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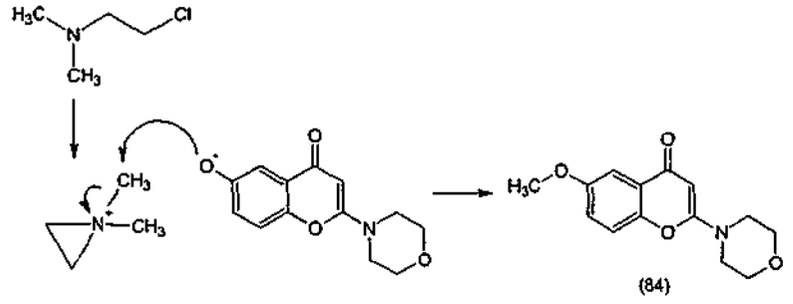
DOCTOR OF PHILOSOPHY
ON..... 30 March 2004 .....
.....[redacted].....
Sec. Research Graduate School Committee

ADDENDUM

p 11 Figure 1.8: Replace (H_2C) with ($CH_2)_3$ in the sulfonamide side chain.

p 50: Comment: In regards to the mechanism through which 6-methoxy-2-morpholinochromone (84) is formed as an undesired side product, it is possible that some (if not all) of the 2-*N,N*-dimethylaminoethylchloride cyclises under the reaction conditions used and that the resulting aziridinium salt may be the alkylating agent. Aryl oxide nucleophilic attack of the aziridinium salt at either ring methylene group would give the required product (83) while attack at either methyl group would afford the side product (84) (refer figure below).



(84)

pp 117-118: Substitute (92) for (94).

p 131: Comment: Silica Gel 60 (0.063-0.200 mm) (Merck) was the adsorbent used for normal phase chromatography.

p 160: Add at the end of paragraph 1: Alkylation of (69) to yield (83) also resulted in the formation of 6-methoxy-2-morpholinochromone (84) as a side product. This was isolated by RP-HPLC as a white solid (6.3 mg). δ (300 MHz; $CDCl_3$; Me_4Si) 7.54 (1H, d, *J* 3.0, ArH), 7.29 (1H, s, ArH), 7.24 (1H, d, *J* 3.0, ArH), 6.51 (1H, s, CH), 3.88 (1H, s, OCH_3), 3.86 (4H, t, *J* 4.8, CH_2CH_2), 3.68 (4H, t, *J* 5.0, CH_2CH_2). Mass spectrum (ESI) m/z 262 $[M + H]^{+\bullet}$.

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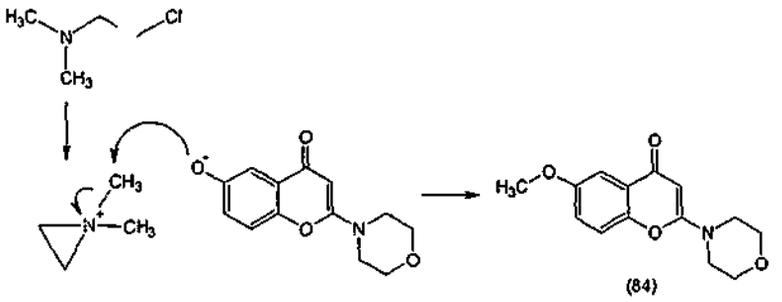

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SYNTHESIS AND STRUCTURE-ACTIVITY STUDIES OF ANTIPLATELET 2-MORPHOLINOCHROMONES

Belinda Maree Abbott

B.Sc. (Honours)

A thesis submitted in fulfillment of the
requirements for the degree of
Doctor of Philosophy.

Department of Medicine (Box Hill Hospital)
Faculty of Medicine
Monash University

December 2003

TABLE OF CONTENTS

Statement of Originality

To the best of the author's knowledge and belief, this thesis contains no material which has been accepted for the award of any other degree or diploma in any University or other institution, and contains no material previously published or written by another person except where due reference is made.

Belinda Abbott

Acknowledgements

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Last but not least, thank you to Damien for your encouragement, patience and support not just during my PhD studies but in everything I do. I look forward to sharing with you what the future holds for us.

Abbreviations

°C	degrees Celsius
$(CH_3CO)_2O$	acetic anhydride
$(F_3CSO_2)_2NPh$	*N*-phenyltrifluromethanesulfonimide
$(F_3CSO_2)_2O$	trifluromethanesulfonic anhydride
2-morpholinochromone	2-(4-morpholinyl)-*4H*-1-benzopyran-4-one
ADA	adenosine deaminase
ADP	adenosine diphosphate
$AlCl_3$	aluminium chloride
AMP	adenosine monophosphate
ATP	adenosine triphosphate
$BF_3.OEt_2$	boron trifluoride diethyl etherate
$BnNH_2$	benzylamine
Br_2	bromine
BuN^+Br^-	tetrabutylammonium bromide
ca.	approximately
cAMP	cyclic 3',5'-adenosine monophosphate
cGMP	cyclic 3',5'-guanosine monophosphate
CH_2Cl_2	dichloromethane
CH_3CN	acetonitrile
$CHCl_3$	chloroform
Cil	cilostamide
CRP	collagen related peptide
CS_2	carbon disulphide
$Cu(OAc)_2$	copper (II) acetate
CuI	copper iodide
DIPA	diisopropylamine
DMAP	*N,N*-dimethlaminopyridine
DMF	*N,N*-dimethylformamide
DMSO	dimethyl sulphoxide

DPPF	1,1-bis(diphenylphosphino)ferrocene
e.g.	for example
EHNA	*erythro*-9-(2-hydroxy-3-nonyl)adenine
EIA	enzyme immunoassay
Et	ethyl
et al	and others
EtOH	ethanol
Fr	Fraction
g	gravities
g	gram
GP	glycoprotein
h	hour(s)
H_2	hydrogen
H_2O	water
H_2SO_4	sulfuric acid
HAI	heat aggregated immunoglobulin
$HClO_4$	perchloric acid
HEPES	4-(2-hydroxyethyl)-1-piperazine-1-ethanesulfonic acid
i.e.	that is
IBMX	1-methyl-3-isobutylxanthine
IC_{50}	concentration at which 50% inhibition of the control occurs
IEX	ion exchange
K_2CO_3	potassium carbonate
K_3PO_4	potassium phosphate
kDa	kilodalton(s)
K_m	Michaelis constant
$KOBu^t$	potassium *tert*-butoxide
LC-MS	liquid chromatography – mass spectrometry
LDA	lithium diisopropylamide
LiHMDS	lithium hexamethyldisilazane; lithium bis(trimethylsilyl)amide
M	molar

mAU	milli-absorbance unit
Me	methyl
MeI	methyl iodide
MeOH	methanol
mg	milligram
Mil	milrinone
min	minute(s)
mL	millilitre
mM	millimolar
mm	millimetre
MS	mass spectrometry
Na_2CO_3	sodium carbonate
NaH	sodium hydride
NaI	sodium iodide
$NaOBu^t$	sodium *tertiary*-butoxide
NaOH	sodium hydroxide
NEt_3	triethylamine
nM	nanomolar
nm	namometre
NMR	nuclear magnetic resonance
OMe	methoxy
PAR4AP	protease-activated receptor 4 agonist peptide
Pd cat.	palladium catalyst
Pd(C)	palladium on activated carbon
$Pd(OAc)_2$	palladium (II) acetate
$Pd(PPh_3)_4$	tetrakis(triphenylphosphine)palladium
$PdCl_2(dppf)$	dichloro[1,1'-bis(diphenylphosphino)ferrocene]palladium
PDE	phosphodiesterase
PDE2	phosphodiesterase 2
PDE3	phosphodiesterase 3 (3A unless otherwise specified)
$PhB(OH)_2$	phenylboronic acid

PI3-kinase	phosphatidylinositol 3-kinase
PMSF	phenylmethylsulfonyl fluoride
PPE	polyphosphoric ester
ppm	parts per million
PRP	platelet rich plasma
PWB	platelet washing buffer
r.t.	room temperature
RIA	radioimmunoassay
RP-HPLC	reverse phase high performance liquid chromatography
s	second(s)
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
t	time
t.l.c.	thin layer chromatography
THF	tetrahydrofuran
TRAP	thrombin receptor agonist peptide
triflate	trifluoromethanesulfonate
Tris	2-amino-2-hydroxymethyl-1,3-propandiol
TxA_2	thromboxane A_2
Upjohn	The Upjohn Company
UV	ultraviolet
V_{max}	maximum velocity
vWf	von Willebrand factor
WP	washed platelets
µL	microlitre
µM	micromolar

Abstract

Platelet aggregation is a critical process in normal haemostasis but when manifested in thrombosis (pathological clot formation) there is a high risk of heart attack or stroke. Compounds which inhibit platelet aggregation represent important pharmacological tools in understanding more about this complex process and may lead to the development of new drugs. Antiplatelet activity for the 2-morpholinochromone class of compounds has been reported in the literature, yet very little evaluation of structure-activity relationships has been described. This thesis aims to explore the synthesis of this class of compounds and the structure-activity relationships that govern their antiplatelet activity, with respect to the inhibition of phosphodiesterase (PDE) and phosphatidylinositol 3-kinase (PI3-kinase) enzyme families.

The first part of this thesis investigates the synthesis of 2-morpholinochromones. Methodology which is concise and readily amenable to the efficient generation of numerous 2-morpholinochromone analogues is described. This has involved the synthesis of key intermediates, using novel approaches to the preparation of the morpholinochromone template. Derivatisation of the key intermediates, including the use of copper and palladium mediated coupling reactions, results in the rapid and efficient generation of 2-morpholinochromone analogues. A diverse but representative library of over 35 known and novel 2-morpholinochromone analogues has been synthesised.

The second part of this thesis describes the development and application of enzyme assays for the evaluation of 2-morpholinochromones. The isolation, purification and characterisation of phosphodiesterase isoforms PDE2 and PDE3 from human platelets is described. Efficient methods for the high throughput screening of compounds against phosphodiesterase isoforms using reverse-phase high performance liquid chromatography have been developed and optimised.

In the third part of this thesis, compounds from the synthesised 2-morpholinochromone library were selected for evaluation against the isolated platelet enzymes of phosphodiesterases 2 and 3 (PDE2 and PDE3) and phosphatidylinositol 3-kinase (PI3-kinase). Platelet aggregation studies were used to correlate the anti-enzyme activity with cellular activity, with the obtained data compared to that reported in the literature. The results of this study have provided valuable insights into the biological activity of the 2-morpholinochromone class. These have included the finding that 2-morpholinochromones can inhibit platelet aggregation by a novel, and as yet undetermined, mechanism of action as well as by PDE3 and PI3-kinase inhibition. In addition, inhibition of PDE2 has been identified as a further novel activity for this class of compounds. Distinct structural motifs for potent inhibition of PDE3, PDE2 and PI3-kinase have been described and opportunities for optimisation are available in each case which are worthy of further investigation.

This thesis offers new avenues for improving the understanding of the mechanism of platelet aggregation as applied to antithrombotic drug design, together with the opportunity for the development of new antiplatelet agents for the prevention or treatment of thrombosis or novel enzyme inhibitors for other pathologies.

Chapter 1
INTRODUCTION

1.1 Platelets and Blood Clot Formation

Platelets are specialised circulating cells which have a critical role in blood clot formation and the cessation of bleeding (haemostasis). Pathological clot formation (thrombosis) can result in the occlusion of a blood vessel supplying a vital organ, such as the heart or brain. Heart attack and stroke are currently major causes of death and disability in Western society.[1,2]

Vascular injury is a trigger of both haemostatic and thrombotic clot formation, exposing vessel contents to adhesive proteins from the endothelial matrix including von Willebrand factor (vWF) and collagen (Figure 1.1). Binding of exposed vWF binding to the glycoprotein (GP) Ib/IX/V surface receptor of circulating platelets causes initial activation, attracting and adhering platelets to the site of injury.[3] Further platelet activation is characterised by shape change, cytoskeletal rearrangement and secretion of granule contents. This is caused by agents such as collagen and thrombin as well as secreted secondary agonists (adenosine diphosphate (ADP), serotonin, thromboxane A_2 (TxA_2)). These compounds have a multitude of effects apart from platelet activation, including further platelet recruitment (aggregation), initiation of the coagulation cascade, release of vasoconstrictive agents and recruitment of other cell types. All of these responses have a role in life saving cessation of bleeding or life threatening vascular occlusion.[4]

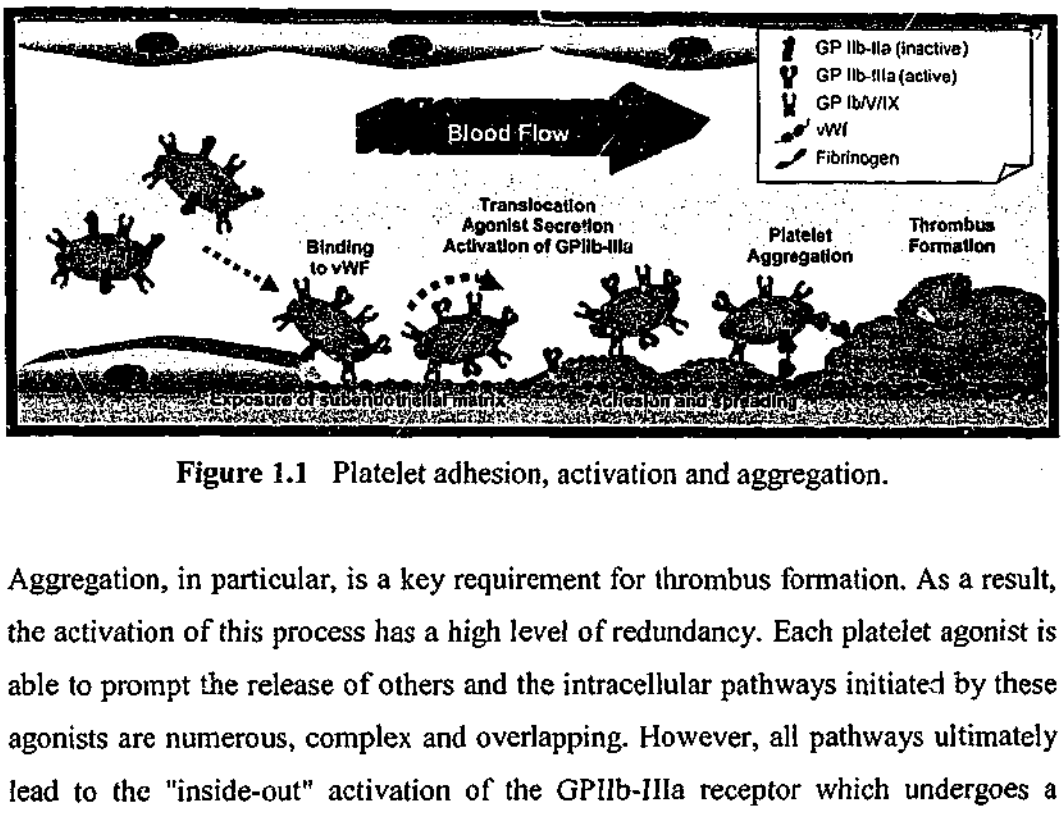


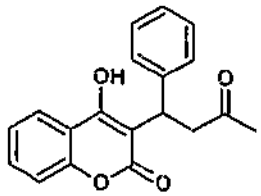
Figure 1.1 Platelet adhesion, activation and aggregation.

Aggregation, in particular, is a key requirement for thrombus formation. As a result, the activation of this process has a high level of redundancy. Each platelet agonist is able to prompt the release of others and the intracellular pathways initiated by these agonists are numerous, complex and overlapping. However, all pathways ultimately lead to the "inside-out" activation of the GPIIb-IIIa receptor which undergoes a conformational change to become competent to bind fibrinogen. As fibrinogen is a dimeric structure, one molecule can form a bridge between two platelets. As each platelet has many GPIIb-IIIa receptors, many fibrinogen bridges result in a cross-linked platelet aggregate.

There are three approaches to antithrombotic therapy for the prevention or treatment of pathological blood clots. These include anticoagulants, thrombolytic agents and antiplatelet agents.

1.2 Anticoagulants

Anticoagulants such as heparin and warfarin (Figure 1.2) have been used for over 50 years for the treatment and prevention of thromboembolism, particularly in high-risk situations such as following major orthopaedic surgery.



Warfarin

Figure 1.2 Anticoagulant.

Unfractionated heparin remains the preferred antithrombotic treatment during percutaneous coronary intervention and is relatively reliable and inexpensive.[5] The naturally-occurring anticoagulant antithrombin, after binding to heparin (a co-factor protein found on endothelial cells), inactivates the coagulation factors thrombin and clotting factor Xa equally (1:1 ratio).[5] Parenteral administration of unfractionated heparin increases inhibition of these coagulation factors.

Warfarin is widely used for the prevention of deep vein thrombosis, pulmonary embolism and stroke and is orally available. The activation of many clotting factors in the coagulation cascade requires carboxylation of glutamic acid residues, which is a Vitamin K dependent process. Warfarin acts by blocking the regeneration of the reduced form of Vitamin K. As a result, clotting factors cannot be activated and the coagulation cascade does not occur.[6,7]

However, there are significant drawbacks to warfarin and heparin, including a need for laboratory monitoring, associated bleeding complications and the high incidence of ischaemic cardiovascular events regardless of treatment.[1,8]

More recent anticoagulation treatments include low molecular weight heparin which is shorter in length and thus less able to bind thrombin, resulting in selective inactivation of factor Xa relative to thrombin.[5] Direct thrombin inhibitors such as hirudin, bivalirudin (a semi-synthetic hirudin fragment) and argatroban act independently of antithrombin but, like the heparins, cannot be administered

orally.[5,8] With agents currently in clinical trials, the development of safer and easier to use anticoagulants continues.[8]

1.3 Thrombolytic Agents

Thrombolytic (or fibrinolytic) therapy involves the use of pharmacological agents to dissolve a formed clot and is particularly useful in acute thrombotic events such as stroke and heart attack. Drugs used for this purpose include intravenous administration of streptokinase, a proteolytic drug, or recombinant tissue plasminogen activator (t-PA), which converts plasminogen to plasmin which in turn breaks down the fibrin of the clot.[3,9] Further development of this type of treatment is limited unless the risk of hemorrhagic complication, most critically intracranial hemorrhage, is not increased with treatment potency.[10]

1.4 Antiplatelet Drugs

To inhibit the activation and aggregation of platelets, there are three areas which have been targeted pharmacologically (Figure 1.3);

(i) Inhibition of agonist binding to cell surface receptors e.g. ADP receptor antagonists

(ii) Blocking of intracellular signalling pathways e.g. cyclooxygenase (COX) inhibitors, phosphodiesterase (PDE) inhibitors

(iii) Prevention of the binding of dimeric fibrinogen to the integrin GPIIb-IIIa receptor by GPIIb-IIIa antagonists

There are currently a number of antiplatelet drugs in clinical use which are known to inhibit these three different parts of the aggregation process (Figure 1.3). These include clopidogrel (ADP antagonist), aspirin (COX inhibitor), cilostazol (PDE inhibitor) and aggrastat (GPIIb-IIIa antagonist). While certain compounds inhibit a particular platelet pathway (e.g. aspirin), others block aggregation regardless of the agonist present and are considered to be global inhibitors (e.g. phosphodiesterase and GPIIb-IIIa inhibitors).

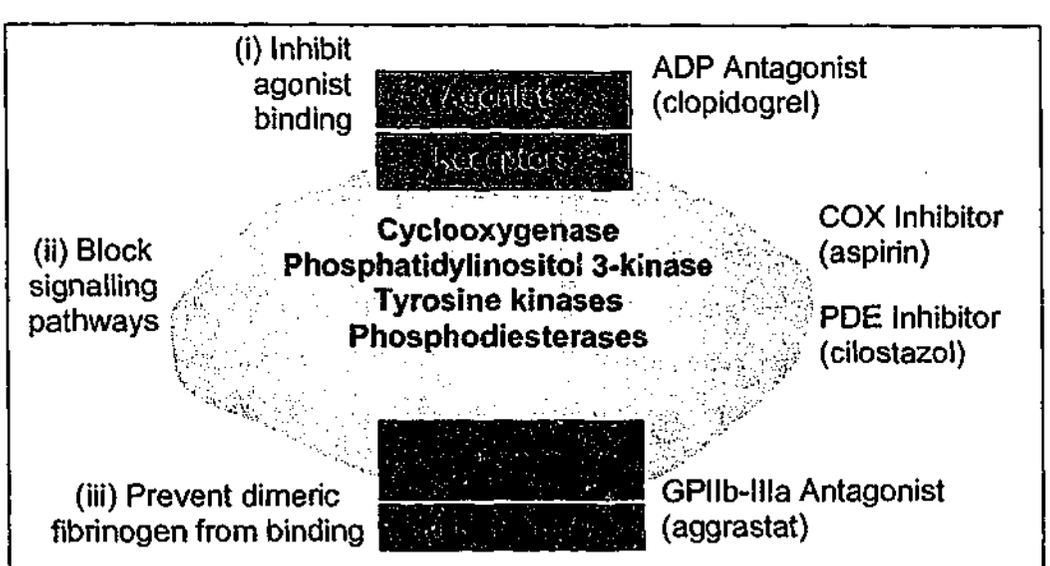


Figure 1.3 Inhibition of platelet activation and aggregation.

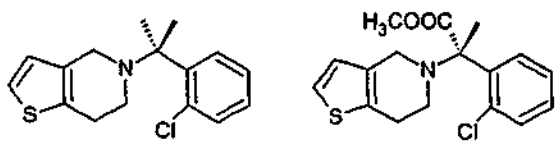
1.4.1 Inhibition of Agonist Binding

An obvious target for inhibition of platelet aggregation is to block the endogenous agonists which induce this process. While a variety of pathways have been examined, including ADP antagonists, serotonin antagonists, thrombin and GPIb/V/IX inhibitors, only ADP receptor blockers have found clinical application so far.

Adenosine Diphosphate Antagonists

Adenosine diphosphate is one of the secreted secondary agonists released from platelet granules upon activation, and thus has a role in the amplification of the platelet aggregation signal. It binds to three receptors on the platelet surface including $P2X_1$, $P2Y_1$ and $P2Y_{12}$ (or $P2T_{AC}$).[4] Thienopyridines, such as ticlopidine or clopidogrel (Figure 1.4), are ADP antagonists for the $P2Y_{12}$ receptor although both compounds first require metabolism by the liver.[4,11] Binding irreversibly to a receptor results in the connected intracellular signalling pathway being blocked for the life of the platelet (approximately seven to ten days[12]). Both these orally active

compounds are approved for use for minimisation of stent thrombosis as well as the secondary prevention of stroke, myocardial infarction and peripheral vascular disease. However, ticlopidine has a number of potentially serious side effects including neutropenia (white cell reduction), aplastic anemia (defective red blood cell production), diarrhea, nausea, jaundice, skin rashes and thrombotic thrombocytopenic purpura (haemorrhage under the skin).[4] It has been largely replaced by clopidogrel which has an improved profile, more rapid onset and longer duration of action although still with some bleeding event problems.[5,13]

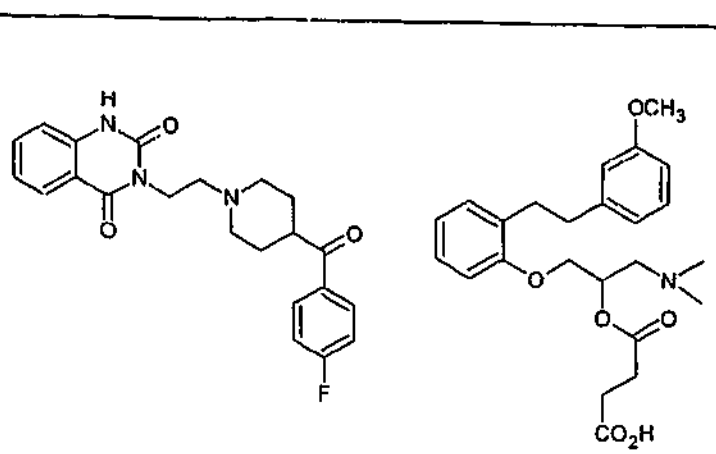


Ticlopidine Clopidogrel

Figure 1.4 Adensosine Diphosphate Antagonists.

Serotonin Antagonists

Upon activation, platelets also release serotonin and the binding of this agonist to the serotonin receptor has an accelerating effect on activation. Serotonin binds to the platelet G_q-coupled receptor 5-HT_{2A} and leads to calcium signalling.[4] Serotonin antagonists which have been used to target other therapeutic areas, such as the ketanserin (Figure 1.5), have demonstrated an antiplatelet side effect.[14] Related compounds such as sarpogrelate (Figure 1.5) are receiving more attention as potential antithrombotics.[15]



Ketanserin

Sarpogrelate

Figure 1.5 Serotonin Antagonists.

Thrombin Inhibitors

Thrombin is the most potent platelet stimulator and has multiple actions across the haemostatic pathway, including the cleavage of fibrinogen to fibrin for thrombus stabilisation. It has effects on the vascular endothelium, smooth muscle cells, the coagulation cascade and platelets. Heparin and hirudin, as well as other peptide and non-peptide inhibitors still under development, represent an important area in antithrombotic research.[1,5] However, as a result of the numerous critical functions of thrombin, inhibitory compounds may have serious side effects. Protease-activated receptors 1 and 4 (PAR1 and PAR4) are the receptors for thrombin, which mediate platelet responses at low and high concentrations of thrombin respectively.[4] Selective inhibition of these seven transmembrane domain receptors is being examined to broaden the applicability of drugs operating by this mechanism.

GPIb/V/IX Inhibition

Platelet adhesion to endothelium bound vWF has also been shown to be critically dependent on the GPIb/V/IX receptor, particularly during conditions of rapid blood flow and/or high shear rates.[12] The implication that this is the sole pathway of shear-induced clot formation, which is particularly important in atherosclerotic disease

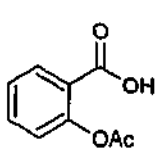
states, suggests that antiplatelet agents for this particular condition could be developed that do not compromise normal low-shear haemostasis. Two approaches have been taken, the first involves development of receptor blocking antibodies against GPIb/IX/V while the second approach involves developing recombinant fragments of vWF that express the GPIb/IX/V binding region and which may act as antagonists of vWF.[1]

However, it has also been suggested that compounds blocking adhesion, rather than aggregation, may undermine normal haemostasis and result in excessive bleeding as seen in the two genetic conditions of von Willebrand disease (where vWF is dysfunctional or not expressed) and Bernard-Soulier syndrome (GPIb/IX/V deficiency).[16] The true potential of this method of antithrombotic therapy awaits results from clinical trials.

1.4.2 Blocking of Signalling Pathways

Cyclooxygenase Inhibitors

Thromboxane A_2 (TxA_2) is another of the secondary agonists released by the activated platelets to encourage aggregation by binding to its corresponding receptor. Thromboxane A_2 is synthesised from arachnidonic acid by the cyclooxygenase pathway in the cell.[4] Aspirin (Figure 1.6), known principally for its analgesic properties, works as an intracellular antiplatelet agent by irreversibly binding to the prostaglandin G/H synthase through acetylation of a serine residue at 529 of the enzyme.[4,11] The irreversible binding disables the cyclooxygenase activity of the enzyme and thus the production of TxA_2 and the resulting pro-aggretory effect for the life of the platelet. Due to its relative safety, low cost and community acceptance, it remains the favoured choice for the chronic prevention of thrombosis.[5] However, even though platelet secretion and aggregation is reduced, aspirin remains a relatively weak platelet inhibitor which does not affect the majority of platelet responses.[16]



Aspirin

Figure 1.6 Cycloxygenase Inhibitor.

Phosphodiesterase Inhibitors

Platelet signalling cascades are known to be generally inhibited by increased intracellular levels of cyclic 3',5'-adenosine monophosphate (cAMP) and cyclic 3',5'-guanosine monophosphate (cGMP), presenting a method for the global inhibition of platelet aggregation.[17] Degradation of cAMP and cGMP is carried out by phosphodiesterases. Three phosphodiesterase isoforms have been confirmed in platelets; PDE2, PDE3 and PDE5. In platelets, cGMP is synthesised by guanylyl cyclase, which can be stimulated by nitric oxide released by nitrovasodilators such as nitroprusside. Degradation of cGMP is carried out by the cGMP-specific cyclic nucleotide phosphodiesterase PDE5 and PDE2. Synthesis of cAMP is completed by adenylyl cyclase while degradation occurs by the phosphodiesterases PDE2 and PDE3. Increases in platelet cAMP levels can be achieved by stimulating adenylyl cyclase, by prostaglandins or low concentrations of adenosine, or by inhibiting phosphodiesterases.[4] Increased levels of cAMP and cGMP, through associated protein kinases, appears to activate enzymes which inhibit platelet activation.[18]

The pyrimidopyrimidine derivative dipyridamole (Figure 1.7) is a phosphodiesterase inhibitor which has been used as an antiplatelet agent, usually in conjunction with aspirin or warfarin. However, there is little evidence to suggest that it is effective when used in this way or that it has independent activity.[4,11] Milrinone and cilostazol inhibit PDE3, the most abundant isoform present in platelets.[11,19] Cilostazol, a quinolinone derivative, is currently approved by the United States of America Food and Drug Administration (FDA) for the treatment of intermittent claudication (lameness) due to lower-extremity peripheral arterial occlusive disease

(PAOD).[19] However, drugs of this type have shown a poor side effect profile, including cardiac effects. This is generally thought to be due to the wide distribution of different subtypes of phosphodiesterases. Headache, diarrhea, palpitations and dizziness are also known side effects of these treatments.[4]

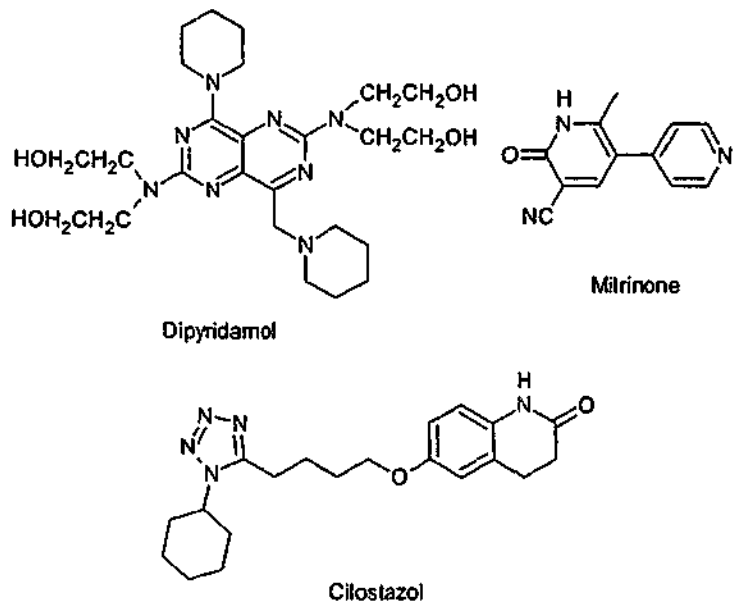


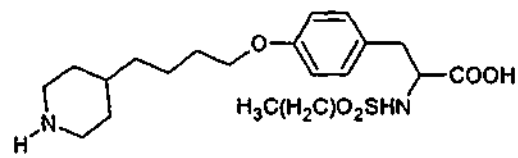
Figure 1.7 Phosphodiesterase Inhibitors.

1.4.3 Preventing Binding of Fibrinogen

GPIIb-IIIa Antagonists

Inside-out signalling leads to the activation of the integrin GPIIb-IIIa receptor, allowing the subsequent binding of dimeric fibrinogen required for the formation of platelet aggregates. As a result, global inhibition of aggregation can also be achieved by inhibiting the binding of fibrinogen to the GPIIb-IIIa receptor. Extensive research led to the discovery of antibody and peptide blockers of this interaction and the drug treatments for acute coronary artery disease which followed have been studied in numerous large multicentre clinical trials and reviewed at length.[4,5,11,16,20]

Glycoprotein IIb-IIIa receptor antagonists represent potent inhibitors of platelet aggregation with abciximab (c7E3, ReoPro), eptifibatide (integrilin) and tirofiban (aggrastat) currently in clinical use.[5] Abciximab is the Fab fragment of a human-murine chimeric monoclonal antibody while eptifibatide is a synthetic cyclic heptapeptide based on the Lys-Gly-Asp (KGD) motif of the snake venom disintegrin barbourin. Tirofiban (Figure 1.8) is an RGD-based peptidomimetic analogue of tyrosine and represents the first non-peptide GPIIb-IIIa antagonist to be marketed.[11]



Tirofiban

Figure 1.8 GPIIb-IIIa Antagonist.

While these glycoprotein IIb-IIIa receptor antagonists have been shown to be beneficial in an acute setting, they are limited due to their need for intravenous administration and possible bleeding complications. The second generation of compounds that have been developed are orally active and are administered as prodrugs with metabolic conversion to the active forms. Sibrafiban, xemilofiban and orbofiban (Figure 1.9) were the first three of these agents to undergo major Phase III clinical trials but, following a mix of poor efficacy and side effect profiles which included bleeding, their development was discontinued.[11,16,21] Despite these initial disappointing results, a number of GPIIb-IIIa antagonists remain in clinical trials and new compounds continue to be developed.

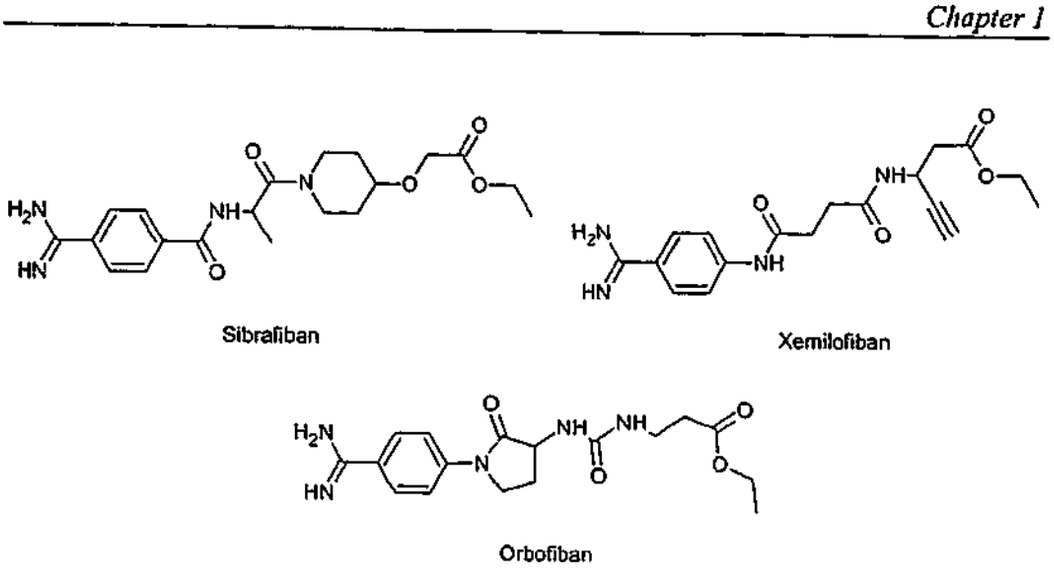


Figure 1.9 Oral GPIIb-IIIa Antagonists.

1.5 Antiplatelet Agents – The Next Generation

There are a number of problems with the current selection of antiplatelet drugs. Possibly the most significant drawback is the narrow therapeutic window. That is, there is little difference between the therapeutic dose and the dose that causes side effects such as bleeding. There is also a large difference in responses to drugs and dosages from patient to patient. A particular treatment which works well in one patient may not work at all in another, or the drug may stop having its therapeutic effect over time. Therefore, ongoing individual monitoring is required on a regular basis, which is inconvenient and inefficient. Additionally, many of the current antithrombotic drugs are only available intravenously, restricting their use to an acute clinical setting.

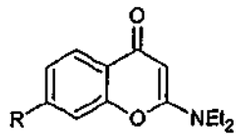
Further research and development producing the next generation of drugs is likely to target pathways which are more important to pathological clot formation (rather than normal haemostasis), particularly as new mechanisms of thrombosis are identified.[22] Together with the development of assays to better measure the inhibition of the targeted enzyme(s), this will enable more selective drugs to be developed. Extensive research is also being done on improved global inhibitors with reduced side effects

while another area of interest is to be able to identify the particular problem pathway in an individual patient and target it specifically.

1.6 2-Morpholinochromones – A New Class of Antiplatelet Agents?

1.6.1 Biological Activity of 2-Aminochromones

As interest in the role of platelets in haemostasis and thrombosis increased in the early 1980s, efforts were directed to synthesising antiplatelet agents which were significantly more active than aspirin in inhibiting platelet function. Although the chromone structure was poorly represented among antiplatelet drugs,[23,24] a longstanding interest in 2-(dialkylamino)-chromone derivatives prompted Mazzei *et al* to test their compounds as *in vitro* inhibitors of platelet aggregation.[25] When compared to aspirin, a number of the chromones tested showed improved activity against particular agonists (being ADP, collagen or arachidonic acid), although none of the chromones tested were more active than aspirin against all three agonists. Encouraging results for 2-(diethylamino)-chromones with electron releasing substituents at C-7 (such as hydroxyl, methoxy and methyl) (Figure 1.10) were targeted for further investigation.[26,27]

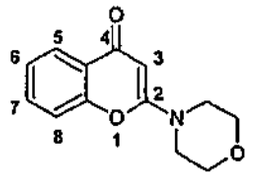


R = OH, OCH_3, CH_3

Figure 1.10 2-(Diethylamino)-chromones.

In 1990, The Upjohn Company published a patent regarding the antiatherosclerotic and antithrombotic properties of 1-benzopyran-4-ones and 2-amino-1,3-benzoxazine-4-ones.[28] It claimed these compounds to be useful as atherosclerotic agents and also to be inhibitors of cell proliferation and/or platelet aggregation. The majority of these compounds were derivatives of 2-(4-morpholinyl)-*4H*-1-benzopyran-4-one, U-67154

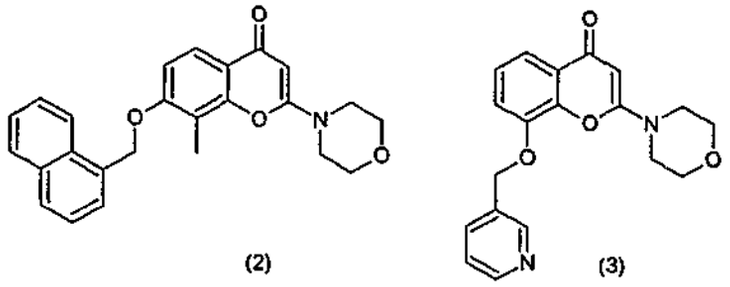
(1) (Figure 1.11), and three years later the laboratories of Upjohn and Lilly jointly published a study of the antiproliferative and antichemotactic properties of this compound.[29] Assays *in vitro* using fibroblasts and smooth muscle cells found that for U-67154 the concentration at which 50% inhibition of the control occurs (IC_{50}) was between 120-200 μM for inhibition of cell proliferation and migration, which was not due to cytotoxicity and was fully reversible upon compound removal.



(1)

Figure 1.11 U-67154.

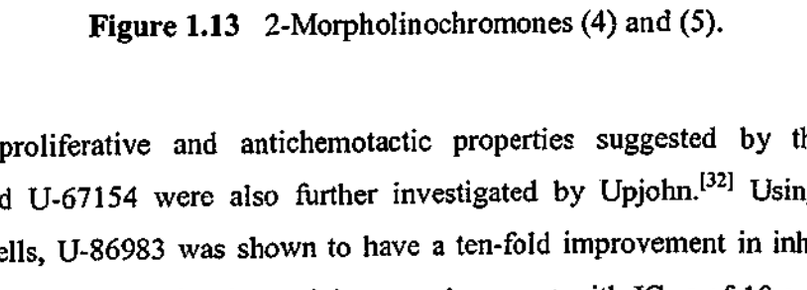
Upjohn followed this work with a description of analogues such as U-84569, 8-methyl-2-(4-morpholinyl)-7-(1-naphthylenylmethoxy)-*4H*-1-benzopyran-4-one (2) (Figure 1.12).[30] This compound showed potential as an antithrombotic agent and was unusual because it inhibited platelet aggregation regardless of the agonist used. Experimental work suggested this occurred by elevation of cAMP levels, though not by direct stimulation of adenylate cyclase. Measured platelet phosphodiesterase activity showed U-84569 had an IC_{50} of 400 nM while U-86983, 2-(4-morpholinyl)-8-(3-pyridinylmethoxy)-*4H*-1-benzopyran-4-one (3) (Figure 1.12) had an IC_{50} of 2 μM. The weakest inhibitor was the parent compound U-67154 with an IC_{50} of 1mM. This rank order of potency was also found to be true for platelet aggregation assays, suggesting that this antiplatelet effect occurred as a result of raised cAMP levels by inhibition of low K_m-cAMP phosphodiesterase (PDE3).



(2) (3)

Figure 1.12 U-84569 (2) and U-86983 (3).

An array of compounds similar to U-84569 were synthesised and evaluated by Upjohn.[31] The parent compound U-67154 had an IC_{50} of ca. 140 μM for ADP-induced platelet aggregation. The introduction of a benzyloxy group to C-7 with C-8 methyl (4) (Figure 1.13) improved the IC_{50} greater than threefold to 40 μM. U-84569 was found to have an IC_{50} of 13 μM, when a C-3 methyl was added activity was lost (IC_{50} >75 μM). A 7-(4-methyl-1-piperazinyl)ethyloxy derivative (5) (Figure 1.13) was found to be the most effective with an IC_{50} of 0.85 μM. Removal of the C-8 methyl was detrimental in this case (IC_{50} 35 μM), whereas a methyl group at C-6 was tolerated (IC_{50} 1.4 μM).

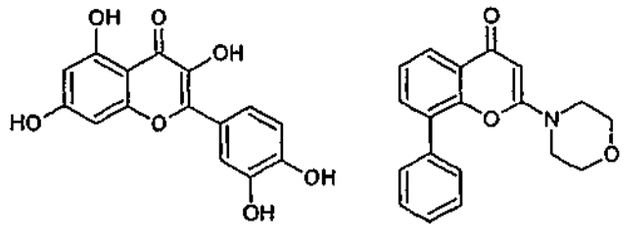


(4) (5)

Figure 1.13 2-Morpholinochromones (4) and (5).

The antiproliferative and antichemotactic properties suggested by the parent compound U-67154 were also further investigated by Upjohn.[32] Using smooth muscle cells, U-86983 was shown to have a ten-fold improvement in inhibition of migration and antiproliferative activity over the parent with IC_{50}s of 10 and 3.5 μM respectively.

Lilly Research Laboratories also synthesised and tested a number of 2-aminochromones for antiproliferative activity, based on the activity of the flavonoid quercetin (Figure 1.14), a phosphatidylinositol 3-kinase inhibitor.[33] One of these analogues was LY294002, 2-(4-morpholinyl)-8-phenyl-*4H*-1-benzopyran-4-one (6) (Figure 1.14), which was reported to completely and specifically abolish PI3-kinase activity.[33] With an IC_{50} of 1.4 μM, LY294002 had significant improvement in PI3-kinase inhibition over the unsubstituted parent compound LY292223, also known as U-67154 (1), which had an IC_{50} of 5 μM.



Quercetin (6)

Figure 1.14 Quercetin and LY294002 (6).

The role of PI3-kinase in intracellular signal transduction continues to be investigated, particularly in regard to using isoform selectivity to treat inflammation and cancer. For this cell signalling research, LY294002 is one of the most widely used compounds.[34-36] However, the importance of PI3-kinase in platelet adhesion, particularly under the conditions of high shear, has also been demonstrated.[37] PI3-kinase generates phosphorylated lipid second messengers which stimulate platelet adhesion under blood-flow conditions and, as platelet adhesion is a necessary step in the formation of a thrombus, PI3-kinase inhibitors such as LY294002 are indicated as potential antithrombotics.

1.6.2 Summary and Objectives

The 2-morpholinochromones reported in the literature have shown a number of different interesting and useful properties, including inhibition of platelet aggregation, smooth muscle cell proliferation and migration at a cellular level. At an enzyme level, they have demonstrated PI3-kinase and PDE inhibition. Yet structural differences between these compounds vary only slightly. Those differences can be classified by the structural substitutions made to the parent compound. Structures which have substitutions at the C-8 position have been made by both Lilly and Upjohn. Of these, one has been reported as a PI3-kinase inhibitor (LY294002) and the other as an antiproliferative agent (U-86983). Upjohn compounds possessing a C-8 methyl group with another C-7 substitution (U-84569, (4), (5)) have demonstrated antiplatelet effects using ADP as an agonist. One of these (U-84569) has been implicated as exerting these antiplatelet effects through phosphodiesterase inhibition. Some compounds lacking the C-8 methyl have relatively reduced activity against ADP-induced platelet aggregation. An alternate C-6 methyl substitution on (5) has been reported to be tolerated in ADP-induced platelet aggregation yet further investigation into these types of structures has not been reported.

Significant efficacy was observed for the 7-(4-methyl-1-piperazinyl)ethyloxy derivative (5) in a canine model of thrombosis without side effects. Enthusiasm for compound development was slowed, however, by observed cardiac effects of platelet PDE3 inhibitors in general (e.g. milrinone) coupled with haemodynamic effects seen early on in the more potent 2-morpholinochromone analogues.[38] However, determination of the many sub-types of PDEs, three of which are expressed in platelets (PDE2, 3A and 5) and the capacity for selective isoform inhibition as shown for PDE2 (*erythro*-9-(2-hydroxy-3-nonyl)adenine or EHNA), PDE3 (cilostazol), PDE4 (rolipram) and PDE5 (sildenafil) has given renewed hope for isoform selective PDE inhibitors as antithrombotics. The cellular mechanism for the antiproliferative effects of 2-morpholinochromones has not been determined to date but, interestingly, would be consistent with either PDE or PI3-kinase inhibition. In addition, ongoing study and characterisation of the isoforms of PI3-kinase is showing that different

isoforms have distinct roles within cells.[39,40] Therefore, isoform selective inhibitors of PI3-kinase, as well as PDE, have great potential for both research and therapeutic purposes.

While numerous 2-morpholinochromones described by Upjohn have antiplatelet activity, no direct assessment of PDE structure-activity has been reported. Since the original report, no further structure-activity data has been published on LY294002, despite its importance as a PI3-kinase inhibitor. Given the importance for discovering new PI3-kinase and PDE inhibitors with enzyme and sub-type selectivity, further evaluation of 2-morpholinochromones from that perspective was required. This work reports on the novel syntheses for 2-morpholinochromones from each of the four described classes and biological evaluation of these compounds.

The objectives of this thesis are to

(i) Investigate the synthesis of known and novel 2-morpholinochromones

(ii) Develop biochemical assays for these compounds

(iii) Evaluate anti-enzyme activity of compounds and correlate to cellular activity

(iv) Discover potential new avenues for the development of antiplatelet agents

Chapter 2

SYNTHETIC STUDIES OF LY294002 – A PHOSPHATIDYLINOSITOL 3-KINASE INHIBITOR

2.1 Introduction

The synthetic morpholinochromone, LY294002 [2-morpholin-4-yl-8-phenyl-*4H*-chromen-4-one, (6)], together with the fungal metabolite wortmannin (Figure 2.1), has been an important pharmacological tool used to delineate the critical role that phosphatidylinositol 3-kinases play in the regulation of intracellular signalling pathways.[35] Despite the extraordinary amount of biological data that has been generated using LY294002, there has been only a single, limited structure-activity study[41] since the original report.[33] Remarkably, numerous compounds of the morpholinochromone class have been described by the Upjohn company as antiplatelet and antiproliferative agents[42] yet, in spite of the potential connection between these cellular responses and PI3-kinase inhibition, no direct assessment of the PI3-kinase activity of these compounds has ever been described.

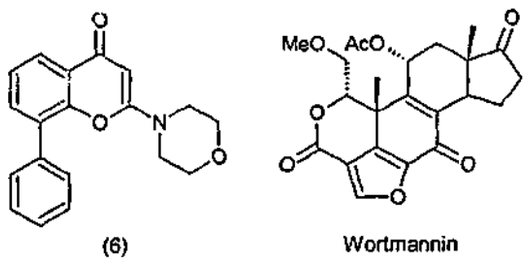


Figure 2.1 LY294002 (6) and Wortmannin.

The determination of structure-activity relationships surrounding LY294002 is emerging as an important task, given that more recent evidence suggests that it is not as selective against PI3-kinase as first thought. Inhibition of other enzymes in the effective concentration range against PI3-kinase has now been shown.[43] Also, LY294002 has demonstrated only moderate potency and selectivity against

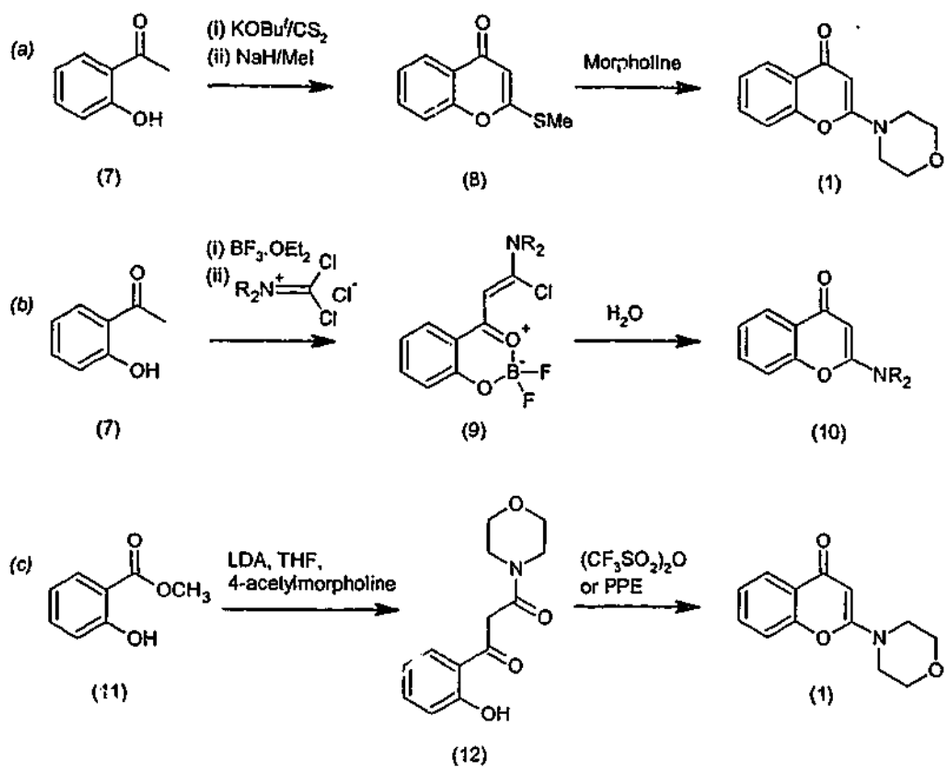
individual isoforms of PI3-kinase.[35] In complex with Class I PI3-kinase p110γ, LY294002 makes contacts with residues that are conserved across the Class I isoforms,[44] which may explain this lack of isoform selectivity. As various PI3-kinase isoforms have now been shown to have distinct roles within cells,[39,40,45,46] isoform selective inhibitors will be required to define the roles of each isoform and to identify the associated cellular responses.

Against this background, it was felt that further evaluation of 2-morpholinochromone analogues was warranted. This requires methodology for the rapid generation of analogues which would be suitable for simultaneous multiple synthesis and high throughput preliminary enzyme inhibition screening.

Synthetic methodology has been pursued for the direct and high yielding preparation of the PI3-kinase inhibitor LY294002. However, synthetic methods that are concise and readily amenable to the efficient generation of analogue libraries are required to facilitate detailed investigation of this important family of enzymes, and here those syntheses are reported.

The great bulk of syntheses of 2-aminochromones have come from two synthetic strategies. Thiochromone precursors (8) have been utilised in preparing a variety of 2-aminochromones (Scheme 2.1*a*), by Bantick and Suschitzky,[47] Roma *et al*[48] and also by Vlahos *et al* in the preparation of LY294002.[33] Yields from these pathways are generally quite poor, although they offer the potential to alter the 2-amino moiety at a late stage in the synthesis. Workers at Upjohn developed a methodology that utilised phosgeniminium chloride reagents to prepare a wealth of compounds (Scheme 2.1*b*).[49] They also developed an alternate synthetic route in which 2-aminochromones were prepared by condensation/dehydration conditions analogous to classical flavone syntheses (Scheme 2.1*c*).[50] Preparation of 2-morpholinochromones under this scheme was critically dependent upon development of a cyclodehydration step from the precursor salicylacetamide (12). Initial attempts to achieve this transformation using acid or base catalysis had failed due to preferential elimination of the morpholinyl group. However, cyclodehydration could

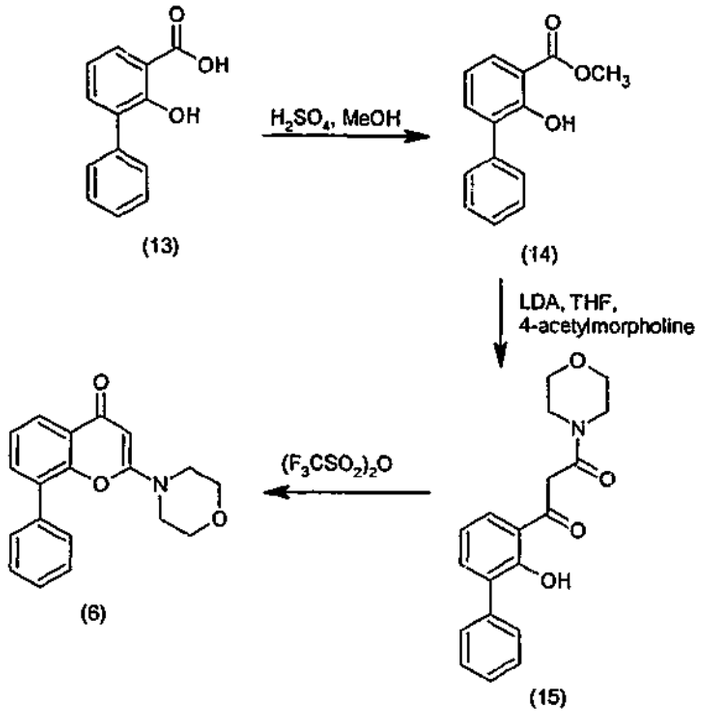
be completed under neutral conditions using trifluoromethanesulfonic anhydride or polyphosphoric ester.



Scheme 2.1

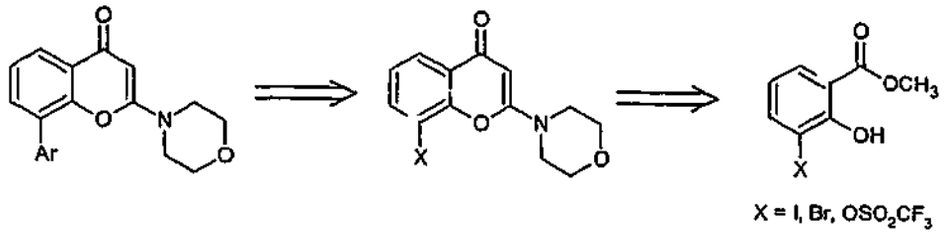
The excellent yields and mild conditions of this condensation/dehydration method seemed to be a more suitable synthesis than those used previously and was applied to the direct and high yielding preparation of LY294002 in this laboratory.[51] This direct synthesis of LY294002 was achieved using the methyl ester of the commercially available 3-phenylsalicylic acid (14) condensed with the lithium diisopropylamide (LDA) generated anion of 4-acetylmorpholine to yield the salicylacetamide (15) (Scheme 2.2). Cyclodehydration with trifluoromethanesulfonic anhydride yielded LY294002 (6) in good yield (44%) and identical by thin layer chromatography (t.l.c.), m.p., HPLC and proton nuclear

magnetic resonance (NMR) to commercial material (Calbiochem). This method was well suited to multigram scale synthesis of LY294002.



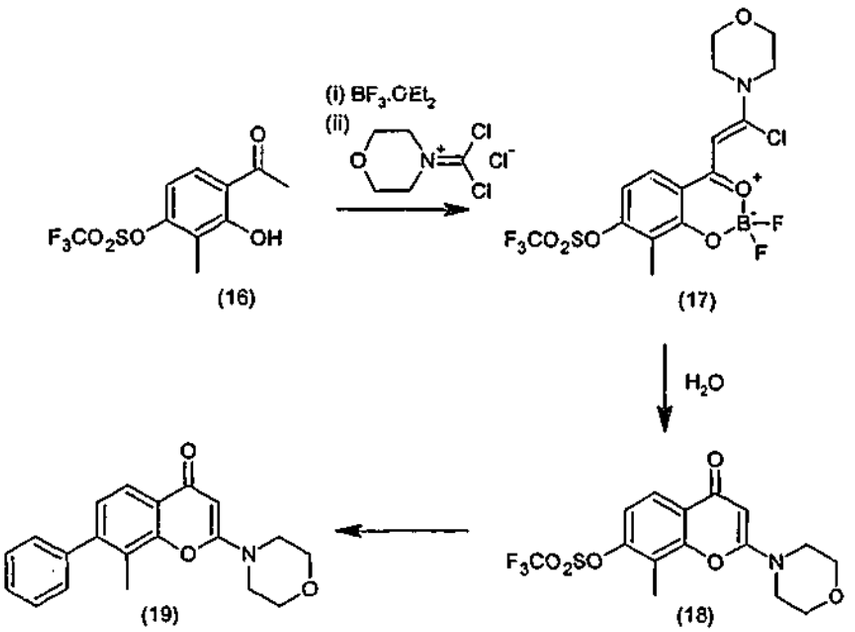
Scheme 2.2

While this synthesis of LY294002 was a significant advance on the literature methods, it was preferred to have methods amenable to the ready synthesis of analogues in which the aryl moiety was varied. As such, it was desirable to generate functionalised 2-morpholinochromones from which the biaryl moiety could be created in the final step. A Suzuki coupling based strategy was the obvious candidate to achieve this, with 8-halogen or 8-trifluoromethanesulfonate substituted 2-morpholinochromones as substrates. The following retrosynthetic analysis was determined (Scheme 2.3).



Scheme 2.3

In support of this concept was the example reported by Gammill *et al* in their 1990 patent, which outlined the synthesis of an LY294002 analogue using palladium-catalysed coupling and a trifluoromethanesulfonate intermediate (Scheme 2.4).[28]



Scheme 2.4

As well as providing a convenient synthesis of LY294002 and analogues, it was envisaged that this general methodology could also provide a versatile strategy for the generation of a diverse library of 2-morpholinochromones. For example, a number of 2-morpholinochromone precursors with trifluoromethanesulfonate substitution in different positions could also be reacted in Suzuki couplings. It was also understood that trifluoromethanesulfonate and halogen precursors could be converted to substituents other than aryl groups by other coupling methodologies.

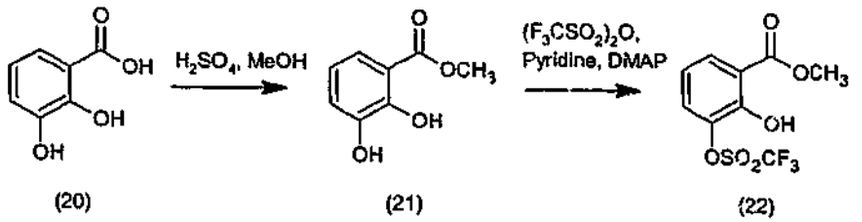
Additionally, the trifluoromethanesulfonates could be reacted to generate boronate or hydroxy substituents, which would again expand the types of couplings which could be undertaken. This would allow efficient synthesis of a very diverse library of analogues for examination in bioassays.

2.2 Synthesis of the Key Trifluoromethanesulfonate Intermediate

The trifluoromethanesulfonate (triflate) group is widely used in synthesis due to its excellent leaving group properties. The high degree of stability of aryl triflates makes them excellent candidates for electrophillic substitution reactions in the *ortho* and *para* positions, including nitration, halogenation and sulfonation. However, it was the discovery of the ability of vinyl and aryl triflates to cross-couple with other compounds to form new carbon-carbon bonds that increased their application in synthetic chemistry greatly.[52]

Advantages for using trifluoromethanesulfonate chemistry also include mild reaction conditions, high regio- and stereoselectivity, good yields and tolerance of other functional groups. A large range of reaction partners can be used, including organometallic reagents and unsaturated systems. The main drawback is the relatively high expense of the triflating reagents.[52] Phenols are the typical starting materials for the synthesis of aryl triflates. Room temperature reaction with trifluoromethanesulfonic anhydride in the presence of a base, such as triethylamine or pyridine, usually results in an excellent yield of the triflate product.

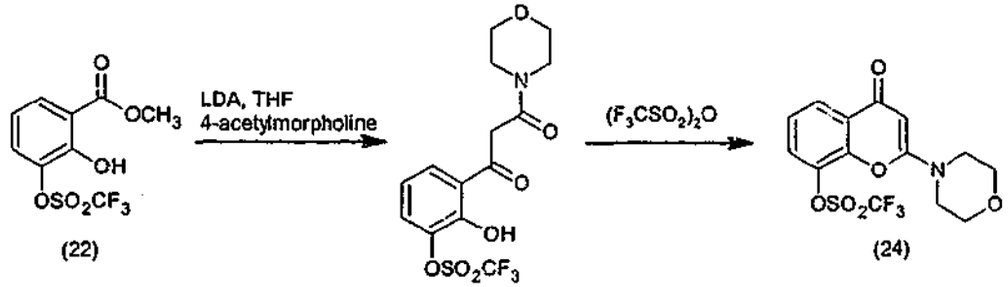
As such, methyl 2,3-dihydroxybenzoate (21) was prepared by esterification of 2,3-dihydroxybenzoic acid (20) in 87% yield, then treated with 1.1 equivalents of trifluoromethanesulfonic anhydride in the presence of 4-(dimethylamino)pyridine and pyridine. The desired methyl 2-hydroxy-3-{[(trifluoromethyl)sulfonyl]oxy}benzoate (22) was synthesised with a yield of 76% (Scheme 2.5). No formation of the 2-trifluoromethanesulfonate side product was observed, presumably due to the influence of the adjacent ester.



(20) (21) (22)

Scheme 2.5

Applying the methods of Morris *et al*[150], the ester (22) was successfully condensed with the LDA-generated anion of 4-acetylmorpholine to give the corresponding salicylacetamide (23) in 75% yield (Scheme 2.6). Column chromatography was unable to completely remove unreacted 4-acetylmorpholine from the salicylacetamide (23), so this was carried through the next step. The key cyclodehydration step was performed with trifluoromethanesulfonic anhydride to give the desired triflate product (24) in good yield following recrystallisation (60%).

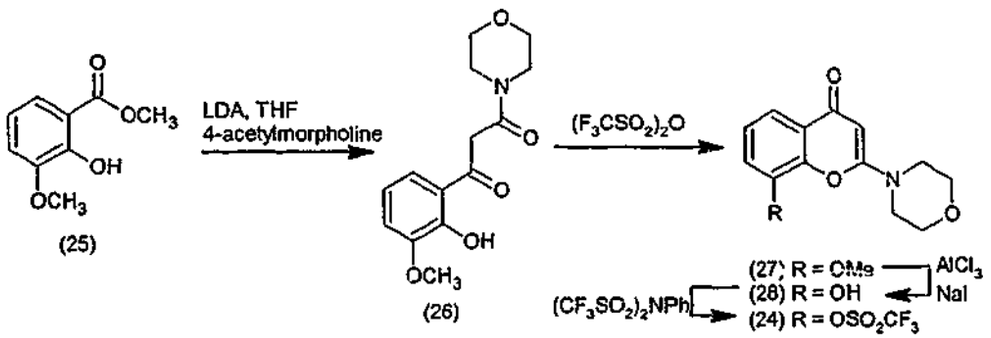


(22) (23) (24)

Scheme 2.6

The formation of the 2-morpholinochromone (24) is most easily characterised by proton NMR in the downfield chemical shift of the uncyclised starting material CH_2 at ca. 4.1 ppm to the 3-CH of the cyclised product at ca. 5.5 ppm and the associated change in integration from 2 to 1 equivalent protons. Characterisation of the trifluoromethanesulfonate (24) was also carried out by t.l.c., melting point and mass spectrometry. By t.l.c., the product (R_f 0.65) could be distinguished from the starting salicylacetamide (R_f 0.75) where the eluent was 20:1 dichloromethane:methanol.

An alternative pathway which was potentially a more time and cost efficient synthesis of the trifluoromethanesulfonate (24) was envisaged by triflation of 8-hydroxy-2-morpholinochromone (28). 3-Methoxysalicylic acid (25) was a significantly less expensive starting material than 2,3-dihydroxybenzoic acid (20) and did not require a 3-hydroxy protection step following esterification. Preparation of 8-methoxy-2-morpholin-4-yl-*4H*-chromen-4-one (27) by the same method of Morris *et al* was accomplished very successfully (Scheme 2.7). The products did not require chromatographic purification but could be precipitated or recrystallised relatively quickly with high purity and yield (63% overall).



Scheme 2.7

Ether cleavage is a widely used reaction in organic synthesis, particularly for the deprotection of hydroxy groups in pharmaceutical and natural product chemistry, and many different reagents are available. In recent years, boron tribromide is often the reagent of choice for ether cleavage as it can be used under mild conditions without affecting other functional groups, avoiding strongly acidic or basic conditions. Iodotrimethylsilane often has a different ether-cleavage pattern from boron halides and the two approaches can complement each other. Aluminium chloride, a Lewis acid, is an inexpensive industrial scale chemical which is also used in the laboratory for the selective cleavage of methoxy groups adjacent to aldehyde or ketone groups.[53]

Demethylation of the methoxy intermediate (27) to the corresponding phenol (28) proved more difficult than initially predicted. Despite initial success on a small scale

using boron tribromide,[54] later attempts to perform this reaction on a similar or larger amount of material showed a competing ring opening of the morpholinyl group. Demethylation using iodotrimethylsilane was attempted without success, as was refluxing the compound (27) with aluminium chloride. However, by the addition of sodium iodide to the aluminium chloride with refluxing, as suggested for flavones by Horie *et al*,[54] demethylation was obtained in 65% yield. Unfortunately it was very difficult to separate the relatively insoluble 8-phenol (28) from the aluminium salts. Attempts to isolate the product by extraction with organic solvent, precipitation by pH adjustment and column chromatography were all unsatisfactory, particularly on larger scale.

Formation of the trifluoromethanesulfonate (24) from the phenol (28) was achieved using the milder and selective, but expensive, triflating reagent *N*-phenyltrifluoromethanesulfonimide and was characterised by m.p., proton NMR and t.l.c as identical to the previously synthesised material. An attempt to use of trifluoromethanesulfonic anhydride under basic conditions had resulted in decomposition of the 8-phenol (28).

2.3 Suzuki Coupling using the Key Trifluoromethanesulfonate Intermediate

Cross-coupling of an organic electrophile with an organometallic reagent is a widely-used reaction for carbon-carbon bond formation. Direct coupling of aryl triflates occurs with organocuprates, but highly reactive lithium compounds cleave the sulfur-oxygen bond only. For reaction with the less reactive compounds (organotin, -zinc, -boron, -aluminum or -silicon) catalysis by transition metals such as palladium and nickel is required. The boron compounds have been preferred for reaction due to their ease of preparation, low toxicity and easy handling.[52]

The Suzuki reaction is the well known palladium-catalysed cross-coupling reaction of alkenyl or aryl trifluoromethanesulfonate with organoboron compounds (alkyl, aryl or alkenyl) in the presence of a base. The mechanism involves oxidative addition, transmetallation and reductive elimination sequences (Figure 2.2)[55] and

the base is thought to cause acceleration of the transmetallation step, possibly due to formation of intermediate oxo-palladium complexes.[52] Suitable catalysts include tetrakis(triphenylphosphine)palladium [$Pd(PPh_3)_4$] and dichloro[1,1'-bis(diphenylphosphino)ferrocene]palladium (II) [$PdCl_2(dppf)$]. Best results are obtained from anhydrous salts (potassium phosphate or carbonate) in polar solvents (dioxane, tetrahydrofuran) or with aqueous solutions of sodium carbonate in dimethoxyethane.[52]

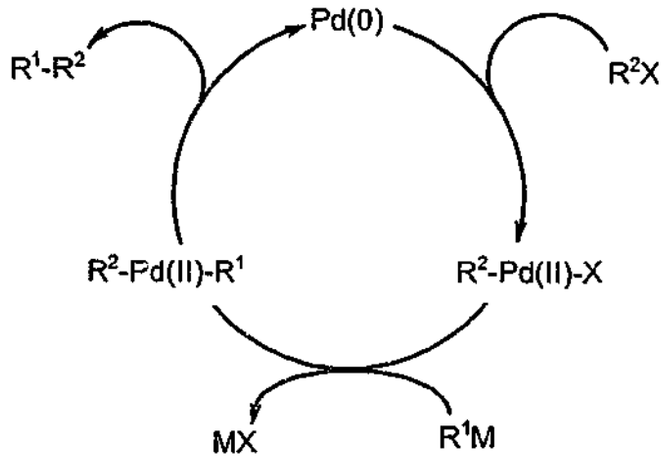


Figure 2.2 Palladium catalyst cycle.

In the first instance, the trifluoromethanesulfonate (24) was coupled with phenylboronic acid according to the method of Badone *et al*[56] (Table 2.1). This involved using water as a non-toxic solvent with efficient "ligandless" catalysts, such as palladium diacetate, in the presence of the phase transfer reagent tetrabutylammonium bromide. While the yield obtained was modest (12%), improvement in the purification of the product (6) (Figure 2.3) was thought likely to increase the yield. Significant losses occurred during the isolation, with analytical HPLC suggesting the product conversion more likely to be approximately 30%.

The key trifluoromethanesulfonate intermediate (24) was then subjected to Suzuki coupling reactions with other arylboronic acids under a variety of conditions to prepare LY294002 analogues, as recorded in Table 2.1 and Figure 2.3. This included

the methodology of Percec *et al*,[57] which applied a more traditional solvent and catalyst (toluene and tetrakis(triphenylphosphine)palladium) resulting in increased yields of 47-63%, compared with the method of Badone *et al*. A third strategy involved the use of acetonitrile (a somewhat polar organic solvent but still with a relatively low boiling point) under similar conditions to those used initially, as suggested by Badone *et al*,[56] which gave yields of 68% and 37%.

Table 2.1 Preparation of LY294002 and analogues.

#	Arylboronic acid	Suzuki coupling conditions	Yield (%)
(6)	Phenylboronic acid	$Pd(OAc)_2$, K_2CO_3, BuN^+Br^-, H_2O, 70°C, 4 h[56]	12
(29)	2-(Trifluoromethyl)phenyl boronic acid	$Pd(PPh_3)_4$, $Na_2CO_{3\,(aq)}$, toluene, 90°C, 20 h[57]	59
(30)	*o*-Tolylboronic acid	$Pd(PPh_3)_4$, $Na_2CO_{3\,(aq)}$, toluene, 90°C, 20 h[57]	47
(31)	2-Chlorophenylboronic acid	$Pd(PPh_3)_4$, $Na_2CO_{3\,(aq)}$, toluene, 90°C, 20 h[57]	63
(32)	4-Fluorophenylboronic acid	$Pd(OAc)_2$, K_2CO_3, CH_3CN, 80°C, 20 h[56]	68
(33)	4-Phenoxyphenylboronic acid	$Pd(OAc)_2$, K_2CO_3, CH_3CN, 80°C, 20 h[56]	37

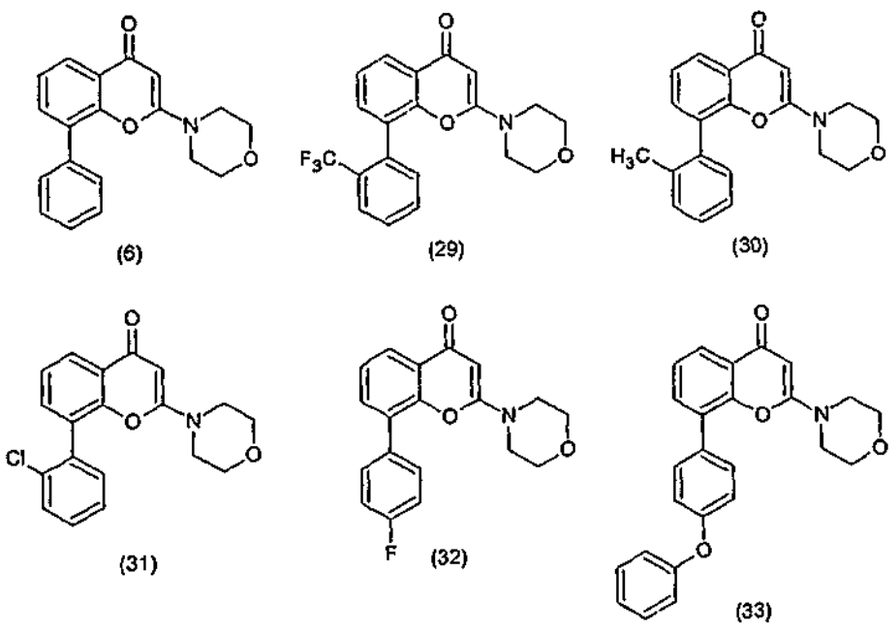
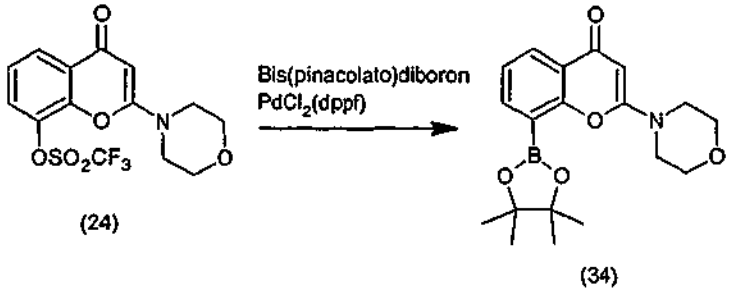


Figure 2.3 LY294002 and analogues formed through Suzuki coupling.

2.4 Suzuki Coupling using a Boronate Intermediate

In an extension of the strategy being used, the 2-morpholinochromone was also made the boronate partner of the Suzuki coupling reaction. While this requires an additional reaction step, doing so serves to expand the breadth of LY294002 analogues accessible as the broad range of aryl halides available can be utilised as substituents.

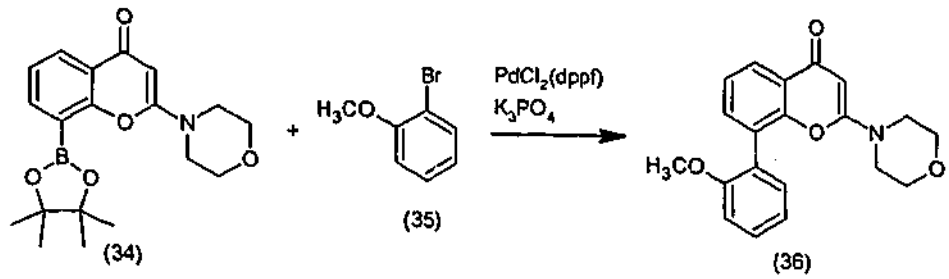
Treatment of the trifluoromethanesulfonate (24) with bis(pinacolato)diboron in the presence of dichloro[1,1'-bis(diphenylphosphino)ferrocene]palladium (II) [$PdCl_2$(dppf)] according to the method of Ishiyama *et al* [58] yielded the boronate ester (34) in 69% yield (Scheme 2.8).



Scheme 2.8

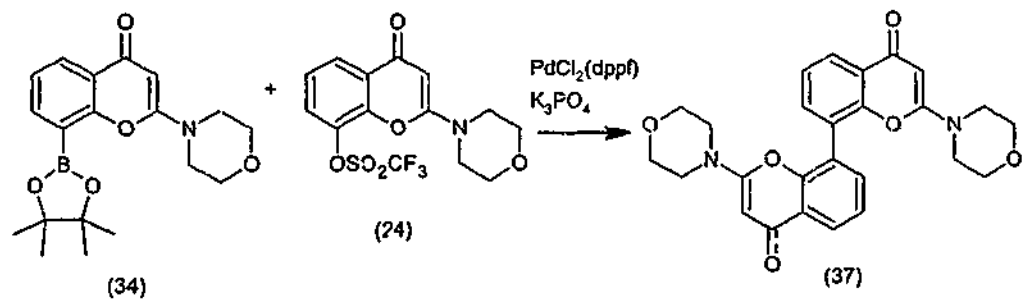
It was difficult to get the reaction to go to completion and it was also very difficult to completely separate the starting material from the product by chromatography. Initial attempts to purify by RP-HPLC resulted in cleavage of the boronate ester to the corresponding phenol under the weakly acidic conditions.

However, the usefulness of this type of Suzuki coupling strategy was demonstrated by the reaction of the boronate ester (34) with 2-bromoanisole (35) using $PdCl_2$(dppf) and potassium phosphate in dioxane (Scheme 2.9) according to the method of Ishiyama *et al.*[58] This resulted in the synthesis of 8-(2-methoxyphenyl)-2-morpholin-4-yl-*4H*-chromen-4-one (36) albeit in modest yield (13%).



Scheme 2.9

Analysis of the crude material by mass spectrometry resulted in the identification of a molecular ion of m/z 458 $[M + H]^{+\bullet}$. This was thought to possibly be due to a bichromone side product (37) from an unwanted Suzuki coupling of the boronate ester (34) with the trifluoromethanesulfonate (24) which had not been completely removed in the previous step (Scheme 2.10). Such a reaction would significantly limit the yield of the desired compound.



Scheme 2.10

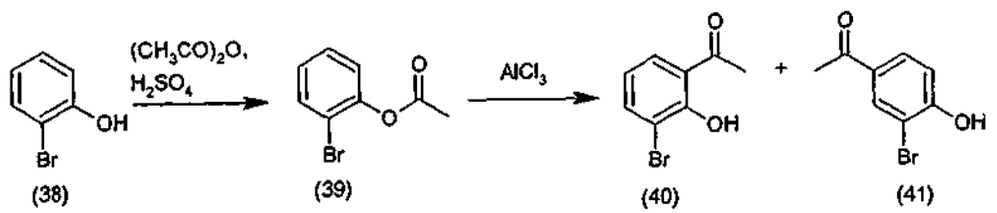
The importance of a highly purified boronate ester led to an extensive evaluation of chromatography eluent systems using thin layer chromatography. A solvent mix of 3:1 chloroform:tetrahydrofuran as eluent gave the best separation and was successfully applied to obtain the pure boronate ester (34), which was utilised further as described in the next chapter.

2.5 Synthesis of Bromo Intermediates

In the course of this work, 8-bromo-2-morpholinochromone was described by Chiosis *et al* as a precursor to LY294002 analogues, prepared via Heck-type couplings.[41] In that report, the synthesis utilised the thiochromone method of Roma *et al* (as described in Scheme 2.1*a*) but starting from 2-bromophenol (38). An 8-bromo-2-morpholinochromone was thought to be an additional means of achieving a key intermediate for Suzuki couplings.

Initial acetylation of 2-bromophenol (38) to afford compound (39) did not go to completion using diisopropylethylamine and acetyl chloride but was successful when stirred overnight with acetic anhydride and sulphuric acid (Scheme 2.11). However, great difficulty was found with the *ortho*-Fries rearrangement. Yields of the intermediate 3-bromo-2-hydroxyacetophenone were very poor as *para*-acylation (41) was found to strongly predominate over *ortho*-acylation (40). The Fries rearrangement was conducted by the addition of aluminium chloride to 2-bromophenyl acetate (39) with heating at 120°C overnight, with a 64% yield and an approximate 3:1 ratio of *para:ortho* being the best result obtained (Scheme 2.11).

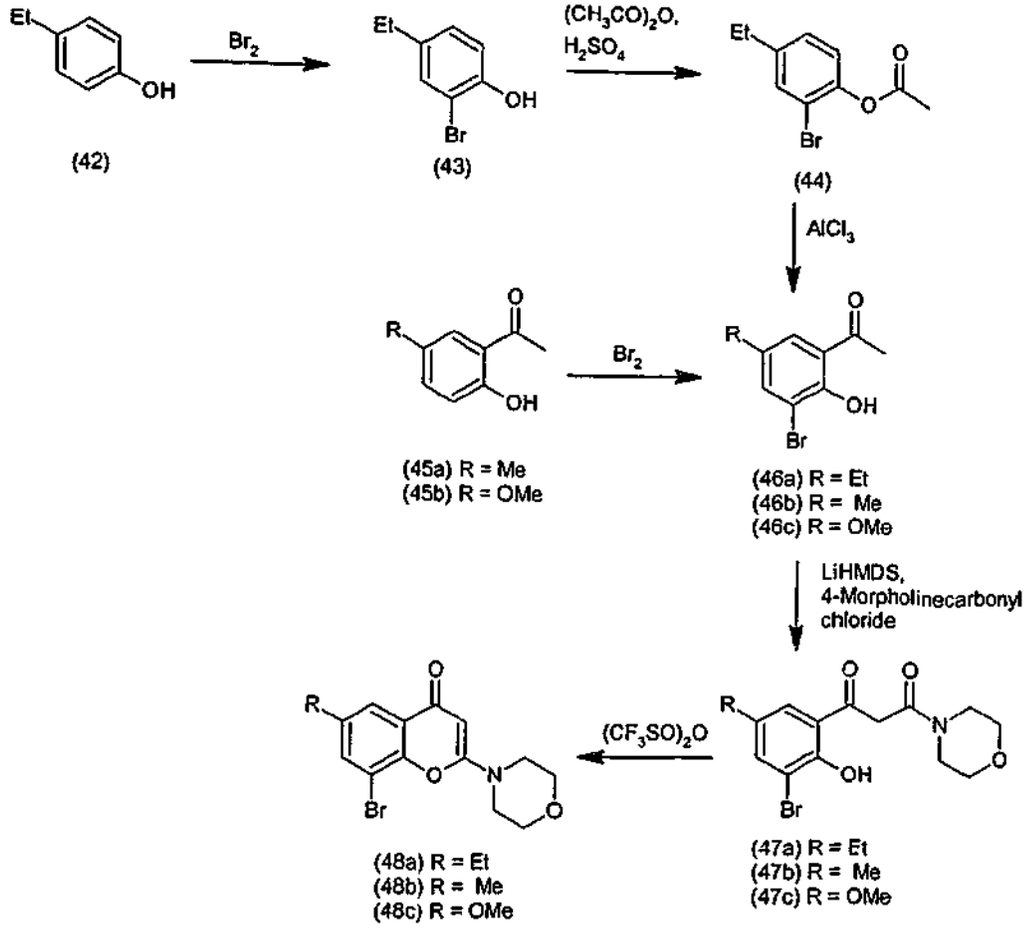
However, as it was difficult to completely purify the desired *ortho* isomer (40) from the *para* isomer (41) by chromatography, it was decided that this synthesis was not a practical way of proceeding. Different conditions, such as heating with polyphosphoric acid[59] or with zirconium chloride in dichloromethane,[60] resulted in degradation of the starting material or no reaction respectively.



(38) (39) (40) (41)

Scheme 2.11

As an alternative, 3-bromo-2-hydroxyacetophenones blocked at the 5-position were investigated (Scheme 2.12). Bromination of 4-ethylphenol (42) followed by acetylation and Fries rearrangement was successful in producing 1-(3-bromo-5-ethyl-2-hydroxyphenyl)ethanone (46a) in 26% overall yield. For the 5-methyl and 5-methoxy cases (46b-c), bromination of a precursor acetophenone (45b-c) gave excellent yields (74% and 97% respectively).



Scheme 2.12

In order to access the desired salicylacetamide intermediates, C-alkylation of the 2-hydroxyacetophenones (46a-c) was required. C-alkylation competes with O-alkylation of 2-hydroxyacetophenones, and the course of alkylation is dependent on the substrate acetophenone, the electrophile and the base. In classical flavone

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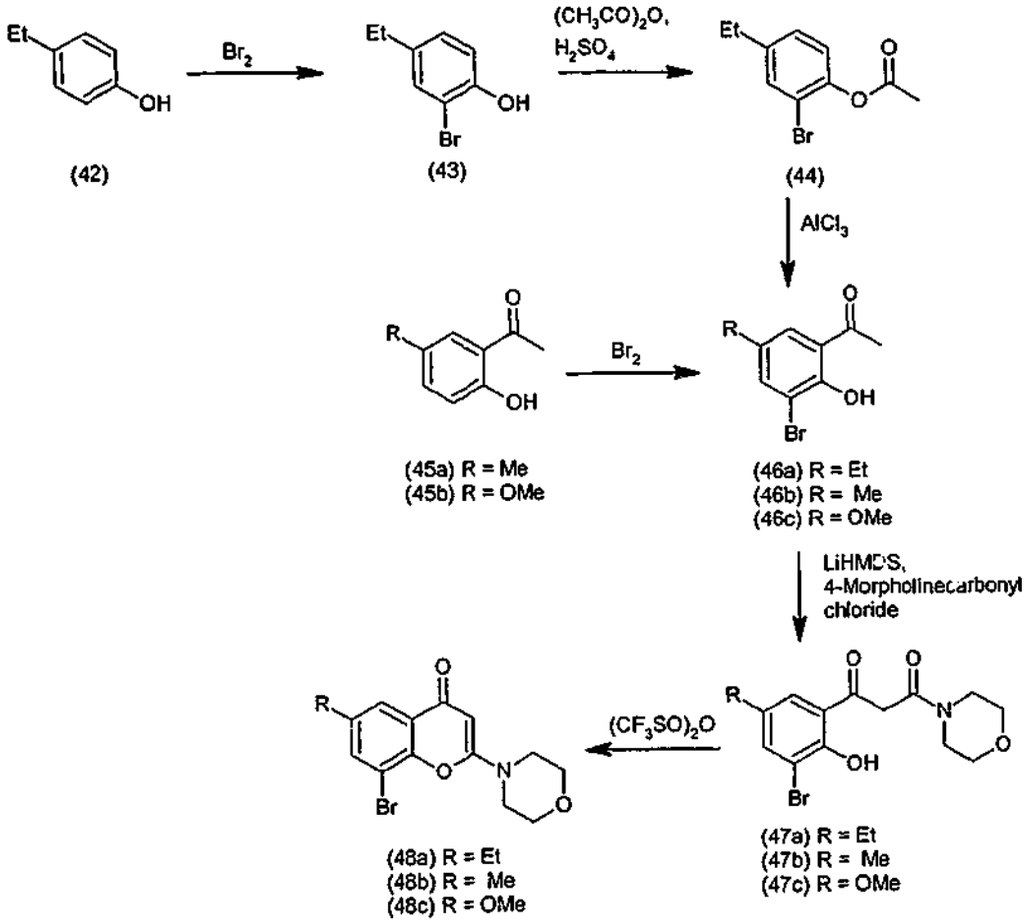

Scheme 2.12

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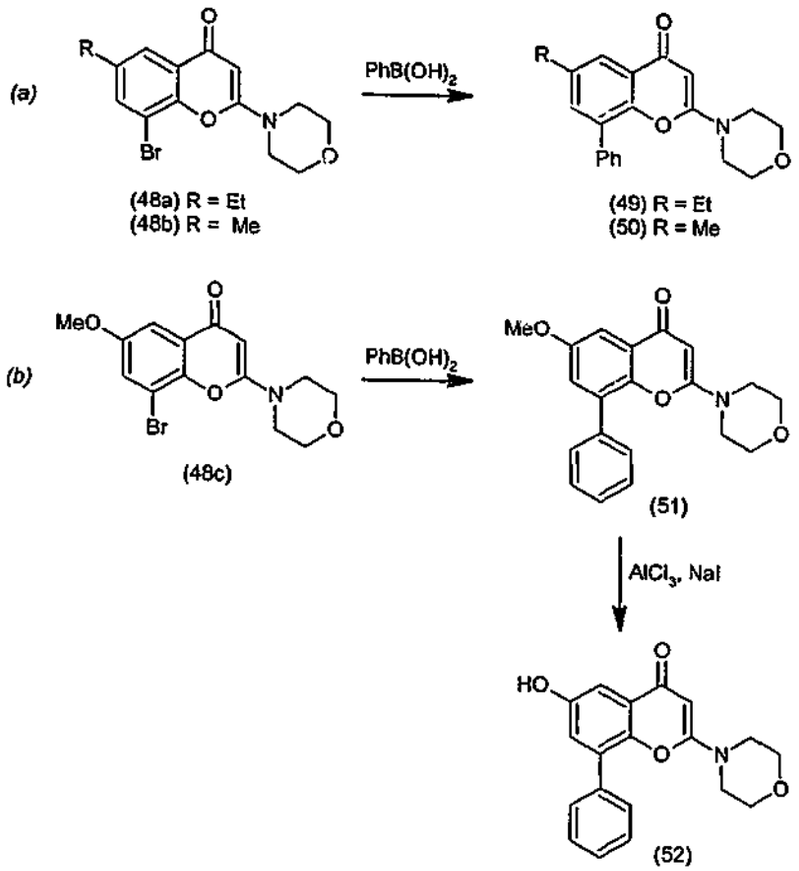
synthesis, O-alkylation is followed by an intramolecular rearrangement, known as the Baker-Venkataraman rearrangement.[61] Direct C-alkylation often provides better yields than a two step procedure.

Cushman and Nagarathnam have previously shown excellent levels of C-alkylation in the synthesis of flavones using lithium bis(trimethylsilyl)amide to form the enolate anion.[62] In this case, condensation of the enolates with 4-morpholinecarbonyl chloride gave good yields of the desired salicylacetamides (47a-c) in 52-58% yield (Scheme 2.12) with typical proton NMR resonances as described in Section 2.2. One particular advantage of this methodology, over that which was salicylic ester based, is the absence of 4-acetylmorpholine contamination in the products.

The resulting salicylacetamides (47a-c) then underwent cyclodehydration with trifluoromethanesulfonic anhydride to yield 6-substituted-8-bromo-2-morpholinochromones (48a-c) in 25-66% yield. Purification was completed by column chromatography where required.

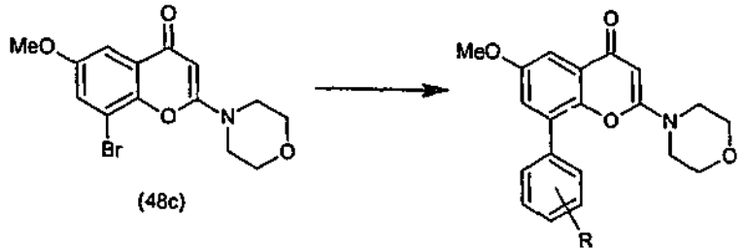
2.6 Suzuki Coupling using Bromo Intermediates

As with the trifluoromethanesulfonate, the 6-substituted-8-bromo-2-morpholinochromones (48a-c) readily underwent Suzuki couplings. For these reactions, the method of Ishiyama *et al* with $PdCl_2$(dppf) and potassium phosphate in dioxane was used.[58] The 6-ethyl and 6-methyl analogues of LY294002 (compounds 49 and 50 respectively) were prepared in this manner (Scheme 2.13*a*) in respective yields of 42% and 30%. The 6-methoxy analogue (51) was prepared on a larger scale, with a yield of 75%, which enabled demethylation with aluminium chloride and sodium iodide to give the corresponding 6-hydroxy analogue (52) in 42% yield following purification by RP-HPLC (Scheme 2.13*b*). Each of the four products (49-52) had proton NMR and MS data consistent with the proposed structures.



Scheme 2.13

Suzuki couplings were also performed using the 6-methoxy-8-bromo-2-morpholinochromone (48c) with 2-, 3- and 4-chloroboronic acids to obtain the *ortho*-, *meta*- and *para*-chloro substitution on the pendant ring (53, 54 and 55 respectively) in yields of 48-75% (Scheme 2.14).



Scheme 2.14

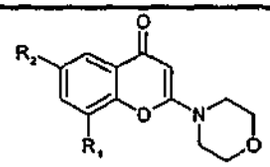
2.7 Summary and Conclusions

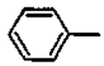
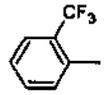
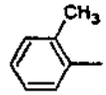
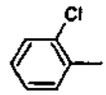
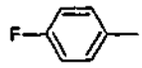
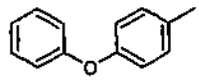
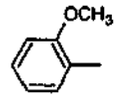
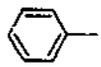
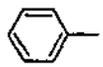
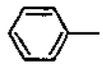
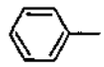
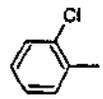
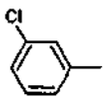
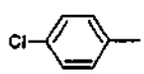
In summary, the synthetic chemistry studies detailed here have identified concise and adaptable routes to the generation of LY294002 and LY294002 analogues. The methods encompass use of either salicylate esters or 2-hydroxyacetophenones as precursors with access to aryl triflate, aryl boronate or aryl halide intermediates for Suzuki couplings.

It should be noted that while a variety of conditions were employed for the various Suzuki couplings performed in this study, the chosen reaction conditions did not appear to markedly affect the reaction yield, although they have not been fully optimised. Interestingly, it was found that the method using palladium diacetate and potassium carbonate in acetonitrile was effective, whilst being convenient in terms of cost and simplicity of work-up.

The methodologies which have been developed, together with the collection of commercially available aryl halides and arylboronic acids, allows access to an extensive library of LY294002 analogues. The 14 compounds described in this chapter and summarised in Table 2.2 are but a small subset of those that should be available by these methodologies.

Table 2.2 Summary of 2-morpholinochromone analogues synthesised.



R_1	R_2	Compound #
	H	(6)
	H	(29)
	H	(30)
	H	(31)
	H	(32)
	H	(33)
	H	(36)
	Et	(49)
	Me	(50)
	OMe	(51)
	OH	(52)
	OMe	(53)
	OMe	(54)
	OMe	(55)

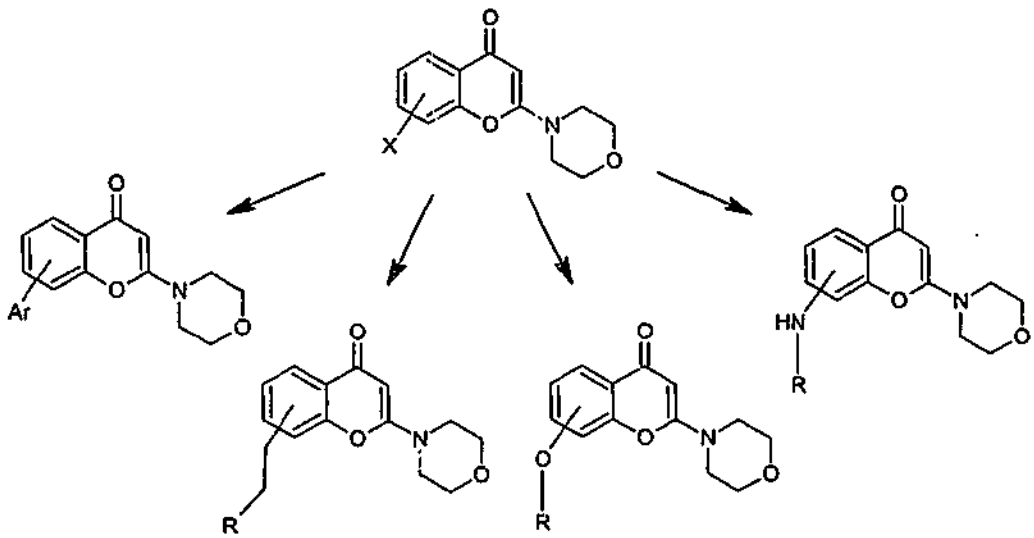
The inclusion of particular substituents may impose constraints on the interaction with PI3-kinases or phosphodiesterases that favour one isozyme or enzyme over others. These constraints might be based on (a) steric factors, such as the bulky phenoxyphenyl substituent; (b) conformational influences, such as the ortho-substituents or (c) rely on additional points of interaction via functional substituents. The distinction of PI3-kinase inhibition from the phosphodiesterase activity reported for these compounds will also be important as these two cellular signalling enzymes have a number of overlapping pathways. The methodology presented here coupled to recent advances in screening techniques has provided a means to perform these investigations.

Chapter 3
SYNTHETIC STUDIES OF "EXTENDED CHAIN" AND OTHER 2-MORPHOLINOCHROMONE ANALOGUES

3.1 Introduction

In the previous chapter, the synthesis of LY294002 and LY294002 analogues was described using key intermediates which had trifluoromethanesulfonate, boronate or bromo functionality at the C-8 position. This functionality allowed the formation of biaryl compounds through Suzuki couplings to provide 2-morpholinochromones with 8-aryl substitutions.

The success of the synthetic methodology used to date could also be applied to produce positional isomers of the compounds, with the key reactive group placed at different positions on the 2-morpholinochromone backbone. Such key intermediate isomers would allow aryl substitution to take place at different positions of the 2-morpholinochromone than at the C-8 position. In addition, all of the key intermediates would be available for synthesis of analogues in which the biaryl moiety was replaced by alternate linker groups such as aromatic alkanes, ethers and amines, enabling the study of the structure-activity relationships of this class to be broadened (Scheme 3.1). In recent years, significant advances have been made in the synthesis of such compounds, particularly using palladium catalysed reactions, and it was hoped that these strategies could be applied to this class of compounds as a means to efficiently generate an array of compounds.



Scheme 3.1

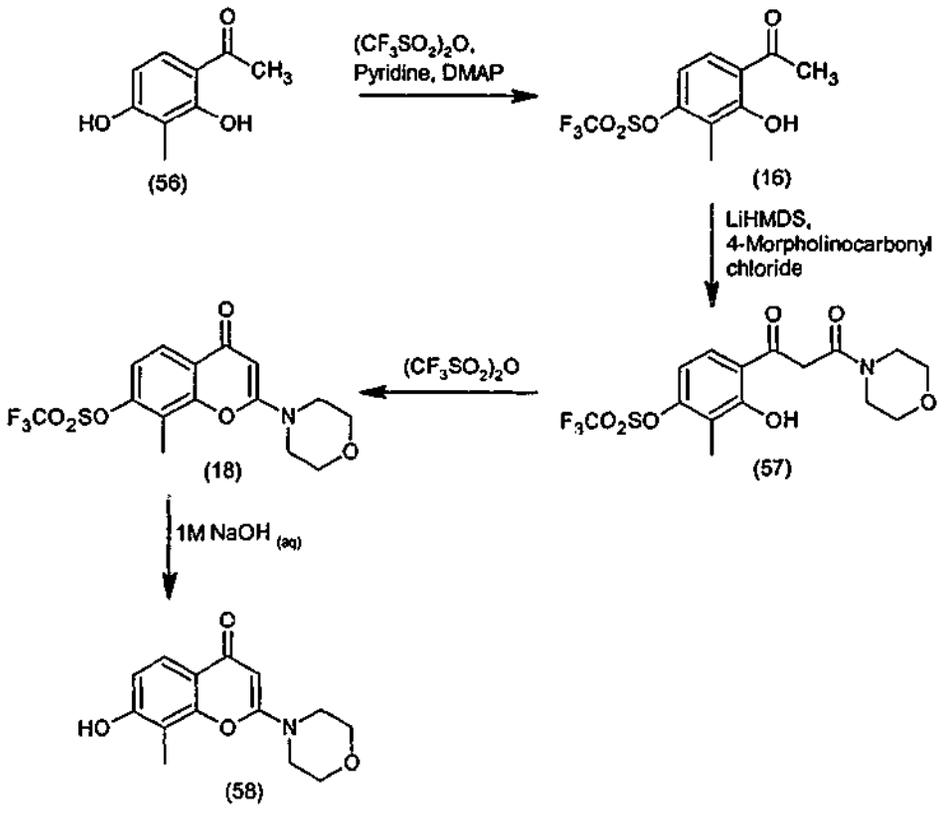
3.2 Synthesis of Key Intermediates

In preparing the trifluoromethanesulfonate-substituted intermediates, the general philosophy adopted was the same as that described in the previous chapter. Depending on the most readily available precursor, either the appropriate salicylate ester or the 2-hydroxyacetophenone was chosen for condensation reactions to synthesise the corresponding 2-morpholinochromones. In addition, the trifluoromethanesulfonates were identified as being convenient precursors to the corresponding phenols, which were thought to also be potentially useful intermediates.

3.2.1 8-Methyl-7-Trifluromethanesulfonate and 8-Methyl-7-Hydroxy Intermediates

Synthesis of the 8-methyl-7-trifluoromethanesulfonate required the strategy used to synthesise the 8-bromo-2-morpholinochromones (Scheme 3.2), based on the C-alkylation of flavones by Cushman and Nagarathnam.[62] Protection of commercially obtained 2',4'-dihydroxy-3'-methylacetophenone (56) was completed in 77% yield using trifluoromethanesulfonic anhydride in the presence of pyridine and 4-

dimethylaminopyridine as the catalyst. The C-alkylation of the trifluoromethanesulfonate protected acetophenone (16) using LiHMDS and 4-morpholinocarbonyl chloride, followed by trifluoromethanesulfonic anhydride cyclodehydration gave the key trifluoromethanesulfonate intermediate (18) with reaction yields of 89% and 37% respectively. Formation of the 2-morpholinochromone was again easily characterised by proton NMR in the downfield chemical shift of the uncyclised intermediate CH_2 at ca. 4.1 ppm to the 3-CH of the cyclised product at ca. 5.5 ppm and the associated change in integration from 2 to 1 equivalent nuclei.



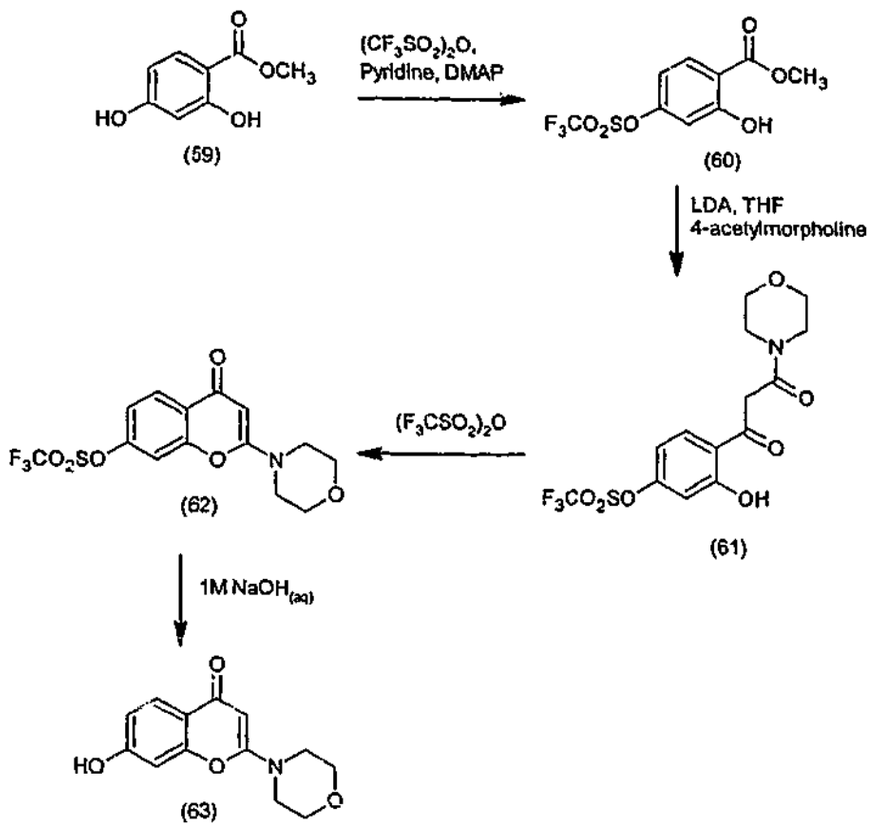
Scheme 3.2

The phenol (58) was initially obtained by deprotection of the trifluoromethanesulfonate using anhydrous conditions with sodium *tert*-butoxide in tetrahydrofuran. Under these conditions, the reaction could not be forced to completion and required purification by chromatography to give the phenol (58) in

35% yield. More successful was the treatment of the trifluoromethanesulfonate with 1 M sodium hydroxide until the deprotection appeared complete, as determined by analytical high performance liquid chromatography (Scheme 3.2). The pH was then lowered using 1 M hydrochloric acid until the product precipitated. Yields of greater than 95% of the phenol (58) were obtained in this way. Removal of the trifluoromethanesulfonate group resulted in the appearance of a broad singlet at approximately 10.4 ppm in the proton NMR spectra, corresponding to the deprotected phenolic proton.

3.2.2 7-Trifluromethanesulfonate and 7-Hydroxy Intermediates

For the 7-trifluoromethanesulfonate intermediate, the starting material of 2,4-dihydroxybenzoate (59) was commercially available so a route similar to the one used to synthesise the key 8-trifluoromethanesulfonate intermediate (24) was followed (Scheme 3.3). Initial protection of the 4-phenol was again completed by reaction with trifluoromethanesulfonic anhydride with pyridine and 4-dimethylaminopyridine to give the desired product (60) in 57% yield. The dark orange oil was of sufficient purity to be reacted in the next step with the enolate of 4-acetylmorpholine, which had been formed by the addition of LDA in tetrahydrofuran at 0°C. Chromatography gave the salicylacetamide (61) in 88% yield which was cyclodehydrated to the trifluoromethanesulfonate 2-morpholinochromone (62) in 84% yield. Successful triflate deprotection was completed by 1 M sodium hydroxide and the product (63) isolated in 49% yield by lowering the pH and extracting into ethyl acetate.



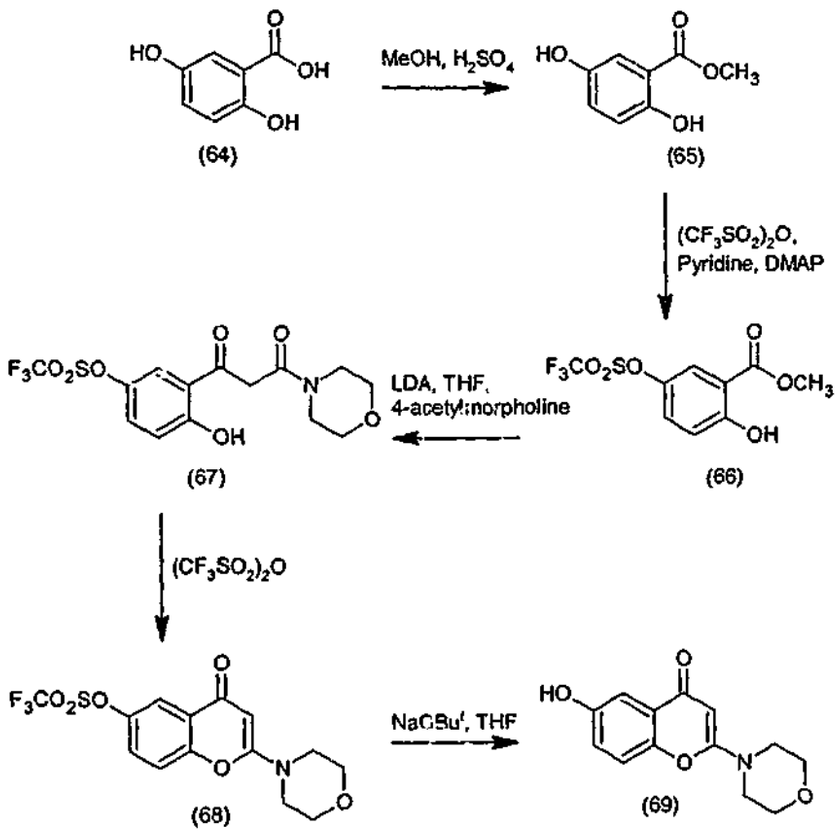
Scheme 3.3

As compared to the 8-methyl substituted compounds, these key intermediates demonstrated the shift of the NMR signal from ca. 4.1 to 6.3 ppm upon cyclisation of the trifluoromethanesulfonate. The phenol proton gave a characteristic signal at ca. 10.5 ppm in the proton spectrum.

3.2.3 6-Trifluromethanesulfonate and 6-Hydroxy Intermediates

The 6-substituted analogues were prepared from the commercially available 2,5-dihydroxybenzoic acid (64) through the methods used in the previous section for the 7-substituted intermediates.

Esterification of the acid (64) in 87% yield was followed by trifluromethanesulfonate protection of the 5-phenol by trifluoromethanesulfonic anhydride with pyridine and 4-dimethylaminopyridine in 93% yield. Both products (65 and 66) were carried through without chromatographic purification. Reaction with the enolate of 4-acetylmorpholine resulted in the salicylacetamide (67) which was chromatographed to obtain an oil (3.1 g). The resulting oil crystallised overnight and was then washed with diethyl ether to remove contaminating 4-acetylmorpholine to give 16% yield of product (67) from the first crop. Cyclodehydration of the first crop with trifluoromethanesulfonic anhydride followed by chromatography gave the trifluromethanesulfonate precursor (68) in 36% yield (Scheme 3.4).



Scheme 3.4

The 6-hydroxy precursor (69) was made under anhydrous conditions, suspending the trifluoromethanesulfonate (68) in tetrahydrofuran with addition of sodium *tert*-

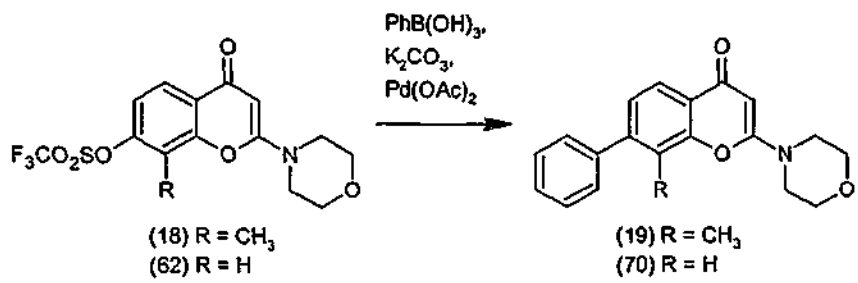
butoxide. In this case, the methodology was effective with the desired product (69) obtained in 75% yield (Scheme 3.4).

3.3 Derivatisation

3.3.1 Suzuki Coupling

Suzuki coupling has been described in the previous chapter using a trifluoromethanesulfonate or bromo key intermediate and arylboronic acids to produce LY294002 and analogues. This reaction was again applied using the two 7-trifluoromethanesulfonates (18) and (62), coupling with phenylboronic acid to give positional analogues of LY294002.

Treating the 8-methyl-7-trifluoromethanesulfonate (18) in acetonitrile with phenylboronic acid, potassium carbonate and palladium diacetate at reflux overnight gave the product (19) in 47% yield after chromatography (Scheme 3.5).



Scheme 3.5

The 7-trifluoromethanesulfonate (62) was treated similarly, except tetrahydrofuran was used as the solvent. In this case, the reaction could not be forced to completion. It was very difficult to obtain product which was uncontaminated by the starting material, which resulted in a low yield (6%) of the pure compound (70) after two chromatographic steps (Scheme 3.5).

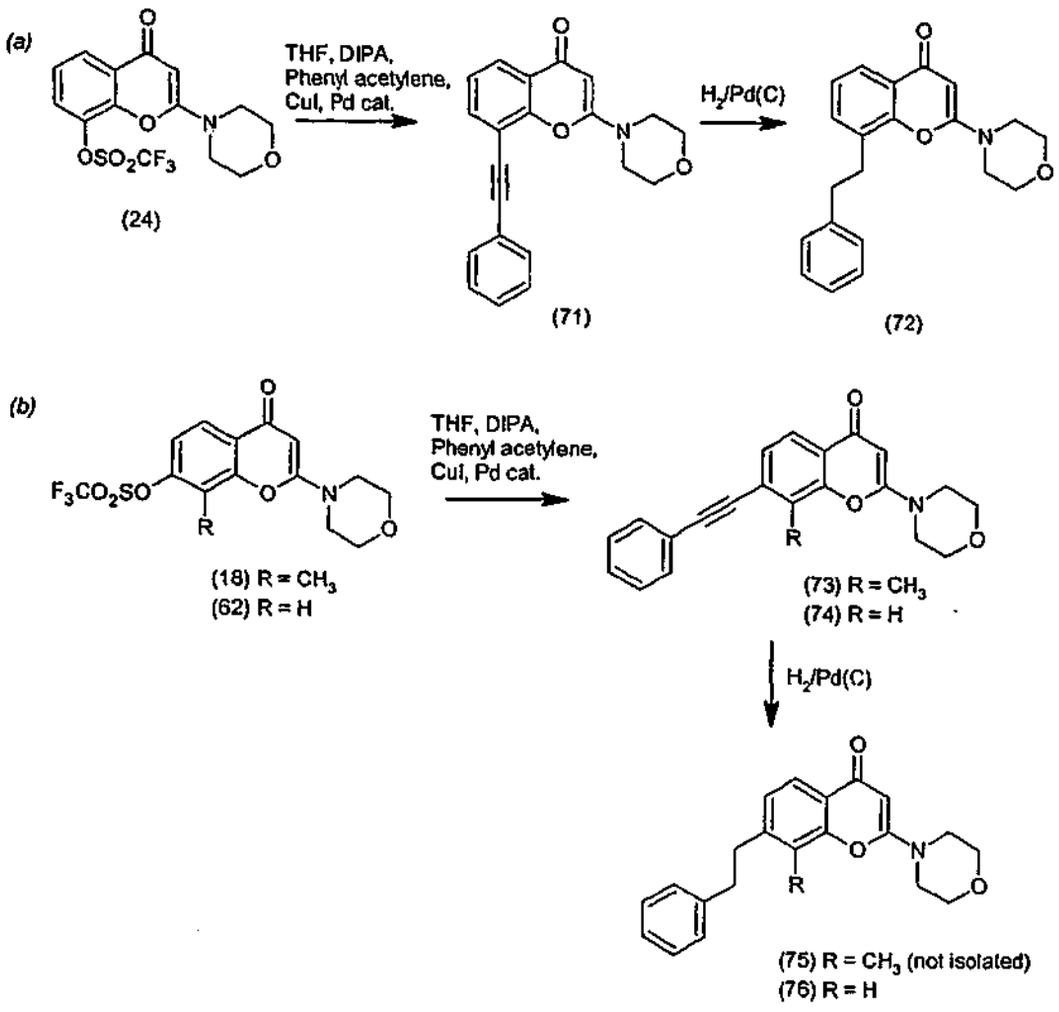
3.3.2 Sonogashira Reaction

Vinyl or aryl triflates can form new carbon-carbon bonds when cross-coupled with unsaturated systems, such as alkenes (Heck reaction), alkynes (Sonogashira reaction), allenes and ketene silyl acetals. These require the addition of a suitable palladium catalyst. In the presence of a palladium and copper catalyst, aryl triflates combine with monosubstituted alkynes to yield the conjugated alkyne.[52]

In the 1990 patent, Gammill *et al*[28] described the synthesis of 2-morpholinochromones using this process, followed by subsequent reduction of the alkyne by palladium-catalysed hydrogenation to yield the corresponding saturated compounds.

Adapting this methodology, the trifluoromethanesulfonates (18, 24 and 62) were dissolved in 1:1 tetrahydrofuran and diisopropylamine (Scheme 3.6*a* and 3.6*b*). Nitrogen was bubbled through the solution and phenyl acetylene, copper iodide and palladium triphenylphosphine dichloromethane were added. Refluxing the reaction overnight, followed by chromatographic purification gave the corresponding alkynyl 2-morpholinochromones (Scheme 3.6). Yields for two of the compounds (71 and 74) were satisfactory (30% and 71% respectively) but losses in purification by RP-HPLC resulted in a low yield (12%) for the third product (73).

While the three alkynyl compounds were of interest in themselves, hydrogenation using palladium on carbon additionally yielded two of the corresponding alkyl analogues (72 and 76) (Scheme 3.6*a* and 3.6*b*). Low yields were attributed to the small amounts of starting alkynyl material available and the difficulties in purification by normal phase chromatography or RP-HPLC, this was particularly the case for the 8-methyl-7-alkyl derivative (75) which was not isolated from the reaction mixture at all.



Scheme 3.6

3.3.3 O-Alkylation

Reaction of hydroxyl intermediates with primary alkyl halides, in the presence of a base such as sodium hydroxide or potassium carbonate, results in the formation of ethers. This is completed by S_N2 displacement of a halide ion by the phenolic 2-morpholinochromone nucleophile.

With the key 8-methyl-7-hydroxy intermediate (58) in hand, elaboration of the hydroxy chromone was performed as described by Gammill *et al.*[42] The hydroxy intermediate was treated with 1,2-dibromoethane in 50% aqueous sodium hydroxide

using the phase transfer reagent tetrabutylammonium hydrogen sulfate. The resulting solid was obtained in 51% yield after washing and was then reacted with N-methylpiperazine in chloroform. Purification by reverse-phase high performance liquid chromatography resulted in the known product (5) in 43% yield (Figure 3.1). This yield was less than the literature report (83%) possibly due to the small scale used (70 mg of starting material compared with 4 g in the literature).[42] Characterisation by proton NMR, mass spectrometry and melting point was in accordance with the literature values.[31]

For the synthesis of U-86983 (3), to the 8-hydroxy morpholinochromone (28) in acetonitrile was added potassium carbonate and 3-(bromomethyl)pyridine hydrobromide according to the method of Morris *et al.*[49] Reverse-phase high performance liquid chromatography gave the desired compound in 8% yield (Figure 3.1). The synthesis was improved when 3-(chloromethyl)pyridine hydrobromide was used, resulting in a yield of 19% following purification by RP-HPLC.

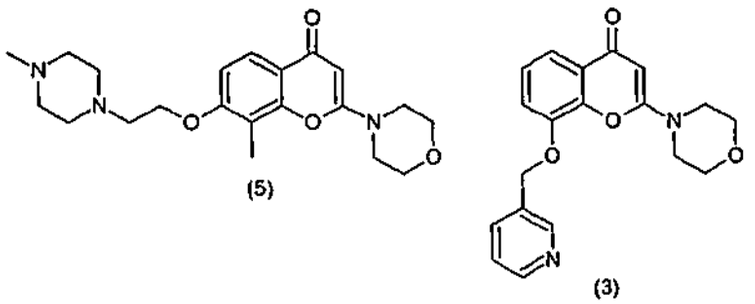


Figure 3.1 2-Morpholinochromones (5) and U-86983 (3).

The derivatisation conditions used to produce the literature compounds (5) and U-86983 (3) from their respective hydroxyl intermediates were then applied to the other key intermediates to form analogous alkylated compounds (77-79) with yields of between 20-49% for these syntheses (Figure 3.2).

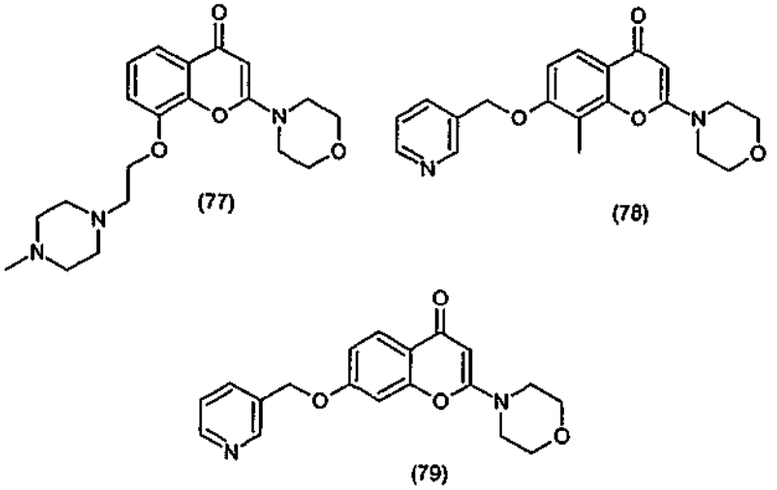


Figure 3.2 2-Morpholinochromones (77), (78) and (79).

Benzylation of the hydroxyl compounds under the same conditions resulted in a benzyloxy analogue series (4, 80 and 81) with yields from 6-91% (Figure 3.3). While an effective means of purifying products, RP-HPLC generally also resulted in significant losses in yield as seen for compounds (80) and (81). This may be due to irreversible adsorption onto the C_{18} silica of the guard column.

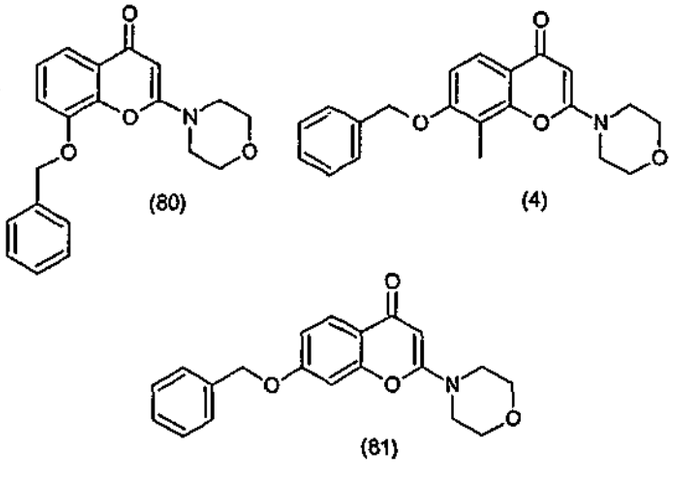
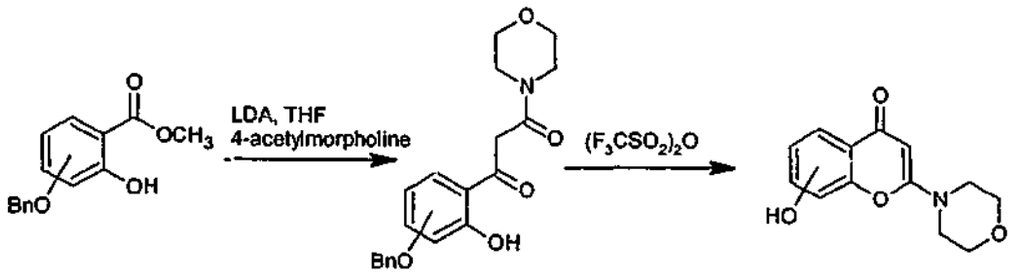


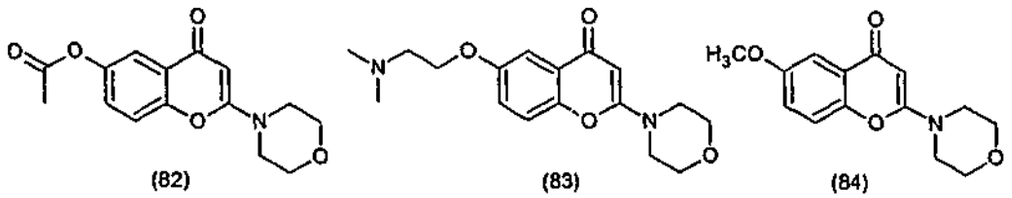
Figure 3.3 2-Morpholinochromones (80), (4) and (81).

Synthesis of benzylated compounds was also attempted through the direct benzyloxy protection of the hydroxyl starting material, instead of triflate protection. This strategy was successful until the final cyclisation step using trifluoromethanesulfonic anhydride, where the compound was debenzylated (Scheme 3.7). Ironically, this is potentially an efficient means of producing the key hydroxyl intermediates.



Scheme 3.7

Reactions of the 6-hydroxy intermediate (69) were also studied, in this case with a view to using these substitutions to modify some physical properties of these compounds, such as increasing their aqueous solubility. The 6-hydroxyl intermediate (69) was acetylated in dichloromethane using acetic anhydride with triethylamine (Figure 3.4), obtaining a 15% yield of the pure product (82) following extraction. The 6-hydroxyl intermediate (69) was also dissolved in *N,N*-dimethylformamide containing potassium carbonate, followed by the addition of 2-*N,N*-dimethylaminoethylchloride. The desired alkylated product (83) was purified by chromatography in 38% yield (Figure 3.4). Unexpectedly, a side product was recovered in significant amount, which was identified by proton NMR and MS as 6-methoxy-2-morpholinochromone (84) (Figure 3.4). No ready explanation for this result is available and it was not further investigated– it may be that the alkyl chloride was contaminated with a methylating reagent, perhaps in the preparation of the 2-dimethylaminoalkyl halide.



(82) (83) (84)

Figure 3.4 2-Morpholinochromones (82), (83) and (84).

The O-alkylations completed on the key intermediates are summarised in Table 3.1.

Table 3.1 2-Morpholinochromone analogues synthesised by O-alkylation.

#	Reaction conditions	Yield (%)
(3)	3-(Bromomethyl)pyridine hydrobromide, K_2CO_3, CH_3CN	8
(5)	1. 1,2-Dibromoethane, tetrabutylammonium hydrogen sulfate, 50 % $NaOH_{(aq)}$ 2. *N*-Methylpiperazine, $CHCl_3$	51 43
(77)	1. 1,2-Dibromoethane, tetrabutylammonium hydrogen sulfate, 50 % $NaOH_{(aq)}$ 2. *N*-Methylpiperazine, $CHCl_3$	48 49
(78)	3-(Bromomethyl)pyridine hydrobromide, K_2CO_3, CH_3CN	22
(79)	3-(Bromomethyl)pyridine hydrobromide, K_2CO_3, CH_3CN	20
(80)	Benzyl bromide, K_2CO_3, CH_3CN	13
(4)	Benzyl bromide, K_2CO_3, CH_3CN	91
(81)	Benzyl bromide, K_2CO_3, CH_3CN	6
(82)	Acetic anhydride, triethylamine, CH_2Cl_2	15
(83)	2-*N*, *N*-dimethylaminoethylchloride hydrochloride, K_2CO_3, DMF	38
(84)	2- *N*, *N*-dimethylaminoethylchloride hydrochloride, K_2CO_3, DMF	12

3.3.4 Copper-catalysed O-Arylation

The Ullman ether synthesis has been extensively used for the formation of diaryl ethers. However, this reaction is limited by the stoichiometric or greater quantities of the copper complex required, harsh reactions conditions (125-220°C in neat phenol or solvents such as pyridine or *N,N*-dimethylformamide (DMF)), and low yields for unactivated aryl halides. In 1997, a general copper-catalysed synthesis of diaryl ethers was reported from aryl halides (preferentially iodides over bromides) with phenols which overcomes many of the problems of the Ullman synthesis.[63,64] The use of cesium carbonate was found to be critical for improved reaction and allowed a

more non-polar solvent to be used at lower temperatures. A number of copper catalysts were useful and it was reported that the addition of 1-naphthoic acid was important for less soluble phenoxides.[63] The method was found to be particularly suitable for unactivated aryl halides and *ortho*-substituted phenols.

Biaryl ethers have also been reported to be readily synthesised from phenols through the use of phenylboronic acids and cupric acetate by Chan and Evans.[65,66] Mild conditions of room temperature with an amine base, together with apparent broad applicability to a large variety of substrates and being able to tolerate many sensitive function groups were reported to be the best features of this methodology.

Through this strategy, biaryl ether analogues (85-87) of 2-morpholinochromones were synthesised (Figure 3.5). Reaction of the 8-hydroxy intermediate (28) with phenylboronic acid, in dichloromethane and with triethylamine as the base, over 48 h was followed by work-up. The small amount of crude material available suggested that purification by RP-HPLC would be appropriate. A relatively low yield of 8% of the product (85) was the result, although in sufficient amount for characterisation and testing. Repeating this procedure with the 7-hydroxy (63) also gave a poor yield of the product (86) of 3%.

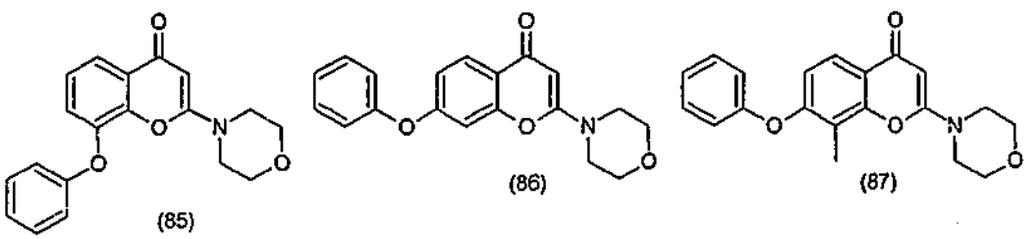
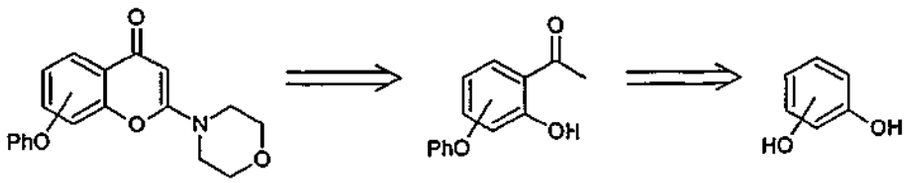


Figure 3.5 2-Morpholinochromones (85), (86) and (87).

As a result, for the arylation of the 7-hydroxy-8-methyl intermediate (58) a number of changes were made to the reaction and purification conditions. This included the addition of molecular sieves, changing solvents from dichloromethane to acetonitrile, heating and changing from RP-HPLC to normal phase column chromatography.

Unfortunately, these changes did not appear to make a significant difference in the yield, with the desired product (87) being obtained in 8% yield (Figure 3.5).

The yields of these products were quite poor and, in some cases, insufficient for full characterisation but did provide sufficient materials for preliminary assay screening. In the wake of these low yields, products identified for further evaluation could be synthesised through an alternative method. This possibly may include the use of a biaryl ether as the starting material for the 2-morpholinochromone product. The following retrosynthetic analysis is suggested (Scheme 3.8). This could be achieved by Ullman ether synthesis of a suitable phenol followed by acylation and Fries rearrangement, methodology which was used by Hölzl *et al* in the preparation of hydroxydiphenyl ether compounds.[67] The resulting acetophenone could then be used to synthesise the corresponding 2-morpholinochromone using the established procedures described previously.



Scheme 3.8

3.3.5 Amination

Like diaryl ethers, diarylamines are commonplace and often form the active moiety in molecules with medicinally important properties. The synthesis of these types of compounds is often difficult, where reactions involving nitration, reduction or substitution are often incompatible with other functional groups. These compounds also often require the use of protection and deprotection steps.

Amine substituted compounds were notably absent from the Upjohn studies. It was desired that amine analogues would be part of the 2-morpholinochromone library for this structure-activity work.

The palladium-catalysed conversion of aryl bromides and iodides to aniline derivatives under mild conditions has been established in recent years. However, in 1997, Buchwald *et al* and Hartwig *et al* extended this arylamination process to trifluoromethanesulfonates.[68,69] Such reactions were completed using $Pd(OAc)_2$, 2,2'-bis(diphenylphosphino)-1,1'-binaphthyl (BINAP) or 2,2'-bis[bis(4-methylphenyl)phosphino]-1,1'-binaphthyl (Tol-BINAP), $NaOBu^t$ and toluene at 80°C by Buchwald *et al*[68] while Hartwig *et al* used palladium (II) dibenzylidieneacetone or $Pd(OAc)_2$, BINAP, $NaOBu^t$ and toluene at 100°C.[69] This trifluoromethanesulfonate-based chemistry was established due to the availability of precursors, the simple conversion of phenols to trifluoromethanesulfonates, the directed aromatic chemistry of protected phenols and the demonstrated value of trifluoromethanesulfonates in Stille and Suzuki couplings.

The 8-trifluoromethanesulfonate (24) was dissolved in tetrahydrofuran and added to a suspension of sodium tertiary-butoxide and dichloro[1,1'-bis(diphenylphosphino)ferrocene]palladium (II) in tetrahydrofuran. No identifiable material could be isolated after heating at 100°C overnight with benzylamine. The same reaction was also attempted with aniline, also without success. Benzylamination of the 7-trifluoromethanesulfonate (62) under the same conditions was also unsuccessful. The only new formed product identified in these studies was the phenol (63), presumably the result of competing hydrolysis by sodium *tertiary*-butoxide. One attempt to overcome this was made by exchanging the base used to cesium carbonate, however no reaction was achieved in this case.

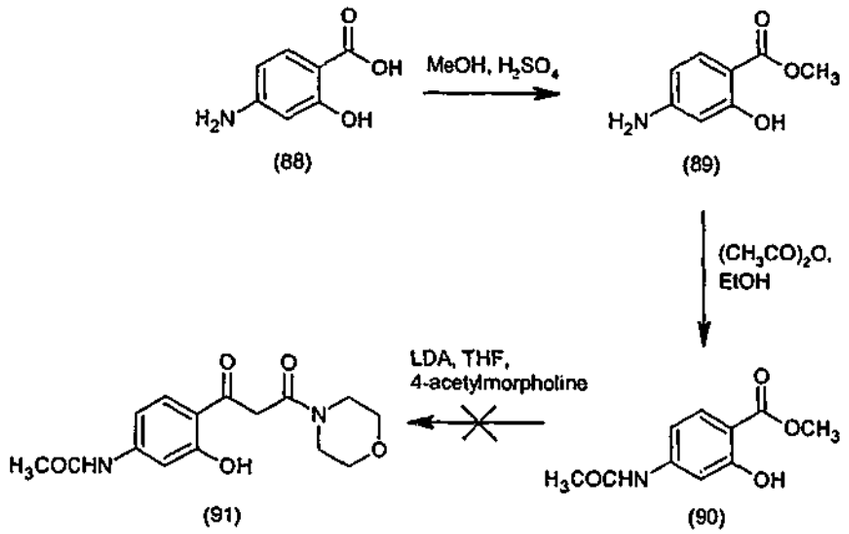
A further alternative was to subject the 8-bromo-6-methyl intermediate (48b) described in the previous chapter with benzylamine using sodium *tertiary*-butoxide and dichloro[1,1'-bis(diphenylphosphino)ferrocene]palladium (II) in tetrahydrofuran. Again, no product was obtained with this aryl halide substrate.

The lack of success of these methods was disappointing, however, the compounds were considered so desirable as to warrant attempted synthesis by other methods.

Morris *et al* had previously described the synthesis of the 7-acetamido morpholinochromone using salicylacetamides methodology in good yield.[50] The difference for the synthesis of this 2-morpholinochromone was the method of cyclodehydration, which was best completed using polyphosphoric ester (PPE) rather than trifluoromethanesulfonic anhydride. For Morris *et al*, PPE gave a 44% yield over a 10% yield of the deacylated product when trifluoromethanesulfonic anhydride was used.

The 7-acetamido morpholinochromone that was synthesised by Morris *et al*[50] was targeted as a useful key intermediate as it could be deprotected and reacted to make amino analogues. The commercially available 4-aminosalicylic acid (88) underwent Fisher esterification to give the ester (89) using methanol and concentrated sulphuric acid (Scheme 3.9). The amino group was protected by reaction with acetic anhydride in ethanol to give the corresponding N-acetyl derivative (90).

Surprisingly, the synthesis of the related salicylacetamide (91) was not successful (Scheme 3.9), despite repeated attempts and Morris *et al* reporting a 80% yield.[50] Reaction with the lithium enolate of 4-acetylmorpholine resulted in only starting material being identified from chromatography. Visually, it appeared that the reaction was perhaps quenching on addition of the salicylic ester (90). A number of different conditions were attempted, including heating, using different rates of ester addition and amounts of tetrahydrofuran to alter the concentration of reactants, but without success.

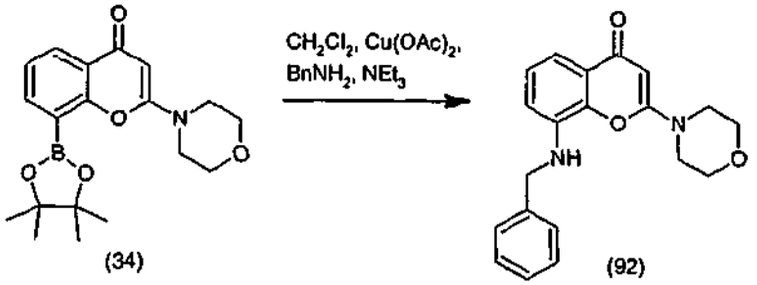


Scheme 3.9

Numerous strategies had been attempted for the synthesis of amine morpholinochromone analogues, with disappointing results. However, success was obtained through the combination of two methodologies which had earlier proved successful for this 2-morpholinochromone series. Chan *et al* had reported that phenylboronic acids were not only efficient O-arylating agents but were also N-arylating agents in the presence of copper diacetate.[65] While the method of Chan *et al*[65] had given quite low yields for the O-arylations completed in this series (3-8%), the desired compounds had been isolated in sufficient amount for structure-activity studies. As the 8-boronate intermediate (34) had been previously synthesised, as described in the previous chapter, it was decided to attempt N-arylation of this compound.

The 8-boronate (34) was dissolved in dichloromethane containing crushed molecular sieves, followed by the addition of copper diacetate, benzylamine and triethylamine. Reverse phase high performance liquid chromatography resulted in the purified product (92) in 21% yield (Scheme 3.10). A significant amount of the 8-hydroxy (28) compound was also recovered, it was thought that the acidic RP-HPLC conditions were degrading the unreacted 8-boronate intermediate. The successful synthesis of

this compound was significant as it was the first example of a 2-morpholinochromone of this type. Further work by Kinacia involves the preparation of the other amino analogues by this methodology.[70]



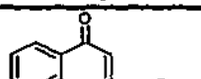
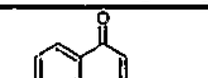
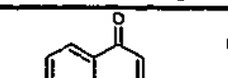
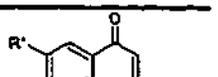
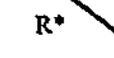
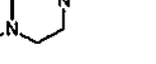
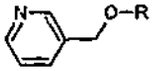
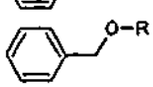
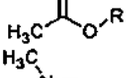
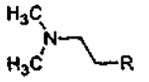
Scheme 3.10

3.4 Summary and Conclusions

Synthesis of key intermediates, including four trifluoromethanesulfonates and their corresponding hydroxyl intermediates with their reactive group in different positions on the chromone, as well as 8-boronate and 8-bromo intermediates, has allowed a number of different known and novel analogues of the three compounds of interest (LY294002 (6), (5) and U-86983 (3)) to be synthesised for structure-activity studies. The twenty-two compounds which have been synthesised as described in this chapter are summarised in Table 3.2 and are representative of the possible analogues which should be accessible using the strategies described.

The syntheses in some cases were very low yielding and a sample of analytical purity could not always be obtained. However, sufficient material was available for characterisation by proton NMR and MS as well as for preliminary biological evaluation. In the cases where such compounds have notable activity an optimal synthesis may be pursued, perhaps by synthetic routes tailored to the specific compounds. Even so, some of the synthetic strategies have been shown to be very useful, in particular the development of the boronate-substituted chromone has allowed some of the difficulties in synthesising amino-substituted derivatives to be overcome.

Table 3.2 Summary of 2-morpholinochromone analogues synthesised in Chapter 3.

R* \ R				
	Compound #			
	(6)[#]	(18)	(70)	
	(71)	(73)	(74)	
	(72)		(76)	
	(77)	(5)		
	(3)	(78)	(79)	
	(80)	(4)	(81)	
				(82)
				(83)
H_3CO-R	(27)[#]			(84)
	(85)	(87)	(86)	
	(92)			

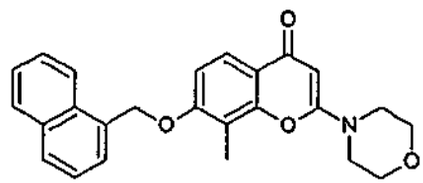
The synthesis of these compounds was described in Chapter 2.

Chapter 4

DEVELOPMENT OF PHOSPHODIESTERASE ISOFORM SEPARATION AND ACTIVITY ASSAYS

4.1 Introduction

Inhibition of low K_m phosphodiesterase activity by 2-morpholinochromone compounds was first reported by workers at The Upjohn Company in 1993. In that report, Benjamin *et al* examined a series of 2-aminochromones and found that one of them, U-84569 (2) (Figure 4.1) was a potent inhibitor of the low K_m cAMP-dependant phosphodiesterase (PDE3) with an IC_{50} of 300 nM.[30]



(2)

Figure 4.1 U-84569.

However, the phosphodiesterase assay methodology used by Benjamin *et al* was limited in a number of ways. Assay conditions utilised a very high substrate concentration of 100 μM [^{3}H]-cAMP and the enzyme source was unpurified platelet lysate. The method of detection was indirect, where the products obtained from the isotopically-labelled cyclic nucleotide substrate were subjected to 5'-nucleotidase treatment followed by chromatographic separation to be able to measure the resulting [^{3}H]-nucleoside. Finally, while the control, 1-methyl-3-isobutylxanthine (IBMX), showed dose-dependant inhibition of cAMP hydrolysis, the dose-response curves of the three 2-morpholinochromones U-67154 (1), U-84569 (2) and U-86983 (3) evaluated did not demonstrate dose-dependent inhibition at low concentrations (Figure 4.2). No indication of replicates of these experiments was given. In part, these limitations reflect the technical shortcomings of the era.

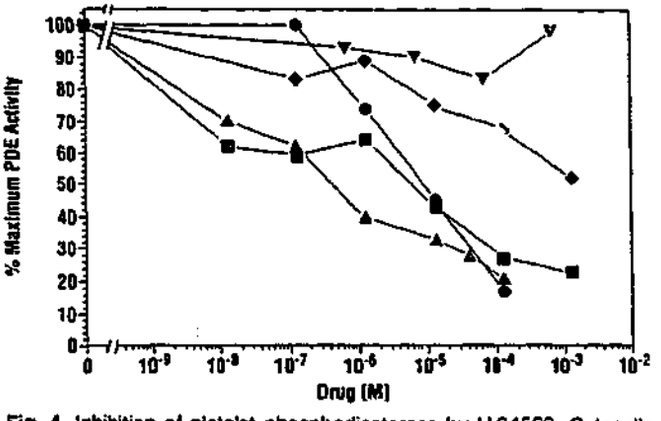


Fig. 4. Inhibition of platelet phosphodiesterase by U-84569. Cytosolic human phosphodiesterase activity (low K_m enzyme) was determined in the presence or absence of increasing concentrations of U-84569, U-86983, IBMX or U-67154. Data are presented as percentage of maximal phosphodiesterase activity. ▼, PGI_2; ◆, U-67154; ●, IBMX; ■, U-86983; ▲, U-84569.

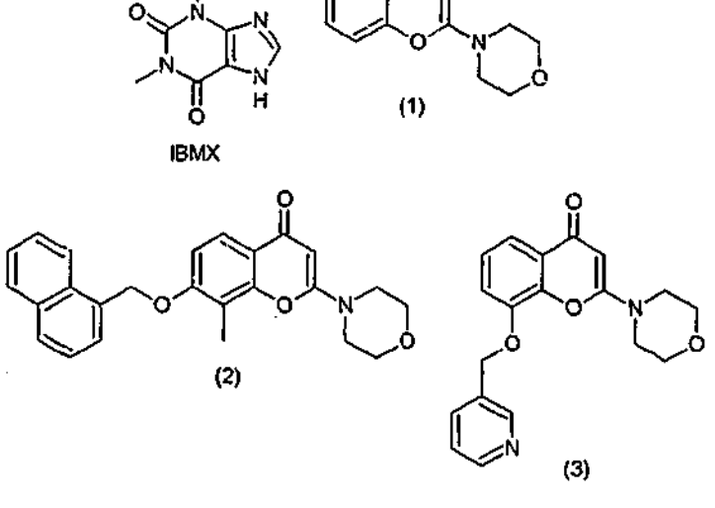


Figure 4.2 IBMX, U-67154 (1), U-84569 (2) and U-86983 (3) with "Fig. 4. Inhibition of platelet phosphodiesterase by U-84569" as reported by Benjamin *et al.*[24]

By separating the phosphodiesterase isoforms, a more accurate assessment of the inhibitory properties of compounds can be made. Anion exchange chromatography can be used to purify platelet phosphodiesterases for their characterisation and study, In 1976 Hidaka and Asano reported the resolution of three peaks of enzyme activity from human platelet protein supernatant.[18,71] These are currently identified as

PDE2, PDE3 and PDE5.[18] Typically, phosphodiesterases are obtained from platelets[72] or from bovine cardiac tissue.[73,74] Two genes that express isozymes of PDE3 have been identified.[18] Mature PDE3A, initially cloned from human cardiac cDNA, is often a soluble cytosolic enzyme (as it is in platelets) and is the major cAMP phosphodiesterase in cardiac muscle. In all cells in which it has been characterised, PDE3B has been membrane-bound. A major problem with the study and characterisation of PDE3, regardless of the source used, is that the purification is both low yielding and the product unstable.[18,75]

Optimum conditions for phosphodiesterase assays are different depending on the isoform used. PDE3 has been characterised as a low K_m PDE, with submicromolar K_m values of 0.1 μM – 0.8 μM for cAMP typically reported.[76] It also has a high affinity for cGMP but hydrolyses cAMP much more rapidly (with a four to ten fold higher V_{max}), resulting in cGMP acting as a competitive inhibitor of cAMP hydrolysis. PDE3 was initially known as "cGMP-inhibited cAMP phosphodiesterase". Milrinone and cilostamide, a cilostazol analogue, are known selective inhibitors of PDE3A (Figure 4.3).

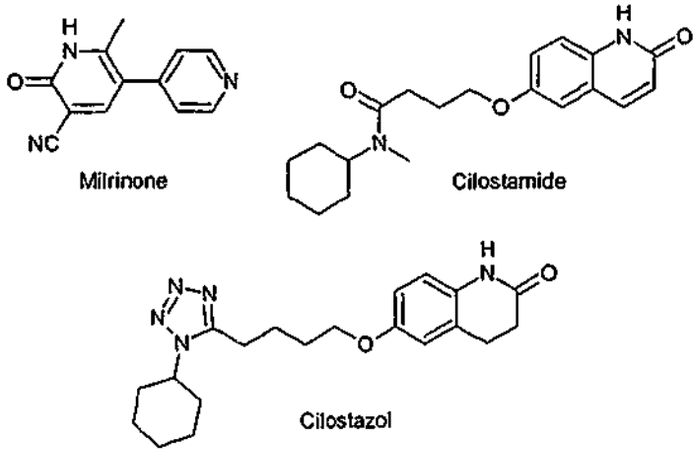
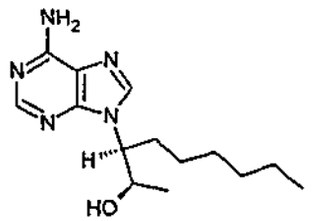


Figure 4.3 Inhibitors of PDE3.

PDE2, in contrast, has a high K_m (range of 30-50 μM cAMP).[76] It can hydrolyse cAMP and cGMP comparably, with each cyclic nucleotide stimulating the hydrolysis

of the other. It has been shown to be important in degrading cAMP where cGMP levels are high (5 μM to 10 μM), with cGMP stimulating cAMP hydrolysis allosterically, and was initially called "cGMP-stimulated phosphodiesterase".[18] To date, the adenosine deaminase inhibitor *erythro*-9-(2-hydroxy-3-nonyl)adenine or EHNA (Figure 4.4) is the only compound known to selectively inhibit PDE2 over the other phosphodiesterase isozymes.



EHNA

Figure 4.4 Inhibitor of PDE2.

Experimentally, the K_m differences between isoforms imply that, depending on substrate concentration used, assays can be tailored to express a measure of PDE2 or PDE3 activity.

Assays of phosphodiesterase activity are typically performed by radiometric methods that use tritium-labelled cAMP as the substrate. A common method, which was used by Upjohn, involved coupling the phosphodiesterase reaction to 5'nucleotidase and measure changes in [^{3}H]-adenosine.[30,77] An alternative is radioimmunoassay (RIA) where the decrease in [^{3}H]-cAMP is monitored using a cAMP-specific antibody.[73,78] Although these methods are sensitive, both involve the expense of isotopically-labelled compounds, dedicated equipment and the inconvenience of the additional care which must be taken while they are being used.

Other methods of measuring phosphodiesterase activity include enzyme immunoassay (EIA) where cAMP from the assay competes with enzyme-conjugated cAMP (instead of radiolabelled cAMP as for RIA) for the binding to the antibody. This method is also sensitive but requires much sample manipulation and is quite

expensive when using the commercially available kits.[78] Fluorescence can also be used; fluorescent anion sensors increase in fluorescence intensity with increases in adenosine monophosphate (AMP) or fluorescent cAMP derivatives decrease in intensity when hydrolysed to AMP.[79,80] Levels of phosphate can also be detected by changes in absorbance, either by again coupling the phosphodiesterase reaction to 5'nucleotidase and measuring changes in the resulting phosphate levels[81] or by using phosphate chemosensors to detect AMP.[82] While EIA, fluorescence and phosphate sensing methods all avoid the use of isotopes, they all require specialist preparation or purchase of expensive substrates or other reagents with many of them also requiring dedicated equipment.

Reverse-phase high performance liquid chromatography, apart from being used to separate and quantitate isotopically-labelled products, has also been used to assay unlabelled phosphodiesterase substrates and products. However, each RP-HPLC run is reasonably time-consuming (approximately 30-40 min for cAMP to elute) and the method is less sensitive than many of the other methods already described.[74,83] Developments of HPLC methods have been hindered by difficulties in quantitation, limited application to biological materials and poor reproducibility due to baseline fluctuations which were in a large part thought to be due to the use of organic solvent.[83] However, Ogata's use of aqueous solvent with isocratic elution gave a less sensitive but simple, economical and reproducible method.[83]

In order to evaluate the synthetic 2-morpholinochromones described in the previous chapters, a methodology for the efficient separation of the phosphodiesterase isoforms, with an interest in PDE3 isolation in particular, was desired. Suitable phosphodiesterase assays were also required and would attempt to overcome some of the difficulties described above and preferably be inexpensive, robust and applicable to high-throughput screening.

4.2 Bovine Cardiac Tissue Phosphodiesterases: Isolation and Activity Studies

Nichols and Morimoto have described purification of PDE3 from a commercial preparation of bovine heart (Sigma).[73] It was thought that this was a ready and reliable source of phosphodiesterase from which inhibitor screening could be easily initiated. In the first instance, an attempt to isolate PDE3 from the bovine cardiac tissue was made by adapting the experimental protocol used by Nichols and Morimoto.[73]

4.2.1 Chromatographic Separation of Crude Complex from Bovine Heart

Crude phosphodiesterase from bovine cardiac tissue was obtained from Sigma and eluted through a MonoQ IEX column from which a UV trace was obtained (Figure 4.5), with similar methods to those of Nichols and Morimoto.[73]

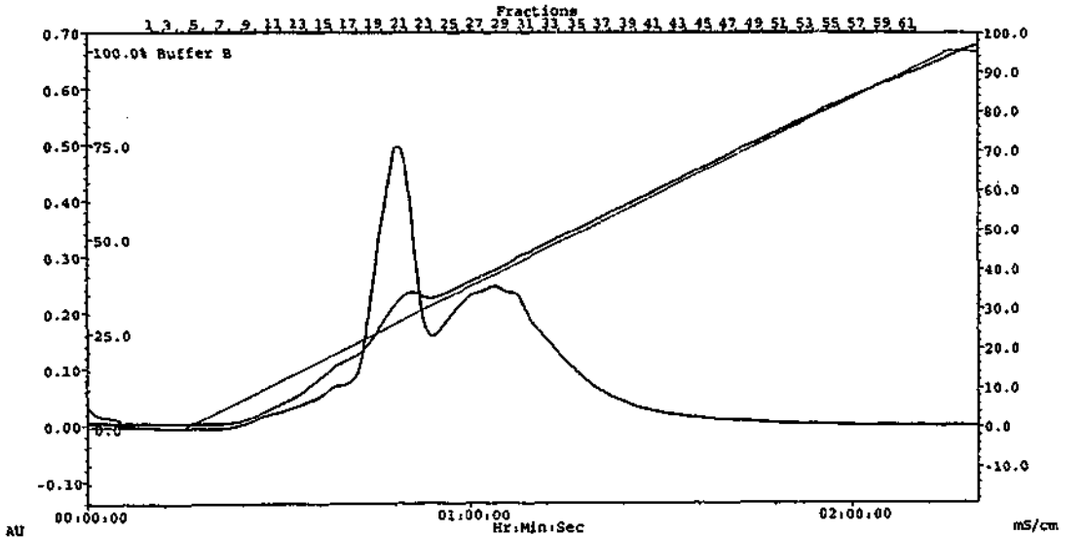


Figure 4.5 UV trace from ion-exchange chromatography of crude bovine phosphodiesterase.

4.2.2 Bovine Phosphodiesterase Assay Conditions and RP-HPLC Analysis

To identify phosphodiesterase activity in the separated fractions, the phosphodiesterase assay of Nichols and Morimoto[73] was adapted to allow evaluation by RP-HPLC. To 325 µL of HEPES (4-(2-hydroxyethyl)-1-piperazine-1-ethanesulfonic acid) buffer, was added a protein fraction (125 µL) followed by 50 µL of 300 µM cAMP. This resulted in a final concentration of 30 µM cAMP, which enabled UV detection of cAMP and the resulting AMP at 254 nm after RP-HPLC. Aliquots (100 µL) were removed from the room temperature reaction at 0, 5 and 10 minutes. Termination by the addition of perchloric acid ($HClO_4$), as reported by Nichols and Morimoto, was found to interfere with analysis by RP-HPLC. Denaturing the enzyme by immersing the microtube containing the reaction mixture in boiling water for 3 minutes, as suggested by Spoto *et al*,[74] was a great improvement in reaction termination technique.

Following termination of the enzymatic reaction, any cAMP and AMP that were present in the assay were separated using a Hewlett Packard Zorbax Eclipse XBD-C_6 5 µM (4.6 x 150 mm) column and aqueous phosphate buffer (0.2 M $(NH_4)H_2PO_4$) with isocratic elution as suggested by Ogata *et al.*[83] Phosphodiesterase activity was measured as the AMP peak area (mAU) from detection at 254 nm.

4.2.3 Bovine Phosphodiesterase Assay RP-HPLC Results

Aliquots which had been removed from the assay and terminated at the 5 minute point were analysed for AMP by RP-HPLC. From plotting the AMP peak area (mAU) of each fraction, two broad peaks of activity were identified (Figure 4.6).

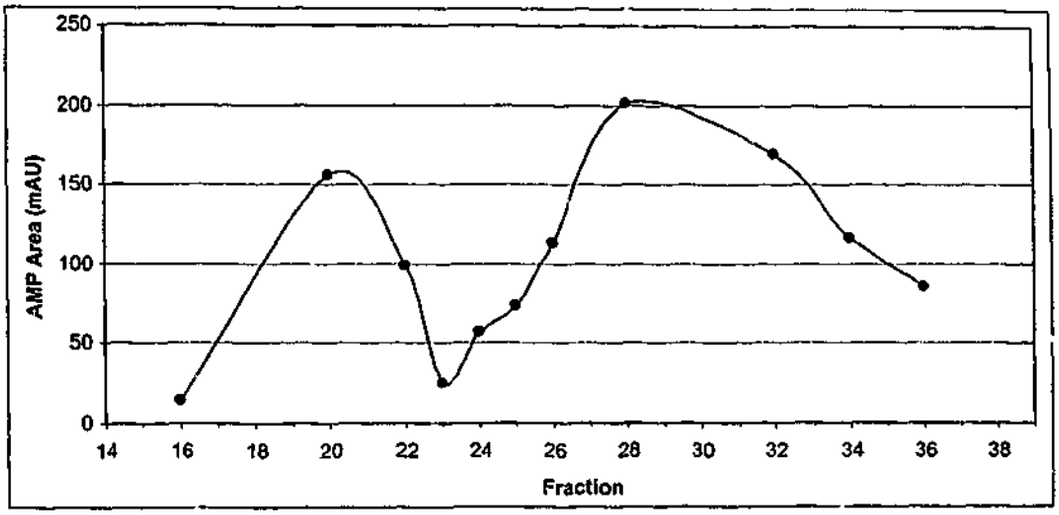


Figure 4.6 Amount of AMP produced in 5 minutes as an indication of phosphodiesterase activity in each fraction obtained from initial purification.

Fractions 27-36 from the initial purification were pooled and the re-run on the MonoQ IEX column, then re-assayed for PDE activity (Figure 4.7).

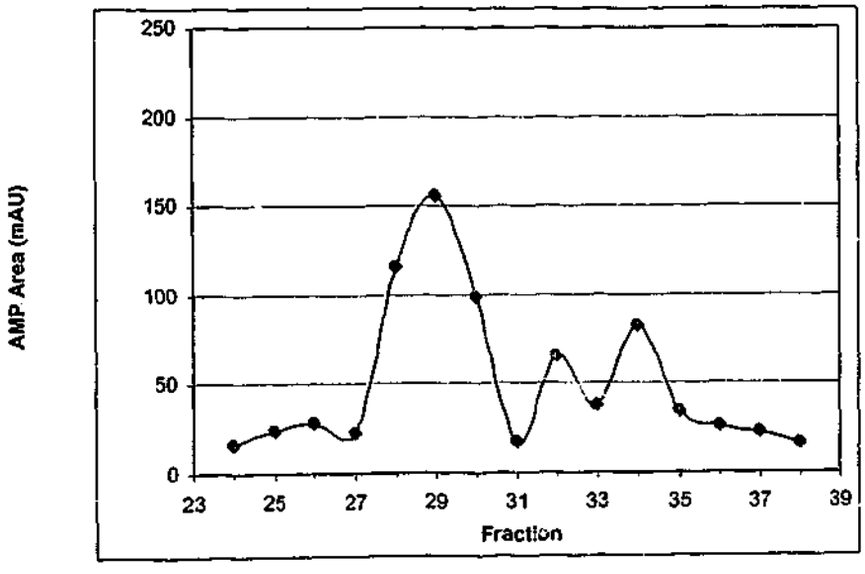


Figure 4.7 Amount of AMP produced in 5 minutes as an indication of phosphodiesterase activity in each fraction obtained from a second purification step of Fractions 27-36.

Fractions which resulted in peaks of PDE activity following the second purification step (Fractions 29, 32 and 34) were assayed in the presence of dimethyl sulfoxide (DMSO) and cilostamide (Figure 4.8). As hydrolysis was not inhibited by cilostamide in any of these three fractions, it was indicated that PDE3 was not present. This was not entirely unexpected as the cAMP concentration being used, which was required for detection following RP-HPLC, was more favourable to PDE2 rather than PDE3 activity. However, no inhibition of hydrolysis was observed when assays were conducted in the presence of the PDE2 inhibitor EHNA either (Figure 4.8).

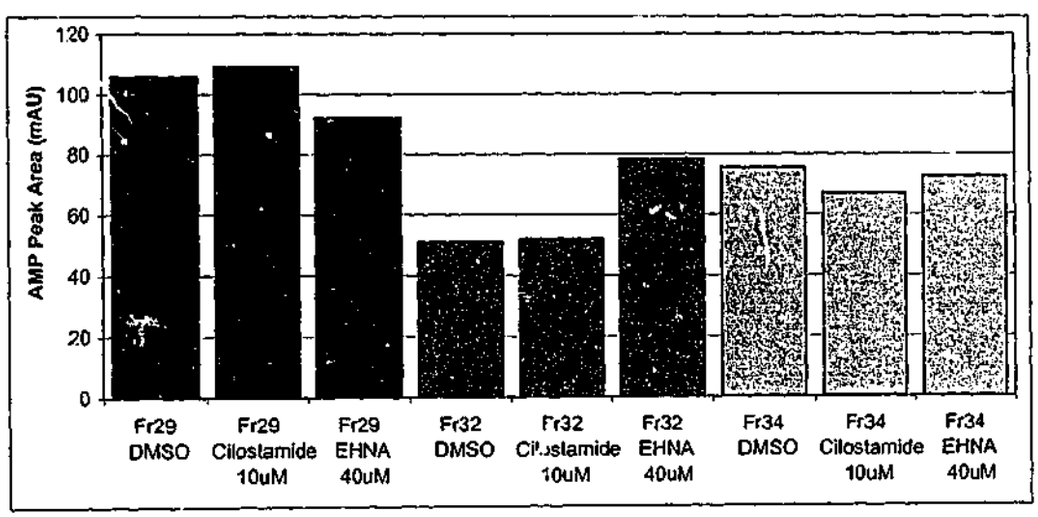


Figure 4.8 Amount of AMP produced in 5 minutes as an indication of PDE activity in Fractions 29, 32 and 34 in the presence of DMSO, cilostamide and EHNA.

Fraction 29, which was observed to have the greatest activity in the earlier screen (Figure 4.7), was further investigated (Figure 4.9). It was noted that enzyme activity was linear over 5 minutes under the conditions of these assays. Theophylline (Figure 4.10), a non-specific PDE inhibitor, was observed to inhibit Fraction 29 and therefore indicative of the presence of phosphodiesterase(s). The only specific inhibitor seen to have a obvious effect was the PDE1 inhibitor vinpocetine (Figure 4.10), which suggested that this fraction contained PDE1.

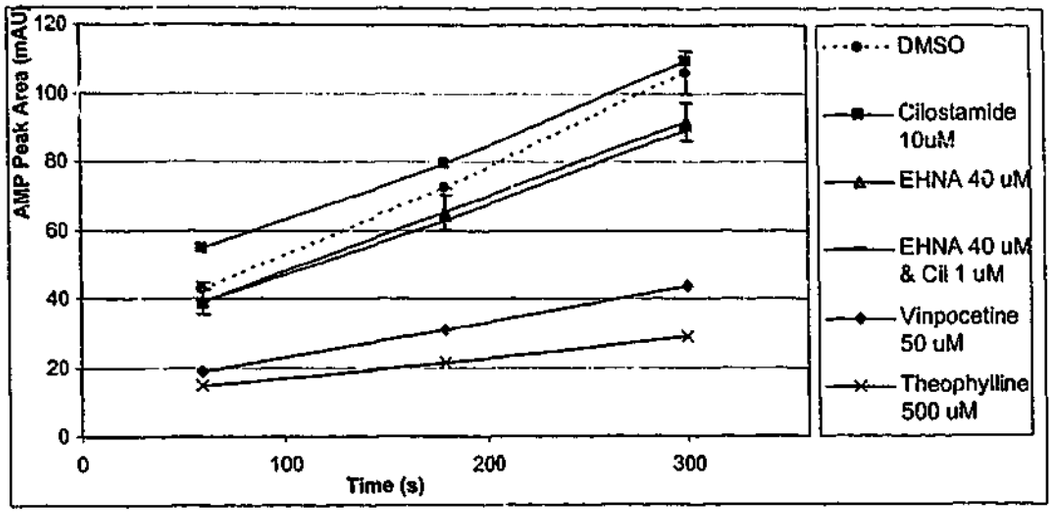


Figure 4.9 Amount of AMP produced in 1, 3 and 5 minutes as an indication of PDE activity in Fraction 29 in the presence of DMSO and PDE inhibitors.

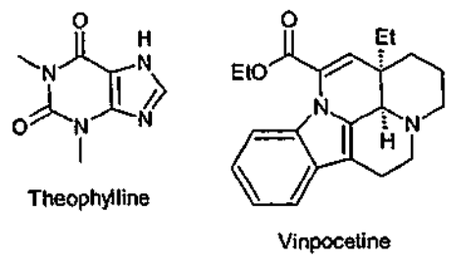


Figure 4.10 Inhibitors of phosphodiesterases.

The identification of PDE1 activity was consistent with the report of Nichols and Morimoto who identified PDE1 as being the major isoform in the bovine heart preparation. However, they had also observed some PDE3 activity eluting later in the gradient. Unfortunately, no equivalent activity was observed in the fractions of this purification after the observed PDE1 activity. A second purification step was also conducted on Fractions 16-26 of the initial purification (Figure 4.6) and, as expected because these fractions eluted before the observed PDE1 activity, no inhibition by cilostamide was observed either.

4.2.4 Bovine Phosphodiesterases: Summary and Conclusions

The isolation of PDE3 from crude bovine heart complex purchased from Sigma did not prove to be as straightforward and reliable as was anticipated. While PDE1 was identified and characterised, no indication of the PDE3 isoform was observed.

Possible reasons for being unable to identify the PDE3 isoform include;

(i) Assay conditions which were inappropriate for optimal PDE3 activity and inhibition

(ii) PDE3 activity being lost through degradation of the isoform during purification or while being stored at –20°C

(iii) An adapted experimental protocol which was inadequate to isolate the desired isoform

(iv) PDE3 not being present in the unpurified crude material

As a consequence, together with the fact that human phosphodiesterase was of more relevance than bovine phosphodiesterase, it was decided to investigate the isolation of phosphodiesterases from human platelets.

4.3 Human Platelet Phosphodiesterases: Isoform Isolation

While not as conveniently available as when ordering from a commercial supplier such as Sigma, human blood can be relatively easily obtained from a consenting donor and platelets can be consequently isolated. Platelet lysis and subsequent ultracentrifugation yields a supernatant containing cytosolic proteins which can be separated by ion-exchange chromatography in a manner similar to that attempted for the crude bovine cardiac material. For this attempt at human platelet phosphodiesterase isolation, protocols similar to those of Dickinson *et al* were followed.[72]

Assays to measure phosphodiesterase activity were based on previous experiments conducted with the isolated bovine fractions and further developed as required.

While some limitations relating to the conditions for UV detection after RP-HPLC were recognised, the method was retained due to the possibility of the level of sensitivity being sufficient to identify human PDE3 activity even under sub-optimal assay conditions.

4.3.1 Platelet Isolation

Human blood was collected in theophylline from healthy volunteers (maximum 400 mL per donor) who had not received antiplatelet medication in the previous two weeks. Platelets were isolated using a modified method of Baezinger and Majerus[84] then were washed and lysed according to Dickenson *et al.*[72] Centrifugation of the platelet lysate resulted in a supernatant containing soluble cytosolic proteins such as phosphodiesterase isoforms.

4.3.2 Chromatographic Separation of Soluble Human Platelet Cytosol

Phosphodiesterase activities were separated from the soluble protein supernatant over 120 minutes using ion-exchange chromatography on a DEAE-Sepharose Fast Flow column (5 mL) using the method of Dickenson *et al* with some modifications.[72] A sample UV trace of the separation is shown (Figure 4.11).

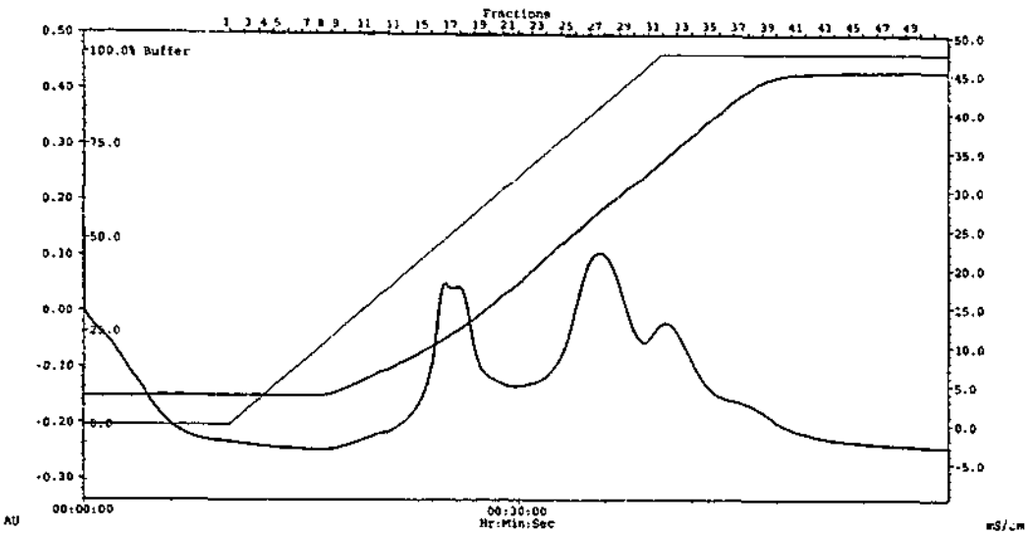


Figure 4.11 UV trace from ion-exchange chromatography of crude human platelet cytosol over 1 hour.

4.3.3 Visualisation of Separated Platelet Protein Fractions using Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

In order to check the complexity of the separated protein fractions, they were visualised using SDS-PAGE, according to the method of Laemmli.[85] Protein samples, obtained from collected Fractions 9-40 were separated and then stained. Fractions 18-39 are shown in Figure 4.12.

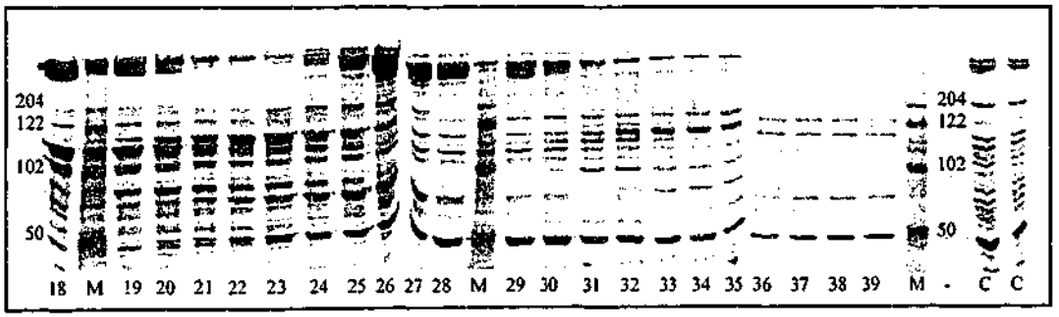


Figure 4.12 SDS-PAGE of human platelet protein in Fractions 18-39 following ion-exchange chromatography (C=control, M=molecular weight marker).

Fractionation of the crude material had occurred by the ion-exchange chromatography but, in comparison with the crude material (C), many of the fractions remained relatively complex. However, the fractions contained, importantly, bands corresponding to the expected molecular weight of ca. 122-125 kDa for PDE3A and ca. 105 kDa for the PDE2 subunits (where the homodimer has a molecular mass of ca. 240 kDa).[76]

4.4 Human Platelet Phosphodiesterase Activity Studies

4.4.1 Platelet Phosphodiesterase Assay Conditions and RP-HPLC Analysis

Similar assay conditions and RP-HPLC protocols to those used for the bovine phosphodiesterase activity assays were employed. The assay consisted of 350 μL of HEPES buffer, with protein fraction (100 μL) added followed by 50 μL of 300 μM cAMP. Aliquots were removed and terminated at 1, 3, 5 and 10 minutes.

4.4.2 Platelet Phosphodiesterase Assay RP-HPLC Results

A peak corresponding to AMP was identified by RP-HPLC (Figure 4.13). This peak increased over time in the enzyme assay, suggesting it was AMP being formed by phosphodiesterase hydrolysing cAMP. Fractions 29-35 were identified for further investigation. Care in analysis had to be taken because another close running peak was observed in the RP-HPLC chromatogram. This peak was found to remain unchanged over the time course and was later identified as corresponding in intensity to the UV absorbing component from ion-exchange chromatography of the platelet cytosolic proteins.

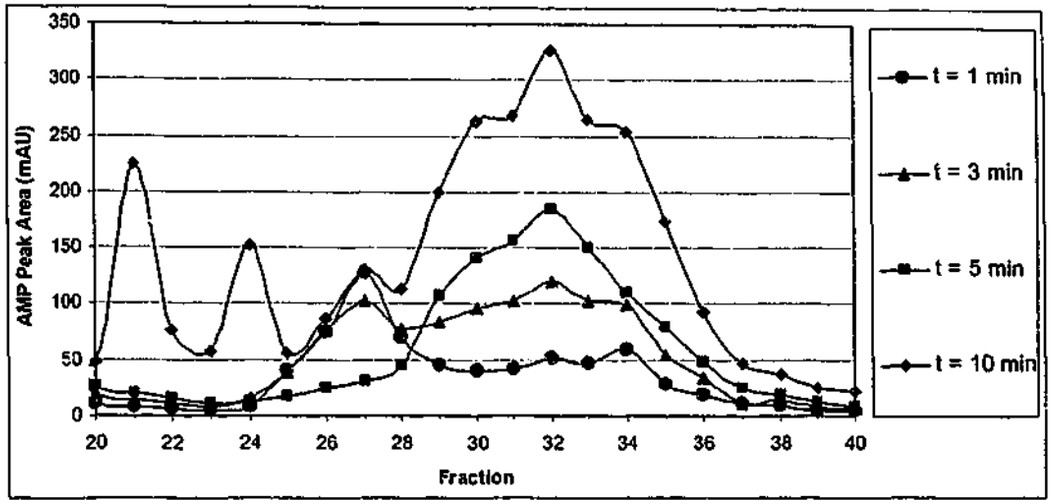


Figure 4.13 Amount of AMP produced after 1, 3, 5 and 10 minutes as an indication of human phosphodiesterase activity following initial purification.

Plotting the AMP peak area (mAU) in Fractions 29-35 against time showed the enzyme was working in the linear range between 1 and 10 minutes (Figure 4.14).

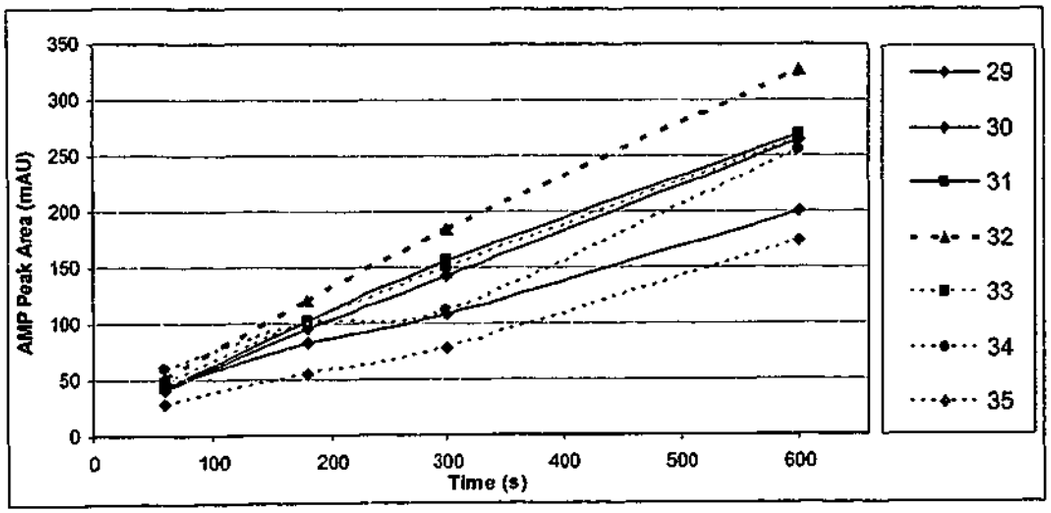


Figure 4.14 Amount of AMP produced as an indication of phosphodiesterase activity over time in Fractions 29-35.

Fractions 29-35 were each tested in the presence of DMSO, 100 μM milrinone (PDE3 inhibitor) and 40 μM EHNA (PDE2 inhibitor), with an assay termination time

of 5 minutes selected as this was within the linear range (Figure 4.15). DMSO appeared to have no inhibitory effects. EHNA was found to consistently inhibit the activity of Fractions 29-31, indicative of PDE2 activity, and inhibited Fractions 32-35 to a lesser extent. This suggested that the PDE2 was in the presence of another PDE. Milrinone did not inhibit the activity of Fractions 29-32 but showed some inhibition of Fractions 33-35. By analogy with the activity profile obtained by Dickinson, it was hypothesised that Fractions 33-35 contained PDE3 contaminated by PDE2.

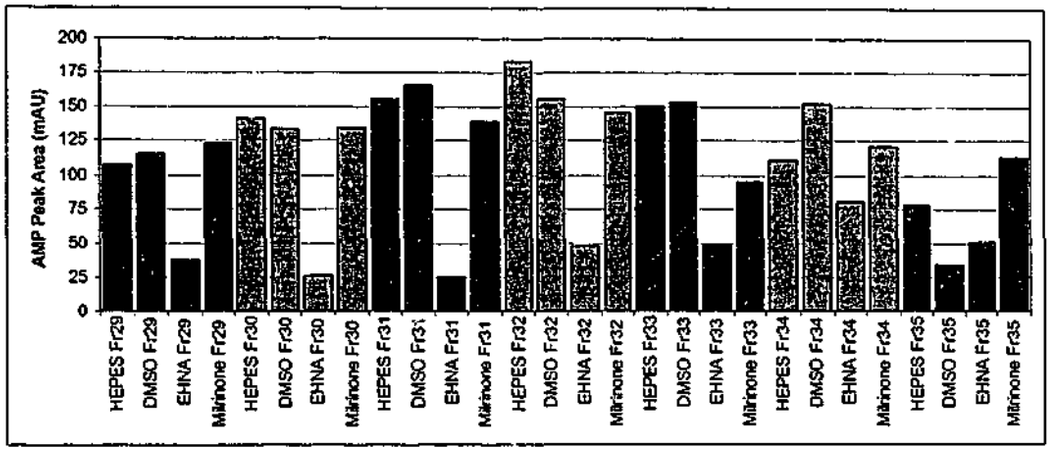


Figure 4.15 Inhibition of phosphodiesterase in Fractions 29-35 by EHNA and milrinone.

It is important to note that these assay conditions utilise 30 μM cAMP, a relatively high concentration of cAMP which is optimum for PDE2 activity. This compromises the expression of PDE3 activity, where any traces of PDE2 become the predominant activity observed. It was thought that PDE2 would not be as active where cAMP concentration was low, thus any AMP formed would be predominantly due to hydrolysis by PDE3. This would allow PDE3 activity to be evaluated without the interference of PDE2 activity.

At this stage, a method for the evaluation of PDE3 activity at low cAMP concentration was urgently needed and the RP-HPLC protocol currently available lacked the sensitivity required. In the following section, the use of an alternative

methodology to achieve evaluation of PDE3 activity at low cAMP concentration is described.

4.4.3 Platelet Phosphodiesterase Assay: Optimisation for PDE3 by Enzyme Immunoassay

For a PDE3 assay containing a low cAMP concentration of 0.5 μM (which is sixty fold less than that used for the PDE2 assay), the cAMP peak could only just be detected following RP-HPLC and, unfortunately, any hydrolysed AMP could not be observed at all. As a result, an enzymeimmunoassay kit using an anti-cAMP antibody was investigated as a possible means to sensitively measure changes in cAMP.

Assays were conducted under the same conditions as for the RP-HPLC analysis, with the exception that the final concentration of cAMP used was 0.5 μM. Under these conditions, the level of cAMP was similar in DMSO and milrinone for each of the Fractions 29-31 (Figure 4.16). However, in the presence of only DMSO, the cAMP level was decreased by approximately 40% for Fraction 32 and to a much greater extent for Fractions 33-35, resulting in approximately 80% decrease in cAMP (Figure 4.16). The presence of milrinone completely blocked the decrease in cAMP in these fractions, indicative of PDE3 being inhibited. Therefore, the PDE3 activity was determined to be located in Fractions 33-35, corresponding to the hypothesis from earlier results (Figure 4.15).

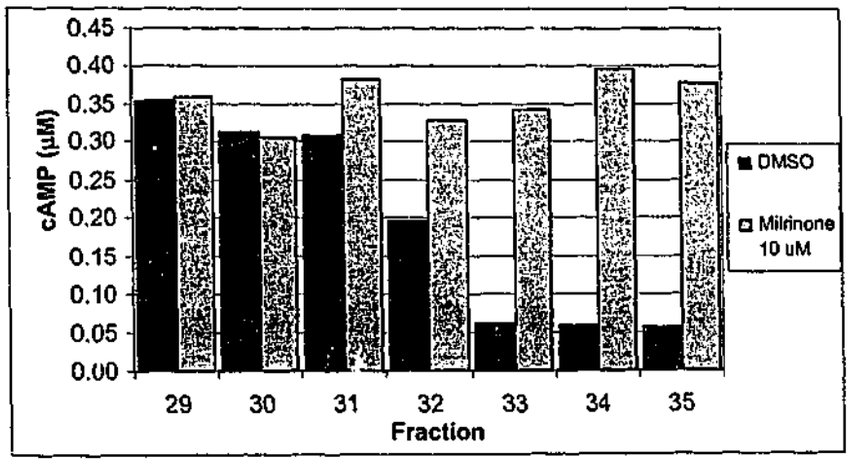


Figure 4.16 Phosphodiesterase activity in Fractions 29-35 when in the presence of DMSO or milrinone.

While successful in isolating PDE3 activity, the EIA method holds significant drawbacks as a means of evaluating compounds as potential inhibitors. It was time-consuming and expensive to complete an EIA, using an entire kit and involving much sample manipulation on a 96-well plate scale after all of the corresponding activity assays, only to find that the enzyme did not behave as expected. The major problem relates to finding initial assay conditions and then being able to titrate the enzyme activity to maintain linearity.

Essentially, the PDE3 activity is unknown at the beginning of the activity assays before the enzymeimmunoassay is used to determine the results. Completing an EIA to characterise the enzyme (as was undertaken earlier and shown in Figure 4.16) before screening is unfeasible given the length of time for EIA incubation alone (more than 4 hours) and also particularly due to the suspected instability of PDE3, even when stored at -20°C. While RP-HPLC is rapid and convenient, estimating PDE3 activity based on results obtained under PDE2 conditions is also fraught with difficulty and likely to be inaccurate. However, the consequences of not knowing the current level of PDE3 activity can result in using enzyme which has become inactive or an amount of enzyme which is too high. Detection of 85% consumption of cAMP (which was the EIA result seen in Figure 4.16) is almost certainly outside the linear

range of the enzyme activity and it would be very difficult to determine IC_{50} values from results such as these. These issues would make such a technique cumbersome for compound screening and prone to a high failure rate.

4.4.4 Human Phosphodiesterases: Summary

Human phosphodiesterases of interest, namely PDE2 and PDE3, were successfully isolated using ion-exchange chromatography. Although the protein fractions obtained were still a relatively complex mixture when visualised by SDS-PAGE, the pooled fractions gave satisfactory results when assayed for activity and could be inhibited with appropriate compounds.

Determination of PDE2 activity can be completed by measuring AMP produced from cAMP hydrolysis using a RP-HPLC Zorbax Eclipse XBD-C_6 column, where activity can be inhibited by EHNA and not by milrinone. Under the high cAMP conditions required for PDE2 activity and RP-HPLC detection, possible PDE3 activity which was partially inhibited with milrinone was also observed. Under these conditions, an assay time up to ten minutes still appears to be within the linear range for both isozymes.

However, the low concentration of cAMP required for optimum PDE3 activity meant that any resulting AMP could not be detected by RP-HPLC under the chromatographic conditions used. EIA was able to detect changes in the requisite low levels of cAMP, this technique was used to confirm PDE3 activity under conditions where inhibition by milrinone was observed. It appears likely that the same choice of assay conditions were what confounded the identification of PDE3 in the bovine heart sample. In spite of this, the numerous drawbacks of the EIA technique still make an improved RP-HPLC assay protocol, similar to that used for PDE2, a more attractive alternative for high-throughput compound screening. A particular strength of the RP-HPLC method was the capacity to directly identify formation of the product, with high reproducibility.

4.5 Advancement in Human Platelet Isolation and Activity Assay Protocols

The methodology developed to date had been successful in achieving the primary goal - producing batches of purified PDE3 and PDE2 which could be assayed under appropriate conditions to test inhibitors. However, there were still a number of practical limitations that would make an inhibitor screening program difficult.

These limitations included;

(i) Contamination of PDE3 by PDE2

(ii) Amount of manual handling required to complete assays and analysis

(iii) Speed and reproducibility of the chromatography run

(iv) Extent of additional sample manipulation and expense required for EIA, particularly in view of the reported instability of the enzyme.

4.5.1 Modification to Phosphodiesterase Isolation Protocol

The development of assay procedures to characterise the phosphodiesterase isoforms as described to date, in particular the completion of time courses, resulted in the isolated PDE2 and PDE3 being consumed rapidly. As a consequence, further batches of platelet protein were required to be purified to enable assay development and compound screening. The opportunity for improvement of this isolation methodology was also recognised, with a need for improved purification of the phosphodiesterases identified.

As suggested by the initial SDS-PAGE and activity assays (Figure 4.12 and Figure 4.13 respectively), improved separation of the PDE2 and PDE3 isoforms was desirable. To attempt this, later batches of human platelet phosphodiesterase were isolated using an extended gradient over 2 hours. A representative UV trace is shown in Figure 4.17.

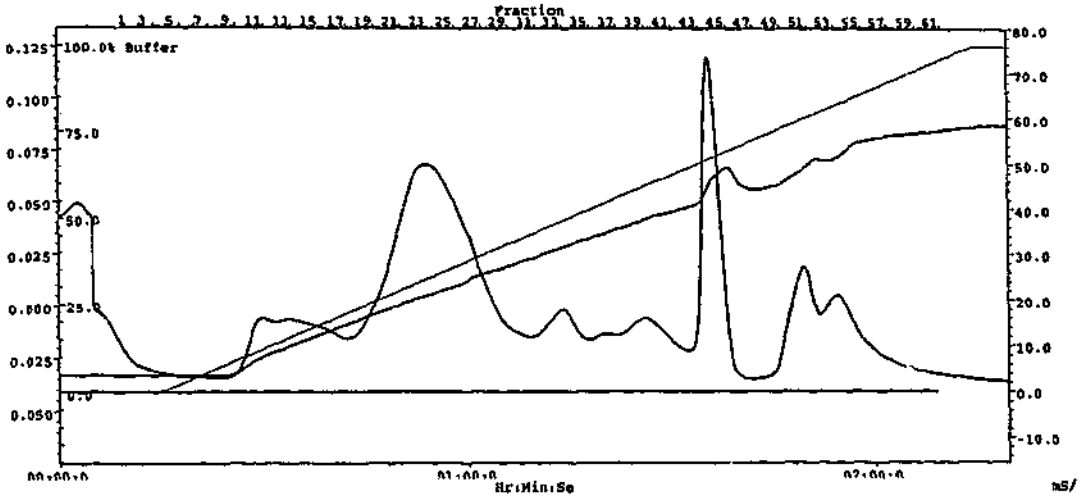


Figure 4.17 UV trace from ion-exchange chromatography of crude human platelet cytosol over 2 hours.

Plotting the peak area of AMP (mAU) produced by each protein fraction, using 45 µL HEPES, 45 µL protein fraction and 10 µL cAMP, produced two peaks of activity (Figure 4.18) which appeared to be much more distinct than that which was seen for the first batch (Figure 4.13). The benefits of this improved resolution included removal of the component that was visible in the UV trace and eluted near AMP in the RP-HPLC profile. An apparent improvement in PDE3 stability was also observed later on. It didn't, however, eliminate the unwanted PDE2 activity in the later fractions where 30 µM cAMP was used but PDE3 activity anticipated.

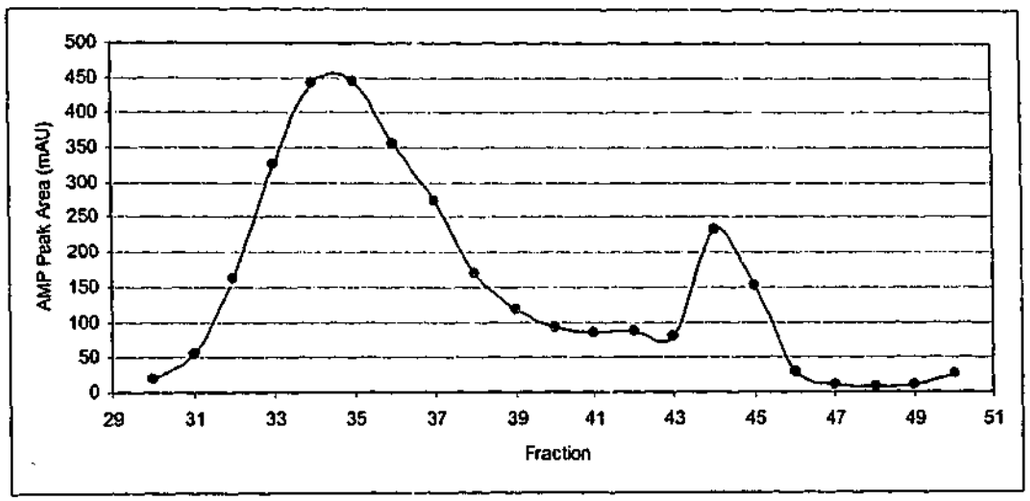


Figure 4.18 Amount of AMP produced as an indication of phosphodiesterase activity following initial purification.

The peaks were thought to be indicative of PDE2 and PDE3 activity respectively, since the assay conditions were promoting PDE2 activity (high cAMP). This was later proved to be the case using appropriate inhibitors (refer to Figure 4.19) against Fractions 31-39 for PDE2 and Fractions 43-46 for PDE3.

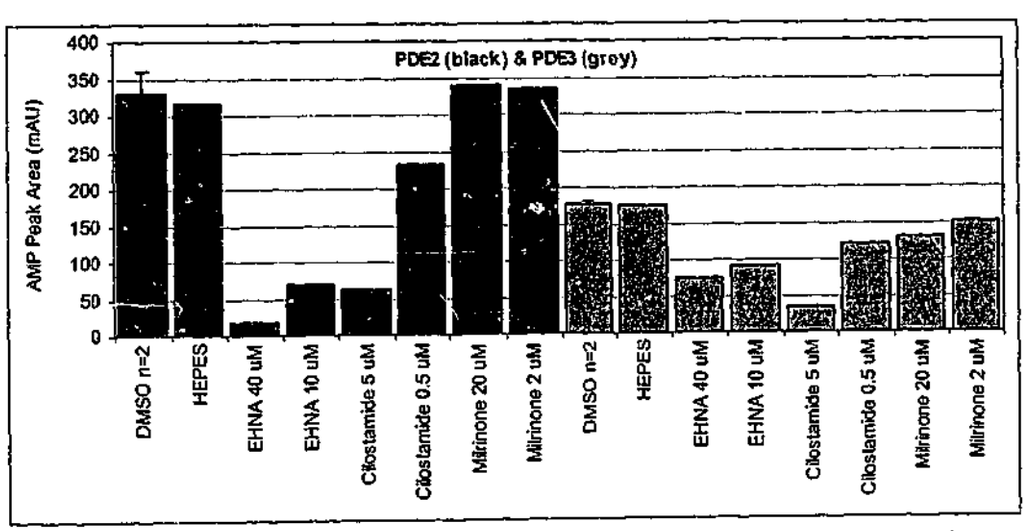


Figure 4.19 Inhibition of PDE2 (black bars) and PDE3 (grey bars) activity in pooled fractions by EHNA, cilostamide and milrinone.

4.5.2 Changes to PDE Assays: Manual Handling Practices

Further improvements to the assay protocols were also attempted. Reasons for this were primarily due to the high rate of isolated phosphodiesterase consumption but also due to the amount of handling and time involved.

Where fractions were being initially screened for phosphodiesterase activity following ion-exchange chromatography, ten minutes was chosen to be the assay endpoint rather than completing time courses. Additionally, an increased volume of the PDE fraction relative to the total volume was also used (such as 45% of the total assay volume rather than 20%, the latter being the assay conditions used to give the results in Figure 4.18 and 4.19). These changes would enable rapid visualisation of any phosphodiesterase activity occurring in the fractions, without large amounts of the enzyme being consumed before screening of the 2-morpholinochromone library had even begun.

For inhibitor screening, it was decided that time course assays would be also avoided where possible to avoid rapid consumption of the isolated enzymes and that for the majority of assays, five minutes would be selected as an appropriate assay end point (which would be in the linear range of activity according to data obtained thus far).

However, a much greater amount of time-consuming manual handling was being undertaken in using microtubes to conduct each individual phosphodiesterase assay and this practice was obviously not going to be suitable for high-throughput screening. Using a 96 well-plate placed in a microplate shaker was found to be advantageous as a greater number of assays could be carried out simultaneously and the plate shaker enabled consistent mixing and incubation at 37°C. Heating at 100°C was part of the method used for reaction termination by Dickenson *et al*, so the 96-well plate was transferred to an 100°C oven for 3 minutes as it was no longer practical to use boiling water for termination.

While the assays could be very successfully carried out in the 96-well plate in the incubator and terminated by transferring the plate to an oven, some manual handling was still involved in transferring the contents of each well to a vial for RP-HPLC analysis. It can be envisaged that this step could be avoided in a longer-term screening program by the use of an autoinjector which would accept 96 well plates, this would allow assays to be performed, terminated and analysed without any individual assay transfers.

4.5.3 Changes to PDE2 Assays: Effect of cGMP Addition

Phosphodiesterase assays were completed to investigate the effect of cGMP, which activates PDE2, to allow improved analysis of activity by RP-HPLC. The addition of 1 µL of 1 mM cGMP, resulting in a final assay concentration of 10 µM, resulted in a ca. 60% increase of activity which could still be completely inhibited by EHNA (Figure 4.20, refer to black bars). As cGMP is a known allosteric stimulator of PDE2, this was considered further evidence of PDE2 activity. The addition of 10 µM cGMP to PDE2 assays was adopted as part of the standard protocol to ensure maximal PDE2 activity.

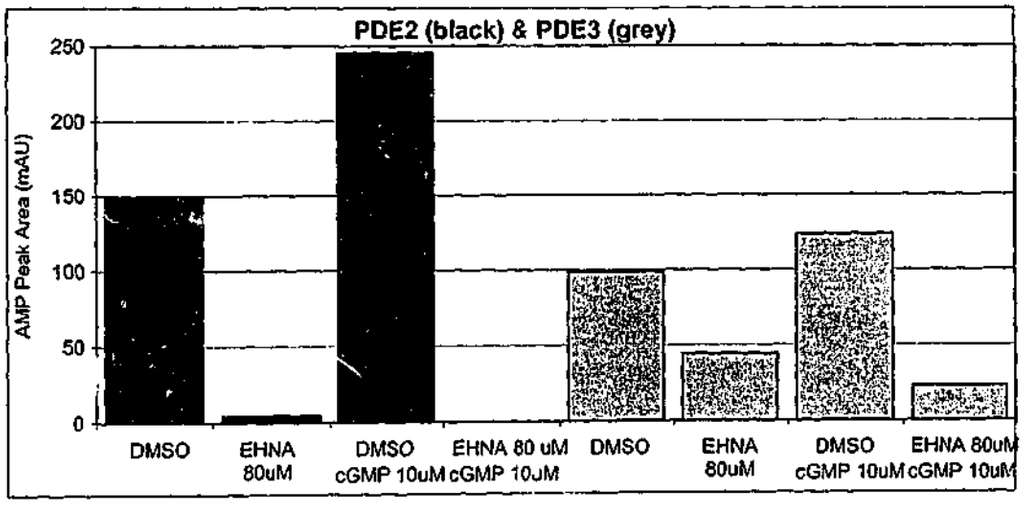


Figure 4.20 Stimulation of PDE activity by cGMP and inhibition by EHNA.

However, the presence of 10 μM cGMP also resulted in a small (ca. 20%) increase in activity in the pooled fractions that were thought to be PDE3, where PDE3 is not supposed to be stimulated by cGMP at all. This result was not entirely unexpected and thought to be due to the second peak of PDE3 activity being contaminated by PDE2. The PDE2 inhibitor EHNA was not able to completely inhibit the activity as it did with the PDE2 assays, regardless of whether cGMP was present or not, and this was thought to be evidence of unaffected PDE3 activity.

The amount of contamination appeared to vary with each batch purified. However, contamination by PDE2 was not considered to be a problem when optimum conditions (no cGMP and low cAMP concentration) were used to assess PDE3 activity, as PDE2 was not active under those conditions. This was evidenced where cAMP was 0.5 μM in the EIA studying PDE3 activity and no cAMP hydrolysis was observed in the PDE2 Fractions 29-31 (Figure 4.16). PDE2 contamination was only a problem where PDE2 conditions were being used to assess PDE3 activity. This again highlighted the need for an assay protocol and method of analysis suited to a low cAMP concentration. However, in summary, the inclusion of cGMP represented a marked improvement in PDE2 activity assays.

4.5.4 Changes to PDE3 Assays: Optimisation for RP-HPLC Analysis

The problem with assaying for PDE3 by RP-HPLC came down to detection sensitivity. An attempt was made to optimise the chromatography to gain a 20 to 30 fold increase in sensitivity for AMP.

Waters Australia Pty Ltd kindly evaluated AMP, cGMP and cAMP elution through a number of columns, including Atlantis, Xterra RP and Xterra MS, at high and low pH using 0.2 M $(NH_4)H_2PO_4$ and acetonitrile (98:2). A recommendation of the Xterra RP column (4.6 mm x 150 mm, 3.5 μM) was made as it showed the best retention and was able to separate AMP well away from the void. This C_{18} column has a polar group embedded in the stationary phase which assists in keeping it

performing well in a highly aqueous mobile phase. It was suggested that a smaller bore column, such as the 2.1 mm x 150 mm column, would be more sensitive. Further investigation of the narrower column suggested gradient elution using acetonitrile would decrease elution times.

In the laboratory, it was confirmed that the Waters Xterra RP_{18} 3.5 μM 2.1 x 150 mm column gave an improvement in sensitivity which allowed the detection of AMP formed under optimum PDE3 assay conditions of 0.5 μM cAMP. Gradient elution also allowed a decrease in run time. The column was equilibrated with the aqueous phosphate buffer with 0.3 mL/minute flow rate. Elution of AMP was completed isocratically in phosphate buffer over 5 minutes, with cAMP eluting by a gradient over a further 5 minutes using phosphate buffer and acetonitrile (peaking at 90:10) which was followed by a return to the starting conditions for a 10 minute equilibration. Representative chromatograms of this RP-HPLC assay in the presence (a) and absence (b) of an inhibitor are shown in Figure 4.21.

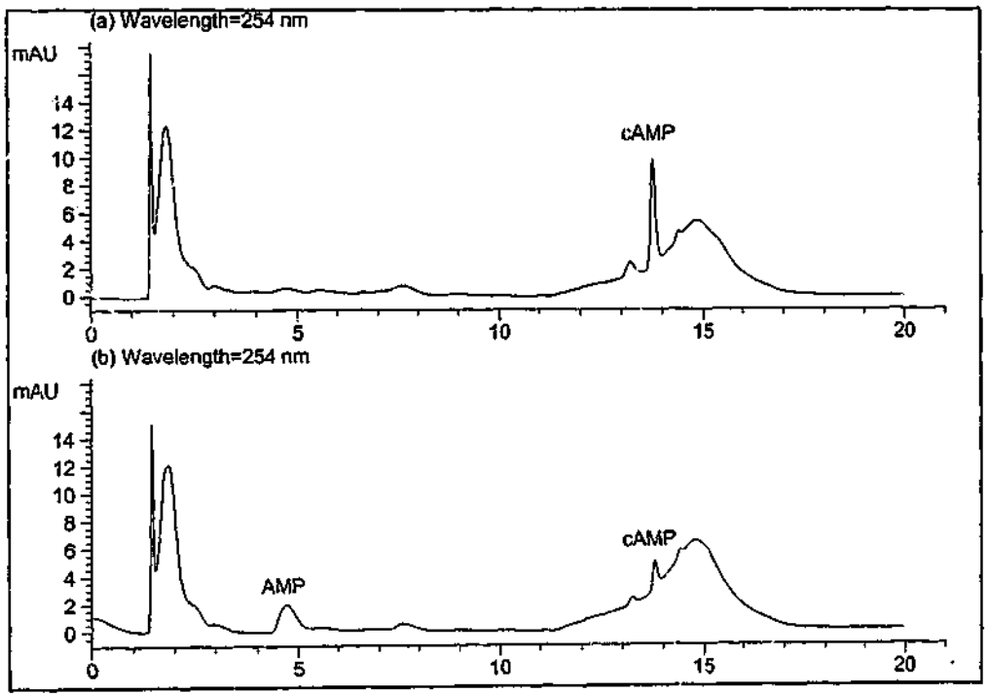


Figure 4.21 RP-HPLC chromatograms (a) in the presence of an inhibitor and (b) without an inhibitor present.

The Xterra RP_{18} column was also successful for decreasing the run time for PDE2 analysis, where the column was equilibrated with aqueous phosphate buffer and acetonitrile (99:1) with 0.3 mL/minute flow rate. A 20 minute gradient elution method was used where the column was run for 3 minutes at the initial conditions, then the gradient rises to phosphate buffer and acetonitrile (90:10) over 5 minutes where it remains for a further 2 minutes, followed by a return to the starting conditions and equilibrating for the remaining 10 minutes.

The use of organic solvent in small quantity (no greater than 10% acetonitrile) greatly decreased run time to 15 minutes for both phosphodiesterase isoform assays and did not appear to greatly hinder quantitation or reproducibility of the AMP, as suggested by Ogata *et al.*[83] Some fluctuation was seen in the elution time of cAMP (ca. 13 to 18 minutes) particularly when the RP-HPLC system was run overnight and it was thought that this was due to room temperature fluctuation, a feature which might be avoided by the introduction of a column heater into the HPLC system. The reduced run time together with the reduced bore of the column with subsequent lower flow rate also gave the Xterra RP column the advantage of using a reduced solvent volume, resulting in increased efficiency.

4.6 Summary and Conclusions

Evaluation of methods for the efficient separation of PDE3 found that the methods of Dickinson *et al*[72] were suitable for obtaining this isoform from human platelets. The PDE3 activity is clean providing appropriate assay conditions are used (i.e. low cAMP concentration), insofar as it is blocked by milrinone and cilostamide and not affected by EHNA. Using these methods, PDE2 was also able to be separated with activity which was inhibited by EHNA but not by milrinone or cilostamide, to a significant degree, and which was also able to be stimulated by the addition of cGMP.

A convenient process for screening fractions for activity has been developed which utilises RP-HPLC for the detection of AMP formed through the enzymatic hydrolysis

of cAMP. Appropriate choice of column and conditions resulted in a protocol which was appropriately time efficient (15-20 minutes per analytical run) with inexpensive running costs for high-throughput screening. The choice of column and gradient elution conditions was also able to allow small amounts of AMP to be detected, giving the sensitivity required to evaluate PDE3 under the appropriate assay conditions (low cAMP concentration).

With stability of the PDE3 being an issue, a key advantage to this methodology is that it allows ongoing evaluation of the enzyme (whether it be PDE3 or PDE2) in real-time so that activity can be monitored throughout the experimental procedure. It also appears likely that commercial bovine cardiac phosphodiesterase may be a source of the PDE3 enzyme, if purified and assayed under the appropriate conditions, such as those which have now been developed.

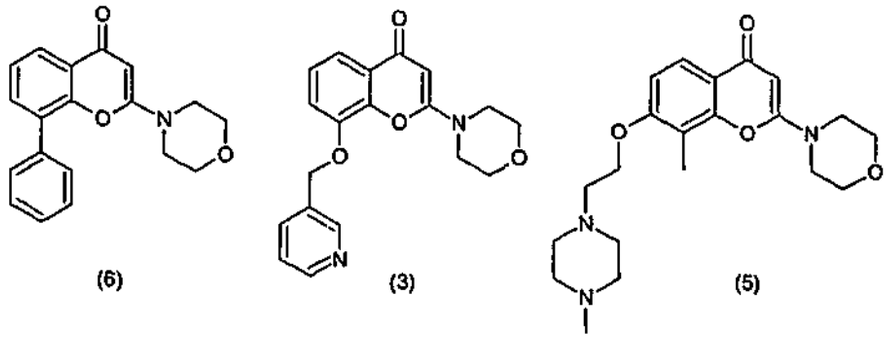
Chapter 5

EVALUATION OF 2-MORPHOLINOCHROMONES LY294002, U-86983 AND (5) AS INHIBITORS OF PLATELET PHOSPHATIDYLINOSITOL 3-KINASES AND PHOSPHODIESTERASES.

5.1 Introduction

As described in Chapter 1, 2-aminochromones have been shown to possess potent and useful activity at the enzyme level, in cellular assays and *in vivo*. In particular, compounds with a morpholinyl group at the 2 position have been shown to have antithrombotic,[30,31] antiplatelet[31,38] and antiproliferative[29,32] effects as well as being phosphodiesterase inhibitors[30,38] and phosphatidylinositol 3-kinase inhibitors.[33]

While the most significant of these synthetic 2-morpholinochromones is LY294002 (6) (Figure 5.1), which has been used as one of the tools to delineate the role that phosphatidylinositol 3-kinases (PI3-kinases) play in the regulation of intracellular signalling pathways,[35] other properties have been reported for 2-morpholinochromones. These are exemplified by the compounds U-86983 (3) and (5) (Figure 5.1). U-86983, developed by the Upjohn Laboratories, has been described as an *in vitro* and *in vivo* inhibitor of rat vascular smooth muscle cell migration which may be useful for the study of proliferative processes such as restenosis.[32] Upjohn also developed potent antiplatelet agents including compound (5), which was reported to be effective in animal models of thrombosis.[31] The antiplatelet activity of compounds structurally similar to (5) had earlier been attributed to inhibition of "low Km-phosphodiesterase" (PDE3).[30]



(6) (3) (5)

Figure 5.1 2-Morpholinochromones LY294002 (6), U-86983 (3) and (5).

While the inhibitory properties of LY294002 against PI3-kinase isoforms have been well characterised,[37,40,86] no assessment of activity against phosphodiesterases had been reported; nor had the inhibitory properties of U-86983 and (5) against PI3-kinases or purified phosphodiesterases been evaluated. Until such studies were done, the cellular mechanisms for the antiplatelet and antiproliferative effects of 2-morpholinochromones could not be safely attributed, particularly as such activities are consistent with either PDE or PI3-kinase inhibition. Moreover, the presence of two cAMP-phosphodiesterase isoforms[18] and four Class I PI3-kinase isoforms[41] in platelets suggested the potential to discover compounds with improved selectivity which could possibly be exploited therapeutically. By initially studying these three compounds, it was hoped that the structural features that render compounds of this class either as PDE3, PDE2 or PI3-kinase inhibitors could be distinguished and that connections to their reported cellular activities could be made.

The results of PDE and PI3-kinase enzyme assays could also provide a new basis for understanding the antiplatelet effects of 2-morpholinochromones. Literature reports have described clear distinctions between the effects of PI3-kinase inhibition (for example, by LY294002[37]) and those of PDE3 inhibition (for example, by milrinone and cilostazol[19]) upon platelets and it was expected that such differences would be similarly discernable using selective 2-morpholinochromones in platelet aggregation studies. Specifically, it was anticipated that PI3-kinase inhibitors would be effective against only a limited range of agonists for which the PI3-kinase pathway has been

shown to be critical. This would include agonists such as adenosine diphosphate (ADP), collagen related peptide (CRP) and heat aggregated IgG (HAI) but not thrombin or its agonist peptides (thrombin receptor agonist peptide and protease-activated receptor agonist peptide i.e. TRAP and PAR4AP). In contrast, PDE3 inhibition is considered a global method of inhibiting platelet aggregation and as such blocks all typical platelet agonists, including thrombin and its agonist peptides (TRAP and PAR4AP), high dose collagen and thromboxane A_2 (TXA_2). It was hoped that a correlation between the activity in platelets and the activity against the isolated enzyme would hold true for the 2-morpholinochromone series.

Thus the objective of the work presented in this chapter was to assess the inhibitory potency of the three literature compounds of interest (Figure 5.1) against platelet cAMP phosphodiesterases and PI3-kinases, and to correlate the results to their inhibitory potency in platelet aggregation assays.

The inhibition of phosphodiesterase activities of these three compounds was assessed in direct comparison with known inhibitors using the methods developed and described in detail in the previous chapter. Inhibition of PI3-kinase activity by these compounds was assessed by scientists at Kinacia Pty Ltd. Using assays of the PI3-kinase activity immunoprecipitated from platelet lysates, an evaluation of inhibitory potency against platelet Class I PI3-kinase was performed, in parallel with evaluation of proprietary compounds prepared by Kinacia scientists.

PI3-kinase activity can be obtained from a variety of sources, including bovine brain as described by Vlahos *et al* in the original report of LY294002.[33] More recently, production of recombinant protein has allowed the measurement of activity against specific PI3-kinase isoforms.[86] In the present study, the PI3-kinase activity was derived by immunoprecipitation of the enzyme from platelets using a sepharose-linked antibody to the p85 regulatory domain of the enzyme. In platelets, this regulatory domain is linked to possibly three Class Ia catalytic subunits, p110α, p110β and, as recently described, p110δ.[87] As such, the assay was not expected to

discriminate between Class Ia isoforms, except for p110γ which is not associated with the p85 regulatory domain but with the p101 domain.[88] Continuing work, not described here, is using immunoprecipitation with isoform selective antibodies or recombinant protein to perform assays of isoform selectivity.

Platelet aggregation studies were performed using standard protocols[89-91] using washed platelet suspensions as well as platelet rich plasma. The compounds were tested under a variety of conditions using agonists such as ADP, CRP, TRAP and PAR4AP. The potency of inhibitors in platelet aggregation studies are well known to be influenced by the activation state of the platelets and can vary markedly depending upon the donor and handling of the platelets. Therefore it is important in making comparisons of compounds to evaluate compounds under the same conditions with a single platelet preparation. The results presented here show single experiments representative of a number of repeat experiments, unless otherwise specified.

5.2 Results of Enzyme Assays

5.2.1 PDE3 Assays

The three literature 2-morpholinochromones LY294002 (6), U-86983 (3) and (5), were evaluated as inhibitors of PDE3 activity in comparison to the well-characterised PDE3 inhibitors of milrinone and cilostamide. EHNA, the selective PDE2 inhibitor, was included as a negative control. The results are shown in Figure 5.2.

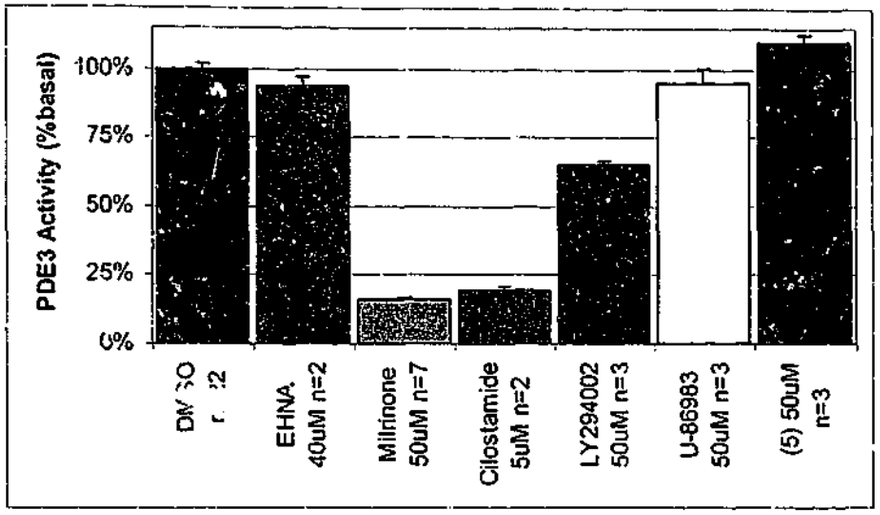


Figure 5.2 Inhibition of PDE3 Activity.

The PDE3 selective inhibitors milrinone (at 50 μM) and cilostamide (at 5 μM) were found to give near complete inhibition of PDE3 activity, in accord with the literature. At 50 μM concentrations, PDE3 activity was reduced by approximately 35% by LY294004, but not greatly affected by U-86983 and (5) (less than 10%).

Dose-response curves were completed for LY294002, in comparison with milrinone and cilostamide. An IC_{50} of approximately 100 μM was determined for LY294002 while the IC_{50}s for milrinone and cilostamide were found to be ca. 0.6 μM and 0.25 μM respectively (Figure 5.3).

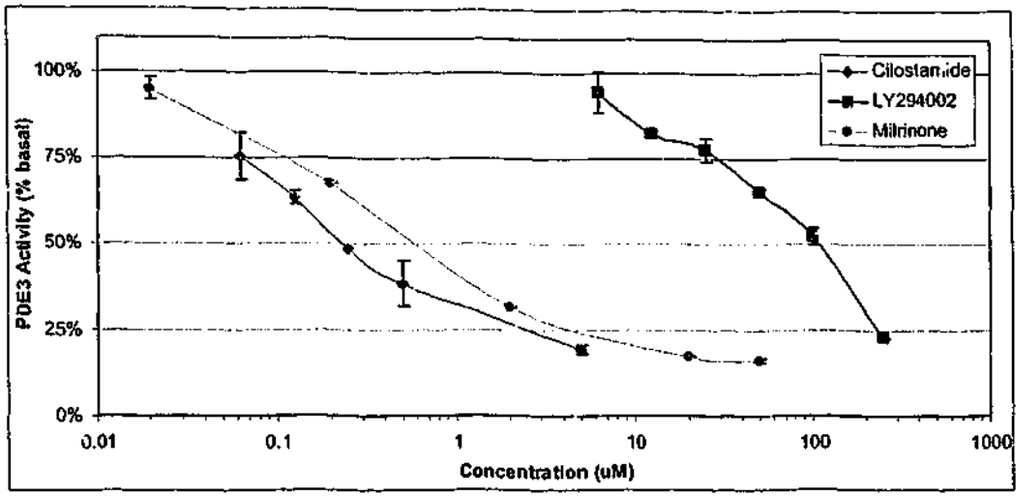


Figure 5.3 Dose-response curves of cilostamide, LY294002 and milrinone ($n \geq 2$).

5.2.2 PDE2 Assays

For assays evaluating the three 2-morpholinochromones for inhibition of PDE2 activity, the known PDE2 inhibitor EHNA was used as a positive control. The activity of EHNA at 40 μM was confirmed, with greater than 85% reduction in PDE2 activity (Figure 5.4).

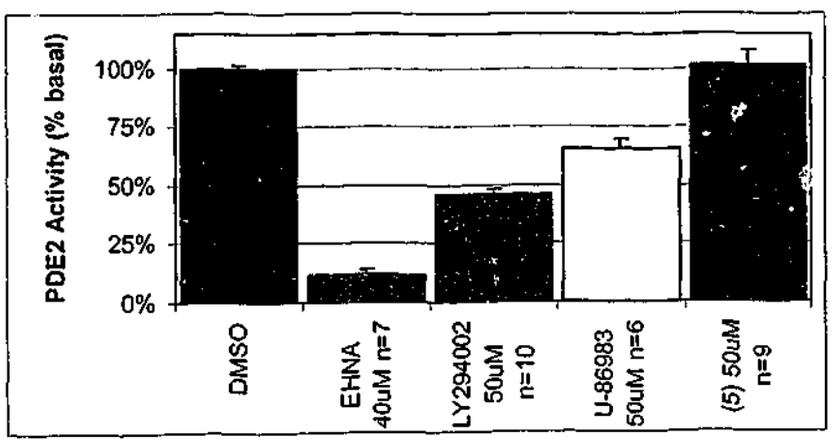


Figure 5.4 Inhibition of PDE2.

Of the three 2-morpholinochromones tested at 50 μM, LY294002 was again the most potent inhibitor, with PDE2 activity reduced by over 50%. U-86983 gave ca. 35% reduction in activity but no inhibition was observed for (5).

Dose-response curves were completed for EHNA, LY294002 and U-86983 from which approximate IC_{50}s of 4 μM, 35 μM and 250 μM respectively were determined (Figure 5.5).

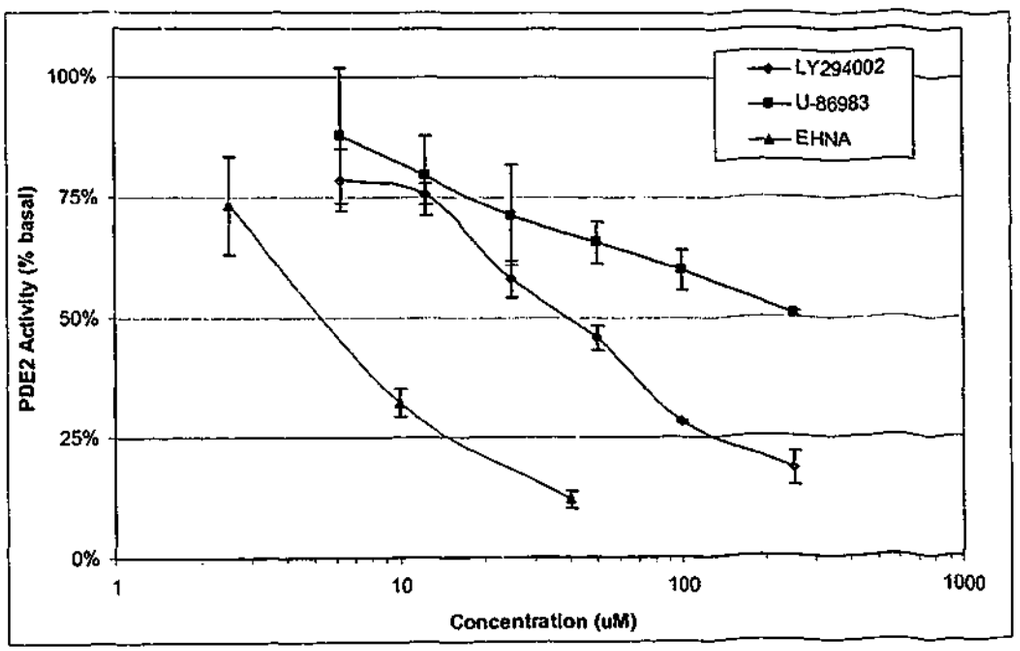


Figure 5.5 Dose-response curves of LY294002, U-86983 and EHNA.

5.2.3 Phosphatidylinositol 3-Kinase Assays

The inhibition of PI3-kinase activity by LY294002 is well established, and is used as a positive control in the screening programs of Kinacia. Unfortunately the PI3-kinase assay is rather laborious and subject to technical limitations which make it difficult to derive quantitative data. The data presented here shows results from a single assay representative of multiple determinations. Qualitatively, the results clearly demonstrate the relative potencies of the compounds.

The IC_{50} for LY294002 (6) against PI3-kinase activity was found to be between 1 and 3 μM (Figure 5.6), which is consistent with the published data for the inhibition of Class 1 PI3-kinase isoforms. U-86983 (3) was found to be more potent than LY294002, with an IC_{50} of between 0.3 and 1 μM, while compound (5) was a very poor inhibitor of PI3-kinase with an IC_{50} of greater than 10 μM (Figure 5.6).

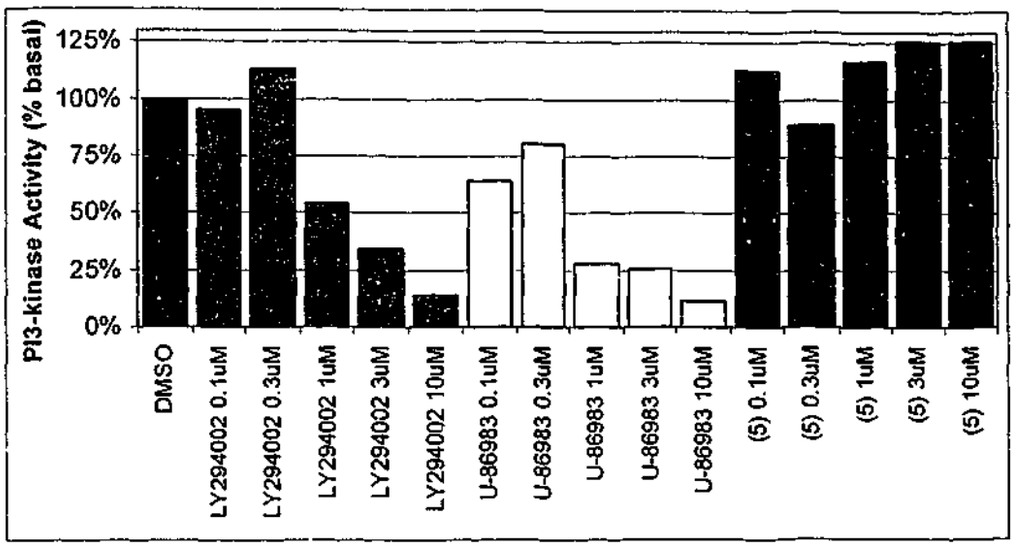


Figure 5.6 PI3-kinase inhibition by LY294002, U-86983 and (5).

5.3 Studies of Platelet Aggregation

In the wake of these enzyme assay results, a series of platelet aggregation experiments were performed in an attempt to correlate the isolated enzyme data with cellular activity.

Firstly, U-86983 (3) was found to poorly inhibit PDE3, contrary to an earlier report which described an IC_{50} of 2 μM.[30] However, it was found to potently inhibit PI3-kinase with an IC_{50} 0.3 – 1 μM. From the enzyme evaluation, U-86983 would be expected to be a very poor inhibitor of TRAP or PAR4AP induced platelet aggregation (which is not dependent of PI3-kinase), but would be anticipated to have reasonable potency against CRP induced aggregation (which is dependent on PI3-kinase). In other words, U-86983 would be expected to have LY294002 effects.

Secondly, compound (5) was found to have no inhibitory activity at PDE3 at 50 μM and only limited PI3-kinase activity, so any antiplatelet activity would have to originate from another mechanism. The reported activity of (5) had been examined only under one condition, against ADP in PRP. In order to evaluate the activity of (5), further studies of platelet aggregation, including using the PDE3 inhibitor cilostamide for direct comparison, were warranted.

5.3.1 Results of Platelet Aggregations

Human washed platelets (WP) were incubated for 1 minute at 37°C in the presence of the test compound in DMSO and then challenged with CRP (0.3 – 0.5 μg/mL) or PAR4AP (50 – 125 μM). The effect of the compounds on WP aggregation induced by CRP is shown in Figure 5.7, while the effect of compounds on WP aggregation induced by PAR4AP is shown in Figure 5.8. Representative traces are shown, at or near the 50% inhibition concentration for the 2-morpholinochromones tested. The known PDE3 inhibitor cilostamide was used as a positive control.

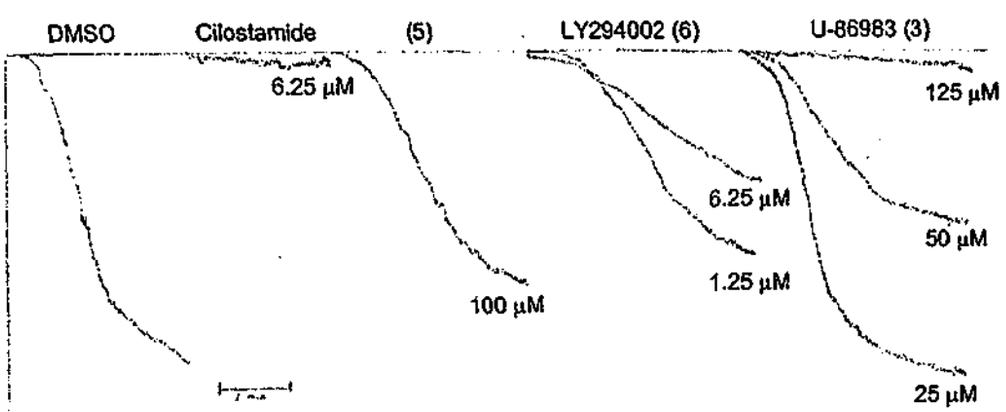


Figure 5.7 Effect of inhibitors on washed platelet aggregation induced by CRP.

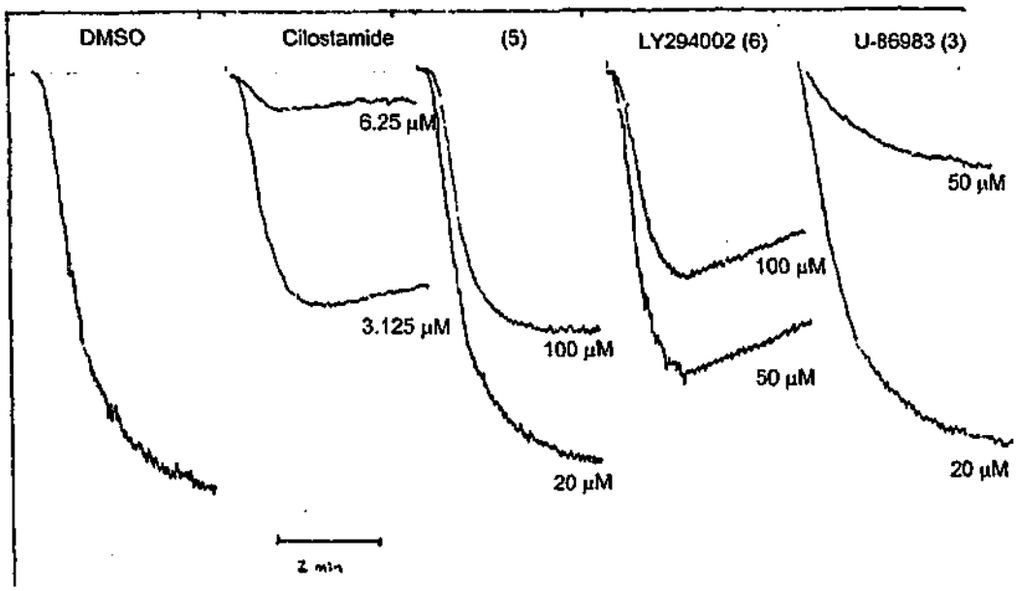


Figure 5.8 Effect of inhibitors on washed platelet aggregation induced by PAR4AP.

LY294002 was found to behave as reported in platelet aggregation studies, effectively inhibiting CRP-induced aggregation (Figure 5.7) but only poorly blocking aggregation induced by PAR4AP, even at 100 µM doses (Figure 5.8).

U-86983 (3) was less effective than LY294002 as a platelet aggregation inhibitor when CRP was used (Figure 5.7). The activity was expected to be comparable as both compounds have been shown to be PI3-kinase inhibitors and the CRP agonist is thought to utilise a PI3-kinase dependent pathway. When PAR4AP was the agonist (Figure 5.8), both U-86983 and LY294002 were poor inhibitors of aggregation. Additional data indicated that U-86983 was also a moderate inhibitor of HAI-induced aggregation (which is PI3-kinase dependant like CRP) and a poor inhibitor of TRAP-induced aggregation (which, like PAR4AP, is not PI3-kinase dependant).[92]

Compound (5) was found to be a poor inhibitor of washed platelets when challenged with CRP (Figure 5.7), PAR4AP (Figure 5.8) and also by TRAP.[92] This was not unexpected, given the low potency of (5) in the isolated enzyme assays for PDE2,

PDE3 and PI3-kinase, but was in marked contrast to the activity reported in the literature.[31] The inhibition of platelet aggregation by this compound under literature conditions, using PRP with the agonist ADP (8 μM),[31] was attempted and compound (5) was indeed found to be active, displaying an IC_{50} of approximately 10 μM under the conditions used (Figure 5.9), and in a manner comparable to the PDE3 inhibitor cilostamide.

This confirmation of activity similar to that reported suggests that while compound (5) can inhibit aggregation, this occurs only in PRP but not in WP and by a mechanism other than inhibition of PDE2, PDE3 or PI3-kinase.

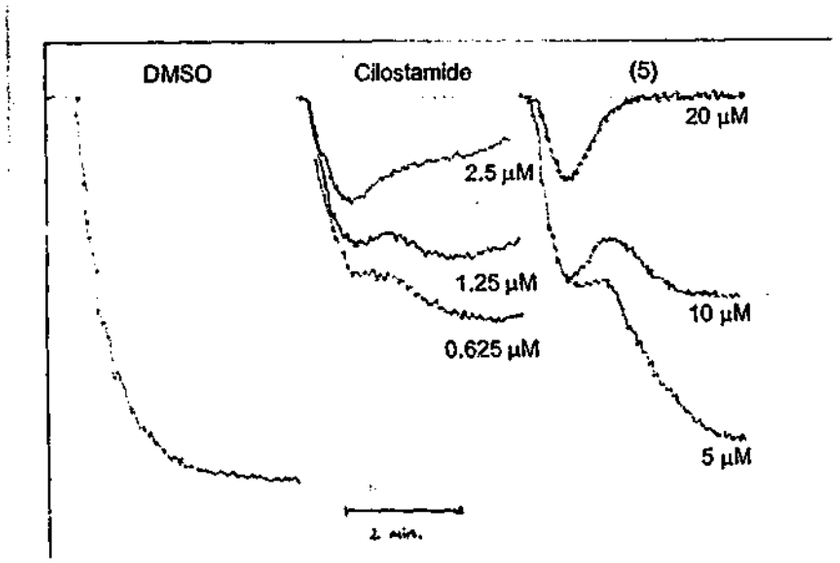


Figure 5.9 Effect of inhibitors on PRP aggregation induced by ADP.

5.4 Discussion

5.4.1 Biological Evaluation of LY294002

As the use of LY294002 has become widespread in recent years, a number of researchers have sought to further characterise its level of selectivity. LY294002 has been reported to be non-selective across the PI3-kinase isoforms, most recently by

Sadhu *et al*[86] and the results obtained in this study largely match those in that report. In this study, the p85 assay indicated an IC_{50} of approximately 1 – 3 μM. Overall, LY294002 shows high selectivity for PI3-kinase and its family members in comparison to other protein kinases.[43] However, with the focus on kinase activities, other possible activities have been overlooked.

In the assays carried out, the PI3-kinase inhibitor LY294002 was also found to be a moderate inhibitor of phosphodiesterases PDE2 and PDE3 with an IC_{50} of approximately 35 μM and 100 μM against each isoform respectively. This is the first report of such activity for LY294002.

Phosphodiesterases and kinases have a number of inhibitors in common, most notably the flavonoid compounds from which LY294002 was derived,[33] and this should not be too surprising given the structural similarity of the purine-based substrates with which these inhibitors compete (cAMP and adenosine triphosphate (ATP) respectively). Indeed, inhibition of PI3-kinase activity has recently been demonstrated for caffeine and theophylline, better known as non-selective PDE inhibitors.[93] While LY294002 only inhibits phosphodiesterase isoforms moderately, the concentrations at which phosphodiesterase inhibition does occur sometimes overlap the concentrations used in cellular studies of PI3-kinase.

For example, LY294002 inhibits many platelet responses at dose levels of 1 – 3 μM, indicative of PI3-kinase dependent events. However, much higher doses are required to inhibit other responses, such as aggregation induced by thrombin, TRAP or PAR4AP, for the latter LY294002 has an IC_{50} of approximately 50 – 100 μM. It seems possible that PDE2 and/or PDE3 inhibition may be a contributing factor to the antiplatelet effect in cellular assays where LY294002 is used at high concentrations.

Overall, LY294002 is still selective for PI3-kinases over PDE2 and PDE3, and in general terms a remarkably selective PI3-kinase inhibitor. Caution will still need to

be applied as recent reports are attributing activity of LY294002 at other loci including casein kinase-2 and the estrogen receptor.[43,94,95]

5.4.2 Biological Evaluation of U-86983

U-86983 has been reported as an antiproliferative compound, it has been evaluated in a number of animal models of restenosis and appears to have significant inhibitory effects on vascular smooth muscle cell migration and proliferation. [32] Until now, no study of its likely enzyme targets has been reported, although its inhibition of platelet phosphodiesterase activity in platelet lysates had been assayed in a separate study.[30] While Benjamin *et al* attributed U-86983 with inhibiting low K_m cAMP-phosphodiesterase (IC_{50} 2 μM),[30] the assay results appear open to interpretation. The assays were completed using an unpurified lysate in the presence of high concentrations of cAMP and yield an inhibitory response which is not dose-dependent (refer to Chapter 4.1).

In this study, evaluation of U-86983 against each isoform showed relatively poor inhibition of PDE2 and PDE3 at 50 μM. Slightly more activity was shown against PDE2, with an IC_{50} of 250 μM.

In contrast, a significant level of activity against PI3-kinase was observed. As an inhibitor of PI3-kinase from p85 immunoprecipitation, U-86983 (IC_{50} 0.3 – 1 μM) was more potent than LY294002 (IC_{50} 1 – 3 μM).

This rank order did not carry through in platelet aggregation studies with LY294002 being a much more effective inhibitor of PI3-kinase dependant CRP-induced aggregation than U-86983. One explanation for this difference may be the relative importance of specific PI3-kinase isoforms in the aggregation process as compared to the expression of activity in the immunoprecipitated enzyme assay. Ongoing studies of both the selectivity of U-86983 for PI3-kinase isoforms and the roles of those isoforms in the platelet aggregation process may elucidate the reasons for the

difference in efficacy. Alternatively it may be that LY294002 has an advantage over U-86983 in terms of other cellular properties, such as cell penetration and sub-cellular localisation. However, the reported effectiveness of U-86983 in other cellular studies suggests a more specific basis for this difference. In related studies performed by Dr. Karen Anderson, U-86983 was found to be equivalent to LY294002 in inhibiting vascular smooth muscle cell proliferation.[96]

Overall, U-86983 is a relatively potent inhibitor of PI3-kinase isoforms and these activities appear to predominate over the phosphodiesterase isoforms. It may be that the preference for specific PI3-kinase isoforms may underpin the observed inhibitory properties in cells, consistent with distinct roles proposed for PI3-kinase isoforms in cell proliferation and migration. The data collected here strongly suggests that PI3-kinase has an important role in the observed antiproliferative activity of this compound.

5.4.3 Biological Evaluation of Compound (5)

In this study compound (5) was found, very surprisingly, to be devoid of activity against the isolated phosphodiesterase enzyme PDE3 at 50 μM. Similarly, no activity was found against PDE2. Against PI3-kinase, compound (5) showed no inhibitory activity at 10 μM. Moreover, (5) showed only very modest inhibition of platelet aggregation, even at a 100 μM assay concentration, when aggregation was induced by a number of platelet agonists including CRP, PAR4AP and TRAP. The antiplatelet activity of (5) was, however, replicated in assays ADP-induced aggregation in platelet rich plasma, as described in the literature.[31]

As such, a striking result of this study is that neither the antiplatelet effects nor any of the associated haemodynamic effects of (5) that have been reported[38] can be attributed to phosphodiesterase inhibition. Compound (5) also had no observed activity against the p110α, p110β and p110δ isoforms of PI3-kinase as shown the p85 assay (Figure 5.6) or against the p110γ isoform in a separate study.[97] Thus, it is

suggested that a novel mechanism of antiplatelet activity for 2-morpholinochromones is being demonstrated.

The question remains that if the activity of (5) is not due to PDE3 inhibition, then what is responsible? The major clue to determining the mechanism of action rests with the differences between platelet aggregations performed in plasma compared with those performed in washed platelet suspensions. One hypothesis is that the mechanism by which compound (5) inhibits platelet aggregation in PRP is by increasing the extracellular levels of adenosine. This could be by inhibiting adenosine uptake, as has been observed for cilostazol, or by the inhibition of adenosine deaminase.

Cilostazol has recently been found to be a dual inhibitor of PDE3 and adenosine uptake.[98,99] Adenosine is an endogenous antithrombotic factor which binds to the G_s-coupled A_{2A} receptor of platelets, resulting in stimulation of adenylyl cyclase and a corresponding increase in the intracellular cAMP. Human plasma normally contains low levels of adenosine because of rapid uptake by platelets and, in particular, erythrocytes.[99] Inhibition of this uptake mechanism results in an increased extracellular level of adenosine which in turn results in the additional stimulation of the A_{2A} receptor.

Adenosine deaminase (ADA) activity, which catalyses the irreversible deamination of adenosine, has also been detected on the surface of haematopoietic cells.[100] Dipyridamole is a currently used therapeutic agent which is known to be involved with the modulation of the ADA enzyme. Flavonoids, related to the morpholinochromone structure, have also been reported as having moderate inhibition of adenosine deaminase.[100] Such inhibition may be another means of increasing of extracellular adenosine to result in an anti-aggregating effect.

Determining the mechanism by which compound (5) exerts its thrombotic action could be the subject of future studies. Certainly, the *in vivo* data suggests an important antithrombotic mechanism which might be further exploited.

5.5 Summary and Conclusions

LY294002 is being used widely in cell signalling and other biological studies, yet in reality not a great deal is known about its selectivity or the molecular basis for its interactions with target enzymes. The recently reported crystal structure determination of LY294002 in complex with the p110γ subunit of PI3-kinase showed LY294002 to have a unique mode of interaction with the kinase.[44] Modified 2-morpholinochromones such as U-86983 may demonstrate a level of kinase isoform selectivity resulting in particular effects on cellular function (perhaps resulting in an antiproliferative rather than an antiplatelet effect). Such results may present new opportunities in the design of isoform-selective PI3-kinase inhibitors.

The observation of phosphodiesterase activity in this class of compounds provokes the same questions as to the mechanism and selectivity of interaction with PDE isoforms. While U-86983 and, in particular, compound (5) did not show PDE inhibition as expected, LY294002 demonstrated inhibition of PDE2 and PDE3 at concentrations that might be relevant in some experimental settings. This is of particular importance when embarking on structural modifications of these compounds. Clearly, a broader base of structure-activity data would be required to establish an understanding of the bioactivity of these compounds. Whether the cellular activity of any of the numerous literature analogues of (5) can be attributed to PDE inhibition, as reported, remains to be seen. The use of isolated PDE assays, rather than platelet cytosol assays or ADP-induced platelet aggregations, has also demonstrated the importance of refined methods of biological evaluation.

As with PI3-kinase, further progress for phosphodiesterase inhibitors will also rely upon the pursuit of isoform selectivity. The determination of the many sub-types of PDEs, three of which are expressed in platelet (PDE2, 3A and 5) and the capacity for selective isoform inhibition as evidenced by EHNA (PDE2), cilostazol (PDE3), rolipram (PDE4) and particularly sildenafil i.e. Viagra (PDE5) demonstrates the capacity for isoform selective phosphodiesterase inhibitors as successful therapeutic agents.

Although not always as expected, 2-morpholinochromones have demonstrated antiplatelet activity through PI3-kinase inhibition, phosphodieste..se inhibition or, in the case of compound (5), through an unknown mechanism of action. The results of this study confirm that there is much to be learned from the continued investigation of the 2-morpholinochromones.

Chapter 6

EVALUATION OF 2-MORPHOLINOCHROMONE ANALOGUES AS INHIBITORS OF PLATELET PHOSPHATIDYLINOSITOL 3-KINASES AND PHOSPHODIESTERASES.

6.1 Introduction

Results from the biological evaluation of LY294002 (6), U-86983 (3) and (5) (Figure 6.1) described in Chapter 5 showed that 2-morpholinochromones demonstrate antiplatelet activity both through PI3-kinase inhibition and phosphodiesterase inhibition, but also that inhibition can occur through an undetermined mechanism.

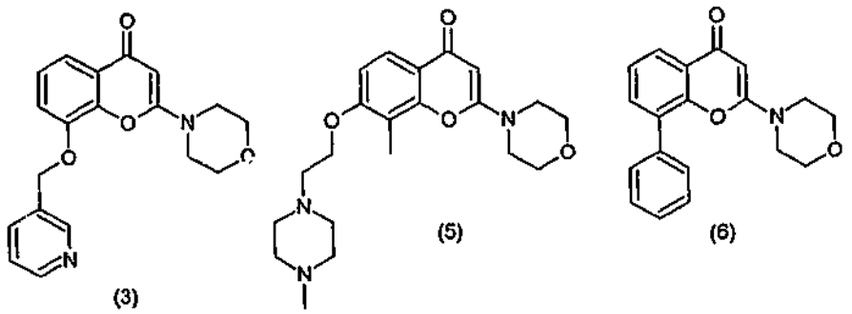


Figure 6.1 U-83983 (3), (5) and LY294002 (6).

While LY294004 (6) was confirmed as a PI3-kinase inhibitor, it was also found to be a moderate inhibitor of PDE2 and PDE3. LY294002 is a better inhibitor of PDE2 than of PDE3, indicating that it may be possible to retain and perhaps even increase selectivity between isoforms. While EHNA is selective for PDE2 over other PDE isoforms, it is also a potent adenosine deaminase inhibitor and this dominates its activity at a cellular level.[101] Currently no truly specific PDE2 inhibitors exist, which is a major reason why the physiological role of PDE2 is not yet well understood. Improvements in the potency and selectivity of 2-morpholinochromones

may lead to a PDE2 selective inhibitor, which in turn may lead to new understanding of the role of the enzyme in platelets and other cells.

U-86983 (3) was found to be a slightly better PI3-kinase inhibitor than LY294002 and was a poor inhibitor of PDE2 and PDE3. As such, it seemed likely that the antiproliferative effects reported in the literature[32] were caused by inhibition of PI3-kinase. The level of potency shown by U-86983 also indicates a structural basis for further modification. It was envisaged that such results may also present new opportunities in the design of isoform-selective kinase inhibitors.

The 2-morpholinochromone (5) was a poor inhibitor of PI3-kinase, PDE2 and PDE3. The latter result was particularly unexpected as the literature reports speculated on antiplatelet effects due to low K_m phosphodiesterase (PDE3) inhibition.[31] Whether the cellular activity of any of the numerous analogues of (5) could be attributed to PDE inhibition, as reported,[30,31] remained to be seen.

The initial results from just these three compounds suggested that there is much to be learned from the continued investigation of the 2-morpholinochromone series. By evaluating the analogues which had been prepared as described in Chapters 2 and 3, it was hoped that structural features that render compounds of this class either as PDE3, PDE2 or PI3-kinase inhibitors could be distinguished.

6.1.1 Compound classification

With numerous compounds to be evaluated, the compound library has been broken into subsets based upon the structural type. In the figures that follow, a colour coding system has been adopted which is summarised in Figure 6.2. The group of compounds in pink includes LY294002 and analogues which have been substituted on the pendant phenyl group. The compounds in yellow are 8-substituted "extended chain" analogues, some of which incorporate a heteroatom in the linking group, such as U-86983. Those in blue are 8-methyl-7-substituted analogues including compound

(5); while those in aqua blue are 7-substituted compounds. The 6,8-disubstituted compounds are in purple and the 6-substituted compounds are in lime green.

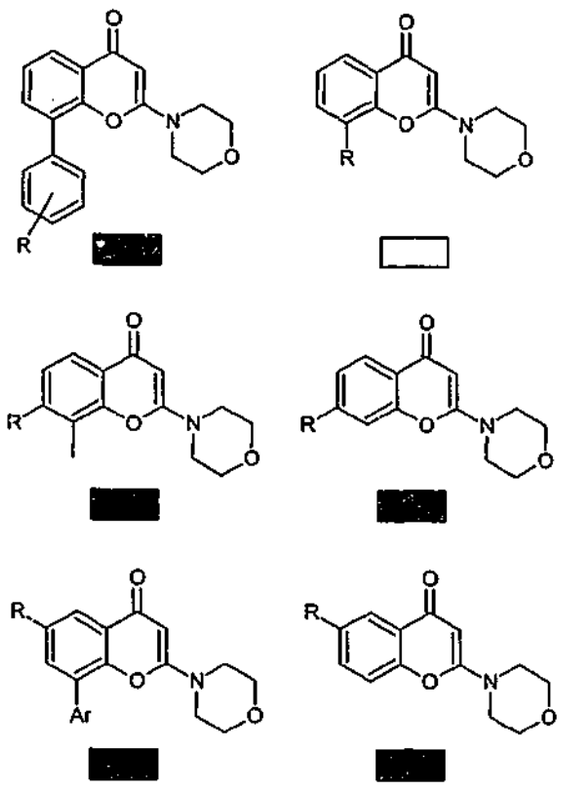


Figure 6.2 Colour coding of structural classes of 2-morpholinochromones.

6.2 PDE3 Screening

Synthesised 2-morpholinochromones were evaluated against isolated PDE3 and their activity compared to the three previously tested literature compounds. Tested compounds mainly included analogues from three different structural classes of 2-morpholinochromones; 8-substituted compounds such as LY294002 and U-86983, 8-methyl-7-substituted compounds like (5) and a third class which was 7-substituted but lacking the 8-methyl group. As when screening against the three literature compounds in the previous chapter, the known PDE3 inhibitors milrinone and cilostamide were used as positive controls while EHNA, a known PDE2 inhibitor,

was the negative control. The results from the evaluation of 21 2-morpholinochromones against PDE3 are shown in Figure 6.3.

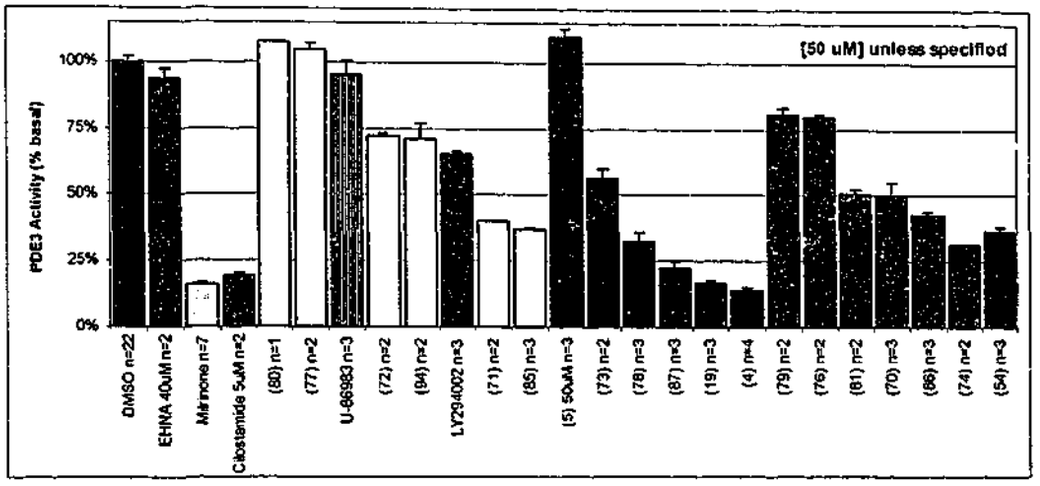


Figure 6.3 Inhibition of PDE3 Activity by 2-Morpholinochromones.

The most notable result was the activity shown by the 8-methyl-7-substituted compounds, reaffirming the original postulate that members of this class of compounds are, in general, inhibitors of PDE3. The piperazinyl substituted compound (5) would appear to be the exception.

In the structural class which was 7-substituted only, inhibition of PDE3 was generally reduced approximately 2 to 4 fold in comparison to the 8-methyl substituted counterparts. The exception to this was the 7-phenylethynyl substituted compound (74), which was nearly two fold more potent than its 8-methyl counterpart (73) (Figure 6.4).

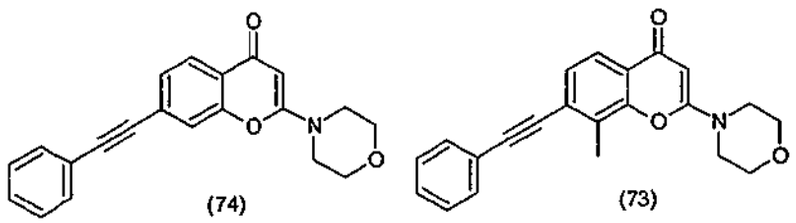


Figure 6.4 Inhibitors of PDE3.

Evaluation of the 8-substituted series of compounds, containing compounds similar to LY294002 and U-86983, showed that changes in substituent may result in marked changes in the ability to inhibit PDE3. Compounds which have a bulky substituent such as U-86983 (3), (77) and (80) (Figure 6.5) have the poorest activity with less than 10% inhibition at 50 μM.

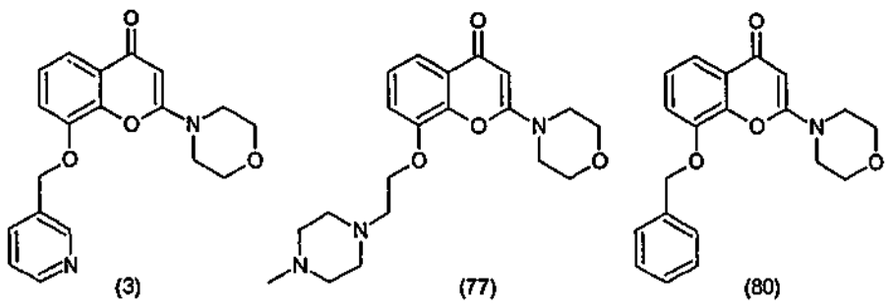


Figure 6.5 Poor inhibitors of PDE3.

Substitution of the ether oxygen in U-86983 (3) to either carbon or nitrogen (72 and 94 respectively) (Figure 6.6) increases inhibition to ca. 25% at the same concentration (50 μM). Shortening the flexible chain to the phenyl substitution of LY294002 (6) also gave a similar improvement to inhibition. Of the 8-substituted compounds evaluated, only two were likely to have IC_{50}s less than 50 μM. They were (71), with a rigid alkynyl phenyl substituent, and (85), which had phenoxy substitution (Figure 6.6).

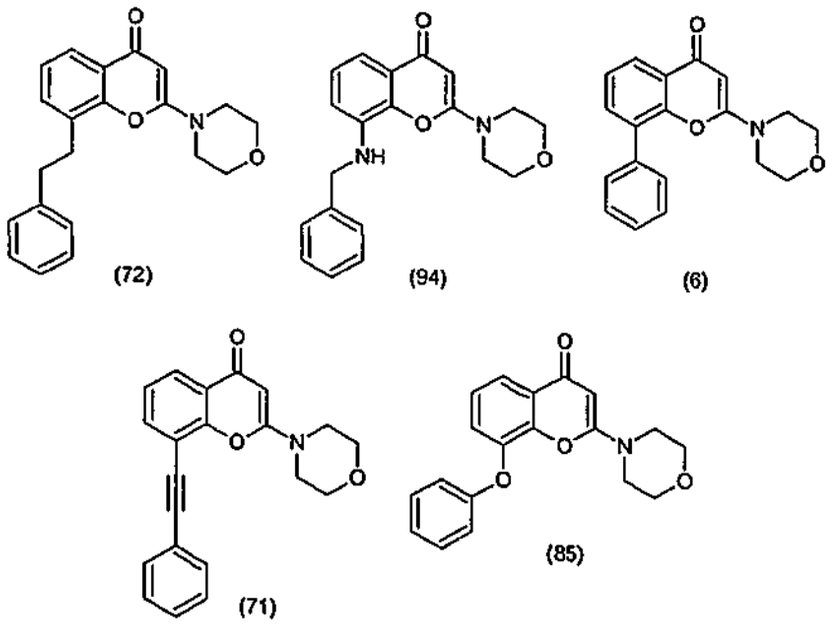


Figure 6.6 Inhibitors of PDE3.

Sufficient enzyme was available to complete a dose-response curve for the best PDE3 inhibitor from the initial screen, compound (4), along with a partial dose-response curve for the analogue (78) (Figure 6.7).

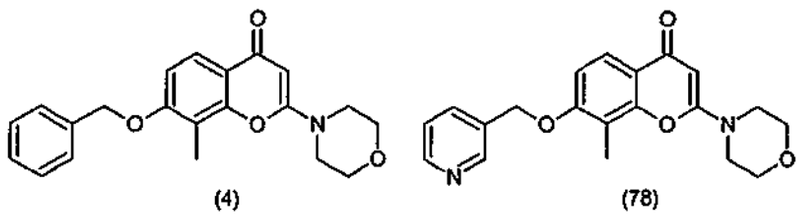


Figure 6.7 Inhibitors of PDE3 used for dose-responses.

The two compounds were plotted against dose-response curves for milrinone, cilostamide and LY294002 (Figure 6.8). Compound (4) displayed an IC_{50} of 0.6 μM, which is equivalent to milrinone. Analogue (78) showed approximately 50% inhibition at 6.25 μM, which was approximately 10 fold less potent than (4). Both these 2-morpholinochromones are much more potent than LY294002 (IC_{50} 100 μM).

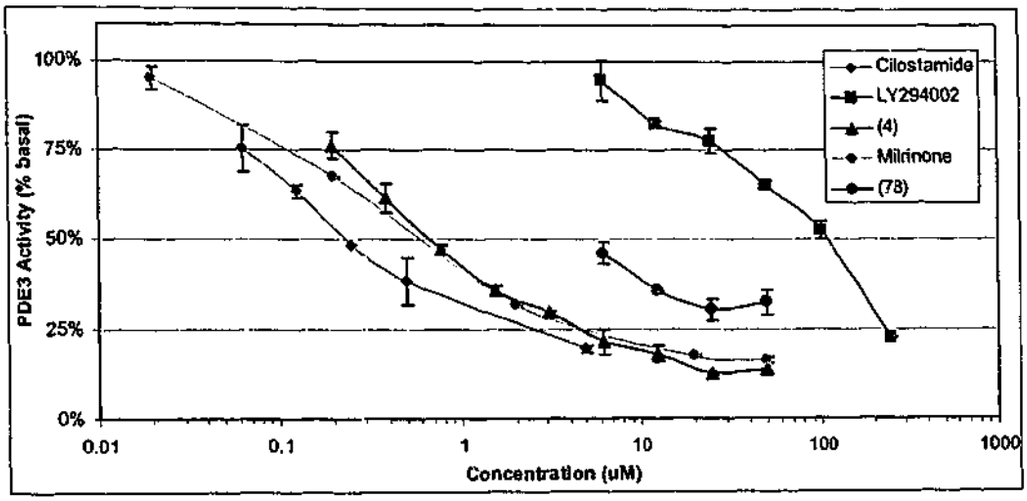


Figure 6.8 Dose-responses of PDE3 Inhibitors (4) and (78).

6.3 PDE2 Screening

For assays evaluating PDE2 activity, sufficient enzyme was available to screen all of the synthesised 2-morpholinochromones using the PDE2 inhibitor EHNA as a positive control. The results from the 2-morpholinochromone screen of 43 compounds against PDE2 are shown in Figure 6.9.

It was observed that the 8-substituted compounds were generally more efficient inhibitors of PDE2 than PDE3. Substitution on the phenyl ring of LY294002 (6), as seen in the pink set, can modify the ability of the compound to inhibit PDE2. Compounds (31) and (33) (Figure 6.10) were estimated to have improved activity relative to LY294002, while the other four compounds tested had an IC_{50} greater than 50 μM.

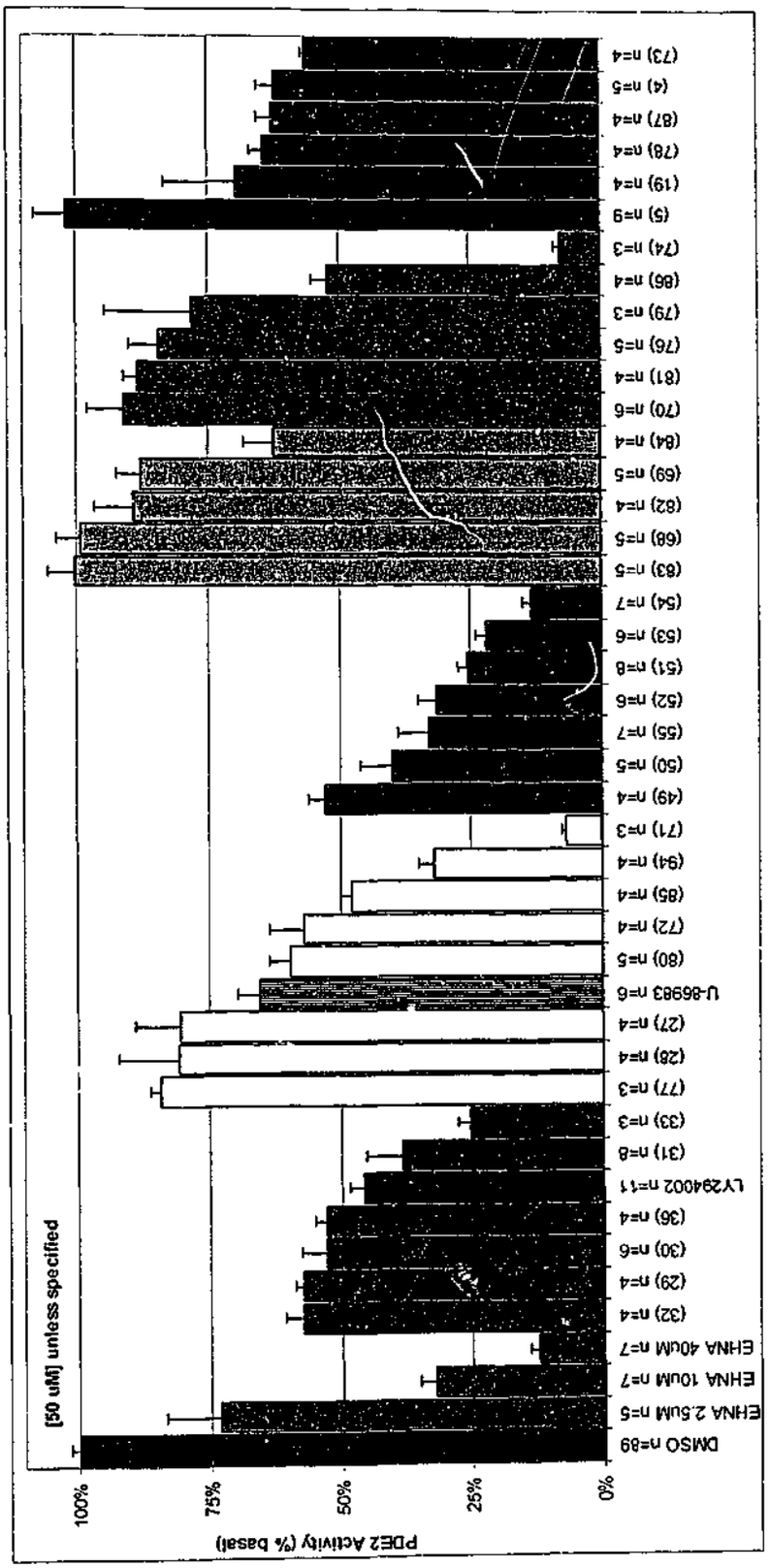


Figure 6.9 Inhibition of PDE2 Activity by 2-Morpholinochromones.

The compound set with more extended substitution in the 8-position than LY294002, as shown in yellow, also showed a mixture of inhibitory potencies. Compounds (92) and (71) were improved over LY294002, the latter was particularly potent, effectively abolishing activity at 50 μM (Figure 6.10). At that concentration, the remaining compounds show no better than 50% inhibition of the enzyme, including U-86983 (3).

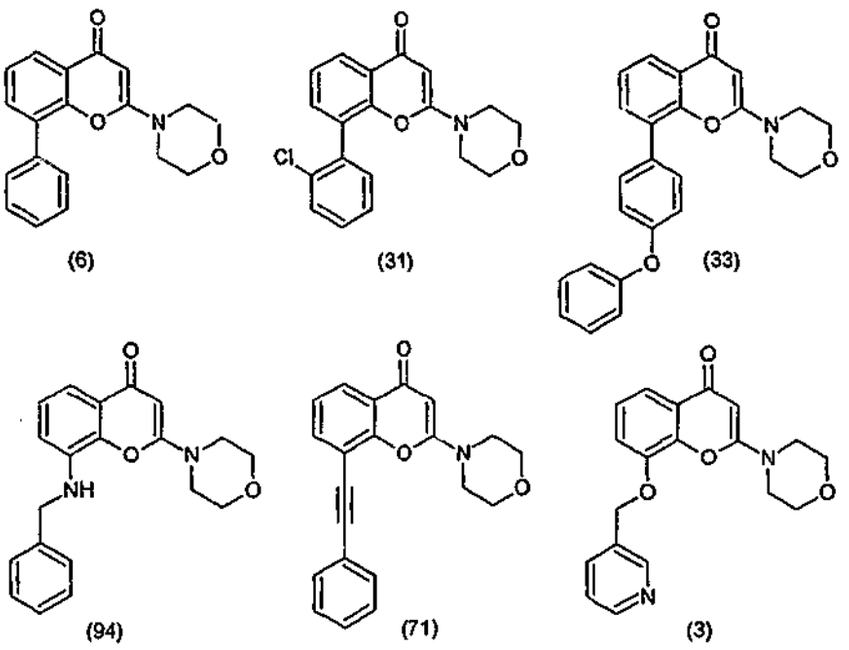


Figure 6.10 Inhibitors of PDE2.

The compound sets which showed potency against PDE3, the 8-methyl-7 substituted analogues (blue set) and to a lesser extent the 7-substituted compounds (aqua set), were not as active against PDE2. All compounds had an IC_{50} of greater than 50 μM except for the 7-alkynyl phenyl derivative (74), which like its structural isomer (71), had very good activity against PDE2 (Figure 6.11).

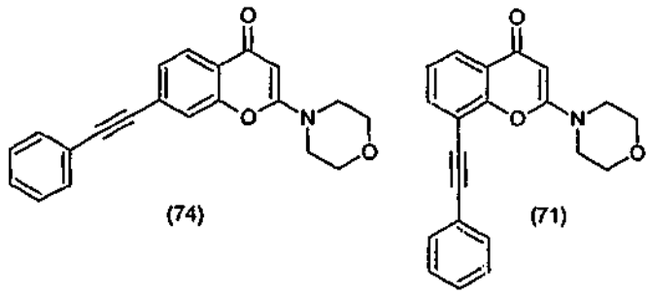


Figure 6.11 Inhibitors of PDE2.

The series of compounds with the best results against PDE2 were found to be the set where there was both 6- and 8-substitution (purple set). The majority of 6,8-disubstituted analogues had improved inhibitory activity against PDE2 compared to LY294002 (IC_{50} 35 μM). In particular, compounds (51) and (52) showed relatively high potency. It should also be noted that without the 8-aryl substitution, as seen for the lime set of 6-substituted compounds, inhibition of PDE2 activity was lost. The activity of compound (51), together with the relative potency of the 8-(3-chlorophenyl)-substituted compound (31), prompted the synthesis and evaluation of analogues (53-55) (Figure 6.12).

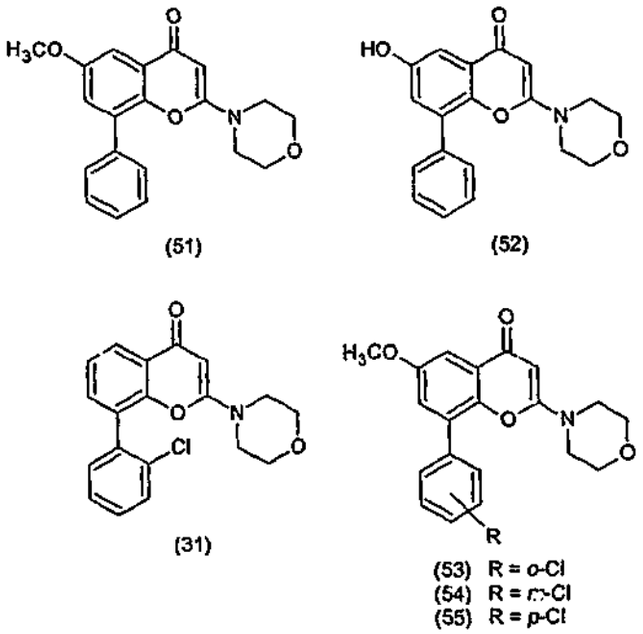


Figure 6.12 Inhibitors of PDE2.

Dose-response curves were completed for a number of the compounds in the purple set (51-55) (Figure 6.12) and also the 8-alkynyl phenyl (71) (Figure 6.11). These dose-response curves were plotted against EHNA, LY294002 and U-86983, with the results shown in Figure 6.13.

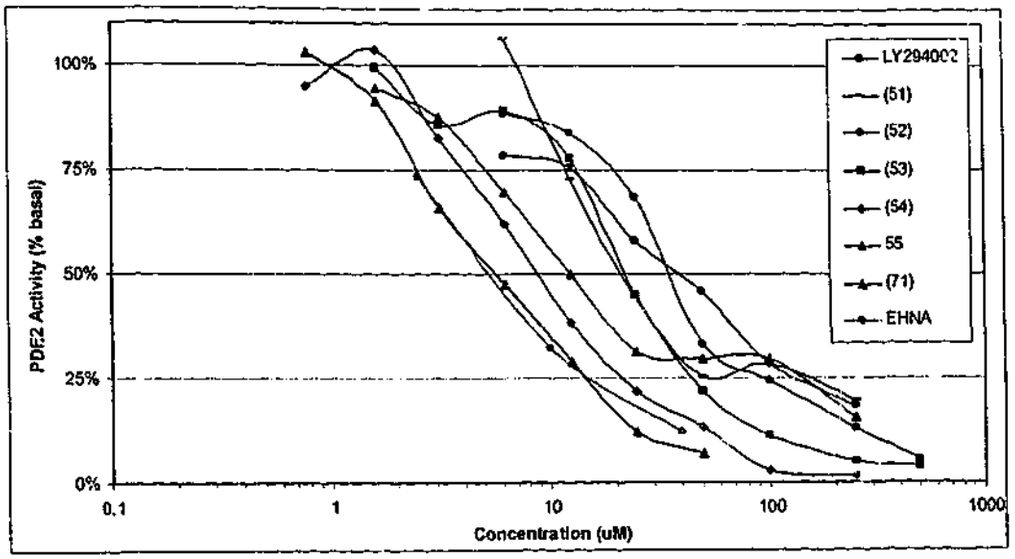


Figure 6.13 Dose-responses of PDE2 Inhibitors.

All of the compounds for which a dose-response was obtained had an IC_{50} less than LY294002 (IC_{50} 35 μM). The 8-alkynyl phenyl compound (71) was found to have an IC_{50} approximate to EHNA of 4 μM, and based upon the initial screen it was expected that the 7-alkyl phenyl compound (74) would also be in this range. The set of compounds which was particularly interesting, however, was the 6, 8-disubstituted compounds which were all improved over LY294002. Improvement in activity was obtained by having 6-methoxy (51) rather than 6-hydroxy (52), when both were substituted by an 8-phenyl. Of the 6-methoxy substituted compounds, the rank order of potency against PDE2 was *ortho*-chlorophenyl (53) > phenyl (51) > *para*-chlorophenyl (55) > *meta*-chlorophenyl (54).

6.4 Phosphatidylinositol 3-Kinase Screening

Test compounds were evaluated by scientists at Kinacia Pty Ltd over the full period of candidature. As described in the previous chapter, in the screening program at Kinacia compounds are typically evaluated as inhibitors of PI3-kinase activity immunoprecipitated from platelets using an antibody to the regulatory p85 subunit. Unfortunately, for the most part insufficient experiments could be done to gather robust, statistically significant data. However, compounds screened through these

methods were evaluated qualitatively, and those which consistently inhibited the enzyme with improved potency over LY294002, used as a positive control in all assays, were then subjected to more detailed evaluation. Representative results are shown for the 2-morpholinochromones tested in this study at or near the concentration at which 50% inhibition was demonstrated and are reported in Figure 6.14. Compounds with PI3-kinase activity above 50% of the control are estimated to have an IC_{50} greater than the given concentration while compounds which result in PI3-kinase activity lower than 50% of the control are estimated to have an IC_{50} lower than the concentration specified.

LY294002 (6), which was used as a control, typically had an IC_{50} between 1 and 3 μM. Substitution on the phenyl group (pink set) mostly resulted in increased inhibition of the enzyme (29, 31-33). Substitution at the C-6 position of LY294002 (purple set) generally had little effect. Removal of the pendant phenyl ring to give only 6-substituted compounds (lime green set) resulted in a loss of inhibition.

However, three 8-substituted compounds emerged as showing potency considerably higher than LY294002. These were analogues of U-86983 (3) (yellow set), namely (80), (85) and (92) (Figure 6.15). These compounds indicated an $IC_{50} \leq 0.1$ μM in the initial screen and were subjected to scrutiny across a narrower concentration range. The IC_{50}s of compounds (80) and (85) were confirmed as approximately 100 nM, while compound (92) proved to be the most potent inhibitor of PI3-kinase from this series of compounds, with an IC_{50} between 10 – 50 nM (Figure 6.16).

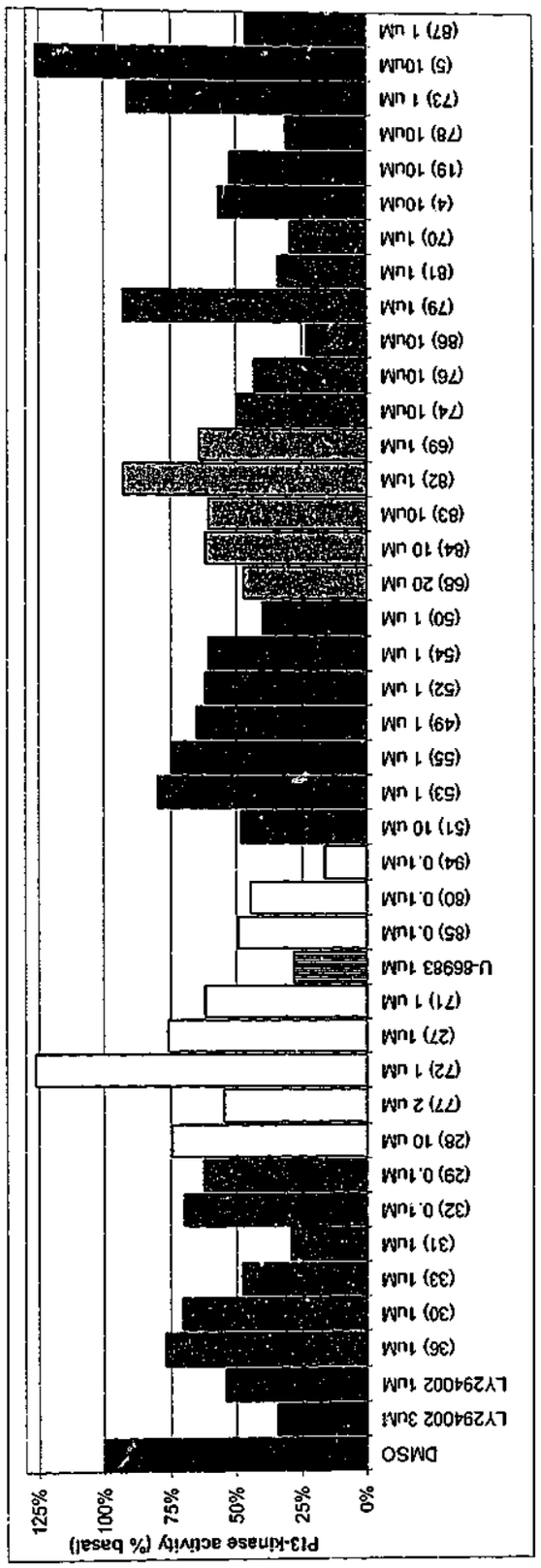


Figure 6.14 Inhibition of PI3-kinase by 2-Morpholinochromones.

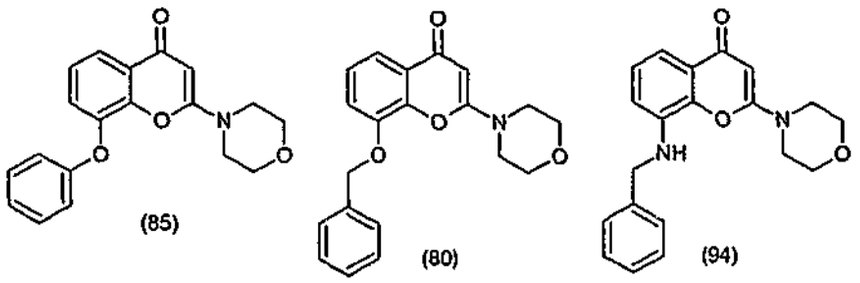


Figure 6.15 Inhibitors of PI3-kinase.

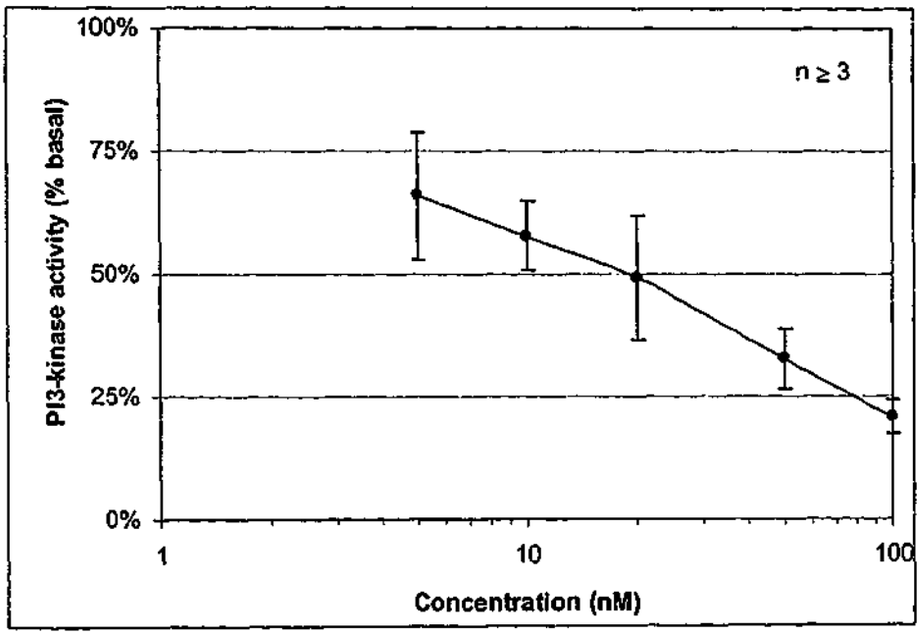


Figure 6.16 Dose-Response Curve for PI3-Kinase Inhibitor (92).

Of the remaining 7-substituted compounds (blue and aqua sets), most were poor inhibitors of PI3-kinase with only compounds (70), (81) and (87) approximating the activity of LY294002.

6.5 Studies of Platelet Aggregations

The determination that compound (5) was inactive against PDE3 had cast doubt on the actual relevance of PDE3 inhibition of this class of compounds to the observed antiplatelet activity. It was very important to determine if the original assertion,

based on the activity of other compounds could be validated. Amongst the compounds described by Upjohn and synthesised for this study, the most potent PDE3 inhibitor tested was compound (4) while compound (78) was of intermediate potency.

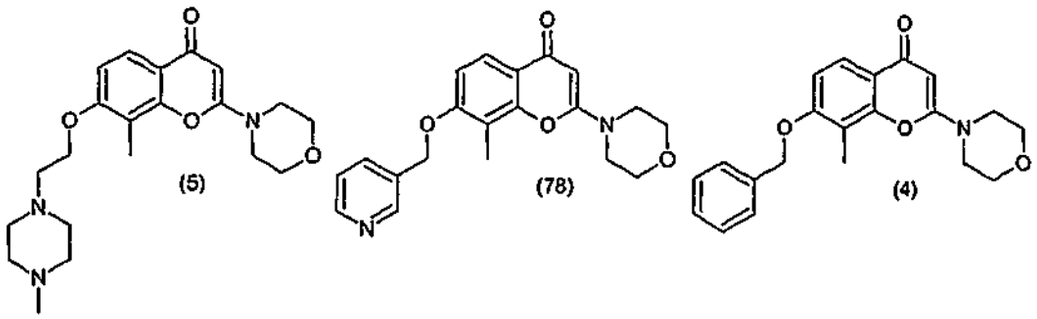


Figure 6.17 Compounds (5), (78) and (4).

Platelet aggregation studies were performed, to determine whether these compounds behaved as PDE3 inhibitors in platelets and also to compare the effects found in washed platelets with those reported by Morris *et al*[31] for inhibition of ADP-induced aggregation from platelet rich plasma.

In ADP-induced aggregation of PRP, using cilostamide as a control, strong inhibition was observed for compound (5) at 20 μM with a similar result for (78) at 25 μM whereas compound (4) required 50 μM to give a comparable level of inhibition (Figure 6.18). This rank order of potency was similar to that reported by Morris *et al.*[31]

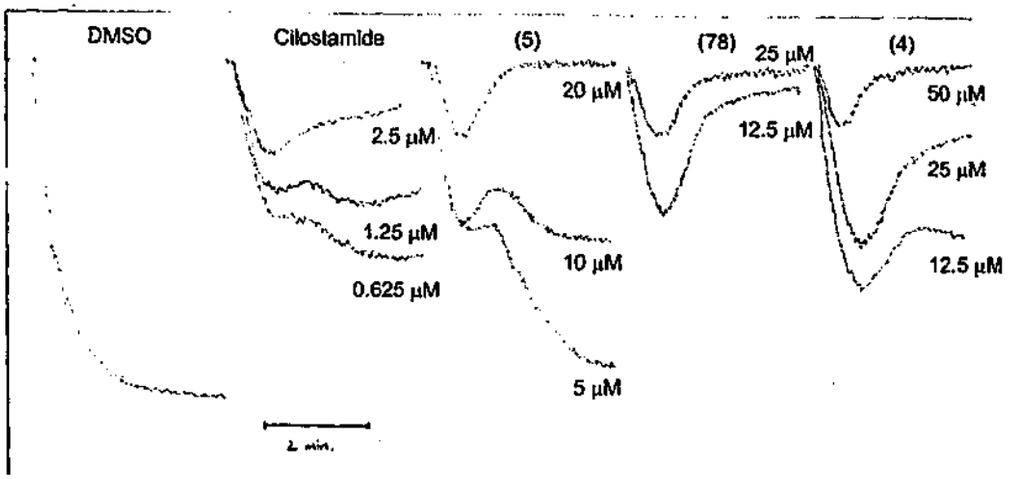


Figure 6.18 Effect of inhibitors on PRP aggregation induced by ADP.

Compounds (4) and (5) were tested for inhibition of PAR4AP-induced aggregation using a washed platelet suspension (Figure 6.19). As shown previously, compound (5) showed very poor inhibition of aggregation. Compound (4) in contrast, showed strong inhibition of platelet aggregation at 25 µM (Figure 6.19).

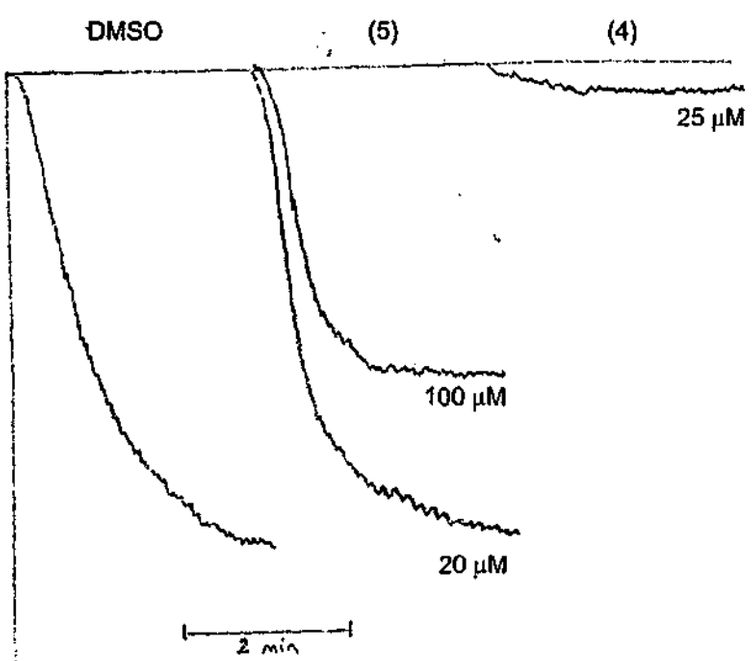


Figure 6.19 Effect of inhibitors on WP aggregation induced by PAR4AP.

The relative potency of these compounds is in contrast to that found in aggregations using PRP (Figure 6.19) and by Morris *et al*[31], but correlated well to the activity found against PDE3 (Table 6.1). As such, compound (4) shows anti-PDE3 activity comparable to milrinone which translates into comparable potency as an inhibitor of platelet aggregation.

In further experiments, Dr. Philip Thompson compared compound (78) and (4) against TRAP-induced washed platelet aggregation.[92] In these assays, compound (4) was approximately five fold more potent than (78), with IC_{50} values of approximately 5 μM and 25 μM respectively (Table 6.1).

Table 6.1 Summary of Compounds (5), (78) and (4).

Compound	ADP-induced aggregation (PRP) Reported IC_{50}[31]	Inhibition of PDE3# Approx. IC_{50}	TRAP-induced aggregation (WP) Approx. IC_{50}
(5)	0.85± 0.3 μM	>50 μM	> 50 μM
(78)	4±2 μM	6.25 μM	25 μM
(4)	40± 24 μM	0.6 μM	5 μM

#PDE3 inhibition (Figures 6.3 and 6.8)

In summary, the platelet aggregation studies have confirmed the antiplatelet activity of 2-morpholinochromones and demonstrated a capacity to distinguish between different mechanistic pathways by the use of different platelet agonists and/or platelet preparations.

Specifically, it was established that compounds (5), (78) and (4) inhibited platelet aggregation in washed platelets consistent with anti-PDE activity. In PRP, however, enhancement to the potency of (5) and (78) indicates an alternate effect. As compound (4) represented the initial lead in the Upjohn program, it appears that the assay methodology adopted has ultimately led away from the optimisation of the inhibition of phosphodiesterase activity, such that PDE3 activity in this class remains unexplored.

6.6 Summary and Conclusions

Screening against the isolated enzymes of PDE2, PDE3 and PI3-kinase, as well as studies of platelet aggregation using selected compounds, has provided a large amount of valuable data which makes a significant contribution to the understanding of the activity of 2-morpholinochromones.

The cellular activity of the numerous analogues of (5) was found to be attributed to PDE3 inhibition, as reported. It would appear that compound (5) was the exception, with a mechanism of action as yet unknown but which could provide an important lead to drug development once determined. With PDE3 inhibition equivalent to milrinone, compound (4) was found to be the lead 2-morpholinochromone of the analogues screened.

Evaluation of 2-morpholinochromones against PDE2 was also performed and yielded two compounds, (54) and (71), of equivalent potency to the non-selective PDE2 inhibitor EHNA. Further optimisation of PDE2 inhibition may yield potent and selective compounds which complement the use of EHNA to give insight into the role of PDE2 in cells, including platelets, which is poorly understood at this time.

The 2-morpholinochromones also contained some highly potent inhibitors of platelet PI3-kinase. Increases in potency over that described for LY294002 of up to 100 fold were obtained by these compounds, with the 8-benzylamino-2-morpholinochromone (92) proving most potent. This proves to be a valuable addition to the development of structure-activity relationships of this important class of enzymes.

Modified 2-morpholinochromones which demonstrate a level of PI3-kinase or phosphodiesterase isoform selectivity have been shown to have particular effects on cellular function in this chapter. Such compounds will allow better understanding of the mechanism of action of these enzymes in platelets and other cells, give insight into their role in disease states and new opportunities for new drug treatments may be

presented as a result. Improving isoform selectivity will be very important to the development of these compounds as PI3-kinase and phosphodiesterase inhibitors.

Chapter 7
CONCLUSIONS AND FUTURE DIRECTIONS

The studies which have been detailed in this thesis have significantly advanced the understanding of the chemistry and biology of 2-morpholinochromones, identifying biochemical and mechanistic rationale for the cellular activity of established compounds, and identifying new opportunities for drug development in a range of areas.

New routes for the synthesis of 2-morpholinochromone analogues have been developed which are concise and adaptable. The methodology described in Chapters 2 and 3 exemplifies the synthesis and utility of trifluoromethanesulfonate, boronate, halide and hydroxyl intermediates which can be derivatised using a variety of transformations to form an extensive and diverse library of 2-morpholinochromones. The Suzuki coupling has been shown to be a very useful reaction for derivatisation, particularly for LY294002 analogues. This was completed using commercially available boronic acids with both trifluromethanesulfonate and halide intermediates. The chemistry of this type of reaction was also able to be extended by using the 2-morpholinochromone as the boronate partner, to facilitate use of the extensive range of halides available for reaction. Numerous analogues were also derived from O-alkylation of the hydroxyl intermediate while other reactions used for derivatisation of the key intermediates included the Sonogashira reaction, copper catalysed O-arylation and amination. The 36 compounds which have been generated form a diverse library which is representative of the vast number of 2-morpholinochromone analogues readily available by the synthetic methodology described. The syntheses that have been developed could also be generalised to the synthesis of many other compounds, not just 2-morpholinochromones.

In Chapter 4, the development of biochemical assays for screening compounds against the phosphodiesterases PDE2 and PDE3 was described. While such assays are reported in existing literature, the adaptation of methodologies was critical to

allowing characterisation of the numerous compounds in this study in a robust and cost-effective way. The major advance of this work was the development of RP-HPLC methodologies for direct detection of AMP at sub-micromolar conditions. This allowed the technique to be used to reliably evaluate inhibition of PDE3 activity, which had significant advantages over traditional methods that rely upon radiolabelling or ELISA-based detection. In particular, this method allowed real time assessment of the activity of the enzyme preparation, which would be very useful in other high throughput screening scenarios. Other parameters optimised included the method for isolation of phosphodiesterases, including the separation and characterisation of the phosphodiesterase isoforms from platelets. Methodology for the high throughput screening of compounds against PDE2 and PDE3 using RP-HPLC was further developed to allow a large number of compounds to be screened using an automated analysis process, saving considerable time and resources.

The large amount of biological assay data presented in Chapter 5 and, in particular, Chapter 6 has been generated to address several issues relating to the activity of 2-morpholinochromones. This has included screening against the isolated enzymes of PDE2, PDE3 and PI3-kinase, as well as studies of platelet aggregation using compounds of interest. Importantly, the results of these assays have provided valuable information in every case.

Firstly, the results provide an insight into the reported antiplatelet activity of these compounds. The 8-methyl 7-substituted compounds were optimal type of structure of those tested for inhibiting PDE3. Of these, compound (4) was shown to have an IC_{50} of ca. 0.6 μM, equivalent to the known PDE3 inhibitor milrinone. This is not the first time such activity has been reported, but it does confirm that proposed activity in a robust assay using a purified enzyme preparation. The reportedly more potent antiplatelet agent compound (5), showed no inhibition of PDE3 at 50 μM. The antiplatelet effects, profound in platelet rich plasma, were abolished when tested in washed platelet suspensions, suggestive of an antiplatelet effect dependent upon plasma factors. This represents another important finding of this work. The compound itself has been shown by others to be an effective antithrombotic in canine

models of thrombosis (albeit with moderate haemodynamic side effects).[38] Determination of the true mechanism by which this compound acts, whether by a pathway linked to adenosine or some other mechanism, could be an important lead to the development of a new therapeutic strategy for the treatment of thrombosis.

Other structural parameters for the expression of PDE3 inhibition have been determined by this first general evaluation of PDE3 activity for this class. The results from the PDE3 screening show that distinct structural motifs favour PDE3 inhibition. Removal of the 8-methyl, for example, usually resulted in a decreased activity against PDE3. Replacement of the benzyloxy substitution by a heterocyclic group was shown to be deleterious to PDE3 inhibition. The optimisation of PDE3 activity and selectivity for this class of compounds has never been attempted but such a process can be readily envisaged, adding a new class of compounds, complementary to the use of milrinone and cilostazol, with which to study the physiology of this enzyme.

Secondly, evaluation of this class of compounds against PDE2 has been performed. The role of PDE2 in platelets and cells in general is poorly understood, not least because no potent selective inhibitors have been described as yet. Such reagents will be highly valuable in this line of research. LY294002 was shown for the first time to have significant inhibitory potency against PDE2. Modification of LY294002 yielded PDE2 inhibitors of equivalent potency to the non-selective PDE2 inhibitor EHNA. The structure type which demonstrated consistent PDE2 inhibition was the 6-methoxy-8-aryl substituted compounds, which lost activity when either the 6- or 8-substitution was removed. Compound (54) was found to be the best PDE2 inhibitor of this class with an IC_{50} of 9 μM. Another compound (71) was in fact the most potent (IC_{50} of 5 μM) of all compounds tested and was found to be equipotent to the reference PDE2 inhibitor EHNA. Compound (71) also showed significant PDE3 inhibition. In general though, PDE2 inhibition was found to be somewhat separated from PDE3 inhibition, and modifications that enhance PDE2 activity, had a deleterious effect on PI3-kinase inhibition. The potential for further elaboration of

these compounds, with which to optimise PDE2 inhibition, warrants detailed investigation.

Thirdly, evaluation of the 2-morpholinochromones against PI3-kinase has similarly been performed, adding to the otherwise shallow pool of structure-activity data for 2-morpholinochromones. Modification of LY294002 has yielded some highly potent inhibitors of PI3-kinase. While 8-substituted compounds did induce some inhibition of PDE isoforms, they were most notably found to be the class of compounds which were the best inhibitors of PI3-kinase. Increases in potency over that described for LY294002 of nearly 100 fold were obtained by modification of that substitution. In particular, the reported antiproliferative agent U-86983, was found to be an inhibitor of PI3-kinase, more potent than LY294002 in isolated enzyme assays. Interestingly, the antiplatelet activity of this compound was found to be less than that of LY294002, suggesting differences in the expression of activity in the platelet. The antiproliferative activity on the other hand matches that displayed by LY294002 closely and, given the absence of anti-PDE activity, strongly suggests that PI3-kinase inhibition is the mechanism by which this drug candidate exerts its effects. The incorporation of a benzylamino group in the 8-position yielded the highly potent compound (92). Further studies of compound (92) as an inhibitor of platelet function in other PI3-kinase related research are warranted. Such studies would include optimisation of the chemical synthesis which was a significant challenge. Kinacia[102] and others[103-105] have recently described an array of compounds that potently inhibit various PI3-kinase isoforms. Compounds such as (92) should prove valuable additions to the development of structure-activity relationships of this important class of enzymes.

Finally, the results of this thesis offer an opportunity for improving the understanding of the mechanism of action of a number of enzymes in platelets, including their role in platelet aggregation. It is postulated that 2-morpholinochromones inhibit platelet aggregation by multiple mechanisms, including by PDE3 inhibition, PI3-kinase inhibition and a third uncharacterised mechanism of action. With improved understanding of these agents comes the opportunity to develop new antiplatelet

drugs. Potent 2-morpholinochromones with a suitable enzyme inhibition profile may yet prove to be effective antithrombotic drugs with reduced side effects. A suitable inhibition profile may include selectivity towards a particular enzyme or isoform, or may have a particular ratio of activities towards one or more targets. This thesis represents a significant advance in the development of such therapies for the prevention or treatment of thrombosis.

Chapter 8
EXPERIMENTAL

8.1 General

Melting points were recorded using a Stuart Scientific Melting Point Apparatus SMP3 and are uncorrected. Proton (^{1}H) and carbon (^{13}C) NMR spectra were recorded on Bruker Avance DRX400 spectrometer (operating at 400 MHz for proton and 100 MHz for carbon) or a Bruker Avance DPX300 spectrometer (operating at 300 MHz for proton and 75 MHz for carbon). All such spectra were recorded in deuteriochloroform ($CDCl_3$) or dimethyl-d_6 sulphoxide ([D_6]DMSO) solution at 30°C. Low resolution electrospray ionisation mass spectra (LRESI-MS) were performed on a Waters Micromass ZQ4000 mass spectrometer employing a scan range of 0-1000 m/z and a cone voltage of 20 V. High resolution mass spectra (HR-MS) were recorded on a Bruker BioApex 47e FTMS fitted with an Analytica Electrospray Source. The samples were measured at a capillary voltage of 75 V. Reverse Phase High Performance Liquid Chromatography (RP-HPLC) was carried out on a Waters Associates Instrument (Milford, MA, USA) comprising a Model 600E system controller and a Model 486 detector. Samples were applied for RP-HPLC according to the method of Thompson and Hearn,[106] and eluted through a Phenomonex Luna C_8 column (20 x 4.5 cm I.D) with detection at 254 nm.

8.2 General Methods

Preparation of Salicylacetamides: Method A

As described by Morris *et al*,[50] to a solution of lithium diisopropylamide [formed *in situ* by treating diisopropylamine (3.2 eq) in tetrahydrofuran with 1.6 M *n*-butyl lithium (3.2 eq)]at 0°C under nitrogen was added 4-acetylmorpholine (1.6 eq) and the solution allowed to stir at 0°C for 1 h. The solution at 0°C was then treated drop-wise with the salicylate ester (1 eq) in tetrahydrofuran. The reaction was stirred at room temperature overnight. The mixture was neutralised with 10% aqueous HCl

and extracted three times with dichloromethane. The combined organic fractions were dried (Na_2SO_4) and the solvent removed to yield the crude product.

Preparation of Salicylacetamides: Method B

To a solution of the 2-hydroxyacetophenone (1 eq) in tetrahydrofuran was added lithium bis(trimethylsilyl)amide (3 eq) at –78°C under nitrogen. The reaction was allowed to come to 0°C and maintained there for 1 h. It was then cooled to –78°C again and 4-morpholinecarbonyl chloride (1.1 eq) was added. The mixture was stirred at room temperature overnight and then poured over ice. The mixture was acidified to pH 3 with 1 M HCl then extracted three times with dichloromethane. The combined extracts were dried (Na_2SO_4), and the solvent was removed *in vacuo* to obtain the crude product.

Preparation of Morpholinochromones: Method C

As described by Morris *et al*,[50] to a solution of the salicylacetamide in dichloromethane (0.2 M) was added trifluoromethanesulfonic anhydride (2–3 eq). After stirring at room temperature overnight, the solvent was evaporated and the residue treated with methanol (10 mL) and stirred at room temperature for 4 h. The solvent was evaporated, and the residue was diluted with half-saturated sodium hydrogen carbonate ($NaHCO_3$) and extracted three times with dichloromethane. The combined organic fractions were washed with saturated sodium chloride (NaCl), dried (Na_2SO_4) and the solvent removed to yield the crude product.

Suzuki Coupling: Method D

2-Morpholin-4-yl-4-oxo-*4H*-chromen-8-yl trifluoromethanesulfonate (1 eq), phenylboronic acid (2 eq), potassium carbonate (4 eq) and butylammonium bromide (2 eq) were combined. Degassed water was added and nitrogen was bubbled through the mixture while quickly adding palladium diacetate (4 mol%). The mixture was then heated at 70°C for 4 h under nitrogen. Upon cooling to room temperature the mixture was extracted three times with ethyl acetate, dried (Na_2SO_4), and the solvent removed *in vacuo* to give the crude product.

Suzuki Coupling: Method E

Nitrogen was bubbled through a mixture of 2-morpholin-4-yl-4-oxo-*4H*-chromen-8-yl trifluoromethanesulfonate (1 eq), lithium chloride (3 eq), 2M aqueous sodium carbonate (2.5 eq) and toluene, then tetrakis(triphenylphosphine)-palladium(0) (5 mol%) was added. The arylboronic acid (1.1 eq) was dissolved in ethanol (1 mL) and injected into reaction flask. The mixture was stirred at 90°C under nitrogen overnight then cooled, filtered and the solid washed with chloroform. The organic filtrate was washed with water, dried (Na_2SO_4) and concentrated *in vacuo* to give the crude compound which was chromatographed with ethyl acetate to obtain the desired product.

Suzuki Coupling: Method F

Nitrogen was bubbled through potassium carbonate (2.5 eq), 2-morpholin-4-yl-4-oxo-*4H*-chromen-8-yl trifluoromethanesulfonate (1 eq) and acetonitrile (50 mL/g). This was followed by the addition of the arylboronic acid (1.2 eq) and palladium diacetate (10 mol%). After heating overnight at 80°C, the mixture was cooled, filtered and the solid washed with acetonitrile (10 mL). The combined washings were evaporated *in vacuo* to give a crude product which was chromatographed using ethyl acetate as eluent to give the purified product.

Suzuki Coupling: Method G

To a solution of 2-morpholin-4-yl-8-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-*4H*-chromen-4-one (1 eq) in dioxane (60 mL/g) was added potassium phosphate (3 eq), dichloro[1,1'-bis(diphenylphosphino)ferrocene]palladium (II) (3 mol%) and the aryl halide (1 eq). The mixture was heated at 80°C under nitrogen for 48 h. The mixture was then cooled to room temperature, filtered and the solid washed with dioxane. The filtrate was concentrated *in vacuo* to yield the crude product which was purified by RP-HPLC.

Suzuki Coupling: Method H

To the 8-bromo-6-substituted-2-morpholin-4-yl-*4H*-chromen-4-one under nitrogen was added potassium phosphate (3–6 eq), phenylboronic acid (1.1–2 eq), dichloro[1,1'-bis(diphenylphosphino)ferrocene]palladium (II) (0.03–0.07 eq) and dioxane (20 mL/mmol). The mixture was refluxed at 85°C over 48 h, cooled to room temperature and filtered. The solvent was removed *in vacuo* and the residue was purified by RP-HPLC unless indicated otherwise.

8.3 Syntheses

Methyl 3-phenylsalicylate (14)

To a stirred suspension of 3-phenylsalicylic acid (13) (3.36 g, 15 mmol) in dry methanol (50 mL) was added drop-wise concentrated sulfuric acid (4.38 g, 43 mmol) over 10 min. The mixture was heated at 80°C for 70 h and the cooled solution was evaporated *in vacuo* to near dryness. The residue was treated with water (60 mL) and extracted with dichloromethane. The combined organic fractions were washed with saturated aqueous $NaHCO_3$ (2 x 30 mL) and water (30 mL), then dried (Na_2SO_4). Removal of the solvent yielded a pale yellow oil (2.65 g, 74%) that crystallised upon standing and was recrystallised from methanol. M.p. 41-42°C (literature m.p. 54-56°C[107]). δ_H (300 MHz; $CDCl_3$; Me_4Si) 11.34 (1 H, s, OH), 7.88 (1 H, d, *J* 7.8, ArH), 7.62-7.36 (6 H, complex m, ArH), 6.98 (1, dd, *J* 8.0, ArH), 3.99 (3 H, s, $COCH_3$). Mass spectrum (ESI) *m/z* 229 $[M + H]^{+\bullet}$).

(4-Morpholinyl)-3-[3'-(2'-hydroxybiphenyl)]-3-oxo-propanamide (15)

Methyl 3-phenylsalicylate (14) (1.48 g, 6.46 mmol) was reacted according to Method A to yield an oil which was purified by chromatography eluting with 0–10% methanol in dichloromethane to yield a yellow oil (2.2 g) contaminated by a small amount of 4-acetylmorpholine. δ_H (300 MHz; $CDCl_3$; Me_4Si) 7.86 (1 H, d, *J* 8.1, ArH), 7.58-7.35 (6 H, complex m, ArH), 7.01 (1 H, dd, *J* 7.8, ArH), 4.17 (2 H, s, CH_2), 3.54-3.43 (8 H, complex m, CH_2CH_2). Mass spectrum (ESI) *m/z* 326 $[M + H]^{+\bullet}$).

2-Morpholin-4-yl-8-phenyl-4H-chromen-4-one (6)

Treatment of (4-morpholinyl)-3-[3'-(2'-hydroxybiphenyl)]-3-oxo-propanamide (15) (2.1 g, 6.46 mmol) with trifluoromethanesulfonic anhydride (2.31 mL, 13.3 mmol) according to Method C yielded a brown solid. The product was recrystallised from ethyl acetate as colorless crystals (0.895g, 44%). M.p. 184°C (literature m.p. 185-187°C[33]). The product co-eluted on thin layer chromatography (t.l.c.) with an authentic sample of LY294002 (Calbiochem). (Found: $[M + H]^{+\bullet}$, 308.1286. $C_{19}H_{17}NO_3$ requires $[M + H]^{+\bullet}$, 308.1281). δ_H (400 MHz; $CDCl_3$; Me_4Si) 8.16 (1 H, dd, *J* 8.0 and 1.6, ArH), 7.54 (1 H, dd, *J* 7.6 and 1.2, ArH), 7.49-7.39 (6 H, m, ArH), 5.50 (1 H, s, CH), 3.71 (4 H, t, *J* 4.8, CH_2CH_2), 3.33 (4 H, t, *J* 4.8Hz, CH_2CH_2); δ_C (100 MHz; $CDCl_3$; Me_4Si) 177.2, 162.6, 150.6, 136.4, 133.5, 130.4, 129.4, 128.4, 128.0, 125.1, 124.8, 123.4, 87.1, 65.9, 44.8. Mass spectrum (ESI) *m/z* 308 $[M + H]^{+\bullet}$.

Methyl 2-hydroxy-3-{[(trifluoromethyl)sulfonyl]oxy}benzoate (22)

To a solution of 2,3-dihydroxybenzoic acid (20) (4.0 g, 26.0 mmol) in methanol (200 mL) was added concentrated sulfuric acid (4.0 g). The reaction was stirred at reflux overnight, then cooled and the solvent was removed *in vacuo*. A precipitate was obtained upon the addition of water (300 mL), this was filtered and dried by vacuum to obtain methyl 2,3-dihydroxybenzoate (21) (3.81 g, 87%) as a mauve solid. M.p. 79-80°C (literature m.p. 80-82°C[108]). δ_H (400 MHz; $CDCl_3$; Me_4Si) 10.88 (1 H, s, OH), 7.33 (1 H, d, *J* 8.0, ArH), 7.08 (1 H, d, *J* 7.6, ArH), 6.77 (1 H, t, *J* 8.0, ArH), 3.92 (3 H, s, $COCH_3$). Mass spectrum (ESI) *m/z* 169 $[M + H]^{+\bullet}$).

To an ice cold solution of methyl 2,3-dihydroxybenzoate (21) (4.9 g, 29.1 mmol) in dichloromethane (125 mL) under nitrogen was added pyridine (4.7 mL, 58.3 mmol) and 4-(dimethylamino)pyridine (0.356 g, 2.91 mmol). Trifluoromethanesulfonic anhydride (5.39 mL, 32.0 mmol) was then added drop-wise by syringe, and the mixture was allowed to stir at room temperature for 48 h. The solution was washed 1 M HCl (2 x 100 mL), then dried (Na_2SO_4) and concentrated *in vacuo*. The crude product was purified by recrystallisation from ethyl acetate to obtain the product as white crystals (6.58 g, 76%). M.p. 93-94°C. (Found: $[M + H]^{+\bullet}$, 301.1435. $C_9H_7F_3O_6S$ requires $[M + H]^{+\bullet}$, 300.9993). δ_H (400 MHz; $CDCl_3$; Me_4Si) 11.20 (1 H,

s, OH), 7.83 (1 H, d, *J* 8.0, ArH), 7.41 (1 H, d, *J* 8.0, ArH), 6.91 (1, dd, *J* 8.0, ArH), 3.99 (3 H, s, $COCH_3$). Mass spectrum (ESI) m/z 301 $[M + H]^{+\bullet}$.

2-Hydroxy-3-(3-morpholin-4-yl-3-oxopropanoyl)phenyl trifluoromethanesulfonate (23)

Treatment of methyl 2-hydroxy-3-{[(trifluoromethyl)sulfonyl]oxy}benzoate (22) (3.62 g, 3.43 mmol) according to Method A yielded the crude product as a yellow oil (5.94 g). This was chromatographed using ethyl acetate as eluent to obtain the desired product (3.58 g, 75%) as a solid contaminated by a small amount of 4-acetylmorpholine. M.p. 138-140°C. (Found: $[M + H]^{+\bullet}$, 398.0514. $C_{14}H_{14}F_3NO_7S$ requires $[M + H]^{+\bullet}$, 398.0521). δ_H (400 MHz; $CDCl_3$; Me_4Si) 7.89 (1 H, d, *J* 7.6, ArH), 7.44 (1 H, d, *J* 8.0, ArH), 6.96 (1 H, dd, *J* 8.0, ArH), 4.10 (2 H, s, CH_2), 3.71-3.42 (8 H, complex m, CH_2CH_2). Mass spectrum (ESI) *m/z* 398 $[M + H]^{+\bullet}$.

2-Morpholin-4-yl-4-oxo-4H-chromen-8-yl trifluoromethanesulfonate (24)

According to Method C, 2-hydroxy-3-(3-morpholin-4-yl-3-oxopropanoyl)phenyl trifluoromethanesulfonate (23) (0.823 g, 2.07 mmol) was treated trifluoromethanesulfonic anhydride (1.25 mL, 7.46 mmol), to yield the desired material after recrystallisation from ethyl acetate (0.469 g, 60%). M.p. 174-175°C. (Found: $[M + H]^{+\bullet}$, 380.0404. $C_{14}H_{12}F_3NO_6S$ requires $[M + H]^{+\bullet}$, 380.0415).δ_H (400 MHz; $CDCl_3$; Me_4Si) 8.17 (1 H, d, *J* 8.0, ArH), 7.48 (1 H, d, *J* 8.0, ArH), 7.38 (1 H, dd, *J* 8.0, ArH), 5.52 (1 H, s, CH), 3.84 (4 H, t, *J* 4.9, CH_2CH_2), 3.57 (4 H, t, *J* 5.0, CH_2CH_2). Mass spectrum (ESI) *m/z* 380 $[M + H]^{+\bullet}$.

Methyl 2-hydroxy-3-methoxybenzoate (25)

To a solution of 2-hydroxy-3-methoxybenzoic acid (5.0 g, 29.7 mmol) in methanol (250 mL) was added concentrated sulfuric acid (5.0 g, 51.0 mmol). The mixture was refluxed over 48 h, cooled and the methanol was removed *in vacuo*. Water was added (200 mL) and a precipitate formed which was filtered and dried under vacuum to yield a white crystalline product (5.15 g, 95%). M.p. 64-66°C (literature m.p. 61-63°C[109]). δ_H (300 MHz; $CDCl_3$; Me_4Si) 10.99 (1 H, s, OH), 7.41 (1 H, d, *J* 8.4,

ArH), 7.02 (1 H, d, *J* 7.8, ArH), 6.80 (1 H, dd, *J* 8.1, ArH), 3.93 (3 H, s, $COCH_3$), 3.88 (3 H, s, OCH_3). Mass spectrum (ESI) *m/z* 183 $[M + H]^{+\bullet}$).

1-(2-Hydroxy-3-methoxyphenyl)-3-morpholin-4-yl-3-oxopropan-1-one (26)

Treatment of methyl 2-hydroxy-3-methoxybenzoate (25) (4.50 g, 24.7 mmol) according to Method A, yielded a tan solid (5.22 g, 86%). M.p. 140-142°C. (Found: $[M + H]^{+\bullet}$, 280.1179. $C_{14}H_{17}NO_5$ requires $[M + H]^{+\bullet}$, 280.1185). δ_H (300 MHz; $CDCl_3$; Me_4Si) 7.39 (1 H, d, *J* 7.5, ArH), 7.06 (1 H, d, *J* 6.9, ArH), 6.85 (1 H, dd, *J* 8.3, ArH), 4.11 (2 H, s, CH_2), 3.88 (3 H, s, OCH_3), 3.68-3.44 (8 H, complex m, CH_2CH_2). Mass spectrum (ESI) *m/z* 280 $[M + H]^{+\bullet}$.

8-Methoxy-2-morpholin-4-yl-4H-chromen-4-one (27)

According to Method C, 1-(2-hydroxy-3-methoxyphenyl)-3-morpholin-4-yl-3-oxopropan-1-one (26) (3.96 g, 14.2 mmol) was treated with trifluoromethanesulfonic anhydride (8.58 mL, 51.0 mmol) to yield an off-white solid (2.84 g, 77%) after recrystallisation with ethyl acetate, m.p. 145-147°C. (Found: $[M + H]^{+\bullet}$, 262.1079. $C_{14}H_{15}NO_4$ requires $[M + H]^{+\bullet}$, 262.1079). δ_H (300 MHz; $CDCl_3$; Me_4Si) 7.69 (1 H, d, *J* 8.1, ArH), 7.24 (1 H, dd, *J* 8.1 and 8.1, ArH), 7.07 (1 H, d, *J* 6.9, ArH), 5.49 (1 H, s, CH), 3.93 (3 H, s, OCH_3), 3.83 (4H, t, *J* 5.0, CH_2CH_2), 3.53 (4 H, m, *J* 4.8, CH_2CH_2). Mass spectrum (ESI) *m/z* 262 $[M + H]^{+\bullet}$.

8-Hydroxy-2-morpholin-4-yl-4H-chromen-4-one (28)

To aluminium chloride (1.53 g, 11.5 mmol) was carefully added sodium iodide (1.76 g, 11.5 mmol) and acetonitrile (15.3 mL), with stirring for 30 min. To this was added 8-methoxy-2-morpholin-4-yl-*4H*-chromen-4-one (27) (1.0 g, 3.83 mmol) and the mixture heated to reflux overnight. Upon cooling, 1 M HCl (10 mL) was added and the mixture heated for a further 2 h. While cooling, the two layers were allowed to separate. The organic layer was collected, dried (Na_2SO_4) and concentrated *in vacuo*. The residue was chromatographed eluting with 0–5% methanol in dichloromethane to obtain a crude orange solid (0.711 g). This was dissolved in 1 M sodium hydroxide (20 mL) and extracted in ethyl acetate to remove contaminating starting

material. To the aqueous phase was added 1 M HCl until a solid precipitated at around pH 5. The precipitate was filtered and dried *in vacuo* to yield an off-white solid (0.616 g, 65%). M.p. 304-305°C (literature m.p. 300°C[28]). (Found: $[M + H]^{+\bullet}$, 248.0923. $C_{13}H_{13}NO_4$ requires $[M + H]^{+\bullet}$, 248.0923). δ_H (400 MHz; [D$_6$]DMSO; Me_4Si) 10.14 (1 H, s, OH), 7.31 (1 H, br m, ArH), 7.11 (2 H, br m, ArH), 5.45 (1 H, s, CH), 3.69 (4 H, br m, CH_2CH_2), 3.49 (4 H, br m, CH_2CH_2). Mass spectrum (ESI) *m/z* 247 $[M + H]^{+\bullet}$.

2-Morpholin-4-yl-4-oxo-4H-chromen-8-yl trifluoromethanesulfonate (24) – Alternate method

8-Hydroxy-2-morpholin-4-yl-*4H*-chromen-4-one (28) (2.70 g, 10.9 mmol) was suspended in acetonitrile (250 mL) to which N-phenyl triflimide (10.7 g, 29.9 mmol) and diisopropylethylamine (5.2 mL, 29.9 mmol) were added. The mixture was stirred for 48 h, then filtered. The filtrate was evaporated *in vacuo* and the resulting material was dissolved in dichloromethane (60 mL). The solution was washed successively with 0.5 M HCl (30 mL), water (30 mL) and 10% aqueous $NaHCO_3$ (30 mL) then dried (Na_2SO_4). Evaporation of the solvent *in vacuo* resulted in the crude material (9.37 g). This was chromatographed in ethyl acetate to obtain the desired product (2.32 g, 32%), which was identical to the material prepared by Method A.

2-Morpholin-4-yl-8-phenyl-4H-chromen-4-one (6) – Alternate Method

2-Morpholin-4-yl-4-oxo-*4H*-chromen-8-yl trifluoromethanesulfonate (24) (0.199 g, 0.525 mmol), was reacted with phenylboronic acid (70.5 mg, 0.578 mmol), according to Method D to give a crude dark orange oil which was chromatographed with 0–5% methanol in dichloromethane yielding the desired product (0.019 g, 12%) which was identical to the material described above.

2-Morpholin-4-yl-8-[2-(trifluoromethyl)phenyl]-4H-chromen-4-one (29)

Treatment of the trifluoromethanesulfonate (24) with 2-(trifluoromethyl)phenylboronic acid (55.1 mg, 0.29 mmol) according to Method E yielded the title compound (58.3 mg, 59%). M.p. 172-173°C. (Found: $[M + H]^{+\bullet}$,

376.1157. $C_{20}H_{16}F_3NO_3$ requires $[M + H]^{+\bullet}$, 376.1160). δ_H (300 MHz; $CDCl_3$; Me_4Si) 8.20 (1 H, dd, *J* 7.8 and 1.8, ArH), 7.80 (1 H, d, *J* 7.8, ArH), 7.65-7.53 (2 H, complex m, ArH), 7.47-7.35 (3 H, complex m, ArH), 5.45 (1 H, s, CH), 3.61 (4 H, t, *J* 5.0, CH_2CH_2), 3.09 (4 H, t, *J* 5.0, CH_2CH_2); δ_C (100 MHz; $CDCl_3$; Me_4Si) 177.0, 162.2, 151.0, 134.9, 133.3, 132.1, 131.5, 128.5, 127.7, 126.2, 125.8, 124.1, 122.9, 87.0, 65.8, 44.5, 29.7 (one signal obscured or overlapping). Mass spectrum (ESI) *m/z* 376 $[M + H]^{+\bullet}$.

8-(2-Methylphenyl)-2-morpholin-4-yl-4H-chromen-4-one (30)

Treatment of the trifluoromethanesulfonate (24) with *o*-tolylboronic acid (39.4 mg, 0.290 mmol) according to Method E yielded the title compound (39.7 mg, 47%). (Found: $[M + H]^{+\bullet}$, 322.1432. $C_{20}H_{19}NO_3$ requires $[M + H]^{+\bullet}$, 322.1443).δ_H (400 MHz; $CDCl_3$; Me_4Si) 8.15 (1 H, m, ArH), 7.45-7.37 (2 H, complex m, ArH), 7.29-7.20 (4 H, complex m, ArH), 5.46 (1 H, s, CH), 3.64 (4 H, m, CH_2CH_2), 3.14 (4 H, m, CH_2CH_2), 2.11 (3 H, s, CH_3). Mass spectrum (ESI) *m/z* 322 $[M + H]^{+\bullet}$.

8-(2-Chlorophenyl)-2-morpholin-4-yl-4H-chromen-4-one (31)

Treatment of the trifluoromethanesulfonate (24) with 2-chlorophenylboronic acid (45.4 mg, 0.290 mmol) according to Method E yielded the title compound (56.7 mg, 63%). M.p. 178-179°C. (Found: $[M + H]^{+\bullet}$, 342.0892. $C_{19}H_{16}ClNO_3$ requires $[M + H]^{+\bullet}$, 342.0897). δ_H (400 MHz; $CDCl_3$; Me_4Si) 8.20 (1 H, br m, ArH), 7.48-7.35 (6 H, complex m, ArH), 5.48 (1 H, s, CH), 3.67 (4 H, br m, CH_2CH_2), 3.23 (4 H, br m, CH_2CH_2); δ_C (75 MHz; $CDCl_3$; Me_4Si) 177.1, 162.2, 150.7, 135.4, 133.7, 133.5, 131.7, 129.6, 129.4, 127.8, 126.9, 125.7, 124.5, 123.0, 86.9, 65.9, 44.5. Mass spectrum (ESI) *m/z* 342 $[M + H]^{+\bullet}$.

8-(4-Fluorophenyl)-2-morpholin-4-yl-4H-chromen-4-one (32)

Treatment of the trifluoromethanesulfonate (24) with 4-fluorophenylboronic acid (88.5 mg, 0.632 mmol) according to Method F yielded the title compound (116.3 mg, 68%). M.p. 120-121°C. (Found: $[M + H]^{+\bullet}$, 326.1192. $C_{19}H_{16}FNO_3$ requires $[M + H]^{+\bullet}$, 326.1192). δ_H (300 MHz; $CDCl_3$; Me_4Si) 8.18 (1 H, dd, *J* 7.8 and 1.8, ArH).

7.55-7.46 (3 H, complex m, ArH), 7.40 (1 H, dd, *J* 7.8 and 7.8, ArH), 7.16 (2 H, dd, *J* 8.7 and 8.7, ArH), 5.52 (1 H, s, CH), 3.74 (4 H, t, *J* 5.0, CH_2CH_2), 3.33 (4 H, t, *J* 5.0, CH_2CH_2); δ_C (100 MHz; $CDCl_3$; Me_4Si) 177.1, 163.8, 162.6, 150.6, 133.4, 132.4, 131.1, 131.0, 129.4, 125.3, 124.8, 123.5, 115.5, 115.3, 87.2, 65.9, 44.8. Mass spectrum (ESI) *m/z* 326 $[M + H]^{+\bullet}$.

8-[4-Phenoxyphenyl]-2-morpholin-4-yl-4H-chromen-4-one (33)

Treatment of the trifluoromethanesulfonate (24) with 4-phenoxyphenylboronic acid according to Method F yielded the title compound (77.9 mg, 37%). M.p. 194-195°C. (Found: $[M + H]^{+\bullet}$, 400.1540. $C_{25}H_{21}NO_4$ requires $[M + H]^{+\bullet}$, 400.1549). δ_H (300 MHz; $CDCl_3$; Me_4Si) 8.16 (1 H, dd, *J* 3.9 and 1.8, ArH), 7.51 (1 H, dd, *J* 3.8 and 1.7, ArH), 7.45-7.41 (2 H, complex m, ArH), 7.39-7.33 (3 H, complex m, ArH), 7.16-7.11 (1 H, complex m, ArH), 7.10-7.03 (4 H, complex m, ArH), 5.59 (1 H, s, CH), 3.71 (4 H, t, *J* 5.0, CH_2CH_2), 3.34 (4 H, t, *J* 5.0, CH_2CH_2); δ_C (100 MHz; $CDCl_3$; Me_4Si) 177.2, 162.6, 157.4, 156.9, 150.7, 133.4, 131.2, 130.9, 129.9, 129.8, 125.0, 124.8, 123.8, 123.4, 119.1, 118.6, 87.2, 66.0, 44.9. Mass spectrum (ESI) *m/z* 400 $[M + H]^{+\bullet}$.

2-Morpholin-4-yl-8-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-4H-chromen-4-one (34)

Nitrogen was bubbled through a stirred suspension of potassium acetate (217.0 mg, 2.215 mmol), bis(pinacolato)diboron (206.2 mg, 0.812 mmol), dichloro[1,1'-bis(diphenylphosphino)ferrocene]palladium (II) (36.2 mg, 0.044 mmol) and dioxane (10 mL). 2-morpholin-4-yl-4-oxo-*4H*-chromen-8-yl trifluoromethanesulfonate (24) (280.0 mg, 0.738 mmol) was dissolved in dioxane (10 mL) and injected into the reaction, which was then heated at 75°C overnight. The mixture was cooled to room temperature and diluted with toluene, washing twice with brine. The solution was dried (Na_2SO_4), filtered and concentrated *in vacuo* to give a brown oil (316.3 mg). This was chromatographed using 95:5 dichloromethane:methanol as the eluent to yield the pure product (182.7 mg, 69%). M.p. 165-166°C. (Found: $[M + H]^{+\bullet}$, 358.1833. $C_{19}H_{24}BNO_5$ requires $[M + H]^{+\bullet}$, 358.1826). δ_H (300 MHz; $CDCl_3$; Me_4Si)

8.26 (1 H, d, *J* 7.8, ArH), 7.98 (1 H, d, *J* 7.2, ArH), 7.31 (1 H, t, *J* 7.5, ArH), 5.58 (1 H, s, CH), 3.80 (4 H, t, *J* 4.7, CH_2CH_2), 3.67 (4 H, t, *J* 4.7 Hz, CH_2CH_2), 1.34 (12 H, s, CH_3). Mass spectrum (ESI) *m/z* 358 $[M + H]^{+\bullet}$.

8-(2-Methoxyphenyl)-2-morpholin-4-yl-4H-chromen-4-one (36)

A solution of 2-morpholin-4-yl-8-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-*4H*-chromen-4-one (34) (100.0 mg, 0.280 mmol) in dioxane (6 mL) was treated with 2-bromoanisole (35) (34.9 μL, 0.280 mmol) according to Method G yielding a white solid (12.6 mg, 13%). M.p. 133-134°C. (Found: $[M + H]^{+\bullet}$, 338.1386. $C_{20}H_{19}NO_4$ requires $[M + H]^{+\bullet}$, 338.1392). δ_H (300 MHz; $CDCl_3$; Me_4Si) 8.17 (1 H, d, *J* 7.8, ArH), 7.65 (1H, d, *J* 8.1, ArH), 7.51 (1 H, dd, *J* 7.7 and 7.7, ArH), 7.44 (1 H, dd, *J* 8.0 and 8.0, ArH), 7.27 (1 H, m, ArH), 7.08 (1 H, dd, *J* 7.4 and 7.4, ArH), 7.01 (1 H, d, *J* 8.1, ArH), 6.84 (1 H, s, CH), 3.75 (3 H, s, OCH_3), 3.73 (4 H, t, *J* 5.1, CH_2CH_2), 3.47 (4 H, t, *J* 4.8, CH_2CH_2); δ_C (100 MHz; $CDCl_3$; Me_4Si) 175.9, 162.8, 156.6, 150.7, 135.5, 131.3, 130.3, 127.5, 125.6, 124.7, 124.3, 120.9, 119.4, 110.9, 86.2, 65.9, 55.6, 45.0. Mass spectrum (ESI) *m/z* 338 $[M + H]^{+\bullet}$.

2-Bromophenyl acetate (39)

To 2-bromophenol (38) (10.1 g, 58.2 mmol) was added acetic anhydride (60 mL) followed by the drop-wise addition of concentrated sulfuric acid (1 mL). The reaction was stirred for 48 hours at r.t. then poured onto ice and extracted with dichloromethane (3 x 50 mL). The dichloromethane was removed from the organic extracts *in vacuo* to give clear oil to which water (100 mL) and diethyl ether (100 mL) were added. The solution was stirred briskly for while sodium hydrogen carbonate was gradually added until saturation was reached. After stirring for 1 hour, the mixture was filtered and the solvent removed from the filtrate in vacuo to yield the desired product (11.2 g, 83%). The product (39) was found to have the same spectral data to that found in the literature.[110]

1-(3-bromo-2-hydroxyphenyl)ethanone (40) and 1-(3-bromo-4-hydroxyphenyl)ethanone (41)

Aluminium chloride (9.96 g) was carefully added to 2-bromophenyl acetate (39) (11.2 g, 52.1 mmol) with stirring and the mixture was heated at 120 °C overnight. After cooling to r.t., dichloromethane was added and the mixture poured onto ice. The organic layer was extracted and dried with sodium sulphate. The solvent was then removed *in vacuo* to obtain a light brown solid which was chromatographed using 9:1 petroleum spirit:ethyl acetate then ethyl acetate as eluent. The desired ortho isomer (40) (1.71g, 15%) was the minor product obtained while the major product was the para isomer (41) (5.50 g, 49%). Both compounds (40) and (41) were characterised by comparison to experimental data found in the literature.[111,112]

2-Bromo-4-ethylphenyl acetate (44)

A solution of 4-ethylphenol (42) (10.5 g, 85.9 mmol) in chloroform (50 mL) at 0°C under nitrogen and was treated drop-wise with bromine (4.5 mL, 87.8 mmol) by syringe and then stirred at 0°C for 3 h. The mixture was allowed to reach room temperature then water was added (50 mL). The organic phase was separated, washed successively with water (50 mL), brine (50 mL) and 10% aqueous $NaHCO_3$ (50 mL) and finally brine again (50 mL), then dried (Na_2SO_4). The solvent was removed *in vacuo* to obtain *2-bromo-4-ethylphenol (43)* as the desired product (15.0 g, 92%) as an oil. δ_H (400 MHz; $CDCl_3$; Me_4Si) 7.28 (1 H, s, ArH), 7.04 (1 H, d, *J* 8.0, ArH), 6.92 (1 H, d, *J* 8.0, ArH), 2.56 (2 H, q, *J* 7.8, CH_2CH_3), 1.20 (3 H, t, *J* 7.6, CH_2CH_3). Mass spectrum (ESI) *m/z* 202 $[M + H]^{+\bullet}$).

To 2-bromo-4-ethylphenol (43) (15.0 g, 74.6 mmol) was added acetic anhydride (90 mL) and concentrated sulphuric acid (3 mL) with stirring at room temperature under a drying tube. Stirring was continued for 48 h then the mixture was poured onto ice and extracted with dichloromethane (3 x 50 mL). The organic phase was washed with saturated aqueous $NaHCO_3$ then dried (Na_2SO_4). Removal of the solvent *in vacuo* gave an oil which was dissolved in diethyl ether (100 mL) and treated with solid $NaHCO_3$. Stirred for 2 h, filtered and removed ether *in vacuo* to give a pale yellow oil (14.72 g, 81%). δ_H (400 MHz; $CDCl_3$; Me_4Si) 7.43 (1 H, s, ArH), 7.14 (1

H, d, *J* 8.0, ArH), 7.02 (1 H, d, *J* 8.2, ArH), 2.62 (2 H, q, *J* 7.6, CH_2CH_3), 2.34 (3 H, s, OCH_3), 1.23 (3 H, t, *J* 7.6, CH_2CH_3). Mass spectrum (ESI) *m/z* 244 $[M + H]^{+\bullet}$).

1-(3-Bromo-5-ethyl-2-hydroxyphenyl)ethenone (46a)

To 2-bromo-4-ethylphenyl acetate (44) (5.0 g, 20.5 mmol) was carefully added aluminium chloride (5 g) with stirring. The mixture was heated at 125°C under a drying tube overnight. Upon cooling, the mixture was dissolved in dichloromethane and poured onto ice. The organic layer was removed, and the aqueous layer extracted with dichloromethane. The combined organic extracts were dried (Na_2SO_4), and the solvent removed *in vacuo* to give a brown crude material (4.30 g). This was chromatographed eluting with 99:1 petroleum spirit:ethyl acetate to obtain the pure product as an oil (1.75 g, 35%). δ_H (400 MHz; $CDCl_3$; Me_4Si) 12.76 (1 H, s, OH), 7.60 (1 H, d, *J* 2.1, ArH), 7.50 (1 H, d, *J* 2.1, ArH), 2.65 (3 H, s, OCH_3), 2.61 (2 H, q, *J* 7.6, CH_2CH_3), 1.24 (3 H, t, *J* 7.6, CH_2CH_3). Mass spectrum (ESI) *m/z* 243 $[M + H]^{+\bullet}$).

1-(3-Bromo-2-hydroxy-5-methylphenyl)ethenone (46b)

To a solution of 2'-hydroxy-5'-methylacetophenone (45a) (5.0 g, 0.033 mol) in chloroform (25 mL) at 0°C under nitrogen was slowly added bromine (1.74 mL, 0.034 mol) by syringe. After 3 h at 0°C the reaction was allowed to reach room temperature then treated with water (25 mL). The organic phase was washed with water, saturated aqueous NaCl, 10% aqueous $NaHCO_3$ and saturated aqueous NaCl again (50 mL of each), then dried (Na_2SO_4). The solvent was removed *in vacuo* and the residue chromatographed using 99:1 petroleum spirit:ethyl acetate as the eluent to obtain the product (5.622 g, 74%). M.p. 86-87°C (literature m.p. 88-89°C[113]). (Found: $[M + H]^{+\bullet}$, 228.9859. $C_9H_9BrO_2$ requires $[M + H]^{+\bullet}$, 228.9864). δ_H (400 MHz; $CDCl_3$; Me_4Si) 12.74 (1 H, s, OH), 7.56 (1 H, s, ArH), 7.49 (1 H, s, ArH), 2.64 (3 H, s, OCH_3), 2.32 (3 H, s, CH_3). Mass spectrum (ESI) *m/z* 229 $[M + H]^{+\bullet}$.

1-(3-Bromo-2-hydroxy-5-methoxyphenyl)ethenone (46c)

A solution of 2-hydroxy-5-methoxyacetophenone (45b) (3.42 g, 20.6 mmol) in acetic acid (15.6 mL) was made, treated with sodium acetate (1.87 g, 22.8 mmol) and cooled to 0°C. A solution of bromine (1.0 mL, 19.6 mmol) in acetic acid (6.24 mL) was added drop-wise by pipette, and the mixture stirred over 48 h. Further bromine (0.5 mL, 9.8 mmol) and sodium acetate (0.935 g, 11.4 mmol) were added and stirring continued overnight. The reaction was poured onto ice and the resulting precipitate was filtered and dried *in vacuo* to give the desired product (4.88 g, 97%). M.p. 76-78°C (literature m.p. 76-76.5[114]). δ_H (300 MHz; $CDCl_3$; Me_4Si) 7.38 (1 H, s, ArH), 7.19 (1 H, s, ArH), 3.80 (3 H, s, OCH_3), 2.64 (3 H, s, $COCH_3$). Mass spectrum (ESI) *m/z* 245 $[M + H]^{+\bullet}$).

1-(3-Bromo-5-ethyl-2-hydroxyphenyl)-3-morpholin-4-yl-3-oxopropan-1-one (47a)

Treatment of 1-(3-bromo-5-ethyl-2-hydroxyphenyl)ethenone (46a) (0.5 g, 2.06 mmol) with lithium bis(trimethylsilyl)amide (6.17 mL, 6.17 mmol) followed by and 4-morpholinecarbonyl chloride (264 μL, 2.26 mmol) according to Method B yielded the desired product (0.861 g) as an oil which was not further purified. (Found: $[M + H]^{+\bullet}$, 356.0517. $C_{15}H_{18}BrNO_4$ requires $[M + H]^{+\bullet}$, 356.0497). δ_H (400 MHz; $CDCl_3$; Me_4Si) 12.34 (1 H, s, OH), 7.64 (2 H, s, ArH); 4.13 (2 H, s, CH_2), 3.52 (4 H, t, *J* 4.8, CH_2CH_2), 3.26 (4 H, t, *J* 4.8, CH_2CH_2), 2.62 (2 H, q, *J* 7.6, CH_2CH_3), 1.24, (3 H, t, *J* 7.6, CH_2CH_3). Mass spectrum (ESI) *m/z* 356 $[M + H]^{+\bullet}$.

1-(3-Bromo-2-hydroxy-5-methylphenyl)-3-morpholin-4-yl-3-oxopropan-1-one (47b)

Treatment of 1-(3-bromo-2-hydroxy-5-methylphenyl)ethanone (46b) (0.5 g, 2.20 mmol) in tetrahydrofuran (12 mL) with 1 M lithium bis(trimethylsilyl)amide in tetrahydrofuran(6.55 mL, 6.55 mmol) and 4-morpholinecarbonyl chloride (280.0 μL, 2.40 mmol) according to Method B, followed by column chromatography using ethyl acetate as eluent yielded the title compound (0.395 g, 52%) as an oil. (Found: $[M + H]^{+\bullet}$, 342.0342. $C_{14}H_{16}BrNO_4$ requires $[M + H]^{+\bullet}$, 342.0341). δ_H (300 MHz; $CDCl_3$; Me_4Si) 7.60 (1 H, s, ArH); 7.57 (1 H, s, ArH), 4.12 (2 H, s, CH_2), 3.72 (4 H,

br m, CH_2CH_2), 3.49 (4 H, t, *J* 4.8, CH_2CH_2), 2.31 (3 H s, CH_3). Mass spectrum (ESI) *m/z* 342 $[M + H]^{+\bullet}$.

1-(3-Bromo-2-hydroxy-5-methoxyphenyl)-3-morpholin-4-yl-3-oxopropan-1-one (47c)

Treatment of 1-(3-bromo-2-hydroxy-5-methoxyphenyl)ethanone (46c) (2.5 g, 10.2 mmol) in tetrahydrofuran (100 mL) was added lithium bis(trimethylsilyl)amide (30.6 mL, 30.6 mmol) and 4-morpholine carbonyl chloride (1.31 mL, 11.2 mmol) according to Method B, followed by column chromatography using ethyl acetate as eluent yielded the title compound as a solid (2.11 g, 58%). M.p. 150-152°C. δ_H (300 MHz; $CDCl_3$; Me_4Si) 7.40 (1 H, s, ArH), 7.38 (1 H, s, ArH), 4.10 (2 H, s, CH_2), 3.79 (3 H, s, OCH_3), 3.66-3.52 (8 H, br m, CH_2CH_2). Mass spectrum (ESI) *m/z* 358 $[M + H]^{+\bullet}$).

8-Bromo-6-ethyl-2-morpholin-4-yl-4H-chromen-4-one (48a)

Treatment of 1-(3-bromo-5-ethyl-2-hydroxyphenyl)-3-morpholin-4-yl-3-oxopropan-1-one (47a) (0.861 g, 2.42 mmol) with trifluoromethanesulfonic anhydride (8.58 mL, 51.0 mmol) according to Method C yielded a tan solid (0.530 g). This was chromatographed using ethyl acetate as the eluent to obtain the pure product (383 mg, 47%). M.p. 162-164°C. (Found: $[M + H]^{+\bullet}$, 338.0380. $C_{15}H_{16}BrNO_3$ requires $[M + H]^{+\bullet}$, 338.0392). δ_H (300 MHz; $CDCl_3$; Me_4Si) 7.91 (1 H, d, *J* 1.8, ArH), 7.60 (1 H, d, *J* 2.1 Hz, ArH), 5.48 (1 H, s, ArH), 3.84 (4 H, t, *J* 5.0, CH_2CH_2), 3.57 (4 H, t, *J* 5.1, CH_2CH_2), 2.70 (2 H, q, *J* 7.8, CH_2CH_3), 1.26 (3 H, t, *J* 7.7, CH_2CH_3.). Mass spectrum (ESI) *m/z* 338 $[M + H]^{+\bullet}$.

8-Bromo-6-methyl-2-morpholin-4-yl-4H-chromen-4-one (48b)

Treatment of 1-(3-bromo-2-hydroxy-5-methylphenyl)-3-morpholin-4-yl-3-oxopropan-1-one (47b) (0.4 g, 1.17 mmol) with trifluoromethanesulfonic anhydride (0.708 mL, 4.21 mmol)) according to Method C above yielded a brown solid (0.272 g). This was chromatographed using ethyl acetate as the eluent to obtain the pure product (94.6 mg, 25%). M.p. 185-186°C. (Found: $[M + H]^{+\bullet}$, 324.0231.

$C_{14}H_{14}BrNO_3$ requires $[M + H]^{+\bullet}$, 324.0235). δ_H (300 MHz; $CDCl_3$; Me_4Si) 7.88 (1 H, s, ArH), 7.58 (1 H, s, ArH), 5.48 (1 H, s, CH), 3.84 (4 H, t, *J* 5.1, CH_2CH_2), 3.56 (4 H, t, *J* 5.0, CH_2CH_2), 2.40 (3 H, s, CH_3). Mass spectrum (ESI) *m/z* 324 $[M + H]^{+\bullet}$.

8-Bromo-6-methoxy-2-morpholin-4-yl-4H-chromen-4-one (48c)

Treatment of 1-(3-bromo-2-hydroxy-5-methoxyphenyl)-3-morpholin-4-yl-3-oxopropan-1-one (47c) (2.11 g, 5.89 mmol) with trifluoromethanesulfonic anhydride (3.57 mL, 21.2 mmol) according to Method C yielded the desired product (1.32 g, 66%) which was not purified any further. M.p. 188-189°C. (Found: $[M + H]^{+\bullet}$, 340.0197. $C_{14}H_{14}BrNO_4$ requires $[M + H]^{+\bullet}$, 340.0184). δ_H (300 MHz; $CDCl_3$; Me_4Si) 7.52 (1 H, s, ArH), 7.38 (1 H, s, ArH), 5.56 (1 H, s, CH), 3.87 (3 H, s, OCH_3), 3.85 (4 H, t, *J* 4.8, CH_2CH_2), 3.59 (4 H, t, *J* 4.8, CH_2CH_2). Mass spectrum (ESI) *m/z* 340 $[M + H]^{+\bullet}$.

6-Ethyl-2-morpholin-4-yl-8-phenyl-4H-chromen-4-one (49)

Treatment of 8-bromo-6-ethyl-2-morpholin-4-yl-*4H*-chromen-4-one (48a) (0.1 g, 0.296 mmol) with phenylboronic acid (43.6 mg, 0.358 mmol) according to Method H yielded the title compound (41.6 mg, 42 %). M.p. 115-116°C. (Found: $[M + H]^{+\bullet}$, 336.1590. $C_{21}H_{21}NO_3$ requires $[M + H]^{+\bullet}$, 336.1599). δ_H (300 MHz; $CDCl_3$; Me_4Si) 7.99 (1 H, d, *J* 2.1 Hz, ArH), 7.50-7.40 (6 H, m, ArH), 6.54 (1 H, s, CH), 3.75 (4 H, t, *J* 4.8, CH_2CH_2), 3.51 (4 H, t, *J* 5.0, CH_2CH_2), 2.79 (2 H, q, *J* 7.8, CH_2CH_3), 1.31 (3 H, t, *J* 7.7, CH_2CH_3); δ_C (75 MHz; $CDCl_3$; Me_4Si) 176.1, 162.9, 148.6, 142.1, 135.7, 134.7, 130.3, 129.3, 128.5, 128.4, 123.3, 120.1, 86.2, 65.8, 45.1, 28.4, 15.4. Mass spectrum (ESI) *m/z* 336 $[M + H]^{+\bullet}$.

6-Methyl-2-morpholin-4-yl-8-phenyl-4H-chromen-4-one (50)

Treatment of 8-bromo-6-methyl-2-morpholin-4-yl-*4H*-chromen-4-one (48b) (95.0 mg, 0.293 mmol) with phenylboronic acid (39.3 mg, 0.322 mmol) according to method H yielded the title compound (28.2 mg, 30%). M.p. 148-149°C. (Found: $[M + H]^{+\bullet}$, 322.1438. $C_{20}H_{19}NO_3$ requires $[M + H]^{+\bullet}$, 322.1443). δ_H (300 MHz; $CDCl_3$; Me_4Si) 7.96 (1 H, s, ArH), 7.49-7.43 (6 H, complex m, ArH), 6.67 (1 H, s, ArH),

3.76 (4 H, t, *J* 5.0, CH_2CH_2), 3.53 (4 H, t, *J* 5.0, CH_2CH_2), 2.50 (3 H, s, CH_3); δ_C (100 MHz; $CDCl_3$; Me_4Si) 176.1, 162.9, 148.6, 135.7, 135.6, 130.2, 129.3, 128.5, 128.4, 124.5, 120.1, 86.3, 65.9, 45.1, 21.0 (one signal obscured or overlapping). Mass spectrum (ESI) *m/z* 322 $[M + H]^{+\bullet}$.

6-Methoxy-2-morpholin-4-yl-8-phenyl-4H-chromen-4-one (51)

Treatment of 8-bromo-6-methoxy-2-morpholin-4-yl-*4H*-chromen-4-one (48c) (0.6 g, 0.176 mmol) with phenylboronic acid (236.4 mg, 0.194 mmol) according to Method H, with chromatographic purification with dichloromethane:methanol (95:5), yielded the title compound (0.440 mg, 75%). M.p. 110-111°C. (Found: $[M + H]^{+\bullet}$, 338.1388. $C_{20}H_{19}NO_4$ requires $[M + H]^{+\bullet}$, 338.1392). δ_H (300 MHz; $CDCl_3$; Me_4Si) 7.57 (1 H, s, ArH), 7.48 (5 H, br m, ArH), 7.23 (1 H, s, ArH), 6.35 (1 H, s, ArH), 3.92 (3 H, s, OCH_3), 3.75 (4 H, br m, CH_2CH_2), 3.46 (4 H, br m, CH_2CH_2); δ_C (100 MHz; $CDCl_3$; Me_4Si) 176.3, 162.7, 156.8, 145.1, 135.5, 131.9, 129.3, 128.6, 123.3, 122.3, 122.0, 105.2, 86.4, 65.9, 56.0, 45.0. Mass spectrum (ESI) *m/z* 338 $[M + H]^{+\bullet}$.

6-Hydroxy-2-morpholin-4-yl-8-phenyl-4H-chromen-4-one (52)

To aluminium chloride (59.3 mg, 0.445 mmol) was carefully added sodium iodide (68.4 mg, 0.445 mmol) and acetonitrile (1.0 mL), with stirring for 30 min. To this was added 6-methoxy-2-morpholin-4-yl-8-phenyl-*4H*-chromen-4-one (51) (50.0 mg, 0.148 mmol) and the mixture heated to reflux overnight. Further aluminium chloride (59.3 mg, 0.445 mmol) and sodium iodide (68.4 mg, 0.445 mmol) was added with refluxing for further 12 h. Upon cooling, the solvent was removed in vacuo and the crude material crudely chromatographed in dichloromethane then dichloromethane:methanol (95:5) to remove any salts. The resulting material was purified by RP-HPLC to result in the solid product (20.3 mg, 42%). M.p. 163-165°C. (Found: $[M + H]^{+\bullet}$, 324.1234. $C_{19}H_{17}NO_4$ requires $[M + H]^{+\bullet}$, 324.1236). δ_H (300 MHz; $CDCl_3$; Me_4Si), 7.46 (7 H, br m, ArH), 6.67 (1 H, s, CH), 3.76 (4 H, br m, CH_2CH_2), 3.56 (4 H, br m, CH_2CH_2), 2.05 (1 H, br s, OH),. Mass spectrum (ESI) m/z 324 $[M + H]^{+\bullet}$.

8-(2-Chlorophenyl)-6-methoxy-2-morpholin-4-yl-4H-chromen-4-one (53)

Treatment of 8-bromo-6-methoxy-2-morpholin-4-yl-*4H*-chromen-4-one (48c) (0.1 g, 0.294 mmol) with 2-chlorophenylboronic acid (50.6 mg, 0.323 mmol) according to Method H, with chromatographic purification with dichloromethane:methanol (95:5), yielded the title compound (51.9 mg, 48%). M.p. 135-137°C. (Found: $[M + H]^{+\bullet}$, 372.0998. $C_{20}H_{18}ClNO_4$ requires $[M + H]^{+\bullet}$, 372.1002). δ_H (300 MHz; $CDCl_3$; Me_4Si) 7.64 (1 H, d, *J* 3.0, ArH), 7.50 (1 H, m, ArH), 7.38 (3 H, br m, ArH), 7.09 (1 H, d, *J* 3.0, ArH), 5.50 (1 H, s, ArH), 3.92 (3 H, s, OCH_3), 3.69 (4 H, br m, CH_2CH_2), 3.23 (4 H, br m, CH_2CH_2). Mass spectrum (ESI) *m/z* 372 $[M + H]^{+\bullet}$.

8-(3-Chlorophenyl)-6-methoxy-2-morpholin-4-yl-4H-chromen-4-one (54)

Treatment of 8-bromo-6-methoxy-2-morpholin-4-yl-*4H*-chromen-4-one (48c) (0.1 g, 0.294 mmol) with 3-chlorophenylboronic acid (50.6 mg, 0.323 mmol) according to Method H, with chromatographic purification with dichloromethane:methanol (95:5), yielded the title compound (67.7 mg, 62%). M.p. 184-186°C. (Found: $[M + H]^{+\bullet}$, 372.0999. $C_{20}H_{18}ClNO_4$ requires $[M + H]^{+\bullet}$, 372.1002). δ_H (300 MHz; $CDCl_3$; Me_4Si) 7.62 (1 H, d, *J* 3.3, ArH), 7.58 (1 H, s, ArH), 7.40 (3 H, m, ArH), 7.16 (1 H, d, *J* 3.0, ArH), 5.52 (1 H, s, ArH), 3.92 (3 H, s, OCH_3), 3.76 (4 H, t, *J* 5.0, CH_2CH_2), 3.36 (4 H, t, *J* 5.0, CH_2CH_2). Mass spectrum (ESI) *m/z* 372 $[M + H]^{+\bullet}$.

8-(4-Chlorophenyl)-6-methoxy-2-morpholin-4-yl-4H-chromen-4-one (55)

Treatment of 8-bromo-6-methoxy-2-morpholin-4-yl-*4H*-chromen-4-one (48c) (0.1 g, 0.294 mmol) with 4-chlorophenylboronic acid (50.6 mg, 0.323 mmol) according to Method H, with chromatographic purification with dichloromethane:methanol (95:5), yielded the title compound (82.8 mg, 75%). M.p. 170-172°C. (Found: $[M + H]^{+\bullet}$, 372.0999. $C_{20}H_{18}ClNO_4$ requires $[M + H]^{+\bullet}$, 372.1002). δ_H (300 MHz; $CDCl_3$; Me_4Si) 7.62 (1 H, d, *J* 2.7, ArH), 7.45 (4 H, b s, ArH), 7.12 (1 H, d, *J* 3.3, ArH), 5.53 (1 H, s, ArH), 3.91 (3 H, s, OCH_3), 3.75 (4 H, t, *J* 4.8, CH_2CH_2), 3.34 (4 H, t, *J* 4.8, CH_2CH_2). Mass spectrum (ESI) *m/z* 372 $[M + H]^{+\bullet}$.

4-Acetyl-3-hydroxy-2-methylphenyl trifluoromethanesulfonate (16)

2',4'-Dihydroxy-3'-methylacetophenone (56) (4.0 g, 24.1 mmol) was dissolved in dichloromethane (125 mL) under nitrogen, to which pyridine (3.89 mL, 48.2 mmol) and 4-dimethylaminopyridine (294.1 mg, 2.41 mmol) were added. The solution was cooled to 0°C and trifluoromethanesulfonic anhydride (4.45 mL, 26.5 mmol) was added drop-wise by syringe. After stirring at r.t. overnight, the reaction was washed with 1M HCl (3 x 50 mL) and extracted. The organic layer was dried with sodium sulphate and evaporated *in vacuo* to give a purple oil (7.13 g). The crude product was adsorbed onto silica gel purified by chromatography with 9:1 petroleum spirit:ethyl acetate as the eluent. A pure white solid (5.50 g, 77%) was obtained. M.p. 58-61°C (literature m.p. >60-64°C[28]). δ_H (300 MHz; $CDCl_3$; Me_4Si) 12.87 (1H, s, OH), 7.69 (1H, d, *J* 8.4, ArH), 6.84 (1H, d, *J* 9.0, ArH), 2.64 (3H, s, CH_3), 2.26 (3H, s, CH_3). Mass spectrum (ESI) m/z 299 $[M + H]^{+\bullet}$.

3-Hydroxy-2-methyl-4-(3-morpholin-4-yl-3-oxopropanoyl)phenyl trifluoromethanesulfonate (57)

Treatment of 4-acetyl-3-hydroxy-2-methylphenyl trifluoromethanesulfonate (16) (2.53 g, 8.50 mmol) in tetrahydrofuran (50 mL) with 1 M lithium bis(trimethylsilyl)amide (25.4 mL, 25.4 mmol) and 4-morpholinecarbonyl chloride (98.8 μL, 8.50 mmol) according to Method B, followed by chromatography in ethyl acetate to give the desired product (3.12g, 89%) as an oil. δ_H (400 MHz; $CDCl_3$; Me_4Si) 7.78 (1H, d, *J* 8.8, ArH), 6.83 (1H, d, *J* 8.8, ArH), 4.08 (2H, s, CH_2), 3.66-3.43 (8H, complex m, CH_2CH_2), 2.22 (3H, s, CH_3). Mass spectrum (ESI) m/z 412 $[M + H]^{+\bullet}$.

8-Methyl-2-morpholin-4-yl-4-oxo-4H-chromen-7-yl trifluoromethanesulfonate (18)

Treatment of 3-hydroxy-2-methyl-4-(3-morpholin-4-yl-3-oxopropanoyl)phenyl trifluoromethanesulfonate (57) (3.20 g, 7.80 mmol) with trifluoromethanesulfonic anhydride (4.7 mL, 28.0 mmol) according to Method C yielded a dark brown oil (2.49 g). This was purified by chromatography using ethyl acetate as the eluent to give the pure product (1.14 g, 37%). M.p. 150-152°C (literature m.p. 151-155°C[28]).

δ_H (400 MHz; $CDCl_3$; Me_4Si) 8.06 (1H, d, *J* 8.8, ArH), 7.23 (1H, m, ArH), 5.49 (1H, s, CH), 3.83-3.50 (8H, complex m, CH_2CH_2), 2.42 (3H, s, CH_3). Mass spectrum (ESI) m/z 394 $[M + H]^{+\bullet}$.

7-Hydroxy-8-methyl-2-morpholin-4-yl-4H-chromen-4-one(58)

To 8-methyl-2-morpholin-4-yl-4-oxo-*4H*-chromen-7-yl trifluoromethanesulfonate (18) (0.506 g, 1.30 mmol) in tetrahydrofuran (125 mL) was added sodium *tert*-butoxide (185.3 mg, 1.90 mmol) which was then stirred for 2 h at r.t. Removed solvent to give a crude oil (0.99 g). Purification by column chromatography eluting with dichloromethane followed by 9:1 dichloromethane:methanol gave the desired product (177.5 mg, 35%). M.p. >250°C (literature m.p. >250°C[28]). δ_H (300 MHz; $[D_6]$DMSO; Me_4Si) 10.35 (1H, bs, OH), 7.58 (1H, d, *J* 8.7, ArH), 6.83 (1H, d, *J* 8.4, ArH), 5.34 (1H, s, CH), 3.72-3.46 (8H, complex m, CH_2CH_2), 2.17 (3H, s, CH_3). Mass spectrum (ESI) m/z 262 $[M + H]^{+\bullet}$.

7-Hydroxy-8-methyl-2-morpholin-4-yl-4H-chromen-4-one (58) - Alternate method

8-Methyl-2-morpholin-4-yl-4-oxo-*4H*-chromen-7-yl trifluoromethanesulfonate (18) (216.6 mg, 0.551 mmol) was added to 1M NaOH (12 mL) with brisk stirring overnight. The walls of the flask were rinsed with 1M NaOH and stirring continued for a further hour. The mixture was acidified to pH 4 with 1 M HCl and a solid precipitated which was removed by filtration to obtain the pure product (140.7 mg, 98%) which was identical to the above by t.l.c, NMR and HPLC.

Methyl 2-hydroxy-4-{[(trifluoromethyl)sulfonyl]oxy}benzoate (60)

To methyl 2,4-dihydroxybenzoate (59) (7.71 g, 46.0 mmol) dissolved in dichloromethane (250 mL) was added pyridine (5.57 mL, 68.0 mmol) and dimethylaminopyridine (0.56 g, 4.6 mmol). The mixture was cooled to 0°C with ice bath and added trifluoromethanesulfonic anhydride (8.5 mL, 50.5 mmol) was added drop-wise by syringe. The ice bath was stirred at r.t. for 48 h. The organic layer was washed three times with 1M HCl (100 mL), dried with sodium sulphate and concentrated to dryness *in vacuo* to yield a dark orange oil (7.83 g, 58%). Proton

NMR indicated that this was 83% desired product, which was co-eluting with the starting material by t.l.c. δ_H (300 MHz; $CDCl_3$; Me_4Si) 11.02 (1H, s, OH), 7.91 (1H, d, *J* 8.7, ArH), 6.89 (1H, d, *J* 2.4, ArH), 6.79 (1H, dd, *J* 8.7 and 2.4, ArH), 3.95 (3H, s, CH_3). Mass spectrum (ESI) m/z 301$[M + H]^{+\bullet}$.

3-Hydroxy-4-(3-morpholin-4-yl-3-oxopropanoyl)phenyl trifluoromethanesulfonate (61)

4-Acetyl-3-hydroxyphenyl trifluoromethanesulfonate (60) (4.0 g, 13.3 mmol) was reacted according to Method A to yield the crude product as a dark orange oil (6.0 g). Chromatographic purification using dichloromethane followed by 20:1 dichloromethane:methanol as eluent yielded the desired product (4.68 g, 88%) as an oil. δ_H (400 MHz; $CDCl_3$; Me_4Si) 7.97 (1H, d, *J* 8.8, ArH), 6.88 (1H, s, ArH), 6.83 (1H, d, *J* 8.8, ArH), 4.07 (2H, s, CH_2), 3.68-3.41 (8H, complex m, CH_2CH_2). Mass spectrum (ESI) m/z 398 $[M + H]^{+\bullet}$.

2-Morpholin-4-yl-4-oxo-4H-chromen-7-yl trifluoromethanesulfonate (62)

The reaction of 3-hydroxy-4-(3-morpholin-4-yl-3-oxopropanoyl)phenyl trifluoromethanesulfonate (61) (1.04 g, 2.62 mmol) with trifluoromethane sulfonic anhydride (1.59 mL, 9.39 mmol) according to Method C gave the desired material (0.835 g, 84%). M.p. 143-146°C. (Found: $[M + H]^{+\bullet}$, 380.0413. $C_{14}H_{12}F_3NO_5S$ requires $[M + H]^{+\bullet}$, 380.0415). δ_H (400 MHz; $CDCl_3$; Me_4Si) 8.21 (1H, d, *J* 8.8, ArH), 7.38 (1H, s, ArH), 7.34 (1H, d, *J* 9.2, ArH), 6.28 (1H, s, CH), 3.85-3.73 (8H, complex m, CH_2CH_2). Mass spectrum (ESI) m/z 380 $[M + H]^{+\bullet}$.

7-Hydroxy-2-morpholin-4-yl-4H-chromen-4-one (63)

To 2-morpholin-4-yl-4-oxo-*4H*-chromen-7-yl trifluoromethanesulfonate (62) (3.04g, 8.01 mmol) was added 1M sodium hydroxide (100 mL) and the mixture was stirred at r.t. over an hour. The mixture was washed with dichloromethane, then acidified with 1M HCl and extracted into ethyl acetate (3 x 30 mL). The combined organic layers were dried with sodium sulphate and concentrated *in vacuo* to give the product (0.967 g, 49%). M.p. >250°C (literature m.p. >250°C[115]). δ_H (300 MHz;

[D_6]DMSO; Me_4Si) 10.48 (1H, s, OH), 7.71 (1H, d, *J* 8.4, ArH), 6.77 (1H, s, *J* 8.7, ArH), 6.76 (1H, s, ArH), 5.34 (1H, s, CH), 3.69-3.43 (8H, complex m, CH_2CH_2). Mass spectrum (ESI) m/z 248 $[M + H]^{+\bullet}$.

Methyl 2,5-dihydroxybenzoate (65)

To 2,5-dihydroxybenzoic acid (64) (3.70 g, 24.0 mmol) in methanol (200 mL) was added concentrated sulfuric acid (4.0 g, 40.8 mmol) followed by refluxing overnight. The methanol was removed *in vacuo*, water (250 mL) was added followed by extraction with dichloromethane. Dried organic layer with sodium sulphate and removed solvent in vacuo to obtain a yellow solid (3.51 g, 87%). M.p. 82-84°C (literature m.p. 85-87°C[116]). δ_H (300 MHz; $CDCl_3$; Me_4Si) 10.38 (1H, s, OH), 7.29 (1H, d, *J* 3.3, ArH), 7.01 (1H, dd, *J* 8.7 and 3.0, ArH), 6.87 (1H, d, *J* 9.0, ArH), 3.92 (3H, s, CH_3). Mass spectrum (ESI) m/z 169 $[M + H]^{+\bullet}$.

Methyl 2-hydroxy-5-{[(trifluoromethyl)sulfonyl]oxy}benzoate (66)

To methyl 2,5-dihydroxybenzoate (65) (3.16 g, 18.8 mmol) in dichloromethane (100 mL) under nitrogen was added pyridine (3.04 mL, 37.6 mmol) and 4-dimethylaminopyridine (0.230 g, 1.88 mmol). The solution was cooled to 0°C with an ice bath and trifluoromethanesulfonic anhydride (3.48 mL, 20.7 mmol) was added drop-wise by syringe. The ice bath was removed and the reaction was stirred at r.t. for 48 h. The organic layer was washed three times with 1M HCl (100 mL), dried with sodium sulphate and concentrated to dryness *in vacuo* to give a yellow oil (5.21 g, 93%). M.p. 83-86°C (literature m.p. 85-87°C[28,117]).δ_H (300 MHz; $CDCl_3$; Me_4Si) 10.85 (1H, s, OH), 7.74 (1H, d, *J* 3.0, ArH), 7.35 (1H, dd, *J* 9.3 and 3.3, ArH), 7.03 (1H, d, *J* 9.6, ArH), 3.98 (3H, s, CH_3). Mass spectrum (ESI) m/z 301$[M + H]^{+\bullet}$.

4-hydroxy-3-(3-morpholin-4-yl-3-oxopropanoyl)phenyl trifluoromethanesulfonate (67)

Methyl 2-hydroxy-5-{[(trifluoromethyl)sulfonyl]oxy}benzoate (66) (5.0 g, 16.7 mmol) was treated according to Method A to yield the crude product as a dark orange oil (6.82 g). Chromatographic purification using ethyl acetate to obtain a

yellow oil (3.08 g) which crystallised overnight. The solid was crushed and washed with ether to remove any 4-acetylmorpholine to give a white powder (1.06 g, 16%). δ_H (400 MHz; $CDCl_3$; Me_4Si) 11.92 (1H, bs, OH), 7.79 (1H, d, *J* 2.8, ArH), 7.41 (1H, dd, *J* 8.0 and 3.0 ArH), 7.07 (1H, d, *J* 9.2, ArH), 4.08 (2H, s, CH_2), 3.73-3.53 (8H, complex m, CH_2CH_2). Mass spectrum (ESI) m/z 398 $[M + H]^{+\bullet}$.

2-Morpholin-4-yl-4-oxo-4H-chromen-6-yl trifluoromethanesulfonate (68)

Treatment of 4-hydroxy-3-(3-morpholin-4-yl-3-oxopropanoyl)phenyl trifluoromethanesulfonate (67) (1.0 g, 2.52 mmol) with added trifluoromethane sulfonic anhydride (1.52 mL, 9.64 mmol) according to Method C resulted in the crude product (0.658 g). This material was chromatographed with ethyl acetate to yield the final product as a yellow solid (0.502 g, 36%). δ_H (300 MHz; $CDCl_3$; Me_4Si) 7.98 (1H, s, ArH), 7.44-7.32 (2H, m, ArH), 5.47 (1H, s, CH), 3.80-3.50 (8H, complex m, CH_2CH_2). Mass spectrum (ESI) m/z 380 $[M + H]^{+\bullet}$.

6-Hydroxy-2-morpholin-4-yl-4H-chromen-4-one (69)

To 2-morpholin-4-yl-4-oxo-*4H*-chromen-6-yl trifluoromethanesulfonate (68) (0.250 g, 0.662 mmol) in tetrahydrofuran (20 mL) was added sodium tertiary-butoxide (95.4 mg, 0.993 mmol). A yellow precipitate formed immediately and the reaction was stirred for 48 h. Solvent was removed *in vacuo* and the resulting solid chromatographed with 1-5% methanol in dichloromethane to obtain the product as a white solid (0.122 mg, 74%). M.p. >250°C (literature m.p. 290-292°C[28]). δ_H (300 MHz; [D_6]DMSO; Me_4Si) 9.72 (1H, s, OH), 7.31 (1H, d, *J* 9.0, ArH), 7.19 (1H, s, ArH), 7.01 (1H, d, *J* 9.0, ArH), 5.42 (1H, s, CH), 3.67-3.45 (8H, complex m, CH_2CH_2). Mass spectrum (ESI) m/z 248 $[M + H]^{+\bullet}$.

8-Methyl-2-morpholin-4-yl-7-phenyl-4H-chromen-4-one (19)

Treatment of 8-methyl-2-morpholin-4-yl-4-oxo-*4H*-chromen-7-yl trifluoromethanesulfonate (18) (200.0 mg, 0.509 mmol) according to Method F yielded the pure product (77.6 mg, 47%). M.p. 190-192°C (literature m.p. 194.5-195°C[28]). δ_H (400 MHz; $CDCl_3$; Me_4Si) 8.02 (1H, d, *J* 8.0, ArH), 7.46-7.36 (3H, m,

ArH), 7.31 (2H, d, *J* 8.0, ArH), 7.24 (1H, d, *J* 8.4, ArH), 5.52 (1H, s, CH), 3.85-3.50 (8H, complex m, CH_2CH_2), 2.32 (s, 3H, CH_3); δ_C (75 MHz; $CDCl_3$; Me_4Si) 177.6, 162.9, 152.5, 146.3, 140.4, 129.2, 128.3, 127.6, 126.4, 123.3, 122.6, 121.7, 87.4, 66.0, 44.8, 13.2. Mass spectrum (ESI) m/z 322 $[M + H]^{+\bullet}$.

2-Morpholin-4-yl-7-phenyl-4H-chromen-4-one (70)

2-Morpholin-4-yl-4-oxo-*4H*-chromen-7-yl trifluoromethanesulfonate (62) (198 mg, 5.25 mmol), phenylboronic acid (78.3 mg, 0.578 mmol), potassium carbonate (208.9 g, 1.32 mmol) and palladium diacetate (4.4 mg, 0.022 mmol) were combined, tetrahydrofuran (5 mL) was added and the reaction was heated at 70°C overnight under nitrogen. Following cooling to r.t., the reaction was filtered and the resulting solid washed with ethyl acetate. From the filtrate, solvent was removed *in vacuo* to give the crude product (232 mg). Purification using chromatography was carried out twice, firstly with dichloromethane and then again in ethyl acetate to give the pure product (9.6 mg, 6%). (Found: $[M + H]^{+\bullet}$, 308.1279 $C_{20}H_{19}NO_4$ requires $[M + H]^{+\bullet}$, 308.1286). δ_H (300 MHz; $CDCl_3$; Me_4Si) 8.20 (1H, d, *J* 8.4, ArH), 7.66-7.41 (7H, m, ArH), 5.53 (1H, s, CH), 3.53-3.87 (8H, complex m, CH_2CH_2). δ_C (100 MHz; $[D_6]$DMSO; Me_4Si) 188.9, 174.8, 166.9, 162.3, 153.7, 144.1, 138.3, 129.9, 128.5, 126.8, 125.1, 123.1, 121.4, 114.6, 886.3, 65.3, 44.4. Mass spectrum (ESI) m/z 308 $[M + H]^{+\bullet}$.

2-Morpholin-4-yl-8-(phenylethynyl)-4H-chromen-4-one (71)

Nitrogen was bubbled through a mixture of 2-morpholin-4-yl-4-oxo-*4H*-chromen-8-yl trifluoromethanesulfonate (24) (91.0 mg, 0.240 mmol), 1:1 tetrahydrofuran/diisopropylamine (3 mL), phenylacetylene (50 μL, 0.455 mmol) and copper iodide (1.0 mg, 0.005 mmol) for 10 min. Bis(triphenylphosphine)palladium(II) chloride was then added and the reaction was refluxed at 100°C under nitrogen overnight. The solvent was removed *in vacuo* to give a crude black material which was dissolved in dichloromethane and adsorbed onto silica. Chromatography using dichloromethane then 1% methanol in dichloromethane as eluent resulted in the product (23.5 mg, 30%). M.p. 193-195°C

(literature m.p. 196-197°C[28]). δ_H (400 MHz; $CDCl_3$; Me_4Si) 8.11 (1H, d, *J* 8.0, ArH), 7.72 (1H, d, *J* 8.0, ArH), 7.50-7.48 (2H, m, ArH), 7.37-7.40 (3H, m, ArH), 7.32 (1H, t, *J* 7.6, ArH), 5.52 (1H, s, CH), 3.85-3.59 (8H, complex m, CH_2CH_2). Mass spectrum (ESI) m/z 332 $[M + H]^{+\bullet}$.

2-Morpholin-4-yl-8-(2-phenylethyl)-4H-chromen-4-one (72)

2-Morpholin-4-yl-8-(phenylethynyl)-*4H*-chromen-4-one (71) (137.7 mg, 0.416 mmol) was dissolved in 8:1 methanol:acetone (90 mL) to which a spoonful of palladium/carbon was added. The reaction was stirred under hydrogen overnight. Filtered, washed with methanol and dried *in vacuo* to obtain a crude product (44.6 mg). Purification by chromatography using ethyl acetate gave the purified product (9.5 mg, 7%). M.p. 107-110°C (literature m.p. 110-112°C[28]). δ_H (400 MHz; $CDCl_3$; Me_4Si) 8.00 (1H, d, *J* 7.6, ArH), 7.36 (1H, d, *J* 6.8, ArH), 7.28-7.18 (4H, m, ArH), 7.09 (2H, d, *J* 7.2, ArH), 5.52 (1H, s, CH), 3.82-3.45 (8H, complex m, CH_2CH_2), 3.11-2.93 (4H, m, CH_2). Mass spectrum (ESI) m/z 336 $[M + H]^{+\bullet}$.

8-Methyl-2-morpholin-4-yl-7-(2- phenylethynyl)-4H-chromen-4-one (73)

Nitrogen was bubbled through 1:1 tetrahydrofuran:diisopropylamine (10 mL), in which was dissolved 8-methyl-2-morpholin-4-yl-4-oxo-*4H*-chromen-7-yl trifluoromethanesulfonate (18) (185.0 mg, 0.470 mmol). Phenylacetylene (62.0 μL, 0.564 mmol), copper iodide (1.9 mg, 0.01 mmol) and bis(triphenylphosphine)palladium(II) chloride (14.0 mg, 0.02 mmol) were successively added. Reaction was refluxed at 100°C overnight then filtered, with the solid being washed with methanol. Solvent was removed from the combined filtrate *in vacuo* to give a dark brown oil (318 mg). This was partially purified using chromatography in dichloromethane followed by 9:1 dichloromethane:methanol. The appropriate fractions were further purified using RP-HPLC to give the purified product (19.3 mg, 12%). M.p. 215°C (char) (literature m.p. 228.5-229.5°C[28]). δ_H (300 MHz; $CDCl_3$; Me_4Si) 7.97 (1H, d, *J* 8.1, ArH), 7.56-7.54 (3H, m, ArH), 7.40-7.37 (3H, m, ArH), 6.67 (1H, s, CH), 3.90-3.71 (8H, complex m, CH_2CH_2). Mass spectrum (ESI) m/z 346 $[M + H]^{+\bullet}$.

8-Methyl-2-morpholin-4-yl-7-(2- phenylethyl)-4H-chromen-4-one (75)

8-Methyl-2-morpholin-4-yl-7-(2- phenylethynyl)-*4H*-chromen-4-one (73) (18.0 mg, 0.151 mmol) was dissolved in 8:1 methanol:acetone (20 mL) to which a spoonful of palladium/carbon was added. Reaction was stirred under hydrogen overnight. After filtering, washing the solids with methanol and drying the combined washings *in vacuo*, a residue was obtained. Purification by RP-HPLC was unable to obtain any products for characterisation.

2-Morpholin-4-yl-7-(phenylethynyl)-4H-chromen-4-one (74)

2-Morpholin-4-yl-4-oxo-*4H*-chromen-7-yl trifluoromethanesulfonate (62) (91.0 mg, 0.24 mmol), phenyl acetylene (50 μL, 0.455 mmol), copper iodide (1.0 mg, 0.005 mmol) and 1:1 tetrahydrofuran:diisopropylamine (3 mL) were combined and nitrogen was bubbled through the mixture. Dichloro[1,1'-bis(diphenylphosphino)ferrocene]palladium (II) (7.0 mg, 0.01 mmol) was added and the reaction was refluxed overnight at 100°C under nitrogen. The solvent was removed *in vacuo* then packed on silica for chromatography in 1% methanol in dichloromethane to obtain the pure product (56.6 mg, 71%). (Found: $[M + H]^{+\bullet}$, 332.1271. $C_{21}H_{17}NO_3$ requires $[M + H]^{+\bullet}$, 332.1286). δ_H (300 MHz; $CDCl_3$; Me_4Si) 8.08 (1H, d, *J* 8.4, ArH), 7.55-7.35 (7H, m, ArH), 5.55 (1H, s, CH), 3.83-3.54 (8H, complex m, CH_2CH_2). Mass spectrum (ESI) m/z 332 $[M + H]^{+\bullet}$.

2-Morpholin-4-yl-7-(2-phenylethyl)-4H-chromen-4-one (76)

2-Morpholin-4-yl-7-(phenylethynyl)-*4H*-chromen-4-one (74) (50.0 mg, 0.151 mmol) was dissolved in 8:1 methanol:acetone (45 mL) to which a spoonful of palladium/carbon was added. Reaction was stirred under hydrogen overnight. After filtering, washing the solids with methanol and drying the combined washings *in vacuo*, a crude product was obtained (67.4 mg). Purification by RP-HPLC obtained the pure product (3.9 mg, 8%). (Found: $[M + H]^{+\bullet}$, 336.1596. $C_{21}H_{21}NO_3$ requires $[M + H]^{+\bullet}$, 336.1599). δ_H (400 MHz; $CDCl_3$; Me_4Si) 8.05 (1H, d, *J* 8.0, ArH), 7.30-7.13 (7H, m, ArH), 6.67 (1H, s, CH), 3.86-3.71 (8H, complex m, CH_2CH_2), 3.08-2.96 (4H, m, CH_2). Mass spectrum (ESI) m/z 336 $[M + H]^{+\bullet}$.

8-Methyl-7-[2-(4-methylpiperazin-1-yl)ethoxy]-2-morpholin-4-yl-4H-chromen-4-one (5)

7-Hydroxy-8-methyl-2-morpholin-4-yl-*4H*-chromen-4-one (63) (140.0 mg, 0.536 mmol) was suspended in 50% aqueous NaOH (1.5 mL) and treated successively with tetrabutylammonium hydrogen sulfate (30.3 mg, 89.3 mmol) and 1,2-dibromoethane (53.0 μL, 6.16 mmol). Reaction mixture was warmed to 60 °C for 2 h then cooled to 0 °C. Precipitate which formed was filtered and washed with 2M NaOH, water and ether. Dissolved solid in hot chloroform, filtered and removed the solvent *in vacuo* to give the pure product (101.0 mg, 51%). δ_H (300 MHz; $CDCl_3$; Me_4Si) 7.97 (1H, d, *J* 8.7, ArH), 6.85 (1H, d, *J* 8.7, ArH), 5.42 (1H, s), 4.39 (2H, t, J 6.0, CH_2), 3.85-3.82 (4H, complex m, CH_2CH_2), 3.69 (2H, t, *J* 6.0, CH_2), 3.51-3.47 (4H, complex m, CH_2CH_2), 2.29 (3H, s, CH_3). Mass spectrum (ESI) m/z 369 $[M + H]^{+\bullet}$.

The resultant 7-(2-bromoethoxy)-8-methyl-2-morpholin-4-yl-*4H*-chromen-4-one (70.0 mg, 0.190 mmol) was suspended in chloroform (1 mL) and *N*-methylpiperazine was added (122.3 μL, 1.10 mmol) upon which the solid dissolved. The reaction was warmed to reflux overnight. After cooling to r.t, chloroform (5 mL) was added followed by a 1:1 mixture of 2M NaOH and saturated NaCl (10 mL). The organic layer was extracted, dried with sodium sulphate and the solvent removed *in vacuo* to give the product as a crude solid (76.8 mg). Purification was completed using RP-HPLC to obtain the pure product (41.0 mg, 43%). M.p. 153.5-154.5°C (literature m.p. 159-159.5°C[28]). δ_H (300 MHz; $CDCl_3$; Me_4Si) 7.95 (1H, d, *J* 8.4, ArH), 6.87 (1H, d, *J* 8.7, ArH), 5.41 (1H, s, CH), 4.19 (2H, t, *J* 5.4, CH_2), 3.83-3.74 (4H, complex m, CH_2CH_2), 3.72 (2H, t, *J* 5.7, CH_2), 3.49-3.46 (4H, complex m, CH_2CH_2), 2.64 (4H, bs, CH_2), 2.47 (4H, bs, CH_2), 2.28 (3H, s, CH_3), 2.24 (3H, s, CH_3). Mass spectrum (ESI) m/z 388 $[M + H]^{+\bullet}$.

2-Morpholin-4-yl-8-(pyridin-3-ylmethoxy)-4H-chromen-4-one (3)

To 8-hydroxy-2-morpholin-4-yl-*4H*-chromen-4-one (28) (100 mg, 0.404 mmol) in acetonitrile (5 mL) was added potassium carbonate (340 mg, 2.43 mmol) followed by 3-(bromomethyl)pyridine hydrobromide (143.0 mg, 2.43 mmol). The reaction was refluxed overnight. Upon cooling, water was added and the mixture was extracted

into ethyl acetate (3 x 30 mL). The organic layer was dried over sodium sulphate and the solvent removed *in vacuo* to give yellow oil which was purified by RP-HPLC to give the pure product (11.0 mg, 8%). M.p. 154-156°C (literature m.p. 160-161°C[28]. δ_H (400 MHz; [D$_6$]DMSO; Me$_4$Si) 8.80 (1H, m, ArH), 8.67 (1H, m, ArH), 8.13 (1H, m, ArH), 7.66 (1H, m, ArH), 7.49 (1H, d, J 7.6, ArH), 7.43 (1H, d, *J* 8.0, ArH), 7.29 (1H, t, *J* 8.0, ArH), 5.51 (1H, s, CH), 5.36 (2H, s, CH_2), 3.66-3.45 (8H, complex m, CH_2CH_2); δ_C (75 MHz; $CDCl_3$; Me$_4$Si) 176.5, 162.6, 146.8, 146.0, 145.8, 143.8, 137.5, 133.6, 124.9, 124.7, 123.1, 118.0, 115.7, 87.1, 68.2, 65.9, 44.9. Mass spectrum (ESI) m/z 339 $[M + H]^{+\bullet}$.

8-[2-(4-Methylpiperazin-1-yl)ethoxy]-2-morpholin-4-yl-4H-chromen-4-one (77)

8-Hydroxy-2-morpholin-4-yl-*4H*-chromen-4-one (28) (44.5 mg, 0.180 mmol) was suspended in 50% aqueous NaOH (1 mL) and treated with tetrabutylammonium hydrogen sulfate (10.2 mg, 30.0 mmol) and 1,2-dibromoethane (178.4 μL, 2.07 mmol). The reaction mixture was warmed to 60°C for 2 h then cooled to 0°C. The precipitate which formed was filtered and washed with 2M NaOH, water and ether. The solid was then dissolved in hot chloroform, filtered and removed solvent *in vacuo* to give the pure product (31.1 mg, 48.3%). δ_H (300 MHz; $CDCl_3$; Me$_4$Si) 7.69 (1H, m, ArH), 7.20 (1H, m, ArH), 7.06 (1H, d, *J* 7.8, ArH), 5.48 (1H, s, CH), 4.39 (2H, t, *J* 5.7, CH_2), 3.82-3.75 (4H, complex m, CH_2CH_2), 3.70 (2H, t, *J* 5.4, CH_2), 3.54 (4H, complex m, CH_2CH_2). Mass spectrum (ESI) m/z 355 $[M + H]^{+\bullet}$.

8-(2-Bromoethoxy)-2-morpholin-4-yl-*4H*-chromen-4-one (30.0 mg, 0.085 mmol) was suspended in chloroform (1 mL) and *N*-methylpiperazine was added (54.5 μL, 0.491 mmol) upon which the solid dissolved. The reaction was warmed to reflux overnight. After cooling to r.t., chloroform (5 mL) was added followed by a mixture of 1:1 2M NaOH and saturated NaCl (10 mL). The organic layer was extracted, dried with sodium sulphate and the solvent removed *in vacuo* to give the product as a crude solid (25.0 mg). Purification was completed using RP-HPLC to obtain the pure product (20.3 mg, 49%). M.p. 168-169°C. δ_H (300 MHz; D_2O; Me$_4$Si) 7.32-7.12 (3H, m, ArH), 5.48 (1H, s, CH), 4.27 (2H, m, CH_2), 3.62-3.44 (18H, complex m, CH_2), 2.73 (3H, s, CH_3); δ_C (100 MHz; [D$_6$]DMSO; Me$_4$Si) 175.0, 161.8, 146.4, 143.0,

124.4, 123.5, 115.8, 115.2, 86.2, 66.6, 65.3, 55.1, 52.1, 49.6, 44.3, 42.2. Mass spectrum (ESI) m/z 374 $[M + H]^{+\bullet}$.

8-Methyl-2-morpholin-4-yl-7-(pyridin-3-ylmethoxy)-4H-chromen-4-one (78)

A suspension of 7-hydroxy-8-methyl-2-morpholin-4-yl-*4H*-chromen-4-one (58) (100.0 mg, 0.382 mmol) in acetonitrile (10 mL) with potassium carbonate (333.4 mg, 2.38 mmol) under nitrogen, was treated with 3-(bromomethyl)pyridine hydrobromide (136.0 mg, 0.537 mmol). The mixture was heated at 60°C overnight then cooled to r.t. Methanol (10 mL) was added and the mixture concentrated to a brown residue which was adsorbed onto silica. Chromatography with 99:1 then 95:5 dichloromethane:methanol was followed by RP-HPLC which gave the desired product (29.6 mg, 22%). M.p. 179-181°C (literature m.p. 182.5-184°C[28]). δ_H (400 MHz; $[D_6]$DMSO; Me_4Si) 8.84 (1H, s, ArH), 8.70 (1H, m, ArH), 8.24 (1H, m, ArH), 7.77-7.73 (2H, m, ArH), 7.19 (1H, d, *J* 8.8, ArH), 5.47 (1H, s, CH), 5.36 (2H, s, CH_2), 3.73-3.50 (8H, complex m, CH_2CH_2), 2.26 (3H, s, CH_3); δ_C (100 MHz; $[D_6]$DMSO; Me_4Si) 175.0, 161.8, 146.4, 143.0, 124.4, 123.5, 115.8, 115.2, 86.2, 66.6, 65.3, 55.1, 52.1, 49.6, 44.3, 42.2. Mass spectrum (ESI) m/z 353 $[M + H]^{+\bullet}$.

2-Morpholin-4-yl-7-(pyridin-3-ylmethoxy)-4H-chromen-4-one (79)

To 7-hydroxy-2-morpholin-4-yl-*4H*-chromen-4-one (63) (100 mg, 0.404 mmol) in acetonitrile (5 mL) was added potassium carbonate (340 mg, 2.43 mmol) followed by 3-(bromomethyl)pyridine hydrobromide (143 mg, 0.566 mmol). The reaction was heated to 70°C overnight. The solid was washed with methanol and filtered. The solvent was removed from the combined washings *in vacuo* to give dark orange crude oil. The material was adsorbed onto silica and purified by chromatography with ethyl acetate then 9:1 dichloromethane:methanol to give product (27.4 mg, 20%). M.p. 188-189°C (literature m.p. 193-194°C[28]). δ_H (300 MHz; $[D_6]$DMSO; Me_4Si) 8.84 (1H, s, ArH), 8.72 (1H, m, ArH), 8.21 (1H, d, *J* 8.1, ArH), 7.84 (1H, m, ArH), 7.72 (1H, m, ArH), 7.24 (1H, s, ArH), 7.07 (1H, d, *J* 9.0, ArH), 5.47 (1H, s, CH), 5.33 (2H, s, CH_2), 3.68-3.42 (8H, complex m, CH_2CH_2); δ_C (100 MHz; $CDCl_3$;

Me_4Si) 174.6, 162.3, 161.3, 154.6, 146.8, 138.3, 133.1, 125.9, 125.3, 124.57, 116.23, 113.5, 101.5, 85.7, 67.1, 65.3, 44.4. Mass spectrum (ESI) m/z 339 $[M + H]^{+\bullet}$.

8-(Benzyloxy)-2-morpholin-4-yl-4H-chromen-4-one (80)

To 8-hydroxy-2-morpholin-4-yl-*4H*-chromen-4-one (28) (84.0 mg, 0.340 mmol) in acetonitrile (2 mL) was added potassium carbonate (295 mg, 1.64 mmol) followed by benzyl bromide (56.6 μL, 0.476 mmol). Heated to 60°C overnight then cooled and removed solvent *in vacuo* to obtain crude material (27.5 mg) which was purified by RP-HPLC. The appropriate fraction was lyphophilised to obtain the pure product (15.1 mg, 13%). M.p. 193-196°C (literature m.p. 192-194°C[28]). δ_H (400 MHz; $[D_6]$DMSO; Me_4Si) 7.47-7.27 (8H, m, ArH), 5.53 (1H, s, CH), 5.30 (2H, s, CH_2), 3.72-3.51 (8H, complex m, CH_2CH_2). Mass spectrum (ESI) m/z 338 $[M + H]^{+\bullet}$.

7-(Benzyloxy)-8-methyl-2-morpholin-4-yl-4H-chromen-4-one (4)

A suspension of 7-hydroxy-8-methyl-2-morpholin-4-yl-*4H*-chromen-4-one (58) (100.0 mg, 0.382 mmol) in acetonitrile (10 mL) with potassium carbonate (333.4 mg, 2.38 mmol) stirring under nitrogen was treated with benzyl bromide (63.7 μL, 0.537 mmol). The mixture was heated at 60°C overnight then cooled to r.t., filtered and the solid washed with acetonitrile (10 mL). The solvent was removed from the combined washings *in vacuo* to give a crude solid (151.0 mg). Purification by chromatography in 9:1 dichloromethane:methanol gave the desired product (121.9 mg, 91%). M.p. 184-186°C (literature m.p. 181.5-182.5°C[28]). δ_H (300 MHz; $CDCl_3$; Me_4Si) 7.97 (1H, d, *J* 9.0, ArH), 7.45-7.32 (5H, m, ArH), 6.96 (1H, d, *J* 8.7, ArH), 5.42 (1H, s, CH), 5.17 (2H, s, CH_2), 3.85-3.47 (8H, complex m, CH_2CH_2), 2.31 (3H, s, CH_3); δ_C (75 MHz; $CDCl_3$; Me_4Si) 177.6, 162.9, 159.7, 153.0, 136.5, 128.6, 128.1, 127.2, 123.8, 116.8, 113.4, 109.1, 86.7, 70.6, 66.0, 44.8. Mass spectrum (ESI) m/z 352 $[M + H]^{+\bullet}$.

7-(Benzyloxy)-2-morpholin-4-yl-4H-chromen-4-one (81)

To 7-hydroxy-2-morpholin-4-yl-*4H*-chromen-4-one (63) (100 mg, 0.404 mmol) in acetonitrile (10 mL) was added potassium carbonate (350 mg, 2.5 mmol) followed

by benzyl bromide (67.3 μL, 0.56 mmol). The mixture was heated to 60°C overnight then cooled and concentrated *in vacuo* to obtain crude material (198 mg) which was purified by RP-HPLC. The appropriate fraction was lyphophilised to obtain the product (12.7 mg). This was dissolved in dichloromethane, dried with sodium sulphate and filtered, with the solvent removed *in vacuo* to give the pure product (8.8 mg, 7%). (Found: $[M + H]^{+\bullet}$, 338.1388. $C_{20}H_{19}NO_4$ requires $[M + H]^{+\bullet}$, 338.1392). δ_H (400 MHz; [D$_6$]DMSO; Me$_4$Si) 7.79 (1H, m, ArH), 7.45-7.32 (5H, m, ArH), 7.19 (1H, s, ArH), 7.03-7.01 (1H, m, ArH), 5.49 (1H, s, CH), 5.18 (2H, s, CH_2), 3.64-3.49 (8H, complex m, CH_2CH_2). Mass spectrum (ESI) m/z 338 $[M + H]^{+\bullet}$.

2-morpholin-4-yl-4-oxo-4H-chromen-6-yl acetate (82)

6-Hydroxy-2-morpholin-4-yl-*4H*-chromen-4-one (69) was suspended in dichloromethane (10 mL) under nitrogen. Triethylamine (37.1 μL, 0.266 mmol) and acetic anhydride (25.1 μL, 0.266 mmol) were added and the reaction was stirred overnight at room temperature. T.l.c indicated incomplete consumption of the starting material so additional triethylamine (37.1 μL, 0.266 mmol) and acetic anhydride (25.1 μL, 0.266 mmol) were added with stirring for a further 24 h. To the mixture was added 1M hydrochloric acid (15 mL) and extraction was completed with dichloromethane (3 x 20 mL). The organic extract was dried with sodium sulphate and the solvent removed *in vacuo* to give the desired product (10.7 mg, 15%). (Found: $[M + H]^{+\bullet}$, 290.1029. $C_{15}H_{15}NO_5$ requires $[M + H]^{+\bullet}$, 290.1028). δ (300 MHz; $CDCl_3$; Me$_4$Si) 7.83 (1H, s, ArH), 7.31 (1H, s, ArH), 5.61 (1H, s, ArH), 6.44 (1H, s, CH), 3.83-3.53 (8H, complex m, CH_2CH_2), 2.31 (3H, s, CH_3). Mass spectrum (ESI) m/z 290 $[M + H]^{+\bullet}$.

6-[2-(dimethylamino)ethoxy]-2-morpholin-4-yl-4H-chromen-4-one (83)

To *N,N*-dimethylformamide (3 mL) and potassium carbonate (83.8 mg, 0.607 mmol) was added 6-hydroxy-2-morpholin-4-yl-*4H*-chromen-4-one (69) (50.0 mg, 0.202 mmol) followed by 2-*N,N*-dimethylaminoethylchloride hydrochloride (43.9 mg, 0.303 mmol). The mixture was stirred under nitrogen at 100°C overnight. The reaction mixture was cooled, poured into water (3 mL) and extracted with

chloroform (2 x 30 mL). The organic extracts were washed with brine (5 mL), dried with sodium sulphate and evaporated in vacuo. The crude compound was packed onto reverse phase silica for RP-HPLC which yielded the product as a yellow oil (24.7 mg, 38%). (Found: $[M + H]^{+\bullet}$, 319.1656. $C_{17}H_{22}N_2O_4$ requires $[M + H]^{+\bullet}$, 319.1658). δ (400 MHz; $CDCl_3$; Me_4Si) 7.53 (1H, d, *J* 3.2, ArH), 7.19 (2H, s, ArH), 5.48 (1H, s, CH), 4.12 (2H, t, *J* 7.2, CH_2), 3.80-3.48 (8H, complex m, CH_2CH_2), 2.73 (2H, t, *J* 7.2, CH_2), 2.32 (6H, s, CH_3). Mass spectrum (ESI) m/z 290 $[M + H]^{+\bullet}$.

2-Morpholin-4-yl-8-phenoxy-4H-chromen-4-one (85)

8-Hydroxy-2-morpholin-4-yl-*4H*-chromen-4-one (28) (200 mg, 0.809 mmol) in dichloromethane (5 mL) was treated with phenylboronic acid (296 mg, 2.92 mmol), cuprous acetate (294 mg, 1.62 mmol) and triethylamine (244 μL, 1.62 mmol) and stirred for 48 h at r.t. After the addition of 1M HCl (3 mL), the mixture was extracted with dichloromethane (3 x 10 mL). The organic extracts were dried with sodium sulphate and the solvent was removed *in vacuo* to give the crude product (89.6 mg). The crude material was adsorbed onto reverse phase silica for RP-HPLC to obtain the purified product. M.p. 187-188°C. (Found: $[M + H]^{+\bullet}$, 324.1229. $C_{19}H_{17}NO_4$ requires $[M + H]^{+\bullet}$, 324.1236). δ_H (300 MHz; $CDCl_3$; Me_4Si) 7.90 (1H, dd, *J* 7.6 and 2.0, ArH), 7.37-7.30 (4H, m, ArH), 7.11 (1H, t, *J* 7.2, ArH), 6.96 (2H, d, *J* 8.0, ArH), 6.44 (1H, s, CH), 3.65-3.38 (8H, complex m, CH_2CH_2). δ_C (100 MHz; $[D_6]$DMSO; Me_4Si) 174.5, 161.4, 157.1, 144.8, 142.7, 130.0, 124.9, 124.4, 123.9, 123.1, 120.1, 116.5, 86.1, 65.1, 44.1. Mass spectrum (ESI) m/z 324 $[M + H]^{+\bullet}$.

2-Morpholin-4-yl-7-phenoxy-4H-chromen-4-one (86)

7-Hydroxy-2-morpholin-4-yl-*4H*-chromen-4-one (63) (0.200 mg, 0.809 mmol), phenylboronic acid (0.296 mg, 2.92 mmol), cuprous acetate (0.294 g, 1.62 mmol), triethylamine (244 μL, 1.62 mmol) and dichloromethane (5 mL) were combined and stirred for 48 h at r.t. Following the addition of 1M HCl (3 mL), the mixture was extracted with dichloromethane (3 x 10 mL). The organic extracts were dried with sodium sulphate and the solvent was removed *in vacuo* to give the crude product (67.9 mg). Purification by RP-HPLC gave the desired compound (8.4 mg, 3%).

(Found: $[M + H]^{+\bullet}$, 324.1231. $C_{19}H_{17}NO_4$ requires $[M + H]^{+\bullet}$, 324.1236). δ_H (400 MHz; $CDCl_3$; Me_4Si) 8.10 (1H, d, *J* 8.4, ArH), 7.43 (2H, m, ArH), 7.24 (1H, m, ArH), 7.10 (2H, d, *J* 7.6, ArH), 6.99 (1H, m, ArH), 6.77 (1H, s, ArH), 5.45 (1H, s, CH), 3.81-3.47 (8H, complex m, CH_2CH_2). Mass spectrum (ESI) m/z 324 $[M + H]^{+\bullet}$.

8-Methyl-2-morpholin-4-yl-7-phenoxy-4H-chromen-4-one (87)

A mixture of 7-hydroxy-8-methyl-2-morpholin-4-yl-*4H*-chromen-4-one (58) (0.30 mg, 0.115 mmol) in acetonitrile (1.2 mL) was treated with, copper acetate (21.0 mg, 0.115 mmol), phenylboronic acid (42.0 mg, 0.344 mmol) and triethylamine (80.0 µL, 0.574 mmol). The reaction was stirred overnight at r.t. and then heated at 80°C for a further 24 h. Upon cooling, the solid was filtered and washed with acetonitrile (5 mL). The filtrate was concentrated *in vacuo* and chromatography in 1-5% methanol in dichloromethane yielded the product (3.0 mg, 8%). (Found: $[M + H]^{+\bullet}$, 338.1388. $C_{20}H_{19}NO_4$ requires $[M + H]^{+\bullet}$, 338.1392). δ_H (300 MHz; $CDCl_3$; Me_4Si) 7.94 (1H, d, *J* 8.7, Ar), 7.36 (2H, dd, *J* 7.5 and 7.4, ArH), 7.14 (1H, dd, *J* 7.5 and 7.5, ArH), 6.98 (2H, d, *J* 7.5, ArH), 6.85 (1H, d, *J* 8.7, ArH), 5.59 (1H, s, CH), 3.88-3.51 (8H, complex m, CH_2CH_2), 2.36 (3H, s, CH_3). Mass spectrum (ESI) m/z 338 $[M + H]^{+\bullet}$.

Methyl 4-(acetylamino)-2-hydroxybenzoate (90)

To 4-aminosalicylic acid (88) (4.0 g, 26.1 mmol) in methanol (110 mL) was carefully added concentrated sulfuric acid (6.8 mL) drop-wise. After refluxing overnight, the solution was cooled and concentrated (20 mL) by removal of the solvent *in vacuo*. Water (80 mL) was added to the concentrated solution, which was then neutralised with sodium hydrogen carbonate. The resulting precipitate was filtered and dried to give methyl 4-amino-2-hydroxybenzoate (89) (3.81 g, 87%) as the product.

To methyl 4-amino-2-hydroxybenzoate (89) in ethanol was added acetic anhydride drop-wise. The mixture was heated at 50°C for 90 min. Following cooling, the mixture was poured into water (20 mL). The mauve precipitate was filtered and dried to give the desired product (0.46 g, 74%).

Both compounds (89 and 90) were confirmed to be identical to those reported in the literature by mass spectrometry and proton NMR[118].

N-[3-hydroxy-4-(3-morpholin-4-yl-3-oxopropanoyl)phenyl]acetamide (91)

Methyl 4-(acetylamino)-2-hydroxybenzoate (90) (0.4 g, 1.9 mmol) was reacted according to Method A to yield an oil which was purified by chromatography eluting with 0–10% methanol in dichloromethane. The only product which could be isolated was a yellow oil (0.28 g) which was identified by proton NMR as starting material.

8-(Benzylamino)-2-morpholin-4-yl-4H-chromen-4-one (92)

To dry dichloromethane (5 mL) with molecular sieves was added the 8-boronate (34) (150.0 mg, 0.420 mmol), followed by the addition of copper (II) acetate (76.3 mg, 0.420 mmol), benzylamine (45.9 μL, 0.420 mmol) and triethylamine (116.0 μL, 0.840 mmol). The reaction was stirred at r.t. overnight with a drying tube. The resulting material was concentrated and the residue purified using RP-HPLC to obtain a yellow solid (30.1 mg, 21%). (Found: $[M + H]^{+\bullet}$, 337.1547. $C_{20}H_{20}N_2O_3$ requires $[M + H]^{+\bullet}$, 337.1552). δ_H (300 MHz; $CDCl_3$; Me_4Si) 7.46-7.18 (7H, m, ArH), 6.85 (1H, d, *J* 7.5, ArH), 5.94 (1H, s, CH), 4.49 (2H, s, CH_2), 3.84-3.52 (8H, complex m, CH_2CH_2). δ_C (100 MHz; [D_6]DMSO; Me_4Si) 175.2, 162.1, 139.6, 136.6, 128.3, 126.6, 126.0, 124.7, 123.8, 122.0, 112.7, 110.2, 86.4, 65.4, 45.7, 44.6. Mass spectrum (ESI) m/z 337 $[M + H]^{+\bullet}$.

8.4 Biological Assay Protocols

Phosphodiesterase Separation Protocol

Human blood was obtained from consenting healthy volunteers (maximum 400 mL) who had not received antiplatelet medication in the previous two weeks. Whole blood was collected in the presence of 70 mM theophylline as an anticoagulant (9:1) in 50 mL tubes. Platelets were isolated and washed using a modified method of Baezinger and Majerus.[84] Platelet rich plasma was separated from the erythrocytes by centrifugation of the whole blood at 200 *g* for 30 min. Platelets were then pelleted

from the PRP by centrifugation at 2,000 *g* for 30 min and the plasma was removed. The platelet pellet was gently washed by resuspension in a tenth of the original volume of whole blood in platelet washing buffer (PWB) made from modified Ca^{2+}-free Tyrode's solution [3.5 mg/mL blood serum albumin (BSA), 50 i.u./mL heparin, 90 μg/mL apyrase and 5 mM Pipes buffer, pH 6.5]. The platelets were then repelleted at 2,000 *g* for 20 min, the PWB was removed and the platelets resuspended for washing in heparin and BSA free PWB. Following the second wash, centrifugation at 2,000 *g* for 20 min and removal of the PWB resulted in a platelet pellet suitable for lysis.

Ice-cold lysis buffer [50 mM Tris, adjusted to pH 6.0 with acetic acid, 1 mM EDTA, 1 mM PMSF, 5 mM 2-mercaptoethanol, 4 μM pepstatin, 20 μM chymotrypsin, 40 μM leupeptin] was added to the platelet pellet (4 mL/100 mL whole blood). An ice-cold Dounce homogeniser was used to mechanically assist in platelet lysis. This was followed by sonication at 0°C which was repeated 6 times for 15 s each time. To remove any remaining intact platelets or granules, the suspension was centrifuged at ca. 19,000 *g* for 20 min. Platelet debris was discarded and the supernatant was ultracentrifuged at ca. 100,000 *g* for 1 h. The soluble protein supernatant was collected and stored on ice anticipating separation.

Phosphodiesterase activities were separated from the soluble protein supernatant over 120 min using ion-exchange chromatography on a DEAE-Sepharose Fast Flow column (5 mL) and a BIORAD BioLogic DuoFlow system. The method of Dickenson *et al* with some modifications.[72] The column was equilibrated with a Tris buffer (50 mM Tris, 1 mM EDTA, adjusted to pH 6.0 with acetic acid). The soluble protein supernatant was diluted to 50 mL using the Tris buffer to ensure low conductivity and then pumped directly onto the column at 5 mL/min. The phosphodiesterase activities were eluted with a linear gradient of increasing the salt concentration to 1M sodium acetate over 2 h with a flow rate of 1 mL/min. All chromatography buffers were stored at 4°C and kept on ice during use. Fractions (2 mL) were collected and stored at 0°C.

Phosphodiesterase Assay

Assays to evaluate phosphodiesterase activity were carried out using thawed aliquots of the desired phosphodiesterase isoform, isolated and identified as described in the Chapter 4. A typical PDE2 assay consisted of 70 μL of HEPES buffer to which 0.5 μL DMSO or inhibitor, 1 μL 1 mM cGMP (final concentration 10 μM) and 20 μL PDE2 were added followed by mixing in an incubator at 37°C for 90 s. The enzymatic reaction was initiated by the addition of 10 μL 300 μM cAMP (final concentration 30 μM) and the reaction terminated after five min by placing the reaction vessel in an oven at 100°C for 3 minutes. Identical conditions were used with PDE3, except with cGMP being omitted and the concentration of cAMP being reduced to 5 μM (final concentration 0.5 μM).

Analysis was carried out RP-HPLC, with an injection of 50 μL of the assay volume onto either a Zorbax Eclipse XBD-C_6 or Xterra RP C_{18} column for PDE2 and only the latter column for PDE3. Peak detection was carried out at 254 nm.

Phosphodiesterase activity was determined as the peak area of AMP (mAU) measurement being reported as a percentage of the DMSO control (where the control without any inhibitor present indicates maximal AMP production under the assay conditions). Unless otherwise stated, all assays were completed at least in triplicate for PDE2 and at least duplicate for PDE3. Data is presented as the mean ± the S.E. IC_{50} is defined as the concentration of inhibitor where 50% of the AMP production has been inhibited as compared to the DMSO control.

Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

A Bio-Rad Mini-Protean II dual slab gel apparatus was used to perform SDS-PAGE, according to the method of Laemmli.[85] A 7.5% resolving gel [0.375 M Tris-HCl, pH 8.8, 0.1% (w/v) SDS, 7.5% (w/v) acrylamide:bisacrylamide (30:0.8), 0.75 mg/mL ammonium persulphate and 0.075 % (v/v) TEMED] was poured into the gel

apparatus and allowed to set for 60 min. A 4% stacking gel [0.125 M Tris-HCl, pH 6.8, 0.1% (w/v) SDS, 4% (w/v) acrylamide:bisacrylamide (30:0.8), 7.5 mg/mL ammonium persulphate and 0.1 % (v/v) TEMED] was applied on top of the set resolving gel and a gel comb inserted. The stacking gel was allowed to set for 60 min prior to insertion of the entire gel into the running apparatus and immersion in SDS-PAGE running buffer [25 mM Tris, pH 8.7, 192 mM glycine and 0.1% (w/v) SDS]. Protein samples, obtained from each of the collected fractions following ion-exchange chromatography, were mixed with an equal volume of 2x Laemmli reducing buffer (0.5 ml 0.25 M Tris-HCl, pH 6.8, 0.2 mL glycerol, 0.6 mL 25% SDS, 0.04 mL Bromophenol Blue, 0.1 mL β-mercaptoethanol) and boiled for 5 min prior to loading onto the gel. Proteins were separated using a constant volume of 200 volts over 2 h. Proteins of known molecular weight were also electrophoresed to enable the estimation of the molecular weights of unknown proteins by comparison of relative mobilities, according to the method described by Shapiro *et al.*[119] The migration of proteins was monitored by Bromophenol Blue contained in the sample buffer, and electrophoresis was terminated once the dye front reached the bottom of the gel. The separated proteins were stained for 30 min with Coomassie Brilliant Blue solution [7% (v/v) acetic acid, 25% (v/v) methanol, 0.1% Coomassie Brilliant Blue] prior to being destained [10% (v/v) acetic acid and 25% v/v) methanol] until the gel background was clear. Gels were washed extensively in water to remove acetic acid before drying under vacuum overnight.

PI3-Kinase Assay

PI3-kinase assays were carried out according to the method of Jackson *et al* with some modification.[89] PI3-kinase activity was derived by immunoprecipitation of the enzyme from platelets using a sepharose-linked antibody to the p85 regulatory domain of the enzyme. The immunoprecipitate was then diluted in kinase assay buffer (20 mM HEPES, pH 7.4, 0.25 mM EDTA, 5 mM $MgCl_2$), then mixed with sonicated phosphatidylinositol 4,5-bisphosphate (30 μg/mL), 1 μL of the test compound or DMSO and [^{32}P]-ATP (50 μM, 1 μCi/nmol) to a final reaction volume

of 100 μL. Following incubation of the enzyme assay for 20 min at r.t., the reaction was stopped with 100 μL of 1 M hydrochloric acid and the phospholipids were extracted with a mixture of 200 μL of chloroform/methanol (1:1) and 500 μL of 2 M potassium chloride. Aliquots (100 μL) of the extract were collected and the radioactivity counted. For more detailed analysis, the ^{32}P-phospholipids were resolved by thin layer chromatography using a chloroform:methanol:acetic acid:water (43:38:5:7) solvent system and detected by a Phosphorimager (Storm 820 Amersham). Individual phospholipids were quantitated by densitometry.

Platelet Aggregation Assay

Platelets were washed according to the method of Schoenwaelder *et al*[90] whereby whole blood (maximum 400 mL per donor) was collected into anticoagulant (acid-citrate-dextrose or ACD consisting of 90 mM sodium citrate, 7 mM citric acid, pH 4.6, 140 mM dextrose and 70 mM theophylline) where the ratio was 9 volumes of blood to one volume of ACD. The blood was centrifuged at 200 *g* for 30 min and the platelet rich plasma removed. Centrifugation of the PRP at 2,000 *g* for 10 min pelleted the platelets which were then resuspended in platelet wash buffer (4.3 mM $NaHPO_4$, 24.3 mM NaH_2FO_4, 4.3 mM K_2HPO_4, pH 6.5, 113 mM NaCl, 5.5 mM glucose, 10 mM theophylline, 0.5% bovine serum albumin) to 10% of the original blood volume. Platelets were stored, until used, in a 37°C water bath.

Washed platelets in PWB were centrifuged at 4000 *g* for 2 min. The PWB supernatant was discarded and the platelets were resuspended in Tyrode's buffer at pH 7.3 containing 1mM $CaCl_2$. To 100 μL of resuspended platelets was added 32 μL of 6.25 μg/mL fibrinogen (to give a final concentration of 0.5 μg/mL) and 268 μL Tyrode's buffer/1mM $CaCl_2$, resulting in a final volume of 400 μL. Platelet samples were placed in a 4 channel aggregometer and incubated at 37°C for 10 min with 1 μL of test compounds at various concentrations or with DMSO as a control. An appropriate agonist (such as ADP, CRP, HAI, TRAP, PAR4AP), at a concentration sufficient to give complete aggregation as earlier determined by a dose-response

curve, was then added to the treated platelets to induce aggregation. Aggregation was monitored over 10 min for each sample, and the extent of aggregation determined as the 0% transmission of control aggregations at t = 4 min.

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