography followed by dilution and crystallisation. Yields, as far as could meaningfully be recorded, are

tabulated in the experimental section. The precursor $^{3}H:^{14}C$ ratio was 8·95:1. Ratios and consequent relative tritium incorporation (retention) figures for the various samples are shown in table 10.

Table 10

Hours (days)	Gliotoxin		Pool Phenylalanine		Protein Phenylalanine	
	$^{3}H:^{14}C$	^{3}H ret.%	$^{3}H:^{14}C$	^{3}H ret.%	$^{3}H:^{14}C$	^{3}H ret.%
24 (1)	6·47	72·3	3·54	39·5	4·97	55·5
48 (2)	4·87	54·4	3·97	44·4	4·85	54·2
72 (3)	4·73	52·8	4·66	52·1	4·84	54·1
96 (4)	4·69	52·4	5·00	55·9	4·90	54·8
120 (5)	4·73	52·8	4·13	46·1	4·67	52·2

Figures showing the incorporations of precursor into gliotoxin, pool phenylalanine and protein phenylalanine are given in table 11. All are based on carbon-14 incorporation.

Table 11

Hours	Gliotoxin % incorp.	Pool Phenylalanine % incorp.	Protein Phenylalanine % incorp.
24	1·3	7·5	19·9
48	1·5	4·2	20·6
72	6·4	2·4	15·5
96	7·0	0·7	16·8
120	6·4	1·0	17·0

Owing to a shortage of L-phenylalanine ammonia-lyase, enzymatic assays of only the two 24-hour mycelial phenylalanine samples and of the 120-hour protein phenylalanine

sample were made. Results from these assays are shown in table 12.

Table 12

Phenylalanine sample	$^{3}H:^{14}C$	Cinnamic acid	
		$^{3}H:^{14}C$	^{3}H ret. %
24-hour, amino-acid pool	3·54	0·25	7·0
24-hour, mycelial protein	4·97	0·55	11·0
120-hour, mycelial protein	4·67	0·36	7·8

Radioscans of paper chromatograms of the boiling-water extract and of the 20%-barium-hydroxide hydrolysate of mycelium harvested after only 24 hours, are shown in fig. 11, together with representations of the ninhydrin-developed chromatograms. The radioactive peak at the baseline in the chromatogram of the boiling-water extract was of particular interest, since it might have provided some further clue about the biosynthesis of gliotoxin, or about the pathway by which the 3'-proton of phenylalanine is lost. Extraction of the neutral and acidified aqueous amino-acid solution (obtained by boiling the 24-hour old mycelium with water, but not purified by chromatography) gave residues with low activity and a $^{3}H:^{14}C$ ratio of about 3:1 (precursor was 8·95:1). About 35 mg of material was extracted with water from baseline portions of paper chromatograms. The residue from this extract was treated with ethanol, and about 5 mg dissolved. The $^{3}H:^{14}C$ ratio of the ethanol extract was 6·3:1, while that of the non-ethanol-soluble fraction was 2·3:1. On acid hydrolysis, the ethanol

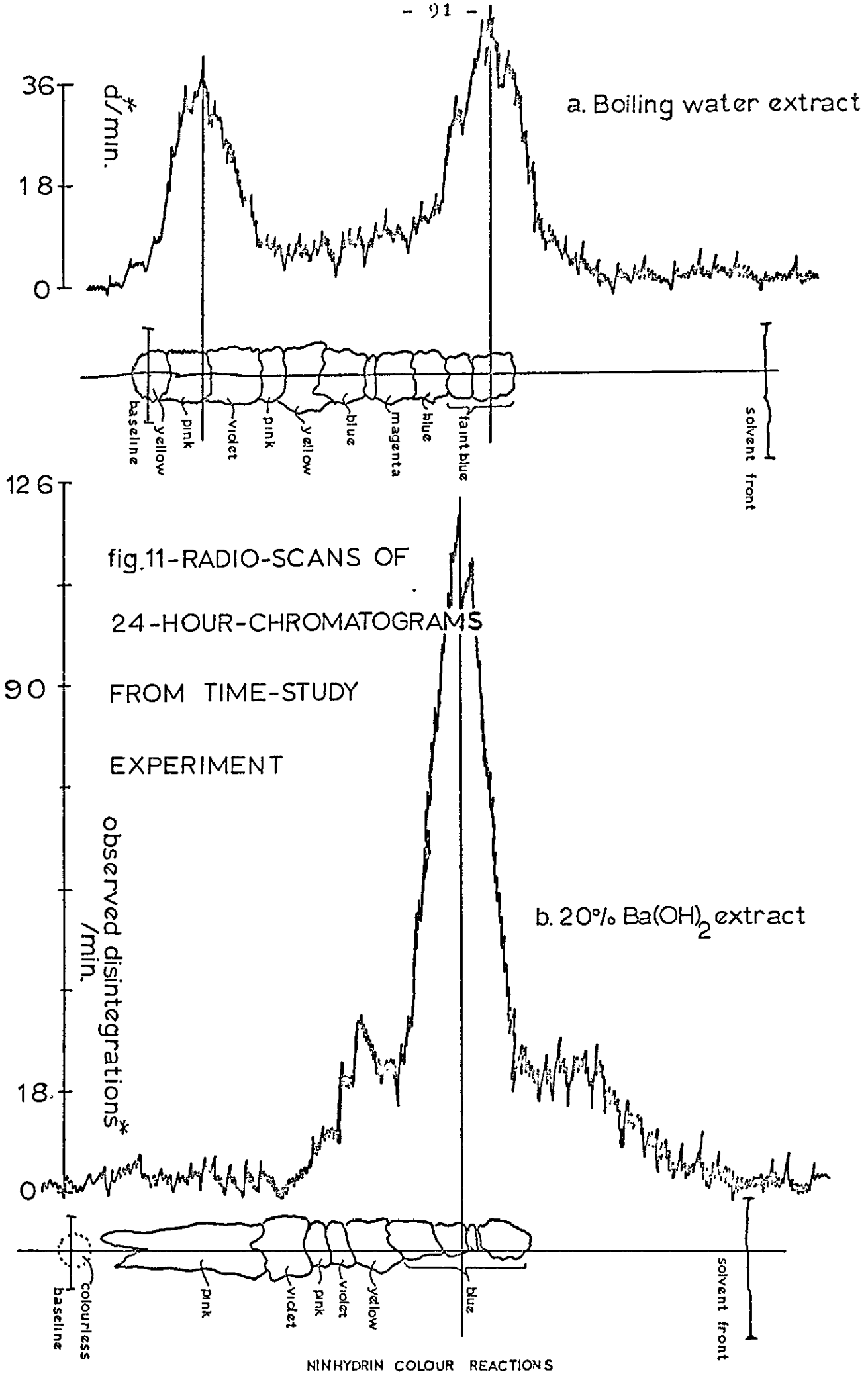


fig.11-RADIO-SCANS OF 24-HOUR-CHROMATOGRAMS FROM TIME-STUDY EXPERIMENT

* not corrected; efficiency unknown

soluble fraction yielded two ninhydrin-positive materials, one at Rf 0·23 and another, in which all the radioactivity was located, at Rf 0·6. Hydrolysis of the water-soluble material gave a smear of many unresolved spots. All radioactivity was located at Rf 0·60.

B.3 Discussion

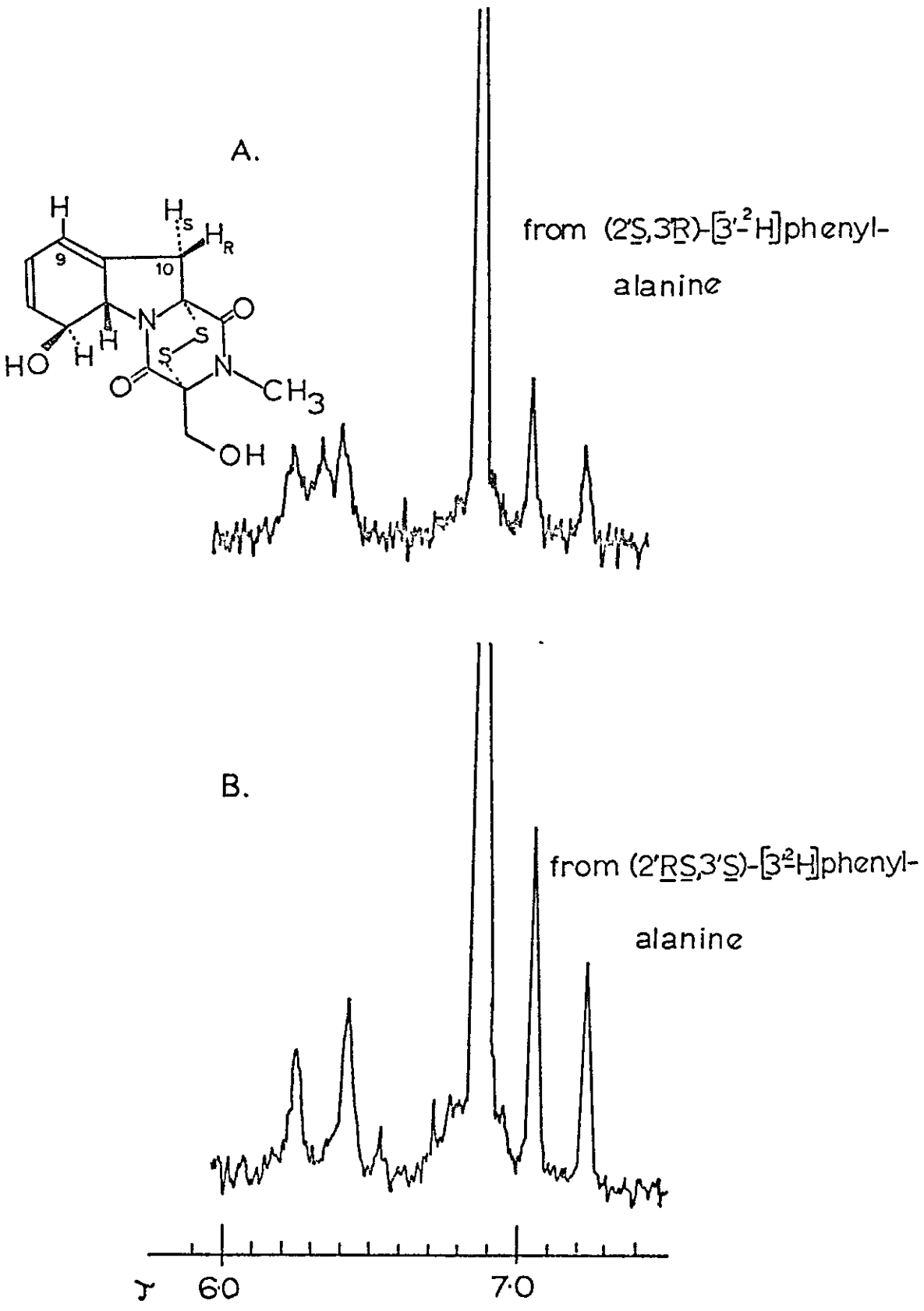
Unless migration of the 2'-proton of phenylalanine can take place during biosynthesis, there is no chance of retention of tritium from (2'R,S)-[1'-^{14}C,2'-^{3}H]phenylalanine occurring on conversion of this precursor into gliotoxin. From table 7 it is apparent that about 5% retention has occurred. Assay of the precursor by the oxazolone route shown in Section B.1 has demonstrated that at least 95% of the label was located at the 2' position. If the remaining 5% were located at the o- or p- positions of the phenyl ring, as postulated, it would be incorporated into gliotoxin (see Section A). The finding that no label from the 2' position is incorporated into gliotoxin is in good agreement with the work of Ryles[9] and of Brannon et al.[90]

Table 7 further shows a relative tritium retention of 49·1% when (2',R,S,3'R,S)-[1'-^{14}C,3'-^{3}H]phenylalanine is converted into gliotoxin. Brannon[90] and Bu'Lock et al.[78] have reported higher incorporations of 3',3' dideuteriated phenylalanine into aranotin and gliotoxin, respectively. In fact the keystone of Bu'Lock's argument that 2',3'-unsaturation (phenylalanine nomenclature) is not a prerequisite of introduction of sulphur, is that complete loss of one deuteron from (2'R,S)-[3',3'-$^{2}H_2$]phenylalanine did not occur during his feedings. The explanation for the apparent discrepancy between the findings in table 7 and those of Bu'Lock (see later assays of mycelial phenylalanines) is that the reaction is not completely stereospecific. As well as (3'R)-tritium loss, some (3'S) label is always

exchanged. Complete loss of (3'R) label seems never to occur. In the (2'R,S)-[3',3'-$^{2}H_{2}$]phenylalanine feedings of Bu'Lock *et al.*[78] the observation was that dideuterio- as well as monodeuteriogliotoxin was formed, which is quite compatible with the results above (tables 7, 8, 9). Since Bu'Lock *et al.* used no internal standard (^{14}C) with which to compare deuterium retention, they could not have detected the partial (3'S)-proton loss.

Feedings of stereoselectively 3'-tritiated phenylalanines (table 7) led to relative ^{3}H retentions of 20·2%, 82·2% and 84·8% on conversion, of (3'R), (3'S) and (3'S) precursors respectively into gliotoxin. Assays of the (3'R) epimer in the precursors are respectively 83·1%, 15·0% and 13·3%. Thus it appears that from the predominantly (3'R)-[3'-^{3}H] precursor all of the (3'S)-[3'-^{3}H]-epimer has been incorporated together with some (3-4%) of the (3'R)-[3'-^{3}H]-epimer. From the predominantly (3'S)-[3'-^{3}H]-precursor some of the (3'S)-[3'-^{3}H]-epimer as well as all of the (3'R)-[3'-^{3}H]-epimer, has been lost. The findings are in good agreement with Bu'Lock's theory[78] but whereas his findings show stereospecific removal of the (3'-*pro*-S) proton of phenylalanine, the results in table 7 show removal of the (3'-*pro*-R) proton. The feeding of (3'S) tritiated precursor was repeated and configurations of all precursors established by assay. One plausible explanation of the anomaly is that the precursor samples sent to Bu'Lock's group, or the derived gliotoxin samples were accidentally interchanged at some stage, either before, during or after the feeding experiment. Alternatively but implausibly odd results may have arisen as a

fig.12-GLIOTOXIN FROM STEREOSELECTIVELY DEUTERIATED PHENYLALANINES 100MHz. N.M.R. SPECTRA AFTER RYLES[137]



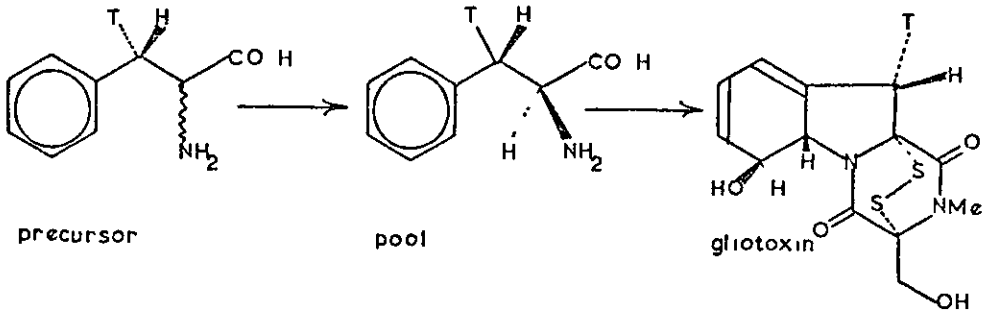
result of comparing a feeding of (2'S,3'R)-[3'-^{2}H]-phenylalanine plus (2'R,S)-[1'-^{14}C]phenylalanine with that of (2'R,S,3'S)-[1'-^{14}C,3'-^{2}H]phenylalanine.

As has already been mentioned in Section A, there has been a certain controversy about the assignment of the C-10 protons in the n.m.r. spectrum of gliotoxin. N.m.r. spectra obtained by Bu'Lock and Ryles of gliotoxin derived from the stereoselectively deuteriated phenylalanines, are shown in fig. 12[137]. Bu'Lock *et al.*[78] have invoked sulphur dishielding to assign the downfield doublet to H_S, whereas in Section A the downfield doublet is ascribed to H_R on the basis of carbonyl dishielding and allylic coupling with H-9. It is clear that if the biosynthesis of the disulphide bridge has no effect upon the 3'-protons of phenylalanine, as postulated[90], then the configuration of the retained label in gliotoxin must be that of the mycelial (pool) phenylalanine, and hence, provided no inversion of the 3' carbon in the pool phenylalanine has occurred, configuration should be the same in the gliotoxin as in the preferentially incorporated precursor; i.e. (S) (Scheme XXX).

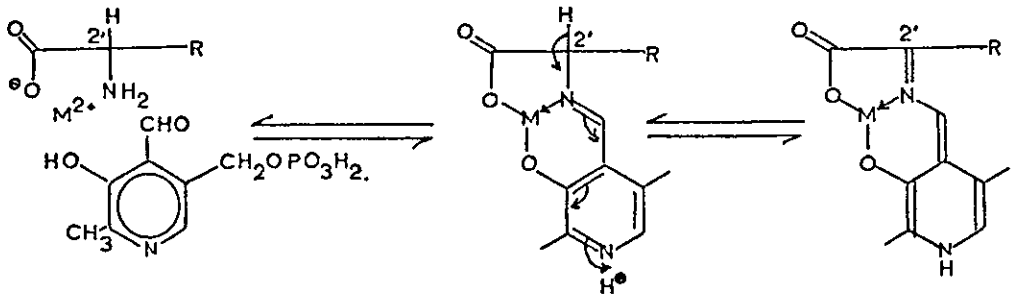
Bu'Lock and Ryles' n.m.r. spectra (fig. 12) show the downfield signal increasing in intensity and thus the "upfield proton" is the one which is labelled. In the light of the author's results and the above argument the downfield signal should be assigned to H_R, this being the configuration at which, in Bu'Lock and Ryles' work, a deuteron is replaced by a proton.

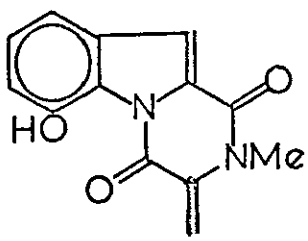
From the results in table 8 it can be seen that the relative tritium retentions for gliotoxin and mycelial

Scheme XXX

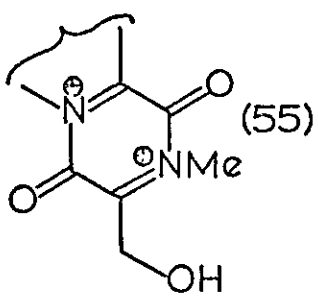


Scheme XXXI

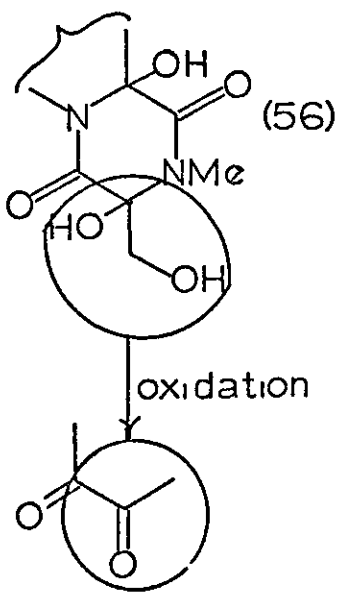




(7)



(55)



(56)

oxidation

(5) & (6) partial structure

phenylalanine in each of the labelled side-chain feedings are closely similar. Retention of tritium from (2'R,S)-[1'-^{14}C,2'-^{3}H]phenylalanine in the mycelial phenylalanine is slightly higher than that in gliotoxin, and corresponds to a retention of 2·5% 2'-tritium, as opposed to complete loss which occurs during gliotoxin biosynthesis. There is no reason why complete loss in the phenylalanine should occur, but since the amino-acids and proteins of micro-organisms exist in a dynamic state[138] the very extensive loss observed is probably due to equilibrium with a pyridoxal complex, as shown in Scheme XXXI[139].

Incorporations of racemic and stereoselectively 3'-tritiated phenylalanines into gliotoxin can be seen from table 8 to be almost identical with the tritium retentions in the corresponding mycelial phenylalanine samples. This finding strongly supports the idea that the mechanism of 3'-tritium loss from phenylalanine is quite independent of gliotoxin biosyntnesis. However the picture is not as simple as appears from table 8. Other factors which evidently affect 3'-tritium loss are apparent from the time-study experiment (table 9), and will be discussed below.

Assay of mycelial phenylalanine samples from the four 3'-tritiated feedings are shown in table 9. Not all of the (3'R) tritium has been removed in any one case. A proportion of (3'R) label, apparently depending on the amount of (3'R) label in the initial precursor, is always retained. This is in good accord with the finding of Bu'Lock *et al.* that some (2'R,S)-[3',3'-$^{2}H_2$]phenylalanine was incorporated

into gliotoxin without deuterium loss[78], but again implies that the (pro-S) rather than the (pro-R) hydrogen at position 10 in gliotoxin is retained. These results are compatible with the idea of stereospecific loss of the (pro-R) proton at the 3'-position of phenylalanine followed at a later stage by the complete sequence of gliotoxin biosynthesis.

Results (table 10) from the time-study experiment show that such gliotoxin as is formed after only 24 hours retains 72% of its tritium. By 48 hours the value has fallen to 54%, and by 72 hours it has reached its final value of ca.53% This is exactly the result expected from the postulated synthetic pathway, gradual loss of phenylalanine (3'R)-tritium down to a constant level.

Close examination has thus failed to shed any light on the biosynthesis of the disulphide bridge in gliotoxin, except to show that loss of the 2'-proton and complete retention of the 3'-protons occurs during the process. The "bioacceptor of the disulphide bridge" (7), put forward by Taylor et al.[59] is thus excluded as a type of intermediate. It seems more likely that biosynthesis occurs either by direct sulphur donation to the species (55) or to the triol (56) by a mechanism analogous to the biosynthesis of cysteine from serine. It is interesting that formation of the triol (56) might well lead to Taylor's metabolites (5) and (6)[10].

On the other hand, these investigations have led to the discovery of an unexpected and apparently unprecedented stereospecific exchange of label at the 3'- position during the metabolism of phenylalanine by T. viride. According to

the results shown in table 10 the metabolic pathway responsible for (3'R)-proton exchange is accompanied by another causing (3'S)-proton exchange during the first 24 hours of growth, since relative tritium retention in pool phenylalanine after this time is less than 50% (39·5%). Actually, even the tritium retention values of 50% and 55% encountered in mycelial phenylalanine from the previous racemic [3'-^{3}H]phenylalanine feeding (table 8) and in the protein phenylalanine from the time-study (table 10) respectively, do not represent retention of all the (3'S) label present. The enzymatic assay figures (tables 9 and 12 respectively) show that 8-10% of (3'R) label always remains. Thus some (3'S)-label is always lost, though to a much lesser extent than tritium in the (3'R)-configuration. Retention of (3'R) tritium may not necessarily indicate retention of the (3'R)-proton under natural circumstances. Retention may be favoured by an isotope effect, which would greatly lower the rate of exchange for (3'R)-labelled species, causing (3'R)-retention of unnatural magnitude. All that is demonstrated by the retention of (3'R)-tritium is that whatever the process responsible for (3'R)-proton removal, it is not essential to any stage of phenylalanine active-transport or metabolism; if it were essential, the (3'R)-tritiated species would either be entirely metabolised along with (3'R)-protium species, or excluded from metabolism altogether.

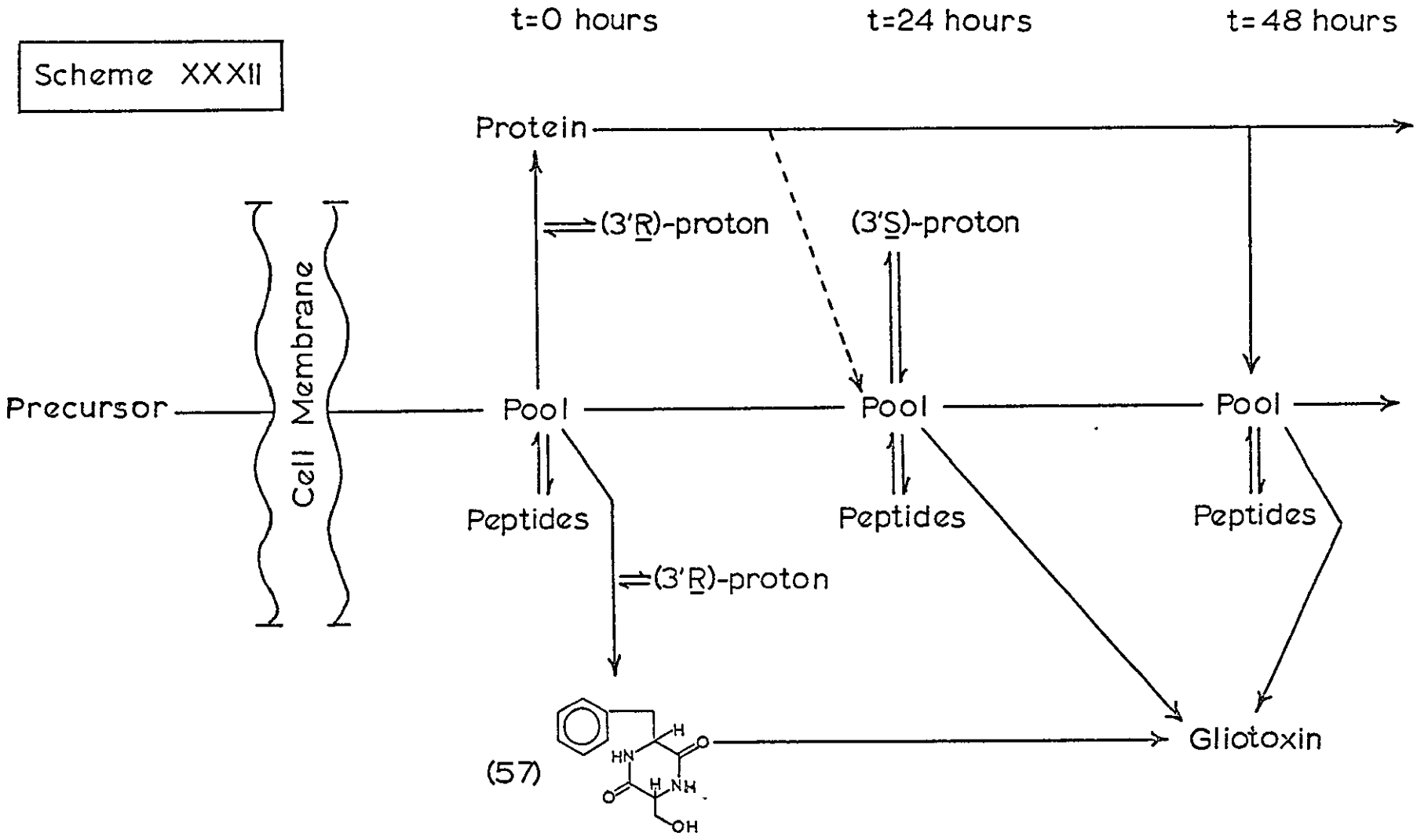
Another observation arising from the time-study is that the process responsible for removal of the (3'R) proton apparently operates on all phenylalanine entering

the cell but to a different extent in each case. For example after 24 hours the gliotoxin, and an ethanol-soluble dipeptide present in the pool both have a $^{3}H:^{14}C$ ratio of about 6·3. The protein phenylalanine, on the other hand has a $^{3}H:^{14}C$ ratio of about 4·9. Phenylalanine in the amino-acid pool has a ratio of about 3·5 (table 10).

No change in the $^{3}H:^{14}C$ ratio of the protein phenylalanine from subsequent samples is observed. All are about 4·9. The amount of ^{14}C activity contained in the protein phenylalanine appears to fall slightly from 24 hours onwards (table 11). Phenylalanine in the amino-acid pool appears to gain 3'-tritium from 24 hours onwards. This may be explained if some labelled phenylalanine from metabolic degradation of the mycelial protein passes into the amino-acid pool. At the same time a great deal of the ^{14}C activity from the pool phenylalanine is apparently lost. A comparable increase in activity in the form of gliotoxin occurs (table 11). The figure for gliotoxin - ^{14}C after 48 hours' growth may be in error because of the difficulty of isolating such a small quantity (16 mg) of the metabolite.

Protein production evidently carries on after 24 hours' growth, since the weight of mycelium obtained in subsequent samples goes on increasing, as does the quantity of material extractable from these samples with barium hydroxide (see Experimental Section). However, according to the carbon-14 incorporation figures (table 11) no phenylalanine from the amino-acid pool is used in protein synthesis after 24 hours' growth. These figures are not

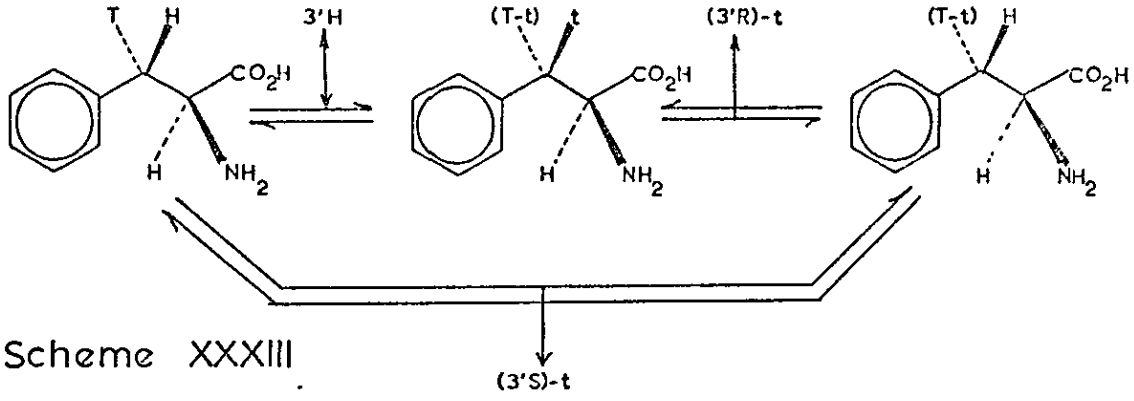
Scheme XXXII



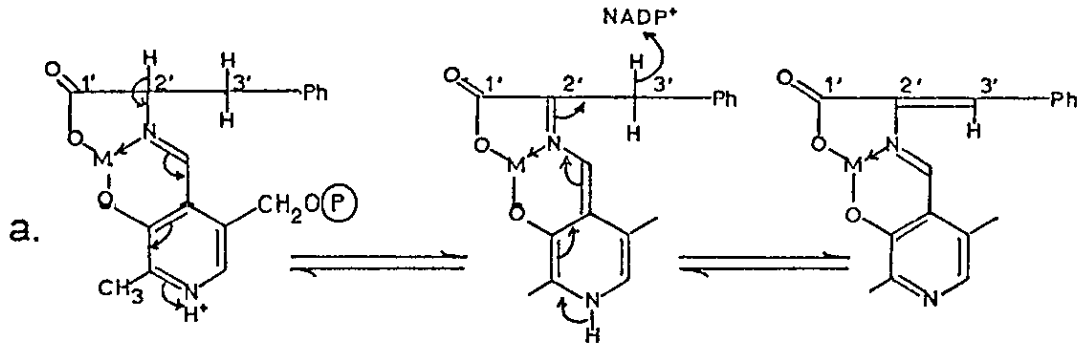
watertight however, since it cannot be known for certain how much if any phenylalanine remains in the mycelium after barium hydroxide extraction.

A scheme which summarises all the above-described phenylalanine interconversions is shown (Scheme XXXII). It is postulated that after passage through the cell wall, the precursor is converted immediately by different routes into protein and into L-seryl-L-phenylalanine anhydride (57). During these conversions the (3'R) proton is exchanged out at different rates. Phenylalanine in equilibrium with pool peptides loses successively more and more (3'S) proton. Finally about 48 hours from inoculation, gliotoxin production is inducted, for which all available (57) and pool-phenylalanine is used. This is a highly simplified picture, and no more than circumstantial evidence of the flimsiest kind is available to show the existence of (57). It will be noted that the $^{3}H:^{14}C$ ratio in the pool phenylalanine and in the pool peptides (water-soluble baseline fraction) after 24 hours are similar (about 3:1), as they should be if an equilibrium of this kind is to be postulated. Loss of (3'S)-tritium from the 24-hour pool phenylalanine could be explained by a non-stereospecific process (which would cause racemisation) in equilibrium with a stereospecific process (which would remove the (3'R) label) as well as by a straightforward stereospecific removal of the (3'S) proton (Scheme XXXIII).

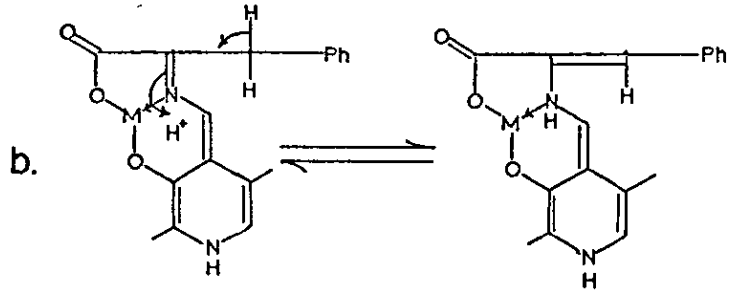
Only speculation is possible about the nature or purpose of the process in which the (3'-pro-R) proton is removed. Several reversible processes are however known which could cause loss of the 3'-protons of phenylalanine.



Scheme XXXIII



Scheme XXXIV



The best-documented are probably those involving the Schiff's base of the amino-acid with pyridoxal phosphate (Scheme XXXIVa and b)[140]. A part of this sequence has already been shown in connection with loss of the 2'-proton of phenylalanine. The first type of process (Scheme XXXIVa) is that responsible for the dehydration of serine or the cleavage of cystathionine by elimination[141]. Normally some good leaving group (water, hydrogen sulphide, homocysteine) is involved but it is possible that hydride removal by a nicotinamide or flavine nucleotide could occur.

Another possibility is enamine-ketimine tautomerism of the pyridoxal-Schiff's base (Scheme XXXIVb.). This could be either spontaneous or enzyme-mediated. The enzyme-mediated reaction should be stereospecific. The spontaneous reaction is favoured in that the enamine tautomer conjugates with both the carboxyl group, and with the phenyl group, but it involves breakage of conjugation with the pyridine ring. It could reprotonate stereoselectively, particularly if the whole complex is bound to a protein, as is generally the case. Intermediacy of a pyridoxal-bound intermediate is made more attractive by the finding that all the protons of alanine are quickly exchanged by incubation with alanine-glutamate transaminase in deuterium oxide[142].

Knox et al. have purified arylpyruvate keto-enol tautomerases which act on phenylpyruvate, 4-hydroxyphenyl-pyruvate[143] and indolylpyruvate[144]. The physiological function of such enzymes is not known and neither is the stereo chemistry of the reaction they produce. As well as arylpyruvates they will accept analogous amino-acids and it is possible that they catalyse the ketimine-enamine

tautomerism shown in Scheme XXXIVb, in Nature.

Pyridoxal phosphate has been implicated in a wide range of reactions involving amino-acids, such as transamination, racemisation, oxidative deamination and cleavage of various beta and gamma substituents. Interestingly, its implication in the active-transport of amino-acids through the cell wall has also been claimed[145]. Spontaneous non-stereospecific proton loss from the 3'-position of phenylalanine bound to pyridoxal phosphate, followed by stereoselective reprotonation, could possibly account for the observed losses and retentions of 3'-tritium.

An alternative type of reaction which could stereospecifically remove 3'-label from phenylalanine is the ammonia-lyase type of reaction already described. The ammonia-lyase reactions so far reported have all involved loss of the (3'S)-proton, so this is not a likely cause of 3'-proton loss in this case. However, reversibility of the ammonia-lyase reaction has been demonstrated[92], and there is the possibility that it could account for the removal of (3'S)-proton observed in the pool phenylalanine 24 hours after inoculation.

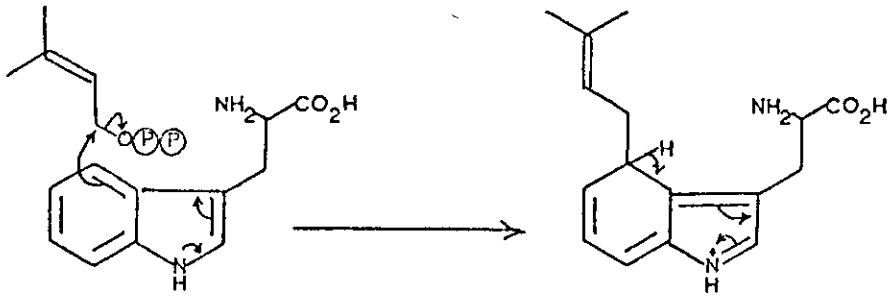
Section C

Biosynthesis of the Clavine Alkaloids

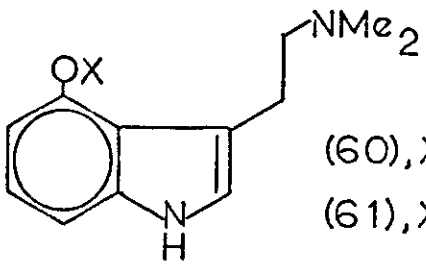
C.1 Introduction

Since it is well established that mevalonate and tryptophan are the starting materials in the biosynthesis of the ergoline skeleton,[147] an interesting question arises as to the actual mechanism by which the isoprene unit derived from mevalonate[148] is attached to tryptophan. Since electrophilic substitution of the indole ring at the 4-position by some mechanism such as in Scheme XXXV is rendered difficult by the low electron-density at this position, an early hypothesis was that dimethylallyl pyrophosphate reacted with 5-hydroxytryptophan, the 4-position being activated by the 5-hydroxy group. This postulate has been disproved by both Baxter[149] and Plieninger[150], who have shown that no loss of label occurs from, respectively, DL-[5-^{3}H]tryptophan or from DL-[5,6-$^{2}H_2$]tryptophan. Only one deuterium atom per molecule is lost from DL-[2,4,5,6,7-$^{2}H_5$]tryptophan and from DL-[4-^{2}H]tryptophan[150].

Since their discovery[151] and determination of the biosynthetic origin[152] of psilocin and psilocybin (60) and (61), Hofman et al. have postulated that a hydroxy or pyrophospho-group at the 4-position of tryptophan may be responsible for indole-isoprene bond formation. A "tail-to tail" condensation is proposed, (see Scheme XXXVI) in the manner of sqalene biosynthesis. However, displacement of an aryl pyrophosphate group is mechanistically unattractive and 4-hydroxylation of the indole ring would seem to imply an N.I.H. shift. This is not observed[150] and Plieninger[153] supports a mechanism in which tryptophan itself participates.



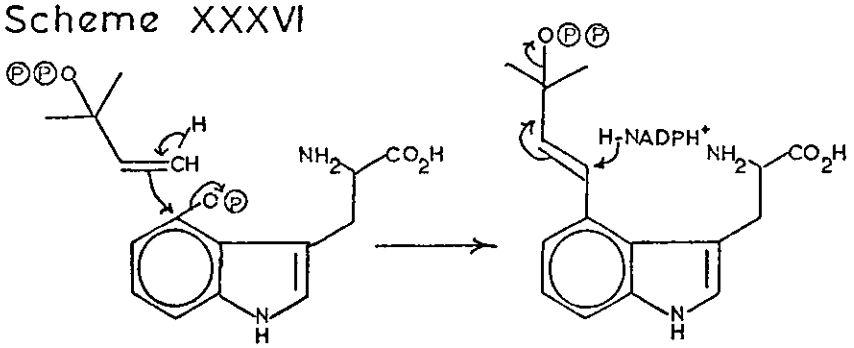
Scheme XXXV

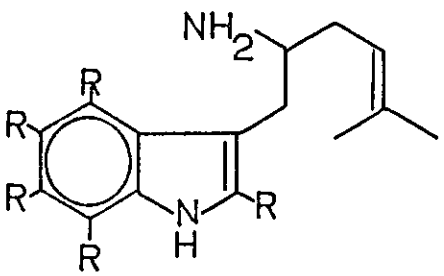


(60), X=H, Psilocin

(61), X=PO_3H_2, Psilocybin

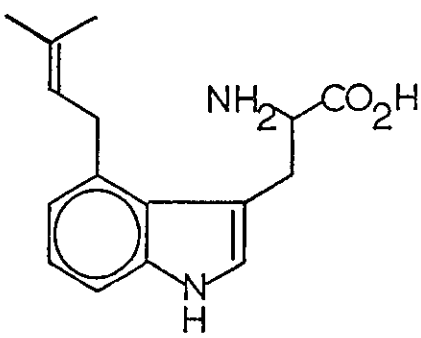
Scheme XXXVI





(62) R=H, 1'-Dimethylallyl-tryptamine

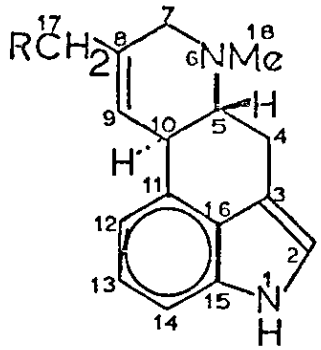
(63) R=T, Weygand's precursor[154]



(64)

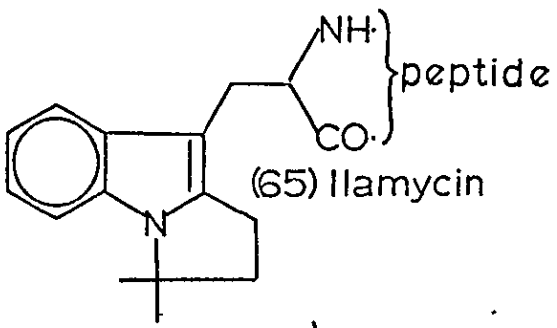
Another mechanism, proposed by Weygand et al.[154] was based on the observation by Gröger et al.[155] that pyridoxal phosphate greatly increases the rate of biosynthesis of clavine alkaloids from tryptophan. Weygand et al.[154] put forward the intermediate 1'-dimethylallyltryptamine (62) and obtained substantial incorporation of 1-(3-[2,4,5,6,7-$^{3}H_{5}$]indolyl)-2-amino-5-methylhex-4-ene (63) into elymoclavine (59). However Plieninger, feeding ^{14}C-labelled 4-dimethylallyltryptophan (64) also obtained very good incorporation into this alkaloid[153]. Weygand et al. have since published the results of competitive experiments showing that ^{3}H-labelled 1-dimethylallyltryptamine is incorporated less readily into clavine alkaloids than ^{14}C-labelled 4-dimethylallyltryptophan. However, both these compounds were incorporated less readily into elymoclavine than was tryptophan itself[156]. These workers have also shown that 4-hydroxytryptophan is apparently not incorporated into the clavine alkaloids[157] thus rendering the theory of Hofmann involving a "psilocybinoid" intermediate still more untenable.

In a recent paper by Bycroft and Landon[158], a totally new mechanism has been postulated which accounts for the introduction of the dimethylallyl group into the 4-position of tryptophan during the biosynthesis of the ergoline skeleton. The same theory can also account for the dimethylallyl groups at the indolic 1 and 2 positions in ilamycin (65) and echinulin (66). It was to test Bycroft's hypothesis (see Scheme XXXVII) that the work in this section was carried out.

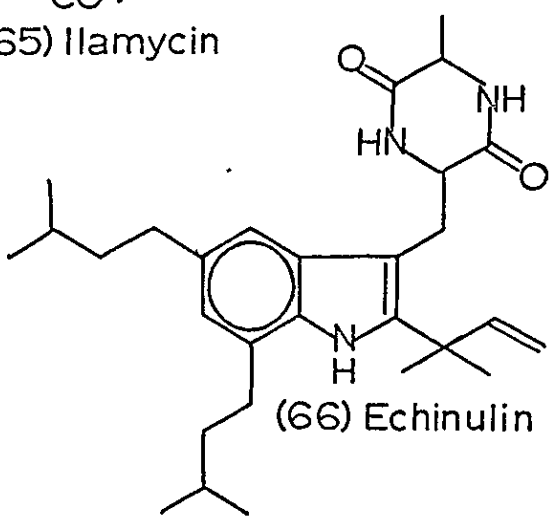


(58), R=H, Agroclavine

(59), R=OH, Elymoclavine

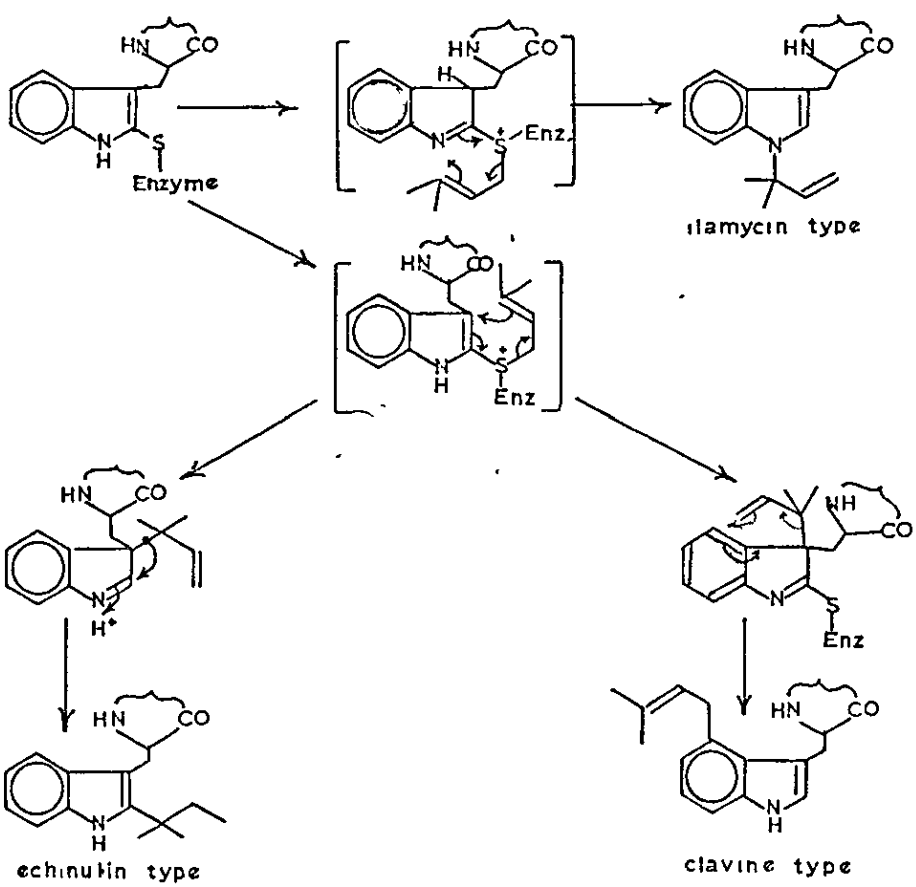


(65) Ilamycin



(66) Echinulin

Scheme XXXVII - Bycroft's hypothesis[158]



C.2 Feeding experiments

Two clavine-producing strains of Claviceps purpurea have been adapted from the original wild strain obtained by Hofmann from the African millet Pennisetum typhoideum Rich.[117] These are Tyler's strain (47A)[159] and Gröger's strain (SD58)[160]. Although Tyler's strain (47A) was kindly supplied by Dr. J. Woolley (Pharmacy Dept., The Polytechnic, Leicester), the only applicable method of work-up (preparative chromatography on impregnated paper) is rather clumsy and tedious. It was more practicable to work with the wild strain, supplied by Dr. A. Hofmann (Sandoz, Basle). Hofmann's culture technique[117] was used, with a slightly modified medium[115]. The clavine alkaloids were extracted with chloroform/isopropanol (3:1) and the crude extract separated on a column of neutral alumina as described by Hofmann[114].

Unlabelled agroclavine and elymoclavine, obtained in this way, were identified by their melting points, specific rotations and by their strong violet colour test with Ehrlich's reagent. All these properties were in good agreement with those reported by Hofmann. The yields of pure alkaloid were extremely variable; only in an early unlabelled incubation were the yields of 0·7 g/litre of each alkaloid, which Hofmann quotes, attained. Yields in the labelled runs appear in Table No. 13. The explanation for the highly variable yields must be that it was impossible to maintain the culture temperature at the 24°C. mentioned by Hofmann. Normal indoor summer temperatures of 25-30°C. gave much poorer yields than the lower temperatures (15-25°C.) prevailing in winter. Since the

alkaloids are sensitive to light, the cultures were kept in the dark as far as possible.

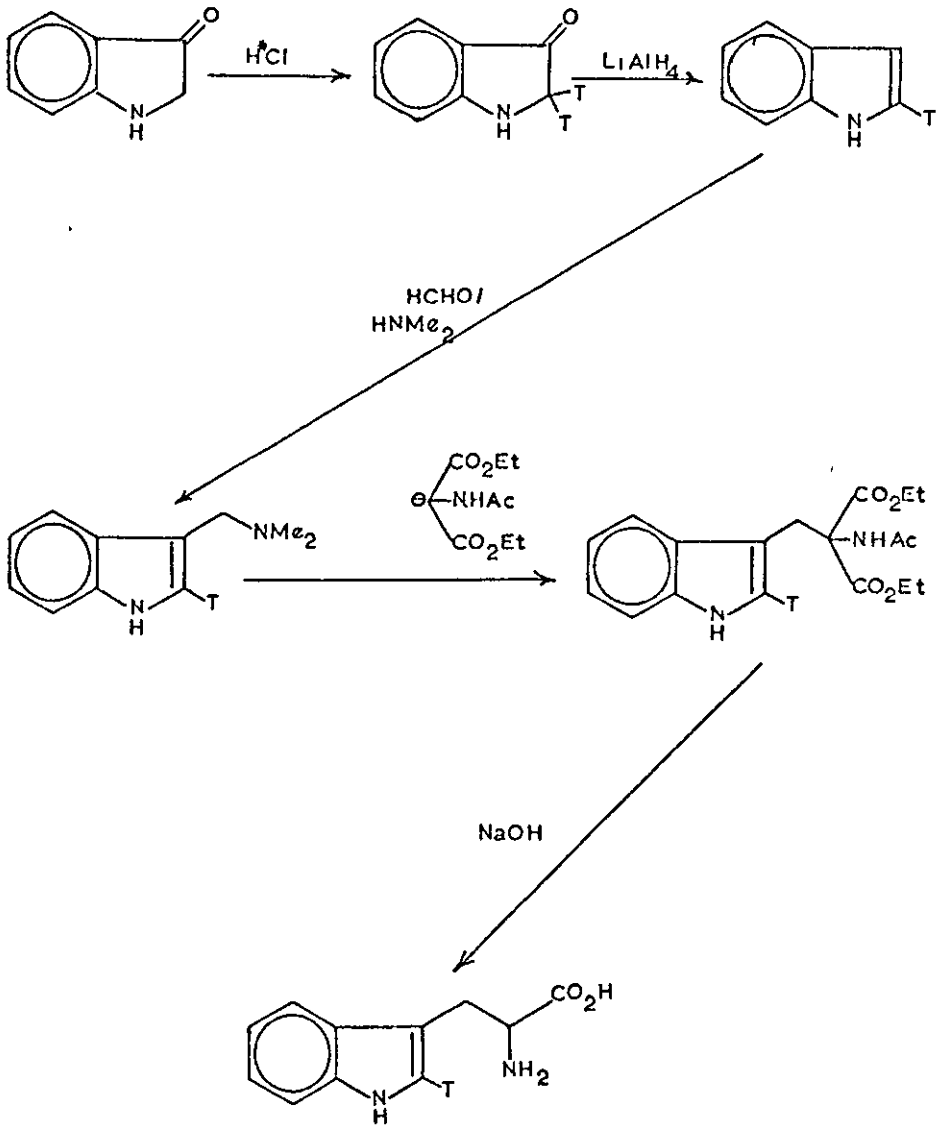
DL-[2-^{3}H]Tryptophan prepared by Dr. S.W. Shah[161] was used for the crucial feeding experiment. That the tritium was located entirely in the indole-2-position had been adequately demonstrated by Shah by degradative procedures, as well as by the unambiguous synthetic route (Scheme XXXVIII). Before use, its specific activity was measured and found to be in reasonable agreement with Shah's value[162], allowing for tritium decay. Chemical and radiochemical purity of the sample were established by running a paper chromatogram, scanning it, and developing with ninhydrin reagent.

Two feeding experiments were carried out in this series. Firstly DL-[3'-^{14}C]tryptophan was administered in order to establish that incorporation was possible under the conditions used. DL-[3'-^{14}C,2-^{3}H]Tryptophan was then fed under similar conditions. The results of the two feedings are shown in table 13 below:

Table 13

Precursor	Acty.*	^{3}H:^{14}C	Alkaloid	Yield	Acty.*	^{3}H:^{14}C	incorp
DL[3'-^{14}C]-tryptophan	9	-	Agro-clavine	450mg	3·6	-	39·5%
			Elymo-clavine	250mg	1·0	-	11·0%
DL[3'-^{14}C,2-^{3}H]-tryptophan	9	5·5:1	Agro-clavine	120mg	0·8	5·3:1	8·9%
			Elymo-clavine	230mg	0·3	5·6:1	3·3%

* total ^{14}C activity; μCi



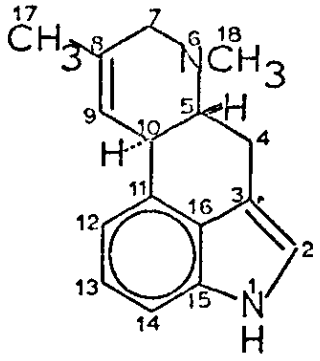
Scheme XXXVIII

Retention of 97% and 101% tritium relative to carbon-14 has occurred during the biosynthesis of agroclavine and elymoclavine, respectively. If the mechanism of introduction of the isopentenyl group into the indole nucleus proceeds *via* Bycroft's hypothetical indole-2-thioether intermediate, total displacement of hydrogen at the indole 2-position would be expected. Thus the above results make Bycroft's theory[158] unlikely at first sight. However, the possibility of total migration of tritium to some other point in the molecule, makes it desirable to establish the position of the tritium in the agroclavine and elymoclavine. A degradation has been sought which specifically removes hydrogen from the 2-position of agroclavine, this being the easier of the two alkaloids to work with.

C.3 Degradative Procedures

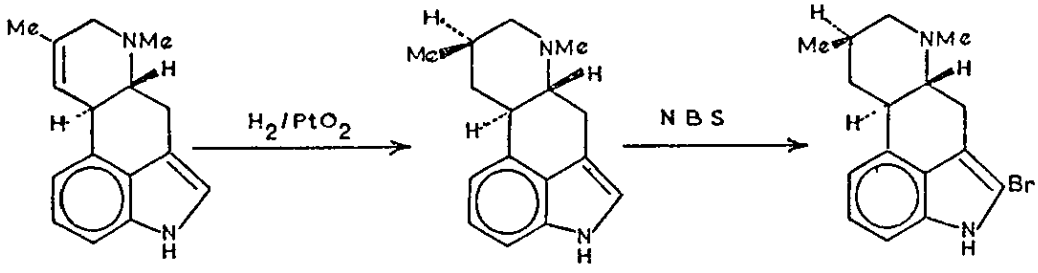
A suitable reaction appeared to be that with N-bromosuccinimide (N.B.S.). Troxler and Hofmann[116] report that treatment of D-lysergic acid NN-diethylamide with this reagent gives the 2-bromo compound in good yield. The 2-bromo-derivative of a 3-substituted indole is easily detected as it gives no colour test with Ehrlich's reagent (p-dimethylaminobenzaldehyde)[116,164].

When agroclavine was treated with a molar equivalent of N.B.S., the well defined product still gave a strong Erlich colour test. N-Bromosuccinimide reacts by a free-radical mechanism with compounds containing an allylic or benzylic hydrogen atom[164]. Inspection of the agroclavine molecule (58) reveals benzylic hydrogens at C-4, allylic hydrogens at C-7 and C-17, and a hydrogen at C-10 which is both allylic and benzylic. At least three atoms of bromine apparently needed to be incorporated into agroclavine before the 2-position was attacked. Characterising and counting the labelled compounds between successive brominations to establish at which stage the tritium was lost, was not practicable with the amount of material available. Since catalytic reduction of agroclavine over platinum oxide is reported to give the single product, festuclavine (67)[125], it was hoped to prepare labelled festuclavine in this way and then brominate the 2-position using N.B.S. It was found that N-acetyl-[3'-^{14}C,2-^{3}H]-tryptophan did not lose tritium when stirred as a methanolic solution with platinum oxide in an atmosphere of hydrogen for 24 hours. Thus the indole-2-position is not labile under these conditions. However, in contrast to the



(58) Agroclavine

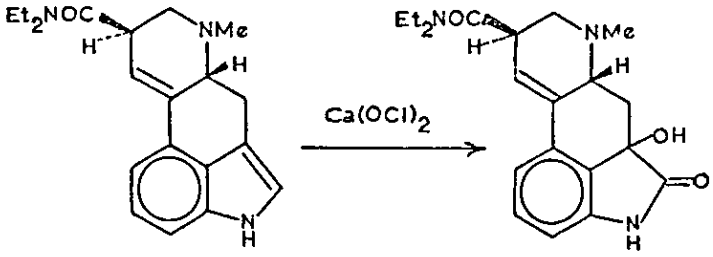
Scheme XXXIX



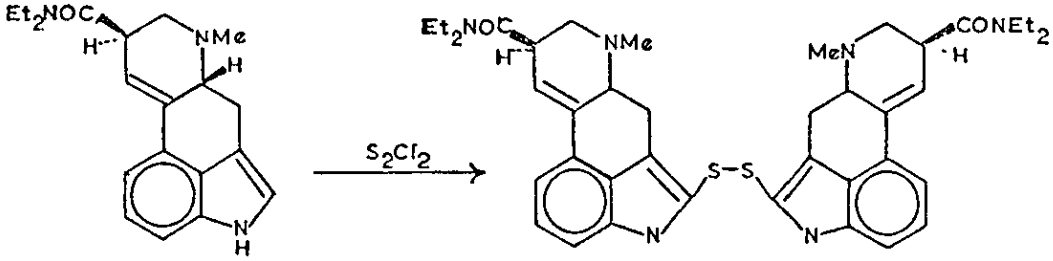
(58)

Festuclavine
(67)

Scheme XL



Scheme XLI



experience of Agurell and Ramstad[125], only agroclavine could be detected with certainty in the reaction mixture after hydrogenating for 24 hours, and this approach (Scheme XXXIX) had to be abandoned.

The failure of the N-bromosuccinimide reaction also ruled out the possibility of using hypochlorite oxidation of the indole ring. Troxler and Hofmann have reported that derivatives of lysergic acid may be oxidised to the corresponding dioxindole compounds (see Scheme XL) using calcium hypochlorite solution[165]. However, this reagent would be expected to react preferentially with the C-9,10 double bond in agroclavine. The double bond in lysergic acid is protected from attack by its hindered position and by conjugation with the indole nucleus.

Another line of approach was represented by the reaction of lysergic acid diethyl amide with di-sulphur dichloride reported by Witkop et al.[119] The reaction is represented in Scheme XLI. Cleavage of the bis-(indol-2-yl)disulphide to give the thio-oxindole is accomplished using zinc in acetic acid.[166] However all attempts to prepare the bis-(indole-2-yl)disulphide of model compounds gave tars, in which only starting material could be detected by thin-layer chromatography. The model compounds used were N-acetyltryptophan, gramine and two analogues of gramine prepared by the Mannich reaction of indole with formaldehyde and, respectively, piperidine[122] and morpholine[123]. Even when reaction conditions other than those of Witkop[120,121] were used, no product of the reaction was isolable by thin-layer chromatography. N-Acetyltryptophan was used as a model compound because its colour with Ehrlich's

reagent is an unmistakeable deep violet; that of gramine and its analogues is a pale pink. Treatment of N-acetyltryptophan with di-sulphur dichloride gave an Ehrlich-positive crude product under all sets of reaction conditions. The reaction was abandoned.

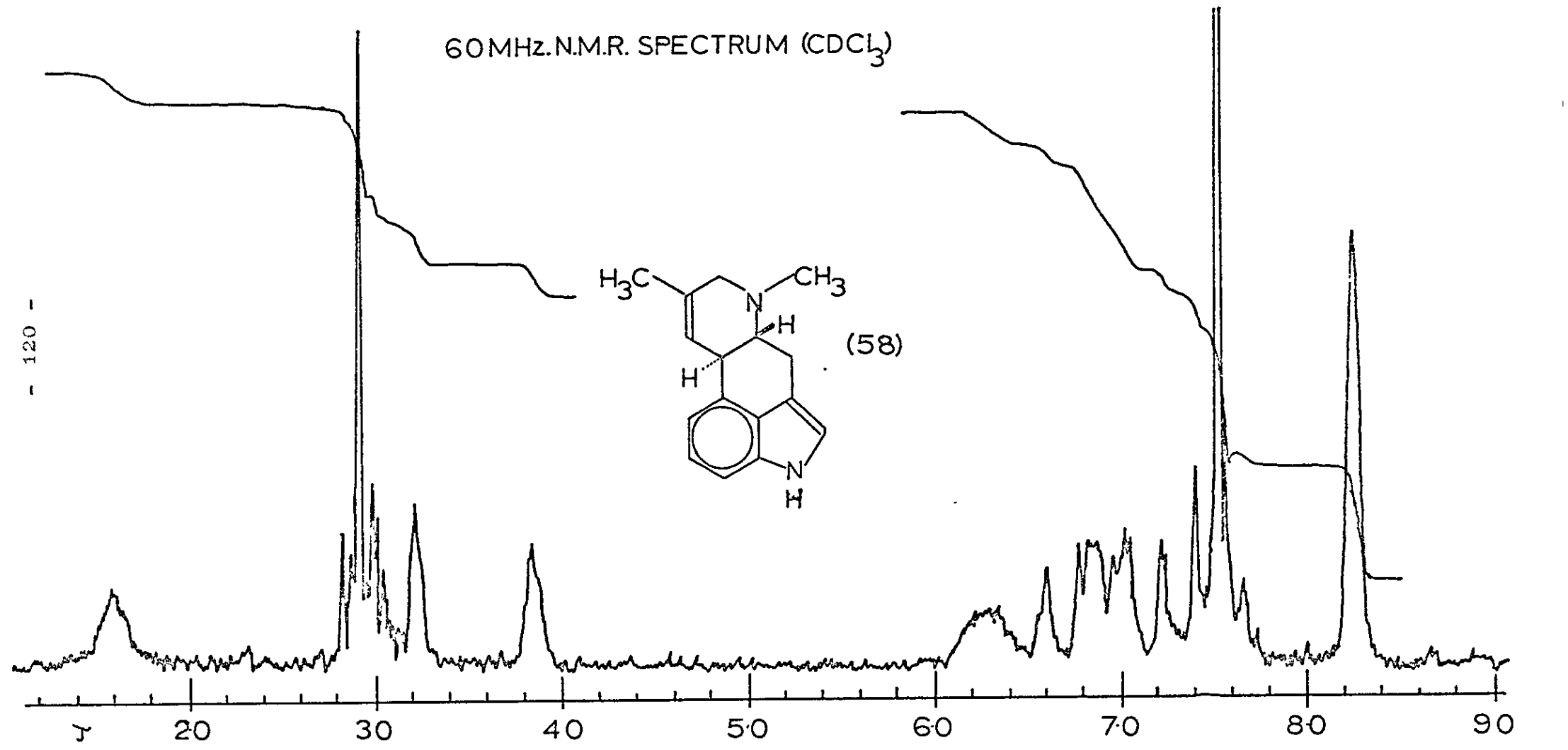
Many other reactions which substitute the 2-position of indoles also attack at some other position in the indole ring. Such reagents as N-bromophthalimide in benzene[167], and N-bromosuccinimide in aqueous acetic acid[168] have not been tried because they are reported to attack the 5 or 6 position of the indole nucleus.

In a more recent paper, Bak et al.[124] have described the hydrogen-deuterium exchange of the ring positions in tryptophan. These workers have claimed that the 2-proton in tryptophan is exchanged much more rapidly than the other ring protons in undiluted deuteriated trifluoroacetic acid at 40°C., and it was hoped that the 2-position of agroclavine could be exchanged in this way. When tryptophan was treated with deuteriotrifluoroacetic acid under the conditions described by Bak et al.[124] for 30 minutes, the integral of the aromatic protons decreased from 5 to 4, and a sharp peak at τ2·8 in the middle of the aromatic region disappeared.

When unlabelled agroclavine was treated with deuteriotrifluoroacetic at 40°C., n.m.r. resolution was so poor that no acceptable spectrum could be run. It was necessary to re-isolate the alkaloid by column chromatography and then run the n.m.r. spectrum in deuteriochloroform. Spectra of agroclavine before and after treatment with deuteriotrifluoroacetic acid are shown (fig. 13 and 14).

fig.13-AGROCLAVINE (58)

60MHz. N.M.R. SPECTRUM ($CDCl_3$)

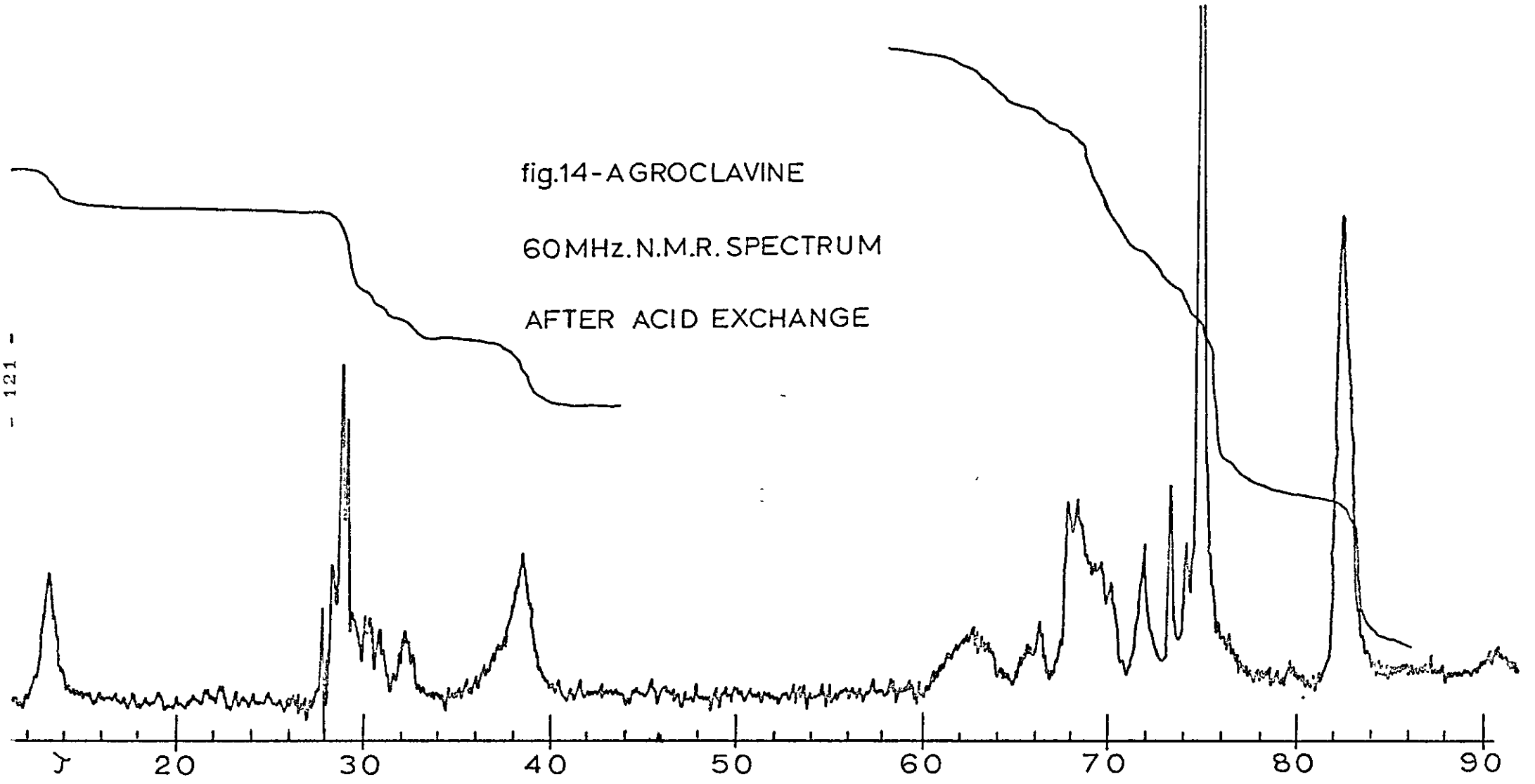


(58)

fig.14 - AGROCLAVINE

60 MHz. N.M.R. SPECTRUM

AFTER ACID EXCHANGE



The aromatic protons are to be found in the region τ2·70-3·30, and the sharp peak at τ3·19 in the unlabelled spectrum is presumably the indole-2-proton. The integral over the aromatic region of the deuteriated spectrum is 2·7. Assignments of the other peaks are given in the Experimental Section.

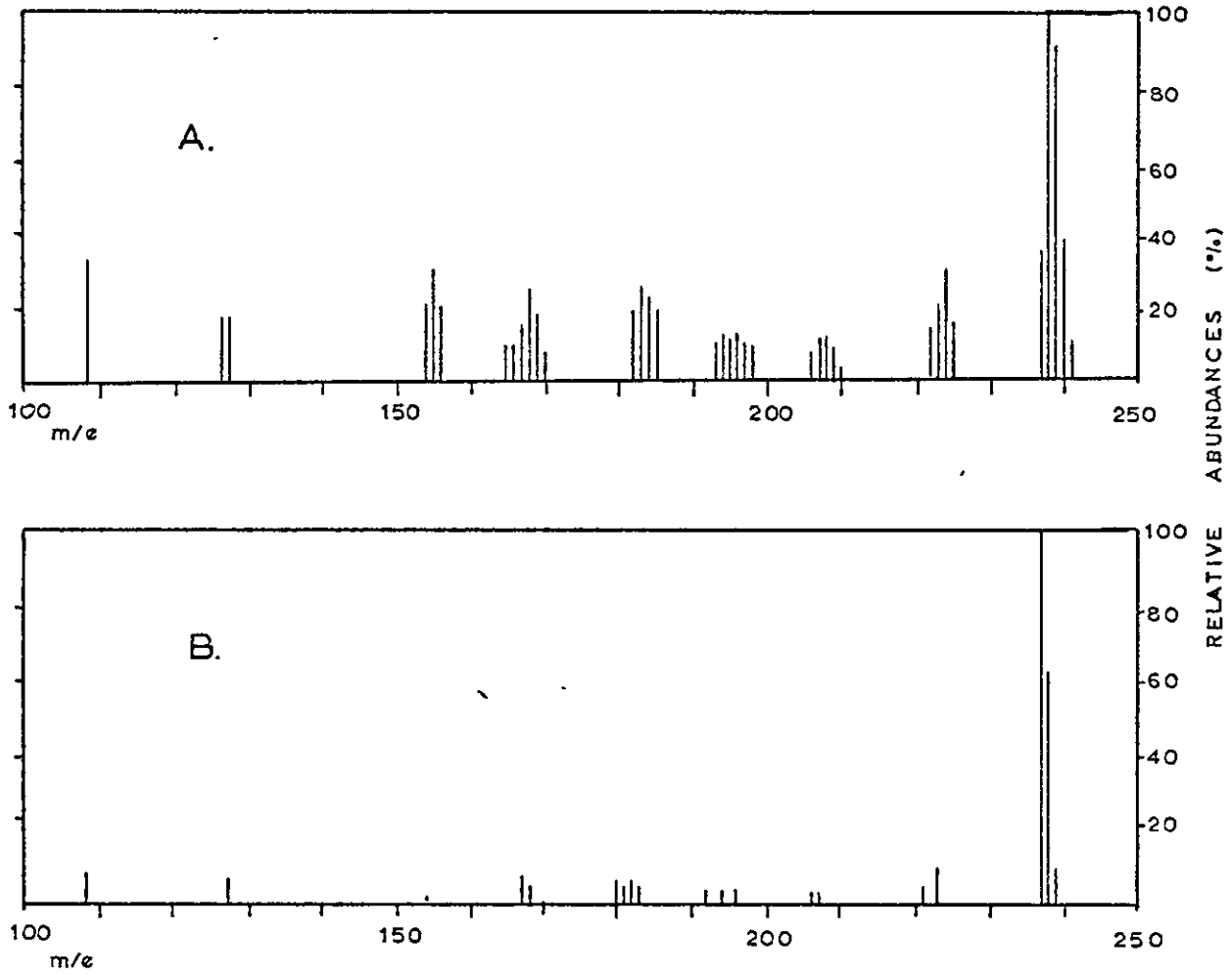
Graphical representations of the mass spectra of agroclavine and deuterium-exchanged agroclavine are shown (fig. 15). The pattern in the region of the molecular ion is complicated by a strong $(M-1)^+$ peak, but it is still evident that a considerable proportion of di-deuteriated as well as mono-deuteriated species are present. Actual percentages of mono-, di- and tri-deuteriated molecules present have been calculated from the peak heights of the M^+, $(M+1)^+$ and $(M-1)^+$ ions (a) assuming that only loss of protium was possible, to give a $(M-1)^+$ but no $(M-2)^+$ ion and (b) assuming that deuterium scrambling took place throughout the molecule and that deuterium loss was predictable on a basis of chance. The calculations are presented in the Experimental Section together with assignments of some of the mass-spectra peaks. Percentages of the various deuteriated species calculated by each method, are tabulated in table 14.

Table 14

Method	d_0	d_1	d_2	d_3
a	23·3%	47·7%	22 3%	6·7%
b	20 2%	55·0%	16·7%	8·1%

fig. 15

COMPARATIVE MASS SPECTRA OF DEUTERIATED (A) & (B) UNDEUTERIATED AGROCLAVINE



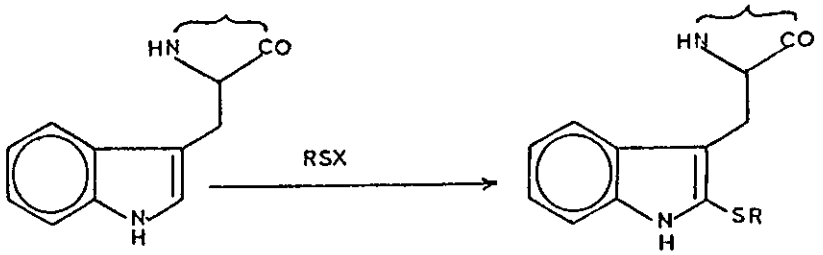
That there is a significant percentage of dideuterioagroclavine present is clear from the number of peaks in the unlabelled spectrum which have corresponding peaks two mass units higher in the labelled spectrum e.g. at m/e 223 (225), m/e 221 (223), m/e 196 (198), m/e 194 (196) etc.

Hydrogen-deuterium exchange was eventually not used to degrade labelled agroclavine, since the positions of the deuterium substitution cannot be ascertained exactly.

Mr. R. Pinchin and Dr. B.W. Bycroft (Nottingham University)[169] have also investigated a number of reagents known to attack indoles or pyrroles in the hope that these might be applicable to the degradation of agroclavine.

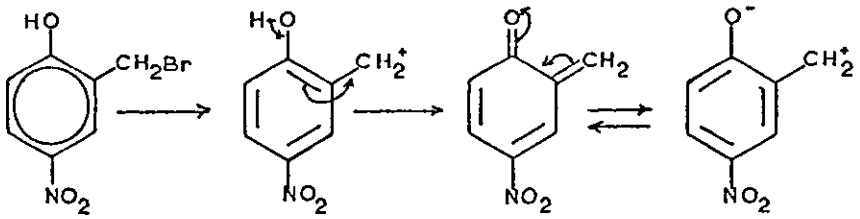
Following on from the work with di-sulphur dichloride described above, Pinchin and Bycroft tried the reaction of methylsulphenyl chloride[170] (Scheme XLII R = methyl, X = Cl) but without obtaining any product in good yield. It was assumed that in the 2-methylthioindole system, as well as in the bis-indole-2-yl disulphide system previously mentioned, back-donation of non-bonding electrons by the sulphur to the indole may account for still greater nucleophilic character, and hence instability in the ring, than is observed in unsubstituted indoles. If the group attached to sulphur at the indole-2-position were strongly electron-withdrawing in character a more stable indole-2-thioether might result. 2,4-Dinitrophenylsulphenyl chloride (Scheme XLII R = 2,4-dinitrophenyl, X = Cl) has been reported to give a stable compound with tryptophan in proteins and peptides[171]. However, when gramine was treated with this reagent, a product was obtained in which

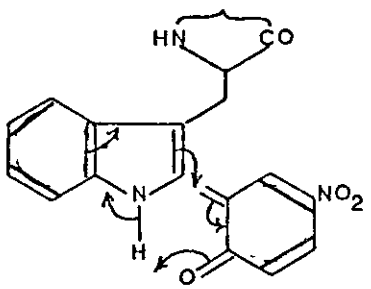
Scheme XLII



(i) R = Me , X=Cl
(ii) R = 2,4-dinitrophenyl , X = Cl
(iii) R=CN, X=SCN

Scheme XLIII - Koshland's reagent





one methyl group per molecule appeared to have been lost (n.m.r.). This product contained a 2,4-dinitrophenyl group (n.m.r.) but its position could not be ascertained.

A recent paper[172] describing attack by thiocyanogen bromide at the meso-position of porphyrins led us to the hope that the indole-2-position of agroclavine might be attacked in the same way to give an indole-2-thiocyanate. Thiocyanogen bromide is known to react readily with pyrroles[173] to give ring-substituted thiocyanates. However, Pinchin and Bycroft report[169] that when gramine is treated with thiocyanogen, formed in situ by the action of bromine on lead (II) thiocyanate[172], only tars were produced.

Koshland[174] has reported a highly specific reaction of tryptophan in peptides and proteins with 2-hydroxy-5-nitrobenzyl bromide. This reagent readily forms a quinone-methide structure[175] which substitutes the 2-position of tryptophan (see Scheme XLIII). Reaction of Koshland's reagent with gramine in acetone gave a yellow oil which turned to a deep brown tar. Only a streak, without succinct spots, was observable on thin layer chromatography.

Section D

Experimental

Notes

(i) Melting points were determined on a Kofler heated-stage apparatus.

(ii) All solvents were distilled before use.

(iii) Absolute chloroform was prepared by washing the technical grade with 2 x its volume of water, followed by distillation from calcium chloride. (A.I. Vogel, Practical Organic Chemistry, 2nd ed. p.174, Longmans Green, London 1951) Solvent purified by distillation from P_2O_5 tends to cause decomposition of clavine alkaloids.

(iv) A Pye Unicam bench pH meter calibrated with buffer solution according to the instruction manual, was used for all accurate pH measurements, as indicated.

(v) Neutral alumina is indicated wherever alumina is used for chromatography.

(vi) A Gallenkamp type 1H400 thermostatic orbital incubator was used for cultures of T. viride.

(vii) All paper chromatograms were developed in a solvent consisting of n-butanol, 6; glacial acetic acid, 2; water, 2. Descending chromatography was used for all preparative papers, ascending for analytical papers.

(viii) Spots of amino-acids were revealed by spraying with minhydrin reagent (0·1% w/v in acetone, 1; n-butanol; 1) and heating at 100°C. in an oven for 5 minutes.

(ix) Wherever "light petroleum" is mentioned, the fraction boiling between 60-80°C. is indicated.

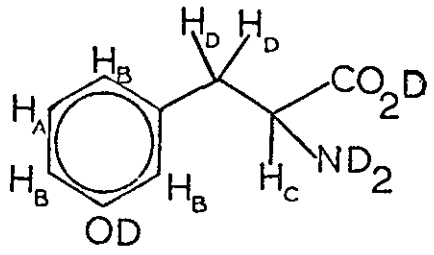
(x) d/min., or disintegrations per minute refers to the number of heavy-isotope nuclei disintegrating per

minute. Correction to this figure from observed counts/minute is carried out using an efficiency value predetermined with the aid of a standard compound of known d/min. One millicurie has been taken throughout to be $2{\cdot}22 \times 10^9$ d/min. Occasional reference to "counts per minute" indicates the disintegrations per minute registered by the Beckmann instrument without correction for efficiency.

D.1 Ring-Labelling of m-Tyrosine and Phenylalanine

DL-[2,4,6-$^2H_3$]-m-Tyrosine

DL-m-Tyrosine (50 mg), obtained from Koch-Light Ltd., Colnbrook, Bucks., was dissolved in a solution of thionyl chloride (148.7 mg) in deuterium oxide (0.5 ml), this being equivalent to 5.0 N-deuterium chloride. The amino-acid solution was transferred to an n.m.r. tube and flushed with dry nitrogen to remove SO_2. The tube was sealed and an n.m.r. spectrum immediately run. Spectra were subsequently run after various periods of heating the tube in a bath of boiling water. The spectrum after fifteen minutes heating (b) is compared with that run immediately after sealing:



τ	form		integral		assigned
	a.	b.	a.	b.	
2·67	(t)	(s)	1·08	1·06	H_A
3.09	(d)	-	2·96	0·49	H_B
5·55	multiplet		1·11	1·11	H_C
6·72	multiplet		1·85	1·85	H_D

After 16 hours' heating (see other figures in Section A) the tube was opened and the m-tyrosine solution evaporated down to dryness. Water was added to the residue and the solution evaporated to dryness again. This was

repeated a total of three times to remove readily exchangeable deuterium. The residue was finally dissolved in the minimum of water, neutralised with ammonia (s.g. 0·880), filtered, and evaporated down to incipient crystallisation when about 2ml. ethanol was added. Crystallisation occurred when the solution was left overnight in the refrigerator.

Recovery - 16·5 mg ; 33%

m.p. - 261-5°C(d) (lit.[100] 275°C.(d))

DL-[2,4,6-$^3H_3$]-m-Tyrosine

m-Tyrosine (100 mg) was dissolved in a solution of thionyl chloride (297·5 mg) in tritiated water (1·0 ml; 200 mCi), this being equivalent to 5N-tritiated hydrochloric acid. The amino-acid solution was transferred to a Carius tube and the SO_2 removed with a stream of dry nitrogen. After sealing and heating in a boiling-water bath for 1 hour, the tube was cooled, opened and the contents evaporated to dryness in vacuo. Readily exchangeable tritium was removed by repeatedly taking up the residue in water and evaporating down again (4 times). Finally the residue was dissolved in a minimum of water, neutralised reduced to near-crystallisation and precipitated with ethanol as in the previous experiment. The procedure was carried out twice altogether (a and b).

a. Recovery 45·2% ; Activity 1·795 mCi/mmole; m.p.263·8°C.

b. Recovery 54·1% ; Activity 2·310 mCi/mmole; m.p.264·9°C.

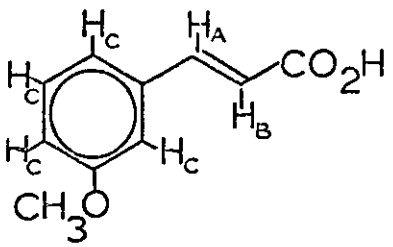
m-Methoxycinnamic acid

DL-m-Tyrosine (100 mg; 0·55 mmole), from the same batch as was used for the exchange labelling (above), was dissolved in 10% sodium hydroxide solution (10 ml) and

stirred in an ice-bath while dimethylsulphate (500 mg; 3·95 mmole) was added in 0·1 g aliquots over 1 hour. The mixture was stirred for a further hour at room temperature and then refluxed for 3 hours. The m-methoxycinnamic acid was precipitated from the cooled reaction mixture by acidification with concentrated hydrochloric acid filtered off and recrystallised from water.

Yield 63.30 mg; 0.39 mmole; 64.4%

m.p. 112-113°C. (lit. [101] 117-118°C.)



n.m.r. spectrum.

($CDCl_3$)

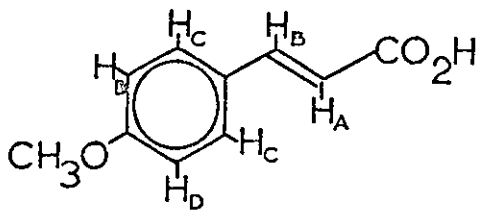
τ	signal	integral	J	assigned
6·19	singlet	3 protons	-	$-OCH_3$
3·63	doublet	1 proton	15·4Hz	H_B
2·98	multiplet	4 protons	-	H_C
2·70	singlet	1 proton	disappears in D_2O-CO_2H.	
2·40	doublet	1 proton	15·4Hz	H_A

p-Methoxycinnamic acid

The preparation was as for m-methoxycinnamic acid, starting from L-p-tyrosine (100 mg) (Koch Light Ltd., Colnbrook, Bucks.). Recrystallisation of the product was from ethyl acetate.

Yield 22·0 mg; 0·13 mmole; 24·4%

m.p. 172-173°C. (lit.[101] 170-174°C.)



n.m.r. spectrum

τ	signal	integral	J	assigned
6·17	singlet	3 protons	-	$-OCH_3$
3·70	doublet	1 proton	15·4Hz	H_A
3·09	doublet	2 protons	8·6Hz	H_D
2·51	doublet	2 protons	8·6Hz	H_C
2·58	singlet	1 proton	-	$-CO_2H$ disappears in D_2O.
2·35	doublet	1 proton	15·4	H_B

o-Methoxybenzaldehyde

Salicylaldehyde (5g; 41 mmole) was dissolved in 10% NaOH (40 ml) and the solution stirred in ice while dimethyl sulphate (10g; 80 mmole) was added in small portions over a period of 2 hours. The reaction mixture was stirred at room temperature for a further hour and finally extracted

with chloroform (3 x 25 ml). After drying the extract over $MgSO_4$ the chloroform was removed *in vacuo* and the residual oil was used to synthesise *o*-methoxycinnamic acid, without further purification.

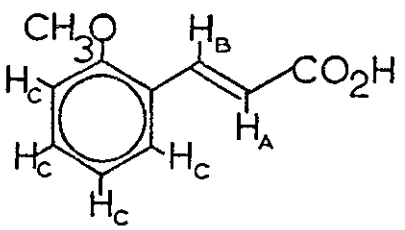
Yield 4·5 g ; 33 mmole; 80%

o-Methoxycinnamic acid[82].

o-Methoxybenzaldehyde (4.5 g; 33 mmole), malonic acid (6·9 g; 66 mmole), anhydrous sodium sulphate (5g), piperidine (6g) in dry pyridine (50 ml) were heated in an atmosphere of dry nitrogen for 2 hours, after which time the reaction mixture was refluxed for a further 2½ hours. The cooled solution was poured into water (1 litre) and extracted with chloroform (4 x 50 ml). The chloroform extract was dried over $MgSO_4$ and the solvent removed *in vacuo*. The residue was recrystallised from ethanol:

Yield 0·6g ; 3.65 mmole; 11%

m.p. 185-187 (Lit.[101] 185-6°C.)



n.m.r. spectrum

τ	integral	signal	J	assigned
6·14	3 protons	singlet	-	$-OCH_3$
3·51	1 proton	doublet	16·3Hz	H_A
3·12-2·48	4 proton -	multiplet		H_C
2·00	1 proton	doublet	16·3Hz	H_B
1·34	1 proton	broad singlet	-	$-CO_2H$

Tritium Exchange from DL-[2,4,6-$^3H_3$]-m-tyrosine

The following experiment was conducted in duplicate. DL-[2,4,6-$^3H_3$]-m-tyrosine (ca.2·0 mg; 100μCi), commercial DL-[1'-^{14}C]phenylalanine (10μCi) and phenylmercury nitrate (2 mg) were dissolved in about 30 ml water, brought to pH3·0 (pH meter) with hydrochloric acid, and transferred to a 50 ml standard flask. The solution was then made up to the mark with deionised water, and sterilised by filtration. Aliquots of 10 ml were transferred to sterile McCartney bottles using a sterile pipette. The McCartney bottles were then sealed and maintained at 30°C. in an incubator, without shaking.

At approximately 2-day intervals, the bottles were opened in a sterile cupboard, and 1ml-aliquots were removed from each. The individual aliquots were then neutralised, evaporated *in vacuo* without heating, transferred to scintillator bottles in solution in ethanolic hydrochloric acid and counted in the usual way.

Despite slight variations due to the presence of some inactive material (e.g. phenylmercury nitrate) the following table shows great stability in the ^3H:^{14}C ratio throughout the experiment.

Hrs.	Series A		Series B	
	*mean ^3H Count/min.	^3H:^{14}C Ratio	*mean ^3H Count/min.	^3H:^{14}C Ratio
3	114,513	11·06;1	72,852	7·39;1
77	205,309	11·45;1	103,480	7·24;1
124	245,498	10·10;1	135,330	6·79;1
173	244,406	10·81;1	149,199	7·08;1
245	306,138	11·04;1	163,414	7·50;1

*Uncorrected disintegrations/minute counted by Beckmann instrument.

[3-^{3}H]Toluene[83]

Commercial m-bromotoluene (B.D.H. Ltd., Poole, Dorset), guaranteed to contain not less than 99·7% of the meta isomer, was used. This material (2·57 g; 15 mmole) was dissolved in dry ether (40 ml) in oven-dried apparatus. An excess of lithium metal (250 mg) was added and the reaction mixture stirred under an atmosphere of dry nitrogen. Formation of a white, cloudy precipitate of lithium bromide indicated that the reaction was in progress. After three hours tritiated water (0.5 ml; 27·8 mmole; 100 mCi) was added by syringe through a serum cap. Stirring was continued for twenty minutes, after which time an excess of water was added in order to destroy any remaining lithium. The reaction mixture was then extracted with ether (3 x 25 ml) and the extracts dried ($MgSO_4$). The ether was fractionated off through a Vigreux column; when the still head reached 40°C. carbon tetrachloride (20 ml) was added and a little of it distilled over at 80°C. in order to remove all the ether. The residual solution was made up to exactly 20 ml with carbon tetrachloride and its concentration measured by n.m.r., comparing the integrals with those of a standard 5% w/vol. toluene solution run under the same conditions.

Yield 1·16 g; 12·61 mmole; 84·1%.

[3-^{3}H]Benzyl bromide[82,83]

The above solution containing 1·16 g (12·61 mmole) of [3-^{3}H]toluene in 20 ml carbon tetrachloride, was transferred to a two-necked flask fitted with a reflux condenser and dropping funnel. The complete unit was positioned over two 150-watt tungsten lamps and wrapped around with

aluminium foil so as to be simultaneously heated and irradiated by the bulbs. When the solvent was refluxing, a 10% w/vol solution of bromine in carbon tetrachloride (7·2 ml; 0·72 g Br; 13·24 mmole) was added dropwise at such a rate that the red colour was instantly discharged. The reaction conditions were maintained for 15 minutes after the bromine had all been added, and then the solution was allowed to cool, washed twice with water and dried ($MgSO_4$). After removing as much CCl_4 as possible the residue was 2·53 g or 117·3%. A yield of 100% was assumed for the following preparation.

Diethyl 2-[3-^{3}H]Benzyl-2-acetamidomalonate[83]

Diethylacetamidomalonate (2·74 g; 12·61 mmole) and a 50% oil slurry of sodium hydride (0·61 g; equivalent to 12·61 mmole) were refluxed together in dry dioxan. After 15 minutes, when the flask was filled with a thick white slurry, [3-^{3}H]benzyl bromide (2·53 g; assumed 12·61 mmole) was transferred down the condenser with a pipette and washed down with a little more dry dioxan. After a further two hours' refluxing the dioxan was distilled off under reduced pressure and the residue run onto a grade III alumina (50 g) column in benzene. The product was obtained as starlike clusters of white needles by eluting with 200 ml benzene, and was recrystallised from light petroleum until a constant activity of 0·810 mCi/mmole was obtained.

Yield 0·644 g; 2·10 mmole; 16·7% m.p. 105-6°C.

(lit.[102] 104-104·2°C.)

DL-[3-^{3}H]Phenylalanine

Diethyl 2-[3-^{3}H]benzyl-2-acetamidomalonate (200 mg; 0·651 mmole) was refluxed for 90 minutes with 5N-hydrobromic acid (2.5 ml). The reaction mixture was then evaporated down *in vacuo*, neutralised and transferred to a column of Dowex 50WX8 cation exchange resin (H^+form). This was eluted with 200 ml quantities of water, 0·75N-, 2N- and 4N-hydrochloric acid. The 2N-hydrochloric acid eluate was found by paper chromatography to contain pure phenylalanine, and was evaporated to dryness *in vacuo*. The residue was dissolved up in a minimum of water, evaporated down to incipient crystallisation and the pH adjusted to 6·0 with sodium hydroxide. The product was allowed to crystallise overnight in the refrigerator.

Yield 52·6 mg; 0·32 mmole; 49·12%

Activity 0·800 mCi/mmole

[2-^{3}H]Toluene

This synthesis was carried out by the same procedure as was used for [3-^{3}H]toluene (above). The starting material was *o*-bromotoluene (B.D.H. Ltd., Poole, Dorset) (2·57 g; 15 mmole).

Yield 1·32 g; 14·4 mmole; 72%

[2-^{3}H]Benzyl bromide

This synthesis was carried out by the same procedure as was used for [3-^{3}H]benzyl bromide (above). The starting material was [2-^{3}H]toluene (1·32 g; 14·4 mmole). The final residue of [2-^{3}H]benzyl bromide was distilled at atmospheric pressure to give a clear colourless liquid, boiling 200-230°C.

Yield 0·943 g; 5·3 mmole; 36·6%

5-[2-^{3}H]Benzyl-3-phenylhydantoin[85].

3-Phenylhydantoin (0·97 g; 5·5 mmole) was stirred at 50°C. for 1 hour with 2M magnesium methyl carbonate[87] in dimethylformamide, after which [2-^{3}H]benzyl bromide (0·943 g; 5·5 mmole) was added dropwise through a serum cap by means of a syringe. The mixture quickly became a deep reddish brown and was stirred for a further 3½ hours at 70°C. It was then dropped slowly into a stirred mixture of 2N-hydrochloric acid (200 ml) and ice (ca.200 g) and extracted with ether (4 x 50 ml), the insoluble solid being filtered off and retained. The ether extract was dried over $MgSO_4$ and evaporated. The drying agent was extracted with dry acetone. The insoluble solid (above) and the residues from all the extracts were pooled; total (4·0 mmole; 73%).

This solid material was dissolved in a little acetone, applied to a column of grade III alumina (50 g) and eluted with acetone. The band corresponding on TLC with authentic material (prepared previously from pure benzyl bromide and examined spectroscopically) was recrystallised from acetone -/light petroleum.

Yield 290 mg; 1·1 mmole; 20%

m.p. 140-145°C. (lit.[103] 173-174°C.)

Activity 1·38 mCi/mmole

DL-[2-^{3}H]Phenylalanine

5-[2-^{3}H]Benzyl-3-phenylhydantoin (266 mg; 1 mmole) was dissolved in dry dioxan (10 ml) and transferred to a Carius tube. Saturated aqueous barium hydroxide (20 ml) was added and the tube was sealed. It was heated in an electric oven at 165°C. for 1 hour. Before heating, the liquid in the tube was yellowish with a fine cloud of white insoluble

material. Afterwards the yellow colour had gone and a great deal more precipitate was in evidence. The tube was opened and the contents neutralised with concentrated sulphuric acid. The barium sulphate was filtered off through celite and the filtrate evaporated to dryness.

Crude yield 175 mg.

This material was taken up in warm water (25 ml), neutralised again with barium hydroxide to pH 7·0 (indicator paper), filtered through celite and extracted with chloroform (3 x 10 ml). The chloroform extract was washed with water and the washings added back to the separated aqueous layer. The chloroform extract contained about 10 mg of a brownish oil, which was discarded. The aqueous layer was boiled down until near to crystallising, then about the same volume (2-3 ml) of ethanol was added and it was allowed to cool.

Yield 51·2 mg; 0·31 mmole; 31%

Activity 1·26 mCi/mmole

D.2 Preparation of Side-Chain Labelled Phenylalanines.

DL-[2'-^{3}H]Phenylalanine

Unlabelled diethyl 2-benzylacetamidomalonate was prepared as described above using reagent grade benzyl bromide. This material (200 mg; 0·63 mmole) was placed in a flask fitted with a reflux condenser and an efficient drying tube, and a solution of thionylchloride in tritiated water (500 mg in 2 ml; 6N; 200 mCi) was added. The mixture was refluxed for 2 hours and then tritiated acid and readily exchangeable tritium were removed by repeatedly evaporating to dryness *in vacuo*, adding water and re-evaporating. The final residue was neutralised to pH 6·5 with the minimum of 4N-sodium hydroxide and finally recrystallised from aqueous ethanol.

Yield 46·7 mg; 0·283 mmole; 45%

Activity 0.557 mCi/mmole

DL-*N*-Benzoyl-[2'-^{3}H;1'-^{14}C]phenylalanine

Diluted [2'-^{3}H;1'-^{14}C] phenylalanine (100 mg; 6·59 x 10^{-4} mCi/mmole ^{14}C; ^{3}H:^{14}C ratio 5·01:1) was dissolved in 4N-sodium hydroxide (1 ml) and the solution stirred in ice, while benzoyl chloride (total 0.3 ml; 1.8 mmole) was added dropwise over ½ hour. The pH was maintained at 9.0 throughout by dropwise addition of 4N-sodium hydroxide (total 1 ml). After stirring for a further ½ hour at room temperature, the reaction mixture was acidified with concentrated hydrochloric acid and the crude product filtered off. Excess benzoic acid was removed by sublimation (130°C; 0·5 mmHg) and the residue recrystallised from water.

Yield 90·5 mg; 0·34 mmole; 55·5%

m.p. 185-7°C. (lit.[104] 187-188°C.)

^{14}C activity 6·65 x 10^{-4} mCi/mmole

^{3}H:^{14}C ratio 1·91:1

Epimerisation of DL-N-Benzoyl-[2'-^{3}H;1'-^{14}C]phenylalanine[105]

a. Using dicyclohexylcarbodiimide

N-Benzoylphenylalanine, prepared as above (50 mg; 0·19 mmole; 6·65 x 10^{-5} mCi/mmole ^{14}C) was dissolved in dry acetonitrile (5 ml) and dicyclohexylcarbodiimide added quickly. The mixture was refluxed for two minutes under anhydrous conditions, and then allowed to cool. Crystals (of dicyclohexylurea) precipitated and were filtered off. The filtrate was treated with one drop of pryidine followed by 5 ml water. The reaction mixture was then evaporated to dryness and recrystallised (with difficutly) from water/ethanol.

Yield 20 mg 40%

m.p. 165-175°C.

^{14}C activity 2·80 mCi/mmole

^{3}H:^{14}C ratio 0·28:1

b. Using acetic anhydride[105]

N-Benzoylphenylalanine (30 mg; 0·11 mmole; 6·65 x 10^{-5} mCi/mmole ^{14}C) was dissolved in dry dioxan (5 ml) and acetic anhydride (0·1 ml; 90 mg; 0·9 mmole) added. The mixture was refluxed for two minutes, then cooled and treated with pyridine and water as before. The reaction product was more easily crystallised.

Yield 15 mg 50%

m.p. 170-176°C.

^{14}C activity 3·26 mCi/mmole

^{3}H:^{14}C ratio 1·10:1

2-Morpholino-2-phenylacetonitrile[106]

Benzaldehyde (10·6 g; 100 mmole) and morpholine perchlorate (20·4 g; 110 mmole) in dry morpholine (100 ml) were stirred for 1 hour at 60°C. in a flask fitted with a reflux condenser. Then sodium cyanide (5·39 g; 110 mmole) dissolved in the minimum of water, was added, and stirring continued at 90°C. for a further hour. The resulting mixture was tipped into 1 litre ice water containing a little hydrochloric acid, and the product precipitated out. It was filtered off, dried, dissolved in benzene and passed through a bed of grade III alumina (about 100 g). Recrystallisation from light petroleum gave colourless needles.

Yield 15·2 g; 76 mmole; 76%

m.p. 68-69°C. (lit.[106] 69-70°C.)

n.m.r.

τ	signal	integral	assigned
2·57	multiplet	5	phenyl group
5·20	singlet	1	acetonitrile 2-proton
6·31	multiplet	4	morpholino protons adjacent O.
7·46	multiplet	4	morpholino protons adjacent N.

2-Morpholino-2-phenyl-[2-^{2}H]acetonitrile[106]

2-Morpholino-2-phenylacetonitrile (10·1 g; 50 mmole) in dry dimethylformamide (50·0 ml) was treated with a 50% suspension of sodium hydride in mineral oil (7·5 g; equivalent to 150 mmole) under an atmosphere of dry nitrogen, at room temperature. A brownish yellow solution

was obtained. The reaction flask was then cooled in ice while deuterium oxide (5·0 ml; 277 mmole) was slowly added. The mixture was stirred for a few minutes and then sufficient thionyl chloride was added to render the mixture acid (pH1-2). About 8·0 ml were required.

The contents of the flask were then tipped into a stirred mixture of 1 litre water, 200 g ice and a little 2N-hydrochloric acid. After extracting with chloroform (3 x 100 ml), the extract was washed with water (2 x 1 litre) to remove dimethylformamide, and dried ($MgSO_4$). The chloroform was removed *in vacuo* and the resulting yellow oil taken up in benzene and passed through grade III alumina to give a colourless product. Recrystallisation from light petroleum gave needles.

Yield 8·75 g; 43·3 mmole; 86·6%

m.p. 68-69°C.

n.m.r.

τ	signal	integral	assigned
2·57	multiplet	5	phenyl group
5·20	singlet	0·15	acetonitrile 2-proton
6·31	multiplet	4)	morpholino group
7·46	multiplet	4)	

Thus the product contained *ca.* 85% deuterium at the acetonitrile 2-position.

In subsequent runs through this preparation, the yields and extents of deuteriation were substantially the same.

(i) Yield 9·02 g; 44·7 mmole; 89·0%

% deuteriated species *ca.* 85%

(ii) Yield 8·80 g; 43·5 mmole; 87·0%

% deuteriated species *ca.* 80%.

[formyl-^{2}H]Benzaldehyde[106]

2-Morpholino-2-phenyl-[2-^{2}H]-acetonitrile (4·8 g; 23·8 mmole) was refluxed for 1 hour with 2N-hydrochloric acid (40 ml), cooled, and the benzaldehyde extracted with dichloromethane (3 x 25 ml). The extracts were pooled and dried ($MgSO_4$). The dichloromethane was removed by distillation through a Vigreux column at normal pressure. Last traces were removed 25°C. *in vacuo*.

Yield 2·72 g; 25·4 mmole; 106%

(Z)-4-[vinyl-^{2}H]Benzylidene-2-phenxloxazolin-5-one

[formyl-^{2}H]Benzaldehyde (2·72; assumed 23·8 mmole), hippuric acid (5·40 g; 31 mmole) and anhydrous potassium carbonate (2·00 g; 14·3 mmole) were placed together in a flask, and acetic anhydride (40 ml) was added. The mixture was swirled gently and put aside in a dark cupboard. After leaving overnight a thick slurry of pale yellow crystals had appeared. These were filtered off, washed with a little aqueous ethanol and dried *in vacuo* over NaOH. Crude yield 3·90 g. Recrystallisation from benzene light petroleum gave pale yellow needles, m.p. 161-163°C. (lit.[107] 164-165°C.)

Yield 3·0 g; 12·0 mmole; 51%

(Z)-[3'-^{2}H]-2'-Benzoylaminocinnamic acid

The above labelled oxazolone (3·0 g; 12·0 mmole) was dissolved in 96% ethanol (60 ml) and aqueous 0·5N-sodium hydroxide (60 ml) added. The mixture was gently warmed on the water bath until the colour was discharged, and then it was allowed to stand at room temperature overnight. The ethanol was removed *in vacuo* and *ca*. 50 ml water added.

The solution was treated with a few drops of ether and then acidified (pH1) with conc. hydrochloric acid. The benzoylaminocinnamic acid precipitated as fine crystals and was filtered off. Recrystallisation from ethyl acetate gave fine colourless needles, m.p. 236-238°C.d. (lit.[108] 225°C(d.)

Yield 2·63; 9·8 mmole; 82%

(2'S,3'R)-plus (2'R,3'S)-N-Benzoyl-[2'-^{2}H]phenylalanine

Z-[3'-^{2}H]-2'-Benzoyl-aminocinnamic acid (2·5 g; 9·4 mmole) was dissolved in 96% ethanol (125 ml) and hydrogenated at room temperature and pressure for 55 minutes in the presence of 0·25 g 10% palladium/charcoal catalyst. The actual uptake of hydrogen was 354 ml (corrected) compared to a theoretical requirement of 228 ml. When hydrogen uptake had ceased, the solution was filtered through Celite and the solvent removed *in vacuo*. The mixture of enantiomeric N-benzoylphenylalanines was recrystallised from water/ethanol.

Yield 2·3 g; 8·6 mmole; 91%

m.p. 186-188°C. (lit.[104] 187-188)

n.m.r. (potassium t-butoxide/D_2O)

τ	signal	J	integral	assigned
2·2-2·9	multiplet	-	10	2 phenyl groups
5·2-5·4	signal mostly obscured by HoD			2' proton
6·7	doublet	5Hz	1·1	3' proton

The n.m.r. integral therefore corresponds to *ca.* 90% deuteriated species.

mass spectrum

m/e	% a	% b
271	1·25	-
270	5·0	0·95
269	1·25	5·72
252	1·25	0·2
251	-	0·79
227	1·25	-
226	6·25	0·92
225	3·75	4·5
224	-	3·03
149	32·8	7·9
148	28·1	7·25
105	100	100
93	4·8	-
92	24·9	6·6
91	5·95	38·0

a. deuteriated material

b. undeuteriated material

The % deuteriated species in the sample is calculated as follows from the M^+ and $M+1^+$ peaks, 271, 270 and 269:-

Ratio of $M+1^+$ to M^+ in unlabelled material $\frac{0·95}{5·72} = 0·166$

Actual contribution to m/e 270 peak by unlabelled material in spectrum of labelled compound is: $0·166 \times 1·25$

% deuteriated species present must be given by

$$\frac{\text{deuteriated species}}{\text{deuteriated + non-deuteriated species}}$$

i.e.
$$\frac{5·0 - 0·166 \times 1·25}{1·25 + 5·0 - 0·166 \times 1·25}$$

$$= \frac{5·0 - 0·166 \times 1·25}{5·0 + 1·25\,(1 - 0·166)}$$

$$= \frac{4·793}{6·04} = 80\%$$

The location of the deuterium may be deduced from the presence, in the labelled spectrum of peaks one mass unit

greater than m/e 91 (tropylium ion), m/e 225 $(M-CO_2)^+$ and m/e 251 $(M-H_2O)^+$. The base peak in both cases is the phenyl acylium ion at m/e 105. In order to provide more material for feeding experiments 10 g ^2H-labelled 2'-benzoylaminocinnamic acid was prepared by the steps described above and converted into labelled N-Benzoyl-phenylalanine by hydrogenation. Hydrogen uptake was 904 ml (corrected), compared to the calculated requirement of 830 ml (37·2 mmole).

(2'S,3'R)- plus (2'R,3'S)- [3'-^{2}H]Phenylalanine

The appropriate benzoyl derivative (3,00g; 11.2 mmole) was refluxed in concentrated hydrobromic acid (48% w/v; 60 ml) for 1 hour. The solution was then cooled and filtered to remove benzoic acid. Yield 1·076 g (theory 1·37 g).

The filtrate was evaporated down to dryness and the residue dissolved in fresh water and evaporated again in vacuo to remove as much acid as possible. The final residue was dissolved in the minimum of hot water, neutralised with 4·0N-sodium hydroxide to pH 6·0 and cooled.

Crude yield of phenylalanine 1·492 g; 9·04 mmole; 80·7%

(2'S,3'R)- plus (2'R,3'S)-N-Chloroacetyl-[3'-^{2}H]phenylalanine[146]

The above crude labelled phenylalanine (1·49 g; 9·04 mmole) was dissolved in the minimum (3·5 ml) of 4N-sodium hydroxide. The solution was then cooled in ice and stirred, while small portions of freshly redistilled chloroacetic anhydride were added (total 4·5 g; 27 mmole). The pH was checked before and after each addition of anhydride and was maintained at 9·0 by addition of 1 ml aliquots of 4N-sodium hydroxide. After the last additions of anhydride

and alkali, stirring was continued for a further 30 minutes on the ice bath.

The reaction mixture was then acidified with concentrated hydrochloric acid to pH1 and extracted with ether (3 x 25 ml). The extracts were dried overnight ($MgSO_4$). In the morning the solution was evaporated to a clear straw-coloured oil, which crystallised on standing for a few minutes. It was recrystallised from water.

Yield 1·610 g; 6·67 mmole; 73·8%

m.p. 128-9°C. (lit.[104] 130-131°C.)

n.m.r. (DMSO-d_6)

τ	signal	J	integral	assigned
1·56	doublet	7·7Hz	1	NH
2·78	singlet	-	5	phenyl protons
5·44	doublet	4·8Hz)	1	2' proton
5·57	doublet	4·8Hz)	)7 7Hz	
5·96	singlet	-	2	$-CH_2Cl$
6·93	doublet	4·8Hz	1·1	3' proton

n.m.r. spectrum of unlabelled N-chloroacetylphenylalanine (DMSO-d_6)

τ	signal	J	integral	assigned
1·56	doublet	10Hz	1	NH
2·78	singlet	-	5	phenyl protons
5·30-5·66	multiplet	-	1	2' proton
6·60-7·29	multiplet	-	2	3' proton

mass spectrum

m/e	% a.	% b.
245	0·15	-
244	0·91	0·16
243	0·62	1·00
242	1·71	0·50
241	0·5	2·82
197	2·60	-
196	0·62	2·0
163	1·77	-
162	0·62	2·1
150	5·9	-
149	47·1	11·3
148	45·2	10·4
147	8·8	21·7
93	17·2	-
92	100	16·5
91	17·6	100

relative abundances

a. deuteriated sample

b. undeuteriated sample

The spectrum is not sufficiently clean to calculate the percentageof deuteriated molecules but substantial peaks occur at one mass unit greater than each of the unlabelled peaks at m/e 243 and m/e 241 (M^+) as well as at m/e 149 $(M-ClCH_2CO-H-CO_2)^+$ and at m/e 91 (tropylium ion). This enrichment pattern indicates that the deuterium must be in the benzylic portion of themolecule.

In a repeat of the synthesis of stereospecifically labelled [3-^{2}H]phenylalanine, the crude product from the hydrolysis of labelled N-benzoylphenylalanine (9·00 g; 33·5 mmole) was chloroacetylated without prior crystallisation.

Overall yeild of N-chloroacetyl-[3-^{2}H]phenylalanines was 6·7 g (27·7 mmole; 83·2%).

Enzymic resolution of N-Chloroacetyl-[3-^{2}H]phenylalanines.[132] (2'S,3'R)- plus (2'R,3'S)-N-Chloroacetyl-[3'-^{2}H]phenylalanine (1·25 g; 5·1 mmole) was dissolved in sufficient aqueous 10% lithium hydroxide to give a pH of 6·5 (BDH narrow-range papers). This solution was made up to 25 ml in a standard flask, with water. Carboxypeptidase A (3 mg as aqueous suspension; 105U.) was added to the solution, which was then shaken well. A polarimeter cell (2·0 cm path length) was filled with the digestion mixture and the rotation of the mixture was measured periodically. Complete conversion was achieved after 3 hours at an ambient temperature of 28°C., as shown by a steady rotation of -0·39°. The solution was allowed to remain at room temperature overnight but no further change in rotation occurred.

The reaction mixture was acidified after 18 hours to pH 1·0 (HCl) and extracted with ether (6 x 25 ml). The ether layers were combined and washed with a little water (2 x 10 ml). The water washings were returned to the aqueous phase. The ether extract was dried overnight ($MgSO_4$). The aqueous fraction was neutralised to pH 6·5 with 10% and filtered to remove denatured enzyme.

(2'RS,3'S)-[3'-^{2}H]Phenylalanine

The ether extract obtained from the enzymic resolution was evaporated down and crystallised to give 0·704 g (95%) of the (2'R,3'S)-N-chloroacetyl-[3'-^{2}H]phenylalanine. This material was refluxed for 1 hour in 6N-hydrochloric acid (10 ml), evaporated to dryness with water and then

taken up again in 10N-hydrochloric acid (3 ml). This latter solution was transferred to a Carius tube, which was sealed and heated in an oven at 180°C. for 20 hours. The tube was then opened and the contents removed treated with charcoal, filtered and evaporated several times with fresh water to remove all possible HCl. The residue was taken up in the minimum of hot water and precipitated by dropwise addition of 4N-sodium hydroxide to pH 6·5.

Yield 178 mg (1·08 mmole; 45%)

(2'S,3'R)-[3'-^{2}H]Phenylalanine

The neutralised aqueous fraction from the enzymic hydrolysis was reduced in volume *in vacuo* and applied to a column of Dowex 1 x 8 cationic resin (40 cm x 2 cm diameter, OH^- form). The column was eluted with water 250 ml until the washings were no longer alkaline, and then with 0·5N, 1·0N and 1·5N aqueous acetic acid solutions. Those fractions which showed a ninhydrin reaction were pooled and evaporated down. The residue was taken up in the minimum of boiling water, ethanol was added and the product allowed to crystallise.

Yield 0·309 g (1·88 mmole; 74%)

$[\alpha]_D^{26}$ -31·5° (lit.[104] -33°)

mass spectrum

m/e	a.	b.	c.
167	0·12	0·06	-
166	4·1	4·43	0·37
165	0·9	1·0	1·95
122	7·7	9·6	-
121	95·0	77·0	5·25

a. (2'S,3'R)-[3'-^{2}H]-phenylalanine

b. (2'RS,3'S)-[3'-^{2}H] phenylalanine

c. unlabelled phenylalanine

m/e	a.	b.	c.
120	20·5	21·2	50·0
105	5·1	8·7	-
104	12·8	13·5	6·3
103	7·7	9·6	15·8
93	26·9	25·1	-
92	87·2	75·1	21·0
91	23·1	23·1	74·0
74	100	100	100

The proportion of deuteriated molecules in sample a.is calculated as follows:

Ratio of $(M+1)^+$ to M^+ peak in unlabelled phenylalanine:

$$\frac{0·37}{1·95} = 0·179$$

True height of peak at m/e 166 in labelled spectrum a:

$$4·1 - 0·179 \times 0·9$$

Total phenylalanine (labelled and unlabelled) present:

$$4·1 + 0·9 - 0·179 \times 0·9$$

proportion of deuteriated molecules in sample a:

$$\frac{4·1 - 0·179 \times 0·9}{4·1 + 0·9(1-0·179)} = \frac{3·94}{4·84} = 81·5\%$$

By the same procedure the proportion of deuteriated species in sample b. is obtained as:-

$$\frac{4·43 - 0·179 \times 1·0}{4·43 + (1 - 0·179)} = \frac{4·25}{5·25} = 81·0\%$$

The presence of strong ions one mass unit greater than those at m/e 165 (M^+); m/e 120 $(M-CO_2-H)^+$; m/e 103 $(M-CO_2-NH_4^+)^+$ and m/e 91 (tropylium ion) shows that the deuterium is associated with the bynzylic portion of the molecule. By contrast there is no peak at m/e 75 in the labelled spectra corresponding to m/e (glycyl cation-radical) in the unlabelled.

In a repeat synthesis of the stereospecifically deuteriated phenylalanines (2'R,3'S) plus (2'S,3'R)-N-Chloroacetyl-[3'-^{2}H]phenylalanine (5 g; 20·5 mmole) was dissolved in 10% brought to pH 6·5 and made up to 100 ml with water. Carboxypeptidase A (12 mg as aqueous suspension; 90 U.) was added to the solution and the reaction was monitored by means of a polarimeter, in the same way as before. The rotation of the solution rose to -0·33° instead of the theoretical value of -0·39° which had previously been observed. Incubation for a further 24 hours with half the original quantity again of enzyme gave no increase in rotation, and the reaction mixture was worked up as before.

Yield of (-)-(2'R,3'S)-N-chloroacetyl-[3'^{2}H]phenylalanine

2·0 g (8·3 mmole; 84%)

$[\alpha]_D^{25}$ = -38·6° (lit. 51°)

This material was recrystallised from water in order to concentrate the (2'R) enantiomer.

Yield 1·4 g (5·81 mmole; 56%)

$[\alpha]_D^{25}$ -48·8° (lit. 51°)

m.p. 123-4°C. (lit. 126°C.)

The purified (2'R)-N-chloroacetylphenylalanine was refluxed with 6N-hydrochloric acid (40 ml) for one hour, cooled, and evaporated to dryness. The residue was taken up in water, neutralised with 4N-sodium hydroxide, boiled down to incipient crystallisation and an equal volume of ethanol was added. On cooling 0·62 g (3·76 mmole; 64·7%) of pure white crystals were obtained. These were transferred to a Carius tube together with 25 ml of 10N-hydrochloric acid. The tube was sealed and heated at

180°C. for 20 hours. It was then cooled and opened. The product was evaporated to dryness, neutralised, treated with ethanol and crystallised in the same way as the (2'R,3'S)-[3'-^{2}H]phenylalanine previously described.

Yield 0·49 g (2·96 mmole; 79%)

(2'S,3'R)-[3'-^{2}H]Phenylalanine

The enzymic digestion product was passed through a column of Dowex 1 x 8 as before, and the phenylalanine eluted with 2N-aqueous acetic acid.

Yield (crude) 1·7 g (10·25 mmole; 100%)

$[\alpha]_D^{25}$ -28·8° (lit. -33°)

This material was recrystallised from water/ethanol.

Yield 0·9g (5·52 mmole; 53·1%)

$[\alpha]_D^{25}$ -32° (lit. -33°)

(2'RS,3'RS)[3'-^{3}H]phenylalanine

(2'R,3'S) plus (2'S,3'R)-[3'^{3}H]phenylalanine was prepared by Dr. S. Narayanaswami by an analogous route to the deuteriated material. The above (125 mg; 0·76 mmole) was sealed in a Carius tube, together with 10N-hydrochloric acid (3 ml), and heated at 180°C. for 20 hours. After this time the tube was cooled and opened. The contents were repeatedly evaporated to dryness, adding fresh water to remove all the hydrochloric acid. The phenylalanine was precipitated by adjusting the pH to 6·5 with 4N-sodium hydroxide, and recrystallised from aqueous ethanol.

Yield 57·1 mg; 45·5%

Activity 1·22 mCi/mmole

(2'RS,3'R)-[3'-^{3}H]Phenylalanine and (2'RS,3'S)-[3'-^{3}H]-Phenylalanine.

These materials were prepared by the procedure described

above for specifically deuteriated phenylalanines. The starting material was (2'R,3'S)-plus (2'S,3'R)-[3'-^{3}H]-phenylalanine prepared by Dr. S. Narayanaswami (0·800 g; 4·85 mmole). The amino-acids were recrystallised and counted before epimerisation to their (2'RS)-forms (last step).

Yields were as follows:

(2'R,3'S)-plus (2'S,3'R)-N-Chloroacetyl-[3'-^{3}H]-phenylalanine

Yield 462 mg; 1·92 mmole; 39·5%

Activity 0·829 mCi/mmole

(2'S,3'R)-[3'-^{3}H]Phenylalanine - from 0·450 g N-chloroacetylphenylalanine

Yield 0·147 g; 0·89 mmole; 96%

Activity 0·784 mCi/mmole

(2'R,3'S)-[3'-^{3}H]Phenylalanine*

Yield 0·115 g; 0·7 mmole; 60% overall

Activity 1·003 mCi/mmole

* In order to keep quantities manageable, 80 mg (0·33 mmole) of (2'R,3'S)-N-chloroacetyl-[3'-^{3}H]phenylalanine obtained from Dr. S. Narayanaswami was added before hydrolysis, hence the slightly higher activity and lower overall yield.

(2'RS,3'R)-[3'-^{3}H]Phenylalanine - from 100 mg (2'S) material

Yield 44·6 mg; 0·27 mmole; 32·5%

Activity 0·785 mCi/mmole

(2'RS,3'S)-[3'-^{3}H]Phenylalanine - from 70 mg (2'R) material

Yield 34·1 mg; 0·21 mmole; 29·5%

Activity 0·910 mCi/mmole

Preparation of potato acetone powder[109]

Potatoes (1800g) were peeled and cut into slices 1-2 mm thick. These were washed well with deionised water and laid out on white plastic trays (5; each 30 x 45 cm). Neomycin sulphate solution (100 ml; 50 mg/litre) was added to each tray, which was then covered with a polythene sheet fixed in place with tape. The trays were placed 45 cm. below a rack of 6 fluorescent tubes, each emitting 500 lumens. Irradiation was maintained for 22 hours, the temperature being ca. 30°C.

The slices were removed from the trays, washed quickly in a bucket of deionised water, and shaken dry. They were then weighed out in 500 g portions into polythene bags, which were fastened and cooled in ice. Total weight was 2 kg.

Each 500 g portion was halved and each half ground separately in a Waring blendor with acetone (300 ml) at -20°C. for 30 seconds. The powders from the two halves were filtered off, pooled and again ground with acetone (300 ml) at -20°C. for 30 seconds. The doubly-treated powder was filtered off, air-dried and sieved (100-mesh). The fraction passing through the sieve was retained and stored at -20°C.

Yield (from 2 kg potatoes) before sieving 293 g

Yield of sieved material 142 g

Preparation of phenylalanine ammonia-lyase solution[109,110]

The above sieved acetone powder (40 g) was stirred with a freshly prepared solution of reduced glutathione (60 mg) in 0·1M-borate Na^+ buffer (400 ml; pH 9·0) at

0°C. for 30 minutes. The mixture was then filtered through 3 thicknesses of surgical gauze. To the stirred filtrate was added 1M-acetate Na^+ buffer (40 ml; pH 5·0) followed by protamine sulphate (170 mg) dissolved in the same acetate buffer (2 ml), and the cloudy solution was stirred for 10 minutes. It was centrifuged (10 min. at 7,000 g) and the supernatant was treated with ammonium sulphate (61 g for ca. 320 ml). After being allowed to equilibrate for 30 minutes the wispy precipitate was centrifuged off (10 min, at 7,000 g) and the supernatant allowed to equilibrate for 30 minutes with more ammonium sulphate (40 g). A precipitate formed, which was centrifuged off (10 min. at 7,000 g). The supernatant was rejected and the precipitate taken up in 0·1M-borate buffer (60 ml) and stored as 4 x 15 ml quantities.

Assay of phenylalanine-ammonia-lyase in prepared solutions[111]

L-[1'-^{14}C]Phenylalanine (2·028 mg; 0·12 mmole; 0·5 uCi) was dissolved and made up to the mark in a 1 ml standard flask, using 0·1M-borate Na^+ buffer (pH 9·0). This solution (0·1 ml) was added to a portion of phenylalanine-ammonia-lyase solution (5 ml) which had been prepared as above and allowed to reach 30°C. in a thermostatically controlled bath. The mixture was stirred at 30°C. for 20 minutes. Then concentrated hydrochloric acid was added to bring the pH to 1·0 and the mixture was extracted with ether (3 x 5 ml). The ether solution was dried ($MgSO_4$), filtered and made up to 25 ml in a standard flask. Quantities of 1, 2, 3 and 5 ml of this solution were pipetted out, evaporated down and counted.

Mean activity/ml observed — 4390 counts/min.

Corrected for efficiency of counting — 7460 d./min.

Activity fed — $1{\cdot}11 \times 10^5$ d./min.

Conversion of available phenylalanine: $\frac{7460}{1{\cdot}11 \times 10^5}$

µg phenylalanine converted/minute $\frac{7460 \times 2{\cdot}028 \times 10^3}{1{\cdot}11 \times 10^5 \times 20}$

$= 6{\cdot}83$ µg/min.

µ moles phenylalanine converted/ minute by 60 ml of solution $= \frac{6{\cdot}83 \times 12}{165}$

$= 0{\cdot}554$ U

$= 554$ mU

(lit.[110] cited ca. 600mU)

Assay of (2'RS,3'RS)-[3'-^{3}H]Phenylalanine

(2'RS,3'RS)-[1'-^{14}C,3'-^{3}H]Phenylalanine (10 mg; ^{14}C. $6{\cdot}17 \times 10^{-4}$ mCi/mmole; ^{3}H:^{14}C ratio 6.45:1) was dissolved in 0·1M-borate Na^+ buffer (2·0 ml; pH 9·0) and added to a portion of phenylalanine-ammonia-lyase solution (15 ml, see preparation above). The mixture was maintained at 30°C. for 1 hour and then left at room temperature overnight. It was then acidified to pH 1·0 using concentrated hydrochloric acid, ether was added to assist denaturation of the enzyme and the mixture was filtered through Celite. The Celite and the aqueous layer were thoroughly extracted with ether and the extracts dried ($MgSO_4$) and evaporated to dryness. The residue was taken up in a little chloroform and unlabelled cinnamic acid (30 mg) added. This solution was transferred to a micro-sublimation tube, the chloroform evaporated and the cinnamic acid sublimed (0·1 mmHg; 120°C.). It was crystallised from aqueous ethanol.

^{14}C activity $5{\cdot}26 \times 10^{-5}$ mCi/mmole

^{3}H:^{14}C ratio 3·07:1

tritium retention relative to ^{14}C 47·5%

Assay of (2'RS,3'R)-[3'-^{3}H]phenylalanine

(2'RS,3'R)-[1'-^{14}C,3'-^{3}H]Phenylalanine (10 mg; ^{14}C $7{\cdot}12 \times 10^{-4}$ mCi/mmole; ^{3}H:^{14}C ratio 5·01:1) was assayed using the same procedure as for feeding IX precursor above. The derived cinnamic acid had the following properties:

^{14}C activity $5{\cdot}62 \times 10^{-5}$ mCi/mmole

^{3}H:^{14}C ratio 4·16:1

tritium retention relative to ^{14}C 83·1%

Assay of (2'RS,3'S)-[3'-^{3}H]phenylalanine

(2'RS,3'S)-[1'-^{14}C; 3'-^{3}H]Phenylalanine (10 mg; ^{14}C $7{\cdot}64 \times 10^{-4}$ mCi/mmole; ^{3}H:^{14}C ratio 4·60:1) was assayed as above. The cinnamic acid had the following properties:

^{14}C activity $5{\cdot}87 \times 10^{-5}$ mCi/mmole

^{3}H:^{14}C ratio 0·69:1

tritium retention relative to ^{14}C 15.0%

Assay of (2'RS,3'S)-[3'-^{3}H]phenylalanine

(2'RS,3'S)-[1'-^{14}C; 3'-^{3}H]Phenylalanine (10 mg; ^{14}C $2{\cdot}50 \times 10^{-4}$ mCi/mmole; ^{3}H:^{14}C ratio 12·6:1) was assayed as above. The cinnamic acid had the following properties:

^{14}C activity $1{\cdot}14 \times 10^{-5}$ mCi/mmole

^{3}H:^{14}C ratio 1·68:1

tritium retention relative to ^{14}C 13·3%

D.3 Production and Reactions of Gliotoxin

Culturing of Trichoderma Viride[18]

Strain no. 1828 NRRL (also known as Gliocladium deliquescens) was obtained from the Commonwealth Mycological Institute, Kew, Surrey, and used for all experiments.

The spores were grown on potato dextrose agar in loose-capped test tubes which were sealed with sellotape before storage in the refrigerator.

The medium was that of Johnson, Bruce and Dutcher[2] i.e. for six litres:-

Sucrose	90 g	
Ammonium sulphate	10 g	
di-Potassium orthophosphate	5 g	
Magnesium sulphate	2·5 g)	) (anhydrous)
Ferric chloride	0·05 g)	
Peptone	0·1 g	

It was made up in deionised water and the pH adjusted to 3·0-3·5 (pH-meter) by adding a few drops of concentrated sulphuric acid. Aliquots (500 ml) of the medium were transferred to 1 litre Erlenmeyer flasks each of which was plugged with cotton wool and autoclaved (20 minutes; 30 lb/in.^{2} gauge).

The mould was subcultured from the potato dextrose slope in one flask of medium for 5 days. Inoculation was then carried out by transferring a loopful of the sub-culture to a fresh flask.

Feeding was accomplished by dissolving the precursor in a known volume of water, sterilising by filtration and transferring the sterile solution to allotted flasks with

a sterile pipette. Eight flasks were grown for each of the m-tyrosine and ring tritiated phenylalanine precursors. Six were used for each of the side-chain tritiated precursors and ten for the time-study.

Work-up of Gliotoxin[18]

After allowing the mould to grow for five days as a shake-culture at 30°C., the mycelium was filtered off at the pump. The medium was then extracted three times with 10% its volume of chloroform. Emulsions were broken by passing them through Celite. The chloroform extract was dried ($MgSO_4$), filtered and evaporated to dryness in vacuo. The residue was washed with methanol and then recrystallised from the same solvent. In each feeding the gliotoxin was recrystallised to constant activity, but usually not more than two recrystallisations were required.

Characterisation

Unlabelled gliotoxin, produced as above, had the following characteristics:

Fine white needles m.p. 180-195°C.(d)
(lit.[2] 221°(d))

$[\alpha]_D^{25}$ chloroform -255° (lit.[2] 270±10° (ethanol))

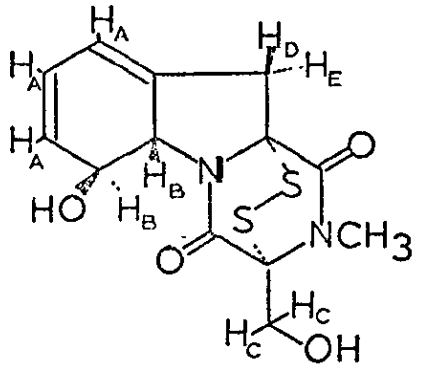
Lassaigne test positive for sulphur and nitrogen.

U.v. λ_{max} 263 nm shoulder at 249 mμ
ϵ 4280 nm

addition of alkali - no change in spectrum.

I.r. (KBr disc)

ν_{max}	3380 cm^{-1} (broad)	(hydrogen bonded OH stretching)
	1663	amide C = O stretching
	1375	
	1240	
	1060	



N.m.r. (100 MHz) Spectrum

τ	signal	integral	J	assigned
4·0-4·3	multiplet	4*	-	H_A*
5·2	singlet	2	-	H_B
5·58	doublet*	1	13Hz)	H_C*
5·81	doublet*	1	13Hz)	
6·28	broadened doublet	1	18Hz	H_D
6·81	singlet	3	-	N-CH_3
7·08	sharp doublet	1	18Hz	H_E

* the integral becomes 3 when D_2O is added, and the AB. quartet due to H_C becomes sharper.

Mass spectrum

m/e	relative abundance	assignment
326	5%	M^+
263	15%	
262	100%	$(M-S_2)^+$
244	27·5%	$(M-S_2-H_2O)^+$
242	32·5%	$(M-S_2-H_2O-H_2)^+$
233	16·3%	$(M-S_2-CO-H)^+$
229	15·0%	$(M-S_2-HCHO-H-H_2)^+$
226	17·6%	$(M-S_2-2H_2O)^+$
217	25%	$(M-S_2-CO-HO)^+$

m/e	relative abundance	assignment
213	16.3%	($M-S_2-HCHO-H_2O-H$)
159	21,3%	(7-hydroxyindole-2-acylium ion-H)
144	22.5%	(indole-2-acylium ion)
143	20%	(indole-2-acylium ion-H)

Anhydrodesthiogliotoxin[12]

Gliotoxin (50 mg; 0·153 mmole) was stirred at room temperature with grade II alumina (2 g) in dry benzene (10 ml) for 12 hours. The solution was then filtered and the alumina extracted with refluxing benzene. The benzene solutions were pooled and evaporated to give a pale yellow solid (24·2 mg). This was chromatographed on grade II alumina (50 g) in benzene to yield a white, or off-white solid m.p. 160-161°C.

Yield 16·0 mg; 0·071 mmole; 46·3%

Lassaigne test - sulphur negative, nitrogen positive

$[\alpha]_D^{25}$ 0·0°

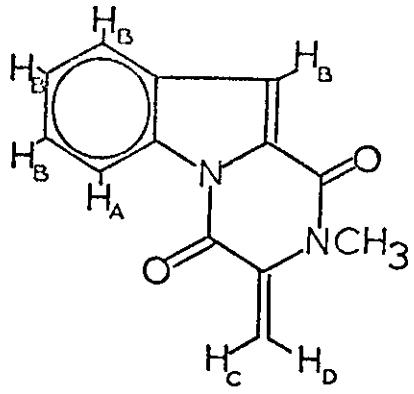
Elemental analysis	found %	calc. %
C	69·27	69·0
H	4·63	4·5
N	12·44	12·4

U.v. λ_{max} 265 nm (ϵ 4,699)

Shoulder λ_{max} 275 nm (ϵ 4,475)

I.r. (chloroform solution)

ν_{max}	2960 cm^{-1}	aryl C-H stretching
	1715)	
	1675)	amide C=O stretching
	1605	
	1390	
	1355	



N.m.r. (60MHz) Spectrum

τ	signal	integral	J	assigned
1·48	doublet	1	7·7Hz	H_A
2·16-2·71	multiplet	4	-	H_B
3·85	doublet	1	1·5Hz	H_C
4·73	doublet	1	1·5Hz	H_D
6·00	singlet	3	-	$N-CH_3$

Mass Spectrum

m/e	relative abundance	assigned
227	20%	$(M+1)^+$
226	100%	$(M)^+$
199	14%	$(M-HCN)^+$
198	13%	$(M-CO)^+$
170	6%	$(M-2CO)^+$
169	10%	$(M-H_2$-acroleyl radical$)^+$
144	15%	indole-2-acylium ion
143	50%	(indole-2-acylium ion-H)
129	20%	
115	60%	indolyl cation-radical
114	20%	
89	10%	
88	15%	

m/e	relative abundance	assigned
85	30%	
83	35%	
55	20%	acroleyl cation
54	12%	

The spectrum published for this compound[12].

m/e	Composition	relative abundance*	assigned
226	$C_{13}H_{10}N_2O_2$	100%	$(M)^+$
199	$C_{12}H_9NO_2$	6·7%	$(M-HCN)^+$
198	$C_{12}H_{10}N_2O$	6·7%	$(M-CO)^+$
197	$C_{12}H_7NO_2$)) $C_{12}H_9N_2O$)	2·7%	$(M-NCH_3)^+$ $(M-HCO)^+$
170	$C_{11}H_{10}N_2$	5·4%	$(M-2CO)^+$
144	C_9H_6NO	31·0%	
143	C_9H_5NO))) $C_{10}H_9N$)	71·0%	
130	C_9H_8N	6·7%	
129	C_9H_7N	28·1%	
116	C_8H_6N	9·4%	
115	C_8H_7N	63·5%	indolyl cation radical

* The relative abundances have been taken from the graphical representation shown in the paper[12]. Bose et al. used the all-glass heated inlet system to introduce their sample into the mass spectrometer, and this system was also used to run the preceding spectrum, in order to obtain comparable results.

Dehydrogliotoxin[7]

Gliotoxin (52 mg; 0·16 mmole) and o-chloranil (38 mg; 0·16 mmole) were refluxed in benzene (3·5 ml) for three hours. The solution was then cooled, concentrated and applied to a

column of silica gel (5 g). The excess of o-chloranil was eluted with benzene, the tetrachlorocatechol with ether-benzene (1:19) and the dehydrogliotoxin with ether-benzene (1:9)

Yield 36·6 mg; 0·12 mmole; 75%

m.p. 181-185°C. (lit.[7] 185-186°C.)

U.v. λ_{max} 273·5 nm (ϵ4,706) peak

λ_{max} 300 nm (ϵ4,073) shoulder

on addition of one drop ethanolic sodium hydroxide, the spectrum became:

λ_{max} 245 nm (ϵ12,200) shoulder

λ_{max} 301 nm (ϵ 7,200) peak

λ_{max} 344 nm (ϵ 2,730) shoulder

I.r. $\underline{\nu_{max}}$

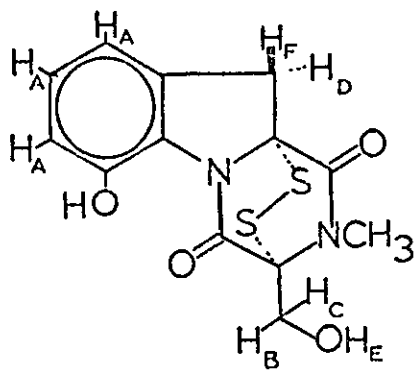
3,500 cm^{-1} sharp phenolic OH

1,675 amide C = O stretching

1,485

1,375

1,060



N.m.r. (100MHz) Spectrum

τ	signal	integral	J	assigned
2·76-3·22	multiplet	3	-	H_A
5·4-5·82	multiplet*	3	-	$H_{B,C,D}$
6·7	doublet	1))*	19Hz	H_F
6·74	singlet	3)	-	$N-CH_3$

* When the solution is treated with a little D_2O the multiplet at τ5·4-5·82 sharpens somewhat and the total integral of the singlet and doublet at τ6·74, 6·7 respectively changes from 5 to 4.

Mass Spectrum

m/e	relative abundance	assigned
324	4%	$(M)^+$
261	16%	
260	100%	$(M-S_2)^+$
258	18%	$(M-S_2-H_2)^+$
243	32%	$(M-S_2-OH)^+$
242	34%	$(M-S_2-H_2O)^+$
231	10%	$(M-S_2-CO-H)^+$
229	16%	$(M-S_2-HCHO-H)^+$
161	38%	
160	32%	7-hydroxyindole-2-acylium ion
134	24%	
133	18%	
132	16%	7-hydroxyindolyl cation

Desthiodihydrogliotoxin[13]

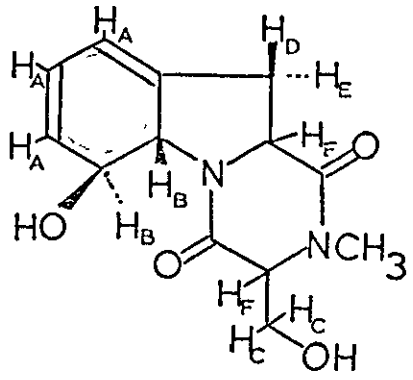
Application of the method of Johnson, Bruce & Dutcher gave a mixture of products not easy to separate, and the

following modified version was used.

Aluminium foil, (0·4 g; 14 mg atom) cut into 1 cm squares was etched with 1N-sodium hydroxide for about 30 seconds, washed quickly with water and amalgamated with 5% mercuric chloride solution for 30 seconds before being removed and washed successively with water, alcohol and ether. Finally it was transferred to a flask containing gliotoxin (70 mg; 0·22 mmole) dissolved in dioxan (25 ml). Water (2 ml; 110 mmole) was added and the mixture allowed to stand under an atmosphere of nitrogen for four hours, after which time the effervescence had almost ceased. The reaction mixture, smelling strongly of hydrogen sulphide, was filtered through Celite to give a clear, colourless solution. The aluminium residue and the Celite were washed well with ethanol and these washings added to the dioxan solution. Evaporation <u>in vacuo</u> gave a white crystalline solid which was recrystallised from <u>ca</u>. 1 ml ethanol.

Yield 18·1 mg; 0·065 mmole; 32·5%

m.p. 244-248°C.(d) (lit.[16] 246-247°C.)
(lit.[13] 243-244°C.)



N.m.r. (100MHz) Spectrum

τ	signal	integral	assigned
4·0-4·3	multiplet	3	H_A
5·2-5·4	multiplet	2	H_B
5·98-6·18	multiplet	2	H_C
7·00	singlet	3	$N-CH_3$
6·92-7·16	multiplet	2	$H_{D,E}$

On addition of a little deuterium oxide, the integral for the multiplet and singlet around τ 7·0 changes from 6 to 5. This is probably due to the primary alcoholic proton. The secondary alcoholic proton and the two methine protons H_F are apparently so broadened as not to appear as peaks at all.

Mass Spectrum

m/e	relative abundance	assigned
265	5%	
264	45%	$(M)^+$
246	20%	$(M-H_2O)^+$
230	50%	$(M-2HO)^+$
229	70%	$(M-H_2O-HO)^+$
228	55%	$(M-2H_2O)^+$
213	30%	
201	35%	
200	25%	
149	65%	
135	60%	
133	100%	
131	50%	

mono-O-Acetylgliotoxin

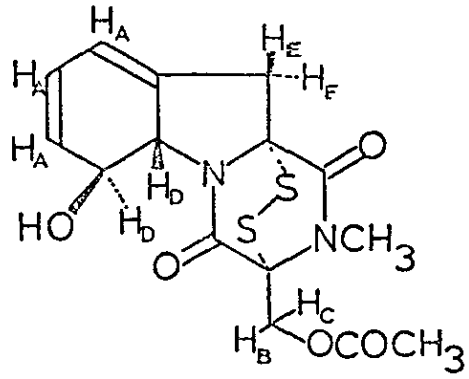
Gliotoxin (100 mg; 0·3 mmole) was dissolved, with warming, in dry benzene (25 ml) and the solution was cooled to room temperature. Pyridine (1·0 ml) was added and the mixture stirred in an ice bath while acetic anhydride (5 ml) was added slowly. When all the anhydride had been added (about 10 minutes) the mixture had turned a pale yellow colour. It was left overnight at room temperature and then evaporated to give a pale green glassy residue (120 mg) which could not be crystallised. This material was taken up in a few drops of chloroform and applied to a silica preparative-thin-layer plate (100 cm x 20 cm) which was developed in benzene/acetone (2:1). The very dense band at Rf 0·70 was picked out by u.v. absorption and removed from the plate. Elution with chloroform followed by evaporation *in vacuo* gave a greenish glassy solid which could not be crystallised, but was examined spectroscopically.

Yield 60 mg; 0·16 mmole; 54%

U.v. λ_{max} 273 nm (ϵ *ca.* 4,500) shoulder

I.r. (gum film on NaCl windows)

ν_{max}	3,400 cm^{-1}	H-bonded OH stretching
	ca. 2,940	acetyl C-H stretching
	1,760	acetyl C=O stretching
	1,710-1,670	gliotoxin C=O stretching
	1,230)	broad bands due
	1,060)	to C-O stretching



N.m.r. (60MHz) Spectrum

τ	signal	integral	J	assigned
4·0-4·2	multiplet	3	-	H_A
5·01	doublet	1	13Hz)	$H_{B\&C}$
5·30	doublet	1	13Hz)	
5·19	singlet	2	-	H_D
6·25	broadened doublet	1	18Hz	H_E
6·90	singlet	3	-	$N-CH_3$
7·09	doublet	1	18Hz	H_F
7·86	singlet	3	-	$-CO-CH_3$

A sharp singlet was also observed at τ 4·42, which disappeared when a drop of D_2O was added - O-H proton.

Reaction of gliotoxin with lithium/liquid ammonia

Gliotoxin (100 mg; 0·3 mmole) was placed in a 100 ml round-bottomed three-necked flask fitted with a stirrer, a solid CO_2 condenser and soda-lime drying tubes. Anhydrous ammonia was distilled into the flask until about 50 ml had condensed, after which the flask was enclosed in insulating material and stirring commenced. Metallic lithium (10 mg; 1·4 mg atom) was introduced through one neck of the flask and brief effervescence occurred followed by formation of a deep blue solution. The

mixture was stirred for 15 minutes and then decolourised with ammonium chloride (500 mg). The ammonia was then allowed to evaporate. The residue was taken up in water (50 ml) and extracted with chloroform (3 x 25 ml). After drying ($MgSO_4$) and removing the chloroform the residue was 75 mg of brownish material. It was chromatographed on a silica column, and the major component was eluted with acetone; 50 mg (gum). This was examined spectroscopically.

U.v. λ_{max} 235 nm (ϵ ca. 5,000) shoulder

λ_{max} 295 nm (ϵ ca. 2,000) shoulder

No change was observed on addition of 1 drop of ethanolic sodium hydroxide.

I.r. ν_{max}

3,400 cm^{-1}	broad band	O-H stretching
ca. 2,940	three bands	C-H stretching
1,670-1,650		C=O (gliotoxin)
1,430		
1,290		
1,130		

N.m.r. Spectrum

τ	signal
4·3	broad singlet
6·0-8·0	humps

After the residue had stood for a few days, the spectrum was run again and this time showed a peak at 2·4 as well as those above.

D.4 Feeding, Amino-acid Extraction and Radioactivity Measurements.

Extraction of mycelial phenylalanine

Mycelium from the side-chain-labelled phenylalanine feedings, which had been allowed to grow for 5 days in a shake culture at 30°C., was havested and extracted as follows. Each batch of 1½ litres of culture medium (2 per Feeding) was filtered using a Buchner funnel. The filtrate was extracted with chloroform to obtain gliotoxin (described above). When each cake of mycelium had been washed with water (250 ml) it was peeled from the filter paper and allowed to dry at atmospheric pressure in a desiccator over calcium chloride. The dry cakes (2 per Feeding) together weighed 10-12 g.

One cake from each feeding was ground in a pestle and mortar with ethanol 20 ml; the fragments and powder were filtered off, and refluxed for 30 minutes with water (50 ml). This aqueous extract was decanted and examined for amino acids by paper chromatography. Except in the last (2'R,S,3'S)-[3'-^{3}H]phenylalanine feeding, none was observed, and the aqueous extracts were discarded.

The fragments of mycelium were then refluxed for 24 hours with a solution of barium hydroxide (10 g) in water (50 ml). After this time, the mixture was cooled and the extract decanted. The fragments of mycelium were washed with water (20 ml), then filtered off and retained. The extract and washings were pooled, neutralised with concentrated sulphuric acid to pH (6·5) and filtered through Celite to give a clear yellow solution. The

above solution was applied to a column of Dowex 50 x 8 resin (H^+ form 50 cm x 2 cm radius). This column was eluted with ca. 300 ml water until the washings were no longer acid, then peptides and amino-acids were obtained by elution with 2N-aqueous ammonia. Chromatographic treatment was, however, found to be unnecessary, and in the last two experiments, ((3'S)-tritiated phenylalanine and time study) it was omitted.

The yellow solution obtained from the neutralised barium hydroxide extract, or from the column of Dowex resin was evaporated in vacuo to dryness, the residue taken up in 5 ml water and sodium-azide (ca. 2 mg) added, to discourage microbial growth. The resulting solution (ca. 0·3 ml per sheet) was applied to two sheets of Whatman No. 1 chromatography paper (each 45 cm x 60 cm), which were then developed (descending method). A reference spot of L-phenylalanine was also applied to the baseline of each sheet. When the chromatograms had developed in the solvent for 7-8 hours they were removed from the tank marked along the solvent front with pencil and dried. A strip bearing the reference spot was cut from the side of each sheet, and developed with ninhydrin reagent. As a check on the regularity of elution along the solvent front, the papers were examined in ultra-violet light and the fluorescing bands, due to degradation products of protein, at Rf 0·3, Rf 0·5 and Rf 0·8 were marked in with pencil. These bands had run level with the baseline in all cases, even where the solvent front appeared to be crooked. A strip was cut across each chromatogram corresponding to

the reference spot of L-phenylalanine and parallel to the baseline. The strips (2 for each Feeding) were heated together with water (3 x 25 ml) on a boiling water bath. The aqueous extracts were decanted, filtered and evaporated down to about 2 ml when unlabelled, reagent grade L-phenylalanine (100 mg) was added. The mixture was crystallised from water/ethanol. In no case did a further recrystallisation alter the specific activity or $^{3}H:^{14}C$ ratio of the product. Examination of the peptide/amino-acid extracts by analytical paper-chromatography showed numerous ninhydrin-active spots, none of which was completely separated. However a radioactive scan of the papers showed in every case a single sharp peak of activity at Rf 0·6 corresponding exactly to an L-phenylalanine reference spot.

Assay of stereospecificity of label in mycelial phenylalanine using phenylalanine-ammonia-lyase.

A crude solution of peptides and amino-acids obtained by barium hydroxide extraction of the mycelium in the manner described was neutralised with concentrated sulphuric acid, filtered evaporated and chromatographed on paper, exactly as described above. Strips bearing the labelled phenylalanine were cut from the papers and extracted with water as before. Residues from the extracts were taken up in ca. 2 ml 0·1M-borate Na^{+} buffer and added to a solution of phenylalanine-ammonia-lyase enzyme (15 ml, prepared from potato tubers as above) which had been allowed to reach 30°C. in a thermostatically-controlled bath. The mixture was maintained at 30°C. for 1 hour and then allowed to

remain at room temperature overnight. The reaction mixtures were worked up in the manner described for the assay of precursors.

Time-study of 3'-tritium loss from phenylalanine

(2'RS,3'RS)-[1'-^{14}C,3'-^{3}H]Phenylalanine (9 mg; ^{14}C 9 μCi; ^{3}H:^{14}C ratio 8·76:1) was dissolved in water (100 ml), sterilised by filtration and measured out into previously prepared and sterilised Erlenmeyer flasks of the usual medium, with a sterile 10 ml pipette. The ten flasks were inoculated from a subculture and then incubated as a shake-culture at 30°C. After each 24 hour period from the time of inoculation two flasks were removed from the shaker and worked up as follows.

The culture medium was filtered and the mycelium washed with water, peeled from the filter-paper and dried in a desiccator as previously described. The filtrate was extracted with chloroform (3 x 100 ml) and the gliotoxin worked up. No gliotoxin could be detected (t.l.c. silica, benzene (2); acetone (1)) in the 24-hour sample, but unlabelled gliotoxin (50 mg) was added to the chloroform residue and the mixture crystallised from methanol in order to detect any trace of active gliotoxin. Weights of the crude chloroform residues and crystallised gliotoxin for the various samples are shown below.

Hours	24	48	72	96	120
$CHCl_3$ extract (mg)	42·8 (oil)	53·8	102·0	118·7	141·9
gliotoxin (mg)	34·9*	16·0	49·0	64·5	32·0

* 50 mg unlabelled gliotoxin added as carrier.

The medium, after extraction with chloroform, was evaporated to about 100 ml, neutralised with saturated barium hydroxide solution and filtered through Celite. The filtrate was evaporated to dryness; the residue was dissolved and made up to 10 ml with water. Paper chromatography of these solutions showed only a faint trace of ninhydrin-positive material, this being at the baseline. No radioactivity could be detected by scanning the chromatograms at maximum sensitivity.

Each batch of dried mycelium was refluxed for 30 minutes with water (50 ml) and for 24 hours with 20% barium hydroxide solution, as previously described. Half-quantities were used to extract the 24-hour mycelium.

After the barium hydroxide extraction, the washed residue was treated with 2N-hydrochloric acid (25 ml) and this was decanted and discarded. The residue was pulverised with water (50 ml), filtered, peeled from the filter-paper and dried in a desiccator. The initial weight of the mycelium samples together with the weights of the dry residues from the water extractions, and weights of the mycelium samples after both extractions are tabulated below. The weight of protein extracted by the barium hydroxide, given by the difference: initial wt. of mycelium - (wt. of H_2O extract + final wt. of mycelium), is also shown:

Hours	24	48	72	96	120
initial mycelium wt.	0·428g	2·746g	3·747g	4·389g	4·872g
H_2O extract-residue	0·098g	0·523g	0·722g	0·380g	0·650g
final mycelium wt.	0·056g	0·616g	1·275g	1·700g	1·882g
inferred protein wt.	0·274g	1·607g	1·750g	2·309g	2·340g

The residues from the extracts were all dissolved in water and made up to 5 ml except for the water extract of the 24 hour sample which was made up to 2 ml. All were protected from microbial degradation by addition of sodium azide (2 mg). Each solution (0·3 ml)* was chromatographed (descending method) on paper, as already described.

*In the case of the 48 hour water extract only 0·2 ml of solution was applied to each chromatogram sheet.

The strips of paper bearing the mycelial phenylalanine were extracted with water, and the extracts evaporated *in vacuo*. The weights of the residues are recorded below:

Hours	24	48	72	96	120
H_2O-extracted Phe.	6·8mg	8·9mg*	8·8mg	7·0mg	6·5mg
$Ba(OH)_2$-extracted Phe.	8·1mg	16·8mg	19·2mg	27·4mg	19·0mg

* from only 0·4 ml of original water-extract.

The labelled phenylalanine was recovered by dilution with unlabelled L-phenylalanine as already described. Assays of the mycelial phenylalanines were carried out, starting from 0·3 ml of each extract per sheet of chromatography paper. Only the two 24 hour phenylalanines and

the 5-day barium hydroxide hydrolysis material were assayed in this way.

All mycelial extracts were examined by paper chromatography. The chromatographs were scanned for radioactivity and the spots revealed with ninhydrin reagent. The barium hydroxide extracts contained only one peak of activity at Rf 0·6, corresponding to a reference spot of authentic L-phenylalanine, but in addition to the phenylalanine peak, the water extracts all had a peak of approximately equal area, at the baseline. For the 24 hour sample, this baseline material was examined as follows. Strips of paper were cut from the baselines of four preparative chromatogram sheets which had been developed in order to obtain the labelled phenylalanine for characterisation and assay. The paper strips, containing the unknown active material were extracted with hot water, the extract filtered and evaporated *in vacuo*. The residue weighed 35·1 mg. It was extracted with hot ethanol; the cooled ethanolic solution was filtered into a 10 ml standard flask and made up to the mark with solvent. 1 ml of this solution was measured into a scintillator bottle, the ethanol was evaporated and the residue counted. Such residue as would not dissolve in ethanol (30·9 mg) was taken up in water and made up to 10 ml in a standard flask. An aliquot (1 ml) was removed, evaporated, and the residue counted. The remaining solutions were evaporated *in vacuo* and the residues refluxed with 6N-hydrochloric acid for 16 hours. The acid was then evaporated and the residues, in a little water, were examined by paper chromatography. The hydrolysate of the

ethanol-soluble fraction showed two ninhydrin positive spots at Rf 0·6 (blue) and Rf 0·23 (pink). There was a little activity detectable by scanning in the spot at Rf 0·6 (phenylalanine). The hydrolysate of the water-soluble fraction showed a great many unresolved ninhydrin-positive spots. The radioactivity scan showed a single strong peak at Rf 0·6.

The remaining aqueous extract of the 24-hour mycelium (0·8 ml) was extracted with ether (3 x 1 ml), and ethyl acetate (3 x 1 ml), then acidified and again extracted with ether (3 x 1 ml). The extracts were all transferred to scintillator bottles, the solvent was evaporated and the residue counted.

Extract	^{14}C activity d/min.	$^{3}H:^{14}C$ ratio
ether	50	2·7 :1
ethyl acetate	79	3·4 :1
ether/acid	95	2·5 :1
ethanol	33	6·3 :1
water	732	2·3 :1

Counting of Radioactivity

A Beckmann Scintillation Counter Type CPM-100 was used for all counting work. Glass screwtop bottles supplied by Beckmann Ltd., were used to hold the samples. After use, the bottles were decontaminated by first soaking for 24 hours in a solution of "Quadralene" laboratory detergent (5% w/v) and then heating the rinsed bottles in a 10% w/v solution of the same detergent for a further 6 hours. The bottles were finally rinsed well in tap water and deionised water and dried in an oven. The plastic screw-caps were discarded after use.

A scintillator solution was used consisting of 2,5 diphenyloxazole, P.O.P. (3·80 g) plus 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene P.O.P.O.P. (0·2 g) dissolved in analytical reagent-grade toluene (1 litre). It was stored under nitrogen in the refrigerator. Compounds to be counted were first dissolved in dry dimethylformamide, which had been distilled from calcium hydride and stored over a molecular sieve (size 4A). For the ring-labelled foodings the volumes used were dimethylformamide (0·1 ml) to scintillator solution (5·0 ml). However at this point difficulties arose with the counting of phenylalanine and the system; dimethylformamide (1 ml) to scintillator solution (10 ml) was used in subsequent work. Clavine alkaloids were counted using the first system.

The Beckmann instrument contains counting channels with pre-selected discriminator bias. In channel I the machine counts tritium with an efficiency of approximately 40% and carbon-14 at about 20% efficiency. In channel II the efficiency of counting tritium is negligible (less than 0·1%) while carbon-14 is counted at about 60% efficiency. Actual efficiency values were obtained by counting n-[1,2-^{3}H]hexadecane and n-[1-^{14}C]hexadecane standards obtained from the Radiochemical Centre, Amersham, Bucks. The activities of these compounds were accurately known; allowance was made for loss of tritium activity due to decay, according to the equation:

$$\log(No/N) = kt^{112},$$

where N_o = number of tritum atoms at time 0 years

N = number of tritium atoms at time t years

k = constant

Efficiencies of counting in each channel were determined by counting weighed samples of the hexadecane standards in the presence of the dimethylformamide/scintillator system. At the outset of the work, standardisation was carried out in the presence of varying quantities of unlabelled substrates, but no significant changes in the efficiencies were observed, provided no more than about 3 mg of substrate was present. Determination of the efficiencies was carried out for each new batch of scintillator solution prepared.

Background counts were ascertained in duplicate for each new day's counting. Dimethylformamide and scintillator solution were measured out into two scintillator bottles and counted. The mean counts/min. for the two bottles in each channel was taken as the background count. Normal background values were approximately as follows:

	a.	b.
channel I	35	40
channel II	12	18

system a. 0·1 ml dimethylformamide plus 5 ml scintillator solution

system b. 1·0 ml dimethylformamide plus 10 ml scintillator solution

The Beckmann Scintillation Counter automatically calculates and prints out the standard error of the population it is counting. All samples were counted to a standard error $\geqslant 5\%$.

Apart from phenylalanine, m-tyrosine and tryptophan all samples could be dissolved in dimethylformamide in quantities ca. 1·0 mg. Samples containing m-tyrosine were weighed directly into the scintillator bottles, treated

with 1·0 ml saturated ethanolic hydrogen chloride and allowed to remain at room temperature for 20 minutes. The bottles were then placed over sodium hydroxide pellets in a desiccator which was evacuated and left at 0·1 mmHg pressure for at least three hours before adding dimethylformamide, when solution occurred without any difficulty. Phenylalanine was treated with ethanolic hydrogen chloride (1·0 ml) and was heated on a boiling water bath for 10 minutes before cooling and placing in the desiccator. All samples were weighed out in duplicate, and all amino-acids were counted at least 6 separate times.

Samples of relatively high activity e.g. undiluted precursors and their intermediates were weighed into a 5 ml standard flask, dissolved in dimethylformamide (solubilised with ethanolic hydrogen chloride where necessary) and made up to the mark with this solvent. From this dimethylformamide solution, 0·1 ml was transferred to a scintillator bottle. Scintillator solution (5 ml) was added and counting carried out in the usual way.

The tryptophan precursor was diluted 1/100 with unlabelled material and acetylated[113]. N-Acetyltryptophan readily dissolves in dimethylformamide.

In order to calculate tritium activity in the presence of carbon-14, the carbon-14 activity is first calculated by dividing the corrected channel II count by the efficiency with which carbon-14 is counted in that channel; i.e. if C^{II} is the corrected count in channel II and E^{C} the efficiency of counting carbon-14 in that channel, then:

$$\frac{C^{II}}{E^{C}} = {}^{14}C \text{ activity (d./min.)}$$

The "spill-over" of carbon-14 counts into channel I is given by the product of the carbon-14 activity multiplied by the efficiency of counting carbon-14 in channel I; i.e. if e^c is the efficiency with which carbon-14 is counted in channel I then the contribution by carbon-14 to the channel I count is:

$$\frac{C^{II}}{E^C} = e^c \text{ counts/min.}$$

The contribution by tritium to the channel I count is given by subtracting the carbon-14 contribution from the total corrected channel I count (C^I)

$$C^I - \frac{C^{II} \times e^c}{E^c} \text{ counts/min.}$$

Tritium activity is calculated by dividing the counts due to tritium by the efficiency with which this isotope is counted in channel 1 (E^H)

$$\frac{C^I - \frac{C^{II} \times e^c}{E^c}}{E^H} = {}^3H \text{ activity (d./min)}$$

Scanning of Radio-chromatograms

All scanning of chromatograms was carried out using a thin-layer plate scanner (Panax Ltd. model RTLS 1A). Since the instrument is intended for scanning thin-layer plates, all paper chromatograms had to be taped on to a 20 x 20 cm glass plate before scanning. No discriminator bias was used. The rate of flow of gas (argon 98%; n-propane 2%) through the windowless geiger tube was 7 ml/min. Slit width 4 mm was used throughout. Chromatograms of precursors were run at scale factor 100 counts/min, time constant 3 sec. speed 120 mm/hour. All other chromatograms were

run at maximum sensitivity (scale factor 3 counts/min.; time constant 100 sec. speed 30 mm/hour). All precursors were checked for radiochemical purity by this means.

D.5 Biosynthesis of the Clavine Alkaloids.

Experimental/Culturing of Claviceps

A culture of Claviceps purpurea (pennisetum)[114] was obtained from Dr. A. Hofmann (Sandoz, Basle) and used for all experiments. The mould was maintained as a slope culture on malt extract agar in loose-capped testtubes which were taped up and stored in a refrigerator when the mould had grown.

The growth medium was as follows[115]:-

per litre of water:		
	Mannitol	50·0g
	Sucrose	50·0g
	Potassium dihydrogen orthophosphate	0·1g
	Magnesium sulphate heptahydrate	0·3g
	Ferrous sulphate heptahydrate	0·01g
	Zinc sulphate heptahydrate	0·0044g
	Succinic acid	5·4g
	Ammonia (s.g. 0·800)	to pH 5·4 (pH meter)

Aliquots of 250 ml medium were transferred to 1 litre Erlenmeyer flasks which were plugged with cotton wool and sterilised in an autoclave (20 minutes; 30 lb/in^2 gauge).

The flasks were inoculated by transferring to each a small piece of mycelium from a malt extract slope. They were allowed to grow in cardboard boxes away from light at room temperature (not more than 25°C.). Feeding of precursors was carried out when the mould had grown for four weeks. After a further four weeks the alkaloids were harvested.

Feeding was accomplished by dissolving the precursor in water (80 ml), sterilising by filtration, and transferring the solution to the flasks with a sterile pipette

(10 ml per flask). Eight flasks (2 litres) were used for each Feeding.

Harvesting of Clavine Alkaloids[114]

The 8-week old culture was filtered through three thickness of surgical gauze to remove particles of mycelium and excess potassium carbonate (20 g) was added to the filtrate to give a final pH of about 10. A mixture of chloroform/isopropanol (3:1) was used to extract the alkaline medium (4 x 250 ml solvent per litre medium). Emulsions formed during the extraction were broken by filtration through a bed (at least 5 cm thick) of acid-washed sand. The pinkish extracts were combined dried ($MgSO_4$) and evaporated to a dark brown oil.

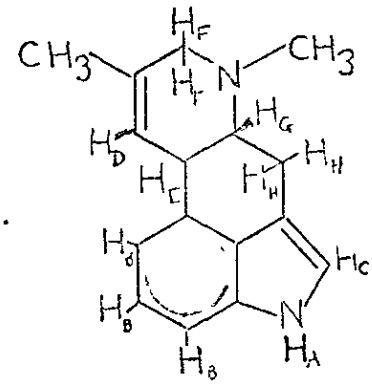
Yield 0·5-0·7 g

Chromatography on neutral alumina (grade III) (100g) gave two distinct fractions upon elution with absolute chloroform. The first to be eluted was a yellow oil with a strong odour of mushrooms (Rf 1·0 on neutral alumina abs. chloroform). This was followed by agroclavine (Rf 0·7, neutral alumina, abs. chloroform) which crystallised on evaporation to white needles. Recrystallisation was from ethyl acetate. The yield was extremely variable due to temperature fluctuation during growth. Range 120 mg - 700 mg.

m.p. 207-210°C. (lit.[114] 210-212°C.)

$[\alpha]_D^{25}$ -180° (pyridine) (lit.[114] -182°)

violet colour with Ehrlich's reagent*



N.m.r.

τ	signal	integral	assigned
1·56	broad singlet	1	H_A
2·77-3·01	multiplet	3	H_B
3·19	singlet	1	H_C
3·80	broad singlet	1	H_D
6·25	hump	1	H_E
6·55-7·36	multiplet	5	H_G,H_F,H_H (prochiral methylene protons)
7·50	singlet	3	$N-CH_3$
8·21	singlet	3	$C-CH_3$

Mass Spectrum

m/e	relative abundance	assignment
239	10·5%	
238	63·0%	$(M)^+$
237	100·0%	$(M-H)^+$
223	10·5%	$(M-CH_3)^+$
221	5·9%	$(M-CH_3-H_2)^+$
207	4·1%	
206	4·1%	
196	4·1%	
194	4·1%	
192	4·1%	

m/e	relative abundance	assignment
183	4·7%	
182	7·6%	
181	5·9%	
180	7·0%	
168	4·7%	
167	8·2%	
154	1·2%	
127	7·0%	
108	8·2%	

A mixture of two compounds was obtained in very low yield by eluting the alumina column with chloroform/methanol (200:1). These compounds (Rf 0·6 and 0·4, neutral alumina, chloroform/methanol (1000:1)) are thought to be the alkaloids setoclavine and isosetoclavine reported by Hofmann[114].

Elution with chloroform/methanol (100:1) gave a product which readily crystallised in tiny pale-green prisms. This was recrystallised from methanol and characterised as elymoclavine.

Yield range 50 mg - 150 mg

m.p. 230-235°C.(d) (lit.[114] 245-247°C.)

$[\alpha]_D^{25}$ -155° (pyridine) (lit.[114] -152°)

violet spot with Ehrlich's reagent*

Repeated recrystallisation gave no noticeable improvement in colour.

The tryptophan precursor was counted as its N-acetyl derivative, as described in the preceeding section. No special procedures were used in the counting of the clavine alkaloids.

*Ehrlich's reagent was prepared by dissolving p-dimethylamino-benzaldehyde (5g) in concentrated hydrochloric acid (25 ml). The compound to be tested, dissolved in a suitable solvent was applied to a filter-paper or dimple-tile and one drop of the reagent added on the tip of a glass rod. No heating was applied; the colour developed within a few seconds.

Bromination of Agroclavine[116]

Agroclavine (50 mg; 0·21 mmole) was dissolved in dry dioxan (2 ml) and a solution of N-bromosuccinimide in dioxan (1·3 ml 0·2M; 0·26 mmole; 24% excess) was added in one portion at room temperature. The mixture was shaken briskly for a few seconds and a yellow floculent precipitate appeared. After heating the reaction mixture at 65°C. for 5 minutes it was a dark red colour. Thin layer chromatography (neutral alumina; benzene) showed a strong spot which was neither agroclavine nor succinimide. The red precipitate was filtered off through Celite and the filtrate partitioned between saturated potassium carbonate solution (10 ml) and chloroform (3 x 5 ml). The chloroform extract was dried ($MgSO_4$), reduced in volume to ca 1 ml and applied to a column of neutral alumina, grade III (10 g). Elution with benzene gave the compound already observed on TLC as white crystals.

Yield 33·1 mg

Ehrlich reaction: strong violet colour

The product from the above reaction (33 mg) in dry-dioxan (2 ml) was treated with N-bromosuccinimide solution (112 mg; 0·6 mmole in 1 ml dioxan) at room temperature. A yellow precipitate was instantly formed, as before. Thin layer chromatography (neutral alumina, benzene) showed

that the starting material disappeared at once and was replaced by a spot Rf 0·5. On heating the reaction mixture at 65°C. for 5 minutes the first artefact disappeared and gave place to a second spot Rf 0·75. The reddish reaction mixture was worked up as described, but no product could be eluted from the alumina column with benzene. However elution with chloroform gave two products in yields of 5 mg each. Neither gave a positive test with Erlich's reagent.

U.v. a. λ_{max}^{nm}: 219(ε 29,000); 260(ε 51,000); 398, 315, 329 (ε ca. 6,700)

b. λ_{max}^{nm}: 219(ε 29,000); 259(ε 38,600); 367,315,326 (ε ca. 7,000)

Agroclavine u.v. spectrum[117]:

λ_{max}^{nm}: 225(ε 28,000), 284(ε 7,590), 293(ε 6,460)

Indoline[118] Spectrum:

λ_{max}^{nm}: 210(ε 32,000), 254(ε 25,000), 306(ε 5,500)

In another experiment, agroclavine (25 mg; 0·1 mmole) was dissolved in dry dioxan (2 ml) and N-bromosuccinimide solution (60 mg; 0·33 mmole in 3 ml dioxan) added. After heating the mixture at 65°C. for five minutes it was partitioned between saturated potassium carbonate solution (15 ml) and chloroform (3 x 10 ml). The chloroform extract was dried ($MgSO_4$), filtered through Celite, reduced in volume and applied to a column of neutral grade III alumina (10 g). Elution with benzene (25 ml) yielded 1·6 mg of a compound which gave no positive Ehrlich test. A second compound with a strong violet Ehrlich reaction, was eluted with absolute chloroform (25 ml).

Yield: 17·5 mg

U.v. 1. λ_{max}: 224(ε45,000); 281(ε13,200) nm

2. λ_{max}: 225(ε35,000); 281(ε17,300) nm

Agroclavine:

λ_{max}: 225(ε28,000); 284(ε7,590); 293(ε6,460) nm

Reaction of Gramine with disulphur dichloride I[119]

Gramine (0·175 g; 1 mmole) and anhydrous trichloroacetic acid (0·4g; 3 mmole) were stirred with benzene (50 ml) until a clear faintly pink solution was obtained. This was cooled to ca. 4°C. and disulphur dichloride solution (5 ml; 15% w/vol.; 0·075 g; 0·55 mmole) was added dropwise. When all the reagent had been added, the solution was a clear yellow colour. After standing at 4°C. for 20 minutes it was dark green but still clear. It was poured into light petroleum (100 ml) but no crystals appeared, only an oil. The light petroleum and benzene were evaporated to give a dark brown tar. Partitioning this tar between 2N-sodium hydroxide (200 ml) and chloroform (10 ml) followed by removing and drying the chloroform layer, gave a clear reddish-brown solution. Evaporation of this solution again gave a brown tar. T.l.c. (neutral alumina; acetone) of this tar showed only one spot, corresponding to starting material.

Reaction of Gramine with disulphur dichloride II[120,121]

Gramine (175 mg; 1 mmole) was dissolved in dry dioxan (25 ml) and ether (25 ml) added to the solution, which was then stirred in an ice bath. disulphur dichloride solution (0·11M in ether; 5 ml; 0·55 mmole) was added dropwise. A white precipitate quickly formed which thickened as the disulphur dichloride was added. The addition took 5 minutes, and stirring was continued for 10 minutes in the absence of

the ice bath. The precipitate was filtered off, but instantly decomposed to a tar. T.l.c. on neutral alumina (acetone) showed a smear from the baseline to Rf 0·70 (gramine spot).

A piperidine[122] and a morpholine[123] analogue of gramine, prepared by Mannich reaction of the appropriate base with indole in the presence of formaldehyde were treated with disulphur dichloride by each of the above procedures, with identical results.

Method II was also carried out using N-acetyltryptophan (50 mg; 0·20 mmole) as starting material. The tar was tested with Ehrlich's reagent and gave a strong violet colour reaction, as does N-acetyltryptophan.

Acid exchange of tryptophan aromatic protons[124]

Recrystallised DL-tryptophan (60 mg) was dissolved in deuterium oxide (1 ml) by adding a few drops of deuteriotrifluoroacetic acid and the solution was transferred to an n.m.r. tube. Another portion of DL-tryptophan (60 mg) was dissolved in pure deuteriotrifluoroacetic acid, prepared by treating trifluoroacetic anhydride with deuterium oxide and then distilling the mixture (b.p. 80-90°C.). This solution was likewise transferred to an n.m.r. tube. The two solutions were stored in the preheating block of the n.m.r. spectrometer (i.e. at 40°C.) and spectra were run of each after intervals of 5 minutes, 25 minutes and 90 minutes from dissolving the samples. The spectrum of the dilute-acid solution remained the same throughout.

N.m.r.

τ	signal	integral	assigned
2·30-3·00	multiplet	5	indole aromatic protons
5·60-5·85	multiplet	1	2' proton
6·50-6·90	multiplet	2	3' protons

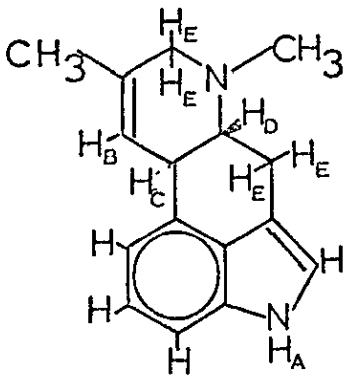
In the spectra of the neat-acid solution, the signals at τ5·60-5·85 and 6·50-6·90 remained the same as in the dilute-acid spectrum. However, even after 5 minutes the signal due to the aromatic protons had altered. A sharp peak at τ2·8 decreased rapidly in height, and a new sharp resonance appeared at τ2·5. Total integrals for the aromatic protons were as follows:

control (dilute-acid) experiment	5·0 protons
5 minutes	4·8 "
25 minutes	4·1 "
90 minutes	2·75 "

Acid exchange of agroclavine aromatic protons

Agroclavine (50 mg) freshly chromatographed on grade III alumina and recrystallised from ethylacetate, was dissolved in neat deuteriotrifluoroacetic acid, prepared as described, and the n.m.r. spectrum was run. Unfortunately the resolution under these conditions was extremely poor and no acceptable spectrum was obtained. After remaining in the spectrometer for 20 minutes at 40°C., the solution was diluted (20 ml water), basified (K_2CO_3) and extracted with chloroform (3 x 10 ml). The residue from the chloroform extract was purified on a column of alumina in the usual manner, and an n.m.r. spectrum of the agroclavine obtained was run. Much of the alkaloid decomposed in the n.m.r. tube

but sufficient could be obtained by rechromatographing, to run a mass spectrum.



N.m.r.

τ	signal	integral	assigned
1·32	broad singlet	1	H_A
2·80-3·25	multiplet	2·7	aromatic protons
3·86	broad singlet	1	H_B
6·25	hump	1	H_C
6·55-7·36	multiplet	5	H_D & H_E
7·50	singlet	3	$N\text{-}CH_3$
8·27	singlet	3	$C\text{-}CH_3$

Mass Spectrum

a. Relative abundances (%) labelled spectrum

b. Relative abundances (%) unlabelled spectrum

m/e	a.	b.
241	10·6	-
240	37·4	-
239	92·5	10·5
238	100·0	63·0
237	35·6	100·0
225	16·0	-
224	29·4	-
223	21·3	10·5
222	14·3	-

m/e	a.	b.
221	-	5·9
210	4·5	-
209	9·5	-
208	12·5	-
207	12·5	4·1
206	8·9	4·1
198	9·8	-
197	10·7	-
196	12·5	4·1
195	11·6	-
194	12·5	4·1
193	10·7	-
192	-	4·1
185	19·6	-
184	23·0	-
183	26·7	4·7
182	20·5	7·6
181	-	5·9
180	-	7·0
169	17·8	-
168	24·0	4·7
167	16·0	8·2
166	10·6	-
165	10·6	-
156	19·6	-
155	30·3	-
154	21·5	1·2
127	17·8	7·0
126	17·8	-
108	34·8	8·2

The proportions of di-, mono- and non-deuteriated molecules in the sample is determined as follows. The patterns of the $(M-1)^+$, $(M)^+$ and $(M+1)^+$peaks in each case are:

m/e	labelled*	unlabelled*
241	0·3	-
240	1·05	-
239	2·6	0·2
238	2·8	1·2
237	1·0	1·9

* peak height: cm

Assuming that there is only loss of protium, never deuterium to give $(M-1)^+$ but not $(M-2)^+$ peaks then the peaks of the labelled spectrum can be assigned.

Let	A represent	M (non-deuteriated agroclavine
	dA "	M (mono-deuteriated agroclavine)
	d_2A "	M (di-deuteriated agroclavine)
	d_3A "	M (tri-deuteriated agroclavine)

m/e	assignment
237	$(A-1)^+$
238	$A^+ + (dA-1)^+$
239	$dA^+ + (d_2A-1)^+ + (A+1)^+$
240	$d_2A^+ + (d_3A-1)^+ + (dA+1)^+$
241	$d_3A^+ + (d_2A+1)^+$

The ratios $(M-1)^+/M^+$ and $(M+1)^+/M^+$, from the spectrum of unlabelled agroclavine, are:

M^+/M^+ : 1:1

$(M-1)^+/M^+$: 1·58:1

$(M+1)^+/M^+$: 0·17:1

If A^+, dA^+, d_2A^+ and d_3A^+ are all related to their derivatives by the same ratios, the equations below can be drawn up.

$1·0 = 1·58A$ let $A = [A^+]$ etc.

$2·8 = A + 1·58dA$

$2·6 = dA + 1·58d_2A + 0·17A$

$1·05 = d_2A + 1·58d_3A + 0·17dA$

$0·3 = d_3A + 0·166d_2A$

Solving these simultaneous equations, values with the following ratios are obtained.

A 23·3%; dA 47·7%; d_2A 22·3%; d_3A 6·7%

If it is assumed that complete scrambling of the label takes place the assignment of the peaks becomes:

m/e	assignment
237	$(A-1)^+ + (dA-2)^+$
238	$A^+ + (dA-1)^+ + (d_2A-2)^+$
239	$dA^+ + (d_2A-1)^+ + (d_3A-2)^+ + (A+1)^+$
240	$d_2A^+ + (d_3A-1)^+ + (dA+1)^+$
241	$d_3A^+ + (d_2A+1)^+$

In this case the ratios of $(M-2)^+$, $(M-1)^+$ and $(M+1)^+$ to M^+ for each species, are:

undeuteriated molecules: let $[A^+] = A$

then $[(A-1)^+] = 1·58$; $[(A+1)^+] = 0·17A$

monodeuteriated molecules: let $[dA^+] = dA$

then $[(dA-2)^+] = \frac{1}{18}\ 1·58dA$; $[(dA-1)^+] = \frac{17}{18}\ 1·58dA$

$[(dA+1)^+] = 0·17dA$

dideuteriated molecules: let $[d_2A^+] = d_2A$

then $[(d_2A-2)^+] = \frac{1}{9}\ 1{\cdot}58d_2A$; $[(d_2A-1)^+] = \frac{8}{9}\ 1{\cdot}58d_2A$;

$[(d_2A+1)^+] = 0{\cdot}17d_2A$

trideuteriated molecules: let $[d_3A^+] = d_3A$

then $[(d_3A-2)^+] = \frac{1}{6}\ 1{\cdot}58d_3A$; $[(d_3A-1)^+] = \frac{5}{6}\ 1{\cdot}58d_3A$;

$[(d_3A+1)^+] = 0{\cdot}17d_3A$

The simultaneous equations obtained, are:

$$1{\cdot}0 = 1{\cdot}58A + 0{\cdot}09dA$$

$$2{\cdot}8 = A + 1{\cdot}49dA + 0{\cdot}18d_2A$$

$$2{\cdot}6 = dA + 1{\cdot}40d_2A + 0{\cdot}26d_3A + 0{\cdot}17A$$

$$1{\cdot}05 = d_2A + 1{\cdot}58d_3A + 0{\cdot}17dA$$

$$0{\cdot}3 = d_3A + 0{\cdot}17d_2A$$

Solving these equations gives values in the following proportions:

A 20·2%; dA 55·0%; d_2A 16·7%; d_3A 8·1%

Stability of the indole-2-position to hydrogenation

DL-[3'-^{14}C; 2-^{3}H]-N-acetyltryptophan (9·1 mg; ^{14}C 1·02 mCi/mmole, ^{3}H:^{14}C ratio 5·5) was dissolved in methanol (10 ml) in a 10 ml two-necked flask fitted with a serum cap. Platinum oxide (Adams' catalyst) (1·0 mg) was added and the mixture stirred in an atmosphere of hydrogen. Aliquots of 1 ml were removed by syringe at 0, 1, 2, 3, 5, 9, 20, 25 and 28 hours. Each aliquot was placed in a scintillator bottle, the methanol was removed *in vacuo* and the residue counted. The results are tabulated below:-

Time (hours)	0	1	2	2	5	9	20	25	28
H_2 uptake (ml at N.T.P.)	0	4	7	9	10	12	14	15	15
^{14}C d./min x 10^3	8·3	8·3	9·3	8·7	8·4	8·7	9·5	7·0	5·1
^{3}H:^{14}C ratio (:1)	5·2	5·3	5·2	5·2	5·2	5·2	5·2	5·1	5·1

Hydrogenation of Agroclavine[125]

Agroclavine (10 mg) was dissolved in methanol (2 ml), Adams' catalyst (1 mg) was added and the mixture stirred under hydrogen at atmospheric pressure for a total of 24 hours. For the first six hours samples were withdrawn (by syringe) at hourly intervals. Thin layer chromatography of these samples (neutral alumina; chloroform 2 benzene 1) showed a strong spot at Rf 0·75 (agroclavine). A weaker spot gradually appeared at Rf 0·28. After six hours fresh platinum oxide (2 mg) was added to the reaction mixture. Hydrogenation was continued and further samples were withdrawn at 7·5 hours (from start of reaction), 11 hours and 24 hours. All samples still had a predominant spot at Rf 0·75 (agroclavine). The remainder of the solution after 24 hours was removed from the hydrogen atmosphere, filtered through Celite and evaporated *in vacuo*. The residue was examined by mass spectroscopy, but only a spectrum of agroclavine could be detected.

Bibliography

1. R. Weindling and O.H. Emerson, Phytopathology, 1936, 26, 1068
2. J.R. Johnson, W.F. Bruce and J.D. Dutcher, J. Amer. Chem. Soc., 1943, 65, 2005
3. W.A. Rightsel, H.G. Schneider, B.J. Sloan, P.R. Graf, F.A. Miller, Q.R. Bartz, J. Ehrlich and E.J. Dixon, Nature, 1964, 204, 1333
4. V. Betina and Z. Baruth, J. Antibiotics (Tokio) Ser. A, 1964, 17, 127
5. N.F. Stanley, Australian J. Sci., 1944, 6, 151
6. J.A. Elvidge and F.S. Spring, J. Chem. Soc., 1949, 2935
7. G. Lowe, A. Taylor and L.C. Vining, J. Chem. Soc. (C), 1966, 1799
8. A. Taylor, "Biochem. Some Foodborne Microbial Toxins" eds. R.I. Mateles and G.N. Wogan, M.I.T., Cambridge, Mass., 1967
9. A.P. Ryles, Ph.D. Thesis, Manchester, 1971
10. M.S. Ali, J.S. Shannon and A. Taylor, J. Chem. Soc. (C), 1968, 2044
11. J.S. Warner, Ph.D. Thesis, Cornell University, 1953
12. A.K. Bose, K.G. Das, P.T. Funke, I. Kugajevsky, O.P. Shukla, K.S. Kanchandani and R.J. Suhadolnik, J. Amer. Chem. Soc., 1968, 90, 1038
13. J.D. Dutcher, J.R. Johnson and W.F. Bruce, J. Amer. Chem. Soc., 1945, 67, 1736
14. J.D. Dutcher and A. Kjaer, J. Amer. Chem. Soc., 1951, 73, 4139
15. J.A. Elvidge and F.S. Spring, J. Chem. Soc., 1949, S135
16. J.R. Johnson and J.B. Buchanan, J. Amer. Chem. Soc., 1953, 75, 2103

17. J.D. Dutcher, J.R. Johnson and W.F. Bruce, J. Amer. Chem. Soc., 1944, 67, 614

18. R.J. Suhadolnik and R.G. Chenoweth, J. Amer. Chem. Soc., 1958, 80, 4391

19. J.D. Dutcher, J.R. Johnson and W.F. Bruce, J. Amer. Chem. Soc., 1944, 66, 617

20. R. Nagarajan, N. Neuss and M.M. Marsh, J. Amer. Chem. Soc., 1968, 90, 6518

21. M.R. Bell, Ph.D. Thesis, Cornell University, 1956

22. J.R. Johnson, J.H. Andreen and A.D. Holley, J. Amer. Chem. Soc., 1947, 69, 2370

23. J.R. Johnson, R.B. Hasbrouck, J.D. Dutcher and W.F. Bruce, J. Amer. Chem. Soc., 1945, 67, 423

24. M.R. Bell, J.R. Johnson, B.S. Wildi and R.B. Woodward, J. Amer. Chem. Soc., 1958, 80, 1001

25. R.L.M. Synge and E.P. White, Chem. & Ind., 1959, 1546

26. R.L.M. Synge and E.P. White, New Zealand J. Agr. Res., 1960, 3, 907.

27. R.H. Thornton and J.C. Percival, Nature, 1959, 183, 63

28. J.W. Ronaldson, A. Taylor, E.P. Wright and R.J. Abraham, J. Chem. Soc., 1963, 3172

29. J. Fridrichsons and A. Mc.L. Mathieson, Acta Cryst., 1965, 18, 1043

30. R. Hodges and J.S. Shannon, Australian J. Chem., 1966, 19, 1059

31. R. Rahman and A. Taylor, Chem. Commun., 1967, 1032

32. W.B. Turner, "Fungal Metabolites" p.327, Academic, London, 1971

33. D. Brewer, R. Rahman, S. Safe and A. Taylor, Chem. Commun., 1968, 1571

34. R. Rahman, S. Safe and A. Taylor, J. Chem. Soc. (C), 1969, 1665

35. S. Safe and A. Taylor, J. Chem. Soc. (C), 1970, 432

36. S. Safe and A. Taylor, J. Chem. Soc. (C), 1971, 1189

37. R. Rahman, S. Safe and A. Taylor, Quart. Rev., 1970, 24, 233

38. E. Francis, R. Rahman, S. Safe and A. Taylor, J. Chem. Soc., Perkin I, 1972, 470

39. R. Hodges, J.W. Ronaldson, A. Taylor and E.P. White, J. Chem. Soc., 1963, 5332

40. H.R. Herrmann, R. Hodges and A. Taylor, J. Chem. Soc., 1964, 4315

41. R. Hodges, J.S. Shannon and A. Taylor, J. Chem. Soc. (C), 1966, 1803

42. R. Hodges, J.W. Ronaldson, J.S. Shannon, A. Taylor and E.P. White, J. Chem. Soc., 1964, 26

43. J.W. Mason and J.G. Kidd, J. Immunol, 1951, 66, 99

44. E.J. Corey and E. Block, J. Org. Chem., 1966, 31, 1663

45. F. Lautenschlaeger, J. Org. Chem., 1966, 31, 1679

46. E.D. Weil, K.J. Smith and R.J. Gruber, J. Org. Chem., 1966, 31, 1669

47. P.M. Pojer and I.D. Rae, Tetrahedron Letters, 1971, 3077

48. T. Hino and T. Sato, Tetrahedron Letters, 1971, 3127

49. H. Poisel and U. Schmidt, Angew. Chem. Int. Ed., 1971, 10, 130.

50. H. Poisel and U. Schmidt, Berichte, 1971, 104, 1714

51. H. Poisel and U. Schmidt, Berichte, 1972, 105, 625

52. E. Ohler, H. Poisel, F. Tataruch and U. Schmidt, Berichte, 1972, 105, 635

53. P.W. Trown, Biochem. Biophys. Res. Commun., 1968, 33, 402

54. M. Ohno, T.F. Spande and B. Witkop, J. Amer. Chem. Soc., 1968, 90, 6521

55. M. Nakagawa, T. Kaneko and H. Yamaguchi, Chem. Commun., 1972, 603

56. R.G. Amiet, F.W. Eastwood and I.D. Rae, Chem. Commun., 1971, 1614

57. J.A. Winstead and R.J. Suhadolnik, J. Amer. Chem. Soc., 1960, 82, 1644

58. A.K. Bose, K.S. Kanchandani, R. Tavares and P.T. Funke, J. Amer. Chem. Soc., 1968, 90, 3593

59. M.S. Ali, R. Rahman and A. Taylor, "Chemistry of Natural Products, 5th Int. Symp.", p.94, I.U.P.A.C. 1968

60. N. Neuss, L.D. Bock, D.R. Brannon, J.C. Cline, D.C. DeLong, M. Gorman, L.L. Huckstep, D.H. Lively, J. Mabe, M.M. Marsh, B.B. Molloy, R. Nagarajan, J.D. Nelson and W.M. Stark, Antimicrobial Agents and Chemotherapy, 1968, 213.

61. N. Neuss, R. Nagarajan, B.B. Molloy and L.L. Huckstep, Tetrahedron Letters, 1968, 4467

62. P.J. Brook and R.E.F. Matthews, New Zealand J. Science, 1960, 3, 591.

63. N.R. Towers and D.E. Wright, New Zealand J. Agr. Res., 1969, 12, 275

64. P.W. Trown, H.F. Lindh, K.P. Milstrey, V.M. Gallo, B.R. Mayberry, H.L. Lindsay and P.A. Miller, Antimicrobial Agents and Chemotherapy, 1968, 225

65. R. Nagarajan, L.L. Huckstep, D.H. Lively, D.C. DeLong, M.M. Marsh and N. Neuss, J. Amer. Chem. Soc., 1968, 90, 2980

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

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

68. J.W. Moncrief, J. Amer. Chem. Soc., 1968, 90, 6517

69. N. Neuss, R. Nagarajan, B.B. Molloy and L.L. Huckstep, Tetrahedron Letters, 1968, 4467

70. E. Vogel and H. Guntner, Angew. Chem. Int. Ed., 1967, 6, 385

71. D. Hauser, H.P. Weber and H.P. Sigg, Helv. Chim. Acta, 1970, 53, 1061

72. H. Minato, M. Matsumoto and T. Katayama, Chem. Commun., 1971, 44.

73. W.B. Geiger, Arch. Biochem. Biophys., 1949, 21, 125

74. D. Brewer, D.E. Hannah, R. Rahman and A. Taylor, Canadian J. Microbiol., 1967, 13, 1451

75. S. Safe and A. Taylor, J. Chem. Soc. Perkin I, 1972, 472

76. J. Fridrichsons and A. McL. Mathieson, Acta Cryst., 1967 23, 439

77. W.D. Jamieson, R. Rahman and A. Taylor, J. Chem. Soc. (C), 1969, 1564

78. J.D. Bu'Lock, A.P. Ryles, N. Johns and G.W. Kirby, Chem. Commun., 1972, 100

79. W.F. Bruce, J.D. Dutcher, J.R. Johnson and L.L. Miller, J. Amer. Chem. Soc., 1944, 66, 614

80. G.W. Kirby and L. Ogunkoya, J. Chem. Soc., 1965, 6914

81. "N.M.R. Spectra Catalogue" Vol. 2 spectrum no. 534, Varian Associates, Palo Alto, California, 1963.

82. I.T. Bruce, Ph.D. Thesis, London 1968

83. D.J. Bennett, Ph.D. Thesis, London 1966

84. W.R. Gretton, private communication to the author.

85. H.L. Finkbeiner and M. Stiles, J. Amer. Chem. Soc., 1964, 86, 961

86. M. Rothstein, J. Amer. Chem. Soc., 1957, 79, 2009

87. H.L. Finkbeiner and M. Wagner, J. Org. Chem., 1963, 28, 216

88. E. Ware, Chem. Rev., 1950, 46, 403

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

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

91. E.E. Conn and P.K. Stumpf, "Outlines of Biochemistry," p. 49, Wiley, New York, 1963

92. E.A. Havir and K.R. Hanson, Biochemistry, 1968, 7, 1904

93. O. Hayaishi, "Oxygenases," p. 133, Academic, New York, 1966

94. G. Guroff, J.W. Daly, D.M. Jerina, J. Renson, B. Witkop, and S. Udenfriend, Science, 1967, 157, 1524.

95. B.J. Auret, D.R. Boyd, P.M. Robinson, C.G. Watson, J.W. Daly and D.M. Jerina, Chem. Commun., 1971, 1585

96. L. Nover and M. Luckner, F.E.B.S. Letters, 1969, 3, 292

97. h. Moody, R.H. Thompson, E. Fattorusso, L. Minale and G. Sodano, J. Chem. Soc. Perkin I, 1972, 18

98. L. Minale, G. Sodano, W.R. Chan and A.M. Chen, Chem. Commun., 1972, 674

99. M. Smith, "Reduction" p. 95, ed. R.L. Augustine, Arnold, London, 1968

100. I. Heilbron and H.M. Bunbury, "Dictionary of Organic Compounds" Vol. III, p.645

101. ibid. p. 282

102. A. Murray and D.Ll. Williams, "Organic Synthesis with Isotopes," p.222, Interscience, New York, 1958

103. F. Beilstein, "Handbuch der organischen Chemie," 24, 388 syst. no. 3591, 4th ed., Springer, Berlin, 1937.

104. ibid., 14, 495 syst. no. 1905

105. H. Matsuo, Y. Fujimoto and T. Tatsumo, Tetrahedron Letters, 1965, 39, 3465

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

107. F. Beilstein, "Handbuch der organischen Chemie," 27, 225, syst. no. 4280, 4th ed. Springer, Berlin, 1937.

108. ibid., 10, 683 syst. no. 1290

109. E.A. Havir and K.R. Hanson, Biochemistry, 1968, 7, 1896

110. E. Haslam and R. Ife, J. Chem. Soc. (C), 1971, 2818

111. P.V. Subba Rao and G.H.N. Towers, "Methods in Enzymology" vol. 17A, Academic, New York, 1970

112. R.E. Lapp and H.L. Andrews, "Nuclear Radiation Physics," 2nd ed. p. 79, Pitman, London, 1956

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

114. A. Hofmann, R. Brunner, H. Kobel and A. Brack, Helv. Chim. Acta, 1957, 40, 1358

115. H. Kaplan, U. Hornemann, K.M. Kelly and H.G. Floss, Lloydia, 1969, 32, 489

116. F. Troxler and A. Hofmann, Helv. Chim. Acta, 1957, 40, 2160

117. A. Stoll, A. Brack, H. Kobel, A. Hofmann and R. Brunner, Helv. Chim. Acta, 1954, 37, 1815

118. A.I. Scott, "Interpretation of the Ultraviolet Spectra of Natural Products," p. 174, Pergamon, London, 1964

119. K. Freter, J. Axelrod and B. Witkop, J. Amer. Chem. Soc., 1957, 79, 3191

120. T. Wieland, O. Weiberg, E. Fischer and G. Hörlein, Annalen, 1954, 587, 146

121. H. Fischer and M. Herrmann, Z. physiol. Chemie 1922, 122, 4

122. L.E. Craig and D.S. Tarbell, J. Amer. Chem. Soc., 1949, 71, 462

123. J.B. Bell and H.G. Lindwall, J. Org. Chem., 1948, 13, 547

124. B. Bak, C. Dambmann and F. Nicolaisen, Acta Chem. Scand., 1967, 21, 1674

125. S. Agurell and E. Ramstad, Lloydia, 1962, 25, 7

126. G.W. Kirby and J. Michael, Chem. Commun., 1971, 415

127. R. Filler, Adv. Heterocyclic Chem., 1965, 4, 95

128. K. Brocklehurst, H.S. Price and K. Williamson, Chem. Commun., 1968, 884

129. W. Steglich, Fortschr. chem. Forsch., 1969, 12, 77

130. A.P. Morgenstein, C. Schutij and W. Th. Nauta, Chem. Commun., 1969, 321

131. G.W. Kirby, J. Michael and S. Narayanaswami, J. Chem. Soc. Perkin I, 1972, 203

132. J.B. Gilbert, V.E. Price and J.P. Greenstein, J. Org. Chem., 1949, 180, 473

133. K.R. Hanson, R.H. Wightman, J. Staunton and A.R. Battersby, Chem. Commun., 1971, 185

134. A.R. Battersby, Accounts Chem. Res., 1972, 5, 148

135. J.T. Holden, "Amino-Acid Pools," p.102, Elsevier 1962

136. P. Chandra and L.C. Vining, Can. J. Microbiol., 1968, 14, 573

137. A.P. Ryles, private communication to the author.

138. A. Meister, "Biochemistry of the Amino-Acids," 2nd ed. p.219, Academic, New York, 1965

139. E.E. Snell, Vitamins Hormones, 1958, 16, 77

140. A.E. Braunstein, "The Enzymes" eds. P.D. Boyer, H. Lardy and K. Myrbeck, Vol. 2 p.113, Academic, New York, 1960

141. A. Meister, "Biochemistry of the Amino-Acids", ed.2 p.402, Academic, New York, 1965

142. T. Oshima and N. Tamiya, Biochem. J., 1961, 78, 116

143. N.S. Constantsas and W.E. Knox, Arch. Biochem. Biophys., 1966, 117, 59

144. R.P. Spencer and W.E. Knox, Arch. Biochem. Biophys., 1962, 96, 115

145. H.N. Christensen, "A Symposium on Amino-Acid Metabolism" p.63, Johns-Hopkins, Baltimore, 1955

146. S.M. Birnbaum, L. Levintow, R.B. Kingsley and J.P. Greenstein, J. Biol. Chem., 1952, 194, 455

147. R. Voigt, Pharmazie, 1968, 23, 285-296; 353-9; 419-436

148. K. Winkler and D. Größer, Pharmazie, 1962, 17, 658

149. R.M. Baxter, S.I. Kandel and A. Okany, Chem. & Ind., 1960, 266

150. H. Plieninger, R. Fischer, G. Keilich and H.D. Orth, Annalen, 1961, 642, 214

151. A. Hofmann, R. Heim, A. Brack, H. Kobel, A. Frey, H. Ott, Th. Petrzilka and F. Troxler, Helv. Chim. Acta, 1959, 42, 1557

152. A. Brack, A. Hofmann, F. Kalberer, H. Kobel and J. Rutschmann, Arch. Pharmaz. Ber. dtsch. pharmaz. Ges., 1961, 294, 230

153. H. Plieninger, R. Fischer and V. Liede, Angew. Chem. Int. Ed., 1962, 1, 399

154. F. Weygand, H.-G. Floss and U. Mothes, Tetrahedron Letters, 1962, 873

155. D. Grüger, H.-J. Wendt, K. Mothes and F. Weygand, Z. Naturforsch., 1959, 146, 355

156. F. Weygand, H.-G. Floss, U. Mothes, D. Grüger and K. Mothes, Z. Naturforsch., 1963, 19b, 202

157. H.-G. Floss and U. Mothes, Z. Naturforsch., 1963, 18b, 866

158. B.W. Bycroft and W. Landon, Chem. Commun., 1970, 967

159. V.E. Tyler and A.E. Schwarting, "Experimental Pharmacognosy", Burgess Publishing Co., Minneapolis, Minnesota, 1962

160. eg. F. Weygand and H.-G. Floss, Angew. Chem. Int. Ed., 1963, 2, 243

161. G.W. Kirby, S.W. Shah and E.J. Herbert, J. Chem. Soc. (C), 1969, 1916

162. S.W. Shah, Ph.D. Thesis, London 1969

163. L.R. Morgan and R. Schunior, J. Org. Chem., 1962, 27, 3696

164. R.O.C. Norman, "Principles of Organic Synthesis" p.475, Wallmer Bros., Birkenhead, Cheshire, 1968

165. F. Troxler and A. Hofmann, Helv. Chim. Acta, 1959, 42, 793-802

166. T. Hino, K. Tsuneoka, M. Nakagawa and S. Akaboshi, Chem. & Pharm. Bull. (Japan), 1969, 17, 550

167. N.I. Putokhin, J. Gen. Chem. U.S.S.R., 1945, 15, 332

168. W.B. Lawson, A. Patchornik and B. Witkop, J. Amer. Chem. Soc., 1960, 82, 5918

169. R. Pinchin, private communication to the author

170. H. Brintzinger, K. Pfannstiel, H. Koddebusch and K. Kling, Berichte, 1950, 83, 87

171. E. Scoffone, A. Fontana and R. Rocchi, Biochemistry, 1968, 7, 971

172. P.S. Clezy and F.R. Fookes, Chem. Commun., 1971, 1268

173. D.S. Matteson and H.R. Snyder, J. Org. Chem., 1957, 22, 1500

174. D.E. Koshland Jr., Y.D. Karkhanis and H.G. Latham, J. Amer. Chem. Soc., 1964, 86, 1448

175. H.R. Horton and D.E. Koshland Jr., J. Amer. Chem. Soc., 1965, 87, 1126