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ISOLATION AND STRUCTURE OF THE METABOLITES FROM DIPLODIA MAYDIS

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ISOLATION AND STRUCTURE OF THE METABOLITES FROM

DIPLODIA MAYDIS

by

STEPHANUS FRANCOIS MARAIS

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JOINT PROMOTOR : PROF T.A. MODRO

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SUMMARY

Investigation of toxic extracts from cultures of the fungus *Diplodia maydis* (Berk.) Sacc. [synonyms *D. zeae* (Schw.) Lév. and *Stenocarpella maydis* (Berk.)] led to the isolation of diplodiatoxin, a known metabolite, as well as four new metabolites *viz.* 3-hydroxydiplodiatoxin stenocarpin, steno-carpin 6-*O*-(4-*O*-methyl-orsellinate) and carpellin. The structure elucidation of the new compounds is based on a detailed analysis of their high-field ^{1}H and ^{13}C nuclear magnetic resonance spectra. The relative configurations were deduced from the magnitude of the proton-proton coupling constants and the results obtained from homonuclear proton-proton nuclear Overhauser effect (n.O.e.) studies. The absolute configuration of stenocarpin 6-*O*-(4-*O*-methylorsellinate) was determined by the 'partial resolution' method of Horeau.

The biosynthetic pathway leading to diplodiatoxin was investigated by incorporation studies with ^{13}C-, ^{2}H and ^{18}O-labelled precursors.

OPSOMMING

'n Ondersoek van toksiese ekstrakte van kulture van *die* skimmel *Diplodia maydis* (Berk.) Sacc. [sinonieme *D. zeae* (Schw.) Lév. en *Stenocarpella maydis* (Berk.)] het gelei tot die isolasie van diplodiatoksien, 'n bekende metaboliet, asook vier nuwe metaboliete nl. 3-hidroksiediplodiatoksien, stenokarpien, stenokarpien 6-*O*-(4-*O*-metielorsellinaat) en karpellien. Die struktuuropklaring van die verbindings is gebaseer op 'n uitgebreide studie van die onderskeie verbindings se hoë-veld ^{1}H- en ^{13}C-kernmagnetiese resonansspektra. Die relatiewe konfigurasie van die verbindings is afgelei van die grootte van die proton-proton koppelingskonstantes en die resultate wat van homokernige proton-proton kern Overhauser effek studies verkry is. Die absolute konfigurasie van stenokarpien 6-*O*-(4-*O*-metielorsellinaat) is deur die 'gedeeltelike resolusie' metode van Horeau bepaal.

Die biosintese van diplodiatoksien is deur middel van inkorporasie van ^{13}C-, ^{2}H- en ^{18}O-gemerkte voorlopers ondersoek.

ACKNOWLEDGEMENTS

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INDEX

CHAPTER 1

INTRODUCTION

Mycotoxins (from Greek, *mykes*: fungus) are fungal metabolites which have an adverse effect on man and his domestic animals. The diseases caused by the ingestion of foods or animal feeds contaminated by these toxic fungal metabolites are commonly called mycotoxicoses and are characterized by their sporadic regional and seasonal occurrence.

Mycotoxins have been part of mankind's environment throughout the ages. The earliest described mycotoxicoses, ergotism, constituted one of the greatest recurring catastrophes of European history over a period of 2000 years. The ergotism epidemic for example of the year 944 in Aquitaine and Limoges in France killed an estimated 40 000 people.[1] The cause of the disease is the parasitic fungus *Claviceps purpurea* which readily infects rye and other grains and grasses, and which produces a group of physiologically-active ergot alkaloids related to lysergic acid.

Massive epidemic outbreaks of alimentary toxic aleukia occurred in Orenburg and other districts of Russia during World War 2.[2-4] *Fusarium sporotrichioides*, producer of the sesquiterpene trichothecene mycotoxins such as T-2 toxin, was implicated in the etiology of the disease which was found to be associated with grain, wheat and barley that had been left on the fields during winter in the war years.

The present international awareness of mycotoxicoses and the interest in the structure, synthesis and biosynthesis of mycotoxins, is attributable to the discovery in 1960 of 'Turkey X' disease, a poultry disease of unknown origin that caused the death of 100 000 turkeys and chickens. The cause

of the disease was subsequently traced to the consumption of Brazilian peanut meal contaminated by aflatoxins,[5,6] a family of highly carcinogenic secondary metabolites produced by the fungi *Aspergillus flavus* and *A. parasiticus*.[7-9]

Humans may be exposed to mycotoxins such as the aflatoxins in several ways: Aflatoxin B_1 produced by the fungus *A. flavus* on a foodstuff such as groundnuts, may be ingested directly by man or may be metabolized into structurally related toxins. Lactating cows for example convert aflatoxin B_1 into the toxic compound aflatoxin M_1, which can enter man's foodchain through the consumption of milk. An alternative route leading to human consumption is contaminated meat and meat products.

The fungus *Diplodia maydis* (Berk.) Sacc. [synonyms *D. zeae* (Schw.) Lév. and *Stenocarpella maydis* (Berk.)] is worldwide one of the most important cob rot pathogens of maize.[10-12] Isolates of this fungus are also responsible for field outbreaks of a neurological disease, called diplodiosis, in cattle and sheep.[13,14]

Although *D. maydis* is encountered worldwide throughout the maize producing areas, outbreaks of diplodiosis are known to occur only in southern Africa.[15] A single instance of a suspected diplodiosis outbreak in Australia has been reported.[16] The lack of recorded outbreaks outside southern Africa can be attributed to factors such as differences in agricultural practices and the variability in the toxigenic potential of *D. maydis*.[15] It is, however disconcerting that toxigenic strains of *D. maydis* have been isolated recently from bulk consignments of maize imported from North and South America where diplodiosis is supposedly absent.[17]

Diplodiosis is a nervous condition of cattle and sheep characterized by ataxia, paresis and paralysis which can result in death.[13,14,17,18] The disease is caused by the

ingestion of mouldy maize infected with *D. maydis*.[19] Diplodiosis has been experimentally induced in cattle and sheep in feeding experiments with *D. maydis* infected maize.[13,17,19] The onset of diplodiosis is indicated by inco-ordination, paralysis and occasional tremors. In the event of continued ingestion of infected maize, these signs become more pronounced and eventually complete muscular paralysis sets in and death soon follows. If on the other hand feeding is discontinued a complete recovery of the animal is possible.[20,21]

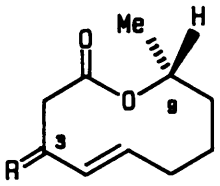
Recent investigations indicate that the ingestion of maize contaminated with *D. maydis*, either naturally infected or culture material, has grave consequences for pregnant ewes as the foetus is either aborted or the lamb is still-born.[22] In those instances where the lamb does survive it shows the typical symptoms of diplodiosis.

D. maydis contamination of maize affects not only ruminants but also poultry such as ducklings and chickens and leads to unacceptable losses and growth retardation. The toxic and erosive effects in poultry poses a potential hazard for the poultry industry with concomitant economic implications.

Some isolates of *D. maydis* known to be toxic to ducklings do not have the ability to induce diplodiosis in ruminants.[19,23] This finding points to the presence of other toxic metabolites in culture material besides the neurotoxin responsible for diplodiosis. The degree of toxicity is to a large degree dependent on the incubation period and temperature of the *D. maydis* cultures. The unidentified neurotoxin survives prolonged exposure to heat at 45°C but in the presence of moisture the toxicity of cultures is diminished by heating at temperatures of 75°C for 48 hours.[17]

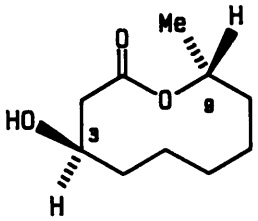
A number of metabolites have been isolated from cultures of different *Diplodia* species and their structures elucidated.

A steroid hydroxylase inhibitor, diplodialide A **(1)** as well as the related metabolites diplodialide B **(2)**, C **(3)** and D **(4)** are produced by *D. pinea*.[24-26]



(1) R=O

(2) R=β-OH, H



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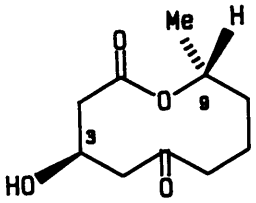


(4)

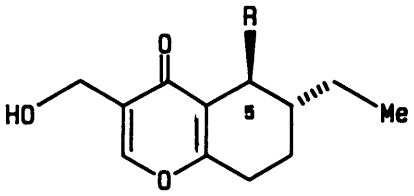
The stereochemistry at C-3 and C-9 of diplodialide D **(4)** was not determined due to insufficient material but was assumed to be the same as in the other three metabolites. The absolute configuration of the other diplodialides was determined as follows.[25] Oxidation of diplodialide B **(2)** gave a product identical to diplodialide A **(1)** whereas diplodialide C **(3)** was identical to the dihydro-derivative of diplodialide B **(2)**. The configuration at C-9 is therefore the same in all three compounds. The absolute configuration at C-9 was determined by the degradation of diplodialide B

(2) to (-)-hexane-1,5-diol, which was converted to the di-*p*-nitrobenzoate. The specific rotation of these two compounds was similar in magnitude but of opposite sign to the values reported for *S*-(+)-hexane-1,5-diol and its di-*p*-nitrobenzoate indicating the 9*R* configuration for diplodialide A, B and C.

The absolute stereochemistry at C-3 of diplodialide B **(2)** was determined by ozonolysis of its acetate to give two acids which were converted to their methyl esters and isolated as their *p*-nitrobenzoates. The products were identified as dimethyl (-)-*p*-nitrobenzoylmalate and the *p*-nitrobenzoyl derivative of methyl 5-hydroxyhexanoate, with the former displaying the same specific rotation as the authentic *S*-(-)-compound prepared from *S*-(-)-malic acid. These results established the 3*S* configuration for diplodialide B **(2)** and the 3*R* configuration for diplodialide C **(3)**. The change in configurational notation is due to the Cahn-Ingold-Prelog sequence rules.

Diplosporin **(5)** is a mycotoxin (LD_{50} 88,4mg/kg) isolated from cultures of *D. macrospora* and the structure of this metabolite was established by physico-chemical techniques.[27,28] A related metabolite, 5-deoxydiplosporin **(6)**, was isolated from the culture medium of *D. macrospora*.[29]

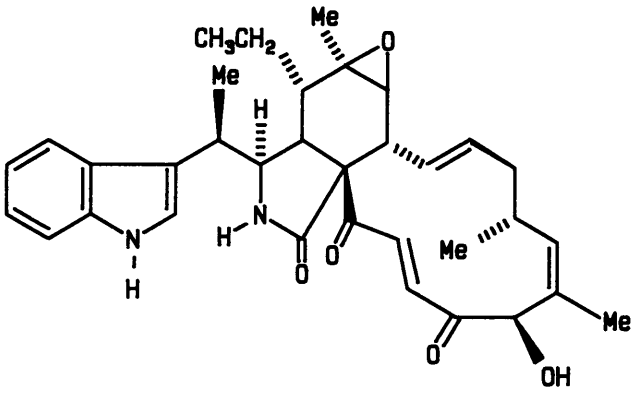
The relative stereochemistry of diplosporin **(5)** was established by a single crystal *X*-ray crystallographic study.[30] The absolute configuration followed when the 'partial resolution' method of Horeau indicated the *S* configuration for the C-5 hydroxy group.[31]



(5) R=OH

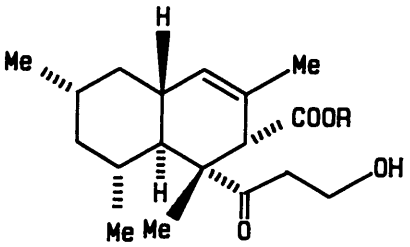
(6) R=H

Chaetoglobusin K (7), another toxic metabolite isolated from *D. macrospora*, exhibits plantgrowth-inhibiting characteristics.[32]



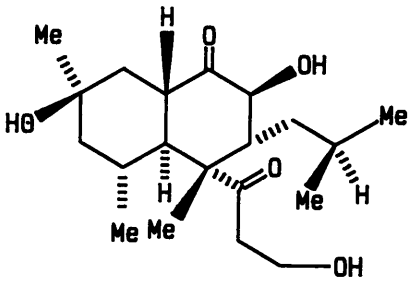
(7)

Diplodiatoxin (8) is the only toxic metabolite isolated from *D. maydis*.[33] It is probably not the major toxin since bioassays revealed that fractions containing this metabolite accounted for only a fraction of the toxicity. Diplodiatoxin has not yet been tested for neurotoxicity in sheep and cattle.



(8) R=H

(9) R=Me



(10)

The relative stereochemistry of diplodiatoxin was determined in a single crystal *X*-ray crystallographic study (**Figure 1**).[34]

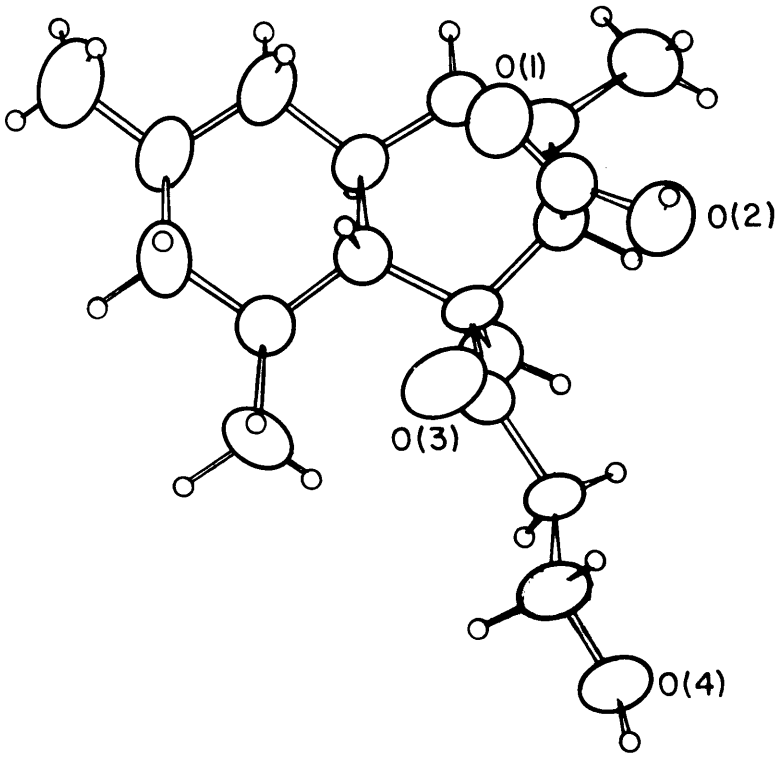
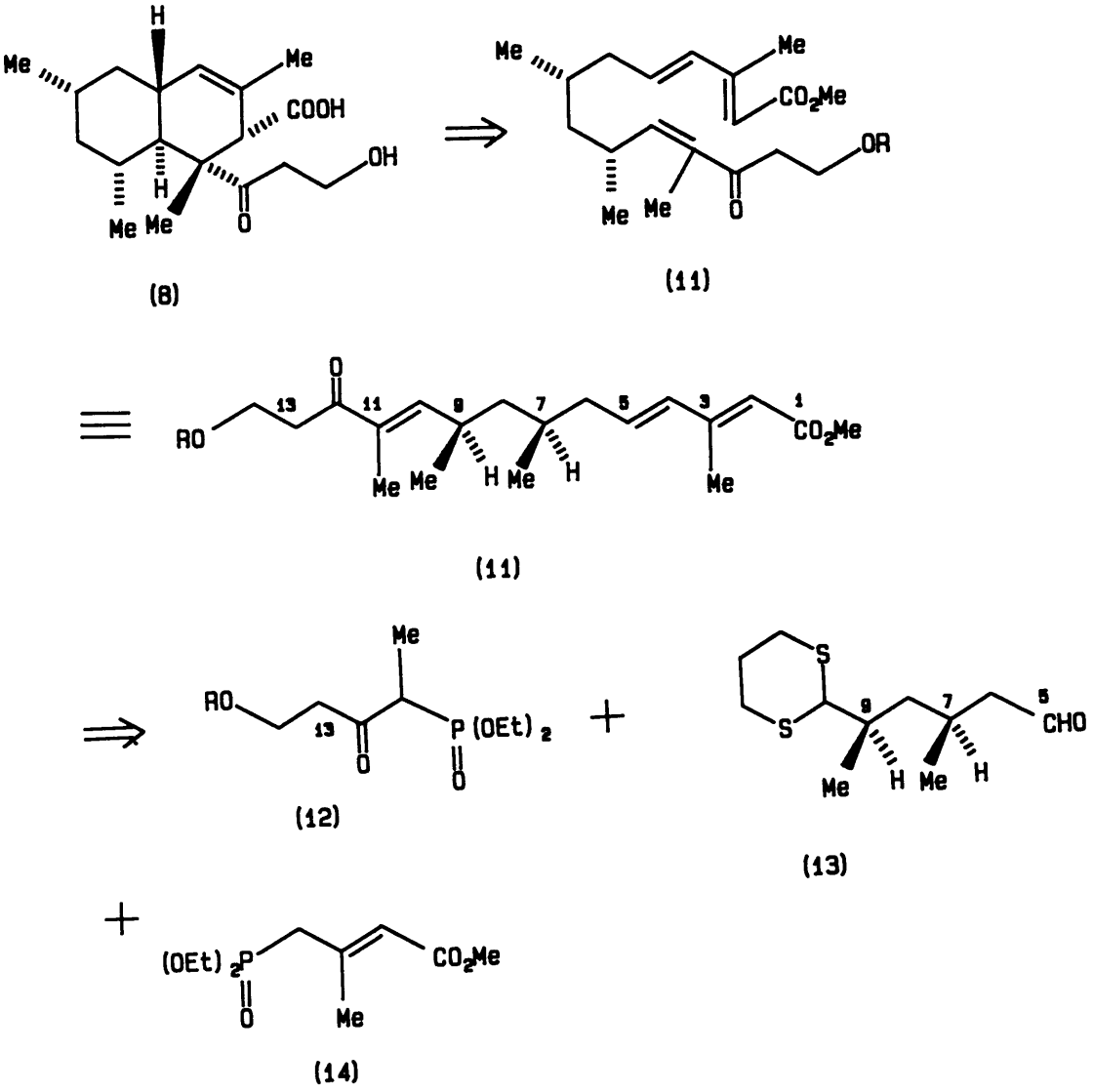


Figure 1 Perspective drawing of diplodiatoxin **(8)** showing the relative stereochemistry.

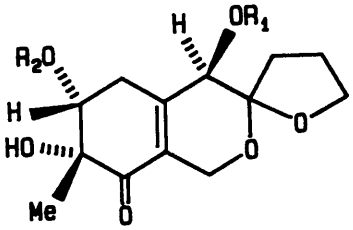
Confirmation of the stereochemistry including the absolute stereochemistry followed from the stereoselective synthesis of natural (+)-diplodiatoxin.[35] A tentative configuration for diplodiatoxin was deduced from the published n.m.r.

data,[33] as well as an extensive comparison of the ^{1}H n.m.r. spectrum of diplodiatoxin with that of betaenone B **(10)**.[36] In the retrosynthetic analysis of diplodiatoxin (**Scheme 1**) the intramolecular Diels-Alder reaction of the (*E,E,E*)-trienone **(11)** forms the key step. Retrosynthetic disconnection of the C-4-- C-5 and C-10-- C-11 bonds in the trienone **(11)** leads to the formulation of three synthons. Although the two phosphonates **(12)** and **(14)** are readily accessible compounds, the half-masked dialdehyde **(13)** with the required stereochemistry, was derived from D-glucose in a 15-step sequence.



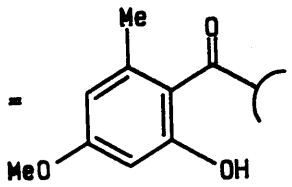
Scheme 1 Retrosynthetic analysis of diplodiatoxin **(8)**

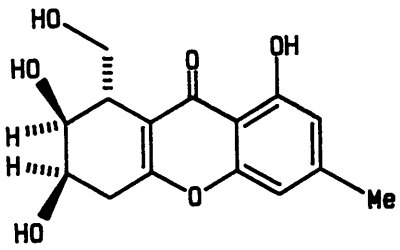
Linkage of the three segments using the Wittig-Horner procedure followed by an intramolecular Diels-Alder reaction produced (+)-diplodiatoxin. Comparison of the c.d. spectrum of the synthetic diplodiatoxin with that of the natural compound, showed that the absolute configuration must be as depicted in (8).



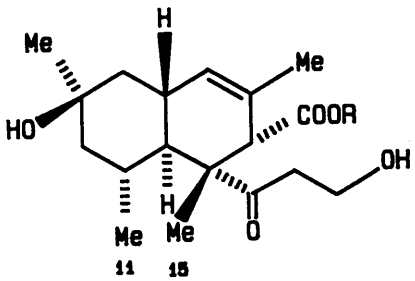
(15) $R_1=R_2=H$

(16) $R_1=H$, R_2 =





(17)



(18)

In the current investigation as described in this thesis, extracts of *D. maydis* cultures were evaluated in a bioassay using day-old ducklings. Fractionation of toxic extracts guided by this bioassay, led to the identification of the known diplodiatoxin **(8)**, a derivative of diplodiatoxin, 3-hydroxydiplodiatoxin **(18)**, and three new compounds. These three compounds, stenocarpin **(15)**, stenocarpin 6-*O*-(4-*O*-methylorsellinate) **(16)**, carpellin **(17)**, as well as diplodiatoxin **(8)** and 3-hydroxydiplodiatoxin **(18)**, proved to be acutely toxic to day-old ducklings. At this stage none of these compounds have been tested in ruminants. The structure elucidation and chemical reactions of stenocarpin **(15)**, stenocarpin 6-*O*-(4-*O*-methylorsellinate) **(16)**, carpellin **(17)** and 3-hydroxydiplodiatoxin **(18)** as well as the biosynthesis of diplodiatoxin **(8)**, form the subject of this thesis.

CHAPTER 2

ISOLATION OF THE TOXIC METABOLITES

Diplodia maydis (Berk.) Sacc. [*Diplodia zea* (Schw.) Lév.] was cultivated in bulk on wet sterile maize kernels. The dried and milled mouldered maize was found to be acutely toxic to day-old ducklings.

This toxic material (22kg) was first extracted in a Waring blender with ethyl acetate to remove mainly non-toxic lipid material and subsequently with a mixture of methanol-water (3:1 v/v). The resulting extract was evaporated under reduced pressure and the residual aqueous solution was subjected to ion exchange chromatography as shown in **Figure 2**. The relevant fractions were monitored by bioassay in day-old ducklings using acute toxicity as the criterium of biological activity. The toxicity was found to be associated mainly with the material obtained in fractions A (15g) and B (58g).

It should be noted that the extracted maize obtained after the final extraction with aqueous methanol was still acutely toxic to day-old ducklings. This result points to the presence of a chemically bonded toxin(s) which is not extracted using the outlined method.

2.1 ISOLATION OF DIPLODIATOXIN (8)

On the basis of the extraction step involving sodium hydrogencarbonate, fraction A (15g) contains organic acids which were separated by column chromatography on silica gel using chloroform-methanol (9:1 v/v) as eluant. Fractions (10ml) were evaluated by thin-layer chromatography (t.l.c.) on silica gel and appropriate fractions combined to give 3 fractions a-c. Fraction c proved toxic to day-old ducklings. It was evident from t.l.c. that fraction c contained a

single mycotoxin which was isolated once again by column chromatography on silica gel using the same eluant as before. Crystallization from chloroform yielded the known metabolite diplodiatoxin **(8)** (880mg) as a white crystalline solid.

2.2 ISOLATION OF STENOCARPIN 6-*O*-(4-*O*-METHYLORSELLINATE) (16), CARPELLIN (17), AND 3-HYDROXYDIPLODIATOXIN (18)

Column chromatography of fraction B was performed on silica gel using first chloroform and subsequently chloroform with increasing amounts of methanol as eluant. Once again fractions (10ml) were evaluated by t.l.c. on silica gel and appropriate fractions combined to give 11 fractions a-k, all of which proved to be acutely toxic to day-old ducklings. Fractions b and c were chromatographed on silica gel using hexane-ethyl acetate (1:1 v/v) as solvent and crystallized from ether-hexane to give stenocarpin 6-*O*-(4-*O*-methylorsellinate) **(16)** (260mg) as a white crystalline compound.

After chromatography on silica gel, using chloroform-methanol (9:1 v/v) as eluant, fractions h and i yielded carpellin **(17)**, a white crystalline compound. Both carpellin and stenocarpin 6-*O*-(4-*O*-methylorsellinate) **(16)** were acutely toxic to day-old ducklings. Fraction j was purified on silica gel using chloroform-methanol (9:1 v/v) as eluant and yielded the toxic 3-hydroxydiplodiatoxin **(18)** as a white solid. The fact that this compound with its carboxylic group was not extracted from the organic layer using sodium hydrogencarbonate could possibly be attributed to its unusual gel forming properties with organic solvents.

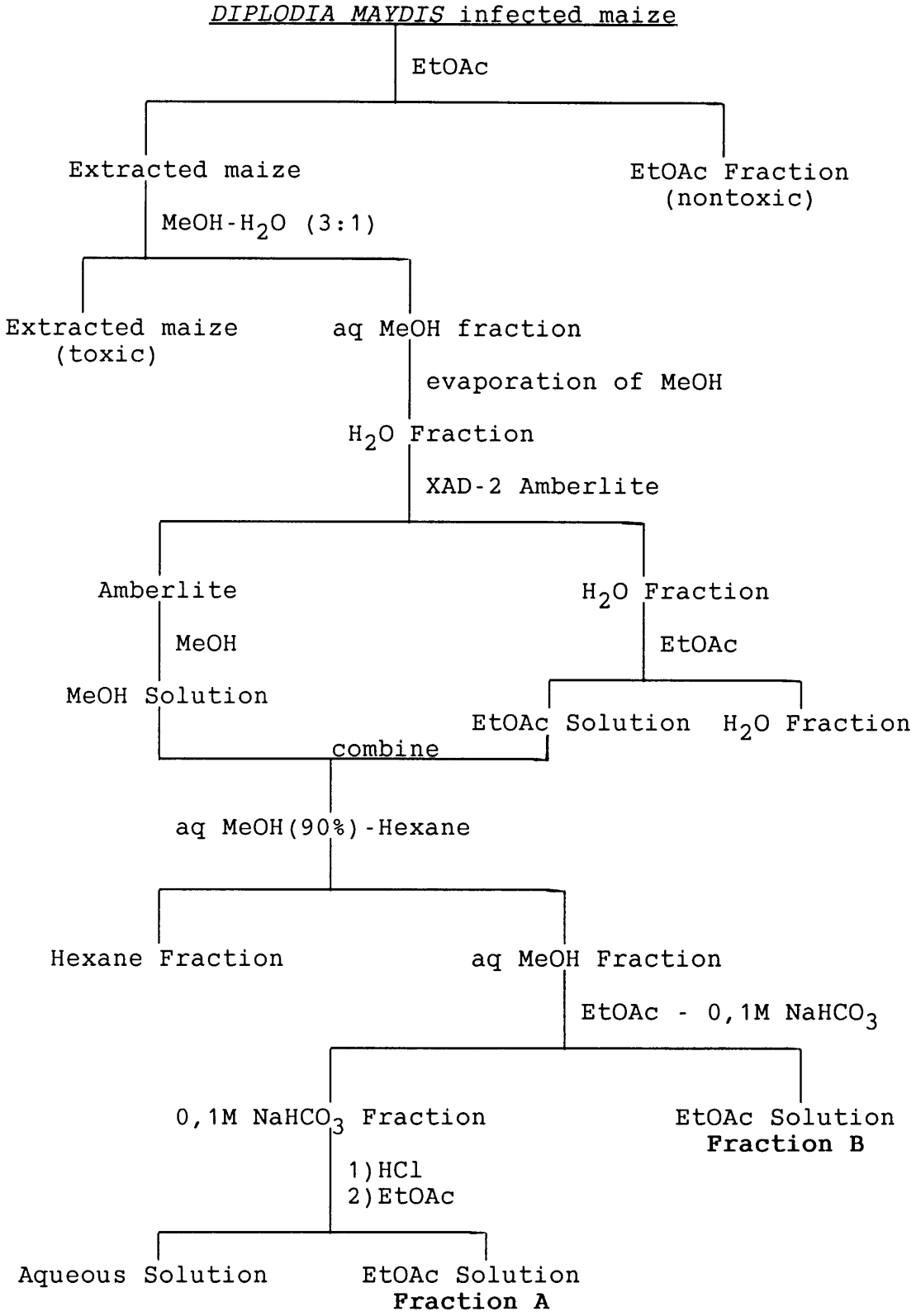


Figure 2 Extraction and fractionation scheme of maize cultures of *Diplodia maydis* (MRC 2829)

2.3 ISOLATION OF STENOCARPIN (15)

Stenocarpin **(15)** was only isolated from the first batch of toxic maize, in which stenocarpin 6-*O*-(4-*O*-methylorsellinate) **(16)** was present as a minor component. In later batches of *D. maydis* infected maize, stenocarpin was completely replaced by much larger quantities of the 6-*O*-(4-*O*-methylorsellinate) derivative. The presence of impurities with very similar chromatographic properties in the fraction containing the stenocarpin metabolite caused great problems in the isolation and purification procedure. As a last resort the material contained in this fraction was acetylated using acetic anhydride and pyridine and stenocarpin was purified as its diacetate derivative **(19)** by chromatography on silica gel using chloroform-acetone (18:1 v/v).

The isolation of stenocarpin and structure elucidation of stenocarpin 4,6-*O*,*O*-diacetate is described as it contributed to the final isolation and structure elucidation of stenocarpin 6-*O*-(4-*O*-methylorsellinate).

2.4 ISOLATION OF DIPLODIATOXIN (8) FROM LIQUID CULTURE MEDIUM

In order to study the biosynthetic origin of the toxic metabolites from *Diplodia maydis* a large number of liquid media as well as a solid maize meal medium were screened for the ability to produce these toxins. In the course of this screening it was found that diplodiatoxin was produced in reasonable quantities on some of the liquid media. None of the other toxins could be detected. On the basis of the yield of diplodiatoxin per liter of medium, potato-dextrose broth (PDB) was judged as the most suitable for biosynthetic studies.

The mycelia were harvested 10 days after inoculation of the potato-dextrose broth with a *D. maydis* infected maize

kernel, macerated with acetone in a Waring blender and the filtrate evaporated. The residual aqueous solution was extracted with ethyl acetate. This ethyl acetate solution was combined with the ethyl acetate extract of the filtered broth. The combined ethyl acetate extracts were concentrated and the residual material subjected to solvent-solvent partitioning as shown in **Figure 3** to give a fraction A containing diplodiatoxin. The diplodiatoxin was purified by column chromatography on silica gel using chloroform-methanol (9:1 v/v) as eluant. Diplodiatoxin **(8)** obtained in the different biosynthetic experiments was converted to the methyl ester **(9)** using diazomethane in order to facilitate the interpretation of the ^{13}C n.m.r. spectra.

TABLE 1 YIELDS AND PHYSICAL DATA OF THE TOXIC METABOLITES

Metabolite	Yield (mg)[a]	Formula	Molecular mass
Stenocarpin 4,6-*O*,*O*-diacetate	6	$C_{17}H_{22}O_8$	354
Stenocarpin 6-*O*-(4-*O*-methyl orsellinate)	12	$C_{22}H_{26}O_9$	434
Carpellin	10	$C_{15}H_{16}O_6$	292
Diplodiatoxin	40 22[b]	$C_{18}H_{28}O_4$	308
3-Hydroxydiplodiatoxin	44	$C_{18}H_{28}O_5$	324

[a] Yields relative to 1kg of dried mouldered maize
[b] Values relative to 1*l* of PDB medium

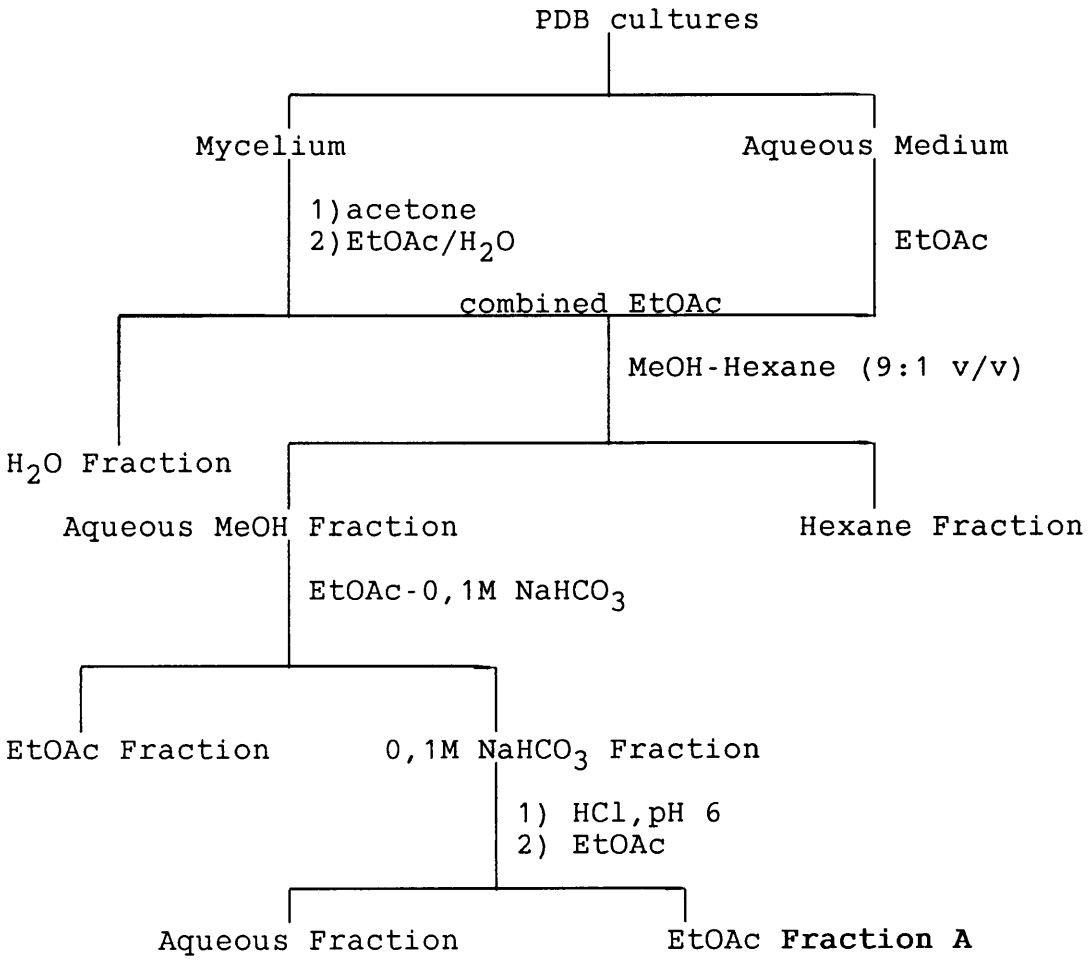


Figure 3 Extraction and fractionation scheme for *Diplodia maydis* cultures grown on potato-dextrose broth

CHAPTER 3

STRUCTURE DETERMINATION OF THE TOXIC METABOLITES

3.1 INTRODUCTION

The strategy for the structure elucidation of the toxic metabolites isolated from maize cultures of *D. maydis* involved the collection of data about the constitution of these metabolites using spectroscopic techniques and chemical reactions.

The molecular formulae were obtained from accurate mass determination of the molecular ion or other significant ions observed in the mass spectra as well as elemental analyses. A detailed study of the ^{1}H and ^{13}C n.m.r. spectra led to the assignment of specific structures and relative configurations to each of these compounds.

N.m.r. spectroscopy is one of the most powerful tools for structural analysis available to the organic chemist. The detailed analysis of both the ^{1}H and ^{13}C n.m.r spectra of the *D. maydis* metabolites was facilitated by the results obtained from a number of n.m.r. techniques.

Assignments based on first-order analysis of the spin systems in the 500,13 MHz ^{1}H n.m.r. spectra were confirmed by homonuclear ^{1}H-$\{^{1}H\}$ decoupling experiments. The 125,76 MHz ^{13}C n.m.r. data, *viz.* chemical shifts, most multiplicities and one-bond (C,H) coupling constants were obtained from proton-decoupled and single-frequency nuclear Overhauser enhanced (n.O.e.) spectra.

The signals of the proton-bearing carbon atoms were correlated with specific proton resonances in two-dimensional (2-D) ^{13}C-$\{^{1}H\}$ heteronuclear shift correlation experiments utilizing the one-bond (^{13}C,^{1}H) spin-spin couplings.

Heteronuclear ^{13}C-{^{1}H} selective population inversion (SPI), a pulsed double resonance n.m.r. technique, was applied routinely in the structure elucidation to determine the long-range (more than one bond) (^{13}C,^{1}H) connectivity patterns, by using long-range (^{13}C,^{1}H) spin-spin couplings.[37-40]

The basic principle of this technique is demonstrated for a simple two-spin AX system of spin-½ nuclei, consisting of a ^{1}H (A part) and a ^{13}C (X part) nucleus. **Figure 4** shows the AX energy level diagram with the relative populations at thermal equilibrium and the allowed single quantum transitions, as well as the corresponding spectrum of the X part.

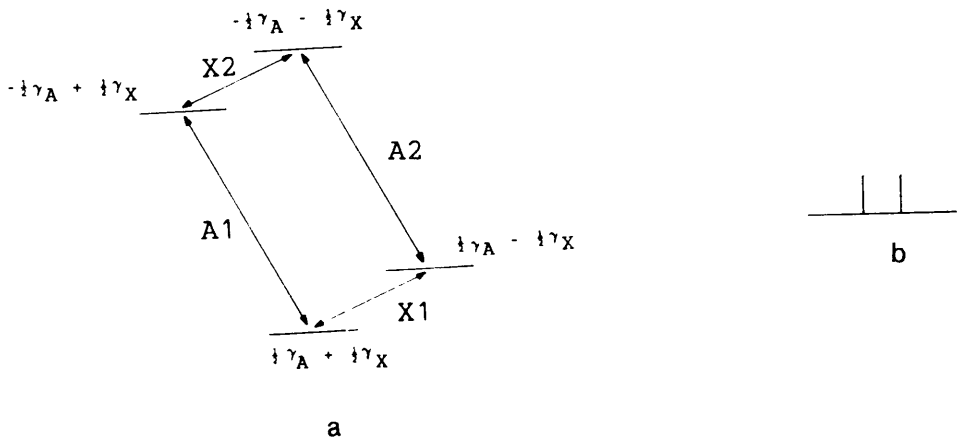


Figure 4 (a) Energy level diagram for an AX spin system at thermal equilibrium and (b) corresponding spectrum of the X part (^{13}C spectrum)

The population difference between two energy levels, and thus the intensity of a transition, is proportional to the gyromagnetic ratio, γ, of the nucleus that changes its spin state during the transition. The relative populations and transition intensities of an AX spin system before and after inversion of the A1 transition are collated in **Table 2.**[40]

TABLE 2 RELATIVE POPULATIONS AND TRANSITION INTENSITIES OF AN AX SPIN SYSTEM BEFORE AND AFTER INVERSION OF THE A1 TRANSITION

	Populations			Intensities	
Energy level	At equilibrium	After inversion of A1 transition	Transition	At equilibrium	After inversion of A1
1	$\frac{1}{2}\gamma_A + \frac{1}{2}\gamma_X$	$-\frac{1}{2}\gamma_A + \frac{1}{2}\gamma_X$	A1 (1 → 3)	γ_A	$-\gamma_A$
2	$\frac{1}{2}\gamma_A - \frac{1}{2}\gamma_X$	$\frac{1}{2}\gamma_A - \frac{1}{2}\gamma_X$	A2 (2 → 4)	γ_A	γ_A
3	$-\frac{1}{2}\gamma_A + \frac{1}{2}\gamma_X$	$\frac{1}{2}\gamma_A + \frac{1}{2}\gamma_X$	X1 (1 → 2)	γ_X	$-\gamma_A + \gamma_X$
4	$-\frac{1}{2}\gamma_A - \frac{1}{2}\gamma_X$	$-\frac{1}{2}\gamma_A - \frac{1}{2}\gamma_X$	X2 (3 → 4)	γ_X	$\gamma_A + \gamma_X$

The energy level diagram and the corresponding spectra shown in **Figure 5** result when the respective spin populations are interchanged through selective population inversion involving the A1 transition. The result of this inversion is that the populations of the two energy levels of the A1 transition have been interchanged. The effect on the population differences however, is much more pronounced. For the two A transitions the population differences are the same, γ_A, except that one is now negative. For the X transitions which previously had a population difference of γ_X, we now find $-\gamma_A + \gamma_X$ for the X1 transition and $\gamma_A + \gamma_X$ for the X2 transition. This result implies that the population differences for the A transitions have been transferred to the X transitions and added to the existing differences. This phenomena is known as polarization transfer.

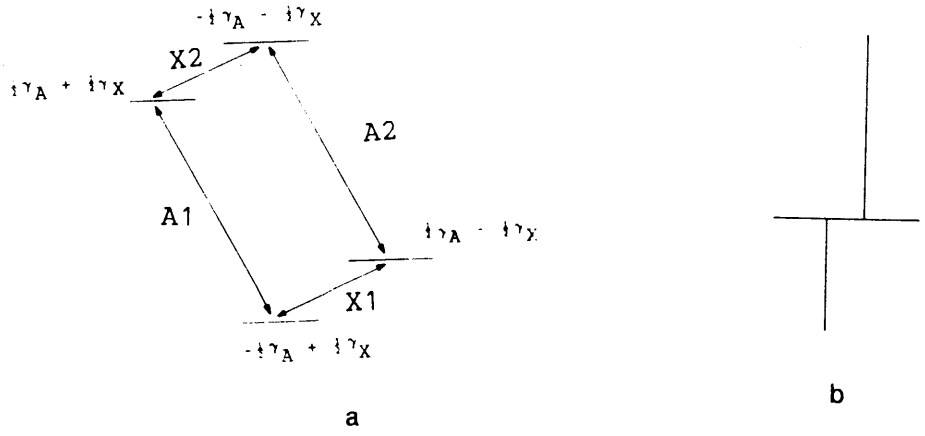


Figure 5 (a) Energy level diagram for an AX spin system after inversion of the A1 transition and (b) corresponding spectrum of the X-part (^{13}C spectrum)

Experimentally, selective population inversion is achieved through a radio-frequency (r.f.) pulse applied at the frequency of the A1 transition such that $\gamma H_2 \cdot \tau = \pi$, where γH_2 is the power level and τ the duration of the π-pulse. As a result, the intensities of the lines in the X-part of the spectrum are changed by an amount proportional to $\pm \gamma_A$ (cf. **Table 2**). In a normal X-{A} SPI experiment maximum signal enhancement factors are $(\gamma_X \pm \gamma_A)/\gamma_X$.

In the case under consideration, where X = ^{13}C and A = ^{1}H, $\gamma_H/\gamma_C \approx 4$ and the application of a π-pulse at the low-field ^{1}H transition (A1) will result in enhancement factors of -3 and +5 for the low field and high field transitions, respectively.

Application of a π-pulse with irradiating power γH_2 = 5Hz at a position 5Hz to the high- or to the low-field of a proton transition generally affects only the carbon atoms two and three bonds removed, as the (C,H) couplings are in the range 0-10Hz.[41] The magnitude of four-bond (C,H) couplings

(ca. 1Hz) precludes their detection under these experimental conditions.[42,43]

Interpretation of the SPI experiments was facilitated by using difference SPI spectroscopy in which a control spectrum is subtracted from the perturbed spectrum so that only changes between the two spectra are obtained.[44]

The SPI technique has been successfully applied to the structure determination and ^{13}C assignments of a number of natural products.[45-50]

The major limitation of the SPI experiment however, is that it is selective: each proton transition in the n.m.r. spectrum must be irradiated individually. This tedious and normally quite impractical task has been circumvented by a two-dimensional (2-D) n.m.r. experiment in which the chemical shift of the ^{13}C and ^{1}H nuclei are correlated using the long-range (^{13}C,^{1}H) coupling constants.[41] In this way the complete two- and three-bond (^{13}C,^{1}H) connectivity pattern for a compound can be determined in one or two experiments.

The concept of two-dimensional (2-D) n.m.r.spectroscopy [40] is best explained by considering a sample which gives rise to only one signal with chemical shift value, v : for instance a solution of chloroform in a deuterated solvent with proton observation.

At the start of the experiment the magnetization vector M is aligned along the z axis as indicated in **Figure 6**. Application of a $(\pi/2)_x$ pulse generates transverse magnetization (coherence) in the xy plane where it begins to precess with frequency v for a time t_1. At the end of the interval t_1, a second $(\pi/2)_x$ pulse is applied and the magnetization is measured as a normal free induction decay (FID).

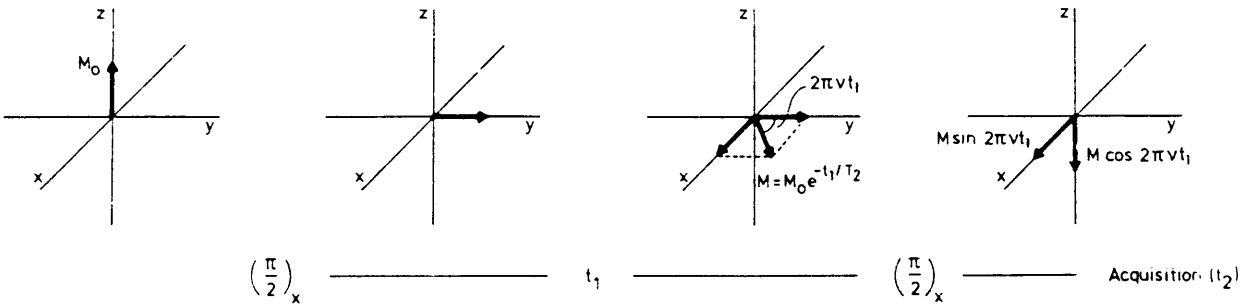


Figure 6 Influence of $\pi/2$ pulses on the magnetization vektor, M_0: Amplitude modulation of an N.M.R. signal can be brought about by varying the interval between two pulses.

If a series of experiments with different values of t_1 is performed, a separate FID is detected in t_2 for each t_1 value.[51] Fourier transformation of each FID with respect to t_2 generates a set of spectra in which the amplitude of the signal oscillates (with frequency ν) as a function of t_1 *viz.* $M \sin 2\pi\nu t_1$. A second Fourier transformation over t_1 of this sine function generates a signal that is centered at ν_1 Hz. The two-dimensional Fourier transform converts the original dataset into a two-dimensional frequency spectrum $f(\nu_1, \nu_2)$ with ν_1 and ν_2 representing the chemical shift, ν of the signal in the two dimensions.

In general experiments are arranged such that the magnetization which evolves with some frequency during t_1, evolves with a different frequency during t_2; the latter frequency will invariably be a normal chemical shift value and could include spin-spin couplings.

In a coupled system of two nuclei e.g. ^1H and ^{13}C the first $(\pi/2)$ pulse creates ^1H magnetization (coherence) which is transferred to the ^{13}C nucleus through $(^{13}C, ^1H)$ coupling by the simultaneous application of a second ^1H $(\pi/2)$ pulse as well as a ^{13}C $(\pi/2)$ pulse. The two-dimensional spectrum which is obtained after Fourier transformation contains a

signal at the coordinates (v_1,v_2) where v_1 represents the ^{1}H chemical shift and v_2 the ^{13}C chemical shift. This is the fundamental scheme for heteronuclear chemical shift correlation. The underlying phenomenon of the technique, the transfer of coherence amongst coupled spins, is most simply understood in the context of the earlier described SPI technique. The technique can be utilized to correlate ^{1}H and ^{13}C chemical shifts through either the one-bond or the two- and three-bond (^{13}C,^{1}H) coupling constants by changing the delay times, which are a function of J(^{13}C,^{1}H), in the pulse sequence.[40]

In contrast to the one-dimensional SPI experiment, excitation is nonselective *i.e.* all resonances of the ^{1}H n.m.r. spectrum are excited at the same time and polarization transfer is t_1-dependant. The elegance of this type of two-dimensional correlation experiment must be seen in the fact that, using only one experiment, connections between two types of nuclei can be established. The correlation character of the method allows an assignment made for one type of nucleus to be transferred immediately to another type.

In the present work the SPI technique as well as the long-range heteronuclear ^{13}C-{^{1}H} chemical shift correlation experiment were used successfully in the structure elucidation of the metabolites of *D. maydis*.

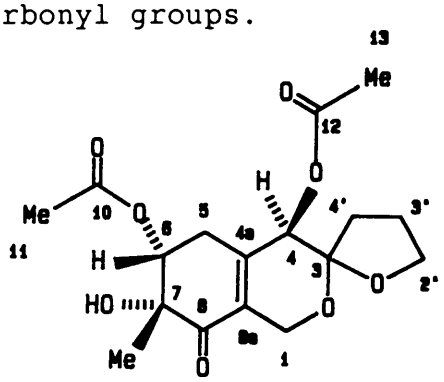
The relative configuration of stenocarpin, stenocarpin 6-*O* (4-*O*-methylorsellinate), carpellin and 3-hydroxydiplodia-toxin was determined by homonuclear ^{1}H-{^{1}H} n.O.e. difference spectroscopy.[52,53] The nuclear Overhauser effect (n.O.e.) is a very useful n.m.r. parameter for chemical structure elucidation and conformational analysis.[54] Irradiation at the resonance frequency of a ^{1}H nucleus produces a perturbation in the observed signal of another ^{1}H nucleus if the decoupled nucleus contributes to the dipolar

relaxation of the observed nucleus. Unlike chemical shifts and coupling constants that depend in part on through-bond effects, n.O.e.s are through-space effects. In an environment providing multiple dipole-dipole relaxation pathways, the n.O.e. between two protons is essentially inversely proportional to the sixth power of the distance between them. However, the ability to observe small n.O.e.s using difference techniques allows one to determine long-range through-space connections between protons.[54]

3.2 STRUCTURE ELUCIDATION OF STENOCARPIN 4,6-*O,O*-DIACETATE (19)

Stenocarpin (16) isolated from the first batch of *D.* maydis infected maize was purified as the diacetate derivative (19) in order to free it from impurities with similar chromatographic properties.

The electron impact mass spectrum of stenocarpin 4,6-*O,O*-diacetate, a colourless oil, showed the apparent molecular ion as a low intensity peak at *m/z* 294. The loss of a 42 mass unit fragment from this ion is consistent with the presence of an acetate group and gives rise to the peak at *m/z* 252. Accurate mass determination of the *m/z* 252 ion established the molecular formula $C_{13}H_{16}O_5$ for this fragment. The ^1H and ^{13}C n.m.r. data indicated the presence of two acetate groups which points to a molecular formula of $C_{17}H_{22}O_8$. The fragment at *m/z* 294 must therefore arise from the molecular ion through the loss of the elements of acetic acid. Absorptions at λ_{max} 236nm (ϵ 4600) are consonant with the presence of an α,β-unsaturated ketone moiety. The i.r. spectrum exhibited an absorbtion maximum at 1740cm^{-1} due to the acetate carbonyl groups.



(19)

The ^1H and ^{13}C n.m.r. data of stenocarpin 4,6-*O,O*-diacetate are collated in Table 3. The signal at δ_H 3,358 in the ^1H

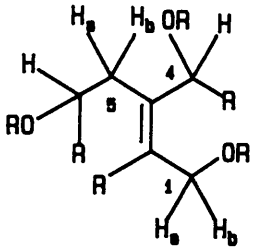
TABLE 3 ^{13}C (125,76MHz) AND ^{1}H (500.13 MHz) N.M.R. DATA OF STENOCARPIN 4,6-*O*,*O*-DIACETATE **(19)**[a]

Atom	δ_C/p.p.m.	*J*/(CH)/Hz	δ_H /p.p.m.	*J*(HH)/Hz
1	58,56T	148,9	a:4,490m	1,7 3,0 3,7 16,6
			b:4,283ddd	0,8 3,4 16,6
3	105,24S			
4	69,20D	150,4	5,170d	1,7
4a	142,89S			
5	32,56T	129,8	a:2,760m	3,4 3,7 3,8 19,0
			b:2,515m	0,8 3,0 2,6 19,0
6	74,88D	154,0	5,309dd	2,7 3,8
7	76,64S			
8	198,69S			
8a	130,96S			
9	23,11Q	129,1	1,365s	
10	170,10S			
11	20,75Q	130,1	2,011s	
12	170,20S			
13	21,04Q	129,3	2,126s	
2'	69,40T	148,3	a: 3,99m	
			b: 3,99m	
3'	23,29T	136,0	a: 2,09m	
			b: 1,92m	
4'	34,21T	128,6	a: 2,02m	
			b: 1,79m	

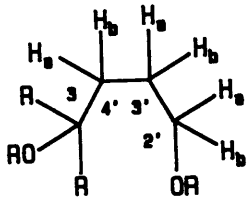
[a] The proton of the C-7 hydroxy group gives rise to a singlet at δ_H 3,358

n.m.r. spectrum which disappeared on addition of deuterium oxide to the sample was assigned to the C-7 tertiary hydroxy group. The three-proton singlet at δ_H 1,365 was assigned to a tertiary methyl group whereas the remaining two three-proton singlets at δ_H 2,011 and 2,126 could be assigned to the methyl peaks belonging to the two acetate groups.

A number of the remaining signals in the ^1H n.m.r. spectrum of stenocarpin 4,6-*O*,*O*-diacetate exhibited fine structure. First-order analyses of these multiplets yielded the values of the proton chemical shifts and proton-proton coupling constants. However a number of resonances are more complex and not well resolved. In this instance the proton-proton connectivity pattern and chemical shift values could be obtained only from a two-dimensional (2-D) (^1H-^1H) chemical shift correlation experiment using the COSY-45 pulse sequence.[40] On the basis of the above results two fragments A and B of the stenocarpin molecule could be constituted as shown in (20) and (21).



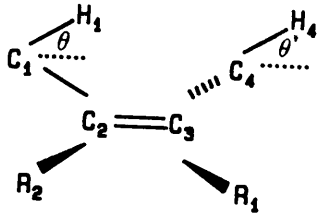
(20)



(21)

Fragment A (20). - The double doublet resonance at δ_H 5,309 served as the starting point in the analysis of this spin system. The chemical shift value indicated that this proton is situated on an oxygen-bearing carbon atom and that this oxygen function is part of an ester moiety e.g. an acetate

group. The chemical shifts of δ_H 2,760 (5-H_a) and δ_H 2,515 (5-H_b) and coupling constant (J 19,0 Hz) of the C-5 methylene protons suggest that this carbon atom is bonded to an sp^2 hybridised carbon atom.[55] As a consequence the observed couplings between the C-1 and C-5 methylene protons are the result of homoallylic couplings i.e. couplings across one double and four single bonds as in the system shown in **(22)**.[56]



(22)

The value of the homoallylic coupling constant varies from 0 to 4 Hz and is dependant on the magnitude of the two allylic angles Θ and Θ' ($J_{H,H'}$ = +4,99 $\sin^2\Theta \sin^2\Theta'$). It must be noted that if one of the angles Θ or Θ' takes up the values of either 0° or 180° the total interaction becomes zero.[56,57]

The chemical shift values of the C-1 methylene protons, δ_H 4,490 (1-H_a) and 4,283 (1-H_b) point to the presence of a C-1 oxygen substituent. The small coupling constant of 1,7Hz observed between 1-H_b and 4-H is once again ascribed to homoallylic coupling. The chemical shift value for the C-4 methine proton, δ_H 5,170 indicates that the C-4 oxygen substituent forms part of an ester moiety i.e. the second acetate ester group.

Fragment B **(21)**. - The proton-proton connectivity pattern of the protons of this isolated spin system, containing six protons, as well as the chemical shift values were determined by 2-D homonuclear correlation spectroscopy using

the COSY-45 sequence. The chemical shift value of the C-2' methylene protons, δ_H 3,99, indicated that these protons are situated on an oxygen-bearing carbon atom. The cross peaks in the COSY spectrum showed that the C-2' protons correlated only with two other methylene protons at δ_H 2,089 and δ_H1,917 which in turn correlated with another pair of methylene protons at δ_H 2,024 and δ_H 1,786.

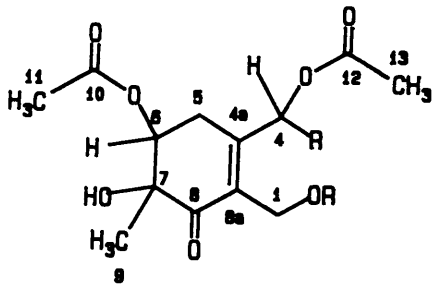
The ^{13}C n.m.r. data of stenocarpin 4,6-*O*,*O*-diacetate (**Table 3**) obtained from single frequency n.O.e. ^{13}C n.m.r. spectra, revealed that the 17 carbon signals in the proton-decoupled ^{13}C spectrum are due to three methyl, five methylene, two methine and seven quaternary carbon atoms. The signals of the proton-bearing carbon atoms were correlated with specific proton resonances in a 2-D ^{13}C-{^1H} chemical shift correlation experiment. These results allowed the assignment of the proton-bearing carbon atoms in fragments A **(20)** and B **(21)**. The magnitude of the observed directly-bonded (C,H) coupling constants (**Table 3**) supports these assignments.[58]

The connections between the different structural units of the stenocarpin molecule were determined by heteronuclear ^{13}C-{^1H} SPI experiments utilizing two- and three-bond (C,H)-couplings. The results obtained from these experiments are collated in **Table 4**. The protons of the tertiary methyl group C-9, served as starting point for the assembly. The three-proton signal at δ_H 1,367, correlated with the resonance at δ_C 23,11, is assigned to the C-9 protons on the basis of the chemical shift value. Application of a π-pulse at a position 5 Hz to low-field of the 9-H resonance in a heteronuclear ^{13}C-{^1H} SPI experiment affected the ^{13}C resonances at δ_C 74,88D, 76,64S and 198,69S. The tertiary nature of the methyl peak requires that it should be situated on the quaternary carbon atom (C-7) which resonates at δ_C 76,64S, indicating an oxygen-substituted carbon atom. The nature of the oxygen function as a hydroxy group was verified by the two-bond upfield deuterium isotope shift of

0,10 p.p.m. observed for this δ_C 76,64S resonance upon addition of a mixture of deuterium oxide and water (1:1 v/v). This quaternary carbon atom, C-7 in turn must be linked to a quaternary sp^2 hybridised carbon atom (δ_C 198,69S, C-8) and to a methine carbon atom substituted by an oxygen atom (δ_C 74,88D, C-6) in order to explain the two- and three-bond (C,H) connectivity pattern determined in the SPI experiment. The signal of the C-6 proton-bearing carbon atom (δ_C 74,88D) has been correlated with the proton resonance at δ_C5,309 in fragment A **(20)**.

Application of a π-pulse ($^*H_2$ = 5 Hz) to the C-6 proton transitions (δ_H 5,309) affected the resonances assigned to C-8 (δ_C 198,69S) as well as those at δ_C 170,10S and 142,89S. Chemical shift connections dictate that the resonance at δ_C 170,10S (C-10) must be due to the sp^2 carbon atom of a carbonyl group linked to an oxygen function (i.e. an acetate group) whereas that at δ_C 142,89 is due to a quaternary sp^2 carbon atom of a carbon-carbon double bond. On the basis of the earlier established proton-proton connectivity pattern of fragment A as indicated in **(20)**, it is evident that the SPI effects observed in this experiment are the result of three-bond (C,H) couplings.

The two- and three-bond (C,H) connectivity pattern demanded by the results of the above two SPI experiments, as well as chemical shift requirements, and the proton-proton connectivity pattern are satisfied only by the partial structure **(23)**.



(23)

TABLE 4 RESULTS FROM SPI EXPERIMENTS ON STENOCARPIN 4,6-*O,O*-DIACETATE

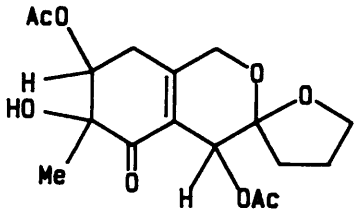
Proton transition irradiated	δ_H/p.p.m	^{13}C resonance affected	Assignment
4-H	5,170d	170,20S	C-12
		142,89S	C-4a
		130,96S	C-8a
6-H	5,309dd	198,69S	C-8
		170.10S	C-10
		142,89S	C-4a
9-H	1.365s	198,69S	C-8
		76,64S	C-7
		74,88D	C-6

Selective irradiation of the C-4 proton transitions (δ_H 5,170 in a SPI experiment affected the signal of a second acetate carbonyl carbon atom (δ_C 170,20S) as well as the signals of the α- (δ_C 142,89S) and β-carbon atoms (δ_C 130,96S) of an α,β-unsaturated ketone moiety. The presence of the latter functionality was inferred from the absorption at 236 nm in the u.v. spectrum.

As the C-4 methine proton exhibited only a small homoallylic coupling (1,7Hz) with one of the methylene protons at C-1,

it is evident that the R group linked to C-4 in the partial structure **(23)** must represent a quaternary carbon atom. The only singlet signal in the ^{13}C n.m.r. spectrum not yet assigned at this stage resonates at δ_C 105,24. The chemical shift value indicates that this carbon atom (C-3) is substituted by two oxygen atoms which must form two separate ether functions as the only hydroxy group present in the molecule has already been assigned to C-7. The two ether functions must link C-3 with C-1 in partial structure **(23)** and with C-2' in fragment B **(21)**. The subsequent formation of the C-3--C-4' linkage based on chemical shift considerations for the C-4' nuclei, generates the C-3 spiro moiety and completes the stenocarpin structure.

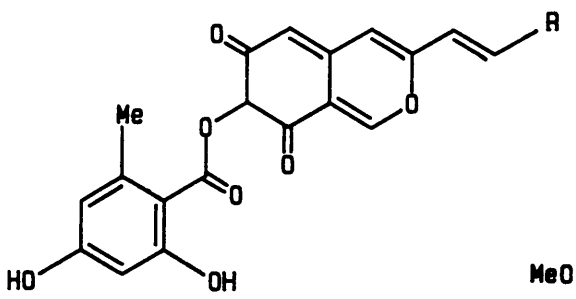
On the basis of the results obtained from the SPI experiments the alternative structure **(24)** can not be excluded at this stage as it is not possible to distinguish between two- and three-bond (C,H) couplings.

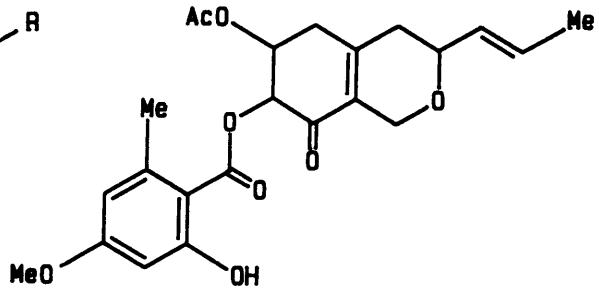


(24)

However on the basis of the n.O.e. connectivity pattern determined for stenocarpin 4,6-*O*,*O*-diacetate (see later) and the long-range (C,H) correlations found for the related metabolite stenocarpin 6-*O*-(4-*O*-methylorsellinate) (see later) the alternative structure **(24)** can be ruled out for the stenocarpin metabolite. The close structural resemblance between the structures of stenocarpin **(15)** and wortmin **(25)**, a toxic metabolite isolated from *Penicillium wortmanni*,[59]

the mitorubrin derivatives **(26)** - **(29)**, isolated from *Hypoxylon fragiforme*[60] and the phytotoxin metabolite ascochitine **(30)** isolated from culture filtrates of *Ascochyta fabae*[61] support the proposed structure **(15)** for stenocarpin.





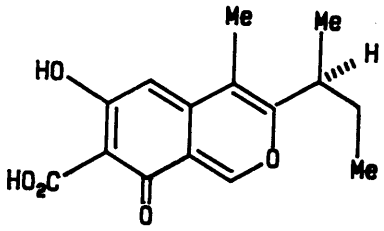
(25)

(26) R=Me

(27) R=CH_2OH

(28) R=CH_2OCOMe

(29) R=CO_2H



(30)

The relative configuration of stenocarpin 4,6-*O,O*-diacetate was deduced from the magnitude of the proton-proton coupling constants as well as the proton-proton nuclear Overhauser effects (n.O.e.s) (see **Figure 7**).

For the basis of this discussion it is assumed that C-4 has the *R* configuration. An appreciable n.O.e. is observed between 4-H (δ_H 5,170) and the C-5 prochiral methylene proton which resonates at δ_H 2,515. As a consequence the signals at δ_H 2,515 and 2,760 can be assigned to the 5*Re* and 5*Si* protons, respectively. When the irradiating frequency in an n.O.e. experiment is applied at δ_H 1,365 (9-H), n.O.e.s

are observed for 6-H (δ_H 5,309) and the 5*Si* proton (δ_H 2,760). In contrast an n.O.e. is observed between 6-H and both the C-5 prochiral methylene protons. These n.O.e.s in conjunction with the proton-proton coupling constants for the protons of ring A in stenocarpin 4,6-*O*,*O*-diacetate prove the relative configuration at the chiral centres in ring A: the C-9 methyl group is *trans* to the C-6 acetoxy group.

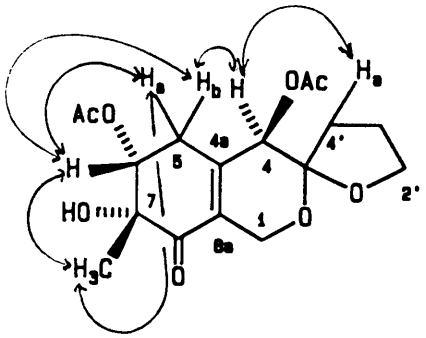
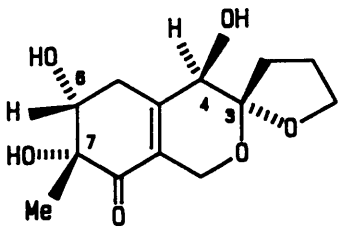
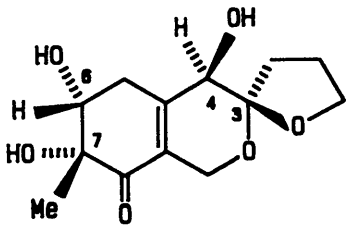


Figure 7 Proton-proton n.O.e. connectivity pattern of stenocarpin 4,6-*O*,*O*-diacetate **(19)**. Irradiation of the resonances due to 4-H, 6-H and 9-H.

The relative configuration of the C-3 spiro carbon atom could not be established by n.O.e. experiments. Although an n.O.e. is observed between 4-H and the C-4' prochiral methylene proton which resonates at δ_H 2,024, no unambiguous assignment of the C-4' methylene protons is available. As such the n.O.e. result can be explained by the deduction that it is the 4'*Si* proton which is affected when C-3 has the *S* configuration whereas in case of the 3*R* configuration it is the 4'*Re* proton for which the n.O.e. is observed.



(32)

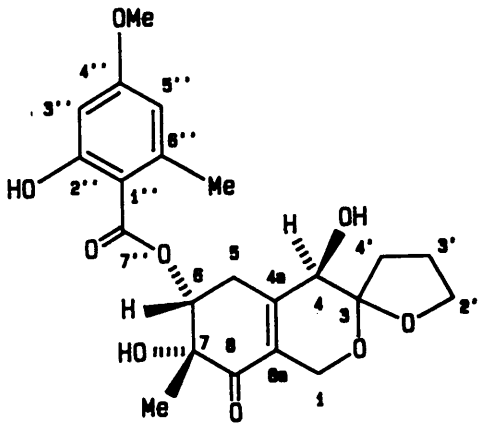


(31)

On the basis of the argument outlined above the relative configuration as shown in **(31)** or **(32)** i.e. 3*S*,4*R*,6*R*,7*R* or 3*R*,4*R*,6*R*,7*R*, or in each case the enantiomers, is assigned to the stenocarpin molecule. The enantiomeric configurations would result if the 4*S* configuration is used.

3.3 STRUCTURE ELUCIDATION OF STENOCARPIN 6-*O*-(4-*O*-METHYLORSELLINATE) (16)

In later batches of *D. maydis* infected maize stenocarpin was completely replaced by much larger quantities of the 6-*O*-(4-*O*-methylorsellinate) derivative. Stenocarpin 6-*O*-(4-*O*-methylorsellinate) **(16)** crystallized from ether-hexane as a white material, m.p.86-88°C, $[\alpha]_D$ + 17,5° (*c* 0,57 in $CHCl_3$). Elemental analysis indicated a molecular formula of $C_{22}H_{26}O_9$ which was confirmed by accurate mass determination on the molecular ion at *m/z* 434. Absorptions at λ_{max}229nm, 262nm and 300nm (ϵ 15100, 11800 and 4400 respectively) are consonant with the presence of the orsellinate moiety in the molecule.[62] The i.r. spectrum exhibited absorption maxima at 1650cm^{-1} and 1680cm^{-1} due to the orsellinate ester and α,β-unsaturated ketone carbonyl groups.



(16)

The structure elucidation of stenocarpin 6-*O*-(4-*O*-methylorsellinate) **(16)** followed the procedure outlined for stenocarpin 4,6-*O*,*O*-diacetate **(19)** (cf. section 3.2). The ^1H and ^{13}C n.m.r. data of the metabolite are collated in **Table 4**. The singlet signals at δ_H 2,537, 3,679 and 11.579 in the

TABLE 5 ^{13}C (125,76MHz) AND ^{1}H (500,13 MHz) N.M.R. DATA OF STENOCARPIN 6-*O*-(4-*O*-METHYLORSELLINATE) (16)[a]

Atom	δ_C /p.p.m.	*J*(CH) /Hz	$\Delta\delta$ /p.p.m.[b]	δ_H /p.p.m.	*J*(HH) /Hz
1	58,85T	148,5		a:4,412m	2,5 3,6 16,5
				b:4,222dd	2,9 16,5
3	106,61S				
4	69,70D	147,5	-0,06	3,595s	
4a	148,18S				
5	33,11T	129,8		*Re*:3,215m	2,9 3,5 3,6 19,6
				Si:2,647m	2,4 2,5 19,6
6	76,49D	157,2		5,677dd	2,3 3,5
7	74,69S		-0,10		
8	199,29S				
8a	128,48S				
9	23,16Q	130,0		1,409s	
2'	69,21T	147,1		a:3,91m[c]	
				b:3,91m	
3'	23,57T	128,7		a:2,04m	
				b:1,90m	
4'	33,58T	131,7		a:2,04m	
				b:2,04m	
1''	104,91S				
2''	165,72S		-0,23		
3''	98,65D	163,1	-0,05	6,253d	2,6
4''	164,12S				
5''	111,29D	161,2		6,179dd	0,8 2,6
6''	143,48S				
7''	170,76S		-0,10		
8''	24,13Q	129,4		2,176d	0,8
4''OMe	55,22Q	144,8		3,735s	

[a] The protons of the C-4, C-7 and C-2'' hydroxy groups appear as singlets at δ_H 2,537, 3,679 and 11,579 respectively,

Table 5 (continued)

b Deuterium isotope shifts in p.p.m. Negative values denote an upfield shift.

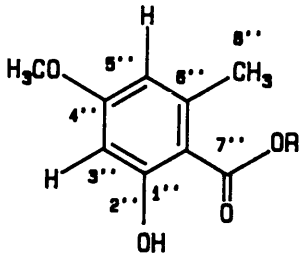
c The chemical shift values of 2'-H, 3'-H and 4'-H were determined by a ^{1}H-{^{1}H} homonuclear chemical shift correlation experiment (COSY-45).

Table 6 RELEVANT ^{1}H N.M.R. DATA OF THE 4,6-*O*,*O*-DIACETATE **(19)** AND 6-*O*-(4-*O*-METHYLORSILLINATE) **(16)** DERIVATIVES OF STENOCARPIN

Atom	δ_H/p.p.m. **(19)**	*J*(HH)/Hz	δ_H/p.p.m. **(16)**	*J*(HH)/Hz
4	5,170d	1,7	3,595s	
5	*Re*:2,760m	3,4 3,7 3,8 19,0	*Re*:3,215m	2,9 3,5 3,6 19,6
	Si:2,515m	0,8 3,0 2,6 19,0	*Si*:2,647m	2,4 2,5 19,6
6	5,309dd	2,7 3,8	5,677dd	2,3 3,5

^{1}H n.m.r. spectrum of **(16)** which disappeared on addition of deuterium oxide to the sample were assigned to the protons of three hydroxy groups. The three-proton singlets at δ_H 1,409 and 3,735 could be assigned to the protons of a tertiary C-methyl and a methoxy group, respectively on the basis of their chemical shifts.

The presence of certain structural units in the 6-*O*-(4-*O*-methylorsellinate) derivative **(16)** was recognized from an analysis of the ^{1}H n.m.r. spectrum as well as the data obtained in a 2-D ^{1}H-{^{1}H} homonuclear chemical shift correlation experiment (COSY-45).



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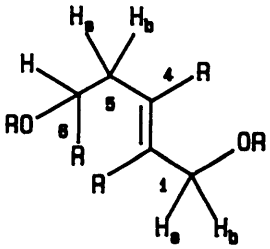
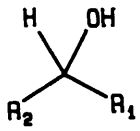


(34)



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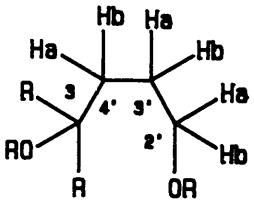


(36)

Fragment A (33). - The presence of a substituted 2,4-dioxygenated benzoate moiety was indicated by the absorption at λ_{max} 262 and 200 nm (ϵ 11800 and 4400, respectively) in the u.v. spectrum of **(16)**.[62] The presence of two *meta*-oriented aromatic protons was evident from the magnitude of the coupling constant (2,6Hz) observed for the resonances at δ_H 6,253 and 6,179. A long-range coupling (J 0,8Hz) was also observed between the aromatic proton which resonates at δ_H 6,179 and the protons of the methyl group located on an sp^2 carbon atom (δ_H 2,176). The one-proton singlet at δ_H 11,579 is characteristic of the proton of an intramolecular

hydrogen-bonded phenolic hydroxy group. The presence of the two aromatic protons *ortho* to the C-4 aromatic methoxy group (δ_H 3,735) was inferred from the chemical shift values and subsequently confirmed by 2-D long range heteronuclear ^{13}C-{^1H} chemical shift correlation and SPI experiments (see later).

Fragment B **(34)**. - The double doublet at δ_H 5,677 assigned to 6-H served, as in the case of stenocarpin 4,6-*O*,*O*-diacetate, as the starting point in the analysis of this spin system. The proton-proton connectivity is based on a ^1H-{^1H} homonuclear chemical shift correlation experiment.

Fragment C **(35)**. - A comparison of the ^1H n.m.r. spectra of the 4,6-*O*,*O*-diacetate and 6-*O*-(4-*O*-methylorsellinate) derivatives of stenocarpin **(Table 6)** showed a characteristic upfield shift of the 4-H resonance on change of the C-4 substituent from an acetoxy to a hydroxy group. The C-4 methine proton appeared as a broad singlet at δ_H 3,595 in the ^1H n.m.r. spectrum of **(16)**. The absence of any correlation peaks for the C-4 proton meant that the connection between fragment C **(35)** and the other protons of this spin system had to be established by indirect means (see later).

Fragment D **(36)**. - This fragment was constituted on the basis of a 2-D ^1H-{^1H} homonuclear chemical shift correlation experiment.

The single frequency n.O.e. ^{13}C n.m.r. spectrum of stenocarpin 6-*O*-(4-*O*-methylorsellinate) revealed that the 22 resonances observed in the proton-decoupled ^{13}C n.m.r. spectrum are due to three methyl, five methylene, four methine and ten quaternary carbon atoms. The ^{13}C n.m.r. spectrum is, with the exception of the resonances of the orsellinate moiety, quite similar to that of the diacetate derivative **(19)**. The signals of the proton-bearing carbon

atoms were correlated with specific proton resonances in a 2-D heteronuclear ^{13}C-{^{1}H} chemical shift correlation experiment (see **Table 7**).

The connections between the different structural units and the substitution pattern of the aromatic ring were determined by heteronuclear ^{13}C-{^{1}H} SPI experiments utilizing the two- and three-bond coupling as well as 2-D long-range ^{13}C-{^{1}H} chemical shift correlation experiments **(Table 7)**.

Selective irradiation of the C-8'' proton transitions in a SPI experiment proved that 8''-H (δ_H 2,176) couples to the carbon atoms which resonate at δ_C 104,91S, 111,29D and 143,48S. The coupling of 8''-H with the unsubstituted sp^2 carbon atom C-5'' (δ_C 111.29D) is through necessity over three bonds and the methyl group must therefore be located at C-6''. The resonance at δ_C 143,48S is assigned to C-6'' on chemical shift considerations. As a consequence the resonance at δ_C 104,91S is assigned to a quaternary sp^2 carbon atom three bonds removed from 8''-H, *i.e.* C-1''.

Irradiation of the C-5'' proton (δ_H 6,179d) in a SPI experiment affected the resonances at δ_C 164,12S, 98,65D, 104,91S (C-1'') and 23,13Q (C-8''). The location of the methoxy group and the assignment of the δ_C 164,12S resonance to C-4'' followed from the three-bond (C,H) coupling observed between the *O*-methyl protons (δ_H 3,735) and C-4'' upon selective irradiation of these protons in a SPI experiment. The *meta*-relationship between the two aromatic protons, 5''-H and 3''-H, and the fact that the two corresponding resonances in the ^{1}H n.m.r. spectrum have been correlated with specific carbon resonances, allows the assignment of the δ_C 98,65D resonance to C-3''. This chemical shift value is characteristic of an unsubstituted sp^2 carbon atom located *ortho* to two sp^2 carbon atoms substituted by oxygen functions. The C-3'' proton in turn

TABLE 7 RESULTS FROM THE LONG RANGE HETERONUCLEAR ^{13}C-{^{1}H} CORRELATION EXPERIMENT ON STENOCARPIN 6-*O*-(4-*O*-METHYLORSELLINATE) **(16)**

Proton transition irradiated	δ_H/p.p.m	^{13}C resonance affected	Assignment
1-H_a	4,412m	148,18S	C-4a
		128,48S	C-8a
1-H_b	4,222dd	148,18S	C-4a
		128,48S	C-8a
		106,61S	C-3
4-H	3,595s	148,18S	C-4a
		128,48S	C-8a
		33,11T	C-5
5-H_{Re}	3,215m	148,18S	C-4a
		128,48S	C-8a
5-H_{Si}	2,647m	148,18S	C-4a
		128,48S	C-8a
		76,49D	C-6
		74,69S	C-7
6-H	5,677dd	199,29S	C-8
		170,76S	C-7''
		148,18S	C-4a
9-H	1,409s	199,29S	C-8
		76,49D	C-6
		74,69S	C-7
3'$H_{a,b}$	2,04m	106,61S	C-3
3''-H	6,253d	165,72S	C-2''
		164,12S	C-4''
		111,29D	C-5''
		104,91S	C-1''

Table 7 (continued)

5''-H	6,179dd	164,12S	C-4''
		104,91S	C-1''
		98,65D	C-3''
		24,13Q	C-8''
8''-H	2,176s	143,48S	C-6''
		111,29S	C-5''
		104,91S	C-1''
4''-OMe	3,735s	164,12S	C-4''
2''-OH	11,579s	165,72S	C-2''
		98,65D	C-3''

couples with the carbon atoms two- (δ_C 165,72S, C-2''; δ_C 164,12S, C-4'') and three-bonds (δ_C 111,29D, C-5''; δ_C 104,91S, C-1'') removed. The assignment of the C-2''resonance was confirmed by irradiation of the hydrogen-bonded phenolic proton (δ_H 11,579) in a SPI experiment which affected the resonances at δ_C 165,72S (C-2'') and 98,65D (C-3'').

The position of the hydroxy bearing carbons can be determined by using deuterium isotope effects. The reported isotope shifts ($\Delta\delta$) observed in the proton-decoupled ^{13}C n.m.r. spectrum of **(16)** are the separations between doubled signals when the exchangeable protons were partially exchanged with deuterium derived from deuterium oxide

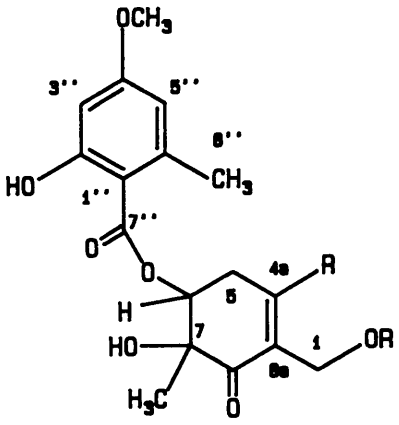
Phenolic compounds show many unusual isotope effects concerning the magnitude, the sign (*i.e.* upfield or downfield shifts), and the long-range nature of the isotope shift.[63] The magnitude of the isotope shift in hydrogen-bonded phenolic compounds for instance, depends on the strength of the hydrogen bond.[64] In general $^2\Delta\delta$ and $^3\Delta\delta$ isotope shifts are negative and $^5\Delta\delta$ is positive[63,64] and this trend is also observed for the resonances of the affected carbons in **(16)**. The isotope effects have a large

potential for assignment purposes of complex structures containing hydrogen-bonded phenolic hydroxy groups.

The observed deuterium isotope shift of C-2'' (δ_C 165,72, -0,23 p.p.m.) results from substitution of the phenolic O-H proton by deuterium and is thus a two-bond effect. The isotope shifts of -0,10 and -0,05 p.p.m. for the δ_C 170,76S and 98,65D (C-3'') resonances are three bond effects and allow the assignment of the former resonance to the C-7'' carbonyl atom of the orsellinate ester moiety.

With the knowledge of the orsellinic ester moiety to hand attention could now be given to the linkage of the different structural units *viz* fragment A **(33)** , B **(34)**, C **(35)** and D **(36)**. For this purpose the tertiary methyl group present in the molecule served as starting point. The 2-D long-range heteronuclear ^{13}C-{^1H} chemical shift correlation experiment established the connectivities between the protons of this methyl group (δ_H 1,409s) and the resonances at δ_C 199,29S, 76,49D and 74,69S. The signal at δ_C 199,29 was assigned to the sp^2 carbon atom of a carbonyl group (C-8). The chemical shift values of the other two resonances indicate that the corresponding carbon atoms carry oxygen substituents. The observed two-bond deuterium isotope shifts ($\Delta\delta$ -0,10 p.p.m.) for the δ_C 74,69S resonance verified the nature of the oxygen function as a hydroxy group. No deuterium isotope shift was observed for the 76,49D resonance, which has been correlated with the proton resonance at δ_H 5,677, the terminus of the spin system in fragment B **(34)**. The proton chemical shift value indicates that the oxygen atom present at C-6 forms part of an ester moiety and this was confirmed by the correlation signal observed between 6-H and the δ_C 170,76S resonance. Additional three-band (C,H) connectivities were also observed between this proton and the resonance at δ_C 199,29S (C-8) and 148,18S, a resonance due to an sp^2 carbon. The (C,H) connectivities between the C-5 methylene proton resonance at δ_H 2,647 and the carbon

resonances at δ_C 148,18S, 128,48S, 76,49D (C-6) and 74,69S (C-7) established the presence of the α,β-unsaturated ketone moiety and the substitution pattern of the six-membered ring as indicated in partial structure **(37)**.



(37)

The singlet methine proton at δ_H 3,595 of fragment D **(36)** has been correlated with the resonance at δ_C 69,70D. The chemical shift value for this carbon atom as well as the two-bond deuterium isotope shift (Δδ -0,07 p.p.m.) observed by this resonance confirms the presence of a hydroxy group at this position. The location of fragment C **(35)** in the structure of **(16)** followed from the two- and three-bond connectivity pattern observed for 4-H and the carbons C-4a (δ_C 148,18S), C-8a (δ_C 128,48S) and C-5 (δ_C 33,11T).

The connection between the partial structure **(37)** and fragment D **(36)** was established by the three-bond (C,H) coupling between the C-1 proton resonance at δ_H 4,222 and the carbon resonance at δ_C 106,16S. The chemical shift value is indicative of a carbon atom substituted by two oxygen atoms both of which form part of ether functions as no deuterium isotope shifts are observed for the δ_C 106,61S resonance.

A comparison of the chemical shift value of C-3' (δ_C 23,57T), which has a single oxygen function two bonds

removed, with that of C-4' (δ_C 33,58T) points to the presence of two oxygen functions two bonds removed from C-4'. Evidence for this supposition was provided by the correlation observed between the resonance at δ_C 106,61S and either the C-3' or C-4' protons. As a consequence the remaining bonds leading to the formation of the spiroketal moiety as well as the stenocarpin 6-*O*-(4-*O*-methylorsellinate) structure were established.

The relative configuration of stenocarpin 6-*O*-(4-*O*-methylorsellinate) was deduced from the magnitude of the proton-proton coupling constants, as well as the proton-proton nuclear Overhauser effects (n.O.e.s)(cf **Figure 8**).

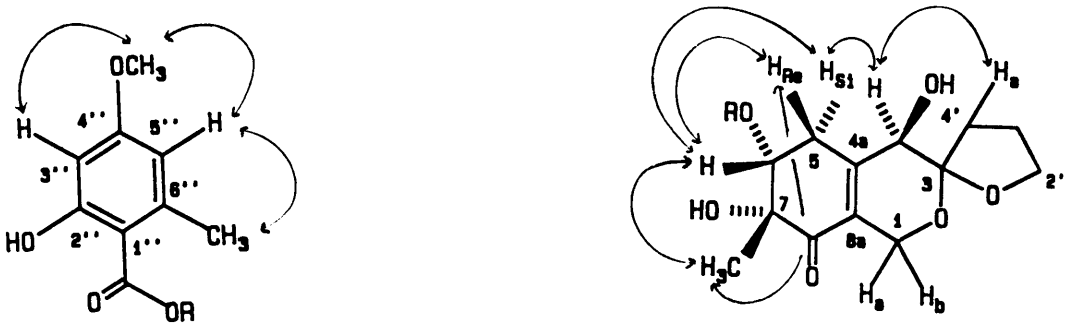


Figure 8 Proton-proton n.O.e. connectivity pattern of stenocarpin 6-*O*-(4-*O*-methylorsellinate) **(16)**.

For the basis of discussion it is assumed, as in the case of stenocarpin 4,6-*O*,*O*-diacetate, that C-4 has the *R* configuration. The observed n.O.e. effects as indicated in **Figure 8** lead to the same conclusions with regard to the relative stereochemistry of the molecule as in the case of the 4,6-*O*,*O*-diacetate derivative. The substitution pattern of the aromatic ring was confirmed by the n.O.e.s observed for the C-3'' and C-5'' protons upon irradiation of the protons of the methoxy group and that for 5''-H when the C-8'' protons were irradiated.

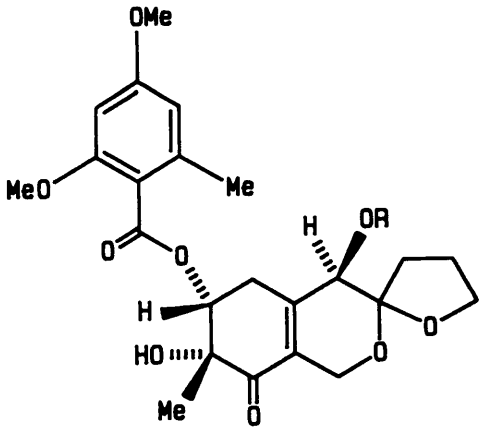
The relative and absolute configuration of the C-3 spiro carbon atom in the stenocarpin molecule can be determined only by *X*-ray crystallography. Attempts to obtain crystals of stenocarpin 6-*O*-(4-*O*-methylorsellinate) **(16)** suited for *X*-ray crystallography proved unsuccessful. Preparation of a chiral derivative of the C-4 hydroxy group by reaction of **(16)** with (-)-*S*-camphanic acid chloride, in order to determine the absolute configuration by *X*-ray crystallography, failed as only starting material was recovered.

The chirality of the C-4 hydroxy group in stenocarpin 6-*O*-(4-*O*-methylorsellinate) **(16)** and thus the absolute configuration of the molecule was determined by the 'partial resolution' method of Horeau. [31,65] This method has been successfully applied to the chirality determination of secondary hydroxy groups in a number of diverse natural products.[66-69]

The esterification of an asymetric alcohol, with the general formula L-CHOH-S, where L is larger than S, with an excess of racemic α-phenylbutyric anhydride, results in the preferential reaction of the hydroxy group with one of the enantiomers of phenylbutyric acid. The excess of the anhydride is hydrolyzed and the α-phenylbutyric acid is recovered and shows optical activity. Horeau has empirically correlated the sign of the recovered acid with the absolute configuration of the starting alcohol. In the event that the α-phenylbutyric acid has a negative optical rotation, and thus the *R* configuration, the starting alcohol is assigned the *S* configuration and *vice versa*.

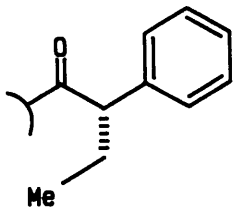
In order to avoid the formation of a diester derivative in the Horeau procedure, the C-2' phenolic hydroxy group present in the stenocarpin 6-*O*-(4-*O*-methylorsellinate) molecule **(16)** was first converted into the methyl ether derivative by treatment with an ethereal solution of

diazomethane. Accurate mass determination on the molecular ion at *m/z* 448 in the electron impact mass spectrum of the product **(38)** gave the molecular formula as $C_{23}H_{28}O_9$. The ^1H n.m.r. spectrum exhibited two three-proton singlets at δ_H 3,728 and 3,746 due to the two aromatic methoxy groups.



(38) R=H

(39) R=



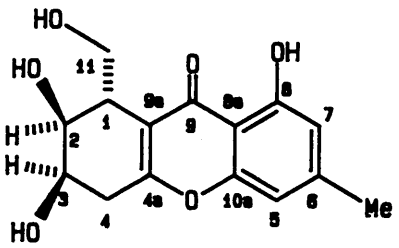
Esterification of the 6-*O*-(2,4-*O*,*O*-dimethylorsellinate) derivative **(38)** with an excess of racemic α-phenylbutyric anhydride and 4-dimethylaminopyridine instead of pyridine, proceeded smoothly leading quantitatively to the 4-*O*-α-phenylbutyrate. Accurate mass determination on the molecular ion at *m/z* 596 indicated the molecular formula of $C_{33}H_{38}O_{10}$ which is in agreement with the formation of a mono-ester derivative **(39)**. The ^1H n.m.r. spectrum of **(39)** clearly shows the presence of two diastereomers in a ratio of 5:4 and the methyl protons of the major diastereomers appear at δ_H 3,746 and 3,728 (C-2'' and C-4''OMe), 2,218 (8''-H) and 1,059 (9-H). The corresponding protons of the minor diastereomers appear at δ_H 3,740 and 3,757, 2,242 and 1,319.

The excess unreacted phenylbutyric anhydride was hydrolized and the recovered α-phenylbutyric acid had $[\alpha]_D$ + 2,8° and

thus the *S* configuration. Stenocarpin and its derivatives must therefore have the 4*R* configuration and consequently either the 3*S*,4*R*,6*R*,7*R* or 3*R*,4*R*,6*R*,7*R* absolute configuration.

3.4 STRUCTURE ELUCIDATION OF CARPELLIN (17)

Carpellin **(17)** crystalized from acetone as a white crystalline solid, m.p. 181-183°C and $[\alpha]_D$ + 6° (*c* 0,52 in MeOH). Accurate mass determination on the molecular ion at *m/z* 292 established the molecular formula as $C_{15}H_{16}O_6$ which was confirmed by elemental analysis. Absorptions at λ_{max} 242 sh, 249 sh, 264 sh and 336nm (ϵ 4700, 14500, 22000 and 21000 respectively) can be attributed to a substitute benzo-γ-pyrone moiety.[62] The i.r. spectrum exhibited absorption maxima at ν_{max} 3300 (OH), and 1660, 1635 and 1600cm^{-1} (γ-pyrone moiety)

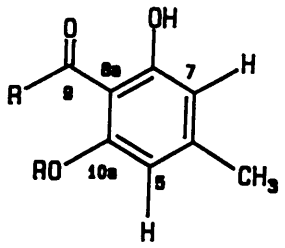


(17)

The ^1H and ^{13}C data of carpellin are collated in **Table 8**. The signals at δ_H 4,719d, 4,736dd, 4,907d and 12,642s which disappeared on addition of deuterium oxide to the sample are assigned to the protons of four hydroxy groups. The signal at δ_H 12,642 is characteristic of an intramolecular hydrogen-bonded phenolic hydroxy group whereas the doublet signals at δ_H 4,719 and 4,907, and the double doublet signal at δ_H 4,736 are due to the protons of secondary and primary hydroxy groups respectively.

The remainder of the signals in the ^1H n.m.r. spectrum of carpellin exhibited fine structure. First order analysis of these multiplets in conjunction with ^1H-{^1H} decoupling experiments yielded the values of the proton chemical shifts

and proton-proton coupling constants. The proton-proton connectivity pattern was established in a 2-D ^{1}H-{^{1}H} homonuclear chemical shift correlation experiment (COSY-45) and identified 2 structural units present in the carpellin structure.



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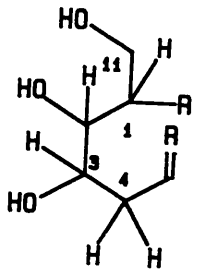


(41)

Fragment A (40). - The chemical shift values of the two *meta*-oriented (*J* 1,5Hz) aromatic protons at δ_H 6,797 and 6,581 indicated that both are located *ortho* to an sp^2 carbon carrying an oxygen atom. One of these oxygen atoms must be part of a phenolic hydroxy group located *peri* to a carbonyl function as the chemical shift value, δ_H 12,642, of this OH proton is characteristic of an intramolecular hydrogen-bonded phenolic hydroxy group. A long-range coupling (*J* 1,0Hz) was also observed between each of these aromatic protons and the protons of the aromatic methyl group.

Fragment B (41). - This fragment is characterized by the number of protons which are located on oxygen bearing carbons. The terminus of this spin system is formed by a double doublet at δ_H 4,763 which has been assigned to the proton of a primary hydroxy group. The value of the coupling constant (*J* 17,6Hz) for the C-4 methylene protons implied that this carbon atom might be linked to an sp^2 hybridised carbon.

Table 8 ^{13}C (125,76MHz) AND ^{1}H (500,13MHz) N.M.R. DATA FOR CARPELLIN (17)[a]

Atom	δ_C /p.p.m.	J(CH) /Hz	$\Delta\delta$ /p.p.m.[b]	δ_H /p.p.m.	J(HH) /Hz
1	42,58D	131,3		2,980m	3,1 3,1 7,9
2	68,51D	144,2	-0,10	4,058m	2,3 3,1 4,0
3	64,29D	139,0	-0,10	4,100m	2,3 5,6 7,1 8,8
4	32,28T	130,4		a:2,753dd	7,1 17,6
				b:2,694dd	8,8 17,6
4a	164,58S		-0,05		
5	107,03D	165,0	+0,08	6,797qd	1,0 1,5
6	146,75S				
7	111,10D	161,5	-0,06	6,581qd	1,0 1,5
8	158,47S		-0,28		
8a	107,44S				
9	181,92S		-0,08		
9a	114,02S				
10a	155,63S				
11	60,35T	142,0	-0,11	a:3,657m	3,2 5,6 11,0
				b:3,411m	5,6 8,0 11,0
12	21,73Q	127,9		2,341dd	1,0 1,0

[a] Solvent DMSO-d_6. The protons of the C-11, C-2, C-3 and C-8 hydroxy groups appear at δ_H4,763dd (J 5,5 5,5Hz), 4,719d (J 4,0Hz), 4,907d (J 5,6Hz) and 12,642s, respectively

[b] Deuterium isotope shifts in p.p.m. Negative values denote an upfield shift.

The ^{13}C n.m.r. data of carpellin obtained from the single frequency n.O.e. ^{13}C n.m.r. spectrum revealed that the 15 signals in the proton-decoupled ^{13}C n.m.r. spectrum are due to one methyl, two methylene, five methine, and seven quaternary carbons. The assignment of specific proton-bearing carbon resonances in fragments A **(40)** and B **(41)** followed from a 2-D heteronuclear ^{13}C-{^{1}H} chemical shift correlation experiment in which the signals of these carbon atoms were correlated with specific proton resonances. The magnitude of the directly bonded (C,H) coupling constants support the assignments.

The location of the hydroxy groups in fragment **(40)** as deduced from an analysis of the ^{1}H n.m.r. spectrum (see above) was confirmed by the deuterium isotope shifts observed for the resonances at δ_C 64,29D (-0,10 p.p.m.), 68,51D (-0,10 p.p.m.) and 60,35T (-0.11 p.p.m.).

The connections between the structural units leading to the carpellin structure were determined from the data obtained in a 2-D long-range heteronuclear ^{13}C-{^{1}H} chemical shift correlation experiment. The results are collated in **Table 9**.

This correlation experiment established the connectivities between the C-1 proton (δ_H 2,980) and the resonances at δ_C 60,35T (C-11), 64,29D (C-3), 114,02S and 181,92S. The last two resonances are assigned to sp^2 hybridised carbon atoms two- and three-bonds removed from 1-H, respectively, on the basis of the correlation observed between 2-H and the carbon resonance at δ_C 114,02S. The resonance at δ_C 181,92 (C-9) is assigned to the carbon atom of the γ-pyrone carbonyl group whereas that at δ_C 114,02 is ascribed to a sp^2 bond carbon atom located α to this carbonyl group.

Table 9 RESULTS FROM THE LONG RANGE HETERONUCLEAR $^{13}C-\{^{1}H\}$ CORRELATION EXPERIMENT ON CARPELLIN (17)

Proton transition irradiated	δ_H/p.p.m.	^{13}C resonance affected	Assignment
1-H	2,980m	181,92S	C-9
		114,02S	C-9a
		64,29D	C-3
		60,35T	C-11
2-H	4,058m	114,02S	C-9a
$4-H_{a,b}$	2,753dd	164,58S	C-4a
	2,694dd	114,02S	C-9a
		64,29D	C-3
5-H	6,797qd	181,92S	C-9
		155,63S	C-10a
		111,10D	C-7
		107,44S	C-8a
7-H	6,581qd	158,47S	C-8
		107,44S	C-8a
12-H	2,341dd	146,75S	C-6
		111,10D	C-7
		107;03D	C-5
8-OH	12,642s	158,47S	C-8
		107,44S	C-8a

The assumption that C-4 might be linked to an sp^2 hybridised carbon atom (see earlier) was confirmed by the correlations observed between the C-4 protons (δ_H 2,753 and 2,694) and the resonances at δ_C 164,58 and 114,02 (C-9a). The chemical shift value of 164,58S is typical of an sp^2 carbon atom substituted by an oxygen atom.

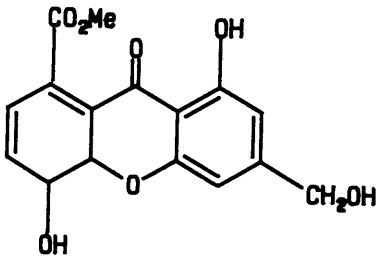
The substitution pattern of the benzene ring in fragment A **(40)** was established by the two- and three-bond (C,H) connectivity pattern. The correlations observed between the methyl protons δ_H 2,341 and the doublet resonances at δ_C 107.03 and 111,10 established the presence of two unsubstituted sp^2 carbon atoms *ortho* to the aromatic methyl group, C-12. In turn the correlations observed between each of the aromatic protons, 5-H (δ_H 6,797) and 7-H (δ_H 6,581) and the resonances at δ_C 155,63 and 158,47, respectively, characteristic of sp^2 carbons bearing an oxygen substituent, confirmed that each aromatic proton is *ortho* to an aromatic carbon atom substituted by an oxygen function. One of these oxygen atoms forms part of an intramolecular hydrogen-bonded phenolic hydroxy group on the basis of the proton chemical shift value (δ_H 12,642).

The assignment of the ^{13}C resonances is based on the following results: A correlation is observed between the hydroxy group proton (δ_H 12,642) and the resonances at δ_C 158,47S (C-8) and 107,44S (C-8a) as well as the two-bond deuterium isotope shift observed for the resonance at δ_C 158,47 ($\Delta\delta$ -0,28 p.p.m.) upon substitution of the OH proton by deuterium. In addition both δ_C 111,10D (C-7, $^3\Delta\delta$ -0,06 p.p.m.) and 107,03D (C-5, $^5\Delta\delta$ +0,08 p.p.m.) exhibit deuterium isotope shifts in line with general trends observed for phenolic substances.[70]

As no isotope shift is observed for the C-10a resonance, it follows that the C-10a oxygen atom is part of an ether

function linking the fragments A **(40)** and B **(41)** and defining the structure of carpellin **(17)**.

Biogenetically the structure of carpellin is related to sydowinol **(42)** isolated from *Aspergillus sydowi*.[71]



(42)

The relative configuration of carpellin was deduced from the magnitude of the proton-proton coupling constants, as well as proton-proton n.O.e.s. The magnitude of the vicinal proton-proton coupling constants for the C-1, C-2, C-3 protons indicates that there are no axial-axial interactions. Hence assuming a half-chair conformation for ring A these protons must have either axial-equatorial or equatorial-equatorial relationships relative to each other.

For the basis of the discussion it is assumed that C-3 has the *R* configuration. An n.O.e. is observed between 3-H and 11-H_b and indicates a *trans* arrangement between the C-3 hydroxy group and the hydroxymethyl group at C-1. This *trans* arrangement was confirmed by the n.O.e. effect between 2-H and 1-H. The n.O.e. effect between the methine protons, 2-H and 3-H indicates a *cis* diol arrangement for the C-2 and C-3 hydroxy groups. It is therefore evident that the substituents of the A-ring of carpellin have the 1-axial, 2-equatorial and 3-axial conformation. On the basis of the above results the 1*S*,2*S*,3*R* conformation, or the enantiomer, is assigned to carpellin.

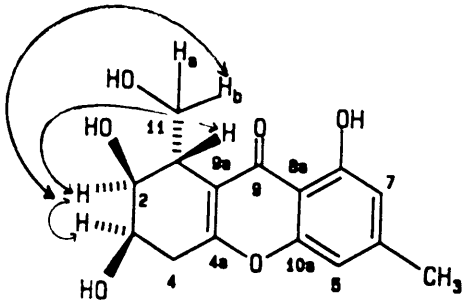
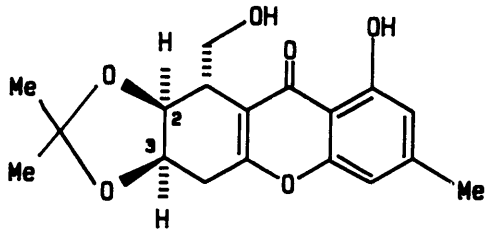


Figure 9 Proton-proton n.O.e. connectivity pattern of carpellin (17).

The presence of three hydroxy groups in ring A militates against the use of the Horeau method for the determination of the absolute configuration of carpellin. A possible solution to this problem involves the acid catalyzed reaction of acetone with the C-11 and C-2 hydroxy groups to form a 2,2-dimethyl-1,3-dioxane ring. In this way the C-3 hydroxy group would be the only hydroxy group available for reaction with the α-phenylbutyric anhydride reagent used in the Horeau method.

In the event a single product was formed which showed the molecular ion at *m/z* 332 in the electron impact mass spectrum. Accurate mass determination of this ion gave the molecular formula as $C_{18}H_{20}O_6$. A comparison of the ^1H n.m.r. data of the reaction product **(43)** (see **Table 10**) with that of carpellin (**Table 8**) shows that the signals of the C-2 (δ_H 4,615) and C-3 (δ_H 4,745) protons experience the greatest shift to lower field and leads to the conclusion that a 1,3-dioxolane derivative involving the C-2 and C-3 oxygen atoms of carpellin is formed in the acid catalyzed reaction with acetone. Analysis of the vicinal proton-proton coupling constants for the protons of the A-ring points to a boat conformation for this ring in **(43)**.

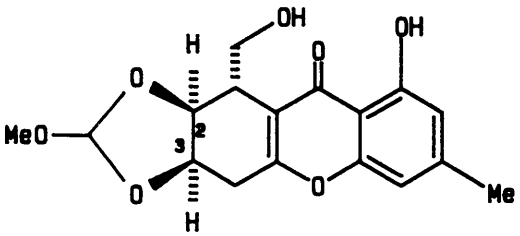


(43)

Table 10 RELEVANT ^{1}H (500,13MHz) N.M.R. DATA FOR **(43)**

Proton	δ_H/p.p.m.	*J*(HH)/Hz
1	3,518m	1,5 5,0 5,0
2	4,615dd	1,5 6,8
3	4,745m	2,0 5,7 6,8
4	a:2,758dd	2,0 17,5
	b:3.081dd	5,7 17,5
11	3,796m	5,0 5,0

An alternative method whereby carpellin is reacted with trimethyl orthoformate leads to the formation of an orthoester derivative **(44)** involving the *cis*-diol system.[72]



(44)

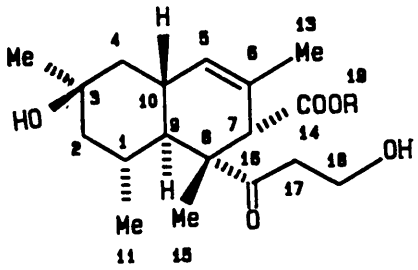
Mass spectrometry of **(44)** gave the molecular ion at *m/z* 334 and accurate mass determination established the molecular

formula as $C_{17}H_{18}O_7$. The ^1H n.m.r. spectrum showed the signals of the C-2 and C-3 protons at δ_H 4,782 and 4,904 respectively (a downfield shift of ~0,7 and 0,8 p.p.m. respectively), thus indicating that the oxygen atoms located at these two positions are also involved in the formation of the orthoester **(44)**. The three-proton singlet at δ_H 3,294 is assigned to the protons of the orthoester methoxy group. Selective deprotection of this orthoester with diisobutyl aluminium hydride did not yield the desired product with one unprotected secondary hydroxy group.[72]

3.5 STRUCTURE ELUCIDATION OF 3-HYDROXYDIPLODIATOXIN (18)

3-Hydroxydiplodiatoxin **(18)** crystallized from chloroform as a white crystalline solid, m.p. 171-173°C and $[\alpha]_D$ + 123° (*c* 0,61 in MeOH). Electron impact mass spectrometry gave the molecular ion peak at *m/z* 324. Accurate mass determination of the *m/z* 306 ion indicated a molecular formula of $C_{18}H_{26}O_4$. When the loss of the elements of H_2O is taken into account the molecular formula for 3-hydroxydiplodiatoxin **(18)** was deduced and subsequently confirmed by elemental analysis as $C_{18}H_{28}O_5$. The u.v. spectrum showed ketone absorption at λ_{max} 288nm (ϵ 305).[62] A sharp band at 1700cm^{-1} in the i.r. spectrum was attributed to the C-16 carbonyl group.

3-Hydroxydiplodiatoxin behaved like a typical carboxylic acid and reaction with an excess diazomethane yielded the methyl ester **(45)**, $C_{19}H_{30}O_5$. The molecular formula as determined by elemental analysis and mass spectrometry for the methyl ester derivatives of diplodiatoxin and 3-hydroxydiplodiatoxin indicate that 3-hydroxydiplodiatoxin has an additional oxygen atom. The structure elucidation of 3-hydroxydiplodiatoxin is thus reduced to locating this extra oxygen in the structure.



(18) R=H

(45) R=Me

TABLE 11 ^{13}C (125,76MHz) AND ^{1}H (500,13MHz) N.M.R. DATA OF 3-HYDROXYDIPLODIATOXIN (18)

Atom	δ_C/p.p.m.	J/(CH)/Hz	δ_H/p.p.m.	J/(HH)/Hz
1	31,56D	122,9	1,663m	
2	49,72T	122,4	a:1,375dd	12,1 13,7
			b:1,545m	2,3 13,9
3	70,35S			
4	45,84T	125,9	a:1,473dd	13,2 13,2
			b:1,717m	13,5
5	127,84D	152,0	5,236s	
6	127,64S			
7	59,23D	129,1	2,672s	
8	52,80S			
9	44,55D	128,8	1,972t	10,1
10	35,23D	120,7	2,181m	
11	22,67Q	125,0	0,500d	6,6
12	31,18Q	125,3	1,186s	
13	22,56Q	125,0	1,603s	
14	176,93S			
15	16,97Q	127,4	1,247s	
16	215,92S			
17	41,60T	123,6	a:2,720m	
			b:2,754m	
18	57,38T	143,9	3,845m	

TABLE 12 ^{13}C (125,76MHz) AND ^{1}H (500,13MHz) N.M.R. DATA OF 3-HYDROXYDIPLODIATOXIN METHYL ESTER **(45)**

Atom	δ_C/p.p.m.	J/(CH)/Hz	δ_H/p.p.m.	J/(HH)/Hz
1	31,42D	126,1	1,661m	
2	49,62T	122,0	a:1,373dd	12,1 13,7
			b:1,56m	2,8
3	70,09S			
4	45,81T	125,2	a:1,472dd	13,1
			b:1,725dd	2,7 13,5
5	128,41D	156,2	5,307s	
6	126,70S			
7	57,85D	130,5	2,693s	
8	52,54S			
9	44,66D	126,3	1,972dd	10,1
10	35,17D	122,9	2,223m	2,1 10,2
11	22,46Q	125,3	0.530d	6,9
12	31,19Q	125,5	1,214s	
13	22,46Q	125,3	1,576d	~1,8
14	172,43S			
15	16,77Q	127,0	1,264s	
16	216,04S			
17	41,46T	124,7	a:2,825m	5,2 5,2 18,8
			b:2,889m	5,2 5,2 18,8
18	57,85T	144,4	3,803m	5,3
19	51,92Q	147,4	3,573s	

The ^{1}H and ^{13}C data of 3-hydroxydiplodiatoxin and its methyl ester are collated in **Tables 11** and **12**. The chemical shifts of the proton-bearing carbon atoms were correlated with specific resonances in a 2-D heteronuclear ^{13}C-{^{1}H} chemical shift correlation experiment.

The structure elucidation was performed on the methyl ester since the signals in the ^{1}H n.m.r. spectrum were better resolved. Comparison of the data with that of diplodiatoxin methyl ester **(9)** (see Section 4.2) shows the close resemblance between the two different compounds. The differences observed in the ^{1}H n.m.r. spectrum of 3-hydroxydiplodiatoxin methyl ester **(45)** are mainly the change in multiplicity and downfield shift (0,346ppm) of the three-proton singlet at δ_H 1,214 (12-H) and the downfield shifts of the methylene protons 2-H_a and 4-H_a. The relevant ^{1}H n.m.r. data are collated in the **Table 13**.

TABLE 13 RELEVANT ^{1}H N.M.R. DATA FOR DIPLODIATOXIN METHYL ESTER **(45)** AND 3-HYDROXYDIPLODIATOXIN METHYL ESTER **(9)**

Atom	Diplodiatoxin methyl ester **(9)**	3-Hydroxydiplodiatoxin methyl ester **(45)**
1	1,305m	1,661m
2	a:0.942m	a:1,373dd
	b:~1,57m	b:~1,56m
4	a:1,030dd	a:1,472dd
	b;1,782dd	b:1,725dd
12	0,868d	1,214s

The ^{13}C n.m.r. spectrum of 3-hydroxydiplodiatoxin methyl ester exhibited 19 signals which are due to five methyl,

four methylene, five methine and five quaternary carbon atoms. In contrast the ^{13}C n.m.r.spectrum of diplodiatoxin methyl ester **(9)** shows the signals of inter alia six methine and four quaternary carbon atoms. The chemical shift value δ_C 70,09 for the additional quaternary carbon atom in the ^{13}C n.m.r. spectrum of 3-hydroxydiplodiatoxin methyl ester is characteristic of an oxygen-bearing carbon atom. The above consideration led to the conclusion that the new metabolite is merely a hydroxy substituted derivative of diplodiatoxin.

The position of the hydroxy group was established by means of a 2-D long-range heteronuclear ^{13}C-{^{1}H} chemical shift correlation experiment. The results are collated in **Table 14.**

The correlations observed between the C-12 proton resonance δ_H 1,214s) and the ^{13}C resonances at δ_C 45,81T (C-4), 49,62T (C-2) and 70,09S (C-3) locate the hydroxy group at C-3 in 3-hydroxydiplodiatoxin methyl ester. The singlet nature of the 12-H signal, in conjunction with this (C,H) connectivity pattern, implies that the two methylene carbon atoms must be three-bonds removed from 12-H and discounts any other location for the hydroxy group but C-3. The remainder of the (C,H) connectivity pattern for **(45)** is in agreement with the assigned diplodiatoxin-type structure.

The relative configuration of 3-hydroxydiplodiatoxin methyl ester was deduced from the proton-proton nuclear Overhauser effects (n.O.e.s) (**Figure 10**). For the basis of the discussion it is assumed that C-11 has the *S* configuration. The n.O.e effects observed between 11-H and both 12-H and 9-H can only arise through a *cis* arrangement of the C-11 and C-12 methyl groups and the C-9 methine proton. The n.O.e effects observed between 11-H and the C-17, C-18 and C-2 methylene protons as well as between 11-H and 15-H did not provide any additional information on the relative

stereochemistry. Appreciable n.O.e.s observed between 15-H and the methine protons 1-H, 10-H and 7-H, indicated a conformation with a *trans* relationship between the methyl groups C-11 and C-15, with 15-H, 10-H and 7-H on the same side of the molecule. As expected this leads to a structure with the same relative configuration as diplodiatoxin. With the knowledge of the absolute configuration of diplodiatoxin to hand [35,73] the specific rotation of diplodiatoxin **(8)**, $[\alpha]_D$ +101°, and 3-hydroxydiplodiatoxin **(18)**, $[\alpha]_D$ +129° indicated that the two compounds have the same absolute configuration.

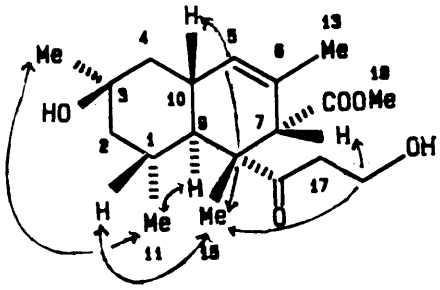


Figure 10 Proton-proton n.O.e. connectivity pattern of 3-hydroxydiplodiatoxin

Table 15 RESULTS FROM THE LONG RANGE HETERONUCLEAR ^{13}C-{^{1}H} CORRELATION EXPERIMENT ON 3-HYDROXYDIPLODIATOXIN METHYL ESTER (45)

Proton transition irradiated	δ_H/p.p.m.	^{13}C resonance affected	Assignment
2-H_b	1,56m	70,09S	C-3
4-H_b	1,725dd	70,09S	C-3
5-H	5,307s	22,46Q	C-13
		57,85D	C-7
7-H	2,693s	22,46Q	C-13
		52,54S	C-8
		128,41D	C-5
9-H	1,972t	16,77Q	C-15
		52,54S	C-8
11-H	0,530d	31,41D	C-1
		49,62T	C-2
12-H	1,214s	45,81T	C-4
		49,62T	C-2
		70,09S	C-3
13-H	1,576d	57,85D	C-7
		126,70S	C-6
		128,41D	C-5
15-H	1,264s	52,54S	C-8
		57,85D	C-7
		216,04S	C-16
17-$H_{a,b}$	a:2,825	52,54S	C-8
	b:2,889	57,85D	C-7
		216,04S	C-16
18-H	3,803m	216,04S	C-16
19-H	3,573s	172,43S	C-14

CHAPTER 4

BIOSYNTHESIS

4.1 INTRODUCTION

A great number of natural products are derived from acetyl-coenzyme A (acetyl-SCoA), one of the basic building blocks of secondary metabolism.[74] In the formation of the fatty acids acetyl-SCoA serves as a starter unit and the carbon-carbon chain is lengthened by condensation with the requisite number of malonyl-SCoA units. The latter are formed from hydrogencarbonate and acetyl-SCoA by an enzyme-mediated carboxylation process which requires biotin as a cofactor. In the condensation reaction of acetyl-SCoA with malonyl-SCoA a concerted loss of carbon dioxide accompanies attack at the carbonyl group of acetyl-SCoA. Experiments with ^{14}C-labelled carbon dioxide have shown that the carbon atom lost as carbon dioxide in the condensation reaction is the same as the one introduced initially by hydrogen carbonate.

Thio-CoA species are not involved in the condensation step, but analogous acyl-thiol ester intermediates are used. The most important of these are the acyl-derivatives of acyl carrier protein (ACP) which possesses a phosphopantotheine side-chain to which the various acyl-groups are joined as thiol esters.

The overall mechanism of the biosynthesis of the fatty acids is shown in **Figure 11**. The cyclical series of reactions probably all take place within the confines of a multi-enzyme complex with individual thiol ester intermediates passing from one enzyme thiol group to the next. In step 1, the acetyl group of acetyl-SCoA is transferred to the acyl carrier protein and in step 2 it is subsequently transferred to the active thiol site of the condensing enzyme. Malonyl-

SCoA is similarly transformed into malonyl-SACP (step 3) and condensation follows (step 4). Stereospecific reduction of the β-keto group involves NADPH as cofactor (step 5) and produces exclusively the 3*R*-hydroxy intermediate. Elimination of water produces the 2*E*-enoyl-SACP species. The cycle is completed by stereospecific reduction of the double bond to produce a saturated acyl-SACP intermediate (step 7). Repetition of this cycle (step 2 to 7 inclusive) utilizing the newly formed acyl intermediate in place of acetyl-SCoA, leads to lengthening of the carbon chain by two carbon atoms every cycle. This process terminates when the chain length reaches C_{16} or C_{18}, yielding palmitic or stearic acids.

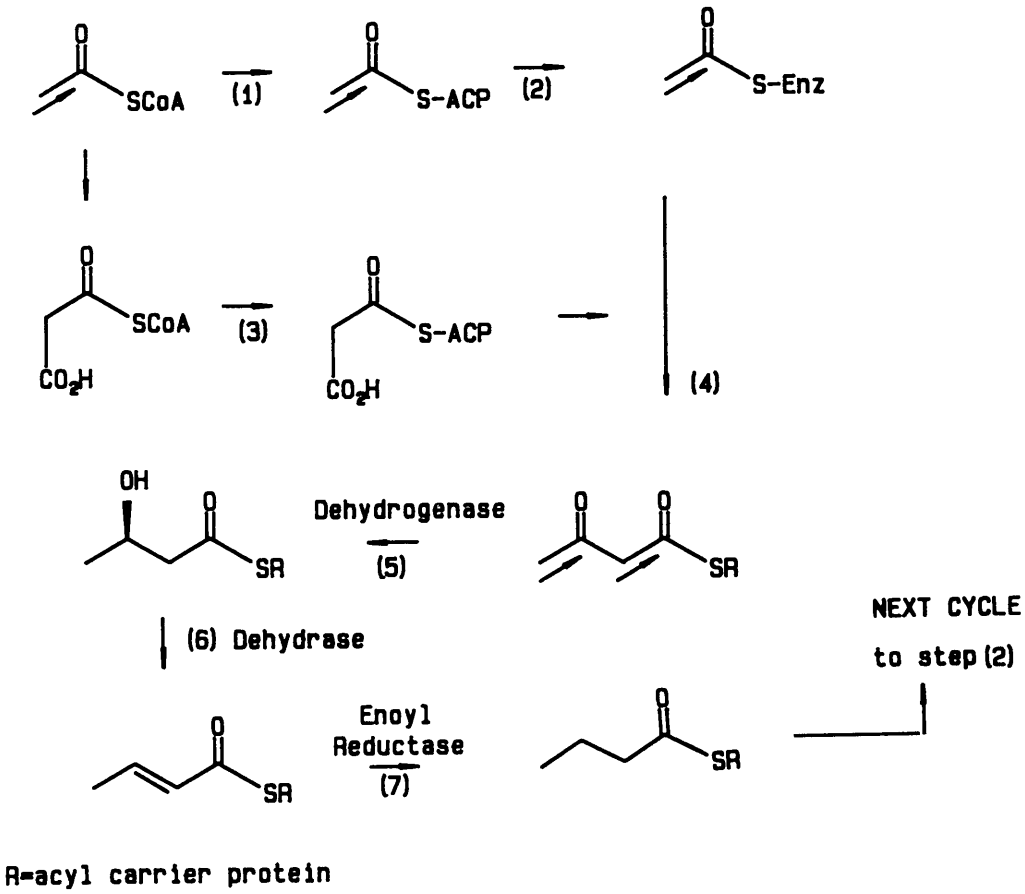
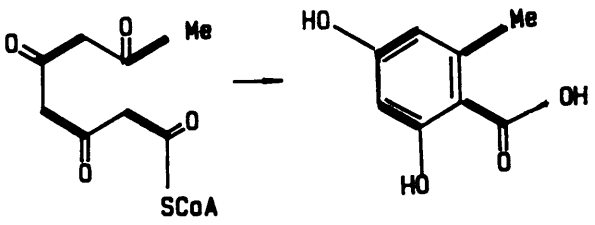


Figure 11 Overall mechanism of the biosynthesis of the fatty acids.

The polyketide pathway is one of the major routes for secondary metabolism.[75] Despite much effort over the nearly 40 years since the recognition of the pathway by Birch, little is known of the exact nature of the intermediates involved in the early stages of polyketide chain-assembly. At its simplest it is thought that poly-β-ketide intermediates are built up by a cyclic process analogous to fatty acid biosynthesis but omitting part of the reduction-elimination-reduction sequence responsible for the loss of acetate oxygen. It has been suggested that the growing β-polyketo-ester chain is stabilized by hydrogen bonding to the multi-enzyme complex or by chelation of its enolate with a metal held by the enzyme.[74] At this stage alkylation of some of the active methylene groups by *S*-adenosyl methionine could occur. Coiling of the growing polyketide chain can lead to intramolecular aldol or Claisen condensations, a process which results in the formation of aromatic compounds and which is exemplified by the formation of orsellinic acid **(47)** *via* the tetraketide **(46)**.



(46) **(47)**

While some aromatic metabolites do retain the full oxygen content of the intermediate β-polyketides, most metabolites show varying degrees of reduction and/or deoxygenation. An increasing body of evidence suggests that this occurs by processes analogous to fatty acid biosynthesis before the initial release of metabolites or intermediates from the

chain-assembly enzymes *i.e.* polyketide chains can be reduced and deoxygenated as chain assembly proceeds and not after assembly has been completed.

The biosynthetic pathway of specific metabolites is nowadays studied through the incorporation of precursors labelled with an appropriate stable isotope such as ^{13}C and ^{2}H. The resulting location of the stable isotope label in the metabolite is determined by n.m.r. techniques.[76] Stable isotope labelling studies have generated much valuable information on both the chain assembly and modification processes.[77,78]

The origin of the carbon skeleton can be studied using ^{13}C labelled precursors such as acetate, propionate mevalonate and methionine. Incorporation of for example [1-^{13}C]- and [2-^{13}C]acetate leads to so-called singly-labelled end-products. A comparison of the intensity of the resonances observed in the proton-decoupled ^{13}C n.m.r. spectrum of the enriched samples with that of the natural abundance sample identifies the enrichment sites, provided the dilution values are not too high. It is of importance to note, however that for comparison purposes, the data should be recorded under virtually identical conditions (*e.g.* solvent concentrations, pulse width and relaxation delay between pulses). The number of enriched sites is indicative of the number of acetate units involved in the biogenesis.

The use of doubly-labelled ^{13}C-precursors such as [1,2-^{13}C]acetate has been one of the major developments in biosynthetic methodology and permits information to be obtained which would have been impossible or at least extremely difficult to obtain by classical radio-isotope labelling techniques.[79,80] The basic concept can be explained as follows.

If we consider a molecule of acetate in which both carbon atoms are entirely ^{13}C, the two adjacent nuclei of spin ½ will couple to each other. If this acetate molecule is incorporated intact into a metabolite, then in any individual molecule, those pairs of carbons derived from an original intact acetate unit must necessarily both be enriched simultaneously and thus will show a mutual (^{13}C, ^{13}C) coupling. In the resultant ^{13}C n.m.r. spectrum, the natural abundance signal is flanked by (^{13}C,^{13}C) coupling satellites. By analyzing the coupling patterns, information is obtained on the way in which the polyketide chain folds prior to condensation and cyclization. The typical values for these (^{13}C, ^{13}C) coupling constants could also be used to categorize the coupled carbons on the basis of their formal hybridisation.[81] If at any stage in the biosynthesis the bond between the two carbons originally derived from an intact acetate unit is broken, then the (^{13}C, ^{13}C) coupling is lost and these carbons then appear simply as enhanced singlets. In this way bond cleavage and rearrangement processes occurring during biosynthesis can be detected.

In the study of the nature of the intermediates on a biosynthetic pathway and in particular elucidating the detailed mechanisms involved in their interconversions, it is essential to determine the biosynthetic origins and fate of the hydrogen and oxygen atoms in the polyketide chain. Hydrogen and oxygen can be monitored directly by n.m.r. using the n.m.r. active isotopes ^{2}H, ^{3}H or ^{17}O. However all these nuclei have distinct disadvantages and a great advance in biosynthetic studies was made by the use of ^{13}C as a reporter nucleus in labelling studies. The presence of ^{2}H one bond removed from a ^{13}C atom can be deduced from the appearance in the proton-decoupled ^{13}C n.m.r. spectrum of a characteristic (^{13}C, ^{2}H) coupling pattern, which is dependent on the number of ^{2}H atoms present, shifted to high-field (the so-called α-shift). Many of the problems

associated with directly attached deuterium are avoided by placing the deuterium label two bonds away from the ^{13}C reporter nucleus as two-bond (^{13}C, ^{2}H) couplings are normally <1Hz. The incorporation of ^{2}H located β to a ^{13}C atom in a precursor is detected by a small characteristic upfield β-isotope shift in the resonance position of the ^{13}C reporter nucleus.[82] The number of such ^{2}H atoms can be deduced from the value of the β-isotope shift.

The use of ^{18}O labelled precursors in biosynthetic studies allows us to study the origin or fate of oxygen atoms. The presence of ^{18}O in the metabolite can be detected by the upfield α isotope shift observed for the ^{13}C nucleus in the ^{13}C n.m.r. spectrum.[62] In this manner information can be obtained on the reduction, deoxygenation and oxidation processes which occur in the biosynthetic pathway and in addition allows us to investigate the oxidation states of possible intermediates.

The use of doubly-labelled precursors such as [1-^{13}C, $^{2}H_3$]- and [1-^{13}C, $^{18}O_2$]acetate has found wide application in biosynthetic studies.[76,84-88].

4.2 INVESTIGATION INTO THE BIOSYNTHESIS OF DIPLODIATOXIN

Diplodiatoxin **(8)** is biosynthetically closely related to versiol **(48)**,[89] a metabolite isolated from *Sporormia affinus*,[90] and *Aspergillus versicolor*,[91]. An analysis of the structures of these compounds suggests that they are formally derived from a linear heptaketide folded as shown in **Figure 12**. The main purpose of the biosynthetic investigation of diplodiatoxin was to confirm the proposed folding pattern and to establish the location of the acetate starter unit.

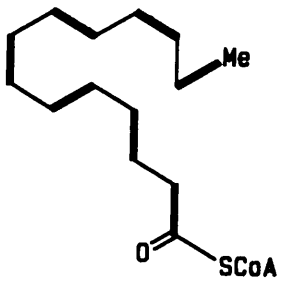
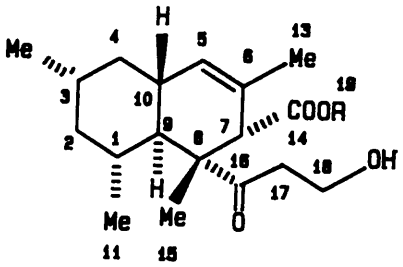
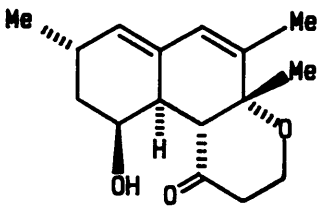


Figure 12 Proposed folding pattern of the heptaketide precursor from which versiol and diplodiatoxin is derived.



(8) R=H

(9) R=Me



(48)

After screening a number of chemically defined media for the production of diplodiatoxin, it was found that the metabolite was produced in adequate yield in stationary

culture using potato-dextrose broth. Studies on the course of fermentation indicated that diplodiatoxin production commenced on day 3 and reached a maximum of 22 $mg.l^{-1}$ 10 days after the inoculation of the medium **(Figure 17)**. Preliminary feeding experiments with [1-^{14}C]acetate as the precursor established conditions which would give a suitable enrichment at each individual acetate-derived carbon atom of diplodiatoxin on feeding ^{13}C-labelled acetate. A satisfactory dilution value* of 27,6 (assuming 7 labelled positions) was obtained by pulsing cultures of *D. maydis* every 24h from day 3 to day 10 with sodium acetate to a total amount of 1,0 $g.l^{-1}$. The absolute incorporation# was 0,04%.

The low yield of 5$mg.l^{-1}$ of diplodiatoxin is ascribed to the inhibitory effect of the added acetate precursors. It was subsequently established that the addition of 2*S*-methionine alleviated the inhibitory effect of added acetate and resulted in improved yields of diplodiatoxin.

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* The dilution value for a radiolabelled precursor is defined in equation1

$$\text{Dilution value} = \frac{\text{specific activity (precursor)} \times m(\text{product})}{\text{specific activity (product)} \quad n(\text{precursor})} \quad \ldots(1)$$

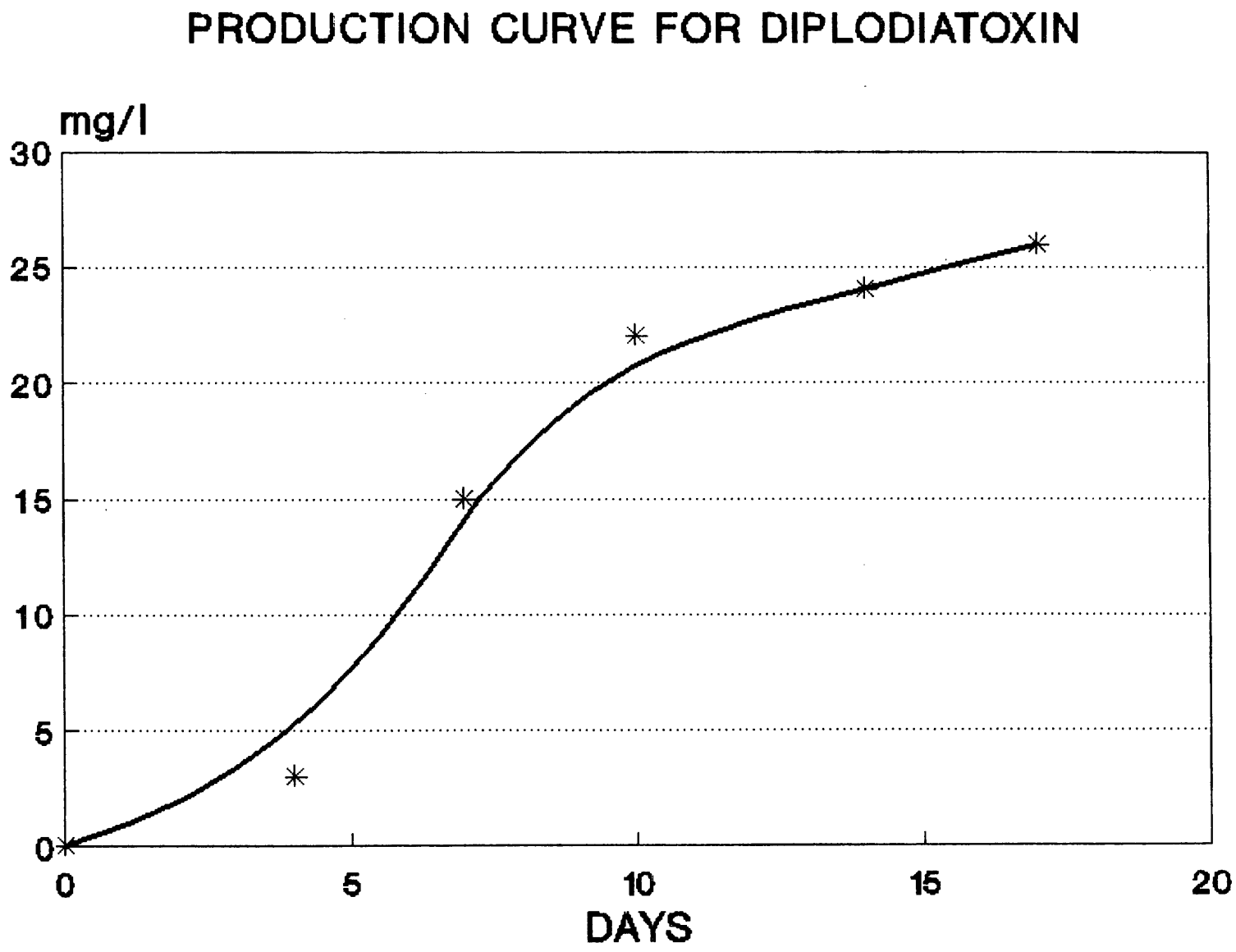
with m and n the respective number of labelled sites.[86]

\# The absolute incorporation is given by equation 2.

$$\text{Incorporation} = \frac{Sm}{Sp} \times \frac{Mm}{Mp} \times 100 \quad \ldots\ldots\ldots\ldots\ldots\ldots(2)$$

with Sm and Sp as the molar specific activity of the metabolite and the precursor respectively and Mm and Mn represents the number of moles of the isolated metabolite and administered precursor respectively.[92]

Figure 17



A prerequisite for biosynthetic studies using ^{13}C isotopes is the unambiguous assignment of the resonances in the natural abundance ^{13}C n.m.r. spectrum of the substance. The assignment of the signals in the proton-decoupled ^{13}C n.m.r. spectrum of diplodiatoxin is presented in **Table 16** and is based on the methodology outlined earlier for stenocarpin and carpellin. The results agree with the assignments reported in the literature.[74] In the biosynthetic experiments the enriched diplodiatoxin was converted to its methyl ester by treatment with diazomethane in order to circumvent the problems of the identical chemical shift values (δ_C 41,81T) of the C-4 and C-17 resonances. In the ^{13}C n.m.r. spectrum of the methyl ester derivative these resonances appear at δ_C 41,80T and 41,62T, respectively (**Table 17**)

The proton-decoupled ^{13}C n.m.r. spectrum of [1-^{13}C]acetate-derived diplodiatoxin methyl ester showed seven enhanced signals attributed to C-2, C-4, C-5, C-7, C-9, C-16 and C-18 whereas the methyl ester of the metabolite derived from [2-^{13}C]acetate showed enhanced signals representative of C-1, C-3, C-6, C-8, C-10, C-14 and C-17. High enrichment factors* were obtained for both the [1-^{13}C]- and [2-^{13}C]acetate derived carbon atoms (average 9,4 and 11.3 respectively). These results point to the involvement of seven acetate units in the formation of the diplodiatoxin molecule.

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*% Enrichment = 1,1 (Enrichment factor) - 1,1. The enrichment factor for a specific carbon atom is obtained by dividing the signal height in the spectrum of the enriched spectrum by the corresponding signal in the natural abundance spectrum recorded under identical experimental conditions.[86]

TABLE 16 ^{13}C (125,76MHz) AND ^{1}H (500,13MHz) N.M.R. DATA FOR DIPLODIATOXIN (8)

ATOM	δ_C/ppm	J/(CH)/Hz	δ_H/ppm	J(HH)/Hz
1	35,98D	124,3	1,329m	
2	45,87T	122,6	a:0,970dd	11,9 12,0
			b:1,57m	
3	33,26D	126,1	1,60m	
4	41,81T	124,5	a:1,023dd	11,7 12,6
			b:1,771m	
5	129,26D	156,0	5,371 brs	
6	126,22S			
7	58,05D	125,8	2,683 brs	
8	52,98S			
9	44,59D	128,2	2,053 brt	9,8
10	40,21D	124,0	1,771m	
11	23,04Q	123,7	0,560 brd	6,9
12	22,08Q	122,9	0,876d	6,4
13	22,56Q	128,4	1,646s	
14	177,13S			
15	16,89Q	126,2	1,246s	
16	215,54S			
17	41,81T	124,5	a:2,930m	3,5 7,5 18,7
			b:2,863m	3,2 5,8 18,7
18	57,63T	142,7	a:3,932m	3,2 7,5 11,6
			b:3,816ddd	3,4 5,8 11,6

TABLE 17 ^{13}C (125,76MHz) AND ^{1}H (500,13MHz) N.M.R. DATA FOR DIPLODIATOXIN METHYL ESTER (9)

Atom	δ_C/ppm	δ_H/ppm	^1J/(CC)/Hz[a]
1	35,92D	1,305m	34,7
2	45,82T	a:0,942m	33,2
		b:1,57m	
3	33,17D	1,60m	33,3
4	41,80T	a:1,030dt	34,3
		b:1,782m	
5	129,01D	5,340 brs	73,7
6	126,21S		73,1
7	58,17D	2,664 brs	57,0
8	52,77S		39,1
9	44,67D	1,970t	34,7
10	40,22D	1,758m	33,8
11	23,03Q	0.533d	
12	22,06Q	0,868d	
13	22,51Q	1,553 brs	
14	172,61S		57,3
15	16,82Q	1,287s	
16	215,99S		39,3
17	41,62T	2,856m	37,0
18	57,99T	a:3,801m	37,7
		b:3,791m	
19	52,02T	3,572s	

[a] Intra-acetate (C,C) coupling in diplodiatoxin methyl ester derived from [1,2-^{13}C]acetate

The arrangement of intact acetate units in diplodiatoxin and thus the folding pattern of the polyketide progenitor was studied by addition of [1,2-^{13}C]acetate to cultures of *D. maydis*. All the signals in the ^{13}C n.m.r. spectrum of the methyl ester derivative of diplodiatoxin derived from [1,2-^{13}C]acetate, with the exception of C-11, C-12, C-13, C-15 and C-19 (the carbon of the methyl ester moiety) exhibited (^{13}C, ^{13}C) spin-spin coupling. The measured ^{1}J(CC) values are given in **Table 17** and prove the presence of the following intact acetate units, C1-C9, C2-C3, C4-C10, C5-C6, C7-C14, C8-C16, C17-C18.

The above results account for the origin of 14 of the carbon atoms in diplodiatoxin. The next step was to confirm the origin of the remaining four carbon atoms by addition of (2*S*)-[*methyl*-^{13}C]methionine to cultures of *D. maydis* as (2*S*)-methionine is an excellent source of one-carbon units in nature. The proton-decoupled ^{13}C n.m.r. spectrum of the enriched diplodiatoxin methyl ester showed enhanced signals for the C-11, C-12, C-13 and C-15 resonances with enrichment factors of 8,9, 15,7, 3,9 and 8,9, respectively. The results prove that all four methyl groups present in diplodiatoxin are derived from alkylation of the appropriate active methylene positions in the heptaketide progenitor by *S*-adenosyl methionine.

The above results confirm the proposed folding pattern of the C_{14} polyketide progenitor of diplodiatoxin as shown in **Figure 12** and established that the methyl carbon of the acetate starter unit, C-7—C-14 is oxidized to a carbonyl group during the biosynthesis. This is in direct contrast to the thioester group of the terminus of the polyketide which is reduced to a hydroxy group.

Attempts to study the fate of the hydrogen atoms during the biosynthesis by incorporation of [1-^{13}C, 2-$^{2}H_3$]acetate had little success. The theoretical location of each possible

deuterium isotope and its associated ^{13}C reporter nucleus is shown in **Figure 13**. In the proton-decoupled ^{13}C n.m.r. spectrum of the methyl ester of diplodiatoxin derived from [1-^{13}C, 2-$^{2}H_3$]acetate only the resonance assigned to C-18 exhibited a low intensity upfield isotope shift ($\Delta\delta$ -0,039 p.p.m.) indicating that a single deuterium atom is retained at C-17. The remainder of the deuterium isotope labels were lost through exchange with the medium at various stages of the biosynthetic pathway.

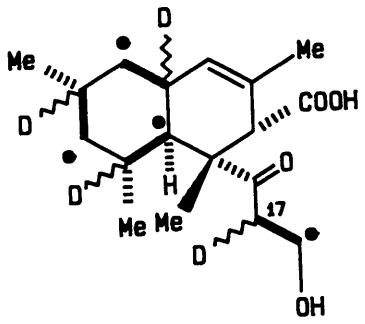

Figure 13 Theoretical location of deuterium labels and its associated carbon reporter nucleus in diplodiatoxin.

The origin of the oxygen atoms was studied by incorporation of [1-^{13}C, $^{18}O_2$]acetate. On the basis of the results obtained earlier with [1-^{13}C]-, [2-^{13}C]-, and [1,2-^{13}C]acetate it is evident that C-14 is derived from C-2 of acetate, the C-14 oxygen atoms are therefore derived from molecular oxygen by an oxidation. The incorporation of ^{18}O from [1-^{13}C, $^{18}O_2$]acetate into diplodiatoxin can be detected by the presence of isotopically shifted resonances in the proton-decoupled ^{13}C n.m.r. spectrum. Only the C-16 resonance exhibited an upfield isotope shift ($\Delta\delta$ -0,047 p.p.m.) indicating that the corresponding carbon-oxygen bond had remained intact throughout the biosynthetic pathway.

The absence of ^{18}O at C-18 could be the result of exchange with the medium in the course of the reduction of the terminal thioester group of the polyketide to the primary hydroxy group.

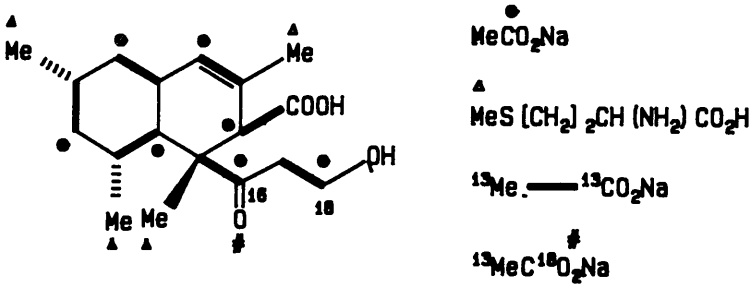

Figure 14 Summary of the biosynthetic origin of the different atoms in diplodiatoxin

4.3 BIOSYNTHESIS OF STENOCARPIN (15) AND CARPELLIN (17)

Attempts to study the biosynthesis of stenocarpin and carpellin were unsuccessful as neither was produced by any of the different media that were screened. The proposed biosynthetic pathway for stenocarpin (15) and carpellin (17) is shown in **Figure 15** and **16**, respectively.

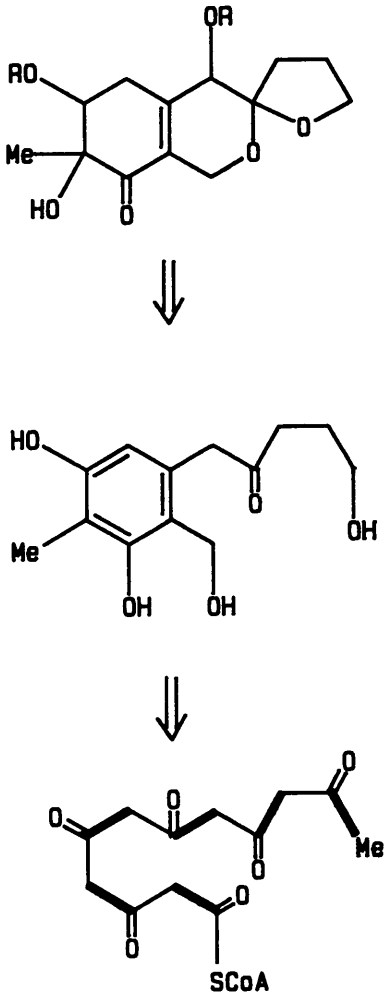

Figure 15 Proposed biosynthetic pathway for stenocarpin (15)

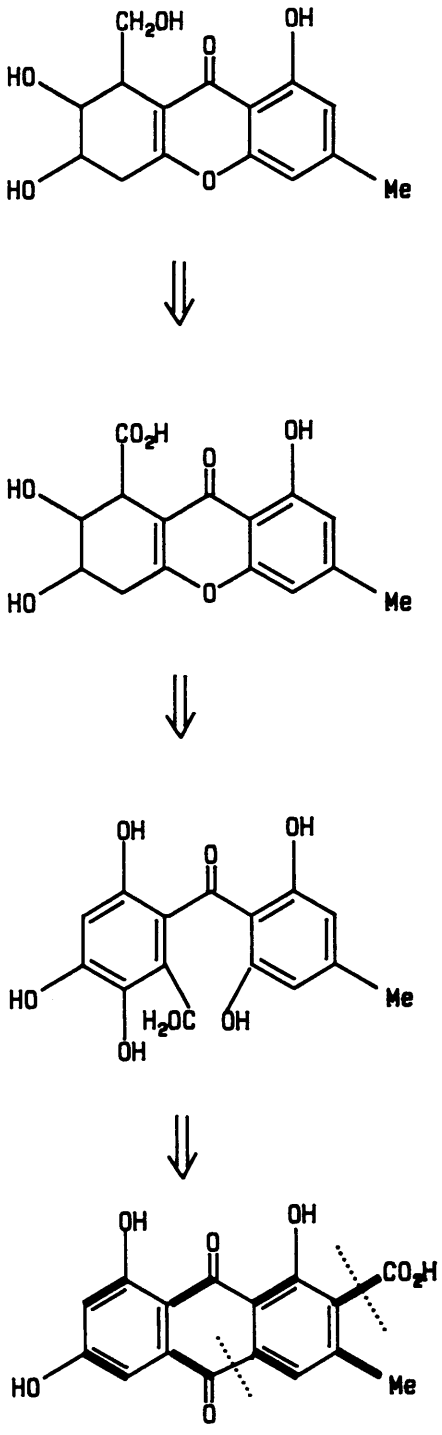

Figure 16 Proposed biosynthetic pathway for carpellin **(17)**

CHAPTER 5

EXPERIMENTAL

Melting points were determined on a Koffler hot-stage apparatus and are uncorrected. Ultraviolet absorptions were measured for solutions in methanol on a Pye Unicam SP 8-100 spectrometer. Infrared spectra were recorded on a Perkin Elmer 237 spectrometer using KBr pills. Mass spectra were taken on a Varian MAT 212 or a MAT 90 double-focussing spectrometer. Nuclear magnetic resonance spectra of [^{2}H]-chloroform solutions were recorded on a Bruker WM-500 spectrometer operating at 500,13MHz for ^{1}H and 125,76MHz for ^{13}C nuclei. Chemical shifts are reported in p.p.m. relative to tetramethylsilane (δ 0,000). The abbreviations s = singlet, d = doublet, t = triplet, m = multiplet and br = broad are used in connection with ^{1}H n.m.r. data. In the case of ^{13}C n.m.r. data, capital letters are used to refer to the patterns resulting from directly-bonded (C,H) couplings [^{1}J(CH)], unless otherwise stated. Optical rotations were measured at 24°C on a Perkin Elmer 241 polarimeter. Radioactivity was measured on a Minaxi Tri-carb 4000.

Thin layer chromatography (t.l.c.) was carried out on precoated silica gel plates (thickness 0,25mm). The t.l.c. behaviour of compounds was monitored using chloroform-methanol (9:1 v/v). Merck silica gel 60 (particle size 0,063-0,200mm) or Macherey Nagel silica gel 60 (0,040-0,063mm) was used for column chromatography. Preparative high-performance liquid chromatography (hplc) was carried out on a Waters Prep LC 500 instrument.

5.1 ISOLATION OF THE METABOLITES

Diplodia maydis (MRC 2829) was grown in bulk on wet sterilized yellow maize kernels for 6-8 weeks at 25°C. The resulting material was acutely toxic to day-old ducklings. The dried, milled, mouldy maize (22kg) was extracted in a Waring blender first with ethyl acetate to remove all the lipid material and subsequently with aqueous methanol (75%).

The aqueous residue which remained after removal of the methanol under reduced pressure, was fractionated by chromatography on macroreticular polystyrene resin (XAD-2) using (a) water and (b) methanol as eluant. The water fraction was partitioned between ethyl acetate and water. The ethyl acetate solution was combined with the methanol fraction obtained from the XAD-2. The solvents were evaporated and the residue partitioned between aqueous methanol and hexane. The methanol was evaporated and the residual material was partitioned between 0,1M sodium hydrogencarbonate and ethyl acetate The ethyl acetate solution was concentrated *in vacuo* to give toxic fraction B (58g). The aqueous layer was acidified with 1M HCl to pH 5 and extracted with ethyl acetate to give toxic material (fraction A ,15g).

5.1.1 Isolation of diplodiatoxin (8).- Fraction A (15g) was purified on a silica column (1,5kg) using chloroform-methanol (9:1 v/v) as eluant to give toxic fractions which contained the known metabolite, diplodiatoxin (880mg), m.p.187° (Lit.[33], m.p.187°), $[\alpha]_D$ + 101° (*c* 0,4 in $CHCl_3$) (lit.[33] $[\alpha]_D$ + 101). Diplodiatoxin was acutely toxic to day-old ducklings.

Fraction B (58g) was chromatographed on a silica gel column (2kg) and eluted first with chloroform and then with chloroform-methanol (9:1 v/v). Fractions (10ml) were collected and on the basis of their t.l.c. behaviour patterns to

give a total of 11 fractions (a)-(k) which were all toxic to day-old ducklings.

5.1.2 Isolation of stenocarpin 6-*O*-(4-*O*-methylorsellinate) **(16)**.- Fractions b and c (3,3g) were chromatographed on silica gel (300g) using hexane-ethyl acetate (1:1 v/v) as eluant to give *stenocarpin 6-O-(4-O-methylorsellinate)* **(16)** (260mg), m.p. 86-88°C (from ether-hexane), $[\alpha]_D$ + 17,5° (*c* 0,57 in MeOH), λ_{max} 229, 262 and 300nm (ϵ 15100, 11800 and 4400, respectively), ν_{max} 1620, 1650, 1680, and 3450cm^{-1}. (Found: C, 61,03; H, 5,90%; M$^+$ 434,159. $C_{22}H_{26}O_9$ requires; C, 60,82; H, 6,03%; M, 434,158).

5.1.3 Isolation of carpellin **(17)**.- Fractions h and i (2,7g) were purified by silica gel chromatography (300g), using chloroform-methanol (9:1 v/v) as eluant to give *carpellin* **(17)** (225mg), m.p. 181-183°C (from acetone), $[\alpha]_D$ + 6° (*c* 0,52 in MeOH), λ_{max} 336, 264sh, 249sh and 242sh nm (ϵ 21000, 22000, 14500 and 4700, respectively), ν_{max} 3300 1660, 1635, 1600 and 1070cm^{-1} (Found: C,61,58; H,5,74%; M$^+$, 292,096. $C_{15}H_{16}O_6$ requires: C,61,64; H, 5,52%, M, 292,095).

5.1.4 Isolation of 3 hydroxydiplodiatoxin **(18)**.- Column chromatography of fraction j (4,86g) on silica gel (500g), with chloroform-methanol (9:1 v/v) as eluant gave *3-hydroxydiplodiatoxin* **(18)** (970mg), m.p. 171°C (from chloroform), $[\alpha]_D$ + 123° (*c* 0,61 in MeOH), λ_{max} 288nm (ϵ 305), ν_{max} 3380, 2970, 2920, 2880 ,and 1700cm^{-1} (Found C, 57,22; H, 7,24%; $C_{18}H_{28}O_5.\frac{1}{2}CHCl_3$ requires: C,57,83; H, 7,47%; M$^+$, 324) Accurate mass determination on the *m/e* 306 ion (loss of H_2O from the molecular ion) yielded 306,182, $C_{18}H_{26}O_4$ requires M 306,183)

5.1.5 Isolation of stenocarpin **(15)**.- Stenocarpin was isolated from a single batch of *Diplodia maydis* infected maize (11kg), in which stenocarpin 6-*O*-(4-*O*-methylorsellinate) was present only in small quantities. In

subsequent batches stenocarpin was entirely replaced by much larger quantities of stenocarpin 6-*O*-(4-*O*-methylorsellinate). The extraction procedure for this batch of maize was carried out in exactly the same manner as before, with the exception of the 0,1M $NaHCO_3$ extraction step of the ethyl acetate solution which was omitted. In this way a toxic fraction (51g) was obtained, which was purified by silica gel chromatography using chloroform-methanol (9:1 v/v) as eluant to yield 12 toxic fractions (a)-(l).

Fraction c (2,24g) was purified by preparative h.p.l.c. on a C_{18} reversed-phase column using methanol-water (1:1 v/v) as eluant to yield four fractions. Fraction b (352mg) which contained stenocarpin was acetylated using acetic anhydride-pyridine (1:2 v/v) and the product purified by column chromatography to yield *stenocarpin 4,6*-O,O-*diacetate* **(19)** (62mg) as a colourless oil, $[\alpha]_D$ + 11,2° (*c* 0,42 in MeOH), λ_{max} 236nm (ϵ 4600), ν_{max} 2940, 1740 and 1225cm^{-1} (Found: *m/e* 294 (M - 60); accurate mass determination on *m/e* 252 (loss of two acetate groups) yielded 252,101, $C_{13}H_{16}O_5$ requires *m/e*, 252,100)

5.1.5 Isolation of diplodiatoxin (8) from culture broths.- Ten aliquots (100ml) of potato-dextrose broth medium (potato-dextrose broth 24g.l^{-1}, yeast extract 1g.l^{-1}) in conical flasks (500ml) were inoculated with whole maize kernels infected with *Diplodia maydis* (MRC 2829) and incubated at 25°C in stationary culture. After 10 days the cultures were filtered, the mycelium extracted with acetone and the solvent removed under reduced pressure. Water was added to the residue and the solution extracted with ethyl acetate. The organic layer was combined with the ethyl acetate solution obtained from the extraction of the aqueous medium with ethyl acetate. The material obtained from the ethyl acetate solution was partitioned between aqueous methanol and hexane. The aqueous methanol was concentrated and the residual material partitioned between ethyl acetate

and 0,1M $NaHCO_3$. The aqueous layer was acidified to pH 6 with 0,1M HCl and extracted with ethyl acetate. The organic solution was concentrated to yield a fraction containing diplodiatoxin. Purification by silica gel chromatography using chloroform-methanol (9:1 v/v) as eluant yielded pure diplodiatoxin **(8)** identical to an authentic sample.

5.2 CHEMICAL TRANSFORMATION OF THE METABOLITES

5.2.1 Methylation of diplodiatoxin **(8)**.- Diplodiatoxin (20mg) in methanol was treated at room temperature with an excess of an ethereal solution of diazomethane for 0,5h. The excess diazomethane was removed in a stream of nitrogen to yield diplodiatoxin methyl ester **(9)** as an oil (19,5mg,95%) (Found: M^+, 322, $C_{19}H_{30}O_4$ requires M, 322).

5.2.2 Methylation of 3-hydroxydiplodiatoxin **(18)**.- 3-Hydroxydiplodiatoxin (50mg) was methylated as in 5.2.1 using diazomethane to yield the methyl ester derivative **(45)** (52mg, 96%) (Found: *m/e* 320,199 (M - 18), $C_{19}H_{28}O_4$ requires *m/e* 320,199).

5.2.3 Methylation of stenocarpin 6-*O*-(4-*O*-methylorsellinate) **(16)**.- Stenocarpin 6-*O*-(4-*O*-methylorsellinate) (25mg) in dry methanol (10ml), was treated with an excess of diazomethane, freshly prepared in dry ether-methanol (1:1 v/v). Excess reagent was removed in a stream of nitrogen to yield *stenocarpin 6-O-(2,4-O,O-dimethylorsellinate)* **(38)** (22mg, 85%), (Found: M $^+$448,172. $C_{23}H_{28}O_9$ requires, M, 448,173),

δ_H 1,410 (3H, s, 9-H), ~1,91 (1H, m, 3'-Hb), ~2,04 (3H, m, 3'-Ha and 4'Ha,b), 2,218 (3H, s, 8''H), 2,679 (1H, m, 5-Hb), 3,210 (1H, m, 5-Ha), 3,637 (1H, d, 7-OH), 3,653 (1H, s, 4-H), 3,728 (3H, s, 4''-OMe), 3,746 (3H,s, 2''-OMe), ~3,91 (2H, m, 2'-Ha,b), 4,189 (1H, m, 1-Hb), 4,398 (1H, m, 1-Ha), 5,412 (1H, dd, 6-H), 6,239 (1H, d, 5''-H), 6,268 (1H, d, 3''-H),

5.2.4 Absolute configuration of stenocarpin 6-*O*-(4-*O*-methylorsellinate) **(16)**.- A solution of α-phenylbutyric anhydride (43mg, 0,14mmol), stenocarpin 6-O-(2,4-O,O-dimethylorsellinate) (38) (22mg, 0,048 mmol), and 4-dimethyl- aminopyridine (30mg, 0,24 mmol) in dichloromethane (30ml) was stirred at room temperature for 1h (t.l.c. control). The excess of anhydride was destroyed by adding water (5ml) and stirring vigorously for another 1h. The aqueous dichloromethane mixture was extracted with 6M sodium hydrogencarbonate (2x10ml), and the organic phase washed with 6M HCl (1x10ml), water (2x10ml), dried ($MgSO_4$) and evaporated. The residual ester **(39)** (24mg, 82,3%) contained no starting material and had $\underline{M}^+$, 594,246 ($C_{33}H_{38}O_{10}$ requires $\underline{M}$, 594,256),

δ_H 0,912 (3H, t, 13-H), 1,059 (3H, s, 9-H), ~1,75-2,20 (6H, m, 3'-Ha,b, 4'-Ha,b and 12-Ha,b), 2,218 (3H, s, 8''-H), 2,554 (1H, m, 5-Hb), ~3,52 (1H, m, 5-Ha), 3,638 (1H, s, 7-OH), 3,728 (3H, s, 4''-OMe), 3,746 (3H, s, 2''-OMe), ~3,90 (2H, m, 2'-Ha,b), 4,201 (1H, m, 1-Ha), 4,351 (1H, m, 1-Hb), 5,107 (1H, m, 1-Ha), 5,218 (1H, dd, 4-H), 5,415 (1H, dd, 6-H), 6,242 (1H, m, 5''-H), 6,275 (1H, m, 3''-H), 7,327-7,259 (6H, m, 15-16H).

The combined sodium hydrogencarbonate extracts were acidified 6M HCl and extracted with dichloromethane to yield α-phenylbutyric acid (18,3mg), $[\alpha]_D$ + 2,8° (*c* 1,8 in toluene)(theoretical* $[\alpha]_D$ + 19,3). The optical yield therefore was 14,5% based on an esterification yield of 100%.

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*The theoretical $[\alpha]_D$ value is calculated according to Eq. 1

$$[\alpha]_D \text{ (theory)} = \frac{+\backslash- 92°}{2a-1} \quad \text{.....Eq 1}$$

where 92° is the specific rotation of pure 2*S*- or 2*R*-phenylbutyric acid (in toluene) and
a =[anhydride]/[alcohol]

5.2.5 Camphanic acid chloride esterification of stenocarpin 6-*O*-(4-*O*-methylorsellinate) **(16)**. (-)-*S*-camphanic acid chloride (9mg) was added to a mixture of stenocarpin 6-*O*-(4-*O*-methylorsellinate) (5mg) and triethylamine (6µl) in dry THF (5ml). The reaction mixture was stirred for 16 h at room temperature and the treated with $NaHCO_3$ (3% solution), extracted with ethyl acetate, washed with saturated NaCl (2x 10ml), water (1x 10ml), dried ($MgSO_4$) and evaporated to dryness. Only unreacted stenocarpin 6-*O*-(4-*O*-methylorsellinate was recovered.

5.2.6 Acetonide formation of carpellin **(17)**.- To carpellin (5mg) in dry acetone (5ml) was added a few drops of perchloric acid and then the reaction mixture was stirred for 2h at room temperature. Crushed ice was added to the reaction mixture which was extracted with ethyl acetate. The organic layer was washed with dilute $NaHCO_3$ (2x 10ml), dried ($MgSO_4$) and evaporated to dryness. The residue was purified by preparative t.l.c. using as eluant chloroform-methanol (97:3 v/v) to yield the acetonide derivative **(43)** (Found: M+, 332,126; $C_{18}H_{20}O_6$ requires M, 332,126),

δ_H 1,235 (3H, s, 14-H), 1,274 (3H, s, 15-H), 2,374 (3H, s, 12-H), 2,758 (1H, m, *J* 2,0Hz and 17,5Hz, 4-H_a), 3,081 (1H, m, *J* 5,7Hz and 17,5Hz, 4-H_b), 3,518 (1H, m, *J* 1,5Hz, 5,0Hz and 5,0Hz, 1-H), 3,796 (2H, m, *J* 5,0Hz and 5,0Hz, 11H), 4,615 (1H, m, *J* 1,5Hz and 6,8Hz, 2-H), 4,745 (1H, m, *J* 2,0Hz, 5,7Hz and 6,8Hz, 3-H), 6,589 (1H, d, 7-H), 6,670 (1H, d, 5-H), 12,407 (1H, s, 8-OH).

5.2.7 Ortho-ester formation of carpellin **(17)**.- A mixture of carpellin (5mg), trimethylorthoformate (3,6mg) and D-10-camphosulphonic acid (catalytic amount), in dichloromethane (1ml) was stirred at room temperature for 24h. The solution was dried ($MgSO_4$), evaporated to dryness and purified by preparative t.l.c. with chloroform-acetone (98:2 v/v) as

eluant to yield **(44)** (Found: $\underline{M}^+$ 334,104; $C_{17}H_{18}O_7$ requires $\underline{M}$, 334,105).

δ_H 2,376 (3H, s, 12-H), 2,791 (1H, m, 4-H_b), 3,131 (1H, m 4-H_a), 3,294 (3H, s, 14-H), 3,785 (1H, m, 11-H_b), 3,857 (1H, m, 11-H_a), 4,782 (1H, m, 2-H), 4,904 (1H, m, 3-H), 5,603 (1H, s, 13-H), 6,593 (1H, s, 7-H), 6,669 (1H, s, 5-H), 12,350 (1H, s, 8-OH).

Selective opening of the ortho-ester was attempted as follows: Diisobutyl aluminium hydride (DIBAH) (142 µl of a 20% solution) was added dropwise to a solution of the ortho-ester **(44)** in dry dichloromethane at -78°C. The reaction was stirred for 30 min at -78°C and then for 15 min at 0°C. The reaction mixture was poured into an aqueous NaOH solution (1M), extracted with ether, dried ($MgSO_4$) and evaporated to dryness. No reaction took place and **(44)** was recovered unchanged.

5.2.8 Carbonate formation of carpellin **(17)**.
Carbonyldiimidazole (12,4mg) dissolved in THF (3ml) was added slowly to carpellin (5mg) in dry THF (2ml) and refluxed for 16h. No reaction occurred and carpellin was recovered unchanged.

5.3 BIOSYNTHESIS OF DIPLODIATOXIN

5.3.1 Production and isolation of diplodiatoxin. Erlenmeyer flasks (10 x 500ml) containing potato dextrose medium (potato-dextrose broth 24g.l^{-1}, yeast extract 1g.l^{-1}) were inoculated with *Diplodia maydis* infected maize kernels and incubated at 25°C in stationary culture. After 10 days the culture was filtered and extracted according to **Figure 3** (Section 2.4.). Fraction A was purified as described in Section 5.1.5 to give diplodiatoxin (22mg), identical to an authentic sample. A production curve showed that production commenced on day 3 and that the maximum production (22mg.l^{-1}) was reached on the 10^{th} day.

5.3.2 Incorporation of labelled precursors

5.3.2.1 Incorporation of labelled precursors in diplodiatoxin.- Aliquots (0,5ml) of a sterile, aqueous solution (35ml) of sodium [1-^{14}C]acetate (1,0g; 24,8µCi/mmol) were added dropwise every 24h for 7 days to 3-day old growths of *D. maydis* on PDB medium. The cultures were harvested on day 10 to give after purification and diazomethane methylation, diplodiatoxin methyl ester (4,7mg; 6,28µCi/mmol), dilution value 27,6 (assuming 7 labelled positions), and an absolute incorporation of 0,04%. Yields are down from the production curve values due to growth inhibition by the acetate precursors. Addition of 2*S*-methionine alleviated the inhibitory effect of the added acetate and resulted in improved yields of diplodiatoxin.

Similar experiments using stable isotope labelled precursors were conducted and the results are collated in **Table 18**.

Table 18

Precursor	Atom % ^{13}C	Amount /mg	Yield/mg diplodiatoxin
Sodium [1-^{13}C]acetate	99	1000	4,6
Sodium [1,2-^{13}C]acetate	91	500	3,5
Sodium [2-^{13}C]acetate	99	250	28[a]
Sodium [1-^{13}C, $^{2}H_3$]acetate	99 (98)[b]	250	32[a]
Sodium [1-^{13}C, ^{18}O]acetate	99 (95)[c]	250	5,0
2*S*-[*methyl*-^{13}C]methionine	99	100	20

[a] 2*S*-methionine added to alleviate the inhibitory effect of the acetate
[b] atom % ^{2}H
[c] atom % ^{18}O

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