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Development of Isatin-based compounds for use in targeted anti-Cancer therapy

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Development of Isatin-Based Compounds for use in Targeted Anti-Cancer Therapy

A thesis submitted in fulfillment of the requirements for the award of the degree

DOCTOR OF PHILOSOPHY

from

UNIVERSITY OF WOLLONGONG

By

Lidia Matesic, B. Med. Chem. (Hons)

School of Chemistry

March 2011
For Mum and Dad
Thesis Declaration

I, Lidia Matesic, declare that the work described in this thesis, submitted in fulfillment of the requirements for the award of Doctor of Philosophy, in the School of Chemistry at the University of Wollongong, is wholly my own work unless otherwise acknowledged or referenced. This document has not been submitted for qualifications at any other academic institution.

Lidia Matesic

March 21, 2011
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Meaning</th>
</tr>
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<tbody>
<tr>
<td>δ</td>
<td>chemical shift</td>
</tr>
<tr>
<td>Ac-Lys-OMe. HCl</td>
<td>$N_a$-acetyl-L-lysine methyl ester hydrochloride</td>
</tr>
<tr>
<td>AcOH</td>
<td>acetic acid</td>
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<tr>
<td>AIBN</td>
<td>2,2'-azobis(2-methylpropionitrile)</td>
</tr>
<tr>
<td>AMB</td>
<td>amphotericin B</td>
</tr>
<tr>
<td>AMC</td>
<td>7-amino-4-methylcoumarin</td>
</tr>
<tr>
<td>anh.</td>
<td>anhydrous</td>
</tr>
<tr>
<td>ArC</td>
<td>aromatic carbon</td>
</tr>
<tr>
<td>ArH</td>
<td>aromatic proton</td>
</tr>
<tr>
<td>aq.</td>
<td>aqueous</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine-5'-triphosphate</td>
</tr>
<tr>
<td>b. p.</td>
<td>boiling point</td>
</tr>
<tr>
<td>bs</td>
<td>broad singlet</td>
</tr>
<tr>
<td>CDK</td>
<td>cyclin-dependant kinase</td>
</tr>
<tr>
<td>CML</td>
<td>chronic myelogenous leukemia</td>
</tr>
<tr>
<td>d</td>
<td>doublet</td>
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<td>DABCO</td>
<td>1,4-diazabicyclo[2.2.2]octane</td>
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<td>dichloromethane</td>
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<td>doublet of doublets</td>
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<tr>
<td>DIPEA</td>
<td>$N$, $N$-diisopropylethylamine</td>
</tr>
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<td>DMAP</td>
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<td>$N$, $N$-dimethylformamide</td>
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<td>dt</td>
<td>doublet of triplets</td>
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<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>EDCI</td>
<td>1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride</td>
</tr>
<tr>
<td>ESI-MS</td>
<td>electrospray ionisation mass spectrometry</td>
</tr>
<tr>
<td>Et$_3$N</td>
<td>triethylamine</td>
</tr>
</tbody>
</table>
Et₂O diethyl ether
EtOAc ethyl acetate
EtOH ethanol
equiv. equivalent(s)
FDA Food and Drug Administration
5-FU 5-fluorouracil
gCOSY gradient correlation spectroscopy
gHMBC gradient heteronuclear multiple bond correlation
gHSQC gradient heteronuclear single quantum correlation
GIST gastrointestinal stromal tumours
h hour(s)
HEPES 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid
ΔHₚ heat of formation
HOBt 1-hydroxybenzotriazole
HPLC high performance liquid chromatography
HREI-MS high resolution electron ionisation mass spectroscopy
HRESI-MS high resolution electrospray ionisation mass spectroscopy
IC₅₀ concentration required to inhibit 50% of the population
J spin-spin coupling constant (NMR)
kDa kilo Dalton
lit. literature
LREI-MS low resolution electron ionisation mass spectroscopy
LRESI-MS low resolution electrospray ionisation mass spectroscopy
m multiplet
m meta
M molar
[M⁺] molecular ion
mAb monoclonal antibody
MAPK mitogen-activated protein kinase
MeOH methanol
min minute(s)
m. p. melting point
MS mass spectrometry
MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxy
methoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium,
inner salt]
m/z mass to charge ratio
NaOAc sodium acetate
NBS N-bromosuccinimide
NH₄OAc ammonium acetate
NMR nuclear magnetic resonance
o ortho
p para
PAI-1 plasminogen activation inhibitor-1
PAI-2 plasminogen activation inhibitor-2
PKA protein kinase A
PBS phosphate buffered saline
PDGFR-β platelet-derived growth receptor-β
Pd(OAc)₂ palladium acetate
PEG poly(ethylene glycol)
PES phenazine ethosulfate
PhMe toluene
P(o-tol)_₃ tris(o-tolyl)phosphine
ppm parts per million
PS petroleum spirit
PTSA p-toluenesulfonic acid
Rf retardation factor
RME receptor-mediated endocytosis
rpm revolutions per minute
RT room temperature
s singlet
SAR structure-activity relationship
sat. saturated
t triplet

~ xiv ~
TBAB  tetrabutylammonium bromide
TBACL  tetrabutylammonium chloride
TBAI  tetrabutylammonium iodide
THF  tetrahydrofuran
THQ  1,2,3,4-tetrahydroquinoline
Tf  transferrin
TfR  transferrin receptor
TLC  thin layer chromatography
TMSCl  trimethylsilyl chloride
TTFMPP  tris(p-trifluoromethylphenyl)phosphine
uPA  urokinase plasminogen activation system
uPAR  urokinase plasminogen activation system receptor
UV-Vis  ultra violet visible
VEGFR-2  vascular endothelial growth factor receptor-2
Publications Arising from this Thesis (to date)

Journal Articles


Conference Abstracts


*Presenter underlined.*


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Abstract

Ligand-targeted drug delivery is currently one of the most challenging areas in pharmaceutical research involving the judicious choice of the drug, linker and targeting ligand. A promising new strategy involves conjugating a cytotoxin with a tumour-targeting protein through an acid-labile linker that is stable at physiological pH. Internalisation at the target tumour site via receptor-mediated processes exposes the conjugate to endosomal and lysosomal pH (4.5-6.0), resulting in selective release of the original cytotoxin inside the tumour cell. This thesis describes the synthesis of a new targeted anti-cancer agent and in particular, the development of the drug and linker component.

Drug development: Isatin (11) and its derivatives are present in numerous natural products including the Australian marine mollusc, *Dicathais orbita*. These derivatives possess a broad spectrum of biological properties including anti-cancer activity. New tricyclic and polycyclic derivatives of isatin (11) were prepared since previous research within our group indicated isatin derivatives exhibit highly potent cytotoxic effects against a variety of human cancer cell lines. To expand the cytotoxic structure-relationship activity of isatin derivatives, the N1-C7 region of isatin (11) was investigated through the addition of a 5- or 6-membered ring (i.e. pyrrolo[3,2,1-**hi**]indole-1,2-dione and pyrrolo[3,2,1-**ij**]quinoline-1,2-dione derivatives respectively) to yield 23 analogues, 18 of which were novel. *In vitro* assays revealed the inclusion of the extra ring reduced cytotoxicity compared to the parent molecules. However, a novel brominated tetracyclic acridine-based isatin, 4-bromo-6H-pyrrolo[3,2,1-*de*]acridine-1,2-dione (144), displayed an IC$_{50}$ value of 3.01 µM against U937 lymphoma cells and was the most cytotoxic
tricyclic/polycyclic isatin derivative synthesised within this project. In general, the
tetracyclic and pentacyclic isatins displayed greater activity than the analogous
tricyclic derivatives.

**Linker development:** The design, synthesis and utility of a series of novel
acid-labile, imine-based linkers, which were attached to a potent \( N \)-alkylisatin-
derived cytotoxin, \( 5,7 \)-dibromo-\( N \)-(\( p \)-methoxybenzyl)isatin (24) was also
investigated. In order to model the conjugation of these acid-labile linkers to the
lysine residues of tumour-targeting proteins, the aryl imines (153-157) were coupled
via their free carboxylic acids to a protected lysine amino acid residue to produce a
novel series of imine-lysine conjugates (158-162). Both the aryl imines (153-157)
and the imine-lysine conjugates (158-162) were stable for an extended period at
pH 7.4 but readily cleaved in aqueous acidic solutions at physiological temperature,
with half-lives ranging from 17.0 to 85.2 min. Observed rates of hydrolysis for the
embedded imine-acid moiety were in the order para-phenylpropionic acid > phenylacetic acid (para > meta) > benzoic acid (meta > para).

The 3-iminoisatins (153-157) and the isatin-lysine conjugates (158-162) were
also assessed for biological activity. 3-Iminoisatins 153-157 displayed cytotoxicity
in the low to sub-micromolar region against U937 cells, while isatin-lysine
conjugates 158-162 all exhibited cytotoxicity in the nanomolar region after being
exposed to U937 cells for 24 h. The meta-substituted isatin-lysine 161 was the most
cytotoxic molecule synthesised within this entire project, with an \( IC_{50} \) value of
165 nM against U937 cells.

**Ligand-targeted drug development:** Finally, the 3-iminoisatin 155 was
conjugated to the tumour-targeting proteins PAI-2 (an endogenous inhibitor of the
urokinase plasminogen activation system) and transferrin (Tf) via amide bond
formation with free lysine residues. UV-Vis spectrophotometry was used to visualise evidence of the isatin-protein conjugates 165 and 166. Upon treating the transferrin receptor over-expressing breast cancer cell line MDA-MB-231 with the isatin-Tf conjugate (166) for 48 h, a significant cytotoxic response (compared to Tf) was observed. At the highest concentration tested (1.56 µM), the conjugate 166 was 47±13% more cytotoxic than the Tf control. This work suggests that imine-linked isatin-protein conjugates possess the potential to be used as new therapeutic agents in the future.
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1.1 General Introduction

Cancer will affect one in two men and one in three women before the age of 85.¹ Today, cancer is the second leading cause of death in the developed world, killing over 39,000 people in Australia in 2005 and accounting for 29% of the total mortalities.¹ People of all ages may be affected by cancer, although incidence generally increases with age. Due to Australia’s increasingly aging population, it is estimated that the number of deaths caused by cancer will increase by 800 people per year.¹ Financially, cancer costs the Australian health system $3.8 billion per year, a total of 7.2% of the entire health system expenditure.² However, mortality rates have decreased and survival rates have improved over recent years. As of 2004, 58% of males and 64% of females in Australia with cancer survive within five years of initial diagnosis compared to just 41% and 53% respectively in 1986.¹ Although this data is promising, there is still a pressing need to improve current cancer therapeutics to further increase survival rates, to reduce the risk of metastatic, or secondary cancers and to mitigate harmful side effects associated with the treatments.

1.2 Molecular Biology of Cancer

Cancer is characterised by a range of abnormal cell behaviour but a key hallmark is the uncontrolled division of cells. Metastasis occurs when a cancerous cell has escaped its primary origin site and invades other sites of the body such as the bones, the brain and the lungs.³ Metastatic cancer cells are able to degrade
membranes and natural barriers in the body to aid their escape. The treatment of these cancers is difficult compared to those that are localised, primarily due to the cells being undetectable at the time of screening.

Although it is a common disease, at the cellular level, the development of cancer is a very rare event. Malignant progression requires multiple genetic modifications and for this reason, the majority of cells do not give rise to cancer. One of the initial steps in the development of malignant tumours is the formation of a benign tumour. Cells in this type of tumour are no longer responsive to the usual growth control signals, and at the same time, lack the ability to invade normal tissue or metastasise. To metastasise, a cell from the benign tumour must detach itself from its initial origin, migrate and invade surrounding tissue, and subsequently degrade the components of the extracellular matrix (ECM) including the basal lamina (Figure 1.1). By disintegrating the membrane, the cancer cells gain access to the blood and lymphatic vessels allowing colonisation at distant sites around the body. This migration is the major cause of cancer related death.
The human body is composed of $10^{15}$ cells and a subset of these, particularly skin, bone marrow and red blood cells, regularly divide and differentiate to repopulate organs and tissues which require cell turnover. There are approximately $10^{12}$ cell divisions per day and molecular mechanisms such as cell cycle checkpoints and apoptotic pathways are in place to govern cell proliferation and cell death. Alterations in these pathways often lead to cancer.

1.2.1 The Cell Cycle and Cancer

Each cell passes through a series of distinct stages that form the cell cycle. This cycle is divided into four phases: the G1, S and G2 phases which constitute interphase, and mitosis, the M phase (Figure 1.2). Mitosis is the process by which duplicated chromosomes are separated into two nuclei, while interphase is the period between cell divisions.
Figure 1.2 Phases of the cell cycle.  

Most cells except e.g. stem cells, spend the majority of their time in the interphase, which allows them to grow and undergo metabolic activities. The S phase is the time in which the cell synthesises DNA. The G1 and G2 phases are rest periods. At this time, cellular DNA passes through numerous cell cycle checkpoints, is inspected for damage, and if necessary, repaired before proceeding to the next phase. When a cell does not respond to cell cycle checkpoints, it proceeds through the cell cycle with damaged DNA giving rise to daughter cells with pre-cancerous characteristics and altered morphology. Through developing further alterations, known as mutations, the cell may develop into a malignant tumour.

1.2.2 Cell Death and Cancer

Cancer is not only characterised by uncontrolled proliferation but also cell immortality. Each day our bodies produce approximately 60 billion cells and each second, almost one million cells will die. This cycle of cell birth and cell death is regulated by mechanisms that control the rates of input and output. Therefore, programmed cell death (referring to strict genetic control) is crucial for the survival
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of almost all multicellular organisms. Physiological or programmed cell death usually occurs through the process of apoptosis, although cell death may also occur through necrosis.

Apoptosis is a series of morphological and biochemical changes that take place in cells that have been “instructed” to die in response to certain stimuli, DNA damage or reaching the end of their lifespan. Changes associated with apoptosis include chromatin condensation, nuclear fragmentation, cell shrinkage and blebbing from the plasma membrane.\textsuperscript{11,12} Apoptotic cells are removed \textit{in vivo} by phagocytosis, whereby the dying cells are “eaten alive” by phagocytes and their “corpses” are recycled back into the body.\textsuperscript{11}

Unlike apoptosis, necrosis is considered to be a passive response to a huge cellular insult\textsuperscript{12} and is free from strict genetic control. A lack of cellular energy production, an imbalance of intracellular calcium flux and the activation of non-apoptotic proteases may induce necrosis. The process of phagocytosis in necrotic cells is much more challenging because the disorderly death does not send “eat me” signals to phagocytes to engulf the cell. This lack of signaling makes it difficult for the immune system to locate and recycle the dead cells.

The cellular features of necrotic cells also differ from apoptotic cells. Necrotic cells feature damaged membrane lipids, random DNA degradation and swelling of cellular organelles.\textsuperscript{12} These organelles then burst releasing harmful contents that potentially damage neighbouring cells. As a result, an inflammatory response occurs. In order to treat cancer chemotherapeutically, pharmaceutical agents have two main strategies to halt cell proliferation or to induce cell death in cancer cells.
1.3 Current Cancer Therapeutics (Non-Selective)

Cancer was considered a terminal disease until the 19th Century when anaesthesia made surgery more feasible. Radiation therapy became the next treatment, but even surgery and radiation combined were insufficient to control metastatic cancer. In 1942 the era of chemotherapy began with the first chemotherapeutic agent, mechlorethamine (1), a nitrogen mustard DNA-alkylating drug used for the treatment of non-Hodgkin’s lymphoma\(^{13}\) (Figure 1.3A). Use of this drug has now discontinued due to its potential application as a chemical warfare agent.\(^{14}\) Although the cancer treated with 1 was in remission for only a few weeks, it proved that systemically administered drugs could induce tumour regression.

A plethora of chemotherapeutics have been developed and clinically employed in subsequent years. Most of these drugs interfere with DNA replication such as the purine- and pyrimidine-based anti-folates 6-mercaptopurine (2) and 5-fluorouracil (5-FU) (3) respectively; DNA-intercalating drugs such as the anthracyclines [e.g. doxorubicin (4) and daunorubicin (5)]; and metal-based DNA-alkylating drugs such as cisplatin (6) and oxaliplatin (Figure 1.3A). Additionally, there are chemotherapeutics that inhibit cell proliferation through interference with microtubule assembly such as vinblastine (7), a tubulin destabiliser and paclitaxel (8), a tubulin stabiliser\(^{15}\) (Figure 1.3B). Microtubules are the basic components of cell structure and play a role in various cellular functions including intracellular transport, maintenance of cell shape, motility and chromosome segregation during mitosis.\(^{16}\) While all of these agents demonstrated anti-proliferative properties, many chemotherapeutic drugs experience resistance after repeated use.\(^{17,18}\)
Chemotherapy largely relies on the premise that cancer cells proliferate at much higher rates than normal cells and are therefore more likely to be killed by a cytotoxic agent.\textsuperscript{3,19} The major drawback of cytotoxins is they have little, if any, specificity. This leads to serious damage of non-tumour tissue\textsuperscript{20} and systemic toxicity, resulting in severe side effects such as hair loss, liver or kidney damage.\textsuperscript{19} Therefore, there was a need to design and develop more targeted drugs with limited
additional systemic effects. It was not until the late 1980s that the era of “targeted therapy” began.\textsuperscript{13} Targets for cancer chemotherapy include signaling molecules, modulators of apoptosis and molecules involved in the promotion of angiogenesis.

### 1.4 Site-Specific Cancer Therapy

Site-specific drug delivery is currently one of the most challenging problems in pharmaceutical research since the drug has to be transported through the systemic circulation, pass complex tissue barriers and be selectively delivered to tumours.\textsuperscript{21} Effective site-specific drug delivery increases the availability of the drug at the target site while reducing its availability at other sites, especially those which display toxicity.\textsuperscript{22} As of 2009, there were 22 Food and Drug Administration (FDA) approved site-specific cancer therapies (nine monoclonal antibodies (mAbs), 12 small molecule drugs and one fusion protein).\textsuperscript{23} Site-specific cancer therapies represent a growing focus area for pharmaceutical companies with more than a 200\% increase in sales in the United States in 2009 compared to 2005.\textsuperscript{23}

Targeting drugs to specific sites is especially important for compounds which are highly toxic, have a narrow therapeutic index and/or limited solubility as these issues can limit the clinical use of potentially highly effective drugs. Site-specific drug delivery can be achieved by: application of the drug directly to the target organ such as dermal or ocular formulations; site-specific bioactivation where the drug is distributed throughout the body but undergoes bioactivation only at the target site\textsuperscript{24} such as kinase inhibitors or; conjugation of a drug to a targeting moiety where a carrier molecule delivers the drug to the tumour site.
1.4.1 Kinase Inhibitors

Cellular division at some point relies on protein kinases. These enzymes transfer a phosphate group from adenosine-5’-triphosphate (ATP) to a tyrosine, serine or threonine residue in a protein. Hence, these kinases can be further divided into tyrosine kinases or serine/threonine kinases. Dysfunctional protein kinase activity has been linked to angiogenesis and various forms of cancer, and thus, kinase inhibitors are appealing therapeutics for the treatment of cancer. The first small molecule kinase inhibitor to be approved by the FDA was imatinib mesylate (Glivec®, Novartis; Gleevec in USA) (9) (Figure 1.4) in May 2001 for the treatment of chronic myelogenous leukemia (CML) and gastrointestinal stromal tumours (GIST). Glivec® was an orphan drug for CML and received the fastest ever FDA approval for an anti-cancer drug. The drug is capable of inhibiting the tyrosine kinase enzyme Bcr-Abl in CML rather than non-specifically inhibiting and killing all rapidly dividing cells.

In January 2006 the FDA approved sunitinib malate (Sutent®, Pfizer) (10) (Figure 1.4) for the treatment of renal cell carcinoma and GIST, particularly for imatinib-resistant or advanced stage GIST. This drug was the first multi-targeted receptor tyrosine kinase inhibitor, targeting vascular endothelial growth factor receptor-2 (VEGFR-2), platelet-derived growth receptor-β (PDGFR-β), stem cell factor receptor, fetal liver tyrosine kinase receptor-3, colony-stimulating factor type 1 and glial cell-line derived neurotrophic factor receptor. Due to its ability to target multiple kinases, may be applicable in the treatment of other cancers. Recently, Phase III trials have demonstrated that the drug also possesses promising cytotoxic activity towards pancreatic neuroendocrine and non-small cell lung cancers.
1.4.2 Site-Specific Cancer Therapy using Conjugates

Further examples of site-specific drug delivery involve targeting receptors or proteins that are over-expressed on tumour cells. Oncology treatments could be significantly improved if a tumour recognising moiety and a cytotoxic agent were attached either directly or via a suitable linker to form a tumour-targeting conjugate. To improve the therapeutic potential of cytotoxic drugs, there has been a significant amount of research in recent years regarding the conjugation of cytotoxins to macromolecules or carrier molecules such as antibodies, proteins and synthetic polymers. In their simplest form, these conjugates consist of the cytotoxin and the macromolecule bound either directly or via a spacer, or linker, which may be cleaved at the target site through enzymatic cleavage (e.g. cathepsin B or esterases) or under acidic conditions to release the active cytotoxin (see Section 1.6) (Figure 1.5).
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Figure 1.5 Schematic representation of a simple drug conjugate.

Tumour-targeting conjugates are essentially prodrugs, which should not be toxic to the host, be stable in circulation and readily cleaved to release the cytotoxic agent once the conjugate has entered the cancer cell. The process of entering the intracellular environment of the targeted cell can be accomplished through receptor-mediated endocytosis (RME). First, ligands are able to bind to specific receptors on the cell surface. These receptors are then gathered into clathrin coated pits and enter the cells efficiently in conjunction with the ligand. Upon internalisation, the vesicles in the receptor-ligand complex are uncoated by an ATP-dependent enzyme. The internalised molecules are then guided to the early (or “sorting”) endosomes and the receptors and ligands dissociate from one another in the acidic environment of the endosomes (pH 5.5-6.8). In many cases, receptors are recycled to the cell surface, while the released ligand is delivered to endosomes or lysosomes (pH 4.5-5.0) for degradation.

The key elements of a successful drug conjugate involve the judicious choice of the drug, the linker and the targeting ligand. The work described here is aimed at the design and development of a new site-specific cancer therapy using:
• an isatin-based anti-cancer agent as the drug;
• acid-labile imine chemistry as the linker;
• and, two tumour-targeting proteins (transferrin and PAI-2) as the targeting ligands.

1.5 Choice of Drug: Isatins as Anti-Cancer Agents

Isatin (1H-indole-2,3-dione) (11) (Figure 1.6) was first discovered by Erdman\textsuperscript{45} and Laurent\textsuperscript{46} in 1840 as a product arising from the oxidation of indigo (12) (Figure 1.6) by nitric and chromic acids. The compound was considered synthetic for almost 140 years until its presence was discovered in fruits of the cannon ball tree, \textit{Couroupita guianensis} Aubl.,\textsuperscript{47} (Figure 1.7A). Isatin (11) is also a component of the secretion from the parotid gland of the \textit{Bufo melanostrictus} (Asian black spined toad) and \textit{Bufo gargarizans} (Asiatic toad) toads from the \textit{Bufo} genus\textsuperscript{48} (Figure 1.7C, D). Derivatives of isatin (11) have also been found in fungi,\textsuperscript{49} \textit{Dicathais orbita}, an Australian marine mollusc\textsuperscript{50} (Figure 1.7E), and in plants from the \textit{Isatis} genus, in particular \textit{Isatis tinctoria} (woad flowers, sometimes incorrectly referred to as \textit{Isatis indigotica}) (Figure 1.7B) which contain isatisine A (13),\textsuperscript{51} an alkaloid for which the total synthesis has only recently been reported.\textsuperscript{52}

\begin{figure}[h]
\centering
\includegraphics[scale=0.5]{structures.png}
\caption{Structures of isatin (11), indigo (12) and isatisine A (13).}
\end{figure}
In humans, isatin (11) is an endogenous molecule. The synthetic and metabolic pathways of 11 are unconfirmed although it is hypothesised that it may be synthesised from tryptophan-rich foods such as meat, dairy and whole grains. The
tryptophan is converted to indole by bacteria from the gastrointestinal tract and then transported to the liver where it is oxidised.\textsuperscript{57,58} Isatin (11) also plays a role in many physiological pathways.

1.5.1 Biological and Pharmacological Effects of Isatin and its Derivatives

Isatin (11) was found to be a major component of tribulin, an endogenous selective inhibitor of monoamine oxidase type B.\textsuperscript{59} Isatin (11) is also a metabolic derivative of adrenaline and a potent antagonist of atrial natriuretic peptide,\textsuperscript{60} the peptide responsible for natriuresis (sodium excretion), diuresis (water excretion) and vasodilatation. Additionally, 11 is anxiogenic (causes anxiety) due to its actions on central benzodiazepine receptors,\textsuperscript{57} which correlates to studies finding increased levels of isatin (11) in rat urine during times of stress such as exposure to acute cold.\textsuperscript{61}

Increased levels of 11 lead to decreased food intake due to the compound increasing serotonin levels, and by directly affecting the medial hypothalamus in the brain.\textsuperscript{62} Therefore, it may be regarded as a satiating agent and interestingly, elevated levels of 11 have been found in the cerebrospinal fluid of humans with the eating disorder bulimia nervosa.\textsuperscript{63}

The synthetic versatility of 11 has led to the preparation of a variety of derivatives. Originally, analogues were synthesised with substituent changes at C2 of the molecule through oxygen alkylation or alterations at C3 using nucleophilic attack, however, in recent years there has been a surge in the synthesis of N-substituted isatins.\textsuperscript{64-68} This has led to isatin derivatives with a broad spectrum of biological properties including anti-inflammatory,\textsuperscript{69} anti-Parkinsonian,\textsuperscript{60}
anti-convulsant,\textsuperscript{70} anti-histaminic,\textsuperscript{71} anti-fungal,\textsuperscript{72} anti-viral,\textsuperscript{73} anti-ulcer\textsuperscript{74} anti-amyloid\textsuperscript{75} and anti-cancer\textsuperscript{71,76} activities.

1.5.2 Isatin as a Cytotoxin

Isatin (11) has been tested against a variety of human cancer cell lines including HL60 (promyelocytic), PC12 (pheochromocytoma) and N1E-115 (neuroblastoma) and was found to be an inhibitor of cell proliferation in all the cell lines investigated.\textsuperscript{69} During apoptosis, 11 caused DNA fragmentation as well as chromatin condensation and reduced cell proliferation by 80\% at a concentration of 100 \( \mu \text{M} \) compared to controls. This effect was concentration dependent as only 20\% cell death was observed at 10 \( \mu \text{M} \).\textsuperscript{69} Further research using human neuroblastoma SH-SY5Y cells confirmed that the effect of 11 on cell proliferation was dose and time dependent.\textsuperscript{60} After 48 h of exposure to 11, cells containing lower concentrations of 11 began to undergo apoptosis, while cells with increased levels of 11 produced a late apoptotic/necrotic response (Figure 1.8). Cells containing 400 \( \mu \text{M} \) of 11 demonstrated the greatest cell death, with an apoptotic response of 82\%.\textsuperscript{60}
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Figure 1.8 Morphological modifications in cell structure of SH-SY5Y neuroblastoma cells. Cells were treated for 48 h with 50 µM (B and E) and 100 µM (C and F) of isatin (11). As a positive control 100 nM (A and D) staurosporine was used. Slides A-C were analysed using light microscopy while slides D-F were analysed with propidium iodide. Arrows show cells with apoptotic morphology.

1.5.3 Isatin Derivatives as Cytotoxins

The cytotoxic nature of isatin derivatives was established over 30 years ago and a comprehensive review of recently synthesised cytotoxic isatin analogues was published by our research group in 2009. Research into the cytotoxic nature of isatins commenced at the University of Wollongong in 2002 when it was discovered that chloroform extracts of the egg masses of the Australian muricid mollusc, *Dicathais orbita*, exhibited cytotoxic activity. Among the components of the extract were tyrindoleninone (14), tyriverdin (15), and 6-bromoisatin (16) (Figure 1.9). While tyrindoleninone (14) was shown to be the most active component in a cell-based assay using human monocyte-like, histiocytic lymphoma cells (U937) ($IC_{50} = 4 \mu M$ against U937 cells, $195 \mu M$ against human mononuclear cells) ($IC_{50} = \text{concentration required to inhibit } 50\% \text{ of the population}$), it was unstable, as was the weakly cytotoxic component, tyriverdin (15).
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Figure 1.9 Components from the egg masses of the Australian mollusc *Dicathais orbita* (14-16); and highly cytotoxic brominated isatin derivatives (17-18).

Attention thus turned to the significantly more stable brominated isatin 16, which was subsequently shown to possess modest cytotoxic activity towards U937 cells (IC$_{50}$ = 84 µM). In an effort to then acquire a variety of brominated isatin motifs, 5,7-dibromoisatin (17) (Figure 1.9) was synthesised and shown to display increased cytotoxicity against U937 cells (IC$_{50}$ = 10.5 µM). In an independent study, the introduction of a 5-bromo substituent to isatin (11) also increased the anti-cancer activity of the parent molecule. In contrast, nitro and methoxy substituents at the C5 position of isatin (11) led to a decrease in cytotoxic activity against U937 cells. Furthermore, cytotoxic activity was enhanced (IC$_{50}$ = 6.76 µM) with the addition of another bromine atom, as in 5,6,7-tribromoisatin (18) (Figure 1.9). Unfortunately, the compound was a side product from the bromination of 6-bromoisatin (16) and proved difficult to produce in large quantities.
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\(N\text{-Alkylated indoles are well known to exhibit anti-cancer activity}^{84,85}\) and the cytotoxic activity of the first \(N\text{-alkylisatin} was described soon after. The \(N\text{-benzyl compound 19} (\text{Figure 1.10})\) induced apoptosis in a panel of human cancer cell lines, but not normal cells, at micromolar concentrations.\(^86\) Further screening by the National Cancer Institute found that the dichlorinated compound 19 exerted a cytostatic effect (inhibiting cell growth by 50-100\%) on 40 out of the 48 cell lines tested, at a concentration of 10 \(\mu\text{M}. At 10\) times this concentration however, the compound exhibited 100\% cytotoxicity on virtually all cell lines, suggesting that this effect may be due to non-specific toxicity.\(^86\)

Since the brominated isatins 16-18 displayed increased potency compared to the parent isatin (11) (IC\(_{50} = 565 \ \mu\text{M}),\(^81\) and it had been demonstrated that \(N\text{-alkylation significantly enhanced the cytotoxicity of 11, Vine et al. synthesised a series of brominated N-substituted isatins (e.g. 20-25) (Figure 1.10) and evaluated their cytotoxicity against a panel of cancer cell lines in vitro.\(^64\) Structure-activity relationship (SAR) studies indicated that the introduction of an \(N\text{-benzyl group with electron withdrawing groups substituted at the meta or para position of the substituent phenyl ring showed increased cytotoxicity compared to ortho derivatives.}

![Figure 1.10](image-url) Early examples of cytotoxic \(N\text{-alkylisatins.} \)
Nine of the 24 compounds synthesised by Vine et al. displayed sub-micromolar IC\textsubscript{50} values and generally demonstrated greater selectivity toward leukemia and lymphoma cell lines over other cell lines tested (e.g. prostate, breast, colon, melanoma). The \textit{N-}(p\text{-methyl})benzyl compound 25 was the most active of the series, displaying an IC\textsubscript{50} value of 490 nM against U937 and human leukemic T cell (Jurkat) cell lines after 24 h.\textsuperscript{64} This effect was enhanced further by at least a factor of two when the incubation time was increased from 24 h to 72 h, making this class of compounds >10 times more active than the conventional chemotherapeutic agents 5-FU (3), vinblastine (7) and paclitaxel (8) against U937 cells.\textsuperscript{79}

Following on from this work, and in order to establish a more comprehensive SAR for the dibrominated \textit{N}-alkylisatins, a family of \textit{N}-phenethyl and \textit{N}-phenacyl isatin derivatives were synthesised (26-30 and 31-35)\textsuperscript{65,87} (Figure 1.11). All five \textit{N}-phenethyl derivatives (26-30) exhibited low to sub-micromolar cytotoxic activity against a panel of human leukemic, lymphoma and carcinoma cell lines, where introduction of a hydrophobic bromo substituent in the \textit{meta} (27) or \textit{para} (28) position yielded the most active compounds.\textsuperscript{65} Interestingly, the corresponding \textit{N}-phenacyl (31-35) derivatives were at least 3-5 times less active against the U937 cells and as a result were not tested against other cell lines.
Further examples of N-substituted isatins that display cytotoxic activity include the dibrominated N-1- and N-2-naphthylmethyl derivatives 36 and 37 (Figure 1.12). The N-naphthylmethyl isatins displayed nanomolar cytotoxicity against U937 and Jurkat cell lines, with IC$_{50}$ values for the N-1-naphthylmethyl derivative 36 of 190 and 910 nM, respectively, along with low micromolar cytotoxicity towards a panel of human carcinoma cell lines, including those derived from breast (MDA-MB-231 and MCF-7), colon (HCT-116) and prostate (PC-3) tissue.$^{65}$ Interestingly, compound 36 represents the most cytotoxic N-alkylisatin reported to date, when compared with other N-alkylisatins tested over 24 h. This suggests that increasing the hydrophobicity of the N-substituent through site-specific placement of an additional aromatic ring is important for enhancing cytotoxic activity.
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Figure 1.12 Cytotoxic 5,7-dibromo-N-naphthylmethylisatin derivatives 36 and 37.

In addition to their potent cell killing ability, N-alkylisatins have also been found to induce G2/M cell cycle arrest (see Section 1.2.1) and dramatically alter lymphocyte morphology\textsuperscript{64,65} (Figure 1.13B) in a similar manner to the tubulin destabiliser vinblastine (7) (Figure 1.13C).

Figure 1.13 Elongated cell morphology after treatment with isatin derivatives. A) DMSO vehicle control; B) N-2-Naphthylmethyl compound 37 at 0.39 µg/mL; C) Vinblastine (7) at 0.39 µg/mL. Magnification 1000 ×\textsuperscript{65}
Considering cells treated with tubulin-interfering chemotherapeutics such as vinblastine (7) and paclitaxel (8) display similar cell morphology to those treated with N-alkylisatins, an array of N-alkylbenzylisatins including the highly potent N-phenethyl (26, 28) and N-naphthylmethyl (36, 37) derivatives were screened for their ability to interfere with microtubule dynamics. A cell-free in vitro tubulin polymerisation assay was performed (Figure 1.14). Vinblastine (7) and paclitaxel (8) were used as a known microtubule destabiliser and stabiliser respectively. Consistent with literature reports,\textsuperscript{16,88} at 10 µM paclitaxel (8) stabilised microtubules while vinblastine (7) was a potent microtubule destabiliser (Figure 1.14). The test compounds, in particular the N-2-naphthylmethyl derivative 37, appeared to be potent microtubule destabilisers at 10 µM (Figure 1.14), as observed by the shift of the curve to the right of the control, indicating a decrease in the rate of tubulin polymerisation. This trend is consistent with reports that structurally similar indole\textsuperscript{84,89} and indolinone\textsuperscript{88,90} compounds inhibit tubulin polymerisation.

\textbf{Figure 1.14} Effects of N-alkylisatins 26, 28, 36 and 37 on the tubulin polymerisation assay.\textsuperscript{65}
The mode of action of cytotoxic isatin derivatives is strongly dependent on the nature of substitution within the molecule (Figure 1.15). While $N$-alkylation results in cytotoxic compounds with nanomolar activity inducing morphological change by interfering with tubulin polymerisation, isatin analogues derivatised at the C2 position exhibit CDK1, CDK2$^{91}$ and GSK3$^{92}$ kinase inhibition. Similarly, isatin derivatives functionalised at the C3 position display kinase inhibitory activity, including inhibition of VEGFR-2, PDGFR-β, fibroblast growth factor receptor and epidermal growth factor receptor.$^{89}$ Derivatisation around the aryl ring generally leads to isatin analogues which induce cancer cell death via apoptosis in the mid to low micromolar range and necrosis in the high micromolar range.$^{79}$

**Figure 1.15** Structure-activity summary of cytotoxic isatin derivatives.$^{79}$

Further sites to explore around the isatin nucleus include the region between N1 and C7, and this forms the basis of the chemistry component within this thesis.
To expand the cytotoxic SAR of the N-alkylated isatins, new derivatives were considered which would partially restrain conformational mobility in the important N-arylalkyl substituent group. To this end, target structures were envisaged which incorporated a new ring between N1 and C7 of the isatin nucleus. In the new target compounds, the original hydrophobic C7-substituent was to be subsumed by a methylene or methine in the new ring (Figure 1.16).

\[ R = \text{H, halogen} \]

**Figure 1.16** General structure of the proposed new target isatin compounds incorporating an additional ring between N1 and C7 of the isatin nucleus.

Restrained conformational flexibility is frequently used within medicinal chemistry in an attempt to increase biological activity. Recent examples of molecules which have benefited from the introduction of rigidity into the structure include the clinically used breast cancer treatment tamoxifen,\(^9\) the macrolide natural product family of epothilones\(^9\) and combretastatin A-4,\(^96\)-\(^98\) which is currently undergoing clinical trials as a cytotoxin and tubulin polymerisation inhibitor. These parent molecules all increased in cytotoxicity when an additional ring system was introduced as shown in Figure 1.17.
Figure 1.17 Structures of tamoxifen, epothilone D and combretastatin A-4 and their more cytotoxic partly rigidified analogues. A) Tamoxifen and its benzoxepin derivative against MCF-7 breast cancer cells; 
B) Epothilone D and its cyclopentene derivative against CCRF-REM leukemia cells; 
C) Combretastatin A-4 and its furazan derivative against SH-SY5Y neuroblastoma cells.

The results from the synthesis of the novel isatin molecules incorporating structural modifications in this area will be discussed in Chapters 2 and 3.
Investigations into the cytotoxic activity and the SAR of the novel isatin derivatives will be discussed in Chapter 6.

1.6 Choice of Linker: Acid-Labile Linkers

Selecting an appropriate linker for the conjugation of a cytotoxin to the carrier molecule is critical for successful drug delivery and release of the drug. Most receptor targeted drugs are delivered via RME, therefore conjugate linkers are required to be acid-labile to ensure hydrolysis in the reduced pH of endosomes and lysosomes (pH 4.5-6.0), while remaining stable at physiological pH (7.4).

The first reported acid-labile linker was the cis-aconityl linker between daunorubicin (5) and Affi-Gel 701 (aminoethyl polyacrylamide beads) and poly(D-lysine). Here, Shen and Ryser reported that the hydrolysis half-life of the cis-aconityl linker on the Affi-Gel 701 conjugate was less than 3 h at pH 4.0 and greater than 96 h at pH 6.0 and above. Over the years the cis-aconityl linker has been utilised to conjugate daunorubicin (5) to monoclonal antibodies (mAbs), and doxorubicin (4) to polymers (Figure 1.18A).

However, the most comprehensively studied acid-labile linker is the hydrazone linker. A hydrazone linker has been used to successfully conjugate anthracyclines such as doxorubicin (4) and daunorubicin (5) to mAbs via the exocyclic C13-keto position (Figure 1.18B). The doxorubicin conjugate shown in Figure 1.18B was found to exhibit stability at physiological temperature and pH, with less than 10% release of 4 after 5 h. At pH 4.5, however, the conjugate released greater than 50% of 4 after a few hours. The acid-labile hydrazone linkage has also been applied to other commercially available therapeutics such as 5-fluorouridine, chlorambucil, daunorubicin (5) and vinblastine (7).
Clinically, the hydrazone linker was used to create Mylotarg® (Wyeth/Pfizer), a conjugate containing the mAb gemtuzumab and the cytotoxic antibiotic ozogamicin (see Section 1.7.1). Mylotarg® was 7000-fold more potent than the analogous amide linker conjugate against the CD33 positive HL-60 cell line in vitro, and 100-fold more cytotoxic than ozogamicin alone.\(^{43}\)

\[\text{Figure 1.18} \text{ Examples of drug conjugates containing acid-labile linkers (highlighted in blue). Doxorubicin (4) conjugated to A) a polymer through a cis-aconityl linker; B) a monoclonal antibody through a hydrazone linker.}\]

Examples of other acid-labile linkers include acetals,\(^{105,106}\) oximes\(^{107}\) and imines.\(^{38,108}\) Imines are not widely used in drug delivery due to their perceived instability,\(^{109}\) however, in recent years stabilised imines have shown great potential
for further development.\textsuperscript{110} Chapter 4 of this thesis will focus on imines as acid-labile linkers for site-specific cancer therapy using conjugates (Figure 1.19).

![Figure 1.19 General schematic of the proposed drug conjugate utilising an imine linker.](image)

### 1.7 Choice of Targeting Ligand: Macromolecular Drug Delivery

Many drugs used in the clinic have serious drawbacks that could be greatly reduced through improved site-specificity and biodegradation protection, leading to decreased side effects. One strategy to overcome these problems is to use macromolecular drug delivery, where a macromolecule such as a protein or an antibody is attached to a drug through a bio-reversible linkage to provide selective drug delivery.\textsuperscript{111} The physiological absorption and distribution of a drug is dependent on the physicochemical properties of the macromolecule, not the drug itself. The advantages of using macromolecules in drug delivery are an improvement in the therapeutic index of a drug by reducing the interactions with non-target tissues and also decreasing premature drug metabolism and excretion.\textsuperscript{112} Other drug delivery systems incorporate the use of polymers,\textsuperscript{113} nanoparticles\textsuperscript{114,115} and liposomes,\textsuperscript{116} which have been widely reviewed.
1.7.1 Antibodies

Antibody-drug conjugates, in particular mAbs which alter cell signaling,\(^3\) are the most commonly used proteins in targeted therapies. They can be cytotoxic to cancer cells in their own right, or conjugated to drugs, bacterial or plant toxins, or radionuclides to improve their efficacy.\(^{117-119}\) By binding to antigens on the surface of cancer cells, the conjugate can be internalised through RME and then the therapeutic agent can be cleaved from the mAb.

More than 20 unconjugated mAbs have been approved for clinical use\(^{120}\) such as trastuzumab (Herceptin\(^{®}\), Genentech/Roche) and lapatinib (Tykerb\(^{®}\), GlaxoSmithKline) for the treatment of HER2-positive breast cancer.\(^{121}\) In contrast, only one mAb-drug conjugate has been granted approval by the FDA to date. Gemtuzumab-ozogamicin (Mylotarg\(^{®}\), Figure 1.20) was approved in 2000 for the treatment of acute myelogenous leukemia.\(^{19,118}\) This conjugate comprised a humanised anti-CD33 hP67.6 mAb attached to the cytotoxic antibiotic ozogamicin (\(N\)-acetyl-\(\gamma\)-calicheamicin) via a hydrazone bond.\(^3\) However, in June 2010, Pfizer voluntarily withdrew Mylotarg\(^{®}\) due to post-approval studies revealing the conjugate did not demonstrate increased survival rates.\(^{122}\)
Currently, there are six mAb-cytotoxin drug conjugates in Phase II or III clinical trials \(^{122}\) (Figure 1.21), with the trastuzumab-emtansine conjugate \(^{121}\) (entry 2, Figure 1.21) in the latest stage of clinical development. In July 2010, a biological license application for this conjugate was filed by Genentech to the FDA. Other preclinical research in progress involves conjugating mAbs with taxanes such as paclitaxel (8) and synthetic analogues of natural products.\(^{24}\)

![Figure 1.20](image.png)

**Figure 1.20** Structure of Mylotarg\(^{®}\). The antibiotic, ozogamicin, is conjugated to the monoclonal antibody, gemtuzumab.

**Figure 1.21** Monoclonal antibody-cytotoxin drug conjugates in Phase II or III clinical trials as of July 2010.\(^{122}\)
The only other immunoconjugates approved by the FDA were the radioimmunoconjugates Zevalin® (ibrutinomab tiuxetan) in 2002 and Bexxar® (tositumomab) in 2003, both for the treatment of B-cell lymphomas. These conjugates contain an anti-CD20 mAb attached to cytotoxic radioactive yttrium-90 and iodine-131, respectively.\(^3,118\)

However, mAbs are expensive, time-consuming to produce and suffer from issues of immunogenicity. Other problems arise with the use of mAb-drug conjugates, including poor penetration into tumours\(^19\) due to their relatively large molecular size. This can be resolved by utilising truncated mAb fragments which have been shown to penetrate tumours more readily than the full-length mAb, although this also has drawbacks as the fragment is cleared from the circulation more rapidly.\(^19\)

### 1.7.2 Transferrin

Non-mAb ligands such as transferrin (Tf) are an alternative option for targeted macromolecular drug delivery. Transferrins are monomeric glycoproteins weighing approximately 80 kDa and consisting of a single polypeptide chain with 670-700 amino acids.\(^123\) Transferrins transport iron to sites of absorption, storage and utilisation, and control levels of free iron in body fluids by binding, sequestering and transporting Fe(III) ions.\(^124\) The advantages of using Tf as a carrier molecule are that it is a commercially available protein and in the case of anti-cancer drug therapy, there is considerable uptake of Tf in tumour tissue due to elevated levels of Tf receptors (TfR) on the cell surface of tumour cells (150,000 – 1,000,000 per cell).\(^125\)

A variety of chemotherapeutics have been conjugated to Tf via cleavable linkers, allowing the release of the cytotoxin within the acidic intracellular tumour
cell environment after RME. The most widely reported examples involve conjugating Tf to doxorubicin (4) in an attempt to decrease the cardiotoxicity associated with the free drug and to combat resistance to this drug. Doxorubicin (4) has been linked to Tf through amide/hydrazone and imine linkers, which all exhibited an increase in efficacy in H-mesothelioma cells when compared to the free drug.

Tf has also been conjugated to the cytotoxin, chlorambucil (Leukeran®), GlaxoSmithKline) through a hydrazone bond (38) (Figure 1.22), which displayed IC₅₀ values 3 to 18-fold lower than those for chlorambucil alone against the MCF-7 (breast) and MOLT4 (leukemia) cell lines.

Recently, the anti-malarial compound artemisinin, derived from *Artemisia annua* (sweet wormwood), proved to also possess cytotoxic activity and was subsequently conjugated to Tf via a hydrazone bond (39) (Figure 1.22). The Tf conjugate contained ~16 molecules of artemisinin and displayed increased cytotoxicity against DU 145 prostate tumour cells compared to free artemisinin.

![Figure 1.22](image-url) Examples of drugs which have been conjugated to Tf.
1.7.3 The Urokinase Plasminogen System

Another alternative and novel strategy which shows promise for the site-specific delivery of cytotoxins involves a different non-antibody macromolecular system, the urokinase plasminogen activation (uPA) system. Tumour progression is strongly associated with pericellular proteolytic activity of the serine proteinase, plasmin.\textsuperscript{130} This is due to unregulated expression of the uPA system, comprising the 53 kDa serine protease uPA, its cell-membrane anchored receptor uPAR and its two endogenous inhibitors PAI-1 and PAI-2.\textsuperscript{131} PAI-1 and PAI-2 both belong to the serpin (serine proteinases inhibitor) family. PAI-1 is thought to be the primary inhibitor of uPA but both PAI-1 and PAI-2 have been shown to inhibit metastasis,\textsuperscript{131,132} although it has also been reported that elevated tumour levels of PAI-1 lead to tumour progression.\textsuperscript{133}

The uPA system catalytically converts inactive plasminogen in circulation to the active proteinase, plasmin by cleaving the arginine-valine bond.\textsuperscript{134} Plasmin at the cell surface catalyses the degradation of proteins in basement membranes and the ECM and therefore assists tumour cell passage to distant areas of the body\textsuperscript{132} (Figure 1.23). A direct correlation exists between the amount of uPA expressed on the surface of cancer cells and the potential for metastasis to occur.\textsuperscript{135-137} The majority of cells under normal physiological conditions do not express uPA activity,\textsuperscript{135} whereas over-expression of the uPA system is observed in numerous malignancies including breast, prostate, endometrial, gastrointestinal, pancreatic and colorectal cancers.\textsuperscript{138}
The uPA system is an appealing target for anti-cancer therapy.\textsuperscript{138} Cell invasion and metastasis could be stopped by inhibiting the catalytic activity of uPA, thus preventing uPA from binding to its receptor,\textsuperscript{131,140} by inhibiting plasmin\textsuperscript{138} or by exploiting the receptor to deliver cytotoxic agents into tumour cells.\textsuperscript{135,141,142} This latter strategy is achievable since PAI-1 and PAI-2 trigger a series of events which internalise the cytotoxic conjugate/inhibitor/uPA/uPAR complex. The complex encounters members of the low density lipoprotein receptor family of endocytosis receptors before it is internalised by RME through clathrin coated pits.\textsuperscript{143} The entire complex is then able to be degraded in the acidic environment of the late endosomes and lysosomes, effluxing the free drug into the cytosol to cause cell death, while the uPAR is recycled back to the cell surface\textsuperscript{143} (Figure 1.24).

While PAI-1 has been exploited to deliver cytotoxic agents to cells,\textsuperscript{135} the targeting ability of PAI-2 is yet to be fully explored. However, PAI-2 has numerous advantages over PAI-1 in terms of targeted cancer therapy. Firstly, it is a very stable protein \textit{in vitro} and \textit{in vivo} and does not revert to an inactive form compared to
PAI-1.\textsuperscript{144} As with PAI-1, PAI-2 is a protein of human origin and it does not elicit an immune response. Additionally, abnormally elevated levels of plasma PAI-2 in late pregnancy are not associated with toxicity, indicating that as a targeting agent, PAI-2 is unlikely to cause adverse effects.\textsuperscript{144} Other benefits related to its function include being limited to uPA inhibition without secondary effects e.g. cell signaling elicited by PAI-1.\textsuperscript{133}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{PAI2-Tox.png}
\caption{PAI-2 conjugated to a toxin targeting the uPA system. The conjugate is internalised by receptor-mediated endocytosis and then cleaved in the acidic environments of the late endosomes or lysosomes releasing the active drug (Tox) into the cytosol and causing cell death.}
\end{figure}

At the commencement of this research project, the only successful targeting of a toxin conjugated to PAI-2 had involved the use of the $\alpha$-emitting radiolabelled isotope ($^{213}$Bi).\textsuperscript{142,145,146} The conjugate selectively caused apoptosis in a prostate cancer cell line (PC3) and did not target or destroy normal tissue which does not express uPA.\textsuperscript{142} In vivo studies on the radioimmunoconjugate also demonstrated
promising activity against breast carcinomas.\textsuperscript{145,146} Investigations into conjugating other toxins, in particular synthetic cytotoxins, were required since problems arise due to the radioactivity, cost, availability and short half-lives of $\alpha$-emitting isotopes.\textsuperscript{144}

In early 2010, our research group reported the first example of a synthetic cytotoxin-PAI-2 conjugate.\textsuperscript{147} The cytotoxic metabolite of 5-FU (3), 2$''$-deoxy-5-fluorouridine, was conjugated to PAI-2 through an ester-labile succinate linker. \textit{In vitro} investigations revealed the PAI-2 conjugate preferentially killed tumour cell lines with elevated levels of uPA expression.\textsuperscript{147} This result confirms that the uPA system demonstrates potential in regard to selectively delivering anti-cancer agents to tumour cells.

The conjugation of a cytotoxic isatin to tumour-targeting proteins via an imine linker (Figure 1.25) will be discussed in Chapter 5.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig1_25.png}
\caption{General schematic of the proposed drug conjugate utilising PAI-2 or Tf as the tumour-targeting ligand.}
\end{figure}
1.8 Aims of Project

Effective targeted delivery of chemotherapeutic agents remains an unsolved problem. It may be possible to selectively deliver the cytotoxic isatin derivatives into tumour cells by conjugating them to a tumour-targeting protein such as Tf or PAI-2. Linking the isatin derivative to a protein with an acid labile group would facilitate hydrolysis of the drug after internalisation and exposure to the acidic environment of the endosomes and/or lysosomes (Figure 1.26). This targeted isatin-imine linker conjugate would limit undesirable systemic toxicity by providing specific delivery of the chemotherapeutic agent to the tumour.

Therefore, the specific aims of this research project were:

- **Drug**: To further explore the cytotoxic SAR of isatin derivatives by synthesising novel isatin scaffolds through the introduction of additional ring systems and measuring their cytotoxicity (Chapters 2, 3 and 6);

- **Linker**: To design and evaluate an appropriate acid-labile, imine linker system to attach cytotoxic isatins to a tumour-targeting protein (Chapter 4) and,

- **Targeting Ligand**: To conjugate the optimal cytotoxic isatin derivative to the tumour-targeting proteins Tf and PAI-2 via the most suitable imine linker and evaluate the conjugate efficacy *in vitro* (Chapters 5 and 6).
Figure 1.26 Proposed mechanism of pH-triggered, intracellular release of active cytotoxic drug inside the tumour cell.
CHAPTER 2

Synthesis of Pyrrolo[3,2,1-\(h\)]indole Derivatives

2.1 Initial Synthetic Targets

Isatin (11) has been derivatised at the C2, C3, C5, C6 and C7 positions, however, there are a few examples of isatins incorporating new ring systems between N1 and C7. As restrained conformational flexibility is frequently used in medicinal chemistry to increase biological activity (see Section 1.5.3), it was envisaged that a new ring system attached to N1-C7 in the isatin could either be 5- or 6-membered, yielding pyrrolo[3,2,1-\(h\)]indole-1,2-dione (6,5,5-fused tricyclic isatins, this chapter) and pyrrolo[3,2,1-\(ij\)]quinoline-1,2-dione derivatives (6,5,6-fused tricyclic isatins, Chapter 3) respectively (Figure 2.1). To facilitate comparison between previously reported \(N\)-alkylisatins and the new tricyclic isatins, \(N\)-alkyl substituents were also to be incorporated into the structure. Modifications to the aromatic ring on isatin would include substituting a halogen (preferably bromine) in the C7 position to retain its cytotoxic activity (Figure 2.2). \(^{81,83}\)

![Figure 2.1 General structures of the pyrrolo[3,2,1-\(h\)]indole-1,2-diones and pyrrolo[3,2,1-\(ij\)]quinoline-1,2-diones.](image-url)
As shown in Figure 2.2, the pyrrolo[3,2,1-\textit{hi}]indole-1,2-diones could be synthesised via two distinct routes. The construction of ring C is possible from the cyclisation of isatin (11) or one of its derivatives via free radical cyclisations or metal-catalysed cross-coupling reactions (route \( A \)) or alternatively, ring B may be prepared from the cyclisation of indoline (40) or one of its derivatives via a Sandmeyer or Stolle isatin synthesis (route \( A \)).

![Pyrolo[3,2,1-\textit{hi}]indole-1,2-diones](image)

\textbf{Figure 2.2} Summary of the proposed synthetic strategies in the formation of pyrrolo[3,2,1-\textit{hi}]indole-1,2-diones.

Herein, the attempted synthesis of novel pyrrolo[3,2,1-\textit{hi}]indole-1,2-diones will be discussed. The biological activity of these pyrrolo[3,2,1-\textit{hi}]indole-1,2-diones including their cytotoxicity, will be discussed in Chapter 6.

\subsection*{2.2 Pyrrolo[3,2,1-\textit{hi}]indole-1,2-dione Derivatives}

The pyrrolo[3,2,1-\textit{hi}]indole ring system was first reported in 1928\textsuperscript{148} but the scaffold was proven to be incorrect in 1939.\textsuperscript{149} The parent compound, pyrrolo[3,2,1-\textit{hi}]indole (41) was not synthesised until 1969,\textsuperscript{150} although the 1,2,4,5-tetramethylpyrrolo[3,2,1-\textit{hi}]indole (42) and 1,2,4,5-tetrahydropyrrolo[3,2,1-
hi]indole (43) derivatives (Figure 2.3) had been reported earlier in 1961.\textsuperscript{151} The first report of 1,2-dihydropyrrolo[3,2,1-hi]indole (44) (Figure 2.3) was described in 1958\textsuperscript{152} and nowadays, the pyrrolo[3,2,1-hi]indole core is present in over 2000 compounds\textsuperscript{153} including anti-convulsants\textsuperscript{154} and a cyclin-dependant kinase (CDK) inhibitor\textsuperscript{155} (45) (Figure 2.3).

While the pyrrolo[3,2,1-hi]indole scaffold is well known in the scientific literature, there are only three examples of pyrrolo[3,2,1-hi]indole-1,2-diones (6,5,5-fused tricyclic isatins) at the time of writing (46-48).\textsuperscript{156-158} This may be attributed to the fact that 6,5,5-fused indole ring systems are highly strained (see Section 2.2.4). However, this strain can be reduced slightly by cyclising an indoline instead\textsuperscript{157} and then dehydrogenating it to form a pyrrolo[3,2,1-hi]indole (Figure 2.4).
Figure 2.4 The three reported pyrrolo[3,2,1-hi]indole-1,2-diones (46-48). A) 8% yield over 2 steps; B) 48% yield over one step; C) 84% yield over one step.

2.2.1 Free Radical Cyclisations

Reactions involving free radicals are important to the pharmaceutical industry and have been heavily researched in recent years as they provide many avenues for the synthesis of heterocycles. Free radicals occur when weak chemical bonds are broken (homolysis) and the atoms receive one bonding electron each. Homolysis is promoted by an initiator in the first stage of the mechanism. Examples of initiators include diatomic halogen molecules, peroxides, and azo compounds such as 1,1′-azobis(cyclohexanecarbonitrile) and azobis(isobutyronitrile) (AIBN). Peroxides are highly reactive and are able to abstract an atom from many organic
molecules,\textsuperscript{161} whereas AIBN is more suitable for weaker bonds such as the Sn-H bond.

Intramolecular free radical reactions are often employed to synthesise 5-membered rings. It was envisaged that the desired tricyclic isatins could be prepared via a free radical cyclisation of $N$-allyl-7-bromoisatin (49) as shown in Scheme 2.1.

\begin{center}
\textbf{Scheme 2.1} Proposed mechanism for the radical cyclisation of $N$-allyl-7-bromoisatin (49) to yield the 5-membered tricyclic isatin 50.
\end{center}

During initiation, AIBN is able to abstract the hydrogen from tributyltin hydride (Bu$_3$SnH), leaving Bu$_3$Sn\textsuperscript{\scriptsize{\ast}} as a free radical (Scheme 2.1). This free radical subsequently undergoes propagation, whereby it can abstract the bromine atom from weak C-Br bonds and facilitate intramolecular ring closure, leading to the formation of a new radical centre which is able to abstract another hydrogen from Bu$_3$SnH,
thus regenerating the Bu$_3$Sn$^+$ free radical. Intramolecular radical reactions such as in Scheme 2.1 are favoured over intermolecular reactions since the double bond acts as a “radical trap” and is in close proximity to the radical.$^{161}$ This results in cyclisations occurring rapidly, even with unactivated double bonds.

2.2.1.1 Attempted ring closure via free radical cyclisation

![Scheme 2.2 Proposed steps in the synthesis of tricyclic isatin 50.](image)

The key precursor to the isatin 50, and to other isatins in this aspect of the project was 7-bromoisatin (55). Although this compound is available commercially, it is costly and only available in small quantities and hence, an efficient multi-gram in-house synthesis was required. 7-Bromoisatin (55) is readily produced from 2-bromoaniline (51) using the Sandmeyer method, the oldest and most frequently used procedure to synthesise isatins.$^{162-164}$ The reaction can be applied to a wide array of substrates, including anilines with electron withdrawing substituents,$^{165}$ heterocyclic amines$^{166}$ and N-alkylanilines.$^{167}$ The Sandmeyer method involves reaction of the appropriate aniline with chloral hydrate and hydroxylamine hydrochloride (or other hydroxylamine salt) in the presence of sodium sulfate to yield the isonitrosoacetanilide intermediate (Scheme 2.3). Exposure of the intermediate to conc. H$_2$SO$_4$ for a short period of time and high temperatures yields the heterocyclic isatin (Scheme 2.3). The synthesis of 7-bromoisatin (55) was
straightforward using this method and its structure was confirmed by $^1$H nuclear magnetic resonance (NMR) spectroscopy, which revealed three aromatic protons at 7.02, 7.51 and 7.78 ppm, together with a NH signal at 11.31 ppm, which corresponded to literature values.$^{168}$ Mass spectrometry (MS) supported the molecular weight of 55 with molecular ion peaks at $m/z$ 225 and 227 ([M]$^+$ and [M + 2]$^+$), corresponding to the $^{79}$Br and $^{81}$Br isotopes within the molecule.

![Diagram](diagram.png)

**Reagents and conditions:** (a) chloral hydrate, Na$_2$SO$_4$, hydroxylamine.HCl, H$_2$O, HCl, 85 ºC, 3 h, 53 = 86%, 54 = 83%; (b) conc. H$_2$SO$_4$, 80 ºC, 15 min, 55 = 47%, 56 = 58%.

**Scheme 2.3** Synthesis of 7-haloisatins via the Sandmeyer method.

The 7-bromoisatin (55) (and 7-iodoisatin (56) which will be discussed later in Section 2.2.2.1.1) was subjected to $N$-alkylation to yield the allyl isatin derivative 49, for subsequent free radical cyclisations to the desired tricyclic isatins. Previously, $N$-alkylated isatins have been successfully produced using an alkyl halide under basic conditions such as sodium hydride, potassium carbonate or cesium carbonate.$^{64,65,74}$ In this case, 55 was treated with sodium hydride to yield the intensely purple coloured anion intermediate 57 (Scheme 2.4) by removal of the acidic proton on the nitrogen. The intermediate 57 is an ambident anion whereby it is possible for $N$- and $O$-alkylation to occur,$^{169}$ although $N$-alkylation is preferred. After the formation of the anion 57, allyl bromide was added and the reaction mixture was heated overnight (Scheme 2.4). A catalytic amount of potassium iodide was added to
facilitate alkylation through in situ formation of allyl iodide, which leads to a more reactive intermediate due to the superior iodine leaving group in the nucleophilic substitution. N-Alkylation of the product is likely to occur via an SN2 reaction mechanism whereby the nucleophilic nitrogen anion attacks the carbon attached to the halide, while the halide ion is displaced (Scheme 2.4).

![Scheme 2.4](image)

**Scheme 2.4** Synthesis of N-allyl-7-bromoisatin (49) via N-alkylation of 7-bromoisatin (55) with allyl bromide.

The allylated isatin 49 was successfully synthesised in high yield and readily characterised using conventional spectroscopic techniques. The 1H NMR spectrum indicated the presence of alkenyl protons at 5.23, 5.26 and 5.99 ppm, which confirm the presence of this functional group. There was the absence of a NH signal in 1H NMR spectrum, however, this would also occur in the case of O-alkylation. Through 13C NMR spectroscopy, signals observed at 158.7 and 182.6 ppm were consistent with the amide carbonyl group at C2 and the ketone at C3 respectively. As such, there was no indication of the O-alkylated product from the gHMBC or 13C NMR spectra. If O-alkylation had occured, an alkoxyimine group would have been present. Compound 49 was then carried through for the ring closing free radical cyclisations.
Although smaller rings should form faster than larger rings in intramolecular free radical cyclisations, Rashatasakhon et al. have described the preferential 6-endo-trig cyclisation (60) of N-allyl-7-bromo-3a-methylhexahydroindolinone (58) compared to the corresponding 5-exo-trig product (59) (Scheme 2.5). According to Baldwin’s rules (see Section 3.2.2), the formation of the 5-exo-trig (59) and 6-endo-trig (60) products are both favourable, however, 5-exo-trig and 6-exo-trig are the most typical cyclisation routes for intramolecular radical cyclisations. With the bromide 58, the 6-endo-trig radical intermediate was thermodynamically more stable than its 5-exo-trig counterpart and hence, it was formed as the major isomer (Scheme 2.5). The yield of the 5-exo-trig product (59) was able to be increased by using a more concentrated solution of Bu\textsubscript{3}SnH (0.1 M cf. 0.01 M).

\[
\text{Reagents and conditions: (a) AIBN, Bu}_3\text{SnH, benzene, reflux, 12 h, 59 = 20\% (optimal conditions), 60 = 89\%.}
\]

\textbf{Scheme 2.5} Reported free radical cyclisation of N-allyl-7-bromo-3a-methylhexahydroindolinone (58), yielding the 5-exo-trig (59) and 6-endo-trig (60) products as minor and major products respectively.

With N-allyl-7-bromoisatin (49), radical cyclisation could lead to the formation of both the 5-exo-trig (50) and 6-endo-trig (61) products (Scheme 2.6). The reaction was attempted several times with varying conditions. Fresh Bu\textsubscript{3}SnH was employed and added dropwise over a long period of time and the equivalents were varied (1.5-4 equiv.), as were the equivalents of the initiator AIBN (0.1-1 equiv.), time
(3-14 h) and solvents (toluene and benzene). In each case, analysis by MS revealed either a complex mixture of products or only a trace of product at \( m/z \) 187. This peak may be attributed to the cyclised products 50 or 61, or \( N \)-allylisatin, which may be formed as a result of the bromine abstraction during the propagation step of the free radical cyclisation (Scheme 2.1). The lack of product/s may be explained by the fact the tricyclic isatins 50 and 61 contain a higher degree of ring strain than the indolinones in Scheme 2.5. Hence, another synthetic strategy had to be employed to produce the desired tricyclic isatins.

![Scheme 2.6: Schematic representation of the attempted radical cyclisations to yield 5- and 6-membered tricyclic isatins.](image)

**Reagents and conditions:** (a) AIBN, Bu₃SnH, benzene, reflux, 14 h.

**Scheme 2.6** Schematic representation of the attempted radical cyclisations to yield 5- and 6-membered tricyclic isatins.

### 2.2.2 Metal-Catalysed Cross-Coupling Reactions

Metal-catalysed cross-coupling reactions have transformed the synthesis of carbon-carbon bonds over the last 30 years and the number of publications regarding these couplings has grown exponentially over this time. Transition metal catalysts allow synthetic entry into compounds which have proven difficult to prepare previously, through the catalyst’s high selectivity and efficiency. Pd catalysts are the most frequently used metal catalysts in synthesis today and form the basis of the Sonogashira, Suzuki, Negishi and Heck cross-coupling reactions (named after Akira Suzuki, Ei-ichi Negishi and Richard F. Heck who were jointly awarded the...
2010 Nobel Prize in Chemistry for the discovery and development of these reactions). Metal-catalysed reactions are appealing in the synthesis of heterocycles due to their ability to yield complicated structures under mild conditions using readily available starting materials.\footnote{177}

The indole scaffold is an abundant feature in many heterocycles and thus is one of the major areas of focus for synthetic organic chemists. For over 100 years, a variety of methods for the synthesis of indoles have been developed. The most well-known method for the construction of indoles is the Fischer indole synthesis which involves heating an aldehyde/ketone and an arylhydrazine in the presence of an acid or acid catalyst.\footnote{178} Other alternatives for indole synthesis include reductive cyclisations,\footnote{179} heteroannulations involving internal alkynes such as the Larock heteroannulation\footnote{180} and the cyclisation of 2-alkynylanilines.\footnote{181}

Within this project, it was decided that the tricyclic isatin scaffold may be successfully synthesised by adapting the cyclisation method for 2-alkynylanilines. If isatins can be considered as masked anilines containing a secondary instead of a primary amine, then cyclisation upon this amine may be viable, leading to the construction of a third ring, i.e. a tricyclic isatin (Scheme 2.7).

\begin{center}
\includegraphics[width=0.8\textwidth]{Scheme_2.7.png}
\end{center}

\textbf{Scheme 2.7} Cyclisation of 2-alkynylanilines to corresponding tricyclic isatins.
2-Alkynylanilines are readily prepared by the Sonogashira reaction and the subsequent cyclisation to an indole can be realised through several avenues. The 2-alkynylanilines may be reacted with a Cu(I) acetylide via the Castro indole synthesis, or be exposed to a base such as sodium ethoxide, or tetrabutylammonium halide salts to yield the corresponding 2-substituted indole. Pd-catalysed cyclisations of 2-alkynylanilines are now routinely reported in the literature and are frequently used in the preparation of indoles. Other less commonly used metal catalysts such as Rh, Zn, Pt, Ir, Hg and In have been reported in recent years as being capable of cyclising 2-alkynylanilines.

2.2.2.1 Sonogashira Reactions

The Sonogashira reaction is the cross-coupling of a vinyl or aryl halide (I, Br or OTf) and a terminal alkyne. It evolved from the Stephens-Castro reaction which involves the cross-coupling of a vinyl or aryl halide with a Cu(I) acetylide. Typically, a Pd(0) catalyst such as Pd/C or Pd(PPh₃)₄ is used in the Sonogashira reaction, however, Pd(II) catalysts such as PdCl₂(PPh₃)₂ or Pd(OAc)₂ can be used since the catalyst may be reduced to Pd(0) through the consumption of the terminal alkyne within the reaction. A Cu(I) halide salt is also required within the Sonogashira reaction, together with an amine such as triethylamine (Et₃N) or diethylamine which serves as a base and may be used as a solvent or co-solvent. A phosphine is also usually included as a ligand for Pd. There are reports of the Sonogashira reaction being performed under copper-, phosphine- and amine-free conditions, under aerobic conditions and in a microwave reactor.
The mechanism for the Sonogashira reaction initially involves the Pd catalyst reacting with a vinyl or aryl halide through an oxidative addition. The complex then reacts with the Cu acetylide produced from the Cu cycle, followed by the conversion from a trans to a cis configuration, before the final Sonogashira product is released through reductive elimination and regeneration of Pd(0) (Figure 2.5).

Figure 2.5 General Sonogashira reaction mechanism.\textsuperscript{194}

2.2.2.1.1 Applying Sonogashira Reactions

In preparation for a Sonogashira reaction, 7-iodoisatin (56) was synthesised in a moderate yield using the Sandmeyer method as described previously for
7-bromoisatin (55) (see Section 2.2.1.1, Scheme 2.3). The structure of the dark red/brown product 56 was confirmed through \textsuperscript{1}H NMR spectroscopy, which indicated an aromatic proton displaying a triplet signal at 6.89 ppm and was ascribed to H5. This proton was directly coupled to doublet signals at 7.49 and 7.94 ppm, representative of the H4 and H6 protons. A NH signal, integrating for one proton was observed at 11.00 ppm. The spectral data coincided with that previously reported in the literature.\textsuperscript{204} The 7-iodoisatin (56) was then subjected to an one-pot Sonogashira/indole cyclisation method based on the conditions of Layek \textit{et al.}\textsuperscript{205} This paper reported that a 5-membered ring could be introduced onto 8-iodo-4-oxo-1,4-dihydroquinoline-3-carboxylic acid ethyl ester (62) to form 2-substituted 6-oxopyrrolo[3,2,1-ij]quinolines (63) (Scheme 2.8). (More information on pyrrolo[3,2,1-ij]quinolines will be discussed in Chapter 3).
Scheme 2.8 Reported one-pot synthesis of 2-substituted 6-oxopyrrolo[3,2,1-ij]quinolines (63).

\[ \text{Reagents and conditions: (a) 10\% Pd/C, PPh}_3, \text{CuI, Et}_3\text{N, EtOH, 80 °C, 2-8 h 45-95\%}. \]
time periods led to a detrimental effect on the yield of 65 (Table 2.1, entry 3 cf. entry 2). The incorporation of Pd(PPh$_3$)$_4$ as a catalyst increased the yield of 65 compared to Pd/C when the reaction was performed in EtOH (Table 2.1, entry 4). The yield of 65 could be further increased by using Pd(PPh$_3$)$_4$ in presence of CH$_3$CN (Table 2.1, entry 6). Using PdCl$_2$(PPh$_3$)$_2$ in CH$_3$CN led to a slight decrease in the yield of 65 (Table 2.1 entry 5 cf. entry 6). The use of Et$_3$N as the solvent also did not increase the yield of 65 (Table 2.1, entry 7). The optimal yield of 65 (69%) was produced utilising Pd(PPh$_3$)$_4$ together with N,N-dimethylformamide (DMF) as the solvent (Table 2.1, entry 8). In retrospect it can be concluded that the descending order of Pd catalyst efficiency in the synthesis of the 7-alkynylisatin 65 is Pd(PPh$_3$)$_4$ > PdCl$_2$(PPh$_3$)$_2$ > Pd/C, while the descending order of solvent efficiency is DMF > CH$_3$CN > Et$_3$N > EtOH.
Table 2.1 Reaction conditions attempted in the synthesis of the alkynylisatin 65.

<table>
<thead>
<tr>
<th>Entry</th>
<th>Catalytic System</th>
<th>Base</th>
<th>Solvent</th>
<th>Temp (°C)</th>
<th>Time (h)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Pd/C (1%), PPh₃ (4%), Cul (10%)</td>
<td>Et₃N</td>
<td>EtOH</td>
<td>80</td>
<td>4</td>
<td>6</td>
</tr>
<tr>
<td>2</td>
<td>Pd/C (2%), PPh₃ (8%), Cul (20%)</td>
<td>Et₃N</td>
<td>EtOH</td>
<td>80</td>
<td>7.5</td>
<td>36</td>
</tr>
<tr>
<td>3</td>
<td>Pd/C (4%), PPh₃ (16%), Cul (24%)</td>
<td>Et₃N</td>
<td>EtOH</td>
<td>80</td>
<td>10</td>
<td>26</td>
</tr>
<tr>
<td>4</td>
<td>Pd(PPh₃)₄ (2%), Cul (14%)</td>
<td>Et₃N</td>
<td>EtOH</td>
<td>80</td>
<td>4</td>
<td>40</td>
</tr>
<tr>
<td>5</td>
<td>PdCl₂(PPh₃)₂ (2%), Cul (5.5%)</td>
<td>Et₃N</td>
<td>CH₃CN</td>
<td>80</td>
<td>4</td>
<td>46</td>
</tr>
<tr>
<td>6</td>
<td>Pd(PPh₃)₄ (2%), Cul (14%)</td>
<td>Et₃N</td>
<td>CH₃CN</td>
<td>80</td>
<td>4</td>
<td>48</td>
</tr>
<tr>
<td>7</td>
<td>Pd(PPh₃)₄ (2%), Cul (14%)</td>
<td>Et₃N</td>
<td>Et₃N</td>
<td>80</td>
<td>4</td>
<td>45</td>
</tr>
<tr>
<td>8</td>
<td>Pd(PPh₃)₄ (2%), Cul (14%)</td>
<td>Et₃N</td>
<td>DMF</td>
<td>80</td>
<td>4</td>
<td>69</td>
</tr>
</tbody>
</table>

*No evidence of the tricyclic product 66 was obtained in any reaction.

As mentioned earlier, the structure of 65 was confirmed through ¹H NMR spectroscopy, which revealed a broad singlet peak at 8.44 ppm, indicating the presence of NH. The ¹³C NMR spectrum showed two signals at 81.4 and 96.9 ppm, which were ascribed to the two C≡C signals in 65 and additionally, the high resolution electron ionisation mass spectrum (HREI-MS) revealed a molecular ion at m/z 247.0634, consistent with the molecular formula (C₁₆H₁₉NO₂[M⁺]). Hence, it was confirmed that 7-iodoisatin (56) had not cyclised to form 66 but alternatively, the novel alkynylisatin 65 had been prepared.

With 65 in hand, metal-catalysed cyclisations were attempted to effect cyclisation to the tricyclic isatin 66 (Scheme 2.9). Following the work of Castro et al.,¹⁸²,¹⁸³ Ezquerra et al. confirmed Cu(I)-mediated intramolecular cyclisations of
2-alkynylanilines were possible in the absence of a Pd catalyst.\textsuperscript{198} The group described how 2 equiv. of CuI in DMF at 100 °C for 3 h promoted the heteroannulation of the 2-alkynylaniline. However, within the current work, the alkynylisatin 65 was unable to be cyclised under these conditions as observed by \textsuperscript{1}H NMR spectroscopy, which revealed the presence of an NH signal at 8.44 ppm. According to Ezquerra et al., longer reaction times are ineffective at improving the yield and instead lead to the formation of side products.\textsuperscript{198} Other metal catalysts such as AgNO\textsubscript{3}, Au(PPh\textsubscript{3})Cl and a PdCl\textsubscript{2}(PPh\textsubscript{3})\textsubscript{2}/CuI system were also investigated since they have been reported as excellent catalysts in the cyclisation of alkynylpyrrolidinones and alkynylpiperidinones.\textsuperscript{206} These reactions also did not yield the desired isatin 66 and this was confirmed by the presence of the NH signal at 8.44 ppm in the \textsuperscript{1}H NMR spectrum, indicating the intermediate alkynylisatin 65 had not reacted. Additionally, MS analysis revealed only the starting material was present.
Scheme 2.9 Attempted metal-catalysed cyclisations of the alkynylisatins 65 and 67.

After several attempts to cyclise alkynylisatin 65, it was decided that the C3 carbonyl on the compound should be protected to avoid any competing reactions and to reduce detrimental ring strain effects on cyclisation. The C3 carbonyl group in isatins is quite reactive and has the opportunity to interfere and stop reactions from proceeding.\textsuperscript{81,207,208} For example, in Pd-catalysed reactions containing PPh\textsubscript{3}, the C3 carbonyl on the isatin may undergo Wittig reactions and/or form a dimerised product.\textsuperscript{209} To obviate this, the C3 carbonyl on 65 can be protected as an ethylene ketal (67) prior to cyclisation. Once the cyclised isatin 68 is obtained, the ethylene ketal should hydrolyse under acidic conditions\textsuperscript{210} to yield the tricyclic isatin 66.

Synthesis of ketals is usually accomplished through reacting an alcohol and a ketone in the presence of an acid catalyst under dehydrating conditions. In the synthesis of the protected alkynylisatin 67, Dean-Stark conditions were used to
remove water formed from the reaction of ethylene glycol and 65 (Scheme 2.9). The reaction led to the protected alkynylisatin 67 in 43% yield. Its structure was confirmed through $^1$H NMR spectroscopy, whereby two triplet signals at 4.31 and 4.53 ppm, integrating for two protons each, were ascribed to the four protons on the ketal protecting group. Furthermore, the $^{13}$C NMR spectrum illustrated only one carbonyl signal, at 174.5 ppm, which was attributed to the C2′ carbonyl. The HREI-MS also revealed a molecular ion at $m/z$ 291.0893, consistent with the molecular formula (C$_{18}$H$_{13}$NO$_3$ [M$^+$]).

Once the protected alkynylisatin 67 was formed, it was exposed to Cu(I) cyclisation conditions$^{198}$ with subsequent heating at 100 ºC for 24 h, but this only gave unreacted 67 as determined by $^1$H NMR spectroscopy. Another attempt to cyclise 67 was made using PdCl$_2$ in CH$_3$CN at 70-75 ºC for 11.5 h. This Pd catalyst is known to cyclise 2-alkynylanilines into 2-substituted indoles$^{211}$ and was successful in cyclising 8-arylethynyl-1,2,3,4-tetrahydroquinolines (69) into the corresponding 2-aryl-5,6-dihydro-4$H$-pyrrolo[3,2-1-ij]quinolines$^{212}$ (70) (Scheme 2.10). However, in our case, the cyclisation of the protected alkynylisatin 67 proved to be unsuccessful as determined by $^1$H NMR spectroscopy, which revealed unreacted starting material.
Reagents and conditions: (a) PdCl$_2$, CH$_3$CN, reflux, 16 h, 70-87%.\textsuperscript{212}

Scheme 2.10 Reported synthesis of 2-aryl-5,6-dihydro-4H-pyrrolo[3,2,1-ij]quinolines (70).\textsuperscript{212}

2.2.2.1.2 A Model Sonogashira Reaction

Since there had been great difficulty in synthesising the 6,5,5-fused tricyclic isatins, it was decided that a model reaction should be performed to prove if Pd-catalysed cyclisation is possible. It was postulated that the difficulty in cyclising the 7-alkynylisatin 65 arises from the fact it contains a secondary amide with an acidic proton and a 6,5-bicyclic scaffold which would introduce further ring strain into the molecule if it were cyclised. In Schemes 2.8 and 2.10, the structures 62 and 69 respectively, were successfully cyclised by Pd but both these compounds contained a 6,6-bicyclic system, which contains a decreased amount of ring strain compared to a 6,5-bicyclic system.\textsuperscript{205,212} To test the hypothesis that indoles may be cyclised under Pd-catalysed conditions, the Sonogashira product 71 was prepared from 2-iodoaniline (52) (Scheme 2.11). The 2-alkynylaniline 71 was synthesised in good yield (72%) using the same method as in the synthesis of the 7-alkynylisatin 65. The $^1$H NMR spectral data for this compound was consistent with those reported in the literature,\textsuperscript{213} and the MS revealed a molecular ion at $m/z$ 193, confirming the molecular weight. The 2-alkynylaniline 71 was then exposed to PdCl$_2$ to yield
2-phenylindole (72) in 74% yield (Scheme 2.11). The structure of this compound was confirmed through $^1$H NMR spectroscopy, which revealed a signal at 8.37 ppm integrating for one proton. This was ascribed to the NH, a secondary amine, as opposed to the primary amine present in the 2-alkynylaniline 71. The remainder of the $^1$H NMR spectral data was also in agreement with those published previously in the literature.

![Scheme 2.11 Synthesis of 2-phenylindole (72).](image)

**Reagents and conditions:** (a) Pd(PPh$_3$)$_4$, CuI, Et$_3$N, DMF, RT, 1 h; (b) phenylacetylene (64), 80 ºC, 4 h, 72% (over 2 steps); (c) PdCl$_2$, CH$_3$CN, 75 ºC, 8 h, 74%.

The cyclisation conditions described above were used successfully by Ezquerra *et al.* in the synthesis of 2-aryl-5,6-dihydro-4H-pyrrolo[3,2,1-ij]quinolines, however, when applied in the current work, the reaction conditions were unsuccessful in cyclising the protected 7-alkynylisatin 67 (Table 2.1). This suggests that Pd-catalysed cyclisations are a viable method in the synthesis of indoles and the compound may contain a secondary amine, as is the case for 2-aryl-5,6-dihydro-4H-pyrrolo[3,2,1-ij]quinolines. The lack of success in cyclising isatins in this project, may be attributed to the ring strain caused by a 6,5,5-fused ring system (see Section 2.2.4). It is possible that the orbitals on the alkynyl bond in the 8-arylethynyl-1,2,3,4-tetrahydroquinolines (69) are within reach of the nitrogen when exposed to cyclisation conditions, but this is not the case with the alkynyl orbitals present in the 7-alkynylisatin 65. The lack of success in forming the desired tricyclic isatins led to
further studies of some other possible metal-catalysed reactions as outlined in the following section.

2.2.2.2 Miscellaneous Metal-Catalysed Reactions

In an attempt to synthesise the dehydrogenated isatin \( \text{74} \), it was envisaged that a \( N \)-vinylisatin derivative such as 7-bromo-\( N \)-vinylisatin (\( \text{73} \)) could undergo an intramolecular Heck reaction (see Section 3.2.2) to form the isatin \( \text{74} \) (Scheme 2.12).

![Scheme 2.12 Proposed synthesis of the tricyclic isatin \( \text{74} \) via an intramolecular Heck reaction of 7-bromo-\( N \)-vinylisatin (\( \text{73} \)).](image)

Previously, it had been shown that a 7-bromo-\( N \)-vinylated indole derivative could cyclise into the corresponding pyrrolo[3,2,1-\( h \)i]indole.\(^{215}\) In the current project, a model vinylation reaction on isatin (\( \text{11} \)) to yield \( N \)-vinylisatin (\( \text{75} \)) was attempted since \( \text{75} \) is not widely reported in literature. \( N \)-Vinylisatin (\( \text{75} \)) cannot be formed by a conventional \( S_N2 \) nucleophilic substitution reaction using a vinyl halide due to the attachment of the halide to an \( \text{sp}^2 \) carbon. However, \( \text{75} \) can be prepared using vinyl acetate and \( \text{NaPdCl}_4 \) as a catalyst under refluxing conditions\(^{216}\) (Scheme 2.13). \( N \)-Vinylisatin (\( \text{75} \)) was synthesised in 42% yield and the structure was confirmed through \(^1\)H NMR spectroscopy, which indicated the vinyl protons were present at 5.17, 5.87 and 6.67 ppm. The vinylation of \( \text{55} \) proved to be unsuccessful and only starting material was present as determined by MS.
Reagents and conditions: (a) vinyl acetate, Na$_2$PdCl$_4$, reflux, 12 h, 75 = 42%, 73 = 0%; (b) 1,2-dibromoethane, Et$_3$N, CH$_3$CN, 70 °C, 6 h, 73 = 0%.

Scheme 2.13 Synthesis of N-vinylisatin derivatives.

The synthesis of 7-bromo-N-vinylisatin (73) was then attempted based on the method by Iddon et al. which utilised a pyrazole derivative with 1,2-dibromoethane to yield the corresponding N-vinylpyrazole.$^{217}$ Analysis by $^1$H NMR spectroscopy revealed a complex mixture of products had formed and no 7-bromo-N-vinylisatin (73) was present. Difficulties in synthesising 73 may arise due to steric hindrance between the bromine atom and the vinyl group.

2.2.3 Other Cyclisation Reactions

The synthesis of 6,5,5-fused tricyclic isatins was also attempted according to the method of Albrecht et al. which involved an iodonium ion intermediate.$^{218}$ Firstly, N-allylisatin (76) was synthesised from isatin (11) and allyl bromide in 69% yield (Scheme 2.14). The structure of this compound was confirmed through $^1$H NMR spectroscopy, which revealed the three allyl protons at 4.37, 5.29 and 5.85 ppm, matching reported data.$^{219}$ Following the method of Albrecht et al., 76 was then reacted with I$_2$, although the procedure proved to be unsuccessful. Analysis by MS and TLC revealed only starting material was present.
Reagents and conditions: (a) NaH, DMF, RT, 20 min; (b) KI, allyl bromide, 65 °C, 18 h, 69% (over 2 steps); (c) I₂, K₂CO₃, CH₃CN, RT, 20 h.

Scheme 2.14 Attempted synthesis of 77 from an iodocyclisation of N-allylisatin (76).

Another strategy to form the 6,5,5-fused tricyclic isatins utilised \(N\)-formylindoline (78) as the starting material (Scheme 2.15). The complex mechanism involves a carbene intermediate, followed by dimerisation before the corresponding isatin is formed. \(N\)-Formylindoline (78) was successfully prepared using the method of Berry et al.\(^{221}\) starting from indoline (40) (Scheme 2.15). The \(N\)-formylindoline (78) was synthesised as a mixture of rotamers (83:17) in a similar ratio to that previously reported,\(^{222}\) although in low yield, which suggests this synthetic method is not ideal for this particular substrate. Its purity and structure were confirmed by \(^1\)H NMR spectroscopy, which coincided with reported data.\(^{223}\) Once 78 had been prepared, it was reacted with oxalyl chloride, \(N,N\)-diisopropylethylamine (DIPEA) and Br₂ (Scheme 2.15) in an attempt to synthesise 46. This reaction had been reported to result in a 79% yield\(^{220}\) although in this case, MS revealed a complex mixture of uncharacterisable products, none of which were identified as the tricyclic isatin 46. The assortment of products observed in the MS may be due to the involvement of the carbene intermediate which is unstable and could yield many products.
Chapter 2 – Pyrroloindole Derivatives

Reagents and conditions: (a) imidazole, TMSCl, DMF, RT, 12 h, 10%; (b) (COCl)_2, DIPEA, Br_2, 35 °C, 1 h.

Scheme 2.15 Attempted synthesis of tricyclic isatin 46 from N-formylindoline (78).

2.2.4 The Stolle Isatin Synthesis

Reagents and conditions: (a) (COCl)_2, reflux, 3 h; (b) AlCl_3, reflux, 3 h.

Scheme 2.16 Overall proposed mechanism in the synthesis of tricyclic isatin 46.

The Stolle isatin synthesis is also a widely used route to produce isatin and its derivatives.\(^{163}\) It involves reacting the appropriate aniline (e.g. 79) with oxalyl chloride to form a chlorooxalylanilide intermediate such as 80, which cyclises to yield the corresponding isatin (11) upon treatment with a strong Lewis acid such as AlCl_3 or boron trifluoride diethyl etherate\(^ {163}\) (Scheme 2.17).

Reagents and conditions: (a) (COCl)_2, reflux, 3 h; (b) AlCl_3, reflux, 3 h.

Scheme 2.17 General scheme for the preparation of isatins via the Stolle procedure.
The Stolle method has been employed in the synthesis of a variety of isatins including N-aryl and polycyclic isatins.\textsuperscript{224} In 1979, Welstead Jr. \textit{et al.} reported the first synthesis of the tricyclic isatin 46 from indoline (40) in 8\% overall yield using the Stolle procedure.\textsuperscript{156} The yield was reportedly increased to 22\% in 1996 by Norman \textit{et al.} by increasing the molar equivalents of oxalyl chloride from 0.5 to 2.0 and using dichloromethane (DCM), rather than ether as the reaction solvent.\textsuperscript{225} However, on closer inspection Norman \textit{et al.}'s yield of 22\% was only for the second synthetic step, from the intermediate 81 to the final product 46. If the yield was calculated from indoline (40), it would have been 15\%. On the contrary, Welstead Jr. \textit{et al.} reported the yield of the intermediate 81 to be 58\% and did not calculate a yield for the second step to the product 46. When the yield of the second synthetic step in Welstead Jr. \textit{et al.}'s synthesis was calculated manually from the manuscript, it was found to be 31\% and the overall yield for 46 from 40 should be 17\%.

Both procedures by Welstead Jr. \textit{et al.} and Norman \textit{et al.} were adapted and repeated within the current work (Scheme 2.18). In the first instance, indoline (40) was heated at reflux with oxalyl chloride in anh. THF for 3.5 h before the solvent was removed by rotary evaporation to yield a sticky red residue. The residue was then dissolved in refluxing anh. DCM and AlCl\textsubscript{3} was added portionwise over 10 min before the reaction mixture was heated at reflux for another 1 h (Table 2.2, entry 1). Analysis by MS revealed a complex mixture of products, with none of them being the desired isatin 46.

The above method was modified by increasing the molar equivalents of AlCl\textsubscript{3} (Table 2.2, entry 2 and 4) and changing to solvents with higher boiling points (CHCl\textsubscript{3} and 1,2-dichloroethane) (Table 2.2, entry 2 and 4) but again, analysis by MS revealed none of the desired tricyclic isatin 46. A small amount of K\textsubscript{2}CO\textsubscript{3} was also
added to oxalyl chloride to remove excess HCl and try to facilitate the cyclisation of indoline (40) (Table 2.2, entry 3). In this case, there was no evidence of the intermediate 81 being formed as determined by MS so the experiment did not proceed.

Many of the previous attempts had not been successful, however, the synthetic method based on the work by Norman et al. finally provided the desired tricyclic isatin 46, albeit in only 6% overall yield (Table 2.2, entry 5). The intermediate 81 was able to be produced without difficulty by using DCM as the reaction solvent and lower temperatures than previous attempts. It was then treated with 5 equiv. of AlCl$_3$ neatly for 20 min at 110 °C until HCl gas ceased evolving, followed by the addition of ice-H$_2$O and further stirring for 2 h at RT. The structure of 46 was confirmed through $^1$H NMR spectroscopy, whereby two methylene signals were present at 3.38 and 4.09 ppm and ascribed to H5 and H4 respectively. The $^1$H NMR spectral data was in accordance with those reported by Norman et al.$^{225}$

**Reagents and conditions:** (a) (COCl)$_2$, DCM, RT, 3 h, 40%; (b) AlCl$_3$, 110 °C, 20 min, 6%; (c) Br$_2$, 95% EtOH, 70-75 °C, 33%.

**Scheme 2.18** General synthetic route for the production of tricyclic isatins 46 and 82 via the Stolle procedure.
Table 2.2 Reaction conditions attempted in the preparation of the tricyclic isatin 46.

<table>
<thead>
<tr>
<th>Entry</th>
<th>Reagents (equiv.)</th>
<th>Solvent</th>
<th>Time (h)</th>
<th>Temp (°C)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>a. indoline (1.0), (COCl)$_2$ (2.0) b. AlCl$_3$ (2.0)</td>
<td>a. THF b. DCM</td>
<td>a. 3.5 b. 1</td>
<td>a. reflux b. reflux</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>a. indoline (1.0), (COCl)$_2$ (2.0) b. AlCl$_3$ (3.0)</td>
<td>a. THF b. CHCl$_3$</td>
<td>a. 3.5 b. 3</td>
<td>a. reflux b. reflux</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>a. indoline (1.0), (COCl)$_2$ (2.0), K$_2$CO$_3$ (0.17)</td>
<td>a. THF</td>
<td>a. 3.5</td>
<td>a. reflux</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>a. indoline (1.0), (COCl)$_2$ (2.0) b. AlCl$_3$ (3.0)</td>
<td>a. THF b. 1,2-dichloroethane</td>
<td>a. 3.5 b. 1.5</td>
<td>a. reflux b. reflux</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>a. indoline (1.0), (COCl)$_2$ (2.0) b. AlCl$_3$ (5.0)</td>
<td>a. DCM b. neat</td>
<td>a. 3.5 b. 0.3</td>
<td>a. RT b. 110°C</td>
<td>6</td>
</tr>
</tbody>
</table>

Since it had been previously reported that bromination at the C5 position on the isatin scaffold resulted in more potent cytotoxicity than the parent molecule, the tricyclic isatin 46 was exposed to Br$_2$ to yield the novel brominated tricyclic isatin 82 through an electrophilic aromatic substitution. The C5 position (which is numbered C7 in the tricyclic isatin 46) is partial to electrophilic halogenation due to the meta-directing C3 carbonyl and the para-directing N1 atom. The tricyclic isatin 46 was dissolved in hot glacial AcOH before Br$_2$ was added, although this reaction yielded a complex mixture of products due to the limited solubility of 46 in the solvent. The bromination of 46 was successful (Scheme 2.18) when it was added to 95% EtOH, which is a known strategy in the synthesis of brominated isatins.

The structure of the novel compound was confirmed through $^1$H NMR spectroscopy, which revealed two aromatic protons at 7.49 and 7.66 ppm, ascribed to H8 and H6 respectively. Analysis by high resolution electrospray ionisation spectrometry (HRESI-MS) resulted in a protonated molecular ion of $m/z$ 251.9655, consistent with the protonated molecular formula of C$_{10}$H$_7$NO$_2$ Br [M+H]$^+$. 

~ 67 ~
Although the tricyclic isatin 46 had been synthesised in low yield, it was thought that indole (83) may be subjected to the same chemical conditions to produce the dehydrogenated tricyclic isatin 74 using the Stolle procedure. The indole intermediate 84 was able to be isolated as a red/brown powder in 19% yield (Scheme 2.19) but on treatment with AlCl$_3$ under neat conditions, no isatin 74 was able to be obtained as the MS revealed the product was once again a complex mixture of uncharacterisable products.

**Reagents and conditions:** (a) (COCl)$_2$, DCM, RT, 3 h, 19%; (b) AlCl$_3$, 110 °C, 20 min.

**Scheme 2.19** Attempted preparation of 74 via the Stolle isatin synthesis.

The problem of cyclising the above indole (83) and indoline (40) compounds using the Stolle procedure may be due to the lack of activating substituents on the benzene ring which could activate the C7 position towards electrophilic aromatic substitution. The Stolle isatin synthesis has resulted in 4,5-dihydro-6,8-dimethoxy-pyrrolo[3,2,1-\textit{h}][indole]-1,2-dione (47) (Figure 2.4B) being produced in a moderate yield (48%)$^{157}$ from 4,6-dimethoxyindoline due to the indole C7 position being activated by the two methoxy substituents on the indoline. The acid chloride intermediate was therefore formed at C7, rather than the traditional N1 position, and cyclisation was completed in the presence of AlCl$_3$. 4,6-Dimethoxy-2,3-diphenyl-1\textit{H}-indole, the precursor to 48, (Figure 2.4C) is so activated by the two phenyl and
two methoxy substituents that AlCl$_3$ is not required to facilitate cyclisation. The cyclisation is completed in 84% yield in the presence of only oxalyl chloride.$^{158}$ Unfortunately, 48 was susceptible to ring opening upon treatment a reducing agent such as NaBH$_4$ or LiAlH$_4$ which confirms the issue of ring strain within 6,5,5-fused ring systems.

This body of work highlights the difficulties in synthesising pyrrolo[3,2,1-$hi$]indole-1,2-diones and may explain why only three of these compounds exist in the literature (Figure 2.4).$^{156-158}$ Conformational analysis at the AM1 level was also performed on the tricyclic isatins 46 and 74 using the molecular modeling program Materials Studio 4.4.$^{226}$ The results indicated that both compounds have a large heat of formation ($\Delta H_f$) of 10.9 kcal/mol and 43.2 kcal/mol respectively (Figure 2.6). In contrast, isatins containing a 6,5,6-fused tricyclic systems such as 85-87 exhibited much lower $\Delta H_f$ values (Figure 2.6), suggesting that they may be easier to synthesise. These results confirm the ring strain within the 6,5,5-fused tricyclic isatins and further verifies why compounds of this structure are very difficult to synthesise. Free radical cyclisation and metal-catalysed cross coupling reactions were unsuccessful in synthesising these tricyclic isatins. The only successful syntheses of pyrrolo[3,2,1-$hi$]indole-1,2-diones arose from adapting literature methods using the Stolle isatin synthesis$^{156,225}$ to synthesise the known pyrrolo[3,2,1-$hi$]indole-1,2-dione (46), followed by electrophilic substitution to yield the novel brominated pyrrolo[3,2,1-$hi$]indole-1,2-dione 82. The results of the evaluation of the cytotoxic and potential DNA intercalatory activity of these two tricyclic isatin derivatives are presented in Chapter 6. Furthermore, four cyclisation precursors described in this chapter (49, 65, 75 and 76), were also tested for their cytotoxicity, which will be described in Chapter 6.
Figure 2.6 Heat of formation values ($\Delta H_f$) for 6,5,5- and 6,5,6-fused tricyclic isatins as calculated by Material Studios 4.4.  

Due to the difficulties encountered in trying to synthesise novel derivatives from this rare class of compounds, it was decided to change focus to the homologous 6,5,6-fused series, of which there is both greater literature precedence and promising results from the conformational analysis as shown in Figure 2.6. The next chapter will therefore focus on the synthesis of the 6,5,6-fused tricyclic isatins, (pyrrolo[3,2,1-ij]quinoline-1,2-diones), and various other novel polycyclic isatin derivatives as a potential new class of (potent) cytotoxic agents.
CHAPTER 3

Synthesis of Pyrrolo[3,2,1-ij]quinoline Derivatives and Other Polycyclic Isatin Derivatives

3.1 Initial Synthetic Targets

As mentioned in Chapter 2, incorporation of an extra ring between N1 and C7 of the isatin nucleus was of interest in order to partially restrain conformational flexibility in the N-substituent group. This chapter will focus on the addition of a 6-membered ring to isatin (11) i.e. 6,5,6-fused tricyclic isatins, known formally as pyrrolo[3,2,1-ij]quinoline-1,2-diones (Figure 3.1). To facilitate the comparison between previously reported N-alkylisatins, the pyrrolo[3,2,1-ij]quinoline-1,2-diones were designed to incorporate similar N-alkyl substituents. Two different routes were considered for the synthesis of the pyrrolo[3,2,1-ij]quinoline-1,2-diones (Figure 3.1), in a similar fashion to the pyrrolo[3,2,1-hi]indole-1,2-diones discussed in Chapter 2. The 6-membered ring C in the pyrrolo[3,2,1-ij]quinoline-1,2-diones was to be approached from isatin (11) via a metal-catalysed cross-coupling reaction (route B) or alternatively, ring B was proposed to be accessed through the cyclisation of a reduced quinoline (88) via a Stolle or Sandmeyer isatin synthesis starting from a quinoline (route A) (Figure 3.1).
In this chapter, the synthesis of pyrrolo[3,2,1-ij]quinoline-1,2-diones and extensions to other benz-fused polycyclic isatins (pyrrolophenanthridinediones and pyrroloacridinediones) will be discussed. The biological activity (including cytotoxicity) of the prepared compounds will be discussed in Chapter 6.

### 3.2 Synthesis of Pyrrolo[3,2,1-ij]quinoline-1,2-dione Derivatives

The indole-based pyrrolo[3,2,1-ij]quinoline nucleus is well known in the scientific literature\(^{205,212,227-231}\) and the ring system occurs in many natural products including alkaloids from the *Amaryllidaceae* (perennial herb and flowering bulb) species\(^{228,232}\) (see Section 3.3.1). Compounds containing a pyrrolo[3,2,1-ij]quinoline scaffold have been reported to exhibit a broad spectrum of biological activities including analgesic, anti-pyretic, anti-inflammatory,\(^ {228}\) anti-hyperlipidemic, anti-hypertensive,\(^ {230}\) anti-convulsant\(^ {154}\) (89), platelet activating factor production inhibition,\(^ {233}\) anti-epileptic, anti-obesity\(^ {234}\) (90) and anti-cancer\(^ {235}\) (91) activities (Figure 3.2A). However, only approximately 50 of the analogous isatin-based pyrrolo[3,2,1-ij]quinoline-1,2-diones have been reported in the literature to date. The
most recognised examples of pyrrolo[3,2,1-ij]quinoline-1,2-diones (92 and 93) exhibit fungicidal activity (Figure 3.2B).\textsuperscript{13,14}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{pyrroloquinoline.png}
\caption{A) Structures of pyrrolo[3,2,1-ij]quinolines with anti-convulsant, anti-obesity and anti-cancer activities; B) Structures of fungicidal pyrrolo[3,2,1-ij]quinoline-1,2-diones.}
\end{figure}

3.2.1 Synthesis of Pyrrolo[3,2,1-ij]quinoline-1,2-dione Derivatives via Route A

3.2.1.1 Attempted Sandmeyer Isatin Synthesis

The Sandmeyer isatin synthesis, which relies on the formation of an isonitrosoamide intermediate, is the most frequently used procedure to synthesise isatins (see Section 2.2.1.1). Applying this method allowed the formation of 7-bromoisatin (55) and 7-iodoisatin (56) in Chapter 2. Using this rationale, it was thought that the pyrrolo[3,2,1-ij]quinoline-1,2-dione (85) could also be prepared from 1,2,3,4-tetrahydroquinoline (THQ) (88) via the Sandmeyer method. THQ (88) may be considered a masked aniline containing a secondary amine, and therefore still has one hydrogen available to participate in the cyclisation.
In our work, the THQ (88) was exposed to chloral hydrate, Na$_2$SO$_4$ and hydroxylamine hydrochloride with heating in an attempt to yield the isonitrosoacetanilide intermediate 94 (Scheme 3.1). However, no intermediate 94 was observed and only starting material 88 was recovered so this approach was not continued. If the reaction had been successful, 94 would have been exposed to conc. H$_2$SO$_4$ and high temperatures to yield the cyclised isatin 85. The difficulty in synthesising the intermediate 94 reveals, perhaps, that steric issues are a factor in the initial secondary amine attack on chloral with a ring-fused aniline. In contrast, N-alkylanilines, which contain a secondary amine but not within a ring-fused system, have been successfully cyclised to the corresponding N-alkylisatins through the Sandmeyer method.\textsuperscript{167}

\textbf{Scheme 3.1} Attempted synthesis of the tricyclic isatin 85 via the Sandmeyer method.

\textbf{Reagents and conditions:} (a) chloral hydrate, Na$_2$SO$_4$, hydroxylamine.HCl, H$_2$O, HCl, 80 °C, 1.5 h; (b) conc. H$_2$SO$_4$, 80 °C, 15 min.
3.2.1.2 Stolle Isatin Synthesis

Scheme 3.2 Stages in the synthesis of tricyclic isatins using the Stolle isatin synthesis.

Due to the success of synthesising pyrrolo[3,2,1-\textit{hi}]indole-1,2-diones using the Stolle isatin synthesis (Chapter 2), this methodology was applied to quinoline derivatives to form pyrrolo[3,2,1-\textit{ij}]quinoline-1,2-diones. A modification of the Stolle isatin synthesis was initially attempted based on a Friedel-Crafts acylation of THQ (88). It has been reported that N-methylisatins (96) may be prepared through Friedel-Crafts acylation of \textit{p}-substituted \textit{N},\textit{N}-dimethylanilines (95) in the presence of oxalyl chloride and a base (Scheme 3.3). This rationale seemed applicable to other secondary aromatic amines and in our case, we chose the secondary aromatic amine, THQ (88). Oxalyl chloride was added dropwise to the base, diazabicyclo[2,2,2]octane (DABCO) (which Cheng \textit{et al.} determined to result in the best yields\textsuperscript{16}), to form a pale yellow salt. A solution of THQ (88) was then added and the reaction mixture was heated at reflux for 20 h in CHCl\textsubscript{3}. Unfortunately, MS analysis on the crude yellow semi-solid product revealed only the starting material 88 was present. The synthesis of the \textit{N}-methylisatin 96 in Scheme 3.3 may be enhanced through the \textit{p}-electron donating methoxy group on the aniline 95, which would lead to an activated system, as opposed to unsubstituted THQ (88) which is possibly not sufficiently activated for Friedel-Crafts acylation.
Reagents and conditions: (a) (COCl)$_2$, DABCO, CHCl$_3$, reflux, 22 h; (b), 5% NaOH, 83% (over 2 steps).\textsuperscript{236}

Scheme 3.3 Reported cyclisation of an aromatic secondary amine to the corresponding isatin via Friedel-Crafts acylation.\textsuperscript{236}

The synthesis of pyrrolo[3,2,1-ij]quinoline-1,2-dione (85) was then attempted by closely following the method described for tricyclic isatin 46 in Chapter 2, to yield the desired tricyclic isatin 85 in 31% yield (Scheme 3.4). The tricyclic isatin 85 was able to be formed in solution, rather than neat conditions, as was the case for the tricyclic isatin 46 in Chapter 2.

Although 85 is a known compound, no account of its synthesis has been reported since 1989\textsuperscript{237} (prepared using the Stolle isatin synthesis) and comprehensive characterisation data was unavailable. Through NMR spectroscopy, the structure of 85 was confirmed. The $^1$H NMR spectrum revealed the absence of an NH proton signal suggesting that cyclisation had occurred. Additionally, the $^{13}$C NMR spectrum exhibited two carbonyl signals at 156.9 and 183.9 ppm, indicative of C2 and C1 respectively and analysis by HREI-MS displayed in a molecular ion peak at
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m/z 187.0638, consistent with the molecular formula of the desired product (C\textsubscript{11}H\textsubscript{9}NO\textsubscript{2}[M+]).

Reagents and conditions: (a) (COCl\textsubscript{2}), THF, reflux, 3.5 h; (b) AlCl\textsubscript{3}, DCM, reflux, 3 h, 31% (over 2 steps); (c) Br\textsubscript{2}, 95% EtOH, 70-75 °C, 78%.

Scheme 3.4 Synthesis of tricyclic isatin 85 and its brominated analogue 92.

The tricyclic isatin 85 was readily brominated through electrophilic substitution to afford 92 in high yield (Scheme 3.4). The isatin 92 was previously synthesised\textsuperscript{238} although no yield or thorough characterisation data were reported. The structure of 92 was confirmed through MS which revealed the molecular ion as isotopic peaks at m/z 265 and 267 ([M]\textsuperscript{+} and [M + 2]\textsuperscript{+}), indicating a bromine atom was present, together with the correct molecular weight. Analysis by \textsuperscript{1}H NMR spectroscopy also revealed two aromatic singlet signals at 7.47 and 7.49 ppm, ascribed to H9 and H7 respectively.

To further investigate the cytotoxic SAR of the 6,5,6-fused tricyclic isatins, structures incorporating N-alkylaryl substituents were desired. The selective addition of an aryl substituent to the cyclised isatin 85 was expected to be difficult, hence the required N-alkylaryl moiety would need to be incorporated into the structure prior to cyclisation. It was envisaged that by starting from quinoline (97), substituents could be added into the C2 position, which upon cyclisation with oxalyl chloride and AlCl\textsubscript{3}, would yield the desired N-alkyl tricyclic isatins. Quinolines substituted at the
2-position are able to be synthesised by reacting quinoline (97) with an organolithium\textsuperscript{239} or Grignard reagent.\textsuperscript{240} Initially, it was thought that this type of reaction would result in the formation of the 4-substituted quinoline\textsuperscript{241} or as a mixture of the 2- and 4-substituted quinolines,\textsuperscript{242} although later it was demonstrated that these nucleophilic substitution reactions only yielded the 2-substituted quinoline.\textsuperscript{239}

Using this rationale, quinoline (97) was reacted with the Grignard reagent, phenylmagnesium bromide (PhMgBr), to form 2-phenyl-1,2-dihydroquinoline (98) (Scheme 3.5). The product has chirality introduced at the C2 position, however, in this project all chiral compounds were used as racemic mixtures as several attempts at separating of the diastereoisomers formed using resolving salts was unsuccessful. 1,2-Dihydroquinolines are known to be air-sensitive and readily oxidise back to the original quinoline,\textsuperscript{243} as well as being susceptible to disproportionation reactions which lead to a mixture of the parent quinoline and the corresponding THQ.\textsuperscript{244} For this reason, the dihydroquinoline 98 was used in further reactions immediately after its preparation, although analysis by NMR spectroscopy and MS revealed the mixture contained the dihydroquinoline 98, together with other quinoline-based products. Purification of the mixture was not attempted due to the instability of 1,2-dihydroquinolines. Upon treatment with oxalyl chloride and AlCl\(_3\), 98 was cyclised to form the novel tricyclic isatin 99 in trace amounts, which may be explained by the use of impure 2-phenyl-1,2,-dihydroquinoline (98). The structure of 99 was verified through \(^{13}\text{C}\) NMR spectroscopy which displayed two carbonyl signals at 157.4 and 182.4 ppm, ascribed to C2 and C1 respectively. Additionally, HRESI-MS revealed a molecular ion of \(m/z\) 262.0882, consistent with the protonated molecular formula of \(\text{C}_{17}\text{H}_{12}\text{NO}_2 [\text{M+H}]^+\).
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Reagents and conditions: (a) 1 M PhMgBr, THF, reflux, 18 h, 90% (crude yield); (b) (COCl)_2, THF, reflux, 3.5 h; (c) AlCl_3, CHCl_3, reflux, 18 h, 99 = 1% (over 2 steps); 101 = 5% (over 2 steps); (d) Na, EtOH, reflux, 2 h, 70%; (e) Br_2, 95% EtOH, 70-75 ºC, 50%.

Scheme 3.5 Synthesis of N-alkyl tricyclic isatins 99, 101 and 102 from quinoline (97).

To prepare the saturated analogue of tricyclic isatin 99, the dihydroquinoline 98 needed to be reduced to 2-phenyl-1,2,3,4-tetrahydroquinoline (100). This reduction was accomplished using a 0.25 M solution of 98 in refluxing EtOH with Na metal (Scheme 3.5).\textsuperscript{240} The THQ 100 was obtained as a yellow/orange oil in good yield and analysis by MS and \textsuperscript{1}H NMR spectroscopy confirmed its structure. The \textsuperscript{1}H NMR spectral data coincided with those reported in the literature.\textsuperscript{244} In a similar fashion to the tricyclic isatin 99, the THQ derivative 100 afforded the tricyclic isatin 101 upon treatment with oxalyl chloride and AlCl_3 (Scheme 3.5). The yield of this compound, although poor, was slightly higher than the analogous unsaturated tricyclic isatin 99. Even though the tricyclic isatin 101 has been synthesised...
previously, only a melting point had been reported as characterisation data. The structure of 101 was readily ascertained through NMR spectroscopy. In the $^1$H NMR spectrum, four multiplets between 2.16-2.66 ppm, integrating for one proton each, were ascribed to the tetrahydro protons H5 and H6. The C5 and C6 signals were observed in the $^{13}$C NMR spectrum at 27.6 and 19.7 ppm, which is typical of methylene signals. Additional confirmation of 101 was provided through HREI-MS which revealed a molecular ion at $m/z$ at 263.0949, consistent with the molecular formula (C$_{17}$H$_{13}$NO$_2$[M$^+$]).

The tricyclic isatin 101 was then exposed to Br$_2$ in 95% EtOH at 70-75 °C to yield the brominated tricyclic isatin 102 in moderate yield (Scheme 3.5). The structure of 102 was confirmed through $^1$H NMR spectroscopy, which exhibited two singlets at 7.50 and 7.59 ppm, attributed to H7 and H9 respectively, and no evidence of H8. Additionally, the structure of 102 was verified through MS which revealed molecular ion peaks at $m/z$ 341 and 343, ([M$^+$] and [M + 2]$^+$), characteristic of the $^{79}$Br and $^{81}$Br isotopes within the molecule.

The brominated analogue of 99 was also desired for inclusion in the SAR study. Bromination through an electrophilic substitution reaction was not viable since the alkene, rather than the aromatic ring in 99 would be preferentially attacked by the bromine. To overcome this obstacle, the bromo substituent needed to be introduced into the molecule at the beginning of the synthesis. This was achieved by starting the reaction sequence from 6-bromoquinoline (103). 6-Bromoquinoline (103) may be synthesised from the bromination of THQ (88) and subsequent reduction to form the quinoline. However, the synthesis is unfavourable since bromination of THQ (88) yields 6-bromo- and 6,8-dibromo-tetrahydroquinoline as a mixture. Furthermore, the ensuing reduction reaction utilises 2,3-dichloro-5,6-
dicyano-1,4-benzoquinone (DDQ), which is capable of liberating HCN gas if the reaction is not performed under scrupulously anhydrous conditions. As an alternative, commercially available 6-bromoquinoline (103) was reacted with the Grignard reagent PhMgBr overnight in refluxing THF to yield 6-bromo-2-phenyl-1,2-dihydroquinoline (104) (Scheme 3.6), together with some other quinoline-based products. As with the previous 1,2-dihydroquinoline 98, the 6-bromo derivative 104 was not purified due to the instability of the compound.

Upon exposure to oxalyl chloride and AlCl₃ under the aforementioned cyclisation conditions, the brominated dihydroquinoline 104 cyclised to form the brominated tricyclic isatin 105 (Scheme 3.6). The yield of 105 was low, similar to the yields of the tricyclic isatins 99, 101 and 102, however, it was obtained in a pure form and in sufficient quantity for biological activity evaluation. HRESI-MS revealed a molecular ion at \( m/z \) 339.9965, consistent with the protonated molecular formula of \( \text{C}_{17}\text{H}_{11}\text{NO}_2\text{Br}[\text{M+H}]^+ \). Analysis of the \( ^1\text{H} \) NMR spectrum revealed the absence of an NH proton signal and the \( ^{13}\text{C} \) NMR spectrum exhibited two carbonyl signals at 181.1 and 157.7 ppm, arising from C1 and C2 respectively, which additionally verified the structure of 105.

![Scheme 3.6](image)

**Reagents and conditions:** (a) 1 M PhMgBr, THF, reflux, 18 h, 66%; (b) (COCl)₂, THF, reflux, 3.5 h; (c) AlCl₃, CHCl₃, reflux, 18 h, 2% (over 2 steps).

**Scheme 3.6** Synthesis of the brominated N-alkyl tricyclic isatin 105.
3.2.2 Synthesis of Pyrrolo[3,2,1-ij]quinoline-1,2-dione Derivatives via Route B

Metal-catalysed cross-coupling reactions are an important class of reactions within organic chemistry and are a valuable tool for the preparation of compounds which have proved difficult to synthesise using alternative methods. In Chapter 2, Sonogashira reactions were discussed in the synthesis of 6,5,5-fused tricyclic isatins. Herein, the synthesis of 6,5,6-fused tricyclic isatins will utilise the Heck reaction, which is the Pd-mediated cross-coupling of an aryl or alkenyl halide (or triflate) with an alkene to form a substituted alkene.\(^{247}\) It is an extremely resourceful procedure in the formation of carbon-carbon bonds and is widely used in heterocyclic synthesis.\(^{248}\) Typically, the Heck reaction is performed under moderate temperatures (> 80 ºC) with an organopalladium catalyst such as Pd/C, Pd(PPh\(_3\))\(_4\), PdCl\(_2\) or Pd(OAc)\(_2\), an olefin, a base such as Et\(_3\)N, K\(_2\)CO\(_3\) or NaOAc, and a ligand such as PPh\(_3\).\(^{173,249,250}\) Furthermore, the addition of tetrabutylammonium salts greatly enhances the reactivity and selectivity of inter- and intramolecular Heck reactions.\(^{251}\) Tetrabutylammonium chloride (TBACl) is far more reactive than tetrabutylammonium bromide (TBAB) and tetrabutylammonium hydrogen sulfate, and the reaction rate is known to increase in proportion to the amount of TBACl used.\(^{252}\) The Heck reaction is also known to proceed at or near RT under ligand-free conditions\(^ {252}\) with enhanced activity and yield and is commonly referred to as “Jeffrey’s ligand-free conditions.”\(^{181}\)

The mechanism of the Heck reaction has some similarities to the Sonogashira mechanism (see Figure 2.6). The first step involves the addition of an alkenyl or aryl halide to the Pd(0) catalyst to form a Pd(II) complex (Figure 3.3). Due to the increase in electrophilicity, the olefin is readily inserted as a \textit{syn}-addition into the Pd complex. The complex is rotated to the \textit{trans} isomer to relieve torsional strain before
the beta-hydride elimination step to release the product. The new Pd(II) complex is destroyed through base-induced reductive elimination to regenerate the Pd(0) catalyst.\textsuperscript{173}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{heck_mechnism.png}
\caption{Mechanism of the Heck reaction.\textsuperscript{249}}
\end{figure}

Many intramolecular Heck reactions have previously been applied to indoles to form tricyclic indoles. These include the cyclisations of 3-alkenyl-4-bromoindoles to form 3,4-annulated indoles\textsuperscript{253,254} and N-alkenyl-2-iodoindoles to form 1,2-annulated indoles.\textsuperscript{255} More relevant to this project was the high yielding cyclisation of the N-allyl-7-bromoindole 106 to form the 1,7-annulated indole 107 as reported by Black \textit{et al.}\textsuperscript{256} (Scheme 3.7). The formation of a 1,7-annulated isatin with an eight-membered ring through an intramolecular Heck reaction has also been reported,\textsuperscript{39} but not with smaller ring sizes.
Reagents and conditions: (a) Pd(OAc)$_2$, P(o-tol)$_3$, Et$_3$N, CH$_3$CN, 100 °C, 15 h, 96%.\textsuperscript{256}

Scheme 3.7 Reported synthesis of a 1,7-annulated indole 107 via an intramolecular Heck reaction.\textsuperscript{256}

Using the rationale from Scheme 3.7, it was envisaged that the synthetic strategy could be applied to the synthesis of the desired 6,5,6-fused tricyclic isatins by starting from \textit{N}-allyl-7-bromoisatin (49). This compound was discussed previously in Chapter 2 where it was used in the attempted synthesis of 6,5,5-fused tricyclic isatins via radical cyclisations. Exposing 49 to an intramolecular Heck reaction could lead to the formation of four isomeric products (86, 87, 108, 109) (Figure 3.4). According to Baldwin’s rules, the formation of the 5-\textit{exo-trig} (108, 109) and 6-\textit{endo-trig} (86, 87) products are both favourable.\textsuperscript{171} Exo ring closure is highly selective for 5-, 6- and 7-membered rings as they are more sterically favourable and have transition states with lower energies than the \textit{endo} products.\textsuperscript{257}
Figure 3.4 Possible products from the intramolecular Heck reaction of the isatin 49.

Firstly, the N-allyl-7-bromoisatin (49) was subjected to Heck conditions similar to those reported by Black et al.\textsuperscript{256} and Keller\textsuperscript{215} (Table 3.1, entry 1). Although 49 may cyclise to yield 5- and 6-membered ring products, the cyclisation of 49 led only to the formation of the 6-\textit{endo-trig} products 86 and 87 (Scheme 3.8). It is thought that this may be due to the bromo and allyl substituents being in a \textit{peri} position which results in the reactive allylic carbons being further away from the organopalladium halide site and therefore, bond formation occurs involving the terminal allylic carbon.\textsuperscript{256} From \textsuperscript{1}H NMR spectroscopic monitoring of the product, a mixture of the 6-membered ring isomers in a 5:4 ratio was present. The isomers 86 and 87 could be isolated by preparative TLC.
Table 3.1 Reaction conditions\textsuperscript{a} attempted for the intramolecular Heck reaction of 49.

<table>
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<th>Catalyst</th>
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<th>Time (h)</th>
<th>Yield (%)</th>
<th>Ratio 86:87</th>
</tr>
</thead>
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<td>P(o-tol)\textsubscript{3}</td>
<td>Et\textsubscript{3}N</td>
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<td>16</td>
<td>5:4</td>
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<td>P(o-tol)\textsubscript{3}</td>
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<td>0</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>Pd(OAc)\textsubscript{2}</td>
<td>P(o-tol)\textsubscript{3}</td>
<td>TBACl, NaOAc</td>
<td>100</td>
<td>18</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>Pd(OAc)\textsubscript{2}</td>
<td>PPh\textsubscript{3}</td>
<td>TBACl, NaOAc</td>
<td>120</td>
<td>18</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>Pd(OAc)\textsubscript{2}</td>
<td>TTFMPP</td>
<td>TBACl, NaOAc</td>
<td>120</td>
<td>18</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>Pd(OAc)\textsubscript{2}</td>
<td>-</td>
<td>TBACl, K\textsubscript{2}CO\textsubscript{3}</td>
<td>90</td>
<td>0.67</td>
<td>17</td>
<td>2:1</td>
</tr>
<tr>
<td>7</td>
<td>Pd(OAc)\textsubscript{2}</td>
<td>-</td>
<td>TBACl, K\textsubscript{2}CO\textsubscript{3}</td>
<td>85</td>
<td>2</td>
<td>52</td>
<td>3:1</td>
</tr>
<tr>
<td>8</td>
<td>Pd(OAc)\textsubscript{2}</td>
<td>-</td>
<td>TBACl, K\textsubscript{2}CO\textsubscript{3}</td>
<td>80</td>
<td>4</td>
<td>5</td>
<td>2.6:1</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Entry 1 was performed in CH\textsubscript{3}CN, entries 2-8 in DMF.

Using 2D NMR (gHMBC), the isomers 86 and 87 could be distinguished. In each case, the C2 carbonyl correlated with the H4 proton within the molecule, however, the C2 carbonyl (153.2 ppm) within the 6H isomer 87 correlated with the H4 proton (6.95 ppm) (Figure 3.5), which integrated for one proton. Hence, the alkenyl bond was in the C4-C5 position, with H6 (3.60 ppm) integrating for two protons. Therefore, it could be deduced that the other compound was the 4H isomer 86, and in this case the C2 carbonyl (157.6 ppm) correlated with H4 (4.62 ppm), which integrated for two protons.
In a bid to increase the yield of the products 86 and 87, tetrabutylammonium iodide (TBAI) was added to improve the reactivity and selectivity of the reaction$^{251}$ (Table 3.1, entry 2). Unfortunately, this attempt was unsuccessful as judged by MS analysis which revealed many unidentified products. Changing the tetrabutylammonium salt to TBACl, which is known for its superior reactivity,$^{252}$ also yielded none of the desired product (Table 3.1, entry 3). MS analysis of the crude products from both of the above reactions revealed a complex mixture of components, with no molecular ion peak at $m/z$ 185, the molecular weight of the tricyclic isatins 86, 87, 108, 109. In Table 3.1, entries 4 and 5, PPh$_3$ and tris($p$-trifluoromethylphenyl)phosphine (TTFMPP) were chosen as the phosphine
ligands respectively, but these were also to no avail – none of the desired products were observed by MS.

Jeffrey’s “ligandless” conditions were also attempted in the cyclisation of **49** (Table 3.1, entries 6-8). The base was also changed from NaOAc to K$_2$CO$_3$ due to its higher reactivity. The advantages of using ligandless conditions in a Heck reaction are the lower reaction temperatures and shorter reaction times necessary, and, encouragingly, higher yields of **86** and **87** were produced (Table 3.1, entries 6 and 7). Upon reacting **49** with Pd(OAc)$_2$, TBACl and K$_2$CO$_3$ at 90 °C for 40 min, a yield of 17% was obtained (Table 3.1, entry 6). According to TLC and $^1$H NMR spectroscopic analysis, a mixture of isomers in a 2:1 ratio was present. The isomers were able to be separated once again through preparatory TLC. By increasing the reaction time to 2 h (Table 3.1, entry 7), the yields of **86** and **87** were increased to 52%. Analysis of the products by $^1$H NMR spectroscopy revealed they were formed in a 3:1 ratio. The reaction time was also extended to 4 h (Table 3.1, entry 8) but then the yields of **86** and **87** decreased to only 5%. Subsequent analysis by $^1$H NMR spectroscopy illustrated that the mixture contained **86** and **87** in a 2.6:1 ratio. It can be concluded that using ligandless conditions during an intramolecular Heck reaction increases the yield of products. In general, longer reaction times lead to decreased yields (Table 3.1, entry 7 > entry 6 > entry 8) with no significant difference in isomer selectivity. It was found that the optimal conditions for the Heck cyclisation were 2 h at 85 °C (Table 3.1, entry 7).
Reagents and conditions: (a) TBACl, K$_2$CO$_3$, Pd(OAc)$_2$, DMF, 85 °C, 2 h. Compounds 86 and 87 synthesised in 52% yield in a mixture of isomers (3:1). Compounds 111 and 112 synthesised in 49% yield in a mixture of isomers (3:2).

Scheme 3.8 Synthesis of tricyclic isatins 86, 87, 111, 112 via an intramolecular Heck reaction.

To obtain the 8-bromo analogues of 86 and 87 for cytotoxicity testing, an electrophilic bromination using Br$_2$ was not applied since the bromine would attack the alkene in 86 and 87 yielding the corresponding dibrominated products. As in the case of 8-bromo-4-phenyl-4H-pyrrolo[3,2,1-ij]quinoline-1,2-dione (105), the bromo substituent needed to be inserted prior to undergoing the Heck cyclisation, even though product yields may be compromised by alternative Pd insertion at the C5-Br position. The required starting material N-allyl-5,7-dibromoisatin (110) was synthesised using the same conditions as for the allylisatins 49 and 76 (Chapter 2). The N-allyl-5,7-dibromoisatin (110) was synthesised in 87% yield and its $^1$H NMR spectral data coincided with those reported in the literature.$^{64}$ Subsequent exposure of the N-allylisatin 110 to the optimal intramolecular Heck conditions (as determined in Table 3.1), gave the brominated tricyclic isatins 111 and 112 in a moderate yield (Scheme 3.8). Analysis by $^1$H NMR spectroscopy revealed the 4H and 6H isomers
(111 and 112) had formed in a 3:2 ratio, with the 4H isomer 111 again being the predominant isomer formed.

The NMR spectral data for the brominated tricyclic isatins 111 and 112 was similar to those observed for the analogous unsubstituted tricyclic isatins 86 and 87. The key difference was the absence of the signal for the H8 proton and the downfield shift (~ 0.14 ppm) of the H7 and H9 proton signals in the 1H NMR spectrum of 111 and 112 when compared to 86 and 87. Confirmation of the molecular formula was observed through HRESI-MS, which exhibited a molecular ion at m/z 263.9659 for the 4H isomer 111, consistent with the protonated molecular formula of \( \text{C}_{11}\text{H}_{7}\text{NO}_{2}\text{Br}^+ \), and m/z 267.9611 for the 6H isomer 112, consistent with the protonated molecular formula of \( \text{C}_{11}\text{H}_{9}\text{NO}_{2}\text{Br}^+ \).

The Heck reaction was also applied to other N-allylated isatins to yield the tricyclic isatins 114, 116, 118 (Scheme 3.9). 7-Bromoisatin (55) was reacted with crotyl bromide to yield 7-bromo-N-crotylisatin (113) in good yield (Scheme 3.9). The structure of this novel compound was verified through 1H NMR spectroscopy, which revealed the absence of the NH proton signal. The correct molecular weight of the product was corroborated through HRESI-MS which exhibited a molecular ion of m/z 279.9969, consistent with the protonated molecular formula of \( \text{C}_{11}\text{H}_{11}\text{NO}_{2}\text{Br}^+ \). The crotyl isatin 113 underwent an intramolecular Heck reaction using identical conditions to those used in Scheme 3.8 to yield the tricyclic isatin 114 in poor yield (Scheme 3.9). The 6-methyl-6H-pyrrolo isomer 114 was formed exclusively as determined by 1D and 2D NMR spectroscopy. Due to the low yield of 114, the brominated analogue of this compound was not attempted.

7-Bromoisatin (55) was also reacted with cinnamyl bromide providing 7-bromo-N-cinnamylisatin (115) in excellent yield (Scheme 3.9). Once more, the
structure of this novel compound was confirmed through NMR spectroscopy and HRESI-MS. Upon undergoing an intramolecular Heck reaction, 115 was able to form 6-phenyl-4H-pyrrolo[3,2,1-ij]quinoline-1,2-dione (116) in poor yield (Scheme 3.9), albeit in higher yield than 114. Interestingly, the tricyclic 116 was formed exclusively as the 4H-pyrrolo isomer with no evidence of the 6H-pyrrolo regioisomer in the 1H and 13C NMR spectra, in contrast to the 6-methyl-6H-pyrrolo tricyclic isatin 114 (Scheme 3.9). The 2D NMR spectral data showed that C6 is a quaternary carbon; hence, it must have cyclised onto the isatin, confirming the formation of 116. Additionally, the LREI-MS data confirmed the loss of a bromine atom from the starting material 115 and the correct m/z peak for the tricyclic isatin 116 parent ion.

The synthesis of the brominated analogue of 6-phenyl-4H-pyrrolo[3,2,1-ij]quinoline-1,2-dione (118) was also attempted. By beginning with 5,7-dibromoisatin (17) and reacting it with cinnamyl bromide, the N-cinnamyl-5,7-dibromoisatin (117) was prepared in essentially quantitative yield (Scheme 3.9). 1H NMR spectral data of the purified compound agreed with those previously reported in the literature.64 However, difficulties were encountered when synthesising the Heck cyclisation product 118 (Scheme 3.9). By applying the same reaction conditions as in previous Heck cyclisations (i.e. 85 °C, 2 h), there was no evidence of the desired product being formed. Extending the reaction time to 4 h also yielded no product. Analysis using MS revealed the isolated crude product was a complex mixture of compounds including the starting material 117. Presumably alternative reactivity at the C5-Br position in 117 was one of the contributing factors to the product mixture complexity.
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Reagents and conditions: (a) NaH, DMF, RT, 20 min; (b) crotyl or cinnamyl bromide, KI, 60 °C, 18 h, 113 = 59% (over 2 steps); 115 = 97% (over 2 steps); 117 = 48% (over 2 steps); (c) TBACl, K$_2$CO$_3$, Pd(OAc)$_2$, DMF, 85 °C, 114 = 2.5 h, 8%; 116 = 2 h, 17%; 118 = 2.4 h.

Scheme 3.9 *N*-Alkylation of isatins 55 and 17 and subsequent intramolecular Heck reactions to yield the tricyclic isatins 114 and 116.

With a range of pyrrolo[3,2,1-*ij*]quinoline-1,2-diones synthesised for future cytotoxicity testing, it was decided that attention should be turned to larger benz fused polycyclic isatins, which would also provide useful information for our SAR.

3.3 Synthesis of Polycyclic Isatins

In order to further explore the isatin scaffold for the development of novel cytotoxins and to incorporate the *N*-benzylic substituent moiety into a fused aromatic ring, attention was turned to extending the quinoline nucleus to benzoquinolines containing a benzo ring fused onto the benzene or heterocyclic ring of quinoline.$^{258}$
Reactions with the 3,4-annulated benzoquinoline, phenanthridine (119), will be discussed in this section. Cyclisation chemistry associated with the 2,3-annulated benzoquinoline, acridine (120), and will be outlined in Section 3.3.2. The synthetic strategies involved in the preparation of isatins based on pyrrolophenanthridines and pyrroloacridines include the Stolle isatin synthesis and metal-catalysed cross-coupling reactions (Figure 3.6). There are also possibilities to form isatins containing a benzophenanthridine nucleus (as depicted by rings E, F and G in Figure 3.6) through the above reactions.

Figure 3.6 Proposed structures and synthetic strategies in the formation of A) pyrrolophenanthridines and B) pyrroloacridines.
3.3.1 Phenanthridine-based Derivatives

The phenanthridine-based isatin derivatives portrayed in Figure 3.6A contain a pyrrolo[3,2,1-\textit{H}]phenanthridine nucleus. The related indole structural core is prevalent in alkaloid metabolites from almost all of the genera in the \textit{Amaryllidaceae} (perennial herb and flowering bulb) family\textsuperscript{208} and include alkaloids such as oxassoanine (123), hippadine (124) and pratorinine (125) (Figure 3.7).\textsuperscript{232,259}

![Figure 3.7 Examples of alkaloids from the Amaryllidaceae family containing a pyrrolo[3,2,1-\textit{H}]phenanthridine nucleus.](image)

3.3.1.1 The Stolle Isatin Synthesis

One compact route to synthesise phenanthridine-based isatins is to apply the Stolle isatin method to phenanthridine (119) (Scheme 3.10). In order for this to succeed, the imine bond in phenanthridine (119) needs to be reduced to allow for subsequent cyclisation with oxalyl chloride and AlCl$_3$. The reduction of 119 was first attempted using tin powder in refluxing HCl,\textsuperscript{260} however, analysis by MS revealed only starting material was present. Replacing tin with NaCNBH$_3$, a reagent known to reduce hydrazones and imines,\textsuperscript{261} yielded 5,6-dihydropyridine (121) in good yield (Scheme 3.10). The structure of 121 was confirmed through LREI-MS which exhibited a molecular ion at \textit{m/z} 181, some 2 atomic mass units higher than that of the starting material, indicating that the imine bond of 119 had been reduced. The
\(^1\)H NMR spectral data also contained a singlet signal at 4.39 ppm, integrating for two protons, which can be attributed to H6.

![Diagram](image)

**Reagents and conditions:** (a) AcOH, EtOH, reflux, 30 min; (b) NaCNBH\(_3\), reflux, 1.5 h, 77% (over 2 steps); (c) (COCl\(_2\)), DMAP, DIPEA, THF, reflux, 3.5 h; (d) AlCl\(_3\), CHCl\(_3\), reflux, 1.5 h, 15% (over 2 steps); (e) Br\(_2\), AcOH, 70-75 °C, 69%.

**Scheme 3.10** Synthesis of the pyrrolophenanthridines 126 and 127 via the Stolle isatin procedure.

When the previously discussed Stolle reaction conditions were applied to 5,6-dihydrophenanthridine (121), the reaction did not proceed and only starting material was recovered from the reaction mixture as determined by MS analysis. In this case, the addition of Et\(_3\)N and a catalytic amount of 4-(dimethylamino)pyridine (DMAP) was employed to facilitate the formation of the oxamide intermediate prior to the Friedel-Crafts acylation to yield phenanthridine-based isatin 126 (Scheme 3.10). Previously, it had been shown that the addition of Et\(_3\)N and DMAP to 5,6-dihydrophenanthridine (121) was necessary to achieve good yields of amide derivatives.\(^{262}\) The yield of the phenanthridine-based isatin 126 was not high, although enough pure material was obtained for future reactions. Confirmation of the structure of 126 through \(^{13}\)C NMR spectroscopy, which revealed signals for the C4 and C5 carbonyl signals at 183.2 and 157.9 ppm respectively, characteristic of the
isatin core. Analysis by HREI-MS indicated a molecular ion at $m/z$ at 235.0625, consistent with the molecular formula of $C_{15}H_9NO_2[M^+]$.

Initially, there were difficulties in brominating the phenanthridine-based isatin 126. The compound was insoluble at 75 ºC in 95% or absolute EtOH. Eventually, bromination proceeded at 75 ºC in glacial AcOH to yield 127 in good yield (Scheme 3.10). The NMR spectral data for this compound appeared similar to 126, except for the absence of the H2 proton in the $^1H$ NMR spectrum. The presence of a bromine atom in the molecule was confirmed by LREI-MS which showed characteristic molecular isotopic peaks at $m/z$ 313 and 315 ([M]$^+$ and [M + 2]$^+$).

### 3.3.1.2 Biaryl Coupling

Another avenue to synthesising phenanthridine-based isatin derivatives is to utilise metal-catalysed cross-coupling reactions. As mentioned in Sections 2.2.2 and 3.2.2, Pd catalysts are very versatile and are able to catalyse an array of important organic chemistry reactions. Pd catalysts are also useful in biaryl coupling reactions where an aryl-aryl bond formation is desired. More specifically, arenes are able to react with aryl halides or triflates to form an aryl-aryl bond. These types of reactions commonly occur as intramolecular cyclisations\textsuperscript{263,264} to form polyarenes such as fullerenes\textsuperscript{265} and phenanthridine-based alkaloids,\textsuperscript{266} including those shown in Figure 3.7.

The pyrrolo[3,2,1-de]phenanthridine scaffold contains a biaryl linkage which may be formed via the Pd-catalysed intramolecular cyclisation of a $N$-alkylindole-based compound (Scheme 3.11).\textsuperscript{228}
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Scheme 3.11 Example of a Pd-catalysed intramolecular biaryl coupling of N-alkylinodole-based compound.\textsuperscript{228}

The biaryl coupling may proceed via a halide on the benzyl moiety as in Figure 3.11 or via a halide on the indole nucleus. Applying this rationale, it was thought that additional novel phenanthridine-based isatins could be prepared through the Pd-catalysed biaryl intramolecular coupling of N-alkylisatins such as 7-iodo-N-(1-naphthylmethyl)isatin (128) and 7-iodo-N-(2-naphthylmethyl)isatin (129). This strategy has also been utilised previously by Garden et al. who synthesised pyrrolophenanthridine derivatives from 7-halo-N-alkylisatins.\textsuperscript{208}

Commencing from 7-iodoisatin (56), 7-iodo-N-(1-naphthylmethyl)isatin (128) was able to be prepared in good yield (Scheme 3.12). Compound identity was confirmed through \textsuperscript{1}H NMR spectroscopy which revealed ten aromatic protons in the 6.90-7.99 ppm region. The \textsuperscript{13}C NMR spectrum gave evidence that N-alkylation had occurred through presence of the N-alkyl CH\textsubscript{2} signal at 42.1 ppm. The next step in the synthesis would typically be the biaryl coupling, but due to the reactivity of the C3 carbonyl in 128, protection of this functional group was required prior to exposure to Pd.\textsuperscript{208,232} The isatin 128 was protected as a cyclic ketal using typical Dean-Stark conditions as described in Section 2.2.2.1.1 and the cyclic ketal derivative 130 was obtained as a light brown powder in high yield (Scheme 3.12). Conversion to the ketal group was verified through \textsuperscript{13}C NMR spectroscopy, which
confirmed the presence of the two ketal carbon signals through a large signal at 66.3 ppm, and the absence of a signal from the carbon of the ketone carbonyl group usually observed at ~ 180 ppm.

\[ \text{Reagents and conditions: } \begin{align*}
(a) & \text{ NaH, DMF, RT, 20 min;} \\
(b) & 1\text{(chloromethyl)}\text{naphthalene, KI, } 60 \degree \text{C,} \\
18 \text{ h, 58\% (over 2 steps);}
(c) & (\text{CH}_3\text{OH})_2, \text{ PTSA, PhMe, reflux, 48 h, 75\%;}
(d) & \text{ Pd(OAc)}_2, \text{ TBAB, NaOAc, DMF, 90 \degree \text{C, 5 h, 86\%;}
(e) & 6 \text{ M HCl/THF (1:1), reflux, 5 h, 67\%;}
(f) & \text{ NBS, CH}_3\text{CN, RT, 18 h.}
\end{align*} \]

Scheme 3.12 Synthesis of pyrrolophenanthridines 131 and 132 through Pd-catalysed biaryl coupling reactions.

Reaction of the isatin 130 with Pd(OAc)$_2$ enables an intramolecular biaryl coupling to take place with the possibility of two regioisomers being formed. If the biaryl coupling occurs between the halide and C2 of the naphthyl moiety in isatin 130, the pyrrolophenanthridine 131 is prepared, whereas coupling via C8 on the naphthyl moiety will result in the formation of a pentacyclic isatin featuring a 7-membered ring (134) (Figure 3.8). It was deduced from examination of the
$^1$H NMR spectrum, that the pyrrolophenanthridine 131 was synthesised with complete regioselectivity and in high yield (Scheme 3.12). The aromatic proton signals for H9’ (7.56 ppm) and H10’ (7.50 ppm) were observed as triplets which were coupled to one another (Figure 3.9). While the isatin containing the 7-membered ring 134 may contain triplet signals for H9’ and H12’, these protons are not coupled to one another and hence, the pyrrolophenanthridine 131 is the only possible regioisomer to contain two triplet signals in the $^1$H NMR spectrum which are coupled to one another.

![Possible regioisomers 131 and 134 from the Pd-catalysed intramolecular biaryl coupling of the isatin 130.](image)

**Figure 3.8** Possible regioisomers 131 and 134 from the Pd-catalysed intramolecular biaryl coupling of the isatin 130.
Under acidic conditions the ketal moiety on the pyrrolophenanthridine 131 was hydrolysed to yield the isatin 132 as a dark red powder in good yield (Scheme 3.12). The red colour of the compound suggested that the ketal had been destroyed and that the C4 carbonyl, an important element in the chromophore of the isatin core, had been restored. The structure of the compound was verified through $^{13}$C NMR spectroscopy, which notably revealed the absence of the ketal carbon signal and the presence of the C4 carbonyl at 182.9 ppm. Bromination of the isatin 132 was unsuccessful due to solubility problems. The isatin derivative 132 was poorly soluble in glacial AcOH, EtOH or CH$_3$CN at 70-75 ºC following the bromination procedures.
described in earlier synthetic schemes. By replacing Br$_2$ with N-bromosuccinimide (NBS) as the source of bromine, the bromination was still ineffective and analysis by MS revealed only the starting material 132 was present.

Pyrrolophenanthridines using 7-ido-N-(2-naphthylmethyl)isatin (129) as the starting material were also prepared. 7-Iodo-N-(2-naphthylmethyl)isatin (129) was synthesised from 7-idoisatin (56) in 2 steps (Scheme 3.13). The structure of the naphthyl compound was confirmed through $^1$H NMR spectroscopy, which showed the absence of an NH proton signal and the presence of a singlet at 5.64 ppm, integrating for two protons and ascribed to the benzylic protons.
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Reagents and conditions: (a) NaH, DMF, RT, 20 min; (b) 2-(bromomethyl)naphthalene, KI, 60 °C, 18 h, 36% (over 2 steps); (c) (CH₂OH)₂, PTSA, PhMe, reflux, 64 h, 92%; (d) Pd(OAc)₂, TBAB, NaOAc, DMF, 90 °C, 5 h, 67%; (e) 6 M HCl/THF (1:1), reflux, 5 h, 90%; (f) NBS, CH₃CN, RT, 18 h, 39%.

Scheme 3.13 Synthesis of pyrrolophenanthridines 136-138 through Pd-catalysed biaryl coupling reactions.

Again the N-naphthylmethylisatin 129 was subjected to Dean-Stark conditions in the presence of ethylene glycol to protect the ketone as a cyclic ketal prior to biaryl coupling. This reaction resulted in excellent yield of 135 (Scheme 3.13) and the ¹³C NMR spectrum lacked a C3 carbonyl signal and displayed a relatively intense signal at 66.3 ppm, arising from the two ketal carbons.

As with the related compound 130, the intramolecular biaryl coupling of 135 could lead to two regioisomers. If the biaryl coupling proceeds via C1 on the naphthyl ring of 135, pyrrolophenanthridine 136 will form, whereas formation of a new bond to C3 on the naphthyl ring of 135 will lead to pyrrolophenanthridine 139 (Figure 3.10). Through ¹H NMR spectroscopy it was ascertained that the
pyrrolophenanthridine 136 was formed exclusively and in good yield (Scheme 3.13). If the pyrrolophenanthridine 139 had been formed, the $^1$H NMR spectrum should have contained two singlets attributed to H8' and H13'. However, aromatic singlet signals were not observed and it could be deduced that the pyrrolophenanthridine 136 had been prepared. The structure of the pyrrolophenanthridine 136 was further verified through $^1$H NMR spectroscopy which revealed a pair of coupled doublets (H8' and H9' at 7.21 and 7.74 ppm respectively) and two triplet signals coupled to each other (H11' and H12' at 7.50 and 7.56 ppm respectively). The regioselectivity of this reaction is in accordance with results for similar reactions reported by Torres et al.\textsuperscript{232}

![Possible regioisomers 136 and 139 from the Pd-catalysed intramolecular biaryl coupling of the isatin 135.](image)

Cleavage of the ketal functionality on 136 under acidic conditions liberated the pyrrolophenanthridine 137 in very high yield (Scheme 3.13). The $^{13}$C NMR spectrum of 137 exhibited a signal at 183.4 ppm, ascribed to the C4 carbonyl and the signal at 66.3 ppm attributed to the ketal carbons had disappeared. The compound 137 was a red powder, which also suggested restoration of the carbonyl. The pyrrolophenanthridine 137 had higher solubility in organic solvents relative to the
pyrrolophenanthridine 132 and consequently was able to be brominated using NBS in CH$_3$CN producing the bromo derivative 138 in moderate yield (Scheme 3.13). Analysis by $^1$H NMR spectroscopy revealed the absence of the H2 proton signal and hence, verified the structure of 138. The LREI-MS also had isotopic molecular ion peaks at $m/z$ 363 and 365 ([M]$^+$ and [M + 2]$^+$), indicative of the brominated pyrrolophenanthridine 138.

3.3.2 Acridine-based Derivatives

Derivatives of acridine (120) exhibit an array of pharmacological properties, and during the early 20$^{th}$ century, anti-bacterials and anti-malarials based on acridine (120) were used clinically. In recent decades attention has turned to the cytotoxic properties of acridine (120) and its derivatives. The 9-anilinoacridine derivative amsacrine (140) was one of the first cytotoxic acridines discovered and due to the planarity of the molecule, its mode of action is primarily as a DNA intercalating agent. Amsacrine (140) and other acridine-based derivatives (141 and 142, Figure 3.11) also express topoisomerase II inhibitory activity, an enzyme which is important for the control of DNA structure, and these compounds also show DNA quadruplex binding selectivity.
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Figure 3.11 Examples of acridine derivatives with cytotoxic activity (140), topoisomerase II inhibitory activity and DNA quadruplex binding selectivity (141 and 142).

To further investigate the cytotoxicity of acridines, it was decided that a pyrroloacridine based on the isatin scaffold would be synthesised. This novel series of acridine derivatives would also serve as an extension into the investigation of benzoquinoline-based isatins and their cytotoxicity could be compared to the phenanthridine-based isatins prepared in Section 3.3.1.

Initially, the imine functionality on acridine (120) was reduced to 9,10-dihydroacridine (122) to allow for subsequent cyclisation. The 9,10-dihydroacridine (122) was synthesised in moderate yield (Scheme 3.14) and $^1$H NMR spectroscopy revealed the presence of the NH proton as a broad singlet at 5.95 ppm, confirming reduction of the 120 to the dihydro compound 122. The remainder of the signals in the $^1$H NMR spectrum coincided with those previously reported in the literature.274
Reagents and conditions: (a) AcOH, EtOH, reflux, 30 min; (b) NaCNBH₃, reflux, 1.5 h, 47% (over 2 steps); (c) (COCl)₂, THF, reflux, 3.5 h; (d) AlCl₃, CHCl₃, reflux, 18 h, 15% (over 2 steps); (e) Br₂, AcOH, 70-75 °C, 41%.

Scheme 3.14 Synthesis of pyrroloacridines 143 and 144 via the Stolle isatin procedure.

The Stolle isatin synthesis method was utilised to cyclise 122 to the pyrroloacridine 143 (Scheme 3.14). There is only one account of this compound in the literature and it was synthesised using the Stolle method. The synthetic procedure contained a limited amount of characterisation data hence, spectroscopic techniques were employed to confirm the structure. As expected for the structure of 143, the signal arising from the amino proton was absent from the ¹H NMR spectrum and the ¹³C NMR spectrum exhibited signals for C1 and C2 at 155.5 and 182.3 ppm respectively. HREI-MS analysis also revealed a molecular ion peak at m/z 235.0633, consistent with the molecular formula (C₁₅H₁₀NO₂ [M⁺]). The pyrroloacridine 143 was subsequently brominated with Br₂ in glacial AcOH generating the brominated pyrroloacridine 144 (Scheme 3.14). Examination of the ¹H NMR spectrum revealed the absence of the H4 proton, indicating that substitution had occurred. The LREI-MS
also showed isotopic molecular ion peaks at $m/z$ 313 and 315 ($[M]^+$ and $[M + 2]^+$), characteristic of the presence of one bromine.
This chapter reported the successful synthesis of 17 novel pyrrolo[3,2,1-ij]quinoline-1,2-diones including one novel acridine- and seven novel phenanthridine-based polycyclic isatins through metal-catalysed cross-coupling reactions and the Stolle isatin synthesis. The facile synthesis of the pyrrolo[3,2,1-ij]quinoline-1,2-diones confirms that this series of compounds have a decreased amount of ring strain compared to the pyrrolo[3,2,1-hi]indole-1,2-diones discussed in Chapter 2. As theoretically predicted using Material Studios software (Section 2.2.4), synthesis of the pyrrolo[3,2,1-ij]quinoline-1,2-diones was more facile and this was a reflection of their lower calculated $\Delta H_f$ values compared to those of pyrrolo[3,2,1-hi]indole-1,2-diones.

Evaluation of the cytotoxic activity of the newly generated 6,5,6-fused tricyclic and polycyclic isatins and two cyclisation precursors (115 and 136) are presented in Chapter 6, along with a preliminary assessment of their possible modes of cytotoxic action by DNA intercalation.

In the succeeding chapter, discussion will focus on the design, synthesis and evaluation of isatin derivatives with acid-labile linkers. These drug-linker compounds were designed to be suitable for attachment to a targeting protein with the ultimate aim of selective delivery and release of the cytotoxic isatin derivative within tumour cells.
4.1 Introduction to Acid-Labile Linkers

Ligand-targeted drug or prodrug delivery is currently one of the most challenging areas in pharmaceutical research and involves the judicious choice of the drug, linker and targeting ligand. The entire conjugate must then be transported intact through the systemic circulation and pass several complex barriers en route to the target site before it becomes activated. In chemotherapy, drug conjugates that are non-toxic to the host, stable in the circulatory system and then readily cleaved at the target site are often used for this purpose. Previously, it was believed that the tumour specificity of anti-cancer agents could be improved by linking the drugs directly to a targeting moiety via non-cleavable amide bonds. However, in many cases the conjugates lacked potency and were less cytotoxic than the unconjugated drugs themselves. Over the past decade however, the significance of the chemical link between the drug and the carrier molecule in relation to its pharmacological activity has come to the forefront. A promising strategy involves conjugating a cytotoxin with a tumour-targeting protein through an acid-labile linker that is stable at physiological pH. Internalisation at the target tumour site via processes such as RME exposes the conjugate to the acidic environment of the endosomes or lysosomes (pH 4.5-6.0), resulting in selective release of the original cytotoxin inside the tumour cell (e.g. Figure 4.1).
Figure 4.1 Schematic representation of a cytotoxin bound to the exposed lysine residues of a tumour-targeting protein via an acid-labile linkage such as an imine, hydrazone or oxime. Upon receptor-mediated endocytosis of the conjugate at the target tumour, the native cytotoxin is selectively released inside the tumour cell.

Over the years, a wide variety of functional groups have been employed in the development of linkers that are stable at physiological pH (7.4), but cleave at endosomal/lysosomal pH including acid-sensitive hydrazones, cis-aconityl groups (see Section 1.6), trityl groups, orthoesters and N-ethoxybenzylimidazoles. The hydrazone linker has, however, been reported to cleave at non-target sites and at pH 7.4 which suggests that this linker may be unstable in the circulatory system. Other less commonly employed acid-labile linkers include acetals, ketals, oximes and imines.

Imine linkers are not widely used in drug delivery due to their perceived
instability, however, recent studies have shown that aromatic imines with extended $\pi-\pi$ conjugation are stable at physiological pH, while readily hydrolysing in mildly acidic solutions (e.g. pH 4.5-6.8). The macrocyclic antibiotic amphotericin B (AMB) has been conjugated to poly(ethylene glycol)s (PEG) of varying lengths through an imine linker (Figure 4.2). All conjugates were stable at physiological pH and temperature, with only 5% of AMB being hydrolysed after 24 h. Conversely, at pH 5.5, AMB was rapidly released and the conjugates exhibited half-lives between 2-45 min. The rate of hydrolysis was dependent on substitution patterns on the benzene ring and not on the molecular weight of the PEG linker.

![Figure 4.2](image-url)
More recently, Müller et al. have described the conjugation of a fluorophore, 7-amino-4-methylcoumarin (AMC), to the self-immolative p-aminobenzzyloxycarbonyl linker system (Figure 4.3), which is activated under acidic conditions to release AMC. At pH 5.0, the imine bond was rapidly cleaved \((t_{1/2} = 17-173 \text{ min})\), whereas at pH 7.4 the 12 compounds displayed greater stability \((t_{1/2} = 8-365 \text{ h})\). For acid-sensitive anti-cancer prodrugs, long-term stability at pH 7.4 \((t_{1/2} > 48 \text{ h})\) is vital and in this case, two of the model AMC conjugates exhibited efficient hydrolysis rates at pH 5.0 together with long-term stability at physiological pH.

![Figure 4.3](structure.png)

**Figure 4.3** Structure of the imine-linked 7-amino-4-methylcoumarin conjugate.

4.2 Initial Synthetic Targets

As described in the Introduction (Chapter 1), our research group has previously described the generation of a series of over 35 highly potent \(N\)-substituted isatin-derived cytotoxins, several of which show nanomolar cytotoxicity against a diverse panel of human cancer cell lines while being inactive towards human mononuclear cells (Figure 4.4). We were interested to develop a strategy to conjugate these potent cytotoxins via a pH-sensitive linker to the exposed lysine residues of tumour-targeting proteins (e.g. Figure 4.1) such as Tf, PAI-2 and the antibody Herceptin, which is...
selective for the HER2 receptor and is overexpressed in 20-30\% of human breast carcinomas.\(^3\)

![Chemical structures](image)

**Figure 4.4** Cytotoxic activity of isatin (11) and its derivatives against the human monocyte-like histiocytic lymphoma U937 cell line.\(^{64,65,81}\)

The various isatin-based cytotoxins developed within the group are derivatised on both the aromatic ring and via \(N\)-substitution.\(^{64,65,81}\) Therefore, the aim of this study was to generate a pH-sensitive linker that did not interfere with these areas of the molecule. Within our research group we have previously conjugated a \(N\)-alkylisatin derivative to PAI-2 and Tf through a succinate linker (Figure 4.5).\(^{291}\) The succinate linker is degraded enzymatically by esterases present within the lysosomes of tumour cells. The limitation of this linker system is that the succinate bond is susceptible to hydrolysis from non-specific esterases.
Therefore, to improve our conjugation method, we decided to create a pH-sensitive linker which would capitalise the available carbonyl group at C3, which is present in all of the above mentioned isatin-derived cytotoxins. This could be achieved using an imine linker. As described later in Chapter 6, the most potent isatin analogues turned out to be the 5,7-dibrominated N-alkylisatins rather than the newly prepared, tricyclic/polycyclic derivatives. For this reason, 5,7-dibromo-N-(p-methoxybenzyl)isatin (24) (Figure 1.10), which has an IC$_{50}$ value of 1.83 µM against U937 cells,$^{64}$ was chosen as the starting cytotoxin, in addition to its ready synthetic availability and structural simplicity, which facilitates product characterisation via NMR spectroscopy. While 24 is less active than the most promising N-substituted-5,7-dibromoisatin cytotoxins (IC$_{50}$ = 1.83 µM vs. < 1 µM respectively),$^{64}$ the bioconjugation strategy presented here should apply equally well to any of the N-substituted isatin-derived cytotoxins, all of which contain an available carbonyl at C3. This strategy is also expected to apply to the bioconjugation of well known anti-cancer agents such as epipodophyllotoxin (145), camptothecin (146) and colchicine (147) to enhance their effectiveness even further.$^{292,293}$ (Figure 4.6).
Chapter 4 – Acid-Labile Linkers

Figure 4.6 Structures of known carbonyl-containing anti-cancer agents (145-147) which could be used for bioconjugation strategies via an imine linker.

Reaction of the isatin-derived cytotoxin 24 with a bifunctional linker such as the anilino carboxylic acids 148-152, would give the aryl imine-linked cytotoxins 153-157 (Scheme 4.1). These compounds, with a free carboxylic acid available for coupling to the exposed lysine residues on a tumour-targeting protein, would be expected to be stabilised by extended resonance delocalisation. Furthermore, it is expected that acid-catalysed hydrolysis of the imine-linked cytotoxins would result in selective release of the original cytotoxin (Figure 4.1). This would be an improvement over several of the existing pH-sensitive bioconjugation strategies, where hydrolysis of the linker generates a modified derivative, which is often of lower potency than the original cytotoxin. This was observed when doxorubicin (4) was conjugated through a N-ethoxybenzylimidazole linker. The modified doxirubicin derivative released under acidic conditions displayed an IC\textsubscript{50} value of ~ 12 µM against human ovarian carcinoma 2008 cells, while doxorubicin (4) exhibited greater than 10-fold lower IC\textsubscript{50} value against the same cell line.\textsuperscript{283}

Prior to development of a cytotoxin-protein conjugate, it is important to conduct preliminary model studies to demonstrate the viability of the proposed selective imine hydrolysis strategy for ultimately releasing the cytotoxin in vivo.
Thus, the cytotoxic isatin derivative 24 was coupled to a N- and C-terminii protected L-lysine amino acid residue via incorporation of the bifunctional, acid-amine linkers (148-152) (Scheme 4.1), and their hydrolytic efficiency was evaluated using UV-Vis spectrophotometry. Herein, is the first instance of the pH-dependent selective release of a potent N-alkylisatin-derived cytotoxin through the acid-catalysed hydrolysis of an imine linker.

4.3 Synthesis of 3-Iminoisatins

The five novel aryl imine derivatives 153-157 were readily prepared by the acid-catalysed reaction of the potent cytotoxin, 5,7-dibromo-N-(p-methoxybenzyl)isatin (24), with a range of commercially available anilino carboxylic acids (148-152), differing in terms of their aromatic substitution patterns and by the length of the linker between the aromatic ring and the carboxylic acid moiety (Scheme 4.1). For this study, para- and meta-benzoic (148, 151), para- and meta-phenylacetic (149, 152) and para-phenylpropionic (150) anilino carboxylic acids were selected. The isatin 24 and the appropriate acids 148-152 were heated at reflux in MeOH or EtOH together with a catalytic amount of AcOH for 1.5 h to yield the imines 153-157. Reactions in this study proceeded in moderate to high yield, with the phenylacetic and phenylpropionic acids (e.g. 154, 155, 157) affording higher yields than 153 and 156 (68-71% cf. 18-56% respectively). Imines 154-157 were produced in higher yields with the higher boiling EtOH rather than MeOH (56-71% cf. 14-18% respectively), while 153 was the only imine to favour MeOH over EtOH (18% cf. 11% respectively). Lack of the AcOH catalyst led to a decrease in chemical yield (18% cf. 13% for 156 when using MeOH as the solvent). There was also no evidence, as seen by
1H NMR spectroscopy, (DMSO-d6) of self-cleavage of the imines 153-157 to the free isatin 24.

Reagents and conditions: a) EtOH, AcOH (cat.), reflux, 1.5 h, 18-71%; (PMB = para-methoxybenzyl).

**Scheme 4.1** Synthesis of the 3-iminoisatins 153-157.

Imines 153-157 were obtained as mixtures of E and Z isomers about the imine bond which were unable to be separated by either TLC or HPLC, as has been reported for other 3-arylimino-2-indolinones. The 1H NMR spectra of the imines 153-157 in DMSO-d6, revealed that most compounds (153-156) were synthesised as approximately 1:1 mixtures of E/Z isomers, with the exception of the meta-phenylacetic imine (157) where the E isomer was favoured over the Z isomer in an approximate 2:1 ratio. The E stereochemistry was assigned to a particular isomer based on the following: the signal from H4 (on the isatin core)
of the $E$ isomer in the $^{1}H$ NMR spectrum was shifted upfield 1.2-1.5 ppm relative to the H4 signals of the $Z$ isomer and the parent compound 24, presumably due to shielding by the ring current on the iminophenyl ring (Figure 4.7). The isomeric ratios of each compound were therefore determined by comparing the size of the integrals from the H4 signals of the $E$ and $Z$ isomers, as has been described previously by our research group for a related series of compounds.\textsuperscript{81} Although some 2-indolinones containing a substituent at C3 have been described as predominantly $E$ isomers,\textsuperscript{76} elsewhere it has been reported that the $E$ and $Z$ isomers of 3-imino-2-indolinones interconvert rapidly in solution at room temperature and that the ratio of isomers is solvent dependent.\textsuperscript{294}

![Figure 4.7 Differences in chemical shift between the E and Z isomers of 3-iminoisatin 153.](image)

The $^{1}H$ NMR spectrum of the para-propionic imine 155 displayed 40 protons, 20 for each isomer. Characteristic peaks included the eight propionic protons ($E$ and $Z$) at 2.55-2.89 ppm and the carboxylic acid proton at 12.13 ppm for both isomers. Similarly, four propionic carbons ($E$ and $Z$) were observed at 30.7-36.5 ppm in the $^{13}C$ NMR spectrum, together with the carboxylic acid carbon at 174.5 ppm for both isomers. Analysis by HRESI-MS confirmed the
molecular weight of 155 by finding a mass of \( m/z \) 526.9722, consistent with the molecular formula (\( C_{24}H_{19}N_2O_{79}Br_{81}[\text{MHCO}_2\text{H}] \)).

The imines 154-157 were formed in good yields (56-71%), whereas 153 was obtained in only 18% yield. This presumably results from the reduced nucleophilicity of the amino moiety in 153, relative to that in 154-157, due to the strong electron withdrawing effect of the para-substituted carboxyl group (Figure 4.11).

### 4.4 Synthesis of Isatin-Lysine Conjugates

The imino acids (153-157) were then coupled to a \( N- \) and \( C- \) protected L-lysine amino acid residue under standard carbodiimide coupling procedures,\(^{295,296}\) utilising \( N,N' \)-dicyclohexylcarbodiimide (DCC), 1-hydroxybenzotriazole hydrate (HOBt) and \( N,N \)-diisopropylethylamine (DIPEA) to yield the desired novel imine-lysine conjugates 158-162 in good to excellent yields (Scheme 4.2).
Once again, the $^1$H NMR spectra (CDCl$_3$) of the isatin-lysine series (158-162) revealed that most compounds (158-161) were synthesised as approximately 1:1 mixtures of $E/Z$ isomers, apart from the meta-phenylacetic analogue (162) where the $Z$ isomer was favoured over the $E$ isomer in an approximate 2:1 ratio, the reverse ratio to that observed for the precursor imine 157. This may, however, be due to the change in NMR solvent from DMSO-$d_6$ with 157 to CDCl$_3$ with 162, as has been observed for other imines.$^{294}$

The $^1$H NMR spectrum of the para-propionic lysine 160 displayed 72 protons, 36 for each isomer. Characteristic peaks included a multiplet at 1.29-1.82 ppm, integrating for 12 protons and ascribed to H3, H4 and H5 for both the $E$ and $Z$ isomers. Additionally, the amide proton (CH$_2$CONH) of 160 was observed at 6.32 ppm for both isomers. In the $^{13}$C NMR spectrum, ten of the

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*Reagents and conditions: a) DCC, HOBr, DIPEA, Ac-LysOMe.HCl, CH$_2$Cl$_2$, RT, 24 h, 41-82% (PMB = para-methoxybenzyl).*

**Scheme 4.2** Synthesis of the isatin-lysine conjugates 158-162.
lysine carbons (C2-C6, E and Z) contained signals between 22.5-52.1 ppm. Analysis by HRESI-MS confirmed the molecular weight of 160 by finding a mass of m/z 779.0896 for the molecular formula C_{34}H_{36}N_4O_6^{79}Br^{81}BrNa [M+Na]^+.

### 4.5 Hydrolysis of 3-Iminoisatins and Isatin-Lysine Conjugates

The rate of acid-catalysed hydrolysis in the imino acids 153-157 as a function of imine absorbance at λ 435 nm over time is shown in Figure 4.8A (middle panel). Analysis of the hydrolysis kinetics conducted at pH 4.5 (37 °C) indicated an exponential first order decay process as evidenced by the ln(A/A_0) versus time plots (Figure 4.8A, bottom panel), which showed a linear relationship at the initial reaction times. No hydrolysis was detected for the derivatives 153-157 when maintained at physiological pH (7.4) in 1 M HEPES buffer at 37 °C over a 240 min period (the first 60 min shown in Figure 4.8A, top panel). Although compounds 153-157 were monitored kinetically over a 240 min period, results are only displayed over a 60 min period as all derivatives reached a plateau in terms of hydrolysis in less than 240 min.
Figure 4.8 Rates of hydrolysis expressed as a function of absorbance ($A/A_o$) over time (pH 7.4 top panel and pH 4.5 middle panel) and the natural log of absorbance ($\ln(A/A_o)$) over time (bottom panel, pH 4.5), measured at $\lambda$ 435 nm in 1 M sodium acetate buffer at 37 °C for (A, middle panel): the imino acid derivatives 153-157 (*$P < 0.05$ for 153 versus 154-157; 155 versus 154, 156 and 157); and (B, middle panel): the imino lysine derivatives 158-162 (*$P < 0.05$ for 158 versus 159-162, 159 versus 160-162, 160 versus 161 and 162).

In addition to the hydrolysis experiments conducted at physiological temperatures (37 °C), selected hydrolyses for 153, 154 and 156 were also conducted at ambient temperature. As expected, the rates of hydrolysis at pH 4.5 for the imino acids 153, 154 and 156 were significantly faster at 37 °C than at room temperature (23 °C) as judged by one-way, repeated measures ANOVA followed by a Tukey post-test (Figure 4.9). No hydrolysis was detected at physiological pH at either 23 °C or 37 °C.
Figure 4.9 Rates of hydrolysis at pH 4.5 expressed as a function of absorbance ($A/A_o$) over time (top panel) and the natural log of absorbance ($\ln(A/A_o)$) over time (bottom panel), measured at $\lambda$ 435 nm in 1 M sodium acetate buffer at both 23 °C and 37 °C for the imino acid derivatives: (A) 153 (*$P < 0.05$); (B) 154 (*$P < 0.05$); and (C) 156 (*$P < 0.05$).

The first order rate constants, $k$, for the imino acids 153-157, determined by non-linear regression, ranged from $0.95-3.99 \times 10^{-2}$ min$^{-1}$ (Table 4.1), which is comparable to those reported in an earlier study by Kalavska et al. of acid-catalysed hydrolysis of some substituted derivatives of 3-phenyliminoxindole at pH 2.0-5.0.$^{297}$ Although it should be noted that these derivatives differ from those herein in that they are neither $N$-alkylated nor halogenated on the isatin aromatic ring, and bear different imino aryl substituents at the C3 position. The fastest hydrolysis rate, as indicated by the largest $k$ value, was observed for the para-phenylpropionic acid derivative 155, with the least amount of resonance stabilisation in the series and a half-life of 17.4 min. Conversely, the smallest $k$ value was found for the most conjugated (stabilised) derivative, the para-benzoic acid derivative 153, which displayed a 4-fold greater half-life of 72.6 min. As discussed earlier, this compound had the lowest yield of
imine formation (18%). In contrast, imines which demonstrated efficient hydrolytic release (154, 156, and 157) were formed in good yields (> 68%). These three imino acid derivatives did not show any significant differences to one another in their hydrolysis rates, as judged by one-way ANOVA, and displayed half-lives ranging from 24.3-29.7 min.

Table 4.1 Rate constants ($k$) and half-lives ($t_{1/2}$) for the hydrolysis of the imino acids 153-157 and the imino lysines 158-162 at pH 4.5.\textsuperscript{a}

<table>
<thead>
<tr>
<th>Imine</th>
<th>$n$</th>
<th>Substitution pattern</th>
<th>$k$ (min$^{-1}$)</th>
<th>$t_{1/2}$ (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>R = OH</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>153</td>
<td>0</td>
<td>para-</td>
<td>0.95 ± 0.0 × 10$^{-2}$</td>
<td>72.6</td>
</tr>
<tr>
<td>154</td>
<td>1</td>
<td>para-</td>
<td>2.85 ± 0.2 × 10$^{-2}$</td>
<td>24.3</td>
</tr>
<tr>
<td>155</td>
<td>2</td>
<td>para-</td>
<td>3.99 ± 0.3 × 10$^{-2}$</td>
<td>17.4</td>
</tr>
<tr>
<td>156</td>
<td>0</td>
<td>meta-</td>
<td>2.33 ± 0.2 × 10$^{-2}$</td>
<td>29.7</td>
</tr>
<tr>
<td>157</td>
<td>1</td>
<td>meta-</td>
<td>2.55 ± 0.2 × 10$^{-2}$</td>
<td>27.2</td>
</tr>
<tr>
<td>R = (CH$_2$)$_4$CH(NHAc)CO$_2$Me</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>158</td>
<td>0</td>
<td>para-</td>
<td>0.81 ± 0.0 × 10$^{-2}$</td>
<td>85.2</td>
</tr>
<tr>
<td>159</td>
<td>1</td>
<td>para-</td>
<td>3.02 ± 0.2 × 10$^{-2}$</td>
<td>23.0</td>
</tr>
<tr>
<td>160</td>
<td>2</td>
<td>para-</td>
<td>4.07 ± 0.2 × 10$^{-2}$</td>
<td>17.0</td>
</tr>
<tr>
<td>161</td>
<td>0</td>
<td>meta-</td>
<td>2.03 ± 0.1 × 10$^{-2}$</td>
<td>34.1</td>
</tr>
<tr>
<td>162</td>
<td>1</td>
<td>meta-</td>
<td>2.10 ± 0.1 × 10$^{-2}$</td>
<td>33.0</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Rates of hydrolysis were measured at 37 °C in 1 M sodium acetate buffer using UV-Vis spectrophotometry ($\lambda$ 435 nm).

A similar trend in the hydrolysis rates was obtained for the imino lysine conjugates 158-162 (Figure 4.8B) with first order rate constants, $k$, ranging from 0.81-4.07 × 10$^{-2}$ min$^{-1}$ (Table 4.1). Again, the fastest hydrolysis rate was
observed for the para-phenylpropionic acid derivative 160 with a half-life of 17.0 min, and the lowest hydrolysis rate was found for the para-benzoic acid derivative 158, which had a 5-fold greater half-life of 85.2 min. In this case, the hydrolysis rates of all but 161 vs. 162 were found to be significantly different from one another, as judged by one-way ANOVA (Figure 4.8B, middle panel), with the para-phenylacetic acid derivative 159 significantly faster than both 161 and 162, with half-lives of 34.1 and 33.0 min respectively, compared to 23.0 min for 159 (Table 4.1). As for the imino acids 153-157, no hydrolysis was detected for the imino lysine derivatives 158-162 maintained at physiological pH (7.4) in 1 M HEPES buffer at 37 °C over a 240 min period (Figure 4.8B, top panel).

The rates of hydrolysis at pH 4.5 for the imino acids and their analogous imino lysine derivatives (e.g. 153 vs. 158, 154 vs. 159, etc) were not significantly different from one another. This can be seen, by comparing the hydrolysis rates of each pair of derivatives (e.g. 153: \( k = 0.95 \times 10^{-2} \text{min}^{-1} \) cf. 158: \( k = 0.81 \times 10^{-2} \text{min}^{-1} \)), which showed no significant difference as judged by one-way, repeated measures ANOVA followed by a Tukey post-test (Figure 4.10). The overlayed graphs in Figure 4.10, clearly show that the distal part of the compounds, whether a carboxylic acid or extended chain with a protected lysine residue, did not significantly contribute to the overall hydrolysis rates observed. Hydrolysis of the various derivatives was found to depend more on the aromatic substitution pattern and linker length.
Figure 4.10 Rates of imine hydrolysis at pH 4.5 expressed as a function of absorbance ($A/A_0$) over time measured at $\lambda$ 435 nm, in 1 M sodium acetate buffer at 37 °C for the imino acid and imino lysine derivatives. The graphs show the data paired in the following combinations: 153 vs. 158; 154 vs. 159; 155 vs. 160; 156 vs. 161; and 157 vs. 162, with *$P > 0.05$ for all five combinations shown.

Acid-catalysed hydrolysis of imines involves the protonation of the imine to give an iminium species, which undergoes nucleophilic attack by water. This gives an unstable hemiaminal intermediate, which upon protonation of the amine nitrogen, readily collapses to the protonated ketone and free amine, with subsequent proton loss furnishing the ketone $^{298}$ (Scheme 4.3).
Scheme 4.3 General mechanism for the hydrolysis of imines to the corresponding carbonyl in the presence of acid. Example 153 is shown above where PABA = para-aminobenzoic acid, R and R’ = isatin nucleus.

The crucial first step in the above mechanism, imine protonation, is largely dependent on the basicity of the imine. In derivatives 153 and 158, the electron withdrawing carbonyl group directly attached to the para-position on the phenyl ring, will reduce the relative basicity of the imine nitrogen through conjugation (Figure 4.11), leading to lower rates of hydrolysis as observed (Figure 4.8, middle panel). Conversely, for the homologous 154 and 159 derivatives, conjugation with the acid or amide carbonyl is disrupted leading to greater availability of the nitrogen lone pair of electrons towards protonation. Furthermore, hyperconjugation in these derivatives could be involved in increasing electron density on the carbon adjacent to the imine nitrogen and thereby its relative basicity. This hyperconjugative interaction could be more significant with the para-propionic derivatives 155 and 160, with the counter delocalising effect of the carbonyl group adjacent to the negative charge on the Ar-CH carbon being removed. The hyperconjugative effects associated with these
groups in the meta-position would not be expected to have a marked effect on the imine basicity.

![Figure 4.11](image)

**Figure 4.11** Resonance contributors of the imine 153 showing extended \( \pi \) delocalisation.

Delocalisation of electron density through the extended conjugation system of 153/158 also gives rise to an additional resonance contributor (compared to those in 154-157 and 159-162) that places the electron density on the oxygen atom of the carboxyl moiety (Figure 4.11). This resonance structure is energetically more favourable than those of the other isatins where the electron density is placed on a carbon atom, and may therefore contribute to the marked increase in stability for 153/158. It is anticipated that this extended delocalisation in 153 and 158 would reduce the susceptibility of the imine to nucleophilic attack by water in the second step of the hydrolysis mechanism (Scheme 4.3).

The analogous meta-substituted derivatives 156/161 would not show the same resonance effects described above, while the phenylacetic acid derivatives 154/159 and 157/162, with an extra carbon between the aromatic ring and carbonyl of the acid or amide would also display less delocalisation of the imine bond. The para-phenylpropionic acid derivatives 155 and 160, with a two carbon
linker between the aromatic ring and carbonyl of the acid/amide, have the least amount of electron delocalisation possible and consequently display the fastest rates of hydrolysis from this series. These results are consistent with complementary studies in the literature on 3-phenyliminoxindole derivatives, where it was found that electron donating groups at the para-position of the aryl ring enhanced the rates of acid-catalysed hydrolysis.\(^{297}\) Conversely, the incorporation of electron withdrawing groups on a series of related aryl imines were found to have a stabilising effect at physiological pH.\(^{289}\) Thus, the incorporation of other electron donating or withdrawing groups would allow for more tunable hydrolysis rates of imine-based compounds.

In conclusion, adjusting the linker length was found to be more important in determining hydrolysis rates in the para-series compared to the meta-series. For both the para-substituted carboxylic acid derivatives 153-155 and the lysine derivatives 158-160, lengthening the linker resulted in a significant increase in the hydrolysis rate. However, the two meta-substituted derivatives in each series (156, 157 and 161, 162), showed no significant difference in their hydrolysis rates. Overall, it appears that electronic effects such as the presence or absence of a conjugated electron withdrawing group may play a larger role in determining the hydrolysis rate than extending the linker.

Using \(N\)- and \(C\)-protected lysine residues as surrogates for the free lysine residues on tumour-targeting proteins, it was successfully demonstrated that these model imine-linked isatin-based cytotoxin conjugates undergo hydrolysis at endosomal/lysosomal pH. These model conjugates were readily prepared and characterised and are stable at physiological pH. With these results in hand, the next part of the project involved conjugating the isatin-based cytotoxin to ligands
such as PAI-2 and Tf.
5.1 Introduction to Cytotoxin-Protein Conjugates

Conventional chemotherapeutics are typically small molecules (< 1000 Da) capable of diffusing into cells within the human body. Unfortunately, these small molecules are characteristically non-specific and will diffuse into healthy and tumour cells indiscriminately, leading to the death of non-tumour tissue and side effects such as hair loss and organ toxicity. To overcome this problem, cytotoxins can be conjugated to macromolecules such as proteins via a linker group that is cleaved intracellularly. As described in the Introduction, although much work has focused on mAbs, they have drawbacks associated with their use and new tumour-targeting ligands are currently being sought.

As described in Section 1.7.3, the uPA system has appeal as a target for cancer treatment. The system’s endogenous inhibitor, PAI-2, was recently attached for the first time to a cytotoxin [2'-deoxy-5-fluorouridine (5-FUdr)] via an esterase-labile succinate linker to form the conjugate (5-FUdrsucc-PAI-2) (Figure 5.1). Up to 7 molecules of 5-FUdrsucc were able to covalently bind to PAI-2 without compromising the inhibitory activity of the protein. On average, the conjugate contained 3 molecules of 5-FUdrsucc, which was sufficient to display a 4-fold increase in \textit{in vitro} cytotoxicity against MDA-MB-231 breast cancer cells when compared to the free drug. The 5-FUdrsucc-PAI-2 conjugate (163) also displayed specificity towards the uPA over-expressing metastatic cell line MDA-MB-231, compared to the low uPA expressing, non-metastatic cell line MCF-7.
Another protein which has been widely exploited for targeting cytotoxins to cancer cells is the iron-ferrying glycoprotein Tf (see Section 1.7.2). One of the most recent studies of drug-Tf conjugates was concerned with the attachment of artemisinin via a hydrazone linker to the $N$-glycosidic chains of Tf\textsuperscript{129} to yield the conjugate 39 (Figure 5.1). Approximately 16 molecules of artemisinin were conjugated to Tf as determined by MALDI-TOF mass spectrometry. The conjugate 39 was lethal to DU 145 prostate tumour cells in a TfR-dependent manner and its cytotoxicity did not depend on cell density.\textsuperscript{129}

![Figure 5.1](image)

**Figure 5.1** Structure of the first cytotoxin-PAI-2 conjugate (5-FUdrsucc-PAI-2 conjugate) (163); artemisinin-Tf conjugate (39).

This chapter describes the novel cytotoxin-protein conjugates formed from the targeting ligands PAI-2 and Tf and an imine-linked isatin-based cytotoxin in an analogous manner to the model studies described in Chapter 4. The designated 3-iminoisatin was conjugated through the available/surface lysine residues of each protein. The 3-iminoisatin was conjugated to Tf via its lysine residues rather than its
Chapter 5 – Isatin-Protein Conjugates

N-glycosidic chains, since this type of conjugation has also proved to result in increased cytotoxicity compared to the free drug alone. The chemical lability of the imine-linked conjugates were assessed under varying pH conditions. The in vitro selectivity and cytotoxicity of the isatin-protein conjugates will be discussed in Chapter 6.

5.2 Synthesis of Isatin-Protein Conjugates

The 3-iminoisatin 155 displayed the most favourable hydrolytic profile and shortest half-life (17.4 min) at λ 435 nm in the model studies described in Section 4.5. For this reason, 155 was selected for conjugation to the two proteins under investigation, PAI-2 and Tf. The ability of the drug conjugate to hydrolyse under acidic conditions was analysed using UV-Vis spectrophotometry.

5.2.1 Conjugation of the 3-Iminoisatin 155 to the Targeting Ligands PAI-2 and Tf

5.2.1.1 Preparation of the Active Ester

The active ester of 155 was prepared using conditions previously reported for cytotoxin-protein conjugates. Briefly, the carboxyl group of 155 was activated by being subjected to DCC/HOBt (similar to the peptide coupling conditions in Scheme 4.1), forming the active ester 164 (Scheme 5.1). As described by Vine, the active ester is readily formed after 30 min and there is no need for the reaction to proceed for 3 h, as reported by Krauer et al. The active ester 164 was used without further purification.
Reagents and conditions: (a) HOBt, DCC, DMF, 0 °C → RT, 30 min.

**Scheme 5.1** Preparation of the active ester 164 from the 3-iminoisatin 155.

5.2.1.2 Conjugation to PAI-2 or Tf

A 20-fold molar excess of the activated ester 164 was added to PAI-2 (or Tf) at pH 8.5 with continual shaking for 3 h, to yield the isatin-PAI-2 (165) or isatin-Tf conjugate (166) (Scheme 5.2). The 20-fold molar excess of 155 was selected to ensure maximum conjugation to the protein. Upon completion of the reaction, the freshly prepared isatin-protein conjugate (165 or 166) was centrifuged at 13,000 rpm for 5 min to remove any precipitated 155 and other insoluble side products such as ureas formed from DCC and HOBt during the peptide coupling. The supernatant (containing the isatin-protein conjugate) was purified by size-exclusion chromatography on a PD10 column and the eluted fractions were stored at 4 °C until required. The protein yield was > 75% after purification for both conjugates.
Reagents and conditions: (a) PAI-2 or Tf, PBS pH 8.5, RT, 3 h.

Scheme 5.2 Preparation of the isatin-protein conjugates 165 and 166.

5.3 Attempted Characterisation and Hydrolysis of Isatin-Protein Conjugates

5.3.1 Isatin-PAI-2 Conjugates

Following the purification of the isatin-PAI-2 conjugate (165) using size-exclusion chromatography, UV-Vis spectrophotometry was used to visualise the eluted fractions for evidence of protein conjugation. Column fractions were monitored at two wavelengths simultaneously, λ 280 nm (corresponding to the absorbance maximum for the protein, PAI-2) and λ 432 nm (corresponding to the absorbance maximum for the isatin that appeared furthest from the protein maximum). The elution profile of the conjugate 165 is shown in Figure 5.2. Fractions 1-5 and 9-11 showed a similar pattern of absorbance readings between the protein and isatin at λ 280 and 432 nm, respectively. The isatin-PAI-2 conjugate (165) eluted in fractions 6-8 as determined by the simultaneous increases in
absorbancies at λ 280 and 432 nm. The concentration of PAI-2 in the conjugate 165 was measured using the Lowry protein determination assay.

![Figure 5.2](image_url)  
**Figure 5.2** Typical size-exclusion chromatography elution profile of the isatin-PAI-2 conjugate (165).

Electrospray ionisation mass spectrometry was employed to further characterise the isatin-PAI-2 conjugate (165). A freshly prepared sample of the conjugate and a sample of PAI-2 in PBS pH 7.4, were dialysed overnight into 10 mM NH₄OAc buffer to yield a final protein concentration of ~10 µM. The results from the mass spectral analyses are presented in Figure 5.3, whereby the data is expressed on a \( m/z \) scale, and the masses have been converted into Da. The mass spectrum of PAI-2 (Figure 5.3A) reveals the expected protein peaks at \( m/z \) 2971.94 and 3184.69, corresponding to a molecular mass of 44,568 Da. This mass is consistent with the molecular weight of PAI-2.\(^{302}\) The isatin-PAI-2 conjugate (165) displayed a very similar mass spectrum to PAI-2 (Figure 5.3B cf. 5.3A), and the weight of the conjugate 165 was calculated to be 44,677 Da. A difference of 109 Da was observed between the mass of the conjugate 165 and PAI-2, however, this does
not correlate with the combined molecular weight of the isatin 24 (425 Da) and the linker (147 Da, total 572 Da). It was thought that the imine-linker may have cleaved from the isatin 155 under the MS conditions and attached to PAI-2, although the mass of the imine-linker is 147 Da, so this also does not correlate to the difference in mass observed between Figure 5.3B and 5.3A. It was postulated that the imine bond may be too unstable under the analysis and therefore altering the mass spectrometry results.\textsuperscript{103} To overcome this, the conjugate 165 was reacted with NaBH\textsubscript{4} in an attempt to reduce the imine bond within the molecule to an amine, which would be more stable when subjected to mass spectrometry. However, the mass spectrum from this experiment did not contain evidence of the conjugate 165 so it cannot be concluded that the amine bond formed at all. Therefore, the results from mass spectral analyses were inconclusive and the method could not be used to determine the number of isatin molecules conjugated to PAI-2.

\textbf{Figure 5.3} A) Mass spectrum of PAI-2. B) Mass spectrum of the isatin-PAI-2 conjugate (165).
Other characterisation methods such as SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) cannot be used effectively due to the molecular weight difference between the protein and the conjugate being too small. To investigate whether the isatin-PAI-2 conjugate (165) was able to be hydrolysed under acidic conditions, a kinetic study using UV-Vis spectrophotometry was employed. Similar to the hydrolysis studies of 153-157 and 158-162 in Chapter 4, the isatin-PAI-2 conjugate (165) was monitored kinetically at λ 435 nm for 240 min at 37 ºC at pH 4 and pH 7. As shown in Figure 5.4, the conjugate 165 was stable at pH 7 for at least 240 min. On the contrary, the conjugate 165 appears to display an inverse hydrolytic profile at pH 4. The sample was stable for approximately 160 min before there was a gradual, yet consistent increase in absorbance. This result is the complete opposite of what it expected when hydrolysis occurs. One explanation for this result may be that there is hydrolysis of the conjugate 165 at pH 4 and that the precipitation of the isatin 24 (or the protein) is interfering with the light path of the spectrophotometer.
Figure 5.4 Results of the kinetic hydrolysis studies for the isatin-PAI-2 conjugate (165) as determined by UV-Vis spectrometry at λ 435 nm. The study was performed at pH 4 and pH 7 for 240 min at 37 °C.

5.3.2 Isatin-Tf Conjugates

The synthesis of the isatin-Tf conjugate (166) was identical to that for the isatin-PAI-2 conjugate (165) and was also purified using size-exclusion chromatography. The collected fractions were visualised by UV-Vis spectrophotometry for evidence of protein conjugation. The elution profile of the conjugate 166 is shown in Figure 5.5. Fractions 5-8 revealed the presence of the conjugate 166 due to the coincident elevated absorbance readings detecting the protein and isatin at λ 280 and 432 nm, respectively. Hence, it was confirmed that the isatin 155 was conjugated to Tf. The concentration of Tf in the conjugate 166 was calculated using the Lowry protein determination assay.
The isatin-Tf conjugate (166), was dialysed overnight into 10 mM NH₄OAc buffer before being injected into the ESI mass spectrometer for analysis. The mass spectrum of the conjugate 166 once again was unclear and the difference in molecular weight between the conjugate 166 and Tf was under 500 Da, indicating that the 3-iminoisatin 155 may not have been attached in the MS sample. Attempts to obtain an improved mass spectrum of the isatin-Tf conjugate (166) by changing mass spectrometer parameters or altering sample matrix conditions such as increasing the NH₄OAc concentration (up to 500 mM) or including various salt solutions were unsuccessful. Incidentally, there are very few examples of MS data reported for drug conjugates in the literature.

Once again, the conjugate was reacted with NaBH₄ in an attempt to reduce the imine bond and form the amino analogue. Analysis by mass spectrometry was inconclusive and the isatin-Tf conjugate (166) could not be identified, therefore it is uncertain whether the imine bond had been reduced. The absorbance values of the conjugate 166 were also investigated pre- and post-dialysis (Figure 5.6). It was hypothesised that the dialysis procedure was possibly degrading the conjugate 166. As shown in Figure 5.6, the absorbance values at λ 280 and 432 nm decrease

**Figure 5.5** Typical size-exclusion chromatography elution profile of the isatin-Tf conjugate (166).
post-dialysis, however, the conjugate 166 remains intact which can be verified by the relative proportion of the isatin (λ 432 nm) to the protein (λ 280 nm).

**Figure 5.6** Pre- and post-dialysis absorbance values at λ 280 and 432 nm for the isatin-Tf conjugate (166).

The isatin-Tf conjugate (166) was monitored kinetically at λ 435 nm for 240 min at 37 °C at pH 4 and pH 7 to assess its ability to hydrolyse under acidic conditions, while remaining stable under physiological conditions. As shown in Figure 5.7, the conjugate 166 was stable at pH 7 for approximately 180 min before the absorbance gradually increased. Similarly, the conjugate 166 appears to be stable at pH 4 for 120 min before the absorbance values increase, creating an inverse hydrolytic profile. This outcome is comparable to the results observed with the isatin-PAI-2 conjugate (165) in Figure 5.4, which suggests a technical issue such as precipitation of the isatin 24 interfering with the light path of the spectrophotometer.
Figure 5.7 Results of the kinetic hydrolysis studies for the isatin-Tf conjugate (166) as determined by UV-Vis spectrometry at $\lambda$ 435 nm using a quartz plate. The study was performed at pH 4 and pH 7 for 240 min at 37°C.
The results described within this chapter illustrate the complexities encountered when working with drug-protein conjugates. It is thought that the conjugation of an isatin to a protein may be hindered due to the size and conformation of the protein, which may conceal possible amino acid sites for conjugation. From Chapter 4, it was reported that the imine bond on the isatin could be hydrolysed when attached to a single amino acid residue (e.g. 160), although with the studies reported in this chapter, significant difficulties were encountered in detecting the hydrolysis of the imine bond when 155 was attached to a protein (i.e. 165 and 166). The resistance of the imine bond to cleave under mildly acidic conditions from an isatin-Tf conjugate was also observed previously by Vine. In Vine’s work, which involved a meta-phenylacetic acid imine linker on a cytotoxic N-alkylisatin derivative, the conjugate was observed to be stable at pH 5.5-7.5 for 20 h at RT and hydrolysis of the imine bond was not detected until pH 3. Additionally, it is postulated that the mass spectral characterisation difficulties encountered may stem from the imine bond within the conjugates, as satisfactory mass spectral data were attained previously for isatin-protein conjugates containing a succinate linker.
The ability of the isatin-Tf conjugate (166) to cleave intracellularly under acidic conditions was further investigated using cell proliferation assays. These results are discussed in the following chapter.
6.1 Overview of Testing Procedures

With 23 new tricyclic/polycyclic isatins and six cyclisation precursors (from Chapters 2 and 3), 10 novel 3-iminoisatin derivatives (Chapter 4) and two of our target isatin-protein conjugates (Chapter 5) in hand, our studies turned to the biological investigation of these compounds. Cytotoxicity testing of the cyclisation precursors (49, 65, 75, 76, 115 and 136), pyrrolo[3,2,1-i]indole-1,2-diones (46 and 82), pyrrolo[3,2,1-ij]quinoline-1,2-diones (85-87, 99, 101, 102, 105, 111, 112, 114 and 116), polycyclic isatins (126, 127, 132, 137, 138, 143 and 144), 3-iminoisatins (153-157) and isatin-lysine conjugates (158-162) were carried out on the U937 lymphoma cell line. This cell line has been utilised previously within our research group\textsuperscript{64,65,81} to analyse the \textit{in vitro} cytotoxicity of isatin derivatives. The human THP-1 leukemic and MDA-MB-231 breast adenocarcinoma cell lines were also employed. These three cell lines were selected as they have differing levels of the receptors involved in endocytosing PAI-2 and T\text{f}, i.e. uPA/uPAR and T\text{f}/T\text{fR} respectively. The receptor expression levels follow the pattern as described by Vine \textit{et al.}\textsuperscript{291} Utilising these cell lines it could be determined if the conjugates were being internalised, in which case their cytotoxicity would be expected to be greater in those cell lines expressing higher concentrations of the relevant receptor, i.e. isatin-PAI-2 conjugate (165) would be expected to exhibit the greatest cytotoxicity against the THP-1 cell line, which expresses the highest level of uPA/uPAR.\textsuperscript{291} Cytotoxicity testing was used to identify active compounds based on their IC\textsubscript{50} values.
(concentration required to inhibit 50% of the population) and also provided a clearer understanding of the SAR of these compounds.

Indolinones, in particular 3-substituted indolinones, are known to possess kinase inhibitory activity.\textsuperscript{79,303} The well known kinase inhibitor sunitinib (10) (Figure 1.4), contains an indolinone core. Sunitinib (10) may also be classed as a 3-substituted isatin and for this reason, the 3-iminoisatins (153-157) and their analogous lysine derivatives (158-162) were selected to be screened as kinase inhibitors. Compounds were tested against protein kinase A (PKA), a kinase which has a role in the regulation of apoptosis and over-expression of the enzyme leads to decreased rates of apoptosis\textsuperscript{304} (see Section 1.4.1). PKA may be used as an initial screen for kinase inhibitory activity as it is a validated drug target already employed in screening for new anti-cancer agents.\textsuperscript{305} As several kinases share a high degree of homology, it is expected that compounds showing activity in this screen will also show activity towards other kinase targets such as the stress-responsive kinases p38 and Jun N-terminal kinases involved in apoptosis, cancer and immune activation.\textsuperscript{306}

A selection of tricyclic/polycyclic isatins were also assessed for their ability to bind to DNA. Anthracycline chemotherapeutics such as doxorubicin (4) and daunorubicin (5) contain a planar tetracyclic core and exert their cytotoxic effect by inserting themselves perpendicularly into DNA without forming covalent bonds.\textsuperscript{307} Similarly, acridines, which contain a planar tricyclic scaffold are known DNA intercalators (see Section 3.3.2), as are some polycyclic phenanthridine and indole derivatives.\textsuperscript{307} As a result, it was of interest to assess the polycyclic isatins synthesised in Chapters 2 and 3 for their DNA-binding ability.
6.1.1 MTS Cell Proliferation Assay

A straightforward and relatively inexpensive method of examining the cytotoxic activity of pure compounds is to use metabolic assays. The CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS assay) was used in this study to determine the number of viable cells in a cellular population through the use of colourimetry. After incubating the cells in the presence of the test compounds for 24 h, a reagent mix was added to detect cell viability. This reagent contained a tetrazolium compound \([3 \text{-}(4,5\text{-dimethylthiazol-2-yl})-5\text{-}(3\text{-carboxymethoxyphenyl})-2\text{-}(4\text{-sulfophenyl})\text{-}2\text{H}\text{-}\text{tetrazolium, inner salt}]\) (MTS) and an electron coupling reagent, phenazine ethosulfate (PES). When combined with PES, MTS yields a red coloured product that is soluble in the culture medium.

The colourless MTS tetrazolium compound is bioreduced into the coloured formazan product by dehydrogenase enzymes in metabolically active cells which produce NADPH or NADH (Figure 6.1). Therefore, the intensity of colour in the assay correlates to the quantity of formazan produced, and is directly proportional to the number of cells living in culture. After an incubation period of 3 h the absorbance was measured in a spectrophotometer at 490 nm, the optimal wavelength for detecting the formazan product. The IC₅₀ values were determined as described in the experimental section.
**Figure 6.1** The colourless MTS tetrazolium salt is bioreduced to the red formazan product by viable cells.

### 6.1.2 Kinase Inhibition Assays

To evaluate the PKA inhibitory activity of the compounds described in Section 6.1, the Promega Kinase-Glo® luminescent assay was employed. This high-throughput assay utilises luminescence to quantify the amount of ATP remaining after a kinase reaction and may be applied to a variety of kinases. As outlined in Figure 6.2, the substrate and ATP react with the kinase to yield a phosphorylated substrate (Reaction 1, Figure 6.2). Any ATP remaining after this reaction then reacts with beetle luciferin in the presence of luciferase to produce one photon of light per turnover (Reaction 2, Figure 6.2). Consequently, the amount of luminescence produced is inversely proportional to kinase activity.
Figure 6.2 The Kinase-Glo® assay reactions sourced from Promega. Reaction 1 illustrates the kinase enzyme reaction while Reaction 2 shows the beetle luciferin/luciferase reaction.

The kinase inhibition assays were performed using an incubation time of 30 min at RT for the kinase enzyme reaction (Figure 6.2, Reaction 1), and 30 min at RT for the luciferase reaction (Figure 6.2, Reaction 2). Known, potent inhibitors of PKA such as staurosporine (167) (IC$_{50}$ = 23 nM) or H89 (168) (IC$_{50}$ = 135 nM) (Figure 6.3) were used as internal standards within the assay.

Figure 6.3 The known PKA inhibitors, staurosporine (167) and H89 (168).
6.1.3 DNA Intercalation Studies

The DNA intercalation assays were performed by Dr. Celine Kelso from the School of Chemistry at the University of Wollongong using electrospray ionisation mass spectrometry. The compounds to be assessed were mixed with Rt1 DNA in a ratio of 1:5 (DNA:drug) so any binding of the drug to DNA would be easily observed. Daunorubicin (5) was used as a control and the binding ability of the drug samples were compared to 5.

6.2 Cytotoxicity Results and Structure-Activity Relationships

The results of the MTS cell proliferation assays on the cyclisation precursors (49, 65, 75, 76, 115 and 136), pyrrolo[3,2,1-\textit{hi}]indole-1,2-diones (46 and 82), pyrrolo[3,2,1-\textit{ij}]quinoline-1,2-diones (85-87, 99, 101, 102, 105, 111, 112, 114 and 116), polycyclic isatins (126, 127, 132, 137, 138, 143 and 144), 3-iminoisatins (153-157), isatin-lysine conjugates (158-162) and isatin-protein conjugates (165 and 166) are outlined in the following sub-sections. The values represent the concentration (µM) required to inhibit 50% cell viability (IC$_{50}$) in the cell lines.

6.2.1 Polycyclic Isatins and Precursors

The results of the MTS cell proliferation assays for the cyclisation precursors (49, 65, 75, 76, 115 and 136) are illustrated in Table 6.1; pyrrolo[3,2,1-\textit{hi}]indole-1,2-diones (46 and 82) and pyrrolo[3,2,1-\textit{ij}]quinoline-1,2-diones (85-87, 99, 101, 102, 105, 111, 112, 114 and 116) are summarised in Table 6.2; results of the polycyclic phenanthridines (126, 127, 132, 137 and 138) and acridines (143 and 144) are outlined in Table 6.3. Results highlighted in red indicate a highly cytotoxic compound (IC$_{50}$ < 10 µM).
Table 6.1 Cytotoxicity results from the MTS assays for the cyclisation precursors (49, 65, 75, 76, 115 and 136) against U937 cells after 24 h incubation.

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC₅₀ (µM)²</th>
</tr>
</thead>
<tbody>
<tr>
<td>49</td>
<td>6.43</td>
</tr>
<tr>
<td>65</td>
<td>15.2</td>
</tr>
<tr>
<td>75</td>
<td>107</td>
</tr>
<tr>
<td>76</td>
<td>125</td>
</tr>
<tr>
<td>115</td>
<td>8.17</td>
</tr>
<tr>
<td>136</td>
<td>29.0</td>
</tr>
</tbody>
</table>

² Values are the mean of triplicates from one experiment.

From Table 6.1, the N-alkylated derivatives 49, 75, 76 and 115 exhibit varying degrees of cytotoxicity. N-Allylisatin (76) was the least cytotoxic derivative, with an IC₅₀ value of 125 µM. The brominated analogue, 49, displayed almost a 20-fold increase in cytotoxicity (IC₅₀ = 6.43 µM) and confirms the importance of a bromo substituent positioned on the isatin core. N-Allyl-7-bromoisatin (49) exhibits similar cytotoxicity to the dibrominated N-allylisatin 110, which was discovered to have an IC₅₀ value of 6.67 µM. The 7-bromo-N-cinnamylisatin derivative 115 was also quite cytotoxic, with an IC₅₀ value of 8.17 µM, although not as active as its dibrominated analogue 117, which has an IC₅₀ value of 2.37 µM. N-Vinylisatin (75) was inactive (IC₅₀ = 107 µM), however, it is anticipated that if the brominated analogue 73 had been successfully prepared, the compound would have been much more cytotoxic.

It is interesting to note that the 7-ethynylisatin 65, which is not N-substituted, displayed an IC₅₀ value of 15.2 µM. While 65 is not considered highly cytotoxic, it reveals that the phenylethynyl moiety on the compound has the capability to significantly increase cytotoxicity relative to the parent molecule, isatin (11)
(IC$_{50}$ = 565 µM). It is expected that the cytotoxicity of 65 would increase further with N-alkylation and bromination at C5. This compound is the first 7-ethynylisatin reported, however, a 7-ethynlooxindole derivative has been shown to exhibit IC$_{50}$ values of 90 nM and > 3 µM against MDA-MB-468 and MDA-MB-231 breast cancer cells respectively, suggesting that this class of compounds are worthy of further investigation.

The spiro-phenanthridine 136 exhibited an IC$_{50}$ value of 29.0 µM, slightly less cytotoxic than the phenanthridine 137 (IC$_{50}$ = 23.4 µM) (Table 6.3). This result is consistent with previous data which revealed that a ketal functionality at the C3 position on isatin is detrimental for cytotoxicity.
Table 6.2 Cytotoxicity results from the MTS assays for the pyrrolo[3,2,1-hi]indole-1,2-diones (46 and 82) and pyrrolo[3,2,1-ij]quinoline-1,2-diones (85-87, 99, 101, 102, 105, 111, 112, 114 and 116) against U937 cells after 24 h incubation.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Substitution</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; (µM)</th>
</tr>
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<tr>
<td><img src="image" alt="Compound" /></td>
<td>R = H</td>
<td>&gt; 577</td>
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<tr>
<td><img src="image" alt="Compound" /></td>
<td>R = Br</td>
<td>111 (± 53)</td>
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<tr>
<td><img src="image" alt="Compound" /></td>
<td>R = H, R&lt;sub&gt;1&lt;/sub&gt; = H</td>
<td>278 (± 23)</td>
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<tr>
<td><img src="image" alt="Compound" /></td>
<td>R = Br, R&lt;sub&gt;1&lt;/sub&gt; = H</td>
<td>8.36 (± 3.5)</td>
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<td><img src="image" alt="Compound" /></td>
<td>R = H, R&lt;sub&gt;1&lt;/sub&gt; = Ph</td>
<td>99.3 (± 3.9)</td>
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<td>15.3 (± 11)</td>
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<td>19.3 (± 0.7)</td>
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<td>R = H, R&lt;sub&gt;1&lt;/sub&gt; = H, R&lt;sub&gt;2&lt;/sub&gt; = Ph</td>
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<td>28.6 (± 2.9)</td>
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<td><img src="image" alt="Compound" /></td>
<td>R = H, R&lt;sub&gt;1&lt;/sub&gt; = CH&lt;sub&gt;3&lt;/sub&gt;</td>
<td>33.2 (± 9.5)</td>
</tr>
</tbody>
</table>

Some interesting conclusions can be drawn from the data presented in Table 6.2 in terms of their SAR. The 6,5,5-fused tricyclic isatin, pyrrolo[3,2,1-hi]indole-1,2-dione (46), was found inactive with an IC<sub>50</sub> value of > 577 µM (beyond testing limits) against U937 cells. The brominated analogue 82 displayed an IC<sub>50</sub> value of 111 µM, a minimum 5-fold increase in cytotoxicity. However, isatin (11) and 5-bromoisatin have IC<sub>50</sub> values of 565 µM and 64.5 µM respectively, against the
same cell line, establishing that the tricyclic analogues 46 and 82 are less active than the parent isatin molecules. Conversely, the 6,5,6-fused tricyclic isatin, pyrrolo[3,2,1-H]quinoline-1,2-dione (85), displayed at least a 2-fold increase in cytotoxicity compared to 46 and is more active than the parent molecule, isatin (11). The brominated derivative 92 exhibited a 33-fold increase in activity when compared to 85. This was the most potent compound in Table 6.1 and the only molecule to be considered highly cytotoxic (IC<sub>50</sub> < 10 μM). This compound was almost 8-fold more active than 5-bromoisatin, suggesting that the addition of a 6-membered ring to the isatin scaffold is beneficial for cytotoxicity, while the addition of a 5-membered ring is detrimental for activity.

Furthermore, the introduction of an alkene into compounds 86 and 87 led to approximately a 6-fold increase in cytotoxicity when compared to the parent molecule 85. However, within the corresponding series of brominated analogues, the saturated compounds were 2-3 times more cytotoxic than the unsaturated derivatives (i.e. 92 vs. 111; 92 vs. 112 and 102 vs. 105). The inclusion of a substituent at the C6 position in 4H-pyrrolo[3,2,1-H]quinoline-1,2-dione (116) resulted in decreased cytotoxicity compared to the parent compound (86), while a substituent at C6 in 6H-pyrrolo[3,2,1-H]quinoline-1,2-dione (114) increased activity compared to its parent compound (87).
The results of C4 substitution within the 6,5,6-fused tricyclic isatin series is inconclusive. These fused compounds may be considered as restrained N-alkylisatins and they were synthesised to allow comparison to the previously reported, highly potent N-alkylisatins. In the case of 85, the addition of a substituent at C4 increased cytotoxicity from 278 µM to 104 µM (compound 101), however, a C4 substituent in 92 decreased activity from 8.36 µM to 15.3 µM (compound 102). N-Benzylisatin (169) was also synthesised within this project for comparison to its fused analogue (101). Cytotoxicity testing of 169 against U937 cells revealed an IC$_{50}$ value of 127 µM, which is similar to the IC$_{50}$ value of 101 (104 µM). This result further confirms the ambiguous effect of N-alkylation (or C4 substitution) on 6,5,6-fused tricyclic isatins with regards to cytotoxicity.
A similar pattern was observed for the polycyclic compounds where the brominated derivatives displayed more cytotoxicity (up to 2.4 times) than their parent molecules (i.e. 127 vs. 126; 138 vs. 137; 144 vs. 143) (Table 6.3). The addition of the extra ring system in 137 increased the cytotoxicity 2-fold compared to 126, while the introduction of an additional ring in derivative 132 decreased cytotoxic activity at least 2.5-fold compared to 126. The polycyclic acridine 143 was greater than 6-fold more cytotoxic than the polycyclic phenanthridine 126 and both acridines (143 and 144) were regarded as highly cytotoxic (IC<sub>50</sub> < 10 µM). Both of these compounds were significantly more cytotoxic than any of the tricyclic derivatives shown in Table 6.1 with the most active compound (144) having an IC<sub>50</sub> value of 3.01 µM against U937 cells. The difference in cytotoxicity between the
parent polycyclic acridine (143) and the parent pyrrolo[3,2,1-$h$i]indole-1,2-dione (46) is at least 80-fold.

Generally, the descending order of cytotoxicity for the compounds described is polycyclic acridines (3.01 - 7.21µM) > polycyclic phenanthridines (18.2 - > 114 µM) > pyrrolo[3,2,1-$i$]quinoline-1,2-diones (8.36 - 278 µM) > pyrrolo[3,2,1-$h$i]indole-1,2-diones (111 - > 577 µM). Bromination at the C5 position of the isatin nucleus increases cytotoxicity relative to the non-brominated analogue in all cases from 2 to 33-fold.

6.2.2 3-Iminoisatins and Isatin-Lysine Conjugates

The 3-iminoisatins (153-157) and lysine conjugates (158-162) prepared in this project were employed as isatin-based linkers and conjugate models respectively. The compounds were also assessed for cytotoxicity at 24 h using the MTS assay as described above. The results are shown in Table 6.4. The cytotoxicity values highlighted in blue indicate compounds with IC$_{50}$ values in the nanomolar range and the value highlighted in red is the most cytotoxic derivative.
Table 6.4 Cytotoxicity results from the MTS assay for 3-iminoisatins (153-157) and isatin-lysine conjugates (158-162) against U937 cells.

<table>
<thead>
<tr>
<th>Imine</th>
<th>n</th>
<th>Substitution</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>R = OH</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>153</td>
<td>0</td>
<td>para-</td>
<td>1.95 (± 0.69)</td>
</tr>
<tr>
<td>154</td>
<td>1</td>
<td>para-</td>
<td>2.13 (± 0.51)</td>
</tr>
<tr>
<td>155</td>
<td>2</td>
<td>para-</td>
<td>0.422 (± 0.09)</td>
</tr>
<tr>
<td>156</td>
<td>0</td>
<td>meta-</td>
<td>3.14 (± 0.39)</td>
</tr>
<tr>
<td>157</td>
<td>1</td>
<td>meta-</td>
<td>0.463 (± 0.08)</td>
</tr>
<tr>
<td>R = (CH₂)₄CH(NHAc)CO₂Me</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>158</td>
<td>0</td>
<td>para-</td>
<td>0.575 (± 0.37)</td>
</tr>
<tr>
<td>159</td>
<td>1</td>
<td>para-</td>
<td>0.642 (± 0.03)</td>
</tr>
<tr>
<td>160</td>
<td>2</td>
<td>para-</td>
<td>0.399 (± 0.11)</td>
</tr>
<tr>
<td>161</td>
<td>0</td>
<td>meta-</td>
<td>0.165 (± 0.06)</td>
</tr>
<tr>
<td>162</td>
<td>1</td>
<td>meta-</td>
<td>0.391 (± 0.13)</td>
</tr>
</tbody>
</table>

The 3-iminoisatins (153-157) are highly cytotoxic (IC<sub>50</sub> < 10 µM), with two derivatives (155 and 157) displaying nanomolar activity towards U937 cells (Table 6.4). The lysine derivatives (158-162) also exhibited potent cytotoxic effects, with all five analogues possessing nanomolar activity and being more cytotoxic than their corresponding imino derivatives (a 19-fold increase in the case of 156 vs. 161). The lysine derivative 161 is the most potent compound (of any class) synthesised within this project with an IC<sub>50</sub> value of 165 nM.
Within the 3-iminoisatin series, the increase in chain length correlated with an increase in cytotoxicity (i.e. 153 < 155 and 156 < 157), however, this trend was not observed within the isatin-lysine series (158-162). In general, meta-substituted compounds were more cytotoxic than their corresponding para-substituted analogues. For example, in the 3-iminoisatin series (153-157), nanomolar cytotoxicity was reached when \( n = 1 \) (IC\(_{50}\) = 463 nM) in the meta series (157) but nanomolar cytotoxicity was not attained until \( n = 2 \) (IC\(_{50}\) = 422 nM) in the para series (155). The same trend was witnessed in the isatin-lysine series (158-162), where the meta analogues were generally more potent than their corresponding para derivatives (i.e. 161 vs. 158; 162 vs. 159). The enhanced cytotoxicity of the isatin-lysine series (158-162) when compared to the 3-iminoisatins (153-157), suggests that increasing the alkyl chain length of 3-iminoisatins leads to a change in intracellular target.

Although the compounds in Table 6.3 were used as linkers and model conjugates with the view to selectively deliver cytotoxins into cells, it appears that some of the 3-iminoisatin derivatives (154, 155, 157) are considerably more cytotoxic than the released parent isatin. In the case of 155, which was the most potent 3-iminoisatin with an IC\(_{50}\) value of 422 nM, the compound is 4 times more cytotoxic than the released isatin (24), which has an IC\(_{50}\) value of 1.83 µM.\(^{64}\) This latter result is unexpected but may represent the beginning of an interesting and novel class of cytotoxic isatin derivatives.

### 6.2.3 Isatin-Protein Conjugates

The MTS assay was also applied to the isatin-PAI-2 (165) and isatin-Tf (166) conjugates to determine whether the cytotoxicity was enhanced as a result of
selective delivery of the isatin into tumour cells. The specificity of the conjugates towards different tumour cell lines was also assessed based on their cytotoxicity.

6.2.3.1 Isatin-PAI-2 Conjugates

The U937 cells (low level expression of uPA/uPAR) and THP-1 cells (high level expression of uPA/uPAR) were treated with isatin-PAI-2 conjugate (165) for 48 h. Selection of this exposure time, rather than the usual 24 h, was to allow for the potential additional time required for RME of the conjugates and release of cytotoxins within the cell due to the molecular size of the conjugate. Figure 6.4 presents an outline of the results of the cytotoxic assays. In both cell lines PAI-2 and the conjugate 165 caused cell proliferation, however, the conjugate resulted in less proliferation than the unconjugated protein in both cases. Based on this result, it cannot be concluded that the conjugate 165 exhibited any cytotoxicity towards the two cell lines in this first preliminary study.

Figure 6.4 Cell viability of PAI-2 and the isatin-PAI-2 conjugate (165) at 48 h. Each data point is the mean of triplicates ± SE. A) Against U937 cells; B) Against THP-1 cells.
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The cytotoxic (or lack of) response of the conjugate 165 was compared between the two cell lines to determine if 165 displayed any specificity between cell lines. In theory, the THP-1 cell line, which over-expresses uPA/uPAR, should exhibit greater cytotoxicity than the U937 cell line, which expresses low levels of uPA/uPAR. However, as illustrated in Figure 6.5, there is only a small difference in the cytotoxicity of the isatin-PAI-2 conjugate (165) on either cell line. This result suggests that either 165 has not been internalised into the tumour cell through RME, or that 165 was not hydrolysed within the endosomes or lysosomes to release the free isatin 24. A related isatin cytotoxin has recently been delivered to uPA/uPAR over-expressing cells using an isatin-PAI-2 conjugate, confirming that it is possible to deliver cytotoxins using this ligand.291 On the other hand, the latter theory may be correct as a previous preliminary study on isatin-PAI-2 conjugates connected through an imine linker revealed that the hydrolysis of the imine and release of the isatin drug did not occur until pH 3,50 approximately 1.5 pH units lower than that found within lysosomes.

![Cell viability of the isatin-PAI-2 conjugate (165) at 48 h against U937 and THP-1 cells, normalised to the PAI-2 control. Each data point is the mean of triplicates ± SE.](image)

**Figure 6.5** Cell viability of the isatin-PAI-2 conjugate (165) at 48 h against U937 and THP-1 cells, normalised to the PAI-2 control. Each data point is the mean of triplicates ± SE.
6.2.3.2 Isatin-Tf Conjugates

The isatin-Tf conjugate (166) was tested for cytotoxicity at 48 h against U937 cells which express low levels of the TfR, and the results are presented in Figure 6.6. Similar to the results found with the PAI-2 conjugate (165) in Figure 6.4A, the isatin-Tf conjugate (166) was less proliferative than the protein (in this case Tf) at the highest concentration tested, however, there was evidence of cell proliferation so the conjugate 166 cannot be regarded as cytotoxic towards this cell line.

![Figure 6.6](image)

**Figure 6.6** Cell viability of Tf and the isatin-Tf conjugate (166) at the highest concentration at 48 h against U937 cells. Data represented is the mean of triplicates ± SE.

With the aid of an inverted light microscope, photographs were obtained of U937 cells after treatment with Tf and the isatin-Tf conjugate (166) (Figure 6.7). Panels A and B in Figure 6.7 illustrate the effects of Tf at 1.56 µM (the highest concentration tested) and 0.78 µM, respectively. The cells are healthy with circular intact membranes and no sign of cell death after a 48 h exposure to Tf. Panels C and D in Figure 6.7 depict the effects of the isatin-Tf conjugate (166) on U937 cells after 48 h of treatment at 1.56 µM and 0.78 µM (equivalent protein concentration), respectively. These two panels exhibit similar cell morphology to U937 cells treated
with Tf (Figure 6.7, panels A and B) and display minimal characteristics of apoptosis.

Figure 6.7 The effects of Tf and the isatin-Tf conjugate (166) on U937 cells at 48 h. Images were viewed at 400 × magnification using light microscopy. A) Tf at 1.56 µM; B) Tf at 0.78 µM; C) Isatin-Tf conjugate (166) at 1.56 µM; D) Isatin-Tf conjugate (166) at 0.78 µM.

Upon treating the TfR over-expressing cell line MDA-MB-231 with the isatin-Tf conjugate (166) for 48 h, a significant cytotoxic response (compared to Tf) was witnessed as shown in Figure 6.8A. The figure also demonstrates for the first time that an imine-linked isatin-protein conjugate is capable of inducing a cytotoxic response and not causing cell proliferation. The data looks promising, although the IC₅₀ value of the conjugate 166 was unable to be determined due to the solubility limitations associated with protein concentration. When compared to Tf at the highest concentration (1.56 µM), the conjugate 166 is 47 ± 13% more cytotoxic, as shown in Figure 6.8B.
Figure 6.8 Cell viability of Tf and the isatin-Tf conjugate (166) against MDA-MB-231 cells at 48 h. Each data point is the mean of triplicates ± SE. A) Dose response curves of Tf vs. isatin-Tf; B) Viability of Tf vs. isatin-Tf at the highest concentration (1.56 µM, equivalent protein concentration).

Photographic images were taken of the Tf and isatin-Tf conjugate (166) upon exposure to MDA-MB-231 cells after 48 h (Figure 6.9). Panels A and B depict Tf at 1.56 µM and 0.78 µM respectively. The effects of treatment with the isatin-Tf conjugate (166) are shown in Figure 6.9, panels C and D at 1.56 µM and 0.78 µM respectively. In panel C, apoptosis of MDA-MB-231 cells is clearly demonstrated by the small and granular appearance of the cells, which can be attributed to chromatin condensation and membrane blebbing. This panel verifies the results from Figure 6.8A, which revealed that the conjugate 166 was cytotoxic towards MDA-MB-231 cells at the highest concentration (1.56 µM), and the enhanced cytotoxic response can be explained due to MDA-MB-231 cells over-expressing the TfR. In panel D of Figure 6.9, the cells do not display characteristics of apoptosis and appear no different than the Tf treated cells in Figure 6.9B. The lack of cytotoxicity at this concentration (0.78 µM) confirms the result observed in Figure 6.8A, which illustrated that the conjugate 166 causes cell proliferation (approximately 150%) towards MDA-MB-231 cells at a concentration of 0.78 µM.
Figure 6.9 The effects of Tf and the isatin-Tf conjugate (166) on MDA-MB-231 cells at 48 h. Images were viewed at 400 × magnification using light microscopy. A) Tf at 1.56 µM; B) Tf at 0.78 µM; C) Isatin-Tf conjugate (166) at 1.56 µM; D) Isatin-Tf conjugate (166) at 0.78 µM.

The comparison of results shown in Figures 6.6-6.9 reveals that MDA-MB-231 cells display much greater cytotoxic specificity than U937 cells when treated with the isatin-Tf conjugate (166). This is proportional to the levels of TfR expressed within the two cell lines. These results suggest that the isatin-Tf conjugate (166) may be more sensitive to RME than the isatin-PAI-2 conjugate (165), which did not display any differences in cytotoxicity between under- and over-expressing uPA/uPAR cell lines (U937 and THP-1 cells respectively). The difference in specificity between the proteins may also be attributed to the cytotoxic payload and the ability of Tf to bind on average more cytotoxins than PAI-2 due to the increased number of lysine residues available for conjugation.291
6.3 Mode of Action Studies

To investigate the mode of action of the compounds synthesised within this project, kinase inhibition and DNA intercalation assays were employed. Molecules with a similar structure to the 3-iminoisatins (153-157) and isatin-lysine conjugates (158-162) exhibit potent kinase inhibitory activity (see Section 1.4.1) and so these compounds were evaluated in kinase inhibition assays. Additionally, a selection of the polycyclic isatins were assessed for their ability to bind to DNA as compounds containing planar polycyclic ring systems are known to be DNA intercalators (see Section 3.3.2).

6.3.1 Kinase Inhibition Assays

The 3-iminoisatins (153-157) and the lysine conjugates (158-162) were assessed for kinase inhibitory activity using the PKA inhibition assay. The results for compounds 153-157 are presented in Table 6.5.
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Table 6.5 PKA inhibitory results for 3-iminoisatins (153-157).

<table>
<thead>
<tr>
<th>Imine</th>
<th>( n )</th>
<th>Substitution</th>
<th>IC_{50} (\mu M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>153</td>
<td>0</td>
<td>para-</td>
<td>42.9 (± 13)</td>
</tr>
<tr>
<td>154</td>
<td>1</td>
<td>para-</td>
<td>ND(^a)</td>
</tr>
<tr>
<td>155</td>
<td>2</td>
<td>para-</td>
<td>20.0 (± 3.4)</td>
</tr>
<tr>
<td>156</td>
<td>0</td>
<td>meta-</td>
<td>52.4 (± 9.6)</td>
</tr>
<tr>
<td>157</td>
<td>1</td>
<td>meta-</td>
<td>46.7 (± 2.2)</td>
</tr>
</tbody>
</table>

\(^a\)ND - not determined.

The 3-iminoisatins (153, 155-157) exhibit modest PKA inhibitory activity (20.0 - 52.4 \( \mu M \)) (Table 6.4). An IC_{50} value could not be determined for 154 due to reproducibility errors. Interestingly, the most active PKA inhibitor (155) was the most cytotoxic 3-iminoisatin in Table 6.3, while the least active PKA inhibitor (156) was also the least cytotoxic 3-iminoisatin in Table 6.3. This suggests there is a relationship between the cytotoxicity of the 3-iminoisatins (153-157) and their mode of action, which appears to be kinase inhibition. Furthermore, kinase assays performed using purified enzymes exhibit 100 to 1000-fold less activity than cell-based assays.\(^{313}\) This confirms the trend observed in Table 6.5, where the 3-iminoisatins (153-157) displayed 10 to 100-fold less activity compared to the cell-based results in Table 6.4.

The parent isatin, (24), was also assessed for PKA inhibitory activity and found to possess an IC_{50} value of 115 \( \mu M \). This result confirms that a carbonyl at C3
is detrimental to kinase activity and explains why most indolinone-based kinase inhibitors (e.g. 10) contain an imine or alkene at the C3 position. It was intriguing to discover that the isatin-lysine conjugates (158-162), which all contain a C3-imino functionality, did not display any PKA inhibitory activity (results are not shown). The absence of PKA inhibitory activity for lysines 158-162 indicates that these molecules possess a different mode of action to the 3-iminoisatins 153-157, and that the lysine chain in 158-162 is responsible for this alternate mode of action. Although compounds 158-162 are not inhibitors of PKA, it is possible that they inhibit other kinases, however, further investigations into the mode of action of 158-162 are required.

In an attempt to establish a possible mode of action for the tricyclic isatins, a model compound (46) was assessed for mitogen-activated protein kinase (MAPK) inhibitory activity. The assay was performed by Dr. Lenka Munoz from the Faculty of Pharmacy at the University of Sydney. The isatin 46 did not exhibit any MAPK inhibitory activity.
6.3.2 DNA Intercalation Studies

A selection of 12 polycyclic isatins (46, 82, 85-87, 92, 101, 111, 116, 126, 127 and 143) were evaluated in the DNA intercalation assays. The mass spectrum of the DNA alone (Figure 6.10A) shows two major peaks at \( m/z \) 1626.4 and 1951.9. Subsequently, the DNA was mixed with daunorubicin (5) and its DNA-binding mass spectrum is shown in Figure 6.10B. Here, neither of the two DNA peaks from Figure 6.10A can be observed, indicating that the \( m/z \) signals in Figure 6.10B are the result of DNA binding with 5. The mass spectrum of DNA + pyrrolo[3,2,1-ij]quinoline-1,2-dione (85) is represented in Figure 6.10C. In this spectrum, the peaks \( m/z \) 1626.4 and 1951.9 are present which correlate to the \( m/z \) signals of DNA alone (Figure 6.10A). Therefore, there is no evidence of 85 acting as a DNA intercalator. The mass spectra of the remaining 11 polycyclic isatins also gave no indication of binding with DNA. It was interesting to note that even though polycyclic phenanthridines and acridines are known to be DNA intercalators,\(^{307}\) the phenanthridines 126 and 127 and the acridine 143 did not display any ability to bind with DNA.
Figure 6.10 Mass spectral results from the DNA intercalation assays. A) DNA alone; B) DNA + daunorubicin (5); C) DNA + compound 85.
7.1 Conclusions

Significant progress was made towards imine-linked isatin-protein conjugates to be used for site-specific drug delivery into tumour cells. As discussed in the Introduction, the key elements of a successful drug conjugate involve the judicious choice of the drug, linker and targeting ligand. The key findings from this study regarding these three aspects are described below.

**Drug:** Synthesis of the pyrrolo[3,2,1-h]indole-1,2-diones (6,5,5-fused tricyclic isatins, Chapter 2) proved challenging. Various synthetic methods such as free radical cyclisations, metal-catalysed cross-coupling reactions and the Sandmeyer isatin method were attempted, all to no avail. The successful synthesis of the tricyclic isatin 46 was attained using the Stolle isatin synthesis and its brominated analogue 82 was prepared through electrophilic substitution. These two compounds were the only two pyrrolo[3,2,1-h]indole-1,2-diones able to be synthesised within this study. Computational chemistry revealed this class of compounds possess high heats of formation because of ring strain effects, consistent with the difficulties encountered during their attempted synthesis.

From the *in vitro* cytotoxicity it was evident that pyrrolo[3,2,1-h]indole-1,2-dione (46) and its brominated analogue 82 were not strongly cytotoxic. In fact, the additional 5-membered ring decreased cytotoxicity compared to the related parent molecules. However, cyclisation precursors *N*-allyl-7-bromoisatin (49) and
7-(phenylethynyl)isatin (65) displayed activity. Derivatives of 65 in particular warrant further investigation since the compound displays an IC\textsubscript{50} value of 15.2 µM and it is neither brominated at the C5 position or N-substituted, both which have been shown in our work to increase cytotoxic activity.

Synthesis of the pyrrolo[3,2,1-\textit{ij}]quinoline-1,2-diones (6,5,6-fused tricyclic isatins, Chapter 3) was much more successful than the pyrrolo[3,2,1-\textit{hi}]indole-1,2-diones. This can be attributed to lower calculated heats of formation for this class of compounds compared to the pyrrolo[3,2,1-\textit{hi}]indole-1,2-diones, associated with the significantly reduced ring strain. Twelve target pyrrolo[3,2,1-\textit{ij}]quinoline-1,2-diones, nine of which were novel, were synthesised together with seven phenanthridine-based isatins and two acridine-based isatins. The quinolines pyrrolo[3,2,1-\textit{ij}]quinoline-1,2-dione (85), 4-phenyl-4\textit{H}-pyrrolo[3,2,1-\textit{ij}]quinoline-1,2-dione (99), 5,6-dihydro-4-phenyl-4\textit{H}-pyrrolo[3,2,1-\textit{ij}]quinoline-1,2-dione (101) and 8-bromo-4-phenyl-4\textit{H}-pyrrolo[3,2,1-\textit{ij}]quinoline-1,2-dione (105) were formed utilising the Stolle isatin synthesis, while 4\textit{H}-pyrrolo[3,2,1-\textit{ij}]quinoline-1,2-dione (86), 6\textit{H}-pyrrolo[3,2,1-\textit{ij}]quinoline-1,2-dione (87), 8-bromo-4\textit{H}-pyrrolo[3,2,1-\textit{ij}]quinoline-1,2-dione (111), 8-bromo-6\textit{H}-pyrrolo[3,2,1-\textit{ij}]quinoline-1,2-dione (112), 6-methyl-6\textit{H}-pyrrolo[3,2,1-\textit{ij}]quinoline-1,2-dione (114) and 6-phenyl-4\textit{H}-pyrrolo[3,2,1-\textit{ij}]quinoline-1,2-dione (116) were attained through an intramolecular Heck reaction. The phenanthridine, 7\textit{H}-pyrrolo[3,2,1-\textit{de}]phenanthridine-4,5-dione (126) was synthesised using the Stolle isatin method, while phenanthridines spiro[benzo[\textit{i}]pyrrolo[3,2,1-\textit{de}]phenanthridine-4,2'-[1,3]dioxolan]-5(7\textit{H})-one (131) and spiro[benzo[\textit{k}]pyrrolo[3,2,1-\textit{de}]phenanthridine-4,2'-[1,3]dioxolan]-5(7\textit{H})-one (136) were formed by a metal-catalysed biaryl coupling reaction. The acridine 6\textit{H}-
pyrrolo[3,2,1-de]acridine-1,2-dione (143) was also prepared by the Stolle isatin method.

*In vitro* cytotoxicity revealed quinoline 85 was inactive, however, its brominated analogue 92 was 33-fold more cytotoxic, displaying an IC₅₀ value of 8.36 µM. Introduction of an alkene into saturated tricyclics increased cytotoxicity when compared to the parent molecule (e.g. 86 and 87 vs. 85). However, within the corresponding series of brominated analogues, the saturated compounds were 2-3 times more cytotoxic than the unsaturated derivatives. The phenanthridine-based isatins were more active than the pyrrolo[3,2,1-ij]quinoline-1,2-diones, although the acridine-based isatins displayed the greatest cytotoxicity of any tricyclic or polycyclic isatin (Figure 7.1). Bromination always yielded more potent analogues (2 to 33-fold). As yet, the cytotoxic mode of action for the tricyclic and polycyclic isatins is unknown; however, the compounds display no ability to intercalate with DNA. The brominated acridine 144 was the lead compound within the entire series, exhibiting an IC₅₀ value of 3.01 µM against U937 cells.
Figure 7.1 Current cytotoxic structure-activity relationships for tricyclic and polycyclic isatin derivatives.

**Linker:** Five novel 3-iminoisatins and their corresponding isatin-lysine conjugates were synthesised in order to evaluate the hydrolytic efficacy of imine linkers. All compounds were prepared as a mixture of $E$ and $Z$ isomers, ranging from ratios of 1:1 to 2:1. At pH 7.4, the imines 153-157 and isatin-lysine conjugates 158-162 were stable for at least 240 min, however at pH 4.5, both series of compounds were capable of hydrolysing the imine bond to release the free isatin 24. Within the 3-iminoisatin series (153-157), the half-lives of the compounds at pH 4.5 ranged from 17.4-72.6 min, while the half-lives of the isatin-lysine conjugates 158-162 were 17.0-85.2 min. The fastest imine hydrolysis rates were observed for the *para*-propionic acid derivatives 155/160, with the least amount of resonance stabilisation in the series. In contrast, the slowest hydrolysis rates were noticed with the *para*-benzoic acid derivatives 153/158, which display the most resonance stabilisation.
When exposed to U937 cells, the 3-iminoisatins \textbf{153-157} and the isatin-lysine conjugates \textbf{158-162} were all highly cytotoxic (< 10 µM). Two of the 3-iminoisatins (155 and 157) displayed nanomolar activity after 24 h, while the five isatin-lysine conjugates (158-162) all exhibited IC$_{50}$ values in the nanomolar region. Isatin-lysine 161 was the most cytotoxic compound synthesised in this entire project, displaying an IC$_{50}$ value of 165 nM, and is the most potent 5,7-dibromo-N-alkylisatin to date.

The cytotoxic mode of action of \textbf{153-157} and \textbf{158-162} was investigated through PKA kinase inhibition assays. The results revealed the isatin-lysines \textbf{158-162} possessed no PKA inhibitory activity, while the 3-iminoisatins \textbf{153-157} displayed modest rates of PKA inhibition (Figure 7.2).
Targeting Ligand: The 3-iminoisatin 155 was conjugated to the tumour-targeting proteins PAI-2 and Tf using standard bioconjugation techniques. Analysis of the two isatin-protein conjugates (165 and 166) by UV-Vis spectrophotometry at λ 280 and 435 nm suggested bioconjugation was successful due to the simultaneous increases in absorbance at these wavelengths. However, attempts at characterising 165 and 166 through MS and hydrolysis studies were inconclusive and revealed that there were problems with detecting 165 and 166 using these methods. On the other hand, when exposed to the TfR over-expressing MDA-MB-231 cell line, the isatin-Tf conjugate (166) displayed both cytotoxic and apoptotic activity at 1.56 µM (the latter evidence by changes to cell morphology), indicating that receptor-mediated endocytosis had occurred, 166 had been
hydrolysed within the endosomes/lysosomes and the free drug 24 had been released. At this concentration, the conjugate 166 was 47±13% more cytotoxic than the Tf control.

7.2 Future Directions

In a bid to further improve potency of our compounds against U937 cells, future work on this project could involve the development of further analogues of the 3-iminoisatins (153-162), in particular derivatives related to the isatin-lysine conjugates (158-162), along with further acridine compounds (143 and 144). In the former case, the addition of the amino acid residue (lysine) increased the cytotoxic activity of our compounds 1.1 to 19-fold, therefore, the effect of appending other amino acids could also be examined. In the latter case, the acridine derivatives were also found to be extremely potent with IC\textsubscript{$50$} values in the range of 3.01-7.21 µM, and may well represent an exciting new class of cytotoxic agents for further development.

The isatin derivatives described in this project could also be tested against a wider range of human cancer cell lines and normal cells to examine the cell specificity of the compounds. Also, further studies such as cell-based kinase assays could be useful to determine the mode of action for these cytotoxins, along with competitive binding studies with tubulin and known tubulin binding agents such as colchicine (147) to characterise the potential binding interaction of the isatin derivatives with tubulin. These studies are currently underway by our collaborators at the University of Wollongong.

Future studies could also include fine-tuning the acid-labile imine linkers (e.g. aliphatic imines, aromatic imines with electron donating/withdrawing
substituents) and evaluating their hydrolytic efficiency using UV-Vis spectrophotometry. The new imine linked isatins could be conjugated via the acid handle to the lysines in the protein Tf (or another tumour-targeting ligand) to produce a site-specific prodrug that would selectively deliver the isatin cytotoxin. The imine linker system could also be applied to other carbonyl-containing cytotoxins such as epipodophyllotoxin (145), camptothecin (146) and colchicine (147). Alternatively, other acid-labile linker systems such as acetals or oximes could be investigated since isatin-Tf based therapies display potential as new therapeutic agents, as described by the results in this thesis.
CHAPTER 8
Experimental

8.1 Chemical Procedures

8.1.1 General

All chemicals were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA), BDH Laboratory Supplies (Poole, England), Bachem (AusPep, Parkville, Australia) or Accela ChemBio (San Diego, CA, USA) and used as supplied. All solvents were AR grade expect DCM which was LR grade and distilled before use. The term petroleum spirit (PS) refers to petroleum spirit with the boiling range of 40-60 °C. When necessary, the purification of solvents and starting materials was carried out using standard procedures. Solvent removal was performed (in vacuo) using a Büchi rotary evaporator at temperatures not greater than 60 °C. Sonication of solutions was performed using a Soniclean 250HT ultrasonic bath (Soniclean, Thebarton, Australia). Sodium hydride was supplied as a 60% dispersion in mineral oil and masses used were calculated appropriately. Celite (Celite 545, particle size 0.02-0.1 mm, Merck) was used to remove metal catalysts from reaction mixtures. Melting points were obtained using a Reichert melting point apparatus and are uncorrected. Geometry optimisation (ΔHf) of tricyclic isatins was performed using VAMP at semi-empirical level (AM1) on Materials Studio 4.4 (Accelrys Inc., San Diego, CA, USA, 2008).
Chromatography

Thin layer chromatography (TLC) on aluminium backed sheets of Merck Silica Gel 60 F$_{254}$ plates was employed to monitor the progress of chemical reactions. Preparative TLC was performed on 20 x 20 cm plates of Merck Silica Gel 60 F$_{254}$ plates. In general, isatins were highly coloured and were visible on the TLC plate; colourless compounds were detected by UV light at $\lambda$ 254 nm. Column chromatography was performed on silica gel 60 (230-400 mesh) under “flash” conditions.\textsuperscript{314} The solvent used in individual chromatographic experiments is indicated and all solvent proportions are given as vol/vol ratios.

Nuclear Magnetic Resonance (NMR) Spectroscopy

Nuclear magnetic resonance (NMR) spectra were acquired on a Varian Unity 300 MHz spectrometer, where proton (\textsuperscript{1}H) and carbon (\textsuperscript{13}C) spectra were obtained at 300 MHz and 75 MHz respectively, or on a Varian Inova 500 spectrometer, where the \textsuperscript{1}H and \textsuperscript{13}C were obtained at 500 MHz and 126 MHz respectively. All spectra were obtained with a probe temperature of 298 K. Spectra were recorded in the deuterated solvent indicated in brackets and were referenced to the residual non-deuterated solvent signal. Chemical shifts (\(\delta\)) were measured in parts per million (ppm). Hydrogen and carbon assignments were made using gradient correlation spectroscopy (gCOSY), gradient heteronuclear single quantum correlation (gHSQC) and gradient heteronuclear multiple bond correlation (gHMBC) spectroscopic techniques. The superscript letter \textsuperscript{a} denotes coincident peaks. The symbol ¶ denotes the carboxylic acid proton signal was not observed. Multiplicities are reported as singlet (s), broad singlet (bs), doublet (d), doublet of doublets (dd), doublet of triplets (dt), triplet (t) and multiplet (m).
Mass Spectrometry

Low resolution electron ionisation mass spectra (LREI-MS) were obtained using a Shimadzu QP5050 spectrometer. Low resolution electrospray ionisation mass spectra (LRESI-MS) were obtained using a Waters Platform LCZ spectrometer. High resolution electron ionisation mass spectra (HREI-MS) were obtained using a Fisons/VG Autospec spectrometer operating with an electron beam of 70 eV, with a source temperature of 250 °C and perfluorokerosene as the internal standard. High resolution electrospray ionisation spectra (HRESI-MS) were obtained using a Waters Q-TOF Ultima spectrometer using leucine enkephalin as the internal standard. The term “MS” refers to LREI-MS unless stated otherwise. Protein samples were analysed using a Waters SYNAPT™ High Definition Mass Spectrometer (HDMS) in +ve mode under nanospray conditions: injection volume of 3 µL, at 200 sampling and 4 extraction cone volts, with a source temperature of 50 °C and a vacuum backing of 3.86 mbar.
8.1.2 Experimental for Chapter 2

Synthesis of 7-bromo-1H-indole-2,3-dione\(^{315}\) (55)

Chloral hydrate (5.01 g, 30.2 mmol) and Na\(_2\)SO\(_4\) (35.0 g, 246 mmol) were dissolved in H\(_2\)O (70 mL) in a 300 mL beaker and heated to 35 °C. 2-Bromoaniline (51) (4.75 g, 3.12 mL, 27.6 mmol) in H\(_2\)O (20 mL) and conc. HCl (3 mL) were warmed and added to the reaction mixture. Hydroxylamine HCl (6.10 g, 87.8 mmol) in H\(_2\)O (27.5 mL) was warmed and added before the mixture was heated at 85 °C for 3 h. The mixture was cooled, filtered and washed with H\(_2\)O (100 mL) to yield the intermediate 53 (5.75 g, 86%) as a fluffy light brown powder.

Conc. H\(_2\)SO\(_4\) (100 mL) was heated in a 1 L beaker to 60 °C. The intermediate 53 was added in portions over 15 min so the temperature did not exceed 65 °C. The reaction mixture was then heated at 80 °C for 15 min before being poured into ice (500 mL). The precipitate was washed with H\(_2\)O (100 mL) to yield 55 (2.94 g, 47%) as a fine dark red powder, m.p. 192-194 °C (lit.\(^{316}\) 191-198 °C), \(R_f\) 0.51 (silica, DCM:MeOH, 9:1).

\(^1\)H NMR (DMSO-\(d_6\), 500 MHz): \(\delta\) 7.02 (t, \(J = 7.5\) Hz, 1H, H5), 7.51 (d, \(J = 7.5\) Hz, 1H, H4), 7.78 (d, \(J = 7.5\) Hz, 1H, H6), 11.31 (bs, 1H, NH). The \(^1\)H NMR spectral data coincided with those reported in the literature.\(^{168}\) LREI-MS: \(m/z\) 225; 227 [M\(^+\)]\(^{79}\)Br; \(^{81}\)Br.
Synthesis of 1-allyl-7-bromo-1H-indole-2,3-dione (49)

A mixture of 7-bromoisatin (55) (250 mg, 1.11 mmol) and NaH (62.3 mg, 1.55 mmol) was dissolved in anh. DMF (6 mL) and stirred under N₂ at RT for 20 min before the addition of KI (37.2 mg, 222 µmol) and allyl bromide (295 mg, 211 µL, 2.44 mmol). The reaction mixture was heated at 60 °C and stirred at this temperature for 18 h. After cooling, EtOAc (25 mL) was added and the resulting solution was extracted with 0.5 M HCl (25 mL) followed by brine (25 mL). The orange organic layer was dried over MgSO₄ and the solvent was removed by rotary evaporation to yield a sticky red residue. The resulting solid was purified by flash chromatography on silica gel (100% DCM) to yield 49 (236 mg, 81%) as orange crystals, m.p. 112-114 °C, Rf 0.44 (silica, DCM).

¹H NMR (CDCl₃, 500 MHz): δ 4.80 (m, J = 3.5 Hz, 2H, H₁'), 5.23 (d, J = 2.0 Hz, 1H, H₃'), 5.26 (d, J = 2.0 Hz, 1H, H₃'), 5.99 (m, 1H, H₂'), 7.00 (t, J = 8.0 Hz, 1H, H₅), 7.59 (d, J = 7.5 Hz, 1H, H₄), 7.69 (d, J = 7.5 Hz, 1H, H₆).

¹³C NMR (CDCl₃, 126 MHz): δ 43.4 (C₁'), 104.5 (ArC), 117.7 (C₃'), 120.9 (ArC), 124.9 (C₄), 125.3 (C₅), 132.0 (C₂'), 144.2 (C₆), 148.0 (C₇a), 158.7 (C₂), 182.6 (C₃).

LREI-MS: m/z 265; 267 [M⁺]⁺Br; ¹⁸¹Br. HREI-MS: m/z calcd for C₁₁H₈NO₂⁺Br [M⁺]: 264.9738; found 264.9736.
Attempted synthesis of 5-methyl-4,5-dihydro-pyrrolo[3,2,1-hi]indole-1,2-dione (50)

**Method A:** According to the method of Mbere, the isatin (80.0 mg, 301 μmol) was dissolved in anh. PhMe (5 mL) and heated at reflux under a N₂ atmosphere. Bu₃SnH (371 mg, 344 μL, 1.28 mmol) and AIBN (52.4 mg, 319 μmol) were dissolved in anh. PhMe (7 mL) and added dropwise over 2 h, and the reaction mixture heated at reflux for another 3 h. The solvent was then removed by rotary evaporation and Et₂O (5 mL) and sat. KF solution (5 mL) were added and the solution stirred at RT for 1 h. The organic layer was separated, washed with H₂O (2 × 5 mL) and dried over MgSO₄. The crude product contained many impurities MS.

**Method B:** According to the method of Rashatasakon et al., the isatin (195 mg, 730 μmol) was heated at reflux in anh. benzene (70 mL). AIBN (12.2 mg, 73.0 μmol) and Bu₃SnH (335 mg, 1.15 mmol) were dissolved in anh. benzene (3 mL) and added dropwise over 1.5 h, before being heated at reflux for 14 h (total volume of benzene was 73 mL to give a 0.1 M solution). The solvent was removed by rotary evaporation but there were difficulties in purifying the product.

**Synthesis of 7-iodo-1H-indole-2,3-dione (56)**

This compound was prepared using the same method as that for 7-bromoisatin (55). Chloral hydrate (6.67 g, 40.3 mmol) and Na₂SO₄ (46.2 g, 325 mmol) were dissolved in H₂O (115 mL) and heated to 35 °C. 2-Iodoaniline (52) (8.00 g, 36.5 mmol) in H₂O (33 mL) and conc. HCl (4.8 mL) were warmed and added to the reaction mixture. Hydroxylamine HCl (8.13 g, 117 mmol) in H₂O (44 mL) was warmed and added
before the mixture was heated at 80 °C for 3 h. The mixture was cooled, filtered and washed with H₂O to yield the intermediate 54 (8.77 g, 83%) as a fluffy beige/tan coloured powder.

Conc. H₂SO₄ (160 mL) was heated to 60 °C. The intermediate 54 was added in portions over 15 min so the temperature did not exceed 65 °C. The reaction mixture was then heated at 85 °C for 15 min before being poured into ice (600 mL). The precipitate was washed with H₂O to yield 56 (5.77 g, 58%) as a dark red/brown powder, m.p. 205-206 °C (lit. 318-208 °C), R₉ 0.56 (silica, DCM/MeOH 9:1).

^1H NMR (DMSO-δ, 500 MHz): δ 6.89 (t, J = 7.5 Hz, 1H, H5), 7.49 (d, J = 7.5 Hz, 1H, H4), 7.94 (d, J = 8.0 Hz, 1H, H6), 11.00 (bs, 1H, NH). The ^1H NMR spectral data coincided with those reported in the literature.²⁰⁴ LREI-MS: m/z 273 [M⁺].

Synthesis of 7-(phenylethynyl)-1H-indole-2,3-dione (65)

According to the methods of Layek et al.²²⁹ and Batchu et al.,¹⁹⁶ 7-idoisatin (56) (100 mg, 367 µmol), Pd(PPh₃)₄ (8.50 mg, 7.35 µmol), CuI (9.60 mg, 36.7 µmol) and Et₃N (92.5 mg, 128 µL, 915 µmol) were added to anh. DMF (1 mL) and stirred at RT for 1 h. Phenylacetylene (64) (56.0 mg, 60 µL, 550 µmol) was added and the reaction mixture was heated at 80 °C for 4 h before being filtered through Celite, diluted with EtOAc (10 mL), then extracted with H₂O (2 × 10 mL) and brine (2 × 10 mL). The organic layer was dried over MgSO₄, filtered and the solvent removed by rotary evaporation. The resulting solid was purified by flash chromatography on silica gel (100% CHCl₃) to yield 65 (62.2 mg, 69%) as a red powder, m.p. 137-139 °C, R₉ 0.72 (silica, DCM/MeOH, 9:1).
\(^1\)H NMR (CDCl\(_3\), 500 MHz): $\delta$ 7.12 (t, $J = 7.5$ Hz, 1H, H5), 7.40 (m, 3H, H3', H4', H5'), 7.58 (m, 3H, H4, H2', H6'), 7.67 (d, $J = 8.0$ Hz, 1H, H6), 8.44 (bs, 1H, NH). \(^{13}\)C NMR (CDCl\(_3\), 126 MHz): $\delta$ 81.4 (C≡C), 96.9 (C≡C), 108.5 (C7 or C3a), 118.1 (C7 or C3a), 121.9 (C1'), 124.0 (C5), 125.4 (C4), 128.9 (C3', C5'), 129.6 (C4'), 132.0 (C2'/C6'), 140.4 (C6), 150.5 (C7a), 158.7 (C2), 182.7 (C3). LREI-MS: m/z 247 [M$^+$]. HREI-MS: m/z calcd for C\(_{16}\)H\(_9\)NO\(_2\) [M$^+$]: 247.0633; found 247.0634.

**Attempted synthesis of 4-phenylpyrrolo[3,2,1-\(hi\)]indole-1,2-dione (66)**

**Method A:** According to the method of Ezquerra et al.,\(^{198}\) 7-phenylethynylisatin (65) (100 mg, 404 µmol) and CuI (212 mg, 808 µmol) were dissolved in anh. DMF (1.62 mL) and heated at 100 °C for 3.5 h. The reaction mixture was filtered through Celite, washed with Et\(_2\)O (25 mL), extracted with brine (25 mL), dried over MgSO\(_4\), filtered and the solvent removed by rotary evaporation. Analysis by TLC and \(^1\)H NMR spectroscopy revealed the crude product contained starting material 65 and no desired product.

**Method B:** According to the method of Jury et al.,\(^{206}\) 7-phenylethynylisatin (65) (40.0 mg, 162 µmol), PdCl\(_2\)(PPh\(_3\))\(_2\) (4.50 mg, 6.48 µmol), Cul (3.10 mg, 16.2 µmol) and Cs\(_2\)CO\(_3\) (79.2 mg, 243 µmol) were dissolved in anh. DMF (1 mL) and heated at 80 °C for 5.5 h. H\(_2\)O (10 mL) was added to the reaction mixture before it was extracted with EtOAc (2 × 10 mL), dried over MgSO\(_4\), filtered and the solvent removed by rotary evaporation. Analysis by TLC and \(^1\)H NMR spectroscopy revealed the crude product contained starting material 65 and no desired product.

**Method C:** The preparation of this compound was attempted according to Method B using 7-phenylethynylisatin (65) (40.0 mg, 162 µmol) and AgNO\(_3\) (5.50 mg,
32.4 µmol) as starting materials. Analysis by TLC and ¹H NMR spectroscopy revealed the crude product contained starting material 65 and no desired product.

**Method D:** The preparation of this compound was attempted according to Method B using 7-phenylethynylisatin (65) (40.0 mg, 162 µmol) and Au(PPh₃)Cl (16.0 mg, 32.4 µmol) in anh. EtOH (1 mL) as starting materials. The reaction mixture was heated at 70 ºC for 5.5 h. Analysis by TLC and ¹H NMR spectroscopy revealed the crude product contained starting material 65 and no desired product.

**Synthesis of 7′-(phenylethynyl)spiro[[1,3]dioxolane-2,3′-indolin]-2′-one (67)**

According to the method of Ribeiro et al., ⁴¹⁹ 7-phenylethynylisatin (65) (300 mg, 1.21 mmol), ethylene glycol (751 mg, 675 µL, 12.1 mmol) and PTSA (30 mg, 10% of starting material weight) were dissolved in PhMe (25 mL) and heated at reflux under Dean-Stark conditions for 48 h. No evidence of product had formed so more ethylene glycol was added (1.50 g, 1.35 mL, 24.2 mmol) and the solution was heated at reflux for a further 24 h before the solvent was removed by rotary evaporation. The resulting solid was purified by flash chromatography on silica gel (100% CHCl₃) to yield 67 (151 mg, 43%) as light golden brown crystals, m.p. 190-192 ºC, R₇ 0.76 (silica, DCM/MeOH, 9:1).

¹H NMR (CDCl₃, 500 MHz): δ 4.31 (t, J = 7.0 Hz, 2H, H4, H5), 4.53 (t, J = 7.0 Hz, 2H, H4, H5), 7.06 (t, J = 7.5 Hz, 1H, H5′), 7.32 (d, J = 8.0 Hz, 1H, H4′), 7.37 (m, 3H, H3′, H4′, H5′), 7.44 (d, J = 7.5 Hz, 1H, H6′), 7.53 (m, 2H, H2′, H6′), 7.72 (bs, 1H, NH). ¹³C NMR (CDCl₃, 126 MHz): δ 66.1 (C4/C5), 83.1 (C=C), 95.2 (C=C), 102.7 (ArC), 106.5 (ArC), 123.4 (C5′), 124.5 (ArC), 125.3 (C4′), 128.7 (C3′,
C5'), a 129.1 (C4'), 131.0 (ArC), 131.9 (C2'/C6'), a 134.1 (C6'), 143.7 (C3'), 174.5 (C2'). LREI-MS: m/z 291 [M⁺]. HREI-MS: m/z calcd for C₁₈H₁₃NO₃ [M⁺]: 291.0895; found 291.0893.

**Attempted synthesis of**

4'-phenyl-2'H-spiro[1,3]dioxolane-2,1'H-pyrrolo[3,2,1-hi]indol]-2'-one (68)

![68](image)

**Method A:** The preparation of this compound was attempted according to the method for 66 using 67 (50.0 mg, 172 µmol), CuI (65.5 mg, 344 µmol) and anh. DMF (700 µL) as starting materials. The reaction mixture was heated at 100 ºC for 24 h, filtered through Celite, washed with Et₂O (15 mL), extracted with brine (15 mL), dried over MgSO₄, filtered and the solvent removed by rotary evaporation to give a brown solid. ¹H NMR spectroscopy revealed no product had formed and only starting material 67 was present.

**Method B:** According to the method of Marchand et al., ²¹² 67 (44.8 mg, 154 µmol) and PdCl₂ (2.73 mg, 15.4 µmol) were dissolved in anh. CH₃CN (1.10 mL) and heated at 70-75 ºC for 11.5 h. The reaction mixture was then filtered through Celite, washed with Et₂O (25 mL), extracted with brine (25 mL), dried over MgSO₄, filtered and the solvent removed by rotary evaporation. ¹H NMR spectroscopy revealed no product had formed and only starting material 67 was present.
Synthesis of 2-(phenylethynyl)aniline (71)

This compound was prepared according to the method for 65 using 2-iodoaniline (52) (2.00 g, 9.13 mmol), Pd(PPh₃)₄ (211 mg, 183 µmol), CuI (244 mg, 1.28 mmol), Et₃N (2.31 g, 3.18 mL, 22.8 mmol) and phenylacetylene (64) (1.40 g, 1.51 mL, 13.7 mmol) as starting materials. The resulting solid was purified by flash chromatography on silica gel (CHCl₃/hexane gradient) to yield 71 (1.26 g, 72%) as a beige powder, m.p. 85-86 °C (lit. 214 89-91 °C), Rₚ 0.57 (silica, CHCl₃).

¹H NMR (DMSO-­d₆, 300 MHz): δ 4.28 (bs, 2H, NH₂), 6.70-6.75 (m, 2H, H4, H6), 7.15 (t, J = 8.7 Hz, 1H, H5), 7.34-7.38 (m, 4H, H3, H3', H4', H5'), 7.52-7.55 (m, 2H, H2', H6'). The ¹H NMR spectral data coincided with those reported in the literature.²¹³ LREI-MS: m/z 193 [M⁺].

Synthesis of 2-phenylindole (72)

According to the method of Iritani et al.,²¹¹ 2-(phenylethynyl)aniline (71) (300 mg, 1.55 mmol) and PdCl₂ (27.5 mg, 155 µmol) were dissolved in dry CH₃CN (10 mL) and heated at 70-75 °C for 8 h. Upon cooling, the reaction mixture was filtered through Celite, washed with Et₂O (100 mL), extracted with brine (100 mL), dried over MgSO₄, filtered and the solvent removed by rotary evaporation. The resulting solid was purified by flash chromatography on silica gel (100% CHCl₃) to yield 72 (223 mg, 74%) as a light brown powder, m.p. 190-192 °C (lit. 316 188-190 °C), Rₚ 0.04 (silica, CHCl₃).

¹H NMR (DMSO-­d₆, 500 MHz): δ 6.84 (s, 1H, H3), 7.13 (t, J = 7.5 Hz, 1H, ArH), 7.20 (t, J = 7.5 Hz, 1H, ArH), 7.33 (t, J = 7.5 Hz, 1H, ArH), 7.41 (d,
$J = 8.0 \text{ Hz, } 1\text{H, ArH}$, 7.45 ($t, J = 7.5 \text{ Hz, } 2\text{H, H3', H5'}$), 7.64 ($d, J = 7.5 \text{ Hz, } 1\text{H, ArH}$), 7.67 ($d, J = 7.5 \text{ Hz, } 2\text{H, H2', H6'}$), 8.37 ($bs, 1\text{H, NH}$). The $^1\text{H NMR}$ spectral data coincided with those reported in the literature.$^{214}$ LREI-MS: $m/z$ 193 [M$^+$.]

**Synthesis of 1-vinyl-1H-indole-2,3-dione (75)**

According to the method of Bayer and Geckeler,$^{216}$ isatin (11) (500 mg, 3.40 mmol), vinyl acetate (7.90 g, 8.46 mL, 91.8 mmol) and Na$_2$PdCl$_4$ (10.0 mg, 34.0 µmol) were heated at reflux for 12 h. Charcoal (34.7 mg, 2.89 mmol) was added and the reaction mixture was stirred at RT for 10 min, then filtered and the solvent removed by rotary evaporation. The starting materials were added again and the procedure was repeated. Et$_2$O (75 mL) was added and the reaction mixture was extracted with H$_2$O ($3 \times 25$ mL), the organic layer was dried over MgSO$_4$, the suspension filtered and the solvent removed by rotary evaporation. The resulting solid was purified by flash chromatography on silica gel (100% DCM) to yield 75 (500 mg, 42%) as an orange powder, m.p. 112-113 °C (lit.$^{216}$ 117 °C), $R_f$ 0.43 (silica, DCM).

$^1\text{H NMR}$ (CDCl$_3$, 500 MHz): δ 5.17 ($d, J = 10 \text{ Hz, } 1\text{H, H2'}$), 5.87 ($d, J = 11 \text{ Hz, } 1\text{H, H2'}$), 6.64-6.69 ($m, 1\text{H, H1'}$), 7.18 ($t, J = 7.5 \text{ Hz, } 2\text{H, H5, H6}$), 7.64 ($dd, J = 3.5, 8.0 \text{ Hz, } 2\text{H, H4, H7}$). $^{13}\text{C NMR}$ (CDCl$_3$, 126 MHz): δ 106.1 (C2'), 111.2 (C5 or C6), 118.0 (C3a), 124.8 (C5 or C6), 125.2 (C1'), 125.9 (C7), 138.7 (C4), 150.0 (C7a), 157.3 (C2), 182.2 (C3). LREI-MS: $m/z$ 173 [M$^+$.]. HREI-MS: $m/z$ caled for C$_{10}$H$_7$NO$_2$ [M$^+$]: 173.0477; found 173.0474.
Attempted synthesis of 7-bromo-1-vinyl-1H-indole-2,3-dione (73)

**Method A:** According to the method for 75, 7-bromoisatin (55) (500 mg, 2.21 mmol), vinyl acetate (5.14 g, 5.50 mL, 59.7 mmol), Na₂PdCl₄ (6.50 mg, 22.1 µmol) and charcoal (22.6 mg, 1.88 mmol) were used as starting materials. MS analysis revealed only starting material 55.

**Method B:** According to the method of Iddon *et al.*, 7-bromoisatin (55) (1.00 g, 4.42 mmol), 1,2-dibromoethane (8.30 g, 3.81 mL, 44.2 mmol), Et₃N (35.8 g, 49.3 mL, 354 mmol) and CH₃CN (49.3 mL) were stirred at 70 °C for 6 h. The solvent was removed by rotary evaporation and DCM (20 mL) was added to the residue. The mixture was extracted with H₂O (20 mL), after which the organic layer was dried over MgSO₄, the suspension filtered and the solvent removed by rotary evaporation. The resulting solid was partially purified by flash chromatography on silica gel using DCM. Analysis by ¹H NMR spectroscopy revealed many impurities were still present in the product.

Synthesis of 1-allylindoline-2,3-dione (76)

This compound was prepared according to the method for 49 using isatin (11) (1.00 g, 6.80 mmol), NaH (381 mg, 9.52 mmol), KI (226 mg, 1.36 mmol) and allyl bromide (1.82 g, 1.30 mL, 15.0 mmol) as starting materials. The reaction mixture was heated at 65 °C for 18 h. The resulting solid was purified by flash chromatography on silica gel (100% DCM) to yield 76 (874 mg, 69%) as a bright red powder, m.p. 85-86 °C (lit.²¹⁹ 88-89 °C), Rᵣ 0.36 (silica, DCM).

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*Chapter 8 - Experimental*
\[ ^1H \text{ NMR} \ (\text{CDCl}_3, \ 500 \text{ MHz}): \delta \ 4.37 \ (d, \ J = 5.0 \text{ Hz}, \ 2\text{H}, \ H_1'), \ 5.29 \ (t, \ J = 5.0 \text{ Hz}, \ 2\text{H}, \ H_3'), \ 5.81-5.88 \ (m, \ 1\text{H}, \ H_2'), \ 6.89 \ (d, \ J = 8.0 \text{ Hz}, \ 1\text{H}, \ H_7), \ 7.12 \ (t, \ J = 8.0 \text{ Hz}, \ 1\text{H}, \ H_5), \ 7.56 \ (t, \ J = 8.0 \text{ Hz}, \ 1\text{H}, \ H_6), \ 7.62 \ (d, \ J = 7.5 \text{ Hz}, \ 1\text{H}, \ H_4). \]

The \(^1\text{H}\) NMR spectral data coincided with those reported in the literature.\(^{219}\) LREI-MS: \(m/z \ 187 \ [\text{M}^+].\)

**Attempted synthesis of**

5-iodomethyl-4,5-dihydropyrrolo[3,2,1-\text{hi}]indole-1,2-dione (77)

According to the method of Albrecht \textit{et al.},\(^{218}\) a mixture of 76 (200 mg, 1.07 mmol), I\(_2\) (1.09 g, 4.28 mmol) and K\(_2\)CO\(_3\) (592 mg, 4.28 mmol) was dissolved in anh. CH\(_3\)CN (6 mL) and stirred under N\(_2\) at RT for 20 h. A sat. solution of sodium thiosulfate (18 mL) was added until the I\(_2\) was consumed. CHCl\(_3\) (20 mL) was added and the solution was extracted with H\(_2\)O (3 \times 20 mL). The organic extract was dried over MgSO\(_4\), filtered and evaporated. MS and TLC analysis revealed only starting material 76 was present.

**Synthesis of N-formylindoline (78)**

\textbf{Method A:} According to the method of Yamada \textit{et al.},\(^{223}\) to a solution of EDCI (1.61 g, 8.40 mmol) in CHCl\(_3\) (4.5 mL) was added formic acid (773 mg, 634 µL, 16.8 mmol) at 0 \textdegree C under N\(_2\). The mixture was stirred for 5 min before a solution of indoline (40) (500 mg, 470 µL, 4.20 mmol) in pyridine (3 mL) was added dropwise over 30 min. The reaction mixture was warmed to RT, stirred for 24 h and then the solvent was removed by rotary evaporation. CHCl\(_3\) (50 mL) was added to the red/pink residue and the reaction mixture was extracted with brine (4 \times 20 mL), the organic layer was dried over
MgSO₄, the suspension filtered and the solvent removed by rotary evaporation. The resulting solid was purified by flash chromatography on silica gel using a hexane/EtOAc gradient followed by a CHCl₃/MeOH gradient. NMR spectroscopy and MS analysis on representative fractions did not reveal any expected product.

**Method B:** According to the method of Berry *et al.*, imidazole (5.15 g, 75.6 mmol) and anh. DMF (2.13 g, 2.26 mL, 29.2 mmol) were stirred under N₂ before the dropwise addition of TMSCl (8.21 g, 9.59 mL, 75.6 mmol). The reaction mixture was stirred at RT for 20 min. Indoline (40) (3.00 g, 2.82 mL, 25.2 mmol) in anh. DMF (11.0 g, 11.7 mL, 151 mmol) was added dropwise and the reaction mixture was stirred at RT for 12 h. A mixture of ice/H₂O:0.1 M HCl (2:1) (50 mL) was added and the reaction mixture was extracted with EtOAc (2 × 50 mL), the organic layer was dried over MgSO₄, the suspension filtered and the solvent removed by rotary evaporation. The resulting solid was purified by flash chromatography on silica gel using a hexane/EtOAc gradient (2:1 to 1:1) to yield 78 (364 mg, 10%) as a lilac powder in a mixture of rotamers (83:17), m.p. 58-59 °C (lit. 58-61 °C), Rf 0.18 (silica, hexane/EtOAc, 2:1).

**¹H NMR** (CDCl₃, 500 MHz): Major rotamer: δ 3.15 (t, J = 8.5 Hz, 2H, H3), 4.06 (t, J = 8.5 Hz, 2H, H2), 7.15-7.27 (m, 4H, H4-H7), 8.93 (s, 1H, CHO). Minor rotamer: δ 3.19 (t, J = 8.5 Hz, 2H, H3), 4.11 (t, J = 8.5 Hz, 2H, H2), 7.04-7.07 (m, 4H, H4-H7), 8.52 (s, 1H, CHO). The ¹H NMR spectral data coincided with those reported in the literature. LREI-MS: m/z 147 [M⁺].
Synthesis of 4,5-dihydropyrrolo[3,2,1-\textit{hi}]indole-1,2-dione (46)

**Method A:** According to the method of Welstead Jr. \textit{et al.}\textsuperscript{156} indoline (40) (1.00 g, 941 µL, 8.39 mmol) and oxalyl chloride (2.13 g, 1.46 mL, 16.8 mmol) were dissolved in anh. THF (20 mL) and heated at reflux for 3.5 h. The solvent was removed by rotary evaporation to yield a blood red residue. The residue was dissolved in anh. DCM (80 mL) and heated at reflux before the addition of AlCl\textsubscript{3} (2.24 g, 16.8 mmol) portionwise over 10 min. The reaction mixture was heated at reflux for a further 1 h, the solution was evaporated, H\textsubscript{2}O (15 mL) was added and the solution was extracted with CHCl\textsubscript{3} (3 × 15 mL) and brine (1 × 15 mL). The organic layer was dried over MgSO\textsubscript{4}, the suspension filtered and the solvent removed by rotary evaporation. MS analysis revealed too many impurities.

**Method B:** The preparation of this compound was attempted according to Method A for 46 with the following modifications: the solution was evaporated and the red residue was dissolved in anh. CHCl\textsubscript{3} (80 mL) and heated at reflux. AlCl\textsubscript{3} was added portionwise over 30 min and the mixture was heated at reflux for another 3 h. MS analysis revealed too many impurities.

**Method C:** The preparation of this compound was attempted according to Method A for 46 with the following modification: K\textsubscript{2}CO\textsubscript{3} (200 mg, 1.45 mmol) was added to oxalyl chloride (2.13 g, 1.46 mL, 16.8 mmol) in anh. THF (10 mL) to remove excess HCl. MS analysis did not reveal the acid chloride intermediate 80 had been produced so the experiment was abandoned.

**Method D:** The preparation of this compound was attempted according to Method A for 46 with the following modification: the solution was evaporated and the red residue was dissolved in anh. 1,2-dichloroethane (80 mL) and heated at reflux. AlCl\textsubscript{3}
was added portionwise over 30 min and the mixture was heated at reflux for another 1.5 h. MS analysis revealed no product had been formed.

**Method E:** According to the method of Meth-Cohn and Goon, N-formylindoline (78) (300 mg, 2.04 mmol) and oxalyl chloride (259 mg, 178 µL, 2.04 mmol) were stirred at RT for 5 min under N₂. Anh. THF (2.10 mL) was added and the reaction mixture was stirred with ice-bath cooling while DIPEA (264 mg, 355 µL, 2.04 mmol) was added dropwise. The mixture was heated to 35 ºC for 1 h and cooled on ice again while Br₂ (163 mg, 52.0 µL, 1.02 mmol) in CHCl₃ (400 µL) was added dropwise. H₂O (10 mL) was then added and the mixture was extracted with Et₂O (2 × 10 mL), washed with 1 M HCl (3 × 5 mL) and H₂O (10 mL). The organic layer was dried over MgSO₄, the suspension filtered and the solvent removed by rotary evaporation. MS analysis revealed many products but no expected product.

**Method F:** According to the method of Norman *et al.*, a solution of indoline (40) (10.0 g, 9.40 mL, 84.0 mmol) in anh. DCM (75 mL) was added to a solution of oxalyl chloride (21.3 g, 14.7 mL, 168 mmol) in anh. DCM (75 mL) slowly over 2 h. The reaction mixture was stirred at RT for a further 3 h, left to stand overnight and then concentrated. Et₂O (200 mL) was added to the residue, the suspension filtered and the filtrate was concentrated to yield 80 (7.04 g, 40%) as a light brown powder. AlCl₃ (21.3 g, 160 mmol) and the acid chloride 80 (6.70 g, 32.0 mmol) were stirred neat in a beaker for 20 min at 110 ºC until all of the gas had been released. The sticky red residue was cooled to RT before the addition of ice-H₂O (500 mL) and further stirring at RT for 2 h. The red solution was extracted with CHCl₃ (2 × 300 mL), the organic layer was dried over MgSO₄, the suspension filtered, the solvent removed by rotary evaporation and triturated with acetone to remove grey impurities. The solution was concentrated and the resulting sticky red residue was purified by flash...
chromatography on silica gel (hexane/EtOAc, 1:1) to yield 46 [845 mg, 15%; overall 6% based on starting indoline (40)] as a red powder, m.p. 195-197 °C (lit.\textsuperscript{225} 203-207 °C), R\textsubscript{f} 0.26 (silica, hexane/EtOAc, 1:1).

\textsuperscript{1}H NMR (DMSO-\textit{d}_6, 500 MHz): \(\delta\) 3.38 (t, \(J = 7.5\) Hz, 2H, H5), 4.09 (t, \(J = 7.5\) Hz, 2H, H4), 6.98 (t, \(J = 7.5\) Hz, 1H, H7), 7.26 (d, \(J = 7.0\) Hz, 1H, H6), 7.48 (d, \(J = 7.0\) Hz, 1H, H8). The \textsuperscript{1}H NMR spectral data coincided with those reported in the literature.\textsuperscript{225} LREI-MS: \(m/z\) 173 [M\textsuperscript{+}].

Synthesis of 7-bromo-4,5-dihydropyrrolo[3,2,1-\textit{hi}]indole-1,2-dione (82)

**Method A:** The cyclised isatin 46 (100 mg, 577 µmol) was dissolved in glacial AcOH (4 mL) and heated to 75 °C. Br\textsubscript{2} (276 mg, 88.7 µL, 1.73 mmol) was added dropwise while the solution was still hot. The reaction mixture was then cooled on ice, filtered and concentrated. MS analysis revealed a variety of products but no starting material or expected product.

**Method B:** The cyclised isatin 46 (100 mg, 577 µmol) was dissolved in 95% EtOH (4 mL) and heated to 75 °C. Br\textsubscript{2} (276 mg, 88.7 µL, 1.73 mmol) was added dropwise while the solution was still hot. The reaction mixture was then cooled on ice, filtered and concentrated. The resulting solid was purified by flash chromatography on silica gel (100% DCM) to yield 82 (47.8 mg, 33%) as dark red crystals, m.p. 162-164 °C, R\textsubscript{f} 0.33 (silica, DCM).

\textsuperscript{1}H NMR (DMSO-\textit{d}_6, 500 MHz): \(\delta\) 3.38 (t, \(J = 7.5\) Hz, 2H, H5), 4.07 (t, \(J = 7.5\) Hz, 2H, H4), 7.49 (s, 1H, H8), 7.66 (s, 1H, H6). \textsuperscript{13}C NMR (DMSO-\textit{d}_6, 126 MHz): \(\delta\) 30.7 (C5), 46.7 (C4), 113.7 (ArC), 115.3 (ArC), 124.7 (ArC), 128.3 (C8), 136.1 (C6), 155.9 (ArC), 159.3 (C2), 183.1 (C1). LREI-MS: \(m/z\) 251; 253
[M$^+$]$^{79}$Br; $^{81}$Br. **HRESI-MS:** $m/z$ calcd for C$_{10}$H$_7$NO$_2$$^{79}$Br [M+H]$^+$: 251.9660; found 251.9655.

**Attempted synthesis of pyrrolo[3,2,1-$h$]indole-1,2-dione (74)**

The preparation of this compound was attempted according to Method F for 46 using indole (83) (5.00 g, 42.7 mmol) and oxalyl chloride (10.8 g, 7.42 mL, 85.4 mmol) as starting materials. The reaction mixture was stirred at RT for a further 3 h, left to stand overnight and then concentrated. Et$_2$O (100 mL) was added to the residue, the suspension filtered and the filtrate was concentrated to yield 84 (1.70 g, 19%) as a red/brown powder.

AlCl$_3$ (5.17 g, 38.8 mmol) and the acid chloride 84 (1.61 g, 7.75 mmol) were stirred neat in a beaker for 20 min at 110 °C until all of the gas had been released. The red/black powder was cooled to RT before the addition of ice-H$_2$O (250 mL) and further stirring at RT for 2 h. The red solution was extracted with CHCl$_3$ ($2 \times 150$ mL), the organic layer was dried over MgSO$_4$, the suspension filtered, the solvent removed by rotary evaporation. Analysis by MS revealed the sample was a complex mixture of products and contained none of the desired product 74.
8.1.3 Experimental for Chapter 3

Synthesis of 5,6-dihydro-4H-pyrrolo[3,2,1-ij]quinoline-1,2-dione (85)

**Method A:** Choral hydrate (1.37 g, 8.26 mmol) and Na$_2$SO$_4$ (9.49 g, 66.8 mmol) were dissolved in H$_2$O (20 mL) and heated at 35 ºC in a 100 mL beaker. THQ (88) (1.00 g, 946 µL, 7.51 mmol) and conc. HCl (820 mg, 707 µL, 22.5 mmol) in H$_2$O (6 mL) were warmed and added, followed by hydroxylamine HCl (1.67 g, 24.0 mmol) in H$_2$O (8 mL). The reaction mixture was heated at 70-80 ºC for 1.5 h, then cooled on ice and filtered. MS analysis on the solid and filtrate revealed mainly starting material 88 so the experiment was abandoned.

**Method B:** According to the method of Cheng,$^{236}$ oxalyl chloride (1.05 g, 720 µL, 8.26 mmol) was added dropwise to a solution of DABCO (1.69 g, 15.0 mmol) in CHCl$_3$ (7.5 mL) to form a pale yellow salt. A solution of THQ (88) (1.00 g, 946 µL, 7.51 mmol) in CHCl$_3$ (40 mL) was added slowly with ice-bath cooling, before the reaction mixture was heated at reflux for 20 h. Upon cooling, H$_2$O (50 mL) was added and the mixture was extracted with CHCl$_3$ (50 mL). The organic layer was dried over MgSO$_4$, the suspension filtered and the solvent removed by rotary evaporation. MS analysis on the yellow semi-solid revealed only starting material 88 was present.

**Method C:**$^{237}$ A mixture of THQ (88) (1.00 g, 946 µL, 7.51 mmol) and oxalyl chloride (1.91 g, 1.27 mL, 15.0 mmol) was dissolved in anh. THF (20 mL) and heated at reflux for 3.5 h. The solution was evaporated and the red residue was dissolved in anh. DCM (80 mL) and heated at reflux. AlCl$_3$ was added portionwise over 30 min and the mixture was heated at reflux for another 3 h. The solution was evaporated, H$_2$O (15 mL) was added and the solution was extracted with CHCl$_3$ (3 × 15 mL) and brine (1 × 15 mL). The organic layer was dried over MgSO$_4$, the suspension filtered
and the solvent removed by rotary evaporation. The resulting solid was purified using flash chromatography on silica gel (100% DCM) to yield 85 (437 mg, 31%) as red crystals, m.p. 195-197 °C (lit.237 198-200 °C), R_f 0.14 (silica, DCM).

^1^H NMR (CDCl_3, 500 MHz): δ 2.04 (m, J = 6.0 Hz, 2H, H5), 2.77 (t, J = 6.0 Hz, 2H, H6), 3.75 (t, J = 6.0 Hz, 2H, H4), 6.99 (t, J = 7.5 Hz, 1H, H8), 7.32 (d, J = 7.5 Hz, 1H, H7), 7.37 (d, J = 7.5 Hz, 1H, H9). ^13^C NMR (CDCl_3, 126 MHz): δ 20.2 (C5), 23.9 (C6), 38.4 (C4), 115.7 (C9a), 121.9 (C6a), 123.0 (C9), 123.2 (C8), 137.1 (C7), 147.6 (C6a'), 156.9 (C2), 183.9 (C1). LREI-MS: m/z 187 [M^+]. HREI-MS: m/z calcd for C_{11}H_{9}NO_2 [M^+]: 187.0633; found 187.0638.

Synthesis of 8-bromo-5,6-dihydro-4H-pyrrolo[3,2,1-ij]quinoline-1,2-dione (92)

According to the method of Vine et al.,^8^ the isatin 85 (150 mg, 801 µmol) was dissolved in 95% EtOH (5 mL) at 70-75 °C. Br_2 (384 mg, 123 µL, 2.40 mmol) was added dropwise while the solution was still hot. The reaction mixture was then cooled, filtered and washed with H_2O and cold absolute EtOH. The resulting solid was purified by flash chromatography on silica gel (100% DCM) to yield 92 (166 mg, 78%) as dark red crystals, m.p. 201-203 °C (lit.238 185-187 °C), R_f 0.17 (silica, DCM).

^1^H NMR (CDCl_3, 500 MHz): δ 2.04 (quintet, J = 5.5, 6.0 Hz, 2H, H5), 2.77 (t, J = 6.0 Hz, 2H, H6), 3.75 (t, J = 5.5 Hz, 2H, H4), 7.47 (s, 1H, H9), 7.49 (s, 1H, H7). ^13^C NMR (CDCl_3, 126 MHz): δ 20.3 (C5), 23.9 (C6), 38.7 (C4), 116.3 (ArC), 117.1 (ArC), 124.3 (ArC), 125.9 (C7), 139.5 (C4), 146.7 (ArC), 156.4 (C2), 182.8 (C1).
LREI-MS: \( m/z \) 265; 267 \([M^+\text{Br}]\); \(^{81}\text{Br}\). HREI-MS: \( m/z \) calcd for \( C_{11}H_{8}NO_2^{79}\text{Br}[M^+] \): 264.9738; found 264.9738.

**Synthesis of 2-phenyl-1,2-dihydroquinoline**\(^{240}\)(98)

Quinoline (97) (6.00 g, 5.49 mL, 46.5 mmol) in anh. THF (20 mL) was added to 1 M PhMgBr in THF (46.5 mL, 46.5 mmol) at 0 °C before being heated at reflux for 18 h. Upon cooling, \( \text{H}_2\text{O} \) (200 mL) was added and the solution was extracted with \( \text{Et}_2\text{O} \) (3 \( \times \) 150 mL). The organic layer was dried over \( \text{MgSO}_4 \), the suspension filtered and the solvent removed by rotary evaporation to yield 98 as an orange/brown oil (8.67 g, 90%), b.p. 155 °C.

**Synthesis of 4-phenyl-4\(\text{H}\)-pyrrolo[3,2,1-\(\text{ij}\)]quinoline-1,2-dione** (99)

Dihydroquinoline 98 (1.04 g, 5.02 mmol) in anh. THF (6 mL) was added portionwise over 30 min to a refluxing solution of oxalyl chloride (1.27 g, 873 \( \mu \)L, 10.0 mmol) in anh. THF (4 mL). The reaction mixture was heated at reflux for 3.5 h before the solvent was removed by rotary evaporation. The dark residue was dissolved in anh. CHCl\(_3\) (30 mL) and heated at reflux. AlCl\(_3\) (2.01 g, 15.1 mmol) was added portionwise over 5 h and the solution was heated at reflux for a further 18 h. The solvent was once again removed by rotary evaporation, the residue was cooled on ice, conc. HCl (10 mL) was added, followed by \( \text{H}_2\text{O} \) (10 mL) and CHCl\(_3\) (50 mL). The phases were separated and the organic layer was washed with \( \text{H}_2\text{O} \) (2 \( \times \) 50 mL), dried over MgSO\(_4\), filtered and the solvent removed by rotary evaporation. The resulting solid was purified by flash chromatography on silica gel.
(100% DCM) to yield 99 (15.7 mg, 1%) as dark red crystals, m.p. 74-76 °C, R_f 0.41 (silica, DCM).

$^1$H NMR (CDCl$_3$, 500 MHz): δ 5.83 (dd, $J = 3.5$ Hz, 9.5 Hz, 1H, H5), 5.88 (d, $J = 3.0$ Hz, 1H, H4), 6.52 (d, $J = 10$ Hz, 1H, H6), 6.99 (t, $J = 8.0$ Hz, 1H, H8), 7.29 (d, $J = 7.5$ Hz, 1H, H7), 7.33 (d, $J = 6.5$ Hz, 1H, H9), 7.37 (m, 3H, H3', H4', H5'), 7.44 (d, $J = 7.5$ Hz, 2H, H2', H6').

$^{13}$C NMR (CDCl$_3$, 126 MHz): δ 56.0 (C4), 114.8 (C9a), 118.0 (C6a), 120.1 (C6), 123.6 (C8), 124.1 (C9), 127.3 (C2'/C6'), 127.4 (C5), 128.7 (C4'), 129.0 (C3'/C5'), 133.7 (C7), 139.2 (C1'), 147.3 (C6b), 157.4 (C2), 182.4 (C1).

LREI-MS: m/z 261[M+].

HREI-MS: m/z calcd for C$_{17}$H$_{12}$NO$_2$ [M+H]$: 262.0868; found 262.0858.

**Synthesis of 2-phenyl-1,2,3,4-tetrahydroquinoline**

Dihydroquinoline 98 (4.52 g, 21.8 mmol) was dissolved in absolute EtOH (87 mL) to give a 0.25 M solution of 98, which was subsequently heated at reflux. Na (9.01 g, 392 mmol) was added portionwise over 1 h and the mixture was heated at reflux for another 1 h. The product was added to H$_2$O (300 mL) and the solution was extracted with Et$_2$O (3 × 100 mL). The organic extract was dried over MgSO$_4$, the suspension filtered and the solvent removed by rotary evaporation. The resulting oil was purified by flash chromatography on alumina gel (100% DCM) to yield 100 as a yellow/orange oil (3.18 g, 70%), b.p. 192 °C (lit. b.p. 176 °C,$^{240}$ 196 °C$^{320}$), R_f 0.38 (silica, DCM).

$^1$H NMR (CDCl$_3$, 500 MHz): δ 1.93-2.02 (m, 2H, H3), 2.73-2.78 (m, 2H, H4), 3.97 (bs, 1H, NH), 4.44 (dd, $J = 2.5$ Hz, 9.0 Hz, 1H, H2), 6.46-6.67 (m, 2H, ArH × 2), 6.94-7.03 (m, 2H, ArH × 2), 7.22-7.41 (m, 5H, H2'-H6'). The $^1$H NMR
spectral data coincided with those reported in the literature.\textsuperscript{244} \textbf{LREI-MS}: $m/z$ 209 [M$^+$].

**Synthesis of 5,6-dihydro-4-phenyl-4\textit{H}-pyrrolo[3,2,1-\textit{i}]quinoline-1,2-dione (101)**

The compound was prepared according to the method for \textsuperscript{99} using \textbf{100} (2.00 g, 9.56 mmol) in anh. THF (8 mL) and oxalyl chloride (2.42 g, 1.66 mL, 19.1 mmol) in anh. THF (12 mL) as starting materials. The reaction mixture was heated at reflux for 3.5 h before the solvent was removed by rotary evaporation. The dark residue was dissolved in anh. CHCl$_3$ (60 mL) and heated at reflux. AlCl$_3$ (3.83 g, 28.7 mmol) was added portionwise over 5 h and the solution was heated at reflux for a further 18 h. The solvent was once again removed by rotary evaporation, the residue was cooled on ice, conc. HCl (20 mL) was added, followed by H$_2$O (20 mL) and DCM (100 mL). The phases were separated and the organic layer was washed with H$_2$O (2 × 50 mL), dried over MgSO$_4$, filtered and the solvent removed by rotary evaporation. The resulting solid was purified by flash chromatography on silica gel (100% DCM) to yield \textbf{101} (120 mg, 5%) as dark red crystals, m.p. 139-140 °C (lit.\textsuperscript{237} 129-130 °C), $R_f$ 0.38 (silica, DCM).

\textbf{1H NMR} (CDCl$_3$, 500 MHz): $\delta$ 2.16-2.22 (m, 1H, H5), 2.26-2.29 (m, 1H, H5), 2.47-2.53 (m, 1H, H6), 2.63-2.66 (m, 1H, H6), 5.47 (s, 1H, H4), 7.05 (t, $J = 7.5$ Hz, 1H, H8), 7.16 (d, $J = 7.5$ Hz, 2H, H2', H6'), 7.27 (t, $J = 7.0$ Hz, 1H, H4'), 7.32 (t, $J = 7.0$ Hz, 2H, H3', H5'), 7.35 (d, $J = 7.5$ Hz, 1H, H7), 7.47 (d, $J = 7.5$ Hz, 1H, H9). \textbf{13C NMR} (CDCl$_3$, 126 MHz): $\delta$ 19.8 (C6), 27.7 (C5), 51.6 (C4), 115.6 (ArC), 122.0 (ArC), 123.2 (C4), 123.2 (C8), 125.4 (C2', C6'), 127.8 (C4'), 128.9
(C3’, C5’), 137.2 (C7), 139.1 (ArC), 147.8 (ArC), 156.8 (C2), 183.6 (C1). **LREI-MS:**
m/z 263 [M⁺]. **HREI-MS:** m/z calcd for C₁₇H₁₃NO₂ [M⁺]: 263.0946; found 263.0949.

**Synthesis of 8-bromo-5,6-dihydro-4-phenyl-4H-pyrrolo[3,2,1-ij]quinoline-1,2-dione (102)**

According to the method of Vine et al., the isatin 101 (65.2 mg, 248 µmol) was dissolved in 95% EtOH (2.60 mL) at 70-75 °C. Br₂ (119 mg, 38.3 µL, 7.44 µmol) was added dropwise while the solution was still hot. The reaction mixture was then cooled, filtered and washed with H₂O and cold absolute EtOH. The resulting solid was purified by flash chromatography on silica gel (100% DCM) to yield 102 (42.3 mg, 50%) as red crystals, m.p. 60-62 °C, Rf 0.82 (silica, DCM/MeOH, 9:1).

**¹H NMR** (CDCl₃, 500 MHz): δ 2.15-2.19 (m, 1H, H5), 2.26-2.29 (m, 1H, H5), 2.50-2.53 (m, 1H, H6), 2.62-2.66 (m, 1H, H6), 5.46 (s, 1H, H4), 7.14 (d, J = 7.5 Hz, 2H, H2’, H6’), 7.28 (m, 1H, H4’), 7.32 (d, J = 7.5 Hz, 2H, H3’, H5’), 7.50 (s, 1H, H7), 7.59 (s, 1H, H9). **¹³C NMR** (CDCl₃, 126 MHz): δ 19.7 (C6), 27.6 (C5), 38.7 (C4), 116.2 (ArC), 116.7 (ArC), 124.2 (ArC), 125.3 (C2’/C6’), 125.9 (C9), 128.0 (ArC), 129.0 (C3’/C5’), 132.3 (C4’), 139.4 (C7), 146.7 (ArC), 156.0 (C2), 182.3 (C1).

**LREI-MS:** m/z 341; 343 [M⁺][⁷⁹Br]; ⁸¹Br. **HRESI-MS:** m/z calcd for C₁₇H₁₃NO₂[⁷⁹Br][M+H]⁺: 342.0130; found 342.0106.
Synthesis of 6-bromo-2-phenyl-1,2-dihydroquinoline (104)

The compound was prepared according to the method for 98 using 6-bromoquinoline (103) (3.00 g, 1.95 mL, 14.4 mmol) in anh. THF (6 mL) and 1 M PhMgBr in THF (14.4 mL, 14.4 mmol) as starting materials. The reaction mixture was heated at reflux for 18 h. Upon cooling, H₂O (70 mL) was added and the solution was extracted with Et₂O (3 × 100 mL). The organic layer was dried over MgSO₄, the suspension filtered and the solvent removed by rotary evaporation to yield 104 as light brown crystals (2.73 g, 66%), m.p. 80-82 °C, Rf 0.56 (silica, CHCl₃).

Synthesis of 8-bromo-4-phenyl-4H-pyrrolo[3,2,1-i]quinoline-1,2-dione (105)

The compound was prepared according to the method for 99 using 104 (1.50 g, 5.21 mmol) in anh. THF (6 mL) and oxalyl chloride (1.32 g, 907 µL, 10.4 mmol) in anh. THF (9 mL) as starting materials. The reaction mixture was heated at reflux for 3.5 h before the solvent was removed by rotary evaporation. The dark residue was dissolved in anh. CHCl₃ (45 mL) and heated at reflux. AlCl₃ (2.08 g, 15.6 mmol) was added portionwise over 5 h and the solution was heated at reflux for a further 18 h. The solvent was once again removed by rotary evaporation, the residue was cooled on ice, conc. HCl (15 mL) was added, followed by H₂O (15 mL) and CHCl₃ (75 mL). The phases were separated and the organic layer was washed with H₂O (2 × 30 mL), dried over MgSO₄, filtered and the solvent removed by rotary evaporation. The resulting solid was purified by flash
chromatography on silica gel (100% DCM) to yield 105 (39.5 mg, 2%) as a dark red powder, m.p. 47-49 °C, Rf 0.79 (silica, DCM).

\[ ^1H \text{ NMR (CDCl}_3, 500 \text{ MHz): } \delta 5.88 (s, 2H, H4, H5), 6.48 (d, J = 8.5 \text{ Hz, } 1H, H6), 7.33-7.39 (m, 3H, H3', H4', H5'), 7.42 (d, J = 7.0 \text{ Hz, } 2H, H2', H6'), 7.79 (d, J = 9.0 \text{ Hz, } 1H, H7), 7.90 (d, J = 8.5 \text{ Hz, } H, H9). \]

\[ ^{13}C \text{ NMR (CDCl}_3, 126 \text{ MHz): } \delta 56.1 \text{ (C4)}, 116.4 \text{ (ArC)}, 119.3 \text{ (ArC)}, 119.8 \text{ (ArC)}, 126.5 \text{ (ArC)}, 127.3 \text{ (C2', C6'}), 127.5 \text{ (C5)}, 128.8 \text{ (C4'}), 129.0 \text{ (C3', C5')}, 129.6 \text{ (ArC)}, 135.8 \text{ (ArC)}, 138.7 \text{ (C1')}, 146.9 \text{ (C6b)}, 157.7 \text{ (C2)}, 181.1 \text{ (C1)}. \]

\[ \text{LREI-MS: } m/z 339; 341 [M^+]+79\text{Br}; 81\text{Br}. \]

\[ \text{HRESI-MS: } m/z \text{ calcd for C}_{17}H_{11}NO_2^{79}\text{Br}[M+H]^+: 339.9973; \text{ found 339.9965.} \]

**Attemped synthesis of 5-methyl-pyrrolo[3,2,1-hi]indole-1,2-dione (109)**

**Method A:** According to the method of Sorensen and Pombo-Villar\(^{321}\), the isatin 49 (200 mg, 752 µmol), NaOAc (247 mg, 3.01 mmol), TBAI (278 mg, 752 µmol), P(o-tol)_3 (22.8 mg, 75.2 µmol) and Pd(OAc)_2 (8.50 mg, 37.6 µmol) were dissolved in anh. DMF (12 mL) and stirred at 110 °C for 18 h. Upon cooling, sat. NaHCO_3 (20 mL) was added and the solution was extracted with EtOAc (2 × 20 mL). The organic layer was dried over MgSO_4, the suspension filtered, and the solvent removed by rotary evaporation. There were difficulties in purifying the product and MS analysis revealed many components present.

**Method B:** The preparation of this compound was attempted according to Method A for 109 with the following modifications: TBACl (209 mg, 75.2 µmol) was used and the mixture was heated at 100 °C for 18 h. MS analysis revealed too many impurities.
Method C: The preparation of this compound was attempted according to Method A for 109 with the following modifications: TBACl (209 mg, 75.2 µmol) and PPh₃ (19.7 mg, 75.2 µmol) were used and the mixture was heated at 120 °C for 18 h. TLC analysis of the crude product indicated many components were present.

Method D: The preparation of this compound was attempted according to Method A for 109 with the following modifications: TBACl (209 mg, 75.2 µmol) and TTFMPP (35.0 mg, 75.2 µmol) were used and the mixture was heated at 120 °C for 18 h. MS analysis revealed too many impurities.

Synthesis of 4H-pyrrolo[3,2,1-ij]quinoline-1,2-dione (86) and 6H-pyrrolo[3,2,1-ij]quinoline-1,2-dione (87)

Method A: According to the method of Keller,²¹⁵ N-allyl-7-bromoisatin (49) (200 mg, 885 µmol), Pd(OAc)₂ (49.6 mg, 221 µmol), P(o-tol)₃ (116 mg, 381 µmol) and Et₃N (1.01 g, 1.38 mL, 10.0 mmol) were dissolved in CH₃CN (15.7 mL) and heated at 100 °C for 15 h. Upon cooling the solvent was removed by rotary evaporation and the resulting solid was purified by flash chromatography on silica gel (100% CHCl₃) and subsequent preparative TLC (silica, 100% CHCl₃) to yield 86 and 87 as dark red crystals (25.5 mg, 16%) in a 5:4 ratio.

Method B: According to the method of Li,³²² the isatin 49 (150 mg, 564 µmol), TBACl (157 mg, 564 µmol), K₂CO₃ (234 mg, 1.69 mmol) and Pd(OAc)₂ (12.7 mg, 56.4 µmol) were dissolved in anh. DMF (7 mL) and heated at 85 °C for 2 h. Upon cooling, DCM (50 mL) was added and the solution was extracted with H₂O.
(3 × 15 mL) and brine (1 × 15 mL). The organic layer was dried over MgSO₄, the suspension filtered and the solvent removed by rotary evaporation. The resulting solid was purified by flash chromatography on silica gel (100% DCM) and subsequent preparative TLC (silica, 100% DCM) to yield 86 and 87 as dark red crystals (54.7 mg, 52%) in a 3:1 ratio, m.p. 194-196 °C, Rₙ 0.31 (4ₐ isomer, silica, CHCl₃), 0.38 (6ₐ isomer, silica, CHCl₃).

¹H NMR (CDCl₃, 500 MHz): 4ₐ-pyrrolo[3,2,1-ij]quinoline-1,2-dione (86):
δ 4.62 (d, J = 8.5 Hz, 2H, H₄), 5.84 (dt, J = 3.5 Hz, 10.5 Hz, 1H, H₅), 6.40 (m, 1H, H₆), 6.93 (dt, J = 2.5 Hz, 7.5 Hz, 1H, H₈), 7.16 (dd, J = 2.0 Hz, 8.0 Hz, 1H, H₇), 7.30 (dd, J = 2.0 Hz, 7.5 Hz, 1H, H₉); 6ₐ-pyrrolo[3,2,1-ij]quinoline-1,2-dione (87):
δ 3.60 (d, J = 2.5 Hz, 2H, H₆), 5.29-5.32 (m, 1H, H₅), 6.95 (m, 1H, H₄), 7.19 (t, J = 7.5 Hz, 1H, H₈), 7.34 (d, J = 8.0 Hz, 1H, H₇), 7.47 (d, J = 7.5 Hz, 1H, H₉).

¹³C NMR (CDCl₃, 126 MHz): 4ₐ-pyrrolo[3,2,1-ij]quinoline-1,2-dione (86): δ 41.2 (C₄), 114.7 (C₉a), 118.3 (C₆a), 121.7 (C₆), 123.2 (C₅), 123.8 (C₈), 124.1 (C₉), 133.7 (C₇), 148.5 (C₆b), 157.6 (C₂), 182.6 (C₁). 6ₐ-pyrrolo[3,2,1-ij]quinoline-1,2-dione (87): δ 24.8 (C₆), 108.5 (C₅), 116.2 (C₉a), 118.0 (C₄), 119.1 (C₆a), 123.6 (C₉), 125.9 (C₈), 136.7 (C₇), 144.6 (C₆b), 153.2 (C₂), 182.8 (C₁). LREI-MS: Both isomers: m/z 185 [M⁺]. HREI-MS: m/z calcd for C₁₁H₇NO₂ [M⁺]: 185.0477; found 185.0471 (4ₐ isomer), 185.0474 (6ₐ isomer).
Synthesis of 1-allyl-5,7-dibromo-1\textit{H}-indole-2,3-dione (110)

This compound was prepared according to the method for 49 using 5,7-dibromoisatin (17) (1.50 g, 4.92 mmol), NaH (165 mg, 6.89 mmol), KI (163 mg, 984 µmol) and allyl bromide (1.31 g, 937 µL, 10.8 mmol) as starting materials. The resulting solid was purified by flash chromatography on silica gel (100% DCM) to yield 110 (1.48 g, 87%) as red crystals, m.p. 98-100 °C, (lit.\textsuperscript{64} 103-105 °C), R\textsubscript{f} 0.84 (silica, DCM/MeOH, 9:1).

\textsuperscript{1}H NMR (CDCl\textsubscript{3}, 300 MHz): \(\delta\) 4.78 (d, \(J = 4.8\) Hz, 2H, H1\textsuperscript{'}), 5.25 (d, \(J = 4.8\) Hz, 1H, H3\textsuperscript{'}), 5.90-6.03 (m, 1H, H2\textsuperscript{'}), 7.69 (s, 1H, H4), 7.85 (s, 1H, H6). The \textsuperscript{1}H NMR spectral data coincided with those reported in the literature.\textsuperscript{64} LREI-MS: \(m/z\) 343; 345; 347 [M\textsuperscript{+}]\textsuperscript{79}Br\textsuperscript{79}Br; \textsuperscript{79}Br\textsuperscript{81}Br; \textsuperscript{81}Br\textsuperscript{81}Br.

Synthesis of 8-bromo-4\textit{H}-pyrrolo[3,2,1-\textit{ij}]quinoline-1,2-dione (111) and 8-bromo-6\textit{H}-pyrrolo[3,2,1-\textit{ij}]quinoline-1,2-dione (112)

These compounds was prepared according to Method B for 86 and 87 using the isatin 110 (150 mg, 435 µmol), TBACl (121 mg, 435 µmol), K\textsubscript{2}CO\textsubscript{3} (181 mg, 1.31 mmol) and Pd(OAc)\textsubscript{2} (9.80 mg, 43.5 µmol) as starting materials. The resulting solid was purified by flash chromatography on silica gel (CHCl\textsubscript{3}/PS, 2:1) and subsequent preparative TLC (silica, 100% DCM) to yield 111 and 112 as dark red crystals (55.8 mg, 49%) in a 3:2 ratio, m.p. 215-217 °C, R\textsubscript{f} 0.56 (4\textit{H} isomer, silica, DCM), 0.67 (6\textit{H} isomer, silica, DCM).
\[\text{H NMR (CDCl}_3, 500 \text{ MHz): 8-bromo-4H-pyrrolo[3,2,1-ij]quinoline-1,2-dione (111): } \delta 4.63 \text{ (d, } J = 2.5 \text{ Hz, 2H, H4), 5.89 \text{ (dt, } J = 3.0 \text{ Hz, 8.0 Hz, 1H, H5), 6.35 \text{ (d, } J = 11 \text{ Hz, 1H, H6), 7.29 \text{ (s, 1H, H7), 7.42 \text{ (s, 1H, H9); 8-bromo-6H-pyrrolo[3,2,1-ij]quinoline-1,2-dione (112): } \delta 3.59 \text{ (m, 2H, H6), 5.30 \text{ (m, 1H, H5), 6.93 \text{ (d, } J = 8.5 \text{ Hz, 1H, H4), 7.50 \text{ (s, 1H, H7), 7.59 \text{ (s, 1H, H9).}}\]

\[\text{C NMR (CDCl}_3, 126 \text{ MHz): 8-bromo-4H-pyrrolo[3,2,1-ij]quinoline-1,2-dione (111): } \delta 41.2 \text{ (C4), 115.8 \text{ (ArC), 116.6 \text{ (ArC), 120.2 \text{ (ArC), 120.8 \text{ (C6), 124.7 \text{ (C5), 126.5 \text{ (C9), 135.8 \text{ (C7), 146.4 \text{ (C6b), 156.8 \text{ (C2), 181.3 \text{ (C1). 8-bromo-6H-pyrrolo[3,2,1-ij]quinoline-1,2-dione (112): } \delta 24.8 \text{ (C6), 108.5 \text{ (C5), 118.2 \text{ (C4), 119.2 \text{ (ArC), 121.4 \text{ (ArC), 122.8 \text{ (ArC), 126.6 \text{ (C9), 139.3 \text{ (C7), 148.5 \text{ (C6b), 158.4 \text{ (C2), 182.0 \text{ (C1).}}\]

\[\text{LREI-MS: Both isomers: } m/z 263; 265 \text{ [M+}^{79}\text{Br], }^{81}\text{Br. HRESI-MS: } m/z \text{ calcd for } C_{11}H_{7}NO_{2}^{79}\text{Br [M+H]}^{+}: 263.9660; \text{ found 263.9659 (4H isomer), } m/z \text{ calcd for } C_{11}H_{9}NO_{2}^{81}\text{Br [M+H]}^{+}: 267.9796; \text{ found 267.9611 (6H isomer).} \]

**Synthesis of (E)-7-bromo-1-(but-2-enyl)-1H-indole-2,3-dione (113)**

This compound was prepared according to the method for 49 using 7-bromoisatin (55) (500 mg, 2.21 mmol), NaH (74.2 mg, 3.09 mmol), KI (73.4 mg, 442 µmol) and crotyl bromide (656 mg, 500 µL, 4.86 mmol) as starting materials. The resulting solid was purified by flash chromatography on silica gel (100% DCM) to yield 113 (363 mg, 59%) as orange crystals, m.p. 71-73 °C, Rf 0.43 (silica, DCM).

\[\text{H NMR (CDCl}_3, 500 \text{ MHz): } \delta 1.69 \text{ (d, } J = 6.0 \text{ Hz, 3H, H4'), 4.73 \text{ (d, } J = 4.5 \text{ Hz, 2H, H1'), 5.61-5.64 \text{ (m, 1H, H2'), 5.74-5.80 \text{ (m, 1H, H3'), 6.99 \text{ (t, } J = 8.0 \text{ Hz, 1H, H5) 7.58 \text{ (d, } J = 7.0 \text{ Hz, 1H, H4), 7.69 \text{ (d, } J = 8.0 \text{ Hz, 1H, H6).}}\]
13C NMR (CDCl₃, 126 MHz): δ 17.8 (C4’), 42.6 (C1’), 104.2 (C3a), 120.7 (C7), 124.6 (C4), 124.7 (C2’), 124.9 (C5), 129.9 (C3’), 143.9 (C6), 147.9 (C7a), 158.5 (C2), 182.7 (C3). LREI-MS: m/z 279; 281 [M⁺]²⁹Br; ²¹Br. HRESI-MS: m/z calcd for C₁₂H₁₁NO₂²⁹Br [M+H]⁺: 279.9973; found 279.9969.

Synthesis of 6-methyl-6H-pyrrolo[3,2,1-ij]quinoline-1,2-dione (114)

The preparation of this compound was attempted according to the method for 86 and 87 using the isatin 113 (161 mg, 575 µmol), K₂CO₃ (239 mg, 1.73 mmol), TBACl (160 mg, 575 µmol) and Pd(OAc)₂ (12.9 mg, 57.5 µmol) as starting materials. The resulting solid was purified by flash chromatography on silica gel (100% DCM) and subsequent preparative TLC (silica, 100% DCM) to yield 114 a dark red powder (9.30 mg, 8%), m.p. 96-97 °C, R₉ 0.45 (silica, DCM).

¹H NMR (CDCl₃, 500 MHz): δ 1.43 (d, J = 7.5 Hz, 3H, CH₃), 3.74 (m, 1H, H6), 5.21 (dd, J = 3.0 Hz, 8.0 Hz, 1H, H5), 6.90 (dd, J = 2.0 Hz, 7.5 Hz, 1H, H4), 7.19 (t, J = 8.0 Hz, 1H, H8), 7.43 (d, J = 8.0 Hz, 1H, H7), 7.45 (d, J = 7.0 Hz, 1H, H9). ¹³C NMR (CDCl₃, 126 MHz): δ 23.6 (CH₃), 29.8 (C6), 114.7 (C5), 116.0 (C9a), 116.3 (C4), 123.4 (C9), 124.6 (C6a), 126.0 (C8), 136.1 (C7), 143.8 (C6b), 153.2 (C1), 182.8 (C2). LREI-MS: m/z 199 [M⁺]. HRESI-MS: m/z calcd for C₁₂H₁₀NO₂²⁹Br [M+H]⁺: 200.0712; found 200.0659.
Synthesis of \((E)\)-7-bromo-1-(3-phenyl-allyl)-1\(H\)-indole-2,3-dione (115)

This compound was prepared according to the method for 49 using 7-bromoisatin (55) (751 mg, 3.33 mmol), NaH (186 mg, 4.66 mmol), KI (111 mg, 670 \(\mu\)mol) and cinnamyl bromide (1.45 g, 1.09 mL, 7.33 mmol) as starting materials.

The resulting solid was purified by flash chromatography on silica gel (100% DCM) to yield 115 (551 mg, 48%) as an orange powder, m.p. 149-151 °C, \(R_f\) 0.33 (silica, DCM).

\(^1\text{H NMR}\) (CDCl\(_3\), 500 MHz): \(\delta\) 4.96 (d, \(J = 6.0\) Hz, 2H, \(H1'\)), 6.30-6.36 (m, 1H, \(H2'\)), 6.66 (d, \(J = 16\) Hz, 1H, \(H3'\)), 7.01 (t, \(J = 7.0\) Hz, 1H, \(H5\)), 7.24 (t, \(J = 7.5\) Hz, 1H, \(H4'\)) 7.30 (t, \(J = 7.5\) Hz, 2H, \(H3', H5'\)), 7.35 (d, \(J = 7.5\) Hz, 2H, \(H2', H6'\)), 7.60 (d, \(J = 7.0\) Hz, 1H, \(H4\)), 7.70 (d, \(J = 8.0\) Hz, 1H, \(H6\)). \(^{13}\text{C NMR}\) (CDCl\(_3\), 126 MHz): \(\delta\) 47.8 (C1'), 104.2 (C3a), 120.8 (C7), 123.0 (C2'), 124.7 (C4), 125.1 (C5), 126.5 (C2', C6'), 128.0 (C4'), 128.6 (C3', C5'), 133.5 (C3'), 136.2 (C1'), 144.0 (C6), 147.7 (C7a), 158.6 (C2), 182.5 (C3). \(\text{LREI-MS}: m/z\) 341; 343 [M\(^+\)]\(^{79}\)Br; \(^{81}\)Br. \(\text{HREI-MS}: m/z\) calcd for C\(_{17}\)H\(_{12}\)NO\(_2\)^{79}Br [M\(^+\)]: 341.0051; found 341.0055.

Synthesis of 6-phenyl-4\(H\)-pyrrolo[3,2,1-ij]quinoline-1,2-dione (116)

The preparation of this compound was attempted according to the method for 86 and 87 using the isatin 115 (130 mg, 380 \(\mu\)mol), K\(_2\)CO\(_3\) (158 mg, 1.14 mmol), TBACl (106 mg, 380 \(\mu\)mol) and Pd(OAc)\(_2\) (8.50 mg, 38.0 \(\mu\)mol) as starting materials. The resulting solid was purified by flash chromatography on silica gel (CHCl\(_3\)/PS, 3:2) and subsequent preparative TLC.
(silica, 100% DCM) to yield 116 as orange/red crystals (17.2 mg, 17%), m.p. 130-132 °C, Rf 0.57 (silica, DCM).

**¹H NMR** (CDCl₃, 500 MHz): δ 4.88 (bs, 2H, H4'), 5.53 (dd, J = 4.0 Hz, 8.0 Hz, 1H, H5), 7.07 (d, J = 8.0 Hz, 1H, H4'), 7.12 (t, J = 7.5 Hz, 1H, H8), 7.25-7.29 (m, 2H, H2'/H6'), 7.36 (t, J = 7.5 Hz, 2H, H3'/H5'), 7.48 (d, J = 7.0 Hz, 1H, H9).

**¹³C NMR** (CDCl₃, 126 MHz): δ 41.7 (C4), 112.8 (C5), 116.2 (ArC), 116.5 (ArC), 123.7 (C9), 126.1 (C8), 127.5 (C6a), 128.3 (C2', C6'), 128.9 (C4'), 129.0 (C3', C5'), 137.5 (C7), 143.4 (C6), 147.6 (ArC), 153.4 (C2), 182.5 (C1). LREI-MS: m/z 261 [M⁺]. HRESI-MS: m/z calcd for C₁₁H₁₂NO₂ [M+H]⁺: 262.0868; found 262.0855.

**Synthesis of (E)-5,7-dibromo-1-(3-phenyl-allyl)-1H-indole-2,3-dione (117)**

![Chemical Structure](image)

This compound was prepared according to the method for 49 using 5,7-dibromoisatin (17) (600 mg, 1.97 mmol), NaH (66.2 mg, 2.76 mmol), KI (91.6 mg, 552 µmol) and cinnamyl bromide (524 mg, 393 µL, 4.33 mmol) as starting materials. The resulting solid was purified by flash chromatography on silica gel (100% CHCl₃) to yield 117 (802 mg, 97%) as red crystals, m.p. 109-111 °C, (lit.⁶⁴ 115-117 °C), Rf 0.80 (silica, DCM/MeOH, 9:1).

**¹H NMR** (CDCl₃, 500 MHz): δ 4.94 (d, J = 5.5 Hz, 2H, H1'), 6.29 (dt, J = 5.5 Hz, 16 Hz, 1H, H2'), 6.64 (d, J = 16 Hz, 1H, H3'), 7.25 (m, 1H, H4'), 7.32 (t, J = 7.5 Hz, 2H, H3', H5'), 7.35 (d, J = 8.0 Hz, 2H, H2', H6'), 7.70 (d, J = 1.5 Hz, 1H, H4), 7.86 (s, 1H, H6). The ¹H NMR spectral data coincided with those reported in the literature.⁶⁴ LREI-MS: m/z 419; 421; 423 [M⁺] ⁷⁹Br, ⁷⁹Br, ⁷⁹Br, ⁸¹Br, ⁸¹Br, ⁸¹Br.
Attempted synthesis of 8-bromo-6-phenyl-4H-pyrrolo[3,2,1-ij]quinoline-1,2-dione (118)

The preparation of this compound was attempted according to the method for 116 using the isatin 117 (150 mg, 356 µmol), K₂CO₃ (148 mg, 1.07 mmol), TBACl (98.9 mg, 356 µmol) and Pd(OAc)₂ (8.00 mg, 35.6 µmol) as starting materials. The reaction mixture was heated at 80 ºC for 2-4 h. Analysis by TLC and MS revealed the product was predominantly starting material 117.

Synthesis of 5,6-dihydropyphenanthridine (121)

Method A: A mixture of phenanthidine (119) (1.00 g, 5.58 mmol) and tin powder (2.52 g, 21.2 mmol, 80-200 mesh) was dissolved in conc. HCl (10 mL) and heated at reflux for 4-18 h. The solution was basified with NaOH solution and extracted with Et₂O (3 × 20 mL). MS analysis revealed only the starting material 119 was present.

Method B: A mixture of phenanthidine (119) (5.00 g, 27.9 mmol) and AcOH (1.68 g, 1.60 mL, 27.9 mmol) were added to absolute EtOH (50 mL) and heated at reflux for 30 min before the addition of NaCNBH₃ (3.33 g, 53.1 mmol). The solution was heated at reflux for a further 1.5 h, cooled and the solvent removed by rotary evaporation. The white residue was basified with aq. NH₃ (100 mL) and extracted with Et₂O (3 × 50 mL). The organic extract was evaporated and the white precipitate was recrystallised from 95% EtOH to yield 121 (3.89 g, 77%) as yellow crystals, m.p. 64-66 ºC (lit. 102-104 ºC), R_f 0.53 (silica, DCM).

¹H NMR (CDCl₃, 500 MHz): δ 3.72 (s, 1H, NH), 4.39 (s, 2H, H6), 6.67 (m, 1H, ArH), 6.82-6.86 (m, 1H, ArH), 7.09-7.12 (m, 2H, ArH × 2), 7.21 (m, 1H, ArH),
7.30-7.33 (m, 1H, ArH), 7.69 (m, 2H, ArH × 2). The $^1$H NMR spectral data coincided with those reported in the literature.$^{323}$ LREI-MS: $m/z$ 181 [M$^+$].

**Synthesis of 7H-pyrrolo[3,2,1-de]phenanthridine-4,5-dione (126)**

**Method A:** The preparation of this compound was attempted according to the method for 85 using 121 (785 mg, 4.33 mmol), oxalyl chloride (1.10 g, 759 µL, 8.66 mmol) and AlCl$_3$ (1.73 g, 13.0 mmol) as starting materials. The product was starting material 85 as determined by MS.

**Method B:** A mixture of 5,6-dihydrophenanthridine (121) (500 mg, 2.76 mmol), oxalyl chloride (701 mg, 482 µL, 5.52 mmol), DMAP (33.7 mg, 276 µmol) and DIPEA (357 mg, 480 µL, 2.76 mmol) was dissolved in anh. THF (10 mL) and heated at reflux for 3.5 h. The solution was evaporated and the residue was dissolved in anh. CHCl$_3$ (40 mL) and heated at reflux. AlCl$_3$ (1.10 g, 8.28 mmol) was added portionwise over 30 min and the solution was heated at reflux for a further 1.5 h. Upon cooling, the solution was extracted with H$_2$O (2 × 25 mL) and brine (1 × 25 mL). The organic extract was dried over MgSO$_4$, filtered and evaporated. The resulting solid was purified using flash chromatography on silica gel (100% DCM) to yield 126 (100 mg, 15%) as dark red crystals, m.p. 212-214 °C, $R_f$ 0.24 (silica, DCM).

$^1$H NMR (CDCl$_3$, 500 MHz): $\delta$ 5.06 (s, 2H, H7), 7.09 (t, $J = 7.5$ Hz, 1H, H2), 7.19 (d, $J = 7.5$ Hz, 1H, H8), 7.33 (t, $J = 7.0$ Hz, 1H, H9), 7.37 (t, $J = 7.5$ Hz, 1H, H10), 7.42 (d, $J = 7.5$ Hz, 1H, H3), 7.76 (d, $J = 8.0$ Hz, 1H, H11), 7.87 (d, $J = 8.0$ Hz, 1H, H1). $^{13}$C NMR (CDCl$_3$, 126 MHz): $\delta$ 42.6 (C7), 116.5 (C3a), 118.9 (C11b), 122.8 (C11), 124.3 (C2), 124.7 (C3), 126.9 (C11a), 128.1 (C8), 128.4 (C7a), 128.7
(C10), 129.6 (C9), 130.7 (C1), 146.6 (C11c), 157.9 (C5), 183.2 (C4). LREI-MS: m/z 235 [M⁺]. HREI-MS: m/z calcd for C₁₅H₉NO₂ [M⁺]: 235.0633; found 235.0625.

Synthesis of 2-bromo-7H-pyrrolo[3,2,1-de]phenanthridine-4,5-dione (127)

Method A: The preparation of this compound was attempted according to the method for 92 using 126 (100 mg, 426 µmol) and Br₂ (205 mg, 66.0 µL, 1.28 mmol) as starting materials. The compound did not dissolve in 95% EtOH (12 mL).

Method B: The preparation of this compound was attempted according to Method A for using 126 (84.0 mg, 357 µmol) and Br₂ (171 mg, 55.0 µL, 1.07 mmol) as starting materials. Absolute EtOH (5 mL) did not dissolve the compound.

Method C: According to the method of Vine et al., 81 7H-pyrrolo[3,2,1-de]phenanthridine-4,5-dione (126) (84.0 mg, 357 µmol) was dissolved in glacial AcOH (5 mL) and heated to 75 °C. Br₂ (171 mg, 55.0 µL, 1.07 mmol) was added dropwise while the solution was still hot. The reaction mixture was then cooled, the precipitated product collected by filtration, and washed with absolute EtOH. The resulting solid was purified by flash chromatography on silica gel (100% CHCl₃) to yield 127 (77.1 mg, 69%) as dark red powder, m.p. 239-241 °C, Rf 0.25 (silica, CHCl₃).

¹H NMR (CDCl₃, 300 MHz): δ 5.08 (s, 2H, H7), 7.22 (m, 1H, H11), 7.38 (m, 2H, H8, H10), 7.53 (s, 1H, H3), 7.70 (m, 1H, H9), 7.98 (s, 1H, H1). ¹³C NMR (CDCl₃, 75 MHz): δ 42.4 (C7), 117.1 (ArC), 117.3 (ArC), 120.7 (ArC), 122.8 (C9), 125.6 (ArC), 126.9 (C3), 128.0 (C11), 128.5 (ArC), 128.7 (C10), 130.1 (C8), 132.8
(C1), 145.2 (C11b’), 156.8 (C5), 181.7 (C4). **LREI-MS**: m/z 313; 315 [M^+\(^{79}\)Br, ^{81}\)Br]. **HREI-MS**: m/z calcd for C\(_{15}\)H\(_8\)NO\(_2\)\(^{81}\)Br [M^+]: 314.9718; found 314.9731.

**Synthesis of 7-iodo-1-(naphthalen-1-ylmethyl)-1H-indole-2,3-dione (128)**

![Chemical structure of 128](image)

This compound was prepared according to the method for 49 using 7-iodoisatin (56) (6.00 g, 22.0 mmol) and 1-(chloromethyl)naphthalene (8.11 g, 6.87 mL, 48.4 mmol) as starting materials. The reaction mixture was heated at 60 °C and stirred at this temperature for 18 h. The resulting solid was purified by flash chromatography on silica gel (100% CHCl\(_3\)) to yield 128 (5.29 g, 58%) as a red powder, m.p. 177-178 °C; R\(_f\) 0.21 (silica, 100% CHCl\(_3\)).

**\(^1\)H NMR** (CDCl\(_3\), 500 MHz): \(\delta\) 5.89 (s, 2H, H1’), 6.90 (t, J = 8.0 Hz, 1H, H5), 7.10 (d, J = 7.0 Hz, 1H, H2’’), 7.38 (t, J = 7.5 Hz, 1H, H3’’), 7.56 (t, J = 7.5 Hz, 1H, H6’’), 7.62 (t, J = 7.5 Hz, 1H, H7’’), 7.72 (d, J = 7.0 Hz, 1H, H4), 7.81 (d, J = 8.5 Hz, 1H, H4’’), 7.92 (dd, J = 3.5 Hz, 8.5 Hz, 2H, H5’’’, H6), 7.99 (d, J = 8.0 Hz, 1H, H8’’’). **\(^{13}\)C NMR** (CDCl\(_3\), 126 MHz): \(\delta\) 42.1 (C1’), 74.1 (C7), 120.6 (C3a), 121.9 (C2’’), 122.3 (C8’’), 125.4 (C4, C5 or C3’’’), 125.5 (C4, C5 or C3’’’), 125.6 (C4, C5 or C3’’’), 126.1 (C6’’), 126.6 (C7’’), 128.1 (C4’’), 129.0 (C5’’’), 130.0 (C8a’’’), 131.0 (C1’’’), 133.9 (C4a’’’), 151.0 (C7a’’’), 151.1 (C6), 158.9 (C2), 182.4 (C3). **LREI-MS**: m/z 413 [M^+]. **HRESI-MS**: m/z calcd for C\(_{19}\)H\(_{12}\)NO\(_2\)I [M+H]^+: 412.9913; found 412.9915.
Synthesis of 7'-iodo-1'-(naphthalen-1-ylmethyl)spiro[[1,3]-dioxolane-2,3'-indolin]-2'-one (130)

According to the method of Ribeiro et al., the isatin 128 (4.96 g, 12.0 mmol), ethylene glycol (14.9 g, 13.4 mL, 240 mmol) and PTSA (496 mg, 10% of starting material weight) were dissolved in PhMe (40 mL) and heated at reflux under Dean-Stark conditions for 24 h. No evidence of product had formed so more ethylene glycol was added (7.45 g, 6.70 mL, 120 mmol) and the solution was heated at reflux for a further 24 h before the solvent was removed by rotary evaporation. The resulting solid was purified by flash chromatography on silica gel (100% DCM) to yield 130 (4.14 g, 75%) as a beige powder, m.p. 236-238 °C, Rf 0.39 (silica, DCM).

**1H NMR** (CDCl3, 500 MHz): δ 4.38 (t, J = 7.0 Hz, 2H, H4, H5), 4.63 (t, J = 6.5 Hz, 2H, H4, H5), 5.77 (s, 2H, H1’’), 6.83 (d, J = 7.5 Hz, 1H, H5’’), 7.05 (d, J = 7.5 Hz, 1H, H2’’), 7.37 (t, J = 7.5 Hz, 1H, H3’’), 7.44 (d, J = 7.5 Hz, 1H, H4’), 7.53 (t, J = 7.0 Hz, 1H, H6’’), 7.60 (d, J = 8.0 Hz, 1H, H7’’), 7.70 (d, J = 8.0 Hz, 1H, H6’), 7.77 (d, J = 8.0 Hz, 1H, H4’’), 7.90 (d, J = 7.5 Hz, 1H, H5’’), 8.00 (d, J = 8.5 Hz, 1H, H8’’). **13C NMR** (CDCl3, 126 MHz): δ 41.9 (C1’’), 66.3 (CH2CH2), 72.8 (C7’), 101.2 (ArC), 122.3 (C2’’), 122.7 (C8’’), 125.2 (C4’), 125.4 (C5’), 125.9 (C3’’), 126.0 (C6’’), 126.5 (C7’’), 127.5 (ArC), 127.8 (C4’’), 129.1 (C5’’), 130.4 (ArC), 131.9 (ArC), 134.0 (ArC), 144.7 (C6’ + ArC), 174.5 (C2’). **LREI-MS:** m/z 457 [M⁺]. **HRESI-MS:** m/z calcd for C21H17NO3I [M+H]⁺: 458.0253; found 458.0233.
Synthesis of spiro[benzo[i]pyrrolo[3,2,1-de]phenanthridine-4,2’-[1,3]dioxolan]-5(7H)-one (131)

According to the method of Torres et al.,\textsuperscript{232} the ketal 130 (2.00 g, 4.37 mmol), Pd(OAc)$_2$ (98.0 mg, 437 µmol), TBAB (1.55 g, 4.81 mmol) and NaOAc (1.79 g, 21.9 mmol) were dissolved in anh. DMF (44 mL) and heated at 90 °C for 5 h. Upon cooling, H$_2$O (200 mL) was added and the solution was extracted with EtOAc (3 × 100 mL). The organic extract was dried over MgSO$_4$, filtered and evaporated. The resulting solid was purified using flash chromatography on silica gel (100% CHCl$_3$) to yield 131 (1.24 g, 86%) as a light brown powder, m.p. 222-224 °C, R$_f$ 0.41 (silica, DCM).

$^1$H NMR (CDCl$_3$, 500 MHz): $\delta$ 4.36 (t, $J$ = 6.5 Hz, 2H, H4, H5), 4.63 (t, $J$ = 6.5 Hz, 2H, H4, H5), 5.27 (s, 2H, H7'), 7.08 (t, $J$ = 7.0 Hz, 1H, H2'), 7.25 (m, 1H, H1' or H3'), 7.50 (t, $J$ = 8.0 Hz, 1H, H10'), 7.56 (t, $J$ = 8.0 Hz, 1H, H9'), 7.72 (d, $J$ = 8.0 Hz, 2H, H1' or H3' + H8'), 7.79-7.84 (m, 3H, H11', H12', H13').

$^{13}$C NMR (CDCl$_3$, 126 MHz): $\delta$ 40.7 (C7'), 65.9 (C4/C5), 103.2 (C3a'), 117.4 (C13b'), 120.0 (C11' or C12' or C13'), 122.3 (ArC), 122.4 (C8'), 123.4 (ArC), 123.5 (C2'), 124.3 (C1' or C3'), 124.5 (C1' or C3'), 125.2 (ArC), 126.4 (C10'), 127.3 (C9'), 128.7 (C11' or C12' or C13'), 128.8 (C11' or C12' or C13'), 130.3 (C7b'), 133.0 (C11a'), 139.3 (C13c'), 183.2 (C5').

LREI-MS: $m/z$ 329 [M$^+$]. HRESI-MS: $m/z$ calcd for C$_{21}$H$_{16}$NO$_3$ [M$^+$]: 330.1130; found 330.1117.
Synthesis of 7H-benzo[i]pyrrolo[3,2,1-de]phenanthridine-4,5-dione (132)

According to the method of Torres et al., the cyclised ketal 131 (1.10 g, 3.34 mmol) was dissolved in 6 M HCl/THF (1:1, 35 mL) and heated at reflux for 5 h. Upon cooling, H2O (200 mL) was added, the solution filtered and washed with more H2O to yield 132 (641 mg, 67%) as a fine dark red powder, m.p. 251-253 °C, Rf 0.29 (silica, DCM).

^1H NMR (CDCl₃, 500 MHz): δ 5.44 (s, 2H, H7), 7.14 (t, J = 7.5 Hz, 1H, H2), 7.45 (d, J = 7.5 Hz, 1H, H1 or H3), 7.62 (t, J = 7.0 Hz, 1H, H10), 7.66 (t, J = 7.5 Hz, 1H, H9), 7.95 (d, J = 8.5 Hz, 1H, H8), 8.01 (t, J = 7.5 Hz, 2H, H11 + H12 or H13), 8.16 (d, J = 9.0 Hz, 1H, H12 or H13), 8.25 (d, J = 8.0 Hz, 1H, H1 or H3). ^13C NMR (CDCl₃, 126 MHz): δ 40.8 (C7), 115.7 (C3a or C13b), 118.7 (C3a or C13b), 119.6 (C11 or C12 or C13), 122.4 (C8), 123.4 (ArC), 124.4 (C2), 124.7 (C1 or C3), 127.0 (C10), 127.8 (C9), 129.2 (C11 or C12 or C13), 129.9 (ArC), 130.2 (C7b), 130.8 (C12 or C13), 133.3 (C11a), 134.2 (ArC), 146.5 (C13c), 157.8 (C4), 182.9 (C5).

LREI-MS: m/z 285 [M⁺]. HRESI-MS: m/z calcd for C₁₉H₁₂NO₂ [M+H]^+: 286.0868; found 286.0829.
Attempted synthesis of 2-bromo-7H-benzo[i]pyrrolo[3,2,1-de]phenanthridine-4,5-dione (133)

**Method A:** The preparation of this compound was attempted according to the method of Vine et al. The isatin 132 (150 mg, 526 µmol) was added to glacial AcOH (5 mL) and heated to 70-75 °C but the compound would not dissolve, even when additional solvent (10 mL) was added. The isatin 132 was not able to dissolve in 95% EtOH or CH$_3$CN at 70-75 °C, so no bromination was observed in these solvents either.

**Method B:** According to the method of Zysman-Colman et al., the cyclised phenanthridine 132 (100 mg, 351 µmol) was dissolved in anh. CH$_3$CN (1 mL) at 0 ºC. NBS (62.5 mg, 351 µmol) in anh. CH$_3$CN (1 mL) was added and the reaction mixture was warmed to RT and stirred for 18 h. DCM (30 mL) was then added and the solution was extracted with H$_2$O (3 × 20 mL). The organic extract was dried over MgSO$_4$, filtered and evaporated. MS analysis on the crude product revealed it only contained the starting material 132 so the experiment was abandoned.

Synthesis of 7-iodo-1-(naphthalen-2-ylmethyl)-1H-indole-2,3-dione (129)

This compound was prepared according to the method for using 7-iodoisatin (56) (2.50 g, 9.16 mmol) and 2-(bromomethyl)naphthalene (3.03 g, 13.7 mmol) as starting materials. The reaction mixture was heated at 60 °C and stirred at this temperature for 18 h. The resulting solid was purified by flash chromatography on silica gel (100% CHCl$_3$) to yield 129 (1.37 g, 36%) as red/brown crystals, m.p. 154-155 °C, R$_f$ 0.41 (silica gel, DCM).
\[ ^1H \text{NMR} \ (CDCl_3, \ 500 \text{ MHz}): \delta \ 5.64 \ (s, \ 2H, \ H1'), 6.88 \ (t, \ J = 7.5 \text{ Hz}, \ 1H, \ H5), 7.38 \ (d, \ J = 7.5 \text{ Hz}, \ 1H, \ H3''), 7.46 \ (dd, \ J = 3.0 \text{ Hz}, \ 6.0 \text{ Hz}, \ 2H, \ H6'', \ H7''), 7.61 \ (s, \ 1H, \ H1''), 7.68 \ (d, \ J = 6.5 \text{ Hz}, \ 1H, \ H4), 7.75 \ (dd, \ J = 3.0 \text{ Hz}, \ 5.5 \text{ Hz}, \ 1H, \ H8''), 7.81-7.85 \ (m, \ 2H, \ H4'', \ H5''), 7.94 \ (d, \ J = 8.0 \text{ Hz}, \ 1H, \ H6). \]
\[ ^13C \text{NMR} \ (CDCl_3, \ 126 \text{ MHz}): \delta \ 43.8 \ (C1'), \ 73.7 \ (C7), \ 120.8 \ (C3a), \ 124.4 \ (C3''), \ 124.9 \ (C1''), \ 125.5 \ (C4), \ 125.7 \ (C5), \ 126.0 \ (C6'' \text{ or } C7''), \ 126.4 \ (C6'' \text{ or } C7''), \ 127.7 \ (C4'' \text{ or } C5'' \text{ or } C8''), \ 127.8 \ (C4'' \text{ or } C5'' \text{ or } C8''), \ 128.7 \ (C4'' \text{ or } C5''), \ 132.7 \ (C4a''), \ 133.3 \ (C8a'' + C2''), \ 150.9 \ (C7a), \ 151.1 \ (C6), \ 159.2 \ (C2), \ 182.4 \ (C3). \]

**LREI-MS:** m/z 413 [M⁺].

**HRESI-MS:** m/z calcd for C₁₉H₁₃NO₂I [M+H]⁺: 413.9991; found 413.9950.

### Synthesis of 7'-iodo-1'- (naphthalen-2-ylmethyl)spiro[[1,3]-dioxolane-2,3'-indolin]-2'-one (135)

According to the method of Ribeiro et al., the isatin 129 (1.10 g, 2.66 mmol), ethylene glycol (4.95 g, 4.45 mL, 79.8 mmol) and PTSA (110 mg, 10% of starting material weight) were dissolved in PhMe (10 mL) and heated at reflux under Dean-Stark conditions for 64 h. Upon cooling, the solvent was removed by rotary evaporation. The resulting solid was purified by flash chromatography on silica gel (100% DCM) to yield 135 (1.12 g, 92%) as a beige powder, m.p. 156-158 °C, R₇ 0.40 (silica, DCM).

\[ ^1H \text{NMR} \ (CDCl_3, \ 500 \text{ MHz}): \delta \ 4.37 \ (\text{quintet}, \ J = 1.5 \text{ Hz}, \ 7.0 \text{ Hz}, \ 2H, \ H4, \ H5), \ 4.63 \ (\text{quintet}, \ J = 1.5 \text{ Hz}, \ 7.5 \text{ Hz}, \ 2H, \ H4, \ H5), \ 5.51 \ (s, \ 2H, \ H1'''), \ 6.82 \ (t, \ J = 8.0 \text{ Hz}, \ 1H, \ H5'), \ 7.35 \ (dd, \ J = 1.5 \text{ Hz}, \ 8.5 \text{ Hz}, \ 1H, \ H3'''), \ 7.41 \ (dd, \ J = 1.0 \text{ Hz}, \ 7.0 \text{ Hz}, \ 1H, \ H4'), \ 7.43 \ (m, \ 2H, \ H6'', \ H7''), \ 7.57 \ (s, \ 1H, \ H1'''), \ 7.71 \ (dd, \ J = 1.0 \text{ Hz}, \ 7.0 \text{ Hz}, \ 1H, \ H4''), \ 7.75 \ (s, \ 1H, \ H1''), \ 7.81 \ (dd, \ J = 1.0 \text{ Hz}, \ 7.0 \text{ Hz}, \ 1H, \ H4''), \ 7.85 \ (m, \ 2H, \ H4'', \ H5''), \ 7.94 \ (d, \ J = 8.0 \text{ Hz}, \ 1H, \ H6). \]

~ 221 ~
8.5 Hz, 1H, H1’’’), 7.76 (m, 1H, H8’’’), 7.81 (m, 2H, H4’’’ or C7’’’), 13C NMR (CDCl₃, 126 MHz): δ 43.7 (C1’’’), 66.3 (C4 + C5), 72.6 (C7’), 101.1 (C3a’’’), 124.5 (C3’’’), 124.7 (C1’’’), 124.9 (C4’), 125.2 (C5’), 125.7 (C6’’ or C7’’’), 126.1 (C6’ or C7’’’), 127.4 (C3’), 127.6 (C4’’ or C5’’ or C8’’’), 127.9 (C4’’ or C5’’ or C8’’’), 128.5 (C4’ or C5’’), 132.6 (C2’’ or C4a’’ or C8a’’’), 133.4 (C2’’ or C4a’’ or C8a’’’), 134.1 (C2’’ or C4a’’ or C8a’’’), 144.4 (C7a’), 144.5 (C6’), 174.5 (C2’’’).

HREI-MS: m/z calcd for C₂₁H₁₇NO₃I [M+H]⁺: 458.0253; found 458.0215.

**Synthesis of spiro[benzo[k]pyrrolo[3,2,1-de]phenanthridine-4,2'-[1,3]dioxolan]-5(7H)-one (136)**

According to the method of Torres et al.,²³² the ketal 135 (1.00 g, 2.19 mmol), Pd(OAc)₂ (49.2 mg, 219 µmol), TBAB (777 mg, 2.41 mmol) and NaOAc (902 mg, 11.0 mmol) were dissolved in anh. DMF (22 mL) and heated at 90 °C for 5 h. Upon cooling, H₂O (100 mL) was added and the solution was extracted with EtOAc (2 × 60 mL). The organic extract was dried over MgSO₄, filtered and evaporated. The resulting solid was purified using flash chromatography on silica gel (100% CHCl₃) to yield 136 (485 mg, 67%) as a beige powder, m.p. 161-163 °C, Rf 0.20 (silica, DCM).

¹H NMR (CDCl₃, 500 MHz): δ 4.36 (t, J = 6.5 Hz, 2H, H4, H5), 4.61 (t, J = 6.5 Hz, 2H, H4, H5), 5.05 (s, 2H, H7’), 7.14 (t, J = 8.0 Hz, 1H, H2’), 7.21 (d, J = 8.5 Hz, 1H, H8’), 7.30 (d, J = 7.5 Hz, 1H, H3’), 7.50 (t, J = 7.5 Hz, 1H, H11’), 7.56 (t, J = 7.5 Hz, 1H, H12’), 7.74 (d, J = 8.0 Hz, 1H, H9’), 7.85 (d, J = 9.0 Hz, 1H, H10’), 8.17 (d, J = 8.5 Hz, 1H, H1’), 8.63 (d, J = 9.0 Hz, 1H, H13’). ¹³C NMR
(CDCl$_3$, 126 MHz): $\delta$ 44.5 (C7'), 66.1 (C4, C5), 119.1 (C13c'), 121.6 (C3a'), 123.3 (C2'), 124.1 (C3'), 125.1 (C8'), 125.3 (C13'), 125.5 (C13b'), 126.1 (C11'), 127.3 (C12'), 129.2 (C9' or C10'), 129.3 (C9' or C10'), 129.4 (C1'), 129.8 (C7a'), 130.2 (C13a'), 134.4 (C9a'), 141.9 (C13d', C4'), 174.3 (C5'). **LREI-MS:** $m/z$ 329 [M$^+$.]

**HRESI-MS:** $m/z$ calcd for C$_{21}$H$_{16}$NO$_3$[M+H]$^+$: 330.1130; found 330.1081.

### Synthesis of 7H-benzo[k]pyrrolo[3,2,1-de]phenanthridine-4,5-dione (137)

According to the method of Torres *et al.*, the cyclised ketal 136 (420 mg, 1.28 mmol) was dissolved in 6 M HCl/THF (1:1, 13.4 mL) and heated at reflux for 5 h. Upon cooling, H$_2$O (80 mL) was added, the solution filtered and washed with more H$_2$O to yield 137 (330 mg, 90%) as a fine dark red powder, m.p. 205-207 °C, $R_f$ 0.19 (silica, DCM).

**$^1$H NMR** (CDCl$_3$, 500 MHz): $\delta$ 5.15 (s, 2H, H7), 7.18 (t, $J = 8.0$ Hz, 1H, H2), 7.24 (d, $J = 8.0$ Hz, 1H, H8), 7.48 (d, $J = 7.5$ Hz, 1H, H3), 7.55 (t, $J = 8.5$ Hz, 1H, H11), 7.60 (td, $J = 1.0$ Hz, 8.5 Hz, 1H, H12), 7.80 (d, $J = 8.5$ Hz, 1H, H9), 7.89 (d, $J = 8.0$ Hz, 1H, H10), 8.37 (d, $J = 8.0$ Hz, 1H, H1), 8.55 (d, $J = 8.5$ Hz, 1H, H13).

**$^{13}$C NMR** (CDCl$_3$, 126 MHz): $\delta$ 44.3 (C7), 117.2 (C13c), 120.2 (C3a), 123.6 (C2), 123.9 (C3), 124.2 (C13b), 124.6 (C13), 124.7 (C8), 126.3 (C11), 127.6 (C12), 129.2 (C10), 129.8 (C7a), 129.9 (C9), 130.8 (C13a), 134.2 (C9a), 135.6 (C1), 149.2 (C13d), 158.8 (C5), 183.1 (C4). **LREI-MS:** $m/z$ 285 [M$^+$.] **HRESI-MS:** $m/z$ calcd for C$_{19}$H$_{12}$NO$_2$[M+H]$^+$: 286.0868; found 286.0860.
Synthesis of 2-bromo-7H-benzo[k]pyrrolo[3,2,1-de]phenanthridine-4,5-dione (138)

The compound was prepared according to Method B for 133 using phenanthridine 137 (100 mg, 351 µmol) and NBS (62.5 mg, 351 µmol) as starting materials. The resulting solid was purified using flash chromatography on silica gel (100% CHCl₃) to yield 138 (49.2 mg, 39%) as a fine dark red powder, m.p. 197-199 °C, R_f 0.38 (silica, DCM).

**¹H NMR** (CDCl₃, 500 MHz): δ 5.09 (s, 2H, H7), 7.20 (d, J = 8.0 Hz, 1H, H8), 7.47-7.57 (m, 2H, H3, H11), 7.63 (m, 1H, H12), 7.81 (d, J = 8.0 Hz, 1H, H9), 7.87 (d, J = 7.0 Hz, 1H, H10), 8.33 (m, 1H, H1), 8.41 (m, 1H, H13). **¹³C NMR** (CDCl₃, 126 MHz): δ 44.2 (C7), 116.6 (ArC), 118.1 (ArC), 122.0 (ArC), 122.7 (ArC), 124.0 (C13), 124.6 (C8), 126.1 (C11), 126.5 (C3), 128.1 (C12), 129.4 (C10), 129.6 (C13a), 130.7 (C9), 134.1 (C9a), 135.6 (ArC), 137.5 (C1), 147.9 (C13d), 157.9 (C5), 181.7 (C4). **LREI-MS**: m/z 363; 365 [M⁺]⁷⁹Br; ⁸¹Br. **HRESI-MS**: m/z calcd for C₁₉H₁₁NO₂⁷⁹Br [M+H⁺]: 363.9973; found 363.9991.

Synthesis of 9,10-dihydroacridine (122)

The compound was prepared according to the method for 121 using acridine (120) (10.0 g, 55.8 mmol), AcOH (6.01 g, 5.73 mL, 100 mmol) and NaCNBH₃ (6.66 g, 106 mmol) as starting materials.

The crude white precipitate was recrystallised from 95% EtOH to yield 122 (4.76 g, 47%) as cream coloured needles, m.p. 171-172 °C (lit. 166-169 °C), R_f 0.67 (silica, CHCl₃).
\textit{1H NMR} (CDCl\textsubscript{3}, 500 MHz): \(\delta\) 4.06 (s, 2H, CH\textsubscript{2}), 5.95 (bs, 1H, NH), 6.67 (d, \(J = 8.0\) Hz, 2H, ArH \(\times 2\)), 6.86 (t, \(J = 7.5\) Hz, 2H, ArH \(\times 2\)), 7.06-7.11 (m, 4H, ArH \(\times 4\)). The \textit{1H NMR} spectral data coincided with those reported in the literature.\textsuperscript{274} LREI-MS: \textit{m/z} 180 [M-H]\textsuperscript{+}.

\textbf{Synthesis of 6H-pyrrolo[3,2,1-de]acridine-1,2-dione}\textsuperscript{275} (143)

The compound was prepared according to the method for \textbf{99} using 9,10-dihydroacridine (122) (4.50 g, 24.8 mmol) in anh. THF (27 mL) and oxalyl chloride (6.30 g, 4.33 mL, 49.6 mmol) in anh. THF (18 mL) as starting materials. The reaction mixture was heated at reflux for a further 3.5 h and the solvent was removed by rotary evaporation. The dark residue was dissolved in anh. CHCl\textsubscript{3} (140 mL) and heated at reflux. AlCl\textsubscript{3} (9.92 g, 74.4 mmol) was added portionwise over 5 h and the solution was heated at reflux for a further 16 h. The solvent was once again removed by rotary evaporation, the residue was cooled on ice, conc. HCl (45 mL) was added, followed by H\textsubscript{2}O (45 mL) and DCM (250 mL). The phases were separated and the organic layer was washed with H\textsubscript{2}O (2 \(\times\) 100 mL), dried over MgSO\textsubscript{4}, filtered and the solvent removed by rotary evaporation. The resulting solid was purified by flash chromatography on silica gel (100% CHCl\textsubscript{3}) to yield 143 (857 mg, 15\%) as dark red crystals, m.p. 204-206 °C, (lit.\textsuperscript{275} 220-221 °C), \(R_f\) 0.40 (silica, CHCl\textsubscript{3}).

\textit{1H NMR} (CDCl\textsubscript{3}, 500 MHz): \(\delta\) 4.21 (s, 2H, H6), 7.01 (t, \(J = 8.0\) Hz, 1H, H4), 7.14-7.22 (m, 3H, H7, H8, H9), 7.45 (t, \(J = 7.5\) Hz, 2H, H5, H10), 7.52 (d, \(J = 7.5\) Hz, 1H, H3). \textit{13C NMR} (CDCl\textsubscript{3}, 126 MHz): \(\delta\) 28.2 (C6), 119.6 (C2a), 123.3 (C10), 123.6 (C3), 126.0 (C7, C8 or C9), 127.0 (C7, C8 or C9), 127.1 (C4 + C7, C8 or C9), 132.3
(C10a), 134.9 (C6a), 136.8 (C5), 145.6 (C5b), 155.5 (C1), 182.3 (C2). LREI-MS: m/z 235 [M⁺]. HREI-MS: m/z calcd for C₁₅H₉NO₂ [M⁺]: 235.0633; found 235.0633.

**Synthesis of 4-bromo-6H-pyrrolo[3,2,1-3d]acridine-1,2-dione (144)**

The compound was prepared according to the method for 127 using 143 (100 mg, 425 µmol), which was dissolved in glacial AcOH (5 mL) and heated to 75 °C. Br₂ (205 mg, 65.9 µL, 1.28 mmol) was added dropwise while the solution was still hot. The reaction mixture was then cooled, the precipitated product collected by filtration, and washed with H₂O. The resulting solid was purified by flash chromatography on silica gel (100% CHCl₃) to yield 144 (54.6 mg, 41%) as a dark red powder, m.p. 190-192 °C, Rf 0.30 (silica, CHCl₃).

**¹H NMR** (CDCl₃, 500 MHz): δ 4.20 (s, 2H, H₆), 7.22 (t, J = 7.5 Hz, 1H, H₅), 7.41-7.47 (m, 3H, H₇, H₈, H₉), 7.55 (t, J = 7.0 Hz, 1H, H₃), 8.57 (d, J = 8.5 Hz, 1H, H₁₀). **¹³C NMR** (CDCl₃, 126 MHz): δ 28.1 (C₆), 116.1 (ArC), 118.9 (ArC), 119.1 (C₁₀), 123.4 (ArC), 123.9 (C₃), 125.7 (C₅), 131.1 (C₇, C₈ or C₉), 131.3, (ArC), 132.5 (C₇, C₈ or C₉), 136.8 (C₇, C₈ or C₉), 145.1 (ArC), 155.4 (C₁), 181.8 (C₂). LREI-MS: m/z 313; 315 [M⁺] ⁷⁹Br; ⁸¹Br. HRESI-MS: m/z calcd for C₁₅H₉NO₂ ⁷⁹Br [M⁺]: 313.9817; found 313.9807.
8.1.4 Experimental for Chapter 4

8.1.4.1 Synthesis of 3-Iminoisatins

General procedure: According to the method by Verma et al.,\textsuperscript{326} activated 3Å sieves (1 g per 100 mg of 24), the isatin 24 (1 equiv.) and EtOH (7.5 mL per 100 mg of 24) (MeOH was used for 153) were sonicated for 10 min. The acid (148-152, 1 equiv.) was added and the mixture sonicated for a further 5 min before the addition of glacial AcOH (200 µL per 100 mg of 24). The reaction mixture was heated at reflux for 1.5 h, the sieves were removed by filtration and the solution was removed by rotary evaporation. The resulting solid was purified by column chromatography on silica gel using a CHCl\textsubscript{3}/MeOH gradient of 100:0 to 95:5 to yield isatins 153-157.

Synthesis of (E and Z)-4-[5,7-dibromo-1-(4-methoxybenzyl)-2-oxoindolin-3-ylideneamino]benzoic acid (153)

Yield 18%. Mixture of E and Z isomers (E:Z 48:52). M.p. 250-252 °C (orange powder). R\textsubscript{f} 0.26 (silica, DCM).

\textsuperscript{1}H NMR (DMSO-\textit{d}\textsubscript{6}, 500 MHz): δ 3.71 (s, 3H, OCH\textsubscript{3}, Z), 3.73 (s, 3H, OCH\textsubscript{3}, E), 5.12 (s, 2H, H1'''', E), 5.30 (s, 2H, H1'''', Z), 6.44 (s, 1H, H4''', E) 6.86 (d, J = 8.0 Hz, 2H, H3''''/H5'''', E), 7.13 (d, J = 7.5 Hz, 4H, H2'/H6', E + Z), 7.18 (d, J = 8.0 Hz, 2H, H2''''/H6'''', Z), 7.25 (d, J = 8.0 Hz, 2H, H2''''/H6'''', E), 7.87 (s, 1H, H4'''', Z), 7.91 (d, J = 8.5 Hz, 2H, H3'/H5', Z), 7.93 (s, 1H, H6'', Z), 8.08 (d, J = 8.0 Hz, 2H, H3'/H5', E),\textsuperscript{13}C NMR (DMSO-\textit{d}\textsubscript{6}, 126 MHz): δ 44.0 (C1'''', E),
44.5 (C1‴‴, Z), 55.6 (OCH3, E), 55.7 (OCH3, Z), 104.2, 104.8, 113.2, 114.6 (C3‴‴/C5‴‴, E), a 114.6 (C3‴‴/C5‴‴, Z), a 114.9, 116.1, 117.6 (C2′/C6′, E), a 119.5 (C3‴‴/C5‴‴, E), a 114.6 (C3‴‴/C5‴‴, Z), 120.6, 125.5, 126.3, 127.7 (C3′/C5′, E), a 128.2 (C2‴‴/C6‴‴, Z), a 128.2 (C2‴‴/C6‴‴, E), a 129.3 (C4′, E), 129.4 (C3′/C5′, Z), a 130.6, a 131.9, 141.1 (C4″, Z), 141.3 (C6″, E), 143.3 (C7a″, E), 144.5 (C7a″, Z), 151.1 (C3″, Z), 152.4 (C3″, E), 153.4 (C4′, Z), 154.0 (C4′, E), 158.1 (C2″, E), 159.1 (C4‴‴, E + Z), a 163.5 (C2″, Z), 167.6 (COOH, Z), 167.8 (COOH, E).  

**LRESI-MS:** m/z 541; 543; 545 [M-H]- 79Br79Br; 79Br81Br, 81Br81Br.  

**HRESI-MS:** m/z calcd for C23H15N2O479Br79Br [M-H]-: 540.9399; found 540.9496.

**Synthesis of (E and Z)-4-[5,7-dibromo-1-(4-methoxybenzyl)-2-oxoindolin-3-ylideneamino]phenyl]acetic acid (154)**

Yield 68%. Mixture of E and Z isomers (E:Z 58:42). M.p. 151-153 °C (red crystals). Rf 0.40 (silica, DCM/MeOH, 9:1).

**1H NMR (DMSO-d6, 500 MHz):** δ 3.57 (s, 2H, H2, Z), 3.65 (s, 2H, H2, E), 3.71 (s, 3H, OCH3, Z), 3.73 (s, 3H, OCH3, E), 5.14 (s, 2H, H1‴‴, Z), 5.30 (s, 2H, H1‴‴, E), 6.60 (d, J = 1.5 Hz, 1H, H4″‘, E), 6.86 (d, J = 9.0 Hz, 2H, H3‴‴/H5‴‴, Z), 6.90 (d, J = 9.0 Hz, 2H, H3‴‴/H5‴‴, E), 7.00 (d, J = 8.5 Hz, 2H, H2′/H6′, E), 7.13 (d, J = 8.5 Hz, 2H, H2‴‴/H6‴‴, Z), 7.17 (d, J = 8.0 Hz, 2H, H2‴‴/H6‴‴, E), 7.25 (t, 4H, J = 7.5 Hz, H2′/H6′ + H3′/H5′, Z), 7.41 (d, J = 8.5 Hz, 2H, H3′/H5′, E), 7.81 (d, J = 2.0 Hz, 2H, H4″, Z + H6″, E), 7.89 (d, J = 2.0 Hz, 1H, H6″, Z).  

**13C NMR (DMSO-d6,**
126 MHz): δ 30.0 (C2, E + Z),^a 44.6 (C1''', E + Z),^a 55.8 (OCH3, E + Z),^a 104.1, 104.8, 112.5, 114.5 (C3'''/C5''', Z),^a 114.7 (C3'''/C5''', E),^a 115.0, 116.1, 117.8 (C2'/C6'),^a 120.7,^a 121.1,^a 125.1, 127.0, 127.6, 128.2 (C2'''/C6''', E),^a 128.3 (C2'''/C6''', Z),^a 129.5, 130.1, 130.5,^a 131.5,^a 133.5, 133.7, 141.1, 142.8 (C7a'', Z), 144.4 (C7a'', E), 146.8, 148.0, 148.9, 158.2 (C2'', Z), 159.1 (C4', E + Z),^a 163.8 (C2'', E), 173.5 (COOH, E + Z).^a LRESI-MS: m/z 557; 559; 561 [M+H]^+ 79Br79Br; 79Br81Br; 81Br81Br. HRESI-MS: m/z calcd for C24H19N2O479Br81Br [M+H]^+: 558.9692; found 558.9678.

Synthesis of (E and Z)-3-[4-[5,7-dibromo-1-(4-methoxybenzyl)-2-oxindolin-3-ylideneamino]phenyl]propanoic acid (155)

Yield 71%. Mixture of E and Z isomers (E:Z 55:45). M.p. 79-81 °C (orange/red crystals). Rf 0.43 (silica, DCM/MeOH, 9:1).

^1H NMR (DMSO-d6, 500 MHz): δ 2.55 (t, J = 7.5 Hz, 2H, H2, E), 2.56 (t, J = 8.0 Hz, 2H, H2, Z), 2.83 (t, J = 7.5 Hz, 2H, H3, Z), 2.89 (t, J = 7.5 Hz, 2H, H3, E), 3.17 (s, 3H, OCH3, Z), 3.73 (s, 3H, OCH3, E), 5.15 (s, 2H, H1''', Z), 5.29 (s, 2H, H1''', E), 6.54 (s, 1H, H4'', E), 6.86 (d, J = 8.0 Hz, 2H, H3''''/H5'''', Z), 6.90 (d, J = 7.5 Hz, 2H, H3''''/H5'''', E), 6.96 (d, J = 7.5 Hz, 2H, H2'/H6', E), 7.13 (d, J = 8.0 Hz, 2H, H2''''/H6'''', Z), 7.18 (d, J = 8.0 Hz, 2H, H2''''/H6'''', E), 7.22-7.24 (m, 4H, H2'/H6' + H3'/H5', Z), 7.38 (d, J = 8.0 Hz, 2H, H3'/H5', E), 7.80 (m, 2H, H4'', Z + H6'', E), 7.88 (s, 1H, H6'', Z), 12.13 (br s, 2H, OH, E + Z). ^13C NMR (DMSO-d6, 126 MHz): δ 30.7 (C3, Z), 30.8
Synthesis of \((E\) and \(Z\))-3-[5,7-dibromo-1-(4-methoxybenzyl)-2-oxoindolin-3-ylideneamino]benzoic acid (156)

Yield 56%. Mixture of \(E\) and \(Z\) isomers (\(E:Z\) 49:51). M.p. 229-231 °C (orange powder). \(R_f\) 0.37 (silica, DCM/MeOH, 9:1).

\(^1\)H NMR (DMSO-\(d_6\), 500 MHz): \(\delta\) 3.71 (s, 3H, OCH\(_3\), \(Z\)), 3.73 (s, 3H, OCH\(_3\), \(E\)), 5.13 (s, 2H, H1''', \(Z\)), 5.30 (s, 2H, H1''', \(E\)), 6.41 (d, \(J = 8.5\) Hz, 2H, H3'''/H5''''', \(E\)), 6.90 (d, \(J = 9.0\) Hz, 2H, H3'''/H5''''', \(Z\)), 7.17 (d, \(J = 8.0\) Hz, 2H, H2''''/H6''''', \(E\)), 7.25 (d, \(J = 8.5\) Hz, 2H, H2''''/H6''''', \(Z\)), 6.86 (d, \(J = 7.5\) Hz, 1H, H4', \(Z\)), 7.34 (d, \(J = 8.0\) Hz, 1H, H4', \(E\)), 7.47 (t, \(J = 8.0\) Hz, 1H, H5', \(Z\)), 7.59 (s 1H, H2', \(E\)), 7.65 (t, \(J = 8.0\) Hz, 2H, H2', \(E + H5', \(Z\)), 7.74 (d, \(J = 7.5\) Hz, 1H, H6', \(E\)), 7.82 (s, 1H, H6'', \(E\)), 7.85 (s, 1H, H4'', \(Z\)), 7.88 (d, \(J = 7.5\) Hz, 1H, H6', \(Z\)), 7.91 (s, 1H, H6'', \(Z\)), 13.13 (br s, 2H, OH, \(E + Z\)). \(^{13}\)C NMR (DMSO-\(d_6\), 126 MHz): \(\delta\) 44.0 (C1''', \(Z\)), 44.5 (C1''', \(E\)), 55.7 (OCH\(_3\), \(E + Z\)), 104.1, 104.7, 114.6
(C3‴/C5‴, E),\(^\text{a}\) 114.6 (C3‴/C5‴, Z),\(^\text{a}\) 114.8, 116.0, 118.3, 120.7, 120.9, 122.0 (C2', Z), 124.5, 125.2, 126.6, 126.8, 126.9, 127.5, 128.1 (C2‴/C6‴),\(^\text{a}\) 128.2 (C7a'', E),\(^\text{a}\) 149.1, 150.4, 151.2, 153.0 (C3'', E),\(^\text{a}\) 158.1 (C2'', Z), 159.0 (C4‴, E + Z),\(^\text{a}\) 163.6 (C2'', E), 167.5 (C3'', Z), 167.8 (COOH, E + Z).\(^\text{a}\) LRESI-MS: \(m/z\) 541, 543; 545 \([\text{M-H}]^{+}\) 79\(^{79}\)Br 79\(^{81}\)Br; 79\(^{81}\)Br 81\(^{81}\)Br. HRESI-MS: \(m/z\) calcd for C\(_{23}\)H\(_{15}\)N\(_2\)O\(_4\) 79\(^{79}\)Br 79\(^{81}\)Br [M-H]: 540.9399; found 540.9424.

Synthesis of (\(E\) and \(Z\))-2-{3-[5,7-dibromo-1-(4-methoxybenzyl)-2-oxoindolin-3-ylideneamino]phenyl}acetic acid (157)

Yield 71%. Mixture of \(E\) and \(Z\) isomers (\(E:Z\) 67:33).

\(\text{M.p.} 182\text{-}184\, ^\circ\text{C}\) (red powder). \(R_f\) 0.33 (silica, DCM/MeOH, 9:1).

\(\text{\textsuperscript{1}H NMR}\) (DMSO-\(d_6\), 500 MHz): \(\delta\) 3.56 (s, 2H, H2, Z), 3.63 (s, 2H, H2, E), 3.71 (s, 3H, OCH\(_3\), Z), 3.73 (s, 3H, OCH\(_3\), E), 5.14 (s, 2H, H1‴, Z), 5.30 (s, 2H, H1‴, E), 6.61 (s, 1H, H4‴, E), 6.86 (d, \(J = 8.0\, \text{Hz}\), 2H, H3‴/H5‴, Z), 6.90 (d, \(J = 8.5\, \text{Hz}\), 2H, H3‴/H5‴, E), 6.94 (d, \(J = 8.5\, \text{Hz}\), 1H, H4‵, E), 7.04 (s, 1H, H2′, E), 7.08 (d, \(J = 7.5\, \text{Hz}\), 1H, H4‵, Z), 7.17 (d, \(J = 8.5\, \text{Hz}\), 2H, H2‴/H6‴, Z), 7.20 (d, \(J = 8.0\, \text{Hz}\), 2H, H6′, E + Z), 7.23 (d, \(J = 8.0\, \text{Hz}\), 2H, H2‴/H6‴, E), 7.28 (t, \(J = 7.5\, \text{Hz}\), 1H, H5′, Z), 7.46 (t, \(J = 7.5\, \text{Hz}\), 1H, H5′, E), 7.78-7.80 (m, 2H, H6″, E + H2′, Z), 7.90 (s, 1H, H4″, Z), 8.00 (s, 1H, H6″, Z), 12.34 (br s, 2H, OH, E + Z).

\(\text{\textsuperscript{13}C NMR}\) (DMSO-\(d_6\), 126 MHz): \(\delta\) 41.2 (C2, E), 41.6 (C2, Z), 43.9 (C1‴, Z), 44.5 (C1‴, E), 55.7 (OCH\(_3\), E + Z),\(^\text{a}\) 104.1, 104.6, 114.5 (C3‴/C5‴, Z),\(^\text{a}\) 114.6
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(C3'''/C5''', E), 115.1, 115.4, 115.9, 116.0, 117.4, 118.6, 118.7, 120.6, 121.8 (C6', E), 125.0, 126.8, 126.9, 127.3, 127.5, 127.8, 128.1 (C2'''/C6''', E), 128.2 (C2'''/C6''', Z), 128.8, 129.3, 129.4, 130.3, 140.6, 141.0, 142.8 (C3'', Z), 144.3, 148.6, 150.3, 152.3 (C3'', E), 158.0 (C2'', Z), 159.0 (C4''', E + Z), 163.7 (C2'', E), 173.0 (COOH, E), 173.1 (COOH, Z). **LRESI-MS:** m/z 555; 557; 559 [M-H] - 79Br-79Br; 79Br81Br; 81Br81Br. **HRESI-MS:** m/z calcd for C_{24}H_{17}N_{2}O_{4}^{79Br81Br} [M-H]$: 556.9535; found 556.9568.

8.1.4.2 Synthesis of Isatin-Lysine Conjugates

**General procedure:** According to the method of Nozaki,295 the 3-iminoisatin (153-157, 1 equiv.) was dissolved in anh. DCM (1 mL per 30 mg of imine) with the aid of sonication. On ice, HOBt (1 equiv.) was added, followed by DCC (1 equiv.), DIPEA (1.1 equiv.) and Ac-Lys-OMe. HCl (1 equiv.). The reaction mixture was warmed to RT and stirred for 24 h. The precipitate was then filtered off and the solvent removed by rotary evaporation. The resulting solid was purified by column chromatography on silica gel using a CHCl₃/MeOH gradient of 100:0 to 95:5 to yield 158-162.
Synthesis of methyl (S,E and S,Z)-2-acetamido-6-[4-[5,7-dibromo-1-(4-methoxybenzyl)-2-oxoindolin-3-ylideneamino]benzamido] hexanoate (158)


Rf 0.41 (silica, CHCl₃/MeOH, 95:5).

**¹H NMR** (CDCl₃, 500 MHz): δ 1.39-1.89 (m, 12H, H3/H4/H5, E + Z), 2.00 (s, 6H, COCH₃, E + Z), 3.42-3.53 (m, 4H, H6, E + Z), 3.73 (s, 3H, COOCH₃, E), 3.75 (s, 3H, COOCH₃, Z), 3.77 (s, 3H, Ar-CH₂-OCH₃, E), 3.79 (s, 3H, Ar-CH₂-OCH₃, Z), 4.59-4.64 (m, 2H, H2, E + Z), 5.24 (s, 2H, H1‴‴‴, E), 5.44 (s, 2H, H1‴‴‴, Z), 6.23 (dd, J = 7.0 Hz, 15 Hz, 2H, NHCOCH₃, E + Z), 6.33 (br s, 1H, ArHCONH, Z), 6.51 (br s, 1H, ArHCONH, E), 6.77 (s, 1H, H4‴‴, E), 6.82 (d, J = 8.5 Hz, 2H, H3‴‴‴/H5‴‴‴, Z), 6.86 (d, J = 8.5 Hz, 2H, H3‴‴‴/H5‴‴‴, E), 7.03 (d, J = 8.5 Hz, 2H, H3‴‴‴/H5‴‴‴, E), 7.06 (d, J = 8.5 Hz, 2H, H3‴‴‴/H5‴‴‴, Z), 7.15 (d, J = 8.5 Hz, 2H, H2‴‴‴/H6‴‴‴, Z), 7.22 (d, J = 8.0 Hz, 2H, H2‴‴‴/H6‴‴‴, E), 7.61 (s, 1H, H6‴‴, E), 7.72 (s, 1H, H4‴‴, Z), 7.81 (d, J = 7.5 Hz, 2H, H2‴‴/H6‴‴, E), 7.84 (s, 1H, H6‴‴, Z), 7.96 (d, J = 8.5 Hz, 2H, H2‴‴/H6‴‴, Z).

**¹³C NMR** (CDCl₃, 126 MHz): δ 22.5 (CH₂, Z), 22.6 (CH₂, E), 23.4 (COCH₃, E + Z), 28.7 (CH₂, E), 29.1 (CH₂, Z), 32.2 (CH₂, E), 32.5 (CH₂, Z), 39.5 (C₆, E), 39.7 (C₆, Z), 44.0 (C₁‴‴‴, Z), 44.6 (C₁‴‴‴, E), 51.9 (C₂, E), 52.1 (C₂, Z), 52.7 (COCH₃, E + Z), 55.5 (Ar-CH₂-OCH₃, E + Z), 104.0, 104.8, 111.4, 114.4 (C₃‴‴‴/C₅‴‴‴, E), 114.4 (C₃‴‴‴/C₅‴‴‴, Z), 115.9, 116.8, 117.4 (C₃‴‴/C₅‴‴, E), 119.4 (C₃‴‴/C₅‴‴, Z), 119.6, 120.6, 125.8 (C₆‴‴, Z), 127.9
(C2''/C6'', E), a 128.2 (C2''''/C6'''', E), a 128.3 (C2''''/C6'''', Z), a 128.4 (C4'', E),
129.1 (C2''/C6'', Z), a 132.1, 133.2, 141.5 (C4'', Z), 142.1 (C6'', E), 142.5 (C7a''', E),
144.2 (C7a''', Z), 146.6, 150.4, 151.4, 151.9, 152.1, 157.6 (C2'''', E), 159.3
(C4'''', E + Z), a 163.6 (C2''', Z), 167.0 (Ar-CONH, E), 167.4 (Ar-CONH, Z), 170.0
(COCH3, Z), 170.5 (COCH3, E), 173.3 (COOCH3, E + Z). a LRESI-MS: m/z 727;
729; 731 [M+H]+; 749; 751; 753 [M+Na]+ 79Br79Br, 79Br81Br, 81Br81Br. HRESI-MS:

Synthesis of methyl (S,E and S,Z)-2-acetamido-6-(2-{4-[5,7-dibromo-1-(4-
methoxybenzyl)-2-oxoindolin-3-ylideneamino]phenyl}acetamido) hexanoate
(159)

Rf 0.33 (silica, CHCl3/MeOH, 95:5).

1H NMR (CDCl3, 500 MHz): δ 1.21-1.82 (m,
12H, H3/H4/H5, E + Z), 1.98 (s, 3H, COCH3, E), 2.02 (s, 3H, COCH3, Z), 3.17-3.27
(m, 4H, H6, E + Z), 3.57 (s, 2H, H2', Z), 3.62 (s, 2H, H2', E), 3.71 (s, 3H, COOCH3,
Z), 3.73 (s, 3H, COOCH3, E), 3.76 (s, 3H, Ar-OCH3, Z), 3.78 (s, 3H, Ar-OCH3, E),
4.51-4.58 (m, 2H, H2, E + Z), 5.27 (s, 2H, H1''', E), 5.43 (s, 2H, H1''''', Z), 5.65 (br
s, 1H, NHCOCH3, Z), 5.72 (br s, 1H, NHCOCH3, E), 6.23 (d, J = 8.0 Hz, 1H,
CH2-CONH, Z), 6.27 (d, J = 8.0 Hz, 1H, CH2-CONH, E), 6.80 (d, J = 1.5 Hz, 1H,
H4‴, E), 6.82 (d, J = 8.5 Hz, 2H, H3‴‴/H5‴‴, Z), 6.85 (d, J = 8.0 Hz, 2H, H3‴‴/H5‴‴, E), 6.97 (d, J = 8.5 Hz, 2H, H2″/H6″ or H3‴/H5‴, Z), 7.11-7.15 (m, 4H, H2‴‴/H6‴‴, Z + H2″/H6″ or H3‴/H5‴, E), 7.22 (d, J = 9.0 Hz, 2H, H2‴‴/H6‴‴, E), 7.37 (d, J = 8.0 Hz, 2H, H2″/H6″ or H3‴/H5‴, E), 7.40-7.46 (m, 2H, H2″/H6″ or H3‴/H5‴, E), 7.59 (d, J = 1.5 Hz, 1H, H6‴, E), 7.69 (s, 1H, H4‴, Z), 7.83 (d, J = 1.5 Hz, 1H, H6‴, Z). \(^{13}\text{C} \text{NMR}\) (CDCl\(_3\), 126 MHz): δ 22.5 (CH\(_2\), E + Z), \(^a\) 23.3 (COCH\(_3\), Z), 23.4 (COCH\(_3\), E), 29.1 (CH\(_2\), E), 30.0 (CH\(_2\), Z), 31.9 (CH\(_2\), E), 32.2 (CH\(_2\), Z), 39.2 (C6, Z), 39.3 (C6, E), 43.5 (C2′, E), 43.7 (C2′, Z), 44.0 (C1‴‴, Z), 44.6 (C1‴‴, E), 52.1 (C2, E + Z), \(^a\) 52.6 (COOCH\(_3\), Z), 52.7 (COOCH\(_3\), E), 55.5 (Ar-CH\(_3\), E + Z), \(^a\) 104.0, 104.7, 114.3 (C3‴‴/C5‴‴, E), \(^a\) 114.4 (C3‴‴/C5‴‴, Z), \(^a\) 115.6, 116.7, 117.4, 118.2, \(^a\) 120.1, 121.1, \(^a\) 125.5, 126.2, 127.7, 128.2 (C2‴‴/C6‴‴, E), \(^a\) 128.2 (C2‴‴/C6‴‴, Z), \(^a\) 128.5, 128.9, 129.9, \(^a\) 130.9, \(^a\) 132.3, 133.3, 141.0, 141.7, 142.1 (C7a‴, Z), 144.0 (C7a‴, E), 148.6, 149.7, 151.9, 157.9 (C2‴, Z), 159.3 (C4‴‴, E + Z), \(^a\) 163.8 (C2‴, E), 170.4 (COCH\(_3\) or CH\(_2\)-CONH, E + Z), \(^a\) 171.2 (COCH\(_3\) or CH\(_2\)-CONH), 171.4 (COCH\(_3\) or CH\(_2\)-CONH), 173.2 (COOCH\(_3\), E + Z), \(^a\)

**HRESI-MS:** \(m/z\) calcd for C\(_{33}\)H\(_{35}\)N\(_4\)O\(_6\)\(^\text{79}\)Br\(^\text{81}\)Br [M+H]\(^+\): 743.0903; found 743.0888.
Synthesis of methyl (S,E and S,Z)-2-acetamido-6-(3-{4-[5,7-dibromo-1-(4-methoxybenzyl)-2-oxoindolin-3-ylideneamino]phenyl}propanamido) hexanoate (160)

Yield 70%. Mixture of E and Z isomers (E:Z 55:45). M.p. 161-163 °C (red powder). Rf 0.42 (silica, DCM/MeOH, 9:1).

\[ ^1H \text{ NMR (CDCl}_3, 500 \text{ MHz)}: \delta 1.29-1.82 \text{ (m, 12H, H}_3/\text{H}_4/\text{H}_5, \text{ E+Z)}, 1.99 \text{ (s, 3H, COCH}_3, \text{ Z)}, 2.03 \text{ (s, 3H, COCH}_3, \text{ E)}, 2.43-2.52 \text{ (m, 4H, H}_2', \text{ E+Z)}, 2.94-3.04 \text{ (m, 4H, H}_3', \text{ E+Z)}, 3.12-3.27 \text{ (m, 4H, H}_2', \text{ E+Z)}, 3.72 \text{ (s, 3H, COOCH}_3, \text{ Z)}, 3.73 \text{ (s, 3H, COOCH}_3, \text{ E)}, 3.76 \text{ (s, 3H, ArHOCH}_3, \text{ Z)}, 3.76 \text{ (s, 3H, ArHOCH}_3, \text{ E)}, 3.72 \text{ (s, 3H, COOCH}_3, \text{ E)}, 4.55 \text{ (t, } J = 4.0 \text{ Hz, 2H, H}_2, \text{ E+Z)}, 5.27 \text{ (s, 2H, H}_1'''', \text{ Z)}, 5.43 \text{ (s, 2H, H}_1'''', \text{ E)}, 5.62 \text{ (br s, 1H, NHCOCH}_3, \text{ Z)}, 5.66 \text{ (br s, 1H, NHCOCH}_3, \text{ E)}, 6.32 \text{ (d, } J = 6.0 \text{ Hz, 2H, CH}_2-\text{CONH, E+Z)}, 6.81 \text{ (d, } J = 4.0 \text{ Hz, 2H, H}_3''''/\text{H}_5''''), 6.83 \text{ (s, 1H, H}_4'''', \text{ E)}, 6.85 \text{ (d, } J = 8.5 \text{ Hz, 2H, H}_3''''/\text{H}_5''''), 6.90 \text{ (d, } J = 7.5 \text{ Hz, 2H, H}_3''''/\text{H}_5''''), 7.10 \text{ (d, } J = 8.0 \text{ Hz, 2H, H}_3''''/\text{H}_5''''), 7.15 \text{ (d, } J = 8.5 \text{ Hz, 2H, H}_2''''/\text{H}_6'''', \text{ E)}, 7.21 \text{ (t, } J = 7.5 \text{ Hz, 4H, H}_2''''/\text{H}_6'''', \text{ E+H}_2''''/\text{H}_6'''', \text{ Z)}, 7.29 \text{ (d, } J = 8.0 \text{ Hz, 2H, H}_2''''/\text{H}_6'''', \text{ Z)}, 7.58 \text{ (s, 1H, H}_6'''', \text{ E)}, 7.68 \text{ (s, 1H, H}_4'''', \text{ Z)}, 7.85 \text{ (s, 1H, H}_6'''', \text{ Z}). \]

\[ ^13C \text{ NMR (CDCl}_3, 126 \text{ MHz)}: \delta 22.5 \text{ (CH}_2, \text{ Z)}, 22.6 \text{ (CH}_2, \text{ E)}, 23.3 \text{ (COCH}_3, \text{ Z)}, 23.4 \text{ (COCH}_3, \text{ E)}, 28.9 \text{ (C}_3', \text{ Z)}, 29.1 \text{ (C}_3', \text{ E)}, 31.6 \text{ (CH}_2, \text{ E)}, 31.7 \text{ (CH}_2, \text{ Z)}, 32.1 \text{ (CH}_2, \text{ Z)}, 32.3 \text{ (CH}_2, \text{ E)}, 38.6 \text{ (C}_2', \text{ Z)}, 38.9 \text{ (C}_2', \text{ E)}, 39.0 \text{ (C}_6, \text{ Z)}, 39.1 \text{ (C}_6, \text{ E)}, 43.9 \text{ (C}_1'''', \text{ Z)}, 44.6 \text{ (C}_1'''', \text{ E)}, 52.0 \text{ (C}_2, \text{ Z)}, 52.1 \text{ (C}_2, \text{ E)}, 52.6 \text{ (COOCH}_3, \text{ E)}}

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+ Z), a 55.5 (Ar-OCH₃, E + Z), a 103.8, 104.6, 114.3 (C3''''/C5'''', Z), a 114.4 (C3''''/C5'''', E), a 115.6, 116.6, 117.9, a 119.8, 121.2 (C3''/C5'', Z), a 125.4 (C6'', Z), 126.5, 128.1, 128.2 (C2'''/C6''', E), a 128.2 (C2'''/C6''', Z), a 128.6, a 128.6, a 128.8, 129.9, 139.4, 140.1, 140.7, 141.5, 141.9 (C7a'', Z), 144.0 (C7a''', E), 146.2, 147.7, 149.2, 151.8, 157.9 (C2''', Z), 159.2 (C4''''', Z), 159.3 (C4''''', E), 163.9 (C2''', E), 170.5 (COCH₃ or CH₂-COHN, E + Z), a 172.4 (COCH₃ or CH₂-COHN), 172.6 (COCH₃ or CH₂-COHN), 173.2 (COOCH₃, E + Z). **LRESI-MS:** m/z 755; 757; 759 [M+H]+ ⁷⁹Br²⁹Br; ⁷⁹Br⁸¹Br; ⁸¹Br⁸¹Br. **HRESI-MS:** m/z calcd for C₃₄H₃₆N₄NaO₆⁷⁹Br⁸¹Br [M+Na]⁺: 779.0879; found 779.0896.

**Synthesis of methyl (S,E and S,Z)-2-acetamido-6-[3-[5,7-dibromo-1-(4-methoxybenzyl)-2-oxoindolin-3-ylideneamino]benzamido] hexanoate (161)**

Yield 78%. Mixture of E and Z isomers (E:Z 47:53). M.p. 74-76 °C (orange crystals). Rf 0.56 (silica, CHCl₃/MeOH, 95:5).

**¹H NMR** (CDCl₃, 500 MHz): δ 1.36-1.91 (m, 12H, H3/H4/H5, E + Z), 1.97 (t, J = 7.0 Hz, 6H, COCH₃, E + Z), 3.41-3.48 (m, 4H, H6, E + Z), 3.72 (s, 3H, COOCH₃, Z), 3.73 (s, 3H, COOCH₃, E), 3.76 (s, 3H, Ar-OCH₃, Z), 3.79 (s, 3H, Ar-OCH₃, E), 4.56-4.62 (m, 2H, H2, E + Z), 5.25 (s, 2H, H1''''', Z), 5.43 (s, 2H, H1''''', E), 6.24 (d, J = 7.5 Hz, 1H, NHCOCH₃, E), 6.28 (d, J = 7.5 Hz, 1H, NHCOCH₃, Z), 6.40 (t, J = 5.5 Hz, 1H, Ar-COHN, Z), 6.55 (t, J = 5.5 Hz, 1H,
Ar-CONH, E), 6.70 (d, J = 1.5 Hz, 1H, H4′′′′, E), 6.82 (d, J = 8.0 Hz, 2H, H3′′′′′/H5′′′′′, Z), 6.86 (d, J = 8.5 Hz, 2H, H3′′′′′/H5′′′′′, E), 7.08 (d, J = 8.0 Hz, 2H, H2′′′′′/H6′′′′′, E), 7.14 (d, J = 9.0 Hz, 2H, H2′′′′′/H6′′′′′, Z), 7.21 (d, J = 9.0 Hz, 2H, H4′′, E + Z), 7.43 (t, J = 8.0 Hz, 1H, H5′′, E), 7.46 (s, 1H, H2′′, E), 7.53 (t, J = 8.0 Hz, 1H, H5′′, Z), 7.54 (d, J = 1.5 Hz, 1H, H2′′, Z), 7.59 (d, J = 1.5 Hz, 1H, H6′′, E), 7.63 (d, J = 8.0 Hz, 1H, H6′′, Z), 7.70 (d, J = 2.0 Hz, 1H, H4′′′′, Z), 7.74 (d, J = 7.5 Hz, 1H, H6′′′′, E), 7.83 (d, J = 2.0 Hz, 1H, H6′′′′, Z). 13C NMR (CDCl3, 126 MHz): δ 22.5 (CH2, Z), 22.6 (CH2, E), 23.3 (COCH3, Z), 23.4 (COCH3, E), 28.7 (CH2, E), 29.0 (CH2, Z), 32.1 (CH2, Z), 32.4 (CH2, E), 39.5 (C6, Z), 39.8 (C6, E), 44.0 (C1′′′′, Z), 44.6 (C1′′′′, E), 51.9 (C2, E), 52.0 (C2, Z), 52.6 (COOCH3, Z), 52.7 (COOCH3, E), 55.5 (Ar-CH2, E + Z), 104.0, 104.8, 114.3 (C3′′′′′/C5′′′′′, Z), 114.4 (C3′′′′′/C5′′′′′, E), 115.8, 116.5 (C2′′, E), 116.7, 118.9 (C5′′, Z), 120.1, 123.0 (C4′′′), 124.7 (C6′′, E), 124.9 (C6′′, Z), 125.6, 126.0, 126.9, 127.8 (C2′′′′′/C6′′′′′, E), 128.1 (C2′′′′′/C6′′′′′, Z), 128.2 (C2′′′′′/C6′′′′′, Z), 128.3 (C4′′′), 128.4 (C6′′, E), 129.1 (C5′′, E), 130.2 (C6′′′, Z), 135.4, 141.3 (C4′′′, Z), 141.9 (C6′′′, E), 142.3 (C5′′, E), 144.1, 148.3, 149.7, 150.4, 152.3, 157.8 (C2′′′, Z), 159.2 (C4′′′′′, Z), 159.3 (C4′′′′′, E), 163.6 (C2′′′, E), 166.9 (Ar-CONH, E), 167.5 (Ar-CONH, Z), 170.4 (COCH3, Z), 170.5 (COCH3, E), 173.2 (COOCH3, E + Z). LRESI-MS: m/z 727; 729; 731 [M+H]+. 79Br79Br, 79Br81Br, 81Br81Br. HRESI-MS: m/z calcd for C32H31N4O679Br81Br [M-H]−: 727.0590; found 727.0380.
Synthesis of methyl (\(S,E\) and \(S,Z\))-2-acetamido-6-(2-[3-{5,7-dibromo-1-(4-methoxybenzyl)-2-oxoindolin-3-ylideneamino}phenyl]acetamido) hexanoate (162)

Yield 82%. Mixture of \(E\) and \(Z\) isomers (\(E:Z\) 37:63). M.p. 194-196 °C (red powder).

\(R_f\) 0.44 (silica, DCM/MeOH, 9:1).

\(^1H\ NMR\) (CDCl\(_3\), 500 MHz): \(\delta\) 1.16-1.69 (m, 12H, H3/H4/H5, \(E + Z\)), 1.98 (s, 3H, COCH\(_3\), \(Z\)), 2.01 (s, 3H, COCH\(_3\), \(E\)), 3.09-3.10 (m, 2H, H6, \(E\)), 3.14-3.24 (m, 2H, H6, \(Z\)), 3.59 (s, 4H, H2’, \(E + Z\)), 3.69 (s, 3H, COOCH\(_3\), \(Z\)), 3.73 (s, 3H, COOCH\(_3\), \(E\)), 4.45 (d, \(J = 3.5\) Hz, 1H, H2’, \(Z\)), 4.53 (d, \(J = 3.5\) Hz, 1H, H2, \(E\)), 5.24 (s, 2H, H1’’’, \(Z\)), 5.43 (s, 2H, H1’’’, \(E\)), 5.68 (br s, 1H, NHCOCH\(_3\), \(E\)), 5.91 (br s, 1H, NHCOCH\(_3\), \(Z\)), 6.20-6.24 (m, 2H, CH\(_2\)HCONH, \(E + Z\)), 6.76 (s, 1H, H4’’’, \(E\)), 6.83 (d, \(J = 8.0\) Hz, 4H, H3’’’/H5’’’’, \(E + Z\)), 6.91 (s, 2H, H2’’, \(E + Z\)), 7.03 (d, \(J = 8.0\) Hz, 1H, H4’’’, \(Z\)), 7.10 (d, \(J = 8.5\) Hz, 4H, H2’’’’/H6’’’’’, \(E + Z\)), 7.21 (d, \(J = 7.5\) Hz, 3H, H4’’’, \(E + H6’’’, \(E + Z\))), 7.40 (t, \(J = 8.0\) Hz, H5’’’, \(Z\)), 7.45 (t, \(J = 7.5\) Hz, 1H, H5’’’, \(E\)), 7.59 (s, 1H, H6’’’, \(E\)), 7.71 (s, 1H, H4’’’, \(Z\)), 7.86 (s, 1H, H6’’’, \(Z\)). \(^{13}C\ NMR\) (CDCl\(_3\), 126 MHz): \(\delta\) 22.4 (CH\(_2\), \(Z\)), 22.5 (CH\(_2\), \(E\)), 23.3 (COCH\(_3\), \(Z\)), 23.4 (COCH\(_3\), \(E\)), 29.1 (CH\(_2\), \(Z\)), 29.9 (CH\(_2\), \(E\)), 31.7 (CH\(_2\), \(Z\)), 32.1 (CH\(_2\), \(E\)), 39.1 (C6, \(Z\)), 39.4 (C6, \(E\)), 43.8 (C2’, \(E\)), 44.0 (C2’, \(Z\)), 44.1 (C1’’’, \(Z\)), 44.6 (C1’’’, \(E\)), 52.1 (C2, \(E\)), 52.3 (C2, \(Z\)), 52.5 (COOCH\(_3\), \(Z\)), 52.6 (COOCH\(_3\), \(E\)), 55.5 (Ar-OCH\(_3\), \(E + Z\))\(^{a}\) 104.1, 104.7, 114.4
(C\textsuperscript{3'}''''/C\textsuperscript{5''''}, E),\textsuperscript{a} 114.4 (C\textsuperscript{3''''''}/C\textsuperscript{5''''''}, Z),\textsuperscript{a} 115.8, 116.2 (C\textsuperscript{2''}, E), 116.9, 118.3, 119.4 (C\textsuperscript{6''}, Z), 119.7, 120.4, 125.7 (C\textsuperscript{2''}, Z), 126.0, 127.1, 127.7, 128.0 (C\textsuperscript{2''''''}/C\textsuperscript{6''''''}, Z),\textsuperscript{a} 128.2 (C\textsuperscript{2''''''}/C\textsuperscript{6''''''}, E),\textsuperscript{a} 128.3,\textsuperscript{a} 128.5, 130.0 (C\textsuperscript{5''}, Z), 130.5 (C\textsuperscript{5''}, E), 135.2, 137.3, 141.2 (C\textsuperscript{6''}, Z), 141.8 (C\textsuperscript{6''}, E), 142.1, 144.1, 148.9, 150.1, 150.3, 152.0, 158.0 (C\textsuperscript{4''''}, E), 159.3 (C\textsuperscript{4''''}, Z), 163.8 (C\textsuperscript{2''''}, E), 170.3 (CH\textsubscript{2}-CONH or COCH\textsubscript{3}), 170.4 (CH\textsubscript{2}-CONH or COCH\textsubscript{3}), 170.7 (CH\textsubscript{2}-CONH or COCH\textsubscript{3}), 171.2 (CH\textsubscript{2}-CONH or COCH\textsubscript{3}), 173.2 (COOCH\textsubscript{3}, E + Z).\textsuperscript{a} \textbf{LRESI-MS:} m/z 763; 765; 767 [M+Na]\textsuperscript{+} 79\textsuperscript{Br}79\textsuperscript{Br}; 79\textsuperscript{Br}81\textsuperscript{Br}; 81\textsuperscript{Br}81\textsuperscript{Br}.

\textbf{HRESI-MS:} m/z calcd for C\textsubscript{33}H\textsubscript{33}N\textsubscript{4}O\textsubscript{6}79\textsuperscript{Br}81\textsuperscript{Br} [M-H]$: 741.0747; found 741.0779.
8.1.5 Experimental for Chapters 5 and 6

8.1.5.1 Synthesis of Isatin-Protein Conjugates

Conjugation of \((E\text{ and } Z)\)-3-\{4\-[5,7\text{-dibromo-1-\{(4\text{methoxybenzyl})-2-oxoindolin-3-ylideneamino\}phenyl\}propanoic acid (155) to PAI-2

Preparation of the active ester (164)

The propionic imine 155 (2.90 mg, 5.00 µmol) was dissolved in anh. DMF (70 µL). On ice, HOBt (690 µg, 5.10 µmol) in anh. DMF (17 µL) was added, followed by DCC (6.20 mg, 30.0 µmol) in anh. DMF (50 µL). The orange reaction mixture was warmed to RT, mixed for 30 min and used without purification. Rf 0.38 (silica, DCM).

Conjugation to PAI-2

A 20-fold molar excess of the active ester 164 (26.0 µL) was added to PAI-2 (474 µL at 4.27 mg/mL) in PBS pH 8.5 and shaken at RT for 3 h. The reaction mixture was then centrifuged at 13,000 rpm for 5 min to remove any precipitate. The conjugate was purified by size-exclusion chromatography (PD-10 column), eluting with PBS pH 7.4. Fractions containing the conjugate (determined by UV-Vis spectrophotometry at λ 280 nm and 432 nm) were pooled and stored at 4 °C for future studies. Protein concentration was determined by the Lowry assay (see Section 8.2.7). Samples of isatin-PAI-2 conjugate (165) (100 µL) were dialysed overnight in 10 mM NH₄OAc buffer in MilliQ H₂O (2 × 2 L) prior to ESI-MS. The samples were analysed using a Waters Synapt™ High Definition Mass Spectrometer (HDMS).
Conjugation of (E and Z)-3-[4-[5,7-dibromo-1-(4-methoxybenzyl)-2-oxoindolin-3-ylideneamino]phenyl]propanoic acid (155) to Tf

The conjugate 166 was prepared according to the method in Section 8.1.5.1 using a 20-fold molar excess of the active ester 164 (19.0 µL) and Tf (831 µL at 3.37 mg/mL). Protein concentration was determined by the Lowry assay (see Section 8.2.7).

Reducing the imine bond to an amine bond in the isatin-PAI-2 conjugate (165)

According to the method of Ding, et al.,286 NaBH₄ (847 ng, 22.4 nmol) was added to a solution of isatin-PAI-2 conjugate (165) (510 µg, 11.2 nmol at 1.46 mg/mL). The reaction mixture was shaken at RT for 15 h before being dialysed against 0.05 M HCl for 4 h and 10 mM NaOAc (× 2). Analysis by ESI-MS did not reveal the desired amino product.

Reducing the imine bond to an amine bond in the isatin-Tf conjugate (166)

The method was the same as the above procedure, using NaBH₄ (545 ng, 14.4 nmol) and a solution of isatin-Tf conjugate (166) (579 µg, 7.19 nmol at 1.45 mg/mL). Analysis by ESI-MS did not reveal the desired amino product.
8.1.5.2 Other Molecules

Synthesis of 1-benzyl-1H-indole-2,3-dione (169)

This compound was prepared according to the method for 49 using isatin (11) (200 mg, 1.36 mmol), NaH (76.0 mg, 1.90 mmol), KI (45.2 mg, 272 µmol) and benzyl bromide (511 mg, 355 µL, 2.99 mmol) as starting materials. The reaction mixture was heated at 75 °C for 18 h. The resulting solid was purified by flash chromatography on silica gel (100% DCM) to yield 169 (212 mg, 66%) as orange crystals, m.p. 127-128 °C (lit.327 132-133 °C), Rf 0.32 (silica, DCM).

1H NMR (CDCl₃, 500 MHz): δ 4.93 (s, 2H, H1'), 6.77 (d, J = 8.0 Hz, 1H, H4 or H7), 7.09 (t, J = 7.0 Hz, 1H, H5 or H6), 7.30-7.35 (m, 5H, ArH × 5), 7.48 (t, J = 7.0 Hz, 1H, H5 or H6), 7.61 (d, J = 7.0 Hz, 1H, H4 or H7). The 1H NMR spectral data coincided with those reported in the literature.327 LREI-MS: m/z 237 [M⁺].
8.2 Biological Procedures

8.2.1 Cell Lines and Cell Culture Conditions

U937 (human, leukemic, monocyte-like, histolytic lymphoma), THP-1 (human, acute monocytic leukemia) and MDA-MB-231 (human, metastatic breast adenocarcinoma) cancer cells were obtained from American Type Culture Collection (ATCC, VA, USA) distributed by Cryosite, NSW, Australia. Cells were regularly cultured in vitro in culture medium consisting of RPMI-1640 medium, along with 2 mM L-glutamine, 5.6% (2 g/L) NaHCO$_3$ and 5% foetal calf serum. The cells were maintained in a Huracell incubator (Kendoro Laboratory Products, Langenselbold, Germany) at 37 °C with a humidified atmosphere containing 5% CO$_2$. Viability of the cells was determined by the trypan blue exclusion method and the number of viable cells was counted with the aid of a haemocytometer. Light microscopy images were obtained using a Leica DC500 12-megapixel high-performance FireWire camera system (Leica Microsystems, AG, Germany) and the Leica IM50 image manager software (Leica Microsystems AG, Heerbrugg, Germany).

8.2.2 MTS Cell Proliferation Assay

Cytotoxicity of the isatin derivatives was determined using the CellTiter 96® AQqous One Solution Cell Proliferation Assay, utilising [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] (MTS) (Promega Co., Madison, WI, USA). Assays were performed in 96-well microplates, consisting of: various dilutions of the test compounds dissolved in a final concentration of 2.5% DMSO, a negative solvent control (2.5% DMSO) and background controls (containing test compounds but no cells) (see Appendix). Penicillin/streptomycin (1%) was added to assays containing protein samples (PAI-2
or Tf). After incubating the test compounds and cells (1.0 x 10^4 cells/well) for 24 h at 37 °C in a 5% CO_2 atmosphere, 20 µL of the CellTiter 96® AQueous One Solution Reagent was added to each well and the assays were incubated at 37 °C for a further 3 h. Assays were read in a Spectromax 250 plate reader at 490 nm using Softmax Pro software (Molecular Devices, USA). The IC_{50} values were determined from sigmoidal dose-response curves (variable slope) using GraphPad Prism 5.00 (GraphPad Software, Inc., San Diego, CA, USA) and are the mean of triplicates of at least two independent experiments.

8.2.3 Tubulin Polymerisation Assay

The *in vitro* tubulin polymerisation assay was obtained from Cytoskeleton Inc. (Jomar Diagnostics, Australia) and the assay was conducted with reagents as described by the manufacturer. Briefly, 5 µL of vehicle control, vinblastine (7), paclitaxel (8) and compounds 26, 28, 36 and 37 at the desired concentrations were incubated with 50 µL of purified bovine neuronal tubulin reaction mix. The rate of polymerisation was monitored kinetically for 1 h at 37 °C using an excitation wavelength of 360±10 nm and fluorescence emission at 440±10 nm.

8.2.4 Hydrolysis Studies

Hydrolysis studies were performed on a Varian Cary 500 Scan UV-Vis-NIR spectrophotometer with 1 cm path length disposable plastic cuvettes. Briefly, 9 µmol of 153-157, 158-162 was dissolved in CH_3CN/H_2O 98.5:1.5 to yield a stock solution of approximately 1 mM. Buffer solutions consisted of 1 M NaOAc adjusted to pH 4.5 and pH 5.3 and 1 M HEPES adjusted to pH 7.4. The pH values of the solutions were measured using a pH Cube pH-mV-Temp Meter (TPS, Springwood, Australia). The
final concentration within the cuvette consisted of stock solution/buffer/H₂O 1:2:2. All solutions were monitored kinetically at λ 435 nm for 4 h at either RT (23 ºC) or 37 ºC. For isatin-protein conjugates, 1 cm path length quartz microcuvettes (Starna Pty Ltd., Baulkham Hills, Australia) were used. The pH values of these samples were adjusted with 10 mM NaOAc buffer. The dependence of the absorption spectra (measured at λ 435 nm) on time, expressed as $A/A_0$ vs. $t$ (where $A =$ absorbance of the sample and $A_0 =$ absorbance at time zero), was used to investigate the kinetics of imine hydrolysis, with all measurements performed on at least two independent occasions. The kinetic parameters $k$ and $t_{1/2}$ were derived by nonlinear regression analysis of the ln ($A/A_0$) versus time plots using GraphPad Prism 5.00 (GraphPad Software, Inc., San Diego, CA, USA), with one-way, repeated measures ANOVA followed by a Tukey post-test to determine whether differences between data sets were significant, with significance set at $P < 0.05$.

### 8.2.5 PKA Kinase Assays

PKA kinase inhibitory activity was determined using the Kinase Glo® Luminescent Kinase Assay (Promega Co., Madison, WI, USA). Assays were performed in white 96-well microtitre plates, consisting of: various dilutions of the test compounds dissolved in 100% DMSO, a negative solvent control (containing kinase enzyme and substrate) and a positive solvent control (containing kinase enzyme and no substrate). ATP (25 µL) was added to all wells. After incubating the plate at RT for 30 min, Kinase-Glo® Reagent (50 µL) was added to all wells and the plate was incubated for a further 30 min at RT. Assays were read on a FLUOstar Optima® Luminometer (BMG Labtech Pty. Ltd., Mornington, Australia).
8.2.6 DNA Intercalation Studies

The DNA used was Rt1, with a length of 16 base pairs, the sequence d(CTC GTC CGA TTC GAT C)•d(GAT CGA ATC GGA CGA G) and a mass of 9763.5 Da. Compounds to be tested were dissolved in CH$_3$CN to yield a 1 mM stock solution. To prepare the samples for analysis, 5 µL of the drug from the stock solution was aliquoted and evaporated before the addition of 79 µL of 100 mM NH$_4$OAc. 1 µL of 1 mM double stranded DNA Rt1 in 100 mM NH$_4$OAc was added to the sample and it was sonicated for 5 min. A further 120 µL of 100 mM NH$_4$OAc was added to the sample prior to analysis. (The final solutions contained 5 µM DNA and 25 µM of drug). The samples were analysed by ESI-MS (-ve) on a Waters Q-TOF Ultima mass spectrometer at a speed of 20 µL/min, at 100 cone volts and a source temperature of 30 ºC.
8.2.7 Protein Concentration Assay (Lowry Assay)

The BioRad® DC protein concentration assay kit (Bio-Rad Laboratories, Pty. Ltd., Gladesville, Australia) was employed to determine the protein concentration of samples. Bovine serum albumin standards (5 µL) ranging from 0-1000 µg/mL and protein samples of unknown concentration (5 µL) were added in duplicate to wells in a 96-well microtitre plate. Reagent A (25 µL) and Reagent B (200 µL) were added to each well and the plate was incubated at RT for 15 min before the absorbance was measured at 750 nm using the Spectromax® 250 UV plate reader (Molecular Devices, Sunnyvale, CA, USA). A standard curve was generated using the Softmax Pro® (Molecular Devices, Sunnyvale, CA, USA) software to calculate protein concentration.
References


References


References


References


References


References


References


References


References


References


References


Setup of the 96-well microplates used in the MTS assay.

- Purple: 100 μL media
- Green: 90 μL cells + 10 μL test compound in 2.5% DMSO
- Yellow: 100 μL cells
- Red: 90 μL cells + 10 μL 2.5% DMSO
- Black: 90 μL media + 10 μL test compound in 2.5% DMSO
Publications

The chapters below contain work from the following published journal articles:

**Chapter 1:**


**Chapter 4:**

**Chapters 4, 5 and 6:**

As the primary supervisor, I, Dr. Danielle Skropeta, declare that the greater part of the work in the articles listed above are attributed to the candidate, Lidia Matesic. For the *Anti-Cancer Agents in Medicinal Chemistry* article, the two first authors, Kara L. Vine and Lidia Matesic, contributed equally. In the above articles, Lidia contributed to the study design and was primarily responsible for data collection, data analysis and data interpretation. The first draft of each manuscript was written by Lidia and she was responsible for responding to the editing suggestions of her co-authors. The co-authors were responsible for assisting in the study design, interpreting data and editing all manuscripts. Lidia has been solely responsible for submitting each manuscript for publication and she has been in charge of responding to reviewers’ comments, with assistance from her co-authors.

Lidia Matesic
Candidate
March 21, 2011

Dr. Danielle Skropeta
Primary Supervisor
March 21, 2011
**N-Phenethyl and N-naphthylmethyl isatins and analogues as in vitro cytotoxic agents**

Lidia Matesic, Julie M. Locke, John B. Bremner, Stephen G. Pyne, Danielle Skropeta, Marie Ranson and Kara L. Vine

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Available online 7 January 2008

**Abstract**—A range of N-phenethyl, N-phenacyl, and N-(1- and 2-naphthylmethyl) derivatives of 5,7-dibromoisatin were prepared by N-alkylation reactions. Their activity against human monocyte-like histiocytic lymphoma (U937), leukemia (Jurkat), and breast carcinoma (MDA-MB-231) cell lines was assessed. The results allowed further development of structure–activity relationships. The compound 5,7-dibromo-N-(1-naphthylmethyl)-1H-indole-2,3-dione 5a was the most potent against U937 cells with an IC50 value of 0.19 μM.

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1. Introduction

Isatin (1H-indole-2,3-dione) 1 is a synthetically versatile molecule which has led to an array of derivatives displaying a broad spectrum of biological properties including anti-cancer activities.1–5 Recently, it has been reported that 5,7-dibromoisatin 2 is significantly more potent in vitro as a cytotoxin than the parent molecule against U937 (human monocyte-like histiocytic lymphoma) cells.6 Other research within our laboratory has indicated that N-benzylation of 5,7-dibromoisatin 2 further increased the cytotoxicity toward these lymphoma cells and was potent against a range of human cancer cell lines including a metastatic breast adenocarcinoma cell line (MDA-MB-231).7 In this context, it was of interest to further investigate the cytotoxicity of N-alkylated 5,7-dibromoisatin analogues by altering the chain length between N-1 and the aryl group, as well as increasing the hydrophobicity of the N-substituent through an extra aromatic ring fusion. Together with this, in vitro screening against various cancer cell lines was carried out in order to establish a more comprehensive structure–activity relationship (SAR). The results are described in this paper.

**2. Results and discussion**

2.1. Chemistry

In order to determine the structural requirements necessary for cytotoxicity, a simple modification to 2 was envisaged with a two-atom linker bridge between the nitrogen and a new aromatic ring, producing N-phenethyl derivatives 4a–e. The phenethyl scaffold was also modified by introduction of another aromatic ring fusion which subsumed the benzylic methylene group in the new ring, to afford the 1-naphthylmethyl derivative 5a. The 2-naphthylmethyl analogue 5b was also synthesized for comparative purposes. With a view to assessing the hydrophobicity requirements, N-phenacyl derivatives 6a–e of 5,7-dibromoisatin 2 were also investigated.

The procedure for the synthesis of the N-phenethyl derivatives 4a–e was based on a combination of the literature methods.8–10 Briefly, 2 was treated with a base such as NaH or K2CO3 which formed an intense purple colored anion (A) that was subsequently reacted with the appropriate aryl alkyl halide 3a–e to give the N-phenethyl derivatives 4a–e in moderate yields.
(Scheme 1). High temperatures are usually required in these syntheses to drive the reactions to completion because the reaction mixtures are prone to crystallization of the impure product at low temperatures.\textsuperscript{8} N-Benzylisatin derivatives had been obtained previously by heating at 80°C,\textsuperscript{7} however a temperature of 50°C was used to produce 4a–e to reduce the risk of substituted styrenes forming as side products from the base catalyzed elimination of the phenethyl bromides 3a–e. The isatin intermediate (A) is also an ambidentate anion which could undergo N- or O-alkylation,\textsuperscript{11} however no evidence for O-alkylation was found through $^{1}$H and $^{13}$C NMR spectroscopic studies.

The N-naphthylmethyl analogues 5a and 5b were prepared in a similar manner to the N-phenethyl series 4a–e, however, the synthesis of the N-phenacyl derivatives 6a–e proved difficult. Attempts to synthesize these compounds included various alkylation protocols and protecting group strategies. A possible competing reaction was a Darzens condensation involving the C3 carbonyl group of the isatin 2. While the Darzens condensation occurs when a ketone or aldehyde reacts with a haloester to form an epoxy ester, it also proceeds with halogenomethylsulfones and halogenoketones such as phenacyl halides.\textsuperscript{12} It has been reported that phenacyl halides preferentially yield a Darzens product rather than the corresponding phenacylisatin,\textsuperscript{13} although in the current study this was not observed in the NMR spectra. The desired compounds 6a–e were obtained in a pure form but in low yields, which were not optimized as the required compounds were obtained in sufficient quantity for cytotoxicity screening.

![Scheme 1](image)

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![Scheme 1](image)

### 2.2. Cytotoxic activity and SAR

The N-phenethyl 4a–e and N-naphthylmethyl derivatives 5a–b were initially tested for cytotoxicity using the MTS cell proliferation assay against three human cancer cell lines including a lymphoma (U937), leukemia (Jurkat), and metastatic breast adenocarcinoma cell line (MDA-MB-231). The results showed that the cytotoxicity of the parent molecule 2 significantly increased through N-alkylation (Table 1), as reported previously for the 5,7-dibromo-N-benzylisatin derivatives.\textsuperscript{7} Compounds 4a–e, which contain a two-carbon linker at the nitrogen, typically increased the anti-proliferative activity 10- to 15-fold against the three tumor cell lines compared to that of the parent brominated isatin 2. Three of the N-phenethyl derivatives, 4a–c, displayed IC\textsubscript{50} values in the sub-micromolar range against U937 cells (e.g., 4a, IC\textsubscript{50} = 0.78 μM, Table 1). Introduction of a hydrophobic bromo substituent in the meta (4b) or para (4c) position yielded the most active compounds in this series, while analogous compounds containing methoxy substituents 4d–e were less cytotoxic. The introduction of a substituent in the meta position (4b and 4d) enhanced

<table>
<thead>
<tr>
<th>Compound</th>
<th>U937</th>
<th>Jurkat</th>
<th>MDA-MB-231</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>10.5</td>
<td>14.3</td>
<td>42.3</td>
</tr>
<tr>
<td>4a</td>
<td>0.78</td>
<td>1.52</td>
<td>4.35</td>
</tr>
<tr>
<td>4b</td>
<td>0.78</td>
<td>1.21</td>
<td>2.72</td>
</tr>
<tr>
<td>4c</td>
<td>0.88</td>
<td>1.15</td>
<td>2.01</td>
</tr>
<tr>
<td>4d</td>
<td>1.07</td>
<td>2.00</td>
<td>5.24</td>
</tr>
<tr>
<td>4e</td>
<td>2.35</td>
<td>2.66</td>
<td>4.51</td>
</tr>
<tr>
<td>5a</td>
<td>0.19</td>
<td>0.91</td>
<td>2.49</td>
</tr>
<tr>
<td>5b</td>
<td>0.74</td>
<td>0.41</td>
<td>2.56</td>
</tr>
<tr>
<td>6a</td>
<td>9.97</td>
<td>nt</td>
<td>nt</td>
</tr>
<tr>
<td>6b</td>
<td>6.36</td>
<td>nt</td>
<td>nt</td>
</tr>
<tr>
<td>6c</td>
<td>9.18</td>
<td>nt</td>
<td>nt</td>
</tr>
<tr>
<td>6d</td>
<td>4.70</td>
<td>nt</td>
<td>nt</td>
</tr>
<tr>
<td>6e</td>
<td>3.52</td>
<td>nt</td>
<td>nt</td>
</tr>
<tr>
<td>Vinblastine</td>
<td>6.88</td>
<td>nt</td>
<td>nt</td>
</tr>
</tbody>
</table>

\( ^{a}\) Values are means of triplicates of at least two independent experiments.

\( ^{b}\) Human monocyte-like histiocytic lymphoma cell line.

\( ^{c}\) Human leukemic T-cell line.

\( ^{d}\) Human metastatic mammary gland adenocarcinoma cell line.

\( ^{e}\) Vine et al.\textsuperscript{6}

\( ^{f}\) Not tested.

\( ^{g}\) Vine et al.\textsuperscript{7}

![Scheme 1](image)

**Scheme 1.** Reagents and conditions: (a) NaH or K\textsubscript{2}CO\textsubscript{3}, DMF, 4°C–rt, 20 min to 3 h; (b) KI, 50°C, 18 h (32–62%).
The activity against the panel of human tumor cell lines (Table 2) indicated that the isatin derivatives 4b-e and 5a-b, while the most cytotoxic against U937 cells, were less active than the N-phenethylmethyl derivatives 4a-e and the metastatic MDA-MB-231 cell line was the least susceptible.

The N-naphthylmethylisatins 5a and 5b demonstrated enhanced cytotoxic activity against the lymphoma (U937) and leukemia (Jurkat) cell lines compared to the N-phenethyl derivatives 4a-e. Both 5a and 5b exhibited sub-micromolar IC_{50} values against these two cell lines, and were the only compounds to do so against Jurkat cells. Compound 5a was the most cytotoxic of all those tested, with an IC_{50} value of 0.19 μM against U937 cells. This compound was four times more potent against U937 cells than the lead N-phenethyl compounds 4a and 4b.

The most active analogue in the N-phenacyl series was 6d, which exhibited an IC_{50} value of 4.70 μM against U937 cells. The parent phenacyl derivative 6a was the least active of the series with an IC_{50} value of 9.97 μM (Table 1). This suggests that the presence of the polar carbonyl group is detrimental to activity. Since the N-phenacylisatins 6a-e were less active than the N-phenethyl 4a-e and N-naphthylmethyl 5a-b series, they were not tested against any other cell lines.

The four most potent derivatives, 4b, 4c, 5a, and 5b, (Table 1) were selected for further cytotoxic screening against a panel of other adherent human tumor cell lines: colorectal (HCT-116), prostate (PC-3), non-metastatic breast (MCF-7), and melanoma (A375). The results in Table 2 indicate that activity against the panel of adherent cell lines varied but all compounds tested had IC_{50} values in the low micromolar range. The lower sensitivity in these adherent cell lines compared to the non-adherent cell lines may be due to reduced cell surface areas leading to decreased drug uptake as well as slower proliferation rates in the former.

### 2.3. Mode of action studies

While performing in vitro cytotoxicity testing, it was observed that the isatin derivatives 4a-e, 5a-b, and 6a-e caused the U937 and, to a lesser extent, the Jurkat cells to undergo elongation. The elongated morphology was more pronounced when the cells were treated with N-naphthylmethyl derivatives 5a and 5b (Fig. 1B). This morphological change was also observed in U937 cells treated with vinblastine (Fig. 1C), a microtubule destabilizer. This suggested that these isatin analogues interfere with microtubule dynamics in a similar fashion to previously reported 5,7-dibromo-N-benzylisatin derivatives. To further investigate the effects of the aforementioned analogues on microtubule formation, a cell-free in vitro tubulin polymerization assay was performed (Fig. 2). The two naphthyl derivatives 5a and 5b were chosen due to their in vitro potency against U937 and Jurkat cells, as well as two representative phenethyl compounds, 4a and 4c. Vinblastine sulfate and paclitaxel were used as a known microtubule destabilizer and stabilizer, respectively. Consistent with the literature reports, at 10 μM paclitaxel stabilized microtubules, while vinblastine sulfate was a potent microtubule destabilizer (Fig. 2). The test compounds, in particular...
Table 3. Inhibition of tubulin polymerization (IC_{50} \mu M) of compounds 4a, 5a, 5b and vinblastine at varying time points

<table>
<thead>
<tr>
<th>Compound</th>
<th>20 min</th>
<th>60 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>4a</td>
<td>4.04</td>
<td>6.60</td>
</tr>
<tr>
<td>5a</td>
<td>4.71</td>
<td>12.0</td>
</tr>
<tr>
<td>5b</td>
<td>2.87</td>
<td>6.47</td>
</tr>
<tr>
<td>Vinblastine</td>
<td>0.95</td>
<td>1.66</td>
</tr>
</tbody>
</table>

*Values are means of duplicates in one experiment.

the 2-naphthyl derivative 5b, appeared to be potent microtubule destabilizers at 10 \mu M (Fig. 2), as observed by the shift of the curve to the right of the control, indicating a decrease in the rate of tubulin polymerization. These results are consistent with reports that structurally similar indole\textsuperscript{16-18} and indolinone\textsuperscript{15,19} compounds inhibit tubulin polymerization.

The three most potent destabilizers, 4a, 5a, and 5b, were chosen for further studies on tubulin polymerization. All of these compounds inhibited the rate of tubulin polymerization in a dose-dependent manner (representative result shown in Fig. 3) and the IC_{50} values are reported in Table 3. Vinblastine displayed similar inhibition in this assay to that reported previously.\textsuperscript{20}

It was of interest to note that in Figure 3, the test compound 5b displayed potent destabilizing effects at higher concentrations (as seen by the shift in the curves to the right of the DMSO control) and stabilized microtubules at lower concentrations (as seen by the shift in the curves to the left of the DMSO control). This phenomenon has also been reported in the case of vinblastine\textsuperscript{14} and suggests that these compounds may bind to more than one binding site on tubulin with different affinities.

3. Conclusions

A number of \textit{N}-phenethyl, \textit{N}-phenacyl, and \textit{N}-naphthylmethyl derivatives of 5,7-dibromoisatin 2 were synthesized and tested against a range of human cancer cell lines. These compounds displayed greater cytotoxicity against non-adherent U937 and Jurkat cells compared to adherent human breast (MDA-MB-231 and MCF-7), colon (HCT-116), prostate (PC-3), and melanoma (A375) tumor cell lines. Cytotoxicity testing showed that 5,7-dibromo-\textit{N}-(1-naphthylmethyl)isatin 5a was the most active compound against all of the cell lines tested and exhibited an IC_{50} value of 0.19 \mu M against U937 cells. The \textit{N}-phenethyl derivatives 4a-c also showed potent sub-micromolar activity against U937 cells. Additionally, the tumor cells displayed elongated cell morphology upon treatment with these compounds, suggesting that these analogues interfere with microtubule dynamics. The results described indicate that these compounds could serve as the basis for the development of a new group of cancer chemotherapeutics.

4. Experimental

4.1. General

4.1.1. Chemistry. All solvents were of AR grade except dichloromethane (DCM) which was of LR grade and distilled before use. The term petroleum spirit (PS) refers to petroleum spirit with a boiling range of 40–60 °C. Solvent removal was performed (in vacuo) using temperatures not greater than 60 °C. Organic halides were purchased from Sigma–Aldrich Chemical Co. and used as supplied. Sodium hydride was supplied as a 60% dispersion in mineral oil and masses used were calculated appropriately. Melting points were obtained using a Reichert melting point apparatus and are uncorrected. Thin-layer chromatography (TLC) on aluminum backed sheets of Merck Silica Gel 60 F\textsubscript{254} plates was employed to monitor the progress of chemical reactions. Preparative TLC was performed on 20 × 20 cm plates. Generally, the isatins were highly colored and visible on the TLC plate; colorless compounds were detected by exposure to UV light at \lambda 254 nm. Column chromatography was performed under ‘flash’ conditions\textsuperscript{21} on silica gel 60 (230–400 mesh). The solvent used in individual chromatographic experiments is indicated and all solvent proportions are given as vol/vol ratios. NMR spectra were acquired on a Varian Unity 300 MHz spectrometer, where proton (\textit{1}H) and carbon (\textit{13}C) spectra were obtained at 300 MHz and 75 MHz, respectively, or on a Varian Inova 500 spectrometer, where the \textit{1}H and \textit{13}C were obtained at 500 MHz and 126 MHz, respectively. All spectra were obtained with a probe temperature of 298 K. Spectra were recorded in CDCl\textsubscript{3} (unless otherwise indicated) and were referenced to the residual non-deuterated solvent signal or TMS. Hydrogen and carbon assignments were also made using gradient correlation spectroscopy (gCOSY), gradient heteronuclear single quantum correlation (gHSQC), and gradient heteronuclear multiple bond correlation (gHMBC) spectroscopic techniques. The superscript letter \textsuperscript{a} denotes coincident peaks. Low resolution electron ionization mass spectra (LREI-MS) were obtained using a Shimadzu QP5050 spectrometer. High resolution electron ionization mass spectra (HREI-MS) were obtained using a Fisons/VG Autospec spectrometer operating with an electron beam of 70 eV, with a source temperature of 250 °C and perfluorokerosene (PFK) as the internal standard. Compounds for testing were >95% pure on the basis of TLC and \textit{1}H NMR analysis.
4.1.2. Cell biology. All cancer cell lines were obtained from the American Type Culture Collection (ATCC, VA, USA). Cells were routinely maintained in RPMI-1640 medium, containing 2 mM L-glutamine, 5.6% (2 g/L) sodium bicarbonate, and 5% fetal calf serum (FCS) (at 37 °C 95% humidified atmosphere and 5% CO2). Adherent cells were detached with sterile trypsin-EDTA, washed with culture media, and resedeed following centrifugation for 5 min at 1600 rpm. The number of viable cells was counted with the aid of a hemocytometer and Trypan blue staining. Cytotoxicity of the isatin derivatives was determined using the CellTiter 96® AQueous One Solution Cell Proliferation Assay, utilizing [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxypyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] (MTS) (Promega Co., Madison, WI, USA) as described previously.6,7 The inhibitory concentration required to inhibit 50% of the metabolic activity of the cell population (IC50) was calculated from sigmoidal dose–response curves using GraphPad Prism 5.00 (GraphPad Software Inc.). Cell images were obtained utilizing [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxypyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] (MTS) (Promega Co., Madison, WI, USA) as described previously.6,7 The inhibitory concentration required to inhibit 50% of the metabolic activity of the cell population (IC50) was calculated from sigmoidal dose–response curves using GraphPad Prism 5.00 (GraphPad Software Inc.). 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Cell images were obtained utilizing [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxypyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] (MTS) (Promega Co., Madison, WI, USA) as described previously.6,7 The inhibitory concentration required to inhibit 50% of the metabolic activity of the cell population (IC50) was calculated from sigmoidal dose–response curves using GraphPad Prism 5.00 (GraphPad Software Inc.). Cell images were obtained utilizing [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxypyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] (MTS) (Promega Co., Madison, WI, USA) as described previously.6,7 The inhibitory concentration required to inhibit 50% of the metabolic activity of the cell population (IC50) was calculated from sigmoidal dose–response curves using GraphPad Prism 5.00 (GraphPad Software Inc.).

(157 mg, 0.11 mL, 0.73 mmol) as starting materials. The
resulting solid was puriﬁed by ﬂash chromatography on
silica gel (CHCl3) to yield 4e (89.5 mg, 62%) as bright
red/orange crystals, mp 176–178 C, Rf 0.53 (silica,
DCM). 1H NMR (500 MHz): d 2.96 (t, J = 8 Hz, 2H,
H2 0 ), 3.78 (s, 3H, OCH3), 4.34 (t, J = 8 Hz, 2H, H1 0 ),
6.83 (d, J = 8.5 Hz, 2H, H300 , H500 ), 7.16 (d, J = 8.5 Hz,
2H, H200 , H600 ), 7.68 (d, J = 2 Hz, 1H, H4), 7.89 (d,
J = 2 Hz, 1H, H6). 13C NMR (126 MHz): d 34.7, 43.1,
55.5, 105.0, 114.4a, 117.1, 121.6, 127.8, 129.2, 130.1a,
145.3, 147.0, 158.1, 158.8, 181.6. HREI-MS: m/z calcd
for C17H13NO3 79Br81Br [M+]: 438.9242; found:
438.9241.
4.2.7. 5,7-Dibromo-1-(1-naphthylmethyl)-1H-indole-2,3dione (5a). A mixture of 5,7-dibromoisatin 2 (101 mg,
0.33 mmol) and NaH (18.0 mg, 0.46 mmol) was dissolved in anhydrous DMF (2.5 mL) and stirred under
nitrogen at rt for 20 min before the addition of KI
(11.0 mg, 0.066 mmol) and 1-chloromethylnaphthalene
(128 mg, 0.11 mL, 0.73 mmol). The reaction mixture
was heated at 60 C and stirred at this temperature for
19 h. After cooling, ethyl acetate (50 mL) was added
and the resulting solution was extracted with 0.5 M
HCl (50 mL) followed by brine (50 mL). The orange organic layer was dried over MgSO4 and the solvent was
removed to yield a sticky red/orange residue. The resulting solid was puriﬁed by ﬂash chromatography on silica
gel [DCM/PS (3:2)] to yield 5a (90.8 mg, 62%) as dark
red crystals, mp 218–219 C, Rf 0.35 (silica, DCM). 1H
NMR (500 MHz): d 5.81 (s, 2H, H1 0 ), 7.06 (d, J = 7 Hz,
1H, H200 ), 7.34 (t, J = 7.5 Hz, 1H, H300 ), 7.53 (t, J = 7 Hz,
1H, H600 ), 7.58 (t, J = 7 Hz, 1H, H700 ), 7.76 (m, 3H, H4,
H6, H400 ) 7.88 (d, J = 8.5 Hz, 1H, H500 ), 7.93 (d,
J = 8.5 Hz, 1H, H800 ). 13C NMR (126 MHz): d 42.8,
105.5, 117.1, 121.3, 121.4, 122.1, 125.3, 126.0, 126.6,
127.5, 128.0, 129.0, 129.8, 130.7, 133.8, 145.3, 146.8,
158.1, 181.2. HREI-MS: m/z calcd for C19H11NO279Br81Br
[M+]: 444.9136; found: 444.9131.
4.2.8. 5,7-Dibromo-1-(2-naphthylmethyl)-1H-indole-2,3dione (5b). The compound was prepared according to
the method for 5a using 5,7-dibromoisatin 2 (50.5 mg,
0.16 mmol) and 2-bromomethylnaphthalene (80.1 mg,
0.36 mmol) as starting materials. The resulting red solid
was puriﬁed by ﬂash chromatography on silica gel
[DCM/PS (3:2)] to yield 5b (46.6 mg, 64%) as dark red
crystals, mp 140–142 C, Rf 0.58 (silica, DCM). 1H
NMR (500 MHz): d 5.56 (s, 2H, H1 0 ), 7.36 (dd,
J = 2 Hz, 8 Hz, 1H, H300 ), 7.47 (m, 2H, ArH · 2), 7.63
(s, 1H, H100 ), 7.74 (d, J = 2 Hz, 1H, H4), 7.76 (m, 1H,
ArH), 7.79 (d, J = 2 Hz, 1H, H6), 7.81 (m, 2H,
ArH · 2). 13C NMR (126 MHz): d 45.1, 105.5, 117.5,
121.7, 124.6, 125.3, 126.4, 126.7, 127.8, 128.0, 128.0,
129.1, 133.0, 133.3, 133.5, 145.6, 147.0, 158.6, 181.5.
HRMS: m/z calcd for C19H11NO279Br81Br [M+]:
444.9136; found: 444.9135.
4.2.9. 5,7-Dibromo-1-(2-oxo-2-phenylethyl)-1H-indole2,3-dione (6a). A mixture of KI (113 mg, 0.68 mmol)
and phenacyl bromide (68.0 mg, 0.34 mmol) was dissolved in anhydrous DMF (0.5 mL) and stirred at
5 C under nitrogen for 5 h, followed by cooling in a

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freezer at 18 C for 20 h.23 A mixture of the 5,7-dibromoisatin 2 (103 mg, 0.34 mmol) and K2CO3 (47.0 mg,
0.34 mmol) or NaH (13.7 mg, 0.34 mmol) was dissolved
in anhydrous DMF (5 mL) and stirred under nitrogen at
4 C for 3 h. This anion solution was added in portions
(0.5 mL) to the phenacyl iodide maintained at 2 C
such that each portion had reacted before the addition
of the next portion (monitored by TLC). The yellow/
brown reaction mixture was stirred at rt for 25 h but
no change in color intensity was observed after 2 h. To
the resulting solution were added water (60 mL) and
1 M HCl (2 mL) to acidify to pH 1. The suspension
was ﬁltered and the precipitate washed with water.
The resulting solid was puriﬁed by ﬂash chromatography on silica gel (DCM) to yield 6a (13.5 mg, 9%) as
bright red/orange crystals, mp 173–175 C, Rf 0.58 (silica, DCM). 1H NMR (500 MHz): d 5.64 (s, 2H, H1 0 ),
7.54 (t, J = 7.5 Hz, 2H, H300 , H500 ), 7.67 (t, J = 7.5 Hz,
1H, H400 ), 7.75 (d, J = 2 Hz, 1H, H4), 7.80 (d,
J = 2 Hz, 1H, H6), 8.00 (d, J = 7.5 Hz, 2H, H200 , H600 ).
13
C NMR (126 MHz): d 48.2, 105.4, 117.1, 121.3,
126.5, 127.6, 128.3a, 129.1a, 134.4, 144.8, 146.9, 158.2,
180.8, 191.3. HREI-MS: m/z calcd for C16H9NO3
79
Br81Br [M+]: 422.8929; found: 422.8928.
4.2.10. 5,7-Dibromo-1-[2-(3-bromophenyl)-2-oxo-ethyl]1H-indole-2,3-dione (6b). This compound was prepared
according to the method for 6a using 5,7-dibromoisatin
2 (305 mg, 1.00 mmol) and 3-bromophenacyl bromide
(278 mg, 1.00 mmol) as starting materials. The reaction
mixture was stirred at 100 C for 16 h.24 The resulting
solid was puriﬁed by ﬂash chromatography on silica
gel (DCM) and subsequent preparative TLC (silica,
DCM) to yield 6b (10.6 mg, 2%) as bright red/orange
crystals, mp 160–162 C, Rf 0.54 (silica, DCM). 1H
NMR (500 MHz): d 5.60 (s, 2H, H1 0 ), 7.44 (t, J = 8 Hz,
1H, H500 ), 7.77 (d, J = 2 Hz, 1H, H4), 7.80 (d, J = 8.5 Hz,
1H, H400 ), 7.81 (d, J = 2 Hz, 1H, H6), 7.93 (d, J = 8.5 Hz,
1H, H600 ), 8.13 (s, 1H, H200 ). 13C NMR (126 MHz): d
48.0, 105.3, 117.3, 121.3, 123.5, 126.6, 127.7, 130.7, 131.2,
135.4, 137.3, 144.8, 146.6, 158.0, 180.6, 190.2. HREI-MS:
m/z calcd for C16H8NO3 79Br79Br81Br [M+]: 500.8034;
found: 500.8037.
4.2.11. 5,7-Dibromo-1-[2-(4-bromophenyl)-2-oxo-ethyl]1H-indole-2,3-dione (6c). 5,7-Dibromoisatin 2(153 mg,
0.5 mmol) and NaH (20.0 mg, 0.5 mmol) were dissolved
in anhydrous DMF (1.25 mL) and stirred at rt under
nitrogen for 20 min before the addition of freshly distilled
trimethylsilyl chloride (81.0 mg, 0.095 mL, 1.5 mmol).25
The reaction mixture was heated at 50 C and stirred at
this temperature for 1 h before the addition of 4-bromophenacyl bromide (139 mg, 0.5 mmol) and further heating at 100 C for 1.5 h. Upon cooling, water (15 mL) was
added, the suspension was ﬁltered, and the precipitate
washed with hot water (90 C) to yield a rust colored compound. The product was recrystallized from glacial
AcOH, ﬁltered, and washed with ice cold water to yield
6c (11.2 mg, 5%) as a light yellow powder, mp 184–
186 C, Rf 0.65 (silica, DCM). 1H NMR (500 MHz): d
5.60 (s, 2H, H1 0 ), 7.70 (d, J = 8.5 Hz, 2H, H300 , H500 ),
7.76 (d, J = 2 Hz, 1H, H4), 7.80 (d, J = 2 Hz, 1H, H6),
7.87 (d, J = 8.5 Hz, 2H, H200 , H600 ). 13C NMR


(126 MHz): δ 47.9, 105.3, 117.2, 121.3, 127.6, 129.6, 129.8, 132.5, 144.7, 146.7, 158.1, 180.7, 190.4. HREI-MS: m/z calcd for C_{16}H_{8}NO_{3}^{79}Br^{81}Br [M^+]: 452.9034; found: 450.9048.  

4.2.12. 5,7-Dibromo-1-[2-(3-methoxyphenyl)-2-oxo-ethyl]-1H-indole 2,3-dione (6d). This compound was prepared according to the method for 6a using 5,7-dibromoisatin 2 (306 mg, 1.00 mmol) and 3-methoxyphenacyl bromide (230 mg, 1.00 mmol) as starting materials. The resulting solid was purified by flash chromatography on silica gel (DCM) to yield 6d (43.5 mg, 10%) as bright orange crystals, mp 205–207°C. 1H NMR (500 MHz): δ 3.88 (s, 3H, OCH_{3}), 5.62 (s, 2H, H1'), 7.20 (d, J = 8 Hz, 1H, H4'), 7.45 (t, J = 8 Hz, 1H, H5'), 7.51 (s, 1H, H2'), 7.58 (d, J = 8 Hz, 1H, H6'), 7.75 (d, J = 2 Hz, 1H, H4), 7.81 (d, J = 2 Hz, 1H, H6). 13C NMR (75 MHz): δ 48.1, 55.5, 105.4, 112.5, 117.1, 120.5, 120.7, 121.3, 127.5, 130.1, 135.0, 144.7, 146.9, 158.1, 160.1, 180.8, 191.2. HREI-MS: m/z calcd for C_{17}H_{11}NO_{4}^{79}Br^{81}Br [M^+]: 450.9055; found: 450.9048.  

4.2.13. 5,7-Dibromo-1-[2-(4-methoxyphenyl)-2-oxo-ethyl]-1H-indole 2,3-dione (6e). This compound was prepared according to the method for 6d using 5,7-dibromoisatin 2 (111 mg, 0.36 mmol) and 4-methoxyphenacyl bromide (111 mg, 0.36 mmol) as starting materials. The reaction mixture was then stirred at rt for 51 h. The suspension was filtered and the precipitate washed with water. The resulting solid was purified by flash chromatography on silica gel (DCM) to yield 6e (18.2 mg, 11%) as bright orange crystals, mp 205–207°C. 1H NMR (500 MHz): δ 3.91 (s, 3H, OCH_{3}), 5.59 (s, 2H, H1'), 7.00 (d, J = 8 Hz, 2H, H3', H5'), 7.74 (d, J = 2 Hz, 1H, H4), 7.80 (d, J = 2 Hz, 1H, H6), 7.98 (d, J = 8 Hz, 2H, H2', H6'). 13C NMR (126 MHz): δ 47.9, 55.9, 105.7, 114.6, 117.2, 121.6, 126.9, 127.7, 130.7, 145.0, 147.3, 158.5, 164.7, 181.2, 186.9. HREI-MS: m/z calcd for C_{17}H_{11}NO_{4}^{79}Br^{81}Br [M^+]: 452.9034; found: 452.9048.

Acknowledgments

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References and notes


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Abstract: Isatin (1H-indole-2,3-dione) and its derivatives demonstrate a diverse array of biological and pharmacological activities including anticonvulsant, antibacterial, antifungal, antiviral and anticancer properties. This broad spectrum of biochemical targets has been facilitated by the synthetic versatility of isatin, which has allowed the generation of a large number of structurally diverse derivatives including analogues derived from substitution of the aryl ring, and/or derivatisation of the isatin nitrogen and C2/C3 carbonyl moieties. The recent FDA approval of the oxindole sunitinib malate, as a kinase inhibitor for the treatment of advanced renal carcinoma and gastrointestinal stromal tumours, underscores the increasing interest in isatins as a new class of antineoplastic agents. In addition to potent kinase inhibition, the mechanism of action of other isatin derivatives includes the inhibition and/or modulation of proteases, translation initiation, neo-vascularisation and tubulin polymerisation. It was therefore the objective of this review to systematically evaluate the cytotoxic and anticancer properties of various substituted isatins and collate these findings to be used as a guide for future structure-activity relationship and mode of action studies. This is the first review to comprehensively discuss the in vitro and in vivo anticancer activities of isatin and its substituted derivatives.

Key Words: Isatin, 2,3-dioxindole, indolone, cytotoxicity, anticancer, enzyme inhibitor, kinase, tubulin.

1. INTRODUCTION

Isatin (1H-indole-2,3-dione) (1) was first discovered by Erdmann and Laurent in 1840 as a product arising from the oxidation of indigo (2) using nitric and chromic acids [1, 2] (Fig. (1)). The compound was considered synthetic for almost 140 years until it was found to be present in plants from the Isatis genus [3], in fruits of the cannon ball tree, Couroupita guianensis Aubl. [4] and in secretions from the parotid gland of the Bufo frog [5]. Various substituted isatins have also been identified in plants [6-8], fungi [9] and marine molluscs [10-13].

![Fig. (1). The chemical structures of isatin (1) and indigo (2).](image-url)

In humans and other mammals, isatin is found as an endogenous molecule. The metabolic pathways of isatin (1) have not yet been sufficiently elucidated; however it has been proposed that it may be synthesised in vivo from tryptophan-rich foods such as meat, dairy and whole grains. In this pathway, it is proposed that tryptophan is converted to indole by bacteria from the gastrointestinal tract and then transported to the liver where it is oxidized [14, 15]. Isatin (1) also plays a role in many physiological pathways, which are beyond the scope of this review (for further information see refs. [16-18]). Herein we review the most recent literature describing the cytotoxic and anticancer activities of isatin analogues derived from either mono-, di-, and tri-substitution of the aryl ring A, and/or those obtained by derivatisation of the isatin nitrogen and C2/C3 carbonyl moieties (Fig. (2)).

![Fig. (2). The various substitution types and patterns possible for the isatin scaffold.](image-url)

2. REVIEWS

Human exploitation of isatin-based chemistry has its genesis in the ancient dye industry which pre-dates Roman times [19, 20]. Isatin (1) and 6-bromoisatin (3b) were intermediates in the production of indigo (2) and Tyrian purple and were most likely also minor pigments in these dyes [21]. It is essentially through the development of synthetic dyes that the modern chemistry of isatin was initiated. The synthetic versatility of isatin has now led to its widespread use as a substrate for organic synthesis. A number of reviews regarding the synthesis and chemistry of isatin [22, 23] and the related oxindoles [24] as well as the utility of isatin as a precursor for the synthesis of other heterocyclic compounds have been published [25-27]. The most recent review to discuss the advances in the use of isatins for organic synthesis was a comprehensive survey by de Silva et al. in 2001 [28]. A number of recent reviews have focused on the biological role of isatin [17, 18] and the range of biological activities displayed by assorted isatins and isatin derivatives [16, 29, 30] including oxindoles and their copper complexes[31, 32]. This review is primarily focused on the cytotoxic and anticancer properties of this chemical class over the period of 2000-2008.
3. CYTOTOXIC AND ANTICANCER ACTIVITIES OF ISATIN DERIVATIVES

3.1. General Modes of Action

The isatin molecule is a versatile moiety and its analogues display diverse types of biological activities [16, 32], including anticancer [33-35]. Many indole-based compounds appear to act as inhibitors of various protein kinase families, particularly receptor tyrosine kinases (RTKs) and serine/threonine-specific protein kinases such as the cyclin-dependent kinases (CDKs). Kinases regulate many intra- and extracellular pathways that control cell growth, differentiation and death, and dysregulation of these enzymes contributes to the development of a range of cancers [36]. Selective small molecule kinase inhibitors are therefore appealing new therapeutics for the treatment of cancer [37]. Isatins that slow or stop the proliferation of cancer cells via inhibition of various protein kinases are discussed in sections 3.4 and 3.5.

The indolic heterocyclic nucleus is also central to a number of tubulin polymerisation inhibitors [38-42]. As isatins are oxidized derivatives of an indolic moiety, many are also found to disrupt microtubule dynamics (see sections 3.3 and 3.5). In addition to the biochemical targets, isatins are also found to intercalate between DNA base pairs (see sections 3.4 and 3.5.2), and inhibit the ribonucleoprotein telomerase [43], which is responsible for maintaining the telomeres of eukaryotic chromosomes. Overexpression of this enzyme is often responsible for the unlimited replicative potential of malignant cells [36].

Finally, the ability to kill cancer cells, or cytotoxicity, of a particular compound is typically defined as the concentration required to inhibit the growth (and ultimately kill) 50% of a cell population in vitro, and is reported as an IC_{50} value. It is important to note, however, that while this standard nomenclature allows for greater comparison of compounds across different structural classes, the use of diverse cell lines, assays (e.g. MTS, MTT, lactate dehydrogenase, sulforhodamine B/KiTox Red, WST-1 and clonogenic) and incubation times always need to be taken into consideration when comparing the potency of the compounds discussed in this review.

3.2. Mono-, Di- and Tri-Substituted Aromatic Isatin Derivatives

The isatin scaffold is a versatile moiety and its success as a new class of antineoplastic agents is supported by the recent FDA approval of the oxindole, sunitinib maleate (Sutent), for the treatment of advanced renal carcinoma [44] and gastrointestinal stromal tumours [45] (see section 3.5.1). Of importance to its activity is the fluorine atom at C5, which is not surprising, as substitution at the 5-position has previously been associated with increased biological activity for a range of isatin-based compounds. For example, in 2007 Vine et al. reported the in vitro cytotoxicity of a range of mono-substituted isatins (3a-g, Fig. (3)) on a human monocyte-like, histiocytic lymphoma (U937) cell line [34]. Structure activity relationship (SAR) studies revealed that substitution at position 5 was favoured over positions 4, 6 or 7, leading to greater cancer cell killing ability. Nitrating at C5 (3f) improved the anticancer activity by a factor of 4, while the addition of a methoxy group (3g) only mildly improved cytotoxicity (i.e. decreasing the IC_{50} by 145 μM) over a 24 h period. Furthermore, halogenation yielded the most active compounds, with 5-bromo-, 5-ido-, and 5-fluoroisatin (3a, 3e and 3d respectively) 5-10 times more active than the unsubstituted parent compound [34]. Increasing the number of electron withdrawing groups on the ring to make combinations of dibromo-, tribromo-, iodo- and nitroisatin derivatives (3h-n) also enhanced the overall activity, against a panel of human cancer cell lines, by up to 100-fold from that of the parent molecule (1). Despite this trend, 6,7-dimethoxyisatin was deemed inactive in a brine shrimp lethality assay (LD_{50} 96 ppm) compared to other substituted heterocyclic isatins [46], suggesting that together with substitution on the aromatic ring A, substitution at N1, C2 and/or C3, may also significantly enhance the molecule’s cytotoxic effect (see sections 3.3 - 3.5).

In terms of its mode of action, isatin itself is proposed to inhibit cancer cell proliferation via interaction with extracellular signal-related protein kinases (ERKs), thereby promoting apoptosis. In 2000, Cane et al. [33] reported that isatin inhibited the proliferation of a human promyelocytic leukemia (HL60) cancer cell line by 80% at a concentration of 0.1 mM, and consequently induced DNA fragmentation and chromatin condensation. Moreover, treatment of N1E-115 neuroblastoma cells with isatin at the same concentration inhibited the phosphorylation of ERK-2, but not ERK-1, by 35% compared to control untreated cells. A subsequent study using human neuroblastoma SH-SY5Y cells confirmed that the effect of isatin on cell proliferation was dose and time dependent [47]. For example, after 24 h of exposure isatin caused 35% detachment (or cell death) of SH-SY5Y cells treated with high concentrations (400 μM) of the compound. Following a 48 h exposure, the percentage of detached cells significantly increased to 82%. Apoptosis was observed in cells exposed to lower concentrations of isatin (50 μM), as indicated by morphological change (including cell shrinkage and nuclear condensation), internucleosomal DNA fragmentation and externalisation of plasma membrane phosphatidylserine. At higher concentrations, (greater than 200 μM) cell death increased even further, however evaluation of membrane permeability by FACS analysis revealed that isatin had significantly damaged the plasma membrane, consistent with necrotic cell death [47]. Such a time and dose dependent switch from apoptosis to necrosis was also observed by Vine et al. whereby 5,6,7-tribromoisatin (3n) was found to be anti-proliferative (and induced apoptosis) at low concentrations (4 μM), but cytotoxic (and induced necrosis) at high concentrations (130 μM) in U937 cells [34]. ERK activation was not tested in this study.

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![Fig. 3](image_url) Selected isatin derivatives with mono-, di- and tri- substitution on ring A.

3.3. N-Alkyl Substituted Isatin Derivatives

N-Alkylated indoles are well known to exhibit anticancer activity [48-50]. However, until recently, little had been reported on the antiapoptotic activity of N-alkyl and N-aryl isatins. In 2003, compound 4 (Fig. (4)), was the first N-alkylisatin described to induce apoptosis in a panel of human cancer cell lines, but not normal cells, at micromolar concentrations [48]. Generally it was found that normal cell lines such as peripheral blood lymphocytes and human mammary epithelial cells were resistant to N-(3,4-dichlorobenzyl)-1H-indole-2,3-dione (4) induced apoptosis, while...
cancer cell lines of lymphoid origin were the most sensitive. Further screening by the National Cancer Institute (NCI) found that the dichlorinated compound exerted a cytostatic effect (inhibiting cell growth by 50-100%) on 40 out of the 48 cell lines tested, at a concentration of 10 μM. At 10 times this concentration however, the compound exhibited 100% cytotoxicity on virtually all cell lines, suggesting that this effect may be due to non-specific toxicity [48].

Later in 2003, Liu et al. [51], identified a class of isatin O-acyl oximes (e.g. compound 5) that selectively inhibited neuronal ubiquitin C-terminal hydrolase (UCH-L1) at sub-micromolar concentrations in the H1299 lung cancer cell line (Fig. (4)). Despite being linked to Parkinson’s disease, UCH-L1 is also expressed in many primary lung tumours and lung cancer cell lines, but not normal lung tissue [52, 53], where it is proposed to be linked to tumour progression upon upregulation [49]. Expression of UCH-L1 also correlates with tumour progression in colorectal cancer [54]. Hence its inhibition by small molecules like compound 5 may be of great therapeutic benefit to lung and colorectal cancer patients in the future.

The increased potency of the halogenated isatins (see section 3.2) and the fact that N-methylation significantly enhanced the cytotoxicity of the parent compound (1) [34] led Vine et al. [35] to synthesise a series of brominated N-substituted isatins (e.g. 6a-d and 7a-n, Fig. (5)) and evaluate their cytotoxicity against a panel of cancer cell lines in vitro [35]. SAR studies indicated that the introduction of an aromatic ring with a one or three carbon atom linker at N1 increased the activity relative to that of the allyl (6a), 2'-methoxyethyl (6b) and 3'-methylbutyl (6c) N-substituted isatins. Furthermore, electron-withdrawing groups substituted at the meta or para position of the substituent phenyl ring were favoured over the ortho orientation. Of the 24 compounds screened, nine displayed sub-micromolar IC50 values and in general demonstrated greater selectivity toward leukemia and lymphoma cell lines over any of the carcinoma cell lines tested. 5,7-Dibromo-N-(p-methylbenzyl)isatin (7f) was the most active compound, inhibiting the metabolic activity of two haematopoietic cancer cell lines by 50% at 0.49 μM. This effect was further enhanced by at least a factor of 2 when the incubation time was increased from 24 h to 72 h, making this class of compounds >10 times more active than the conventional chemotherapeutic agents 5-fluorouracil, paclitaxel and vincristine against U937 cells (Fig. (5B)).

![Fig. (4). Examples of the first synthetic cytotoxic N-substituted isatin-based molecules.](image-url)

![Fig. (5). A Examples of cytotoxic N-substituted 5,7-dibromoisatins; B Viability of U937 cells after treatment with increasing concentrations of the commercial anticancer agents (▲) 5-Fluorouracil, (▲) Vinblastine and (▲) Paclitaxel or the N-alkylisatin (●), 5,7-dibromo-N-(p-trifluoromethylbenzyl)isatin (7e). Briefly, cells were incubated for 24 h at 37 °C with different concentrations of test compound, then analysed for cell viability using the MTS assay. Each data point is a mean of triplicates (± SE) and is expressed as a percent of the DMSO control.](image-url)
Following on from this work, and in order to establish a more comprehensive SAR for the di-brominated N-alkylisatins, Mateis et al. synthesised a family of N-phenethyl and N-phenacyl isatin derivatives (8a-e and 9a-e, Fig. (6)) [55]. All five N-phenethyl derivatives (8a-e) exhibited low to sub-micromolar cytotoxic activity against a panel of human leukemic, lymphoma and carcinoma cell lines where introduction of a hydrophobic bromo substituent in the meta (8b) or para (8e) position yielded the most active compounds [55]. Interestingly, the corresponding phenacyl (9a-e) derivatives were at least 3-5 times less active against the U937 cells and as a result were not tested against other cell lines.

Table 1. Enzyme and Cell Based Inhibitory Activity of the Representative N-Alkylisatins 6d, 7e, 7f, 7j, 7k and 7l on Serine/Threonine (CDK5 and GSK3) and Tyrosine (DYRK1A, JAK1, JAK2 and c-FMS) Kinases

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC50 (μM)</th>
<th>Enzyme</th>
<th>Cell</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DyrK1A&lt;sup&gt;a&lt;/sup&gt; or CDK5&lt;sup&gt;b&lt;/sup&gt; or GSK3&lt;sup&gt;c&lt;/sup&gt;</td>
<td>JAK1&lt;sup&gt;d&lt;/sup&gt; or JAK2&lt;sup&gt;e&lt;/sup&gt; c-FMS&lt;sup&gt;f&lt;/sup&gt;</td>
<td>Ba/F3-TJ2</td>
</tr>
<tr>
<td>6d</td>
<td>&gt;10</td>
<td>&gt;5</td>
<td>0.17</td>
</tr>
<tr>
<td>7e</td>
<td>&gt;10</td>
<td>&gt;5</td>
<td>0.29</td>
</tr>
<tr>
<td>7f</td>
<td>&gt;10</td>
<td>&gt;5</td>
<td>0.29</td>
</tr>
<tr>
<td>7j</td>
<td>&gt;10</td>
<td>&gt;5</td>
<td>1.07</td>
</tr>
<tr>
<td>7k</td>
<td>&gt;10</td>
<td>&gt;5</td>
<td>&lt;0.16</td>
</tr>
<tr>
<td>7l</td>
<td>&gt;10</td>
<td>&gt;5</td>
<td>0.39</td>
</tr>
</tbody>
</table>

<sup>a</sup> For cell based assays, the growth factor dependent myelomonocytic cell line BaF3 was transfected with either pTELJAK2 or pTELJAK3 and the cells selected for factor independent growth. Assays were conducted in the presence 15 μM ATP and performed essentially as described in Ref. [68];<sup>b</sup> DYRK1A: Dual-specificity tyrosine-phosphorylation regulated kinase 1A;<sup>c</sup> CDK5: Cyclin dependent kinase 5;<sup>d</sup> GSK3: Glycogen synthase kinase 3;<sup>e</sup> JAK1: Janus protein tyrosine kinase 1;<sup>f</sup> JAK2: Janus protein tyrosine kinase 2;<sup>g</sup> c-FMS: Colony-stimulating factor-1 receptor.

<sup>1</sup> Assays were performed as described by Leclerc et al. [69].

<sup>2</sup> Assays were performed as described in Ref. [68].
Wnt and hedgehog pathways which, when deregulated, are both involved in several forms of human cancer [63]. In these pathways, GSK3β is known to play a role in the dynamics of the mitotic spindle [64], whereby GSK3β directly phosphorylates several microtubule-associated proteins (MAPs) such as Tau, MAP1β and MAP2, involved in microtubule stabilisation [65]. CDK5 and DYRK1A have also been implicated in the abnormal hyperphosphorylation of the microtubule-binding protein Tau, leading to cytoskeletal disruption [66, 67].

Additionally, the enzymatic activities of the tyrosine kinases JAK1, JAK2 and c-FMS were not altered by the N-alkylisatins 6d, 7e, 7f, 7j, 7k, and 7l, even at the highest concentration tested (Table 1). This was further confirmed by cell based screening in the IL-3-independent, TEL-JAK2 and TEL-JAK3 mouse myeloid (Ba/F3) transfected cell lines, whereby all compounds were equipotent on both cell lines, with the exception of the p-tolyl derivative 7l (Table 1). This indicates that the cytotoxic effect is not likely to be occurring through the JAK signaling pathway. Together, these results suggest that despite the structural similarities that the representative N-alkylisatins share with known kinase inhibitors, tubulin may in fact be the primary target [68]. Inability of this class of compounds to inhibit the mitotic and spindle assembly checkpoint kinases, for example CDK1, the aurora kinases, and the polo-like kinases would further support the theory that N-alkylisatin-induced microtubule disassembly is not a secondary phenomenon.

3.4. C2 Substituted Isatin Derivatives

The best known cytotoxic C2-substituted isatin derivative is indirubin (12a), the red component of indigo pigments used since ancient times for dyeing textiles. This compound was obtained from plants belonging to the genii Indigofera, Iastis and Polygonum, as well as from marine mollusces of the Murex genus [70]. Indirubin is the active constituent of a traditional Chinese antileukemia medicine and the lead structure of a class of compounds that display strong antiproliferative activity via ATP-competitive inhibition of both CDK1 and CDK2 (Fig. (8)) [71-73]. A number of modes of action have been ascribed to the indirubins [74]. These include induction of apoptosis through cell cycle arrest at G2/M, as a result of inhibition of GSK3 [69], c-Src kinase and NF-xB activation and expression [75, 76], as well as activation of the aryl hydrocarbon receptor [77, 78]. Based on a range of mechanistic and crystallographic studies of indirubins with GSK3 and CDK2, a vast number of soluble indirubin derivatives have been synthesised and their biological activity and specificity have been evaluated [72, 79]. The indirubin derivatives 12a-k displayed strong inhibitory activity towards CDK2 with IC$_{50}$ values ranging from 2-0.01 µM, with derivatives 12i and 12k also showing potent activity in suppressing the in vitro growth of MCF-7 cells [71, 72]. Some of the early indirubin derivatives were plagued by poor water solubility. However, the addition of basic diamine side chains, as in derivatives 12i and 12k, markedly improved their water solubility. The incorporation of a quaternary centre into the 3'-position of these polar indirubin derivatives further improved water solubility, while retaining potency towards CDK2.

In addition to indirubin derivatives, the marine environment has yielded an array of other novel C2-substituted indoliones including the alkaloid matemone (13, Fig. (9)). Isolated from the Indian Ocean sponge Iotrochota purpurea, matemone was found to intercalate DNA, to inhibit the division of sea-urchin eggs and to exhibit mild cytotoxicity towards a range of human carcinoma cell lines. Further examples of marine-based isatins have been provided by the sea cucumber-associated fungus Aspergillus fumigatus, which was found to produce several novel prenylated indole diketopiperazine alkaloids, including compound 14 and the three spirotryprostatins C-E (15a-c, Fig. (9)). The prenylated indoles were evaluated for their cytotoxicity with derivative 15c exhibiting the most potent activity with IC$_{50}$ values of 2-3 µM against the human non-small cell lung carcinoma (A549), the human acute lymphoblastic leukaemic (MOLT-4) and HL60 cell lines [80].

A range of 2-arylidenedihydroindole-3-ones (16a-f), or azaaurones, were recently evaluated for their antiproliferative and apoptotic abilities against bladder tumours (Fig. (10)). Azaaurone (16c) was the most active compound, which inhibited the proliferation and induced apoptosis apparently via a fibroblast growth factor receptor (FGFR) 3 dependent pathway in DAG-1 and RT112 bladder tumour cell lines [81].

Other pharmacologically important examples of C2-substituted isatin derivatives are indigo carmine (5,5'-indigodisulfonic acid sodium salt), which is widely used as a non-toxic, inexpensive stain for colonoscopic diagnosis and management of early colorectal cancer [82, 83], and the duocarmycins C1 (17) and C2 (18) (Fig. (11)). The duocarmycins are C2-substituted indoles that are amongst the most potent antitumour antibiotics discovered to date and have been reviewed in detail elsewhere [84-86]. A review of the patent literature on small molecule CDK inhibitors covering the period 2001-2004 includes a diverse range of C2-substituted isatin-derived compounds [87], once again highlighting the clinical significance of this important class of molecules.
3.5. C3 Substituted Isatin Derivatives

The susceptibility of isatin to attack by nucleophiles at C3 has resulted in the generation of a large number of 3-substituted isatins, which is reflected by the vast proportion of biologically active 3-substituted indolin-2-ones reported in the literature relative to the other substitution patterns. For example, a variety of 3-phenylhydrazones (e.g. 19) and thiazoles (e.g. 20) have been shown to slow cancer cell cycle progression via inhibition of CDK2 (Fig. (12)) [61]. Similarly, an array of imidazole containing (e.g. 21 and 22) and pyrrole containing (e.g. 23 and 24) 3-substituted isatins inhibit tubulin polymerisation in a range of cancer cell lines, leading to cell cycle arrest at G2/M and eventual cell death (Fig. (12)) [88, 89]. Furthermore, 3-substituted indolin-2-ones have been designed and synthesised as a novel class of tyrosine kinase inhibitors which exhibit selectivity toward different RTKs. Sun et al. found the pyrrole 24 to be highly specific against the vascular endothelial growth factor (VEGF) (Flk-1) RTK and 3-(substituted benzylidene)indolin-2-ones (25 and 26) containing bulky groups in the phenyl ring at the C3 position showed high selectivity towards epidermal growth factor (EGF) and Her-2 RTKs [37].

3.5.1. 3-Arylidene Derivatives

Indolinones were first reported in 1987 to possess kinase inhibitory activity [90]. Many indolinones such as SU5416 (semaxanib, 27) were discontinued in clinical trials due to dangerous side effects [91], although SU11248, (sunitinib, 28), now marketed as Sutent® by Pfizer, was approved by the FDA in January 2006 for the treatment of gastrointestinal stromal cancers and renal cell carcinoma [92]. The drug is a multiple RTK inhibitor, targeting VEGF receptor-2 (VEGFR-2), platelet-derived growth receptor-β (PDGFR-β), stem cell factor receptor (c-KIT), fetal liver tyrosine kinase receptor-3 [91], colony-stimulating factor type 1 and the glial cell-line derived neurotrophic factor receptor [93]. The indolinone scaffold is a well-known c-KIT RTK inhibitor [94]. Substituents at C4/C5 on the indolinone nucleus and substituents on the 3'- and 4'-positions of the pyrrole ring are regarded as favourable for CDK2 inhibition, while substituents in the 5'-position are beneficial for VEGFR-2 and PDGFR-β inhibition [95]. 3-Substituted indolinones possessing the Z configuration are potent inhibitors of the FGFR, while compounds adopting the E configuration inhibit epidermal growth factor receptor (EGFR) [42].
The indolinone SU5402 (29), encompasses a Z configuration about the alkenyl bond (Fig. (13)). Li et al. synthesised 20 new analogues of 29 bearing the E configuration and assessed them for cytotoxicity [96]. The lead compound, containing a methyl ester on the pyrrole group displayed IC₅₀ values of 98 nM and 65 nM against human lung (A549) and breast (MDA-MB-468) carcinoma cell lines after 72 h.

Mitochondrial damage, caspase activation and apoptosis can be induced in a dose- and time-dependent manner by SU9516 (22) in U937 cells [97]. Apoptosis may also be induced in Jurkat and HL60 leukemic cells treated with 10 μM of 22 for 6 h, or with 5 μM for 24-72 h in RKO and SW480 human colon carcinoma cell lines [62]. Pyrrolyllactone and pyrrolyllactam derivatives of 22 have been synthesised and described as potent CDK2 inhibitors, in particular compounds exhibiting a polar substituent in the 4-position [31, 95].

\[
\begin{align*}
R^1 & \quad R^2 \\
27 & \quad \text{Me} \quad H \\
28 & \quad (\text{CH}_2)_2\text{COOH} \quad \text{H} \\
29 & \quad \text{Me} \quad (\text{CH}_2)_2\text{COOH}
\end{align*}
\]

Fig. (13). Examples of isatin-based kinase inhibitors including the clinically employed sunitinib (28).

Imidazole derivatives of 27 have been demonstrated to possess greater inhibition of EGF at 10 μM than 27 itself (40-55% cf. 30% respectively) [98]. A range of 3-(E)-benzylidene-2-indolinones derived from 27 have been synthesised and their SAR has been evaluated. Compounds incorporating a methoxy group at C6 on the isatin moiety resulted in greater potency against PC-3, MCF-7 and MDA-MB-231 cell lines after 72 h compared to derivatives with a hydrogen or hydroxyl group at C6 [99]. Furthermore, the most active compounds displayed inhibition of tubulin polymerisation at the micromolar level. By combining the most potent derivative with the tubulin binding natural product Combretastatin A-4, the novel compound was able to inhibit tubulin polymerisation at a greater rate, and the cytotoxicity was also enhanced (IC₅₀ = 0.7 nM and 0.9 nM against the MCF-7 and MDA-MB-231 cell lines, respectively). Further investigations into the cytotoxic activity of this compound revealed it to be extremely potent with GI₅₀ values of < 10 nM against 46 out of 53 NCI cell lines [100].

SU6668 (30) is efficacious when administered in athymic mice with tumour xenografts, demonstrating over 75% growth inhibition against a spectrum of tumour types [101]. It also significantly suppresses tumour angiogenesis [102], and is capable of inducing substantial tumour regression, regardless of initial tumour size. The drug was in four separate Phase I trials, but was discontinued due to the superior activity of sunitinib.

Sunitinib (28) displays excellent activity against PDGFR-β and VEGFR-2 (IC₅₀ = 2 nM and 80 nM respectively) and is over 30 times more potent against these kinases than the pyrrolo-containing compounds 30 and 33 [103]. It is now the standard first-line treatment of renal cell carcinoma and is generally well tolerated, although side effects such as hypertension and asthenia still occur and are attributed to the inhibition of VEGF receptors [93]. SU14813 (31) has also emerged as a multiple RTK inhibitor with strong antiangiogenic and antitumour activity. It expresses dose-dependent antitumour efficacy and aids in the regression of human acute myelogenous leukemia (MV4;11), as well as human renal carcinoma (786-O), human colon carcinoma (Colo205 and MV522) and rat glioma (C6) [104]. The compound also displays moderate systemic clearance; a short plasma half-life in mice (t₁/₂ = 1.8 h) and inhibition of tumour growth can be improved when the morpholino derivative 31 is combined with the anti-mitotic chemotherapeutic, docetaxel.

Islam et al. found that indolinones possessing a pyrrole ring, similar in structure to the SU series of compounds (Fig. (13)), are inhibitors of the cAkt2 protein kinase, which is activated by phosphoinositide-dependent kinase-1 through the support of phosphoinositide 3-kinase activity in cells [105]. Activity towards the cAkt2 kinase could be increased almost 400-fold through the addition of a urea group at C5 on the isatin nucleus and a methyl group on the alkene bond at C3. The novel compound named BX-517, was optimised by exploring the C4’ position on the pyrrole ring and lead to potent phosphoinositide-dependent kinase-1 inhibitors with superior pharmacokinetic properties compared to BX-517 [106].

A plethora of other indolinones including 4-cyclicamino and 4-alkynyl-oxindoles have exhibited CDK2 inhibitory activity and potent cytotoxicity, as reviewed by Harris [31]. Indolin-2-ones and azo-indolinones containing a variety of ring systems have been reported to be potent nanomolar inhibitors of the TrkA RTK and low micromolar inhibitors of CDK2 [107], while indolone-pyridine derivatives have been found to be powerful B/Akt protein kinase inhibitors [108].

The novel indolinones BIBF1000 (32) and BIBF1120 (33) induce apoptosis and are excellent compounds for inhibiting colony formation of human myeloid leukemic cell lines in a dose-dependent manner (Fig. (14)). Both compounds have an IC₅₀ value of 8 nM against the leukemic Kasumi-1 cell line and comparable activity against the leukemic Mono-Mac-1 cell line [109]. BIBF1120 (33), inhibits VEGF, FGF and PDGF receptors, displays efficacy in vivo amongst multiple animal models and is currently in Phase III trials for the treatment of non-small cell lung cancer and other cancers [110].

\[
\begin{align*}
R & \quad \text{R} \\
32 & \quad \text{NMe}_2 \\
33 & \quad \text{N}
\end{align*}
\]

Fig. (14). Examples of the BIBF series of isatin-based kinase inhibitors.

Another 3-benzylidene-indolinone, RPI-1 (34, Fig. (15)), has been shown to inhibit the RET RTK (IC₅₀ value = 0.17 μM) and...
display antiproliferative activity, particularly towards a modified mouse embryonic fibroblast cell line NIH3T3 (NIH3T3<br>\(\text{Men2}^{\text{A}}\)) \((\text{IC}_{50}\) value = 3.6 \(\mu\)M after 72 h) \[111\]. Derivatives containing a diphenyl urea functionality were introduced to the 5,6-dimethoxyindolinone core of RPI-1 as diphenyl urea moieties are known to be potent inhibitors of other kinases including CDKs. The lead compound discovered, \(N\)-methyl-3-aryleidobenzylidene-indolin-2-one \((\text{35})\), displayed greater activity against 3 modified NIH3T3 cell lines but was inactive against the RET RTK, suggesting a separate mode of action \[111\] (Fig. (15)).

\[\text{Fig. (15). The 3-benzylidene indolinone RPI-1 (34) and a potent derivative (35).}\]

An indolinone substituted at C3 with an aminomethylene-lysine derivative \((\text{36})\) Fig. (16) displayed cytotoxicity after 48 h towards human colon (DLD-1) and ovarian (PA-1) carcinoma cell lines, as well as towards murine fibroblast cell lines \((\text{IC}_{50}\) values ranged from 10-17 \(\mu\)M) \[112\]. Compounds containing more rigid side chains derived from D-phenylalanine and L-phenylglycine were found to be inactive. The antitumour activity of 3-imidazothiazole-2-indolinones was investigated by Andreani et al. \[88\]. The most active compound \((\text{37})\) possessed a chloride atom at C5 and was the most potent derivative against all cancer cell lines tested (Fig. (16)). It displayed selectivity towards leukemic cell lines and was more active than the commercially available anti-mitotic agent, vincristine.

\[\text{Fig. (16). Examples of the 3-aminomethylene-lysine (36) and 3-imidazothiazole (37) derivatives.}\]

3.5.2. Hydrazones and Imines

Isatin hydrazones have been explored in detail and evaluated for their CDK2 inhibitory activity. A brominated isatin hydrazone containing a p-sulfonamide functionality exhibited an \(\text{IC}_{50}\) value of 60 nM against the CDK2 enzyme and was initially the lead compound in an SAR study by Bramson et al. \[61\]. Their investigation revealed that lipophilic substituents at C4 or oxazoly1 substituents at C5 increased CDK2 inhibitory activity to the low nanomolar range. Equally potent analogues were discovered by fusing the C4/C5 positions with a heterocycle containing a hydrophobic substituent at C4 and a hydrogen bond acceptor at C5. These fused derivatives, together with a 5-(2,6-dimethoxyphenyl)amide analogue displayed notable potency against an array of human cancer cell lines, with most of the \(\text{IC}_{50}\) values in the sub-micromolar region after 72 h.

A range of 5-substituted isatins with a hydrazone functionality at C3 linked to a substituted quinazolone \((\text{38a-d})\) have been synthesised and assessed for their cytotoxic activity (Fig. (17)) \[113\]. Three compounds \((\text{38a, 38c and 38d})\) displayed comparable cytotoxicity to clinically proven thioguanine and 5-fluorouracil. A di-halosubstituted derivative \((\text{38c})\) displayed the most noticeable effects on renal (UO-31 and RXF 393) and neural (U251) cell lines, with the potency attributed to the electronic nature of the molecule. The two mono-halosubstituted compounds \((\text{38a and 38d})\) were slightly less active than the di-halosubstituted derivative \((\text{38c})\), while the analogue containing no halogens \((\text{38b})\) was the least active of the compounds tested.

\[\text{Fig. (17). Cytotoxic isatin-quinazolinone derivatives.}\]

Recently, isatin-based hydrazones \((\text{39a-c})\) have been identified as inhibitors of the protein tyrosine phosphatase Shp2, which plays an important role in cell signalling, cell proliferation, differentiation and migration (Fig. (18)) \[114\]. Compounds containing a carboxylic acid group on the hydrazone aromatic ring and a p-halosulfonamide at the C5 position \((\text{39a and 39b})\) selectively inhibit Shp2 in the low micromolar region. The most active analogue \((\text{39c})\) exhibited an \(\text{IC}_{50}\) value of 0.8 \(\mu\)M against Shp2.

\[\text{Fig. (18). Isatin-based inhibitors of Shp2.}\]

Isatin aminoacetylhydrazones, such as compound \((\text{40})\) (Fig. (19)) and benzoisatin analogues (for example, \text{41}) have been synthesised and are speculated to be DNA intercalating agents \[30\]. However, none of the isatin aminoacetylhydrazones tested were active against porcine testicular cells after 24 h at 250 \(\mu\)g/mL.
Among the analogous benzoisatin compounds, three exhibited cytotoxic activity and a p-methylpiperazinyl derivative (41) resulted in 100% cell death at concentrations between 120-250 μg/mL.

Fig. (19). Isatin aminoacetylhydrazone (40) and benzoisatin (41) derivatives.

Jurancic et al. [115] have reported the cytotoxic effects of isatin-β-thiocarbonyldrazone (42a) and N-ethylisatin-β-thiocarbonyldrazone (42b) on B16 (murine melanoma), HeLa (human cervical carcinoma) and human peripheral blood mononuclear cell lines after 72 h using the MTT assay. N-ethylisatin-β-thiocarbonyldrazone (42b) was three times more potent against the two neoplastic cell lines than 42a (IC50 = 10.4 μM cf. 34.2 μM for B16 and IC50 = 21.9 μM cf. 61.7 μM for HeLa, respectively). Interestingly, 42a displayed greater cytotoxicity against non-stimulated human blood mononuclear cells than 42b (IC50 = 17.6 μM cf. > 47.0 μM, respectively).

Thiosemicarbazones, a hydrazone derivative containing a sulfur atom, are active against several pox viruses. The β-thiosemicarbzone of N-methylisatin is a prophylactic agent for smallpox [116] and was one of the first antiviral agents used clinically. The thiosemicarbazones of isatin and its derivatives have been reported in recent years to also display cytotoxic activity. Fifteen previously reported N3-substituted isatin-3-thiosemicarbazones have been screened for cytotoxicity against brine shrimp (Artemia salina) by Perverz et al., including compounds 42a-e. (Fig. (20)) [117]. Only the 2-methylphenyl (42c), 2-methoxyphenyl (42d) and 3-nitrophenyl (42e) derivatives displayed significant cytotoxic activity. The most active analogue was 42d with a LD50 value of 11 μM against Artemia salina. Further studies into N3-substituted isatin-3-thiosemicarbazones involving one, two or three phenyl substituents have been performed by the above authors [118]. Eleven of the twelve derivatives contained at least one fluoride atom although, intriguingly, the most active compound was the chloro-substituted analogue (42f) with a LD50 value of 11 μM against Artemia salina (Fig. (20)). The remaining compounds exhibited LD50 values of 16-136 μM.

A variety of 5-nitroisatin and 1-morpholino/piperidinomethyl-5-nitroisatin derivatives containing a thiosemicarbazone group (e.g. 42g-k, Fig. (20)) have been synthesised and reported by Karali [119]. Eight analogues were selected as prototypes and evaluated against the NCI’s full panel of tumour cell lines. All of the compounds tested had comparable cytotoxicities to thioguanine and 5-fluorouracil. Amongst the 5-nitroisatin derivatives, phenyl (42g) and 4-nitrophenyl (42h) substituted thiosemicarbazones were more potent than their alkyl (42j) counterparts. In addition, the 1-morpholinomethyl compounds generally exhibited greater cytotoxicity than the unsubstituted compounds. The lead compound contained a 4-chlorophenyl substituted thiosemicarbazone (42k) and was significantly more active than thioguanine and 5-fluorouracil against non-small cell lung cancer and leukemia cell lines [119].

Further investigations into biologically active thiosemicarbazones have led to the synthesis of a range of 5-bromo-3-thiosemicarbazone isatins (e.g. 42l and 42m, Fig. (20)), including heterocyclic thiazolidinone and imidazolidinedione moieties [120]. The introduction of a bromine atom at the C5 position of the isatin ring is known to increase the anticancer activity of the parent molecule [121]. Four model compounds were selected after preliminary screening to be further evaluated against the NCT’s full panel of tumour cell lines. All of the compounds reported here were found to possess at least comparable cytotoxicity to thioguanine and 5-fluorouracil, with the 4-fluorophenyl and 4-nitrophenyl thiosemicarbazone derivatives 42l and 42m showing even greater levels of cytotoxicity.

C3 carbonyl substitution with an imine functionality of the N-alkylated fluorouracilone anti-bacterial isatin derivative, gatifloxacin (which is now banned), gave rise to one compound, containing a sulfonamide attached to pyrimidine (43a) (Fig. (21)) with a GI50 value of 0.269 μM against the human colon carcinoma cell line Colo205 [122]. Additionally, 43a was >20 times more active than the chemotherapeutic drug etoposide against this and the human renal carcinoma cell line A498. A similar derivative, incorporating a 4.5-dimethoxy pyrimidine (43b) displayed a GI50 value of 0.176 μM after 48 h, and was 85 times more potent than etoposide against the non-small lung carcinoma cell line HOP-92.

3-Imino-substituted indolines bearing amino acids have been investigated as kinase inhibitors and cytotoxins. Imino groups incorporating valine (44a and 44b) (Fig. (22)) and threonine (45a and 45b) residues proved to be inactive against the CDK1/cyclin B, CDK5/p25 and GSK-3α/β protein kinases [123]. Conversely, histidine imino groups (46a and 46b) increased kinase activity and subsequent halogenation at C5 on the isatin core yielded extremely potent kinase inhibitors, with the lead compound, a 5-bromo derivative (46b), displaying an IC50 value of 0.37 μM against the CDK5/p25 kinase. Interestingly, none of the analogues synthesised displayed cytotoxicity towards human breast or lung carcinoma or towards a human glioblastoma cell line (SF-268 cells), although
this may be attributed to the presence of the carboxylic acid functionality which may inhibit the entry of the compound into tumour cells.

Fig. (21). Gatifloxacin-isatin derivatives.

Additionally, novel bis-Schiff bases of 5-fluoroisatin (3d) such as 3,3’-[oxybis(4,1-phenylenenitrilo)]bis(1,3-dihydro)-5-fluoro-2H-indol-2-one have been found to be cytotoxic towards the human embryonic cell line (HEL) while a dichlorinated derivative, 3,3’-[methylenebis(2-chloro-3,5-diethyl-4,1-phenylenenitrilo)]bis(1,3-dihydro)-2H-indol-2-one, displayed cytotoxicity at 16 µg/mL against African green monkey kidney (Vero) and HeLa cell lines.

Fig. (22). Imino-isatins incorporating the amino acids valine (44), threonine (45) and histidine (46).

3.3.5. Metal Complexes

Due to cisplatin and other platinum drugs being used clinically for the treatment of several different tumour types, other metal-based compounds have been investigated in order to find drugs with fewer side effects and greater efficacy. Copper-based anti-cancer drugs have been researched thoroughly with the premise that drugs containing endogenous metals may be less toxic to the human body [125]. Copper-induced apoptosis and necrosis has been reported in the gills of fish and the effects can be enhanced when administered in conjunction with other agents. These complexes have the ability to trigger oxidative stress by generating reactive oxygen species, which attack other biomolecules during the oxidation of endogenous substances [32, 126]. Numerous oxindole-copper complexes were reviewed by Cerchiaro and Ferreira in 2006 [32].

Two isatin–diimine copper complexes, Cu(isapn) (47) (Fig. (23)) and Cu(isaepy) 2 (48), have been evaluated against SH-SY5Y neuroblastoma, M14 melanoma and U937 cell lines and were found to induce pro-apoptotic activity involving the mitochondrial pathway and/or copper-dependent oxidative stress [127]. The compounds induce cell cycle arrest by strongly activating the proteins p53 and p21, suggesting this pathway is activated in response to cell damage. Apoptosis of these copper complexes is also caspase-dependent and this seems to be the principal mechanism of cell death [127].

Bis-isatin thiocarbohydrazones have recently been prepared and coordinated to Cu(II) (49a) , Ni(II) (49b), Co(II) (49c) and Zn(II) (49d) ions [128]. All compounds tested displayed cytotoxic activity after 24 h in the brine shrimp lethality bioassay and produced 50% cell death at or below a concentration of 19.1 µg/mL against Ehrlich ascitic carcinoma (EAC) cells. In vivo studies involving female Swiss Albino mice inoculated with EAC cells revealed that all the compounds reduced weight gain, prolonged the mean survival times and reversed the tumour-induced increase in white blood cell counts compared to the control [128].

Isatins containing sulfonamides through an imine or hydrazone linkage have been complexed with Co(II) (for example compound 50a) (Fig. (24)), Ni(II) (example 50b), Zn(II) (example 50c) and Cu(II) (example 50d) ions [129]. By evaluating the complexes using the brine shrimp bioassay, only three complexes (one Ni(II) and two Cu(II)) displayed potent cytotoxicity. The maximum activity observed was a LD50 value of 156 nM.

Ruthenium compounds are known to be stable and have predictable structures in solution and solid state, for this reason are considered to be potential alternatives to platinum-based anti-cancer drugs [130]. The ligands isatin-3-(4-chlorophenyl)thiosemicarbazone (icpl, 51) (Fig. (25)) and N-methylisatin-3-thiosemicarbazone (nmnt, 52) were prepared and complexed to ruthenium-1,10-phenanthroline and 2,2’-bipyridine derivatives through the sulfur atom and imine nitrogen atom [131]. The complexes were tested in vivo against EAC bearing mice and found to increase the life span of the mice by 57-66%. Further investigations by these researchers included the synthesis of the ligand isatin thiosemicarbazone (itsz, 53) and subsequent chelation to ruthenium-1,10-phenanthroline and 2,2’-bipyridine derivatives [130]. The compounds were tested in vitro against leukemic human (MOLT 4/C8 and CEM) and murine (L1210) cell lines. The ruthenium complexes displayed greater activity than their ligands and the 1,10-phenanthroline derivative was the more active of the two with an IC50 value of 3.9 µM against the CEM cell line. Once again, these compounds were evaluated in vivo against EAC bearing mice and found to increase life span and mean survival time compared to the control.

3.5.4. Isoindigo and Derivatives

The isomer of indinium (12a) bearing two indolino moieties is known as isoindigo (54). Glycosyl-isoindigo derivatives (55) have been prepared containing benzyl or acetyl protecting groups on the sugar residue (Fig. (26)) [132]. Protected analogues are speculated to improve cellular penetration and thus, display enhanced biological activity compared to the corresponding non-protected compounds. According to Sassatelli et al., acetylated glycosyl-isoindigo derivatives were more cytotoxic against a panel of human solid tumour cell lines than the analogous benzylated compounds [132]. Further investigations revealed isoindigo (54) was almost inactive towards 10 kinases tested, although benzylated glycosyl-
isoindigos were active especially towards CDK2 (IC_{50} values typically between 0.1-0.2 \mu M) which suggests the protected sugar functionality positively interacts with the ATP-binding sites of the enzymes [133]. Recently, compounds integrating a 7'-azaisoindigo scaffold (56) were synthesised for the first time (Fig. (26)) [134]. These compounds displayed antiproliferative properties against the human buccal carcinoma cell line KB, and exhibit micromolar IC_{50} values against this cell line after 72 h, indicating that the presence of an additional nitrogen atom to the isoindigo nucleus improves cytotoxicity.

Another isoindigo, N-methyl-isoindigotin, more commonly known as meisoindigo (57) was first synthesised in 1982 [135] and was approved by the Chinese government in 2001 for the treatment of chronic mylogenous leukemia (CML) (Fig. (26)) [136]. When combined with hydroxyurea, 57 substantially prolonged median duration of chronic phase and median survival in CML patients compared to meisoindigo and hydroxyurea alone [137]. Meisoin- digo (57) displays a superior side effect profile and increased efficacy compared to indirubin (12a) [138]. These properties may be attributed to meisoindigo’s increased bioavailability and water solubility [136]. In vitro, 57 induces apoptosis in the transformed human vein endothelial cell line, ECV304 [139], and inhibits the growth of NB4, U937, KG1a, K562 and Jurkat leukemic cell lines in a dose- and time-dependent fashion. In animal models meisoindigo (57) is capable of significantly inhibiting Lewis lung carcinoma and Walker carcinosarcoma 256, inhibiting DNA and RNA synthesis in W256 cells and hindering microtubule assembly [138].

Compounds incorporating a spirocycle at C3 on the isatin moiety (often called spiro-oxindoles) are well known for their biological activities and the scaffold is common in alkaloids such as (-)-horsfiline (58), (+)-elacomine (59) [140] and the Gelsemium alkaloids such as 14-acetoxygelsenicine (60) which has an EC_{50} value of 0.25 \mu M against the A431 human epidermoid carcinoma after 48 h (Fig. (27)) [141]. Recently, spiro-oxindoles integrating silyl functionalities have been synthesised [142]. Two of these compounds, one including a tricyclic isatin core 61 and the other a N-benzylisatin derivative 62, were evaluated against human non-small
cell lung adenocarcinoma (A579) and human hepatocellular carcinoma (HepG2) cell lines (Fig. 27). Both compounds displayed comparable cytotoxicity towards the A579 cells after 24 h (EC$_{50} = 42.8$ μM and 42.6 μM respectively). However, when assessed against the HepG2 cell line, 62 exhibited twice the activity of the compound 61 (EC$_{50} = 16.8$ μM and 32.5 μM, respectively).

![Fig. (26). Cytotoxic isindigo derivatives.](image)

Of twenty spiro-oxindoles containing an isoxazolidine ring system, three compounds, 63-65 (Fig. 27), inhibited MCF-7 cell invasion at 10 μM, although no activity was apparent at 1 μM [143]. Yong et al. tested a comparable compound (66) and reported it to be cytostatic with a GI$_{50}$ value of 2.6 μM against MCF-7 cells [144].

Another class of oxindoles which have been evaluated for their anti-proliferative activity are the 3,3-diaryloxindoles (Fig. 28). Natarajan et al. found that this group of compounds deplete intracellular Ca$^{2+}$ stores leading to inhibition of translation initiation [145]. The parent compound, 3,3-diaryloxindole (67a) displayed a GI$_{50}$ of 13 μM against a human lung cancer cell line. By substituting the 5-position on isatin with iodine and sulfonic acid, the activity increased to 8 μM and 9 μM, respectively. The greatest activity was noticed with α-hydroxy and m-t-butyl substituents on the same phenyl ring (67b) resulting in a GI$_{50}$ value of 3 μM. N-Substitution of the parent compound (67a) with alkyl, aryl and electron-withdrawing groups eliminated Ca$^{2+}$ depletion activity and hence no growth inhibitory activity was observed.

A derivative of 3,3-diaryloxindole is oxyphenisatin (67c). Oxyphenisatin acetate was used as a laxative for 40 years before its withdrawal from the Australian and American markets in the 1970s due to hepatotoxicity [146]. No research had been conducted into oxyphenisatin for almost 20 years, until 2007 when Uddin et al. published data showing that the compound and its derivatives were potent antiproliferatives, particularly against the human breast carcinoma MDA-MB-468 cell line [147], a cell line which expresses a high number of EGF receptors. Oxyphenisatin (67c) itself has an IC$_{50}$ of 112 nM against MDA-MB-468 cells. The two hydroxyl groups on 67c are pertinent for activity, and cytotoxicity can be increased (to less than 10 nM) by replacing small and lipophilic substituents in the 6- and/or 7-position of the isatin moiety. The lead analogue was the 6,7-difluoroxyphenisatin which displayed an IC$_{50}$ of 3 nM against MDA-MB-468 cells [147].

![Fig. (27). Examples of biologically active spiro-oxindoles.](image)

### 4. CONCLUSIONS

Isatin is the core nucleus of an array of cytotoxic and anti-neoplastic compounds. A SAR summary for the isatin derivatives discussed in this review is shown in Fig. (29). The mono-di- and tri-aryl ring substituted isatin series formed from derivatisation at X (see section 3.2) is generally found to induce cancer cell death via apoptosis in the mid-low micromolar range and necrosis in the high micromolar range. This is proposed to be linked to a reduction in
ERK activity. Small electron withdrawing groups at positions 5, 6 and/or 7 enhance, but are not essential for, anti-tubulin or anti-kinase activity when found as part of a larger substituted compound (i.e. substitution at X with concomitant substitution at W, Y and Z). This is most likely due to increased cell permeability and hydrophobicity.

**Fig. (28).** Examples of 3,3-diaryloxindoles.

W
- Pyrrole and imidazole groups at W inhibit RTKs and tubulin polymerization, albeit non-specifically.
- Carbonyl at W required (when Y = N-alkyl or N-arylenebenzene) for specific disruption to microtubule dynamics.
- Hydrazones, imine or hydrazide at W results in specific and potent, CDK and RTK inhibition.

X
- Small electron withdrawing groups at X preferred, mono-, di- and tri-halogenation optimal at positions 5, 6 and/or 7.
- Substitution at X is not detrimental for activity.

Y
- 1 – 3 carbon chain and an aromatic ring at Y essential for microtubule destabilisation.
- Small electron withdrawing substituents on the ring preferred.
- *Para* or *meta* substitution favoured over ortho.

Z
- Dimers at Z results in selective GSK3 and CDK1 & 2 inhibitors.
- Compounds containing styrenes with electron withdrawing groups at Z inhibit RTKs.

**Fig. (29).** A cytotoxicity structure-activity summary for isatin derivatives discussed in this review.

N-Alkylation at Y however, with no further substitution at W or Z, often results in cytotoxic compounds with sub-micromolar activity; inducing morphological change, G2/M cell cycle arrest and ultimately cell death via apoptosis (see section 3.3). The compounds discussed in this review appear to be tubulin specific and do not inhibit serine/threonine or tyrosine kinases (see Table 1). A 1-3 carbon chain linker extending to an aromatic ring is optimal for potent microtubule disruption and small electron withdrawing substituents on the ring in the *para* or *meta* position are favoured over the *ortho* position. Most importantly, and together with its specificity, this series of novel molecules are >10 times more active than the conventional cancer chemotherapeutics; 5-fluorouracil, vinblastine and paclitaxel on human monocytic lymphoma cells. Together with the possibility that these compounds may bind to a novel site on tubulin, it suggests that the development of anticancer agents based on the N-alkylisatin scaffold may be beneficial in combination with clinically used anticancer agents for the treatment of multi-drug resistant tumours in the future. Further development and preclinical assessment of this particular series of isatins is therefore warranted.

Substitution at W has resulted in the generation of by far the most structurally and biochemically diverse isatin-based compounds to date (see section 3.5). Yet despite over 1500 cytotoxic C3 substituted compounds reported in the literature, only one has made it to market as an anti-cancer drug. This is primarily due to their non-specific toxicity, as these molecules often target the ATP binding site of multiple kinases, therefore affecting a magnitude of cellular targets. For example, substitution at W on the 3'- and 4'-positions of the pyrrole ring of some arylidine derivatives is regarded as favourable for CDK2 inhibition, while substituents in the 5'-position are beneficial for VEGFR-2 and PDGFR-β inhibition. 3-Substituted indolamines possessing the Z configuration are potent FGFR inhibitors, while compounds adopting the E configuration inhibit EGFR. Despite this, toxic related side effects of C5 substituted isatins may be overcome in the future through the use of a targeted or ligand directed prodrg approach. This promising new strategy would specifically deliver a potent class of isatin-based molecules to cancer cells *via* receptor mediated endocytosis and concentrate it within these cells only after site specific cleavage, ultimately reducing toxicity. Alternatively, further SAR studies may identify sites and substituents necessary to confer target specificity.

Finally, dimerisation at Z often results in ATP-competitive inhibition of CDK1, CDK2 and GSK3, as well as reduction in the activation and expression of c-src kinase and NF-κB (the most renowned modulators being the indirubins, see section 3.4). Although the early indirubin derivatives were plagued by poor water solubility, the addition of basic diamine side chains and the incorporation of a quaternary centre into the 3'-position markedly improved their water solubility, while retaining potency towards CDK2. Such small modifications have led to large advances in the development of this class of molecules as potential new anticancer agents. In general the C2 substituted class of isatins is active in the micro to nanomolar range and extend from kinase inhibitors to potent anticancer antibiotics.

In summary, isatin has already proven to be an excellent scaffold for both the natural and synthetic construction of molecules with interesting biological activities. With the possibility of deriving the N1, C2 and C3 positions, along with substitution on the aromatic ring, the synthetic permutations for isatin are almost endless. Despite the fact that the isatins are well studied compounds, new derivatives are continually being discovered and known isatin-based compounds are constantly being rediscovered in terms of their biological activity. Isatins, in general, are therefore still worthy of further exploration for the purpose of discovering new and exciting molecules with anticancer activity.

**ACKNOWLEDGEMENT**

The studies of the inhibitory activities of five N-alkylisatin against JAK1, JAK2, and c-FMS/CSF-1-R were carried out by Dr. Christopher Burns and colleagues at Cytopia Research Pty Ltd. (Melbourne, Australia). Inhibitory assays against GSK-3, CDK5 and DYRK1A were performed by Olivia Lozach in collaboration with Dr. Laurent Meijer at the CNRS Station Biologique (Roscoff, France). We are grateful to the University of Wollongong for support through the Institute for Biomolecular Science (IBS), a URC Small Grant and a University Cancer Research Grant. We also thank the Illawarra Cancer Carers Inc., Kiama, Minnamurra, and Gerringong Sunrise Rotary, the Robert East Memorial Fund, Prof. P. Clingan, and other private donors for funding assistance. Assoc. Prof. M. Ranson is a recipient of a Cancer Institute NSW Fellowship award.
ABBREVIATIONS

Only abbreviations that appear more than twice in the text are listed here

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>A549</td>
<td>Human non-small cell lung adenocarcinoma</td>
</tr>
<tr>
<td>CDK</td>
<td>Cyclin dependent kinase</td>
</tr>
<tr>
<td>c-KIT</td>
<td>Stem cell factor receptor</td>
</tr>
<tr>
<td>CML</td>
<td>Chronic myelogenous leukemia</td>
</tr>
<tr>
<td>EAC</td>
<td>Ehrlich ascitic carcinoma</td>
</tr>
<tr>
<td>EGF/R</td>
<td>Epidermal growth factor/receptor</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-related protein kinase</td>
</tr>
<tr>
<td>FGFR</td>
<td>Fibroblast growth factor/receptor</td>
</tr>
<tr>
<td>GI50</td>
<td>Concentration required to inhibit the growth a cellular population by 50%</td>
</tr>
<tr>
<td>GSK3</td>
<td>Glycogen synthase kinase 3</td>
</tr>
<tr>
<td>HeLa</td>
<td>Human cervical carcinoma</td>
</tr>
<tr>
<td>HL60</td>
<td>Human promyelocytic leukemia cell line</td>
</tr>
<tr>
<td>IC50</td>
<td>Concentration required for 50% inhibition of a biological or biochemical process in vitro</td>
</tr>
<tr>
<td>Jurkat</td>
<td>Human leukemic T-cell</td>
</tr>
<tr>
<td>LD50</td>
<td>Median lethal dose</td>
</tr>
<tr>
<td>MAP</td>
<td>Microtubule-associated protein</td>
</tr>
<tr>
<td>MCF-7</td>
<td>Human non-metastatic mammary gland adenocarcinoma</td>
</tr>
<tr>
<td>MDA-231</td>
<td>Human metastatic mammary gland adenocarcinoma</td>
</tr>
<tr>
<td>MTS</td>
<td>[3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt]</td>
</tr>
<tr>
<td>MTT</td>
<td>[3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]</td>
</tr>
<tr>
<td>NCI</td>
<td>National Cancer Institute</td>
</tr>
<tr>
<td>PC-3</td>
<td>Human prostate adenocarcinoma</td>
</tr>
<tr>
<td>PDGF/R</td>
<td>Platelet-derived growth/receptor</td>
</tr>
<tr>
<td>RTK</td>
<td>Receptor tyrosine kinase</td>
</tr>
<tr>
<td>SAR</td>
<td>Structure activity relationship</td>
</tr>
<tr>
<td>SF-268</td>
<td>Human glioblastoma</td>
</tr>
<tr>
<td>SH-SY5Y</td>
<td>Human neuroblastoma</td>
</tr>
<tr>
<td>U937</td>
<td>Human monocye-like histiocytic lymphoma</td>
</tr>
<tr>
<td>VEGF/R</td>
<td>Vascular endothelial growth factor/receptor</td>
</tr>
<tr>
<td>WST-1</td>
<td>[2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt]</td>
</tr>
</tbody>
</table>

REFERENCES

Cytotoxic and Anticancer Activities of Isatin and Its Derivatives


Cytotoxic and Anticancer Activities of Isatin and its Derivatives


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Silibinin might impede proliferation and invasion of MDA-MB-231 cells through enhancing the mRNA expression of Lipocalin-2 and thus, impinging on phosphorylation and activation of ERK 1/2. Furthermore, silibinin may cramp cathepsin B-dependent invasion through induction of mRNA levels of cystatin M. Activation of procaspase 3 by silibinin may trigger caspase3-mediated cleavage of NHE1 and subsequently, induction of apoptosis in MDA-MB-231 cells.

C09 GENERATION OF FUSION PROTEIN FUSED TO HBCAG AND ANTI-TUMOR EFFECT IN VIVO

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Background: The epidemiological gender receptor (EGER) play an important role in tumor cell proliferation, differentiation and survival. The type variant III (EGFRvIII) is the most common variation of EGER and is correlated with tumor progression and poor survival. Because EGERvIII is pure in high frequency in several different tumor type and has not been detected in normal tissues, it is an ideal target for tumor specific therapy.

Methods: Using genetic engineering technology, we developed the recombinant fusion protein by inserting foreign epitope, encoding mutant region of EGERvIII (LEEKGKNNYVVDTH) into the immunodominant el loop of the HBcAg. Next, we immunized BALB/c mice with fusion protein, then detect antibody titer in sera. ELISPOT was used to evaluate tumor-specific IFN-γ-secreting cells after vaccination. Using Renca-vIII(+) cells as specific stimulator, cytotoxic activity of splenocytes was examined. BALB/c mice were challenged with Renca-vIII(+) cells after immunization and the protective immune responses against the tumor cell were evaluated.

Results: Pep3-HBcAg/pET-28a(+) recombinant plasmids was constructed successfully as expected. EGERvIII-HBcAg fusion proteins were adequately expressed in Escherichia coli BL-21 (DE3). Western blot analysis demonstrated that the fusion protein could selectively combined with EGERvIII-specific antibody. Using ELISA, we found that the titers of anti-fusion protein antibody in mice immunized with fusion protein were much higher than those immunized with HBcAg or PBS. Further observation showed fusion protein induced a high frequency of IFN-γ-secreting lymphocytes. CD4+ T cells rather than CD8+ T cells were associated with the production of IFN-γ-secreting lymphocytes. Using Renca-vIII(+)-secreting lymphocytes as specific stimulator, we observed remarkable cytotoxic activity in splenocytes from mice immunized with fusion protein, which further indicate that anti-tumor effect of fusion protein is EGERvIII-specific. In vivo study, fusion protein showed resistance to tumor development. These findings demonstrate that EGERvIII-HBcAg fusion protein triggered a protective response against tumor expressing EGERvIII.

Conclusion: In this study, we successfully prepared the EGERvIII-HBcAg fusion protein. Immunization of animals with fusion protein stimulates a Ag-specific humoral response, and confers protective immunity to tumor challenge of EGERvIII(+) tumor cells. We hope our approach will be helpful to the further research into a viable practical tumor vaccine.

C10 PROMISING IN VITRO ANTITUMOR PROPERTIES OF NEWLY SYNTHESIZED CAMPTOTHECIN GLUCURONIDE PRODRUG FOR TARGETED TUMOR THERAPY BY ENZYME/PRODRUG STRATEGY

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Systemic toxicity caused by non-selective mode of action, poor water solubility and interaction with human serum albumin are major serious problems with existing camptothecin drugs. We previously successfully solved most of the problems conjugating camptothecin derivative 9AC with glucuronic acid via a self-immolative linker to obtain a prodrug suitable for enzyme/prodrug tumor targeting therapy. Despite excellent results on animal models, interactions with HSA remained an obstacle for its use in humans. To overcome this problems an experimental anticancer camptothecin derivative 9AC with glucuronic acid via a self-immolative linker to obtain a prodrug suitable for enzyme/prodrug tumor targeting therapy. C10 was most active among the drugs tested against C26 with IC50 ~ 10 - 20 nM. C10 was effective against a panel of tumor cells including H1299 and A549 with IC50 ~ 10 - 20 nM. C10 was effective against a panel of tumor cells including H1299, A549 and DU145 with IC50 ~ 10 - 20 nM.

C11 PH-TRIGGERED RELEASE OF POTENT ISATIN-DERIVED CYTOTOXINS CONJUGATED TO THE TUMOR TARGETING PROTEIN PAI-2

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Introduction: N-Alkylated derivatives of 5,7-dibromosiamin, in particular those containing N-benzyl, N-phenethyl and N-naphthylmethyl moieties (e.g. 1), have been shown to possess sub-micromolar cytotoxicity in vitro against a diverse panel of human cancer cell lines. To increase both the availability of these drugs at their target site and to reduce unwanted side effects, the isatin derivatives were conjugated to free lysine on the targeting protein PAI-2 through an acid labile lysine linker. In order to model the pH–triggered release mechanism of the cytotoxins the isatin derivaties were first conjugated to a single lysine amino acid residue and the hydrolysis of these model conjugates were assessed.

Methods: Five structurally different isatin-lysine conjugates were synthesized using standard peptide coupling techniques. The model conjugates were monitored for hydrolysis by UV-Vis spectrophotometry over a 4 hour period at 37°C at both lysosomal and endosomal pH (4-5), while retaining stability at physiological pH. The isatin conjugates with favourable kinetic profiles were then linked to PAI-2 via its free lysine residues and their hydrolysis monitored by UV-Vis spectrophotometry at various pH conditions. The isatin-PAI-2 conjugates were also examined in vitro against U937 and THP-1 leukemic cell lines for evidence of internalisation and hydrolysis.

Results: All five model isatin-lysine conjugates were capable of cleaving and releasing the free drug under mildly acidic conditions (e.g. pH 5.0-5.5), with varying degrees of stability at physiological pH. Extension of the imine chain length, such as with a C3-propionic acid linker increased stability at pH 7 and was therefore chosen as the linker for the isatin-PAI-2 conjugate. The C3-linked isatin-PAI-2 conjugate displayed the most promising cell assay results, hydrolysis characteristics, and kinetic profile of all the complexes examined.

Conclusions: The results demonstrate that potent isatin-based cytotoxins can be successfully conjugated to free lysines residues on the tumour targeting agent PAI-2 through acid-labile imine linkers. These isatin-PAI-2 conjugates, while displaying stability at physiological pH, are subsequently cleaved under mildly acidic conditions such as endosomal and lysosomal pH. These results strongly suggest that isatin-PAI-2 conjugates warrant further development as a potential new and effective targeted anticancer treatment.

C12 EVALUATION OF ANTITUMOR ACTIVITY AND MOLECULAR MECHANISM OF PENTACYCLIC TRITERPENOID LANTADENE A

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Lantadene A [LA, 22R]-angeloyl-3- oxoolean-12-en-28-acid] a pentacyclic triterpenoid isolated from leaves of theousus weed Lantana camara L. was evaluated for its antitumor activity. LA showed apoptosis in human leukemia HL-60 cell line. Typical morphological changes including cell shrinkage, chromatin condensation and characteristic DNA ladder formation in agarose gel electrophoresis were observed. LA induced marked concentration and time dependant inhibition of cancer cell proliferation with IC50 value of 19,310.10 µg/ml following 48th incubation. Flow cytometric analysis showed suppressed cell proliferation associated with cell cycle arrest in the G0/G1 phase. LA significantly inhibited cell proliferation of HL-60 cells and induced cell apoptosis through down regulating Bcl-2 and up regulating Bax expression. The peptide caspase-3 inhibitors z-DEVD-CHO (NHE2-Asp-Glu-Val-Asp-CHO, 2µM), increased the viability of HL-60 cells, previously treated with LA. The LA was further evaluated for its tumor suppression potential in DMBA/TPA induced squamous cell carcinoma in Swiss albino mice. A significant decrease in the incidence of number of lesions in mice was observed in LA treated groups as compared to DMBA/TPA alone. The RNA of tumors were isolated, after 20 weeks study and evaluated for various signaling factors like p53, p65 and c-jun. Significant increase in the protein levels of c-jun, p65 and p35 by ELISA were observed in DMBA/TPA treated mice tumors whereas less expression was observed in LA treated tumors.
Synthesis and hydrolytic evaluation of acid-labile imine-linked cytotoxic isatin model systems

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ABSTRACT

In this study a series of isatin-based, pH-sensitive aryl imine derivatives with differing aromatic substituents and substitution patterns were synthesised and their acid-catalysed hydrolysis evaluated. These derivatives were functionalised at the C3 carbonyl group of a potent N-substituted isatin cytotoxin and were stable at physiological pH but readily cleaved at pH 4.5. Observed rates of hydrolysis for the embedded imine-acid moiety were in the order para-phenylpropionic acid > phenylacetic acid (para > meta > benzoic acid (meta > para)). The ability to fine-tune hydrolysis rates in this way has potential implications for optimising imine linked, tumour targeting cytotoxin–protein conjugates.

1. Introduction

A promising strategy for anti-cancer drug delivery involves conjugating a cytotoxin with a tumour targeting protein through an acid-labile linker that is stable at physiological pH.4–14 Internalisation at the target tumour site via processes such as receptor mediated endocytosis5 exposes the conjugate to the acidic environment of the endosomes or lysosomes (pH 4.5–6.0), resulting in selective release of the original cytotoxin inside the tumour cell.4,5

Over the years, a wide variety of functional groups have been employed in the development of linkers that are stable at physiological pH (7.4), but cleave at endosomal/lysosomal pH including acid-sensitive hydrazones,6–9 cis-aconityl groups,10 trityl groups,4,11 orthoesters12 and N-ethoxybenzylimidazoles.13 In particular, the antineoplastic anthracenyl doxorubicin and daunorubicin have been conjugated through hydrazone and cis-aconityl linkers.4,6,10,14,15 The hydrazone linker has, however, been reported to cleave at non-target sites16 and at pH 7.4,17 which suggests that this linker may be unstable in the circulatory system. Other less commonly employed acid-labile linkers include acetics,18,19 ketals,19 oximes20 and imines.21,22

The imine group as part of a selective acid-sensitive linker to tumour targeting proteins was of potential interest in our work on potent isatin-based cytotoxins.23–25 The cytotoxicities against U937 lymphoma cells of these compounds, together with that of isatin itself for comparison purposes, is shown in Figure 1.

The most potent isatin-based cytotoxins we have developed are derivatised on both the aromatic ring and via N-substitution.23–25 Therefore the aim of this study was to generate a pH-sensitive linker that did not interfere with these areas of the molecule, but instead capitalised on the available carbonyl group at C-3, which is present in all of our isatin-derived cytotoxins. This could be achieved using an imine linker. Although imine linkers are not widely employed in drug delivery,26 they have gained attention in recent years with several studies showing that aromatic imines with extended π–π conjugation are stable at physiological pH, while readily hydrolysing in mildly acidic solutions (e.g., pH 4.5–6.8).27–31

Figure 1. Cytotoxic activity of isatin and its derivatives against the human monocyte-like histiocytic lymphoma U937 cell line.23–25

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Thus, reaction of our isatin-derived cytotoxins (e.g., 1) with a bifunctional linker such as the anilino carboxylic acids 2a–e, would give the aryl imine linked cytotoxins 3a–e shown in Scheme 1. These compounds, with a free carboxylic acid available for potential coupling via amide formation to the exposed lysine residues on a suitable tumour targeting protein, would be expected to be stabilised by extended resonance delocalisation. Furthermore, acid-catalysed hydrolysis of the imine-linked cytotoxins should result in selective release of the original isatin (1). This would be an improvement over some of the existing pH-sensitive bioconjugation strategies, where hydrolysis of the linker generates a modified derivative of lower potency than the original cytotoxin.13

The benchmark chemical studies required to establish the imine group as a feasible cytotoxin linking option for isatins, together with related model work, are now presented in this paper. In particular, we have prepared the aryl imine derivatives 3a–e of the cytotoxic isatin (1) via reaction with the bifunctional, acid–amine linkers (2a–e) (Scheme 1). The carboxylic moieties in 3a–e were then used to react with the ε-amino group of an Nα,Nε-protected lysine derivative to afford the amides (4a–e), as a preliminary model of such reactions with protein based lysine residues. The hydrolytic efficiencies of the imines (3a–e) and amides (4a–e) were evaluated using UV–vis spectrophotometry. Herein, we report the first instance of the pH-dependent selective release of a potent N-alkylisatin derived cytotoxin through the acid-catalysed hydrolysis of an aryl imine linker.

2. Results

2.1. Synthesis

The five novel aryl imine derivatives 3a–e were readily prepared by the acid-catalysed reaction of the brominated cytotoxin, N-para-methoxybenzylisatin cytotoxin (1), with a range of commercially available anilino carboxylic acids (2a–e), differing in terms of their aromatic substitution patterns and by the length of the linker between the aromatic ring and the carboxylic acid moiety (Scheme 1). For this study, para- and meta-benzoic (2a, 2d), para- and meta-phenylacetic (2b, 2e) and para-phenylpropionic (2c) anilino carboxylic acids were selected. The isatin 1 and the appropriate acids 2a–e were then heated at reflux in MeOH or EtOH together with a catalytic amount of AcOH for 1.5 h to yield the imines 3a–e. Reactions in this study proceeded in moderate to high yield, with the phenylacetic and phenylpropionic acids (e.g., 3b, 3c, 3e) affording higher yields. Imines 3b–e were produced in higher yields with the higher boiling EtOH rather than MeOH, while 3a was the only imine to favour MeOH over EtOH. Lack of the AcOH catalyst led to a decrease in chemical yield. There was also no evidence, as seen by 1H NMR spectroscopy, (DMSO-d6) of self-cleavage of the imines 3a–e to the free isatin 1.

Imines 3a–e were obtained as mixtures of E and Z isomers about the imine bond and could not be separated by either thin-layer chromatography or HPLC, as has been reported for other 3-arylimino-2-indolinones.12 1H NMR spectra of the imines 3a–e in DMSO-d6, revealed that most compounds (3a–d) were synthesised

![Scheme 1. Reagents and conditions: (a) EtOH, AcOH (cat.), reflux, 1.5 h, 18–71%; (b) DCC, HOBt, DIPEA, Ac-Lys-OMe, CH₂Cl₂, rt, 24 h, 41–82% (PMB = para-methoxybenzyl).](image-url)
as approximately 1:1 mixtures of E/Z isomers, apart from the meta-phenylacetic imine (3e) where the E isomer was favoured over the Z isomer in an approximate 2:1 ratio. The E stereochemistry was assigned to a particular isomer based on the following: the signal from H4 (on the isatin core) of the E isomer in the 1H NMR spectrum was shifted upfield 1.2–1.4 ppm relative to the H4 signals of the Z isomer and the parent compound 1, presumably due to shielding by the ring current on the iminophenyl ring. The isomeric ratios of each compound were therefore determined by comparing the size of the integrals from the H4 signals of the E and Z isomers, as has been described previously by our group for a related series of compounds.23 Although some 2-indolinones containing a substituent at C-3 have been described as predominantly E isomers,33 elsewhere it has been reported that the E and Z isomers of 3-imino-2-indolinones interconvert rapidly in solution at room temperature and that the ratio of isomers is solvent dependent.22 The imines 3b–e were formed in good yields (56–71%), whereas 3a was obtained in only 18% yield. This presumably results from the reduced nucleophilicity of the amino moiety in 3a, relative to that in 3b–e, due to the strong electron withdrawing effect of the para-substituted carboxyl group.

The imino acids (3a–e) were then selectively coupled to the protected lysine derivative Ac-Lys-OMe under standard carbodiimide coupling procedures34,35 to give the desired novel imine–lysine model conjugates 4a–e in good to excellent yields (Scheme 1). Once again, the 1H NMR spectra (CDCl3) of the isatin–lysine series (4a–e) revealed that most compounds (4a–d) were synthesised as approximately 1:1 mixtures of E/Z isomers, apart from the meta-phenylacetic analogue (4e) where the Z isomer was favoured over the E isomer in an approximate 2:1 ratio, the reverse ratio to that observed for the precursor imine 3e. This may, however, be due to the change in NMR solvent from DMSO-d6 with 3e to CDCl3 with 4e, as has been observed for other imines.32 All of the imines (3a–e, 4a–e) were fully characterised by 1H and 13C NMR spectroscopy and mass spectrometry.

2.2. Hydrolysis studies

The rate of acid-catalysed hydrolysis in the imino acids 3a–e as a function of imine absorbance at λ = 435 nm over time is shown in Figure 2A (middle panel). Analysis of the hydrolysis kinetics conducted at pH 4.5 (37 °C) indicated an exponential first order decay process as evidenced by the ln(A/As) versus time plots (Fig. 2A, bottom panel), which showed a linear relationship at the initial reaction times. The first order rate constants, k, determined by non-linear regression, ranged from 0.95 to 3.99 × 10⁻² min⁻¹ (Table 1), which is comparable to those reported in an earlier study.36 It should be noted, however, that these derivatives differ from those herein in their hydrolysis rates, as judged by one-way ANOVA, and the pH of the buffers used.

Hydrolysis of the various derivatives was found to depend on the aromatic substitution pattern and linker length and was not influenced by whether the derivatives were in the imino acid or imino lysine form. This can be seen by comparing the hydrolysis rates of each pair of derivatives (e.g., 3a: k = 0.95 × 10⁻² min⁻¹ vs 4a: k = 0.81 × 10⁻² min⁻¹), which showed no significant difference as judged by one-way ANOVA (see Supplementary data).

In addition to the hydrolysis experiments conducted at physiological temperatures (37 °C), selected hydrolysates for 3a, 3b and 3d were also conducted at ambient temperature. As expected, the rates of hydrolysis at pH 4.5 were significantly lower at 23 °C compared to those conducted at 37 °C, while no hydrolysis was detected at physiological pH at either 23 °C or 37 °C (see Supplementary data).

3. Discussion and conclusion

We have reacted various anilino carboxylic acids (2a–e) with the potent cytotoxin para-methoxybenzylisatin (1) to generate a series of aryl imine derivatives (3a–e, Scheme 1) with differing aromatic substitution patterns and linker length. Alkyl imine derivatives are known to hydrolyse at physiological pH, however, aryl imines are significantly more stable and have found recent potential application as pH-sensitive linkers in drug delivery.27–31 Accordingly, the aryl imines we prepared were all found to be stable at physiological pH but readily cleaved at reduced pH. Furthermore, in order to model the conjugation of these acid-labile derivatives to the lysine residues of a tumour-targeting protein, the aryl imines 3a–e were coupled via their free carboxylic acids to a protected lysine amino acid residue to produce a novel series of aryl imine-lysine conjugates 4a–e. These were all found to be stable for an extended period at pH 7.4 but readily cleaved in aqueous acidic solutions at physiological temperature, with half-lives ranging from 17.0 to 85.2 min, consistent with those reported recently for the acid-catalysed hydrolysis of other aryl imines.31

The rates of acid-catalysed hydrolysis of both the imino acids 3a–e and imino lysines 4a–e, followed the expected trend, with the para-phenyl propionic acid derivatives (3c and 4c) displaying the fastest hydrolysis rates, and the para-benzoic acid derivatives (3a and 4a) displaying the lowest rates. Acid-catalysed hydrolysis of imines involves the protonation of the imine to give an iminium species, which undergoes nucleophilic attack by water. This gives an unstable hemiaminal intermediate, which upon protonation of the amine nitrogen, readily collapses to the protonated ketone and free amine, with subsequent proton loss furnishing the ketone.27 The crucial first step in the mechanism, imine protonation, is largely dependent on the basicity of the imine. In derivatives 3a and 4a, the electron-withdrawing carbonyl group 3a–e when maintained at physiological pH (7.4) in 1 M HEPES buffer at 37 °C over a 240 min period (first 60 min shown in Fig. 2A, top panel).

A similar trend in the rate of hydrolysis was obtained for the imino lysine conjugates 4a–e (Fig. 2B) with first order rate constants, k, ranging from 0.81 to 4.07 × 10⁻² min⁻¹ (Table 1). Again, the fastest hydrolysis rate was observed for the para-phenylpropionic acid derivative 4c, which had a fivefold greater half-life of 17.0 min, and the lowest hydrolysis rate was found for the para-benzoic acid derivative 4a, which had a fivefold greater half-life of 85.2 min. In this case, the hydrolysis rates of all but 4d versus 4e were found to be significantly different from one another, as judged by one-way ANOVA, with the para-phenylacetic acid derivative 4b significantly faster than both 4d and 4e, with half-lives of 34.1 and 33.0 min, respectively, compared to 23.0 min for 4b. As for the imino acids 3a–e, no hydrolysis was detected for the imino lysine derivatives 4a–e when maintained at physiological pH (7.4) in 1 M HEPES buffer at 37 °C over a 240 min period (Figure 2B, top panel).

Hydrolysis of the various derivatives was found to depend on the aromatic substitution pattern and linker length and was not influenced by whether the derivatives were in the imino acid or imino lysine form. This can be seen by comparing the hydrolysis rates of each pair of derivatives (e.g., 3a: k = 0.95 × 10⁻² min⁻¹ vs 4a: k = 0.81 × 10⁻² min⁻¹), which showed no significant difference as judged by one-way ANOVA (see Supplementary data).
the imino lysines greater availability of the imine nitrogen lone pair of electrons for conjugation with the acid or amide carbonyl is disrupted leading to extended conjugation (Fig. 3), leading to lower rates of hydrolysis ring, will reduce the relative basicity of the imine nitrogen through electron density on the carbon adjacent to the imine nitrogen and thereby its relative basicity. This hyperconjugative interaction could be more significant with the para-propionic derivatives 3c and 4c, with the counter delocalising effect of the carbonyl group adjacent to the negative charge on the Ar–CH carbon being removed. The hyperconjugative effects associated with these groups in the meta-position would not be expected to have a marked effect on the imine basicity.

Our results are consistent with those from complementary studies on 3-phenyliminoxindole derivatives, where it was found that electron-donating groups at the para-position of the aryl ring enhanced the rates of acid-catalysed hydrolysis. Conversely, the incorporation of electron-withdrawing groups on a series of related aryl imines were found to have a stabilising effect at physiological pH. Thus, it is expected that the incorporation of other derivatives could be involved in increasing electron density on the carbon adjacent to the imine nitrogen and thereby its relative basicity. This hyperconjugative interaction could be more significant with the para-propionic derivatives 3c and 4c, with the counter delocalising effect of the carbonyl group adjacent to the negative charge on the Ar–CH carbon being removed. The hyperconjugative effects associated with these groups in the meta-position would not be expected to have a marked effect on the imine basicity.

Figure 3. Resonance contributors of the imine 3a showing extended π delocalisation.
electron donating or withdrawing groups would allow for more tunable hydrolysis rates of imine-based compounds.

Adjusting the linker length was more important in determining hydrolysis rates in the para-series compared to the meta-series. For both the para-substituted carboxylic acid derivatives 3a–c and the lysine derivatives 4a–c, lengthening the linker resulted in a significant increase in the hydrolysis rate. However, the two meta-substituted derivatives in each series (3b, d and 4b, d), showed no significant difference in their hydrolysis rates. Overall, it appears that electronic effects such as the presence or absence of a conjugated electron-withdrawing group may play a larger role in determining the hydrolysis rate than extending the linker.

In this study we chose the para-methoxybenzylisatin (1), which has an I$_{C_{50}}$ value of 1.83 µM$^{-1}$ against the human monocyte-like histiocytic lymphoma U937 cell line, as our starting cytotoxin due to its ready synthetic availability and structural simplicity, which facilitates product characterisation via NMR spectroscopy. While 1 is less active than our most promising cytotoxins (I$_{C_{50}}$ = 1.83 µM vs 1 µM, respectively), the bioconjugation strategy presented here should equally well apply to any of the N-substituted isatin derived cytotoxins we have developed, all of which contain an available carbonyl at C-3. This strategy is also potentially applicable to the bioconjugation of well known anti-cancer agents such as epipodophyllotoxin, camptothecin and colchicine to enhance their effectiveness even further.$^{10,40}$

In conclusion, a series of novel aryl imine linked isatins (3a–e), which could potentially be coupled to the lysine residues of a tumour targeting protein via standard peptide coupling techniques, were synthesised from the N-substituted isatin-derived cytoxim (1). Compounds (3a–e) and the model lysine coupled derivatives (4a–e) are available for tumour targeting protein conjugates.

4. Experimental

4.1. Synthesis

All chemicals were purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA), BDH Laboratory Supplies (Poole, England) or Bachem (AusPep, Parkville, Australia) and used as supplied. Flash column chromatography$^{31}$ was performed on Silica Gel 60 (230–400 mesh). Thin layer chromatography (TLC) on aluminium backed sheets of Merck Silica Gel 60 F$_{254}$ plates were employed to monitor the progress of chemical reactions. Preparative TLC was performed on a Nicolet Avatar 360 FT-IR spectrometer. NMR spectra were acquired on a Varian Inova 500 spectrometer, where the proton (H) and carbon ($^{13}$C) were obtained at 500 and 126 MHz, respectively. All spectra were obtained with a probe temperature of 298 K. Hydrogen and carbon assignments were also made using gradient correlation spectroscopy (gCOSY), gradient heteronuclear single quantum correlation (gHSQC) and gradient heteronuclear multiple bond correlation (gHMBC) spectroscopic techniques. Low resolution electrospray ionisation mass spectra (ESI) were obtained using a Waters Platform LCZ spectrometer and high resolution ESI spectra (HRMS) on a Waters Q-TOF Ultima spectrometer. Melting points were obtained using a Reichert melting point apparatus and are uncorrected.

4.1.1. General procedure for the synthesis of the imines (3a–e)

Activated 3 Å sieves (1 g per 100 mg of 1) for 15 min. The acid (1 equiv) and EtOH (7.5 mL per 100 mg of 1) were sonicated for 10 min. The mixture was heated at reflux for 1.5 h, the mixture was cooled and filtered, and the solution was evaporated. The resulting solid was purified by column chromatography on silica gel using a CHCl$_3$/MeOH gradient of 100:0 to 95:5 to yield isatins 3a–e.

4.1.1.1. (E and Z)-3-[5,7-Dibromo-1-(4-methoxybenzyl)-2-oxoindolin-3-ylidine amino][benzoic acid (3a). Orange powder (46.5 mg, 18%); mixture of E and Z isomers (E/Z 48:52); mp 250–252 ℃; R$_f$ 0.26 (silica, DCM); IR $\nu_{max}$ 2955 (COOH), 1696 (CO), 1598, 1515, 1446, 1248, 1143, 863, 810 cm$^{-1}$, $\delta_{H}$ (500 MHz; DMSO-$d_6$): 3.71 (s, 3H), 3.73 (s, 3H), 5.12 (s, 2H), 5.30 (s, 2H), 6.44 (s, 1H), 6.86 (d, $J$ = 8.0 Hz, 2H), 6.90 (d, $J$ = 8.0 Hz, 2H), 7.13 (d, $J$ = 7.5 Hz, 4H), 7.18 (d, $J$ = 8.0 Hz, 2H), 7.25 (d, $J$ = 8.0 Hz, 2H), 7.84 (s, 1H), 7.87 (s, 1H), 7.91 (d, $J$ = 8.5 Hz, 2H), 7.93 (s, 1H), 8.08 (d, $J$ = 8.0 Hz, 2H); $^1$H$_C$ (126 MHz; DMSO-$d_6$): 44.0, 44.5, 55.6, 55.7, 104.2, 104.8, 113.2, 114.6, 114.6, 114.9, 116.1, 117.6, 119.5, 120.6, 125.5, 126.3, 127.7, 128.2, 128.2, 129.3, 129.4, 130.6, 131.9, 141.1, 141.3, 143.3, 144.3, 151.1, 152.4, 153.4, 154.0, 158.1, 159.1, 163.5, 165.6, 167.8, 175.0 (ESI-) m/z: 541, 543 [M–H]–, 79Br$^{79}$Br$, 79$Br$^{81}$Br, 81$Br$^{81}$Br; HRMS (ESI-) m/z: calcd for C$_{22}$H$_{13}$N$_2$O$_2$Br$_2$: 540.9399 [M–H]–; found: 540.9496 [M–H]–.

4.1.1.2. (E and Z)-2-[4,5,7-Dibromo-1-(4-methoxybenzyl)-2-oxoindolin-3-ylidine amino][phenylacetic acid (3b). Red crystals (356 mg, 68%); mixture of E and Z isomers (E/Z 58:42); mp 151–153 ℃; R$_f$ 0.40 (silica, DCM/MeOH: 9:1); IR $\nu_{max}$ 2935 (COOH), 1708 (CO), 1516, 1445, 1309, 1247, 1142, 802, 753 cm$^{-1}$, $\delta_{H}$ (500 MHz; DMSO-$d_6$): 3.57 (s, 2H), 3.65 (s, 2H), 3.71 (s, 3H), 3.73 (s, 3H), 5.14 (s, 2H), 5.30 (s, 2H), 6.60 (d, $J$ = 1.5 Hz, 1H), 6.87 (d, $J$ = 9.0 Hz, 2H), 7.60 (d, $J$ = 9.0 Hz, 2H), 7.00 (d, $J$ = 8.5 Hz, 2H), 7.13 (d, $J$ = 8.5 Hz, 2H), 7.17 (d, $J$ = 8.0 Hz, 2H), 7.24 (t, $J$ = 7.5 Hz, 4H), 7.41 (d, $J$ = 8.5 Hz, 2H), 7.81 (d, $J$ = 2.0 Hz, 2H), 7.89 (d, $J$ = 2.0 Hz, 1H); $^1$H$_C$ (126 MHz; DMSO-$d_6$): 44.0, 44.6, 55.8, 104.1, 104.8, 112.5, 114.5, 114.7, 115.0, 116.1, 117.8, 120.7, 121.1, 125.1, 127.0, 127.6, 128.2, 128.3, 129.5, 130.1, 130.5, 131.5, 133.3, 133.7, 141.1, 142.4, 144.8, 146.8, 148.0, 148.9, 158.2, 159.1, 163.8, 173.5, MS (ESI+) m/z: 557; 559; 561 [MH$^+$]; $^{79}$Br$^{79}$Br, $^{79}$Br$^{81}$Br, $^{81}$Br$^{81}$Br; HRMS (ESI+) m/z: calcd for C$_{22}$H$_{13}$N$_2$O$_2$Br$_2$: 558.9692 [MH$^+$]; found 558.9678 [MH$^+$].
14.1.4. (E and Z)-3-[5,7-Dibromo-1-(4-methoxybenzyl)-2-oxoindolin-3-ylideneamino]benzoic acid (3d). Orange powder (144 mg, 56%); mixture of E and Z isomers (E/Z 49:51); mp 229–231 °C; Rf 0.37 (silica, DCM/MeOH 9:1); IR νmax 2950 (COOH), 1719 (CO), 1680, 1515, 1449, 1297, 1250, 1141, 812, 768 cm⁻¹. 4.1.4.1. HRMS (ESI-): m/z: calcd for C₂₃H₂₈NO₅Br²⁻: 526.9799 [M−CO₂H]−; found 526.9722 [M−CO₂H]−.

14.1.5. (E and Z)-2-[3-[5,7-Dibromo-1-(4-methoxybenzyl)-2-oxoindolin-3-ylideneamino]phenyl]acetic acid (3e). Red powder (187 mg, 71%); mixture of E and Z isomers (E/Z 67:33); mp 182–184 °C; Rf 0.33 (silica, DCM/MeOH 9:1); IR νmax 2944 (COOH), 1719 (CO), 1698, 1515, 1449, 1297, 1250, 1141, 812, 768 cm⁻¹. 4.1.5.1. HRMS (ESI-): m/z: calcd for C₂₃H₂₈NO₅Br²⁻: 440.4, 455, 55.7; 104.1, 104.7, 114.6, 114.6, 114.8, 116.0, 118.3, 120.7, 120.9, 122.0, 124.5, 125.2, 126.6, 126.8, 127.5, 128.1, 128.4, 129.3, 129.4, 130.8, 132.1, 140.8, 141.0, 143.1, 144.5, 149.1, 150.4, 151.2, 150.8, 158.9, 159.0, 163.6, 167.5, 167.8. MS (ESI-): m/z: 541; 543; 545 [M−H]−; 79Br²⁻; 81Br²⁻; HRMS (ESI-): m/z: calcd for C₂₃H₂₈NO₅Br²⁻: 540.9399 [M−H]−; found 540.9424 [M−H]−.

14.1.6. General procedure for the synthesis of the imine–lysine conjugates (3a–e). The isatin imine (3a–e, 1 equiv) was dissolved in dry DCM (1 mL per 30 mg of imine) with the aid of sonication. On ice, 1-hydroxybenzotriazole hydrate (HOBt) (1 equiv) was added, followed by N,N-dicyclohexylcarbodiimide (DCC) (1 equiv), N,N-diisopropylethyamine (DIPEA) (1.1 equiv) and (S)-N-acetyl-lysine methyl ester hydrochloride (Ac-Lys-OMe·HCl) (1 equiv). The reaction mixture was warmed to rt and stirred for 24 h. The precipitate was filtered through filtration and the filtrate evaporated. The resulting solid was purified by column chromatography on silica gel using a CHCl₃/MeOH gradient of 100:0 to 95:5 to yield the imine-lysine conjugate 3a–e.

14.2.1.2. Methyl (S,E and S,Z)-2-acetamide-6-[4-[5,7-dibromo-1-(4-methoxybenzyl)-2-oxoindolin-3-ylideneamino]benzamido]hexanoate (4a). Orange crystals (18.7 mg, 50%); mixture of E and Z isomers (E/Z 53:47); mp 165–167 °C; Rf 0.41 (silica, CHCl₃/MeOH 95:5). 4.2.1.1. HRMS (ESI+): m/z: calcd for C₂₃H₂₈NO₅Br²⁻: 79Br²⁻; 81Br²⁻; HRMS (ESI+) m/z: found for C₂₃H₂₈NO₅Br²⁻: 79Br²⁻; 81Br²⁻; HRMS (ESI+) m/z: 779.0879 [M+Na]+; found 779.0896 [M+Na]+.

14.2.1.4. Methyl (S,E and S,Z)-2-acetamide-6-[3-[5,7-dibromo-1-(4-methoxybenzyl)-2-oxoindolin-3-ylideneamino]benzamido]hexanoate (4d). Orange crystals (83.4 mg, 78%); mixture of E and Z isomers (E/Z 47:53); mp 74–76 °C; Rf 0.56 (silica, CHCl₃/MeOH 95:5); IR νmax 2393 (NH), 1734 (CO), 1645, 1547, 1446, 1318, 1249, 1142, 801, 721 cm⁻¹, δC (500 MHz; CDCl₃): 1.36–1.91 (m, 12H), 1.97 (t, J = 7.0 Hz, 6H), 3.41–3.48 (m, 4H), 3.72
(s, 3H), 3.73 (s, 3H), 3.76 (s, 3H), 3.79 (s, 3H), 4.56–4.62 (m, 2H), 5.25 (s, 2H), 5.43 (s, 2H), 6.24 (d, J = 7.5 Hz, 1H), 6.28 (d, J = 7.5 Hz, 1H), 6.70 (d, J = 1.5 Hz, 1H), 6.82 (d, J = 8.0 Hz, 2H), 6.85 (d, J = 8.5 Hz, 2H), 7.08 (d, J = 6.0 Hz, 2H), 7.11 (d, J = 9.0 Hz, 2H), 7.21 (d, J = 9.0 Hz, 2H), 7.43 (t, J = 8.0 Hz, 1H), 7.46 (s, 1H), 7.53 (t, J = 8.0 Hz, 1H), 7.54 (d, J = 1.5 Hz, 1H), 7.59 (d, J = 1.5 Hz, 1H), 7.63 (d, J = 8.0 Hz, 1H), 7.70 (d, J = 2.0 Hz, 1H), 7.74 (d, J = 7.5 Hz, 1H), 8.33 (d, J = 2.0 Hz, 1H).

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Supplementary data

Supplementary data (hydrography graphs of imines 3a–e vs their analogous imino lysines 4a–e at pH 4.5 and hydrography graphs of imines 3a, 3b and 3d at 23 °C vs 37 °C associated with this article can be found, in the online version, at doi:10.1016/j.bjmc.2011.01.015.

References and notes