NORCANTHARIDIN ANALOGUES: PP1 AND PP2A INHIBITION AND POTENTIAL THERAPEUTIC DEVELOPMENT

by

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I hereby certify that the work embodied in this Thesis contains four published papers of which I am a joint author. A copy of each of the papers is attached in the Appendices.

Benjamin Sauer

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ABBREVIATIONS

Abbreviations	
AIDS	Acquired Immunodeficiency Syndrome
ATP	Adenosine Triphosphate
cdk	cyclin dependant kinases
DCM	Dichloromethane
DMF	*N*,*N*-Dimethylformamide
DMSO	Dimethylsulfoxide
EIMS	Electron Impact Mass Spectra
EPI	Endogenous Protein Inhibitor
ESIMS	Electrospray Ionisation Mass Spectra
GCMS	Gas Chromatograph Mass Spectrometer
GI_{50}	Inhibition Concentration 50; drug concentration required to inhibit cell growth by 50% relative to untreated control
HAART	Highly Active Antiretroviral Therapy
HIV	Human Immunodeficiency Virus
HPLC	High Performance Liquid Chromotography
IC_{50}	Inhibition Concentration 50; drug concentration required to inhibit enzyme function by 50%
IR	Infrared
MSD	Mass Selective Detector
NMR	Nuclear Magnetic Resonance
OA	Okadaic Acid
PK	Protein Kinase
PP	Protein Phosphatase
PP1	Protein Phosphatase 1
PP2A	Protein Phosphatase 2A
PPM	Protein Phosphatase Magnesium
PPP	Phosphor-Protein Phosphatases
pRb	Retinoblastoma
PTP	Protein Tyrosine Phosphatase
SAR	Structure Activity Relationship
THF	Tetrahydrofuran
TLC	Thin Layer Chromatography
TS	Total Synthesis
TSG	Tumour Suppressor Genes

ABSTRACT

This study described in this work examines the potential for derivatives of the potent PP1 (IC_{50} 9.0 µM) and PP2A (IC_{50} 3.0µM) inhibitor, norcantharidin, the demethylated cantharidin analogue, and their protein phosphatase inhibition, namely PP1 and PP2A and their cytotoxicity across a range of human cancer cell lines.

A variety of derivatives were examined, paying particular attention to modifications to the anhydride moiety. These included a series of ring opened and ring closed cantharimides, a series of α-hydroxylactams, a series of lactone analogues and derivatives, and a series of heteroatom substituted analogues.

Of the analogues developed, the ring opened and ring closed cantharimides displayed moderate to excellent activity, in cases, an improvement over the lead compound norcantharidin was observed. The ring closed dodecyl-linked bis-analogue (**63**) was the most potent analogue displaying µM potent cytoxicities against all the cell lines examined. Of the ring opened analogues, the morpholino analogues proved most active.

Chapter 1

Introduction

1.1 The Role of Protein Phosphatases

The reversible phosphorylation of regulatory and structural proteins is fundamental to the regulation of many cellular functions. Approximately one third of intracellular proteins are reversibly phosphorylated [1] [2] [3] [4]. A protein's phosphorylation level is tightly regulated by two families of enzymes; Protein Kinases (PK)s, which are responsible for phosphorylation, and Protein Phosphatases (PP)s which are responsible for dephosphorylation.

The phosphorylation process has been simply described as a biological 'on/off switch', with PK responsible for the on, and PP responsible for the off phase of the cycle [5] [6]. However, the delicate balance between PKs and PPs activity allows the phosphorylation level of a specific protein to be adjusted, imparting 'rheostat like' control in key cellular events, including; cell proliferation, gene expression, neurotransmission, cellular signalling and apoptosis [2] [3] [4].

Reversible phosphorylation involves the PKs catalyzed phosphorylation of an amino acid residue of a specific intercellular protein, typically serine/threonine and tyrosine, using Adenosine Triphosphate (ATP) as the phosphoryl donor [2] [6]. Dephosphorylation is catalyzed by PPs [6]. The phosphorylation/dephosphorylation cycle is shown in **Figure 1.1**. The balance between PPs and PKs activity is tightly controlled *in vivo* by extracellular and intracellular signals including hormones, growth factors and metabolites, and also regulated by secondary proteins known as the Endogenous Protein Inhibitors (EPI) [1]. EPIs have evolved to selectively regulate specific PP or PK activity [6]. The activity of both PKs, PPs, and EPIs is also believed to be regulated by protein phosphorylation, further underlining the importance and complexity of the reversible phosphorylation process [7] [8].

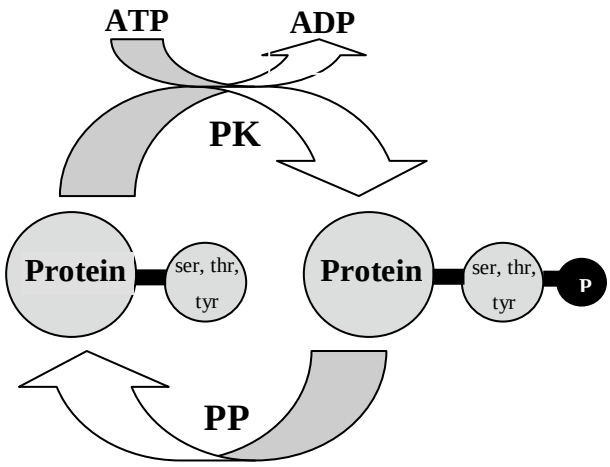


Figure 1.1: Basic Mechanism for the Reversible Phosphorylation.

The phosphorylation level of a specific protein has been shown to have a profound effect on a protein's activity and properties, resulting in changes in protein conformation and, consequently, protein-protein interactions. The change in protein activity can be manifested by initiation or disruption of protein-protein interactions, protein-ligand interactions, the stabilization or marking of proteins for destruction, or by facilitating or inhibiting cellular movement [4] [6]. It is known that the deregulation of protein phosphorylation can be the cause or consequence of a disease or disorder, including; cancer, HIV AIDS, diabetes and Alzheimer's disease [9]. Therefore there is a definite need for the generation of PK and PP inhibitors for therapeutic use. A selected list illustrating disease states occurring from abnormal phosphorylation and the responsible PP or PK is given in **Table 1.1**.

Table 1.1: Disease caused by Mutations in Kinases and Phosphatases.

Disease/disorder	Kinase/Phosphatase
Insulin signalling pathway	1B tyrosine phosphatase
X-linked myotubular myopathy	MTM1 tyrosine phosphatase
Cystic Fibrosis	2C serine/threonine phosphatase
Myotonic muscular dystrophy	Myotonin protein kinase
X-linked agammaglobulinaemia	Bruton tyrosine kinase
Hirschsprungis disease	RET2 kinase
Chronic myelogenous leukaemia	Abelson tyrosine kinase
Leprechanism, diabetes	Insulin receptor kinase

Data taken from [9], [10].

In the past two decades cellular signalling research has focused on the development of PK inhibitors. This is in part due to the fact that PKs out number PPs three to one, but also due to the original belief that PKs were more highly regulated, and consequently of greater importance in the original pathway [11]. Until recently PPs were thought of as house keeping enzymes, with their sole role to reactivate the related kinases [12]. Analysis of the human genome has shown *ca*. 500 protein kinases and 170 protein phosphatases of which only 100 phosphatases have been identified [9]. Therefore, as PPs comprise *ca*. 3% of the human genome, it is now becoming apparent that PP and PK are of equal importance in the phosphorylation process, and hence a need for the generation of inhibitors of PP activity [3] [6].

1.2 Serine/Threonine Protein Phosphatases

1.2.1 Classification of Serine/Threonine Protein Phosphatases

Unlike PKs, which have evolved from a single primordial gene, PPs are a diverse group of enzymes encoded by three major gene families [13]. PPs are defined on the basis of their biological characteristics, substrate specificity, catalytic subunit, and inhibitor studies. Accordingly, the PPs have been subdivided into three main families: the Phosphor-Protein Phosphatase (PPP) and Protein Phosphatase Magnesium dependant (PPM) families of the serine/threonine specific PPs, and the Protein Tyrosine Phosphatase (PTPs) superfamily of the tyrosine and dual specific PPs which are not discussed further in this study [8] [10] [11] [14] [15]. Within each PP family, the catalytic domains are highly conserved, and substrate specificity is determined by regulatory subunits [16]. Since the catalytic domain of each PP is associated with different regulatory subunits producing specific holoenzymes, the number of functional phosphatases is comparable to the number of kinases [13].

In eukaryotic cells up to 98% of all protein phosphorylation occurs on the serine/threonine residues [12]. Initial work on the biochemical classification of the serine/threonine PPs was carried out extensively by Cohen in the late 1970's and early 80's, with identification of two main structural types; Type 1 (PP1) which preferentially dephosphorylates the β–subunit of phosphorylase kinase, and Type 2 (PP2) which preferentially dephosphorylates the α–subunit [1] [12] [17] [18]. Type 1 and Type 2 serine/threonine PPs can also be distinguished by their interaction with the EPIs. That is, Type 1 serine/threonine PPs are selectively inhibited by inhibitor-1 (I_1^{PP1}, IC_{50} = 0.45 nM) and inhibitor-2 (I_2^{PP1}, IC_{50} = 0.8 nM), and Type 2 serine/threonine PPs are selectively inhibited by I_1^{PP2A} (IC_{50} = 4 nM) and I_2^{PP2A} (IC_{50} = 2 nM) [1]. Type 2 phosphatases are further divided by divalent cation requirement; PP2A is spontaneously active in absence of cations, where as PP2B (also known as calcineurin) is Ca^{2+}

dependant, and PP2C Mg^{2+} dependant [12] [18] [19]. Other serine/threonine PPs include PP4, PP5, PP6, and PP7 with multiple isoforms of each identified [11]. A diagrammatic representation of the domain organisation of members of the serine/threonine phosphatase is shown in **Figure 1.2**.

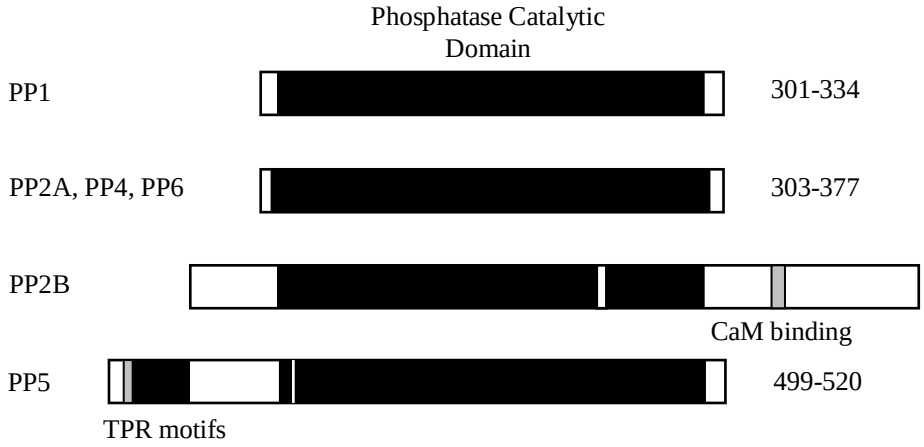


Figure 1.2: Domain Organization of Protein Phosphatases in the Serine/Threonine Family. Figure adapted from [19].

Of the serine/threonine PPs, PP1, 2A, 2B and 2C account for the majority of serine/threonine phosphatase activity [2]. Previous studies have illustrated that the primary amino acid sequences of PP1, PP2A and PP2B are related and show reasonable homology, while PP2C is distinctly different and derived from a separate gene family. It has been shown that the amino acid sequence of each of their catalytic domains is highly conserved; however there is a clear difference in the substrate specificity and regulatory subunits associated with each of the serine/threonine PPs [18]. The differential in substrate specificity of each catalytic subunit is largely determined by the regulatory subunits [16]. It has been hypothesised that the regulatory subunits for a specific catalytic domain also target the activities to defined subcellular compartments, and modulate protein specificity; hence the role of PPs is tightly regulated within normal cells [18].

1.2.2 Protein Phosphatase 1

PP1 consists of a 1:1 complex between a 37 kDa catalytic subunit (PP1c) and a number of different regulatory or targeting subunits (R) [20]. The activity and substrate specificity of PP1 is a direct response to the interaction of PP1c with different R subunits, and is controlled by hormones, growth factors and cellular metabolites, by facilitating the interaction with specific subunits [21]. Thus, the R subunit acts as an activity modulator, targeting subunits and substrates, and is responsible for imparting the specificity associated with each PP1 holoenzyme.

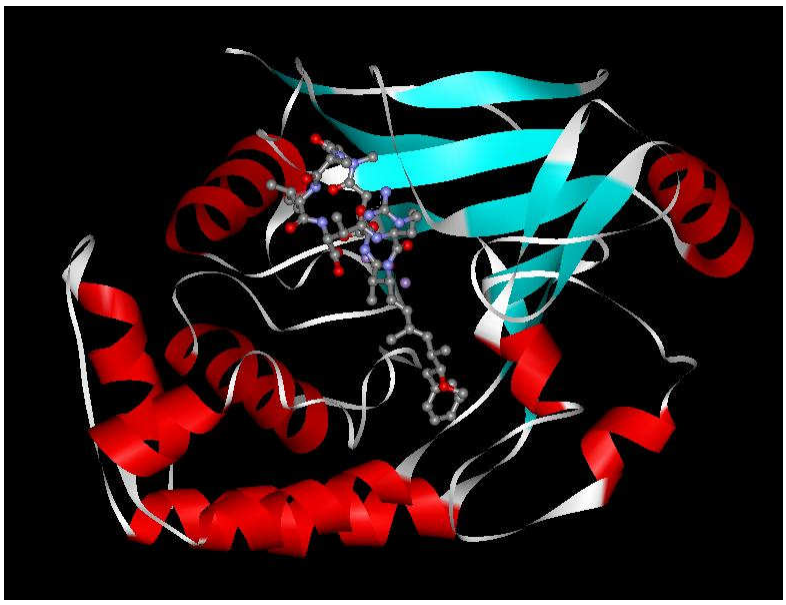


Figure 1.3: PP1 Cerius 2.2 Generated Model

PP1c is a relatively small protein comprising of *ca*. 300 residues, with high structural homology, even between phyla, making it one of the most conserved enzymes discovered [6]. Molecular cloning studies have illustrated that the eukaryotic genomes contains between one (e.g., *Saccharomyces cerevisiae*) and eight (e.g., *Arabidopsis thaliana*) genes that encode the different isoforms of PP1c and typically display 90% overall amino acid homology [22]. Currently, four isoforms of PP1c have been identified and are divided into three distinct types; 1α, 1γ (which includes two splice variants, 1 and 2), and 1δ, all of which cannot be differentiated by substrate specificity, or by their interaction with different R subunits [1]. However, the different isoforms have different subcellular locations, and have varying concentrations, suggesting that a mechanism for generating isoform-specific complexes with specific R subunits may exist [22].

The different R subunits are generally classified into 3 groups; activity modulating proteins, targeting proteins, and specific substrates, which are understood to restrict its activity to its microenvironment and hence its specificity [22]. PP1 activity is believed to be regulated by its interaction with specific endogenous inhibitors including; inhibitor-1 (I_1^{PP1}), inhibitor-2 (I_2^{PP1}), dopamine- and cAMP-regulated phosphoprotein of molecular weight of 32 kDa (DARPP-32), nuclear inhibition of protein phosphatase 1 (NIPP1), C-kinase activated phosphatase of molecular weight of 17 kDa (CPI17), and ribosomal inhibitor of PP1 (RIPP) [1] [7]. A list of PP1 regulatory subunits and their inhibition is shown in **Table 1.2**.

Table 1.2: Composition of PP1 and their Regulatory Subunits.

Catalytic Subunit (No. amino acid residues)	**Regulatory Subunit/Inhibition**	***K_i***
α (300), δ (327), γ_1 (323), γ_2 (337)	I_1^{PP1}	2 nM
	I_2^{PP1}	3 nM
	DARPP-32	1 nM
	R_{GL}	-
	G_L	100 nM
	G_M,	-
	NIPP1	0.2 nM
	RIPP L5, HSP,	20 nM
	RBGP,	-
	p53BP2	1 nM
	PSF	1,000 nM
	CPI17,	
	sds22	25,000 nM
	PPP1R5	100 nM

Inhibition is recorded as inhibition constant (K_i) values.
Data taken from [1]

PP1 plays multiple roles *in vivo* being implicated in the regulation of glycogen metabolism in response to insulin, smooth muscle contraction, protein synthesis, and as a negative regulator of the cell cycle [2] [23]. The regulation of these processes is achieved by the complexation of PP1c and their specific substrates. For example, the PP1 holoenzyme associated with glycogen metabolism, PP1G, consists of PP1c complexed with a regulatory protein G, which directs this holoenzyme towards glycogen particles and the sarcoplasmic reticulum [24]. Similarly, in the

case of myosin specific holoenzyme PP1M, PP1c is bound to myosin and two other subunits including G_M, which facilitates myosin binding [20].

1.2.3 Protein Phosphatase 2A

The general structure of PP2A has been shown to be a heterotrimeric complex *in vivo*. The core of PP2A consists of a heterodimer between a 36 kDa catalytic subunit (PP2Ac), and a 'constant regulatory subunit' of 65 kDa (PR65 or 'a' subunit) which is further complexed to a variable 'b' subunit [25]. The 'a' subunit of the PP2Ac/a dimer is understood to act as a scaffold, allowing the association of PP2Ac with specific b subunits [13]. Final regulation and targeting of the holoenzyme is conferred by differential association with the variable subunit b [25]. Currently, 16 b subunits have been identified and can be divided into three gene families, termed b (52 kDa), b' (53 kDA), and b'' (72 kDa and a 130 kDa splice variant) [2] [24]. The binding of PP2Ac and the b subunits is mutually exclusive.

Molecular cloning studies have identified two isoforms of the PP2A catalytic subunit (PP2Ac), that is; 2Aα and 2Aβ. As with PP1, the PP2A isoforms are highly conserved, with mammalian PP2A isoforms illustrating a >97% homology [12]. Two additional PPs have been shown to be structurally related to PP2A, that is; PP4 and PP6, which are encoded by distinct genes and share 65% and 57% homology to PP2A respectively [12]. It has been argued that PP4 and PP6 should be classified as isoforms of PP2A; however they currently remain classified as distinct families, and as such, are outside the scope of this study [12].

The crystal structure of PP2A has been recently elucidated. The amino acid sequences identity of PP1 and PP2A are highly conserved. Studies indicate that the primary amino acid sequences of PP1 and PP2A show specific similarities in their catalytic subunits, sharing 50% homology and share an overall similarity within the enzyme active site of 43% [11]. A PP2A modelled structure was developed by Chamberlin *et al.* using homology data and the PP1 X-ray structural coordinates [26]. The PP2A model was designed using Homology 2.3 module in the Insight II 2.3.7 graphic molecular modelling program which was developed by Biosym Technologies Inc. [26].

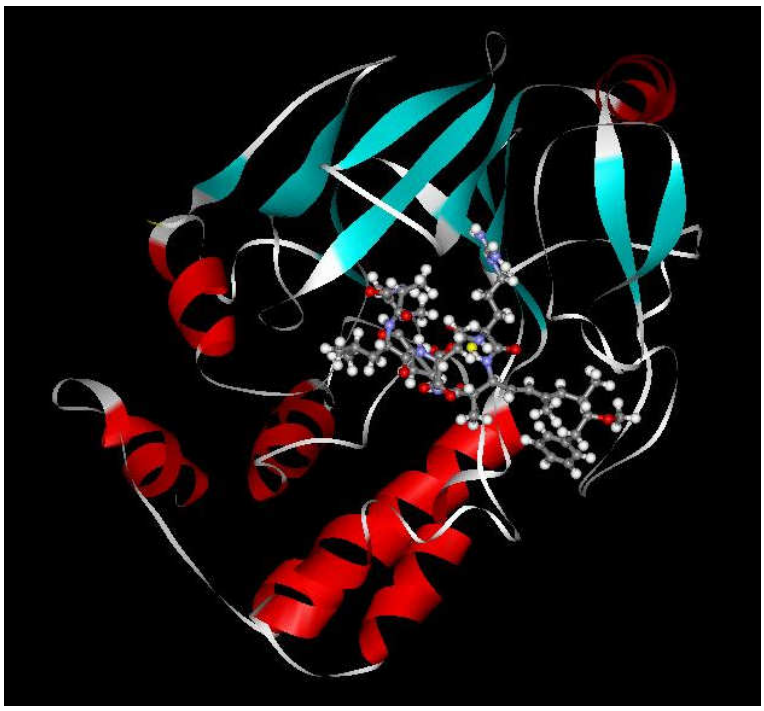


Figure 1.4: PP2A Cerius 2.2 Generated Model

PP2A accounts for up to 1% of cellular proteins and, like PP1, a major portion of serine/threonine PP activity in most cells [4]. PP2A has been identified as a negative regulator of the cell cycle progression, DNA replication, transcription, and translation [23]. PP2A is regulated by the differential expression of several forms of three different subunits, which determine its catalytic activity and its substrate specificity. PP2A also has its own endogenous protein inhibitors, termed I_1^{PP2a} and I_2^{PP2A} [18]. PP2A is also regulated by phosphorylation of its active site. That is, deactivation is achieved by phosphorylating on the threonine and tyrosine residues, with activation attained by methylation of the carboxy-terminus [6]. PP2A is activated by polyanions such as polylysine and histone H1. For a list of PP2A regulatory subunits refer to **Table 1.3**.

Table 1.3: Composition of PP2A and their Regulatory Subunits.

Catalytic Subunit	Regulatory Subunit	K_i
α, β	Aα, Aβ, Bα, Bβ, Bγ, B'α_1,	-
	B'α_2, B'β, B'γ, B'δ, B(RP72),	-
	RP130, B56α, B56β,	-
	I_1^{PP2A}	4 nM
	I_2^{PP2A}	2 nM

Inhibition is recorded as inhibition constant (K_i) values.

Data taken from [1], [6].

1.2.4 Protein Phosphatase 5

The serine/threonine PP PP5 has been shown to possess distinct structural and enzymatic properties. Other members of the serine/threonine PP generally consist of both a catalytic subunit complexed with a regulatory subunit(s) which are responsible for determining their enzymatic activity, substrate specificity and subcellular targeting [27]. PP5 consists of a single 58 kDA polypeptide chain, containing a conserved serine/threonine PP catalytic domain near its C-terminus, and three tetratricopeptide repeat (TPR) domains fused to the N-terminus of the phosphate domain [2] [19] [27]. The TPR domains acts as a scaffold, allowing interaction with proteins such as the heat shock protein 90 (hsp90), the atrial natriuretic peptide receptor, the anaphase-promoting complex, cryptochromes, and PP2A [27]. PP5 has also been shown to have comparably low phosphatase activity *in vitro* [27].

Due to the initial detection of PP5 within the cell nucleus, it was initially described as a nuclear enzyme [27]. However most subsequent reports describe PP5 as being present in the cytoplasm as some immunoreactivity is also present in the cytosol [1] [27]. PP5 appears to be involved in the promotion of cell growth, modulation of glucocorticoid receptor signalling, and termination of responses to oxidative stress [27]. Despite this progress in the identification of the biological functions of PP5, there is limited understanding of its biochemical regulation.

Activation of PP5 can be achieved by proteolysis of the TPR domain by trypsin or other proteases, allowing activation of the PP5 catalytic domain. Therefore the TPR domain is an autoinhibitory domain, however it is assumed that activation of PP5 via proteolysis does not occur *in vivo* [1] [27]. High concentrations of polyunsaturated fatty acids have been shown to relieve the autoinhibition effect by binding to the TPR domain, thus inducing conformational changes [1] [27]. Arachidonic acid has been reported as one of the most effective activators of PP5, however concentrations reported *in vitro* are much higher than those found *in vivo*, and therefore it is not thought to be a physiological activator [27]. Other reported examples include long chain saturated and unsaturated fatty acyl-CoA esters, which have shown activation at physiological concentrations, as well as binding of the C-terminus of hsp-90, which interacts with the TPR domain of PP5 which also results in activation of the enzyme [27].

1.3 Protein Phosphatases and Disease

So, why target protein phosphatases? The fundamental role that PPs play within various signalling pathways of eukaryotic cells allows possible exploitation and/or manipulation of these pathways for therapeutic purposes. There are a variety of human diseases and/or disorders that are a result of irregular cellular signalling, including cancer, diabetes, asthma, immunosuppression and HIV-AIDS.

1.3.1 Diabetes

Diabetes mellitus (sugar diabetes) is a common disorder, affecting an estimated 150 million people world wide in 2002, with these numbers expected to double by 2025 [28]. Diabetes is a carbohydrate metabolism disorder, resulting from an insufficiency in the production or utilization, or both, of insulin. Produced by the pancreas, insulin enables intercellular transport of glucose fulfilling the bodies energy requirements. In the case of diabetes, glucose is unable to, or inefficiently, enter the cell, and hence accumulates in the bloodstream in high concentrations. There are also two main types of diabetes, type 1, or insulin dependent diabetes, and type 2 or non-insulin dependent diabetes. Diabetes may manifest itself at any age, although rare among children and becomes more common with age [28]. Without treatment, recovery is almost never seen, and in fact the condition steadily worsens. Treatment, usually highly successful, is with insulin injections, particularly in children who will require injections for the rest of their lives. Oral hypoglycaemic agents appear to work when there is some active pancreatic islet tissue left, and some investigators claim that dietary control can also be successful [28].

Insulin stimulates protein phosphorylation by activating several well-characterised kinase cascades, primarily protein kinase B (PKB), downstream of the tyrosine phosphorylation of insulin receptor substrate-1 (IRS-1). Insulin also stimulates phosphatidylinositol (PI) 3-kinase activity [29] [30]. However many of the metabolic effects of insulin are mediated through dephosphorylation by, primarily, PP1 and PP2A [29] [30] [31]. Insulin stimulation is accompanied by activation of PP1 and inactivation of PP2A [32] [33]. PP1 activation occurs via phosphorylation of the glycogen associated regulatory subunit, and inactivation of PP2A which is thought to proceed through tyrosine phosphorylation of the catalytic subunit [31]. PP1, primarily the β-isoform, has been shown to target glycogen via interaction with several glycogen-targeting regulatory subunits, which bestow the substrate specificity to PP1 and mediate the specific regulation of PP1 by compartmental intracellular pools [31]. Polymorphisms in the glycogen-targeting subunit of PP1 have been implicated with some forms of type 2 diabetes, and is associated with insulin resistance [31]. Increased PP2A activity is also

believed to be a possible cause of insulin resistance [32] [33]. Therefore sequential activation of PP1 and inactivation of PP2A illustrates a suitable target for development of a therapy for insulin resistance.

1.3.2 Neurological disorders

PP1 and PP2A have been implicated in fundamental signalling process within the brain that control neuronal function. PP1 has been shown to be widely distributed and has multiple roles in neuronal function.

1.3.3 HIV-AIDS

The Human Immunodeficiency Virus (HIV), which causes Acquired Immuno Deficiency Syndrome (AIDS), principally attacks CD4 T-cells which plays a vital part of the human immune system [34]. As a result, the body's ability to resist opportunistic viral, bacterial, fungal, protozoal, and other infection is greatly diminished. *Pneumocystis carinii* pneumonia is the leading cause of death among people suffering from HIV, and neurological complications and dramatic weight loss are characteristic of the end stage of the disease, known as AIDS. HIV can be transmitted sexually; through contact with contaminated blood, tissue, or needles; and from mother to child during birth or via breastfeeding. Full-blown symptoms of AIDS may not develop for more than 10 years after infection.

HIV AIDS afflicts approximately 33.6 million people worldwide [34]. It is known that Highly Active Antiretroviral Therapy (HAART) enormously improves the health of many HIV infected individuals. However resistance to HAART treatment may occur and treatment is often limited by drug toxicity. Recent discoveries have shown that PP2A stimulates HIV replication, and PP2A inhibition blocks HIV-I replication.

1.3.4 Cancer

Cancer is arguably one of modern mans most feared diseases. Roughly 1 in 5 people will die from cancer [35]. This is complicated with the fact that cancer includes a variety of diseases, with several initiating causes, several cofactors and promoters, and with a variety of results and effects on the affected body. However it is understood that a cancer cell no longer responds to the normal social constraints of controlled cell division. These transformed cells grow, divide and continue to proliferate in an uncontrolled manner [36]. These cell cycle abnormalities characterise malignancy.

1.3.4.1 The Cell Cycle

In healthy non-malignant cells, the cell cycle is a highly regulated process allowing the reproduction of cells by duplication of their contents, followed by division into two identical cells. In eukaryotic cells, division occupies only a small part of the cell cycle, during the rest of the time cells are in interphase [35]. It is during interphase that DNA replication and other processes fundamental to cell cycle progression, including cell differentiation, gene expression and protein synthesis occur [37]. For a cell to proceed successfully through the cell cycle it is important that each stage of the cell cycle be completed prior to entry into the next. This is regulated through a series of check points.

The cell cycle is divided into four different phases; first gap phase (G_1), DNA replication (S), a second gap phase (G_2), and mitosis (M), which involves cell division into two identical daughter cells. When a cell enters G_1, normal cellular activity returns, including gene expression and protein synthesis. Many of the new cells formed now differentiate, and do not undergo further division [37]. For the continuing cells, DNA replication occurs in the S phase, during which DNA content doubles and chromosomes are replicated [35] [37]. These cells then enter the G_2 phase before progressing to mitosis. During the M phase, the replicated chromosomes condense and the sister chromatids separate, which is easily seen under a microscope [35]. It is here that division occurs and mitosis is complete when two daughter cells have formed [37]. For a diagrammatic representation of the cell cycle and its appropriate phases refer to **Figure 1.5**.

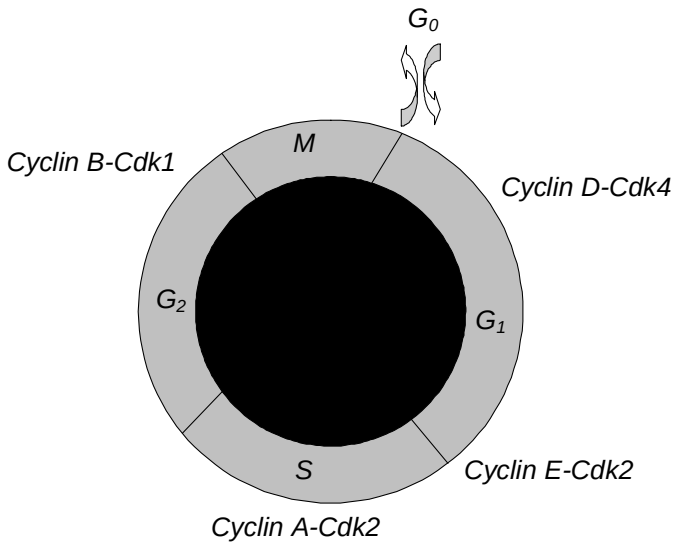


Figure 1.5: The Cell Cycle - Key Enzymes (the cyclins and catalytic cdk unit associated with progression through each phase are shown). Figure adapted from [37].

1.3.4.2 Cell Cycle Regulation

Normal cell progression through the different phases of the cell cycle is primarily controlled by the cyclin dependant kinases (cdks). These kinases comprise of a regulatory (cyclin) and catalytic (cdk) subunit, and allow a cell to pass from one phase to the next, as highlighted by the cyclin-cdk complexes shown in the above figure, and ultimately through a set of check points that monitor completion of critical events such as DNA replication and spindle formation [38]. There are two major check points in which regulate cell cycle progression. Firstly at the end of G_1, known as the restriction point, at which point a cell decides on progression through the cell cycle, and secondly at the G_2/M interphase, where DNA replication is examined to see if it is complete [37].

The regulation of the G_1 and G_2/M check points by the different cyclins-cdks complexes is also governed by the association with cdks inhibitors, especially p16, p21 and p27, and the Tumor Suppression Genes (TSG), primarily Rb and p53 [39]. The cyclin-cdks complexes are responsible for the phosphorylation of inactive enzymes involved in activating the relative cell cycle events. Cyclin D-cdk4 complex pushes cells through the G_1 phase, Cyclin E-cdk2 complex allows cells through late G_1, Cyclin A-cdk2 complex regulates S-phase entry, and Cyclin B-cdk1 complex controls the G_2/M check point (refer to **Figure 1.6**) [39]. If a cellular defect is noted, the activation of the appropriate cyclin-cdk complex is deficient and hence the cell cycle is incomplete. If no defect is detected, the cyclin-cdk complex is activated via phosphorylation, which leads to the activation of a transcription factor by the removal of an inhibitory phosphate present within the transcription factor. Transcription of the specific genes necessary for the next phase of the cell cycle occurs, including the respective cyclin and kinases genes.

1.3.4.3 Phosphorylation, Cyclin-cdk activation and the cell cycle

The role of phosphorylation within the cell cycle is classically illustrated by studies involving gene mutations of PP1 and PP2A in yeast and drosophila [40]. Loss of function mutations of both PP1and PP2A cause a variety of mitosis related defects. In yeast, PP1 mutants are unable to complete anaphase and cannot initiate chromosome segregation, whilst over expression of PP1 is lethal. In drosophila PP1 mutants, death at the larval-pupal boundary is a result of incomplete spindle formation, as well as defective sister chromatid segregation, hyperloidy and excessive chromosome condensation, and a delay in progression to mitosis. In yeast, PP2A mutants are not viable. However, mutants lacking only one PP2A subunit illustrate defects in cell separation, whilst in drosophila it leads to abnormal anaphase resolution.

PP1 and PP2A mediate regulatory control of the cell cycle by modulating the activity of the cdks and the TSG Rb [41] [42]. The activity of the cyclin-cdk complexes is dependant on cyclin kinase binding, phosphorylation level, and their interactions with appropriate inhibitory proteins. Activation of the cyclin-kinase complexes comprises of phosphorylation of the threonine residue (Thr-161) by cdk-activating kinase (CAK), followed by removal of inhibitory phosphates on tyrosine 15 (Y15) and threonine (T14) [39]. The addition of phosphates to Y15 and T14 is catalysed by Wee 1 and Mik 1, while cdc25A, B, or C phosphatases facilitate their removal [39]. PP2A plays a pivotal role in regulating this process, by stimulating Wee 1 activity, inhibiting cdc25 activity, and/or by directly dephosphorylating Thr 161 on the cyclin-cdk complex [39]. The role of PP2A in the cell cycle is shown in **Figure 1.6**.

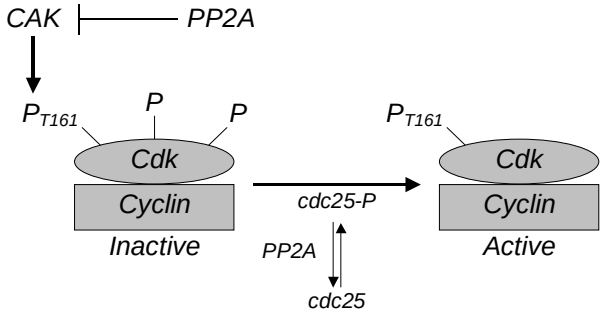


Figure 1.6: PP2A and Cyclin-cdk Activation.

The G_1/S transition check point or restriction point of the cell cycle is regulated by the cyclin D-cdk4 and cyclin E-cdk2 complexes, which mediate their effects by phosphorylating and inactivating the Tumor Suppression Gene (TSG), retinoblastoma (pRb) [39]. The pRb protein controls the movement of cells into the S-phase. Phosphorylation of pRb inhibits interaction with the S-phase transcription factor E2F, facilitating transcription of proteins needed for DNA synthesis and hence continuation of the cell cycle into the S-phase [39]. Dephosphorylation of pRb-P by PP1 blocks continuation of the cell cycle (**Figure 1.7**).

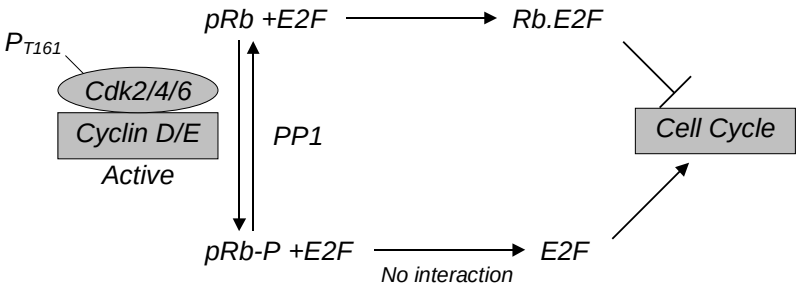


Figure 1.7: PP1 and the Phosphorylation of the Retinoblastoma Protein (pRb).

Both PP1 and PP2A are negative regulators of the cell cycle [39]. Inhibition of PP1 and PP2A abrogates the G_1 and G_2/M check points, forcing cells through the cell cycle prematurely, allowing uncontrolled mitosis. Abnormalities in cell cycle movement and check point abrogation have been described for various PP inhibitors, including Okadaic acid class of natural inhibitors, such as cantharidin, fostriecin, okadaic acid, calyculin A, microcyctin-LR, and tautomycin. In particular, fostriecin and cantharidin have been shown to force cells prematurely through the cell cycle and into mitosis with multiple aberrant mitotic spindles, subsequently inducing cell death.

1.3.4.4 Normal Cell Growth, Tumor Suppression Genes, Oncogenes and the Formation of Cancer:

The cells of multicellular organisms need to be highly specialized, thus control over cell division and growth are important. Cells enter the cell cycle when the need for new cells arise, e.g. in the repair of damaged cells associated with injured tissue. At the completion of this process, the cell cycle terminates. Whilst most embryotic cells have the ability to proliferate, not all adult cells retain this ability. Specialized stem cells are capable of growing in response to environmental factors, such as damage to surrounding cells [37]. Consequently, cell cycle events are in turn regulated by a number of social control genes. A cell that undergoes a mutation, or set of mutations disrupting the social controls associated with division, will divide without regards to the organism as a whole, and may ultimately become cancerous [35]. Two major gene families involved with such mutations involve the TSG and the oncogenes.

The Tumour Suppressor Genes (TSG) are proteins that prevent excessive growth, and include cell cycle regulators (including pRb) and genes for transcription factors (including p53). TSGs are known as negative regulators of cell proliferation. Other examples of TSG include VHL (a protein that regulates RNA polymerase II elongation), and components that regulate signalling pathways (including NF1, NF2, APC, PTEN and the TGF-β receptor) [36]. TSG are responsible for suppressing tumor formation by preventing cellular division when it is not required. The p53 protein is responsible for blocking cell cycle progression if DNA is damaged, and Rb is the gene product associated with retinoblastoma (refer to **Figure 1.7**) [36]. Any mutation that removes or otherwise modifies a check point inhibitor, such as p53, or removes or modifies a transcription inhibitor, such as Rb, will lead to loss of cell cycle regulation and potential tumour formation [38]. For a list of TSG refer to **Table 1.4**.

Table 1.4: Known Tumor Suppression Genes.

Gene	Chromosome	Neoplasm
APC	5q21-22	Familial adenomatosis
BCNS	9q31	Medulloblastoma
BRCA1	17q12-21	Breast and ovarian carcinoma
BRCA2	13q12-13	Breast carcinoma
CMAR/CAR	16q	Breast and prostate carcinoma
DCC	18q21	Colon carcinoma
INHA	Q33-qter	Gonadal tumors
MEN 1	11q13	Parathyroid, pancreatic and pituitary tumors
NF1	17q11.2	Neurofibromatosis type 1
NF2	22q12	Neurofibromatosis type 2
NM23	17q21.3	Neuroblastoma, colon carcinoma
p53	17p13.1	Breast, colon and lung carcinoma, osteosarcoma, etc.
Rb	13q14	Retinablastoma, breast, bladder and lung carcinoma
VHL	3p25	Renal carcinoma and pheochromocytoma.
WT1	11p12	Wilm's tumor

Data taken from [36].

1.3.4.5 Treatment of Malignancy

Traditionally, treatment of malignancy has relied on therapeutics that prevent uncontrolled cellular replication, or by targeting rapidly dividing cells. Radiation therapy is one method used for destroying tumor cells. However there are often difficulties in applying a sufficiently high dose to kill tumor cells without destroying the surrounding normal tissue. Additionally, there are numerous tumours that are resistant to radiation therapy. Whilst the development of chemotherapy revolutionised cancer treatment, these drugs also target normal cells, although normal cells usually have the ability to recover better that tumor cells. Another problem associated with cytotoxic drugs involves the transport or delivery of the therapeutic agent to the tumor cells and specificity. As with radiation therapy, some tumours are resistant to chemotherapy drugs. However, since cancers are a result in mutations involving TSG and oncogenes, methods for stimulating the bodies own immune system being explored have the added advantage of being able to attack secondary tumor as well as the primary tumor. These methods include, but are not limited to, gene replacement therapy and antisense gene therapy, etc [31]. However, as we have seen, it is unlikely that a single gene is responsible for the aberrant cell behaviour of cancer. To counter this there is an increasing number of therapeutic strategies promoting the apoptotic cell death pathway, enhancing treatment of conventional

chemotherapy drugs [31]. Our primary interest in PPs for the treatment of malignancy is based upon the intricate balance between PPs and PKs in regulating cell cycle entry, mitosis and apoptosis pathways [5] [6].

1.4 Inhibitors of the Serine/threonine PPs

Presently there is a variety of both natural and synthetic compounds, which inhibit the activity of the serine/threonine PPs. The natural inhibitors can be divided into two main classes of compounds that regulate or inhibit PP1 and PP2A activity. That is; the endogenous protein inhibitors (EPI), and the okadaic acid (OA) class of compounds.

Six known endogenous protein inhibitors have evolved to selectively regulate the activities of PP1 and PP2A (**Table 1.4**). However, as previously mentioned, the EPI cannot be practically used to investigate the inhibition of PPs, as they are hindered by their poor membrane permeability, high molecular weight, and potential instability [1] [6]. They are also degraded by proteolytic enzymes and can be inactivated by phosphorylation, rendering them ineffective *in vivo* [1] [6].

As a result, the OA class of compounds are of greater potential utility from both a therapeutic and for examination of original transduction viewpoint. The OA class of compound consists of a group of structurally diverse natural toxins and known tumor promoters which have been implicated in altering the phosphorylation state of proteins. The OA class of compounds comprises of a diverse structural array of secondary metabolites from a variety of biological sources, and have been shown to mediate their effects by potently inhibiting the serine/threonine PPs. Consequently, the development of OA class of compounds is important in further understanding the phosphorylation process, as well as examining inhibitor specificity, potency and availability for possible therapeutic purposes [3] [9] [12]. The OA class of compounds comprises of OA (**1**), calyculin-A (**2**), tautomycin (**3**), microcystin-LR (**4**), fostriecin (**5**), and cantharidin (**6**). For a list of OA class of compounds and their IC_{50} values for PP1 and PP2A inhibition, refer to **Table 1.5**.

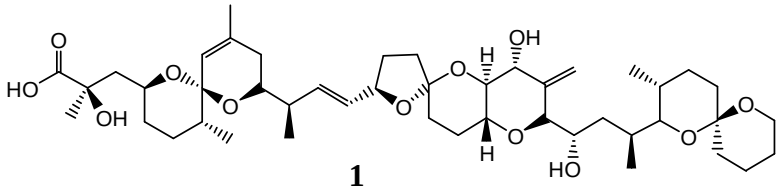
Table 1.5: OA Class of Compounds and Associated PP1 and PP2A Inhibition IC_{50} Values

Compound	PP1 (nM)	PP2A (nM)
Okadaic acid (**1**)	42	0.51
Calyculin (**2**)	2.0	0.50
Tautomycin (**3**)	0.3	1.0
Microcystin-LR (**4**)	1.7	0.04
Fostriecin (**5**)	131000	3.4
Cantharidin (**6**)	1780	260

Data adapted from [11].

Many of these toxins, though they have been shown to be cytotoxic to cancer cells, have been shown to be too toxic for clinical use, and at certain concentrations may act as tumor promoters [12] [43]. Fostriecin and cantharidin on the other hand have not been shown to promote tumor activity, and are undergoing clinical assessment [43]. Furthermore, with the exception of cantharidin and tautomycin, most of these natural toxins are not available on a large scale, hindering clinical trials and analogue development.

1.4.1 Okadaic acid



1

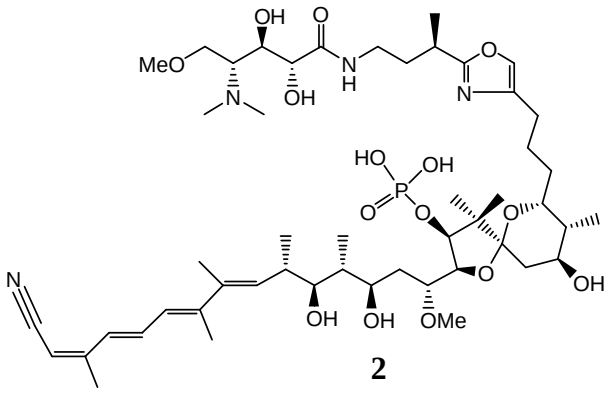
Okadaic acid (**1**) was the first, and is arguably the most well known of the serine/threonine PPs inhibitors [44] [45]. OA has been shown to be a potent inhibitor of PP1 (IC_{50} = 42 nM) and PP2A (IC_{50} = 0.51 nM), exhibiting *ca.* 100 fold PP2A selectivity [11]. Originally isolated by Tachibana *et al.* from extracts of the marine sponge *Halichondria okadai*, OA was found to be produced by several species of marine dinoflagellets in a symbiotic relationship [12] [44] [46]. OA is also the causative agent of diarrhetic shellfish poisoning [12] [47].

Okadaic acid is an acyl polyketide derivative with a highly functionalized carbon skeleton containing 17 stereogenic centers, 3 spiroketal moieties, 3 alcohol groups and a terminal carboxylic acid moiety. OA has also been shown to adopt a cyclic shape with a hydrophobic tail in solution [46]. To date there have been a number of structure activity relationship (SAR)

studies and total syntheses of OA reported. Initial total synthesis (TS) were carried out by Isobe *et al.* in 1986, which included a 106 step complex synthesis [48]. This was followed by Forsyth *et al.* in 1996, which was carried out the total synthesis in half the number of steps, as well as illustrating a 2 fold better yield and improved synthetic flexibility [49]. Still, the total synthesis of OA is difficult and time consuming.

Reported SAR data has illustrated that esterification, or complete removal of the terminal carboxylate, totally removes PP activity [12]. Similarly, acetylation of C-2, C-7, C-24 and C-27 producing okadaic acid tetraacetate, also rendered the compound inactive [12]. Therefore the acidic group was determined as necessary for binding. The 2-hydroxy moiety was also found to be crucial for binding, with modification or removal significantly hindering activity; however the 7-hydroxy is not necessary for binding [46].

1.4.2 Calyculin-A



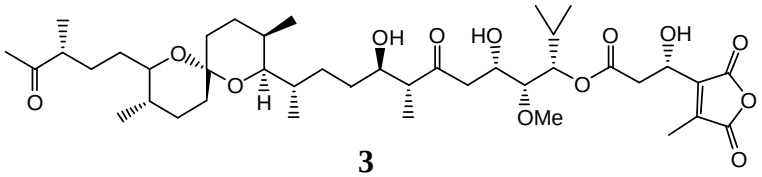
2

Calyculin-A (**2**) was first identified as an anti-tumor agent, and was isolated from the marine sponge *Discodermia calyx* [12] [47]. Calyculin-A has been shown to be a competitive inhibitor of PP1 (IC_{50} = 2 nM) and PP2A (IC_{50} = 0.5 nM), exhibiting only a 4 fold selectivity for PP2A [11]. Calyculin-A consists of a spiroketal moiety with a phosphate group attached, as well as amine, amide, hydroxy- and cyano- functionality. Other important structural features include the conjugated double bonds. The actual source organism of Calyculin-A is not known, as the spiroketal moiety is typical of secondary metabolites produced by micro-organisms, as with OA [12].

Additional Calyculin analogues isolated from the marine sponge *Discodermia calyx* include; Calyculin-B to -H. The observed modifications to the original Calyculin-A structure include

removal of the C-32 methyl carbon, as well as double bond orientation at the C-2,3 and C-6,7 olefins. These modifications have little effect on the inhibitory activity against PP2A. Other important analogues include the dephosphorylated analogue, dephosphonocalyculin, which has been shown to be a potent inhibitor of PP1 (3.0 nM) and PP2A (8.2 nM) [50]. This was of particular interest because the phosphate group was initially believed to be the primary mode of binding to the active site of the protein. However, the insignificant reduction in activity indicates that there are other important interactions necessary for binding. To date, there have been a number of TS of the Calyculin family reported, however limited published inhibition data has hindered SAR examination [51] [52].

1.4.3 Tautomycin

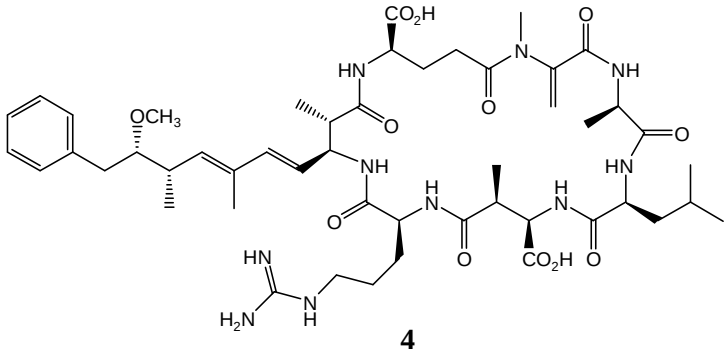


3

Tautomycin (**3**) was first isolated in 1987 from a broth of soil organisms from a previously undescribed species of Streptomyces, *Streptomyces spiroverticillatus,* during routine screening for antibiotics and has been reported to induce a morphological change in human leukemia cells (K562) [53]. Tautomycin, like OA, composes of a highly functional polyketide chain but with fewer stereogenic centers (13), a spiroketal moiety, and terminal anhydride functionality. In solution, tautomycin is also known to exist as a tautomeric mixture of the anhydride and the diacid, hence the basis of its name [12] [54]. Tautomycin exhibits structural similarities to OA including the spiroketal ring, as well as a dialkymaleic anhydride similar to that of cantharidin.

However unlike the majority of the OA class of compounds, tautomycin exhibits *ca*. 3.3 fold selectivity for PP1 (IC_{50} = 0.3 nM) over PP2A (IC_{50} = 1 nM), making it the first OA inhibitor to display PP1 selectivity [11]. Although the structural basis for this observation is not completely understood, TS and SAR data suggests that it may originate from the hydrophobic segment between C-1 and C-16 [12]. TS and SAR data of tautomycin was first published by Oikawa *et al* in 1994 [55]. Further studies have since been reported [56] [57] [58]. However unlike the rest of the OA metabolites from marine algae and micro-organisms, tautomycin can be also produced by soil organisms on a large scale.

1.4.4 Microcystin-LR



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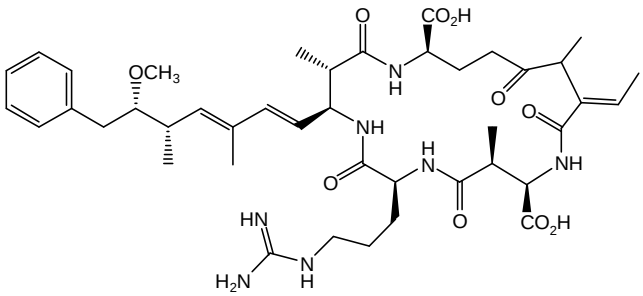

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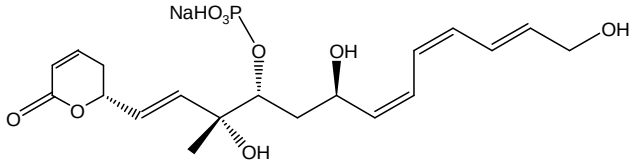
Microcystin-LR (**4**) is a heptapeptide toxin belonging to the class of natural toxins known as microcystins of which greater than 60 are known. There is also a similar class of PP inhibitors, the nodularins (**7**), which are cyclic pentapeptides. Both families of cyclic peptides are produced by cyanobacteria (blue green algae), which has been responsible for numerous human and animal poisonings [12] [59]. Microcystin-LR has been identified as the major toxin associated with Northern Hemisphere species of blue green algae *Microcystis aeruginosa*. Microcystin-LR is a potent inhibitor of PP1 (IC_{50} = 1.7 nM) and PP2A (IC_{50} = 0.04 nM), exhibiting *ca*. 50 fold PP2A selectivity [11].

Microcystins have several structural features in common including a cyclic polypeptide structure, of the general structure cyclo(D-Ala-L-X-D-erythro-β-methyl-isoAsp-L-Y-Adda-D-isoGlu-N-methyldehydro Ala), where X and Y are variable L-amino acids, and an Adda side chain. Nodularin's lacks the variable L-amino acids, and D-Ala while the *N*-methyldehydro-Ala of the microcystins is replaced by the methyl analogue, *N*-dehydroaminobutyric acid [12]. Over 60 different microcystins and 10 nodularins have been identified and biologically evaluated as PP inhibitors [59]. SAR data and the total synthesis of microcystin LR were published in 2003 [59] [60].

Notable modifications to the Microcystin skeleton include; esterification of the Glu- residue resulting in significant loss of inhibition, similarly modification of the Adda side chain resulting in loss of inhibition [59]. The *trans*-double bond system is also crucial for binding, however replacement of the C9 methoxy with a hydroxy- or acetoxy- has little to no effect [59]. Furthermore, the crystal structure of PP1 and microcystin-LR has been determined in 1995 [61]. This has further allowed determination of structural features necessary for binding.

1.4.5 Fostriecin



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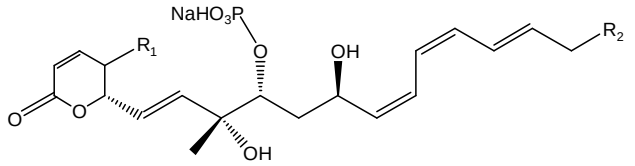


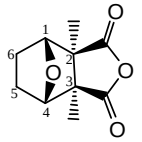
8, $R_1 = R_2 = H$
9, $R_1 = R_2 = OH$

Fostriecin (**5**) is a novel antibiotic isolated from *Streptomyces pulveraceus* along with its two related analogues, PD 113,270 (**8**) and PD 113,271 (**9**), was discovered during a routine anti-tumor screening program [62]. Fostriecin has been shown to be active *in vitro* against leukaemia (L1210, IC_{50} = 0.46 μM), lung, breast and ovarian cancer [63] [64]. Fostriecin is notable as the most selective PP2A inhibitor (IC_{50} = 3.4 nM) known, with a >40,000 fold selectivity over PP1 (IC_{50} = 131,000 nM) [11]. However phase 1 clinical trials have not been promising as blood concentration required for anti-tumor activity could not be reached due to instability of fostriecin in blood plasma and the unpredictable purity in the clinical supply of the natural product [12] [43].

Fostriecin is a phosphate ester comprising a δ-lactone ring, conjugated double bond systems, and primary, secondary and tertiary alcohols. The total synthesis of fostriecin was first completed in 2001 by Boger [65] and since then a number of total syntheses and SAR studies have been published [63] [64] [66]. SAR data has shown that removal of the phosphate ester, or disturbance to the δ-lactone ring resulted in significant loss of cytotoxicity, which suggests that

both are important for the biological activity of fostriecin [12]. It has also been shown that removal of the primary alcohol has also been shown to have little effect on the cytotoxicity of fostriecin [12].

1.5 Cantharidin



6

Cantharidin (*exo*,*exo*-2,3-dimethyl-7-oxobicyclo[2.2.1]heptane-2,3-dicarboxylic acid anhydride) is a natural toxin isolated from the Meloidae family of Coleoptera (Blister bettles), which includes approximately 1500 known species of beetles. The blister beetles employ cantharidin as a copulatory gift and as a defensive agent against predation of their eggs [11] [67]. Cantharidin has also been utilised in traditional medicines for over 2000 years, and also has the infamous reputation as the active component of 'Spanish fly', a supposed aphrodisiac [11] [68] [67]. However due to its toxicity, the use of cantharidin within modern medicine has been limited.

The use of cantharidin in traditional medicine dates back as early as 306 BC in ancient Chinese prescriptions, and has been listed as a drug under the name Mylabris in *Materia Medica* since 50 AD [67]. Traditional uses of cantharidin include the treatment of furuncles, piles, as an abortifacient, and in the treatment of dropsy [67]. The use of cantharidin as an aphrodisiac has been extensively reported in literature. This a result of urogenital tract irritation, with severe priapism in men and pelvic engorgement in women, allowing for the perceived aphrodisiac properties of cantharidin [11]. In modern medicine, cantharidin has been employed topically in the treatment of warts and molluscum since the 1950s with no reported ill effect [67].

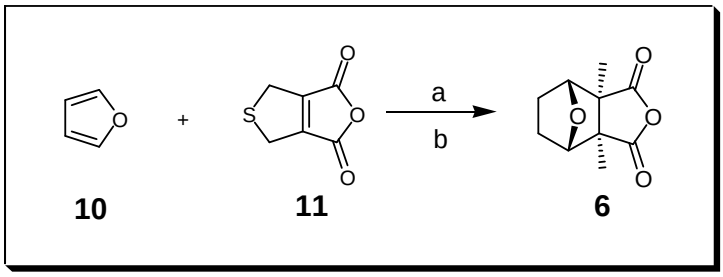
More importantly with respect to this thesis, cantharidin has been employed as an anti-tumor agent, particularly in the treatment of hepatoma and oesophageal carcinoma as early as 1264 AD [43]. In recent studies, cantharidin has been shown to be active against cervical, tongue, ginival, neuroblastoma, bone, leukaemia, ovarian, and colon cancers [11]. Interestingly, in contrast to most other anticancer drugs, it has been shown that cantharidin does not induce myelosuppression, which affects a patients immuno-response system, but in fact stimulates the production of white blood cells [11] [43]. Despite such qualities, cantharidins nephrotoxicity has halted its use in modern oncology, and 1962 cantharidin was removed from the US drug market

by the Food and Drug Administration (FDA). Despite this, cantharidin it is still available in Canada and is nevertheless being used as a traditional medicine and as an aphrodisiac [67].

Cantharidin has been identified as a toxin, as skin exposure can induce blistering and ingestion or injection may be lethal. Cantharidin has been responsible for numerous animal and human poisonings which is a result of the occurrence of blister bettles in cattle feed, and incorrect dosage or use as an aphrodisiac [67] [69] [70]. The maximum dosage of cantharidin tolerated by the body is only 10-65 mg, which would result in renal failure and liver damage, often resulting in death between 12 to 24 hours [67] [71]. Cantharidin poisoning is often characterized by nausea, hematemesis, severe abdominal pain, blistering, dysphagia, mucosal erosion and haemorrhage of gastrointestinal tract, diarrhoea, lumbar pain, coma or death [11] [67].

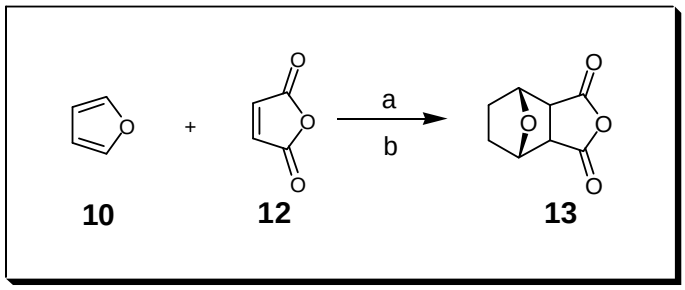
Cantharidin is a potent and competitive inhibitor of PP1 (IC_{50} = 1.78 μM) and 2A (IC_{50} = 0.26 μM), exhibiting *ca*. 10 fold PP2A selectivity [72]. It is the smallest and most drug like of the OA class of compounds, and offers the simplest structure. The anhydride moiety of both tautomycin and cantharidin is thought hydrolyse *in vivo* to the diacid [12].

Until the late 1980's, the TS of cantharidin proved problematic. The obvious route through a simple [4 + 2] Diels-Alder cycloaddition reaction furan (**10**) and dimethylmaleic anhydride has proved elusive. This is believed to be a result of the electron-donating methyl groups of dimethylmaleic anhydride, decreasing its dienophilicity, and the aromaticity of furan, hindering it a poor Diels-Alder diene [73]. Other avenues have also proved difficult and cumbersome. In 1942, Ziegler *et al.* succeeded in the TS of cantharidin (**6**), although only managed isolating a 2% yield from a complex reaction mixture. Finally, the total synthesis of cantharidin was achieved in 1980 by Dauben *et al*. via a high-pressure [4 + 2] Diels-Alder cycloaddition reaction between furan and (**11)**, followed by hydrogenation at 15 kbar with Raney nickel catalyst (refer to **Scheme 1.1**) [73]. The use of (**11**) as a dienophile assists in overcoming the electronic and steric problems associated with Diels-Alder reactions involving dimethylmaliec anhydride [73].



Scheme 1.1: Reagents and Conditions; (a) DCM 15 kbar, (b) Raney Ni, EtOAc.

The TS of norcantharidin (**13**), the demethylated analogue, is substantially simpler, completed in 2 steps commencing with a simple Diels-Alder reaction of furan (**10**) and maliec anhydride (**12**), followed by 4 atmospheres hydrogenation with Pd/C catalyst (refer to **Scheme 1.2**). The ease in synthesis makes norcantharidin a prime target for analogue development. Perhaps more importantly, it has been shown that the two methyl groups are not essential for anti-tumor activity or for the stimulation of bone marrow, but are associated with cantharidins nephrotoxicity [68]. Norcantharidin exhibits comparable PP1 (IC_{50} = 2.0 μM) and PP2A (IC_{50} = 0.5 μM) inhibition to cantharidin, as well as retaining the favourable property of not inducing myelosuppression [72]. Just as important is the fact that the associated nephrotoxicity associated with cantharidin is diminished.



Scheme 1.2: Reagents and Conditions; (a) Ether, 24 hrs, (b) Acetone, Pd-C, 4 atm, 24 hr.

1.6 Binding Interactions of the OA inhibitors with PP1 and PP2A

Knowledge of the structure and mode of action of the PP1 and PP2A active sites is important in understanding their interactions with OA class of inhibitors. The elucidation of PP1/microcystin-LR complex by Golberg *et al.* and PP1/tungsten complex by Egloff *et al.* from recombinant PP1 was achieved using X-ray crystallography, offering notable information in relation to the PP1 active site and the requirements for binding [61] [74]. PP1 active site consists of an α–helical domain including 9 α-helices and a β-sheet domain including 14 β-strands comprised of 3β-sheets [17]. The core of the catalytic subunit is comprised of a pair of

mixed β-sheets arranged in parallel to form a β-sandwich type arrangement [74]. One of these sheets is joined to the structural core unit comprised of a β-α-β-α-β structure, which is bound to the two metal ions, typically Fe^{2+} or Mn^{2+}, which are bridged by a water molecule at the active site, as illustrated in **Figure 1.8** [20].

Solution based NMR studies support this model as the conformation of microcystin-LR does not change significantly from its solution structure, and is further supported by computer modelling studies [17] and computer modelling studies support the PP1-microcystin model [75] [76]. Comparable results have also been reported in relation to other members of the OA class of natural inhibitors. For example, okadaic acid has been shown to take on a cyclic shape in solution similar to microcystin-LR due to intramolecular hydrogen bonding [46].

The dephosphorylation mechanism involves the two metal ions acting as ligands for the phosphate oxygens, the generation of a hydroxide ion which proceeds to acts as a nucleophile, whilst H125 acts as a proton donor for the alcohol leaving group [20]. The other residues are thought to orientate and stabilize the pentacoordinate phosphate group, including R96, N124 and R221 [20].

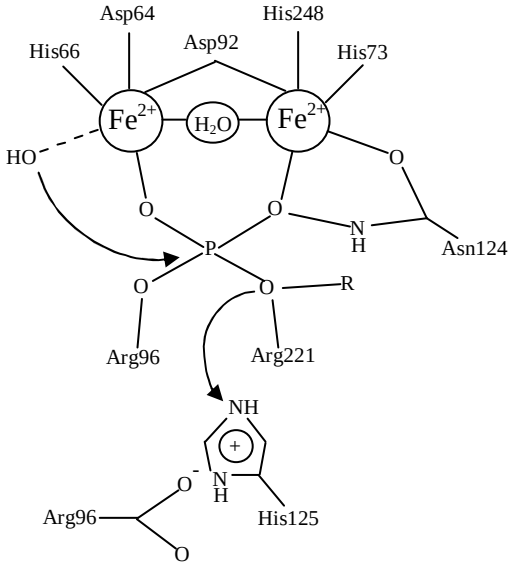


Figure 1.8.: PP1 Active Site.
Figure adapted from Lee [20].

PP2A modelling studies have relied on its 50% shared homology with PP1 [26]. Chamberlin *et al.* of University of California have developed a PP2A model based on PP1 crystal coordination's and docking studies. These advances have allowed detailed ligand docking

studies to be performed on both PP1 and PP2A, forming a more target based approach to the development of small molecule inhibitors of the Serine/threonine PP [14] [26]. With the diverse array of structural features, functionality and biosynthesis reported in relation to the OA class of serine/threonine phosphatase inhibitors it is easy to under look possible similarities in binding interactions. This has provided evidence for a common binding domain and modes of interactions between the PP1 and PP2A active sites and the members of the OA class of natural inhibitors. This includes the need for an 'acidic tail', a 'hydrophobic/peptidomimetic segment' with the exception cantharidin, and a section which mimics a peptide for binding to a phosphatase peptide domain.

The 'acid tail', such as a phosphate group (e.g., calyculin-A) or a dicarboxylate group (e.g., cantharidin and tautomycin), is hypothesised to bind to the bimetal active site. The methyl groups adjacent to the diacid group are thought to mimic the phosphorylated threonine residue, the natural substrate of the enzymes. They are also thought to be a contact point in binding, or that it places conformational restrictions on the molecule, such that it pre-organises it to a favourable conformation for binding. The 'hydrophobic/peptidomimetic segment' binds to a hydrophobic cleft in the enzyme [26]. McCluskey *et al.* have also identified an acidic groove within the active site that may be accessible to analogues possessing basic side chains [14].

1.7 References

1. Herzig, S., Nemann, J., *Effects of Serine/Threonine Protein Phosphatases on Ion Channels in Excitable Membranes.* Physiological Reviews, 2000. **80**(1): p. 173-210.
2. Barford, D., *Structural Studies of Reversibe Protein Phosphorylation and Protein Phosphatases.* Biochemical Society Transactions, 1999. **27**: p. 751-766.
3. Klumpp, S., Krieglstein, J., *Serine/Theronine Protein Phosphatases in Apoptosis.* Current Opinion in Pharmacology, 2002. **2**: p. 458-462.
4. Cohen, P., *The origins of protein phosphorylation.* Nature Cell Biology, 2002. **4**(May): p. 127-130.
5. Brautigan, D.L., *Flicking the Switches: Phosphorylation of Serine/Threonine Protein Phosphatases.* Cancer Biology, 1995. **6**: p. 211-217.
6. Sheppeck, J.E., Gauss, C., Chamberlin, R. A., *Inhibition of the Ser-Thr Phosphatases PP1 and PP2A by Naturally Occuring Toxins.* Bioorganic & Medicinal Chemistry, 1997. **5**(9): p. 1739-1750.
7. Oliver, C., J., Shenolikar, S., *Physiological Importance of Protein Phosphatase Inhibitors.* Frontiers in Bioscience, 1998. **3**: p. 961-972.
8. Garcia, A., Cayla, X., Guergnon, J., Dessauge, F., Hospital, V., Rebollo, M. P., Fleischer, A., Rebollo, A., *Serine/theronine Protein Phosphatases PP1 and PP2A are Key Players in Apoptosis.* Biochimie, 2003. **85**: p. 721-726.
9. Cohen, P., *The Role of Protein Phosphatases in Human Health ad Disease.* Eurpean Journal of Biochemistry, 2001. **268**: p. 5001-5010.
10. Barford, D., *The Mechanism of Protein Kinase Regulation by Protein Phosphatases.* Molecular Communications, 2001. **29**(4): p. 385-391.
11. McCluskey, A., Sakoff, J. A., *Small Molecule Inhibitors of Serine/Threonine Protein Phosphatases.* Mini Reviews in Medicinal Chemistry, 2001. **1**: p. 43-55.
12. Honkanen, R.E. and T. Golden, *Regulators of Serine/Threonine Protein Phosphatase at the Dawn of the Clinical Era?* Current Medicinal Chemistry, 2002. **9**: p. 2005-2075.
13. Zolnierowicz, S., *Type 2A Protein Phosphatases, the Complex Regulator of Numerous Signaling Pathways.* Biochemical Pharmacology, 2000. **60**: p. 1225-1235.
14. McCluskey, A., Ackland, S. P., Gardiner, E., Walkom, C. C., Sakoff, J. A., *The Inhibition of Protein Phosphatases 1 and 2A: A New Target for Rational Anti-Cancer Drug Design?* Anti-Cancer Drug Design, 2001. **16**: p. 291-303.
15. Kennelly, P.J., *Protein Phosphatases- A Phylogenetic Perspective.* Chemical Reviews, 2001. **101**: p. 2291-2312.
16. Bollen, M., Beullens, M., *Signaling be Protein Phosphatases in the Nucleus.* Trends in Cell Biology, 2002. **12**(3): p. 138-145.
17. Homles, C.F.B., Maynes, J. T. , Pereault, K. R., Dawson, J. F. , James, M. N. G., *Molecular Enzymology Underlying Regulation of Protein Phosphatase-1 by Natural Toxins.* Current Medicinal Chemistry, 2002. **9**: p. 1981-1989.
18. Peirce, M.J., Munday, M. R., Peachell, P. T., *Role of Protein Phosphatases in the Regulation of Human Mast Cell and Basophil Function.* The American Physiological Society, 1999. **277**: p. 1021-1028.
19. Cohen, P.T.W., *Novel Protein Phosphatases; Variety is the Spice of Life.* Trends in Biochemical Sciences, 1997. **22**: p. 245-251.
20. Lee, E.Y.C., Zhang, L., Zhao, S., Wei, Q., Zhang, J., Qi, Z. Q., Belmonte, E. R., *Phosphorylase Phosphatase: New Horizons for an Old Enzyme.* Frontiers in Bioscience, 1999. **4**: p. 270-285.
21. Herve, J.C., Sarrouilhe, D., *Modulation of Junctional Communication by Phosphorylation: Protein Phosphatases, the Missing Link in the Chain*. Biology of the Cell, 2002. **94**: p. 423-432.

22. Bollen, M., *Combinatorial Control of Protein Phosphatase-1*. Trends in Biochemical Sciences, 2001. **26**(7): p. 426-431.
23. Denu, J.M., Stuckey, J. A. , Saper, M. A. , Dixon, J. E., *Form and Function in Protein Dephosphorylation*. Cell, 1996. **87**: p. 361-364.
24. Turowski, P., Lamb, N. J. C., *The Analysis of Protein Phosphatases.* Cell Biology Protein Phosphatases, 2003: p. 1-2.
25. Strack, S., Westphal, R. S., Colbran, R. J., Ebner, F. F., Wadzinski, B. E., *Protein Serine/Threonine Phosphatases 1 and 2A Associate with and Dephosphorylate Neurofilament.* Molecular Brain Research, 1997. **49**: p. 15-28.
26. Gauss, C., Sheppeck, J. E., Nairn, A, C., Chamberlin, R., *A Molecular Modeling Analysis of the Binding Interactions Between the Okadaic Acid Class of Natural Product Inhibitors and the Ser-Thr Phosphatases, PP1 and PP2A.* Bioorganic & Medicinal Chemistry, 1997. **5**(9): p. 1751-1773.
27. Ramsey, A.J., Chinkers, M., *Identification of Potential Physiological Activiators of Protein Phosphatase 5*. Biochemistry, 2002. **41**: p. 5625-5632.
28. Blair, L., McGough, N., *Dibetes.* 2002.
29. Munro, S., Cuthbertson, D. J. R., Cunningham, J., Sales, M., Cohen, P. T. W., *Human Skeletal Muscle Expresses a Glycogen-Targeting Subunit of PP1 that is identical to the Insulin-Sensitive Glycogen -Targeting Subunit (G.Sub.L) of Liver (Protein Phosphatase 1)*. Diabetes, 2002. **51**(3): p. 591-598.
30. Cazzoli, R., Carpenter, L., Biden, T. J., Schmitz-Peiffer, C., *A Role for Protein Phosphatase 2A Activity, but not Atypical Protein Kinase C(zeta), in the Inhibition of Protein Kinase B/Akt and Glycogen Synthesis by Palmitate*. Diabetes, 2001. **50**(10): p. 2210-2218.
31. Sakoff, J., A., McCluskey, A., *Protein Phosphatase Inhibition: Structure Based Design. Towards New Therapeutic Agents.* Current Pharmaceutical Design, 2004. **10**(10): p. 1139-1159.
32. Begum, N., Ragolia, L., *cAMP Counter-regulates Insulin-mediated Protein Phosphatase-2A Inactivation in Rat Sketeal Muscle Cells.* The Journal of Biological Chemistry, 1996. **271**(49): p. 31166-31171.
33. Lehtihet, M., Honkanen, R. E., Sjoholm, A., *Inositol hexakisphosphate and Sulfonylureas Regulate beta-cell Protein Phosphatases.* Biochemical and Biological Research Communications, 2004. **316**: p. 893-897.
34. Corperation, M., *Aids.* Microsoft Encarta, 2002.
35. Alberts, B., Bray, D., Lewis, J., Raff, M., Roberts, K., Watson, J, D., *Molecular Biology of the Cell.* 1989.
36. Franks, L., M., Teich, N, M., *Cellular and Molecular Biology of Cancer.* 1997.
37. Knox, B., Ladiges, P., Evans, B., *Biology.* 1995.
38. Sherr, C.J., *The Pezcoller Lecture: Cancer Cell Cycles Revisited*. Cancer Research, 2000. **60**: p. 3689-3695.
39. Sakoff, J.A., McCluskey, A., Ackland, S. P., Sim, A., *Protein Phosphatase inhibitors as Anti-Cancer Drugs.* Manuscript (un-published), 2003.
40. Wera, S., Hemmings, B. A., *Serine/theronine Protein Phosphatases.* Biochemistry Joural, 1995. **311**: p. 17-29.
41. Stein, G.S., Baserga, R., Giordano, A., Denhardt, D. T, *The Molecular Basis of Cell Cycle and Cell Growth Control.* 1999.
42. Durfee, T., Becherer, K., Chen, Y., Yeh, S. H., Yang, Y., Kilburn, A. E., Lee, W. H., WElledge, S. J., *The Retinoblastoma Protein Associates with the Protein Phosphatase Type 1 Catalytic Subunit.* Genes and Development, 1993. **7**: p. 555-569.
43. Sakoff, J.A., Ackland, S. P., Baldwin, M. L., Keane, M. A., McCluskey, A., *Anticancer Activity and Protein Phosphatase 1 and 2A Inhibition of a New Generation of Cantharidin Analogues.* Investigation of New Drugs, 2002. **20**: p. 1-11.

44. Tachibana, K., Scheuer, P, J., Tsukitani, Y., Kikuchi, H., Van Engen, D., Clardy, J., Gopichand, Y., Schitz, F, J., *Okadaic Acid, a Cytotoxic Polyether from Two Marine Sponges of the Genus Halichondria*. Journal of the American Chemical Society, 1981. **103**(9): p. 2469–2471.
45. Schmitz, F., J., Prasad, R, S., Gopichand, Y., Hossain, M, B., Van Der Helm, D., Schmidt, P, J., *Acanthifolicin, a New Episulfide-Containing Polyether Carboxylic Acid from Extracts of the Marine Sponge Pandaros Ccanthifolium.* Journal of the American Chemical Society, 1981. **103**(9): p. 2467–2469.
46. Dounay, A.B., Forsyth, C. J., *Okadaic Acid: The Archetypal Serine/Threonine Protein Phosphatase Inhibitor.* Current Medicinal Chemistry, 2002. **9**: p. 1939-1980.
47. Cohen, P., Holmes, C. F. B. , Tsukitani, T., *Okadaic Acid a New Probe for the Study of Cellular Regulation.* Trends in Biochemical Sciences, 1990. **15**: p. 98-102.
48. Isobe, M., Ichikawa, Y., *Total Synthesis of a Marine Toxic Polyether, Okadaic Acid.* Yuki Gosei Kagaku Kyokaishi, 1986. **44**(12): p. 1155-1168.
49. Forsyth, C.J., Sabes, S, F., Urbanek, R, A., *An Efficient Total Synthesis of Okadaic Acid.* Journal of the American Chemical Society, 1996. **119**: p. 8381-8382.
50. Matsunaga, S., Wakimoto, T., Fusetani, N., Suganuma, M., *Isolation of Dephosphonocalyculin A from the Marine Sponge, Discodermia Calyx.* Tetrahedron Letters, 1997. **38**: p. 3763-3764.
51. Ogawa, A., K., Armstrong, R, W., *Total Synthesis of Calyculin C.* Journal of the American Chemical Society, 1998. **120**: p. 12435-12442.
52. Smith, A., B., Friestad, G, K., Duan, J, J., Barbosa, J., Hull, K, G., Iwashima, M., Qiu, Y., Spoors, P, G., Bertounesque, E., Salvatore, B, A., *Total Synthesis of (+)-Calyculin A and (-)-Calyculin B.* Journal of Organic Chemistry, 1998. **63**: p. 7596-7597.
53. Cheng, X., F., Kihara, T., Kusakabe, H., Magae, J., Kobayashi, Y., Fang, R, P., Ni, Z, F., Shen, Y, C., Ko, K., Yamaguchi, I., Isono, K., *A New Antibiotic, Tautomycin.* Journal of Antibiotics, 1987. **40**: p. 907-909.
54. Oikawa, H., *Synthesis of Specific Protein Phosphatase Inhibitors, Tautomycin and Tautomycetin Towards Structure-Activity Relationship Study.* Current Medicinal Chemistry, 2002. **9**: p. 2033-54.
55. Oikawa, H., Oikawa, M., Ueno, T., Ichihara, A., *Total Synthesis of Tautomycin: Efficient Aldol Coupling of Two Large Subunits.* Tetrahedron Letters, 1994. **35**: p. 4809-4812.
56. Ichikawa, Y., Tsuboi, K., Jiang, Y, M., Naganawa, A., Isobe, M., *Total Synthesis of (+)-Tautomycin.* Tetrahedron Letters, 1995. **36**: p. 7101-7104.
57. Shimizu, S., Nakamura, S., Nakada, M., Shibasaki, M., *Total Synthesis of (+)-Tautomycin.* Tetrahedron, 1996. **52**: p. 13363-13408.
58. Sheppeck, J.E., Liu, W., Chamberlin, A. R., *Total Synthesis of the Serine/Threonine-Specific Protein Phosphatase Inhibitor Tautomycin.* Journal of Organic Chemistry, 1997. **62**: p. 387-398.
59. Gulledge, B.M., Aggen, H. B. , Huang, H. B. , Chamberlin, A. R., *The Microcystins and Nodularins: Cyclic Polypeptide Inhibitors of PP1 and PP2A.* Current Medicinal Chemistry, 2002. **9**: p. 1991-2003.
60. Costerison, *An Approach to the Total Synthesis of the Protein Phosphatase 1 and 2A inhibitor Microcystin LR.* Diss., 2003.
61. Goldberg, J., Huang, H, B., Kwon, Y, G., Greengard, P., Nairn, A, C., Kuriyan, J., *Three-Dimensional Structure of the Catalytic Subunit of Serine/threonine phosphatase-1.* Nature, 1995. **376**(6543): p. 745-753.
62. Stampwala, S., S., Bunge, R, H., Hurley, T, R., Willmer, N, E., Branklewicz, A, J., Steinman, C, E., Smitka, T, A., French, J, C., *Novel antitumor agents CI-920, PD 113,270 and PD 113,271. II. Isolation and characterization.* Journal of Antibiotics, 1983. **36**: p. 1601-1605.

63. Kazuyuki, M., Masahiro, I., Hitoni, K., Satoko, M., Taskeshi, I., *Total Synthesis of an Antitumor Antibiotic, Fostriecin*. Journal of the American Chemical Society, 2003. **125**: p. 8238-8243.
64. Lewy, D.S., Gauss, C. M. , Soenen, D. R. , Boger, D. D., *Fostriecin: Chemistry and Biology*. Current Medicinal Chemistry, 2002. **9**: p. 2005-2032.
65. Boger, D., Ichikawa, S., Zhong, W., *Total Synthesis of Fostriecin (CI-920)*. Journal of the American Chemical Society, 2001. **123**: p. 4161-4167.
66. Wang, Y., G., Kobayashi, Y., *Formal Total Synthesis of Fostriecin*. Organic Letters, 2002. **4**(26): p. 4615-4618.
67. Moed, L., Shwayder, T. A. , Chang, M. W., *Cantharidin Revisited*. Arch Dermatol, 2001. **137**: p. 1357-1360.
68. Wang, G., *Medical uses of Mylabris in Ancient China and Recent Studies*. Journal of Ethnopharmacology, 1989. **26**: p. 147-62.
69. McCluskey, A., Keane, M. A., Mudgee, L., Sim, A. T. R., Sakoff, J. A., *Anhydride Modified Cantharidin Analogues. Is Ring Opening Important in the Inhibition of Protein Phosphatase 2A?* European Journal of Medicinal Chemistry, 2000. **35**: p. 957-964.
70. McCluskey, A., Taylor, C. , Quinn, R. J., *Inhibition of Protein Phosphatase 2A by Cantharidin Analogues*. Bioorganic and Medicinal Chemistry Letters, 1996. **6**(9): p. 1025-1028.
71. Honkanen, R.E., *Cantharidin, Another Natural Toxin that Inhibits the Activity of Serine/Threonine Protein Phosphatases Types 1 and 2A*. Federation of European Biochemistry Society Letters, 1993. **330**: p. 283-286.
72. Sakoff, J., A. and A. McCluskey, *Protein Phosphatase Inhibition:Structure Based Design. Towards New Therapeutic Agents*. 2003.
73. Dauben, W., G., Kessel, C, R., Takemura, K, H., *Simple, Efficient Total Synthesis of Cantharidin via a High-Pressure Diels-Alder Reaction*. Journal of the American Chemical Society, 1980. **102**: p. 6893-6894.
74. Egloff, M., Cohen, P, T, W., Reinemer, P., Bardford, D., *Crystal Structure of the Catalytic Subunit of Human Protein Phosphatases 1 and its Complex with Tungstate*. Journal of Molecular Biology, 1995. **254**: p. 942-959.
75. Trogen, G., B., Annila, A., Eriksson, J, E., Konttelt, M., Meriluoto, J., Sethson, I., Zdunek, J., Edlund, U., *Conformational Studies of Microcystin-LR using NMR Spectroscopy and Molecular Dynamics Calculations*. Biochemistry, 1996. **271**: p. 3197-3205.
76. Bagu, J.R., Sonnichsen, F. D., Williams, D., Anderson, R. J., Sykes, B. D., Holmes, C. F. B., *Comparison of the Solution Structures of Microcystin-LR and Motuporin*. Nature Structural Biology, 1995. **2**: p. 114-116.

Chapter 2

Lead Selection and Analogue Development

2.1 Introduction

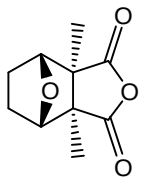
Developments in life sciences, technology and data analysis have significantly changed the field of medicinal chemistry and drug design. This progress include, but are not limited to; advances in molecular modelling, development of new synthetic strategies and approaches (such as combinatorial chemistry, parallel and solid phase synthesis), new screening methods (such as virtual and high throughput screening), human genome research, and the promise of proteonomics. However these developments have not accelerated the number of new chemical entities (NCEs) entering the market place, and have emphasized the need for the efficient assembly of small molecules as potential modulators for therapeutics and drug design [1]. The need for functional pharmacology has forced the pharmaceutical industry to move from serendipity based to rational based research, utilising well-designed target based approaches including *in vitro*, *in vivo* and *in silico* screening [1] [2].

With respect to the regulation of PP1 and PP2A activity, the elucidation of the PP1 active site and the availability of extensive SAR data has aided the design of small molecule inhibitors [3] [4]. Although pivotal roles have been implied for both PP1 and PP2A in controlling the cell cycle, little progress of their use as therapeutic targets has occurred, perhaps a result of the ubiquitous nature of these signalling proteins. Examination of PP1/PP2A inhibitors in cell growth assays highlights their potential utility as anticancer agents. However the correlation between *in vivo* PP1 and PP2A inhibition and *in vitro* cytotoxicity is a confluence of the physical and chemical properties of the molecules being evaluated, such as cell permeability, lipophilicity, pK_a, H-bonding and H-donor effects. For example, microcystin-LR, a potent PP1 and PP2A inhibitor, is highly water soluble, but lacks a means of rapid cellular uptake which has significantly reduced *in vivo* cytotoxicity [5]. This is in contrast to the response shown by okadaic acid and calyculin A, which both display broad a spectrum cytotoxicity and are referred to as universal apoptogens [3].

Poor pharmacological properties, coupled with their toxicity, has stymied the development of the majority of the OA class of natural inhibitors as anticancer agents, with fostriecin and cantharidin the exceptions holding the best therapeutic promise. Large scale analogue

development of the majority of the OA class of natural inhibitors has also been restricted due to limited supplies and/or difficult synthetic routes, with the exception of tautomycin and cantharidin [3]. Furthermore, it has been shown that certain members of the OA class of inhibitors act as tumor promoters including okadaic acid (mouse skin assay), although only at extremely high concentrations relative to the IC_{50} (100-300 μM), calyculin A (mouse skin assay), microcystin-LR (rat liver), while others have fail to demonstrate these effects under similar conditions, such as tautomycin (mouse skin assay) which induces apoptosis [3]. The contradictory response of tumour promotion/inhibition shown by the OA class of compounds in which inhibit the same family of enzymes is not completely understood, however it may be simply due to potency, expression of PP isoforms in the assays used, physical properties of the inhibitor, or a combination of factors [3].

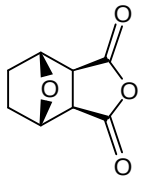
Evaluation of cantharidin (**6**) has highlighted numerous features that make it an excellent lead compound for drug development. Cantharidin has been used for almost a millenium as an anticancer agent, possesses known toxicity, and is synthetically simple and amenable to synthetic manipulation. Additional favourable aspects include excellent lipophilicity, it is not a substrate for P-glycoprotein (cell lines over expressing this protein remain sensitive to cantharidin), it does not require intracellular activation, or induce the unfavourable property of myelosuppression, as is the case with most chemotherapy drugs [6] [7]. In recent studies, cantharidin has been shown to be active *in vitro* against tongue, cervical, ginival, mucoepidermoid carcinoma, adenocystic carcinoma, neuroblastoma, bone, leukaemia, ovarian and colon cancer cell lines, with GI_{50} values ranging between 1.3-15 μM [8] [9]. However, cantharidin has been shown to display nephrotoxicity, possesses a low therapeutic index, illustrates multi-modal toxicity, and displays an irritant effect on urinary organs, which has been related to the aphrodisiac effects [4] [10].



6

Norcantharidin (**13**), the demethylated analogue of cantharidin, has comparable PP1 and PP2A activity, along with a similar cytotoxicity profile. As with cantharidin, norcantharidin is active *in vitro* against cervical, ovarian, hepatoma, laryngocarcinoma, osteocarcinoma, leukaemia cell

lines with GI_{50} values ranging between 13-47 μM [9]. In clinical trials, norcantharidin has been shown to increase the mean survival time of 285 patients suffering primary hepatoma from 4.7 to 11.1 months, and the yearly rate of survival from 17 to 30%, compared with 102 patients with conventional chemotherapy treatments (5FU, hydroxycamptotherine, vincristine, thiophosphoramide and mitomycin) [10]. Furthermore, norcantharidin has been shown to posses many of cantharidins favourable properties, but surprisingly, not only did it not induce myelosuppression or little to no urinary tract irritation, but it induces haemopoiesis (bone marrow stimulation) [10]. Norcantharidin also has the beneficial property of significantly reduced nephrotoxicity and a simple total synthesis making it a suitable lead for analogue development [6] [10].



13

2.2 Background

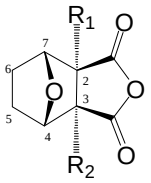
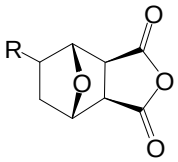
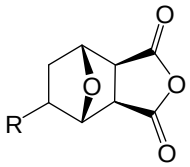
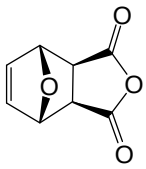
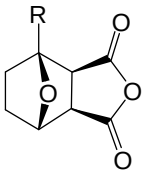
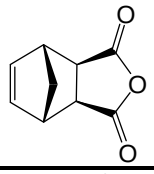
2.2.1 Current Development of Cantharidin Analogues

The main focus in developing cantharidin analogues is to examine the effect of structural modification on PP1 and PP2A activity. Reported modifications include; removal of one or both of the methyl groups at either C2 and/or C3; the addition of alkyl groups to the bicyclo[2.2.1]heptane skeleton, a 5,6-double bond; modification of the anhydride moiety by ring opening or substitution; modification of the etheral bridgehead, and a combination of the described modifications.

Skeletal modification, such as removal of one or both of the 2,3-methyl groups, as with palsonin (**14**) and norcantharidin (**13**) respectively, exhibited no significant effect on the inhibitory properties [4]. However, removal of both methyl carbons significantly aids synthetic ease. Inclusion of a double bond at the 5,6-positions (**19**) has little effect on inhibition of PP1, but similarly promotes synthetic ease. Alkyl substitutions at the 5,6-position (**15**-**18**) considerably reduces PP1 and PP2A activity, whilst improving selectivity and inhibition of PP2B [4]. Introduction of alkyl substituents at 1- or 4-position (**20**) is detrimental to PP1 and PP2A inhibition [4]. Modification to the 7-oxobridgehead, including substitution with a methylene carbon (**21**), heteroatom such as sulphur or nitogen, or with complete removal of the

bridgehead, has generally resulted in a loss of inhibition. This group is thought to be involved in H-bonding with the active site. For examples and results of skeletal modifications and their effect on PP1 and PP2A inhibition refer to **Table 2.1**.

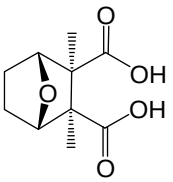
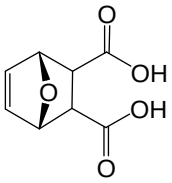
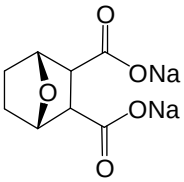
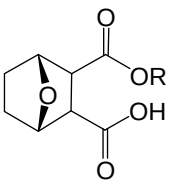
Table 2.1: Effects of Skeletal Modification on PP1 and PP2A inhibition (recorded as IC_{50} or % inhibition measured at 100 μM compound concentration).

Compound				Protein Phosphatase Inhibition		
				PP1	PP2A	PP2B
		R_1	R_2			
	6	Me	Me	1.78 μM [a]	0.26 μM [a]	N/R
	14	Me	H	656 nM [b]	120 nM [b]	N/R
	13	H	H	93% [c]	90% [c]	39% [c]
		R				
	15	$PhCO_2CH_2$		N/R	N/R	11.0 μM [d]
	16	$Ph(CH_2)_3CO_2CH_2$		N/R	N/R	1.2 μM [d]
		R				
	17	$AcOCH_2$		94% [c]	81% [c]	24% [c]
	18	$BzOCH_2$		76% [c]	81% [c]	44% [c]
	**19**			91% [c]	90% [c]	46% [c]
		R				
	20	CH_2OAc		23% [c]	7% [c]	30% [c]
	**21**			N/R	48.1% [e]	N/R

[a] Data taken from [11]; [b] Data taken from [12]; [c] Data taken from [13]; [d] Data taken from [14]; [e] Data taken from [15]; N/R = not reported.

Studies have shown that the ring opened dicarboxylic acid analogues (**22**) are only slightly weaker PP2A inhibitors. However the bis-sodium salt (**23**) showed improved inhibition (0.17 μM), but no explanation was supplied, with the generation of the dicarboxylic acid anticipated under assay conditions [4]. This is most likely a result of how the assay was conducted. Molecular modelling studies have shown that the slightly reduced inhibition illustrated by the dicarboxylic analogues is most likely a direct result of increased conformational flexibility compared to the parent anhydride [15]. Similarly, the mono-ester analogues (**24-26**) displayed a significant loss of PP2A inhibition as well as reduced PP1 inhibition; however modest selectivity towards PP1 was observed. More interestingly, the mono-esterified analogues showed comparable inhibition to the parent anhydride, suggesting that at least one acid group is needed for binding. For examples and results of anhydride ring opening and their effect on PP1 and PP2A inhibition refer to **Table 2.2**.

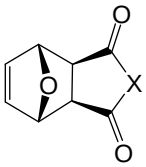
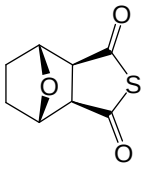
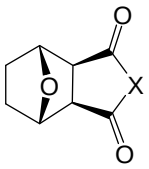
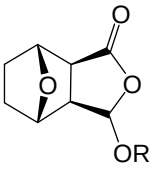
Table 2.2: Effects of Anhydride Ring Opening on PP1 and PP2A inhibition (recorded as IC_{50} or % inhibition measured at 100 μM compound concentration).

Compound			Protein Phosphatase Inhibition	
			PP1	PP2A
	**22**		N/R	92-95% [a] (53 nM) [b]
	**22b**		N/R	80% [c] (300 μM) [c]
	**23**		N/R	(0.17 μM) [d]
		R		
	24	Me	(4.7 μM) [e]	(0.41 μM) [e]
	25	Et	(3.0 μM) [e]	(0.45 μM) [e]
	26	n-Pr	(4.8 μM) [e]	(0.47 μM) [e]

[a] Data taken from [16]; [b] Data taken from [17]; [c] Data taken from [18]; [d] Data taken from [19] ; [e] Data taken from [11]; N/R = not reported.

Substitution of the oxygen in the anhydride moiety had mixed results. Replacement with nitrogen (**27**) typically results in reduced inhibition, whilst replacement with sulphur, as in endothall thioanhydride (**29**), a commercially available agricultural insecticide, has been shown to improve PP2A activity [20]. Other heteroatom substitutions include the formation of the cantharimides (**30**-**33**), which involved substitution at the anhydride position with primary and secondary amines, as well as a variety of amino acids. Amino acids with acidic, basic and non-polar residues were examined, with the basic amino acid, such as D- and L-histidine (**32**, **33**) displaying comparable PPs inhibition to the parent anhydride. Cantharimides exhibiting non-polar and acidic amino acid residues showed poorer PP1 and PP2A inhibition. Selective reduction of one of the carbonyl carbons has also received attention. For examples and results of anhydride modification and their effect on PP1 and PP2A inhibition refer to **Table 2.3.**

Table 2.3: Effects of Anhydride Modification on PP1 and PP2A inhibition (recorded as IC_{50} or % inhibition measured at 100 μM compound concentration).

Compound			Protein Phosphatase Inhibition	
			PP1	PP2A
		X		
	27	NH	N/R	15% [a]
	28	S	N/R	97% [a]
	**29**		N/R	608 nM [b]
		X		
	**30**	D-Tyr	101 μM [c]	112 μM [c]
	31	L-Tyr	570 μM [c]	245 μM [c]
	32	D-His	3.22 μM [c]	0.81 μM [c]
	33	L-His	2.82 μM [c]	1.35 μM [c]
		R		
	**34**	H		
	35	Me	>1000 μM [d]	>1000 μM [d]
	36	Et	746 μM [d]	55 μM [d]
	37	n-Pr	>1000 μM [d]	>1000 μM [d]

[a] Data taken from [3]; [b] Data taken from [21]; [c] Data taken from [22]; [c] Data taken from [11]; N/R = not reported.

The ongoing development of SAR data occurs through the design and testing of libraries of compounds for PP1 and PP2A inhibition, cytotoxicity against cell lines and molecular modelling studies. However, it has been noted that modification of the parent skeleton of cantharidin and its analogues has not provided potent analogues, with few exceptions, showing that cantharidin tolerates little in the way of modification whilst retaining PP1 and PP2A activity [23].

The result of these studies has lead to the identification of several structural features of which have been shown to be important for PP1 and PP2A activity. The 2,3-methyl groups are thought to play a role in PP1 and PP2A inhibition as they mirror the methyl groups in larger toxins, such as tautomycin, and have been hypothesised to position the reactive group, however they are not crucial for binding (**4**, **8**). The 7-oxo group has been found to be required for binding. It is thought to act as a hydrogen donor or acceptor at the active site. The anhydride moiety is also an important feature; however ring opening of the anhydride moiety to the dicarboxylic acid (**16**), or the ability to form at least one carboxylic acid close to the 7-oxa position, as with the monoester (**19**-**21**), is also tolerated. In general, structure activity data of the important features required for inhibition has been summarised in **Figure 2.1**.

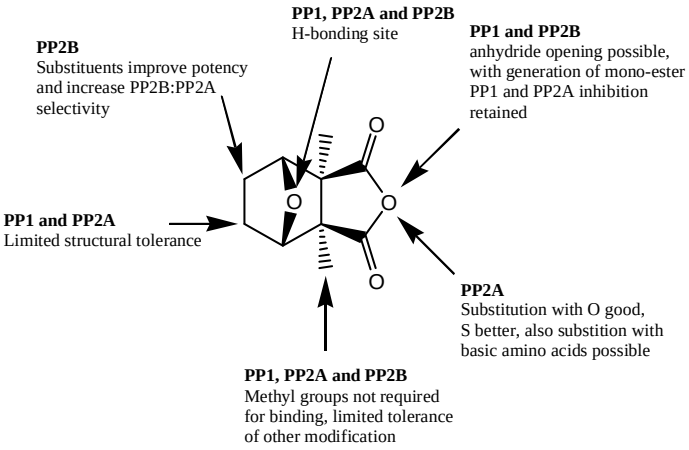


Figure 2.1: Cantharidin Pharmacophore
Figure taken from [4]

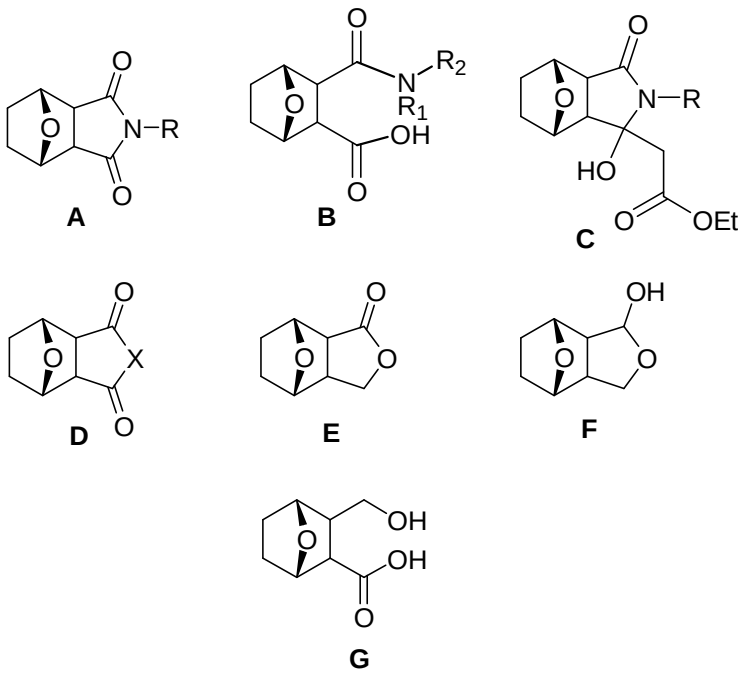
Previous studies from our laboratory have illustrated that the presence of an acidic moiety is important for PP inhibition. It has also been hypothesised that ring opening prior to binding is an important feature. However the generation of a series of ring opened analogues possessing a monoester displayed comparable PP2A selectivity and potency, questioned the necessity for both carboxylates to be present for PP1 and PP2A inhibition [23]. Therefore the ability to form a minimum of one free carboxylate is thought to be required to maintain PP1 and PP2A activity [9]. It is thus conceivable that a minimal pharmacophore of the bicycle[2.2.1]heptane skeleton include a single carboxylate, and a basic side chain would lead to the development of a new series of PP1 and PP2A inhibitors [23].

These new developments have formed the synthetic focus of this study, with the generation of norcantharidin analogues possessing at least one carboxylate, and either a neutral, basic or acidic side chain with an ability to interact with the acidic groove of the active site will generate NCEs exhibiting PP1 and PP2A activity and good cytotoxicity.

2.3 Project Aims

2.3.1 Target Selection

Extending McCluskey's minimal pharmacophore approach requires the synthesis and evaluation of target libraries at the periphery and core of this model. This allows the generation of new targets from rationally designed libraries for further analogue development. Hence a series of target compounds were designed and synthesised as part of this thesis. The proposed analogues include: development of a series of ring closed (**A**) and ring opened cantharimides (**B**), possessing a range of substituted primary and secondary amines, including amino acids with acidic and basic functionality; development of a series of α-hydroxylactams (**C**), similarly possessing a range of neutral, acidic and basic functionality; substitution of the oxygen of the anhydride with a heteroatom (**D**); and the formation of a selectively reduced lactone/lactol (**E**/**F**) and ring opened analogue (**G**). Libraries (**A**-**G**) will be evaluated for ability to inhibit PP1 and PP2A, and as potential cytotoxic agents against a panel of tumour cell lines.



The ring closed cantharimides (**A**), previously reported by McCluskey *et. al.*, illustrate an interesting synthetic target [22]. This is primarily a result of their retained PPs activity, their

unique nature relative to the natural inhibitors, as well as the ease of chemical synthesis and addition to current library diversification. Furthermore, it is not yet known whether they hydrolyse *in vivo* like anhydride moiety of cantharidin and tautomycin, primarily due to the replacement of oxygen with a primary amine. Interestingly, from the analogues reported in literature, only those possessing a free carboxylate and a basic side chain maintained potency. Therefore, we targeted the investigation of the effect on removal or moving of the carboxylate away from the 7-oxa, and addition of; hydrophobic tails, acidic and basic residues, polar moieties and aromatic rings, in order to explore the chemical space and or binding requirements of the cantharimide family.

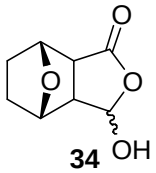
Similarly the ring opened cantharimides (**B**), which were previously unreported, satisfy many of the requirements for a new synthetic target. This is primarily due to their ease of chemical synthesis, but more importantly, they possess a free carboxylate and a basic side chain, requirements needed for the related ring closed cantharimides. Furthermore, if *in viv*o ring-opening of the ring closed analogues is inhibited, these analogues may prove to retain the activity of the parent anhydride. Their development also aids in library diversification, and allows possible further synthetic modification. In this thesis, we investigated the effect of the addition of; hydrophobic tails, acidic and basic residues, polar moieties and aromatic rings, in order to explore the chemical space and or binding requirements of the ring opened cantharimide family.

The development of the α-hydroxylactams (**C**) accesses previously unexplored chemical space in relation to PPs and anticancer activity, however similar chemistry has been reported in literature [24]. The development of this family of compound is important due to the structural diversity compared to current cantharidin analogues, and its interesting synthetic chemistry. Furthermore, the α-hydroxylactams represent a third generation family within our group, and allow further investigation into what pharmacological modifications will be tolerated whilst maintaining potency. Investigation of the removal or moving of one of the carboxylates away from the 7-oxa, and addition of hydrophobic tails, acidic and basic residues, polar moieties and aromatic rings were approached in order to explore the chemical space and or binding requirements of the α-hydroxylactam family for binding. Furthermore, the presence of a lactam moiety is expected to have a significant effect on ring opening.

The development of norcantharidin analogues with the oxygen of the anhydride moiety substituted by a heteroatom (**D**), either nitrogen or sulphur was investigated to evaluate the

effect of facile ring opening. That is, what effect, if any does replacement of the oxygen have on the rate of ring opening, and what implication this has on PP1/PP2A inhibition and anti-cancer activity.

The development of the lactone (**E**) and lactonol (**F**) analogues was of interest due to the complete removal of one of the carbonyl carbons, and to investigate the inhibition of the ring opening. This is significant due to recent findings of our research group, with the development of (**34**) containing a selectively reduced carbonyl carbons to a secondary alcohol. Previous studies in our laboratory have shown that (**34**) possesses interesting PP and anticancer activity, especially the selective targeting of colon rectal carcinoma. Screening of analogues (**E**, **F**) will investigate the importance of the secondary alcohol, as well as the removal of the second carbonyl carbon. They may also prove to be good lead compounds for the generation of new analogues with the introduction of etheral side chains.



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The development of the ring opened analogue (**G**) was important due to the resemblance of the bis-sodium salt (**18**) and mono-esterified (**22-24**) analogues, and the removal of one carbonyl carbon (**G**), may also be used in the generation of new analogues including mono-esterified or etheral analogues.

2.4 References

1. Walker, M.J.A., Barrett, T., Guppy, L. J., *Functional Pharmacology: the Drug Discovery Bottleneck.* Drug Discovery Today: Targets, 2004. **3**(5): p. 208-215.
2. Friedrich, C.M., Paterson, T. S., *In Silico Predictions of Target Clinical Efficacy.* Drug Discovery Today: Targets, 2004. **3**(5): p. 216-222.
3. Honkanen, R.E. and T. Golden, *Regulators of Serine/Threonine Protein Phosphatase at the Dawn of the Clinical Era?* Current Medicinal Chemistry, 2002. **9**: p. 2005-2075.
4. McCluskey, A., Sakoff, J. A., *Small Molecule Inhibitors of Serine/Threonine Protein Phosphatases.* Mini Reviews in Medicinal Chemistry, 2001. **1**: p. 43-55.
5. Laidley, C.W., Cohen, E., Casida, J. E., *Protein Phosphatases in Neuroblastoma Cells:[H3] Cantharidin Binding Site in Relation to Cytotoxicity.* Journal of Pharmacuetical Experimental Theory, 1997. **280**: p. 1152-1158.
6. Sakoff, J., A., Howitt, I, J., Ackland, S, P., McCluskey, A., *Serine/Threonine Protein Phosphatase Inhibition Enhances the Effect of Thymidylate Synthase Inhibition.* Cancer Chemotherapy and Pharmacology, 2004. **53**: p. 225-232.
7. Sakoff, J.A., Ackland, S. P., Baldwin, M. L., Keane, M. A., McCluskey, A., *Anticancer Activity and Protein Phosphatase 1 and 2A Inhibition of a New Generation of Cantharidin Analogues.* Investigation of New Drugs, 2002. **20**: p. 1-11.
8. Efferth, T., Rauh, R., Kahl, S., Tomicic, M., Bochzelt, H., Tome, M. E., Briehl, M. M., Bauer, R., Kaina, B., *Molecular Modes of Action of Cantharidin in Tumor Cells.* Biochemical Pharmacology, 2005. **69**: p. 811-818.
9. McCluskey, A., Ackland, S. P., Bowyer, M. C., Baldwin, M. L., Garner, J., Walkom, C., Sakoff, J, A., *Cantharidin Analogues: Synthesis and Evaluation of Growth Inhibition in a Panel of Selected Cell Lines.* Bioorganic Chemistry, 2003. **31**: p. 68-79.
10. Wang, G., *Medical uses of Mylabris in Ancient China and Recent Studies.* Journal of Ethnopharmacology, 1989. **26**: p. 147-62.
11. McCluskey, A., Bowyer, M. C., Collins, E., Sim, A. T. R., Sakoff, J, A., Baldwin, M. L., *Anhydride Modified Cantharidin Analogues: Synthesis., Inhibition of Protein Phosphatases 1 and 2A and Anticancer Activity.* Bioorganic and Medicinal Chemistry Letters, 2000: p. 1687-1690.
12. Li, Y.M., C. Mackintosh, and J.E. Casida, *Protein Phosphatase 2A and its Cantharidin/Endothall Thioanhydride Binding Site.* Biochemistry Pharmacology, 1993. **46**: p. 1435.
13. Sodeoka, M., Baba, Y., Kobayashi, S., Hirukawa, N., *Structure-Activity Relationship of cantharidin derivatives to protein phosphatases 1, 2A, and 2B.* Bioorganic Medicinal Chemistry, 1997. **7**(14): p. 1833-1836.
14. Tatlock, J.H., Linton, M. A., Hou, X. J., Kissinger, C. R., Pelletier, L. A., Showalter, R. E., Tempczyk, A., Villafranca, J. E., *Structure-Based Design of Novel Calcineurin (PP2B) Inhibitors.* Bioorganic Medicinal Chemistry Letters, 1997. **7**(8): p. 1007-1012.
15. McCluskey, A., Keane, M. A., Mudgee, L., Sim, A. T. R., Sakoff, J. A., *Anhydride Modified Cantharidin Analogues. Is Ring Opening Important in the Inhibition of Protein Phosphatase 2A?* European Journal of Medicinal Chemistry, 2000. **35**: p. 957-964.
16. Honkanen, R.E., *Cantharidin, Another Natural Toxin that Inhibits the Activity of Serine/Threonine Protein Phosphatases Types 1 and 2A.* Federation of European Biochemistry Society Letters, 1993. **330**: p. 283-286.
17. Li, Y.M., Casida, J. E., *Cantharidin-Binding Protein: Identification as Protein Phosphatase 2A.* Proceedings from the National Academy of Science (USA), 1992. **89**: p. 11867-11870.

18. McCluskey, A., Taylor, C., Quinn, R. J., *Inhibition of Protein Phosphatase 2A by Cantharidin Analogues.* Bioorganic and Medicinal Chemistry Letters, 1996. **6**(9): p. 1025-1028.
19. Enz, A., Zenke, G., Pombo-Viliar, E., *7-Oxa[2.2.1]bicycloheptane-2,3-dicarboxylic acid Derivatives as Phosphatase Inhibitors.* Bioorganic Medicinal Chemistry Letters, 1997. **7**: p. 2513.
20. Knapp, J., Boknik, P., Huke, S., Gombosova, I., Linck, B., Luss, H., Muller, F, U., Muller, T., Nacke, P., Schmitz, W., Vahlensieck, U., Neumann, J., *Contractility and Inhibition of Protein Phosphatases by Cantharidin.* Genaral Pharmacology, 1998. **31**(5): p. 729-733.
21. Laidley, C.W., Dauben, W. G., Guo, Z, R., Lam, J. Y. L., *2-Carboxymethylendothal Analogues as Affinity Probes for Stabilized Protein Phosphatase 2A.* Bioorganic Medicinal Chemistry Letters, 1999. **7**(12): p. 2937-2944.
22. McCluskey, A., Walkom, C., Bowyer, M. C., Ackland, S. P., Gardiner, E., Sakoff, J. A., *Cantharimides: A New Class of Modified Cantharidin Analogues Inhibiting Protein Phosphatases 1 and 2A.* Bioorganic and Medicinal Chemistry Letters, 2001. **11**: p. 2941-2946.
23. McCluskey, A., Ackland, S. P., Gardiner, E., Walkom, C. C., Sakoff, J. A., *The Inhibition of Protein Phosphatases 1 and 2A: A New Target for Rational Anti-Cancer Drug Design?* Anti-Cancer Drug Design, 2001. **16**: p. 291-303.
24. Murray, W.V., Lalan, P., Gill, A., Addo, M. F., Lewis, J. M., Lee, D. K. H., Wachter, M. P., Rampulla, R., Underwood, D. C., *Substituted Pyrrolidin-2-one Biphenyltetrazoles as Angiotensin II Antagonists.* Bioorganic and Medicinal Chemistry Letters, 1993. **3**(2): p. 369-374.

Chapter 3

3.1 Biological Assays – Experimental

3.1.1 Introduction

In addition to robust and reliable chemistry, the efficient development of biologically active molecules also depends on specific and streamlined biological screening processes. In relation to PP1 and PP2A inhibitors, both radioactive and non-radioactive assays have been reported, however non-radioactive assays are favoured as a result of reduced costs and increased safety and throughput [1]. Baykov first reported a non-radioactive malachite green assay in 1988 [2]. However, variation in inhibition results have been shown over a wide range of concentration, as well as dependence on assay conditions, eg., temperature, choice of substrate. For this study, a malachite green assay as described by Gupta was adopted [1]. This was due to its increased robustness, less variance on assay conditions, as well as the simple commercial availability.

A variety of cancer cell lines were also used for *in vitro* cytotoxicity. These cell lines were cultured in appropriate media, and maintained at the Mater Hospital in Newcastle, Australia. All compounds were initially screened at 100 μM (unless specified otherwise) drug concentration against HT29 & SW480 (Human colon carcinoma), MCF-7 (breast), A2780 (ovarian), H460 (lung), A431 (skin), DU145 (prostrate), Be2-C (neuroblastoma), SJ-G2 (glioblastoma) G401 (Human Caucasian kidney carcinoma, Wilm's Tumor), cell growth inhibition. Where warranted, a full GI_{50} dose response curve was obtained. This was typically conducted for those analogues displaying 100% inhibition at 100 μM drug dose. A GI_{50} value is the drug concentration required to inhibit cell growth by 50% relative to a control. It should also be noted that all the described biological testing was carried out by Dr Sakoff and Gilbert research group at the Marter Hospital.

3.1.2 Protein Phosphatase Inhibition

Serine/threonine phosphatase assay kit, including phosphorylated hexapeptide (Lys-Arg-pThr-Ile-Arg-Arg) purified PP1 (rabbit skeletal muscle), PP2A (human red blood cells), were purchased from Upstate Biotechnology, Lake Placid, NY. Concentrations of PP1, PP2A and substrate were 0.3 mU/well, 30 mU/well, and 200 μM, respectively. Reactions were initiated by the addition of 5 μL of substrate to a mixture containing 5 μl of enzyme, 10 μl buffer (50 mM Tris-HCl, pH 7.0, 100 M CaCl2) and 10 μl inhibitor. Total well volume was 30 μl. Prior to the addition of substrate, the inhibitor and enzyme were preincubated for 10 min. Reactions were incubated at RT for 60 min. Reactions were halted via the addition of malachite green solution

(50 μl), with UV/vis absorbance readings taken at 650 nm after 10 min developing time. Subsequent inhibitor dose response curve of percentage enzyme activity verse drug concentration of drug required to inhibit enzyme activity by 50%. Data represents the mean (±SEM) IC_{50} of at least three independent replicates.

3.1.3 Cell Cultures and Stock Solutions

All stock solutions were stored at -20 ºC and prepared as follows; Cantharidin (Biomol, USA), and synthesised analogues, 10 mM solution in phosphate buffered saline (PBS). Cell lines were cultured at 37 °C under 5% CO_2 in air. The A2780 (human ovarian carcinoma) and HT29 (human colon carcinoma) cell lines were maintained in Dulbecca's modified Eagle's medium (DMEM) (Trace Biosciences, Australia) supplemented 10 mM sodium bicarbonate. The G401 (human kidney carcinoma), were maintained in McCoys (Trace Biosciences). The H460 (human lung carcinoma) cell line was maintained in RPMI 1640 supplemented with glucose and pyruvate. All cultured media was further supplemented with foetal bovine serum (10%), penicillin (100 IU/ml), streptomycin (100 μg/ml) and glutamine (4 mM). Cells were passaged every 3-7 days and all cell lines were routinely tested and found to be mycoplama free.

3.1.4 *In Vitro* Growth Inhibition Assay

Cells in logarithmic growth were transferred to 96–well plates. Cytotoxicity was determined by plating cells in triplicate in 100 μL medium at a density of 2,500–3,500 cells/well for all cell lines. On day 0, (24 h after plating) when the cells were in logarithmic growth, 100 μL medium with or without the test agent was added to each well. After drug exposure growth inhibitory effects were evaluated using the MTT (3-[4,5-dimethyltiazol-2-yl] 2,5-diphenyl-tetrazolium bromide) assay and absorbance read at 540 nm. The IC_{50} was the drug concentration at which cell growth is 50% inhibited based on the difference between the optical density values on the day 0 and those at the end of drug exposure [3].

3.2 Chemistry - Experimental

3.2.1 General Instrumentation

Melting points (Mp) were recorded on a Stuart Scientific SMP1 melting point apparatus and are uncorrected.

Infrared (IR) spectra (υ max cm^{-1}) were recorded on a Perkin-Elmer Paragon 1000 Fourier-transform IR spectrophotometer. Samples were applied as a thin film to a NaCl plate and spectra recorded at 4 cm^{-1} resolution.

Low resolution Electron Impact Mass Spectra (EIMS, 70 eV) were recorded on either a Shimadzu QP-5050A Gas Chromatograph Mass Spectrometer (GCMS) or QP-2100 GCMS. Samples were prepared by dissolving 1 mg of sample in approximately 1 mL of solvent (usually HPLC grade chloroform, methanol or acetone), injecting 1 μL, and the resultant gas chromatograph and mass spectra recorded.

Low resolution Electrospray Ionisation Mass Spectra (ESIMS) were recorded on an Agilent Series 1100 Mass Selective Detector (MSD). Samples were prepared by dissolving 1 mg of sample in approximately 1 mL of solvent, usually High Performance Liquid Chromatography (HPLC) grade acetonitrile or methanol, diluting to 5 μg/mL, injecting 10 μL, and the resultant gas chromatograph and mass spectra recorded.

Nuclear Magnetic Resonance (NMR) spectra (both ^{1}H and ^{13}C spectra) were recorded on a 300 MHz Bruker Avance-300DPX NMR spectrometer. Chemical shifts were reported in parts per million (δ ppm). For proton spectra recorded in deuterated solvents ($CDCl_3$, DMSO-d_6 or CD_3OD), and the residual solvent peaks were used as the internal reference with 7.25, 2.5, and 3.35 ppm respectively. Similarly, for carbon spectra the residual solvent peaks for chloroform, dimethyl sulfoxide (DMSO) or methanol were used as internal reference at 77.0, 39.7, and 49.0 ppm respectively. Multiplicities for proton spectra are abbreviated: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, b = broad. Coupling constants were reported in Hertz (Hz). Distortionless Enhancement by Polarisation Transfer (DEPT) was used to assign the carbon spectra.

3.2.2 Chromatography

Analytical Thin Layer Chromatography (TLC) was performed on Merck aluminium back TLC sheets pre-coated with silica gel 60 F_{254} (0.2 mm layer thickness). Developed plates were visualized through exposure to UV light, followed by exposure to either anisaldehyde, permanganate or ammonium molydbate dips combined with heat.

All traditional flash chromatography was carried out using a modified method described by [4] using Aldrich silica gel, 200-400 mesh 60 Å, and solvents as indicated. Automated flash

chromatography was carried out an ISCO Combi-Flash retrieve system employing redisep prepacked silica columns and solvents as indicated. All short column chromatography was carried out using a modified method described by [4] using Merck silica gel, (60 PF), and solvents as indicated. Recrystalisation of compounds was by the method of vapour diffusion using solvents as indicated.

3.2.3 Reagents and Solvents

Many reagents were commercially available (Sigma/Aldrich,) and were used as supplied. All bulk solvents were distilled prior to use. When necessary, solvents were purified and/or dried according to procedures set out in [5]. Acetone was dried by distillation from sodium iodide. *N*,*N*-Dimethylformamide (DMF) was dried by distillation from calcium sulphide and storing over activated sieves under nitrogen. Tetrahydrofuran (THF) was dried by distillation from sodium / benzophenone under nitrogen. Methanol was distilled from magnesium and iodide under nitrogen. Toluene was distilled from sodium / benzophenone under nitrogen.

3.2.4 Reaction Conditions

All reactions requiring dry solvent were performed using oven dried glassware (130 °C/24 hrs) and under nitrogen or argon atmosphere, or where applicable, using a calcium chloride drying tube. Syringes and needles were oven dried (130 °C) and cooled in an evacuated desiccator prior to use. All organic extracts were dried over anhydrous $MgSO_4$ or Na_2SO_4 as indicated. Solvents were removed *in vacuo* using a Buchi rotatory evaporator. Residual solvents removed using high vacuum. Reactions were carried out using traditional glassware, reaction station vessels, including Radley Carosel or Buchi Syncore Reactor as specified.

3.3 References

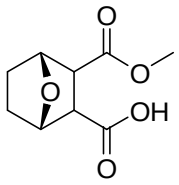
1. Gupta, V., Ogawa, A. K., Du, X., Houk, K. N., Armstrong, R, W., *A Model for Binding of Structurally Diverse Natural Product Inhibitors of Protein Phosphatases PP1 and PP2A.* Journal of Medicinal Chemistry, 1997. **40**: p. 3199-3206.
2. Baykov, A.A., Evtushenko, O. A., Avaeva, S. M., *A Malachite Green Procedure for Orthophosphate Determination and its use in Alkaline Phosphate-Based Enzyme Assay*. Analytical Biochemisrty, 1988. **171**: p. 226-270.
3. Sakoff, J., A., Ackland, S, P., *Thymidylate Synthase Inhibition Induces S-phase Arrest, Biphasic Mitochondrial Alterations and Caspase-Dependent Apoptosis in Leukaemia Cells.* Cancer Chemotherapy and Pharmacology, 2002. **46**(6): p. 477-487.
4. Still, C.W., Kahn, M. , Mitra, A., *Rapid Chromatographic Technique for Preparative Separations with Moderate Resolution*. The Journal of Organic Chemistry, 1978. **43**(14): p. 2923–2925.
5. Perrin, D., Aramagero, W. L. F., Perrin, D. R., *Purification of Laboratory Chemicals.* 1996(4th Rev.).

Chapter 4

Development of Ring-Closed Cantharimide as Potential PP1 and PP2A Inhibitors

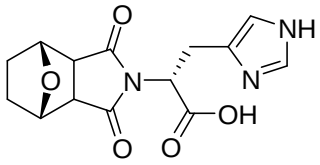
4.1 Introduction

The ring opening of norcantharidin (**6**) and tautomycin (**3**) to the dicarboxylic form has been described as an important characteristic for PP1 and PP2A activity [1]. However ring-opened norcantharidin analogues developed by our group such as the monoester (**24)**, possess only one free carboxylate, display retained PP1 activity and a slight increase in PP2A selectivity, undermines the necessity of *in vivo* ring opening for activity [2]. Furthermore, the recent development of ring-closed cantharimde analogues, including lead compound, the D-histidine cantharimide (**32)**, further challenge the importance of a dicarboxylic acid, and forces us to re-evaluate the necessary components for PP1 and PP2A inhibition [3].



PP1 IC_{50} = 4.7 μM
PP2a IC_{50} = 0.41 μM

24



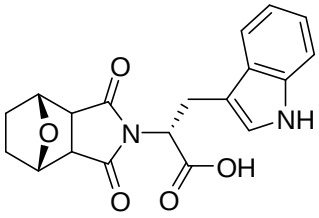
PP1 IC_{50} = 3.22 μM
PP2a IC_{50} = 0.81 μM

32

McCluskey *et. al.* has reported modelling data suggesting that compounds with the etheral bridge head of cantharidin (the 7-oxa position), a minimum of a single free carboxylic acid group, and a basic residue could give rise to new PP1 and PP2A inhibitors [3]. This is based on data suggesting that the single carboxylic acid of the ring opened analogues, such as the monoester, still binds in the active site of PP1 and PP2A, with the alkyl chain of the ester being proximal to an acidic groove in the proteins active site. Although, more recent work as a result of this thesis suggests that, especially with PP2A that there is a small molecule binding pocket (at least one) in addition to the primary catalytic binding site [4] [5]. More importantly, in respect to the cantharimides, the major amino acids in this groove are acidic and, consequently, analogues possessing a basic residue may lead to retained or improved PP1/PP2A

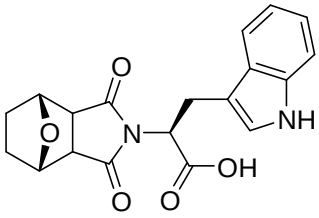
inhibition [3] [6]. Additionally, McCluskey hypothesised a correlation between PP2A selectivity and potency, and cytotoxicity against a number of tumor cell lines [6].

Bioassay results of reported ring-closed cantharimide analogues developed by our research group have shown strong support of McCluskey's hypothesis, with four analogues (**30-33**) possessing a basic residue displaying good to excellent PP1 and PP2A activity, as well as exhibiting a general selectivity for PP2A. As yet no ring-closed cantharimide analogues possessing neutral or acidic residues have afforded a potent PP1 or PP2A inhibitor [6]. Also, no difference was generally observed between different diastereomers, the exception being **30** which exhibited a 5 fold PP2a activity over **31** [6] [3].



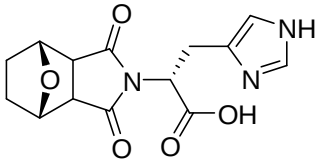
PP1 IC_{50} = 101 μM
PP2a IC_{50} = 112 μM

30



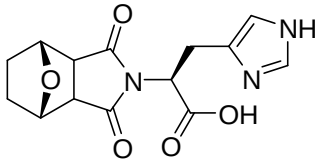
PP1 IC_{50} = 570 μM
PP2a IC_{50} = 112 μM

31



PP1 IC_{50} = 3.22 μM
PP2a IC_{50} = 0.81 μM

32



PP1 IC_{50} = 2.82 μM
PP2a IC_{50} = 1.35 μM

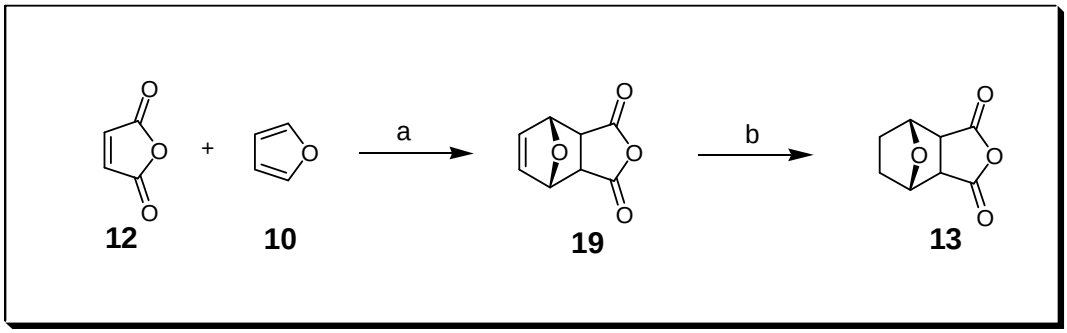
33

The main aim of this chapter was the development of ring-closed cantharimide analogues for screening and to further examine the McCluskey's hypothesis. The range of amine and amino-acid substituents chosen, similar to the analogues described in Chapter 5 (ring-opened analogues), represent a diverse range of size, flexibility and spatial distribution, including simple aliphatic chains and aromatic rings, alcohols, primary and secondary amines, as well as acidic and basic amino-acids. These analogues were designed to examine the effects of skeletal modifications on the resulting PP inhibition and, consequently, anticancer activity.

4.2 Results and Discussion – Chemical Synthesis

4.2.1 Synthesis of Norcantharidin

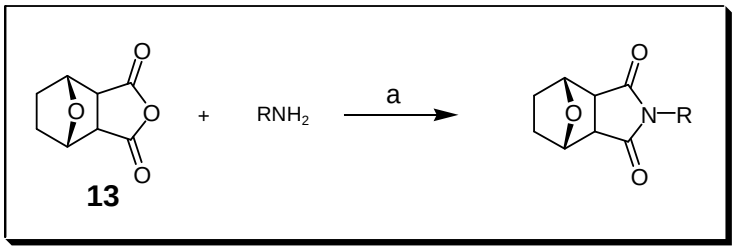
The generation of the commercially available lead compound norcantharidin (**13**) was carried out using a simple and economical 4 + 2 π Diels Alder condensation reaction, by stirring at room temperature in diethylether forming the 5,6-dehydro analogue (**19**), followed by hydrogenation with 10% Pd/C catalysis in acetone, with recrystallisation from ethylacetate afforded pure (**13**). Both reactions are high yielding > 82%, with the yield limiting step being the recrystallisation from ethylacetate. Other solvent systems can be used, for example pure diethylether or hexane/ethylacetate mixtures which may improve yield, however purity is reduced and hence ethylacetate is the preferred recrystalisation solvent. A general reaction scheme is described in **Scheme 4.1**.



Scheme 4.1: Reagents and Conditions; (a) Et_2O, RT, 48 h; (b) Acetone, H_2, Pd-C 4 atm, RT, 24 h.

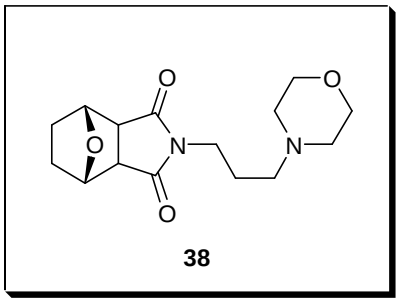
4.2.2 General Synthesis of the Alkyl, Phenyl and Benzyllic Ring-Closed Norcantharimide Analogues

The generation of the alkyl, phenyl, and benzyllic ring closed cantharimide analogues were carried by the treatment of (**13**) with an appropriate primary amine in the presence of triethylamine in THF at reflux for 36 hours. This facilitated a condensation reaction to provide a range of cyclic imides [3] [7]. A general reaction scheme is depicted in **Scheme 4.2**.



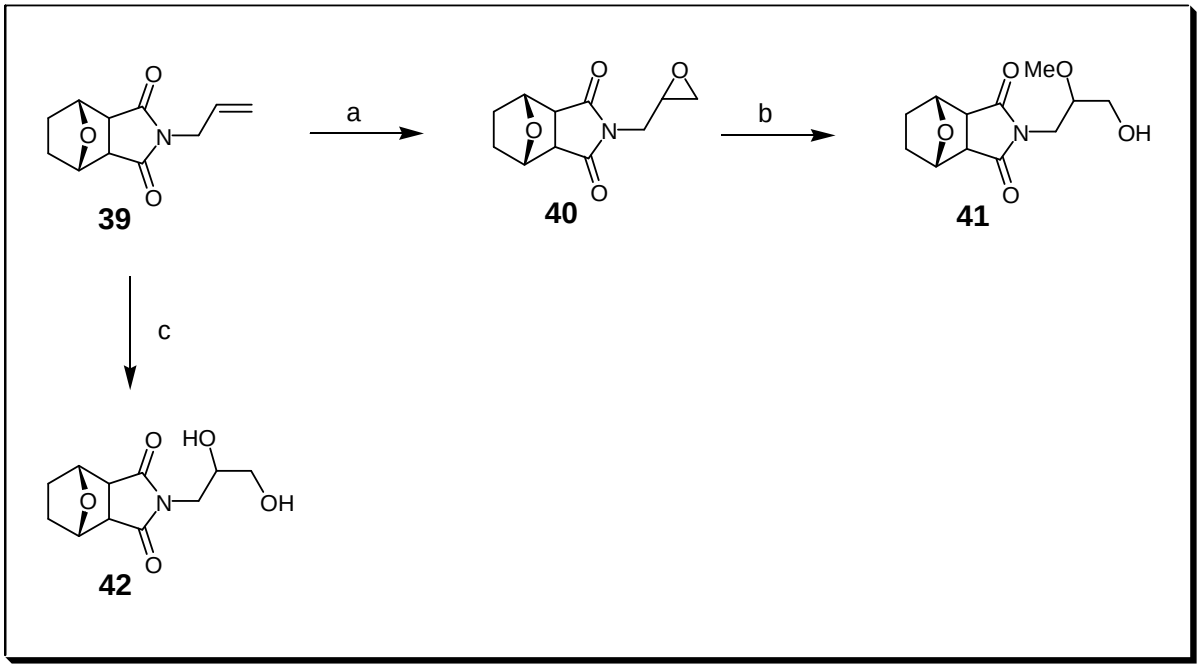
Scheme 4.2: Reagents and Conditions; (a) NEt_3, THF reflux, 36 h.

Yields generally ranged from poor to excellent, with only the *N*-propylmorphilino (**38**) displaying poor yield. The general synthesis was simplified due to the precipitation of the product typically at room temperature, which proved much simpler than methods described by Lin *et. al.*, whom employed high pressure approaches [8]. This allowed the robust and rapid generation of a variety of analogues for screening.



4.2.3 General Synthesis of the Allylic Derived Ring Closed Cantharimide Analogues

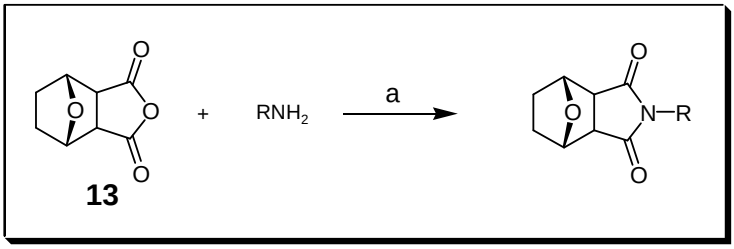
The *N*-allyl analogue (**39**) allowed easy functionalisation to either; (i) the diol (**42**) (using osmium tetroxide) or, (ii) the methoxy alcohol analogue (by firstly treating (**39**) with *m*CPBA forming the epoxide (**40**), followed by methanolysis upon treatment with (1*S*)-(+)-10-camphorsulfonic acid to the methoxy alcohol (**41**)). A general scheme is depicted in **Scheme 4.3**.



Scheme 4.3: Reagents and Conditions; (a) *m*CPBA, RT, 16 h (b) (1*S*)-(+)-10-Camphorsulfonic acid / MeOH; (c) OsO_4 / NMO, acetone / H_2O, 80 ºC, 16 h.

4.2.4 General Synthesis of the Amino acid Ring Closed Cantharimide Analogues

The generation of the aminoacid ring closed cantharimide analogues was carried out using a method described by Zhou *et. al.* [9]. This involved stirring norcantharidin (**13**) and the relevant amino acid at reflux in dry DMF overnight affording the respective pure cantharimide analogue in moderate to good yields. A general reaction scheme is depicted in **Scheme 4.4**.



Scheme 4.4: Reagents and Conditions; (a) DMF, reflux, 24 h.

4.3 Results and Discussion – Biological Assay

Cytotoxicity screening was carried out against a panel of human cancer cell lines grown routinely by our research group; including HT29 and SW480 (colorectal carcinoma), G401 (kidney carcinoma), MCF-7 (breast adenocarcinoma), A2780 (ovarian carcinoma), H460 (lung carcinoma), A431 (epidermoid carcinoma), DU145 (prostate carcinoma), BE2-C (neuroblastoma) and SJ-G2 (glioblastoma). Where warranted, GI_{50} values were determined. That data is shown in **Table 4.1** through **4.4**.

Firstly, the cytotoxicity of a variety of simple alkyl cantharimide analogues was analysed, as well as cantharidin (**6**) and norcantharidin (**13**) as controls, as shown in **Table 4.1**. As expected, the controls, cantharidin (**6**) and norcantharidin (**13**), display broad spectrum anticancer activity with (**6**) displaying a 10-fold improved potency against its demethylated analogue. The simple alkylated cantharimide analogues generally exhibited poor to moderate activity in relation to controls (**6**) and (**13**), with the notable exception being the long alkyl chain analogues (**48**-**50**) which displayed improved broad spectrum cytotoxicity, particularly against HT29 (colon), in comparison to the shorter and branched or cyclic alkyl analogues. This is most likely a response of improved cellular uptake due to the lypophilic alkyl chain; however the octadecyl analogue (**51**) did not follow this trend, suggesting further increases in chain length may not lead to improved activity. Analogues (**39**, **54**), possessing a terminal double bond, displayed variable results, with the allyl analogue (**39**) displayed moderate selectivity for MCF-7 (breast adenocarcinoma) cells, as well as promising GI_{50} of 80 ± 12 µM, and the but-3-ene analogue exibited poor inhibition; however both analogues allow a handle for further analogue development.

Table 4.1: Cytotoxicity of cantharidin (**6**), norcantharidin (**13**) and a variety of simple alkylated norcantharimides (**43**-**54**) in a panel of human cancer cell lines. Cytotoxicity levels are first expressed as % inhibition at 100 µM drug concentration (*in italics*) and, if potent, as Growth Inhibition, GI_{50} µM (**in bold**)

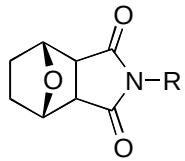
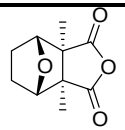
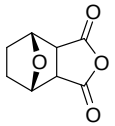
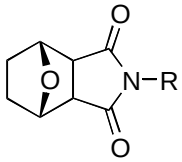
Compound	R	HT29	G401	SW480	MCF-7	A2780	H460	A431	DU145	BE2-C	SJ-G2
											
6		**32 ± 0.1**	**-**	**4.5 ± 0.3**	**7.5 ± 0.4**	**4.4 ± 0.3**	**3.3 ± 0.2**	**2.9 ± 0.2**	**2.1 ± 0.3**	**3.7 ± 0.6**	**1.7± 0.1**
13		**57 ± 5**	**-**	**44 ± 6**	**68 ± 4**	**38 ± 1**	**45 ± 3**	**31 ± 1**	**28 ± 3**	**43 ± 6**	**23± 3**
43	Et	*<10*	*17.5*	*<10*	*<10*	*<10*	*<10*	*<10*	*<10*	*<10*	*<10*
44	*n*-Pr	*16 ±11*	*13.8*	*45 ±44*	*<10*	*<10*	*<10*	*<10*	*<10*	*<10*	*<10*
45	*n*-Bu	*<10*	*65*	*<10*	*<10*	*<10*	*<10*	*<10*	*<10*	*<10*	*<10*
46	*n*-Hexyl	*<10*	*22.3*	*<10*	*<10*	*<10*	*<10*	*<10*	*<10*	*<10*	*<10*
47	*n*-Octyl	*70 ±11*	*39.7*	*65 ±4*	*88 ±18*	*50 ±10*	*<10*	*61 ±3*	*44 ±11*	*68 ±16*	*54 ±11*
48	*n*-Decyl	*20 ±1*	-	*59 ±0*	*44 ±4*	*44 ±7*	*>100*	*52 ±5*	*83 ±4*	*3 9 ±4*	*72 ±2*
49	*n*-Dodecyl	*25 ±4*	-	*55 ±2*	*52 ±8*	*40 ±4*	*53 ±4*	*35 ±4*	*66 ±4*	*40 ±6*	*58 ±2*
50	*n*-Tetradecyl	*19 ±0*	-	*52 ±4*	*43 ±7*	*35 ±4*	*66 ±7*	*47 ±3*	*73 ±2*	*50 ±4*	*49 ±5*
51	*n*-Octadecyl	*<10*	-	*<10*	*<10*	*<10*	*<10*	*<10*	*<10*	*<10*	*<10*
52	*s*-Bu	*<10*	*26.8*	*<10*	*<10*	*<10*	*<10*	*<10*	*<10*	*<10*	*<10*

Table 4.1 (Continued): Cytotoxicity of cantharidin (**6**), norcantharidin (**13**) and a variety of simple alkylated norcantharimides (**43-54**) in a panel of human cancer cell lines. Cytotoxicity levels are first expressed as % inhibition at 100 µM drug concentration (*in italics*) and, if potent, as Growth Inhibition, GI_{50} µM (**in bold**)

Compound	R	HT29	G401	SW480	MCF-7	A2780	H460	A431	DU145	BE2-C	SJ-G2
											
53	*c*-Hexyl	*<10*	*22.3*	*<10*	*<10*	*<10*	*<10*	*<10*	*<10*	*<10*	*<10*
39	Allyl	*19 ± 9*	-	*50 ± 26*	*90 ± 14*	*67 ± 12*	*20 ± 8*	*32 ± 5*	*15 ± 17*	*62 ± 5*	*2 ± 29*
					80 ± 12						
54	But-3-enyl	*<10*	-	*<10*	*<10*	*<10*	*<10*	*<10*	*<10*	*<10*	*<10*

NB: A % inhibition value of <10% indicates that the compound failed to influence cell growth when compared with untreated controls.

Given the promising data associated with simple alkyl cantharimides, the effect of additional functionality was explored, in the hope that this would have an impact on the ability of these analogues to penetrate the cell membrane hence improving their resultant cytotoxicity. **Table 4.2** shows the cytotoxicity of a variety of alkyl substituted ring-closed cantharimide analogues possessing alcohol, methoxy, epoxide, morpholine moeties and, in the case of (**62-63**), dimeric norcantharimides, against a panel of human cancer cell lines. The cytotoxicity displayed by the introduction of epoxy and alcoholic ranged from poor (**41**, **57-59**), to moderate (**40**, **55-56**), to good activity for the 1,2-diol (**42**) analogue. GI_{50} values for the diol were also determined for four cell lines (SW480, MCF-7, A2780 and BE2-C with GI_{50}'s of 62 ± 2, 46 ± 4, 59 ± 2 and 70 ± 4 µM, respectively) and also modest levels of activity at the remaining five cell lines (*ca*. 45% at 100 µM). The dimeric norcantharimides displayed moderate to excellent activity, with the bis-analogue together with the dodecyl linker (**63**) displaying broad spectrum activity in excess of norcantharidin (**13**), with GI_{50} values ranging from 8.3 ± 0.7 to 60 ± 6 µM across the tumor cell lines screened. Due to the encouraging results displayed by the introduction of oxygen bearing functionality, cantharimide analogues possessing morpholine functionality were developed, however, the morpholine analogues (**38**, **60-61**) failed to display any significant activity.

Table 4.2: Cytotoxicity of a variety of functionalised ring-closed norcantharimide (**55-63**) analogues in a panel of human cancer cell lines. Cytotoxicity levels are first expressed as % inhibition at 100 µM drug concentration (*in italics*) and, if potent, as Growth Inhibition, GI_{50} µM (**in bold**).

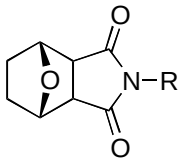
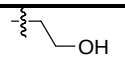
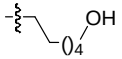
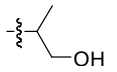
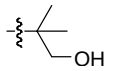
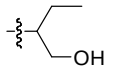
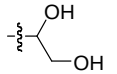
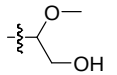
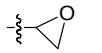
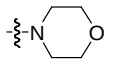
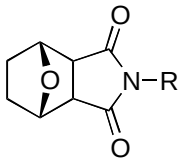
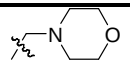
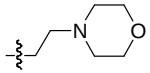
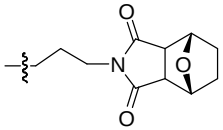
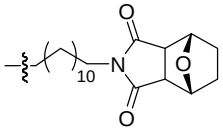
Compound	R	HT29	G401	SW480	MCF-7	A2780	H460	A431	DU145	BE2-C	SJ-G2
											
55		*31 ±12*	-	*18 ±3*	*45 ±22*	*15 ±2*	*16 ±3*	*<10*	*<10*	*<10*	*<10*
56		*21 ±4*	-	*16 ±2*	*51 ±13*	*18 ±2*	*14 ±1*	*16 ±3*	*<10*	*22 ±9*	*12 ±5*
57		*<10*	*10.4*	*<10*	*<10*	*<10*	*<10*	*<10*	*<10*	*<10*	*<10*
58		*<10*	*16.1*	*<10*	*<10*	*<10*	*<10*	*<10*	*<10*	*<10*	*<10*
59		*<10*	*16.9*	*<10*	*<10*	*<10*	*<10*	*<10*	*<10*	*<10*	*<10*
42		*39 ±12*	-	*69 ±29*	*>100*	*>100*	*32 ±11*	*60 ±19*	*67 ±24*	*73 ±7*	*27 ±5*
		>100		**62 ± 2**	**46 ± 4**	**59 ± 2**	**>100**	**>100**	**>100**	**70 ± 4**	**>100**
41		*12 ±4*	-	*<10*	*33 ±3*	*<10*	*<10*	*<10*	*<10*	*<10*	*<10*
40		*25 ±3*	-	*30 ±6*	*10 ±16*	*40 ±6*	*17 ±5*	*40 ±9*	*22 ±5*	*20 ±13*	*25 ±4*
60		*29 ±5*	*9.2*	*29 ±1*	*12 ±4*	*7 ±2*	*18 ±0*	*30 ±5*	*3 ±5*	*39 ±8*	*3 ±2*

Table 4.2 (Continued): Cytotoxicity of a variety of functionalised ring-closed norcantharimide (**55-63**) analogues in a panel of human cancer cell lines. Cytotoxicity levels are first expressed as % inhibition at 100 µM drug concentration (*in italics*) and, if potent, as Growth Inhibition, GI_{50} µM (**in bold**).

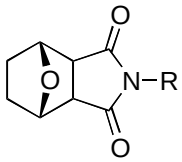
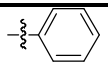
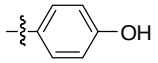
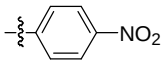
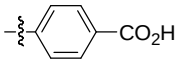
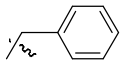
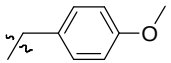
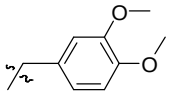
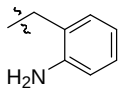
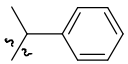
Compound	R	HT29	G401	SW480	MCF-7	A2780	H460	A431	DU145	BE2-C	SJ-G2
											
61		*<10*	*11.6*	*<10*	*<10*	*<10*	*<10*	*<10*	*<10*	*<10*	*<10*
38		*<10*	-	*<10*	*<10*	*<10*	*<10*	*<10*	*<10*	*<10*	*<10*
62		*10 ±7*	-	*16 ±5*	*52 ±41*	*31 ±26*	*18 ±7*	*10 ±5*	*13 ±5*	*10 ±8*	*10 ±8*
63		**8.3 ± 0.7**	-	**24 ± 4**	**18 ± 0**	**19 ± 1**	**31 ± 7**	**18 ± 4**	**60 ± 6**	**17 ± 4**	**43 ± 10**

NB: A % inhibition value of <10% indicates that the compound failed to influence cell growth when compared with untreated control

Given the results displayed by the simple and substituted alkylated cantharimides, the effect of adding substituted aromatic groups was next examined. **Table 4.3** shows the cytotoxicity of a variety of cantharimides, displaying either phenyl or benzyl moieties with a varying degree of functionality (**64-72**) against a panel of human cancer cell lines. In most cases the cytotoxicity displayed by the addition of aromatic moiety showed no significant effect or selectivity between the different cell lines. Furthermore, the phenyl analogues cytotoxicity results correspond with data reported by Lin *et. al*., who reported the synthesis and anticancer activity of cantharidin analogues [8]. The addition of a variety of functional groups including hydroxyl, methoxy, nitro, carboxy and amino groups also had no effect on cytotoxicity.

Due to results described by McCluskey *et.* al. (2001), the growth inhibition of a variety of amino-acid substituted ring-closed cantharimide analogues (**73-86**) was screened against HT29 and G401 as shown in **Table 4.4**[3]. The growth inhibition displayed generally exhibited no significant effect, or selectivity, between the two cell lines, with the exception of the D-histidine analogue (**32**). Compound (**32**) displayed moderate (49.8%) and excellent G401 (>100%) inhibition for HT29 and G401 respectively, and displayed a moderate selectivity for G401 kidney tumour cells (~2 fold). Compound (**32**) also showed an improvement in growth inhibition over norcantharidin, which exhibited inhibition value of 50.3% for G401. As discussed, the presence of the basic residue of the D-histidine, provides possible favourable interactions with acidic residues within the active site as predicted by the McCluskey *et. al.* model. However, protein phosphate inhibition studies were disappointing with no inhibition of PP1, PP2A and PP5 at 100 μM.

Table 4.3: Cytotoxicity of a variety of benzyl substituted ring-closed norcantharimide (**64-72**) analogues in a panel of human cancer cell lines. Cytotoxicity levels are first expressed as % inhibition at 100 µM drug concentration.

Compound	R_1	HT29	G401	SW480	MCF-7	A2780	H460	A431	DU145	BE2-C	SJ-G2
											
64		63 ± 4	-	78 ± 24	>100	90 ± 11	50	25 ± 3	24 ± 3	54 ± 9	42 ± 6
65		47 ± 8	-	21 ± 3	22 ± 4	15 ± 3	12 ± 1	27 ± 0	<10	14 ± 6	44 ± 4
66		60 ± 4	-	44 ± 11	>100	53 ± 11	37 ± 0	13 ± 4	13 ± 1	45 ± 5	23 ± 7
67		<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
68		74.1	62.7	-	-	-	-	-	-	-	-
69		45 ± 4	22.8	24 ± 4	64 ± 10	20 ± 1	23 ± 6	14 ± 5	<10	29 ± 2	11 ± 3
70		45 ± 8	-	29 ± 9	71 ± 22	29 ± 11	31 ± 15	<10	12 ± 6	11 ± 5	<10
71		33 ± 10	15.7	13 ± 4	41 ± 26	18 ± 5	21 ± 7	<10	<10	13 ± 1	<10
72		9.3	16.6	-	-	-	-	-	-	-	-

NB: A % inhibition value of <10% indicates that the compound failed to influence cell growth when compared with untreated control

Table 4.4: Cytotoxicity of a variety of amino-acid substituted ring-closed norcantharimide (**32,73-86**) analogues in a panel of human cancer cell lines. Cytotoxicity levels are first expressed as % inhibition at 100 µM drug concentration.

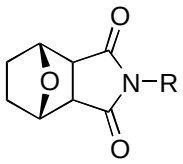
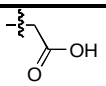
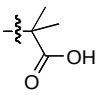
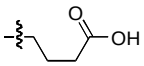
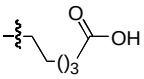
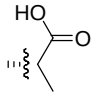
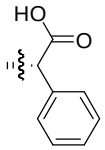
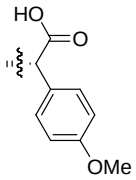
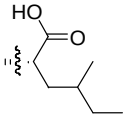
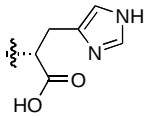
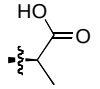
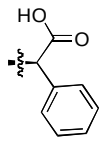
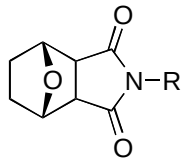
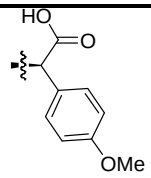
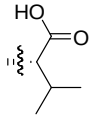
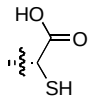
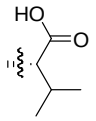
Compound	R	HT29	G401
			
73		4 ± 4.5	17.5 ± 6.2
74		<0	9.6 ± 2.4
75		<0	<0
76		7 ± 4.4	23.6 ± 14.4
77		2 ± 7.4	19.6 ± 10.1
78		5.6 ± 3.0	6.9 ± 4.1
79		<0	18.0 ± 6.6
80		11.3 ± 8.5	16.4 ± 7.7
32		49.8 ± 5.3	>100
81		15.7 ± 14.0	16.4 ± 4.2
82		0.5 ± 8.2	<0

Table 4.4 (Continued): Cytotoxicity of a variety of amino-acid substituted ring-closed cantharimide (**73-85, 32**) analogues in a panel of human cancer cell lines. Cytotoxicity levels are first expressed as % inhibition at 100 µM drug concentration.

Compound	R	HT29	G401
			
83		13.1 ± 6.3	8.8 ± 2.4
84		<0	<0
85		<0	<0
84		<0	<0

NB: A value of <10% indicates that the compound failed to influence cell growth when compared with untreated controls

4.2.1 General Summary

Basic modification of the parent norcantharidin (**13**) has allowed the development of a new series of novel norcantharimides, including simple alkylated, morpholine, aromatic and bis-analogues, and a range of amino-acid analogues possessing either acidic or basic residues which displayed modest to good cytotoxicities. The most potent analogues contained either a C8-12 alkyl chains (**42-45**), an allyl group (**49**), a 1,2-diol moiety (**56**), a dodecyl-linked bis structure (**63**), or an amino-acid possessing a basic residue (**32**) which corresponds to data described by McCluskey *et. al.* [6]. Of the analogues generated the dodecyl-linked bis-analogue was the most potent analogue displaying µM potent cytoxicities against all the cell lines examined at levels that improve on the lead norcantharidin (**13**).

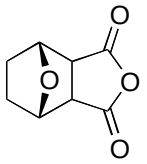
Further skeletal development of these analogues may lead to other interesting analogues, such as the investigation of analogues with longer carbon chain with varying degrees of functionality including diol functionality at various sections along the chain. Also, investigating other linkers

with or without functionality for the bis-analogues may lead to other interesting and active analogues. Also, as none of the ring-closed cantharimide analogues, including the above mentioned, proved to be significant PP1c, PP2A and PP5 inhibitors, suggesting a possible different mode of action than the lead compound, norcantharidin (**13**). This may be a result of a completely different mode of action, or an as yet undiscovered mechanism. It should also be noted, that ring opening of these analogues may not have occurred *in vivo*.

4.4 Experimental – Synthesis of the Cantharimides

4.4.1 Synthesis of Norcantharidin

4,10-Dioxatricyclo[5.2.1.02,6]decane-3,5-dione (13):



13

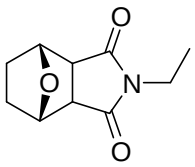
To a stirring solution of maleic anhydride (**12**) (17 g, 0.17 mol) in ether (50 mL) was added furan (**10**) in excess. The reaction mixture was stoppered and stirred overnight at room temperature (approx. 20 °C). The white precipitate was collected and air-dried for 2 minutes. Samples were stored in refrigeration (4 °C). Approximately 5 g of the white solid was dissolved in dry acetone and hydrogenated at 4 atm overnight with 10% Pd/C catalyst. After completion the catalyst was removed by filtration with filter aid (celite), and concentrated *in vacuo* to obtain crude (**13**). Recrystallisation from ethylacetate/petroleum spirit afforded pure (**13**) (4.1 g/81%) as a white solid. Final analysis was carried out using GCMS and NMR.

^{1}H NMR ($CDCl_3$): δ 5.09 (2H, q), 3.55 (1H, s), 3.20 (1H, s), 1.68, 1.95 (4H, q),
^{13}C NMR ($CDCl_3$): δ 171.0, 80.1, 50.6, 28.1.
GCMS: 168 [M], 41 (100%);

4.4.2 General Synthesis of Allylic Cantharimides.

To a solution of (**13**) (500 mg, 2.98 mM) in dry THF (20 mL) was added ~1 equivalent of the respective amine (~2.98 mM). The reaction mixture was then heated at reflux for approximately 36 hrs using a Büchi Syncore Reactor. The reaction mixture was then cooled to room temperature (approx. 20 °C), and the precipitate was collected by filtration, and washed with ~8 mL cold THF. In the case of a precipitate not forming, the sample was concentrated *in vacuo*. Final analysis carried out using GCMS and NMR.

*Synthesis of 4-Ethyl-10-oxa-4-azatricyclo[5.2.1]decane-3,5-dione (**43**):*

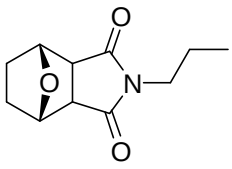


43

The previously reported *N*-ethyl substituted analogue **43** was synthesised according to the general method (**4.4.2**) using ethylamine (70%) and norcantharidin **13** affording white solid (472 mg, 81%). The determined melting point corresponds to literature reports [10].

Mp: 166-169°C;
^{1}H NMR (DMSO-d_6): δ 4.81 (2H, dd, *J* = 2.4, 3.1 Hz), 3.46 (2H, q, *J* = 7.1 Hz), 2.80 (2H, s), 1.80 (2H, m), 1.55 (2H, m), 1.09 (3H, t, *J* = 7.1 Hz);
^{13}C NMR (DMSO-d_6): δ 176.9, 79.0, 49.9, 33.9, 28.5, 12.85;
ESI-MS: 196 [M+H], 195 [M], 127 (100%);
IR (neat): 2991, 2946, 1693, 1446, 1406, 1344, 1311, 1229, 1207, 1190, 1151, 1018, 887, 825, 807 cm^{-1};
HR-MS (m/z): calc $C_{10}H_{13}NO_3$: 195.08756.

*Synthesis of 4-Propyl-10-oxa-4-azatricyclo[5.2.1]decane-3,5-dione (**44**):*

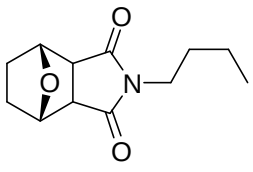


44

The previously reported *N*-propyl substituted analogue **44** was synthesised according to the general method (**4.4.2**), using *N*-propylamine and norcantharidin **13** affording white solid (500 mg, 80%). The determined melting point corresponds to literature reports [10].

Mp: 76-77°C;
^{1}H NMR (DMSO-d_6): δ 4.83 (2H, dd, *J* = 2.2, 3.1 Hz), 3.40 (2H, t, *J* = 7.3 Hz), 2.82 (2H, s), 1.81 (2H, m), 1.56 (2H, m), 0.84 (3H, t, *J* = 7.3 Hz);
^{13}C NMR (DMSO-d_6): δ 177.2, 79.0, 49.9, 40.6, 28.6, 20.9, 11.1;
ESI-MS: 210 [M+H], 209 [M], 141 (100%);
IR (neat): 2970, 2883, 1695, 1438, 1406, 1345, 1310, 1212, 1205, 1188, 1150, 1026, 914, 886, 826, 789 cm^{-1};
HR-MS (m/z): calc $C_{11}H_{15}NO_3$: 209.10519.

*Synthesis of 4-Butyl-10-oxa-4-azatricyclo[5.2.1]decane-3,5-dione **(45)**:*

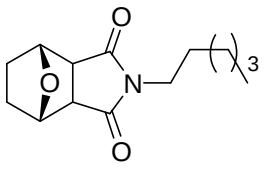


45

The previously reported *N*-butyl substituted analogue **45** was synthesised according to the general method (**4.4.2**), using *N*-butylamine and norcantharidin **13**, affording a white solid (818 mg, 93%). The determined melting point corresponds to literature reports [10].

Mp: 82-83°C;
^{1}H NMR (DMSO-d_6): δ 4.82 (2H, dd, *J* = 2.2, 3.2 Hz), 3.41 (2H, t, *J* = 7.3 Hz), 2.81, (2H, s), 1.80 (2H, m), 1.57-1.45 (4H, m), 1.23 (2H, m), 0.86 (3H, t, *J* = 7.3 Hz);
^{13}C NMR (DMSO-d_6): δ 177.2, 79.0, 49.8, 38.8, 29.6, 28.6, 19.9, 13.5;
ESI-MS: 224 [M+H], 223 [M], 181 (100%);
IR (neat): 2958, 2874, 1695, 1436, 1401, 1368, 1344, 1309, 1188, 1142, 1031, 999, 917, 890, 826 cm^{-1};
HR-MS (m/z): calc $C_{12}H_{17}NO_3$: 223.12084.

*Synthesis of 4-Hexyl-10-oxa-4-azatricyclo[5.2.1]decane-3,5-dione (**46**):*

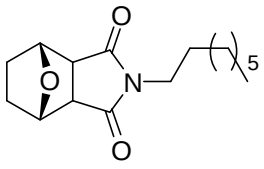


46

The previously reported *N*-hexyl substituted analogue **46** was synthesised according to the general method (**4.4.2**), using *N*-hexylamine and norcantharidin **13**, affording colourless oil (654 mg, 87%). Analysis by TLC of the crude product showed no traces of starting material or other impurities. The product was not further purified.

^{1}H NMR (DMSO-d_6): δ 4.80 (2H, m), 3.39 (2H, t, *J* = 7.3 Hz), 2.80 (2H, s), 1.80 (2H, m), 1.57-1.43 (4H, m), 1.21 (6H, m), 0.81 (3H, t, *J* = 7.3 Hz);
^{13}C NMR (DMSO-d_6): δ 177.2, 79.0, 49.8, 39.0, 31.2, 28.5, 27.4, 26.2, 22.4, 13.9;
ESI-MS: 252 [M+H], 251 [M], 168 (100%);
IR (neat): 2954, 2859, 1700, 1437, 1400, 1372, 1185, 1002, 888, 825 cm^{-1};
HR-MS (m/z): calc $C_{14}H_{21}NO_3$: 251.15214.

*Synthesis of 4-Octyl-10-oxa-4-azatricyclo[5.2.1]decane-3,5-dione (**47**)*



47

The previously reported *N*-octyl substituted analogue **47** was synthesised according to the general method (**4.4.2**), using *N*-octylamine and norcantharidin **13**, affording a white solid (714 mg, 86%). The determined melting point corresponds to literature reports [10].

Mp: 36-37°C;

^{1}H NMR (DMSO-d_6): δ 4.81 (2H, m), 3.39 (2H, t, *J* = 7.3 Hz), 2.80 (2H, s), 1.78 (2H. m), 1.58-1.43 (4H, m), 1.21 (10H, m), 0.82 (3H, t, *J* = 7.3 Hz);

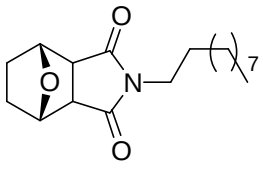
^{13}C NMR (DMSO-d_6): δ 177.2, 79.0, 49.8, 39.1, 31.6, 29.0, 28.9, 28.5, 27.5, 26.6, 22.5, 14.0;

ESI-MS: 280 [M+H], 279 [M], 168 (100%);

IR (neat): 2927.1, 2855.8, 1700.1, 1437.1, 1401.1, 1371.4, 1347.0, 1308.2, 1185.8, 888.2, 825.6 cm^{-1};

HR-MS (m/z): calc $C_{16}H_{25}NO_3$: 279.18344.

*Synthesis of 4-Decyl-10-oxa-4-azatricyclo[5.2.1]decane-3,5-dione (**48**)*

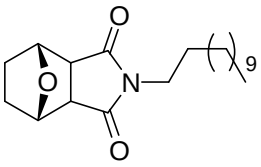


48

The previously reported *N*-decyl substituted analogue **48** was synthesised according to the general method (**4.4.2**), using *N*-decylamine and norcantharidin **13**, affording a white solid (623 mg, 68%). The determined melting point corresponds to literature reports [10].

Mp: 30-31°C;
^{1}H NMR ($CDCl_3$): δ 4.84 (2H, t, *J* = 2.1 Hz), 3.44 (2H, t, *J* = 7.3 Hz), 2.81 (2H, s), 1.82-1.85 (2H, m), 1.52-1.59 (4H, m), 1.26 (14H, s), 0.88 (3H, t, *J* = 6.7 Hz);
^{13}C NMR ($CDCl_3$): δ 177.2, 79.1, 50.5, 39.2, 32.0, 29.5, 29.3, 29.1, 28.7, 27.6, 26.7, 22.7, 14.0;
ESI-MS: 308 [M+H], 307 [M], 168 (100%);
HR-MS (m/z): calc $C_{18}H_{29}NO_3$: 307.21474.

*Synthesis of 4-Dodecyl-10-oxa-4-azatricyclo[5.2.1]decane-3,5-dione (**49**)*

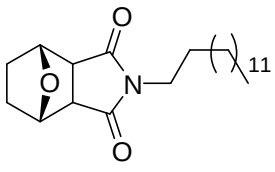


49

The previously reported *N*-dodecyl substituted analogue **49** was synthesised according to the general method (**4.4.2**), using *N*-dodecylamine and norcantharidin **13**, affording a white solid (820 mg, 82%).

Mp: 36-37°C;
^{1}H NMR ($CDCl_3$): δ 4.85 (2H, q, *J* = 2.3 Hz), 3.44 (2H, t, *J* = 7.3 Hz), 2.83 (2H, s), 1.85-1.82 (2H), 1.60-1.50 (4H, m), 1.52-1.59 (4H, m), 1.24 (20H, s), 0.87 (4H, m);
^{13}C NMR ($CDCl_3$): δ 176.7, 78.5, 49.3, 38.5, 29.0, 28.9, 28.8, 28.5, 28.0, 27.0, 26.1, 22.1, 13.5;
ESI-MS: 336 [M+H], 335 [M], 168 (100%);
HR-MS (m/z): calc $C_{20}H_{33}NO_3$: 335.24604.

*Synthesis of 4-Tetradecyl-10-oxa-4-azatricyclo[5.2.1]decane-3,5-dione (**50**)*



50

The *N*-tetradecyl substituted analogue **50** was synthesised according to the general method (**4.4.2**), using *N*-tetredecylamine and norcantharidin **13**, affording a white solid (856 mg, 79%).

Mp: 45-46°C;

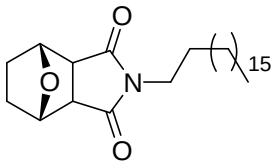
^{1}H NMR ($CDCl_3$): δ 4.84 (2H, q, *J* = 2.2 Hz), 3.42 (2H, t, *J* = 7.3 Hz), 2.82 (2H, s), 1.83 (2H, quin, *J* = 4.5 Hz), 1.56 (2H, m), 1.23 (20H, s), 0.86 (2H, m);

^{13}C NMR ($CDCl_3$): δ 176.7, 78.5, 49.3, 38.5, 29.1, 29.0, 28.5, 28.0, 26.1, 22.1, 13.5;

ESI-MS: 364 [M+H], 365 [M], 168 (100%);

HR-MS (m/z): calc $C_{22}H_{37}NO_3$: 363.27734.

*Synthesis of 4-Octadecyl-10-oxa-4-azatricyclo[5.2.1]decane-3,5-dione (**51**)*



51

The *N*-octadecyl substituted analogue **51** was synthesised according to the general method (**4.4.2**), using *N*-octadecylamine and norcantharidin **13**, affording a white solid (1.00 g, 80%).

Mp: 62-64°C;

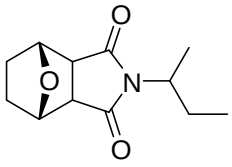
^{1}H NMR ($CDCl_3$): δ 4.86 (2H, q, *J* = 2.3 Hz), 3.54 (2H, t, *J* = 6.8 Hz), 2.86 (2H, s), 2.33 (2H, t, *J* = 7.4 Hz), 1.83-1.91 (4H, m), 1.58 (2H, m);

^{13}C NMR ($CDCl_3$): δ 177.1, 176.7, 78.5, 49.3, 37.5, 30.4, 28.0, 22.0, 13.8;

ESI-MS: 420 [M+H], 419 [M], 168 (100%);

HR-MS (m/z): calc $C_{26}H_{45}NO_3$: 419.33994.

*Synthesis of 4-sec-Butyl-10-oxa-4-azatricyclo[5.2.1]decane-3,5-dione (**52**)*



52

The *N*-(sec)-butyl substituted analogue **52** was synthesised according to the general method (**4.4.2**), using *sec*-butylamine and norcantharidin **13** affording a white solid (654 mg, 98%).

Mp: 44-46°C;

^{1}H NMR (DMSO-d_6): δ 4.82 (2H, s), 4.03 (1H, m), 2.77 (2H, m), 1.92-1.80 (m), 1.64-1.52 (m), 1.30 (3H, d, *J* = 7.0 Hz), 0.79 (3H, t, *J* = 7.5 Hz);

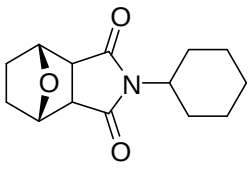
^{13}C NMR (DMSO-d_6): δ 177.5, 79.2, 50.1, 49.5, 28.6, 25.9, 17.5, 10.9;

ESI-MS: 224 [M+H], 223 [M], 168 (100%);

IR (neat): 2968, 2878, 1696, 1462, 1398, 1367, 1307, 1214, 1188, 888, 824 cm^{-1};

HR-MS (m/z): calc $C_{12}H_{17}NO_3$: 223.12084.

*Synthesis of 4-Cyclohexyl-10-oxa-4-azatricyclo[5.2.1]decane-3,5-dione (**53**)*

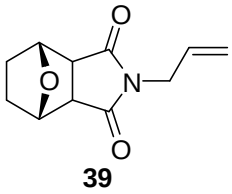


53

The *N*-cyclohexyl substituted analogue **53** was synthesised according to the general method (**4.4.2**), using cyclohexylamine and norcantharidin **13** affording a white solid (604 mg, 81.5%).

Mp: 97-99°C;
^{1}H NMR (DMSO-d_6): δ 4.78 (2H, m), 3.86 (1H, m), 2.73 (2H, s), 2.05 (2H, m), 1.77 (4H, m), 1.53 (6H, m), 1.16 (4H, m);
^{13}C NMR (DMSO-d_6): δ 177.3, 79.1, 51.9, 49.4, 28.6, 28.6, 25.8, 25.0;
ESI-MS: 250 [M+H], 249 [M], 168 (100%);
IR (neat): 2922, 2853, 1695, 1456, 1398, 1373, 1348, 1308, 1258, 1198, 1150, 1039, 1000, 894, 826 cm^{-1};
HR-MS (m/z): calc $C_{14}H_{19}NO_3$: 249.13649.

*Synthesis of 4-Allyl-10-oxa-4-azatricyclo[5.2.1]decane-3,5-dione (**39**)*

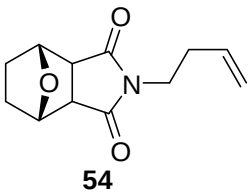


39

The *N*-allyl substituted analogue **39** was synthesised according to the general method (**4.4.2**), using 1-aminopropyl-2-ene and norcantharidin **13**, affording a white solid (463 mg, 75%).

Mp: 116-117°C;
^{1}H NMR ($CDCl_3$): δ 5.71-5.76 (1H, m), 5.16-5.22 (2H, m), 4.88-4.90 (2H, m), 4.08 (2H, dd, *J* = 4.0, 1.3 Hz), 2.89 (2H, s), 1.85-1.88 (2H, m), 1.55-1.62 (2H, m);
^{13}C NMR ($CDCl_3$): δ 176.0, 129.8, 116.9, 78.4, 49.4, 40.3, 28.0;
ESI-MS: 208 [M+H], 207 [M], 168 (100%);
HR-MS (m/z): calc $C_{11}H_{13}NO_3$: 207.08954.

*Synthesis of 4-But-3-enyl-10-oxa-4-azatricyclo[5.2.1]decane-3,5-dione (**54**)*



54

The but-3-enyl substituted analogue **54** was synthesised according to the general method (**4.4.2**), using 1-aminobut-3-ene and norcantharidin **13**, affording a white solid (204 mg, 31%).

Mp: 64-65°C;

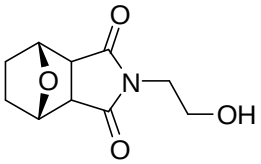
^{1}H NMR (DMSO-d_6): δ 5.65 (1H, m), 4.99 (2H, m), 4.66 (2H, m), 3.37 (2H, t, *J* = 6.9 Hz), 3.00 (2H, s), 2.17 (2H, q, *J* = 6.9 Hz), 1.61 (4H, br s);

^{13}C NMR (DMSO-d_6): δ 177.1, 78.6, 59.5, 49.4, 41.3, 27.9;

ESI-MS: 222 [M+H], 221 [M], 168 (100%);

HR-MS (m/z): calc $C_{12}H_{15}NO_3$: 221.10519.

*Synthesis of 4-(2-Hydroxypropyl)-10-oxa-4-azatricyclo[5.2.1]decane-3,5-dione (**55**)*

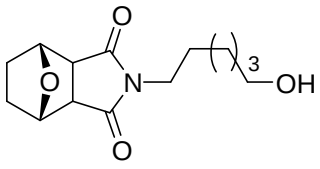


55

The previously 2-hydroxyethyl substituted analogue **55** was synthesised according to the general method (**4.4.2**), using 2-hydroxyethylamine and norcantharidin **13** affording a white solid (503 mg, 80%). The determined melting point corresponds to literature reports [10].

Mp: 164-166°C;
^{1}H NMR (DMSO-d_6): δ 4.86 (2H, q, *J* = 2.1 Hz), 3.70 (2H, t, *J* = 4.8 Hz), 3.64 (2H, q, *J* = 3.6 Hz), 3.01 (1H, br s), 2.89 (2H, s), 1.84 (2H, m), 1.61 (2H, m);
^{13}C NMR (DMSO-d_6): δ 177.1, 78.6, 59.5, 49.4, 41.3, 27.9;
ESI-MS: 212 [M+H], 168 (100%);
HR-MS (m/z): calc $C_{11}H_{15}NO_4$: 211.08446.

*Synthesis of 4-(6-Hydroxyhexyl)-10-oxa-4-azatricyclo[5.2.1]decane-3,5-dione (**56**)*

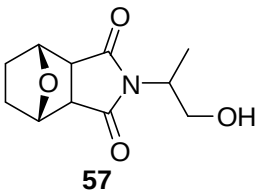


56

The 6-hydroxyhexyl substituted analogue **56** was synthesised according to the general method (**4.4.2**), using *N*-6-hydroxyhexylamine and norcantharidin **13** affording a white solid (330 mg), yield 62%.

Mp: 56-57°C;
^{1}H NMR (DMSO-d_6): δ 4.80 (2H, m), 3.54 (2H, m), 3.41 (2H, m), 2.82 (2H, s), 2.15 (2H, m), 1.57 (2H, m), 1.48 (2H, m), 1.27 (4H, m);
^{13}C NMR (DMSO-d_6): δ 179.1, 79.5, 68.9, 63.0, 49.6, 28.4, 22.1;
ESI-MS: 240 [M+H], 72 (100%);
IR (neat): 3447, 2953, 1696, 1350, 1305, 1190, 1149, 1057, 996, 979, 882, 827, 576 cm^{-1};
HR-MS (m/z): calc $C_{12}H_{17}NO_4$: 267.14706.

*Synthesis of 4-(2-Hydroxy-1-methylethyl)-10-oxa-4-azatricyclo[5.2.1]decane-3,5-dione (**57**)*

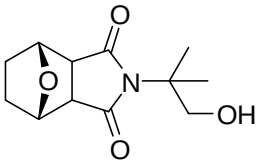


57

The 2-hydroxy-1-methylethyl substituted analogue **57** was synthesised according to the general method (**4.4.2**), using 2-amino-propan-1-ol and norcantharidin **13** affording a white solid (352 mg, 49%)

Mp: 104-107°C;
^{1}H NMR (DMSO-d_6): δ 4.84 (2H, m), 4.26 (1H, m), 3.80 (1H, m), 3.70 (1H, m), 2.83 (2H, s), 2.14 (1H, s), 1.84 (m), 1.58 (2H, m), 1.28 (3H, d, *J* = 7.1 Hz);
^{13}C NMR (DMSO-d_6): δ 177.9, 79.3, 63.5, 50.4, 49.7, 49.6, 28.6, 28.5, 13.8;
ESI-MS: 226 [M+H], 168 (100%);
IR (neat): 3500-3250, 2959, 1768, 1685, 1465, 1400, 1375, 1310, 1190, 1134, 1062, 1038, 1000, 886, 825 cm^{-1};
HR-MS (m/z): calc $C_{11}H_{15}NO_4$: 225.10011.

*Synthesis of 4-(2-Hydroxy-1,1-dimethylethyl)-10-oxa-4-azatricyclo[5.2.1]decane-3,5-dione (**58**)*

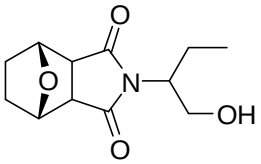


58

The 2-hydroxy-1,1-dimethylethyl substituted analogue **58** was synthesised according to the general method (**4.4.2**), using 2-amino-2-methyl-propan-1-ol and norcantharidin **13** affording a pale yellow solid (328 mg, 46%).

Mp: 134-136°C;
^{1}H NMR (DMSO-d_6): δ 4.78 (2H, dd *J* = 2.3, 3.1 Hz), 3.71 (2H, s), 3.52 (1H, br s), 2.72 (2H, s), 1.78 (2H, m), 1.52 (2H, m), 1.39 (6H, s);
^{13}C NMR (DMSO-d_6): δ 179.1, 79.5, 68.9, 63.0, 49.6, 28.4, 22.1;
ESI-MS: 240 [M+H], 168 (100%);
HR-MS (m/z): calc $C_{12}H_{17}NO_4$: 239.11576.

*Synthesis of 4-(1-Hydroxymethylpropyl)-10-oxa-4-azatricyclo[5.2.1]decane-3,5-dione(**59**)*

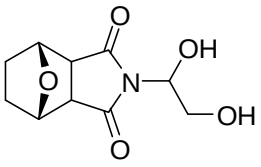


59

The 1-hydroxymethylpropyl substituted analogue **59** was synthesised according to the general method (**4.4.2**), using 2-amino-butan-1-ol and norcantharidin **13** affording a pale yellow oil (349 mg, 49%). Analysis by TLC of the crude product showed no traces of starting material or other impurities. The product was not further purified.

^{1}H NMR (DMSO-d_6): δ 4.79 (2H, s), 4.05 (1H, m), 3.87 (1H, m), 3.63 (1H, m), 3.01 (1H, br s), 2.81 (2H, q *J* = 11.6 Hz), 1.79-1.38 (6H, m), 0.79 (3H, t *J* = 7.4 Hz);
^{13}C NMR (DMSO-d_6): δ 178.2, 79.2, 62.1, 56.5, 49.6, 49.4, 28.5, 28.4, 20.7, 10.3;
ESI-MS: 240 [M+H], 168 (100%);
HR-MS (m/z): calc $C_{12}H_{17}NO_4$: 239.11576.

*Synthesis of 4-(2,3-Dihydroxypropyl)-10-oxa-4-azatricyclo[5.2.1.02,6]decane-3,5-dione (**42**)*

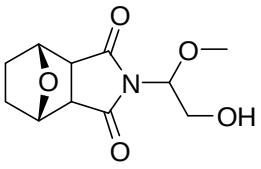


42

The 2,3-dihydroxypropyl analogue **42** was synthesised according to the general method described in Scheme 4.3 (**4.2.3**) by adding 120 mg of a 2.5% OsO_4 solution in *t*-BuOH (0.012 mmol, 0.5 mol%) dropwise to a stirred solution of the allyl analogue (**39**) (500 mg, 2.41 mmol), *N*-methylmorpholine-*N*-oxide (310 mg, 2.65 mmol) in acetone/water (5:2 mL). The resulting solution was heated at 80ºC for 16 h before being diluted ether (100 mL) and washed with water (3 x 20 mL). The organic layer was concentrated *in vacuo* to afford a brown solid. Flash chromatography (70% EtOAc/hexanes) afforded a pale white solid which was recrystallised from EtOAc to afford a white crystalline solid (197 mg, 34%).

^{1}H NMR (DMSO-d_6): δ 4.73 (1H, d, *J* = 4.6 Hz), 4.66 (2H, s), 4.51 (1H, t, *J* = 5.7 Hz), 3.63 (1H, q, *J* = 5.7 Hz), 3.23-3.32 (2H, m), 3.00 (2H, s), 1.62 (4H, s);
^{13}C NMR (DMSO-d_6): δ 177.4, 78.3, 67.9, 63.9, 49.3, 41.8, 27.8;
ESI-MS: 242 [M+H], 168 (100%);
HR-MS (m/z): calc $C_{14}H_{20}N2O_4$: 241.09502.

*Synthesis of 4-(3-Hydroxy-2-methoxypropyl)-10-oxa-4-azatricyclo[5.2.1.02,6]decane-3,5-dione (**41**)*

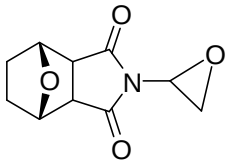


41

The methoxylated analogue **41** was synthesised according to the general method described in Scheme 4.3 (**4.2.3**) by adding (1S)-(+)-10-camphorsulfonic acid (15 mg, 0.06 mmol) to a stirred solution of epoxide (**40**) (220 mg, 0.98 mmol) in methanol (4mL). The resultant solution was warmed to 35°C and stirred for 16 h before being concentrated *in vacuo* affording a clear oil. Flash chromatography (70% EtOAc/hexanes) afforded a white solid (195 mg, 78%).

Mp: 95-96°C;
^{1}H NMR (DMSO-d_6): δ 4.88-4.89 (2H, m), 3.94-3.96 (1H, m), 3.60-3.68 (2H, m), 3.38 (3H, s), 3.33-3.39 (2H, m), 2.91 (2H, s), 1.85-1.88 (2H, m), 1.60-1.63 (2H, m);
^{13}C NMR (DMSO-d_6): δ 177.0, 78.6, 73.5, 67.7, 58.7, 49.4, 41.7, 28.0;
ESI-MS: 256 [M+H], 168 (100%);
HR-MS (m/z): calc $C_{12}H_{16}N2O_4$: 255.11067.

*Synthesis of 4-Oxirananylmethyl-10-oxa-4-azatricyclo[5.2.1.02,6]decane-3,5-dione (**40**)*



58

The oxirananylmethyl substituted analogue **40** was synthesised according to the general method described in Scheme 4.3 (**4.2.3**) by adding *m*-chloroperbenzoic acid (2.15 g, 77% in water, 9.66 mmol) in one portion to a cooled and stirred solution at 0°C of the allyl analogue (**39**) (1.0 g, 4.83 mmol) in DCM (20 mL). The resulting solution was warmed to room temperature and stirred for 16 h before being diluted with DCM (30 mL). This was washed with $NaHCO_3$ (3 x 10 mL, saturated). The organic layer was dried (Na_2SO_4), filtered and concentrated in vacuo affording a white solid (1.08 g, 76%).

Mp: 83-84°C;

^{1}H NMR (DMSO-d_6): δ 4.82 (2H, m), 3.65 (4H, t, *J* = 4.7 Hz), 3.48 (2H, t), 2.81 (2H, s), 2.37 (4H, m), 2.30 (2H, t, *J* = 5.0 Hz), 1.81 (2H, m), 1.70 (2H, s), 1.55 (2H, m);

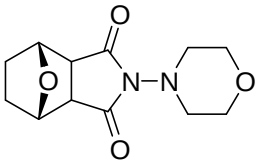
^{13}C NMR (DMSO-d_6): δ 177.2, 79.0, 66.8, 55.8, 53.4, 51.3, 49.9, 37.2, 28.5, 24.2;

ESIMS: 295 [M+H], 168 (100%);

IR (neat): 2958, 1695, 1443, 1404, 1348, 1310, 1257, 1187, 1115, 1070, 997, 916, 888, 824 cm^{-1};

HR-MS (m/z): calc $C_{15}H_{22}N2O_4$: 294.15796.

*Synthesis of 4-Morpholin-4-yl-10-oxa-4-azatricyclo[5.2.1.02,6]decane-3,5-dione (**60**)*



60

The *N*-morphilino substituted analogue **60** was synthesised according to the general method (**4.4.2**) using *4*-aminomorpholine and norcantharidin **13** affording a yellow solid (324 mg, 43%).

Mp: 169-171°C;

^{1}H NMR (DMSO-d_6): δ 4.78 (2H, m), 3.71 (4H, t, *J* = 4.7 Hz), 3.18 (4H, t, *J* = 4.5 Hz), 2.73 (2H, s), 1.78 (2H, m), 1.53 (2H, m);

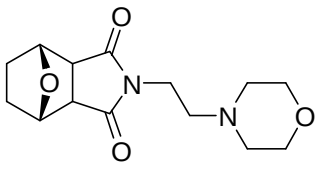
^{13}C NMR (DMSO-d_6): δ 175.1, 79.1, 66.7, 51.2, 47.9, 28.5;

ESI-MS: 253 [M+H], 168 (100%);

IR (neat): 3585, 3479, 2961, 2925, 2863, 1716, 1456, 1376, 1362, 1309, 1270, 1200, 1110, 896, 875, 821, 753 cm^{-1};

HR-MS (m/z): calc $C_{12}H_{16}N_2O_4$: 252.1101.

*Synthesis of 4-(2-Morpholin-4-ylethyl)-10-oxa-4-azatricyclo[5.2.1.02,6]decane-3,5-dione (**61**)*

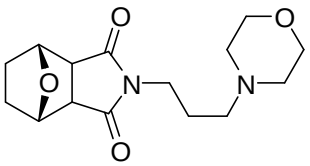


61

The *N*-ethylmorphilino substituted analogue **61** was synthesised according to the general method (**4.4.2**) using 4-ethylaminomorpholine and norcantharidin **13** affording an orange brown solid (306 mg, 37%).

Mp: 109-111°C;
^{1}H NMR (DMSO-d_6): δ 4.82 (2H, m), 3.60 (4H, t, *J* = 4.5 Hz), 3.55 (2H, t, *J* = 4.5 Hz), 2.84 (2H, s), 2.48-2.43 (6H, m), 1.80 (2H, m), 1.56 (2H, m);
^{13}C NMR (DMSO-d_6): δ 177.1, 79.0, 67.0, 55.1, 53.4, 49.9, 36.6, 28.6;
ESI-MS: 281 [M+H], 168 (100%);
IR (neat): 2997, 1698, 1404, 1207, 1160, 1115, 1006, 825, 668 cm^{-1};
HR-MS (m/z): calc $C_{14}H_{20}N_2O_4$: 280.14231.

*Synthesis of 4-(3-Morpholin-4-ylpropyl)-10-oxa-4-azatricyclo[5.2.1.02,6]decane-3,5-dione (**38**)*

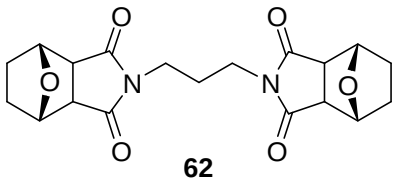


38

The *N*-propylmorphilino substituted analogue **38** was synthesised according to the general method (**4.4.2**) using 4-propylaminomorpholine and norcantharidin **13** affording a orange/brown oil (58 mg, 7%). Analysis by TLC of the crude product showed no traces of starting material or other impurities. The product was not further purified.

^{1}H NMR (DMSO-d_6): δ 4.82 (2H, m), 3.65 (4H, t, *J* = 4.7 Hz), 3.48 (2H, t), 2.81 (2H, s), 2.37 (4H, m), 2.30 (2H, m), 1.81 (2H, m), 1.70 (2H, s), 1.55 (2H, m);
^{13}C NMR (DMSO-d_6): δ 177.2, 79.0, 66.8, 55.8, 53.4, 51.3, 49.9, 37.2, 28.5, 24.2;
ESI-MS: 295 [M+H], 168 (100%);
IR (neat): 2958, 1695, 1443, 1404, 1348, 1310, 1257, 1187, 1115, 1070, 997, 916, 888, 824 cm^{-1};
HR-MS (m/z): calc $C_{15}H_{22}N_2O_4$: 294.15796.

*Synthesis of Bis-3,6-epoxycyclohexane-1,2-dicarboximido)-trimethyl (bis-norcantharimide-propyl linker) (**62**)*

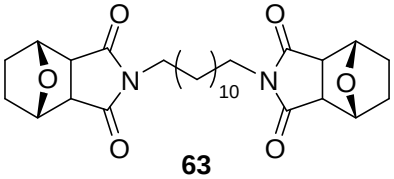


62

The bis-norcantharimide-propyl linker analogue **62** was synthesised according to the general method (**4.4.2**) using 4-propylaminomorpholine and norcantharidin **13** affording a yellow solid (892 mg, 80%).

Mp: 159-160°C;
^{1}H NMR (DMSO-d_6): δ 4.86 (2H, m), 3.43 (2H, m), 2.86 (2H, s), 1.85 (4H, m), 1.58 (2H, quin);
^{13}C NMR (DMSO-d_6): δ 176.5, 78.5, 49.4, 35.6, 28.0, 24.7;
ESI-MS: 375 [M+H], 168 (100%);
HR-MS (m/z): calc $C_{19}H_{22}N_2O_6$: 374.14779.

*Synthesis of Bis-3,6-epoxycyclohexane-1,2-dicarboximido)-dodedecylmethyl (bis-norcantharimide-dodecyl linker) (**63**)*

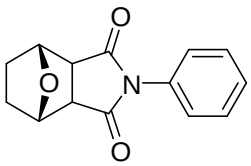


63

The bis-norcantharimide-dodecyl linker analogue **63** was synthesised according to the general method (**4.4.2**) using 4-propylaminomorpholine and norcantharidin **13** affording a white solid (924 mg, 62%).

^{1}H NMR (DMSO-d_6): δ 4.85 (2H, q, *J* = 2.2 Hz), 3.43 (2H, t, *J* = 7.3 Hz), 2.84 (2H, s), 1.85 (2H, m), 1.59-1.51 (4H, m), 1.22 (10H, m);
^{13}C NMR (DMSO-d_6): δ 176.6, 78.5, 49.3, 38.5, 28.9, 28.8, 28.5, 27.0, 26.0;
ESI-MS: 501 [M+H], 168 (100%);
HR-MS (m/z): calc $C_{28}H_{40}N_2O_6$: 500.28864.

*Synthesis of 4-Phenyl-10-oxa-4-azatricyclo[5.2.1.02,6]decane-3,5-dione (**64**)*



64

The phenyl analogue **64** was synthesised according to the general method (**4.4.2**), using aminobenzene and norcantharidin **13** affording a bone coloured solid (572 mg, 79%).

Mp: 171-173°C;

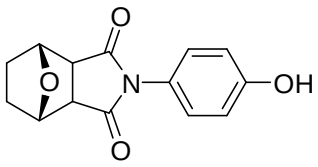
^{1}H NMR ($CDCl_3$): δ 7.42-7.45 (2H, m), 7.25-7.28 (2H, m), 4.97 (2H, q, *J* = 1.1 Hz), 3.01 (2H, s), 1.87-1.90 (2H, m), 1.63-1.65 (2H, m);

^{13}C NMR ($CDCl_3$): δ 175.7, 128.5, 128.1, 125.0, 78.9, 49.5, 28.1;

ESI-MS: 244 [M+H], 168 (100%);

HR-MS (m/z): calc $C_{14}H_{13}NO_3$: 243.08954.

*Synthesis of 4-(4-Hydroxyphenyl)-10-oxa-4-azatricyclo[5.2.1.02,6]decane-3,5-dione (**65**)*



65

The hydroxyphenyl analogue **65** was synthesised according to the general method (**4.4.2**), using 4-aminophenol and norcantharidin **13** affording a white solid (**65**) (347 mg), yield 45%.

Mp: 172-173°C;

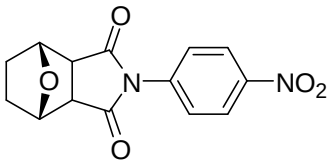
^{1}H NMR ($CDCl_3$): δ 7.11 (2H, d, *J* = 8.1 Hz), 6.86 (2H, d, *J* = 0.9 Hz), 4.98 (2H, q, *J* = 0.9 Hz), 3.01 (2H, s), 1.56-1.62 (4H, m);

^{13}C NMR ($CDCl_3$): δ 181.5, 127.4, 115.4, 78.9, 49.4, 28.1;

ESI-MS: 260 [M+H], 168 (100%);

HR-MS (m/z): calc $C_{14}H_{13}NO_4$: 259.08954.

*Synthesis of 4-(4-Nitro-phenyl)-10-oxa-4-azatricyclo[5.2.1.02,6]decane-3,5-dione (**66**)*



66

The nitrophenyl analogue **66** was synthesised according to the general method (**4.4.2**), using 4-aminonitrobenzene and norcantharidin **13** affording an orange yellow solid (652 mg, 76%).

Mp: 207-209°C;

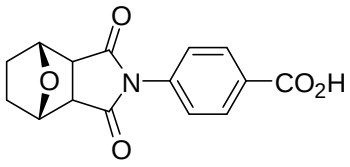
^{1}H NMR ($CDCl_3$): δ 8.29 (2H, d, *J* = 7.1 Hz), 7.55 (2H, d, *J* = 7.1 Hz), 4.99 (2H, m), 3.07 (2H, s), 1.90-1.94 (2H, m), 1.66-1.69 (2H, m);

^{13}C NMR ($CDCl_3$): δ 174.9, 146.5, 136.8, 126.4, 123.7, 79.1;

ESI-MS: 289 [M+H], 168 (100%);

HR-MS (m/z): calc $C_{14}H_{12}N_2O_5$: 288.07462.

*Synthesis of (4-(3,5-Dioxo-10-oxa-4-aza-tricyclo[5.2.1.02,6]dec-4-yl)-benzoic acid (**67**)*



67

The benzoic acid analogue **67** was synthesised according to the general method (**4.4.2**), using 4-aminobenzoic acid and norcantharidin **13** affording a white solid (307 mg, 36%).

Mp: 275-277°C;

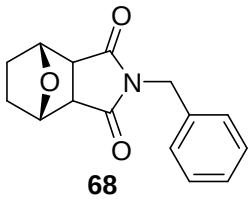
^{1}H NMR ($CDCl_3$): δ 8.13 (2H, d, *J* = 8.4 Hz), 7.30 (2H, d, *J* = 8.1 Hz), 4.96 (2H, q, *J* = 1.9 Hz), 3.00 (2H, s), 1.88 (2H, m), 1.63 (2H, m);

^{13}C NMR ($CDCl_3$): δ 175.3, 160.1, 134.3, 132.2, 129.8, 125.3, 78.9, 49.5, 28.0;

ESI-MS: 288 [M+H], 168 (100%);

HR-MS (m/z): calc $C_{15}H_{13}NO_5$: 287.07937.

*Synthesis of 4-Benzyl-10-oxa-4-azatricyclo[5.2.1.02,6]decane-3,5-dione (**68**)*

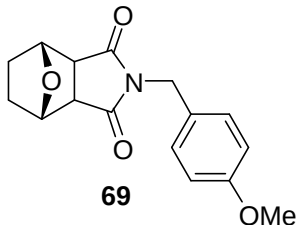


68

The previously reported benzyl substituted analogue **68** was synthesised according to the general method (**4.4.2**) from benzylamine and norcantharidin **13**. Recrystallisation from ethylacetate/petroleum spirit gave pale yellow crystals (697 mg, 91%). The determined melting point corresponds to literature reports [10].

Mp: 114-116°C;
^{1}H NMR (DMSO-d_6): δ 7.22 (5H, m), 4.81 (2H, m), 4.56 (2H, s), 2.81 (2H, s), 1.77 (2H, m), 1.54 (2H, m), 1.21 (2H, m);
^{13}C NMR (DMSO-d_6): δ 176.7, 135.3, 128.4, 127.8, 127.5, 78.9, 49.8, 42.3, 28.4;
ESI-MS: 258 [M+H] (100%);
IR (neat): 3009, 2959, 1652, 1601, 1581, 1496, 1430, 1399, 1340, 1308, 1177, 998, 887 cm^{-1};
HR-MS (m/z): calc $C_{15}H_{15}NO_3$: 257.21576.

*Synthesis of 4-(4-Methoxybenzyl)-10-oxa-4-azatricyclo[5.2.1.02,6]decane-3,5-dione (**69**)*

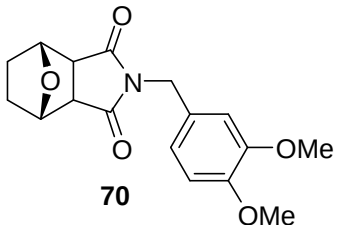


69

The 4-methoxybenzyl substituted analogue **69** was synthesised according to the general method above (4.4.2) from 4-methoxybenzylamine and norcantharidin **13** afforded crude yellow oil. Recrystallisation from ethylacetate/petroleum spirit gave yellow crystals (778 mg, 91%).

Mp: 77-80°C;
^{1}H NMR (DMSO-d_6): δ 7.26 (2H, d, *J* = 8.7 Hz), 6.81 (2H, d, *J* = 8.7 Hz), 4.87 (2H, m), 4.56 (2H, s), 3.77 (3H, s), 2.85 (2H, s), 1.84 (2H, m), 1.60 (2H, m);
^{13}C NMR (DMSO-d_6): δ 176.8, 159.2, 129.7, 127.8, 114.0, 79.0, 55.2, 50.0, 42.0, 28.6;
ESI-MS: 288 [M+H], 168 (100%);
HR-MS (m/z): calc $C_{12}H_{17}NO_4$: 287.11576.

*Synthesis of 4-(3,4-Dimethoxybenzyl)-10-oxa-4-azatricyclo[5.2.1.02,6]decane-3,5-dione (**70**)*

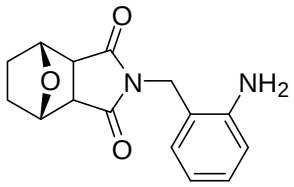


70

The 3,4-dimethoxybenzyl substituted analogue **70** was synthesised according to the general method (**4.4.2**) from 3,4-dimethoxybenzylamine and norcantharidin **13** affording a white solid (718 mg, 76%).

Mp: 155-157°C;
^{1}H NMR (DMSO-d_6): δ 6.89 (2H, m), 6.77 (1H, m), 4.87 (2H, q, *J* = 2.4 Hz), 4.56 (2H, s), 3.84 (3H, s), 3.83 (3H, s), 2.86 (2H, s), 1.84 (2H, m), 1.60 (2H, m);
^{13}C NMR (DMSO-d_6): δ 176.3, 148.5, 148.1, 127.6, 120.1, 110.9, 110.7, 78.5, 55.3, 49.5, 41.7, 28.0;
ESI-MS: 318 [M], 168 (100%);
HR-MS (m/z): calc $C_{17}H_{19}NO_5$: 317.12632.

*Synthesis of 4-(2-Amino-benzyl)-10-oxa-4-aza-tricyclo[5.2.1.02,6]decane-3,5-dione (**71**)*

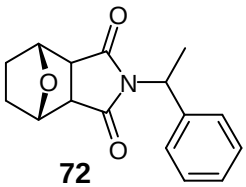


71

The 2-aminobenxyl analogue **71** was synthesised according to the general method (**4.4.2**) from 2-aminomethylphenylamine and norcantharidin **13** affording a yellow solid (349 mg, 43%).

Mp: 159-160°C;
^{1}H NMR (DMSO-d_6): δ 7.09-7.00 (2H, m), 6.68 (1H, d), 6.54 (1H, t), 4.71 (2H, s), 3.84 (2H, s), 2.74 (2H, s), 1.54-1.46 (4H, m);
^{13}C NMR (DMSO-d_6): δ 174.1, 146.8, 129.8, 128.9, 118.7, 116.1, 115.3, 79.8, 53.7, 29.0;
ESI-MS: 273 [M], 168 (100%);
HR-MS (m/z): calc $C_{15}H_{16}N_2O_3$: 272.11609.

Synthesis of 4-(1-Phenylethyl)-10-oxa-4-azatricyclo[5.2.1.02,6]decane-3,5-dione ***(72)***



72

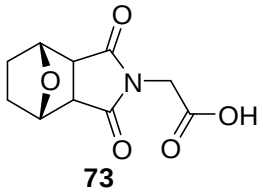
The phenylethyl analogue **72** was synthesised according to the general method (**4.4.2**) from 2-aminomethylphenylamine and norcantharidin **13** affording a yellow solid (414 mg, 51%).

Mp: 159-160°C;
^{1}H NMR (DMSO-d_6): δ 7.09-7.00 (2H, m), 6.68 (1H, d), 6.54 (1H, t), 4.71 (2H, s), 3.84 (2H, s), 2.74 (2H, s), 1.54-1.46 (4H, m);
^{13}C NMR (DMSO-d_6): δ 174.1, 146.8, 129.8, 128.9, 118.7, 116.1, 115.3, 79.8, 53.7, 29.0;
ESI-MS: 272 [M+H], 168 (100%);
HR-MS (m/z): calc $C_{15}H_{16}N_2O_3$: 271.12359.

4.4.3 General Synthesis of Amino acid and Acidic amine substituted ring-closed cantharimides analogues

To a solution of norcantharidin (500 mg, 2.98 mM) in dry DMF (20ml) was added the respective amine (2.98 mM). The reaction mixture was heated at reflux for 36 hrs using a Radley Carousel Reactor, cooled to room temperature, diluted with ethylacetate (30 mL), and washed with saturated aqueous ammonium chloride solution (6x20 mL). The combined organic phase was dried ($MgSO_4$), filtered, and concentrated *in vacuo*. Reaction progression was monitored by TLC, and in cases by GC-MS. Final analysis was by NMR.

*Synthesis of (3,5-Dioxo-10-oxa-4-azatricyclo[5.2.1.02,6]dec-4-yl)acetic acid (**73**)*



73

The ethanoic acid substituted analogue **73** was synthesised according to the general method above (**4.4.3**) from aminoethanoic acid and norcantharidin **13**. Recrystallisation from EtOAc/hexanes affording white crystals, (463 mg, 69%).

Mp: 132-135°C.

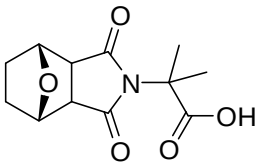
^{1}H NMR (DMSO-d_6): δ 4.81 (2H, s) 3.46 (2H, q, *J* = 7.1 Hz), 2.80 (2H, s), 1.80 (2H, m), 1.55 (2H, m), 1.09 (3H, t, *J* = 7.21 Hz);

^{13}C NMR (DMSO-d_6): δ 176.9, 176.0, 79.0, 49.9, 33.9, 28.5, 27.8;

ESI-MS: 226 [M+H], 225 [M], 127 (100%);

HR-MS: calc 225.05986.

*Synthesis of 2-(3,5-Dioxo-10-oxa-4-azatricyclo[5.2.1.02,6]dec-4-yl)-2-methylpropionic acid (**74**)*

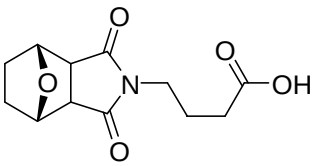


74

The N-2'-(2methylpropanoic acid) substituted analogue **74** was synthesised according to the general method (**4.4.3**) from 2-amino-2-methylpropionoic acid and norcantharidin **13**. Recrystallisation from EtOAc/hexanes affording white crystals (490 mg, 65%).

Mp: 221-224°C.
^{1}H NMR (DMSO d_6): δ 4.68 (2H, m), 3.00 (2H, s), 1.59 (4H, m), 1.52 (6H, s);
^{13}C NMR (DMSO d_6): δ 177.3, 173.2, 78.7, 59.8, 49.0, 27.8, 23.3;
GCMS: 253 [M], 208 (100%);
HR-MS: calc 253. 092716.

*Synthesis of (3,5-Dioxo-10-oxa-4-azatricyclo[5.2.1.02,6]dec-4-yl)butyric acid (**75**)*



75

The N-butyric acid substituted analogue **75** was synthesised according to the general method (**4.4.3**) from 4-aminobutyric acid and norcantharidin **13**. Recrystallisation from EtOAc/hexanes affording white crystals (339mg, 45%).

Mp: 151-154°C.

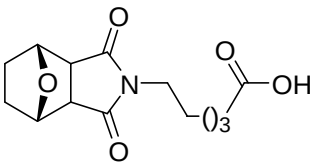
^{1}H NMR (DMSO d_6): δ 4.84 (2H, m), 3.53 (2H, t, *J* = 6.8 Hz), 2.84 (2H, s), 2.32 (2H, t, *J* = 7.4), 1.86 (4H, m), 1.57 (2H, m);

^{13}C NMR (DMSO d_6): δ 177.2, 177.1, 79.2, 50.0, 38.1, 30.9, 28.6, 22.7;

GCMS: 253 [M], 68 (100%);

HR-MS: calc 253.092716.

*Synthesis of 6-(3,5-Dioxo-10-oxa-4-azatricyclo[5.2.1.02,6]dec-4-yl)hexanoic acid (**76**)*



76

The N-hexanoic acid substituted analogue **76** was synthesised according to the general method (**4.4.3**) from 6-aminohexanoic acid and norcantharidin **13**. Recrystallisation from EtOAc/hexanes affording white crystals (368 mg, 44%).

Mp: 103-106°C.

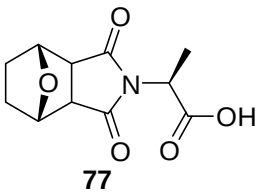
^{1}H NMR (DMSO d_6): δ 4.66 (2H, m), 3.29 (2H, t, *J* = 7.1 Hz), 2.99 (2H, s), 2.14 (2H, t, *J* = 7.3 Hz), 1.61 (4H, m), 1.43 (4H, m), 1.19 (2H, m);

^{13}C NMR (DMSO d_6): δ 177.4, 174.2, 78.4, 49.3, 37.8, 33.4, 27.9, 26.8, 25.5, 23.9;

GCMS: 281 [M], 68 (100%);

HR-MS: calc 281.081273

*Synthesis of 2-(3,5-Dioxo-10-oxa-4-azatricyclo[5.2.1.02,6]dec-4-yl)propionic acid (**77**)*



77

The D-alanine substituted analogue **77** was synthesised according to the general method (**4.4.3**) from D-alanine and norcantharidin **13**. Recrystallisation from EtOAc/hexanes affording white crystals (363 mg, 51%).

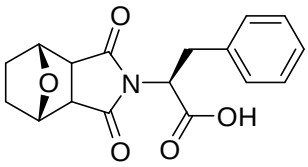
Mp: 151-155°C.

^{1}H NMR (DMSO d_6): δ 4.77 (2H, m), 4.71 (1H, q, *J* = 7.2 Hz), 3.02 (2H, m), 1.76 (2H, m), 1.62 (2H, m), 1.41 (3H, d, *J* = 7.2 Hz);

^{13}C NMR (DMSO d_6): δ 176.5, 170.4, 78.4, 50.9, 49.2, 28.0, 14.0;

HR-MS: calc 239.08932.

*Synthesis of 2-(3,5-Dioxo-10-oxa-4-azatricyclo[5.2.1.02,6]dec-4-yl)-3-phenylpropionic acid (**78**)*



78

The D-phenylalanine substituted analogue **78** was synthesised according to the general method (**4.4.3**) from D-phenylalanine and norcantharidin **13**. Recrystallisation from EtOAc/hexanes affording white crystals (507 mg, 54%).

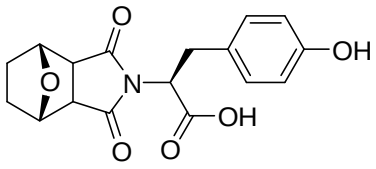
Mp: 202-205°C.

^{1}H NMR (DMSO d_6): δ 9.21 (1H, bs), 7.00, (2H, d, *J* = 8.3 Hz), 6.67 (2H, d, *J* = 8.3 Hz), 3.26 (4H, m), 3.09 (3H, m), 1.67 (4H, m);

^{13}C NMR (DMSO d_6): δ 176.5, 169.4, 155.7, 129.8, 127.1, 115.0, 78.1, 53.7, 49.0, 33.7, 27.9;

HR-MS: calc 315.11872.

*Synthesis of 2-(3,5-Dioxo-10-oxa-4-azatricyclo[5.2.1.02,6]dec-4-yl)-3-(4-hydroxyphenyl)propionic acid (**79**)*



79

The D-tyrosine substituted analogue **79** was synthesised according to the general method (**4.4.3**) from D-tyrosine and norcantharidin **13**. Recrystallisation from EtOAc/hexanes affording slightly pale yellow crystals (572 mg, 58%).

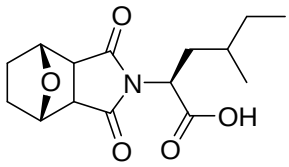
Mp: 158-161°C.

^{1}H NMR (DMSO d_6): δ 7.18 (2H, d, *J* = 7.3 Hz), 7.09 (2H, d, *J* = 7.3 Hz), 4.73 (1H, q, *J* = 4.6, 11 Hz), 4.65 (1H, m), 4.50 (1H, m), 3.30 (2H, m), 3.10 (2H, d, *J* = 11 Hz), 2.98 (1H, d, *J* = 7.2 Hz), 2.89 (1H, d, *J* = 7.2 Hz), 1.56 (4H, m);

^{13}C NMR (DMSO d_6): δ 176.5, 169.3, 137.0, 128.9, 128.1, 126.4, 78.1, 53.4, 49.0, 48.9, 33.2, 28.0, 27.8;

HR-MS: calc 331.11963.

*Synthesis of 2-(3,5-Dioxo-10-oxa-4-aza-tricyclo[5.2.1.02,6]dec-4-yl)-4-methylhexanoic acid (**80**)*



80

The D-isoleucine substituted analogue **80** was synthesised according to the general method (**4.4.3**) from D-isoleucine and norcantharidin **13**. Recrystallisation from EtOAc/hexanes affording slightly pale yellow crystals (385 mg, 46%).

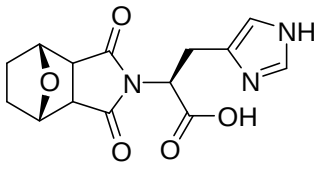
Mp: 114-117°C.

^{1}H NMR (DMSO d_6): δ 4.70 (2H, m), 4.23 (1H, m), 3.09 (2H, m), 2.20 (1H, m), 1.62 (4H, m), 1.35 (2H, m), 0.94 (3H, d, *J* = 6.7 Hz), 0.80 (3H, m);

^{13}C NMR (DMSO d_6): δ 176.9, 169.2, 78.6, 56.5, 48.9, 38.7, 27.9, 24.5, 16.6, 10.6;

HR-MS: calc 295.12981

*Synthesis of 2-(3,5-Dioxo-10-oxa-4-azatricyclo[5.2.1.02,6]dec-4-yl)-3-(1H-imidazol-4-yl)propionic acid (**32**)*

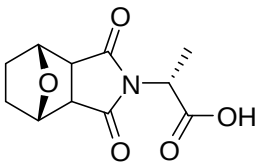


32

The D-histidine substituted analogue **32** was synthesised according to the general method above (**4.4.3**) from D-histidine and norcantharidin **13**. Recrystallisation from EtOAc/hexanes affording slightly pale yellow crystals (445 mg, 49%).

Mp: 238-241°C.
^{1}H NMR (DMSO d_6): δ 7.54 (1H, m), 6.70 (1H, s), 4.62 (2H, m), 3.20 (1H, m), 2.95 (2H, m), 2.72 (1H, s), 1.59 (4H, m);
^{13}C NMR (DMSO d_6): δ 176.6, 169.4, 134.5, 132.9, 78.2, 52.7, 49.1, 49.0, 28.0, 27.9, 25.3.;
HR-MS: calc 305.102746.

*Synthesis of 2-(3,5-Dioxo-10-oxa-4-azatricyclo[5.2.1.02,6]dec-4-yl)propionic acid (**81**)*



81

The L-alanine substituted analogue **81** was synthesised according to the general method above (**4.4.3**) from L-alanine and norcantharidin **13**. Recrystallisation from EtOAc/hexanes affording slightly pale yellow crystals (370 mg, 52%).

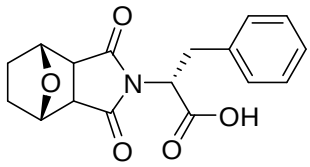
Mp: 189-91°C.

^{1}H NMR (DMSO d_6): δ 4.77 (2H, m), 4.71 (1H, q, *J* = 7.2 Hz), 3.02 (2H, m), 1.76 (2H, m), 1.67 (2H, m), 1.46 (3H, d, *J* = 7.2 Hz).

^{13}C NMR (DMSO d_6): δ 178.6, 178.4, 172.4, 80.5, 51.2, 51.1, 29.4, 29.3, 14.5.

HR-MS: calc 239.08932.

*Synthesis of 2-(3,5-Dioxo-10-oxa-4-azatricyclo[5.2.1.02,6]dec-4-yl)-3-phenylpropionic acid (**82**)*

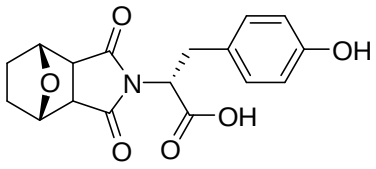


82

The L-phenylalanine substituted analogue **82** was synthesised according to the general method above (**4.4.3**) from L-phenylalanine and norcantharidin **13**. Recrystallisation from EtOAc/hexanes affording slightly pale yellow crystals (516 mg, 55%).

Mp: 208-211°C;
^{1}H NMR (DMSO d_6): δ 9.10, 6.91 (2H, d, *J* = 8.4 Hz), 6.57 (2H, d, *J* = 8.4 Hz), 4.62 (2H, m), 3.00 (2H, m), 1.60 (4H, m);
^{13}C NMR (DMSO d_6): δ 176.5, 176.4, 169.4, 155.7, 129.8, 127.1, 115.0, 78.1, 53.7, 49.0, 48.9, 32.3, 28.0, 27.8;
HR-MS: calc 315.11872.

*Synthesis of 2-(3,5-Dioxo-10-oxa-4-azatricyclo[5.2.1.02,6]dec-4-yl)-3-(4-hydroxyphenyl)propionic acid (**83**)*

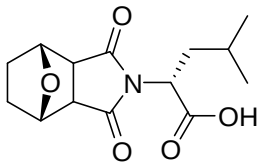


83

The L-tyrosine substituted analogue **83** was synthesised according to the general method above (4.4.3) from L-tyrosine and norcantharidin **13**. Recrystallisation from EtOAc/hexanes affording slightly pale yellow crystals (503 mg, 51%).

Mp: 162-165°C;
^{1}H NMR (DMSO d_6): δ 7.18 (2H, d, *J* = 7.3 Hz), 7.09 (2H, d, *J* = 7.3 Hz), 4.73 (1H, q, *J* = 4.6, 11 Hz), 4.65 (1H, m), 4.50 (1H, m), 3.30 (2H, m), 3.10 (2H, d, *J* = 11 Hz), 2.98 (1H, d, *J* = 7.2 Hz), 2.89 (1H, d, *J* = 7.2 Hz), 1.56 (4H, m);
^{13}C NMR (DMSO d_6): δ 176.5, 169.3, 137.0, 128.9, 128.1, 126.4, 78.1, 53.4, 49.0, 48.9, 33.2, 28.0, 27.8;
HR-MS: calc 331.11963.

*Synthesis of 2-(3,5-Dioxo-10-oxa-4-azatricyclo[5.2.1.02,6]dec-4-yl)-4-methylpentanoic acid (**84**)*

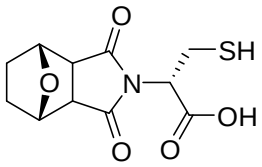


84

The L-leucine substituted analogue **84** was synthesised according to the general method (**4.4.3**) from L-leucine and norcantharidin **13**. Recrystallisation from EtOAc/hexanes affording slightly pale yellow crystals (402 mg, 48%).

Mp: 187-190°C;
^{1}H NMR (DMSO d_6): δ 4.69 (2H, m), 4.52 (1H, dd, *J* = 4.4, 11.2), 3.09 (2H, q, *J* = 7.4, 20.8 Hz), 1.95 (1H, m), 1.70-1.59 (4H, m), 1.34 (2H, m), 0.80 (6H, t, *J* = 6 Hz);
^{13}C NMR (DMSO d_6): δ 176.8, 170.1, 78.5, 50.3, 49.2, 49.0, 36.0, 27.9, 27.8, 24.0, 23.1, 20.8;
HR-MS: calc 281.12879.

Synthesis of 2-(3,5-Dioxo-10-oxa-4-azatricyclo[5.2.1.02,6]dec-4-yl)-3-mercaptopropionic acid ***(85)***



85

The L-cysteine substituted analogue **85** was synthesised according to the general method (**4.4.3**) from L-cysteine and norcantharidin **13**. Recrystallisation from EtOAc/hexanes affording white crystals (460 mg, 57%).

Mp: 272-275°C;
^{1}H NMR (DMSO d_6): δ 4.77 (2H, m), 4.71 (1H, q, *J* = 7.2 Hz), 3.05 (2H, m), 3.02 (2H, m), 1.76 (2H, m), 1.67 (2H, m).
^{13}C NMR (DMSO d_6): δ 178.6, 178.4, 172.4, 80.5, 51.2, 51.1, 29.4, 24.3.
HR-MS: calc 271.049873.

4.5 References

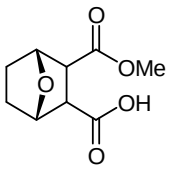
1. Honkanen, R.E. and T. Golden, *Regulators of Serine/Threonine Protein Phosphatase at the Dawn of the Clinical Era?* Current Medicinal Chemistry, 2002. **9**: p. 2005-2075.
2. McCluskey, A., Keane, M. A., Mudgee, L., Sim, A. T. R., Sakoff, J. A., *Anhydride Modified Cantharidin Analogues. Is Ring Opening Important in the Inhibition of Protein Phosphatase 2A?* European Journal of Medicinal Chemistry, 2000. **35**: p. 957-964.
3. McCluskey, A., Walkom, C., Bowyer, M. C., Ackland, S. P., Gardiner, E., Sakoff, J. A., *Cantharimides: A New Class of Modified Cantharidin Analogues Inhibiting Protein Phosphatases 1 and 2a*. Bioorganic and Medicinal Chemistry Letters, 2001. **11**: p. 2941-2946.
4. Gauss, C., Sheppeck, J. E., Nairn, A, C., Chamberlin, R., *A Molecular Modeling Analysis of the Binding Interactions Between the Okadaic Acid Class of Natural Product Inhibitors and the Ser-Thr Phosphatases, PP1 and PP2A.* Bioorganic & Medicinal Chemistry, 1997. **5**(9): p. 1751-1773.
5. Hill, T.H., Stewart, S. G., Gilbert, J., Ackland, S, P., Sauer, B., Sakoff, J, A., McCluskey, A., *Norcantharidin, Synthesis and Anticancer Activity:Synthesis of New Norcantharidin Analogues and their Anticancer Evaluation*. Bioorganic Medicinal Chemistry, 2007. **15**: p. 6126-6134.
6. McCluskey, A., Ackland, S. P., Gardiner, E., Walkom, C. C., Sakoff, J. A., *The Inhibition of Protein Phosphatases 1 and 2A: A New Target for Rational Anti-Cancer Drug Design?* Anti-Cancer Drug Design, 2001. **16**: p. 291-303.
7. Hill, T.H., Stewart, S. G., Ackland, S, P., Gilbert, J., Sauer, B., Sakoff, J, A., McCluskey, A., *Norcantharidin Analogues: Synthesis, Anti-cancer Activity and Protein Phosphatase 1 and 2A Inhibition. Evidence for a Small Molecule Binding Pocket.* Bioorganic Medicinal Chemistry Letters, 2007. **15**: p. 6126-6134.
8. Lin, P., H., Huang, H, S., Lin, C, C., Lee, L, W., Lin, P, Y., *Effects of Cantharidinimides on Human Carcinoma Cells.* Chemical and Pharmaceutical Bulletin, 2004. **52**: p. 855-857.
9. Zhou, Z., H., Zhang, Z, B., Chen, R, U., *Synthesis of Phosphonodipeptide derivatives of exo-7-oxabicyclo[2.2.1]heptane-2,3-dicarboxylic anhydride.* Phosphorus, Sulfur and Silicon and the Related Elements, 1999. **152**: p. 45-52.
10. Grogen, C.H., Rice, L. M., *Bicyclic Imides and Isoindolines.* Journal of Medicinal Chemistry, 1963. **6**(6): p. 802-804.

Chapter 5

Development of Ring Opened Cantharimides as Potential PP1 and PP2A Inhibitors

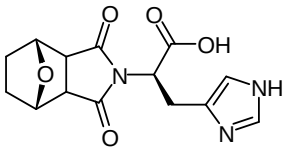
5.1 Introduction

As previously noted the ring opening of cantharidin to the dicarboxylic form has been described as an important characteristic for PP1 and PP2A activity, however the previous development of ring-opened analogues, such as the monoester (**24**) and ring-closed cantharimides (**Chapter 4**) including (**32**), both undermine the importance of this mechanism for binding [1] [2]. The data presented herein raises some questions about the earlier studies, and challenges the original idea that ring opening anhydride species is crucial in maintaining inhibition of PP1 and PP2A. Furthermore, more recent molecular modelling data reported by McCluskey *et. al*, and bioassay results strongly suggests that the original hypothesis of cantharidin analogues binding within PP2A's active site is erroneous [3]. Additional support for this revised hypothesis has been forthcoming from Shan's laboratory [4]. Shan has demonstrated that cantharidin is not a competitive inhibitor of PP2A and this data supports the modelling findings from McCluskey's laboratory [3].



PP1 = 4.7 μM; PP2A = 0.41 μM

24



PP1 = 3.22 μM; PP2A = 0.81 μM

32

Given the lack of success in anhydride modifications, and these recent developments in both enzyme kinetics and molecular modelling, the main focus of this chapter is to further examine the McCluskey hypothesis, and to generate a number of ring-opened cantharimide analogues for screening. The range of amine substituents chosen, is similar to **Chapter 4** (ring-closed analogues), and represents the diverse range of the pool available, including aliphatic chains, aromatic rings, alcohols, primary and secondary amines, as well as morpholine moieties. Substituents also varied in size, flexibility and spatial distribution. Analogues were initially

screened for phosphatase and carcinoma cell line inhibition. The PP1 and PP2A IC_{50} and cell line GI_{50} (μM) of successful candidates were also determined.

5.2 Results and Discussion – Chemical Synthesis

The generation of the ring-opened cantharimides, via an elegantly simple one step modified Gabriel synthesis, was developed by this laboratory. Commencing with the easily synthesised norcantharidin (**13**), subsequent treatment with one equivalent of the relevant amine at room temperature in dry THF at room temperature, generated the respective ring-opened cantharimide analogue precipitating overnight with generally good yields. In a few isolated cases the ring closed analogues could be detected in trace amounts, the degree to which this occurred was predicted by the inherent nucleophilicity of the starting amine. Highly nucleophilic amines showed a propensity towards generating the ring closed analogues, fortunately in this instance this was the exception rather that the rule.

Reaction rates varied as did yields, however generally the quicker the reaction the better the yield; typically a function of amine nucleophilicity. Reaction yields were also largely affected by the quality of the precipitation. That is with slower precipitation, the larger needle crystals formed, typically resulting in better yields. Limiting factors inhibiting the types of amines used included steric bulk associated with the amine lone pair of electrons, and was also dependant on whether a primary or secondary amine was being generated, as shown by the attempted synthesis of morpholine-3,5-dione, and solubility, such as 2-aminoisobutyric acid. The synthesis of the 3,5-dione analogue has since been carried out in our laboratory, and required modest heat. Refer to **Figure 5.1** for a proposed reaction mechanism.

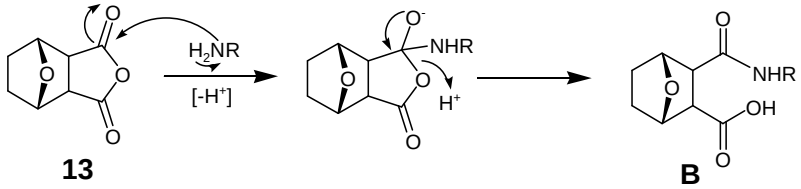


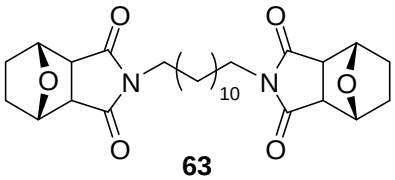
Figure 5.1: Possible mechanism for the formation of ring opened cantharimides

5.3 Results and Discussion – Biological Assay

5.3.1 Protein Phosphatase and Growth Inhibition

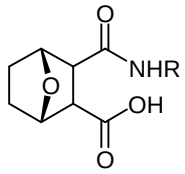
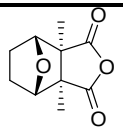
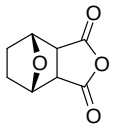
As in previous chapters two main areas of biological investigations were pursued: protein phosphatase inhibition (PP1 and PP2A) with initial screening conducted at 100 μM drug concentration, and cytotoxicity against an extended panel of human carcinoma cell lines, again at 100 μM drug concentration. These cell lines included; HT29 and SW480 (colorectal carcinoma), G401 (kidney carcinoma), MCF-7 (breast adenocarcinoma), A2780 (ovarian carcinoma), H460 (lung carcinoma), A431 (epidermoid carcinoma), DU145 (prostate carcinoma), BE2-C (neuroblastoma) and SJ-G2 (glioblastoma). Where warranted both IC_{50} and GI_{50} determinations were undertaken. For results refer to **Tables 5.1** through **5.3**.

Firstly, the PP1 and PP2A inhibition and cytotoxicity of a variety of simple ring-opened cantharimides possessing alkyl substitutes (**86-89**) were analysed, as well as cantharidin (**6**) and norcantharidin (**13**) as controls as shown in **Table 5.1**. As previously described, the controls, cantharidin (**6**) and norcantharidin (**13**), display broad spectrum anticancer activity with (**6**) displaying a 10-fold improved potency against its demethylated analogue. Of the compounds displayed in **Table 5.1**, the most significant analogues proved to be the piperidine (**89**) and cyclopentyl (**88**). The piperidine analogue was by far the most intriguing we have produced so far; displaying more potent phosphatase inhibition results than the parent anhydride (**13**), and generally displayed an increase in cytotoxicity than either the parent anhydride the ring-closed (**Chapter 4**) bis-analogue (**63**) across the cell lines evaluate. The cyclopentyl analogue (**88**) also displayed comparable cytotoxicity to the parent anhydride (**13**) and also displayed moderate PP2A selectivity. The ethyl (**86**) and propyl (**87**) analogues showed moderate to poor phosphatase and growth inhibition.



63

Table 5.1: Inhibition of PP1 and PP2A, and Growth Inhibition of cantharidin (**6**), norcantharidin (**13**) and a variety of functionalised ring-opened alkylated cantharimides (**86-89**) in a panel of human cancer cell lines. Inhibition levels of PP1 and PP2A are first expressed as % inhibition at 100 M drug concentration (*in italics*) and, if potent as IC_{50} µM (**in bold**). Cytotoxicity levels are first expressed as % inhibition at 100 µM drug concentration (*in italics*) and if potent, as Growth Inhibition, GI_{50} µM (**in bold**).

Compound	R	IC_{50} (µM)		GI_{50} (µM)								
		PP1	PP2A	HT29	SW480	MCF-7	A2780	H460	A431	DU145	BE2-C	SJ-G2
												
6		**11 ± 2.0**	**1.2 ± 0.1**	**32 ± 0.1**	**4.5 ± 0.3**	**7.5 ± 0.4**	**4.4 ± 0.3**	**3.3 ± 0.2**	**2.9 ± 0.2**	**2.1 ± 0.3**	**3.7 ± 0.6**	**1.7± 0.1**
13		**9.0 ± 1.4**	**3.0 ± 0.4**	**57 ± 5**	**44 ± 6**	**68 ± 4**	**38 ± 1**	**45 ± 3**	**31 ± 1**	**28 ± 3**	**43 ± 6**	**23± 3**
86	Et	*72 ±2.2*	*61 ±9.1*	*12 ±3.3*	*5 ±3.1*	*12 ±2.9*	*13 ±2 .9*	*5 ±4.9*	*6 ±1.9*	*<0*	*2 ±6.1*	*9 ±5.1*
87	*n*-Pr	*70 ±2.6*	*59 ±6.4*	*8 ±2.9*	*4 ±2.6*	*10 ±3.2*	*14 ±2.6*	*5 ±6.2*	*9 ±2.2*	*<0*	*3 ±2.6*	*8 ±6.1*
88	Cyclo-pentyl	*90 ±3.2*	*87 ±1.4*	*89 ±0.9*	*>100*	*93 ±0.9*	*87 ±1.3*	*>100*	*96 ±1.3*	*>100*	*>100*	*98 ±5.6*
		18 ± 1.5	**17 ± 1.0**	**44 ± 3.0**	**27 ± 4.5**	**48 ± 2.5**	**56 ± 1.0**	**29 ± 4.3**	**32 ± 2.0**	**33 ± 4.0**	**31 ± 2.0**	**31± 1.5**
89	Piperidine	**3.0 ± 0.5**	**4.4 ± 0.2**	**14 ± 1.2**	**9.3 ± 0.4**	**14 ± 0.9**	**12 ± 1.0**	**19 ± 1.7**	**14 ± 0.2**	**16 ± 0.3**	**14 ± 0.3**	**18± 1.9**

NB: A value of 0% indicates that the compound failed to influence enzyme inhibition when compared with untreated controls (i.e., the higher the value the greater the enzyme inhibition).

Given the promising data displayed in **Table 5.1**, the effect of additional functionality was then explored in the hope that this would have an impact on the ability of these analogues to penetrate the cell membrane, hence improving their resultant cytotoxicity. **Table 5.2** shows the phosphatase inhibition and cytotoxicity of a variety of simple ring-opened cantharimides possessing functionalised substituent's (**90**-**102**). Of the compounds displayed in **Table 5.2**, several proved to be significant phosphatase inhibitors, as well as displaying good to excellent cytotoxicity to the panel of cell lines, including; the diol (**91**), piperidinyl ethanol (**92**), morpholine (**93**), thiomorpholine (**94**), piperazine (**95**) and dimethylmorpholine (**97**). Interestingly, all of these analogues also exhibit improved cytotoxicty to the parent anhydride (**13**).

Of particular significance, the morpholine (**93**) and thiomorpholine (**94**) analogues display significant PP2A selectivity (>3 fold selectivity), and display comparable cytotoxicity to the methylated anhydride (**6**). The 4-aminomorpholine (**98**) and 4-ethylaminomorpholine (**99**) analogues also displayed moderate phosphatase activity; however GI_{50} values were not determined. The slight reduction in activity may be a result of moving the morpholine moiety away from the carboxyl group, whilst also increasing the size and possible spatial hindrance. The 2-aminopropanol (**90**) analogue also displayed promising phosphatase activity and cytotoxicity. The pyrrolidinyl ethylamine (**100**), piperidinyl ethylamine (**101**) and piperazinyl ethylamine (**102**) displayed poor phosphatase and growth inhibition. From these results, it is apparent that the introduction of an oxygen, sulphur or nitrogen to the ring opened skeleton has a significant effect on the phosphatase and cytotoxicity, whilst increasing structural diversity of the ring opened analogues. Furthermore, the alcoholic analogues allow a handle for further modifications.

Table 5.2: Inhibition of PP1 and PP2A, and Growth Inhibition of a variety of functionalised ring-opened cantharimides (**90**-**102**) in a panel of human cancer cell lines. Inhibition levels of PP1 and PP2A are first expressed as % inhibition at 100 M drug concentration (*in italics*) and, if potent as IC_{50} µM (**in bold**). Cytotoxicity levels are first expressed as % inhibition at 100 µM drug concentration (*in italics*) and if potent, as Growth Inhibition, GI_{50} µM (**in bold**)

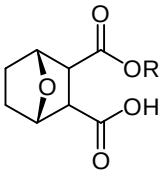
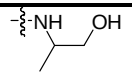
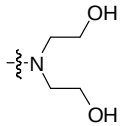
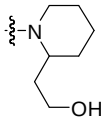
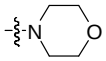
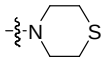
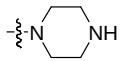
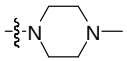
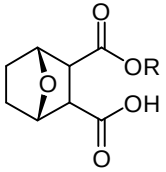
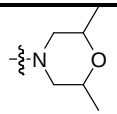
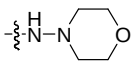
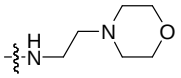
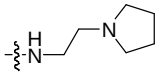
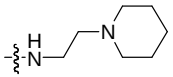
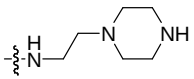
Compound	R	PP1	PP2A	HT29	SW480	MCF-7	A2780	H460	A431	DU145	BE2-C	SJ-G2
												
90		*82 ± 2.6*	*84 ± 9.8*	*98 ± 3.6*	*>100*	*>100*	*>100*	*95 ± 4.9*	*96 ± 1.9*	*>100*	*>100*	*99 ± 5.1*
91		*>100*	*99 ± 1.3*	*>100*	*>100*	*>100*	*>100*	*>100*	*>100*	*>100*	*>100*	*>100*
		5 ± 1.0	**3 ± 1.0**	**29 ± 0**	**18 ± 0**	**35 ± 1.0**	**37 ± 0.5**	**20 ± 1.5**	**24 ± 1.5**	**25 ± 0**	**22 ± 0.5**	**19 ± 1.5**
92		*>100*	*98 ± 1.2*	*>100*	*>100*	*>100*	*>100*	*99 ± 1.7*	*>100*	*>100*	*>100*	*>100*
		5 ± 1.0	**4 ± 0.4**	**19 ± 0.5**	**12 ± 2.9**	**17 ± 1.0**	**18 ± 0.5**	**14 ± 0.8**	**17 ± 0.8**	**17 ± 0.3**	**15 ± 0**	**16 ± 0**
93		**13 ± 2.0**	**2.8 ± 0.1**	**7.4 ± 2.1**	**5.5 ± 0.1**	**7.7 ± 1.8**	**8.3 ± 0.4**	**18 ± 2.8**	**8.5 ± 1.3**	**14 ± 0.3**	**6.0 ± 0.6**	**11 ± 0.6**
94		*>100*	*>100*	*>100*	*>100*	*>100*	*>100*	*99 ± 1.7*	*>100*	*>100*	*>100*	*>100*
		3.2 ± 0	**5 ± 0.4**	**10 ± 1.0**	**4.0 ± 0.5**	**7.0 ± 0.8**	**8.0 ± 0.8**	**12 ± 1.3**	**8.4 ± 0.3**	**11 ± 1.0**	**4.7 ± 0.4**	**9.4 ± 0.7**
95		**32 ± 2.0**	**9.3 ± 0.8**	**17 ± 0**	**17 ± 0.9**	**20 ± 1.7**	**24 ± 2.5**	**21 ± 1.6**	**23 ± 3.0**	**29 ± 1.5**	**2.0 ± 0.7**	**13 ± 3.0**
96		Insol.	Insol.	Insol.	Insol.	Insol.	Insol.	Insol.	Insol.	Insol.	Insol.	Insol.

Table 5.2 (Continued) Inhibition of PP1 and PP2A, and Growth Inhibition of a variety of functionalised ring-opened cantharimides (**90**-**102**) in a panel of human cancer cell lines. Inhibition levels of PP1 and PP2A are first expressed as % inhibition at 100 M drug concentration (*in italics*) and if potent as IC_{50} µM (**in bold**). Cytotoxicity levels are first expressed as % inhibition at 100 µM drug concentration (*in italics*) and if potent, as Growth Inhibition, GI_{50} µM (**in bold**)

Compound	R	PP1	PP2A	HT29	SW480	MCF-7	A2780	H460	A431	DU145	BE2-C	SJ-G2
												
97		**9.3 ± 1.2**	**4.4 ± 0.1**	**19 ± 0.9**	**15 ± 1.3**	**24 ± 2.7**	**24 ± 1.5**	**24 ± 0.9**	**21 ± 0**	**25 ± 0**	**18 ± 3.6**	**20 ± 0.7**
98		**44 ± 6.3**	**18 ± 0.3**	*29 ±5.3*	*29 ±1.0*	*12 ±3.8*	*7.0 ±1.8*	*18 ±0.4*	*36 ± 4.9*	*3.1 ±5.0*	*39 ±8.1*	*30 ±2.4*
99		**31 ± 2.9**	**43 ± 1.0**	*37 ±13*	*14 ±1.6*	*7.5 ±3.0*	*14 ±1.2*	*10 ±3.7*	*23 ±5 .6*	*17 ±6.9*	*26 ±20*	*19 ±4.8*
100		*< 0*	*< 0*	*3.9 ±3.0*	*2.4 ±3.1*	*5.9 ±6.6*	*8.4 ±2.8*	*7.7 ±4.8*	*6.3 ±2.8*	*< 0*	*< 0*	*5.7 ±3.3*
101		*< 0*	*< 0*	*13 ±4.7*	*5.1 ±8.3*	*13 ±1.8*	*11 ±0.6*	*1.2 ± 7.7*	*4.8 ±3.4*	*< 0*	*2.6 ±1.4*	*< 0*
102		*< 0*	*< 0*	*4.0 ±6.5*	*4.0 ±1.9*	*7.0 ±4.7*	*7.0 ±0.9*	*3.0 ±3.2*	*4.0 ±3.1*	*< 0*	*< 0*	*< 0*

NB: A value of 0% indicates that the compound failed to influence enzyme inhibition when compared with untreated controls (i.e., the higher the value the greater the enzyme inhibition).

Given the promising data displayed in **Table 5.2**, we next examined the effect of the addition of aromatic functionality on the phosphatase inhibition and resultant cytotoxicity. **Table 5.3** shows the phosphatase inhibition and cytotoxicity of a variety of simple ring-opened cantharimides possessing aromatic functionality (**103-109**). The 4-pentyloxyphenylamine (**105**) analogue displayed excellent PP1 (>100%) and PP2A (97.5%) inhibition, with IC_{50} values for PP1 (12.5 µM) and PP2A (10 µM) comparable to results obtained for cantharidin (**6**) and norcantharidin (**13**). The indanylamine (**107**) analogue displayed good phosphatase inhibition with no significant PP1 or PP2A selectivity illustrated. The indanylamine (**85**) analogue also possessed an excellent IC_{50} (µM) values for PP1 (25.5 µM) and PP2A (22.5 µM), which is comparable to results obtained for cantharidin (**6**) and norcantharidin (**13**). The analogues possessing basic amine residues (**104, 108**-**109**) yielded moderate to poor phosphatase inhibition (65-29%).

Table 5.3: Inhibition of PP1 and PP2A, and Growth Inhibition of a variety of aromatic ring-opened cantharimides (**103-109**) in a panel of human cancer cell lines. Inhibition levels of PP1 and PP2A are first expressed as % inhibition at 100 M drug concentration (*in italics*) and, if potent as IC_{50} µM (**in bold**). Cytotoxicity levels are first expressed as % inhibition at 100 µM drug concentration (*in italics*) and if potent, as Growth Inhibition, GI_{50} µM (**in bold**)

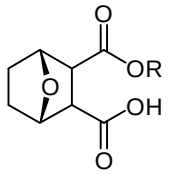
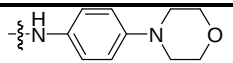
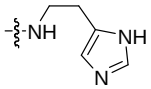
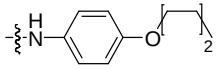
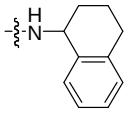
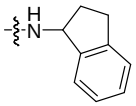
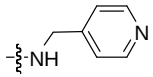
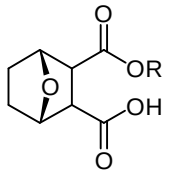
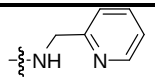
Compound	R	PP1	PP2A	HT29	SW480	MCF-7	A2780	H460	A431	DU145	BE2-C	SJ-G2
												
103		**6.5 ± 2.4**	**7.9 ± 0.4**	*23 ±1.6*	*29 ±0.7*	*20 ±2.7*	*18 ±3.8*	*19 ±3.1*	*21 ±3 .9*	*17 ±1.6*	*13 ±4.1*	*21 ±2.3*
104		*35 ±5.5*	*56 ±8.7*	*21 ±4.6*	*33 ±5.3*	*17 ±2.0*	*20 ±1 .9*	*25 ±5.1*	*32 ±2.5*	*14 ±3.0*	*23 ±2.3*	*27 ±3.7*
105		*>100*	*98 ±1.4*	*77 ±1.5*	*72 ±4.2*	*94 ±2.1*	*59 ±1.6*	*39 ±4.0*	*67 ±3 .3*	*56 ±5.5*	*55 ±1.3*	*75 ±2.8*
		13 ± 1.5	**10 ± 2.0**									
106		*49 ±0.2*	*56 ±11*	*60 ±1.7*	*93 ±2.8*	*60 ±2.3*	*53 ±1. 3*	*98 ±3.1*	*78 ±2.6*	*84 ±4.8*	*96 ±1.8*	*85 ±6.0*
107		*95 ±7.8*	*79 ±2.4*	*44 ±2.1*	*60 ±4.3*	*29 ±3.6*	*31 ±2.4*	*51 ±5.1*	*54 ±4 .7*	*45 ±3.9*	*52 ±5.2*	*58 ±4.3*
		26 ± 3.5	**23 ± 1.5**									
108		*46 ±3.8*	*29 ±4.1*	*23 ±3.3*	*26 ±3.6*	*17 ±3.6*	*15 ±1 .7*	*12 ±4.7*	*16 ±5.5*	*1.0 ±1.9*	*12 ±0.4*	*15 ±1.6*

Table 5.3 (Continued): Inhibition of PP1 and PP2A, and Growth Inhibition of a variety of aromatic ring-opened cantharimides (**103**-**109**) in a panel of human cancer cell lines. Inhibition levels of PP1 and PP2A are first expressed as % inhibition at 100 M drug concentration (*in italics*) and if potent as IC_{50} µM (**in bold**). Cytotoxicity levels are first expressed as % inhibition at 100 µM drug concentration (*in italics*) and, if potent, as Growth Inhibition, GI_{50} µM (**in bold**)

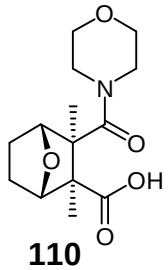
Compound	R	PP1	PP2A	HT29	SW480	MCF-7	A2780	H460	A431	DU145	BE2-C	SJ-G2
												
109		*65 ±2.4*	*50 ±3.1*	*32 ±1.4*	*49 ±5.0*	*27 ±2.9*	*25 ±1 .1*	*42 ±1.5*	*24 ±4.5*	*19 ±1.8*	*28 ±1.9*	*37 ±2.9*

NB: A value of 0% indicates that the compound failed to influence enzyme inhibition when compared with untreated controls (i.e., the higher the value the greater the enzyme inhibition).

5.3.2 General Summary

The nucleophilic ring opening of the parent norcantharidin (**13**) with a series of amines has allowed the generation of a variety of novel norcantharidin analogues that are more potent than the original lead compounds. Both the phosphatase inhibition and cytotoxicity were shown to be enhanced. In particular, further evaluation of substituted morpholino analogues should be investigated. Furthermore, since cantharidin (**6**) displays greater potency, PP2A selectivity and cytotoxicity than the demethylated analogue (**13**), it is also hypothesised that the generation of cantharidin based variants of **89**, **91**, **92** and **94**, may lead in to the development of novel potent analogues. For example, the synthesis of the morpholine cantharidin analogue (**110**) since developed by our laboratory, is more potent in all aspects than the parent cantharidin (**6**) and related morpholino-norcantharimide displayed in **Table 5.2** [5].

Table 5.4: Inhibition of PP1 and PP2A, and Growth Inhibition of the Morpholino Cantharimide analogue (**110**) in a panel of human cancer cell lines.

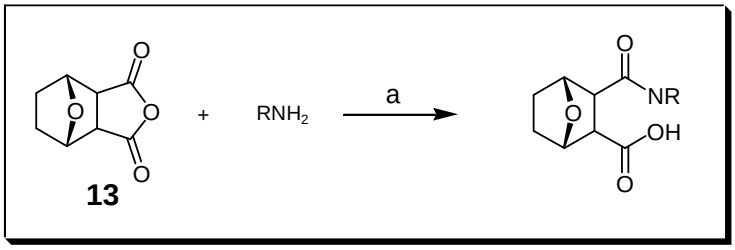
PP1	PP2A	HT29	SW480	MCF-7	A2780	H460	A431	DU145	BE2-C	SJ-G2
										
					110					
6 ±2.2	*0.8 ±0.1*	*3 ±0.0*	*4 ±0.1*	*6 ±0.2*	*5 ±0.4*	*3 ±0.1*	*3 ±0.1*	*2 ±0.0*	*3 ±0.3*	*2 ±0.1*

NB: A value of 0% indicates that the compound failed to influence enzyme inhibition when compared with untreated controls (i.e., the higher the value the greater the enzyme inhibition).

5.4 Experimental Synthesis of the Ring-opened Cantharimides

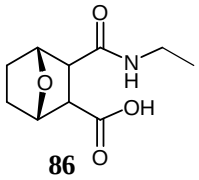
5.4.1 General Reaction Scheme

To a solution of (**13**) (200 mg, 1.19 mM), in dry THF (20 mL), was added 1 equivalent of the respective amine (1.19 mM). The reaction mixture was then stirred at room temperature for approximately 24 hrs using a Radley Carousel Reactor. The reaction mixture was cooled in an ice-water bath, and the precipitate was collected by filtration, and washed with cold THF (4-8 °C). The solid filtrate was then dried under high vacuum, and final analysis carried out using LCMS and NMR. A general reaction scheme is described in **Scheme 5.1**.



Scheme 5.1: Reagents and Conditions; (a) THF RT, 24h.

Synthesis of 3-Ethylcarbamoyl-7-oxabicyclo[2.2.1]heptane-2-carboxylic acid (**86**):

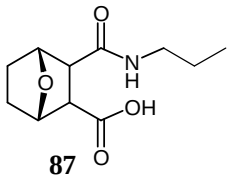


86

The N-ethyl substituted analogue **86** was synthesised according to the general method (**5.4.1**) using ethylamine (70%) and norcantharidin **13** affording pure yellow/white crystals (134.8 mg/0.632 mM), yield 53%.

Mp: 123-126°C;
^{1}H NMR (DMSO): δ 7.24 (1H, bs), 4.69 (1H, bs), 4.45 (1H, d, *J* = 3.7 Hz), 2.98 (1H, m), 2.77 (2H, s), 1.47 (4H, m), 0.95 (3H, t, *J* = 7.2 Hz);
^{13}C NMR (DMSO): δ 176.9, 174.8, 82.4, 81.2, 57.4, 56.7, 37.2, 32.7, 32.5, 29.4, 18.4;
ESI-MS (m/z): 258, [M+2Na] (100%), 236 [M+Na], 196 [M-17];
HR-MS (m/z): calc $C_{10}H_{15}NO_4$; 213.10385.

Synthesis of 3-propylcarbamoyl-7-oxabicyclo[2.2.1]heptane-2-carboxylic acid (**87**):

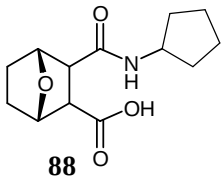


87

The N-propyl substituted analogue **87** was synthesised according to the general method (**5.4.1**) using propylamine and norcantharidin **13** affording pure white crystals (270.0 mg/1.19 mM), yield 100%.

Mp: 131-134°C;
^{1}H NMR (DMSO): δ 7.26 (1H, bs), 4.71 (1H, bs), 4.46 (1H, d, *J* = 3.7 Hz), 3.00 (1H, m), 2.77 (2H, s), 1.57 (4H, m), 1.47 (2H, q, J = 7.2 Hz), 0.95 (3H, t, *J* = 7.2 Hz);
^{13}C NMR (DMSO): δ 176.9, 174.7, 82.4, 81.1, 57.4, 56.6, 37.2, 32.7, 32.5, 18.4;
ESI-MS (m/z): 273, [M+2Na] (100%), 250 [M+Na], 210 [M-17];
HR-MS (m/z): calc $C_{11}H_{17}NO_4$; 227.12855.

Synthesis of 3-cyclopentylcarbamoyl-7-oxabicyclo[2.2.1]heptane-2-carboxylic acid (**88**):



88

The N-cyclopentane substituted analogue **88** was synthesised according to the general method (**5.4.1**) using propylamine and norcantharidin **13** affording pure white crystals (264.1 mg/1.04 mM), yield 88%.

Mp: 169-171°C;

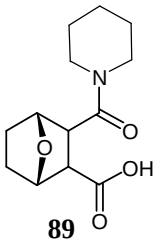
^{1}H NMR (DMSO): δ 7.30 (1H, d, *J* = 7.1 Hz), 4.82 (1H, d, *J* = 3.3 Hz), 4.56 (1H, d, *J* = 3.7 Hz), 4.11 (1H, m), 2.91 (2H, m), 1.60 (12H, m);

^{13}C NMR (DMSO): δ 176.3, 173.8, 82.7, 80.7, 56.8, 55.5, 54.0, 36.3, 36.1, 32.8, 32.3, 27.4;

ESI-MS (m/z): 298, [M+2Na], 276 [M+Na] (100%), 254 [M+H], 236 [M-17];

HR-MS (m/z): calc $C_{13}H_{19}NO_4$; 253.12945.

Synthesis of 3-(Piperidine-1-carbonyl)-7-oxa-bicyclo[2.2.1]heptane-2-carboxylic acid (**89**):



89

The piperidine analogue **89** was synthesised according to the general method (**5.4.1**) using piperidine and norcantharidin **13** affording (252.1 mg/0.99 mM), yield 83%.

Mp: 152 – 154°C;

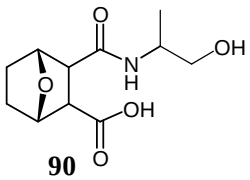
^{1}H NMR (DMSO): δ 7.31 (1H, d, *J* = 7.1), 4.83 (1H, d, *J* = 3.3 Hz), 4.57 (1H, d, *J* = 3.7 Hz), 4.11 (1H, m), 2.94 (2H, m), 1.60 (12H, m);

^{13}C NMR (DMSO): δ 176.5, 173.8, 82.7, 80.8, 56.8, 55.7, 53.9, 36.3, 36.1, 35.2, 32.8, 32.3, 27.4;

ESI-MS (m/z): 298, [M+2Na], 276 [M+Na] (100%), 254 [M+H], 236 [M-17];

HR-MS (m/z): calc $C_{13}H_{19}NO_4$; 253.12945.

Synthesis of 3-(2-Hydroxy-1-methylethylcarbamoyl)-7-oxa-bicyclo[2.2.1]heptane-2-carboxylic acid (**90**):

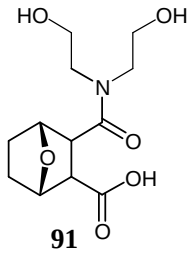


90

The *2-hydroxy-1-methyl* analogue **90** was synthesised according to the general method (**5.4.1**) using 2-amino-propan-1-ol and norcantharidin **13** affording pure light yellow crystals (72.1 mg/0.30 mM), yield 25%.

Mp: 132-134ºC;
^{1}H NMR (DMSO): δ 7.27 (1H, bs), 4.70 (1H, bs), 4.47 (1H, d, *J* = 3.7 Hz), 3.00 (1H, m), 2.77 (2H, s), 2.87 (1H, m), 1.57 (2H, q, *J* = 7.2 Hz), 1.01 (3H, t, *J* = 7.2 Hz);
^{13}C NMR (DMSO): δ 176.8, 174.6, 82.0, 81.1, 57.5, 56.6, 37.1, 32.8, 32.5, 18.4;
ESI-MS (m/z): 288, [M+2Na], 266 [M+Na] (100%), 244 [M+H], 236 [M-17];
HR-MS (m/z): calc $C_{11}H_{17}NO_5$; 243.10836.

Synthesis of 3-[Bis-(2-hydroxyethyl)-carbamoyl]-7-oxa-bicyclo[2.2.1]heptane-2-carboxylic acid (**91**):

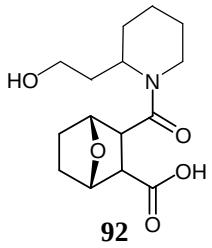


91

The N-diethanol analogue **91** was synthesised according to the general method (**5.4.1**) using diethanolamine and norcantharidin **13** affording pure white crystals (187.6 mg/ 0.69 mM), yield 58%.

Mp: 149-152C;
^{1}H NMR (DMSO): δ 7.26 (1H, bs), 4.72 (1H, bs), 4.45 (1H, d, *J* = 3.7 Hz), 3.00 (1H, m), 2.77 (2H, s), 1.57 (4H, m), 1.47 (4H, t, *J* = 7.2 Hz);
^{13}C NMR (DMSO): δ 175.8, 175.0, 82.2, 80.8, 63.1, 62.5, 56.7, 54.5, 52.4, 52.3, 32.8, 31.9;
ESI-MS (m/z): 318, [M+2Na] (100%), 296 [M+Na], 274 [M+H], 256 [M-17];
HR-MS (m/z): calc $C_{12}H_{19}NO_6$; 273.12385.

Synthesis of 3-[2-(2-Hydroxyethyl)-piperidine-1-carbonyl]-7-oxa-bicyclo[2.2.1]heptane-2-carboxylic acid (**92)**:

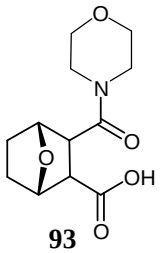


92

The 2-hydroxyethylpiperidine analogue **92** was synthesised according to the general method (**5.4.1**) using 2-ethanolpiperidine and norcantharidin **13** affording pure white crystals (291.4 mg/ 0.98 mM), yield 82%.

Mp: 162-164°C;
^{1}H NMR (DMSO): δ 7.29 (1H, d, *J* = 7.1), 4.85 (1H, d, *J* = 3.3 Hz), 4.57 (1H, d, *J* = 3.7 Hz), 4.11 (3H, m), 2.94 (4H, m), 1.60 (12H, m);
^{13}C NMR (DMSO): δ 176.5, 173.8, 82.7, 80.8, 56.8, 55.7, 53.9, 47.8, 36.3, 36.1, 35.2, 32.8, 32.3, 30.2, 27.4;
ESI-MS (m/z): 342, [M+2Na], 320 [M+Na], 298 [M+H] (100%);
HR-MS (m/z): calc $C_{15}H_{23}NO_5$; 297.16294.

Synthesis of 3-(Morpholine-4-carbonyl)-7-oxa-bicyclo[2.2.1]heptane-2-carboxylic acid (**93)**:

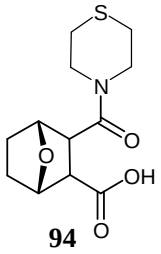


93

The morpholine analogue **93** was synthesised according to the general method (**5.4.1**) using 4-aminomorpholine and norcantharidin **13** affording (2.42 mg/ 0.94 mM), yield 80%.

Mp: 156ºC;
^{1}H NMR (DMSO): δ 4.67 (2H, bs), 3.59 (4H, bs), 2.83 (4H, bs), 2.79 (2H, s), 1.50 (4H, bs);
^{13}C NMR (DMSO): δ 173.2, 78.9, 65.4, 65.4, 52.7, 44.5, 28.8;
ESI-MS (m/z): 300, [M+2Na] (100%), 278 [M+Na], 256 [M+H], 238 [M-17];
HR-MS (m/z): calc $C_{12}H_{17}NO_5$; 255.10936.

Synthesis of 3-(Thiomorpholine-4-carbonyl)-7-oxa-bicyclo[2.2.1]heptane-2-carboxylic acid (**94)**:

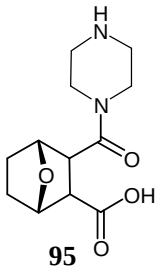


94

The thiomorpholine analogue **94** was synthesised according to the general method (**5.4.1**) using 4-aminothiomorpholine and norcantharidin **13** affording affording pure white (196.8 mg/0.73 mM), yield 61%.

Mp: 203-205°C;
^{1}H NMR (DMSO): δ 4.65 (1H, m), 3.77 (2H, m), 3.51 (2H, m), 3.16 (1H, d, *J* = 9.6 Hz), 3.00 (1H, d, *J* = 9.5 Hz), 2.51 (2H, m), 1.52 (2H, m);
^{13}C NMR (DMSO): δ 176.1, 173.5, 82.0, 81.5, 56.7, 52.3, 51.8, 47.8, 32.8, 32.2, 30.5, 30.3;
ESI-MS (m/z): 316, [M+2Na] (100%), 394 [M+Na], 272 [M+H], 254 [M-17];
HR-MS (m/z): calc $C_{12}H_{17}NO_4S$; 271.09423.

Synthesis of 3-(Piperazine-1-carbonyl)-7-oxa-bicyclo[2.2.1]heptane-2-carboxylic acid (**95)**:

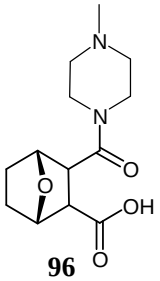


95

The piperazine analogue **95** was synthesised according to the general method (**5.4.1**) using piperazine and norcantharidin **13** affording (146.1 mg/0.58 mM), yield 48%.

Mp: 146-148ºC;
^{1}H NMR (DMSO): δ 4.65 (2H, bs), 3.61 (4H, bs), 2.84 (4H, bs), 2.79 (2H, s), 1.50 (4H, bs);
^{13}C NMR (DMSO): δ 173.5, 78.9, 65.4, 56.7, 52.8, 44.7, 28.9;
ESI-MS (m/z): 299, [M+2Na] (100%), 377 [M+Na], 255 [M+H], 237 [M-17];
HR-MS (m/z): calc $C_{12}H_{18}N_2O_4$; 254.13394.

Synthesis of 3-(4-Methyl-piperazine-1-carbonyl)-7-oxa-bicyclo[2.2.1]heptane-2-carboxylic acid (**96**):



96

The methylpiperazine analogue **96** was synthesised according to the general method (**5.4.1**) using 4-methylpiperazine and norcantharidin **13** affording (126.1 mg/0.47 mM), yield 40%.

Mp: 228-230ºC;

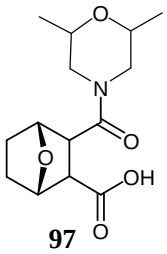
^{1}H NMR (DMSO): δ 4.63 (2H, m), 3.84 (2H,m, br), 3.12 (1H, d *J* = 9.5 Hz), 2.98 (1H, d *J* = 9.5 Hz), 2.34 (1H, m br), 2.16 (4H, s), 1.51-1.46 (4H, m)

^{13}C NMR (DMSO): δ 172.1, 169.3, 77.8, 77.6, 53.9, 53.7, 52.7, 48.4, 45.4, 28.7, 28.3;

ESI-MS (m/z): 313, [M+2Na] (100%), 291 [M+Na], 269 [M+H], 251 [M-17];

HR-MS (m/z): calc $C_{12}H_{18}N_2O_4$; 268.14398.

Synthesis of 3-(2,6-Dimethyl-morpholine-4-carbonyl)-7-oxa-bicyclo[2.2.1]heptane-2-carboxylic acid (**97**):



97

The dimethylmorpholine analogue **97** was synthesised according to the general method (**5.4.1**) using 2,6-dimethylmorpholine and norcantharidin **13** affording (226.1 mg/0.80 mM), yield 67%.

Mp: 165-167°C

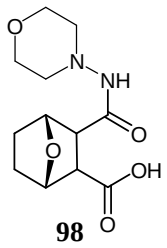
^{1}H NMR (DMSO): Not determined

^{13}C NMR (DMSO): Not determined

ESI-MS (m/z): 328, [M+2Na] (100%), 306 [M+Na], 284 [M+H], 266 [M-17];

HR-MS (m/z): calc $C_{14}H_{21}NO_5$; 283.32283.

Synthesis of 3-(Morpholin-4-ylcarbamoyl)-7-oxa-bicyclo[2.2.1]heptane-2-carboxylic acid (**98**):



98

The 4-aminomorpholine analogue **98** was synthesised according to the general method (**5.4.1**) using 4-aminomorpholine and norcantharidin **13** affording (136.2 mg/0.50 mM), yield 42%.

Mp: 156-158℃

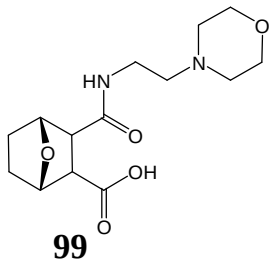
^{1}H NMR (DMSO): Not determined

^{13}C NMR (DMSO): Not determined

ESI-MS (m/z): 315, [M+2Na] (100%), 293 [M+Na], 271 [M+H], 253 [M-17];

HR-MS (m/z): calc $C_{12}H_{18}N_2O_5$; 270.28273.

Synthesis of 3-(2-Morpholin-4-ylethylcarbamoyl)-7-oxabicyclo[2.2.1]heptane-2-carboxylic acid (**99**):



99

The 4-ethylmorpholine analogue **99** was synthesised according to the general method (**5.4.1**) using 4-ethylaminomorpholine and norcantharidin **13** affording pure white crystals (193.7 mg/0.65 mM), yield 55%.

Mp: 116-119°C;

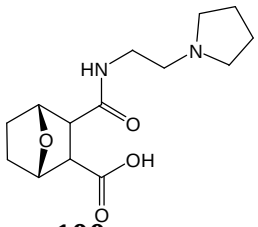
^{1}H NMR (DMSO): 6.96 (1H, bs), 4.72 (2H, m), 3.60 (2H, m), 4.10 (2H, m), 2.91 (4H, m), 2.21 (2H, m), 1.60 (10H, m);

^{13}C NMR (DMSO): δ 177.0, 175.2, 82.5, 82.3, 81.2, 70.1, 69.9, 60.8, 56.9, 39.4, 32.9, 32.5, 21.5;

ESI-MS (m/z): 343 [M+2Na], 321 [M+Na], 299 [M+H], 281 [M-17] (100%);

HR-MS (m/z): calc $C_{14}H_{22}N_2O_5$; 298.33473.

Synthesis of 3-(2-Pyrrolidin-1-yl-ethylcarbamoyl)-7-oxa-bicyclo[2.2.1]heptane-2-carboxylic acid (**100**):

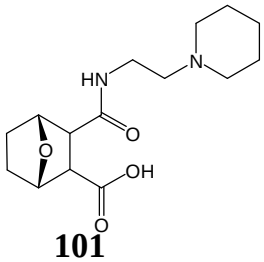


100

The ethylpyrrolidine analogue **100** was synthesised according to the general method (**5.4.1**) using ethylaminopyrrolidine and norcantharidin **13** affording pure white crystals (317.5 mg/1.12 mM), yield 94%.

Mp: 134-136°C;
^{1}H NMR (DMSO): 7.01 (1H, bs), 4.65 (2H, m), 3.59 (2H, m), 4.11 (2H, m), 2.91 (4H, m), 1.60 (12H, m);
^{13}C NMR (DMSO): 177.1, 175.4, 82.5, 82.1, 81.5, 70.3, 69.9, 60.0, 57.3, 39.4, 33.0, 32.6, 21.3;
ESI-MS (m/z): 327, [M+2Na], 305 [M+Na], 283 [M+H], 265 [M-17] (100%);
HR-MS (m/z): calc $C_{14}H_{22}N_2O_4$; 282.34485.

Synthesis of 3-(2-Piperidin-1-ylethylcarbamoyl)-7-oxabicyclo[2.2.1]heptane-2-carboxylic acid (**101**):



101

The ethylpiperidine analogue **101** was synthesised according to the general method (**5.4.1**) using ethylaminopiperidine and norcantharidin **13** affording pure white crystals (158.8 mg/0.54 mM), yield 45%.

Mp: 64-67°C;

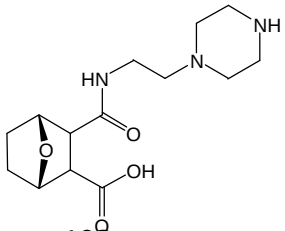
^{1}H NMR (DMSO): 6.98 (1H, bs), 4.65 (2H, m), 3.60 (2H, m), 4.11 (2H, m), 2.92 (4H, m), 1.61 (12H, m), 1.49 (2H, m);

^{13}C NMR (DMSO): δ 177.1, 175.0, 82.7, 82.4, 81.0, 70.0, 69.7, 60.8, 57.0, 39.5, 33.8, 32.9, 32.5, 21.4;

ESI-MS (m/z): 319 [M+Na], 297 [M+H], 279 [M-OH] (100%);

HR-MS (m/z): calc $C_{15}H_{24}N_2O_4$; 296.35785.

Synthesis of 3-(2-Piperazin-1-yl-ethylcarbamoyl)-7-oxa-bicyclo[2.2.1]heptane-2-carboxylic acid (**102**):



102

The ethylpiperazine analogue **102** was synthesised according to the general method (**5.4.1**) using ethylaminopiperazine and norcantharidin **13** affording pure off white crystals (261.7 mg/0.88 mM), yield 74%.

Mp: 83-86°C;

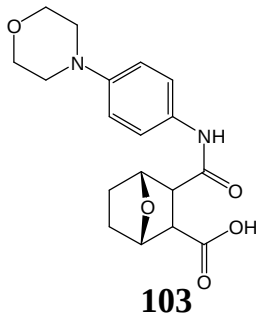
^{1}H NMR (DMSO): 6.96 (1H, bs), 4.65 (2H, m), 3.59 (2H, m), 4.11 (2H, m), 2.91 (4H, m), 1.60 (12H, m);

^{13}C NMR (DMSO): 177.0, 175.3, 82.4, 82.4, 81.4, 70.2, 69.9, 60.1, 57.2, 39.3, 32.9, 32.6, 21.4;

ESI-MS (m/z): 342 [M+2Na], 320 [M+Na], 298 [M+H], 280 [M-OH] (100%).

HR-MS (m/z): calc $C_{14}H_{24}N_3O_4$; 297.35485.

Synthesis of 3-(4-Morpholin-4-yl-phenylcarbamoyl)-7-oxa-bicyclo[2.2.1]heptane-2-carboxylic acid (**103**):

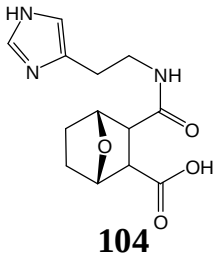


103

The 4-morpholinephenyl analogue **103** was synthesised according to the general method (**5.4.1**) using 4-morpholin-4-yl-phenylamine and norcantharidin **13** affording pure white crystals (201.8 mg/58 mM), yield 48%.

Mp: 176-178°C;
^{1}H NMR (DMSO): δ 10.9 (1H, bs), 9.35 (1H, s), 7.36 (1H, d, *J* = 8.9 Hz), 6.85 (1H, d, 8.9 Hz), 4.76 (1H, d, *J* = 2.1 Hz), 4.60 (1H, d, *J* = 3.7 Hz), 2.88-3.02 (6H, m), 1.47-1.56 (4H, m);
^{13}C NMR (DMSO): δ 173.1, 169.6, 148.0, 132.5, 121.2, 116.3, 79.6, 77.7, 67.0, 54.4, 52.5, 50.0, 29.8, 29.3,
ESI-MS (m/z): 392 [M+2Na], 370 [M+Na], 348 [M+H], 330 [M-OH] (100%).
HR-MS (m/z): calc $C_{18}H_{22}N_2O_4$; 347.16140.

Synthesis of 3-[2-(1H-Imidazol-4-yl)-ethylcarbamoyl]-7-oxa-bicyclo[2.2.1]heptane-2-carboxylic acid (**104**):

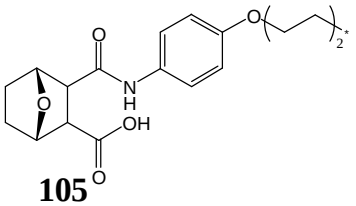


104

The ethylimidazole analogue **104** was synthesised according to the general method (**5.4.1**) using (1H-imidazol-4-yl)-ethylamine and norcantharidin **13** affording pure cream crystals (172 mg/0.62 mM), yield 52%.

Mp: 163-165°C;
^{1}H NMR (DMSO): Not determined
^{13}C NMR (DMSO): Not determined
ESI-MS (m/z): 324 [M+2Na], 302 [M+Na], 280 [M+H], 262 [M-OH] (100%);
HR-MS (m/z): calc $C_{13}H_{27}N_3O_4$; 279.29362.

Synthesis of 3-(4-Pentyloxyphenylcarbamoyl)-7-oxabicyclo[2.2.1]heptane-2-carboxylic acid (**105**):



105

The pentyloxyphenyl analogue **105** was synthesised according to the general method (**5.4.1**) using 4-pentyloxyphenylamine and norcantharidin **13** affording pure white crystals (216.7 mg/0.62 mM), yield 52%.

Mp: 162-164°C;

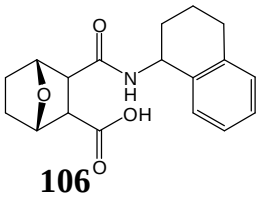
^{1}H NMR (DMSO): δ 11.97 (1H, bs), 9.54 (1H, s), 7.50 (2H, d, *J* = 8.9 Hz), 6.93 (2H, d, *J* = 9.0 Hz), 4.87 (1H, m), 4.71 (1H, m), 4.00 (2H, t, *J* = 6.4 Hz), 3.06 (2H, m), 1.66 (6H, m), 1.46 (4H, m), 0.99 (3H, t, *J* = 7.0 Hz);

^{13}C NMR (DMSO): δ 176.2, 172.8, 158.5, 135.3, 124.8, 118.3, 82.7, 80.8, 71.5, 57.3, 55.6, 32.9, 32.4, 31.7, 25.8, 17.8;

ESI-MS: 392 [M+2Na], 370 [M+Na] (100%), 348 [M+H], 330 [M-17];

HR-MS (m/z): calc $C_{19}H_{25}NO_5$; 347.41212.

Synthesis of 3-(Indan-1-ylcarbamoyl)-7-oxabicyclo[2.2.1]heptane-2-carboxylic acid (**106**):



106

The tetrahydronapthalene analogue **106** was synthesised according to the general method (**5.4.1**) using 1,2,3,4-Tetrahydronaphthalen-1-ylamine and norcantharidin **13** affording white crystals (203.4 mg/0.67 mM), yield 57%. NMR shows a mixture of diastereoisomers.

Mp: 149-151°C;

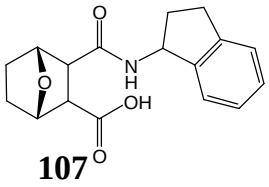
^{1}H NMR (DMSO): δ 7.73 (1H, d, *J* = 7.8 Hz), 7.48 (1H, d, *J* = 7.8 Hz), 7.05-7.23 (10H, m), 4.71 (2H, bs), 4.51 (2H, dd, *J* = 13.9, 4.1 Hz, 2H), 4.13 (1H, bs), 3.51 (1H, bs), 2.82-2.89 (4H, m), 2.68 (4H, q, *J* = 6.7 Hz), 1.45-1.83 (m, 20H);

^{13}C NMR (DMSO): δ 173.5, 173.4, 171.1, 171.0, 138.5, 138.4, 137.9, 137.8, 129.7, 129.5, 129.4, 129.2, 128.0, 127.4, 126.7, 78.8, 79.5, 77.9, 77.8, 67.9, 54.0, 53.7, 53.0, 52.9, 47.2, 47.1, 30.5, 29.7, 29.6, 29.4, 26.5, 20.9, 20.7, 19.6;

ESI-MS (m/z): 360 [M+2Na], 338 [M+Na] (100%), 316 [M+H];

HR-MS (m/z): calc $C_{18}H_{21}NO_4$; 315.16.

Synthesis of 3-(Indan-1-ylcarbamoyl)-7-oxa-bicyclo[2.2.1]heptane-2-carboxylic acid (**107**):

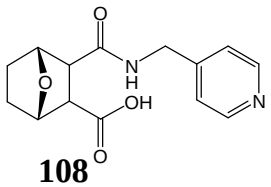


107

The indane analogue was synthesised according to the general method (**5.4.1**) using Indan-1-ylamine and norcantharidin **13** affording white crystals (**107**) (198.7 mg/0.67 mM), yield 55%.

Mp: 205-206°C;
^{1}H NMR (DMSO): 7.87 (1H, m), 7.22 (3H, m), 4.98 (1H, m), 4.84 (1H, m), 4.63 (1H, m), 2.98 (2H, m), 2.80 (1H, m), 1.77 (10H, m);
^{13}C NMR (DMSO): δ 181.0, 178.5, 138.6, 128.4, 125.9, 76.0, 74.7, 54.9, 31.3, 28.9, 27.7;
ESI-MS (m/z): 346 [M+2Na], 324 [M+Na] (100%), 302 [M+H];
HR-MS (m/z): calc $C_{17}H_{19}NO_4$; 301.34253.

Synthesis of 3-[(Pyridin-4-ylmethyl)-carbamoyl]-7-oxabicyclo[2.2.1]heptane-2-carboxylic acid (**108**):

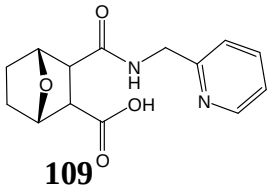


108

The para-substituted pyridine analogue **108** was synthesised according to the general method (**5.4.1**) using C-Pyridin-4-ylmethylamine and norcantharidin **13** affording pure white crystals (300.8 mg/1.09 mM), yield 91%.

Mp: 196-198°C;
^{1}H NMR (DMSO): δ 8.44 (2H, m), 8.05 (1H, m), 7.24 (2H, m), 4.74 (1H, m), 4.55 (1H, m), 4.23 (2H, m), 2.91 (2H, m), 1.47 (4H, m);
^{13}C NMR (DMSO): δ 176.3, 174.9, 153.3, 152.6, 126.1, 82.7, 80.8, 56.9, 55.9, 45.2, 32.9, 32.4;
ESI-MS (m/z): 321 [M+2Na], 299 [M+Na] (100%), 277 [M+H], 259 [M-17];
HR-MS (m/z): calc $C_{17}H_{19}NO_4$; 276.28943.

Synthesis of 3-[(Pyridin-2-ylmethyl)-carbamoyl]-7-oxabicyclo[2.2.1]heptane-2-carboxylic acid (**109**):



109

The ortho-substituted pyridine analogue **109** was synthesised according to the general method (**5.4.1**) using C-Pyridin-2-ylmethylamine and norcantharidin **13** affording pure white crystals (150.2 mg/0.54 mM), yield 46%.

Mp: 153-155°C;

^{1}H NMR (DMSO): δ 8.45 (1H, m), 8.03 (1H, m), 7.71 (1H, m), 7.32 (1H, m), 7.22 (1H, t), 4.73 (1H, d), 4.54 (1H, d), 4.30 (2H, dq), 2.96 (1H, d), 2.87 (1H, d), 1.45 –1.56 (4H, m);

^{13}C NMR (DMSO): δ172.6, 171.0, 158.4, 148.5, 136.6, 122.1, 120.9, 78.7, 76.9, 53.4, 53.1, 28.9, 28.7;

ESI-MS (m/z): 321 [M+2Na], 299 [M+Na] (100%), 277 [M+H], 259 [M-OH];

HR-MS (m/z): calc $C_{17}H_{19}NO_4$; 276.28943.

5.5 References

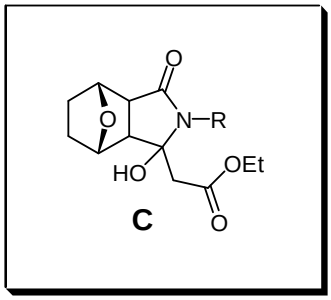
1. Honkanen, R.E. and T. Golden, *Regulators of Serine/Threonine Protein Phosphatase at the Dawn of the Clinical Era?* Current Medicinal Chemistry, 2002. **9**: p. 2005-2075.
2. McCluskey, A., Keane, M. A., Mudgee, L., Sim, A. T. R., Sakoff, J. A., *Anhydride Modified Cantharidin Analogues. Is Ring Opening Important in the Inhibition of Protein Phosphatase 2A?* European Journal of Medicinal Chemistry, 2000. **35**: p. 957-964.
3. McCluskey, A., Walkom, C., Bowyer, M. C., Ackland, S. P., Gardiner, E., Sakoff, J. A., *Cantharimides: A New Class of Modified Cantharidin Analogues Inhibiting Protein Phosphatases 1 and 2A.* Bioorganic and Medicinal Chemistry Letters, 2001. **11**: p. 2941-2946.
4. Shan, H., Cai, Y., Zeng, W., Chen, H., Fan, B., Liu, X., Xu, Z., Wang, B., Xian, L., *Cytotoxicity of Cantharidin Analogues Targeting Protein Phosphatase 2A.* Anti-cancer Drugs, 2006. **17**: p. 905-911.
5. Hill, T.H., Stewart, S. G., Sauer, B., Gilbert, J., Ackland, S, P., Sakoff, J, A., McCluskey, A., *Hetrocyclic Substituted Cantharidin and Norcantharidin Analogues-Synthesis, Protein Phosphatase (1 and 2A) Inhibition, and Anti-cancer Activity*. Bioorganic Medicinal Chemistry Letters, 2007. **17**: p. 3392-3397.

Chapter 6

Development of α-Hydroxylactam Derivatives as Potential PP1 and PP2A Inhibitors

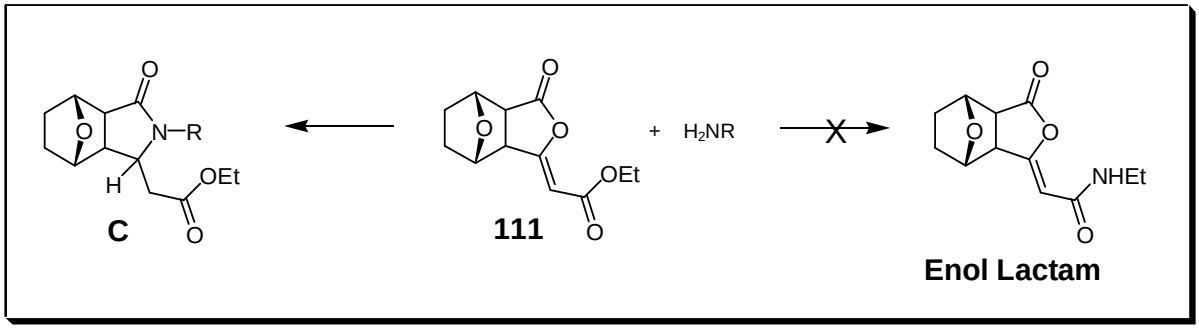
6.1 Introduction

Recent evaluation of the cantharidin SAR data has highlighted a number of structural features which may lead to the development of new phosphatase inhibitors, including the presence of the ethereal bridgehead of cantharidin, at least one of the carboxyl groups, and the possible interaction of a basic residue [1] [2]. The synthesis of the α-hydroxylactam analogues (**C**) or allows the investigation of moving one of the carboxylates away from the 7-oxa bridgehead. Furthermore the synthesis of the α-hydroxylactam analogues allows the investigation of previously unexplored chemical space in relation to phosphatase activity, such as the presence of the α-hydroxy moiety instead of the parent anhydride or related ring-closed cantharimide, and the direct comparison with the latter due to the addition of similar amine reagents possessing hydrophobic tails, acidic and basic residues, and in turn increasing the structural diversity of our cantharidin analogue libraries.



C

The initial aim of this chapter was the trans-amination of the enol-lactone analogue (**111**) to form the corresponding amide analogue for biological screening (Refer to **Scheme 6.1**). However NMR studies of initial trials indicated the nucleophilic addition of the amine to the lactone ring in preference to the ester side chain to form the corresponding lactam and removal of the enol double bond to form the α-hydroxyl. This will be further described in the following section (**Section 6.2**).

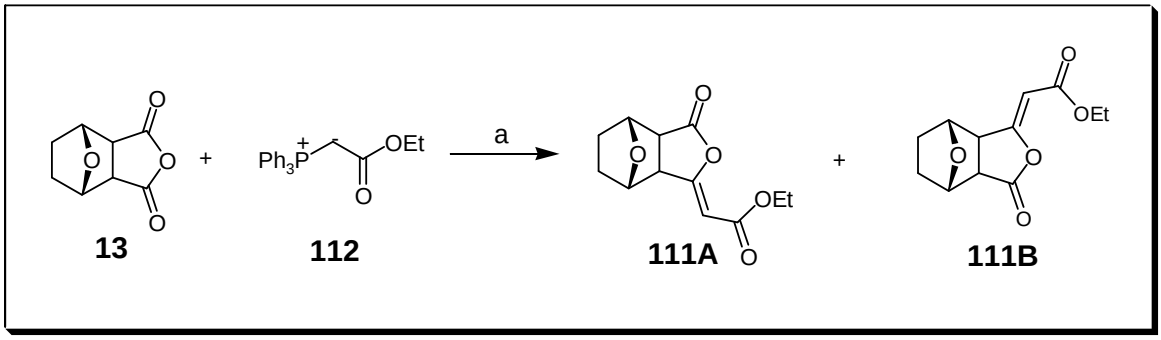


Scheme 6.1 – Preferential Formation of the α-hydroxylactam in lue of the Trans-aminated Enol Lactam

Due to the unexpected chemistry and the structural similarity to the previously described cantharimides reported in **Chapter 4** (ring-closed analogues) and **Chapter 5** (ring-opened analogues), a range of α-hydroxylactam analogues were developed for screening. The amine reagents chosen were similar to the previous described chapters, including aliphatic amines, aromatic substituted amines, amino-alcohols, as well as the morpholino amines which has shown interesting activity with the cantharimide analogues. These modifications allowed variation in size, flexibility, spatial distribution and functionality of the modification, as well as possible handles for further manipulation. These analogues were designed to examine the effects of skeletal modifications on the resulting PP inhibition and, consequently, anticancer activity.

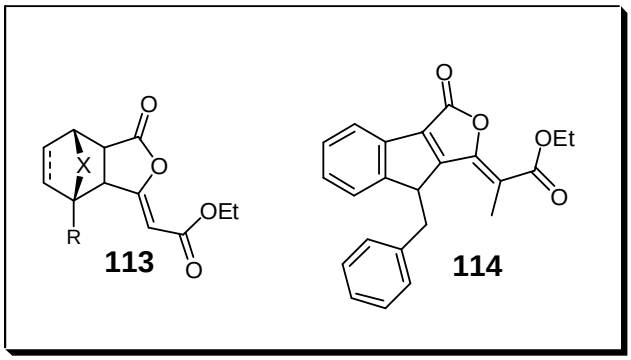
6.2 Results and Discussion – Chemical Synthesis

Synthesis of the α-hydroxylactam library commenced with the readily accessible norcantharidin (**13**). Treatment of **13** with the phosphorane (**112**) (generated by treatment of ethyl bromoacetate with triphenyl phosphine followed by HBr removal with NaOH) facilitated the rapid installation of the enol lactone ester moiety yielding **111** via the Wittig reaction. It should be noted that attack of either carbonyl carbon of the parent anhydride is possible with the formation of either enantiomer (**111A** and **111B**) as a racemic mixture which were not separated. Refer to **Scheme 6.2**.



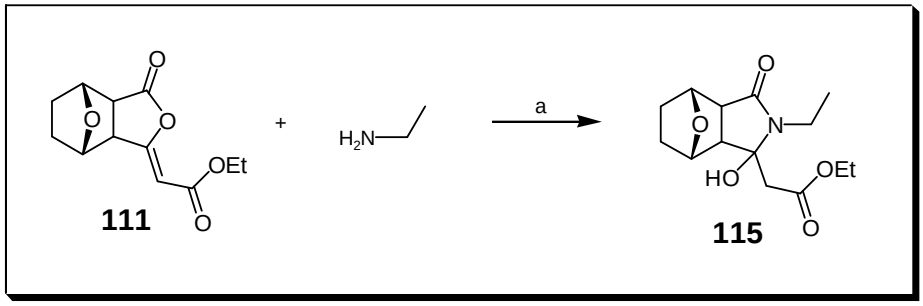
Scheme 6.2: Reagents and Conditions; (a) Dry toluene, RT, 24 h.

Similar Wittig chemistry involving anhydrides to form enol lactones **113** and **114** has been previously described by Miki *et. al* and in particular Murray *et. al.* who both reported comparable structural analogues to our enol lactone [3] [4].



The further treatment of **111** with ethylamine in dry toluene at room temperature was shown to illustrate rapid (< 24 hrs) and complete conversion as shown by TLC analysis (the appearance of a single spot with a much reduced R_f). Concentration *in vacuo* afforded a yellow crystalline solid which on spectroscopic examination interestingly displayed an OH peak in the IR at

3373 cm^{-1} (broad) which was further supported by the observation of the [M-H_2O] ion by GCMS. Subsequent H^1 and C^{13} NMR analysis revealed the presence of two ethyl groups, and the formation of the sp^3 hybridised quaternary carbon indicated by the downfield signal (~90 ppm) on the C^{13} spectra and absence of the expected number of sp^2 hybridised methylene carbons, indicating the formation of the α-hydroxy. All evidence clearly points to the facile nucleophilic addition of the amine reagent to generate the α-hydroxylactam **115** as shown in **Scheme 6.3**.



Scheme 6.3: Reagents and Conditions; (a) Dry toluene, RT, 24 h.

The unexpected nucleophilic attach of the electrophilic carbonyl carbon of the lactone ring and subsequent formation of the α-hydroxylactam in preference to the direct transamination of the ester was most likely due to steric effects hindering reaction rates. The further nucleophilic attach of the amide nitrogen via the α,β-unsaturated carbonyl carbon (i.e., via a modified Michaels reaction) was catalysed by the proton transfer forming the α-hydroxylactam. Examination of NMR data showed no doubling of peaks around the hydroxyl carbon indicating the formation of a single enantiomer. Refer to **Figure 6.1** for a proposed reaction mechanism.

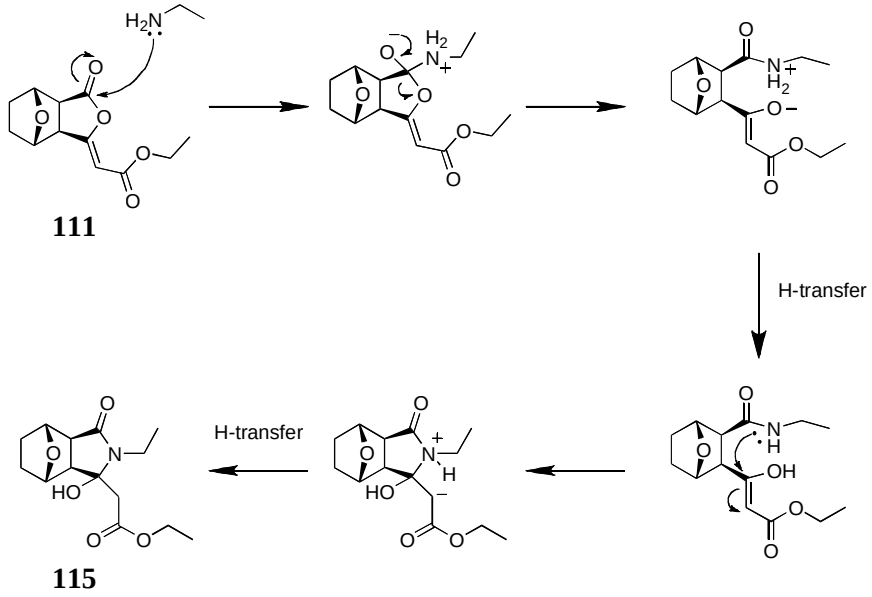
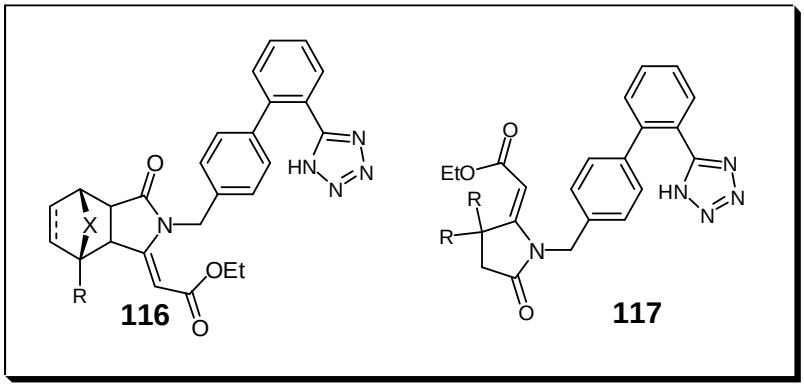


Figure 6.1: Possible mechanism for the formation of the α-hydroxylactam **115**.

This directly contrasts the products described by Murrey *et.al*. Interestingly, Murray synthesised a variety of substituted pyrrolidine-2-one biphenyltertrazoles **116** and **117** for screening as an antagonist of the angiotension II receptor [3]. This involved the treatment of the parent anhydride with 2-[(4-aminomethyl)phenyl]phenyltetrazole in pyridine at 40 – 100ºC over molecular sieves, allowing the formation of the enol lactam analogues via nucleophilic substitition. This significant difference in chemistry is most likely due to the presence of the non-protic nonpolar solvent used in our reactions being 'dry' toluene. The formation of the α-hydroxylactone moiety as characterised in our studies allows significant differentiation and a handle for further synthetic development.

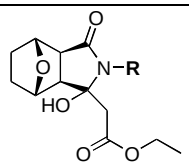


6.3 Results and Discussion – Biological Assay

With our libraries of α-hydroxylactams in hand we set about determining their biological activity. As before our primary interest was in the investigation of the protein phosphatase 1 and 2A inhibition and cytotoxicity. Interestingly, in all instances we observed no protein phosphatase inhibition, suggesting that the modifications made have removed the inhibitory action usually associated with norcantharidin analogues. However, given our original hypothesis that a carboxylate was required for protein phosphatase inhibition, the masking as the ethyl ester may be responsible for this.

The cytoxoicty data suggests that this is not the case, as previous studies from our group has shown rapid cleavage by endogenous esterases and potent cytotoxicity as a result of protein phosphatase inhibition. The lack of protein phosphatase inhibition suggests that these α-hydroxylactams are more correctly thought of as norcantharidimide analogues rather than norcantharidin analogues. **Table 6.1** shows the growth inhibition effects of N-alkyl substituted alpha-hydroxylactams (**115, 118-124**) on HT29 (colon) and G401 (kidney) cell lines. These studies were conducted at a single drug concentration of 100 μM.

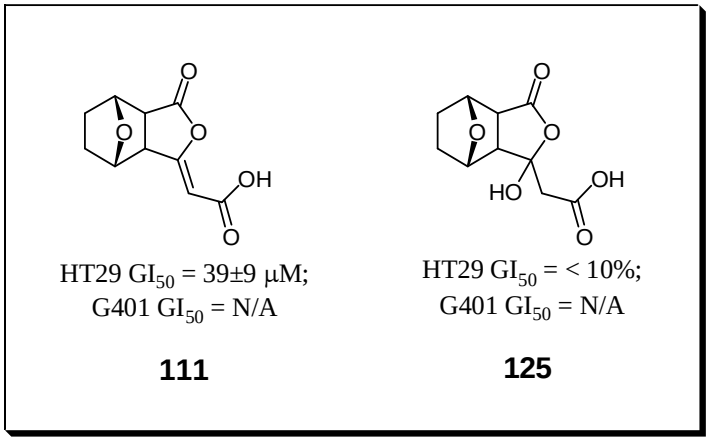
Table 6.1: Growth inhibition (%) of a variety of alkyated α-hydroxylactam (**115**, **118-124**) analogues on HT29 (colon) and G401 (kidney) cell lines at 100 μM drug concentration.



Compound	R	HT29 (colon)	G4010 (kidney)
115	Et	-	14±1.8
118	*n*-Pr	<10	20±3.0
119	*n*-Bu	<10	16±0.5
120	*n*-Hexyl	11±5.4	35±2.8
121	*n*-Octyl	33±12.8	28±1.5
122	*sec*-Butyl	<10	20±1.3
123	*c*-Hexyl	<10	17±2.0
124	Hexanol	10±4.5	<10

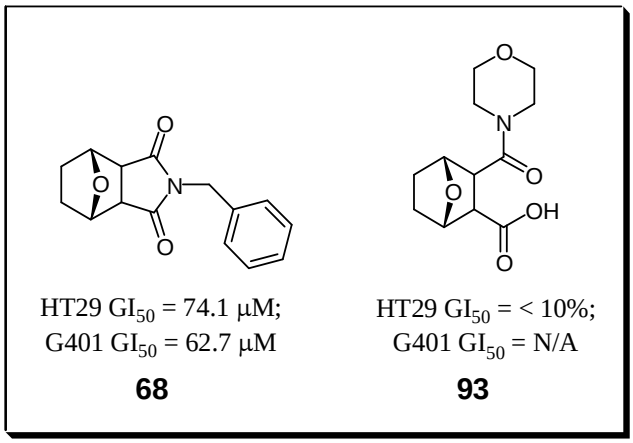
NB: A value of <10% indicates that the compound failed to influence cell growth when compared with untreated controls (i.e., the higher the value the greater cytotoxicity).

Whilst the growth inhibition is essentially non-existent, and somewhat surprising, they correlated well with poor phosphatase (<0%) inhibition. It should be noted that with an increase in the alkyl chain length as with analogues (**120-121**), display slightly improved cytotoxicity in comparison to the shorter and branched or cyclic alkyl analogues. This corresponds with the results displayed by the ring closed cantharimide analogues, which is most likely a response of improved cellular uptake due to the lypophillic alkyl chain. It appears that moving the carboxylate and the presence of the hydroxyl has a significant effect on cytotoxicity and phosphatase activity. Further examination of this effect was examined by our laboratory with the synthesis of the α-hydroxylactone (**125**), enforcing the negative effect presence of the hydroxyl on both cytotoxicity and phosphatase activity.



Scott Stewart, Tim Hill, Jennette Sakoff & Adam McCluskey – unpublished data.

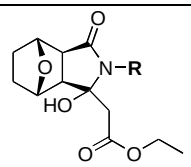
As can be seen from **Table 6.2** the introduction of terminal functionality in the N-substituent (**126-135**), such as alcohol, amine, morpholine and aromatic groups, had essentially no effect on cytotoxicity. Again, this contrasts some of the activity displayed by the ring closed norcantharimides as well as the more promising data shown by the ring opened analogues, further illustrating the effect of the introduction of the α-hydroxy substituent on removing activity. In particular, structural modifications such as the introduction of an aromatic ring, such as the ring closed benzylic (**68**) analogue, or the introduction of extra oxygen functionality, such as morpholine (**93**) analogue, described in Chapter 4 and Chapter 5 respectively, were shown to improve protein phosphatase inhibition and cytotoxicity in the parent ring opened analogues. Unfortunately, these styles of modifications have little to no effect on cytotoxicity of the α-hydroxylactone analogues.

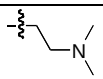
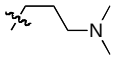
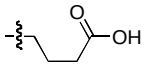
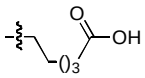
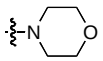
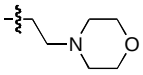
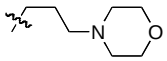
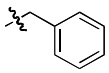
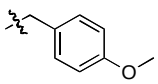
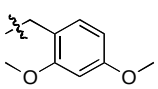


HT29 GI_{50} = 74.1 μM; G401 GI_{50} = 62.7 μM — **68**

HT29 GI_{50} = < 10%; G401 GI_{50} = N/A — **93**

Table 6.2: Growth inhibition (%) of a variety of functionalised α-hydroxylactam (**126**-**135**) analogues on HT29 and G401 cells at 100 μM drug concentration.



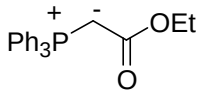
Compound	R	HT29 (colon)	G401 (kidney)
126		11±4.7	19±5.0
127		<10	19.8±3.0
128		19±6.4	17±1.6
129		<10	15±0.8
130		15.2±4.7	19.3±1.6
131		10±7.6	16±2.1
132		<10	<10
133		<10	14±1.8
134		<10	20±3.0
135		<10	16±3.0

NB: A value of <10% indicates that the compound failed to influence cell growth when compared with untreated controls (i.e., the higher the value the greater the growth inhibition).

6.4 Experimental – Synthesis of Lactam derivatives

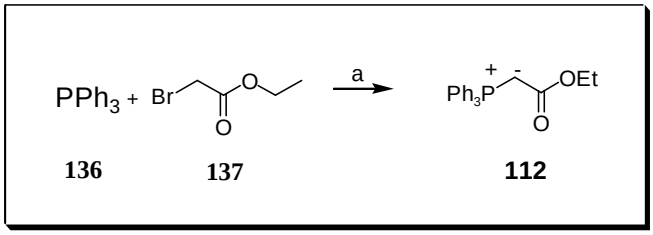
6.4.1 Synthesis of the Ylid

(Triphenyl-λ^5-phosphanylidene)-acetic acid ethyl ester (112):



112

To a solution of triphenylphosphine (**136**) (15.0 g, 0.06 mol) in anhydrous toluene (50 mL) was added ethylbromoacetate (**137**) (9.5 g, 0.06 mol). The mixture was stirred at RT for 72 hrs. The resultant white product was filtered, and then washed with cold toluene (5 mL) and petroleum spirit (5 mL), affording crude phosphorane salt. A general reaction scheme is shown in **Scheme 6.4**.



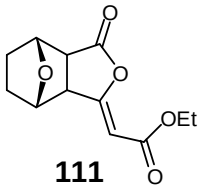
136 **137** **112**

Scheme 6.4: Reagents and Conditions; (a) Dry toluene, NaOH, RT 72 hrs

The crude ylid was dissolved in dichloromethane (60 mL) and placed in a separatory funnel containing 40 mL of water. A 5 mL aliquot of 3M NaOH was added to the funnel and shaken vigorously until the aqueous layer had obtained a pH of >10. The organic layer was eluted and the aqueous layer extracted with dichloromethane (2 x 30 mL). The organic layers were combined and dried with $MgSO_4$, and solvent removed *in vacuo*. To the resultant oil was added chilled petroleum ether (5 mL), and stored in the freezer with occasional swirling until white crystals formed. A further addition of cold petroleum ether (10 mL) was added, and product filtered to afford pure (**112**) (16.4 g, 82%).

6.4.2 Enol-lactone derivate

*(5-Oxo-4,10-dioxa-tricyclo[5.2.1.02,6]dec-3-ylidene)acetic acid ethyl ester (111)***:**



111

To a solution of norcantharidin (**13**) (2.0 g, 0.01 mol) in anhydrous toluene was added the ylid (2.83g, 0.01 mol). The mixture was stoppered and stirred at RT for 24 hrs, and concentrated *in vacuo* affording crude (**111**). The crude product was purified using flash chromatography (EtOAc/petrol; 1:3) and concentrated *in vacuo* afforded pure (**111**) (1.46 g, 65%).

^{1}H NMR ($CDCl_3$): δ 5.61 (1H, s), 4.83 (2H, dd, J= 4.3 Hz), 4.12 (2H, q, *J* = 7.2 Hz), 3.69 (1H, d, *J* = 7.8 Hz), 2.88 (1H, d, *J* = 7.9 Hz), 1.75 (2H, m), 1.55 (2H, m), (3H, t, *J* = 7.3 Hz);

^{13}C NMR ($CDCl_3$): δ 173.6, 168.7, 166.5, 97.1, 82.8, 79.9, 60.13, 48.4, 48.2, 28.2, 28.0, 14.1;

GCMS: 239 [M+1] (1%), 238 [M] (7%), 170 (100%);

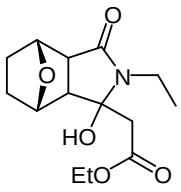
HR-MS: 238.07981;

IR: 3089, 2986, 2956, 2909, 2874, 1816, 1704, 1660.

6.4.3 General Synthesis of α-hydroxylactam derivatives

To a mixture of (**111**) (100 mg/0.42 mmol) in dry toluene (10 mL), was added the respective amine (~0.42 mM). The reaction mixture was then stirred for 24 hrs using a radley carousel reactor at room temperature, and concentrated *in vacuo*. Reaction progression was monitored by TLC, and final analysis by GCMS and NMR. All samples were dried under high vacuum.

Synthesis of (4-Ethyl-3-hydroxy-5-oxo-10-oxa-4-aza-tricyclo[5.2.1.02,6]dec-3-yl)acetic acid ethyl ester (**115**)



115

The *N*-ethyl substituted analogue **115** was synthesised according to the general method (**6.4.3**) using ethylamine (70%) and the enol-lactone **111** affording pure pale yellow crystals (120 mg, 99%). Analysis by TLC and GCMS showed no traces of starting materials. The product was not further purified.

Mp: 72-74ºC;

^{1}H NMR ($CDCl_3$): δ 4.82 (1H, d, *J* = 5.1 Hz), 4.68 (1H, d, *J* = 4.3 Hz), 4.08 (2H, dq, *J* = 7.1 Hz), 3.19 (2H, dq, *J* = 7.2 Hz), 2.81 (2H, m), 2.61 (2H, m), 2.61 (2H, m), 1.71 (2H, m), 1.48 (2H, m), 1.20 (3H, t, *J* = 7.1 Hz), 1.07 (3H, t, *J* = 7.2 Hz);

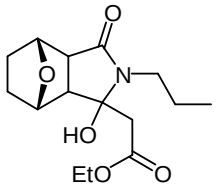
^{13}C NMR ($CDCl_3$): δ 173.3, 169.1, 89.8, 78.3, 77.9, 60.8, 51.5, 50.2, 43.3, 34.1, 29.4, 26.8, 14.4, 14.0;

GCMS: 266 [M-17] (6%), 265 [M-18] (35%), 196 (100%);

HR-MS: 283.14362;

IR: 3373, 2981, 2878, 2459, 2247, 1738, 1673, 1651, 1456, 1418, 1373, 1344, 1188, 1130, 1087, 1030, 934, 897, 820, 754, 668.

Synthesis of (3-Hydroxy-5-oxo-4-propyl-10-oxa-4-aza-tricyclo[5.2.1.02,6]dec-3-yl)-acetic acid ethyl ester (**118**)



118

The *N*-propyl substituted analogue **118** was synthesised according to the general method (**6.4.3**) using *N*-propylamine and the enol-lactone **111** affording pure pale tan crystals (128 mg, 99%). Analysis by TLC and GCMS showed no traces of starting materials. The product was not further purified.

Mp: 42-44ºC;

^{1}H NMR (DMSO): δ 5.1 (1H, s), 4.68 (2H, d, *J* = 5.1 Hz), 4.09 (2H, q, *J* = 7.1 Hz), 3.58 (1H, d, *J* = 7.3 Hz), 3.34 (2H, m), 3.10 (1H, m), 2.80 (1H, m), 2.64 (1H, m), 1.70 (2H, m), 1.51 (4H, m), 1.22 (3H, t, *J* = 7.1 Hz), 0.81 (3H, t, *J* = 7.4 Hz);

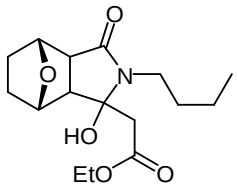
^{13}C NMR (DMSO): δ 176.3, 167.1, 160.9, 91.3, 82.5, 79.2, 59.5, 49.4, 46.4, 41.9, 28.8, 28.3, 19.4, 14.3, 11.0;

GCMS: 280 [M-17] (5%), 279 [M-18] (31%), 210 (100%);

HR-MS: 297.15989;

IR: 3359, 2982, 2887, 1743, 1658, 1456, 1381, 1345, 1187, 1131, 1039, 997.

Synthesis of (4-Butyl-3-hydroxy-5-oxo-10-oxa-4-aza-tricyclo[5.2.1.02,6]dec-3-yl)acetic acid ethyl ester (**119**)

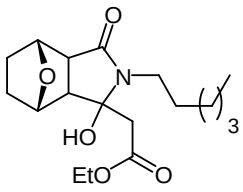


119

The *N*-butyl substituted analogue **119** was synthesised according to the general method (**6.4.3**) using *N*-butylamine and the enol-lactone **111** affording pure tan yellow oil (130 mg, 99%). Analysis by TLC and GCMS showed no traces of starting materials. The product was not further purified.

^{1}H NMR ($CDCl_3$): δ 4.86 (1H, d, *J* = 5.2 Hz), 4.71 (1H, d, *J* = 4.4 Hz), 4.12 (2H, dq, *J* = 7.2 Hz), 3.12 (2H, m), 2.85 (2H, m), 2.62 (2H, m), 1.73 (2H, m), 1.54 (4H, m), 1.31 (2H, m), 1.26 (3H, t, *J* = 7.2 Hz), 0.88 (3H, t, *J* = 7.2 Hz);
^{13}C NMR ($CDCl_3$): δ 173.5, 169.2, 89.8, 78.5, 78.1, 60.9, 51.5, 50.2, 43.4, 39.5, 31.4, 29.5, 26.7, 20.5, 14.1, 13.7;
GCMS: 294 [M-17] (5%), 293 [M-18] (24%), 41 (100%);
HR-MS: 311.16978;
IR: 3361, 2979, 2870, 1742, 1662, 1443, 1374, 1347, 1191, 1130, 1038, 998.

Synthesis of (4-Hexyl-3-hydroxy-5-oxo-10-oxa-4-aza-tricyclo[5.2.1.02,6]dec-3-yl)acetic acid ethyl ester (**120**)

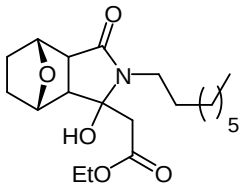


120

The *N*-hexyl substituted analogue **120** was synthesised according to the general method (**6.4.3**) using *N*-hexylamine and the enol-lactone **111** affording pure yellow oil (143 mg, 99%). Analysis by TLC and GCMS showed no traces of starting materials. The product was not further purified.

^{1}H NMR ($CDCl_3$): δ 4.89, (1H, d, *J* = 5.2 Hz), 4.69 (1H, d, *J* = 4.4 Hz), 4.09 (2H, m), 3.17 (2H, m), 3.09 (2H, m), 2.81 (2H, m), 2.61 (2H, m), 1.72 (2H, m), 1.48 (4H, m), 1.21 (6H, m), 0.81 (3H, m);
^{13}C NMR ($CDCl_3$): δ 173.3, 169.0, 89.5, 77.9, 77.7, 60.5, 51.6, 50.0, 43.5, 31.2, 29.0, 28.9, 26.8, 26.7, 22.3, 13.9, 13.7;
GCMS: 322 [M-17] (1%), 321 [M-18] (6%), 234 (100%);
HR-MS: 339.20442;
IR: 3360, 2980, 2871, 1740, 1659, 1443, 1373, 1349, 1189, 1131, 1038, 998.

Synthesis of (3-Hydroxy-4-octyl-5-oxo-10-oxa-4-aza-tricyclo[5.2.1.02,6]dec-3-yl)acetic acid ethyl ester (**121**)



121

The *N*-octyl substituted analogue **121** was synthesised according to the general method (**6.4.3**) using *N*-octylamine and the enol-lactone **111** affording yellow oil (178 mg). Analysis by TLC and GCMS showed presence of starting material. The product was purified by gradient recrystallisation in petroleum spirit (40-60°C), affording pure clear crystals (117 mg, 76%).

Mp: 57-59 ºC

^{1}H NMR ($CDCl_3$): δ 4.87, (1H, d, *J* = 5.3 Hz), 4.68 (1H, d, *J* = 4.3 Hz), 4.09 (2H, m), 3.17 (2H, m), 3.09 (2H, m), 2.81 (2H, m), 2.61 (2H, m), 1.72 (2H, m), 1.48 (6H, m), 1.21 (6H, m), 0.81 (3H, m);

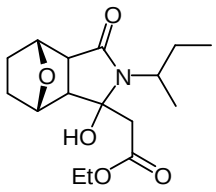
^{13}C NMR ($CDCl_3$): δ 173.2, 169.1, 89.5, 77.9, 77.7, 60.5, 51.6, 50.0, 43.5, 31.2, 29.0, 28.9, 26.8, 26.7, 26.6, 24.1, 22.3, 13.9, 13.7;

GCMS: 350 [M-17] (1%), 349 [M-18] (4%), 262 (100%);

HR-MS: 367.24387;

IR: 3362, 2978, 2869, 1741, 1660, 1445, 1374, 1347, 1190, 1129, 1036, 998.

Synthesis of (4-sec-Butyl-3-hydroxy-5-oxo-10-oxa-4-aza-tricyclo[5.2.1.02,6]dec-3-yl)acetic acid ethyl ester (**122**)



122

The *N*-(*sec*)-butyl substituted analogue **122** was synthesised according to the general method (**6.4.3**) using *sec*-butylamine and the enol-lactone **111** affording pure off white crystals (131 mg, 99%). Analysis by TLC and GCMS showed no traces of starting materials. The product was not further purified.

Mp: 43-46ºC;

^{1}H NMR ($CDCl_3$): δ 4.84 (1H, m), 4.70 (1H, m), 4.12 (2H, q, *J* = 7.2 Hz), 3.15 (1H, m), 2.83 (2H, m), 2.55 (2H, m), 1.73 (4H, m), 1.51 (2H, m), 1.23 (6H, m), 0.80 (3H, m);

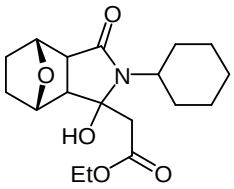
^{13}C NMR ($CDCl_3$): δ 173.5, 169.3, 90.5, 78.9, 78.3, 60.8, 54.4, 51.6, 50.0, 43.7, 29.5, 6.8, 26.4, 18.6, 14.1, 11.8;

GCMS: 294 [M-17] (5%), 293 [M-18] (23%), 238 (100%);

HR-MS: 311.16978;

IR: 3360, 2975, 2876, 1738, 1661, 1444, 1373, 1343, 1188, 1130, 1036, 997.

Synthesis of (4-Cyclohexyl-3-hydroxy-5-oxo-10-oxa-4-aza-tricyclo[5.2.1.02,6]dec-3-yl)acetic acid ethyl ester (**123**)



123

The *N*-cyclohexyl substituted analogue **123** was synthesised according to the general method (**6.4.3**) using cyclohexylamine and the enol-lactone **111** affording off white solid (180 mg). Analysis by TLC and GCMS showed presence of starting material. The product was purified using speedy column chromatography (hexane/ether; 1:25), and concentrated *in vacuo* affording pure off white crystals (100 mg, 71%).

Mp: 114-116°C;

^{1}H NMR ($CDCl_3$): δ 4.84 (1H, d, *J* = 5.2 Hz), 4.71 (1H, d, *J* = 4.2 Hz), 4.14 (2H, m), 3.07 (2H, m), 2.82 (2H, m), 2.58 (2H, m), 2.23 (2H, m), 1.70 (4H, m), 1.50 (6H, m), 1.25 (3H, t, *J* = 7.1 Hz), 1.18 (4H, m);

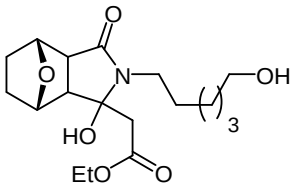
^{13}C NMR ($CDCl_3$): δ 173.3, 169.3, 90.6, 78.8, 78.2, 60.9, 53.1, 51.8, 49.9, 43.4, 30.4, 29.5, 29.4, 26.8, 26.2, 25.1, 14.1;

GCMS: 320 [M-17] (3%), 319 [M-18] (17%), 238 (100%);

HR-MS: 339.18962;

IR: 3330, 2981, 2933, 2853, 1735, 1676, 1536, 1443, 1371, 1311, 1253, 1189, 1128, 1045, 1031, 998.

Synthesis of [3-Hydroxy-4-(6-hydroxy-hexyl)-5-oxo-10-oxa-4-aza-tricyclo[5.2.1.02,6]dec-3-yl]-acetic acid ethyl ester (**124**)

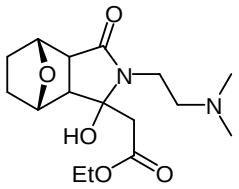


124

The *N*-hexanol substituted analogue **124** was synthesised according to the general method (**6.4.3**) using 6-aminohexan-1-ol and the enol-lactone **111** affording pure yellow oil (146 mg, 98%). Analysis by TLC and GCMS showed no traces of starting materials. The product was not further purified.

^{1}H NMR ($CDCl_3$): δ 4.86 (1H, d, *J* = 5.0 Hz), 4.71 (1H, d, *J* = 4.1 Hz), 4.12 (2H, q, *J* = 7.1 Hz), 3.58 (2H, t, *J* = 6.2 Hz), 3.41 (1H, s), 3.14 (2H, m), 2.84 (2H, m), 2.63 (2H, m), 1.79 (2H, m), 1.52 (6H, m), 1.30 (4H, m), 1.23 (3H, t, *J* = 7.1 Hz);
^{13}C NMR ($CDCl_3$): δ 173.6, 169.2, 89.9, 78.5, 78.0, 62.6, 60.9, 51.5, 50.1, 43.4, 39.4, 32.4, 29.4, 29.1, 26.8, 26.6, 25.1, 14.4;
GCMS: 356 [M+1] (2%), 338 [M-17] (18%), [M-18] (45%), 250 (10%), 31 (100%);
HR-MS: 355.19974;
IR: 3500-3250, 2936, 2859, 1738, 1682 1536, 1443, 1371, 1311, 1253, 1189, 1128, 1045, 1031, 998.

Synthesis of [4-(2-Dimethylamino-ethyl)-3-hydroxy-5-oxo-10-oxa-4-aza-tricyclo[5.2.1.02,6]dec-3-yl]acetic acid ethyl ester (**126**)



126

The *N*-dimethylaminoethyl substituted analogue **126** was synthesised according to the general method (**6.4.3**) using dimethylethylamine and the enol-lactone **111** affording crude (**126**) (126 mg). Analysis by TLC and GCMS showed traces of starting materials. The product was purified using flash chromatography (methanol/dichloromethane; 1:40), and concentrated *in vacuo* affording pure off white crystals (59 mg, 43%).

Mp: 76-78 ºC;

^{1}H NMR ($CDCl_3$): δ 5.03 (1H, d, *J* = 4.5 Hz), 4.71 (1H, d, *J* = 4.3 Hz), 4.08 (2H, m), 3.64 (1H, m), 2.91 (1H, m), 2.69 (4H, d, *J* = 6.7 Hz), 2.25 (6H, s), 2.17 (1H, m), 1.70 (2H, m), 1.48 (2H, m), 1.46 (4H, m), 1.17 (2H, t, *J* = 7.1 Hz);

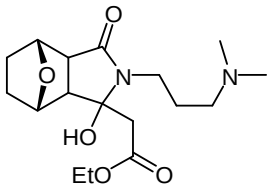
^{13}C NMR ($CDCl_3$): δ 173.6, 169.4, 87.5, 78.5, 77.9, 60.6, 56.8, 53.1, 50.1, 46.2, 45.0, 38.0, 28.4, 28.2, 14.2;

GCMS: 309 [M-17] (1%), 308 [M-18] (5%), 263 (10%), 58 (100%);

HR-MS: 326.18295;

IR: 3440, 2985, 2875, 2831, 2781, 1732, 1700, 1675, 1464, 1420, 1370, 1311, 1266, 1132, 1080, 1030, 999.

Synthesis of [4-(3-Dimethylamino-propyl)-3-hydroxy-5-oxo-10-oxa-4-aza-tricyclo[5.2.1.02,6]dec-3-yl]acetic acid ethyl ester (**127**)

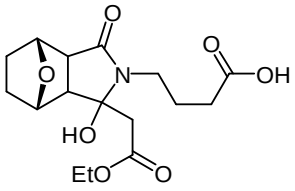


127

The *N*-dimethylaminopropyl substituted analogue **127** was synthesised according to the general method (**6.4.3**) using dimethylpropylamine and the enol-lactone **111** affording pure yellow oil (145 mg, 99%). Analysis by TLC and GCMS showed no traces of starting materials. The product was not further purified.

^{1}H NMR ($CDCl_3$): δ 4.94 (1H, d, *J* = 4.5 Hz), 4.68 (1H, d, *J* = 4.3 Hz), 4.03 (2H, m), 3.41 (1H, m), 2.91 (1H, m), 2.57 (4H, d, *J* = 6.7 Hz), 2.49 (1H, m), 2.23 (2H, m), 2.15 (2H, s), 2.12 (6H, s) 1.67 (2H, m), 1.46 (4H, m), 0.95 (2H, t, *J* = 7.1 Hz);
^{13}C NMR ($CDCl_3$): δ 173.4, 169.5, 89.3, 78.4, 77.4, 60.6, 55.0, 53.0, 50.3, 45.9, 43.9, 37.2, 28.5, 28.3, 22.0, 14.1;
GCMS: 323 [M-17] (2%), 322 [M-18] (11%), 84 (64%), 58 (100%);
HR-MS: 340.20495;
IR: 3444, 2980, 2873, 2827, 2782, 1732, 1702, 1673, 1464, 1418, 1370, 1311, 1266, 1190, 1132, 1080, 1029, 1001.

Synthesis of 4-(3-Ethoxycarbonylmethyl-3-hydroxy-5-oxo-10-oxa-4-aza-tricyclo[5.2.1.02,6]dec-4-yl)butyric acid (**128**)



128

The *N*-butyric acid substituted analogue **128** was synthesised according to the general method (**6.4.3**) using 4-aminobutyric acid and the enol-lactone **111** affording crude yellow/orange oil (117 mg). The product was purified using flash chromatography (1.5% methanol in dichloromethane) and concentrated *in vacuo* affording pure white crystals (84 mg, 58%).

Mp: 109-111°C;

^{1}H NMR ($CDCl_3$): δ 4.78 (1H, m), 4.72 (1H, m), 4.15 (2H, q, *J* = 7.1 Hz), 3.64 (2H, m), 3.48 (1H, m), 2.71 (1H, d, *J* = 7.4 Hz), 2.34 (2H, t, *J* = 7.2 Hz), 1.83 (8H, m), 1.59 (2H, m), 1.28 (3H, t, *J* = 7.1 Hz);

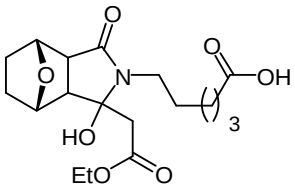
^{13}C NMR ($CDCl_3$): δ 177.4, 176.6, 167.2, 160.5, 92.0, 82.7, 79.4, 59.8, 49.6, 46.6, 39.5, 30.6, 28.9, 28.4, 21.2, 14.4;

GCMS: 324 [M-17] (3%), 323 [M-18] (33%), 254 (42%), 236 (76%), 41 (100%);

HR-MS: 341.14925;

IR: 3500-3000, 2984 , 1738 , 1717 , 1698, 1682, 1622, 1463, 1417, 1330, 1292, 1147, 1096, 1045, 1005.

Synthesis of 6-(3-Ethoxycarbonylmethyl-3-hydroxy-5-oxo-10-oxa-4-aza-tricyclo[5.2.1.02,6]dec-4-yl)hexanoic acid (**129**)



129

The *N*-hexanoic acid substituted analogue **129** was synthesised according to the general method (**6.4.3**) using 6-aminohexanoic acid and the enol-lactone **111** affording crude yellow oil (139 mg). The product was purified using flash chromatography (methanol/dichloromethane; 1:50) and concentrated *in vacuo* affording pure of white crystals (94 mg, 61%).

Mp: 126-128°C;

^{1}H NMR ($CDCl_3$): δ 4.77 (1H, m), 4.71 (1H, m), 4.16 (2H, q, *J* = 7.2 Hz), 3.63 (1H, d, *J* = 7.3 Hz), 3.45 (2H, m), 2.69 (1H, d, *J* = 7.3 Hz), 2.32 (2H, t, *J* = 7.2 Hz), 1.78 (2H, m), 1.58 (6H, m), 1.32 (2H, m), 1.28 (3H, t, *J* = 9.8 Hz);

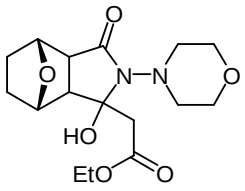
^{13}C NMR ($CDCl_3$): δ 176.4, 167.3, 160.9, 91.6, 82.7, 79.3, 59.8, 49.6, 46.6, 40.3, 33.7, 28.9, 28.5, 26.1, 25.9, 24.2, 14.4;

GCMS: 352 [M-17] (5%), 351 [M-18] (12%), 264 (73%), 251 (68%), 41 (100%);

HR-MS: 369.18232;

IR: 3500-3000, 2950, 2875, 1742, 1716, 1699, 1622, 1464, 1447, 1418, 1330, 1287, 1143, 1046, 1002.

Synthesis of (3-Hydroxy-4-morpholin-4-yl-5-oxo-10-oxa-4-aza-tricyclo[5.2.1.02,6]dec-3-yl)acetic acid ethyl ester (**130**)



130

The *N*-morpholine substituted analogue **130** was synthesised according to the general method (**6.4.3**) using 4-aminomorpholine and the enol-lactone **111** affording crude yellow oil (117 mg). The product was purified using flash chromatography (1% methanol in dichloromethane) and concentrated *in vacuo* affording pure off white crystals (67 mg, 48%).

Mp: 131-133°C;

^{1}H NMR ($CDCl_3$): δ 4.89 (1H, d, *J* = 5.0 Hz), 4.74 (1H, d, *J* = 4.2 Hz), 4.15 (2H, m), 3.71 (4H, bs), 2.96 (1H, m), 2.69 (2H, m), 2.54 (1H, m), 1.78 (2H, m), 1.51 (2H, m), 1.24 (3H, t, *J* = 7.1 Hz);

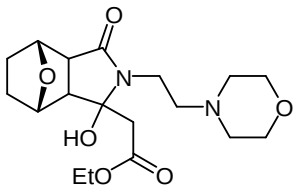
^{13}C NMR ($CDCl_3$): δ 172.2, 169.9, 89.1, 78.1, 77.8, 67.2, 60.8, 52.3, 49.8, 48.9, 42.6, 29.5, 27.0, 14.1;

GCMS: 323 [M-17] (1%), 350 [M-18] (2%), 168 (40%), 86 (100%);

HR-MS: 340.1583;

IR: 3500-3000, 2961, 2856, 1970, 1740, 1701, 1675, 1459, 1418, 1372, 1305, 1245, 1188, 1117, 1076, 1004.

Synthesis of [3-Hydroxy-4-(2-morpholin-4-yl-ethyl)-5-oxo-10-oxa-4-aza-tricyclo[5.2.1.02,6]dec-3-yl]-acetic acid ethyl ester (**131**)

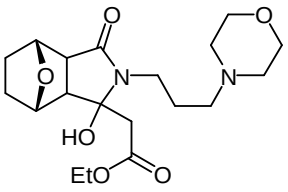


131

The *N*-ethylmorpholine substituted analogue **131** was synthesised according to the general method (**6.4.3**) using 4-ethylaminomorpholine and the enol-lactone **111** affording crude yellow oil (117 mg). The product was purified using flash chromatography (methanol/dichloromethane; 1:20) and concentrated *in vacuo* affording pure yellow oil (101 mg, 65%).

^{1}H NMR ($CDCl_3$): δ 5.01 (1H, d. *J* = Hz), 4.65 (1H, d, *J* = Hz), 4.07 (2H, m), 3.65 (4H, m), 2.64 (6H, m), 2.44 (2H, m), 1.67 (2H, m), 1.47 (2H, m), 1.17 (3H, t, *J* = Hz);
^{13}C NMR ($CDCl_3$): δ 173.5, 169.2, 87.8, 78.6, 77.9, 66.5, 60.6, 55.6, 53.5, 53.0, 50.1, 45.8, 37.3, 28.4, 28.1, 14.0;
GCMS: 351 [M-17] (1%), 350 [M-18] (5%), 100 (100%);
HR-MS: 368.19274;
IR: 3500-3000, 2959, 2855, 1969, 1738, 1703, 1674, 1456, 1418, 1372, 1305, 1245, 1188, 1117, 1075, 1018, 1004.

Synthesis of [3-Hydroxy-4-(3-morpholin-4-yl-propyl)-5-oxo-10-oxa-4-aza-tricyclo[5.2.1.02,6]dec-3-yl]acetic acid ethyl ester (**132**)

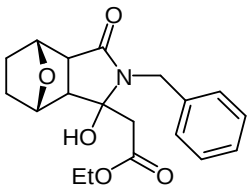


132

The *N*-propylmorpholine substituted analogue **132** was synthesised according to the general method (**6.4.3**) using 4-propylaminomorpholine and the enol-lactone **111** affording crude orange/brown oil (142 mg). The product was purified using flash chromatography (methanol/dichloromethane; 1:40) and concentrated *in vacuo* affording pure white crystals (119 mg, 74%).

Mp: 169-172°C;
^{1}H NMR ($CDCl_3$): δ 4.91 (1H, d, *J* = 4.6 Hz), 4.68 (1H, d, *J* = 4.4 Hz), 4.06 (2H, m), 3.69 (4H, m), 3.43 (1H, m), 2.94 (1H, m), 2.62 (4H, d, *J* = 9.7 Hz), 2.50 (2H, m), 2.42 (4H, bs), 2.16 (2H, m), 1.66 (4H, m), 1.48 (2H, m), 1.19 (3H, t, *J* = 7.1 Hz);
^{13}C NMR ($CDCl_3$): δ 173.6, 169.4, 89.3, 78.5, 77.6, 66.1, 60.7, 54.0, 53.0, 52.7, 50.3, 45.6, 36.8, 28.5, 28.2, 20.9, 14.1;
GCMS: 365 [M-17] (3%), 364 [M-18] (22%), 126 (45), 100 (100%);
HR-MS: 382.21232;
IR: 3500-3000, 2981, 2954, 2873, 2833, 2787, 1732, 1682, 1463, 1444, 1403, 1370, 1350, 1308, 1255, 1219, 1189, 1148, 1076, 1038, 1007.

Synthesis of (4-Benzyl-3-hydroxy-5-oxo-10-oxa-4-aza-tricyclo[5.2.1.02,6]dec-3-yl)acetic acid ethyl ester (**133**)

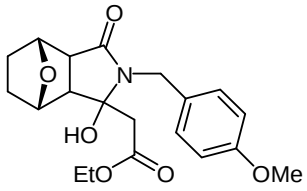


133

The *N*-benzyl substituted analogue **133** was synthesised according to the general method (**6.4.3**) using benzylamine and the enol-lactone **111** affording crude brown oil (127 mg). The product was purified using flash chromatography (hexane/ether; 1:5) and concentrated *in vacuo* affording pure white crystals (104 mg, 72%).

Mp: 120-122°C;
^{1}H NMR ($CDCl_3$): δ 7.28-7.08 (5H, m), 4.81 (1M, d, *J* = 4.3 Hz), 4.74 (1H, d, *J* = 4.2 Hz), 3.86 (2H, m), 3.75 (2H, s), 2.76 (2H, s), 1.69 (2H, m), 1.45 (2H, m), 1.06 (3H, t, *J* = 7.1 Hz);
^{13}C NMR ($CDCl_3$): δ 173.9, 169.1, 143.0, 138.1, 128.4, 127.6, 126.9, 126.6, 89.7, 78.4, 77.8, 60.5, 51.8, 50.4, 46.3, 43.7, 42.2, 29.2, 27.1, 13.8;
GCMS: 328 [M-17] (5%), 327 [M-18] (20%), 258 (23%), 91 (100%);
HR-MS: 345.15973;
IR: 3330, 2983, 2957, 2877, 2836, 1733, 1687, 1674, 1615, 1586, 1513, 14443, 1411, 1372, 1345, 1304, 1247, 1187, 1148, 1129, 1034, 999.

Synthesis of [3-Hydroxy-4-(4-methoxy-benzyl)-5-oxo-10-oxa-4-aza-tricyclo[5.2.1.02,6]dec-3-yl]acetic acid ethyl ester (**134**)



134

The *N*-methoxybenzyl substituted analogue **134** was synthesised according to the general method (**6.4.3**) using 4-methoxybenzylamine and the enol-lactone **111** affording crude yellow oil (148 mg). The product was purified using flash chromatography (100% ether) and concentrated *in vacuo* affording pure off white crystals (95 mg, 60%).

Mp: 129-131°C;

^{1}H NMR ($CDCl_3$): δ 7.17 (2H, d, *J* = 8.5 Hz), 6.78 (2H, d, *J* = 8.5 Hz), 4.87 (1H, d, *J* = 5 Hz), 4.79 (1H, d, *J* = 4.7 Hz), 3.9 (2H, m), 3.74 (3H, s), 2.83 (2H, m), 1.75 (2H, m), 1.61 (2H, m), 1.13 (3H, t, *J* = 7.2 Hz);

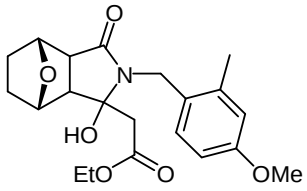
^{13}C NMR ($CDCl_3$): δ 174.0, 169.1, 158.7, 130.2, 129.1, 113.8, 89.9, 78.5, 78.0, 60.7, 55.2, 51.6, 50.4, 43.5, 41.8, 29.5, 26.8, 13.9;

GCMS: 358 [M-17] (2%), 357 [M-18] (10%), 121 (100%);

HR-MS: 375.17856;

IR: 3500-3250, 2983, 2957, 2877, 2836, 1733, 1687, 1674, 1615, 1586, 1513, 14443, 1411, 1372, 1345, 1304, 1247, 1187, 1148, 1129, 1034, 999.

Synthesis of [3-Hydroxy-4-(2-methyl-4-methoxybenzyl)-5-oxo-10-oxa-4-aza-tricyclo[5.2.1.02,6]dec-3-yl]acetic acid ethyl ester (**135**)



126

The *N*-2-methyl-4-methoxybenzyl substituted analogue **135** was synthesised according to the general method (**6.4.3**) using 2-methyl-4-methoxybenzylamine and the enol-lactone **111** affording crude yellow/brown oil (138 mg). The product was purified using flash chromatography (hexane/ether; 1:10) and concentrated *in vacuo* affording pure slight yellow crystals (99 mg, 66%).

Mp: 135-138 ºC;

^{1}H NMR ($CDCl_3$): δ 7.16 (2H, d, *J* = 8.4 Hz), 6.80 (3H, m), 4.87 (1H, d, *J* = 5 Hz), 4.79 (1H, d, *J* = 4.7 Hz), 3.9 (2H, m), 3.74 (3H, s), 2.83 (2H, m), 2.74 (3H, m), 1.75 (2H, m), 1.61 (2H, m), 1.13 (3H, t, *J* = 7.2 Hz);

^{13}C NMR ($CDCl_3$): δ 174.2, 173.5, 169.4, 142.4, 128.6, 128.3, 127.3, 126.6, 104.0, 90.6, 78.9, 77.9, 60.9, 52.6, 51.9, 50.3, 43.7, 29.6, 27.0, 21.0, 14.3;

GCMS: 342 [M-17] (4%), 341 [M-18] (19%), 168 (35%), 105 (100%);

HR-MS: 389.18356;

IR: 3500-3250, 2984, 2960, 2877, 2836, 1733, 1690, 1674, 1614, 1586, 1513, 14443, 1411, 1372, 1345, 1304, 1187, 1148, 1129, 1033, 997.

6.5 References

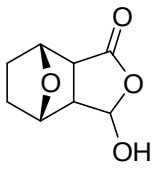
1. McCluskey, A., Walkom, C., Bowyer, M. C., Ackland, S. P., Gardiner, E., Sakoff, J. A., *Cantharimides: A New Class of Modified Cantharidin Analogues Inhibiting Protein Phosphatases 1 and 2a*. Bioorganic and Medicinal Chemistry Letters, 2001. **11**: p. 2941-2946.
2. McCluskey, A., Ackland, S. P., Gardiner, E., Walkom, C. C., Sakoff, J. A., *The Inhibition of Protein Phosphatases 1 and 2A: A New Target for Rational Anti-Cancer Drug Design?* Anti-Cancer Drug Design, 2001. **16**: p. 291-303.
3. Murray, W.V., Lalan, P., Gill, A., Addo, M. F., Lewis, J. M., Lee, D. K. H., Wachter, M. P., Rampulla, R., Underwood, D. C., *Substituted Pyrrolidin-2-one Biphenyltetrazoles as Angiotensin II Antagonists.* Bioorganic and Medicinal Chemistry Letters, 1993. **3**(2): p. 369-374.
4. Miki, Y., Hachiken, H., Sugimoto, Y., Yanase, N., *Reaction of 1-Benzylidole-2,3-dicarboxylic Anhydride with Wittig Reagents.* Hetrocycles, 1997. **45**(9).

Chapter 7

Development of Lactone Derivatives as Potential PP1 and PP2A Inhibitors

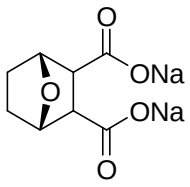
7.1 Introduction

The removal of one of the carbonyl carbons, as with the hydroxylactone (**34**), or ring opening to form the disodium salt (**23**), has been shown to display good to comparable phosphatase activity in relation to the parent anhydride (**13**) respectively [1, 2]. In this chapter we describe the complete removal of one of the carbonyl carbons to form the lactones (**138**-**139**), as well as the selective reduction of the remaining carbonyl carbon to form the lacton-ol derivative (**140**). We also synthesised the ring opened mono-sodium salt derivative (**141**) of the parent lactone.



PP1 IC_{50} = 24 μM
PP2a IC_{50} = 5 μM

34



PP1 IC_{50} = N/A
PP2a IC_{50} = 0.17 μM

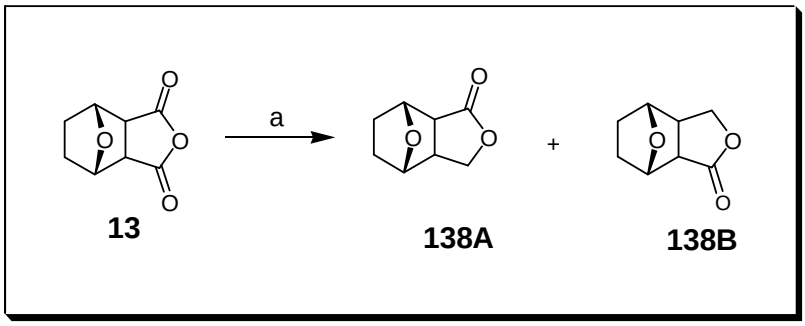
23

The described targets illustrate interesting structural modifications, and should attain valuable information in relation to the cantharidin pharmacophore, specifically on the requirement of both of the carbonyl carbons, as well the inhibition of ring opening to the dicarboxylic form, on phosphatase and anticancer activity. Furthermore, they also provide a possible handle for future analogue development.

7.2 Results and Discussion – Chemical Synthesis

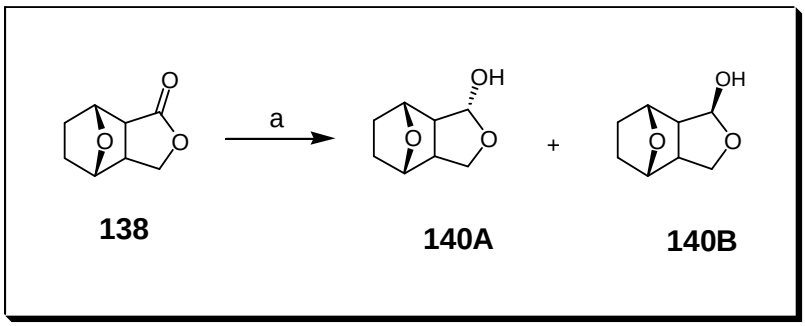
The generation of the lactone (**138**-**139**) and lacton-ol (**140**) analogues was carried out by chemistry previously described by Sprague *et. al.*, who reported the synthesis of 7-ozabicyclo[2.2.1]heptane analogues as inhibitors of thromboxane A_2/PGH_2 [3]. However, these modifications to the parent anhydride had not been biologically addressed in relation to phosphatase of anti-cancer activity.

The target removal a single carbonyl group of the parent anhydride (**13**, **19**) to form the lactones (**138**-**139**), was carried out by the selective reduction using sodium borohydride at reduced temperature (0ºC). It should be noted that attach of either equivalent carbonyl carbon of the parent anhydride is possible, which could lead to the formation of either enantiomer as a racemic mixture (**Scheme 7.1**). However this was not investigated due to poor biological activity displayed.



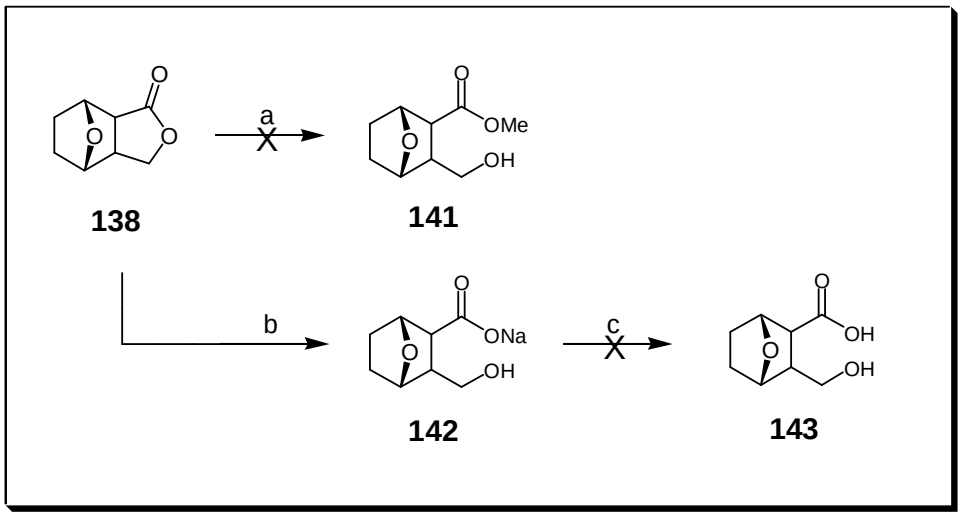
Scheme 7.1: Reagents and Conditions; (a) $NaBH_4$, THF, 0ºC.

The selective reduction of the remaining carbonyl group on the lactone (**138**) to form the alcohol (**140**), was carried out using DIBAL at -78ºC in dry toluene. Reaction completion was confirmed by carbon NMR, which substantiated the absence of carbonyl peaks, and the presence of a down field sp^3 hybridised methane carbon (δ 102.6 ppm). Examination of NMR data (both proton and carbon) showed doubling of peaks, indicating the formation of a both diasteriomers (**Scheme 7.2**). Carbon NMR denotes the formation of approximately a 1:3 formation ratio of either diastereomer, however these were not isolated and hence stereochemistry of the alcohol group was not resolved. Furthermore, as discussed above, it is also possible that the lactone starting material was racemic, therefore further enatiomers may also have been formed. Again, due to poor biological activity, this was not further investigated.



Scheme 7.2: Reagents and Conditions; (a) DIBAL, dry toluene, -78ºC.

The attempted ring opening of the lactone (**138**) to form the methoxylate ester analogue (**141**) was trialled by stirring the lactone in methanol in the presence of triethylamine at room temperature, however NMR analysis showed no conversion starting material. This was possibly due to the reversible intramolecular ring closing being thermodynamically favoured, and may have been overcome by changing the reaction condition (such as reduced temperature), but was not further attempted. Instead, ring opening to form the mono-sodium salt (**142**) was achieved by simply stirring the lactone in methanolic sodium hydroxide. The mono-sodium (**142**) adduct was titrated slowly with 0.1M HCl to attempt to form the free acid (**143**), however, the ring closed lactone (**138**) was again formed.

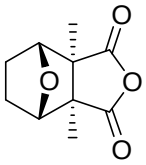
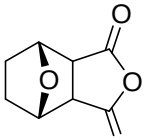
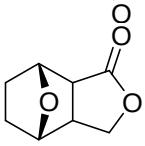
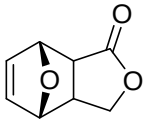
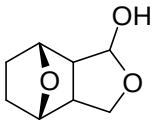
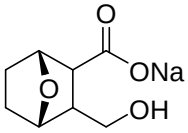


Scheme 7.3: Reagents and Conditions; (a) MeOH, TEA, RT, 24hrs (b) NaOH, MeOH, RT, 2hrs, (c) MeOH, HCl, 0ºC ,1hrs.

7.3 Results and Discussion – Biological Assay

As shown in **Table 7.1**, the growth inhibition on HT29 (Human colon carcinoma) and G401 (Human kidney carcinoma) cells of cantharidin (**6**), the demethylated norcantharidin (**13**) and the lactone analogues (**138-140, 142**) was determined. The data clearly shows that the removal of one or both of the carbonyl groups reduces the activity significantly, highlighting the importance of the second carbonyl carbon for maintaining activity.

Table 7.1: Growth Inhibition (%) of cantharidin (**6**), norcantharidin (**13**) and the lactone analogues (**138-140, 142**) on HT29 (colon) and G401 (kidney) cell lines. Cytotoxicity levels are first expressed as % inhibition at 100 µM drug concentration (*in italics*) and, if potent, as Growth Inhibition, GI_{50} µM (**in bold**)

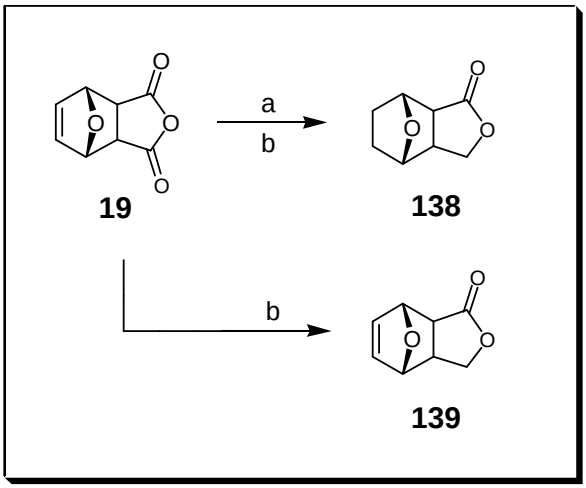
Compound		HT29		G401	
		Mean	SE	Mean	SE
	**6**	**3.2**	0.1	N/D	N/D
	**13**	**57**	5	N/D	N/D
	**138**	*<10*	6.3	*17.1*	1.2
	**139**	*<10*	6.4	*11.1*	5.0
	**140**	*<10*	8.1	*15.3*	6.4
	**142**	*<10*	2.4	*22.0*	12.7

NB: A value of <10% indicates that the compound failed to influence cell growth when compared with untreated controls (i.e., the higher the value the greater the growth inhibition).

7.4 Experimental – Synthesis of the Lactone derivatives

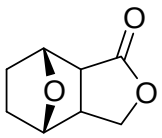
7.4.1 General Synthesis of the Lactone Analogues

To a stirring solution of the parent lactone (1 g) in dry THF (25 mL), 1 eq. of sodium borohydride in dry THF was slowly added at 0ºC. The resultant mixture was stirred under a calcium chloride drying tube for approx. 4 hrs. The solvent was removed under vacuum and the solid residue added slowly with stirring to ice. The resulting mixture was acidified to pH3 with conc. HCl and extracted with DCM (6 x 20 mL). The combined extracts were dried ($MgSO_4$) and concentrated *in vacuo*.



Scheme 7.4: Reagents and Conditions; (a) H_2, 5% Pd/C, Acetone 4 atm; (b) $NaBH_4$, THF 0ºC, 4 hrs

*Synthesis of 4,10-Dioxa-tricyclo[5.2.1.02,6]decan-3-one **(138)**:*

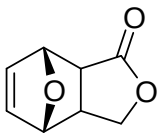


138

The lactone analogue **138** was synthesised according to the general method (**7.4.1**) using norcantharidin **13** affording white solid (709.5 mg, 77%).

Mp: 152-154ºC;
^{1}H NMR (DMSO-d_6): δ 4.84 (1H, d), 4.51 (1H, d), 4.40 (1H, t), 4.09 (1H dd), 2.75 (1H, t), 2.69 (1H, m), 1.76 (2H, m), 1.53 (2H, m);
^{13}C NMR (DMSO-d_6): δ 177.5, 82.4, 79.7, 72.3, 49.7, 43.4, 28.8, 27.6;
GCMS: 154 [M], 70 (100%);
IR (neat): 3025, 2991, 2974, 2958, 2918, 2883, 1770, 1484, 1460, 1394, 1315, 1276, 1245, 1196, 1139, 1074, 1055, 1016, 968, 924, 899, 856, 815;
HR-MS (m/z): calc $C_8H_{10}O_3$: 154.05826

Synthesis of 4,10-Dioxa-tricyclo[5.2.1.02,6]dec-8-en-3-one **(139)**



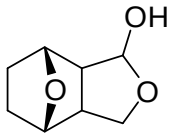
139

The 5-6,unsaturated analogue **139** was synthesised according to the general method (**7.4.1**) using **13** affording white solid (805.9 mg, 89%).

Mp: 161-163ºC;
^{1}H NMR (DMSO): δ 6.45 (2H, m), 5.28 (1H, s), 4.97 (1H, s), 4.50 (1H, t, J = 9.1 Hz), 4.20 (1H, m), 2.82 (1H, m), 2.73 (1H, m);
^{13}C NMR (DMSO): δ 175.9, 136.8, 136.7, 84.3, 82.1, 71.6, 48.1, 41.9;
GCMS: 152 [M], 70 (100%);
IR (neat): 3024, 2995, 2974, 2960, 2882, 1771, 1484, 1459, 1394, 1315, 1276, 1245, 1196, 1139, 1074, 1055, 1016, 968, 925, 899, 856;
HR-MS (m/z): calc $C_8H_8O_3$: 152.04863.

7.4.2 Synthesis of the α-hydroxy analogue

Synthesis of 4,10-Dioxa-tricyclo[5.2.1.02,6]decan-3-ol **(140)**



140

To a stirring solution of the parent lactone (**139)** (250 mg/1.623 mmol) in dry toluene (mL) maintained at -78 °C using a liquid nitrogen/acetone ice bath was added a 1 M solution of DIBALH in dry toluene (3.42 mL/3.42mmol) slowly over a 10 minute period. Stirring at -78 °C was continued for 30 minutes, after which the reaction was quenched with a solution of acetic acid (215.7 mg/3.595 mmol) in toluene (10 mL). The reaction mixture was allowed to warm to -30 °C, and then carefully treated with 10% HCl (32 mL), allowing the reaction temperature to rise to 0 °C. The aqueous phase was separated, and extracted with Chloroform (7 x 30 mL). The combined extracts were washed with 5% $NaHCO_3$ (22 mL), dried with $MgSO_4$, and concentrated *in vacuo*, yielding a mixture of diastereoisomer (169.0 mg, 67%) in an approximately a 1:3 ratio (B/A).

Mp: 133-134°C;

^{1}H NMR (DMSO): δ 4.50 (1H, d), 4.35 (1H, d), 4.10 (1H, t), 3.78 (1H, dd), 2.94 (1H, bs), 2.49 (1H, m), 1.63 (2H, m), 1.44 (2H, m);

^{13}C NMR (DMSO): A δ 102.6, 81.4, 78.52, 71.8, 56.4, 47.8, 28.8, 28.4;

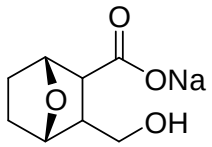
B δ 98.7, 78.5, 77.9, 68.8, 53.3, 48.9, 28.6, 27.3;

GCMS: 155 [M-H], 41 (100%);

HR-MS (m/z): calc $C_8H_{11}O_3$: 156.08655.

7.4.3 Synthesis of the ring opened lactone analogue

Synthesis of 3-Hydroxymethyl-7-oxa-bicyclo[2.2.1]heptane-2-carboxylic sodium salt **(142)**



142

To a stirring solution of the parent lactone (**138**) (250 g/1.623 mmol) in MeOH (73 mL) was added sodium hydroxide (16.3 mL) slowly over 5 minutes. The reaction was stirred at RT for 2 hours, after which the solvent was removed in vacuo. Ethyl acetate (20 mL) was added to the resultant residue and triturated for 20 minutes. The solid product was collected and washed with 4 mL ethyl acetate yielding (214.8 mg , 68%).

Mp: 173-174°C;

^{1}H NMR (DMSO): δ 4.63 (1H, d), 4.37 (1H, d), 3.49 (1H, m), 3.36 (1H, m), 2.54 (1H, d), 2.15 (1H, m), 1.63 (2H, m), 1.59 (2H, m), 1.41 (2H, m);

^{13}C NMR (DMSO): δ 179.8, 80.8, 78.9, 63.1, 55.0, 51.2, 30.1, 30.0;

GCMS: 154 [M-17], 41 (100%);

HR-MS (m/z): calc $C_8H_{11}O_3$: 194.06873.

7.5 Reference

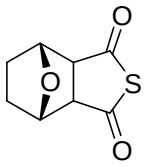
1. McCluskey, A., Keane, M. A., Mudgee, L., Sim, A. T. R., Sakoff, J. A., *Anhydride Modified Cantharidin Analogues. Is Ring Opening Important in the Inhibition of Protein Phosphatase 2A?* European Journal of Medicinal Chemistry, 2000. **35**: p. 957-964.
2. Enz, A., Zenke, G., Pombo-Viliar, E., *7-Oxa[2.2.1]bicycloheptane-2,3-dicarboxylic acid Derivatives as Phosphatase Inhibitors.* Bioorganic Medicinal Chemistry Letters, 1997. **7**: p. 2513.
3. Sprague, P.W., Heikes, J. E., Gougoutas, J. Z., Malley, M. F., Harris, D. N., Greenberg, R., *Synthesis and in Vitro Pharmacology of 7-Oxabicyclo[2.2.1]heptane Analogues of Thromboxane A_2/PGH_2.* Journal of Medicinal Chemistry, 1985. **28**: p. 1580-1590.

Chapter 8

Development of Heteroatom Substituted Anhydrides as Potential PP1 and PP2A Inhibitors

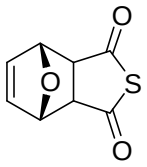
8.1 Introduction

Substitution of the central oxygen atom of the anhydride moiety with a heteroatom, such as sulphur or nitrogen, has received moderate attention in literature. Endothall thioanhydride (**29**) and the 5-6 unsuturated analogue (**28**) have been screened against PP2A (608 nM and 97% respectively), with little effect on PP2A activity in comparison to cantharidin [1]. The PP1 activity of these two cantharidin analogues has not been reported. Substitution with nitrogen, as with (**27**), appears to have a negative effect on PP2A inhibition (15%) [2]. Similarly its PP1 inhibition has not been reported.



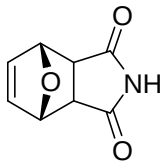
PP2a IC_{50} = 608 μM

29



PP2A inhibition = 97%

28



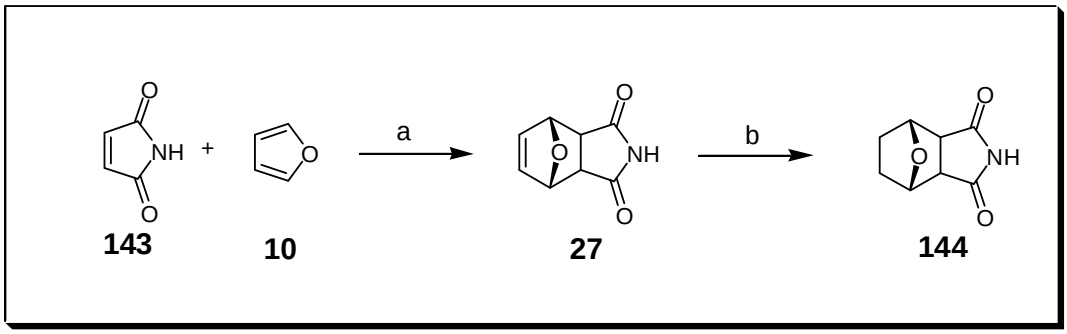
PP2A inhibition = 97%

27

One of the aims of this section was to extend our norcantharidin analogue library, and to examine the effect of substitution of the central oxygen of the anhydride moiety on PP1 and PP2A activity. Furthermore, the generation of heteroatom substituted analogues may allow the development of second generation analogues through potential ring opened adducts [1].

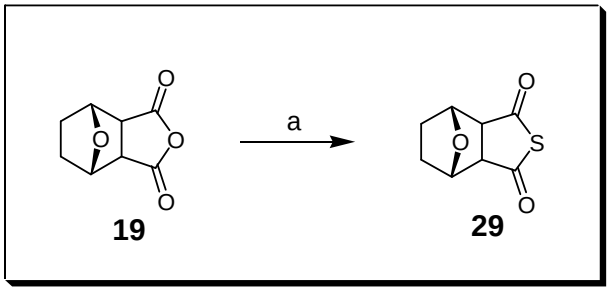
8.2 Results and Disscussion – Chemical Synthesis

Compounds (**27**) was synthesised using a method described by Kwart *et. al*., via a simple diels alder reaction involving furan and maleimide in a closed vessel [3]. This was converted to the 5,6-unsaturated analogue (**144**) in moderate yields using the previously described hydrogenation procedure. Refer to **Scheme 8.1**.



Scheme 8.1: Reagents and Conditions; (a) Et_2O, RT, 48h; (b) Acetone, 4 atm, RT, 24h.

Compound (**29**) was synthesised with moderate yields from (**13**), using 1.5 eq. $Na_2S.9H_2O$ and tetrabutylammonium iodide as the phase transfer catalysis. Refer to **Scheme 8.2**. Both reactions are simple and require little monitoring. Although moderate yields are obtained, both could be used in the generation of second generation analogues after ring opening.

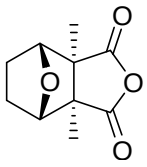
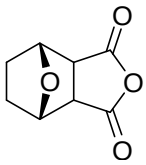
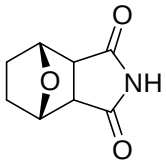
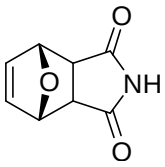
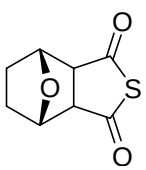


Scheme 4.2: Reagents and Conditions; (a) Et_2O, RT, 48h; (b) Acetone, 4 atm, RT, 24h.

8.3 Results and Discussion – Biological Assay

As shown in **Table 8.1** the growth inhibition of HT29 (Human colon carcinoma) and G401 (Human kidney carcinoma) cells of cantharidin (**6**), the demethylated norcantharidin (**13**) and the nitrogen substituted analogues (**27, 144**) was determined. The data for the sulphur substituted analogue (**29**) is to follow (*tf*). The data clearly shows that the substitution of the central oxygen with nitrogen reduces the activity significantly, highlighting the importance of ring-opening for maintaining activity.

Table 8.1: Cytotoxicity of cantharidin (**6**), norcantharidin (**13**) and the heteroatom substituted analogues (**27, 29, 143**) analogues on HT29 (colon) and G401 (kidney) cell lines. Cytotoxicity levels are first expressed as % inhibition at 100 µM drug concentration (*in italics*) and if potent, as Growth Inhibition, GI_{50} µM (**in bold**)

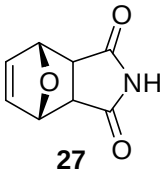
Compound		HT29		G401	
		Mean	SE	Mean	SE
	**6**	**3.2**	0.1	N/D	N/D
	**13**	**57**	5	N/D	N/D
	**144**	*15.8*	6.6	*9.9*	9.9
	**27**	*26.2*	6.1	*24.3*	12.1
	**29**	*tf*	-	*tf*	-

tf = data to follow

NB: A value of <10% indicates that the compound failed to influence cell growth when compared with untreated controls (i.e., the lower the value the greater the growth inhibition).

8.4 Experimental – Synthesis of hetroatom derivatives

Synthesis of 10-Oxa-4-azatricyclo[5.2.1.02,6]dec-8-ene-3,5-dione (**27**)

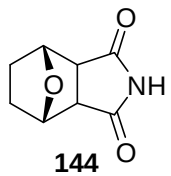


27

To saturated furan water solution was added maleimide in an amount such that a 50% excess of furan was present. This reaction was heated in a sealed glass tube for 10 hours at 90 °C. The crystalline product was separated upon cooling and recrystallised from boiling water to yield pure (**27**) (52%). Final analysis was carried out using GCMS and NMR.

Mp: 160-162 °C;
^{1}H NMR ($CDCl_3$): δ 6.52 (2H, s), 5.15 (2H, s), 2.89 (2H, s);
^{13}C NMR ($CDCl_3$): δ 179.8, 137.6, 82.2, 50.1;
ESI-MS: 166 [M+H], 165 [M] (100%);
IR (neat): 3147.0, 3028.0, 1774.5, 1715.6, 1399.4, 1355.8, 1288.1, 1205.2, 1190.0, 1143.3, 1091.2, 1020.2, 936.8, 897.3. 856.1;
HR-MS (m/z): calc $C_{16}H_{25}NO_3$: 165.04827.

Synthesis of 10-Oxa-4-azatricyclo[5.2.1.02,6]decane-3,5-dione (**144**)



144

5 g of (**27**) was dissolved in dry acetone and hydrogenated overnight at 4 atm with 10% Pd/C catalyst. After completion the catalyst was removed by filtration with filter aid (kenite), and concentrated *in vacuo* to obtain crude (**144**). Recrystallisation from ethylacetate/petroleum spirit (4:1) afforded pure (**144**) (4.1 g/87%). Final analysis was carried out using GCMS and NMR.

Mp: 180-182 °C;

^{1}H NMR ($CDCl_3$): δ 4.65 (2H, m), 3.29 (2H, bs), 2.93 (2H, s);

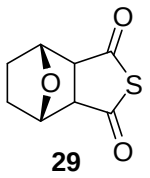
^{13}C NMR ($CDCl_3$): δ 179.0, 78.3, 50.8, 27.9;

ESI-MS: 168 [M+H], 167 [M] (100%);

IR (neat): 3054.1, 3006.0, 2779.8, 1755.3, 1703.8, 1674.5, 1403.4, 1360.1, 1306.1, 1262.0, 1239.9, 1201.6, 1006.9, 947.3, 922.8, 900.1, 819.1;

HR-MS (m/z): calc $C_{16}H_{25}NO_3$: 167.05937.

Synthesis of 10-Oxa-4-thiatricyclo[5.2.1.02,6]decane-3,5-dione (**29**)



29

To approximately 20 mL of 1:1 DCM/deionised water was added 500 mg of (**13**). Approximately 1.5 equivelent of $Na_2S.9H_2O$ (~1 g) and 100 mg of tetrabutylammonium iodide were added and stirring maintained at RT for 5 hours. This reaction was monitored by ^{1}H NMR and TLC. Recrystallisation from ethylacetate/petroleum spirit (4:1) afforded pure (**29**) (411 g/87%). Final analysis was carried out using GCMS and NMR.

Mp: 74-76 °C;
^{1}H NMR ($CDCl_3$): δ 4.96 (2H, s), 3.41 (2H, m), 1.74 (2H, m), 1.42 (2H, m);
^{13}C NMR ($CDCl_3$): δ 207.0, 84.6, 66.5, 31.8;
ESI-MS: 185 [M+H], 184 [M] (100%);
HR-MS (m/z): calc $C_{16}H_{25}NO_3$: 184.01473.

8.5 References

1. Laidley, C.W., Dauben, W. G., Guo, Z, R., Lam, J. Y. L., *2-Carboxymethylendothal analogues as affinity probes for stabilized protein phosphatase 2A.* Bioorganic Medicinal Chemistry Letters, 1999. **7**(12): p. 2937-2944.
2. Honkanen, R.E. and T. Golden, *Regulators of Serine/Threonine Protein Phosphatase at the Dawn of the Clinical Era?* Current Medicinal Chemistry, 2002. **9**: p. 2005-2075.
3. Kwart, H., Burchuk, I., *Isomerism and Adduct Stability in the Diels-Alder Reaction. I. The Adducts of Furan and Malemide.* Journal of the American Chemical Society, 1952. **74**(12): p. 3094–3097.

Chapter 9

Conclusion and Future Directions

9.1 Conclusions

The aim of this study was the synthesis of new norcantharidin analogues for the development of further SARs data, specifically PP1 and PP2A inhibition, in the hope of developing novel therapeutic agents. Previous studies have shown that the anhydride moiety displays moderate tolerance to structural modification in relation to phosphatase activity. These include, but are not limited to; (i) ring opening of the anhydride, (ii) substitution of the central anhydride oxygen, (iii) selective reduction of one of the carbonyl carbons. Therefore, this study has targeted structural modifications to the anhydride moiety, such as; the introduction of a heteroatom to the central anhydride position, removal of one or both of the carbonyl carbons, and ring opening of the anhydride. This was to further examine the tolerance to the described modifications, as well as develop possible leads for future work.

Based on the described targeted modifications, a series of compound families were synthesised and screened for PP1 and PP2A inhibition, as well as cytotoxicity. These families include: a series of ring closed (**Chapter 4**) and ring opened cantharimides (**Chapter 5**), possessing of alkyl, cyclic, aromatic, and, in the case of the former, functionalised by the nucleophilic substitution of amino-acids; a series of α-hydroxylactams (**Chapter 6**), possessing an ester side chain, and similarly, possessing a range of alkyl, cyclic and benzyl functionality; the lactone/lactonol and mono carboxylate ring opened analogues (**Chapter 7**); and the heteroatom substituted thio and amide anhydrides (**Chapter 8**).

The ring closed cantharimides, which had been previously reported by McCluskey *et. al.*, illustrated an interesting synthetic target [1]. This was primarily a result of their retained PPs activity, their unique nature relative to the natural inhibitors, their ease of chemical synthesis and readily available starting materials. Furthermore, it is not yet known whether they hydrolyse *in vivo* like anhydride moiety of cantharidin and tautomycin, primarily due to the replacement of oxygen with a primary amine. Of the ring closed cantharimides synthesied and assayed in this study, the most potent analogues contained either a C8-12 alkyl chains (**42**-**45**), an allyl group (**49**), a 1,2-diol moiety (**56**), a dodecyl-linked bis structure (**63**), or an amino-acid possessing a basic residue (**32**) which corresponds to data described by McCluskey *et. al.* [2]. The dodecyl-

linked bis-analogue (**63**) was the most potent analogue displaying µM potent cytoxicities against all the cell lines examined at levels that improve on the lead norcantharidin (**13**).

Further skeletal development of these analogues may lead to other interesting analogues, such as the investigation of analogues with longer carbon chain with varying degrees of functionality including diol functionality at various sections along the chain. Also, investigating other linkers with or without functionality for the bis-analogues may lead to other interesting and active analogues. Also, as none of the ring-closed cantharimide analogues, including the previous mentioned, proved to be significant PP1c, PP2A and PP5 inhibitors, suggesting a possible different mode of action than the lead compound, norcantharidin (**13**). This may be a result of a completely different mode of action, or an as yet discovered mechanism. It should be further iterated, that ring opening of these analogues may not have occurred *in vivo*.

Similarly the ring opened cantharimides, which were previously unreported, satisfy many of the requirements for a new synthetic target. This was primarily due to their ease of chemical synthesis and readily available starting material, but more importantly, they possess many of the requirements needed for the related ring closed cantharimides, as well as providing a handle for further modifications. More importantly, evidence that the *in viv*o ring-opening of the ring closed analogues is inhibited; these analogues may prove to retain the activity of the parent anhydride. Of the ring opened analogues, several shown to display good to excellent activity. In particular, further evaluation of substituted morpholino analogues should be investigated. Furthermore, since cantharidin (**6**) displays greater potency, PP2A selectivity and cytotoxicity than the demethylated analogue (**13**), it is also hypothesised that the generation of cantharidin based variants of **89**, **91**, **92** and **94**, may lead in to the development of novel potent analogues. For example, the synthesis of the morpholine cantharidin analogue (**110**) since developed by our laboratory, is more potent in all aspects than the parent cantharidin (**6**) and related morpholino-norcantharimide displayed in **Table 5.2** [3].

The development of the α-hydroxylactams accesses previously unexplored chemical space in relation to PPs and anticancer activity, however similar chemistry has been reported in literature [4]. The development of this family of compound is important due to the structural diversity compared to current cantharidin analogues, and its interesting synthetic chemistry. Furthermore, the α-hydroxylactams represented a third generation family within our group. Investigation of the removal or moving of one of the carboxylates away from the 7-oxa, and addition of hydrophobic tails, acidic and basic residues, polar moieties and aromatic rings were approached

in order to explore the chemical space and or binding requirements of the α-hydroxylactam family for binding. However, it appears that moving the carboxylate and the presence of the hydroxyl has a significant effect on cytotoxicity and phosphatase activity.

The development of the lactone and lactonol analogues was of interest due to the complete removal of one of the carbonyl carbons, and to investigate the inhibition of the ring opening of the lactone. The development of the ring opened lactone analogue was important due to the resemblance of the bis-sodium salt (**18**) and mono-esterified (**19**-**21**) analogues, as well as in the generation of new analogues including mono-esterified or etheral analogues. However, screening showed that complete removal of one, and the selective reduction of the remaining carbonyl carbon has a significant effect on activity. Furthermore, the mono-sodium analogue also displayed poor activity, possible due to *in vivo* ring closing.

The development of norcantharidin analogues with the oxygen of the anhydride moiety substituted by a heteroatom, either nitrogen or sulphur was investigated to evaluate the effect of facile ring opening. That is, what effect, if any does replacement of the oxygen have on the rate of ring opening, and what implication this has on PP1/PP2A inhibition and anti-cancer activity. The displayed activities have shown that replacement with sulphur appears to retain activity; where as the replacement with nitrogen has a negative effect on activity, hinting towards inhibition of ring opening *in vivo*. Therefore, further investigation of ring opened thio anhydride analogues may lead to novel analogues possessing retained activity.

These finding have allowed us to revisit the cantharidin pharmacophore discussed in **Chapter 2**, in particular relation to the anhydride moiety (**Figure 9.1**). As noted, substitution of the central oxygen atom of the anhydride is possible, with both sulphur and some amino acids. Also the dodecyl-linked bis-analogue (**63**) prove a potent inhibitor. The ring opening to form the mono-amide analogues, as in the case of the morpholino derivative, also displayed retained activity. The formation of a α-hydroxyl appears to have a significant negative effect on activity, as does the removal of one or both of the anhydride carbonyl groups.

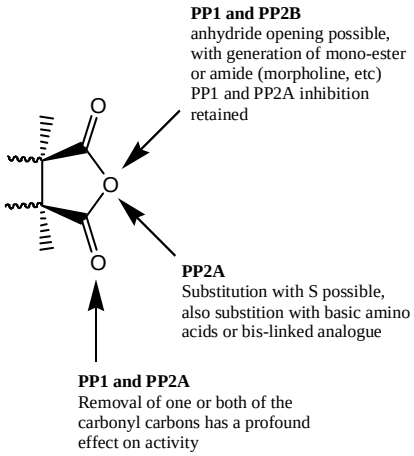


Figure 9.1: Modifications to the Cantharidin Pharmacophore

Figure modified from [5]

9.2 References

1. McCluskey, A., Walkom, C., Bowyer, M. C., Ackland, S. P., Gardiner, E., Sakoff, J. A., *Cantharimides: A New Class of Modified Cantharidin Analogues Inhibiting Protein Phosphatases 1 and 2a*. Bioorganic and Medicinal Chemistry Letters, 2001. **11**: p. 2941-2946.
2. McCluskey, A., Ackland, S. P., Gardiner, E., Walkom, C. C., Sakoff, J. A., *The Inhibition of Protein Phosphatases 1 and 2A: A New Target for Rational Anti-Cancer Drug Design?* Anti-Cancer Drug Design, 2001. **16**: p. 291-303.
3. Hill, T.H., Stewart, S. G., Sauer, B., Gilbert, J., Ackland, S, P., Sakoff, J, A., McCluskey, A., *Hetrocyclic Substituted Cantharidin and Norcantharidin Analogues-Synthesis, Protein Phosphatase (1 and 2A) inhibition, and Anti-cancer Activity.* Bioorganic Medicinal Chemistry Letters, 2007. **17**: p. 3392-3397.
4. Murray, W.V., Lalan, P., Gill, A., Addo, M. F., Lewis, J. M., Lee, D. K. H., Wachter, M. P., Rampulla, R., Underwood, D. C., *Substituted Pyrrolidin-2-one Biphenyltetrazoles as Angiotensin II Antagonists.* Bioorganic and Medicinal Chemistry Letters, 1993. **3**(2): p. 369-374.
5. McCluskey, A., Sakoff, J. A., *Small Molecule Inhibitors of Serine/Threonine Protein Phosphatases.* Mini Reviews in Medicinal Chemistry, 2001. **1**: p. 43-55.

Appendix 1

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Bioorganic & Medicinal Chemistry Letters 17 (2007) 3392–3397

Heterocyclic substituted cantharidin and norcantharidin analogues—synthesis, protein phosphatase (1 and 2A) inhibition, and anti-cancer activity

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Received 10 February 2007; revised 21 March 2007; accepted 29 March 2007
Available online 2 April 2007

Abstract—Norcantharidin (**3**) is a potent PP1 (IC_{50} = 9.0 ± 1.4 μM) and PP2A (IC_{50} = 3.0 ± 0.4 μM) inhibitor with 3-fold PP2A selectivity and induces growth inhibition (GI_{50} ~45 μM) across a range of human cancer cell lines including those of colorectal (HT29, SW480), breast (MCF-7), ovarian (A2780), lung (H460), skin (A431), prostate (DU145), neuroblastoma (BE2-C), and glioblastoma (SJ-G2) origin. Until now limited modifications to the parent compound have been tolerated. Surprisingly, simple heterocyclic half-acid norcantharidin analogues are more active than the original lead compound, with the morphilino-substituted (**9**) being a more potent (IC_{50} = 2.8 ± 0.10 μM) and selective (4.6-fold) PP2A inhibitor with greater in vitro cytotoxicity (GI_{50} ~9.6 μM) relative to norcantharidin. The analogous thiomorpholine-substituted (**10**) displays increased PP1 inhibition (IC_{50} = 3.2 ± 0 μM) and reduced PP2A inhibition (IC_{50} = 5.1 ± 0.41 μM), to norcantharidin. Synthesis of the analogous cantharidin analogue (**19**) with incorporation of the amine nitrogen into the heterocycle further increases PP1 (IC_{50} = 5.9 ± 2.2 μM) and PP2A (IC_{50} = 0.79 ± 0.1 μM) inhibition and cell cytotoxicity (GI_{50} ~3.3 μM). These analogues represent the most potent cantharidin analogues thus reported.

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Appendix 2

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Bioorganic & Medicinal Chemistry 15 (2007) 6126–6134
Bioorganic & Medicinal Chemistry

Norcantharimides, synthesis and anticancer activity: Synthesis of new norcantharidin analogues and their anticancer evaluation

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Received 20 March 2007; revised 12 June 2007; accepted 13 June 2007
Available online 20 June 2007

Abstract—A range of amines was reacted with norcantharidin (**2**) to provide the corresponding norcantharimides (**9**–**43**). Treatment of norcantharidin with allylamine afforded the corresponding allyl-norcantharimide (**20**) which was amenable to epoxidation (mCPBA, **22**) and subsequent ring opening ($MeOH/H^+$; **23**) or alternatively, osmylation (OsO_4/NMO; **24**). These simple synthetic modifications of **2** facilitated the development of a novel series of norcantharimides displaying modest to good broad spectrum cytotoxicity against HT29 and SW480 (colorectal carcinoma); MCF-7 (breast adenocarcinoma); A2780 (ovarian carcinoma); H460 (lung carcinoma); A431 (epidermoid carcinoma); DU145 (prostate carcinoma); BE2-C (neuroblastoma); and SJ-G2 (glioblastoma). Analogues possessing a C_{10}, C_{12} or C_{14} alkyl chain or a C_{12} linked bis-norcantharimide displayed the highest levels of cytotoxicity.

0968-0896/$ - see front matter
doi:10.1016/j.bmc.2007.06.034

Appendix 3

CHEMMEDCHEM

DOI: 10.1002/cmdc.200800192

Norcantharidin Analogues: Synthesis, Anticancer Activity and Protein Phosphatase 1 and 2A Inhibition

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*Cantharidin (**1**) and its derivatives are of significant interest as serine/threonine protein phosphatase 1 and 2A inhibitors. Additionally, compounds of this type have displayed growth inhibition of various tumour cell lines. To further explore both of these inhibition pathways, a number of amide–acid norcantharidin analogues (**15–26**) were prepared. Compounds **23** and **24**, containing two carboxylic acid residues, showed good PP1 and PP2A activity, with IC_{50} values of ~15 and ~3 μM, respectively. Substituted aromatic amide analogues **45**, **48**, **49**, **52**, **53**, and **54** also displayed good PP1 and PP2A inhibition, with IC_{50} values in the range of 15–10 μM (PP1) and 11–5 μM (PP2A). However, bulky* ortho *substituents on the aromatic ring caused the aromatic ring to be skewed from the NCO planarity, leading to a decrease in PP1 and PP2A inhibition. A number of analogues, **20**, **22**, **25** and **46**, showed excellent tumour growth inhibition, with **46** in particular being more potent than the lead, norcantharidin **2**.*

Appendix 4

Letters in Drug Design & Discovery, 2009, 6, 1-7

Synthesis of 4-substituted-3-hydroxy-5-oxo-10-oxa-4-azatricyclo[5.2.1]dec-3-yl Acetic Acid Ethyl Esters as Norcantharidin Analogues

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Received August 26, 2008: Revised September 22, 2008: Accepted September 23, 2008

Abstract: A novel library of eighteen 4-substituted-3-hydroxy-5-oxo-10-oxa-4-azatricyclo[5.2.1]dec-3-yl acetic acid ethyl esters was generated in high yield in two steps from norcantharidin, a known protein phosphatase 1 and 2A inhibitor that displays good anticancer activity. Interestingly these analogues are bereft of anticancer and protein phosphatase activity, but possess the attributes needed for medicinal agents and could be used as scaffolds in other targets.

Keywords: Norcantharidin, Wittig reaction, Protein phosphatase 1 and 2A, Anticancer, Drug design scaffold.

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