THE BIOSYNTHESIS OF DOTHISTROMIN

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ABSTRACT

This thesis is concerned with the biosynthesis of dothistromin (2,3,3a,12a tetrahydro-2,3a,4,6,9 pentahydroxy-anthra[2,3-b]furo[3,2-d]furan-5,10 dione) by the fungus Dothistroma pini. The biosynthesis of related secondary metabolites is reviewed and as a working hypothesis it is proposed that dothistromin is solely acetate-derived.

In the preliminary phases of the investigation strains of the organism giving high yields of the metabolite were sought and isolated from natural sources. Some growth media were tested for their ability to support growth, promote sporogenesis and sustain high yields of dothistromin. A medium containing malt and dried whole yeast was chosen. The growth characteristics of the organism in this medium were studied and the temporal relationship between growth and pigment production for a variety of cultural conditions was found. The findings of these experiments suggested times when it would be favourable to add possible precursors.

Incorporation studies with [1-^{14}C]-sodium acetate revealed that dothistromin incorporated isotope from this precursor and disclosed that the lipids heavily incorporated the label. Subsequent experiments were concerned with examining the effects that precursor concentration, time of precursor addition and time of metabolite harvesting had on the isotope enrichment and yield of dothistromin.

It was found that the optimisation of these two parameters were mutually exclusive processes and compromise conditions which were compatible with obtaining both reasonably good enrichments and yields of dothistromin had to be selected.

Initially attempts were made to determine the distribution of isotope in dothistromin, which had incorporated isotopically labelled acetate, by chemical degradation. Potassium tertiary-butoxide/water cleavage of the anthraquinone ring of the pentamethyl derivative of dothistromin labelled by [1-^{14}C]-acetate yielded 1,4-dimethoxybenzene which had a molar specific radioactivity that was 0.33 times that of the starting material. This finding was consistent with the formation of dothistromin from nine molecules of acetate.

Subsequently ^{13}C-NMR techniques were used to determine the distribution of isotope in dothistromin derived from [1-^{13}C] and [2-^{13}C]-acetates. Pulsed Fourier transform ^{13}C-NMR spectra of the monoethyl acetal derivative of dothistromin were obtained using broad-band proton decoupling and off-resonance proton decoupling. By comparison with the ^{13}C-NMR spectra of a number of model compounds the resonances in the spectrum of the dothistromin derivative were assigned in most cases to specific carbon atoms and in a few instances to two or three alternatives.

The ^{13}C-NMR spectra of the dothistromin derivatives which had been enriched by isotope from the carbon-13 labelled acetates showed nine resonances with intensities enhanced by enrichment

from the carboxyl carbon of acetate, eight from the methyl carbon and one resonance of uncertain origin. The distribution of isotope in the anthraquinone moiety of the molecule was consistent with its formation from a polyketide precursor but this was not proven because of the equivocal assignment of some of the NMR signals. The distribution of isotope in the furofuran ring moiety and its relation to the distribution in the anthraquinone part was the same as that reported by others for corresponding structures in aflatoxin B_1 and sterigmatocystin.

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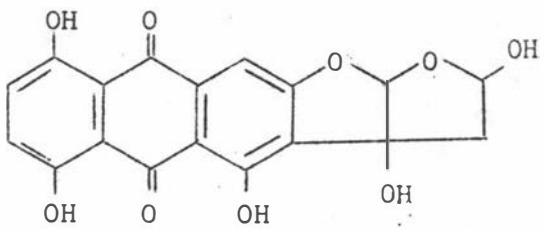
CHAPTER 1

Introduction

1.1 Biosynthetic origin of dothistromin

This thesis is concerned with the biogenesis of the hydroxyanthraquinone, dothistromin(1-1), by the fungus Dothistroma pini. This compound, which was isolated and characterised by Bassett et al. (1970), conforms to the currently accepted criteria for a product of secondary metabolism (Bu'Lock, 1961). Its production so far seems to be confined to a single species and it has no obvious function in the general metabolism of the organism.



(1-1)

The biosynthetic origins of a large variety of secondary metabolites have been determined (Richards and Hendrickson, 1964; Thomson, 1971; Turner, 1971; Geissman, 1972; Geissman, 1973). From this body of evidence it has become clear that certain classes of compounds may reasonably be expected to be derived from certain small molecules which are intermediates of general metabolism, e.g. acetyl - coenzyme A (acetyl-CoA), mevalonic acid, shikimic acid and a variety of amino acids, etc. On the basis of this information it could be predicted that the anthraquinone portion of the dothistromin molecule is likely to be derived from acetyl-CoA.

Although there is less evidence for the origin of bis-difuran rings in natural products, it suggests that this part of dothistromin might also be formed from acetyl-CoA (Biollaz et al., 1968a, 1968b, 1970)

1.2 Acetyl-coenzyme A

Acetyl-CoA stands at the hub of intermediary metabolism (Spenser, 1968) and as might be expected, prodigious numbers of secondary metabolites are generated either directly or indirectly from two-carbon units at the oxidation level of acetic acid. Elucidation of the central role of acetyl-CoA has shown it to be a key intermediate in three major metabolic pathways of primary metabolism, each of which also finds its counterpart in the biogenetic routes to secondary metabolites (Figure 1-1). The formation of long chain fatty acids by successive addition of malonyl-coenzyme A (malonyl-CoA) to an acetyl-CoA starter molecule is paralleled, with modifying reactions, by the pathways leading to the polyketomethylene chain precursors (known as polyketides) of acetate derived secondary metabolites (Birch, 1967).

A second pathway of primary metabolic importance leads from acetyl-CoA to mevalonic acid, the progenitor in steriod biosynthesis. In secondary metabolism the mevalonic acid route leads to terpenes and other naturally occurring polyisoprenoids. The third process of acetate metabolism important to this discussion is the tricarboxylic acid cycle and its shunt into the glyoxylic acid cycle (Krebs and Lowenstein, 1960).

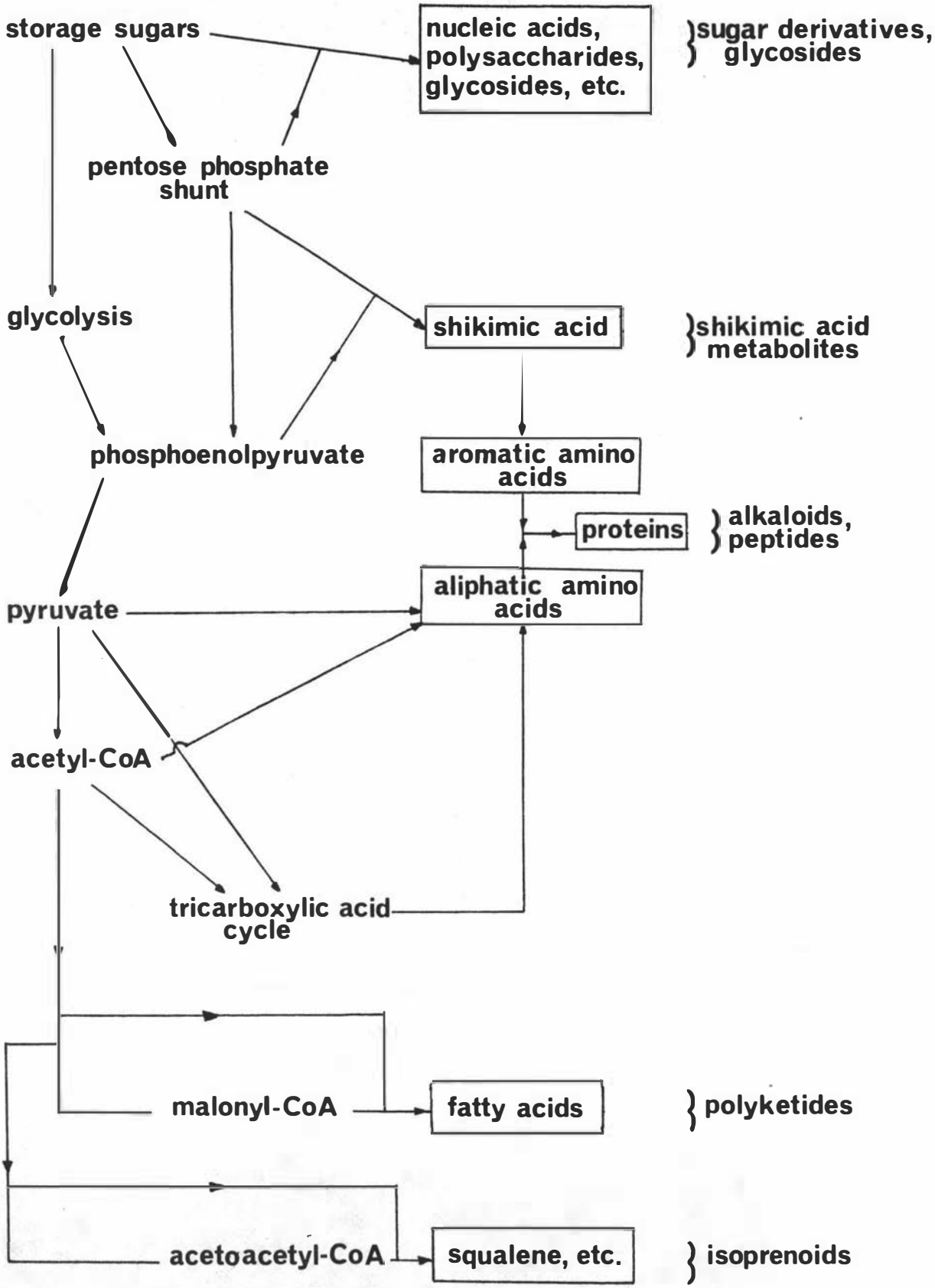


Figure (1-1) The main flow of carbon metabolism (left), the main products of primary synthesis (boxes) and the corresponding categories of secondary metabolites (right).

Intermediates from both metabolic cycles can serve directly or indirectly as precursors of numerous secondary metabolites.

An important fact, usually neglected in a discussion on sources of metabolic acetate, is that acetyl-CoA is not only derivable from carbohydrates via the glycolytic pathway and by β-oxidation of long chain fatty acids, but it is also a product of the catabolism of several amino acids, e.g. tyrosine, tryptophan, lysine, leucine and isoleucine (Meister, 1967). Such versatility of origin of an important metabolite underlines the need for careful design of experiment and for cautious interpretation of incorporation data.

1.3 The acetate hypothesis for the biosynthesis of aromatic compounds.

Following the demonstration of the conversion of dehydroacetic acid to orcinol and of diacetylacetone to a naphthalene derivative (Collie and Myers, 1893), Collie (1907) hypothesised that "multiple keten" compounds, produced by linear condensation of acetic acid residues, were the biological precursors of naturally occurring aromatic compounds.

Some time later it was shown that fatty acids are formed *in vivo* by successive condensation of acetic acid residues (Rittenberg and Bloch, 1945). Following the identification of acetyl-CoA as the biological two-carbon residue (Lynen *et al.*, 1951), the detailed sequence of reactions in fatty acid biosynthesis was worked out (Summarised by Lynen *et al.*, 1968).

Extrapolating from the knowledge about fatty acid biosynthesis available at the time, Birch and Donovan (1953) independently reformulated the acetate hypothesis for the biosynthesis of aromatic compounds. They suggested that head to tail condensation of acetyl-CoA residues, as in fatty acid biosynthesis, would form a linear polyketomethylene acid (polyketide) if, unlike fatty acid biosynthesis, the reduction of the carbonyl groups did not occur. Then, it was postulated, the polyketide could cyclise in mechanistically acceptable ways to give phenolic products. The discovery, subsequently, of the role of malonyl-CoA (derived biologically by carboxylation of acetyl-CoA) as the chain propagating unit in fatty acid biosynthesis (Wakil, 1958) led to a modification of the acetate hypothesis (Lynen, 1959).

As an example, the formation of the fungal metabolite endocrocin(1-2) according to the acetate hypothesis is shown in Figure 1-2 and contrasted with the sequence of reactions known to occur in the biosynthesis of fatty acids with the same number of carbon atoms.

1.4 Stages in the biosynthesis of polyketide-derived metabolites

For the purpose of discussing polyketide biogenesis it is convenient to distinguish between (a) the assembly processes, (b) cyclisation and (c) modifying processes.

1.4.1 Assembly processes (enzymes forming polyketides)

Little work has been reported on the possible enzymatic system(s) involved in polyketide formation. This situation

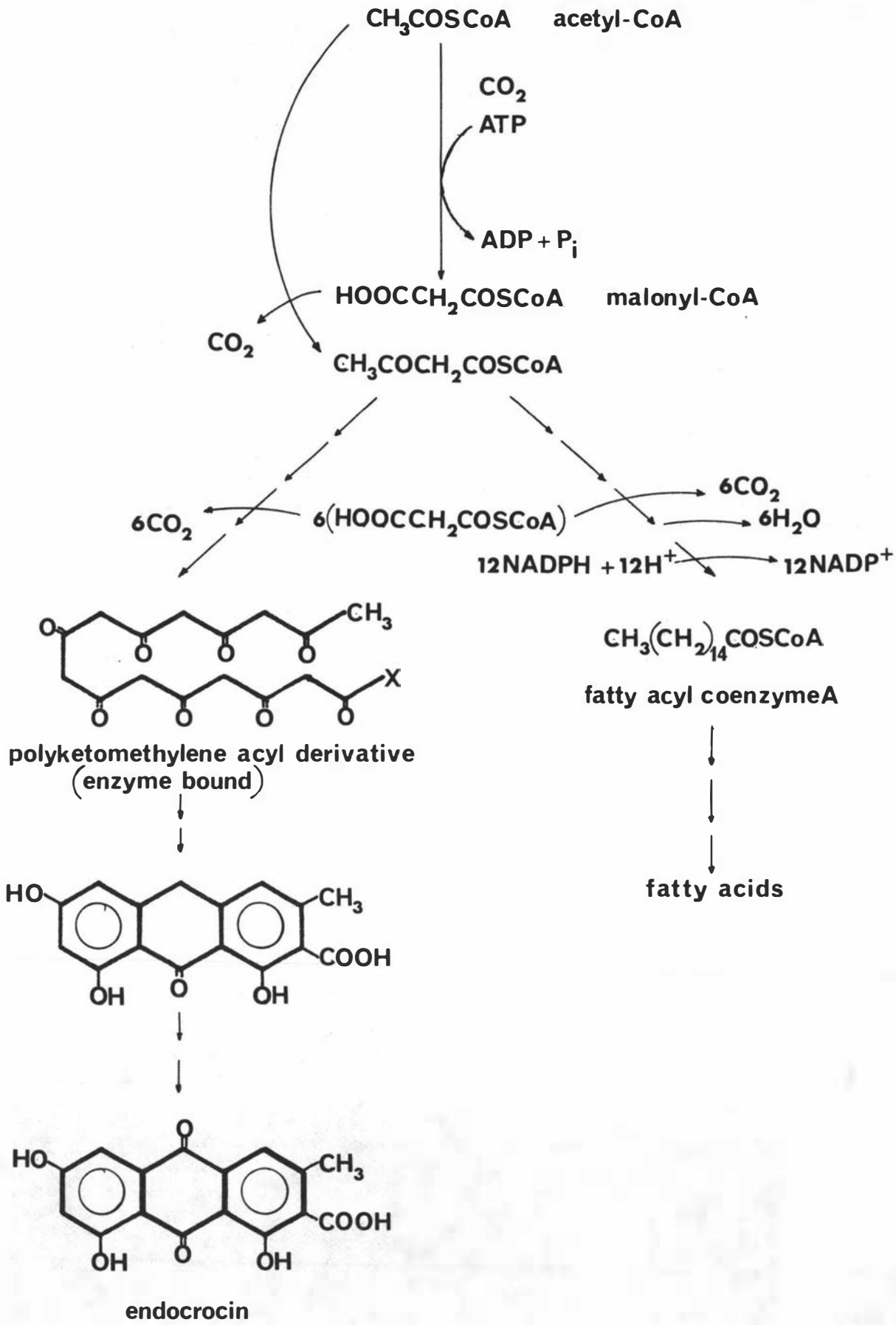


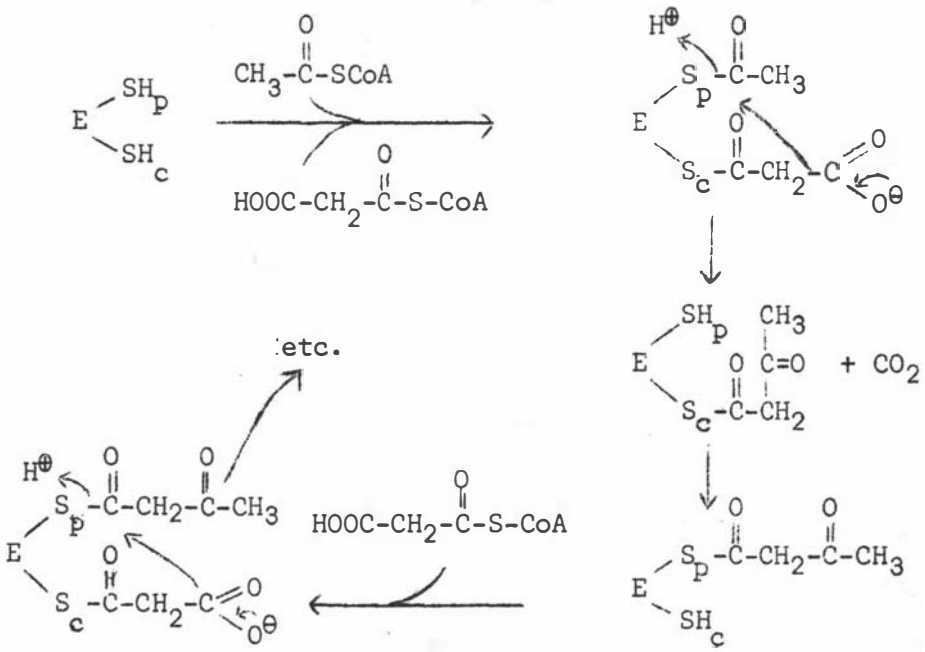
Figure (1-2) Comparison of the biosynthesis of a 16 carbon fatty acid and 16 carbon secondary metabolite.

seems to be due to the relative instability of isolated enzymes of fungal origin catalysing polyketide formation.

Gatenbeck and Hermodsson (1965) prepared an enzyme solution with 30 fold purification from Alternaria tenuis with the capacity to synthesise alternariol(1-3). Synthesis was found to require acetyl-CoA and malonyl-CoA; malonyl pantetheine also served as an effective precursor in place of malonyl-CoA. Light (1967) found that when [1,3-^{14}C]-malonyl-CoA was added to cell free extracts of Penicillium patulum radioactively labelled 6-methylsalicyclic (1-4) acid was produced. Chemical degradation of the product revealed that one-third of the radioactivity was in the carboxyl group which is consistent with an acetyl-polymalonyl biosynthetic origin. Two reports, emphasising the relationship of fatty acid and polyketide biosynthesis, were those of Yalpani et al. (1969) and Nixon et al. (1968) who have found with yeast and pigeon liver fatty acid synthetase, respectively, that triacetic acid lactone is produced from acetyl-CoA and malonyl-CoA in the absence of NADPH while in its presence normal fatty acid biosynthesis is resumed. Probably the most significant work was the isolation of a distinct multienzyme complex for the synthesis of 6-methylsalicylic acid(1-4) (Dimroth et al., 1970). An extract was prepared from Penicillium patulum, grown in submerged culture, which converted acetyl-CoA and malonyl-CoA into 6-methylsalicylic acid(1-4) in the presence of NADPH. A 100 fold purification gave a single particle (MW, 1.1-1.5 x 10^6) which catalysed the complete conversion. Inhibition studies

with iodoacetamide and N-ethylmaleimide showed that it had similar properties to fatty acid synthetase. However, in the latter stages of the purification, this multienzyme complex was separated from the fatty acid synthetase complex. Incubation with acetyl-CoA and malonyl-CoA in the absence of NADPH gave triacetic acid lactone as a derailment product, indicating that in 6-methylsalicylic acid(1-4) synthesis reduction of one of the carbonyls occurs before condensation with the last malonyl-CoA.

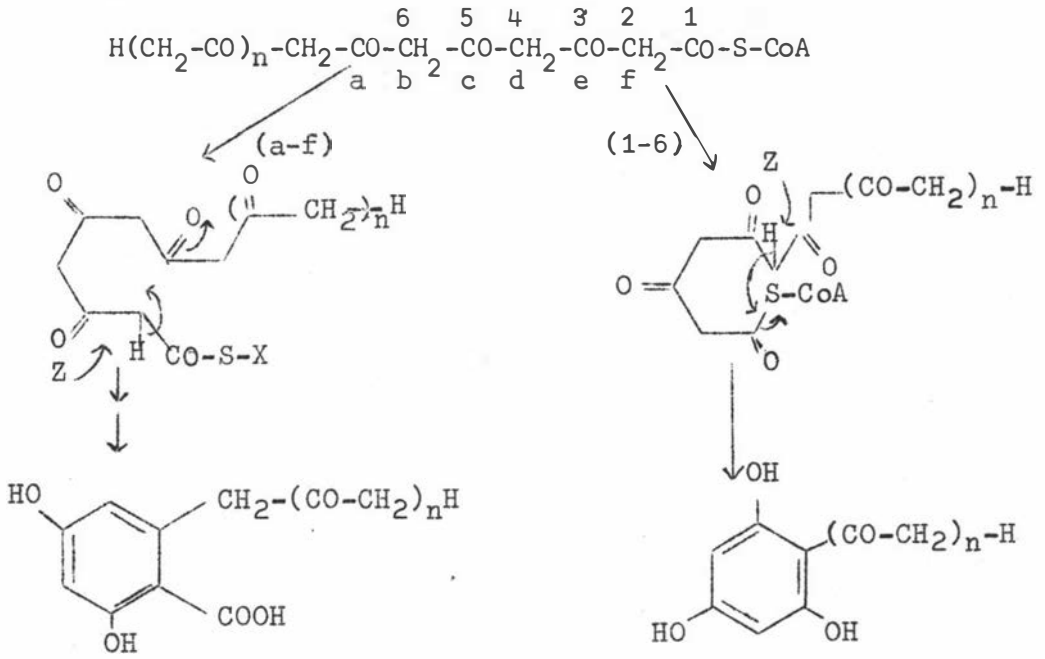
On the basis of these findings a hypothetical reaction scheme was proposed for 6-methyl salicylic acid biosynthesis, in analogy to that proposed for the operation of fatty acid synthetase. In this scheme (see below) the multienzyme synthetase is visualised as having two types of thiol groups, called "central" (c) and "peripheral" (p). The starter acetyl group is bonded, as its thiol ester, to the "peripheral" thiol group and the chain extending malonyl groups are bonded to the "central" ones. Condensation between the nucleophilic methylene group of the malonyl residue and the electrophilic carbonyl group of the acetyl residue, together with elimination of carbon dioxide and thiol, in what is believed to be a concerted reaction (lynen, 1972), would yield the acetoacetyl derivative of the "central" thiol group. Following transfer of the acetoacetyl group to the "peripheral" thiol and formation of a thiol ester between, the now vacant, "central" thiol group and another malonyl residue, the events just described would be repeated until a polyketide of the required length was generated.



1.4.2 Cyclisation processes

There is little evidence concerning stages beyond malonyl-CoA in the assembly process. The cyclisation probably follows the termination of the polyketide chain and can be thought of as a stabilisation reaction occurring in a matrix of specific topography which determines the character of the products (Bu'Lock, 1965a). The presence of the doubly-activated methylene groups and reactive carbonyl groups make possible two kinds of cyclisation reactions. Thus an enolate anion may attack a keto group intramolecularly to yield cyclised products of either O- or C-acylation. Both reactions occur with facility in forming 6-membered rings, the C-alkylation (aldol or Claisen ester condensation) in this case leading to a phenol ring. Two major routes lead to two families of phenols, the acylphloroglucinol and orsellinic acid derivatives

distinguished by their aromatic substituent patterns as shown.



ORSELLINIC ACIDS ACYLPHLOROGLUCINOLS

A number of chemical models for the cyclisation of polyketides have been prepared (Money, 1970; Scott et al., 1971; Hay and Harris, 1972; and earlier publications of these groups). Base catalysed cyclisation of these model compounds yielded a variety of products, corresponding to both aldol and Claisen ester condensations. Since the corresponding cyclisations that occur in vivo usually lead to unique products, these reactions are probably under enzymatic control. In some substances, such as orsellinic acid,(1-5), all the oxygen atoms except those involved in the cyclisation step, are retained (Gatenbeck and Mosbach, 1959).

That these phenolic oxygens were genuinely derived from the acetate carboxyl group was shown by using [1-^{14}C, ^{18}O]-acetic acid (Gatenbeck, 1958a). In other compounds the absence of the oxygen substituent implied partial reduction of the

polyketide chain had occurred. This was attributed to reduction of the carbonyl group in the polyketide intermediate to an alcohol followed by dehydration (Birch and Donovan, 1953). Some supporting evidence has come from studies of purified 6-methylsalicylic acid synthetase (Dimroth et al., 1970, c.f. Staunton, 1970). When this enzyme was incubated with acetyl-CoA and malonyl-CoA in the absence of NADPH, triacetic acid lactone was produced rather than 6-methylsalicylic acid (1-4). Such removable oxygen atoms are presumably not required for stabilization, folding or cyclisation of the chain. In other metabolites only partial cyclisation occurs with the remaining moiety being stabilised by partial or complete reduction.

1.4.3 Modifying processes

1.4.3.1 Alkylation

A number of polyketide-derived secondary metabolites have been found to possess carbons in addition to those provided by acetate (Richards and Hendrickson, 1964). These extra carbons can be bonded either to oxygen or to carbon and can range in oxidation level from saturated hydrocarbon to carboxyl carbon. In the case of single carbon additions, this is usually derived from the S-methyl group of methionine. However it should be noted that C-methyl groups in some polyketides arise because propionyl-CoA and 2-methylmalonyl-CoA have been used in place of acetyl-CoA and malonyl-CoA, respectively, at certain stages in the assembly of the polyketide chain

(Corcoran and Darby, 1970). Less commonly the additional carbons are isopentenyl groups, derived from mevalonic acid.

Concerning the timing of C-methylation, the evidence to hand suggests, in the case of polyketides, the chain is alkylated prior to cyclisation (summarised by Lederer, 1969 and Money, 1973). On the other hand firm evidence for O-methylation prior to cyclisation is lacking, while there is ample evidence for such alkylation following polyketide cyclisation.

1.4.3.2 Oxygen addition and removal

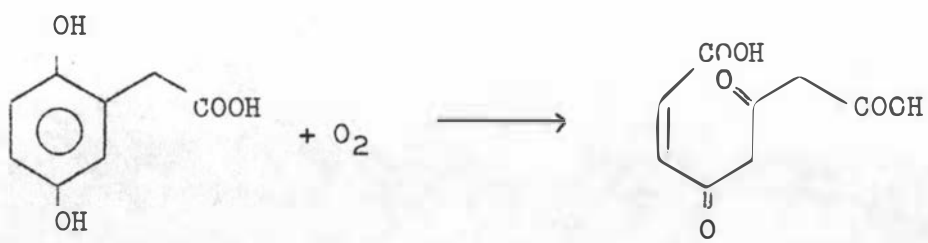
The lack of oxygen in positions where they might be expected, if the polyketide precursor was cyclised without modification, is a common finding among secondary metabolites. Since proven examples of direct removal of oxygen substituents from aromatic rings are rare, it is presumed that these oxygens have been removed by reduction and then dehydration of carbonyl groups in the enzyme-bound polyketide, prior to cyclisation. An example of the latter process, previously mentioned, is the biosynthesis of 6-methylsalicylic acid(1-4)

The presence of additional oxygen atoms on polyketide skeletons is also a common occurrence. In the majority of cases the extra oxygen was probably introduced by monooxygenase catalysis (Hayaishi, 1968 and 1969), since only single atom additions have occurred. These enzymes catalyse reactions where one mole of oxygen is required to add one atom of oxygen to the

substrate and the other atom is converted to water, the hydrogen being provided by a suitable donor, e.g. NADPH. A recent finding of probable significance has been the demonstration that arene oxides are produced from aromatic substrates by liver microsomal monoxygenases (Jerina et al., 1970).

Less commonly observed among secondary metabolites are products of the possible catalytic activity of dioxygenases. These enzymes catalyse the addition of both atoms of molecular oxygen to a substrate and the reaction does not require a hydrogen donor. Usually the substrates are aromatic molecules having either 1,2-dihydroxy or 1,4-dihydroxy substitution and the dioxygenation results in the cleavage of the aromatic ring (Hayaishi, 1968). Two well known examples of dioxygenases are pyrocatechase and homogentisic acid oxygenase which catalyse the following reactions, respectively.

catechol cis, cis-muconic acid



homogentisic acid maleyl acetoacetic acid

Examples of this type of transformation among aromatic metabolites of polyketide origin have been reviewed (Thomas, 1965).

1.4.3.3 Decarboxylation

A feature of the acetate-polymalonate pathway is that the terminal carboxyl group of the hypothetical polyketomethylene may take part in a wide variety of reactions, one of the commonest being decarboxylation. It is often the case that the actual decarboxylase enzymes may be extracted from moulds by relatively simple methods, although attempts to isolate other enzymes of secondary metabolism, have in the past, proved less successful.

Although decarboxylation is a common biochemical reaction, the non-oxidative removal of a carboxyl group directly attached to an aromatic ring has been demonstrated only in a few cases. Among phenolic acids the decarboxylation of 2,3-dihydroxybenzoic acid has been studied with cell free preparations obtained from a certain strain of A.niger (Terui et al., 1952) and the conversion of 2,4-dihydroxybenzoic acid into resorcinol(1-6) was shown with intact cultures of an Aspergillus species (Halvorson, 1963). Similarly 3,4-dihydroxybenzoic acid has been found to be converted into catechol by several moulds (Butkewitsch, 1924).

On the other hand, phenolic acids that are formed by acetate-polymalonate condensations, e.g. 2 hydroxy-6-methyl-benzoic acid and orsellinic acid(1-5) in contrast to the above, seem not to be substrates for any of the decarboxylase systems described, even though such acids function as precursors to a large number of acetate-polymalonate derived compounds, in the formation of which a step of decarboxylation is inferred from structural evidence or from the results of radioactive

tracer studies. It has been only recently that decarboxylase enzymes have been purified which decarboxylate simple acetate-polymalonate derived aromatic acids, e.g. Orsellinic acid(1-5) from Gliocladium roseum (Pettersson, 1965) and 6-methylsalicylic acid(1-4) in Penicillium patulum (Light, 1969).

1.5 Coupling of separate polyketide chains

An alternative to folding and cyclisation of a single polyketide chain to form aromatic compounds is the possibility of coupling between two preformed polyketides. Since the initial demonstration of such a process for citromycetin (1-7) by Gatenbeck and Mosbach (1963), several biosyntheses of polyketides in this way have been established, e.g. mollisin (1-8) (Seto et al., 1973a), rotiorin(1-9) (Holker et al., 1964a) and sclerin(1-10) (Tokorayama and Kubota, 1971).

This mode of cyclisation indicates that in certain cases a polyketide could be economically constructed by the coupling of prefabricated molecules composed of few acetyl units which would remain bound to the enzyme surface (Lynen, 1967). The recent isolation of triacetic lactone (Acker et al., 1966; Bentley and Zwitkowits, 1967) from a strain of P.stipitatum suggests the wide availability of such units in living systems.

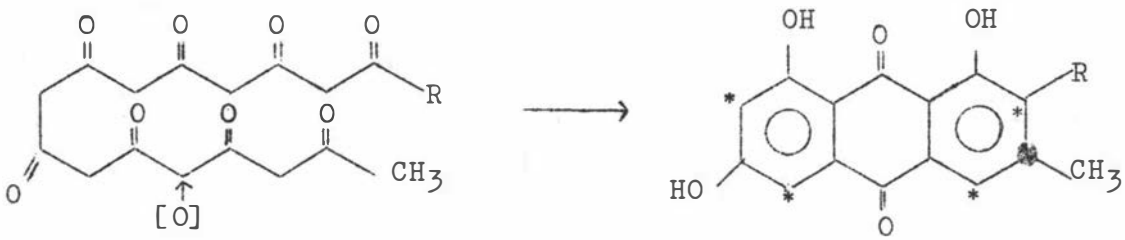
Application of separate chain mechanisms to cometabolites may suggest that branching of a common biosynthetic route might be merely the result of the condensation of two common poly-β-keto intermediates in different directions. The possibility

of diversity in polyacetyl chain formation and its cyclisation in living cells implies that the presence of the same constructive units in the molecules may not necessarily mean the same biosynthetic path.

1.6 Biosynthesis of anthraquinones

1.6.1 Fungal

In the main, fungal anthraquinones conform to the emodin pattern, arising from suitable folding and condensation of an octaketide or longer chain (Shibata, 1967).



R = OH, O-alkyl

endocrocin(1-2) R = COOH

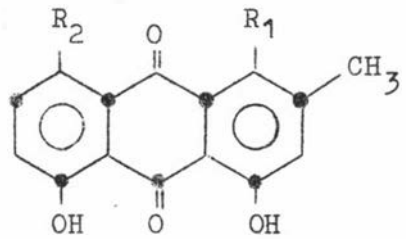
emodin(1-11) R = H

Numerous variations of this basic structure exist, resulting from O-methylation, side-chain oxidation, chlorination, decarboxylation, dimerisation and the introduction or omission of nuclear hydroxy groups, while in endocrocin(1-2) the terminal carboxyl group is retained. In a few cases the usual β-methyl group is replaced by a propyl substituent, e.g. nalgiovensin(1-12) (Raistrick and Ziffer, 1951) and ptilometric acid(1-13) (Powell and Sutherland, 1967).

Other variations in the carbon skeleton can be attributed

to folding of the polyketide chain in different ways prior to cyclisation. Thus condensation of the above octaketide chain (R=OH) could lead to the formation of 1,3,6,8-tetrahydroxyanthraquinones such as solorinic acid(1-14), averufin (1-15), nidurufin(1-16) and versicolorin A(1-17). It must be noted however, that to form the anthraquinone nucleus from the cyclisation of a linear acetate-polymalonate chain it is necessary to introduce a second quinone oxygen irrespective of different possibilities of chain folding.

Proof of the acetate-malonate origin of fungal anthraquinones was first demonstrated with helminthosporin(1-18) using [1-^{14}C]-acetate as a substrate (Birch et al., 1958).



	R_1	R_2
helminthosporin(1-18)	H	OH
islandicin(1-19)	OH	H

Kuhn-Roth oxidation of the radioactively labelled anthraquinone yielded acetic aid bearing one-seventh of the total radioactivity. Extensive investigations of the anthraquinone pigments of Pencillium islandicin were carried out by Gatenbeck (1958b, 1960). Firstly, emodin(1-11) was investigated with [1-^{14}C] and [2-^{14}C]-acetic acid; again Kuhn-Roth oxidation produced acetic acid showing one-seventh of the emodin activity(1-11, heavy dot), more extensive degradation ultimately involving the bromopicrin cleavage showed methyl labels at the sites marked with asterisks (page 14). A complete degradation of islandicin(1-19 ;

R_1=OH, R_2=H) from carboxyl-labelled acetate confirmed the labelling pattern shown (heavy dots).

Gatenbeck (1960) also used [1-^{14}C, ^{18}O]acetate to study the origin of the oxygen atoms of islandicin(1-19) and emodin (1-11) and found, as expected, that three oxygen atoms of islandicin(1-19) and 4 oxygen atoms of emodin were derived from the carbonyl group of acetate. These experiments, however, did not reveal whether the anthraquinone nucleus was formed from a single polyketide chain or from two preformed benzene derivatives (Tatum, 1944) in accord with the "two-chain theory" for heptaketides (Vanek and Soucek, 1962). The problem was resolved when it was demonstrated (Gatenbeck, 1962) in the case of islandicin, that using [^{14}C]malonate only one "starter" acetate unit is used and not two as required by a "two-chain theory".

1.6.2 Higher Plants

Higher plant anthraquinones fall into two classes; those with substituents in only one aromatic ring as in alizarin(1-20) and those with substituents in both aromatic rings, e.g. chrysophanol(1-21) and emodin(1-11). It now appears that these two classes are biologically distinct.

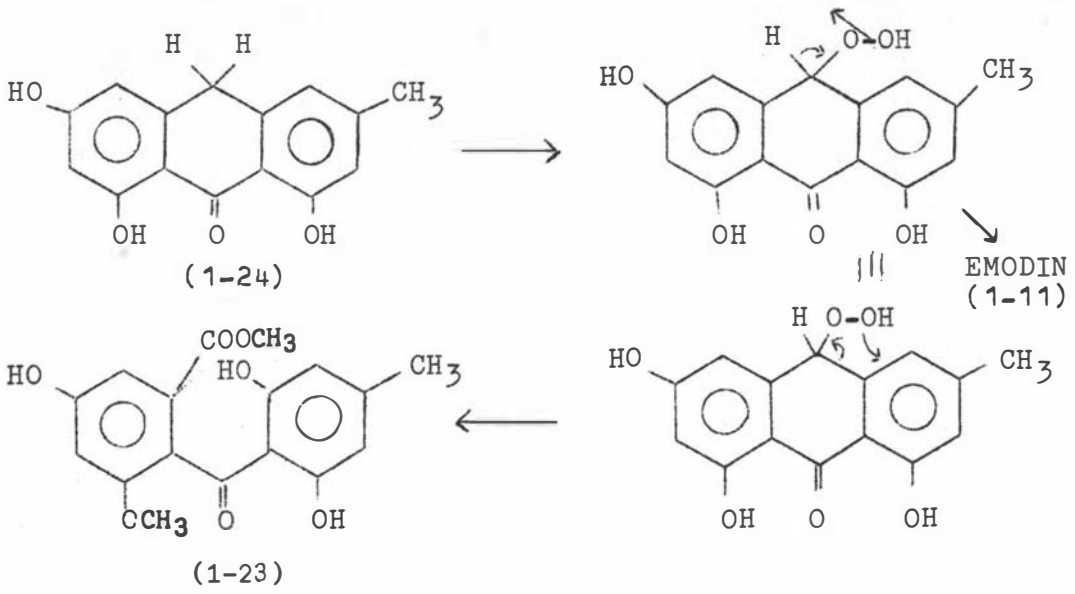
It has recently been shown (Leistner, 1971; Fairbairn and Muhtahi, 1972) that, in contrast to the findings with alizarin(1-20) which is constructed from shikimic, mevalonic and presumably 2-oxoglutaric acids (Burnett and Thomson, 1967;

Leistner and Zenk, 1971; Robins and Bentley, 1972), the biosynthesis of chrysophanol(1-21) and emodin(1-11) (examples of the second class) in Rhamnus fragula and Rumex alpinus or in Rumex obtusifolius, occur via the acetate-polymalonate pathway. In a related study (Curtis et al., 1971) of plant anthraquinone biosynthesis it was shown that, in spite of the absence of ring A hydroxy-groups in pachybasin(1-22), the biosynthetic pathway to this compound in Phoma foveata involved acetate and malonate units only.

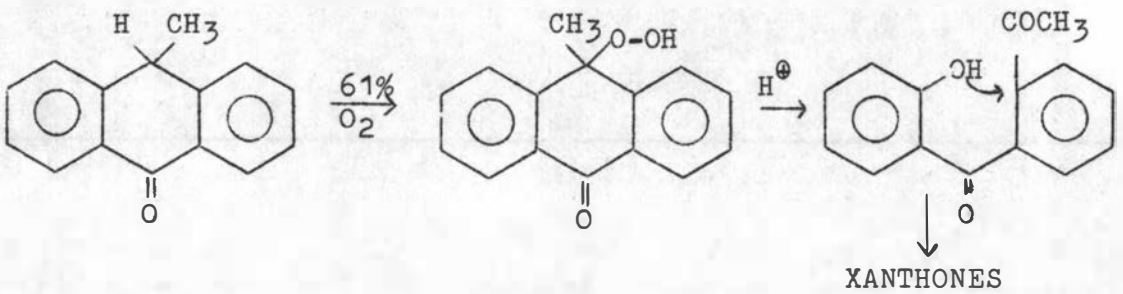
Until recently anthraquinones were regarded as metabolic end products and they are accumulated by certain organisms in relatively large amounts. However in some fungi they are metabolised further, notably in ergot (Claviceps purpurea) (Franck, 1969) and Aspergillus terreus (Curtis et al., 1972).

1.7 Possible role of substituted anthrones in secondary metabolism

To explain the formation of some anomalous phenolic compounds such as sulochrin(1-23) Money (1963) proposed an emodin anthrone(1-24) intermediate. Biological peroxidation of the reactive mesomethylene position would permit an acceptable rearrangement mechanism leading to either emodin(1-11) or sulochrin(1-23).

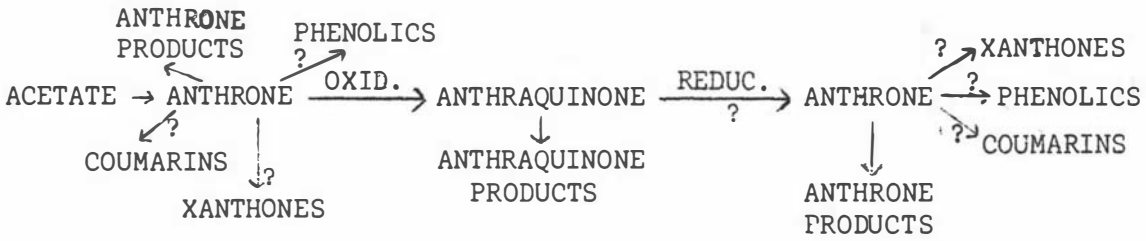


Experiments, where [^{14}C]acetate and [^{14}C]emodin were fed to Claviceps purpurea (summarised by Franck, 1969), established that ergochromes were biosynthesised from anthraquinones. Since oxidative opening of anthraquinone rings required vigorous reagents, such as chromic acid or strong base, and resulted in complete cleavage to benzoic acids, and since anthraquinones were found to be inert to peracids under Baeyer-Villiger oxidation conditions (thought to be a model for the biological reaction), Franck concluded that anthraquinones may not be the immediate cyclised precursors of ergochromes. On the other hand model experiments with anthrones showed the readily available hydroperoxide derivatives could be ring-opened to give benzophenone derivatives in line with the proposal of Money.



If this process was a model of events occurring *in vivo*, then xanthone formation could occur by the well documented (Taylor and Battersby, 1967) biological process of phenol coupling of the benzophenone derivatives.

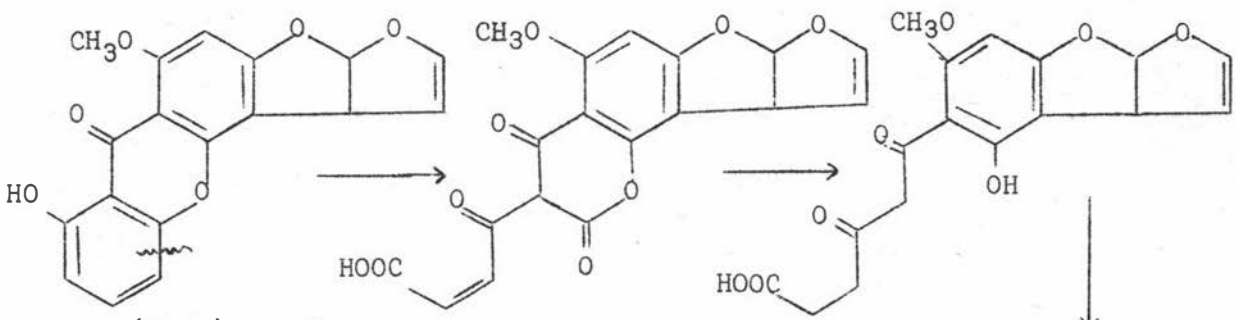
Thus it is suggested that, in addition to their undoubted role as intermediates between polyketides and anthraquinones, anthrones could have a separate existence as intermediates (by reduction) between anthraquinones and other secondary metabolites.



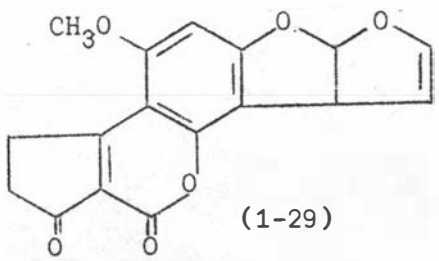
1.8 Biosynthesis of metabolites with a furo[2,3-b]benzofuran ring system

At present 30 natural products are known which have a furo[2,3-b]benzofuran ring system. In 12 of these this ring system is fused to a coumarin derivative (aflatoxins and parasiticol(1-25)), in 6 to a xanthone (sterigmatocystins and aspertoxin(1-26)) and in 9 to an anthraquinone (aversin(1-27), versicolorins(3) and dothistromins(5)) (summarised by Moss, 1972, plus 3 aflatoxins recently characterised by Heathcote and Hibbert, 1974).

Following publication of the tentative structures of aflatoxin B_1 (Asao et al., 1963; Hartley et al., 1963; van der Merwe et al., van Dorp et al., 1963) it was pointed out by Moody (1964) that the modes of forming carbon skeletons summarised in the acetate hypothesis and the isoprene rules (Ruzicka, 1963), did not lead to a simple hypothesis and three possible alternatives were suggested. By 1964 several compounds were found that could be incorporated into aflatoxins (Adye and Mateles, 1964). In the same year Holker and Underwood (1964b) proposed a scheme to explain a possible biosynthetic relationship between sterigmatocystin(1-28), a metabolite found in Aspergillus versicolor, and aflatoxins.

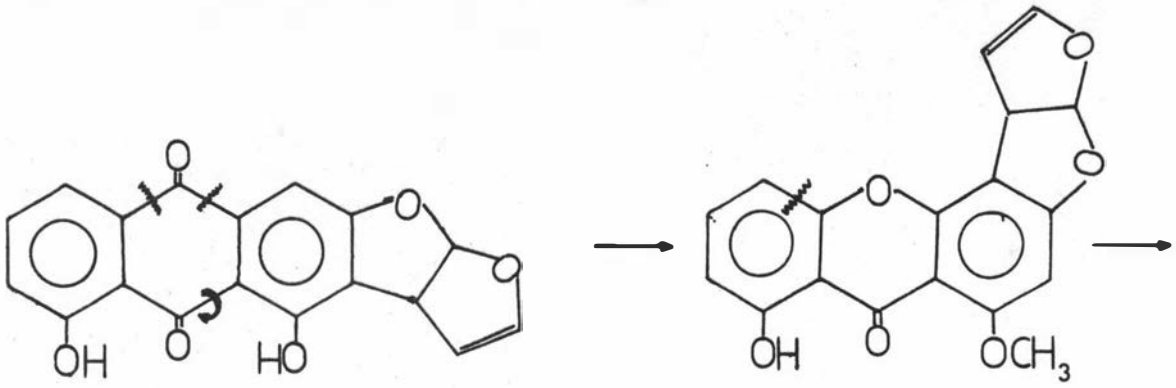


(1-28)

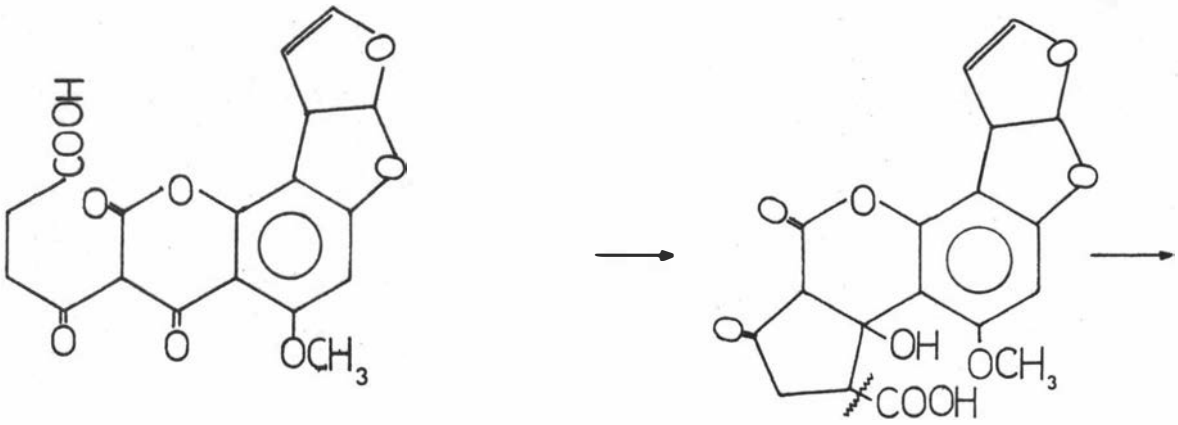


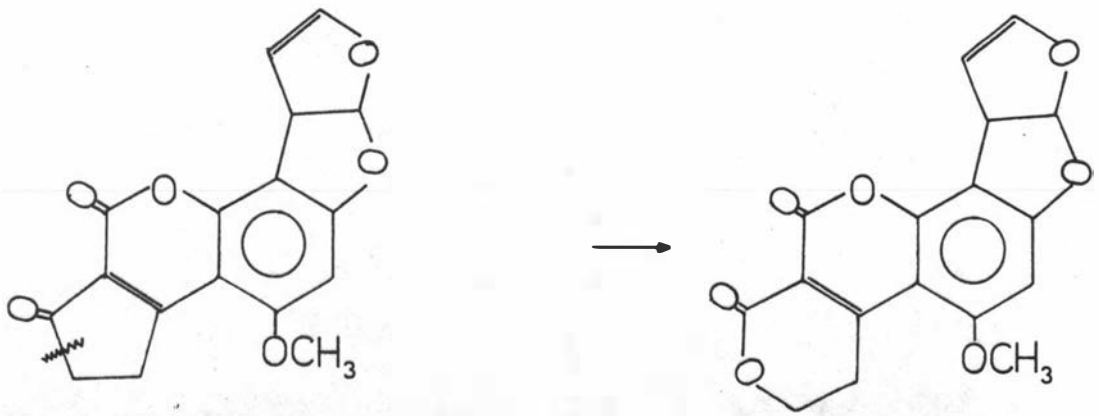
(1-29)

Shortly after, Thomas (1965) proposed a possible pathway for the conversion of a linear difuroanthraquinone into firstly sterigmatocystin(1-28) and then into aflatoxin B_1(1-29) (Figure 1-3). However, Holker and Underwood (1964b) failed to find any incorporation of [^{14}C]-sterigmatocystin into aflatoxin by either A.flavus or A.parasiticus and in addition



ANTHRAQUINONE **XANTHONE**





AFLATOXIN-B_1 **AFLATOXIN-G_1**

Figure (1-3) A possible relationship between anthraquinones of the versicolorin type, xanthones of the sterigmatocystin type and aflatoxins (after Thomas, 1965).

found A.versicolor did not produce detectable quantities of aflatoxin nor A.flavus and A.parasiticus sterigmatocystin(1-28).

Experimental support for a difuroxanthone precursor in aflatoxin biosynthesis was provided by Elsworthy et al. (1970) who showed that 5-hydroxysterigmatocystin(1-30) (Holker and Kagal, 1968) could be incorporated into aflatoxins B_2 (1-31) and G_2(1-32) and by Hsieh et al. (1973) who showed that sterigmatocystin(1-28) could be incorporated into aflatoxin B_1.

Incorporation experiments (Donkersloot et al., 1968 , Hsieh and Mateles, 1970), followed by degradative studies (Biollaz et al., 1968a and 1970) revealed that aflatoxin B_1(1-29) incorporated/from [1-^{14}C]- and [2-^{14}C]-sodium acetate. **radioactivity** By chemical degradation the radioactivity of 10 strategically placed carbons was measured directly and an intriguing labelling pattern was disclosed. The distribution of activity follows, for the most part, the alternating pattern characteristic of polyketide biogenesis, but with two notable exceptions. The benzylic carbon in the bisdifuran ring system and the aromatic carbon directly bonded to it were both found to be derived from the methyl carbon of acetate and a pair of adjacent carbons in the cyclopentanone ring were both derived from the acetate carboxyl group. The distibution of activity across the labelled positions was uniform, which was consistent with a biosynthesis from a single polyketide chain.

To account for these findings (figure 1-4) Biollaz et al., (1970) proposed that a C-18 polyhydroxynaphthacene (2), the

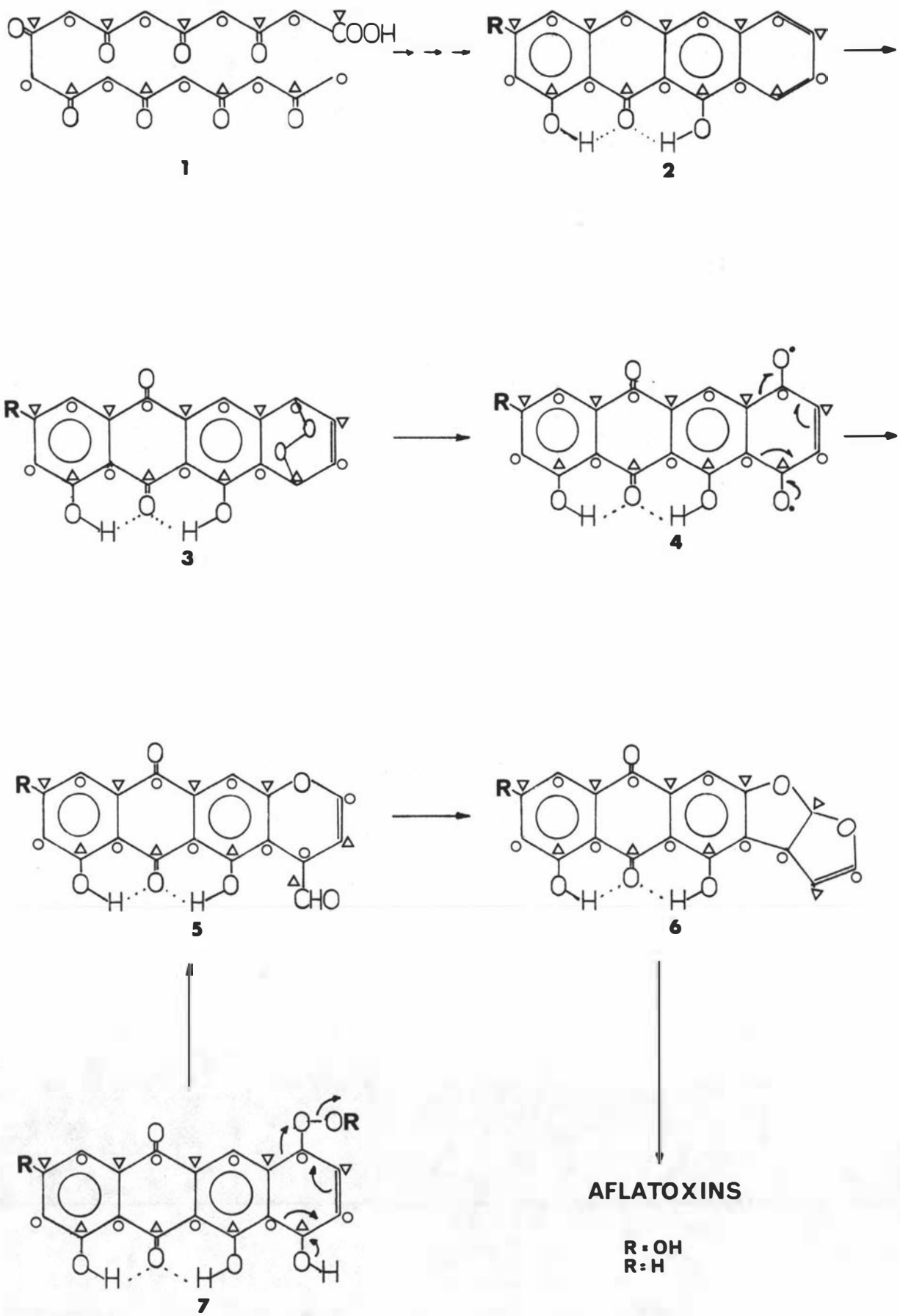


Figure (1-4) A possible biogenetic origin of the fused difuran ring system (after Biollaz et al., 1970).

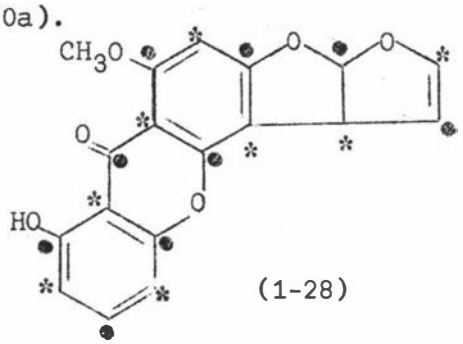
first cyclisation intermediate, was derived from a nona-acetyl (1) chain from which 2 oxygen atoms had been removed. This was oxidised to the endoperoxyanthraquinone (3) which in turn rearranged via the diradical (4) or zwitterion to the aldehyde (5). An alternative endoperoxidation route leading to the same pyran (5) could also feasibly result from a 1,4 addition of a hydroperoxide to the naphthacenequinone (7), with subsequent peroxide rearrangement. The former proposal was thought to be most likely as most biological oxidations involving radicals show evidence for the intermediacy of singlet oxygen. Formation of the two terminal rings (6) could possibly be brought about by an isomerisation of (5), similar to that found in the *in vitro* synthesis of aflatoxin B_1(1-29), leading to such linear bisdifuroanthraquinones as the versicolorins, aversin(1-27) and dothistromin.

The endoperoxide rearrangement suggested by Biollaz *et al.*, as a mechanism for generating the fused difuran ring system, although an elegant hypothesis, is without precedent amongst known chemical rearrangements and is still unsubstantiated.

Further feeding experiments were carried out to test the scheme. For example, compound (2, R=H) and the corresponding phenol (2, R=OH), labelled in each case with tritium, were administered to *A.flavus*, but no incorporation of isotopic label from these early precursors in aflatoxins was found.

More recently the biosynthetic origin of the 17 carbon

atoms in the carbon skeleton of sterigmatocystin(1-28) was determined using carbon-13 enrichment studies (Tanabe et al., 1970a).



(1-28)

* $^{13}CH_3COOH$

• $CH_3{}^{13}COOH$

The continuous wave carbon-13 NMR spectrum for sterigmatocystin (1-28) derived from [1-^{13}C]acetate showed nine carbon resonances of enhanced intensity. Similarly eight carbons showed enhancement when derived from [2-^{13}C]acetate. This labelling pattern (above) is in agreement with that found by Holker and Mulheirn (1968) from chemical degradation of [^{14}C]-sterigmatocystin and supports the novel biogenetic hypothesis that sterigmatocystin(1-28) is an early precursor of the aflatoxins. The carbon-13 result failed, however, to support the observation (Holker and Mulheirn, 1968) that the xanthone and difuran moieties are labelled to a different extent.

1.9 Coexistence of 18 carbon and 20 carbon metabolites

The coexistence of metabolites likely to be derived from a common precursor and differing in their number of carbon atoms is not uncommon. Aucamp and Holzapfel (1970) have found that A.versicolor (Vuill.). Tiraboschi produces sterigmatocystin(1-28), versicolorin C(1-33) and averufanin (1-34) and A.nidulans, sterigmatocystin(1-28), versicolorin C (1-33), averufin(1-15) and nidurufin(1-16). Heathcote and

Dutton (1969) have reported the coexistence of averufanin(1-34), versicolorin C(1-33) and aflatoxin B_1(1-28) in A.flavus.

Aucamp and Holzapfel (1970) suggest that the fact that versicolorin C(1-33) and sterigmatocystin(1-28) have been shown to coexist in these fungi, adds further weight to the suggestion by Holker and Kagal (1968) that sterigmatocystin (1-28) may be biologically derived from a difuroanthraquinone or related anthrone.

2.0 Bioconversion of averufin to aflatoxins

Thomas (reported in Moss, 1972) has proposed another pathway based on the possibility that the linear 6 carbon side chain of averufin(1-15) may be a precursor of the 4 carbon difuran side chain of the versicolorins and aversin(1-27) (Figure 1-5). It is suggested that oxidation of the averufin side chain at 4 carbon would result in chain folding followed by what appears to be a standard carbonium ion rearrangement initiated by the oxidative removal of a terminal acetyl group. This rearrangement gives the characteristic difuran side chain with the expected labelling pattern.

Support for this has resulted from mutant studies (Donkersloot, et al., 1972) with A.parasiticus ATCC 15517 impaired in aflatoxin biogenesis where significant quantities of averufin(1-15) is produced. The authors concluded that 20 carbon rather than 18 carbon intermediates predominate in aflatoxin biosynthesis or that since one of the steps in

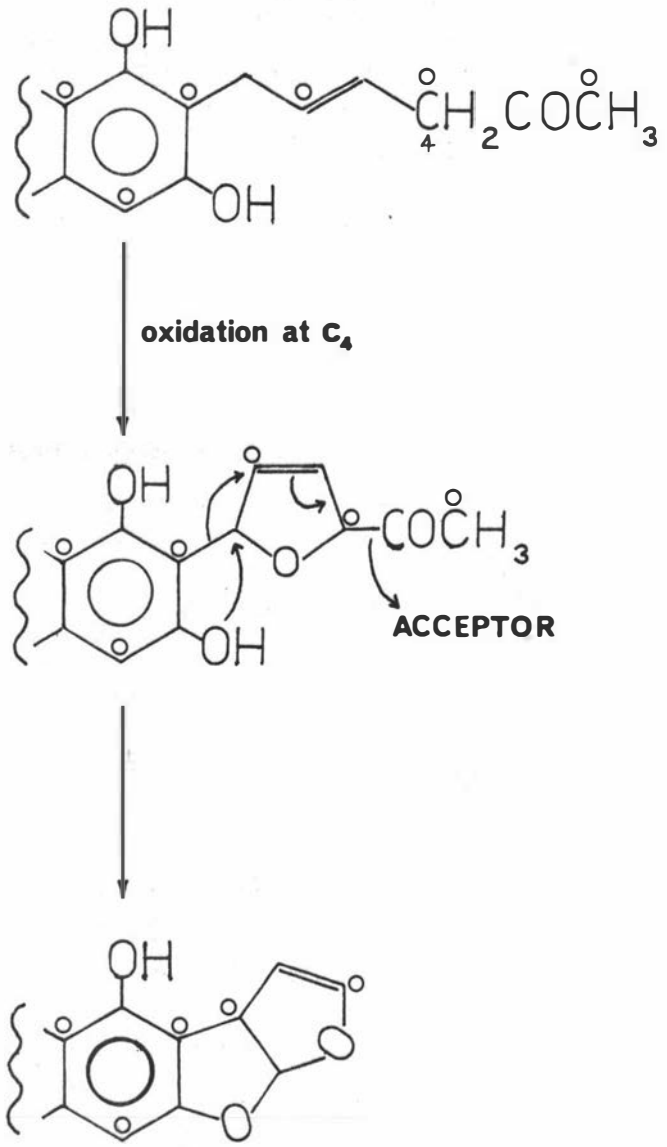


Figure (1-5) A possible mechanism for deriving the difuran ring system from a linear 6 carbon chain (after Thomas, quoted in Moss, 1972).

aflatoxin synthesis has been blocked in the averufin producing mutant, an additional acetyl group is added before the polyketide is released from the multienzyme complex, or alternatively, averufin(1-15) arises from a completely independent pathway, greatly enhanced in the mutant. A continuation of this work (Lin *et al.*, 1973) revealed that averufin(1-15) and aflatoxins coexist in the mycelium of wild type *A.parasiticus* and that the same organism readily metabolises [^{14}C]-averufin to [^{14}C]-aflatoxin B_1.

These results lead to a working hypothesis that aflatoxins arise through the condensation of one acetyl-CoA and 9 malonyl-CoA molecules to form a 20 carbon polyketide. This unstable intermediate is modified either during or after its formation and cyclisation, and appears as a 20 carbon polyhydroxyanthraquinone in the cytoplasm. Subsequent steps leading to a sterigmatocystin type compound could occur according to the recent Thomas scheme.

As has already been noted, good evidence is available for the final conversion of 5-hydroxysterigmatocystin(1-30) and sterigmatocystin(1-28) into aflatoxin (Elsworthy *et al.*, 1970; Hsieh *et al.*, 1973).

2.1 The oxidative coupling hypothesis for aflatoxin biosynthesis

The most recent proposal for the biosynthesis of aflatoxins is based on the condensation of two preformed polyketide chains (Heathcote et al., 1973). From the incorporation studies of possible radioactive precursors and keeping in mind the labelling pattern found in aflatoxin B_1(1-29), it is suggested that acetoacetate is oxidatively coupled to a preformed anthraquinone molecule (Figure 1-6).

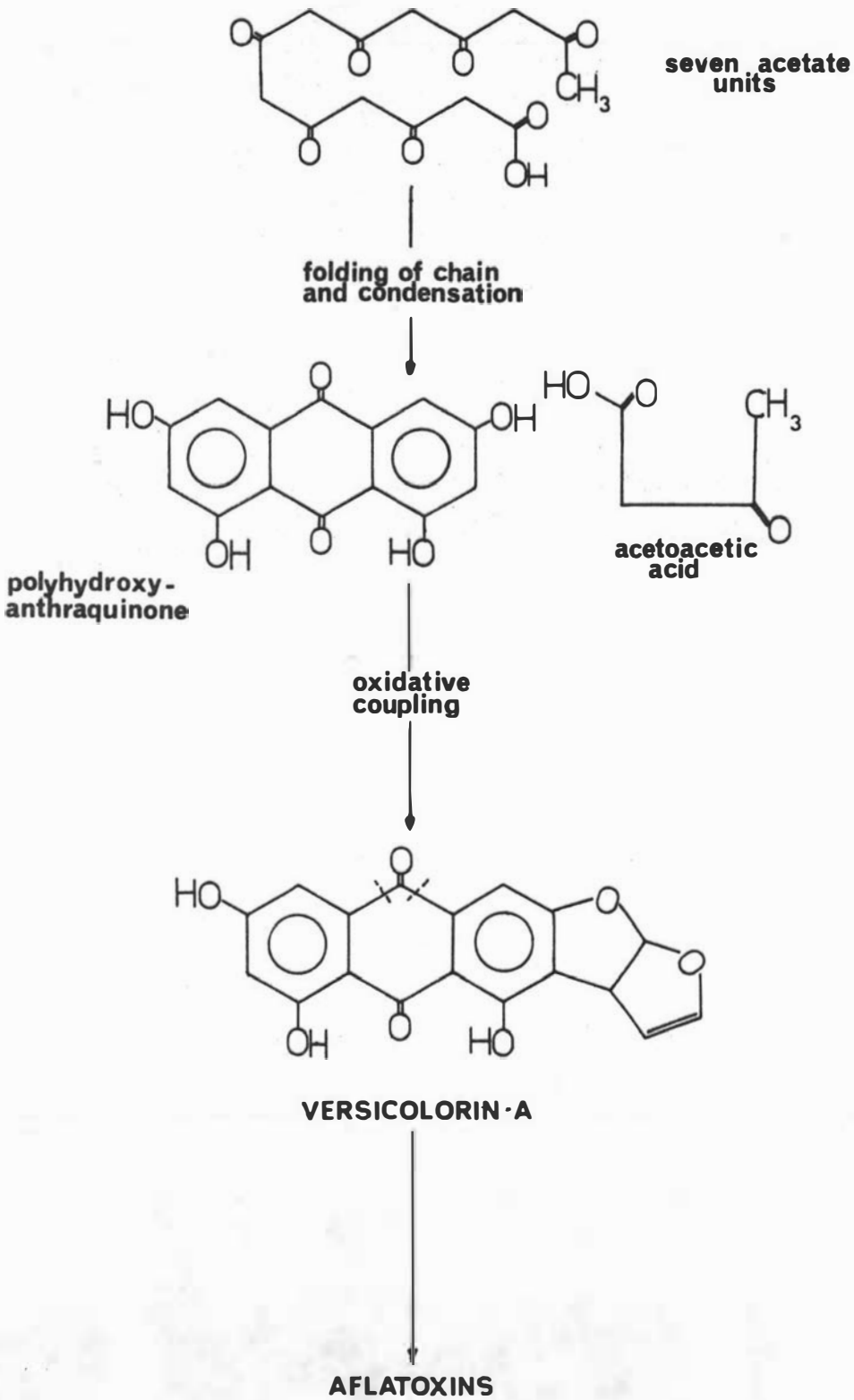


Figure (1-6) A possible route for the biosynthesis of aflatoxins via the acetate pathway and oxidative coupling of acetoacetate (after Heathcote et al., 1973).

CHAPTER TWO

Growth of the organism and production of dothistromin

2.1 The organism

Dothistroma pini is a primary pathogen that invades and kills pine foliage. It sporulates shortly after the death of these tissues, with the formation of groups of minute black stromata, which emerge through the dead epidermis. These contain the conidial masses and are often associated with a red staining of the substrate (Gadgil, 1967), which has led to the common names for the disease of 'red band' or 'red spot'. These lesions on the needles constitute the most striking disease symptom. Dothistroma pini was first reported by Hulbary (1941) as the causative agent of red needle blight in Pinus nigra var. Austriaca.

Dothistroma pini is widely spread, having been isolated in Europe, India, Russia, Canada, U.S.A and more importantly in exotic pine plantations of East Africa, New Zealand and Chile where forestry is based to a large extent on highly susceptible species.

This pathogen was first identified in New Zealand on Pinus radiata D. Don in 1964 (Gilmour, 1965), from an initial locus of infection near Tokoroa. Infections have been recorded on twenty five species and varieties of pine (Gilmour, 1967a, 1967b) since then.

To date three varieties of Dothistroma pini have been described (summarised by Gibson, 1972). There is, however,

still some doubt about the validity of these varieties, and some workers, such as Gadgil (1967), feel that the characters by which they are distinguished are insufficiently reliable, despite the careful controls imposed by the authors in the course of their observations.

In 1966 Funk and Parker described evidence showing Dothistroma pini to be the imperfect form of the ascomycete Scirrhia pini.

Dothistroma pini has no special nutrient requirements and can grow on agar medium containing simple carbohydrates, nitrogen salts, phosphates and major mineral elements (Ivory, 1967b). Growth is comparatively slow. The inability of Dothistroma pini to survive appreciable periods on natural substrate in competition with other saprophytes, combined with its growth rate and lack of special nutrients, make it an excellent example of the type of pathogen defined by Garrett (1950) as an ecologically obligate parasite, where the host provides the shelter of a non-competitive habitat rather than some special nutrient factor.

2.2 Isolation

The problems of isolation from the natural state are, in the main, concerned with separating the disease causing organism from numerous saprophytic species. These saprophytes frequently invade necrotic tissue behind the advancing front of the parasitic mycelium giving rise to secondary infection either during the natural biological process (Booth, 1971) or

during transport of the infected sections to the laboratory.

Preliminary studies on growth supporting, sporogenic medium were carried out using re-isolated Dothistroma pini cultures obtained from the Forest Research Institute, (F.R.I.), Rotorua. Most of these cultures produced acceptable levels of dothistromin both on solid media, from which an initial indication as to the biochemical potentialities for producing pigment was obtained, and in liquid culture isolated from good stock culture.

It was, however, necessary at times to isolate the fungus from the natural state for a continuation of this investigation.

2.2.1 Isolation from plant tissue

Diseased pine needles showing evidence of fruiting stromata with red banded regions, were collected from a plantation of infected Pinus radiata within the Kohitere Forest near Levin. Individual needles were subjected to pre-isolation treatment to facilitate satisfactory isolation of the pathogen. The most common method and the one finally adopted was to place diseased needles in an improvised humidity chamber (see experimental).

In Dothistroma pini asexual spores or conidia are borne on aerial conidiophores and are characteristically ejected in a gelatinous mass or spore horn under the conditions of the humidity chamber. Such spore horns, once identified, can easily be transferred into a drop of sterile water, identified microscopically and subsequently streaked out onto potato dextrose agar (PDA) in petri dishes (pre-isolation plates).

Many more conidia were extruded by gently teasing the lesioned area with a pair of mounted needles. Separation of accompanying saprophytes was greatly simplified by the inclusion of penicillin and streptomycin in PDA as antibacterial supplements.

2.2.2 Single spore isolation

Following microscopic identification of secondary conidia, after approximately two weeks incubation at 18°C, conidia from uncontaminated colonies on those plates showing a low degree of saprophytic contamination were removed and used as inoculum for a serial dilution series ranging from a dilution of 1 to $1/10^4$. This was then streaked onto 5% malt agar plates (isolation plates). Growth of isolated colonies arising from single spores were observed (Plate A) on the plates inoculated with the more dilute spore suspensions.

Following isolation of the fungus it was necessary to re-isolate from the isolation plates so as to give a clearly defined basis for the starting point of future studies on the isolate (Booth, 1971). There are various methods of isolating a single spore, namely:

(i) before germination,

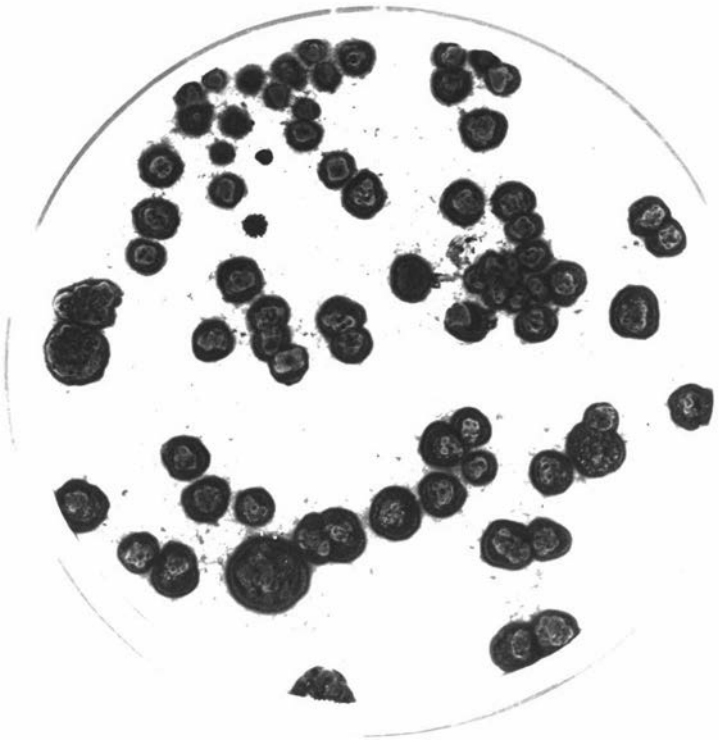
(ii) at germination,

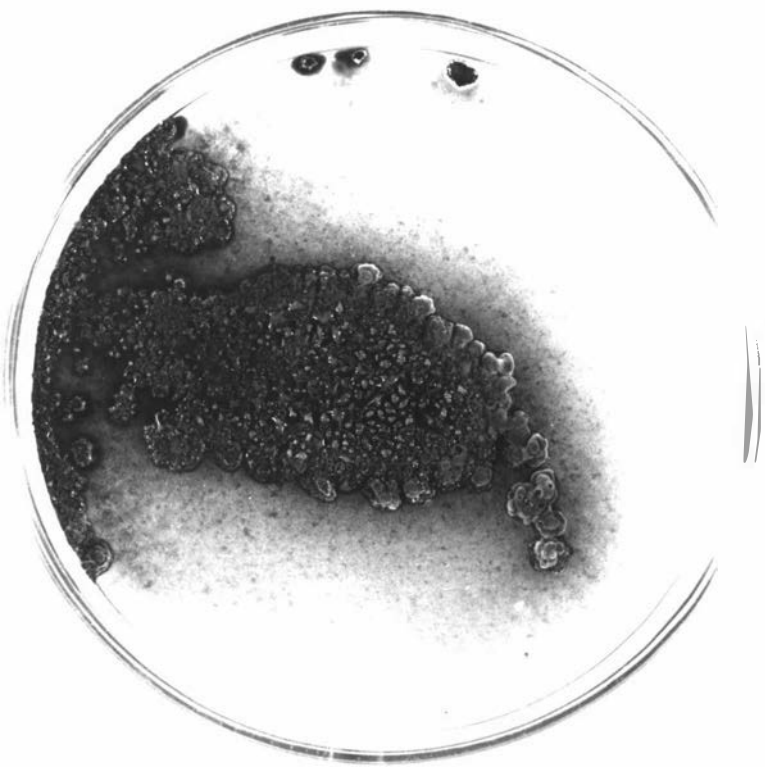
(iii) isolation of individual colonies derived from a single spore.

The final alternative was found most convenient. Isolated colonies were removed with a scalpel and dispersed in sterile

Plate A: Single colonies of *Dothistroma pini*

Plate B: Growth of *Dothistroma pini* on malt agar





water. Cultivation on fresh 5% malt agar plates (re-isolation plates) gave good growth and good dothistromin production with no evidence of contamination (Plate B).

2.3 Maintenance

Although the strain of Dothistroma pini isolated showed no evidence of becoming attenuated under artifical growth conditions, it did lose the ability to produce pigment on repeated subculturing and did, at times, give rise to what appeared to be purely vegetative, non-sporulating forms.

It, therefore, became necessary to maintain a viable inoculum in a non-growing condition to provide cultures with a minimum of genetic change.

Culture maintenance and the provision of a continuing source of the organism, preferably from a single isolate, involves three problems; (a) keeping the organism viable, (b) keeping it free from contamination and (c) maintaining the biochemical property which is being studied. The last of these requirements is the most difficult to fulfil, for fungi are extremely variable organisms - strains obtained from different sources, while appearing morphologically identical, will not necessarily behave in the same way biochemically. In order to keep an organism alive at ambient temperatures it must be transferred periodically to fresh medium. However, repeated subculturing of fungi on nutrient agar can lead to selection of dominant mutant strains or pure vegetative non-sporulating forms, and attenuation of the mould

under artifical conditions.

It is necessary then that transfers be as infrequent as possible. For this reason and also to reduce the chance of variation within the culture, the growth of stock culture should be kept to a minimum. For most experiments fungi are grown in liquid media but for storage purposes a solid medium is more convenient.

Although growth studies on Dothistroma pini have been made by several workers (Ivory, 1967a, 1967b; Sanders, 1969), no work has been carried out on spore maintenance. For this investigation the three most popular methods were used.

2.3.1 Agar Slant

The agar slant or periodic transfer method is the most generally employed for maintaining fungal cultures. This involved the preparation of 5% malt slants in McCartney bottles and the transfer of spores from old cultures to fresh slants of a suitable substrate at regular 2 monthly intervals.

While agar slants are easily prepared, convenient to use and instantly available for examination and comparison, it was found that the ability of the fungus to produce pigment decreased with increased number of transfers.

2.3.2 Soil Culture

There are two ways of preparing soil cultures in common use, (i) inoculation of dried soil with a small volume of

spore suspension, followed by immediate drying and (ii) use of large volumes of inoculum or moistened soil followed by incubation. The first of these obviously permits no growth and would maintain conidia introduced into the soil. The second, by permitting growth, would maintain propagative cells and mycelium at least one generation removed from the original inoculum.

The former method, used for Dothistroma pini, is the method most commonly employed for soil maintainance of fungi as outlined by Greene and Fred (1934). A concentrated spore suspension (2 cm^3) obtained from subcultures of the F.R.I. stock culture was added to 5 grams of sterilised orchard loam soil which was then dried at room temperature and stored at 4^oC (Cormack, 1951). It was subsequently noted that the cultures became non-viable after approximately 15 months, failing to germinate on 5% malt agar medium.

2.3.3 Lypophilisation (Raper and Alexander, 1945)

The main virtues of this method for fungal maintenance are,

(i) culture viability is greatly prolonged,

(ii) new cultures arise from spores originally processed,

(iii) simultaneous preparation of multiple tubes provides a source of uniform inoculum over an indefinite period, and

(iv) strain variation is minimised through infrequent cultivation.

However, preparation of Dothistroma pini lyophils using a sugar suspending medium did require more effort than the other methods. This method is also disadvantageous in contrast with the soil method in that lypophilised cultures may be used only once, whereas the soil preparation had the advantage of one tube supplying a continuing source of inoculum.

2.4 Extraction and purification of dothistromin

For the routine extraction and subsequent purification of dothistromin from Dothistroma pini cultures it was necessary to evolve efficient procedures which allowed either analysis of whole cultures or small culture samplings.

2.4.1 Extraction

Initially, with 100 cm^3 and 300 cm^3 malt cultures, the mycelium was separated from the medium by filtration. However, the viscous nature of the culture medium after 8 and 11 days incubation, respectively, made filtration impractical on a routine basis, prior to solvent extraction.

The procedure adopted for extraction of the whole culture entailed first acidifying the medium to pH 1-3 and then macerating the mycelium in a blender in the presence of 20 cm^3 and 50 cm^3 of ethyl acetate respectively to prevent foaming.

Brunt (1970) found that dothistromin was extractable to

varying degrees with butanol, chloroform, ethyl acetate and hexane. Ethyl acetate was used in the ratio of one volume to one volume of the culture medium. The same procedure was adopted by Gallagher (1971) but it was pointed out that during extraction in a separating funnel, care was required to overcome the formation of stable emulsions. It was suggested that using excess ethyl acetate would overcome this problem. However, the overall procedure was time consuming and therefore inconvenient for a routine system.

It was found during this investigation that a better method was to extract the blended mixture three times with 3 volumes of ethyl acetate. Although this gave an increased volume of ethyl acetate solution, it was a comparatively rapid extraction method. There was no emulsion formation even with vigorous shaking in a separating funnel. On evaporation of the dried extract a red solid was obtained.

Because of the large volume involved, extraction of the Fermacell culture (20 litres) required modification of the above method in so far as the culture medium (in one litre aliquots) was extracted only once without prior maceration of the mycelium. The large volume of ethyl acetate extract (60 litres) was reduced using a Cyclone evaporator.

2.4.2. Chromatography

Dothistromin was purified from the red solid, obtained as described above from shaken cultures, by preparative thin-layer

chromatography and column chromatography using silica gel.

2.4.3 Differential solubilities

The purification of relatively large amounts of dothistromin by chromatographic methods was found to be very time consuming. Therefore, an alternative procedure based on the differential solubility of the pigments in the red solid was investigated. It is well known that most polyhydroxyanthraquinones have a very low solubility in most and especially non-polar solvents.

The possibility of differentially extracting the impurities was tested by triturating the crude red solid from 100 cm^3 and 300 cm^3 cultures, in ethyl acetate. The resulting suspension of red solid was centrifuged and the residue washed twice with diethyl ether and twice with hexane to remove remaining lipids and cometabolites. Mass spectrometry of the insoluble red, amorphous powder revealed that besides dothistromin, it contained deoxydothistromin (2-1) and bisdeoxydehydrodothistromin(2-2) (Danks and Hodges, 1974).

An alternative method then tried was to use chloroform in place of ethyl acetate and to wash the centrifuge pellet three times with ether. The product in this case was found to be largely dothistromin contaminated by a small amount of deoxydothistromin(2-1). It was not possible to separate these two compounds. As dothistromin has a small degree of

solubility in these solvents it was recovered from the washings by preparative thin-layer chromatography.

This latter method was used for all total culture extractions. However, in those experiments where the culture was sampled at intervals, thin-layer chromatography was retained as the method of dothistromin purification.

2.5 Identification of dothistromin

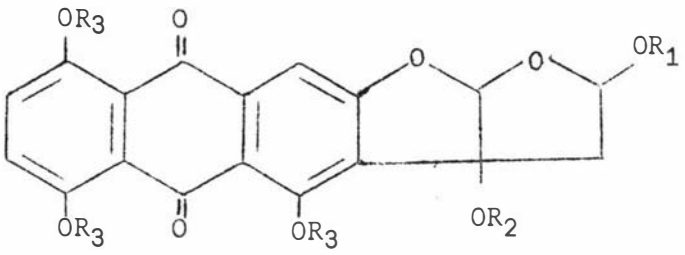
To unequivocally establish that the red powder was dothistromin the ethyl acetal tetramethyl ether derivative was prepared in a two step reaction procedure (Gallagher and Hodges, 1972).

2.5.1 Dothistromin ethyl acetal

Treatment of purified dothistromin, dissolved in ethanol with a catalytic amount of thionyl chloride gave a quantitative conversion to the ethyl acetal(2-3).

2.5.2. Dothistromin ethyl acetal tetramethyl ether

Methylation of the ethyl acetal (3-3) with dimethyl sulphate and potassium carbonate in refluxing acetone yielded a mixture of two products which were separated by thin-layer chromatography. The first of these, of lower R_f, was identified as ethyl acetal trimethyl ether (2-4). The second compound, of higher R_f, was obtained in higher yield and was shown to be ethyl acetal tetramethyl ether (2-5) (see experimental).



	R_1	R_2	R_3
(2-3)	Et	H	H
(2-4)	Et	H	Me
(2-5)	Et	Me	Me

2.6 Estimation of dothistromin in culture extracts

Purified dothistromin in ethanol gave an ultraviolet-visible absorption spectrum very similar to that of 1,4,5-trihydroxyanthraquinone. The visible spectrum was in good agreement with that reported by Gallagher and Hodges (1972). Although it is known that deoxydothistromin (2-1) coexists with the major metabolite (Gallagher, 1971), extinction coefficients were calculated assuming total homogenity for the purpose of estimating dothistromin production. Dothistromin concentrations were calculated from the visible absorption maximum at 490 nm (ε = 8,400).

The losses involved in the differential solubility method of purification precluded its use as a quantitative method for estimating dothistromin concentration in small samples. An alternative method used was to purify the dothistromin in the ethyl acetate extract by thin-layer chromatography on

0.2 mm thick silica gel plates developed with ethyl acetate - chloroform (60:40). The dothistromin containing band was eluted from the gel, the eluate evaporated to dryness and the red solid dissolved and made up to volume in ethanol. The dothistromin concentration for the total culture was calculated from the absorbance at 490 nm.

2.7 Cultivation and cultural description

The aim of studying the growth characteristics of Dothistroma pini under laboratory conditions was to find a suitable solid medium that, besides supporting good growth, would also allow good sporulation. In turn, a concentrated spore inoculum would lead to pigment production in good yield from liquid culture.

In order to carry out successful and meaningful biolabelling studies with carbon-13 and carbon-14 enriched precursors, it was necessary to (i) have a good pigment producing culture to obtain a high dothistromin yield, (ii) be able to determine the onset of pigment production as accurately as possible and on achieving this, (iii) predict the onset of pigment production.

2.7.1 Growth on solid media

Preliminary subculturing by streaking plates with dilute spore suspensions from F.R.I. samples showed that Dothistroma pini did not grow as a smooth mycelial mat. Instead these

multispored inoculations yielded individual colonies which did not merge during any part of the growth phase. Consequently the yield of spores per plate culture was low and this technique was changed to one of flooding the surface of the plate with concentrated spore suspensions by means of a syringe. This resulted in greater spore loads per plate.

While 5% malt agar alone induced satisfactory sporulation a marked improvement was obtained by adding 0.5% dried whole yeast. On this medium conidial production was at a maximum when the culture mat took on a moist shiny appearance (Plate B) as verified by microscopic examination. This modified medium was used for all subculturing. Spores were sown by lightly spraying the sterile medium with inoculum from a syringe. This method has the advantage of not only giving rapid plate coverage but also decreasing the risk of contamination from air-borne contaminants, since it can be done by just raising the lid of the petri dish sufficiently to allow the syringe needle to be inserted.

2.7.1.1. Cultural description

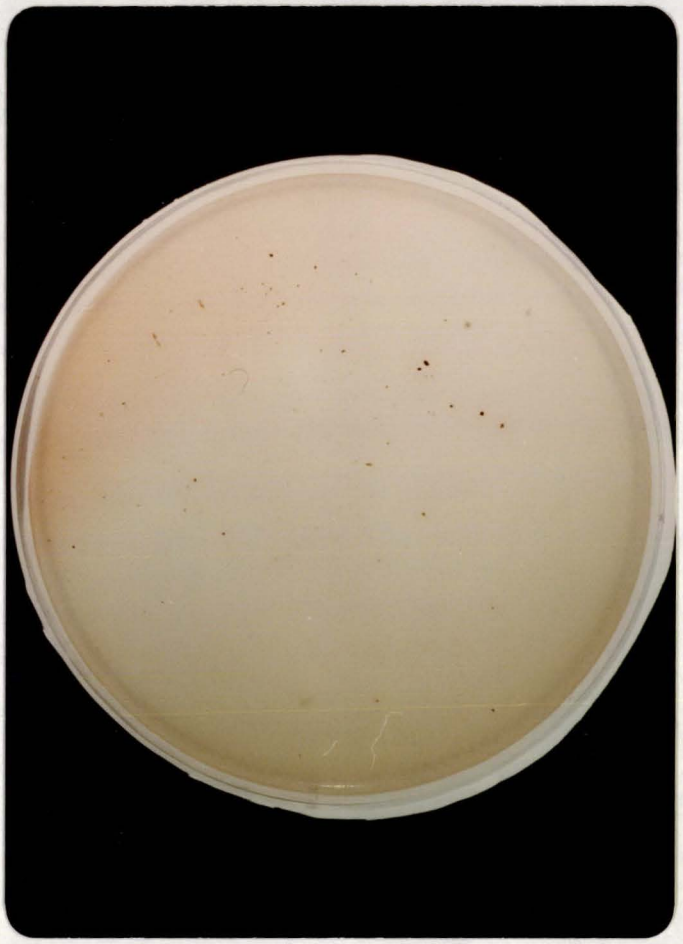
Cultures grown from spores on 5% malt, 0.5% dried whole yeast extract appear as appressed hyaline colonies to the unaided eye after 3-4 days at 18°C (Plate C). Pigment production appears to be initiated after 5-6 days and as production increases it begins to diffuse into the surrounding agar (Plate D). After 9-11 days a marked increase in growth and concurrent pigment production is observed (plate E).

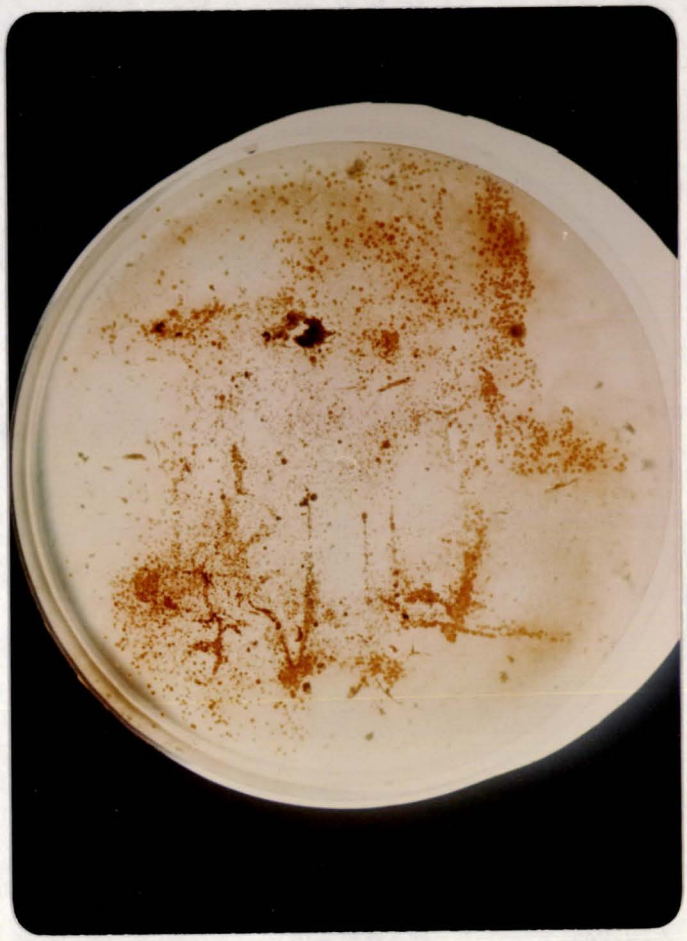
Plate C:
Growth of Dothistroma pini
after 3-4 days incubation

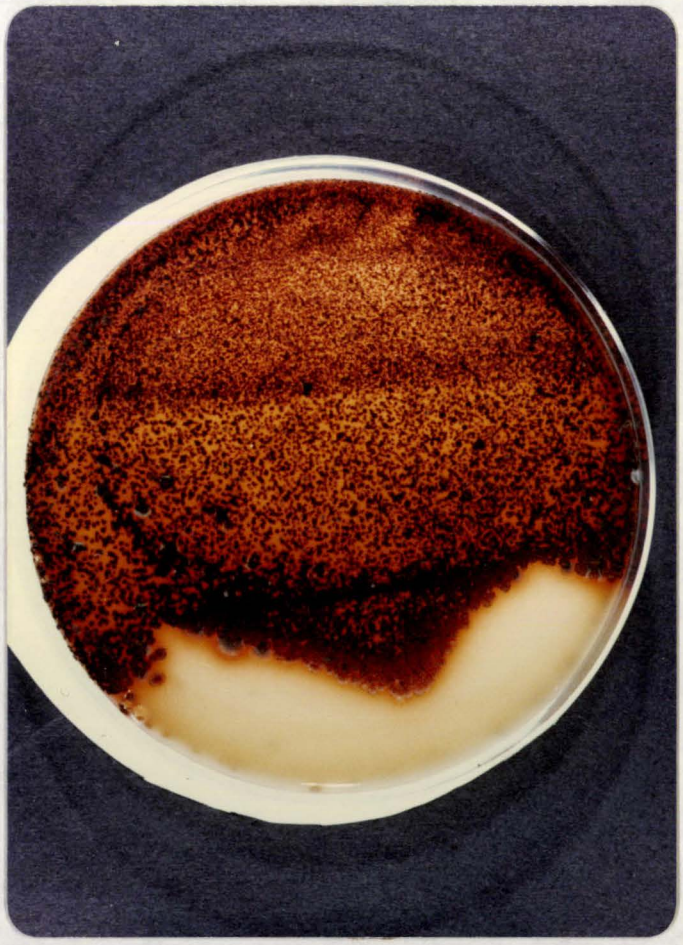
Plate D:
Growth of Dothistroma pini
after 5-6 days incubation

Plate E:
Growth of Dothistroma pini
after 9-11 days incubation

Plate F:
Growth of Dothistroma pini
after 16-20 days incubation







The mycelium does not spread broadly, instead, the colonies gradually become hemispherical, stromatoid and darkly coloured on production of conidia after 16-20 days (plate F).
At this stage deposits of red solid appear at the periphery of the growing mycelium. About 22-28 days after inoculation white, aerial mycelium begins to form.

2.7.3 Growth in liquid culture

2.7.3.1 Surface culture

When a static liquid nutrient medium is inoculated with fungal spores or mycelium, the mycelium grows over the surface of the culture to form what is variously referred to as a felt, a pad or a mat. The two major objections to this type of culture is that growth is inhomogeneous so that the mat contains mycelium at various stages of development and in a variety of environments.

Conditions at the surface of the mat tend to be more aerobic in contrast to the growing mycelium, at, or even below the surface of the liquid medium where the environment is partly anaerobic. However, it is only the latter two growing regions that are best supplied with nutrient. Secondly, and as was found with Dothistroma pini surface cultures, not all spores float, with some adhering to the sides of the flask or settling to the bottom of the culture flask.

Both modified Raulin's and 5% malt medium surface cultures showed little mycelial growth and pigment production, so

this method of growth was discontinued.

2.7.3.2 Shaken culture (submerged)

In this technique the medium is shaken after inoculation with spores or mycelium so that growth occurs in the body of the liquid. The advantages over surface cultures are that nutrient uptake is more efficient, giving more rapid growth and that growth is usually homogeneous enough for the culture to be used directly in metabolic studies.

In shake cultures each colony is exposed to a uniform environment in all spatial directions hence the typical colony is a globose structure. Even under shaken conditions it may be difficult to obtain sufficiently homogeneous growth for physiological experiments, especially in those organisms in which the mycelium tends to form pellets rather than loose aggregates (conditions at the centre of a pellet will be very different from those at the surface).

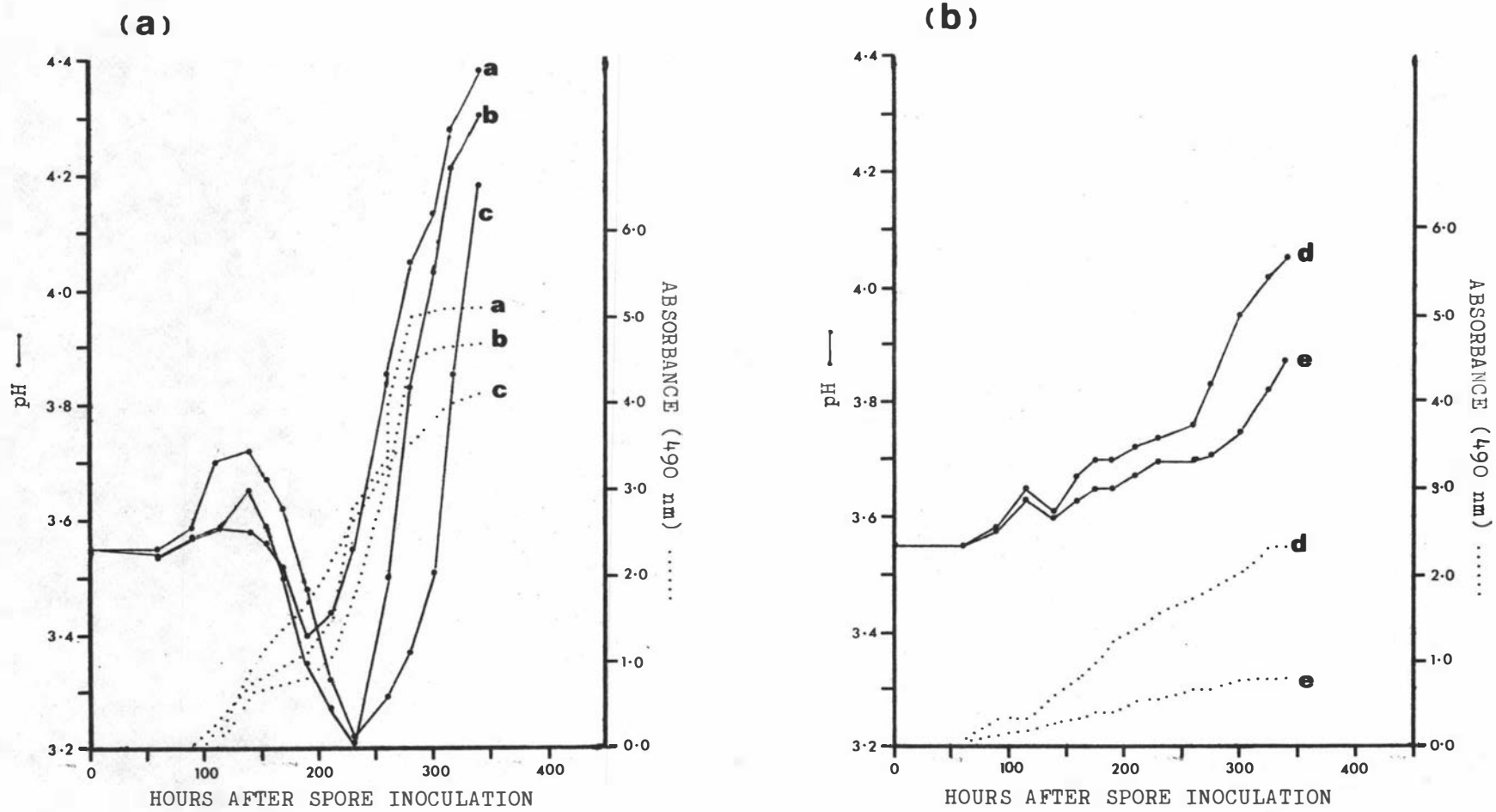
2.7.3.2a Modified Raulin's medium

Both malt (Gallagher, 1971) and synthetic Raulin's medium (Brunt, 1970) have been previously described as useful for the production of dothistromin in submerged culture. The effect on dothistromin production of using various sugars in place of sucrose in the Raulin's medium was examined. The hydrogen ion concentration was monitored in 200 cm^3 shake cultures grown in 500 cm^3 er lenmeyer flasks following

inoculation to see whether the buffering capacity was adequate as the results reported by Brunt (1970) suggested an early loss of buffering capacity in this medium. Total growth was assessed by measuring mycelial dry weight, with pigment production being measured as previously described.

It was evident that 50 hours after inoculation (Graph 2-1) all flasks showed an apparent loss of buffering capacity. The initial alkaline shift in pH after 80 hours may reflect citrate utilisation. In those flasks having glucose, sucrose and fructose as the major carbon source a subsequent drop in pH was observed after 140 hours, possibly reflecting an accumulation of organic acids as a result of sugar metabolism (Graph 2-1a). In contrast, those flasks containing ribose and maltose as the major carbon source did not show any significant change in pH after this time (Graph 2-1b). However all cultures underwent a rapid alkaline shift after 240 hours. The cause of these pH changes was not investigated.

The mycelial dry weight was measurable in those flasks with glucose, sucrose and fructose after 60 hours and in those flasks containing maltose and ribose, 150 hours after spore inoculation. This increased at an apparently linear rate until 300 hours after inoculation when the rate became zero. The sugars differed markedly in their ability to suport growth, with ribose being the sugar least effective.



Graph 2-1 Relation between pH, pigment production and time for the growth of Dothistroma pini in Raulin's medium modified to contain the following sugars as carbon source; (a) glucose, (b) sucrose, (c) fructose (d) maltose and (e) ribose.

The carbon sources tested can be divided into three groups. The first, which supports good growth of the fungus, contains sucrose, glucose and fructose. The second group supporting moderate growth would include maltose, while ribose only supported poor growth.

Dothistromin production was just detectable after 50 hours and increased at a linear rate in the case of ribose and maltose. The overall yield in all cases was very low with the best yields being obtained from glucose; approximately 2.5 mg per 100 cm^3 of culture medium. While there appeared to be some correlation between pH change and pigment production using glucose, sucrose and fructose, there was no such correlation in those cultures containing ribose and maltose. Similarly there was no apparent correlation between pigment production and mycelial growth, as was found by Bassett and Buchanan (1969).

2.7.3.2b Malt medium

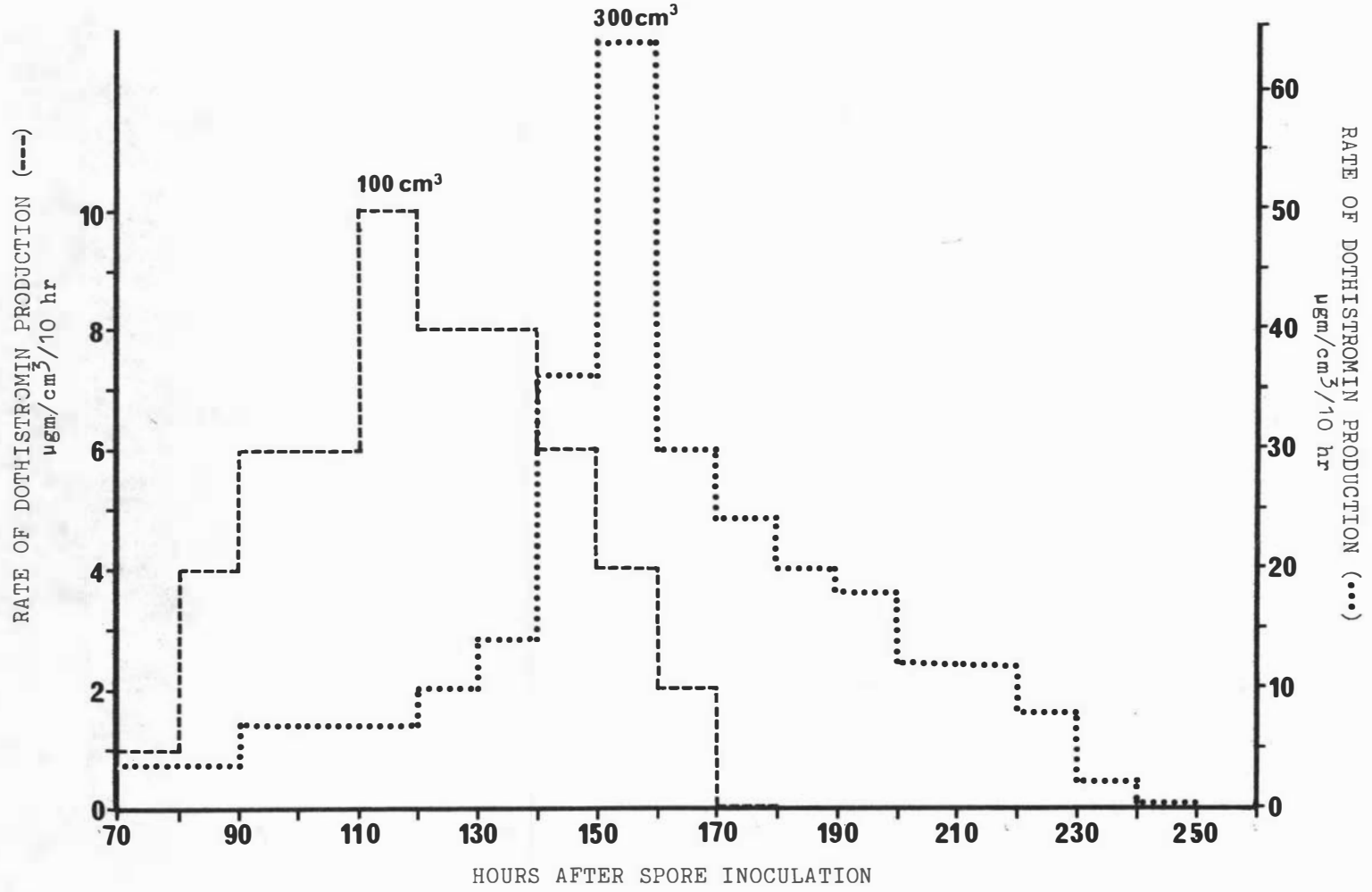
Gallagher and Hodges (1972) first reported the use of a malt medium, consisting of 10% w/v malt, 0.5% dried whole yeast and cholesterol (20 mg/litre), as a satisfactory growth medium for dothistromin production. Work in this investigation showed that comparable dothistromin production could be obtained using 5% malt

and 0.5% dried whole yeast medium without cholesterol.

Initial studies used one litre er lenmeyer shaker flasks containing 300 cm^3 of sterilised malt medium. This volume was such that in these flasks the surface area of the medium was the maximum that their shape would allow for good aeration. Pigment production proved to be better than that found in Raulin's medium, with a final dothistromin concentration ranging from about 0.2-0.3 mg per cm^3 over all flasks.

A continuous measurement of pigment concentration revealed that production began at about 80-90 hours after spore inoculation and reached a maximum rate after 150-160 hours. 100 cm^3 malt cultures in 250 cm^3 er lenmeyer flasks showed a similar rate profile (Graph 2-2). However, dothistromin production was at a maximum only 110-120 hours after spore inoculation. These cultures showed a dothistromin yield of about 0.05-0.15 mg per cm^3 of culture medium.

One disadvantage of the larger cultures was the formation of surface foam which was known to adversely affect aeration of the medium. In an attempt to prevent this, 0.5 cm^3 sterile polyethylene glycol was added to autoclaved flasks before inoculation with spores.



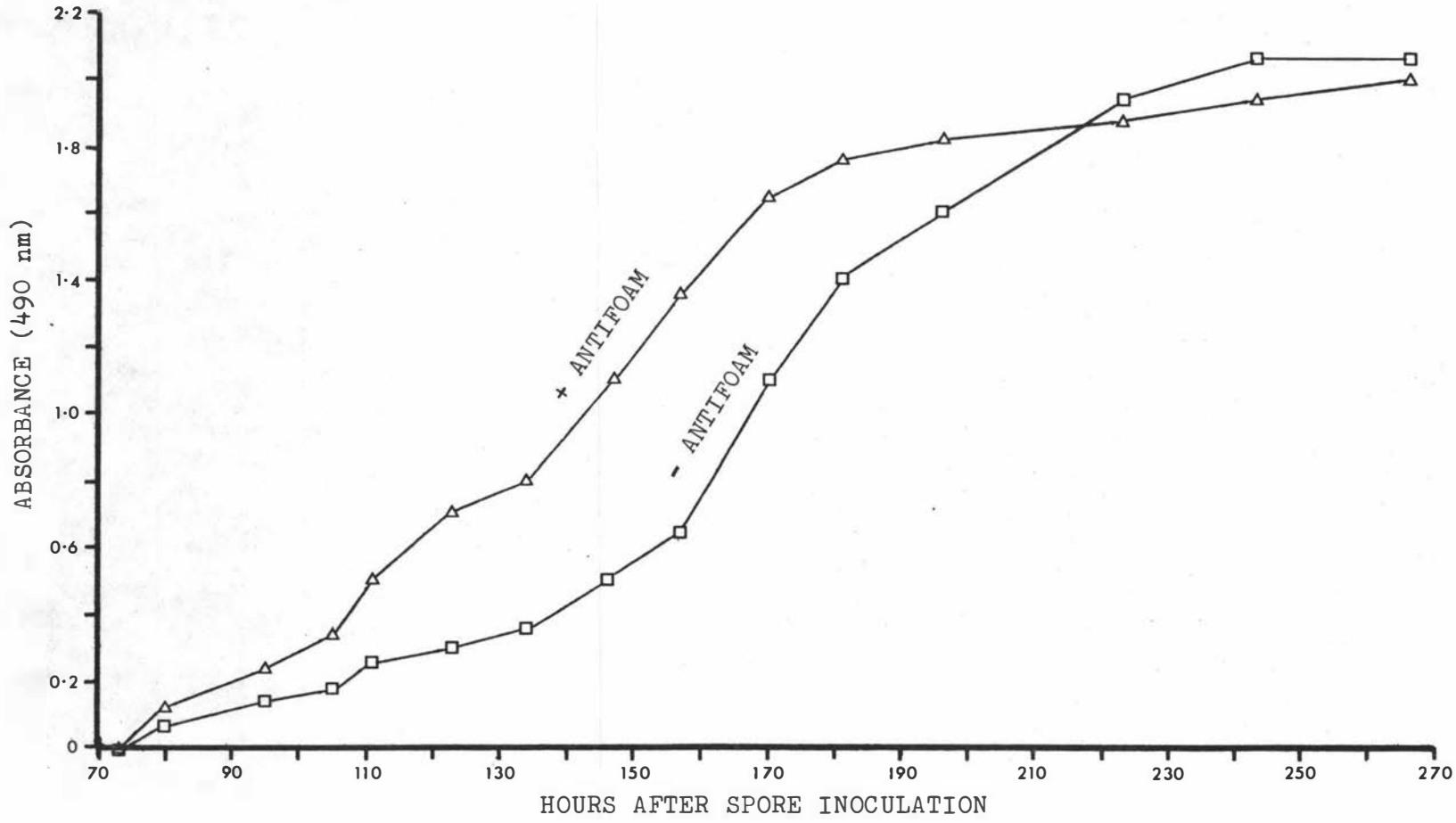
Graph 2-2 Rate of dothistromin production versus time from inoculation in 100 cm^3 (---) and 300 cm^3 (••••) malt cultures of Dothistroma pini. Yield of dothistromin was calculated using ϵ_{490} = 8400.

Duplicate experiments using the same spore concentrations, were carried out to determine whether this surface active agent affected pigment production in any way. Graph 2-3 shows that the rate of pigment production in the flasks containing antifoam was increased relative to the control over the early stages of pigment production period, which may be a direct result of improved aeration. However, the final yield of pigment remained the same in both cases. Foaming was not a problem in 100 cm^3 cultures.

2.7.4 Correlation of pH with pigment production

At this stage of the study it was apparent that growth of *Dothistroma pini* in malt medium gave yields of dothistromin per unit volume superior to those obtained using modified Raulin's medium and that there was a definite period during the growth phase where the rate of pigment production was highest. However, the data above do not give any indication as to the impending onset of pigment production.

The time elapsing from the time of inoculation to the appearance of pigment did vary by about 5-10 hours in 300 cm^3 cultures and by about 5 hours in 100 cm^3 cultures. This variation is probably due to the inability to control such variables as the age of the spore inoculum, size of the inoculum and small variations in the composition of the nutrient medium between experiments or even between individual



Graph 2-3 Time course of pigment production in 300 cm^3 malt cultures of Dothistroma pini with and without antifoam. Each valve shown represents the average of two flasks.

flasks. It therefore became important to find some sort of indicator of impending pigment production, in order to be able to add an isotopically labelled precursor just prior to its onset.

The nature of the metabolic activities of moulds is such that the pH of the environment of a growing culture will not remain constant for long. The pH of the culture medium exerts control over fungal growth by affecting the availability of certain metallic ions, cell permeability and enzymatic activity within the cell (Raper and Fennell, 1965). These changes in pH are, in the main, associated with the uptake and excretion of certain anions and cations and with the accumulation in the medium of organic acids, especially glucuronic, pyruvic, citric and succinic acids formed by the metabolism of carbohydrates. Whatever the cause, the ultimate pH of the culture medium depends on the relative rate and occurrence of these pH changing processess and the extent to which the medium is buffered.

A study was made to find any possible correlation between changes in pH and pigment production in 100 cm^3 malt cultures. Three hundred cm^3 cultures do not offer any advantages over 100 cm^3 cultures in terms of pigment production, indeed smaller volumes are more satisfactory for labelling experiments in that dilution is decreased and so the remaining work concentrated on events occurring in 100 cm^3 cultures.

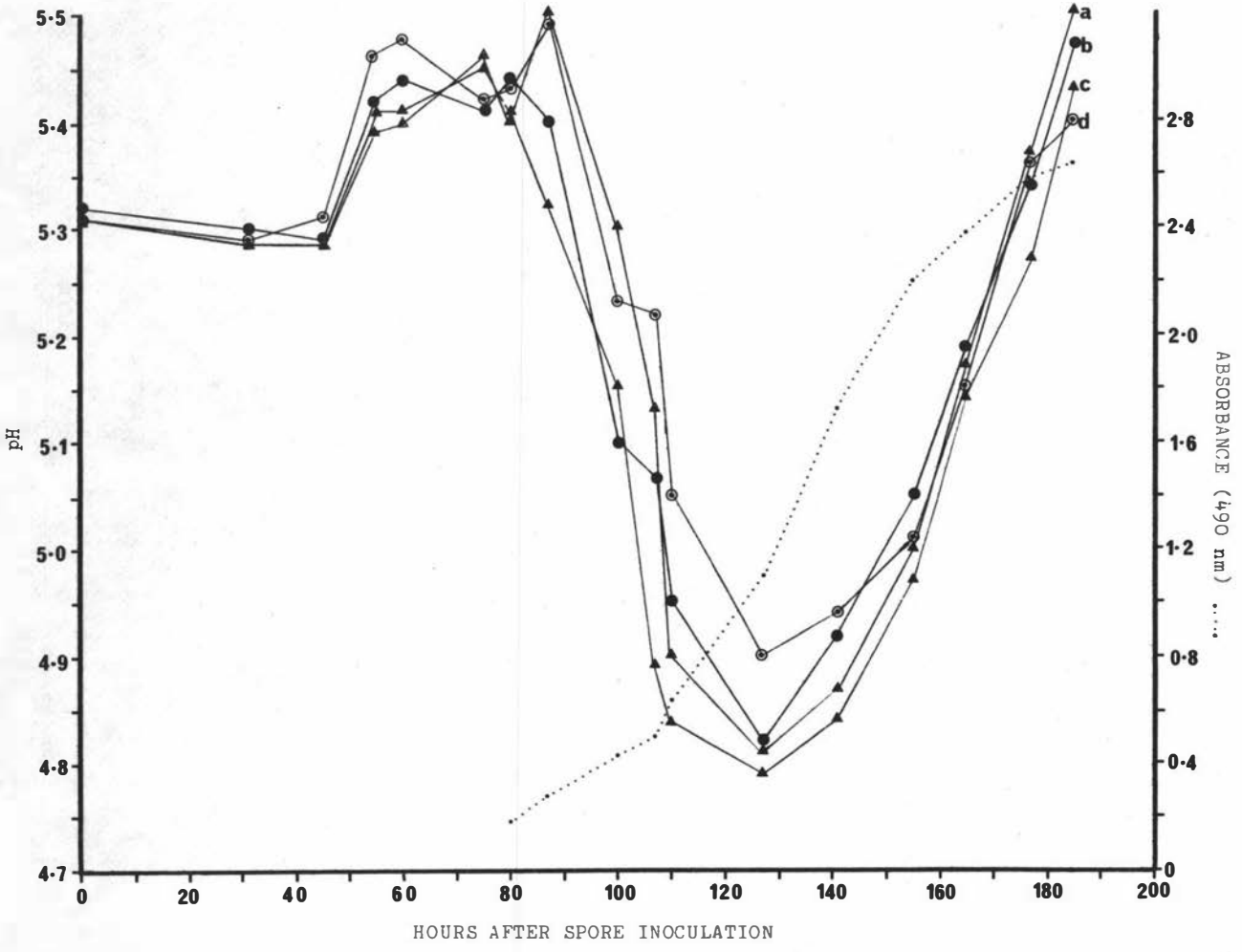
2.7.4.1 Measurement of pH

The Graph (2-4) of pH of the medium against time was similar to that found in the modified Raulin's medium when sucrose, glucose and fructose were used as the major carbon sources. After 60 hours the pH was found initially to increase slightly, followed by several small but rapid pH changes before dropping markedly after 90 hours to a minimum value. The pH then increased again as rapidly as it had dropped.

Pigment production was measurable shortly before the marked drop in pH (approximately 80 hours) and increased up to 180 hours after spore inoculation, the most rapid production being between 100-160 hours. Similar pH profiles were observed when this experiment was repeated four times (Graph 2-4). Thus there appears to be definite phases of metabolic activity during the growth of Dothistroma pini under these conditions and dothistromin production seems to be occurring only during some of these phases.

There is an accumulating body of evidence, of which the experiments of Borrow et al. (1961, 1964a, 1964b) and Bu'Lock et al. (1965b, 1965c) may be cited, that growth and metabolism of a fungus in submerged culture pass through distinct phases.

Initially, during what Borrow et al. refer to as the "balanced phase" and Bu'Lock as the "tropophase", the organism grows in an exponential manner with uptake of essential nutrients per cell occurring at a constant rate.



Graph 2-4 Time course of pH change (flask a, ▲ ; flask b, ● ; flask c, ▲ and flask d, ⊙) and pigment production (·····) in four equivalent 100 cm^3 malt cultures of Dothistroma pini. Each valve shown for pigment production represents the average of the four flasks.

Production of secondary metabolites rarely occurs during this phase which ends with the exhaustion of one of the nutrients, usually nitrogen or phosphorus. At this point in nitrogen-limited cultures, cell replication ceases, a variety of metabolic changes occur and the production of secondary metabolites commences. The organism has then entered what Bu'Lock calls the "idiophase", i.e. the phase during which the species specific metabolites appear, which continues until the carbon source is exhausted and autolysis sets in. For nitrogen-limited cultures, Borrow et al. divided the idiophase into the "storage phase", during which cell dry weight continues to increase due to accumulation of fat and carbohydrate and production of secondary metabolites commences, and the "maintenance phase" during which dry weight is constant but uptake of glucose and production of secondary metabolites continues. In fungal cultures in which nutrients other than nitrogen are exhausted, Borrow et al. also distinguish a "transition phase" between the balanced phase and storage phase, during which all proliferation continues at a reduced rate.

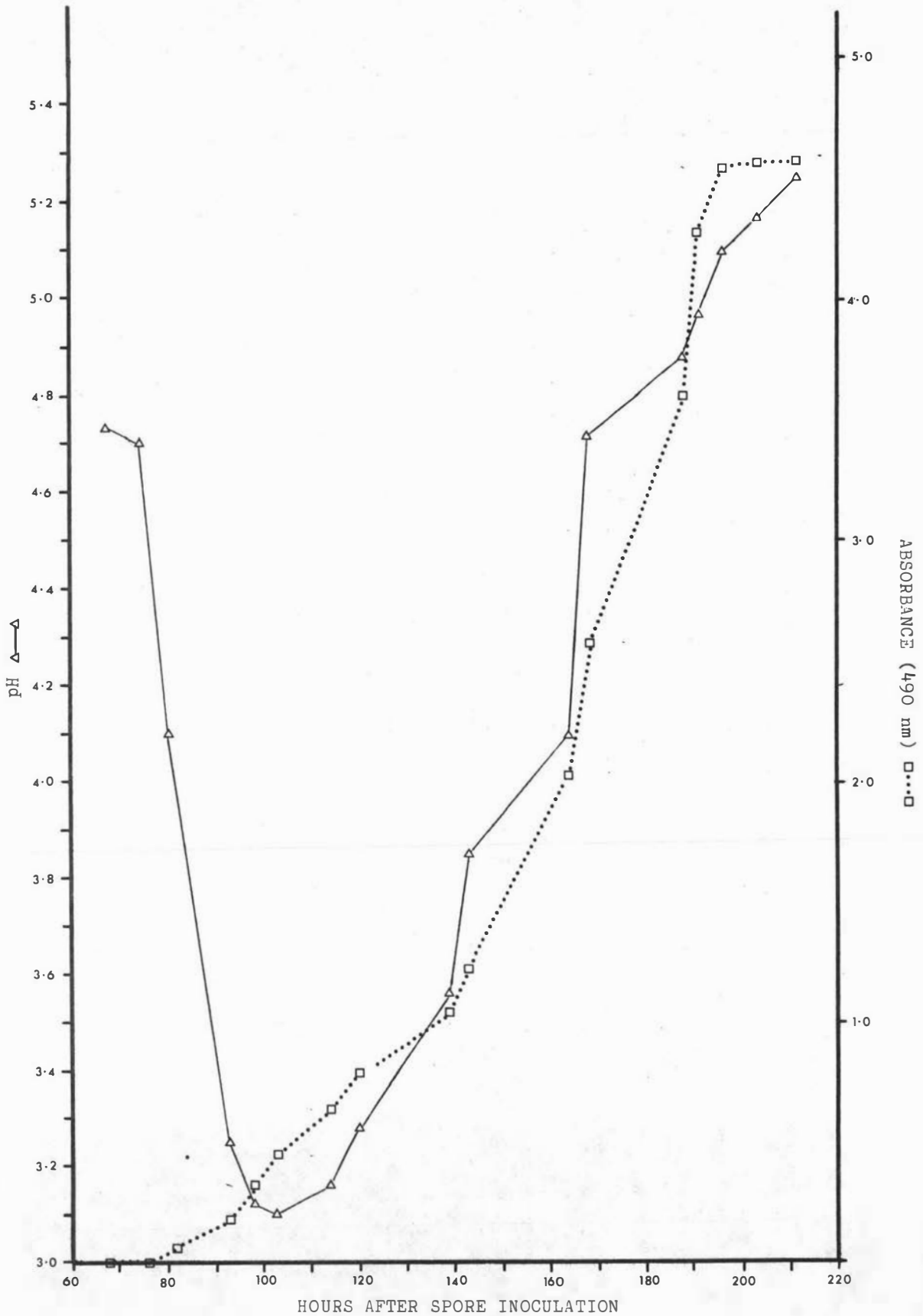
In accord with these ideas the period of fungal growth up to about 80 hours after spore inoculation (graph 2-4) would correspond to the "tropophase" and thereafter during secondary metabolite production it would correspond to the "idiophase".

2.7.5 Fermacell culture

This involves vigorous stirring and the passage of air or oxygen through the medium. Because of the efficient agitation and aeration, the process may be readily scaled up and is the most satisfactory method for large-scale production of fungal metabolites.

For chemical degradation studies with dothistromin it became necessary to obtain the red pigment in gram quantities. Twenty litres of aerated malt medium was used in the Fermacell apparatus and inoculated with a concentrated spore suspension.

After about 75 hours there began a significant drop in pH, reaching a minimum of pH 3.1 at 103 hours after inoculation. Then a shift to alkaline pH was recorded similar to that found in shaken cultures. Pigment production was measurable after 81 hours and by 103 hours the culture took on a reddish-brown colouration. Under these growth conditions the commencement of pigment production coincided with the acid shift in pH and increased at a rapid rate for a further 110 hours (Graph 2-5).



Graph 2-5 Time course of pH change and pigment production in a 20 litre malt culture of Dothistroma pini.

CHAPTER 3

Isotopic labelling of dothistromin

3.1 Approach to the study of biosynthesis

In a complete analysis of the biosynthesis of a particular metabolite a logical progression of investigation would be to (i) establish the identity of the metabolite, (ii) examine the efficiency of simple compounds as precursors on the basis of known biosynthetic relationships; such investigations constitute one of the main uses of isotopic tracers, (iii) look for intermediates between the simple precursors and the metabolite (the use of auxotrophic mutants alone or in combination with tracer and enzymatic techniques is probably the best technique for the detection of intermediates), (iv) then pursue mechanistic studies of individual steps (sequential analysis) perhaps using specifically labelled intermediates and isolating enzymes.

Logically then, as no previous biosynthetic studies had been carried out on dothistromin, the investigation began by testing simple molecules as possible precursors. Then, if a suitable precursor was found, a study of the distribution of its carbon atoms in the carbon skeleton of dothistromin would be required to establish whether the precursor was either acting as a source of a general carbon pool from which the true precursor was formed or being specifically used.

3.2 Criteria used to assess biosynthesis

Before describing the investigation of the incorporation

of [^{14}C]-labelled acetate into dothistromin, by Dothistroma pini, the criteria used to assess the effectiveness of precursors will be defined and discussed. Brown (1972) has discussed some aspects of this in detail, in particular the use of dilution value, percentage incorporation, relative isotope content, and the effect of label randomisation on the interpretation of these values.

3.2.1 Dilution value

Dilution value, (A_1/A_2), is defined as the ratio of molar specific radioactivity of the precursor (A_1), to the molar specific radioactivity of the product (A_2). For the purpose of discussing the significance of dilution value as a method of assessing a precursor, consider a simple biosynthetic pathway involving the conversion of some precursor B into a product X via several intermediates. If the pool of X is such that X is present in isolatable quantities, then the dilution value for this conversion will increase in proportion to the size of the X pool. If pools exist for each intermediate as well, then the specific radioactivity of X will be less, again in relation to the pool sizes, and the dilution value will be greater.

In a more complex case where intermediates leading to X have more than one metabolic fate the dilution value will be greater again.. In both cases additional factors such as membrane permeability and the rate of membrane transport for the externally supplied precursor relative to the rate at which it is supplied internally can also affect dilution.

The advantages in calculating the dilution value compared to other criteria are that the product need not be recovered quantitatively and precise dilution values are easily obtained in that the only values needed are the specific radioactivity of two compounds that can be purified to constant specific radioactivity. However, it has been shown (McCalla and Neish, 1959; Neish, 1958) that dilution values are very sensitive to variation in the dose of precursor at low dose rates, i.e. when the ratio of endogenous precursor to added precursor is large.

3.2.2 Percentage incorporation (P.I.)

The second criterion used is defined by the relation

$$P.I. = \frac{A_2 M_2}{A_1 M_1} \times 100$$

where A_1 = specific radioactivity of precursor,

A_2 = specific radioactivity of product,

M_1 = moles of administered precursor,

M_2 = moles of recovered product.

Therefore to obtain P.I. it is necessary to estimate the total radioactivity of both precursor and product. While A_1 and M_1 are readily determined, M_2 requires either quantitative recovery of the product or a good selective assay and it is necessary to have the product radiochemically pure in order to determine A_2. P.I. values can only be regarded as minimal as a result of the losses incurred during purification of the metabolite under investigation. Never-the-less, in cases where only a few, relatively clear cut purification steps in

near complete recovery need be undertaken and adequate purity is thereby achieved, P.I. can be of great value, especially for the comparison of label incorporation into related compounds.

3.2.3 Relative isotope content (R.I.C.)

Another way of expressing the incorporation of an isotopically labelled precursor which is closely related to dilution value is the relative isotope content (Hsieh and Mateles, 1970). In this instance the precursor-product relationship is defined as the ratio of the specific radioactivity of the product to the specific radioactivity of the precursor; R.I.C. = A_2/A_1, where A_2 and A_1 are as defined previously.

Whereas P.I. represents the percentage of isotopic atoms in the precursor which have been recovered in the product, i.e. it shows the material economy of the process, R.I.C. indicates the number of moles of precursor incorporated per mole of product and hence the precursor-product relationship.

3.2.4 Carbon-14 enrichment

Another way of expressing precursor effectiveness is enrichment. This is defined, in the case of carbon-14 for example, as

$$\text{Enrichment} = \frac{(\text{Actual specific radioactivity of product})\ (100)}{(\text{Maximum possible specific radioactivity of product})}$$

or,

$$\text{Enrichment} = \frac{(\text{Number of atoms of } {}^{14}C/\text{mole of product})\ (100)}{(\text{Total number of atoms of carbon/mole of product})}$$

This expression is useful where the possibility of carrying out carbon-13 tracer experiments by carbon-13 NMR is assessed by means of carbon-14 tracer experiments. It is also used in this thesis when the extent of carbon-13 incorporation into dothistromin is assessed by mass spectrometry.

For the purpose of these enrichment calculations the assumptions were made that both carbons of sodium acetate contributed equally to the biosynthesis of dothistromin (18 carbons) and that there was no randomisation of the isotopic atoms into positions derived from unlabelled atoms. The maximum possible specific radioactivity of the product is then 9 times the specific activity of the precursor when singly labelled acetate is used.

3.2.5 The effect of label randomisation

A factor which must be kept in mind when considering the significance of the various measures of incorporation previously mentioned is the possibility of label redistribution or randomisation during the conversion of the precursor to the product. Ideally, if a precursor is converted to a product by a direct route and if there are no reversible, degradative, side reactions *in vivo* (e.g., a reversible decarboxylation reaction), or reversible side reactions involving symmetrical intermediates, the position of the label in the product should be entirely in accordance with that predicted on the basis of structural and metabolic considerations. However, it is generally unwise to draw conclusions about the localisation of the label in the product, especially

when the precursor is metabolically active and can have more than one metabolic fate, without resorting to chemical degradation to establish the position of the label. However, if one is administering an isotopically labelled precursor with an elaborated carbon skeleton, which need undergo only minor changes to give rise to the product, then valid conclusions may be drawn without degradation.

3.3 Biosynthetic precursor of dothistromin

In the absence of previous biosynthetic investigations of the origin of the carbon skeleton of dothistromin, the choice of possible precursor(s) was guided by published information on the biosynthesis of anthraquinones and aflatoxins which has been surveyed in Chapter 1.

In a study of the relative contribution of acetate and glucose to the biosynthesis of aflatoxins, Hsieh and Mateles (1970) found that (i) acetate was preferentially incorporated into aflatoxins despite the presence of glucose in a concentration 4 times higher than that of acetate, (ii) acetate appeared to inhibit competitively the incorporation of carbon atoms from glucose into aflatoxins and (iii) when the molar ratio of acetate to glucose was 1:4 or higher, the newly formed aflatoxin was almost exclusively derived from acetate, giving an R.I.C. value close to the theoretical maximum of 9.

On the basis of this information it appeared possible that the dothistromin carbon skeleton was entirely derived

from acetate via a single polyketide chain so radioisotope studies were conducted using [^{14}C]-sodium acetate.

Shikimic acid seemed less likely to be a precursor of dothistromin in view of studies on the incorporation of labelled compounds into aflatoxins (Adye and Mateles, 1964) produced by Aspergillus flavus. These results showed that the two principle compounds, aflatoxin B_1 and aflatoxin G_1 incorporate [7-^{14}C]-shikimic acid 126 times and 26 times less efficiently than [1-^{14}C]-sodium acetate, respectively. It was proposed at the time that failure to incorporate shikimic acid might be attributed to difficulties in transporting it into the cell (Gross and Fein, 1960).

In addition, it is now established that although some aromatic natural products in fungi have been shown to be shikimic acid derived (Turner, 1971), anthraquinones from the same organisms appear to be derived by the acetate-polymalonate route (Shibata, 1967; c.f. Mosbach, 1969). In contrast both pathways are operative in the biosynthesis of plant anthraquinones (reviewed in Chapter 1). However in no case has an anthraquinone been found to be solely derived from shikimic acid. Always the biosynthesis has been of a mixed shikimate, acetate-polymalonate origin. Since the metabolic pathways normally involved in the conversion of acetate carbon to shikimate (via the tricarboxylic acid or glyoxylic acid pathways) include a large number of intermediates, many of which are common to other metabolite pathways, a very large dilution of acetate carbon, so directed, would be

expected relative to its more direct use via the acetate-polymalonate pathway. Consequently it would be expected that the biosynthesis of dothistromin by such a mixed pathway would be readily detected, using [^{14}C]-acetate as precursor, by the lack of isotope in that portion of the molecule derived from shikimic acid.

3.4 Carbon-14 biolabelling of dothistromin

To proceed with the investigation of the possible biogenetic route leading to dothistromin it was important to obtain carbon-14 labelled material of high specific radioactivity. Taking into consideration the cost of carbon-14 enriched precursors, and the need to have enough radioactive product to permit extensive chemical degradation, it became necessary to study variables associated with maximising the specific radioactivity and radiochemical yield of the product.

Preliminary studies, using 100 cm^3 malt cultures of Dothistroma pini, showed that pigment began to form after 73-80 hours of growth and reached a maximum concentration at 110 hours after spore inoculation. It was presumed that by adding carbon-14 labelled precursors at various times a time would be found where label incorporation would be maximal.

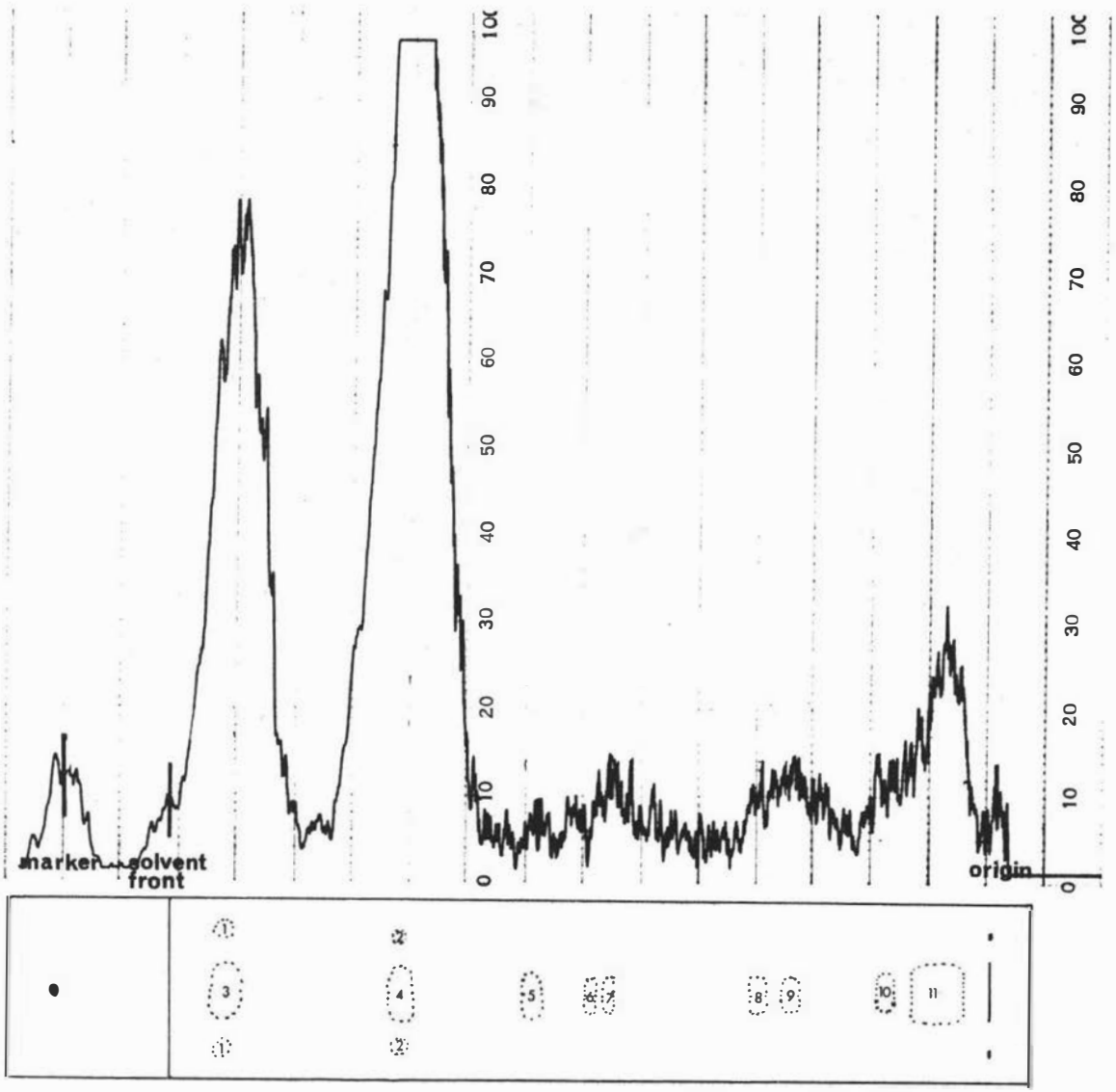
3.5 Preliminary examination of the incorporation of [1-^{14}C]-sodium acetate into pigment extracts

A preliminary experiment was done to see whether

dothistromin could be labelled from [^{14}C]-acetate and to determine whether other labelled compounds would be removed by the procedure used to extract dothistromin. A sample of [1-^{14}C]-sodium acetate (300 µmole, 9.01 x 10^{-1} Ci/M) was introduced into a 100 cm^3 malt culture of Dothistroma pini at the time when pigment production was first detected visually (73-78 hours). The culture was incubated at 18°C for an additional 5 days. The crude residue from an ethyl acetate extract of the culture was analysed by tlc on silica gel using the solvent hexane:diethyl ether:acetic acid (80:20:1 v/v). Eight major bands were detected by inspection with daylight and with UV light. A radioactivity scan of the same tlc plate (Figure 3-1) revealed that some radioactivity was incorporated into the dothistromin fraction and into other coloured bands, but the major amount of radioactivity on the chromatogram resided in two UV (350 nm) visible bands at R_f 0.92 and 0.68.

Considering the nature of the precursor used and the composition of the chromatography solvent employed, it seemed possible that the components giving rise to the two major radioactive bands could be lipid material. The same culture extract was analysed again alongside authentic samples of tripalmitin and palmitic acid using the same technique. Palmitic acid was commonly found to be a component of impure pigment samples by mass spectrometry (m/e 256).

Analysis of the chromatoplate revealed that the triglyceride and fatty acid standards had moved to positions



Reproduction of the thin layer chromatogram in alignment with the scan of its radioactivity

1. tripalmitin standard (R_f=0.92)
2. palmitic acid standard (R_f=0.68)
3. unknown triglyceride(?) (R_f=0.92)
4. unknown fatty acid(?) (R_f=0.68)
5. 'red band' (R_f=0.52)
6. 'orange band' (R_f=0.43)
7. 'yellow band' (R_f=0.38)
8. 'orange band' (R_f=0.25)
9. 'orange band' (R_f=0.18)
10. 'yellow band' (R_f=0.12)
11. Dothistromin containing band (R_f=0.1)

Figure 3-1 Silica gel t.l.c. radiochromatograph of the crude ethyl acetate extract of dothistroma pini fed [1-^{14}C]-sodium acetate.

which corresponded well with the radioactive peaks at R_f 0.92 and 0.68 respectively. It therefore seemed possible, although not by any means proven, that these two bands were of lipid origin. It was also evident that even though the radioactive precursor was added to the culture at a time when dothistromin production was rapidly increasing there was still a significant degree of lipid biogenesis. Ward and Packter (1974) have also reported the independence of fatty acid and simple phenol biosynthesis.

These results demonstrated that it was important that lipid material be quantitatively removed from pigment samples before a meaningful carbon-14 measurement could be obtained. As a routine check to see whether in fact this had been achieved, purified dothistromin samples were monitored by the means described above. In each case the samples proved to be free of any detectable, visible or radioactive contamination.

The possibility that the dothistromin band and a radioactive contaminant might have coincided, using the solvent system previously described, was checked by analysing the sample with an additional two solvent systems. No other radioactive compounds were detected.

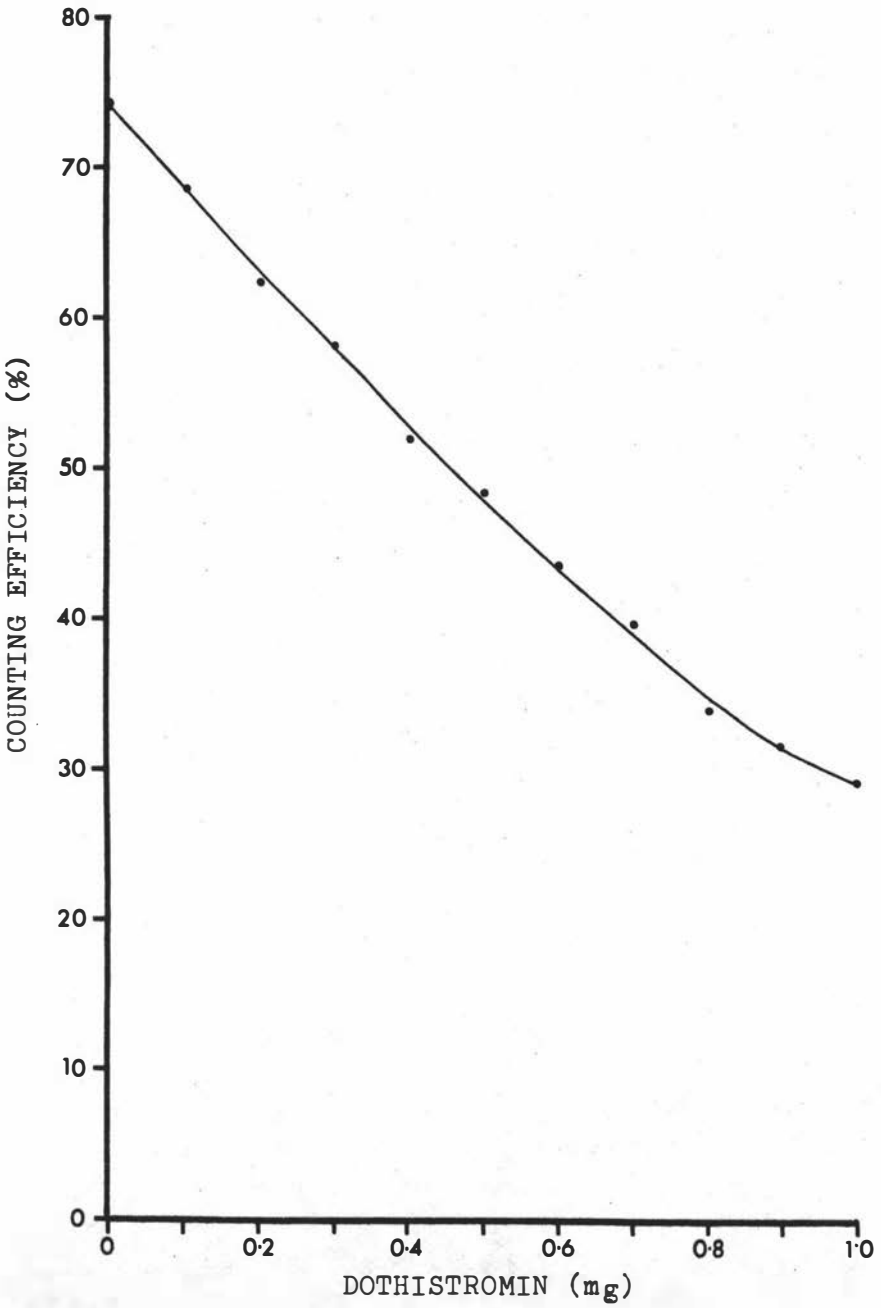
3.6 Determination of radioactive counting efficiency for dothistromin

Before the specific radioactivity of [^{14}C]-dothistromin produced in the labelling studies could be determined by liquid

scintillation counting on a routine basis it was necessary to be able to correct for the quenching of the scintillation process caused by dothistromin. The efficiency with which a radioactive sample is counted by liquid scintillation usually varies from sample to sample because of the different degrees of quenching produced by the samples. The decrease in counting efficiency which occurs is produced either by nonradiative energy transfer from the excited fluor molecules to the sample molecules (chemical quenching) or by sample molecules absorbing the light emitted by the fluor molecules (colour quenching) (Funt and Hetherington, 1962; Birks, 1964; Yang and Lee, 1969).

Colour quenching is most severe with red and yellow coloured compounds as the photomultiplier tubes commonly used in liquid scintillation counters are most sensitive to the blue end of the spectrum. Since dothistromin is an intensely red compound, it was expected to cause considerable colour quenching and thus it was essential to determine the counting efficiency of each sample. This was carried out by the construction of an empirically determined quench correction curve from a standard quenched series of samples of known absolute radioactivity. Details of this method can be found in the experimental section.

From these results the absolute counting efficiency was calculated and plotted against the amount of added dothistromin (Graph 3-1). This graph was used for the calculation of the absolute specific radioactivities of dothistromin samples from



Graph 3-1 Quench curve for dothistromin

culture extracts.

3.7 Effect of time of precursor addition on the specific radioactivity of dothistromin

The initial set of radiotracer experiments was devised to determine the effect on the final specific radioactivity of dothistromin of adding radioactive sodium acetate at different times during the growth cycle. The development of Dothistroma pini in submerged culture in malt media appears to be a metabolically phased process (Chapter 2).

It was noted that if pigment was being synthesised during the apparent log phase, it was occurring at a level below the limits of the method of detection used. Only when the culture had entered an apparent stationary phase, characterised by a sudden decrease then an increase in pH, did dothistromin production become measurable. It seemed logical to expect that the best time to add the labelled precursor would be at some time near the beginning of pigment formation, since general metabolic activity would be reduced with the decline in growth rate and the precursor would have the greatest opportunity of entering the pathway of dothistromin biosynthesis relative to its use for other metabolic purposes.

In a series of separate experiments (see experimental) [1-^{14}C]- and [2-^{14}C]-sodium acetate were added aseptically to a series of Dothistroma pini shake cultures, derived from the same inoculum, at different times of growth, ranging from

before pigment production (48 hours) to near completion of pigment production (144.5 hours). The pH was monitored until the commencement of pigment production and was used as an indicator for the beginning of this process. Labelled precursor was added (300 μmoles) at a time far in advance of the detectable beginning of dothistromin production to allow for the possibility of the formation of some intermediate during the log phase, which was subsequently used for dothistromin biosynthesis.

It was expected that these experiments would show that there was a time of precursor addition which led to the formation of product with maximal specific radioactivity. This maximum was observed when the radioactive tracer was added at 77 hours and 90 hours, respectively, after spore inoculation (Graph 3-2).

These findings could be the net result of the effect of several processes. Precursor added prior to the start of dothistromin production would be used for other metabolic purposes and the pool of radioactive acetate would be reduced. This decrease would continue during pigment production, perhaps to the extent that the pool of radioactivity was depleted before pigment production had ceased. Thus the specific radioactivity of the dothistromin would be minimal at the earliest time of precursor addition and could be expected to increase with later additions. At later times of precursor addition a stage would be reached when pigment synthesis had started before precursor addition which could

lead to a decrease in the specific radioactivity attained at the end of each experiment. Further, the situation could be reached where the pool of radioactive acetate existed after pigment synthesis had ceased. In this phase of the experiment the specific radioactivity of the product would be expected to decrease from a maximum value.

Variation in the rate of utilisation of acetate for other metabolic purposes, at different stages of the growth cycle, may be superimposed on the above events and further affect the time of precursor addition which leads to a product of maximal specific activity.

In addition, the results show that [2-^{14}C]-sodium acetate realised approximately 30% greater enrichment of dothistromin than did [1-^{14}C]-sodium acetate under the same conditions, as might be expected remembering that the carboxyl group of acetate is more metabolically mobile than the methyl group.

The results summarised in Tables 3-1 to 3-6 show that, overall, the enrichment of dothistromin was low in both sets of experiments. This can be partly explained by the fact that the molar ratio of sodium acetate added to dothistromin produced was only 12, compared to the theoretical minimum of 9. Because of competition for the acetate pool by other metabolic processes, obviously the amount of acetate added was insufficient to maintain a constant supply of highly labelled precursor during the production phase. Graph 3-2 shows the averaged isotope incorporation and dothistromin production for the three experiments with each of the species of labelled acetate.

TABLE 3-1

Incorporation of [1-^{14}C]-sodium acetate into dothistromin: experiment 1

Flask Number	Addition of Carbon-14 (hrs)[a]	Dothistromin Concentration (mg/100 cm^3)	Specific Activity of Dothistromin (x10^{-4} Ci/M)	Dilution Value (fold)	R.I.C. (x10-3)	P.I.[b] (x10^{-3}%)	Enrichment (x10-2%)
1	48.0	-	2.07	531	1.88	15.18	2.10
2	65.3	-	2.20	500	2.00	16.10	2.22
3	77.0	0.5	2.70	407	2.46	19.80	2.73
4	90.0	2.0	2.75	400	2.50	20.17	2.78
5	97.3	3.0	2.37	464	2.16	17.38	2.40
6	105.0	3.6	1.29	852	1.17	9.46	1.30
7	114.0	5.0	1.06	1038	0.96	7.77	1.07
8	127.0	6.1	0.97	1134	0.88	7.11	0.98
9	138.0	7.8	0.86	1279	0.78	6.31	0.87
10	144.5	9.0	0.49	2245	0.45	3.59	0.49

[a] hours after spore inoculation

[b] specific activity of precursor = 1.10 x 10^{-1} Ci/Mole

TABLE 3-2

Incorporation [1-^{14}C]-sodium acetate into dothistromin: experiment 2

Flask Number	Addition of Carbon-14 (hrs)[a]	Dothistromin Concentration (mg/100 cm^3)	Specific Activity of Dothistromin (x10^{-4} Ci/M)	Dilution Value (fold)	R.I.C. (x10^{-3})	P.I.[b] (x10^{-3}%)	Enrichment (x10^{-2}%)
1	48.0	-	1.99	543	1.84	15.65	2.05
2	65.3	-	2.17	498	2.01	17.01	2.23
3	77.0	0.5	2.57	421	2.38	20.21	2.64
4	90.0	2.1	2.70	400	2.50	21.23	2.78
5	97.3	3.0	2.29	472	2.12	18.01	2.35
6	105.0	3.7	1.29	838	1.19	10.14	1.33
7.	114.0	5.2	1.08	1000	1.00	8.49	1.11
8	127.0	6.3	0.95	1138	0.88	7.47	0.98
9	138.0	8.0	0.77	1404	0.71	6.10	0.79
10	144.5	9.5	0.54	2002	0.50	4.25	0.56

[a] hours after spore inoculation

[b] specific activity of precursor = 1.081 x 10^{-1} Ci/Mole

TABLE 3-3

Incorporation of [1-^{14}C]-sodium acetate into dothistromin: experiment 3

Flask Number	Addition of Carbon-14 (hrs)[a]	Dothistromin Concentration (mg/100 cm^3)	Specific Activity of Dothistromin (x10^{-4} Ci/M)	Dilution Value (fold)	R.I.C. (x10^{-3})	P.I.[b] (x10^{-3}%)	Enrichment (x10^{-2}%)
1	48.0	-	1.98	569	1.76	14.95	1.95
2	65.3	-	2.12	531	1.88	16.00	2.09
3	77.0	0.5	2.61	431	2.32	19.70	2.58
4	90.0	2.1	2.70	417	2.40	20.38	2.66
5	97.3	3.0	2.34	481	2.08	17.66	2.31
6	105.0	3.7	1.26	894	1.12	9.51	1.24
7	114.0	5.2	1.01	1115	0.90	7.62	1.00
8	127.0	6.3	0.92	1224	0.82	6.94	0.91
9	138.0	8.0	0.72	1564	0.64	5.44	0.71
10	144.5	9.5	0.47	2396	0.42	3.55	0.46

[a] hours after spore inoculation

[b] specific activity of precursor = 1.126 x 10^{-1} Ci/Mole

TABLE 3-4

Incorporation of [2-^{14}C]-sodium acetate into dothistromin: experiment 4

Flask Number	Addition of Carbon-14 (hrs)[a]	Dothistromin Concentration (mg/100 cm3)	Specific Activity of Dothistromin (x10^{-4} Ci/M)	Dilution Value (fold)	R.I.C. (x10^{-3})	P.I.[b] (x10^{-3}%)	Enrichment (x10^{-2}%)
1	50.0	-	2.34	381	2.63	21.89	2.92
2	65.0	-	2.61	341	2.93	24.41	3.25
3	78.0	0.5	3.11	286	3.50	29.10	3.88
4	90.5	2.1	3.29	271	3.69	30.77	4.10
5	98.0	3.5	2.72	328	3.05	25.44	3.39
6	105.0	3.8	1.56	571	1.75	14.59	1.95
7	114.0	4.9	1.24	719	1.39	11.60	1.55
8	127.0	6.3	1.15	775	1.28	10.76	1.43
9	138.0	8.1	0.99	900	1.11	9.26	1.23
10	144.0	9.3	0.59	1510	0.66	5.52	0.74

[a] hours after spore inoculation

[b] specific activity of precursor = 0.891 x 10^{-1} Ci/Mole

TABLE 3-5

Incorporation of [2-^{14}C]-sodium acetate into dothistromin: experiment 5

Flask Number	Addition of carbon-14 (hrs)[a]	Dothistromin concentration (mg/100 cm^3)	Specific Activity of Dothistromin (x10^{-4} Ci/M)	Dilution value (fold)	R.I.C. (x10^{-3})	P.I.[b] (x10^{-3}%)	Enrichment (x10^{-2}%)
1	50.0	-	2.20	409	2.45	21.19	2.72
2	65.0	-	2.52	357	2.80	24.27	3.11
3	78.0	0.5	3.02	298	3.36	29.10	3.73
4	90.5	2.4	3.29	274	3.65	31.68	4.06
5	98.0	3.7	2.71	332	3.01	26.10	3.35
6	105.0	3.9	1.49	604	1.66	14.35	1.84
7	114.0	4.9	1.12	804	1.24	10.79	1.38
8	127.0	6.3	1.08	833	1.20	10.40	1.33
9	138.0	8.0	0.86	1047	0.96	8.28	1.06
10	144.0	9.7	0.49	1837	0.54	4.72	0.60

[a] hours after spore inoculation

[b] specific activity of precursor = 0.900 x 10^{-1} Ci/Mole

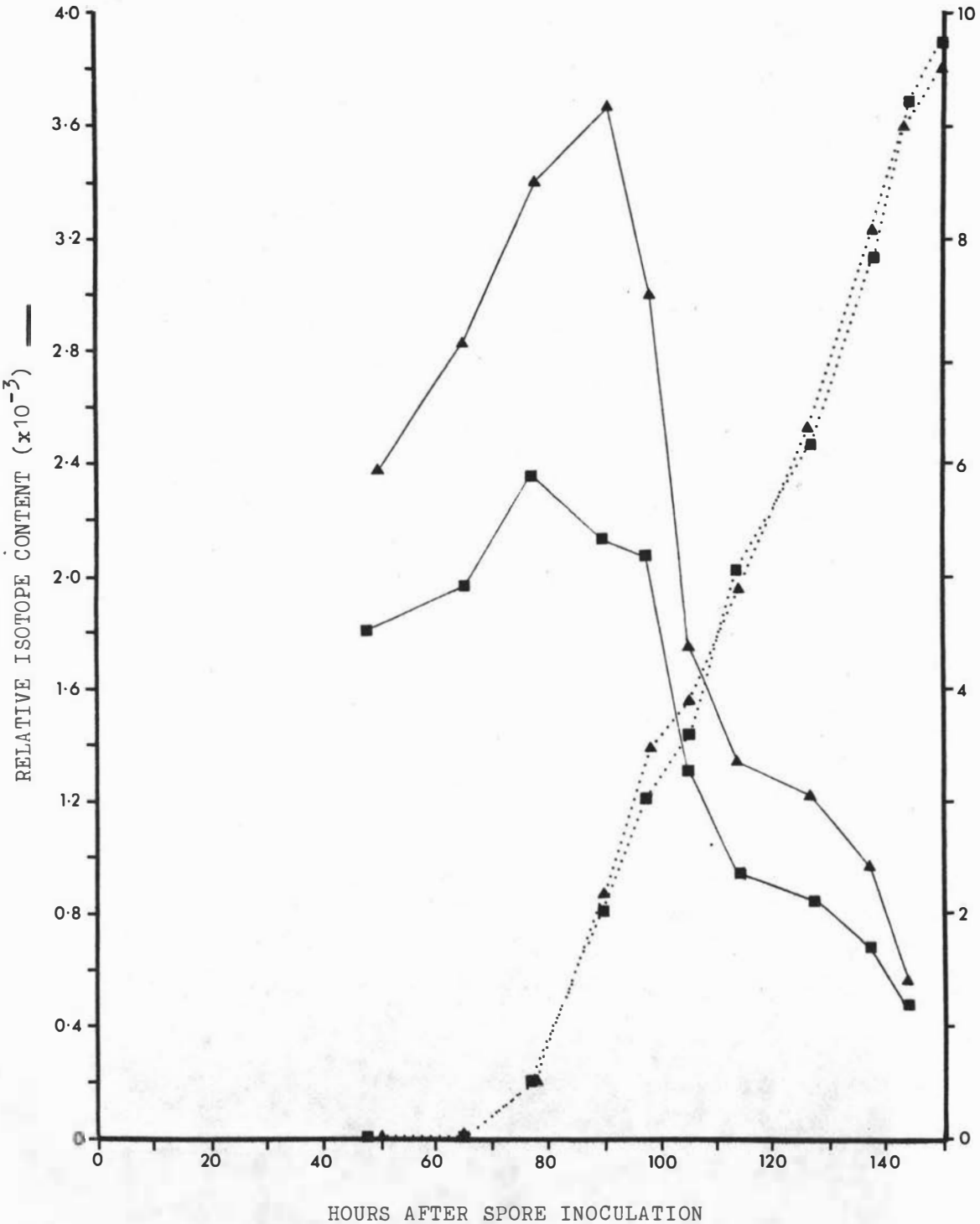
TABLE 3-6

Incorporation of [2-^{14}C]-sodium acetate into dothistromin: experiment 6

Flask Number	Addition of Carbon-14 (hrs)[a]	Dothistromin Concentration (mg/100 cm^3)	Specific Activity of Dothistromin ($\times 10^{-4}$ Ci/M)	Dilution Value (fold)	R.I.C. ($\times 10^{-3}$)	P.I.[b] ($\times 10^{-3}$%)	Enrichment ($\times 10^{-2}$%)
1	50.0	-	2.11	416	2.40	20.67	2.69
2	65.0	-	2.43	361	2.77	23.80	3.08
3	78.0	0.4	2.98	295	3.39	29.19	3.77
4	90.5	2.1	3.20	274	3.65	31.34	4.05
5	98.0	3.3	2.70	325	3.08	26.45	3.42
6	105.0	3.9	1.71	513	1.95	16.75	2.16
7	114.0	4.8	1.22	720	1.39	11.95	1.54
8	127.0	6.4	1.04	844	1.19	10.19	1.32
9	138.0	8.1	0.72	1219	0.82	7.05	0.91
10	144.0	9.6	0.45	1951	0.51	4.41	0.57

[a] hours after spore inoculation

[b] specific activity of precursor = 0.878×10^{-1} Ci/Mole



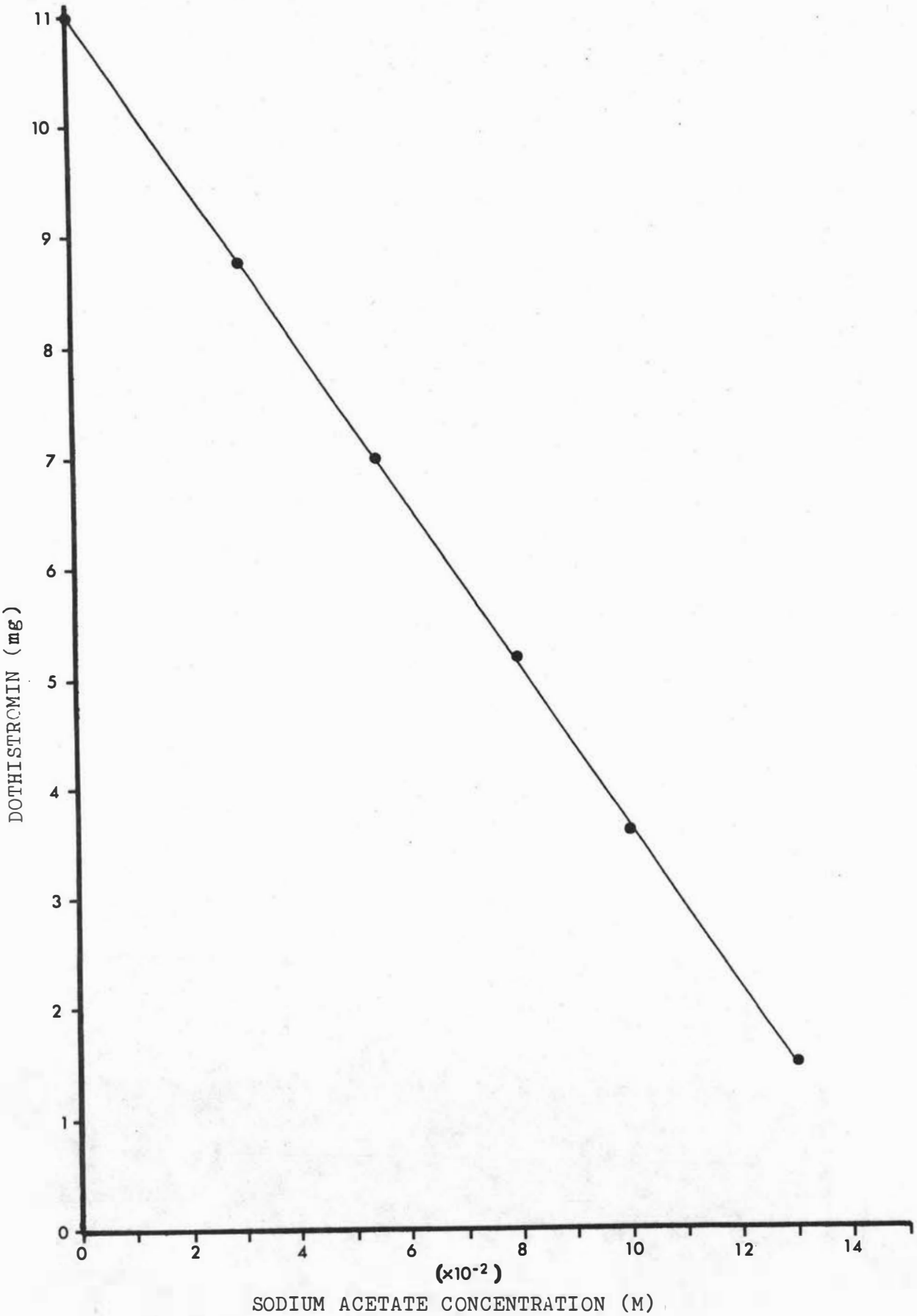
Graph 3-2 Incorporation of [1-^{14}C]-sodium acetate (■) and [2-^{14}C]-sodium acetate (▲) into dothistromin from 100 cm^3 malt cultures of Dothistroma pini.

3.8 Effect of acetate concentration on dothistromin elaboration.

In view of the disappointingly low percentage incorporation of [^{14}C]-sodium acetate into dothistromin shown by the previous experiments, an attempt was made to find a way of improving it. The effect of increasing the administered dose of sodium acetate on dothistromin yields was investigated.

In this experiment a set of 6 x 100 cm^3 *Dothistroma pini* cultures were inoculated with a spore suspension and incubated as before. About 10 hours after the onset of pigment production, solutions of unlabelled sodium acetate were added to 5 flasks to give (a) final concentrations in the range zero to 13×10^{-2} M. After an additional 4 days growth the cultures were analysed for total dothistromin content. As it appeared from the previous experiments that radioactive acetate was most efficiently incorporated into dothistromin about 10 hours after the onset of pigment production, i.e., 85 hours after spore inoculation, this was the timing used in this and subsequent experiments.

The results, summarised in Graph 3-3, showed that there existed an inverse linear dependence between dothistromin biosynthesis and acetate concentration in the medium. As the acetate concentration was increased a dramatic decrease in pigment yield was observed, to the extent that at an acetate concentration of 10×10^{-3} M pigment production was decreased by 64%.



Graph 3-3 Effect of sodium acetate concentration on the yield of dothistromin in 100 cm^3 malt cultures of Dothistroma pini.

3.9 Effect of acetate concentration on the incorporation of [^{14}C]-acetate into dothistromin

In view of the above findings the effect of acetate concentration on the dilution, R.I.C., P.I. and enrichment values for dothistromin derived from [^{14}C]-acetate was studied. The experiments described before were repeated, but this time each culture was fed varying amounts of sodium acetate from a stock solution where the specific radioactivity of [1-^{14}C]-sodium acetate (10.36×10^{-3} Ci/M) and [2-^{14}C]-sodium acetate (9.0×10^{-3} Ci/M) in separate stock solutions was constant. Final culture concentrations ranged from 10×10^{-3} M to 100×10^{-3} M.

The results (Tables 3-7 and 3-8) showed that an increasing sodium acetate concentration resulted in a concurrent increase in R.I.C. at the expense of rapidly decreasing dothistromin yield. The decrease in yield is reflected by the decreasing P.I.. Dilution values are an indication of the relative efficiency of [^{14}C]-acetate as a precursor of dothistromin. The decrease in these values with increasing sodium acetate concentration is as expected since the exogenous acetate would make an increasing contribution to the pool of biological precursor (presumable acetyl-coenzyme A) of dothistromin, relative to endogenous sources. However, the results obtained with the two highest concentrations of acetate used indicated that there was a limiting concentration beyond which no further improvement in the dilution values could be obtained. This may be due to the saturation of either some enzyme catalysing

TABLE 3-7

Effect of acetate concentration on the incorporation of [1-^{14}C]-sodium acetate into dothistromin

Flask Number	Moles of Sodium Acetate ($\times 10^{-3}$M)	Yield of Dothistromin (mg)	Specific Activity of Dothistromin ($\times 10^{-3}$ Ci/M)	Dilution Value[a] (fold)	R.I.C. ($\times 10^{-2}$)	P.I. ($\times 10^{-2}$%)	Enrichment ($\times 10^{-1}$%)
1	10	12.5	0.36	28.78	3.47	11.64	3.9
2	20	11.5	0.49	21.14	4.73	7.33	5.3
3	40	9.11	1.06	9.77	10.23	6.27	11.4
4	60	7.67	1.71	6.06	16.50	5.66	18.3
5	100	2.68	1.73	5.99	16.70	1.67	18.6

[a] Specific activity of precursor = 10.36×10^{-3} Ci/Mole

TABLE 3-8

Effect of acetate concentration on the incorporation of [2-^{14}C]-sodium acetate into dothistromin

Flask Number	Moles of Sodium Acetate ($\times 10^{-3}$M)	Yield of Dothistromin (mg)	Specific Activity of Dothistromin ($\times 10^{-3}$ Ci/M)	Dilution Value[a] (fold)	R.I.C. ($\times 10^{-2}$)	P.I. ($\times 10^{-2}$%)	Enrichment ($\times 10^{-1}$%)
1	10	12.27	0.52	17.37	5.76	19.00	6.4
2	20	11.16	0.60	14.92	6.70	10.05	7.4
3	40	.92	1.09	8.26	12.10	7.27	16.5
4	60	7.81	1.56	5.77	17.33	6.07	19.2
5	100	4.57	1.78	5.06	19.76	2.43	22.0

[a] Specific activity of precursor = 9.0×10^{-3} Ci/Mole

a reaction in the pathway of dothistromin synthesis or perhaps a membrane transport mechanism.

A comparison of these results with the results obtained in the experiments using unlabelled sodium acetate shows the overall yield of dothistromin to be slightly greater in the latter experiments. It can be noted that again enrichment is greater for [2-^{14}C]-sodium acetate.

3.10 The specific radioactivity of dothistromin in relation to the time of its isolation.

In view of the findings of the above experiments and keeping in mind that the main objectives were to obtain as much dothistromin as possible and to obtain dothistromin with a high specific radioactivity, it was evident that conditions would have to be chosen which were not optimal for either of the objectives. As a satisfactory compromise between the opposing trends of the yield of dothistromin and its specific radioactivity, the following experimental conditions were chosen.

For all subsequent biosynthetic experiments sufficient isotopically labelled acetate was added to 100 cm^3 malt cultures to give a final concentration of 10×10^{-3} M, at about 10 hours after the onset of pigment production (i.e. approximately 85 hours after spore inoculation) and the cultures were incubated a further 4 days after sodium acetate addition.

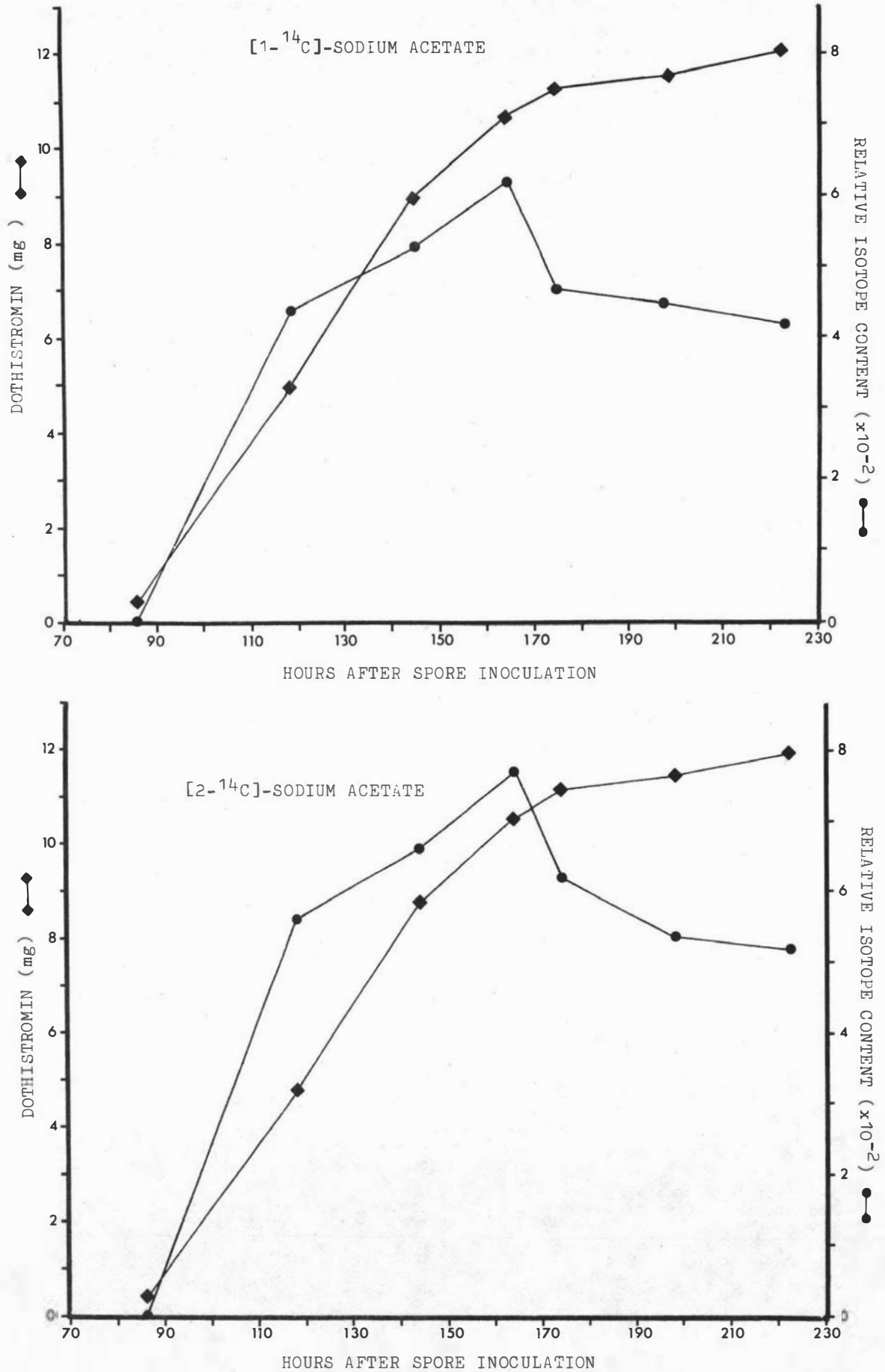
Since the acetate concentration chosen above was probably insufficient to maintain a continuous supply of exogenous labelled precursor for the duration of the period of pigment production, the relation between the specific radioactivity of dothistromin and the time of its isolation was investigated. [1-^{14}C]-sodium acetate (1.17 x 10^{-2} Ci/M) and [2-^{14}C]-sodium acetate (9.594 x 10^{-3} Ci/M) were added to separate cultures of Dothistroma pini under the conditions specified above and the R.I.C. and specific radioactivity of the dothistromin produced were monitored with respect to time. (Table 3-9, Graph 3-4).

The specific radio activity of dothistromin reached a maximum about 78 hours after precursor addition with both [1-^{14}C]- and [2-^{14}C]-sodium acetate and decreased thereafter. The R.I.C. values of the dothistromin samples with highest specific radioactivity were 0.062 and 0.077 from [1-^{14}C]- and [2-^{14}C]-sodium acetate, respectively. The values were a significant improvement on those attained in previous experiments with this concentration of acetate and at the same time the yield of dothistromin was only slightly less. Therefore, it was advantageous, under the experimental conditions chosen, to isolate the dothistromin at a time before pigment synthesis had ceased.

TABLE 3-9

Yield and R.I.C. of [^{14}C]-dothistromin produced by 100 cm^3 cultures of Dothistromin pini

Labelled precursor	Hours after Spore Inoculation	Specific Activity of Dothistromin ($\times 10^{-4}$ Ci/M)	Dothistromin Concentration (mg/100 cm^3)	Relative Isotope Content ($\times 10^{-2}$)
[1-^{14}C]-sodium acetate (S.A.=1.171x10^{-2} Ci/M)	86.00	-	0.5	-
	118.15	5.09	5.0	4.35
	144.30	6.21	9.0	5.30
	164.00	7.29	10.7	6.23
	174.45	5.54	11.3	4.73
	198.45	5.22	11.6	4.46
	222.45	4.91	12.1	4.19
[2-^{14}C]-sodium acetate (S.A.=9.594x10^{-3} Ci/M)	86.00	-	0.4	-
	118.15	5.40	4.8	5.63
	144.30	6.30	8.8	6.57
	164.00	7.34	10.6	7.65
	174.45	5.85	11.2	6.10
	198.45	5.18	11.5	5.40
	222.45	4.90	12.0	5.10



Graph 3-4 Yield and relative isotope content of [^{14}C]-dothistromin produced by 100 cm^3 malt cultures of Dothistroma pini in the presence of 0.01M [1-^{14}C]-sodium acetate and 0.01M [2-^{14}C]-sodium acetate, 86 hours after spore inoculation.

It probably would not have been necessary to pursue the labelling studies to the extent described in this chapter in order to obtain carbon-14 labelled dothistromin with a suitably high specific radioactivity to permit location of the labelled atoms by chemical degradation. However, as will become apparent in Chapter 7, the production of dothistromin with the enrichment finally attained in these experiments (0.47% and 0.57%, respectively) was crucial to the success of the experiments concerned with the investigation of the biosynthesis of dothistromin using carbon-13 labelled precursors.

CHAPTER 4

Attempted determination of the biolabelling pattern in [^{14}C]-dothistromin by chemical degradation

4.1 Method of chemical degradation

When the problem of the biosynthesis of dothistromin was first considered, the possibility of solving it by the carbon-13 tracer techniques, then available, seemed remote and the high cost of the labelled precursors at the time was also discouraging. Therefore, the problem was initially approached with the aim of elucidating dothistromin biosynthesis using carbon-14 tracer techniques and chemical degradation of the molecule to locate labelled carbon atoms. Some results, reported in this chapter, were obtained by these means before it became apparent from reports of improved carbon-13 NMR techniques and from concomitant decreases in the cost of carbon-13 labelled precursors that it would be feasible to tackle the problem by carbon-13 tracer techniques.

Two approaches to the chemical degradation of dothistromin were considered. One of these, concerned with isolating the carbon atoms of the bifuran rings, was to be modelled on the sequence of reactions used by Buchi's group to determine the labelling pattern in the similarly structured portion of aflatoxin B_1(1-29) (Biollaz *et al.*, 1968a, 1970). The other was to make use of the recently devised procedure for cleaving methoxyanthraquinones (Davis and Hodge, 1971b; Davis *et al.*, 1973). In the event only the latter approach was pursued to

the stage of obtaining useful results before the change to carbon-13 tracer techniques was made.

4.2 Potassium tertiary-butoxide cleavage of anthraquinones

Although fission of non-enolisable carbonyl compounds has been carried out with reagents such as sodium and potassium hydroxide mixtures at 200°C (Lock and Rodiger, 1939) and soda lime in toluene (Hamlin and Weston, 1959), the reagent of choice is potassium tertiary-butoxide:water (molar ratio 10:3) in an aprotic solvent (Swan, 1948; Gassman et al., 1967). Davis and Hodge (1971b) examined the cleavage of a range of mostly monosubstituted benzophenones with the reagent formed by adding water (3 equivalents) to potassium tertiary-butoxide (10 equivalents) in 1,2-dimethoxyethane. The effects of substituents on the direction and rate of overall cleavage was investigated and a mechanism involving the formation of benzoate anion and aryl carbanion cleavage products was proposed.

The same workers (Davis and Hodge, 1971b) have shown that anthraquinone, several methoxyanthraquinones and 1- and 2-chloroanthraquinones are cleaved in high yield to afford mixtures of benzoic acids and/or phthalic acids when treated with an excess of the butoxide-water reagent in monoglyme at 85°C. From their results a general scheme for anthraquinone cleavage was proposed (Figure 4-2) in which four possible pathways for cleavage exist. Paths "a" and "b" afford a pair of benzoic acids in equal yield, while "c" and "d" result in a mixture

of phthalic acids and non-acid fragments, giving overall, a mixture of four benzoic acids and two phthalic acids.

It seemed most likely that cleavage of anthraquinones took place in two distinct stages (Figure 4-3), (i) cleavage at one carbonyl group forming a benzophenone 2-carboxylic acid and (ii) cleavage of the salt at either side of the remaining carbonyl group.

Their results (Davis and Hodge, 1971a) suggested that cleavage rates were affected by the position and nature of the substituents. With benzophenones, the rates of cleavage of the methoxy-substituted benzophenones decreased in the order ortho-OMe>H>para-OMe with the position of the meta-OMe in the series uncertain. Substituents favoured cleavage of the bond to the ring bearing the substituent in the order ortho-OMe>ortho-COO>meta-OMe>H>para-OMe. Extrapolation of these results to anthraquinones then would make α-methoxy-anthraquinones more reactive towards butoxide cleavage than anthraquinone itself. This was found. For 1-methoxyanthraquinone (Figure 4-3, R_1=OMe) the acid products were benzoic acid and 3-methoxybenzoic acid; phthalic acid was a minor product. This meant that following cleavage first at "a", cleavage of the resulting benzophenone was favoured at "b" rather than at "c" as might have been expected from the substituent effects found for substituted benzophenones, with the result that the major products were benzoic acids.

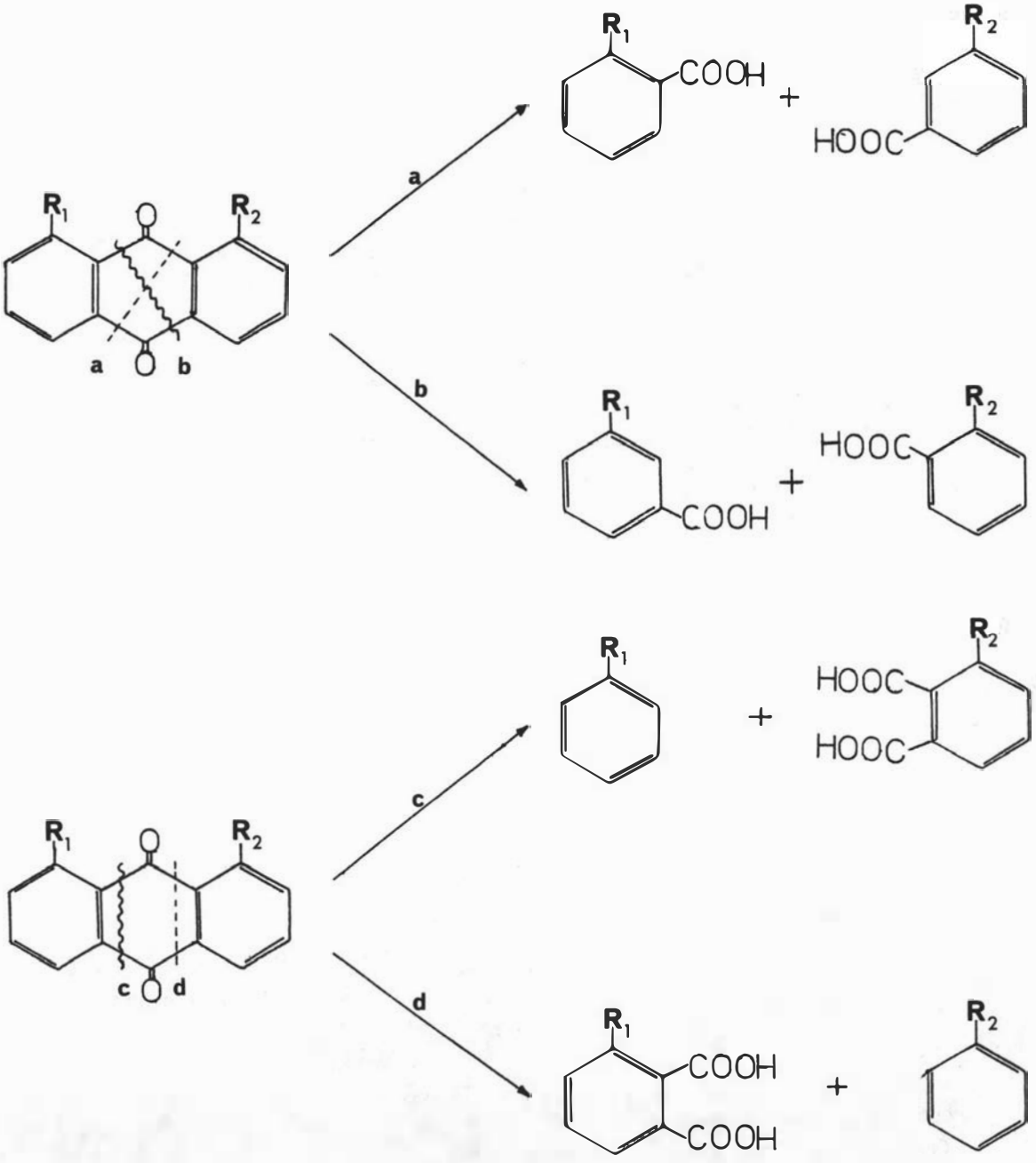


Figure 4-2 Mechanism for the potassium tertiary-butoxide cleavage of anthraquinone (after Davis and Hodge, 1971)

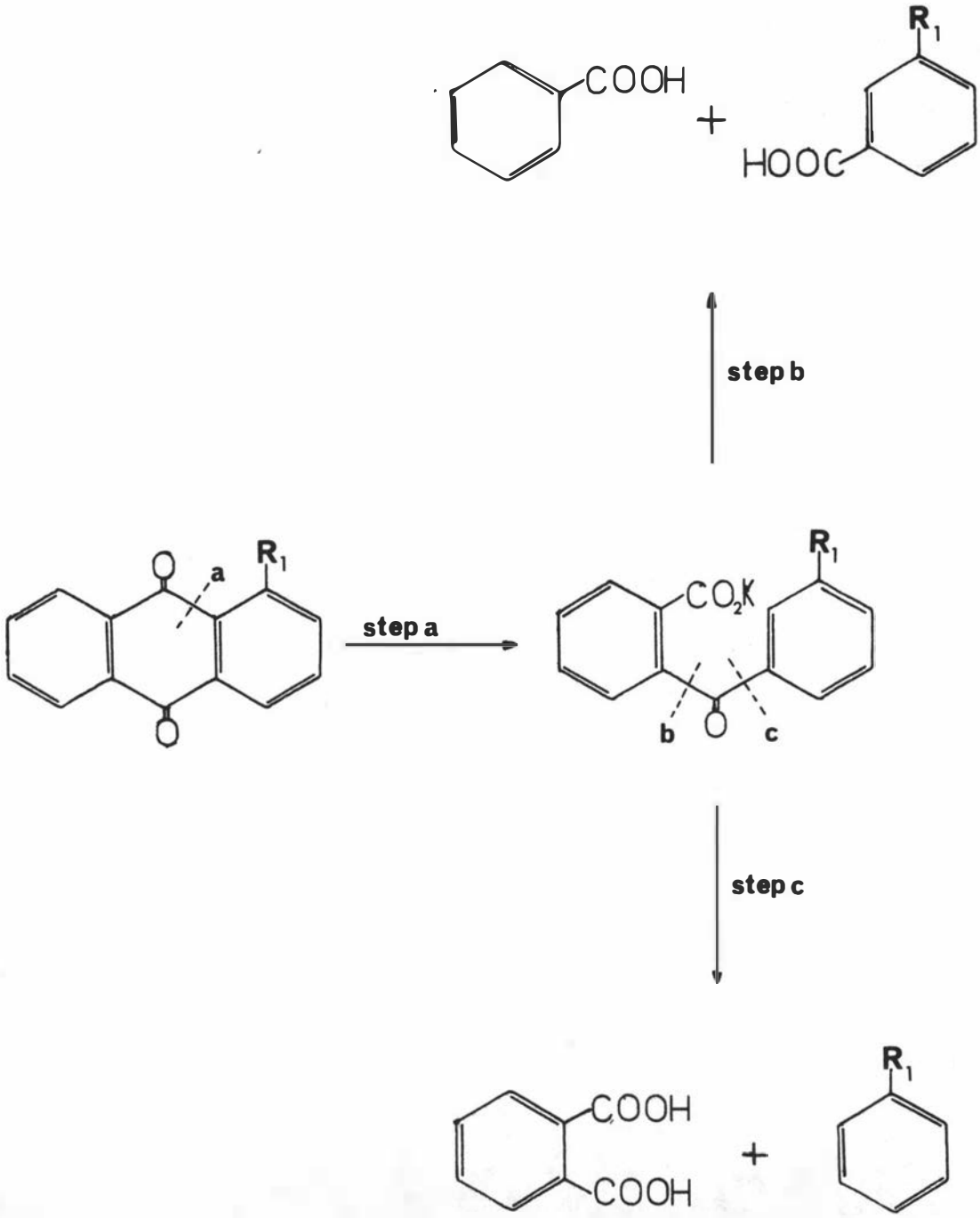


Figure 4-3 Mechanism for the potassium tertiary-butoxide cleavage of 1-methoxyanthraquinone (R=OMe) (after Davis and Hodge, 1971).

4.2.1 Optimisation of the reaction conditions

Before committing the limited amount of carbon-14 labelled dothistromin to degradation, the cleavage reaction was tried on a few representative methoxyanthraquinones in order to find conditions which might give the best yield of cleavage products.

The mixture of acids produced by cleavage of each anthraquinone was converted to their trimethyl silyl (TMS) esters (Sweeley et al., 1963) and analysed by gas liquid chromatography (glc). The results are shown in Table 4-2 and the retention volumes of a series of authentic TMS esters used to identify the reaction products are given in Table 4-1.

The first experiments were conducted using vacuum dried potassium tertiary-butoxide synthesised from singly and then doubly distilled tertiary-butanol which had been refluxed over barium oxide (Swan, 1948).

The results summarised in Table 4-2 show clearly that in comparison to the findings of Davis and Hodge (1971b), the yields of acids isolated were low. However, the acids identified by glc and the relative proportions were fully in accord with their results. More careful purification of the tertiary-butanol had little effect on the yield of cleavage products. An improvement was found, however, when the potassium tertiary-butoxide prepared from triply distilled tertiary-butanol, was sublimed immediately prior to use (Table 4-2). It was concluded from this that the decreased acid yield in the previously described experiments may have been due to traces of water in an otherwise

TABLE 4-1

Glc of the TMS esters of some aromatic acids

Standard compound	Adjusted retention volume (min)[a]
Benzoic acid	4.6
Phthalic acid	7.3
3,5-dimethoxybenzoic acid	9.7
2,5-dimethoxybenzoic acid	10.9
2-methoxybenzoic acid	14.4
3-methoxybenzoic acid	17.0
4-methoxybenzoic acid	20.1

[a] Adjusted relative to the retention volume of benzene, nitrogen flow rate = 40 cm^3/min.

TABLE 4-2

Potassium tertiary-butoxide cleavage of some substituted anthraquinones

Substituent(s)	Reaction time(hrs)[a]	Yield of acids (%) I[d]	II[e]	III[f]	IV[g]	lit.[gh]	Acids produced	Composition of the acid fraction(%)[c]
H	4	30	30	72	94	98	Benzoic Phthalic	96 4
1-methoxy	2	28	29	63	91	91	Benzoic 3-methoxybenzoic Phthalic	44 50 6
1,3-dimethoxy	2	33	34	68	92	94	Benzoic Phthalic 3,5-dimethoxybenzoic unidentified	46 6 44 4
1,4-dimethoxy	2	37	36	75	97	98	Phthalic unidentified	94 6
1,8-dimethoxy	4[b]	35	35	71	90	89	2-methoxybenzoic 3-methoxybenzoic unidentified	48 49 3

[a] Reactions carried out at reflux temperature (approx. 85°)

[b] Reaction carried out at 20°

[c] Determined by glc as the TMS ester

[d] Potassium tertiary-butoxide made using singly distilled tertiary-butanol

[e] Potassium tertiary-butoxide made using doubly distilled tertiary-butanol

[f] Sublimed potassium tertiary-butoxide

[g] Commercial potassium tertiary-butoxide

[h] Davis and Hodge (1971b)

water sensitive reaction.

At this stage a supply of commerical potassium tertiary-butoxide (Kodak) became available. Under identical reaction conditions, improved yields were obtained comparable to literature values (Davis and Hodge, 1971b). Thus it was apparent that the success of the reaction was greatly dependent on the purity of the potassium tertiary-butoxide used.

4.3 Preparation of dothistromin methyl acetal tetramethyl ether

Carbon-14 enriched dothistromin methyl acetal tetramethyl ether (4-3) was synthesised from [^{14}C]-dothistromin, obtained as described in Chapter 3 from [1-^{14}C]-sodium acetate, in two steps (Gallagher and Hodges, 1972).

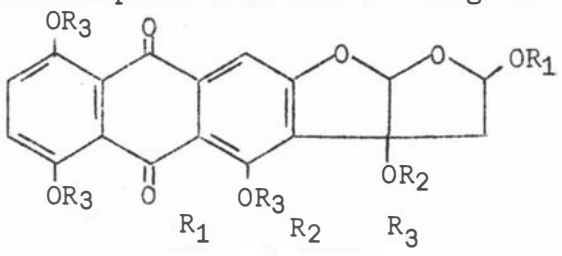
(i) Methylation of the hemiacetal with methanol and thionyl chloride gave [^{14}C]-dothistromin methyl acetal(4-1). The visible spectrum, as expected, was identical to that of dothistromin. The identity of the new compound was confirmed by accurate mass measurement m/e 386.0620.

The mass spectrum contained a molecular ion (M) at m/e 386, with other major ions at m/e 357, 325, 309 and 272. The base peak was observed at m/e 299 corresponding to the loss of a neutral fragment (verified by metastable peaks) of m/e 58 (C_3H_6O) from m/e 357.

Loss of a formyl radical (CHO$^{\cdot}$) from the molecular ion giving m/e 357 (Gallagher, 1971) is not uncommon and has been

shown to occur on electron impact of hydroxyanthraquinones (Ritchie et al., 1964), alkoxyanthraquinones (Bowie, 1970), benzofurans (Reed and Reid, 1963) and furan itself.

(ii) Methylation of the remaining hydroxyl groups was achieved using dimethyl suphate, potassium carbonate and acetone under reflux giving mainly [^{14}C]-dothistromin methyl acetal tetramethyl ether(4-3), m.p. 186-189°C, with some dothistromin methyl acetal trimethyl ether(4-2). These two compounds were separated by tlc and isolated therefrom. A radioactivity scan of the separated components is shown in Figure 4-4.



	R_1	R_2	R_3
(4-1)	Me	H	H
(4-2)	Me	H	Me
(4-3)	Me	Me	Me

The electronic absorption spectrum of 4-3 was observed to bear little resemblance to that found for dothistromin, λ_{max} (ethanol) 225, 273, 420 nm (10^{-3} ε respectively 26.7, 20.0 5.89).

The mass spectrum agreed with that reported by Gallagher (1971), with the molecular ion being observed at m/e 442 ($C_{23}H_{22}O_9$). The base peak at m/e 367 ($C_{20}H_{15}O_7$) results from loss of a neutral fragment, C_3H_8O (m/e 60), from a daughter ion at m/e 427. The m/e 427 ion arises from the molecular ion by loss

of a formyl radical (CHO$^{\cdot}$, m/e 29).

The proton NMR of dothistromin methyl acetal tetramethyl ether(4-3) showed signals arising from the non-aromatic difuran ring system protons at δ2.66 (2H, triplet) (ppm downfield from TMS) which were assigned to the methylene group. Signals at δ3.12 and 3.18 (3H, singlet) were assigned to the aliphatic methoxyl groups. In contrast, 3 singlets at δ3.93, δ3.96 and δ4.06 (3H each) were assigned to the aromatic methoxyl groups. A doublet at δ5.18 (1H) was consistent with the acetal proton bonded to carbon 2 (see page 91) since it had a coupling of J=4.5 Hz. The other acetal proton, bonded to carbon 12a, was assigned to the singlet at δ6.15 (1H).

Aromatic signals at δ7.25 (2H, singlet) and δ7.35 (1H, singlet) are due to protons on carbons 7 and 8 and 11 respectively. This was entirely in agreement with previous findings (Gallagher, 1971).

The component having a lower R_f value on tlc was shown to be dothistromin methyl acetal trimethyl ether(4-2). Mass spectroscopy showed a molecular formula $C_{22}H_{20}O_9$ (MW 428). Fragmentation of the molecular ion followed the pattern characteristic of all dothistromin-like molecules giving an intense ion at m/e 399($M^{+\cdot}$ -CHO$^{\cdot}$); $C_{21}H_{19}O_8^{+}$. Loss of a neutral fragment, m/e 58 (C_3H_6O), from m/e 399 gives rise to the base peak at m/e 341 ($C_{18}H_{13}O_7$). The UV spectrum showed 3 typical absorption bands: λ_{max} (ethanol) 226, 275 and 407 nm (10^{-3} ε respectively 26.3, 19.6, 5.82). The NMR spectrum was identical

to that previously reported (Gallagher, 1971) and in particular, lacked the benzylic methoxyl resonance at δ3.18.

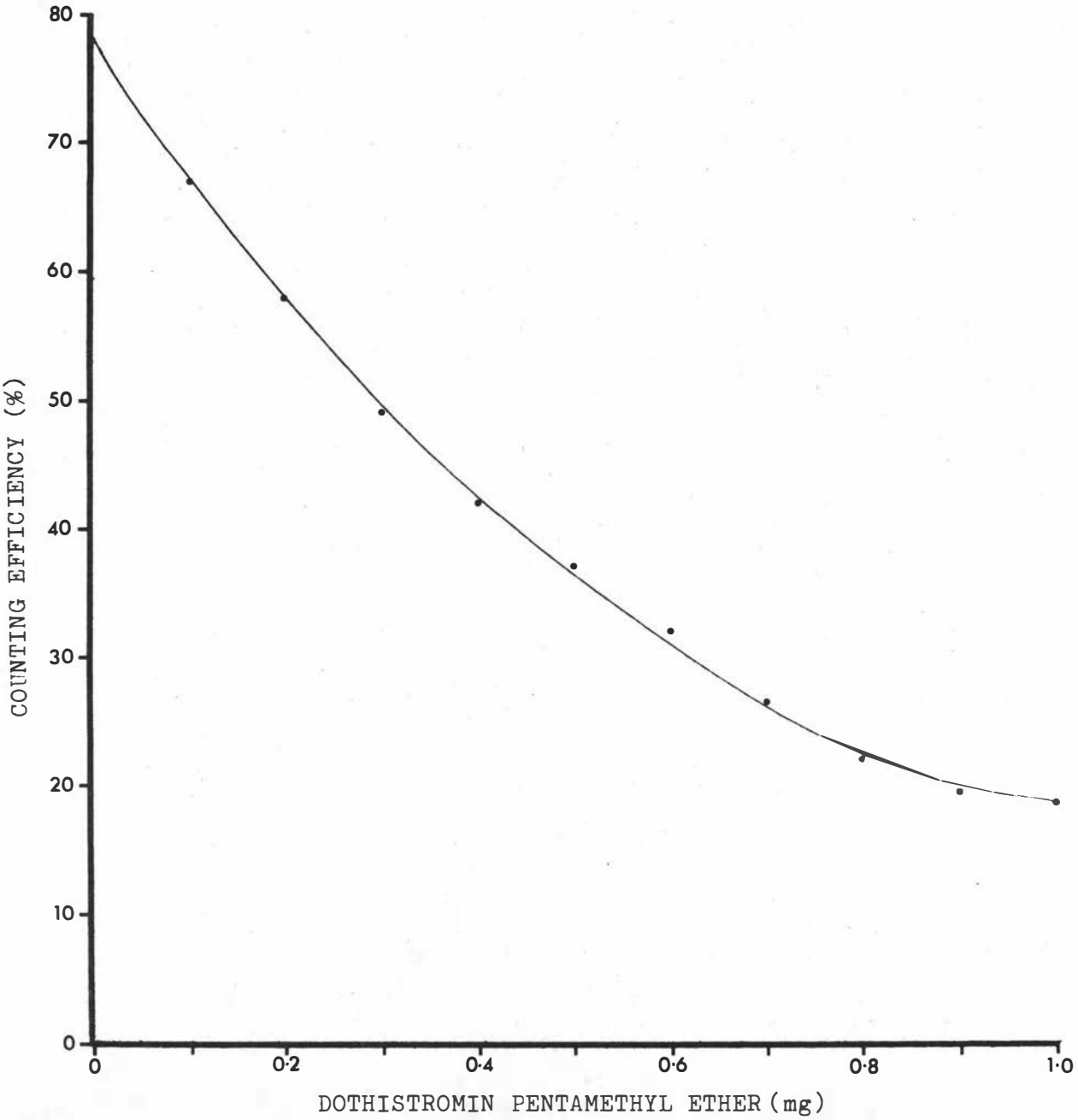
As with dothistromin, it was necessary to construct a quench correction curve for dothistromin methyl acetal tetramethyl ether(4-3) before any radioactivity measurements could be made (Graph 4-1).

The final specific radioactivity of [^{14}C]-dothistromin methyl acetal tetramethyl ether was found to be 3.7×10^{-5} Ci/M after being crystallised to a constant specific radioactivity.

4.3.1 Cleavage of [^{14}C]-dothistromin methyl acetal tetramethyl ether and analysis of the products

For the potassium tertiary-butoxide cleavage of 4-3, reaction conditions of 4 hours at reflux temperature, using the same reagents described previously, were chosen. Workup of the neutral fraction gave a product in low yield. Identification of the product by glc (Figures 4-5a, 4-5b), melting point, UV absorption and mass spectrometry showed it to be identical to 1,4-dimethoxybenzene.

The [^{14}C]-1,4-dimethoxybenzene residue was crystallised five times from water. This crystalline material (0.75 mg) was assayed for radioactivity using the toluene/PPO/POPOP scintillation fluid previously described. The specific ·oactivity, corrected for background and machine counting ·ncy, was found to be 1.24×10^{-5} Ci/M.



Graph 4-1 Quench curve for dothistromin pentamethyl ether.

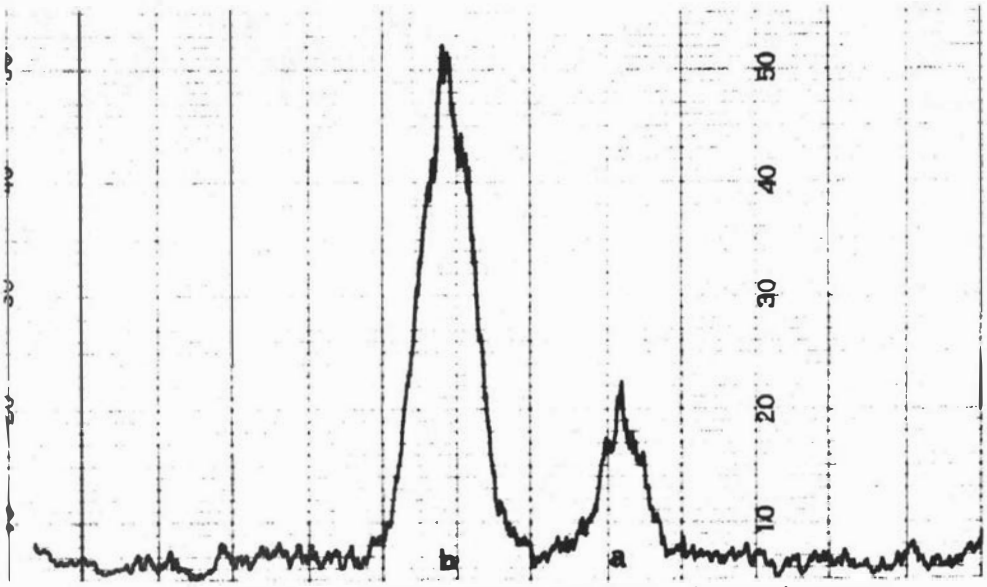


Figure 4-4 Radioactivity scan of the chromatogram of the (a) tetramethyl- and (b) pentamethyl-derivatives of [^{14}C]-dothistromin.

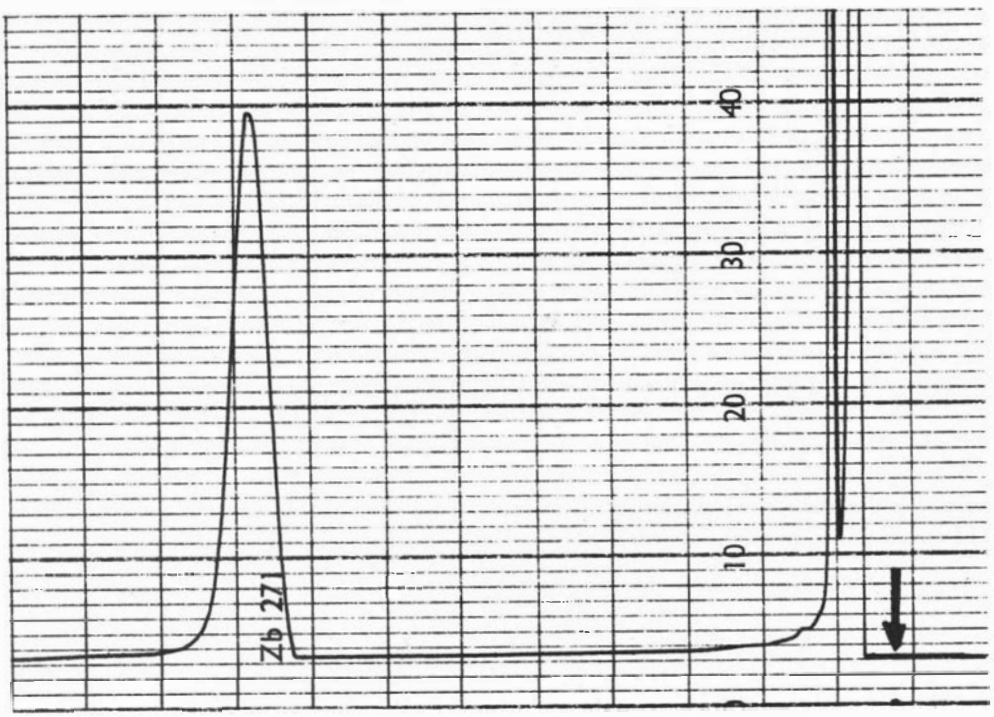


Figure 4-5a Glc of 1,4-dimethoxybenzene.

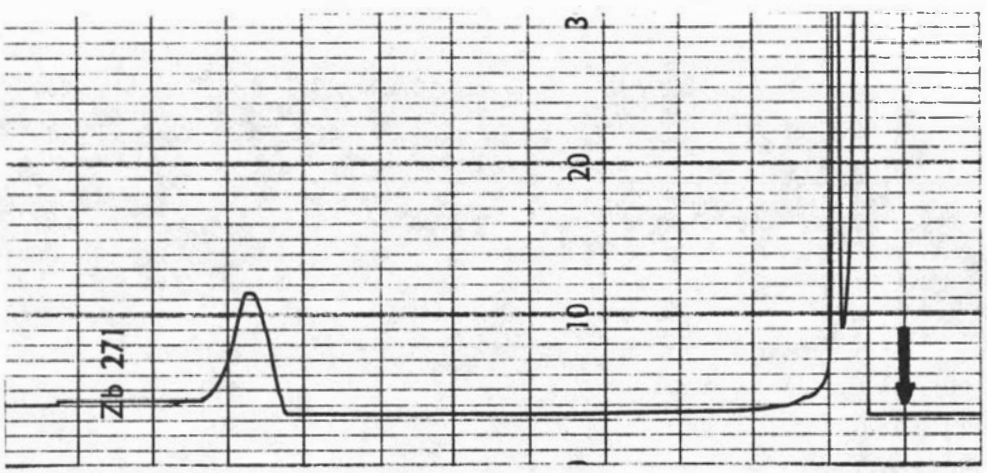
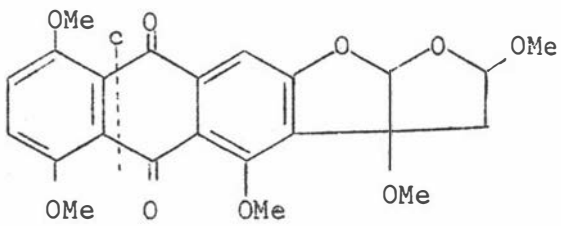


Figure 4-5b Glc of [^{14}C]-1,4-dimethoxybenzene.

No purified product could be obtained from the acid fraction. The tar like appearance of this fraction suggested that extensive polymerisation had taken place. Such side reactions are often the case in alkyl-anthraquinones (Hodge, 1973) and dothistromin has other reactive centres as well as the anthraquinone ring, e.g. the methylene group in the terminal furan ring, where some competitive reaction could occur. The 1,4-dimethoxybenzene could only have been produced by cleavage of dothistromin at "c".



The ratio of the molar specific radioactivity of 1,4-dimethoxybenzene to dothistromin methyl ether tetramethyl ether is 0.33. This finding is consistent with the formation of dothistromin from an 18 carbon acetate-polymalonate precursor, but of course does not prove this possibility. Carbon by carbon degradation and disclosure of an alternating sequence of carbon-14 labelled atoms of the same specific radioactivity would be required to fulfil the hypothesis. Unfortunately, because of its symmetry, 1,4-dimethoxybenzene would not give the desired information if it were degraded in this way.

CHAPTER 5

Carbon-13 tracer studies

5.1 ^{13}C-NMR and the study of biosynthesis

The elucidation of biosynthetic pathways by carbon-13 tracer techniques is particularly attractive, mainly because of the decreased number of chemical manipulations required to complete the analysis after isolation of the metabolite. In many cases, the need for degradative schemes to isolate specific centres in the molecule under study is completely eliminated, although in some cases a few interconversions may be necessary. This situation contrasts sharply with that for studies utilising radioactive isotopes for which it is essential to chemically isolate the individual carbon atoms of the original molecule to establish the labelling pattern. These additional chemical transformations are not only time consuming to develop and to carry out, but also can introduce errors. For example, in biosyntheses which incorporate two or more types of biogenetic unit to different extents, the differences in isotope concentrations may be difficult to assess because of the error introduced by the degradative sequence.

For biosynthetic studies the degree of incorporation of an isotopic label into the metabolite under study by the producer organism places the most severe limitation on the choice of carbon-13 tracer technique. A second disadvantage is the difficulty of making precise quantitative assessments of the distribution of enrichment at a number of non-equivalent centres, e.g. in those cases in which a

labelled atom is scrambled among several centres because of randomisation in a precursor. The intensity of a specific proton decoupled carbon-13 signal in the NMR spectrum depends on the efficiency of the decoupling and the magnitude of the nuclear Overhauser enhancement (N.O.E.) assuming that other operative parameters are optimised, e.g. phasing and sweep rate. Thus resonances in different regions of the spectrum may exhibit different intensities, even for carbons bearing hydrogen atoms. Pronounced intensity differences are observed for signals arising from carbons not bonded to hydrogen. There is an additional complication for intensity measurements of signals from neighbouring carbons which are both enriched because of ^{13}C-^{13}C coupling; if the chemical shifts are nearly equivalent there may be overlapping of the components of each multiplet.

Under ideal spectral conditions (in particular a high signal to noise (S/N) ratio) it has been suggested that an isotope enrichment of a specific carbon atom in a metabolite as little as 1.2% (compared with 1.1% natural abundance of ^{13}C) may be sufficient to assess the extent of biolabelling. However, where sample size is small and/or circumstances of spectral measurement do not lead to a high S/N ratio, such small differences could not be used with confidence. In non-ideal situations then, enrichments of greater than 0.1% above natural abundance would be necessary. A final important limitation is that the sensitivity of ^{13}C-NMR is such that samples of the order of several milligrams

represent the approximate lower limit. Consequently radioactive labelling will be the method of choice for systems in which the quantity of metabolite is severely limited.

5.2 Methods of detection

Two detection methods have been employed in the work reported in the literature to date, the indirect assessment of ^{13}C content by examination of ^{13}C satellites of coupled proton signals and the direct measurement of the ^{13}C spectrum of the ^{13}C-enriched material by continuous wave and pulsed Fourier transform - NMR.

Although the latter technique is far superior, measurements of satellite spectra have led to the successful elucidation of biosynthesis in 6 systems, thus establishing the value of this approach. The first metabolite studied by the former technique was griseofulvin(5-1) (Tanabe and Detre, 1966), this was followed by fusaric acid(5-1) (Desaty et al., 1968), sepedonin(5-3) (McInnes et al., 1968; Wright et al., 1969), variotin(5-4) (Tanabe and Seto, 1970a), piericidin A(5-5) (Tanabe and Seto, 1970b) and mollisin(1-8) (Tanabe and Seto, 1970c).

Satellite analysis, however, has additional limitations. Since individual ^{13}C-^{1}H satellites of specific carbons must be unequivocally identified, difficulties arise with the more complex proton spectra where there are fewer separately resolved satellite bands; carbons lacking protons, of course, cannot be detected except in the simplest cases because the

long-range ^{13}C-^{1}H couplings are small. Thus, measurement for all labelled centres in a given molecule may not be possible. The satellites for any proton resonance which is well shifted from other absorptions are readily detected if reasonably concentrated solutions can be examined. Small amounts of proton-containing impurities can be troublesome and care has to be taken to confirm that the measured band is indeed a satellite signal rather than an impurity peak or a spinning side band of a stronger absorption in the spectrum; if both satellite signals for a given ^{13}C-^{1}H band are observable the characterisation of the satellites is straight forward. In many instances, however, only one of the two satellites is in an uncluttered region of the spectrum.

5.3 Direct measurement

5.3.1 Continuous wave - NMR (CW-NMR)

Since the first natural-abundance ^{13}C-NMR spectra were reported in 1957 by Lauterbur and Holm there have been several advances in techniques and instrumentation. All these improvements have focused on increasing sensitivity, since the combination of lower intrinsic magnetic moment and natural abundance (1.1%) makes the carbon signal 1/5700 as sensitive to detection as a proton signal. At an applied field of 23.5 KG, protons absorb at 100 MHz and carbon nuclei at 25.10 MHz. With respect to biosynthetic experiments, CW-NMR offered the first chance to view carbons directly. The elucidation of the biosynthesis of ^{13}C-enriched secondary metabolites using CW-NMR includes

Radicinin(5-7) (Tanabe et al., 1970a), Sterigmatocystin(1-28) (Tanabe et al., 1970b), Asperlin(5-8) (Tanabe et al., 1971), Ochratoxin(5-9) (Yamazaki et al., 1971; Maebayaski et al., 1972), Sepedonin(5-3) (McInnes et al., 1971), Cephalosporin C (5-10)(Neuss et al., 1971) and Shanorellin(5-11) (Wat et al., 1972).

5.3.2 Pulsed Fourier transform - NMR

The experimental advances made in the late 1960s allowed carbon-13 NMR spectroscopy to be utilised in practical studies of organic systems. The development of pulsed Fourier transform (PFT)-NMR during this time has made versatile ^{13}C-NMR studies not only practical but also nearly comparable with ^{1}H-NMR in terms of experimental ease and quality of results.

The great inefficiency of conventional frequency or field sweep NMR (CW-NMR) is the fact that at any given instant only one frequency is being observed. Thus for ^{13}C-NMR at 23.5 kG, where the chemical shift range for most molecules covers 5000 Hz (approx. 200 ppm), each 1-Hz-wide resonance line would be observed only 1/5000 of the time the spectrum was scanned. The rest of the time would be spent observing other peaks or looking at the baseline. The only solution to the inefficiency of single frequency observation is to excite all ^{13}C nuclei in the sample simultaneously and observe the total response of the sample as can be done in the FT mode.

A short radiofrequency pulse does not produce excitation at one frequency but instead excites a finite bandwidth of

frequencies. If the pulse is short enough (~50 μsec) then the bandwidth of excitation can be >5000 Hz, sufficient to simultaneously excite all ^{13}C-nuclei in a sample. In order to strongly excite the sample in this short period of time very high r.f. power is required.

Both pulsed FT and conventional sweep mode NMR employ time-averaging to improve signal to noise (S/N) ratios of spectra. In CW-NMR it is not uncommon for several hundred or several thousand 50 to 250 second scans to be accumulated. In PFT-^{14}C-NMR as many as 10^6 pulses spaced at 0.1 second to several seconds apart might be used. Since the S/N ratio of a spectrum improves with the square root of the number of scans or pulses, the advantage of the PFT method can be expressed in terms of a much shorter time to achieve a given S/N ratio than that required in a frequency or field sweep experiment.

The sensitivity advantage gained over CW-NMR in a single pulse experiment is proportional to $(F/\Delta)^{\frac{1}{2}}$ where F is the total chemical shift range and Δ is the linewidth of the narrowest signal. For ^{13}C-NMR the theoretical increase in sensitivity is of the order of $(5000\ Hz/0.5\ Hz)^{\frac{1}{2}}$ or approximately 100. In practice, sensitivity enhancements of 10 are achieved routinely. Compared to the other methods, PFT-NMR decreases the analytical time by 100 fold. For this reason PFT-NMR has been the method of choice for studying ^{13}C-enriched secondary metabolites since about 1972 (Table 5-1).

TABLE 5-1

Summary of ^{13}C-enriched metabolites analysed by PFT-^{13}C-NMR

Prodigiosin(5-12)	Cushley et al., 1971
Antibiotic X-537A(5-13)	Westley et al., 1972
Palmitoleic acid(5-14)	Burlingame et al., 1972
Vitamin B_{12}(5-15)	Battersby et al., 1971 Brown et al., 1972 Scott et al., 1972a, 1972b
Aureothin(5-16)	Yamazaki et al., 1972
Virescenosides (2) (5-17a,b)	Polonsky et al., 1972
Pyrrolnitrin(5-18)	Martin et al., 1972
Protoporphyrin-1X(5-19)	Battersby et al., 1972
Helicobasidin(5-20)	Tanabe et al., 1973a
Avenaciolide(5-21)	Tanabe et al., 1973b
Asperetin(5-22)	Cattel et al., 1973
Dihydrolatumicidin I(5-23)	Seto et al., 1973a
Streptovaricin(5-24)	Milavetz et al., 1973
Ochrephilone(5-25)	Seto and Tanabe, 1974
Hirsutic acid C(5-26) Complicatic acid(5-27)	Feline et al., 1974
Tenellin(5-28)	McInnes et al., 1974
Ascochlorin(5-29)	Tanable and Suzuki, 1974
Penicillic acid(5-30)	Seto et al., 1974
Trichothecolone(5-31)	Hanson et al., 1974
Multicolic acid(5-32) Multicolosic acid(5-33)	Gudgeon et al., 1974

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Pyrrolnitrin(5-18)	Martin et al., 1972
Protoporphyrin-1X(5-19)	Battersby et al., 1972
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Multicolic acid(5-32) Multicolosic acid(5-33)	Gudgeon et al., 1974

CHAPTER 6

The carbon-13 spectral assignments of dothistromin ethyl acetal

6.1 Carbon-13 spectrum of dothistromin ethyl acetal

In the first instance the success of a biosynthetic experiment using carbon-13 enriched precursors depends upon the correct assignment of the ^{13}C resonance signals in the ^{13}C-NMR natural abundance spectrum of the metabolite being studied. Because of its insolubility in commonly used NMR solvents, it was necessary to convert dothistromin to the more readily soluble ethyl acetal derivative. This derivative was chosen because it was easy to prepare in good yield, an important consideration given the limited amount of material available with a significant carbon-13 enrichment, and because it provided a ^{13}C-NMR spectrum in which the individual signals were well resolved.

Before discussing the proposed ^{13}C-NMR assignments of dothistromin ethyl acetal(2-3) it is necessary to discuss briefly some characteristic features of ^{13}C-NMR spectra and some analytical techniques pertinent to assignments. In particular, factors determining peak heights and the use of new coupling techniques as assignment aids in PFT-^{13}C-NMR will be discussed. These points have been reviewed by Stothers (1972) and Levy and Nelson (1972).

6.2 Carbon-13 peak areas : integration of signal intensity

In the ^{13}C-NMR spectrum, several important factors combine in making this type of measurement less useful.

As no direct correlation is found between signal intensity and the number of carbon nuclei associated with each peak, this limits the usefulness of proton-decoupled ^{13}C-NMR spectra for quantitative analysis. For this reason, such measurements are not usually attempted in routine ^{13}C-NMR studies.

This loss of correlation between signal intensity and the number of nuclei is due mainly to (i) differing longitudinal spin-lattice relaxation times and (ii) variable nuclear Overhauser effects. These two factors will be discussed under assignment aids.

6.3 Assignment aids

6.3.1 Proton broad-band decoupling and nuclear Overhauser enhancement (N.O.E.)

Since geminal ^{13}C-^{1}H coupling constants range from 100 to 250 Hz, interpretation of ^{13}C-NMR spectra can be complicated by overlapping ^{13}C-^{1}H multiplets. A method introduced to overcome these difficulties in PFT-^{13}C-NMR spectroscopy, by simplifying the spectra and increasing sensitivity, is the technique of proton broad-band decoupling. This technique not only requires a radio-frequency field, applied perpendicular to the magnetic field and equal to the carbon-13 Larmor frequency, but also a second radio-frequency (continuous or pulsed) such that it covers the entire range of the Larmor frequencies of the protons. Under these conditions all the ^{13}C-^{1}H multiplets collapse (double resonance). A singlet therefore appears for each non-equivalent carbon atom and the signal to noise ratio

is thus increased as a result of multiplet collapse and also by the N.O.E.

The N.O.E. (Overhauser, 1953) is based on the fact that the natural population of the proton spin levels is changed by double resonance stimulation with the second radio-frequency field. The spin state with the higher energy frequency has a higher population than that with the lower energy. Subsequent relaxation processes also influence the occupation of spin levels of other nuclei by dipole-dipole interaction. If the spin levels couple as, for example, in the case of geminal and vicinal nuclei the N.O.E. is particularly strong. This proton broad-band decoupling of ^{13}C-^{1}H multiplets leads to a distinct increase in signal intensities.

The magnitude of the Overhauser enhancement depends on the nature and environment of a specific carbon; the enhancement may be variable for carbons in the same molecule. Because maximum enhancement occurs only if dipolar coupling dominates and because relaxation mechanisms reduce the observed increase such that the N.O.E. effect vanishes in the limit, then addition of a paramagnetic species to a sample would reduce the N.O.E.

La Mar (1971a, 1971b), Freeman et al., (1971) and Natusch (1971) have shown experimentally and theoretically that under these conditions the N.O.E. can be quenched without strong broadening of the carbon-13 resonance lines. Then this technique could be useful for precise quantitative intensity measurements.

6.3.2 "Off-resonance" decoupling

Although proton broad-band decoupling simplifies the ^{13}C-NMR spectrum and increases the sensitivity, this technique has the disadvantage of suppressing coupling information of value for the assignment of the signals. Because in some fully coupled ^{13}C-NMR spectra the couplings can be complex, "off-resonance" partial decoupling is a valuable aid to the assignment of carbon-13 resonances in such cases. The sensitivity obtained by this technique compares well with that from proton broad-band decoupling.

Again, this is a double resonance method (Wenkert et al., 1969; Reich et al., 1969) but here the second radio-frequency is a few hundred Hz outside the range of the proton Larmor frequencies ("off-resonance") instead of inside this range. Under these circumstances vicinal and long-range couplings collapse, resulting in a small comparative decrease in the signal to noise ratio. What is then observed is geminal ^{13}C-^{1}H coupling. Characteristically, and depending on the frequency range of the second field, the geminal ^{13}C-^{1}H couplings decrease to 10-50 Hz with the result that a spectrum of intense multiplets of first order is observed. For example, a methyl group is represented in such a ^{13}C-NMR spectrum by a quartet of signals, methylene groups by triplets, methine carbons by doublets and quaternary carbons by singlets.

6.3.3 Longitudinal relaxation times (T_1)

If relaxation processes are controlled by intramolecular C-H dipole-dipole interactions, the longitudinal relaxation time (T_1) of a carbon-13 atom (Freeman and Hill, 1970; 1971; Allerhand et al., 1971) should depend in a characteristic manner on the number of hydrogen atoms directly bonded to that carbon (Kuhlmann et al., 1970).

While the theory (Bloch, 1946; Becker, 1969) and actual measurement (reviewed by Levy and Nelson, 1972) of T_1 is not relevant to the present discussion, the physical meaning is significant. In the case of 2-ethyl pyridine (Freeman and Hill, 1970), T_1 increased as the number of directly bonded hydrogen atoms decreased, e.g.

$T_1(\overset{\diagup}{\underset{\diagdown}{=}}C-)$	>	$T_1(\overset{\diagup}{\underset{\diagdown}{=}}C-H)$	>	$T_1(CH_2)$	>	$T_1(CH_3)$
68 sec		21-23 sec		20 sec		14.5 sec

As the nuclear Overhauser enhancement decreases with increasing T_1 in the absence of ^{13}C-^{1}H coupling, this would explain why carbon-13 signals of quaternary and carbonyl carbon atoms give the lowest intensities in proton broad-band decoupled PFT-^{13}C-NMR spectra. Conversely methyl groups appear as the most intense signals. This general rule can be influenced by other factors such as steric considerations, molecular tumbling, atomic interaction, etc., but applied intelligently it can be a valuable adjunct to signal assignment when this is not possible on a chemical shift basis.

6.4 Carbon-13 spectral assignments of dothistromin ethyl acetal*

As very few quinones and only one anthraquinone derivative have been investigated by ^{13}C-NMR (see Stothers, 1972) it was necessary, for the interpretation of the spectrum of dothistromin ethyl acetal(2-3) to attempt to assign signals by correlation with the spectra of some substituted anthraquinone, furobenzofuran and benzene model compounds.

For ease of explanation the assignment of each distinct line (Table 6-1) in the partial spectrum (Figure 6-1) will be discussed under the following headings:

6.4.3 Carbonyl carbons (185-188 ppm)

6.4.4 Aromatic carbons (102-165 ppm)

6.4.4.1 Aromatic carbons directly bonded to oxygen (156-165 ppm)

6.4.4.2 Aromatic carbons directly bonded to hydrogen (102-136 ppm)

6.4.4.3 Aromatic carbons not bonded to hydrogen or oxygen (102-136 ppm)

6.4.5 Aliphatic carbons (0-85 ppm, 102 ppm, 117 ppm)

Peaks in Figure 6-1 marked with a cross signify the presence of impurity. It is probable that these peaks are mainly due to the presence of deoxydothistromin ethyl acetal(6-5).

* According to the present convention, chemical shifts down field from the TMS methyl carbon resonance signal are stated as positive, in analogy with the proton δ scale.

TABLE 6-1

Carbon-13 chemical shifts of dothistromin ethyl acetal

Chemical shift (ppm)	Relative peak intensity	Multiplicity[a]	No. of bonded hydrogen atoms
188.685	0.43	S	0
185.234	0.29	S	0
165.589	0.36	S	0
159.951	0.48	S	0
157.405	0.59	S	0
156.495	0.69	S	0
136.184	0.36	S	0
129.757	0.45	D	1
129.151	0.48	D	1
123.391	0.43	S	0
117.571	0.40	D	1
112.114	0.45	S	0
111.750	0.29	S	0
111.144	0.38	S	0
106.233	0.52	D	1
101.474	0.60	D	1
85.558	1.00	S	0
62.540	0.76	T	2
43.723	0.45	T	2
15.227	0.32	Q	3

[a] Singlet(S), Double(D), Triplet(T), Quartet(Q)

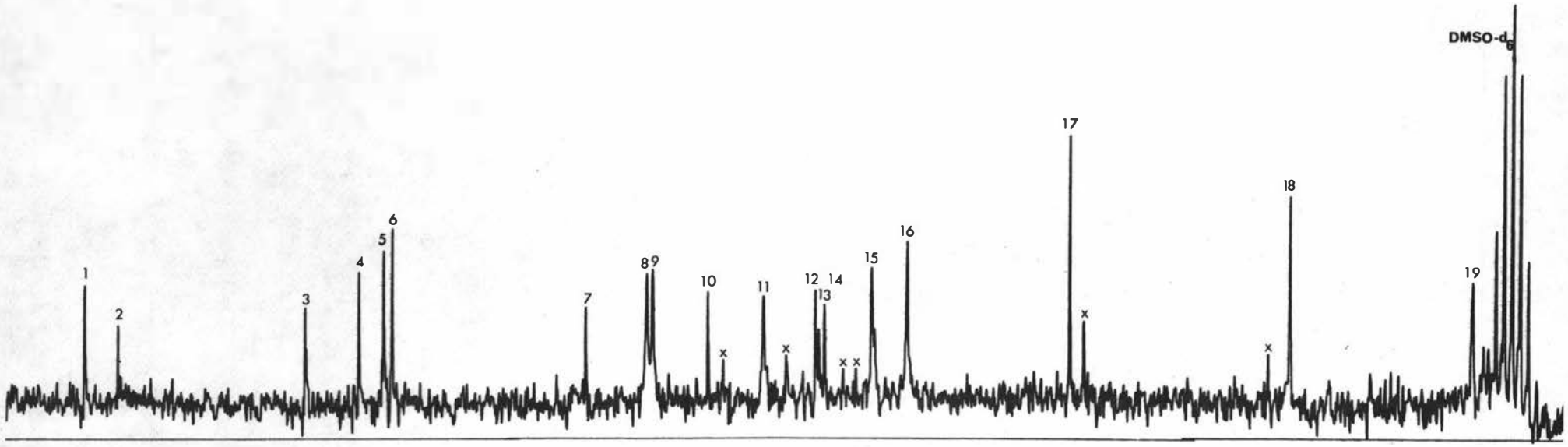
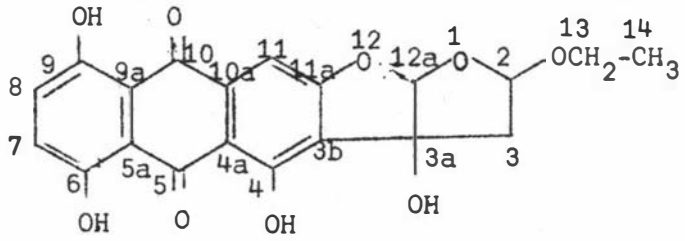


Figure 6-1 The proton noise-decoupled PFT-^{13}C-NMR spectrum (25.2 MHz) of dothistromin ethyl acetal.

6.4.1 Nomenclature for dothistromin

The ring numbering for dothistromin ethyl acetal adopted in this chapter is that found in Chemical Abstracts (1974), namely -



6.4.2 Additivity calculations for substituted anthraquinones

In the absence of any previous ^{13}C-NMR study into chemical shifts of anthraquinones it was necessary to develop an empirical method whereby the carbon-13 chemical shifts of several substituted anthraquinones (Table 6-2) could be calculated. The most successful additivity calculations, using a single set of compounds (Table 6-3), were based on substituent parameters of toluene, anisole and acetoxybenzene and using the chemical shifts of anthraquinone itself as a reference. In this way the chemical shift of most anthraquinone carbons could be calculated with some degree of accuracy (See Table 6-2).

Because of the success in using the additivity effects of simple substituted benzene derivatives in assigning carbon-13 chemical shifts of substituted anthraquinones, this method was used for assignments of aromatic carbons in dothistromin ethyl acetal (2-3). The method of calculation for both model anthraquinones and 2-3 is shown on page 97.

TABLE 6-2

Carbon-13 Chemical shifts[1,2,3] of some substituted anthraquinones

---

1 Relative to TMS

2 Top values for each carbon are the observed shifts

3 Bottom values for each carbon are the calculated shifts

a,b,c,d,e,f Assignments could be reversed

x Spectrum provided and analysed by Mr H.Gottlieb

Y Signal not readily apparent

z Signal of weak intensity

Compound	Carbon number 1	2	3	4	4a	5	6	7	8	8a	9	9a	10	10a
anthraquinone[x]	127.0	133.9	133.9	127.0	133.3	127.0	133.9	133.9	127.0	133.3	182.8	133.3	182.8	133.3
1-methoxy-	160.2	119.6[a]	134.9[b]	117.9[a]	135.6[c]	127.0[d]	133.0[e]	132.4[e]	126.3[d]	134.1[bc]	181.5[f]	121.4	183.3[f]	134.9[bc]
	158.7	119.5	134.9	119.3	134.3	127.0	133.9	133.9	127.0	133.3		118.9		133.3
1,4-dimethoxy-	153.6	119.9	119.9	153.6	122.4	125.8	132.7	132.7	125.8	133.7	182.7	122.4	182.7	133.7
	151.0	120.5	120.5	151.0	119.9	127.0	133.9	133.9	127.0	133.3		119.9		133.3
1,5-dimethoxy-	159.6	119.6[a]	135.0	116.7[a]	137.3	159.6	119.6[a]	135.0	116.7[a]	137.3	182.6	119.7	182.6	119.7
	158.7	119.5	134.9	119.3	134.3	158.7	119.5	134.9	119.3	134.3		118.9		118.9
1,8-dimethoxy-	159.5	118.9[a]	133.8	118.1[a]	134.8	118.1[a]	133.8	118.9[a]	159.5	124.1	184.0[b]	124.1	182.7[b]	134.8
	158.7	119.5	134.9	119.3	134.3	119.3	134.9	119.5	158.7	118.9		118.9		134.3
1,2,5,8-tetramethoxy-	147.1	157.4	123.3[a]	115.2[a]	128.7	152.9[b]	119.4[c]	118.6[c]	152.4[b]	122.9[d]	183.0[e]	125.3[d]	182.1[e]	122.4[d]
	144.3	151.2	120.5	120.3	126.6	151.0	120.5	120.5	151.0	119.9		119.9		119.9
1,3-dimethoxy-2-methyl-(6-4)	160.6	120.4[z]	163.0	105.1	133.0[a]	127.5[b]	133.4[c]	133.3[c]	126.9[b]	135.3[a]	185.4[d]	129.1[z]	181.3[d]	134.8[a]
	160.4	114.3	167.3	104.8	132.4	127.0	133.9	133.9	127.0	133.3		111.1		133.3
1-acetoxy-	150.3	129.9	134.8	125.7	135.3	127.0[a]	134.3[b]	133.4[b]	127.2[a]	134.1[y]	181.8[c]	132.7[z]	182.3[c]	134.1[y]
	150.2	127.7	135.4	124.9	134.8	127.0	133.9	133.9	127.0	133.3		127.1		133.3
2-acetoxy-	120.1	155.3	128.4	129.3	131.2	127.3	134.3[a]	134.1[a]	127.3	133.5[b]	182.0[c]	135.2	182.2[c]	133.4[b]
	120.8	157.1	127.7	128.5	131.2	127.0	133.9	133.9	127.0	133.3		134.8		133.3
1,4-diacetoxy-	148.3	131.0	131.0	148.3	126.2	126.9	134.1	134.1	136.9	133.4	181.5	126.2	181.5	133.4
	148.1	129.2	129.2	148.1	128.6	127.0	133.9	133.9	127.0	133.3		128.6		133.3
1,5-diacetoxy-	150.1	129.6	135.0	125.8	136.0	150.2	129.6	135.0	125.8	136.0	y	y	y	y
	150.2	127.7	135.4	124.9	134.8	150.1	127.7	135.4	124.9	134.8		127.1		127.1

TABLE 6-3

Carbon-13 substituent effects of substituted benzenes

Substituent	Position[a]			
	C-1	Ortho	Meta	Para
CH_3[b]	+ 8.9	+0.7	-0.1	-2.9
CH_2OH[c]	+12.3	-1.7	-0.3	-1.3
$COCH_3$[b]	+ 9.1	+0.1	0.0	+4.2
$OCOCH_3$[c]	+23.2	-6.2	+1.5	-2.1
OCH_3[b]	+31.4	-14.4	+1.0	-7.7
OH[b]	+26.9	-12.7	+1.4	-7.3

[a]Relative to TMS.

[b]From Levy and Nelson(1972).

[c]From Stothers(1972).

TABLE 6-4

Carbon-13 substituent effects[a] of substituted acetophenones

Subst.	C-1	C-2	C-3	C-4	C-5	C-6
2-OCH_3	- 9.0	+30.8	-16.2	+1.3	-7.9	+1.9
2-OH	-17.5	+34.1	- 9.0	+4.0	-9.0	+2.3

[a]Relative to acetophenone (Levy and Nelson,1972)

6.4.3 Carbonyl carbons

The carbonyl carbon has been the most investigated of all carbons by ^{13}C-NMR and has chemical shifts ranging from 150 to 220 ppm downfield from TMS (reviewed by Levy and Nelson 1972).

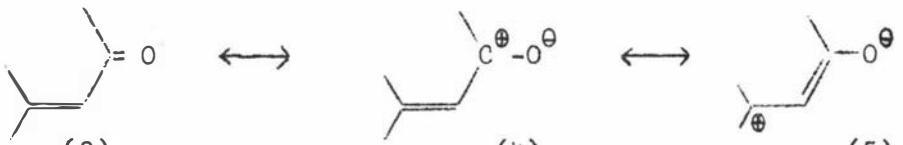
The low field position of carbonyl carbon resonances relative to those of other types of carbon atoms appears to be primarily due to a polarisation of the electrons in the associated pi bond (1) toward the more electronegative atom (2), i.e. due to a significant contribution of (2) to the overall electronic distribution.

(1) (2)

Although carbonyl groups have distinctive shifts depending on the adjacent functionality, there is significant overlap between various types. As a group, the carbonyl carbons of aliphatic ketones absorb the farthest downfield of all carbonyls (204-215 ppm) (Jackman and Kelly, 1970).

Alkyl substitution at the α-carbon causes a downfield shift of 2-13 ppm suggesting an inductive effect on the pi bond toward the oxygen, makes form (2) less important. Placement of an sp^2 centre α to the carbonyl carbon causes a marked upfield shift (shielding) of about 10 ppm, for instance in α,β-unsaturated systems. Ketone carbonyl resonances can be expected in the range 190-210 ppm

(Grutzner et al., 1970; Marr and Stothers 1965, 1967). This behaviour is general for aryl and α,β-unsaturated carbonyl derivatives and may be rationalised in terms of decreased electron deficiency at the carbonyl carbon because of electron release from the conjugated pi system. Thus form (5) will have a tendency to nullify the effect of (4) with respect to the electron density at the carbonyl carbon.



(3) (4) (5)

6.4.3.1 Carbonyl shifts in substituted anthraquinones

Relative to the benzophenone carbonyl resonance (194.8 ppm) the signals due to the anthraquinone carbonyl carbons are shielded by approximately 10 ppm and all have chemical shifts in the range 181.0 to 184.0 ppm (Table 6-2). Stothers, (1972) has noted that this extra shielding of the quinone carbonyl, relative to benzophenone, is probably due to increased cross conjugation resulting from the coplanarity of the carbonyl group and the aromatic rings in the quinone.

Carbons 5 and 10

The carbon-13 NMR natural abundance spectrum of 2-3 exhibited two low field, low intensity signals (peaks 1 and 2) at 188.689 and 185.234 ppm respectively. In the off-resonance decoupled spectrum of 2-3 these signals were still singlets.

The assignment of each signal is straight forward. One would expect that intramolecular hydrogen bonding, involving the phenolic hydrogen and carbonyl oxygen, would cause an increased polarisation of the carbonyl bond. This would then lead to a substantial deshielding of both quinone carbonyls of (2-3) relative to the carbonyl of anthraquinones lacking peri-hydroxyl groups, since the hydroxyl groups are ideally orientated for strong interaction. A similar effect is noted for ortho-hydroxybenzaldehyde and ortho-hydroxyacetophenone shielded +6.2 and +8.4 ppm respectively, relative to the parent compounds (Dhami and Stothers, 1965a).

Because of greater hydrogen bonding, therefore greater deshielding, peak 1 (188.689 ppm) is assigned to carbon 5 and peak 2 (185.234 ppm) to carbon 10 of 2-3.

6.4.4 Aromatic carbons (102-165 ppm)

Carbon-13 resonances for aromatic hydrocarbons are generally found in the range 110 to 135 ppm downfield from TMS (benzene = 128.5 ppm). However, the shifts of the aryl carbons alter dramatically when there are polar substituents (Lauterbur, 1958), within a range of approximately 70 ppm. Lauterbur also showed from a study of the methyl derivatives of iodobenzene (Lauterbur, 1963a), aniline (Lauterbur, 1963b), dimethylaniline (Lauterbur, 1963b) and nitrobenzene Lauterbur, 1963c) that the shielding effects of substituents tended to be additive in polysubstituted aromatic

systems except when the substituents were in an ortho relationship. This suggests that steric interference between neighbouring groups upsets their interactions with the ring such that the deviation from additivity may provide a measure of the steric hindrance.

Further work by Savitsky (1963), Maciel and Natterstad (1965) and Dhami and Stothers (1965a, 1965b, 1966) on other polysubstituted aromatic systems have permitted an extensive analysis of the additivity relations. These results showed that aromatic carbons bonded directly to a substituent had the widest range of chemical shifts, namely 96.7-168.3 ppm. In contrast, those aromatic carbons which were meta to the substituent bearing carbon were least affected by the substitution; shift range 127.0-131.6 ppm. The ortho and para carbons are affected to a greater extent by substitution with chemical shift differences of 20-25 ppm relative to their shifts in the parent hydrocarbon.

6.4.4.1 Aromatic carbons directly bonded to oxygen (156-165 ppm)

Aryl carbons directly bonded to oxygen show a downfield shift because of deshielding (Table 6-3). Similar downfield shifts are observed for acetoxy and methoxy substituted anthraquinones (Table 6-2). Here the shifts are from 20 to 30 ppm downfield of their position in the spectrum of anthraquinone. To summarize, for these anthraquinone derivatives all such resonances fall in the range 147 to 160 ppm.

Dothistromin ethyl acetal(2-3) has four aryl carbons directly bonded to oxygen, three of these being in the α position and the fourth in a β position. These four carbons would be expected to be deshielded by the oxygen such that the resonances fall in the above mentioned range. The carbon-13 natural abundance spectrum of 2-3 shows four signals at 165.589 (peak 3), 159.951 (peak 4), 157.405 (peak 5) and 156.495 ppm (peak 6) within this range. The off-resonance decoupled spectrum of the same compound showed that these signals arose from carbon atoms which were not bonded to hydrogen since they all remained as singlets.

Carbons 6 and 9

The resonances of carbons 6 and 9 would be expected to have nearly equivalent chemical shifts; only two of the four signals qualify, namely 157.405 ppm (peak 5) and that at 156.495 ppm (peak 6). Individual assignments of these two signals were not possible with the information at hand.

An important feature of the application of ^{13}C chemical shift data to structural elucidation is the existence of simple additive relationships which correlate the shieldings within specific families of compounds with a limited number of structural parameters (see Levy and Nelson, 1972). Using such additivity rules it was possible to assign the remaining two resonances.

Carbon 11a

Dealing with carbon 11a first, it should be possible to predict its chemical shift knowing the chemical shifts of the appropriate carbon atoms in anthraquinone, phenol, anisole and benzyl alcohol.

Begining with the chemical shift of carbon 2 of anthraquinone, namely 133.9 ppm; add to this +31.4 ppm for an O- alkyl substituent (anisole), add +1.4 ppm for a carbon meta to a hydroxyl group (phenol) and add -1.7 ppm for a carbon ortho to a methylene hydroxy group (benzyl alcohol). This gives 165.0 ppm in good agreement with peak 3 (165.589 ppm).

Carbon 4

By elimination, peak 4 at 159.951 ppm is due to carbon 4. A calculation of the chemical shift of carbon 4 using anthraquinone, benzyl alcohol, anisole and hydrogen bonded ortho-hydroxyacetophenone (Table 6-4), gives 160.4 ppm in good agreement with peak 4.

6.4.4.2 Aromatic carbons directly bonded to hydrogen (101-136 ppm)

Comparison of the off-resonance decoupled and broad-band decoupled spectra of dothistromin ethyl acetal(2-3) revealed five signals within the aromatic shift region (101-136 ppm) which were due to carbon atoms bonded to a single hydrogen atom (doublets in the off-resonance experiment) (Table 6-1).

Carbons 7 and 8

Again it would be expected that carbons 7 and 8 would have similar chemical shifts and that each carbon would exhibit an upfield shift relative to anthraquinone being in a position ortho to the phenolic group (Table 6-3).

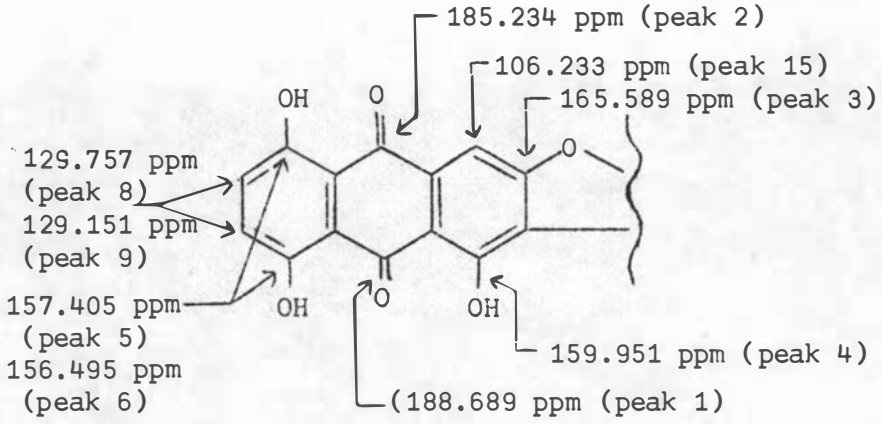
The chemical shifts of these two atoms can be calculated using data from the spectra of anthraquinone and ortho-hydroxyacetophenone. The appropriate carbon in anthraquinone resonates at 133.9 ppm. The chemical shift difference (relative to acetophenone) for carbon 3 of ortho-hydroxyaceto-phenone is -9 ppm, while that for carbon 4 is +4 ppm. The net shift difference is -5 ppm and the calculated chemical shift is then approximately 128.9 ppm. This is in good agreement with the signals at 129.757 ppm (peak 8) and 129.151 ppm (peak 9), (hydroxybenzophenone could have been a better model than hydroxyacetophenone but the information for this compound was not available), which are thus assigned to carbons 7 and 8. Individual assignment of these signals could not be made from the data at hand.

Carbon 11

The only other aromatic carbon bonded to hydrogen is at position 11. Application of the additivity principle again using anthraquinone, anisole, benzyl alcohol and phenol as model compounds yields a calculated chemical shift for this carbon of 105.0 ppm. In the ^{13}C-NMR broad-band spectrum of

rubiadin dimethyl ether(6-4). there is a signal at 105.1 ppm which has a high relative intensity indicating that it is possibly bonded to hydrogen (no off-resonance decoupling data available) and can be unambigously assigned to carbon 4 (Table 6-2). This follows from calculation of its chemical shift by adding the substituent effects observed on the appropriate carbon atoms in anthraquinone, anisole and toluene (Table 6-3) which gives a value of 104.8 ppm. The ^{13}C-NMR spectrum of rubiadin dimethyl ether(6- 4) shows only one signal in this region (Table 6-2). Since replacement of the phenolic hydroxyl by a methoxyl group in ortho hydroxyacetophenone only causes a chemical shift difference of +1.1 ppm in the carbon atom para to these groups (Dhami and Stothers, 1965a) then the signal at 106.233 ppm (peak 15) in the spectrum of dothistromin ethyl acetal(2-3) is assigned to carbon 11.

To briefly summarise the 9 shift assignments so far using stereochemical arguments, chemical shifts of model compounds, additive relationships and off-resonance decoupling results:



6.4.3.3 Aromatic carbons not bonded to hydrogen or oxygen (102-136 ppm)

There are five quaternary carbon atoms in 2-3, namely carbons 3b, 4a, 5a, 9a, 10a. As a group these atoms can be assigned to the following five signals in this region of the ^{13}C-NMR natural abundance spectrum, i.e. 136.184 (peak 7), 123.391 (peak 10), 112.114 (peak 12), 111.750 (peak 13) and 111.144 ppm (peak 14), since none of these signals showed splitting in the off-resonance decoupled spectrum. Further support for this arises from the low signal intensities which are characteristic of quaternary carbon atoms due to the low N.O.E. contribution.

Carbon 3b

Dealing with carbon 3b; adding the chemical shift differences due to substituents (using again ortho-hydroxyacetophenone, benzyl alcohol and anisole as models) to the chemical shift of the corresponding carbon of anthraquinone gives approximately 122.8 ppm. This calculated shift is only about 0.6 ppm away from the closest resonance at 123.391 which is thus assigned to carbon 3b. In rubiadin dimethyl ether (6- 4), a similar carbon gives a chemical shift of 120.40 ppm.

Carbon 10a

In those model substituted anthraquinones found in Table 6-2 where either or both rings carry one α substituent,

the quaternary bridge carbon meta to this substituent appears to be slightly deshielded relative to the corresponding carbon of anthraquinone, with chemical shifts ranging from approximately 134-138 ppm. In addition such carbon atoms generally show a greater signal intensity than those carbons ortho to α substituents. This feature can be explained by an apparent longitudinal relaxation "relief" through the adjacent aromatic hydrogen which results in additional nuclear Overhauser enhancement. Chemical shift calculation by adding the effects of substituents found in the model compounds, described previously, to the appropriate chemical shift for the same carbon in anthraquinone gives a value of 135.3 ppm. From this information carbon 10a is readily assigned to peak 7 at 136.184 ppm downfield from TMS.

Carbons 4a, 5a and 9a

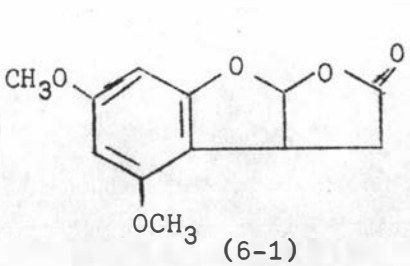
The three signals in this group which remain to be assigned have quite similar chemical shifts. The bridgehead carbons 5a, 9a, 4a are in similar chemical environments and would thus be expected to resonate at similar frequencies. Calculation of the chemical shifts on the basis of the additivity of substituent effects using anthraquinone, ortho-hydroxy-acetophenone, benzyl alcohol and anisole gives values which disagree (118.1 ppm for carbons 5a and 9a and 107.8 ppm for carbon 4a) with the chemical shift of the observed signals. Similar calculations for the bridge carbons of the model compounds in Table 6-2 yield values which, in the main, differ from those observed and it would seem that the relationship cannot be extended to carbons of this type. Thus individual assignment

of these three signals at 112.114, 111.750 and 111.144 ppm cannot be made at present.

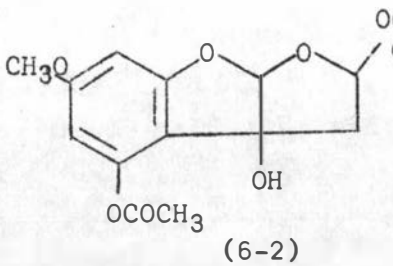
6.4.5 Aliphatic carbons (0-85 ppm, 102 ppm, 117 ppm)

Saturated carbons absorb at high field and over a fairly broad range of frequencies, from -2 to +43 ppm (Grant and Paul, 1964). Introduction of a hydroxyl group causes a pronounced downfield shift by 30-50 ppm of the signal from the carbon to which it is bonded (Roberts et al., 1970). In cyclopentane, for example, an oxygen substituent causes the carbon resonance to shift from 25.6 ppm (Burke and Lauterbur, 1964) to 68.4 ppm (Maciel and Savitsky, 1965; Weigert and Roberts, 1970).

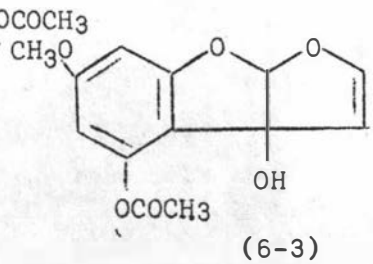
Unfortunately ^{13}C-NMR data for the difuran system such as that in 2-3 are not available. To assist with the assignment of signals arising from the carbon atoms of the difuran ring portion of 2-3, the ^{13}C-NMR spectra of the substituted furobenzofurans 6-1, 6-2, 6-3 were obtained (Tables 6-5, 6-6 and 6-7 respectively)



(6-1)



(6-2)

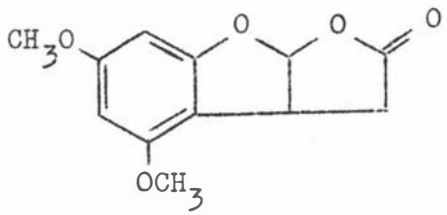


(6-3)

The signals from these three compounds were assigned as follows. The resonances at 174.17, 170.31, 170.25 and 169.76 ppm were readily assigned to the lactone carbonyl of 6-1, the aryl

TABLE 6-5

Carbon-13 shift assignments of 2,3,3a,8a-tetrahydro-2-oxo-4,6-dimethoxyfuro[2,3-b]benzofuran(6-1)*



Chemical shift[a] (ppm)	Relative peak intensity	Multiplicity[d]	Assignment
174.17	0.14	S	2
162.84	0.19	S	6,7a
158.96	0.14	S	6,7a
156.80	0.14	S	4
108.25	0.58	D	8a
105.39	0.22	S	3b
92.77	1.00	D	5,7
88.94	0.93	D	5,7
55.50	0.75	M[b]	9,10
55.23	0.85	M[b]	9,10
40.45	0.52	D	3a
32.09	0.53	M[c]	3

[a]Relative to TMS.

[b]Signal overlap.

[c]Multiplicity uncertain.

[d]Singlet(S), Doublet(D), Multiplet(M).

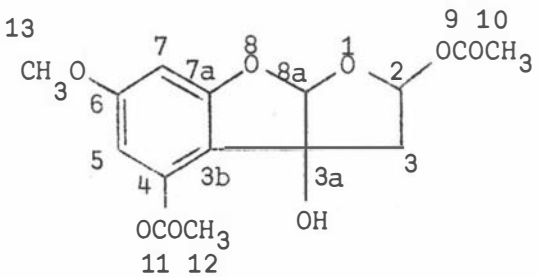
*Gift from Dr R.T. Gallagher (N.Z.D.S.I.R.)

TABLE 6-6

Carbon-13 shift assignments of

2,4-Diacetoxy-3a-hydroxy-6-methoxy-2,3,3a,8a-

tetrahydrofuro 2,3-b benzofuran(6-2).



Chemical shift[a] (ppm)	Relative peak intensity	Assignment
170.25	0.36	11
169.76	0.32	9
163.46	0.47	6,7a
161.03	0.38	6,7a
147.57	0.36	4
115.50	0.75	8a
113.14	0.50	3b
102.71	0.96	5
98.46	1.00	2,3a
95.43	0.95	7
85.48	0.99	2,3a
55.84	0.58	13
43.29	0.67	3
21.04	0.53	10,12

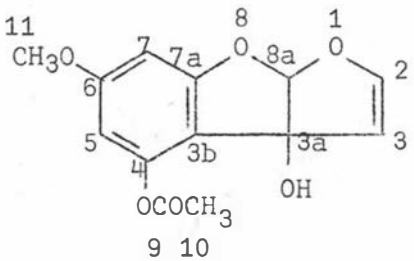
[a]Relative to TMS.

TABLE 6-7

Carbon-13 shift assignments of

3a-Hydroxy-4-acetoxy-6-methoxy-3a,8a-dihydroxy-

furo 2,3-b benzofuran(6-3).



Chemical shift[a] (ppm)	Relative peak intensity	Assignment
170.31	0.21	9
162.61	0.39	6,7a
161.40	0.20	6,7a
148.85	0.75	2
147.09	0.30	4
117.20	0.79	8a
113.32	0.20	3b
105.74	0.71	3
102.34	1.00	5
95.25	0.96	7
90.28	0.64	3a
55.84	0.60	11
20.92	0.40	10

[a]Relative to TMS.

acetate carbonyl of 6-3 and the aryl acetate carbonyl and hemiacetal acetate carbonyl, respectively, of 6-2, according to the considerations previously outlined (section 6.4.3). Next the aromatic carbons bonded to oxygen were assigned on the basis of the reduced signal intensity expected from carbons not directly bonded to hydrogen and of the downfield shift produced by an oxygen substituent. Signals at 162.84, 158.96 and 156.80 ppm in the spectrum of 6-1 could be assigned collectively to carbons 4,6 and 7a and this was supported by the absence of splitting of these signals in the off-resonance decoupled spectrum of 6-1. In the spectrum of 6-2, resonances at 163.46 and 161.03 ppm, were assigned to carbons 6 and 7a while the signal at 147.57 ppm could only be due to carbon 4. The latter assignment is in accord with the shift differences observed for aromatic carbons when a methoxyl group is replaced by an acetoxyl group (Table 6-3). Similarly signals with chemical shifts of 162.61 and 161.40 ppm in the spectrum of 6-3 were assigned to carbons 6 and 7a and that at 147.09 ppm to carbon 4. Assignment of carbon 4 to that signal and not to the one at 148.85 ppm follows from the low intensity of the 147.09 ppm resonance and from the similar chemical environment for the corresponding carbon in 6-2 which has a chemical shift of 147.57 ppm.

Turning to the high field signals, those at 21.04 and 20.92 ppm in the spectrum of 6-2 and 6-3 respectively were assigned to the methyl carbons of the acetate residues, by analogy with the resonance of this type of carbon in ethyl acetate (20.3 ppm) (Stothers, 1972) and by virtue of the

absence of a corresponding signal in the spectrum of 6-1.

By correlation with the chemical shift of the α carbon of isopropyl benzene (38.9 ppm), the signal at 40.45 ppm in the spectrum of 6-1 was assigned to carbon 3a. Further support for this assignment was provided by the split of this signal into a doublet in the off-resonance decoupled spectrum of 6-1 and the absence of related signals in the spectra of 6-2 and 6-3.

The signal at 32.09 ppm in the spectrum of 6-1 must be due to the resonance of carbon 3. The signal is split in the off-resonance decoupled spectrum, but the multiplicity of the signals was not readily discerned. The ^{13}C-NMR of butyrolactone has been reported (Johnson and Jankowski, 1972) and the chemical shift of the carbon corresponding to carbon 3 of 6-1 was given as 27.7 ppm. This does not correlate well but butyrolactone is not an ideal model compound.

The signal at 43.29 ppm in the spectrum of 6-2 was then assigned to carbon 3, being the only unassigned signal in this region and because corresponding signals were lacking in the spectra of 6-1 and 6-3.

By analogy with the chemical shift of the methoxyl carbon of anisole (54.7 ppm), signals at 55.50 and 55.23 in the spectrum of 6-1 and at 55.84 in the spectra of both 6-2 and 6-3 were assigned to the methoxyl carbons in these compounds.

Continuing with the spectrum of 6-1, of those signals which remained to be assigned, three, with resonances at 88.94, 92.77 and 108.25 ppm, were split into doublets in the off-resonance decoupled spectrum and therefore, could only be assigned to carbons 5, 7 and 8a. Calculation of the chemical shifts of carbons 5 and 7, by addition of the substituent effects observed with the model compounds anisole and isopropyl benzene (Stothers, 1972), gave values of 92 ppm. Therefore, these two carbons were together assigned to the signals at 92.77 and 88.94 ppm. By elimination, carbon 8a is assigned to the signal at 108.25 ppm. The signal at 105.39 ppm, which is not split in the off-resonance decoupled spectrum, must then be due to carbon 3b.

Returning to the spectra of 6-2 and 6-3; signals at 115.50 and 117.20 ppm, respectively, were assigned to carbons 8a in these molecules on the following grounds. Introduction of an hydroxyl group at the benzylic carbon of isopropyl benzene was found to cause a change in the chemical shift of the β carbons of +7.3 ppm (from 24.2 - 31.5 ppm) (Stothers, 1972; Johnson and Jankowski, 1972). Using the chemical shift of carbon 8a of 6-1 and adjusting it by the chemical shift difference just noted gave a value of 115.5 ppm, in close agreement with the two signals observed in the spectra of 6-2 and 6-3. The relative intensity of these signals was in keeping with that expected of carbons bearing hydrogen atoms.

The signal at 148.85 ppm in the spectrum of 6-3 has no counterpart in the spectra of the other two compounds and hence it probably arises from a part of the molecule which is unique to 6-3, i.e. the doubly bonded carbons. Carbons 2 of furan and benzofuran are reported to have chemical shifts of 143.05 and 145.1 ppm respectively (Johnson and Jankowski, 1972). Assignment of carbon 2 of 6-3 to this signal is then reasonable.

There are three sets of signals with similar chemical shifts in the spectra of 6-2 and 6-3 (113.14 and 113.32; 102.71 and 102.34; 95.43 and 95.25 ppm, spectrum signals of 6-2 first). These signals probably arise from parts of these two molecules which have similar chemical environments, i.e. the aromatic ring. The three carbons which have not been assigned signals are 3b, 5 and 7. Calculation of the chemical shift of carbon 3b by adding the chemical shift differences produced in benzene, ortho to an acetoxyl group (-6.2 ppm), ortho to a methoxyl group (-14.4 ppm) and para to the same group (-7.7 ppm) to the chemical shift of C-1 of benzyl alcohol (140.8 ppm) (Johnson and Jankowski, 1972) gives a value of 112.5 ppm. This is in reasonable agreement with the resonances at 113.136 and 113.318 which are then assigned to carbons 3b. The intensities of these signals are low in accord with expectation for carbons having no bonded hydrogen atoms.

Similar calculations for carbons 5 and 7 yielded values of 99.9 and 97.3 ppm respectively. The latter value agrees

reasonably well with the position of the observed resonances at 95.43 and 95.25 ppm (in 6-2 and 6-3, respectively) and these are then assigned to carbon 7. Although the agreement is again, not good in the case of carbons 5, they are assigned to the signals at 102.71 and 102.34 ppm in the absence of another pair of signals with more appropriate chemical shifts. Both sets of signals are of high relative intensity as would be expected of resonances from hydrogen bearing carbon atoms.

Two signals, at 105.74 and 90.28 ppm, in the spectrum of 6-3 remained to be assigned to carbon 3 and 3a. Carbons 3 in the ^{13}C-NMR spectra of furan (Johnson and Jankowski, 1972) and benzofuran (Okuyama and Fuemo, 1974) were found to have chemical shifts of 109.7 and 106.9 ppm respectively. Although these two compounds are not ideal models for the appropriate part of 6-3, they are considered to have enough relevance to permit a choice between the two signals of concern. Consequently carbon 3 is assigned to the signal at 105.74 ppm. Then, by elimination, the signal at 90.28 ppm is alloted to carbon 3a. Carbon-13 NMR data from a suitable model for carbon 3a could not be found.

Still remaining to be assigned are signals for carbons 2 and 3a of 6-2. The signals at 98.46 and 85.48 ppm must be assigned to carbons 2 and 3a but in the absence of off-resonance decoupled information for this compound a choice could not be made between these two.

Carbons 13 and 14

The signals at 62.640 ppm (peak 18) and 15.227 ppm are readily assigned to the methylene and methyl carbons respectively of the ethyl residue by virtue of their multiplicities in the off-resonance decoupled spectrum and by reference to the chemical shifts of the appropriate carbon signals in the spectrum of diethyl acetal (60.6 and 15.3 ppm) (Johnson and Jankowski, 1972).

With this information to hand the four remaining signals in the spectrum of dothistromin ethyl acetal (2-3) could be assigned as follows.

Carbon 12a

The signal at 117.571 ppm (peak 11), which split to a doublet in the off-resonance decoupled spectrum, (thereby indicating the carbon concerned was directly bonded to a single hydrogen atom), was assigned to carbon 12a since its chemical environment is quite similar to those of carbons 8a in 6-2 and 6-3 (115.50 and 117.20 ppm, respectively).

Carbons 3 and 3a

The signal at 85.558 ppm (peak 17), which was not split in the off-resonance decoupled spectrum, was assigned to carbon 3a; this is the only signal at high field not coupled to hydrogen. The signal at 43.723 ppm was assigned to carbon 3 in analogy with carbon 3 of 6-2 (43.29 ppm). Since the 43.723 ppm signal (peak 19) was partly

obscured by resonances arising from the DMSO-d_6 solvent, the multiplicity of the corresponding signal in the off-resonance decoupled spectrum was not readily discerned.

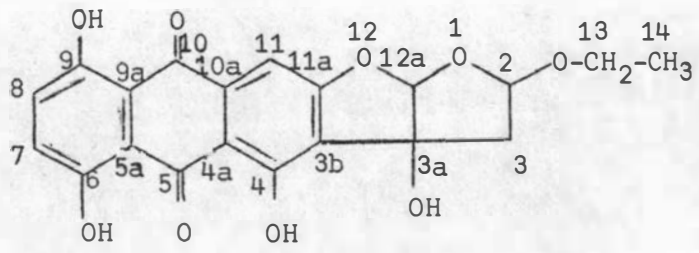
Carbon 2

The signal remaining to be assigned had a chemical shift of 101.474 ppm. This signal split to a doublet in the off-resonance decoupled spectrum and thus arises from a carbon bonded to a single hydrogen atom. By elimination this signal could be assigned to carbon 2. However the signal at 106.18 ppm was previously assigned to carbon 11 on the basis of a chemical shift calculation which gave a value of 104.8 ppm. Both signals split to doublets in the off-resonance decoupled spectrum so the assignments could be reversed. Results are summarised in Table 6-8.

TABLE 6-8

Carbon-13 shift assignments of dothistromin ethyl acetal

Chemical shift (ppm)	Assignment
188.689	5
185.234	10
165.589	11a
159.951	4
157.405	6,9
156.495	6,9
136.184	10a
129.757	7,8
129.151	7,8
123.391	3b
117.571	12a
112.114	4a,5a,9a
111.750	4a,5a,9a
111.144	4a,5a,9a
106.233	11(2)
102.474	2(11)
85.558	3a
62.640	13
43.723	3
15.227	14



CHAPTER 7

Biosynthesis of dothistromin

7.1 Production of carbon-13 labelled dothistromin

When it became apparent from carbon-14 feeding studies that carbon-13 tracer techniques were possible and then economically feasible and access to instrumentation became practical, the chemical degradation was abandoned in its favour since it appeared possible to obtain a much more complete picture of dothistromin biosynthesis in the time available using these techniques.

[1-^{13}C]-sodium acetate (90 atoms percent) and [2-^{13}C]-sodium acetate (90.5 atoms percent) were added, in separate experiments, to Dothistroma pini shake cultures about 10 hours after the visible onset of pigment production as described in the experiments with carbon-14 labelled precursors. Each culture was extracted with ethyl acetate, 7 days after spore inoculation and the dothistromin purified by the differential solubility method described before, except that no attempt was made to recover dothistromin removed by the procedures. The yields of dothistromin quoted in Table 7-1 represent precipitated dothistromin only. The success of each experiment was monitored by measuring the carbon-13 enrichment of each purified sample by mass spectrometry.

7.2 Use of mass spectrometry for determining carbon-13 enrichment

Since the electron-impact fragmentation patterns of many classes of natural products are now known, mass spectrometry can be used in conjunction with heavy isotopes in biosynthetic studies (Biemann, 1962; Budzikiewicz et al., 1967; Milne, 1971: Ratner, 1972). A study of the fragmentation pattern of isotopically enriched metabolites can yield the labelling pattern without recourse to chemical degradation. Prerequisites for such an undertaking is that the metabolite under investigation be thermally stable at the mass spectrometer probe inlet temperatures and that a reasonably simple fragmentation pattern is obtained. Some studies of biosynthesis using ^{2}H, ^{14}N or ^{18}O enriched precursors by this method have been reviewed by Tanabe, (1973).

Unfortunately, the second criterion for analysis of the carbon-13 enriched metabolite using mass spectrometry, is not met in the case of dothistromin. Besides the uncertainity in the fragmentation pathway of the difuran side chain (Gallagher and Hodges, 1972), the anthraquinone moiety itself shows little tendency to fragment under electron-impact as reported for other anthraquinones (Budzikiewicz et al., 1967).

This aside, the mass spectrometric analysis of carbon-13 enriched secondary metabolites such as dothistromin is still useful for determining a minimal isotopic enrichment which would indicate whether there was sufficient isotope in the metabolite

to allow its distribution to be determined by ^{13}C-NMR. This measurement is achieved by determining the $\frac{M+1}{M}$ ratios in the mass spectrum of the enriched metabolites, where M represents the intensity of the molecular ion and M+1, the intensity of the peak 1 atomic mass unit higher than M.

From the known elemental composition ($C_xH_yO_z$) of the molecule under investigation it is possible to calculate the theoretical ratio of $\frac{M+1}{M}$ due to the natural occurrence of isotopes of the constituent elements, using equation (i) (Beynon and Williams, 1963),

$$\frac{P_{M+1}}{P_M} = \frac{x\,C}{100-C} + \frac{yH}{100-H} + \frac{zO_1}{100-O_1-O_2} \qquad \ldots\ldots\ldots(i)$$

where P_{M+1} is the probability of obtaining a combination of mass (M+1) by incorporation of a single atom of ^{13}C or ^{2}H or ^{17}O. P_M is the probability that the combination contains no heavy isotopes of any sort where x, y, z are the number of ^{12}C, ^{1}H and ^{16}O, respectively, and C, H, O_1 and O_2 are the percentage natural abundance of ^{13}C, ^{2}H, ^{17}O and ^{18}O, respectively.

Knowing, from measurement, the value of the term on the left of equation (i) and the values of the second and third terms on the right hand side, the value of C (the percent carbon-13) can be calculated. This will be a minimum value for those carbon atoms which have been enriched by incorporation of ^{13}C from the labelled precursor since the enrichment has been calculated as though the isotope were evenly distributed among all carbons in the molecule (in the absence of information about

TABLE 7-1

Production of carbon-13 enriched dothistromin

Experiment	[^{13}C]-sodium acetate	Yield of Dothistromin[a] (mg)	Carbon-13 enrichment[b] (%)
1/1	[1-^{13}C]-	10.0[c]	4.5
3/3	"	12.0	2.1
4/4	"	20.0	1.7
4/5	"	7.0	0.7
5/7	"	12.0	2.3
5/8	"	4.0	2.4
5/9	"	7.0	2.5
6/12	"	18.0[c]	2.6
6/12	"	20.0[c]	2.8
6/14	"	7.0[c]	2.7
6/15	"	5.0[c]	2.7
2/2	[2-^{13}C]-	3.0[d]	2.4
4/6	"	20.0[d]	2.1
5/10	"	10.0[d]	2.5
6/16	"	12.0[d]	2.6
7/17	"	7.0[d]	2.2
7/18	"	8.0[d]	2.4

[a] Approximate values only (see text)

[b] Calculated by mass spectrometry

[c] Used in ^{13}C-NMR study

[d] Used in ^{13}C-NMR study

the actual distribution), whereas only some of the carbon atoms may be enriched in fact. Thus it is assumed that the carbon-13 tracer experiment will be practicable if this minimum enrichment value is above the limits acceptable for the detection of any signal enhancement in ^{13}C-NMR spectra resulting from carbon-13 enrichment.

Equation (i) was encoded into Focal language and the P.D.P. 8E computer programmed to calculate enrichments directly from the mass spectral parameters.

7.3 Carbon-13 NMR spectra of dothistromin enriched from [^{13}C]-acetate

For ^{13}C-NMR analysis carbon-13 enriched dothistromin (see Table 7-1) samples were converted to the ethyl acetal derivative. Because of the small amounts of material, the products were analysed by ^{13}C-NMR without purification.

The PFT-^{13}C-NMR proton noise-decoupled spectra* of the enriched dothistromin ethyl acetal samples, dissolved in DMSO-d_6, are shown in Figures 7-1 and 7-2.

* The author would like to acknowledge the kind assistance of Dr G.W.A. Milne (National Heart and Lung Institute, N.I.H., Bethesda, U.S.A.) in analysing the carbon-13 enriched samples by PFT-^{13}C-NMR.

7.3.1 Spectrum of dothistromin ethyl acetal enriched from [1-^{13}C]-sodium acetate

In the spectrum (Figure 7-1, Table 7-2) of dothistromin ethyl acetal enriched from [1-^{13}C]-sodium acetate, it is readily seen that the signals assigned to 3, 4, 5, 6 or 9, 7 or 8, 5a or 9a or 4a, 10a, 11a and 12a are of enhanced height relative to the remaining signals when this spectrum is compared with the natural abundance spectrum (Table 7-2).

A number of signals (denoted by X) are evident in this spectrum and the spectrum of dothistromin ethyl acetal enriched from [2-^{13}C]-acetate, which are not observed in the spectrum of the unlabelled derivative. Further, many of these additional signals are not common to the spectra of the enriched samples. Hence these signals could arise from ^{13}C enriched carbon atoms of contaminating deoxydothistromin ethyl acetal. Since no ^{13}C-NMR natural abundance spectrum of deoxydothistromin ethyl acetal is available to enable these additional signals to be assigned to this compound, the possibility must be allowed for these signals to arise from non-anthraquinoid products of the derivative making reaction. However the derivatives of the two species of enriched dothistromin were formed under near identical reaction conditions and this latter possibility would seem less likely.

Leaving consideration of the enhanced signal at 129.757 ppm until later, the carbons marked by an asterisk in the formula of dothistromin (1-1) appear to be enriched with ^{13}C derived from the carboxyl group of acetate. Three of the signals with

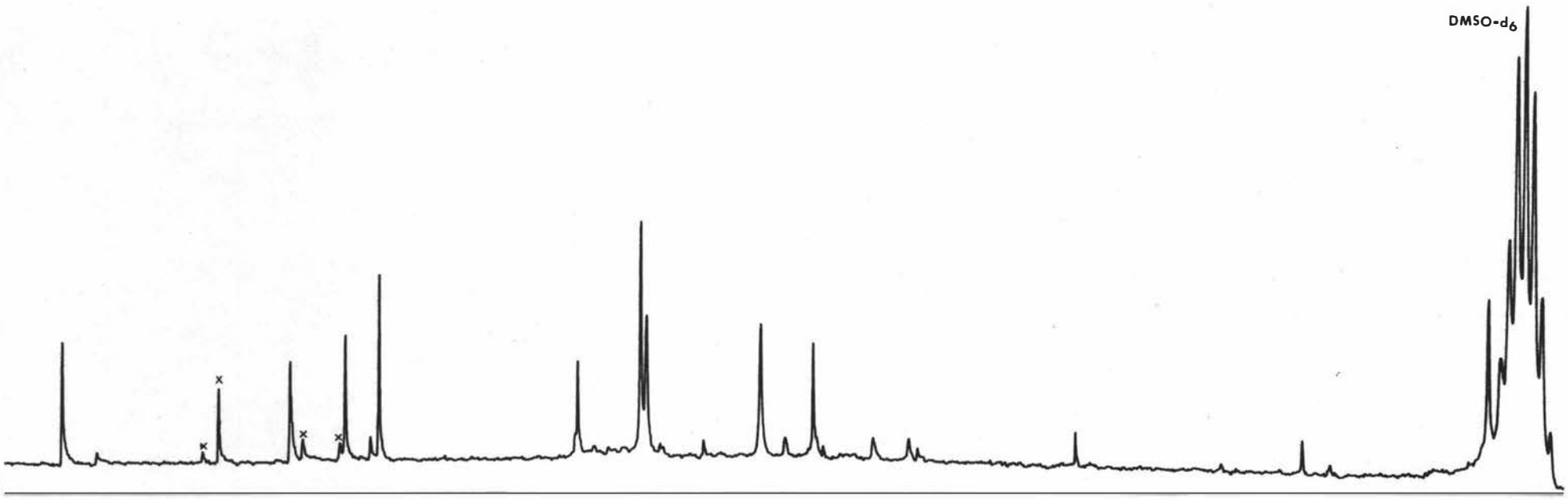
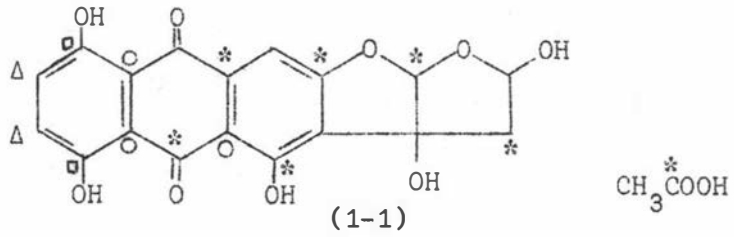


Figure 7-1 The proton noise-decoupled PFT-^{13}C-NMR spectrum (25.2 MHz) of dothistromin ethyl acetal enriched from [1-^{13}C]-sodium acetate.

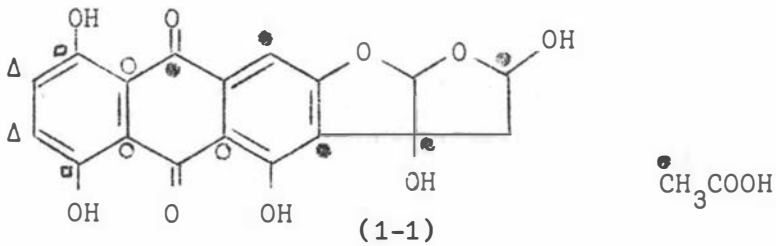
enhanced intensity could not be assigned unequivocally to a single carbon atom, but their assignment could be limited to a few possibilities indicated by like symbols.



(1-1)

7.3.2 Spectrum of dothistromin ethyl acetal enriched from [2-^{13}C]-sodium acetate

Again, in the spectrum of dothistromin ethyl acetal enriched from [2-^{13}C]-sodium acetate (Figure 7-2, Table 7-2) those signals assigned to carbons 2, 3a, 3b, 4a or 5a or 9a, 6 or 9, 7 or 8, 10 and 11 appear enhanced (Table 7-2).



(1-1)

Therefore the carbon atoms indicated by filled circles in (1-1) appear to be derived from the methyl group of acetate. There are 4 signals with enhanced intensities for which definite assignments could not be made and the possible origins of these signals are shown again by like symbols.

It is apparent that the signal at 129.757 ppm is of enhanced intensity in the spectra of dothistromin ethyl acetal enriched by both species of [^{13}C] acetate. This situation could arise if acetate giving rise to the carbon producing this signal was converted to an intermediate in which the carboxyl and methyl carbons of acetate became biologically indistinguishable, e.g. acetate was converted to a symmetrical intermediate. Further one of the two indistinguishable carbons would have to be eliminated in the course of dothistromin biosynthesis, since there is only one signal of enhanced intensity in the spectra of the compound enriched from both species of [^{13}C] acetate. A second possibility is that an impurity is present which fortuitously gives rise to a resonance with the same chemical shift as the signal concerned. As mentioned before, other signals attributed to impurities were observed in the spectra of the ^{13}C enriched samples, which had not been purified because of the consequent loss of already scarce material. Because there are these possibilities the biosynthetic origin of the carbon giving this signal must remain uncertain.

Thus six of the seven carbon atoms of dothistromin for which ^{13}C-NMR spectrum signal assignments could not be definitely made are derived from acetate, three from the carboxyl carbon and three from the methyl carbon, while the origin of the seventh carbon is obscure.

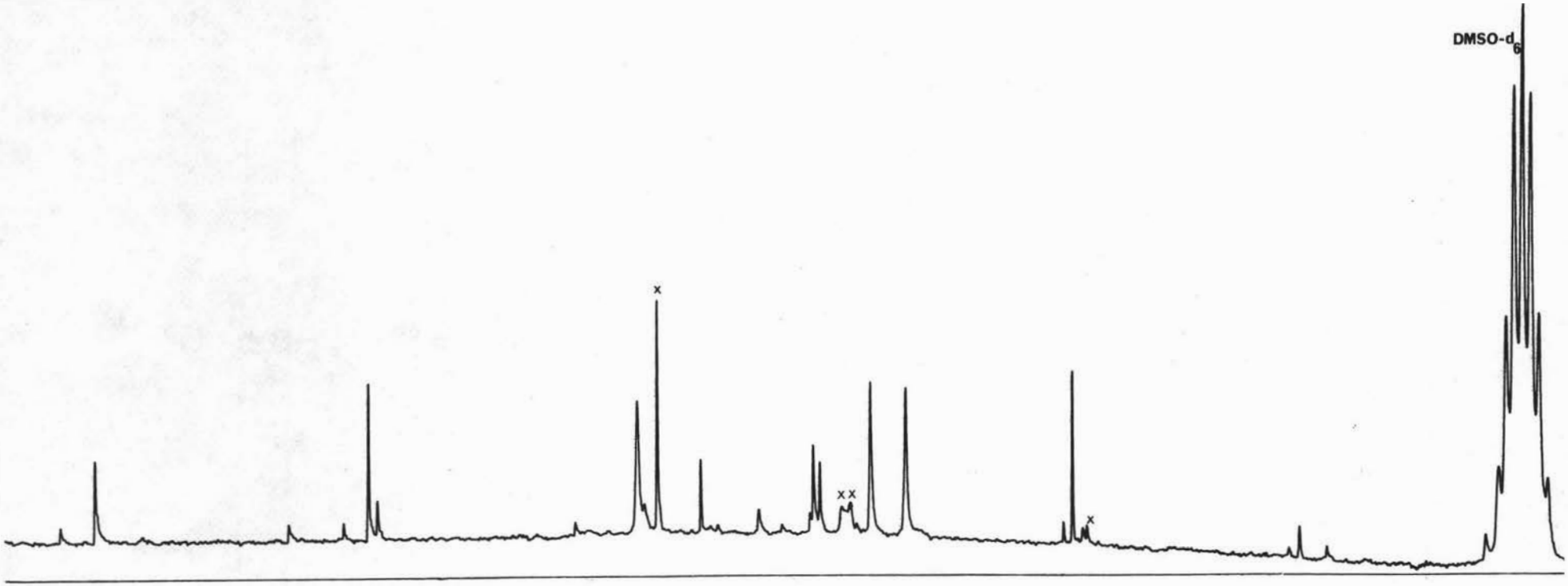


Figure 7-2 The proton noise-decoupled PFT-^{13}C-NMR spectrum (25.2 MHz) of dothistromin ethyl acetal enriched from [2-^{13}C]-sodium acetate.

TABLE 7-2

Carbon-13 chemical shifts of dothistromin enriched from [1-^{13}C]- and [2-^{13}C]-acetate

Chemical shift[a] (ppm)	Assignments	[1-^{13}C]-acetate[b]	[2-^{13}C]-acetate[b]
188.62	5	6.6	0.84
185.11	10	0.97	7.2
165.57	11a	6.8	1.2
159.91	4	6.3	0.88
157.28	6,9	1.0	6.8
156.39	6,9	6.8	1.5
136.08	10a	6.5	0.98
129.62	7,8	11.8	6.8
129.05	7,8	6.8	1.6
123.29	3b	0.96	4.4
117.54	12a	8.0	1.5
111.96	4a,5a,9a	6.6	1.37
111.58	4a,5a,9a	1.6	8.2
111.07	4z,5a,9a	0.67	4.9
106.18	11(2)	0.94	7.7
102.39	2(11)	0.89	6.2
85.49	3a	0.83	4.3
62.55	13	1.00	1.00
43.68	3	8.8	1.9
14.72	14	not recorded	

[a] Chemical shifts from enriched spectra

[b] Peak heights were measured and were normalised to the signal at 62.55 ppm in each spectrum (this signal is assigned to O-CH_2-CH_3). The figures shown are a ratio of the normalised peak heights in the enriched spectra to those in the natural abundance spectrum

7.3.3 ^{13}C-^{13}C coupling in spectra of ^{13}C enriched dothistromin

Although direct ^{13}C-^{13}C coupling is not usually observed in natural abundance ^{13}C-NMR spectra, because the intensity of the coupled signals is only 0.55% of the main non-coupled signal, the coupling between adjacent carbons may become apparent when these carbon atoms are both enriched with carbon-13. An instance where such coupling has been observed was with sterigmatocystin(1-28) which had been enriched by biosynthesis from [2-^{13}C]-acetate (Tanabe et al., 1970 and Tanabe, 1973). In the ^{13}C-NMR spectrum of this compound the signals arising from carbons 3a and 3b were split with Jcc=48 Hz. This observation confirmed the finding that these two carbon atoms were both enriched by isotope from [2-^{13}C]-acetate.

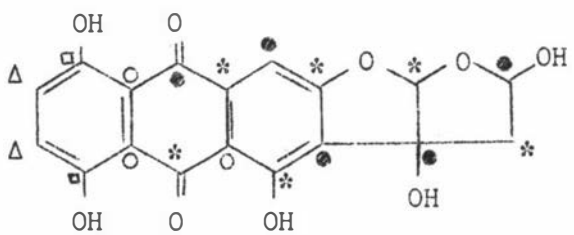
Coupling interactions between directly bonded carbon-13 atoms have only been investigated in a few instances (summarised by Levy and Nelson, 1972) using doubly labelled compounds. From the limited number of examples tested it appears that the magnitude of the coupling between adjacent carbon-13 centres (Jcc) is approximately correlated with the s character of the orbitals making up the bond. In particular, $J_{1,7}$ for toluene, benzyl alcohol and benzyl chloride has been found to be 44.2, 47.7 and 47.8 Hz respectively (Ihrig and Marshall, 1972).

In the spectrum of dothistromin ethyl acetal enriched by isotope from [2-^{13}C]-sodium acetate the signal at 85.193 ppm,

assigned to carbon 3a, shows coupling with Jcc=approximately 50 Hz. Further, the signal at 123.291 ppm (assigned to carbon 3b) shows just discernable splitting with coupling of Jcc = approximately 50 Hz. These splittings were not evident in the spectrum of the metabolite biosynthesised from [1-^{13}C]-acetate. The magnitude of the coupling is consistent with that reported for atoms of this type. This then confirms the previous finding that the carbon atoms giving rise to these split signals were both derived from the methyl group of acetate.

7.4 Biosynthesis of dothistromin from acetate

The findings of the previous sections are summarised below:



Here the filled circles indicate carbon atoms which have been found to be derived from the methyl carbon of acetate and asterisks, those derived from the carboxyl carbon. The open squares indicate a pair of atoms, one derived from the methyl carbon and the other from the carboxyl carbon of acetate, which could not be assigned definitely to one of a pair of resonances in the ^{13}C-NMR spectrum. The open circles show 3 carbons found to be derived from 2 methyl carbons and 1

carboxyl carbon of acetate, for which an assignment could not be chosen definitely from among three signals in the ^{13}C-NMR spectrum. The open triangles betoken 2 atoms, of which one is provided by the carboxyl carbon of acetate while the origin of the other is uncertain, which could be assigned to either of a pair of resonances in the ^{13}C-NMR spectrum.

The alternating pattern of labelling from [1-^{13}C] and [2-^{13}C]-acetates found in half of the anthraquinone ring of dothistromin and the derivation of 3 out of 6 of the remaining carbons from each species of [^{13}C]-acetate suggest a polyketide origin for this moiety. However this remains unproven because of the uncertain origin of the remaining carbon (7 or 8) in the anthraquinone ring. If this carbon atom is derived from a symmetrical intermediate, in the way previously discussed, then this mode of anthraquinone ring formation would be unique among the ways of forming these rings now known for a large number of examples. Thus it is felt the second possibility, that the fortuitous superimposition of a resonance from an impurity is masking the enrichment of the signal by carbon-13 from the methyl group of acetate, will be found to be the correct explanation of the findings. This situation would be clarified by using [1,2-^{13}C]-sodium acetate as a dothistromin precursor (the use of doubly labelled acetate was first exploited by Seto et al., 1973a, 1973b). If the anthraquinone ring system is formed solely from a polyketide precursor then 7 pairs of resonances emanating from the carbon atoms comprising it

would show ^{13}C-^{13}C coupling. The labelling pattern found among the carbons of the bifuran rings and the fused aromatic ring of dothistromin is the same as that established for the corresponding parts of sterigmatocystin (Tanabe et al., 1970 and Tanabe, 1973) and aflatoxin B_1 (Biollaz et al., 1970). While this pattern does not shed any light on the actual mechanism of formation of this ring system or permit a choice to be made from among the various postulates for a mechanism, it is another piece of evidence which, added to the rest, suggests a general mechanism of formation for compounds of this type. Again the use of doubly labelled acetate as a precursor could give a pattern of carbon-carbon spin couplings which might confirm one of the postulated mechanisms and deny the others.

The coexistence of 18 carbon difuroanthraquinones and 20 carbon anthraquinones bearing non-rearranged linear 6 carbon alkyl substituents among the secondary metabolites of Dothistroma pini (Danks and Hodges, 1974), Aspergillus versicolor and Aspergillus nidulans (Aucamp and Holzapfel, 1970) and Aspergillus flavus (Heathcote and Dutton, 1969) may be a clue to the formation of the difuran ring system. Already some evidence is extant for the conversion of averufin(1-15), a 20 carbon anthraquinone of the type just mentioned, to aflatoxin B_1(1-29) by Aspergillus parasiticus (Lin,et al., 1973). An investigation of any precursor-product relationship between these two classes of compounds with Dothistroma pini might therefore be fruitful.

EXPERIMENTAL

Melting points (m.p.) were determined on a Kofler Hot-Stage microscope and are uncorrected.

Ultraviolet and visible absorption spectra were determined in 95% ethanol solution on a UNICAM SP800, PERKIN-ELMER 402 or PERKIN-ELMER 124 spectrophotometer.

Infra-red spectra were recorded as Nujol mulls on a PERKIN-ELMER 720 spectrophotometer or BECKMAN IR-20 spectrophotometer.

The proton nuclear magnetic resonance (^{1}H-NMR) spectra were obtained using a JEOL C-60 HL spectrometer. Chemical shifts are given in parts per million (ppm) downfield from tetramethylsilane (TMS) as an internal standard; coupling constants (J) are given in Hertz (Hz). The abbreviations s,d,t,q and m refer to singlet, doublet, triplet, quartet, and multiplet respectively. The NMR data are given by listing in order the chemical shift, number of protons, multiplicity and coupling constants.

PFT-^{13}C-NMR spectra were recorded on a Varian XL-100-12 spectrometer (25.2 MHz) equipped with FT-100X Fourier Transform accessory and an 8K 620L computer or Bruker HFX-90/6 spectrometer at 22.63 MHz equipped with a Fabritek FT-1083 computer. Chemical shifts are given ppm downfield from TMS.

Mass spectra were obtained with an AEI MS902 double-focussing high-resolution mass spectrometer interfaced with a P.D.P.8E computer. All spectra were obtained by use of the direct insertion probe. Where data from a mass spectrum is quoted, this is generally for the most prominent peaks in the upper mass region of the spectrum.

Radioactivity was measured with a PACKARD (Model 3375) Tri-Carb liquid scintillation spectrometer. Amplification = 50% channel width = 50-1000.

Experimental for chapter 2

Preparation of potato dextrose agar (PDA) (containing antibiotic)

Potato extract (Oxoid L101)	4 gm
Dextrose	20 gm

were dissolved in one litre of hot distilled water. To this 15 gm of agar (Davis) was added, soaking for 15 minutes before autoclaving for 15 minutes at 15 psi and 121°C.

Antibacterial supplements:

(a) Benzyl penicillin BP sodium salt (Glaxo)

One half phial (30 mg; 5×10^4 units) was dissolved in 100 cm^3 sterile distilled water and stored at 4°C. This was added to the nutrient agar so as to give a final concentration of 50 units/cm^3.

(b) Streptomycin sulphate (Glaxo)

One quarter phial (250 mg; 1.9×10^5 units) was dissolved in 19 cm^3 sterile distilled water and stored at 4°C. One cm^3 was used per 100 cm^3 PDA medium.

Both antibiotics were added to cooled, sterilised PDA medium before pouring plates.

Preparation of 5% malt medium

(a) Liquid growth medium

Desiccated malt extract (partly defined below)	50 gm
Dried whole yeast (D.Y.C.)	5 gm

were mixed in 1 litre of hot distilled water and stirred vigorously for 5 minutes before dispensing, in 100 cm^3 aliquots, into 250 cm^3 erlenmeyer flasks or 300 cm^3 aliquots, in the case of 1 litre erlenmeyer flasks. Flasks were plugged with embossed milk filters (Cresta) and the contents sterilised for 15 minutes at 15 psi and 121°C.

In the experiment (see page 47) testing the effect of antifoam agent on dothistromin production, 0.5 cm^3 of sterilised polyethylene glycol was added aseptically to 300 cm^3 of sterilised malt medium before spore inoculation.

(b) Solid growth medium

To prepare 5% malt agar, 15 gm of agar was added to 1 litre of the liquid growth medium and this was sterilised and dispensed into petri dishes. For agar slants, nutrient agar was dispensed into McCartney bottles and the caps loosely fitted before autoclaving. The bottles were left to cool on a sloping rack to produce a solid slant.

Wander Malt Extract (Type 500 LDE)

Constitution*

(i)	Moisture	Less than 5%
(ii)	Reducing sugars as hydrated maltose	75-85%
(iii)	Non-reducing sugars as sucrose	3-6%
(iv)	Protein	Not less than 6%
(v)	Ash	Less than 2%
(vi)	Diastatic power	Less than 6° Lintner
(vii)	pH of a 10% solution	Not less than 4.8

* Wander (Aust.) Pty. Ltd., Melbourne, Australia

Preparation of the modified Raulin's medium

The following were dissolved, in the given order, in one litre of hot distilled water:

Sucrose	29.10 gm
D(+) tartaric acid	3.32 gm
KH_2PO_4	0.50 gm
NH_4NO_3	3.38 gm
K_2CO_3	0.50 gm
$MgSO_4$	0.32 gm
$CaSO_4$	0.20 gm
$ZnSO_4.7H_2O$	0.075 gm
$FeSO_4.7H_2O$	0.075 gm
Thiamine-HCl	6.25×10^{-4} gm

The pH was adjusted to pH 3.5 with sodium hydroxide before addition of citrate phosphate buffer.

Citrate-disodium hydrogen phosphate buffer solution (McIlvaine)

Citric acid monohydrate (0.1 M) 21.01 gm per litre

Disodium hydrogen phosphate (0.2 M) 28.40 gm per litre

One litre of buffer at pH 3.5 was prepared by mixing 298 cm^3 0.2 M Na_2HPO_4 with 702 cm^3 of 0.1 M citric acid.

A volume of 250 cm^3 of McIlvaine's buffer was added to the Raulin's medium (1 litre) and thoroughly mixed before distributing 200 cm^3 aliquots into 500 cm^3 erlenmeyer flasks for autoclaving.

In those flasks where glucose, fructose, maltose and ribose were the main carbon source, sugars were added to the nutrient medium in the same molar concentrations as sucrose.

Mycelial weight

Culture samples were withdrawn from the culture at different times and filtered through Whatman No. 1 filter paper. The mycelial pellet and filter paper was dried at 50°C to a constant weight. Results are tabulated below.

Time [a]	Carbon Source				
	Sucrose	Glucose	Maltose	Fructose	Ribose
61	0.04[b]	0.03	0.05	-	-
90	0.58	0.48	0.31	-	-
115	0.99	0.71	0.60	-	-
138	1.42	0.98	0.92	-	-
156	1.79	1.31	1.32	0.05	0.05
170	2.05	1.69	1.71	0.37	0.15
190	2.55	2.00	2.00	0.69	0.32
210	3.05	2.57	2.39	1.00	0.43
230	3.46	3.07	2.74	1.31	0.50
260	3.80	3.41	2.99	1.63	0.62
280	4.30	3.89	3.23	1.89	0.71
300	4.50	4.12	3.44	2.30	0.80
320	4.52	4.18	3.48	2.31	0.81
340	4.56	4.21	3.50	2.33	0.83

[a] Hours after spore inoculation

[b] Weight of mycelium in gms

Isolation of Dothistromin pini

(a) Pre-isolation

A sample of extensively infected pine needles was collected from young Pinus radiata stands within the Kohitere Forest, following visual identification of the disease.

Sporulation of the pathogen within the host tissue was brought about by incubation of the pine needles in an improvised humidity chamber consisting simply of 2 glass microscope slides in the bottom of an enclosed glass petri dish supported by several discs of moist filter paper. The petri dish was left at room temperature (22^{o}-25^{o}C) for 48 hours, a sufficient period of time for production of conidia, if the fungus was viable within the lesion.

Spore horns produced under these conditions were selectively removed by needle, with more spores being extruded by gently teasing the lesioned area with a pair of mounted needles with the aid of a dissecting microscope and transferred into a drop of sterile water. The spore suspension was streaked onto PDA containing antibotics, using a nichrome wire loop.

(b) Isolation

After about two weeks incubation at 18^{o}C, those individual colonies furthest from any contamination were removed by needle and subjected to a serial dilution. Four test tubes, each containing 0.9 cm^3 distilled water, were sterilised. One

tenth cm^3 was transferred from tube 1 to tube 2 mixing thoroughly, this was repeated for the remaining tubes giving a dilution range of 1 to $1/10^4$. The spore suspension of each tube was used to streak 5% malt agar plates.

(c) Re-isolation

Single colonies arising from single spore growth on the isolation plates were removed with a scapel and used to inoculate re-isolation plates in the same manner and so give rise to spores free from contamination.

Maintenance of Dothistroma pini

(a) Culture maintenance on agar slants

Concentrated spore suspensions were streaked onto malt slants. The McCartney bottles were stored at $4^{o}C$. Spore mobilisation was achieved by flooding each culture with sterile water.

(b) Soil maintenance of Dothistroma pini

Dry soil collected from within the campus was ground to a fine loam and dispensed, in 5 gm portions, into a series of 10 cm^3 test tubes. The tubes were plugged with cotton wool and autoclaved for 1 hour at 15 psi and $121^{o}C$.

Two cm^3 aliquots of concentrated spore suspensions were added aspectically to each tube and the contents mixed to give an even spore distribution. The tubes were dried at room temperature and stored at $4^{o}C$. Spore mobilization was achieved by adding a soil sample to a small volume of sterile water and dispensing in the normal manner into malt agar plates after soaking for 15 minutes.

(c) Lypophilisation of Dothistroma pini

(i) Suspending medium: Glucose (14%) and peptone (14%) were dissolved in distilled water and sterilised separately. Equal quantities were mixed before use.

(ii) Ampoules: These were made from 10 cm x 0.5 cm diameter Pyrex test tubes which were constricted about 2 cm from the open end, making certain that a uniform glass thickness was maintained at the drawn section so that a short thickened neck was produced. A small protective cotton wool plug was inserted below the point of constriction to prevent contamination of the contents when the ampoule was opened.

Spores from malt agar plates were mobilised with the suspending medium and drawn off by means of a sterile syringe. Approximately 0.25 cm^3 of the spore suspension per ampoule was injected past the cotton wool plug and freeze dried for 4 hours under vacuum (0.05 mm Hg). Ampoules were sealed under vacuum, and stored at $4^{o}C$.

(iii) Method of revival: A volume of sterile water equal to the original volume of the spore suspension was placed in the opened ampoule at room temperature. After 30 minutes the cotton plug was removed and the spore suspension was streaked out onto nutrient agar.

(a) Method for sowing Dothistroma pini onto nutrient agar

The procedure adopted was to sow concentrated Dothistroma pini spore suspensions by flooding standard 5% malt agar plates from a sterile syringe and incubating at 18°C. All slant cultures were inoculated by streaking the malt surface using a nichrome wire loop.

(b) Spore mobilisation from nutrient agar

Following sporulation of the fungus after about 16 days, plates were flooded with approximately 5 cm^3 distilled water and the spores were dislodged by brushing the mycelial mat with a sterile glass rod. Spore suspensions were drawn off by syringe and bulked in a sterilised conical flask.

(c) Procedure for sterilisation of liquid shake cultures

Shake culture flasks containing nutrient media were enclosed with eight layers of embossed milk filter (Cresta) which were fixed with a wide, tightly fitting, rubber band. This was similarly covered with paper and finally with aluminium foil to protect the filter during autoclaving for 15 minutes at 15 psi and 121°C. The two outer layers were removed following autoclaving.

(d) Spore inoculation of liquid culture

Inoculation was by sterile glass syringe through the filter using a flamed 4-6 inch 18 gauge needle. Milk filters showed self sealing properties.

Bulk extraction of crude pigment from submerged Dothistroma pini culture

(a) Modified Raulin's medium

After 18 days incubation at 18°C on a rotatary shaker (New Brunswick, model G-10), the mycelium of the 200 cm^3 culture was separated on Whatman No.1 filter paper and as no pigment could be extracted from this fraction, this was discarded. The filtrate was twice extracted with 3 volumes of ethyl acetate in a separating funnel, washed with water and dried over anhydrous magnesium sulphate before evaporating to dryness. Dothistromin was purified from the residue by thin-layer chromatography as described below.

(b) Malt medium

(i) Shaker flasks: One hundred cm^3 and 300 cm^3 cultures, incubated in 250 cm^3 and 500 cm^3 erylenmeyer flasks for 8 and 11 days respectively, at 18°C on a rotatory shaker, were transferred to a commerical blender and acidified to pH 1-3 with dilute hydrochloric acid (monitored by pH meter). To this, 20 cm^3 and 50 cm^3 of ethyl acetate was added respectively, before homogenising for 2 minutes at medium speed. The homogenate, quantitatively transferred to 500 cm^3 and 1000 cm^3 separating funnels, respectively, with washing, was extracted with 3 volumes of ethyl acetate until the organic phase was free of pigmentation (usually 3 extractions were required). The bulked extract was washed with water (3x100 cm^3

in both cases) and dried over anhydrous sodium sulphate for 1 hour. After filtering the extract, the solvent was evaporated under vacuum (40°-60°C) leaving a red amorphous solid.

(iii) Fermacell culture: Twenty litres of 15 days old culture was extracted in 1 litre portions following acidification. Each portion was extracted only once with 3 volumes of ethyl acetate. The resulting 60 litres of solution was concentrated, without prior drying in a Cyclone evaporator using super-heated steam. Final concentration to a red gum was achieved on a rotatory evaporator.

Purification of dothistromin

(a) Thin-layer chromatography (preparative)

Preparative thin-layer chromatography (tlc) on tlc grade silica gel (0.5-0.75 mm thick) of an ethyl acetate solution of the crude extract gave a main red band at approximately R_f 0.4-0.5 when developed with ethyl acetate-chloroform(60:40). When it was apparent that the red band had separated from a number of lesser coloured cometabolite bands it was scraped off while still wet with solvent, into ethyl acetate and the mixture was partitioned with water in a separating funnel to remove the silica gel. After drying, the organic phase was concentrated under vacuum until a red amorphous powder remained. This powder could not be induced to crystallise from a number of solvents and appeared as a single spot following tlc with a variety of solvent mixtures.

(b) Column chromatography

Columns (13 cm x 1.5 cm) were packed with a slurry of silica gel 100-200 mesh in ethyl acetate-chloroform(60:40). The crude extract was layered onto the column as a solution in the eluting solvent and the column was eluted with the same solvent. The dothistromin containing eluate was worked up as above.

Purification of dothistromin by differential solubilities from total culture extracts

(a) To the dried ethyl acetate culture extract a minimum volume of fresh ethyl acetate was added and the red solid titurated in the solvent. The resulting suspension of red solid was transferred to a centrifuge tube by Pasteur pipette and centrifuged for 30 seconds. The supernatant was drawn off and the residue washed twice with diethyl ether and twice with hexane, each washing being spun down. The dried residue gave a red-orange amorphous powder containing the bulk of the dothistromin in a near pure form. The ethyl acetate, diethyl ether and hexane washes were bulked and evaporated to dryness. The residual dothistromin was purified by preparative tlc.

(b) Ethyl acetate was substituted for chloroform and the same procedure adopted but only washing with diethyl ether (3 times). Tlc showed the resulting amorphous red solid to be chromatographically pure.

Purification of dothistromin from culture samplings

Usually 3 cm^3 of culture was twice extracted with 3 volumes of ethyl acetate in a 25 cm^3 separating funnel. The product in the extract was chromatographed twice on 0.2 mm thick silica gel plates as above. The red solid obtained was dissolved and made up to a known volume in ethanol for absorbance measurements.

Ultraviolet and visible absorption spectrum of dothistromin

A weighed sample of purified dothistromin was made up in ethanol and the extinction coefficient determined assuming total homogeneity, λ_{max} (ethanol): 230, 255, 268, 280, 478, 490, 508 and 523 nm (10^{-3} ε respectively 19.00, 10.95, 12.00, 13.75, 8.10, 8.40, 6.79 and 5.76)

All quantitative measurements for dothistromin content were estimated from the visible absorption maximum at 490 nm ($\varepsilon = 8.4 \times 10^3$).

pH measurement of culture medium

A 3 cm^3 aliquot of culture medium was removed by means of a syringe at predetermined times and the pH measured on a calibrated pH meter using a combination electrode. The pH of each sample was measured three times and averaged. The same sample was then analysed for dothistromin content.

Samplings (10 cm^3) from the fermacell cultures was achieved through a sterilised valve at the base of the vessel.

Dothistromin ethyl acetal (2-3)

Treatment of dothistromin (50 mg) dissolved in anhydrous ethanol (50 cm^3) with a catalytic amount of thionyl chloride for 3 hours at room temperature gave the ethyl acetal. Evaporation of the solvent under vacuum gave a solid which was purified by tlc (silica gel) using the solvent ethyl acetate-chloroform (60:40). There was a single band, R_f = 0.75, which was eluted from the gel and evaporation of solvent left a red powder (53 mg, quantitative).

The amorphous red powder crystallised from ethanol as small dark red plates, m.p. 203-205°, lit. 197-210° (Gallagher, 1971), phase change (needles) at ca. 170°. It had the following spectral characteristics which were identical to those reported for dothistromin ethyl acetal.

λ_{max} (ethanol) 231, 263, 275, 283, 467(sh), 478, 490, 509, 523 nm. Found: M (mass spectrometry), 400.0793. $C_{20}H_{16}O_9$ requires M, 400.0794. Mass spectrum, m/e (relative intensity): 400(22) (M^+), 371(22), 355(6), 325(10), 309(6), 299(100), 283(10), 272(10).

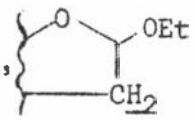
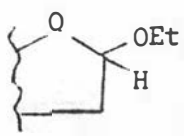
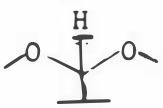
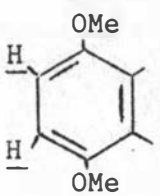
Dothistromin ethyl acetal trimethyl ether(2-4) and -tetramethyl ether(2-5)

Dothistromin ethyl acetal (53 mg) was refluxed for 12 hours in acetone (60 cm^3) in the presence of dimethyl sulphate (1 cm^3) and potassium carbonate (1 gm). Removal of the solvent gave a yellow-brown gum which could be resolved into two

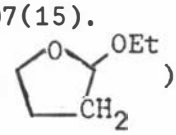
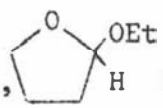
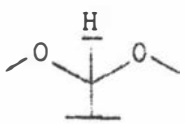
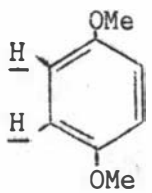
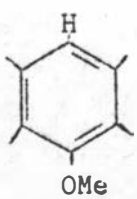
yellow spots by tlc (R_f = 0.47 and 0.56) using ethyl acetate-chloroform (60:40).

These two products were purified by column chromatography (silica gel, 100-200 mesh) when eluted with an ethyl acetate-chloroform (60:40) mixture.

The R_f =0.47 derivative (10 mg) was found to have properties identical to those reported for dothistromin ethyl acetal trimethyl ether. It crystallised as yellow needles from ethanol, m.p. 248-258°, lit., 252-254°(Gallagher, 1971) and had the following spectral features. λ_{max} (ethanol) 223, 273 and 420 nm. Found: M (mass spectrometry), 442.1260. $C_{23}H_{22}O_9$ requires M, 442.1263. Mass Spectrum, m/e (relative intensity): 442(20) (M^+), 427(5), 424(10), 413(5), 409(5), 397(10), 353(10), 341 (100), 327(10).

^{1}H-NMR ($CDCl_3$), 0.82 (3H, t, J=7Hz, -O-CH_2-$\underline{CH_3}$); ca. 2.63 (2H, m, ); 3.5 (2H, m, -O-$\underline{CH_2}$-CH_3); 3.90, 3.93, 3.97 (3H each, s, aromatic -OCH_3); 5.3 (1H, d, J=4.5 Hz ); 6.05 (1H, s, ); 7.25 (2H, s, ); 7.ss (1H, s, ).

It was noted that the solid was light sensitive turning from yellow to green upon exposure to daylight.

The major product (ca. 30 mg), R_f = 0.56, crystallised from ethanol as yellow needles, m.p. 184-188°. It had the following physical properties. λ_{max} (ethanol) 235, 272 and 428 nm. Found: M (mass spectroscopy), 456.1417. $C_{24}H_{24}O_9$ requires M, 456.1417. Mass spectrum, m/e (relative intensity) 456(50) (M^+), 441(16), 427(73), 426(13), 425(17), 424(36), 413(25), 412 (12), 411(35), 410(13), 409(21), 408(15), 397(15), 396(15), 395(48), 393(11), 383(44), 382(56), 381(37), 38 (13), 379(27) 378(13), 367(100), 365(4), 355(12), 355(12), 353(23), 352(15), 351(34), 350(11), 341(55), 340(11), 339(28), 337(30), 335(15), 327(10), 325(16), 323(18), 321(14), 311(55), 309(15), 307(15). ^{1}H-NMR ($CDCl_3$), 0.83 (3H, t, J=7 Hz, O-CH_2-CH_3); 2.63, m,  ; 3.5 (2H, m, OCH_2-CH_3), 3.18 (3H, s, benzylic OMe), 3.93, 3.95, 4.05 (3H each, s, aromatic -OCH_3); 5.27 (1H, d, J=4.5 Hz,  ; 6.1 (1H, s, ), 7.25 (2H, s, ), 7.33 (1H, s, ).

These properties were consistent with those reported for dothistromin ethyl acetal tetra methyl ether (Gallagher, 1971).

Experimental for chapter 3

[^{14}C]-precursors

[1-^{14}C]-sodium acetate (specific radioactivity, 63 mCi/M) and [2-^{14}C]-sodium acetate (specific radioactivity, 56 μCi/M) were purchased from the Radiochemical Centre, Amersham. The contents of each vial were dissolved in 1 cm^3 of distilled water which had been made slightly alkaline with sodium hydroxide and stored at 4°C.

When applicable the radioactive precursor was diluted with the appropriate amount of a known solution of unlabelled anhydrous sodium acetate and thoroughly mixed (vortex mixer) prior to measurement of final specific radioactivity.

Liquid scintillation counting solvents

Three different scintillation solvents were used;

(i) A toluene based scintillation solvent, used for determining the radioactivity of dothistromin methyl acetal tetramethyl ether(2-5), contained 4 gm of 2,5-diphenyl oxazole (PPO) and 0.2 gm of 1,4-bis[2-(5 phenyl oxazolyl)]benzene (POPOP) per litre of absolute toluene.

(ii) A 1,4-dioxan based scintillation solvent, used for [^{14}C]-dothistromin assays, contained 7 gm PPO, 0.05 gm POPOP and 50 gm naphthalene per litre of 1,4-dioxan. The 1,4-dioxan was carefully purified by percolating through a column of freshly activated aluminium oxide followed by distillation.

(iii) A Triton-X100 based scintillation solvent was used for determining the specific radioactivity of [1-^{14}C]- and [2-^{14}C]-sodium acetate solutions and consisted of two volumes of the toluene/PPO/POPOP mixture described under (i), combined with 1 volume of Triton-X100 (Rohm and Haas Co., Philadelphia).

Preparation of standard quench curves

Into 11 tared scintillation bottles was carefully pipetted 0.25 cm^3 of [1-^{14}C]-hexadecane (specific radioactivity. 1.016 μCi/gm, purchased from the Radiochemical Centre, Amersham) and the weight determined by difference. To each bottle was added 9 cm^3 of the 1,4-dioxan scintillation fluid which was then counted 5 times for 5 minutes. Then samples, in the range of 0.1 to 1.0 cm^3, of a solution of purified dothistromin in 1,4-dioxan (1 mg/cm^3) were added to 10 of these bottles. All 11 bottles were made up to a final volume of 10 cm^3 with 1,4-dioxan and were recounted as before.

The absolute counting efficiency for each bottle was calculated from the ratio of the second count to the first count and the known specific radioactivity of the [1-^{14}C]-hexadecane.

The quench correction curve was obtained by plotting counting efficiency against the amount of added dothistromin.

The same procedure was adopted, but using the toluene based scintillation solvent, to obtain a quench correction curve for dothistromin methyl acetal tetramethyl ether(4-3).

Incorporation of [^{14}C]-acetate into dothistromin

Each 100 cm^3 malt culture was incubated at 18^{o}C on a rotatory shaker as previously described until about 10 hours after the start of pigment production, about 85 hours after inoculation. At this time the cultures were removed from the shaker and a sterile sample of the radioactive precursor added.

The aqueous solutions of radioactive precursor were sterilised as they were added to the cultures by filtration through a Millipore filter (type GSWP 013 00, 0.22 µm pore size), mounted in a Swinny filter holder which was fitted to a 5 cm^3 all glass syringe. The transfer was made quantitative by subsequently injecting 3 aliquots of 2 cm^3 distilled water from the same syringe.

Following the desired period of incubation (usually 4 days after addition of label) the radioactive dothistromin was isolated and purified from the pigment fraction as previously described.

Purification of [^{14}C]-dothistromin

(a) From total culture extract

The red amorphous solid obtained by extraction of the total culture, as described previously, was dissolved in a minimum volume of ethyl acetate and layered onto a silica gel column (13 cm x 0.5 cm dia.). The column was eluted with 50 cm^3 diethyl ether and then ethyl acetate-chloroform(60:40). Radioactive purity was monitored with a Packard (model 7200)

radiochromatogram strip scanner.

(b) From culture samples

[^{14}C]-dothistromin in ethyl acetate extracts of 3 cm^3 of culture medium was purified by tlc.

Quantitation of [^{14}C]-dothistromin

The amount of dothistromin in Dothistroma pini culture samples was estimated after extraction and purification, from the increase in absorbance at 490 nm (molar extinction coefficient of dothistromin at 490 nm = 8400). Purified dothistromin was dissolved and made up to 10 cm^3 with 95% ethanol in a volumetric flask and the absorbance of this solution measured.

The same solution, plus washings from optical cells, was evaporated to dryness in vacuo and the residue was taken up in 3 cm^3 of 1,4-dioxan. This solution was transferred, together with a 1 cm^3 washing, to a scintillation counting bottle and 9 cm^3 of a 1,4-dioxan based scintillation solvent was then added.

The scintillation bottle was placed in the dark for 1 hour before measuring the radioactivity. Specific radioactivities (Ci/mole) of dothistromin were calculated, after correction for background count, using the quench correction curve. For the determination of the specific radioactivity of purified dothistromin from total culture extracts, about

0.1-0.2 mg of compound was accurately weighed into a tared scintillation counting bottle for counting as before.

Evaluation of radioactive ethyl acetate extracts by radiochromatogram scanning

300 µmoles of [1-^{14}C]-sodium acetate (9.01 x 10^{-1} Ci/M) was added to a 100 cm^3 malt culture after 77 hours incubation. Five days after acetate addition the culture was extracted as before. Tlc of the radioactive residue (20 cm x 5 cm plate, 0.2 mm layer) gave 8 major bands when developed with hexane-diethyl ether-acetic acid (80:20:1, v/v).

The distribution of radioactivity was analysed using a Packard model 7200 radiochromatogram scanner (see Figure 3-1). Tripalmitin and palmitic acid standards, developed with the crude ethyl acetate extracts, were identified by spraying tlc plates with a solution of 2',7'-dichlorofluorescein in ethanol. Under these conditions tripalmitin and palmitic acid standards coincided with the major radioactivity bands at R_f 0.92 and 0.68, respectively.

Effect of time of precursor addition on the specific radioactivity of dothistromin

In 6 sets of experiments, 10 x 100 cm^3 malt cultures (each set) were inoculated with equal volumes of the same spore suspension. All flasks were incubated as described before and the pH of each culture was monitored to determine impending dothistromin

production. For each set of experiments, small volumes of [1-^{14}C]-sodium acetate (experiments 1 to 3) and [2-^{14}C]-sodium acetate (experiments 4 to 6) were diluted with unlabelled sodium acetate to give a final concentration of 300 μmoles/cm^3.

The specific radioactivities of these solutions were:

Experiment 1	1.10×10^{-1} Ci/M
Experiment 2	1.081×10^{-1} Ci/M
Experiment 3	1.126×10^{-1} Ci/M
Experiment 4	0.891×10^{-1} Ci/M
Experiment 5	0.900×10^{-1} Ci/M
Experiment 6	0.878×10^{-1} Ci/M

Radioactive precursor (1 cm^3) was added to each culture in the series at a time interval 48 to 144.5 hours after spore inoculation (Tables 3-1 to 3-6). Where the radioactive precursor was added after dothistromin production had begun, a 3 cm^3 culture sample was removed immediately before addition of the label and assayed for dothistromin. All flasks were extracted 150 hours after spore inoculation and the specific radioactivity of dothistromin determined.

Effect of acetate concentration on dothistromin elaboration

Five, 100 cm^3 malt cultures were inoculated with varying concentrations of unlabelled sodium acetate after 85 hours incubation. An additional flask served as a blank. The final flask concentrations of sodium acetate ranged from 0 to 13×10^{-2} M.

Four days after acetate addition the dothistromin content of each flask was determined as previously described and plotted against the amount of sodium acetate added (Graph 3-3).

Effect of acetate concentration on the incorporation of [^{14}C]-acetate into dothistromin

Two stock solutions of sodium acetate were prepared and a sample of [1-^{14}C]-sodium acetate was added to one and [2-^{14}C]-sodium acetate to the other. The final specific activity of each solution was 10.36×10^{-3} Ci/M and 9.0×10^{-3} Ci/M, respectively. Aliquots of each solution was added, aseptically to 5 culture flasks in separate experiments but all derived from the same spore inoculum The final concentrations of sodium acetate per flask ranged from 10×10^{-3} M to 100×10^{-3} M. Dilution value, R.I.C., P.I. and enrichment were calculated from the results (see Tables 3-7 and 3-8).

The specific radioactivity of dothistromin in relation to the time of its isolation

To two 100 cm^3 malt cultures of Dothistroma pini and their duplicates was added 1 millimole of [1-^{14}C]-sodium acetate (1.17×10^{-2} Ci/M) and [2-^{14}C]-sodium acetate (9.59×10^{-4} Ci/M) after 86 hours incubation. This gave a culture concentration of 10×10^{-3} M sodium acetate. At various times (Table 3-9) 3 cm^3 aliquots were removed and

the concentration, specific radioactivity and R.I.C. of purified [^{14}C]-dothistromin determined. For the first measurement the sample was removed 5 minutes after addition of precursor.

Experimental for chapter 4

Preparation of potassium tertiary-butoxide

(a) Purification of tertiary-butanol

Three methods of preparing anhydrous tertiary-butanol were used.

(i) Commercial tertiary-butanol (Riedel-De Haen) was refluxed for 12 hours over barium oxide and distilled under nitrogen before use.

(ii) Commercial tertiary-butanol was refluxed over sodium wire (approximately 0.3 gm sodium to 100 cm^3 alcohol) until about two-thirds of the metal had dissolved. Anhydrous alcohol was distilled twice from sodium through a 30 cm fractionating column packed with dried glass beads. The tertiary-butanol was stored in tightly stoppered bottles. Quantities of solvent needed for experiments were obtained by redistilling the solvent from freshly prepared sodium wire and discarding the first 10% of the distillate.

(iii) Same as (ii) but distilled three times.

(b) Potassium metal

The oxidised metal surface was shaved off with a knife edge under xylene which had been previously dried over sodium. The cleaned metal was removed from the solvent, blotted rapidly with tissue paper and introduced into a tared beaker containing dry xylene.

(c) Potassium tertiary butoxide

Three methods of preparation were used.

(i) Weighed potassium metal was introduced into a flat bottomed flask previously flushed with nitrogen and containing sufficient purified tertiary-butanol to completely react with the metal (approximately 6 cm^3 of alcohol to 200 mg of potassium). The flask was reflushed, with dry nitrogen and the reaction mixture was refluxed with moisture protection, for 30 minutes. When all the potassium had reacted, excess solvent was removed under reduced pressure and the residue was heated under vacuum (150°C at 12 mm Hg) in an oil bath until the solid appeared dry and then allowed to cool. The potassium tertiary-butoxide was stored in a dry box under nitrogen.

(ii) The same product previously described was sublimed (150°C, 8 mm Hg) onto a cold finger in a dry box prior to use.

(iii) Commercial potassium tertiary-butoxide was obtained from Kodak Ltd., and stored under nitrogen.

All potassium tertiary-butoxide transfers were carried out in a dry box.

Purification of monoglyme

1,2-Dimethoxyethane was refluxed, with moisture protection, over calcium hydride for 6 hours before being fractionally distilled under nitrogen. On redistillation, the liquid was stored in a tightly stoppered bottle over type 4A molecular sieves.

Cleavage of anthraquinones

The base catalysed cleavage reactions were carried out on approximately 1.0-2.0 millimoles of anthraquinone and methoxyanthraquinones. The routine procedure adopted for each reaction and the product workup was as follows:

Each methoxyanthraquinone was dissolved in a minimum volume of monoglyme and the solution added to a stirred mixture of potassium tertiary-butoxide (10 M equivalents) in monoglyme, under nitrogen, in a 25 cm^3 flat bottom flask with ground glass joints fitted with a side arm and water cooled condenser with calcium chloride drying tube.

Three mole equivalents of water was quantitatively added to the vigorously stirred reaction mixture as a 10% solution in monoglyme, when the reaction mixture took on a grey colour. The reaction was carried out under a continuous flow of dry nitrogen which necessitated adding additional monoglyme at various times to maintain the initial volume. On completion of the cleavage process excess brine was added and the reaction mixture extracted 3 times with 3 volumes of ethyl acetate in a separating funnel. The extracts were bulked and washed with water, dried over anhydrous sodium sulphate and evaporated under vacuum to give the neutral fraction.

The basic reaction mixture was acidified with concentrated hydrochloric acid and the aqueous fraction extracted 3 times with 3 volumes of ethyl acetate. The bulked extracts were washed successively with saturated aqueous potassium

hydrogen carbonate and then water before drying over sodium sulphate. Concentration of the solution under reduced pressure, afforded the phenolic fraction. Potassium hydrogen carbonate extractives were isolated by cautiously acidifying the bulked washings with concentrated hydrochloric acid and extracting three times with 3 volumes of ethyl acetate. The bulked ethyl acetate extracts were washed with water, dried and evaporated, giving the acidic fraction.

Gas-liquid chromatography (glc)

Glc analyses were undertaken on a Varian aerograph, series 1700, gas chromatograph with dual column system and flame ionisation detectors. The signal from the detector was recorded on a flat-bed Servoscribe Potentiometric 0.2 mV to 20V slave recorder with floating input.

Separation of the TMS esters of the acidic fraction was carried out on a 1.5 m x 0.6 mm (O.D.) Silanised glass column packed with 5% S.E.52 (silicone gum rubber) on Chromosorb W (AW-DMCS). The column packing was prepared using the funnel coating method of McNair and Bonelli (1969). Dry nitrogen was used as the carrier gas. The flow rate was adjusted to give optimum operating conditions.

The injection port and detector temperatures were maintained at 190°C and 200°C, respectively, while the column temperature range was from 150-155°C.

Silylation of acid fraction and standards

Chromatographically pure TMS esters of substituted benzoic acids (Table 4-1), for use as chromatographic standards and the TMS esters of the acid fraction were prepared using the method of Sweeley et al., (1963). The dry residue from the acid fraction was dissolved in 1.0 cm^3 anhydrous pyridine (dried over potassium hydroxide pellets) and treated with 0.2 cm^3 of hexamethyl disilazane (Pierce Chemical Co.) and 0.1 cm^3 trimethyl chlorosilane (Pierce Chemical Co.) in screw capped glass vial. The mixture was shaken vigorously for 30 seconds and allowed to stand for 5 minutes before directly injecting known volumes into the glc.

The products on storage in a desiccator over phosphorus pentoxide at room temperature, showed no change in composition over the period of use.

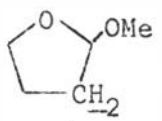
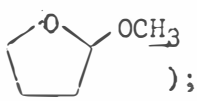
Dothistromin methyl acetal (4-1)

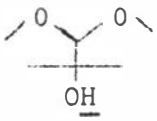
Dothistromin (50 mg) was completely dissolved in hot methanol (60 cm^3). Following addition of freshly distilled thionyl chloride,(5 drops) the orange solution was stirred for 3 hours at room temperature. Evaporation of the solvent under vacuum gave a red solid which appeared homogeneous by tlc (silica gel) developed with ethyl acetate-chloroform (60:40) (R_f = 0.77). Column chromatography on silica gel 100-200 mesh, using the same solvent, gave a red amorphous powder (50 mg) which resisted crystallisation from several common solvents. λ_{max} (ethanol) 467(sh), 478, 490, 509, 523 nm. Found: M (mass spectrometry), 386.0620. $C_{19}H_{14}O_9$ requires M, 386.0622 Mass spectrum m/e (relative intensity): 386(40) (M^+), 357(27), 325(19), 309(6), 299(100), 272(10).

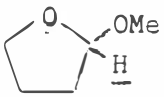
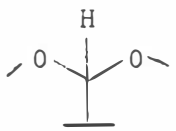
Dothistromin methyl acetal trimethyl ether (4-2) and -tetramethyl ether (4-3)

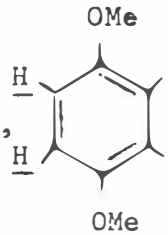
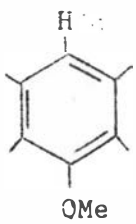
Dothistromin methyl acetal (50 mg), dimethyl sulphate (1 cm^3), anhydrous potassium carbonate (1 gm) and dry acetone (60 cm^3), were heated under reflux for 12 hours. After filtering, evaporation of the filtrate yielded an oil which on tlc with the solvent mixture ethyl acetate-chloroform (60:40), gave 2 yellow spots (R_f = 0.44 and 0.67). Column chromatography on silica gel (100-200 mesh) using the same solvent, separated the oil into a faster moving compound (4-3) (36 mg) and a slower moving compound (4-2) (12 mg). The slower moving minor component had the following physical

properties. λ_{max} (ethanol) 226, 275, 407 nm (10^{-3} ε respectively 26.3, 19.6, 5.82). Found: M (mass spectrometry), 428.1097. $C_{22}H_{20}O_9$ requires M, 428.1107. Mass spectrum m/e (relative intensity): 428 (30) (M^+), 410(8), 413(5), 399(36) 397(9), 395(4), 383(3), 381(6), 378(8), 378(6), 367(5), 353(7), 341(100) 327(8), 311(6).

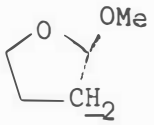
^{1}H-NMR ($CDCl_3$), 2.61 (2H, m ); 3.11 (3H, s, );

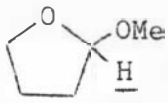
3.62 (1H, s, ); 3.98, 3.92, 3.89 (3H, s, Aromatic -OCH_3);

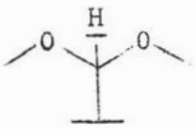
5.19 (1H, d, J=2.5 Hz ); 6.14 (1H, s, );

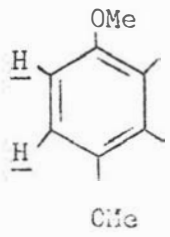
7.25 (2H, s, ); 7.30 (1H, s, )

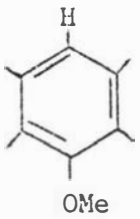
The major, faster moving yellow fraction, crystallised from methanol giving bright yellow needles, m.p. 186-189°, lit. 181-192° (Gallagher, 1971). λ_{max} (ethanol) 225, 273, 420 nm (10^{-3} ε respectively 26.7, 20.0, 5.89). ν_{max} (nujol) 2850, 2650, 2550, 200, 1860, 1665, 1650, 1585, 1570, 1485, 1470, 1410, 1355, 1305, 1220, 1150, 1110, 1010, 980, 950, 910, 875, 840, 750; Found: M (mass spectrometry), 442.1285. $C_{28}H_{22}O_9$ requires M, 442.1262. Mass spectrum m/e (relative intensity): 442(100) (M^+), 427(19), 413(66), 411(35), 410(42), 395(43), 394(14), 382(18), 381(53), 379(48), 367(33), 351(21), 341(23).

^{1}H-NMR ($CDCl_3$) 2.66 (2H, triplet, J=4.5 Hz, 

); 3.12, 3.18 (3H, s, aliphatic -OCH_3); 3.93, 3.96, 4.06 (3H, s, aromatic -OCH_3); 5.18 (1H, d, J=4.5 Hz 

); 6.15 (1H, s, 

); 7.25 (2H, s, 

); 7.35 (1H, s, 

).

Experimental for chapter 6

Preparation of model anthraquinones

(i) 1-methoxyanthraquinone (ii) 1,4-dimethoxyanthraquinone

(ii) 1,5-dimethoxyanthraquinone (iv) 1,8-dimethoxyanthraquinone

(v) 1,2,5,8-tetramethoxyanthraquinone

These compounds were formed from the appropriate hydroxyanthraquinone with dimethyl sulphate in refluxing acetone over anhydrous potassium carbonate. The reaction mixtures were filtered and the filtrate reduced under vacuum. The residues were all crystallised twice from ethanol. Melting points and ^{1}H-NMR were consistent with literature values. The PFT-^{13}C-NMR spectra were run in $CDCl_3$. Results are tabulated in Table 6-2.

(vi) 1,3-dimethoxy-2-methylanthraquinone (Rubiadin dimethyl ether (6-4))

Gift from Dr R.T. Gallagher. PFT-^{13}C-NMR spectral data in in Table 6-2.

(vii) 1-acetoxyanthraquinone

(viii) 2-acetoxyanthraquinone

(ix) 1,4-diacetoxyanthraquinone

(x) 1,5-diacetoxyanthraquinone

These compounds were formed from the appropriate hydroxyanthraquinone by refluxing in acetic anhydride and pyridine. The reaction mixtures were reduced under vacuum. Residues were all crystallised

twice from acetic acid. Melting points and ^{1}H-NMR were consistent with published results. The PFT-^{13}C-NMR spectra were run in $CDCl_3$. Results are tabulated in Table 6-2.

PFT-^{13}C-NMR of unenriched dothistromin ethyl acetal

The ^{13}C-NMR spectrum was measured in DMSO-d_6 at 25.2 MHz (Varian XL-100-12); data set = 8k, pulse width = 40 µsec; sweep width = 500 Hz; sweep off set = 32008 Hz; number of scans = 22,563. The spectrum was obtained on approximately 130 mg in 1 cm^3 of DMSO-d_6 in a 5 mm tube.

Experimental for chapter 7

[^{13}C]-precursors

[1-^{13}C]-sodium acetate (90 atoms percent) and [2-^{13}C]-sodium acetate (90.5 atoms percent) were purchased from Prochem Ltd., England.

Addition of carbon-13 enriched precursors to Dothistroma pini cultures

The precursors were weighed out, in 83 mg portions, into sterile test tubes and dissolved in 3 cm^3 distilled water. Solutions were added aspectically to 100 cm^3 malt cultures, about 10 hours after the visible onset of pigment production as described before. After 4 days additional incubation, [^{13}C]-dothistromin was purified by the differential solubilities method. Residual enriched dothistromin from organic washings were bulked and left.

PFT-^{13}C-NMR of dothistromin ethyl acetal enriched from [1-^{13}C]-sodium acetate and [2-^{13}C]-sodium acetate

The ^{13}C-NMR spectra (25.2 MHz) were measured in DMSO-d_6; data set = 8K, pulse width = 30 µsec; sweep width = 5000 Hz; sweep offest = 32008 Hz; number of scans = 63,200 ([1-^{13}C]-sodium acetate) and 67,896 ([2-^{13}C]-sodium acetate). The spectra were obtained on approximately 60 mg in 1 cm^3 of DMSO-d_6 in 5 mm tubes.

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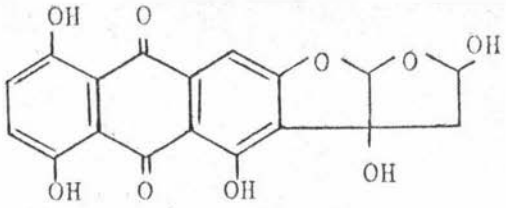

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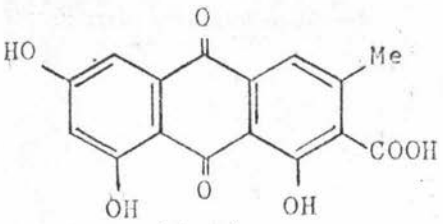

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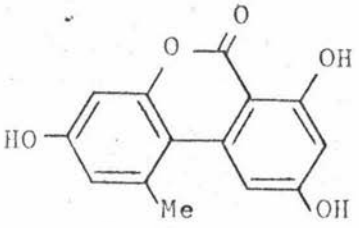

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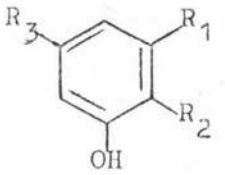

	R_1	R_2	R_3
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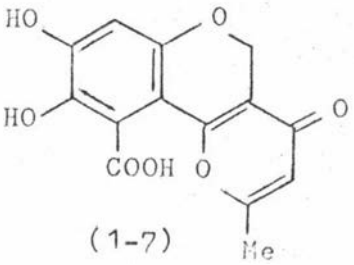
(1-5)	Me	COOH	OH
(1-6)	OH	H	H



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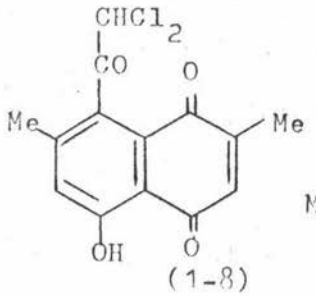

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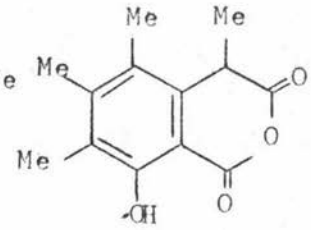

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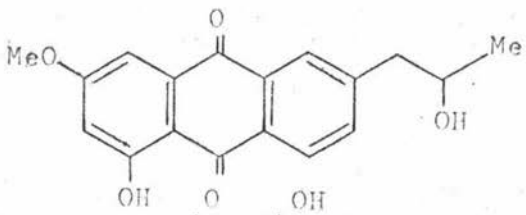

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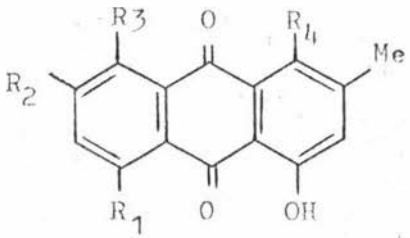
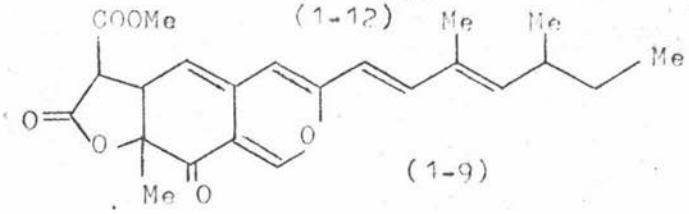


	R_1	R_2	R_3	R_4
(1-11)	OH	OH	H	H
(1-18)	OH	H	OH	H
(1-19)	OH	H	H	OH
(1-21)	OH	H	H	H
(1-22)	H	H	H	H



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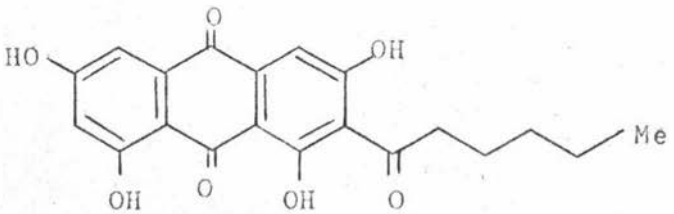

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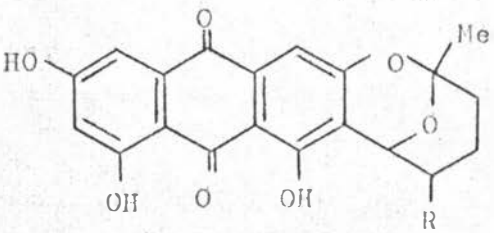
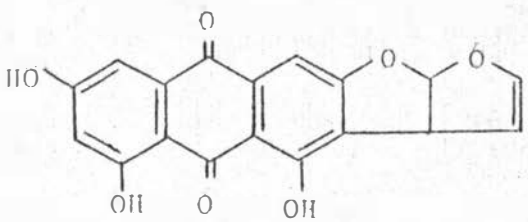


	R
(1-15)	H
(1-16)	OH



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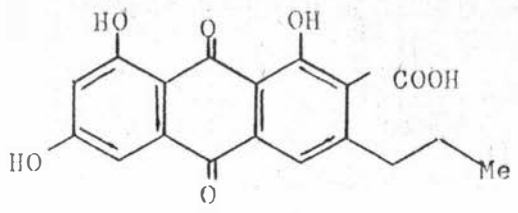

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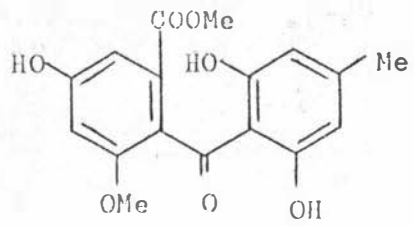

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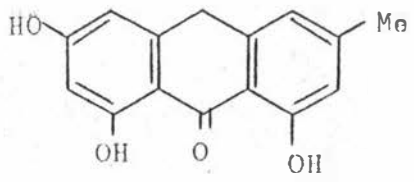

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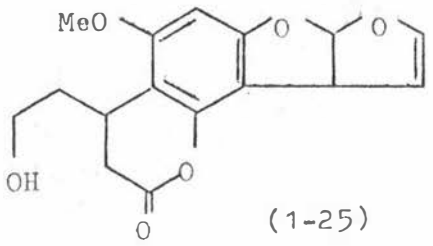

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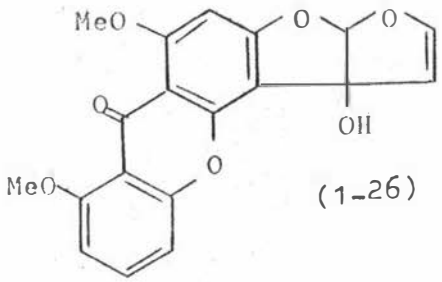

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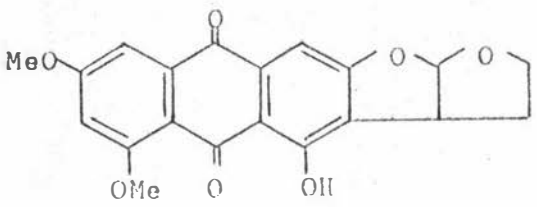

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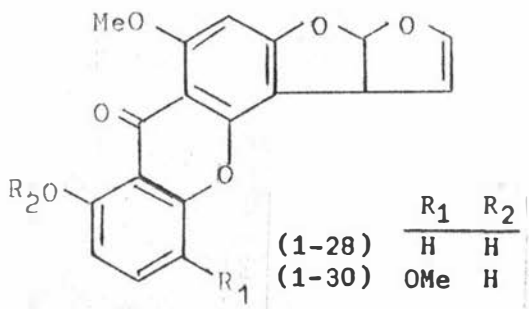
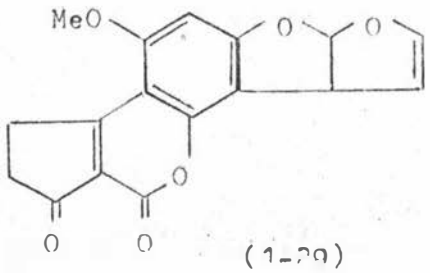


	R_1	R_2
(1-28)	H	H
(1-30)	OMe	H



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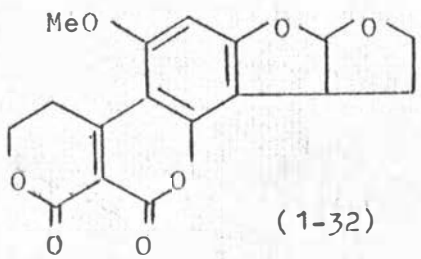

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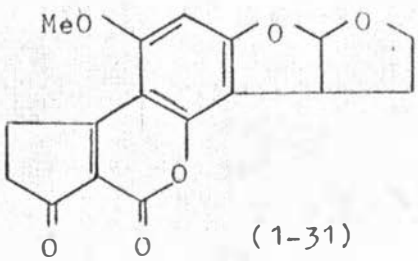

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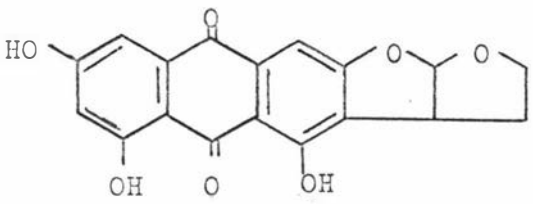

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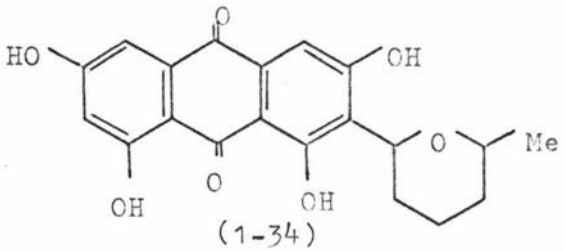

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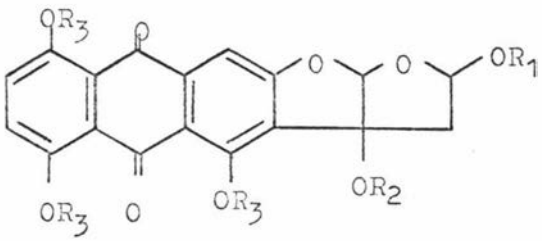
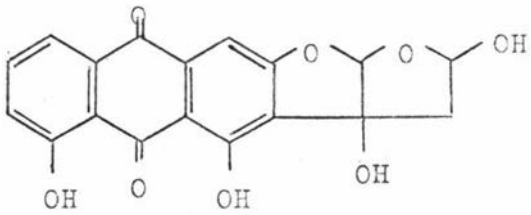


	R_1	R_2	R_3
(2-3)	Et	H	H
(2-4)	Et	H	Me
(2-5)	Et	Me	Me



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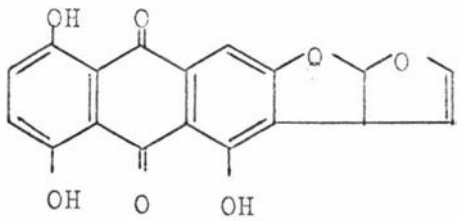

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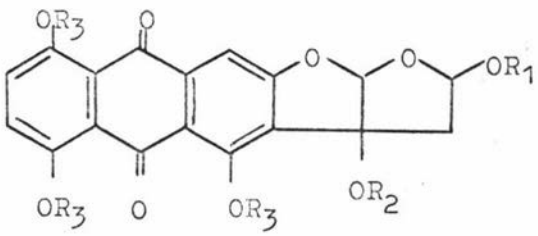
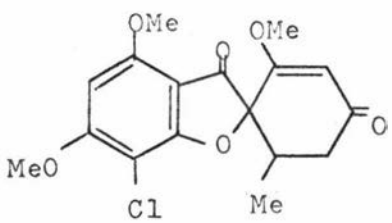


	R_1	R_2	R_3
(4-1)	Me	H	H
(4-2)	Me	H	Me
(4-3)	Me	Me	Me



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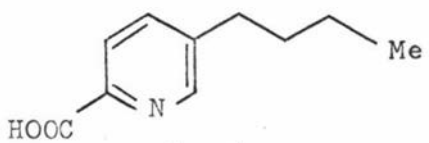

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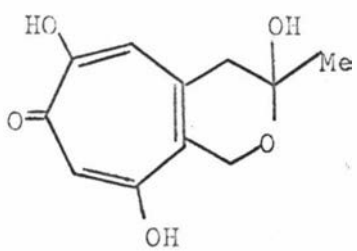

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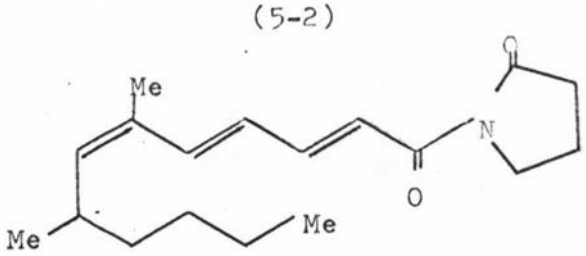

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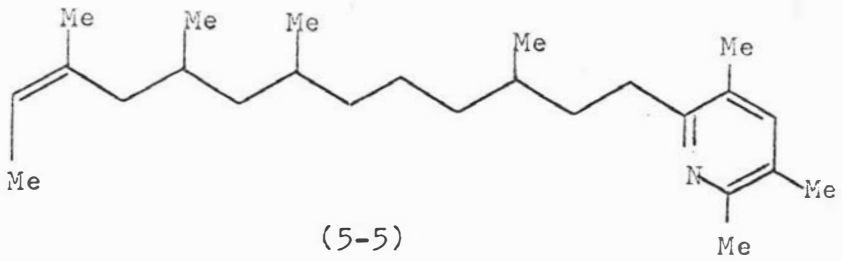

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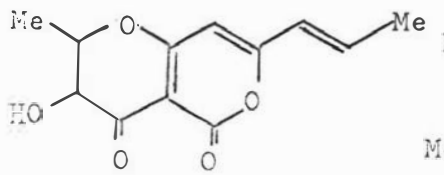

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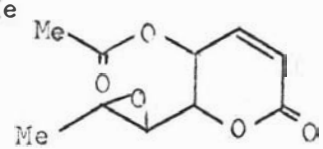

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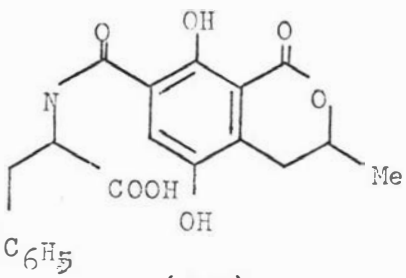

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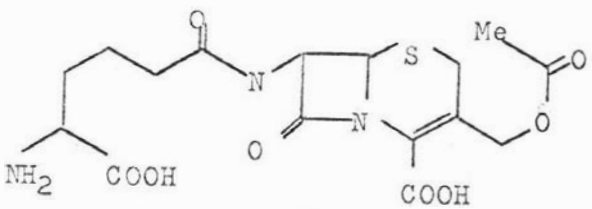

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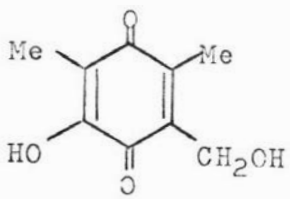

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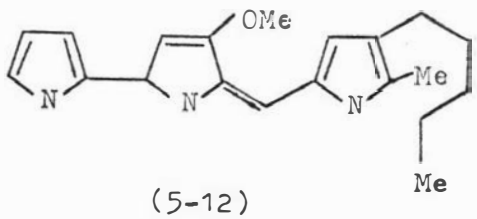

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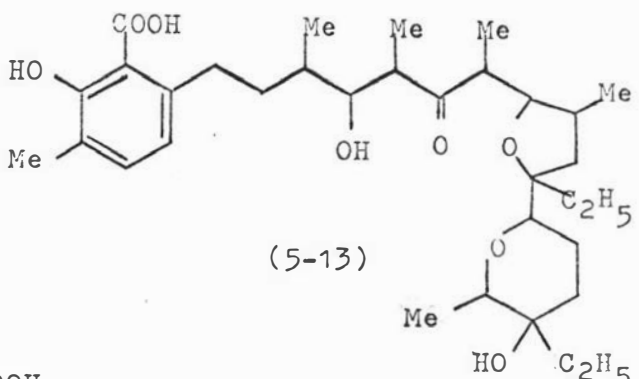


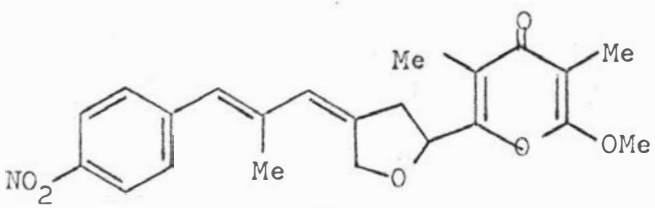
(5-13)

$CH_3(CH_2)_5CH{=}CH(CH_2)_7COOH$

(5-14)

see Scott et al., 1972a, 1972b.

(5-15)



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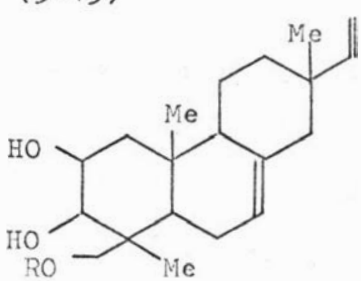
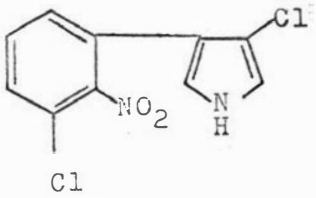


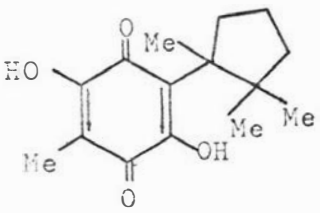
	R
(5-17a)	H
(5-17b)	β-D-altropyranosyl



(5-18)

see Battersby et al., 1972.

(5-19)



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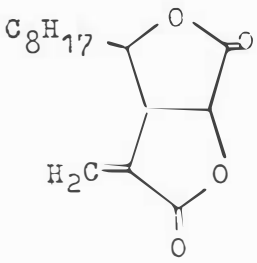

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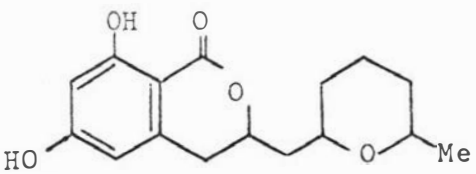

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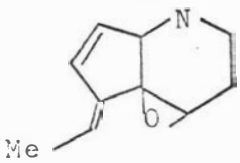
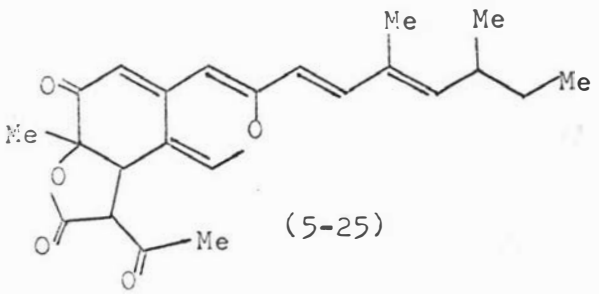


(5-23)

see Milavetz et al., 1973.

(5-24)



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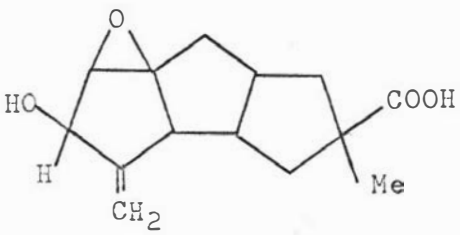

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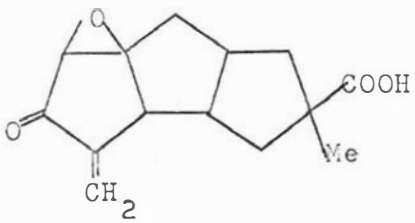

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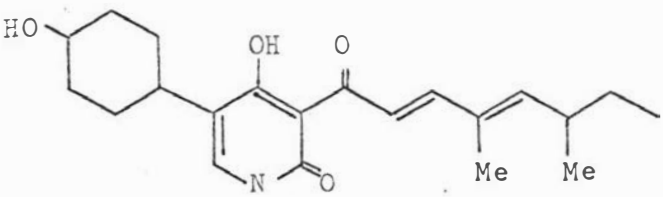

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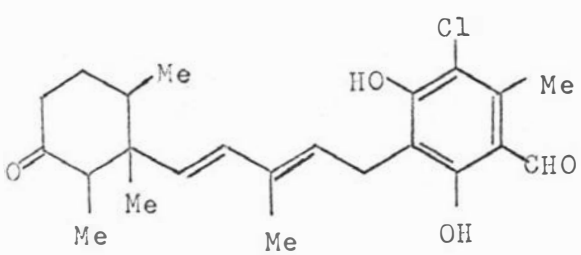

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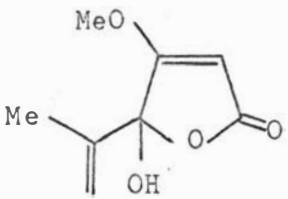

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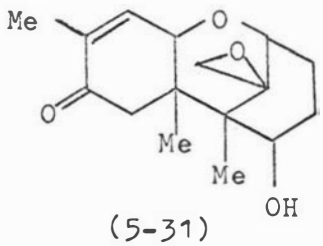

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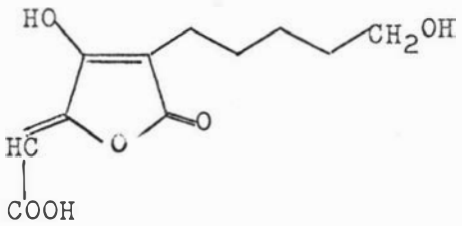

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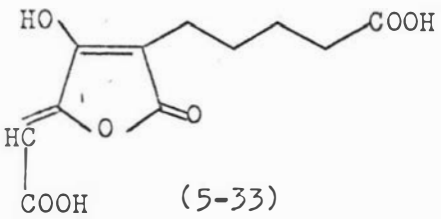

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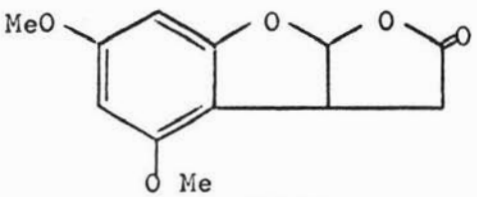
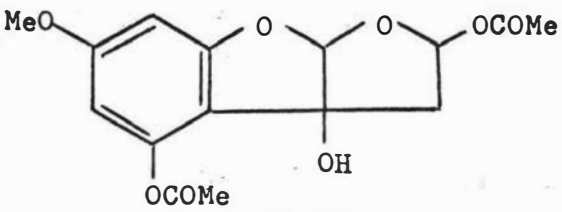


(6-1)



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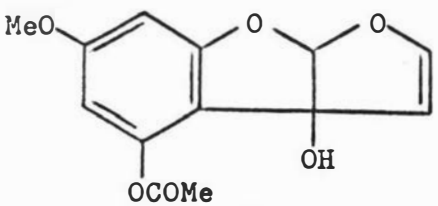

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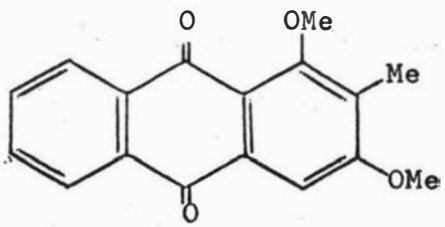

(6-4)