THE STRUCTURE OF DOTHISTROMIN

A Thesis

Presented to Massey University

in Partial Fulfilment of the Requirements

for the Degree of

Doctor of Philosophy

by

R.T. GALLAGHER

Massey University

July 1971

To Ruth,

and Tracey and David

ABSTRACT

The fungus Dothistroma pini Hulbary is a needle pathogen of Pinus radiata and other pines, producing a necrotic disease commonly known as Dothistroma needle blight. The fungus is widely distributed in major pine forests throughout the world; it was reported in New Zealand on P. radiata in 1964.

The species P. radiata, P. ponderosa, and P. nigra (laricio) are all grown extensively in New Zealand and unfortunately are all highly susceptible to the disease. Because forestry plays an important role in the New Zealand economy, the disease is of economic significance to New Zealand. The disease can be controlled by spraying with insoluble compounds of copper.

Following a suggestion that D. pini might produce a toxin responsible for host cell death, a red pigment was isolated from D. pini cultures and shown to be toxic to Chlorella pyrenoidosa, a unicellular green alga. This thesis is concerned with a detailed investigation into the nature and characterisation of the red pigment.

D. pini was cultured in the laboratory on an aqueous malt medium, and red pigment extracted from the cultures and purified by thin layer chromatography was shown by mass spectroscopy to be a mixture of two closely related compounds of molecular formula $C_{18}H_{12}O_9$ and $C_{18}H_{12}O_8$. The former compound which was present in greater amount, was named dothistromin, and the latter was named deoxydothistromin. Both dothistromin and deoxydothistromin were shown to be present in extracts of D. pini infected P. radiata needles.

A detailed chemical investigation using chemical reactions (including the classical degradative technique of zinc dust distillation), derivative formation, infrared spectroscopy, electronic absorption spectroscopy, nuclear magnetic resonance spectroscopy,

and mass spectroscopy, allowed elucidation of the structure of dothistromin. Dothistromin was shown to be a tri-α-hydroxyanthraquinone onto which was fused a substituted tetrahydrodifuro ring system.

A major feature of the structure of dothistromin is the substituted tetrahydrofuro[2,3-b]benzofuran moiety. Fungal metabolites known to incorporate this structural feature include the toxic and potently carcinogenic aflatoxins, and the carcinogenic sterigmatocystin. A discussion on the possible carcinogenicity of dothistromin, its co-metabolites, and artefacts is included.

The strong green-yellow fluorescence of solutions of the red pigment and dothistromin, when irradiated with ultraviolet light is attributable to the 1,4-dihydroxyanthraquinone chromophoric nucleus of dothistromin.

Another important structural feature of dothistromin is the reactive hemiacetal group, allowing dothistromin to undergoe facile acid catalysed mono-alkylation and mono-acetylation.

The probability that in solution dothistromin exists as a complex equilibrium mixture, was discussed.

The mass spectrum of dothistromin shows a characteristic loss of the formyl radical CHO$^{\bullet}$ (m/e 29), and the neutral fragment C_2H_4O (m/e 44). The same loss of a formyl radical, and a homologous neutral fragment was also shown by a number of dothistromin derivatives. Two fragmentation schemes were proposed to rationalise the mass-spectral fragmentation of dothistromin.

During the course of the investigation, a number of crystalline, optically active derivatives of dothistromin were prepared; these included dothistromin penta-acetate and dothistromin ethyl ether tetra-acetate. The structure and absolute configuration of a crystalline heavy atom derivative of dothistromin was determined

by an x-ray crystallographic diffraction study. This confirmed the structures proposed in this thesis, and also allowed the absolute configuration of the cis-fused furo rings of dothistromin to be deduced.

Deoxydothistromin was assigned one of two structures, and the nature of other co-metabolites was briefly considered. The synthesis and biosynthesis of dothistromin was also discussed.

Acknowledgements

I wish to express my sincerest thanks to my supervisor, Professor R. Hodges, for helpful discussion and encouragement throughout the course of this work, and for giving up so much of his valuable time to instruct me in the technique of mass spectroscopy.

I am indebted to Professor R.D. Batt for arranging the project for this thesis.

My thanks to Dr. K.W. Jolley (and, earlier, Dr. D.F.S. Natusch of the Applied Chemistry Division, D.S.I.R.) for running NMR spectra.

To various other members of the academic and technical staff of the Department of Chemistry and Biochemistry of Massey University, I express my gratitude, for useful discussion and assistance.

I would like to express my appreciation to Dr. C. Bassett and Miss M. Buchanan of the Forest Research Institute, Rotorua, and Mr. P.J. Brunt, for considerable help and advice on various aspects of *D. pini* culture.

I thank Professor T.N. Waters, his wife Dr. J.M. Waters, and Mr. C.A. Bear, of the Department of Chemistry, University of Auckland, for the X-ray diffraction study which they carried out on a derivative of dothistromin prepared during this work.

Finally, I would like to thank Miss J. Thompson, who typed this thesis.

TABLE OF CONTENTS

List of Figures

List of Schemes

List of Tables

INTRODUCTION

The fungus Dothistroma pini Hulbary is a needle pathogen of Pinus radiata and other pines, producing a necrotic disease commonly known as Dothistroma needle blight (Thyr and Shaw, 1964; Funk and Parker, 1966; Gadgil, 1967). The disease is characterised by production of red bands on infected needles. Attack by D. pini is followed by premature casting of the infected needles with a resultant retardation of growth, and sometimes even by death of the infected tree, in the case of severe, repeated defoliation.

D. pini is widely distributed and has been recorded in North and South America (Canada, U.S.A.), Africa (Kenya, Rhodesia), Europe (France, Spain, United Kingdom, Russia), Asia (India), and Australasia (New Zealand).

D. pini was first identified in New Zealand on P. radiata in 1964, near the town of Tokoroa (Gilmour, 1965).

It would appear that most species of the genus Pinus are more or less susceptible to attack by D. pini. The species P. radiata, P. ponderosa, and P. nigra (laricio) are all grown extensively in New Zealand and unfortunately are all highly susceptible to the disease (Gilmour, 1967). Because of the important role that forestry plays in the New Zealand economy, the disease is of economic significance to New Zealand.

The disease can be successfully controlled by aerial spraying with aqueous suspensions of insoluble copper compounds, such as copper oxychloride and cuprous oxide, and it has recently been shown that spraying every three to four years will keep the

disease in check. It has been found that P. radiata becomes highly resistant to attack by D. pini at age approximately twenty years, and thus spraying is not necessary for stands of trees over the age of twenty years (Gilmour and Noorderhaven, 1970; Gadgil, 1970).

Since early observations had indicated that certain trees appeared to possess a natural resistance to the disease, it was thought that a good method of combating the disease would lie in the production of resistant seedling stock with which to replenish milled forest. Unfortunately, large-scale experimentation in New Zealand has failed to yield any so-called "resistant" seedlings (Basset, 1970).

Because of the possibility that the pathogen may in time adapt itself to the age-resistance factor of P. radiata, and since spraying with copper will only control the disease and not eradicate it, it is desirable that more should be known about the detailed relationship between host and pathogen In particular, the factors which are involved in host resistance to attack, and the mechanism resulting in needle death, appear worthy of detailed investigation.

Following a suggestion that D. pini might produce a toxin responsible for host cell death (Gadgil, 1967), Bassett and Brunt (1968) isolated a red pigment from D. pini cultures, which was shown to be toxic to Chlorella pyrenoidosa, a unicellular green alga. Thus, when an ethyl acetate extract of a laboratory culture of D. pini was concentrated and subjected to thin layer chromatography on silica gel G using chloroform : ethyl acetate (40:60), a number of coloured bands appeared on the plate. Each of these

bands was removed, and the adsorbed component recovered with ethanol and assayed against C. pyrenoidosa, by an antibiotic disc technique (Bassett et al., 1967). A red pigment obtained from a bright red band on the TLC plate accounted for practically all of the "toxicity" to C. pyrenoidosa. This same red pigment was obtained from P. radiata needles infected with D. pini, and showed the same toxicity to C. pyrenoidosa as the crude ethyl acetate extract. Attempts to demonstrate toxicity of the extract to P. radiata needles have not yet been successful, but cultures of P. attenuata tissue were killed when antibiotic discs, treated as in the Chlorella test were placed on the medium beside the culture (Bassett, pers. commun.).

This thesis is concerned with the elucidation of the molecular structure of the red pigment implicated as a toxin. A literature check revealed that a number of people have observed the production of a red pigment by D. pini, but no significant chemical investigation into the red pigment appears to have been carried out. Thus, Funk and Parker (1966) noted that the internal material of the ascus, and sometimes the ascospores, became coloured by a purplish-red stain when mounted in dilute potassium hydroxide with a portion of the stroma, whilst Sanders (1969) obtained purplish red coloured potassium hydroxide and sodium hydroxide extracts of fungal plate cultures, and speculated that the purplish colour with sodium hydroxide suggested anthraquinones or benzoquinones.

RESULTS AND DISCUSSION

CHAPTER 1. ISOLATION AND PURIFICATION OF THE RED PIGMENT

Laboratory Culture of Dothistroma pini

Dothistroma pini was cultured in the laboratory to produce quantities of red pigment as required during the course of the project. Culture was successfully carried out in shake flasks containing culture medium inoculated with a water spore suspension of D. pini spores. With a heavy inoculum, red pigment production appeared to have reached a maximum some six to ten days after inoculation.

Two culture media were tried - (1) an aqueous malt solution (10% w/v) containing yeast, and (2) a modified Raulins medium containing thiamine HCl and casein. The malt solution appeared to give better results than the Raulins medium (i.e. higher pigment yield) and because of its simplicity and cheapness, was used in all large scale laboratory culture work for this thesis.

Extraction of Red Pigment from D. pini Cultures

The red pigment was extracted from D. pini cultures with ethyl acetate, such extracts having a characteristic orange colour. Removal of the ethyl acetate under vacuo gave an amorphous red powder, which was examined by TLC on silica gel G; this revealed the expected presence of a red pigment as the major component of the crude extract.

Ethyl acetate appears to be the solvent of choice for extraction of the wet culture slurry, being a good solvent for the

red pigment and sufficiently miscible with water to give reasonable penetration of the fungal growth components. However, care is required in the extraction procedure to avoid the formation of stable emulsions which are extremely difficult to deal with. The procedure outlined in the experimental section helps to avoid this difficulty.

Purification of Red Pigment by Preparative TLC

The desired red pigment was obtained in mg quantities from the crude red pigment extract by preparative TLC on silica gel G with ethyl acetate : chloroform (60:40) as solvent. The red pigment travelled as an intensely red coloured band, Rf ~0.40 (superior resolution was obtained using unlined tanks).

The colour of the red pigment band on TLC plates was found to vary dramatically on different occasions, although giving rise to the same red pigment upon recovery (as determined by mass spectroscopy). This colour variation appeared to be related to variation in pH of the system. Thus under slightly basic or neutral conditions, the pigment travels as a blood-red coloured band whereas under acid conditions, it tends to travel as an orange to orange-red coloured band. Variation in acidity of adsorbent on different batches of plates, of culture extract, and of developing solvent could all result in variation of pH at the site of adsorption of the red pigment on the TLC adsorbent. Such pH variation can cause colour changes to take place under the above chromatographic conditions, with compounds such as α-hydroxyanthraquinones, for example, by altering the hydrogen bonding of the α-hydroxyl protons with the carbonyl groups, and

also by altering the chelation with certain metal ions such as Ca^{2+} and Mg^{2+} present in the silica gel G adsorbent.

The red pigment was recovered from the TLC plate, as outlined in the experimental section, to give an amorphous red powder. This purified red pigment appeared to be homogeneous when further examined by TLC with silica gel G.

Isolation of Red Pigment from Infected Needles

An ethyl acetate extract of *D. pini* infected *Pinus radiata* needles obtained from a forest area heavily infected with *D. pini*, had the same characteristic orange colour as ethyl acetate extracts of *D. pini* laboratory cultures, and gave rise to the same red band on TLC. Mass spectral examination of the red pigment from prep. TLC of the needle extract confirmed identity with the red pigment from laboratory cultures.

Attempts at Crystallisation of the Red Pigment

Having obtained the red pigment as a homogeneous, amorphous red powder by prep. TLC of ethyl acetate culture extracts, attempts were made to obtain it in a crystalline state.

A number of different organic solvents, including ethyl acetate, chloroform, acetone, benzene, toluene, methyl ethyl ketone, methyl isobutyl ketone, and combinations of these, were tried, but crystalline material was not obtained. On several occasions, plate-like crystalline material appeared to be deposited, but microscopic examination revealed only amorphous deposits of indefinite shape. When the alcohols methanol or ethanol were used as solvents, poorly defined crystalline material of wide

melting point was sometimes obtained, but this was found later to be due to chemical reaction between the pigment and the alcohol, to produce methoxy- and ethoxy-derivatives.

CHAPTER 2. EXAMINATION OF THE RED PIGMENT BY MASS SPECTROSCOPY

The "homogeneous" red pigment obtained by prep. TLC of *D. pini* culture extracts was examined by mass spectroscopy, using the direct insertion probe. The line diagram derived from the mass spectrum, which was obtained at a source temperature of ca. 150° above room temperature, is shown in Fig. 1.

The upper peak in the spectrum appeared at a position of m/e 372, and clearly represented a molecular ion. The next peak appeared at position m/e 356. The separation of these two peaks by amount m/e 16 corresponded to the difference of an oxygen atom - confirmed (see later) by mass measurement of the two peaks. Since the loss of an oxygen atom from an ion is not common in the mass spectroscopy of organic compounds, it was proposed that the ion at m/e 356 was also a molecular ion. Using the "defocusing technique" for metastable ion detection (see appendix), it was shown that while there was an intense signal for metastable ions of m/e 372 decomposing to ions of m/e 354, no signal was observed for metastable ions of m/e 372 fragmenting to ions of m/e 356. Thus, there was no evidence to suggest that the peak at m/e 356 arose from fragmentation of the molecular ion at m/e 372, thus supporting the proposition that the m/e 356 ion was also a molecular ion.

Close examination of the spectrum revealed that there appeared to be two series of fragment ion peaks, separated by amount m/e 16, one series arising by fragmentation of the second molecular ion at m/e 356. The twenty most significant and intense peaks

in the upper region of the spectrum were mass measured, and the results, shown in Table I, allowed division of a number of the peaks into two series derived from the molecular ions at m/e 372 and m/e 356.

Metastable peaks were observed on chart for the transitions

$$C_{18}H_{12}O_9^{+\cdot} \xrightarrow[*]{-CHO^{\cdot}} C_{17}H_{11}O_8^{+} \xrightarrow[*]{-C_2H_4O} C_{15}H_7O_7^{+}$$

m/e	372	29	343	44	299

Dothistromin and Deoxydothistromin

From the above data it was concluded that the red pigment was in fact composed of two major compounds of molecular weight 372 ($C_{18}H_{12}O_9$) and 356 ($C_{18}H_{12}O_8$); the name Dothistromin was proposed for the former compound which gave the most intense spectrum, and Deoxydothistromin for the latter compound.

Structural Conclusions from the Mass Spectral Data

A consideration of the molecular formula for dothistromin, $C_{18}H_{12}O_9$, shows that dothistromin is a highly oxygenated, highly unsaturated molecule; the formula requires thirteen "double bond equivalents". The mass spectrum showed a number of intense doubly charged ions, consistent with a highly unsaturated system, probably aromatic and/or quinonoid in nature.

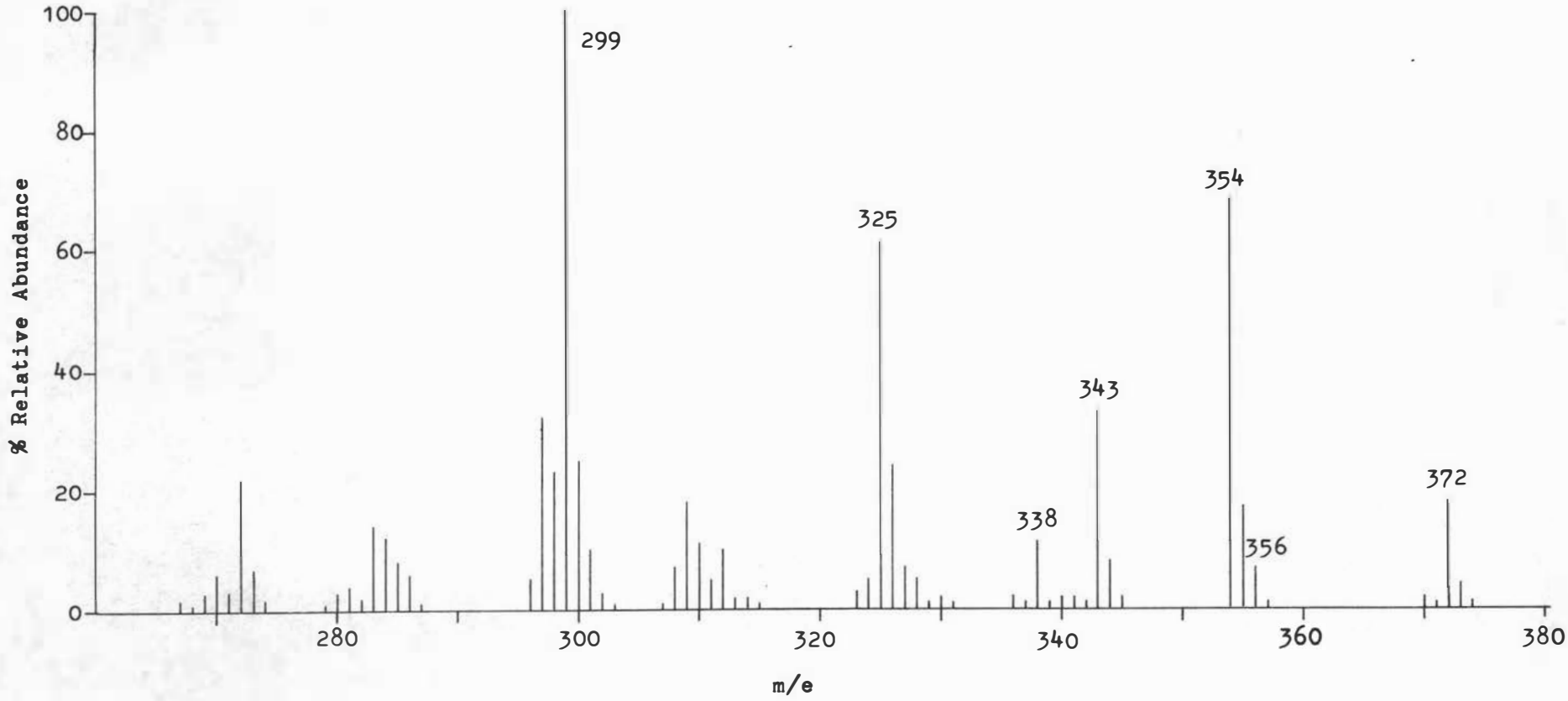


Fig. 1

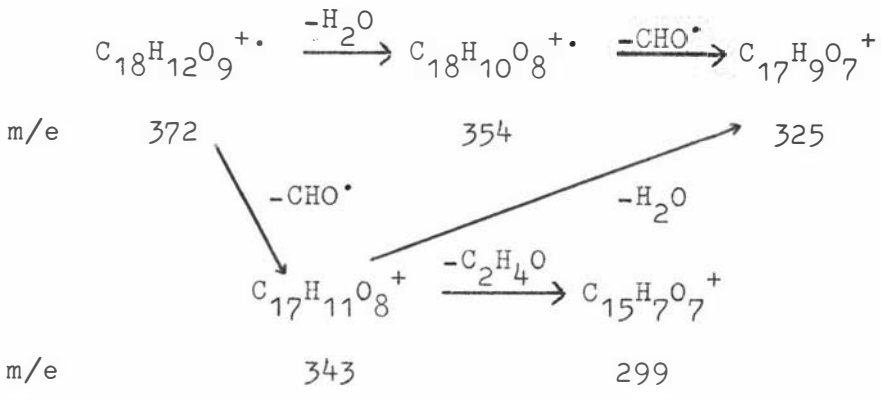
Mass Spectral Line Diagram of Homogeneous Red Pigment

Table I

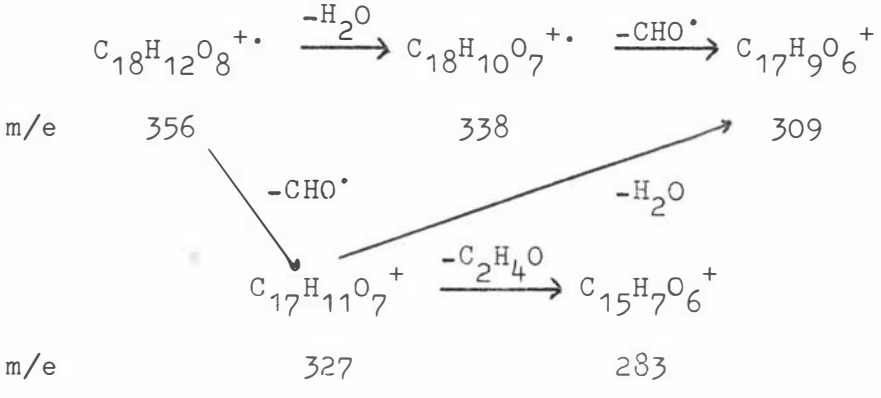
Molecular Formulae of Ions Observed in Upper Mass Region of Mass Spectrum of Homogeneous Red Pigment

Ion	Molecular Formula	m/e 372 series	m/e 356 series
372	$C_{18}H_{12}O_9$	$C_{18}H_{12}O_9$	
356	$C_{18}H_{12}O_8$		$C_{18}H_{12}O_8$
354	$C_{18}H_{10}O_8$	$C_{18}H_{10}O_8$	
343	$C_{17}H_{11}O_8$	$C_{17}H_{11}O_8$	
338	$C_{18}H_{10}O_7$		$C_{18}H_{10}O_7$
328	$C_{16}H_8O_8$	$C_{16}H_8O_8$	
327	$C_{17}H_{11}O_7$		$C_{17}H_{11}O_7$
326	$C_{17}H_{10}O_7$	$C_{17}H_{10}O_7$	
325	$C_{17}H_9O_7$	$C_{17}H_9O_7$	
312	$C_{16}H_8O_7$		$C_{16}H_8O_7$
310	$C_{17}H_{10}O_6$		$C_{17}H_{10}O_6$
309	$C_{17}H_9O_6$		$C_{17}H_9O_6$
299	$C_{15}H_7O_7$	$C_{15}H_7O_7$	
298	$C_{16}H_{10}O_6$		
297	$C_{16}H_9O_6$	$C_{16}H_9O_6$	
283	$C_{15}H_7O_6$		$C_{15}H_7O_6$
281	$C_{16}H_9O_5$		$C_{16}H_9O_5$
272	$C_{14}H_8O_6$	$C_{14}H_8O_6$	
256	$C_{14}H_8O_5$		$C_{14}H_8O_5$

The mass spectral data indicated the following ion fragmentation schemes for dothistromin and deoxydothistromin:



dothistromin ion fragmentation



deoxydothistromin ion fragmentation

The loss of the formyl radical CHO$^{\cdot}$ from the molecular ion of dothistromin, or from the ion at m/e 354 arising from the loss of water from the molecular ion, was a major point of interest as it was known that certain substituted anthraquinones and oxygenated aromatic compounds lost this radical as a major fragmentation process.

The ready loss of H_2O from the molecular ion could indicate the presence of an aliphatic hydroxyl group.

The loss of the neutral fragment C_2H_4O of m/e 44 from the ion at m/e 343 to give the base peak at m/e 299 in the spectrum was also of considerable interest, as there were few significant peaks in the spectrum below the base peak, indicating particular stability of the base peak ion. Thus the loss of the neutral m/e 44 fragment could well be of informative and diagnostic value from a structural elucidation viewpoint. Similar conclusions could be reached for deoxydothistromin.

The similarity of the mass spectral fragmentation patterns of dothistromin and deoxydothistromin, and the chromatographic behaviour of the red pigment as a homogeneous compound, suggested that dothistromin and deoxydothistromin were closely related and probably differed only in their number of hydroxyl groups.

CHAPTER 3. ATTEMPTS TO SEPARATE DOTHISTROMIN AND DEOXYDOTHISTROMIN

Examination of Red Pigment by TLC

In an attempt to separate the two components dothistromin and deoxydothistromin of the red pigment, various TLC adsorbents and solvent systems were investigated. Preliminary experiments revealed that aluminium oxide, cellulose powder, kieselghur, and polyamide were for one reason or another, unsuitable. Silica gel on the other hand, gave superior compact spots with a number of solvent systems. Solvent systems tried with both silica gel G and H (Merck), included combinations with acetic acid, e.g. $CHCl_3$: EtOAc : HOAc = 36:54:10; acetone : HOAc = 9:1; benzene : ethyl formate : HOAc = 75:24:1. Continuous development with solvents such as 100% $CHCl_3$ which gave the red pigment a low Rf value were tried; also, "wedge shaped" adsorbent layers were used with silica gel H and $CHCl_3$: EtOAc : HOAc = 36:54:10, with multiple development. Unfortunately, none of the systems tried appeared to give a separation of dothistromin and deoxydothistromin.

Examination of Red Pigment by Counter Current Distribution (CCD)

With the failure to obtain a separation of dothistromin and deoxydothistromin by TLC, other separative methods were considered. Of these, counter current distribution (CCD) appeared to be worthy of investigation. (A brief account of CCD, with references, is given in the appendix to this thesis).

If dothistromin and deoxydothistromin possessed phenolic hydroxyl groups such as are found in hydroxyanthraquinones for

example, and they differed in the number of these hydroxyl groups that they possessed, then it should be possible to separate them by partition between an organic solvent and an aqueous system of appropriate pH.

Preliminary, small scale CCD experiments were carried out in a rack of test tubes, to allow evaluation of systems for use in a Craig CCD machine. An ethyl acetate solution of the red pigment was subjected to the CCD extraction procedure with aqueous base systems including potassium acetate, borax, universal buffer mixtures, and phosphate buffers. The aim was to use a system with an aqueous phase of basic pH, to give a distribution coefficient (K) value of approximately 1.0. At the same time however, a pH as near to neutrality as possible is desirable, to avoid excessive degradation of the red pigment components by prolonged contact with base and air. Also, the stability of the emulsion formed between a solvent such as ethyl acetate and an aqueous buffer, when shaken together, appears to increase markedly with increasing pH, and emulsion formation makes it difficult to carry out a separation on a Craig-type CCD apparatus, especially when the emulsion has a high separation time (i.e. high stability).

When a crude ethyl acetate culture extract was shaken with potassium acetate as buffer solution ($\sim$0.1M, pH $\sim$10.0), the resulting aqueous phase often had a low pH, in the range pH 5 to 6. This indicated that the ethyl acetate extract was considerably acidic; it was necessary to neutralise this acidity by several equilibrations with aqueous buffer solution, before carrying out a CCD run.

A borax buffer (0.025M borax, pH ~9.3) appeared to have a complexing effect with the red pigment, as it gave a much more intensely red coloured aqueous phase than the other buffers, even with these buffers at pH 9.6 - 10.0.

CCD Run 1:

In the first run, called "CCD Run 1", on the hand-operated 120 tube Craig CCD machine, ethyl acetate was used for the upper phase and a borax buffer pH 9.3 was used for the lower phase. Using a crude ethyl acetate culture extract (which had been previously "washed to neutrality" with buffer) for this run, three major bands were observed in the tubes after 120 transfers: (i) a bright red coloured band (i.e. aqueous phase bright red, upper ethyl acetate phase orange) centred around tube no. 30, (ii) an extended yellow-orange "leading band", centred on tube no. 112, and (iii) a dark purple coloured band near the origin, centred on tube no. 5. Examination of the TLC behaviour and the visible electronic spectra of the upper ethyl acetate phases of these bands clearly indicated that the band centred on tube no. 30 contained the same red pigment observed on TLC during the preliminary work of this thesis shown to contain dothistromin and deoxydothistromin. The "trailing edge" tubes of this central band however, had a noticeable yellowish tinge to them, and this showed up in the visible spectra as a shoulder of increased intensity in the 460 nm region. A plot of tube number versus absorbance at 492 nm and 464 nm is shown in Fig. 2(a). The difference between the spectra of the tubes is slight and a plot of the ratio of the peak absorbances versus tube number, shown in Fig. 2(b), gives a better indication of the presence of more

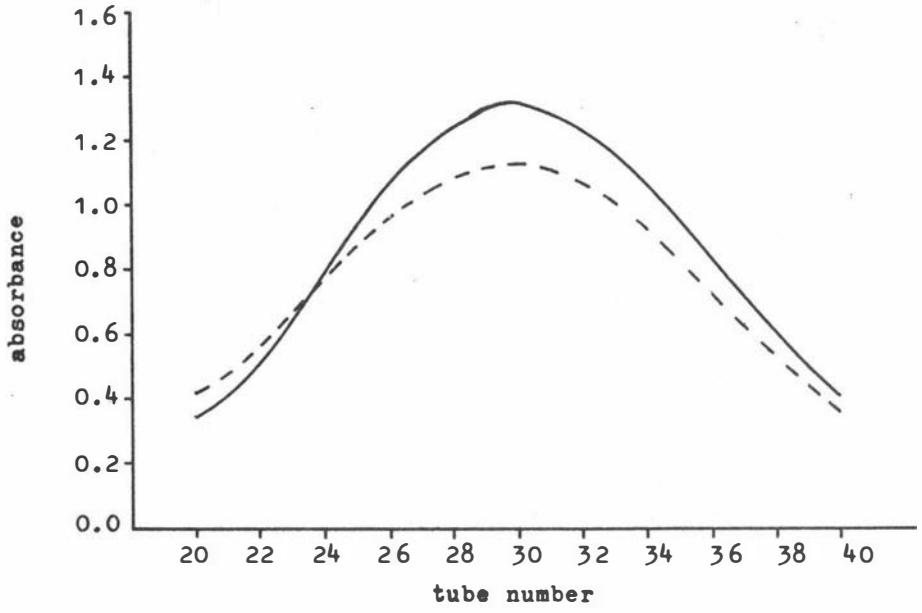


Fig. 2(a)

Plot of Tube Number versus Absorbance at 492 nm (—), and 464 nm (---), CCD Run 1

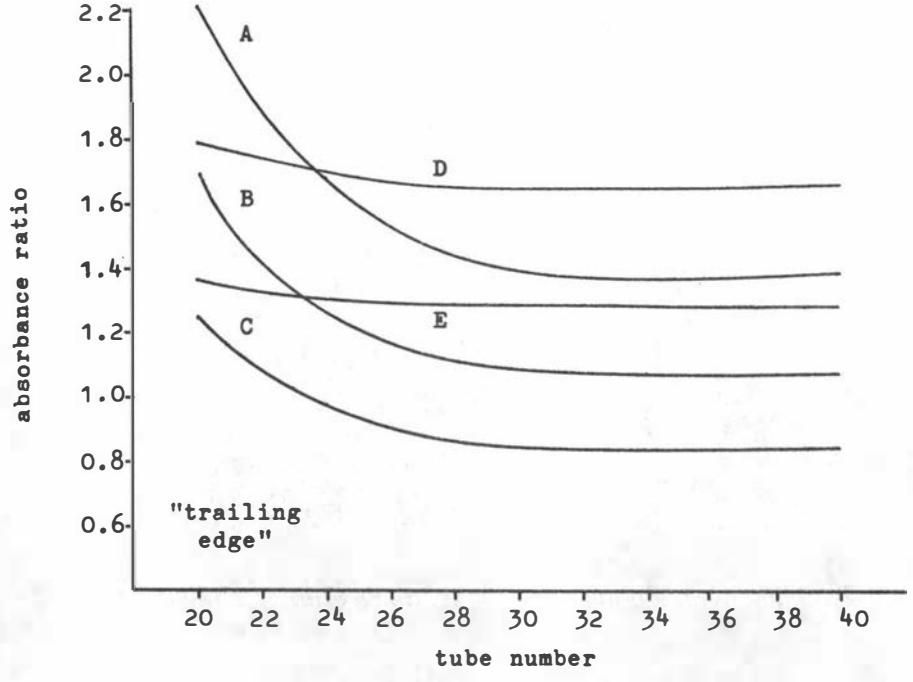


Fig. 2(b)

Plot of Ratio of Peak Absorbances versus Tube Number, CCD Run 1

A 464 nm : 527 nm D 492 nm : 527 nm
B 464 nm : 514 nm E 492 nm : 514 nm
C 464 nm : 492 nm

than one compound, since the ratio changes considerably towards the trailing edge of the band.

CCD Run 2:

Having demonstrated the presence of more than one compound in the red pigment band known to contain dothistromin and deoxydothistromin, a second CCD run was carried out in an endeavour to obtain a K value closer to 1.0, and thus an increased separation of the compounds in this band. To do this it was necessary to change to a phosphate buffer to obtain a higher pH than is possible with borax.

Using a phosphate buffer of pH 10.1, three major bands were obtained in CCD run 2, from the same pigment extract as used for CCD run 1. This time the dothistromin-containing band was centred around tube no. 56. This gave a K value of approximately 1.0. A plot of absorbance at 492 nm versus tube number (Fig. 3(a)), and a plot of the ratio of the absorbance at 492 nm to that at 464 nm (Fig. 3(b)) again indicated the presence of more than one compound in the central band.

The visible electronic spectra for tubes no. 56 to 63 were very similar, and as the absorbance ratio for these tubes lay on the straight line portion of the ratio plot (Fig. 3(b)), the tube contents for this group were pooled together, and worked up to yield an orange gum. An attempt was made to clean this up further by prep. TLC, using a silica gel G TLC plate pre-developed in methanol and reactivated. When the plate was developed with the normal ethyl acetate : chloroform (60:40) solvent, the compound ran for a short distance as an intensely purple-red coloured band. This band, which spread all the way to the origin,

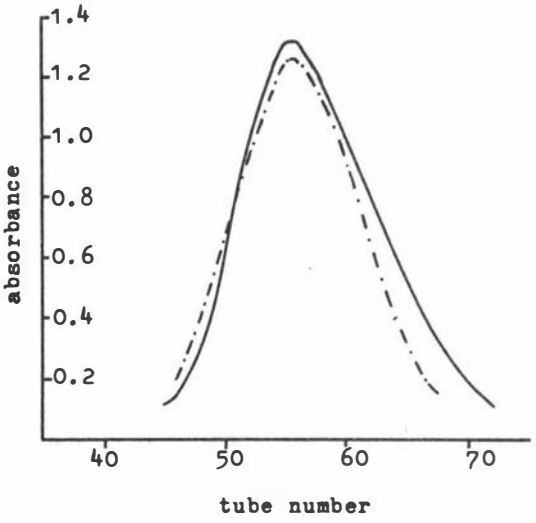


Fig. 3(a)

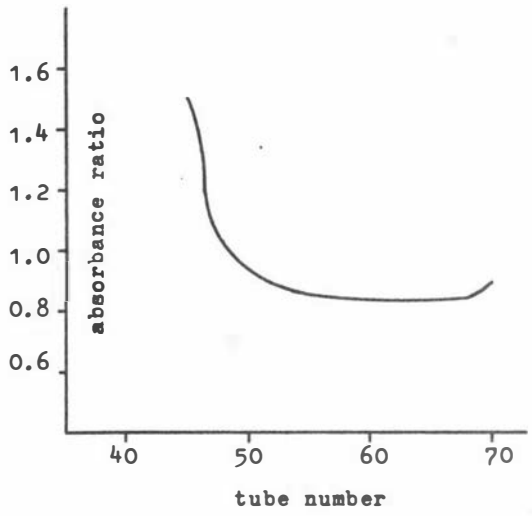


Fig. 3(b)

(— absorb. at 492 nm)

(--- calculated absorb. for k = 1.0)

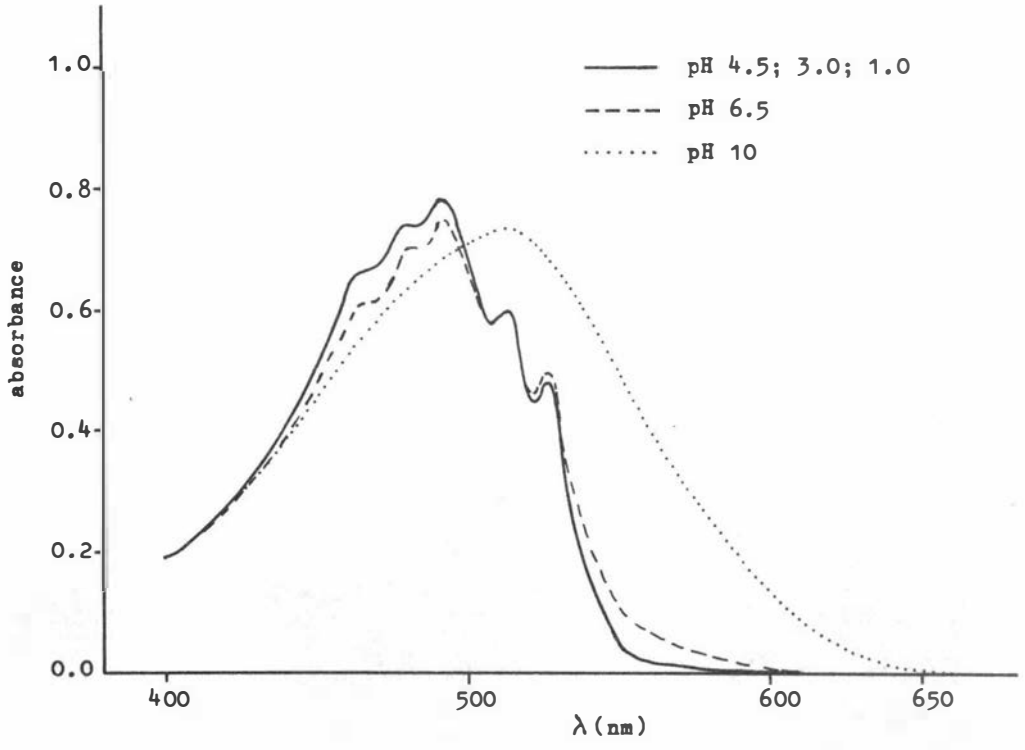


Fig. 4

Visible Electronic Spectrum of Pigment From Tube No. 55, CCD Run 2

could not be persuaded to run further even with more polar solvents, and in fact it was found that the compound had undergone virtually irreversible combination with the silica gel adsorbent. A possible explanation of this effect lay in the ability of the red pigment to complex with certain metal ions, especially Ca^{2+} and Mg^{2+} (see page 31), and to react chemically with alcohols (see page 72).

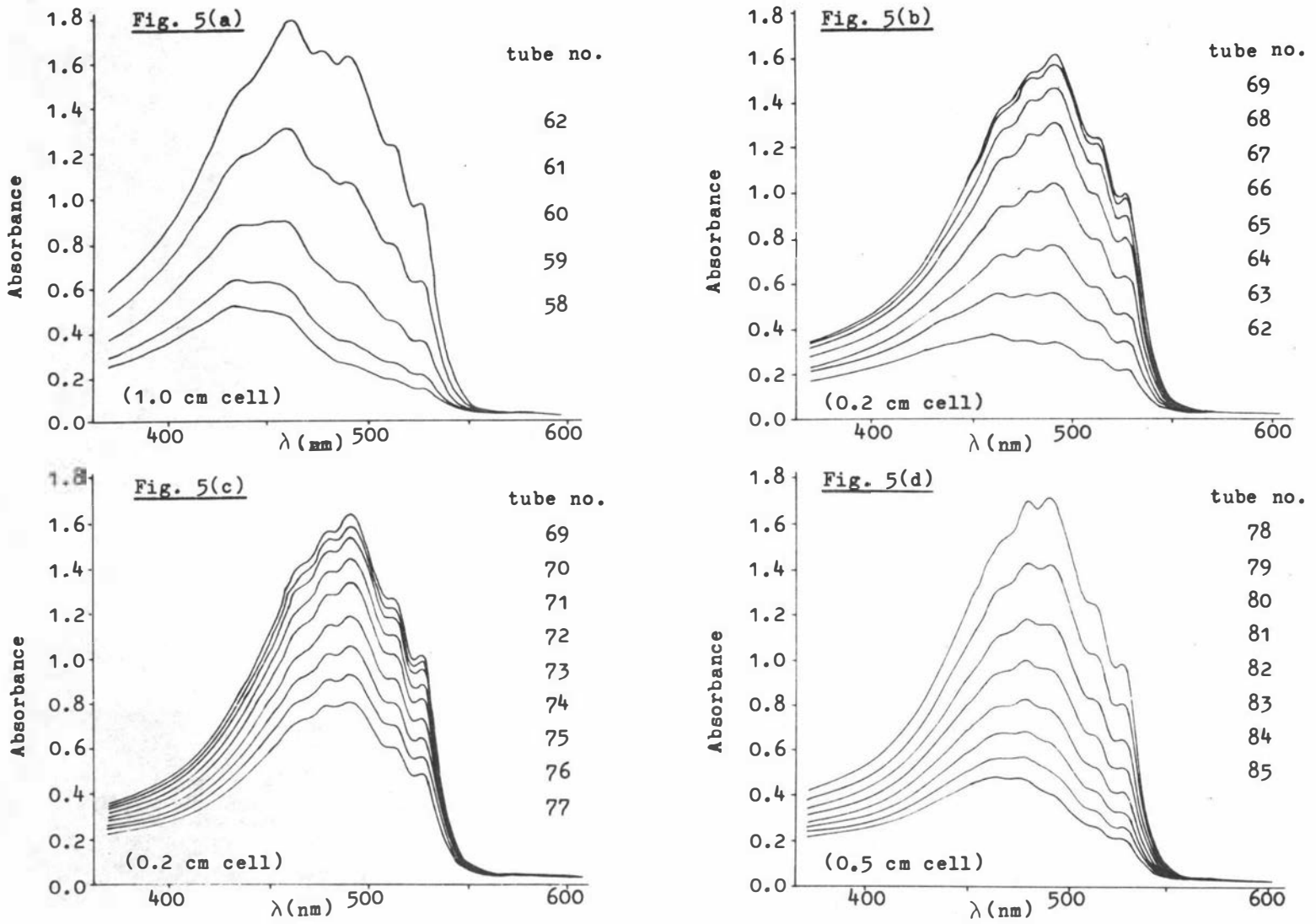
With the loss of the possibly pure pigment from tubes no. 56 to 63 by irreversible chromatographic adsorption, the contents of tube no. 55 were examined in the hope that this would yield substantially pure pigment. Work-up gave an orange-red amorphous powder, the visible electronic spectrum of which (in ethanol) is shown in Fig. 4. An ε value was calculated for λmax. at 492 nm; this was ε = 6,500 (at pH $\leq$ 4.5), and ε = 6,250 at pH 6.5. A study of the variation of absorbance in ethanol, versus pH showed that over the range pH 1.0 to 4.5, pH had no significant effect on the intensity of absorbance or on the shape of the absorption curve. At a pH of 6.5, the intensity of the peaks at 464, 480, and 492 nm drop a little, at 514 nm the intensity stays approximately the same, at 527 nm it increases a little, and it increases significantly from 530 nm onwards. This coincides with a very noticeable difference in colour of the ethanolic solution - at the lower pH's it is typically orange in colour, whereas at pH 6.5 or higher it becomes red in colour and loses its orange fluorescence. At higher pH's (e.g. pH 10) aqueous solutions and ethanol solutions of the pigment show only a smooth curve with no fine structure. Ethyl acetate phases equilibrated with the aqueous solutions however, show characteristic peaks on the absorption curves,

which remain virtually unaltered with change in pH of the aqueous phase. A practical result of this observation is that during the course of a CCD run with ethyl acetate as upper phase, progress of the distribution can be followed at any stage simply by removal of an aliquot of the ethyl acetate phase of any tube desired, followed by its return after having determined the visible spectrum, without any pH adjustments being required.

CCD Run 3:

This run was carried out in an attempt to obtain a larger quantity of purified red pigment for a more detailed chemical investigation. The pigment used for this run was obtained from a culture extract which had been partially purified by column chromatography on silica gel H. A phosphate buffer, pH 10.1, was used for the aqueous phase, and ethyl acetate for the upper phase. As with CCD runs 1 and 2, a central red pigment band was obtained with a K value of approximately 1.0. The visible electronic spectra of the ethyl acetate phase from each of the tubes of this band - tube no. 58 to tube no. 84 - were recorded, and are shown in Fig. 5(a) to Fig. 5(d). Examination of these spectra showed the presence of a "leading edge" and a "trailing edge" component.

Several tubes from the band were selected for examination by mass spectroscopy. Tube no. 70 was found to give a spectrum characteristic of normal red pigment containing dothistromin and deoxydothistromin peaks at m/e 372, 356, 354, 343, 338, 325, 299, etc. Tube no. 59 gave a spectrum in which the peak at m/e 356 was of significantly increased intensity relative to the peak at m/e 372 (ratio of m/e 356 : m/e 372 approximately 1.7:1.0), compared to spectra of the red pigment obtained ex prep. TLC



Visible Electronic Absorption Spectra of Ethyl Acetate Phase from CCD Run 3, Tubes 58-84

(ratio of m/e 356 : m/e 372 approximately 1.0 : 2.5). Thus the compound from tube no. 59 appeared to be enriched with deoxydothistromin, and thus a partial separation of deoxydothistromin had been achieved. The spectrum clearly showed a much greater relative abundance of the peaks at m/e 283 and m/e 327 compared to the normal red pigment spectra. Mass measurement showed the following peak compositions: m/e 327 = $C_{17}H_{11}O_7$, m/e 309 = $C_{17}H_9O_6$, m/e 283 = $C_{15}H_7O_6$. Also, metastable peaks verified the transitions:

$$C_{17}H_{11}O_7 \xrightarrow[*]{-C_2H_4O} C_{15}H_7O_6$$

m/e 327 m/e 44 m/e 283

$$C_{17}H_{11}O_7 \xrightarrow[*]{-H_2O} C_{17}H_9O_6$$

m/e 309

Further conclusions were later made on the structure of deoxydothistromin from the absorption curves recorded above - see pages 35 to 38.

Tube 81 gave a mass spectrum with typical red pigment peaks, but also with a strong peak at m/e 368; mass measurement showed this to correspond to $C_{20}H_{16}O_7$, and a metastable peak existed for the transition:

$$C_{20}H_{16}O_7 \xrightarrow[*]{-C_3H_6O} C_{17}H_{10}O_6$$

m/e 368 m/e 58 m/e 310

This could indicate the existence of a compound of formula $C_{20}H_{16}O_7$ as a leading edge component, and this could be an ethyl ether or dimethyl ether derivative of a compound of formula $C_{18}H_{12}O_7$.

CCD on a Steady State Distribution Machine

With the above partial separation of dothistromin and deoxydothistromin obtained, it was decided to carry out a further CCD run, on an automated steady state distribution machine. The advantage of the steady state CCD apparatus is that in this apparatus, it is possible to maintain any desired band of interest in the central portion of the tube bank, and to concentrate this band from a crude extract by a continual feed "steady state" process. It is then possible to gradually widen the band and separate it into its various components even when they are very closely related (Fleetwood, 1962; Alderweireldt, 1961; Quickfit and Quartz Ltd).

Red pigment was subject to CCD in such an apparatus with ethyl acetate as upper solvent phase, and a phosphate buffer, pH 10.35, as lower phase. After the distribution had been allowed to proceed overnight, it was noted that the red pigment band was travelling in a narrow band of high K value. Attempts to circumvent this behaviour by increasing the ratio of lower : upper phase transfers were unsuccessful. A check on the pH of the aqueous phase showed this to be pH 8.0, thus explaining the anomalous partition ratio. The drop in pH appeared to be due to excessive hydrolysis of the ethyl acetate phase, the buffer capacity being ultimately exhausted. This same problem had not been observed in the previous work (i.e. CCD runs 1, 2 and 3) where each run had been carried out in under 12 hours; the run with the steady state machine had been carried out for some 32 hours. The reason for the longer time is that the automatic machine has built in minimum program time factors for each portion

of the distribution motion, which can readily be bettered in the case of manual equipment using solvent systems which do not form troublesome emulsions.

With the ethyl acetate/aqueous buffer system shown to be unsuitable for the automatic equipment, other solvent systems were investigated.

n-Butanol/aqueous buffers: n-Butanol appeared to be a moderately good solvent for the red pigment. However, a test tube rack experiment revealed that it required a buffer of surprisingly high pH to extract any significant colour from the butanol phase. Whereas the n-butanol solution, whilst in equilibrium with neutral aqueous solvents, is the same characteristic orange colour as ethyl acetate solutions of the extract, at alkaline pH's it is a beautiful wine-red colour, the colour remaining in the n-butanol phase until high pH (pH ~11) buffer systems are used. Thus the alkali metal salt derivatives of the red pigment appear to partition favourably into n-butanol at pH's up to pH 11. This could be associated with the high solubility of water in n-butanol - at 25°C the solubility of water in n-butanol is 20.5% and the solubility of n-butanol in water is only 7.5%. Ethyl acetate has a reverse solubility order: 8% ethyl acetate in water and only 3.3% water in ethyl acetate.

It was found that the partition ratio K could be adjusted to a somewhat more favourable value by addition of sodium benzoate. Thus a K value of 1.5 was obtained in a test tube rack experiment using n-butanol/0.25M phosphate buffer pH 10.5 containing 0.1M sodium benzoate.

Methyl isobutyl ketone (MIBK)/aqueous buffers: MIBK is a better

solvent for the red pigment than n-butanol, and it is possible to extract nearly all the colour out of an MIBK solution with a phosphate buffer of pH 10.0, whereas to do this with n-butanol a buffer of pH >11.0 is required.

Using a phosphate buffer of pH 9.4, a K value of 1.0 was obtained in a test tube rack experiment with a solution of red pigment in MIBK. At this pH, the time required for the MIBK and aqueous layers to separate is very short - only 20-25 secs. Thus the MIBK system would appear to be a good system to use for any large scale CCD work on the red pigment, especially when carrying out a high number of transfers.

Attempts at a more complete separation of dothistromin and deoxydothistromin by CCD were not pursued further however, in spite of the above encouraging results, as a still more profitable line of investigation was revealed by work carried out concurrently on acetylation.

CHAPTER 4. THE HYDROXYANTHRAQUINONE CHROMOPHORE OF DOTHISTROMIN

Hydroxyanthraquinones show characteristic reversible oxidation-reduction colour changes with alkaline dithionite and give characteristic colour reactions with alkali and other reagents (Shibata, 1950; Rodd, 1956; Thomson, 1957). Similarities in the colour reactions with different hydroxyanthraquinones often indicate similarities in the hydroxyl group substitution pattern.

A number of colour reactions and properties of the red pigment indicated that dothistromin was a hydroxyanthraquinone.

Reversible Reduction-Oxidation with Alkaline Dithionite

The purple colour of an alkaline solution of the red pigment was slowly discharged to yellow by shaking with alkaline sodium dithionite, and was restored to purple by shaking with air. This reversible oxidation-reduction colour change reaction with alkaline dithionite is characteristic of anthraquinones and hydroxyanthraquinones (Rodd, 1956; Fieser and Fieser, 1963; Powell *et al.*, 1967). Some comparative tests carried out in the laboratory are summarised in Table II.

The orange colour of an ethyl acetate solution of the red pigment was also immediately discharged to pale yellow on shaking with Adams catalyst over hydrogen.

The above results showed that dothistromin contained a quinonoid system, its behaviour being consistent with that of hydroxyanthraquinones.

Colour with Alkali

The red pigment (purified by TLC or CCD) formed an intense purple coloured solution with aqueous sodium hydroxide. This reaction appears to indicate a 1,2- or 1,4-dihydroxy substitution in an anthraquinone nucleus. Thus, whilst alizarin (1) and anthragallol-3-methyl ether (2) give a purple solution with sodium hydroxide, xanthopurpurin (3), rubiadin (4) and anthragallol-2-methyl ether (5) give a red solution (Nicholls, 1948).

A comparative test carried out in the laboratory with several hydroxyanthraquinones supported this result, and is summarised in Table III.

Colour with Various Metal Ions in Ethanol

The purified red pigment gave an intense magenta coloured solution with ethanolic magnesium acetate, this colour indicating an anthraquinone with α-hydroxyl groups (Shibata, 1950; Sutherland and Wells, 1967). The same colour was obtained with alkaline solutions of calcium and magnesium ions and the red pigment. Comparative tests were carried out with several hydroxyanthraquinones and ethanolic solutions of several metal salts, and the results are shown in Table IV. The very close similarity between the colours produced by the red pigment and 1,4-dihydroxyanthraquinone (quinizarin) (6) suggested that dothistromin possessed a 1,4-dihydroxy substitution arrangement in one of the aromatic rings of the anthraquinone nucleus.

Table II

Action of Alkaline Sodium Dithionite on Hydroxyanthraquinones

Anthraquinone	Colour with 1.0 N NaOH	Colour with alk. sodium dithionite	Colour on shaking with air
1,4-di-OH	purple	orange-yellow	purple
1,8 " "	red	golden-yellow	red
1,2 " "	purple-blue	pale-orange	purple
2,6 " "	orange	no colour change	-
1,2,4-tri-OH	red	" " "	-
1,2,5,8-tetra-OH	blue	pale-yellow	purple
red pigment	purple	yellow	purple
anthraquinone	pale-yellow (not sol.)	blood red	pale-yellow

Table III

Colour of Hydroxyanthraquinones with Aqueous 1.0 N NaOH

Anthraquinone	Colour
1,2-di-OH	intense purple
1,4-di-OH	" "
red pigment	" "
1,2,4-tri-OH	" red-purple
1,8-di-OH	" red
rubiadin	" red
2,6-di-OH	orange
1,2,5,8-tetra-OH	intense indigo

Table IV

Colour of Hydroxyanthraquinones with Various Metal Ions in Ethanol

Anthraquinone	Ethanol	Mg (II) acetate	Ni (II) nitrate	Cu (II) acetate	Co (II) nitrate	Al (III) chloride
(ethanol)	col.	col.	v. pale green	v. pale green	pale pink	col.
red pigment	orange	magenta	red	mauve	red	scarlet
1,4-di-OH	pale yellow	magenta	red	mauve	red	scarlet
1,2- " "	yellow	purple	red	purple	red	red
1,8- " "	pale yellow	red	orange	red	orange	orange
1,2,4-tri-OH	orange	red	magenta	red	red	orange-red
2,6-di-OH	v. pale yellow	pract. col.	pract. col.	pract. col.	pale peach	pract. col.

Metal Salt and Chelate Complex Formation

The above colour reactions demonstrate the ability of hydroxyanthraquinones (and the red pigment) to form salts with alkali metal ions, and also the ability to form metal chelate complexes with a variety of metal ions (Rodd, 1956). Whilst salts of the 1,2-dihydroxyanthraquinone (alizarin) (1) type have the greatest stability, salts of the less acidic 1,4-dihydroxy-anthraquinone type are easily decomposed, e.g. the dipotassium salt of 1,4-dihydroxyanthraquinone is decomposed by CO_2 (Perkin, 1879).

Although the literature abounds with references on spectroscopic studies of chelate complex formation between hydroxyanthraquinones and a whole host of metal ions, the actual structures of the chelate complexes are by no means completely established (e.g. see Suemitsu, 1963; Srivastava and Banerji, 1967).

Fluorescence

Solutions of the red pigment in acetic acid (or ether or ethanol) exhibited an intense green-yellow fluorescence under near UV irradiation. Such fluorescence is characteristic of the 1,4-dihydroxyanthraquinone (6) nucleus - see below. Table V summarises the results of examination of solutions of a number of hydroxyanthraquinones for fluorescence. From the observations recorded in the table it was concluded that dothistromin possessed a 1,4-dihydroxyanthraquinone nucleus.

The generalisation that strong fluorescence of a solution of a hydroxyanthraquinone in acetic acid is characteristic of the quinizarin (6) nucleus, is based on empirical observations.

Table V

Fluroescence of Hydroxyanthraquinone Solutions under irradiation with UV light (λ 350 nm)

Anthraquinone	Et_2O Soltn.	Ethanolic $Mg(OAc)_2$ soltn.
1,4-di-OH	intense yellow-green	intense scarlet red
1,8-di-OH	v. weak yellow-green	intense orange
1,2-di-OH	faint orange	v. weak orange-red
2,6-di-OH	no obs. fluorescence	v. weak green
1,2,4-tri-OH	intense yellow-green	intense orange
1,2,5,8-tetra-OH	strong yellow-orange	-
red pigment	intense yellow-green	intense scarlet red
dothistromin ethyl ether (49)*	intense yellow green	intense scarlet red

* see page 72

Thus Raistrick, Robinson and Todd (1934) studied the fluorescence of the solutions in acetic acid of 15 hydroxyanthraquinones and concluded that compounds containing the 1,4-dihydroxyanthraquinone nucleus gave a characteristic fluorescence. Howard and Raistrick (1955) claimed that "in the intervening years (since 1934) a number of other polyhydroxyanthraquinones of established structure have been examined and have confirmed the validity of the generalisation". Since then, numerous other citations have appeared in the literature to further support the generalisation (e.g. Thomson, 1957; Dave et al., 1959; Sutherland and Wells, 1967; Powell et al., 1967).

The author of this thesis believes that a rationalisation of the observation that solutions of 1,4-dihydroxyanthraquinones are strongly fluorescent, whereas solutions of anthraquinone itself, and hydroxyanthraquinones with other hydroxyl group substitution patterns are not, can be proposed from a detailed consideration of the phenomenon of fluorescence. A discussion on this is presented in the Appendix, under the heading "The Fluorescence of 1,4-dihydroxyanthraquinone".

The Visible Absorption Spectra

The visible electronic absorption spectrum of the purified red pigment in ethanol showed a remarkable similarity to those reported for anthraquinones with three α-hydroxyl groups - see Table VI.

It is well known (Morton and Earlam, 1941; Briggs et al., 1952; Birkinshaw, 1955; Scott, 1964) that in the visible absorption spectra of hydroxyanthraquinones, the major absorption band due to the quinonoid nucleus is greatly affected by the number of

Table VI

Visible Electronic Absorption Spectra Data on Some α-Hydroxyanthraquinones

Anthraquinone	λmax (nm)	Reference
Red Pigment	478, 490, 509, 523	-
1,4,5-tri-OH	480, 490, 510, 525	Scott (1964)
Helminthosporin (2-Me-4,5,8-tri-OH	480, 490, 510, 525	Birkinshaw (1955)
Islandicin (2-Me-1,4,5-tri-OH)	466-470, 492, 513, 527	" "
Calenarin (2-Me-1,4,5,7-tetra-OH)	489, 508, 515-525	" "
1,2,4,5,7-penta-OH	473, 487, 496, 517, 532	Powell et al. (1967)
Cynodontin (2-Me-1,4,5,8-tetra-OH)	518, 545, 558	Birkinshaw (1955)

free α-hydroxyl groups, but little affected by the presence of β-hydroxyl groups. Thus anthraquinone itself has a visible absorption band at 405 nm. This 405 nm absorption is susceptible to increasing fine structure, intensity increases, and shifts to longer wavelengths by introduction of α-hydroxyl groups; the greater the number of α-hydroxyl groups, the longer the wavelength of absorption. In fact, the position of absorption and pattern of fine structure are of diagnostic value, especially for three and four α-hydroxyl groups. Thus, as can be seen in Table VI, 1,4,5-trihydroxyanthraquinone, Helminthosporin, and Islandicin, all anthraquinones with three α-hydroxyl groups, all have very similar absorption bands. By comparison, Cynodontin, which has four α-hydroxyl groups, shows even further bathochromic displacement of the quinonoid absorption than the above tri-α-hydroxyanthraquinones.

Notwithstanding the fact that the red pigment was shown in the early part of this investigation to be a mixture of two compounds, dothistromin and deoxydothistromin, the above spectral similarity was such as to strongly suggest that dothistromin was a substituted anthraquinone with three α-hydroxyl groups. In view of the very close chemical similarity of dothistromin and deoxydothistromin (identical TLC behaviour, similar mass spectral fragmentation), it was considered at this stage of the investigation that deoxydothistromin would also have either the same hydroxyanthraquinone chromophoric moiety as dothistromin, or an anthraquinone nucleus with one less α-hydroxyl group (deoxydothistromin appeared to have one hydroxyl group less than dothistromin, as shown by acetylation experiments - see later).

Deoxydothistromin could not have a β-hydroxyl group in the anthraquinone nucleus, as this would result in significantly different behaviour between deoxydothistromin and dothistromin, on TLC and CCD. If deoxydothistromin was a di-α-hydroxyanthraquinone, then it could have a 1,4-, a 1,5- or a 1,8-dihydroxyanthraquinone substitution pattern, and be otherwise identical to dothistromin. It would be difficult to detect small amounts of the 1,4-dihydroxy compound in the presence of dothistromin, since the absorption band and fine structure of both 1,4-dihydroxy- and 1,4,5-trihydroxyanthraquinone are somewhat similar. However, in the case of a 1,5- or 1,8-dihydroxyanthraquinone compound, the difference would be more noticeable, because of the significantly lower wavelength absorption of these dihydroxyanthraquinones. Published data for these hydroxyanthraquinones are shown in Table VII.

On the basis of this data, an experiment was carried out in which small aliquots of a concentrated solution of 1,4-dihydroxyanthraquinone were added to a solution of the red pigment in ethyl acetate, the visible spectrum being recorded after each addition. The experiment was repeated again, but this time with aliquots of 1,8-dihydroxyanthraquinone. The results showed that whereas the addition of 1,4-dihydroxyanthraquinone caused a gradual change of fine structure without significant change in shape of the curve, the addition of 1,8-dihydroxyanthraquinone caused a dramatic change in the region below 490 nm, but no change at all from 490 nm upwards. In fact, the "synthetic" curves showed a remarkable similarity to the absorption curves recorded for tubes 61 and 60 ex CCD run 3. This clearly

Table VII

Visible Electronic Absorption Spectra Data for Di-α- and Tri-α-hydroxyanthraquinones

Anthraquinone	λmax (nm)	Reference
1,8-di-OH	411, 421, 431, 446 456	Morton and Earlam (1941)
1,5-di-OH	397, 418, 437	" " " "
1,4-di-OH	459, 474, 486, 498, 508, 520	" " " "
1,4,5-tri-OH	405, 480, 490, 510, 525	Scott (1964)

demonstrated that the pigment from tubes 54-62 (approx.) ex CCD run 3 contained dothistromin plus a significant proportion of a 1,5- or 1,8-dihydroxyanthraquinone, this anthraquinone being, in all probability, deoxydothistromin. This follows, since mass spectral fragmentation of the contents of tube 59 ex CCD run 3 had shown a preponderance of deoxydothistromin over dothistromin (see page 21).

Zinc Dust Distillation

Zinc dust distillation of the red pigment at 400°C under an atmosphere of hydrogen, gave a low yield of a pale yellow coloured product which was highly fluorescent under UV irradiation (λ 350 nm).

The zinc dust distillation product was examined by TLC. An aluminium oxide G TLC plate developed with n-pentane (Biernoth, 1968) showed the distillation product as an unresolved smear from Rf 0.75 to Rf 0.85, whilst anthracene, phenanthrene and naphthalene appeared as spots on the same plate with Rf values of 0.75, 0.75 and 0.85, respectively. A cellulose TLC plate impregnated with DMF and developed with isooctane (Sawicki et al., 1964; White and Howard, 1967) showed four fluorescent spots from the distillation product, Rf's 1.00, 0.90, 0.84 and 0.75. On the same plate, acenaphthene, anthracene, phenanthrene and fluoren-9-one appeared at Rf values of 0.93, 0.75, 0.75 and 0.51 respectively. These results clearly indicated that the distillation product was a complex mixture, and it was decided to examine this by GLC, with a view to separating the major components by prep. GLC.

A QF-1 column operated at 180°C showed two major peaks for

the zinc dust distillation product of retention times (Rt times) 4.2 min and 6.4 min, and minor peaks at Rt 8.5, 9.5 and 11.5 min. Under the same conditions, phenanthrene, terphenyl, and pyrene gave peaks with Rt's of 4.2, 11.5 and 14.5 min respectively. The component compounds of the distillation product were collected on a preparative scale; they all showed intense blue fluorescence under UV irradiation (λ 350 nm), and all gave UV absorption spectra very similar to that of anthracene.

On the basis of mass spectral examination and their UV spectra, the following identifications were made:

Rt 4.2 min component - anthracene: The molecular ion at m/e 178 was shown to be $C_{14}H_{10}^{+\cdot}$, and a metastable peak showed the transition

$$C_{14}H_{10}^{+\cdot} \xrightarrow[*]{-C_2H_2} C_{12}H_8^{+\cdot}$$

m/e	178	26	152

Also, a doubly charged molecular ion appeared at a position of m/e 89. These features and the mass spectral pattern were co-incident with those for anthracene.

Further, the Rf value by TLC, the Rt value by GLC, the fluorescence under UV irradiation (λ 350 nm) and the UV absorption spectrum in EtOH were all indistinguishable from that of anthracene. Phenanthrene, which has very similar mass spectral and chromatographic behaviour, has, in the absorbed state, a darker blue fluorescence than the white-blue fluorescence of anthracene, and has a signficantly different UV absorption spectrum to that of anthracene (Jaffé and Orchin, 1962).

Rt 6.4 min component - 2-methyl anthracene: The similarity of the UV absorption spectrum of this component to that of anthracene, its strong fluorescence under UV irradiation, and its longer retention time on GLC compared to anthracene, left little doubt that this component was a substituted anthracene.

Mass spectral examination revealed a molecular ion $C_{15}H_{12}^{+\cdot}$ and a spectrum consistent with a methyl anthracene. Since there are three possible methyl anthracenes, 1-, 2-, and 9-methyl anthracene, it was necessary to distinguish between these.

Authentic 1-methyl anthracene was prepared from the zinc dust distillation of 1-chloro-4-methyl anthracene (Fischer and Sapper, 1911) which was prepared by acylation of phthalic anhydride with p-chlorotoluene, followed by condensation of the benzoylbenzoic acid formed with conc. H_2SO_4 containing 20% SO_3 (Heller and Schulke, 1909). 2-methyl anthracene, kindly supplied by L.H. Briggs (1970), was purified prior to use, by prep. GLC.

The UV absorption spectra of anthracene and its three monomethyl derivatives, although very similar, show small yet significant differences (Peters, 1957; Cherkasov, 1959; Jaffé and Orchin, 1962; Gelus et al., 1962; Taga, 1963). Thus, 1-, 2-, and 9-methyl anthracenes have long wavelength band maxima bathochromically displaced from that of anthracene, by 3.2 nm, 0.9 nm, and 9.4 nm, respectively. 1- and 2-methyl anthracene can also be distinguished by differences in the fine structure of their low intensity longwavelength maxima in the region 310-335 nm, and by the presence of a shoulder on the low wavelength side of the band at 250 nm of 1-methyl anthracene. A comparison of the UV absorption spectra of the component from GLC with the spectra of

anthracene and 1- and 2- methyl anthracene clearly revealed that the distillation product was 2-methyl anthracene, consistent with all the above spectral features.

Further evidence to substantiate the identity of the Rt 6.4 min component from GLC, was obtained by NMR spectroscopy. Thus, Carruthers et al. (1967) showed that the NMR chemical shifts (τ values, in $CDCl_3$, w.r. to TMS) of the methyl protons of 1-methyl anthracene are 7.30, 2-methyl anthracene 7.48, and 9-methyl anthracene, 7.11. The NMR spectra of authentic 1- and 2-methyl anthracenes were run in $CDCl_3$/TMS, and methyl group proton shifts were observed at τ 7.29 and τ 7.45 respectively, in excellent agreement with Carruthers' values. An NMR spectrum of the Rt 6.4 min component, using 37 sweeps with a CAT, showed a methyl group signal at τ 7.47, whilst admixture with 1-methyl anthracene at similar concentration gave an additional methyl signal at τ 7.20, thus confirming the identification of the zinc dust distillation product component as 2-methyl anthracene.

Rt 8.5 min and 9.5 min components: The similarity of the UV absorption spectra of these components to that of anthracene, the strong fluorescence under UV irradiation, and the longer retention times on GLC indicated that both components were substituted anthracenes. Mass spectral examination of the Rt 8.5 min component showed a molecular ion $C_{16}H_{14}^{+\cdot}$, with a strong fragment peak at m/e 191, shown to be $C_{15}H_{11}$, a metastable peak confirming the transition

$$C_{16}H_{14}^{+\cdot} \xrightarrow{-CH_3^{\cdot}} C_{15}H_{11}^{+\cdot}$$

m/e	206	15	191

However, very little data on the mass spectra of ethyl- and di-methyl anthracenes was available, and this necessitated production of authentic materials for direct comparison.

1- and 2-acetyl anthracene were prepared by Friedel-Crafts acylation of anthracene with acetyl chloride (Gore, 1957; Manecke and Storck, 1962; Gore and Thadani, 1966 and 1967). The 1- and 2- isomers from this reaction did not separate cleanly by fractional crystallisation, TLC indicating some cross-contamination of both isomers. GLC on a QF-1 column clearly separated the two isomers, and showed that the 1-isomer from crystallisation contained ~5% 2-isomer, and that the 2-isomer from crystallisation contained ~1-2% 1-isomer. The impure 2-isomer was subject to Wolf-Kishner reduction (Waldmann and Marmorstein, 1937; Klemm et al., 1963), and the crystalline product (2-ethyl anthracene) was examined by mass spectroscopy. Its mass spectral similarity to that of the Rt 8.5 min component showed that this zinc dust distillation component was almost certainly an ethyl anthracene, rather than a di-methyl anthracene. Lack of material and time precluded further investigation of this component, and also of the Rt 9.5 min and 11.5 min components.

Rt 16 min component - formula $C_{17}H_{12}O$: During prep. GLC of the zinc dust distillation product, a small amount of a material fluorescing intense dark green under UV irradiation, Rt ~16 min was collected. Mass spectral examination allowed determination of the molecular formula, $C_{17}H_{12}O$. In view of the fact that this was only one carbon atom less than in the dothistromin molecule

($C_{18}H_{12}O_9$), effort was devoted to obtaining more of this component, since structural elucidation could then account for almost the entire carbon skeleton of dothistromin. Initially, attempts were made to improve the relative yield of the component from zinc dust distillation by carrying out the distillation at lower temperatures, i.e. under less drastic conditions. Thus at 340°C, distillation gave mostly sublimed red pigment, with very little fluorescent product. At 380°C, distillation gave a product with virtually the same GLC peak pattern as the 400°C distillation product, with however, slightly increased amounts of the higher Rf products. Distillation at 370°C gave essentially the same results as obtained at 380°C, but longer times were required for the products to distill out of the zinc dust zone. It thus appeared that the product ratio could not be significantly altered by lowering the distillation temperature.

Distillations were attempted using the same technique, but with cadmium dust, aluminium powder, iron powder and copper bronze powder replacing the zinc dust. With 1,2,4-trihydroxyanthraquinone as a test compound, all these materials gave some fluorescent hydrocarbon, but none appeared to be as efficient or gave as clean products (most products were contaminated by much unreduced hydroxyanthraquinone) as the zinc dust. Any further attempts to obtain increased yields of the $C_{17}H_{12}O$ product were therefore abandoned.

Interpretation of the Results of Zinc Dust Distillation

The distillation of complex organic compounds, especially natural products, with zinc dust is a classical method of

elucidating the basic skeleton of the molecules under investigation. In spite of the fact that the yields from the reaction are invariably very low, and that the high temperature required for the reaction often leads to excessive bond breaking and rearrangements, the technique has been used with considerable success with phenols, quinones and alkaloids (Valenta, 1963). Thus, zinc dust distillation played a significant role in the elucidation of the structure of the antibiotic terramycin - the basic skeleton of terramycin was revealed from the isolation of naphthacene (2.5% yield) on zinc dust distillation of a terramycin degradation product (Hochstein et al., 1953). Kögl (1935) used the method in a systematic investigation of fungal pigments.

In the case of hydroxyanthraquinones, numerous examples of the isolation of 2-methyl anthracene from zinc dust distillation are known, and a number of these are recorded in a book by Thomson (1957).

In view of the low yields of products obtained from zinc dust distillation, it is important to ensure that certain impurities capable of giving significant yields of polycyclic aromatic hydrocarbons are not present in the compound under investigation. Thus, phthalate plasticizers can condense under the conditions of the zinc dust distillation to give anthracene and substituted anthracenes (Clar, 1964). A careful check by TLC was made for the presence of phthalate plasticizers in the red pigment used for the zinc dust distillation, comparative tests being carried out with several common phthalate plasticizers. This investigation revealed the complete absence of phthalate plasticizers in the pure red pigment used for the

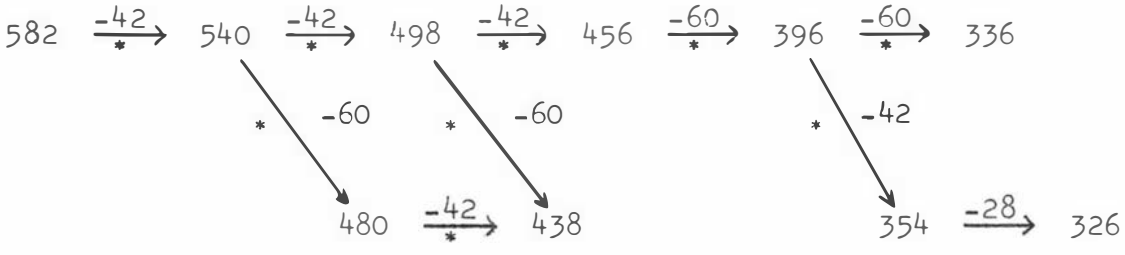
distillation work.

Identity of the Chromophoric Moiety of Dothistromin

Consideration of all the results described up to this stage show that dothistromin is a β-alkyl-tri-α-hydroxyanthraquinone.

CHAPTER 5. ACETYLATION OF THE RED PIGMENT. DOTHISTROMIN PENTA-ACETATE AND DEOXYDOTHISTROMIN TETRA-ACETATE

Red pigment, obtained by prep. TLC of *D. pini* extract, was subject to acid catalysed acetylation with acetic anhydride. The reaction product, a pale yellow gum,‡ was examined by mass spectroscopy. The mass spectrum, a line diagram of which is shown in Fig. 6, revealed the presence of two series of related peaks, one arising from a molecular ion at m/e 582, the other from a molecular ion at m/e 524. These molecular ions clearly corresponded to dothistromin penta-acetate (MW 582) and deoxydothistromin tetra-acetate (MW 524). Thus, the fragmentation paths of the molecular ion m/e $582^{+\cdot}$ involved multiple losses of the elements of ketene, C_2H_2O (m/e 42), and the elements of acetic acid, $C_2H_4O_2$ (m/e 60).



fragmentation of dothistromin penta-acetate

‡ The observation that acetylation of dothistromin/deoxydothistromin pigment mixture resulted in a marked colour change from red pigment to pale yellow material, was clear evidence that acetylation of phenolic hydroxyl groups in the chromophoric moiety of the molecule had occurred.

The loss of the elements of ketene (m/e 42) is characteristic of aromatic acetates, whilst the loss of the elements of acetic acid (m/e 60) is characteristic of aliphatic acetates (Budzikiewicz et al., 1967; Thomas, 1970). In this connection, a number of acetoxy substituted aromatic compounds and anthraquinones (compounds (10) to (18), page 56), prepared in the laboratory as model compounds during this investigation, were examined by mass spectroscopy. In all cases, the mass spectra of these compounds showed successive losses of the elements of ketene, with no evidence for the loss of the elements of acetic acid. In the same way, the mass spectra of daunomycin tetraacetate, a naphthacene quinone with four nuclear aromatic acetate groups, showed the successive loss of four ketene groups from the molecular ion: m/e 594, m/e 552 ($M-C_2H_2O$), m/e 510 ($M-2xC_2H_2O$), m/e 468 ($M-3xC_2H_2O$), and m/e 426 ($M-4xC_2H_2O$) (Arcamone et al., 1968).

The loss of ketene from aryl acetates is stated to most likely occur through a four-centre intermediate, e.g. (7) rather than a six-centre one, e.g. (8) (Shapiro and Tomer, 1969; Thomas, 1970). In the case of acetates of monocyclic aliphatic alcohols, by far the most dominant process is 1,2-elimination of acetic acid. Similarly, 1,4-diacetoxy-cyclohexane shows an intense M-120 peak due to a double 1,2-elimination of acetic acid (Budzikiewicz et al., p.469, 1967).

Compounds with two adjacent aliphatic acetoxy groups, one of which is a benzylic acetate group, can lose acetic acid (m/e 60) followed by a loss of m/e 59, or, they can lose acetic acid followed by loss of ketene, e.g. some diacetoxy derivatives

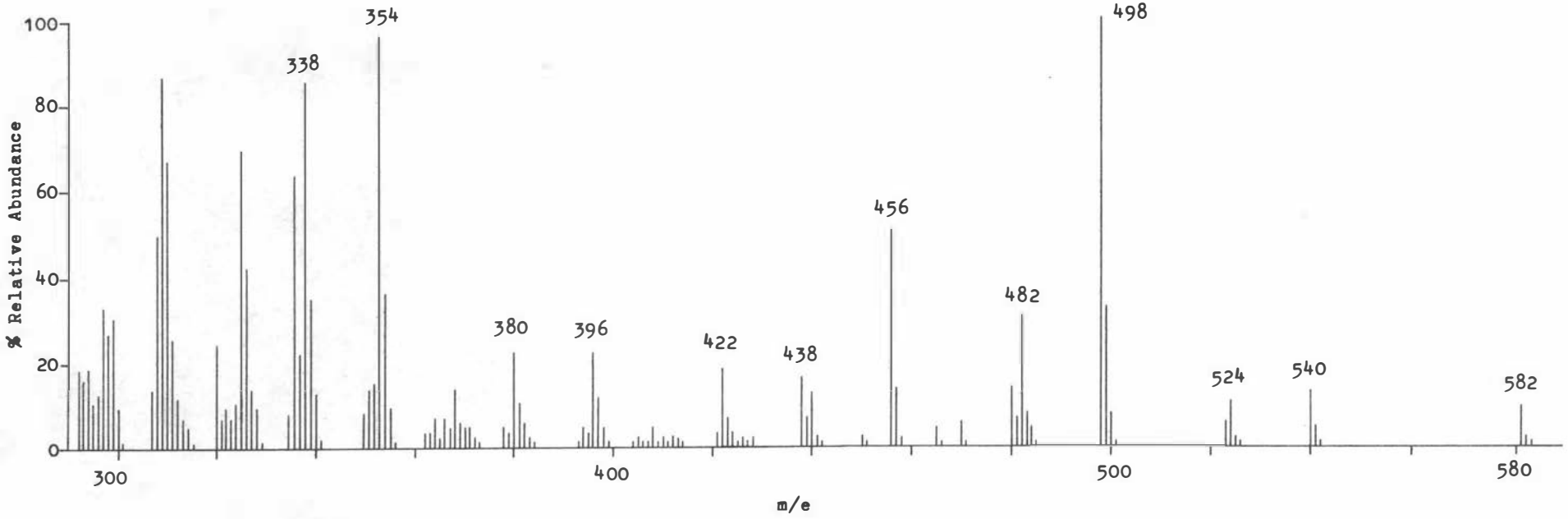
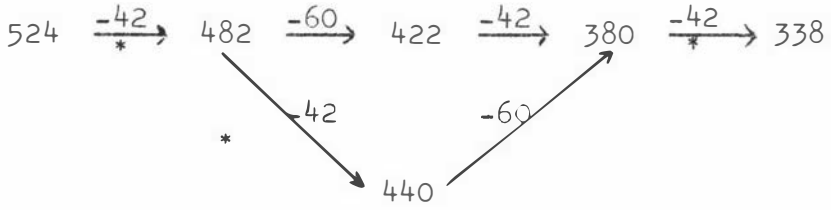


Fig. 6

Mass Spectral Line Diagram of Acetylation Product of Red Pigment

of flavan-3,4-diols show this behaviour (Drewes, 1968).

The scheme for the fragmentation of dothistromin penta-acetate can thus be seen to be consistent with this derivative having three aromatic acetate groups and two aliphatic acetate groups. In the same way, the fragmentation paths of deoxy-dothistromin tetra-acetate indicated that this derivative could have two (or three) aromatic acetate groups and two (or one) aliphatic acetate groups:



fragmentation of deoxydothistromin tetra-acetate

Having demonstrated the presence of dothistromin penta-acetate and deoxydothistromin tetra-acetate in the crude acetylation product, attempts were made to separate the acetates by prep. TLC. Using silica gel G and benzene : acetic acid (100:9), the crude yellow gum was separated into a bright yellow-orange coloured band of high Rf (Rf ~0.70) and a very pale yellow coloured band (appearing as a dark absorbing band under UV irradiation, λ 350 nm) of low Rf (Rf ~0.30). Close examination revealed in fact that both of these bands were partially resolved "doublets", i.e. they both consisted of two very closely running bands. The compounds giving rise to the bands were unstable on dry TLC plates, darkening in colour if allowed to remain on the dry plates for any appreciable length of time.

The yellow coloured high Rf material was examined by mass spectroscopy and appeared to give a molecular ion at m/e 498 corresponding to dothistromin triacetate, and also a molecular ion at m/e 482 corresponding to deoxydothistromin triacetate.

The pale yellow coloured low Rf bands proved to be the major products. The upper of the two close bands was present in greater amount. Mass spectral analysis of the compound from this band showed that it was in fact dothistromin penta-acetate. Crystallisation of the penta-acetate from $CHCl_3$/hexane gave pale yellow needles, m.p. 196-198°C, and mass spectral examination of this crystalline material revealed pure dothistromin penta-acetate, free from contamination by deoxydothistromin tetra-acetate, see Fig. 7. Thus a complete separation of dothistromin from deoxydothistromin had been achieved.

The lower of the bands at Rf 0.30 on TLC appeared to be a mixture of deoxydothistromin tetra-acetate (MW 524), dothistromin tetra-acetate, and dothistromin penta-acetate. Crystalline material could not be coaxed to form from this mixture, nor could the components be resolved by TLC.

In order to circumvent undue delay in achieving the goal of this thesis, the tetra-acetates and tri-acetates were not further investigated. Instead, effort was concentrated on examination of the crystalline dothistromin penta-acetate.

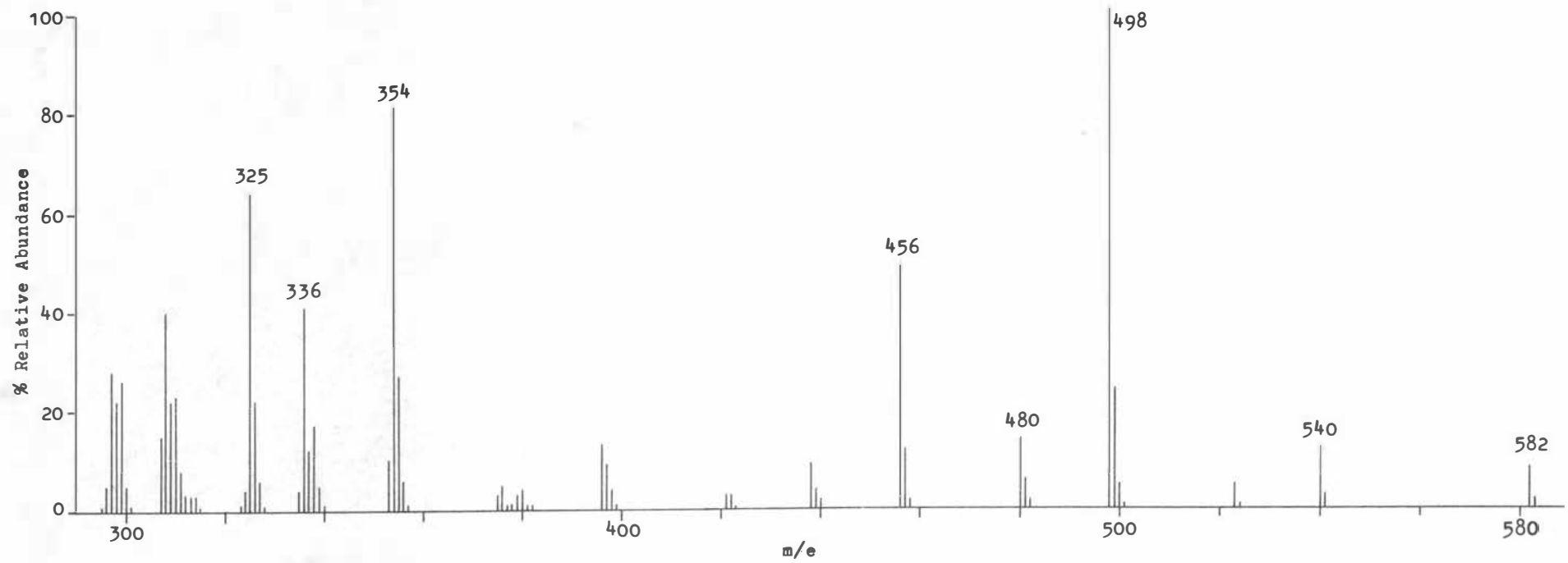


Fig. 7

Mass Spectral Line Diagram of Dothistromin Penta-acetate

CHAPTER 6. THE STRUCTURE OF DOTHISTROMIN PENTA-ACETATE

As discussed in the previous section, the mass spectrum of the pale yellow crystalline dothistromin penta-acetate, m.p. 196-198°, was consistent with the presence of three aromatic and two aliphatic acetate groups. The absorption spectrum (in ethanol) of this penta-acetate showed absorption consistent with it being an alkoxy substituted tri-α-acetoxyanthraquinone, the evidence for this being discussed in some detail on pages 58 to 61.

Additional evidence for the presence of aromatic and aliphatic acetate groups was given by the infrared spectrum (in $CHCl_3$), which showed a broad high-intensity ester absorption peak in the region 1740-1790 cm^{-1}, centred at 1770 cm^{-1}, due to absorption by aromatic and aliphatic acetate carbonyls. In addition, there was a sharp carbonyl band at 1680 cm^{-1}, assigned to the quinone carbonyls, and peaks at 1620 cm^{-1} and 1590 cm^{-1}, assigned to aromatic double bonds.

Further study by mass spectroscopy, using the metastable defocusing technique (see Appendix), indicated a one step loss of the elements of acetic anhydride, $C_4H_6O_3$ (m/e 102), from the molecular ion and also from a number of fragment ions

e.g. $M^{+\cdot} \xrightarrow[*]{-C_4H_6O_3} m^{+\cdot}$

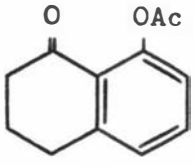
m/e	582	102	480

Such a loss is consistent with an o-acetoxybenzyl acetate type of structure. Thus Lucidin triacetate (9) shows this one step loss. That polynuclear acetoxy aromatics and anthraquinones do not exhibit this loss was shown by mass spectral examination of

the acetoxy compounds (10)-(18) described on page 56, the only important fragmentations in the mass spectra being the successive losses of the elements of ketene. Thus the m/e 102 loss observed for dothistromin penta-acetate is consistent with the presence in this derivative of a benzylic acetate group substituted ortho to a nuclear acetoxy group.

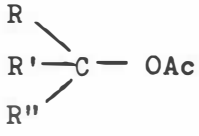
The NMR spectrum of dothistromin penta-acetate (Fig. 8) was very informative. Thus signals (in $CDCl_3$, ppm from TMS) were observed for three aromatic protons at δ7.58 (1H), δ7.42 (2H), five acetate groups at δ2.45 (6H), δ2.42 (3H), δ2.00 (3H) and δ1.67 (3H), and "multiplets" at δ6.5 (2H) and δ2.82 (2H). Apart from confirming that the derivative was a penta-acetate, the spectrum also showed that there were two identical non-coupled aromatic protons and one other aromatic proton.

The chemical shifts of the acetate groups were most interesting, as clearly three of the acetate groups were in very similar environments, the other two being quite different, the group at δ1.67 ppm in fact appearing at remarkably high field position. An extensive literature search revealed that of several hundred chemical shift values located for various aromatic acetate groups, those of type I occurred at an average shift value of δ2.45 ppm, all falling inside the limits δ2.52-δ2.37 ppm,



I

II



III

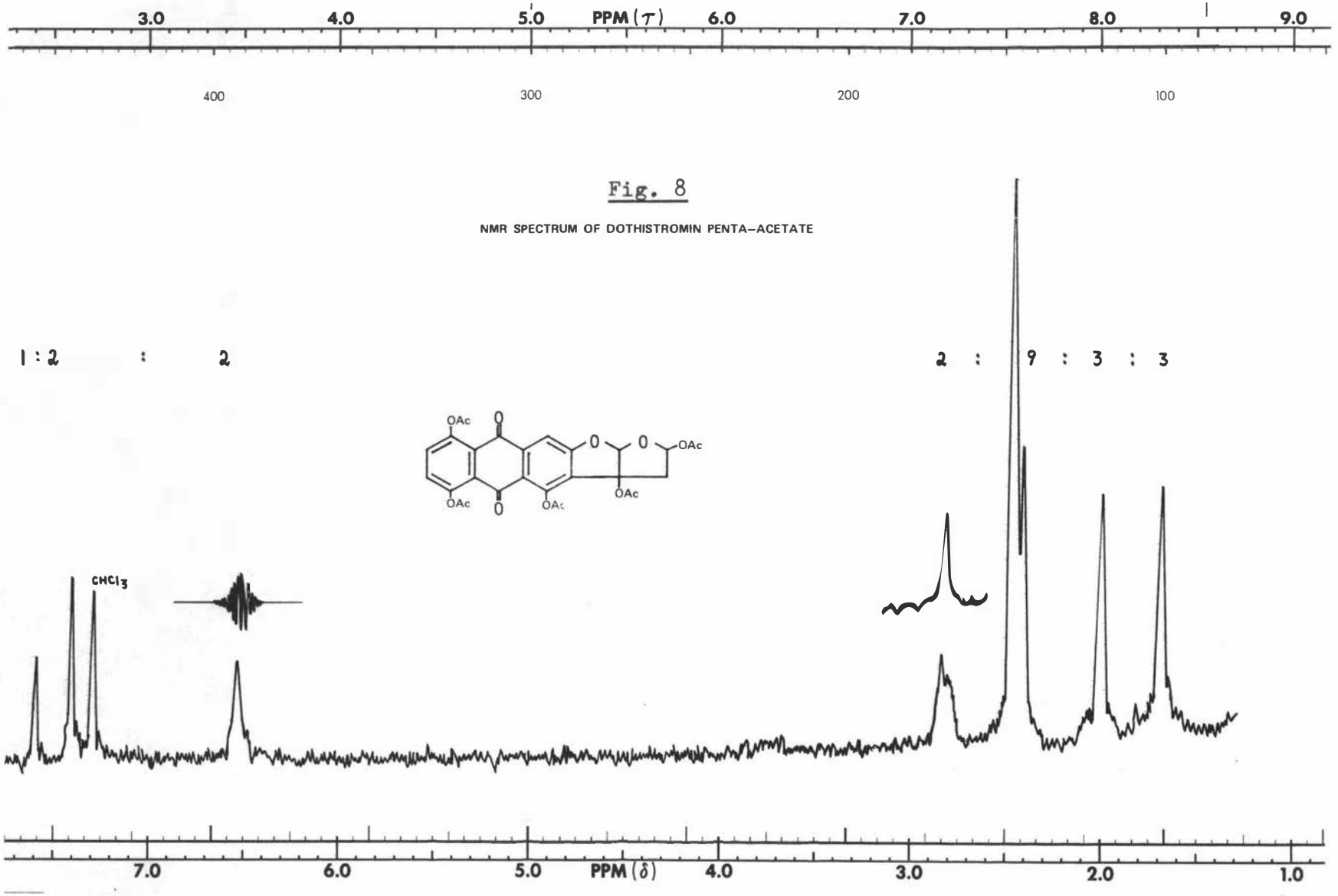


Fig. 8

NMR SPECTRUM OF DOTHISTROMIN PENTA–ACETATE

most of them well inside these limits. For α-acetoxy-anthraquinones, the values were consistently near δ2.45 ppm. On the other hand, aromatic acetates of type II appeared over a very much wider range of shift values, being more dependent on ring substituents than α-acetoxyanthraquinones. However, a typical value of around δ2.3 ppm was often noted, with many acetate shifts in this group falling in the range δ2.3-δ2.4 ppm, a few occasionally appearing as high upfield as δ1.9 ppm. Acetoxyanthraquinones with β-acetate groups typically had acetate shifts in this range, as did acetate groups attached to unsaturated bonds not necessarily part of an aromatic system. Aliphatic acetates of type III, appeared typically in the range δ2.0-δ1.9 ppm.

The chemical shift values of the acetate groups of a number of aryl acetates (10), (11), (12) and acetoxyanthraquinones (13)-(18), prepared in the laboratory, were determined, and are recorded in Table VIII. They all occurred well within the appropriate ranges discussed above.

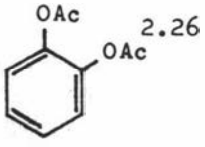
From these results it is clear that the NMR chemical shift values of acetate groups can be of considerable diagnotic value.

The NMR data for three of the acetate groups and for the aromatic protons of dothistromin penta-acetate was consistent with the partial structure (19) for dothistromin penta-acetate, there being excellent agreement for the shift values of the α-acetate groups and the two equivalent aromatic protons with the corresponding shift values for 1,2,5,8-tetra-acetoxyanthraquinone (20), one of the reference compounds prepared during the course of this thesis. The benzylic (i.e. aliphatic type)

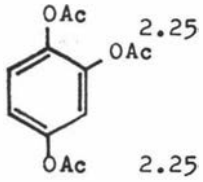
Table VIII

Chemical Shifts of Acetate Groups of Some Aryl Acetates and Acetoxyanthraquinones, in $CDCl_3$

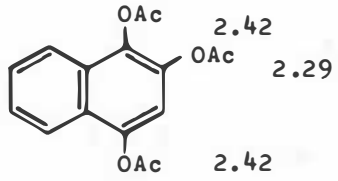
(δ values, ppm from TMS)



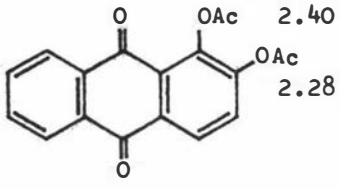
(10)



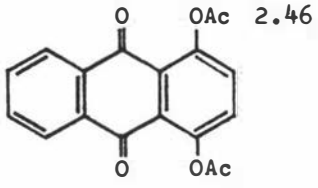
(11)



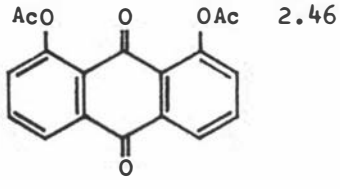
(12)



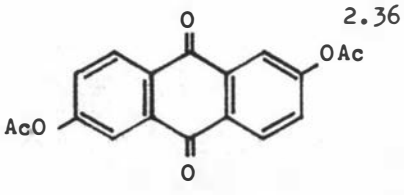
(13)



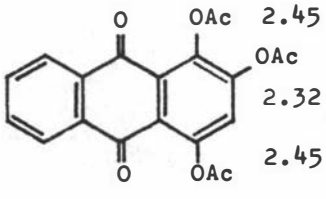
(14)



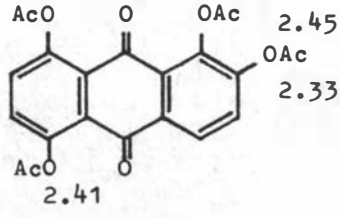
(15)



(16)



(17)



(18)

acetate group in the partial structure (19) would account for the acetate signal at δ2.00 ppm in the spectrum of dothistromin penta-acetate.

The acetate signal at δ1.67 ppm in the spectrum of the penta-acetate appeared to indicate shielding of an acetate group by an aromatic ring. Such shielding has been observed (Hillis and Horn, 1965) in some C-glycosyl flavonoid acetates, e.g. 2",3",4",6"-tetra-O-acetyl-ß-D-glucopyranozyl benzene (21), in which the 2"-acetyl group appears at δ1.78 ppm, and vitexin tetra-acetate (22) in which the 2"-acetyl group appears at δ1.67 ppm. It was proposed that the 2"-acetyl group in (21) prevents coplanarity of the aromatic and pyranosyl rings, so that the molecule exists chiefly in two identical conformations with the planes of the two rings roughly perpendicular to each other, and with the 2"-acetyl group over the plane of the phenyl ring in the diamagnetic region of its field.

With the above shielding phenomena being held as a requirement for any structure proposed for dothistromin penta-acetate, possible structures for the penta-acetate based on partial structure (19) were examined with the aid of Dreiding models. Only the structures (23) and (24) were found to satisfy the above criteria and all the other facts observed on dothistromin penta-acetate.

The presence of two acetal protons (H'a', H'd', see (25)) in these structures would account for the 2-proton "multiplet" in the NMR spectrum at δ6.5 ppm, whilst the methylene group would account nicely for the 2-proton multiplet at δ2.82 ppm. The technique of spin decoupling was used to show that one of

the superimposed proton signals at δ6.5 ppm (H'a') was coupled to the methylene signal at δ2.82 ppm, and that the other (H'd') was in fact a sharp uncoupled singlet.

The "angular" isomer structure (24) for the penta-acetate was ruled out on the basis of the following evidence: (i) the NMR chemical shift values of β-acetate groups in acetoxyanthraquinones are normally in the range δ2.3-δ2.4 ppm, whereas α-acetate groups in acetoxyanthraquinones occur consistently lower downfield, nearer δ2.45 ppm; (ii) the chemical shift value δ7.58 ppm for the single aromatic ring proton in the penta-acetate is in excellent agreement with that (δ7.58 ppm) for 1-O-acetyl-3-O-methyl rubiadin (30) (see page 61). The corresponding proton in (24) would be expected to occur at a considerably different position, as it does (δ7.88 ppm) for 3-O-acetyl-1-O-methyl rubiadin (29) (see page 61); (iii) the electronic absorption spectra of dothistromin penta-acetate favoured structure (23) and not (24).

It is well established (Astill and Roberts, 1953, and refces. therein; Pusey and Roberts, 1963) that the UV absorption spectrum of a fully acetylated hydroxyquinone resembles that of the parent unsubstituted quinone. Similarly, Jurd (1962) in a discussion on the spectral properties of flavonoid compounds, states that acetylation of a phenolic hydroxyl group effectively nullifies its effect on the absorption, and the spectrum of a fully acetylated polyhydroxyflavone or flavonol, is, therefore, similar to that of flavone itself, e.g. quercetin penta-acetate (λ max 300, 252 nm), flavone (λ max 297, 250 nm). In the same way, the acetate of a partially methylated or glycosidated

polyhydroxyflavone or flavonol has a spectrum which resembles that of the parent methoxyflavone. Similar results apply to aurones and chalcones.

It has been shown (Brockmann and Müller, 1959; Kuntsmann and Mitscher, 1966) that polyacetoxyanthraquinones possess visible spectra remarkably like that of anthraquinone itself, the main difference being a shift of the visible maxima to higher wavelength in the acetylated derivatives. This was amply confirmed by examination of the spectra of the acetoxyanthraquinones (13)-(17) prepared in the laboratory as part of this investigation. The spectral data, recorded in Table IX, shows that the shift in the near-visible maxima of 326 nm of anthraquinone, is approx. 6-7 nm per α-acetate group present, the presence of β-acetate groups having no significant effect. By comparison, the near-visible band maxima at 348 nm for dothistromin penta-acetate would correspond fairly well to a tri-α-acetoxyanthraquinone (326+3x7 = 347 nm). However, the low wavelength region 225-300 nm is considerably different from that of anthraquinone. It does in fact resemble that of 2-methoxyanthraquinone (Morton and Earlam, 1941; Pusey and Roberts, 1963), rather than that of anthraquinone itself. To further define the structure from the absorption spectra, the model compounds (26) and (27) were desired, but were unavailable. Their synthesis was not attempted, as the naturally occurring anthraquinone, rubiadin-1 methyl ether (28) (see Thomson, 1957, p.166) was readily accessible by extraction of the bark of Coprosma Australis (Briggs and Dacre, 1948), and its conversion to the acetates (29) and (30) was a moderately easy task.

Table IX

Electronic Absorption Spectra of Some Acetoxyanthraquinones

	Acetoxyanthraquinone	λmax (nm) (in EtOH)			Δλ vis. (nm)	-OAc Groups α-	β-
	anthraquinone	254	270(sh)	326	-	-	-
(13)	1,2-di-OAc	254	274(sh)	332	6	1	1
(14)	1,4-di-OAc	252	270(sh)	339	13	2	0
(15)	1,8-di-OAc	252	270(sh)	339	13	2	0
(16)	2,6-di-OAc	258	276(sh)	326	0	0	2
(18)	1,2,5,8-tetra-OAc	254	270(sh)	344	18	3	1
	1,2,4,5,7-penta-OAc‡ (in $CHCl_3$)	259	279(sh)	347	21	3	2
	dothistromin penta-acetate	238, 272, 280, 348					

‡ spectrum of this compound obtained from Powell *et al.* (1967)

Thus the bark of C. Australis was extracted with acetone to give rubiadin-1 methyl ether (28); acetylation with acetic anhydride/pyridine gave the desired acetate (29). Rubiadin (31) was obtained from (28) by hydrolysis with conc. H_2SO_4 at elevated temperature in a sealed tube. Methylation of (31) with dimethyl sulphate/an. potassium carbonate in refluxing acetone gave a mixture of rubiadin-3 methyl ether (32), and some dimethyl ether (33). Column chromatography allowed separation of pure (32), which was acetylated to give the desired acetate (30).

The absorption spectra of acetates (29) and (30) were determined (in ethanol) and compared with that of dothistromin penta-acetate - see Fig. 9. As can be seen from the spectra, the low wavelength regions (225-300 nm) of compounds (29) and (30) are distinctly different, as expected, and dothistromin penta-acetate is quite similar to that of (30) in this region. Further, the near-visible band at 335 nm in the spectrum of (30) would be expected to shift upfield to approx. (335 + 12 to 14) nm = approx. 347 to 349 nm upon introduction of two further α-acetate groups (giving compound (26)). The corresponding band in dothistromin penta-acetate occurs at 348 nm, and thus there can be little doubt from this spectral evidence that dothistromin penta-acetate does indeed have the linear structure (23).

The proposed structure (23) possesses three asymmetric centres, and it was anticipated that provided racemisation of the molecule had not occurred during the isolation or acetylation procedure the penta-acetate would exhibit optical activity. The derivative was in fact found to be strongly laevorotatory, with $[\alpha]_D$-142° ($CHCl_3$). It was expected that the difuro rings of

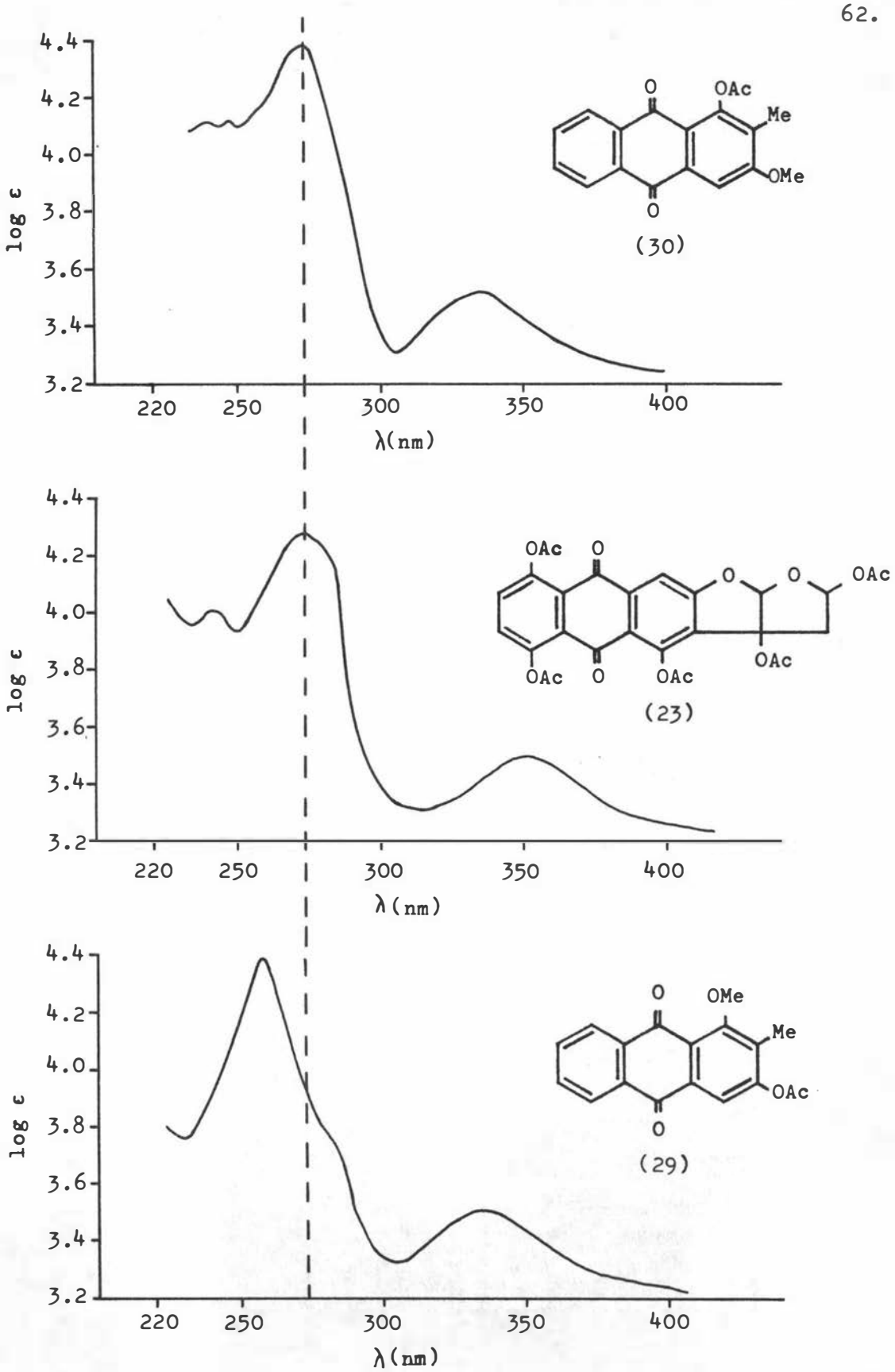


Fig. 9

Electronic Absorption Spectra of Dothistromin Penta-acetate and Model Compounds (29) and (30)

(23) would be in the cis-fused rather than the considerably more strained and energetically unfavourable trans-fused configuration.

A literature search for compounds reported to possess tetrahydrofuro-, dihydrofuro-, or furo-[2,3-b] benzofuran systems revealed several other naturally occurring compounds with this structural feature. These were the sterigmatocystins, the aflatoxins, aversin, and the versicolorins. Because of the known high toxicity and potent carcinogenicity of some of these compounds, and their structural relationship to dothistromin, they are of especial importance in this study and are discussed in the appendix to this thesis, under the title "Sterigmatocystin, the Aflatoxins, Aversin and Versicolorin". Most of the published chemistry of these fungal metabolites is very new, with the first structure being reported in 1962, and a major portion of the papers appear from 1968 onwards.

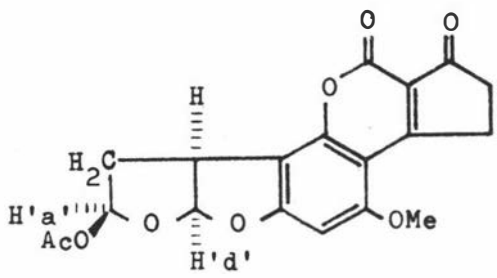
Some NMR data which appeared in the literature recently on two epimeric acetoxy aflatoxin B_1 derivatives (34) and (35), and two synthetic epimeric acetoxy tetrahydrofuro[2,3-b] benzofurans (36) and (37), is shown in Table X. The corresponding data are also shown for dothistromin penta-acetate (23). It can be seen that the chemical shift of the acetate group in the aflatoxin B_1 acetate (34) and the synthetic acetate (36) is in excellent agreement for the corresponding acetate group (δ1.67 ppm) in dothistromin penta-acetate (23), these "endo" acetate groups showing long-range shielding by the magnetic anisotropy of the aromatic ring. In the acetates (35) and (37), the "exo" acetate groups are not so shielded, and appear at the expected position near δ2.0-δ2.1 ppm. The NMR data for the acetal protons and

Table X

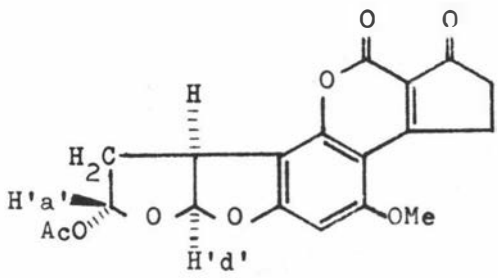
NMR Data for

2-acetoxy-tetrahydrofuro[2,3-b]benzofurans

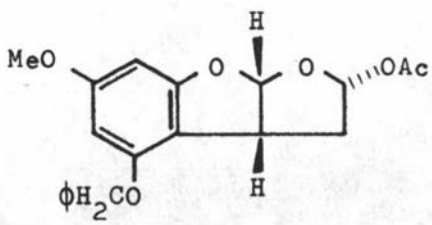
Compound	-OAc	H'a'	H'd'	$-CH_2$	Refce
(34)	1.68 ( )	6.38	6.52	2.45	Dutton & Heathcote (1968)
(34)	1.74 ( )	6.46	6.55	2.50	Wiley et al. (1969)
(35)	2.12 ( )	6.37	6.55	2.50	" " " "
(36)	1.66 ( )	6.35	6.35	2.50	Büchi & Weinreb (1971)
(37)	2.02 ( )	6.35	6.35	2.45	" " "
(23)	1.67	6.5	6.5	2.8	



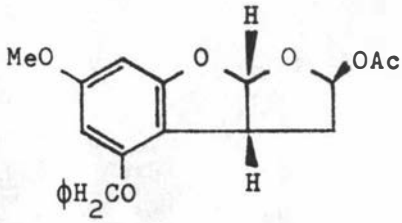
(34)



(35)



(36)



(37)

methylene protons also show excellent agreement, giving further substantial confirmation for the structure (23) for dothistromin penta-acetate.

CHAPTER 7. THE STRUCTURE OF DOTHISTROMIN

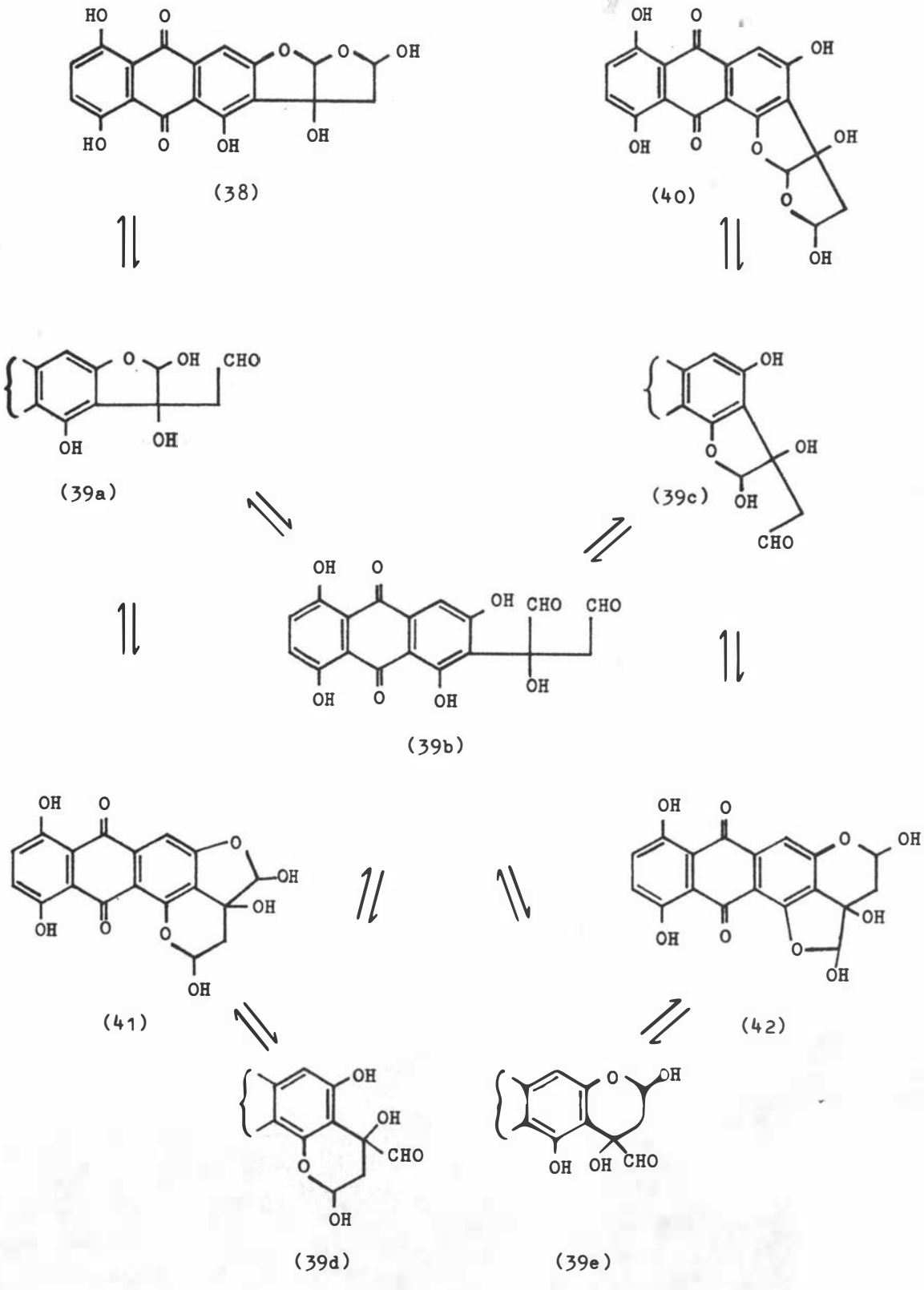
With the structure of dothistromin penta-acetate (23) established, it appeared logical to propose the pentacyclic hemiacetal structure (38) for dothistromin.

Dothistromin Equilibrium Mixture in Solution

In solution it is possible that (38) would be in equilibrium with the tricyclic open-chain dialdehyde (39b), the pentacyclic hemiacetal (40), the tetracyclic aldehydes (39a), (39c), (39d), and (39e), and the pentacyclic bishemiacetals (41) and (42), as shown in scheme 1. The situation with these isomers is somewhat analogous to that of the aldoses which exist in solution as an equilibrium mixture of the α- and β-pyranose forms, the α- and β-furanose forms, and an open chain aldehyde form.

Evidence for the existence of the acyclic aldehyde form of aldoses includes chemical reactions, polarography, and UV absorption spectroscopy; in most cases the proportion of the acyclic form is extremely small, $< 0.1\%$ (Rodd, 1967; Pigman and Isbell, 1968; Angyal, 1969). Similarly, simple γ- and δ-hydroxy aldehydes exist predominantly as cyclic hemiacetals (Fuson, 1962).

The only recorded examples of acyclic forms of hydroxy-tetrahydrofuro[2,3-b]benzofuran systems are the hemiacetal derivatives of aflatoxin and sterigmatocystin, which under alkaline conditions give rise to salts, e.g. (43), (44) (Büchi *et al.*, 1967; Pohland *et al.*, 1968; Rance and Roberts, 1970). The charge on the phenoxide anions, being conjugated with carbonyl groups, accounts for a pronounced bathochromic shift observed in



Scheme 1

Dothistromin Equilibrium Mixture in Solution

the UV absorption spectra of the ionised derivatives.

The problem of determining the composition of the equilibrium mixture of dothistromin in solution (e.g. ethyl acetate culture extracts or solvent solutions of purified dothistromin), is a complex one indeed. This can be appreciated when it is realised that it is only recently (mid 60's) that the composition of the equilibrium mixture of a sugar in solution was determined (Angyal, 1969). The various structures shown in scheme 1 involve a number of different substitution patterns in the pentasubstituted aromatic ring, and thus it would appear possible to differentiate between these by absorption spectroscopy (UV, IR, NMR).

(1) UV/visible absorption spectra: Comparison of the absorption spectrum of dothistromin solutions with suitable model compounds such as (45), (46), and (47), could allow distinction between (38) (similar to (45)), (40) (similar to (46)), and (41) and (42) (both similar to (47)).‡ Since the differences in the absorption spectra would be fairly small, a pure sample of dothistromin free of contamination by deoxydothistromin would appear desirable. Unfortunately, none of the model compounds (45), (46) and (47) are readily available, and the synthesis of each of these is somewhat difficult,

‡ The addition of base such as aqu. sodium carbonate to ethanolic solutions of (45), (46) and (47) would allow easy distinction of (46) from (45) and (47) due to the bathochromic shift which would occur in the spectrum of (46) (but not of (45) or (47)) because of ionisation of the acidic β-hydroxyl group to give the phenoxide ion. However, the same test could not be applied to dothistromin since each of the forms (38), (40), (41) and (42) would be in equilibrium with a small amount of a form with a β-hydroxyl group, and addition of the base would shift the equilibrium to a common stable phenoxide ion species.

especially for (46) and (47) which require the use of selective protecting groups.

(2) Infrared absorption spectra: The vibration of the two carbonyl groups in a quinone appear not to be closely coupled, and whilst anthraquinone itself and symmetrically substituted derivatives give a single carbonyl stretching frequency absorption, certain unsymmetrically substituted quinones give two carbonyl bands. The effect is very marked with amino- and hydroxy-derivatives. Indeed, it is well known (Flett, 1948; Tanaka, 1958; Bloom et al., 1959; Bellamy, 1966 and 1968), that the presence of α-hydroxyl groups in an anthraquinone has a profound effect on the carbonyl stretching absorption, an intense C=O band appearing at much lower frequency than normal. Correlations have been made between the number of α-hydroxyl groups and the carbonyl absorption frequencies. Thus Bloom et al. (loc. cit.) examined the spectra of 59 anthraquinone derivatives and arrived at the grouping scheme shown in Table XI.

Table XI

IR Carbonyl Frequencies of Anthraquinones with α-Hydroxyl Groups

α-Hydroxyl Groups	>C=O, cm^{-1}
No α-OH	1678-1653
1-OH	1675-1647 and 1637-1621
1,4- and 1,5-$(OH)_2$	1645-1608
1,8-$(OH)_2$	1678-1661 and 1626-1616
1,4,5-$(OH)_3$	1616-1592
1,4,5,8-$(OH)_4$	1592-1572

The α-hydroxyl groups are very strongly hydrogen bonded to their adjacent carbonyl groups, and the carbonyl frequency shift is attributed to a "conjugated-chelation effect", in which the donor-acceptor properties of the chelating centres are increased by resonance.

Another extensive infrared correlation with 80 different anthraquinones was made by Tanaka (loc. cit.), using solutions in dioxane as well as solid state nujol mulls as used by Bloom et al. Tanaka discussed separately the effects of substitution on both the free and the chelated carbonyl frequencies. Unfortunately, the presence of methoxy groups complicates the correlations, and introduces a number of anomalies.

As the compounds examined in the above mentioned studies do not include substitution patterns similar enough to be useful as model compounds for (38)-(42) in the proposed dothistromin equilibrium mixture, it would be necessary to prepare suitable model compounds before conclusions could be made from examination of the infrared spectrum of a solution or mull of dothistromin. Even if this were done, the results may not be conclusive.

(3) NMR absorption spectra: The NMR absorption spectra of (38), (40), (41) and (42) would be expected to show considerable similarities, since all four structures have two acetal protons, one of which is coupled to a methylene group, the other uncoupled; they all have a benzylic hydroxyl group, and the aromatic ring substitutions are similar.

The α-hydroxyl groups in the anthraquinone nucleus are strongly intramolecularly hydrogen bonded, and should give rise to sharp signals at approx. δ11-δ14 ppm from TMS (Moore and Scheuer, 1966; Kuntsmann and Mitscher, 1966) - c.f. intramolecularly hydrogen bonded phenols, which show signals in the range δ10.5-δ16 ppm (Jackman and Sternhell, 1969); thus it should be possible to distinguish (38) from (40), (41) and (42) by examination of the signals in this area of the spectrum. If integration corresponds to 3 protons, this would indicate negligible contributions due to structures (40), (41) and (42); an intermediate value between 2 and 3 protons would give an indication of the relative contribution of form (38), whilst a value of 2 protons for the integration would indicate negligible contribution from form (38). This investigation was not carried out on a quantitative basis because of the unavailability of dothistromin of sufficient purity.

Structural Evidence from Chemical Reactions

From the above discussion it can be seen that it would be very difficult to determine the composition of the dothistromin equilibrium mixture in solution. [This same problem has not been encountered with any of the related naturally occurring furo[2,3-b] benzofuran compounds reported to date, since none of these have had a second free alternative phenolic OH group present at which ring closure could also occur].

Dothistromin reacts chemically as if it had structure (38); i.e. under acid catalysed acetylation conditions it gives the

penta-acetate (23), and acid-catalysed reaction with alcohols gives the mono-alkyl ether derivatives (48), (49), (50), which can be further acylated or methylated to polyacetoxy and polymethoxy derivatives (see below). Despite the fact that α-hydroxyl groups in hydroxyanthraquinones are very strongly hydrogen bonded (chelated) with the adjacent carbonyl group, and that β-hydroxyl groups are chemically more reactive than α-hydroxyl groups in hydroxyanthraquinones (i.e. more acidic, more easily acetylated and alkylated) and therefore that there should be faster ring shutting for β-hydroxyl groups than α-hydroxyl groups, it cannot be concluded from this that dothistromin must have structure (38), because the system is an equilibrium system. However, for a number of chemical reactions, the effective structure of dothistromin can be regarded as (38).

Formation of Ether Derivatives of Dothistromin - Mono-alkylation and Mono-acetylation of the Hemiacetal Group

Acid catalysed etherification of the red pigment with methanol, ethanol and n-butanol readily afforded the expected mono-methyl, -ethyl and -n-butyl ethers, respectively. The derivatives were purified by prep. TLC with silica gel G. The following properties of these derivatives showed that structures (48), (49), and (50) could be assigned to them:

(i) the derivatives had higher R_f values on TLC (silica gel G) than the red pigment (dothistromin), suggesting formation of a less polar derivative;

(ii) the derivatives could not be extracted from solution in diethyl ether by aqu. Na_2CO_3, but were extracted with

aqu. NaOH, showing that no β-hydroxyl groups were present in the anthraquinone nucleus, or could be formed by the action of aqu. Na_2CO_3;

(iii) the visible absorption spectra in ethyl acetate were superimposable on that of the starting material, showing that the chromophoric moiety had not been altered;

(iv) the mass spectra were consistent with the conversions (38) → (48), (38) → (49), and (38) → (50) having occurred (see below);

(v) acetylation of the derivatives with acetic anhydride/pyridine afforded the corresponding tetra-acetates (51), (52), and (53). The ethyl ether tetra-acetate (52) was obtained as a pure, crystalline compound, and detailed investigation of this compound (see page 79) confirmed its structure as (52), and thus structure (49) for the precursor compound.

In a similar manner, acid-catalysed acetylation using acetic acid as the acylating reagent, gave the mono-acetate (54), its structure following from similar considerations to those applied to the mono-ethers described above.

Analogous reactions are well known in sugar chemistry, and have also been recorded for aflatoxins B_{2a} and G_{2a} (Pohland *et al.*, 1968; Dutton and Heathcote, 1968 and 1969), with synthetic aflatoxin B_1 hemiacetal (Büchi *et al.*, 1967), and with a synthetic hemiacetal furo[2,3-b] benzofuran (Büchi and Weinreb, 1971).

Mass Spectroscopy of Dothistromin and its Mono-alkyl Ether Derivatives. Loss of the Formyl Radical $C\dot{H}O$ (m/e 29) and the Fragment C_2H_4O (m/e 44)

With the availability of the mono-alkyl ether derivatives (48), (49), and (50), and the mono-acetate (54), of dothistromin, it was possible to propose fragmentation mechanisms based on structure (38).

As discussed earlier (page 12), two significant features of the mass spectrum of dothistromin are the loss of a formyl radical $C\dot{H}O$ (m/e 29) from the molecular ion (and from the molecular ion after it had lost the elements of water), and the loss of the neutral fragment C_2H_4O (m/e 44).

A number of substituted anthraquinones and oxygenated aromatic compounds have been shown to lose the formyl radical in the mass spectrometer. Thus, the successive losses of $C\dot{H}O$ and/or CO, have been shown by hydroxyanthraquinones (Beynon, 1960; Ritchie et al., 1964) and alkoxyanthraquinones (Bowie et al., 1970). Significant losses of $C\dot{H}O$ have been recorded in the spectra of some quinones known as the Tanshinones (Hayashi et al., 1970). Comantherin, a condensed aromatic pyrone, has been shown to lose $C\dot{H}O$ from the molecular ion to give the base peak in its spectrum (Kent et al., 1970), various derivatives of comantherin also showing intense peaks in their spectra due to the loss of $C\dot{H}O$. Benzofurans often give M-$C\dot{H}O$ peaks (see Budzikiewicz et al., 1967, p.622; Reed and Reid, 1963), and elimination of the formyl radical from the molecular ion gives the base peak in the spectrum of furan. Simple aromatic species can also lose the formyl radical, e.g. diaryl ethers (Budzikiewicz et al., 1967, p.250),

m-dimethoxybenzene, m-methoxy phenol (Barnes and Occolowitz, 1963; Thomas, 1970) and resorcinol and phenol (Aczel and Lumpkin, 1960).

The model anthraquinone compounds (28) and (32) prepared during the course of this thesis, were examined by mass spectroscopy and both compounds gave spectra exhibiting strong loss of the formyl radical from the molecular ion.

From the above examples it can be seen that dothistromin possesses several structural features which are known to be conducive to the loss of the formyl radical in the mass spectrometer.

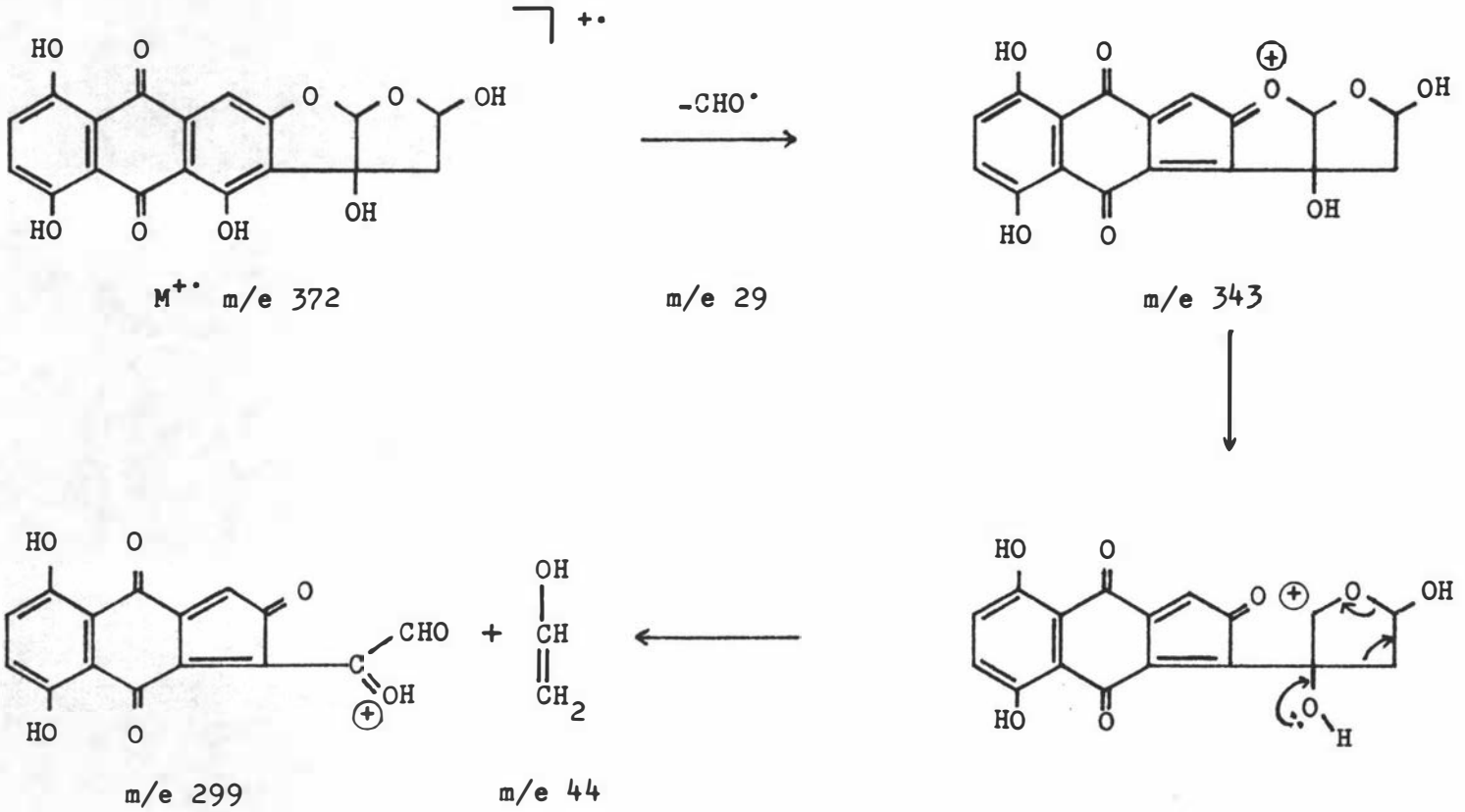
Thomas (1970) proposed the tentative structural formulae (56), (57) for the ions (m/e 95) resulting from the loss of the formyl radical from m-methoxy phenol (55). Since the formyl radical could come from either the hydroxy or the methoxy groups, and in order to account for the alternative modes of further fragmentation of the m/e 95 ion by loss of formaldehyde or water (both evidenced by metastable peaks), Thomas proposed that the formyl radical could be derived in part from each group to give the ions (56) and (57).

To check the feasibility of Thomas' proposal, the deuterated compound (58) was prepared in the laboratory by methylation of resorcinol with CD_3I. The mass spectrum of (58) was consistent with the formation of ions of type (56) (M-$\dot{C}HO$) and also of type (57) (M-$\dot{C}DO$).‡

‡ The most intense fragment ion in the mass spectra of (59), also isolated from methylation of resorcinol with CD_3I, was due to the loss of $\dot{C}DO$, and was consistent with the formation from m-dimethoxybenzene (60), of the stable quinonoid structure (61), as proposed by Barnes and Occolowitz (1963).

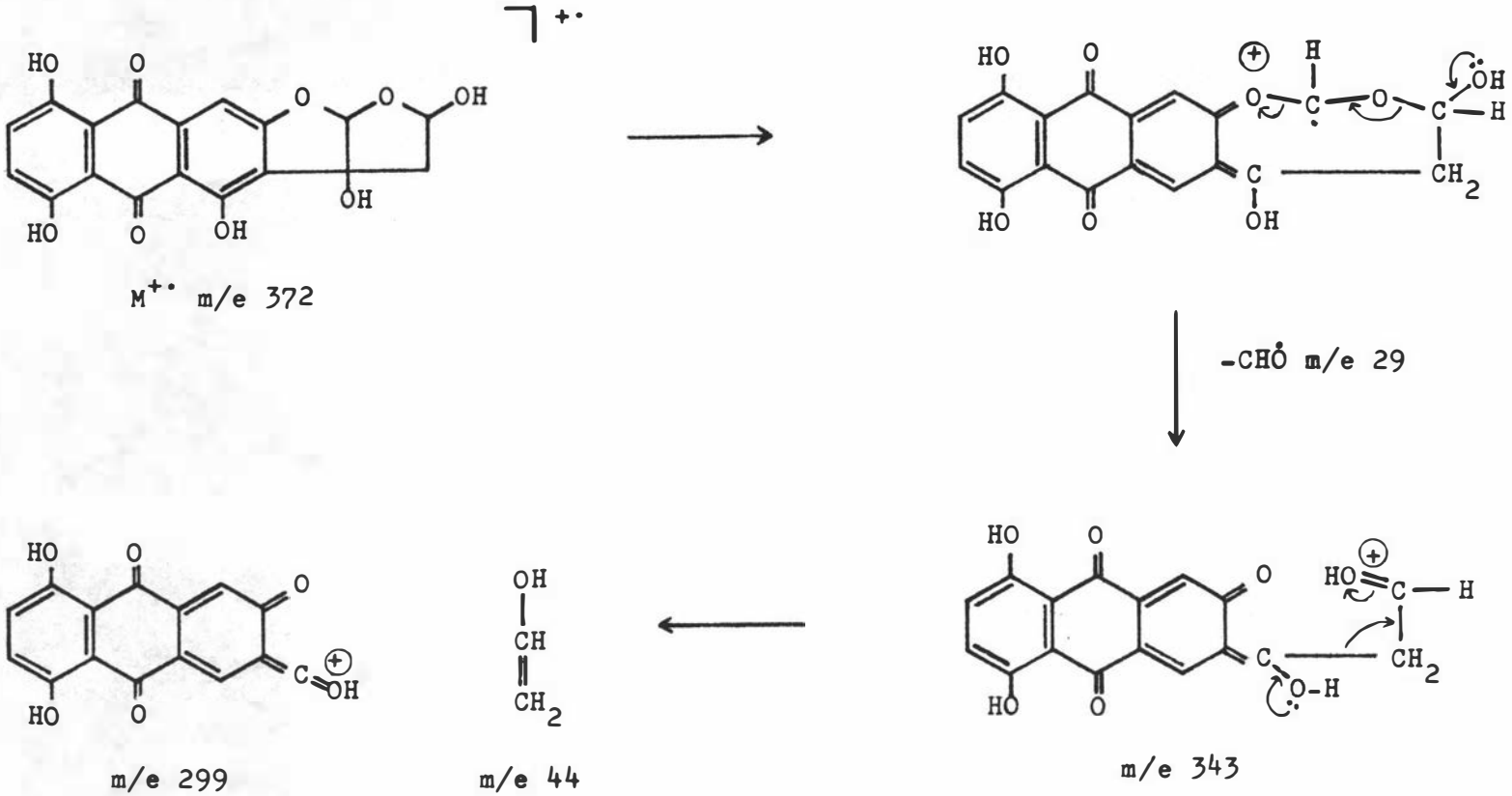
A possible scheme for the fragmentation of dothistromin based on the above results is shown in scheme 2, together with an alternative possibility, scheme 3. Any scheme for the fragmentation of dothistromin must not only explain the loss of the formyl radical $\dot{C}HO$, but also, the subsequent loss of the neutral fragment C_2H_4O (m/e 44); both schemes proposed do this. Evidence that the C_2H_4O fragment from the fragmentation of dothistromin was in fact derived from the hemiacetal carbon and the adjacent methylene carbon atoms was given by the mass spectra of the ether derivatives (48), (49) and (50). The spectra of these derivatives showed respectively, loss of neutral fragments (verified by metastable peaks) of m/e 58 (= 44 + 14), 72 (44 + 28) and 100 (44 + 56), instead of the fragment of m/e 44 as observed for dothistromin.

Very recently, some mass spectral data (no mechanisms or fragmentation schemes proposed) on the aflatoxins has been published (Haddon et al., 1971). This showed that the aflatoxins B_1, B_2, G_1, G_2, M_1, and aspertoxin, all give intense molecular ions, followed by sequential losses of $\dot{C}HO$ and CO. Whilst it is possible that in the case of these compounds fragmentation with loss of the formyl radical occurs from the unsaturated lactone portion of the molecule, this appears unlikely, in view of the fact that aflatoxin M_1 shows a striking increase in the abundance of the peak due to loss of $\dot{C}HO$, compared to aflatoxin G_1. The only difference between aflatoxin M_1 and G_1 is a benzylic hydroxyl group present in aflatoxin M_1, absent in aflatoxin G_1. It would be difficult to rationalise a fragmentation scheme in the light of this result, without involving the furan rings. On



Scheme 2

Mass Spectral Fragmentation of Dothistromin



Scheme 3

Alternative Mass Spectral Fragmentation of Dothistromin

the other hand, the aflatoxins do possess an aromatic ring with oxygen substitution meta- to a methoxyl group, and on the basis of the discussion above, this feature could give rise to the formyl radical loss.

To further investigate the mass spectral fragmentation of dothistromin, the model compounds (62), (63), (64) and (65) could be examined. These should be reasonably accessible compounds (see discussion on synthesis of dothistromin, page 102), since several closely related analogues have been recently synthesised (e.g. Büchi and Weinreb, 1971).

Dothistromin Ethyl Ether Tetra-acetate

As mentioned on page 73, acetylation of the mono-alkyl ether derivatives of dothistromin with acetic anhydride/pyridine afforded the corresponding tetra-acetates. The ethyl ether tetra-acetate (52) was obtained as a pure, crystalline compound, m.p. 193-194.5°C. The absorption spectrum of this compound in EtOH was practically identical to that of the penta-acetate (23), confirming the identity of the chromophoric systems of these two derivatives of dothistromin. High resolution mass spectroscopy confirmed the molecular formula $C_{28}H_{24}O_{13}$ (MW 568), and the low resolution spectra showed the following fragmentation:

$$(M)\ 568 \xrightarrow[*]{-42} 526 \xrightarrow[*]{-42} 484 \xrightarrow[*]{-42} 442 \xrightarrow[*]{-60} 382$$

This indicated the presence of three aromatic acetate groups and one aliphatic acetate group. The NMR spectrum of the ethyl ether tetra-acetate (Fig. 10) showed singlet peaks at δ7.50 (1H) and δ7.37 (2H) which can be assigned to three aromatic protons; a singlet at δ6.48 (1H), the H'd' acetal proton (see (66)); a

NMR SPECTRUM OF DOTHISTROMIN ETHYL ETHER TETRA–ACETATE

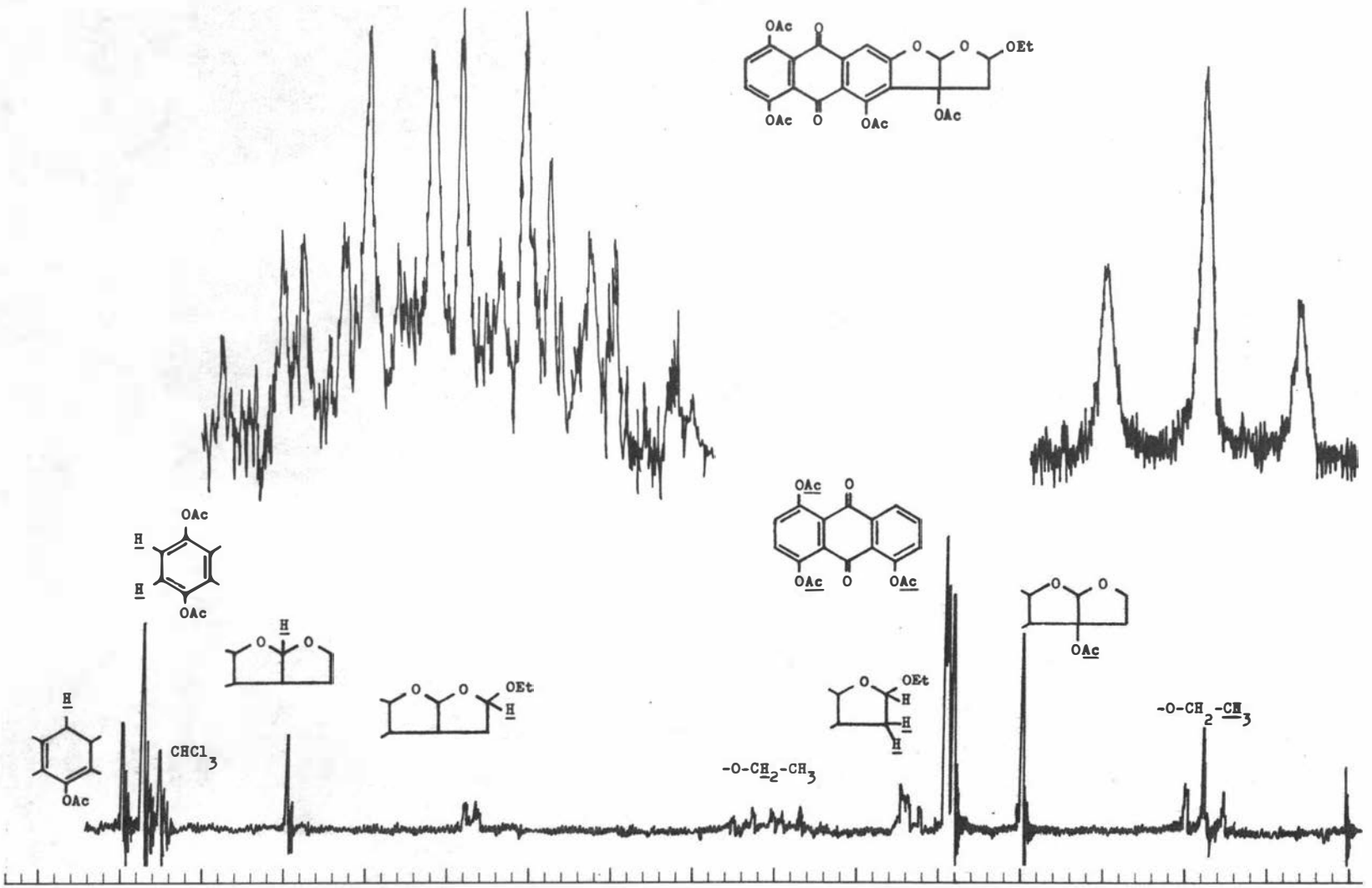


Fig. 10

multiplet at δ5.36 (1H) the H'a' acetal proton; a multiplet at ~δ3.5 (2H), the methylene protons of the ethoxy group; a multiplet at δ2.70 (2H), the methylene protons H'b', H'c'; singlets at δ2.45 (3H), δ2.44 (3H) and δ2.42 (3H), the aromatic acetate groups; a singlet at δ1.99 (3H), the benzylic acetate; a triplet at δ0.89 (3H), the methyl group of the ethoxy group. The spectrum clearly showed the acetal proton H'd' as a sharp singlet with the same chemical shift as in dothistromin penta-acetate, but now the acetal proton H'a' had moved markedly up-field to δ5.35. Chemical shifts of δ5.35 and δ5.30 are recorded for similar acetal protons in ethoxy derivatives of aflatoxin B_1 and G_1 respectively (Dutton and Heathcote, 1969). In these same compounds the methyl protons of the ethoxyl group had a chemical shift of δ0.94, the ethoxyl methylene protons appeared at δ3.51, whilst the acetal protons at the difuro ring junction had chemical shifts of δ6.54 and δ6.58.

The above evidence convincingly establishes the structure of the ethyl ether tetra-acetate derivative as (52). The chemical shift value (δ0.89) for the methyl protons of the ethoxy group in the NMR spectrum (52) indicates shielding by the aromatic ring, since the methyl protons of ethanol (in $CDCl_3$) normally appear at δ1.22 (Varian, 1962). This proves the configuration of the ethoxy group in this compound to be "endo" to the ring system; the observed aromatic ring shielding is not possible for an ethoxy group in the "exo" position.

Non-equivalent Methylene Protons

In the NMR spectrum of (52) (Fig. 10) it was found that the

signal at ~δ3.5 due to the methylene protons of the ethoxy group does not appear as a simple quartet, as in the case of ethanol, but as a multiplet with considerable fine structure. This effect arises from magnetic non-equivalence of the two methylene protons and has been observed to occur in a number of compounds with ethoxy groups attached to a centre of asymmetry (Whitesides et al., 1964; Martin and Martin, 1966; Bullock et al., 1967; McFarlane and Nash, 1969; Pavlik and Filipescu, 1969; Sidall and Stewart, 1969; Taddei, 1970). Closer examination of the spectrum of (52) revealed in fact that the multiplet was a quartet of quartets arising from the ABX_3 proton system (see inset, Fig. 10).

It has been shown (Sidall and Stewart, 1969) that three conditions must be fulfilled before non-equivalence results in an observable chemical shift between two species. These are (i) there must be no symmetry element between the species on the NMR time scale; (ii) a field gradient (electric and/or magnetic) must exist between the species; and (iii) there must be no molecular motion(s) that simulates a symmetry operation too closely.

It can also be shown that observable non-equivalence does not necessarily require molecular asymmetry; it is sufficient that there be no symmetry plane between the geminal species. Numerous examples of methylene proton non-equivalence in molecules that are not asymmetric are known; a good example is that of acetal (Shafer et al., 1961).

All of the derivatives of the ethyl ether of dothistromin for which NMR spectra were determined, exhibited non-equivalent methylene protons of the ethoxy group. Not only is the ethoxy

group attached to an asymmetric centre in these derivatives, but also, there is the presence of a suitably located aromatic ring providing a source of a large anisotropic magnetic field.

Methylation of Dothistromin

Before the structure of dothistromin was elucidated, attempts were made to methylate dothistromin, in an endeavour to produce crystalline derivatives for the structural investigation.

Methylation of dothistromin with dimethyl sulphate in refluxing acetone over anhydrous K_2CO_3 gave a yellow-orange coloured solution, which was examined by TLC on silica gel and shown to be a complex mixture of yellow and orange coloured compounds. Two major fractions were isolated from a 7.0 hr reaction time product by prep. TLC. These fractions were shown by mass spectroscopy to contain compounds of formula $C_{23}H_{22}O_9$ (MW 442) and $C_{22}H_{20}O_9$ (MW 428) respectively; clearly these were penta-methyl ether and tetra-methyl ether derivatives of dothistromin. Unfortunately, both fractions were contaminated by appreciable amounts of other methylated compounds, and could not be crystallised nor further purified by prep. TLC. Other methylating reagents, e.g. methyl iodide in dimethyl formamide/silver oxide, produced similar results.

When the structure of dothistromin had been elucidated, it was possible to propose an explanation for the above behaviour, in terms of the presence of the reactive hemiacetal group in dothistromin. Clearly, this required blocking before the molecule was subjected to the basic methylating conditions. This was achieved by acid-catalysed ether (acetal) formation, described on page 72.

Methylation of Dothistromin Mono-Ethyl- and Mono-Methyl-Ethers

Various methyl ether derivatives of the ethyl acetal (49) and the methyl acetal (48) were prepared, for further chemical investigations on dothistromin. Table XII shows the methylating reagents and reaction conditions used, and the products identified. Discussion on the reactions, the products formed and proof of structures, follows under the appropriate reaction number heading.

Methylation Reaction I: The bright yellow coloured solution from this reaction gave, by prep. TLC, two major compounds. The first of these, of higher R_f, was a non-crystalline orange-red coloured pigment (67) shown by mass spectroscopy to have molecular formula $C_{22}H_{20}O_9$ (MW 428). Its fragmentation pattern showed a characteristic loss of the formyl radical m/e 29 from the molecular ion followed by loss of a neutral fragment m/e 72 (c.f. identical behaviour of (49)) to give an intense fragment ion m/e 327. This fragmentation pattern also showed that the benzylic hydroxyl group was not methylated - as will be shown later, methylation of the benzylic hydroxyl group causes a profound change in the fragmentation pattern of dothistromin and its derivatives. If it could be assumed that the phenolic hydroxyl group remaining unmethylated in (67) would be more difficult to methylate than the other two phenolic hydroxyl groups in dothistromin, then structure (67), consistent with the above evidence, could be assigned to this compound.

The second compound, of lower R_f on TLC, was obtained in higher yield from reaction II, and it was shown to have structure (68).

Table XII

Summary of Methylation Reactions

Reaction Number	Reactant	Methylating Reagents	Conditions	Products Identified
I	(49)	$DMSO_4$/acetone/an.K_2CO_3	reflux, 0.5 hr	(67), (68)
II	(49)	" / " / "	" 6.0 hr	(68), (69)
III	(67)	MeI/DMF/$Ba(OH)_2.8H_2O$	" 2.0 hr	(69), and [(67, R_2 = Me) or (68)]
IV	(68)	CD_3I/DMF/$Ba(OH)_2.8H_2O$	" 2.0 hr	(70)
V	(49)	" / " / "	" 20.0 hr	(71), (75), (76), (77)
VI	(48)	$DMSO_4$/acetone/an.K_2CO_3	" 6.0 hr	(78), (79) + others

Methylation Reaction II: After isolation by prep. TLC and purification by column chromatography, the major product (68) from this reaction was obtained as yellow needles, m.p. 252-254°C ($CHCl_3$/n-hexane), $[\alpha]_D^{20}$ - 182°. Mass spectroscopy showed a molecular formula $C_{23}H_{22}O_9$ (MW 442), and a fragmentation of the molecular ion due to the loss of the formyl radical $\dot{C}HO$ (m/e 29), followed by the neutral fragment C_4H_8O (m/e 72) (c.f. identical behaviour for the ethyl acetal (49)) to give an intense fragment ion $C_{18}H_{13}O_7$. The NMR spectrum showed signals due to the aromatic protons at δ7.28 (2H), δ7.13 (1H), acetal protons at δ6.07 (1H), δ5.32 (1H), three aromatic methoxyl groups at δ3.98, δ3.94, δ3.91 (each 3H), the non-equivalent methylene protons of the ethoxy group at ~δ3.5, the ring methylene protons at ~δ2.62 (coupled to the acetal proton at δ5.32), and the methyl proton triplet of the endo ethoxy group at δ0.82 (shielded by the aromatic ring).

Also isolated from the above reaction was the fully methylated derivative (69). The NMR spectrum of this compound was similar to that given by (68), except for the appearance of a new aliphatic methoxyl signal at δ3.17, clearly the benzylic methoxyl group. The mass spectrum of this compound was however more complex than that of (67), (68), or any other derivative of dothistromin prepared up to this stage. This behaviour was assumed to be due to the fact that the benzylic group was methylated in this derivative, although it was not immediately obvious why this should have such a profound effect. The surprising feature of the mass spectrum of (69) was the fragmentation:

$$M^{+\cdot} \xrightarrow{-\dot{C}HO} M_1^{+} \xrightarrow{-C_3H_8O} M_2^{+}$$

m/e 456 m/e 29 m/e 427 m/e 60 m/e 367

in which loss of the neutral fragment C_3H_8O (m/e 60) occurs, compared with the loss of the neutral fragment C_4H_8O (m/e 72) in the case of (68). In addition, other competitive fragmentation processes assumed equal or greater importance in the spectrum of (69) compared to the relatively simple spectra of (48), (49), (50), (67), and (68).

Methylation Reaction III: The unexpected mass spectral fragmentation of (69) was considered worthy of closer examination, and thus consideration was given to the preparation of derivatives with deuterium labelled methyl groups at selected positions.

The first step was to check the action of MeI/DMF/$Ba(OH)_2 \cdot 8H_2O$ as a methylation system on dothistromin derivatives. Thus, the dimethyl ether ethyl acetal (67), from reaction I, was methylated with this reagent. The product was shown by mass spectroscopy to correspond to a mixture of compounds of MW 456 and MW 442, corresponding to the fully methylated ether (69), and an ethyl ether trimethyl ether [(67, R_2 = Me), or (68)].

Methylation Reaction IV: Having shown the suitability of the MeI reagent for methylation of dothistromin ether derivatives, the ethyl acetal trimethyl ether (68) was methylated using CD_3I. This gave a product, which after purification by prep. TLC, was shown by mass spectroscopy to be the expected deuterated compound (70), MW 459. The spectrum showed intense peaks at m/e 430 ($M^{+\cdot}$ - $\dot{C}HO$), and m/e 370 ($M^{+\cdot}$ - $\dot{C}HO$ - C_3H_8O (m/e 60)), showing that the loss of the C_3H_8O (m/e 60) fragment in the mass spectrum of (69) did not involve the protons of the benzylic methoxyl group. As with the unlabelled derivative (69), the spectrum showed other important competitive fragmentations. A noteable

feature was the loss of CD_3OH from the molecular ion of (70); (69) showed a corresponding loss of CH_3OH.

Methylation Reaction V: Methylation of the ethyl acetal (49) with CD_3I gave, after column chromatography, a major product assigned structure (71), a smaller amount of the fully methylated compound (75), and minor products (76), (77).

The structure of the major product was proven to be (71) from the following evidence: (i) the MW by mass spectroscopy was 451, corresponding to either (71), (72), (73) or (74); (ii) the R_f value on TLC was significantly higher than that of the methylated derivative (68) thus eliminating (72); (iii) the addition of aqu. NaOH gave a purple colouration indicating the presence of an α-hydroxyl group in the anthraquinone nucleus; (iv) the mass spectral fragmentation pattern was very different to that of the methylated derivative (68); (v) the NMR spectral data were completely consistent with (71), but not (72), (73) or (74). In $CDCl_3$, it showed signals due to aromatic protons at δ7.28 (2H) and δ7.10 (1H), acetal protons at δ6.08 (1H,s) and δ5.30 (1H,d), non-equivalent methylene protons of the ethoxy group at ~δ3.42; ring methylene protons at ~δ2.65, and the methyl proton triplet of the ethoxy group at δ0.86 ppm. A sharp singlet at δ13.19 (1H) was due to the strongly hydrogen bonded α-hydroxyl group in the anthraquinone nucleus. The lone aromatic proton in the penta-substituted aromatic ring of (72), (73) and (74) might be expected to appear nearer δ7.3 ppm rather than δ7.10 ppm as observed above; c.f. the equivalent proton of (68) which appears at δ7.33 ppm. The less likely (73) and (74) can be rejected also on the appearance of the two adjacent aromatic

protons in the tetra-substituted aromatic ring as a sharp singlet in the above spectrum, indicating their magnetic equivalence.

In addition, the hydroxyl group in the major product was shown to be in the penta-substituted aromatic ring and not the tetra-substituted ring, by the following experiment. The NMR spectrum was determined in dimethylacetamide (DMAc) as solvent, and then a small quantity of 1.0M aqu. NaOH was added. The resultant formation of the phenoxide anion caused a marked shift of the signal due to the lone aromatic proton, of approx. 0.53 ppm, and a significant, but much smaller shift, of approx. 0.21 ppm of the singlet due to the two equivalent aromatic protons. As the use of this technique (Brown, 1964; Highet and Highet, 1965; Corrie et al., 1970) on hydroxyanthraquinones does not appear to have been recorded before, the effect of base on the anthraquinone, rubiadin-3 methyl ether (32), was studied. This showed a shift of approx. 0.27 ppm for the signal of the lone proton para- to the hydroxyl group, and a very much smaller shift for the signals of the protons in the other aromatic ring. Other solvents were investigated, but DMAc was found to be the only acceptable solvent for examination of hydroxyanthraquinones by this technique.

The fully methylated (75), MW 468, gave a spectrum similar to that of (69) and (70), but a fragmentation $M^{+\cdot}$ - m/e 29 - m/e 63 was observed, rather than that of $M^{+\cdot}$ - m/e 29 - m/e 60 as observed for (69) and (70). Also, (75) showed the loss of CD_3OD from the molecular ion instead of CD_3OH as in the case of (70), and CH_3OH from (69).

The minor products, of MW 434 and 418, were assigned

structures (76) and (77) respectively, both showing fragmentations of $M^{+\cdot}$ - m/e 29 - m/e 46. It appeared that the MW 418 compound was a derivative of the deoxydothistromin impurity in the starting material.

Methylation Reaction VI: Column chromatography of the reaction product afforded two major components, shown to be the penta-methyl ether (78) and the tetra-methyl ether (79), by mass spectroscopy and NMR spectroscopy. Although (78) was obtained as a crystalline compound (yellow needles ex $CHCl_3$/n-hexane), $[\alpha]_D^{20}$ - 90° ($CHCl_3$), the melting point was not sharp, despite repeated crystallisation. The possibility that this compound was an epimeric mixture, i.e. a mixture of α- and β-methyl acetal epimers, was considered; however, no evidence of this could be found by TLC or NMR, but examination by NMR did reveal that the compound crystallised with ½ molecule $CHCl_3$ per molecule.

The tetra-methyl ether (79) gave a mass spectrum which showed an intense fragmentation due to $M^{+\cdot}$ - m/e 29 - m/e 58 (identical to that of (48)) to give an intense ion of m/e 341. The NMR spectrum showed three aromatic methoxyl groups as well as the acetal methoxy group.

Other minor methylation products were produced in this methylation reaction (e.g. compounds of MW 410, 426, and 440), but these were not further investigated.

The unexpected mass spectral fragmentation processes of the fully methylated dothistromin derivatives were interesting, because unlike the derivatives (48), (49), (50), (54), (67), (68), (72), and (79), they could not be rationalised in terms of either of the fragmentation schemes, scheme 1 or scheme 2,

proposed for dothistromin. However, detailed fragmentation schemes for these ethers were not proposed in this thesis, as the above preliminary investigation revealed that still further information was required.

Heavy Atom Derivatives of Dothistromin for an X-Ray Diffraction Study

Attempts were made to introduce a heavy atom into the dothistromin molecule for the purposes of obtaining a crystalline derivative for structure determination by an x-ray diffraction study. The first attempts were aimed at substitution of a bromine atom into the crystalline derivatives dothistromin penta-acetate (23) and dothistromin ethyl ether tetra-acetate (52). These attempts were not successful, and thus attention was turned to producing derivatives from dothistromin, using reagents containing a halogen atom.

Dothistromin p-bromobenzyl ether (80): This non-crystalline derivative was obtained by heating dothistromin in molten p-bromobenzyl alcohol, the structure following from mass spectral considerations. Acetylation of this derivative resulted in loss of the p-bromobenzyl group.

Dothistromin chloro-acetate (81): Treatment of dothistromin with molten chloroacetic acid gave the non-crystalline chloro-acetate (81).

Dothistromin chloro-acetate tetra-acetate (82): The chloro-acetate (81) was acetylated to give the chloro-acetate tetra-acetate (82). The NMR spectrum of this derivative in $CDCl_3$ showed signals due to aromatic protons at δ7.56 (1H), δ7.38 (2H), acetal protons at δ6.57 (1H) and δ6.53 (1H), methylene protons (chloroacetate group) at δ3.64 (2H), ring methylene protons at δ2.88 (2H), aromatic acetoxy groups at δ2.45 (6H) and δ2.41 (3H), and a benzylic acetoxy group at δ2.00 (3H). In addition, the spectrum showed that the crystalline derivative contained 1 mole of toluene of crystallisation per molecule, sharp peaks being observed at δ7.19 (5H) and δ2.33 (3H, methyl group) in the precise positions recorded for toluene in $CDCl_3$ (Varian, 1962).

The crystals of this derivative were fragile and of irregular shape, did not exhibit a sharp melting point, and did not appear suitable for an x-ray diffraction study.

Dothistromin iodo-acetate tetra-acetate (83): The above chloro-acetate tetra-acetate derivative was converted to the iodo-acetate tetra-acetate (83) by halogen exchange with sodium iodide. The mass spectrum and the NMR spectrum were completely consistent with structure (83) for this derivative, the NMR spectrum showing that this derivative also crystallised from toluene with 1 molecule of toluene of crystallisation per molecule of derivative. The crystals were small and irregular shaped, and did not appear to be suitable for x-ray structural analysis.

Dothistromin bromo-ethyl ether (84): 2-Bromoethanol was prepared from ethylene oxide and HBr according to the method of

Thayer et al. (1932). Dothistromin reacted with this to give the bromo-ethyl ether (84). The mass spectral fragmentation of this derivative was analogous to that of dothistromin ethyl ether (49). The compound gave small orange-red needles from various solvents, but they did not appear suitable for x-ray structural analysis.

Dothistromin bromo-ethyl ether tetra-acetate (85): Acetylation of the bromo ethyl acetal (84) afforded pale-yellow crystals from diethyl ether, m.p. 168-174°C, $[\alpha]_D^{20}$ - 103° ($CHCl_3$). Mass spectroscopy and NMR spectroscopy gave data completely consistent with structure (85). This derivative was found to be suitable for x-ray structural analysis.

Confirmation of Structure and Absolute Configuration of Dothistromin Bromo-Ethyl Ether Tetra-Acetate (85), by an X-Ray Diffraction Study

An x-ray diffraction study of the above derivative was carried out in the department of chemistry at Auckland University by C.A. Bear, J.M. Waters, and T.N. Waters. Crystals for the analysis were selected from a crop of small, pale yellow prisms crystallised from $CHCl_3/Et_2O$.

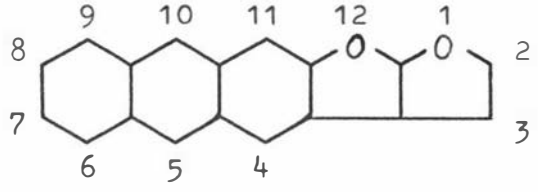
The study confirmed the structure (85), and further established the absolute configuration, as shown in (86). The final R factor obtained, after taking into account anisotropic vibrational factors for the atoms, was 11.9%.

Systematic Nomenclature for Dothistromin and its Derivatives

A number of furo[2,3-b]benzofuran-containing fungal

metabolites have now been isolated, and several synthetic furo[2,3-b]benzofuran derivatives have also been prepared. Waiss et al. (1968) suggested a nomenclature system based on the common linear difuro-ring structure. Thus, for example, they name aflatoxin B_1 ((103), R = H) (appendix, page 143) as 6-methoxy-difurocoumarone, and versicolorin A ((108), $R_1 = R_2 = R_3 = H$) (appendix, page 144), as 7,9,10-trihydroxydifuroanthraquinone. Whilst this nomenclature may have some attractions, it suffers from several very serious limitations. It does not conform to accepted systematic nomenclature systems (e.g. IUPAC), it does not specify the position of fusion of the furan rings with the remainder of the skeletal framework, and nor does it specify the manner in which the furan rings are fused to one another.

Chemical Abstracts (Subject Index, 67, July-Dec., 1967) list the ring system below as shown,



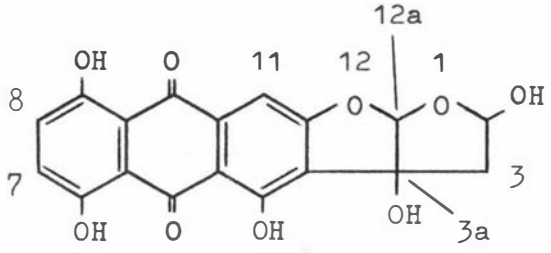
anthra[2,3-b]furo[3,2-d]furan

and index versicolorin A as: anthra[2,3-b]furo[3,2-d]furan-5,10-dione,3a,12a-dihydro-4,6,8-trihydroxy.

On this basis it can be seen that an acceptable systematic name for dothistromin‡ is: 2,3,3a,5,10,12a-hexahydro-2,3a,4,6,9-pentahydroxy-anthra[2,3-b]furo[3,2-d]furan-5,10-dione. The structure would be systematically depicted as shown, with the

‡ that is, if dothistromin had structure (38) - see discussion pages 66 to 72.

heterocyclic oxygen atom of the outer furo ring in the top right hand corner of the diagram:



Systematic Structural Representation and Numbering System for Dothistromin

For convenience, brevity, ease of readability and in line with accepted practice, the common names for dothistromin derivatives were used throughout the discussion section of this thesis.

Configuration, Optical Activity and Racemisation of Dothistromin

The absolute configuration of the fused furan rings of dothistromin, form (38), must be as shown in (87), i.e. the same as that of the bromo-ethyl ether tetra-acetate (85), (shown in (86)), as determined by x-ray structural analysis. Epimerisation of the asymmetric centre of the benzylic carbon atom in dothistromin can not occur in solution, even though dothistromin is undoubtedly in equilibrium with the open chain dialdehyde (39b) in solution. In contrast, optically active hemiacetal derivatives of aflatoxins and sterigmatocystin have been racemised within a few minutes by dissolution in aqueous alkali (Büchi et al., 1967; Rance and Roberts, 1970). In these cases, the benzylic carbon atom was not blocked, and thus racemisation by enolisation of the ring-opened dialdehyde form rapidly occurred. Ring closure of

(39b) undoubtedly gives *cis* fused rather than the much more strained *trans* fused furo rings (see, e.g. Knight *et al.*, 1966). This maintains the relative stereochemistry of the acetal carbon atom C_{12a} at the ring junction, and hence the various derivatives of dothistromin produced during this investigation, including the bromo-ethyl ether tetra-acetate (85), must have the same absolute configuration about this ring junction.

The configuration of the hemiacetal group may vary from "endo" to "exo" depending on the conditions under which the particular species under consideration was isolated, racemisation of the free hemiacetal centre readily occurring.

It is worth noting that all the crystalline derivatives of dothistromin examined for optical activity are strongly laevorotatory in solution. Thus the penta-acetate (23) has $[\alpha]_D^{20}$ - 142° ($CHCl_3$); the ethyl ether tetra-acetate (52) has $[\alpha]_D^{20}$ - 156° ($CHCl_3$); the penta-methyl ether (78) has $[\alpha]_D^{20}$ - 90° ($CHCl_3$); the ethyl ether trimethyl ether (68) has $[\alpha]_D^{20}$ - 182° ($CHCl_3$); the bromo-ethyl ether tetra-acetate (85) has $[\alpha]_D^{20}$ - 103° ($CHCl_3$).

CHAPTER 8. MISCELLANEOUS REACTIONS OF DOTHISTROMIN AND ITS DERIVATIVES

Degradation of Dothistromin to Salicylic Acid

When ethyl acetate solutions of *D. pini* pigment extract are extracted with aqu. Na_2CO_3, a beautiful deep-red coloured aqu. phase is obtained, and most of the colour is removed from the organic solvent phase. It was noted early on in the investigation that this colour fades in time, until eventually the aqu. solution becomes almost colourless. It was concluded at the time that some form of oxidation was occurring, and that this was degradative, as addition of reducing agents such as sodium hydrosulphite or stannous chloride did not restore the colour.

When hydrogen peroxide was added to the deep-red coloured aqu. Na_2CO_3 solution, the colour was found to fade much more rapidly, the solution typically becoming colourless in several hours. Solvent extraction of the aqu. phase subsequently yielded salicylic acid. Careful experiments on purified pigment ex CCD, and a "blank" run were then carried out; these experiments indicated that the salicylic acid was an authentic degradation product.

Whilst it is well known that similar oxidative degradation of xanthones gives substituted salicylic acids (Roberts, 1960), it is difficult to see how such a compound arises from dothistromin. The possibility exists that the salicylic acid in fact arises from deoxydothistromin, present in the solution with dothistromin. Experiments with D_2O (replacing H_2O), labelled dothistromin, and partially methylated derivatives of dothistromin could

provide an answer to the identity of the precursor of the salicylic acid.

Hydrolysis of the Acetal Group of Dothistromin Penta-Methyl Ether (78)

Hydrolysis of the methyl acetal group of (78) with acetone and aqu. 10% H_2SO_4 at 80-90°C for 24 hr, gave the hemiacetal derivative (88). The new compound had a lower R_f value than the starting material on TLC, as expected, and mass spectroscopy confirmed the molecular formula as $C_{22}H_{20}O_9$. Its mass spectrum was quite different from that of the dothistromin tetra-methyl ether derivative (79) in which the benzylic hydroxyl group is free.

Oxidation of the Hemiacetal (88) to the Lactone (89)

Oxidation of the hemiacetal (88) with sodium dichromate in glacial acetic acid allowed isolation of a compound of higher R_f value on TLC than the starting material. Mass spectral examination revealed the expected molecular formula $C_{22}H_{18}O_9$ for the lactone (89). Similar oxidations of a versicolorin hemiacetal derivative (Hamasaki et al., 1967), and of clerodin hemiacetal (Barton et al., 1961) have been reported.

CHAPTER 9. DEOXYDOTHISTROMIN AND OTHER CO-METABOLITES OF DOTHISTROMIN

When laboratory cultures of D. pini are extracted with ethyl acetate, a number of co-metabolites are extracted together with dothistromin. Thus, TLC examination of a crude ethyl acetate culture indicates the presence of a number of coloured compounds apart from the major coloured metabolite, dothistromin. It would appear that some of these coloured co-metabolites also have a hydroxyanthraquinone chromophore, with at least one α-hydroxyl group in the anthraquinone nucleus, as they give a purple colouration with alkali.

One of the major co-metabolites is deoxydothistromin; this travels with dothistromin on TLC. The molecular formula for deoxydothistromin was established as $C_{18}H_{12}O_8$. On the basis of all the evidence accumulated during the thesis investigation (TLC, mass spectroscopy, CCD, absorption spectroscopy, chemical reactions) it is concluded that deoxydothistromin has either structure (90) or (91). Thus mass spectroscopy (see pages 12-13; page 23) shows that deoxydothistromin fragments by loss of a formyl radical followed by loss of the same neutral fragment C_2H_4O (m/e 44) lost by dothistromin, whilst absorption spectroscopy (see pages 35-38) is consistent with either (90) or (91), but clearly not (92) or (93).

A yellow metabolite was identified during the CCD investigation (see page 23), formula $C_{20}H_{16}O_7$, MW 368. This same pigment was detected in culture extracts, and thus appears to be a genuine co-metabolite and not an artefact. It appears on TLC

at a higher R_f value than dothistromin. This could be a dimethyl di-deoxy derivative of dothistromin. Its mass spectrum showed the loss of a neutral fragment C_3H_6O (m/e 58) from the molecular ion to give an ion at m/e 310 (verified by a metastable peak).

Some culture extracts have given red pigment whose mass spectrum has shown a small amount of a compound with a molecular ion of composition $C_{18}H_{10}O_9$ (MW 370), in an otherwise normal dothistromin/deoxydothistromin mass spectrum. This could be the lactone (94), occurring as a co-metabolite or an oxidation artefact.

By analogy with the aflatoxins, sterigmatocystins and versicolorins (see appendix) it would not seem unreasonable to predict that the "saturated" derivative (95) and the "unsaturated" derivative (96) of dothistromin might be isolated as metabolites of D. pini. The "saturated" derivative (95) (MW 356) has the same formula, $C_{18}H_{12}O_8$, as deoxydothistromin, whilst the "unsaturated" derivative (96) (MW 354), could be present as a co-metabolite, or it could be produced as an artefact by dehydration of the hemiacetal derivative. It would be difficult to detect this compound in the presence of dothistromin by mass spectroscopy, since dothistromin itself loses the elements of water in the mass spectrometer to give an ion at m/e 354.

CHAPTER 10. POSSIBLE CARCINOGENICITY OF DOTHISTROMIN ITS CO-METABOLITES, AND ARTEFACTS

In view of the potent carcinogenicity of some of the aflatoxins, and carcinogenicity of sterigmatocystin (see Appendix), it appears that the dihydrofuro[2,3-b]benzofuran (97) moiety, or the tetrahydro derivative (98) of this, could be an important feature conferring or contributing carcinogenic properties on suitable polycyclic molecules containing this feature. For this reason, dothistromin, its co-metabolites, and its various artefacts and derivatives should be handled and treated with the utmost caution, as recommended by Goldblatt (1969) in the case of the aflatoxins. The same comment applies to cultures, culture extracts, spores, and spore suspensions of D. pini.

Although dothistromin itself has a hemiacetal group in the furobenzofuran system, and this feature appears to nullify the carcinogenic activity of aflatoxin, i.e. aflatoxins B_{2a} and G_{2a} which have such a feature, do not exhibit the potent carcinogenicity of aflatoxins B_1 and G_1, it should be borne in mind that elimination of the hemiacetal group by dehydration would produce the unsaturated derivative (96). This unsaturated derivative, analogous to aflatoxin B_1 and sterigmatocystin, could well be a metabolite of D. pini under certain culture conditions, or of different strains of D. pini than those used in the present investigation.

CHAPTER 11. THE SYNTHESIS AND BIOSYNTHESIS OF DOTHISTROMIN

The Synthesis of Dothistromin

It should be possible, in principle, to synthesise dothistromin by first preparing the required substituted tetrahydrofuro[2,3-b]benzofuran and then to form the anthraquinone nucleus on this by suitable acylation and cyclisation reactions. The substituted tetrahydrofuro[2,3-b]benzofurans are now reasonably accessible synthetic compounds, largely because of the work of Büchi *et al.* on the synthesis of the aflatoxins (see Appendix) and Roberts and co-workers on the synthesis of O-methylsterigmatocystins and O-methyl aversin (see Appendix). Thus, after synthesis of the substituted tetrahydrofuro[2,3-b]benzofuran, Friedel-Crafts acylation with 3,6-dimethoxyphthalic anhydride, followed by acid catalysed cyclisation, as in the usual classical method of formation of the anthraquinone nucleus, could then be attempted. However, the lability of the hemiacetal group, and the tetrahydrofurofuran system itself to the rigorous acidic conditions of the final ring closure could pose a major problem with this method. A far more promising approach to this stage of the synthesis would be to apply the method used in the recent synthesis of O-methylaversin (Holmwood and Roberts, 1971). In this synthesis, an acid chloride functional group is introduced into the substituted tetrahydrofuro[2,3-b]benzofuran, which is then converted into the desired anthraquinone by a new type of polyhydroxyanthraquinone synthesis (Davies *et al.*, 1969; Hassall and Morgan, 1970), in which the final ring closure is achieved

under alkaline conditions. It would probably be helpful to use the lactone derivative of the tetrahydrofuro [2,3-b] benzofuran during this stage of the synthesis (see synthesis of O-methyl-sterigmatocystin, Rance and Roberts, 1970).

An alternative possibility is to first synthesise the anthraquinone nucleus, and then to construct the difuro ring system on this.

With the recent report of the conversion of sterigmatocystin into dihydroaspertoxin (Hutchinson and Holzapfel, 1971) now to hand, it would appear that introduction of the benzylic hydroxyl group could well be carried out as the final step of the total synthetic sequence. This would have the dual advantage of simplifying the synthesis of the required substituted tetrahydro-furo [2,3-b] benzofuran, and avoiding problems associated with the lability of benzylic hydroxyl groups.

The Biosynthesis of Dothistromin

The biosynthesis of the aflatoxins has been investigated (Biollaz *et al.*, 1970; Elsworthy *et al.*, 1970). Thus Biollaz *et al.* showed by degradative studies on radioactive aflatoxin B_1 prepared by fermentation of added [1-^{14}C]- and [2-^{14}C]-acetates and of methyl-^{14}C-methionine, that the carbon skeleton is derived entirely from acetic acid, and suggested the intermediary of a single polyacetate chain. A hypothetical pathway was proposed, in which the formation of a difurohydroxyanthraquinone from an endoperoxyanthraquinone is postulated to occur.

Biosynthetic studies (Tanabe *et al.*, 1970) with ^{13}C enrich-ed acetate on sterigmatocystin, showed, by the use of ^{13}C-NMR

spectra, the biogenetic origin of all the carbon atoms in this metabolite. Thus, sodium [1-^{13}C] acetate (56%) and sodium [2-^{13}C] acetate (61%) were incorporated in separate cultures to give the ^{13}C labelled sterigmatocystins.

From the above work on the aflatoxins and sterigmatocystin, and from consideration of the fact that a number of fungal anthraquinones have been shown to be acetate derived, it seems likely that dothistromin is completely acetate derived. It would be interesting to know whether labelling studies on dothistromin reveal a pattern consistent with the involvement of an endoperoxy-anthraquinone as proposed by Biollaz *et al*. (*loc. cit.*) for the aflatoxins. The elegant ^{13}C technique described above, circumvents the older, tedious and time-consuming degradative technique for label determination, and is clearly the method of choice for proving the biogenetic origin of the carbon atoms in a metabolite such as dothistromin.

GENERAL SUMMARY AND CONCLUSIONS

The fungus *Dothistroma pini*, a needle pathogen of *Pinus radiata* and other pines, produces a number of coloured metabolites when cultured on an aqueous malt medium. One of these metabolites, a red pigment extractable with ethyl acetate, had previously been implicated as a toxin due to its toxicity to *Chlorella pyrenoidosa* (a unicellular green alga). The same red pigment was also obtainable from pine needles collected from naturally infected trees. This thesis was concerned with a detailed investigation into the nature and characterisation of the red pigment.

The investigation first established that the red pigment was a mixture of two closely related compounds of molecular formulae $C_{18}H_{12}O_9$ and $C_{18}H_{12}O_8$ (as determined by high resolution mass spectroscopy). The former, which was present in greater amount, was named dothistromin, and the latter was named deoxydothistromin.

A detailed chemical investigation revealed that dothistromin was a substituted trihydroxyanthraquinone. Thus, by its reversible oxidation-reduction with alkaline dithionite, its colour reaction with alkali, its colour reactions with metal ions in solution, its fluorescence properties, its visible absorption spectra, and its degradation to 2-methylanthracene on zinc dust distillation, dothistromin was shown to possess a β-alkyl-tri-α-hydroxyanthraquinone chromophoric moiety.

Acetylation of dothistromin allowed isolation of crystalline, optically active dothistromin penta-acetate, whose formula was

conclusively established as (23). Numerous other derivatives of dothistromin were prepared, and these included the crystalline derivatives dothistromin ethyl ether tetra-acetate, formula (52), dothistromin ethyl ether trimethyl ether, formula (68), and dothistromin penta-methyl ether, formula (78). The properties of these derivatives, and the properties and reactions of dothistromin allowed postulation of structure (38) for dothistromin. The probability that in solution dothistromin exists as a complex equilibrium mixture (see Scheme 1) is discussed in some detail in Chap. 7.

An outstanding feature of the structure of dothistromin, is the presence of a substituted furo[2,3-b]benzofuran moiety. A number of fungal metabolites are known to possess this feature, and they are all related to the potently carcinogenic and toxic aflatoxins. The structures of the aflatoxins (and the related fungal metabolites sterigmatocystin, aversin, versicolorin, and derivatives of these) have only recently been determined, and it is fortunate that data on some acetate derivatives of a few of the aflatoxins was published during the course of this investigation, as this data greatly facilitated deduction of the structure of dothistromin penta-acetate. In view of the close structural similarity of dothistromin to sterigmatocystin, and to a lesser extent, to the aflatoxins, a discussion (Chap. 10) on the possible carcinogenicity of dothistromin, its co-metabolites, and artefacts, is included.

A noteworthy structural feature of dothistromin is the reactive hemiacetal group: dothistromin undergoes facile acid-catalysed mono-alkylation and mono-acetylation.

A number of heavy atom derivatives of dothistromin were prepared. The structure and absolute configuration of one of these, dothistromin bromo-ethyl ether tetra-acetate (85), was determined by an x-ray crystallographic diffraction study. This confirmed the structures proposed in this thesis, and also allowed the absolute configuration of the cis-fused furo rings of dothistromin to be deduced. Dothistromin and its derivatives have the same absolute configuration as the aflatoxins.

The mass spectrum of dothistromin and a number of its derivatives show an interesting and characteristic fragmentation due to loss of the formyl radical CHO· (m/e 29) and, in the case of dothistromin, loss of the neutral fragment C_2H_4O (m/e 44). Loss of a homologous neutral fragment was also characteristically shown by a number of the dothistromin derivatives. Fragmentation schemes (Scheme 2 and Scheme 3) were proposed to rationalise the fragmentation of dothistromin.

The structure of deoxydothistromin was shown to be either (90) or (91) (see Chap. 9), by consideration of all the data obtained during the structural investigation of dothistromin, despite the fact that attempts to separate dothistromin and deoxydothistromin by thin layer chromatography were not successful. A partial separation was however achieved by the technique of counter current distribution. The structures of other co-metabolites of dothistromin are also discussed in Chap. 9.

The synthesis and biosynthesis of dothistromin is briefly discussed in Chap. 11. Attempts at the synthesis of dothistromin could well be aimed at any of the methyl derivatives prepared in this investigation.

EXPERIMENTAL SECTION

GENERAL

Melting points were determined on a Kofler hot-stage microscope and are corrected. Ultraviolet and visible absorption spectra were recorded on a Unicam SP800 spectrophotometer or a Perkin-Elmer Model 402 spectrophotometer. Infrared spectra were recorded on a Beckman IR-20 spectrophotometer. Optical rotations were determined for $CHCl_3$ solutions at 20° on a Hilger and Watts Standard Polarimeter.

Mass spectra were obtained with an AEI MS902 double-focusing high-resolution mass spectrometer. Low resolution spectra were recorded at a resolution of ca. 1000 ppm, and accurate mass measurements were made by the peak matching technique on the oscilloscope, at a resolving power of ca. 10,000. Unless otherwise stated, all spectra were run by use of the direct insertion probe. Where data from a mass spectrum are quoted, this is generally for the most prominent peaks in the upper mass region of the spectrum; m* denotes transitions for which satisfactory metastable peaks were observed on chart.

Nuclear magnetic resonance (NMR) spectral measurements were determined on a JEOL JNM-C-60HL high resolution nuclear magnetic resonance spectrometer or a Varian HA-60 NMR spectrometer. The spectral data are given in the following order: position of peak (δ value in ppm downfield from TMS), number of protons, peak multiplicity, and assignment. The peak multiplicities are abbreviated by s, d, t, q, and m, which refer to singlet, doublet, triplet, quartet, and multiplet, respectively.

Thin layer chromatography (TLC) was carried out on 0.2 mm thick layers of Merck silica gel G unless otherwise stated. For preparative TLC (prep. TLC), the same adsorbent was used at a thickness of approx. 1 mm, on square glass plates 20 cm x 20 cm; the prep. TLC plates were activated by drying for at least 8 hr at 120°.

Gas liquid chromatography (GLC) was carried out using a Varian Aerograph Series 1740 instrument, with an FID detector. A 5% QF-1/Chromosorb W column ($\frac{1}{4}$" diam., 6' long, stainless steel) was used for both analytical GLC and prep. GLC, with a N_2 flow rate of 77 ml/min. For prep. GLC, a 1:10 stream splitter was connected to the input of the FID detector.

PREPARATION OF REFERENCE COMPOUNDS AND REAGENTS

1-Chloro-4-methylanthraquinone: see page 40. The anthraquinone was obtained as thin yellow needles ex toluene, m.p. 164°, lit. value 164-5° (Heller and Schulke, 1909), 163-4° (Mellier, 1956).

1-Methylanthracene: see page 40. Zinc dust distillation of 1-chloro-4-methylanthraquinone at 400°C under an atmosphere of hydrogen gave 1-methylanthracene, white needles ex EtOH, m.p. 84-88°, lit. value 85-86° (Fischer and Sapper, 1911), 84-85° (Klemm et al., 1963).

1- and 2-Acetylanthracene: see page 42. The Friedel-Crafts acylation product gave yellow crystals from benzene, which were shown by TLC and GLC to be a mixture of two compounds. Mass spectral examination showed only one molecular ion, m/e 220, consistent with the mixture being 1- and 2-acetylanthracene. Repeated fractional crystallisation gave 1-acetyl- and 2-acetyl-

anthracene, both isomers being slightly cross-contaminated, as shown by GLC (Rt 1-isomer, 5.5 min; Rt 2-isomer, 7.8 min, column at 225°C). The 2-acetylanthracene, purified by prep. TLC, had m.p. 191-193°, lit. value 195.5-196° (Gore and Thadani, 1966). Mass spectrum, m/e (%): 220 (76) (M^+), 205 (77), 177 (100), 176 (50), 151 (21), 150 (15), 88.5 (24), 88 (42), m*: 220 → 205 → 177 → 151.

2-Ethylanthracene: see page 42. The 2-ethylanthracene from the Wolf-Kishner reduction had an Rt of 8.5 min on GLC at 180°C. Mass spectrum, m/e (%): 206 (100) (M^+), 191 (47), 189 (13), 95.5 (10), m*: 206 → 191.

1,2-Diacetoxybenzene (10): see page 55. Prepared by acetylation of catechol in 10% aqu. NaOH, with Ac_2O (Chattaway, 1931). Mass spectrum, m/e (%): 194 (14) (M^+), 152 (29), 110 (100), m*: 194 → 152 → 110.

1,2,4-Triacetoxybenzene (11), and 1,2,4-triacetoxynaphthalene (12): see page 55. Prepared by the Thiele acetylation of p-benzoquinone and 1,4-naphthoquinone, respectively (Burton and Praill, 1952). Mass spectrum (11), m/e (%): 252 (7) (M^+), 210 (14), 168 (41), 126 (100), m*: 252 → 210 → 168 → 126 → 97. Mass spectrum (12), m/e (%): 302 (8) (M^+), 260 (12), 218 (27), 176 (100), 175 (15), 147 (7), m*: 302 → 260 → 218 → 176 → 147.

1,2-diacetoxyanthraquinone (13), 1,4-diacetoxyanthraquinone (14), 1,8-diacetoxyanthraquinone (15), 2,6-diacetoxyanthraquinone (16), 1,2,4-triacetoxyanthraquinone (17), and 1,2,5,8-tetraacetoxyanthraquinone (18): These compounds were all prepared from the appropriate hydroxyanthraquinone by acid catalysed acetylation (a few drops of conc. H_2SO_4 added to a soln. of the hydroxy-

anthraquinone in Ac_2O). All the acetates showed the expected mass spectral fragmentation. Mass spectra: see page 47 and page 52; NMR: see page 55; absorption spectra: see page 59;

Rubiadin-1 methyl ether (28): see page 59. Damp bark (460 g) ex C. Australis was extracted with acetone, and the product worked up according to the method of Briggs and Dacre (1948), to give fine yellow needles (820 mg) ex HOAc, m.p. 292°, lit. m.p. 291° (Jones and Robertson, 1930), 302° (Briggs and Dacre, loc. cit.). λ max (EtOH): 240, 245, 281, 360. Mass spectrum, m/e (%): 268 (100) (M^+), 253 (45), 251 (23), 250 (33), 239 (31), 225 (11), 223 (13), 222 (19), m*: 268 → 250, 268 → 239.

Rubiadin-1 methyl ether-3-acetate (29): Acetylation of (28) with Ac_2O/pyridine gave the acetate (29), yellow needles from EtOAc, m.p. 183°, lit. m.p. 174° (Jones and Robertson, 1930), 183° (Briggs and Dacre, 1948). NMR ($CDCl_3$), 7.88 (1H, s, Ar-H), 3.95 (3H, s, $-OCH_3$), 2.38 (3H, s, β-OAc), 2.25 (3H, s, $-CH_3$). Absorption spectra, see page 61. Mass spectrum, m/e (%): 310 (100) (M^+), 268 (100), 253 (59), 251 (39), 250 (51), 239 (43), 238 (22), m*: 310 → 268 → 150, 268 → 239.

Rubiadin (31): Rubiadin-1 methyl ether (28), (250 mg), was heated with conc. H_2SO_4 (5 ml) in a sealed glass tube at 150°C for 12 min, the cooled product was poured into water, the olive green ppte. collected, dissolved in HOAc, treated with decol. charcoal and filtered, to give orange-yellow micro prisms (100 mg, 42%), m.p. 290°, lit. m.p. 290° (Jones and Robertson, 1930), 302° (Briggs and Nicholls, 1949). Mass spectrum, m/e (%): 254 (100) (M^+), 236 (2), 226 (6), 225 (6), 199 (4), 198 (2), 197 (4), m*: 254 → 236 → 226, 254 → 225.

Rubiadin-3 methyl ether (32): Methylation of rubiadin (31) with dimethyl sulphate in refluxing acetone over an. K_2CO_3 for 12 min, gave a yellow solid shown by mass spectroscopy to be a mixture of two components, MW 268 and 282. Column chromatography (using TLC grade silica gel G) with toluene allowed separation of a yellow compound, homogeneous by TLC. Crystallisation from toluene gave yellow needles of (32), m.p. 190-192°, lit. m.p. 186° (Jones and Robertson, 1930). λ max (EtOH): 242, 246, 276, 335, 394, 414. Mass spectrum, m/e (%): 268 (100) (M^+), 253 (10), 251 (10), 250 (33), 239 (24), 238 (22), 225 (7), m* 268 → 250, 268 → 239.

Rubiadin-3 methyl ether-1-acetate (30): Acetylation of (32) with Ac_2O/pyridine gave the acetate (30), yellow needles from ethyl acetate, m.p. 198-200°, lit. m.p. 200° (Jones and Robertson, 1930). NMR ($CDCl_3$), 7.59 (1H, s, Ar-H), 3.98 (3H, s, $-OCH_3$), 2.49 (3H, s, α-OAc), 2.14 (3H, s, $-CH_3$). Absorption spectra, see page 61. Mass spectrum, m/e (%): 310 (5) (M^+), 268 (100), 267 (10), 253 (9), 251 (10), 250 (53), 239 (22), 238 (17), m*: 310 → 268 → 250, 268 → 239.

Resorcinol mono-methyl ether-d_3 (58) and Resorcinol di-methyl ether-d_6 (59): Resorcinol (3.0 g, 27.3 mmole) in acetone (25 ml) containing an. K_2CO_3 (2.07 g, 15.0 mmole) and CD_3I (2.0 g, 13.8 mmole) was heated under reflux for 6 hr. The acetone was evapt'd under vacuo, the product acidified and extracted with benzene. The benzene layer was washed with H_2O to remove unchanged resorcinol, then ext'd with aqu. NaOH, the NaOH layer acidified and ext'd with benzene to give, after evapt'n of benzene and distillation under vacuo, the desired methyl ether-d_3 (58)

(1.02 g, 8.0 mmole). From the initial benzene layer was obtained the dimethyl ether-d_6 (56) (0.34 g, 2.4 mmole), total product yield 75% (based on CD_3I). Mass spectrum (58), m/e (%): 127 (100) (M^+), 126 (4), 125 (1), 98 (7), 97 (14), 96 (10), 95 (42), 81 (19), m*: 127 → 98, 127 → 97, 127 → 96, 127 → 95. Mass spectrum (59), m/e (%): 144 (100) (M^+), 143 (4), 142 (1), 115 (5), 114 (21), 113 (4), 112 (15), 98 (17), 80 (20), m*: 144 → 115, 144 → 114, 144 → 113, 144 → 112.

2-Bromoethanol: 2-bromoethanol, b.p. 47-52°/14 mm Hg, lit. b.p. 47-51°/15 mm (Thayer et al., 1932), was obtained from ethylene oxide and HBr. NMR ($CDCl_3$), ca. 3.9 (2H, m, $-CH_2-OH$), ca. 3.5 (2H, m, $-CH_2-Br$). Mass spectrum, m/e (%): 126 (13) ($M+2^+$), 124 (13) (M^+), 97 (11), 96 (8), 95 (17), 94 (8), 93 (6), 45 (85), 44 (34), 31 (100).

LABORATORY CULTURE OF DOTHISTROMA PINI

Two isolates of D. pini were used for culture in the laboratory. One isolate was provided by the Forest Research Institute, Rotorua, from their stock collection. A second isolate was obtained by Mr. P.J. Brunt from diseased Pinus radiata needles from Te Wera State Forest in Taranaki.

Stock cultures were maintained by streaking spores on 10% malt agar plates, and subculturing at intervals, to ensure viable cultures.

To prepare the plates, 25 g of agar (Difco) was added to 250 ml of 10% malt medium (see below), and poured into screw capped bottles, which were autoclaved for 15 min at 15 psi and 120° C. After cooling to a temp. just below 40° C, the malt

agar was poured into sterile petri dishes.

Aqueous Malt Medium: An aqu. malt soln. (10% w/v) was made up by dissolving the required amount of malt ("Wander Brand Dried Malt", type 500 LDE, ex A. Wander N.Z. Ltd., Christchurch), in hot distilled water stirred by a magnetic stirrer. When the malt had dissolved, yeast (5 g/litre) (DYC "Active" Yeast, Dominion Yeast Co., Auckland) was added, followed by cholesterol (20 mg/litre). After ca. 10 min further stirring, the medium was poured into conical flasks.

Modified Raulins Medium: The following compounds were dissolved in 4 litres of warm water: sucrose (116.5 g), D+ tartaric acid (13.3 g), KH_2PO_4 (2.0 g), NH_4NO_3 (13.5 g), K_2CO_3 (2.0 g), $MgCO_3$ (1.3 g), $CaSO_4$ (0.8 g), $ZnSO_4 \cdot 7H_2O$ (0.3 g), $FeSO_4 \cdot 7H_2O$ (0.3 g), thiamine HCl (2.5 mg). The pH of the soln. was then adjusted to pH 3.5 by the addition of NaOH, and then 1 litre of McIlvaines buffer was added. The McIlvaines buffer was prepared by dissolving Na_2HPO_4 (14.2 g) in H_2O (500 ml) and citric acid (15.6 g) in H_2O (750 ml) and mixing 298 ml of the Na_2HPO_4 soln. with 702 ml of the citric acid soln. to give 1 litre of buffer, pH 3.5. After stirring for ca. 10 min, the medium was poured into conical flasks and to each flask was added fat-free casein (100 mg/100 ml medium).

Inoculation and Culture: The conical flasks containing culture medium were autoclaved at 15 psi and 120° C for 15 min. Inoculation was carried out by adding a water suspension of *D. pini* spores (prepared by agitating malt-agar plate cultures of *D. pini* with sterile water). The culture flasks were then shaken continuously on a gyrotory mechanical shaker at 12-18°,

until ready for harvest. With a heavy inoculum, red pigment production appeared to have reached a max. within 6-10 days.

Extraction of Red Pigment from D. pini Cultures. Excess ethyl acetate was added to the culture flask so that the flask could just be shaken rapidly on the gyrotory shaker without spillage occurring, and the flasks shaken for ca. 8 hr. After standing overnight, the orange coloured ethyl acetate layer was carefully decanted off, a further quantity of fresh ethyl acetate added to the flask, and the above extraction process repeated. After this second extraction, the total aqu. mixture was transferred to a large separating funnel, an equal volume of distilled H_2O added, the funnel shaken gently for ca. 2-3 min, and the residual ethyl acetate layer separated. The total ethyl acetate extract was then washed repeatedly with aliquots of distilled H_2O, filtered through a large fluted filter paper, and the ethyl acetate evaporated under vacuo over the water bath, 40-50° C, using a rotary evaporator. This gave an amorphous red powder, which contained a high percentage of the desired red pigment (as shown by TLC).

Purification of Red Pigment by Prep. TLC: Approx. 40 mg of red pigment extract in ethyl acetate was applied to a silica gel coated plate, as a narrow band less than 5 mm wide, by repeated streaking with a fine pipette. When the plates had been developed (EtOAc:$CHCl_3$ = 60:40) sufficiently to resolve the red pigment from other components in the extract, the red band of adsorbent, Rf 0.4, was scraped off whilst still wet with solvent, into ethyl acetate. The resultant slurry was then shaken vigorously with distilled H_2O in a separating funnel, the orange

coloured ethyl acetate layer separated and washed with distilled H_2O. The ethyl acetate soln. was then filtered and/or centrifuged; subsequent evaporation of the ethyl acetate under vacuo gave purified amorphous red pigment.

Counter Current Distribution: A 120 tube hand-operated Craig CCD apparatus with a lower phase (stationary phase) volume of 20 ml, and an upper phase (mobile phase) volume of 22.0 ml was used. The tubes were numbered consecutively from the feed-in end of the tube bank.

The borax buffer used for CCD run 1 was a 0.025M borax soln. made 0.1M w.r. to NaCl; the phosphate buffer, used for CCD run 2, was prepared from $K_3PO_4.H_2O$ (460 g) dissolved in 2 litre H_2O, to which was added ca. 1.7 litre 1.0M HCl.

Visible Absorption Spectra of Red Pigment: Red pigment ex tube no. 15, CCD Run 2, see page 20, had λmax (EtOH): 478, 490, 509, 523. At pH 6.5, and assuming a homogeneous sample containing only compound of MW 372, calculated log ε values are: 3.81, 3.84, 3.75 and 3.68, respectively.

Examination of the Red Pigment by Mass Spectroscopy: Found: M, 372·0478. $C_{18}H_{12}O_9$ requires M, 372·0480. M_2, 356·0527, $C_{18}H_{12}O_8$ requires M, 356·0531. Also found, m, 354·0373, $C_{18}H_{10}O_8$ = 354·0375; 343·0462, $C_{17}H_{11}O_8$ = 343·0453; 338·0430, $C_{18}H_{10}O_7$ = 338·0426; 325·0348, $C_{17}H_9O_7$ = 325·0348; 312·0268, $C_{16}H_8O_7$ = 312·0258; 310·0474, $C_{17}H_{10}O_6$ = 310·0476; 309·0395, $C_{17}H_9O_6$ = 309·0398; 299·0180, $C_{15}H_7O_7$ = 299·0192; 298·0460, $C_{16}H_{10}O_6$ = 298·0477; 297·0395, $C_{16}H_9O_6$ = 297·0399; 283·0240, $C_{15}H_7O_6$ = 283·0243; 281·0454, $C_{16}H_9O_5$ = 281·0449; 272·0318, $C_{14}H_8O_6$ = 272·0321; 256·0395, $C_{14}H_8O_5$ = 256·0372.

Zinc Dust Distillation: An apparatus was constructed to enable the zinc dust distillation to be carried out under closely controlled conditions. Four 60 watt electric heating elements were bolted to an aluminium block which had a hole drilled through it to take a pyrex tube, and also a small hole to take a 0-520° C thermometer. A 15 mm diam. pyrex tube, ca. 40 cm long, was sealed at one end, and a B14 cone sealed to the other end. A glass fitting with a B14 socket was designed which allowed H_2 gas to flow into the pyrex tube and out an exit tube at the same end. A trap inserted in the exit line, consisted of a Dreschel bottle containing glass beads and toluene, and a glass coil immersed in a container of liquid air.

The compound to be distilled (ca. 30 to 50 mg) was thoroughly mixed with 100 times its weight of zinc dust (May and Baker Zinc Powder) and the mixture tamped down in the closed end of the pyrex tube. The same quantity of pure zinc dust was then tamped down on top of this, followed by a short plug of glass wool. The tube was then inserted through the aluminium block so that only the pure zinc dust zone was within the block. The apparatus was flushed with H_2 gas, the H_2 gas flow then turned to a slow rate, and the heating elements turned on. After the zinc dust zone had been held at 400° C for ca. 5-10 min, the compound/zinc dust zone was moved into the block, so that both zones were heated at 400° C. Typically, fluorescent material began to condense on the cool walls of the pyrex tube outside the block, and the distillation was complete within 20 min. The pyrex tube was then cut open and the condensed fluorescent material recovered by solution in a suitable solvent (e.g. n-pentane,

EtOH). No fluorescent material was detected in the exit line traps.

Prep. GLC of Zinc Dust Distillation Product: GLC on the QF-1 column operated at 180° C allowed separation of the following peaks from the zinc dust distillation product of homogeneous red pigment: Rt 4.2 min, 6.4 min (major components), and Rt 8.5, 9.5, 11.5 and 16 min (minor components).

Rt 4.2 min component: Found: M (mass spectrometry), 178·0780. $C_{14}H_{10}$ requires M, 178·0782. Mass spectrum, m/e (%): 178 (100) (M^+), 177 (8), 176 (16), 152 (7), 151 (7), 150 (5), 89 (22) (M^{2+}), 76 (19) (152^{2+}), m*: 178 → 152.

Rt 6.4 min component: Found: M (mass spectrometry), 192·0935. $C_{15}H_{12}$ requires M, 192·0939. Mass spectrum, m/e (%): 192 (100) (M^+), 191 (34), 190 (9), 189 (19), 165 (11) ($M-C_2H_3$).

Rt 8.5 min component: Found: M (mass spectrometry), 206·1093. $C_{16}H_{14}$ requires M, 206·1095. Mass spectrum, m/e (%): 206 (100) (M^+), 191 (63) ($M-CH_3$), m* 177 (206 → 191).

Rt 9.5 min component: Mass spectrum, m/e (%): 206 (100) (M^+).

Rt 16 min component: Found: M (mass spectrometry), 232·0886. $C_{17}H_{12}O$ requires M, 232·0888. Mass spectrum, m/e (%): 232 (100) (M^+), 231 (12), 203 (16), 202 (20), 116 (12) (M^{2+}).

Detection of phthalates by TLC: Silica gel G plates, developed with CH_2Cl_2, or EtOAc : $CHCl_3$: HOAc = 40:60:1. Sprayed with a mixture (1:1) of 4N aqu. H_2SO_4 and 20% resorcinol in EtOH, and heated in an oven at 120° C for 10 min. Yellow spots were obtained with phthalate esters, phthalic acids, or phthalic anhydride. The colour intensified to a dark orange by exposure of the above sprayed plates to ammonia vapour in a glass tank.

With CH_2Cl_2 as developer, dibutyl phthalate had Rf 0.55; dimethyl phthalate, Rf 0.52; dothistromin, Rf 0.0. With EtOAc : $CHCl_3$: HOAc solvent, dibutyl phthalate had Rf 0.50; dimethyl phthalate, Rf 0.45; phthalic acid, Rf 0.08; phthalic anhydride, long smear; dothistromin, Rf 0.41.

Acetylation of Dothistromin: Homogeneous red pigment (50 mg) ex prep. TLC was dissolved in AR grade Ac_2O (50 ml). H_2SO_4 (1 drop) was then added to the orange coloured solution, the solution warmed gently on the water bath, with shaking, until golden yellow in colour, and allowed to stand for 8 hr at room temp. Excess NaOAc was then added to neutralise the H_2SO_4, the reaction product poured into H_2O (200 ml) and shaken vigorously for several hours. The aqu. mixture was ext'd with Et_2O (2 x 50 ml), the Et_2O extract washed with H_2O and evapt'd under vacuo, to give a pale yellow gum. Examination by mass spectroscopy revealed this product to be a mixture of polyacetylated compounds, the principle components having MW's 582 and 524, see page 46 and Fig. 6. Mass spectrum, m*: 582 → 540 → 498 → 456 → 396 → 354, 524 → 482 → 440, 480 → 438 → 396, 498 → 438, 380 → 338 → 309. Purification by prep. TLC using as solvent benzene : HOAc = 100:9, allowed separation into the constituent components, as described on page 49. The bands on the still wet prep. TLC plates were harvested by scraping them immediately into flasks of solvent. The major component, Rf 0.30 (upper band of doublet) was obtained as a pale yellow gum (26 mg), and identified as dothistromin penta-acetate (23). Crystallisation from $CHCl_3$/hexane gave pale yellow needles, m.p. 196-198° C (some phase change at 140-150°), $[\alpha]_D$ - 142° (C 0.17). Found: M (mass spectrometry), 582.1014.

$C_{28}H_{22}O_{14}$ requires M, 582·1009. λmax (EtOH) 238, 272, 280, 348 nm (ε = 8,500; 18,400; 17,100; 3,300), see Fig. 9. IR, see page 52; NMR, see page 53 and Fig. 8. Mass spectrum - see line diag., Fig. 7; m* (defocussing technique), 540, 582 → 480; 496 → 456; 480, 498, 54 → 438; 438, 456, 498 → 396; 420, 438, 480 → 378; 396, 456 → 354.

Dothistromin methyl ether (48): To red pigment (50 mg, ex prep. TLC) in MeOH (50 ml, dry), was added $SOCl_2$ (5 drops), and the orange solution stirred for 2 hr. Evapt'n under vacuo and purification by prep. TLC (EtOAc : $CHCl_3$ = 60:40) gave an amorphous red compound, Rf 0.60. Found: M (mass spectrometry), 386·0630. $C_{19}H_{14}O_9$ requires M, 386·0637. Also, found, 357·0608, $C_{18}H_{13}O_8$ = 357·0610; 299·0201, $C_{15}H_7O_7$ = 299·0192. Mass spectrum, m/e (%): 386 (38) (M^+), 357 (25), 325 (8), 309 (4), 299 (100), 272 (8), m* 386 → 357 → 299. Also, deoxydothistromin methyl ether impurity, found: M, 370·0686, $C_{19}H_{14}O_8$ requires M, 370·0688; 341·0652, $C_{18}H_{13}O_7$ = 341.0661; mass spectral peaks 370 (1.7) (M^+), 341 (4.0), 283 (6.8).

Dothistromin ethyl ether (49): Prepared by the same method described above for (48), but with EtOH replacing MeOH. The red compound, Rf 0.60, was crystallised from EtOH/H_2O to give small red needles, m.p. 197-210° (some crystals melting much earlier than others). Found: M (mass spectrometry), 400·0795. $C_{20}H_{16}O_9$ requires M, 400·0794. Mass spectrum, m/e (%): 400 (15) (M^+), 371 (20), 355 (4), 325 (9), 309 (4), 299 (100), 283 (9), 272 (9), m*: 400 → 371 → 325 → 283, 371 → 299. Deoxydothistromin ethyl ether impurity peak: 384 (0.5) (M^+).

Dothistromin n-butyl ether (50): Prepared as above, but n-butanol

replacing MeOH. Obtained a dark red amorphous powder, Rf 0.60. Found: M (mass spectrometry), 428·1108. $C_{22}H_{20}O_9$ requires M, 428·1107. Mass spectrum, m/e (%): 428 (10) (M^+), 399 (20), 383 (2), 325 (10), 309 (5), 299 (100), 283 (8), 272 (8), m*: 428 → 399 → 325, 399 → 299.

Dothistromin ethyl ether tetra-acetate (52): The ethyl ether (49) was dissolved in Ac_2O, pyridine (Ac_2O:pyd. 5:1), and allowed to stand for 8 hr. Evapt'n of the solvents from the golden yellow soln. gave a pale yellow gum, which crystallised from $CHCl_3$/hexane as the ethyl ether tetra-acetate (52), pale yellow needles, m.p. 193-194.5°, $[\alpha]_D$ - 156° (C 0.18). Found: M (mass spectroscopy), 568·1222. $C_{28}H_{24}O_{13}$ requires M, 568·1227. λ max (EtOH): 238, 276, 282, 348 (, 10,800; 19,500; 19,300; 3,400). NMR - see page 79 and Fig. 10. Mass spectrum, m/e (%): 568 (12) (M^+), 526 (14), 484 (100), 442 (31), 413 (9), 382 (59), 371 (12), 355 (15), 354 (15), 353 (39), 336 (12), 325 (22), 310 (5), 309 (17), 308 (19), 307 (14), 299 (41), 298 (15), 297 (15), 283 (8), m*: 568 → 526 → 484 → 442 → 382 → 353.

The same derivative was obtained by heating the ethyl ether (49) under reflux with Ac_2O and an. NaOAc for 6 hr. The product was poured into aqu. NaOAc to hydrolyse the Ac_2O, then ext'd with Et_2O, the Et_2O soln washed with aqu. $NaHCO_3$, and the solvent removed under vacuo.

Dothistromin n-butyl ether tetra-acetate (53): This derivative was prepared by the action of Ac_2O/pyridine on the methyl ether (50). Found, M (mass spectroscopy), 596. $C_{30}H_{28}O_{13}$ requires M, 596. Mass spectrum, m/e (%): 596 (12) (M^+), 554 (12), 512 (100), 470 (27), 410 (44), 354 (21), 353 (24), 325 (44), 309 (19),

308 (18), 307 (16), 299 (55), 298 (19), 297 (19), 283 (11), m*: 596 → 554 → 512 → 470 → 410 → 353.

Dothistromin mono-acetate (54): Addition of a catalytic quantity of $SOCl_2$ to a solution of dothistromin in HOAc, and isolation of the red product by prep. TLC (EtOAc : $CHCl_3$ = 60:40, Rf 0.6) gave amorphous dothistromin mono-acetate (54). Found, M (mass spectrometry), 414·0586. $C_{20}H_{14}O_{10}$ requires M, 414·0587. Mass spectrum, m/e (%): 414 (70) (M^+), 372 (2), 355 (19), 354 (20), 343 (11), 336 (3), 325 (74), 311 (9), 310 (8), 309 (15), 308 (11), 299 (100), 298 (14), 297 (14), 283 (6), 272 (10), m*: 414 → 372, 414 → 354 → 336, 343 → 299.

Methylation Reaction I: (see page 84). The reaction product, a bright yellow solution, was poured into aqu. K_2CO_3, ext'd with EtOAc, the EtOAc extract washed with H_2O, and evaporated to dryness under vacuo, to give a yellow-orange gum which was separated into two major components, Rf 0.65 and Rf 0.50, by prep. TLC (EtOAc : $CHCl_3$ = 60:40).

Component 1: Rf 0.65: Dothistromin dimethyl ether ethyl ether (67): Found: M (mass spectrometry), 428·1099. $C_{22}H_{20}O_9$ requires M, 428·1107. Also found, 399·1080, $C_{21}H_{19}O_8$ = 399·1079; 327·0505, $C_{17}H_{11}O_7$ = 327·0504. Mass spectrum, m/e (%): 428 (40) (M^+), 399 (41), 383 (5), 353 (3), 339 (4), 327 (100), 163.5 (4) (327^{2+}), m* 428 → 399 → 327.

Component 2: Rf 0.50: Dothistromin ethyl ether trimethyl ether (68): see methylation reaction II, below.

Methylation Reaction II: (see page 85 and page 86). Prep. TLC (EtOAc : $CHCl_3$ = 60:40) allowed sept'n of the reaction product into two major components, Rf 0.5 and Rf 0.7.

Component 1: Rf 0.5: Dothistromin ethyl ether trimethyl ether (68): Column chromatography (TLC grade silica gel G, using EtOAc : $CHCl_3$ = 60:40), allowed isolation of the ether (68), yellow needles ex $CHCl_3$/hexane, m.p. 252-254°C (phase change at 200-220°), $[\alpha]_D$ - 182° (C 0.56). Found: M (mass spectroscopy), 442·1263. $C_{23}H_{22}O_9$ requires M, 442·1263. NMR ($CDCl_3$) - see page 86. Mass spectrum, m/e (%): 442 (14) (M^+), 427 (3), 424 (6), 413 (46), 409 (2), 397 (8), 353 (7), 341 (100), 327 (8), 170.5 (3) (341^{2+}), m* 442 → 424, 442 → 413 → 341.

Component 2: Rf 0.7: Dothistromin ethyl ether tetramethyl ether (69): This derivative was obtained as a pale yellow gum. Found: M (mass spectroscopy), 456·1419. $C_{24}H_{24}O_9$ requires M, 456·1420. Also found, 421·1387, $C_{23}H_{23}O_8$ = 427·1392; 421·1154, $C_{23}H_{20}O_8$ = 424·1158; 367·0807, $C_{20}H_{15}O_7$ = 367·0817. NMR ($CDCl_3$), 7.33 (1H, s, Ar-H), 7.25 (2H, s, 2xAr-H), 6.07 (1H, s, acetal proton), 5.25 (1H, m, acetal proton), 4.03, 3.93, 3.92 (each 3H, s, aromatic-OMe's), 3.17 (3H, s, benzylic-OMe), 2.64 (2H, m, -C-$\underline{CH_2}$-CH-), 0.81 (3H, t, -$OCH_2\underline{CH_3}$). Mass spectrum, m/e (3): 456 (51) (M^+), 441 (17), 427 (74), 426 (13), 425 (28), 424 (36), 413 (26), 412 (13), 411 (36), 410 (13), 409 (21), 408 (15), 397 (15), 396 (15), 395 (49), 393 (11), 383 (45), 382 (57), 381 (38), 380 (13), 379 (27), 378 (13), 367 (100), 365 (40), 355 (12), 353 (23), 352 (15), 351 (34), 350 (11), 340 (11), 341 (55), 339 (28), 337 (30), 335 (15), 327 (11), 325 (17), 323 (19), 321 (15), 311 (55), 309 (15), 307 (15), m*: 456 → 427 → 367.

Methylation Reaction III: (see page 85 and page 87). The reaction mixture was poured into H_2O, extracted with $CHCl_3$, and purified by prep. TLC to yield an orange gum. The mass spectrum

of this product revealed it to be a mixture of two compounds of MW 456 and 442.

Methylation Reaction IV: (see page 85 and page 87). The ether (68), (20 mg), was added to $Ba(OH)_2.8H_2O$ (200 mg) in DMF (2 ml), and stirred at room temp. for 5 min. CD_3I (1 g) was then added, and the mixture refluxed for 2 hr. After pouring into H_2O, and extraction with $CHCl_3$, purification by prep. TLC afforded dothistromin ethyl ether tetramethyl ether-d_3 (70). Found: M (mass spectroscopy), 459. $C_{24}H_{21}D_3O_9$ requires M, 459. Mass spectrum, m/e (%): 459 (92) (M^+), 444 (23), 430 (100), 428 (25), 424 (45), 414 (40), 409 (25), 408 (13), 398 (35), 395 (38), 384 (38), 379 (25), 378 (22), 370 (67), 367 (23), 354 (15), 351 (15), 341 (40), 339 (22), 337 (16), m*: 459 → 430 → 370, 459 → 424, 444 → 414.

Methylation Reaction V: (see page 85 and page 88). Column chromatography of the reaction product on TLC grade silica gel G, using $CHCl_3$ as eluant allowed fractionation into two major (Rf 0.6, Rf 0.45)‡ and two minor components (Rf 0.95, Rf 0.95).‡

Component 1, Rf 0.6. Dothistromin ethyl ether trimethyl ether-d_9 (71): This component was intense orange in colour. Found: M (mass spectroscopy), 451. $C_{23}H_{13}D_9O_9$ requires M, 451. NMR ($CDCl_3$) - see page 88. Mass spectrum, m/e (%): 451 (100) (M^+), 422 (24), 419 (19), 406 (19), 405 (20), 387 (16), 376 (56), 359 (23), 343 (10), 333 (52), m*: 451 → 422 → 376, 451 → 419, 451 → 406, 451 → 405 → 333.

Component 2, Rf 0.45. Dothistromin ethyl ether tetramethyl ether

‡ Rf values on TLC with $CHCl_3$ as eluant

-d_{12} (75): This yellow compound was obtained as a gum, and is the d_{12}-labelled analogue of the ether (69). Found, M (mass spectroscopy), 468. $C_{24}H_{12}D_{12}O_9$ requires M, 468. Mass spectrum, m/e (%): 468 (100) (M^+), 450 (19), 439 (88), 434 (28), 432 (23), 404 (35), 403 (19), 393 (26), 388 (14), 387 (18), 386 (14), 376 (60), 360 (19), 350 (30), 348 (14), m*: 468 → 439 → 376, 468 → 432, 468 → 423.

Minor Components, Rf 0.95: This orange gum contained dothistromin diethyl ether ethyl ether-d_6 (76), found, M (mass spectroscopy), 434; $C_{22}H_{14}D_6O_9$ requires M, 434, and the deoxydothistromin derivative (77), M 418. Mass spectrum, m/e (%): 434 (100) (M^+), 418 (17) (M^+), 405 (32), 389 (25), 370 (13), 359 (49), 344 (15), 343 (19), 342 (25), 326 (15), 316 (25), m*: 434 → 405 → 359, 434 → 389 → 343, 405 → 342.

Methylation Reaction VI: (see page 85 and page 90). The two major products from this reaction had Rf values (EtOAc : $CHCl_3$ = 60:40) of 0.40 (major product) and 0.30, and were separated by column chromatography on TLC grade silica gel G, with $CHCl_3$: EtOAc = 1:1.

Component 1, Rf 0.40, Dothistromin pentamethyl ether (78): This was obtained as yellow crystals from $CHCl_3$/hexane, m.p. 156-179°; yellow needles ex MeOH/H_2O, m.p. 181-192°, $[\alpha]_D$ - 90° (C 0.40). Found, M (mass spectroscopy), 442·1259. $C_{23}H_{22}O_9$ requires M, 442·1262. Also, 413·1235, $C_{22}H_{21}O_8$ = 413·1235; 381·0967, $C_{21}H_{17}O_7$ = 381·0974; 367·0813, $C_{20}H_{15}O_7$ = 367·0817; 351·0870, $C_{20}H_{15}O_6$ = 351·0868. NMR ($CDCl_3$): 7.30 (1H, s, Ar-H), 7.21 (2H, s, 2xAr-H), 6.11 (1H, s, acetal proton), 5.18 (1H, m, acetal proton), 4.04, 3.95, 3.92 (each 3H, s, aromatic - OMe's),

3.17, 3.10 (both 3H, s, aliphatic -OMe's), 2.66 (2H, m, -$\underline{CH_2}$-CH-). NMR (acetone-d_6): 7.93 (0.5H, s, $CHCl_3$ of crystlln.), 7.38 (2H, s, 2xAr-H), 7.08 (1H, s, Ar-H), 6.16 (1H, s, acetal proton), 5.18 (1H, d, acetal proton), 3.95 (3H, s, Ar-OMe), 3.88 (6H, s, 2xAr-OMe), 3.14, 3.04 (both 3H, s, aliphatic -OMe's), 2.69 (2H, m, -$\underline{CH_2}$-CH-). Mass spectrum, m/e (%): 442 (100) (M^+), 427 (28), 425 (14), 413 (93), 411 (52), 410 (41), 397 (24), 395 (52), 394 (19), 381 (72), 379 (48), 367 (52), 365 (26), 351 (31), 341 (35), 337 (19), 335 (13), m*: 442 → 413 → 367.

Component 2, Rf 0.3, dothistromin tetramethyl ether (79): Found, M (mass spectroscopy), 428·1099. $C_{22}H_{20}O_9$ requires M, 428·1107. Also, 399·1072, $C_{21}H_{19}O_8$ = 399·1079; 341·0659, $C_{18}H_{13}O_7$ = 341·0661. NMR ($CDCl_3$): 7.17 (2H, s, 2xAr-H), 7.16 (1H, s, Ar-H), 6.04 (1H, s, acetal proton), 5.19 (1H, d, acetal proton), 3.98, 3.92, 3.89 (each 3H, s, aromatic -OMe's), 3.62 (1H, s, benzylic -OH), 3.11 (3H, s, aliphatic -OMe), 2.61 (2H, m, -$\underline{CH_2}$-CH-). Mass spectrum, m/e (%): 428 (32) (M^+), 410 (10), 413 (7), 399 (38), 397 (9), 395 (6), 383 (5), 381 (8), 379 (10), 378 (8), 367 (7), 353 (9), 341 (100), 327 (10), 311 (6), m*: 428 → 399 → 341.

DOTHISTROMIN HEAVY ATOM DERIVATIVES

Dothistromin p-bromobenzyl ether (80): Red pigment heated in excess molten p-bromobenzyl alcohol at 80-100° C for 6 hr, followed by removal of the p-bromobenzyl alcohol by distillation under vacuo, gave the ether (80). This p-bromobenzyl ether was remarkably insoluble in the usual solvents used for the red pigment and its derivatives, and the amorphous red powder could not be induced to crystallise. Found: M (mass spectroscopy), 540.

$C_{25}H_{17}O_9Br$ requires M, 540. Mass spectrum, m/e (%): 542 (62) ($M+2^+$), 540 (62) (M^+), 513 (32), 511 (32), 497 (5), 495 (5), 354 (9), 325 (20), 299 (100), m*: 542 → 513, 540 → 511.

Dothistromin Chloroacetate (81): see page 91. Treatment of red pigment with molten $CH_2ClCOOH$ for 4 hr, followed by removal of the excess $CH_2ClCOOH$ by distillation under vacuo and purification by prep. TLC (EtOAc : $CHCl_3$ = 60:40), gave (81) as an amorphous red powder. Found, M (mass spectroscopy) 448. $C_{20}H_{13}O_{10}Cl$ requires M, 448. Mass spectrum, m/e (%): 450 (22) ($M+2^+$), 448 (58) (M^+), 354 (46), 336 (6), 325 (100), 309 (32), 308 (35), 299 (63), 298 (21), 297 (21), 283 (10), 272 (11), 162.5 (5), 154.5 (9), m*: 448 → 354 → 336.

Dothistromin chloroacetate tetra-acetate (82): Acetylation of the chloroacetate (81) (150 mg) with Ac_2O/pyridine (9:1) for 3 hr, followed by isolation of the product and purification by prep. TLC, allowed isolation of a yellow gum which gave small, pale yellow crystals (126 mg) of (82) (solvated with 1 molecule of toluene/molecule - see NMR) from toluene, m.p. 121-124° C. Found, M (mass spectroscopy), 616·0608. $C_{28}H_{21}O_{14}Cl$ requires M, 616·0620. NMR ($CDCl_3$), see page 92. Mass spectrum, m/e (%): 618 (1) ($M+2^+$), 616 (3) (M^+), 576 (4), 574 (9), 534 (36), 532 (100), 492 (24), 490 (59), 438 (24), 430 (11), 397 (11), 396 (11), 378 (9), 354 (76), 336 (88), 325 (64), 309 (41), 308 (77), 307 (21), 299 (21), 298 (21), 297 (30), m*: 574 → 532 → 490.

Dothistromin iodoacetate tetra-acetate (83): The chloroacetate tetra-acetate (82) was refluxed in acetone sat'd with NaI, for 6 hr. At the end of the reaction a white granular ppte. had formed. The acetone was evapt'd off under vacuo, and the yellow

solid dissolved in Et_2O and ext'd with H_2O. Evapt'n of the Et_2O gave a yellow solid which yielded small irregular shaped crystals from toluene, of (83) (toluene solvate), m.p. behaviour like "liquid crystals", m.p. range 85-120°. Found, M (mass spectroscopy) 708. $C_{28}H_{21}O_{14}I$ requires M, 708. NMR ($CDCl_3$): 7.60 (1H, s, Ar-H), 7.36 (2H, s, 2xAr-H), 7.20 (5H, s, Ar-H, toluene of crystlln.), 6.54 (1H, s, acetal proton), 6.52 (1H, m, acetal proton), 3.31 (2H, s, $-OCOCH_2Cl$), 2.90 (2H, m, $-\underline{CH_2}-CH-$), 2.45 (6H, s, 2xα-OAc's), 2.40 (3H, s, 1xα-OAc), 2.36 (3H, s, $-CH_3$ of toluene of crystlln.), 2.01 (3H, s, benzylic -OAc). Mass spectrum, m/e (%): 708 (0.5) (M^+), 666 (2), 624 (15), 582 (10), 438 (38), 396 (8), 378 (8), 354 (37), 336 (100), 325 (30), 308 (45), 297 (11), m*: 708 → 666 → 624 → 582.

Dothistromin bromo-ethyl ether (84): To red pigment in 2-bromo ethanol, stirred, was added a catalytic quantity of $SOCl_2$. After stirring for 4 hr, then standing overnight, the red soln. was poured into excess Et_2O, and the resultant soln. washed with aqu. $NaHCO_3$, then repeatedly with H_2O until free of 2-bromo ethanol. Evapt'n of the Et_2O under vacuo, followed by column chromatography (TLC grade silica gel G, EtOAc : $CHCl_3$ = 60:40), allowed isolation of the ether (84). Fine red crystals were obtained from toluene, m.p. 230-235° (phase change 222-225°). Found, M (mass spectroscopy) 477·9899. $C_{20}H_{15}BrO_9$ requires M, 477·9900. Mass spectrum, m/e (%): 480 (27) ($M+2^+$), 478 (27) (M^+), 451 (10), 449 (10), 340 (11), 325 (17), 299 (100), 283 (7), 272 (7), m*: 480 → 451 → 299, 478 → 449 → 299.

Dothistromin bromo-ethyl ether tetra-acetate (85): see page 93. Acetylation of the bromo-ethyl ether (84) with refluxing

Ac_2O/NaOAc for 3 hr, and isolation of the product gave a yellow-brown gum; purification by prep. TLC (benzene : EtOH = 50:2, plate developed twice) and crystallisation from $CHCl_3/Et_2O$ gave small yellow prisms, m.p. 168-174°, $[\alpha]_D$ - 103° (C 0.12). Found, M (mass spectroscopy) 648·0305. $C_{28}H_{23}O_{13}Br$ requires M, 648·0302. NMR ($CDCl_3$), 7.46 (1H, s, Ar-H), 7.32 (2H, s, 2xAr-H), 6.45 (1H, s, acetal proton), 5.38 (1H, d, acetal proton), ca. 3.7 (2H, m, $-O\underline{CH_2}CH_2Br$), ca. 3.1 (2H, m, $-OCH_2\underline{CH_2}Br$), 2.7 (2H, m, $-\underline{CH_2}-CH-$), 2.43 (6H, s, 2xα-OAc's), 2.39 (3H, s, α-OAc), 1.97 (3H, s, benzylic -OAc). Mass spectrum, m/e (%): 650 (5) ($M+2^+$), 648 (5) (M^+), 606 (9), 604 (9), 564 (100), 562 (100), 522 (40), 520 (40), 462 (23), 460 (23), 433 (10), 431 (10), 353 (63), 325 (47), 309 (28), 308 (36), 299 (58), 298 (23), 297 (23), 109 (53), 107 (53), m*: 648 → 606 → 564 → 522 → 462 → 353, 520 → 460 → 353.

Dothistromin tetramethyl ether (88): see page 98. Found, M (mass spectroscopy) 428·1108. $C_{22}H_{20}O_9$ requires M, 428·1107. Mass spectrum, m/e (%): 428 (38) (M^+), 413 (12), 399 (13), 396 (11), 382 (21), 381 (16), 380 (15), 379 (12), 378 (10), 367 (100), 365 (27), 353 (32), 351 (26), 339 (12), 341 (16), 339 (29), 337 (21).

Oxidation of hemiacetal (88) to the lactone (89): The hemiacetal (88) (~5 mg) in HOAc (1.0 ml), was treated with $Na_2Cr_2O_7$ (10 mg) in the same solvent (1.0 ml), for 12 hr at 20°. H_2O (10 ml) was then added, the aqu. mixture ext'd with Et_2O (10 ml) and EtOAc (10 ml), the combined extracts dried, filtered, evapt'd under vacuo and purified by prep. TLC (EtOAc : $CHCl_3$ = 60:40), to give ca. 5 mg of the lactone (89), as a yellow-

orange gum. Found: M (mass spectroscopy), 428·1108. $C_{22}H_{20}O_9$ requires M, 428·1107. Mass spectrum, m/e (%): 428 (38) (M^+), 413 (12), 399 (13), 396 (11), 382 (21), 381 (16), 380 (15), 379 (12), 378 (10), 367 (100), 365 (27), 353 (32), 351 (26), 339 (12), 341 (16), 339 (29), 337 (21).

APPENDIX

Metastable Ions - Detection by the Defocusing Technique

The condition for the ion beam to pass centrally through the electrostatic analyser and monitor slit in a double focusing mass spectrometer is

$$\frac{\text{Energy of ion beam}}{\text{ESA Voltage x Ionic Charge}} = \frac{V}{E} = \text{Constant}$$

where V = ion accelerating voltage, and E = electrostatic analyser voltage. In the double focusing instrument E and V can normally be adjusted relative to one another to establish the correct ratio for proper focusing. This is so in the AEI MS9 double focusing mass spectrometer used for mass spectroscopy during the work of this thesis. The circuitry is such that once the correct ratio is established for central transmission of the ions, then any alteration of the ion accelerating voltage (as required for mass measurement by the peak matching technique, or for changing the mass range) causes a corresponding alteration in E so that the ratio remains the same and the transmission conditions are unaltered.

For metastable transitions $m_1^+ \longrightarrow m_2^+ + (m_1-m_2)$ occurring in the field free region preceding the electrostatic analyser, the energy of any daughter ions m_2^+ formed is not sufficient for transmission through the electrostatic analyser, and in normal operation of the mass spectrometer these ions are lost from the

beam. By arranging the circuitry so that E can be adjusted independently of V if required, it is possible to alter the ESA transmission. If this is done, and the ratio V/E is raised above normal by the factor m_1/m_2, the main ion beam is defocused and lost, whilst only daughter ions m_2^+ formed from decomposition in the field free region, are transmitted into the magnetic analyser and collected. Thus if the magnetic analyser is set to collect ions of mass m_2 at normal V/E, and V is then changed continuously, a series of ions m_2^+ will be collected at various ratios V/E. A change in V to a value V_1 to bring into focus a particular group of m_2 ions indicates that these ions arose from the metastable transition $m_1^+ \longrightarrow m_2^+ + (m_1-m_2)$, where $m_1/m_2 = V_1/V$. Since V, V_1 and m_1 are known, m_2 can be readily calculated. In this way, a number of precursor ions are often identified for each fragment ion, and it is thus possible to obtain a precursor ion spectrum for each fragment ion in the normal mass spectrum.

References:

(1) J.H. Beynon, R.A. Saunders, and A.E. Williams, The Mass Spectra of Organic Molecules, Elsevier, Amsterdam, 1968.

(2) M. Barber and R.M. Elliot, Twelfth Annual Conference on Mass Spectrometry and Allied Topics, ASTM Committee E-14, Montreal, Canada, 1964, p.150.

Counter Current Distribution

Counter Current Distribution (abbreviated CCD)‡ is a stepwise multiple extraction procedure for fractionation of a solute by distributing it into a number of cells containing two immiscible (or partially immiscible) solvents. The process can be treated mathematically with considerable precision, and calculations made by use of the binomial theorem (Goodall, 1964; Craig and Craig, 1956). Typically, high numbers of extractions and transfers are needed for the separation of complicated solute mixtures, and an apparatus was developed whereby many extractions and transfers are accomplished quantitatively in one operation. This apparatus is known as the "Craig counter current distribution apparatus", after Craig (1944). An excellent comprehensive account of CCD is given by Craig and Craig (1956).

‡ The term counter-current implies that the two immiscible phases move across one another in opposite directions. It is now commonly used in connection with Craigs (1944) apparatus, so that it is not strictly accurate, since in the Craig apparatus only one phase moves (Goodall, 1954).

The Fluorescence of 1,4-Dihydroxyanthraquinone

Electronically Excited States of Organic Molecules and Luminescence

Absorption of visible or ultraviolet light by an organic molecule in the ground state raises the molecule to an excited electronic state.‡ These excited states are of quite high energy - absorption at 200 nm corresponds to 143.2 kcal/mole, and the excited molecules are thus quite labile. The excited states may be deactivated by radiative and nonradiative processes, and these processes are conveniently represented by a modified Jablonski diagram, see Fig. 11 (Kan, 1966; Turro, 1967).

Deactivation by radiation (emission): The simplest process of deactivation is a luminescent emission from the excited singlet state to the singlet ground state, of a light quantum of the same, or substantially the same, frequency as the quantum which produced the excitation. This is the phenomenon of fluorescence. A second type of emission occurs through deactivation by radiation from the excited triplet state to the singlet ground state, and is called phosphorescence.

Deactivation by radiationless processes: Two important types of radiationless processes are internal conversion - the intramolecular radiationless interconversion between different electronic states of like multiplicity, i.e. singlet-singlet or triplet-

‡ The electronic ground state of most organic molecules is a singlet state (all electrons spin paired), and the excited electronic states are singlet and triplet states. For every excited state arising from a single electron promotion there is a singlet state and a triplet state; the triplet state is always of lower energy than its corresponding singlet state.

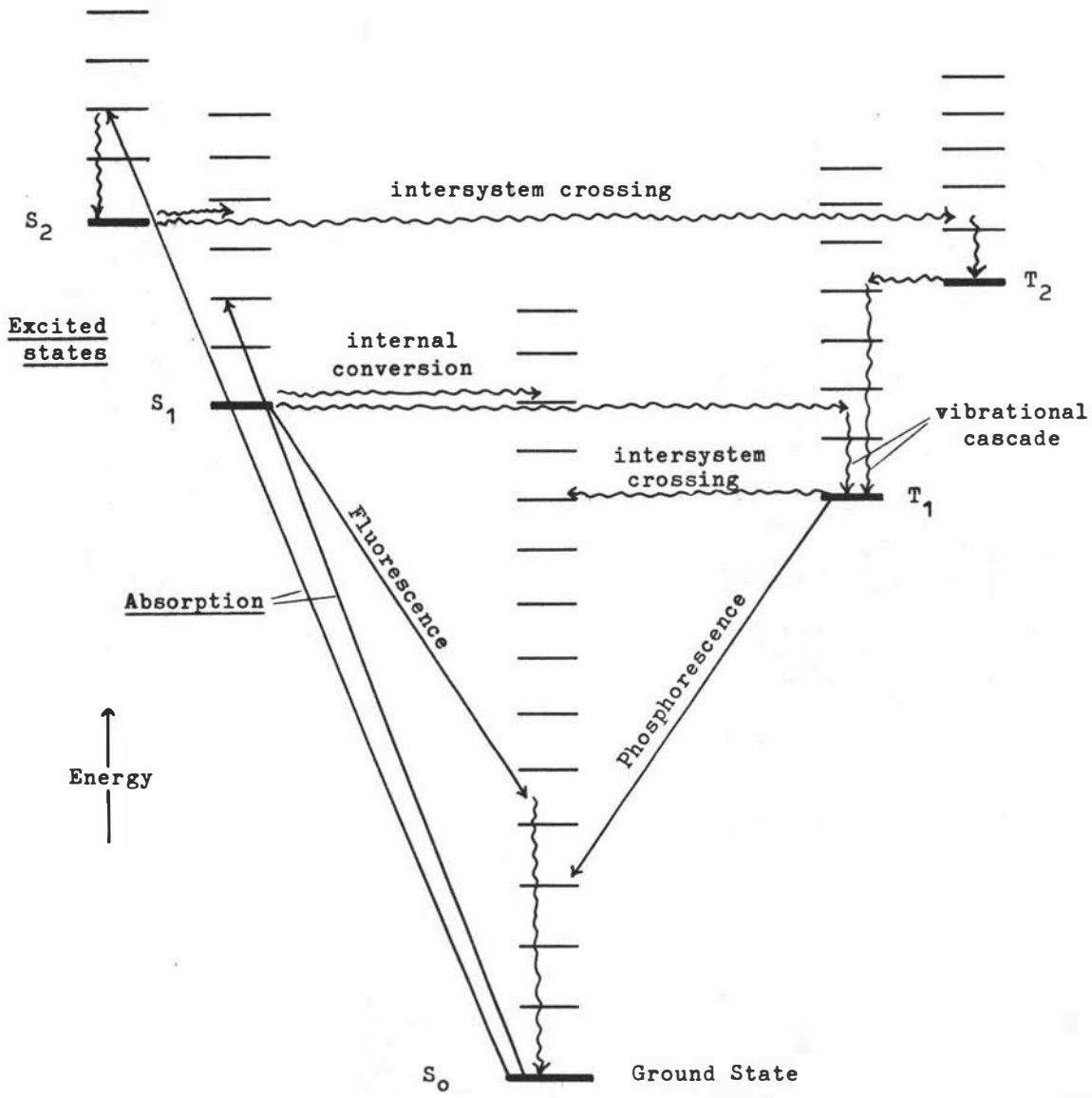


Fig. 11

Modified Jablonski diagram, showing processes which may occur on absorption of light by an organic molecule in the singlet electronic ground state. Solid lines indicate processes involving absorption or emission of light. Wavy lines indicate nonradiative processes. S_o is the ground state, S_1, S_2 are excited singlet states, and T_1, T_2 are excited triplet states. (Only electronic and vibrational levels are shown in this diagram).

triplet processes ($S_1 \rightsquigarrow S_0$, $T_2 \rightsquigarrow T_1$, Fig. 11) and intersystem crossing - the intramolecular radiationless interconversion between singlet and triplet states ($S_1 \rightsquigarrow T_1$, $T_1 \rightsquigarrow S_0$, Fig. 11). Excesses of vibrational energy which result from these radiationless processes are carried away rapidly as heat by collisions with surrounding molecules, via rapid vibrational "cascades". Other radiationless processes include chemical reactions (which includes "predissociation"), and sensitisation - direct electronic-energy transfer from the excited molecule (donor) to an acceptor molecule.

Whereas π,π^* states generally have large singlet-triplet energy separations, those for n, π^* states are generally small; also, n, π^* excited states have longer lifetimes than π,π^* states. Both these factors account for the fact that intersystem crossing occurs with a much greater probability from an n, π^* excited state than from a π,π^* state. Thus, if the lowest excited singlet state is n, π^*, intersystem crossing is enhanced at the expense of fluorescence, usually removing fluorescence as a major pathway for the loss of excitation energy (Hercules, 1966).

Fluorescence and molecular structure (Jaffé and Orchin, 1962; Turro, 1965; Hercules, 1966; Kan, 1966; West, 1967): Most organic compounds do not fluoresce, quenching occurring by one or more of the various competitive deactivation processes described above.

For polyatomic molecules, deactivation by predissociation is often important. Clearly, if the electronic excitation energy of the polyatomic molecule is much greater than the bond

strength of the weakest bond linkage, then the probability of predissociation will be relatively high. This would appear to be the main reason for the non-fluorescence of compounds whose absorption bands are in the far UV - the high energy ($\sim$150 kcal/mole) of excitation exceeds certain bond energies in the molecule. Thus, saturated hydrocarbons, ethers, alcohols, and acids do not show UV fluorescence.

Functional groups which introduce a long wavelength n-π^* transition into the absorption spectrum of a molecule tend to reduce the fluorescence efficiency of that molecule to zero (Hercules, 1966). In many aromatic carbonyl compounds the lowest energy electronic transition is of n, π^* character. Thus, benzophenone, acetophenone, and anthraquinone do not show measurable fluorescence, but exhibit intense phosphorescence. However, aromatic aldehydes or ketones containing substituents capable of hydrogen-bonding with the carbonyl oxygen (e.g. OH, NH_2) may fluoresce, the hydrogen bond removing the perturbing influence of the non-bonding electrons on the oxygen atom. Thus, anthraquinone is non-fluorescent, whereas 1,4-dihydroxyanthraquinone fluoresces (see below); benzoic acid is not significantly fluorescent, whereas salicylic acid (orthohydroxybenzoic acid) is strongly fluorescent. Similar effects of hydrogen bonding by solvents can be observed on a number of aromatic aldehydes and ketones, and heterocyclic nitrogen compounds.

The majority of fluorescent organic compounds contain cyclic arrangements of conjugated chains. The conjugated chain is important as a factor conducive to fluorescence, because of the relatively low energy of excitation associated with absorption

bands of long wavelength.

Compact planar ring structures also appear to favour fluorescence. The compactness induces a rigidity of the molecule as a whole; in non-rigid structures low frequency vibrations increase the probability of deactivating radiationless transitions (West, 1967). Molecular rigidity can be achieved in various ways, e.g. fused rings, chelation, and adsorption on a surface.

Fluorescence is favourable in aromatic and condensed hydrocarbons since these compounds absorb at relatively long wavelength and have very rigid structures. Of course, substituents in the active chromophore of these compounds should not have weak bonds which would be susceptible to predissociation.

The fluorescence of 1,4-dihydroxyanthraquinone: As stated above, anthraquinone does not show measurable fluorescence, whereas 1,4-dihydroxyanthraquinone does. The explanation for this behaviour is as follows.

In the ground state, anthraquinone dissolved in alcohol is stable and shows no tendency to react. However in the excited state anthraquinone rapidly abstracts a hydrogen atom from the alcohol and is reduced to 9,10-dihydroxyanthracene, oxidising the alcohol to an aldehyde. This photo reduction of anthraquinone is a rapid, efficient reaction having a quantum efficiency approaching unity (Bolland and Cooper, 1954; Dearman and Chan, 1966; Hercules, 1966). Anthraquinone has a low intensity n, π^* absorption band at 405 nm, and the anthraquinone molecule is thus first excited to the n, π^* singlet state corresponding to this absorption, which then converts to the n, π^* triplet state, which undergoes chemical reaction.

α-Hydroxyanthraquinones on the other hand, have intense π,π* absorption bands in the visible region, the wavelength of these bands increasing with the number of α-hydroxyl groups present (see page 33 for discussion and references). These intense π,π* bands apparently "bury" the low intensity n, π* carbonyl absorption band expected in this region. In the case of 1,4-dihydroxyanthraquinone, this species has an upper wavelength limit to its long wavelength π,π* band at 520 nm (Morton and Earlam, 1941; Scott, 1964; El Ezaby et al., 1970). Thus, 1,4-dihydroxyanthraquinone has its lowest excited singlet state (S_1) at significantly lower energy than anthraquinone, and moreover, this is a π,π* state in contrast to that of anthraquinone which is n, π*. Further, 1,4-dihydroxyanthraquinone also has a compact planar ring structure which should have a high degree of rigidity because of the chelate rings formed by strong intramolecular hydrogen bonding with both carbonyl groups. In this case then, quenching by internal conversion and predissociation should be unfavourable, and intersystem crossing to the triplet state should also be unfavourable, with the result that 1,4-dihydroxyanthraquinone can reasonably be expected to fluoresce in suitable solvents.

In the case of the mono-α- and other di-α-hydroxyanthraquinones, the upper wavelength limit of the long wavelength π,π* absorption band occurs at lower wavelength (i.e. higher energy) than 1,4-dihydroxyanthraquinone. For example, 1,5- and 1,8-dihydroxyanthraquinone have limits of 437 nm and 457 nm respectively. Thus higher energy excitation is required for fluorescence to be possible, and therefore there is a greater chance of quenching by interanl conversion and predissociation, compared to 1,4-dihydroxyanthraquinone. However, whilst this would account for diminished fluorescence efficiency, it appears more likely that the main reason for non-

fluorescence in the case of these species could be deactivation by a considerably more efficient intersystem crossing, from the singlet π,π^* level to the triplet manifold. This can best be seen by consideration of the electronic state diagrams for the various molecules concerned. Although insufficient data is available in the literature to construct complete energy level diagrams of the lower electronic states of the anthraquinones under discussion, the type of diagram, depicting the relative qualitative order of the states, can be drawn up, as shown in Fig. 12. If the assumption is made that mono-α-hydroxy and 1,8-dihydroxyanthraquinones have n,π^* states at approx. the same energy levels as in the parent anthraquinone,‡ then the triplet n,π^* state could lie just below the level of the singlet π,π^* state, allowing highly efficient intersystem crossing ($S_1 \rightarrow T_2$). Thus, it has been shown that intersystem crossing between states of different orbital character (i.e. $^1n,\pi^* \leftrightarrow {}^3\pi,\pi^*$, or $^1\pi,\pi^* \leftrightarrow {}^3n,\pi^*$) takes place at a considerably greater rate than between states with the same orbital character (i.e. $^1n,\pi^* \leftrightarrow {}^3n,\pi^*$, or $^1\pi,\pi^* \leftrightarrow {}^3\pi,\pi^*$) (see S.K. Lower and M.A. El-Sayed, Chem.Rev., 66, 199 (1966)). This intersystem crossing would be followed by rapid internal conversion to the lowest lying triplet state, the π,π^* state.

‡ This assumption is not unreasonable, in view of the behaviour of these compounds in the infrared region - see discussion, page 69; thus, e.g., 1,8-dihydroxyanthraquinone shows a chelated and a free carbonyl group in its IR spectrum.

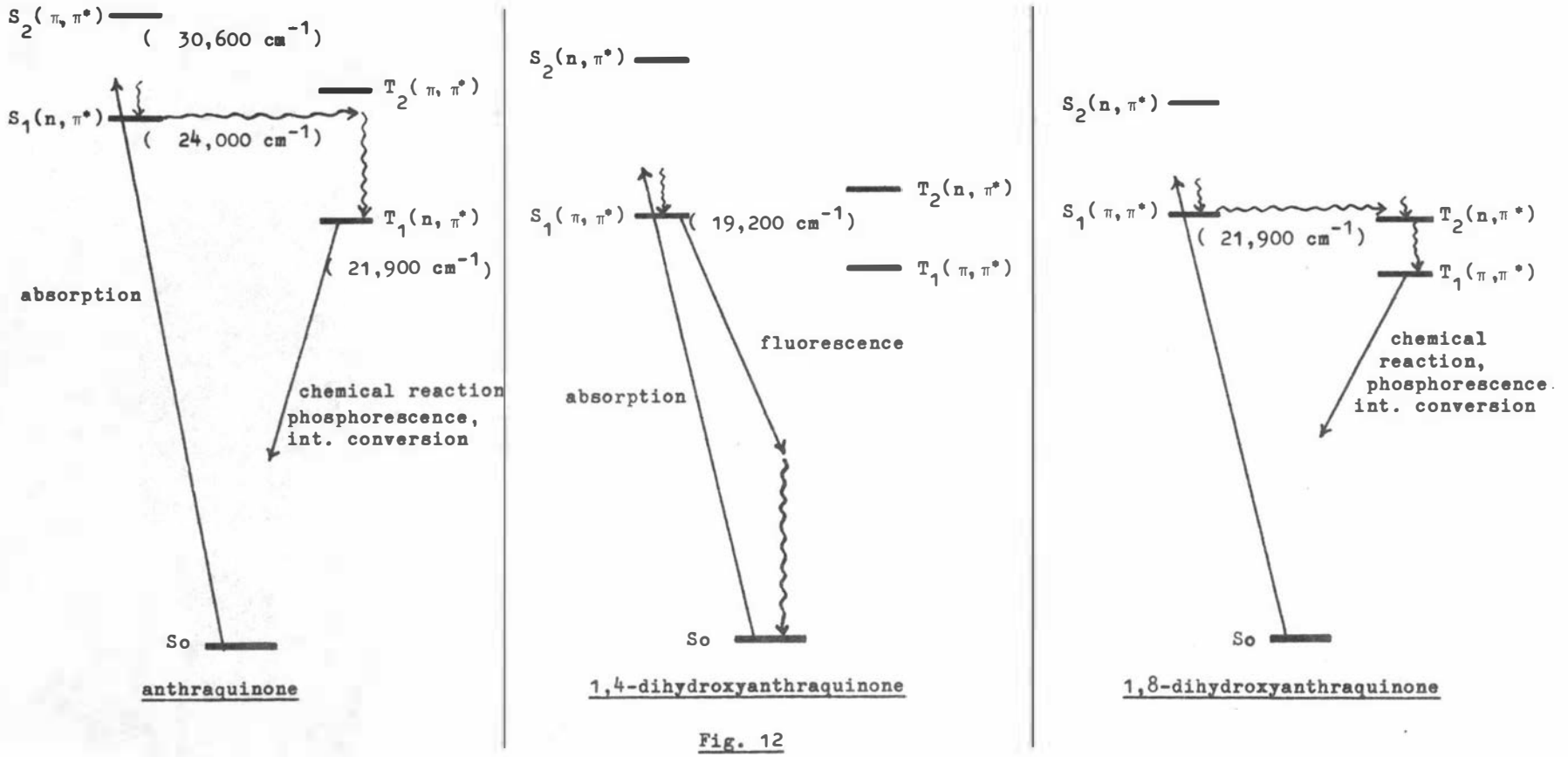


Fig. 12

Energy Level Diagram Types for Electronic States of Anthraquinones

Sterigmatocystin, the Aflatoxins, Aversin, and Versicolorin

A number of compounds possessing a dihydro- or tetrahydrofuro[2,3-b]benzofuran structural feature are now known. These include sterigmatocystin, the aflatoxins, aversin and versicolorin. These compounds are all fungal metabolites, and the published chemistry on them is very new, a major portion appearing over the last few years.

Sterigmatocystin

Sterigmatocystin ((99), R_1 = Me, R_2 = H) was the first known natural product to contain the dihydrofuro[2,3-b]benzofuran ring system (Bullock et al., 1962). Several other naturally occurring derivatives of sterigmatocystin have since been isolated: O-methylsterigmatocystin ((99), $R_1 = R_2$ = Me) (Burkhardt and Forgacs, 1968); 5-methoxysterigmatocystin (100) (Holker and Kagal, 1968); aspertoxin (101) (Rodricks et al., 1968; Waiss et al., 1968); dihydro-O-methylsterigmatocystin (102) (Cole et al., 1970); demethylsterigmatocystin ((99), $R_1 = R_2$ = H) (Elsworthy et al., 1970).

The synthesis of (±)-dihydro-O-methyl sterigmatocystin (Rance and Roberts, 1969), and (±)-O-methyl sterigmatocystin (Rance and Roberts, 1970), and the conversion of sterigmatocystin into dihydroaspertoxin (Hutchinson and Holzapfel, 1971) have been reported. Also, an x-ray analysis has confirmed the structure for sterigmatocystin (Tanaka et al., 1970).

Sterigmatocystin is toxic and carcinogenic (Dickens et al., 1966; Purchase and van der Watt, 1968; Lillehoj and Ciegler, 1968; Abedi and Scott, 1969).

The Aflatoxins

The aflatoxins are highly toxic and exceedingly carcinogenic fungal metabolites produced by a number of *Aspergillus* and *Penicillium* species. A comprehensive book has been published on the aflatoxins (Goldblatt, 1969), and several reviews have appeared (Wogan, 1966; Matthew, 1970). "Official" analytical methods for the determination of the aflatoxins, as food mycotoxins, have been proposed, and work in this field continues to be actively investigated (see, e.g. Stoloff, 1971).

The two major aflatoxins are aflatoxin B_1 ((103), R = H), and aflatoxin G_1 ((105), R = H); the structures shown for these were proposed by Asao and Büchi *et al*. in 1963 and confirmed by the same workers in 1965. Since then a number of other aflatoxins have been identified; these include aflatoxin B_2 ((104), $R_1 = R_2 = H$); B_{2a} ((104), $R_1 = OH$, $R_2 = H$); G_2 ((106), $R_1 = R_2 = H$); G_{2a} ((106), $R_1 = OH$, $R_2 = H$); M_1 ((103), R = OH); and M_2 ((104), $R_1 = H$, $R_2 = OH$) (see above reviews), and aflatoxins GM_1 ((105), R = OH) and B_3 (Heathcote and Dutton, 1969).

The total synthesis of aflatoxin B_1 (Büchi *et al*., 1967) and aflatoxins M_1 and G_1 (Büchi and Weinreb, 1969 and 1971) have been reported. The structures of aflatoxins B_2 and G_1 have been confirmed by x-ray crystallography (van Soest and Peerdeman, 1964; Cheung and Sim, 1964); no determination of the absolute configuration was made by this method. The absolute configuration of aflatoxin B_1, and hence of B_2, has been determined (Brechbühler *et al*., 1967) by chemical degradation to an optically active aliphatic acid whose absolute stereochemistry was known. Since the circular dichroism curves of

aflatoxin B_1 and G_1 are essentially superimposable, and G_1 may be hydrogenated to G_2, these workers proposed that the G series also had the same absolute stereochemistry of the hydrofuran ring system as for the B series.

Aversin

The anthraquinone aversin ((107), $R_1 = R_3 = Me$, $R_2 = H$) was reported in 1963 as a metabolite of a variant strain of A.versicolor (Bullock et al., 1963). The synthesis of (±)-O-methylaversin has just recently been reported (Holmwood and Roberts, 1971).

Versicolorin

The anthraquinone pigments versicolorin A ((108), $R_1 = R_2 = R_3 = H$), and versicolorin B ((107), $R_1 = R_2 = R_3 = H$) were isolated from A.versicolor (Hamasaki et al., 1965 and 1967). Versicolorin C, a third anthraquinone pigment isolated from the same fungus, was shown to be a racemate of versicolorin B. The isolation of the anthraquinone versiconol (109) was reported in 1969, and it was proposed that this could be a precursor of versicolorin C (Hatsuda et al., 1969). 6-Deoxyversicolorin A was reported in 1970, and it appears that this could be the biological precursor of sterigmatocystin (Elsworthy et al., 1970). Finally, very recently, the isolation of 6,8-O-dimethylversicolorin A ((108), $R_1 = H$, $R_2 = R_3 = Me$) has been reported (Hatsuda et al., 1971).

REFERENCES

Z.H. Abedi and P.M. Scott, J.Assoc.Offic.Anal.Chem., 52, 963 (1969).

T. Aczel and H.B. Lumpkin, Anal.Chem., 32, 1819 (1960).

F.C. Alderweireldt, Anal.Chem., 33, 1920 (1961).

S.J. Angyal, Angewandte Chemie, 8, 157 (1969).

F. Arcamone, G. Franceshi, P. Orezzi, S. Penco, and R. Mondelli, Tetrahedron Lett., 3349 (1968).

T. Asao, G. Büchi, M.M. Abdel-Kader, S.B. Chang, E.L. Wick, and G.N. Wogan, J.Amer.Chem.Soc., 85, 1706 (1963); idem, ibid, 87, 882 (1965).

B.D. Astill and J.C. Roberts, J.Chem.Soc., 3302 (1953).

C.S. Barnes and J.L. Occolowitz, Aust.J.Chem., 16, 219 (1963).

D.H. Barton, H.T. Cheung, A.D. Cross, L.M. Jackman, and M. Martin-Smith, J.Chem.Soc., 5061 (1961).

C. Bassett, R.T. Sherwood, J.A. Kepler, and P.B. Hamilton, Phytopathology, 57, 1046 (1967).

C. Bassett and P.J. Brunt, pers.commun., (1968). See also, P.J. Brunt, An Investigation into the Extracellular Enzymes Produced by Dothistroma Pini Hulbary and Their Possible Relation to Pathogenicity, M.Sc. Thesis, Massey University, N.Z. (1970).

C. Bassett, pers.commun., (1970).

L.J. Bellamy, The Infrared Spectra of Complex Molecules, Methuen and Co. Ltd., London, (1966).

L.J. Bellamy, Advances in Infrared Group Frequencies, Methuen and Co. Ltd., London, (1968).

J.H. Beynon, Mass Spectrometry and Its Applications to Organic Chemistry, Elsevier, Amsterdam, p.360 (1960).

G. Biernoth, J.Chromatog., 36, 325 (1968).

M. Biollaz, G. Büchi, and G. Milne, J.Amer.Chem.Soc., 92, 1035 (1970).

J.H. Birkinshaw, Biochem.J., 59, 485 (1955).

H. Bloom, L.H. Briggs, and B. Cleverly, J.Chem.Soc., 178 (1959).

J.L. Bolland and H.R. Cooper, Proc.Roy.Soc. (Lond.), 225A, 405 (1954).

J.H. Bowie, P.J. Hoffmann, and P.Y. White, Tetrahedron, 26, 1163 (1970).

S. Brechbühler, G. Büchi, and G. Milne, J.Org.Chem., 32, 2641 (1967).

L.H. Briggs and J.C. Dacre, J.Chem.Soc., 564 (1948).

L.H. Briggs and G.A. Nicholls, J.Chem.Soc., 1241 (1949).
L.H. Briggs, G.A. Nicholls, & R.L. Paterson, J.Chem.Soc., 1718 (1952).
L.H. Briggs, Dept. of Chemistry, Auckland University, N.Z. (1970).

H. Brockmann and W. Müller, Ber., 92, 1164 (1959).

J.M. Brown, Tetrahedron Lett., 2215 (1964).

G. Büchi, D.M. Foulkes, M. Kuron, G.F. Mitchell, and R.S. Schneider, J.Amer.Chem.Soc., 89, 6745 (1967).

G. Büchi and S.M. Weinreb, J.Amer.Chem.Soc., 91, 5408 (1969); idem, ibid, 93, 746 (1971).

H. Budzikiewicz, C. Djerassi, and D.H. Williams, Mass Spectrometry of Organic Compounds, Holden-Day Inc., San Francisco, (1967).

E. Bullock, J.C. Roberts, and J.G. Underwood, J.Chem.Soc., 4179 (1962).

E. Bullock, D. Kirkaldy, J.C. Roberts, and J.G. Underwood, J. Chem.Soc., 829 (1963).

E. Bullock, E.E. Burnell, and B. Gregory, Chem.Comm., 193 (1967).

H.J. Burkhardt and J. Forgacs, Tetrahedron, 24, 717 (1968).

H. Burton and P.F.G. Praill, J.Chem.Soc., 755 (1952).

W. Carruthers, H.N.M. Stewart, P.G. Hansell, and K.M. Kelly, J.Chem.Soc.(C), 2607 (1967).

F.D. Chattaway, J.Chem.Soc., 2495 (1931).

A.S. Cherkasov, Optics and Spectroscopy, 6(4), 315 (1959).

K.K. Cheung and G.A. Sim, Nature, 201, 1185 (1964).

E. Clar, Polycyclic Hydrocarbons, Academic Press, N.Y. (1964).

R.J. Cole, J.W. Kirksey, and H.W. Schroeder, Tetrahedron Lett., 3109 (1970).

J.E.T. Corrie, G.H. Green, E. Ritchie, and W.C. Taylor, Aust.J. Chem., 23, 133 (1970).

L.C. Craig, J.Biol.Chem., 155, 519 (1944).

L.C. Craig and D. Craig in Technique of Organic Chemistry, A. Weissberger ed., Vol.III, 2nd Edit., Interscience, N.Y., p.149 (1956).

K.G. Dave, B.S. Joshi, A.V. Patwardhan, and K. Venkataraman, Tetrahedron Lett., 22 (1959).

J.S. Davies, V.H. Davies, and C.H. Hassall, J.Chem.Soc.(C), 1873 (1969).

H.H. Dearman and A. Chan, J.Chem.Phys., 44, 416 (1966).

F. Dickens, H.E.H. Jones, and H.B. Waynforth, Brit.J.Cancer, 20, 134 (1966).

S.E. Drewes, J.Chem.Soc.(C), 1140 (1968).

M.F. Dutton and J.G. Heathcote, Chem. and Ind. (Lond.), 418 (1968); idem, ibid, 983 (1969).

G.C. Elsworthy, J.S. Holker, J.M. McKeown, J.B. Robinson, and L.J. Mulheirn, Chem.Comm., 1069 (1970).

M.S. El Ezaby, T.M. Salem, A.H. Zewail, and R. Issa, J.Chem.Soc. (B), 1293 (1970).

L.F. Fieser and M. Fieser, Topics in Organic Chemistry, Reinhold Pub. Corp., N.Y., p.20 (1963).

D. Fischer and A. Sapper, C.A., 2648 (1911).

J.G. Fleetwood, Chem. and Process Eng., 43, 106 (1962).

M.St.C. Flett, J.Chem.Soc., 1441 (1948).

A. Funk and A.K. Parker, Can.J.Bot., 44, 1171 (1966).

R.C. Fuson, Reactions of Organic Compounds, Wiley, N.Y., p.396 (1962).

P.D. Gadgil, N.Z.J.Bot., 5, 498 (1967).

P.D. Gadgil, N.Z.J.Bot., 8, 303 (1970).

M. Gelus, J.M. Bonnier, and P. Traynard, Compt.Rend., 255, 2576 (1962); C.A., 58, 5160b (1963).

J.W. Gilmour, N.Z.For.Service For.Res.Inst.Rept.for 1964, 63 (1965).

J.W. Gilmour, Plant Disease Reptr., 51, 727 (1967).

J.W. Gilmour and A. Noorderhaven, N.Z.For.Service For.Res.Inst. For.Pathology Report No.29 (unpublished), Nov. (1970).

L.A. Goldblatt, Ed., Aflatoxin, Academic Press, N.Y. (1969).

R.R. Goodall, J.Royal Inst.Chem., Sept., p.466 (1954).

P.H. Gore, J.Org.Chem., 22, 135 (1957).

P.H. Gore and C.K. Thadani, J.Chem.Soc.(C), 1729 (1966); idem, ibid, 1498 (1967).

N.F. Haddon, M. Wiley, and A.C. Waiss, Jr., Anal.Chem., 43, 268 (1971).

T. Hamasaki, Y. Hatsuda, N. Terashima, and N. Renbutsu, Agr.Biol. Chem., 29, 166 (1965); idem, ibid, 31, 11 (1967).

C.H. Hassall and B.A. Morgan, Chem.Comm., 1345 (1970).

Y. Hatsuda, T. Hamasaki, M. Ishida, and S. Yoshikawa, Agr.Biol. Chem., 33, 131 (1969); idem, ibid, 35, 444 (1971).

T. Hayashi, Y. Inouye, M. Ohashi, and H. Kakisawa, Org.Mass Spectrometry, 3, 1293 (1970).

J.G. Heathcote and M.F. Dutton, Tetrahedron, 25, 1497 (1969).

G. Heller and K. Schulke, C.A., 318 (1909).

D.M. Hercules, Fluorescence and Phosphorescence Analysis, Interscience, J.Wiley and Sons Inc., N.Y. (1966).

R.J. Highet and P.F. Highet, J.Org.Chem., 30, 902 (1965).

W.E. Hillis and D.H.S. Horn, Aust.J.Chem., 18, 531 (1965).

F.A. Hochstein, C.R. Stephens, L.H. Convor, P.P. Regna, R. Pasternack, P.N. Gordon, F.J. Pilgrim, K.J. Brunings, and R.B. Woodward, J.Amer.Chem.Soc., 75, 5455 (1953).

J.S.E. Holker and S.A. Kagal, Chem.Comm., 1574 (1968).

G.M. Holmwood and J.C. Roberts, Tetrahedron Lett., 833 (1971).

B.H. Howard and H. Raistrick, Biochem.J., 59, 475 (1955).

R.D. Hutchinson and C.W. Holzapfel, Tetrahedron, 27, 425 (1971).

L.M. Jackman and S. Sternhell, Applications of Nuclear Magnetic Resonance Spectroscopy in Organic Chemistry, Pergamon Press, Germany, 2nd Edit., p.216-218 (1969).

H.H. Jaffé and M. Orchin, Theory and Applications of Ultraviolet Spectroscopy, J. Wiley and Sons Inc., N.Y., p.533 (1962).

E.T. Jones and A. Robertson, J.Chem.Soc., 1699 (1930).

L. Jurd, in The Chemistry of Flavonoid Compounds, T.A. Geissman, Ed., Pergamon Press, Lond., p.107 (1962).

R.O. Kan, Organic Photochemistry, McGraw-Hill Book Co., N.Y. (1966).

R.A. Kent, I.R. Smith, and M.D. Sutherland, Aust.J.Chem., 23, 2325 (1970).

L.H. Klemm, A.J. Kohlik, and K.B. Desai, J.Org.Chem., 28, 625 (1963).

J.A. Knight, J.C. Roberts, and P. Roffey, J.Chem.Soc.(C), 1308 (1966).

F. Kögl and W.B. Deijs, Ann., 515, 14 (1935), and earlier refces.

M.P. Kuntsmann and L.A. Mitscher, J.Org.Chem., 31, 2920 (1966).

E.B. Lillehoj and A. Ciegler, Mycopathol.Mycol.Appl., 35, 373 (1968).

G. Manecke and W. Storck, C.A., 56, 7235d (1962).

M.L. Martin and G.J. Martin, Bull.Soc.Chim.France, 2117 (1966).

C.P. Mathew, Chem. and Ind. (Lond.), 913 (1970).

W. McFarlane and J.A. Nash, Chem.Comm., 524 (1969).

M.T. Mellier, Ann.Chim. (Paris), 12, 666 (1955); C.A., 50, 12808f (1956).

R.E. Moore and P.J. Scheuer, J.Org.Chem., 31, 3272 (1966).

R.A. Morton and W.T. Earlam, J.Chem.Soc., 159 (1941).

G.A. Nicholls, Natural Products of the Coprosma Genus and Certain Aspects of Anthraquinone Chemistry, Ph.D. Thesis, Auckland University, N.Z. (1948).

J.W. Pavlik and N. Filipescu, Tetrahedron Lett., 4631 (1969).

Perkin, J.Chem.Soc., 76, 433 (1879).

D. Peters, J.Chem.Soc., 646, 4182 (1957).

W. Pigman and H.S. Isbell, Advances in Carbohydrate Chemistry, 23, 11 (1968).

A.E. Pohland, M.E. Cushmac, and P.J. Andrellos, J.Assoc.Offic. Anal.Chemists, 51, 907 (1968).

V.H. Powell, M.D. Sutherland, and J.W. Wells, Aust.J.Chem., 20, 535 (1967).

I.F.H. Purchase and J.J. van der Watt, Food Cosmet.Toxicol., 6, 555 (1968).

D.F.G. Pusey and J.C. Roberts, J.Chem.Soc., 3542 (1963).

Quickfit and Quartz Ltd, Stone, Staffordshire, England: Technical Manual, Steady State Distribution Machine.

H. Raistrick, R. Robinson, and A.R. Todd, Biochem.J., 28, 559 (1934).

M.J. Rance and J.C. Roberts, Tetrahedron Lett., 277 (1969); idem, ibid, 2799 (1970).

R.I. Reed and W.K. Reid, J.Chem.Soc., 5933 (1963).

E. Ritchie, W.C. Taylor, and J.S. Shannon, Tetrahedron Lett., 1437 (1964).

J.C. Roberts, J.Chem.Soc., 785 (1960).

E.H. Rodd, The Chemistry of Carbon Compounds, Vol.IIIB, Elsevier, Amsterdam, p.1373 (1956).

E.H. Rodd, The Chemistry of Carbon Compounds, Vol.IF, 2nd Edit., Elsevier, Amsterdam, Chap.23 (1967).

J.V. Rodricks, E. Lustig, A.D. Campbell, and L. Stoloff, Tetrahedron Lett., 2975 (1968).

E. Sanders, Studies on Dothistroma pini Hulbary, M.Sc. Thesis, Auckland University, N.Z. (1969).

E. Sawicki, T.W. Stanley, W.C. Elbert, and J.D. Pfaff, Anal.Chem., 36, 497 (1964).

A.I. Scott, Interpretation of the Ultraviolet Spectra of Natural Products, Pergamon, Frankfurt, p.286-293 (1964).

P.R. Shafer, D.R. Davis, M. Vogel, K. Nagarajan, and J.D. Roberts, Proc.Nat.Acad.Sci., U.S.A., 47, 49 (1961).

R.H. Shapiro and K.B. Tomer, Org.Mass Spectrometry, 2, 579 (1969).

S. Shibata, M. Takido, and C. Tanaka, J.Amer.Chem.Soc., 72, 2789 (1950).

T.H. Sidall and W.E. Stewart, Progress in NMR Spectroscopy, Pergamon, 5, Chap.2 (1969).

K.C. Srivastava and S.K. Banerji, Aust.J.Chem., 20, 1385 (1967).

L. Stoloff, J.Assoc.Offic.Anal.Chemists, 54, 305 (1971).

R. Suemitsu, Agr.Biol.Chem., 27, 1 (1963).

M.D. Sutherland and J.W. Wells, Aust.J.Chem., 20, 515 (1967).

F. Taddei, J.Chem.Soc.(B), 653 (1970).

J. Taga, C.A., 59, 5943d (1963).

M. Tanabe, T. Hamasaki, H. Seto, and Le Roy Johnson, Chem.Comm., 1539 (1970).

O. Tanaka, Chem.Pharm.Bull.Japan, 6, 18 (1958).

N. Tanaka, Y. Katsube, Y. Hatsuda, T. Hamasaki, and M. Ishida, Bull.Chem.Soc.Japan, 43, 3635 (1970).

F.K. Thayer, C.S. Marvel, and G.S. Miers, Org.Synthesis, Col.Vol.I, p.111 (1932).

C.B. Thomas, J.Chem.Soc.(B), 430 (1970).

R.H. Thomson, Naturally Occurring Quinones, Butterworths, Lond., Chap.4, p.158 (1957).

B.D. Thyr and C.G. Shaw, Mycologia, 56, 103 (1964).

N.J. Turro, Molecular Photochemistry, W.A. Benjamin, Inc., N.Y. (1967).

Z. Valenta, in Techniques of Organic Chemistry, A. Weissberger ed., Interscience, N.Y., Vol.XI, part 2, Chap.XI, p.633 (1963).

T.C. Van Soest and A.F. Peerdeman, Koninkl.Ned.Akad.Wetenschap. Proc.Ser.B., 67, 469 (1964).

Varian NMR Catalogue, Varian Associates, Palo Alto, California, Vol.1 (1962), and Vol.2 (1963).

A.I. Vogel, Practical Organic Chemistry, Longmans, Lond., 3rd Edit., Chap.IV, p.740 (1961).

A.C. Waiss, Jr., M. Wiley, D.R. Black, and R.E. Lundin, Tetrahedron Lett., 3207 (1968).

H. Waldmann and E. Marmorstein, C.A., 31, 2598 (1937).

W. West, in Technique of Organic Chemistry, A. Weissberger, ed., Interscience, N.Y., Vol.IX, Chap.VI (1967).

R.H. White and J.W. Howard, J.Chromatog., 29, 108 (1967).

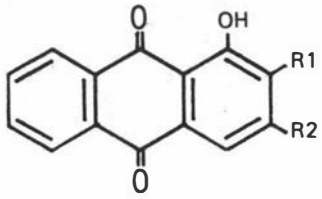
G.M. Whitesides, D. Holtz, and J.D. Roberts, J.Amer.Chem.Soc., 86, 2628 (1964).

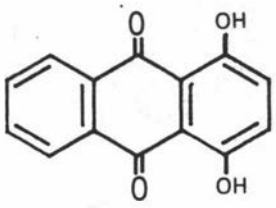
M. Wiley, A.C. Waiss, Jr., and N. Bennett, J.Assoc.Offic.Anal. Chemists, 52, 75 (1969).

G.N. Wogan, Bact.Reviews, 30, 460 (1966).

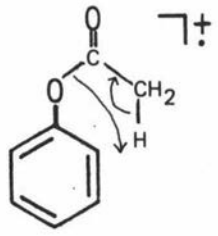
FORMULAE

	R1	R2
(1)	OH	H
(2)	OH	OMe
(3)	H	OH
(4)	Me	OH
(5)	OMe	OH

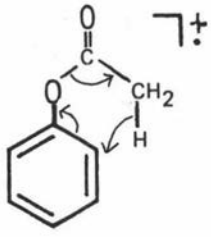




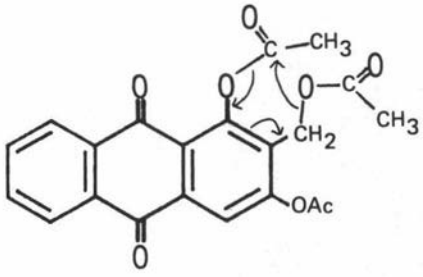
(6)



(7)



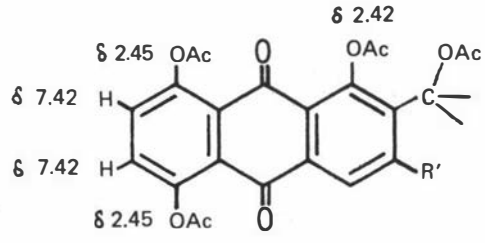
(8)



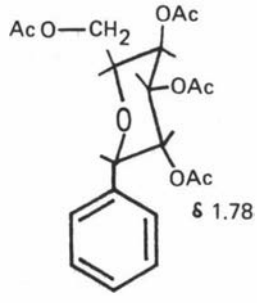
(9)

see page 56

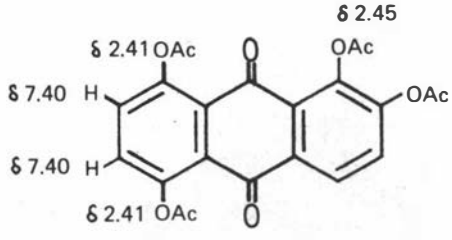
(10) – (18)



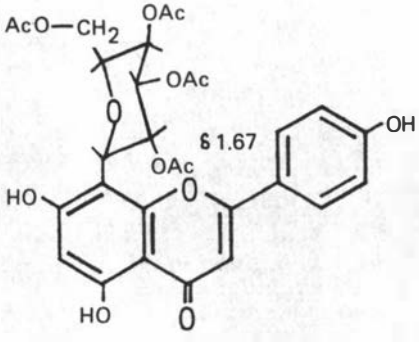
(19)



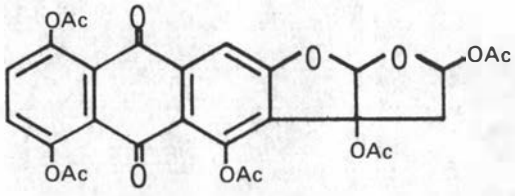
(21)



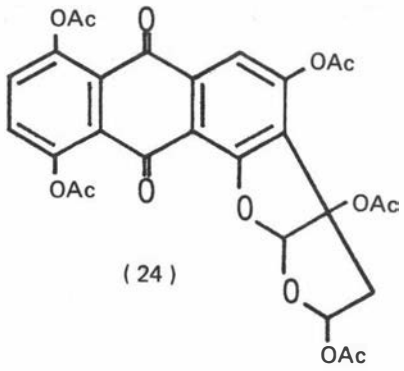
(20)



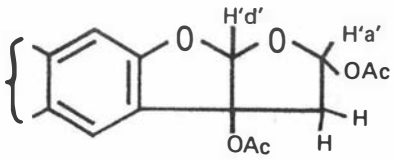
(22)



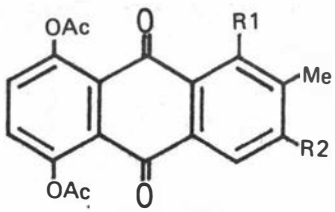
(23)



(24)

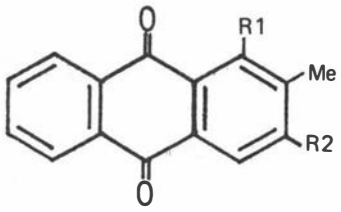


(25)



	R1	R2
(26)	OAc	OMe
(27)	OMe	OAc

	R1	R2
(28)	OMe	OH
(29)	OMe	OAc
(30)	OAc	OMe
(31)	OH	OH
(32)	OH	OMe
(33)	OMe	OMe



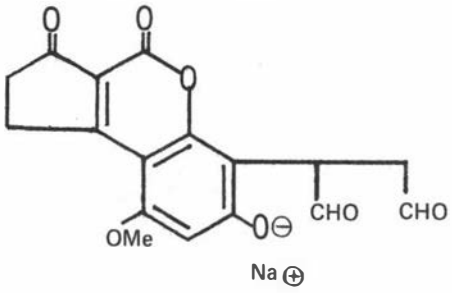
(28)–(33)

see page 64

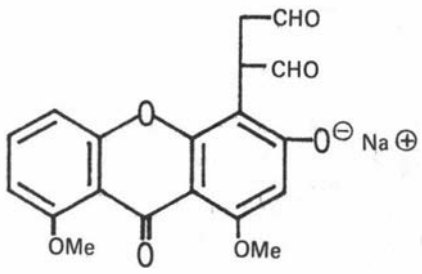
(34)–(37)

see page 67

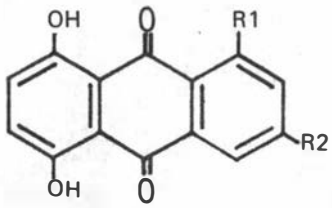
(38)–(42)



(43)



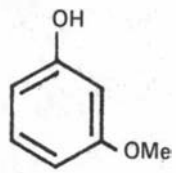
(44)



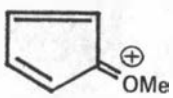
	R1	R2
(45)	OH	OMe
(46)	OMe	OH
(47)	OMe	OMe

see page 155

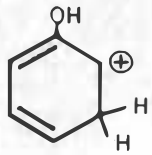
(48)–(54)



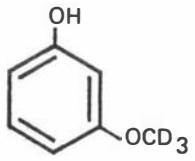
(55)



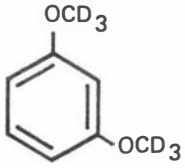
(56)



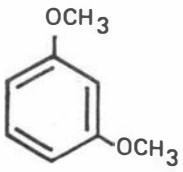
(57)



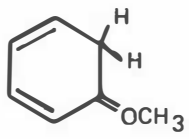
(58)



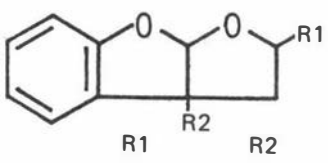
(59)



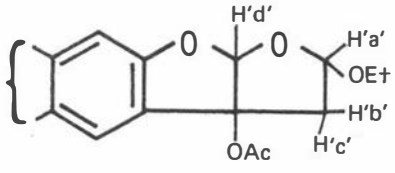
(60)



(61)



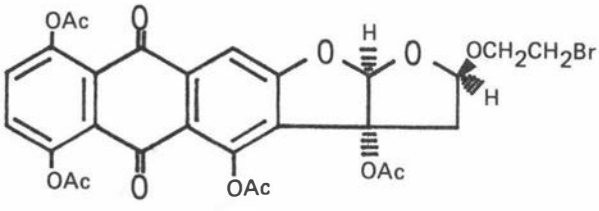
	R1	R2
(62)	H	H
(63)	OH	H
(64)	H	OH
(65)	OH	OH



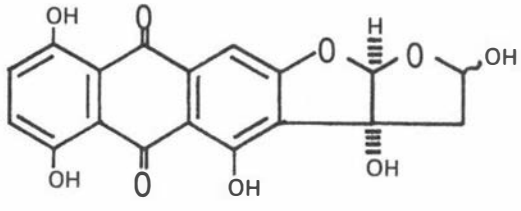
(66)

see page 155

(67)–(85)



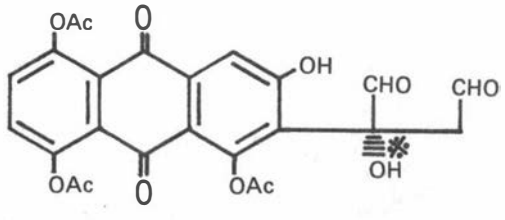
(86)



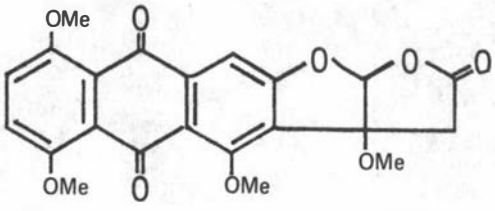
(87)

see page 155

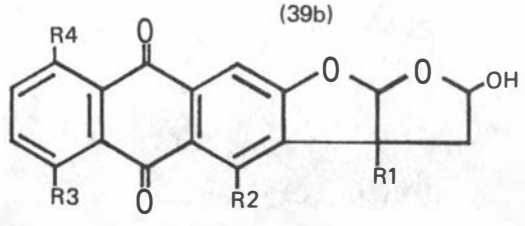
(88)



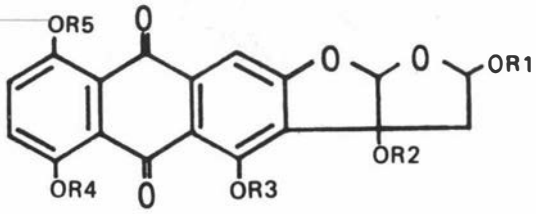
(39b)



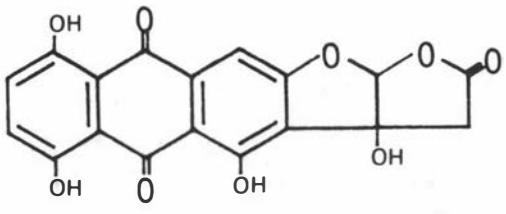
(89)



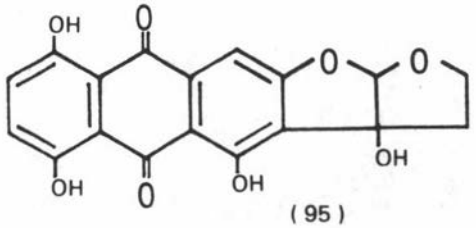
	R1	R2	R3	R4
(90)	OH	OH	OH	H
(91)	OH	OH	H	OH
(92)	OH	H	OH	OH
(93)	H	OH	OH	OH



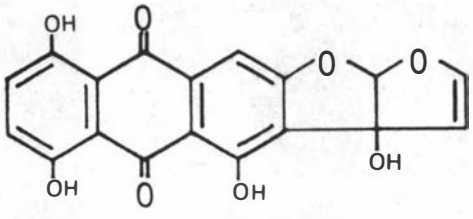
Compound No.	R_1	R_2	R_3	R_4	R_5
(23)	Ac-	Ac-	Ac-	Ac-	Ac-
(38)	H	H	H	H	H
(48)	Me	H	H	H	H
(49)	Et	H	H	H	H
(50)	n-Bu	H	H	H	H
(51)	Me	Ac	Ac	Ac	Ac
(52)	Et-	Ac	Ac	Ac	Ac
(53)	n-Bu	Ac	Ac	Ac	Ac
(54)	Ac	H	H	H	H
(67)	Et	H	H	Me	Me
(68)	Et	H	Me	Me	Me
(69)	Et	Me	Me	Me	Me
(70)	Et	$Me\text{-}d_3$	Me	Me	Me
(71)	Et	$Me\text{-}d_3$	H	$Me\text{-}d_3$	$Me\text{-}d_3$
(72)	Et	H	$Me\text{-}d_3$	$Me\text{-}d_3$	$Me\text{-}d_3$
(73)	Et	$Me\text{-}d_3$	$Me\text{-}d_3$	H	$Me\text{-}d_3$
(74)	Et	$Me\text{-}d_3$	$Me\text{-}d_3$	$Me\text{-}d_3$	H
(75)	Et	$Me\text{-}d_3$	$Me\text{-}d_3$	$Me\text{-}d_3$	$Me\text{-}d_3$
(76)	Et	$Me\text{-}d_3$	(H, H, $Me\text{-}d_3$)		
(77)	Et	$Me\text{-}d_3$	(H, $Me\text{-}d_3$, desoxy)		
(78)	Me	Me	Me	Me	Me
(79)	Me	H	Me	Me	Me
(80)	$p\text{-}BrC_6H_4CH_2$	H	H	H	H
(81)	CH_2ClCO	H	H	H	H
(82)	CH_2ClCO	Ac	Ac	Ac	Ac
(83)	CH_2ICO	Ac	Ac	Ac	Ac
(84)	CH_2BrCH_2	H	H	H	H
(85)	CH_2BrCH_2	Ac	Ac	Ac	Ac
(88)	H	Me	Me	Me	Me



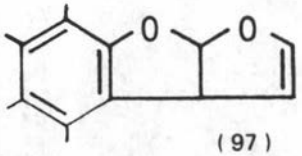
(94)



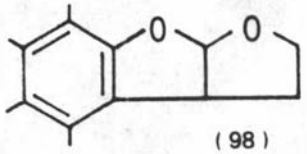
(95)



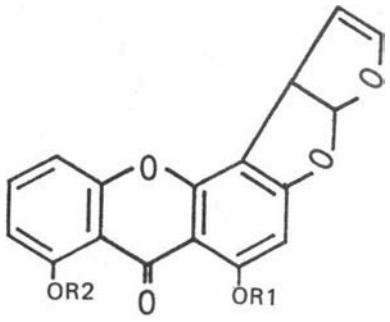
(96)



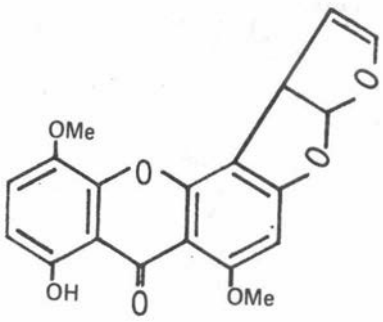
(97)



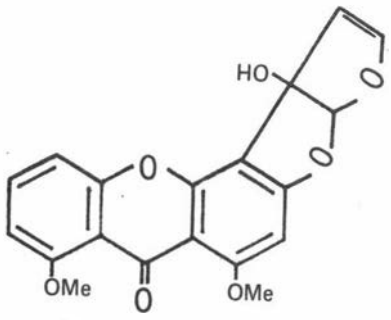
(98)



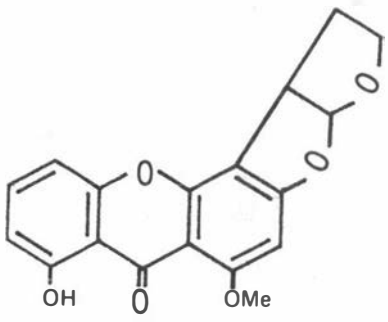
(99)



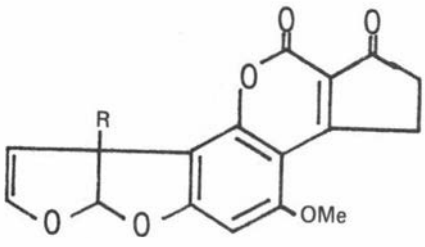
(100)



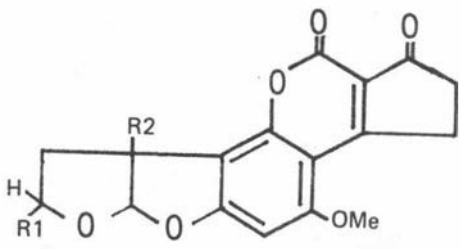
(101)



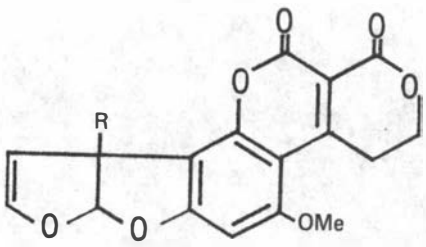
(102)



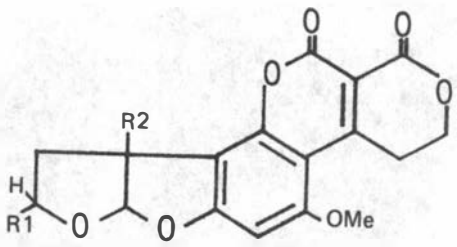
(103)



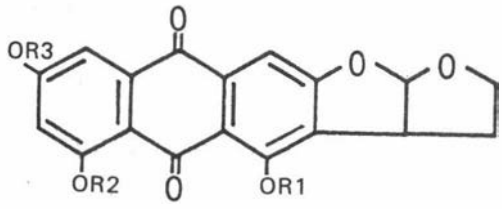
(104)



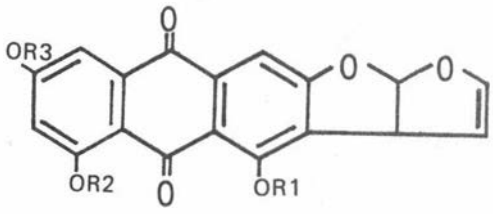
(105)



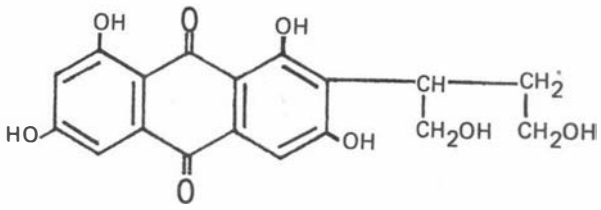
(106)



(107)



(108)



(109)

Publications

Publications arising as a result of the work of this thesis:

C. Bassett, M. Buchanan, R.T. Gallagher and R. Hodges, "A toxic difuroanthraquinone from *Dothistroma pini*", *Chem. and Ind.* (Lond.), 1659 (1970).

C.A. Bear, J.M. Waters, and T.N. Waters, and R.T. Gallagher and R. Hodges, "X-Ray Determination of the Molecular Structure of a Derivative of Dothistromin, a Fungal Toxin Implicated in Pine Needle Blight", *Chem. Comm.*, 1705 (1970).