Bacterial Autoinducer Derived 4-Quinolones as Novel Immune Modulators

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Abstract

Immunological disorders, including those of an autoimmune nature, cause chronic morbidity and disability. Common conditions, such as rheumatoid arthritis and psoriasis as well, as the less commonly occurring multiple sclerosis and myasthenia gravis are known to have an autoimmune basis. These disorders are not well controlled with current therapies. The main problems include the lack of specificity of action thereby leading to a number of adverse drug effects. For example, severe nephrotoxicity is associated with tacrolimus and ciclosporin; newer monoclonal antibody therapies have the potential for immunological toxicity. Thus the search for alternative immune modulating agents is a well sought after objective.

Recently, the quorum sensing signal molecules, *N*-(3-oxododecanoyl)-L-homoserine lactone (OdDHL) and 2-*n*-heptyl-3-hydroxy-4(1*H*)-quinolone (PQS), isolated from *Pseudomonas aeruginosa*, were found to possess the ability to modulate the immune response. Structure activity relationship (SAR) studies using OdDHL as the lead molecule had been undertaken but no work has been done to maximise the potential of PQS. The immune modulatory activity of PQS suggests that an evaluation of its synthetic analogues may provide better understanding of the structural components that influence its activity and may lead to the development of novel therapeutic agents. In order for a SAR study to be attempted with PQS a more efficient synthetic route has to be developed to obtain practical quantities of this material.

To address these issues, the research in this thesis firstly investigates a number of synthetic routes towards the preparation of PQS and its synthetic analogues. The successful synthetic routes yielded analogues with alterations to the main structural components of PQS, namely the alkyl side chain, *N*'-substitution, 3-hydroxyl group and substitution on, and in, the carbocyclic ring. Structural alterations were made singularly or in combination in these areas of the molecule. Besides these quinolone-based analogues, a number of quinazolinone-based variants were also elaborated.

All analogues were characterised for identity and purity, then tested for immune modulatory activity in a concanavalin A mitogen stimulated murine cell proliferation assay and their cytotoxicity using a dye exclusion assay. Bioactivity was shown to be dependent on the length of the 2-akyl side chain, with extensions to the chain more tolerated than a reduction. The nature of the substitution at the 3-position has an influence on activity, whereas a proton on the nitrogen is not essential. The less polar and more lipophilic molecules displayed increased bioactivity. The core quinolone structure can tolerate insertion of extra heteroatoms the heterocyclic ring and substitution of chlorine on the carbocyclic ring, while retaining an acceptable level of activity. A similar trend was observed in the quinazolinone derivatives.

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Abbreviations

Δ	Heat
μM	Micromolar
Ac	Acetyl
Ac_2O	Acetic anhydride
AcOH	Acetic acid
AHL	*N*-Acyl homoserine lactone
AI	Autoinducer
Anal.	Elemental analysis
aq	Aqueous
BHL	*N*-Butanoyl-L- homoserine lactone
BNF	British National Formulary
br	Broad
^{t}Bu	*tert*-butyl
BuLi	Butyllithium
°C	Degrees centigrade
$CDCl_3$	Deuterated chloroform
CDI	1,1'-Carbonyldimidazole
CF	Cystic fibrosis
CMI	Cell mediated immunity
CNS	Central nervous system
ConA	Concanavalin A
CSF	Colony-stimulating factor
CTCM	Complete cell culture medium
d	Doublet
DCCI	1,3-Dicyclohexylcarbodiimide
DCE	Dichloroethane
DCM	Dichloromethane
DCU	N,N'-Dicyclohexylcarbodiimide
DIPEA	Diisopropylamine
DHFR	Dihydrofolate reductase
DKP	Diketopiperazine

DMAP	4-Dimethylaminopyridine
DMARD	Disease-modifying antirheumatic drug
DMF	Dimethylformamide
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
E	Electrophile
Et	Ethyl
EtOH	Ethanol
FK506	Tacrolimus
FKBP	FK506 binding protein
FKBP12	FK binding protein-12
For	Formyl
g	Gram(s)
GCSF	Granulocyte-colony stimulating factor
h	Hour(s)
HAQs	4-Hydroxy-2-alkylquinolines
HBSS	Hanks' balanced salt solution
Hexamine	Hexamethylenetetramine
HHL	*N*-Hexanoyl-L-homoserine lactone
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IMDP	Inosine monophosphate dehydrogenase
IMPDH	Inosine 5'-monophosphate dehydrogenase
IR	Infrared
LDA	Lithium diisopropylamide
m	multiplet
M	Molar
mAb	Monoclonal antibody
Me	Methyl
MeOH	Methanol
MHC	Major histocompatibility complex
Min	Minute(s)

mL	Millilitre
mm	Millimetre
MMF	Mycophenolate Mofetil
mmol	Millimole
mol	Moles
m.p.	Melting point
MS	Mass spectroscopy
mTOR	Mammalian target of rapamycin
m/z	Mass/charge ratio
NaOH	Sodium hydroxide
NK	Natural killer
NMP	*N*-Methylpyrrolidone
NMR	Nuclear magnetic resonance
NT	Not toxic
OdDHL	*N*-(3-Oxododecanoyl)-L-homoserine lactone
OHHL	*N*-(3-Oxohexanoyl)-L-homoserine lactone
P. aeruginosa	*Pseudomonas aeruginosa*
Ph	Phenyl
PLC	Preparative thin-layer chromatography
$POCl_3$	Phosphorus oxychloride
PPE	Polyphenyl ether
PQS	*Pseudomonas* quinolone signal
PPTS	Pyridine *p*-toluenesulphonate
PTK	Protein tyrosine kinase
q	Quartet
RNA	Ribonucleic acid
QS	Quorum sensing
QSSM	Quorum sensing signal molecules
R_f	Retention factor
s	Singlet
S	Cytostatic
SAR	Structure activity relationship
t	Triplet
TBI	Total body irradiation

T^{C1}	Type 1 Cytotoxic T cell
T^{C2}	Type 2 Cytotoxic T cell
TFA	Trifluroacetic acid
TGF-β	Transforming growth factor-β
T^{H1}	Type 1 T-Helper cell
T^{H2}	Type 2 T-Helper cell
THF	Tetrahydrofuran
TLC	Thin layer chromatography
TMS	Tetramethylsilane
TNF-α	Tumour necrosis factor alpha
TNF-β	Tumour necrosis factor beta
TOF	Time of flight
p-TSA	*p*-toluene sulphonic acid
UHP	Urea-hydrogen peroxide
Z	Benzyloxycarbonyl

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Chapter 1: Introduction

Chapter 1

Introduction

The human immune system provides a defence against a wide range of pathogenic organisms, such as bacteria, viruses and parasites. It consists of physical barriers, including skin and mucous membranes, non-specific cellular defences, referred to as the innate response, and targeted cellular responses, specific to the invading organism, which forms the adaptive response. Disorders of the immune system may be due to immune deficiency, hypersensitivity and autoimmunity. Autoimmunity occurs when the distinction between self and non-self, crucial to the function of the immune response, is lost and there is an immune response mounted against the body's own tissues. These disorders may be treated by the use of immune suppressants, such as steroids, ciclosporin and cytokine inhibitors, although these therapies are not ideal, due to the potential for serious adverse drug reactions. Due to this, the identification and development of novel immune modulating therapies is warranted. The recent discovery of the ability for signal molecules, involved in the bacterial communication method known as quorum sensing, to modulate the immune system has led to interest in investigating this ability further.

1.1 Immune system

The immune system serves as a defence against microbes which possess the ability to cause harm to a host organism via a diverse range of pathogenic mechanisms, concurrently avoiding responses that cause damage of host tissues. To combat this wide range of pathogenic mechanisms, the immune response utilises complex and varied processes to eradicate or control organisms that present a threat.

The immune system consists of two main parts, defined by the speed and specificity of the response mounted. These two parts are not independent and there is a great deal of interaction between the two but they remain distinct

in their components and their mode of response. These two parts of the immune system are termed the innate and adaptive responses.

1.1.1 Innate Immunity

Innate immunity consists of relatively non-specific elements that operate against nearly all substances that threaten the body and are able to act rapidly in providing the initial host response. These elements include physical or chemical barriers and cellular defences[1]. The lack of specificity of the response of innate cellular defence means that the innate response may lead to harm being caused to the host. A level of innate immunity passed from mother to child is present for a time following birth.

1.1.1.1 Physical and chemical barriers

These can be considered as the first line of defence employed by the host against infection. The main function of these barriers is to prevent access of foreign or infectious bodies to the host and may not commonly be seen as part of the immune system.

1.1.1.1.1 Physical Barriers

Physical barriers mainly comprise of the skin and mucous membranes. Most substances and organisms cannot gain access to the host through intact skin, but will be able to through areas of damage where the integrity of the skin is compromised. Mucous membranes are present on the respiratory, genitourinary and gastrointestinal tracts and aid in immunity by trapping organisms, thereby preventing them entering the host. The organisms are then removed towards external openings via ciliated epithelial cells.

1.1.1.1.2 Innate Chemical Factors

Chemical factors, such as an acidic pH, fatty acids and hydrolytic enzymes can prevent entry through hair follicles and sebaceous glands. The presence of various hydrolytic enzymes in saliva, low pH of the stomach and proteolytic enzymes in the small intestine also contributes to a hostile environment for microorganisms[1].

Additionally, small molecules and proteins, such as interferons and components of the complement system[2] that are found in the serum contribute to non-specific immune defences. Activation of complement components produced in response to various organisms can induce an enzymatic cascade, which will target the membrane of pathogenic organisms, leading to their death.

1.1.1.1.3 Cellular defences

If an invading organism gains access to the body, past the chemical and physical barriers referred to above, the next line of defence involves a range of specialised cells intended to remove the foreign body.

Although the innate response is not antigen-specific, it still exhibits the ability to distinguish between foreign molecules and self. Phagocytes can recognise pathogen-associated molecular patterns that are present on microbes but not on host cells. Examples of these are found on Gram negative bacteria (lipopolysaccharide), Gram positive bacteria (lipotechoic acid) and yeast cells (mannans).

The ability for the innate immune response to have this recognition is one factor that shows the close relationship between the innate and adaptive response. The pattern-recognition receptor can recognise the broad patterns on microbes and can then present the processed product onto the antigen specific T cells.

The innate response is largely confined to extracellular organisms, as the majority of the processes required to remove the pathogen needs exposure to the surface of the organism. Hence the innate system is not able to recognise, or remove, intracellular organisms such as viruses.

1.1.1.1.3.1 Granulocytes

Neutrophils

One of the most important aspects of the innate immune response is the recruitment and activation of neutrophils at the site of the infection, in order to remove the pathogenic organism[3]. Neutrophils are normally constantly present in the blood stream and can localise on a site of infection through a multistep process involving proinflammatory mediators, adhesion molecules, chemo attractants and chemokines[4].

Macrophages

Macrophages are highly phagocytic towards microbes that have been bound by immunoglobulins (Ig) or complement. They are mobilised following the recruitment of neutrophils and remain at sites of inflammation and infection for extended periods[4].

Eosinophils

The main role of eosinophils is to protect the host from parasitic infections. Infections of this type cause the production of IgE, which coats the cell. Eosinophils have low affinity receptors for IgE and bind to the cell surface, secreting highly cytotoxic materials onto the surface of the invading cells.

Natural Killer Cells

Natural killer (NK) cells have two methods of recognising abnormal cells. They are able to bind to targets coated with antibody, leading to a cytotoxic response. Additionally, they can interact with major histocompatibility (MHC) class 1, normally expressed on all nucleated cells. If they are not able to bind to the MHC, the NK cells lyse the target[5]. This is achieved by the secretion of perforins onto the surface of the adhered cell, leading to the formation of pores in the cell membrane. Granzymes are injected through these pores, initiating apoptosis[1].

Basophils and Mast Cells

Basophils and mast cells are the initiators of immediate hypersensitivity reactions. This is achieved through the release of histamine and other mediators, leading to tissue inflammation, oedema and smooth muscle contraction.

1.1.1.1.4 Cell Targeting

For the cells involved in the innate immune response to be effective they need to be targeted to the site of infection. This may be achieved internally by the interaction of cellular receptors or externally through signals that influence the direction of these cells, such as adhesion molecules and chemokines.

1.1.2 Adaptive Immunity

Adaptive immunity is a more specific form of immunity, involving an encounter with a foreign substance leading to the production of a specific immune response to that foreign agent. The ability to mount such an immune response against specific invaders is present from birth but requires the initial exposure to foreign material, termed an antigen. The specificity of the adaptive immune response means that there is much less chance of damage to the host as the response is targeted against the foreign body.

Alongside the specificity of this response, another important feature is the memory that is retained following previous exposure to foreign material. The length of time that this is retained varies but may last the lifetime of the individual. This memory allows the host to respond more rapidly, and to a greater extent, following re-exposure to a foreign material.

The two major types of cell involved in acquired immunity are T-lymphocytes, originating from the thymus, and B-lymphocytes, originating in the bone marrow.

1.1.2.1 T-Lymphocytes

There are a number of different types of T-lymphocyte and these are distinguished by the expression of the glycoproteins CD4 and CD8 on the cell surface. The majority of T cells present in circulation are $CD4^+$ (60 – 70%) with the rest being $CD8^+$. These two types of T cells will develop into subsets with distinctly different function after exposure to antigen[6]. $CD4^+$ T cells develop into two different types of T helper cells (T^{H1} and T^{H2}) following the stimulation of an antigen and the type of T helper cell they become depends on the cytokines present at the site of infection[7]. At the beginning of an immune response a T^H cell can exhibit characteristics of both T^{H1} and T^{H2} but after prolonged time the response will be predominately one of the two types. T helper cells are characterised by the immune products they release and T^{H1} generally support cell mediated immune response, whereas T^{H2} aid in humoral, anthelmintic and allergic responses. $CD8^+$ T cells can develop into cytotoxic T cells, designated either T^{C1} or T^{C2} cells[8].

Acquired immunity that is regulated by T-lymphocytes is termed cell-mediated immunity (CMI). In order for the T cell to recognise an infected host cell, thereby recognising both self and non-self, the cell surface glycoproteins, MHC molecules, are made use of. Class I MHCs are those that have been synthesised within the cell[9], whereas class II MHCs have been proteolytically processed following ingestion.

1.1.2.2 B-Lymphocytes

B cells develop from stem cells and their development depends on stromal cells and their release of IL-7 and through their maturation and development will assemble an antigen binding component within the B cell receptor. There are two types of B-lymphocytes, T dependent, requiring T cells to produce antibodies, and T independent[1].

Antigens may activate both T and B cells and this will cause the B cell to mature under the influence of helper T cells. Following exposure to the antigen, random mutations in the antigen binding region of the B cell will take place[4]. If these mutations cause less affinity with the antigen, these cells will

not receive important growth signals and will die. If the mutations increase the affinity for the antigen the cell will proliferate and dominate the responding cells[10]. This process allows the development and retention of B cell memory[11].

T cell independent responses are possible when a B cell is stimulated by a polymeric antigen. The somatic mutation does not happen in these responses and the resulting memory is therefore weak[4].

1.1.2.3 Cytokines and Chemokines

Cytokines and chemokines are secreted proteins whose functions include growth, differentiation and activation. They regulate the nature of the immune response and control many factors in the immune cellular response.

Cytokines and chemokines are also involved in allergic responses and play a roll in, amongst other things, mast cell growth and histamine release.

1.1.2.3.1 Cytokines

Cytokines are involved in almost every area of immunity and inflammation. Their influence extends across innate immunity, antigen presentation, cellular recruitment and adhesion molecule production.

A cascade response can be seen following the production of cytokines. The function of a cytokine can vary depending on its source, target and phase in the immune response in which the cytokine is presented. The cytokines produced when responding to an immune incident can determine the nature of the immune response, whether it is cytotoxic, allergic, cell mediated or humoral.

Cytokines may be divided into interleukins (ILs), interferons (IFNs), colony-stimulating factors (CSFs) and tumour necrosis factors (TNFs). Examples of where a range of cytokines act within the immune response and their results are shown in Table 1.1. Important members of this class of molecule are further discussed below.

Table 1.1 Areas and results of cytokine action within the immune system

Form of immunity	*Cytokine*	*Result*
Innate	IL-1, IL-6, IFN-α, TNF-α, IFN-γ	Acute phase response in liver
Cellular	IL-2, 10, 12, 18, 23, IFN-γ	T cell proliferation Macrophage activation
Humoral and allergic	IL-3, IL-4, IL-5, IL-9, IL-10, IL-13 IL-25, TNF-α	IgE production Inflammatory cell recruitment

Interleukin-2

Interleukin-2 (IL-2) expression is important in the proliferation of T cells. IL-2 is able to allow the proliferation of T cells that will be specific to the antigen that incites the immune response. T cells are initially stimulated, causing the release of IL-2, which then leads to the T cell proliferation.

Tumour Necrosis Factor

Tumour necrosis factor (TNF) consists of two homologous proteins TNF-α and TNF-β, produced primarily by mononuclear phagocytes and lymphocytes respectively. TNFs bring about antitumour immunity and are able to initiate direct cytotoxic effects on cancerous cells. They are also able to induce adhesion molecules, activate neutrophils therefore mediating chemotaxis, adherence and degranulation.

Interferons

Interferons (IFN) are a group of cytokines that are so called due to their ability to "interfere" with viral growth. There are three types of interferons, IFN-α, IFN-β and IFN-γ. IFN-α has a significant antiviral activity through inhibiting viral replication, protecting uninfected cells and stimulating antiviral immunity. IFN-β is similar in biological effect to IFN-α. IFN-γ has a weak antiviral activity but is the primary cytokine responsible for cell-mediated immunity[12]. It

induces the accumulation of macrophages at the site of cellular immune responses, allowing these macrophages to remove intracellular pathogens.

1.1.2.3.2 Chemokines

Chemokines are small molecules whose prime function is to induce chemotaxis in a range of cells, including neutrophils, lymphocytes and eosinophils. New functions for chemokines are still being discovered, extending their role beyond chemotaxis. Haemostatic functions associated with the adaptive immune response, such as lymphocyte trafficking and haematopoiesis, are now recognised to involve the action of chemokines. Chemokine receptors can bind to more than one ligand, allowing an overlap of function. Table 1.2, shows the main functions of chemokines divided into inflammation and homeostasis but the functions of many known chemokines remain unknown. Chemokines can be divided into four sub-families dependent on the positioning of their N-terminal cysteine residues.

Table 1.2 Functions of chemokine action within the immune system

Physiological function	***Chemokine***
Inflammation	CCL1, CCL2, CCL3, CCL4, CCL5, CCL7, CCL8, CCL11, CCL13, CCL17, CCL20, CCL22, CCL2, CCL26, CCL28, CXCL1, CXCL2, CXCL3, CXCL8, CXCL9, CXCL10, CXCL11, CXCL16, CX3CL1
Homeostasis	CCL17, CCL18, CCL19, CCL20, CCL21, CCL22, CCL25, CCL27, CCL28, CXCL13, CXCL14
Unknown	CCL6, CCL9, CCL10, CCL12, CCL14, CCL15, CCL16, CCL23, XCL1, XCL2, CXCL4, CXCL5, CXCL6, CXCL7, CXCL12, CXCL15

1.2 Immunopathology

The immune system is able to protect the host organism against the threat posed by a large range of pathogens and other environmental challenges. It is extremely adaptive and able to account for some deficiency in its function. Despite this it is still liable to fail and may do this in three main ways. The balance of the immune system may be restored though immune modulation.

1.2.1 Immunodeficiency

When the immune system is unable to defend the host against pathogenic organisms it may be seen to have become deficient. The causes may be genetic in nature, secondary to disorders such as leukaemia or non-immunological abnormalities, or the result of cytotoxic chemotherapy in the treatment of malignancy.

An example of how immunodeficiency may be treated is the use of filgrastim, which is recombinant human granulocyte-colony stimulating factor (GCSF), in the case of neutropenia occurring due to chemotherapy[13].

1.2.2 Hypersensitivity

Hypersensitivity occurs when the response to an allergen, or non-microbial antigen, is out of proportion and causes an adverse reaction above and beyond the capability of the original stimulus. The term “allergy” refers to the symptoms and signs that are present with exposure to allergens. This type of disease may be termed atopic in nature. There appears to be a genetic element to atopic illness[14]. The most serious example of this type of immunopathology is the massive immune response of anaphylaxis which can be caused by a seemingly innocuous material. Other types of atopic disease include hay fever, asthma and eczema.

1.2.3 Autoimmunity

The immune system normally differentiates between self and non-self. In some cases this distinction can break down, leading to an immune response directed at the host, termed autoimmunity. Autoimmune disease is when recognition of self as non-self leads to pathological consequences, involving antibody, complement, immune complexes and cell-mediated immunity.

A growing number of diseases are thought to have an autoimmune basis, although it is not always straightforward to class diseases as such. The cause of autoimmune disease is thought to be multifactorial, with possible genetic bases, environmental factors and the involvement of a number of potential triggering factors. Two main ways in which autoimmune diseases develop are the bypassing of unreactive T cells or the stimulating of autoreactive T cells.

1.2.3.1 Genetic Factors

Genetic factors are important in the development of autoimmune conditions[15], although the area is not currently well understood. The majority of autoimmune conditions appear to be polygenic[16] and therefore are difficult to study effectively. There are a small number of conditions in which a single mutant gene is responsible, such as polyendocrinopathy syndrome type 1[17].

1.2.3.2 Environmental Factors

Environmental factors also play a central role in the initiation of autoimmune conditions, with viruses being the main focus of attention. Again, this area is not fully understood currently and the role of environmental factors in the development of autoimmune conditions needs further investigation.

Infection appears to be a major environmental factor in the development of autoimmune conditions, as can be seen from epidemiological and clinical data[18]. The mechanisms that have been suggested to cause autoimmunity as a result of virus infection are polyclonal lymphocyte

activation[18], antigen mimicry[19-21] and increased immunogenicity of organ autoantigens following inflammation due to infection[22,23].

Another example of infection causing autoimmune disease is the development of Chagas' disease during infection with *Trypanosoma cruzi*, causing autoimmune myocardial damage. The antigens of the pathogen bind to host cells, causing antibodies and T cells to lyse these cells.

1.2.3.3 Division of autoimmune diseases

Autoimmune diseases can be divided according to the dominant mechanism involved in pathogenesis: antibody, immune complex formation or effector mechanisms of cell-mediated immunity.

Antibody-mediated autoimmune diseases include haemolytic anaemia, when antibodies react with red blood cells, and myasthenia gravis, where the target for the antibodies is the acetylcholine receptor at the neuromuscular junction.

Systemic lupus erythematosus is an example of immune complex-mediated autoimmune disease. The disease attacks many organs of the body and the central nervous system, due to the unnaturally high levels of anti-DNA antibodies produced, possibly due to enhanced helper T cell activity or the inability of phagocytic complexes to clear immune complexes.

T cell-mediated autoimmune diseases include Type 1 diabetes mellitus, involving the destruction of insulin producing β-islet cells by inflammation and rheumatoid arthritis, in which the synovium can be stimulated to produce pro-inflammatory cytokines and enzymes that break down tissue This results in inflamed joints and destruction of cartilage and bone[1].

1.2.3.4 Treatment of autoimmune diseases

The treatment of autoimmune disorders is currently based in suppression of the immune response to lessen the damage caused by the self directed immune response.

The most commonly used current immune modulating drugs have been isolated from soil bacteria and soil fungi. The potential for a drug developed

from a human pathogen may allow a more targeted effect and reduce the side effects that are associated with immune therapy.

1.3 Immune Therapies

1.3.1 Irradiation

A non-drug method for immune suppression therapy is the use of radiation. This can be in the form of total body irradiation (TBI) or irradiation of relevant areas of the body and the immune system, such as total lymphoid irradiation.

TBI is used for the conditioning of patients before they undergo haematopoietic cell transplantation. After the exposure 80 % of lymphocytes undergo intermitotic death, including B lymphocytes and the precursors to all T cells. There is an additional effect in the ability for cells to localise to sites around the body.

Total lymphoid irradiation is used in treatment of Hodgkin's disease, solid organ graft rejection and severe cases of rheumatoid arthritis. The local irradiation does not increase susceptibility to infection but does produce lymphopenia and affects hypersensitivity responses.

1.3.2 Immune modulating drugs

There are a number of drugs in clinical use which modulate the immune response. The majority of these are for the area of immune suppression and are commonly used following organ transplant or in the treatment of autoimmune conditions. There are also immune stimulants but the use of these in a clinical setting is more limited.

1.3.2.1 Immune Suppressants

Immune suppressants can be divided into groups based on their principal mode of action although some agents may have modes of action that can be categorised in more than one area. These mechanisms are limited to five main groups; regulation of gene expression, alkylation, inhibition of *de novo* purine

synthesis, inhibition of pyrimidine synthesis and kinase and phosphatase inhibitors [24]. Table 1.3 summarises the various drugs currently used and groups them in relation to their mode of action

Table 1.3 Mechanisms of action in immunosuppressive drugs

Mechanism of Action	***Drug***
Regulation of gene expression	Glucocorticoids
Alkylation	Cyclophosphamide
Kinase and phosphatase inhibitors	Ciclosporin Tacrolimus Sirolimus
Inhibition of de novo purine synthesis	Azathioprine Mycophenolate Mofetil Mizoribine Methotrexate
Inhibition of de novo pyrimidine synthesis	Leflunomide Brequinar Methotrexate

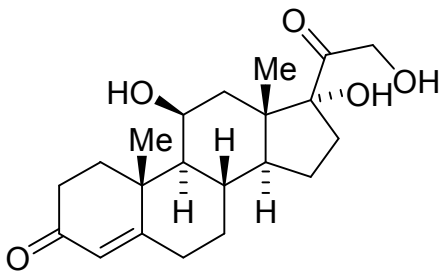
More recently there has been the introduction of another group of immune modulating drugs, these having their action through cytokine inhibition.

1.3.2.1.1 Glucocorticosteroids

Glucocorticosteroids are hormones that have a wide range effect on the immune system and inflammatory response. The first steroid to be available was cortisone, a naturally occurring hormone, in 1949. Subsequent to the discovery of cortisol, alterations to its structure produced glucocorticoids with greater potency and duration of action. Steroids have both an immune suppressant action and an anti-inflammatory action. There is a range in

potency, ranging from the least potent, hydrocortisone, to dexamethasone, which is 30 times more potent. Glucocorticosteroids have effects on target gene transcription[25], alteration of circulating levels of neutrophils, eosinophils, macrophages and lymphocytes[26] and inhibition of pro-inflammatory cytokines[27]. The diverse mode of action of the glucocorticosteroids makes them useful for the treatment of dermatological, allergic and autoimmune disorders.

Examples of glucocorticosteroids include hydrocortisone **1.1**, used for the treatment of rheumatic disease and inflammatory bowel disease and prednisolone **1.2**, indicated for asthma and prevention of organ rejection following transplantation.



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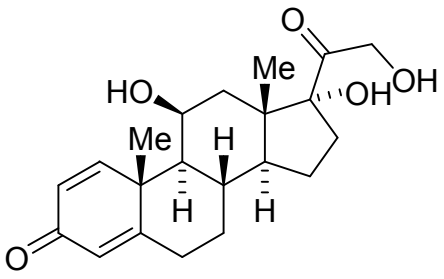


1.2

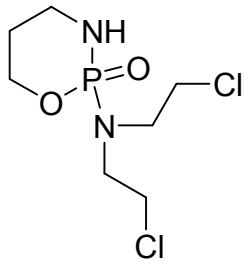
The diversity of the mode of action unfortunately leads to complications, with adverse effects associated with this class of drugs. These adverse reactions most commonly include hypertension, weight gain, glucose intolerance, gastritis, adrenal suppression, diabetes and osteoporosis. The use of steroids in children is also associated with growth failure.

This wide range and frequent occurrence of side effects means there needs to be a risk versus benefit approach to the use of steroids. The usual approach is to use sufficient quantities to control the disease and then reduce the dose to the lowest possible to limit the side effects.

1.3.2.1.2 Cyclophosphamide

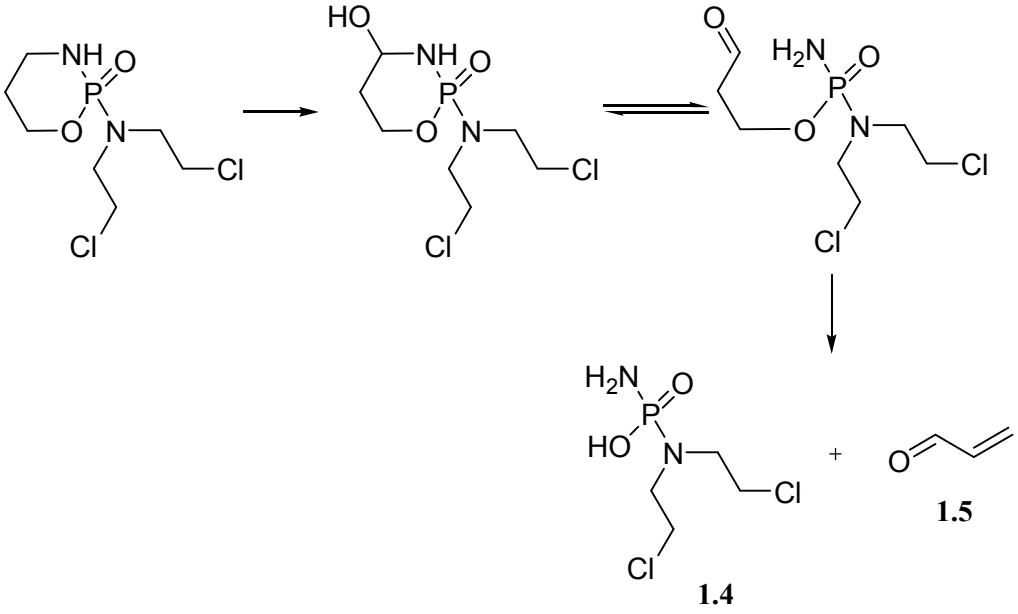
Cyclophosphamide **1.3** is an alkylating agent, which can form transition complexes with the 7-nitrogen atom of guanine and other components of DNA[24]. This leads to DNA fragmentation, mutations and cell death[27].

Cyclophosphamide was first investigated for use as chemical warfare agents and following World War II research was carried out regarding their potential therapeutic effects.



1.3

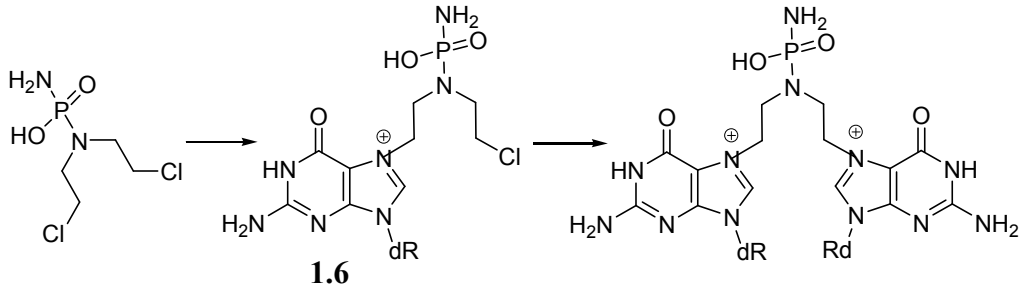
When given orally cyclophosphamide is relatively inactive until the cleavage of the phosphorus-nitrogen linkage, eventually leading to the formation of the phosphoramide mustard **1.4** and the metabolite acrolein **1.5**, shown in scheme 1.1.



1.4 **1.5**

Scheme 1.1 Formation of phosphoramide mustard from cyclophosphamide

The phosphoramide mustard **1.4** is an alkylating agent[28] and is able to crosslink the DNA base guanine **1.6** (scheme 1.2) thereby inhibiting DNA replication, suppressing cell division or triggering apoptosis. Cyclophosphamide's main effect is on B cells, suppressing cellular immunity and antibody production[24].



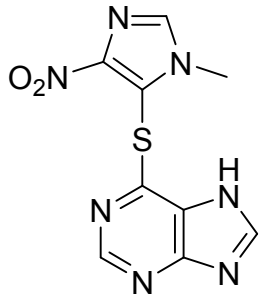
1.6

Scheme 1.2 The crosslinking of guanine by phosphoramide mustard

The drug's main clinical use is as a cytotoxic agent in oncology and haematology. It is used for its immune modulating effect prior to bone marrow transplantation, following organ transplantation or for severe rheumatoid arthritis. Cyclophosphamide causes severe systemic side effects including alopecia, cardiotoxicity and leukopenia. During its use, regular blood counts need to be undertaken to ascertain any damage on the production of cells. Also, the active metabolite acrolein **1.5** can cause severe haemorrhagic cystitis. Due to its high toxicity, cyclophosphamide is only used if other therapies have proved unsuccessful.

1.3.2.1.3 Azathioprine

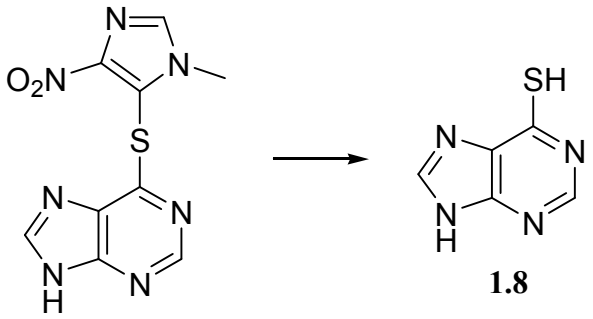
Azathioprine **1.7** was one of the first generation of immunosuppressive drugs to be used in the area of organ transplant. It was originally synthesised in 1949 and was later found to have a range of effects on the immune system[29-31].



1.7

Azathioprine is metabolised to 6-mercaptopurine **1.8** after oral administration (Scheme 1.3). The metabolism of azathioprine has been reviewed by Chan *et*

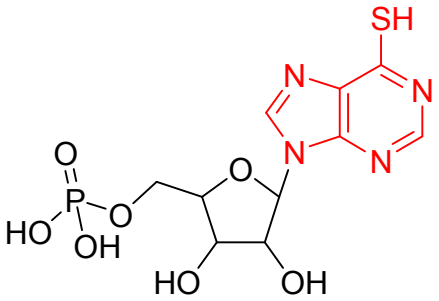
al.[32]. 6-Mercaptopurine is a purine analogue, causing effects on purine synthesis, and is incorporated into DNA in a non-specific way, leading to the death of rapidly dividing cells in the bone marrow and intestine[27]. This was originally developed to be a drug in its own right but it was found that its imidazol derivative, azathioprine, was less toxic.



1.7 **1.8**

Scheme 1.3 The formation of 6-mercaptopurine via the metabolism of azathioprine

As a purine analogue it will form 6-thioinosine monophosphate **1.9**, inhibiting the production of purine nucleotides and, therefore, inhibiting DNA replication.



1.9

Azathioprine is used for transplant recipients and to treat a number of autoimmune conditions, commonly when a corticosteroid does not provide sufficient control.

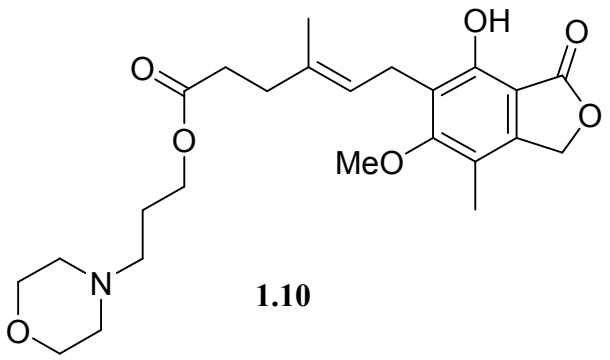
Even though azathioprine is less toxic than it predecessor, 6-mercaptopurine, it still carries with it a large range of adverse effects. Severe myelosuppression is the most common side effect observed. An additional active metabolite of Azathioprine, 6-thioinosinic acid, is able to be

incorporated into both RNA and DNA nucleic acids. As a result there is widespread chromosomal breakage in a large number of cell types. There is also the possibility of hepatic toxicity, as the drug is metabolised by the liver.

Azathioprine is widely used following organ transplant and is used to treat a range of autoimmune disorders, mainly rheumatoid arthritis. Patients who are having treatment with azathioprine have to undergo regular monitoring due to the large risk of myelosuppression. The drug's generality of action is the main limitation in its use.

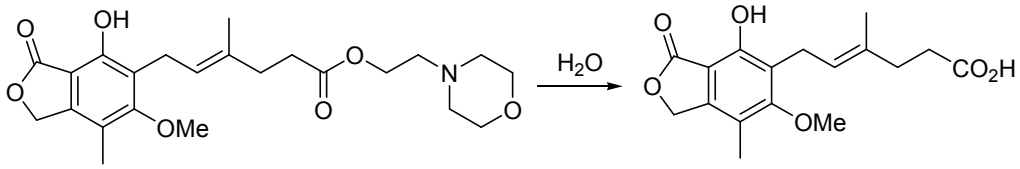
1.3.2.1.4 Mycophenolate Mofetil

Mycophenolate mofetil (MMF) **1.10** is a pro drug of mycophenolic acid **1.11** that inhibits the enzyme inosine monophosphate dehydrogenase (IMDP) types I and II [33], which leads to the inhibition of guanosine nucleotide synthesis without incorporating into DNA[27].



1.10

MMF **1.10** undergoes ester hydrolysis in the liver to yield mycophenolic acid **1.11**, the active metabolite of the drug, shown in scheme 1.4. Mycophenolic acid is now available in medicinal form as Myfortic® produced by Novartis.



1.10 **1.11**

Scheme 1.4 Hydrolysis of Mycophenolate Mofetil

In the S phase of the cell cycle, guanine monophosphate is produced via the pathway shown in Fig 1.1[34]. During the activation of T cells, the activity of types I and II inosine monophosphate dehydrogenase enzymes increases tenfold.

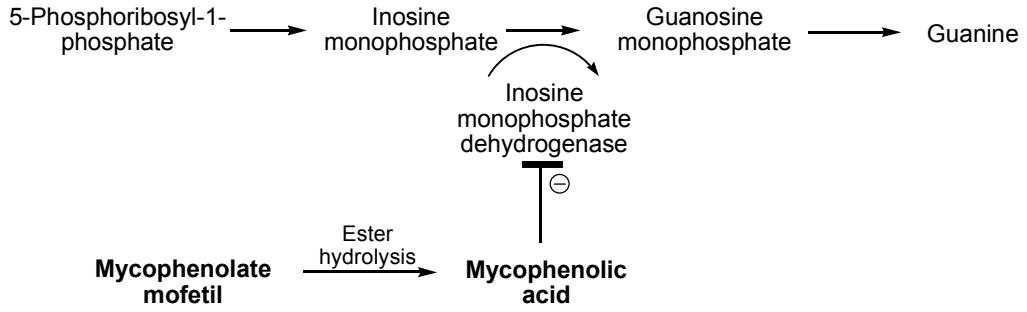


Figure 1.1 Mechanism of action of Mycophenolate Mofetil

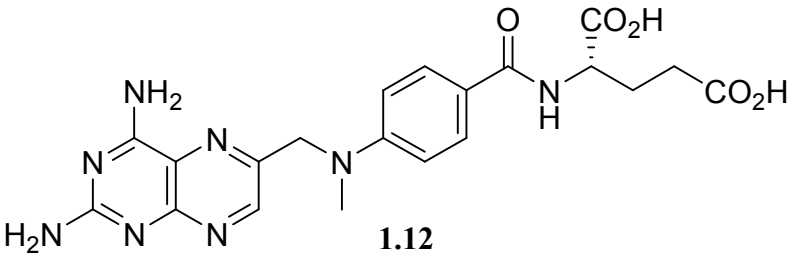
Following the ester hydrolysis, mycophenolic acid non-competitively and reversibly inhibits types I and II inosine monophosphate dehydrogenase activity, leading to an inhibition of DNA synthesis in the G_2 phase of the cell cycle [34]. MMF inhibits the proliferation of T-lymphocytes and antibody production by B-lymphocytes, as these processes are dependent on *de novo* purine synthesis for DNA replication. In addition, it prevents glycosylation of adhesion proteins, inhibiting migration of lymphocytes to areas of inflammation[27].

Mycophenolate mofetil is classified by the British National Formulary as an antiproliferative immune suppressant. It is licensed for the prophylaxis of acute rejection in renal or cardiac transplantation, when it is used in combination with ciclosporin and corticosteroids[13].

Clinical trials have shown that MMF is useful in decreasing the incidence and severity of acute rejection episodes but does not significantly affect the overall long-term rate of graft survival. There is evidence showing that MMF is a better option than azathioprine in a number of combination therapies, such as ciclosporin or tacrolimus, and reduces the risk of acute rejection episodes.

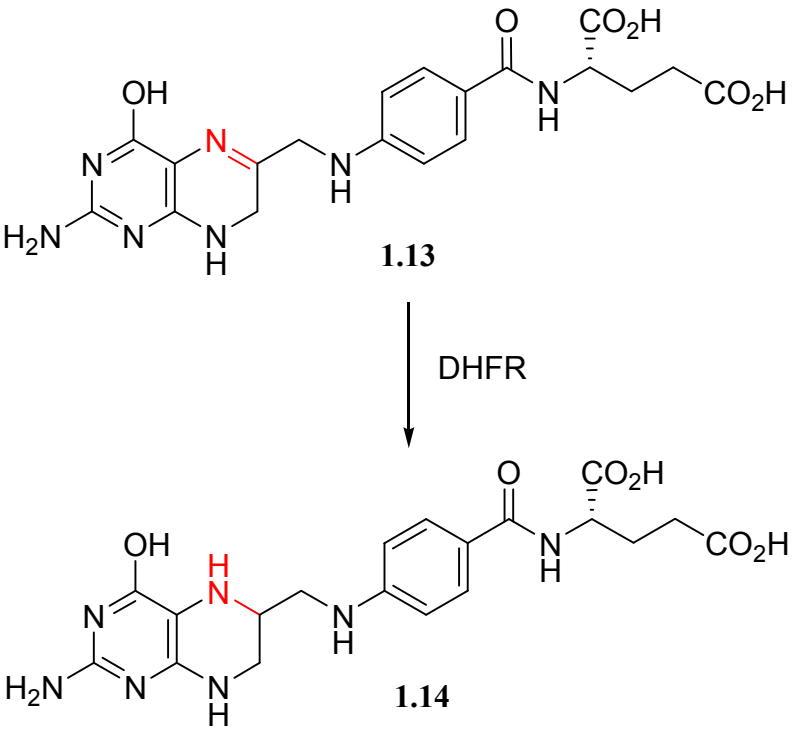
1.3.2.1.5 Methotrexate

Methotrexate **1.12** was synthesised initially for the treatment of malignancies and was subsequently found to have an effect on the immune system.



1.12

Methotrexate is a folate antagonist, inhibiting dihydrofolate reductase. The synthesis of thymidine nucleotides involves the conversion of dihydrofolate **1.13** to tetrahydrofolic acid **1.14** by dihydrofolate reductase (DHFR), shown in scheme 1.5.



1.13

1.14

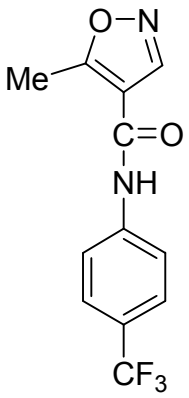
Scheme 1.5 Conversion of dihydrofolate to tetrahydrofolic acid by dihydrofolate reductase

Methotrexate is a dihydrofolate analogue, possessing a very similar structure to dihydrofolate, and competitively inhibits dihydrofolate reductase and prevents DNA replication and proliferation of lymphocytes. This inhibition prevents the regeneration of the active tetrahydrofolate from the inactive dihydrofolate and results in cessation of nucleotide synthesis[24]. This action kills cells that are in the S-phase of the cell cycle, in which DNA is synthesised, but has no effect on non-proliferating cells[27]. Methotrexate may also be considered to be an inhibitor of *de novo* pyrimidine synthesis.

Methotrexate is used to treat severe uncontrolled psoriasis, rheumatoid arthritis and malignant disease[13]. There is a risk of potentially fatal blood dyscrasias and liver cirrhosis even with low dose Methotrexate, thus requiring ongoing monitoring of blood counts while undergoing therapy.

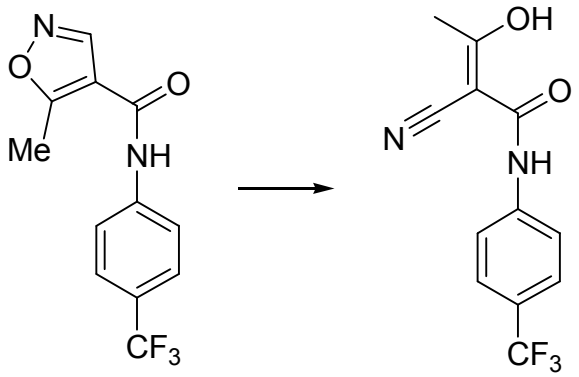
1.3.2.1.6 Leflunomide

Leflunomide **1.15** was originally developed as an agricultural pesticide and its anti-inflammatory and immune suppressant capabilities were discovered sometime later.



1.15

Leflunomide is an isoxazole derivative that acts as prodrug for its open-ring metabolite, AA771726 **1.16** (Scheme 1.6). Leflunomide is advantageous as it exhibits less gastrointestinal toxicity than the active metabolite.



1.15 **1.16**

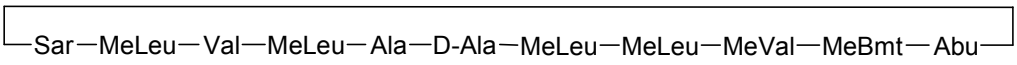
Scheme 1.6 Metabolism of leflunomide

This metabolite inhibits dihydroorotate dehydrogenase[35] causing an inhibition of pyrimidine nucleotide synthesis[36-38] which in turn leads to a suppression of lymphocyte proliferation.

Leflunomide is used as a disease-modifying antirheumatic drug (DMARD). The side effects associated with Leflunomide include bone marrow suppression and an increase in the risk of infection and malignancy[13].

1.3.2.1.7 Ciclosporin

Ciclosporin **1.17** is a lipophilic cyclic undecapeptide and was discovered by Sandoz during screening for materials with antibiotic properties produced the fungi, *Hypocladium inflatum gams*.



1.17

Ciclosporin inhibits T cell activation at an early step, causing failure to activate the transcription of genes which normally function in coordinating the immune response, such as those encoding the cytokines. As a result, a primary effect that is largely confined to the T cells leads to a process in which a therapeutic useful immune suppression is the outcome.

Ciclosporin was one of the first drugs to offer a selective, T cell targeted, immune suppression. This was advantageous as the myelosuppression

caused complications in azathioprine based therapies and had reduced the number of transplantations being carried out. The discovery and implementation of ciclosporin reinvigorated the area of transplantation.

Ciclosporin's exact mechanism of action was not known for a number of years following its discovery and use. The mode of action was subsequently found to involve targeting calcineurin and that ciclosporin only becomes active after interaction with the immunophilin, cyclophilin, to form an inhibitory immunophilin-drug complex. This then binds with calcineurin, a phosphatase, and effectively brings together two proteins that would not normally interact. This inhibits the up-regulation of various cytokines, mainly IL-2, which is essential for the proliferation and maturation of T cells. Ciclosporin also affects the production of interferon gamma (IFN-γ), essential for the activation of macrophages.

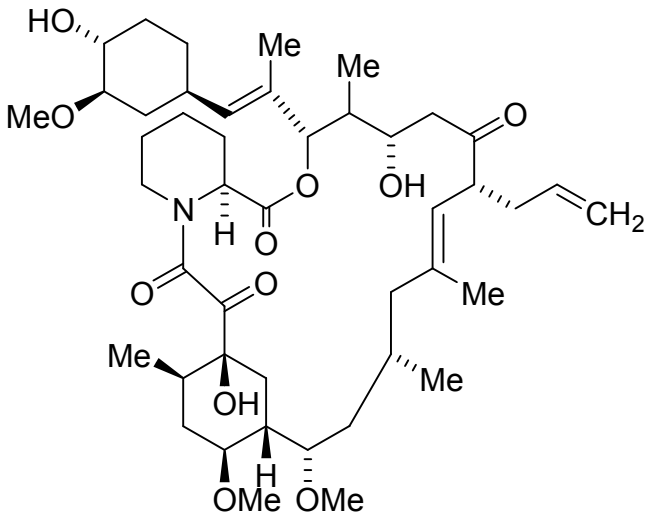
Ciclosporin is indicated for organ transplantation, alone and in combination with other immunosuppressant therapy, bone-marrow transplantation, nephrotic syndrome, rheumatoid arthritis, atopic dermatitis and psoriasis.

The main limitation for the use of ciclosporin is its side effect profile, mainly its nephrotoxicity. There are a range of side effects associated with ciclosporin, largely due to three main mechanisms. Firstly, it activates endothelin-converting enzyme, thereby increasing production of ET-1. This causes systemic hypertension, renal vasoconstriction and decreases glomerular filtration rate. Secondly, ciclosporin induces apoptosis of renal tubular epithelial cells, leading to drop out of tubular lining cells. Lastly, it augments production of transforming growth factor-β (TGF-β) which causes tubular interstitial fibrosis.

Despite its side effects ciclosporin is a potent immune suppressant and has been effective in improving the outcome of organ transplants. However it is increasingly being used in combination with other therapies in order to keep the dose of ciclosporin as low as possible, thereby minimising the associated side effects.

1.3.2.1.8 Tacrolimus

Tacrolimus **1.18**, also known as FK506, is a macrolide produced by the bacteria *Streptomyces tsukubaensis,* an organism found in the Tsukuba area of northern Japan[39]. The first studies on the compound were carried out in 1988 by Ochiai *et al*[40].



1.18

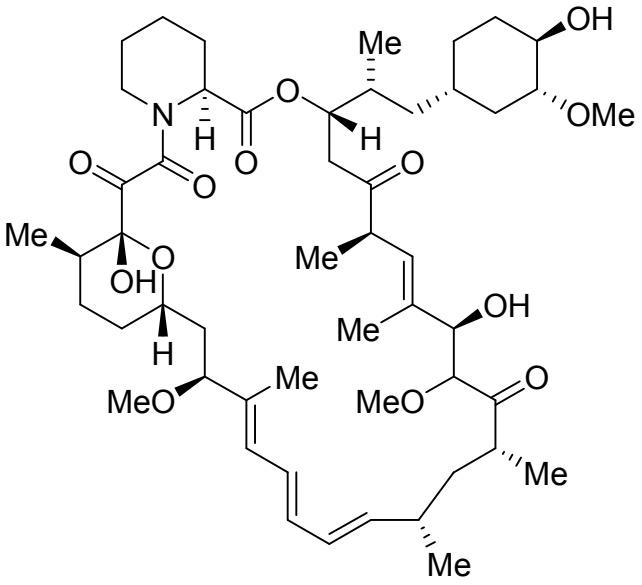
Tacrolimus has a similar mode of action to ciclosporin, despite bearing little similarity structurally, in that it is a calcineurin inhibitor. Tacrolimus inhibits the production of IL-2, resulting in the decreased proliferation of activated T cells[41-43]. Tacrolimus interacts with FK-binding proteins (FKBPs), with the resulting complex able to bind to calcineurin, thereby inhibiting its phosphatase activity[41-43]. This leads to a range of effects that interfere with T cell receptor-dependent cell activation. Tacrolimus is also able to affect the corticoid receptor[42], providing an additional site of action.

The advantage of tacrolimus over ciclosporin is an increased potency[44,45] meaning there is the ability for clinical combination therapies using corticosteroids to be tapered more rapidly and the ability to be more useful as a monotherapy.

The side effects of tacrolimus are the limiting factor in its use, especially in the chronic use. It exhibits the same side effect profile as ciclosporin.

1.3.2.1.9 Sirolimus

Sirolimus **1.19** was first isolated from a strain of *Streptomyces hygroscopicus* collected from Rapa Nui (Easter Island)[46], hence its other name of rapamycin. Sirolimus was initially found to have potent anti-fungal activity[47] but subsequent studies have shown it possesses impressive antitumour, antiproliferative and immune suppressive properties[48].



1.19

Sirolimus is a lipophilic macrocyclic lactone and is structurally very similar to tacrolimus but has a distinctly different mode of action. It forms an immune suppressive complex with the intracellular protein FK binding protein-12 (FKBP12). Through this, sirolimus inhibits a number of cytokine-mediated signal transduction pathways, in particular the mammalian target of rapamycin (mTOR). This action stops the progression of T cells from the G phase to the S phase of the cell cycle. The action effectively stops the proliferative response of lymphocytes caused by IL-2[49].

Sirolimus is used for prophylaxis of organ rejection in kidney allograft recipients. Initially it is used in combination with ciclosporin and corticosteroid and subsequently only with corticosteroid.

Sirolimus is potentially hepatotoxic, teratogenic and will cause renal impairment. It also has a range of effects on blood counts, including

thrombocytopenia, neutropenia and leucopenia. Sirolimus does not exhibit the characteristic nephrotoxicity of the calcineurin inhibitors[50].

1.3.2.1.10 Cytokine Inhibitors

This class of immune modulating agents are genetically engineered proteins and monoclonal antibodies (mAb), and are used to treat severe rheumatoid arthritis that has failed to respond to two DMARDs, juvenile idiopathic arthritis where there has been a failure to respond to, or tolerate, methotrexate; severe active Crohn's disease when treatment with immune modulating agents or corticosteroids has failed or not tolerated; and moderate to severe chronic psoriasis, where the patient is unresponsive to other systemic therapy and photochemotherapy.

These differ from the therapies already described as they are recombinant antibody therapies. They mainly inhibit the pro-inflammatory cytokine, TNF-α which is a key mediator in chronic inflammatory conditions[51]. These agents are able to offer an effective treatment for conditions such as rheumatoid arthritis, with little associated side effects. There is a potential for allergic reactions when treatment is initiated due to the presence of murine elements in the some of the antibodies.

Infliximab is a combination of human constant and murine variable region IgG monoclonal antibody which is able to bind TNF-α and TNF-β as well as be able to lyse TNF producing cells[52]. It has shown efficacy in the treatment of rheumatoid arthritis and Crohn's disease.

Entanercept consists of two recombinant human TNF-receptor chains fused with IgG_1[53]. This inhibits TNF activity at cell receptor, competitively binding and inactivating free TNF.

Adalimumab is a recombinant IgG monoclonal anti-TNF-α antibody that is able to bind with high affinity with TNF-α. The advantage of adalimumab is that its composition is fully human and should therefore not cause allergic problems that are seen with the other TNF inhibitors[54].

Anakinra inhibits the activity of IL-1, therefore differing from the other cytokine inhibitors. It is able to treat rheumatoid arthritis but is not recommended unless it is part of a controlled long-term clinical study.

The major adverse effects are injections site reactions, increased susceptibility to infection, lymphoproliferative diseases, demyelinating diseases and aggravation of congestive heart failure[54].

1.3.2.2 Search for new immune suppressant therapies

There is a common origin of many of the immune suppressants reviewed in that they are derived from natural sources. These are either steroids or cytokines of human origin or the isolation of active compounds from fungal and bacterial sources. The latter source remains of interest in the search for novel immune modulating agents[55], due to the observed ability for bacteria to exert an effect on the host's immune response[56]. The high toxicity associated with existing immune suppressants necessitates the search for new agents. A major way in which bacteria can avoid the immune response is the control of virulence factors due to a form of cell-to-cell communication termed quorum sensing.

1.4 Quorum sensing

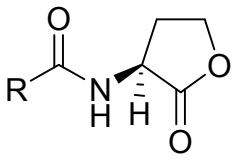
Quorum sensing (QS) is a form of intercellular communication used by bacteria for controlling gene expression in response to population density. It was known that bacteria were able to respond to external stimuli, such as changes in pH, temperature or osmolarity, and that this response was controlled due to the stimulation, or repression, of various target genes but it was not thought that there was any coordination within bacterial populations. It is now known that bacterial populations can communicate and coordinate their actions, as a population.

Quorum sensing was first observed in the bioluminescent marine bacteria *Vibrio fischeri*[57] and *Vibrio harveyi*[58]. It was observed that these bacteria only produced light when there was a high population density. This was part of a symbiotic relationship between the bacteria and the Hawaiian bobtail squid, *Euprymna scolopes*[59], enabling the production of light for the avoidance of predators.

Quorum sensing was initially thought to be a factor in a few obscure examples of bacterial species but it has now been established that most, if not all, bacteria can perceive and respond to neighbouring microbial populations through quorum sensing systems.

1.4.1 Quorum sensing system in Gram positive bacteria

Quorum sensing systems generally consist of a small, diffusible signalling molecule, commonly known as a quorum sensing signal molecule (QSSM), and a transcriptional activator protein. Gram-positive bacteria commonly produce amino acids and short peptides as signalling molecules, whereas Gram-negative bacteria produce small molecules, predominantly *N*-acyl homoserine lactones (AHLs), which have the general structure **1.20**, where R relates to differences in length, substitution and saturation in the side chain.



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The essentials of a simple quorum sensing system are shown in Fig. 1.2[60].

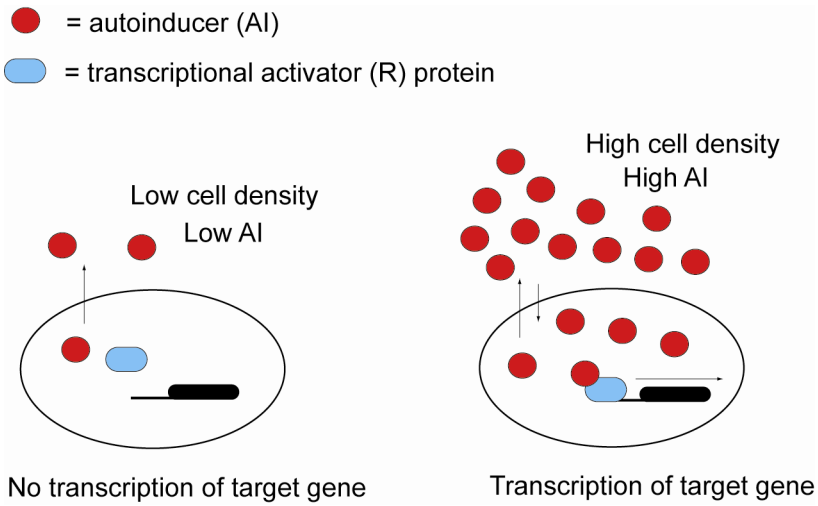


Figure 1.2 Quorum sensing in Gram-negative bacteria

At low cell density bacteria produce a basal level of signalling molecule, termed the autoinducer (AI), which diffuses out of the cell down a concentration gradient, although there are active transport mechanisms used in some cases. As the population increases in size, the concentration of extracellular AI increases. When an optimum population density is reached the AI reaches a threshold concentration and binds to a transcriptional R protein, causing its activation and subsequent modulation of specific target genes.

The AHL signal molecules are commonly synthesised by the LuxI-type AHL synthases and interact with LuxR-type transcriptional regulator proteins. Due to this interaction the expression of target genes are activated. This is illustrated by the QS system of *Vibrio fischeri* as, shown in Fig. 1.3

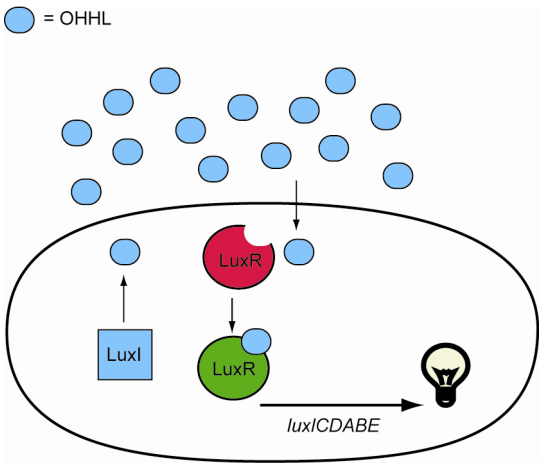


Figure 1.3 Quorum sensing system of *Vibrio fischeri*

The *luxI* produces the AHL signal molecule, *N*-(3-oxohexanoyl)-L-homoserine lactone (OHHL)[58], and, when the population is of the required size, OHHL binds to and activates the LuxR protein. LuxR is then able to induce transcription of the required *luxI* operon, thereby allowing bioluminesence[61,62].

There are a vast assortment of classes of chemical signals that are employed in quorum sensing, with individual bacteria able to produce more than one type of signalling molecule and complex, hierarchical circuits existing to integrate and process sensory information. Inter-species communication is also observed, possibly for either synergistic or competitive ends.

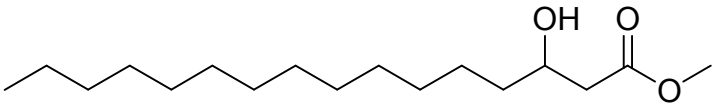
These chemical signals control a large range of phenotypes across a vast range of bacterial species. Some examples of those present in Gram-negative bacteria are summarised in Table 1.4.

Table 1.4 Examples of phenotypes controlled by quorum sensing

Organism	***Signal Molecule***	***Phenotype***
Aeromonas hydrophila	*N*-butanoyl-L-homoserine lactone (BHL)	Exoprotease production[63]
Agrobacterium tumefaciens	*N*-(3-oxooctanoyl)-L-homoserine lactone (OOHL)	Ti plasmid conjugation[64-66]
Burkholderia cepacia	*N*-octanoyl-L-homoserine lactone (OHL)	Protease production[67]
Erwinia stewartii	*N*-(3-oxohexanoyl)-L-homoserine lactone (OHHL)	Capsular polysaccharide biosynthesis, virulence[68]
Escheria coli	Unknown	Cell division, attachment, lesion formation[69-71]
Pseudomonas aerofaciens	*N*-hexanoyl-L-homoserine lactone (HHL)	Phenazine antibiotic biosynthesis[72,73]
Rhizobium etli	Unknown	Restriction of number of nitrogen fixing nodules[74]
Rhodobacter sphaeroides	*N*-tetradec-7-enoyl-L-homoserine lactone (7-*cis*-TDHL)	Prevents bacterial aggregation[75]
Vibrio anguillarum	Unknown	AHL production[76]

1.4.2 Additional classes of signalling molecule

Although the majority of quorum sensing signalling molecules produced by Gram-negative bacteria are AHLs, there is also evidence of other classes of compound produced. *Ralstonia solanacearum*, a plant pathogen, produces 3-hydroxy-palmitic acid methyl ester **1.21**, involved in regulating virulence[77] and *Pseudomonas aureofaciens* produces butyrolactones[78] **1.22**. Diketopiperazines (DKPs) **1.23** have been shown to be produced by a range of bacteria, including *Pseudomonas fluorescens*, *Enterobacter agglomerans* and *Citrobacter freudii*[79]. These are unlikely to activate LuxR-based biosensors strongly but are able to antagonise the action of AHLs[79]. However, their role in cell-to-cell signalling has not been established.



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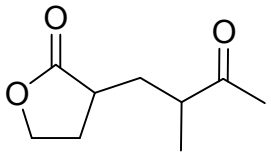

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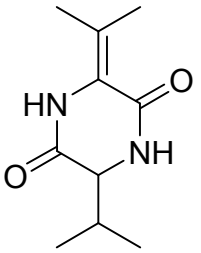


1.23

1.4.3 Novel target for antimicrobial therapy

The identification of quorum sensing systems and QSSMs has highlighted a possible target for the development of novel antimicrobial therapies. The interference or antagonism of QSSMs that control virulence factors could potentially remove the pathogenicity from those bacteria.

This has already been demonstrated in a range of bacteria, where autoinducer analogues have inhibited the activation of R proteins[63,76,80,81].

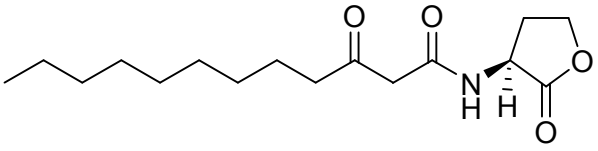
Further options for targeting QS systems is the degradation of AHLs via the use of enzymes[82] or interference with the biosynthesis of QSSMs[83-86].

1.4.4 Quorum sensing in *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is an opportunistic pathogen that causes morbidity in immunocompromised patients and is a particularly prevalent pathogen in cystic fibrosis (CF), where it is the main perpetrator of decline in lung function and mortality in the disease. Its pathogenic effects are due to its ability to secrete extracellular virulence factors, including proteases and haemolysins, as well as being able to form biofilms, which provide protection from host defences during colonisation. The bacteria may be present with no effect in a healthy individual but will cause damage once the ability of the immune system declines.

1.4.4.1 QSSMs produced by *Pseudomonas aeruginosa*

P. aeruginosa was initially found to produce two major signalling molecules, *N*-(3-oxododecanoyl)-L-homoserine lactone (OdDHL) **1.24** and *N*-butanoyl-L-homoserine lactone (BHL) **1.25** and two minor ones, *N*-(3-oxohexanoyl)-L-homoserine lactone (OHHL) **1.26** and *N*-hexanoyl-L-homoserine lactone (HHL)[87-89] **1.27**. All of these molecules are members of the AHL family.



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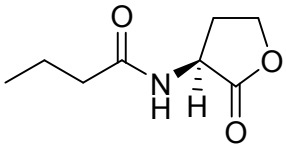

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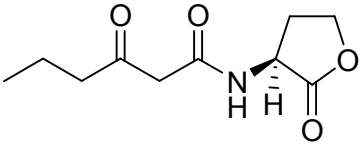

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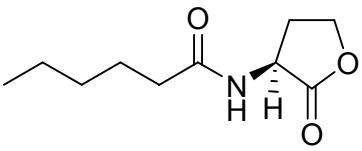


1.27

The structural differences between these molecules allow for specificity in the overall quorum sensing system and the expression of genes associated with the individual AHLs.

1.4.4.1.1 AHLs in *Pseudomonas aeruginosa* quorum sensing

The quorum sensing system for *P. aeruginosa* is one of the most studied and best understood QS systems. Research into the system has shown that there is the potential for more complexity in QS than was first thought.

It has been shown that at least two discrete quorum sensing systems exist, both regulated by different AHLs, and that these systems are able to exert an influence over the other[90], as shown in Fig 1.4.

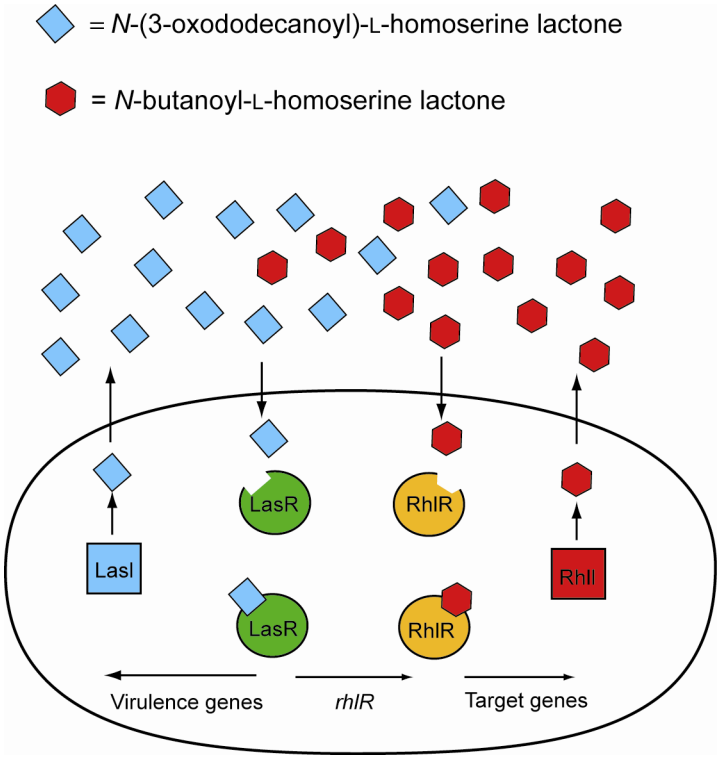


Figure 1.4 Hierarchical *P. aeruginosa* quorum sensing system

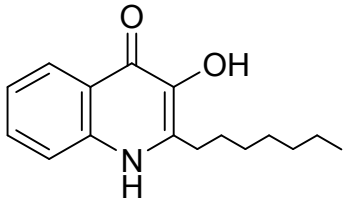
LasI causes the synthesis of *N*-(3-oxododecanoyl)-L-homoserine lactone which then binds to and activates the transcriptional activator protein LasR[91,92], which regulates the production of virulence factors including LasA protease, alkaline protease, elastase and exotoxin A[92,93]. The *las* system also exerts transitional control over the *rhl* system[90].

RhlR is another LuxR-type protein which is responsive to *N*-butanoyl-L-homoserine lactone[94,95], the synthesis of which is directed by RhlI[89]. This leads to the production of more virulence factors such as pyocynin, hydrogen cyanide, cytotoxic lectins, rhamnolipid and siderophores[89,96-99]. There is

evidence of the potential of hierarchical cascades within quorum sensing systems[90].

1.4.4.2 *Pseudomonas* Quinolone Signal

Subsequent to the discovery of the AHLs produced by *P. aeruginosa*, it was then discovered that another type of signal molecule was produced by *P. aeruginosa* during the analysis of *N*-butyryl-L-homoserine lactone[100]. This molecule belonged to the 4-quinolone family of compounds and its structure was identified as 2-*n*-heptyl-3-hydroxy-4(1*H*)-quinolone **1.28**. The compound was designated the name *Pseudomonas* quinolone signal (PQS) by Pesci *et al*[100].

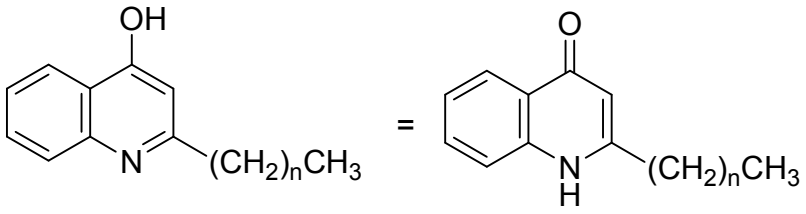


1.28

PQS was initially found to induce *lasB* and was dependent on the other quorum sensing systems for its biosynthesis and bioactivity[100].

Molecules bearing quinolone structures, termed "Pyo" compounds, had previously been isolated from *P. aeruginosa* cultures and investigated as antibiotic substances[101,102]. PQS was first isolated and identified in 1959[103]. The discovery of PQS was evidence of small, heterocyclic, molecules being present as quorum sensing signals other than AHLs.

Since the initial identification of PQS it has been shown that *P. aeruginosa* produces a range of similar molecules, such as the PQS precursor 2-heptyl-4(1*H*)-quinolone (HHQ) and 2-nonyl-4(1*H*)-quinolone. These compounds have been collectively termed 4-hydroxy-2-alkylquinolines (HAQs)[104]. The HAQs include 2-alkyl-4(1*H*)-quinolones **1.29**, 2-alkyl-3-hydroxy-4(1*H*)-quinolones **1.30**, N oxides **1.31** and derivatives with unsaturated alkyl side chains **1.32** and **1.33**.



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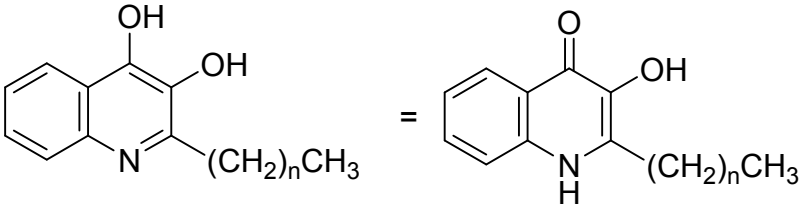

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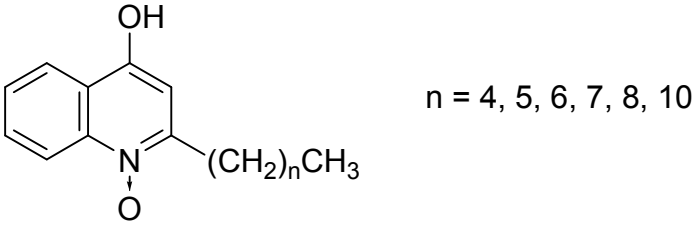

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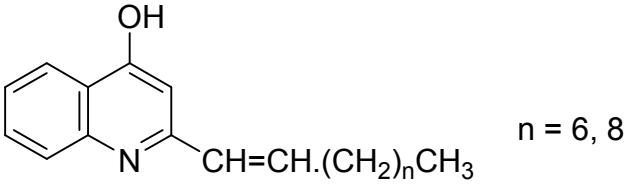

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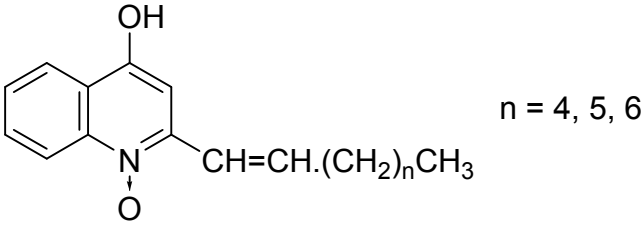

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1.4.4.2.1 The Role of PQS in *Pseudomonas aeruginosa* Quorum Sensing

It has been shown that the synthesis and bioactivity of PQS are controlled by the *las* and *rhl* QS systems respectively. This suggests that PQS is not involved in sensing population density, rather it is synthesised in response to population

density as other systems instigate the transcription of the genes required for its production and has an influence later in the cell cycle[105].

It has been shown that PQS production is dependent on the *las* system, and that PQS itself has an integral part in the *rhl* system[106], directly influencing the expression of *rhlI*[105].

PQS controls the expression of *lasB*[100] which is responsible for producing LasB elastase, a major virulence factor for *P. aeruginosa*. It may have a role in the adherence of *P. aeruginosa* and the formation of biofilms[106] with regards to infection in the lungs of CF patients although the mechanisms through which it does this are not yet known.

1.4.5 Quorum sensing signal molecules as immune modulators

It was hypothesised that since bacteria such as *P. aeruginosa* were using QSSM, small molecules able to readily diffuse across cell membranes[107], to coordinate their virulence, it may be possible that the signalling molecules themselves have a direct effect on the immune system of the host[56]. It can be seen that this ability for the bacterial population to remain undetected by the host until the population was of a required size as evidence of this. Additionally, it has been shown that OdDHL was able to stimulate dose-dependent interleukin 8 (IL-8) production by lung epithelial cells[108], showing the possibility of these molecules to have a direct effect on biological systems. This provided preliminary evidence that there is a possible direct effect from QSSM on the immune response.

1.4.5.1 Immune modulation by *N*-Acylhomoserine lactones (AHLs)

Two of the AHLs produced by *P. aeruginosa*, OdDHL and OHHL, have been tested *in vivo* in a range of assays in order to determine whether these molecules are capable of modulating the immune system.

OdDHL and OHHL were chosen in order to compare the difference effects of a long chain AHL compared to that with a short chain. OdDHL was found to exert a significant immune modulating activity *in vitro*, without

exhibiting any cytotoxicity. OHHL was not found to have any significant effects[56].

It has also been shown that OdDHL also has a direct effect on fibroblasts[109,110], respiratory epithelial cells[108,109,111], arterial smooth muscle cells[112,113], and that its effects on the immune system include specific effects on monocytes[110], neutrophils[110] and lymphocytes[114,115].

1.4.5.1.1 SAR study on OdDHL

A structure activity relationship (SAR) has been undertaken using OdDHL as the lead molecule in which the areas altered were the side chain **A**, the 3-oxo-acyl moiety **B** and the hetero ring **C** [114] Fig. 1.5.

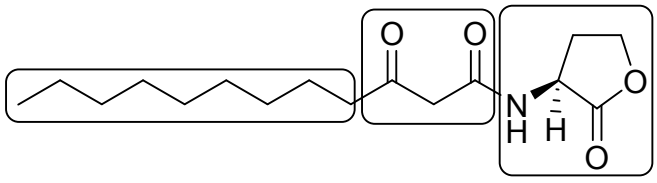


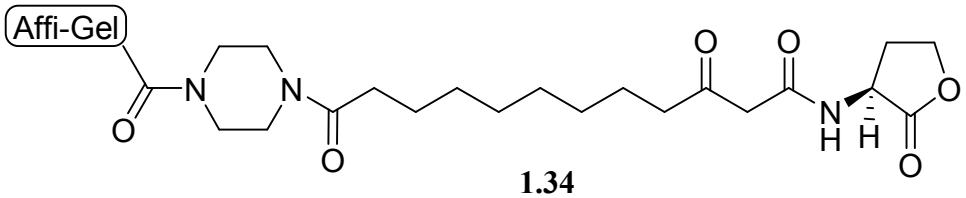
Figure 1.5 Areas altered in SAR study of OdDHL

In the course of this study, twenty-four analogues of OdDHL were synthesised and evaluated in a range of biological assays to ascertain their ability to modulate murine and human leukocyte proliferation and TNF-α secretion.

The SAR study found the following structural attributes that are were important in exerting its immune modulatory action. A C_{12} side chain is optimal, although C_{13} and C_{14} chains retained similar activities and a branched chain is slightly less active. A reduction in activity when the chain is unsaturated close to the 3-oxo-acyl moiety shows that flexibility in the side chain is important. Polar groups on the side chain reduce the activity significantly, suggesting that the lipophilic nature of the molecule is important, probably due to its solubility and its ability to enter cells. Analogues in which the 3-oxo was replaced with 3-hydroxyl retained activity but replacing the homoserine lactone ring with other heterocyclic ring systems produced inactive

molecules. It was noted, however, that OdDHL had inferior potency than ciclosporin or dexamethasone in cell proliferation assays.

The exact target and mode of action for OdDHL is not yet known, although some work in this area has been carried out. Dr. Christopher Harty, in his research thesis, used affinity chromatography in attempting to identify the immunophilin bound by OdDHL. Affinity chromatography involves loading the drug molecule onto a solid support, such as an Affi-Gel, allowing the screening of biological products. This technique has been used to identify the binding proteins for a range of drug molecules. Terminal **1.34** and mid-chain linked **1.35** matrices were prepared, treated with murine splenocytes and bound proteins identified using tricene sodium dodeylsulfate polyacrylamide gel electrophoresis (SDS-PAGE).



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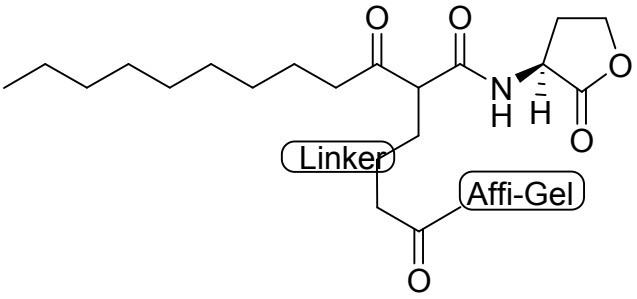


1.35

This identified two bound proteins that showed homology with murine calgranulin A and B. Further work confirmed calprotectin as a putative molecular target for OdDHL[116], although there is some doubt as too whether calgranulin is the specific immunological target for OdDHL due to its lack of specificity in binding.

Ritchie *et al*. found that the effect that OdDHL has on T cells is a combination of factors. These include OdDHL affecting early events in T cell

activation, acting on intracellular targets and it does not induce death in T-lymphocytes at concentrations needed to suppress T cell proliferation[117].

1.4.5.2 Immune modulation by PQS

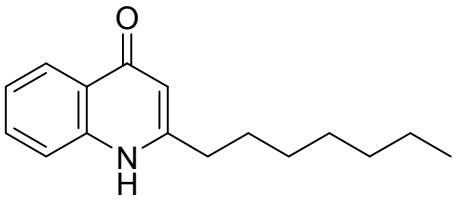
PQS was found to modulate the immune system and when compared in a range of immunological assays by Hooi *et al*, OdDHL and PQS were found to modulate the immune response in distinctly different ways[118]. PQS was more potent in suppressing lymphocyte response to ConA (IC_{50} 0.98 μM compared to 18.24 μM for OdDHL) in the absence of cytotoxicity. Also, PQS was shown to be more potent than OdDHL in suppressing T cell proliferation (IC_{50} 1.73 and 44.47 μM respectively). PQS exerted its antiproliferative action without any effect on cell viability, whereas at higher concentrations OdDHL began to affect cell viability[118].

Results suggested that OdDHL prevents proliferation by acting upstream of IL-2 secretion whereas PQS inhibits proliferation downstream of IL-2 production, in a manner similar to rapamycin. Also, in contrast to the suppressive effect OdDHL has on TNF-α secretion, PQS has an inhibitory effect[118].

Based on these results Hooi *et al.* concluded that the abilities of these compounds to modulate the immune response could possibly be harnessed to treat immunological disease[118]. The different modes of action exhibited by PQS and OdDHL also raise the possibility of the compounds having a synergistic relationship with regards to immune modulation.

1.4.5.2.1 Structure activity relationship study of PQS

No SAR data is available for PQS. The only information known prior to this project has come from the testing of the precursors in the existing PQS synthesis. This showed that 2-*n*-heptyl-4(*1H*)-quinolone **1.36** and 3-formyl-2-*n*-heptyl-4(*1H*)-quinolone **1.37** do not possess any activity in the immunological assays.



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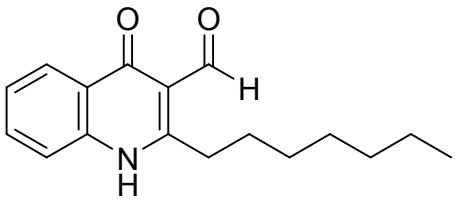


1.37

The areas in which alteration could be made in the first stages of a SAR are shown in Fig. 1.6. The alkyl chain (**A**), substitution at the 3-position (**B**), the substitution on the nitrogen (**C**) and the possible introduction of substitution onto the aromatic ring (R) are all possible areas of exploitation in an SAR study.

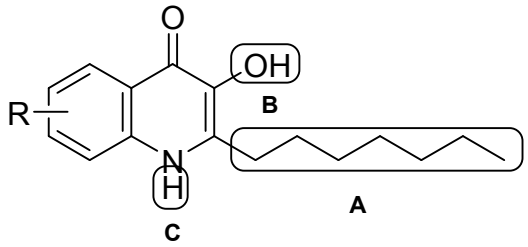


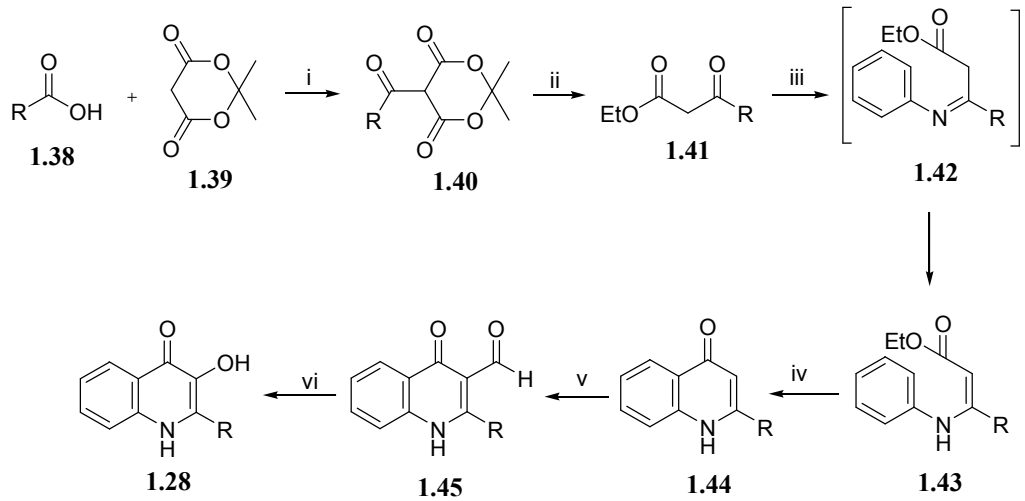
Figure 1.6 Potential areas of alteration in SAR study of PQS

Through these alterations it may be possible to identify the essential pharmacophores that need to be present within the PQS structure, to exert optimum immune modulatory activity.

Examples of alteration would take the form of finding the optimum chain length, shown by the change in activity of lengthening or shortening the chain. Differing functionality at the 3-position may show the importance of having hydrogen bonding capability at this position, or the possible steric effects of certain groups. Substitution on the nitrogen, such as a methyl group, would investigate the importance of hydrogen bonding in this part of the molecule. For example, if the N-methyl PQS analogue showed reduced activity, it may be postulated that the hydrogen atom present on the nitrogen is an important structural component in the PQS activity on the immune system.

1.5 Synthesis of PQS

While PQS has been synthesised previously[100] the synthesis reported is not satisfactory for the purposes of carrying out a SAR study. The existing synthesis is shown in scheme 1.7.



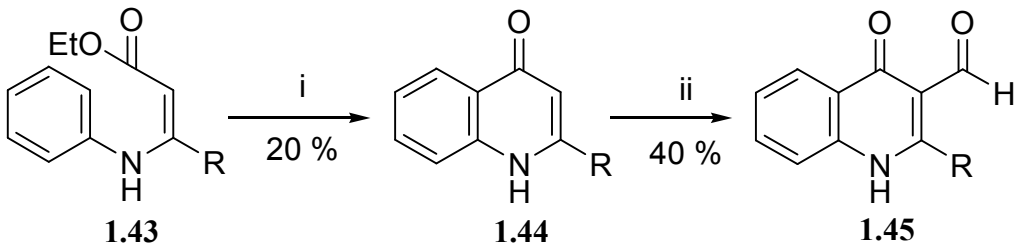
Reagents and conditions: (i) DCCI, DMAP, DCM; (ii) EtOH, Δ; (iii) Aniline, *p*-TSA, C_6H_6, Δ; (iv) Diphenyl ether, 240 °C; (v) Hexamine, TFA, Δ; (vi) H_2O_2, EtOH, $NaOH_{aq}$

Scheme 1.7 Existing synthesis of PQS

Meldrum's acid **1.38** is acylated using octanoic acid **1.39** in the presence of 1,3-dicyclohexylcarbodiimide (DCCI) and catalytic 4-dimethylaminopyridine (DMAP). This 5-acyl Meldrum's acid **1.40** is then heated at reflux in ethanol to form a β-keto ester **1.41**. Acid-catalysed condensation of the β-keto ester with aniline forms the imine **1.42**. The imine isomerises to the thermodynamically more stable enamine **1.43**, which undergoes a cyclo-condensation when heated in a high boiling solvent, such as diphenyl ether, forming 2-heptyl-4(1*H*)-quinolone **1.44**. A formyl group is introduced at the 3-position using hexamethylenetetramine (hexamine) in the presence of trifluoroacetic acid (TFA), giving **1.45**. The formyl group is then transformed to a hydroxyl group via a Baeyer Villiger oxidation, using hydrogen peroxide, forming PQS **1.28**.

This synthesis is unsuitable due to the large number of steps involved, coupled with low yields at certain steps, most noticeably in the thermal

cyclisation of the enamine **1.43** and formylation of the 2-alkyl-4(1*H*)-quinolone **1.44**, shown in scheme 1.8 (yields of 20 % and 44 % respectively).



1.43 **1.44** **1.45**

Reagents and conditions: (i) Diphenyl ether, 240 °C; (ii) Hexamine, TFA, Δ

Scheme 1.8 Low yielding steps in existing PQS synthesis

The synthetic route also affords little opportunity to quickly and simply introduce any variation in the course of the synthesis following the first couple of steps, and no option of producing a synthon. A synthon is a molecule with a basic structure which can be altered readily, thereby enabling the creation of a number of analogues in a short time. If it is not possible to produce a synthon molecule, another preferable option could be to have a synthetic route that involves fewer steps to achieve a final molecule and be able to introduce variation in those steps. Therefore, in order to undertake an effective SAR study, there needs to be the development of a new suitable synthetic method for producing PQS and its related analogues.

1.6 Aims of the thesis

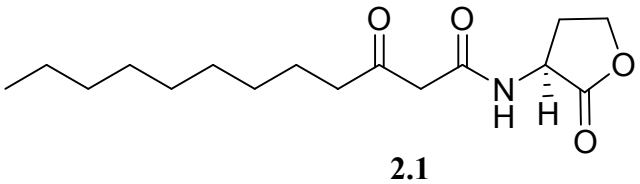
- The main aim of the research programme is to use PQS as the lead molecule in a structure activity relationship study in order to determine the essential structural features required for biological activity. The effect of alterations to the structure will be assessed using immunological assays

- Development of a new facile route for the synthesis of PQS and other structurally related molecules and use these methods to produce a library of compounds.

Chapter 2

Synthesis of 4-Quinolone Based Analogues of 2-Heptyl-3-Hydroxy-4(1*H*)-Quinolone (PQS)

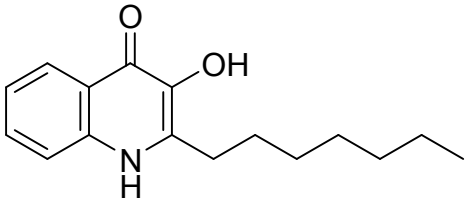
The ability for bacterial quorum sensing molecules to suppress the immune system was first identified in *N*-(3-oxododecanoyl-)-L-homoserine lactone (OdDHL)[56] **2.1**.



2.1

This molecule is produced by *Pseudomonas aeruginosa* and is one of a range of similarly structured homoserine lactone based signal molecules, although this was the only one to display an ability to modulate the immune system. It was shown that OdDHL can exert a significant effect on the immune response of mammalian cells in vitro[56]. In a murine splenocyte assay OdDHL significantly reduces the ability of lymphocytes to respond to the stimulant concanavalin A (ConA). This assay gives a good indication with regards to immune suppression capability in humans.

Lately, another quorum sensing signal molecule produced by *P. aeruginosa*, 2-heptyl-3-hydroxy-4(1*H*)-quinolone (PQS) **2.2** was identified by Pesci et al[100].



2.2

PQS was also found to possess an immune modulatory capability[118]. When compared in a range of immunological assays OdDHL and PQS were found to

modulate the immune response in a distinctly different ways. Most importantly, PQS was more potent in suppressing T-cell proliferation (IC_{50} 0.98 μM compared to 18.24 μM for OdDHL)[118]. PQS exerted its antiproliferative effect without any effect on cell viability, whereas at higher concentrations OdDHL began to affect cell viability. Results suggested that OdDHL prevents proliferation by acting upstream of IL-2 secretion whereas PQS is preventing proliferation downstream of IL-2 production, in a manner similar to rapamycin. Also, whereas OdDHL has a suppressive effect on TNF-α secretion, PQS has a contrasting inhibitory effect[118].

An initial structure activity relationship (SAR) study, using OdDHL as the lead molecule has been undertaken[114]. Twenty four alterations were made to the structure and the compounds were assayed for their ability to suppress leukocyte proliferation in both murine and human cells as well as TNF-α secretion by lipopolysaccharide (LPS) stimulated human leucocytes. The areas altered were the side chain (**A**), the 3-oxoacyl moiety (**B**) and the hetero-ring (**C**) as shown in Fig. 2.1.

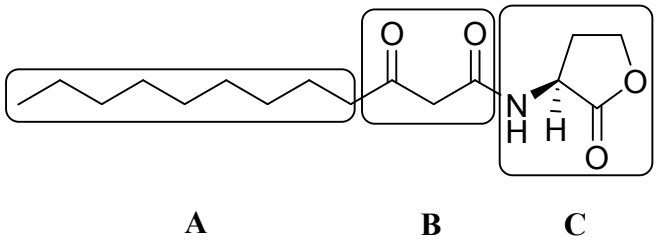


Figure 2.1 Areas of OdDHL altered in SAR study

It was found that the optimal compounds were those with alkyl chain length between C_{11} – C_{13} and either a 3-oxo or 3-hydroxy group. Analogues with monosaturation and a terminal nonpolar substituent on the side chain were also potent immune suppressive agents. Those compounds that lacked the homoserine lactone ring did not have L-configuration at the chiral centre and those with polar substituents did not have significant activity. The successful compounds that were taken on to testing of TNF-α secretion indicated the suitability of these compounds for further investigation[114].

In this way the SAR study was important in establishing the pharmcophores within the structure, that is the areas essential for its biological activity.

The evidence that PQS has immune modulating activity, allied to the supporting evidence from the SAR study of OdDHL, heavily suggested that it would be valuable to investigate further the immune modulatory activity of this quorum sensing signal molecule.

The structural components of the molecule that can be altered are shown below in Fig 2.2. These areas are the 2-alkyl chain (**A**), the 3-hydroxy (**B**) and substitution on the nitrogen (**C**). In addition to this there could be substitution introduced onto the carbocyclic ring

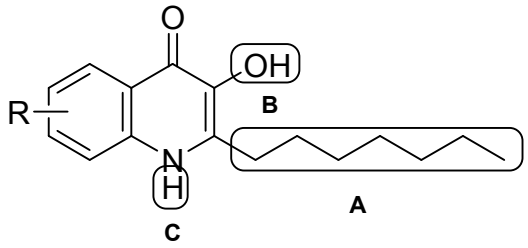
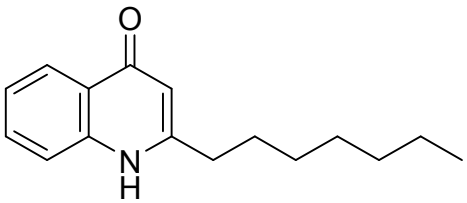


Figure 2.2 Structural components of PQS that can be altered for SAR study

Results from the SAR study would provide information regarding the important structural elements present in the molecule for optimum activity, thereby identifying the pharmacophore, and eventually it may be possible to produce a molecule that has improved immune activity than the original PQS.

The only information relating to structure and activity is that the two precursors of PQS in the synthetic pathway, 2-heptyl-4(1*H*)-quinolone **2.3** and 3-formyl-2-heptyl-4(1*H*)-quinolone **2.4**, had no activity.



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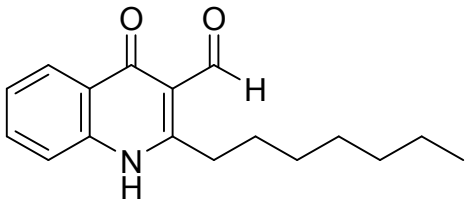


2.4

An issue to consider in future development of these compounds would be their quorum sensing ability. The ideal molecule would potently suppress the

immune system but have no effect with regards the activation of quorum sensing pathways.

The target immunophilins for PQS are not known and hopefully the SAR may also provide some insight into the nature of the interactions between the target receptor and PQS and its analogues. This information will be far from conclusive, in terms of target, but can be used as the initial steps towards understanding the receptor-molecule interaction.

Due to the disadvantages of existing immune modulators, outlined in chapter 1, new and novel molecules are of importance in this field. The ability of the PQS quorum sensing molecule to modulate the immune system warrants further research in developing facile syntheses of this type of molecule.

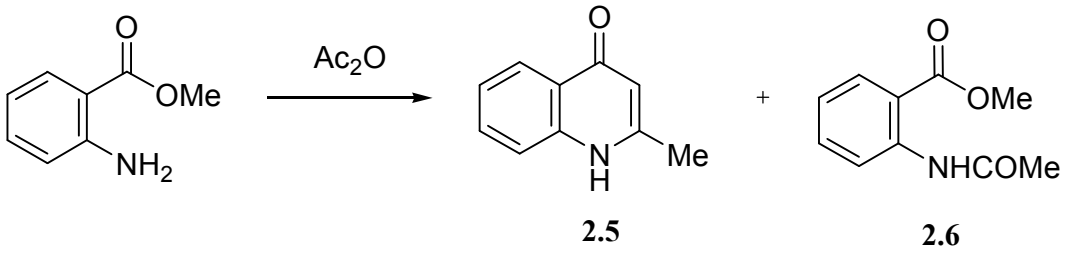
2.1 Synthesis of *Pseudomonas* Quinolone Signal and its analogues

The existing quinolone based drug molecules have already been reviewed in Chapter 1. This review will deal with the synthetic methods reported in literature to elaborate the 4-quinolone core structure present in PQS.

Though a number of examples of 2-methyl and 2-phenyl substituted 4-quinolones are reported in the literature, there is little information on 2-alkyl substituted analogues apart from 2-methyl derivatives.

2.1.1 Review of literature methods of 4-quinolone synthesis

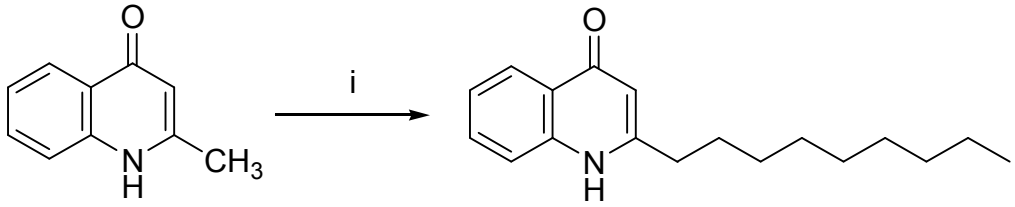
The simplest method reported in the literature for the production of 2-methyl-4(1*H*)-quinolone **2.5** is the condensation of methyl anthranilate with acetic anhydride, scheme 2.1, producing 2-methyl-4(1*H*)-quinolone in one step, though an amide ester of the methyl anthranilate **2.6** is also produced as a side product.



2.5 **2.6**

Scheme 2.1 Synthesis of 2-methyl-4(1*H*)-quinolone using methyl anthranilate

The production of the 2-methyl compound to the SAR study is important as the 2-methyl substituent can be elongated to a longer alkyl chain, for example via the use of *n*-octyl bromide in the presence of sodium amide[119], scheme 2.2.



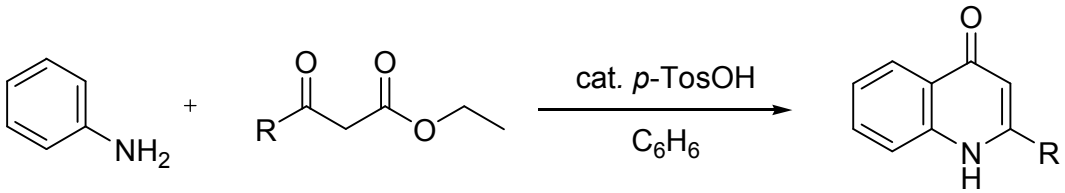
2.7

Reagents and conditions: (i) $NaNH_2$, NH_3 in H_2O, *n*-octyl bromide

Scheme 2.2 Conversion of 2-methyl-4(1*H*)-quinolone to 2-nonyl-4(1*H*)-quinolone

In this example the chain is lengthened to a nonyl chain to give **2.7** but this could easily be used to set the desired length of the alkyl chain after the quinolone ring system was formed. It is more convenient though, if possible, to have the desired alkyl chain in place when the quinolone is initially formed.

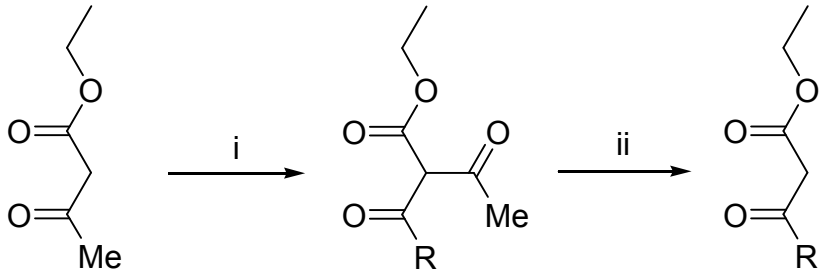
The paper in which the quorum sensing attribute of PQS was described used a synthetic route based on that published by Somanathan *et al*[120]. They synthesised a number of 2-alkyl-4(1*H*)-quinolones **2.9** by the condensation of β-keto esters **2.8** with aniline, scheme 2.3.



2.8 **2.9**

Scheme 2.3 Condensation of aniline with a β-keto ester to produce 2-alkyl-4(1*H*)-quinolone

In this case the requisite β-keto esters were produced by the acylation of ethyl acetoacetate **2.10** with an acid chloride in the presence of sodium hydride, followed by treatment with sodium ethoxide, causing deacetylation[121-123], allowing the alkyl chain to be incorporated as required, as shown in scheme 2.4.



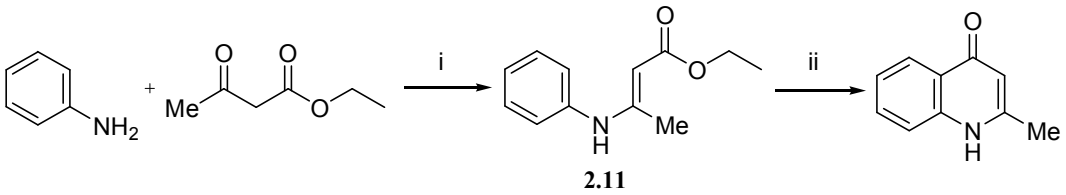
2.10

Reagents and conditions: (i) NaH, RCOCl; (ii) NaOEt in EtOH

Scheme 2.4 Production of β-keto ester using an acid chloride

A limitation with this method of quinolone production, with regards to the demands of this project, is the absence of the hydroxy, or indeed any suitable substitution, at the 3-position meaning that it needs to be introduced onto the already formed quinolone product.

The condensation of a β-keto ester with aniline, seen in scheme 2.3, is also used in the Conrad-Limpach synthesis, the difference being that the enamine product of the condensation **2.11** is isolated and heated in diphenyl ether, a high boiling point solvent, to produce the 4-quinolone. The quinolone is recovered by the addition of petroleum ether, due to differential solubility. This has been used to produce 2-methyl-4-quinolone[124].

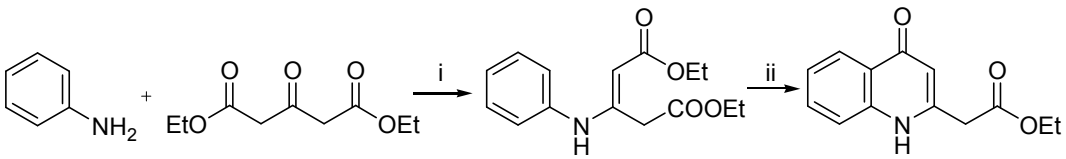


2.11

Reagents and conditions: (i) Glacial AcOH, C_6H_6; (ii) Diphenyl ether, 200 °C

Scheme 2.4 Formation of 2-methyl-4(1*H*)-quinolone

A similar method has been used to produce 2-ester functionalised substitution on the resulting 4-quinolone[125]. In this case the β-keto ester is replaced by diethyl 3-oxoglutarate **2.12** and this is heated in chloroform under Dean-Stark conditions and the intermediate enamine product **2.13** is then heated in excess Polyphosphoric acid (PPA) to form the 4-quinolone **2.14**.

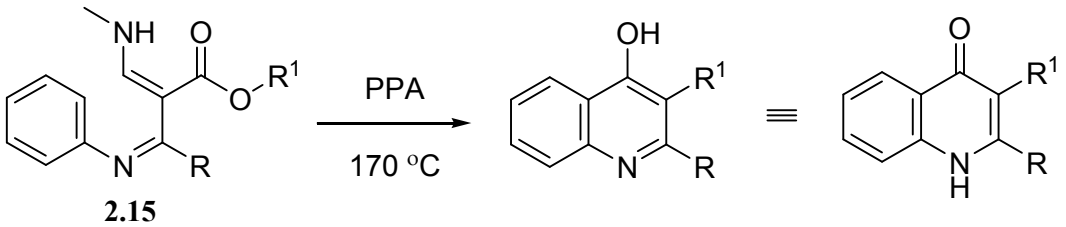


2.12 **2.13** **2.14**

Reagents and conditions: (i) cHCl, $CHCl_3$, Δ; (ii) PPA, Δ

Scheme 2.6 Synthesis of 2-ester functionalised 4-quinolones

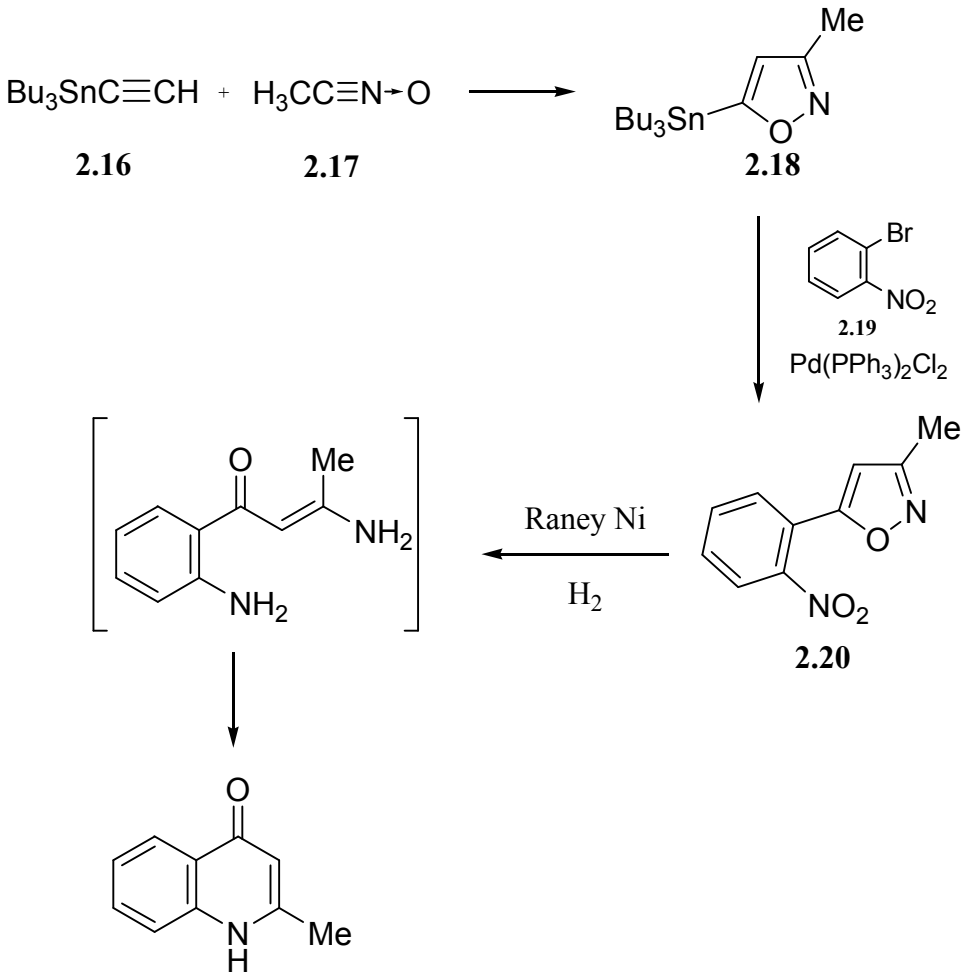
This use of PPA to allow ring closure was initially reported in 1961[126] and concentrated on the conversion of a number of esters **2.15** into the quinolone by heating in PPA at 170 °C.



2.15

Scheme 2.7 Formation of a 2,3-disubstituted 4-quinolone using PPA

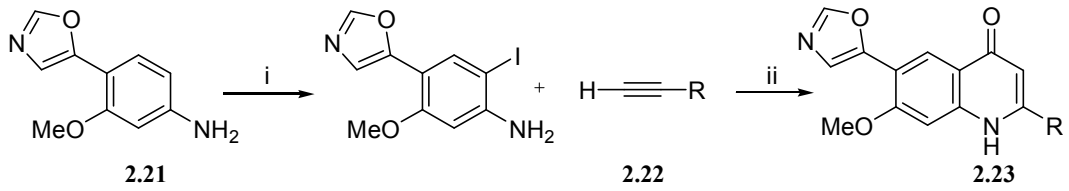
Another method for the production of 2-methyl-4(1*H*)-quinolone involves the initial synthesis of 5-(tributylstannyl)isooxazoles **2.18** using tributylethynylstannane **2.16** and *N*-acetonitrile oxide **2.17**. This forms a bicyclic structure **2.20** when reacted with 3-bromonitrobenzene **2.19** using a palladium catalyst, which is then catalytically hydrogenated over Raney nickel at pressure to obtain 2-methyl-4(1*H*)-quinolone[127].



Scheme 2.8 Formation of 2-methyl-4(1*H*)-quinolone

Quinolone based molecules have also been investigated in relation to inhibition of inosine 5'-monophosphate dehydrogenase (IMPDH)[128], seeking to find specific IMPDH type II selectivity. This study produced 4-quinolones with substitution at the 2, 6 and 7-positions. Two synthetic routes were detailed, both taking 3-methoxy-4-(oxazol-5-yl)benzenamine **2.21** as the starting point, leading to the substitution in the final quinolone molecule as described above. The first step involves the iodination of the aromatic nucleus followed by

reaction with a suitable alkyne **2.22** in the presence of a palladium chloride/triphenyl phosphine catalyst to form the desired quinolone **2.23**[128], scheme 2.9.

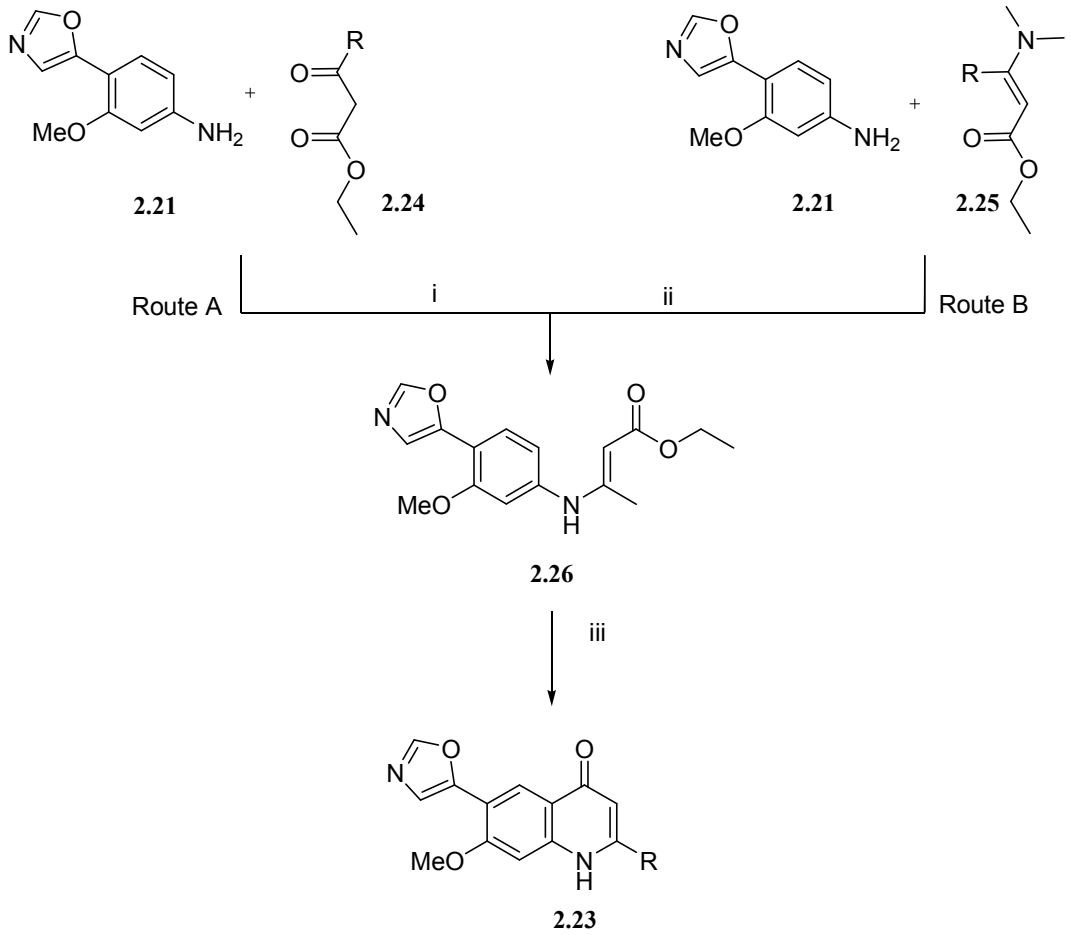


2.21 **2.22** **2.23**

Reagents and conditions: (i) I_2, pyridine, CH_2Cl_2, AgOTf; (ii) $Cl_2Pd(PPh_3)_2$, CO, Et_2NH, Δ

Scheme 2.9 Formation of highly substituted 4-quinolone structure

An alternative method has also been reported for the production of **2.23** and is similar to that mentioned previously. It uses either a β-keto ester **2.24** (Route A) in refluxing toluene in the presence of toluene-*p*-sulphonic acid (*p*-TSA) or 3-(dimethylamino)but-2-enoate **2.25** in refluxing dichloromethane (Route B), in the presence of pyridine *p*-toluenesulphonate (PPTS). Both of these routes produce the same enamine product **2.26**. This enamine is then heated in xylene to close the ring and give the 7-methoxy-6-(oxazol-5-yl)-2-alkyl-4(1*H*)-quinolone **2.23**, scheme 2.10.

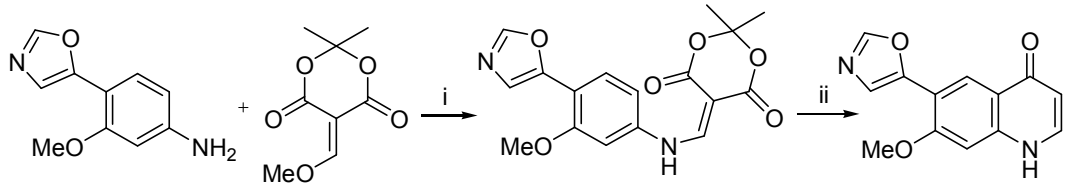


2.21 **2.24** **2.21** **2.25** **2.26** **2.23**

Reagents and Conditions: (i) *p*-TSA, toluene, Δ; (ii) CH_2Cl_2, PPTS, Δ;

Scheme 2.10 Two different routes to the formation of a substituted 4-quinolone

A 4-quinolone without 2-substitution was also produced by a third method, again using 3-methoxy-4-(oxazol-5-yl)benzenamine **2.21** as the starting point, reacting it with 5-(methoxymethylene)-2,2-dimethyl-1,3-dioxane-4,6-dione **2.27** and then heating the resulting product in diphenyl ether[128], scheme 2.11. This differs to the other methods described, as there is no substitution of the heterocyclic ring on the molecule produced **2.28**.

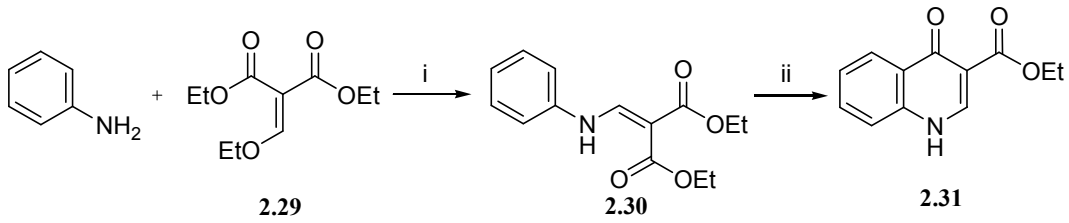


2.21 **2.27** **2.28**

Reagents and conditions: (i) MeOH, rt, 15 min; (ii) Ph_2O, 200 °C, 2.5 h

Scheme 2.11 Formation of 6,7-disubstituted 4-quinolone

Another method to produce quinolones possessing ester, or nitro, functionality at the 3-position has been reported. Here aniline is reacted, neat, with diethyl ethoxymethylenemalonate **2.29**, forming an arylaminomethylenemalonate **2.30**. This can then be heated in Dowtherm A, a high boiling solvent, to around 250 °C to form ethyl 1,4-dihydro-4-oxo-3-carboxylate **2.31**, as seen in scheme 2.12, which then precipitates out of solution on cooling.

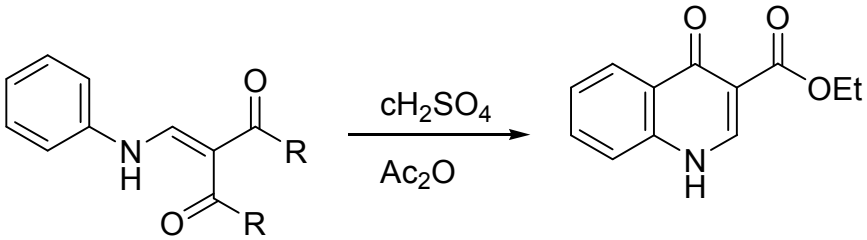


2.29 **2.30** **2.31**

Reagent and conditions: (i) Reactants mixed neat; (ii) Dowtherm A, 250 °C
Scheme 2.12 Formation of ethyl 1,4-dihydro-4-oxo-3-carboxylate

This introduces functionality at the 3-position in a short number of steps, but there is no substitution at the 2-position and this means that an alkyl chain would need to be introduced via metallation for it to be useful for this SAR study.

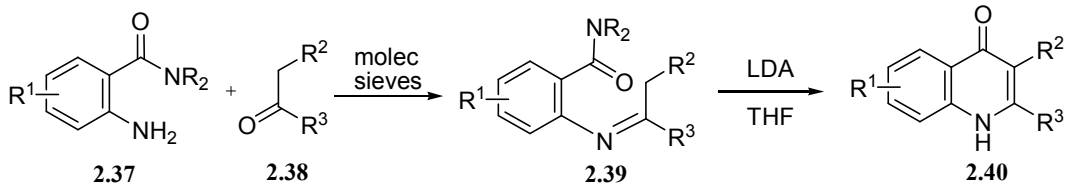
As described above, most commonly the ring closure to a 4-quinolone is reported via thermal methods, that is the use of a high boiling solvent. There is evidence that the ring closure can be carried out using a combination of acetic anhydride and concentrated sulphuric acid. A number of acetoacetate anils **3.35** have been cyclised in this way, producing 3-substituted-4-quinolones **2.36**, scheme 2.14.



2.35 **2.36**

Scheme 2.14 Cyclisation using sulphuric acid and acetic anhydride

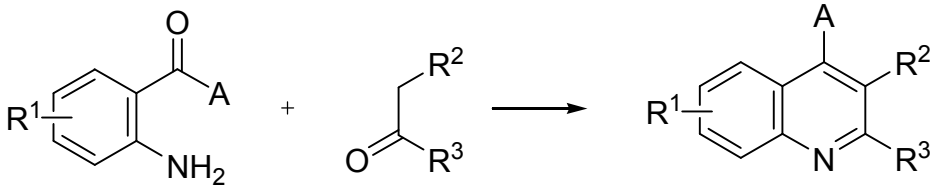
As well as using aniline derivatives to produce quinolones, *o*-amino carbonyl compounds have also been used. In one method, an anthranilamide **2.37** is combined with a ketone **2.38** to produce the intermediate imine **2.39**, which is then treated with lithium diisopropylamide (LDA) in THF, producing a 4-quinolone with 2- and 3-substitution[129] **2.40**, scheme 2.15. The substitution, in this example, consisted of alkyl chain, ethyl ester and phenyl substitution.



2.37 **2.38** **2.39** **2.40**

Scheme 2.15 Formation of 2,3-disubstituted-4(1*H*)-quinolone

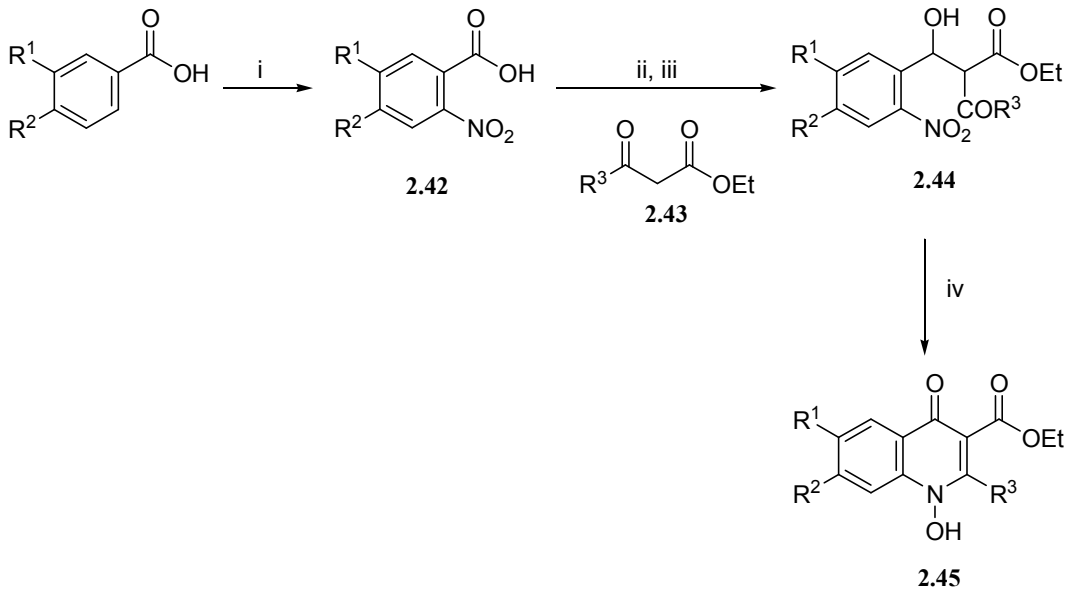
This is a modification of the von Niementowski synthesis, which itself is similar to both the Friedländer and Pfitzínger methods. The nature of the A group, in the molecule **2.41**, changes in relation to the method being shown[129] in scheme 2.16.



2.41

Scheme 2.16 Synthesis of 4-quinolone and derivatives. Methods distinguished by nature of A group; A = H, R (Friedländer); A = CO_2Na (Pfitzínger); A = OH, OR (von Niementowski)

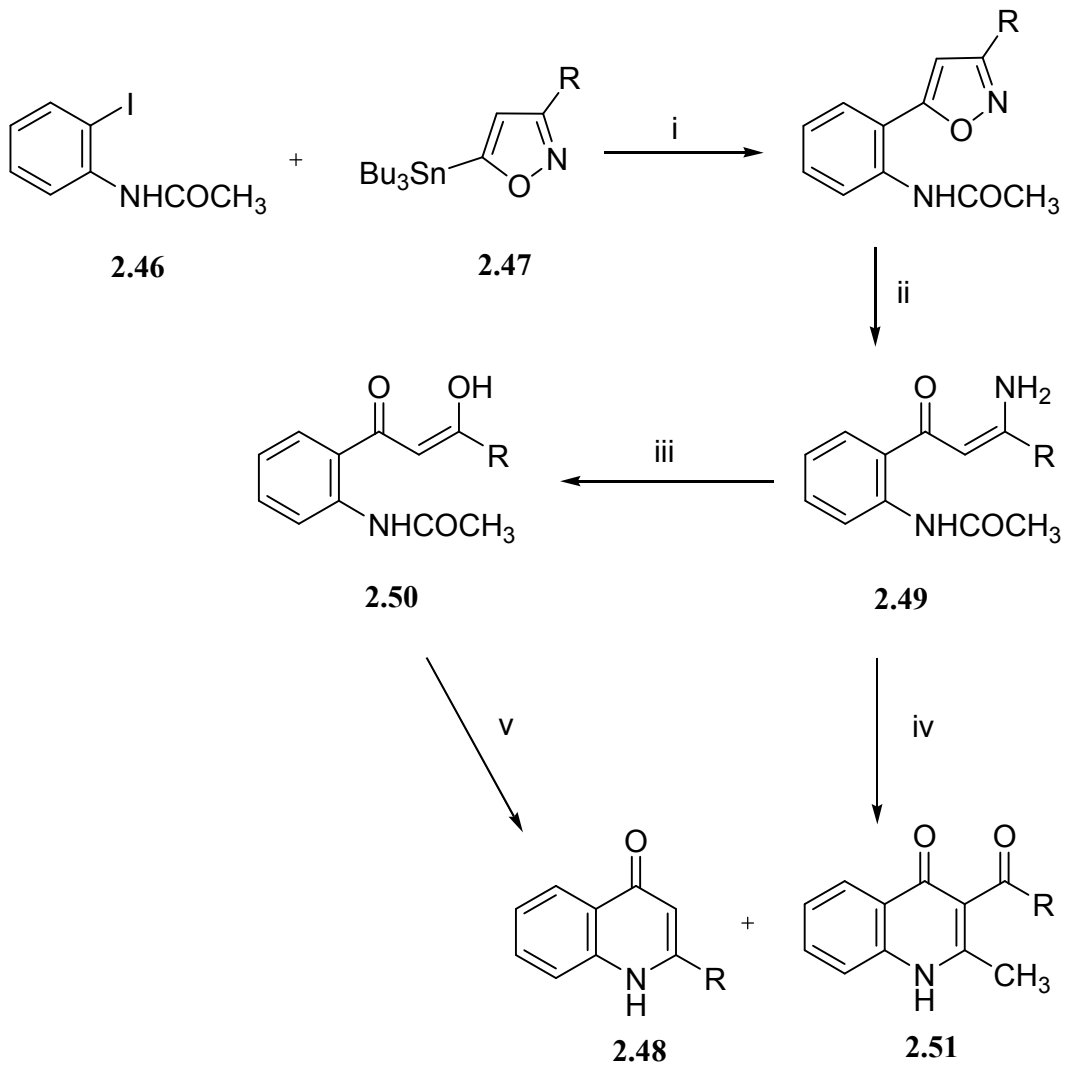
A method similar, in some ways, to those discussed before is where a nitrobenzoic acid **2.42**, produced by using a benzoic acid, is used. The acid is the converted into an acid chloride and is condensed with a β-keto ester **2.43** to give the compound **2.44**. This diketo ester can then be converted to an *N*-hydroxy-2-substituted-4-quinolone **2.45**, possessing ester functionality at the 3 position, by mild catalytic hydrogenation[130], scheme 2.17.



Reagents and conditions: i) HNO_3/H_2SO_4; ii) $SOCl_2$, urea/toluene; iii) Mg, EtOH/toluene; iv) H_2, Pd-C, EtOH, 1 bar

Scheme2.17 Formation of *N*-hydroxy-2-substituted quinolone

The Heck-Stille coupling has been utilised in the production of substituted 4-quinolones[131]. The reaction involves the palladium catalysed coupling of a stannyl compound **2.47** with methyl 2-iodoacetanilide **2.46**, or similar iodobenzene, followed by a catalytic reduction to open the oxazole ring. Depending on the substitution present in the system, the intermediate could either be cyclised directly to the quinolone **2.48** or undergo a transformation before cyclisation, scheme 2.18.



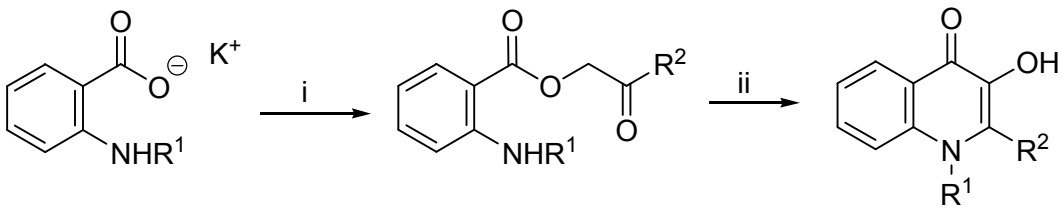
Reagents and conditions: (i) 2-iodoacetanilide, $Pd_2(dba)_3$, $AsPh_3$, dioxane, 50 °C; (ii) Raney-Ni, dioxane (R = Ph) or methanol (R = Me); (iii) HOAc, NaOAc, 110 °C; (iv) HOAc, NaOAc, 110 °C or EtOH, H_2O, HCl; (v) Δ, 140 °C

Scheme 2.18 Formation of 4-quinolones using Heck-Stille coupling

A 4-quinolone was formed from **2.49** (R = Me) by either treating with acidic aqueous ethanol or refluxing in acetic acid in the presence of sodium acetate. If the R = Ph the reflux in acetic acid merely converted the amino group into a hydroxyl **2.50**, which then required to be heated to around 140 °C to form the 4-quinolone. There was a mixture of compounds produced, one with the R group at the 2-position **2.48**, which would be preferred in the case of this project, or the R group as a component of an ester at the 3-position **2.51**, which

unfortunately constitutes the higher percentage of product formed in the reaction.

Quinolones with 2-substitiution and a hydroxyl group at the 3-position **2.54**, ideal for our SAR study, have also been reported and are synthesised by the method shown in scheme 2.19[132]. Anthranilic acids are used as starting materials and are esterified using either chloroketones or bromoketones, in the presence of potassium bromide, indicating that the potassium salt **2.52** is involved in the reaction. These anthranilate derivatives **2.53** are then heated either in *N*-methylpyrrolidone (NMP) or polyphosphoric acid (PPA), for times ranging from 10 min to 15 h. The length of time was dependent on the nature of the substitution present in the system. It was found that using NMP as the reaction solvent is more suitable than PPA, in terms of yield and purity. The substitution present on the molecules produced was altered at the 2-position and the nitrogen but was limited to methyl and phenyl substitution, no alkyl chain substitution was included. It was also revealed that increased size of substituents at the 1- and 2-positions, would cause a decrease in the yield of the final quinolone.

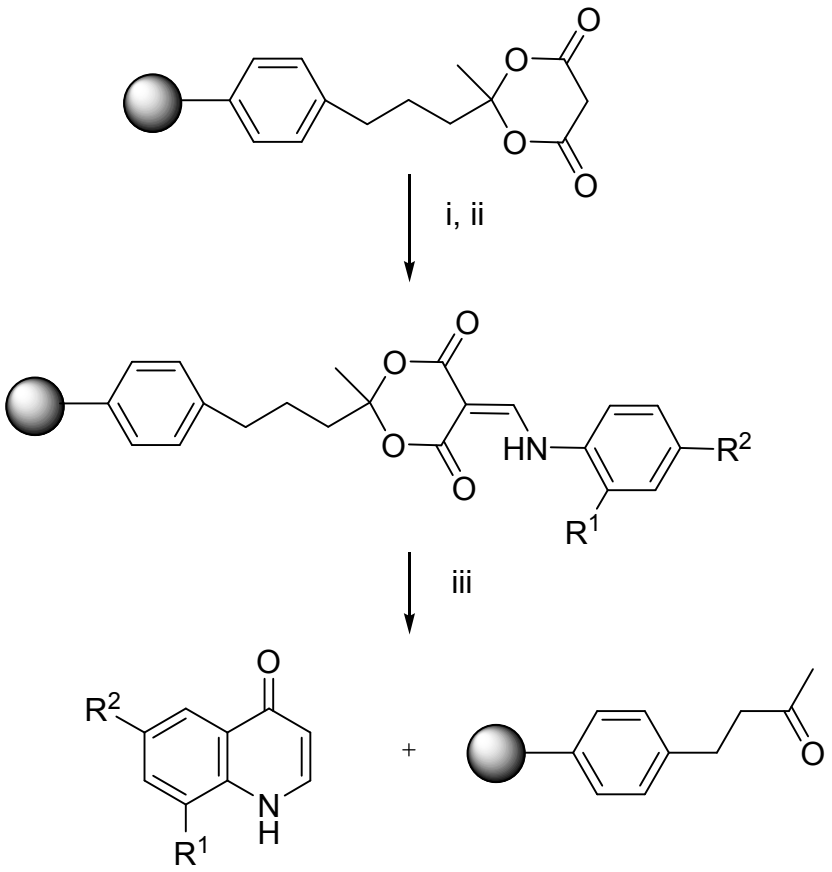


2.52 **2.53** **2.54**

Reagents and conditions: (i) XCH_2COR, DMF, 20 – 50 °C; (ii) PPA or NMP

Scheme 2.19 Formation of *N*-substituted-2-substituted-3-hydroxy-4(1*H*)-quinolone

4-Quinolones disubstituted in the 6- and 8-positions **2.55** have been produced using solid phase synthetic methods[133]. A polymer-supported cyclic ester was reacted with an aniline derivative and then heated to 240 °C to give the 4-quinolone **2.55** and return a polymer bound ketone **2.56**, which can then be recycled in another reaction. The quinolones produced from this method do not have any substitution at the 2 and 3 positions.



2.55 **2.56**

Reagents and conditions: (i) $HC(OEt)_3$, reflux, 6 h; (ii)

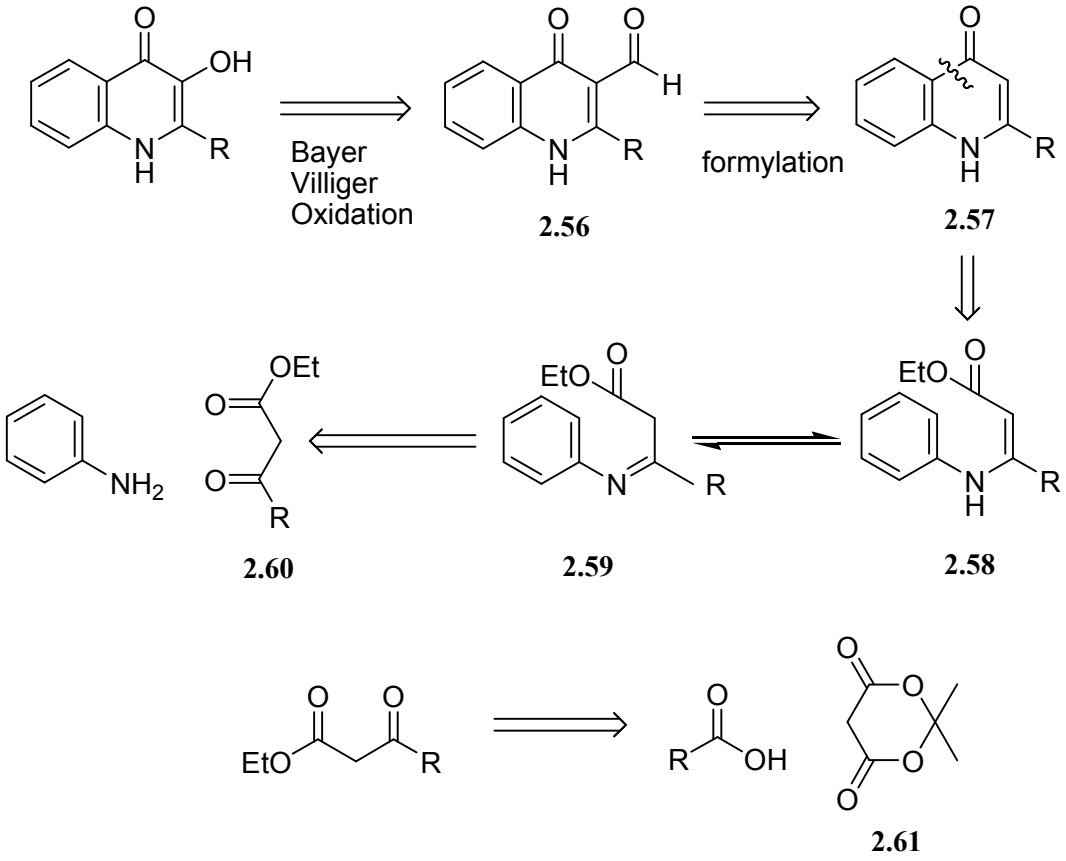
Scheme 2.20 Solid phase synthesis of disubstituted 4-(1*H*)-quinolone

2.1.2 Retrosynthetic analysis of PQS

The synthesis reported by Pesci *et al*[100] can be rationalised if a retrosynthetic approach to PQS is considered and this can be seen in scheme 2.21.

PQS has a 4-quinolone structure with a heptyl side chain and a hydroxyl group at the 3-position. It would be possible to have this hydroxyl introduced via a Baeyer Villiger oxidation on a formylated quinolone **2.56**, produced from the 2-heptyl-4(1*H*)-quinolone **2.57**. A disconnection at the C-C bond shown affords an enamine intermediate **2.58**, which will also exist in the less stable imine form **2.59**. Considering the imine form of this intermediate, it becomes obvious that a condensation reaction between aniline and a suitable β-keto ester **2.60** would provide a suitable synthetic route. β-Keto esters may be

formed by the acylation of Meldrum's acid **2.61** with a carboxylic acid, followed by alcoholysis of the acylated derivative.



Scheme 2.21 Retrosynthetic approach of PQS

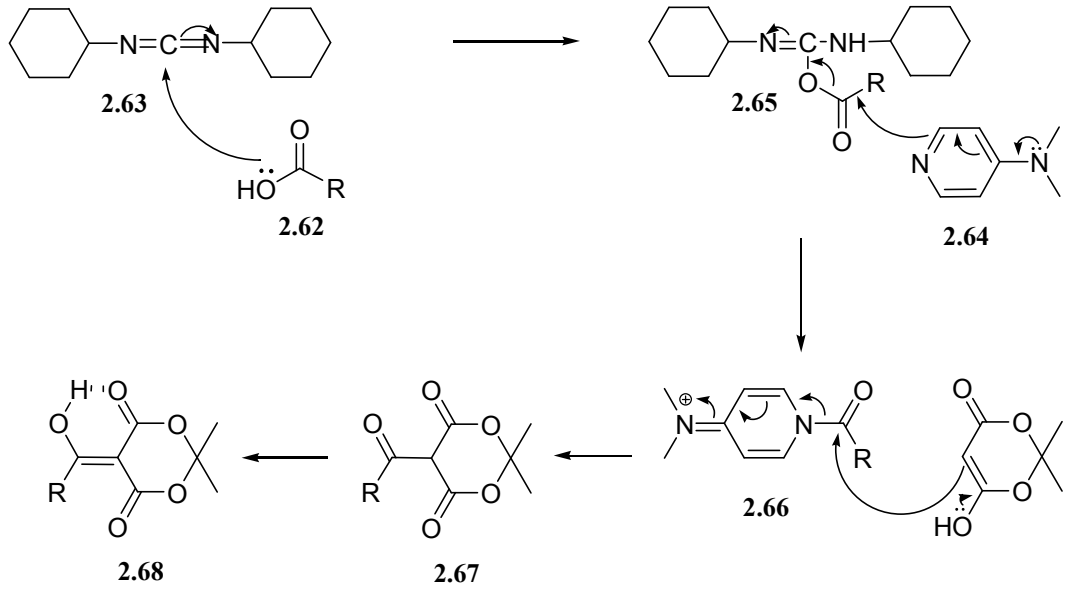
2.2 Synthesis of PQS via Existing Method

The existing method was evaluated in the lab to identify problems and issues that arise at each step of the synthetic route.

2.2.1 Formation of β-keto esters by acylation of 2,2-dimethyl-1,3-dioxane-4,6-dione (Meldrum's acid)

Meldrum's acid is initially acylated using the appropriate carboxylic acid **2.62**. The acid determines the length of the final carbon side chain at the 2-position of the quinolone. An acid containing one more carbon than required in the side chain needs to be used e.g. for a pentyl side chain, hexanoic acid is used. The

carbonyl in the acid needs to be activated to make it more electrophilic. The method involves 1,3-dicyclohexylcarbodiimide (DCCI) **2.63** with a catalytic amount 4-dimethylaminopyridine (DMAP) **2.64**. The mechanism for the 5-acyl derivative formation is shown in scheme 2.22.



Scheme 2.22 Acylation of Meldrum's acid

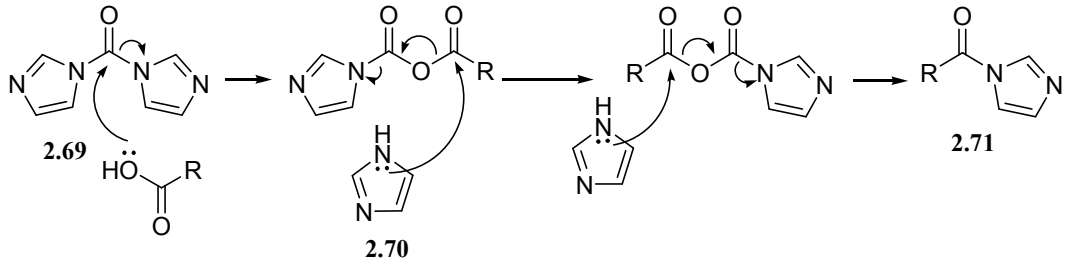
Initially the condensation of the carboxylic acid with DCCI affords *o*-acylisourea **2.65,** which is intercepted by the nucleophilic attack of DMAP to give the highly reactive *N*-acyl pyridinium species **2.66**. The latter is the effective acylating agent and delivers the desired product by regenerating DMAP. As the DMAP is continually being regenerated it needs to be added in only catalytic amounts. The product **2.67** exists in its H-bonded enolic form **2.68**.

2.2.1.1 Alternative method for acylation of Meldrum's acid using 1,1'-carbonyldimidazole (CDI)

Alternatively the acylation may be achieved using 1,1'-carbonyldimidazole (CDI) as shown in schemes 2.23 and 2.24.

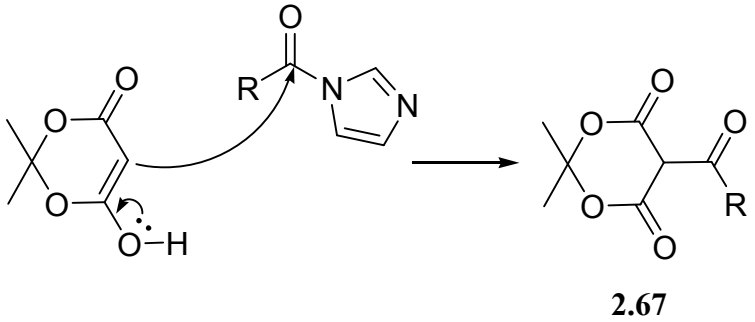
The carboxylic acid is activated in a similar manner by the use of 1,1'-carbonyldimidazole (CDI) **2.69**, shown in scheme 2.23. There is electrophilic

attack on the carbonyl of the CDI, liberating an imidazole ring **2.70** and starts a process that produces an acylated imidazole species **2.71** with an activated carbon in the carbonyl.



Scheme 2.23 The activation of carbon by the production of an imidazole species

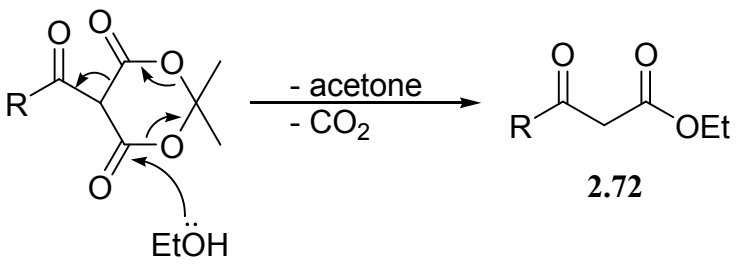
The imidazole species makes the carbon more electrophilic and is therefore open to attack by the enolised form of Meldrum's acid, forming 5-acyl Meldrum's acid **2.67**, scheme 2.24.



Scheme2.24 Formation of 5-acyl Meldrum's acid from imidazole

2.2.2 Formation of β-keto ester

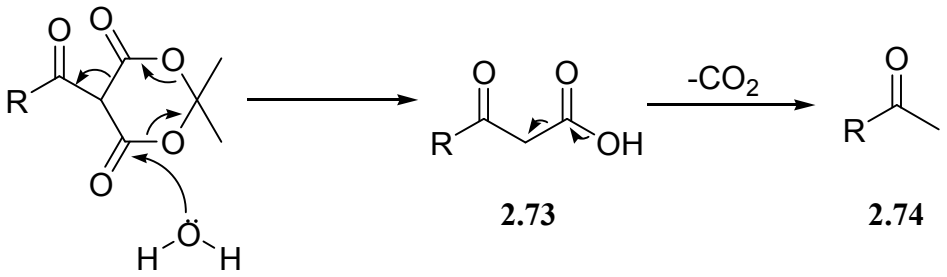
The alcoholysis of Meldrum's acid with ethanol at reflux gives the corresponding β-keto ester **2.72**. The mechanism involves the nucleophilic attack of ethanol on one of the endocyclic carbonyls, liberating acetone and carbon dioxide, and producing the β-keto ester, as shown in scheme 2.25. The ring carbonyl is much more electrophilic than the exocyclic carbonyl due to the latter being deactivated due to the positive inductive effect of R.



2.72

Scheme 2.25 Formation of β-keto ester

Care was taken to use anhydrous ethanol as if there is water present in the system there will be some β-keto acid formed **2.73**, which will then convert to the corresponding methyl ketone **2.74**, as shown in scheme 2.26. However, if formed, this can be removed using column chromatography.

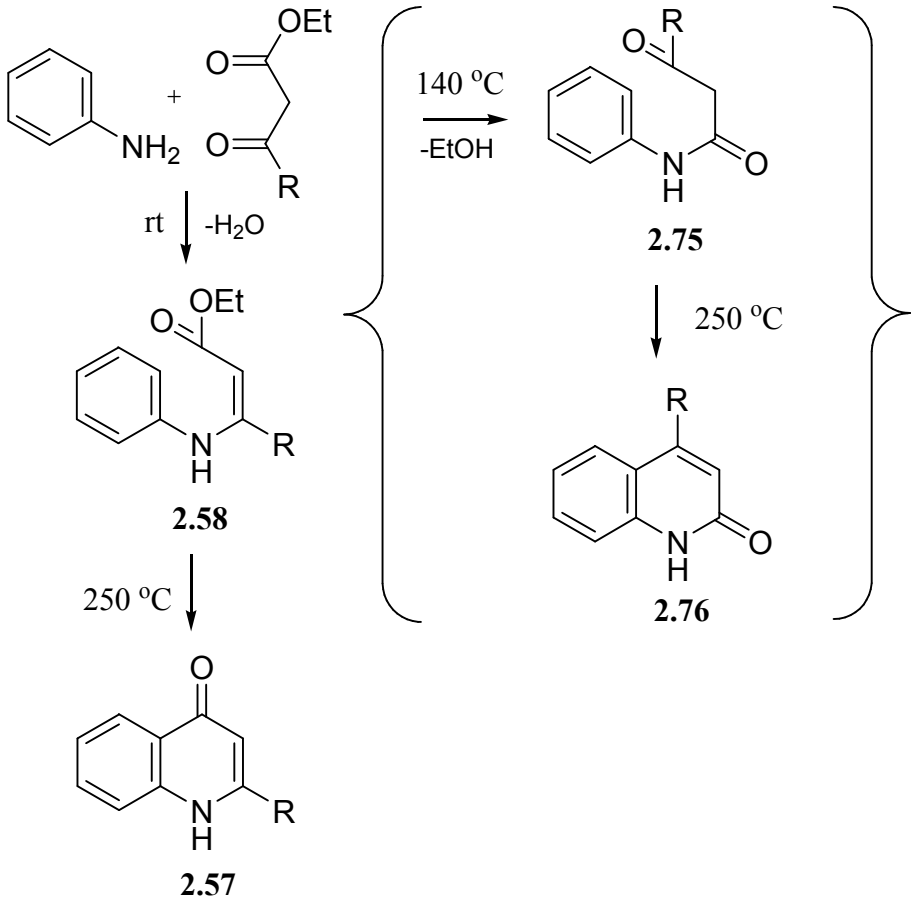


2.73 **2.74**

Scheme 2.26 Formation of a methyl ketone

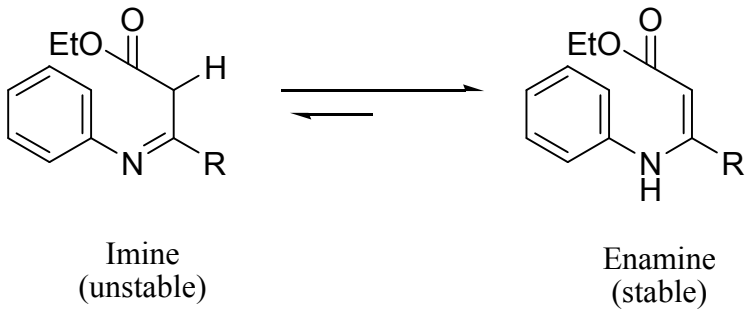
2.2.3 Formation of 4-quinolone

When reacted with aniline at low temperatures the β-keto ester gives the kinetic product **2.58**, which when cyclised, gives the 4-quinolone **2.57**. When higher temperatures are used, the thermodynamic product **2.75** is given, producing a 2-quinolone **2.76** when cyclised, scheme 2.27. The literature value for the difference in temperature is stated to be approximately 140 °C, but in our hands there appears to be a difference in the proportions of 4- and 2-quinolone produced even with the relatively small difference in temperature such as provided by using benzene instead of toluene.



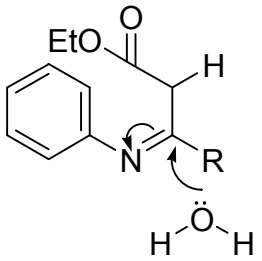
Scheme 2.27 Formation of thermodynamic (2-quinolones) and kinetic product (4-quinolones)

The low temperature directs the attack by the nitrogen lone pair to the more electrophilic ketone carbonyl rather that the ester carbonyl. The product has two resonance forms, the imine and enamine (scheme 2.28). The imine form is unstable and the product is stabilised by the enamine form.



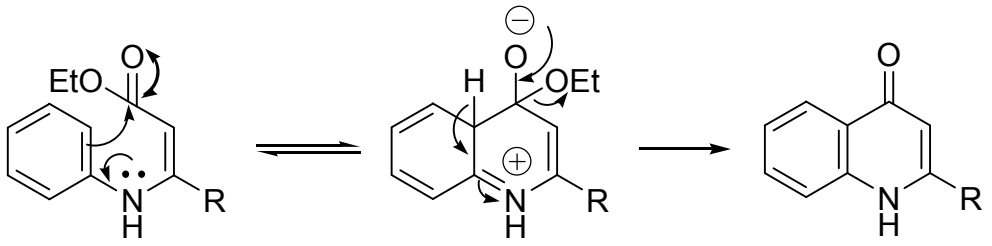
Scheme 2.28 Resonance forms of product **2.58**

The imine is also liable to attack from water, thereby reversing the reaction, scheme 2.29. For this reason, Dean Stark apparatus is used to remove any water formed during the reaction, thereby reducing the chances of this hydrolysis.



Scheme 2.29 Hydrolysis of imine

Heating at reflux in diphenyl ether (b.p. 250 ^{o}C) provides the energy required to close the ring, forming the 4-quinolone **2.57**, scheme 2.30. The reaction proceeds by the nucleophilic attack of the aromatic ring on the ester carbonyl. This is a high-energy process, as it requires the disruption of the aromaticity of the ring hence the high temperature needed to accomplish the reaction.



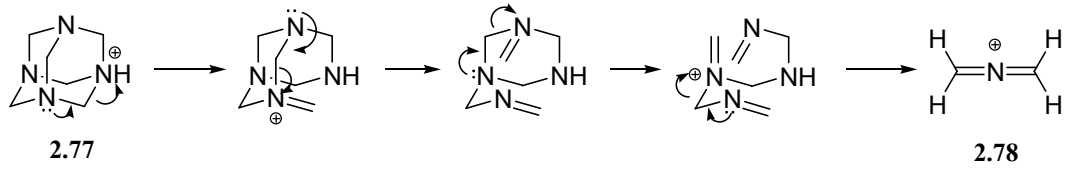
2.57

Scheme 2.30 Mechanism of thermal ring closure

To complete the synthesis of PQS, a hydroxy group needs to be introduced at the 3-position. For this, initially a 3-formyl group was introduced as discussed below.

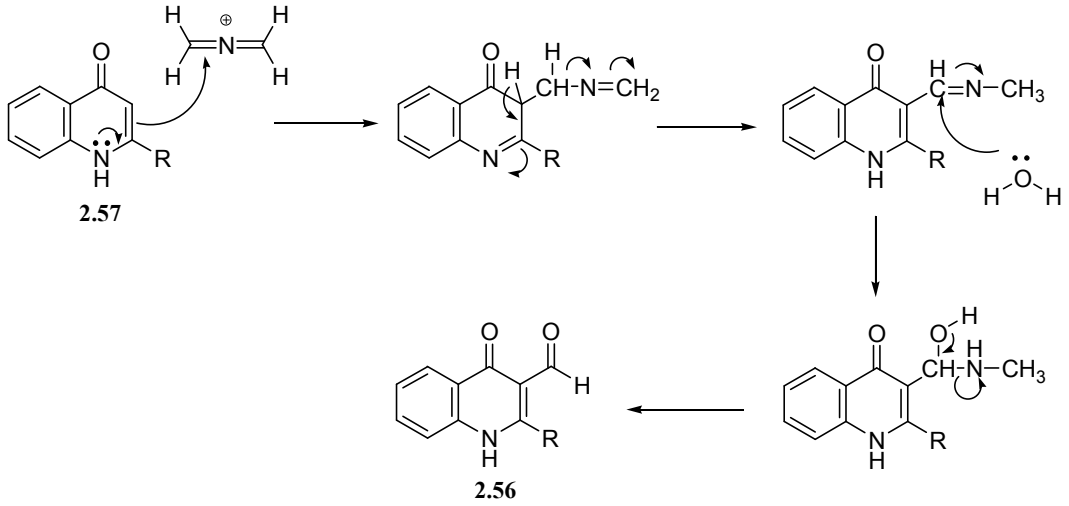
2.2.4 Formylation of 4-quinolone

A method for formylation found in the literature, the Duff Reaction[134], involves hexamine **2.77** in the presence of trifluroacetic acid (TFA)[100,135]. The mechanism of the reaction is shown in scheme 2.31. The protonation of the hexamine leads to the eventual release of the reactive species **2.78**.



Scheme 2.31 Formation of ammonium ion from hexamine

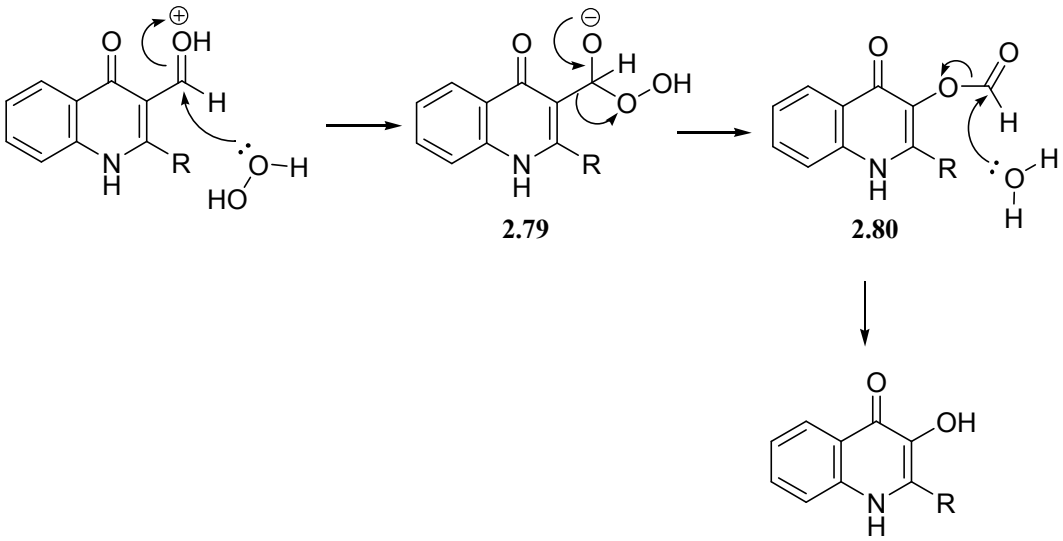
The species **2.78** then reacts with the quinolone **2.57**, at the 3-position on the ring, to give the formylated product **2.56** following aqueous work-up, scheme 2.32. This step only produces a 44 % yield of the formylated 4-quinolone.



Scheme 2.32 Formylation of 4-quinolone by hexamine in presence of TFA

2.2.5 Conversion of Formyl Group to Hydroxyl via Baeyer Villiger Oxidation

The conversion of the formyl group to the required hydroxyl is via a Baeyer Villiger oxidation and involves the use of hydrogen peroxide. This attacks the protonated carbonyl of the formyl group forming intermediate **2.79** which rearranges to form the bond between the 3-position carbon of the quinolone and the oxygen, replacing the C-C bond with a C-O bond, and producing the formate **2.80**. This formate is then hydrolysed in the presence of water to yield the hydroxyl group and the product PQS.



Scheme 2.33 Conversion of formyl to hydroxyl via Baeyer Villiger oxidation

Although this method delivers the final product, it does so only in small yield. The problems with this synthetic route include a large number of steps, some of which are low yielding. If adopted, this method will require synthesis of each different analogue from scratch, as there is no room to alter a core molecule to produce a number of analogues quickly.

2.3 Exploration of alternative synthetic routes to PQS and its analogues

The problems that need to be addressed with regard to a new method for the synthesis of PQS and related 4-quinolone analogues are as follows:

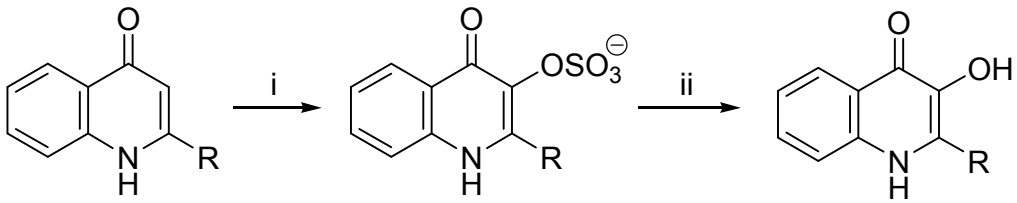
- Large number of steps in synthetic route. It would be advantageous to reduce these, if possible.
- Steps that are low yielding
- Formylation step that does not work effectively and is low yielding
- An intermediate synthon that could deliver analogues with fewer steps

Examining the synthesis of PQS that has been described above certain problems can be identified and possible improvements that could be suggested.

2.3.1 Alternative Methods for Formylation

The formylation step was critical but low yielding and a difficult reaction in practice. Two possible approaches could be considered. One would be to investigate different methods of introducing the formyl, or acyl, group onto the quinolone structure. Another option would be to devise a synthetic route which delivers a suitable group at the 3-position that can be later transformed into an OH group. In both cases ideally, for the synthesis of PQS, the group present at the 3-position would be a hydroxyl group, thereby negating the need for further synthetic steps to convert the group into the hydroxyl but there may be advantages gained, with regards to the structure activity relationship study, if the functional group was one that would allow conversion into a number of different groups and thus allow swift analogue production.

A method of doing this is by the use of the Elbs oxidation, shown in scheme 2.34, in which a peroxodisufate ion reacts with the quinolone anion, forming a sulfate ester **2.81**. This ester can be hydrolysed using an acid catalysed hydrolysis, forming a quinolone with the desired hydroxyl functionality[136].



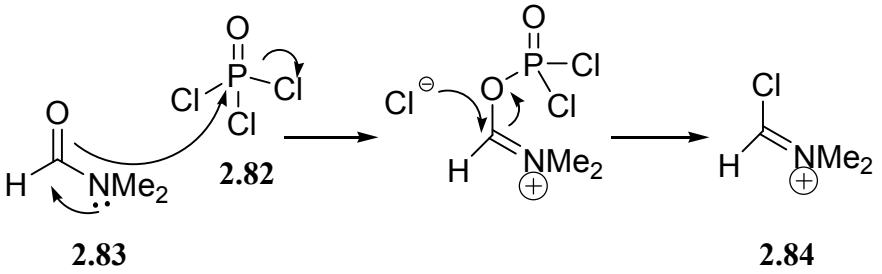
2.81

Reagents and conditions: (i) $S_2O_4^{2-}/OH^-$; (ii) H_2O/H^+

Scheme 2.34 Introduction of hydroxyl group via Elbs oxidation

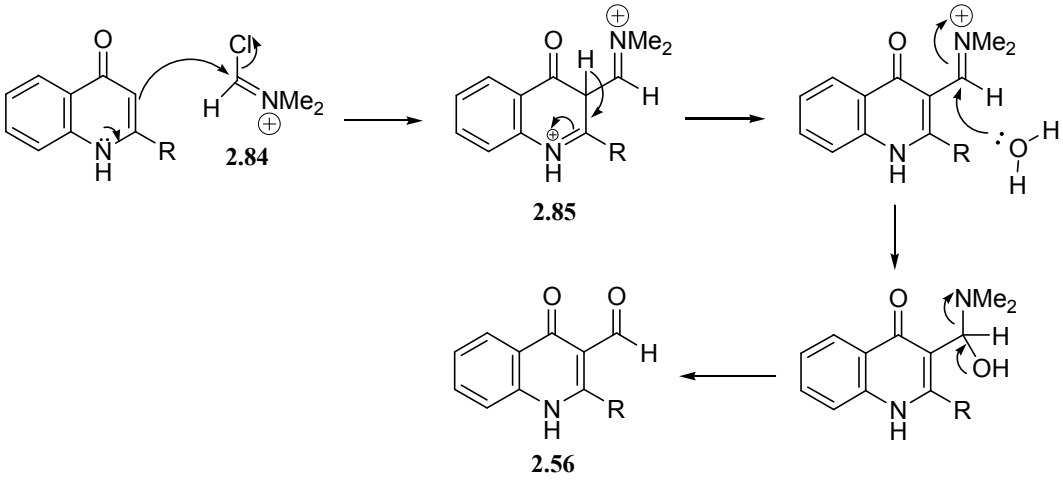
2.3.1.1 Vilsmeier Reaction

A commonly used method for formylation is the Vilsmeier reaction; therefore the suitability of this method was investigated. The mechanism is shown in schemes 2.34 and 2.35. There is the initial formation of the Vilsmeier iminium cation **2.84** via the reaction between phosphorus oxychloride ($POCl_3$) **2.82** and the solvent, dimethylformamide (DMF) **2.83**.



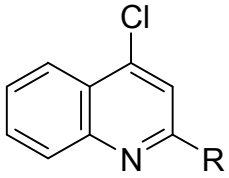
Scheme 2.34 Formation of Vilsmeier cation

This iminium cation **2.84** could then react with the quinolone at the 3-position to form a more stable iminium salt **2.85**, due to the conjugation between the heterocyclic nitrogen and the iminium group. A work up involving aqueous Na_2CO_3 would hydrolyse the imine salt, giving the formylated product **2.56**.



Scheme 2.35 Vilsmeier reaction followed by hydrolysis

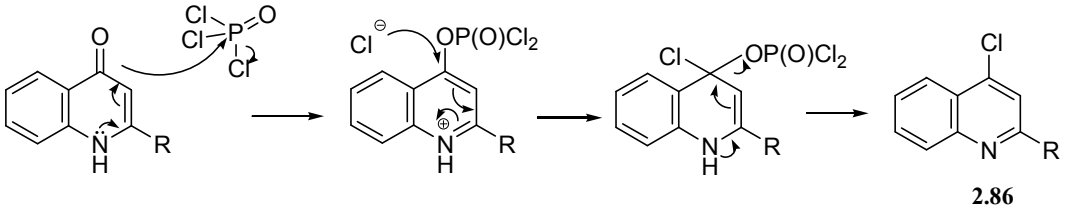
Two different methods of the Vilsmeier reaction were attempted[137,138] but unfortunately did not yield the desired formylated product. Spectroscopy of the product suggests that, instead of formylating at the 3-position, there was chlorination at the 4-position, producing **2.86**.



2.86

If there had been formylation at the 3-position there should be a proton observable for both the formyl group and that attached to the nitrogen, these were not present. Additionally, in the mass of the compound produced did not match that of the expected product. Having an additional CHO, the mass of the formylated product should have been 271 but MS showed that it had the characteristic pattern to show the relative occurrences of Cl mass prominent around 261. The formation **2.86** was not totally unexpected as $POCl_3$ is often used as a chlorinating agent, although this is mostly when it is used on its own as a primary reactant.

Initially it was thought that the method chosen did not give sufficient opportunity for the Vilsmeier complex to form in the reaction vessel and therefore the reaction proceeded as though the quinolone was reacting with $POCl_3$ (scheme 2.36).



Scheme 2.36 Chlorination of quinolone

An alteration to the method, allowing prior mixing of $POCl_3$ and DMF, thereby giving an adequate opportunity for the complex to form, was attempted but the chlorinated product was again produced. It is possible that the Vilsmeier reaction would exclusively form 3-formyl product from *N*-methyl quinolone as the proton is missing to provide the 4-chloro derivative.

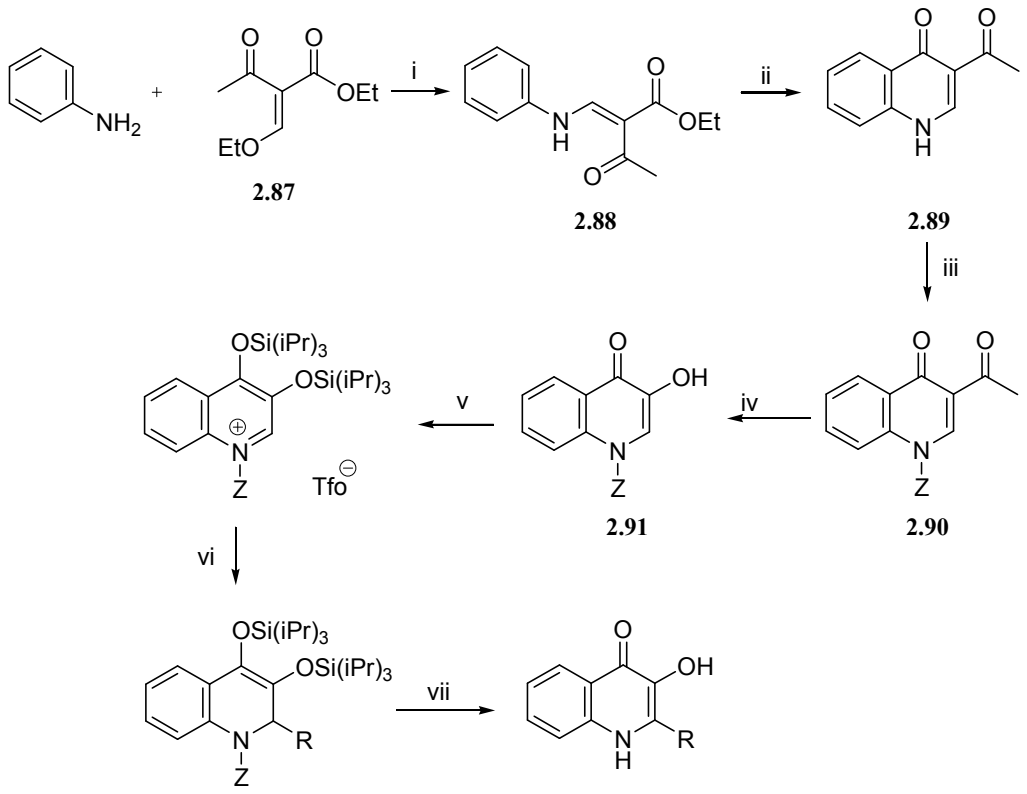
2.3.2 Exploring alternative methods for cyclisation

In the existing method used to produce PQS, in the cyclisation step, the aromaticity of the carbocyclic ring needs to be disrupted to allow the ring closure that forms the bicyclic quinolone structure. An aromatic structure is very stable and therefore a large amount of energy needs to be introduced to the system to allow it to be overcome, hence the use of a solvent with a high boiling point. Diphenyl ether was used in this case. If reactive functional groups were present on the required positions of the benzene ring and allowed cyclisation without disrupting the aromatic system of the benzene ring, the ring closure would theoretically require less energy, proceed more readily and may prove preferential in terms of ease of execution and yields.

Another issue of concern with the use of diphenyl ether is the recovery of the quinolone compound following the reaction. This is achieved via differential solubility of the quinolone between diphenyl ether and petroleum ether (60 – 80 °C) and recovery is low. When the process is scaled up it becomes problematic in relation to the volume of petroleum ether required. Considering these issues, alternative methods were attempted to form a quinolone ring system without the need for thermal methods of cyclisation.

2.3.2.1 Synthesis of 2-alkyl-3-hydroxy-4(1*H*)-quinolone via 3-Acetyl-4(1*H*)-quinolone

Literature shows a method for the synthesis of an ester substituted quinolone that may be able to be altered to produce an acylated 4-quinolone. The synthesis involves a ring closure via the use of concentrated sulphuric acid and acetic anhydride. In addition this quinolone may be used as a synthon molecule that will allow the introduction of a variety of side chains at the 2-position. In order to ensure the addition of the alkyl chain is directed specifically to the 2-position the nitrogen would need to be protected using a suitable group.



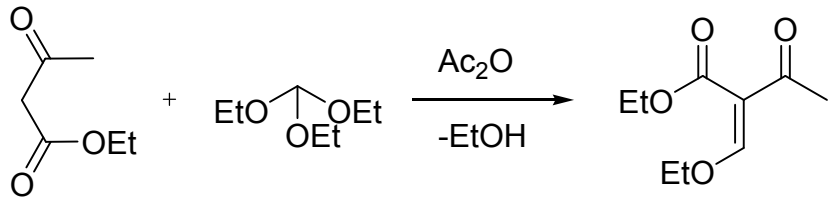
Reagents and Conditions: (i) Δ; (ii) CH_2SO_4/Ac_2O; (iii) ZCl; (iv) H_2O_2 in H_2O, $NaOH_{(aq)}$, EtOH, 6 h; (v) $(iPr)_3SiOTf$, rt, 1h; (vi) RMgBr, CH_2Cl_2/THF, rt (vii) 10% PdC, H_2, EtOH, rt

Scheme 2.37 Proposed synthesis of PQS and its analogues via acylated 4-quinolone synthon

Aniline is reacted with ethyl 2-(ethoxymethylene)-3-oxobutanoate **2.87**, forming **2.88**, which undergoes a ring closure to the 3-acetyl-4(1*H*)-quinolone **2.89**. This 4-quinolone can then be protected, using the benzyloxycarbonyl protecting group, to furnish **2.90** followed by the conversion of the 3-acetyl group to the 3-hydroxy derivative **2.91** via a Baeyer Villiger oxidation. Protection of both the 3- and 4-hydroxy groups using triisopropylsilyl triflate allows the introduction of the alkyl side chain at the 2-position via the use of an appropriate Grignard reagent[139]. Hydrogenolysis removes all the protecting groups in one step, affording the desired 2-alkyl-3-hydroxy-4(1*H*)-quinolone.

2.3.2.1.1 Synthesis of ethyl 2-(ethoxymethylene)-3-oxobutanoate

The first step in this synthesis is the production of the ethyl 2-(ethoxymethylene)-3-oxobutanoate **2.87** and this is done by the condensation of ethyl acetoacetate **2.92** with triethyl orthoformate **2.93** in the presence of acetic anhydride, scheme 2.38.

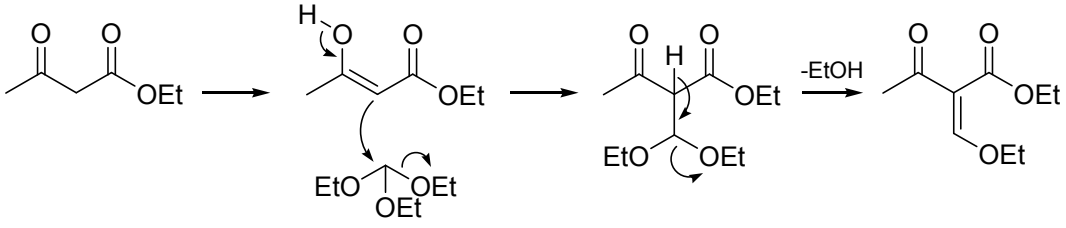


2.92 **2.93** **2.87**

Scheme 2.38 Formation of ethyl 2-(ethoxymethylene)-3-oxobutanoate

The reactants are mixed and heated to 140 °C. As the reaction proceeds, ethanol is produced as the by product and reacts with the acetic anhydride, forming ethyl acetate. It is thus easy to monitor the reaction, the reaction having stopped when no more ethyl acetate is produced. As well as this, if the ethyl acetate is distilled off as it is produced it will drive the reaction forward.

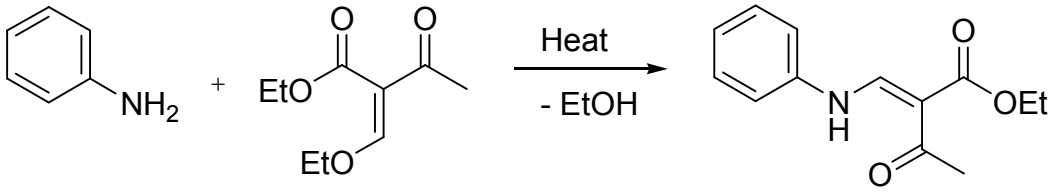
The reaction proceeds via the mechanism shown in scheme 2.39. The reaction is initiated via the enolic form of the ethyl acetoacetate, leading to a Michael type attack on the triethyl orthoformate. The product was then purified using vacuum distillation, producing a straw coloured, viscous liquid.



Scheme 2.39 Mechanism of formation of ethyl 2-(ethoxymethylene)-3-oxobutanoate

2.3.2.1.2 Synthesis of the arylaminomethylenemalonate

As discussed earlier, aniline is mixed with ethyl 2-(ethoxymethylene)-3-oxobutanoate **2.87** and heated to 130 °C for 3 h, forming an arylaminomethylenemalonate **2.88**, scheme 2.40. After the allotted time the reaction mixture is cooled to room temperature and then further cooled, allowing the malonate to solidify as an amber solid. The appearance of the solid suggests an amorphous nature, rather than crystalline.



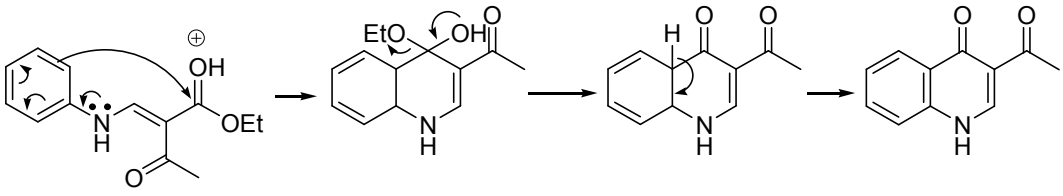
2.87 **2.88**

Scheme 2.40 Formation of arylaminomethylene malonate

Also, it is obvious that there will be two isomers of the arylaminomethylene malonate due to the presence of the double bond. The NMR confirmed this, with two separate signals showing for each expected proton that integrates to the expected value.

2.3.2.1.3 Synthesis of 3-acyl-4(1*H*)-quinolone

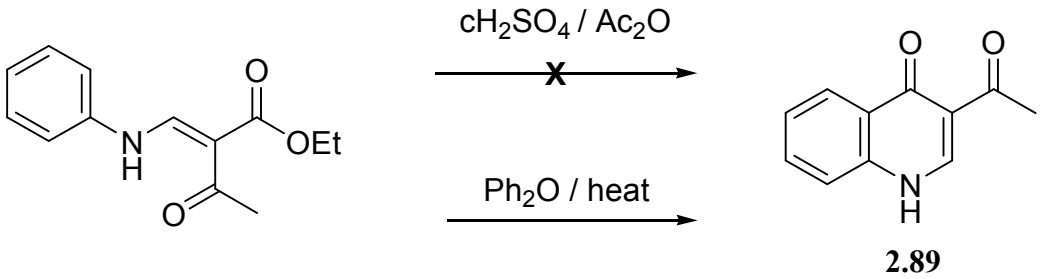
It was stated in the literature that the ring closure to the quinolone could be achieved by means of a reaction involving cH_2SO_4 and acetic anhydride[140]. This would proceed through the mechanism shown in scheme 2.41 and start with the acid protonating one of the carbonyls, leading to electrophilic attack by the aromatic electrons.



Scheme 2.41 Proposed mechanism of acid catalysed 3-acetyl-4(1*H*)-quinolone ring closure

Disappointingly this was not achieved. After the initial reaction, the solution is neutralised and chilled, prompting a white solid to precipitate. This white solid was shown to be, in the main, returned starting material by NMR.

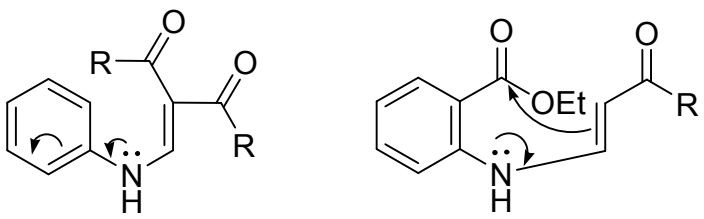
However, refluxing in diphenyl ether as used in the original method produced the desired compound **2.89** using petroleum ether to precipitate the solid out of solution gave a better recovery in this example, most likely due to the absence of an alkyl chain aiding the molecule's solubility in polar solvents.



2.89

Scheme 2.42 Comparing methods of ring closure

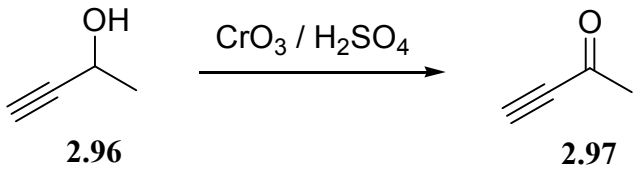
Another method reported in literature proposed a different route to produce 3-acetyl-4(1*H*)-quinolone without using a thermal method to effect ring closure. Also, the route dealt with another issue with the original synthesis in relation to the ring closure step. As mentioned before for the ring closure to take place with a mono-substituted benzene derivative the aromaticity of the benzene ring has to be overcome as shown in **2.94**. If there is an additional ester substituent at the ortho-position on the benzene ring, this would be a lower energy process.



2.94 **2.95**

Scheme 2.43 Differences between mechanisms of 4-quinolone ring closure with and without ortho-substituents

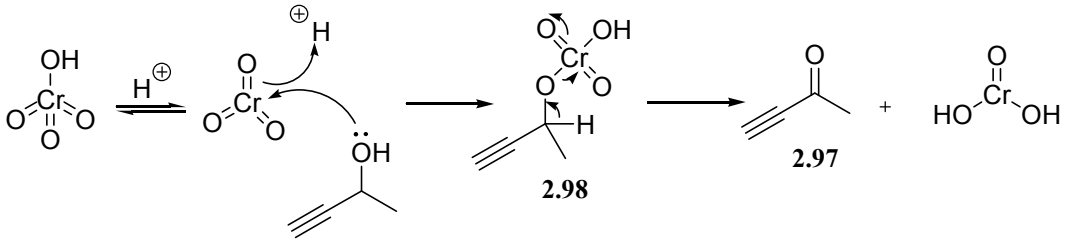
To this end, structure **2.95** was synthesised as follows. The commercially available 3-butyn-2-ol **2.96** was oxidised using chromic acid to form 3-butyn-2-one **2.97**. The NMR showed the loss of the hydroxyl and other proton, as well as a shift in the alkyne proton.



2.96 **2.97**

Scheme 2.44 Formation of 3-butyn-2-one by oxidation of 3-butyn-2-ol

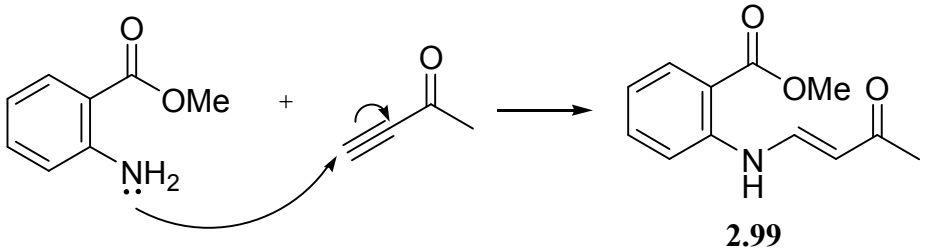
The oxidation proceeds according to the mechanism shown in scheme 2.45. The reaction starts with the formation of $HCrO_4^-$ ions, Cr(VI). In the presence of acid this forms a chromate ester with the alcohol. This ester decomposes by elimination of the $HCrO_3^-$, (CrIV), yielding the ketone product.



Scheme 2.45 Chromic acid oxidation of 3-butyn-2-ol

The Cr(IV) subsequently reacts with a Cr(VI) species to yield two Cr(V). These Cr(V) species can oxidise alcohols in the same way and are therefore reduced to Cr(III). Due to this there is an observable colour change as the reaction proceeds caused by orange Cr(VI) being converted into green Cr(III).

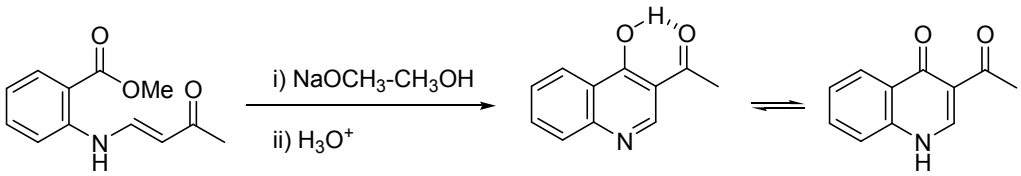
When 3-butyn-2-one is reacted with methyl anthranilate in methanol and left to stand, it forms an enamine **2.99**[141], scheme 2.46. This molecule would be able to undergo ring closure without having to disrupt the aromaticity of the benzene ring.



2.99

Scheme 2.46 Formation of methyl 2-(3-oxobut-1-enylamino)benzoate

The literature described a method forming the 3-acetyl-4(1*H*)-quinolone in the presence of base, specifically sodium methoxide[141].



2.89

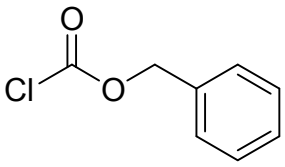
Scheme 2.47 Proposed ring closure of methyl 2-(3-oxobut-1-enylamino)benzoate

The reaction was carried out but the product was not sufficiently recovered, partly due to the low solubility of the quinolone causing difficulty in the purification by column chromatography. The reaction was attempted using sodium hydride, a stronger base, to see if this would cause the reaction to proceed more smoothly, but this proved equally unsuccessful. However, we were able to produce sufficient quantities of **2.89** by the method detailed in the scheme.

2.3.2.1.4 *N*-Protection of 3-Acetyl-4(1*H*)-quinolone

As mentioned before it was thought that this quinolone could be used as a synthon, allowing the manipulation of the 2-position and altering of the side chain. To enable the 2-position to be selectively targeted in any reaction the nitrogen would have to be suitably protected. This protecting group could then be removed at a later stage to afford the NH.

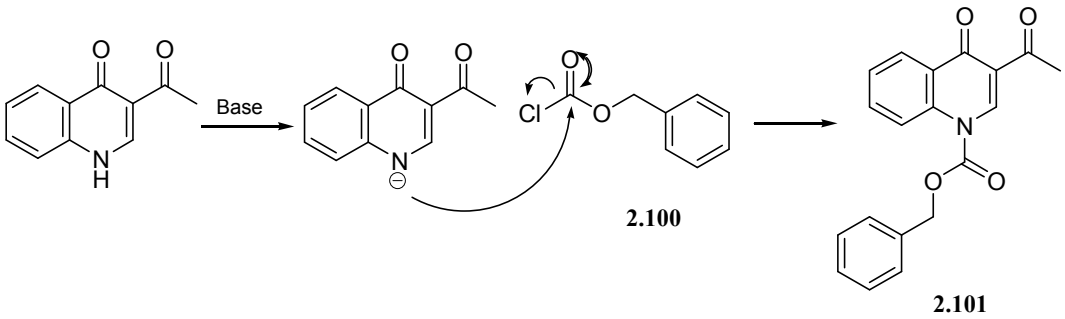
The use of protecting groups is used widely within peptide and other synthetic chemistry areas. The protecting group chosen in this case was the carboxybenzyl (Z) group. This is used for the protection of amines and is introduced using benzyl chloroformate **2.100**.



2.100

Literature shows that this group can be used to protect the nitrogen of 4-quinolones with ethyl esters and nitro groups[142] present at the 3-position and it was decided to investigate its possible use in this example.

A suitable base should theoretically be able to remove the proton attached to the nitrogen, followed by the addition of benzyl chloroformate **2.100**. The negative charge on the nitrogen, caused by the removal of the proton, would attack the carbonyl on the benzyl chloroformate and chlorine would leave. This would then deliver *N*-protected 3-acetyl-4(1*H*)-quinolone **2.101**, scheme 2.48.



Scheme 2.48 *N*-Z protection of 3-acetyl-4(1*H*)-quinolone

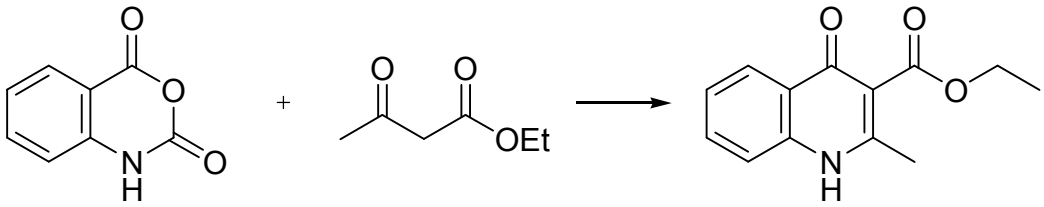
Three bases with varying strengths were tried in this reaction, in an attempt to introduce the Z group. Sodium hydride was used initially, diisopropylamine (DIPEA) in the second attempt and then sodium methoxide in the third attempt. Unfortunately none of these were able to cause the reaction to complete satisfactorily. A reason that the reaction does not proceed is due to the acetyl

group allowing the negative charge from the nitrogen to delocalise and therefore become less reactive. This delocalisation of charge is not as apparent with 4-hydroxyquinolone, hence its ability to be *N*-Z protected.

This was disappointing and consequently the rest of the route could not be attempted. Due to this, alternative syntheses towards the target structure, via 3-ester substituted 4-quinolones were sought.

2.3.3 Synthesis of 2-Alkyl-3-methoxycarbonyl-4(1*H*)-quinolone

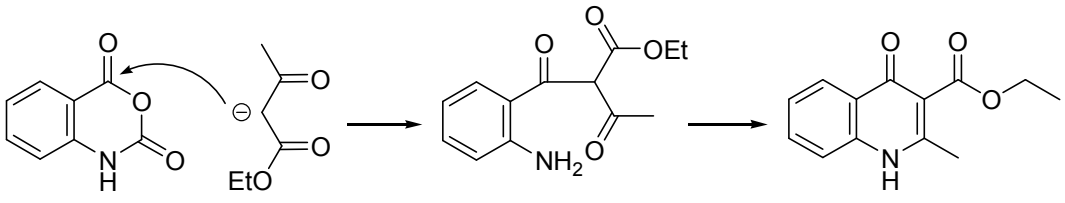
It was reported that the reaction of isatoic anhydride **2.102** and ethyl acetoacetate **2.103** yielded 2-methyl-3-ethoxycarbonyl-4(*1H*)-quinolone **2.104**, as shown in scheme 2.49.



2.102 **2.103** **2.104**

Scheme 2.49 Formation of 2-methyl-3-ethoxycarbonyl-4(1*H*)-quinolone

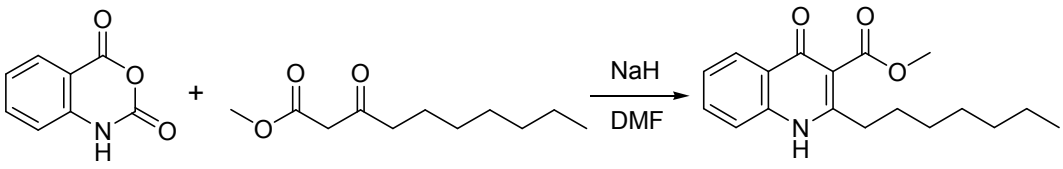
It was originally thought that the production of this molecule would take two steps as shown in scheme 2.50, the first involving the nucleophilic opening of the heterocyclic ring of the isatoic anhydride, using sodium hydride to abstract a methylene proton, followed by a refluxing in ethanol to cause the ring closure to the quinolone.



2.102 **2.103** **2.105** **2.104**

Scheme 2.50 Mechanism of 2-methyl-3-ethoxycarbonyl-4(1*H*)-quinolone formation

In reality the reaction went in one step and did not require the isolation of the ring opened intermediate **2.105**. This was rather encouraging as using appropriate β-keto ester the desired alkyl chain can be installed at the 2-position. Indeed, when methyl 3-oxodecanoate was used **2.106**, instead of the ethyl acetoacetate, to give 4-quinolone with C_7 side chain **2.107**, present in PQS, was the result.



2.106 **2.107**

Scheme 2.51 Formation of methyl 2-heptyl-1,4-dihydro-4-oxo-3-carboxylate using isatoic anhydride

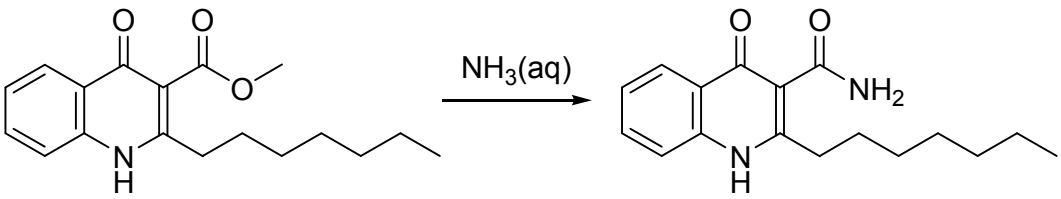
Although this molecule still has a group at the 3-position that would need to be transformed to an OH group to afford PQS, the quinolone core structure is assembled in only one step. This is a clear advantage over the original method, requiring four steps in order to produce the similar formylated quinolone.

2.3.3.1 Transformation of 3-Methyl Ester Functionality to Deliver Other Derivatives

The methyl ester at the 3-position could be altered to give other functional groups at that position. These new analogues with different functional groups could then be assessed to see their effect on the activity of the molecules in biological assay.

2.3.3.1.1 Formation of 3-carboxamide

Conversion of the methyl ester into a carboxamide **2.108** with the use of ammonia solution was attempted.



2.108

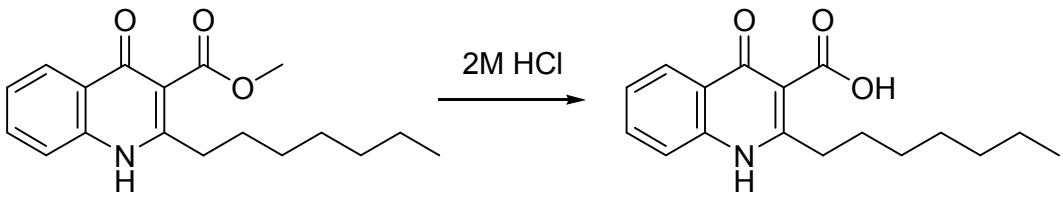
Scheme 2.52 Conversion of methyl ester to carboxamide

This was not successful, largely due to solubility problems associated with the quinolone. Initially the compound was placed in 33 % ammonia solution and stirred overnight but the solid never sufficiently dissolved to allow it to react with the ammonia and therefore there was a return of the starting material. In the second attempt a small amount of methanol was added to the solution, in order to allow the solid methyl ester to dissolve. This again was stirred overnight and then the solvent removed under vacuum to leave a white solid. This solid was shown to be the starting material, due to the presence of the methyl singlet in the NMR at δ 3.76.

2.3.3.1.2 Formation of Carboxylic Acid

This ester can be used to produce 2-heptyl-1,4-dihydro-4-oxo-3-carboxylic acid **2.109**. This can be achieved either by acid or base hydrolysis. The use of a base such as sodium hydroxide would theoretically be able to saponify the ester. This method was attempted, using aqueous sodium hydroxide but the reaction did not produce the carboxylic acid. An explanation for the failure for this reaction to work may be that the use of the base may cause N-negative charge to feed into the carbonyl and therefore will prevent attack by the hydroxide ion which is required for the saponification to occur.

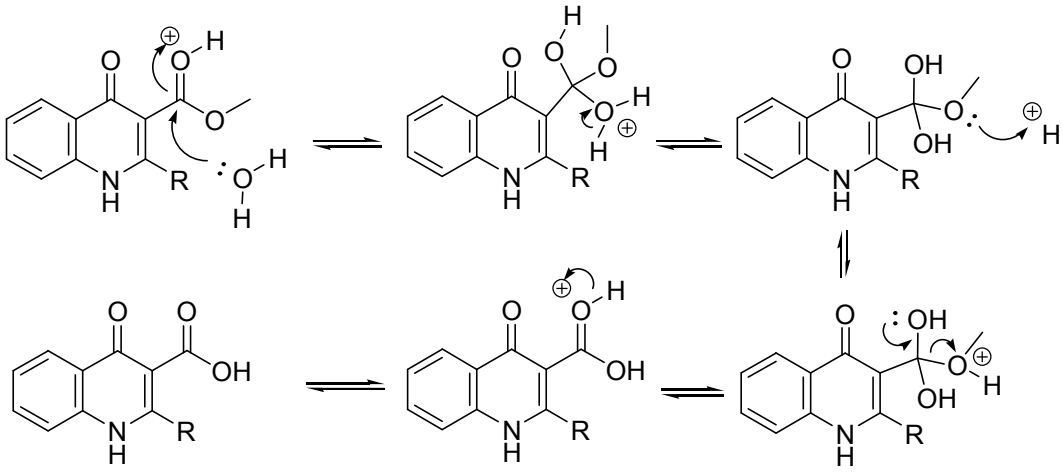
This was eventually achieved by heating the methyl ester in 2M HCl solution, causing the hydrolysis of the ester and forming the desired carboxylic acid functionality.



2.109

Scheme 2.53 Conversion of methyl ester to carboxylic acid

The mechanism for this reaction is shown in scheme 2.54 and begins with the protonation of the carbonyl leading to an attack by water leading to the formation of the carboxylic acid and methanol.

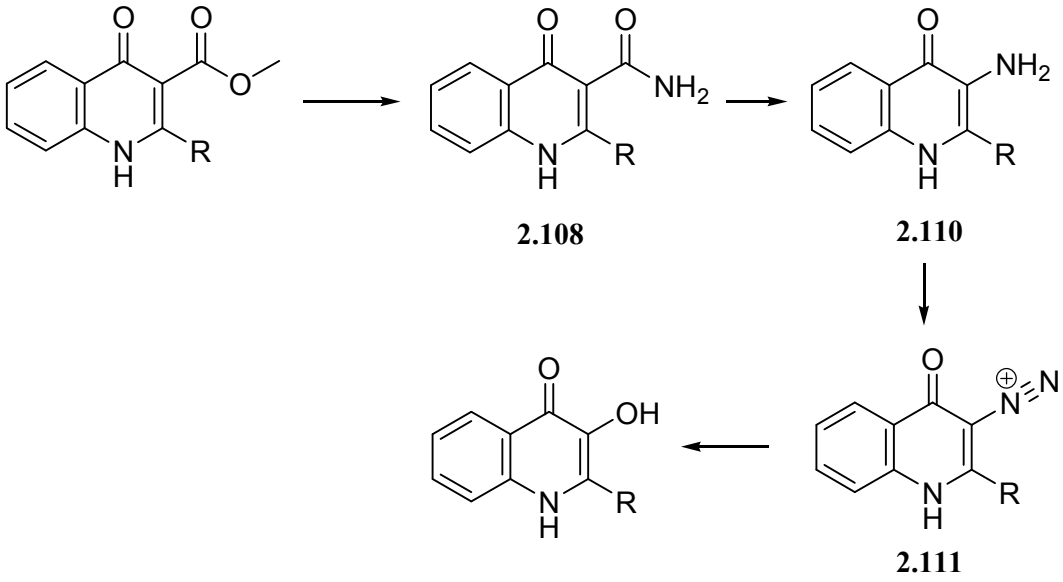


Scheme 2.54 Mechanism of acid catalysed hydrolysis

The product was obtained without the need for purification via a column, as is the method involving the chloroketones. This is highly desirable, as the quinolones with substituents at the 3 position become very insoluble, making it extremely difficult to carry out chromatography. Therefore if other methods of purification are available, this is desirable.

It is theoretically possible to transform the 3-CO_2Me to 3-OH via a conversion route of ester to carboxamide **2.108**, then to amino **2.110**, then diazo **2.111** and finally OH. This would also be valuable in the fact that it produces a number of other analogues for testing in biological assays. Of main interest would be 2-heptyl-3-amino-4(1*H*)-quinolone **2.110**, as NH_2 is an

isostere of OH, possessing similar hydrogen bonding properties and also similar in size.



Scheme 2.55 Conversion of methyl ester to hydroxyl via other potentially useful functional groups

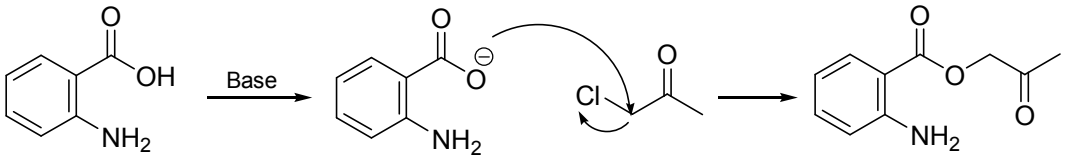
Considering the number of steps involved and the solubility of quinolone, this approach was abandoned and alternative methods for the synthesis of PQS and its analogues were explored.

2.4 Alternative Method for Synthesis of PQS and its Analogues

2.4.1 Synthesis of 2-methyl-3-hydroxy-4(1*H*)-quinolone

The literature records a facile two step synthetic route to 2-methyl-3-hydroxy-4-(1*H*)-quinolone starting from anthranilic acid[132,143].

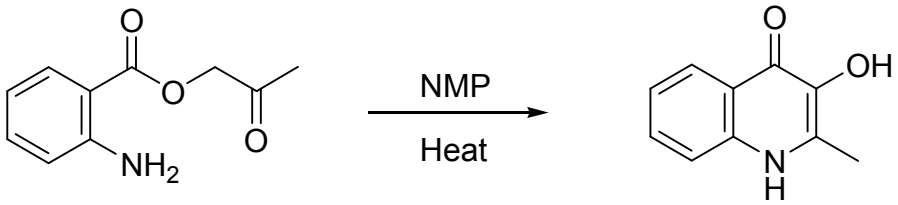
Esterification of anthranilic acid with a α-chloroketone in DMF, in the presence of a base, furnishes **2.112**, as seen in scheme 2.56[132].



2.112

Scheme 2.56 Esterification of anthranilic acid

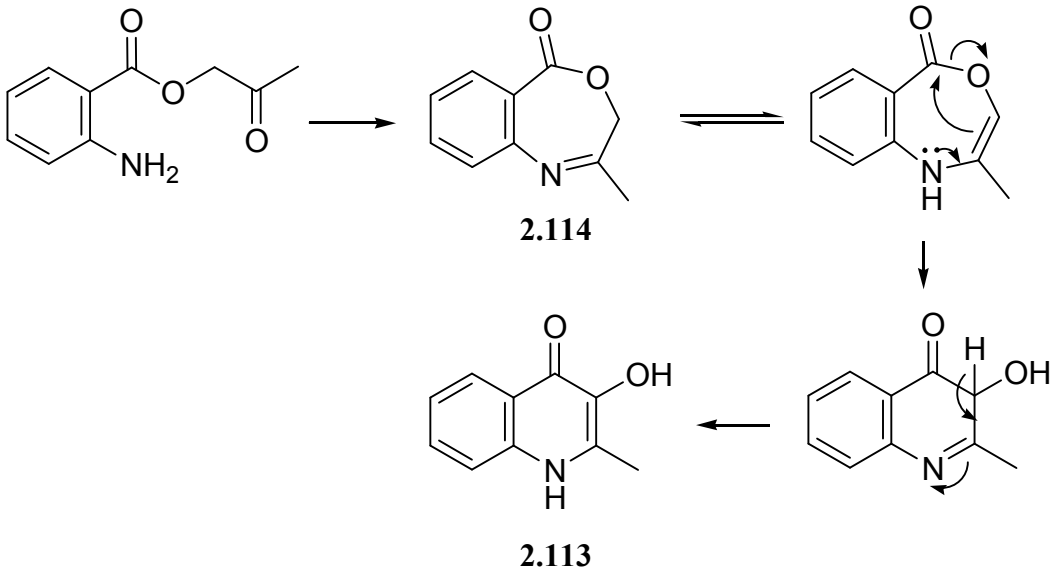
This can then be heated in *N*-methylpyrrolidinone, a high boiling dipolar aprotic solvent, to produce 2-methyl-3-hydroxy-4(*1H*)-quinolone **2.113**, scheme 2.57.



2.113

Scheme 2.57 Formation of 2-methyl-3-hydroxy-4(1*H*)-quinolone

The mechanism for this step, as shown in scheme 2.58, involves a seven membered ring intermediate **2.114** formation initially, which then undergoes a rearrangement to yield the 4-quinolone **2.113**.



2.114

2.113

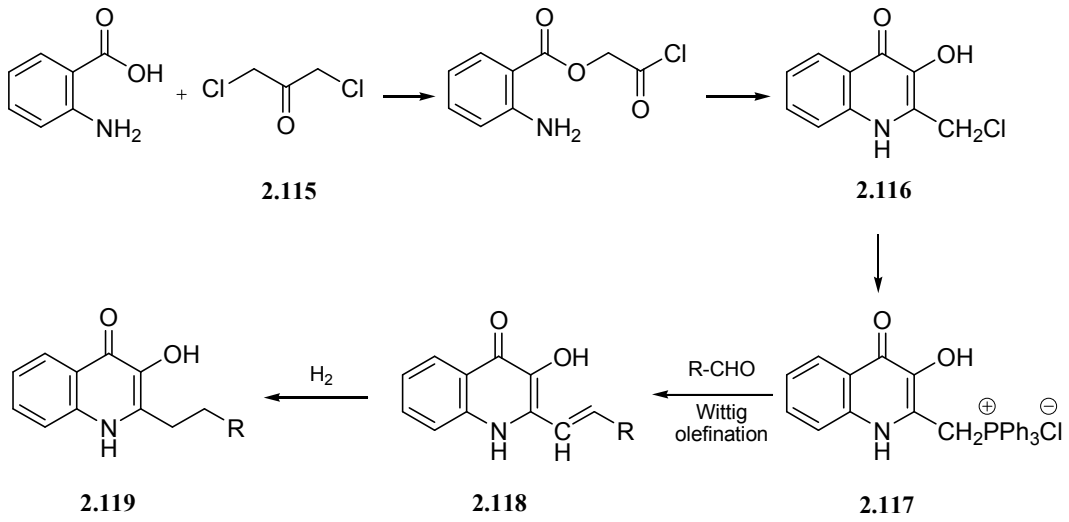
Scheme 2.58 Mechanism of 2-methyl-3-hydroxy-4(1*H*)-quinolone formation

This shows there is still the need for a high boiling point solvent but the method allows the recovery of the material in a more convenient and clean manner. Polyphosphoric acid (PPA), literature boiling point 300 °C, may also be used to effect ring closure. PPA is less convenient to use in practice, in terms of product recovery.

The production of 2-methyl-3-hydroxy-4(1*H*)-quinolone was carried out as a pilot synthesis, to evaluate the literature method. The reaction proceeded well, in desirable yields at both steps and with ease of recovery of the product. Chloroacetone was a commercially available starting material. However other α-chloroketones, with longer alkyl chains are not commercially available.

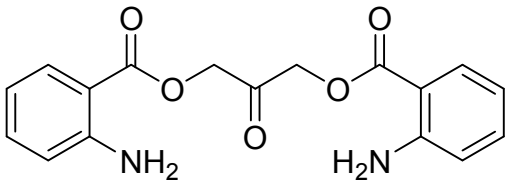
2.4.2 Introduction of Halogen on Alkyl side chain

It was thought if dichloroacetone **2.115** was used in the place of chloroacetone, this would give a quinolone product with CH_2Cl substituent at 2-position **2.116**. This would allow the manipulation of the side chain via a phosphonium derivative **2.117** produced using triphenylphosphine, which in turn can react with an aldehyde of suitable length to afford the desired alkyl chain, via Wittig olefination, albeit unsaturated **2.118**. The latter can be hydrogenated to achieve the saturated chain **2.119**.



Scheme 2.59 Introduction of an alkyl chain via Wittig olefination

The reaction did not proceed as desired, using the same conditions as that for the chloroacetone product. Due to the reactivity of the chloride substituents, dimeric product **2.120** was formed. This dimer could not be easily identified by using proton NMR, due to its symmetry causing the protons to be equivalent and therefore only producing one set of signals. The main distinguishing feature was no signal present for the CH_2 next to the chlorine. The structure was finally confirmed with the use of MS, the mass being 328, rather than 227.



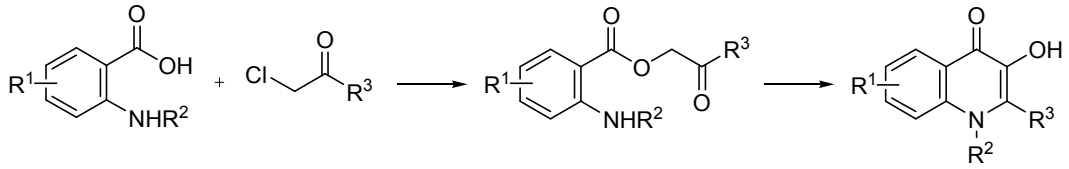
2.120

The reaction was repeated without heating and with an excess of the dichloroacetone, in an attempt to ensure that esterification was the most prevalent reaction that could take place in the reaction. The reaction was also repeated by reacting anthranilic acid with DIPEA, a weaker base than sodium hydride, and adding this dropwise to a solution of dichloroacetone, thereby allowing the deprotonated acid to react with the dichloroacetone and minimising the levels of free anthranilic acid available to react with the acylated product. Despite these attempts to control aspects of the reaction, the dimer was still the only product formed instead of the desired material.

2.4.3 Extension of the Method Using Other α-Chloroketones

2.4.3.1 General synthesis of PQS analogues

As the only derivative reported in literature produced by the method shown above in schemes 2.56 and 2.57 was 2-methyl-3-hydroxy-4(1*H*)-quinolone[132] it was decided to investigate the possible extension of this reaction, with the use of longer chain α-chloroketones, or other halogenated ketones. The use of derivatives of anthranilic acid would allow the substitution on the carbocyclic ring, as detailed in scheme 2.60.



Scheme 2.60 General method for producing substituted 4-quinolones using α-chloroketones

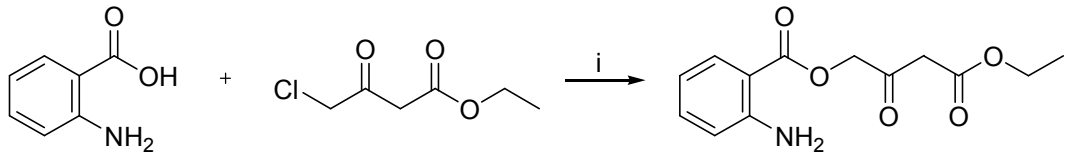
Thus the method had great scope and would allow production of analogues with choice of substitution on the carbocyclic ring, at the 2-position and also on the heterocyclic nitrogen.

2.4.3.2 Esterification of Anthranilic Acids with α-Haloketones

The range of α-chloroketones available commercially is unfortunately small. Bromoketones were investigated to give substitution at the 2 position but these compounds gave much less satisfactory products than the chloroketones, in terms of ease of purification and quality of the material. Bromoketones are likely to be more reactive than the corresponding chloroketones. This may prove a problem with forming these O-alkylated intermediates cleanly due to the increased potential for side reactions. Products produced with the use of α-chloroketones did not require any purification after the initial reaction and simply precipitated out of solution upon dilution with cold water.

2.4.3.2.1 Reaction With 4-Chloroacetoacetate

Commercially available chloroketone ethyl 4-chloroacetoacetate **2.121** was expected to deliver the ester **2.122**.



2.121 **2.122**

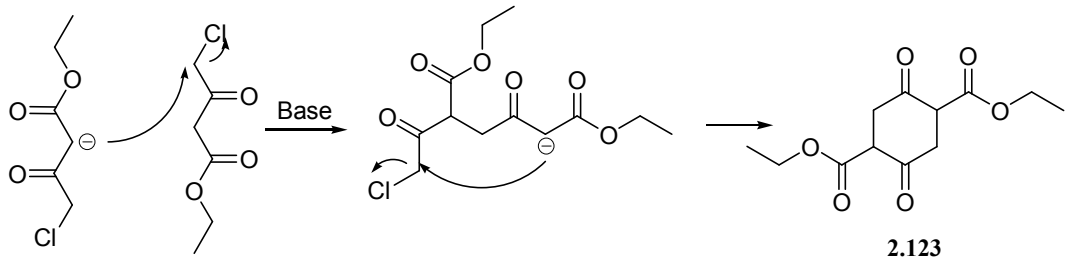
Reagents and Conditions: (i) Potassium carbonate, DMF, heat

Scheme 2.61 Reaction of ethyl 4-chloroacetoacetate with anthranilic acid

The reaction proceeded satisfactory and produced **2.122** as a colourless solid. The product was identified due to having the protons present in the aromatic region of the NMR spectra, allied with 2 singlets produced by the CH_2 protons present at the 4 and 5-positions on the ester side chain.

2.4.3.2.2 Formation of side product

Besides **2.122**, a second product was also produced. This was isolated by recrystalisation from ethyl acetate/hexane and identified, using NMR and mass spectrometry as diethyl 2,5-dioxocyclohexane-1,4-dicarboxylate[144] **2.123** and possibly via the mechanism shown scheme 2.62.



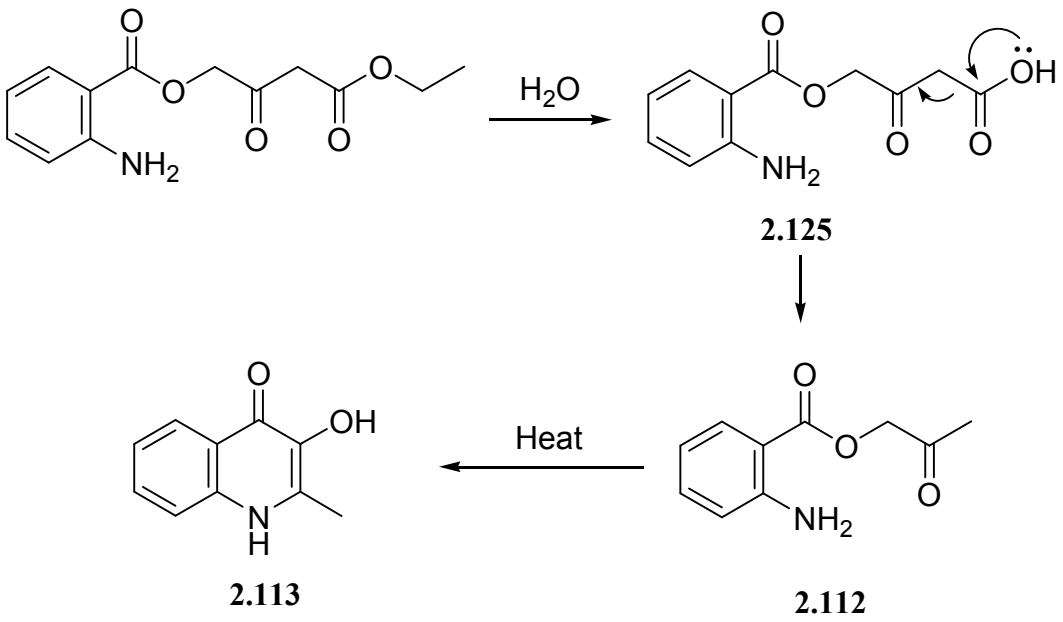
Scheme 2.62 Formation of dicarboxylate side product

The base, potassium carbonate removes the proton to generate an anion **2.124**, leading to a nucleophilic attack on one of the chloroketone displacing chlorine. This intermolecular reaction is then followed by an intramolecular one, similar in nature, leading again to a nucleophilic attack and the displacement of the second chlorine remaining on the molecule and forming **2.123**.

2.4.3.2.3 Ring closure of ester product 2.122

When the ester **2.122** was heated in NMP to effect ring closure, the expected material, with the ethyl ester at the 2-position, was not formed. The NMR spectrum showed no signals for the ethyl protons. The material produced was actually 2-methyl-3-hydroxy-4(1*H*)-quinolone **2.113**. The production of this material can be rationalised as shown in scheme 2.63. If there was any water

present in the NMP there is the potential for the ester to be hydrolysed, forming a β-keto acid **2.125**. The latter, in the high boiling solvent, was decarboxylated, yielding ethyl 2-aminobenzoate. This will then obviously yield 2-methyl-3-hydroxy-4(1*H*)-quinolone, as seen before, after undergoing ring closure.

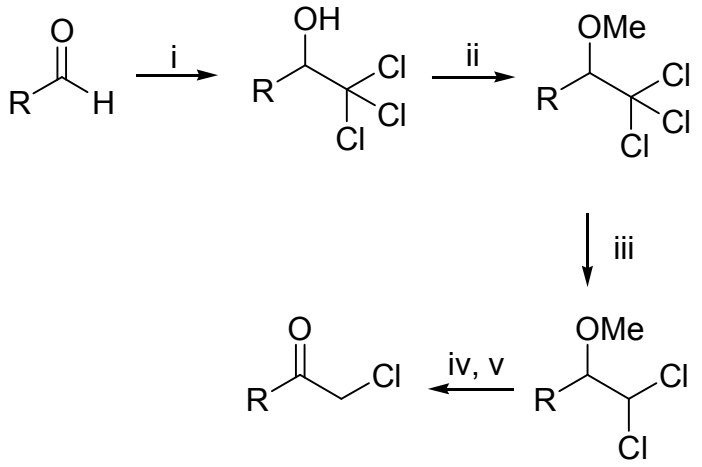


Scheme 2.63 Hydrolysis and decarboxylation occurring during heating

2.4.3.3 Synthesis of α-Chloroketones

Due to the high reactivity of the α-bromoketones, seen in reactions attempted with this type of compounds, it was decided to use α-chloroketones exclusively.

Though these α-chloroketones are known to be important as staring materials in a range of synthetic methods but convenient methods for their production have not always been forthcoming. α-Chloromethyl ketone has been formed via the chlorination of methyl ketone or the reaction of an acyl chloride with diazomethane[145]. Shono *et al*[146] proposed what they referred to as a simple, straight forward method but this involved the formation of a trichloroether type of molecule, followed by an electroreductive reaction to furnish a dichloro species, which then had to be converted to a mono chloro ketone via an additional two steps.

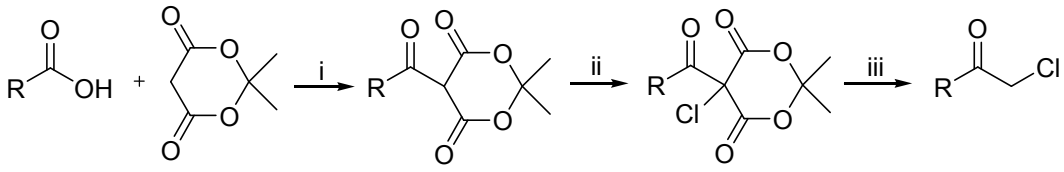


Reagents and conditions: (i) + e, CCl_4, $CHCl_3$, DMF; (ii) NaH, CH_3I, THF; (iii) + e, $CH_3OH - H_2O$; (iv) KOH, C_2H_5OH; (v) 10 % H_2SO_4

Scheme Formation of α-chloroketone using electroreduction

2.4.3.3.1 Synthetic Methods for α-Chloroketones

Two methods were investigated in order to produce α-chloroketones with the required side chains. The first of these involved acylating Meldrum's acid **2.61** with an appropriate carboxylic acid in the presence of DMAP and DCCI. This 5-acylated Meldrum's acid **2.67** is then treated by heating at reflux in sulfuryl chloride scheme 2.64. The excess sulfuryl chloride was removed under vacuum to yield 5-chloro-5-acyl Meldrum's acid **2.126**. Hydrolysis with 1M HCl, liberated the chloroketone[147] **2.127**.



2.66 **2.126** **2.127**

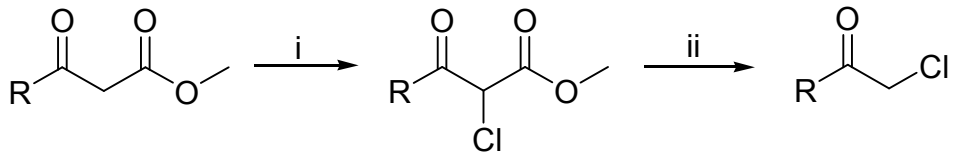
Reagents and conditions: (i) DMAP, DCCI, DCM; (ii) SO_2Cl_2; (iii) 1 M HCl, heat

Scheme 2.64 Formation of α-chloroketone using Meldrum's acid

This is unsatisfactory method for the production of α-chloroketones, as acylated Meldrum's acids were difficult to purify or store for periods of time,

due to the instability of the ring structure. A preferable method, involving the use of β-keto esters as the starting material, was used. While the β-keto esters are produced using some of the above methods, namely acylation of Meldrum's acid, they are easier to purify and store for longer periods of time.

Thus α-chloroketones with varying chain length were produced by using sulfuryl chloride and β-keto esters of appropriate chain lengths. This is shown in scheme 2.65.



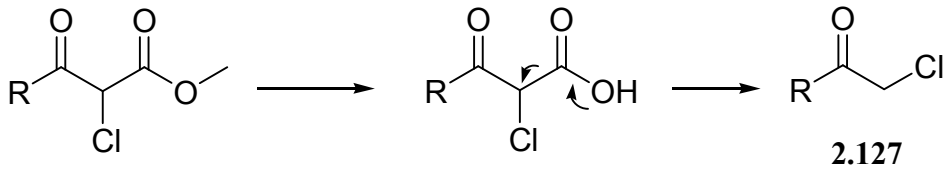
2.128 **2.127**

Reagents and conditions: (i) SO_2Cl_2 (ii) cH_2SO_4, H_2O, Δ

Scheme 2.65 Synthesis of α-chloroketones using β-keto esters

The sulfuryl chloride was added to the relevant β-keto esters neat, stirring at 0°C, then left to stir overnight at room temperature. After this the remaining sulfuryl chloride was removed under vacuum yielding the chlorinated β-keto ester **2.128** as a yellow oil, in a quantative yield. The oil was used without purification in the next step. There was an observable colour change in the course of the reaction, as the original solution was colourless and clear, whereas the product was yellow. The product was readily identified by NMR, which showed a loss of proton, with a singlet for the CH in the chlorinated product being evident in the ^1H NMR spectrum. The signal for this proton occurs at a high PPM because it is between two carbonyls and chlorine. The chlorine is an electron withdrawing group and will therefore cause the proton to be less shielded. Additionally, the electrons will be able to delocalise into the π electron system.

In the final step, the α-chloroketone **2.127** is produced from this chlorinated β-keto ester by hydrolysis of the methyl ester with concomitant decarboxylation of the carboxylic acid produced **2.129**.



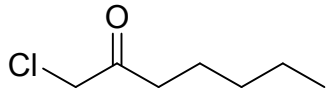
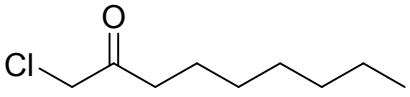
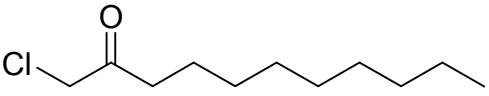
2.128 **1.129** **2.127**

Scheme 2.66 Hydrolysis and decarboxylation of chlorinated β-keto ester

This transformation was attempted in two different ways. The first method attempted was the heating at reflux in a mixture of tetrahydrofuran (THF), water and concentrated sulphuric acid but this required a significant time for the reaction to take place (approx. 40 h).The alternative was a method in which the compound was heated at reflux in 50 % concentrated sulphuric acid/water mixture and the desired chloroketone was afforded after approximately 2 h and could be extracted into a suitable organic solvent. The two methods are comparable in terms of yield but the reduced reaction time of the latter meant it was more convenient as a routine method.

A number of α-chloroketones were thus produced using this method and are listed in Table 2.1. All compounds were obtained essentially in quantative yield.

Table 2.1 α-Chloroketones produced

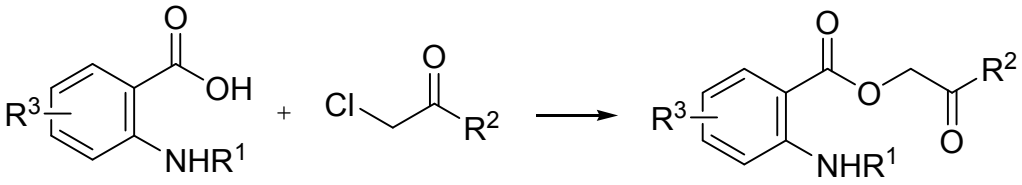
Compound	*Structure*
2.130	
2.131	
2.132	

It was found that methyl esters were better for producing the α-chloroketones than ethyl esters, in terms of yields and the nature of the product recovered.

2.4.3.4 Esterification of Anthranilic Acid Derivatives with α-Chloroketones

The α-chloroketones was then used to react with a range of substituted anthranilic acid, or substituted anthranilic acid to obtain a variety of PQS analogues in the same way that was shown for the production of the methyl compound.

A number of substituted anthranilic acids are commercially available and one named either anthranilic acid derivatives or 2-aminobenzoic acid derivatives. Those derivatives allow production of a number of structures with different substitution in various the positions on the carbocyclic ring.



2.133 **2.134**

Reagents and conditions: (i) Potassium carbonate, DMF, heat

Scheme 2.67 General synthesis of *O*-alkylated anthranilic acid derivatives

The α-chloroketone was added to a mixture of the anthranilic acid derivative **2.133** that had been stirred with potassium carbonate in DMF. The potassium carbonate removes the acidic proton, allowing the chloroketone to *O*-alkylate the anthranilic acid forming compounds of general structure **2.134**. The initial reaction to remove the proton is carried out at 90 °C and the following reaction involving the chloroketone is initially at room temperature but is then heated to 50 °C to complete the reaction. The product is simple to recover as it precipitates out after dilution with ice water that is roughly 10 times the volume.

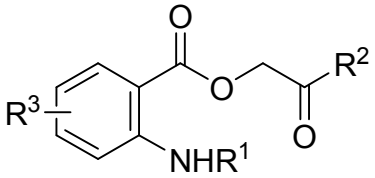
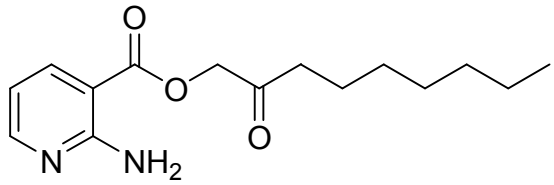


Table 2.2 *O*-alkylated anthranilic acids produced

Compound	R^1	R^2	R^3	*Yield*
2.112	H	CH_3	H	61
2.135	H	C_5H_{11}	H	36
2.136	H	C_7H_{15}	H	75
2.137	H	C_7H_{15}	6-Cl	70
2.122	H	$CH_2CO_2C_2H_5$	H	31
2.138	CH_3	C_7H_{15}	H	28

In addition to the compounds shown in the table above, 2-aminopyridine-3-carboxylic acid was also used to produce 2-oxononyl 2-aminopyridine-3-carboxylate **2.139**.

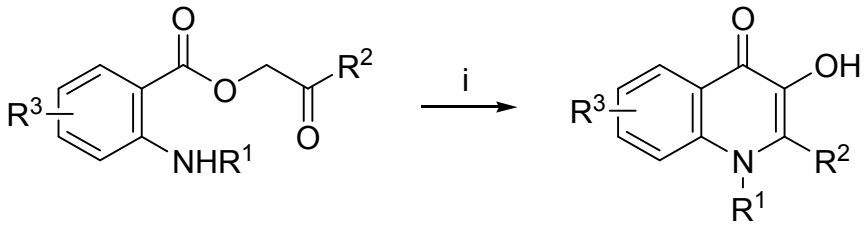


2.139

This compound provides the opportunity to introduce a heterocyclic atom into the carbocyclic ring, bringing potential changes to the molecule's properties[148] which are further discussed below.

2.4.3.5 Cyclisation of Anthranilates

The anthranilates produced are cyclised to form the 3-hydroxy-4(1*H*)-quinolone structure **2.140** by heating for varying times in the high boiling point solvent, NMP. As previously mentioned this method still requires the use of a high boiling point solvent but the recovery and quality of the compounds produced are preferable to the original method, where diphenyl ether was used.



2.140

Reagents and conditions: (i) NMP, heat

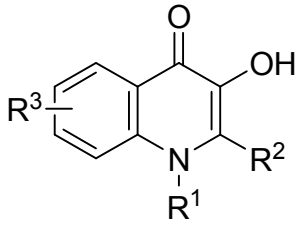
Scheme 2.68 General synthesis of substituted 3-hydroxy-4(1*H*)-quinolones (PQS analogues)

In terms of reaction times required for ring closure, it was found that as the length of the side chain increased, the reaction time required increased. This is due to the electron donating effect of the straight alkyl chain substituents feeding into the carbonyls and making them less electrophilic and therefore less reactive, meaning that the reaction will proceed at a slower rate. There may also be some steric effects of the chain, as the chain increases in length it becomes more flexible and more likely to cause steric hindrance in the intramolecular reaction process required for ring closure.

Again, a main advantage of this method is the convenience of purification of the product. The quinolone precipitates out of solution on addition of ethyl acetate and the cooling in ice. Due to the problems that are involved with solubility of the quinolone, the purification of these compounds is difficult. The use of column chromatography is not suitable for quinolone compounds that have substitution in the 3-position, as these are sparingly soluble in traditional organic solvents.

2.4.4 PQS analogues synthesised

The method outlined in schemes 2.67 and 2.68 proved to be very valuable in the production of a number of PQS analogues **2.140**, with alterations not only in the length of alkyl chain at the 2-position but also in the substitutions at the N and in the carbocyclic ring. The analogues produced are shown in the table below.



2.140

Table 2.3 PQS analogues produced

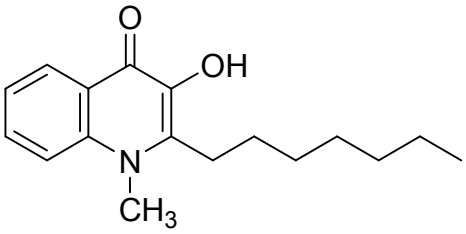
Compound	*R^1*	*R^2*	*R^3*	*Yield %*
2.141	H	C_1H_3	H	73
2.142	H	C_5H_{11}	H	78
2.143	H	C_7H_{15}	H	59
2.144	H	C_7H_{15}	6-Cl	47
2.145	Me	C_7H_{15}	H	28

2.4.5 Variations in General Method

Some of the compounds produced by this new method differed in their ease of synthesis slightly. These are discussed below.

2.4.5.1 *N*-Methyl PQS

The *N*-methyl compound, 2-heptyl-3-hydroxy-1-methyl-4(*1H*)-quinolone **2.146** was not as convenient to produce as the other compounds. The yield of the *O*-alkylated *N*-methyl anthranilic acid derivative was lower then the other results, as seen in table 2.2, as was the yield obtained after heating in NMP. This was largely due to it not precipitating out of solution on the addition of ethyl acetate, as the other compounds had. This meant the recovery of the product from the high boiling solvent had to be attempted in an alternative manner.

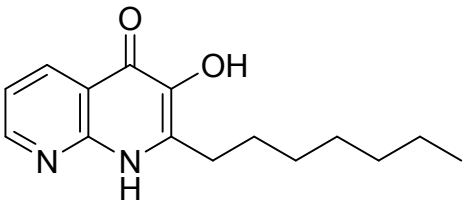


2.146

In order to obtain the product the ethyl acetate was removed under vacuum and as much of the NMP was removed as possible, through a combination of high vac evaporation and by a stream of nitrogen gas, leaving a thick, viscous liquid. A small amount of ethyl acetate was added to this liquid and the solution was chilled, causing the product to form off-white crystals. This affected the yield and caused it to be lower than that of the other analogues produced using this method.

2.4.5.2 Naphthyridinone Based Analogue

This problem of the product not precipitating upon the addition of ethyl acetate also occurred with the synthesis of 2-heptyl-3-hydroxy-1,8-naphthyridin-4(1*H*)-one **2.147**, containing an extra nitrogen in the 8-position. There is evidence to suggest that the introduction of extra heterocyclic atoms in a molecule is potentially better for increased bioactivity. The additional nitrogen could lower the pK_a of the molecule, meaning that it would be more available at physiological pH[148]. This would be possible without increasing the bulk of the molecule and thereby having little effect on the receptor interaction. An attempt was made to recrystalise the compound using ethyl acetate in a manner similar to that of the *N*-methyl compound **2.146** but this did not produce satisfactory results.



2.147

2.5 Extended Use of New Method

Subsequent to the completion of this project the method outlined above has been utilised to produce a further number of compounds in the School of Pharmacy, Nottingham, including further chain length alterations, including a C2 and C3 chain, as well as methyl substitution on the carbocyclic ring.

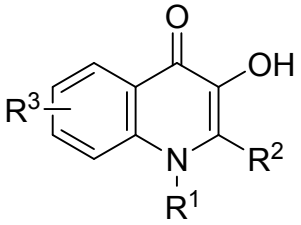


Table 2.4 Additional substituted 4-quinolones produced

Compound	R^1	R^2	R^3	*Yield %*
2.148	H	C_2H_5	H	77
2.149	H	C_3H_7	H	59
2.150	H	C_7H_{15}	6-Me	57

As well as advantages in the production of a wide range of analogues, there is also the advantage in the fact that there is no purification required to obtain material in terms of column chromatography. This is important if the SAR identifies a suitable molecule for further investigation in the way of biological assays, formulation or production. Column chromatography can be convenient in a lab setting when dealing with up to a few gram scale but becomes a problem when the reaction is scaled up, in terms of size of column and subsequent volumes of solvent required.

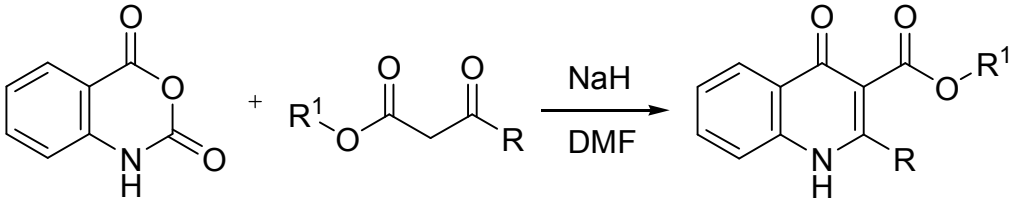
2.6 Summary of Results

A facile synthetic route to PQS and its analogues has been developed along with a facile, one step synthesis to produce a 3-substituted-4(1*H*)-quinolone

This project has demonstrated how the synthesis of analogues of PQS may be achieved in a number facile synthetic routes, allowing the manipulation of the required areas of the molecule, namely:

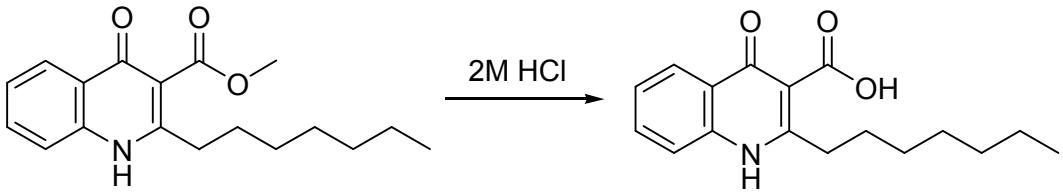
- The 2-alkyl chain
- The 3-position substitution
- *N*-substitution
- Substitution on the carbocyclic ring
- Introduction of additional heterocyclic atoms into the molecule

2-Alkyl-4(1*H*)-quinolones with ester functionality at the 3-position can be synthesised from isatoic anhydride and a β-keto ester in one step. There is the ability to alter both the 2-alkyl chain and the ester by changing the β-keto ester. This reaction produces the quinolone without the need for purification.



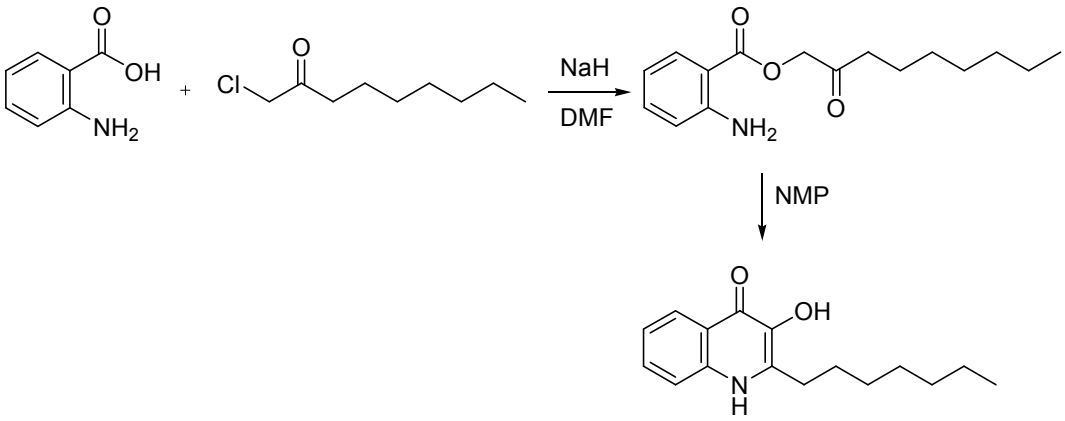
Scheme 2.69 Formation of 2-alkyl-3-ester-4(1*H*)-quinolones

The ester at the 3-position has the potential to be transformed into other groups, such as carboxylic acid and carboxamide. The conversion to carboxylic acid was achieved by refluxing in 2M HCl, thereby hydrolysing the ester.



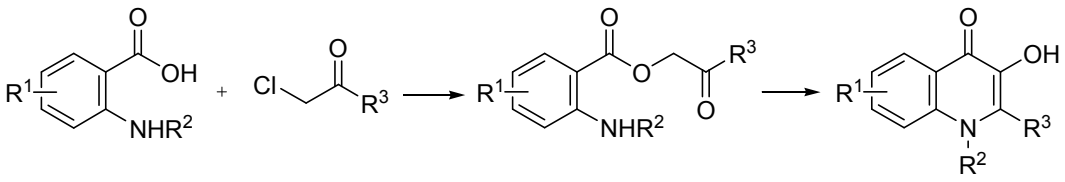
Scheme 2.70 Conversion of ester into carboxamide

PQS can be synthesised by the alkylation of anthranilic acid and cyclisation with NMP in a facile, two step synthesis.



Scheme 2.71 Facile two step synthesis of PQS

The method allows quick, simple synthesis of a number of analogues by simply employing appropriate starting materials. Diversity can be introduced, at the 2-position, on the nitrogen and also in the carboxylic ring.



Scheme 2.72 General synthesis of PQS analogues

This method was used to produce a range of analogues in a short period of time. These analogues included an example of each of the 3 areas shown above being altered, as well as the introduction of a heterocyclic atom into the carbocyclic ring.

These synthetic routes have enabled the production of 4-quinolone molecules with alterations made at each of the areas targeted in the original aims and objectives of the project.

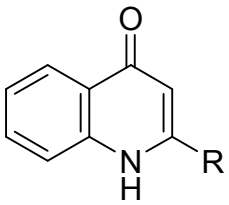
The method established in this work is more convenient and better than that reported previously. These 4-quinolone based analogues of PQS that have been subsequently tested in a range of biological assays to establish their immune modulatory activity, discussed further in Chapter 4.

Chapter 3

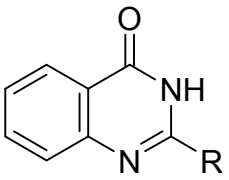
Quinazolinone Based Analogues of PQS

3.1 Quinazolinone based immune modulatory agents

As discussed in the previous chapter, there is evidence to suggest that the introduction of extra heterocyclic atoms in a molecule is potentially better for increased bioactivity along with altering physiochemical factors, such as solubility[148]. They also may allow a more simple method of synthesis and these changes would be possible without increasing the bulk of the molecule and thereby minimising effect on the receptor interaction. Analogues were synthesised and evaluated with an additional nitrogen at the 8-position in the carbocyclic ring. This concept was further extended by incorporating an extra nitrogen at the 3-position in the hetero ring of the 4-quinolone structure **3.1**, giving rise to the well known quinazolinone ring structure **3.2**. Quinazolinones were chosen as they possess a closely related structure and had previously been exploited as drug molecules[149-153].



3.1



3.2

Using quinazolinones it would be possible to introduce a variety of substitution in the core structure in the areas shown in figure 1.1. It would be possible to alter the length and nature of the 2-alkyl chain (R^1), change the functionality at the 3-position (R^2) and to introduce substitution into the carbocyclic ring (R^3).

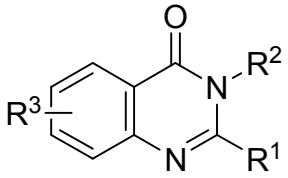


Figure 1.1 Structural areas of quinazolinones that can be altered for SAR study

These alterations could be achieved individually or in combination and their effects assessed within biological assays. This structure activity relationship (SAR) study would thus provide a number of new molecules that can be assayed for their immune modulatory properties.

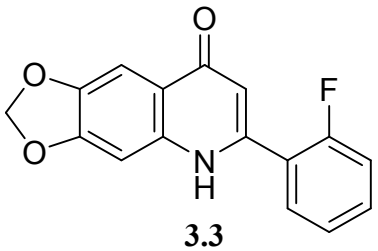
It was also intended to use the quinazolinone structure as a surrogate structure, expecting that alterations will mimic the relative equivalents in the original 4-quinolone system.

An example of this would be observing the effect of altering the length of the side chain and seeing if the activity rises or falls. It would be of interest to see if the change in activity parallels the 4-quinolone analogues.

3.1.1 Quinazolinones as Quinolone Surrogate systems

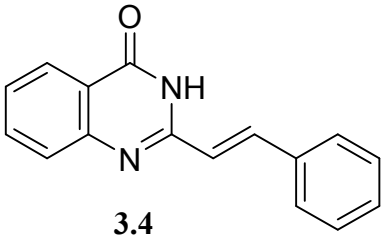
Quinazolinones have been used previously as a suitable replacement for the 4-quinolone structure.

Xia *et al.*[154]investigated fluorinated 2-phenyl-4-quinolone derivatives with regards their antimitotic antitumour activity. The lead molecule for the study was 2'-fluoro-6,7-methylenedioxy-2-phenyl-4-quinolone **3.3**, a potent antimitotic antitumour agent.

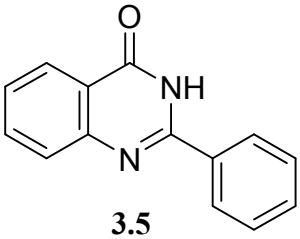


3.3

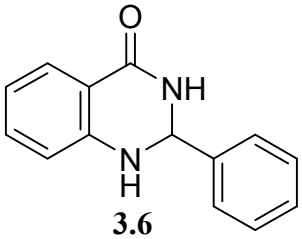
In the course of this study, three quinazolinone structures, **3.4**, **3.5** and **3.6**, were compared with the lead molecule.



3.4

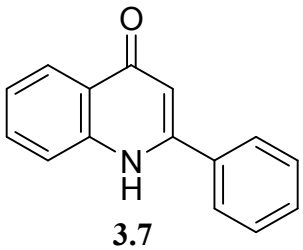


3.5



3.6

These molecules were assayed for their antitubulin activity and were compared with compounds bearing the 4-quinolone structure **3.7**. This was undertaken to provide an insight into the important structural elements required for antitubulin activity. Specifically these areas were the presence of an N hydrogen and the nature of the substitution at the 2-position.



3.7

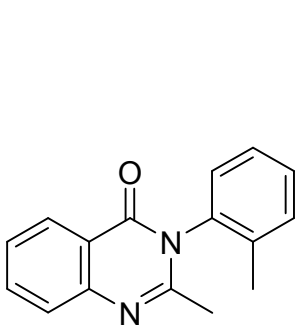
In this case, it was found that the presence of a hydrogen on the 1-N was important for the retention of activity and that 2-styryl group showed enhanced activity when compared with the 2-phenyl.

3.2 Literature Review

A number of quinazolinones of varying complexity in structure have been reported to have a range of medicinal properties.

For example, methaqualone **3.8** has sedative effects, 2-alkyl/aryl-3-arylhydrazino-4(3*H*)-quinazolinones **3.9** possess antibacterial activity[152,153], as well as central nervous system (CNS) effects similar to that of monoamine-oxidase inhibitors[153]. Quinazolinylpyrimidinediones have been found to have anti-inflammatory activity[151,155,156]. Derivatives of 2,3-dihydro-2-(aryl)-quinazolinone (DHQZ) **3.10** show some antitumour effects[150,157] and various 2-

methyl quinazolinone analogues have been synthesised that possess antifolate properties[158].



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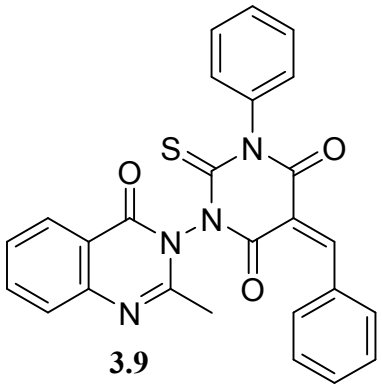

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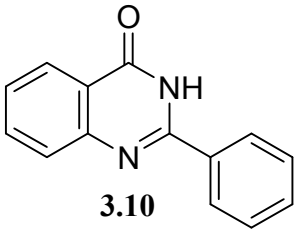
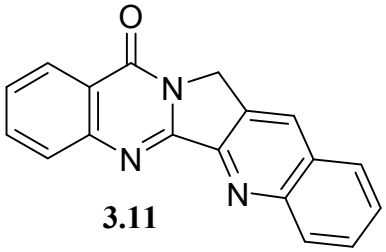


3.10

Quinazolinone alkaloids are present in nature and a number of these have been isolated and identified. Some of these naturally occurring quinazolinones are known to have medicinal properties, including luotonin A[159] **3.11**, which has cytotoxic properties[160,161], and tryptanthrin[162] **3.12**, which has been shown to have antibacterial properties[163], inhibit nitric oxide and prostaglandin E_2 synthesis[164].



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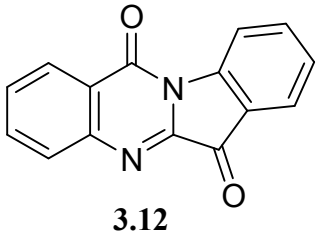


3.12

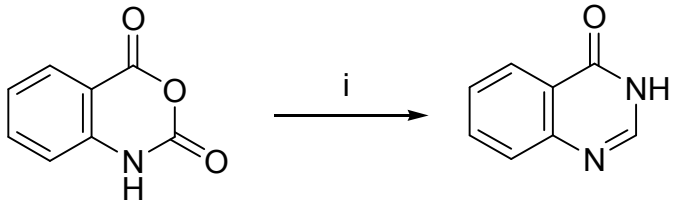
It was thus encouraging to notice that a number of quinazolinone based structures have been exploited as drug candidates. In order to elaborate quinazolinone structures that mimic PQS molecule it was imperative to review the literature to exploit the synthetic routes for their preparation.

3.2.1 Synthesis of Quinazolinones

There are a number of different methods for producing this class of molecules in the literature. However, this review will only concentrate on the synthesis of 3-substituted 2-alkyl-4(*3H*)-quinazolinones as these are the molecules that are closely related to PQS.

3.2.1.1 Review of literature methods of quinazolinone synthesis

A simple, unsubstituted quinazolinone **3.13** can be synthesised using isatoic anhydride **3.14** by treating it with formamidine acetate[165], scheme 3.1. More complex quinazolinones can be formed by introducing substitution onto the isatoic anhydride[166].

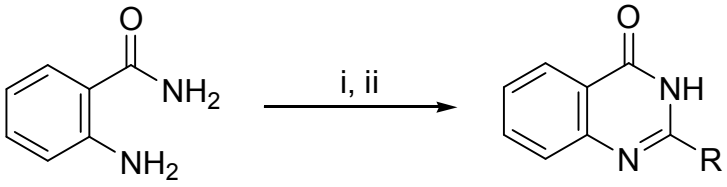


3.14 **3.13**

Reagents and conditions: (i) Formamidine acetate

Scheme 3.1 Formation of quinazolin-4(3*H*)-one using isatoic anhydride

4-Quinazolinones with 2-substution can be produced simply and quickly. Carboxylic acid quinazolinone derivatives **3.15** can be conveniently synthesised by reacting anthranilamide **3.16** with a range of anhydrides and oxalates, including benzoic anhydride, succinic anhydride, glutaric anhydride and diethyl oxalate, followed by a cyclisation using aqueous sodium hydroxide[167,168], as shown in scheme 3.2.



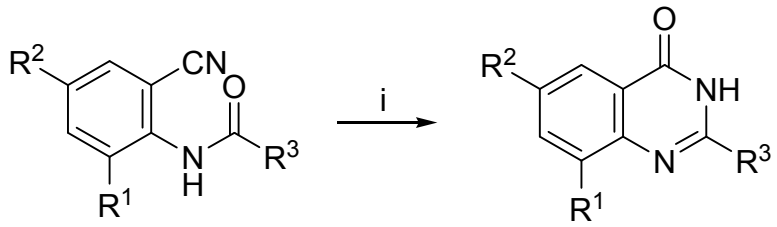
3.15 **3.16**

R = Ph, $(CH_2)_2COOH$, CO_2Et

Reagents and conditions: (i) various anhydrides or oxalate; (ii) aq NaOH

Scheme 3.2 Formation of quinazolinone carboxylic acid derivatives

A further simple way to form substituted quinazolin-4(3*H*)-ones **3.17** is by oxidative hydration of *o*-aminobenzonitriles **3.18** using urea-hydrogen peroxide (UHP) that will cyclise in the same step[169,170], scheme 3.3.

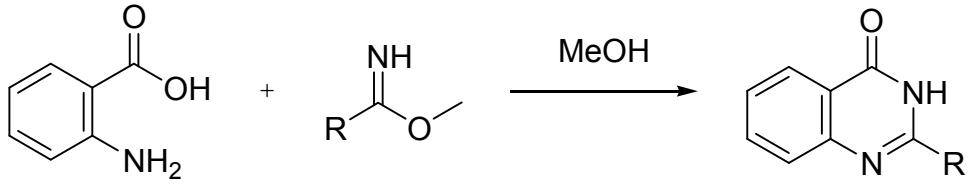


3.18 **3.17**

Reagents and conditions: (i) UHP, cat. K_2CO_3, acetone: water, Δ

Scheme 3.3 Synthesis of quinazolin-4(3*H*)-ones using *o*-aminobenzonitriles

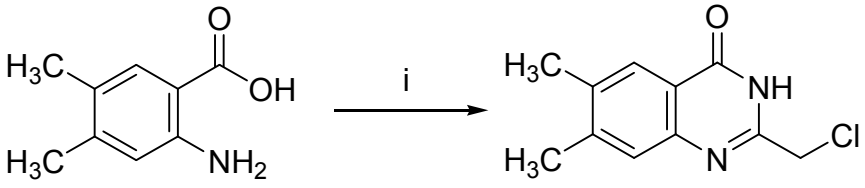
The reaction of anthranilic acid **3.19** with a variety of pre-formed imidates **3.20** in methanol, scheme 3.4, is an additional method for the production of simple 2-substituted quinazolinones **3.21**.



3.19 **3.20** **3.21**

Scheme 3.4 Formation of simple 2-substituted quinolone from anthranilic acid and an imidate

A number of quinazolinones with a functionalised 2-alkyl substituted are also reported. It is possible to synthesise a 6,7-disubstituted quinazolinone with a chloromethyl present at the 2-position **3.22** by cyclising a substituted anthranilic acid derivative **3.23** with chloroacetonitrile in methanol in the presence of catalytic sodium methoxide, scheme 3.5.

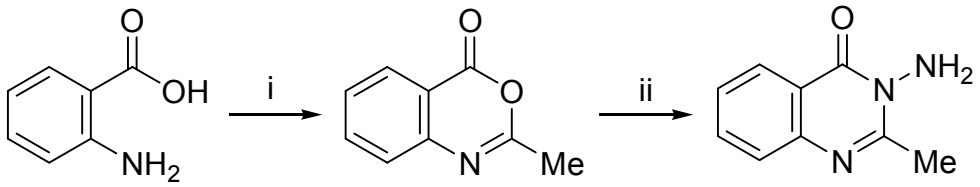


3.22 **3.23**

Reagents and conditions: (i) $ClCH_2CN$, MeONa, MeOH

Scheme 3.5 Formation of 2-chloromethyl quinazolinone

For the purpose of this project, the production of 2, 3-disubstituted-4-quinazolinones is important. The predominant method for the production of 2, 3-disubstituted-4-quinazolinones in literature is the acylation of anthranilic acid **3.19**, or one of its derivatives[171,172], followed by cyclisation, frequently forming a benzoxazinone intermediate[173] **3.24**. This also allows he introduction of substitution at the 3-position[174], via the use of nitrogen dinucleophiles. In this example, shown in scheme 3.6, hydrazine is used to produce **3.25**.

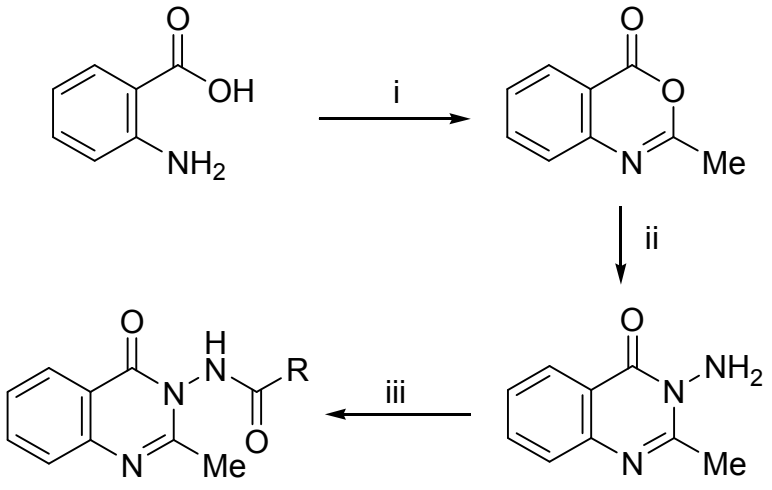


3.19 **3.24** **3.25**

Reagents and conditions: (i) Ac_2O, reflux; (ii) NH_2NH_2

Scheme 3.6 Production of 2-methyl-3-amino-4-quinazolinone

This simple reaction can be extended to alter the nature of substitution at the 2-position through lithiation of the molecule. The example below shows this being carried out on 3-(pivaloylamino)-2-methyl-4(3*H*)-quinazolinone **3.26** (R = *t*Bu) or 3-(acetylamino)-2-methyl-4(3*H*)-quinazolinone **3.26** (R = Me)[174], synthesised as shown in scheme 3.7.

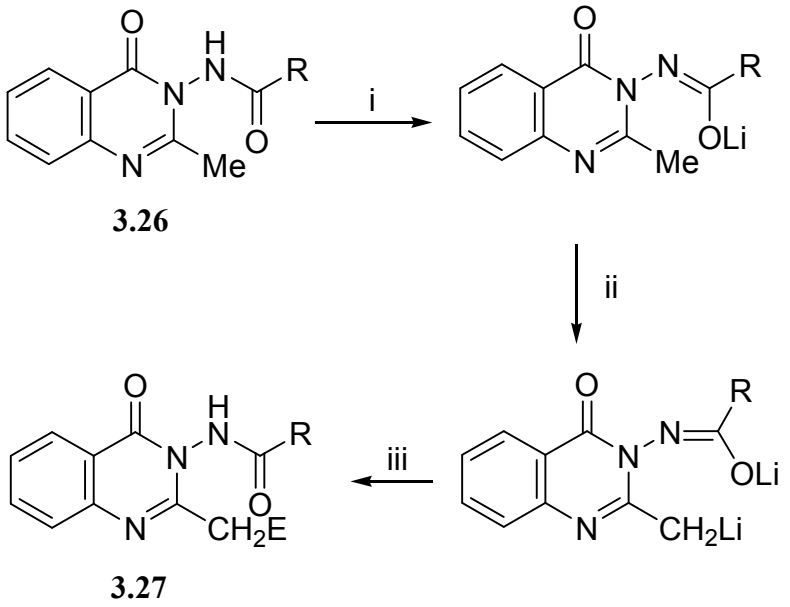


3.26

Reagents and conditions: (i) Ac_2O, reflux; (ii) NH_2NH_2; (iii) acylating agent (pivaloyl chloride or Ac_2O)

Scheme 3.7 Formation of 3-acylamino quinazolinones

The derivative **3.26** is then treated twice with butyl lithium (BuLi) as shown in scheme 3.8. The BuLi first lithiates the amide carbonyl and then causes lithiation at the 2-methyl. Following this, it is then possible to insert an electrophile at the 2-position forming **3.27**.



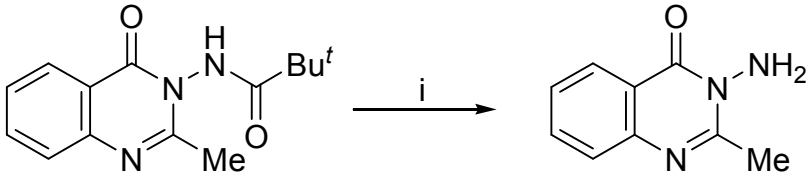
3.26

3.27

Reagents and conditions: (i) BuLi, THF, - 78 °C; (ii) BuLi, THF, - 78 °C; (iii) electrophile, - 78 °C

Scheme 3.8 Introduction of an electrophile to substituted 4-quinazolinone via lithiation

The acylated quinazolinone can also be easily converted back to a 3-amino substituent by refluxing in aqueous sodium hydroxide[174], scheme 3.9.

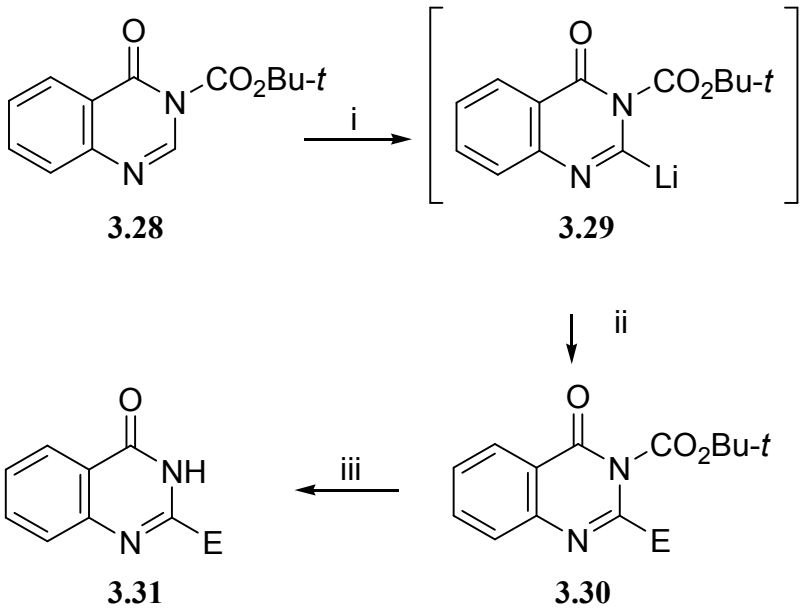


Reagents and conditions: (i) NaOH, MeOH, H_2O, reflux

Scheme 3.9 Deacylation of substituted quinazolinone

In this example, the pivaloyl group is being used as a protecting group, thus allowing the aforementioned lithiation to introduce variation into the molecule at the 2-position.

This type of reaction can also be carried out on a quinazolinone **3.28** unsubstituted at the 2-position, as shown in scheme 3.10. Again it involves the use of BuLi at low temperature[175], having first protected the 3-N with a group such as *t*-butoxycarbonyl **3.29**. An electrophile can then be introduced at the 2-position, forming **3.30**, and the quinazolinone subsequently deprotected[176] to leave 2-substituted molecule **3.31**.

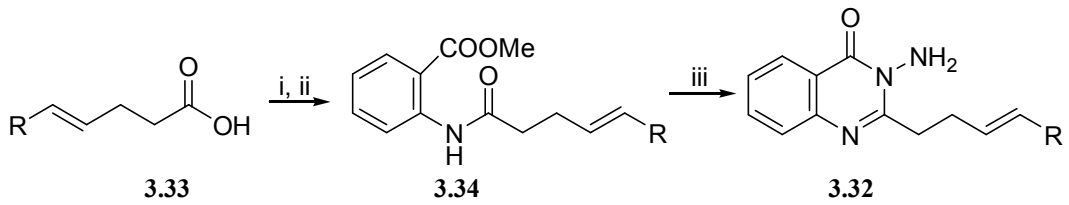


3.28 **3.29** **3.30** **3.31**

Reagents and conditions: (i) BuLi, THF, - 78 °C; (ii) Electrophile, H_3O^+; (iii) TFA, CH_2Cl_2

Scheme 3.10 Addition of electrophile to protected quinazolinone

Atkinson *et al*[177] state that the production of 2-alkyl-3-amino-quinazolinone **3.32** allows insertion of a double bond into the alkyl side chain when the quinazolinone ring is constructed in the way shown in scheme 3.11. It begins with the production of an acid chloride from a carboxylic acid **3.33**, using thionyl chloride, which is then reacted with methyl anthranilate to form the product **3.34**. This can then be cyclised in ethanol, with hydrazine allowing the insertion of the amino functionality at the 3-position.

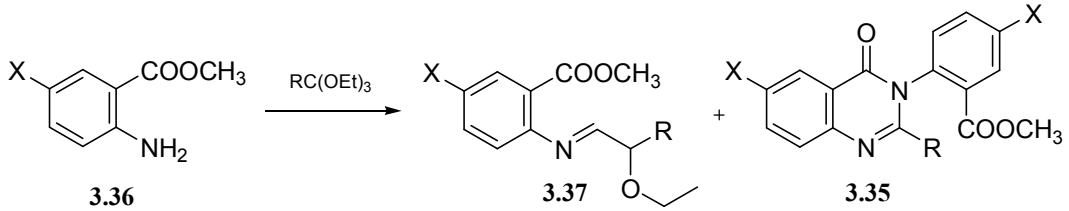


3.33 **3.34** **3.32**

Reagents and conditions: (i) $SOCl_2$; (ii) methyl anthranilate; (iii) NH_2NH_2, EtOH

Scheme 3.11 Production of 2-alkenyl-3amino-4-quinazolinone

3-Aryl substituted quinazolinones **3.35** can be formed, as shown in scheme 3.12, by premixing an anthranilate **3.36** and an ortho ester, followed by reflux and addition of the anthranilate ester to the refluxing ortho ester[178]. This will cause the production of a side product of formimidate **3.37** in cases where there is an alkyl chain present as the R group. The yield of quinazolinone can be increased by varying the ratio of orthoformate/anthranilate, a smaller ratio giving a better yield.

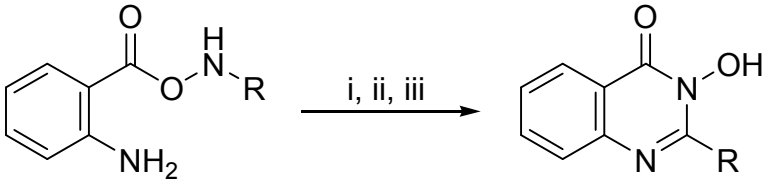


3.36 **3.37** **3.35**

Scheme 3.12 Formation of 3-phenyl substituted quinazolinone from ortho ester

3.2.1.1.1 3-Hydroxy and 3-amino-4-quinazolinones

Suitable derivatives of anthranilic acid may be used to form quinazolinones. Reddy *et al*[179] found that 2-methyl-3-hydroxy-4-quinazolinone **3.38** was formed when an equimolar mixture of *O*-(2-aminobenzoyl) hydroxylamine **3.39** and triethyl orthoformate, was heated with triethyl orthoacetate, scheme 3.13. They also produced a number of quinazolinone molecules by reacting aoryl derivatives of *O*-(2-aminobenzoyl)-*N*-aroylhydroxylamines in decalin or polyphenyl ether (PPE). The molecules produced were largely limited to those with substituted aromatic groups at the 2 position and the details relating to 2-alkyl substituted derivatives, more relevant to this study, were not investigated.

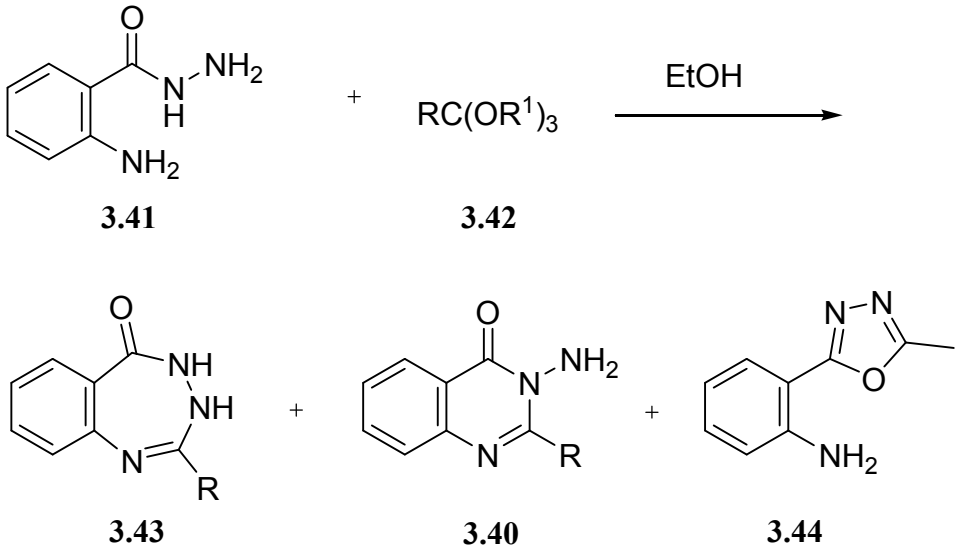


3.39 **3.38**

Reagents and conditions: i) $HC(OEt)_3$/benzene; ii) $CH_3C(OEt)_3$/decalin; iii) PPE

Scheme 3.13 Formation of 2-substituted-3-hydroxy-4-quinazolinone

It has also been noted that 2-substituted 3-amino-4-quinazolinones **3.40** have been formed by refluxing anthranoyl hydrazine **3.41** with orthoesters[180] **3.42**, as shown in scheme 3.14. This also produces the two side products, a benzotriazepinone **3.43** and an oxadiazole **3.44**. This method came about as an observation that quinazolinones were produced in the attempted synthesis of benzotriazepinones.



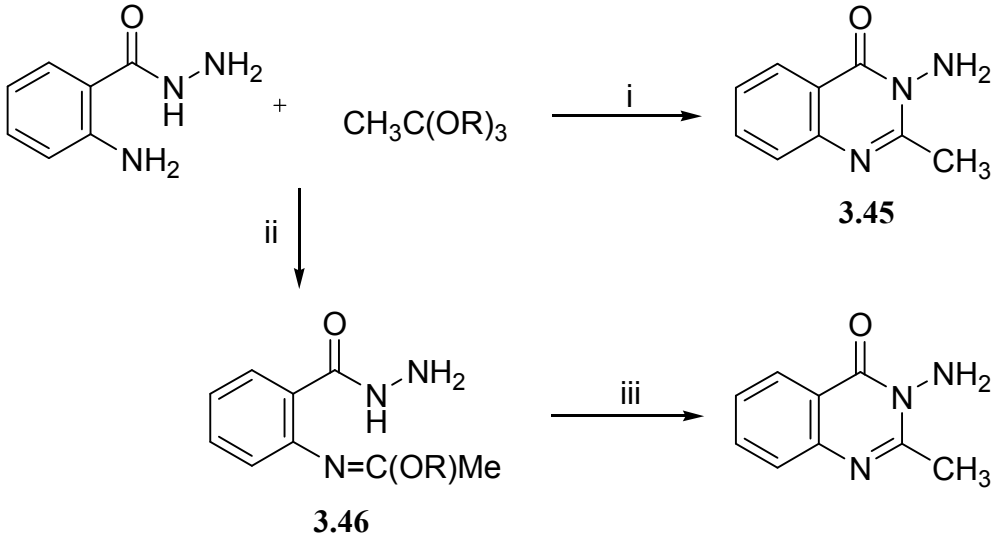
3.41 **3.42**

3.43 **3.40** **3.44**

Scheme 3.14 Production of 2-substituted-3-amino-4-quinazolinones as a by product in the production of benzotriazepinones

They also observed that the ring closure was dependent on the temperature of reflux. The quinazolinone is the thermodynamic product and is formed at higher temperatures. In ethanol the 4-quinazolinone **3.45** was formed, whereas in benzene only the imidate **3.46** was produced, which could then be cyclised

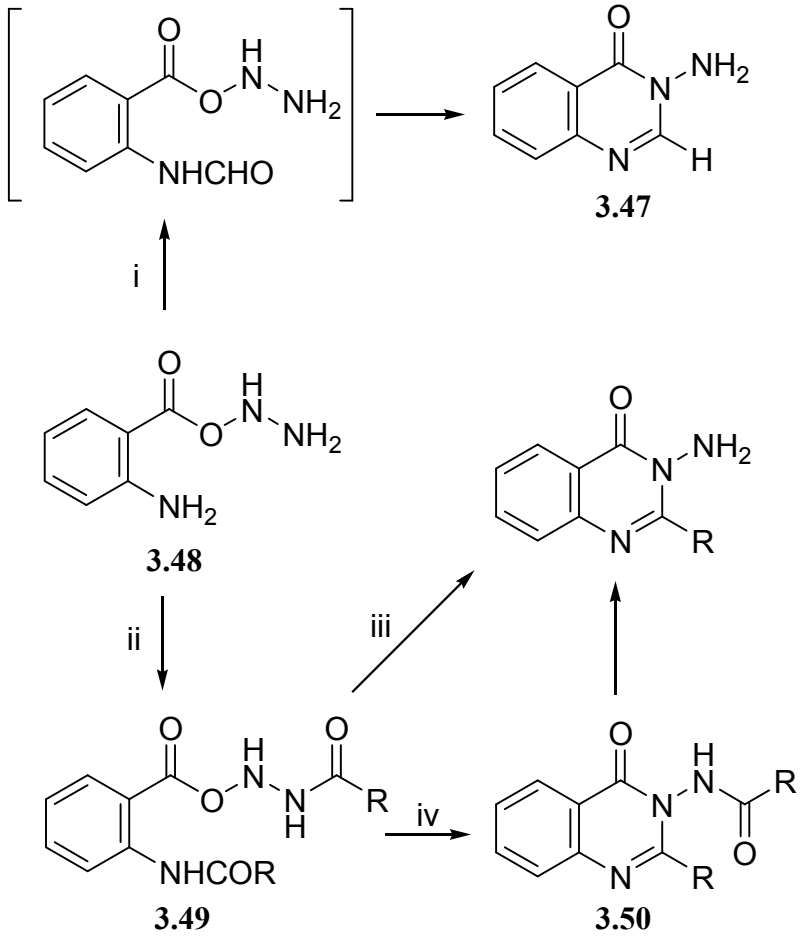
by heating in a suitable solvent, such as ethanol or higher boiling point solvent, scheme 3.15. These reactions do not occur if aprotic solvents are used.



Reagents and conditions: (i) EtOH, heat; (ii) C_6H_6, heat; (iii) EtOH or high boiling solvent, heat

Scheme 3.15 Formation of the thermodynamic and kinetic products of 2-aminobenzohydrazide

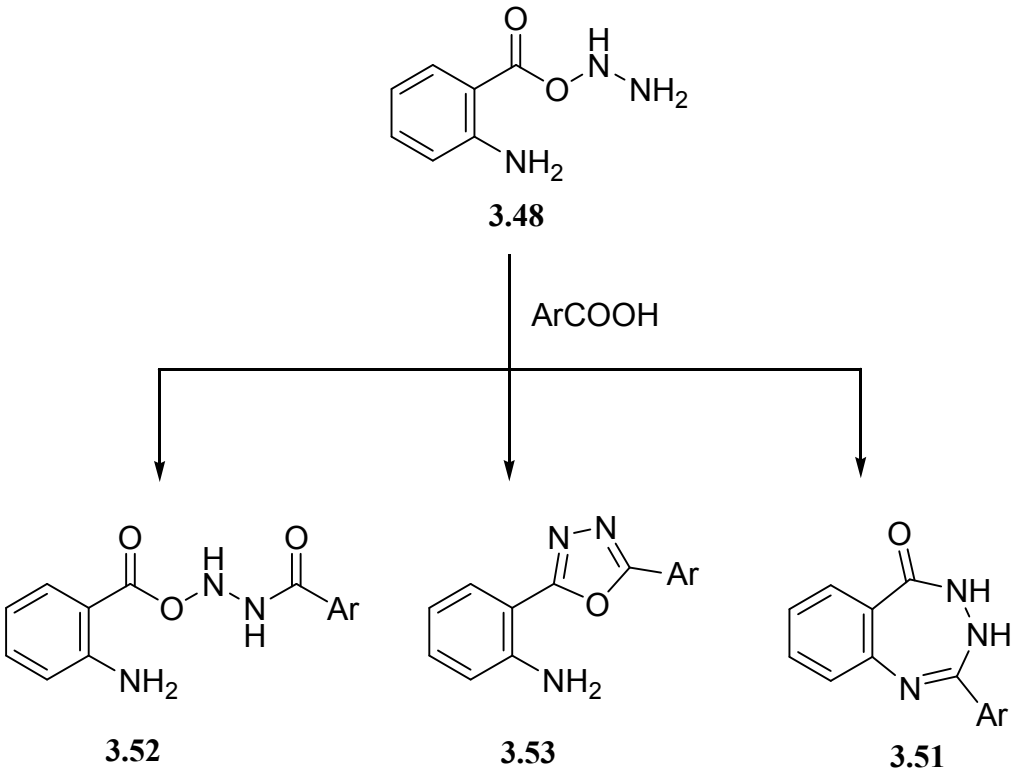
3-Amino-4-quinazolinone **3.47** was shown to form in one step when 2-aminobenzoylhydrazine **3.48** was treated at reflux with formic acid[181]. When acetic acid was substituted for the formic acid, firstly 1-(2-acetylaminobenzoyl)-2-acetlyhydrazine **3.49** is formed, which can be directly converted to 3-aminoquinazolin-4-one in one step upon treatment with concentrated sulphuric acid or in two steps via 3-acetamido-2-methylquinazolinone intermediate **3.50**.



Reagents and conditions: (i) HCOOH; (ii) RCOOH; (iii) cH_2SO_4; (iv) xylene, *p*-toluene sulphonic acid

Scheme 3.16 Products when 2-aminobenzohydrazide is reacted with different carboxylic acids

It was also shown that similar reactions to those outlined above, using aromatic acids there was not any formation of a quinazolinone molecule, rather a benzotriazepinone **3.51** was the main product formed, accompanied by two other minor products[181] **3.52** and **3.53**, scheme 3.17.



Scheme 3.17 Reaction of 2-aminobenzoylhydrazine with aromatic acids

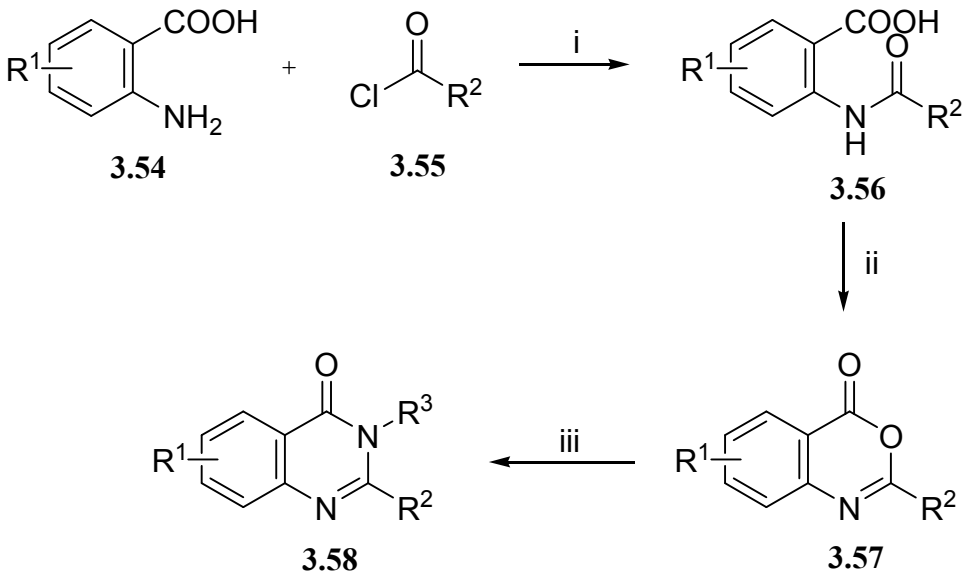
There thus exists a large amount of literature relating to the production of quinazolinones and there is the potential to provide a number of useful molecules in this series within a short period of time.

3.3 Synthesis of quinazolinones

The aim of the general synthetic route was to produce a number of analogues that have structural similarity to PQS in a simple fashion but with the ability to introduce diversity in a straightforward manner.

3.3.1 General synthetic route

The general route that was used for the elaboration of the PQS based quinazolinone structure is shown below in scheme 3.18.

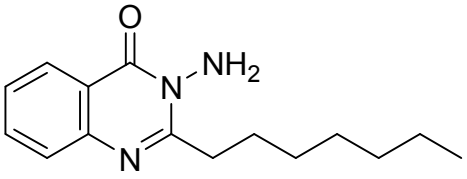


Reagents and conditions: (i) NaOH, H_2O; (ii) Ac_2O, Δ; (iii) R^3NH_2, EtOH, Δ

Scheme 3.18 General method for the production of 4-quinazolinone molecules

An anthranilic acid derivative **3.54** is initially acylated using an acid chloride **3.55**, forming **3.56**. This can then be cyclised to form a benzoxazinone **3.57**, which when reacted with a hydrazine derivative forms a 2,3-substituted quinazolinone **3.58**.

This general method was used to produce a number of analogues. The formation of 2-*n*-heptyl-3-amino-4(3*H*)-quinazolinone **3.59** is discussed in detail and any modifications to the method will be described in relation to other derivatives.



3.59

The decision to produce 2-*n*-heptyl-3-amino-4(3*H*)-quinazolinone **3.59** was taken as NH_2 is an isostere of OH. This means that NH_2 has the same valency as OH and allows the character of the molecule to be changed in a controlled way[182].

Also, the NH_2 group will allow the retention of any hydrogen bonding possible at this position. This is potentially important as the two immediate precursors of PQS, 2-*n*-heptyl-4(1*H*)-quinolone and 2-*n*-heptyl-3-formyl-

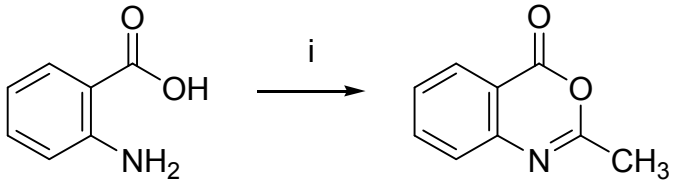
4(1*H*)-quinolone, lack functional groups capable of hydrogen bonding and have been shown to be inactive in the relevant biological assays.

3.3.2 Formation of 4-Quinazolinones via benzoxazinone

The initial step required in the scheme was the formation of the benzoxazinone intermediate, which has been extensively used in the production of 4-quinazolinones. Interestingly, they have also been used in the synthesis of 2-quinolones[183].

These heterocycles have their own therapeutic effects, including the inactivation of chymotrypsin[184,185] and inhibition of serine protease[149]. These molecules can be synthesised in a number of ways.

The best established method is described by Errede *et al*[186,187], based on work done by Bogert and Seil[188], delivering 2-methylbenzoxazinone **3.14**, by refluxing anthranilic acid **3.13** in acetic anhydride, is shown below.

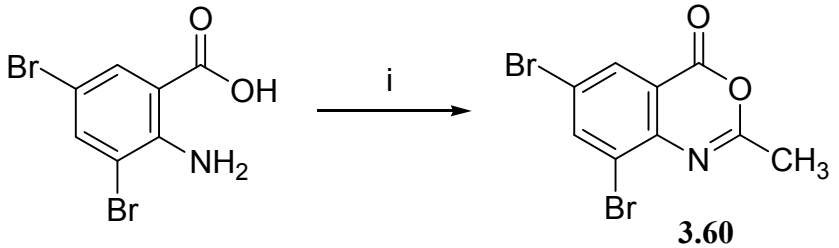


3.13 **3.14**

Reagents and conditions: (i) Ac_2O, Δ

Scheme 3.19 Production of 2-methyl-benzoxazinone

This synthesis of the benzoxazinones can also tolerate substitution on the carbocyclic ring, as demonstrated by the production of a 6,8-dibromo-2-methyl-4-quinazolinone[152] **3.60** is produced if the reaction in scheme 3.20 is undertaken.

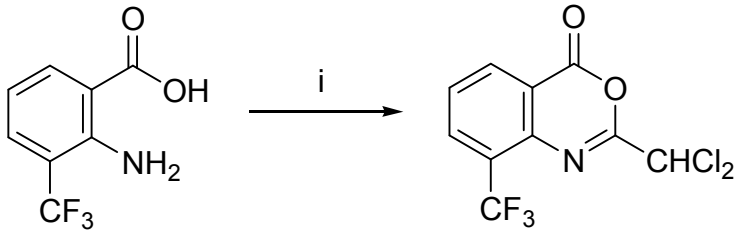


3.60

Reagents and conditions: (i) Ac_2O, Δ

Scheme 3.20 Synthesis of substituted benzoxazinone using acetic anhydride

In another example the acetic anhydride was replaced by dichloroacetyl chloride, thereby giving the 2-dichloromethylbenzoxazinone. An 8-triflouromethyl compound **3.62** was produced from the corresponding anthranilic acid[189] **3.61** using this method.

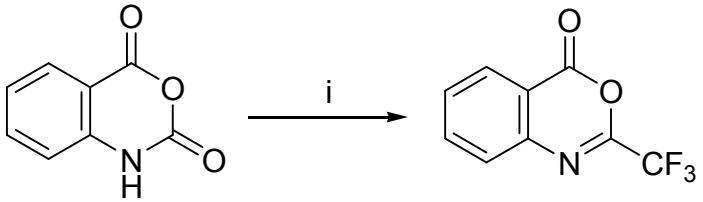


3.61 **3.62**

Reagents and conditions: (i) Dichloroacetyl chloride, Δ

Scheme 3.21 Synthesis of substituted benzoxazinone using dichloroacetyl chloride

There are alternatives for the production of the benzoxazinones, such as the use of isatoic anhydride **3.63** instead of anthranilic acid, which is heated at reflux in pyridine. An anhydride is used to supply the substitution at the 2-position, in the example shown in scheme 3.22 a trifluoroacetic anhydride is used to form **3.64**.

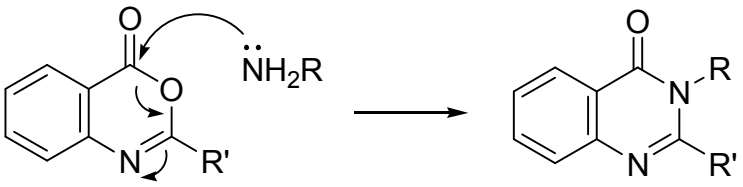


3.63 **3.64**

Reagents and conditions: (i) $(CF_3CO)_2O$, pyridine, Δ

Scheme 3.22 Formation of 2-(trifluoromethyl)-4*H*-benzoxazinone

In the second step, attack by nitrogen nucleophiles, such as hydrazines, aniline[190] and amines[191], on the 4-carbonyl of the benzoxazinone produces the desired 4-quinazolinone. This allows substitution to be introduced at the 3-position of the resulting quinazolinone **3.65**.



3.65

Scheme 3.23 Attack of nitrogen nucleophile on benzoxazinone

The substituents will have a dominant bearing on how reactive the benzoxazinone carbonyl is and it appears to be the electronic contribution in particular of the ring substituents that dominate. The order of reactivity was seen to follow the trend of the electronic contribution of the substituents through either induction, resonance or a combination of the two[187].

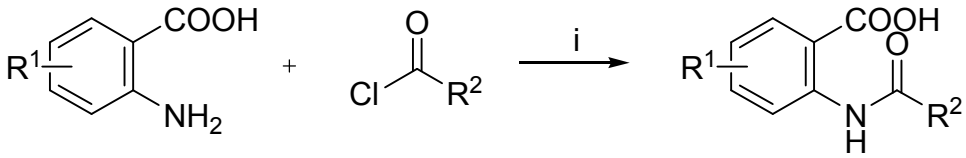
Conversely, if there is an N-N type molecule acting as the nucleophile, the factor that determines which nitrogen attacks seems to be the steric effects, rather than nucleophilicity. The least hindered nitrogen attacks the carbonyl, rather than the most nucleophilic.

This information is valuable if a range of nitrogen nucleophiles are used in the formation of quinazolinones and may go towards explaining differing yields of final product.

Thus the benzoxazinone molecule was very useful as there was potential to introduce diversity into the system at specific points by simply changing the nature of the starting materials. It has been shown that they will undergo ring opening with different nucleophiles[192-194] allowing incorporation of substitution at the 3-position. This in turn will generate a number of molecules for the SAR study.

3.3.3 Acylation of anthranilic acid

Substituted anthranilic acid **3.54** can allow substitution on the carbocyclic ring in **3.56**, whereas changing the acid chloride **3.55** provides the alteration in the side chain.



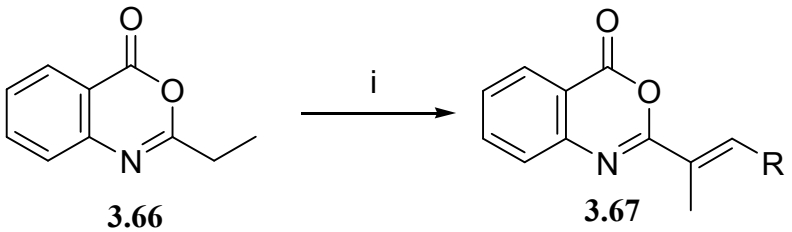
3.54 **3.55** **3.56**

Reagents and conditions: (i) NaOH, H_2O

Scheme 3.24 General method for *N*-acylation of anthranilic acid derivatives

3.3.3.1 Alteration of alkyl chains

It was decided to alter the side chain early in the process, so that it would be in place when the ring closure was complete, rather than attempting to alter its nature following the ring closure. The latter is possible using an aldehyde with zinc chloride to convert an ethyl side chain **3.66** to that with an unsaturated or aromatic nature[190] **3.67**. This method would allow more complex substitution if required later on in the SAR study.



3.66 **3.67**

Reagents and conditions: (i) RCHO, $ZnCl_2$

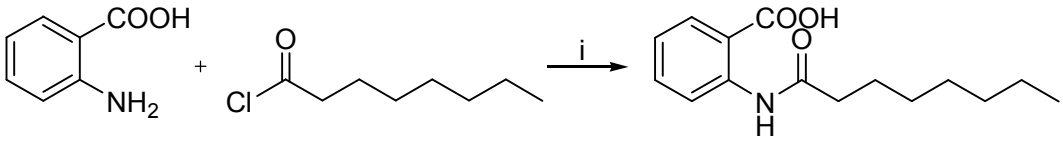
Scheme 3.25 Alteration of benzoxazinone side chain using zinc chloride

3.3.3.2 Formation of *N*-octanoylanthranilic acid

In order to introduce C_7-side chain at the 2-position, mimicking that present in PQS, anthranilic acid is acylated in a Schotten-Bauman type reaction, with

octanoyl chloride **3.68** that is added dropwise to a solution of anthranilic acid in aqueous NaOH, scheme 3.26.

This solution is extracted with ethyl acetate and following removal of the solvent and trituration with petroleum ether, allows the *N*-octanoylanthranilic acid **3.69** to be collected as a white solid in a 65 % yield.

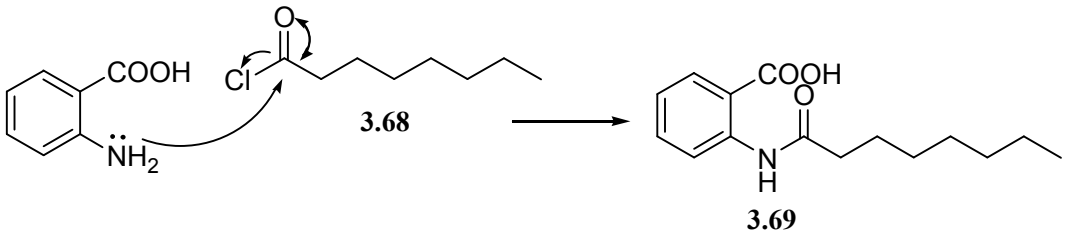


3.68 **3.69**

Reagents and conditions: (i) NaOH, H_2O

Scheme 3.26 Formation of *N*-octanoyl anthranilic acid

The mechanism for the reaction proceeds as shown in scheme 3.27, lone pair on the nitrogen attacks the electrophilic carbonyl on the octanoyl chloride **3.68**, forming *N*-octanoylanthranilic acid. The H^1 NMR shows the expected broad peak for NH, present at δ 11.03.



3.69

Scheme 3.27 Acylation of anthranilic acid with octanoyl chloride

It is also possible to produce this molecule in a reaction using pyridine at 0 °C[187] but the use of sodium hydroxide compared to pyridine is preferable due to toxicity considerations. The method using pyridine was attempted but produced a product that was extremely inferior to that produced by the method explained above.

When commercially available, acid chlorides were used to give alkyl chain substitution. The method can be extended to other substitutions with the

required acid chlorides **3.71** being prepared by treating carboxylic acids **3.70** with thionyl chloride at reflux temperature for 2 h[187].

3.3.3.3 *N*-Acylated Anthranilic acids produced

A number of molecules, containing differing substituents both in terms of length of side chain and on the benzene ring, were synthesised in this way.

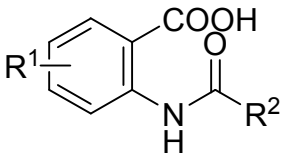


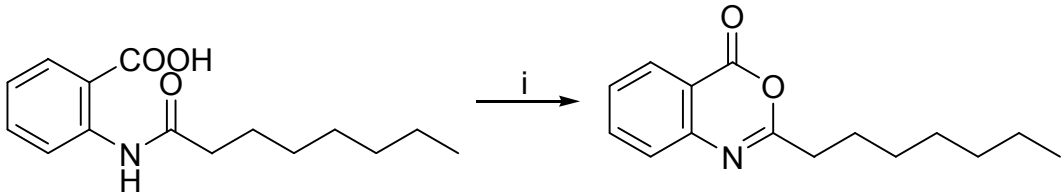
Table 3.1 *N*-Acylated anthranilic acids produced

Compound	R^1	R^2	*Yield (%)*
3.74	H	C_5H_{11}	63
3.69	H	C_7H_{15}	65
3.75	H	C_9H_{19}	67
3.76	4-Cl	C_5H_{11}	70
3.77	4-Cl	C_7H_{15}	69
3.78	4-Cl	C_9H_{19}	68

3.3.4 Formation of 2-alkyl benzoxazinone

The next step in the synthesis involves cyclisation of the anthranilate **3.69**, via dehydration, forming a benzoxazinone **3.79** intermediate.

Two dehydrating agents, acetic anhydride[174,187,194] and thionyl chloride[187], were assessed with acetic anhydride yielding a substantially cleaner more satisfactory product.

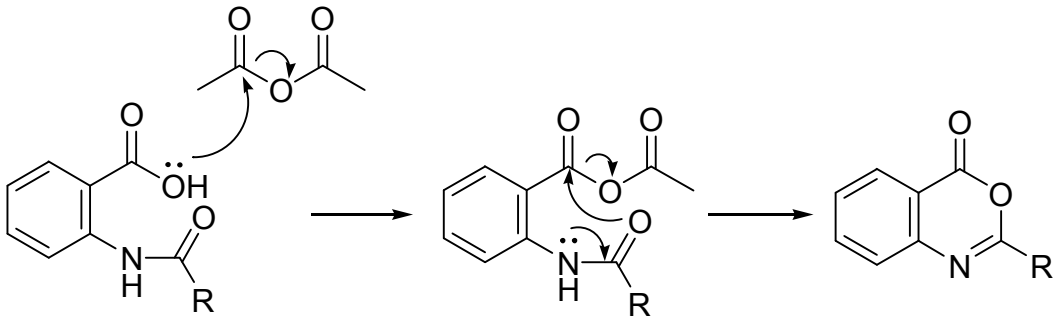


3.69 **3.79**

Reagents and conditions: (i) Ac_2O, Δ

Scheme 3.28 Formation of 2-heptyl benzoxazin-4-one

The reaction proceeds according to the mechanism shown in scheme 3.29. The carboxylate anion on the anthranilate **3.69** attacks a carbonyl on the acetic anhydride. The intramolecular cyclisation is driven by the lone pair on the nitrogen and the proximity of the mixed anhydride functional group. These two steps occur in the course of the reaction, with the product **3.79** being afforded after the removal of the acetic anhydride.



Scheme 3.29 Benzoxazinone ring closure

3.3.4.1 Benzoxazinones produced

A number of substituted benzoxazinones were synthesised using this general route. They were all produced in quantitative yield and used without further purification in the next step.

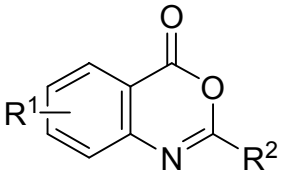


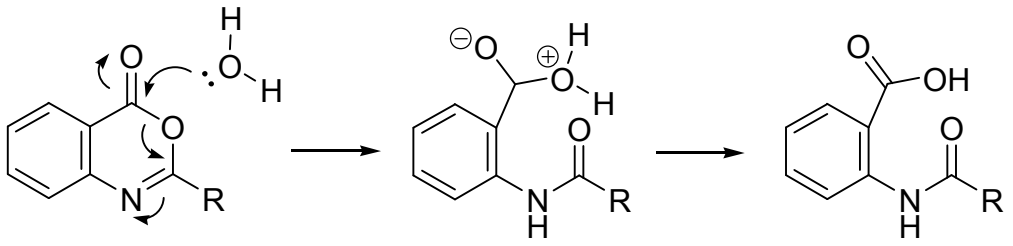
Table 3.2 Benzoxazinones produced

Compound	R^1	R^2
3.80	H	C_5H_{11}
3.79	H	C_7H_{15}
3.81	H	C_9H_{19}
3.82	6-Cl	C_5H_{11}
3.83	6-Cl	C_7H_{15}
3.84	6-Cl	C_9H_{19}

3.3.4.2 Instability of benzoxazinones

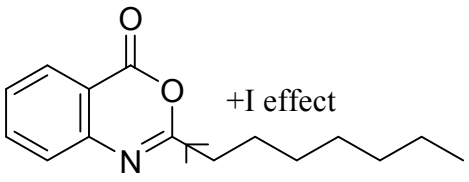
One of the main reasons that these molecules were not purified due to their potential to degrade when exposed to water.

It is a report that the benzoxazinones are liable to hydrolysis by water and that 2-alkylbenzoxazinones increase in stability relating to the length of the side chain[194]. The actual extent to which this hydrolysis occurs varies greatly across a range of molecules. A molecule of water would be able to open the benzoxazinone ring by attacking the intracyclic carbonyl and effectively hydrolysing the cyclic ester, as shown in scheme 3.30. Care was therefore taken in storage and utilisation of these molecules.



Scheme 3.30 Hydrolysis of benzoxazinone

As mentioned before, it has also been suggested that the nature of the substitution on the benzoxazinone can modulate the reactivity of the carbonyl, in that electron donating groups cause the carbonyl to be less electrophilic and reduce the reactivity of the benzoxazinone carbonyl to nucleophilic attack[185]. This is desirable in terms of stability on storage, or if they are to be final molecules themselves, but may be a factor when these molecules are altered for further analogue production.



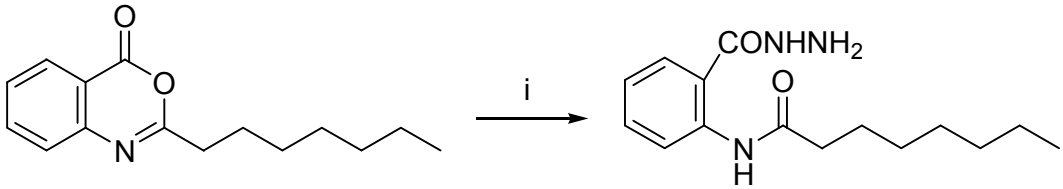
An alkyl chain will act as an electron donor (+I effect), as shown above, which suggests that the longer the chain gets, the more difficult a reaction and

insertion of the group that will constitute substitution at the 3-position of the corresponding quinazolinone. This did not present a problem with the molecules that were produced in this study but may have a bearing on further investigations to extend the number of analogues in this series.

3.3.5 Formation of 2-*n*-heptyl-3-amino-4(3*H*)-quinazolinone

The next stage in the synthesis requires the substitution to be introduced to the molecule to provide a suitable quinazolinone molecule.

When the benzoxazinone **3.79** reacts with hydrazine at room temperature, in tetrahydrofuran (THF), scheme 3.31, it yields an uncyclised form of the desired product, 2-(octanamido)benzohydrazide **3.84**, along with a highly coloured, fluorescent material, which could not be satisfactorily characterised.



3.79 **3.84**

Reagents and conditions: (i) NH_2NH_2, THF

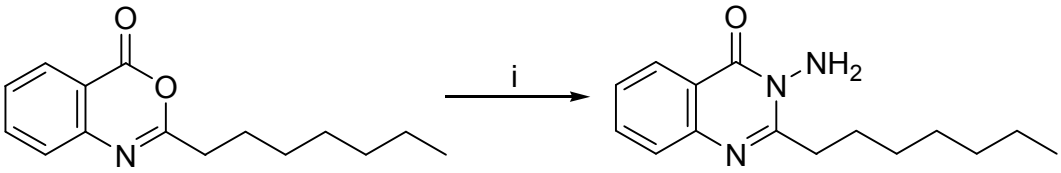
Scheme 3.31 Formation of 2-(octanamido)benzohydrazide

Even though the quinazolinone was not produced, this method would have had value if the uncyclised form could be recovered in a suitable quantity and then further reacted to form the quinazolinone. Unfortunately, the uncyclised form of the compound was produced in an 8 % yield, meaning that the method was not suitable for use.

This implied that there required to be more energy put into the system to allow the ring closure to occur. Another contributing factor is that it is desirable to keep the number of steps to achieve the desired final molecule to a minimum.

Fortunately, refluxing the benzoxazinone **3.79** in anhydrous ethanol in the presence of excess hydrazine, as shown in scheme 3.32, produced the

required molecule[152,178,195], 2-*n*-heptyl-3-amino-4(3*H*)-quinazolinone **3.85**. It is also possible to carry out this reaction using pyridine[153].



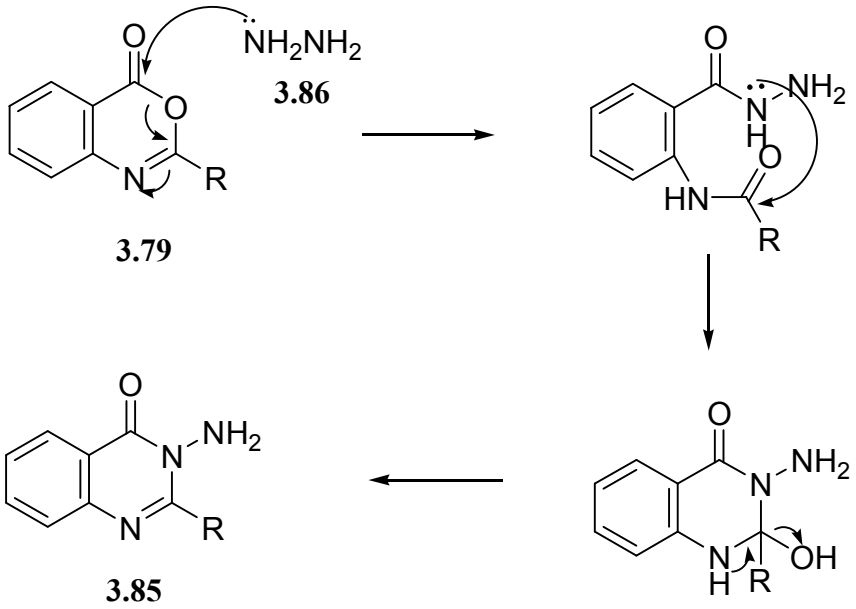
3.79 **3.85**

Reagents and conditions: (i) NH_2NH_2, EtOH, Δ

Scheme 3.32 Formation of 2-*n*-heptyl-3-amino-4(3*H*)-quinazolinone

The production of 2-*n*-heptyl-3-amino-4(3*H*)-quinazolinone was supported by a signal for the NH_2 protons at δ 4.91 in the H^1 NMR spectrum and by it showing *m/z* 260.2 in positive electrospray MS. The NH_2 signal disappeared when the ^1H NMR was recorded in the presence of deuterium oxide (D_2O).

The mechanism for the reaction is as shown below in scheme 3.33. It begins with an intermolecular reaction between the hydrazine **3.86** and the benzoxazinone **3.79**, and then proceeds with an intramolecular step, causing the ring closure. Firstly, the lone pair on the hydrazine nitrogen attacks at the carbonyl on the ring, causing the ring to open. The lone pair of the newly produced hydrazide nitrogen then instigates an intramolecular attack towards the amide carbonyl, causing the ring to close again. A loss of the water molecule, forming 2-*n*-heptyl-3-amino-4(3*H*)-quinazolinone **3.85**, is driven by extension to conjugation. The solid obtained was triturated with hexane and purified using column chromatography.



Scheme 3.33 Formation of 2-*n*-heptyl-3-amino-4(3*H*)-quinazolinone

There is evidence reported that temperatures of 150 °C – 250 °C are required to afford ring closure to the quinazolinone, although the temperature seems to vary in terms of what substitution is present in both starting materials. To illustrate this with some examples, the relative reactivities, in relation to substitution at R in the scheme above would be, (R = CF_3) > (R = H) > (R = CH_3) > (R = NH_2) ≈ (R = Ph)[187].

An alternative mechanism where hydrazine attacks the 2-position[178,187] is hindered by the +I effect of the 2-alkyl substituent.

There is also the possibility that this molecule may be formed by first reacting the anthranilate with hydrazine, then causing ring closure by refluxing in acetic acid[195] but this is probably more relevant for a reaction that does not proceed to completion in one step and would require further ring closure.

3.3.6 Use of general method

Initially the compounds 2-*n*-heptyl-3-amino-4(3*H*)-quinazolinone and 2-*n*-heptyl-3-hydroxy-4(3*H*)-quinazolinone were synthesised and their biological activity assessed. This was done to ascertain whether this route was a fruitful one to pursue.

The compounds showed initial activity that, while below that of PQS, was comparable to OdDHL, discussed further in chapter 4. These results were encouraging enough to continue with the synthesis of a number more compounds in this series.

3.3.6.1 4-Quinazolinones produced

This general method was used to produce a number of molecules with little alteration to that outlined above. Molecules that included chlorine substitution on the carbocyclic ring produced very clean products following recrystalisation.

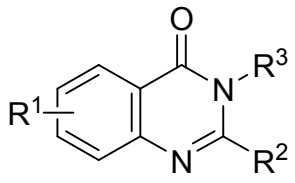


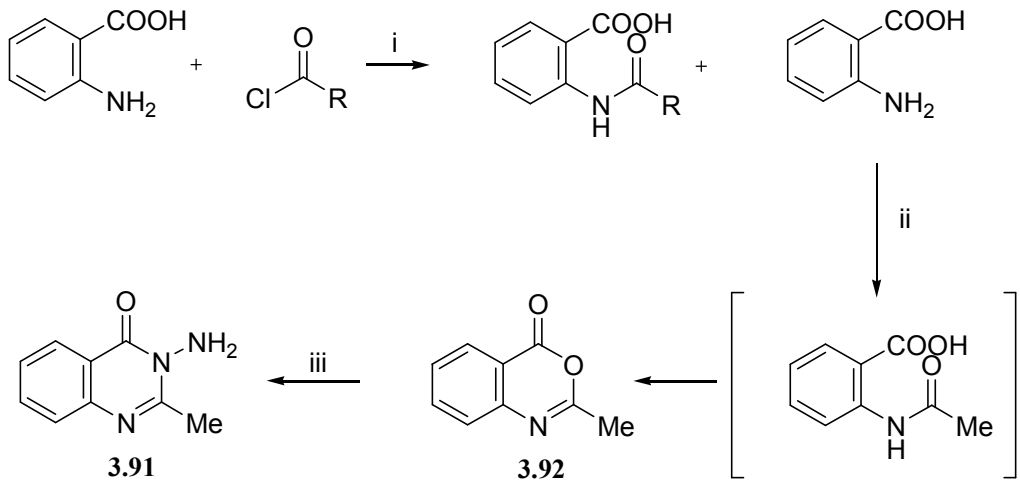
Table 2.3 Substituted 4-quinazolinones synthesised

Compound	R^1	R^2	R^3	*Yield (%)*
3.87	H	C_7H_{15}	OH	29
3.85	H	C_7H_{15}	NH_2	38
3.88	H	C_5H_{11}	NH_2	47
3.89	H	C_9H_{19}	NH_2	46
3.90	6 - Cl	C_7H_{15}	NH_2	78

3.3.6.1.1 Formation of 2-methyl-4-quinazolinone as a side product

There was a formation of a 2-methyl derivative **3.91** as a side product during the formation of the longer alkyl chain molecules These occurred due to there being some of the anthranilic acid, or derivative, not being acylated during the course of the first step of the reaction, thereby meaning that a 2-methylbenzoxazinone **3.92** was formed in the following step and this was not removed. As these were not purified, these were carried on to the next step and

the hydrazine would react in the same way to form the 2-methyl derivative **3.91**, which would then be isolated as part of purification.



3.91 **3.92**

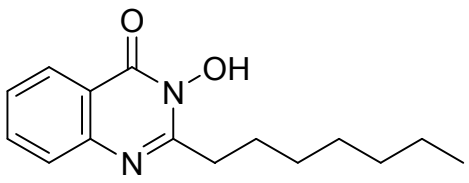
Reagents and conditions: (i) NaOH, H_2O; (ii) Ac_2O, Δ; (iii) NH_2NH_2, EtOH, Δ

Scheme 3.34 Formation of 2-methyl-3-amino-3*H*-quinazolin-4-one analogue side product

The compounds, however, are useful in the SAR study, as they provide molecules with a minimal alkyl side chain and can be valuable in showing the role that an alkyl side chain plays.

3.3.6.2 Formation of 2-*n*-heptyl-3-hydroxy-4(3*H*)-quinazolinone

The production of 2-*n*-heptyl-3-hydroxy-4(3*H*)-quinazolinone **3.87** is obviously of value in this study and the investigation of the surrogate system offered by the quinazolinones as this most closely resembles PQS.



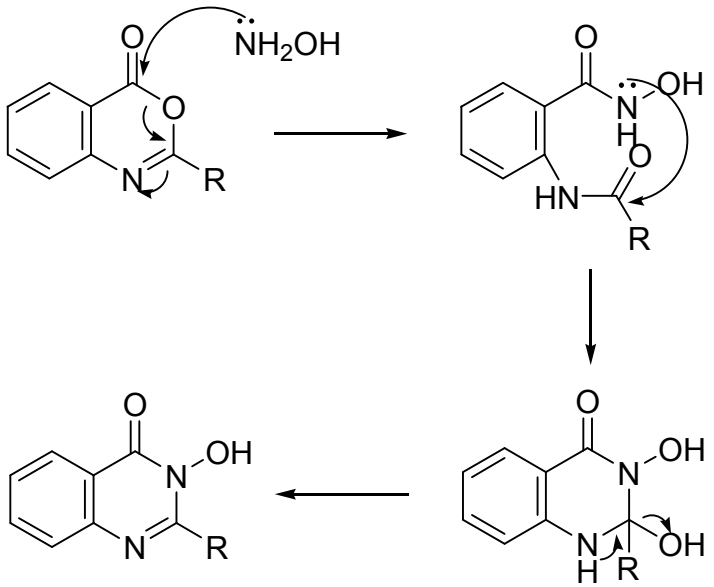
3.87

The production of 2-*n*-heptyl-3-hydroxy-4(3*H*)-quinazolinone differed from the established route for the addition of the hydroxy group as there is not a commercially available hydrazine derivative (NH_2OH or hydroxylamine)

suitable to do the straight reaction. The hydrochloride salt is available, which requires the free base to be liberated prior to the reaction. This was done by using sodium ethoxide by dissolving sodium metal in ethanol. This caused the formation of sodium chloride that needed to be removed by filtration prior to the addition of 2-heptyl-4-benzoxazinone.

Although the reaction did take place it did not proceed as successfully as the previously described examples and the desired product was obtained in a low yield of 29 %.

After the liberation of the free base, the mechanism is very similar to that for the previously described for 2-*n*-heptyl-3-hydroxy-4(3*H*)-quinazolinone and is shown in scheme 3.35.

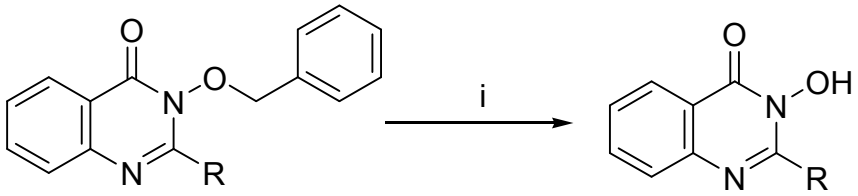


Scheme 3.35 Mechanism for formation of 2-*n*-heptyl-3-hydroxy-3*H*-quinazolin-4-one

The lower yield is possibly due to a reduction of the amount of free hydroxylamine in the reaction mixture, caused by inevitable losses during the treatment with sodium methoxide and subsequent filtration. There is also the possible reduction in the reactivity of the nitrogen lone pair in the hydroxylamine, due to the relative higher electronegativity of the oxygen (α effect), compared to the nitrogen present in hydrazine. This would cause the lone pair to be less nucleophilic.

3.3.6.2.1 Alternative method for production of 2-*n*-heptyl-3-hydroxy-4(3*H*)-quinazolinone

An alternative method, that was not attempted, that potentially could allow easier purification and increased recovery of the product is shown in scheme 3.36. This method would involve the use of *o*-benzylhydroxylamine to produce **3.93**. The benzyl group could then be removed, via hydrogenolysis, to obtain the desired hydroxyl group at the 3-position.



3.93

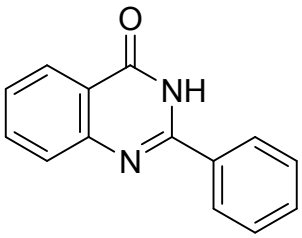
Reagents and conditions: (i) Pd/C, H_2, EtOH

Scheme 3.36 Proposed alternative method for the production of 2-alkyl-3-hydroxy-4*H*-quinazolinone

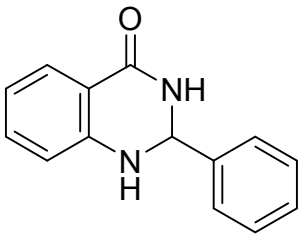
3.3.7 1, 2-Dihydro derivatives of 4-quinolones

Reduced activity of 2-*n*-heptyl-3-hydroxy-4(3*H*)-quinazolinone as compared to PQS may be, in part, to the absence of hydrogen on the N^1. The presence of the hydrogen on the nitrogen is potentially significant. Having the nitrogen unsubstituted retains the aromaticity, and therefore the rigidity, of the molecule but evidence suggests that the presence of a proton on the nitrogen significantly affects the activity.

Quinazolinones have been previously investigated in relation to their potential antitumour activity. In the course of this investigation the molecules **3.94** and **3.95** were produced[154] and assayed for their inhibition of tubulin polymerization[150]. The molecule with the hydrogen present on the nitrogen was significantly more active than that which did not (IC_{50} 14 μM compared with > 40)[154].



3.94



3.95

This change in activity is obviously dependent on the nature of the active site interaction with the molecule and whether hydrogen bonding is significant in this.

3.3.7.1 Method of reduction

The introduction of the hydrogen onto the nitrogen requires a reduction across the 1, 2-double bond present in the heterocyclic ring. Similar reactions have been carried out using the reducing agents including sodium borohydride and sodium cyanoborohydride[196].

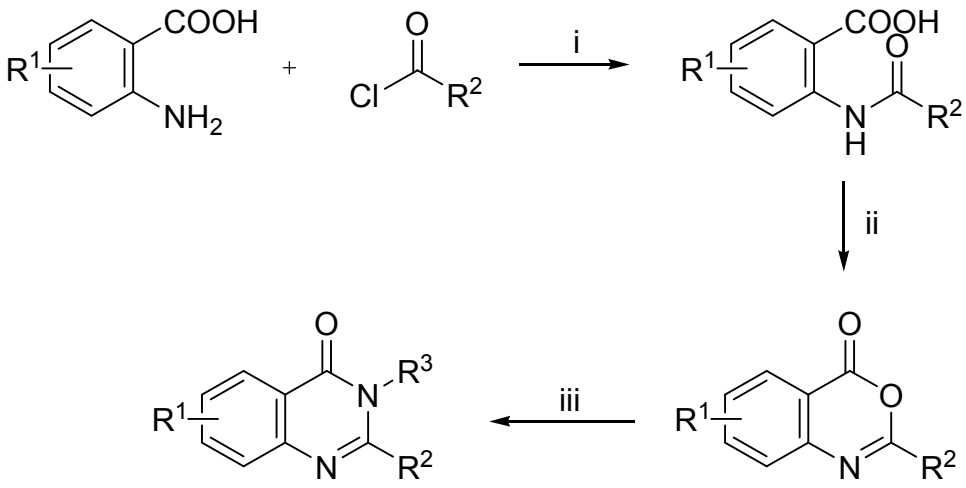
Theoretically there are three main alternatives:

- Hydrogenation of the double bond, using a palladium catalyst. In this method the hydrogen is absorbed onto the palladium surface and the π electrons of the nitrogen-carbon double bond are able to bind and each atom to take on a hydrogen.
- Sodium borohydride can be used in methanol. This allows the formation of sodium methoxide, creating basic conditions and causing a hydride ion to attack the carbon and reducing the double bond onto the nitrogen.
- Using sodium cyanoborohydride[196] in acidic conditions causes a protonation of the nitrogen. The pH of the reaction must be maintained at pH 3-4 to ensure success in the reaction. The hydride from the cyanoborohydride attacks the carbon and the charge is equalised onto the nitrogen, causing the reduction of the double bond. Potential cleavage of the carbon-nitrogen bond has been reported when using this method[197] but has also shown success in similar systems[196]

It would be of value to investigate this type of structural change in the quinazolinone series and its effect on immune modulatory activity. It would provide an insight into the importance of hydrogen bonding at this point if the molecule. Additionally, there is the removal of the aromaticity of the system, reducing rigidity and allowing greater movement of the ring, which may be important in its binding interactions with the target immunophilin.

3.4 Summary of Results

A number of substituted 2-alkyl-3-substituted quinazolinones were produced using a facile three step synthetic route.



Reagents and conditions: (i) NaOH, H_2O; (ii) Ac_2O, Δ; (iii) R^3NH_2, EtOH, Δ

Scheme 3.37 Facile three step synthesis of 2-alkyl-3-substituted quinazolinones

This method allows the introduction of a variety of substituents into the core structure of the quinazolinone, in the areas shown above, in a quick and simple way. This has allowed the synthesis of a number of analogues in the system that acts as a surrogate system for the 4-quinolone based compounds. This system was significantly more convenient with regards to production of

molecules than that of the 4-quinolone. The quinazolinone series of molecules can be quickly and simply produced, using inexpensive starting materials.

This series of molecules has allowed the number of analogues in the SAR study to be increased in number in a short period of time. It may also provide information with regards to similar alterations in the 4-quinolone series. The immune modulatory properties of these new 4-quinazolinone based derivatives were investigated as detailed and discussed in chapter 4.

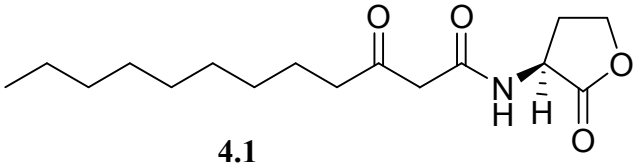
Chapter 4

Immune Modulatory Activity of PQS Analogues: Structure Activity Relationship Studies

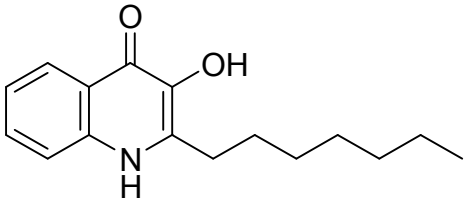
4.1 Natural products in immune therapy

Immune modulation is the alteration of the body's immune response via the use of external stimuli. This is important in the range of diseases and conditions that occur due to an inappropriate immune response initiated by the host. This can take the form of the rejection of a transplanted organ, hypersensitivity to allergens or autoimmunity, where the host mounts an immune response to its own tissues. In these cases it is imperative to suppress the immune response to obtain a favourable outcome and the agents currently in use in this area have been reviewed in Chapter 1. A number of these compounds have been directly or indirectly derived from natural products e.g. Tacrolimus, also known as FK506, a macrolide produced by the bacteria *Streptomyces tsukubaensis*[39] and Sirolimus first isolated from a strain of *Streptomyces hygroscopicus*[46]. The discovery that signal molecules involved in the bacterial communication process, known as quorum sensing, possessed immune modulating capability[56] has led to the possible exploitation of these molecules with the view of developing immune therapies[114].

The quorum sensing signal molecules (QSSMs), *N*-(3-oxododecanoyl)-L-homoserine lactone (OdDHL) **4.1** and 2-*n*-heptyl-3-hydroxy-4(1*H*)-quinolone (PQS) **4.2**, produced by the opportunistic human pathogen *Pseudomonas aeruginosa*, have been found to modulate the immune system in distinctly different ways[118].



4.1



4.2

While an initial structure activity relationship (SAR) study has been undertaken using OdDHL as a lead molecule[114], no such information regarding PQS is known. Its ability to potently inhibit the immune response warrants this area being investigated.

4.2 Structure activity relationship studies

The aim of a structure activity relationship (SAR) study is to establish which parts of the molecule are important to biological activity. In order to carry out an SAR study a lead molecule is first identified and then a number of analogues that vary slightly from the lead molecule are synthesised. These can then be assessed with regards to their biological activity and compared to the lead molecule.

Drug molecules will interact with their target receptor through binding interactions such as ionic bonding, van der Waals interactions, hydrogen bonding and dipole-dipole interactions. The functional groups present on the molecule can possibly engage in these types of interactions but these may not occur in practice. A method for establishing the importance of the potential interactions is to alter the structure e.g. by converting a hydroxyl into either an ether or ester. If the activity remains similar to the lead molecule, the group altered does not play a large role in the interactions but if the activity is reduced then it can be concluded that the group has a major influence.

In order to ascertain what functional units within the PQS structure were important for its binding to immunophilin targets it is essential to carry

out a SAR study. The areas that might be considered to have potential for binding to a receptor in PQS are highlighted in Fig. 4.1. The alkyl chain (**A**), substitution at the 3-position (**B**), the substitution on the nitrogen (**C**) and the possible introduction of substitution onto the carbocyclic ring (R) are all possible areas of exploitation in an SAR study.

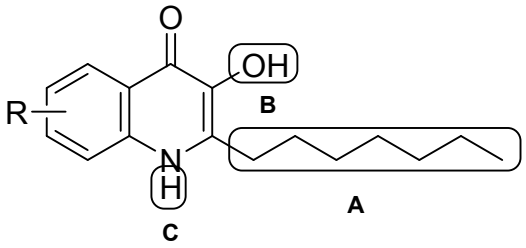


Figure 4.1 Potential areas of alteration in SAR study of PQS

A number of the PQS analogues produced were assayed to ascertain their activity in relation to the inhibition of T cell proliferation. In order to gain an accurate appreciation of this effect it was important to also ascertain their cytotoxicity. These assays are outlined below. Through these assays it is possible to gain an idea of what areas of the PQS molecule are important for its action as an immune modulator and begin to optimise the structure. The results obtained from the murine splenocyte proliferation assay do not correlate directly with the compounds' cellular physiological activity; rather it is an indicator of pharmacological activity. The term EC_{50} represents the plasma concentration required for obtaining 50% of a maximum effect *in vivo*.

4.2.1 Proliferation Assay

The concanavalin A (ConA) mitogen-stimulated proliferation of murine spleen cells was used to assess the effect of the PQS analogues on T cell activation and proliferation. Proliferation was assessed by the incorporation of [^{3}H]thymidine into DNA. Eight-week-old female mice were obtained from Harlan (Biscester, Oxon, United Kingdom) and given food and water ad libitum. Spleen cell suspensions were prepared by removing the spleens and placing them into Hanks' balanced salt solution (HBSS). The spleens were chopped with scissors and forced through a Falcon 70μm pore size nylon cell

strainer (2350; Beeton Dickinson, Franklin Lakes, N.J.) with the plunger from a 5 ml syringe to produce a single cell suspension. Pellets of the cells were produced by centrifugation and erythrocytes were lysed using 0.017 M Tris-0.144 M ammonium chloride, pH 7.2. Leukocytes were washed with HBSS twice and resuspended in complete cell culture medium (CTCM). The cell suspension was incubated for 48 h at 37°C in 5% CO_2/air in the presence of the PQS analogues and ConA, which stimulates the proliferation of the cells. Following this time, 20 µl of [^{3}H]thymidine in CTCM was added and the mixture incubated for a further 24 h. Cells were harvested onto fibreglass filters with a Packard filtermate 196 harvester. The [^{3}H]thymidine is incorporated into any proliferating leukocytes and, following harvesting and the addition of 20 µl scintillation fluid (Microscint-O), the proliferation can be quantified using a Packard Topcount scintillation counter.

The compounds were tested in duplicate (n = 2) over a concentration range of 0.3 to 1000 µM with the EC_{50} calculated from the resulting dose-response curves. The dose-response curve for PQS is shown below (figure 4.2). Wells containing DMSO alone provided a control and the solvent was found to have a negligible effect on inhibition of proliferation.

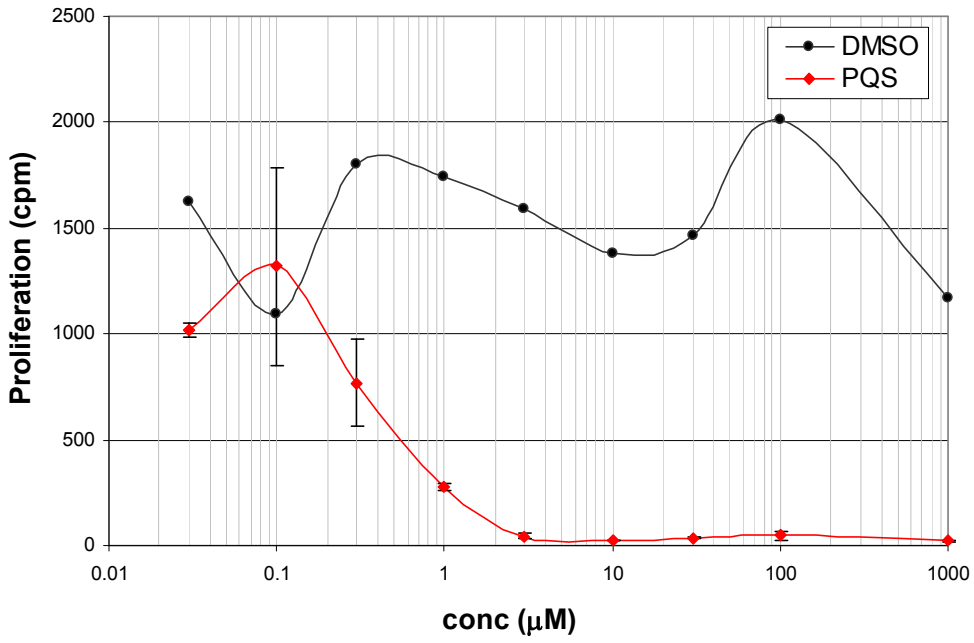


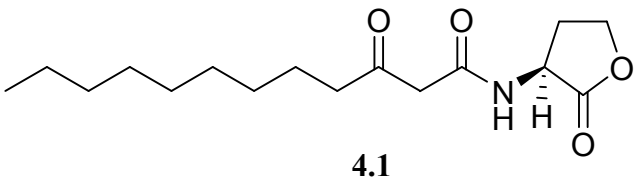
Figure 4.2 The effect of PQS and DMSO on the ConA stimulated proliferation of murine splenocytes

4.3 SAR studies

Systematic alterations to the areas indicated in Fig. 4.1 were carried out to obtain new analogues of PQS.

4.3.1 Alteration of side chain

It can be seen from evidence reported previously that the length of an alkyl chain can influence the pharmacological activity of a molecule. A related example to illustrate this is the difference in antiproliferative activity between OdDHL **4.1** and OHHL **4.3** [56,81]. The only difference between the two molecules is alkyl chain length, yet OdDHL possesses a potent antiproliferative activity in a murine splenocyte assay, whereas OHHL is inactive. An alkyl chain will aid in the lipophilicity of a compound, the longer chain providing increased lipophilicity.



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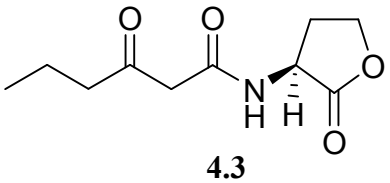


4.3

In relation to altering the alkyl side chain of the original PQS structure, three structures were tested. These included 2-methyl-3-hydroxy-4(1*H*)-quinolone and 2-*n*-pentyl-3-hydroxy-4(1*H*)-quinolone produced in the course of this study, via the synthetic methods discussed in Chapter 2. Additionally, 2-*n*-

nonyl-3-hydroxy-4(1*H*)-quinolone[1] was tested, in order to allow the comparison of both shortening and lengthening the alkyl chain. The results are summarised in Table 4.1.

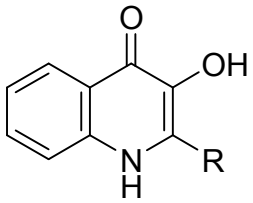


Table 4.1 Immunological data for compounds with alkyl chain length alterations

Compound	***R***	***EC_{50} (μM)***
2.143 (PQS)	C_7H_{15}	0.082
2.141	CH_3	15.5
2.142	C_5H_{11}	0.9
4.4	C_9H_{19}	0.023

It is clear that the alkyl chain is important, due to the drop in activity with **2.141**. The results show that the molecule can tolerate a shortening or lengthening of the alkyl side chain and still retain a useful activity. The optimum chain length of those compounds tested appears to be C_9.

The dose-response curves shown in figure 4.3 demonstrate the similarity in activity of PQS and 2-*n*-nonyl-3-hydroxy-4(1*H*)-quinolone **4.4**.

[1] Kindly donated by Mr Alex Truman of the Institute of Infection, Immunity and Inflammation, University of Nottingham

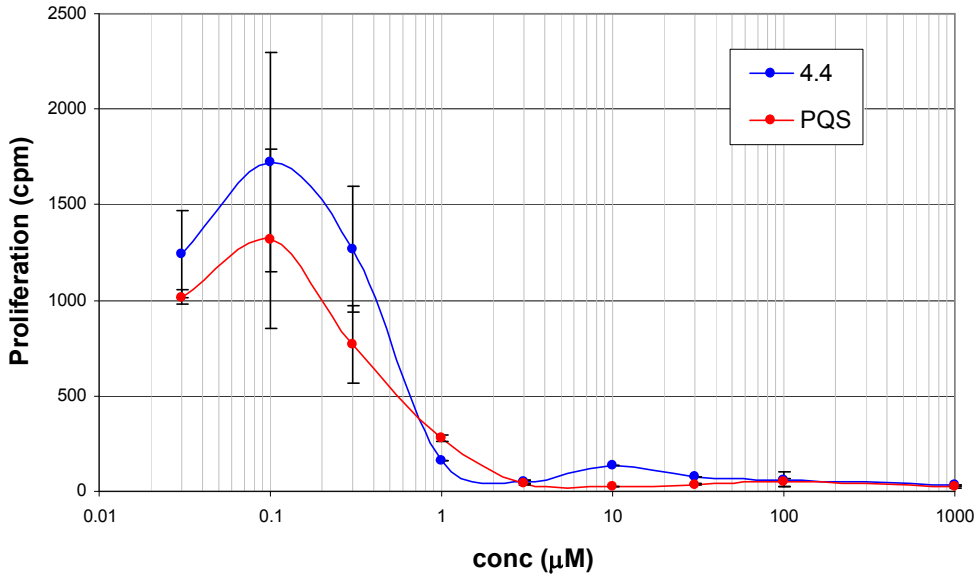
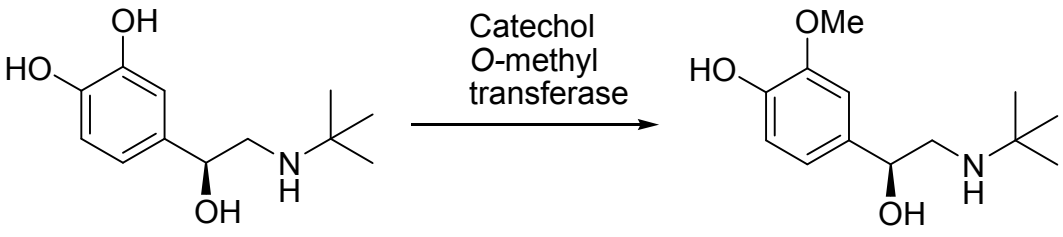


Figure 4.2 Influence of PQS and 2-*n*-nonyl-3-hydroxy-4(1*H*)-quinolone **4.4** on ConA mitogen-driven proliferation of murine spleen cells

4.3.2 *N*-substitution

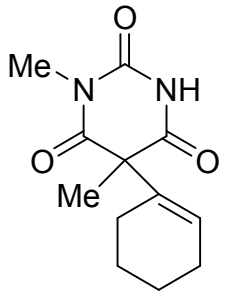
When the nitrogen is present on PQS and its derivatives is replaced with a methyl group it removes the capability for hydrogen bonding. A similar example to this can be seen when considering terbutaline **4.5**, where its anti-asthmatic, β_2-agonist activity is lost when the molecule is metabolised to its methyl ether[198] **4.6**, as seen in scheme 4.1.



4.5 **4.6**

Scheme 4.1 Metabolism of noradrenalin to its inactive methyl ether

Methylation also decreases the polarity of the molecule, thereby making it more lipophilic and enabling greater membrane permeability. This is important in classes of drugs such as hypnotics and antiepileptics, such as hexabarbitone **4.7**.



4.7

The hydrogen being replaced by the methyl group will prevent any tautomerism occurring in the molecule and will therefore provide clues to which tautomer interacts more strongly with the receptor.

The *N*-methyl derivative shows a drop in activity compared with PQS. This decrease is not large and suggests that the hydrogen bonding interaction with the target, involving this area of the molecule, while useful, is not key to its activity. It also suggests that permeability of the molecule is important for its action.

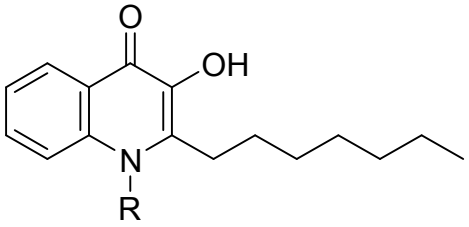
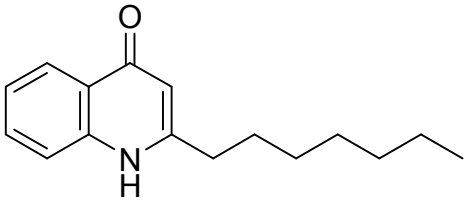


Table 4.2 Immunological data for *N*-methyl PQS

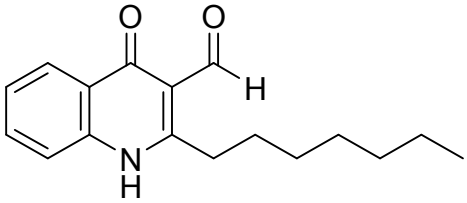
Compound	***R***	***EC_{50} (μM)***
2.143 (PQS)	H	0.082
2.145	CH_3	0.2

4.3.3 Alteration of the hydroxyl functional group

A hydroxyl groups present in a drug molecule can be commonly involved in hydrogen bonding with the target receptor. A hydroxyl group will act as a hydrogen bond donator. Converting a hydroxyl to an ether or an ester will change the nature of the bonding. An ester, for example, will act as a hydrogen bond acceptor, interacting if a hydrogen is present on the receptor, rather than a hydrogen bond donator in the vein of the hydroxyl. It had been seen previously that the immediate two precursors in the original synthetic route for PQS, 2-*n*-heptyl-4(1*H*)-quinolone **4.8** and 2-*n*-heptyl-3-formyl-4(1*H*)-quinolone **4.9**, did not have any activity in murine splenocyte antiproliferation assays. This suggests that the interaction of the hydroxyl group with the target is important.



4.8



4.9

The method chosen for this study was to replace the hydroxyl with an ester group at the 3-position. This was achieved in the production of **2.107** and **2.104**, having the ester as a lone alteration to structure and in combination with the reduction of the side chain, along with a 3-carboxylic acid derivative **2.109**.

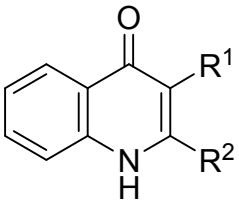


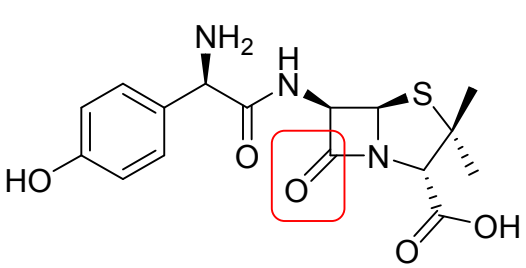
Table 4.3 Immunological data for compounds with altered 3-substitution

Compound	R^1	R^2	EC_{50} *(μM)*
2.143 (PQS)	OH	C_7H_{15}	0.082
2.107	$COCH_3$	C_7H_{15}	2.9
2.104	COC_2H_5	CH_3	199.0
2.109	COOH	C_7H_{15}	12.4

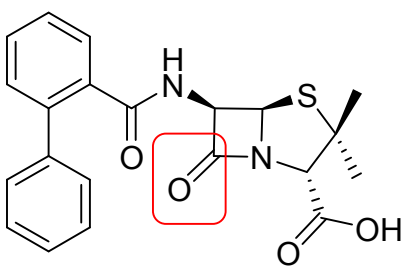
While there is the potential for hydrogen bonding with an ester the nature of this bond will be different. When a hydroxyl group is present, this acts as a hydrogen bond donator, whereas the ester will act as a hydrogen bond acceptor. The results show that the activity drops when the hydroxyl is replaced with an ester **2.107**. The activity also drops when a carboxylic acid, another hydrogen bond acceptor, is present at the 3-position **2.109**. These results suggest that the nature of the hydrogen bonding at this position is an important consideration, with hydrogen bond donators allowing a stronger interaction between the receptor and the molecule. Compound **2.104**, where the ester at the 3-position is present with a methyl at the 2-position, confirms that the 2-alkyl chain is important for activity.

4.3.4 Additions and insertions involving the carbocyclic ring

Lead molecules do not always interact with all possible binding areas of the receptor and also may not be chemically optimal in terms of solubility and lipophilicity. The addition of extra functional groups can allow the interaction with areas of the binding site that are not involved with the lead molecule and also the addition of groups to affect the polarity of the molecule will have an effect on the solubility. Additional groups may also affect the stability of molecules which may take the form of increased resistance to hydrolysis or the combating of enzymes that metabolise the drug. This can be seen when comparing amoxicillin **4.10** with diphenicillin **4.11**. Amoxicillin is susceptible to β-lactamase enzymes and the addition of an additional phenyl group sterically inhibits the access of these enzymes to the β-lactam ring and therefore limiting the enzyme's hydrolytic activity[198].



4.10



4.11

The effect of additions to the carbocyclic ring were investigated initially by the addition of a chlorine at the 6-position in the quinolone structure. Chlorine substitution has been shown to affect binding and metabolism in drug molecules[199]. The activity for the 6-chloro compound is lower than that of PQS but is still useful, showing a tolerance for this nature of substitution.

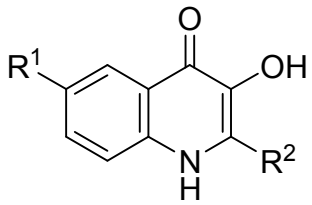


Table 4.4 Immunological data for compounds with chloro substitution

Compound	R^1	R^2	EC_{50} *(μM)*
2.143	H	C_7H_{15}	0.082
2.144	Cl	C_7H_{15}	0.6

4.3.5 Heterocyclic ring structure

The introduction of additional heteroatoms into a drug molecule can bring about changes in its physiochemical properties[148]. The addition of an extra heteroatom into the heterocyclic ring was achieved by introducing a nitrogen at the 3-position, giving rise to the synthesis of a number of quinazolinone structures.

The quinazolinone series demonstrated less activity than that of the equivalent quinolone molecules, PQS having an EC_{50} of 0.98 μM, although the activity was better than OdDHL (EC_{50} of 4.5 μM) in the case of the 3-amino derivatives.

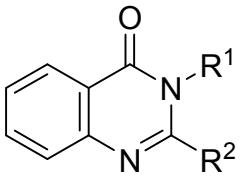


Table 4.5 Immunological data for compounds with additional heterocyclic atoms present in the ring system

Compound	R^1	R^2	EC_{50} *(μM)*
3.85	NH_2	C_7H_{15}	2.2
3.87	OH	C_7H_{15}	10.8
3.91	NH_2	CH_3	400.0
3.88	NH_2	C_5H_{11}	5.5
3.89	NH_2	C_9H_{19}	9.3

The alkyl chain length is important, demonstrated by the lack of activity of **3.91** which possesses a reduced alkyl chain, compared with that of **3.85**. A C_7 chain shows the best activity with a drop in activity when going to C_5 and C_9. This contrasts with the results for the 4-quinolones, where the activity was slightly raised with a longer alkyl chain.

Interestingly, the replacement of OH with NH_2 appears to increase potency, as shown by comparing **3.87** and **3.85**. NH_2 is an isostere of OH and allows the retention of hydrogen bonding, without changing other factors greatly, such as the steric effects. This difference in activity can also be seen in comparing the dose response curves from the murine splenocyte assay, figure 4.3.

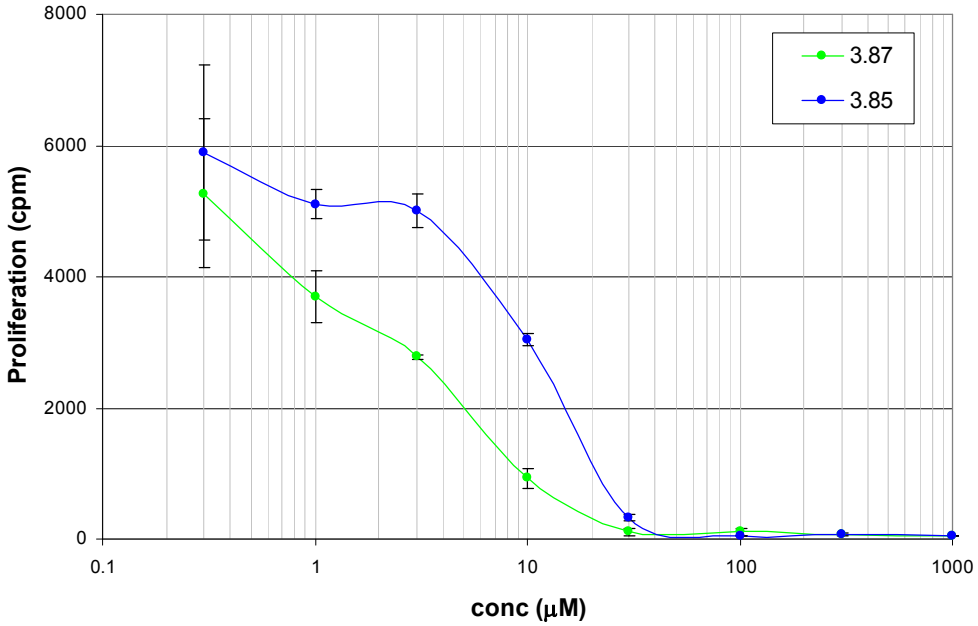
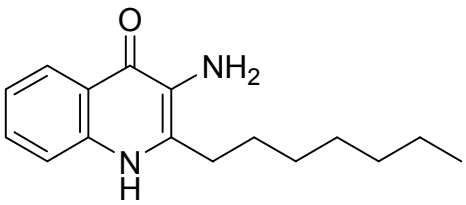


Figure 4.3 Influence of 3-substituent on the quinazolinone ring system. 2-*n*-Heptyl-3-amino-4(3*H*)quinazolinone **3.87** shows a greater activity than 2-*n*-hydroxy-3-amino-4(3*H*)quinazolinone

If these results transfer over to the quinolone series of molecules it suggests that the production of 2-*n*-heptyl-3-amino-4(1*H*)-quinolone **4.12** would offer promising results.



4.12

The introduction of chlorine onto the carbocyclic ring was also investigated by producing 6-chloro-2-heptyl-3-amino-4(3*H*)-quinazolinone and this can be compared to **3.85** to see the effect on biological activity.

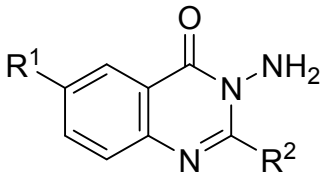


Table 4.5 Immunological data for chloro substituted quinazolinone

Compound	R^1	R^2	EC_{50} *(μM)*
3.85	H	C_7H_{15}	2.2
3.90	Cl	C_7H_{15}	20.3

It can be seen that the introduction of chlorine onto the carbocyclic ring is not well tolerated and causes a ten-fold drop in activity. This contrasts with the 4-quinolone series of molecules, where antiproliferative activity was not adversely affected by the introduction of chlorine onto the carbocyclic ring.

4.4 Toxicity Assay

The cytotoxicity of the PQS analogues towards murine spleen cells was assessed by dye exclusion after 24h. The murine spleen cells were incubated with the compounds for 24 h at 37°C in 5% CO_2/air. The cells were then stained with 0.4% trypan blue, a dye that is incorporated into dead cells, leaving viable cells unstained. The viable cells are then counted. This is an improvement over lactate dehydrogenase (LDH) release, a method that has been used in this area previously, as the assay can also detect cytostatic compounds. The toxicity value relates to the concentration at which toxicity is first observed. There is also the possible cytostatic (S) effect observable in the compounds too.

The toxicity data is important to provide an idea of the influence, initially, on cell proliferation in the murine splenocyte assay and also in future considerations to do with the compounds potential use as a drug molecule. The ideal result regarding the toxicity assay is for a compound to be non-toxic (NT) but if this is not the case, a large window exists between the EC_{50} and the concentration at which the compound is toxic. This allows the evaluation of the potential for assessment of what is known as therapeutic window, if the two

concentrations above are too close there is the increased possibility of toxic effects being observed if it was used as a drug molecule. The results of the toxicity assay are shown in table 4.6 in comparison to the EC_{50}.

Table 4.6 Toxicity data for PQS analogues. Some compounds are non-toxic (NT) while others demonstrated a possible cytostatic (S?) effect

Compound	*EC_{50} (μM)*	*Toxicity (μM)*
2.141	15.5	NT
2.142	0.9	S
2.144	0.6	40 S?
2.145	0.2	10
2.104	199.0	750 S?
2.107	2.9	70
2.109	12.4	120
3.85	2.2	50
3.87	10.8	3.87
3.88	5.5	100
3.89	9.3	300
3.90	20.3	300
3.91	400.0	NT
4.4	0.13	600 S?

4.5 Summary of results and conclusions

From these results it can be seen that certain areas of the PQS molecule are important for biological activity, as well as the introduction of additional heteroatoms causing a reduction of activity;

- The alkyl chain present at the 2-position is important for activity, shown by the 2-methyl derivative having largely reduced activity. The molecule can tolerate changes of chain length by either addition or subtraction of two carbons to the chain.

- The proton on the nitrogen does not appear to have a large influence on activity.
- The polarity and lipophilicity of the molecule has an influence on its activity.
- The 3-hydroxyl functionality is important, as demonstrated by the observation that the precursors in the synthetic route lacking this group were inactive. There is the possibility for some alteration of the functionality at the 3-positon, such as the use of a methyl ester, while retaining a useful amount of activity but the introduction of an acid reduces activity even more. The concomitant reduction in size of the 2-alkyl chain reduces activity greatly.
- The 4-quinazolinone structures are roughly ten times less potent than that of the 4-quinolones but still retain a useful level of activity, comparable to that of OdDHL
- The production of 2-alkyl-3-amino-4(1*H*)-quinolones may provide potent biologically active molecules.

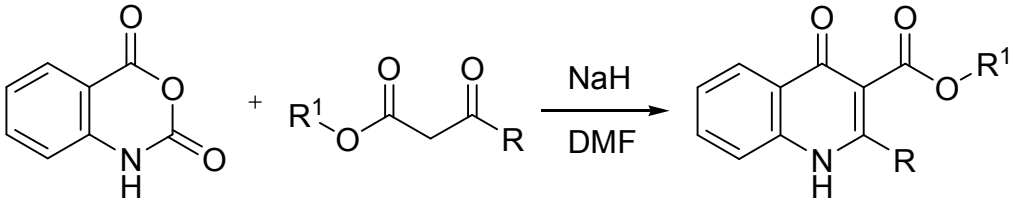
Chapter 5

Conclusion and Future Work

5.1 Conclusion

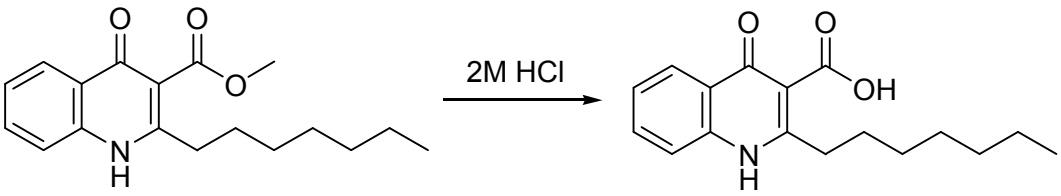
This project has demonstrated how the synthesis of analogues of 2-n-heptyl-3-hydroxy-4(1*H*)-quinolone (PQS) may be achieved in a number facile synthetic routes, allowing the exploitation certain areas of the molecule. These areas include the 2-alkyl chain, 3-position substitution, *N*-substitution, substitution on the carbocyclic ring and the introduction of additional heteroatoms into the molecule

2-Alkyl-4(1*H*)-quinolones with ester functionality at the 3-position can be synthesised from isatoic anhydride and a β-keto ester in one step. There is the ability to alter both the 2-alkyl chain and the ester by changing the β-keto ester. This reaction produces the quinolone without the need for purification.



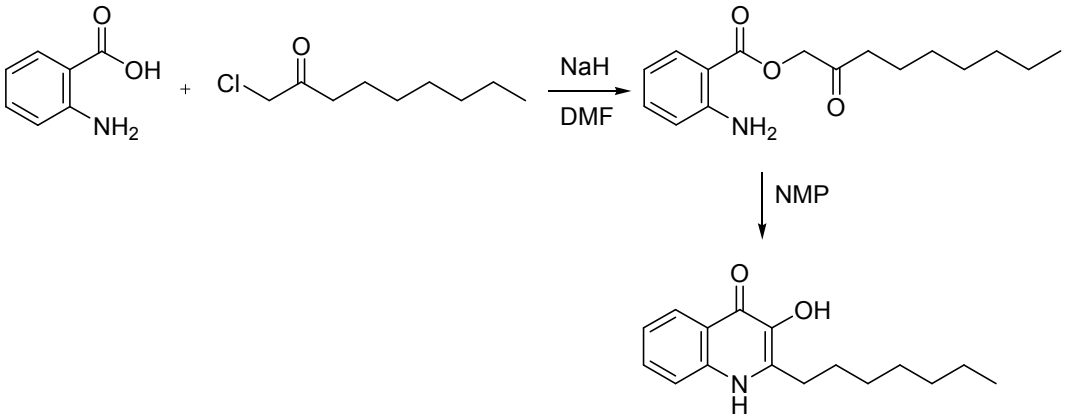
Scheme 5.1 Formation of 2-alkyl-3-ester-4(1*H*)-quinolones

The ester at the 3-position can be transformed into a carboxylic acid.



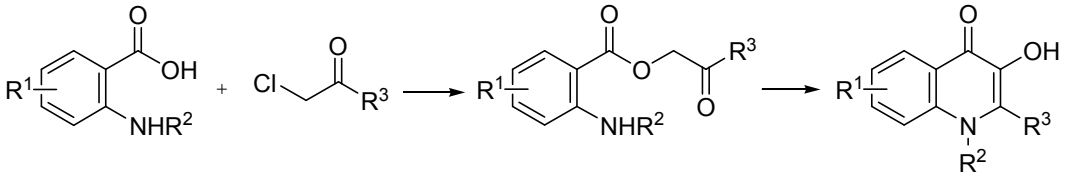
Scheme 5.2 Conversion of ester into carboxamide

PQS can be synthesised by the alkylation of anthranilic acid and cyclisation with NMP in a facile, two step synthesis.



Scheme 5.3 Facile two step synthesis of PQS

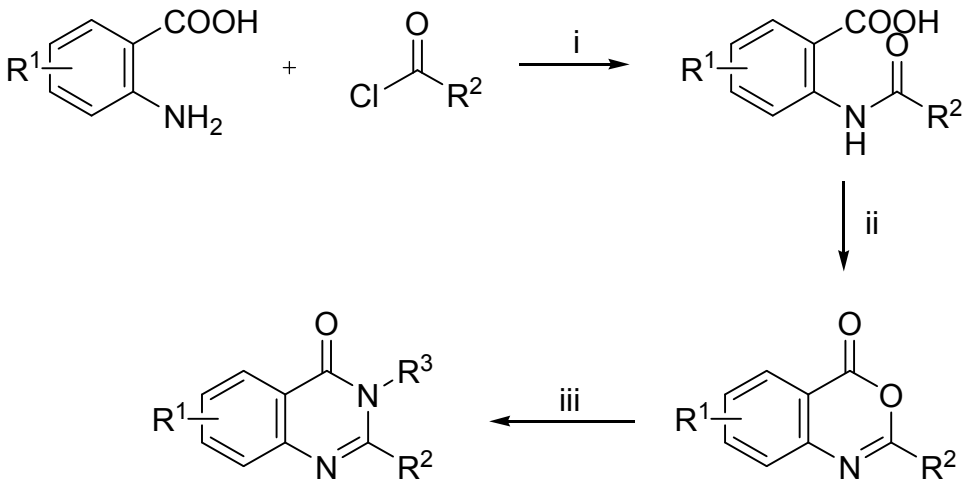
The method allows quick and simple synthesis of a number of analogues by simply employing appropriate starting materials. Diversity can be introduced, at the 2-position, on the nitrogen and also in the carboxylic ring.



Scheme 5.4 General synthesis of PQS analogues

These synthetic routes have enabled the production of 4-quinolone molecules with single or combined systematic alterations made at each of the areas targeted in the aims and objectives of the project.

A number of substituted 2-alkyl-3-substituted quinazolinones were produced using a facile three step synthetic route.



Reagents and conditions: (i) NaOH, H_2O; (ii) Ac_2O, Δ; (iii) R^3NH_2, EtOH, Δ

Scheme 5.5 Facile three step synthesis of 2-alkyl-3-substituted quinazolinones

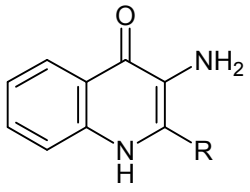
This allows the introduction of a variety of substituents into the core structure of the quinazolinone in a quick and simple way.

These molecules were assessed for immune modulatory activity in a murine splenocyte assay. From this it was seen that certain areas of the PQS molecule are important for biological activity and that the introduction of additional heteroatoms caused a reduction in activity.

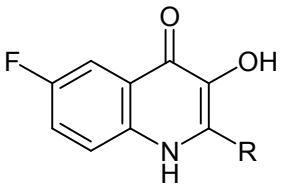
The alkyl chain present at the 2-position is important for activity, shown by 2-methyl-3-hydroxy-4(1*H*)-quinolone having greatly reduced activity. The molecule can tolerate changes of chain length by either addition or subtraction of two carbons to the chain. The proton on the nitrogen does not have a large influence on activity, due to the better activity seen with the *N*-methyl derivative. The polarity and lipophilicity of the molecule influences its activity. The 3-hydroxy group is important for activity but there is the possibility for some alteration of the substitution at the 3-positon, such as the use of a methyl ester, while retaining an acceptable amount of activity but the introduction of an acid reduces activity further. The 4-quinazolinone structures are roughly ten times less potent than that of the 4-quinolones but still retain a useful level of activity, comparable to that of OdDHL.

5.2 Future Work

Future work in this area would include the production of additional analogues containing further alteration of the 2-alkyl chain. These alterations would include further alterations to length, reduction of flexibility by producing unsaturated chains and substitution on and within the alkyl chain. Further substitution at the 3-position would include the production of 2-*n*-heptyl-3-amino-4(1*H*)-quinolone **5.1**, as the results for the 3-amino quinazolinones suggest this would possess potent activity. Substitution of further groups around the carbocyclic ring would involve the introduction of both electron withdrawing, such as fluorine **5.2**, and donating groups, such as a methoxy **5.3**, in order to assess their effect on activity. These groups would also be rotated round the carbocyclic ring.



5.1



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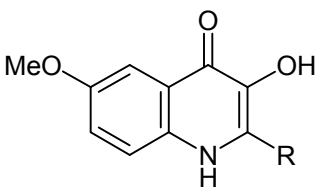


5.3

Analogues which yielded promising results in the initial murine splenocyte assays would be taken on to testing with human PBMC cells in order to more fully see the potential effects on human immune response.

The identification of a target immunophilin for PQS, potentially via the use of affinity matrices, would be of great value. The identification of a target would allow a more targeted approach to analogue design through the use of molecular modelling.

Chapter 6

Experimental Section

6.1 Materials

Starting materials were purchased from Aldrich Chemical Co., Dorset and Lancaster Synthesis Ltd., Morecambe. Deuterated chloroform and deuterated dimethylsulfoxide were purchased from Goss International Ltd., England. Methanol-d_4 was purchased from Cambridge Isotope Laboratories, Andover. The solvents used were HPLC grade. Ethanol was dried by refluxing with magnesium turnings and iodine ($Mg(OEt)_2$), redistilled and stored over molecular sieves (3 A 1-2 mm beads).

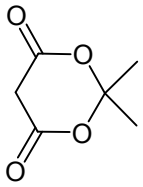
6.2 General analytical procedures

Melting points were determined on a Kofler Hot Stage and are uncorrected. ^1H NMR and ^{13}C NMR spectra were recorded on a Bruker AMX-250 spectrophotometer operating at 250 MHz, using tetramethylsilane as an internal standard, and 62.9 MHz respectively. Chemical shifts are reported relative to an internal $CDCl_3$ standard on a broad band decoupled mode, and assignments were done using a DEPT pulse sequence. Infrared spectra through the range 4000-600 cm^{-1} were obtained with KBr discs or as thin films using an Avatar 360 Nicolet FT-IR spectrophotometer. Elemental analyses were obtained on a Perkin-Elmer 240 D elemental analyser.

Thin-layer chromatography (TLC) was preformed using Merck silica gel 60 GF_{254} precoated (0.2 mm) alumina plates. Preparative thin-layer chromatography (PLC) was preformed using Merck silica gel 60 GF_{254} coated (1.0mm) glass plates (20 cm x 20 cm). Column chromatography was preformed using Merck Kieselgel 60 (230-400 mesh) silica

6.3 Chapter 2

2,2-Dimethyl-1,3-dioxane-4,6-dione (Meldrum's Acid) (2.61)

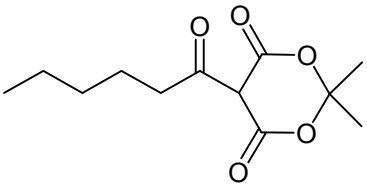


Concentrated H_2SO_4 (0.4 mL) and acetone (10 mL) were added to a stirred solution of malonic acid (13.0 g, 0.125 mol) in acetic anhydride (15 mL). The solution is stirred at room temperature for 1 h Scratching then caused the product to precipitate, which was subsequently filtered under suction, washed with ice-cold water, and dried under vacuum in a dessicator to yield a white, crystalline solid.

Mp: 90-91 ^{o}C (Lit: 94-96 ^{o}C).

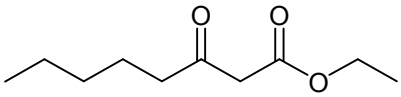
^{1}H NMR ($CDCl_3$) δ 1.80 (6H, s, CH_3), 3.61 (2H, s, $COCH_2CO$)

5-Hexanoyl meldrum's Acid (2.67)



N, N'-Dicyclohexylcarbodiimde (0.454 g, 2.2 mmol) was added to a stirred solution of hexanoic acid (2.32 g, 2 mmol) and 4-dimethylaminopyridine (0.269 g, 2.2 mmol) in dry dichloromethane (30 mL). The mixture was stirred at room temperature for 1 h and Meldrum's acid (2 mmol) was added. The stirring was continued at room temperature overnight. The solvent was removed under reduced pressure and the residue redissolved in ethyl acetate and filtered. The filtrate was washed with 1 M HCl (2 x 20mL) and dried over $MgSO_4$. The solvent was removed under reduced pressure yielding a yellow oil, which was mixed with acetone (3 ml) and chilled in a fridge. The solid impurity was then removed by micro filtration and washed with cold acetone. The acetone was then evaporated under vacuum to yield the product as a yellow oil in 93 % yield. This was used without purification in the next step.

Ethyl 3-oxooctanoate (2.72)



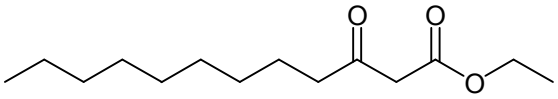
A solution of 5-hexanoyl Meldrum's acid (1.09g, 4.24 mmol) in dry ethanol (30 ml) was heated under reflux overnight. The solvent was evaporated under vacuum and residue redissolved in ethyl acetate. The solution was sequentially washed with a saturated solution of sodium bicarbonate, 1 M $KHSO_4$ and saturated brine solution. Drying over $MgSO_4$ and concentration under vacuum yielded the β-keto ester as an oil. The product was then purified using column chromatography, producing a 63 % yield.

TLC; R_f = 0.25 (10 % ethyl acetate, hexane).

^{1}H NMR ($CDCl_3$) δ 0.77 (3H, t, *J* = 1.97 Hz, 8-H CH_3), 1.16 (7H, m, $OCH_2\mathit{CH_3}$, 7-H & 6-H), 1.48 (2H, quintet, *J* = 14.52 Hz, $COCH_2\mathit{CH_2}$), 2.42 (2H, t, *J* = 7.28 Hz, 4-H), 3.31 (2H, s, $COCH_2CO$), 4.06 (2H, m, $O\mathit{CH_2}CH_3$)

^{13}C NMR ($CDCl_3$) δ 13.87, 14.10 (2 x CH_3), 22.39, 23.15, 43.00, 49.33, 53.41, 61.33 (6 x CH_2), 167.28, 202.99 (2 x 4°)

Ethyl 3-oxododecanoate

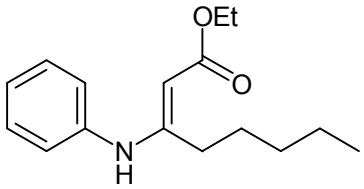


Yield = 79 %.

TLC; R_f = 0.29 (10 % ethyl acetate, hexane).

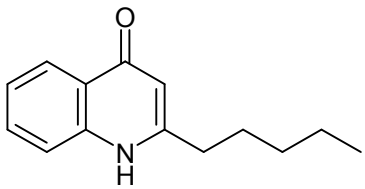
^{1}H NMR ($CDCl_3$) δ 0.84 (3H, t, *J* = 6.87 Hz, 12-H), 1.22 (15H, m, $OCH_2\mathit{CH_3}$ & 11-H to 6-H $(CH_2)_6$), 1.82 (2H, m, 5-H), 2.49 (2H, t, *J* = 7.43 Hz, 4-H), 3.40 (2H, s, 2-H), 4.13 (2H, m, $CO\mathit{CH_2}CH_3$)

Ethyl 3-anilino-2-octanoate (2.58)



A solution of aniline (0.354 g, 3.8 mmol), ethyl 3-oxooctanoate (0.92 g, 3.8 mmol) and toluene-*p*-sulfonic acid (30 mg) in dry benzene (40 mL) was heated under reflux for 24 h using Dean-Stark apparatus for the azeotropic removal of water. The solution was filtered to remove the toluene-*p*-sulfonic acid and solvent was removed under vacuum. The product was yielded as an oil, which was used without purification in the next step.

2-Pentyl-4(1*H*)-quinolone (2.57)



The crude product of ethyl 3-anilino-2-octanoate (1.70 g, 6.5 mmol) was added drop wise to refluxing diphenyl ether (10 mL) and heated for 30 min. The solution was cooled to room temperature and then diluted with petroleum ether (b.p. 60-80 ^{o}C; 80 ml) until product began to precipitate out as a fine crystalline solid. The petroleum ether was decanted off and the solid was washed with further petroleum ether to remove any residual diphenyl ether. Product was a pale orange, crystalline solid in 50 % yield.

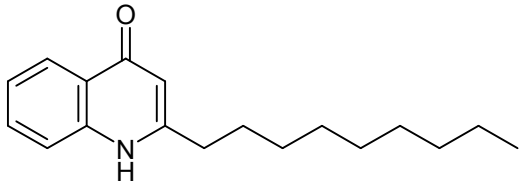
TLC; R_f = 0.46 (5 % methanol, DCM).

^{1}H NMR ($CDCl_3$) δ 0.94 (3H, t, *J* = 6.56 Hz, 5'-H CH_3), 1.34 (4H, m, $(CH_2)_2$), 1.86 (2H, m, 2'-H), 2.87 (2H, t, *J* = 7.74 Hz, 1'-H), 6.43 (1H, s, 3-H), 7.48 (1H, m, 8-H), 7.74 (1H, t, *J* = 7.51 Hz, 5-H), 8.05 (1H, d, *J* = 8.32 Hz, 7-H), 8.53 (1H, d, *J* = 8.04 Hz, 6-H), 13.18 (1H, s, NH)

^{13}C NMR ($CDCl_3$) δ 13.88 (CH_3), 22.34, 28.68, 31.28, 34.29 (4 x CH_2), 108.12 (Ar CH), 118.51, 123.59 (Ar 2 x CH), 124.89 (4°), 125.25, 131.79 (Ar 2 x CH), 140.56, 155.20, 178.79 (3 x 4°)

(MS-ES) *m/z* 216.12 (MH^+, $C_{14}H_{17}NO$ requires *m/z* 216.29)

2-Nonyl-4-(1*H*)-quinolone



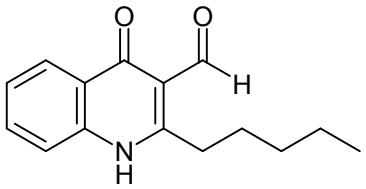
Yield = 76 %.

TLC; R_f = 0.23 (5 % methanol, DCM).

^{1}H NMR ($CDCl_3$) δ 0.99 (3H, t, *J* = 6.67 Hz, 9'-H CH_3), 1.32 (12H, m, $(CH_2)_6$), 1.87 (2H, m, 2'-H), 2.87 (2H, t, *J* = 7.12 Hz, 1'-H), 6.42 (1H, s, 3-H), 7.48 (1H, t, *J* = 7.27 Hz, 6-H), 7.74 (1H, t, *J* = 7.37 Hz, 8-H), 8.02 (1H, d, *J* = 8.17 Hz, 7-H), 8.53 (1H, d, *J* = 7.93 Hz, 5-H), 13.21 (1H, s, NH)

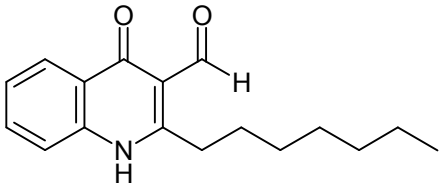
^{13}C NMR ($CDCl_3$) δ 14.10 (CH_3), 22.65, 24.90, 29.16, 29.27, 29.35, 29.46, 31.84, 34.40 (8 x CH_2) 108.09 (Ar CH), 118.66, 123.55 (Ar 2 x CH), 124.97 (4°), 125.19, 131.75 (Ar 2 x CH), 140.74, 155.48, 178.90 (3 x 4°)

3-Formyl-2-pentyl-4(1*H*) quinolone (2.56)[135]



A mixture of 2-pentyl-4(1*H*)-quinolone (0.2 g, 0.93 mmol) and hexamine (0.065 g, 0.465 mmol) in trifluroacetic acid (6 mL) was stirred under nitrogen at reflux for 27 h. Methanol (7.5 mL) and water (7.5 mL) were added and heating continued for 1 h. 3 M HCl (3 mL) was added and heating continued for 30 min. The mixture was allowed to cool. The precipitate formed on cooling was filtered under suction and washed with water. Product was then purified using column chromatography. This method proved unsuccessful in producing the required molecule.

3-Formyl-2-heptyl-4(1*H*) quinolone[137,138]



Method A

A solution of 2-heptyl-4(1*H*)-quinolone (121.5 mg, 0.5 mmol) and dimethylformamide (DMF) (46.05 mg, 0.63 mmol) in 1,2-dichloroethane (10 mL) was cooled to 0 ^{o}C. Phosphorus oxychloride (92.98 mg, 0.626 mmol) was added slowly with stirring. The mixture was then heated under reflux for 2 h. The solution was cooled, poured over crushed ice and neutralized with sodium acetate. The organic layer was separated and the aqueous layer extracted with ether. The organic phases were combined and washed with sodium hydrogen carbonate and dried over magnesium sulphate. The solvent was removed under vacuum to yield the product as a brown liquid. TLC; R_f = 0.76 (5 % methanol, DCM)

^{1}H NMR ($CDCl_3$) δ 0.79 (3H, t, *J* = 6.32 Hz, 7'-H CH_3), 1.25 – 1.53 (8H, m, $(CH_2)_4$), 1.66 – 1.77 (2H, m, 2'-H), 2.85 (2H, t, *J* = 7.72 Hz, 1'-H), 7.31 (1H, s, 3-H), 7.37 – 7.48 (1H, m, 7-H), 7.51 – 7.67 (1H, m, 6-H), 7.97 (1H, d, *J* = 8.419 Hz, 8-H), 8.08 (1H, d, *J* = 8.40 Hz, 5-H), 8.26 (1H, s, NH)

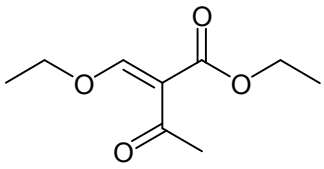
(MS-ES) *m/z* 261.98 & 263.91 (MH^+, $C_{17}H_{21}NO_2$ requires 272.16)

Method B

DMF (0.88 mmol) was cooled in an ice-salt bath and phosphorus oxychloride (0.22 mmol) was added with stirring over a period of approximately 30 min. To this a solution of 2-heptyl-4(1*H*)-quinolone (0.2 mmol) in DMF (0.3 mmol) was added over the period of about 1 h, ensuring that the temperature did not rise above 10 ^{o}C. This viscous solution was brought to 35 ^{o}C and was stirred for 1 h. Crushed ice is then added to the vessel, producing an aqueous solution. The solution was transferred to a flask containing crushed ice and to this was added an aqueous solution of sodium hydroxide with stirring. The solution was rapidly brought to its boiling point and allowed to cool to room temperature, after which it was placed in a refrigerator overnight. The aqueous phase was washed with ether and the solvent removed to yield a yellow oil.

Despite a difference in colour this was shown to be the same product as produced by first method attempted for the Vilsmeier by ^{1}H NMR and TLC. TLC; R_f = 0.76 (5 % methanol, DCM)

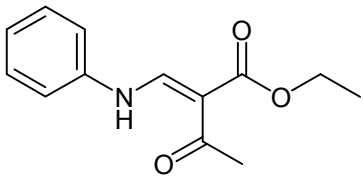
Ethyl-2-(ethoxymethylene)-3-oxobutanoate (2.87)



Ethyl acetoacetate (26.03 g, 0.2 mol), triethyl orthoformate (44.46 g, 0.3 mL) and acetic anhydride (60 mL) were mixed together and heated to 140 ^{o}C. The ethyl acetate formed during the reaction was distilled off and the reaction is stopped whenever no more ethyl acetate is produced. The product was then collected as a straw coloured oil using vacuum distillation, in a 72 % yield (26.76 g)

^{1}H NMR ($CDCl_3$) δ 1.27 – 1.43 (6H, m, 2 x $COOCH_2\mathit{CH_3}$), 2.35 (3H, apparent doublet, $COCH_3$), 4.18 – 4.34 (4H, m, 2 x $COO\mathit{CH_2}CH_3$), 7.66 (1H, apparent doublet, CH)

Arylaminomethylenemalonate (2.88)

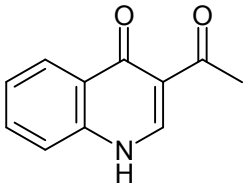


Ethyl-2-(ethoxymethylene)-3-oxobutanoate (11.20 g, 60 mmol) was added neat to aniline (5.20 g, 60 mmol), causing the evolution of heat. This was fitted with an air condenser heated at 130 ^{o}C for 6 hr. This was allowed to cool to room temperature and then further cooled to 0 °C, causing formation of the product, as an orange solid, in a 95 % yield (13.38 g).

TLC; R_f = 0.56 (50 % ethyl acetate, hexane).

^{1}H NMR ($CDCl_3$) δ 1.37 (3H, t, *J* = 7.12 Hz, $COCH_2\mathit{CH_3}$), 2.58 (3H, s, $COCH_3$), 4.29 (2H, q, J = 7.121 Hz, $CO\mathit{CH_2}CH_3$), 7.17 – 7.29 (3H, m, Ar), 7.40 – 7.45 (2H, m, Ar), 8.54 (1H, apparent doublet, CH), 12.79 (1H, apparent doublet, NH)

3-Acetyl-4(1*H*)-quinolone (2.89)



Method A

Methanolic sodium methoxide was prepared from sodium (0.29 g) and methanol (20 mL). Methyl 2-(3-oxobut-1-enylamino)benzoate (0.548 g, 2.5 mmol) was added and heated at reflux for 10 min. Following this the reaction mixture was poured into cold water (70 mL) and washed with dichloromethane (3 x 20 mL)[140]. The aqueous layer was then acidified causing the formation of a yellow precipitate. A suitable product was not able to be recovered from this.

Method B

Methyl 2-(3-oxobut-1-enylamino)benzoate (0.377 g, 1.7 mmol) was added to a suspension of sodium hydride (0.0413 g, 1.7 mmol) in dimethylformamide, an air condenser was fitted, and this was heated at 70 ^{o}C for 2h. DMF was removed and water was added and acidified with 2M HCl and extracted with ethyl acetate (2 x 20 mL). On removal of solvent a brown oil was left.

Method C

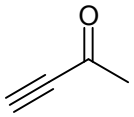
The arylaminomethylenemalonate **2.88** (1.123 g, 4.8 mmol) was added to boiling diphenyl ether (15 mL) and this was heated at reflux for 30 min. The solution was allowed to cool and petroleum ether (60 – 80 ^{o}C) added to precipitate crude product. This was further washed with petroleum ether (3 x 50 mL) producing the product as a beige solid in a 54 % yield (0.491 g)

^{1}H NMR ($CDCl_3$) δ 2.62 (3H, s, $COCH_3$), 7.43 – 7.47 (1H, m, 6-H), 7.63 (1H, dd, *J* = 0.38 & 8.20 Hz, 8-H), 7.71 – 7.75 (1H, m, 7-H), 8.24 (1H, dd, *J* = 1.17 & 8.09 Hz, 5-H), 8.52 (1H, s, 2-H), 12.52 (1H,br s, NH)

^{13}C NMR ($CDCl_3$) δ 36.24 (CH_3), 123.02 (4°), 123.40, 129.70, 131.07 (3 x aromatic CH), 133.20 (4°), 137.05, 144.02 (2 x CH), 148.78, 181.27, 202.58 (3 x 4°)

(MS-ES) *m/z* 188.07 (MH^+, $C_{11}H_9NO_2$ requires *m/z* 188.06)

But-3-yn-2-one (2.97)

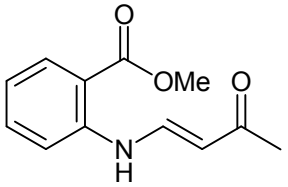


A solution of CrO_3 (10.1 g) in H_2SO_4/H_2O was added slowly over 30 min to a stirring solution of butyn-2-ol (5 ml, 68 mmol) in sulphuric acid/water (5.4 ml/ 18 ml) at 0 ^oC. The solution was stirred for 4 h, ensuring that the temperature did not rise above 10 ^oC, then dichloromethane (25 mL) added. The reaction mixture was washed with saturated sodium bicarbonate solution extracted with dichloromethane (2 x 20 mL) and dried over magnesium sulphate. The dichloromethane was distilled off, to leave the product as a clear, colourless solution.

^1H NMR ($CDCl_3$) δ 2.41 (3H, s, CH_3), 5.73 (1H, s, CH)

IR 1687 (C=O), 2097 (C≡O), 3287 (CH) cm^{-1}

Methyl 2-(3-oxobut-1-enylamino)benzoate (2.99)[141]



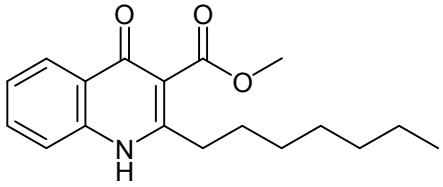
Methyl anthranilate (5.14 g, 34 mmol) and 3-butyn-2-one (2.31 g, 34 mmol) were dissolved in methanol, ensuring that the temperature did not rise above 20 ^oC. The solution was allowed to warm to room temperature and left to stand in a tightly stoppered flask for 20 h. Water was added dropwise following this time, forming an immiscible, brown liquid. When this brown layer was dried the product was formed as a crystalline solid in a10 % yield (0.775 g).

^1H NMR ($CDCl_3$) δ 2.22 (3H, s, CO*CH₃*), 4.02 (3H, s, COO*CH₃*), 5.40 (1H, d, *J* = 8.12 Hz, CO*CH*CHNH), 13.0913 (1H, br d, *J* = 10.80 Hz, NH)

^{13}C NMR ($CDCl_3$) δ 29.8 (CH_3), 52.31 (COO*CH₃*), 100.08 (CH), 113.59 (Aromatic), 115.72 (4°), 121.45, 132.117, 134.17 (3 x aromatic), 139.65 (NH*CH*CH), 142.81 (4°), 167.02 (*C*OOCH$_3$), 198.41 (CO)

(MS-ES) *m/z* 219.98 (MH^+, $C_{12}H_{13}NO_3$ requires *m/z* 220.09)

Methyl 2-heptyl-1,4-dihydro-4-oxo-3-carboxylate (2.107)



Ethyl 3-oxodecanoate (1.13 g, 5 mmol) was added to a suspension of sodium hydride (0.12 g, 5mmol) in dry DMF (20 mL), causing the liberation of hydrogen gas. To this a solution of isatoic anhydride (0.816 g, 5 mmol) in dry DMF was added drop wise and stirred under nitrogen overnight. After this, the solvent was removed under vacuum and the remaining solvent triturated with 1M HCl, yielding the product as a white solid.

Mp. 170 – 171 °C

IR 1725 (Ester C=O), 3272 (NH) cm^{-1}

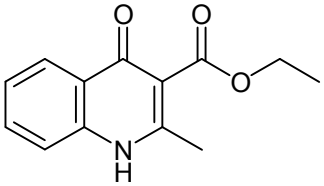
^{1}H NMR (DMSO) δ 0.86 (3H, t, *J* = 6.98 Hz, 7'-H), 1.25 – 1.31 (8H, m, $(CH_2)_4$), 1.65 (2H, quintet, *J* = 7.63 Hz, 2'-H), 2.62 (2H, t, *J* = 7.99 Hz, 1'-H), 3.35 (3H, s, COO*CH*$_3$), 7.33 – 7.37 (1H, m, 6-H), 7.57 (1H, d br, *J* = 7.57 Hz, 8-H), 7.66 – 7.70 (1H, m, 7-H), 8.05 (1H, dd, *J* = 1.22 & 8.08 Hz, 5-H), 11.80 (1H, s br, NH)

^{13}C NMR (DMSO) δ 14.40 (CH_3), 22.49, 28.70, 29.09, 29.33, 31.52, 32.36 (6 x CH_2), 52.20 (COO*CH*$_3$), 114.90 (4°), 118.90, 124.18 (2 x aromatic CH), 124.92 (4°), 125.42, 132.74 (2 x aromatic CH), 139.74, 153.02, 167.89, 174.10 (4 x 4°)

(MS-ES) *m/z* 302.18 (MH^+, $C_{18}H_{23}NO_3$ requires *m/z* 302.17)

Anal. calcd. for $C_{18}H_{23}ON$: C, 71.73; H, 7.69; N, 4.65. Found: C, 70.46; H, 7.36; N, 4.65.

Ethyl 1,4-dihydro-2-methyl-4-oxoquinoline-3-carboxylate (2.104)



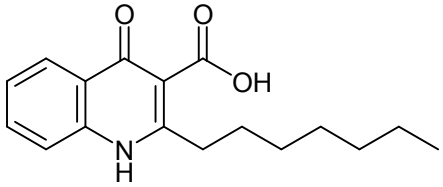
^{1}H NMR ($CDCl_3$) δ 1.27 (3H, t, *J* = 7.09 Hz, $COCH_2$*CH*$_3$), 2.39 (3H, s, CO*CH*$_3$), 4.23 (2H, q, *J* = 7.15 Hz, CO*CH*$_2$$CH_3$), 7.33 (1H, td, *J* = 0.88 & 7.91

Hz, Ar), 7.54 (1H, br d, *J* = 8.33 Hz, Ar), 8.05 (1H, dd, *J* = 1.23 & 8.06 Hz, 5-H)

^{13}C NMR ($CDCl_3$) δ 14.69 ($COOCH_2\mathit{CH_3}$), 21.22 (CH_3), 60.09 ($COO\mathit{CH_2}CH_3$), 113.88 (4°), 122.06, 122.65, 125.19 (3 x aromatic CH), 126.09 (4°), 130.92 (aromatic CH), 168.94, 173.39 (2 x 4°)

(MS-ES) *m/z* 232.11 (MH^+, $C_{13}H_{13}NO_3$ requires *m/z* 232.09)

2-Heptyl-1,4-dihydro-4-oxo-3-carboxylic acid (2.109)



Method A

2-Heptyl-4(1H)-quinolone-3-methyl ester (0.09 g, 3 mmol) was dissolved in hot methanol (12 mL), to this 1 M sodium hydroxide (3 mL) was added and heated overnight. Removal of some of the solvent caused the precipitation of a white solid, which was showed to be starting material by NMR.

Method B

2-Heptyl-4(1H)-quinolone-3-methyl ester was heated at reflux overnight in 2 M HCl. After the reaction mixture had cooled, the precipitate was collected under suction and dried over silica.

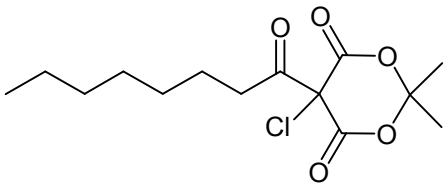
TLC; R_f = 0.54 (5 % methanol, DCM)

^{1}H NMR (DMSO) δ 0.86 (3H, t, *J* = 6.95 Hz, $(CH_2)_6\mathit{CH_3}$), 1.26 – 1.41 (8H, m, $(CH_2)_4$), 1.66 (2H, m, 2'-H), 2.63 (2H, t, *J* = 7.63 Hz, 1'-H), 7.57 (1H, m, 6-H), 7.78 (1H, br d, *J* = 8.04 Hz, 8-H), 7.88 (1H, m, 7-H), 8.25 (1H, dd, *J* = 1.29 & 8.17 Hz, 5-H), 13.04 (1H, s, NH)

^{13}C NMR (DMSO) δ 14.40 (CH_3), 22.52, 28.84, 29.60, 29.75, 31.61, 33.91 (6 x CH_2), 106.17 (4°), 119.22 (Ar CH), 123.42 (4°), 125.60, 126.30, 134.44 (3 x Ar CH), 138.62, 162.92, 166.76, 179.52 (3 x 4°)

(MS-ES) *m/z* 286.14 (MH^-, $C_{17}H_{21}NO_3$ requires *m/z* 286.15)

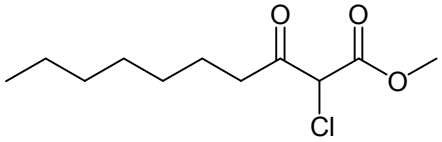
5-Chloro-5-Hexanoyl Meldrum's acid (2.126) [147]



5-hexanoyl meldrum's acid (0.750 g, 3.1 mmol) was heated at reflux in excess sulfuryl chloride (15 mL) for 2 h. The sulfuryl chloride was removed under vacuum to yield the product as a pale yellow oil.

^{1}H NMR ($CDCl_3$) δ 0.92 (3H, t, *J* = 6.80 Hz, $(CH_2)_5CH_3$), 1.35 (4H, m, $(CH_2)_2$), 1.67 (2H, m, CH_2CH_2CO), 1.85, 1.90 (6H, 2 x s, 2 x CH_3), 2.96 (2H, t, *J* = 7.20 Hz, CH_2CH_2CO)

Methyl 2-chloro-3-oxodecanoate (2.128)



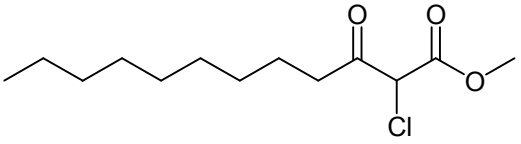
Sulfuryl chloride (0.615 mL) was added neat to methyl 3-oxodecanoate (1.5 g, 7.5 mmol) at 0 °C. This was then stirred at room temperature overnight. On removal of solvent the product was afforded as a yellow oil in a 95 % yield (1.68 g)

Yield: 95 %

R_f – 0.75 EtOAc/hexane 50/50

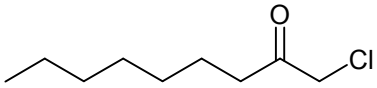
^{13}C NMR ($CDCl_3$) δ 14.07 (CH_3), 22.59, 23.48, 28.83, 28.95, 31.62, 38.99 (6 x CH_2), 53.73 ($COOCH_3$), 60.74 (COC(Cl)CO), 165.63(COOCH$_3$), 199.07 (COC(Cl))

Methyl 2-chloro-3-oxododecanoate



^{1}H NMR ($CDCl_3$) δ 0.90 (3H, t, *J* = 6.6 Hz, CH_3), 1.28 – 1.32 (12H, m, $(CH_2)_6$), 1.62 – 1.72 (2H, m, 5-H), 2.85 (2H, t, *J* = 7.20 Hz, 4-H), 3.94 (1H, s, 2-H)

1-Chlorononan-2-one (2.131)



Method A

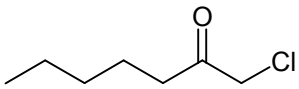
5-chloro-5-octanoyl-Meldrum's acid was heated at reflux in water for 2 h. Following this, there was extraction of the reaction mixture with ethyl acetate[147]. This method proved unsuccessful in producing the product in desired quality

Method B

Methyl 2-chloro-3-oxodecanoate (1.06 g, 6 mmol) was heated at reflux in a mixture of concentrated sulphuric acid (10 mL) and water (10 mL) for 2 h. The reaction mixture was extracted with dichloromethane and solvent removed under vacuum. This yielded the product as a yellow oil that was used without purification in the next step.

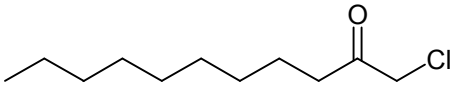
^{1}H NMR ($CDCl_3$) δ 0.90 (3H, t, *J* = 6.74 Hz, CH_3), 1.30 – 1.33 (8H, m, $(CH_2)_4$), 1.60 – 1.67 (2H, m, 4-H), 2.60 (2H, t, *J* = 7.36 Hz, 3-H), 4.01 (2H, s, 2-H)

1-Chloroheptan-2-one (2.130)



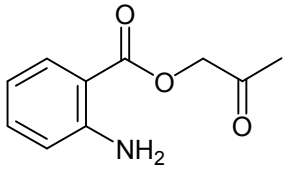
^{1}H NMR ($CDCl_3$) δ 0.95 (3H, t, *J* = 6.81 Hz, CH_3), 1.30 – 1.40 (4H, m, $(CH_2)_2$), 1.63 – 1.75 (2H, m, 4-H), 2.83 (2H, t, *J* = 7.43 Hz, 3-H), 4.38 (2H, s, 2-H)

1-Chloroundecan-2-one (2.132)



^{1}H NMR ($CDCl_3$) δ 0.90 (3H, t, *J* = 6.60 Hz, CH_3), 1.28 – 1.32 (12H, m, $(CH_2)_6$), 1.64 – 1.71 (2H, m, 4-H), 2.85 (2H, t, *J* = 7.27 Hz, 3-H), 3.94 (2H, s, 2-H)

2-Oxopropyl 2-aminobenzoate (2.112)[132]



To a solution of anthranilic acid (1.646 g, 12 mmol) in DMF (40 mL) was added potassium carbonate (1.176 g, 8.6 mmol) and this heated at 90 °C, with stirring for 1 h. After this time the solution was cooled to 20 °C, chloroacetone (0.924 g, 10 mmol) added, stirred at room temperature for 30 min and then at 50 °C for 30 min. The mixture was poured into iced water (approx. 100 mL), forming the product as a yellow solid (1.17 g, 61 %)

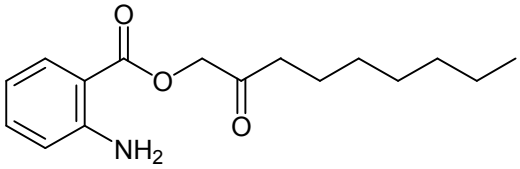
Mp. 77 – 78 °C

^{1}H NMR ($CDCl_3$) δ 2.26 (3H, s, CO*CH$_3$*), 4.85 (2H, s, COO*CH$_2$*CO), 5.72 (2H, s br, NH_2), 6.67 – 6.71 (2H, m, 4-H and 6-H), 7.28 – 7.34 (1H, m, 5-H), 7.96 (1H, dd, *J* = 1.73 & 8.35 Hz, 3-H)

^{13}C NMR ($CDCl_3$) δ 26.28 ($COOCH_2CO$*CH$_3$*), 68.33 (COO*CH$_2$*$COCH_3$), 109.66 (4°), 116.43, 116.74, 134.69 (3 x aromatic) 150.83(4°), 167.19(**C**$OOCH_2COCH_3$), 202.56($COOCH_2$*C*OCH_3)

(MS-ES) *m/z* 194.03 (MH^+, $C_{10}H_{11}NO_3$ requires *m/z* 194.07)

2-Oxononyl-2-aminobenzoate (2.136)



Mp. 59 °C

Yield – 75 %

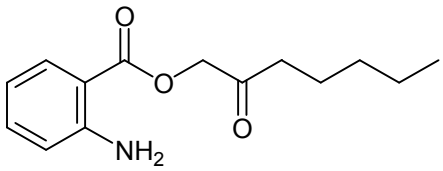
R_f – 0.65 EtOAc/hexane 50/50

^{1}H NMR ($CDCl_3$) δ 0.90 (3H, t, *J* = 6.74 Hz, CH_3), 1.25 – 1.34 (8H, m, $(CH_2)_4$), 1.62 – 1.70 (2H, m, $COCH_2$*CH$_2$*), 2.52 (2H, t, *J* = 7.49 Hz, CO*CH$_2$*CH_2), 4.85 (2H, s, COO*CH$_2$*CO), 5.71 (2H, s br, NH_2), 6.67 – 6.71 (2H, m, 4-H & 6-H), 7.29 – 7.34 (1H, m, 5-H), 7.96 (1H, dd, *J* = 1.71 & 8.33 Hz, 3-H)

^{13}C NMR ($CDCl_3$) δ 14.08 (CH_3), 22.60, 23.31, 29.03, 29.14, 31.68, 38.93 (6 x CH_2), 67.98 (COO*CH_2*CO), 109.81, 115.65 (2 x 4°), 116.41, 116.72, 131.41, 134.61 (4 x aromatic CH), 150.77 (4°), 167.20 (*C*OOCH$_2$CO), 204.86 (COOCH$_2$*C*O)

(MS-ES) *m/z* 278.17 (MH^+, $C_{16}H_{23}NO_3$ requires *m/z* 278.17)

2-Oxoheptyl-2-aminobenzoate (2.135)



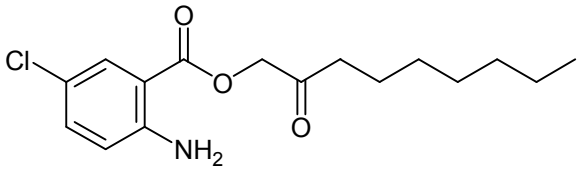
TLC; R_f = 0.61 (50 % ethyl acetate, hexane)

^{1}H NMR ($CDCl_3$) δ 0.92 (3H, t, *J* = 6.86 Hz, CH_3), 1.34 (4H, m, $(CH_2)_2$), 1.67 (2H, m, COCH$_2$*CH_2*), 2.52 (2H, t, *J* = 7.34 Hz, CO*CH_2*CH$_2$), 4.85 (2H, s, COO*CH_2*CO), 5.74 (2H, br s, NH_2), 6.69 (2H, m, Ar), 7.32 (1H, m, Ar), 7.96 (1H, dd, *J* = 1.62 & 8.32 Hz, 3-H)

^{13}C NMR ($CDCl_3$) δ 13.92 (CH_3), 22.44, 22.98, 31.34, 38.89 (6 x CH_2), 67.99 (COO*CH_2*CO), 109.08 (4°), 116.41, 116.73, 131.41, 134.62 (4 x aromatic CH), 150.77 (4°), 167.21, 204.87 (2 x CO)

(MS-ES) *m/z* 249.82 (MH^+, $C_{14}H_{17}NO_2$ requires *m/z* 250.14)

2-Oxononyl-2-amino-5-chlorobenzoate (2.137)



Mp. 91-93 °C

Yield – 69.7 %

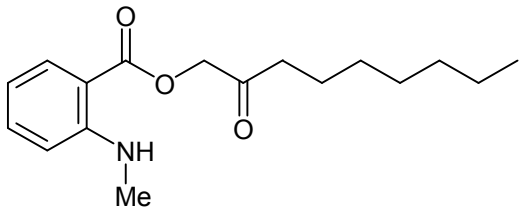
TLC; R_f = 0.64 (50 % ethyl acetate, hexane)

^{1}H NMR ($CDCl_3$) δ 0.89 (3H, t, *J* = 7.01 Hz, $(CH_2)_6$*CH_3*), 1.29 – 1.32 (8H, m, $(CH_2)_4$), 1.65 (2H, m, *CH_2*CH$_2$CO), 2.50 (2H, t, *J* = 7.47 Hz, CH$_2$*CH_2*CO), 4.86 (2H, s, COO*CH_2*CO), 5.74 (2H, s br, NH_2), 7.25 (1H, dd, *J* = 2.55 & 8.82 Hz, 5-H), 7.92 (1H, d, *J* = 2.53 Hz, 6-H), 7.96 (1H, br d, *J* = 2.52 Hz, 3-H)

^{13}C NMR ($CDCl_3$) δ 14.08 (CH_3), 22.60, 23.35, 29.02, 29.13, 31.63, 38.89 (6 x CH_2), 68.10 (COO*CH_2*CO), 110.54 (4°), 118.11 (Ar CH), 120.82 (4°), 130.55, 134.62 (Ar CH), 149.27, 166.26, 204.26 (3 x 4°)

(MS-ES) *m/z* 312.13 & 314.13 (MH^+, $C_{16}H_{22}NO_3$ requires *m/z* 312.13 & 314.13)

2-Oxononyl-2-(methylamino)benzoate (2.138)



Mp. 39 – 41 °C

Yield – 27.7 %

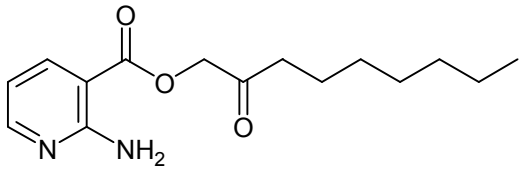
TLC; R_f = 0.7 (50 % ethyl acetate, hexane)

^{1}H NMR ($CDCl_3$) δ 0.89 (3H, t, *J* = 7.00 Hz, $(CH_2)_6$*CH_3*), 1.29 – 1.31 (8H, m, $(CH_2)_4$), 1.65 (2H, m, *CH_2*CH_2CO), 2.51 (2H, t, *J* = 7.38 Hz, CH_2*CH_2*CO), 2.51 (2H, t, *J* = 7.38 Hz, CH_2*CH_2*CO), 2.93 (3H, d, *J* = 5.04 Hz, NH*CH_3*), 4.82 (2H, s, COO*CH_2*CO), 6.62 – 6.66 (1H, m, 4-H), 6.70 (1H, d, *J* = 8.3 Hz, 6-H), 7.41 – 7.45 (1H, m, 5-H), 7.55 (1H, s br, *NH*CH_3), 8.00 (1H, dd, *J* = 1.59 & 8.03 Hz, 3-H)

^{13}C NMR ($CDCl_3$) δ 14.08 (CH_3), 22.61, 23.30, 29.04 (3 x CH_2), 29.14 (NH*CH_3*), 29.57, 31.64, 38.91 (3 x CH_2), 67.96 (COO*CH_2*CO), 108.85 (4°), 110.82, 114.48, 131.78, 135.18 (3x aromatic CH), 152.29, 167.72, 205.06 (3 x 4°)

(MS-ES) *m/z* 292.19 (MH^+, $C_{17}H_{25}NO_3$ requires *m/z* 292.18)

2-Oxononyl-2-aminopyridine-3-carboxylate (2.139)



Mp. 82 – 83 °C

Yield – 49.7 %

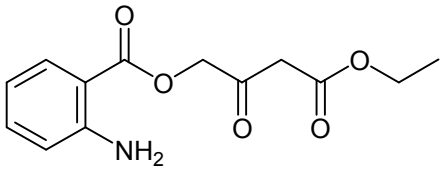
TLC; R_f = 0.45 (50 % ethyl acetate, hexane)

^{1}H NMR ($CDCl_3$): δ 0.89 (3H, t, *J* = 7.03, $(CH_2)_6CH_3$), 1.29 – 1.31 (8H, m, $(CH_2)_4$), 1.66 (2H, m, CH_2CH_2CO), 2.50 (2H, t, *J* = 7.38 Hz, CH_2CH_2CO), 4.88 (2H, s, $COOCH_2CO$), 6.67 (1H, dd, *J* = 4.76 & 7.80 Hz, 5-H), 8.24 (1H, dd, *J* = 1.8 & 7.82 Hz, 4-H), 8.27 (1H, m, 3-H)

^{13}C NMR ($CDCl_3$) δ 14.08 (CH_3), 22.60, 23.34, 29.02, 29.12, 31.62, 38.90 (6 x CH_2), 68.17 ($COOCH_2CO$), 105.43 (aromatic CH), 112.87 (4°), 140.46, 154.16 (2 x aromatic CH), 159.52, 166.15, 203.99 (3 x 4°)

(MS-ES) *m/z* 279.16 (MH^+, $C_{15}H_{22}N_2O_3$ requires 279.16)

3-(Ethoxycarbonyl)-2-oxopropyl-2-aminobenzoate (2.122)

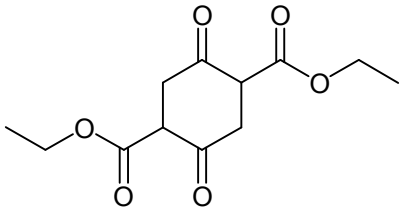


Yield – 31 %

^{1}H NMR ($CDCl_3$) δ 1.29 (3H, t, *J* = 7.14 Hz, $COCH_2CH_3$), 3.60 (2H, s, $COCH_2CO$), 4.22 (2H, q, *J* = 7.13 Hz, $COCH_2CH_3$), 4.97 (2H, s, $COOCH_2CO$), 5.72 (2H, s br, NH_2), 6.67 – 6.71 (2H, m, 4-H & 6-H), 7.30 – 7.34 (1H, m, 5-H), 7.93 (1H, dd, *J* = 1.69 & 8.46 Hz, 3-H)

^{13}C NMR ($CDCl_3$) δ 14.04 (CH_3), 46.22, 61.73, 67.81 (3 x CH_2), 116.43, 116.74, 131.31, 134.81 (3 x Ar CH), 150.92, 166.98, 167.50, 197.54, 206.00 (5 x 4°)

Diethyl 2,5-dioxocyclohexane-1,4-dicarboxylate (2.123)

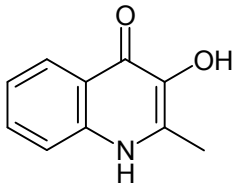


^{1}H NMR ($CDCl_3$) δ 1.34 (6H, t, *J* = 7.13 Hz, 2 x $COOCH_2CH_3$), 3.21 (4H, s, 2 x CH_2), 4.27 (4H, q, *J* = 7.14 Hz, 2 x $COOCH_2CH_3$), 12.22 (2H, s, 2 x CH)

^{13}C NMR ($CDCl_3$) δ 14.23 (2 x $COOCH_2CH_3$), 28.54 (2 x CH_2), 60.73 (2 x CH), 93.26 (2 x $COOCH_2CH_3$), 168.45, 171.30 (4 x 4°)

(MS-ES) *m/z* 255.08 (MH^-, $C_{12}H_{16}O_6$ requires *m/z* 255.25)

2-Methyl-3-hydroxy-4(*1H*)-quinolone (2.141)[132]



A solution of anthranilate (0.386 g, 2 mmol) in 1-methyl-2-pyrrolidinone (NMP) (2mL) was heated at reflux for 10 min. The reaction was then cooled, ethyl acetate (8 mL) added and stirred at 0 °C for 30 min. The product formed as a beige precipitate and was collected by filtration in a 73 % yield (0.255 g)

Mp. 296 – 299 °C (decomp)

TLC; R_f = 0.19 (5 % methanol, DCM)

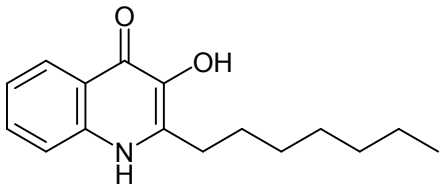
IR (KBr) 1639 (C=O), 2920 (NH), 3252 (OH) cm^{-1}

^{1}H NMR (DMSO) δ 2.37 (3H, s, CH_3), 7.22 (1H, m, 6-H), 7.50 – 7.56 (2H, m, 7-H & 8-H), 8.10 (1H, dd, *J* = 1.00 & 8.25 Hz, 5-H), 11.53 (1H, s, NH)

^{13}C NMR (DMSO) δ 14.52 (CH_3), 118.08, 121.96 (2 x aromatic CH), 122.77 (4°), 124.99, 130.43 (2 x aromatic CH), 132.12, 137.73, 138.55 (4°), 169.08 (CO)

(MS-ES) *m/z* 176.08 (MH^+, $C_{10}H_9NO_2$ requires *m/z* 176.06)

2-*n*-heptyl-3-hydroxy-4(1*H*)-quinolone (PQS) (2.143)



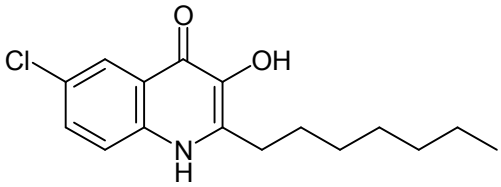
Yield – 59 %

(MS-ES) *m/z* 260.15 (MH^+, $C_{16}H_{21}NO_2$ requires *m/z* 260.16

^{1}H NMR (DMSO) δ 0.84 (3H, t, *J* = 6.99 Hz, $(CH_2)_6CH_3$), 1.23 – 1.33 (8H, m, $(CH_2)_4$), 1.67 (2H, m, 2'-H), 2.73 (2H, t, *J* = 7.85 Hz, 1'-H), 7.22, (1H, m, Ar), 7.53 (2H, m, Ar), 8.09 (1H, d, *J* = 8.12 Hz, Ar) 11.43 (1H, br s, NH)

^{13}C NMR (DMSO) δ 14.39 (CH_3), 22.53, 28.29, 28.59, 8.95, 29.25, 31.64 (6 x CH_2), 118.27, 121.99 (2 x aromatic CH) 122.65 (4°), 124.97, 130.36 (2 x aromatic CH), 135.99, 137.81, 138.31 (3 x 4°), 169.28 (CO)

6-Chloro-2-heptyl-3-hydroxy-4(1*H*)-quinolone (2.144)



Mp. 250 – 252 °C

Yield – 47 %

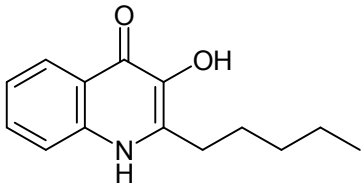
TLC; R_f = 0.59 (5 % methanol, DCM)

^{1}H NMR (DMSO) δ 0.84 (3H, t, *J* = 6.93 Hz, 7'-H), 1.28 (8H, m, 3'-H – 6'-H), 1.64 (2H, m, 2'-H), 2.72 (2H, t, *J* = 7.87 Hz, 1'-H), 7.56 (2H, m, 7-H & 8-H), 8.03 (1H, br d, *J* = 2.04 Hz, 5-H), 11.64 (1H, s, NH)

^{13}C NMR (DMSO) δ 14.40 (CH_3), 22.52, 28.21, 28.62, 28.92, 29.24, 31.64 (6 x CH_2) 120.73, 123.63 (4°) 123.66 (Ar CH), 126.64 (4°) 130.55 (Ar CH), 136.21, 136.81, 138.74 (3 x 4°), 168.19 (CO)

(MS-ES) *m/z* 292.11 & 294.11 (MH^-, $C_{16}H_{20}ClNO_2$ requires 292.12 & 294.12)

2-*n*-Pentyl-3-hydroxy-4(1*H*)-quinolone (2.142)



Mp. 214 – 216 °C

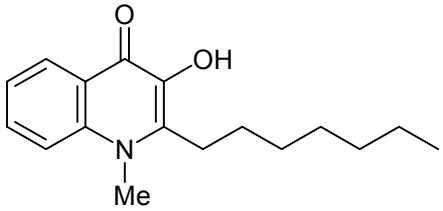
TLC; R_f = 0.37 (5 % methanol, DCM)

^{1}H NMR (DMSO) δ 0.87 (3H, t, *J* = 4.79 Hz, 5'-H), 1.33 (4H, m, 3'-H & 4'-H), 1.68 (2H, m, 2'-H), 2.73 (2H, t, *J* = 7.55 Hz, 1'-H), 7.22 (1H, m, 7-H), 7.53 (2H, m, 6-H & 8-H), 8.10 (1H, br d, *J* = 8.11 Hz, 5-H), 11.44 (1H, s, NH)

^{13}C NMR (DMSO) δ 14.36 (CH_3), 22.36, 27.95, 28.53, 31.47 (CH_2), 118.23, 121.97 (Ar CH), 122.67 (4°), 124.95, 130.44 (Ar CH), 135.99, 137.82, 138.31 (3 x 4°) 169.33 (CO)

(MS-ES) *m/z* 230.11 (MH^-, $C_{14}H_{17}NO_2$ requires *m/z* 230.13)

2-Heptyl-3-hydroxy-1-methyl-4(1*H*)-quinolone (2.145)



2-Oxononyl-2-(methylamino)benzoate (1.455 g, 0.5 mmol) was dissolved in NMP (0.4 mL) and heated at reflux for 1 h 15 min. This was cooled to room temperature and ethyl acetate added. This was placed in an ice bath and stirred for 30 min. Ethyl acetate and as much of NMP as possible was removed under vacuum. Ethyl acetate (0.2 mL) was added and the solution chilled in a fridge, forming the product as off-white crystals in a 28 % yield.

Mp. 106 – 107 °C

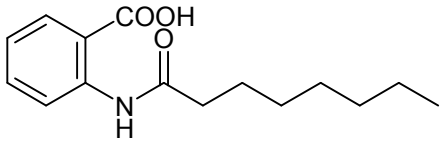
^{1}H NMR (DMSO) δ 0.86 (3H, t, *J* = 6.78 Hz, 7'-H), 1.34 (8H, m, 3'-H – 6'-H), 1.57 (2H, m, 2'-H), 2.97 (2H, t, *J* = 7.78 Hz, 1'-H), 3.82 (3H, s, N*CH*$_3$), 7.32 (1H, m, 7-H), 7.66 (1H, m, 6-H), 7.78 (1H, br d, *J* = 8.80 Hz, Ar), 8.23 (1H, dd, *J* = 1.49 & 8.10 Hz, 5-H)

^{13}C 14.44 (CH_3), 22.55, 26.88, 28.13, 28.93, 29.40, 31.70 (6 x CH_2), 35.00 (NCH_3), 117.08, 122.30 (2 x Ar CH), 123.51 (4°), 125.49, 131.17 (2 x aromatic CH), 138.63, 139.12, 139.25 (3 x 4°), 168.43 (C=O)

(MS-ES) *m/z* 259.14 (MH^-, $C_{15}H_{20}N_2O_2$ requires *m/z* 259.33)

6.4 Chapter 3

***N*-Octanoyl Anthranilic Acid (3.69)**



Method A

A solution of anthranilic acid (0.6857g, 5 mmol) in pyridine (50 mL) was well stirred and cooled to 0 ^{o}C in an ice-salt bath. A solution of octanoyl chloride (0.895g, 5.5 mmol) in pyridine was added drop wise and the solution was stirred for 2 h. Pyridine was removed under vacuum leaving a mixture of solid

and liquid. This was triturated with a mixture of ethyl acetate/ether, giving pyridine hydrochloride as a solid, which was removed by filtration. The filtrate was washed with 2M HCl and saturated brine solution, then dried over $MgSO_4$. Removal of solvent produced an orange syrup that was unsuitable for further work.

Method B

Anthranilic acid (6.875g, 50 mmol) was added to a solution of sodium hydroxide (2 g, 50 mmol) in water and was well stirred at 0 ^oC. To this octanoyl chloride (8.95 g, 55 mmol) and sodium hydroxide solution (2 g, 50 mmol) were added drop wise over 30 min, with stirring. The solution was stirred for 30 min, removed from the ice bath and stirred for a further 30 min. A precipitate formed during this time. Ethyl acetate was added to the flask and the solution was acidified with conc. HCl, with stirring, until the aqueous layer appeared clear. The aqueous layer was removed. The organic layer was dried over $MgSO_4$ and solvent removed to yield a waxy solid. This was triturated with petroleum ether (b.p. 60-80 ^oC), yielding a crystalline solid, in 66 % yield, which was collected under suction and dried in a dessicator, under vacuum. Product is a beige, crystalline solid

Mpt = 91 – 92 ^oC

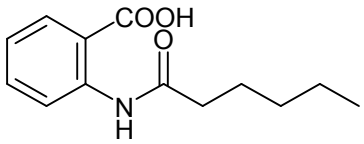
TLC; R_f = 0.68 (50 % ethyl acetate, hexane).

IR (KBr) 1673 (amide C=O), 1692 (amide C=O), 3334 (NH_2) cm^{-1}

^1H NMR ($CDCl_3$) δ 1.01 (3H, t, *J* = 1.70 Hz, 8'-H CH_3), 1.44 (8H, q, *J* = 6.64 Hz, $(CH_2)_4$), 1.83 (2H, m, 3'-H), 2.54 (2H, m, 2'-H CO*CH_2*), 7.24 (1H, m, 4-H), 7.72 (1H, m, 6-H), 8.25 (1H, dd, *J* = 1.54 & 8.02 Hz, 5-H), 8.88 (1H, dd, *J* = 0.74 & 8.49 Hz, 3-H), 10.91 (1H, s, COOH), 11.05 (1H, s, NH)

^{13}C NMR ($CDCl_3$) δ 14.08 (CH_3), 22.63, 25.56, 29.02, 29.16, 31.67, 38.74 (6 x CH_2), 114.05 (4°), 120.60, 122.64, 131.74, 135.58 (4 x Ar CH), 142.07, 172.10, 172.86 (3 x 4°)

_N_-Hexanoyl Anthranilic Acid (3.74)

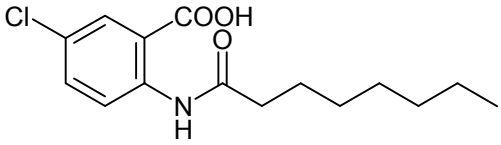


Yield: 70 %

TLC; R_f = 0.31 (50 % ethyl acetate, hexane)

^{1}H NMR ($CDCl_3$): δ 0.95 (3H, t, *J* = 2.25 Hz, CH_3), 1.36 – 1.45 (4H, m, $(CH_2)_2$), 1.76 – 1.83 (2H, m, $COCH_2CH_2$), 2.50 (2H, t, *J* = 7.46 Hz, $COCH_2CH_2$), 6.69 – 6.73 (1H, m, Ar H), 7.13 – 7.17 (1H, m, Ar H), 7.61 – 7.65 (1H, m, Ar H), 8.80 (1H, dd, *J* = 0.88 & 8.53 Hz, Ar H), 10.97 (1H, br s, NH)

4-Chloro-_N_-Octanoyl Anthranilic Acid (3.77)



Yield: 68 %

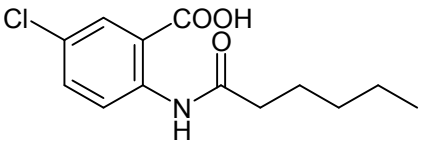
TLC; R_f = 0.28 (50 % ethyl acetate, hexane)

^{1}H NMR ($CDCl_3$): δ 0.90 (3H, t, *J* = 6.44 Hz, CH_3), 1.31 – 1.41 (8H, m, $(CH_2)_4$), 1.74 – 1.86 (2H, m, $COCH_2CH_2$), 2.52 (2H, t, *J* = 7.42 Hz, $COCH_2CH_2$), 7.58 (1H, dd, *J* = 2.63 & 9.08 Hz, 5-H), 8.12 (1H, d, *J* = 2.60 Hz, 3-H), 8.77 (1H, dd, *J* = 3.44 & 9.09, 6-H), 11.06 (1H, s, NH)

^{13}C NMR ($CDCl_3$) δ 14.11 (CH_3), 22.67, 25.45, 29.35, 29.42, 31.87, 38.67 (6 x CH_2), 118.27 (4°), 121.98 (aromatic CH), 127.59 (4°), 131.18, 135.35 (2 x aromatic CH), 140.67, 171.08, 172.64 (3 x 4°)

(MS-ES) *m/z* 295.74 & 297.64 (MH^-, $C_{15}H_{20}ClNO_3$ requires 297.78)

4-Chloro-_N_-hexanoyl anthranilic acid (3.76)

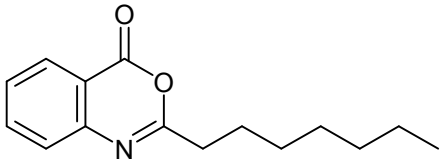


Yield: 63 %

^{1}H NMR ($CDCl_3$) δ 0.92 (3H, t,), 1.13 – 1.18 (4H, m, $(CH_2)_2$), 1.51 – 1.55 (2H, m, $COCH_2CH_2$), 2.21(2H, t, *J* = 7.45, $COCH_2CH_2$), 7.25 (1H, dd, *J* = 2.63 & 9.05 Hz, 5-H), 7.83 (1H, d, *J* = 2.60 Hz, 6-H), 8.50 (1H, d, *J* = 9.05 Hz, 5-H), 11.11 (1H, br s, NH)

^{13}C NMR ($CDCl_3$) δ 13.80 (CH_3), 22.19, 24.92, 31.11, 38.37 (4 x CH_2), 117.89 (4°), 119.52 (aromatic CH), 130.67 (4°), 130.74, 133.49 (2 x aromatic CH), 140.30, 149.49, 169.13 (3 x 4°)

2-*n*-Heptyl-benzo [*d*] [1,3]-oxazin-4-one (3.79)



Method A

A solution of n-octanoyl anthranilic acid (1 g, 3.8 mmol) in thionyl chloride (20 mL) was heated under reflux for 2 h. Thionyl chloride was removed under vacuum yielding a viscous, brown oil that was unsatisfactory to take on in the synthesis.

Method B[187,188]

A solution of *N*-octanoyl anthranilic acid (1 g, 3.8 mmol) in acetic anhydride (20 mL) was heated under reflux for 2 h. Acetic anhydride was evaporated under high vacuum to yield the product as a yellow oil in 93 % yield. Residual acetic acid was removed by placing in a dessicator, under vacuum, with KOH.

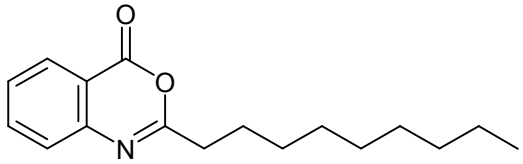
R_f – 0.68 EtOAc/hexane 50/50

^{1}H NMR ($CDCl_3$) δ 0.86 (3H, t, *J* = 6.80 Hz, 7'-H CH_3), 1.34 (8H, m, $(CH_2)_4$), 1.81 (2H, m, 2'-H), 2.66 (2H, t, *J* = 7.88 Hz, 1'-H), 7.50 (2H, m, 6-H & 8-H), 7.77 (1H, m, 7-H), 8.16 (1H, dd, *J* = 1.23 & 7.86 Hz, 5-H)

^{13}C NMR ($CDCl_3$) δ 14.41 (CH_3), 22.95, 26.51, 29.69, 29.41, 31.99, 35.22 (6 x CH_2), 117.19 (4°), 126.89, 128.43, 128.73, 136.78 (3 x aromatic CH), 146.82, 160.18, 163.64 (3 x 4°)

(MS-ES) *m/z* 246.26 (MH^+, $C_{15}H_{19}NO_2$ requires *m/z* 246.32)

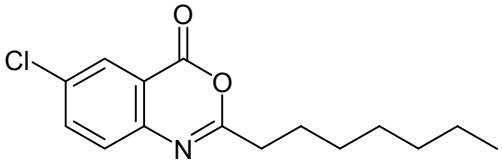
2-Nonyl-4*H*-benzo[d][1,3]oxazin-4-one (3.81)



^{1}H NMR ($CDCl_3$) δ 0.88 (3H, t, *J* = 6.23 Hz, 9'-H CH_3), 1.27 – 1.37 (12H, m, $(CH_2)_6$), 1.77 – 1.89 (2H, m, 2'-H), 2.69 (2H, t, *J* = 7.46 Hz, 1'-H), 7.46 – 7.58 (2H, m, 6-H & 8-H), 7.76 – 7.83 (1H, m, 7-H), 8.18 (1H, dd, *J* = 1.13 & 7.86 Hz, 5-H)

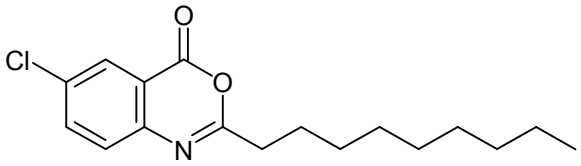
^{13}C NMR ($CDCl_3$) δ 14.11 (CH_3), 21.33, 22.67, 26.16, 29.11, 29.26, 29.26, 29.41, 31.86, 34.87 (8 x CH_2), 116.85 (4°), 126.54, 128.08, 128.39, 136.51 (3 x aromatic CH), 146.48, 159.83, 163.32 (3 x 4°)

6-Chloro-2-heptyl-4*H*-benzo[d][1,3]oxazin-4-one (3.83)



^{13}C NMR ($CDCl_3$) δ 14.45 (CH_3), 22.98, 26.45, 29.28, 29.42, 32.01, 35.20 (6 x CH_2), 118.37 (4°), 128.14, 128.57 (2 x aromatic), 134.11 (4°), 137.11 (aromatic), 145.34, 159.18, 164.00 (3 x 4°)

6-Chloro-2-nonyl-4*H*-benzo[d][1,3]oxazin-4-one (3.84)



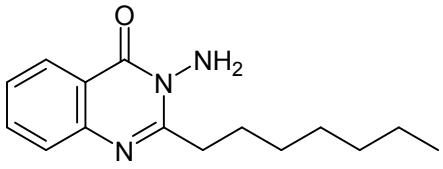
Mp. 51-53 °C

TLC; R_f = 0.51 (50 % ethyl acetate, hexane)

^{1}H NMR ($CDCl_3$) δ 0.89 (3H, t, *J* = 6.23 Hz, 9'-H CH_3), 1.25 – 1.43 (12H, m, $(CH_2)_6$), 1.80 – 1.85 (2H, m, 2'-H), 2.70 (2H, t, *J* = 7.55 Hz, 1'-H), 7.51 – 7.55 (1H, m, 5-H), 7.73 – 7.67 (1H, m, 7-H), 8.17 (1H, br d, *J* = 2.43 Hz, 8-H)

^{13}C NMR ($CDCl_3$) δ 14.11 (CH_3), 21.35, 22.67, 26.07, 29.23, 29.38, 31.85, 34.82 (8 x CH_2), 117.79 (4°), 127.76, 128.02 (2 x CH), 133.73 (4°), 136.75 (CH), 144.88, 158.57, 163.61 (3 x 4°)

2-*n*-Heptyl-3-amino-4(3*H*)quinazolinone (3.85)



Method A

A solution of 2-*N*-heptyl-benzo[*d*][1,3]-oxazin-4-one (1.31 g, 5.35 mmol) and hydrazine monohydrate (0.268 g, 5.35 mmol) in tetrahydrofuran (THF) (15 mL) was stirred at room temperature for 2 h. The solvent was removed under vacuum to yield a bright pink oil that solidified on cooling to form a crystalline solid. The solid was triturated with ether, giving a white precipitate that was collected by filtration. The white precipitate was found to be the uncyclised form of the desired product.

Method B

A solution of 2-*N*-heptyl-benzo[*d*][1,3]-oxazin-4-one (0.494 g, 2 mmol) and hydrazine monohydrate (0.4 ml) in ethanol (20 mL) was stirred at reflux overnight. The solvent was removed under reduced pressure, yielding a yellow, crystalline solid. This was triturated with hexane and the resulting solid was recrystallised from EtOH/water to give beige, needle crystals in 38 % yield.

Mp. 74 – 75 °C

IR (KBr) 1591.17 (C=N), 1664 (amide C=O), 3203, 3301 (NH_2) cm^{-1}.

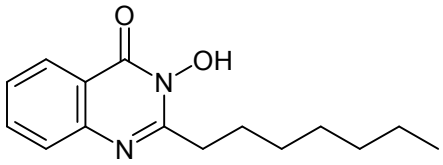
^{1}H NMR ($CDCl_3$) δ 0.93 (3H, t, *J* = 6.71 Hz, CH_3), 1.43 (8H, m, $(CH_2)_4$), 1.86 (2H, m, 2'-H), 3.06 (2H, t, *J* = 7.99 Hz, 1'-H), 4.92 (2H, s, NH_2), 7.41 (1H, m, 6-H), 7.75 (2H, m, 8-H & 7-H), 8.27 (1H, dd, *J* = 1.02 & 8.02 Hz, 5-H)

^{13}C NMR ($CDCl_3$) δ 14.84 (CH_3), 22.63, 27.217, 29.06, 29.46, 31.74, 34.62 (6 x CH_2), 119.98 (4°), 126.26, 126.47, 127.10, 134.27 (4 x aromatic CH), 147.02, 158.55, 161.95 (3 x 4°)

(MS-ES) *m/z* 260.2 (MH^+, $C_{15}H_{21}N_3O$ requires *m/z* 260.35)

Anal. calcd. for $C_{15}H_{21}N_3O$: C, 69.47; H, 8.16; N, 16.20. Found: C, 69.35; H, 8.26; N, 16.29.

2-*n*-Heptyl-3-hydroxy-4(3*H*)quinazolinone (3.87)



Sodium metal was dissolved in ethanol (20 mL). Hydroxylamine hydrochloride (4 mmol) was added and stirred for 15 min. The solid was removed by filtration and 2-*N*-heptyl-benzo[*d*][1,3]-oxazin-4-one (0.988 g, 4 mmol) was added to the filtrate and refluxed for 48 h. The solvent was removed under vacuum to yield a viscous yellow oil, which when triturated with ether yielded a beige crystalline solid. This was collected by filtration. Product produced in 29 % yield.

Mp. 215 - 220 °C

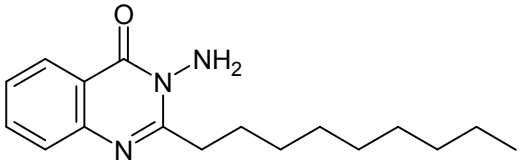
IR (KBr) 1588 (C=N), 1665 (amide C=O), 3432 (OH) cm^{-1}

^1H NMR (MeOH-d_4) δ 1.03 (3H, t, *J* = 6.81 Hz, 7'-H CH_3), 1.41 (8H, m, $(CH_2)_4$), 1.85 (2H, m, 2'-H), 2.55 (2H, t, *J* = 7.80 Hz, 1'-H), 5.06 (1H, s, NOH), 7.18 (1H, m, 6-H), 7.49 (1H, m, 8-H), 8.18 (1H, dd, *J* = 1.57 & 7.81 Hz, 7-H), 8.65 (1H, m, 5-H)

^{13}C NMR (MeOH-d_4) δ 14.83 (CH_3), 24.08, 24.57, 27.35, 30.56, 30.75, 33.31, 40.00 (6 x CH_2), 120.88, 123.79 (2 x aromatic CH), 125.48 (4°), 132.50, 132.79 (2 x aromatic CH), 141.68, 174.67, 175.23 (3 x 4°)

(MS-ES) *m/z* 261.58 (MH^+, $C_{15}H_{20}N_2O_2$ requires *m/z* 261.33)

2-*n*-Nonyl-3-amino-4(3*H*)-quinazolinone (3.89)



Yield = 46 %

Mpt = 57 – 58 ^oC

(MS-ES) *m/z* 286.37 (MH^+, $C_{17}H_{25}N_3O$ requires *m/z* 287.40)

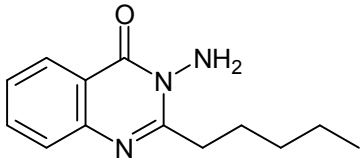
IR (KBr) 1593 (C=N), 1683 (amide C=O), 2849, 2923 (NH_2) cm^{-1}

^1H NMR ($CDCl_3$) δ 0.88 (3H, t, *J* = 6.27 Hz, CH_3), 1.27 – 1.46 (12H, m, $(CH_2)_6$), 1.74 – 1.88 (2H, m, CH_2), 3.02 (2H, t, *J* = 7.71 Hz, CH_2), 4.86 (2H, s,

NH_2), 7.41 – 7.47 (1H, m, 7-H), 7.64 – 7.69 (2H, m, 6-H & 8-H), 8.23 (1H, dd, *J* = 0.99 & 8.01 Hz, 5-H)

^{13}C NMR ($CDCl_3$) δ 14.12 (CH_3), 22.17, 22.68, 27.18, 29.29, 29.40, 29.49, 31.88, 34.64 (8 x CH_2), 119.96 (4°), 126.21, 126.42, 127.11, 134.22, (4 x Ar), 147.03, 155.50, 161.92 (3 x 4°)

2-*n*-Pentyl-3-amino-4(3*H*)-quinazolinone (3.88)



Yield = 47 %

Mpt = 60 – 61 ^{o}C

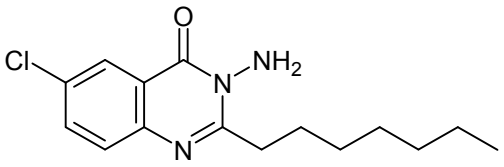
IR (KBr) 1593 (C=N), 1686 (amide C=O), 2928, 2959 (NH_2) cm^{-1}

^{1}H NMR ($CDCl_3$) δ 0.95 (3H, t, *J* = 6.9 Hz, 5'-H), 1.40 – 1.50 (4H, m, $(CH_2)_2$), 1.79 – 1.91 (2H, m, 2'-H), 3.03 (2H, t, *J* = 1.67 Hz, 1'-H), 4.89 (2H, s, NH_2), 7.42 – 7.49 (1H, m, 7-H), 7.66 – 7.78 (2H, m, 6-H & 8-H), 8.22 – 8.26 (1H, m, 5-H)

^{13}C NMR ($CDCl_3$) δ 14.40 (CH_3), 22.86, 27.26, 32.04, 35.01 (4 x CH_2), 120.39, (4°), 126.62, 126.85, 127.55, 14.63 (3 x Ar CH), 147.46, 158.83, 12.31 (3 x 4°)

(MS-ES) *m/z* 232.07 (MH^+, $C_{13}H_{17}N_3O$ requires *m/z* 232.14)

6-Chloro-2-*n*-heptyl-3-hydroxy-4(3*H*)-quinazolinone (3.90)



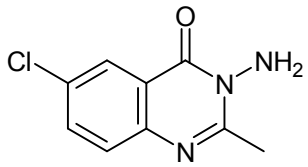
Yield = 78 %

^{1}H NMR ($CDCl_3$) δ 0.91 (3H, t, *J* = 6.48 Hz, CH_3), 1.41 (8H, m, $(CH_2)_4$), 1.84 (2H, m, 2'-H), 3.03 (2H, t, *J* = 7.72 Hz, 1'-H), 4.88 (2H, s, NH_2), 7.65 (2H, m, 7-H & 8-H), 8.24 (1H, d, *J* = 2.24 Hz, 5-H).

^{13}C NMR ($CDCl_3$) δ 14.49 (CH_3), 23.03, 27.42, 29.45, 29.82, 32.13, 34.98 (6 x CH_2), 121.38 (4°), 126.10, 129.31 (2 x aromatic), 132.34 (4°), 135.08 (aromatic), 145.97, 159.11, 161.32 (3 x 4°)

Anal. calcd. for $C_{15}H_{20}ClN_3O$: C, 61.32; H, 6.86; N, 14.30. Found: C, 61.35; H, 6.80; N, 14.33.

6-Chloro-2-methyl-3-hydroxy-4(3*H*)-quinazolinone (3.91)



Mp. 168 °C

^{1}H NMR ($CDCl_3$) δ 2.72 (3H, s, CH_3), 4.91 (2H, s, NH_2), 7.60 (1H, d, *J* = 8.70 Hz, 5-H), 7.68 (1H, dd *J* = 2.42 & 8.73 Hz, 7-H), 8.21 (1H, d, *J* = 2.24, 8-H)

^{13}C NMR ($CDCl_3$) δ 22.25 (CH_3) 121.07 (4°), 125.78, 128.70 (2 x Ar), 132.06 (4°), 134.79 (Ar), 145.48, 155.75, 160.62 (3 x 4°)

Anal. calcd. for $C_9H_8N_3OCl$: C, 51.56; H, 3.85; N, 20.04. Found: C, 51.28; H, 3.95; N, 19.63.

Chapter 7

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Appendix

A1 Raw Data for murine splenocyte assays

Well	Test material	Conc (μM)	cpm			cpm		cpm%
						Mean	SD	Mean
X1	**3.85**	1000	52	53		52.5	0.71	1.0
X2	**3.85**	300	62	63		62.5	0.71	1.2
X3	**3.85**	100	63	152		107.5	62.93	2.0
X4	**3.85**	30	147	68		107.5	55.86	2.0
X5	**3.85**	10	1031	817		924.0	151.32	17.6
X6	**3.85**	3	2745	2805		2775.0	42.43	52.9
X7	**3.85**	1	3980	3411		3695.5	402.34	70.4
X8	**3.85**	0.3	6071	4473		5272.0	1129.96	100.5
X11	**3.87**	1000	47	38		42.5	6.36	0.8
X12	**3.87**	300	97	59		78.0	26.87	1.5
X13	**3.87**	100	51	50		50.5	0.71	1.0
X14	**3.87**	30	367	297		332.0	49.50	6.3
X15	**3.87**	10	2970	3111		3040.5	99.70	57.9
X16	**3.87**	3	5183	4832		5007.5	248.19	95.4
X17	**3.87**	1	5266	4944		5105.0	227.69	97.3
X18	**3.87**	0.3	4952	6838		5895.0	1333.60	112.3
X11	**3.88**	1000	47	32		39.5	10.61	5.7
X12	**3.88**	300	51	36		43.5	10.61	6.2
X13	**3.88**	100	42	55		48.5	9.19	6.9
X14	**3.88**	30	47	49		48.0	1.41	6.9
X15	**3.88**	10	1154	1414		1284.0	183.85	184.0
X16	**3.88**	3	2731	4123		3427.0	984.29	491.0
X17	**3.88**	1	4149	3885		4017.0	186.68	575.5
X18	**3.88**	0.3	5415	4093		4754.0	934.80	681.1
X9	**Positive**		5841	5161	5099			
X19	**Positive**		4760	5220	5409	5248.3	359.52	100.0
X10	**Negative**		46	78	33	52.3	23.16	1.0
X20	**Dexamethasone**	**0.5**	77	98	131	102.0	27.22	1.9

Well	Test material	Conc (μM)	cpm			cpm		cpm%
						Mean	SD	Mean
X1	**3.89**	1000	36	29		32.5	4.95	0.6
X2	**3.89**	300	32	39		35.5	4.95	0.7
X3	**3.89**	100	44	108		76.0	45.25	1.5
X4	**3.89**	30	506	890		698.0	271.53	13.6
X5	**3.89**	10	2833	2576		2704.5	181.73	52.5
X6	**3.89**	3	3744	3531		3637.5	150.61	70.6
X7	**3.89**	1	5785	4348		5066.5	1016.11	98.4
X8	**3.89**	0.3	5243	4572		4907.5	474.47	95.3
X11	**3.9O**	1000	34	34		34.0	0.00	0.7
X12	**3.9O**	300	139	99		119.0	28.28	2.3
X13	**3.9O**	100	869	960		914.5	64.35	17.8
X14	**3.9O**	30	1655	1941		1798.0	202.23	34.9
X15	**3.9O**	10	4205	2794		3499.5	997.73	67.9
X16	**3.9O**	3	4217	4889		4553.0	475.18	88.4
X17	**3.9O**	1	5625	4909		5267.0	506.29	102.3
X18	**3.9O**	0.3	5111	3946		4528.5	823.78	87.9
X9	**Positive**		4816	4922	5262			
X19	**Positive**		4952	5132	5821	5150.8	364.92	100.0
X10	**Negative**		35	18	25	26.0	8.54	0.5
X20	**Dexamethasone**	**0.5**	82	57	49	62.7	17.21	1.2

Well	Test material	Conc (μM)	cpm			cpm		cpm%
						Mean	SD	Mean
X1	**3.91**	1000	25	19		22.0	4.24	0.4
X2	**3.91**	300	3027	3742		3384.5	505.58	64.1
X3	**3.91**	100	3802	4283		4042.5	340.12	76.6
X4	**3.91**	30	4667	6406		5536.5	1229.66	104.9
X5	**3.91**	10	9976	5153		7564.5	3410.38	143.3
X6	**3.91**	3	7230	4149		5689.5	2178.60	107.8
X7	**3.91**	1	6127	4978		5552.5	812.47	105.2
X8	**3.91**	0.3	5497	5039		5268.0	323.85	99.8
X11	**2.141**	1000	33	35		34.0	1.41	0.6
X12	**2.141**	300	51	45		48.0	4.24	0.9
X13	**2.141**	100	36	63		49.5	19.09	0.9
X14	**2.141**	30	1816	1194		1505.0	439.82	28.5
X15	**2.141**	10	2816	2893		2854.5	54.45	54.1
X16	**2.141**	3	3674	4951		4312.5	902.98	81.7
X17	**2.141**	1	5509	4085		4797.0	1006.92	90.9
X18	**2.141**	0.3	4613	4355		4484.0	182.43	85.0
X11	**2.104**	1000	31	13		22.0	12.73	32.8
X12	**2.104**	300	758	661		709.5	68.59	1059.0
X13	**2.104**	100	3656	5547		4601.5	1337.14	6867.9
X14	**2.104**	30	4055	5840		4947.5	1262.19	7384.3
X15	**2.104**	10	4811	4400		4605.5	290.62	6873.9
X16	**2.104**	3	4825	5615		5220.0	558.61	7791.0
X17	**2.104**	1	4417	4443		4430.0	18.38	6611.9
X18	**2.104**	0.3	4648	5276		4962.0	444.06	7406.0
X9	**Positive**		6764	4951	5415			
X19	**Positive**		5305	5144	4091	5278.3	867.36	100.0
X10	**Negative**		42	27	43	37.3	8.96	0.7
X20	**Dexamethasone**	**0.5**	63	66	65	64.7	1.53	1.2

Well	Test material	Conc (μM)	cpm			cpm		cpm%
						Mean	SD	Mean
X1	**2.107**	1000	37	42		39.5	3.54	0.8
X2	**2.107**	300	76	47		61.5	20.51	1.2
X3	**2.107**	100	55	94		74.5	27.58	1.4
X4	**2.107**	30	85	49		67.0	25.46	1.3
X5	**2.107**	10	358	544		451.0	131.52	8.8
X6	**2.107**	3	3917	2203		3060.0	1211.98	59.5
X7	**2.107**	1	10572	4512		7542.0	4285.07	146.7
X8	**2.107**	0.3	3793	8252		6022.5	3152.99	117.1
X11	**2.109**	1000	37	31		34.0	4.24	0.7
X12	**2.109**	300	94	95		94.5	0.71	1.8
X13	**2.109**	100	66	35		50.5	21.92	1.0
X14	**2.109**	30	893	800		846.5	65.76	16.5
X15	**2.109**	10	2696	4065		3380.5	968.03	65.7
X16	**2.109**	3	6416	6088		6252.0	231.93	121.6
X17	**2.109**	1	4214	5060		4637.0	598.21	90.2
X18	**2.109**	0.3	5590	5610		5600.0	14.14	108.9
X9	**Positive**		5366	4949	4864			
X19	**Positive**		6109	5112	4453	5142.2	561.32	100.0
X10	**Negative**		74	36	33	47.7	22.85	0.9
X20	**Dexamethasone**	**0.5**	75	85	80	80.0	5.00	1.6

Well	Test material	Conc (μM)	cpm			cpm		cpm%
						Mean	SD	Mean
X1	**2.144**	1000	52	48		50.0	2.83	0.9
X2	**2.144**	300	67	71		69.0	2.83	1.3
X3	**2.144**	100	77	492		284.5	293.45	5.2
X4	**2.144**	30	238	79		158.5	112.43	2.9
X5	**2.144**	10	96	50		73.0	32.53	1.3
X6	**2.144**	3	73	56		64.5	12.02	1.2
X7	**2.144**	1	277	333		305.0	39.60	5.5
X8	**2.144**	0.3	4087	4414		4250.5	231.22	77.3
X11	**2.142**	1000	94	33		63.5	43.13	1.2
X12	**2.142**	300	129	137		133.0	5.66	2.4
X13	**2.142**	100	54	57		55.5	2.12	1.0
X14	**2.142**	30	54	58		56.0	2.83	1.0
X15	**2.142**	10	24	39		31.5	10.61	0.6
X16	**2.142**	3	50	67		58.5	12.02	1.1
X17	**2.142**	1	1210	1219		1214.5	6.36	22.1
X18	**2.142**	0.3	3849	3846		3847.5	2.12	70.0
X11	**2.145**	1000	49	36		42.5	9.19	0.8
X12	**2.145**	300	47	78		62.5	21.92	89.3
X13	**2.145**	100	59	50		54.5	6.36	77.9
X14	**2.145**	30	38	64		51.0	18.38	72.9
X15	**2.145**	10	44	60		52.0	11.31	74.3
X16	**2.145**	3	52	67		59.5	10.61	85.0
X17	**2.145**	1	221	130		175.5	64.35	250.7
X18	**2.145**	0.3	1356	1520		1438.0	115.97	2054.3
X1	**4.4**	1000	30	25		27.5	3.54	0.6
X2	**4.4**	300	265	58		161.5	146.37	3.4
X3	**4.4**	100	77	68		72.5	6.36	1.5
X4	**4.4**	30	76	64		70.0	8.49	1.5
X5	**4.4**	10	39	37		38.0	1.41	0.8
X6	**4.4**	3	37	82		59.5	31.82	1.3
X7	**4.4**	1	56	88		72.0	22.63	1.5
X9	**Positive**		6048	5851	4879			
X19	**Positive**		5107	6254	4848	5497.8	625.65	100.0
X10	**Negative**		73	126	36	78.3	45.24	1.4
X20	**Dexamethasone**	**0.5**	70	111	69	83.3	23.97	1.5