FLAVONES AND RELATED COMPOUNDS AS INHIBITORS OF PROTEIN TYROSINE KINASES.

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SUMMARY.

Aberrant tyrosine protein kinase activity has been implicated in the formation and maintenance of malignancy and so presents a potential target for cancer chemotherapy. Quercetin, a naturally occuring flavonoid, inhibits the tyrosine protein kinase encoded by the Rous sarcoma virus but also exhibits many other effects. Analogues of this compound were synthesised by the acylation of suitable 2-hydroxyacetophenones with appropriately substituted aromatic (or alicyclic) acid chlorides, followed by base catalysed rearrangement to the 1-(2-hydroxyphenyl)-3-phenylpropan-1,3-diones. Acid catalysed ring closure furnished flavones.

The majority of the 1-(2-hydroxyphenyl)-3-phenylpropan-1,3-diones were shown by NMR to exist in the enol form. This was supported by the crystal structure of 1-(2-hydroxy-4-methoxyphenyl)-3-phenylpropan-1,3-dione. In contrast, 1-(4,6-dimethoxy-2-hydroxyphenyl)-3-phenylpropan-1,3-dione did not exhibit keto-enol tautomerism in the NMR spectrum and was shown in its crystal structure to assume a twisted conformation.

Assessment of the biological activity of the analogues of quercetin was carried out using whole cells and the kinase domain of the tyrosine protein kinase encoded by the Abelson murine leukaemia virus, pt*abl*50 kinase.

Single cell suspension cultures and clonogenic potential of murine fibroblasts transformed by the Abelson Murine leukaemia virus (ANN-1 cells) did not indicate the existence of any struture activity relationship required for cytotoxicity or cytostasis. No selective toxicity was apparent when the "normal" parent cell line, (3T3), was used to assess the cytotoxic potential of quercetin. The IC_{s50} for these compounds were generally in the region of 1-100μM.

The potential for these compounds to inhibit pt*abl*50 kinase was determined. A definite substitution requirement emerged from these experiments indicating a necessity for substituents in the A ring or in the 3-position of the flavone nucleus. Kinetic data showed these inhibitors to be competitive for ATP.

Keywords. tyrosine protein kinase inhibitors, flavones, structure/activity relationship, chemotherapy, structural analysis.

An apple a day.....

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Tables

ABBREVIATIONS

Adenosine diphosphate	ADP
Adenosine triphosphate	ATP
Broad singlet	brs
Broad doublet	br d
Chronic myelogenous leukaemia	CML
Concentration of inhibitor	[I]
Concentration of compound producing 50% inhibition of growth or enzyme reaction.	IC_{50}
Concentration of substrate	[S]
Correlation spectroscopy	COSY
Counts per minute	cpm
Cyclic adenosine monophosphate	cAMP
Cytosine triphosphate	CTP
Dimethyl formamide	DMF
Dimethyl sulphoxide	DMSO
Diacylglycerol	DAG
Diethyl ether	ether
Dimethylbenz-(a)-anthracene	DMBA
Dulbecco's Modified Eagle Medium	DMEM
Endoplasmic reticulum	ER
Epidermal Growth Factor	EGF
Estimated standard deviation	ESD
Ethylene diamine tetracetic acid	EDTA
Figure	Fig.
Foetal calf serum	FCS
Guanosine triphosphate	GTP
Hours	hrs
Immunoglobulin	Ig

Inhibition constant	Ki
Infra-red	IR
Inositol	I
Inositol-1,4-bisphosphate	$I_{1,4}P_2$
Inositol phosphate	IP
Inositol-1,3,4,5-tetrakisphosphatase	$I_{1,3,4,5}P_4$ase
Inositol-1,3,4,5-tetrakisphosphate	$I_{1,3,4,5}P_4$
Inositol-1,3,4-trisphosphatase	$I_{1,3,4}P_3$ase
Inositol-1,3,4-trisphosphate	$I_{1,3,4}P_3$
Inositol-1,4,5-trisphosphatase	$I_{1,4,5}P_3$ase
Inositol-1,4,5-trisphosphate	$I_{1,4,5}P_3$
Insulin-like growth factor	IGF
Kilodaltons	Kd
Literature	lit.
Maximum velocity of enzyme reaction	Vmax
Melting point	m.pt.
Michaelis Constant	Km
Nuclear Overhauser effect	nOe
Nuclear magnetic resonance	NMR
Nicotine adenine dinucleotide	ßNAD
Observed	obs.
Philadelphia chromosome	Ph'
Phosphatidylinositol	PI
Phosphatidylinositol-4,5-bisphosphate	PIP_2
Phosphatidylinositol-4-phosphate	PIP
Phospholipase C	PLC
Phosphodiesterase	PDE
Phosphoprotein	pp

Platelet derived growth factor	PDGF
Piperazine-N,N'-bis[2-ethanesulfonic acid]	PIPES
Protein	p
Protein kinase C	PKC
Tris-(hydroxymethyl)aminomethane	Tris
Rous sarcoma virus	RSV
Seconds	secs
Temperature sensitive	ts
Tetrahydrofuran	THF
Tetradecanoyl phorbol acetate	TPA
Thin layer chromatography	tlc
Tumour bearing rabbits	TBR
Tyrosine protein kinase	TPK
Ultra violet	UV

CHAPTER ONE.

TYROSINE PHOSPHORYLATION AND CELLULAR TRANSFORMATION.

Francis Peyton Rous first reported the observation of an "infectious" cancer in 1911 and was able to attribute this to a virus [1]. This virus is an RNA tumour virus which encodes four genes, three of which encode viral proteins but the fourth, the "onc" gene, is unnecessary for the virus. This "onc" gene is designated src and the product of this gene is responsible for the transformation of cells infected with RSV [2].

The src gene encodes a phosphoprotein of 60 000 daltons, termed pp60$^{v\text{-}src}$ [3, 4, 5, 6, 7]. pp60$^{v\text{-}src}$ is a protein kinase [5, 8] that is capable of phosphorylating the immunoglobulin heavy chain in immunoprecipitates. Although an initial report identified this as phosphorylation of a threonine residue in the Ig heavy chain [8] this is now known to be incorrect. pp60$^{v\text{-}src}$ has the unusual kinase activity capable of phosphorylating proteins on tyrosine residues [9, 10].

There is much evidence to show that tyrosine phosphorylation by pp60$^{v\text{-}src}$ is responsible for the transformation of cells infected with RSV. Many mutants of RSV have been isolated that are temperature sensitive in their ability to transform infected cells *in vitro*, and some of these temperature sensitive (ts) mutants have been used

experimentally to investigate the correlation between the activity of $pp60^{v\text{-}src}$ and cellular transformation. Temperature sensitive mutants of RSV are incapable of transforming infected cells at the non-permissive temperature but do so at the permissive temperature. Certain mutants are non-tumourigenic in three week old chickens (whose body temperature is 42°C) and do not form foci in clonogenic assays at 41°C (the non-permissive temperature) but do form foci at 35°C, the permissive temperature [11].

Sefton *et al* [12] used one such mutant, the tsLA29(PR-RSV-A) isolated originally by Wyke and Linial [13] to show that the phosphorylation of tyrosine by $pp60^{v\text{-}src}$ was essential for the transformation of cells by RSV. The level of phosphotyrosine in cells infected with the ts mutant was compared to that of the cells infected with a wild-type RSV, when both these infected cells were incubated at 36°C and 41°C and the degree to which these cells were transformed at these temperatures was compared, using 2-deoxyglucose uptake as a measure of transformation. At the permissive temperature (36°C) the rate of uptake of 2-deoxyglucose by the wild-type RSV infected cells and the ts mutant infected cells was similar. Although the abundance of phosphotyrosine in the ts mutant infected cells was less than that of the wild-type RSV infected cells it was five times higher than in uninfected cells and this correlated with the amount of protein kinase activity that could be immunoprecipitated from a lysed cell

preparation, suggesting that even at 36°C the $pp60^{v\text{-}src}$ of the ts mutants was not fully active. At the non-permissive temperature, 41°C, the cells infected with the wild-type RSV showed no decrease in protein kinase activity, and although the rate of uptake of 2-deoxyglucose was decreased it was still much greater than that of the ts mutant infected cells. At the non-permissive temperature the ts mutant infected cells contained a similar amount of phosphotyrosine, exhibited similar immunoprecipitable protein kinase activity and rate of uptake of 2-deoxyglucose as the non-infected cells. These changes were evident within 60 minutes of the shift from permissive to non-permissive temperatures. This suggests that there are enzymes present in the cell that act as tyrosine phosphatases.

Protein tyrosylphosphatases have been identified in a number of cell systems [14], [15], [16], [17] and these phosphatases can be inhibited by Zn^{2+} [14], and by orthovanadate [15], [16].

If uncontrolled tyrosine phosphorylation is responsible for the transformation of cells then an inhibitor of tyrosine phosphatases would disturb the balance between the kinase and phosphatase and this could lead to transformation of the cells treated with a tyrosine phosphatase inhibitor.

J K Klarlund [18] was able to show changes in the morphology of a normal cell line in tissue culture, along with an increase

in 2-deoxyglucose uptake. Although the addition of 1mM sodium orthovanadate lead to cell death within 6-12hrs the cell line could tolerate lower concentrations of sodium orthovanadate (37.5 μM) for upto a month.

So it seems that uncontrolled phosphorylation of tyrosine residues in proteins is responsible for the transformation of certain cells.

Tyrosine phosphorylation as a protein modification was unknown until it was identified in polyoma virus middle-T antigen immunoprecipitates, [19] by two dimensional gel electrophoresis. Threonine and tyrosine do not separate well by electrophoresis in one dimension, so after the initial separation has been carried out by electrophoresis the gels are chromatographed in a second dimension to further separate the threonine and tyrosine residues.

Phosphorylation as a protein modification is well documented (for review see C S Rubin and O M Rosen [20]). By far the most abundant phosphoamino acid present in the cell is phosphoserine; this accounts for approximately 90% of the cell's phosphoamino acids, phosphothreonine accounting for only 10% of the phosphoamino acids. Prior to the discovery of phosphotyrosine in RSV infected tumour cells phosphotyrosine had not been detected in normal cells. Only one in three thousand phosphoamino acids in a normal cell is accounted for

by phosphotyrosine! [12], even in cells transformed by RSV where there is a seven to ten fold increase in phosphotyrosine this phosphoamino acid accounts for only 0.3% of the phosphoamino acids in the cell.

Uninfected chicken cells contain DNA sequences that are related to the transforming gene of RSV [21]. DNA from other species (calf and murine DNA were tested) also showed some homology to the viral src gene although this homology was not as great as that of chicken DNA [22]. These genes encode for two proteins which are very similar both structurally and functionally [23], [24], [25], [26], [27], although they are not identical. The difference appears to reside in the carboxy terminus of the protein; 19 amino acids in the carboxy end of $pp60^{c\text{-}src}$ have been replaced in $pp60^{v\text{-}src}$ by 12 unrelated amino acids [28].

As mentioned above the src gene and its encoded protein serve no function to the virus and much evidence suggests that this gene was incorporated into a non-oncogenic virus during infection of a chicken by that virus. Hanafusa and co-workers, [29], [30], [31] using transformation defective mutants of RSV, (those viruses which have partial deletions in the src gene and which are incapable of transforming chicken fibroblasts) have shown that after a long latency period following injection of transformation defective RSV tumours have developed distant from the site of injection and these tumours can produce fully

transforming RSV. This new virus was designated "recovered Avian Sarcoma Virus".

OTHER TYROSINE PROTEIN KINASES

It has been suggested that the acutely transforming RSV was generated, after a latency period, from an avian leukosis virus infection of chickens [32].

$pp60^{v-src}$ is not the only tyrosine specific protein kinase known; RSV is a member of a family of retroviridiae whose transforming gene products encode proteins that are tyrosine specific protein kinases (TPKs). RSV is probably the best characterised of these viruses which are listed in Table 1.1.

The growth factor receptors and insulin receptor are transmembrane proteins. The epidermal growth factor (EGF) receptor is a single chain membrane glycoprotein [40], whereas the insulin receptor is more complex consisting of two subunits of MW 125 000 (αsubunits) and two subunits of MW 90 000 (β subunits) [41, 42].

SOURCE	ONCOGENE	PROTEIN	REFERENCE
Rous sarcoma virus	src	$pp60^{v\text{-}src}$	9
Abelson murine leukaemia virus	abl	$pp120^{gag.abl}$	33
Fujinami sarcoma virus	fps	$pp140^{gag.fps}$	34
McDonough feline sarcoma virus	fms	$p180^{gag.fms}$	35
Gardner-Rasheed feline sarcoma virus	fgr	$p70^{gag.actin.fgr}$	
Y73 avian sarcoma virus	yes	$pp90^{gag.yes}$	
Avian erythro-blastosis virus	erb-B	$p44^{erb\text{-}B}$	36
Simian sarcoma virus	sis	$p28^{sis}$	37
From a chemically transformed human cell line	met	??	38
from a rat neuro-blastoma induced by ethyl-nitroso-urea.	neu	??	39

Table 1.1. Tyrosine protein kinases and their sources.

When EGF binds to its cell surface receptor the receptor becomes phosphorylated on tyrosine [43] and is then internalised as a hormone-receptor complex. This complex then initiates a cell response and is ultimately degraded in lysosomes.

The β-subunit of the insulin receptor is responsible for tyrosine protein kinase activity when insulin binds to the receptor [44], [45], [46]. Closely related to the insulin stimulated tyrosine protein kinase of the insulin receptor is the stimulation of a tyrosine protein kinase activity in the insulin-like growth factor receptor by insulin-like growth factor 1 (IGF 1). IGF-1 is a polypeptide homologous to human pro-insulin [47].

Thus although phosphorylation of proteins on tyrosine residues is a rare event when compared to serine and even threonine phosphorylation, there is abundant evidence to suggest that it is intimately involved in the regulation of cell growth in response to hormonal stimuli.

Two of the viral tyrosine protein kinases are related to the EGF receptor and the platelet derived growth factor (PDGF). p28sis shows extensive homology to PDGF [48], [49] and the v-erb-B oncogene shows homology with the EGF receptor [50].

ACTIVATION OF $pp60^{v\text{-}src}$.

Serine and threonine protein kinases are dependent on cyclic-AMP (c-AMP) for their activation and utilise only ATP as a phosphate donor [51], [20]. $pp60^{v\text{-}src}$ is independent of c-AMP for its activation [52] and is able to transfer phosphate to its protein substrate from a number of phosphate donors, ATP and

GTP being the most common [53]. The phosphorylation of one of the substrates of $pp60^{v\text{-}src}$ can be enhanced by the addition of Ca^{2+} and a phospholipid, either phosphatidylserine or phosphatidylinositol [54] in a manner similar to the activation of protein kinase C [55, 56].

SUBSTRATES

Since tyrosine phosphorylation has been implicated in both the initial transformation of the cell and the maintenance of the transformed phenotype in cells infected with RNA tumour viruses whose oncogenes encode tyrosine protein kinases [57, 12] a knowledge of the substrates for these enzymes will help elucidate the pathways through which a cell may be transformed and malignancies initiated.

$pp60^{v\text{-}src}$, the Rous Sarcoma Virus transforming gene product, is itself phosphorylated on serine [58, 25] and tyrosine residues [9, 59]. $pp60^{c\text{-}src}$, the cellular analogue of $pp60^{v\text{-}src}$, is also phosphorylated on serine and tyrosine but is unable to transform cells even when expressed at levels sufficient to produce the same amount of tyrosine phosphorylation as $pp60^{v\text{-}src}$. It is therefore important not only to elucidate the substrates for tyrosine protein kinases but also the substrate specificity between the cellular and viral proteins, since this is inherent to separating the normal function of the protein from its role in carcinogenesis.

$pp60^{v\text{-}src}$ is phosphorylated on serine-17 in Rous Sarcoma Virus-transformed chicken cells and this phosphorylation is catalysed by a cyclic AMP dependent protein kinase [58]. The tyrosine phosphorylation of the enzyme is self-catalysed, i.e. the enzyme is capable of autophosphorylation which in $pp60^{v\text{-}src}$ occurs at tyrosine-416 [60] and this amino acid lies within the kinase domain of the enzyme. $pp60^{c\text{-}src}$ has an identical amino acid sequence in its kinase domain but tyrosine-416 is not phosphorylated [61].

Site-specific mutagenesis resulting in a protein where the amino acid residue at 416 is phenylalanine instead of tyrosine has been carried out by Snyder *et al* [62, 63]. Comparison of this mutant $pp60^{v\text{-}src}$ with the wild type enzyme showed that the phosphorylation of the tyrosine residue had no effect on the kinase activity of the enzyme, nor did it have any effect on its ability to transform cells. However when cells transformed by this mutant were implanted into mice their tumorigenic effect was decreased approximately one thousand fold. So the autophosphorylation of the enzyme may have an effect on tumour formation.

Perhaps the most notable change in cells transformed by retroviral tyrosine protein kinases is the marked disruption of cell morphology. This change in cell shape lead investigators to study the cell membrane in an attempt to discover any

elevation of phosphotyrosine in RSV-transformed cells. The typically ruffled appearance of RSV-transformed cells is due to the disruption in organisation of microfilaments such as vinculin, actin and fibronectin which are responsible for maintaining the integrity of cell shape [64], [65]. This disruption may be due either to an association of the enzyme with the microfilaments or to tyrosine phosphorylation of these cellular components by $pp60^{v\text{-}src}$. Sefton *et al* [66] showed vinculin to be a substrate for $pp60^{v\text{-}src}$ *in vivo* and demonstrated an eight fold increase of phosphotyrosine in vinculin from RSV-transformed chick cells compared to vinculin from normal uninfected chick cells. This may be as a result of the increased expression of the viral protein compared with the normal cellular protein. However there is a novel phosphotyrosyl linkage in vinculin in RSV- transformed cells suggesting that although both $pp60^{c\text{-}src}$ and $pp60^{v\text{-}src}$ have the same enzymatic activity there is a difference in their substrate specificity.

In normal cells the levels of phosphotyrosine are very low and accounts for only 0.03% of the total phosphoaminoacids present in the cell [9] making detection of these proteins difficult; however, by using antibodies to phosphotyrosine it is possible to detect phosphotyrosyl proteins in both normal and transformed cells. P A Maher *et al* [67] have shown that in normal cells phosphotyrosyl proteins are concentrated in the adhesion plaques of cells and at intercellular junctions. This

evidence, taken in conjunction with the elevation of tyrosine phosphorylation in vinculin, suggests that one mechanism through which tyrosine protein kinases transform cells is through the focal adhesion plaques of cells.

The observation that fibronectin degradation occurred at sites of expression of $pp60^{v\text{-}src}$ [68] also suggests that the enzyme has a membrane associated site of action.

Fibronectin molecules can assemble into fibrils, bind to cells and link them to other fibrils in the extracellular matrix; fibronectin is also responsible for attaching cells in tissue culture to the dish in which they are grown [69]. This disruption of fibronectin may well be responsible for the anchorage-independent growth of tumour cells and could be an important factor in the invasiveness of tumours.

Both vinculin and fibronectin are modified by the expression of $pp60^{v\text{-}src}$. Are these effects linked in some way or is one merely a fortuitous event associated with the high levels of the enzyme?

Fibronectin has been shown to be associated with an adhesion plaque protein complex in developing embryos [70]. This fibronectin- protein complex is termed the fibronectin receptor. Present in this receptor is a distinct binding site for talin (a cytoskeletal protein present in adhesion plaques)

lending even more weight to the hypothesis that this receptor complex is extensively involved with the anchorage of cells. The fibronectin complex is phosphorylated in cells that have been transformed by oncogenes encoding tyrosine protein kinases [71]. The fibronectin complex may be a substrate for $pp60^{v\text{-}src}$ but there is no conclusive evidence to support this.

$pp60^{v\text{-}src}$ not only phosphorylates cytoskeletal proteins, it also phosphorylates the cell surface insulin receptor [72].

THE INSULIN RECEPTOR

Many growth factors stimulate phosphorylation of their receptors on binding of the appropriate ligand. The increased phosphoprotein content is due to an increase in phosphotyrosine, phosphoserine and phosphothreonine [73].

Although tyrosine phosphorylation of $pp60^{v\text{-}src}$ is not necessary for the activity of this enzyme [63, 74] there is evidence to suggest that activation of receptor kinases, such as the insulin receptor kinase, is dependent on tyrosine phosphorylation [75, 76]. If $pp60^{v\text{-}src}$ is activating the insulin receptor kinase by tyrosine phosphorylation this would circumvent its requirement for insulin and may be responsible for initiating an unwarranted cell response, for example cell division.

CALPACTINS

One of the first substrates for pp60$^{v\text{-}src}$ to be described was a 36kd cellular protein, p36 [77]. The phosphorylation of this protein is an early event in transformation of chicken cells. Temperature- sensitive (ts) mutants, of RSV were used to infect chicken embryo fibroblasts. These ts mutants will produce the transformed phenotype in cells when incubated at the permissive temperature, but on shifting to the non-permissive temperature the cells will revert to the "normal" phenotype. Using two dimensional gel electrophoresis Radke and Martin were able to show that within twenty minutes of shifting the infected cells to the permissive temperature a phosphoprotein of 36kd was detectable.

Erikson and Erikson [78] identified a 34kd protein as a specific substrate for pp60$^{v\text{-}src}$ *in vivo* and *in vitro*. This protein was phosphorylated on tyrosine and serine *in vivo*, whereas *in vitro* experiments using isolated pp60$^{v\text{-}src}$ showed that the protein was phosphorylated only on tyrosine.

Radke *et al* [79] and Erikson and Erikson [78] identified a non-phosphorylated form of p36 and p34 in transformed and normal cells. It appears that only 10-15% of this protein is phosphorylated in transformed cells.

Erikson and Erikson [78] have shown that this protein is

present in normal cells of other species and on transformation of those cells with RSV this protein is phosphorylated. Normal fibroblasts of these species (mouse, vole and rat cells were used) showed greatly reduced or undetectable levels of this phosphoprotein.

The identification of this substrate for $pp60^{v\text{-}src}$ raises many questions, the most important being the function of this protein, since without knowledge of the protein's function the significance of its phosphorylation by $pp60^{v\text{-}src}$ is lost. Are these two reports of this substrate discussing the same protein or is there a family of proteins whose molecular weights are in the range 34-39kd? Is the serine phosphorylation of the protein important to its function? Where is the protein localised in the cell i.e. is it a membrane bound protein or is it cytosolic?

Greenberg and Edelman [80], [81] and Nigg *et al* [82], using antibodies to p34, have shown that this protein is located at the inner surface of the plasma membrane. Nigg *et al* identified another protein of the same group, this one named p39, [83]. This group of proteins has been termed "calpactins" by Glenney [84]. The calpactin family can be subdivided into Calpactin I, including p34, p36 and p39, and Calpactin II, which is probably p35, [85]. Calpactin has the ability interact with the cytoskeletal proteins actin and spectrin [86], [87] this interaction requiring millimolar concentrations of Ca^{2+}.

Although tyrosine phosphorylation of calpactin occurs readily *in vitro* it may be greatly enhanced by the addition of millimolar Ca^{2+}, and while the Km was unchanged the apparent Vmax is increased [54]. This sensitivity for Ca^{2+} could be increased by the addition of either phosphatidylserine (PS) or phosphatidylinositol (PI) from millimolar to micromolar concentrations . This is reminiscent of the activation of protein kinase C by Ca^{2+} and phospholipid.

p36 is covalently linked to the fatty acid myristic acid, possibly through an amide linkage, but the function of this acylation is obscure[88].

PHOSPHATIDYLINOSITOL PHOSPHORYLATION.

While remaining in the cell membrane the next proposed substrate for $pp60^{v\text{-}src}$ is not a cytoskeletal protein.

Phosphatidylinositol-4,5-bisphosphate (PIP_2) has recently been recognised as a precursor to second messengers that are arguably of more importance than Ca^{2+}. Hydrolysis of PIP_2 yields inositol trisphosphate (IP_3) and diacylgylcerol (DAG), (for reviews see M J Berridge and R F Irvine [89], and M J Berridge [90]).

PIP_2 is generated by the phosphorylation of

phosphatidylinositol-4-phosphate (PIP) which is itself formed by the phosphorylation of phosphatidylinositol (PI), see Figs. 1.1 and 1.2. Both of these phosphorylation steps are catalysed by specific kinases, PI kinase and PIP kinase. PIP_2 can be converted back to PIP and ultimately to PI by the action of phosphomonoesterases. So it seems that the phosphorylation and dephosphorylation of PIP forms a futile cycle. However, when an agonist binds to its cell surface receptor PIP_2 is diverted out of this futile cycle towards a phosphodiesterase, phospholipase C (PLC) which hydrolyses PIP_2 to DAG and inositol-1,4,5-trisphosphate ($I_{1,4,5}P_3$).

As mentioned above both DAG and $I_{1,4,5}P_3$ can act as second messengers. IP_3 mobilises cellular Ca^{2+} from the endoplasmic reticulum. This Ca^{2+} increase activates the Ca^{2+}/calmodulin kinase to phosphorylate its protein substrates so eliciting the cellular response.

DAG activates protein kinase C which then phosphorylates specific cellular proteins. Thus, this also leads to a cellular response.

Once the second messengers have activated their targets they are recycled, by a number of biochemical steps, to PI.

$I_{1,4,5}P_3$ is hydrolysed to inositol-1,4-bis- phosphate (I1,4P2) by inositol-1,4,5-trisphosphatase ($I_{1,4,5}P_3$ase).

I1,4P2 is then hydrolysed to inositol phosphate (IP) by inositol-1,4-bisphosphatase and inositol is ultimately generated by the hydrolysis of IP by inositol phosphatase, (IPase).

DAG is converted to phosphatidic acid (PA) by phosphorylation and then interacts with CTP to form cytosine diphosphate-DAG, CDP-DAG. This CDP-DAG complex then recombines with inositol to replenish the PI pool.

The apparent simplicity and economy of this cycle in producing cell responses is rapidly becoming complicated by evidence showing second messenger effects of the intermediates of the cycle.

Phosphatidic acid, generated by the phosphorylation of DAG with ATP, has a "growth factor-like" action [91], by stimulating Ca^{2+} release from intracellular stores, it induces the expression of the c-fos and c-myc proto-oncogenes, and stimulates DNA synthesis.

Although $I_{1,4,5}P_3$ is the initial inositol trisphosphate formed from hydrolysis of PIP_2, it appears now that other isomers are also generated. Inositol-1,3,4-trisphosphate ($I_{1,3,4}P_3$) has been detected in cholinergically stimulated parotid glands. $I_{1,4,5}P_3$ was also generated in this system (its appearance being immediate) whereas the formation of

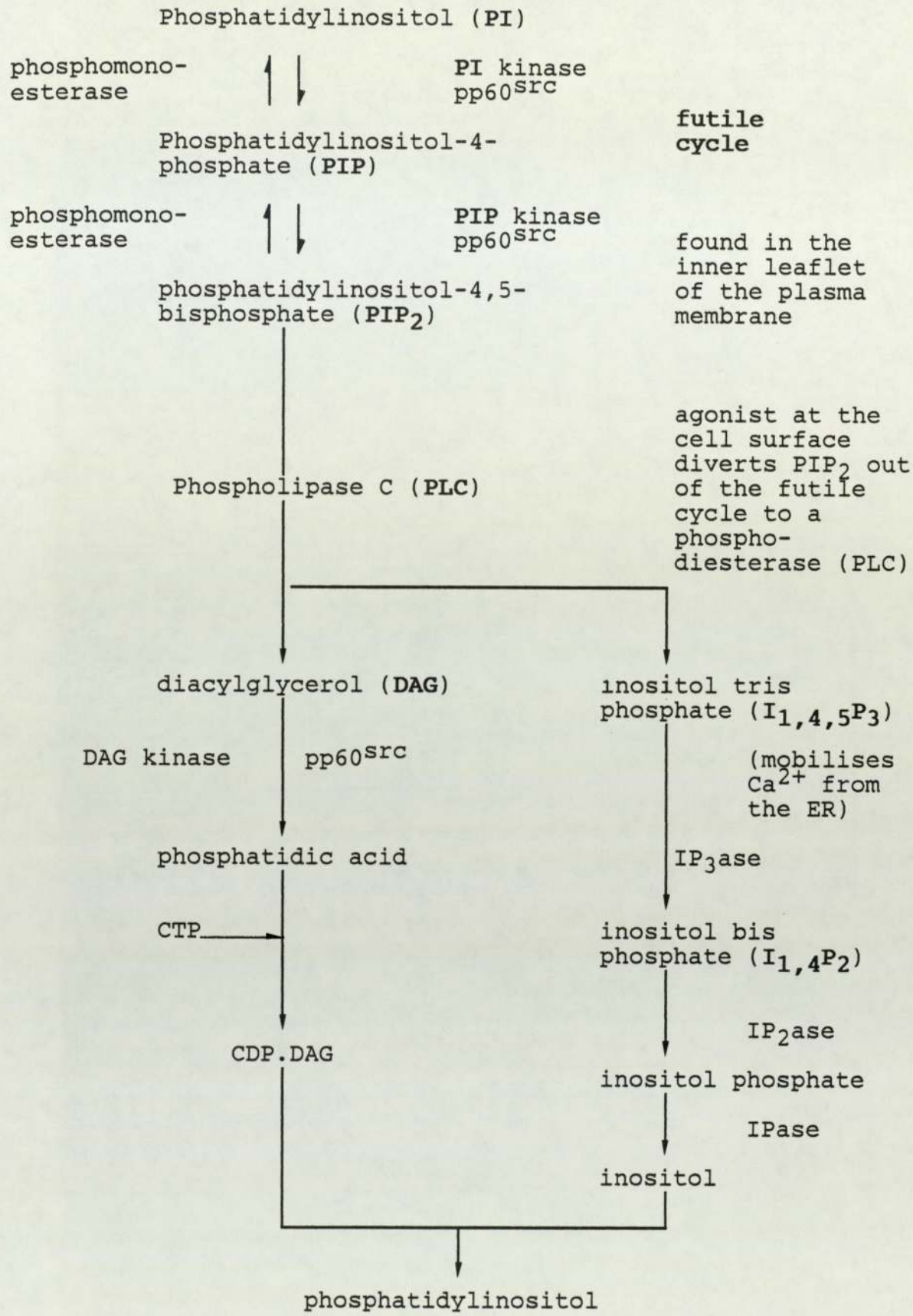


Fig.1.1 The phosphatidylinositol pathway.

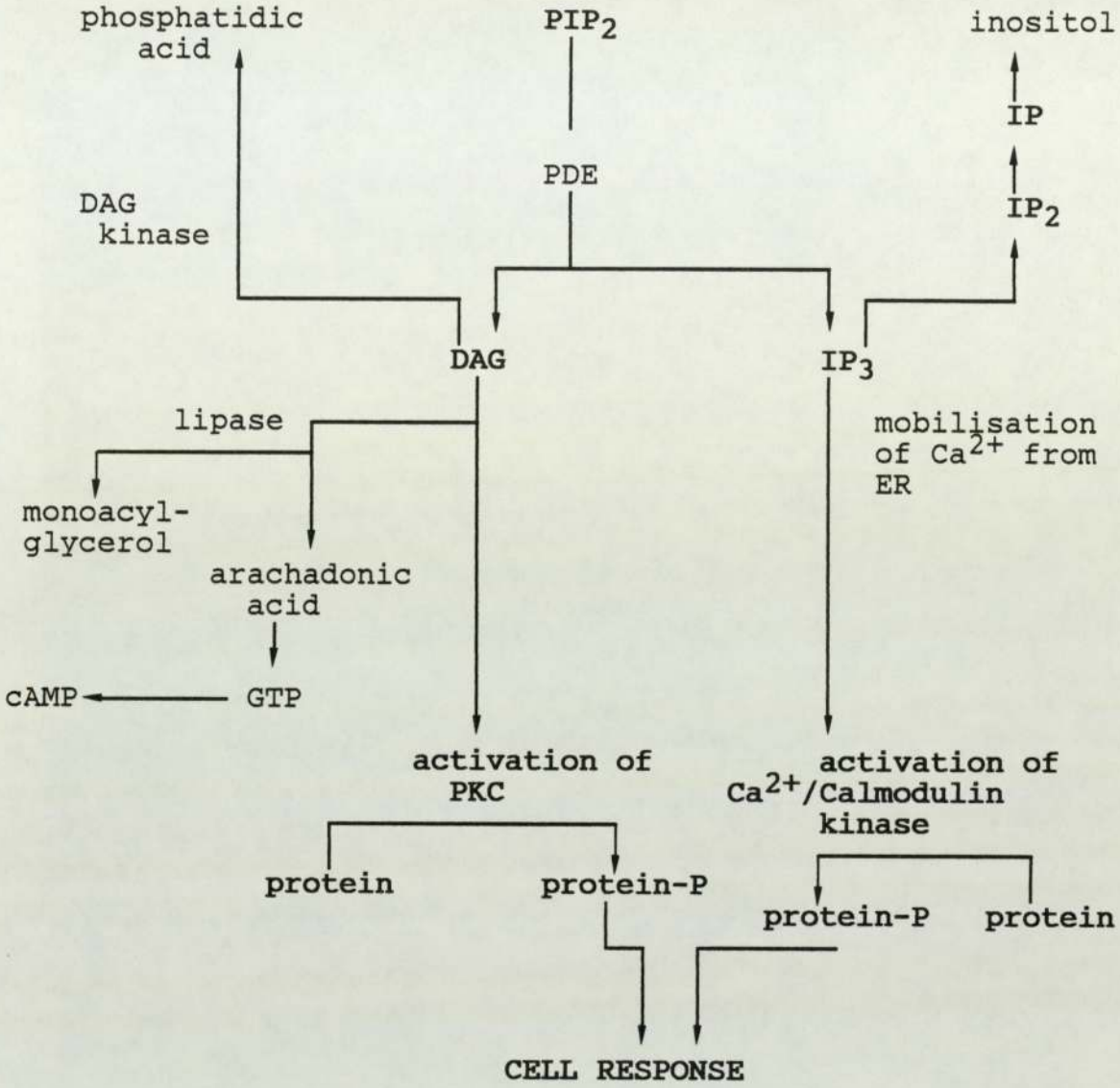


Fig. 1.2. Mechanisms by which IP_3 and DAG elicit the cellular response to an external stimulus.

$I_{1,3,4}P_3$ was delayed [92].

$I_{1,3,4}P_3$ appears to be generated by dephosphorylation at the 5-position of inositol-1,3,4,5-tetrakisphosphate, ($I_{1,3,4,5}P_4$), by inositol-1,4,5-trisphosphate 5-phosphatase, ($I_{1,4,5,}P_3$ 5ase). Inositol-1,3,4,5-tetrakisphosphate is generated as rapidly as $I_{1,4,5}P_3$ in certain cells and tissues, and could be the product of the reaction between $I_{1,4,5}P_3$ and a novel ATP-dependent kinase [93]. $I_{1,3,4,5}P_4$ and $I_{1,3,4}P_3$ are both now believed to have some second messenger activities. $I_{1,3,4,5}P_4$ can regulate the influx of Ca^{2+} from the external medium into sea urchin eggs [94]. $I_{1,3,4}P_3$ mobilises Ca^{2+} from intracellular stores in a permeabilised Swiss 3T3 cell preparation [95].

So now the scheme looks considerably more complex than it did initially, compare Figs.1.1 and 1.2 with Fig. 1.3.

Reports have suggested that tyrosine protein kinases were acting at some stages in the PI cycle. Sugimoto *et al* [96] reported that $pp60^{v\text{-}src}$ was acting as a PI and PIP kinase and also as a DAG kinase, and so being capable of generating cellular responses by activating both protein kinase C and the Ca^{2+}/calmodulin kinase, and also of producing the "growth factor-like" actions of phosphatidic acid. Macara *et al* [97] showed that immunoprecipitated p68v-ros (the oncogene of the UR-2 avian sarcoma virus) not only had tyrosine kinase activity

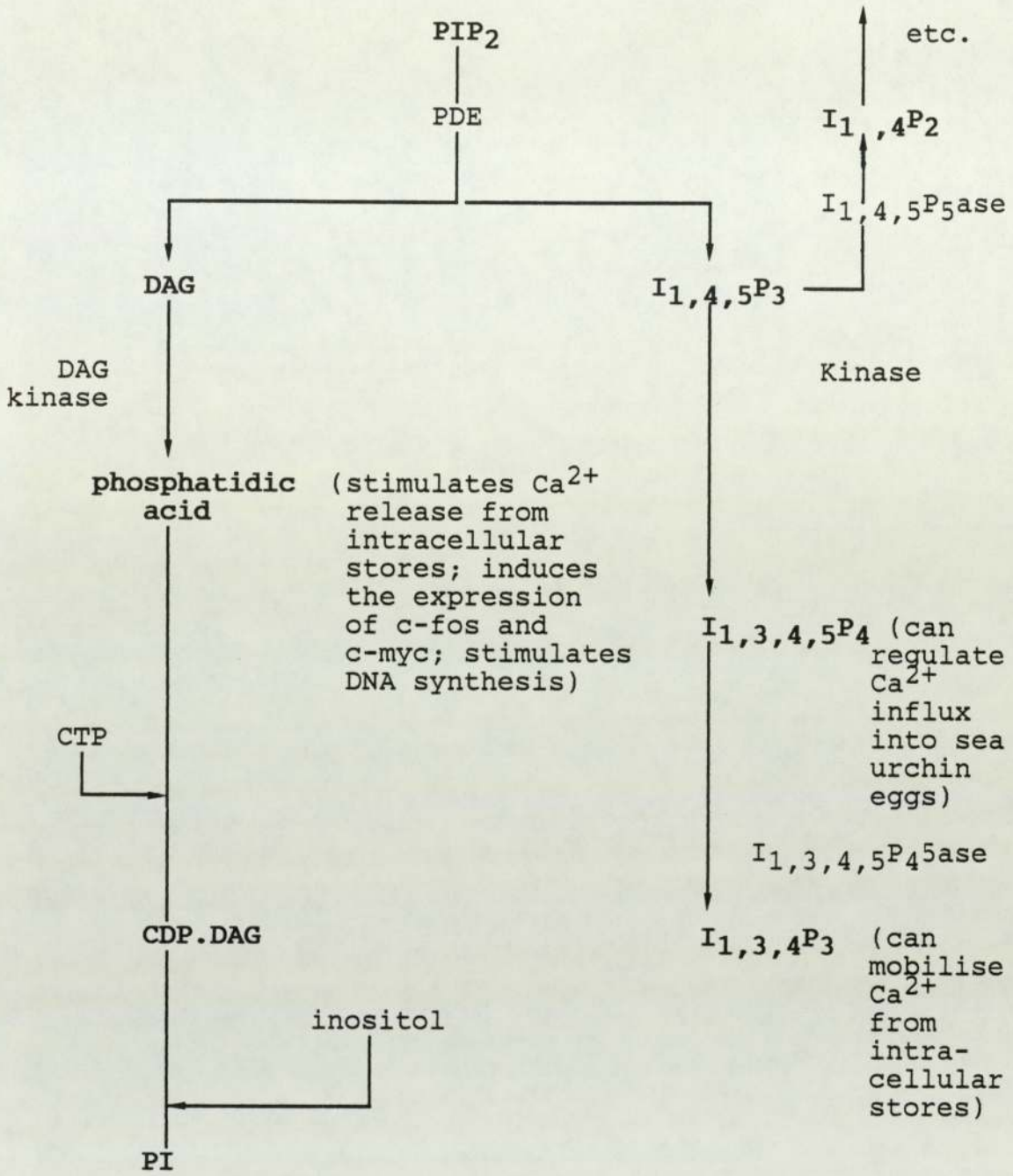


Fig. 1.3 Additional second messengers generated by the phosphatidylinositol cycle.

but also showed PI kinase activity. Machicao and Wieland, [98] using preparations of the insulin receptor from human placenta (the insulin receptor acts as a tyrosine protein kinase when insulin binds to it , in a manner similar to the EGFR), demonstrated the phosphorylation of PI when insulin was added to the preparation, suggesting that the insulin receptor-associated tyrosine protein kinase was also acting as a PI kinase.

Other workers in this field have disputed these results and shown that tyrosine protein kinase activity could be separated from the PI kinase activity. MacDonald *et al*, [99] using two transformed cell lines (one transformed by the RSV and containing high levels of the $pp60^{v\text{-}src}$ named RS-1 cells, and the other cell line was transformed by the Moloney murine leukaemia virus, containing pp56, another tyrosine protein kinase, named LSTRA cells), showed that the increase in rates of phosphorylation of peptide tyrosine in the two cell lines was not parallelled by an increase in phosphorylation of PI, PIP or DAG. Thompson *et al* [100] using A431 cells (these cells have a high concentration of the membrane receptor for EGF, this EGF-receptor acts as a tyrosine protein kinase when EGF binds to it) were able to separate the tyrosine protein kinase and PI kinase activity. There was no competition between angiotensin II (a small peptide substate) and PI for the tyrosine protein kinase and PI kinase activities of the EGF-receptor. Fry *et al*, [101] using NIH3T3 murine fibroblasts

transformed by the Abelson Murine Leukaemia virus (ANN-1 cells), found that an increase in tyrosine protein kinase activity in these cells was not accompanied by a corresponding increase in the stimulation of PI-kinase activity. Although immunoprecipitates of the Abelson kinase did show some PI-kinase activity *in vitro*, this level was extremely low and not considered to be of physiological significance.

It was postulated that the Abelson kinase might phosphorylate a PI-kinase and in this manner stimulate PI turnover. Whitman *et al* [102] demonstrated a PI kinase activity associated with the polyoma middle-T antigen. $pp60^{v\text{-}src}$ is known to associate with the polyoma middle-T antigen, and since more recent results suggest that tyrosine protein kinase and PI kinase activity can be separated in a number of systems, this result might suggest that it is the complex of a tyrosine protein kinase and the polyoma middle-T antigen that forms a PI kinase.

So although there is no discreet answer to the identity of the PI kinases there appears to be a connection between tyrosine protein kinases and this elegant second messenger system.

PHOSPHOTYROSINE AND NUCLEAR PROTEINS

The majority of the investigations carried out on the

tyrosine protein kinases have centred on the cell membrane, since this appears to be the subcellular location of $pp60^{v\text{-}src}$ and the receptors for growth factors such as insulin and PGDF are membrane proteins, while the plasma membrane is the location for the substrates of $pp60^{v\text{-}src}$. Details are now emerging of another location of $pp60^{v\text{-}src}$.

The phosphotyrosine content of normal cells is in the region of 0.03% of total phosphoaminoacids present; in transformed cells that figure is 0.3% so the difficulty in detecting phosphotyrosyl proteins is considerable. Resh and Erikson [103] described a novel location of both $pp60^{v\text{-}src}$ and $pp60^{c\text{-}src}$. RSV-infected cells (field vole cells and chicken embryo fibroblasts) were used to investigate the subcellular location of $pp60^{v\text{-}src}$. The cells were homogenised, separated into three fractions by differential centrifugation and probed with an antibody to pp60src, α p60src. α p60src is a highly specific antibody to the src protein, [104], particularly for the amino-terminal portion of pp60src, [103]. The distribution of $pp60^{v\text{-}src}$ between the nuclear fraction and the plasma membrane and membraneous organelles was found to be approximately equal. This was in contrast to the results obtained when cells were probed with serum from tumour bearing rabbits (TBR serum) containing an antibody to $pp60^{v\text{-}src}$. The results obtained with TBR serum showed the majority of the $pp60^{v\text{-}src}$ to be associated with the plasmsa membrane fraction.

The study was repeated probing uninfected chicken embryo fibroblasts with bothαp60src and TBR serum. Although $pp60^{c\text{-}src}$ was present in quantities thirty to fifty times lower than the quantities of $pp60^{v\text{-}src}$ in RSV-transformed cells usingαp60src, it was still possible to identify $pp60^{c\text{-}src}$ and this protein showed a similar interaction to nuclear membranes to $pp60^{v\text{-}src}$.

Recent work by Bell *et al* [105] has identified phosphotyrosyl proteins present in the nucleus of Abelson transformed NIH 3T3 fibroblasts which bind specifically to murine DNA.

Although it is not yet known what is the rôle, if any, of tyrosine phosphorylation of these nuclear proteins in regulating their biological activity, or if it actually does regulate their biological activity, there is a growing body of evidence implicating tyrosine protein kinases with signalling pathways involving the nucleus.

Blat *et al* [106] have described an increase in the phosphorylation of non-histone proteins in cells infected with a ts mutant of RSV when these cells are incubated at the permissive temperature compared to those cells incubated at the non-permissive temperature. The non-histone proteins have intrinsic protein kinase activities and it was found that the kinase activities of these proteins from cells incubated at 37°C was four fold higher than the same proteins from cells

incubated at 41°C (the non-permissive temperature). Phosvitin, casein and histone were used as substrates for these assays, phosvitin being the best substrate.

Analysis of these non-histone chromosomal proteins identified a large increase in the phosphorylation of a group of proteins of molecular weight in the region of 80kd, and an increase in two other proteins, 60 and 68kd [106]. The 80kd phosphoproteins did not contain phosphotyrosine, but the 60 and 68kd proteins were relatively stable to alkali suggesting that the phosphate linkage was to a tyrosine residue.

$pp60^{v\text{-}src}$ phosphorylates DNA topoisomerase I *in vitro*. This phosphorylation of the enzyme results in a loss of over 90% of the ability of the enzyme to relax supercoiled DNA *in vitro*, [107]. However, this tyrosine phosphorylation of topoisomerase I appears not to be of physiological significance [108] since topoisomerase I activity of 3T3 cells is not decreased when these cells are transformed with either the Abelson murine leukaemia virus or the Rous Sarcoma virus, and incubation of topoisomerase I from either rat liver or HeLa cells showed only 2% of the topoisomerase I molecules to be phosphorylated.

Growth factors and hormones have a specific receptor in the cell membrane. Many of these receptors, on binding of the appropriate ligand, autophosphorylate on tyrosine and are

internalised. The EGF receptor is one such enzyme with kinase associated activity. Among its substrates are the progesterone receptor subunits,[109], and these receptor subunits are phosphorylated on tyrosine residues only. The phosphorylation of steroid receptors appears to be involved in the migration of the receptor to the nuclear binding site [110].

The oestradiol receptor is also a phosphoprotein, phosphorylation being required for the binding of oestradiol to the receptor [111]. This phosphorylation of the oestradiol receptor is catalysed by a cytosolic kinase. The binding of oestradiol *in vitro* can be stimulated in the presence of the kinase by addition of Ca^{2+} and calmodulin can also activate this kinase. Analysis of the phosphorylation of the oestradiol receptor shows only tyrosine phosphorylation, [112]. On migration of the receptor-hormone complex to the nuclear site the action of a phosphatase inactivates the complex which is then released into the cytoplasm [113].

So again tyrosine phosphorylation is implicated with events in the nucleus. Perhaps tyrosine protein kinases are involved in signalling pathways between the membrane and the nucleus. The existence of cells transformed by the RSV that are tumourigenic in athymic mice, express $pp60^{v\text{-}src}$ and yet exhibit the morphology of uninfected cells [114] may support this concept, although the apparently normal morphology of the cell line (European field vole fibroblasts) may be due to a defect

in the host cell's gene product.

CELLULAR DIFFERENTIATION AND TYROSINE PROTEIN KINASES

All the cells of the body are ultimately derived from that one cell formed at the moment of conception. This cell divides continually and as the embryo developes distinct cell types may be observed and eventually discreet organs are formed. This is the most fundamental model of differentiation, (for a recent review of differentiation see Leo Sachs in Cancer Research[115]). Dead cells of a particular tissue are continually being replaced from a pool of pluripotent cells which are induced to differentiate by quite complex factors. Once cells have differentiated terminally, that is have reached a point at which they are committed to become a particular cell type, there is a limited number of cell divisions left before the cell dies. If for some reason this differentiation pathway becomes blocked or differentiation is not induced the cells may continue to divide in an uncontrolled manner potentially leading to the formation of a tumour or in the case of the circulatory system a leukaemia. The mechanisms of regulating differentiation of cells is therefore of great interest.

Normal cellular homologues of retroviral oncogenes have been implicated in the regulation of differentiation [116], [117], [118].

The pluripotent cell line p19S18O1A1 (or O1A1) has a low level of tyrosine protein kinase activity attributable to c-src, the cellular homologue of $pp60^{v\text{-}src}$. This level of phosphotyrosine increases slightly on treatment of the cells with retinoic acid, a differentiating agent [119]. However, as the cells differentiated, became attached to their substrate and developed neuritic processes this elevation of $pp60^{c\text{-}src}$ activity increases to eight to ten times that of the parent O1A1 cells [120] and was similar to that of cells transformed by the RSV.

The hypothesis that $pp60^{c\text{-}src}$ is more important in neuronal differentiation or neuronal function than in cell proliferation [121] is supported by observations by Iba *et al* [122] of neuroretinal tissue from chick embryos. When a neuroretinal monolayer culture was maintained *in vitro* for one week and assayed for $pp60^{c\text{-}src}$ kinase activity, this activity was found to be four times less than that of the tissue from which it was derived (7 day chick embryos) although it was comparable to that of chick embryo fibroblasts; the same tissue from older embryos (10 day embryos) showed a ten fold increase in kinase activity compared to that of chick embryo fibroblasts. So it would appear that $pp60^{c\text{-}src}$ may play a role in regulating the differentiation of certain neuronal tissues.

Tyrosine protein kinases have been implicated in the differentiation of cells other than neuronal tissue. When the

human leukaemic cell line HL60 was treated with g-interferon (IFN-g), which is capable of inducing differentiation in certain neoplastic cells [123], tyrosine kinase activity could be detected in triton extracts of the cells two days after treatment [124] and this was coincident with the onset of differentiation, although the identity of the tyrosine protein kinase was not elucidated.

A tyrosine protein kinase that has been identified in HL60 cells differentiating along the monocyte pathway is the c-fms proto-oncogene [125]. Further evidence for the involvement of tyrosine protein kinases in differentiation is given by other observations of HL60 cells. Dimethyl sulphoxide and tetradecanoylphorbol acetate (TPA) were used to induce the differentiation of these cells to granulocytes and monocytes respectively and changes in phosphotyrosine content were observed [126]. The phosphotyrosine content of differentiated cells had dropped from 1.5±0.2% (the level in untreated cells) to 0.1±0.1% of the phosphoamino acid content in the cell despite the fact that tyrosine kinase activity increased differentiation. This was explained by an increase in protein phosphotyrosine phosphatase activity corresponding to differentiation.

The heat shock protein HSP90 associates with the receptors for progesterone, oestrogen, androgen and glucocorticosteroid. These proteins can complex with $pp60^{v\text{-}src}$, [127] the fes and fgr

tyrosine protein kinases [128].

Despite the early observations that retroviral tyrosine protein kinases were responsible for the formation of tumours in birds, their role in human malignancy is very much more complicated. High levels of tyrosine protein kinase activity will not necessarily lead to tumour formation. Indeed certain normal tissues show higher tyrosine protein kinase activity than transformed cells; for instance blood platelets express high levels of $pp60^{c\text{-}src}$ [129] and normal resting lymphocytes have higher tyrosine protein kinase activity than proliferating cells or leukaemic blood cells [130] while neural tissues express high levels of $pp60^{c\text{-}src}$ in tissue culture [131].

Perhaps this high concentration of proto-oncogenes in normal cells is involved in the regulation of differentiation and in membrane-nucleus signal transduction, while in transformed cells the aberrant activity of tyrosine protein kinase may be attributable to mutations or chromosomal translocations.

TYROSINE PROTEIN KINASE INVOLVEMENT IN HUMAN TUMOURS.

Tyrosine phosphorylation in proteins was first recognised in polyoma virus middle-T antigen immunoprecipitates [19] and shortly afterwards the transforming gene product of the RSV was found to have the unusual enzymatic activity of phosphorylating

tyrosine residues in proteins [9], [10]. Despite the involvement of phosphotyrosine in animal malignancies is there any significance of tyrosine phosphorylation of proteins to human tumour development and growth? Recent observations suggest that there is.

Chromosomal rearrangements are known to be associated with different cancers. For example, the c-myc gene is translocated from its normal position on chromosome 8 to chromosome 14 in Burkitt's Lymphoma, [132] [133]. Another chromosomal abnormality in human tumours is the Philadelphia chromosome (Ph'). This chromosome represents a deletion of the long arm of chromosome 22 [134] and has been identified in the leukaemic cells of over 90% of chronic myeloid leukaemia patients [135]. This translocation is used to diagnose the leukaemia presented as chronic myeloid leukaemia (CML). However the Ph' chromosome has also been found in the leukaemic cells of 2-3% of the patients with acute lymphoblastic leukaemia (ALL).

The c-abl oncogene is translocated to the Ph' chromosome in CML Ph' positive patients and this translocation has been mapped to the breakpoint cluster region on chromosome 22 [136]. This c-abl translocation has also been observed in some Ph' positive ALL patients [137].

The transcription of these gene sequences produces an abl-protein that is distinguishable in the different forms of

leukaemia from the normal c-abl protein [138], [139] and this unique form of the c-abl tyrosine kinase can distinguish Ph' positive CML from Ph' positive ALL [140].

Another tyrosine kinase, the neu oncogene product, has recently been connected with human breast cancer. Gene amplification of the neu oncogene was observed in breast cancer and the extent of amplification correlated inversely with overall survival time and time to relapse [141].

Tyrosine protein kinases have been shown to be responsible for the malignant phenotype in chicken cells *in vitro* and recent work indicates altered gene products, due to chromosomal translocation, and gene amplification of certain members of the tyrosine kinase family in human malignancies. These proteins, therefore, are a potential target for chemotherapy, although designing a molecule that will specifically inhibit or interact with the malignant form of a particular tyrosine kinase poses a problem of considerable size.

TYROSINE PROTEIN KINASE INHIBITORS

A number of natural products have been shown to inhibit tyrosine protein kinase activity. These include quercetin, genistein and staurosporine. Amiloride, a potassium sparing diuretic, also has activity against tyrosine specific protein kinases.

Staurosporine, a microbial alkaloid, is an extremely potent inhibitor of $pp60^{v\text{-}src}$, with an IC_{50} of 6.4nM, [142]. However, there does not appear to be any specificity associated with staurosporine since this compound also inhibits protein kinase C with an IC_{50} of 2.7nM, and a c-AMP dependent protein kinase with an IC_{50} of 8.2nM. The manner in which it inhibits these kinases is unknown.

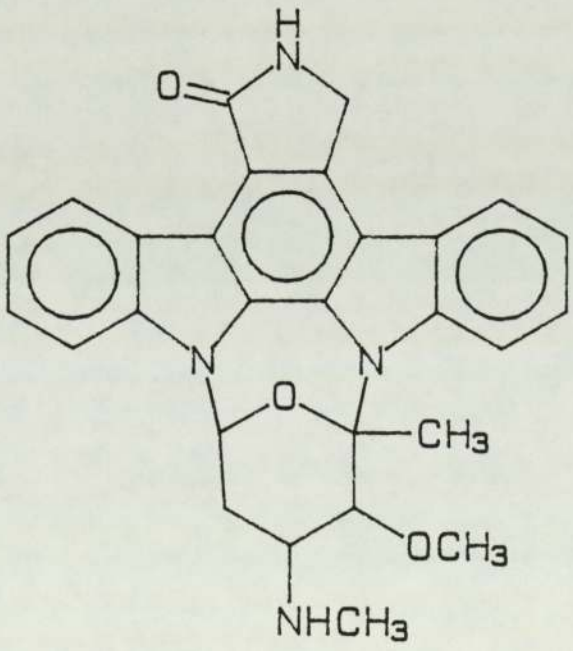


Fig. 1.4. Staurosporine.

Amiloride, Fig. 1.5, is also known to inhibit tyrosine specific protein kinases [143].

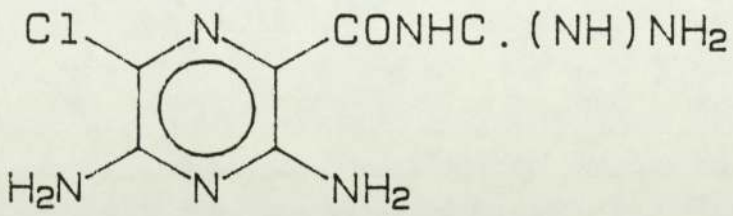


Fig. 1.5 Amiloride.

Genistein, 5,7,4'-trihydroxyisoflavone (Fig. 1.6),isolated from a fermentation broth of Pseudomonas sp., inhibits the EGF-receptor kinase both in vitro and in vivo [144]. This inhibition is competitive for ATP, but genistein showed little activity against serine and threonine protein kinases suggesting that there is some scope for selectivity.

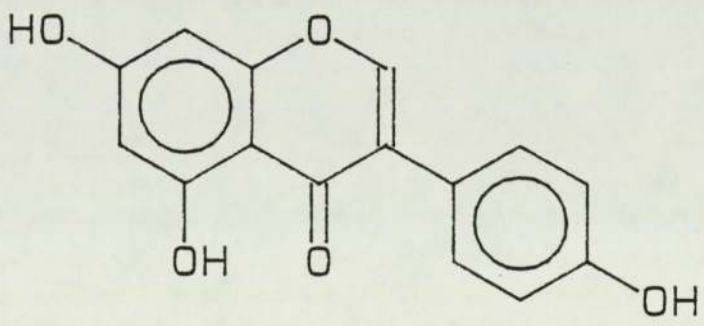


Fig. 1.6 Genistein.

Quercetin (Fig. 1.7), 3,3',4',5,7-pentahydroxy- flavone, is a naturally occurring product widely distributed in the plant kingdom. It has been detected in certain varieties of lettuce, red cabbage, kale and green garlic [145].

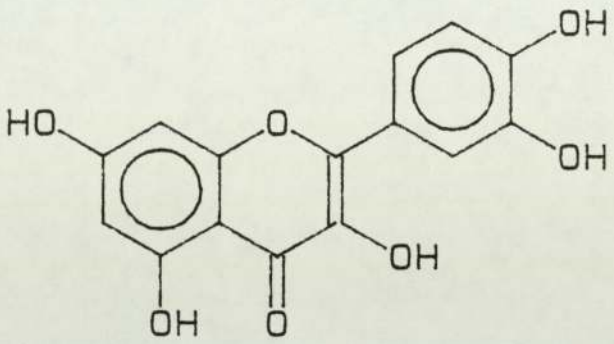


Fig. 1.7. Quercetin.

Quercetin was observed to inhibit a c-AMP independent protein kinase activity in Erlich Ascites tumour cells [146] and two years later it was shown to inhibit $pp60^{v\text{-}src}$ *in vitro* (using a partially purified preparation of $pp60^{v\text{-}src}$) and *in vivo* [147].

However, this inhibition of $pp60^{v\text{-}src}$ is only one of quercetin's many effects. Quercetin inhibits 3',5'-c-AMP phosphodiesterases [148], cytochrome P450 activity in rat liver microsomes [149], Na^+,K^+ ATPase [150], lactate transport in HeLa cells [151], and neutrophil phospholipase A_2 [152]. Other flavonoids are known to inhibit lipoxygenase and cyclooxygenase *in vitro* [153], glyoxalase I *in vitro* [154] and arachidonate

5-lipoxygenase [155]. Quercetin has also been found to be mutagenic, but not carcinogenic in rats [156] and to be genotoxic in cultured mammalian cells [157].

The mechanism through which quercetin inhibits $pp60^{v\text{-}src}$ appears to involve competition of quercetin with the phosphate donor and this could be either ATP, GTP or CTP [147], although why this should be is not immediately obvious since there is no structural similarity between quercetin and either ATP, GTP or CTP. Molecular orbital calculations carried out by Ferrel *et al* [158] on a series of flavone compounds suggest that the distribution of charge over the pyrone ring of flavones is similar to that of the pyrimidine ring of c-AMP. This result may explain why quercetin can act as a competitive inhibitor for a nucleotide and may explain why genistein, an iso-flavone, also appears to be an ATP competitor. However, the doses required to inhibit $pp60^{v\text{-}src}$ activity have no effect on c-AMP dependent protein kinase activity. Further conflicting evidence is given by Cochet *et al* [159]. Quercetin inhibited a c-AMP independent protein kinase that utilised either ATP or GTP as phosphate donor but had no effect on another c-AMP independent protein kinase that could utilise only ATP. These anomalies may be due to differences in the nucleotide binding region of the enzyme although these results may indicate that similar charge distributions in flavones and adenine are purely coincidental, and have no real bearing on the mechanism of action of quercetin in these systems.

Quercetin itself is not an ideal chemotherapeutic agent due to its lack of selectivity, however it may be used as a "lead" compound in the design of agents as potential inhibitors of tyrosine protein kinases. Although quercetin has quite diverse effects, recent results suggest that this natural product and other flavones may be important in the prevention of some forms of cancer. Quercetin and other flavonols have been shown to inhibit the tumour promoting activity of teleocidin and 12-O-tetradecanoylphorbol-13-acetate (TPA) on mouse skin treated with the carcinogen dimethylbenz-(a)-anthracene (DMBA) when topically applied, although it had no effect when given orally [160, 161].

Since quercetin has so many diverse actions the aim of this project was to synthesise a series of analogues of this flavonoid to elucidate any potential structure activity relationship towards inhibition of protein tyrosine kinases.

CONCLUSIONS

There is a growing body of evidence implicating tyrosine kinase involvement with human malignancy. Since evidence from animal systems has shown aberrant tyrosine kinase activity to be responsible for the maintenance of the transformed phenotype these enzymes present an ideal target, potentially, for chemotherapy. The advantages of such treatment include a

degree of selective toxicity with regard to the growth factor receptor tyrosine kinases since not every cell in the body has these receptors. The potential disadvantage associated with inhibiting the aberrant tyrosine kinases involved in the maintenance of malignancy is that of chronic treatment. However with improved drug design and a better understanding of these enzymes the possible side effects of such therapy could be kept to a minimum.

CHAPTER TWO

THE PREPARATION OF ANALOGUES OF QUERCETIN

Quercetin, 3,3',4',5,7-pentahydroxyflavone, (Fig. 2.1) has been shown to inhibit the transforming gene product of the Rous sarcoma virus by Graziani *et al* [147]; however, this is just one of the many effects of this compound.

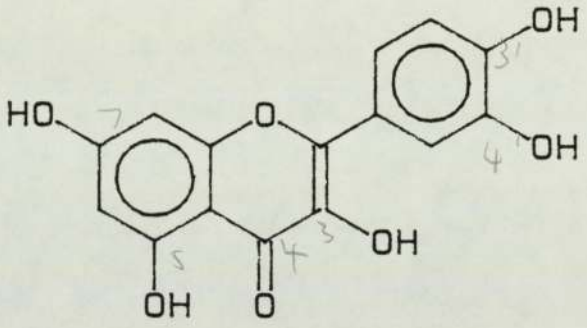


Fig. 2.1 Quercetin. (93)

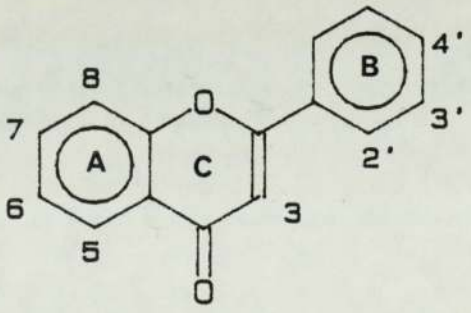


Fig. 2.2 Numbering System for Flavone.

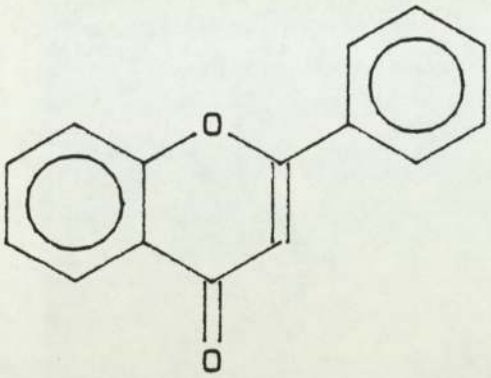
The aim of this project was to synthesise a series of analogues of quercetin to seek a more selective inhibitor and to elucidate any structure-activity relationships that there might be. In order to carry out this study, it was decided to

prepare:-

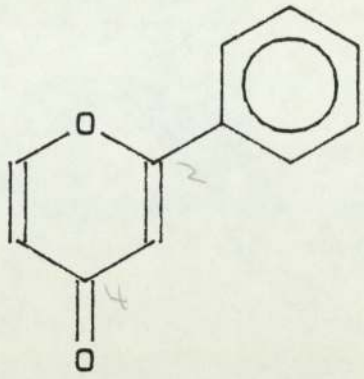
1. A series of flavones variously subsitituted in the 3, 3', 4', 5 and 7 positions, i.e. the substitution positions of quercetin, to determine the substituent requirement for activity of the compounds.
2. A series of 2-phenylpyran-4-ones, to determine the basic structural skeleton requirements, i.e. to determine if the "A" ring of the flavone nucleus is necessary for activity
3. A series of 2-cyclohexylchromones, to examine the requirement for aromaticity in this position.
4. Thioflavone, that is to substitute sulphur for oxygen in the heterocycle, to investigate the effect of altering it on the activity of the compound

These target compounds are shown below, in Fig.2.3.

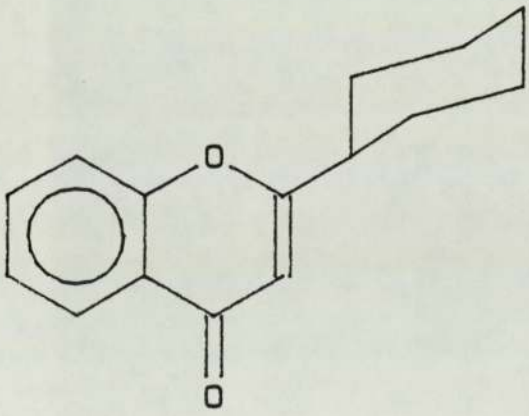
There are many methods for the preparation of flavones cited in the chemical literature. Banerji and Goomer [162] prepared the corresponding propan-1,3-diones by the lithiation of *o*-hydroxyacetophenone and then treating this complex with benzoyl chloride at -78°C, as shown in Scheme 2.1. The propan-1,3-diones were then treated with a mineral acid to eliminate water and cyclise the compound to yield the flavone.



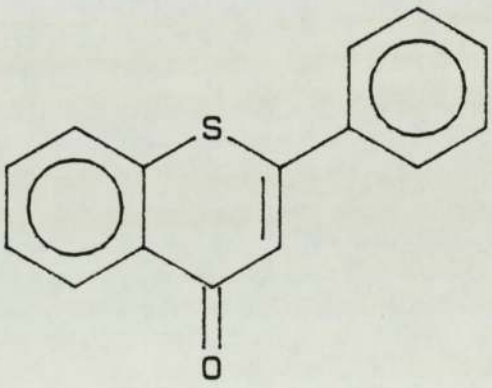
Flavone (variously substituted)



2-phenylpyran-4-one

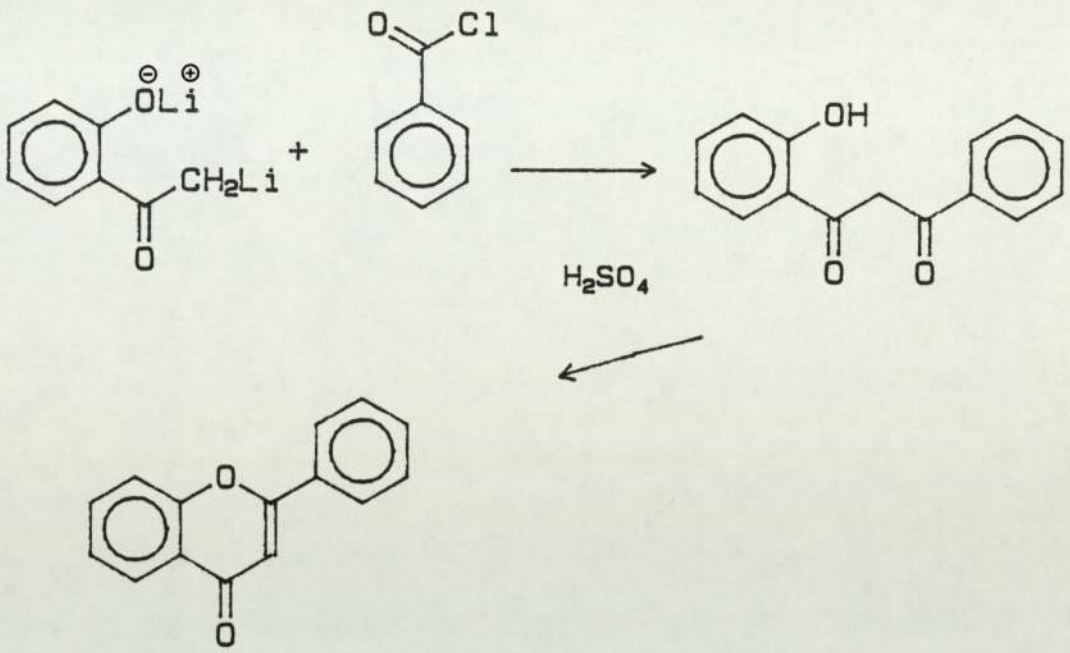


2-cyclohexylchromone



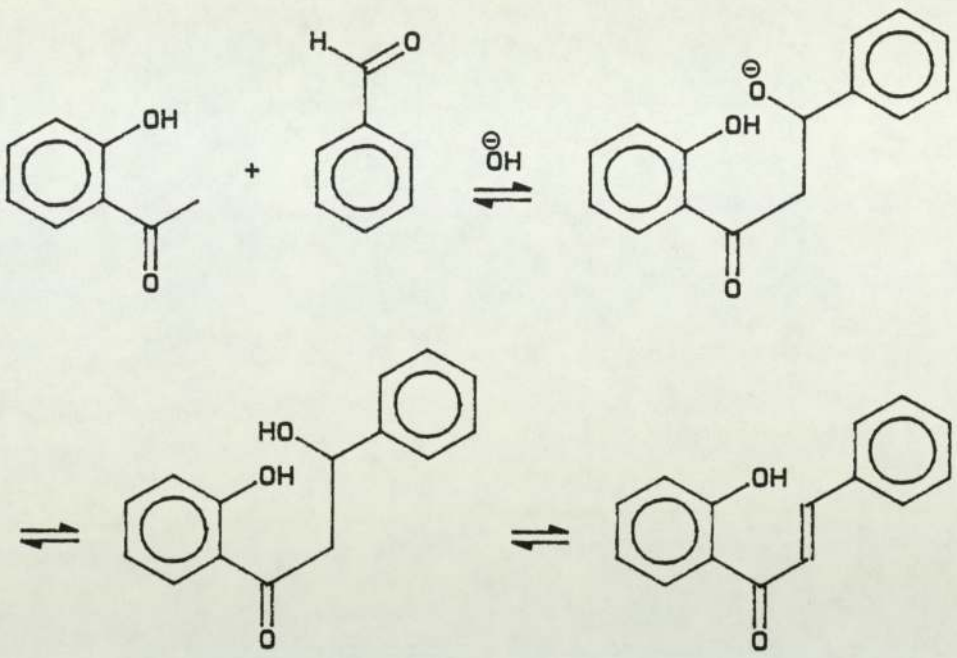
Thioflavone

Fig. 2.3 The target compounds.



Scheme 2.1. Flavone preparation by the method of Banerji and Goomer.

Gupta *et al* [163] synthesised flavanones by the condensation of *o*-hydroxyacetophenone with benzaldehyde in alcoholic potassium hydroxide, generating the chalcone, (the Claisen-Schmidt reaction). Cyclocondensation of the chalcone in ethanol containing a trace of sulphuric acid furnished the flavanone, as shown in Scheme 2.2.



Scheme 2.2. Claisen-Schimdt reaction for the preparation of 2-hydroxychalcone.

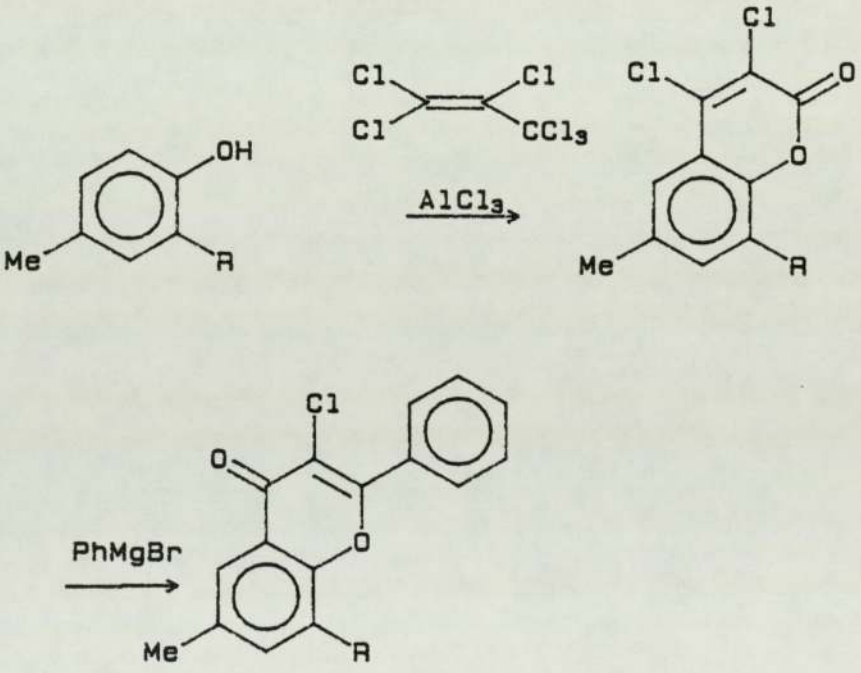
Flavanones may be oxidised to the corresponding flavone; Fatma *et al* [164] obtained 5,7,4'-trimethoxyflavone by treating the flavanone with a catalytic amount of iodine and a trace of sulphuric acid in DMSO at 100°C.

Mulvagh *et al* [165] isolated 3-methylflavone in 24% yield as well as *homo* iso-flavone (41%) when 3-benzylidene chroman-4-one was treated with benzyl chloride and potassium carbonate in DMF at 100°C.

Meyer-Dayan *et al* [166] obtained flavones on the oxidation of flavylium salts with thallium trinitrate in methanol. Kasahara *et al* [167] used phenoxy palladation followed by

elimination of hydridopalladium to generate flavone from the sodium salt of 2-hydroxychalcone.

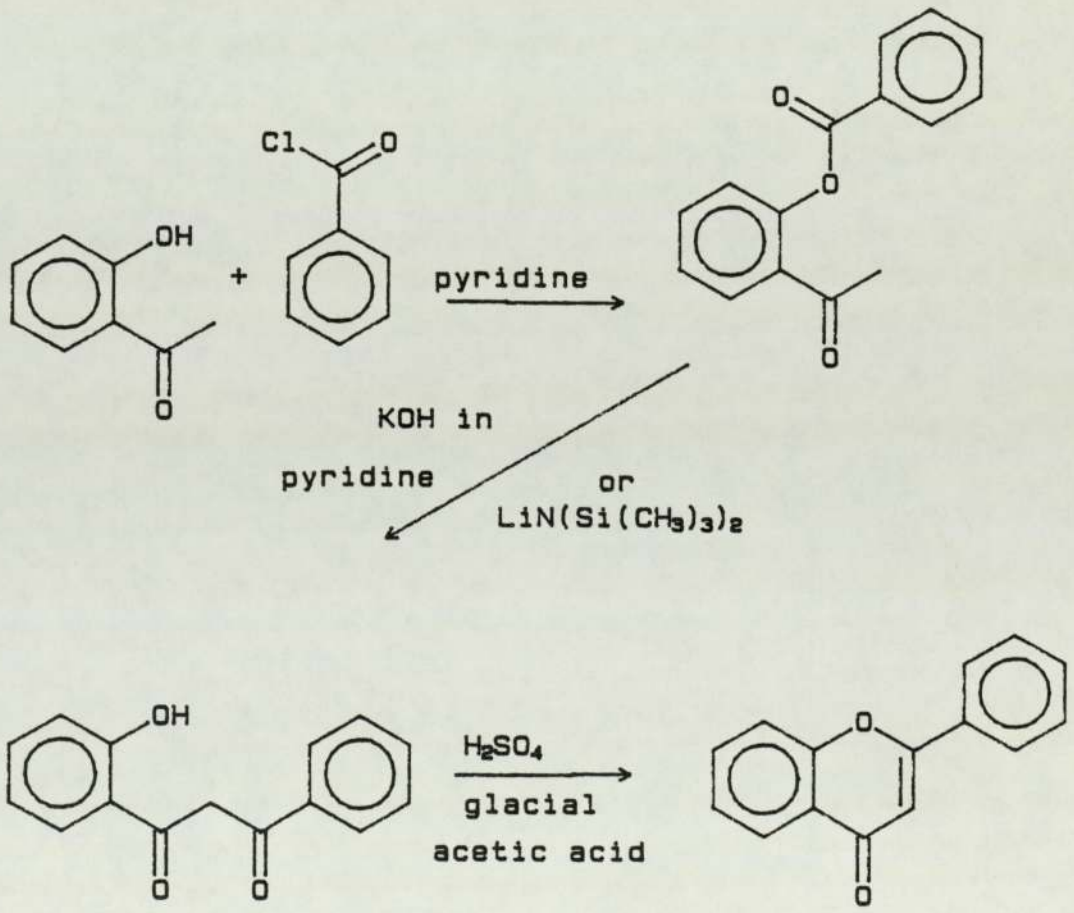
Newman and Ferrari [168] synthesised 3-chloroflavone, as outlined in Sheme 2.3, by treating 3,4-dichlorochroman- 2-one with phenyl magnesium bromide. The 3,4-dichloro- chroman-2-one was prepared by treating 2-hydroxy- benzophenone with hexachloropropene and aluminium trichloride in nitroethane. When 3-chloroflavone was treated with cuprous cyanide 3-cyanoflavone was obtained which furnished flavone when the nitrile was hydrolysed with hot acetic acid.



Scheme 2.3. Flavone synthesis by the method of Newman and Ferrari.

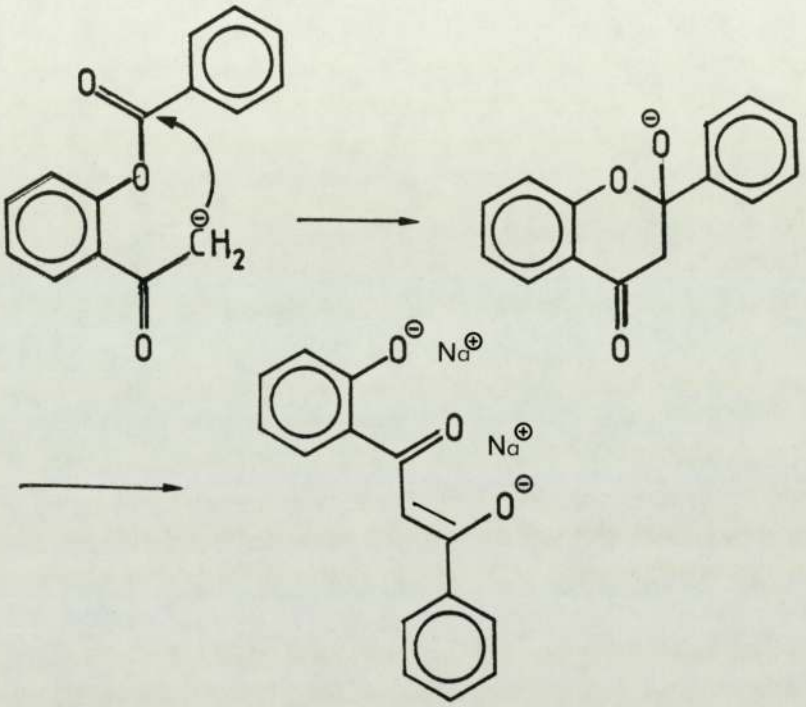
In this study, though, flavones were synthesised generally by the method of Wilson Baker [169], since this method was the most accessible and has been widely used .

2-Hydroxyacetophenone was smoothly acylated at oxygen with benzoyl chloride in pyridine to yield 2-benzoyloxyacetophenone. Base-catalysed rearrangement of this ester to 1-(2-hydroxyphenyl)-3-phenylpropan-1,3-dione was effected using powdered potassium hydroxide in pyridine. The final step in the synthesis of flavone was achieved by the acid catalysed dehydration of the 1-(2-hydroxyphenyl)-3-phenylpropan-1,3-dione using a catalytic amount of concentrated sulphuric acid in glacial acetic acid, as shown in Scheme 2.4.



Scheme 2.4. Flavone synthesis by the method of Baker.

The conditions for the rearrangement of the ester to the propan-1,3-dione at first sight appear to be ideal for the hydrolysis of the ester by nucleophilic attack of the hydroxide anion at the carbonyl carbon. However, the hydroxide anion acts as a base rather than as a nucleophile and generates the enolate and it is this species which acts as a nucleophile towards the ester carbonyl as shown in Scheme 2.5.



Scheme 2.5. Mechanism for the rearrangement of 2-(benzoyloxy)-acetopheneone to 2'-hydroxyphenyl-3-phenyl-propan-1,3-dione.

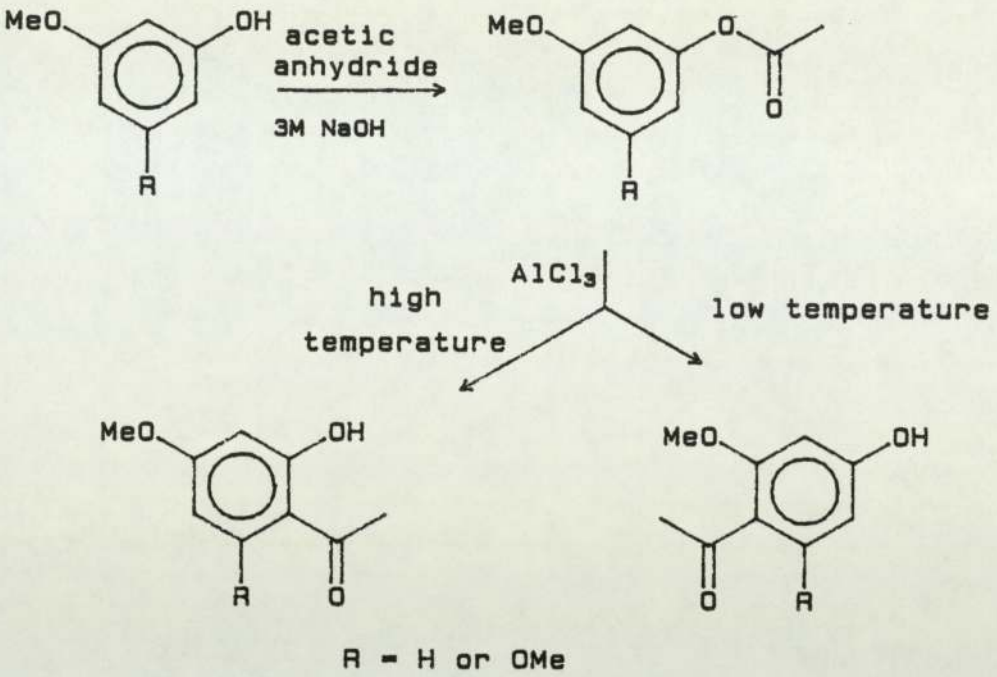
This reaction has been called, although misleadingly, a Claisen condensation although this strictly applies only to the condensation between two ester moieties.

Flavones bearing substituents in the 3' and 4' positions were easily approached using suitably substituted benzoyl

chlorides.

Preparation of flavones substituted in the 5 and 7 positions proved to be more difficult. In order to incorporate groups into these positions, modification of the 2-hydroxyacetophenone was required. A common method of preparing 2-hydroxyacetophenones with appropriate oxygen functions is by the Fries rearrangement of the corresponding phenyl acetate, as illustrated in Scheme 2.6. This reaction is analogous to the Friedel-Crafts reaction although it is quite distinct from it, (for review see [170], [171], [172]). Perhaps it should be mentioned here that Fries was not the first chemist to observe the rearrangement of phenyl acetates to hydroxyacetophenones. Eykmann reported the preparation of 2-methyl-4-hydroxyacetophenone in 1904 [173] and later the formation of 2-hydroxy-4-methylacetophenone at a higher temperature [174] when 3-methylphenol and acetyl chloride were treated with zinc chloride.

Acylation of an aromatic ring under the conditions of a Friedel-Crafts reaction leads predominantly to electrophilic substitution onto a ring carbon (for reviews see: Olah [175], Norman and Taylor [176] and Gore [177]). However, in the presence of strong nucleophilic groups in the ring such as in a phenol, acylation will occur at the oxygen [178]. In the case of *O*-acylation, the phenyl ester can be rearranged to either an *ortho* or a *para* substituted aryl ketone by the Fries rearrangement, depending on the conditions employed.



Scheme 2.6. The Fries Rearrangement.

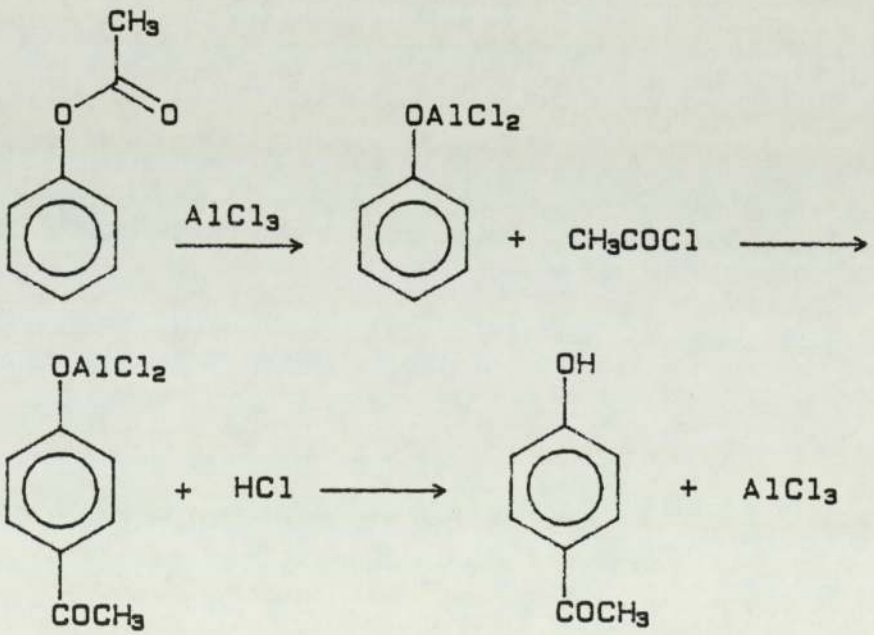
This rearrangement uses a strong Lewis acid such as aluminium trichloride or, less commonly, zinc chloride, boron trichloride or iron (III) chloride. Lewis acids such as aluminium tribromide and boron tribromide are unsuitable catalysts because of the nucleophilicity of the bromide ions formed; indeed, this property can be exploited in the cleavage of ethers (for review see M V Bhatt and S U Kulkarni [179]).

As with Friedel-Crafts reactions, the presence of an electron-withdrawing or *meta* directing group in the aromatic ring prevents the reaction and it has been reported that a nitro or benzoyl group either *ortho* or *para* to the hydroxyl group will inhibit the reaction [180]. In the present study, it

was found that 3-nitrophenyl acetate was an unsuitable substrate for this reaction. The possible explanations for this are:

1. That the electron density at the carbonyl oxygen reduced to such an extent that complexation with the Lewis acid does not occur.
2. That the nucleophilicity of the carbon *ortho*- to the acetoxy function has been decreased.

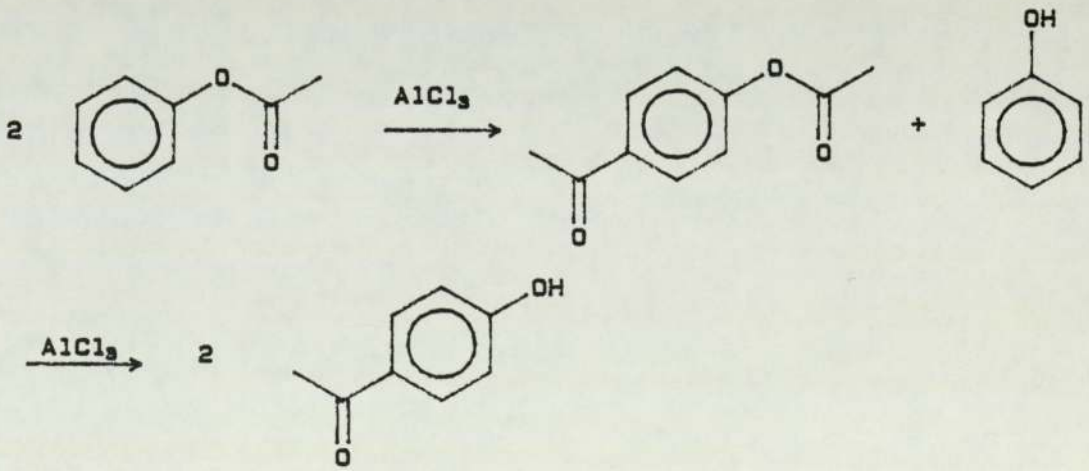
The mechanism of the Fries rearrangement has been the subject of much study and debate. At least three mechanisms have been suggested and evidence produced to support each different mechanism (for review see A H Blatt [170]). In the pathway proposed by Skraup and Poller [181], decomposition of the



Scheme 2.7. Mechanism of the Fries Rearrangement proposed by Skraup and Poller

phenol ester yields the acid chloride and an aluminium dichloride-phenolate complex. The acid chloride is then subjected to nucleophilic attack by the ring carbon of the phenolate complex as shown in Scheme 2.7. Further evidence for this has been given by Cox [182].

Evidence for the second mechanistic proposal is furnished by the observations of Rosenmund and Schnurr [180] that *para*-substituted hydroxyketones may be formed by the Fries reaction at lower temperatures (ca. 30°C), whereas the formation of *ortho*-substituted hydroxyketones requires higher temperatures, usually in excess of 140°C. Since *para* substitution may occur, this suggests that this particular rearrangement takes place *via* an intermolecular mechanism owing to the geometrical disposition of the reacting moieties. In fact, it has been shown in a cross-over experiment that one molecule of ester can serve as the acyl donor for a second molecule [180], see Scheme 2.8.



Scheme 2. 8. Mechanism of the Fries Rearrangement proposed by Rosenmund and Schnurr

A third possible mechanism which has been postulated by Auwers and Mauss suggests that a true intramolecular rearrangement is involved in the preparation of a hydroxy ketone by the Fries rearrangement [183], [184]. Since there is evidence to support all three proposed mechanisms, it may be possible that different mechanisms are acting under different conditions.

Since the preparation of flavones substituted in the 5 and 7 position was essential if any structure activity relationship was to be elucidated, it was necessary to prepare 4- and 6-substituted 2-hydroxyacetophenones.

The procedure recommended by Blatt [170] for the preparation of substituted 2-hydroxyacetophenones requires the phenyl acetate to be ground with a molar equivalent of aluminium chloride (up to 25% excess $AlCl_3$ may be required, depending on the grade of the reagent) and heated strongly without a solvent for thirty minutes. In the present study, an intractable black tar was obtained when 3,5-dimethoxyphenyl acetate was subjected to these conditions. Since similar reactions have been reported [185], [186] using either nitrobenzene or nitromethane as solvent, the procedure was repeated in nitrobenzene. This, too, produced an amorphous solid suspended in the solvent.

A possible explanation for the failure of

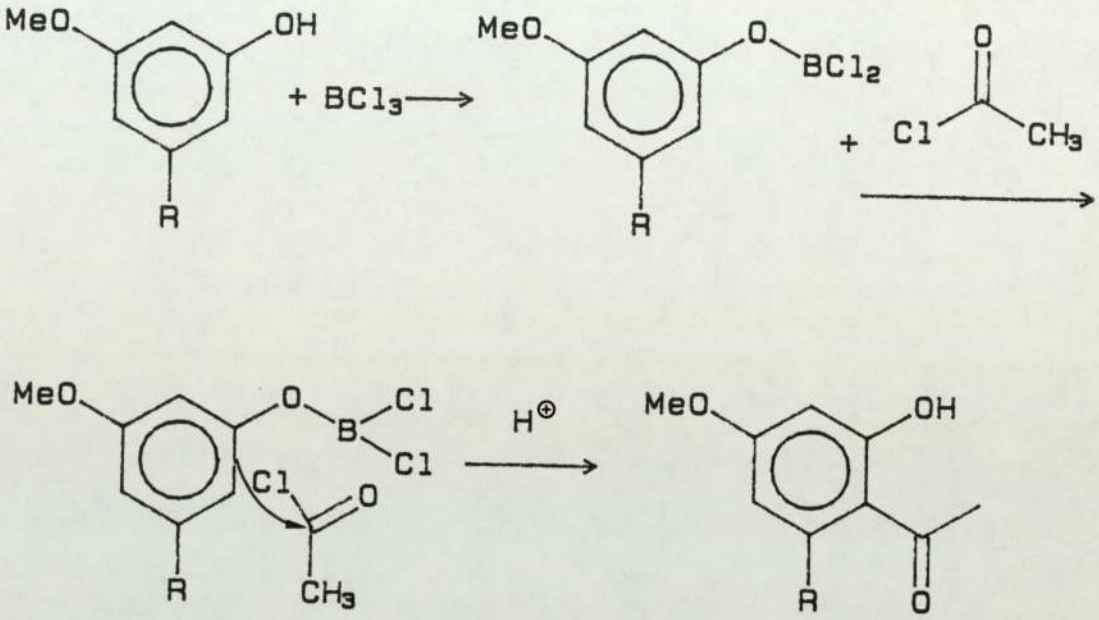
3,5-dimethoxyphenyl acetate to furnish an identifiable product under these conditions could be attributable to the increase in activity of the carbon *ortho* to the hydroxyl group owing to the electron donating effects of the methoxy functions both *ortho* and *para* to this position.

The need for such an apparently difficult rearrangement was obviated by modification of a procedure devised by Piccolo *et al* [187] which rendered these compounds accessible *via* a facile one pot synthesis.

2-hydroxy-4-methoxy- and 4,6-dimethoxy-2-hydroxy-acetophenones (cpds. **2** and **1** resp.) were prepared, in 78% yield ,by treating the corresponding phenol with BCl_3 in CH_2Cl_2 followed by addition of acetyl chloride, as described in the experimental section. This method furnished these compounds in one step from the phenol whereas the Fries rearrangement required the initial preparation of the phenyl acetate, therefore the synthesis of the acetophenones by this method is more efficient than by the Fries rearrangement. The proposed mechanism is shown in Scheme 2.9.

The Lewis acid, BCl_3, complexes onto the phenolic oxygen to generate a phenoxy-boron complex. On addition of acetyl chloride the carbonyl group also complexes with the boron via donation of an electron pair from the oxygen to the p-orbital of the boron. The carbon *ortho* to the phenolic oxygen is then the subject of an electrophilic attack by the carbonyl carbon

with subsequent loss of chloride. The six membered cyclic intermediate is then hydrolysed on the addition of dilute hydrochloric acid.



R = H or OMe

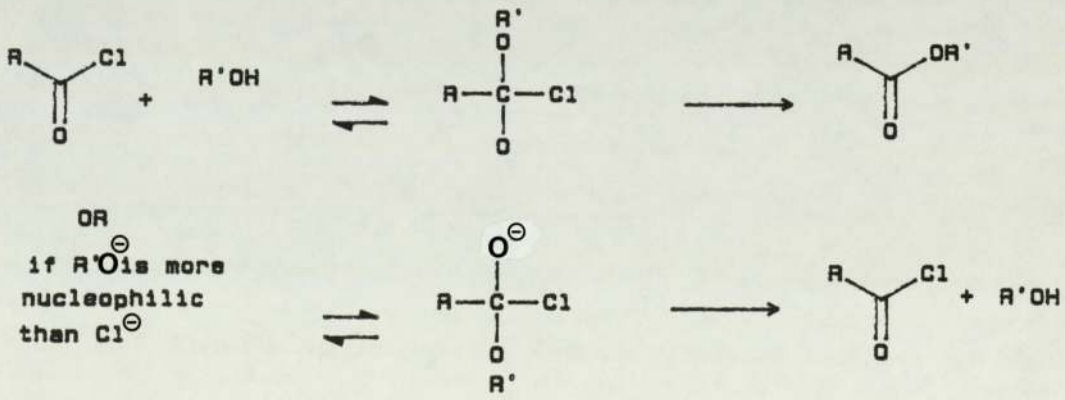
Scheme 2.9. Proposed mechanism for the preparation of 2-hydroxy-4-methoxyacetophenone from 3-methoxyphenol and acetyl chloride.

This proposed mechanism may support one of the possible mechanisms for the Fries rearrangement, that is, by treating a phenyl acetate with $AlCl_3$ the corresponding acid chloride is generated along with aluminium phenoxide, the acid chloride is then the subject of a nucleophilic attack by the aromatic ring. Complexation of the BCl_3 with the phenol in a suitable solvent before adding the acyl chloride renders the initial acylation

in the classical Fries rearrangement superfluous. There is one potential drawback with this procedure, although in this case it is an advantage. According to the mechanism, only *ortho* substitution will occur, so this reaction is not of use for the preparation of *para*-substituted phenol ketones.

The above method is ideal for the preparation of oxygen functionalised 2-hydroxyacetophenones. However, the preparation of these acetophenones containing nitrogen functional groups was also required. The Fries rearrangement, being a Friedel-Crafts reaction, is unsuitable for the preparation of nitro substituted 2-hydroxyacetophenones due to the electron withdrawing effect of these groups [178]. Although references to the preparation of 2-hydroxy-4-nitrophenol by the Fries rearrangement appear in the literature [188], [189] no experimental details are given. The method used to prepare 2-hydroxy-4-methoxyacetophenone and 2-hydroxy-4,6-dimethoxy-acetophenone was unsuccessful in the attempted preparation of 2-hydroxy-4-nitroacetophenone even under much more vigorous conditions ($AlBr_3$ in nitrobenzene at reflux for 24 hours). It was expected that 2-hydroxy-4-nitroacetophenone could be prepared by a photochemical reaction, the photo-Fries rearrangement, (for review see D Bellus and P Hrdlovic. [190]) from 3-nitrophenyl acetate, albeit in low yields (8-10%), in a mann er similar to the rearrangement of p-nitrophenyl-p-nitrobenzoate [191]. However due to the lack of access to an ultraviolet lamp of high enough energy it was not possible to prepare this compound.

Acylation of both 2-hydroxy-4-methoxyacetophenone and 4,6-dimethoxy-2-hydroxyacetophenone with benzoyl chloride required catalysis with 4-(*NN*-dimethylamino)pyridine (DMAP). However, when a more powerful acylating agent, 4-nitrobenzoyl chloride, was employed catalysis with DMAP was required only for the acylation of the dimethoxyacetophenone (although the ease of acylation of 2-hydroxy-4-methoxyacetophenone was improved by utilising DMAP). The simplest explanation of these effects is the reduced ability of the phenol to form the phenoxide anion in the presence of the electron donating methoxy groups, despite their being meta to the phenol. Another explanation could lie with collapse of the tetrahedral intermediate in the acylation reaction. DMAP forms the acylpyridinium species with the acid chloride and the pyridinium anion is a far better leaving group than a chloride ion, since it is more nucleophilic. 4,6-dimethoxy-2-hydroxyacetophenone is more nucleophilic than 2-hydroxy-acetophenone, therefore this species could be a better leaving



group than chloride from the tetrahedral intermediate yielding

the starting materials rather than the desired product, as shown above.

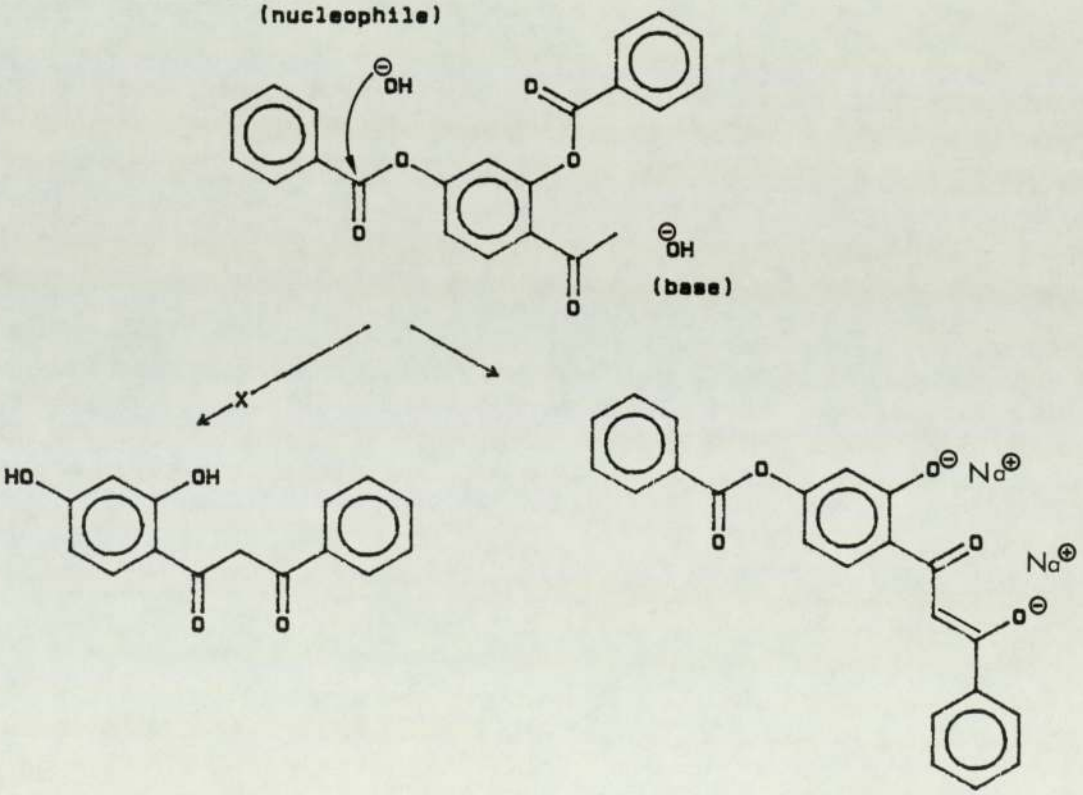
The phenyl esters were generally recrystallised from methanol and for the majority of the compounds described this solvent was quite adequate. However recrystallisation of 4,6-dimethoxy-2-(4-nitrobenzoyloxy)-acetophenone (21) with methanol resulted in partial methanolysis yielding 4,6-dimethoxy-2-hydroxy-acetophenone and methyl 4-nitro-benzoate; chloroform/ light petroleum (boiling range 60-80°C) was therefore used to recrystalllise this compound. 2-Benzoyloxy-4,6-dimethoxyacetophenone (19) was also recrystallised using these solvents. More importantly, this susceptibility of the ester group to nucleophilic attack suggested that the rearrangement of these esters with potassium hydroxide in pyridine might not be viable. A non-nucleophilic base was therefore sought to rearrange these compounds.

Among these bases are 1,4-diaza-[2,2,2]bicyclooctane, (DABCO), diazabicylcoundecene, (DBU), *NN*-diisopropyl-ethylamine, 2,6-dimethylpyridine, potassium *t*-butoxide, lithium hexamethyldisilazide, lithium diisopropylamide and sodium hydride. Lithium hexamethyldisilazide was chosen from among these because of its ease of use and was generated *in situ* under nitrogen at -78°C by the addition of n-butyl lithium to hexamethyldisilazane in dry tetrahydrofuran (THF).

An alternative method for the preparation of flavone with

hydroxyl groups in the 5 and 7 positions was by the acylation of 2,4-dihydroxy- or 2,6-dihydroxyacetophenone. Two equivalents of the suitably substituted acid chloride were used to acylate the required dihydroxyacetophenone to furnish the diester. Initially it was thought that, under the rearrangement conditions, the benzoyl group in the 4 or 6 position would be hydrolysed to yield the free hydroxyl while the benzoyloxy group in the 2-position would be rearranged to give the 1-(2-hydroxyphenyl)-3-phenylpropan-1,3-dione. However, no hydrolysis was observed.

From this observation it appears that NaOH in pyridine is a poor nucleophile but a good base. The reaction is displayed in Scheme 2.10.



Scheme 2. 10. Rearrangement versus hydrolysis of 2,4-dibenzoyloxyacetophenone

Despite this apparent stability of the ester to nucleophilic attack under the conditions of the rearrangement, when the flavone is prepared in the usual manner by the acid catalysed cyclocondensation of the dione, dissolution of the product in aqueous base leads to hydrolysis within ten minutes. The acid and the hydroxyflavone can then be separated by suspending the reaction mixture in aqueous sodium hydrogen carbonate and filtering to remove the flavone.

Functionalisation of the 3-position posed some interesting synthetic problems. A hydroxyl group can be introduced into this position using the method of Costa *et al* [192]. The most acidic proton in flavone is the proton on the 3-carbon. If flavone is treated with lithium di-isopropylamide in THF at -78°C under N_2 the 3-lithioflavone is generated. Treatment of this complex with trimethylborate furnishes the borate ester which can then be cleaved oxidatively with hydrogen peroxide to yield 3-hydroxyflavone (**82**). The general applicability of this method is limited by the acidity of the proton on the 3-carbon. When this procedure was applied to 4'-methoxyflavone (**49**) and 3'-methoxyflavone (**50**), no reaction took place and the original flavones were recovered. The most plausible explanation for this is that it is due to the electron-donating effects of the oxygen functions increasing the electron density over the pyrone ring and decreases the acidity of the proton at the 3-position. Owing to the insolubility of the 4'-nitro and 3'-nitroflavones (**60** and **59**) in THF, it was not possible to

test this hypothesis, although 4'-chloroflavone (54) would be an interesting substrate for this reaction.

Since a chlorine atom is approximately isosteric with a hydroxyl group, synthesis of a 3-chloroflavone was of interest. This was achieved by treating flavone in carbon tetrachloride with sulphuryl chloride as a chlorinating agent in an electrophilic substitution reaction. A slight excess of sulphuryl chloride was used in this procedure and the reaction not only yielded 3-chloroflavone (83) but also the addition product, 2,3,3-trichloroflavanone (84). The two products were initially separated by column chromatography using silica and chloroform, and 2,3,3-trichloroflavanone was purified by preparative thin layer chromatography. Although this reaction has been reported in the literature, [193], [194] a tetrachloroflavanone, 2,3,3,6-tetrachloroflavanone, was isolated as well as the mono substituted flavone when flavone was treated with sulfuryl chloride in sulfolane, whereas only the mono and trisubstituted products were isolated in the present study, when CCl_4 was the solvent.

Using these methods a variety of substituted flavones were prepared and modification of these compounds yielded further analogues.

Aminoflavones were generated by the reduction of appropriately substituted nitroflavones with tin (II) chloride in ethanol in the presence of a trace of concentrated

hydrochloric acid. There are a number of potentially useful procedures for reducing nitro functions, these include:-

1. Catalytic hydrogenation; the most common procedure uses palladium on charcoal as a catalyst in an atmosphere of hydrogen (for review see Rylander [195]).
2. Hydrazine and Raney nickel [196].
3. The use of sulphides, such as sodium hydrogen sulphide, ammonium sulphide, or polysulphides. This reaction is named the Zinin Reduction, (see Porter [197]).
4. Metals and acid, or salts of metals in low oxidation states, such as tin (II) chloride, zinc, or iron filings.

Although reduction of a nitro group under an atmosphere of hydrogen with a catalyst such as platinum, ruthenium or palladium may be of great use in other systems; in the present study, it was not considered as a potentially useful reaction since the C2-C3 double bond could also be reduced under these conditions.

The presence of a carbonyl group in flavone rendered hydrazine and Raney nickel useless for this procedure since it was postulated that the carbonyl group of the 4-position could be the subject to nucleophilic attack by the hydrazine and so form the hydrazone. However a literature report of this reaction [198] described the formation of azoxyflavone rather

than the hydrazone. Even so, this report supports the unsuitability of this method for the formation of amines in this system.

While there is no chemical reason why an inorganic sulphide may not be used in these reactions to form an amine, owing to their unpleasant odour and lack of ease of handling it was decided that this procedure would not be used.

The specificity of tin (II) chloride in ethanol with a trace of acid to the reduction of the nitro group in this system, together with its ease of handling and short reaction time made these conditions most practically useful.

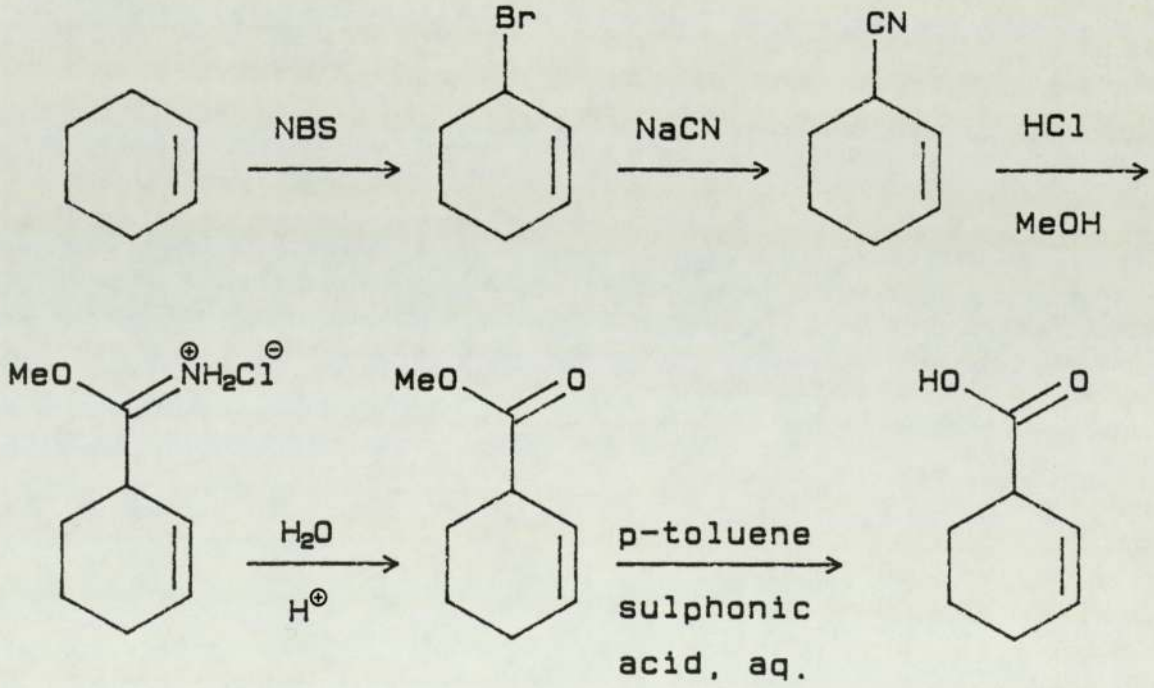
Flavones substituted with hydroxyl functions were initially prepared by the demethylation of the corresponding methyl ether using hydrobromic acid in glacial acetic acid. However this reaction required over seven days to go to completion and required frequent replenishment with hydrogen bromide. The use of BBr_3 in dichloromethane at room temperature furnished these compounds within twelve hours.

In addition to the preparation of flavones for this study, preparations of 2-cyclohexylchromones were attempted.

An initial preparation of 2-cyclohexylchromone (48) followed the same procedure as that to prepare flavone. The acylation reaction produced a dark yellow oil which was then

subjected to rearrangement by sodium hydroxide in pyridine. The compound did not rearrange to the 1-(2-hydroxyphenyl)-3-cyclohexylpropan-1,3-dione but was the subject of a nucleophilic attack by the hydroxyl anion which hydrolysed the ester to the original phenol and cyclohexyl carboxylic acid. Employing a hindered base, potassium *tert* butoxide, furnished the rearranged product as another dark yellow oil. Ring closure was effected with concentrated sulphuric acid in glacial acetic acid.

The preparation of 2-cyclohexylchromones with hydroxyl functions in the cyclohexyl ring proved very much more difficult and required the synthesis of cyclohexenecarboxylic acids. Cyclohex-2-ene carboxylic acid (88) was prepared from cyclohexene as shown in Scheme 2.11.



Scheme 2. 11. Preparation of cyclohex-2-ene carboxylic acid from cylcohexene

Free radical bromination of cyclohexene with *N*-bromosuccinimide yielded 3-bromocyclohexene (85). Substitution of a cyano group for the bromo atom was attempted in ethanol. However, the mass spectrum of the product of this reaction indicated that instead of the desired substitution taking place the bromo atom had been replaced with a hydroxyl group. The reaction was repeated using sodium iodide as a catalyst; however this also yielded cyclohex-2-enol. The preparation was repeated using the procedure of Davies and Whitman [199], stirring two equivalents of dry powdered sodium cyanide with one equivalent of 3-bromocyclohexene in dry *N*-methyl pyrrolidinone for 90 minutes at 20°C. This method proved most successful although all reactants were required to be completely dry since, in the presence of a trace of water, cyclohex-2-enol would be generated. It is evident from the NMR spectrum of one reaction that a mixture of 3-cyanocyclohexene (86) and cyclohex-2-enol was obtained. In the spectrum of a pure sample 3-cyanocyclohexene, the chemical shifts in $CDCl_3$ are δ 5.75 for the multiplet produced by the two vinyl protons, δ 3.25 for the multiplet produced by the CH.CN proton and δ 1.9, multiplet which is generated by the remaining cyclohexyl protons. The spectrum of the mixture of 3-cyanocyclohexene and cyclohex-2-enol shows an additional peak at δ 4.8 which is produced by the C<u>H</u>.OH grouping although the hydroxyl proton itself is not apparent in the spectrum.

The Pinner Synthesis furnished the hydrochloride salt of

cyclohex-2-enylimino methyl ester by bubbling dry hydrogen chloride through a refluxing solution of dry 3-cyanocyclohexene in methanol for six hours and stirring overnight. Quenching with crushed ice afforded the methyl ester of cyclohex-2-ene carboxylic acid acid 87. The free acid was obtained by hydrolysing the ester with aqueous toluene-4-sulphonic acid. At this stage, the double bond could be oxidised to yield the diol in a mixture of configurations, alternatively the double bond could be looked upon as a protected diol, in which case the oxidation would not be carried out until the cyclohexenylchromone had been formed. The latter course was chosen, and the acid chloride was formed by boiling the acid in CCl_4 with one equivalent of thionyl chloride.

Acylation of 2-hydroxyacetophenone requires base catalysis. In the case of acylation with cyclohex-2-enecarbonyl chloride the presence of base, albeit pyridine, catalysed not only the acylation reaction but also the migration of the double bond into conjugation with the carbonyl group. The product of the reaction was shown by ^{13}C NMR to be a mixture of 2-(cyclohex-2-en-1-carboxy)acetophenone, 2-(cyclohex-1-en-1-carboxy) acetophenone and a very small quantity of the acid.

Not surprisingly, the conditions of the rearrangement procedure were not conducive to producing the desired product and an intractable brown oil was formed which, by thin layer chromatography, proved to be a mixture of a large number of

compounds.

Among the desired target compounds was a series of 2-phenylpyran-4-ones. The rationale for the synthesis of these compounds was to ascertain what rôle, if any, the A ring of flavone had in the activity of these compounds against tyrosine protein kinases.

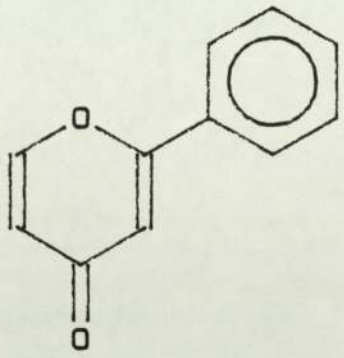
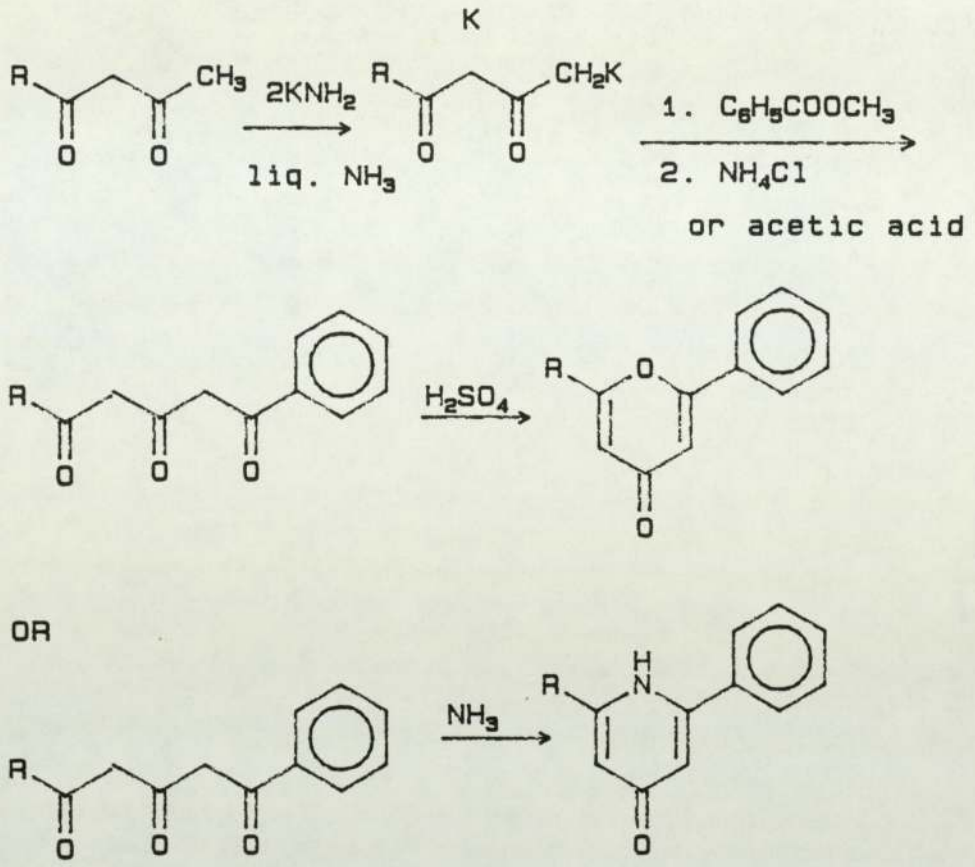


Fig.2.4. 2-Phenylpyran-4-one.

The standard method for the synthesis of pyran-4-ones is by the acid-catalysed dehydration of the corresponding 1,3,5-triketone. The method of Light and Hauser [200] is shown in scheme 2.12.

The dipotassium salt of a ß-diketone was generated with potassium amide in liquid ammonia and then treated with two equivalents of an ester. The product of this reaction is the 1,3,5-triketone which, when treated with a strong mineral acid, will yield the pyran-4-one. If the cyclisation is carried out with ammonia the product of the reaction is the 4-pyridone.

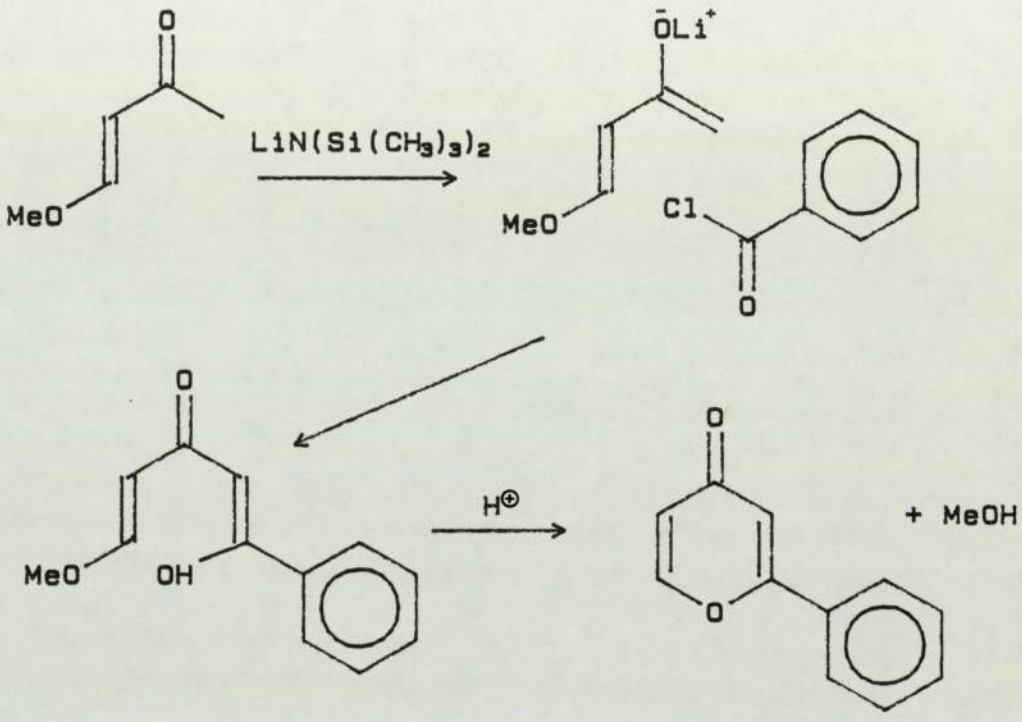


Scheme 2.12. Synthesis of 2-phenylpyran-4-ones.

Although there are literature procedures for the synthesis of 2-phenylpyran-4-ones, these compounds tend to be substituted in the 6-position with a methyl group. Initially, it was intended to attempt the synthesis of 2-phenylpyran-4-one itself. Koreeda and Akagi [201] reported a "convenient" synthesis of substituted pyran-4-ones including that of 2-phenylpyran-4-one.

The method of Koreeda and Akagi utilised a

ß-methoxy-ß-enone, such as *trans*-4-methoxybut-3-en-2-one, and an acid chloride as the starting materials. The lithium enolate of the enone is generated by treating it with lithium hexamethyldisilazide in THF at -78°C. The acid chloride is then added to the reaction and the carbonyl carbon is then subject to a nucleophilic attack by the lithium enolate complex, as shown in scheme 2.13:-



Scheme 2.13. Synthesis of 2-phenylpyran-4-one.

The enol that was generated in this reaction was then treated with a trace of trifluoroacetic acid in benzene to furnish the 2-phenylpyran-4-one. In the present work, however, the reaction between the lithium enolate and the acid chloride produced a red solid which, according to thin layer chromatography, was a mixture of eight or nine compounds.

Since it was possible that the enol was unstable on silica gel, the NMR spectrum was studied. This spectrum prooved to be uninterpretable.

The procedure was repeated using 4-methoxypent-3-en-2-one (91) which was prepared by the *O*-methylation of pentane-2,4-dione with dimethylsulphate in acetone and potassium carbonate. This enone would furnish the 2-methyl-6-phenylpyran-4-one (**92**), which can be prepared by other methods (see below), however, due to the expense of *trans*-4-methoxybut-3-en-2-one, it was decided to use this less expensive starting material in this reaction initially to verify the "effectiveness" of the procedure.

In this study, utilising the reaction conditions of Koreeda and Akagi the overall yield of 2-methyl-6-phenylpyran-4-one was 2.5%. The intermediate enol was isolated by preparative thin layer chromatography and the pyrone was furnished by treating the enol with trifluoroacetic acid in toluene.

In order to investigate the significance of the nature of the heterocycle in the ability of these compounds to inhibit tyrosine protein kinases it was desired to prepare a series of thioflavones, see fig 2.3.

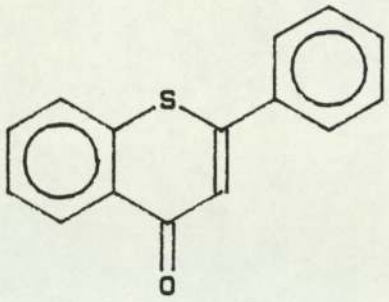
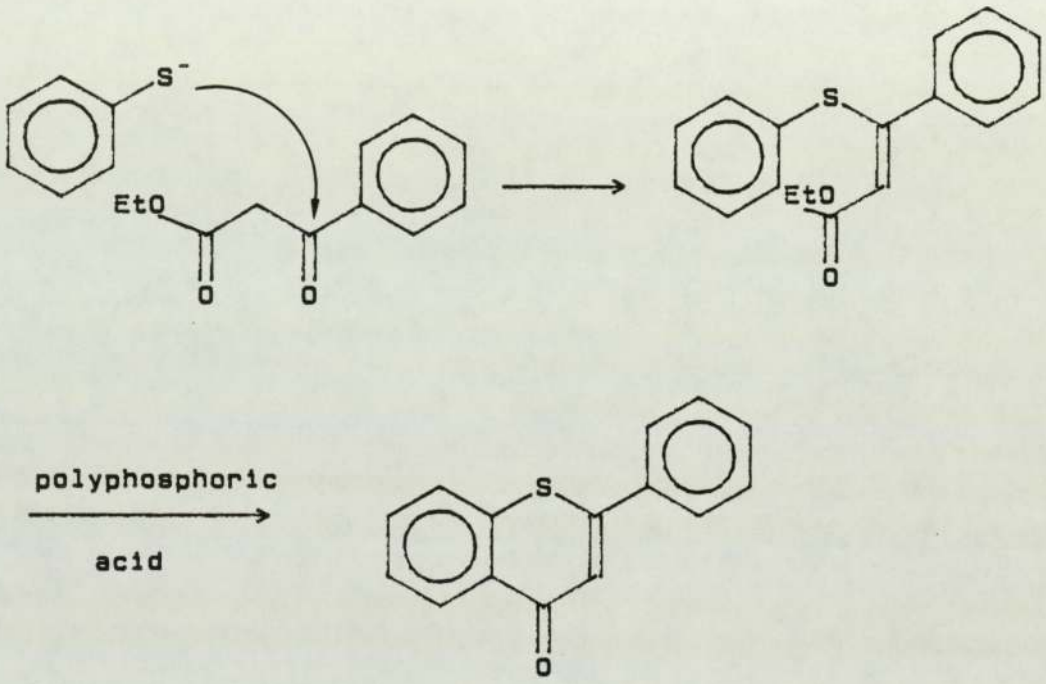


Figure 2.3. Thioflavone.

Bossert [202] prepared thioflavone by the condensation of thiophenol and ethylbenzoyl acetate in polyphosphoric acid by the route shown in Scheme 2.14.



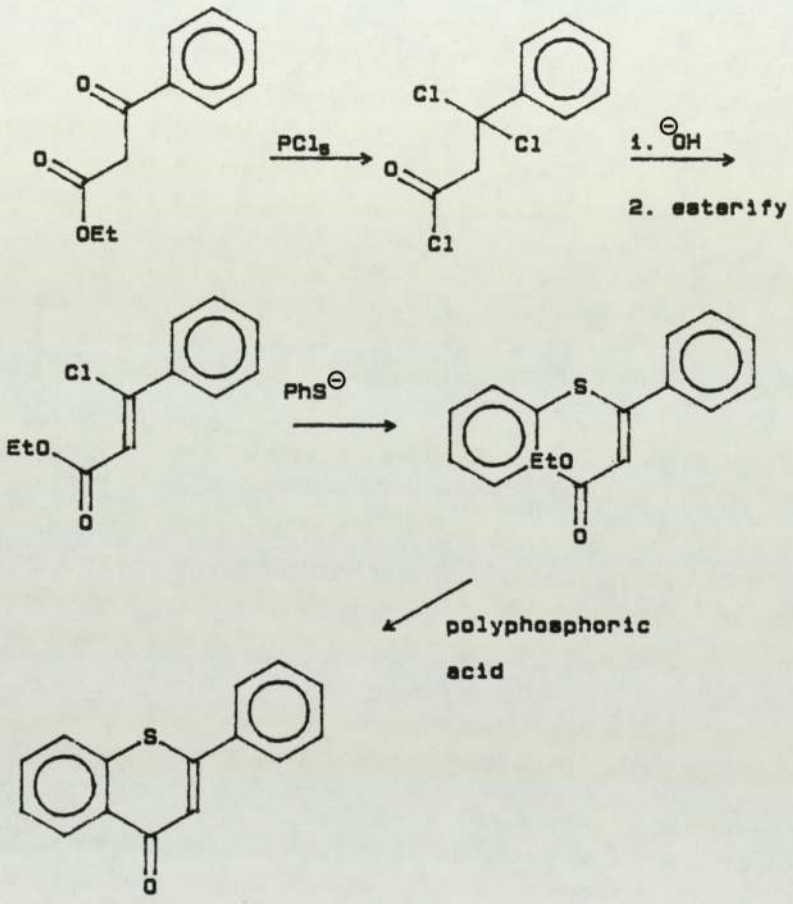
Scheme 2.14. Thioflavone Synthesis.

The initial nucleophilic attack of the thiophenol generates a thioether as shown. An internal Friedel-Crafts acylation, catalysed by polyphosphoric acid, then furnishes the thioflavone.

This method could also be used to generate thiochromones

from thiophenol and the appropriate ß-oxo ester.

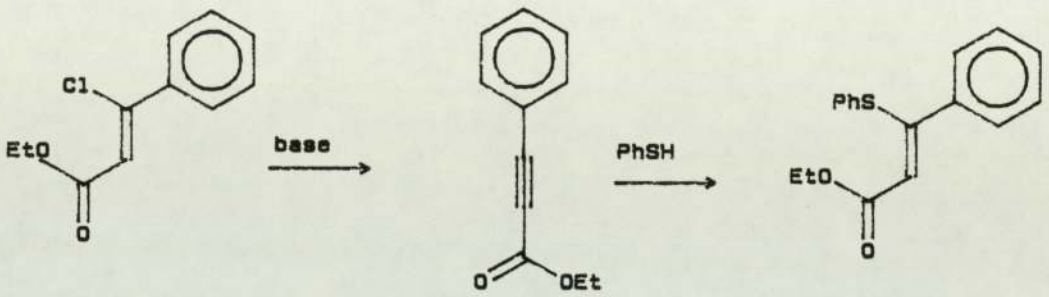
In this study the synthesis of thioflavone was attempted by the nucleophilic attack of thiophenol on ethyl ß-chlorocinnamate using sodium ethoxide as a base to generate the thiophenoxide, as shown in Scheme 2.15.



Scheme 2.15. Postulated preparation of thioflavone from thiophenol and ethyl ß-chlorocinnamate.

ß-chlorocinnamic acid (89) was generated by treating ethyl benzoyl acetate with phosphorus pentachloride in phosphorus oxychloride to furnish the gem-dihalide. Elimination of hydrogen chloride yielded the ß-chlorocinnamic acid.

Reactions of this type are well documented in the chemical literature, (for a review see [203]). There are many potential mechanisms for this rearrangement, however an addition-elimination or an elimination-addition mechanism would appear most favoured. The elimination-addition mechanism requires base catalysis as shown in Scheme 2.16.



Scheme 2.16. Elimination-addition mechanism for nucleophilic attack at a vinylagous carbon.

It was thought that this reaction would proceed using thiophenol as a nucleophile and ethyl ß-chlorocinnamate as the vinyl ketone.

However, instead of isolating the required thioether it appeared from the NMR spectrum that ethoxide had acted as nucleophile instead of the thiophenol and ethyl ß-ethoxycinnamate was prepared instead of the desired thioether, and, according to the NMR, both the *cis* and *trans* isomers were obtained, this supports the elimination-addition

mechanism for this reaction.

Why the ethoxide anion acts as the nucleophile in this system as opposed to thiophenol, which should be a far better nucleophile, may be explained on the grounds of steric hindrance. Since the ethoxide anion is very much smaller than the thiophenoxide anion its approach to the C atom α to the phenyl ring will not be hindered by the bulk of that ring, while approach of the thiophenoxide will be hindered, not only sterically, but also by mutual repulsion of the π-systems of the two phenyl rings.

By these methods a total of thirty seven compounds were prepared that were suitable for testing as potential tyrosine kinase inhibitors. These compounds are listed in Table 2.1.

Table 2.1. The synthetic analogues of quercetin.

Compound	R^3	R^5	R^7	$R^{3'}$	$R^{4'}$
47	H	H	H	H	H
49	H	H	H	H	OMe
50	H	H	H	OMe	H
51	H	H	H	OMe	OMe
57	H	H	OMe	H	H
64	H	OMe	OMe	H	H
62	H	OMe	OMe	H	NO_2
65	H	H	OMe	H	NO_2
82	OH	H	H	H	H
70	H	H	H	H	OH
71	H	H	H	OH	H
69	H	H	H	OH	OH
83	Cl	H	H	H	H
54	H	H	H	H	Cl
53	H	H	H	Cl	H
55	H	H	H	Cl	Cl
52	H	H	H	F	H
56	H	H	H	H	Br
58	H	H	H	H	NO_2
59	H	H	H	NO_2	H
63	H	H	H	NO_2	Cl
61	H	H	H	OMe	NO_2
60	H	H	H	NO_2	OMe
77	H	H	H	H	NH_2
78	H	H	H	NH_2	H
75	H	H	H	OMe	NH_2
76	H	H	H	NH_2	OMe
72	H	H	H	NO_2	$N(CH_3)_2$
73	H	H	H	NH_2	$N(CH_3)_2$
74	H	OMe	OMe	H	NH_2
67	H	H	H	NO_2	OH
68	H	H	H	OH	NO_2
66	H	H	PhCOO	H	H
80	H	H	OH	OMe	H

Also prepared were 2-cyclohexylchromone (48) and 2,3,3-trichloroflavanone (84).

CHAPTER THREE

ON THE KETO-ENOL TAUTOMERISM OF THE 1-(2-HYDROXYPHENYL)-3-PHENYLPROPAN-1,3-DIONES

In the course of characterising the intermediates in the synthesis of substituted flavones, it was observed that the propan-1,3-diones exhibited some interesting NMR spectroscopic details.

In the 60MHz ^1H NMR spectrum of 1-(2-hydroxyphenyl)-3-phenylpropan-1,3-dione (**32**) in $CDCl_3$, there was no signal given by the methylene protons. These protons would be expected to resonate as a singlet in the region δ 4.3-4.7. The 400MHz spectrum however, did show a signal at δ 4.63 but the integral of this signal showed it to correspond to 0.13 H, not the 2H that would be expected. On searching downfield, three singlets could be observed at δ11.93, δ 12.10 and δ15.54. A singlet was also apparent at δ 6.84.

1-(2-hydroxyphenyl)-3-phenylpropan-1,3-dione exhibits keto-enol tautomerism and so can exist in several forms, three of which are shown in Fig. 3.1.

This keto-enol tautomerism explains the appearance of the very low-field signal at δ15.45 and that of the vinylic proton at δ6.84. The chemical shifts of the enol proton, the vinylic

proton and the methylene protons are similar to those reported for 1,3-diphenylpropan-1,3-dione [237]. The appearance of the

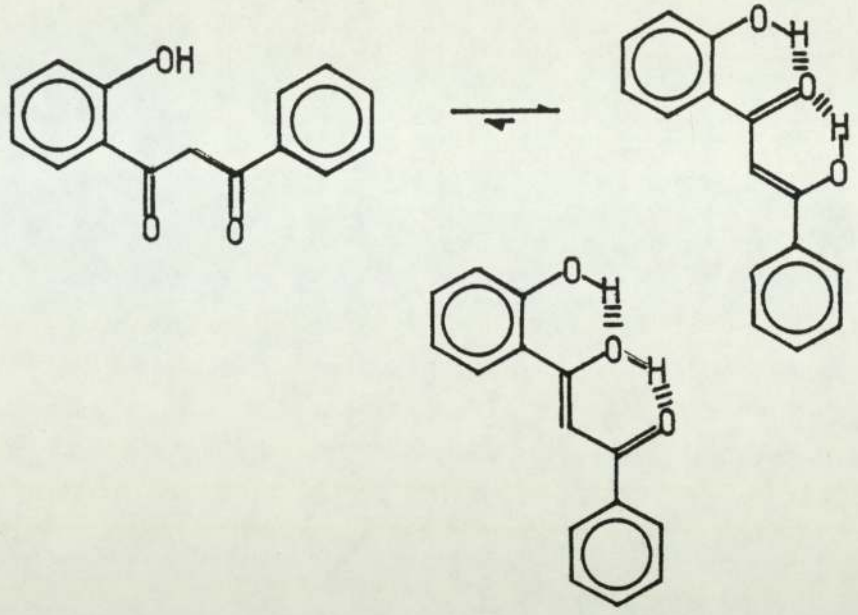


Fig. 3.1. Keto-enol tautomerism of 1-(2-hydroxyphenyl)-3-phenylpropan-1,3-dione.

signal at δ4.63 indicates that this compound exists in an equilibrium between the two forms and, as determined by the relative integrals, the energetically most favourable form is that of the enol.

The majority of the 1-(2-hydroxyphenyl)-3-phenylpropan-1,3-diones prepared in this study exhibited this effect in the NMR spectra, to a greater or lesser extent. The percentage enolisation being in the region of 90% for most of those compounds studied. This value is comparable to that for 1,3-diphenylpropan-1,3-dione [238].

Figure 3.2. The 400MHZ H^1 NMR spectrum of 1-(2-hydroxy-4-methoxyphenyl)-3-phenylpropan-1,3-dione (29) in $CDCl_3$.

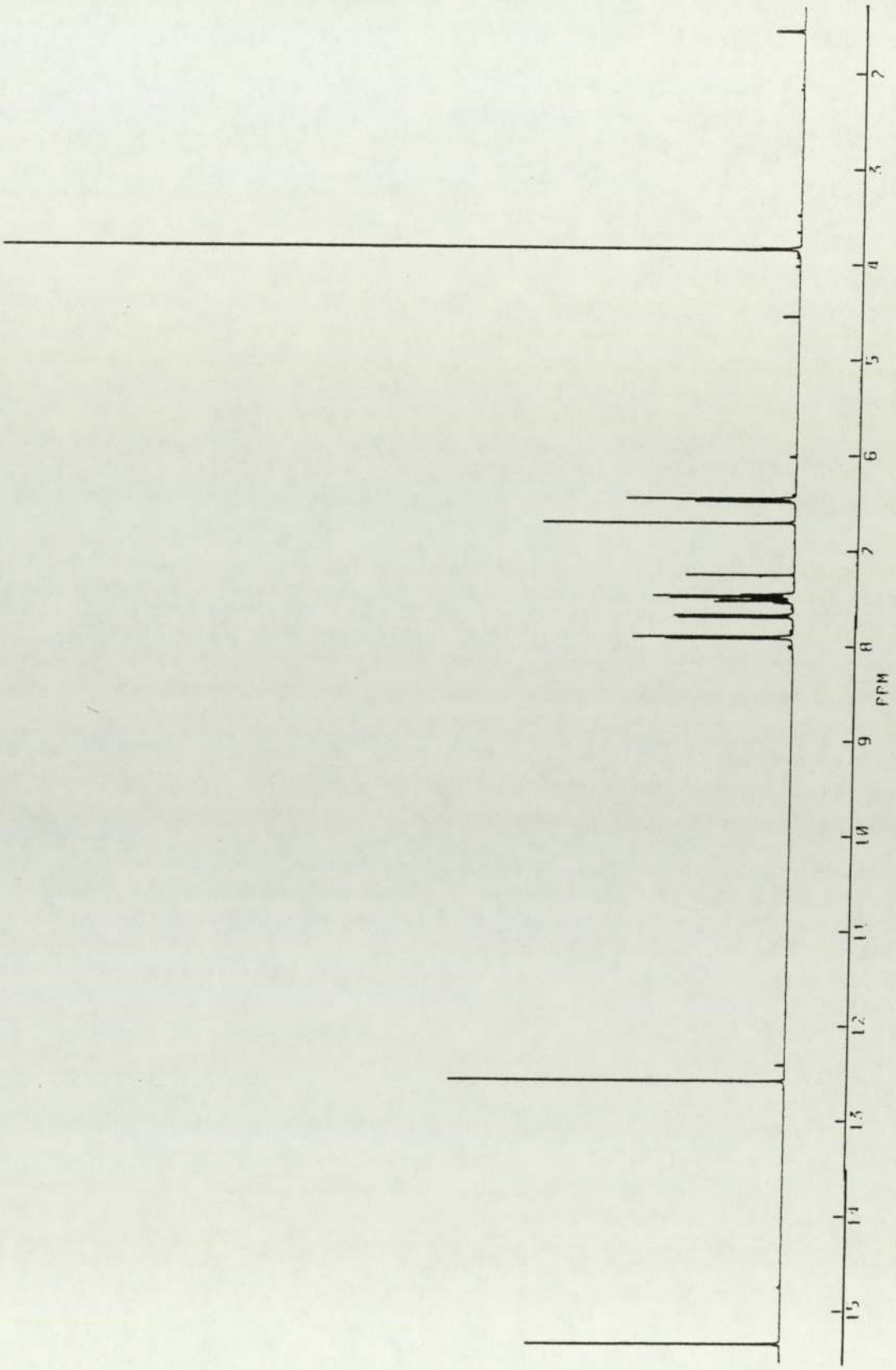


Figure 3.3. The 400MHZ H^1 NMR spectrum of 1-(4,6-dimethoxy-2-hydroxyphenyl)-3-phenylpropan-1,3-dione (28) in $CDCl_3$.

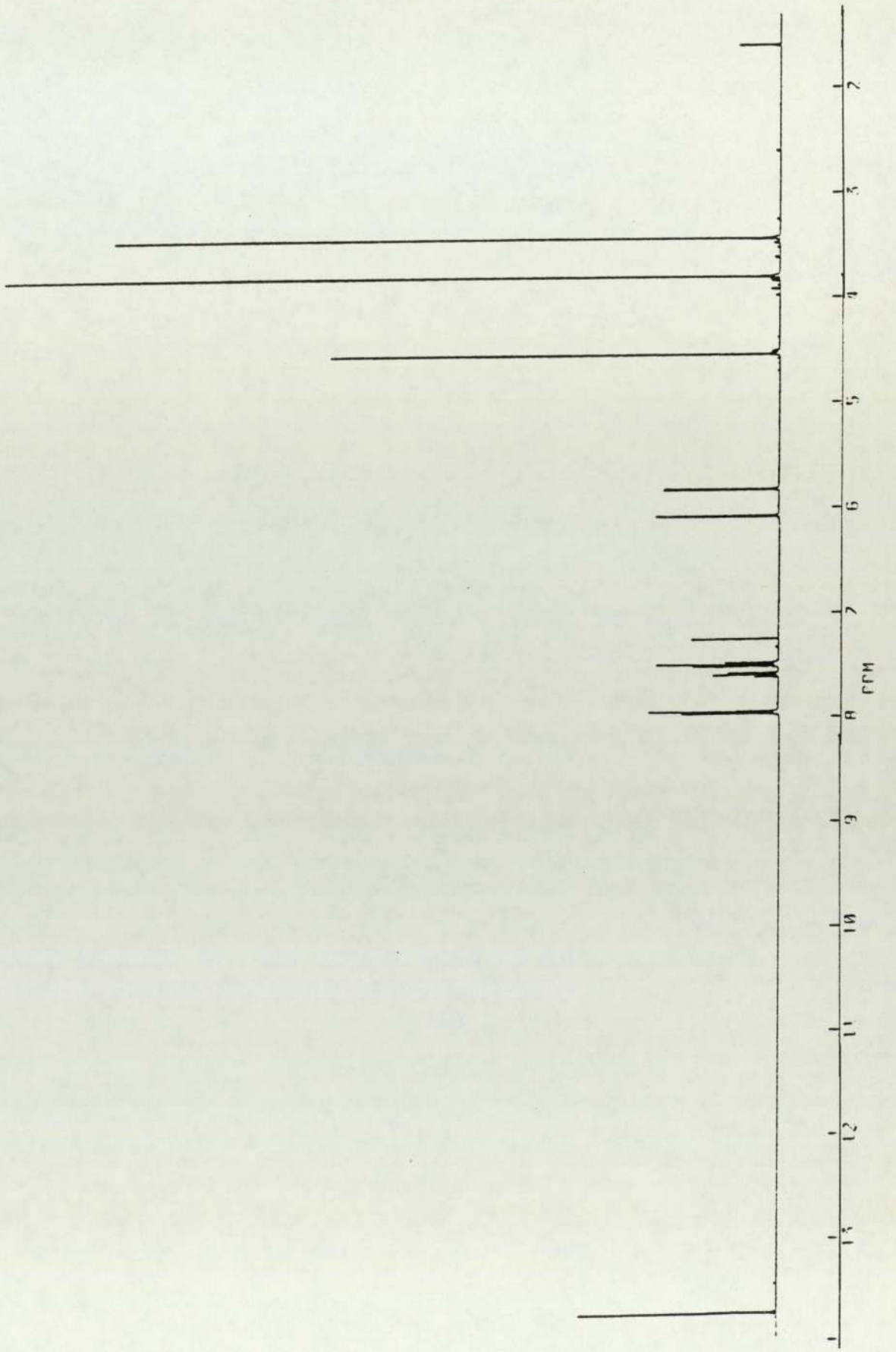


Figure 3.4a. The crystal structure of 29

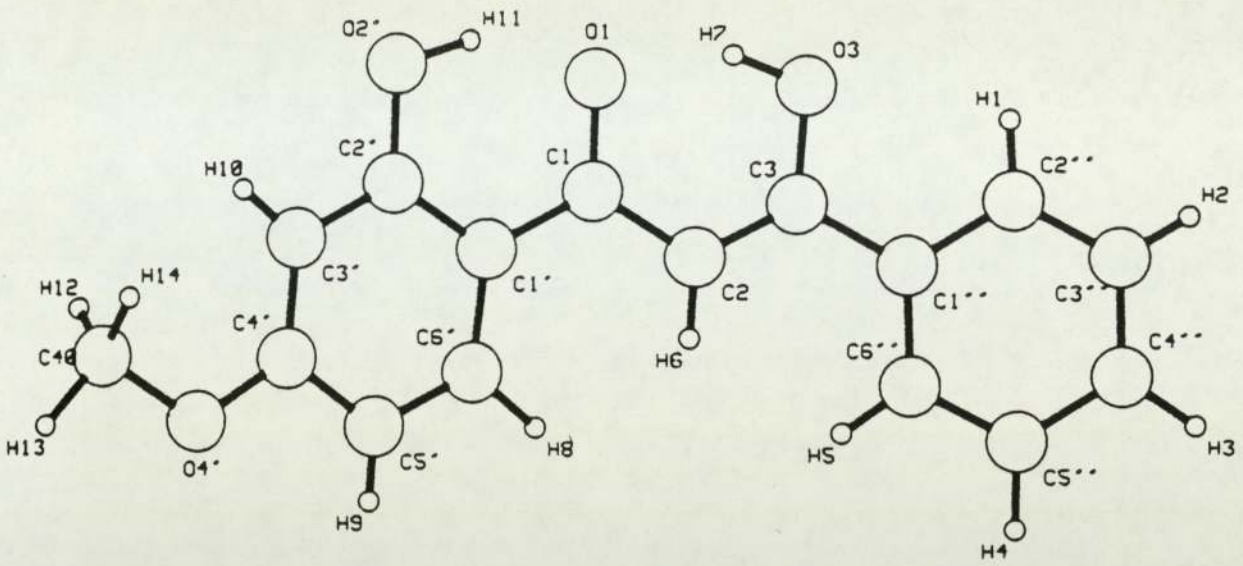
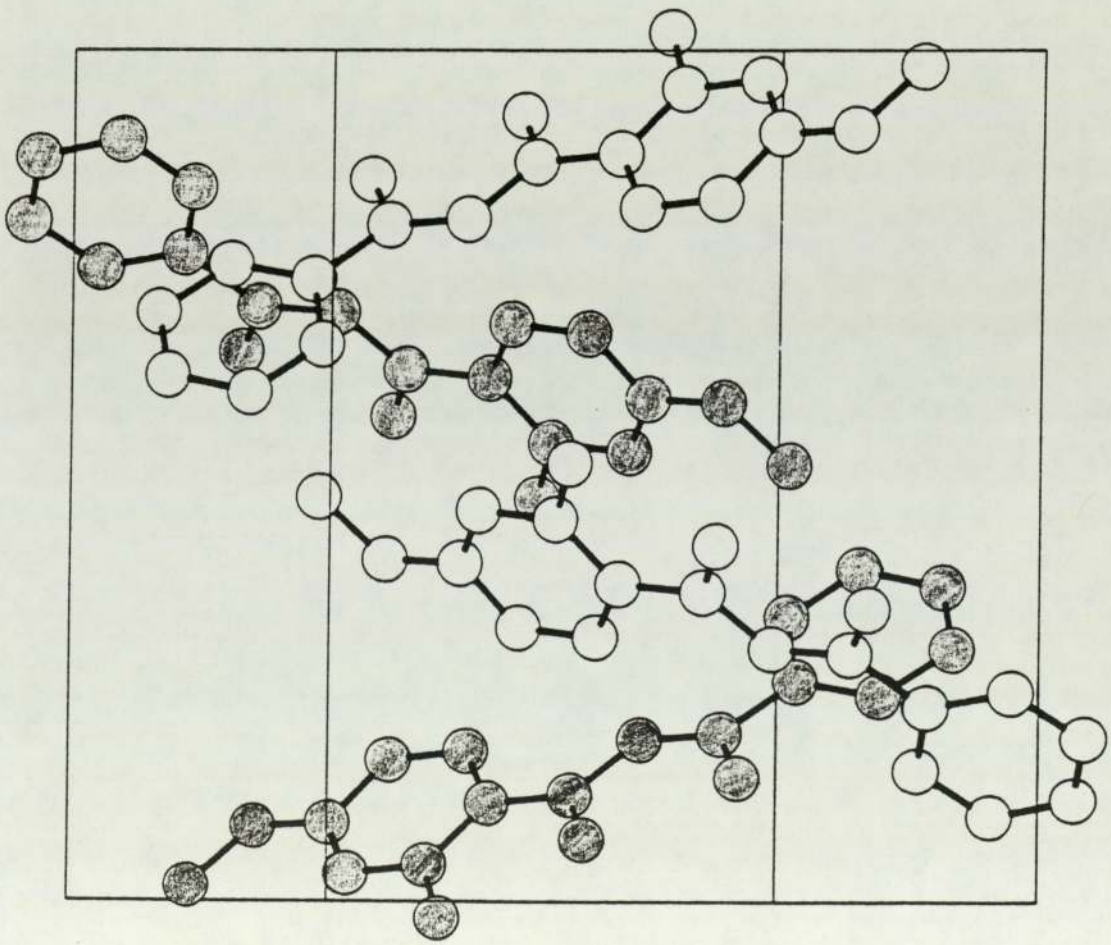


Figure 3.4b. The molecular packing of 29



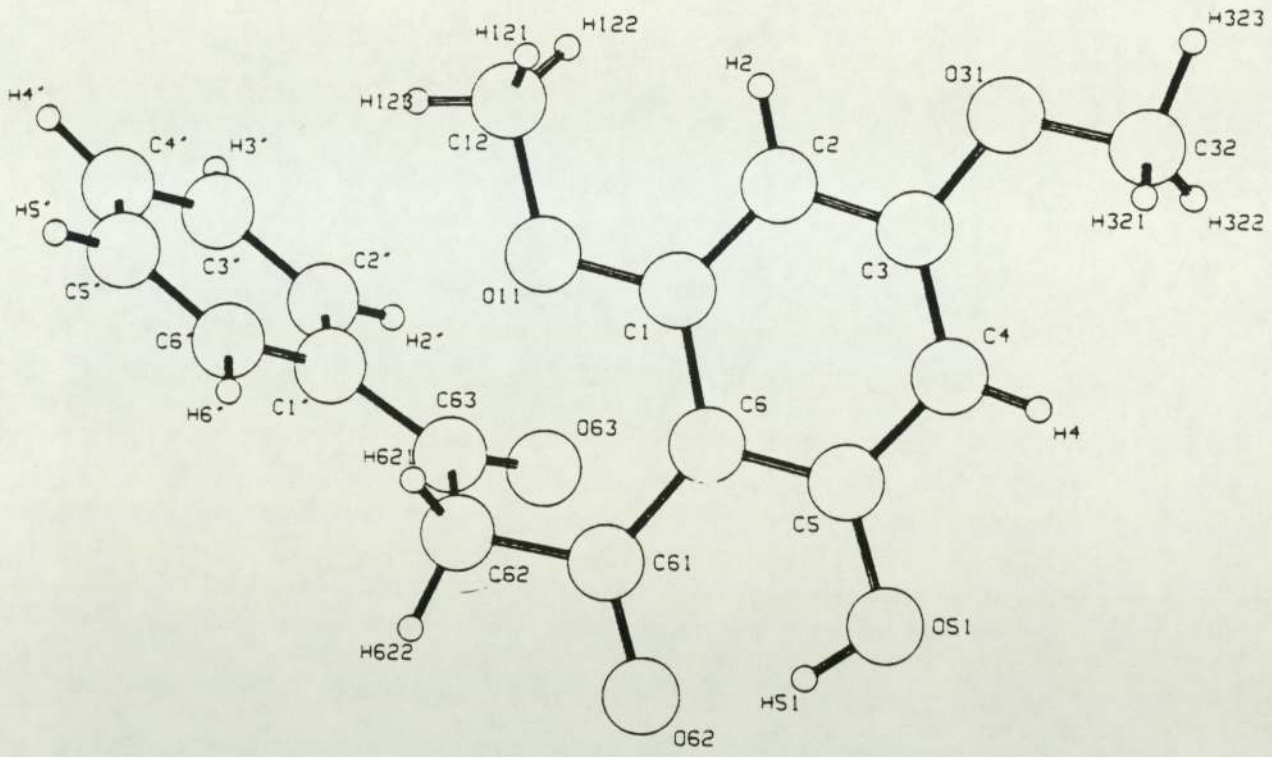
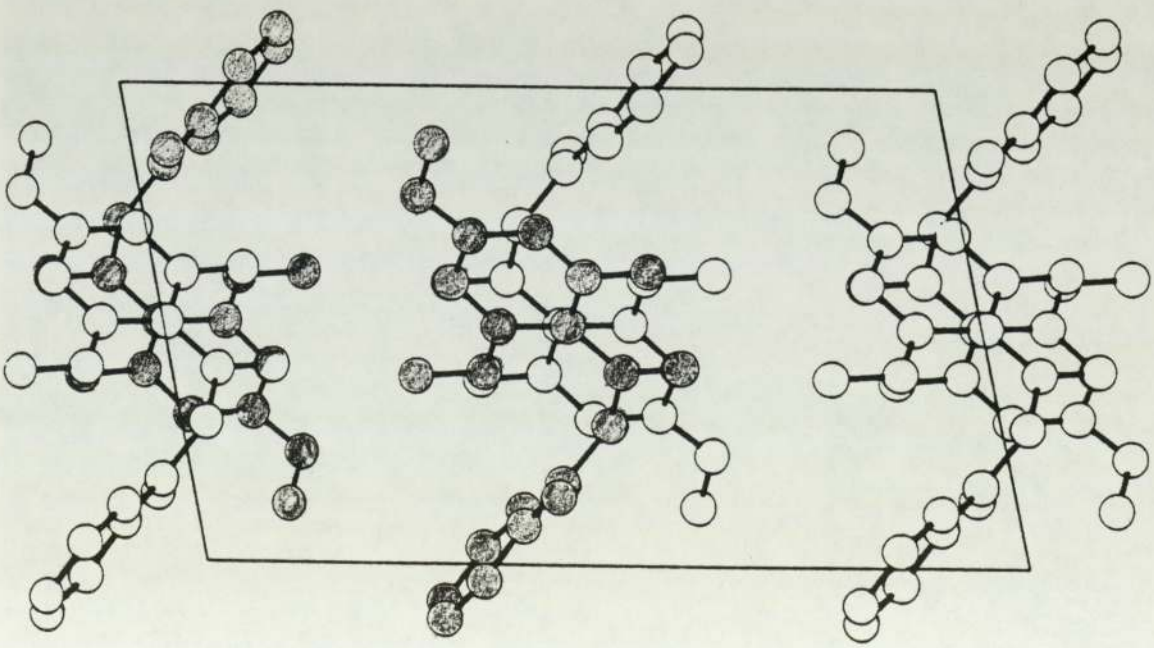


Figure 3.4a. The crystal structure of **28**

Figure 3.4b. The molecular packing of **28**



Enolisation of these compounds may be an effect of dissolution. This hypothesis could be tested by solid-state NMR or by X-ray crystallography. Unfortunately, when these compounds were recrystallised fine yellow amorphous powders were usually obtained. However, when 1-(2-hydroxy-4-methoxyphenyl)-3-phenylpropan-1,3-dione (29) was recrystallised from methanol large transparent brown crystals were formed which proved suitable for X-ray crystallography.

The NMR spectrum of this compound in solution in $CDCl_3$ showed greater than 90% enol form, as shown in Fig. 3.2.

The enol form of this compound can be written as two different tautomers, as shown in Fig.3.1. The crystal structure of this compound would not only clarify whether the compound existed in the enol form in solid state but also which tautomeric form the enolised compound took.

An analogue of **29**, 1-(2-hydroxy-4,6-dimethoxyphenyl)-3-phenylpropan-1,3-dione (**28**), exhibited no enolisation in the NMR spectrum, see Fig 3.3.

When this compound was recrystallised from methanol large transparent yellow hexagons were formed and these, too, proved suitable for crystallography.

The crystal structures of these compounds are shown in

Fig. 3.4. (29) and 3.5. (28).

Experimental

The propan-1,3-diones were synthesised as described in Chapter Seven. Well formed needle-like crystals of 1-(2-hydroxy-4-methoxyphenyl)-3-phenylpropan-1,3-dione were grown by recrystallisation from methanol; hexagonal plates of 1-(2-hydroxy-4,6-dimethoxyphenyl)-3-phenylpropan-1,3-dione (28) were also grown by recrystallisation from methanol.

Crystal Data

1-(2-hydroxy-4-methoxyphenyl)-3-phenylpropan-1,3-dione (29)

$C_{16}H_{14}O_4$, M=270.3 Monoclinic, a = 10.622(2), b = 12.795(5), c = 10.748(3), ß = 111.77(1), V = 1356.6 Å^3 (by least squares refinement on diffractometer angles for 25 automatically centred reflections, λ =0.71069Å) space group $P2_1/a$, Z = 4, Dx = 1.321 gml^{-1}, Crystal dimensions = 0.60 x 0.55 x 0.325 mm, μ(Mo-Kα) = 0.57 cm^{-1}.

1-(4,6-dimethoxyphenyl-2-hydroxy)-3-phenylpropan-1,3-dione (28)

$C_{17}H_{16}O_5$, M = 300.3 Monoclinic, a = 9.503(2), b = 9.924(2), c = 16.125(3) Å, ß = 102.22(2)°, V = 1486.3 Å^3 (by least squares refinement on diffractometer angles for 25 automatically centred reflections, λ = 0.71069Å) space group $P2_1/c$, Z = 4, Dx = 1.439gml^{-1}. Crystal dimensions = 0.80 x 0.48 x 0.64 mm,

μ(Mo-Kα)=0.64cm^{-1}.

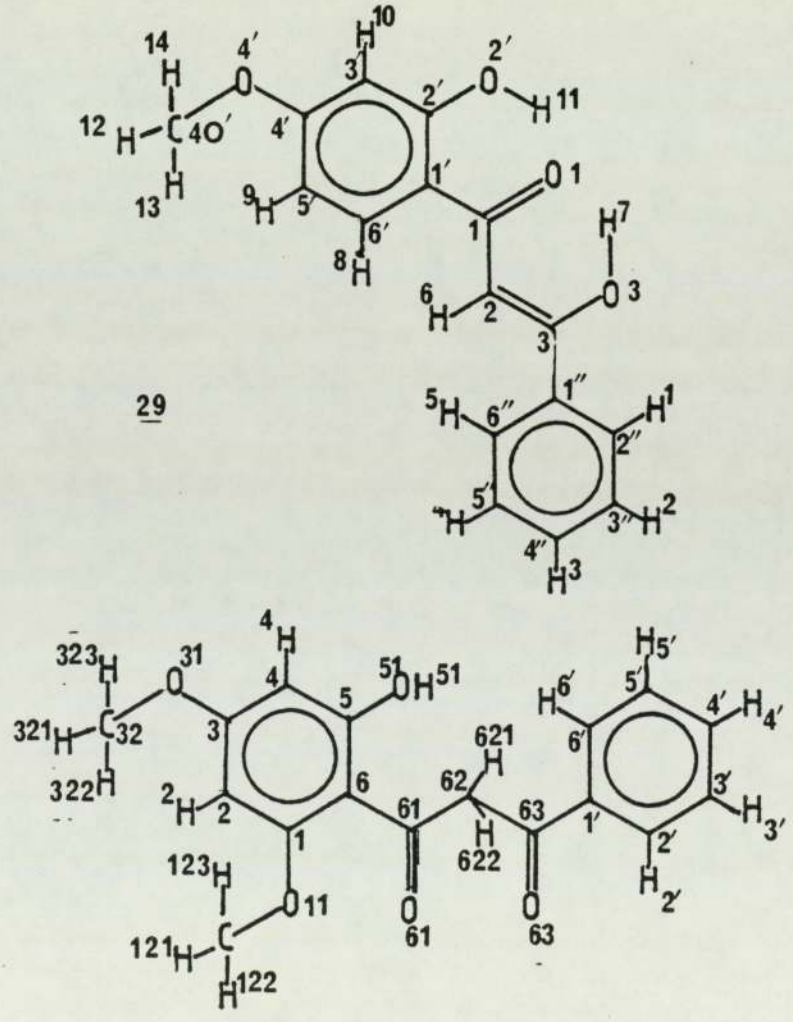
Data Collection and Processing-CAD4 diffractometer, $\omega/2\theta$ mode with scan width = 1.25 + 0.35 tanθ, scan speed 1.25-6.7 deg min^{-1}, graphite-monochromated Mo-Kα radiation. No decomposition or movement of the crystal was detected during data collection and refinement.

Structure Analysis and Refinement:- the structure of 1-(2-hydroxy-4-methoxyphenyl)-3-phenylpropan-1,3-dione was solved by the use of SHELX [239] direct methods; 2 671 unique reflections (merging R = 0.0926) measured between $2° \leq \theta \leq 26°$ for +h, $\pm$k, $\pm$l giving 1 939 with $|F_o| > 3\sigma(F_o)$; all hydrogen atoms were located from a difference electron density map; reflections were weighted according to $W = K/\sigma^2(Fo)$ where K, which should approach unity if there are no serious systematic errors, equalled 2.3867, R = 0.058 and Rw = 0.0571. At termination of refinement no positional parameter shifted by more than 0.006 ESD and the final difference electron-density map showed no feature greater than 0.19 e Å^{-3}.

1-(2-hydroxy-4,6-dimethoxyphenyl)-3-phenylpropan-1,3-dione was solved by the use of MULTAN78 direct methods [240] and refined by the use of SHELX [239] least squares; 2 970 unique reflections (merging R = 0.0254) measured between $2° \leq \theta \leq 26°$ for h, $\pm$k, $\pm$l, giving 2 442 with $|Fo| > 3\sigma(Fo)$; all hydrogen atoms were located from a difference electron density map; reflections were

weighted according to $W = K/\sigma^2(Fo)$ where K equalled 3.195, R = 0.0423 and Rw = 0.0413. At termination of refinement no positional parameter shifted by more than 0.014 ESD and the final difference electron-density map showed no feature greater than 0.20 e $Å^{-3}$.

The numbering schemes are shown in Fig. 3.6. The physical dimensions and parameters of 29 are shown in the Tables 3.1-3.6. and of 28 are shown in tables 3.7-3.12.



29

28

Fig 3.6. Numbering Schemes

TABLE 3.1 Positional Parameters (fractional co-ordinates x10^4) for 1-(2-hydroxy-4-methoxyphenyl)-3-phenylpropan-1,3-dione with ESD in parentheses.

	x	y	z
C(1)	2 766(3)	1 296(2)	3 899(3)
C(2)	2 278(3)	1 960(2)	2 747(3)
C(3)	2 902(3)	2 027(2)	1 862(3)
C(1'')	2 423(3)	2 654(2)	621(3)
C(2'')	2 981(4)	2 483(3)	-353(3)
C(3'')	2 516(4)	3 033(3)	-1 536(4)
C(4'')	1 533(4)	3 764(3)	-1 761(4)
C(5'')	1 001(4)	3 965(3)	- 796(4)
C(6'')	1 444(3)	3 411(3)	388(3)
C(1')	2 050(3)	1 198(2)	4 821(3)
C(2')	2 440(3)	445(2)	5 853(3)
C(3')	1 743(4)	340(3)	6 704(3)
C(4')	660(4)	958(3)	6 567(3)
O(C4')	311(6)	184(5)	8 437(5)
C(5')	255(4)	1 713(3)	5 573(4)
C(6')	937(4)	1 824(3)	4 727(3)
O(1)	3 831(2)	756(2)	4 114(2)
O(3)	4 048(2)	1 499(2)	2 035(2)
O(2')	3 498(2)	-206(2)	6 049(2)
O(4')	96(2)	912(2)	7 350(2)
H(1)	3 614(28)	1 945(21)	-186(26)
H(2)	2 813(39)	2 858(28)	-2 192(38)
H(3)	1 056(35)	4 129(26)	-2 623(36)
H(4)	212(35)	4 422(27)	950(35)
H(5)	1 065(28)	3 574(21)	1 028(28)
H(6)	1 423(30)	2 261(21)	2 595(26)
H(7)	4 209(35)	1 068(26)	2 803(35)
H(8)	669(46)	2 480(33)	4 045(42)
H(9)	-437(40)	2 081(29)	5 472(39)
H(10)	2 112(32)	-98(24)	7 348(32)
H(11)	3 959(42)	73(32)	5 427(40)
H(12)	1 250(40)	404(26)	8 983(35)
H(13)	-501(44)	231(31)	8 849(40)
H(14)	342(51)	-581(40)	8 067(48)

Table 3.2 Bond distances of 1-(2-hydroxy-4-methoxyphenyl)-3-phenylpropan-1,3-dione with ESD in parentheses.

BOND	DISTANCE
C(1)-C(2)	1.431(4)
C(1)-C(1')	1.461(4)
C(1)-O(1)	1.272(3)
C(2)-C(3)	1.349(4)
C(2)-H(6)	0.94(3)
C(3)-C(1'')	1.476(4)
C(3)-O(3)	1.343(3)
C(1')-C(2'')	1.399(4)
C(1'')-(C6'')	1.375(4)
C(2'')-C(3'')	1.374(5)
C(2'')-H(1)	0.93(3)
C(3'')-H(2)	0.90(4)
C(3'')-C(4'')	1.356(5)
C(4'')-H(3)	0.99(4)
C(4'')-C(5'')	1.377(5)
C(5'')-H(4)	0.98(3)
C(5'')-C(6'')	1.378(4)
C(6'')-H(5)	0.94(3)
C(1')-C(2')	1.411(4)
C(1')-C(6')	1.400(4)
C(2')-O(2)	1.351(3)
C(2')-C(3')	1.380(4)
C(3')-H(10)	0.86(3)
C(3')-C(4')	1.359(4)
C(4')-O(4')	1.362(3)
O(4')-C(O4')	1.430(5)
C(O4')-H(12)	0.99(4)
C(O4')-H(13)	1.11(4)
C(O4')-H(14)	1.06(5)
C(4')-C(5')	1.386(5)
C(5')-H(9)	0.85(4)
C(5')-C(6')	1.364(4)
C(6')-H(8)	1.08(4)
O(3)-H(7)	0.95(4)
O(2')-H(11)	1.03(4)

Table 3.3.Interatomic angles for 1-(2-hydroxy-4-methoxy-phenyl)-3-phenylpropan-1,3-dione with ESD in parentheses.

ATOMS	BOND ANGLE
C(1')-C(1)-C(2)	121.7(3)
O(1)-C(1)-C(2)	119.5(2)
O(1)-C(1)-C(1')	118.8(2)
C(3)-C(2)-C(1')	122.2(3)
H(6)-C(2)-C(1)	114(2)
H(6)-C(2)-C(3)	123(2)
C(1'')-C(3)-C(2)	125.3(3)
O(3)-C(3)-C(2)	121.8(3)
O(3)-C(3)-C(1'')	112.9(2)
C(2'')-C(1'')-C(3)	119.5(3)
C(6'')-C(1'')-C(3)	122.1(2)
C(6'')-C(1'')-C(2'')	118.4(3)
C(3'')-C(2'')-C(1'')	120.4(3)
H(1)-C(2'')-C(1'')	117(2)
H(1)-C(2'')-C(3'')	123(2)
C(4'')-C(3'')-C(2'')	120.5(3)
H(2)-C(3'')-C(2'')	119(2.5)
H(2)-C(3'')-C(4'')	120(2.5)
C(5'')-C(4'')-C(3'')	119.9(3)
H(3)-C(4'')-C(3'')	126(2)
H(3)-C(4'')-C(5'')	114(2)
C(6'')-C(5'')-C(4'')	120.3(4)
H(4)-C(5'')-C(4'')	123(2)
H(4)-C(5'')-C(6'')	116(2)
C(5'')-C(6'')-C(1'')	120.4(3)
H(5)-C(6'')-C(1'')	122(2)
H(5)-C(6'')-C(5'')	118(2)
C(2')-C(1')-C(1)	121.1(3)
C(6')-C(1')-C(1)	122.7(3)
C(6')-C(1')-C(2')	116.2(2)
C(3')-C(2')-C(1')	120.9(3)
O(2')-C(2')-C(1')	121.9(2)
O(2')-C(2')-C(3')	117.2(3)
C(4')-C(3')-C(2')	120.9(3)
H(10)-C(3')-C(2')	113.5(2)
H(10)-C(3')-C(4')	125(2)
C(5')-C(4')-C(3')	119.8(3)
O(4')-C(4')-C(3')	125.3(3)
O(4')-C(4')-C(5')	114.9(3)
H(12)-C(O(4'))-O(4')	103(2)
H(13)-C(O(4'))-H(12)	121(3)
H(14)-C(O(4'))-O(4')	110(3)
H(14)-C(O(4'))-H(12)	109(4)
H(14)-C(O(4'))-H(13)	110(4)
C(6')-C(5')-C(4')	119.8(3)
H(9)-C(5')-C(4')	120(3)

Table 3.3.Interatomic angles for 1-(2-hydroxy-4-methoxy-phenyl)-3-phenylpropan-1,3-dione with ESD in parentheses (cont...)

ATOMS	BOND ANGLE
H(9)-C(5')-C(6')	121(3)
C(5')-C(6')-C(1')	122.4(3)
H(8)-C(6')-C(1')	120(2)
H(8)-C(6')-C(5')	118(2)
H(7)-O(3)-C(3)	106(2)
H(11)-O(2')-C(2')	105(2)
C(O(4'))-O(4')-C(4')	117.7(3)

Table 3.4 Hydrogen bond contact distances and angles for 1-(2-hydroxy-4-methoxyphenyl)-3-phenylpropan-1,3-dione.

HYDROGEN BOND	ANGLE AT H(°)	O·····O DISTANCE (Å)
O(3)-H(7)···O(1)	151	2.517
O(2')-H(11)··O(1)	148	2.552

Table 3.5. Deviations of non-hydrogen atoms from the least squares plane through the C' phenyl ring of 1-(2-hydroxy-4-methoxyphenyl)-3-phenylpropan-1,3-dione. (Atoms used in the defining plane are marked with an asterisk).

ATOMS	DEVIATIONS
*C(1')	-0.0036
*C(2')	0.0014
*C(3')	0.0030
*C(4')	-0.0050
*C(5')	0.0026
*C(6')	0.0017
C(1)	-0.0366
C(2)	-0.2163
C(O(4'))	0.0503
O(1)	0.0659
O(3)	-0.1692
O(2')	-0.0021
O(4')	-0.0096

The equation for the plane is given by :-

$$0.6114x + 0.6643y + 0.4299z + 4.1395 = 0.$$

Table 3.6 Torsion Angles for 1-(2-hydroxy-4-methoxyphenyl)-3-phenylpropan-1,3-dione.

ATOMS	ANGLE
C(1')-C(1)-C(2)-C(3)	-177.5
O(1)-C(1)-C(2)-C(3)	0.8
C(2)-C(1)-C(1')-C(2')	171.8
C(2)-C(1)-C(1')-C(6')	-7.1
O(1)-C(1)-C(1')-C(2')	-6.5
O(1)-C(1)-C(1')-C(6')	174.6
C(1)-C(2)-C(3)-C(1'')	176.9
C(1)-C(2)-C(3)-O(3)	-2.2
C(2)-C(3)-C(1'')-C(2'')	-165.9
C(2)-C(3)-C(1'')-C(6'')	14.4
O(3)-C(3)-C(1'')-C(2'')	13.4
O(3)-C(3)-C(1'')-C(6'')	-166.4
C(3)-C(1'')-C(2'')-C(3'')	177.7
C(6'')-C(1'')-C(2'')-C(3'')	-2.5
C(3)-C(1'')-C(6'')-C(5'')	-178.5
C(2'')-C(1'')-C(6'')-C(5'')	1.7
C(1'')-C(2'')-C(3'')-C(4'')	1.5
C(2'')-C(3'')-C(4'')-C(5'')	0.4
C(3'')-C(4'')-C(5'')-C(6'')	-1.2
C(4'')-C(5'')-C(6'')-C(1'')	0.1
C(1)-C(1')-C(2')-C(3')	-178.6
C(1)-C(1')-C(2')-O(2')	1.1
C(6')-C(1')-C(2')-C(3')	0.4
C(6')-C(1')-C(2')-O(2')	-180.0
C(1)-C(1')-C(6')-C(5')	178.5
C(2')-C(1')-C(6')-C(5')	-0.4
O(2')-C(2')-C(3')-C(4')	-1.79.4
C(2')-C(3')-C(4')-C(5')	-0.8
C(2')-C(3')-C(4')-O(4')	179.7
C(3')-C(4')-C(5')-C(6')	0.8
O(4')-C(4')-C(5')-C(6')	-179.7
C(3')-C(4')-O(4')-C(O(4'))	2.5
C(5')-C(4')-O(4')-C(O(4'))	-176.9
C(4')-C(5')-C(6')-C(1')	-0.1

Table 3.7. Positional parameters (fractional co-ordinates x10^4) o 1-(2-hydroxy-4,6-dimethoxyphenyl)-3-phenylpropan-1,3-dione with ESD in parentheses.

	x	y	z
C(1)	5 031(2)	2 920(1)	5 792(1)
C(2)	4 117(2)	2 418(2)	6 266(1)
C(3)	3 137(2)	1 411(1)	5 934(1)
C(4)	3 047(2)	912(2)	5 127(1)
C(5)	3 978(2)	1 424(1)	4 649(1)
C(6)	5 003(2)	2 442(1)	4 952(1)
C(61)	5 964(2)	2 902(2)	4 420(1)
C(62)	7 085(2)	3 977(2)	4 690(1)
C(63)	8 313(2)	3 508(2)	5 386(1)
C(1')	9 195(2)	4 514(1)	5 957(1)
C(2')	10 356(2)	4 064(2)	6 566(1)
C(3')	11 149(2)	4 966(2)	7 135(1)
C(4')	10 789(2)	6 307(2)	7 100(1)
C(5')	9 656(2)	6 764(2)	6 498(1)
C(6')	8 870(2)	5 884(2)	5 922(1)
O(11)	5 984(1)	3 923(1)	6 075(1)
C(12)	6 081(3)	4 428(2)	6 917(1)
O(31)	2 306(1)	996(1)	6 472(1)
C(32)	1 224(2)	5(2)	6 185(1)
O(51)	3 857(1)	902(1)	3 866(1)
O(62)	5 878(1)	2 414(1)	3 697(1)
O(63)	8 587(1)	2 314(1)	5 460(1)
H(2)	4 161(17)	2 727(16)	6 810(11)
H(4)	2 402(19)	237(17)	4 880(11)
H(12A)	5 152(24)	4 790(19)	6 973(13)
H(12B)	6 400(21)	3 667(19)	7 332(12)
H(12C)	6 808(20)	5 101(18)	6 986(11)
H(32A)	478(23)	382(20)	5 747(14)
H(32B)	1 646(22)	-802(21)	6 040(13)
H(32C)	771(25)	-139(21)	6 713(16)
H(62A)	6 641(17)	4 785(16)	4 843(10)
H(62B)	7 487(18)	4 193(16)	4 207(11)
H(O51)	4 545(27)	1 358(23)	3 659(14)
H(C2')	10 587(20)	3 118(18)	6 591(11)
H(C3')	11 898(22)	4 629(18)	7 530(13)
H(C4')	11 323(22)	6 922(20)	7 536(14)
H(C5')	9 385(22)	7 736(21)	6 470(12)
H(C6')	8 094(20)	6 228(18)	5 474(11)

Table 3.8 Bond distances of 1-(2-hydroxy-4,6-dimethoxyohenyl)-3-phenylpropan-1,3-dione with ESD in parentheses.

ATOMS	DISTANCES
C(1)-C(2)	1.368(2)
C(1)-C(6)	1.429(2)
C(1)-O(11)	1.358(2)
C(2)-C(3)	1.393(2)
C(3)-C(4)	1.378(2)
O(31)-C(3)	1.355(2)
C(4)-C(5)	1.387(2)
C(5)-C(6)	1.417(2)
C(5)-O(51)	1.348(2)
C(6)-C(61)	1.453(2)
C(61)-C(62)	1.506(2)
C(61)-O(62)	1.249(2)
C(62)-C(63)	1.510(2)
C(63)-C(1')	1.490(2)
C(63)-O(63)	1.213(2)
C(1')-C(2')	1.386(2)
C(1')-C(6')	1.393(2)
C(2')-C(3')	1.386(3)
C(3')-C(4')	1.373(3)
C(4')-C(5')	1.365(3)
C(5')-C(6')	1.374(3)
C(12)-O(11)	1.432(2)
O(31)-C(32)	1.427(2)
C(2)-H(2)	0.92(2)
C(4)-H(4)	0.94(2)
C(62)-H(62A)	0.90(2)
C(62)-H(62B)	0.96(2)
C(2')-H(C2')	0.96(2)
C(3')-H(C3')	0.91(2)
C(4')-H(C4')	0.99(2)
C(5')-H(C5')	1.00(2)
C(6')-H(C6')	0.98(2)
C(12)-H(12A)	0.98(2)
C(12)-H(12B)	1.00(2)
C(12)-H(12C)	0.95(2)
C(32)-H(32A)	0.96(2)
C(32)-H(32B)	0.95(2)
C(32)-H(32C)	1.040(3)
O(51)-H(O51)	0.92(3)

Table 3.9. Interatomic angles for 1-(2-hydroxy-4,6-dimethoxy-phenyl)-3-phenylpropan-1,3-dione.

ATOMS	BOND ANGLE(°)
C(6)-C(1)-C(2)	121.5(1)
O(11)-C(1)-C(2)	122.4(1)
O(11)-C(1)-C(6)	116.1(1)
C(3)-C(2)-C(1)	119.9(1)
C(4)-C(3)-C(2)	121.6(1)
O(31)-C(3)-C(2)	113.9(1)
O(31)-C(3)-C(4)	124.5(1)
C(5)-C(4)-C(3)	118.3(2)
C(6)-C(5)-C(4)	122.9(1)
O(51)-C(5)-C(4)	116.2(1)
O(51)-C(5)-C(6)	120.9(1)
C(5)-C(6)-C(1)	115.9(1)
C(61)-C(6)-C(1)	124.6(1)
C(61)-C(6)-C(5)	119.5(1)
C(62)-C(61)-C(6)	123.1(1)
O(62)-C(61)-C(6)	120.3(1)
O(62)-C(61)-C(62)	116.6(1)
C(63)-C(62)-C(61)	112.1(1)
C(1')-C(63)-C(62)	119.9(1)
O(63)-C(63)-C(62)	119.4(1)
O(63)-C(63)-C(1')	120.8(1)
C(2')-C(1')-C(63)	118.8(1)
C(6')-C(1')-C(63)	122.6(1)
C(6')-C(1')-C(2')	118.6(2)
C(3')-C(2')-C(1')	120.1(2)
C(4')-C(3')-C(2')	120.2(2)
C(5')-C(4')-C(3')	120.2(2)
C(6')-C(5')-C(4')	120.2(2)
C(5')-C(6')-C(1')	120.6(2)
C(12)-O(11)-C(1)	118.7(1)
C(32)-O(31)-C(3)	118.6(1)
H(2)-C(2)-C(1)	120(1)
H(2)-C(2)-C(3)	120(1)
H(4)-C(4)-C(3)	124(1)
H(4)-C(4)-C(5)	118(1)
H(62A)-C(62)-C(61)	110(1)
H(62A)-C(62)-C(63)	112(1)
H(62B)-C(62)-C(61)	108(1)
H(62B)-C(62)-C(63)	107(1)
H(62B)-C(62)-H(62A)	107(1)
H(2')-C(2')-C(1')	119(1)
H(2')-C(2')-C(3')	121(1)
H(4')-C(4')-C(3')	119(1)
H(4')-C(4')-C(5')	121(1)

Table 3.9. Interatomic angles for 1-(2-hydroxy-4,6-dimethoxy-phenyl)-3-phenylpropan-1,3-dione (cont...)

ATOMS	BOND ANGLE(°)
H(5')-C(5')-C(4')	121(1)
H(5')-C(5')-C(6')	119(1)
H(6')-C(6')-C(1')	120(1)
H(6')-C(6')-C(5')	120(1)
H(12A)-C(12)-O(11)	110(1)
H(12B)-C(12)-O(11)	108(1)
H(12B)-C(12)-H(12A)	111(2)
H(12C)-C(12)-H(12B)	110(2)
H(32A)-C(32)-O(31)	110(1)
H(32B)-C(32)-O(31)	110(1)
H(32B)-C(32)-H(32A)	116(2)
H(32C)-C(32)-O(31)	103(1)
H(O51)-O(51)-C(5)	103(1)

Table 3.10 Hydrogen bond contact distance and angle for 1-(2-hydroxy-4,6-dimethoxyphenyl)-3-phenylpropan-1,3-dione.

HYDROGEN ATOM	ANGLE AT H (°)	O-O DISTANCE Å
O(51)-H(51)····O(62)	155.6	2.497

Table 3.11.Deviations of non-hydrogen atoms from the least-squares plane through the C1 phenyl ring system. (The atoms used in the plane calculation are marked with an *)

ATOM	DEVIATION(Å)
*C(1)	0.0004
*C(2)	0.0025
*C(3)	-0.0046
*C(4)	0.0038
*C(5)	-0.0009
*C(6)	-0.0011
C(61)	-0.0383
C(62)	-0.0425
C(63)	-1.3408
C(1')	0.0381
C(2')	0.0168
C(3')	-0.0131
C(4')	0.0323
C(5')	0.0011
C(6')	-0.0556
O(11)	-2.3580

The equation for the plane is given by:-

$-0.6745x + 0.7005y - 0.2329z - 3.0610 = 0.$

Deviation of non-hydrogen atoms from the least squares plane through the C1' phenyl ring.

ATOM	DEVIATION (Å)
*C(1')	0.0094
*C(2')	-0.0022
*C(3')	-0.0047
*C(4')	0.0044
*C(5')	0.0030
*C(6')	-0.0099
C(61)	0.6824
C(62)	0.0409
C(63)	0.0919
H(2)	-0.0652

The equation for the plane is given by :-

$-0.6130x - 0.1558y + 0.7746z + 0.0652 = 0.$

Table. 3.12. Torsion angles for 1-(2-hydroxy-4,6-dimethoxy-phenyl)-3-phenylpropan-1,3-dione

ATOMS	ANGLE(°)
C(6)-C(1)-C(2)-C(3)	0.4
O(11)-C(1)-C(2)-C(3)	178.4
C(2)-C(1)-C(6)-C(5)	0.0
C(2)-C(1)-C(6)-C(61)	-178.3
O(11)-C(1)-C(6)-C(5)	-178.2
O(11)-C(1)-C(6)-C(61)	3.5
C(2)-C(1)-O(11)-C(12)	3.0
C(6)-C(1)-O(11)-C(12)	-178.9
C(1)-C(2)-C(3)-C(4)	-.9
C(1)-C(2)-C(3)-O(31)	179.5
C(2)-C(3)-C(4)-C(5)	1.0
O(31)-C(3)-C(4)-C(5)	-179.4
C(2)-C(3)-O(31)-C(32)	177.6
C(4)-C(3)-O(31)-C(32)	-2.1
C(3)-C(4)-C(5)-C(6)	-.6
C(3)-C(4)-C(5)-O(51)	179.7
C(4)-C(5)-C(6)-C(1)	.2
C(4)-C(5)-C(6)-C(61)	178.5
O(51)-C(5)-C(6)-C(1)	-179.9
C(5)-C(6)-C(61)-O(62)	.8
C(6)-C(61)-C(62)-C(63)	70.1
O(62)-C(61)-C(62)-C(63)	-110.5
C(62)-C(62)-C(63)-C(1')	-156.4
C(61)-C(62)-C(63)-O(63)	25.3
C(62)-C(63)-C(1')-C(2')	-177.1
C(62)-C(63)-C(1')-C(6')	5.1
O(63)-C(63)-C(1')-C(2')	1.2
O(63)-C(63)-C(1')-C(6')	-176.5
C(63)-C(1')-C(2')-C(3')	-176.5
C(6')-C(1')-C(2')-C(3')	1.3
C(63)-C(1')-C(6')-C(5')	175.6
C(2')-C(1')-C(6')-C(5')	-2.1
C(1')-C(2')-C(3')-C(4')	.0
C(2')-C(3')-C(4')-C(5')	-.6
C(3')-C(4')-C(5')-C(6')	-.1
C(4')-C(5')-C(6')-C(1')	1.5

The crystal structures confirm that 29 exists in the enol form in solid state and that 28 exists in the keto form.

The enol system in 29 is planar and the precise tautomeric form can be elucidated from the relative bond lengths:-

BOND	DISTANCE
C1'-C1	1.461±4
C1-C2	1.431±4
C2-C3	1.349±4

The C1'-C1 bond is not a true C-C single bond; in paraffinic compounds a C-C single bond is 1.541±3Å [235]. The shortening of this bond is due to the conjugation of the carbonyl double bond with the phenyl ring. This may also explain why the C1-C2 bond is shortened since the enol system shows extensive conjugation. The C2-C3 bond is comparable with a true C-C double bond; 1.337±6Å is the accepted international value for this bond [235]. Further evidence to support this tautomeric form is given by the C-O double bond and the C-O single bond lengths:-

BOND	DISTANCE
C1-O1	1.273±3
C3-O3	1.343±3

The C-O double bond (C1-O1) is slightly longer than expected for a ketonic C-O double bond. This may be attributable to an electronic effect of the H-bonding from the phenolic OH to the carbonyl oxygen. The C-O single bond is

comparable to the value of a shortened C-O single bond (for instance in a carboxylic acid) of 1.36±1Å [235].

It is interesting to note, however, that the phenyl ring attached to the 3-C is not co-planar with the enol system, but at an angle of 14°. While it might initially be thought that strict co-planarity would improve conjugation of the π-bonds of the enol system with the π-clouds of the phenyl ring, consideration of the interatomic distances between the vinyl proton and the aromatic protons *ortho* to the enol system explain this effect. Even at an angle of 14° the contact distance between H6 (the vinyl proton) and H5 (one of the *ortho* protons) is 2.308Å. If the phenyl ring were co-planar with the enol system then these protons would be unacceptably close.

Figure 3.4b. shows the molecular packing in the unit cell. It appears that there is parallel stacking of the phenyl rings, although interpretation of the packing of the molecule from this diagram is limited.

In contrast to the enol system seen in **29**, the crystal structure of **28** shows no such tautomerism, although the carbonyl oxygen atom *ortho* to the phenolic OH is H-bonded to that OH. The C-C single bond lengths of the propan-1,3-dione system are similar to those in **29** although they are not identical.

BOND in 29	DISTANCE Å	BOND in 28	DISTANCE Å
C1'-C1	1.461±4	C6-C61	1.453±2
C1-C2	1.431±4	C61-C62	1.506±2
C3-C1''	1.476±4	C63-C1'	1.490±2

Again these bonds are not true C-C single bonds, but this could be due to the conjugation of the phenolic OH to the carbonyl oxygen of C61. The C62-C63 bond length is 1.510±2, this is significantly longer than the other C-C bond lengths in the propan-1,3-dione system, especially when compared to the C61-C62 bond. Why this should be so is obscure.

The C61-O62 bond length in **28** is comparable to the C1-O1 bond length in **29**, 1.249±2 Å and 1.272±3 Å respectively. The C63-O63 bond length is slightly shorter than expected , 1.213±2 Å compared to 1.23±1 for a ketonic C-O double bond [235]. This may be due to conjugation of the carbonyl with the phenyl ring, a shortened C-O double bond in a conjugated system having a value of 1.207±6 Å in international tables.

Figure 3.5b. shows the molecular packing of **28**. Again parrallel stacking of phenyl rings is evident. The atoms in one of the molecules have been shaded to facilitate interpretation of this diagram.

The 400MHz NMR spectrum of 1-(2-hydroxy-4,6-dimethoxy-phenyl)-3-phenyl propan-1,3-dione did not prove facile to interpret. The spectrum is shown in Fig.3.4. The difference in the chemical shifts of the two O-methyl groups at first

appears surprising since their environments initially appear to be similar, and comparing the chemical shifts of the two methyl groups in the propan-1,3-dione to those in the acetophenone show that there is indeed an unusual difference in the chemical shifts:-

COMPOUND	CHEMICAL SHIFT OF OMe GROUPS	
2-benzoyloxy-4,6-dimethoxy-acetophenone	3.73	3.75
1-(2-hydroxy-4,5-dimethoxy-phenyl)-3-phenylpropan-1,3-dione	3.79	3.43

It appears that one of the O-methyl groups is being shielded and this causes it to resonate further upfield than the other O-methyl group. Contemplation of the crystal structure and data supplies a reasonable explanation for this effect. Since this compound does not exhibit any keto-enol tautomerism the molecule is not constrained in a planar form about the propan-1,3-dione system. The O-methyl group on the in the 6-position lies very close to the phenyl ring attached to the 3-C of the propan-1,3-dione system. There is a contact distance of 2.969Å between one of the protons of the O-methyl group (H123(C)) and C6' of the phenyl ring. This is close enough for the protons of the O-methyl group to be within the pi-cloud of the phenyl ring and so be shielded by it.

These two molecules and their respective structures give an interesting comparison between the keto and enol form of the

1-(2-hydroxyphenyl)-3-phenylpropan-1,3-diones. It is possible that steric hindrance is the reason why **28** exists in the keto form in preference to the enol form. There is a contact distance of 2.018Å between H6 and H8 in **29**, therefore it is reasonable to assume that the size of the O-methyl group in this position would cause so much distortion of the enol system as to make it energetically unfavourable.

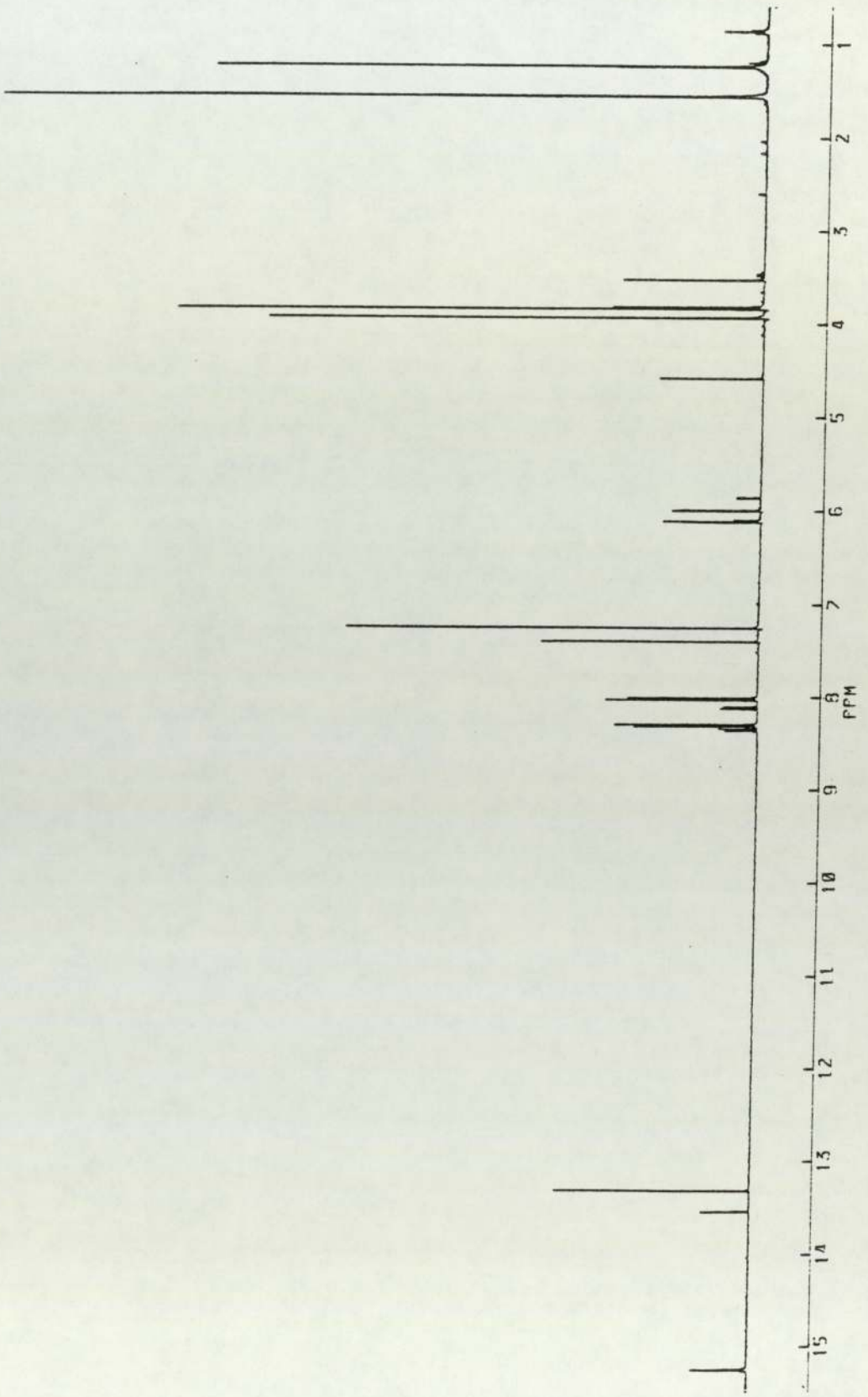
The presence of two strongly electron donating substituents both *ortho* and *para* to the propan-1,3-dione moiety may increase the electron density in this region making the enol tautomer less favourable. Some evidence to support this may be seen in the NMR spectrum of 1-(2-hydroxy-4,6-dimethoxyphenyl)-3-(4-nitrophenyl)-propan-1,3-dione, (**31**).

The 300MHz NMR spectrum of **31** (Figure 3.7.) shows two distinct forms of this compound, both the keto and enol form. The presence of the nitro group in the *para* position of the 3-phenyl ring may stabilise the enol form by pulling electrons out of this system, and so counteracting the effects of the two O-methyl groups to some extent.

The NMR spectrum shows **31** to be 80% enolised, therefore both steric and electronic effects may be the cause of the **28** being seen in the keto form.

The NMR spectra of the majority of the 1,3-diphenylpropan-

Figure 3.7. The 400MHz H^1 NMR spectrum of 1-(4,6-dimethoxy-2-hydroxyphenyl)-3-(4-nitrophenyl)-propan-1,3-dione



1,3-diones were consistent with theoretical predictions. The NMR spectrum of the rearrangement of 2,6-dibenzoyloxy-acetophenone, however, prooved somewhat complex.

As mentioned in Chapter Two, in an attempt to introduce substituents into the 5 and 7 positions of the flavone, 2,6-dihydroxyacetophenone was treated with two equivalents of benzoyl chloride. The diester was rearranged using powdered sodium hydroxide in pyridine, and initially it was expected that the benzoyloxy group in the 6 position would be hydrolysed while that in the 2-position would rearrange. The NMR spectrum proved that this was not the case, since signals from two separate phenyl rings could be assigned, consequently the structure in Fig. 3.8. was proposed.

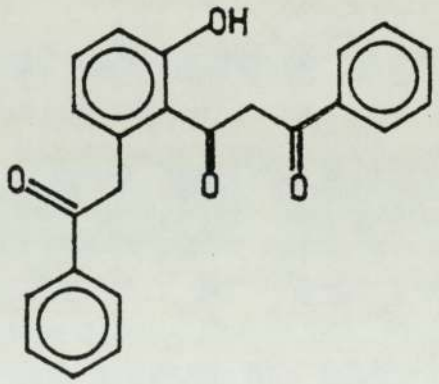


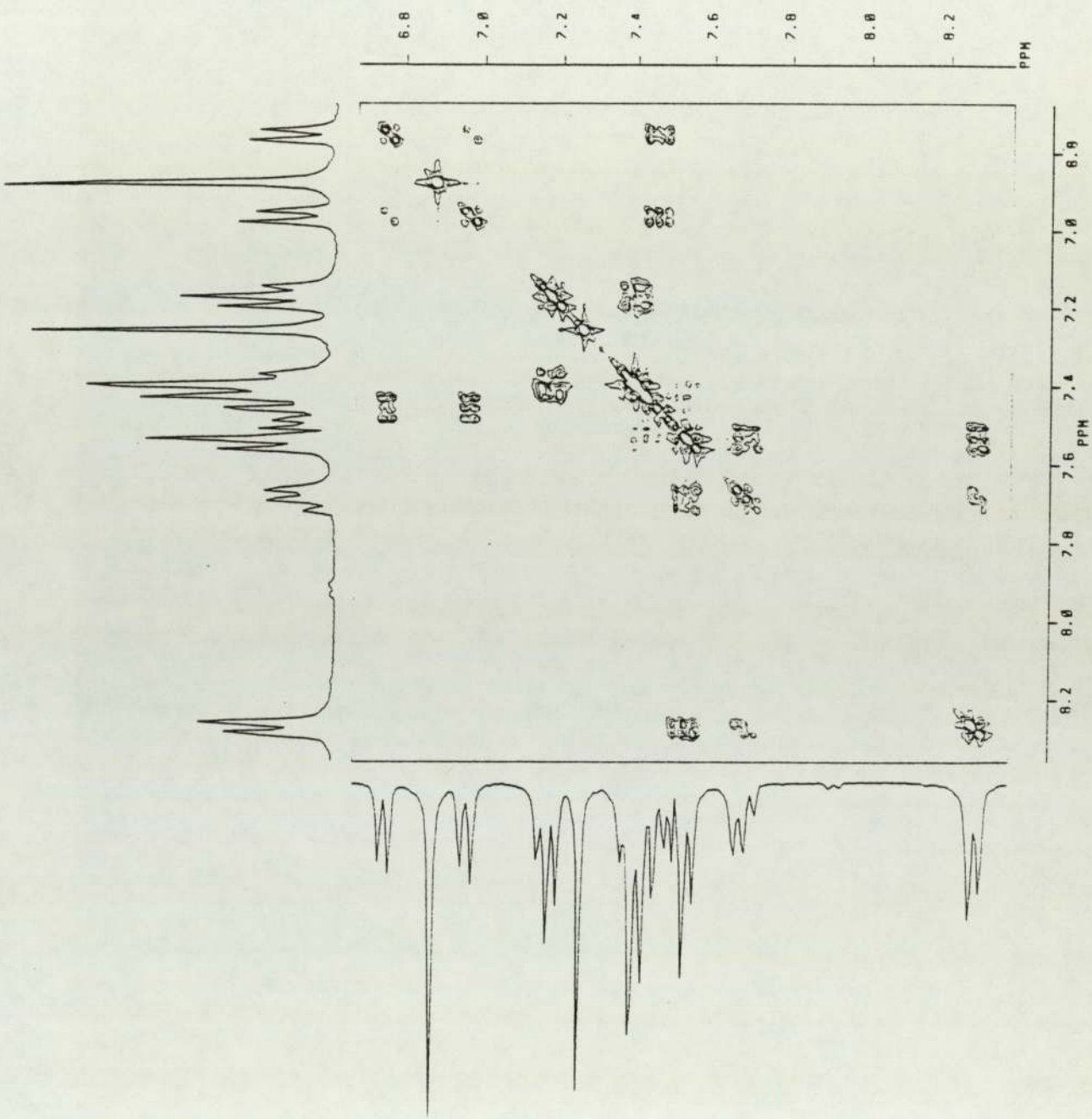
Fig. 3.8. 1-(2-hydroxy-6-benzoyloxyphenyl)-3-phenylpropan-1,3-dione (46)

Since the two phenyl rings appear to be in similar electronic environments, it would be reasonable therefore to predict that the chemical shifts of the protons of the phenyl rings would be similar. This was not confirmed by the 400 MHz NMR spectrum. Comparing this spectrum with that of 1-(2-hydroxyphenyl)-3-phenylpropan-1,3-dione it was found that one set of protons *ortho* to a carbonyl group appeared at 8.28ppm, 0.1 ppm further downfield than expected. This could be explained by the electron withdrawing effect of an additional benzoyloxy group being present *ortho* to the propan-1,3-dione system. However, on further interpretation of the spectrum it appeared that the other set of *ortho* protons is unusually upfield, at 7.4 ppm. The individual aromatic protons could be identified in the spectrum although assigning the protons to their respective phenyl rings could not be done on the basis of coupling constants owing to the ambiguity of the *J* values found.

Correlation spectroscopy, (COSY), of this compound aided the interpretation of the single dimension NMR spectrum of this compound. The COSY of **46** is shown in Fig. 3.9. This spectrum shows that the downfield aromatic protons can all be assigned to the same phenyl ring and so with the upfield aromatic protons.

This still does not allow complete interpretation of the

Figure 3.9. The correlation spectrum of 1-(2-hydroxy-6-benzoyloxyphenyl)-3-phenylpropan-1,3-dione.



spectrum, however, since one of the phenyl rings appears to be in an unusually shielded environment.

The orientation of the phenyl rings in the molecule should explain the shielding of one of the rings seen in the spectrum. There are a number of potential orientations of the two phenyl rings and these are shown in Fig. 3.10.

The first orientation shows the z,z' ring to be at right angles to the enol system and so the z,z'protons may lie in a

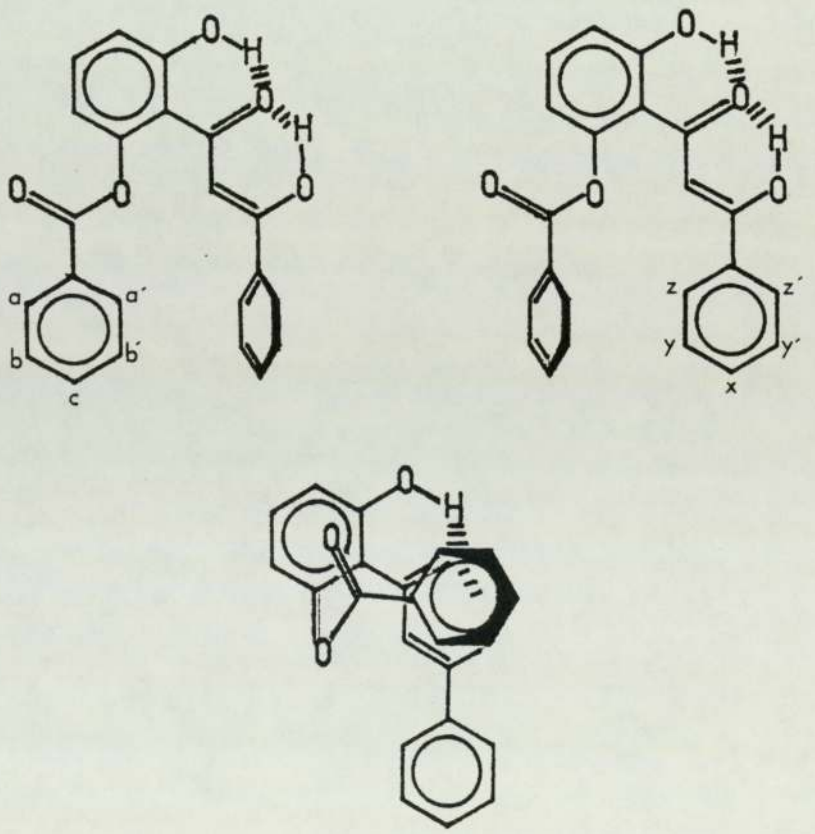


Fig 3.10. The potential orientations of the phenyl rings of 1-(2-hydroxy-6-benzoyloxyphenyl)-3-phenylpropan-1,3-dione (46).

shielding cone produced by the pi-bond of the carbonyl group giving an unusually upfield chemical shift. The second orientation shows the a,a' phenyl ring at right angles to the carbonyl group, again lying the a,a' protons in a shielding cone. The third possible orientation shows the benzoyl group positioned so that the phenyl ring lies over the enol system. In this last orientation the a,a' protons would be shielded by the π-cloud over the enol system.

In order to verify which, if any, of these possibilities is correct a number of NMR experiments were performed. If the z,z' phenyl ring is in plane with the enol system then the z,z' protons would be expected to experience a nuclear Overhauser effect (nOe) from the vinylic proton.

Irradiation of the proton at δ 6.87 did indeed produce an nOe, however both sets of protons *ortho* to the carbonyl groups showed an enhancement although there was a far greater effect on the protons at δ 7.4 compared to that at δ 8.3. This suggests that the protons generating the upfield signal are closer in space to the vinyl protons than the protons generating the downfield signal, although these protons are also close enough to the vinyl proton to experience an nOe.

This experiment rules out one of the potential

orientations of the phenyl rings. If the a,a' phenyl ring were at right angles to the carboxylate system then no nOe would be experienced by the a,a' protons when the vinyl proton was irradiated.

If the z,z' phenyl ring were at right angles to the carbonyl system then no nOe would be expected. If, however, this phenyl ring were co-planar with the enol system then a large nOe should be seen. Should this be the case then the interpretation of the spectrum is complicated still further since this does not explain why these protons should be shielded.

Consideration of the crystal structure of 1-(2-hydroxy-4-methoxyphenyl)-3-phenylpropan-1,3-dione shows that the phenyl ring attached to the 3 carbon is out of plane by 14°, so this may explain why an nOe is seen at both the z,z' and the a,a' protons.

To determine if the a,b,c phenyl ring is orientated in such a way that it lies over the top of the enol system another nOe difference experiment was carried out. This time the enolic OH, at δ 15.5, was irradiated. This, too, produced an ambiguous result. Saturation transfer of energy from the enolic OH to the phenolic OH picked up the protons on the 1 C phenyl ring. However the largest nOe observed was again at the upfield protons, although the downfield protons also appear to

be stimulated but to a much smaller extent.

While these results suggest that the a,b,c phenyl ring may lie in a position in which the protons may be shielded by the π-cloud of the enol system, none of the evidence is conclusive and it would appear that the only possible way of solving this structural problem is by X-ray crystallography.

CHAPTER FOUR

THE BIOLOGICAL EFFECTS OF THE ANALOGUES OF QUERCETIN ON INTACT CELLS

The involvement of tyrosine protein kinases in the initial transformation of certain cells in animals and in tissue culture coupled with their involvement in the maintenence of that malignant phenotype present these enzymes as suitable targets for chemotherapy.

Quercetin was shown to inhibit the transforming gene product of the Rous Sarcoma virus by Graziani *et al* [147] in 1983. However, this is just one of the many activities of this compound. Quercetin inhibits a cAMP independent protein kinase activity in Erlich Ascites tumour cells [146], 3',5'-cAMP phosphodiesterases [148], cytochrome P450 activity in rat liver microsomes [149], Na^+,K^+ ATPase [150], lactate transport in HeLa cells [151],and neutrophil phospholipase A_2 [152],while other flavonoids are known to inhibit lipoxygenase and cyclooxygenase *in vitro* [153], glyoxalase I *in vitro* [154], arachidonate 5-lipoxygenase [155]. Quercetin has also been found to be mutagenic, but not carcinogenic in rats [156]and to be genotoxic in cultured mammalian cells [157] . Quercetin is not therefore the ideal candidate as a potential chemotherapeutic agent.

In the present study a series of analogues of quercetin were prepared in order to investigate any structure activity

relationship there might be in an attempt to design rationally a specific inhibitor of tyrosine protein kinase.

The effect of these compounds on intact cells was investigated using two murine cell lines, 3T3 fibroblasts and ANN-1 cell. The 3T3 cell line is derived from murine fibroblasts and are considered to be "normal" cells, that is they exhibit density dependent growth and will not form colonies. In addition to this, these cells are anchorage dependent in their growth. ANN-1 cells[239] are derived from 3T3 fibroblasts that have been transformed by infection with the Abelson murine leukaemia virus. These cells are anchorage independent, density independent and readily form colonies when grown in medium containing agar. These cell lines present a convenient method for comparing the effects of these compounds on a "normal" cell to the effects on a transformed cell.

A dose response curve was first obtained using ANN-1 cells for quercetin between 10-250μM. This dose response curve is shown in Fig. 4.1. The IC_{50} (that dose which inhibits the growth of treated cells compared to control cells by 50%) from this graph is in the range 40-60μM.

In order to rapidly estimate the potency of those compounds which had been synthesised these analogues were tested at one concentration using ANN-1 cells. The concentration utilised was 50μM, a bar-chart summarising the results of these experiments is shown in Fig 4.2.

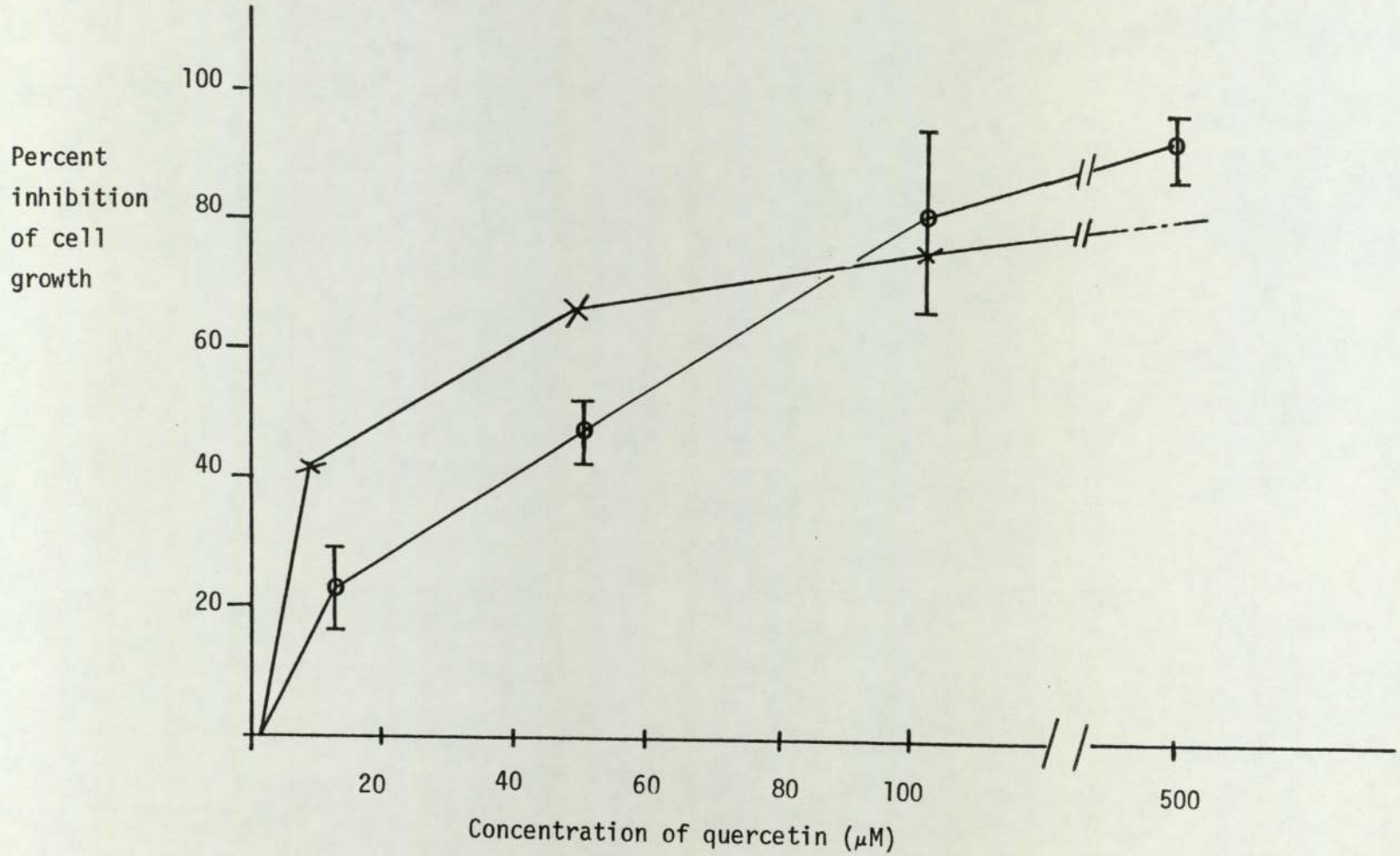


Fig.4.1. The dose response curves for quercetin using ANN-1 cells (**O**) and 3T3 cells (x).

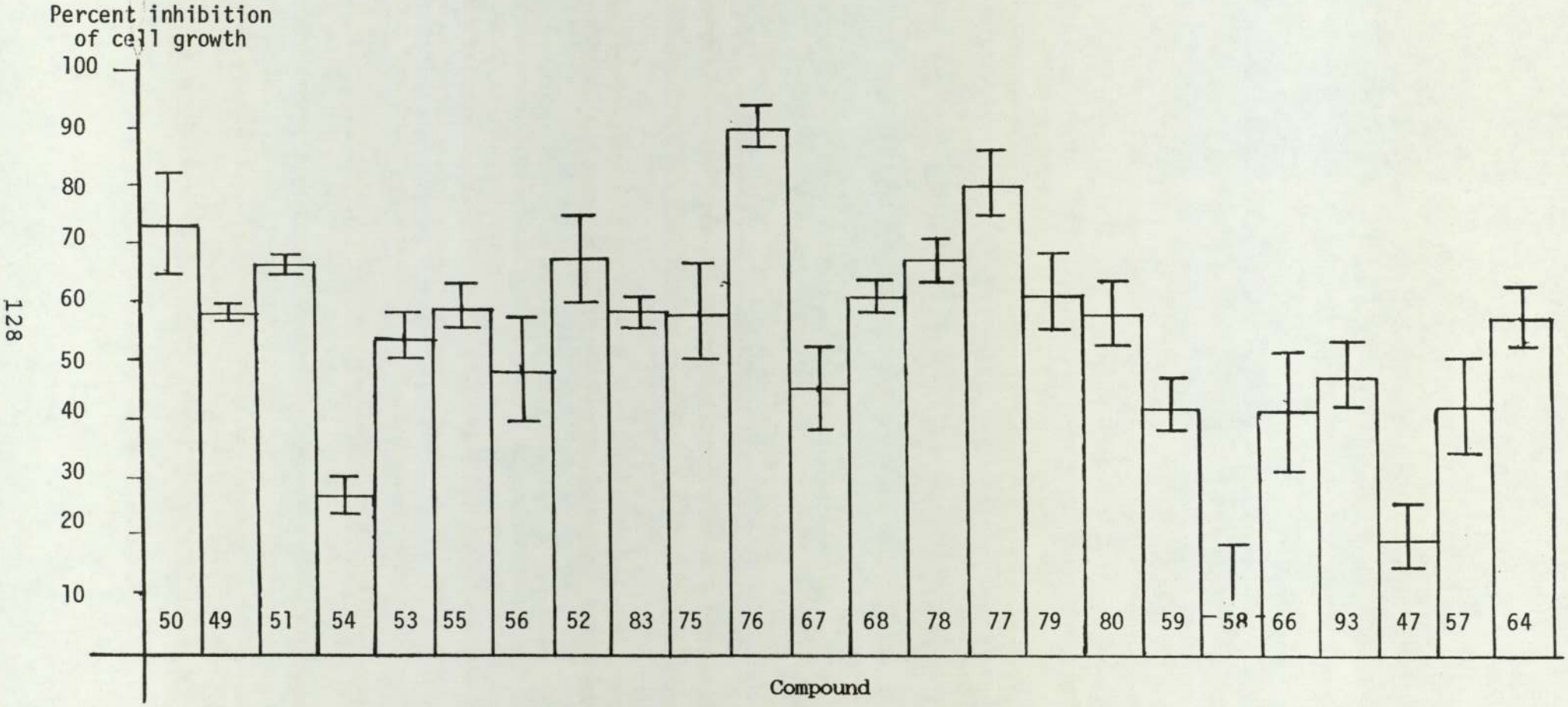


Fig. 4.2. A comparison of the percentage inhibition (± standard deviation) of ANN-1 cell growth by the compounds at a concentration of 50μM.

A range of toxicities was observed from 5% ± 9.8 for 4'-nitroflavone to 90% ± 3 (n=3) for 4'-methoxy-3'-amino-flavone. It is interesting to note that the isomer of 76, 4'-amino-3'-methoxyflavone shows 59% ± 3 (n=3) inhibition of cell growth at this concentration.

Dose response curves of 15 compounds were obtained and the IC_{50} of these compounds (expressed as a range) are shown in Table 4.1.

Table 4.1 The IC_{50} of the analogues of quercetin expressed as a range of concentrations.

Compound No	Substituent(s) and position	$IC_{50}\mu M$	n=
76	4'-MeO-3'-NH_2	0.8-2.5	3
75	4'-NH_2-3'-MeO	20-35	3
50	3'-MeO	18-30	2
55	3',4'-$(Cl)_2$	5-20	2
51	3',4'-$(MeO)_2$	13-30	1
49	4'-MeO	10-25	2
77	4'-NH_2	10-20	2
47	Flavone	100-140	2
82	3-OH	8-15	2
71	3'-OH	28-34	2
69	3',4'-$(OH)_2$	8	1
70	4'-OH	14	1
52	3'-F	25-40	2
57	7-MeO	45	1
64	5,7-$(MeO)_2$	22-30	2

These compounds show a range of toxicities, with IC_{50} which range from 0.8-2.5 μM to 100-140μM. Flavone acetic acid showed no inhibition of the growth of ANN-1 cells, nor did there appear to be any alteration of cell morphology. (Certain

of the compounds proved insoluble at this concentration and so no data were collected. These compounds are 4'-nitro-3'-methoxyflavone, 4'-methoxy-3'-nitroflavone and 4'-dimethyl-amino-3'-nitroflavone).

These results were obtained using a single-cell suspension of ANN-1 cells. Single-cell suspension assays do not discriminate between cytotoxicity and cytostasis. In order to determine if the compounds tested were in fact cytotoxic clonogenic assays were carried out as described in the Materials and Methods section.

ANN-1 cells were grown in medium containing 0.5% agar, and the plating efficiency of the cells under these conditions in this study ranged from 15-40%. (Conditions for maximal plating efficiency were not determined). The results of the clonogenic assays are shown below in Table 4.2

Table 4.2 The clonogenic potentials of ANN-1 cells after treament with quercetin and analogues.

Compound	Conc. μM	Percentage inhibiton of colony formation
Quercetin (93)	1	0
	10	90
	50	95
Flavone (47)	1	-
	10	0
	50	90
5,7-$(MeO)_2$- (51)	0.1	24
	10	48
	50	82
3'-Fluoro- (52)	0.1	0
	5	0
	50	33
7-MeO- (57)	0.1	0
	10	0
	50	0

It would appear from these results that quercetin, flavone and 5,7-dimethoxyflavone are cytotoxic, their ICs_{50} are lower than the results obtained from the single cell suspension assays:

Compound	IC_{50} for single cell susp. assay μM	IC_{50} for clonogenic assay. μM
Quercetin	40-50	5
Flavone	100-140	30
5,7-$(MeO)_2$	22-30	12

7-methoxyflavone and 3'-fluoroflavone appear to be cytostatic only, in the clonogenic assay there is no inhibition of colony formation.

No structure-activity relationship for cytotoxicity or cytostasis is apparent from these results. There is no particular substitution pattern that is required for activity; those compounds substituted in the A ring only appear to be equitoxic to those substituted in the B ring.

More interestingly perhaps is the lack of selective toxicity between ANN-1 cells and 3T3 fibroblasts. The dose response curve in Fig. 4.1 shows quercetin to be equitoxic to both cell lines.

These results raise questions about the potential use of these types of compounds for the treatment of human malignancy, since there does not appear to be any difference in toxicity between the "normal" cell line and the transformed cell line.

Questions arise about the design of these experiments. Since the aberrant activity of a tyrosine-specific protein kinase has been shown to be responsible not only for the initial transformation of the cell but also for the maintenance of the transformed phenotype [12], is cytotoxicity or cytostasis, then, necessarily the best end-point by which to judge the activity of these compounds?

Hunter *et al* showed, by experiments using cells that had been transformed by a mutant of RSV which encoded a $pp60^{v\text{-}src}$, that was temperature sensitive in its activity, that the

activity of the protein was responsible for the maintenance of the transformed phenotype. Cells cultured at 36°C (the temperature at which the kinase encoded by this virus is active) showed elevated phosphotyrosine levels and increased 2-deoxyglucose uptake. When these cells were incubated at 42°C, the temperature at which the kinase is inactive, the level of phosphotyrosine fell to a level only very slightly higher than that uninfected cells within 60 minutes. This fall corresponded with a decrease in 2-deoxyglucose uptake suggesting that the activity of the tyrosine kinase was responsible for the maintenance of the transformed phenotype. The drop in phosphotyrosine levels was not caused by inactivating $pp60^{v\text{-}src}$ since the substrate proteins were already phosphorylated on tyrosine. The activity of tyrosine phosphatases was responsible for the removal of phosphate from substrates and so reversal of the malignant phenotype.

It might be argued, therefore, that if a compound were acting as a tyrosine protein kinase inhibitor in an intact cell parameters other than cytotoxicity or cytostasis should be measured, shortly after treatment of the cells with the compound. It might be expected that there would then be morphological changes in the cells, perhaps a flattening of the cell and the appearance of anchorage dependent growth, similar to the growth characteristics of the parent cell line, the 3T3 fibroblast.

When ANN-1 cells were grown in the presence of quercetin

and its analogues there were changes in cell morphology. However these changes were not those that would have been predicted from the reasoning above and are illustrated in the photographs below. These photographs were all taken at the same magnification (20 times by 8 times magnification). It can be seen quite clearly that there is an alteration in the cell shape. Treated cells (Figure 4.3b) appear to be much bigger than controls (Figure 4.3a and b) and far from appearing to flatten out these cells seem to be even more spherical. Non-transformed 3T3 fibroblasts are shown for comparison with the untreated ANN-1 cells to exhibit the marked changes in cell morphology. The 3T3 cells are very difficult to see clearly and this illustrates the morphological changes.

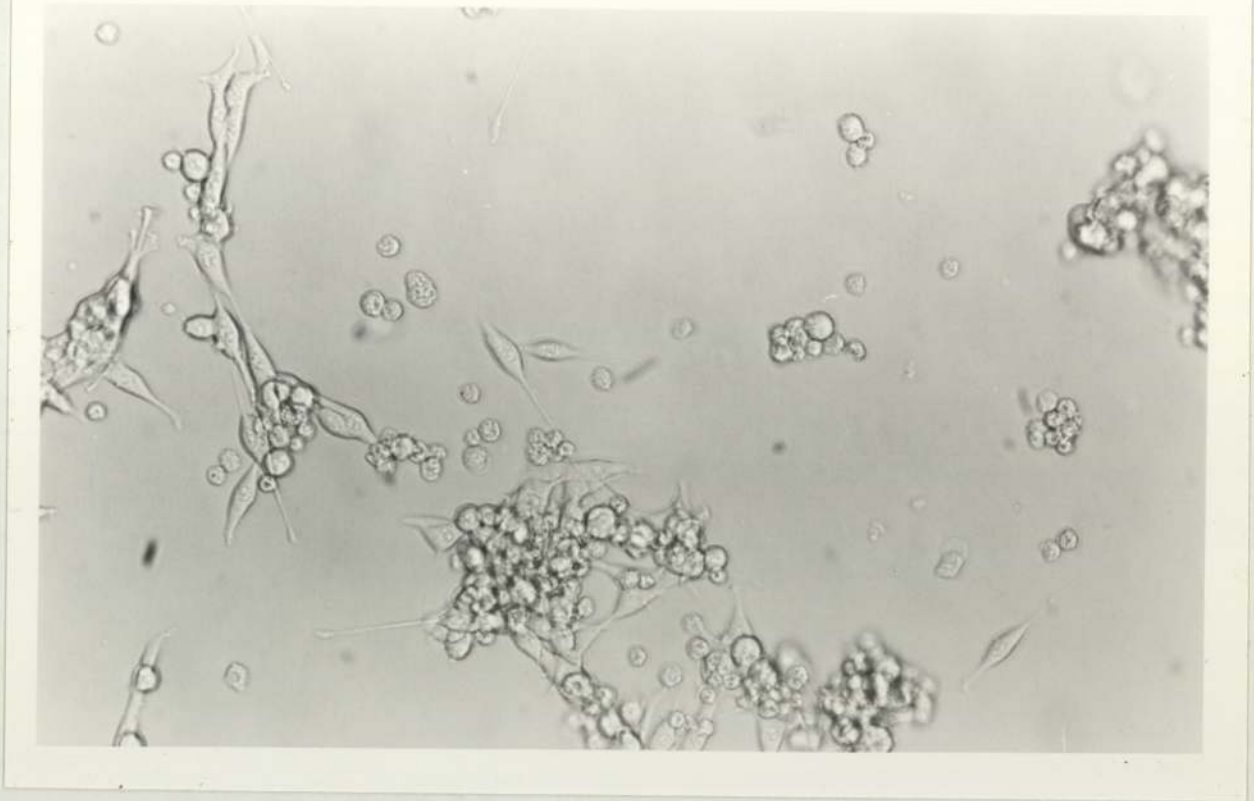
To investigate further this effect cells were grown on a multi-well slide and stained with Harris's haematoxylin as described in Materials and Methods. Photographs of these slides are shown below (Fig.4.4). Many of the cells treated with compounds appear to be multinucleate; this would account for the increase in cell size although this result raises more questions about the mechanism of action of these compounds and the manner in which the ANN-1 cells die.

Do these cells die polyploid or is this effect produced by the compounds? Have the cells become blocked in a specific phase of the cell cycle? These questions could be answered by further experiments. Flow-cytometry may clarify whether the compounds are producing a block in a specific phase of the cell

cycle; treatment of the cells with a cytotoxin known not to produce a block in a specific phase of the cell cycle may reveal how these cells usually die.

Figure 4.3a. ANN-1 cells, untreated; (i) cells at $5x10^4$ cells/ml, and (ii) cells at $5x10^5$ cells/ml.

(i)



(ii)

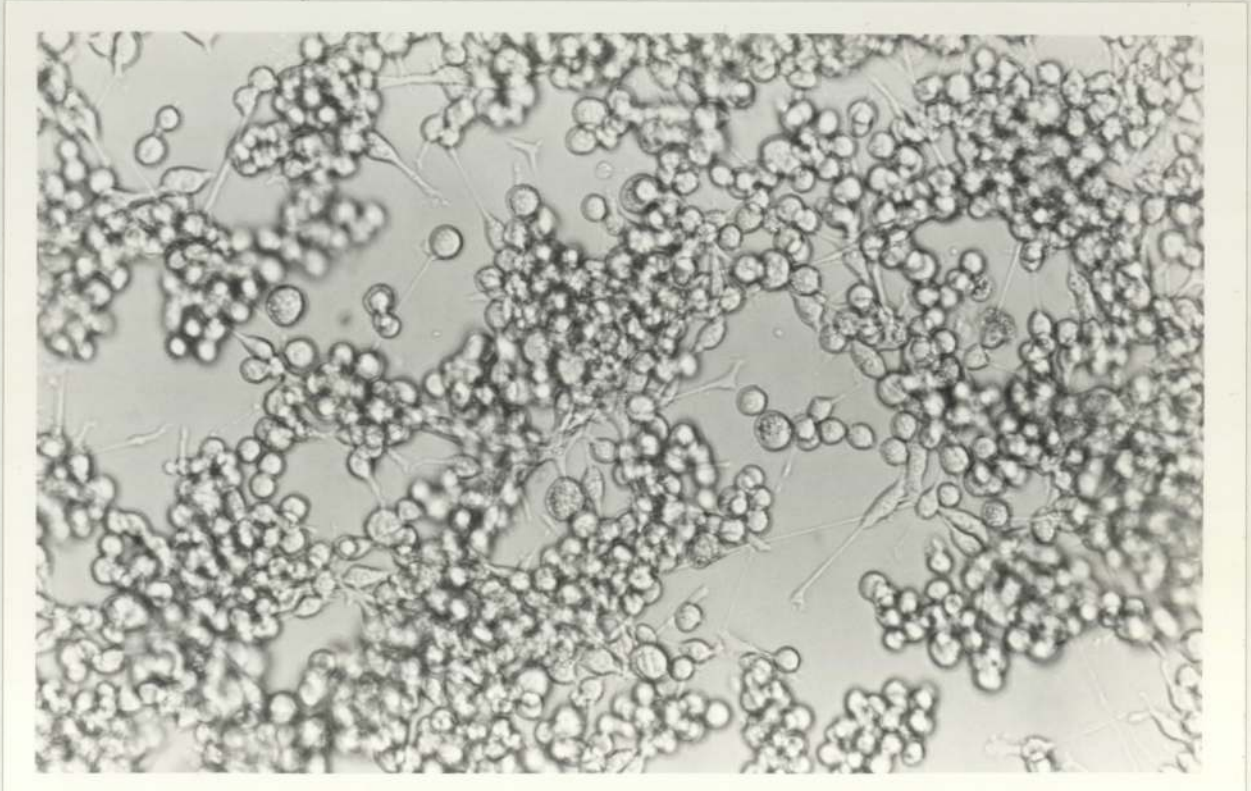
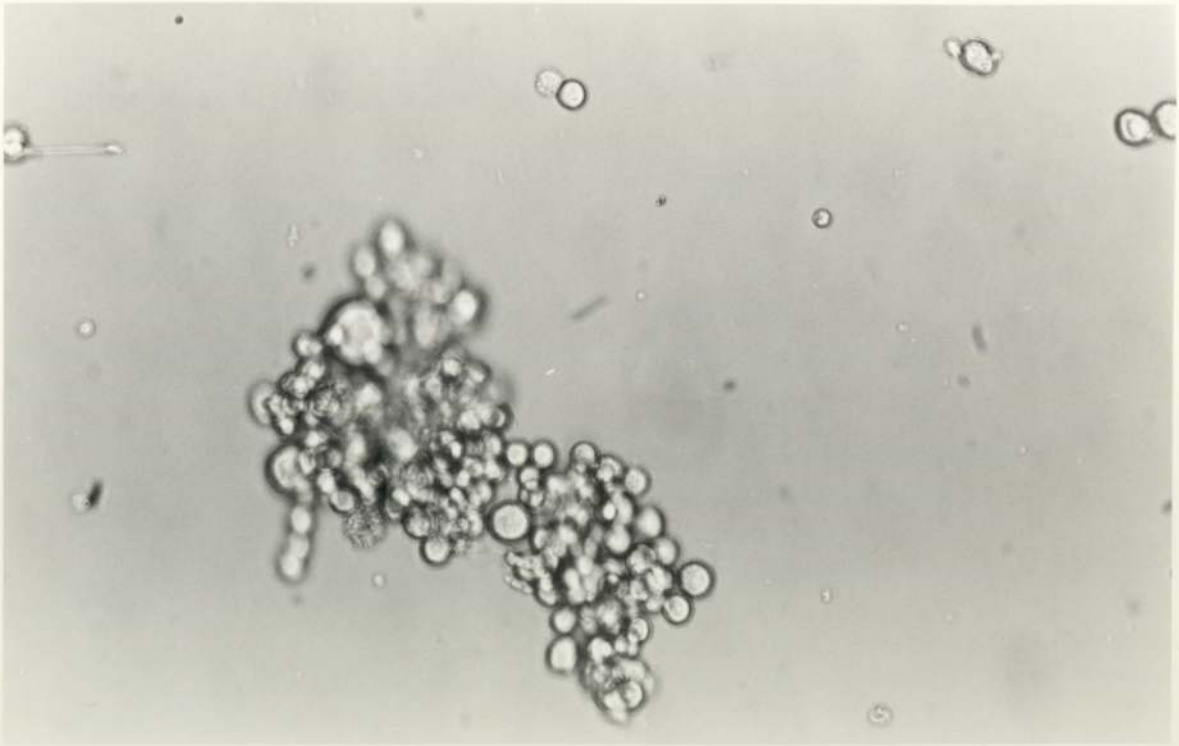
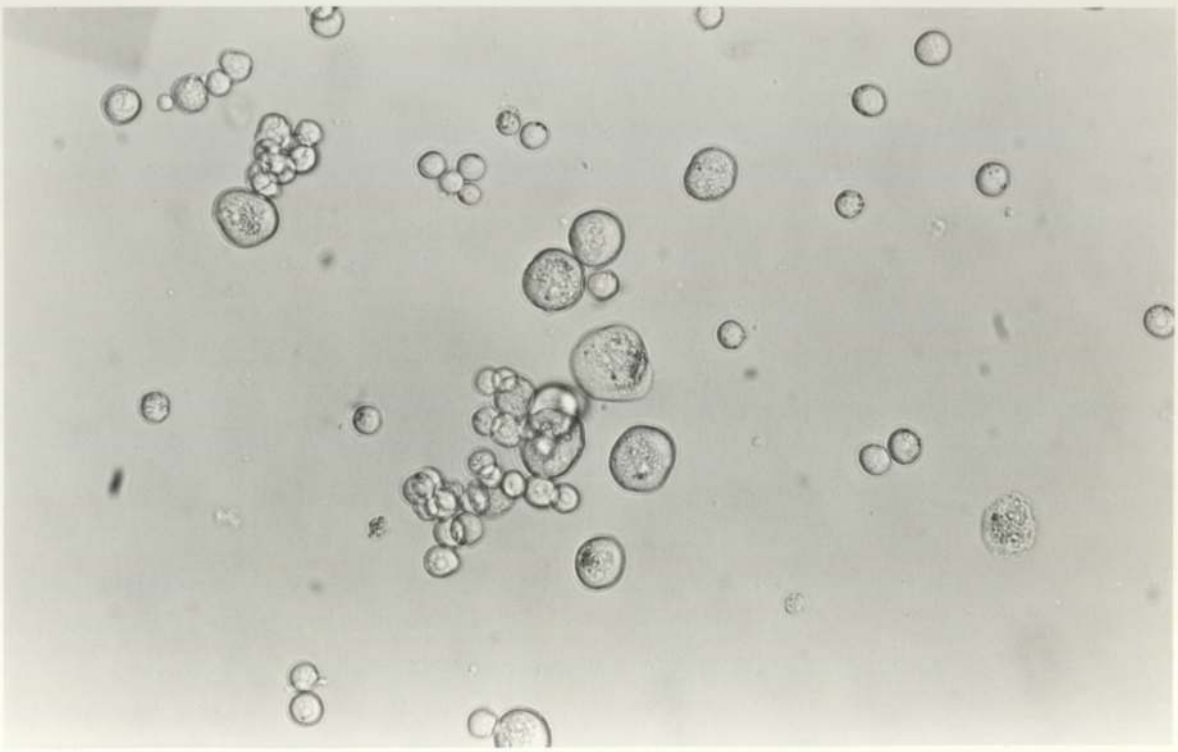


Figure 4.3b. ANN-1 cells treated with (i) quercetin (ii) flavone; (iii) 4'-methoxy-3'-aminoflavone.

(i)



(ii)



(iii)

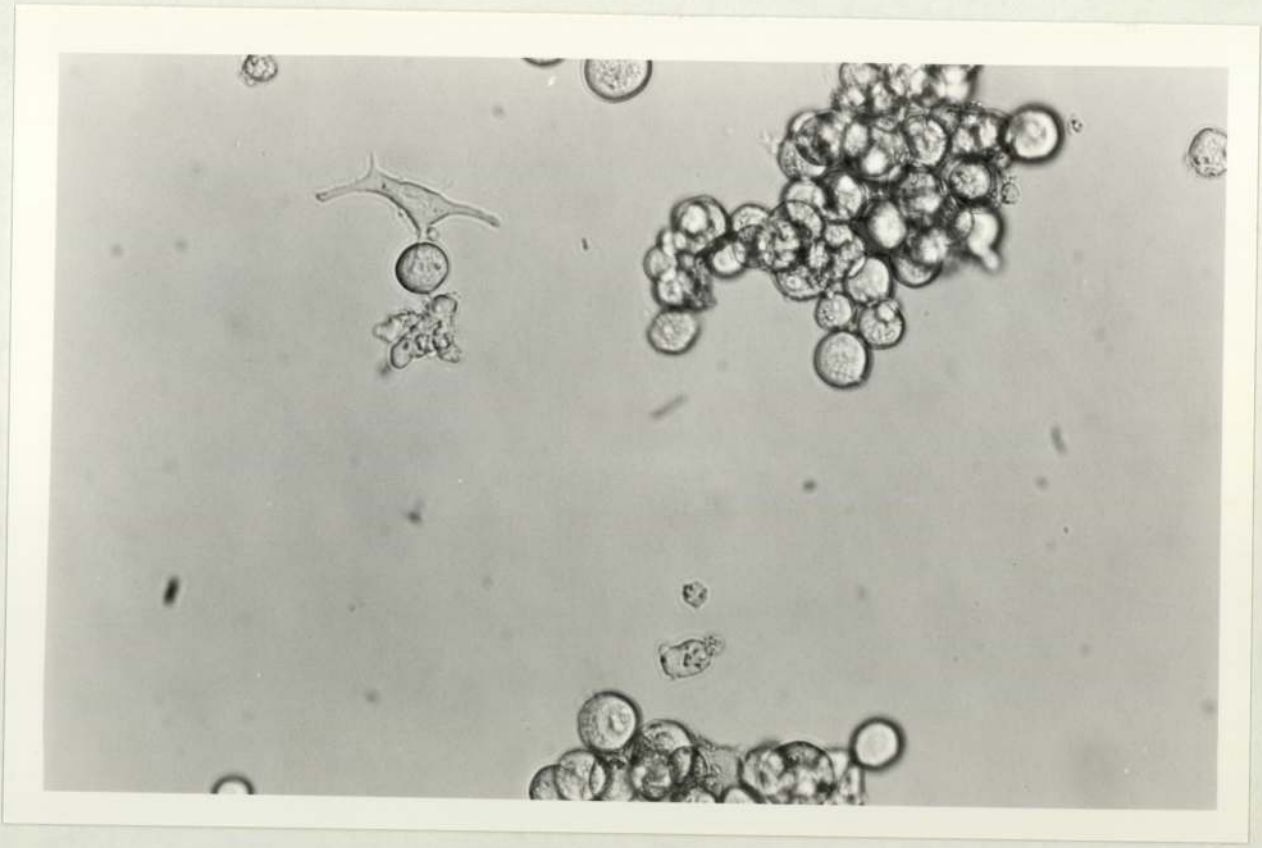


Figure 3.4c. The parent cell line, 3T3.

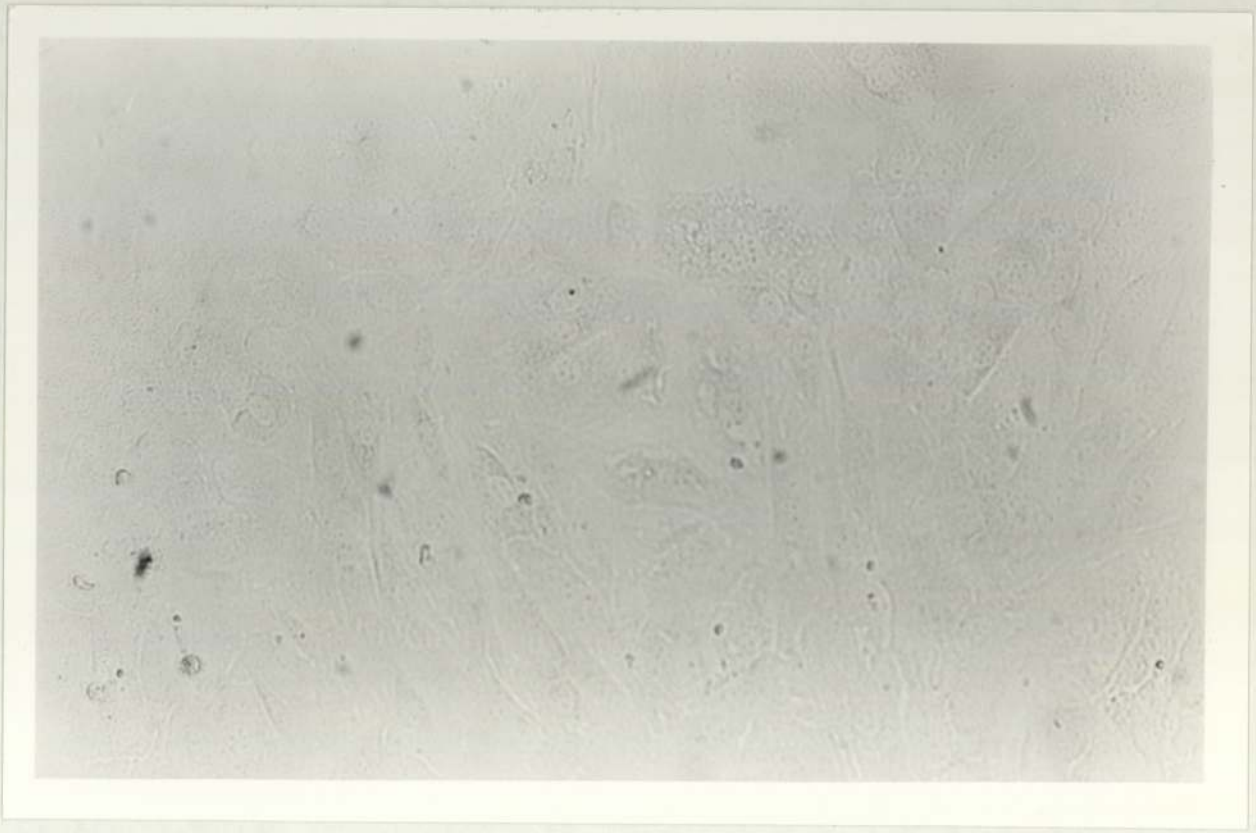
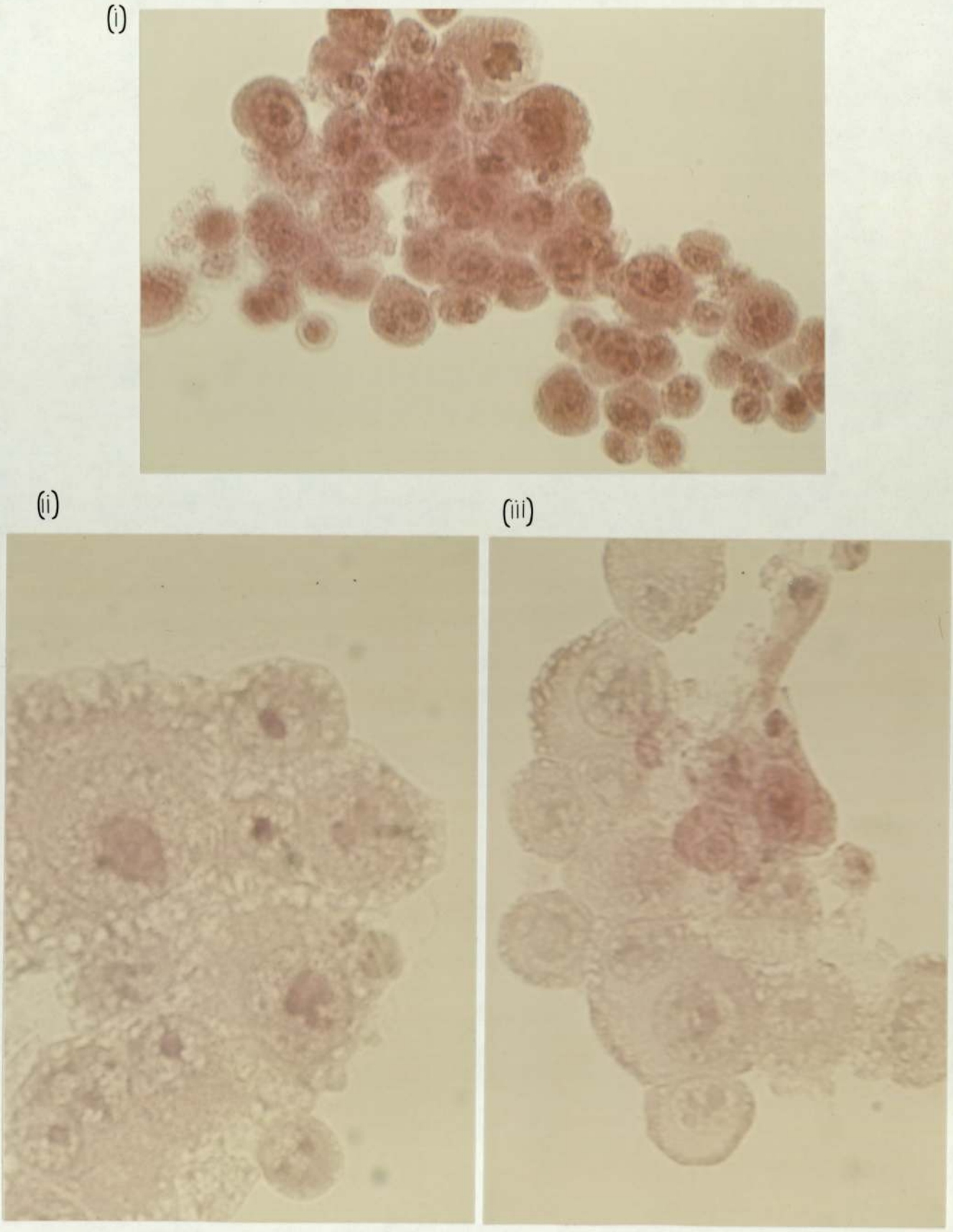


Figure 4.4. ANN-1 cells stained with Harris's haematoxylin, (i) untreated cells, (ii) cells treated with 3'-fluoroflavone, (iii) cells treated with 4'-methoxy-3'-aminoflavone.



CHAPTER FIVE

STRUCTURE ACTIVITY RELATIONSHIPS FOR QUERCETIN AND ANALOGUES IN THE INHIBITION OF THE ABELSON PROTEIN KINASE

In 1983 Graziani *et al* [147] showed that quercetin inhibited the transforming gene product of the Rous Sarcoma virus, $pp60^{v\text{-}src}$. Owing to the promiscuity of this molecule in exerting a multitude of effects on biological systems, it was the aim of this project to synthesise a series of analogues of quercetin in order to investigate any structure/activity relationship there might be towards the inhibition of tyrosine protein kinase and hence design a potentially specific inhibitor of these enzymes.

The biological activity of the compounds prepared was assayed by two means:-

1. By estimation of their effects towards whole cells in tissue culture using two cell types, the ANN-1 cell (a murine fibroblast that has been transformed by the Abelson murine leukaemia virus [239]) and the parent cell line, NIH3T3.

2. By their ability to inhibit an isolated tyrosine protein kinase. The kinase domain of the Abelson protein, pt*abl*50, was used.

pt*abl*50 kinase has been cloned from DNA sequences encoding the kinase domain of the Abelson protein using a vector which allows their expression in E. Coli. [238]. The protein was a gift of Dr. J G Foulkes (formerly of NIMR, London).

Angiotensin I was used as a substrate for this enzyme in these assays. The use of angiotensins as substrates for tyrosine protein kinases has been described by Wong and Goldberg [233, 234]. Although this decapeptide is not a natural substrate for the Abelson kinase, it can be phosphorylated on tyrosine by the Abelson kinase. Angiotensin I is commercially available and has certain properties, such as a degree of solubility in trichloroacetic acid which will allow it to be used as a substrate for a tyrosine kinase in a cell extract, making it an attractive reagent.

The amino acid sequence of this peptide is

Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu

The phosphate donor in this reaction was ATP although this kinase is also capable of using GTP. The magnesium salt of γ-[^{32}P]-ATP was used in this reaction since pt*abl*50 kinase activity requires the presence of Mg^{2+} [241].

Prior to assaying the analogues of quercetin for their

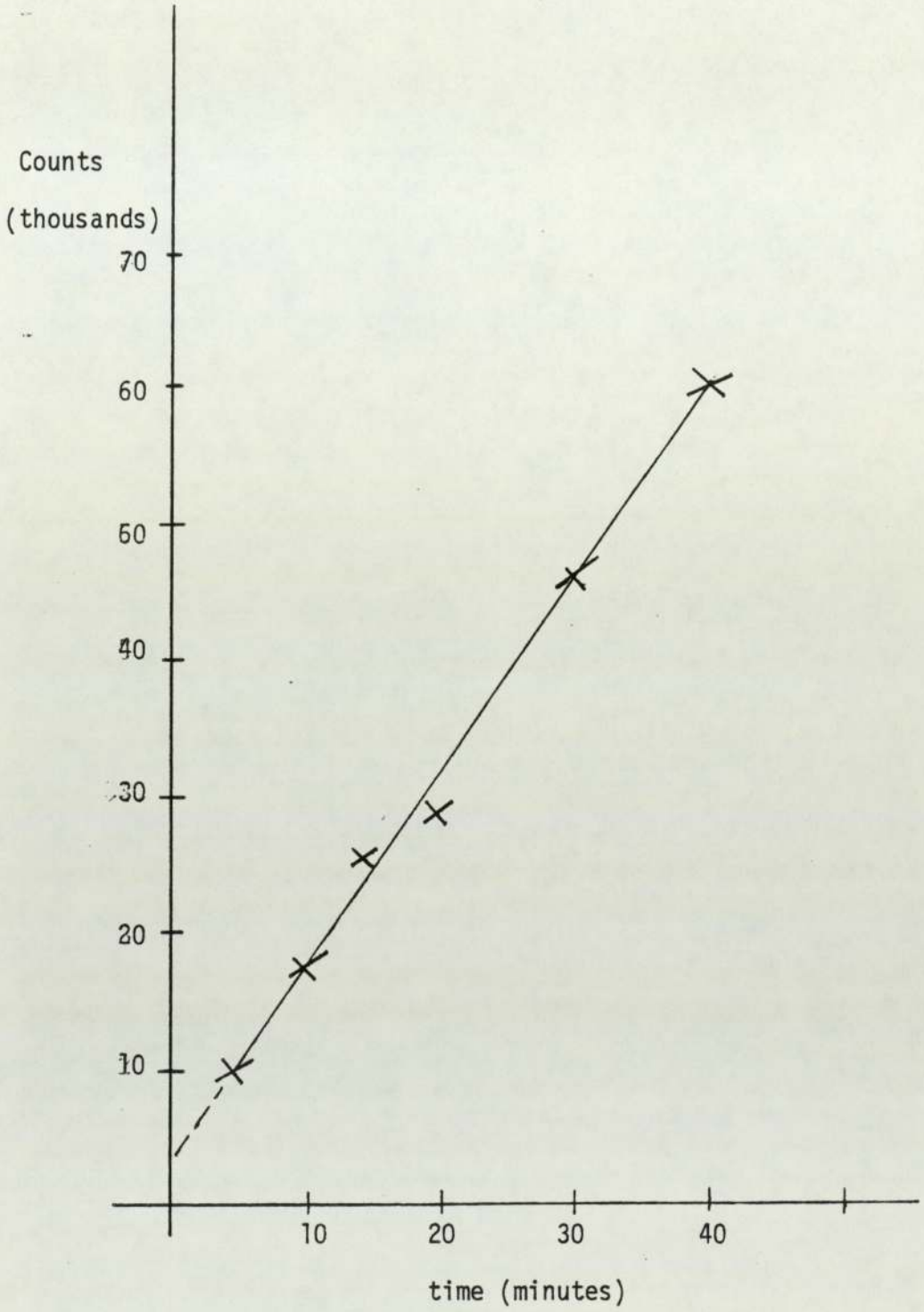


Fig. 5.1. A plot of counts incorporated into angiotensin I against time to show that the reaction is linear for at least 40 minutes.

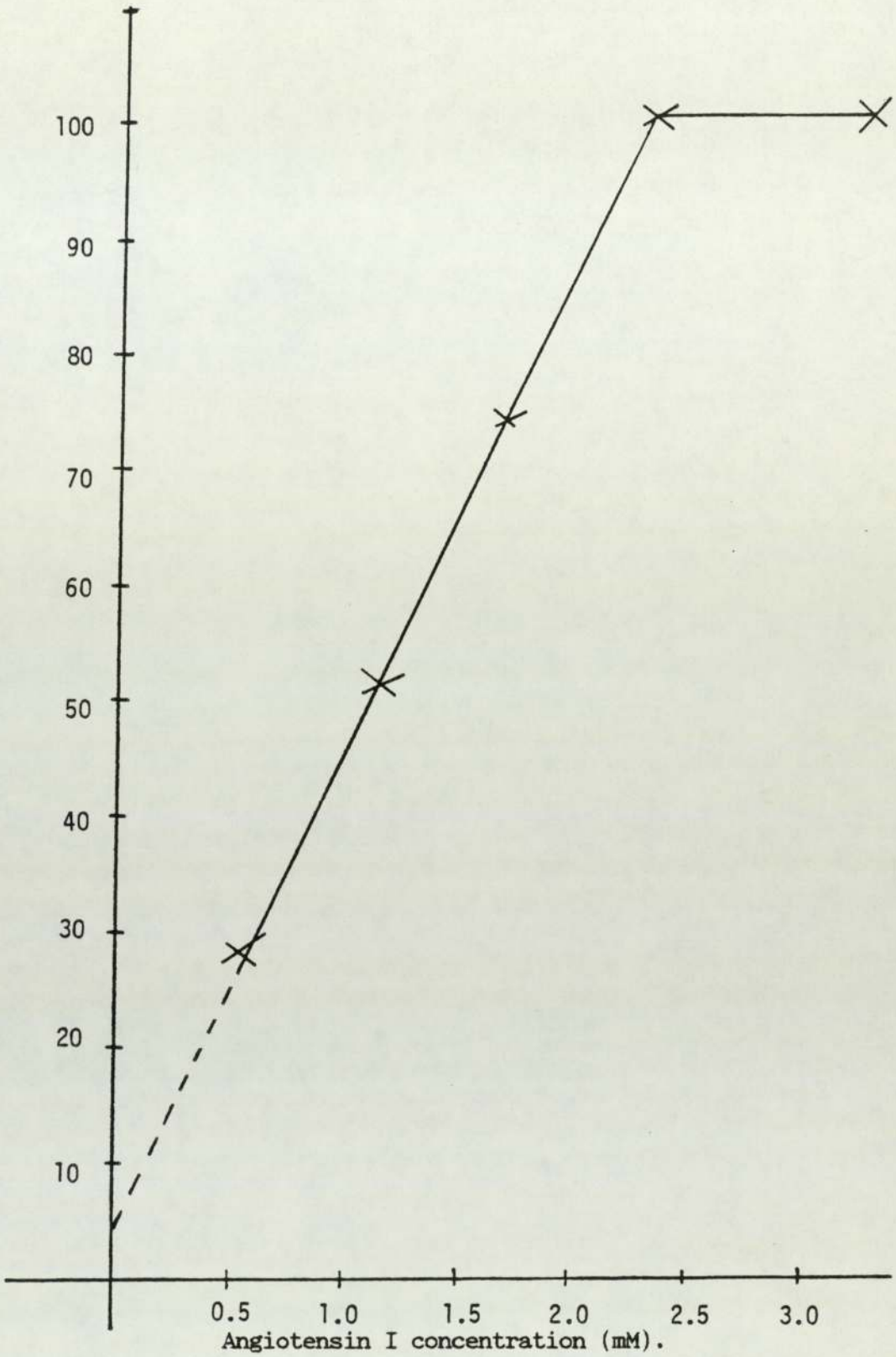
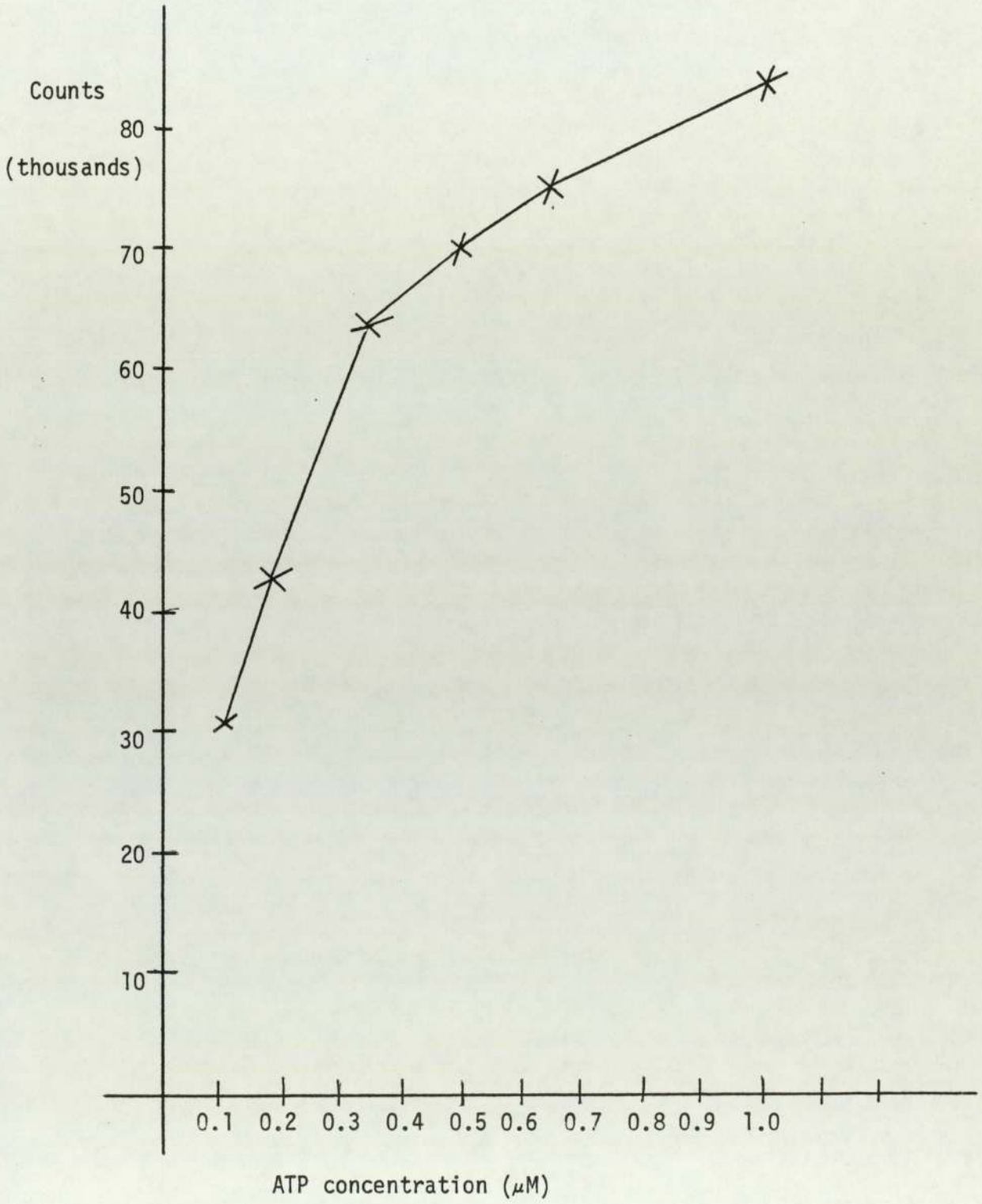


Fig. 5.2. Angiotensin I concentration v ^{32}P-phosphate incorporation to determine rate-limiting and saturating concentrations of angiotensin I for the phosphorylation reaction

Fig. 5.3. A plot of counts incorporated into angiotensin I against ATP concentration to investigate the effect of varying the phosphate donor concentration.



potential to inhibit this tyrosine kinase the kinetic properties of the enzyme were investigated.

Incorporation of phosphate into angiotensin I in an assay using 10µl angiotensin I 3mg/ml, 10µl 0.3mM MgATP, and 10µl pt*abl*50 kinase diluted 1:100 with enzyme buffer, was linear for at least 40 minutes, as shown in the graph on fig 5.1.

Rate-limiting and saturating concentrations of the substrates were determined. Angiotensin I saturated the enzyme at 1.96mM, (final concentration in the assay) but the reaction rate increased linearly up to this concentration, see figure 5.2.

The ATP concentration that saturated the enzyme was 0.65µM, but 0.1-0.33µM was rate-limiting in an assay utilising 1.96mM angiotensin I, as shown in figure 5.3.

The standard assay to investigate the potential of the synthesised compounds to inhibit the kinase consisted of 10µl angiotensin I 3mg/ml; 10µl 30mM Mg^{2+}, 0.3mM [^{32}P]-ATP; 10µl pt*abl*50 kinase diluted 1:100 with enzyme buffer and 5µl of the inhibitor in DMSO in assay buffer; (the final concentration of DMSO in the reaction was 5% and this showed no effect on the ezyme). The mixture was incubated for thirty minutes and then quenched with 10% phosphoric acid. Under these conditions the peptide is protonated and so bears a positive charge. 100µl of

the reaction mixture were spotted onto phosphocellulose paper. This paper is negatively charged so the protein binds to it. The papers were then washed extensively in 6% acetic acid which removes the excess γ-[^{32}P]-ATP.

Five of the compounds tested showed any inhibition of the kinase. Their IC_{50}s are shown in Table 5.1

Table 5.1 The IC_{50}s of the compounds showing activity against the Abelson kinase.

COMPOUND	IC_{50} (μM)
Quercetin	11
7-methoxyflavone	96
3-hydroxyflavone	139
flavone acetic acid	174
3-chloroflavone	360

Flavone, 4'-amino-3'-methoxyflavone and 4'-methoxy-3'-aminoflavone showed no activity against the enzyme at or below 500μM. A structure/activity relationship begins to emerge from these data. It appears that a substituent is required in the 3-position or in the A ring. However, 5,7-dimethoxyflavone does not show any significant activity against the enzyme, although this could be explained by steric effects at the site in the enzyme were these compounds are exerting their effect. This hypothesis could be tested by assaying the activity of 5,7-dihydroxyflavone.

To determine the manner in which these compounds were inhibiting the activity of pt*abl*50 kinase the kinetics of this inhibition were studied. (Due to the poor solubility of 3-chloroflavone, the kinetics of the inhibition of the kinase by this compound were not investigated).

To ascertain whether quercetin and the synthetic analogues were acting by an effect on the substrate, angiotensin I, a series of assays were performed at one concentration of the inhibitor but at a range of angiotensin I concentrations that were rate limiting. A double reciprocal plot of velocity against substrate concentration, a Lineweaver-Burke plot [246], was produced from the results of these experiments. This graph is shown in Fig. 5.4.

The Michaelis-Menten [244] equation describing the kinetics of an enzyme reaction is given by

$$v=\frac{Vmax \times [S]}{Km + [S]}$$

where v is the velocity of the reaction

Vmax is the maximum velocity of the reaction that can be attained by increasing substrate concentrations

[S] is the substrate concentration

Km is the Michaelis constant

(A full derivation of this equation is given by Dixon and Webb [245]).

The Michaelis-Menten equation can be rearranged to :-

$$\frac{1}{v} = \frac{1}{Vmax} + \frac{km}{Vmax} \times \frac{1}{[S]}$$

which is analogous to the equation for a straight line,

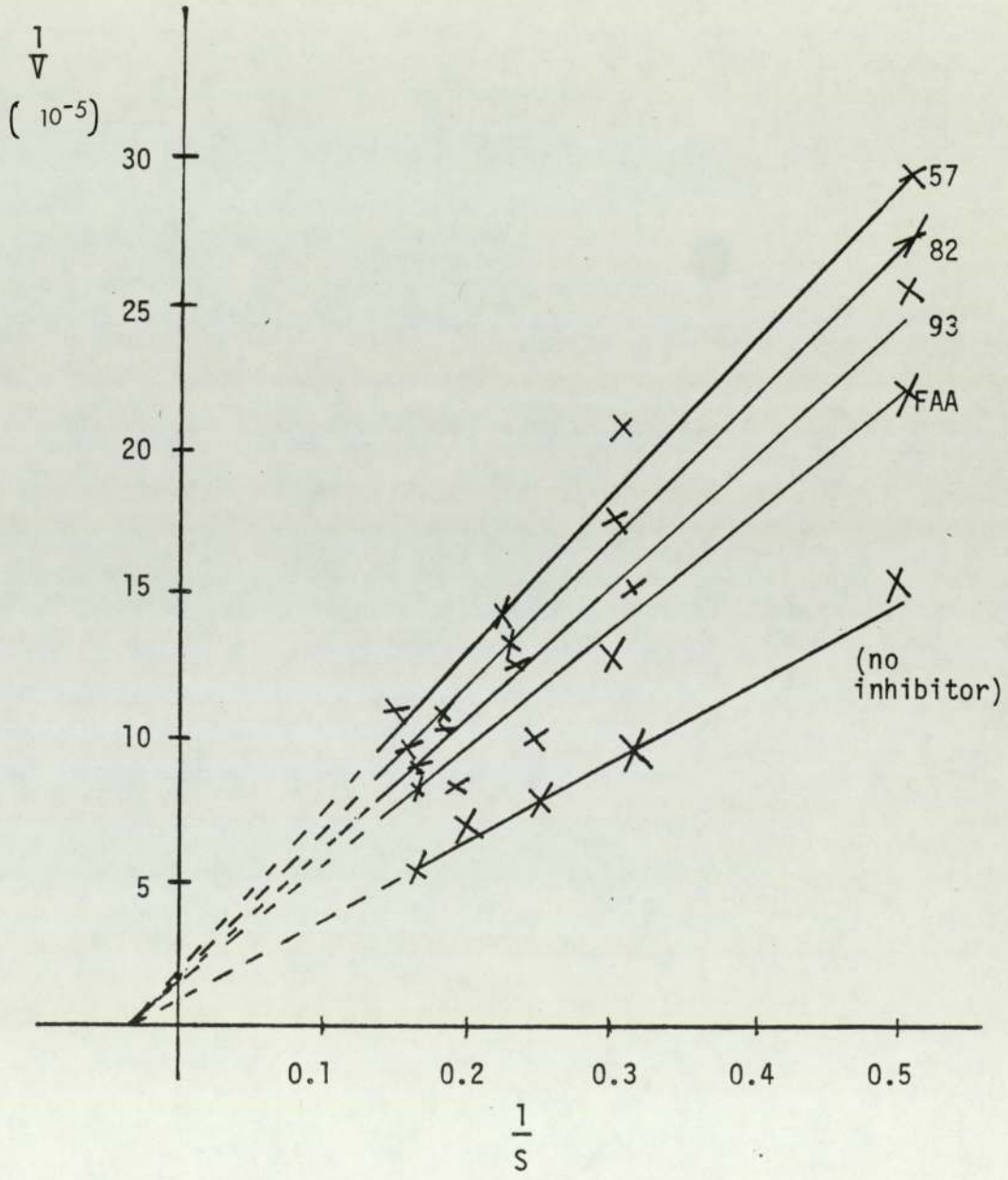
$$y = mx + c$$

The intercept on the y axis in the graph in Fig.5.4. corresponds to the value c, which according to the linear transformation of the Michaelis-Menten equation is 1/Vmax. The slope of the line is represented in the equation by Km/Vmax. However, Km may be determined from the graph since the intercept on the x axis is -1/Km.

The Km for angiotensin I in this system is 0.83mM, and the Vmax is 1.66×10^5 pmoles $min^{-1}\mu g^{-1}$ kinase. It will be seen from the graph that in the presence of an inhibitor the Km for the reaction remains the same but the Vmax is decreased. Since Km is unchanged it can be concluded that the inhibitors are not competing with this substrate for its binding site in the enzyme, the decrease in Vmax suggests that the inhibitors are binding to the enzyme in such a way that increases in the concentration of angiotensin I does not remove the inhibitor.

Fig. 5.4. A Lineweaver-Burke plot for the phosphorylation reaction utilising rate-limiting angiotenin I concentrations but saturating ATP concentrations.

(57 = 7-methoxy flavone, 82 = 3-hydroxyflavone
93 = quercetin, FAA = flavone acetic acid)



Therefore these inhibitors are non-competitive for angiotensin I.

The assay was repeated using a saturating concentration of angiotenin I but a rate-limiting concentration of ATP. The Lineweaver-Burke plot of this data is shown in Fig. 5.5. The Km for ATP in this system is 24.4μM. Foulkes *et al* [238], using angiotensin II as phosphate acceptor, found the Km for ATP to be dependent on the concentration of substrate.

The graph in Fig.5.5 shows the Vmax under these conditions remains constant, however the Km increases in the presence of an inhibitor. These results indicate that the compounds are competetive for ATP in their inhibition of the enzyme. The Kms apparent for the reaction in the presence of the inhibitors are listed in Table 5.2

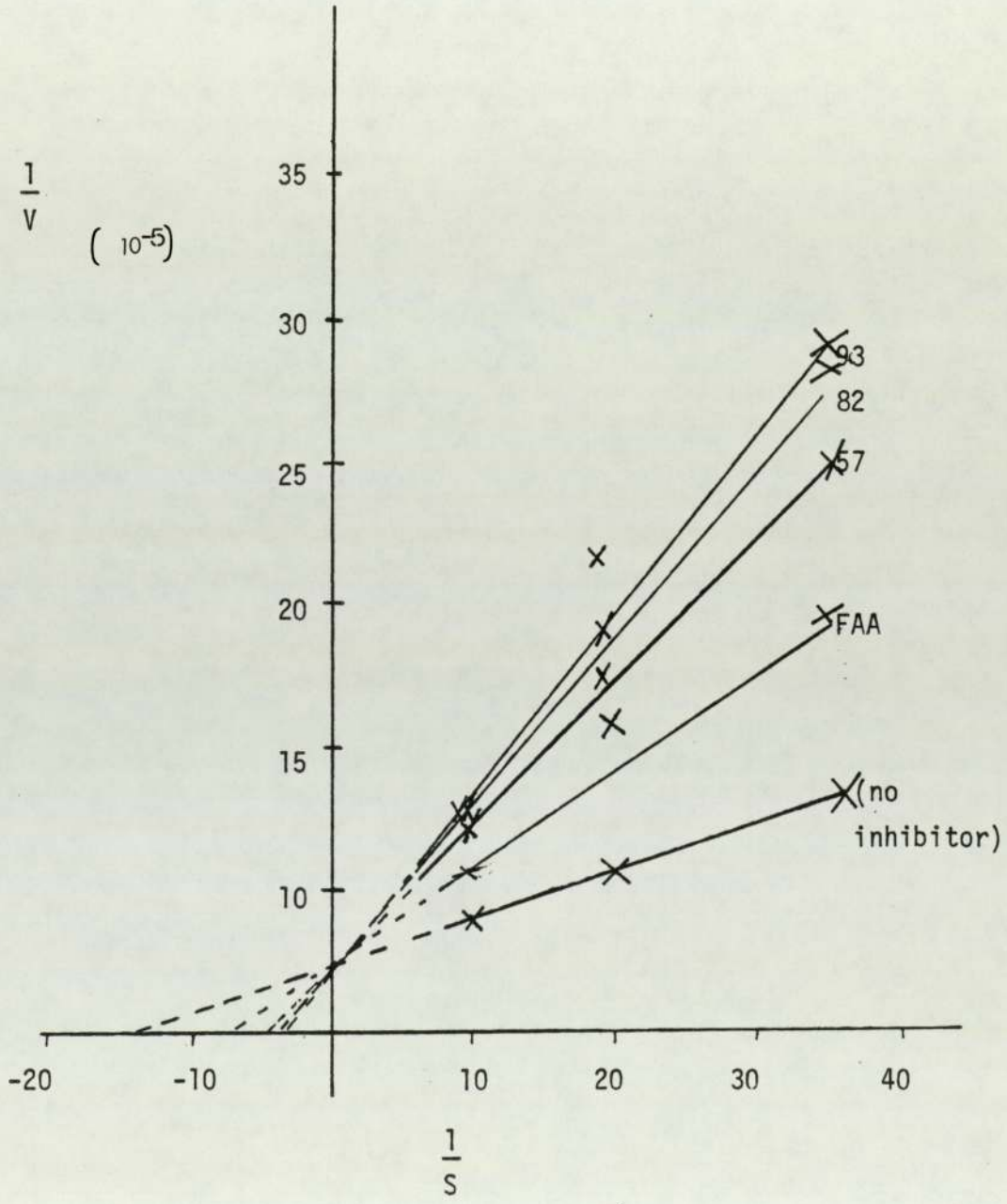
Table. 5.2 The Km for ATP for pt*abl*50 kinase in the presence of quercetin and analogues.

Inhibitor	Concentration of inhibitor(μM)	Km(μM)
-	24.4	
quercetin	10	91
3-hydroxyflavone	150	87
7-methoxyflavone	100	66.6
flavone acetic acid	150	50

An equation analogous to the Michaelis-Menten equation

Fig. 5.5. A Lineweaver-Burke plot for the phosphorylation reaction utilising rate-limiting concentrations of ATP but saturating concentrations of angiotensin I.

(57 = 7-methoxyflavone, 82 = 3-hydroxyflavone,
93 = quercetin, FAA = flavone acetic acid)



can be derived in which the concentration of a competitive inhibitor as well as substrate concentration appears. This equation is given below

$$v = \frac{Vmax}{1 + \frac{Km}{[S]}\left(1 + \frac{[I]}{Ki}\right)}$$

Linear transformation of this equation gives

$$\frac{1}{v} = \frac{1}{Vmax} + \frac{Km}{Vmax} * \left(1 + \frac{[I]}{[Ki]}\right) * \frac{1}{[S]}$$

When $1/v = 0$ the intercept on the x axis gives $-1/Km$ (Km apparent in the presence of an inhibitor) and hence Ki (the inhibition constant) of the inhibitor can be determined either from the slope of the plot,

$$slope_{1/s} = \frac{Km}{Vmax} * \left(1 + \frac{[I]}{Ki}\right)$$

or from the intercept on the x axis,

$$-\frac{1}{Km_{app}} = -\frac{1}{Km\left(1 + \frac{[I]}{Ki}\right)}$$

rearranging this equation for Ki gives

$$Ki = \frac{I}{\frac{Km_{app}}{Km} - 1}$$

Table 5.3 gives the inhibition constants for the four compounds whose kinetics were studied.

Table 5.3 The inhibition constants for quercetin and analogues.

Inhibitor	Ki (μM)
Quercetin	3.66
3-hydroxyflavone	39
7-methoxyflavone	58
flavone acetic acid	143

Although these are the results of only one experiment and repeat experiments are required to verify these data, comparison of the Ki for quercetin against pt*abl*50 kinase and against $pp60^{v\text{-}src}$ show these values to be similar. Graziani *et al* [147] calculated the Ki for quercetin to be 6-11μM.

While a competitive inhibitor of ATP may appear to be an unlikely candidate as a potential anti-tumour agent, there does appear to be some specificity of quercetin between cyclic-AMP dependent and cyclic-AMP independent protein kinases. Graziani *et al* [147] stated that concentrations of quercetin that inhibited cyclic-AMP independent protein kinases had no effect on cyclic-AMP dependent protein kinases.

Genistein, 5,7-dihydroxy isoflavone, inhibits the src protein kinase, the kinase activity of the EGF receptor and $pp110^{gag\cdot fes}$ [144]. This inhibition was competitive towards ATP, the Ki for genistein against the EGF receptor kinase was 3.7μg/ml (13.7μM). At 100μg/ml, (370μM) this compound had little effect on the threonine and serine kinase activity of protein kinase C.

There are many protein kinases other than tyrosine-specific protein kinases that are cyclic-AMP independent, such as protein kinase C, and it might be expected that these enzymes would be affected by an ATP analogue, but the results of Akiyama *et al* indicate that there is potentially an inhibitor specific for a cAMP-independent tyrosine-specific protein kinase.

Further investigations must be carried out to determine if any specificity between tyrosine-specific protein kinases and other cyclic-AMP independent protein kinases can be achieved.

CHAPTER SIX

CONCLUSIONS

Chapter Two describes the synthesis of ninety two compounds, thirty five of which were analogues of quercetin. The potential cytotoxicity of 30 compounds was tested using single cell suspension cultures and clonogenic assays as described in Chapter Four, and the ability, or otherwise, of the compounds to inhibit a tyrosine protein kinase was investigated by means of the kinetic experiments recounted in Chapter Five.

The flavones were synthesised by the method of Baker [169], by acylation of appropriate 2-hydroxyacetophenones with suitably substituted aromatic acid chlorides, followed by base catalysed rearrangement to 1-(2-hydroxyphenyl)-3-phenylpropan-1,3-diones which furnished the flavone when treated with concentrated acid.

2-hydroxy-4-methoxy- and 4,6-dimethoxy-2-hydroxy-acetophenone were prepared by the acylation of the respective phenol with acetyl chloride catalysed by boron trichloride. These compounds were more accessible by this reaction than by the more usual Fries rearrangement.

2-cyclohexyl chromone was prepared by the same method as the flavones; however an attempt to prepare

2-cyclohex-2-enyl chromone by the same means proved unsuccessful.

The 1-(2-hydroxyphenyl)-3-phenylpropan-1,3-diones exhibited extensive enolisation in their NMR spectra. The crystal structure of **29** confirmed that this was not a dissolution effect. Compound **28** however existed in the keto form in its NMR spectrum. This compound assumes a twisted conformation as shown by its crystal structure. The reason for the disparity between the extent of enolisation of these two compounds may be due to steric hindrance caused by the methoxy group in the 6-position of **28** or, perhaps more likely, to the increased electron density in the enol system caused by the extra electron donating group.

The results of the assays of *in vitro* cytotoxicity do not suggest the existence of a structure/activity relationship nor a selective toxicity towards transformed cells compared with the "normal" parent cell line. However, the data obtained using the cloned enzyme, pt*abl*$_{50}$ kinase, indicate a requirement for the presence of substituents either in the 3-position or in the A ring of the flavone nucleus for these compounds to inhibit this tyrosine-specific protein kinase, as shown in Fig. 6.1

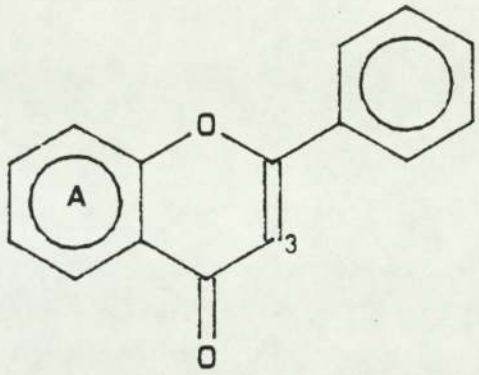


Fig. 6.1 Apparent substitution pattern required for a flavone to be an inhibitor of tyrosine protein kinase.

The results presented in this study suggest that the cytotoxicity or cytostasis elicited by the compounds is independent of the ability of the compounds to inhibit the tyrosine protein kinase expressed by the ANN-1 cells. This is supported by the initial results of the experiments which used 3T3 fibroblasts. Quercetin appears to be equitoxic to both cell lines whereas flavone acetic acid is not cytotoxic or cytostatic to ANN-1 cells but is able to inhibit the enzyme *in vitro*.

The questions which should now be addressed are:-

a. What is the mode of action by which these compounds bring about cell death?
b. Is it possible to design a compound that will selectively inhibit a tyrosine-protein kinase but will not be a non-selective cytotoxic agent.

Cells in culture represent a convenient model by which

to test compounds for cytotoxicity, cytostasis and/or other effects and allows a comparison of data with data from a cell-free model, such as an isolated enzyme. This comparison is limited though and it must be remembered that cell culture is only a model and so suffers the limitations of any model. For example, flavone acetic acid which does not inhibit the growth of ANN-1 cells below 500μM has activity against the MAC 13 and 26 colon carcinomas in NIMR mice [236]; 4'-methoxy-3'-aminoflavone, the most cyctotoxic of the compounds tested, is currently being tested against the MAC 13 carcinoma in this department and from initial results this compound appears to be as active as FAA against this tumour and as non toxic to the control mice.

This project has many leads to investigate further:-

a. To determine the therapeutic index for and the possible mechanism of action of 4'-methoxy-3'-aminoflavone against the MAC 13 tumour.
b. Further elucidation of the structure/activity relationship of these compounds towards the Abelson protein.
c. Whether the compounds show any specificity towards tyrosine protein kinases compared with serine or threonine protein kinases, or if there is specificity for cAMP-independent protein kinases compared with cAMP-dependent protein kinases.

d. Whether those compounds that inhibit the enzyme have any effect on the phosphoamino acid content of the cell.

The basis for this project is in the field of experimental chemotherapy, however the author believes that the results presented here will allow further investigation of these target enzymes and that ultimately a selective inhibitor of tyrosine-protein kinases will play a useful clinical role in the treatment of human malignancy.

CHAPTER SEVEN

EXPERIMENTAL

All melting points were recorded on an Electrothermal Digital melting point apparatus and are uncorrected.

NMR data was obtained from a Varian EM360A 60MHz except were stated. 300MHz NMR data was obtained by Dr. M C Perry (Dept. of Chemical Engineering and Applied Chemistry, Aston University) from a Bruker AC300 300MHz and 400MHz NMR data was obtained by Drs. A T Harrison and O W Howarth (SERC NMR Spectroscopic Service, Univ. of Warwick) from a WH400 400MHz.

Mass spectral data was obtained from a VG Micromass 12B by Karen Farrow (Dept. Pharm Sci., Aston Univ); high resolution mass spectral data was obtained from SERC Spectroscopic Centre, Swansea.

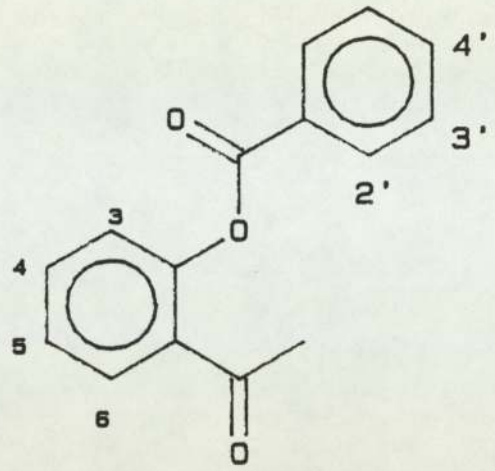
Infra red and ultra violet spectra were recorded on a Pye-Unicam SP200 spectrophotometer, and a Pye-Unicam SP2000 ultra violet spectrophotometer, respectively.

Elemental microanalyses were performed by Butterworths Laboratories, Teddington, Middlesex; and Elemental Microanalysis, Okehampton, Devon.

2-hydroxy-4,6-dimethoxyacetophenone (1) was prepared from 3,5-dimethoxyphenol in 78% yield in the following manner;-

BCl_3 (50ml of 1M in CH_2Cl_2, 0.05mol) was placed in a two necked round bottom flask under nitrogen and cooled to -10°C in an ice/salt bath. 3,5-dimethoxyphenol (7.7g, 0.05mol) was then added to this solution in CH_2Cl_2 (50ml). The mixture was allowed to stir for 5mins before the addition of acetyl chloride (3.9g, 0.05mol). The reaction mixture was then warmed and allowed to reflux for three hours. The mixture was then carefully quenched by adding an excess of 1M hydrochloric acid and then the reaction was left to stir for an hour. Separation and concentration of the organic layer yielded a red solid which was then dissolved in CH_2Cl_2 (50ml) and filtered through a silica plug. Removal of the solvent under reduced pressure afforded 2-hydroxy-4,6-dimethoxyacetophenone (7.7g, 78% yield) m.pt. 82°C, lit. 82-83°C [204]; NMR $CDCl_3$ δ 2.56 (3H s $COCH_3$) 3.78 (3H s OMe) 3.8 (3H s OMe) 5.85 (1H d J 2 Hz 3) 6.0 (1H d J 2 Hz 5) 13.9 (1H s OH).

2-hydroxy-4-methoxyacetophenone (2) was prepared in a similar manner giving 78% yield also, m.pt. 50°C, lit. 48°C [205]; NMR $CDCl_3$ δ 2.50 (3H s $COCH_3$) 3.75 (3H s OMe) 6.30 (1H d $J_{3,5}$ 2 Hz 3) 6.37 (1H dd $J_{5,6}$ 9 Hz $J_{5,3}$ 2 Hz 5) 7.54 (1H d $J_{5,6}$ 9 Hz 6) 12.72 (1H s OH).



2-benzoyloxyacetophenone (3) was prepared by treating benzoyl chloride (4.9g, 0.035mol) with 2-hydroxyacetophenone (3.4g, 0.025mol) in pyridine (20mls). After stirring for 30mins at room temperature, the reaction was monitored by tlc ($CHCl_3$) and when the reaction had gone to completion the mixture was poured onto an excess of 1M hydrochloric acid and crushed ice. The solid was filtered off and recrystallised from methanol. The yield after recrystallisation was 78%, m.pt. 89-90°C, lit. 87-88°C [206]; NMR $CDCl_3$ δ 2.4 (3H s $COCH_3$) 7.6-7.0 (6H m 3',4',5', 3, 4, 5,) 7.7 (1H dd $J_{6,5}$ 8 Hz $J_{6,4}$ 2 Hz 6) 8.15 (2H dd $J_{2',3'}$ 6 Hz $J_{2',4'}$ 2 Hz 2's).

The following compounds were prepared in an analogous manner:-

2-(3-fluorobenzoyloxy)acetophenone (4) in 74% yield, m.pt. 65-66°C; found C69.58%, H 4.33%, $C_{15}H_{11}FO_3$ requires C 69.76%, H

4.29%; NMR $CDCl_3$ δ 2.51 (3H s $COCH_3$) 8.0-7.1 (8H m ArH); m/z, m^+ 258, 123 (100%), 107, 95.

2-(3,4-dimethoxybenzoyloxy)acetophenone (5) in 84% yield, m.pt. 126-7°C, lit. 129°C [207]; found C 67.98%, H5.39%, $C_{17}H_{16}O_5$ requires C 67.99%, H 5.37%; NMR $CDCl_3$ δ 2.5 (3H s $COCH_3$) 3.92 (6H s 2OMe) 7.9-6.8 (7H m ArH).

2-(4-methoxybenzoyloxy)acetophenone (6) in 85% yield, m.pt. 116°C, lit. 113-114°C [207]; NMR $CDCl_3$ δ 2.46 (3H s $COCH_3$) 3.82 (3H s OMe) 6.94 (2H d $J_{3',2'}$ 8 Hz 3', 5') 7.56-7.03 (3H m 3, 4, 5,) 7.8 (1H dd $J_{6,5}$ 8 Hz $J_{6,4}$ 2 Hz 6) 8.13 (2H d $J_{2',3'}$ 8 Hz 2', 6').

2-(3-methoxybenzoyloxy)acetophenone (7) in 53% yield, m.pt. 52°C, found C 71.11%, H 5.22%, $C_{16}H_{14}O_4$ requires C71.10%, H5.22%; NMR $CDCl_3$ δ 2.46 (3H s $COCH_3$) 3.76 (3H s OMe) 7.90-6.90 (8H m ArH); m/z, m^+ 270, 152, 135 (100%), 107,92, 77.

2-(4-nitrobenzoyloxy)acetophenone (8) in 65% yield, m.pt. 90°C, lit. 99-100°C [208]; NMR $CDCl_3$ δ 2.50 (3H s $COCH_3$) 7.67-7.10 (3H m 3, 4, 5) 7.88 (1H dd $J_{6,5}$ 6 Hz $J_{6,4}$ 2 Hz 6) 8.36 (4H s 2', 3', 5', 6').

2-(3-nitrobenzoyloxy)acetophenone (9) in 75% yield, m.pt. 94°C, lit. 99-100°C [208]; NMR $CDCl_3$ δ 2.50 (3H s $COCH_3$) 7.9-7.15 (5H m 5', 3, 4, 5, 6) 8.6-8.4 (2H m 4', 6') 8.98 (1H t

J 2 Hz 2').

2-(4-chlorobenzoyloxy)acetophenone (10) in 75% yield, m.pt. 94°C, lit. 92-3°C, [209]; found C 65.65%, H 4.22%,, $C_{15}H_{11}ClO_3$ requires C 65.58%, H4.03%; NMR $CDCl_3$ δ 2.5 (1H s $COCH_3$) 7.5-7.1 (3H m 3, 4, 5) 7.42 (2H d $J_{3',4'}$ 8 Hz 3', 5') 7.80 (1H dd $J_{6,5}$ 7 Hz $_{6,4}$ 1.5 Hz 6) 8.08 (2H d $J_{2',3'}$ 8 Hz 2', 6').

2-(4-bromobenzoyloxy)acetophenone (11) in 73% yield, m.pt. 127°C; found C 56.24%, H 3.56%, $C_{15}H_{11}BrO_3$ requires C 56.45%, H 3.47%; NMR $CDCl_3$ δ 2.5 (3H s $COCH_3$) 7.9-7.1 (4H m 3, 4, 5, 6) 7.57 (2H d $J_{3',4'}$ 8 Hz 3' 5') 7.97 (2H d $J_{3',4'}$ 8 Hz 3',5'); m/z, m^+ 318/320, 183/185 (100%), 155/157, 104, 92,76.

2-(3,4-dichlorobenzoyloxy)acetophenone (12) in 78% yield, m.pt. 84°C, found C 58.09%, H 3.26%, $C_{15}H_{10}Cl_2O_3$ requires C 58.27%, H 3.26%; NMR $CDCl_3$ δ 2.52 (3H s $COCH_3$) 7.15 (1H dd $J_{3,4}$ 8 Hz $J_{3,5}$ 2 Hz 3) 7.50-7.3 (2H m 4,5) 7.56 (1H d $J_{5',6'}$ 8 Hz 5') 7.83 (1H dd $J_{6,5}$ 8 Hz $J_{6,4}$ 2 Hz 6) 7.92 (1H dd $J_{6',5'}$ 8 Hz $J_{6',2'}$ 2Hz 6') 8.26 (1H d $J_{2',6'}$ 2 Hz 2'), m/z, m^+ 308/310/312, 173/175/177 (100%), 145/145/149, 75.

2-(4-chloro-3-nitrobenzoyloxy)acetophenone (13) in 55% yield; m.pt. 99-100°C, found C 56.36%, H 3.16%, $C_{15}H_{10}ClNO_5$ requires C 56.35%, H 3.15%; NMR $CDCl_3$ δ 2.53 (3H s $COCH_3$) 7.98-7.18 (4H m 3, 4, 5, 6) 8.65 (1H d $J_{2'6'}$ 2 Hz 2'); m/z m^+ 319/321,

184/186 (100%), 138/140, 110/112, 92, 75.

2-cyclohexylcarbonyloxyacetophenone (14) in 51% yield, low melting solid/liquid, found C 72.84%, H7.75%, $C_{15}H_{18}O_3$ requires C 73.14%, H 7.36%, NMR $CDCl_3$ δ 2.13-1.11 (11H m cyclohexyl protons) 2.5 (3H s $COCH_3$) 7.00 (1H dd $J_{3,4}$ 8 Hz $J_{3,5}$ 2Hz 3) 7.5-7.18 (2H m 4,5) 7.7 (1H dd $J_{6,5}$ 8Hz $J_{6,4}$ 2 Hz 6); m/z, m^+ 246, 130, 121 (100%), 83.

2-(4-methoxy-3-nitrobenzoyloxy)acetophenone (15) in 86% yield, m.pt. 139-140°C, found C 60.83%, H 4.20%, N 4.16%, $C_{15}H_{13}NO_6$ requires C 60.95%, H 4.15%, N 4.44%, NMR $CDCl_3$ δ 2.5 (3H s $COCH_3$) 4.0 (3H s OCH_3) 7.14 (1H d $J_{5',6'}$ 10 Hz 5') 7.73-7.23 (3H m 3,4,5) 7.83 (1H dd $J_{6,5}$ 7 Hz $_{6,4}$ 2Hz 6) 8.3 (1H dd $J_{6',5'}$ 8 Hz $J_{6',2'}$ 2 Hz 6') 8.62 (1H d $J_{2',6'}$ 2 Hz 2'); m/z, m^+ 315, 180 (100%), 133, 120, 105, 92, 76, 43.

2-(3-methoxy-4-nitrobenzoyloxy)acetophenone (16) in 76% yield, m.pt. 108-109°C; found C 60.78%, H 4.13%, N 4.49%, $C_{16}H_{13}NO_6$ requires C 60.95%, H 4.15%, N 4.44%; NMR (300MHz) $CDCl_3$ δ 2.54 (3H s $COCH_3$) 4.03 (3H s OMe) 7.25 (1H dd $J_{3,4}$ 8.0 Hz $J_{3,5}$ 1.1 Hz 3) 7.41 (1H ddd $J_{5,3}$ 1.1 Hz $J_{8.14}$ $J_{5,6}$ 7.4Hz 5) 7.63 (1H ddd $J_{4,5}$ 8.14 Hz $J_{4,3}$ 8.0 Hz $J_{4,6}$ 1.6 Hz 4) 7.82-7.92 (4H m 6, 2', 5', 6'); m/z, m^+ 315, 180 (100%), 105, 76.

2-(3-chlorobenzoyloxy)acetophenone (17) in 81% yield, m.pt. 84°C; found C 65.68%, H 4.05%, Cl 12.95%, $C_{15}H_{11}ClO_3$ requires C

65.58%, H 4.03%, Cl 12.90%; NMR (400MHz) $CDCl_3$ δ 2.53 (3H s $COCH_3$) 7.22 (1H dd $J_{3,4}$ 8.10 Hz $J_{3,5}$ 1.16 Hz 3) 7.38 (1H ddd $J_{5,6}$ 7.7 Hz $J_{4,5}$ 7.6 Hz $J_{5,3}$ 1.16 Hz 5) 7.46 (1H dd $J_{5',6'}$ 7.76 Hz $J_{5',4'}$ 8.05 Hz 5') 7.59 (1H ddd $J_{4,5}$ 7.6 Hz $J_{4,3}$ 8.10 Hz $J_{4,6}$ 1.67 Hz 4) 7.61 (1H ddd $J_{4',5'}$ 8.05 Hz $J_{4',2'}$ 2.17 Hz $J_{4',6'}$ 1.10 Hz 4') 7.87 (1H dd $J_{6,5}$ 7.7 Hz $J_{6,4}$ 1.6 Hz 6) 8.08 (1H ddd $J_{6',2'}$ 2.72 Hz $J_{6',4'}$ 1.10 Hz $J_{6',5'}$ 7.7 Hz 6') 8.17 (1H dd $J_{2',6'}$ 2.72 Hz $J_{2',4'}$ 2.17 Hz 2'); m/z, m^+ 274/276, 156/158, 139/141, 92 (100%).

2-(4-nitrobenzoyloxy)-4-methoxyacetophenone (**18**) was prepared by the acylation of 2-hydroxy-4-methoxyacetophenone (4.98g, 0.03mol) with 4-nitrobenzoyl chloride (7.4g, 0.04mol) in pyridine (15ml) using 4-(*NN*-dimethylamino)pyridine (366mg, 0.003mol) as catalyst. When the reaction was complete (after 10 mins) it was poured onto an excess of 1M hydrochloric acid and crushed ice. The solid was filtered off and recrystallised from methanol. The yield after recrystallisation was 68%; m.pt. 141°C, lit. 135-137°C, [210]; NMR $CDCl_3$ δ 2.45 (3H s $COCH_3$) 3.83 (3H s OMe) 6.66 (1H d $J_{3,5}$ 2 Hz 3) 6.76 (1H dd $J_{5,6}$ 9 Hz $J_{5,3}$ 2 Hz 5) 7.80 (1H d $J_{6,5}$ 9 Hz 6) 8.28 (4H s 2',3',5',6').

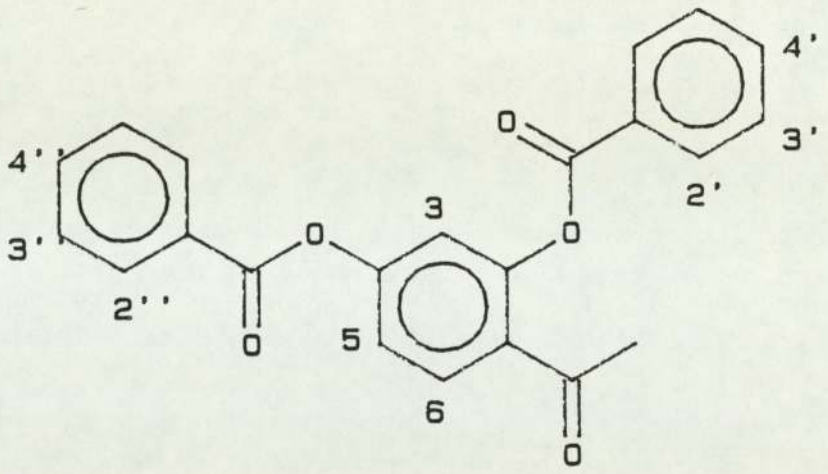
The following compounds were made in an analogous manner:

2-benzoyloxy-4,6-dimethoxyacetophenone (**19**) in 70% yield, recrystallised from $CHCl_3$/60-80 Pet. ether, m.pt. 92-93;

found C67.72%, H5.38%, $C_{17}H_{16}O_5$ requires C67.99%, H5.37%; NMR $CDCl_3$ δ 2.41 (3H s $COCH_3$) 3.73 (3H s OMe) 3.75 (3H s OMe) 6.32 (2H s 3,5) 7.3-7.7 (3H m 3',4',5') 8.05 (2H dd $J_{2',3'}$ 7Hz $J_{2',4'}$ 2 Hz 2's); m/z, m^+300, 285, 105 (100%), 77.

2-benzoyloxy-4-methoxy acetophenone (**20**) in 84% yield, a yellow oil, m/z m^+ obs. 270.0891, $C_{16}H_{14}O_4$ requires 270.0980, 105 (100%), 77.0397; NMR $CDCl_3$ δ 2.45 (3H s $COCH_3$) 3.78 (3H s OMe) 6.70 (1H d $J_{3,5}$ 2 Hz 3) 6.75 (1H dd $J_{5,3}$ 8 Hz J 2 Hz 5) 7.53 (3H m 3',4' 5') 7.81 (1H d $J_{6,5}$ 8 Hz 6) 8.15 (2H dd $J_{2',3'}$ 9 Hz $J_{2',4'}$ 2Hz 2's).

2-(4-nitrobenzoyloxy)-4,6-dimethoxyacetophenone (**21**) in 67% yield, recrystallised from ethyl acetate / 60-80 pet. ether; m.pt. 159-160°C; found C 59.16%, H 4.37%, N 4.05%, $C_{17}H_{15}NO_7$ requires C 59.13%, H 4.37%, N 4.05%; NMR $CDCl_3$ δ 2.45 (3H s $COCH_3$) 3.80 (3H s OMe) 3.86 (3H s OMe) 6.31 (1H d $J_{3,5}$ 2 Hz 3) 6.38 (1H d $J_{5,3}$ 2 Hz 5) 8.26 (4H s 2',3',5',6'); m/z, m^+ 345, 330 (100%), 314, 286, 256, 150, 137, 104, 76.



2,4-dibenzoyloxyacetophenone (22) was prepared by acylation of 2,4-dihydroxyacetophenone (7.6g, 0.05mol) with benzoyl chloride (16.8g, 0.12mol) in pyridine (20ml). When the reaction was complete the mixture was poured onto an excess of 1M hydrochloric acid and crushed ice. The product was filtered off and recrystallised from methanol. The final yield was 83%; m.pt. 85.2 °C; found C 73.24%, H 4.47%, $C_{22}H_{13}O_5$ requires C 73.32%, H 4.47%; NMR $CDCl_3$ δ 2.53 (3H s $COCH_3$) 7.18 (1H d $J_{3,5}$ 2 Hz 3) 7.37 (1H dd $J_{5,6}$ 8 Hz $J_{5,3}$ 2 Hz 5) 7.63-7.40 (6H m 3',4',5',3'',4'',5'') 7.90 (1H d $J_{6,5}$ 8 Hz 6) 8.26-8.06 (4H m 2',6',2'',6''); m/z, m^+ 360, 105 (100%), 77, 52, 43.

The following compounds were made in an analogous manner:

2,6-dibenzoyloxyacetophenone (23) in 63% yield; m.pt. 108°C; found C 73.32%, H 4.58%, $C_{22}H_{13}O_5$ requires C 73.32%, H 4.47%; NMR $CDCl_3$ δ 2.45 (3H s $COCH_3$) 7.7-7.1 (9H m 3, 4, 5, 3', 4', 5', 3'', 4'', 5'') 8.14 (4H m 2', 2'', 6', 6''); m/z, m^+ 360, 105 (100%), 77.

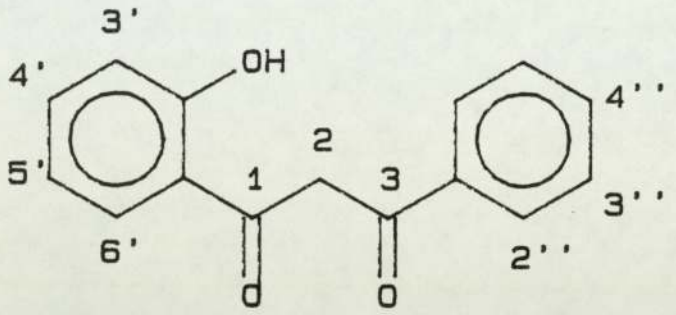
2,4-di(4-methoxybenzoyloxy)acetophenone (**24**) in 72% yield; m.pt. 119°C; found C 68.38%, H 4.79%, $C_{24}H_{20}O_7$ requires C 68.56%, H 4.79%; NMR (400MHz) $CDCl_3$ δ 2.54 (3H s $COCH_3$) 3.880 (3H s OMe) 3.882 (3H s OMe) 6.97 (2H d $J_{3',4'}$ or $_{3'',4''}$ 9 Hz 3',5' or 3'',5'') 7.17 (1H d $J_{3,5}$ 2 Hz 3) 7.24 (1H dd $J_{5,6}$ 9 Hz $J_{5,3}$ 2 Hz 5) 7.93 (1H d $J_{6,5}$ 9 Hz 6) 8.12 (2H d $J_{2'',3''}$ 9 Hz 2''s) 8.14 (2H d $J_{2',3'}$ 9 Hz 2',6'); m/z m^+ 360, 105 (100%), 77, 52, 43.

2,4-di(4-nitrobenzoyloxy)acetophenone (**25**) in 74% yield, m.pt. 150°C; found C 58.49%, H 3.26%, N 6.39%, $C_{22}H_{14}O_9N_2$ requires C 58.67%, H 3.13%, N 6.22%; NMR (300MHz) $CDCl_3$ δ 2.57 (3H s $COCH_3$) 7.25 (1H d $J_{3,5}$ 2 Hz 3) 7.35 (1H dd $J_{5,6}$ 9Hz $J_{5,3}$ 2 Hz 5) 8.02 (1H d $J_{6,5}$ 9Hz 6) 8.364 (4H s 2', 3', 5', 6' or 2'', 3'', 5'', 6'') 8.368 (4H s 2', 3', 5', 6' or 2'', 3'', 5'', 6''); m/z, m^+ 450, 300, 149, 120 (100%), 105, 76, 43.

2,4-di(3-nitrobenzoyloxy)acetophenone (**26**) in 68% yield; m.pt. 186°C; found C 58.50%, H 3.21% N 6.50%, $C_{22}H_{14}O_9N_2$ requires C 58.67%, H 3.13%, N 6.22%; NMR (300MHz) $CDCl_3$ δ 2.59 (3H s $COCH_3$) 7.29 (1H d $J_{3,5}$ 2 Hz 3) 7.38 (1H dd $J_{5,6}$ 8.5 Hz $J_{5,3}$ 2 Hz 5) 7.70 (2H dt J 8Hz 5', 5'') 8.03 (1H d $J_{6,5}$ 8.5 Hz 6) 8.55-8.50 (6H m 2', 4', 6', 2'', 4'', 6''); m/z m^+ 450, 150 (100%), 104, 76, 43.

2,4-di(3-methoxybenzoyloxy)acetophenone (**27**) in 81% yield;

m.pt. 67-68°C; found C 67.68%, H 5.00%, $(C_{24}H_{20}O_7)_3MeOH$ requires C 67.79%, H 5.00%; NMR $CDCl_3$ δ 2.53 (3H s $COCH_3$ 3.83 (6H s 2OMe) 6.9-7.9 (11H m ArH); m/z, m^+ 420, 136 (100%), 108, 93.



1-(2-Hydroxy-4,6-dimethoxyphenyl)-3-phenylpropan-1,3-dione (28) was prepared from 2-benzoyloxy-4,6-dimethoxyacetophenone (3.45g, 0.01 mol) using lithium hexamethyldisilazide which was prepared *in situ* by adding n-butyl lithium (1.28g, 0.02mol) to hexamethyldisilazane (3.22g, 0.02 mol) in THF at -78°C under N_2; 2-benzoyloxy-4,6-dimethoxyacetophenone was added to the base in THF (20mls) gradually over 15 mins. The reaction was then allowed to reach room temperature and was then quenched with an excess of 1M hydrochloric acid. The solid was collected and washed with water before being recrystallised from methanol. The yield after recrystallisation was 63%, m.pt. 151°C; found C 67.96%, H 5.28%; $C_{17}H_{16}O_5$ requires C 67.99%, H 5.37%; NMR $CDCl_3$ δ 3.43 (3H s OMe) 3.78 (3H s OMe) 4.51 (2H s $COCH_2CO$ 5.78 (1H d $J_{3',5'}$ 2 Hz 3') 6.02 (1H d $J_{5',3'}$ 2 Hz 5') 7.4-7.6 (3H m 3'', 4'', 5'') 7.8-8.0 (2H m 2'',

6'') 13.67 (1H s OH); m/z m^+ 300, 283, 269, 181, 105 (100%), 77.

The following compounds were prepared in an analogous manner:-

1-(2-Hydroxy-4-methoxyphenyl)-3-phenylpropan-1,3-dione (**29**) in 37% yield, m.pt. 138°C; found C 71.10%, H 4.97%, $C_{16}H_{14}O_4$ requires C 71.10%, H 5.22%; NMR $CDCl_3$ δ 3.76 (3H s OMe) 4.5 (0.16H br.s $COCH_2CO$) 6.42 (2H dd $J_{5',6'}$ 9 Hz $J_{5',3'}$ 2 Hz 5') 6.53 (1H d $J_{3',5'}$ 2 Hz 3') 6.62 (0.88H s COCHCO) 7.4-7.6 (3H m 3',4',5') 7.60 (1H d $J_{6,5}$ 9 Hz 6) 7.7-7.9 (2H m 2',6') 12.5 (1H s phenolic OH) 15.4 (0.88H s enolic OH); m/z m^+ 300, 283, 269, 181, 105 (100%), 77.

1-(2-Hydroxy-4-methoxyphenyl)-3-(4-nitrophenyl)propan-1,3-dione (**30**) in 48% yield; m.pt. 200°C; found C 60.71%, H 4.13%, N 4.44%, $C_{16}H_{13}NO_6$ requires C 60.95%, H 4.15%, N 4.44%; NMR (300MHz) $CDCl_3$ δ 4.61 (0.08H s $COCH_2CO$) 6.48 (1H d $J_{3',5'}$ 2.44 Hz 3') 6.51 (1H dd $J_{5',6'}$ 8.9 Hz $J_{5',3'}$ 2.4 Hz 5') 6.78 (0.92H s COCHCO) 7.70 (1H d $J_{6',5'}$ 8.9 Hz 6') 8.08 (2H d $J_{2'',3''}$ 8.8 Hz 2''s) 8.33 (2H d $J_{3'',2''}$ 8.8 Hz 3''s) 12.20 (0.08H s phenolic OH of keto form) 12.44 (0.92H s phenolic OH of enol form) 15.2 (0.92H s enolic OH); m/z m^+ 315, 298, 285, 193, 150 (100%), 104, 76.

1-(2-Hydroxy-4,6-dimethoxyphenyl)-3-(4-nitrophenyl)propan-1,3-dione (31) in 53% yield; m.pt.192°C; found C 59.06%, H 4.40%, N 3.85%, $C_{17}H_{15}NO_7$ requires C 59.13%, H 4.37%, N 4.05%; NMR (300MHz) $CDCl_3$ δ 3.53 (0.6H s OMe keto) 3.82 (0.6H s OMe keto) 3.85 (2.4H s OMe enol) 3.94 (2.4H s OMe enol) 4.59 (0.4H s $COCH_2CO$ keto) 5.87 (0.2 H d $J_{5',3'}$ 2.4 Hz 5'keto) 6.006 (0.8H d $J_{5',3'}$ 2.4 Hz 5'enol) 6.11 (0.2H d $J_{3',5'}$ 2.4 Hz 3' keto) 6.127 (0.8H d $J_{3',5'}$ 2.4 Hz 3' enol) 7.41 (0.8H s COCHCO) 8.03 (1.6H d $J_{2'',3''}$ 8.9Hz 2'' enol) 8.127 (0.4H d $J_{2'',3''}$ 8.9 Hz 2'' keto) 8.317 (1.6H d $J_{3'',2''}$ 8.9 Hz 3'' enol) 8.36 (0.4H d $J_{3'',2''}$ 8.9 HZ 3'' keto) 13.33 (0.8H phenolic OH of enol form) 13.55 (0.2H phenolic OH of Keto form) 15.26 (0.2H enolic OH); m/z, m^+ 345, 328, 314, 181 (100%), 167, 150, 137, 121, 104.

1-(2-Hydroxyphenyl)-3-phenylpropan-1,3-dione (32) was prepared by adding potassium hydroxide (1.7g, 0.03 mol), which had been finely powdered in a pre-heated mortar, to 2-benzoyloxyacetophenone (4.8g, 0.02 mol) in pyridine (50mls)at 50°C, and the mixture was then allowed to stir for 15-20mins. The mixture was then poured into a slight excess of 1M hydrochloric acid. from methanol. Yield after recrystallisation was 63%, m.pt. 124°C, lit. 117-120°C [211]; NMR (400MHz) $CDCl_3$ δ 4.63 (0.13H s $COCH_3CO$), 6.84 (0.94H s COCHCO) 6.92 (1H ddd $J_{5',4'}$ 7.0 Hz $J_{5',6'}$ 8.0 Hz $J_{5',3'}$ 1.1 Hz 5') 7.00 (1H dd $J_{3',4'}$ 8.3 Hz $J_{3',5'}$ 1.1 Hz 3') 7.46 (1H

ddd $J_{4',5'}$ 7.0 Hz $J_{4',3'}$ 8.3 Hz $J_{4',6'}$ 1.6 Hz 4') 7.48 (2H dd $J_{2'',3''}$ 8.0Hz $J_{3'',4''}$ 7.3 Hz 3'') 7.56 (1H tt $J_{4'',3''}$ 7.3 Hz $J_{4'',2''}$ 1.6 Hz 4'') 7.786 (1H dd $J_{6',5'}$ 8.0 Hz $J_{6',4'}$ 1.6 Hz 6') 7.94 (2H dd $J_{2'',3''}$ 8.0 Hz $J_{2'',4''}$ 1.6 Hz 2'') 11.93 (0.06H s phenolic OH of keto form) 12.10 (0.94H s phenolic OH of enol form) 15.54 (0.94H s enolic OH).

The following compounds were prepared in an analogous manner:-

1-(2-Hydroxyphenyl)-3-(4-chlorophenyl)propan-1,3-dione (**33**) in 43% yield, m.pt. 120°C, lit. 122.4°C [209]; NMR $CDCl_3$ δ 6.7 (1H s COCHCO) 6.8-7.4 (3H m 3',4',5') 7.37 (2H d $J_{2'',3''}$ 8 Hz 3''s) 7.66 (1H dd $J_{6',5'}$ 7 Hz $J_{6',4'}$ 1 Hz 6) 7.79 (2H d $J_{3'',2''}$ 8 Hz 2''s) 11.95 (1H s phenolic OH) 15.5 (1H br.s enolic OH).

1-(2-hydroxyphenyl)-3-(3-chlorophenyl)propan-1,3-dione (**34**) in 56% yield, m.pt. 117-118°C, lit. 115°C [212]; NMR (400MHz) $CDCl_3$ δ 4.61 (0.1H s $COCH_2CO$) 6.80 (0.95H s COCHCO) 6.93 (1H ddd $J_{5',4'}$ 7.1 Hz $J_{5',6'}$ 8.1 Hz $J_{5',3'}$ 1.0 Hz 5') 7.0 (1H dd $J_{3',4'}$ 8.2 Hz $J_{3',5'}$ 1.0 Hz 3') 7.42 (1H dd $J_{5'',6''}$ 8.3 Hz $J_{5'',4''}$ 7.9 Hz 5'') 7.50 (1H ddd $J_{6'',5''}$ 8.3 Hz $J_{6'',4''}$ 1.0 Hz $J_{6'',2''}$ 2.0 Hz 6'') 7.60 (1H ddd $J_{4',5'}$ 7.1 Hz $J_{4',3'}$ 8.2 Hz $J_{4',6'}$ 1.5 Hz 4') 7.79 (1H dd $J_{6',5'}$ 8.12 Hz $J_{6',4'}$ 1.5 Hz 6') 7.80 (1H ddd $J_{4'',5''}$ 7.9 Hz $J_{4'',6''}$ 1.0 Hz $J_{4'',2''}$ 2 Hz 4'') 7.89 (1H t J 2.0 Hz 2'') 12.00 (1H s phenolic OH) 15.43 (1H s enolic OH).

1-(2-hydroxyphenyl)-3-(3,4-dichlorophenyl)propan-1,3-dione (35) in 47% yield, m.pt. 155-7°C, found C 58.10%, H 3.18%, $C_{15}H_{10}Cl_2O_3$ requires C 58.27%, H 3.26%; NMR (400MHz) $CDCl_3$ δ 4.59 (0.14H s $COCH_2CO$) 6.78 (0.93H s COCHCO) 6.93 (1H ddd $J_{5',4'}$ 7.1 Hz $J_{5',6'}$ 8.16 Hz $J_{5',3'}$ 1.0 Hz 5') 7.00 (1H dd $J_{3',4'}$ 8.4 Hz $J_{3',5'}$ 1 Hz 3') 7.48 (1H ddd $J_{4',5'}$ 7 Hz $J_{4',3'}$ 8.4 Hz $J_{4',6'}$ 1.5 Hz 4') 7.56 (1H d $J_{5'',6''}$ 8.5 Hz 5'') 7.75 (1H dd $J_{6'',5''}$ 8.5 Hz $J_{6'',2''}$ 2.1 Hz 6'') 7.77 (1H dd $J_{6',5'}$ 8.16 Hz $J_{6',4'}$ 1.5 Hz 6') 8.00 (1H d $J_{6'',2''}$ 2.1 Hz 2'') 11.80 (0.07H s phenolic OH keto) 11.95 (0.93H s phenolic OH enol) 15.43 (0.93H s enolic OH); m/z, m^+ 308/310/312, 173/175/177 (100%), 121.

1-(2-hydroxyphenyl)-3-(4-bromophenyl)propan-1,3-dione (36) in 42% yield, m.pt. 142°C; found C 56.75%, H 3.54%, $C_{15}H_{11}BrO_3$ requires C 56.45%, H 3.47%; NMR (400MHz) $CDCl_3$ δ 4.59 (0.18H s $COCH_2CO$) 6.79 (0.9H s COCHCO) 6.92 (1H ddd $J_{5',4'}$ 7.0 Hz $J_{5',6'}$ 8.1 Hz $J_{5',3'}$ 1Hz 5') 6.99 (1H dd $J_{3',4'}$ 8.5 Hz $J_{3',5'}$ 1 Hz 3') 7.46 (0.91H ddd $J_{4',5'}$ 7.0 Hz $J_{4',3'}$ 8.5 Hz $J_{4',6'}$ 1.6 Hz 4' keto) 7.49 (0.09H ddd $J_{4',5'}$ 7.0 Hz $J_{4',3'}$ 8.5 Hz $J_{4',6'}$ 1.6 Hz 4' enol) 7.61 (1.82H d $J_{3'',2''}$ 8.6 Hz 3'' enol) 7.63 (0.18H d $J_{3'',2''}$ 8.5 Hz 3'' keto) 7.75 (0.91 H dd $J_{6',5'}$ 8.1 Hz $J_{6',4'}$ 1.6 Hz 6' enol) 7.78 (1.82H d $J_{2'',3''}$ 8.6 Hz 2'' enol) 7.79 (0.09H dd $J_{6',5'}$ 8.1 Hz $J_{6',4'}$ 1.6 Hz 6' keto) 7.86 (0.18H d $J_{2'',3''}$ 8.5 Hz 2'' keto) 11.86 (0.09H s phenolic OH keto) 12.02 (0.91H s phenolic OH enol) 15.48

(0.91H s enolic OH).

1-(2-Hydroxyphenyl)-3-(4-chloro-3-nitrophenyl)propan-1,3-dione (37) in 54% yield, m.pt. 196°C, lit. 189-190°C [213]; NMR (300MHz) $CDCl_3$ δ 4.65 (0.2H s $COCH_2CO$) 6.84 (0.9H s COCHCO) 6.94 (1H ddd $J_{5',4'}$ 7.1 Hz $J_{5',6'}$ 8.1 Hz $J_{5',3'}$ 1 Hz 5') 7.01 (1H dd $J_{3',4'}$ 8.4 Hz $J_{3',5'}$ 1 Hz 3') 7.50 (1H ddd $J_{4',5'}$ 7.1 Hz $J_{4',3'}$ 8.4 Hz $J_{4',6'}$ 1.47 Hz 4') 7.67 (1H d $J_{5'',6''}$ 8.5 Hz 5'') 7.78 (1H dd $J_{6',5'}$ 8.1 Hz 6') 8.05 (1H dd $J_{6'',5''}$ 8.5 Hz $J_{6'',2''}$ 2.2 Hz 6'') 8.40 (1H d $J_{6'',2''}$ 2.2 Hz 2'') 11.85 (0.9Hz s phenolic OH) 15.40 (0.9H s enolic OH).

1-(2-hydroxyphenyl)-3-(4-methoxyphenyl)propan-1,3-dione (38) in 66% yield; m.pt 111°C, lit 111°C [211]; NMR $CDCl_3$ δ 3.8 (3H s OMe) 4.55 (0.2H s $COCH_2CO$) 6.7 (0.9H s COCHCO) 6.93 (2H d $J_{3'',2''}$ 10 Hz 3''s) 7.2-7.8 (4H m 3',4',5',6') 7.87 (2H d $J_{2'',3''}$ 10 Hz 2''s).

1-(2-hydroxyphenyl)-3-(3-methoxyphenyl)propan-1,3-dione (39) in 70% yield; m.pt. 84-85°C; found C 70.74%, H 5.26%, $C_{16}H_{14}O_3$ requires C 71.10%, 5.22%; NMR (400MHz) $CDCl_3$ δ 3.878 (3H s OMe) 4.615 (0.22H s $COCH_2CO$) 6.815 (0.89H s COCHCO) 6.917 (1H ddd $J_{5,4}$ 7 Hz $J_{5,6}$ 8.1 Hz $J_{5,3}$ 1.0 Hz 5) 7.00 (1H dd $J_{3,4}$ 8.4 Hz $J_{3,5}$ 1.0 Hz 3) 7.086 (1H ddd $J_{6',5'}$ 8.0 Hz $J_{6',2'}$ 0.7 Hz $J_{6',4'}$ 2.5 Hz 6') 7.39 (1H dd $J_{5',6'}$ 8.0 Hz $J_{5',4'}$ 7.8 Hz 5') 7.460 (1H dd $J_{2',6'}$ 0.7 Hz $J_{2',4'}$ 1.4 Hz 2') 7.463 (1H ddd $J_{4,5}$ 7 Hz $J_{4,3}$ 8.4 Hz $J_{4,6}$ 1.6 Hz 4) 7.50 (1H ddd J

$J_{4',5'}$ 7.8 Hz $J_{4',6'}$ 2.5 Hz $J_{4',2'}$ 1.4 Hz 4') 7.768 (1H dd $J_{6,4}$ 1.6 Hz $J_{5,6}$ 8.1 Hz 6) 11.933 (0.11H s phenolic OH of keto form) 12.077 (0.89H s phenolic OH of enol form) 15.54 (0.89 s enolic OH); m/z, m^+ 254 (100%), 163, 135, 121, 92.

1-(2-hydroxyphenyl)-3-(3,4-dimethoxyphenyl)propan-1,3-dione (40) in 62% yield, m.pt. 115°C, lit. 115°C [214]; NMR (400MHz) $CDCl_3$ δ 3.90 (0.63H s OMe keto) 3.94 (0.63H s OMe keto) 3.945 (2.37H s OMe enol) 3.96 (2.37H s OMe enol) 4.58 (0.42H s $COCH_2CO$) 6.75 (0.79H s COCHCO) 6.90 (0.79H ddd $J_{4',5'}$ 7.0 Hz $J_{5',6'}$ 8.1 Hz $J_{5',3'}$ 1.0 Hz 5' enol) 6.91 (0.21H ddd $J_{5',4'}$ 7.0 Hz $J_{5',6'}$ 8.0 Hz $J_{5',3'}$ 1.0 Hz 5' keto) 6.913 (0.21H d J 8.5Hz 5'' keto) 6.925 (0.79H d $J_{5'',6''}$ 8.5 Hz 5'' enol) 6.973 (0.21H dd $J_{3',4'}$ 8.6 Hz $J_{3',5'}$ 1.0Hz 3' keto) 6.975 (0.79H dd $J_{3',4'}$ 8.4 Hz $J_{3',5'}$ 1.0 Hz 3' enol) 7.435 (0.79H ddd $J_{4',5'}$ 7.0 Hz $J_{4',3'}$, 8.4 Hz $J_{4',6'}$ 1.5 Hz 4' enol) 7.44 (1H d $J_{2'',6''}$ 2.1 Hz 2'') 7.47 (0.21H ddd $J_{4',5'}$ 7.0 Hz $J_{4',3'}$ 8.6Hz $J_{4',6'}$ 1.6 Hz 4' keto) 7.56 (0.79H dd $J_{6'',5''}$ 8.5Hz $J_{6'',2''}$ 2.1 Hz 6'' enol) 7.63 (0.21H dd $J_{6'',5''}$ 8.3 Hz $J_{6'',2''}$ 2.0 Hz 6'' keto) 7.75 (0.79H dd $J_{6',5'}$ 8.1Hz $J_{6',4'}$ 1.5 Hz 6' enol) 7.80 (0.21H dd $J_{6',5'}$ 8.0 Hz $J_{6',4'}$ 1.6 Hz 6' keto) 11.96 (0.21H s phenolic OH keto) 12.09 (0.79H s phenolic OH enol) 15.85 (0.79H s enolic OH).

1-(2-hydroxyphenyl)-3-(3-nitrophenyl)propan-1,3-dione (41) in 72% yield; m.pt. 150°C, lit. 157°C [208]; NMR (400MHz) $CDCl_3$ δ 4.70 (0.26H s $COCH_2CO$) 6.90 (0.87H s COCHCO) 6.95 (1H ddd $J_{5',6'}$ 8.1 Hz $J_{5',4'}$ 7.0 Hz $J_{5',3'}$ 1 Hz 5') 7.01 (1H dd $J_{3',4'}$ 8.5 Hz $J_{3',5'}$ 1 Hz 3') 7.50 (1H ddd $J_{4',5'}$ 7.0 Hz $J_{4',3'}$ 8.5 Hz $J_{4',6'}$ 1.5 Hz 4') 7.69 (1H t J 8.0 Hz 5'') 7.815 (1H dd $J_{6',5'}$ 8.1 Hz $J_{6',4'}$ 1.5 Hz 6') 8.27 (1H ddd J_o 8 Hz J_m 1.9 Hz J_m 1 Hz 4'' or 6'') 8.38 (1H ddd J_o 8 Hz J_m 1.9 Hz J_m 1 Hz 4'',6'') 8.74 (1H t J 1.9 Hz 2'') 11.9 (1H br.s phenolic OH) 15.43 (0.87H br.s enolic OH).

1-(2-hydroxyphenyl)-3-(4-nitrophenyl)propan-1,3-dione (42) in 58% yield; m.pt. 195°C, lit. 198-201°C [215]; NMR (400MHz) $CDCl_3$ δ 6.90 (1H s COCHCO) 6.95 (1H ddd $J_{5,'4'}$ 7.1 Hz $J_{5',6'}$ 8.1 Hz $J_{5',3'}$ 1.0 Hz 5') 7.03 (1H dd $J_{3',4'}$ 8.5 Hz $J_{3',5'}$ 1 Hz 3') 7.51 (1H ddd $J_{4',5'}$ 7.1 Hz $J_{4',3'}$ 8.5 Hz $J_{4',6'}$ 1.6 Hz 4') 7.79 (1H dd $J_{6',5'}$ 8.1 Hz $J_{6',4'}$ 1.6 Hz 6') 8.09 (2H d $J_{2'',3''}$ 8.9 Hz 2'') 8.33 (2H d $J_{3'',2''}$ 8.9 Hz 3'') 11.92 (1H s phenolic OH) 15.30 (1H s enolic OH).

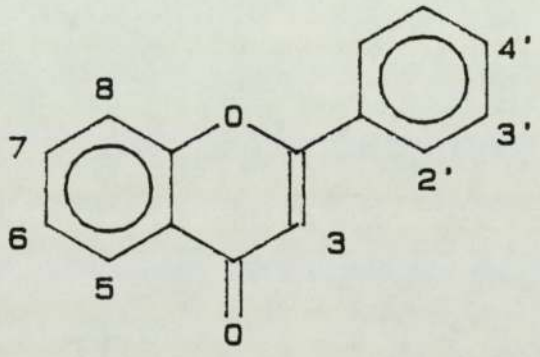
1-(2-hydroxyphenyl)-3-(3-methoxy-4-nitrophenyl)propan-1,3-dione (43) in 54% yield; m.pt. 188-189°C; found C 60.55%, H 4.21%, N 4.16%; $C_{16}H_{13}NO_6$ requires C 60.95%, H 4.15%, N 4.44%; NMR (300MHz) $CDCl_3$ δ 4.05 (3H s OMe) 6.84 (1H s COCHCO) 6.94 (1H dd J_o 8 Hz J_o 7.2 Hz 5') 7.02 (1H d J 8.3 Hz 3') 7.48-7.52 (2H m 6'',4') 7.65 (1H d $J_{2'',6''}$ 1.2 Hz 2'') 7.77

(1H dd $J_{6',5'}$ 8 Hz $J_{6',4'}$ 1.4 Hz 6') 7.90 (1H d $J_{5'',6''}$ 8.5 Hz 5'') 11.89 (1H s phenolic OH) 15.38 (1H s enolic OH); m/z, m^+315, 180 (100%), 121, 76.

1-(2-hydroxyphenyl)-3-(4-methoxy-3-nitrophenyl)propan-1,3-dione (44) in 50% yield, m.pt. 171°C; found C 61.09%, H 4.28%, N 4.37%, $C_{16}H_{13}NO_6$ requires C 60.95%, H 4.15%, N 4.44%; NMR (400MHz) $CDCl_3$ δ 4.05 (3H s OMe) 4.61 (0.18H s $COCH_2CO$) 6.8 (0.9H s COCHCO) 6.94 (1H ddd $J_{5',3'}$ 1 Hz $J_{5',6'}$ 8.1 Hz $J_{5',4'}$ 7.2 Hz 5') 7.01 (1H dd $J_{3',4'}$ 8.4 Hz $J_{3',5'}$ 1 Hz 3') 7.19 (1H d $J_{5'',6''}$ 8.9 Hz 5'') 7.48 (1H ddd $J_{4',3'}$ 8.4 Hz $J_{4',5'}$ 7.2 Hz $J_{4',6'}$1.6Hz 4') 7.78 (1H dd $J_{6',5'}$ 8.1 Hz $J_{6',4'}$ 1.6 Hz 6') 8.14 (1H dd $J_{6'',5''}$ 8.9 Hz $J_{6'',2''}$ 2.3 Hz 6'') 8.42 (1H d $J_{2'',6''}$ 2.3 Hz 2'') 11.8 (0.1H phenolic OH, Keto form) 11.95 (0.9H phenolic OH enol form) 15.63 (0.9H enolic OH); m/z, m^+ 315, 298, 285, 180, 121 (100%), 76.

1-(2-hydroxy-4-benzoyloxyphenyl)-3-phenylpropan-1,3-dione (45) in 52% yield, m.pt. 171°C, lit. 167°C [216]; NMR (400MHz) $CDCl_3$ δ 4.63 (0.2H s $COCH_2CO$) 6.80 (0.9H s COCHCO) 6.84 (1H dd $J_{5,6}$ 8.9 Hz $J_{5,3}$ 2.3 Hz 5) 6.89 (1H d $J_{3,5}$ 2.3 Hz 3) 7.5 (5H m 3''s, 3'''s, 4''or 4''') 7.66 (1H tt J_o 7.4 Hz J_m1.3Hz 4'' or 4''') 7.85 (1H d J8.9Hz 6) 7.94 (2H dd J_o 8.4 Hz J_m 1.3 Hz 2'' or 2''') 8.19 (2H J_o 8.4 Hz J_m 1.2 Hz 2'' or 2''') 12.16 (0.1H s phenolic OH of keto form) 12.36 (0.9H s phenolic OH of enol) 15.45 (0.9H s enolic OH).

1-(2-hydroxy-6-benzoyloxyphenyl)-3-phenylpropan-1,3-dione (46) in 64% yield, m.pt. 184°C; found C 73.38%, H 4.52%, $C_{22}H_{16}O_5$ requires C 73.32%, H 4.47%; NMR (400MHz) $CDCl_3$ δ 6.75 (1H dd J_o 7.8 Hz J_m 0.9 Hz 3' or 5') 6.87 (1H s COCHCO) 6.95 (1H dd J_o 8.45 Hz, J_m 0.9Hz 3' or 5') 7.16 (1H dd J_o 8.11 J_o 7.6 3'' or 3''') 7.37 (1H tt J_o 7.3 Hz J_m 1.2 Hz 4'' or 4''') 7.40 (1H dd J_o 7.3 Hz J_m 1.2 Hz 2'' or 2''') 7.45 (1H t J 8.2Hz 4') 7.52 (1H dd J_o 7.9 Hz J_o 7.6 Hz 3'' or 3''') 7.68 (1H tt J_o 7.4 Hz J_m 1.17 Hz 4'' or 4''') 8.26 (1H dd J_o 8.3 Hz J_m 1.2 Hz 2'' or 2''') 11.91 (1H s phenolic OH) 15.73 (1H s enolic OH); m/z, m^+ 360, 238, 105 (100%), 77.



Flavone (47) was prepared by dissolving 1-(2-hydroxyphenyl)-3-phenylpropan-1,3-dione (2.4g, 0.01mol) in glacial acetic acid (30ml) which was then brought to boiling point. H_2SO_4 (1ml) was then added and the reaction heated for an hour. On completion of the reaction a fluorescent spot is visible on t.l.c. with R.f. 0.2 in $CHCl_3$. The reaction mixture was poured onto crushed ice and allowed to reach room temperature. The solid was filtered, washed repeatedly with water, dried and

then recrystallised from 60-80 pet. ether to give fine white crystals. m.pt. 94°C, lit. 96-97°C [168]; NMR $CDCl_3$ δ 6.73 (1H s 3) 7.66-7.20 (6H m 3',4',5',6,7,8) 7.93-7.76 (2H m 2',6') 8.20 (1H m $J_{5,6}$ 8 Hz 5).

The following compounds were made in an analogous manner:

2-cyclohexylchromone (48) in 46% yield as a brown oil; NMR (300MHz) $CDCl_3$ δ 1.25-2.08 (10H m cyclohexyl protons) 2.48-2.56 (1H m 1') 6.18 (1H s 3) 7.36 (1H ddd $J_{6,5}$ 8.0 Hz $J_{6,7}$ 7.0 Hz $J_{6,8}$ 1.0Hz 6) 7.42 (1H ddd $J_{8,7}$ 8.5 Hz $J_{8,6}$ 1.0 Hz 8) 7.64 (1H ddd $J_{8,7}$ 8.5 Hz $J_{7,6}$ 7.0 Hz $J_{7,5}$ 1.7 Hz 7) 8.17 (1H dd $J_{5,6}$ 8.0 Hz $J_{5,7}$ 1.7 Hz 5); m/z m^+ obs. 228.1147 (100%), $C_{15}H_{16}O_2$ requires 228.1150, 213, 199, 187, 173, 160, 147, 121, 92, 77; max. 1740, 1610 cm^{-1}.

4'-methoxyflavone (49) in 64% yield; recrystallised from 60-80 pet.ether; m.pt. 161°C, lit 156-157°C [217]; NMR $CDCl_3$ δ 6.68 (1H s 3) 6.96 (2H d $J_{3',2'}$ 9 Hz 3',5') 7.60-7.20 (3H m 6,7,8) 7.81 (2H d $J_{2',3'}$ 9 Hz 2'6') 8.18 (1H d $J_{5,6}$ 8 Hz 5).

3'-methoxyflavone (50) in 60% yield, recrystallised from ethyl acetate; m.pt. 132-133 °C, lit. 130-131°C [217]; NMR $CDCl_3$ δ 3.66 (3H s OMe) 6.7 (1H s 3) 7.82-6.82 (7H m 2',4',5',6',6,7,8) 8.16 (1H brd $J_{5,6}$ 8 Hz 5).

3',4'-dimethoxyflavone (51) in 73% yield, recrystallised from ethyl acetate; m.pt.155°C, lit. 155°C [214]; NMR $CDCl_3$ δ 3.88 (6H s OMe's) 6.63 (1H s 3) 6.88 (1H d $J_{5',6'}$ 8 Hz 5') 7.66-7.16 (5H m 2', 6', 6, 7, 8) 8.15 (1H brd $J_{5,6}$ 7 Hz 5).

3'-fluoroflavone (52) in 72% yield; recrystallised from 60-80 pet. ether; m.pt. 106-107°C, lit. 101-102°C [227]; NMR (300MHz) $CDCl_3$ δ 6.79 (1H s 3) 7.23 (1H dddd $J_{HH\ 5',6'}$ 8.3 Hz $J_{HH\ m}$ 2.6 Hz $J_{HH\ m}$ 1.0 Hz $J_{HF\ 4',5'}$ 8.3 Hz 4') 7.42 (1H ddd $J_{HH\ 6,5}$ 8.0 Hz $J_{HH\ 6,7}$ 7.0 Hz $J_{HH\ 6,8}$ 1.0 Hz 6) 7.49 (1H ddd $J_{HH\ 5',6'}$ 8.4 Hz $J_{HH\ 5',4'}$ 8.3 Hz J_{HF} 5',3' 5.8 Hz 5') 7.56 (1H dd $J_{HH\ 8,7}$ 8.6 Hz $J_{HH\ 8,6}$ 1.0 Hz 8) 7.62 (1H ddd $J_{HH\ m}$ 2.6 Hz $J_{HH\ m}$ 1.0 Hz $J_{HF\ 2',3'}$ 9.7 Hz 2') 7.69 (1H ddd $J_{HH\ m}$ 2.6 Hz $J_{HH\ m}$ 1.0 Hz J_{HH} 6',5' 8.4 Hz 6') 7.70 (1H ddd $J_{HH\ 7,6}$ 7.0 Hz $J_{HH\ 7,8}$ 8.6 Hz $J_{HH\ 7,5}$ 1.7 Hz 7) 8.22 (1H dd $J_{HH\ 5,6}$ 8.0 Hz $J_{HH\ 5,7}$ 1.7 Hz 5).

3'-chloroflavone (53) in 64% yield; m.pt. 128°C; NMR (300MHz) $CDCl_3$ δ 6.75 (1H s 3) 7.40 (1H ddd $J_{6,5}$ 8.0 Hz $J_{6,7}$ 7.0 Hz $J_{6,8}$ 1.0 Hz 6) 7.42 (1H dd $J_{4',5'}$ 8.12 Hz $J_{',6'}$ 7.4 Hz 5') 7.45 (1H ddd $J_{4',5'}$ 8.12 Hz J_{m} circa 1.7 Hz J_{m} circa 1.5 Hz 4') 7.54 (1H dd $J_{8,7}$ 8.6 Hz $J_{8,6}$ 1.0 Hz 8) 7.68 (1H ddd $J_{7,8}$ 8.6 Hz $J_{7,6}$ 7.0 Hz $J_{7,5}$ 1.6 Hz 7) 7.74 (1H ddd $J_{6',5'}$ 7.4 Hz J_{m} circa 1.7 Hz J_{m} circa 1.5 Hz 6') 7.87 (1H dd J_{m} circa 1.7 Hz and 1.5 Hz 2') 8.18 (1H dd $J_{5,6}$ 8.0 Hz $J_{5,7}$ 1.6 Hz 5); λ max. ($CHCl_3$) 290, 255, lit. 288.3, 254.5 [228].

4'-chloroflavone (54) in 73% yield, recrystallised from ethyl acetate; m.pt. 192-193°C, lit. 188-189°C [209]; NMR $CDCl_3$ δ 6.7 (1H s 3) 7.61-7.1 (3H m 6,7,8) 7.43 (2H d $J_{2',3'}$ 10 Hz 2',6') 7.8 (2H d $J_{3',2'}$ 10 Hz 3',5') 8.18 (1H dd $J_{5,6}$ 6 Hz $J_{5,7}$ 2 Hz 5).

3',4'-dichloroflavone (55) in 63% yield, m.pt. 217°C, found C 62.11%, H 2.83%, $C_{15}H_8Cl_2O_2$ requires C 61.11%, H 2.83%; NMR (300MHz) $CDCl_3$ δ 6.77 (1H s 3) 7.43 (1H ddd $J_{6,7}$ 8.1 Hz $J_{6,5}$ 7.9 Hz $J_{6,8}$ 1 Hz 6) 7.57 (1H dd $J_{6,8}$ 1 Hz $J_{8,7}$ 7.3 Hz 8) 7.60 (1H d $J_{5',6'}$ 8.3 Hz 5') 7.72 (1H ddd $J_{7,6}$ 8.1 Hz $J_{7,8}$ 7.3Hz $J_{7,6}$ 1.6 Hz 7) 7.73 (1H dd $J_{2',6'}$ 2.1 Hz $J_{6',5'}$ 8.3 Hz 6') 8.03 (1H d $J_{2',6'}$ 2.1 Hz 2') 8.22 (1H dd $J_{5,7}$ 1.6 Hz $J_{5,6}$ 7.9 Hz 5); m/z m^+ 290/292, 262/264, 199/201, 170/172, 120 (100%), 92.

4'-bromoflavone (56) in 73% yield, m.pt. 184.5-186°C, lit. 178°C [225]; found C 59.65%, H 3.12%, $C_{15}H_9BrO_2$ requires C 59.82%, H 3.01%; NMR (400MHz) $CDCl_3$ δ 6.75 (1H s 3) 7.38 (1H ddd $J_{7,6}$ 7.0 Hz $J_{6,5}$ 8.0 Hz $J_{6,8}$ 6) 7.52 (1H dd $J_{8,7}$ 8.6Hz $J_{8,6}$ 1 Hz 8) 7.6 (2H d $J_{2',3'}$ 8.7 Hz 2's or 3's) 7.67 (1H ddd $J_{7,8}$ 8.6 Hz $J_{7,6}$ 7.0Hz $J_{7,5}$ 1.6 Hz 7) 7.74 (1H d $J_{3',2'}$ 8.7 Hz 2's or 3's) 8.12 (1H dd $J_{5,6}$ 8.0 Hz $J_{5,7}$ 1.6 Hz 5).

7-methoxyflavone (57) in 62% yield, recrystallised from 60-80 pet.ether; m.pt.110°C, lit. 107-108°C [218]; NMR $CDCl_3$ δ 3.86 (3H s OMe) 6.66 (1H s 3) 6.80-7.00 (2H m 6,8) 7.4-7.55 (3H m

3',4',5') 7.65-7.9 (2H m 2',6') 8.05 (1H d $J_{5,6}$ 8 Hz 5).

4'-nitroflavone (58) in 72% yield, recrystallised from ethyl acetate; m.pt. 242°C, lit. 244-6°C [215]; NMR (300MHz) $CDCl_3$ δ 6.89 (1H s 3) 7.45 (1H ddd $J_{6,7}$ 7.0 Hz $J_{6,5}$ 8.0 Hz $J_{6,8}$ 1.0 Hz 6) 7.60 (1H dd $J_{8,7}$ 8.6 Hz $J_{8,6}$ 1.0 Hz 8) 7.74 (1H ddd $J_{7,6}$ 7.0 Hz $J_{7,8}$ 8.6 Hz $J_{7,5}$ 1.6 Hz 7) 8.10 (2H d $J_{2',3'}$ 8.3 Hz 2's) 8.23 (1H dd $J_{5,6}$ 8.0 Hz $J_{5,7}$ 1.6 Hz 5) 8.37 (2H d $J_{3',2'}$ 8.3 Hz 3's).

3'-nitroflavone (59) in 71% yield, recrystallised from ethoxyethanol/water; m.pt. 197°C, lit. 201-202 °C [219]; NMR (300MHz) $CDCl_3$ δ 6.90 (1H s 3) 7.46 (1H ddd $J_{5,6}$ 7.96 Hz, $J_{6,7}$ 7.05 Hz, $J_{6,8}$ 1.0 Hz, 6) 7.63 (1H dd $J_{7,8}$ 8.49 Hz, $J_{8,6}$ 1.0 Hz 8) 7.73 (1H dd $J_{4',5'}$ 8.1 Hz $J_{5',6'}$ 8.0 Hz 5')7.76 (1H ddd $J_{7,8}$ 8.49 Hz, $J_{7,6}$ 7.05 Hz, $J_{7,5}$ 1.7 Hz, 7) 8.22 (1H ddd $J_{5',6'}$ 8.0 Hz, $J_{6'2'}$ 1.8 Hz, $J_{6'4'}$ 1.0 Hz 6') 8.39 (1H ddd $J_{4',5'}$ 8.1 Hz, $J_{4',6'}$ 1.0 Hz, $J_{4',2'}$, 2.2 Hz 4') 8.81 (1H ddd $J_{2',4'}$ 2.2 Hz, $J_{2',6'}$ 1.0 Hz 2').

4'-methoxy-3'-nitroflavone (60) in 95% yield, recrystallised from ethyl acetate; m.pt. 229°C; found C 64.58%, H 3.73%, N 4.89%, $C_{16}H_{11}NO_5$ requires C 64.64%, H 3.72%, N 4.71%; NMR (300MHz) $CDCl_3$ δ 4.05 (3H s OMe) 6.76 (1H s 3) 7.23 (1H d $J_{5',6'}$ 8.80 Hz 5') 7.44 (1H ddd $J_{6,5}$ 7.97 Hz $J_{6,7}$ 7.20 Hz, $J_{6,8}$ 1.0 Hz, 6) 7.58 (1H dd $J_{8,7}$ 8.4 Hz $J_{8,6}$ 1Hz 8) 7.72 (1H

ddd $J_{7,8}$ 8.4 Hz $J_{7,6}$ 7.20 Hz $J_{7,5}$ 1.66 7) 8.06 (1H dd $J_{6',2'}$ 2.3 Hz, $J_{6',5'}$ 8.80 Hz 6') 8.21 (1H dd $J_{5,6}$ 7.97 Hz $J_{5,7}$ 1.66 Hz 5) 8.45 (1H d $J_{2',6'}$ 2.3Hz 2'); m/z, m^+ 297, 269, 250, 221, 152, 120, 92, 76; ν max. (nujol) 1620, 1530, 1350 cm^{-1}.

3'-methoxy-4'-nitroflavone (**61**) in 82% yield, m.pt. 241°C, found C 64.49%, H 3.59%, N 4.74%, $C_{16}H_{11}NO_5$ requires C 64.64%, H 3.72%, N 4.71%; NMR (300MHz) $CDCl_3$ δ 4.07 (3H s OMe) 6.85 (1H s 3) 7.46 (1H ddd $J_{6,7}$ 7.0 Hz $J_{6,5}$ 8.9 Hz $J_{6,8}$ 1.0 Hz 6) 7.55-7.6 (3H m 6', 2', 8) 7.77 (1H ddd $J_{7,6}$ 7.0 Hz $J_{7,8}$ 8.5 Hz $J_{7,5}$ 1.7 Hz 7) 7.96 (1H d $J_{5',6'}$ 8.9 Hz 5') 8.23 (1H dd $J_{5,6}$ 8.0Hz $J_{5,7}$ 1.7 Hz 5); m/z m^+ 297, 267, 252, 165, 152, 120 (100%), 92, 76.

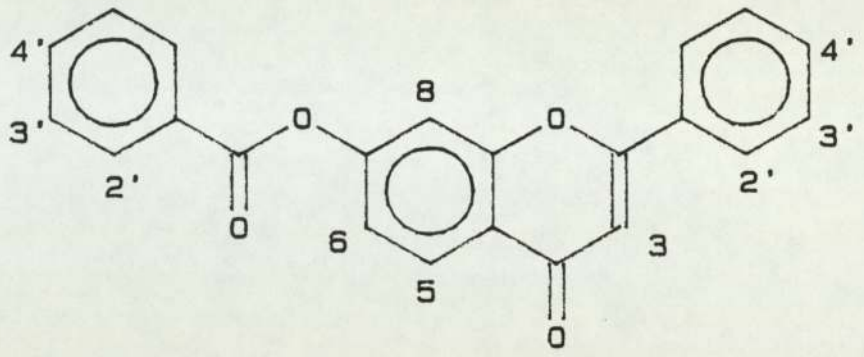
5,7-dimethoxy-4'-nitroflavone (**62**) in 67% yield, m.pt. 199-200°C; NMR (300MHz) $CDCl_3$ δ 3.90 (3H s OMe) 3.94 (3H s OMe) 6.38 (1H d $J_{8,6}$ 2 Hz 8) 6.57 (1H d $J_{6,8}$ 2 Hz 6) 6.74 (1H s 3) 8.02 (2H d $J_{2',3'}$ 9 Hz 2',6') 8.33 (2H d $J_{6',2'}$ 9 Hz 3', 5'); m/z, m^+ obs. 327.0739 (100%) $C_{17}H_{13}NO_6$ requires 327.0742, 310, 298, 281, 252, 151.

4'-chloro-3'-nitroflavone (**63**) in 59% yield, m.pt. 221°C, lit. 227°C [213]; NMR (300MHz) $CDCl_3$ δ 6.85 (1H s 3) 7.45 (1H ddd $J_{6,7}$ 7.0 Hz $J_{6,5}$ 8.0 Hz $J_{6,8}$ 1.0 Hz 6) 7.59 (1H dd $J_{8,7}$ 8.5 Hz$J_{8,6}$ 1.0 Hz 8) 7.72 (1H d $J_{5',6'}$ 8.5 Hz 5') 7.74 (1H ddd $J_{7,6}$ 7.0 Hz $J_{7,8}$ 8.6 Hz $J_{7,5}$ 1.7 Hz 7) 8.05 (1H dd $J_{6',2'}$ 2.2

Hz $J_{6',5'}$ 8.5 Hz 6') 8.22 (1H dd $J_{5,6}$ 8.0 Hz J 5,7 1.7 Hz 5) 8.45 (1H d $J_{2',6'}$ 2.2 Hz 2').

5,7-dimethoxyflavone (64) in 63% yield, recrystallised from 60-80 pet ether; m.pt. 151°C, lit. 142-143 °C [216]; NMR $CDCl_3$ δ 3.86 3H s OMe), 3.9 (3H s OMe) 6.32 (1H d $J_{8,6}$ 2 Hz 8) 6.52 (1H d $J_{6,8}$ 2 Hz 6) 6.6 (1H s 3) 7.6-7.4 (3H m 3',4',5') 7.95-7.7 (2H m 2',6').

7-methoxy-4'-nitroflavone (65) in 64% yield, recrystallised from $CHCl_3$/60-80 pet.ether; m.pt. 218°C; found C 64.61%, H 3.90%, N 4.75%, $C_{16}H_{11}NO_5$ requires C 64.64%, H 3.73%, N 4.71%; NMR (300MHz) $CDCl_3$ δ 3.93 (3H s OMe) 6.83 (1H s 3) 6.97 (1H d $J_{8,6}$ 2.2 Hz 8) 7.01 (1H dd $J_{6,5}$ 8.8 Hz $J_{6,8}$ 2.2 Hz 6) 8.07 (2H d $J_{2',3'}$ 6.9 Hz 2's) 8.13 (1H d $J_{5,6}$ 8.8 Hz 5) 8.36 (2H d $J_{3',2'}$ 6.9 Hz 3'); m/z, m^+ 297 (100%), 269, 254, 208, 196, 180, 150, 122, 107, 84.



7-benzoyloxyflavone (66) in 73% yield, m.pt. 163°C, found C76.91%, H 4.11%, $C_{22}H_{14}O_4$ requires C 77.18%, H4.12%; NMR (400MHz) $CDCl_3$ δ 6.82 (1H s 3) 7.29 (1H dd $J_{6,5}$ 8.6 Hz $J_{6,8}$ 2.14 Hz 6) 7.5-7.56 (5H m 3"s,4' or 3''s,4'') 7.67 (1H tt $J_{3',4',5' \text{ or } 3'',4'',5''}$ 7.45 Hz $J_{4',2' \text{ or } 4'',2''}$ 1.32 Hz 4' or 4'') 7.91 (2H dd $J_{2',3' \text{ or } 2'',3''}$ 7.6 Hz $J_{2',4' \text{ or } 2'',4''}$ 2 Hz 2' or 2'') 8.22 (2H dd $J_{2',3' \text{ or } 2'',3''}$ 8.4 Hz $J_{2',4' \text{or } 2'',4''}$ 1.3 Hz 2' or 2'') 8.29 (1H d $J_{5,6}$ 8.68 Hz 5); m/z m^+ 342, 105 (100%), 77.

4'-hydroxy-3'-nitroflavone (67) was prepared in the following manner:

4'-methoxy-3'-nitroflavone (594mg, 0.002mol) was dissolved in 50ml dichloromethane and BBr_3 (1.5g, 0.006mol) was added. The mixture was left to stir for 16 hours and then quenched with a minimum of 10% aqueous NaOH. The mixture was acidified with concentrated hydrochloric acid and extracted with

dichloromethane and concentrated. The yield was 53%; m.pt. 196°C; m/z m^+ 283.0485 (100%) observed, $C_{15}H_9NO_5$ requires 283.0479, 255, 237, 208, 152, 120, 116, 92; NMR (400MHz) $CDCl_3$ δ 6.79 (1H s 3) 7.33 (1H d $J_{5',6'}$ 8.9 Hz 5') 7.44 (1H ddd $J_{6,7}$ 7.00 Hz $J_{6,5}$ 8.0 Hz $J_{6,8}$ 1 Hz 6) 7.61 (1H dd $J_{7,8}$ 8.5 Hz $J_{6,8}$ 1 Hz 8 7.73 (1H ddd $J_{7,6}$ 7.00 Hz, $J_{7,8}$ 8.5Hz $J_{7,5}$ 1.6 Hz) 8.1 (1H dd $J_{6',5'}$ 8.9 Hz $J_{6',2'}$ 2.3 Hz 6') 8.74 (1H d $J_{2',6'}$ 2.3 Hz 2').

3'-hydroxy-4'-nitroflavone (68) was prepared in an analogous manner in 48% yield; m.pt. 217°C; NMR d_6-DMSO δ 6.8 (1H s 3) 7.2-8.2 (6H m 2', 6', 5, 6, 7, 8) 8.5 (1H d $J_{5',6'}$ 8 Hz 5'); m/z, m^+ 283.0490 (100%) observed, $C_{15}H_9NO_5$ requires 283.0479, 255, 237, 208, 152, 120, 116, 92.

3',4'-dihydroxyflavone (69) was prepared in a similar fashion in 61% yield; m.pt. 233-235°C, lit. 243°C [229]; NMR (300MHz) $CDCl_3$ δ 6.83 (1H s 3) 7.01 (1H d $J_{5',6'}$ 8.4 Hz 5') 7.43 (1H ddd $J_{6,7}$ 7.0 Hz $J_{6,5}$ 8.0 Hz $J_{6,8}$ 1.0 Hz 6) 7.48 (1H dd $J_{2',6'}$ 2.2 Hz $J_{6',5'}$ 8.4 Hz 6') 7.54 (1H d $J_{2',6'}$ 2.2 Hz 2') 7.58 (1H dd $J_{8,7}$ 8.6 Hz $J_{8,6}$ 1.0 Hz 8) 7.71 (1H ddd $J_{7,6}$ 7.0 Hz $J_{7,8}$ 8.6 Hz $J_{7,5}$ 1.7 Hz 7) 8.22 (1H dd $J_{5,6}$ 8.0 Hz $J_{5,7}$ 1.7 Hz 5).

4'-hydroxyflavone (70) was prepared by dissolving 4'-methoxyflavone, (504mg, 0.002mol), in glacial acetic acid (10mls) and adding HBr in glacial acetic acid (10mls 30%wt%).

The mixture was brought to reflux and monitored by tlc. Frequent further additions of HBr in glacial acteic acid were required to demethylate the ether. When the reaction was complete (after seven days) the mixture was quenched with 10% NaOH, reacidified with 1M hydrochloric acid and extracted with dichloromethane. Concentration of the organic layer yielded a solid which was recrystallised from ethyl acetate; m.pt. 265°C, lit. 269-271°C [217]; NMR (400MHz) $CDCl_3$ δ 6.74 (1H s 3) 6.97 (2H d $J_{3',2'}$ 8.9 Hz 3') 7.40 (1H dd $J_{6,5}$ 8.2 Hz $J_{6,7}$ 9.4 Hz 6) 7.57 (1H d $J_{8,7}$ 8.3 Hz 8) 7.68 (1H ddd $J_{7,6}$ 9.4Hz $J_{7,8}$ 8.3 Hz $J_{5,7}$ 1.6 Hz 7) 7.85 (2H d $J_{3',2'}$ 8.9 Hz 2') 8.22 (1H dd $J_{5,6}$ 8.2 Hz $J_{5,7}$ 1.6 Hz 5).

3'-hydroxyflavone (71) was prepared in an analogous manner in 60% yield, m.pt. 202°C, lit. 209°C [217]; NMR (400MHz) $CDCl_3$ δ 6.84 (1H s 3) 7.03 (1H m 4') 7.39 (1H t J 7.9Hz 5') 7.41-7.45 (2H m 2',6) 7.50 (1H m 8) 7.56 (1H br.d J_{circa} 8 Hz 6') 7.72 (1H ddd $J_{7,5}$ 1.6 Hz J_o 7 Hz J_o 8.5 Hz 7) 8.23 (1H dd $J_{5,7}$ 1.6 Hz $J_{5,6}$ 8.0 Hz 5).

4'-dimethylamino-3'-nitroflavone (72) was prepared by heating 4'-chloro-3'-nitroflavone (752 mg, 2.5 mmol) to reflux in aqueous dimethylamine 40 wt % solution (50 mls) for 12 hrs. The mixture was poured onto crushed ice and hydrochloric acid and the mixture neutralised. The solid was filtered off and recrystallised from ethoxyethanol/ water. Yield was 62%; m.pt. 219-220; found C 65.7%, H 4.4%, N 8.6%, $C_{17}H_{14}N_2O_4$ requires C

65.8%, H 4.54%, N 9.0%; NMR (400MHz) $CDCl_3$ δ 3.01 (6H s $2CH_3$) 6.71 (1H s 3) 7.09 (1H d $J_{5',6'}$ 9.0 Hz 5') 7.41 (1H ddd $J_{6,7}$ 7.0 Hz $J_{6,5}$ 7.9 Hz $J_{6,8}$ 1.0 Hz 6) 7.56 (1H dd $J_{8,7}$ 8.6 Hz $J_{6,8}$ 1.0Hz 8) 7.70 (1H ddd $J_{7,5}$ 1.7 Hz $J_{7,6}$ 7.0Hz $J_{7,8}$ 8.6Hz 7) 7.85 (1H dd $J_{6',2'}$ 2.3 Hz $J_{6',5'}$ 9.0 Hz 5') 8.2 (1H dd $J_{5,6}$ 7.9 Hz $J_{5,7}$ 1.7 Hz 5) 8.40 (1H d $J_{6',2'}$ 2.3 Hz 6'); ν max. (nujol) 1660, 1620 cm^{-1}; m/z, m^+ 310 (100%), 293, 265, 235, 165, 92.

3'-amino-4'-dimethylaminoflavone (**73**) was prepared in 85% yield by the reduction of 4'-dimethylamino-3'-nitroflavone (155mg, 0.5mmol) with stannous chloride (560mg, 2.5mmol) in ethanol (20mls) and conc. hydrochloric acid (1ml). The mixture was heated to reflux and stirred for one hour. After cooling and concentrating the mixture was basified using an excess of 10M NaOH and extracted with dichloromethane. The organic extract was concentrated and yielded a dark yellow oil. NMR (300MHz) $CDCl_3$ δ 2.70 (3H s CH_3) 2.72 (3H s CH_3) 4.02 (2H brs NH_2) 6.72 (1H s 3) 7.05 (1H d $J_{5',6'}$ 8.2 Hz 5') 7.24 (1H s 2') 7.32 (1H d $J_{6',5'}$ 7.9 Hz 6') 7.38 (1H d $J_{6,5}$ 7.8 Hz 6) 7.52 (1H d J 8.2Hz 8) 7.66 (1H t J 7.0Hz 7) 8.20 (1H dJ 7.8 Hz 5); ν_{max} (nujol) 3400, 3300, 1670, 1620 cm^{-1}; m/z m^+ 280.1211 (100%) (calculated 280.1211) 265, 250, 237, 121, 104, 84.

4'-amino-5,7-dimethoxyflavone (**74**) was prepared in a similar fashion in 36% yield, m.pt. 223°C; NMR (400MHz) $CDCl_3$ δ 3.88 (3H s OMe) 3.93 (3H s OMe) 4.10 (2H brs NH_2) 6.34 (1H d $J_{6,8}$

2.33 Hz 6) 6.53 (1H d $J_{8,6}$ 2.33 Hz 8) 6.72 (2H d $J_{3',2'}$ 8.75 Hz 3') 7.67 (2H d $J_{2',3'}$ 8.75 Hz 2'); m/z m^+ obs 297.0995 calc. 297.1001 (100%), 280, 251, 224, 151, 134, 117.

4'-amino-3'-methoxyflavone (**75**) was synthesised by reducing 3'-methoxy-4'-nitroflavone, (430mg, 0.0015 mol), with stannous chloride (1.7g, 0.0075 mol) in conc. hydrochloric acid (20mls). The mixture was heated for an hour and then cooled to 0°C. The precipitate was collected and recrystallised from ethyl acetate; yield 75%; m.pt. 186°C; found C 71.61%, H 5.02%, N 5.20%, $C_{16}H_{13}NO_3$ requires C 71.89%, H 4.90%, N 5.24%; NMR d_6-DMSO δ 3.9 (3H s OMe) 5.3 (2H s NH_2) 6.7 (1H s 3) 6.8 (1H dd $J_{8,7}$ 7 Hz $J_{8,6}$ 1 Hz 8) 7.4-7.7 (5H m 2',5',6',6,7) 8.05 (1H dd $J_{5,6}$ 7 Hz $J_{5,3}$ 1.5 Hz 5);m/z, m^+ 267, 252, 224, 147 (100%), 92, 77; ν max (nujol) 3400, 3300, 1620 cm^{-1}.

The following compounds were prepared in a similar fashion:-

3'-amino-4'-methoxyflavone (**76**) in 45% yield; m.pt.181°C; found C 71.89%, H 5.04%, N 5.53%; $C_{16}H_{13}NO_3$ requires C 71.89%, H 4.90%, N 5.24%; NMR (400MHz) $CDCl_3$ δ 3.88(3H s OMe) 3.99 (2H br.s NH_2) 6.67 (1H s 3) 6.83 (1H d $J_{5',6'}$ 8.4 Hz 5') 7.23 (1H d $J_{2',6'}$ 2.2 Hz 2') 7.29 (1H dd $J_{6',5'}$ 8.4 Hz $J_{6',2'}$ 2.2 Hz 6') 7.36 (1H ddd $J_{6,5}$ 7.9 Hz $J_{6,7}$ 7.0 Hz $J_{6,4}$ 0.8Hz 6) 7.49 (1H dd $J_{8,7}$ 8.6 Hz $J_{8,6}$ 0.8 Hz 8) 7.63 (1H ddd $J_{7,6}$ 7.0 Hz $J_{7,8}$ 8.6 Hz $J_{7,5}$ 1.7 Hz 7) 8.18 (1H dd $J_{5,6}$ 7.9 Hz $J_{5,7}$ 1.7

Hz 5); m/z, m^+267, 252 (100%), 224, 132, 92.

4'-aminoflavone (77) in 56% yield; m.pt. 224°C, lit. 233°C [230]; NMR (300MHz) $CDCl_3$ δ 4.10 (2H br s NH_2) 6.68 (1H s 3) 6.74 (2H d $J_{2',3'}$ 8.7 Hz 3's) 7.37 (1H ddd $J_{6,7}$ 7.0 Hz $J_{6,5}$ 8.0 Hz $J_{6,8}$ 1.0 Hz 6) 7.51 (1H dd $J_{8,7}$ 8.5 Hz $J_{8,6}$ 1.0 Hz 8) 7.65 (1H ddd $J_{7,8}$ 8.5 Hz $J_{7,6}$ 7.0 Hz $J_{7,5}$ 1.7 Hz 7) 7.74 (2H d $J_{3',2'}$ 8.7 Hz 2's) 8.20 (1H dd $J_{5,6}$ 8.0 Hz $J_{5,7}$ 1.7 Hz 5).

3'-aminoflavone (78) in 62% yield; m.pt. 237°C; found C 75.65%, H 4.62%, N 5.95%, $C_{15}H_{11}NO_2$ requires C 75.93%, H 4.67%, N 5.90%; NMR (300MHz) $CDCl_3$ δ 4.79 (2H brs NH_2) 6.76 (1H s 3) 6.82 (1H ddd $J_{4',5'}$ circa 7.0 Hz J_m 2.4 Hz J_m 1.0 Hz 4') 7.26-7.30 (2H m 5',6') 7.39 (1H ddd $J_{6,7}$ 7.0 Hz $J_{6,5}$ 8.0 Hz $J_{6,8}$ 1.0 Hz 6) 7.54 (1H dd $J_{8,7}$ 8.6 Hz $J_{8,6}$ 1.0 Hz 8) 7.68 (1H ddd $J_{7,6}$ 7.0 Hz $J_{7,8}$ 8.6 Hz $J_{7,5}$ 1.6 Hz 7) 8.21 (1H dd $J_{5,6}$ 8.0 Hz $J_{5,7}$ 1.6 Hz 5); m/z m^+ 237 (100%), 209, 117, 92.

4'-azidoflavone (79) was prepared from 4'-aminoflavone in 63% yield in the following manner:-

4'-aminoflavone (592mg, 0.0025mol) was dissolved in 5M hydrochloric acid (5mls) at room temperature, this soln was then cooled to below 0°C in an ice/salt bath. Sodium nitrite (190mg, 0.0027mol) was dissolved in a minimum quantity of water (5ml) and gradually added to the amine over 15 minutes, maintaining the temperature below 0°C. This mixture was then left to stir for a further 15 minutes. Sodium azide (650mg,

0.01mol) was then added to the diazotised mixture over 30 minutes. The reaction mixture was then left stirring for 30 minutes before being poured onto crushed ice and gently basified with concentrated ammonium hydroxide. The precipitate was collected, washed with ice-water and recrystallised from methanol; m.pt. 143°C, found C 68.13%, H 3,40%, N 16.06%, $C_{15}H_9N_3O_2$ requires C 68.42%, H 3.44%, N 15.96%; NMR $CDCl_3$ δ 6.66 (1H s 3) 7.04 (2H d $J_{3',2'}$ 8 Hz 3',5') 7.6-7.0 (3H m 6,7,8) 7.8 (2H d $J_{2',3'}$ 8 Hz 2',6') 8.1 (1H dd $J_{5,6}$ 8 Hz $J_{5,3}$ 2 Hz 5); max 2100, 1640, 1600cm^{-1}; m/z, m^+ 263, 235 (100%), 221, 117, 76.

3'-azidoflavone (80) was prepared in an analogous manner in 76% yield, recrystallised from methanol; m.pt. 105°C; found C 68.14%, H 3.35%, N 16.25%, $C_{15}H_9N_3O_2$ requires C 68.42%, H 3.44%, N 15.96%; NMR $CDCl_3$ δ 6.68 (1H s 3) 7.9-7.0 (7H m 2',5',6',6,7,8) 8.2 (1H dd $J_{5,6}$ 7 Hz $J_{5,3}$ 1 Hz 5); ν max (nujol) 2100, 1630, 1600 cm^{-1}; m/z, m^+ 263, 235 (100%), 221, 180, 117, 92, 76).

7-hydroxy-3'-methoxyflavone (81) was prepared by dissolving 7-(3-methoxybenzoyloxy)-3'-methoxyflavone (840mg, 0.002 mol) in 20% aqueous KOH (15mls). The mixture was warmed gently and stirred for an hour. The mixture was then poured onto an excess of 1M hydrochloric acid and crushed ice. After the mixture had reached room temperature the precipitate was collected and suspended in saturated sodium bicarbonate

solution and warmed for 30mins, filtered and the precipitate was recrystallised from ethanol; yield 74%; m.pt. 237°C, lit. 238-240°C [220]; NMR (300MHz) d_6-DMSO δ 3.83 (3H s OMe) 6.40 (1H brs 8) 6.49 (1H br.d $J_{5,6}$ 9 Hz 6) 6.66 (1H s 3) 7.10 (1H dd $J_{4',5'}$ 8.2 Hz J_m 2.5 Hz 4') 7.43 (1H dd $J_{5',4'}$ 8.2 Hz $J_{5',6'}$ 7.8 Hz 5') 7.45 (1H br.s 2') 7.53 (1H d $J_{6',5'}$ 7.8 Hz 6') 7.59 (1H d $J_{6,5}$ 9 Hz 5).

3-hydroxyflavone (82) was prepared in 86% yield by treating flavone (2.22g, 0.01 mol) with lithium di-isopropylamide (1.07g, 0.01 mol) at -78°C. Lithium di-isopropylamide was generated *in situ* by the addition of n-butyl lithium solution (6.25ml of 1.6M solution in hexanes, 0.01 mol) to diisopropylamine (1.01g, 0.01 mol) in 20mls dry THF at -78°C. To the lithioflavone was added trimethyl borate (1.04g, 0.01 mol) and the mixture was allowed to stir for 30 minutes after which acetic acid (0.9g, 0.015 mol) was added followed by hydrogen peroxide (1.2mls of 30 wt.% in water). The reaction mixture was then allowed to reach room temperature, washed with saturated sodium bicarbonate solution and extracted with ethyl acetate. When the solvent had been removed under reduced pressure the resulting solid was recrystallised from ethanol; m.pt. 165°C, lit. 169°C [192]; NMR (400MHz) $CDCl_3$ δ 7.39 (1H ddd $J_{6,7}$ 7.0 Hz $J_{6,5}$ 8 Hz $J_{6,8}$ 1 Hz 6) 7.41-7.57 (5H m 5,8,3's and 4') 7.68 (1H ddd $J_{7,5}$ 1.6 Hz $J_{7,6}$ 7.0 Hz $J_{7,8}$ 8.6 Hz 7) 8.24 (2H m 2's).

3-chloroflavone (83) was prepared by treating flavone (2.22g, 0.01mol) with a slight excess of sulphurylchloride (1.5g, 0.011mol) in CCl_4 (30mls) and stirring for 24 hrs. 3-chloroflavone was isolated in 83% yield by column chromatography using chloroform on silica (rf 0.4-0.5); m.pt. 123-124°C, lit. 122-124°C [226]; NMR (400MHz) $CDCl_3$ δ 7.41 (1H ddd $J_{6,8}$ 1.0 Hz $J_{6,7}$ 7.0 Hz $J_{6,5}$ 8.0 Hz 6) 7.48 (1H dd $J_{8,6}$ 1.0Hz $J_{8,7}$ 8.6 Hz 8) 7.50-7.54 (3H m 3's and 4') 7.68 (1H ddd $J_{7,6}$ 7.0 Hz $J_{7,8}$ 8.6 Hz $J_{7,5}$ 1.6 Hz 7) 7.88 (2H m 2's) 8.24 (1H dd $J_{5,6}$ 8.0 Hz $J_{5,7}$ 1.6 Hz 5).

2,3,3-trichloroflavanone (84) (50mg, 1.5%), was isolated by column chromatography from the reaction to prepare 3-chloroflavone, (rf 0.2); m.pt. 102-104°C; found C 54.89%, H 3.01%, $C_{15}H_9Cl_3O_2$ requires C 54.99%, H 2.77%; NMR (400MHz) $CDCl_3$ δ 7.22 (1H dd $J_{8,7}$ 8.7 Hz $J_{8,6}$ 1 Hz 8) 7.32 (1H ddd $J_{6,7}$ 7.0 Hz $J_{6,5}$ 8.0 Hz $J_{6,8}$ 1 Hz 6) 7.56-7.48 (3H m 3's and 4') 7.69 (1H ddd $J_{7,6}$ 7.0 Hz $J_{7,8}$ 8.7 Hz $J_{1.7}$ Hz 7) 8.02-8.00 (2H m 2's) 8.14 (1H dd $J_{5,6}$ 8.0Hz $J_{5,7}$ 1.7 Hz 5); ν max. (nujol) 1718, 1610 cm^{-1}; λ max. 260nm; m/z, m^+ 330/328/326, 291/293, 255/257, 206, 136, 120, 105, 92, 77.

3-bromocyclohexene (85) was prepared by bromination of cyclohexene in the following manner:-

N-bromosuccinimide (17.9g, 0.1 mol) was added to cyclohexene (24.6g, 0.3 mol) that had been previously

distilled, in CCl_4 (100 mls). Benzoyl peroxide (300mg, 1.24 mmol), which had been dried by pressing between two filter papers, was added and the mixture was warmed gently on a water bath until a vigorous reaction started. The mixture was removed from the heat until the reaction had modified and was then refluxed for two hours. The mixture was cooled and filtered to remove the succinimide which was then washed three times with CCl_4 (20mls). The excess cyclohexene and the CCl_4 were removed by distillation. Distillation of the residue yielded 3-bromocyclohexene. This required further distillation using a fractionating column to obtain pure 3-bromocyclohexene. Overall yield of 3-bromocyclohexene was 62%, boiling range 60-63°C at 10mm Hg, lit. 66-67°C at 20mm Hg [206]; NMR $CDCl_3$ δ 1.6-2.4 (6H m 4,4',5,5',6,6') 4.7-4.9 (1H m 3) 5.8-5.9 (2H m 1, 2).

3-cyanocyclohexene (**86**) was prepared from 3-bromocyclohexene (4g, 0.025mol) by the addition of dry sodium cyanide (2.5 g, 0.05mol) in dry N-methylpyrolidinone (20mls). The mixture was allowed to stir at room temperature overnight and then poured onto water. 3-cyanocyclohexene was extracted into ether and purified by distillation. Overall yield was 82%, boiling range 70-72°C at 10 mm Hg, lit. 89°C at 23 mm Hg [199]; NMR $CDCl_3$ δ 1.5-2.1 (6H m 4, 4', 5, 5', 6, 6') 3.2 (1H m CHCN) 5.9-5.6 (2H m CH·CH).

Methylcyclohex-2-ene carboxylate (87) was prepared from

3-cyanocyclohexene in the following manner:-

3-cyanocyclohexene (3.21g, 0.03 mol) was dissolved in dry methanol (50mls) in a two-necked round-bottomed flask. Dry HCl was generated by dripping sulphuric acid (concentrated) onto ammonium chloride. The HCl was dried by passing through sulphuric acid (concentrated) and was then bubbled into the methanolic solution of 3-cyanocyclohexene. The mixture was refluxed for 6-8 hours during which time a white precipitate formed. After eight hours the gas flow was stopped and the mixture allowed to stir at room temperature overnight. The mixture was poured onto an excess of crushed ice and extracted with ether. The product was purified by distillation, and final yield was 42%; boiling point 60°C at 10 mm Hg, lit. 64-65°C at 12mm Hg, [199]; NMR $CDCl_3$ δ 1.5-2.1 (6H m 4, 4', 5, 5', 6, 6') 3.4 (1H m CH·COO) 3.6 (3H s Me) 5.8 (2H s CH·CH).

Cyclohex-2-ene carboxylic acid (88) was prepared by treating methylcyclohex-2-ene carboxylate (1.26g, 0.01 mol) with p-toluenesulphonic acid (200mg, 0.01mol), in 1,4-dioxan/water (9:1, 10mls) and heating to reflux for three hours. The mixture was washed with brine and then the acids were extracted with sodium carbonate solution. The extractions were acidified with and then extracted with ether. Cylcohex-2-ene carboxylic acid was isolated by distillation. The final yield was 57%, b.pt. 125°C at 10 mm Hg, lit. 130°C at 14mm Hg [199]; NMR $CDCl_3$ δ 1.6-2.1 (6H m 4, 4', 5, 5', 6, 6') 3.0-3.1 (1H m CH·COO) 5.8 (2H br.s CH·CH) 11.5 (1H br.s COOH).

ß-chlorocinnamic acid (89) (prop-2-en-3-chloro-3-phenyl carboxylic acid) was synthesised by dissolving ethyl benzoyl acetate (48g, 0.25 mol) in phosphorous oxychloride (70mls) and adding PCl_5 (104 g, 0.5 mol) and heating. When the evolution of HCl had ceased, the phosphorous oxychloride was removed by distillation and the residue was poured carefully onto an excess of crushed ice and allowed to stir overnight. The mixture was basified by the addition of sodium carbonate, charcoal was added and the whole was heated until the solid had dissolved. The solution was filtered and acidified with concentrated hydrochloric acid. The ß-chlorocinnamic acid was filtered off and recrystallised from a small quantity of ethanol. Yield was 28%, m. pt. 136-7°C, lit. 139-140°C [221]; NMR $CDCl_3$ δ 6.5 (1H s COCHCCl) 7.1-7.8 (5H m ArH) 10.4 (1H br.s COOH).

methyl ß-chlorocinnamate (90) was furnished in 78% yield by heating ß-chlorocinnamic acid (1.82g, 0.01 mol) in methanol (30mls) with concentrated sulphuric acid (1ml) for two hours. The methanol was removed under reduced pressure and the residue dissolved in chloroform, washed with water and dried over sodium sulphate. Removal of the chloroform under reduced pressure gave a fluid brown oil. NMR $CDCl_3$ δ 3.8 (3H s OMe) 6.5 (1H s COCHCCl) 7.2-7.8 (5H m ArH).

4-methoxypent-3-en-2-one (91) was prepared by the methylation

of 2,4-pentanedione (25g, 0.25mol) with dimethylsulphate (31.5g, 0.25mol) in acetone (200mls) containing anhydrous potassium carbonate (73.5g, 0.75mol), and heated to reflux for 24hrs. Most of the acetone was removed under reduced pressure and the residue poured onto water. The mixture was acidified and extracted with ethyl acetate. 4-methoxypent-3-en-2-one was isolated in 30% yield as a clear liquid. NMR $CDCl_3$ δ 2.12 (3H s CH_3) 2.23 (3H s CH_3) 3.6 (3H s OMe) 5.4 (1H br.s CH·CO), lit. NMR $CDCl_3$ δ 2.12 (3H s CH_3 (5)) 2.24 (3H s CH_3 (1)) 3.65 (3H s OMe) 5.51 (1H s CH·CO) [222]; m/z, m^+ 114, 99 (100%), 71, 43.

2-(4-methoxyphenyl)-6-methylpyran-4-one (92) was isolated in 2.5% yield in the following manner:-

Lithium hexamethyldisilazide was prepared by adding n-butyl lithium (1.28g, 0.02mol) to hexamethyldisilazane (3.22g, 0.02mol) in dry THF (20mls) at -78°C and then stirred for 5 mins prior to the dropwise addition of 4-methoxypent-3-en-2-one (2.28g, 0.02mol) in THF (10mls) over 25 minutes. This mixture was stirred for twenty minutes before the addition of 4-methoxybenzoyl chloride (1.7g, 0.01mol) in THF (10mls). After stirring at -78°C for thirty minutes the mixture was allowed to reach ambient temperature, treated with a saturated solution of ammonium chloride and then extracted with ether. Removal of the ether under reduced pressure yielded a residue which was then dissolved in toluene (20mls) and trifluoroacetic acid (1ml) was added. This mixture was allowed to stir overnight, the toluene removed under reduced

pressure and the residue separated into two main fractions by dry column chromatography using first chloroform and then methanol. The second fractions was further separated using preparative thin layer chromatography ($CHCl_3$:MeOH, 9:1). The fluorescent band of Rf 0.2-0.4 was isolated and yielded 2-(4-methoxyphenyl)-pyran-4-one when washed extensively with chloroform. NMR $CDCl_3$ δ 1.9 (3H s Me) 3.8 (3H s OMe) 6.1 (1H br.s COCH) 6.5 (1H br.s COCH) 6.9 (2H d *J* 8Hz 3',5') 7.8 (2H d *J* 8Hz 2',6'), lit. NMR $CDCl_3$ δ 2.33 (s 3H) 3.84 (s 3H) 6.11 (d 1H) 6.57 (d 1H) 6.95 (d 2H) 7.67 (d 2H) [223]; m/z, m^+ 216, 188, 135, 76, 43 (100%).

(1-(2-hydroxyphenyl)-3-cyclohexylpropan-1,3-dione and 1-(2-hydroxyphenyl)-3-(3-fluorophenyl)propan-1,3-dione were not purified before use in the next step of the syntheses).

CHAPTER EIGHT

BIOLOGICAL MATERIALS AND METHODS

Tissue Culture

All tissue culture work was carried out using sterile techniques in a "Gelair" or "Envair" laminar air-flow cabinet that had been previously sterilised with UV light and sprayed with alcohol.

ANN-1 cells (3T3 murine fibroblasts that have been transformed by the Abelson murine leukaemia virus) were originally established by C D Scher and R Siegler[242], and were a kind gift of Dr. J Gordon Foulkes, (NIMR, London). The cells were cultured in Dulbecco's modified Eagle medium (DMEM) (GIBCO Europe) supplemented with 10% foetal calf serum (FCS) (Imperial Labs, Salisbury; GIBCO, Paisley, Scotland; or FLOW, Irving, Scotland;) (complete medium) under an atmosphere of 10% CO_2 in air, 100% humidity, at 37°C.

Cell stocks were stored in liquid nitrogen in complete medium containing 10% DMSO. in aliquots of 1-2 x 10^6 per ml

3T3 murine fibroblasts were obtained from Flo Laboratories and were cultured in a one:one mixture of DMEM, Waymouths medium (GIBCO) supplemented with 10% FCS. Confluent cultures

were re-seeded to the appropriate density by removing and discarding the medium, rinsing the cells with 10mls Ca^{2+} and Mg^{2+} free phosphate buffered saline, and with 1ml of trypsin/EDTA solution. 1ml of typsin/ EDTA solution was again added and the cultures incubated at 37°C for 3 mins, or until the cells had detached from the surface of the culture flask. 2mls of complete medium was added and the cell suspension divided and plated out into a fresh flask at the desired density.

P160 cells (established from NIH3T3 cells which transformed by the Abelson murine leukaemia virus, expressing the $p160^{gag \cdot abl}$ protein)(a kind gift of Dr. A. Gebhardt, NIMR, London)) were cultured in DMEM supplemented with 10% FCS and passaged by removing the medium from the cells, adding 5mls of Ca^{2+}/Mg^{2+} free phosphate buffered saline to the flask and then incubating the culture for 5 mins to permit cell detachment. The supernatent was then pooled with the original medium, centrifuged and the pellet resuspended in 1ml complete medium and then plated out at the desired density.

Assay of the inhibition of growth

ANN-1 cells seeded at a density of $2x10^4$ cells/ml were treated with a range of drug concentrations. Each drug was dissolved in DMF, the final concentration of which in the medium was 0.5%v/v. This concentration had no effect on the

growth of ANN-1 cells. Cultures were incubated for three days, harvested and counted.

Assays of the clonogenic potential of ANN-1 cells

ANN-1 cells were treated with a range of drug concentrations as described for growth inhibition assays. After three days cells were harvested, centrifuged, and the cell pellet resuspended in 1ml fresh complete medium. The resultant single cell suspension was then diluted to give a range of cell densities from $2x10^6$ to $2x10^3$/ml. A 0.5% solution of agar in complete medium was prepared and kept at 44°C before addition of the cells, (0.5ml to 19.5ml agar/medium). 4mls of cell suspension was added to each of three wells in a six well culture vessel giving a final cell number of $2x10^5$ to $2x10^2$ cells per well. Cultures were incubated under a 5% CO_2 atmosphere for 10 days, stained with a 0.5% solution of iodonitrotetrazolium violet, and colonies counted.

Staining

Cells were grown on Miles "Lab-Tek" tissue culture chamber/slides as desribed for growth inhibition assays. The media was removed and the multiwell tray separated from the slide. The cells were fixed with absolute alcohol by immersion for 2 minutes in a 100% alcohol bath. The slides were then

immersed for 2 mins each time in 90% and 70% alcohol, then distilled water. The slides were then rinsed with distilled water, immersed in 1% acid alcohol for 7-10 seconds, rinsed with water and then immersed in Harris's haematoxylin [231] for 10 mins. After this the slides were rinsed once with distilled water and then placed in Scotts tap water substitute for 30 secs, rinsed twice with distilled water and then immersed in Navy eosin [232] for 20 secs. The slides were then placed in absolute alcohol for 60 secs, then in fresh absolute alcohol for a further 60 secs, before being immersed in xylene for 1 min. The slides were placed in fresh xylene for a further minutes.

Assay of Tyrosine Protein Kinase

The inhibition of tyrosine kinase activity was assayed by measurement of the incorporation of ^{32}P from γ-[^{32}P]-MgATP into angiotensin I by pt*abl*50 (the kinase domain of the Abelson protein which was a kind gift from Dr. J. Gordon Foulkes [241].

The general assay procedure consisted of 10ul of pt*abl*50 kinase (diluted 1:100 in enzyme buffer) 10ul angiotensin I (SIGMA) (3mg/ml in assay buffer) 5ul inhibitor in 35% DMSO in assay buffer (final concentration of DMSO in the assay was 5%v/v which had no effect on enzyme activity) and the reaction was iniatiated with 10ul 30mM Mg^{2+}, 0.3mM γ-[^{32}P]-ATP (Amersham International or prepared by the recipe which

follows) (600cpm/pmol-10000cpm/pmol). After incubating the mixtures at 30°C for 30 minutes the assays were quenched by the addition of 100ul 10%$^{v/v}$ phosphoric acid. 100ul of the reaction mixture was then spotted onto phosphocellulose paper (p81, Whatman) 2cm^2. The papers were washed extensively with 6%$^{v/v}$ acetic acid, once with acetone and then dried and counted.

The kinase assay could be carried out in a using cell lysate from ANN-1 cells. ANN-1 cells, 1x10^7 cells, were lysed using 1ml of lysis buffer and incubating for 10mins. The suspension was centrifuged and the pellet discarded. The lysate was then diltued 1:10 with enzyme buffer and used in the same manner as pt*abl*50. The reaction was quenched with 5% trichloroacetic acid. The Eppendorf tubes were then centrifuged and within 15 mins 100ul from each sample was spotted onto p81 and washed as before.

Buffers used in the tyrosine protein kinase assay.

Assay buffer:-

50mM PIPES, pH 7.5;

0.1mM EDTA;

0.05%$^{v}/_{v}$ Brij 35

Enzyme buffer:-

50mM PIPES, pH 7.5;

0.1mM EDTA;

0.05%$^{v}/_{v}$ Brij 35;

1mM dithiothreitol;

0.1mg/ml bovine serum albumin ,

Lysis buffer:-

50mM PIPES, pH 7.5;

0.2mM EDTA;

50mM NaF;

100uM Sodium vanadate;

1% triton;

1mM dithiothreitol;

Preparation of γ-[^{32}P]-ATP

γ-[^{32}-P]-ATP was prepared by the method of Johnson and Walseth [243].

Glycerophosphate dehydrogenase (75-150 U/mg), triosephosphate isomerase (5000 U/mg), glyceraldehyde-3-phosphate dehydrogenase (40-100 U/mg), 3-phosphoglycerate kinase (1000 U/mg) and lactate dehydrogenase (700-1400 U/mg) were obtained, sulphate free, from Sigma.

The reaction medium consisted of the following:-

500mM Tris pH 9.0

300mM $MgCl_2$

2.4mM L-α-gylcerophosphate

100mM dithiothreitol

10mM ß-NAD

2mM ADP

40mM pyruvate

The enzyme mixture consisted of:-

20ul glcerophosphate dehydrogenase (60u/ml)

20ul triosephosphate isomerase (50u/ml)

20ul glyceraldehyde-3-phosphate dehydrogenase (80u/ml)

20ul 3-phosphoglycerate kinase (45u/ml)

10ul lactate dehydrogenase (550u/ml)

55ul 100mM Tris pH 9.0

10ul 100mM dithiothreitol

45ul distilled water.

The reaction was initiated by incubating ^{32}P-orthophosphate, reaction medium and enzyme mixture in a 5:4:1 ratio at room temperature until >95% or more of the inorganic phosphate has been converted into ATP. The reaction was monitored at 5 minute intervals by the removal of 5ul aliquots of the mixture. These aliquots were added to 1ml of 50mM KH_2PO_4, 25ul were withdrawn and counted. A small amount of activated charcoal was added to the remaining KH_2PO_4 mixture and the whole vortexed. 25ul was removed and counted by liquid scintillation. When greater than 95% of the radioactivity could be removed by vortexing with charcoal the reaction vessel was placed in a beaker of freshly boiled water and allowed to stand for 10 mins.

The γ-[^{32}P]-ATP was purified by chromatography using a 0.7 x 4cm sephadex column using a 100mM triethylamine, 400mM

triethylamine (pH 8.0) gradient buffer. 2ml samples were collected, counted, and those containing the radioactivity were pooled and freeze dried.

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