

STRUCTURE ELUCIDATION AND SYNTHESIS OF

DIFFERENTIATION INDUCING FACTOR (DIF)

FROM DICTYOSTELIUM DISCOIDEUM

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ABSTRACT

Structure Elucidation and Synthesis of Differentiation Inducing Factor from Dictyostelium discoideum

Developing Dictyostelium amoebae, under conditions of food shortage, aggregate by chemotaxis to cyclic-AMP, forming a papilla of cells that successively transforms into a cylindrical slug and finally into a fruiting body consisting of a cellular stalk supporting a mass of spore cells. Within the slug 'prestalk' and 'prespore' cells can be recognised, arranged in an anterior/posterior pattern with 'prestalk' cells in the anterior. It is the cell pattern formation that is of interest in Dictyostelium development. A dialysable factor termed DIF (Differentiation-Inducing Factor) was discovered that was found to induce differentiation of amoebae into stalk cells.

Large-scale productions of Dictyostelium, over a period of two years, generated less than 0.1 mg DIF from which the basic covalent structure was determined by microchemical analysis, mass spectrometry and proton n.m.r. spectroscopy, as a new compound type.

The exact chemical structure could not be determined by physico-chemical analysis due to lack of natural material and number of isomeric possibilities.

Several possible isomers were synthesised by various routes and one compound proved to be identical to the natural material with respect to its spectroscopic properties, HPLC retention times and biological activity. An active minor species of DIF was also synthesised. A bulk synthesis (50 mg) of the major active species was undertaken for further biological characterisation.

A number of structural analogues of DIF were synthesised to investigate structural requirements for a putative receptor. Deuterium labelled DIF was also generated which was found to be stable. This provides a means of synthesising radio-labelled DIF for future receptor, immunological and metabolism studies. DIF is the first endogenous morphogen to be fully characterised and this should open up the field for studying chemical signalling in embryological development.

In memory

Bob & Barbara Knight

If a man will begin with
certainties, he shall end
in doubt, but if he will
be content to begin with
doubts he shall end in
certainties.

Francis Bacon
(1561 - 1626)

The Advancement of Learning

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ABBREVIATIONS

a.m.u.	atomic mass unit
cAMP	cyclic adenosine monophosphate
D.B.E.	double bond equivalents
DCI	Desorption Chemical Ionisation
DEI	Desorption Electron Impact
3,5-DHA	3,5-dihydroxyanisole
DIF	Differentiation Inducing Factor
EI-MS	Electron Impact-Mass Spectrometry
FAB	Fast Atom Bombardment
FT-IR	Fourier Transform Infra-Red
GC-MS	Gas Chromatography Mass Spectrometry
^1H -N.M.R.	Proton Nuclear Magnetic Resonance
HPLC	High Pressure Liquid Chromatography
2,4,6 TMAP	2,4,6-Trimethoxyacetophenone

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CHAPTER I

INTRODUCTION

1.1 Biological Development

Development involves the generation of new cell types in particular spatial patterns. This often occurs in small regions of embryonic tissue, eg. limb buds, in which there is evidence for some sort of signalling between the cells. This evidence has come from experiments involving transplantation of cells to other areas of the developing embryo. The transplanted cells differentiate according to their new position. This does, however, require knowledge of embryo fate maps to predict what the tissue would have developed into if left undisturbed. It has also been shown that the transplanted cells can redirect local cells to new fates. Other evidence arises from size regulation, in which embryos of differing sizes still produce properly proportioned adults.

The chemical nature of these signals, termed 'morphogens' and the dynamics of the system are essentially unknown, although it has been suggested that low molecular weight signals may be involved. Dictyostelium is a most useful model system for studying such morphogenetic signalling. It shows many phenomena seen in the development of higher embryos including formation of patterns of cell types, size regulation and regulation after surgery.

The biological system of Dictyostelium is very simple since it contains only two cell types, not hundreds. Furthermore as the cells exist as 'free-living' embryos they are easily accessible for biological and developmental study. There is also an abundance of material available. The following work discusses the life cycle of Dictyostelium and the discovery of the morphogen DIF and its subsequent characterisation.

1.2 Historical Aspects

It was the German mycologist, O. Brefeld, who first discovered cellular slime moulds in 1869. However, it was not until 1880 that the unique nature of the asexual life cycle was understood, chiefly due to the studies of the French mycologist, P. van Tieghem (1). Perhaps the most significant work after this was that of A. Arndt. Arndt made the first time-lapse film of the slime mould Dictyostelium mucoroides and published his observations in 1937 (2).

The modern era began with the discovery of a new species of cellular slime mould, Dictyostelium discoideum by K. B. Raper in 1935 and the subsequent publication of this research in 1940 (3,4).

This modern era has had several trends. Some researchers have been concerned with the discovery and characterisation of new species, with ecological problems and with problems involving species interaction. Others have been concerned with the development of biology and the various important aspects of the biochemistry of slime mould development. The molecular approach was given a boost by the development of axenic strains, thus eliminating the confusion of the chemistry of bacterial food supply (5,6,7).

In most studies there has been a combination of research at the molecular and cellular level and have included such areas as genetics, gene action, chemotaxis, cell adhesion and different aspects of differentiation including pattern formation. It is this area of differentiation research that provides the backbone for this biochemical study.

1.3 Life Cycle of Dictyostelium discoideum

Dictyostelium discoideum has two distinct life cycles: sexual and asexual. The latter is easy to reproduce in the laboratory and has been the focus of almost all experimental studies.

One of the unusual aspects of the life cycle is that Dictyostelium discoideum feed first as separate amoebae and, after a period of starvation, enter a social, multicellular stage. In this respect they are quite different from the majority of multicellular animals and plants that start as a fertilized egg or spore and grow at the same time as they differentiate and undergo morphogenesis. In cellular slime moulds growth is completely separate from differentiation (with the exception of a few late cell divisions).

The starvation reaction of aggregation requires chemotaxis; the amoebae stream into central collection points (Fig 1). In D. discoideum this process is mediated by chemoattraction to cyclic AMP (cAMP) pulses which are relayed out from a pacemaker cell or groups of cells.

The amoebae not only increase their production of cAMP during aggregation but there is an a priori increase in cAMP receptor sites and the phosphodiesterase that converts cAMP to the chemotactically inactive 5'-AMP. There is also an increase in cell adhesiveness (8,9). The centre of the aggregate

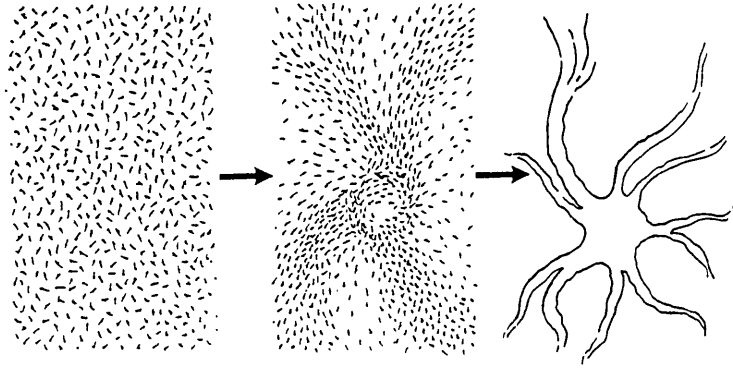


Fig. 1. Isolated amoebae aggregating in response to chemoattraction to cAMP.

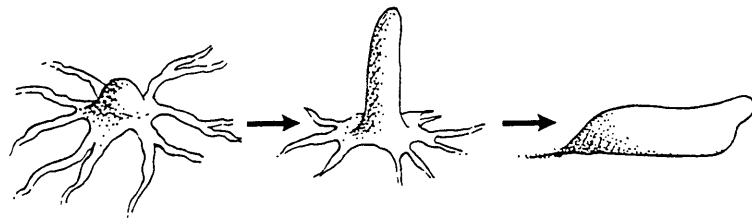


Fig. 2. Aggregated amoebae produce a migrating slug.

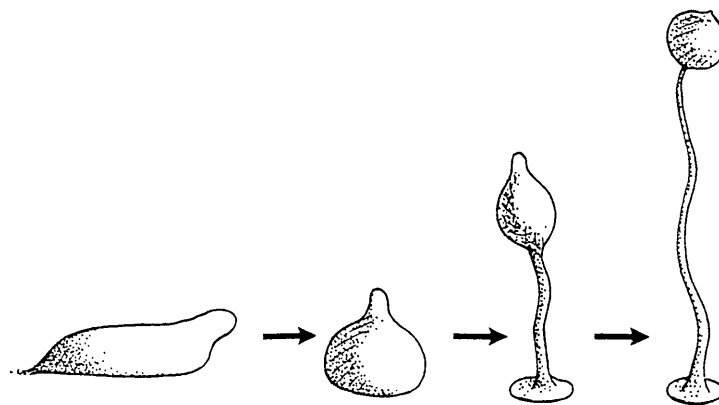


Fig. 3. Production of final fruiting body.

is at first a hemispherical mound of cells, but later, after about twelve hours, a papilla forms on top of the aggregate and by extension the aggregate transforms into an elongated cylinder, with a tapering anterior end. If the environmental conditions are favourable, the slug may construct a fruiting body in situ or assume a horizontal orientation and migrate (Fig 2). The migrating slug, motile grex or pseudoplasmodium has discernible anterior and posterior ends. As it migrates it secretes a slime sheath which is left behind as a collapsed tube.

During this period of migration, it is evident that there are two zones of cells. Initially shown by Raper (4), the anterior quarter of the slug contains cells predestined to become stalk cells. These 'prestalk' cells can be stained differentially with a number of histochemical stains, showing them to be quite distinct from the posterior three-quarters of the slug which are 'prespore' cells (10,11). These two cell types display a number of biochemical differences that anticipate their presumptive fates. Thus far, the most efficacious means of distinguishing the cell types has been the use of fluorescent-labelled antispore antibodies (12) or the use of isotope-labelled fucose which is only incorporated into the prespore cells (13).

There has been considerable interest in the environmental factors that promote migration and induce culmination or the formation of the final fruiting body. It has been shown that dessication induces fruiting (4). The presence of high concentration of solutes shortens the duration of

migration, with electrolytes being more effective than non-electrolytes (14). Overhead light was shown to be a strong stimulus for culmination (15). Finally it has shown that the presence of ammonia (which is produced by cell catabolism) at non-toxic concentrations, favours migration, while the absence of it favours fruiting (16).

At the beginning of culmination the slug tip stops forward motion and points upward, while the posterior end continues to move so that all the cells are gathered directly under the papilla. At this stage, the stalk cells begin to form near the tip at the upper end of the prestalk zone. Stalk cells are formed by a progressive vacuolization of the cells, during which time cellulose cell walls are deposited. When the differentiation is complete, the cells die (17). Potential new stalk cells arise from a band of prestalk cells around the stalk tip. These prestalk cells are oriented at right angles to their direction of upward movement and can be seen to be secreting non-starch polysaccharide at their centripetal end; they secrete the stalk cylinder itself before moving to the apex to become stalk cells (1).

Initially, the movement of cells causes the apical stalk to be pushed down through the zone of prespore cells, causing the whole cell mass to bulge laterally. Once the stalk reaches the base, a torus of cells originating from the posterior end of the previous migrating slug turns into vacuolate, stalk-like cells to form the basal disc, hence the name D. discoideum. If there is no interruption of the addition to the tip of the stalk it continues to elongate

and, in doing so, the whole prespore mass is elevated into the air. This stage is often referred to as a 'sorogen' and the mature fruiting body a 'sorocarp'. Early on in this upward voyage, the prespore mass develops into fully differentiated spores; each prespore cell condenses and forms a complex cellulose coat which is capsule-shaped. The final fruiting body has a slender, tapering stalk with a terminal, globular or lemon-shaped spore mass or sorus (Fig 3).

1.4 The Discovery of DIF

There has been great interest in the factors that induce differentiation. First there was evidence that cAMP induces stalk cell differentiation in cells that have not undergone normal development (18,19), but it was subsequently discovered that cAMP was also a factor in spore differentiation (20,21). Since then, there has been evidence for an additional factor, termed a 'morphogen', that is involved in stalk cell differentiation (22,23). Town et al (20) showed that cAMP could induce differentiation of isolated amoebae into stalk cells and that the efficiency of this conversion was enhanced by some sort of cell interaction. The two observations of the density dependence of stalk cell induction and increased conversion of single cells at high density could have two possible interpretations. Either an inducing factor was present or the induction was cell contact-dependent. To distinguish between these two possibilities, a test of synergism was set up to see if cells undergoing stalk cell differentiation at high density could enhance the frequency of stalk cell formation in a low density population, separated from them by a Cellophane membrane. The low density population had an induction frequency of 0.5%, whereas the same cells plated over a dense layer of 'helper' cells gave an induction frequency of 40 - 80% (20). This strongly suggested that cells undergoing stalk-cell differentiation at high density secrete a dialysable factor which enhances cAMP-induced differentiation in isolated cells.

The characterisation of the dialysable factor was taken further by Town and Stanford (22). The factor, named Differentiation-Inducing Factor (DIF), was obtained by spreading washed amoebae on a layer of cellophane supported over a reservoir containing a solution of Bonner's salts (pH 6.0), 1 mM cAMP, and streptomycin $100 \mu\text{g ml}^{-1}$. Another layer of cellophane was placed over the cells to produce a bilayer. The preparation was incubated in a dark, humid atmosphere at 22°C for 48 hours. DIF was collected in the salt solution. The assay was based on the ability of DIF to induce stalk cell differentiation in isolated cells. 1% agar plates were poured containing 10 mM cAMP, 5mM 4-morpholineethanesulphonic acid (MES), 2mM CaCl and $1 \mu\text{g}$ pyridoxal phosphate. Vegetative cells were plated at a density of $1.0 - 2.5 \times 10^3 \text{ cells cm}^{-2}$. Plates were incubated as mentioned above. A representative number of cells were examined by phase-contrast microscopy or fixed with osmium vapour and scored (22). The DIF assay was later modified and standardised (see methods).

Early studies showed that the stalk cell formation was absolutely dependent on exogenous cAMP. The differentiation process was inhibited by up to 50% with 50 mM KCl or 30 mM NaCl. It was found that DIF binds to unsilanized glass and to some plastics. Ultrafiltration and gel filtration were used to give an indication of molecular weight. DIF passed through Amicon UM2 filters but was retained by UM05. DIF was excluded from Sephadex G15 but penetrated G25. These results suggested a molecular weight of 1000 - 2000.

At pH 6.0 DIF bound to DEAE-cellulose, but not to CM-cellulose. DIF was found to be sensitive to a crude mixture of glycosidases, but was unaffected by micrococcal nuclease and Pronase. DIF was sensitive to mild periodate oxidation using 5 mM sodium periodate at 21⁰ C pH 5.5. The effect of temperature showed that DIF was heat-stable between pH 5 - 10 but very labile at acid pH. At pH 2 about 80% of the biological activity was destroyed in 30 mins at 95⁰ C. These observations indicated that the biological activity of DIF was due to an oligosaccharide moiety containing terminal (acid labile)'sialic acid residues. It was subsequently found that neuraminidases from Clostridium perfringens and Vibrio cholerae destroyed DIF activity. Town and Stanford made the assumption that DIF was part oligosaccharide in nature and carried out further studies to glean more information about its structure.

It was reasoned that a charged oligosaccharide would interact with a specific cell-surface receptor. Hence free saccharides were used to inhibit DIF action. Sialic acid, L-fucose and N-acetyl-D-galactosamine all showed comparative levels of inhibition. D-fucose, D-glucuronic acid, D-galacturonic acid, D-glucose, D-galactose, D-mannose, N-acetyl-D-mannosamine and N-acetyl-D-glucosamine were ineffective.

The effect of a variety of glycosidases was investigated. The inhibitory effect of the neuraminidases was presumed to be due to removal of a terminal sialic acid residue.

α -L-fucosidases from bovine epididymis and from C. lampas also showed inhibition, thus suggesting both fucose and sialic acid residues in the molecule. β -galactosidase and

α -N-acetylglucosaminidase had no effect on DIF activity. A range of proteases was used in activity experiments. Trypsin, pepsin, carboxypeptidase Y, Pronase, proteinase K and papain showed no depletion of activity. Interestingly, alkaline phosphatase did cause loss of biological activity. No inhibitory products were produced and enzymic destruction was prevented by 5 mM inorganic phosphate. The relatively high level of enzyme required to destroy DIF activity was thought to be due to the presence of phosphate, from cAMP hydrolysis, in the crude DIF solution. The interaction of DIF with agarose-coupled lectins was investigated. No interaction was exhibited with agarose or lentil lectin couple to agarose. Ricin agglutinin showed some retardation of DIF on elution through the agarose column. Soybean lectin bound about half the activity but could be eluted with N-acetylgalactosamine. A similar result was found with the fucose-binding protein from Lotus. The affinity of DIF for a sialic acid-binding lectin from Limulus, ie. limulin, was investigated in solution as the lectin was not available in an immobilised form. DIF was preincubated with the lectin prior to assay. Limulin inhibited 90% of the biological activity. The lectin did not have any differentiation-inducing activity. It was therefore concluded that a specific lectin-DIF interaction reduces free DIF levels and hence stalk cell differentiation. However, it was borne in mind that the lectin may be reacting directly with the cells and thus reducing stalk cell differentiation by some other mechanism.

The fact that DIF is found in the aqueous dialysate implies it has some hydrophilic moiety on the aglycone part of the molecule. However, its interaction with glass and plastic surfaces and its retardation during chromatography on Bio-Gel P2 suggests hydrophobic moieties. It was further found that the activity could be extracted from dried preparations with acetone or chloroform/methanol (2 : 1).

The experimental approaches in this Town and Stanford study consistently suggested the presence of saccharide moieties in DIF, more specifically, essential sialyl, L-fucosyl and N-acetyl-galactosamine residues. The requirement of a phosphate group for DIF activity was interesting, because the involvement of sugar phosphate residues in other cell-macromolecule recognition processes had recently been reported (24,25). Although the evidence for the presence of three sugars in DIF was very persuasive, it was indirect. Definitive proof of the presence of these sugars had to await purification and further chemical studies.

Gross et al, in 1981, suggested that DIF was a non-polar molecule (23). They showed that DIF was readily extracted from aqueous solution into petroleum ether. Their view was further supported by the behaviour of DIF during chromatography on silica gel. It was found that DIF migrated with R_f values similar to those of neutral lipids. This hydrophobic nature of DIF cast some doubts on the conclusions of Town and Stanford that the molecule contained phosphate and

sialic residues both of which would be expected to confer charge on it. High pressure liquid chromatography was used to purify ether extracted material and a single peak was obtained which implied that DIF was a single molecular species or a number of closely related species.

Gross et al went on to question the role of DIF and proffered the idea that it functions as an activator of prestalk cell formation in a patterning mechanism of the kind proposed by Wolpert et al (26).

Thus it was expected that DIF would accumulate at the time when the pattern was established. This was found to be the case.

It was found that charting the development of cells of strain V12M2 on agar, they completed aggregation after about nine hours. The tip formation occurred between nine and twelve hours and the formation of the sorocarp was completed within twenty-four hours. It was shown that cell-associated DIF accumulated between nine and twelve hours, with similar kinetics to the prespore-specific enzyme UDP-galactose polysaccharide transferase. Thereafter the level of DIF remained fairly constant during slug migration and fruiting.

The concept of DIF being an activator of prestalk cell formation had other consequences. It predicted that DIF levels should be higher in the anterior, prestalk, zone than in the posterior, prespore, zone. Furthermore, DIF should be requisite for the formation of prestalk cells not just for the production

of mature stalk cells. Thirdly, DIF should not be required for the pathway of spore formation and ought to have a role as an inhibitor of this pathway. Indeed it was shown that addition of small amounts of DIF-containing extracts inhibited both prespore and spore cell formation (23).

Kay et al showed that it was possible to obtain efficient spore or prespore cell differentiation in dilute cell populations (23,27), which negated the idea that spore formation required an unknown inducer or cell contact in addition to cAMP. Kay further suggested that cAMP was the only inducer required for spore formation. However, as it had previously been shown that stalk cells were induced by cAMP and DIF, it could be concluded that cAMP does not discriminate between stalk and spore differentiation. Therefore DIF is the only pathway-specific inducer active in sporogenous strains. He therefore proposed that DIF by favouring the stalk pathway, controlled the choice between stalk and spore cell formation.

It was shown that isolated cells of the sporogenous strains HM18 and HM29 were directed from spore to stalk formation by partially purified DIF preparations in the presence of cAMP.

Brookman et al (28) showed that the sensitivity of the DIF assay could be increased at least four-fold by modifying the salt solution and submerging the cells directly onto the surface of tissue culture dishes.

At this time, a unit of DIF activity was defined as that amount which induces the formation of 1% stalk cells.

The same group investigated the levels of DIF in aggregation-defective mutants, HM8 and HM11. There was initially a small amount of DIF but no subsequent increase in the DIF level of the kind observed in the wildtype strain.

This suggested that DIF production was a post-aggregative event. They further showed that, in fast-shaken cell suspensions, extracellular cAMP strongly stimulated DIF production as well as the accumulation of other post-aggregative products (29-31). However, it was not determined whether cAMP induces DIF by activating a pre-existing enzyme or by stimulating the synthesis of intermediate enzymes involved in DIF synthesis.

Kay and Jermyn had managed to further purify DIF by using two HPLC steps and stated that it had a molecular weight of less than 500 and was active at concentrations of less than 10^{-10} M (32). This purer DIF was shown to suppress spore formation in sporogenous amoebae and redirected the cells to stalk cell formation, although it was not clear when this step might occur during normal spore differentiation (33,36). It was suggested that if DIF is to control the differentiation of prestalk and prespore cells, it ought to directly inhibit prespore cell formation. Prespore cells could be recognised by using a specific antibody (35,36) and it was shown that DIF completely inhibits prespore cell differentiation when present from the time of plating.

Kay and Jermyn also investigated the role of DIF within a morphogenetic field. This is a small area of embryonic tissue in which there is social action between cells. The morphogenetic field is capable of reorganising itself into a properly proportioned miniature when some part is removed by surgery. Dictyostelium exhibits this capacity to a remarkable degree and it has long been known that prespore isolates can regulate to reform a new prestalk region (4). When this occurs, a proportion of the prespore cells re-differentiate into prestalk and, ultimately, into stalk cells (35,36). Thus, to investigate whether DIF was capable of inducing a re-differentiation, slugs were disaggregated after eighteen hours of development and incubated as separated cells in the presence of cAMP. Under these conditions, 50-60% of cells stained as prespores, but on addition of DIF, most cells were redirected to stalk formation and a rapid loss of prespore staining was observed (32).

It has also been suggested that ammonia has a significant role in the formation of stalk and spore cells. Ammonia accumulates during development and has been shown to have a delayed effect on the transition from the motile grex to culmination (16). Evidence that ammonia plays an important role comes from the study of the mutant strain KY3, isolated originally by Yanagisawa (37). This mutant forms slugs that fail to culminate under normal circumstances. The phenotype appears to result from a high sensitivity to ammonia. Sussman and co-workers have proposed that ammonia, acting antagonistically with cAMP, controls all morphogenetic movements after aggregation. They further suggested that the

interplay of ammonia and cAMP controls the timing of terminal differentiation and that the spatial distribution of cAMP and ammonia in the slug may control the choice of the slug cells between entering the prestalk or prespore pathway of differentiation (38,39).

As previously mentioned, there is little to support the notion that cAMP is pathway-specific (27). The evidence for a pathway-specific effect of ammonia is equivocal. Sternfeld and David studied the role of ammonia in the differentiation of submerged clumps of cells (40). Previously, Sternfeld and Bonner had shown that mature spores and stalks would differentiate in submerged cultures, but only in an atmosphere of pure oxygen. The latter study found that the density dependence for differentiation could be circumvented by using a conditioned medium, which was shown to contain ammonia and another factor. Ammonia was required for differentiation but resulted in both spore and stalk cells. In the absence of an atmosphere of pure oxygen, ammonia was found to antagonise nonspecifically the induction of differentiation by cAMP in submerged cultures (42). However, Gross et al (23) found that 2 - 5 mM ammonia shifted the spore:stalk ratio of sporogenous mutants in favour of spore differentiation. Ammonia also appears to be one of the requirements for spore formation in a mutant exhibiting a decreased requirement for cell contact during differentiation (43). Thus the evidence for a role of ammonia in directing cells towards spore differentiation is strongly suggestive but far from conclusive. Furthermore, considering

the lack of specificity of cAMP in inducing later differentiation, it is highly likely that ammonia is exerting its effects through blocking cAMP production, was originally suggested by Schindler and Sussman (16,44).

Gross et al provided evidence that ammonia was a second morphogen that acted antagonistically to DIF and that the choice of differentiation pathway was a consequence of intracellular pH (45). Amoebae of strain V12M2 and its sporogenous mutant HM18 were fed aliquots of conditioned medium free of DIF and were incubated at densities where they would produce enough DIF to form substantial numbers of stalk cells. No inhibition of stalk cell formation was detected at pH 6.2, but strong inhibition occurred at pH 7.5. In HM18 spore formation was stimulated concomitant with the stalk cell inhibition. Their interpretation of the pH dependence of inhibition was that the inhibitory substance is the neutral form of a weak base. Ammonia was a likely candidate, since large amounts are released by the developing amoebae (46). In addition, inhibitory activity co-distilled with ammonia at pH 5.0 and pH 10.0 and was destroyed by incubation with glutamate dehydrogenase mix of enzymes for which ammonia is a substrate (16).

Ammonia and other weak bases are thought to influence various biological processes by diffusing into the cell where they become protonated and cause a rise in the intracellular pH (47).

It appeared that the choice of differentiation pathway in Dictyostelium depended on intracellular pH; a high pH (induced by ammonia) inhibited stalk and enhanced spore formation and low pH (probably mediated by DIF) had the reverse effect. Other weak bases such as trimethylamine, methylamine, imidazole and procaine mimicked the effect of ammonia and weak acids had the opposite effect. Exposure of Hm18 amoebae to increasing concentrations of propanoate at pH 5.0 caused progressive diversion from spore to stalk pathway. Acetate, butanoate and methylpropanoate were also effective. Reduction in the extracellular pH shifted the pattern of differentiation toward stalk formation.

The fact that DIF has such a high potency, ie. it is effective at very low concentrations, implies that it does not simply act as a weak acid. A fashionable thought is that it acts as an inhibitor of proton export from the cell. Proton transport out of the cell usually occurs by electroneutral change with other monovalent cations (48) or by an energy-dependent electrogenic process (49). Plasma membrane ATP-dependent proton pumps have been demonstrated in fungi (50) (including Physarum, an acellular slime mould), as well as in algae and higher plants (51). The synthetic oestrogen, diethylstilbestrol, has been shown to be a potent inhibitor of these plasma membrane H^+ -ATPases (52).

Gross et al tested the effect of diethylstilbestrol on stalk and spore cell differentiation (45). They found that very low concentrations could divert cells from the spore to the stalk pathway. It also mimicked the action of DIF by inducing stalk cell formation when added to isolated cells

of the V12M2 strain. A second proton pump inhibitor, zearalenone, a naturally-occurring oestrogen (53), also mimicked the actions of DIF. These results supported the idea that DIF acted by lowering the intracellular pH by inhibiting an ATP-dependent proton pump.

Until 1983 all studies with DIF had been with rather crude preparations. Kay et al attempted to purify DIF further in sufficient quantities for both biological experiments and sensitive physical techniques.

To produce more DIF it was necessary to scale up production. Strain V12M2 was replaced by Ax2 which was grown axenically in 40 litre fermentors or 20 litre aspirators. At high cell densities DIF production was slow, so incubation was continued for 3 - 5 days after the cells were plated in order to obtain maximum yields of DIF. The released DIF could be readily adsorbed by the non-polar resin XAD-2 which was thenceforth included in the incubation medium. The DIF could be easily eluted from the resin with ethanol. The yield from this procedure was about 50 - 100 units/ 10^6 cells.

Kay et al found that DIF could be extracted from water with hexane at neutral or acidic pH, but not at very alkaline pH. This suggested that DIF was a non-polar material that became charged at alkaline pH.

This behaviour of DIF was exploited in its purification by extracting first with hexane at alkaline pH, to remove basic and neutral lipids, and then at neutral pH to extract DIF.

After three HPLC steps, DIF was purified some 2000-fold over the XAD-2 eluate with an apparent recovery of about 2% (54).

Previous purifications using reverse-phase HPLC had resolved DIF as a single, major activity, DIF-1 (23). The largest scale production revealed the existence of four minor species with bio-activity, DIF 2 - 5. Between them they constituted about 4% of the activity recovered from the first HPLC step. Further HPLC of these minor species confirmed them as distinct from DIF-1 (Table 1). All minor activities were more polar than DIF-1.

Kay et al attempted to characterise DIF-1 by looking at some physical and chemical properties after discussions in these laboratories.

Earlier experiments had suggested that DIF had a molecular weight of 1000 - 2000 (22). Gel permeation HPLC using a styrene/divinylbenzene matrix and eluting with tetrahydrofuran showed that DIF eluted after such steroids as progesterone and estradiol, indicating a molecular weight of less than 500.

	Relative recovery from HPLC-1	Retention time HPLC-1	Retention time HPLC-2	Specific activity (estimate) U/mg $\times 10^6$	Sensitivity to borohydride % activity remaining
DIF-1	95.8	96	34	120	5
DIF-2	2.7	63.5	27.5	2.4	1
DIF-3	1.4	51	24.3	0.2	11
DIF-4	1.4	51	20.3	0.02	120
DIF-5	0.1	30	13.5	n.d	52

Table 1. Comparison of some chemical and biochemical parameters of the major DIF species and minor DIF species.

Various chemical treatments (summarised in Table 2) showed that DIF-1 was stable to both acid and alkali which probably precluded the presence of a carboxylic acid or an activated amine (55). Sodium borohydride treatment destroyed activity, which is strongly suggestive of a carbonyl group (56). Base-catalysed acetylation with pyridine/acetic anhydride destroyed some activity. This could have indicated the presence of a hydroxyl group or it could be due to the enolisation and reaction of the carbonyl group. The activities of DIF-2 and DIF-3 were also destroyed by borohydride. DIF-4 was resistant to borohydride and DIF-5 was partially resistant.

Town and Stanford (22) had argued that DIF contained a sialic acid and a fucose residue. However, the extraction of DIF into hexane would be unusual for a molecule containing a high proportion of sugar. Kay et al confirmed that periodate oxidation destroys some biological activity but the mild conditions, which should have been sufficient to destroy the sialic acid residue, had only a minor effect. In addition, DIF-1 was found to be completely resistant to fucosidase and neuraminidase under conditions similar to those previously reported to destroy it. These experiments argue against DIF having essential sugar residues in its structure.

The work that follows in this thesis explains how the structure DIF was determined and the chemical characterisation of DIF.

Treatment	Reacts with	Activity Remaining %
1M KOH		90
1M HCl		98
CH ₃ OH/HCl	-COOH	59
CH ₃ OH/BF ₃	-COOH	92
CH ₃ OH/AC ₂ O	-C(NH ₂)COOH	101
Pyridine/AC ₂ O	-C-OH	26
Borohydride	C=O	5
Periodate (mild)	Sialic acid	83
Periodate (harsh)	many	17

Table 2. Effects of chemical treatments on DIF-1 biological activity.
(Reproduced from Ref. 54 by permission).

CHAPTER II

INTRODUCTION

Aspects of Structure Elucidation

2.1 Introduction to structure elucidation

Such is the inquisitiveness of the human mind that no natural phenomenon may escape scrutiny and subsequent investigation. Whether it is the motions of the stars or the force of gravity or self-replicating deoxyribonucleic acid, 'Cause and Effect' must be the most fundamental 'raison d'être' for the scientist.

Man's quest for knowledge has found a niche in biochemistry: the chemistry of life. The ultimate goal would therefore be the total characterisation of all the molecules involved in living systems. To realise this goal scientists have invented and developed sophisticated tools and machines that will help them ultimately elucidate the structure of all biomolecules.

Twenty-five years ago, the characterisation of one bioactive molecule, its precursors and metabolites would have been a lifetime's research, a magnum opus. Today, as instrumentation is evolving with protean rapidity, it is possible to accomplish this and more in a few years.

The late 60's and 70's were the halcyon days as far as structure elucidation was concerned. Today the discovery of a novel molecule is a relatively scarce occurrence. Many of the recent structure elucidations have centred on unusual gene products, sequencing biopolymers or studying proteins with considerable homology to already-characterised molecules. The structure elucidation of alkaloids and secondary metabolites provides the greatest chemical variety and perhaps the greatest demand on the scientist's resources, intuition, creativity of thought and logical rationalisation and interpretation of data.

Structure elucidation is the supreme mental challenge for the chemist and biochemist.

The structure elucidation of bio-organic molecules falls into three areas. One area is that of X-ray crystallography; this technique requires single crystals and therefore, relatively large, in biological terms, quantities of substances of the order of milligrammes or more. The suitability of this technique for the structure elucidation of biomolecules will be discussed later.

The second area is that of solution methods to determine the structure of biopolymers, such as DNA, RNA and proteins. Very sensitive and rapid methods have been developed to determine the sequence of bases (in DNA and RNA) and amino acids (in peptides and proteins). However, these methods only determine the primary structure, although in a limited number of cases the secondary and tertiary structure has been determined by X-ray crystallography.

The third area encompasses all compounds that are either not standard biopolymers or suitable crystals cannot be obtained. This includes not only those compounds, which no matter how much is available, refuse to crystallise, but also those cases where crystallisation is effectively impossible because of a limited supply of the substance. This area includes many physiologically important compounds such as hormones, vitamins, neuropeptides, toxins, antibiotics and secondary metabolites. Twenty-five years ago such problems would have required tedious and demanding chemical degradation, and most could not be tackled at all. Recently a number of powerful methods for their solution have evolved most notably mass spectrometry and nuclear magnetic resonance spectroscopy. Together with ultra-violet spectroscopy and infrared spectroscopy the chemist and biochemist has sufficient analytical artillery to elucidate the structure of an unknown biomolecule; the major proviso being that there is enough pure material for adequate analyses. Consequently, it is of paramount importance that a carefully thought out strategy is adopted, ie. non-destructive analyses are performed before destructive or semi-destructive ones, and following non-destructive analysis the sample must be prudently partitioned between a variety of 'destructive' experiments in order to maximise the information returns.

The following dissertation is intended as a discussion based upon experience gained in this research of the usefulness and shortcomings of these four techniques and how to make most judicious use of them in structure elucidation.

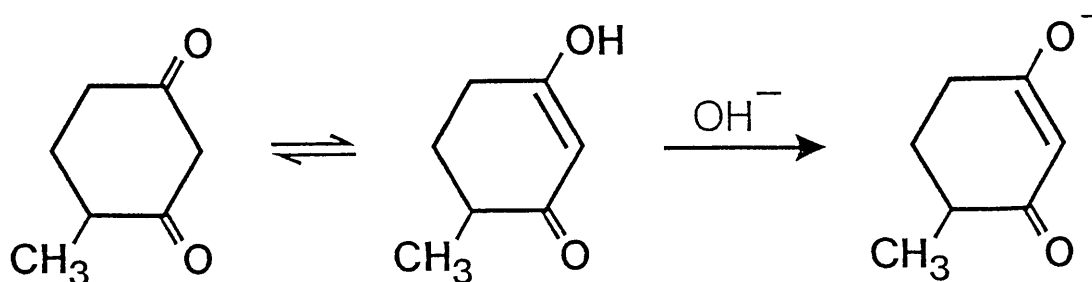
2.2 Ultra-violet spectroscopy

Electronic absorption spectroscopy in ipso rarely, if ever, could provide a complete solution to a structure elucidation problem. It is, however, an essential adjunct to other forms of physical measurement and to chemical studies.

A U.V. scan of the material under study is usually a useful place to commence especially if the molecular formula has been determined in a structure elucidation problem. Firstly the technique is non-destructive, which is obviously helpful if there is a very limited amount of material available. Secondly the technique is relatively sensitive, although this is a function of the chromophore and extinction coefficient. It is usually necessary to have pure material. This helps to avoid misinterpretation of the spectrum, as even minor contaminants may contain chromophores with a high ϵ thus producing spurious absorbance bands or even masking bands produced by the material itself.

Various generalizations can be made about the shape of the absorption curves; intense absorption in the range 200-300 nm ($\epsilon = 10,000-20,000$) suggests the conjugation of at least two chromophores, which may or may not be the same. Intense absorption beyond 300 nm suggests further extension of conjugation. Very low-intensity bands in the region 270-350 nm ($\epsilon = 10-200$) are likely to arise from $\pi-\pi^*$ transition of the carbonyl function. The presence of medium-intensity bands at 210-300 nm ($\epsilon = 5,000-16,000$) commonly indicates aromatic derivatives with polar substitution. Aromatic compounds

possessing several substituents with mobile electrons, or a group which considerably extends the conjugation (an auxochrome) may give rise to electron transfer bands at higher wavelengths and enhanced intensity. Sometimes, as in the case of poly-ynes, the shape of the absorption curve is so characteristic that electronic spectral studies can be used as a diagnostic even when studying very small amounts of material. In certain chromophoric systems, the application of various criteria may eliminate some structures from the possible alternatives. Some compounds show variation in structure in different solvents and those with ionisable groups are affected by change in pH. Such changes are often manifested by alterations in the shape of the absorption spectral curve. Cyclic β -diketones exist almost entirely in the enol form in polar solvents, while in non-polar solvents the diketo form prevails. 4-Methyl-1,3-cyclohexanedione shows in cyclohexane a low-intensity spectrum expected from a simple β -dicarbonyl compound. In ethanol the spectrum is that of an enone with a β -hydroxyl substituent $\lambda_{\text{max}}^{255}$ ($\epsilon = 12,600$). In basic solution the enolic form may ionise to the enolate anion which absorbs near 280 nm ($\epsilon = 20,000$). (Scheme 1).



Scheme 1

Such environmental dependence may sometimes provide diagnostic evidence leading to the recognition of particular functional groups such as phenols or aromatic amino substituents. Knowledge of the molecular formula from mass spectrometry or combustion analysis can make the interpretation of the U.V. spectrum of an unknown less complicated. From the molecular formula the number of double bond equivalents (D.B.E.) can be calculated (see later) which is useful in assigning general structures to unknowns, eg. if there are less than four D.B.E.'s the unknown will not contain a benzene ring.

Hence, U.V. gives a superficial inkling as to which functional groups may be present in terms of conjugation. It is then necessary to turn to more specific techniques for a more definitive analysis of the molecular structure.

2.3 Infrared spectroscopy

For the chemist or biochemist the greatest value of infrared spectroscopy has been the contribution to the elucidation of molecular structure, particularly in the recognition of functional groups and their environment which may not be readily visible in the ultraviolet. The advent of nuclear magnetic resonance spectroscopy has made the study of alkyl, aryl and other hydrogen-containing moieties less dependent on infrared absorption, but there are often instances when both techniques are of equal value.

The infrared spectrum, especially the 'fingerprint' region, is perhaps most efficacious for establishing identity between an organic substance and a compound of known structure, by empirical comparison of the traces.

Fortunately, most carbon-carbon skeletal vibrations have frequencies under 1300 cm^{-1} . Consequently, the higher frequency vibrations of the substituent groups will tend to be localised or isolated and not be unduly affected by the rest of the molecule. Their absorption bands are thus diagnostic for the presence or absence of the group. Generally, X-H stretching and multiple-bond stretching vibrations are effectively localised and absorb in what is termed the 'group frequency region' ($4,000\text{--}1,350\text{ cm}^{-1}$). This is most reliable for spectra-structure correlations. Below about $1,350\text{ cm}^{-1}$ the occurrence of complex and less easily predictable interactions between various low frequency vibrations produces an absorption pattern or fingerprint more characteristic of the molecule as a whole than of its individual parts.

Infrared data on band assignment is normally given in the form of correlation charts and correlation tables. These charts enable all structural units to be listed which are likely to be responsible for the absorption observed at a particular frequency. The list would be reduced by considering other data available on the compound, for example, its origin, its elemental composition and any alternative spectral evidence. The tables are then consulted, these give other important frequencies of structural units occurring elsewhere in the infrared region. A search at such frequencies in the spectrum will usually either support or eliminate a molecular feature short-listed from the correlation charts.

More often than not the sampling method is selected for its convenience rather than its suitability. Yet the extra time and effort involved in preparing and using a solution instead of, say, a Nujol mull can easily be regained in the greater ease and certainty of spectral interpretation. Powdered solids scatter too much radiation, especially at high infrared frequencies, to give good spectra. Only when the air spaces between particles are replaced by a medium of more comparable refractive index may a solid, in general, be used. Medicinal liquid paraffin (Nujol) is suitable, a smooth homogenous paste is made and termed a Nujol mull. No attempt is made at the reference beam to compensate for the C-H bands of the paraffin. If these interfere with the interpretation, a halogenated medium, eg. hexachloro-1,3-butadiene will leave the C-H region clear.

An alternative answer to this problem of scattering is to disperse the solid in a matrix of pure, dry alkali halide such as potassium bromide, compressed into a disc. This is usually effected by loading the sample onto the KBr in a volatile solvent such as chloroform or diethyl ether. The solvent evaporates quickly leaving the sample microcrystallised within or in the surface of the matrix. The major practical difficulty is the extreme affinity of the powdered halide for atmospheric moisture. Misleading hydroxyl bands are usually visible in disc spectra at 3,450 and 1,640 cm^{-1} .

Samples that are liquid can be examined as a thin film between two polished rocksalt windows. Solutions of solids or liquids in organic solvents are contained in sealed cells of known pathlength, compensated against pure solvent in the reference beam. Dead regions of solvent absorption are avoided by minimizing the pathlength, eg. about 0.1 mm. Non-polar solvents of simple structure are preferred to minimize association effects and interfering bands.

The advent of Fourier transform infrared spectroscopy has made far infrared more accessible and has considerably speeded up and improved the sensitivity of spectroscopy in the infrared region in general. However, as was the experience with DIF in this thesis, sensitivity of FT-IR is limited and problems associated with sample impurities and even sample recovery limit the efficacy of the method.

The great advantage of FT spectroscopy is speed. Since the whole spectrum is contained in an interferogram which is recorded within one second, this is effectively the scan time. Even adding the computing and plotting time, the overall time to obtain a spectrum is very short compared to the 10 minutes or so required by conventional methods to obtain similar resolution. Essentially to achieve a given resolution each element or observation point, of the spectrum must be examined for a given time; in the conventional method each element is examined consecutively, whereas in the interferometer all the elements are examined simultaneously and later sorted out by computation.

In conventional instruments the radiation is usually brought to a focus on a slit, and it is essentially the image of the slit that is seen by the detector; a very fine slit gives good resolving power since only a narrow spread of frequencies fall on the detector at any moment, but the total energy passing through the instrument is severely limited, requiring high-gain and 'noisy' amplifiers. In FT work, parallel beams are used throughout and there is no need to focus the radiation. No slit is required and all source energy passes through the instrument consequently amplifiers are less critical, and the resolving power is governed solely by the mirror transverse and computer capacity. The availability of a computer for FT-IR analysis means that several scans of the sample may be added into the computer memory to improve signal-to-noise ratio. The spectrum output may be modified to suit the user by removing solvent peaks, correcting baseline drift, expanding parts of the spectrum, etc. Perhaps, more importantly, this

manifests itself in enhancing sensitivity. Conventional IR methods, in practice, require milligrams of material for useful spectra whereas FT-IR can routinely be performed on samples of a few hundred micrograms. This is obviously important when considering the structure elucidation of natural products where, more often than not, only microgramme quantities are available. It must be further borne in mind when planning a protocol for structure elucidation that IR can be destructive, ie. it may not be possible to retrieve the sample from a mull or KBr disc without gross contamination. Thus, non-destructive nuclear magnetic resonance spectroscopy may be of more value in many circumstances.

2.4 Nuclear magnetic resonance spectroscopy

2.4.1. ^1H -n.m.r.

The elucidation of the structure of organic compounds is perhaps the most spectacular application of nuclear magnetic resonance spectroscopy (n.m.r.), since its availability to organic chemists from the mid 1960's. As previously mentioned, in ultraviolet spectroscopy, the information is usually obtained about one or two structural features of a complex molecule (eg. chromophoric groups). In infrared spectroscopy all parts of the molecule contribute to give a complex spectrum. N.m.r. spectroscopy lies between these two extremes; many more structural features contribute to the spectrum than to a U.V. spectrum, but it is usually less complex than an IR spectrum. There is a reasonable likelihood that assignments can be made to most, if not all, bands in a pure compound. The approach to the interpretation of these three types of spectra is empirical; extensive use is made of correlation tables and collections of spectra of known compounds.

For proton spectra the solvent used should not contain hydrogen. Ideally, carbon tetrachloride would be used but many compounds are insoluble in this solvent, so deuterated solvents such as CDCl_3 , D_2O , CD_3COCD_3 , CD_3SOCD_3 , etc. are used.

The magnetic field experienced by the nucleus is not usually identical to that applied. This arises because the electrons magnetically screen the nucleus. The screening effect bears a relationship to the chemical environment of the magnetic

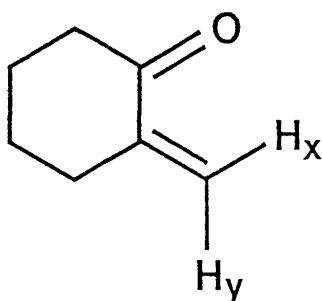
nucleus, and to the type of bonding involved. Hence, chemically different nuclei of hydrogen, for example, experience slightly different values of the magnetic field and consequently absorb at slightly different frequencies.

Modern instruments possess an electronic integrating facility, which enables the areas to be measured and presented as a series of plateaux on the same chart as the absorption curves. The height of a plateau, measured in millimetres, is proportional to the area under the curve and hence to the number of protons causing the absorption. The characteristic positions of the bands in an n.m.r. spectrum are referred to as chemical shifts.

There are several factors that will affect chemical shift values and these can be useful in interpreting spectra.

Electron-withdrawing groups attached to the molecular system have an inductive effect so de-shield the nucleus, while electron-repelling groups cause shielding of the nucleus.

There are effects due to magnetic anisotropy. With π -electron compounds, an element of symmetry is lacking. A neighbouring nucleus, which may not be directly chemically bonded to the π -system, may be shielded or de-shielded, depending only upon its orientation in space with respect to the π -bond system, eg. in the compound below, the proton labelled H_x absorbs at a lower field than the otherwise very similar proton H_y since the former lies in the deshielding zone



of the carbonyl group. It is worth noting that inductive effects are experienced only through chemical bonds whereas the consequences of magnetic anisotropy can be detected across space within the molecular framework.

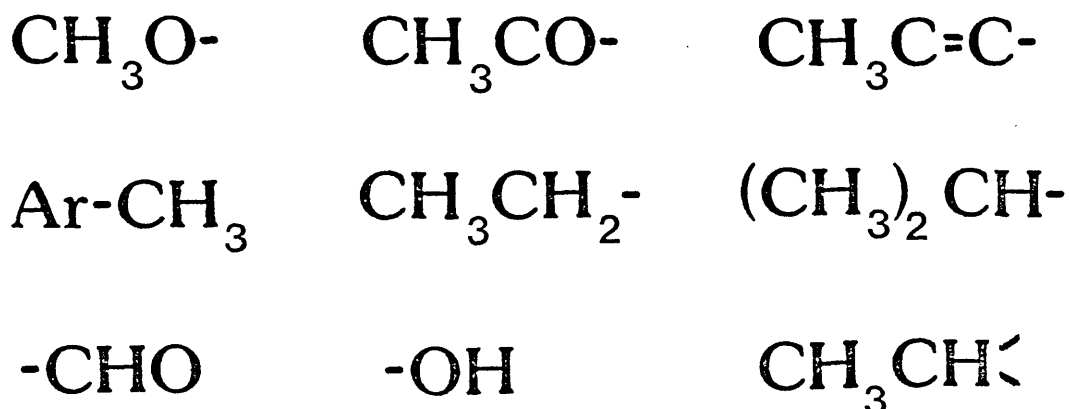
In dilute solution (0.05-0.5M), in CCl_4 or CDCl_3 , the chemical shifts of protons attached to carbon atoms are independent of concentration and temperature variations. In stark contrast protons of $-\text{OH}$, $>\text{NH}$ and $-\text{SH}$ groups exhibit a substantial concentration and temperature effect due to hydrogen bonding; intramolecular hydrogen bonds are less affected than intermolecular hydrogen bonds by changes in concentration. These temperature and concentration effects had considerable bearing on the determination of functional groups present in the DIF structure. The signal due to protons attached to electronegative atoms of this type are most easily detected by deuterium exchange. The solution of the compound is shaken for a few minutes with D_2O , and the spectrum redetermined. The proton signals disappear since absorptions due to deuterium are not detectable with the magnetic field and oscillator parameters appropriate for proton spectra. The deuterated sample may, however, exhibit an extra resonance at δ 5 due

to HDO. All types of proton absorptions may be sensitive to solvent changes and such shifts can be very useful in spectral interpretations.

Aromatic compounds account for a high proportion of products met in the field of structure elucidation. It is often possible to deduce the aromatic substitution pattern by a casual inspection of the aromatic region of the spectrum although this was not possible in the case of DIF. Various patterns are encountered with mono-, di-, tri-, etc. substituted benzenes and these are well categorised in basic texts on n.m.r. or organic molecules.

A major advantage of the technique over some other analytical methods is that the sample under investigation is chemically unaffected, although it may be lost by adsorption on glass surfaces when small quantities are studied. (This is discussed later in 'Planning a Strategy').

Proton n.m.r. has several applications within the sphere of structure elucidation. The most obvious and widely exploited application is that of functional group analysis. The following can be easily recognised, given a reasonable amount of a single pure compound ($\approx 200 \mu\text{g}$) for ^1H -n.m.r. :



Less common applications are those of molecular weight determination, quantitative analysis and rate processes. As previously stated, the amount of material available for analysis is a major criterion. Ideally, for a clear n.m.r. spectrum at least 0.25 mg is required although weak spectra can be obtained from 0.1 mg of sample but require several days of scanning. If very small amounts of material are available it is often necessary to use extremely pure solvents, ie. it may be necessary to re-distill the solvents before use. Also, as in the case of the DIF, spectrum acquisition must be made over 6-24 hours rather than a few minutes.

Two-dimensional (2-D) n.m.r. is attracting increasing interest. It is becoming a powerful tool in structure determination. However, it is still rather complicated and time consuming to record the spectra. 2-D-n.m.r. is therefore used in structure determination for very complex structures or to resolve complicated spectra where reasonable amounts of sample and equipment time are available.

2.4.2. ^{13}C -n.m.r.

The isotopic abundance of ^{13}C is only 1.11% and the nucleus has a relatively small intrinsic magnetic moment. At 2.3 T, for example, the resonance frequency of the ^{13}C nucleus is 25 MHz, whereas for ^1H it is 100 MHz. Thus the sensitivity of a ^{13}C measurement is approximately 10^{-4} of that for a proton. Notwithstanding these difficulties, advances in instrument design allied with noise decoupling and pulse Fourier Transform methods now allow routine observations of ^{13}C magnetic resonance spectra.

The major advantage of ^{13}C over ^1H magnetic resonance spectroscopy is the increased separation of chemical shift signals. This improvement is a powerful aid to structural analysis, however, as in other forms of n.m.r. spectroscopy, chemical shift data are best acquired by observing model systems rather than through theoretical considerations.

In ^1H -n.m.r. spectroscopy signal intensities are normally presented in the form of an integrated trace superimposed over the spectrum, hence the number of protons per signal can be deduced. Correlations between relative peak intensities in ^{13}C n.m.r. spectra are not so reliable, particularly where quaternary carbon atoms are concerned, and the data is displayed in numerical rather than graphical form.

^1H and ^{13}C spectroscopic techniques are complementary but, whereas the former method has a number of applications to the solution of relatively simple problems, the shift resolution possible with ^{13}C n.m.r. spectroscopy makes it especially suited for the study of polymers and compounds containing many carbon atoms. Nevertheless, ^{13}C n.m.r. finds some use in less complex examples particularly when information on the nature of quaternary carbon atoms is required.

The chemical shifts of carbon atoms depend in part upon molecular geometry; this has proved useful in conformational and configurational analyses, eg. axial methyl carbon atoms attached to cyclohexane rings resonate 5 ppm upfield from

those of equatorial analogues and also the α -carbon signals of cis-alkenes are appreciably shielded relative to those of the corresponding trans-isomers.

The major drawback of ^{13}C n.m.r. spectroscopy is that upwards of 25 mg of material is required to produce a reasonable spectrum. In biological terms this is a vast amount, hence the technique has found relatively few applications in biological studies, however the technique has proved indispensable to the organic synthetic chemist.

As a slight offshoot from structure elucidation studies, ^{13}C n.m.r. spectroscopy has found a minor role in biosynthetic studies.

The incorporation of labelled precursors by living organisms has offered a long established method for the study of the biogenesis of complex molecules. The use of radio-isotopes, such as ^{14}C , is rendered tedious by many factors, not least the need finally to selectively degrade the natural product to establish the position of the label. However, if the ^{13}C n.m.r. spectrum of the metabolite has been previously analysed vis á vis the chemical shifts of the carbon atoms, it is a simple matter to locate in situ the positions of enrichment in molecules obtained from feeding experiments with precursors containing the ^{13}C isotope.

2.5 Mass spectrometry

As with n.m.r., only in the past 25 years or so have chemists and biochemists begun to use mass spectrometry to help solve structural problems. There has been a rapid development of the technique in the past fifteen years and it now plays a vital role in almost all bioorganic chemistry laboratories.

2.5.1. Molecular formula determination

The most general use of mass spectrometry in natural product chemistry is for the determination of molecular weight (low resolution) and molecular formula (high resolution). A prerequisite for such determination by electron impact (EI) is that the intact molecule can be vapourised prior to ionisation. The current upper limit of molecular weight determination by EI mass spectrometry is around 1500, taking into account favourable volatility and thermal stability. The advantages of molecular formula determination up to this region should not be underplayed, especially in the cases of non-crystalline substance available in microgram or nanogram quantities, where no alternative approach can be adopted.

2.5.2. Structural information by derivatisation and mixture analysis

Mass spectrometry has had an integral role to play in biochemical structure elucidation. The structural analysis of mediators of biological phenomena and pathophysiological conditions, etc. owes a great deal to the application of the technique.

Mass spectrometry has helped to elucidate or clarify the processes controlling analgesia, asthma, anaphylaxis, rickets, etc. Very recently it has also been useful in comparing synthetic molecules designed for clinical use, such as insulin and interferon, with the naturally derived material.

Of the half dozen or so techniques available for structure elucidation, mass spectrometry has shown itself to be the most versatile and universally applicable. Originally, however, mass spectrometry was the technique used by chemists to confirm the structures of low molecular weight organic compounds. The requirement for EIMS of sample volatility and thermal stability precluded the analysis of most biologically derived polar species such as peptides and carbohydrates. It is therefore necessary to convert such molecules to volatile derivatives rendering them more amenable to EI analysis.

To increase the volatility of a compound it is necessary to eliminate both inter- and intramolecular hydrogen bonding and ionic interaction. In the case of peptides it is necessary to acetylate free amino groups and permethylate acidic hydrogens, to produce a chloroform-soluble species. This acetyl-permethyl derivative is not only sufficiently volatile but the fragmentation is directed to the peptide bond. This manifests itself in the production, under EI-MS analysis, of N-terminal sequence ions. Consequently the sequence of the peptide can be determined. A similar approach is used for carbohydrate structure elucidation. The use of derivatisation for lower molecular weight compounds is less well categorised but is often useful for looking at functional groups.

One of the major advantages of mass spectrometry is in the analysis of mixtures. A sample can be fractionally volatilised into the ion source over a temperature range of 50-350 C. Impurities arising from reagents, etc. are removed at low temperature hence some degree of separation is achieved by temperature adjustments. Mixture analysis, as part of a deliberately planned strategy, is a major advantage of MS over classical peptide sequencing, where the rate-limiting step is isolating highly purified peptides. This method was used widely in the structure elucidation of the enkephalins, adipokinetic hormone and several other peptides. Since the technique is independent of compound class, unusual residues in peptides and carbohydrates can be analysed as well as low molecular weight species.

2.5.3. Ionisation methods

2.5.3.1. Electron impact

Electron impact is a highly energetic form of ionisation, the upshot being that fragmentation invariably arises. This may lead to little or no molecular ion formation. To overcome this, some 'softer' forms of ionisation have been developed to yield more molecular weight information. However, in the study of small peptides, ie. less than 15 residues, EI is the only method that can guarantee sequence information by fragmentation at every peptide bond. This gives a perspective and contrast to Fast Atom Bombardment (FAB) where fragmentation does not always occur. Thus, although FAB has a great sensitivity requiring about 1 nanomole or 1 μ g, if more material is available ie. more than 25 nanomoles, then EI and sometimes Chemical

Ionisation (CI) should be preferred with respect to confidence in the generation of sequence information.

2.5.3.2. Chemical ionisation

The ionisation in this technique is mediated through a reagent gas ion (R), usually methane or ammonia. The gas is ionised to generate primary ionising species such as $(R)^+$ and thermal electrons. The sample is then protonated by this charged species, which can act as a Lewis or Bronsted acid, normally because the ion is further stabilised by collisions, to form a quasimolecular ion $(M + H)^+$. Accordingly, less internal energy is transferred, which increases the probability of observing the $(M + H)^+$ ion compared to EI. Fragmentation is also directed toward the C- as well as the N- terminus.

2.5.3.3. Fast atom bombardment

The technique of Fast Atom Bombardment requires the sample to be loaded in a glycerol or thioglycerol matrix onto an obliquely aligned metallic target. The matrix is bombarded with accelerated argon or xenon ions or atoms.

In FAB, a long lived beam of ions is generated and intense quasi-molecular ions $(M + H)^+$ $(M - H)^-$ are observed. Fragmentation, to give useful information about structure, may occur, but not with the predictability of EI methods.

Molecular ion data can be obtained at the 10 pmol level but for some degree of fragmentation 1 nmol is required. Certainly for peptide analysis, FAB complements EI and it is important to have a variety of ionisation modes available for analysis of unknowns.

Certain chemical structures such as benzylic or aliphatic alcohols eliminate water readily under EI therefore it would be worthwhile applying FAB which is more likely to show a molecular ion.

2.5.4. Application

Within the sphere of structure elucidation, mass spectrometry has had a revolutionary impact. Prior to the development of FAB, the technique was instrumental in the structure elucidation of the enkephalins and the leukotrienes. Since FAB has been available, novel high mass peptides, in particular neuropeptides, and high mass oligosaccharides have been sequenced.

The use of mass spectrometry has often been a definitive means of structure elucidation, eg. in FAB-mapping; but in other cases the information generated has been less clear cut, although fragmentation pathways have confirmed or indicated the presence of some functional groups. In these latter cases the combination of mass spectrometry with spectroscopic analyses has provided the necessary information to complete the structure elucidation jigsaw.

Its sensitivity and multiplicity of uses renders it a technique no serious chemist or biochemist should be without.

2.6 Planning a strategy

The tenet for structure elucidation is a trade-off between maximising physicochemical data and minimising handling losses. Therefore, planning a strategy to make most judicious use of a limited sample is tantamount to success.

Initially, the question of sample purity should be addressed. How the sample has been 'worked-up' from its source can provide useful information, eg. gel filtration may give an indication of molecular weight. Knowledge of which purification techniques had been employed may indicate whether the sample is a single species or a group of closely related compounds, as seen in this DIF study. HPLC is usually an imperative preparative technique before physicochemical analysis.

Certain experiments such as acid/base stability and chemical inactivation can be performed on partially pure samples or side fractions from HPLC, although it is vital that a sensitive and specific bioassay is available.

It is important at an early stage to determine the molecular formula as this may well dictate which techniques are employed at later stages in the analysis and possibly give an indication of the class of compound, eg. if there is a low ratio of carbon to oxygen one may surmise that part or all of the molecule may be carbohydrate.

The molecular formula can be determined using mass spectrometry. Although this is a destructive technique, it is 'cost effective'

when considering data obtained against expenditure of sample. FAB-MS, DCI or DEI may often provide a means of molecular weight determination and hence accurate mass measurement. From the molecular formula, the number of double bond equivalents (D.B.E.) can be calculated using the formula $\frac{1}{2} (2n_4 + n_3 - n_1 + 2)$, where n_4 is the number of tetravalent atoms, n_3 is the number of trivalent atoms and n_1 is the number of univalent atoms in the molecular formula.

It is advisable to then accumulate spectroscopic data, eg. ultra-violet spectroscopy data by analysing the sample in a volatile solvent, eg. methanol. Addition of acid may produce a hypsochromic shift ie. λ_{max} shifts to a shorter wavelength. similarly a bathochromic shift may occur on addition of base. This may give an indication of an auxochrome or a phenolic group in the sample. It is probably not possible to determine the molar extinction coefficients if there is insufficient sample to weigh, but this can be determined at a later stage once specific quantitation is possible.

If the sample is in a pure form, ^1H -n.m.r. is the next logical stage. As the technique is non-destructive there should be no reservations as far as analysis of the whole sample is concerned, the only losses incurred may arise from transferring samples between vessels. The sample should be shaken with D_2O or TFAD to exchange any acidic protons after the initial analysis. This is useful if the chemical structure contains a carboxylic acid or phenolic group. If there are still unassigned functional groups, infra-red spectroscopy may be used provided that the percentage of total sample used is small or that the sample is retrievable.

EI-MS analysis on small aliquots of the unknown should confirm or discount certain functional groups. These can be further investigated by specific derivatisation and labelling. Using 1:1 deuterium labelling, eg. $\text{CH}_3\text{CO}/\text{CD}_3\text{CO}$ or $\text{CH}_3\text{I}/\text{CD}_3\text{I}$ certain functional groups can be recognised from the isotopic pattern produced under MS analysis, ie. a doublet three mass units apart will be seen in the spectrum. These can be useful in distinguishing low intensity signals from background signals.

All the collated data should provide ample evidence to deduce the chemical structure, however, in some cases, eg. DIF, such analyses may be inconclusive.

In this project the above idealised scheme was not fully applicable as the data from the analyses on DIF generated three or four possible structures. In light of the limited natural material available, these could only be distinguished by synthesis and subsequent comparison with the data from the natural material.

As analytical techniques become more sensitive and specific, a complete analysis can be achieved on less natural material. As far as low molecular weight compounds are concerned, 0.5 mg seems to be about the lower limit for a complete, unequivocal structure elucidation where the compound class is known. As described in this thesis, it is nevertheless possible to elucidate the structure of an unknown on far less material (ie. $\approx 50\mu\text{g}$) by a proper combination of advanced analytical strategy, coupled with chemist's intuition and synthesis.

CHAPTER III

RESULTS AND DISCUSSION

3.1 Structure Elucidation

3.1.1. Preliminary perspectives

As in many cases of attempted structure elucidation the overriding criterion for success, partial success, or failure has been the amount of pure material available for analysis. The organic chemist is used to achieving an effective micro-analysis on tens of milligrams of unknown compound. When a total analysis and characterisation must be achieved on a 1000-fold less material, a much more innovative and ingenious approach must be undertaken rather than a routine regimen of chemical analyses.

In 1982, this laboratory became involved in the early chemical characterisation of DIF, by advising on chemical micro-treatments prior to testing for biological activity (54).

The compound was found to be stable to both acid and base treatment, apparently impervious to acetylation in pyridine or methanol with acetic anhydride, but was "destroyed", at least in part, by reduction with sodium borohydride. Some conclusions on the chemistry of DIF could be deduced from these experiments, eg. it presumably did not contain an α -amino group or a hydroxyl group but it probably contained a carbonyl function.

Such deductions are fraught with assumptions and the project began in earnest in these laboratories with the realisation that detailed and expert chemical and spectroscopic study would be required to even get a handle on the structure of DIF.

The work began by repeating the chemical stability experiments. Unfortunately, interpretation based on the bioactivity data is suspect for a number of reasons, not least that the chemical treatment chosen must allow rapid and complete removal of the reagents and quantitative recovery of the sample. Thus a treatment which destroys half or two-thirds of the bioactivity had to be regarded as suspect and only a 'full activity' or 'full destruction of activity' result could be regarded as valid.

These early definitive experiments again suggested a carbonyl moiety in the molecule (borohydride reduction), the absence of -NH_2 or -OH functions (either partial or full activity after treatment with $(\text{CH}_3\text{CO})_2\text{O}$ /pyridine or methanol respectively) and the absence of esters, lactones or other unstable linkages (acid/base stability).

In this type of project there is no easy way of estimating the amount of sample available or indeed its purity. Early 'best guesses' of the quantities used in pilot chemical experiments were based on an assumption that a compound with, say, 0.1 nanomolar activity would be a potent biochemical and could be equated to '1 unit'. A further

guess at 1000 for the molecular weight showed that micro-scale chemical experiments were carried out on something of the order of a few femtomoles to picomoles of DIF. Clearly a scale-up was required for further chemical and spectroscopic analysis and a target of 1 - 10 microgrammes was chosen initially.

3.1.2. Spectroscopic and spectrometric studies

Approximately one-tenth of all subsequent DIF preparations was screened for U.V. activity; only a fraction was used due to losses on transfer and adsorption to the cuvette. Unfortunately, the data were variable, there being no consistent $\lambda_{\max}$ between preparations, but rather broad absorbances between 260 and 290 nm with $\lambda_{\max}$ varying usually between 273 nm and 280 nm.

Whether the U.V. spectrum was derived from DIF or from some impurity had to await more definitive analysis. All that could be concluded was that if DIF had a U.V. $\lambda_{\max}$ in the 270-280 nm range then this would argue for at least an α,β - unsaturated carbonyl functionality, possibly supplemented by an auxochrome.

There had previously been some discrepancy regarding the approximate molecular weight of DIF. Early experiments with crude DIF in aqueous solution suggested a molecular weight of 1000 - 2000 (22). Gel permeation HPLC was used, with commercially available steroids as standards and references, to get a more accurate estimate of the

molecular weight. Nevertheless, anomalous elution of standards could only narrow the problem down to suggest a species of less than 500 daltons.

Since the early isolation experiments on DIF were from aqueous solution, it was initially assumed that the new ionisation technique of Fast Atom Bombardment (FAB) (57) would be appropriate for DIF analysis by mass spectrometry.

No result was obtained in either positive or negative ion modes, although experience in other areas of analysis suggested that this could be a reflection of the state of purity of the sample rather than its overall polarity and amenability to the FAB method (57).

Therefore, it was decided to risk a complete DIF preparation on an Electron Impact mass spectrometric run using a temperature gradient to allow further purification of the sample.

A weak spectrum was produced which nevertheless showed a potential molecular ion cluster of signals based on m/z 306. The isotope ratio observed for the cluster was m/z 306 : 308 : 310 approximately 9 : 6 : 1.

Chlorine (^{35}Cl and ^{37}Cl in a ratio of 3 : 1) and bromine (^{79}Br and ^{81}Br in a ratio of 1 : 1) both have an appreciable natural abundance of their heavier isotopes,

which leads to very characteristic molecular ion clusters at M , $M+2$, $M+4$ etc. (Fig. 4).

Analysis of the peak ratios from the EI spectrum were indicative of a compound containing two chlorine atoms.

These signals were clearly not derived from known impurities, eg. phthalates and the connectivity between the spectrum and DIF was further established by observing the same signals in successive preparations of DIF.

A better quality EI spectrum was later obtained on a larger preparation, showing more clearly some of the fragmentation in the molecule and this spectrum is illustrated in Fig. 5.

The Electron Impact spectrum of DIF shows a molecular ion at m/z 306. The molecular ion cluster exhibits the characteristic pattern for the presence of two chlorine atoms in the molecule, ie. ions at m/z 306 : 308 : 310 were in the ratio 9 : 6 : 1.

Accurate mass measurement of several of the ions in the spectrum, in the EI mode (see later) showed the absence of nitrogen in the molecule.

Initial interpretation of the major signals in the spectrum led to the following observations. The fragment ion at m/z 288 can only arise via rearrangement through loss of water. m/z 263 can only correspond to a loss of 43 a.m.u.

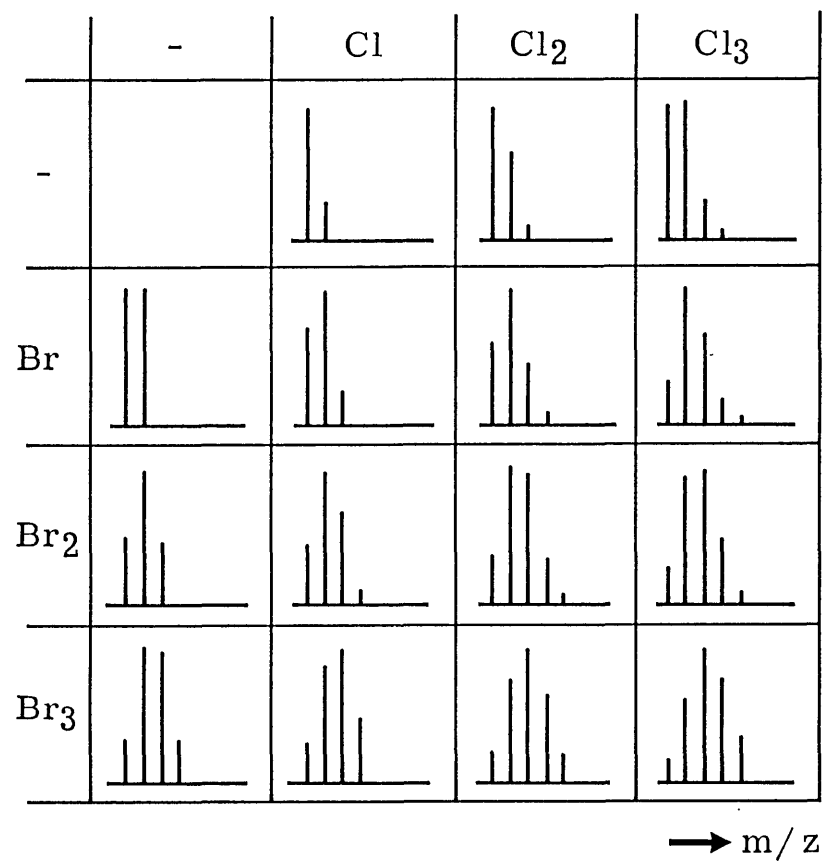


Fig. 4. Molecular cluster ions arising from compounds containing one, two or three chlorine or bromine atoms.

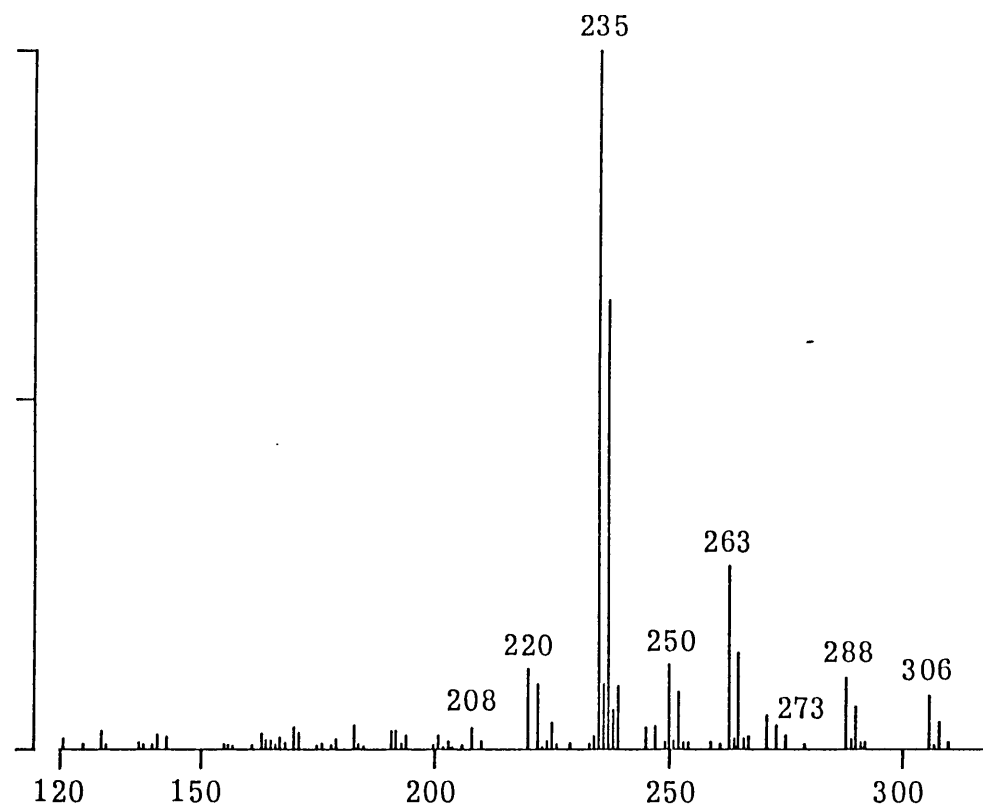


Fig. 5. Electron Impact mass spectrum of natural DIF-1

from the molecular ion and can be a loss of a propyl radical $C_3H_7^\bullet$ or of C_2H_3O ; these were differentiated later by accurate mass measurement. m/z 273 was interpreted as a loss of $CH_3^\bullet$ from the m/z 288 fragment. The signal at m/z 250 was interpreted as a McLafferty rearrangement/elimination of C_4H_8 from a pentyl ketone, again using mass measurement information (see later). Loss of 71 mass units produced the base peak of the spectrum at m/z 235, which is interpreted as an α -cleavage with loss of a pentyl radical. A less abundant ion was seen at m/z 220 corresponding to a net loss of 86 mass units. This seemed likely to have arisen via loss of methyl from the m/z 235 ion although a concerted loss from the molecular ion at m/z 306 is not discounted. All these fragments conserve the 'dichloro-isotope' ratio.

Interestingly, the spectrum displays no major peaks below m/z 200. This implies that DIF has a chemically stable nucleus of about 200 daltons; furthermore, absence of the loss of Cl as a fragment suggests that chlorine is bonded to this stable nucleus.

An investigation of the possibility that chlorine was attached via a stable linkage was made using chemical hydrolytic procedures, and utilising chloramphenicol, containing a 1,1 substituted carbon, as a standard reference compound.

The mass spectrum of a base-treated DIF preparation was identical to that shown in Fig. 5, whereas base treatment of chloramphenicol, under the same conditions, led to the loss of chlorine from the molecule followed by further destruction presumably via a carbene intermediate. It was concluded that the chlorine in DIF was unlikely to be present as CHCl or CCl_2 .

3.1.3. Borohydride reduction of DIF

The reaction between DIF and sodium borohydride destroys bioactivity. This suggests that the chemical structure probably contains a carbonyl function that is presumed to be essential for activity.

An EI spectrum was determined for BH_4^- - reduced DIF and is illustrated in Fig. 6. The most important information is derived from the observed molecular ion at M/z 308 (^{35}Cl isotopes) & shows the presence of only one carbonyl function in the DIF molecule, via the addition of two hydrogen atoms on reduction to give a secondary alcohol.

Examination of the fragment ions in the spectrum showed the losses anticipated from the spectrum from non-reduced DIF (Fig. 5) and since no novel fragments were generated from the newly formed alcohol, it was inferred that the carbonyl function was outside the main, non-fragmenting nucleus of DIF.

M/z 290 is assigned to loss of water, although this can now occur as a straight 1,4 - elimination of an exocyclic alcohol, as well as via the presumed involvement of the ring in the non-reduced DIF case. Indeed, in a later experiment it proved possible to separate two products by GC-MS, one corresponding to the already eliminated reduced product.

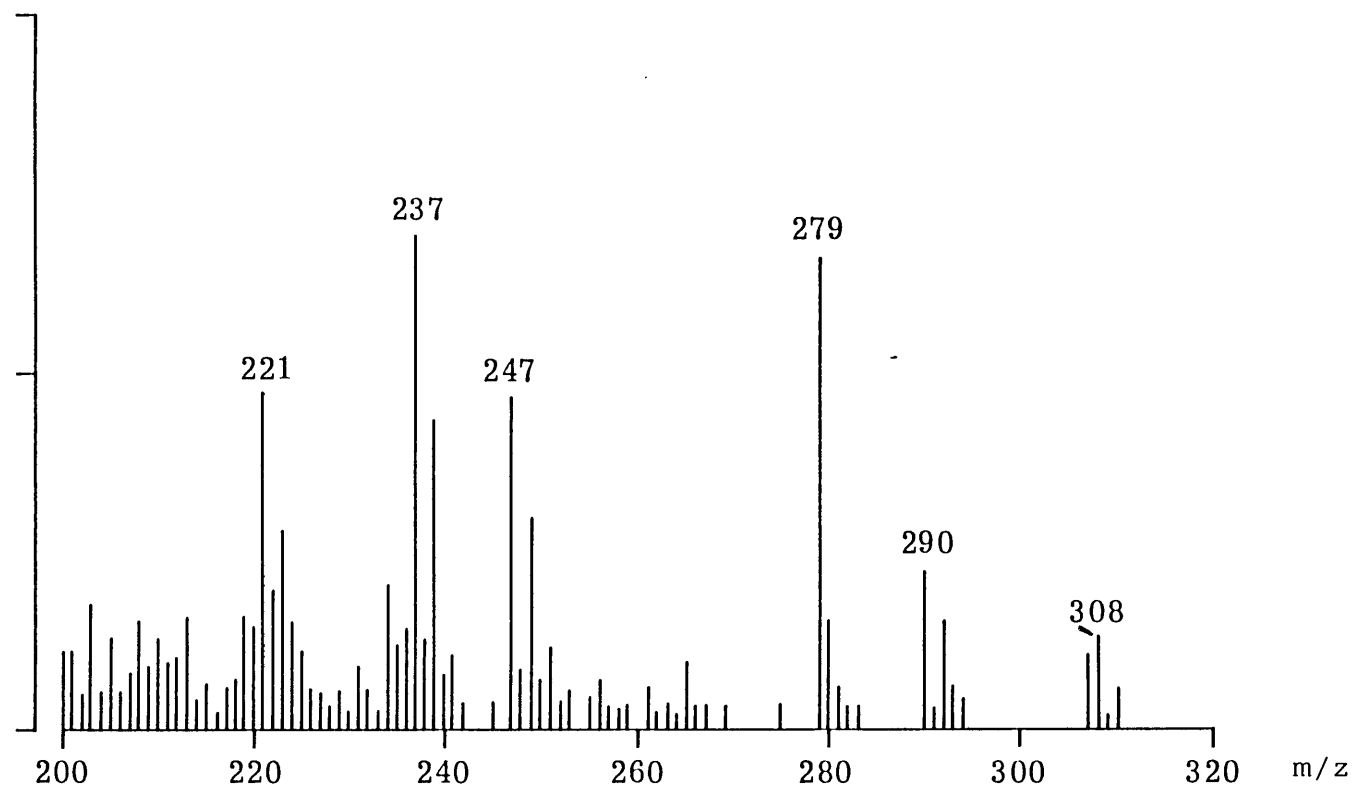


Fig. 6. Electron Impact mass spectrum of borohydride-reduced DIF-1.

The m/z 279 impurity is seen in adjacent spectra and does not contain the chlorine isotope ratio. m/z 247 and 221 correspond to losses of $C_3H_7^\bullet$ and $C_5H_9^\bullet$ from m/z 290, and m/z 237 arises via the loss of $C_5H_{11}^\bullet$ from the molecular ion.

In an attempt to determine the number of hydroxyl groups present in the reduced DIF molecule a small sample was converted to the trimethylsilyl derivative. The TMS derivative of reduced DIF produced a very weak EI spectrum after purification using GC-MS. The major fragmentation observed was loss of 90 a.m.u. $((CH_3)_3SiOH)$ which was expected since a 'new' hydroxyl group had been generated in the molecule. There were, however, no further losses of 90 a.m.u. suggesting that DIF contains only one alcohol function with the requisite hydrogen on the 2 position to allow 1,4-elimination of trimethylsilanol.

3.1.4. DIF Methylation

Diazomethane (CH_2N_2) is widely used in mass spectrometry to reduce the polarity of compounds under study and hence increase volatility and as a means of quantitating certain functional groups present in a molecular structure. It has an advantage over trimethylsilyl (TMS) derivatives in that the increase in mass is relatively small; it is conceivable that by making TMS derivatives of polyhydroxy compounds the resulting molecular ion may be beyond the mass range of the instrument.

Fig. 7 shows the EI spectrum of the DIF dimethyl compound produced by reaction with diazomethane. It is noticeable that the molecular ion signal is considerably stronger relative to fragment ions than in the underivatized DIF spectrum (Fig. 5).

The molecular ion at m/z 334 indicates that two methyl groups have been incorporated into the molecule indicating the presence in DIF of acidic hydroxyl groups, presumably either two carboxylic acids, two phenols or one of each. As expected there is no longer a signal due to the loss of H_2O but the equivalent loss of methanol is not observed. This indicates that there is no readily accessible hydrogen adjacent to the O-substitution position. The first major fragment ion at m/z 303 corresponds to a loss of $\text{CH}_3\text{O}^\bullet$ (31 a.m.u.). The signal at m/z 291 is analogous to the m/z 263 signal in DIF, ie. γ -cleavage of an aliphatic ketone. The McLafferty rearrangement ion at m/z 278 is

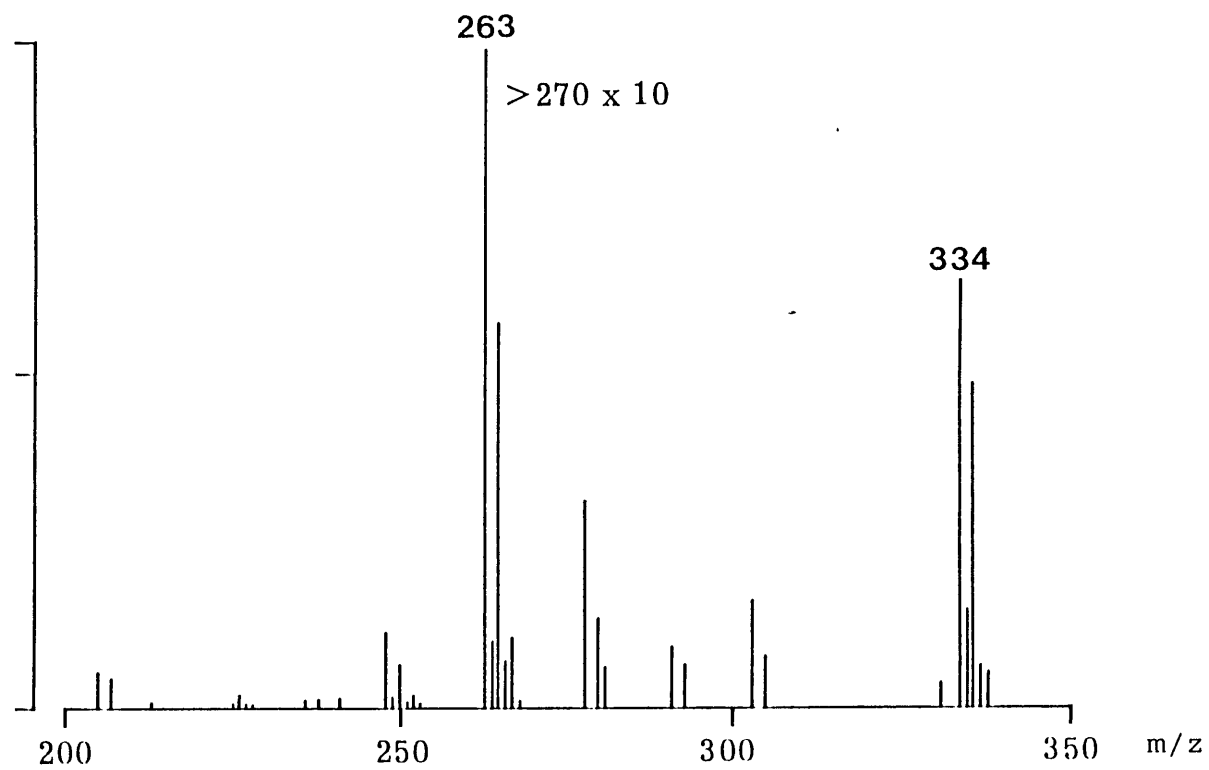


Fig. 7. Electron Impact mass spectrum of dimethyl DIF-1 after reaction with diazomethane

still prominent and the major fragment ion m/z 263 now corresponds to loss of 71 mass units ($C_5H_{11}^{\cdot}$) from the molecular ion. A signal at m/z 248 indicates a loss of $CH_3^{\cdot}$ from the m/z 263 fragment ion. Two relatively weak fragment ions have become apparent; m/z 236 which corresponds to a loss of 98 mass units from the molecular ion. This can be explained by loss of $C_5H_{11}CO$ with a hydrogen transfer to the charged ion. The other fragment ion m/z 205 corresponds to a loss of $CH_3O^{\cdot}$ from the m/z 236 fragment ion.

The straightforward conclusion from this experiment would be that there are two acidic hydroxyls in the molecular structure. However, a second component in the methylated DIF product was also observed at a later retention time on the GC-MS (Fig. 8). The spectrum showed a clear molecular ion at m/z 348, containing the correct chlorine isotope ratio. A small fragment ion at m/z 305 is interpreted as loss of $C_3H_7^{\cdot}$. Fragment ions at m/z 292 and m/z 277 corresponded to losses of C_4H_8 (McLafferty rearrangement) and $C_5H_{11}^{\cdot}$ respectively. However, a 'new' fragment has arisen at m/z 249 which corresponds to a loss of 99 mass units. This could result from the loss of $C_5H_{11}CO$. In natural DIF and the dimethyl ether, the major fragment ions at m/z 235 and m/z 263 respectively arise from the production of an acylium ion that is stabilised through conjugation to a ring system. The inference drawn from the appearance of this 99 mass unit loss is that the stabilisation effect has been reduced by a methylene insertion between the ring and carbonyl

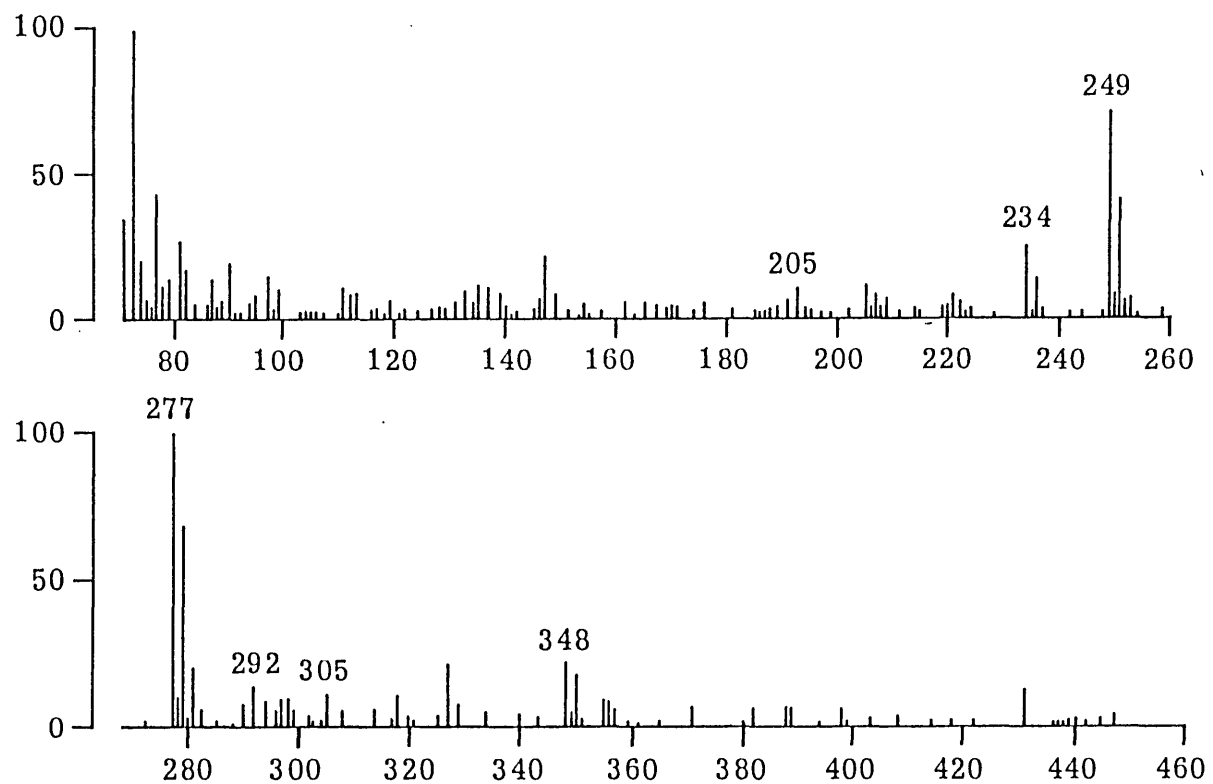


Fig. 8. Electron Impact mass spectrum of trimethyl DIF-1 after gas chromatography.

function. The alternative explanation may have been that another hydroxyl had appeared through keto-enol tautomerism of the carbonyl group. However, this could be discounted as the resulting methyl ether would not allow the McLafferty rearrangement to occur. This supposed trimethyl DIF was a relatively minor component and therefore assumed to be a by-product. Hence the dimethyl derivative was used for interpretation.

It was necessary to distinguish between carboxyl and phenol groups. A usual test for phenolic moieties is methylation using potassium carbonate and iodomethane. Mass spectral analysis after reaction times varying from one hour to twenty-four hours did not indicate that methylation had occurred, ie. m/z 306 remained unchanged. The conclusion that the DIF structure did not contain phenolic hydroxyls was questionable however, since whilst simple phenols in for example tyrosine were shown to be readily methylated, experiments with polyphenolic compounds such as resorcinol and phloroglucinol showed a very slow methylation rate.

An alternative approach to answer the phenol/acid question using esterification in methanol HCl again showed no change in m/z 306 in the DIF EI spectrum. This data was felt to be more reliable, ie. in showing the absence of carboxyl groups, although experience with some hydroxy acids showed very slow methylation rates at room temperature. The result was therefore not definitive at this stage but suggested that there were non-carboxylic hydroxyls in DIF.

3.1.5. High resolution mass measurement

The fragmentation pattern observed during EI mass spectrometry of DIF contained some ions that could arise through only one possible fragmentation, eg. m/z 288 corresponding to the loss of a molecule of water. However, other fragment ions required accurate mass measurement to definitively determine the particular chemical loss, eg. m/z 263 could arise from loss of $C_3H_7^{\cdot}$ or $C_2H_3O^{\cdot}$. In addition it was important to determine the molecular formula for the intact molecule.

High resolution mass measurement was performed on the major fragment ions using perfluorokerosene as an internal standard, and manually peak-matching selected ions, in order to minimise error. The molecular ion m/z 306 had three calculated compositions within ± 3.0 ppm of that observed, $C_{25}H_5$, $C_{20}H_6N_2O_2$ and $C_{13}H_{16}O_4Cl_2$. The first two do not contain chlorine or are chemical nonsense and are therefore discounted. The m/z 306 ion, found to have an accurate mass of 306.0424, was thus determined to correspond to $C_{13}H_{16}O_4Cl_2$ (Table 3). The ion at m/z 263 had four calculated compositions within 5 ppm. However, two required an atom of sulphur in the structure and did not contain two chlorine atoms. Another composition required four chlorine atoms leaving $C_{10}H_9O_4Cl_2$ as the composition of m/z 263. (Table 3). This, therefore, corresponds to the loss of $C_3H_7^{\cdot}$ from the molecular ion. Further mass measurements showed that the ion m/z 250 arose from a loss of C_4H_8 from the molecular ion at

elemental composition								
m/z	C	H	N	O	Cl	ppm	ACC mass	Conclusion fragment lost
306	13	16	0	4	2	-1.0	306.0424	M ⁺
288	13	14	0	3	2			-H ₂ O
263	10	9	0	4	2	-0.9	262.9876	-C ₃ H ₇ [•]
250	9	8	0	4	2	-1.0	249.9798	-C ₄ H ₈
235	8	5	0	4	2	0.8	234.9563	-C ₅ H ₁₁ [•]
220	7	2	0	4	2	-4.6	219.9329	-C ₆ H ₁₄ [•]

Table 3. Elemental composition and accurate mass measurement of the major fragment ions in the Electron Impact mass spectrum of DIF.

m/z 306. This is a McLafferty rearrangement and has a particular structural requirement for the fragmentation to occur (see later). m/z 235 was due to a loss of $C_5H_{11}^{\cdot}$ (see Table 3). Finally, the peak at m/z 220 was determined as a loss of $C_6H_{14}^{\cdot}$ which may require two separate fragmentations.

At this point it is also possible to determine the number of double bond equivalents in the DIF molecule. By applying the equation (see section 2.6)

$$\frac{1}{2} (2n_4 + n_3 - n_1 + 2)$$

one arrives at

$$\frac{1}{2} (2 \times 13 + 0 - 18 + 2) = 5 \text{ DBE.}$$

This is a strong indication that DIF has an unsaturated ring system within its structure, corresponding to that deduced from the absence of fragments below m/z 200 in the E.I. mass spectrum. By subtracting the now known single carbonyl, 4 double bond equivalents now remain to be identified.

Desorption chemical ionisation mass spectrometry was used to confirm that m/z 306 is indeed the molecular weight. The technique is less likely to cause fragmentation as the energy causing ionisation is less than that of the electrons in EI-MS.

As previously stated, DCI produces quasimolecular ions. When run in the positive mode, the molecular ion was found to be 307 a.m.u. $(M+H)^+$ and in the negative mode the ion produced was $(M - H)^-$ 305 a.m.u. Hence the molecular weight was confirmed as 306 a.m.u.

3.1.6. Nuclear Magnetic Resonance spectroscopy

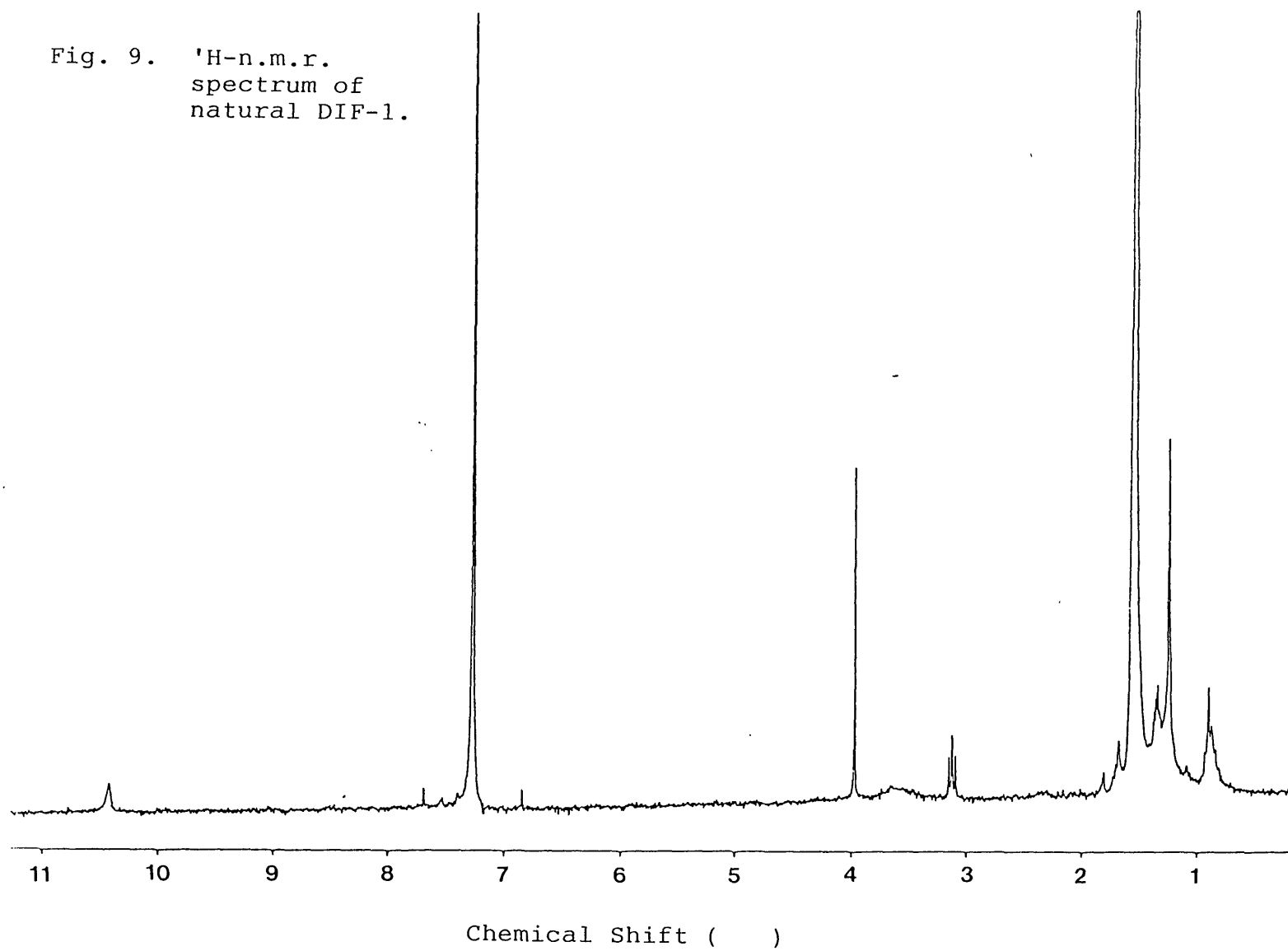
Nuclear Magnetic Resonance spectroscopy is one of the best ways of determining the possible presence of aromatic or other conjugated protons in a molecule. However, the sample requirement is considerable, and an early attempt to obtain a DIF NMR spectrum on a total preparation gave only solvent impurity signals. It was necessary, therefore, to scale-up the DIF preparation and the sample used for NMR was later determined as about 60 μ g. Several spectra were determined, the best quality one being shown in Fig. 9.

There are several aspects of the spectrum that must be considered. Broadly, the whole spectrum is of very low intensity. This was presumed to have arisen because of the very limited amount of material available for analysis.

Furthermore, it was not possible to state categorically which signals were from the sample and which were from solvent and sample impurities. Some solvent impurities were eliminated by running the blank solvent, eg. CHCl_3 in the CDCl_3 at δ 7.24.

Below δ 2.0 it was practically impossible to distinguish particular multiplets which may correspond to methyls, methylenes, etc. A low intensity distorted triplet occurred at δ 3.10. This was strongly suggestive of a methylene adjacent to a strong electron-withdrawing group such as a carbonyl function. The singlet at δ 3.96 integrated as approximately three to two ratio with the δ 3.10 signal,

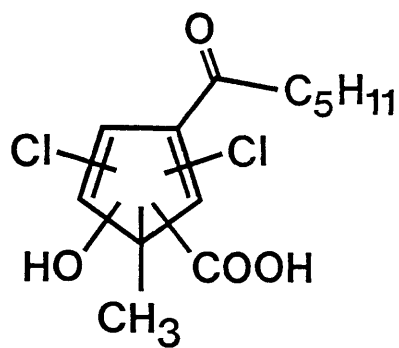
Fig. 9. ¹H-n.m.r.
spectrum of
natural DIF-1.



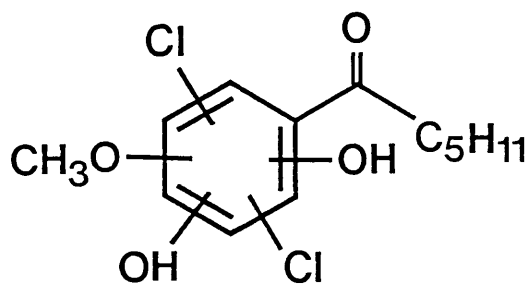
suggesting an 'isolated' methyl group such as methoxy for the δ 3.96 signal. The preliminary U.V. studies had suggested that there may be an unsaturated ring system; however, there was no evidence of any aromatic protons which would appear between δ 6.0 and δ 9.0. Finally a small, broad peak appeared in some spectra at δ 10.32 but the integration did not give a clear indication of the number of protons in the signal. This suggested there may be an aromatic hydroxyl or carboxylic acid in the molecule. The sample was shaken with D₂O which resulted in the disappearance of the peak at δ 10.32 which would be expected if the signal did arise from a hydroxyl or acid moiety.

Decoupling experiments were limited or impossible due both to the weakness of the spectrum (obtained over 18 hours) and complexity of the δ 0 - 2 region, but irradiation at δ 3.10 led to the collapse of a presumed quintet at δ 1.67 to a triplet. This data together with the chemical analysis suggested the presence of a -CO-CH₂-CH₂- unit in the structure.

The upshot of the chemical and physicochemical analyses was the number of hydroxyls in the molecule was still unclear. Nevertheless, two general structures (Fig. 10) were emerging from the data. One based on a cyclopentadiene ring system and the other based on a benzene ring. On balance, the latter was favoured from the chemical and spectroscopic data but also biosynthetic grounds, and possible methods for synthesis were explored. However, a definite spectroscopic proof of structure was still lacking at this stage



Cyclopentadiene nucleus



Benzene nucleus

Fig. 10. Two possible nuclear structures for DIF-1 from physicochemical data derived from the natural material.

and these experiments were also continued.

Fourier Transform Infrared spectroscopy was carried out on half the sample recovered from the NMR solvent by blowing down under nitrogen. The spectrum was contaminated, probably by plasticizers, again preventing a discrimination between the cyclopentadienyl (acid-containing) and benzene (hydroxyl-containing) nuclear structures (Fig. 10).

Collisional activation MS-MS on a triple quadrupole, using the dimethyl derivative of DIF, confirmed the proposed structural unit of DIF as a substituted benzene unit, via successive losses from m/z 263 of CH_3 and CO which cannot be achieved rationally from the cyclopentadienyl structure, which also has to lose OCH_3 or CH_3 followed by O at some point.

The dimethyl DIF derivative was subjected to catalytic hydrogenation to investigate the stability and resistance to reduction of the molecule. Under conditions (see Methods) that would easily destroy leukotriene B_4 activity (58), DIF was unaltered chemically and there was no reduction in bioactivity. This result was indicative of a very stable ring system and again provided good evidence for a benzene ring nucleus.

There was very little natural DIF remaining after these chemical and physicochemical treatments. The remainder was stored for comparison with synthesised material. Synthesis was now vital as the only means of finally elucidating the complete structure of DIF.

3.1.7. Biosynthetically favourable pathways

There have been reviews on natural product biochemistry (59-63), some giving more emphasis to alkaloids, others to isoprene derived compounds, etc. An alternative approach has been to review natural products from a particular source, eg. marine organisms (64) or fungi (65). Overall, natural products have been discussed from a variety of perspectives.

Natural phenolic compounds can arise from two major biosynthetic pathways; the polyketide pathway or the shikimate pathway. Phenolic compounds occur in other biosynthetic pathways, but these are usually by way of an enzymic hydroxylation or oxygenation.

The shikimate pathway tends to produce compounds with hydroxyls on adjacent carbon atoms in ring systems, whereas the polyketide produces hydroxyls ortho- to each other. This can readily be explained by the fact that polyketides are condensation products of acetate molecules. The biogenetic concept was first realised by Collie (66) on the basis of some interesting transformations observed with polyacetyl compounds. However, it was not until 1953 that Birch (67) re-examined the concept and laid down most of the modern views of polyacetate biosynthesis. He pointed out its broad scope and great usefulness and also accumulated experimental support by studies with radioactive tracers. Fig. 11 shows how different derivatives can arise from a linear poly- β -ketomethylene.

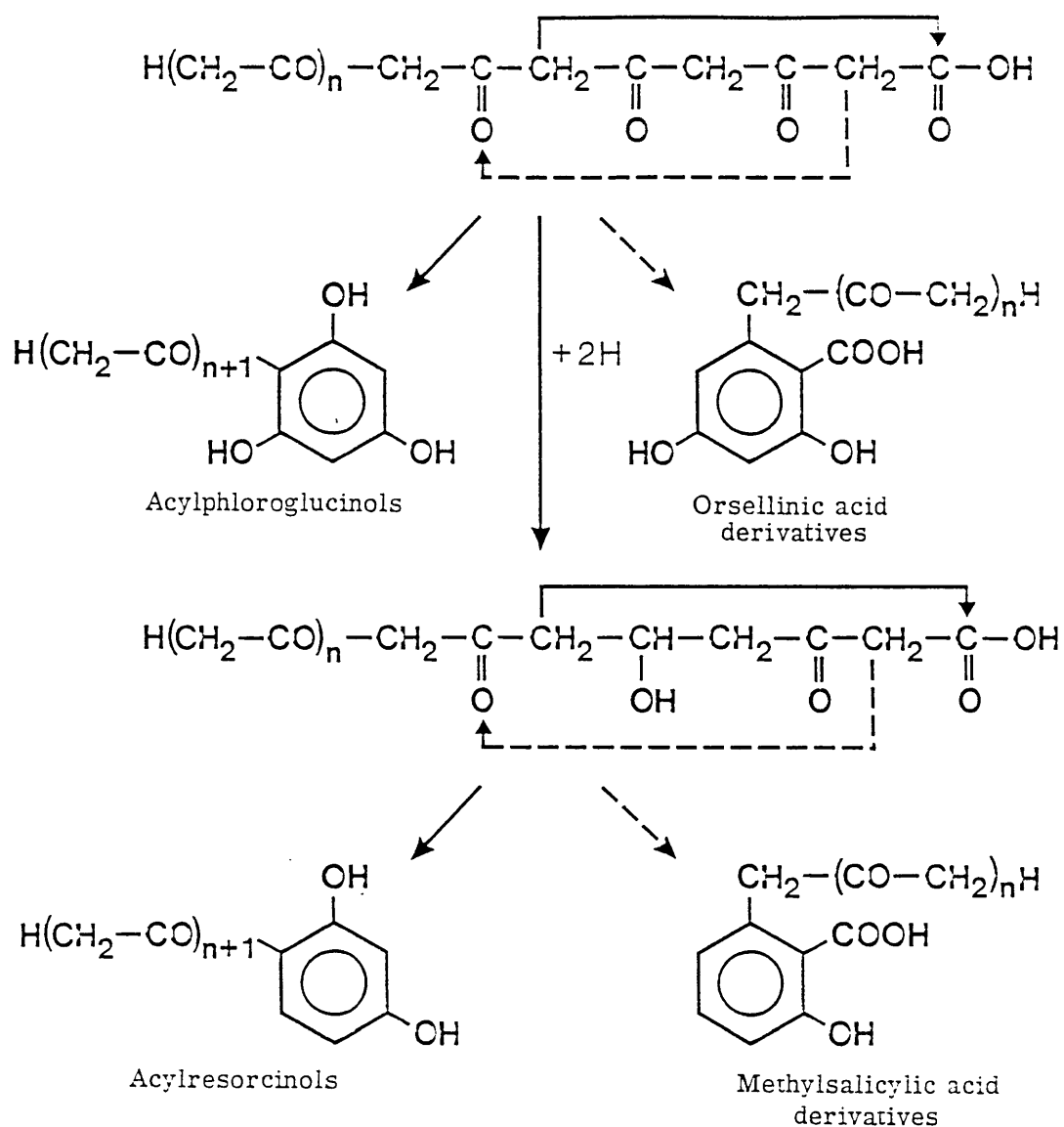


Fig. 11. The polyacetate rule: pathways to polyphenolic compounds.

3.1.8 Searching for a DIF-like nucleus

To get some idea on the relative positions of hydroxyls on the benzene ring, a range of commercially available hydroxy ketones, hydroxy aldehydes and hydroxy benzoic acids were tested for bioactivity.

A simple chlorinating solution was prepared and the various phenols were chlorinated. This would serve two purposes; firstly, the products could be visualised more easily on the mass spectrometer due to the molecular ion clusters and, secondly, if the original compound had chlorine in it, these analogues may be active in the bioassay system if they are chlorinated. Table 4 lists some of the compounds tested under bioassay and analysed for similar fragmentation patterns to DIF. The results from the bioassay were very inconclusive. The only compounds to show DIF activity were 2,4-dichlorophenoxyacetic acid (a plant growth regulator) and to a lesser extent 2,4,6-trihydroxybenzoic acid. It was impossible to say whether the lack of bioactivity in the majority of the compounds was due to the absence of an alkyl chain or free hydroxyls and chlorine in the same molecule.

However, the mass spectra of these compounds were much more revealing. 3,5-Dichloro-2,4,6-trimethoxyacetophenone gave a strong peak at m/z 263 with the correct chlorine isotope ratio. Here was a sign that one of the **compounds** had a similar nucleus to DIF. The 2,4,6-positioning of oxygen atoms was in accordance with the compounds being derived from a polyacetate biosynthesis.

2,3,4-trimethoxyacetophenone

2,3-dichloro-4,5,6-trimethoxyacetophenone

3,4,5-trimethoxyacetophenone

2,6-dichloro-3,4,5-trimethoxyacetophenone

2,4,6-trimethoxyacetophenone

3,5-dichloro-2,4,6-trimethoxyacetophenone

2,4,6-trimethoxybenzaldehyde

2,4,6-trihydroxybenzoic acid

1,3,5-trihydroxybenzene (phloroglucinol)

1-chloro-2,4-dinitrobenzene

3,4-dichloronitrobenzene

2,4-dichlorophenoxyacetic acid

2,4,5-trihydroxybutyrophenone

Table 4. Some phenolic compounds analysed by bioassay and EI-MS to determine the ring substitution pattern in DIF.

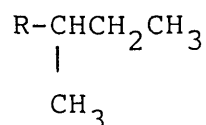
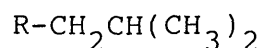
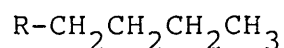
This revelation left a few questions, concerning the structure of DIF, unanswered. If there was a methoxy group, was it ortho or para to the carbonyl function? Was the C_5H_{11} alkyl moiety a straight chain or could it be an isopentyl or even a tert-butyl chain? The latter seemed very unlikely, as it was difficult to show a facile loss of 43 mass units ($C_3H_7^\bullet$) from the structure.

These discrepancies could only be clarified by synthesis of all possible isomers!

3.1.9 DIF-2

While the structure elucidation of DIF was being undertaken some preliminary experiments were done on a chemically related species designated DIF-2 (54). Kay et al had shown that DIF-2 was more polar than DIF-1 by separation on HPLC (Fig. 12). The molecular ion was 14 mass units less than DIF-1 which implied it may be lower in a homologous series. Indeed, the fragmentation pattern indicated that DIF-2 had a C_4H_9 alkyl chain, as opposed to DIF-1 which contained a C_5H_{11} . The EI mass spectrum is shown in Fig. 13. Ions corresponding to γ -cleavage, McLafferty rearrangement and loss of C_4H_9 to produce the acylium ion were present as in DIF-1 but there was no fragment ion present due to loss of water, ie. one would expect an ion at 274 a.m.u. This raised the question, does the fragmentation of the alkyl chain affect the fragmentation of the DIF nucleus with respect to loss of water? The answer to this question would, perhaps, explain or provide a mechanism for the generation of this fragment ion.

There were three possible isomers for a C_4H_9 alkyl chain shown below.



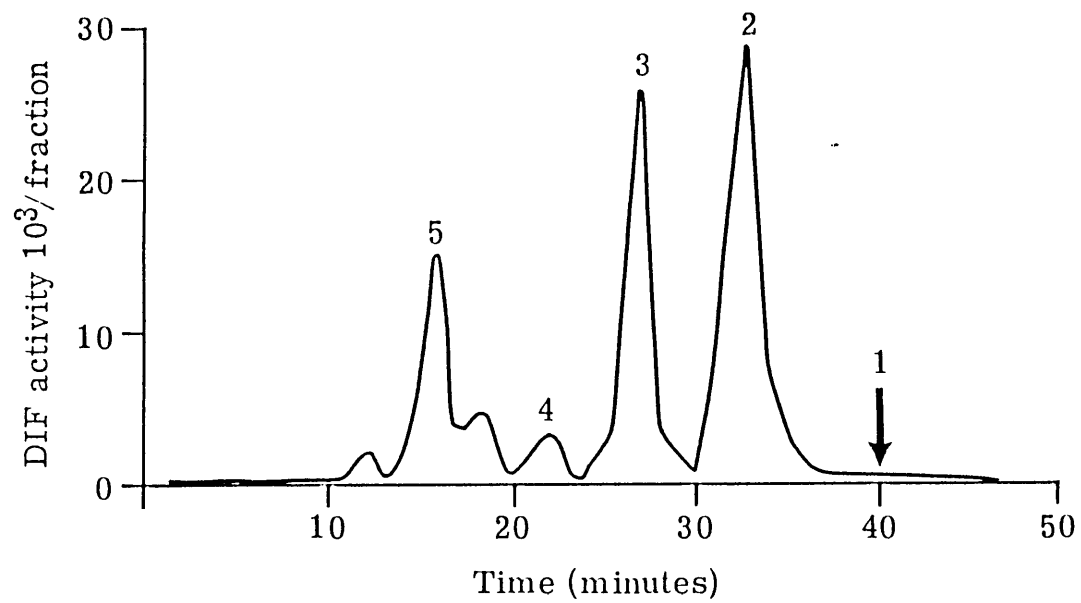


Fig. 12. Separation of minor DIF species by HPLC.
(DIF-1 elutes at position 1).

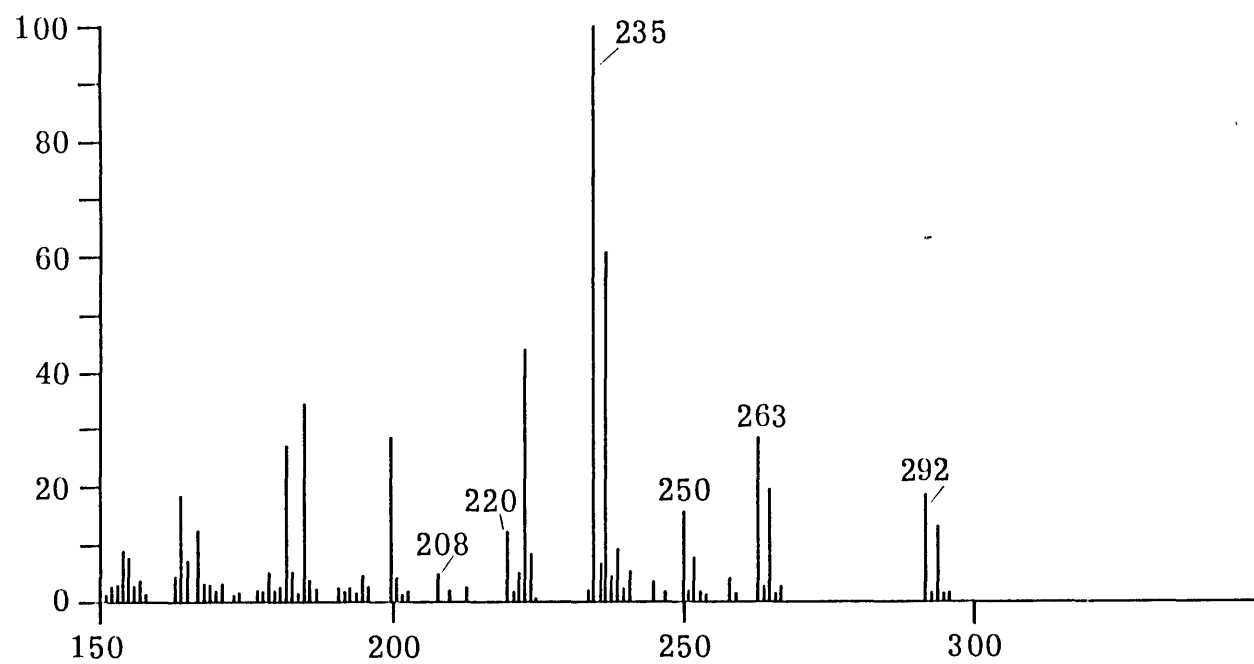


Fig. 13. Electron Impact mass spectrum of DIF-2.

As suggested for DIF-1, it would be necessary to synthesise all these isomers to definitively state which structure was correct.

3.2 Syntheses

The availability of reagents was the main criterion for the choice of synthetic method used. Apart from the Grignard synthesis (see below) the reagents for DIF-2 synthesis were more readily available at the outset of this study than those for DIF-1. As new materials became commercially available they were either incorporated into the current synthesis or a new synthetic method was adopted.

3.2.1 Synthesis I : Grignard synthesis

One of the aspects of the DIF-1 structure that was not clear was whether the C_5H_{11} was in the normal C_5 configuration or the isopentyl configuration. It was thought that the isopentyl structure would be favoured as a result of isoprenylation of a precursor and subsequent hydrogenation of the double bond. The facile loss of 43 mass unit also suggested an isoalkyl chain. The occurrence of isoprenoid derivatives of orcinol has been well characterised (68,69). Therefore, the isopentyl derivative was used initially. The reaction scheme was intended to generate a C_5H_{11} benzylic alcohol, oxidise with activated manganese dioxide and finally chlorinate. This should produce the same

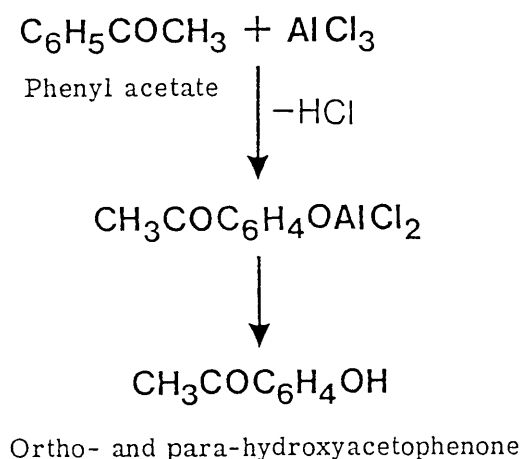
spectrum as DIF dimethyl ether.

The organic products were separated from the magnesium salts by precipitation and solvent extraction. The products were analysed by GC-MS and it was found that the major component had a molecular ion of m/z 250, 18 mass units less than that predicted. Either the product had readily eliminated water to produce a styrene type compound, or the process of gas chromatography had caused water to be eliminated. The spectrum showed a loss of 43 mass units which was concurrent with γ -cleavage or loss of isopropyl moiety. The product was mixed with activated MnO_2 but no visible reaction occurred and no change in mass spectrum was recorded. Chlorination produced an isotopic pattern which suggested that four chlorines had been introduced into the molecule which confirms the presence of an aliphatic double bond. This was a similar result to that observed when DIF-1 was reduced with borohydride. It was therefore necessary to find an alternative method of ring acylation.

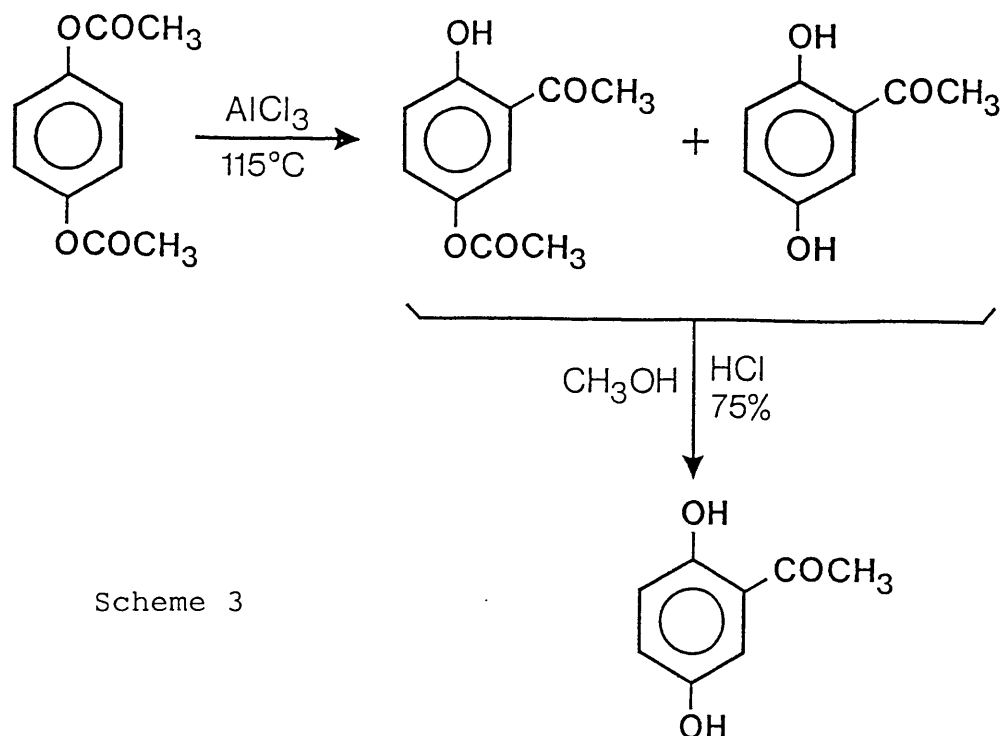
3.2.2 Synthesis II : Fries Reaction

3.2.2.1 Theory and usage

In 1880, Fries discovered that an acyl derivative of a phenol, when heated with aluminium chloride is converted into the isomeric σ or p-hydroxy ketone or more often into a mixture of both. The material is in the reaction mixture as the aluminium chloride salt and the hydroxy compound is liberated on hydrolysis with ice and hydrochloric acid. The reaction is outlined below. (Scheme 2).

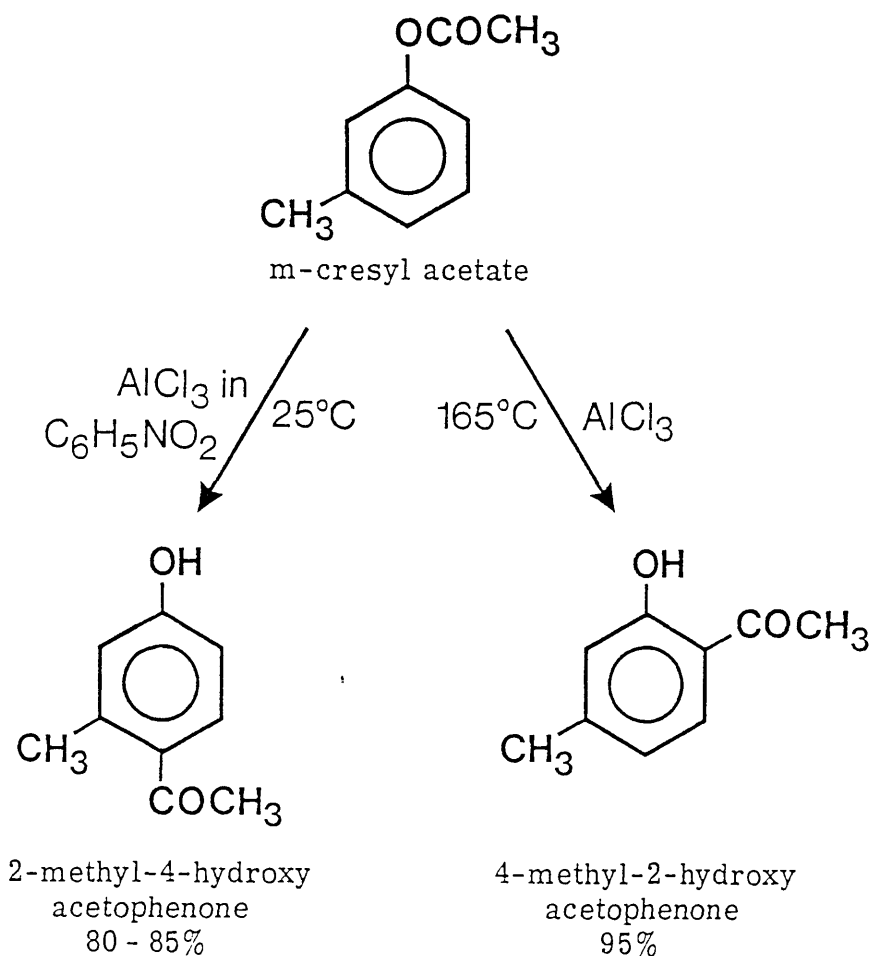


An improved procedure was developed by Reynolds et al (70) as part of their synthesis of vinylhydroquinone diacetate. The Fries rearrangement incorporated in the synthesis was as follows:- (Scheme 3)



The advantages of this method are that it is easily reproducible, the yields are higher and a purer product is obtained. Any unconverted starting material is removed as water-soluble hydroquinone.

m-Hydroxy ketones occur rarely as products of the Fries reaction. The major variation is in the relative amounts of ortho and para isomers. The ratio varies with solvent and amount of catalyst and is particularly influenced by the temperature at which the reaction takes place. This can be illustrated by the behaviour of m-cresyl acetate, which can be converted into either of two products in good yield by adjustment of temperature. (Scheme 4).

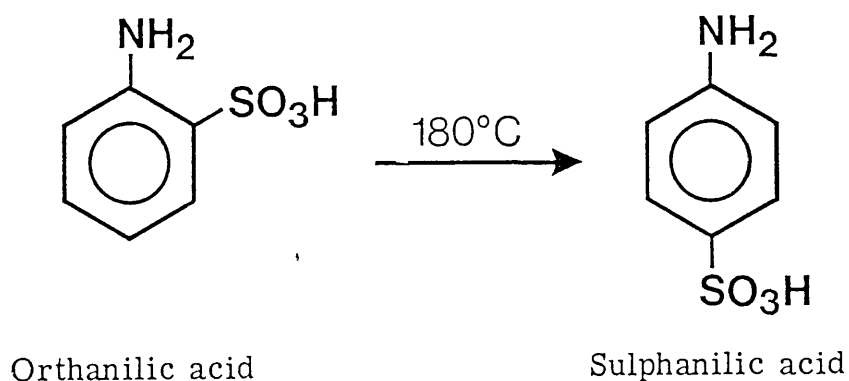


Scheme 4

Generally, a low temperature favours para substitution and a high temperature favours ortho substitution.

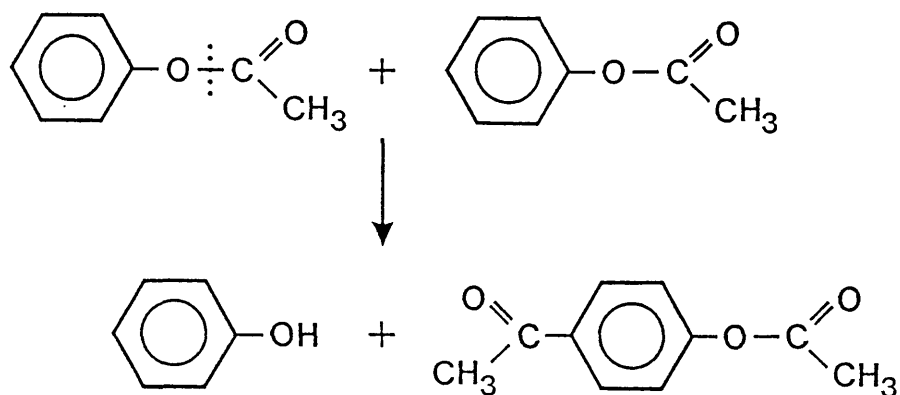
This orientation is opposite to that encountered in Friedel-Crafts condensation with substituted hydrocarbons, where the extent of attack in a hindered ortho position decreases with rise in temperature and hence the Fries reaction is not adequately interpreted on the hypothesis that the ester is merely cleaved to the phenoxyaluminium chloride and acetyl chloride, with interaction of the components as in a usual Friedel-Crafts reaction. That the *o*-hydroxy ketone, in the form of the aluminium chloride complex, accumulates at a high temperature in preference to the

σ -isomer contrasts with the rearrangement of the heat-labile orthanilic acid to the more heat stable para-isomer, sulphanic acid. (Scheme 5).



Scheme 5

It appears unlikely that the Fries reaction proceeds by intramolecular migration of the acyl group, for the primary low-temperature product is not the σ -isomer expected by analogy. A more plausible mechanism is that of an intermolecular acylation, ie. substitution in one molecule of an acyl group originating from another, with the formation of the p-hydroxy ketone as the normal product because of the greater accessibility of the p-position. (Scheme 6).



Scheme 6

In analogy with the known reversibility of other reactions of the Friedel-Crafts type, it would be inferred that the formation of the σ -hydroxy ketone at high temperatures may result from reversion of the normal p-compound to the original ester and a re-introduction of the acyl group in the σ -position. It is known that the Fries reaction can be reversed by acids and that σ -hydroxy ketones can be isomerised to the σ -isomer by aluminium chloride at high temperatures.

The σ -isomer may predominate at high temperatures because of its greater stability. This can be attributed to the presence of a chelate ring in the aluminium chloride complex produced in the reaction. This interpretation is supported by the fact that the free σ -hydroxy ketones are chelated.

Consequently, the σ -hydroxy ketones tend to be more volatile than the p-hydroxy isomers.

The distinct differences in properties associated with presence or absence of chelation facilitate separation of the mixtures ordinarily obtained in Fries reactions. The σ -isomer can often be removed selectively by steam distillation, or by fractional distillation. If the substances are slightly volatile the p-isomer can usually be separated by crystallization. Hence the Fries reaction has practical preparative value and provides a convenient route to both σ - and ρ -acyl derivatives of phenols. However this preparative value cannot be exploited if the products of synthesis contain both σ - and ρ -hydroxyls.

3.2.2.2 Synthesis of DIF-2

The previous attempt at synthesis of a DIF-like compound would have produced a fully methylated species. A compound became commercially available which could be acylated and still retain two free hydroxyls. The compound, 3,5-dihydroxyanisole (3,5 DHA) (phloroglucinol monomethyl ether) is shown in Fig. 14).

The first synthesis to be tried with this compound was the esterification with isovaleroyl chloride ($(\text{CH}_3)_2\text{CHCH}_2\text{CH}_2\text{COCl}$) this would ultimately lead to the synthesis of DIF-2 with an isobutyl side chain. (The higher homologue isocaproyl chloride was not commercially available at this time).

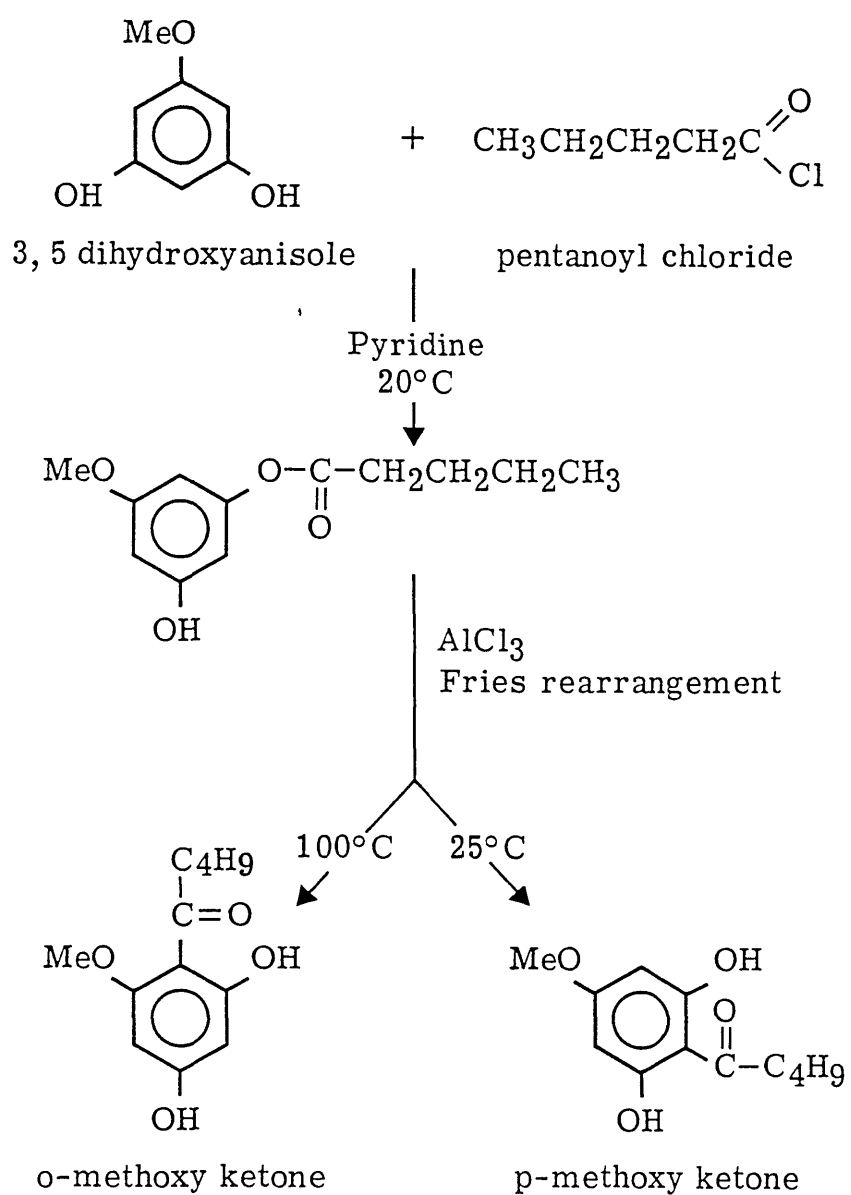


Fig. 14. Synthesis of the pentyl ketones of 3,5 - dihydroxyanisole via the Fries reaction.

One equivalent of 3,5-DHA and isovaleroyl chloride were reacted in pyridine for one hour over a steam bath (100°C). Excess pyridine was removed from the reaction mixture by rotary evaporation. The products were analysed by both direct probe EI-MS and by GC-MS.

GC-MS showed that there were three compounds in the final mixture, unreacted 3,5-DHA, a monoester and a diester. The latter two were in approximately equal amounts.

The monoester spectrum showed some characteristic fragments; loss of $\text{CH}_3^{\cdot}$, $\text{C}_2\text{H}_5^{\cdot}$, $\text{C}_3\text{H}_7^{\cdot}$ and $\text{C}_4\text{H}_9^{\cdot}$. The most facile loss would be from cleavage of the ester bond. This should result in a large peak at m/z 139 but the smaller fragment, COC_4H_9 , can produce a charged acylium ion, therefore although a m/z 139 fragment is produced it is uncharged and not detected by the spectrometer. A large peak at m/z 85 was seen corresponding to the afore-mentioned COC_4H_9 acylium ion. A sample of the products was chlorinated after Fries rearrangement and analysed by EI-MS and GC-MS. Table 5 shows a comparison of the fragments from natural DIF-2 and synthetic DIF-2. The pattern of losses of methyls and water molecules was different. Furthermore, from these fragmentation patterns it is clear that synthetic DIF-2 is not the same as natural DIF-2. (Fig. 13).

Natural DIF-2	m/z	Synthetic DIF-2
molecular ion	292	molecular ion
--	277	$M - CH_3^\bullet$
$M - C_2H_5^\bullet$	263	-
	259	$277 - H_2O$
McLafferty	250	McLafferty
$263 - H_2O$	245	-
-	244	$259 - CH_3^\bullet$
$M - C_4H_9^\bullet$	235	$M - C_4H_9$
$235 - CH_3^\bullet$	220	$235 - CH_3^\bullet$

Table 5. Fragment ions from natural DIF-2 and a synthetic DIF-2 compound 4.

The chlorinated mixture was purified on HPLC by R R Kay. From several peaks only two bioactivities were found. The major peak of activity was re-run on HPLC and found not to elute in the same position as natural DIF-2. However, it did possess very high bioactivity compared with natural DIF-2. Clearly the isobutyl ketone was not the correct side chain in DIF-2, but the fact that there was high bioactivity may provide information on the exact structural requirements for a putative receptor. It was not clear from the HPLC whether the minor activity corresponded to a different isomer or a monochloro compound.

Having already discounted the sec-butyl isomer on the premise that the McLafferty rearrangement would produce an ion at m/z 264 (loss $H_2C = CH_2$) and this is not seen in natural DIF-2, it was necessary to synthesise the n-butyl ketone of 3,5-DHA.

In order to reduce the amount of diester produced, an excess of the phenol was used.

Valeroyl chloride (pentanoyl chloride) was used to make the n-butyl ester of 3,5-DHA (Fig. 14). It was noted that the 3,5-DHA contained a condensation product of two molecules of 3,5-DHA. EI-MS analysis of the n-butyl ester, showed that the mono and diester had been produced and also the mono-ester of the contaminated diphenylether. The esterification had produced a white crystalline precipitate and a yellow liquor.

It has been reported that a relatively low temperature favours the para-hydroxy ketone (71). Thus, the crystals were filtered from the liquor and the Fries rearrangement reaction was kept at 25°C. The products separated into the organic layer after neutralisation of the AlCl₃. A sample was removed and chlorinated. The EI-MS spectrum showed two major products, a trichloro, 3,5 DHA (m/z 242) and a trichloro n-butyl ester of 3,5 DHA, m/z 326. Both had the characteristic 27:27:9:1 isotope ratio for a trichloro compound (Fig. 4). It was thought that the reaction had not proceeded appreciably and that these signals were 'swamping' the chlorinated ketone signal. The ester could have been removed by acid or base-catalysed hydrolysis but this would have no effect on the trichloro 3,5-DHA. The Fries reaction was done on the liquor. It was noticeable that the liquor had a sweet, pungent smell, whereas the product, after Fries, did not smell sweet at all.

A sample was removed and chlorinated as before. Table 6 summarises the complex EI-spectrum. The various components were separated and analysed by GC-MS. The dichloro ketone (m/z 292) had an identical spectrum to natural DIF-2.

The chlorinated mixture was run on HPLC. 1 ml fractions were collected, dried down and bioassayed. A major peak of activity was detected at $t_R = 41-43$ mins. This coincided with a peak having a DIF-2 spectrum. A minor activity was detected at $t_R = 28-29$ which corresponded to a monochloro product. Therefore, the dichloro n-butyl ketone of 3,5 DHA had been shown to have considerable bioactivity

m/z	
326 (q)	trichloroester/ketone
308 (s)	diketone/diester
292 (t)	M ⁺ dichloro C ₄ H ₉ ketone 3,5-DHA
263 (t)	292 - C ₂ H ₅ [•]
258 (d)	M ⁺ monochloro ketone 3,5-DHA
250 (t)	292 - C ₃ H ₆ McLafferty
242 (q)	trichloro 3,5-DHA
235 (t)	292 - C ₄ H ₉ [•]
229 (d)	258 - C ₂ H ₅ [•]
224 (s)	ester/ketone
216 (d)	258 - C ₃ H ₆ McLafferty
208 (t)	292 - COC ₄ H ₉ [•] H transfer
201 (d)	258 - C ₄ H ₉ [•]
174 (d)	258 - COC ₄ H ₉ H transfer
167 (s)	224 - C ₄ H ₉ [•]
140 (s)	3,5-DHA

Table 6. Major fragment ions from the Electron Impact mass spectrum of the chlorinated products of the Fries reaction.

and an identical mass spectrum to DIF-2. There was no reason to question whether this was truly DIF-2. Much emphasis had been placed on synthesising DIF-2, although DIF-1 was the real goal. Toward the end of these studies on DIF-2 a new synthetic method was applied to DIF synthesis. As previously stated neither caproyl chloride or isocaproyl chloride were commercially available so a new method had to be found exploiting a different functional moiety during synthesis. This occurred in the Hoesch synthesis, a variation on the Fries reaction.

3.2.3 Synthesis III : Hoesch synthesis

3.2.3.1 Theory and usage

The Hoesch synthesis is the condensation of a nitrile with a phenol, polyhydric phenol or a phenolic ether to produce a hydroxyaryl or alkoxyaryl ketone (72-74).

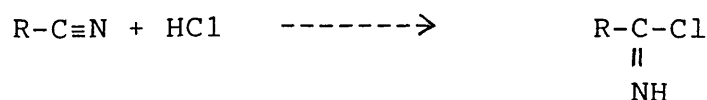
Equimolar quantities of such reactants are dissolved in cold, dry ether. Zinc chloride or ferric chloride is used as a catalyst and dry hydrogen chloride is introduced.

This synthesis, which is an extension of the Gatterman aldehyde reaction (75,76), is closely related to several well-known procedures leading to aryl ketones, such as the Fries (77) and the Nencki (78) reactions.

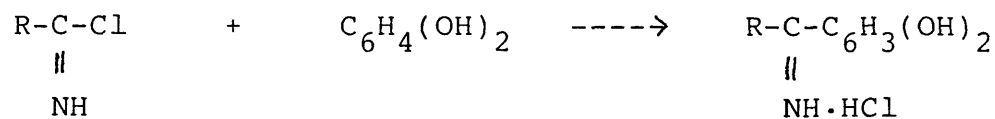
The Hoesch synthesis has proved to be the most convenient synthetic method for certain polyhydroxyacylphenones and polyhydroxybenzophenones. The Hoesch reaction has been used for the identification of nitriles by producing solid derivatives with phloroglucinol (79).

The reaction appears to involve three separate steps:

1) Formation of imino chlorides



- 2) Interaction of imino chlorides with phenol to give Ketimine hydrochlorides



- 3) Hydrolysis of Ketimine hydrochlorides to ketones.



Various procedures are described involving different time factors for the completion of the reaction. Sometimes the reaction mixture has been held at 0°C for a few hours to a few days (see experimental and discussion).

Dry ether is the most suitable solvent in terms of high yield of product. Glacial acetic acid is a possible substitute for ether and it is a better solvent for imino chlorides (80) but the yields of ketone are usually lower. Other solvents have been used successfully including chloroform/ether, methyl acetate and ethyl bromide (81). Acetic anhydride, dilane, amyl ether and benzene have been reported as unsuitable (81).

Anhydrous zinc chloride is a desirable though not indispensable catalyst (81,82). Generally the yields are better if a catalyst is used (81). Ferric chloride sometimes has advantages over zinc chloride, but

comparative experiments have not been sufficiently numerous to make it possible to predict which is to be preferred. Aluminium chloride, a more powerful catalyst, is sometimes necessary.

When the reaction is complete the hydrolysis and isolation of the ketone may be accomplished in a number of ways. If the Ketimine hydrochloride is very insoluble, it may be filtered from the ether and hydrolyzed. It has been reported that the Ketimine hydrochlorides may be converted into the less soluble sulphates by dissolving the hydrochlorides in water and adding dilute sulphuric acid or aqueous ammonium sulphate (72).

the reaction mixture containing the Ketimine hydrochloride can be treated with water and the ether layer removed. The aqueous solution is then heated and the ketone which separates is filtered or extracted with a solvent.

Table 7 lists the ketones, reported in the literature, synthesised by the Hoesch method with relevance to the project herein.

Reactants	Products	Ref.
Orcinol		
CH_3CN	2,4-dihydroxy-6-methylacetophenone	3a
$\text{C}_6\text{H}_5\text{CH}_2\text{CH}_2\text{CN}$	2,4-dihydroxy-6-methyl- β -phenylpropriophenone	71
Orcinol monomethyl ether		
CH_3CN	2-hydroxy-4-methoxy-6-methylacetophenone & 4-hydroxy-2-methoxy-6-methylacetophenone	3a
Phloroglucinol		
CH_3CN	2,4,6-trihydroxyacetophenone	3a,57
$\text{C}_2\text{H}_5\text{CN}$	2,4,6-trihydroxypropriophenone	84
$\text{n-C}_3\text{H}_7\text{CN}$	2,4,6-trihydroxybutyrophenone	84,85
$\text{iso-C}_3\text{H}_7\text{CN}$	2,4,6-trihydroxyisobutyrophenone	84
$\text{n-C}_4\text{H}_9\text{CN}$	2,4,6-trihydroxyvalerophenone	86
$\text{iso-C}_4\text{H}_9\text{CN}$	2,4,6-trihydroxyisovalerophenone	86
$\text{n-C}_5\text{H}_{11}\text{CN}$	2,4,6-trihydroxycaprophenone	69b,84
$\text{iso-C}_5\text{H}_{11}\text{CN}$	2,4,6-trihydroxyisocaprophenone	84
$\text{CH}_3\text{OCH}_2\text{CN}$	2,4,6-trihydroxy- ω -methoxyacetophenone	19,57b

Table 7. Polyhydroxyphenones synthesised via the Hoesch Reaction (83)

Reactants	Products	Ref.
Phloroglucinol monomethyl ether		
$n\text{-C}_3\text{H}_7\text{CN}$	4,6-dihydroxy-2-methoxybutyrophenone	85a
$\text{C}_6\text{H}_5\text{CN}$	2,6-dihydroxy-4-methoxybenzophenone	95
$\text{CH}_3\text{OCH}_2\text{CN}$	2,4-dihydroxy- ω , 6-dimethoxyacetophenone	96
Phloroglucinol dimethyl ether		
CH_3CN	2-hydroxy-4,6-dimethoxyacetophenone	18
$\text{C}_6\text{H}_5\text{CN}$	2- and 4-hydroxy-2,6-dimethoxybenzophenone	98
$\text{CH}_3\text{OCH}_2\text{CN}$	ω ,2,4-trimethoxy-6-hydroxyacetophenone & ω ,2,6-trimethoxy-4-hydroxyacetophenone	97
Phloroglucinol trimethyl ether		
CH_3CN	2,4,6-trimethoxyacetophenone	13,18

Table 7 (continued)

3.2.3.1 DIF-1 synthesis : Isopentyl side chain

The idea that DIF-1 biosynthesis involved an isoprenylation step (see 3.2.1) had not been discounted and it was thought that DIF-2, with an n-butyl side chain, may arise from a demethylation of DIF-1. Consequently, the first Hoesch synthesis was intended to produce the isoamyl ketone of 3,5 DHA.

The required nitrile, isohexanonitrile, was not commercially available so it was necessary to synthesise it from the alkyl bromide. The synthesis yielded 0.2 moles of the nitrile (22%). The product was analysed by IR spectroscopy. A peak at 2250 cm^{-1} indicated the presence of a nitrile group. However, the smell of the product suggested some isonitrile was also present.

The nitrile and 3,5 DHA were reacted, as shown in Fig. 15. The production of the Ketimine chloride required 72 hours. At the end of the reaction a sample of the Ketimine was analysed by EI-MS. A molecular ion was seen at 237 a.m.u. Fragmentations corresponding to loss of $\text{C}_3\text{H}_7^\cdot$ and $\text{C}_5\text{H}_{11}^\cdot$ were also noted.

Some of the precipitate and supernatant did not readily dissolve in boiling water. Up to 20% (final conc. v:v) methanol was used to dissolve the water-insoluble products. Hydrolysis of the solution produced three components; an orange oil, that separated on filtering and solidified on cooling; a feathery, off-white precipitate that

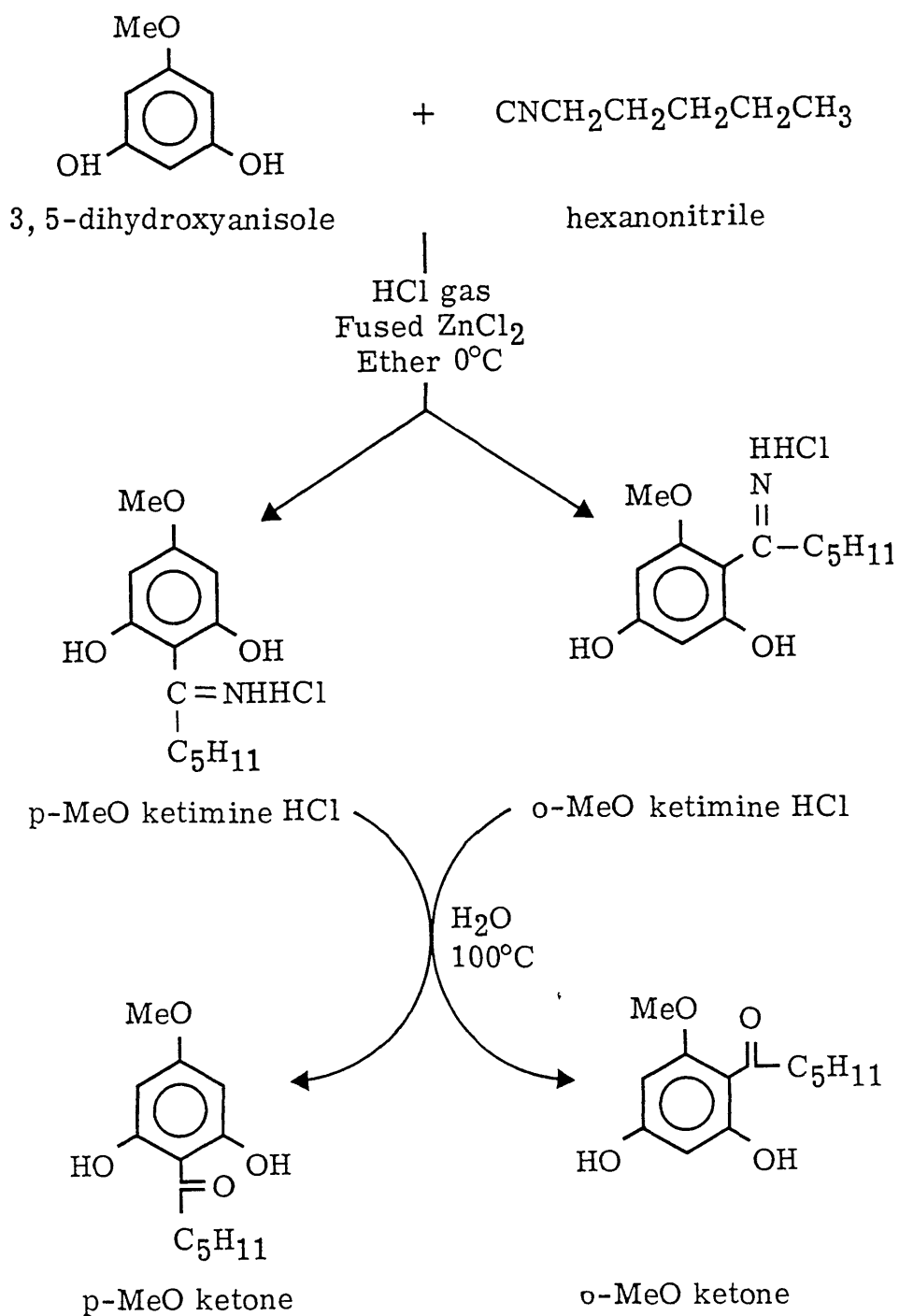


Fig. 15. Synthesis of the pentyl ketones of
 3,5 - dihydroxyanisole via the
 Hoesch reaction.

developed as the filtrate cooled and the yellow liquor itself. The off-white precipitate was analysed by EI-MS and found to have a characteristic fragmentation pattern. The molecular ion was detected at m/z 238. Major fragment ions occurred at m/z 195, 182, 167 and 152, these corresponded to loss of $C_3H_7^{\cdot}$, loss of C_4H_8 (McLafferty), loss of $C_5H_{11}^{\cdot}$ and loss of $CH_3^{\cdot}$ from 167, respectively.

A contaminating peak at 140 was also apparent; this corresponded to the unreacted 3,5 DHA. A sample was chlorinated and shown to have a DIF-1-like spectrum, however, this was contaminated with monochloro peaks and trichloro peaks. This mixture was purified on HPLC and analysed by EI-MS, GC-MS and bioassay.

HPLC of the mixture produced a number of peaks, two of which had DIF-1-like spectra ($t_R = 32-35$ (Peak 1) and $t_R = 40-42$ (peak 2)). These spectra showed peaks at m/z 306 (molecular ion), 263, 250, 235 and 220. The fragments corresponded to the same losses as the unchlorinated ketone, only 68 mass units higher. The EI spectra showed that the two compounds had the same fragmentation pattern but the relative abundance of these fragment ions was different. It seemed that two different isomers had been synthesised. U.V. analysis showed that peak 1 had a very different absorbance curve to natural DIF-1, whereas peak 2 had a similar curve but not identical to DIF-1.

Bioassay of all the HPLC fractions showed a major activity that coincided with peak 2 and a minor activity that

coincided with a monochloro compound. Within the sensitivity range of the bioassay it appeared that the 'other isomer' was completely inactive.

GC-MS separated the two peaks and gave a clear indication of the differences in fragmentation of the two isomers; in peak 1, the molecular ion is about twice as abundant as the ion resulting from a McLafferty rearrangement, whereas in peak 2 they are about the same size. The ratios of m/z 235:263 also differ in the two isomers. Over and above this data, DIF-1 had not been synthesised. None of the spectra indicated a fragment ion due to loss of water. It was originally thought that this may be an artefact, but since it appeared in DIF and DIF derivatives, it was clearly related to the DIF molecule. The pattern of synthesis of DIF-1 looked like following the pattern of DIF-2. In both cases an isoalkyl side chain analogue had been shown to possess bioactivity but exhibit different fragmentation patterns. Both eluted very closely to their respective natural analogues but did not co-elute. Of the remaining four isomers with C_5H_{11} side chains the methyl on the α -carbon could be discounted as it would give a different McLafferty rearrangement. The two dimethyl propane isomers could also be discounted as neither would produce an $M - C_3H_7^{\cdot}$ in preference to an $M - C_2H_5^{\cdot}$ or $M - CH_3^{\cdot}$. Consequently, the n-pentyl side chain seems the only further candidate for the DIF-1 structure.

3.2.3.3 DIF-1 synthesis: n-Pentyl side chain

The n-pentyl ketone derivative of 3,5-DHA was synthesised in the same manner as the isopentyl derivative (Fig. 15), except that it was unnecessary to synthesise the nitrile from the alkyl bromide. Once again, the reaction was allowed to proceed for 72 hours. After hydrolysis, several grams of the orange solid were produced. This material had an extremely unpleasant smell and had to be kept in a fume cupboard. The strong colour of this material suggested extended conjugation, and was initially thought to be a diketone product. The yield of off-white precipitate was very low (about 30 mgs).

GC-MS analysis of this product showed the presence of two major components. Both components had similar mass spectra to the isopentyl ketone, ie. loss of $C_3H_7^+$, C_4H_8 and $C_5H_{11}^+$. However, there was now a strong ion at m/z 220 which corresponded to the loss of a molecule of water. A low abundance ion was also detected at m/z 205 which corresponds to loss of CH_3^+ from the 210 fragment ion. An ion of m/z 177 suggested that the 220 fragment ion could also lose $C_3H_7^+$. There was no evidence of a loss through a McLafferty rearrangement, nor a loss of $C_5H_{11}^+$ from the m/z 220 ion.

The two peaks themselves had very similar mass spectra. The only discernible differences between the two were the ratios of the molecular ion to the ion arising from McLafferty rearrangement, and the loss of CH_3 (m/z 152)

from the major fragment ion at m/z 167. The mass spectrum of the first peak that eluted from the GC showed the m/z 238 molecular ion was more abundant than the ion at m/z 182 (McLafferty loss of C_4H_8) while in the second eluted peak the ion at m/z 182 was greater than m/z 238. The former eluting peak had a significant fragment ion at m/z 152 whereas this ion was barely detectable above the baseline in the later eluting peak.

The ratio of the two components was approximately 6:1, the earlier eluting peak being more abundant.

It was clear that both the expected isomers had been synthesised (Fig. 15) but it was still not possible to state definitively which was the para-isomer and which was the ortho-isomer.

Chlorination of the ketone mixture produced a mixture of mono-, di- and trichloro derivatives. Analysis by EI-MS gave a spectrum very similar to natural DIF (m/z 306, 288, 263, 250, 235 and 220) although the peak at m/z 250 was rather low compared with the molecular ion. This would be expected as the majority of the dichlorinated material produced a low abundance ion at m/z 250. The dichloro signals were of low abundance compared to those arising from higher chlorinated species.

The initial intention was to purify the two isomers such that they could be distinguished by n.m.r. Unfortunately,

the usual running conditions of the HPLC (see Methods) could not entirely resolve the two unchlorinated components. Even changing the propanol concentration to 25% did not produce any significant change in the HPLC profile. A considerable number of HPLC runs were performed on the ketone, since it was hoped that side fractions could be used. After 75 runs it seemed clear that this procedure was not 'cost effective' as far as time and solvents were concerned.

It was subsequently found that chlorination of the ketone mixture enabled the two components to be resolved by the original HPLC conditions.

It was decided to scale up the synthesis to produce more of the off-white precipitate and also reduce the amount of orange product - the supposed diketone.

The reaction was scaled up three-fold. More off-white material was collected but it was subsequently found that the orange product also contained a large amount of ketone (m/z 238).

The unchlorinated orange material was run on the HPLC and, although there were a few more components, the two 'monoketones' were resolved from other components but not from each other. It was felt that if the orange material was chlorinated, it was likely that a considerable number of peaks would be produced and this would render purification of the two chlorinated ketone isomers much more difficult. Therefore, the two unchlorinated ketones were collected and chlorinated. The chlorinated ketone mix produced several

peaks corresponding to the various mono-, di- and tri-chlorinated isomers.

Fortunately, the two dichloro ketones were completely resolved from the other components but occurred in low amounts relative to the higher chlorinated species. It soon became clear that, although this method of two HPLC purifications produced pure samples of the potential DIF isomers, it would take a considerable time to produce enough material for n.m.r. It was therefore intended to chlorinate the orange material and, if necessary, attempt to create running conditions that would separate the two isomers.

It was rather surprising that the products of chlorination of the orange material gave an HPLC profile that enabled the two components with DIF-like mass spectra to be isolated.

Before a large production of these two isomers could be effected a new compound became commercially available - n-hexanoyl chloride. Considering the Fries reaction had been successful in the synthesis of DIF-2, it was thought that the yield of ketones could be enhanced by adopting a 'proven' synthesis. It ought to be stated that the structure of DIF-1 was thought more likely to be the para-methoxy derivative. Therefore the Fries reaction was kept at 25°C which favours the para-isomer. As expected the reaction produced more of the para-isomer than the other isomer but the conversion of the ester to the ketone was not very effective. The products from the rearrangement contained about 10% ketone and 90% ester. Several options were available;

the mixture could be treated again with AlCl_3 to effect further conversion or the ester could be removed by base hydrolysis or this particular synthetic method could temporarily be shelved as a separation of two DIF-1-like compounds had been achieved from the Hoesch synthesis. The last option was chosen.

The HPLC profile of the chlorinated orange product showed several species. In the past, the EI spectrum of the products had shown mainly higher chlorinated species, this was thought to be due to using a too highly concentrated chlorinating solution. The solution was diluted and it soon became apparent that it was possible to produce a high proportion of the desired dichloro derivatives by gradual chlorination. Over-chlorination was also reduced by dissolving the sample in a large volume of chloroform. The fact that the chlorinating solution was immiscible with the sample solution afforded a more 'gentle' reaction.

The two peaks with DIF-like mass spectra eluted at $t_R = 30\text{-}32\text{mins}$ (major component, designated peak-1) and $t_R = 38\text{-}40\text{ mins}$ (minor component, designated peak-2). HPLC fractions were analysed by bioassay. Fig. 16 shows the bioactivity in the HPLC fractions. A minor activity was shown corresponding to an unresolved peak at $t_R = 26\text{ mins}$ which was thought to correspond to a monochloro derivative. The major activity corresponded to the later-eluting peak-2. This followed a similar pattern to DIF-2 (section 3.2.2.2).

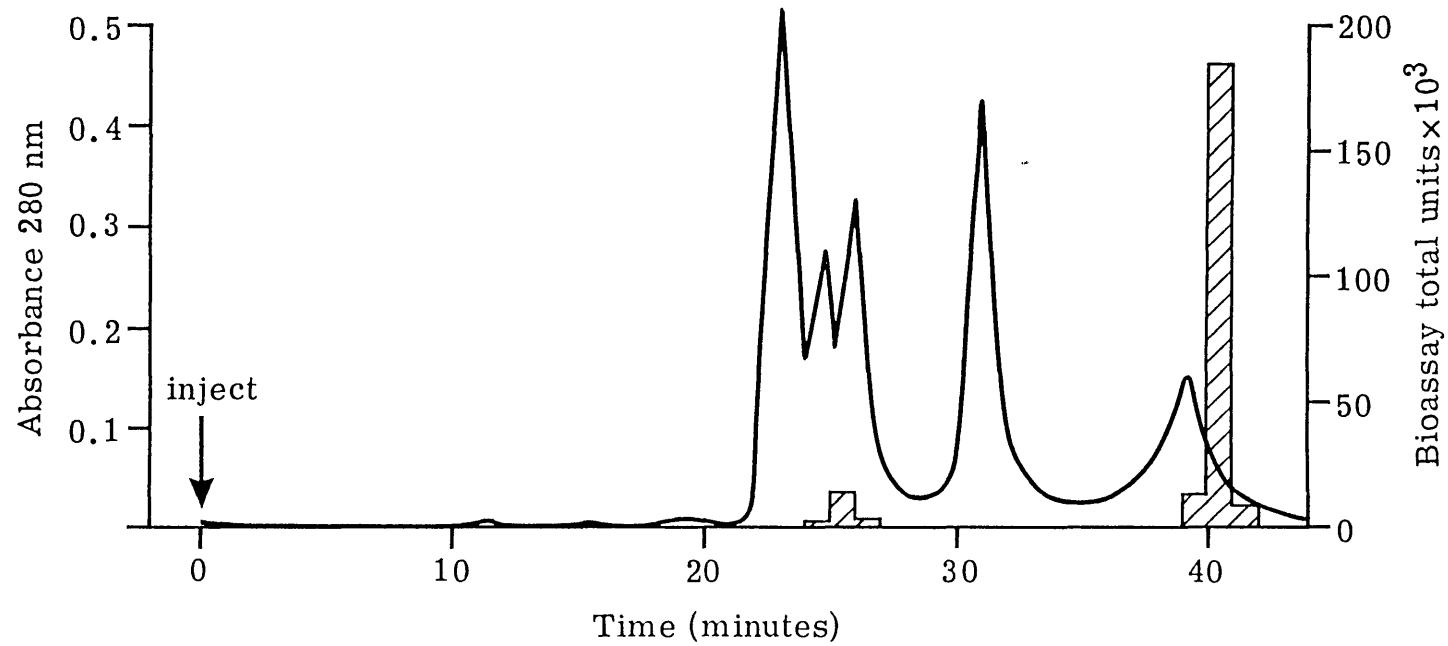


Fig. 16. HPLC elution profile of chlorinated pentyl ketones of 3,5 - DHA, exhibiting DIF activity.

The most interesting point to arise from analysing these two dichloro peaks was the difference in their U.V. spectrum. Fig. 17 shows the U.V. spectra of peak-1 and peak-2 run in methanol. There is clearly a considerable difference in the spectra which appears to be attributable to the position of an O-methyl group. Peak-1 had two peaks of absorbance ($\lambda_{\max}$) at 335 and 258 nm. Peak-2 had an absorbance peak at 279 nm. It is not possible to determine the extinction coefficient for these two isomers until enough material is available to make a solution of known concentration.

The evidence from EI-MS, GC-MS, U.V. and bioassay gave considerable support to the implication that peak-2 and DIF-1 are the same compound. The statement is not unequivocal and it would be necessary to compare ^1H -n.m.r. spectra of the natural compound and the synthetic material. It was clearly obvious that milligrams of material would provide the answer to the remaining question ... Is DIF-1 the ortho or para-methyl derivative of the dichloro, n-pentyl ketone of phloroglucinol (1,3,5 trihydroxybenzene)?

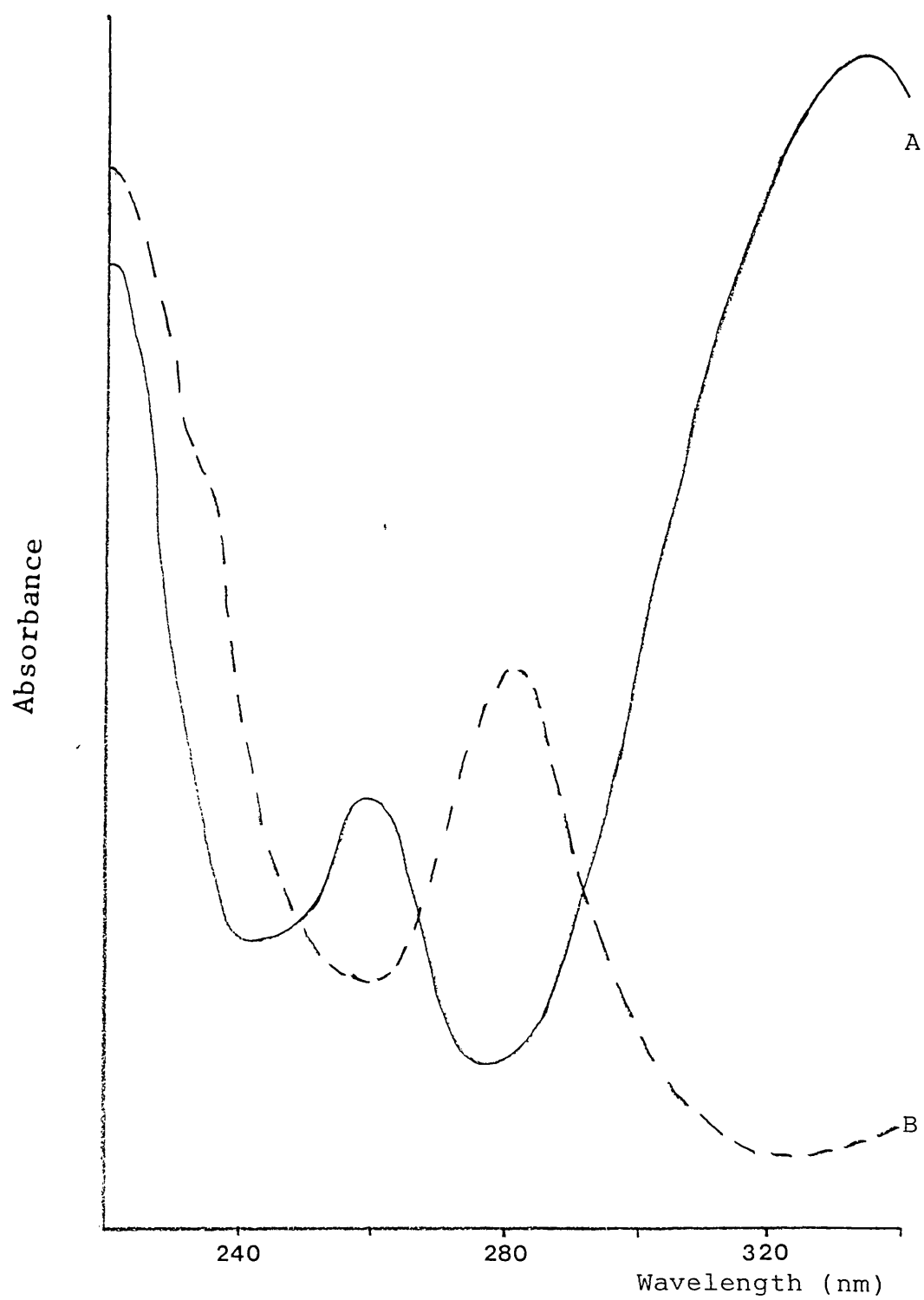


Fig. 17. Ultraviolet spectra of the two isomers of synthetic DIF-1.

A - ortho-methoxy derivative

B - para-methoxy derivative (DIF-1).

3.3 Purification and characterisation of DIF-1

3.3.1 Proton-n.m.r. analysis of synthetic DIF's

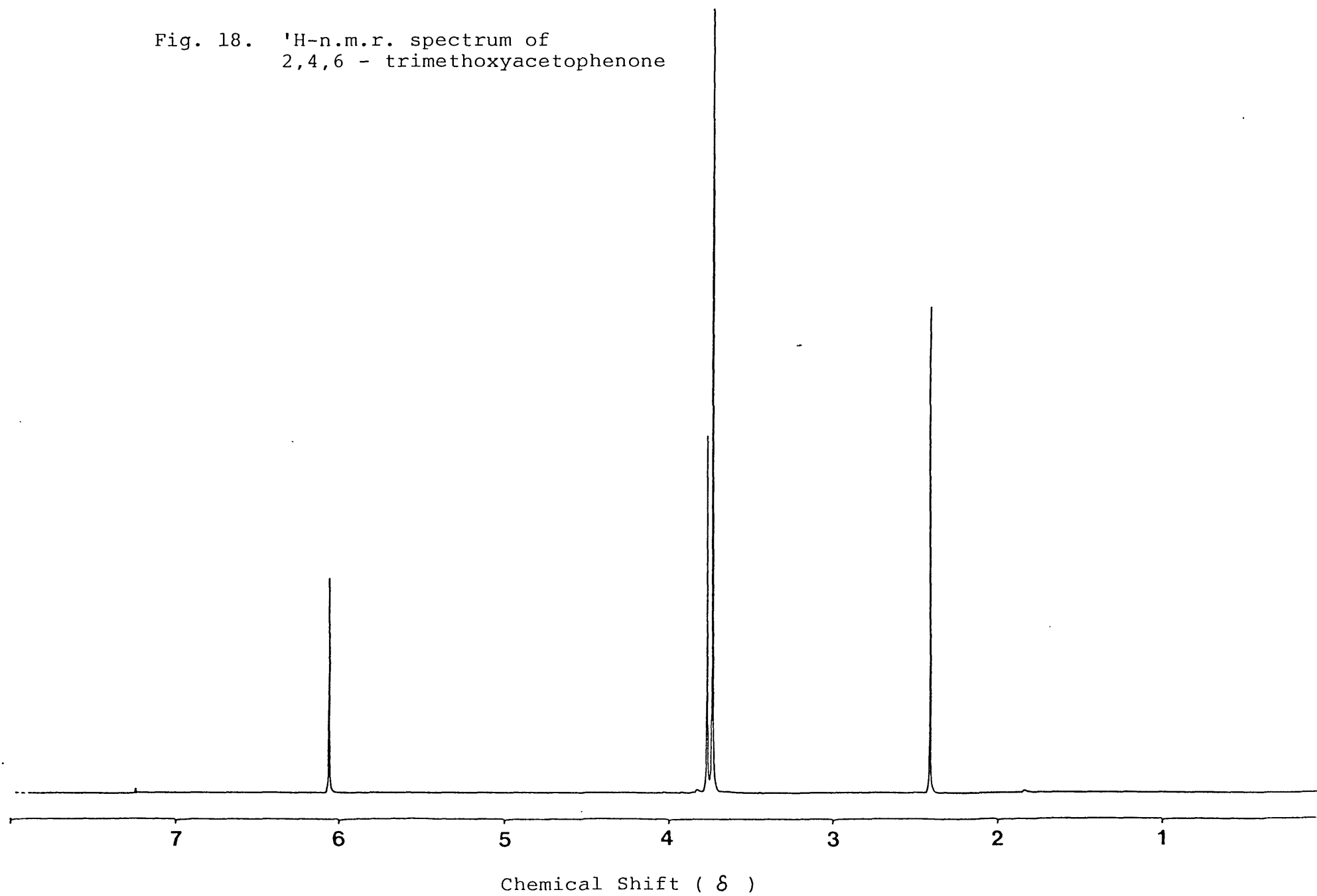
The task of collecting enough of peak-2 for ^1H -n.m.r. was a laborious one. Each HPLC run generated about 20 μg of the desired material. The target was 2 mg for a good ^1H -n.m.r. spectrum. In tandem with this, the other, inactive isomer was also collected for ultimate comparisons, etc.

Finally enough of each purified peak was produced to effect a ^1H -n.m.r. To distinguish between an ortho and para-methoxy signal it would be useful to find a compound that could be used as a standard. One of the early materials purchased in an attempt to find a DIF-like nucleus (Table 3) provided the requirements of such a standard.

2,4,6-Trimethoxyacetophenone (2,4,6-TMAP) contains three methoxy groups, two ortho to the methyl ketone and one para to the said ketone. Integration of these signals should distinguish between these isomeric methoxy groups.

Fig 18 shows the ^1H -n.m.r. spectrum of 2,4,6-TMAP. There are four major signals, all of which are singlets which renders the spectrum very easy to interpret. The signal at δ 2.41 integrates as 3 protons and corresponds to the methyl adjacent to the carbonyl function. The signal at δ 6.06 integrates as 2 protons and corresponds to the two aromatic protons. The signal at δ 3.71 integrates as 6 protons and corresponds to the two equivalent methyls

Fig. 18. ^1H -n.m.r. spectrum of
2,4,6 - trimethoxyacetophenone



ortho to the carbonyl function. Lastly, the signal at δ 3.77 integrates as 3 protons and corresponds to the para-methoxy group. Consequently a para-methoxy substituent has a greater chemical shift than its ortho isomer. Figs. 19 and 20 shows the ^1H -n.m.r. spectra of peak-1 and peak-2 respectively.

Fig. 19 shows the ^1H -n.m.r. spectrum of peak-1. The signals are as follows: δ 14.0, 1H singlet, D_2O exchangeable, δ 7.24 (solvent CDCl_3), δ 6.40 1H broad singlet D_2O exchangeable, δ 3.90 singlet Ar-OCH_3 , δ 3.05 2H triplet ArCOCH_2 - , δ 1.66 4H multiplet CH_2 , δ 1.30 2H multiplet CH_2CH_3 and δ 0.87 3H triplet CH_3CH_2 . The spectrum clearly shows two exchangeable hydroxyls in different environments. This argues strongly for the case of an ortho-methoxy compound.

Fig. 20 shows the spectrum of peak-2. It was fortunate that peak-2 contained a small contamination of peak-1 and that the signals from peak-1 are distinguishable from its isomer. The signals at δ 14.0 and δ 6.6 are from the hydroxyls in peak-1 whereas the signal at δ 10.32 integrates as two protons corresponding to equivalent hydroxyls ortho to the carbonyl function. The singlet at δ 3.96 corresponds to the para methoxy group. The contaminating ortho-methoxy group occurs at δ 3.91. The greater chemical shift of a para methoxy moiety is more clearly shown in Fig. 18. The remaining signals correspond to similar proton resonances found in peak-2 but they are further complicated by the contamination of a small amount of peak-1.

Fig. 19. ^1H -n.m.r. of the synthetic ortho isomer
of DIF-1 (inactive species).

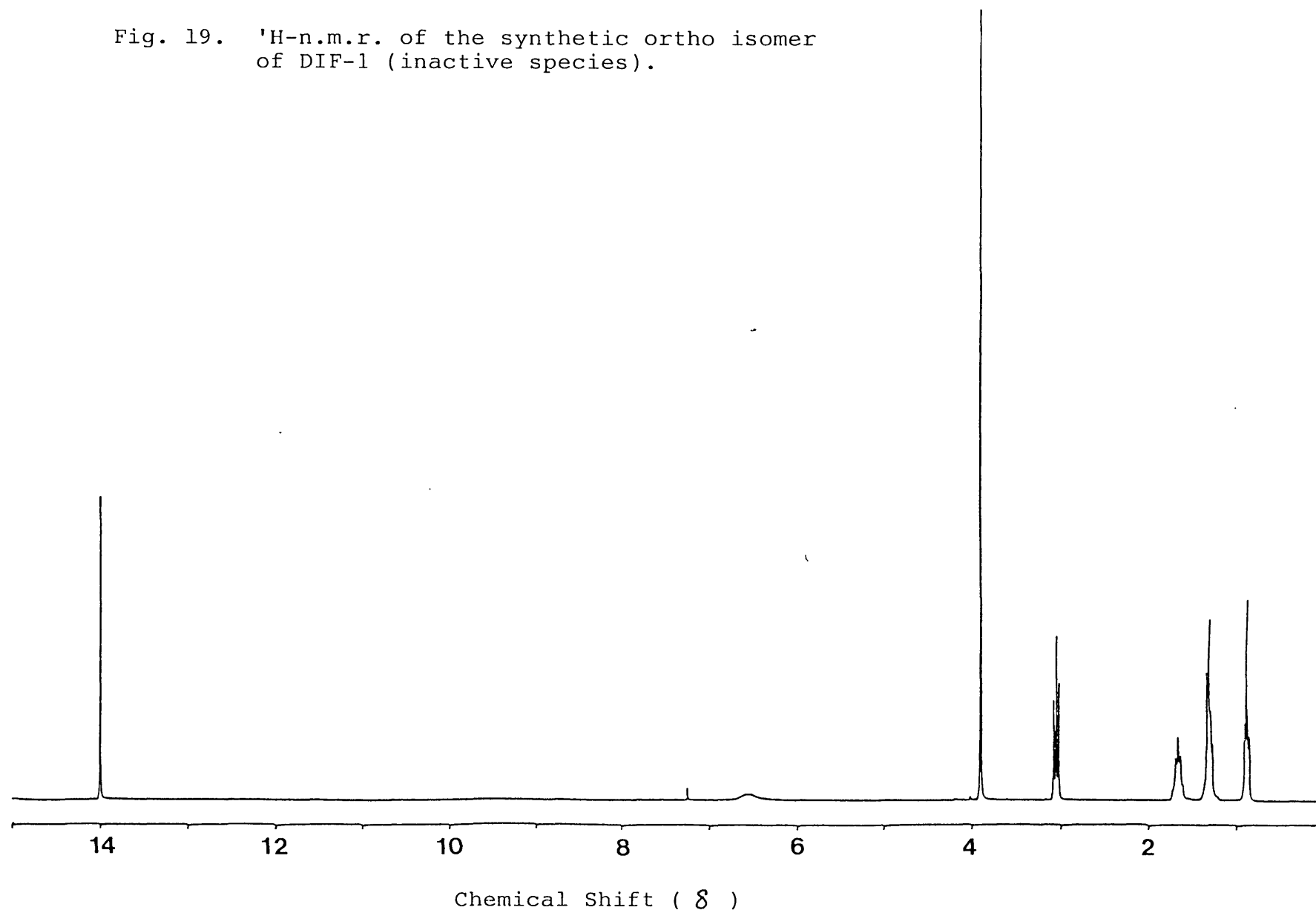
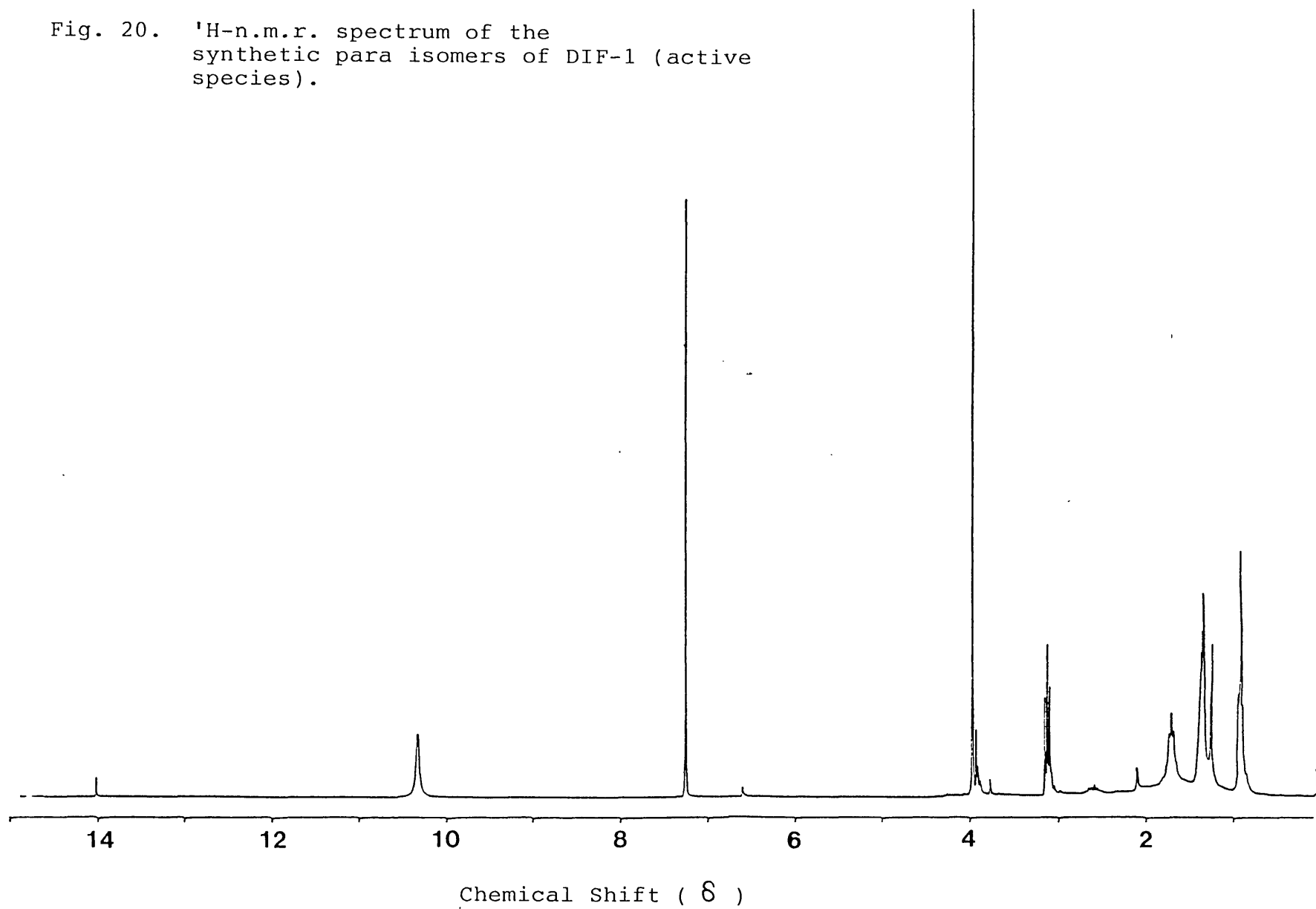
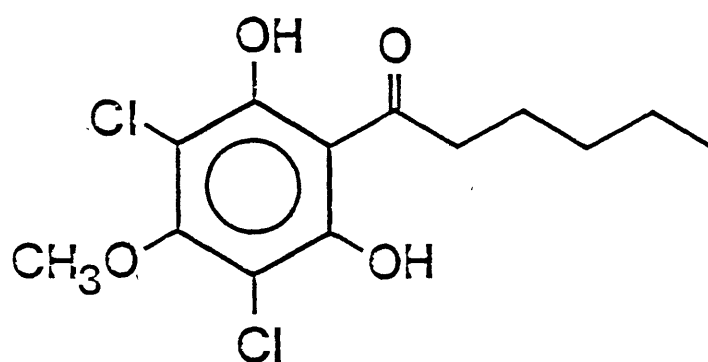
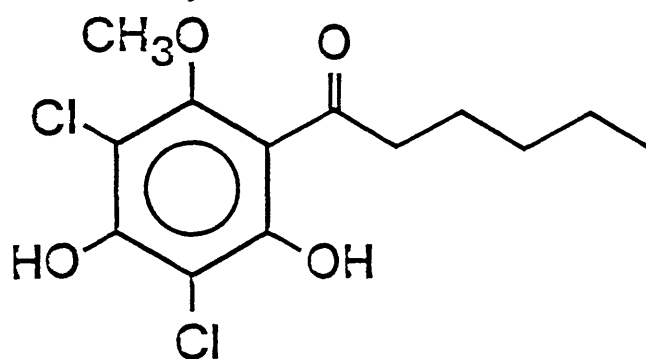


Fig. 20. ¹H-n.m.r. spectrum of the synthetic para isomers of DIF-1 (active species).





p-methyl derivative (peak-2)



o-methyl derivative (peak-1)

Fig. 21. Ortho and para-methyl derivatives of the pentyl ketone of 1,3,5 - trihydroxybenzene: The two DIF-1 isomers.

Comparison of these two spectra shows unequivocally that the active compound, peak-2, is the paramethoxy derivative. The structure is shown, with the ortho methoxy derivative, in Fig.21.

3.2.2 Mass spectrometric analyses

EI-MS analysis of the two isomers showed that the two peaks were identical in their fragmentation pathways and that they could be distinguished by the abundance of the fragment ion arising from the McLafferty rearrangement. Fig.22 shows the mechanism of this fragmentation. There are certain structural requirements necessary for this cleavage to occur. Fig.22 shows that an electron-withdrawing group is required, such as a carbonyl group, and a γ -hydrogen must be available such that a six membered transition state can be produced and the transfer of a hydrogen radical can occur (84). The ortho methoxy isomer has a low abundance m/z 250 ion and the para methoxy isomer has a m/z 250 ion as abundant as the molecular ion. The difference can be explained by the stability of the hydrogen bond between the carbonyl group and an ortho hydroxyl. The para-isomer has two hydroxyls that can hydrogen bond, this renders the hydrogen bond slightly less stable than the bond in the ortho-isomer where only one position of hydrogen bonding can occur. Since the 'free' carbonyl is required for the McLafferty rearrangement, the para-methoxy structure will allow a more facile rearrangement than the ortho-methoxy isomer. The difference in the abundance of these fragment ions can be used as a diagnostic for either the ortho methoxy or para methoxy isomers in subsequent analogues.

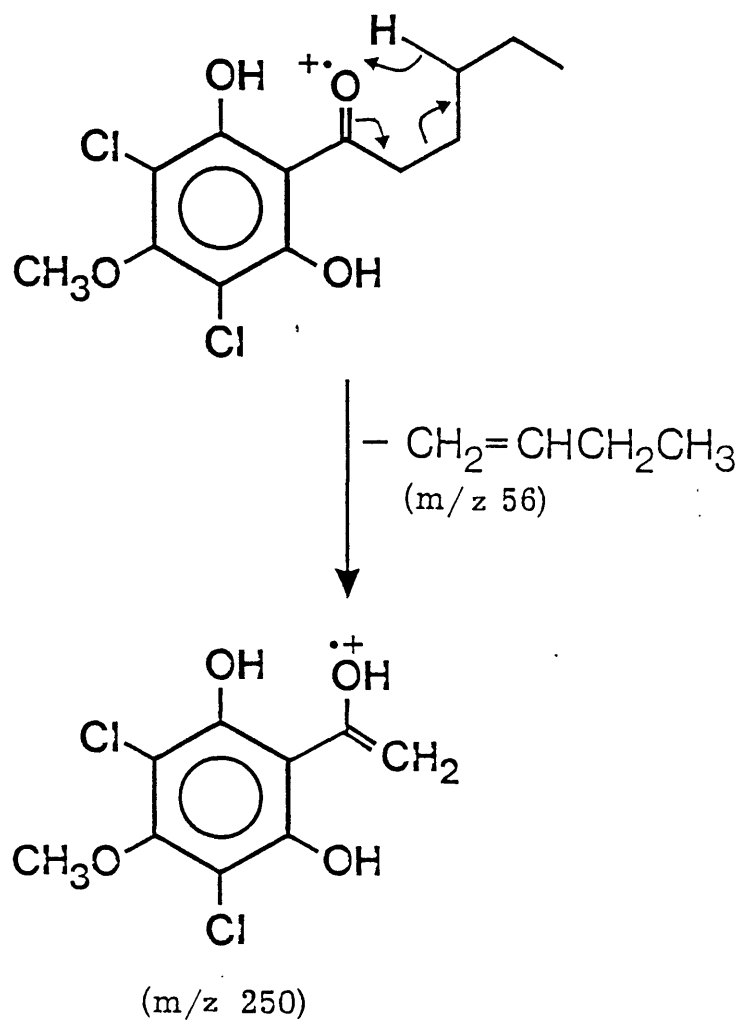


Fig. 22. Mechanism of McLafferty rearrangement of DIF-1 during EI-MS analysis.

GC-MS analysis of a mixture of the two isomers showed that the two components could be readily separated by GC and the computer generated spectra clearly showed the differences, albeit small, in the relative abundances of the relevant fragment ions.

A useful test of identity between a natural and synthetic compound is co-elution on either HPLC or GC. Approximately equal amounts of natural DIF-1 and synthetic DIF-1 were mixed and subjected to GC-MS. The compounds eluted as a single peak, which had an EI-mass spectrum identical to the individually analysed compounds run under the same conditions.

Before the ^1H -n.m.r. had finally distinguished between the two isomers it had been impossible to state categorically which mass spectrum had been produced from which isomer. This is an excellent example of where two analytical techniques have been used synergistically, in analytical symbiosis, to define and elucidate the very fine detail of a structure elucidation problem.

3.3.3 Ultra-violet analyses

Fig. 16 shows the considerable difference in the absorption spectra of the two isomers. To explain this apparent dichotomy it is necessary to follow some rules that govern the principal absorption band in substituted benzene derivatives (85). For the structure $R-C_6H_4-COX$, if X = alkyl residue the parent chromophore has a calculated λ_{max} of 246 nm (in ethanol). An σ -hydroxyl adds 7 nm, a p -methoxy adds 25 nm and the m -chloro has no effect on the absorbance. This gives a calculated λ_{max} for the para-isomer of 278 nm. The ortho-isomer should have the same λ_{max} since $-OH$ and $-OMe$ are equivalent in these calculations. However, the structure of the ortho-methoxy isomer (Fig. 21) may result in the ortho hydroxyl group being very acidic, since it is hydrogen-bonded to the juxtaposed carbonyl group. At a neutral pH it may effectively behave as a deprotonated hydroxyl. This has the effect of increasing the calculated λ_{max} by 53 nm to 331 nm which is seen in the U.V. spectrum.

In acid (M/10 HCl) the λ_{max} shifts from 278 nm to 273 nm for the para-methoxy isomer. The ortho-methoxy isomer spectrum changes more dramatically. The high absorbance at 335 nm is reduced considerably and a new maximum appears at 281 nm and this fits in with the conversion of an $-O^-$ to $-OH$.

In base (M/10 NaOH) there is no apparent change in the U.V. spectrum of the ortho-methoxy isomer. The other isomer shows a broader absorption maximum which is shifted to red by about 8 nm.

A similar example of this acid/base behaviour has been cited (85,86). A base-induced redshift is diagnostic of phenolic compounds in which the phenolic hydroxyl group is para (but not ortho or meta) to a conjugated ring substituent, such as ketone or allyl group (86).

The hypsochromic and bathochromic shifts in the U.V. spectrum of synthetic are the same as those exhibited by natural DIF-1.

The presence of highly acidic hydroxyl, indicated in the U.V. spectrum, is supported by the ^1H -n.m.r. spectrum which showed a signal at δ 14.1 that can be attributed to such an acidic proton.

Before DIF-1 had been synthesised quantitation of the natural material had proved to be difficult. Once sufficient material had been produced for ^1H -n.m.r., ie. an amount that could be weighed, accurately, it was possible to determine the molar extinction coefficient and thereby provide a means of spectroscopic quantitation. By using standard dilutions of synthetic DIF-1 the extinction coefficient, ϵ , was calculated as 4300 ± 500 for the absorbance maximum at 278 nm.

3.3.4 X-ray crystallography

Synthetic DIF-1 was found to have crystallised from deuterated chloroform in a closed vessel over some four weeks. The gross appearance was yellow-brown monoclinic crystals with some browning due to oxidation occurring in parts of the sample. A non-oxidised crystal was removed and crushed to produce a suitably-sized fragment for crystallographic analysis. Fragments from the same crystal were analysed by EI-MS to confirm their identity and purity. The crystal fragments were found to have an identical electron impact mass spectrum to material purified by HPLC.

Under crystallographic analysis a total of 2953 independent reflections were measured (to $\theta = 50^\circ$), of which 668 were judged to be 'unobserved' ($F < 3\sigma(F)$). The structures were solved by direct methods, and least squares refinement reached $R = 0.048$ ($R_W = 0.039$).

Two crystallographically independent molecules were observed which were found to have no chemically significant differences between them. These arose from the two possible intramolecular hydrogen-bond positions between the carbonyl group and each of the ortho-hydroxyls. Fig. 23 shows the structure of DIF-1 and confirms it as that deduced from physiocochemical data. The other crystallographic structure shows the carbonyl group juxtaposed to the other ortho hydroxyl and the structure is superimposable on the structure in Fig. 23.

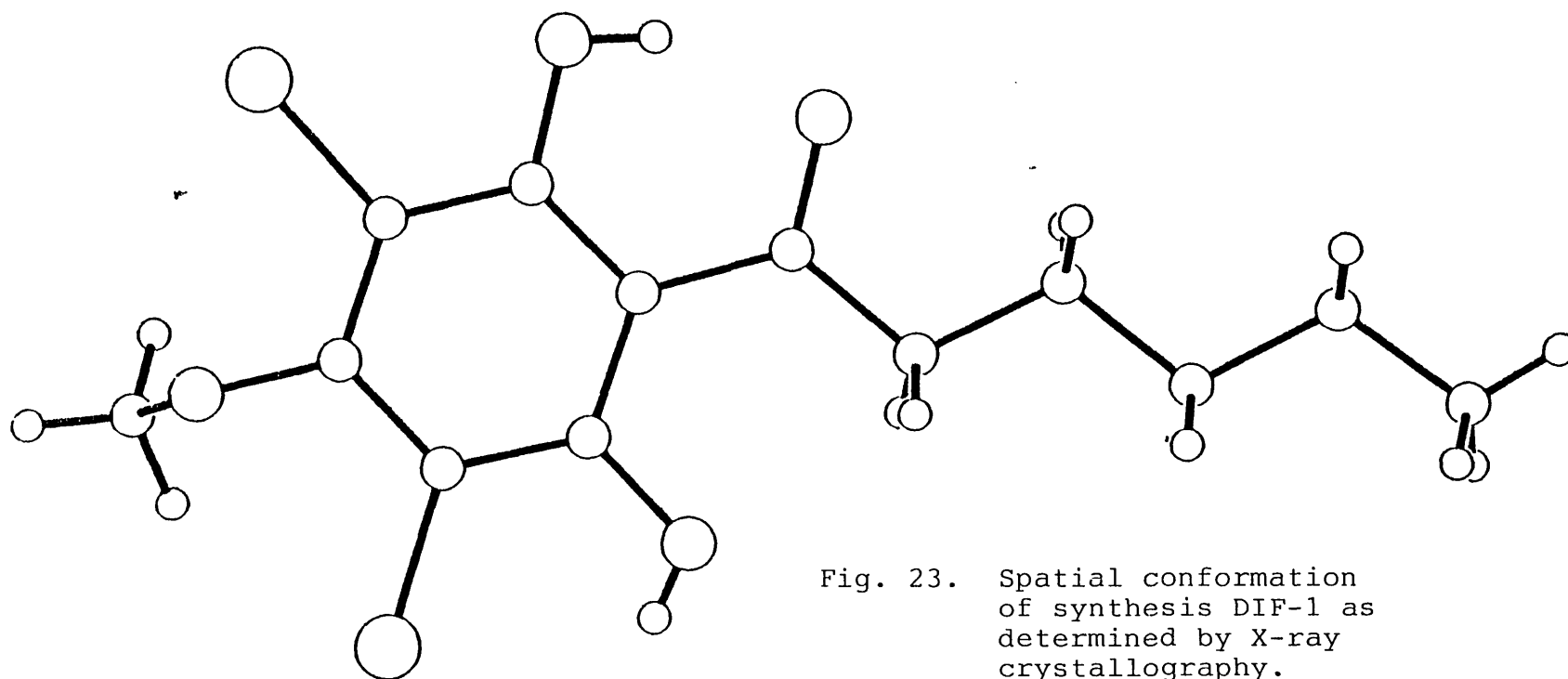


Fig. 23. Spatial conformation of synthesis DIF-1 as determined by X-ray crystallography.

The crystal data obtained is summarised in Table 8

Formula	=	$C_{13}H_{16}Cl_2O_4$
crystals	=	monoclinic
a	=	13.329
b	=	7.505
c	=	29.490
β	=	103.29°
U	=	2871.0 Å (at 19°C)
space group	=	$P 2_1/n$ (No. 14)
Z	=	8

Table 8. X-ray crystallographic data generated
from a crystal of DIF-1

3.3.3 DIF Bioassay

As a result of determining the molar extinction coefficient, it was now possible to accurately measure small amounts of DIF for the purpose of calculating its specific activity in the bioassay.

Before absolute quantitation the specific activity of natural DIF-1 was estimated at about 120×10^6 units mg^{-1} (87). a number of titrations were carried out to determine the specific activity. Over the space of ten weeks fourteen specific activity determinations were carried out on three samples of purified synthetic DIF-1. The bioassay varied considerably from day to day, although different samples run on the same day gave very similar results. The specific activity was calculated as $138.84 \times 10^6 \pm \text{S.D } 82.42 \times 10^6$ units μmole^{-1} (452.3×10^6 units mg^{-1}). The original estimate was based on trying to weigh about 20 μg of material.

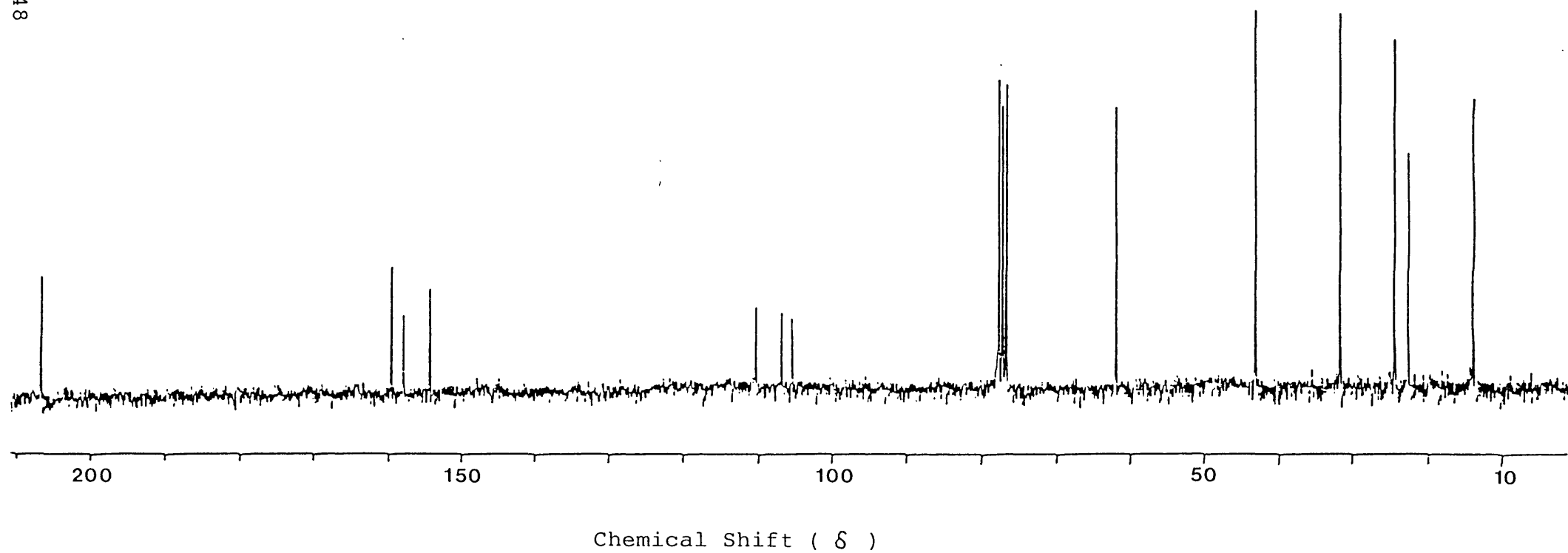
This figure can be used to determine the relative potencies of synthetic analogues.

3.3.6 Bulk preparation of DIF and its ortho-methyl isomer

Once the HPLC conditions for purification of the two chlorinated ketones had been optimised, it was possible to collect several milligrams of material by repeatedly running aliquots of a stock mixture of the two isomers. When presented with a novel class of chemical compound there are many screens it can undergo to test for a bioactivity other than its natural use. To be able to effect a wide range of biological assays, etc it is necessary to have a large amount of material. Some eight weeks were consumed with synthesising and purifying 50 mg of each isomer for such tests. (At time of writing, the results from these tests were unavailable). With this amount of material it was possible to run a ^{13}C -n.m.r. spectrum of the ortho-methoxy isomer. It was not possible to run a spectrum of the bioactive isomer but the ^{13}C -n.m.r. spectrum shown in Fig.24 should serve as a reference for similar compounds. From the spectrum all thirteen carbon atoms can be accounted for. The signals are interpreted as follows: the signal at δ 206 corresponds to the ketonic carbon atom. The two signals at δ 159 and δ 157 correspond to aromatic carbon atoms bonded to chlorine. The signal at δ 154 arises from the aromatic carbon bonded to the carbonyl group. The three signals at δ 105, δ 107 and δ 110 arise from the remaining three aromatic carbon atoms. The triplet around δ 77 is due to the solvent CDCl_3 . The signal at δ 62 is due to the aromatic O-methyl group. One might expect this signal to be shifted in the para isomer, as there will be less electronegative influence from the

Fig. 24. ^{13}C - n.m.r. of synthetic ortho isomer of DIF-1

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adjacent carbonyl. The signal at δ 43 arises from the methylene to the carbonyl group. The three signals at δ 31, δ 24, and δ 22 correspond to the three remaining methylenes in the alkyl chain and finally the signal at δ 14 corresponds to the terminal methyl group in the alkyl chain. The para-methoxy isomer would be expected to have a very similar spectrum, however, analogues that have different numbers of carbon atoms or altered functional groups will accordingly have different spectra.

^{13}C -n.m.r. data has been published on a metabolite from a brown alga which corresponds to an unchlorinated analogue of the para-methoxy isomer but contains a long unsaturated alkyl chain (88). However, the aromatic and ketonic carbons are very similar to the compound described herein.

3.4 Analogues of DIF

3.4.1 Introduction

Once a new bioactive molecule has been purified and its structure elucidated, it is very common to investigate its interaction with its receptor. If a preparation of the receptor is available various binding studies can be done to determine stoichiometry, affinity of binding and various binding constants, etc. If such receptor preparations are not available an alternative approach is to alter the structure of the 'substrate' either by chemical or enzymic degradation, or by chemical modification. From such experiments it is possible to determine 'a minimal structural requirement' or which functional groups are necessary for activity.

There are many examples of chemical modification being used for structure elucidation and, by virtue of the particular reactions, have shown that particular functional groups are vital for activity. This was shown to be the case with DIF; methylation by diazomethane was used to quantitate hydroxyl groups. The dimethyl ether of DIF was biologically inactive. If the compound is relatively simple to synthesise, it is possible to gain considerable information about structurally important functional groups by synthesising analogues. This approach is rather impractical as far as large peptides are concerned although smaller peptides, which are available commercially, are often synthesised with an amino acid alteration or substitution (89, 90). In the case of DIF-1, the synthesis is straightforward and a number of analogues can be produced.

From the structure of DIF, there are four functionalities which can be altered through new synthesis or post-synthesis modification. These are as follows: a) phenolic hydroxyls; b) halide; c) carbonyl group; d) alkyl chain. the more immediate derivatives will be discussed in turn.

a) Phenolic hydroxyls

The DIF-1 structure already possesses a modification to one of the hydroxyl groups on the ring, ie. a para-O-methyl. It has already been shown that the Hoesch synthesis produces two isomers, differing only by the position of this methyl group. The structure elucidation and synthesis of DIF-1 has demonstrated that the position of this methyl is very relevant to biological function as the ortho-methyl has only 0.136% of DIF activity.

It has also been shown that methylation of the ortho-hydroxyls further reduced bioactivity. It can, therefore, be argued that the hydroxyl groups are a prerequisite for activity.

It is possible to synthesise a dimethoxy analogue. There are two possible routes. Firstly, DIF can be methylated using diazomethane for a limited reaction time, although in practice the product tended to be the trimethoxy derivative. Alternatively, the synthesis could be performed using dimethoxyphloroglucinol, which is commercially available.

The desmethyl derivative can be synthesised by using phloroglucinol as the phenolic reagent. This, of course, would only produce one isomer.

Finally, para-deoxy DIF can be synthesised using orcinol (3,5-dihydroxy-toluene) as the phenolic reagent. Both the ortho and para-methyl derivatives could be made in the same reaction.

More indirect modifications could be made, eg. the para-ethoxy derivative, acetylated derivatives, etc. Higher ethers may prove more difficult to synthesise.

b) Halide functional group

DIF contains two chlorine atoms. Their function is, as yet, unknown, although one can speculate on the possible function.

Chlorine produces a negative inductive effect and a positive mesomeric effect. It may be that the strong electronegativity stabilises a deprotonated hydroxyl group. It may be that chlorine affects solubility in the cell membrane or simply a charge cloud of particular size is required for interaction with the putative receptor.

It has also been shown that the monochloro species exhibits some bioactivity, and that the deschloro analogue has a very minor activity.

The chlorine could be replaced by bromine or iodine by using bromine water or an iodine solution instead of chlorine water.

Furthermore, deshalo, monohalo and dihalo derivatives can be made of all the other structural analogues.

c) Carbonyl group

There are really only two simple modifications that can be performed on the carbonyl group. The more straightforward of the two is reduction. This can be achieved by borohydride and has been shown to destroy bioactivity. However, it has been shown that the resultant benzylic alcohol eliminates water very readily to produce a styrene type derivative. It is perhaps worth noting that if the alcohol was stable, a chiral centre would have been introduced into the molecule, producing two enantiomers which may have had different 'bioactivities'. The other modification is to eliminate the ketone altogether by synthesising a C₆H₁₃ alkyl chain via a Friedel-Crafts alkylation. Although, from the borohydride reaction, it seems clear that the carbonyl group is essential for activity of DIF.

d) Alkyl side chain

Varying the length of the alkyl side chain has already been shown to affect activity. cf. DIF-1 and DIF-2.

It is likely that the alkyl chain is important for solubility in a hydrophobic environment or may participate as a 'spacer' within the binding site of a receptor. The former seems more likely since, DIF may need to enter through a cell membrane to react with an intracellular receptor.

The drawback of producing a range of homologues of different chain length is that each one requires a whole new synthesis

ab ovo. The ease with which this can be achieved depends on the availability of the requisite nitriles. Straight chain nitriles are now commercially available, whereas isoalkyl nitriles must be synthesised from the alkyl bromides.

From a biological point of view there is very little difference in bioactivity between the alkyl and isoalkyl isomers as shown by both DIF-1 and DIF-2.

The more highly branched analogues may be more difficult to synthesise due to steric interactions. There are many more analogues of the DIF structure that can be produced, based on different functional group substitution around the ring. A long-term synthetic programme would be required to cover all these possibilities. Consequently, this research has only been concerned with the above four categories, although alterations in the carbonyl group seem well characterised.

3.4.2 Synthesis and analysis of alkyl side chain homologues

For convenience, a three symbol abbreviation was developed for the alkyl chain homologues; the first symbol is either 'o' or 'p' to denote the ortho or para isomer. The second and third figures, eg. '7C' denote the number of carbons in the alkyl chain (not including the carbonyl group). Thus, the seven-carbon homologue of the ortho isomer is designated o7C.

The synthesis of homologues with 3 to 8 carbon atoms in the alkyl chain was essentially the same. All the nitriles, when reacted with 3,5-DHA, produced the characteristic blood-red colouration of the ketimine chloride. Each ketimine was analysed by EI-MS, before hydrolysis, to confirm whether the initial reaction had occurred. The three components produced after hydrolysis, were all analysed by EI-MS. In all cases the water-insoluble orange solid was found to contain the highest yield of ketone.

In each synthesis, the amount of 3,5-DHA was standardised, 2.5g (0.0179 moles) was used and a molar equivalent of the nitrile was used. The approximate yield of ketone varied from 13-65%. This did not include small amounts present in the liquor.

Each ketone mix was chlorinated such that the EI mass spectrum showed a ratio of 75:25 of the dichloro:monochloro species. Further chlorination tended to produce over-chlorinated species. After chlorination the mixture was dried down by rotary evaporation. The sample was weighed and a 10 mg ml⁻¹ solution was made by addition of 35% propan-1-ol in 5% acetic acid. The mixture

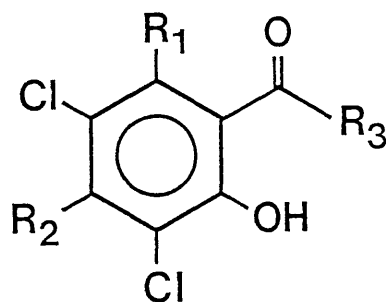
was run on HPLC and the dichloro isomers were collected separately in pear-shaped flasks. Monochloro isomers were not resolved under these elution conditions and were not collected. Each HPLC run produced about 50 μ g of the para-isomer and 200 μ g of the ortho-isomer. It was necessary to collect about 3 mg of each isomer for ^1H -n.m.r. analysis. Each isomer was also analysed by EI-MS, GC-MS and U.V.

The ^1H -n.m.r. proved to be the only clear-cut means of determining whether the samples were contaminated with each other or any other species.

Samples were also subjected to the DIF bioassay and the specific activity and relative activity (to DIF-1) were determined.

The physiocochemical data for each compound are presented and discussed and then the homologous series will be discussed as a whole.

The general structure for the homologues is shown below



For each different isomer R_1 , R_2 and R_3 will be cited as well as the designated symbol. In all cases R_3 is the normal form of the alkyl chain.

Table 9 shows the data generated from both the U.V. analysis and ^1H -n.m.r. spectroscopy. The U.V. absorption curves for the two series of isomers (ortho and para) were very similar. The ortho-methoxy homologues all showed the characteristic absorbance at around 335 nm and 260 nm. Similarly, there was no significant difference amongst the para-methoxy homologues. The length of alkyl chain clearly does not affect absorption profiles. The compounds had similar extinction coefficients at their respective λ_{max} . Although ϵ was not determined for every isomer, standard concentrations (1 mg ml^{-1}) generated absorption maxima of similar intensity.

The ^1H -n.m.r. spectra of the two homologous series differed very slightly within each series. The '3C' and '4C' analogues did not contain a signal at $\delta 1.30$. The differences between the two series was identical to those borne out by comparing synthetic DIF and the ortho-methoxy isomer. The ortho isomers produce two different signals due to the phenolic hydroxyls; one shifted to $\delta 14.0$, indicative of a strongly hydrogen-bonded proton and the other at $\delta 6.5$ (para-hydroxyl) which behaves spectroscopically as a normal phenol. The phenolic hydrogens in the para-methoxy isomers both experience some degree of hydrogen-bonding but since they are equivalent this intramolecular bonding is less intense.

	R ₁	R ₂	R ₃	max in CH ₃ OH (nm)	Chemical Shift δ
o3C	OMe	OH	C ₃ H ₇	335 260	13.98(s, 1H) , 0.48(s, 1H) , 3.92(s, 3H) , 3.03(t, 2H) , 1.73(m, 2H) , 0.98(m, 3H) ,
p3C	OH	OMe	C ₃ H ₇	279	10.31(s, 2H) , 3.96(s, 3H) , 3.10(t, 2H) , 1.72(m, 2H) , 0.98(m, 3H)
o4C	OMe	OH	C ₄ H ₉	334 260	14.00(s, 1H) , 6.48(s, 1H) , 3.92(s, 3H) , 3.05(t, 2H) , 1.68(m, 4H) , 0.90(m, 3H) ,
p4C	OH	OMe	C ₄ H ₉	278	10.32(s, 2H) , 3.96(s, 3H) , 3.11(t, 2H) , 1.67(m, 4H) , 0.90(m, 3H)
o5C	OMe	OH	C ₅ H ₁₁	334 260	14.01 (s, 1H), 6.49(s, 1H) , 3.90(s, 3H) , 3.05(t, 2H) , 1.66(m, 4H) , 1.30(m, 2H)
p5C	OH	OMe	C ₅ H ₁₁		10.32(s, 2H) , 3.96(s, 3H) , 3.10(t, 2H) , 1.68(m, 4H) , 1.32(m, 2H) , 0.89(m, 3H)
o6C	OMe	OH	C ₆ H ₁₃	334 259	14.03(s, 1H) , 6.47(s, 1H) , 3.92(s, 3H) , 3.08(t, 2H) , 1.68(m, 4H) , 1.28(m, 4H)
p6C	OH	OMe	C ₆ H ₁₃	278	10.34(s, 2H) , 3.96(s, 3H) , 3.11(t, 2H) , 1.69 (m, 4H) , 1.33(m, 4H) , 0.90(m, 3H)
o7C	OMe	OH	C ₇ H ₁₅	334	14.02(s, 1H) , 6.49(s, 1H) , 3.92(s, 3H) , 3.07(t, 2H) , 1.68(m, 4H) , 1.30(m, 6H) , 0.87(m, 3H)
p7C	OH	OMe	C ₇ H ₁₅	277	10.35(s, 2H) , 3.96(s, 3H) , 3.13(t, 2H) , 1.61(m, 4H) , 1.29(m, 6H) , 0.88(m, 2H)
o8C	OMe	OH	C ₈ H ₁₇	334 260	14.03(s, 1H) , 6.47(s, 1H) , 3.92(s, 3H) , 3.07(t, 2H) , 1.68(m, 4H) , 1.26(m, 8H) , 0.87(m, 3H)
p8C	OH	OMe	C ₈ H ₁₇	277	10.35(s, 2H) , 3.96(s, 3H) , 3.12(t, 2H) , 1.68(m, 4H) , 1.26(m, 8H) , 0.85(m, 3H)

Table 9. U.V. and ¹H-n.m.r. data from alkyl
side chain analogues of DIF-1

The signals at δ 3.96 (para-isomers) and δ 3.92 (ortho-isomers) were highly conserved throughout the series, such that given an unknown within this series or a higher homologue it should be possible to state whether it is an ortho or para isomer.

The triplet at δ 3.10 (para) and δ 3.05 (ortho) could be used in a similar way as the O-methyl singlet as a diagnostic, although this may be slightly less reliable. The difference may be attributed to the extent of stabilisation of the hydrogen-bond between the phenolic hydroxyl and the carbonyl group. The hydrogen bonding in the para isomer is less strong than in the ortho isomer, therefore the carbonyl group has a slightly stronger inductive effect on its adjacent aliphatic carbon atom in the para-isomer. This results in the two protons on the α -carbon being marginally more acidic than their ortho isomer counterparts, hence the greater chemical shift.

The signals arising from the aliphatic protons seem unaffected by the ring isomerisation. However, the terminal methyl has slightly more acidic protons in the 3C isomers (δ 0.98) than in the 8C isomers (δ 0.85). Again this can be explained by proximity to a carbonyl moiety.

Table 10 shows the mass spectral data for these series of homologues. The particular structures of these homologues all lend themselves to a highly regular fragmentation pattern. There seems to be five facile cleavages most of which concern the alkyl ketone side chain. All the homologues produce fragment ions at m/z 263, 235 and 220 in relatively high abundance. The m/z 250 fragment ion, arising from the McLafferty rearrangement

Compound	Major fragment ions	
	m/z (relative abundances)	%
o3C	278 (11.5) , 263 (3.80) 247 (5.95) , 235 (100) , 220 (34.5)	
p3C	278 (15.8) , 263 (8.2) , 250 (3.16) , 245 (4.59) , 235 (100) , 220 (17.1)	
o4C	292 (12.1) , 263 (18.45) , 250 (3.48) , 235 (100) , 220 (36.4)	
p4C	292 (13.3) , 263 (27.7) , 250 (13.6) , 235 (100) , 220 (21.5)	
o5C	306 (10.4) , 288 (9.17) , 263 (13.6) , 250 (4.75) , 235 (100) , 220 (23.7)	
p5C	306 (12.7) , 288 (13.3) , 263 (25.9) , 250 (14.6) , 235 (100) , 220 (12.9)	
o6C	320 (8.86) , 302 (8.23) , 263 (19.8) , 250 (6.65) , 235 (100) , 220 (25.6)	
p6C	320 (11.1) , 302 (11.4) , 273 (8.07) , 263 (26.6) , 250 (17.4) , 235 (100) ,	
o7C	334 (6.96) , 316 (7.49) , 263 (17.4) , 250 (7.59) , 235 (100) , 220 (36.7)	
p7C	334 (14.7) , 316 (12.7) , 263 (32.8) , 250 (22.6) , 235 (100) , 220 (10.9)	
o8C	348 (7.02) , 330 (7.36) , 263 (19.3) , 250 (6.88) , 235 (100) , 220 (27.1)	
p8C	348 (13.2) , 330 (8.23) , 263 (34.2) , 250 (24.8) , 235 (100) , 220 (10.1)	

Table 10. Major fragment ions arising from EI-MS analysis of alkyl side chain analogues of DIF-1.

is also common to all homologues, although the intensity of the ion is lower in the ortho isomers but increases with increasing chain length. The homologues containing five carbons and above, all readily lose a molecule of water. This implies that the physical size and spatial configuration of the alkyl chain, in relation to the hydroxyl groups, is important in this particular fragmentation.

There is no indication that any of these isomers lose a methyl group in preference to the loss of water.

The mass spectral data enforces the argument that these phenols have very stable nucleus structures, since no appreciable or significant fragmentation appears below m/z 208.

In a similar way to the ^1H -n.m.r. profiles, the mass spectra of different isomers and homologues can be used as a diagnostic for structures with this type of ring substitution. Fig.25 shows a direct comparison of the two isomers of the six carbon homologues. The relative sizes of peaks at m/z 250 and m/z 220 are the ones of diagnostic interest.

All in all, these two homologous series show remarkably similar physiocochemical characteristics. However, from a biological perspective they are vastly different. The bioassay data will be presented and discussed after considering a few other analogues.

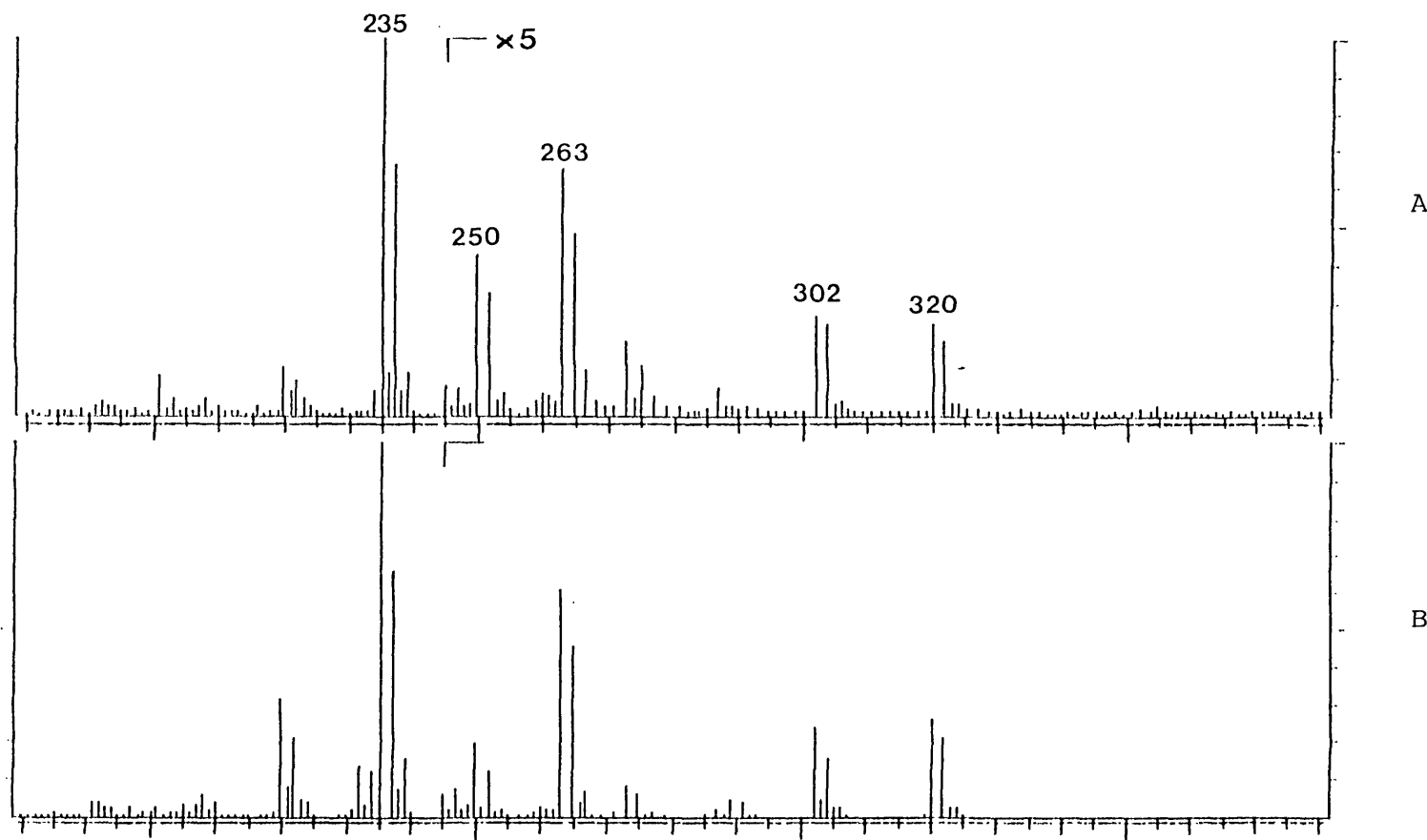


Fig. 25. Direct comparison of EI mass spectra of 6C - analogues of DIF showing differences in relative abundance of major fragment ions.

A - para-methoxy 6C analogue

B - ortho-methoxy 6C analogue.

3.4.3 Synthesis and analysis of simple ring analogues

Whereas the alkyl chain homologues required 'nouvelles synthèses', the halo-derivatives of DIF could be produced from the ketone mix resulting from hydrolysis of the Hoesch ketimine (Fig. 15). Using a 5mM solution of bromine water, three successive brominations resulted in an approximately equal amount of mono- and dibromo-products. Further addition of bromine water resulted in over bromination.

In a similar manner to chlorinated compounds, brominated compounds have a characteristic ion cluster due to the natural abundance of the two major isotopes (Fig. 4). A monobromo compound will exhibit a doublet, two mass units apart, of equal abundance. A dibromo derivative produces a triplet, again each signal two mass units apart, in the ratio 1:2:1. The two dibromo isomers were purified on HPLC and analysed in the same fashion as other analogues.

Table 11 shows all the spectroscopic data and mass spectral data from the two dibromo-DIF isomers. The presence of bromine instead of chlorine seems to have caused a slight (2nm) red shift in both absorption curves which is consistent with theoretical values of meta-bromo compounds (85). The para isomer appears to have a similar molar extinction coefficient as the chloro compound.

The ^1H -n.m.r. of the dibromo derivatives was essentially the same as the chloro analogues except that the most acidic protons, or the phenols, were shifted downfield. This is perhaps unusual, as one would expect the presence of chlorine to cause an increase

in chemical shift, due to its strong inductive effect. the signal due to the isolated methoxy group was shifted in the opposite direction. The conclusion one could draw is that the effect on the methoxy compound is due to the inductive effect and that on the hydrogen-bonded hydroxyl is due to a mesomeric effect.

The monobromo and dibromo isomers all showed the characteristic isotope pattern, fragmentation pattern and abundance of ions as seen with the equivalent chloro compounds during mass spectrometric analysis. It was noticeable that generally the abundance of ions arising from fragmentation of the alkyl chain was lower than that for the corresponding chloro compounds. The bioassay data are discussed later.

Iodination of the ketone proved to be considerably more difficult. After three iodinations only 2% of the monoiodo derivative was produced. The concentration of iodine was increased ten-fold and after two hours a good monoiodo EI spectrum was obtained. No diiodo-DIF was detected even at higher source temperatures in the mass spectrometer. However, the monoiodo compound gave a good spectrum on GC-MS, and showed the characteristic fragmentation pattern. Signals were seen at m/z 364, 346, 321, 308, 293 and 278.

In light of the difficulties in iodination no further tests were performed on the material.

The position of the O-methyl group is clearly important. Whether it acts in a physical size capacity or acts to restrict hydrogen

bonding, a methyl group is preferred in the para position for bioactivity. What would be the effect if no methyl group at all?

A desmethyl DIF was synthesised. This, at first, proved very difficult. It was thought this may be due to the two molecules of water of crystallisation present in the commercial sample of phloroglucinol. Nevertheless, it was possible to produce sufficient ketone for further experimentation by increasing the reaction time for the original ketimine formation to seven days.

Chlorination of the trihydroxy ketone also proved difficult, at first there seemed to be some oxidation as well as over-chlorination. The chlorinating solution was diluted five-fold. To produce a good yield of the dichloro compound, seven chlorinations were required. Since there is no O-methyl group the problem of separating dichloro isomers does not arise, it is merely necessary to separate the monochloro compound from the dichloro product.

U.V. analysis of the desmethyl compound (des-Me 5C) showed two maxima of equal intensity at 326 nm and 284 nm. Once again, comparison of standard solutions of the desmethyl DIF and synthetic DIF showed that the molar extinction coefficient for the 284 nm band was similar to the 278 nm band in the synthetic DIF material.

The ^1H -n.m.r. (Table 11) showed a two proton signal at δ 10.48 (cf Fig. 20 para methoxy homologue) which corresponded to the

	R ₁	R ₂	R ₃	max	n.m.r. δ	Major fragment ions m/z (% abundance)
o5CBr ₂	OMe	OH	C ₅ H ₁₁	336 252	14.12 (s, 1H) , 6.54 (s, 1H) , 3.87 (s, 3H) , 3.09 (t, 2H) , 1.68 (m, 4H) , 1.31 (m, 2H) , 0.89 (t, 3H)	396 (8.90) , 378 (9.59) , 353 (14.6) , 340 (4.45) , 325 (100) , 310 (27.4)
p5CBr ₂	OH	OMe	C ₅ H ₁₁	280	10.41 (s, 2H) , 3.192 (s, 3H) , 3.11 (t, 2H) , 1.68 (m, 4H) , 1.35 (m, 2H) , 0.89 (t, 3H)	396 (16.8) , 378 (20.9) , 353 (29.5) , 340 (17.1) , 325 (100) , 310 (13.4)
desMe5C	OH	OH	C ₅ H ₁₁	326 284	10.48 (s, 2H) , 6.37 (s, 1H) , 3.09 (t, 2H) , 1.68 (m, 4H) 1.34 (m, 2H) , 0.90 (t, 3H)	292 (13.2) , 274 (8.54) , 249 (24.3) , 236 (14.7) , 231 (4.85) , 221 (100)
o5C desoxy	Me	OH	C ₅ H ₁₁	n.d.	10.8 (s, 1H) , 6.32 (s, 1H) , 2.83 (t, 2H) , 2.49 (s, 3H) , 1.70 (m, 4H) , 1.29 (m, 2H) , 0.88 (t, 3H)	290 (7.93) , 272 (2.70) , 247 (4.01) , 234 (5.11) , 219 (100)

Table 11. Ultraviolet, n.m.r. and mass spectrometric data from analysis of brominated and ring isomers of DIF-1.

s - singlet

t - triplet

m - multiplet

two equivalent, hydrogen-bonded ortho-hydroxyls. A singlet at δ 6.37 showed that the para-hydroxyl behaves as a 'normal' phenol. As expected, the singlet due to an O-methyl group was absent. The other signals arising from the alkyl chain were very similar to those in the methylated analogues (Table 9).

The mass spectrum of desmethyl-DIF showed the usual fragmentation pattern. The fragment ion arising from loss of water was relatively smaller than in the p5C. The McLafferty fragment ion was more abundant than the molecular ion. This has been a characteristic of para isomers. It can be reasoned that the presence of a methyl group ortho to the ketone allows the carbonyl function to hydrogen-bond more tightly to the ortho hydroxyl rendering the carbonyl group less capable of accepting a proton for the enol ion. There is, of course, no loss of CH_3^+ from the acylium ion (m/e 221). The bioassay will demonstrate how important the O-methyl group is in terms of activity.

A question arose as to the importance of the O-methyl group. It has been shown that the position around the aromatic ring has a marked effect on the physiocochemical and biological properties of these compounds. What would be the effect if the O-methyl was replaced by a C-methyl, ie. para-desoxy DIF?

In a similar manner to phloroglucinol, orcinol (3,5 dihydroxy toluene) possesses water of crystallisation. The production of the ketimine took four weeks. No blood-red coloration was observed, but EI-MS analysis of the reaction mixture showed a slow increase in the amount of ketimine produced over the reaction time.

Hydrolysis of the ketimine did not produce a water-soluble oil. The precipitate, on cooling, was shown to contain the required ketone but in low yield (8%).

Chlorination produced three major products, two monochloro isomers and a single dichloro species. The mono chloro compounds were not completely resolved under the standard HPLC conditions.

¹H-n.m.r. of the purified dichloro compound indicated the presence of two hydroxyls in different environments. One at δ 10.80 which would appear to be hydrogen-bonded to the carbonyl group and one 'normal' phenol at δ 6.32. A singlet arising from the aromatic methyl occurred at δ 2.49. The remaining signals from the alkyl protons were similar to other '5C' compounds.

The conclusion from this analysis is that this was an ortho-methyl derivative and that the para-methyl compound had not been synthesised.

The mass spectrum showed the characteristic fragmentation of a 5C compound, ie, loss of H₂O, C₃H₇[•], C₄H₈ and C₅H₁₁[•]. There was no evidence of a loss of CH₃[•] from the ring system.

All the structural analogues of DIF show remarkable physioco-chemical homology. Although this group can be divided into the ortho isomers and para isomers and within each subset the homology is even greater. With this in mind it is possible to put into perspective the structure and function of these analogues in terms of bioactivity.

3.4.4 Differentiation-inducing activity of DIF analogues

In the process of characterising synthetic DIF-1, one of the most important studies was the determination of the specific activity (Section 3.3.4). The activity cited was the mean of some fourteen separate assays. There was a considerable variation in activity from assay to assay ranging from 61.5×10^6 units μmole^{-1} to 327.8×10^6 units μmole^{-1} . The cause of this variation is not known. Each assay is based on the mean of 4-6 plates that are counted under the microscope. It is thought that daily temperature fluctuations may be instrumental in this variation or the amount of endogenous DIF present within the cells might affect the assay.

It was not possible to assay all the analogues on one particular day, therefore the relative activity of the isomers was determined with respect to the activity of DIF-1 itself on the same day. This was felt to be a legitimate approach since on the same day, identical samples produced closely correlated results.

The results are presented in tabulated form (Table 12) and graphical form (Fig. 26).

The relative activities varied between 0.0004% and about 50%, therefore each reading was multiplied by 1000 and plotted as $\log_{10}$ (% activity). The limits and $\log_{10}$ (mean activity) were plotted against the number of carbon atoms in the alkyl side chain. Two separate curves are shown, the ortho-methoxy homologous series and the para-methoxy homologous series.

Analogue	% relative activity	$\bar{x} \cdot 10^3$	$\log_{10}(\bar{x} \times 10^3)$
p3C	2.31 , 2.05 , 3.56	2,640	3.421
p4C	33.8 , 37.0 , 51.7	40,800	4.611
p5C	100	100,000	5.000
p6C	14.4 , 13.1 , 15.3	14,270	4.154
p7C	2.19 , 2.50 , 2.45	2133	3.329
p8C	0.0426 , 0.0244 0.0354	34.13	1.533
o3C	0.0035 , 0.0031 0.0075	4.70	0.672
o4C	0.0066 , 0.0076 0.016	10.07	1.003
o5C	0.136 , 0.194, 0.100	143.3	2.156
o5C	0.288 , 0.332 0.540	383.3	2.584
o7C	0.0013 , 0.00044 0.0044 , 0.0123	2.047	0.311
o8C	0.0015 , 0.0015 0.0013 , 0.0010	1.325	0.122

Table 12. Bioactivity of alkyl side analogues
of DIF-1

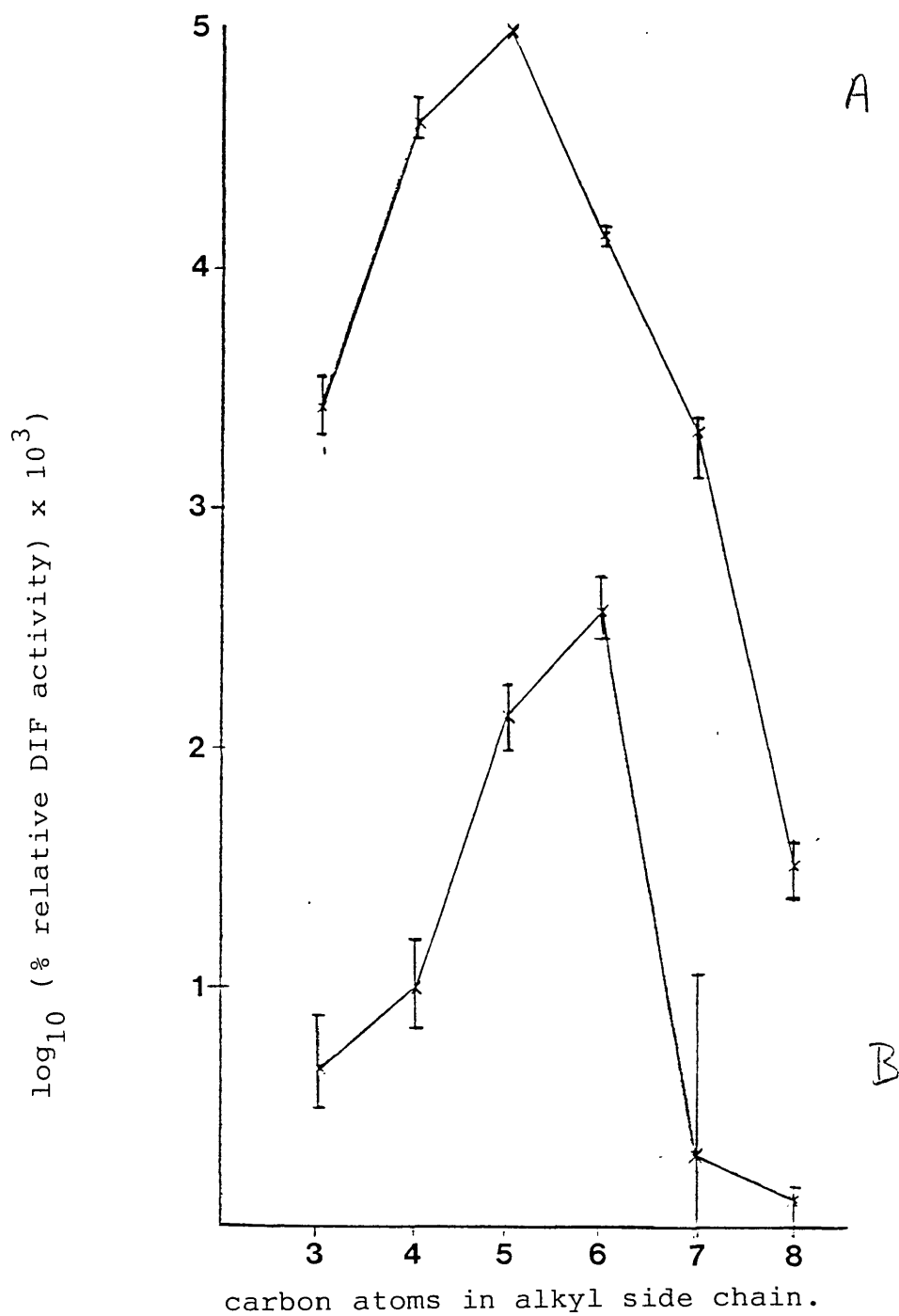


Fig. 26. Graph showing the effect of the length of the alkyl side chain in DIF-1 on activity.

$$\text{Activity} = \pm \log_{10} (\text{S.D.} \times 10^3)$$

A = para methoxy homologues

B = ortho methoxy homologues

Despite daily variations in the bioassay, the results give a clear account of the effect of shortening or lengthening the alkyl side chain.

The bioactivity decreases in the para-methoxy series if the number of carbon atoms in the alkyl chain changes but there is still at least 1% DIF activity in all these homologues except for the p8C which has a very low bioactivity. The shape of this curve suggests that the function of the alkyl side chain is not absolutely specific. It is more likely to function as having an effect on the overall solubility of the molecule or as a means of passing through a membrane rather than having a specific interaction with part of a receptor or acting as a spacer molecule. If the alkyl side chain were specifically involved in binding, one would expect the bioactivity to decline much more severely as the structure was modified.

The ortho-isomer homologue curve demonstrates that the position of the methoxy group is very important for bioactivity as there is a decrease of two orders of magnitude in most of the pairs of isomers. However, the o6C seems to be an anomaly. It has significantly greater bioactivity than its o5C homologue. It may be that the loss of bioactivity encountered by lack of a para-methoxy group or two ortho hydroxyls is compensated by having a more hydrophobic tail.

There was considerably more variation in the assay results from the ortho homologous series. It is thought that this may arise from using compounds at close to lethal concentration

and counting stalk cells at low cell density. One assay of o7C has some twenty-seven times the activity of another assay of the same material.

The brominated analogues produced an interesting result. The paramethoxy dibromo analogue exhibited DIF activity between 78.3% and 98.9% (mean 85.7%) of DIF-1 itself. Whereas the ortho isomer had relative activity between 0.22% and 0.28% (mean 0.24%) which is twice as high as the ortho dichloro compound. It is possible to argue that the reduced activity recorded by the para-isomer arises from some distortion of planarity of the molecule due to the bulkiness of the bromine atoms or that the asymmetric charge distribution generated by the highly electronegative chlorine is sufficient to produce a specific ionic interaction within a receptor binding site, whereas the bromine, which is less electronegative, may not have the required inductive strength.

The increase in activity between the ortho methoxy halo-analogues is more difficult to explain. The weaker inductive effect of bromine may render the hydrogen-bonded ortho-hydroxyl slightly less acidic which may be important since, in the para methoxy isomer, the acidity of both ortho hydroxyls is reduced as there are two possible lowest energy states for hydrogen bonding.

The diiodo compounds could not be synthesised by the methodology used in the bromo analogue synthesis. It would probably have had even less bioactivity due to distortion of the ring planarity and a weaker inductive effect.

Bioassay of the desmethyl analogue produced relative activities from 0.10% to 0.54% (mean 0.39%). This was greater than all the ortho methoxy analogues. This would suggest that the methyl group in the para position has some form of interaction or may effectively block an interaction, which, otherwise, may cause a different orientation of the effector molecule within its receptor binding site or alter dissociation constants, etc.

To determine exactly whether it is the paramethoxy or the two ortho hydroxyls that are required for receptor site interaction it would be necessary to synthesise the two dimethoxy isomers. One would have a free para-hydroxyl and two methylated ortho-hydroxyls the other would have one free ortho hydroxyl. From their particular specific activities one would gain a better understanding of the molecule's receptor site interactions.

The ortho desoxy analogue had a relative specific activity of about 0.035% which was about 25% of its O-methyl analogue. This would implicate that particular oxygen atom as having some function. It was unfortunate that the para-desoxy compound was not synthesised as this may have given a greater understanding of role of the O-methyl functional group.

3.4.5 Summary of DIF analogue studies

At the outset of these studies, it was intended to synthesise a variety of analogues of DIF-1 such that a picture could be built up of the structural requirements of the putative DIF-1 receptor. By altering the chemical structure of the functional groups, it has been possible to speculate about particular molecular interactions within the receptor binding site.

Variation in the length of the alkyl side chain reduced activity, but not to an extent that one would expect if it were involved in a specific interaction. It seems likely that the alkyl chain functions as a means of entering through a cell membrane, to react with an internal receptor, or that it functions as an 'anchor' within the phospholipid bilayer while the aromatic moiety interacts with a protein binding site.

Activity is destroyed by reduction of the carbonyl group.

The carbonyl group could have two functions; it may interact directly with an amino or hydroxyl group or can behave in an electron-withdrawing capacity to render an aromatic hydroxyl more acidic or deprotonate it altogether. The carbonyl may interact with the guanidino functional group of arginine or the electron-deficient carbon atom may interact with the carboxylate anion from glutamate or aspartate. A good example of such interactions is exhibited by carboxypeptidase A (91) as it cleaves the C-terminal amino acid from a polypeptide

chain. Within a programme of structural analogue syntheses, it would be useful to synthesise the thio-carbonyl analogue. This would not be sufficiently electronegative to deprotonate an ortho-hydroxyl and thereby it would be possible to determine the probable function of this functional group.

The data cited does not give a clear indication whether the reduction of activity in the ortho methoxy isomers arises due to the lack of a methyl group in the para position or the lack of two ortho hydroxyl groups. The result from the desmethyl analogue would tend to favour the former. It would be worthwhile looking at the effect of enlarging this ether to determine whether it was behaving as a blocking group or as a size-specific functional moiety.

The dimethyl derivatives would provide further information regarding the function of both ortho hydroxyls. One could speculate about the activity of two such analogues. The di-ortho-methoxy would have a lower activity than the ortho-methoxy isomer but the other isomer, which simply has one 'blocked' hydroxyl, would be expected to have an activity greater than all the ortho isomers and the desmethyl isomer.

The unexpected result arising from bioassay of o6C may occur because the marginally longer alkyl chain can allow a little more flexibility of the substrate within its binding site and a more effective receptor/substrate interaction than is occurring than in the o5C analogue.

The effect of bromine in the molecule, instead of chlorine was not surprising. The para methoxy dibromo analogue was almost as bioactive as DIF itself. One would conclude that this reduction in activity is possibly due to a slight distortion in the ring system due to steric hindrance from a bulkier substituent or that the bromine is not sufficiently electronegative to enhance the overall charge distribution in the molecule which would tend to deprotonate a phenolic hydroxyl. Had the diiodo compounds been produced, the activity would probably have been further reduced for the reasons stated above.

Many aspects of these basic structural alterations could have been taken a stage further - a second phase of structural analogues synthesis. All the compounds cited could have been tested in their monochloro and deschloro form. The limitation was an inadequate HPLC protocol for their purification. Isoalkyl isomers could have been synthesised for both the homologous series. The shortcomings of this have been explained as the difficulty in producing suitable nitriles for the initial Hoesch reaction. The isoalkyl isomers of DIF-1 and DIF-2 seemed to have very similar bioactivities although slightly less than the normal compounds. One might expect this trend to occur throughout the two homologous series. The synthesis of other branched analogues would depend on the availability of the alkyl halide and whether the branching caused any steric hindrance. One might expect the derivative with a methyl group on the α -carbon to have a high bioactivity although this produces a change in the spatial orientation of the side chain. Bromo analogues could be produced for all the alkyl homologues. One would expect to see a similar trend in the para-methoxy

series where the bromo compound has about 75% of the activity of its chloro counterpart. In the other homologous series one might expect bromine to enhance the activity of the homologue in the same way as the o5C. It may be possible to generate fluorinated analogues, although the process of introducing the F^+ ion by electrophilic addition may prove difficult.

A whole gamut of ring isomers could also be produced. There are six different positions on the aromatic ring for dichloro isomers; 2,3; 2,4; 2,5; 2,6; 3,4; 3,5. For a monomethoxy dihydroxy compound, 2,6 and 3,5 (DIF) can only produce two isomers, the remaining ring substitutions can produce three, therefore, there are sixteen possible ring isomers (Fig. 10, benzene nucleus). Four of the parent phenols are commercially available in partially or fully methylated form, but the 2,3,5 and 2,3,6 phenols are not readily available commercially, and would therefore need to be synthesised via an alternative route. The DIF analogue derived from a 3,5 dihydroxy-4-methoxy ketone would have the requisite para-methoxy and two free hydroxyls, but these are in a slightly different juxtaposition. However, this may provide an indication of the flexibility of the protein/substrate interaction within the binding site.

Just as a range of functional group isomers were synthesised for DIF-1, each different ring isomer would present the same possibility.

Whilst searching for the original ring substitution pattern, the various compounds (Table 3) were tested for inhibition

of stalk cell differentiation. It is likely that one or more of these ring analogues may be an effective inhibitor. This might provide a means of labelling or isolating the putative DIF-1 receptor.

3.5 Production of labelled DIF-1

The major advantages of producing a radiolabelled compound are those of facilitated detection and quantitation. Prior to producing labelled DIF, the only effective means of detection were by bioassay and to a limited extent by mass spectrometry. Both these methods preclude the investigation of DIF with the putative receptor or immunological studies, since both techniques rely on DIF being in a chemically intact form, although mass spectrometry can be used to investigate metabolites which are, by definition, chemically altered. Labelled material provides the facility to screen for DIF in more complex systems and to look at its interaction with proteins (receptors and immunoglobulins) and nucleic acids. Labelled material would expedite the analysis of DIF metabolism. It is likely that several labelled compounds could be isolated from Dictyostelium discoideum once it had been treated exogenously with labelled DIF.

There would appear to be three routes by which labelled DIF could be produced.

1. One of the starting products of the Hoesch synthesis could be labelled with ^3H or ^{14}C . Neither the phenol nor the nitrile is readily available in labelled form. Thus, a customised synthesis would be necessary, although it would be straightforward to produce labelled 3,5-dihydroxy-anisole via the 0-methyl group. In practice, the yields from the Hoesch reaction are fairly low and it would therefore be more cost effective to insert a label at a later stage in the synthesis.

2. The chlorinated desmethyl analogue could be synthesised, bearing in mind the susceptibility of the trihydroxyketone to oxidation by chlorine water. The para-methyl group could be added in a labelled form via $^{14}\text{CH}_2\text{N}_2$ or CT_3I (tritiated methyl iodide).

However this is more likely to produce the ortho methyl isomer, hence the products would have to be purified by HPLC. Moreover, the methylation has to be very limited as the molecule readily produces a trimethoxy derivative with excess methylating agent. Despite these drawbacks this could be effected and it does provide the best route for ^{14}C labelling.

3. The easiest and quickest means of labelling, and the method chosen here, is to utilise the relatively high acidity of the two protons on the α -carbon atom to the carbonyl group. Under strong basic conditions these two protons are accessible and amenable to a tritium exchange via a base-induced keto-enol tautomerism. The unknown parameter was how readily the tritiated label would re-exchange under neutral conditions.

This method of proton exchange was initially tried with deuterated reagents for reasons of cost, ease of handling and rapid EI-MS analysis. The DIF sample was 'washed' twice with CDCl_3 to remove traces of CHCl_3 . Reaction with 10% NaOD for 30 mins at 20°C was thought to be sufficient for keto-enol tautomerism and concomitant deprotonation. Subsequent neutralisation with DCl would allow reversion to the ketone form and stabilisation of the product $\text{R-CO-CD}_2\text{-C}_4\text{H}_9$. Extraction

with CDCl_3 allows easier EI-MS analysis. It was important to shake the sample with H_2O to exchange the phenolic protons and test the lability of the introduced deuterium atoms.

EI-MS analysis showed that the reaction had not proceeded to produce 100% d_2DIF . There was a distribution of peaks from m/z 306 to m/z 312, but the major peak was at m/z 308. This was twice as abundant as m/z 307 and five times as abundant as m/z 306. The fragmentation pathway showed that one deuterium was lost in the elimination of water, ie. a signal at m/z 289. A small fraction of the total deuterium was being lost in the γ -cleavage, ie. 264 = 265 m/z in abundance. This could be due to proton transfer down the alkyl chain to the β -carbon atom although this effect was very minor. One deuterium was lost in the McLafferty rearrangement which was not entirely expected. Fig. 22 shows that the protons on the α -carbon are conserved on the charged ion. Therefore, the fragment ion at m/z 251 can only be explained by the loss of a deuterium atom on the β -carbon. As expected, there was no alteration in the isotope patterns of the fragment ions at m/z 235 and 220.

The same sample was re-run two weeks later to assess the stability of the deuterium labelled material. The spectrum was almost identical to that run previously which suggests that 'non-induced' proton exchange is not a major concern with respect to the stability of the labelled compound. Although

it has not been undertaken, it seems fair to assume that these observations can be extended to the tritium-labelled material, and, consequently, this would appear to be a clean, effective means of generating radiolabelled DIF.

CHAPTER IV

DISCUSSION AND CONCLUSIONS

Determining the chemical identity of an unknown molecule is not the sole consideration in a structure elucidation problem. Just as relevant are the routes taken, the choice of experiments, the analysis of ambiguous data, the objective appraisal of results and the piecing together of the chemical jigsaw.

When the first samples of DIF were analysed physicochemically, it was not known whether the data produced was real or artefact or whether it was arising from the unknown compound or from contaminants. The first U.V. analyses exhibited broad absorption peaks with differing maxima. This may have been due to a strongly absorbing chromophore present as an impurity in the sample. The largest preparation of DIF barely produced enough material for an adequate ^1H -n.m.r. analysis (Fig. 9). The 60 μg of sample required long scan times and generated spectra with low intensity signals. Many of these signals could not be assigned and had ambiguous integration ratios. The signal at δ 10.3, which was ultimately important in the determination of the ring substitution pattern, was often not detected and did not integrate as two protons. It was not possible to determine the structure of the alkyl chain in DIF by n.m.r. due to many contaminating peaks below δ 2.5.

The consistency of the mass spectrometric data was the guide indicating that DIF was at least being observed in the MS experiments, since the spectrum was reproducible and not due to solvent impurities such as phthalates, etc.

Using a range of sophisticated analytical techniques, the final nuclear structure was narrowed down to two possibilities (Fig. 10). The cyclopentadienyl nuclear structure was eliminated using collision activation mass spectrometry (MS-MS). This technique showed that the daughter ions produced by focussing on the base peak of fully methylated DIF (m/z 263) could not arise from the cyclopentadienyl structure. The observed losses of $\text{CH}_3^\bullet$ and CO confirmed the presence of a benzene nucleus in the DIF structure.

The lack of natural material made a complete structure elucidation impossible. The ring substitution pattern and the structure of the alkyl chain were still unknown. There were 32 possible isomers of the aromatic nucleus with either an n-pentyl or isopentyl side chain. Early mass spectrometric studies on a number of chlorinated and unchlorinated polyhydroxy benzenes (Table 3) had suggested that a 2,4,6 trihydroxy ring substitution pattern might be a possible basic structure for DIF. This pattern would also be favourable on biosynthetic grounds arising from polyacetate biosynthesis (Fig. 11). (92, 93).

n-Butyl, isobutyl, n-pentyl and isopentyl ketones of 3,5 dihydroxyanisole were synthesised. The dichloro derivatives of these ketones was also synthesised. The exact structures of DIF-1 and DIF-2 were confirmed by comparison of analytical data from the natural and synthetic compounds (94).

The chemical structure of DIF-1 was determined as:

3,5 - dichloro - 2, 6 - dihydroxy - 4 - methoxyhexanophenone.

The chemical structure of DIF-2 was determined as (95):

3,5 - dichloro - 2, 6 - dihydroxy - 4 - methoxypentanophenone.

Both these compounds are totally novel, although phenolic ketones occur widely as natural products (60,65, 92, 93, 96-99).

One of the major interests in these compounds is the presence of chlorine. Some 700 halometabolites have been characterised and many reviews exist that cite these compounds from their respective sources (64, 65, 100-105). Among better known chloro-organics are chlortetra-cycline, chloramphenicol and griseofulvin. However, many other chlorinated metabolites possess antimicrobial activity, a few show anti-tumour action, others are potent enzyme inhibitors or function as plant auxins.

It would obviously be interesting to test DIF for antibiotic, anti-tumour activity and possible enzyme inhibition, however this would become a major research project and would require large amounts of material (≈ 1 gm). The structure elucidation and synthesis now opens up this opportunity.

The mechanism of in vivo chlorination in Dictyostelium is unknown, although it seems likely that a chloroperoxidase may be responsible for this reaction (106). Although an extensive programme of research had not been undertaken, preliminary studies had failed to find a chloroperoxidase in the slime mould using

antichloroperoxiase antibodies (107). There is a possible explanation for this observation; there are considerable similarities between fatty acid synthesis and polyketide synthesis. Both involve combining C_2 carbon units to produce either saturated alkyl chains or polyketonic chains. The total synthesis of the simple fatty acids occurs on a multi-functional protein (108). Fig. 26 shows a hypothetical biosynthetic route for the synthesis of DIF-1. Generation of the twelve-carbon hexa ketide may take place on one part of the protein and then chlorination, reduction, cyclisation and methylation can all take place sequentially while the substrate is attached to the enzyme. The chloroperoxidase activity may be within the multi-functional protein and therefore inaccessible to recognition by antibodies.

DIF-2 may arise either through biosynthesis via an initial coupling of acetyl CoA to propanoyl CoA or by a C-demethylation during or after chain biosynthesis. DIF-2 may occur as a metabolite of DIF-1, although it would be difficult to find a reason for C-demethylation unless a methyl transfer was occurring to alkylate DNA, which may be the way DIF exerts its biological action.

One of the minor DIF species, DIF-3 (Fig. 12) was found to be a monochloro compound after mass spectrometric analysis. This would argue against biohalogenation at such an early stage in the biosynthetic pathway. However monochloro-DIF may be a metabolite, in which case, the suggested biosynthesis is still applicable. Such theories could be ratified once labelled DIF-1 is available.

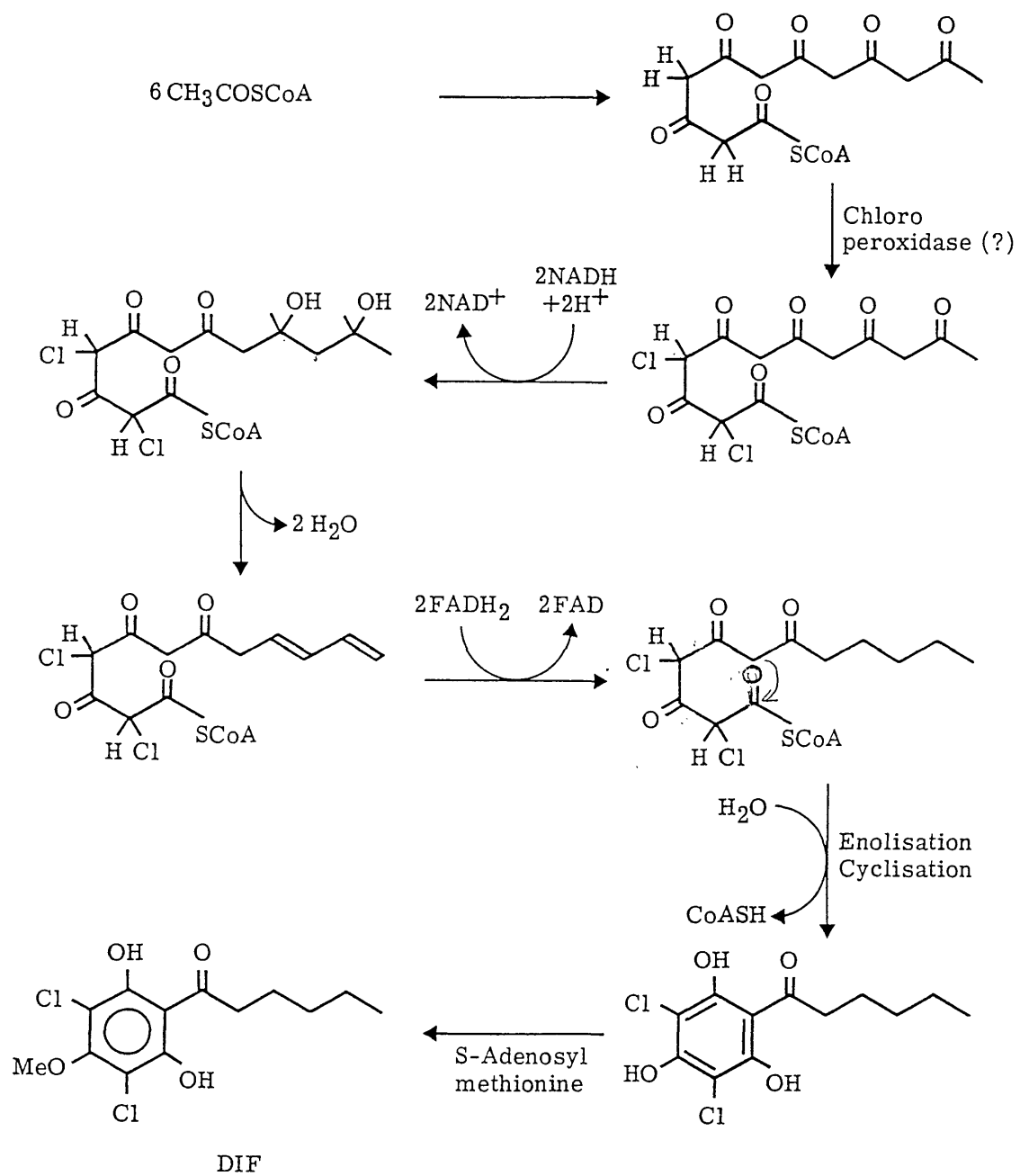


Fig. 27. Hypothetic biosynthetic pathway of DIF-1 from Acetyl-CoA.

The results and conclusions generated from the structural analogue studies have given a strong indication of the importance of the various functional groups in the DIF structure. This is not a complete study but it provides the basis of a more extensive analogue synthesis programme. It is possible that one or some of these analogues may have an inhibitory effect on DIF activity. By investigating the kinetics of inhibition, more data regarding the putative receptor can be generated which would afford a better understanding of DIF mode of action.

The physicochemical data and subsequent structure elucidation of DIF has provided a means of detection and quantitation, the production of labelled DIF will broaden such studies and facilitate the research at the cellular and molecular level.

DIF is the first endogenous morphogen to be chemically characterised and this will help us to understand chemical signalling during development in a simple biological system. This will allow developmental biology to be studied at the tissue, cellular, protein and molecular level. The whole concept of embryology, cell patterning and fate maps now has a chemical perspective.

CHAPTER V

MATERIALS AND METHODS

MATERIALS

Chloramphenicol, 1-chloro dinitrobenzene, iodomethane, iso-amylbromide and sodium borohydride were obtained from Sigma Chemical Co., Poole, Dorset.

Butyronitrile, Diazald, hexanonitrile, isovaleroyl chloride, octanonitrile, orcinol, 2,3,4-trimethoxyacetophenone, 3,4,5-trimethoxyacetophenone, 2,4,6-trimethoxyacetophenone, 2,4,6-trimethoxybenzaldehyde, 2,4,6-trihydroxybenzoic acid, valeroyl chloride and deuterated solvents CDCl_3 , D_2O and TFAD were purchased from Aldrich Chemical Co, Gillingham, Dorset.

BSTFA, TMCS and pyridine were purchased from Pierce.

3,5-dihydroxyanisole, phloroglucinol and 2,4,5-trihydroxybutyrophenone were bought from Fluka, Glossop, Derbyshire.

Heptanonitrile, n-hexanoyl chloride, nonanitrile, valeronitrile were obtained from Lancaster Synthesis, Morecambe, Lancs.

METHODS

A. ANALYTICAL

Ultraviolet spectroscopy

U.V. spectra were recorded in methanol (10 mm pathlength) (0.5-1.0 ml) on a Cary 210 scanning spectrophotometer. Scans were run from 340-230 nm. The spectra were calibrated with a holmium filter.

Electron impact mass spectrometry

Mass spectrometry was carried out on a Kratos MS50 mass spectrometer fitted with a high field magnet. The electron impact (EI) mode was used. Samples were loaded in chloroform into a hollow quartz tip; the accelerating voltage was 8 kV with 70 eV electron beam energy. Samples were analysed at either 120° or 150° C source temperature. Spectra were recorded on U.V. sensitive paper and counted manually.

Fast atom bombardment mass spectrometry

FAB mass spectra were obtained on a VG Analytical High Field ZAB IF mass spectrometer operated in the positive and negative modes at an accelerating voltage of 8kV. The atom gun was operated at 8-10 kV and xenon was used as the bombarding gas. Spectra were recorded on U.V. sensitive oscillographic paper and were counted manually. The instrument was scanned in a mass controlled mode and linear scans were performed at a scan rate of 600 a.m.u. min⁻¹.

Samples were dissolved in methanol and loaded into a mixture of glycerol and monothioglycerol (1:1, v:v, 1 μ l) on the stainless steel target, between 0.5-10 μ g of sample was loaded.

Desorption Electron Impact

Samples were run on same machine as for DCI. The sample was desorbed from the rhenium wire loop by heating at 500 mA sec⁻¹, and applying increasingly stronger bursts of current, eg. 100 mA, 200 mA, 400 mA.

Desorption Chemical Ionisation

DCI was performed on a Finnegan MAT 4500 quadrupole mass spectrometer fitted with INCOS 2000 data system. Samples were adsorbed onto a rhenium wire loop and desorbed off at 500 mA sec⁻¹ using ammonia as reagent gas. Quasi-molecular ions (M+H)⁺ and adducts (M+NH₄)⁺ were produced. This was performed in the positive and negative modes.

Gas Chromatography/Mass Spectrometry

Gas chromatography/mass spectrometry (GC/MS) was performed on a Finnigan MAT 4500 gas chromatography/quadrupole mass spectrometer interfaced to an INCOS 2000 data system. Samples were run on a 30m SE54 fused silica capillary column of internal diameter 0.25 mm. This was routed through the separator oven and directly into the mass spectrometer ion source (maintained at 150°C). Helium was used as carrier gas at a constant head pressure of 20 p.s.i. (138 kPa). A Grob-type injector was used in the splitless mode.

Nuclear Magnetic Resonance

Proton-N.M.R.

^1H -n.m.r. was carried out on a Bruker WM250 250 MHz spectrometer. CDCl_3 was used as a reference, producing a single peak at 7.24 ppm. Samples were dissolved in 0.5 mls CDCl_3 and placed in 5 mm n.m.r. tubes. The sweep width was 16 ppm - -2 ppm.

^{13}C -n.m.r.

Samples for ^{13}C -n.m.r. were also run in CDCl_3 . A sweep width of 220-0 ppm was employed.

X-Ray Crystallography

A suitably-sized crystal was fragmented and X-ray crystallographic measurements were carried out on a Nicolet R3m/Eclipse S140 diffractometer system with graphite monochromated $\text{Cu-K}\alpha$ radiation. Integrated intensities in one quadrant were measured using the ω - scan technique. The structure was solved by direct methods.

High Pressure Liquid Chromatography (HPLC)

HPLC was carried out on a Waters instrument fitted with a reverse-phase μ Bondapak C_{18} column, (semi-preparative 30 cm x 0.78 cm).

Solvents were of 'Analytical Reagent' quality; acetic acid and propan-1-ol were used directly from the bottle. Aqueous solvents were degassed on a water pump before use.

The column eluate was monitored at 254 nm and 280 nm.

DIF Bioassay

The DIF bioassay was carried out by Dr R. Kay at the ICRF and later at the MRC Lab of Molecular Biology, Cambridge.

Up to 5 μ l of DIF in ethanolic solution is added to 2 ml of 10 ml MES (K^+ salt pH 6.2), 2 mM NaCl, 10 mM KCl, 1 mM $CaCl_2$, 1 mM cAMP, 0.2 mg ml^{-1} streptomycin sulphate, 15 μ g ml^{-1} tetracycline, in a 5 cm diameter sterilin tissue culture dish. Washed cells of strain V12M2 were resuspended in NS (20 mM NaCl, 20 mM KCl, 1 mM $CaCl_2$) is added to the dishes at a density of $7.5 \times 10^2 \text{ cm}^{-2}$ and allowed to differentiate in the dark at 22°C. After 2-3 days, amoebae and stalk cells are scored by phase-contrast microscopy.

Mass Measurement

Mass measurement was achieved on the Kratos MS5D using perfluorokerosene as a reference. Accurate masses were measured for molecular ion and major fragment ions.

Determination of Molar Extinction Coefficient (E) for DIF

1 mg of DIF was dissolved in 1.0 ml methanol. Duplicate dilutions were made of 1/100 and 1/20 from the 1.0 mg ml⁻¹ stock. The absorbance was measured at λ_{max} 278 nm. Methanol was used to zero the spectrophotometer. From the absorbance values, using the Beer-Lambert law, the molar extinction coefficient was determined.

B. SYNTHESSES

Grignard Synthesis

A dry, 100 ml round bottom flask is fitted with a 100 ml dropping funnel and a reflux condenser, on top of which is placed a CaCl_2 drying tube.

0.73g (0.03 moles) granulated magnesium, 10 mls dry diethyl ether, 0.315 mls 1-bromo-3-methylbutane (isoamylbromide) and a magnetic stirrer is added to the flask. The flask is kept at 4°C in water/ice bath.

If the reaction does not commence within 5 mins, 0.2 ml CH_3I a crystal of iodine is added. Once the formation of the organometallic has begun, 3.66 mls (0.018 moles) of isoamylbromide is added dropwise over 10 mins. The reaction mixture is stirred for a further 20 mins.

1g (0.005 moles) 2,4,6-trimethoxybenzaldehyde is added slowly to the mixture. A dark brown, viscous product is formed. 10 mls H_2O and 10 mls dilute HCl are added to neutralise the Grignard salt and precipitate inorganic salts.

The products are placed in a separating funnel and 20 mls CHCl_3 is added. This produces three immiscible layers. The two organic layers are separated and concentrated by rotary evaporation. Products are analysed by EI-MS.

Fries Reaction

Ester Formation

1.4g dihydroxyanisole was dissolved in 6 ml dry pyridine. 1 molar equivalent of alkyl acid chloride was added dropwise with continuous stirring. After 15 mins 10 ml H₂O was added. The organic layer was collected and the pyridine removed by rotary evaporation. The products were analysed by EI-MS.

Fries Rearrangement

6g of anhydrous AlCl₃ was put into a 100 ml 3-necked, round bottom flask. 20 ml of dichloromethane was added and the slurry was stirred. The ester was dissolved in 20 ml CH₂Cl₂ and added dropwise over 60 mins. The mixture boils vigorously as HCl is evolved. The colour turns amber. The mixture is stirred for a further 4 hours and then allowed to stand overnight.

The AlCl₃ was neutralised by adding 20 ml dilute HCl followed by 20 ml H₂O. the organic layer was separated from the aqueous layer and washed twice with water. The products were analysed by EI-MS.

Hoesch Synthesis

Ketimine Synthesis

2.5 g dihydroxyanisole (DHA), 2.0 mls hexanonitrile, 25 mls cold, dry ether and about 2 g fused, powdered zinc chloride were put into a 125 ml Dreschel flask. The flask and contents were placed in an ice/salt bath. HCl gas was bubbled through the solution for 20 mins. A red or orange colouration indicated that reaction was proceeding. The reaction mix was stored at 4 °C for 18 hours. HCl gas was again bubbled through the mixture for 20 mins. The flask was kept at 4 °C for 48-72 hours to allow a precipitate (ketimine chloride) to form.

Hydrolysis of Ketimine

Nitrogen was bubbled through the mixture to remove HCl gas. The blood-red supernatant and precipitate were washed twice with cold ether. The supernatant and precipitate were dissolved in 100 mls boiling water. The solution was transferred to a 250 ml round bottom flask on a heating mantle. Any material that did not dissolve in the water could be dissolved with up to 20 mls methanol. The solution was heated under reflux for 2 hours. The solution was filtered in a heated Buchner funnel. The filtrate was collected and allowed to cool. A feathery, off-white precipitate was produced which can be collected by another filtration. The hot solution may contain an immiscible orange oil. On exposure to air this solidifies to produce amorphous orange clusters. Both the white and orange precipitates contain the ketone.

This synthesis was used for all alkyl homologues (C_3 , C_4 , C_6 , C_7 , C_8).

DHA was substituted by phloroglucinol to make the desmethyl analogue and orcinol was used to make the desoxy analogue.

Preparation of Isohexanonitrile

0.5 moles of KCN (33g) was warmed in 35 ml of water. 0.45 moles of 1-bromo-4-methylbutane in $((CH_3)_2CHCH_2CH_2Br)$ in 75 ml of ethanol was added slowly into a round bottom flask. The reagents were refluxed for 4 hours. A brown colouration developed and NaBr crystallised out. The NaBr was filtered off and washed with MeOH. The nitrile can be collected by fractional distillation.

At $65^\circ C$ the methanol was distilled off. Subsequently two layers formed. The lower, aqueous layer is discarded. The upper layer is fractionated. The nitrile distilled over at $152^\circ - 160^\circ C$. This was collected and redistilled. The fraction distilling at $152^\circ - 154^\circ C$ was collected.

Chlorination

Chlorine gas (BDH) was bubbled through 100 mls of 10 mM HCl for two minutes. The chlorine water was added to an equal volume of the ketone in chloroform (approx. 1% solution) in a separating flask. The flask was shaken by hand vigorously

for 1 min. and the layers were allowed to separate. The aqueous layer was removed by aspiration. A sample of the yellow organic layer was analysed by EI-MS. The ratio of the molecular ions of the monochloro- and dichloro derivatives was recorded. The chlorination procedure was repeated until the ratio is 1:3 ($\text{Cl}_1:\text{Cl}_2$).

The isomers were separated by HPLC.

Bromination and Iodination

A 5 mM solution of the halogen is made and an equal volume is added to a 1% solution of the ketone in chloroform. The analysis of the products is identical to that of the chloro-derivatives.

Purification of Halogenated Ketones

The mixture of products from the halogenation of the ketones was separated by reverse-phase HPLC. An isocratic elution was used of 35% propan-1-ol in 5% acetic acid. The column was eluted at 1.0 ml min^{-1} . The column eluate was monitored at 254 and 280 nm. 1.0 ml fractions or whole peaks were collected. The desoxy derivative required an isocratic elution of 28% propan-1-ol in 5% acetic acid for optimum resolution of components.

C. CHEMICAL

Acid Hydrolysis

DIF was dissolved in 2M hydrochloric acid and incubated at 60°C for 2 hours.

Base Hydrolysis

DIF was dissolved in 2M sodium hydroxide and incubated at 60°C for 2 hours.

Borohydride Reduction

The sample in methanol was treated with sodium borohydride (1.0 mg ml^{-1}) and incubated at room temperature for 60 mins. The reaction was stopped and excess borohydride neutralised with acetic acid. The reduced sample was extracted with CHCl_3 .

Borodeuteride Labelling

The procedure was repeated as above but a 1:1 mixture of $\text{NaBH}_4/\text{NaBD}_4$ was used.

Reaction with Diazomethane

Ethereal diazomethane was freshly prepared in a millimole-size Wheaton generator (Pierce and Warringer U.K. Limited) by the method of Fales et al (109) and allowed to react with a methanolic solution of DIF for 30 mins. Excess reagents were removed under nitrogen.

Catalytic Hydrogenation

Sample was dissolved in methanol and Platinum dioxide was added. Hydrogen was bubbled through the mixture. The procedure was carried out at one atmosphere for one hour and subsequently at two atmospheres for two hours. LTB_4 was used as a positive control. LTB_4 was converted to $\text{h}_8 - \text{LTB}_4$.

Deuterium-Labeling of DIF

0.1 mg OF DIF was dried under vacuum. The sample was redissolved in CDCl_3 twice. 0.4 ml 100% D_2O and 0.1 ml 40% NaOD was added. After 30 min. at room temperature the solution was neutralised by the addition of 0.2 ml 20% DC;. The labelled material was extracted with 2 x 0.5 ml CDCl_3 . The sample was shaken with 0.5 ml H_2O . The aqueous layer was discarded and the product analysed by EI-MS.

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