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Marine natural products isolation, screening and analogue synthesis

Ana Zivanovic

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Marine natural products isolation, screening and analogue synthesis

A thesis submitted in (partial) fulfillment of the requirements for the award of the degree of

DOCTOR OF PHILOSOPHY

from

University of Wollongong

School of Chemistry
March, 2012
Thesis Declaration

I, Ana Zivanovic, declare that the work described in this thesis, submitted in fulfilment 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.

Ana Zivanovic

March 23, 2012
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<tr>
<td>δ</td>
<td>chemical shift</td>
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<tr>
<td>Ac</td>
<td>acetyl</td>
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<td>anh.</td>
<td>anhydrous</td>
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<td>EC₅₀</td>
<td>half maximal effective concentration</td>
</tr>
<tr>
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<td>gHMBC</td>
<td>gradient heteronuclear multiple bond correlation</td>
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<td>high performance liquid chromatography</td>
</tr>
<tr>
<td>HREI-MS</td>
<td>high resolution electron impact mass spectrometry</td>
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</table>
HRESI-MS  high resolution electrospray ionisation mass spectrometry
HTS  high-throughput screening
IC_{50}  concentration required to inhibit 50% of the population
IHMRI  Illawarra Health and Medical Research Institute
J  spin coupling constant (NMR)
JAK 3  janus kinase 3
LCFA  long chain fatty acid
lit.  literature
LREIMS  low resolution electron impact mass spectrometry
m  multiplet
m  meta
[M+]  molecular ion
MAPK  mitogen-activated protein kinase
Me  methyl
min  minute(s)
MNP  marine natural products
m. p.  melting point
MS  mass spectrometry
mTOR  mammalian target of rapamycin
MTS  [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium, inner salt]
m/z  mass to charge ratio
NIST  National Institute of Standards and Technology
NMR  nuclear magnetic resonance
NP  natural products
NW  North West
o  ortho
p  para
PBS  phosphate buffered saline
PDA  photodiode array
PKA  protein kinase A
PKB  protein kinase B
PKC  protein kinase C
PDGFR  platelet-derived growth receptor
PI3K  phosphatidylinositol 3-kinase
PMB  para-methoxybenzyl
ppm  parts per million
PTK  protein tyrosine kinase
PUFA(s)  polyunsaturated fatty acid (s)
Rf  retention factor
ROCK  Rho-associated protein kinase
ROV  remotely operated vehicle
RP-TLC  reverse phase thin layer chromatography
Rt  retention time
RT  room temperature
RTK  receptor tyrosine kinase
s  singlet
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>SAR</td>
<td>structure-activity relationship</td>
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<tr>
<td>Sat.</td>
<td>saturated</td>
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<tr>
<td>Ser</td>
<td>serine</td>
</tr>
<tr>
<td>SERPENT</td>
<td>Scientific and Environmental ROV Partnership Using Existing Industrial Technology</td>
</tr>
<tr>
<td>t</td>
<td>triplet</td>
</tr>
<tr>
<td>TLC</td>
<td>thin layer chromatography</td>
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<td>threonine</td>
</tr>
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<td>tyrosine</td>
</tr>
<tr>
<td>UV</td>
<td>ultra violet</td>
</tr>
<tr>
<td>VEGFR-2</td>
<td>vascular endothelial growth factor receptor 2</td>
</tr>
</tbody>
</table>
Publications arising from this thesis

Journal Articles (published to date)

1. Ana Zivanovic, Natalie J. Pastro, Jane Fromont, Murray Thomson, Danielle Skropeta, Kinase inhibitory, haemolytic and cytotoxic activities of three deep-water sponges from the North Western Australia and their fatty acid compositions, Natural Product Communications 2011, 6 (12), 1921-1924.


5. Ana Zivanovic, 4-(4,6-Dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium Chloride, Synlett, 2012, 23(16): 2426-2427

Conference Abstracts

Oral Presentations

6. Zivanovic, A.; Matesic, L.; Skropeta, D., Isatin derivatives as potential cAMP dependant protein kinase A (PKA) inhibitors October 2011: Royal Australian Chemical Institute Natural Products Chemistry Group Annual One-Day Symposium, Wollongong, NSW.

7. Zivanovic, A; Matesic, L; Skropeta, D., 3-Substituted-N-alkylated-5,7-dibromoisatin derivatives as potential cAMP dependant protein kinase A (PKA) inhibitors (3 minute student poster talk), Jun 2011: 27th International Symposium on the Chemistry of Natural Products/ 7th International Conference on Biodiversity, Brisbane, QLD.


Poster Presentations


12. Zivanovic, A.; Matesic, L.; Skropeta, D., 3-Substituted-N-alkylated-5,7-dibromoisatin derivatives as potential cAMP dependant protein kinase (PKA) inhibitors, June 2011: 27th International Symposium on the Chemistry of Natural Products / 7th International Conference on Biodiversity, Brisbane, QLD.


17. Zivanovic, A.; Skropeta, D., Biological activity and sterol and fatty acid composition of three deep-water sponges from North-Western Australia, November 2009: *Royal Australian Chemical Institute Natural Products Chemistry Group Annual One-Day Symposium*, Newcastle, NSW.

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Abstract

Most drugs used today demonstrate their bioactivity by acting as receptor antagonists or as enzyme inhibitors. Due to the significant role of enzymes in the activity of a number of disease processes, and their structural conformation that is easily accessible to inhibition by small drug like molecules, they are widely investigated as drug targets. The first step in the preparation of an enzyme inhibitor is defining the target enzyme whose function is going to be interrupted or blocked in a particular disease or infection.

c-AMP Dependent protein kinase A (PKA) is an important enzyme involved in the regulation of a large number of physiological processes. As such, the inhibition of PKA has become an attractive drug target in a number of areas, in particular in immune function and for memory disorders. Interestingly, despite PKA being one of the most well characterised of all protein kinases, there are as yet no PKA inhibitors reported from marine sources.

The natural environment has provided a wealth of chemically diverse, bioactive compounds that are responsible for over half of the medications currently available for a multitude of diseases. Herein, ten algal species from South East Australia assigned as *Prionitis linearis*, *Corallina vancouveriensis*, *Codium fragile*, *Codium dimorphum*, *Colpomenia sinuosa*, *Padina* sp., *Phyllospora comosa*, *Sargassum vestitum*, *Sargassum* sp. and *Ulva lactuca* were analysed. Extracts of different polarity were generated from each sample and tested for their PKA inhibitory activity, haemolytic activity and brine shrimp cytotoxicity giving novel results on bioactivity from marine algae. There are no literature data so far on the kinase inhibitory activities of extracts isolated from these algal samples and for most of the samples haemolytic data are also reported for the first time. Furthermore, the chemical (fatty acid and sterol) composition of the ten algal extracts were also analysed and compared.

The bioactivity profiles of the alga samples were strongly species dependent, with no apparent correlation between PKA inhibition, haemolysis and/or brine shrimp lethality. In general, the non-polar fractions isolated from all alga samples were found to give potent biological results and displayed more activity, in particular greater PKA inhibitory activity, compared with the polar fractions. The biological activity is reflected in the different algal divisions, with the order of PKA inhibitory activity identified as follows: brown seaweeds > red seaweeds > green seaweeds. The main constituents of the non-polar fractions were identified as fatty acids (FAs) and sterols in all algal samples. The FA composition was dominated by saturated FAs, hexadecanoic acid and unsaturated 9,12-octadecadienoic acid were the most predominant acids, while cholesterol was found to be the major sterol of all ten algal species investigated.

The deep sea is one of the most biodiverse habitats on the planet and deep-sea sponges are the largest source of new deep-water natural products reported and are found to contain a
rich diversity of new and unusual sterols. In this study four deep-sea sponges from North Western Australia assigned to the genera *Haliclona*, *Sarcotragus*, *Ircinia* and *Geodia* were investigated for their biological activities in a variety of assays (for PKA inhibition, haemolytic activity and brine shrimp toxicity assay). The chemical composition of the extracts of different polarity obtained from all three samples were also determined and compared.

Overall the sponge *Haliclona* sp. displayed greater haemolytic activity than PKA inhibitory activity, along with low levels of brine shrimp lethality with the bioactivity mainly found in the non-polar fractions. The *Ircinia/Sarcotragus* sponge species exhibited higher PKA inhibitory activity than the activity found in the other two assays, with the two non-polar fractions displaying activity greater than 80%. The trend of the bioactive non-polar fractions continued for the *Geodia* sponge species where the non-polar extracts showed both potent PKA inhibitory activity (97%) and brine shrimp lethality (88%), with modest haemolytic activity observed for several fractions. Most of the identified compounds in those fractions were fatty acids and sterols and contributed to a partial fatty acid and sterol profile of this deep-sea demosponge. A total of 29 fatty acids and 23 sterols were identified, with the highest proportions found in *Ircinia/Sarcotragus* sp. > *Haliclona* sp. > *Geodia* sp. There was no apparent correlation amongst the sterol composition of the three deep-sea sponges. The sterol profile varied across the sponge species either in type of sterols or in relative percentages. This work, has however uncovered the rich potential of deep-sea sponges as sources of bioactive metabolites. In addition, this study has laid the foundations for the future investigation and assessment of kinase inhibitory potential of metabolites from both shallow and deep water invertebrates.

Another aspect of drug discovery, in addition to isolating natural products, is modification of the isolated metabolites from nature and their structural modification towards improved bioactivity. Development of natural products includes modification of functional groups, their removal, introduction of novel groups and stereocenters to the molecule, or occasionally radical changes of the basic scaffold. Although the structural diversity in nature is extraordinary, synthetic chemistry methods can provide novel structure types which cannot be obtained from natural biosynthetic methods.

Isatin derivatives were previously investigated in our research group for cytotoxicity against a range of human cell lines and found to display potent activity, causing decreased cell proliferation in all cell lines studied. Previous literature research of isatin-indole compounds has also demonstrated that these scaffolds are also important for kinase inhibitory activity, as evidenced by the large number of indole based kinase inhibitors approved for use and currently in clinical trials. Hence, it was of interest to further develop our isatin-based structures and test them for potential protein kinase A inhibitory activity. The aim was to determine the structural
requirements and the position of the different groups on the isatin molecule necessary for PKA inhibitory activity. Herein, the synthesis of 41 novel imino isatin derivatives and 14 alkenyl isatin analogues and evaluation of their cytotoxicity, PKA and PKB inhibitory activity is presented, followed by a detailed analysis of their structure-activity relationships. Of a total of 77 isatin derivatives prepared four isatin derivatives displayed potent PKA inhibitory activity (65-86%) and six displayed potent PKB inhibitory activity (57-84%). Compounds 178, 208, 209 and 246 displayed the highest PKA inhibitory activities and their IC₅₀ values were determined as 43.5, 51.8, 38.5, 52.2 µM respectively (at 100 µg/mL). Four isatin derivatives (175, 176, 177 and 208) were active in both PKA and PKB assay. Three isatins (163, 187, 206) displayed selective activity over PKB than PKA, while two compounds (178, 209) were more selective towards PKA. In addition the newly developed isatin derivatives also exhibited potent cytotoxicity against human leukemic monocyte lymphoma cell lines U937 in the micromolar range. This work suggests that isatin derivatives possess a real potential to be further developed into PKA and PKB inhibitors in the future.
Chapter 1

Introduction
1.1 Drug discovery

Modern drugs and traditional medicines are largely based on natural products (NPs). Nature has been the cornerstone of drug development with 70% of all drugs on the market connected with natural products, while only 30% of novel drugs have been purely synthetic.\(^1\) Discovery of novel natural product-based drugs includes isolation of new metabolites from various natural sources such as plants, marine organisms and bacteria and also involves structurally inspired development of previous identified natural compounds. Pharmaceutical industries have tried to develop better novel drug sources using combinatorial chemistry and high throughput screening on molecular targets; however natural products are still found to be unique and irreplaceable.\(^2,^3\)

Research shows that nature derived compounds have better drug like properties than compounds prepared by combinatorial chemistry, e.g. 65% of compounds from the Dictionary of Natural Products have no violations of Lipinski’s “rule of five”, which is often used to determine drug-likeness of chemical compounds.\(^4^-^6\) Additionally, naturally occurring metabolites display greater biochemical specificity and chemical diversity than synthetic drugs (Table 1.1).\(^7,^8\) Statistical comparison of natural product derived compounds and synthetically produced drugs shows obvious differences in their structural properties. Natural derived compounds generally have more sp\(^3\) hybridized bridgehead atoms, stereogenic centres, number of rings and oxygen content than synthetic compounds and more favourable chemical complexity.\(^9\)

<table>
<thead>
<tr>
<th>Table 1.1: Some fundamental differences between biosynthesis and synthesis.(^8)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Building blocks</strong></td>
</tr>
<tr>
<td><strong>Strategy</strong></td>
</tr>
<tr>
<td><strong>Scaffold diversity</strong></td>
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<tr>
<td><strong>Functional group tolerance</strong></td>
</tr>
<tr>
<td><strong>Novel motifs</strong></td>
</tr>
<tr>
<td><strong>C–H activation</strong></td>
</tr>
<tr>
<td><strong>Stereocontrol</strong></td>
</tr>
</tbody>
</table>

1.2 Natural products as a source of novel drugs

Records of the use of natural products in traditional medicine dates back to ancient times worldwide.\(^10\) In Greece, South America, Egypt, China and Rome, plants were widely used as a source of both nutrition and medicine.\(^10,^11\) However, <10% of the 300,000 to 500,000 plant species are used by humans and animals as foods, while more are used for medicinal purposes (Fig. 1.1).\(^12\) More than 3000 plant species have been reported to be used for the treatment of cancer where 67% of the effective anticancer drugs today can be traced back to natural origin.\(^13\) Approximately two thirds of the human population generally consume plant-derived traditional medicines for their primary health care as reported by the World Health Organization (WHO).\(^14\)
Phytotherapy or study of the use of natural product generated extracts as medicines is not related to history only, but has also gained attention and implementation in drug therapy in the last few decades. The great importance of natural products is due to the fact that they are the major source of novel drugs.\textsuperscript{6}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{chemopreventive_herbs.png}
\caption{Widely used herbs with chemopreventive efficacy.\textsuperscript{15}}
\end{figure}

Natural products embody organic compounds, which are obtained from plants, microorganisms, invertebrates and vertebrates.\textsuperscript{16} Nature can be viewed as a productive biochemical factory for the biosynthesis of both primary and secondary metabolites because natural molecules have numerous different biological functions (Fig. 1.2).\textsuperscript{16} Primary metabolites play a pivotal role in the metabolism and reproduction of cells and they are crucial for the survival and metabolic processes of an organism, while secondary metabolites are specific for some limited range of species and they mainly appear to be superfluous to an organism. Primary metabolites include carbohydrates, proteins, nucleic acids, fatty acids, steroids and lipids and they occur through fundamental biosynthetic pathways. Secondary metabolites are found to be connected with an organism’s interaction with its environment, producing novel chemical compounds that promote their survival.\textsuperscript{17,18} Secondary metabolites are present in small amounts and usually work by inhibiting or stimulating biological processes in other organisms and are involved in the interactions between organisms.\textsuperscript{19} Therefore, these metabolites often attracted scientific interest due to both the large number of different structures produced by nature and their diverse range of biological activities. In addition, they represent the individuality of the species at a chemical level because they are regularly found to be unique to a particular
Initially it was believed that secondary metabolites, which are found to have an array of biological activities, were metabolic waste products. However, further research showed that secondary metabolites serve for important defence, communication, and predation purposes.\textsuperscript{14}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{biogenesis.png}
\caption{Outline of the biogenesis of natural products.\textsuperscript{16}}
\end{figure}

As previously stated, Nature has provided the majority of drugs in use today, and natural products (NP) are currently considered the most successful source of drugs ever, with nearly one-third of the top-selling drugs in the world being natural products or their derivatives.\textsuperscript{19,21} The discovery of new natural compounds can lead to new ways of enhancing life, such as through the discovery of new classes of drugs with novel modes of actions. Important given the growing drug tolerance and resistance in microorganisms and cancer cells.\textsuperscript{22} In the period from 2005 to 2010 19 natural product based compounds (Table 1.2) were approved for use on the market including seven NPs, 10 semi-synthetic NPs and two NP-derived drugs.\textsuperscript{17}
Unlike morphine, quinine has been replaced by superior, syn
tthetic analogues such as the drug pamaquine. Penicillin (3)
was isolated from the culture broth of Penicillium notatum
as a bioactive principle inhibiting the growth of Gram-
positive bacteria. Soon thereafter when research of natural
products had become popular, the isolation of new compounds
including salicin from Salix alba, strychnine and brucine from
Strychnos nux-vomica, colchicine from Colchicum autumnale,
cafeine from Coffea arabica, nicotine from Nicotiana tabacum,
atropine from Atropa belladonna and cocaine from
Erythroxylum coca. Some of the most well known natural drugs
and medicines used in the health industries are the antibiotic
erythromycin, the antifungal agent amphotericin B, and
the immunosuppressive agents cyclosporine A and FK506.
Vinca alkaloids, vincristine sulphate and vinblastine sulphate,
are also the first plant derived anticancer agents to be approved for use
in cancer.
Figure 1.3: Some of the earliest isolated and characterised drugs obtained from Nature: a) quinine from *Cinchona* bark; b) morphine from the plant *Papaver somniferum*; c) penicillin from the bacteria *Penicillium notatum*.

Pharmaceutical companies use plant extracts in high-throughput screening (HTS) and test them on a range of receptor based assays for drug discovery. Extracts typically contain a complex mixture of compounds and it is a challenge to isolate a pure bioactive principle, especially as the activity of extracts has occasionally been found to be due to the synergy of the mixture, while the pure compounds may be inactive. In addition, in the process of discovering novel bioactive metabolites, it may also occur that an isolated compound is a known compound that was identified, characterised and analysed previously. Many natural products have complex structures that cannot be prepared in the laboratory and therefore it is necessary to further develop natural product screening to discover compounds more amenable for large scale synthesis.

1.3 Marine natural products as sources of novel drugs

Almost 75% of the Earth’s surface is covered by oceans and this huge quantity of marine environment is a vast and fertile area presenting a large opportunity for research. For thousands of years, natural products were obtained from terrestrial organisms but around 50 years ago, with the advent of scuba technology, scientists started to explore the marine environment. Of the estimated 153,000 known natural products found so far, 30,000 compounds are obtained from marine organisms and there are still many more to be discovered. Furthermore, of the accounted 34 fundamental phyla of life, 32 occur in the sea while only 17 occur on land. While there are many natural products that have been developed into drugs, only a few are from marine sources because the interest in marine natural products (MNP) had a
much later start.\textsuperscript{31} The marine environment is still unexplored as a rich source of novel drugs, but extremely promising as a source of new drug candidates.\textsuperscript{11} Approximately half of the novel MNPs reported in the literature are biologically active.\textsuperscript{33} The 60-70 years of activity in the MNP field has resulted in 4796 distinct genera/species being recorded in the MarinLit database as sources of novel MNPs.\textsuperscript{34} With the advent of new technologies which allow for easier collection of marine organisms, HTS and combinatorial chemistry, marine organisms are revealing themselves to be a vast, untapped source of new biologically active natural products.\textsuperscript{35} Kong et al. analysed the relationship between novel chemical structures of marine products and scaffolds of these compounds with time (Fig. 1.4) in the period of 1965-2000 and found that the ratio linearly increased over time.\textsuperscript{36} The authors calculated that the trend would continue in the future proving that MNPs are a rich source of novel metabolites.\textsuperscript{36}

![Figure 1.4: Time-dependent variation (x axis) of the ratios of novel structures (in red) and scaffolds (in blue) derived from marine natural products.\textsuperscript{36}](image)

Although, the diversity of life in terrestrial organisms and the terrestrial environment is extraordinary, the greatest biodiversity is found in the world’s oceans, which have the highest number of species.\textsuperscript{37} Many of these marine organisms produce natural products that are structurally unique with various biological activities that can not be found in terrestrial organisms. In addition, some marine metabolites show extraordinary biological activity at very low concentrations.\textsuperscript{38} Many marine organisms are soft bodied and slow-moving organisms that usually lack physical protective systems. Consequently, in order to protect themselves from predators and to adapt to different environmental conditions they produce unique metabolites for chemical defence, of which many display potent bioactivity and have pharmaceutical potential.\textsuperscript{39,40} Marine secondary metabolites have been found to play different functions including a defensive role, antifouling and growth inhibition.\textsuperscript{41} There are two types of defences that plants and marine organisms use to protect themselves from predators; constitutive and
induced chemical defence. Constitutive chemicals are constantly produced whereas the induced defence occurs only with changes in environment. Activated chemical defence is a type of induced defence and occurs within seconds of damage of marine species. Marine organisms, react to stress and injuries by producing biologically active and toxic compounds, typically by enzyme transformation of inactive precursors. Marine organisms produce compounds with unique structures as a result of the specific environmental conditions with low temperatures, changeable salinity and pressure and strong effects of bacteria and pathogens.

The quantitative and qualitative effects of marine metabolites on herbivores was investigated and it was determined that primary metabolites are the same for terrestrial plants and marine organisms, while they differ in the composition of the secondary metabolites. For instance, marine occurring compounds are rich in halogens, unusual nucleosides and new peptides. An important characteristic of marine natural compounds is that marine organisms produce products which are released into the surrounding water area and diluted. For those compounds in order to display noticeable bioactivity they have to be very potent.

The question of how compounds produced by marine organisms are correlated to human health has often been posed. Production of antibacterial compounds in marine organisms is related to the fact that marine sessile organisms receive their dietary uptake by filtering seawater, which contains a high concentration of bacteria. They need to protect themselves from these bacteria and other microorganism and hence produce antibacterial constituents. Sponges occasionally grow together which creates competition for a place and the sponge that produces more chemicals to harm the other sponge has the advantage. The chemicals involved in this type of defence are mostly found to be active against human cancer.

The first significant discovery of biologically active compound from a marine source was reported in 1950 by Bergman et al., which encouraged further investigation into the MNP field. Spongouridine (4) and spongothymidine (5) (Fig. 1.5) were isolated from the Caribbean sponge Tethya crypta (Tethyllidae). Structural development of these metabolites led to the synthesis of approved antiviral drugs, ara-A (6) and ara-C (7) (Fig. 1.5), which are still in use today.

**Figure 1.5:** Bioactive compounds isolated from marine Caribbean sponge Tethya crypta (4 and 5) and their synthetic analogues (6 and 7)
Nowadays, hundreds of patents which describe novel bioactive MNPs have been filed and approximately 10-15 different MNPs are in clinical trials at this time. Currently, there are five approved drugs isolated from marine organisms (Table 1.3). Of the five marine derived drugs on market, four are Food and Drug Administration (FDA)-approved in the US Pharmacopeia while the fifth Yondelis is approved by the European Agency and is completing Phase III clinical studies in the US for approval.

Table 1.3: Marine derived drugs: approved and in clinical trials.

<table>
<thead>
<tr>
<th>Clinical status</th>
<th>Compound name</th>
<th>Marine Organism</th>
<th>Chemical class</th>
<th>Disease area</th>
</tr>
</thead>
<tbody>
<tr>
<td>Approved</td>
<td>Cytarabine, Ara-C</td>
<td>Sponge</td>
<td>Nucleoside</td>
<td>Cancer</td>
</tr>
<tr>
<td></td>
<td>Vidarabine, Ara-A</td>
<td>Sponge</td>
<td>Nucleoside</td>
<td>Antiviral</td>
</tr>
<tr>
<td></td>
<td>Ziconotide</td>
<td>Cone snail</td>
<td>Peptide</td>
<td>Pain</td>
</tr>
<tr>
<td></td>
<td>Yondelis (ET-743)</td>
<td>Tunicate</td>
<td>Alkaloid</td>
<td>Cancer</td>
</tr>
<tr>
<td></td>
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<td>Sponge</td>
<td>Macrolide</td>
<td>Cancer</td>
</tr>
<tr>
<td>Phase III</td>
<td>Soblidotin (TZT 1027)</td>
<td>Bacterium</td>
<td>Peptide</td>
<td>Cancer</td>
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<tr>
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<td>Alkaloid</td>
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<tr>
<td></td>
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<td>Fungus</td>
<td>Diketopiperazine</td>
<td>Schizophrenia</td>
</tr>
<tr>
<td></td>
<td>Plitidepsin</td>
<td>Tunicate</td>
<td>Depsipeptide</td>
<td>Cancer</td>
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<td></td>
<td>Elisidepsin</td>
<td>Mollusc</td>
<td>Depsipeptide</td>
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<td></td>
<td>PM1004</td>
<td>Nudibranchc</td>
<td>Alkaloid</td>
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<td></td>
<td>Tasilodin (ILX-651)</td>
<td>Bacterium</td>
<td>Peptide</td>
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<td></td>
<td>Pseudopterosins</td>
<td>Soft Coral</td>
<td>Diterpene glycoside</td>
<td>Cancer</td>
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<td>Phase I</td>
<td>Bryostatin 1</td>
<td>Bryozoa</td>
<td>Polyketide</td>
<td>Wound healing</td>
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<tr>
<td></td>
<td>Hemisterlin (E7974)</td>
<td>Sponge</td>
<td>Tripeptide</td>
<td>Cancer</td>
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<tr>
<td></td>
<td>Marizomib (NPI-0052)</td>
<td>Bacterium</td>
<td>Beta-lactone-gamma lactam</td>
<td>Cancer</td>
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</table>

Vidarabine was approved in 1976 for the treatment of acute keratoconjunctivitis, recurrent epithelial keratitis caused by herpes simplex virus type 1 and 2, and superficial keratitis. Cytarabine (6, described earlier) was approved by the FDA in 1969 and it appears in two formulations. Conventional cytarabine is used for lymphocytic, acute myelocytic and meningeal leukemia while liposomal cytarabine is approved for the treatment of lymphomatous meningitis. Ziconotide (8) is a natural product compound developed from the ω–conotoxin MVIIA peptide isolated from the venom of the marine snail Conus magus, which was found to specifically block Ca,2.2 (neuronal calcium) channels. It was approved in 2004 for the treatment of severe chronic pain in patients with cancer or AIDS (Fig. 1.6). Mangrove tunicate Ecteinascidia turbinata yielded trabectedin (ET-743 (9), Yondelis, Fig. 1.6) which was approved for use as an anticancer agent against soft tissue sarcoma in Europe 2007. In November 2010 Halaven (eribulin mesylate, 10) was approved for metastatic breast cancer by the FDA (Table 1.2, Fig. 1.6). Eribulin mesylate is a synthetic analogue of the complex marine natural product halichondrin B found in the pacific sponge Halichondria okadai.
Figure 1.6: Marine natural products: cytarabine, vidarabine, ziconotide and ecteinascidin 743.

Marine natural products have been found to exhibit a range of activities including antitumor, anticancer, antiplasmodial, antileukemic, biofouling, anti-inflammatory, antitubercular, antiviral, chemical signalling, venoms, antifouling agents, biological means of protection from harmful environments and many other activities.\textsuperscript{53-55} Sponges are the dominant source of new MNP s reported annually (accounting for almost 40%), followed by coelenterates (also referred to as cnidarians) and microorganisms (Fig. 1.7).\textsuperscript{19,56}
1.3.1 Marine algae

Among marine natural products, marine algae are one of the most widespread species estimated to be more than 15,000. Algae are known to produce a range of novel compounds and in particular novel lipid patterns as a result of their adaptation to different environmental conditions. Seaweeds have been used as a food source due to their nutritional value, mostly in Asian countries where they have been popular for a long time, but nowadays all around the world as well. Algae have become very popular in human consumption as a result of their low calorie content, high levels of vitamin B12, iron, essential fatty acids, proteins, antioxidants, minerals, essential and free amino acids and ability to stabilize blood sugar levels. The interest in marine algae rose significantly at the beginning of the eighties and since then continues in a similar trend. A number of promising anticancer compounds have been isolated from algae including stypoldione (11) from the brown alga *Stypopodium zonale*, which was found to inhibit polymerisation of microtubules and the cytotoxic dehydrothysiferol squalene derivatives, thyresenol A (12) and B (13) isolated from the red alga *Laurencia viridis*, active against a number of human breast cancer cell lines (Fig. 1.8).
A very important characteristic of marine algae is that they can be readily collected. Algae are located in easily accessible coastal areas and access to a large amount of algal material is available. Locations along the New South Wales coast of Australia have been identified as ‘biodiversity hotspots’, providing access to a wide variety of marine life for MNP study.  

1.3.2 Marine sponges

Sponges have been found to be the greatest source of novel MNPs reported every year. Sponges are extremely widespread among marine organisms, with over 7000 species described, including both shallow and deep-water sponges that are found to inhabit depths of over 8000 m, with some deep-water species displaying carnivorous behaviour. Their investigation started with discovery of arabinoside analogues from the sponge *Tethya crypta* in the 1950s. So far, most of the bioactive metabolites have been reported from shallow-water species, however deep-water sponges have also produced important bioactive compounds and represent a wealthy new source of biologically and structurally interesting molecules. Sponges possess great chemical diversity and produce a variety of bioactive compounds including hymenialdisine (14, Fig. 1.9), a potent inhibitor of CDK-1, isolated from *Axinella verrucosa* and *Acanthella aurantiaca*; cytotoxic β-carboline (15) and nortopsentins A-C (16-18) from *Hyrtiosreticulatus* and *Spongosorites ruetleri* respectively (Fig. 19); (Z)-stellettic acid C with telomerase inhibitor activity from the sponge *Stelleta* sp. (19, Fig. 1.9) and girolline from *Pseudaxinyssa cantharella*, which was found to be an inhibitor of protein synthesis (20, Fig. 1.9).

![Figure 1.9: Bioactive metabolites isolated from marine sponges.](image.png)
1.3.3 Deep-sea marine natural products

The deep-sea is classified to be the environment under the depths beyond the euphotic zone (the bright ocean layer, upper 200-300m). It is one of the most biodiverse and species-rich habitats on the planet, rivalling that of coral reefs and rainforests. The deep sea covers 70% of the earth’s surface and constitutes an important frontier in the progressive search for new sources of biological and therapeutic agents. The number of species inhabiting the world’s deep oceans is estimated to be as high as 10 million, which is 50 times greater than the number of marine species so far reported. However of the 30,000 MNPs reported so far, less than 2% come from deep-water marine organisms. The investigations to date have shown deep-sea organisms to be a promising source of new and unexplored chemical diversity for drug discovery. Of the various deep-sea phyla studied so far, deep-sea sponges have proven to be the richest source of novel metabolites, accounting for over 60% of those reported, with over half exhibiting significant cytotoxicity towards a range of human cancer cell lines. Furthermore, the deep-sea environment is characterised by a high proportion of single rare species. More than half of these single deep-sea species are new to science.

![Figure 1.10: World ocean bathymetric map. The vast oceans cover 70% of the world’s surface, with 95% greater than 1000 m deep.](image)

Living conditions of deep-sea organisms are significantly different from the environmental factors under which other marine organisms live. They live under extreme conditions where those differences include low temperatures, high pressure and absence of light (Fig. 1.10). The extreme conditions are assumed to be responsible for the production of unique secondary metabolites as their primary metabolic pathways can also be affected and changed. Furthermore, as a result of having to adapt to distinctive surroundings, deep-sea marine organisms have greater diversity of species than shallow-water species (Fig. 1.11).
As the depth increases the temperature decreases, down to -4°C at depths greater than 2000 m. Pressure at those great depths is extremely high and reaches up to 1000 atm or even more at the deepest parts. It is believed that below 250 m, light does not penetrate therefore causing deep-sea species to live in the dark with no vision abilities required, while chemoreception and mechanoreception becomes more important. There are also variations in the other living conditions including salinity of 35%, pH values usually around 8, and deep-water currents are slower compared to the shallow-water values. As a result, all these specific conditions cause unique processes enabling deep-sea organisms to survive in this environment and may result in the production of structurally different metabolites. Interest in deep-sea exploration has increased and new bioactive compounds have been reported including three novel cytotoxic compounds (21-23) from the deep-sea fungus *Phialocephala* sp., rossinones A (24) and B (25) with anti-inflammatory, antibacterial and antiviral activity from the deep-sea ascidian *Aplidium* sp. and levantilide A (26) from a deep-sea *Micromonospora* strain, which displayed antiproliferative activity against several tumour cell lines (Fig. 1.12).
Chapter 1

Introduction

Figure 1.12: Bioactive metabolites isolated from deep-sea marine organisms.

Deep-sea metabolites have been reported from a range of marine phyla along with a range of microorganisms such as archaea, bacteria and fungi. As for shallow-water organisms, the deep-water organisms have also proven to be a prolific source of metabolites from a diverse variety of structural classes including alkaloids (in particular bromoindoles), amino acids, polyketides, fatty acids, macrolides, terpenes, sterols, glycosides, and products of mixed biosynthetic pathways. The approval of eribulin mesylate (10) in 2010 by FDA, which is a synthetic analogue of a compound firstly isolated from the shallow-water Japanese sponge *Halichondria okadai* but also found in the deep sea marine sponge *Lissodendoryx* sp. from New Zealand, has significantly encouraged further exploration of deep-sea bioactive metabolites.\(^{52,88}\)

Deep sea MNPs have been derived from all parts of the world and from various environmental conditions. Expanded access to submersibles and remotely operated vehicle (ROV) technology through new collaborations, including with the oil and gas industry, are providing researchers today with the equipment they need to further discover and analyse deep-sea fauna and their natural products.\(^{89,90}\) Employing sophisticated ROV technology for deep-sea collections, as opposed to the more commonly used trawling operations, provides the opportunity to:

a) examine species interactions *in situ*, which are often important indicators of the likelihood of bioactive compound production;
b) select specific samples, such as those that are abundant in the area, increasing the chance of a successful scale-up procedure later on; and 

c) perform sampling with minimal environmental consequences.

The investigation of deep-sea MNPs shows that the most of the deep-sea metabolites were found in depths ranging from 100–400 m (50%), while only 10% were collected from depths of 500–600 m (Fig. 1.13). Only 8% of the compounds reported were obtained at depths below 1000 m, due to the greater difficulty in accessing bathyl and abyssal depths. Around 60% of the deep-sea natural products are reported to possess bioactivity, with over half exhibiting significant cytotoxicity towards a range of human cancer cell lines. This raises the question of whether deep-sea fauna are a richer source of bioactive metabolites than their shallow-water counterparts.

![Figure 1.13: Geographic origins of reported novel deep-sea natural products.](image)

**1.4 Drug synthesis based on natural product scaffolds**

Another aspect of drug discovery, in addition to isolating components from natural products, is the alteration of isolated metabolites from Nature and their structural adjustment towards improved bioactivity. Development of natural products includes modification (or removal) of functional groups, introduction of novel groups and stereocenters into the molecule, or occasionally more radical changes of the basic scaffold. Synthetic chemistry modifications of molecules can involve further structural diversity that can not be obtained from natural biosynthetic methods. NPs usually show excellent bioactivity and selectivity but might have poor pharmacokinetics, hence synthesis can be used to improve activity and decrease toxicity.
Three previously mentioned approved drugs, cytarabine, vidarabine and eribulin mesylate are all synthetic analogues developed from naturally occurring metabolites (Table 1.3).

Curcumin (27) is the major pigment obtained from turmeric and it was found along with several of its analogues to display moderate cytotoxicity (Fig. 1.14), while several synthetic analogues of curcumin exhibited more potent anti-androgenic activities against two human prostate cancer cell lines.\textsuperscript{91,92} Further development is underway because curcumin analogues were found to be more potent than the currently available drug for the treatment of prostate cancer, hydroxyflutamide.\textsuperscript{92} Betulinic acid (28) was isolated from the plant Syzygium claviflorum and found to display anti HIV-1 activity (Fig. 1.14).\textsuperscript{93} Dihydrobetulinic acid (29), which was obtained through hydrogenation of betulinic acid, exhibited a more potent anti-HIV activity.\textsuperscript{94} Encouraged by the initial results Kashiwada \textit{et al.} continued further modifications of betulinic and dihydrobetulinic acid to give compounds that displayed significant activity in acutely infected H9 lymphocytes (EC\textsubscript{50} < 3.5 x 10\textsuperscript{-4} \, \mu M).\textsuperscript{94} In addition, many other research groups are also engaged in the modification of 28 with the aim to achieve more potent compounds.\textsuperscript{95} Soler \textit{et al.} synthesized a novel series of betulinic acid derivatives and several members of this new series exhibited anti-HIV-1 activities in the nanomolar range in CEM-4 (T-lymphoblastic leukemia) and MT-4 (human T cell lymphotropic) cell lines.\textsuperscript{96}

\textbf{Figure 1.14:} Natural product based structures on which commercial drugs have been synthesized.

Not only are drugs made by modifying natural products but some natural products that are difficult to isolate are produced synthetically. Yondelis (9), isolated from a marine tunicate, is an example of a compound produced semi-synthetically. Yondelis is an anti-tumour drug approved for the treatment of soft tissue sarcoma.\textsuperscript{97} Due to the difficulties in the collection of large quantities of the producing organism and isolation of the desired compound, it is now prepared semi- synthetically from the microbial product cyanosafracin B.\textsuperscript{97} Along with the total synthesis of bioactive natural compounds, derived total synthesis is also used as a method that includes preparation of less complex structures than the target molecule. An example of a complex structure of a marine metabolite is halichondrin B (30), isolated from both shallow and deep-water sponges (section 1.3.3). It displayed several bioactivities and 30 is now considered
for further clinical development. However, due to limited sponge material and diminutive amounts in which 30 was obtained, other methods for the preparation of these components were explored. Aicher et al. synthesized eribulin mesylate (10, section 1.3), which is the shortened and simplified version of halichondrin B and equally active as the parent compound. (Fig. 1.15). Eribulin mesylate has been approved for breast cancer treatment in 2010, while halichondrin B is not approved as a drug.

![Halichondrin B](image)

**Figure 1.15**: The naturally occurring compound halichondrin B from sponge *Halichondria okadai*, which can now be obtained through synthesis.

### 1.5 Enzymes as drug targets

Most drugs used today demonstrate their bioactivity by acting as receptor antagonists or as enzyme inhibitors. If the drug acts as an antagonist it is interfering with an agonist in the body, while enzyme inhibitors interfere with the normal enzyme function. Nowadays, it is estimated that enzyme inhibitors make up almost half of the drugs in clinical use. Due to the significant role of enzymes in the activity of a number of disease processes, and their structural conformation that is easily accessible to inhibition of small drug like molecules, they are widely investigated as drug targets. The first step in the preparation of an enzyme inhibitor is defining which function of the target enzyme is going to be interrupted or blocked in a particular disease or infection.
1.6 Protein kinase inhibitors

Protein kinases are enzymes that encompass all enzymes that catalyse the chemical transfer of a phosphate group from a high energy molecule such as adenine triphosphate (ATP) to a specific substrate (amino acid) (Fig. 1.17). The human genome encodes for approximately 518 different protein kinases, which are divided into different kinase families on the basis of their selectivity for substrates. They are one of the largest enzyme families in the human body (Fig. 1.16). The covalent attachment of a phosphate group to a substrate requires a free hydroxyl moiety, and there are three amino acids that can provide this; serine, threonine and tyrosine. Therefore, serine/threonine (Ser/Thr) kinases will recognise and attach a phosphate group to a serine or threonine amino acid, while the tyrosine-specific protein kinase family will phosphorylate a protein at a tyrosine moiety. The vast majority of kinases are Ser/Thr kinase.
Protein kinases are important in almost every major pathway in eukaryotic cells and they play a central role in cellular activities and regulation, signal transduction in signal transmission pathways. Moreover, kinases have important roles in memory metabolism, cell growth, apoptosis, immune response, gene expression, oncogenesis, differentiation, controlling cell differentiation, proliferation, metabolism, DNA damage repair, cell motility, response to external stimuli and apoptosis. As a result, deregulation of kinases has been identified to be a main cause in an increasing list of diseases. Thus, a major aim of several pharmaceutical companies is to discover potent and selective inhibitors of kinases relevant to cancer, e.g. protein kinase C (PKC) and cyclin dependent kinases (CDKs). Kinases are also important in other diseases such as Alzheimer’s, e.g. glycogen synthase kinase 3β (GSK3β). Consequently, protein kinase inhibitors have emerged as an important class of targeted therapeutic agents.

Anticancer treatments involving kinase enzymes can be specifically targeted to cancer cells because numerous kinases have been found to be misregulated in cancerous cells. The current focus on kinases is in the development of drugs with lower side effects than previous cancer treatments, which traditionally focused on DNA replication and chromosome regulation and thus also affected many healthy human cells. The development of kinase inhibitors has been predicted to be a major driver of pharmaceutical growth with more than 130 kinase inhibitors reported to be in either Phase I or Phase II clinical trials, the majority of these being tested for
their potential as cancer treatments. Nowadays, it is estimated that one-third of pharma drug discovery programs target kinases. Knowledge of signalling pathways and the ways in which a cell respond to external signals have led to protein kinases becoming important targets for anti-cancer drug design. Imatinib (31) is the first small tyrosine kinase inhibitor that was approved for clinical use and with the further approval of other inhibitors such as erlotinib (32) and gefitinib (33) it was shown that these small molecule kinase inhibitors can be effective drugs (Fig. 1.18).

Kinases can exist in both active and inactive conformations. While there are many similarities in the active state, kinases exhibit many differences in an inactive state. The catalytic subunit of kinases consists of the smaller N-terminal lobe that contains five β-sheets and one α-helix; the C-helix and C-terminal lobe that is mostly α-helix. A hinge segment is between the two lobes (Fig. 1.19). Important regions of the ATP binding site include the hydrophobic purine-binding cavity, the hinge region, the P loop and the activation loop. ATP binds in the cleft where the adenine ring forms hydrogen bonds with the kinase “hinge” region, the segment that connects amino- and carboxyl-terminal kinase domains. The ribose and tri-phosphate groups bind to the hydrophilic channel extending to the substrate binding site that features conserved residues that are essential to catalysis.

![Figure 1.19: Structural basis for the action of small molecule kinase inhibitors – ATP binding region.](image)

All kinases have a catalytic subunit that binds ATP. Therefore, the main problem in targeting kinases is their selectivity. Staurosporine, an inhibitor of many kinases, inhibits 104 of 113 kinases tested by Fabian et al. Many inhibitors failed in drug trials because of the lack of selectivity. Imatinib is found to inhibit other kinases in addition to its primary drug target which indicates that an inhibitor doesn’t require absolute selectivity for an acceptable safety profile but it is essential to know its off-target effects. Despite concerns for kinase selectivity it has been established that kinase specific inhibitors can be developed. Kinase inhibitors achieve their
specificity by either directly targeting points that are structurally divergent in kinases or by exploiting differences in the conformational flexibility of the ATP-binding site. All kinase inhibitors that are in clinical use bind at the ATP binding site except one that targets the mammalian target of rapamycin (mTOR). Most kinase inhibitors that are marketed or are in development, inhibit multiple kinases. Among compounds with similar scaffolds some can be very specific toward one particular kinase, while others can display low selectivity and bind to several kinases.

The majority of kinase inhibitors are ATP competitive and bind to the hinge region with one to three hydrogen bonds (Fig. 1.19). Even though the ATP binding site exhibits great similarities among protein kinases, some of the enzymes display differences in the binding pocket. For instance, the ACG protein kinase subfamily that includes PKA, protein kinase B (PKB or Akt) and Rho-associated protein kinase (ROCK) have a specific phenylalanine residue in the adenine binding site (Fig. 1.20). Phenylalanine distinguishes the adenine pocket of the AGC subfamily. It is found on the C-terminal chain of the protein kinase catalytic domain, which folds back into the N-lobe across the catalytic cleft. As a result, the phenylalanine aromatic ring shields one side of the adenine pocket and affects the protein-ATP or protein-kinase inhibitor contact. Competitive inhibitors recognize the active form of an enzyme. Typical for these inhibitors is that they have a heterocyclic ring that mimics ATP. The other types of inhibitors are compounds that recognize the inactive enzyme conformation. The most selective inhibitors are allosteric inhibitors because they bind to the allosteric site of enzyme which is unique for the particular kinase.

**Figure 1.20:** View of PKA in complex with ATP. Phenylalanine is located on the C-terminal polypeptide chain (dark blue), which folds back into the N-lobe, shields the entrance side of the adenine pocket.
1.6.1 cAMP-Dependant protein kinase (PKA)

cAMP-Dependant protein kinase (protein kinase A, PKA) is completely characterised and the simplest protein kinase enzyme, which was the one of the first to be discovered, sequenced and cloned. Its simplicity is related to the mechanism by which it is activated. In the inactive state PKA is a tetramer, consisting of two regulatory and two catalytic subunits (Fig. 1.21). Two catalytic subunits bind to a regulatory subunit dimer to form an inactive holoenzyme complex.

G-protein coupled receptors (GPCR) are one of the largest gene families of signalling proteins. When a GPCR is activated by extracellular hormones, it stimulates Ga subunits and becomes active and activates enzyme adenylyl cyclase that synthesizes cyclic-AMP (cAMP) from ATP (Fig. 1.21). Activation of cAMP signalling involves binding of an extracellular signalling ligand to the GPCR which through G proteins regulates one of several isoforms of adenylyl cyclase leading to generation of cAMP. cAMP binds to the inactive holoenzyme PKA, in particular to its regulatory subunit which causes dissociation of the tetramer PKA complex giving R-dimer complex and two monomeric catalytic subunits. The monomeric catalytic subunit is the active form of the PKA enzyme. The free catalytic subunits are able to catalyse the transfer of ATP to a specific substrate (Ser or Thr protein residue).

PKA is an ubiquitous enzyme involved in the phosphorylation of a wide range of proteins, ion channels and transcription factors. It has been demonstrated to regulate a number of physiological processes including cardiovascular function, steroid biosynthesis, reproductive function, myogenesis, adipocyte metabolism, exocytotic processes and immune function. PKA was also found to have a key role in memory processes. The cAMP-PKA pathway has been linked to the promotion of malignant phenotypes of head and neck squamous cell
carcinoma and demonstrated to be activated in a range of tumours.\textsuperscript{124} Conversely, PKA inhibitors have been found to display both \textit{in vitro} and \textit{in vivo} antitumour activity against various human cancer cell lines and to enhance monocyte function in HIV-infected patients.\textsuperscript{125} Thus, it is becoming increasingly apparent that the ability to selectively inhibit PKA provides a new way of potentially modulating cancer, immune function, and memory disorders such as Alzheimer’s disease, Parkinson’s disease and schizophrenia.\textsuperscript{122,123,126}

The most well known PKA inhibitors are staurosporine (34) from \textit{Streptomyces} sp., plant-derived polyphenols such as ellagic acid (35) and piceatannol (36) and the synthetic isoquinolinesulfonamides such as H-89 (37) and H-9 (38) (Fig. 1.22). Many of these inhibitors have been derived from Nature, however, the marine environment has remained relatively unexplored in this area, with no marine-derived PKA inhibitors yet reported.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figures/fig1.22.png}
\caption{Known protein kinase A inhibitors.}
\end{figure}

\subsection*{1.6.2 Protein kinase B (Akt kinase)}

Akt belongs to a group of serine-threonine protein kinases and is component of the phosphatidylinositol 3-kinase (PI3K)/AKT signalling pathway (Fig. 1.23).\textsuperscript{127} Akt is activated by the phosphoinositide 3-phosphate-dependent kinases PDK1 and PDK2, receptor tyrosine kinases and integrins.\textsuperscript{127,128} Akt belongs to the AGC family of kinases, which consists of 16 kinase families that have related catalytic domains, and shares high homology with PKA and PKC. The PI3K/AKT signalling pathway is found to have a significant role in the organism and its interruption leads to apoptosis in cancer cells.\textsuperscript{129} Akt kinase has an important role in cellular processes that are involved in the formation and growth of cancer, including cell migration, proliferation, transcription, glucose metabolism and apoptosis and its overexpression has been detected in a number of cancer types.\textsuperscript{130}
Since its report in 2002 it has received over 2000 citations in the literature and is certainly one of the prime targets in the development of new kinase inhibitors. Figure 1.24 shows some of the reported Akt kinase inhibitors.

**Figure 1.23**: PI3K/Akt signalling pathway.\(^{131}\)

**Figure 1.24**: Akt (PKB) kinase inhibitors.\(^{132-134}\)
1.7 Kinase inhibitors from marine invertebrates

The most well-known kinase inhibitor from a marine source is bryostatin-1 (43), which was first isolated from the marine bryozoan *Bugula neritina* (Fig. 1.25).\textsuperscript{135,136} Bryostatin-1 is unique in that its mechanism involves the activation and subsequent down-regulation of protein kinase C (PKC) and has been shown to inhibit tumour growth and invasion and also angiogenesis.\textsuperscript{137} Bryostatin-1 has been involved in over 80 Phase I, II and III combinatorial anticancer clinical trials during the past two decades.\textsuperscript{138} In addition, Bryostatin-1, in combination with Taxol (Paclitaxel), was granted Orphan Drug Designation by the US FDA for oesophageal cancer in 2001.\textsuperscript{139} Currently, 43 is in clinical trials of various phases for Alzheimer’s disease, metastatic solid tumours, advanced kidney cancer, relapsed acute myelogenous leukaemia, metastatic pancreatic cancer, lung cancer and ovarian and cervical cancer.\textsuperscript{140}

![Bryostatin-1 (43)](image1)

![11-Hydroxystaurosporine (44)](image2)

**Figure 1.25**: Marine natural product based kinase inhibitors.

The efficacy of the natural product staurosporine (34) as a PKC inhibitor has been known since last century when the alkaloid was isolated from the bacteria *Streptomyces stauroporeus* and shown more recently to have an IC\textsubscript{50} value of 2.7 nM against PKC.\textsuperscript{141} More recently, derivatives of 34 have also been shown to be powerful inhibitors of PKC including 11-hydroxystaurosporine (44), which was the first indolo[2,3-α]carbazole to be identified from a marine organism.\textsuperscript{142} Isolated from the marine tunicate species *Eudistoma*, 44 reported an IC\textsubscript{50} value of 2.2 nM against PKC, approximately 30% more active than the parent compound staurosporine, both of which inhibit PKC by blocking the ATP catalytic domain.\textsuperscript{142} Also highly
cytotoxic, 44 has been incorporated into pharmaceuticals patented to inhibit hair growth and tested for use as an anticancer agent although these have been limited by the high toxicity of staurosporine at therapeutic doses caused by cross-inhibition of other critical enzymes.\textsuperscript{135,142,143}

In 1994, the sponge Xestospongia sp. collected in waters off the Papua New Guinea coast, furnished xestocyclamine A (45) bearing a novel skeleton and found to inhibit PKC with an IC\textsubscript{50} value of 4 μg/ml (Fig. 1.26).\textsuperscript{144} Xestocyclamine A and its pure enantiomer (-)-xestocyclamine A are considered critical PKC inhibitors for use in the development of anticancer drugs and there are many research groups focused on synthesising the stereochemically complex marine alkaloids.\textsuperscript{145,146} (Z)-Axinohydantoin (46) and debromo-Z-axinohydantoin (47) are two PKC inhibitors with respective IC\textsubscript{50} values of 9.0 and 22.0 μM that were isolated from the marine sponge Stylotella aurantium.\textsuperscript{147} These novel compounds were isolated during a scale-up collection of the PKC inhibitors, hymenialdisine (48) and debromohymenialdisine (49) from the same sponge species.\textsuperscript{147} Hymenialdisine is found to inhibit a range of kinases. Five novel sesquiterpene derivatives, frondosins A-E (50-54), were isolated from the marine sponge Dysidea frondosa and shown to have inhibitory activity against PKC with reported IC\textsubscript{50} values of 1.8, 4.8, 20.9, 26.0 and 30.6 μM respectively (Fig. 1.26).\textsuperscript{148} Frondosins A-E were also reported to be inhibitors of interleukin-8 in the low micromolar range.\textsuperscript{148} More recently (-)-frondosin A and D have shown comparable activity against the HIV virus.\textsuperscript{149}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure126.png}
\caption{PKC inhibitors isolated from marine sponges.}
\end{figure}
As previously mentioned, poor selectivity is a common problem among kinase inhibitors due to the ATP binding site, where many inhibitors exert their actions, being conserved amongst the majority of kinase families. Fascaplysin (55, Fig. 1.27), a red pigment isolated from the marine sponge Fascaplysinopsis sp., has the major distinguishing feature because it is a selective inhibitor of CDK-4. Fascaplysin exhibits an IC$_{50}$ value of 0.35 μM against the CDK-4/cyclin D complex, while IC$_{50}$ values against other kinases were comparably much higher. Fascaplysin (55) has an indole moiety which is also present in 11-hydroxystaurosporine (44) described earlier (Fig. 1.27). The indole moiety has also been found in meridianin (e.g. 56) and granulatamide (e.g. 57) derivatives, isolated from marine organisms that have been found to inhibit various kinases. These results indicate that a number of indole compounds are potent kinase inhibitors, hence suggesting that the indole nucleus is required for kinase inhibition.

![Fascaplysin (55) IC$_{50}$ = 0.35 μM (CDK-4/cyclin D)](image)

![Meridianin F (56) IC$_{50}$ = 2.5 mmol/l (PKA)](image)

![Isogranulatamide (57) IC$_{50}$ = 0.1 μM (Chk1)](image)

**Figure 1.27:** Indole based kinase inhibitors isolated from marine organisms.

### 1.8 Isatin-indole based kinase inhibitors

A range of indole and isatin-based compounds have been developed as inhibitors of various protein kinase families, predominantly receptor tyrosine kinases (RTKs) and serine/threonine-specific protein kinases such as CDKs. Research of indole and isatin derivatives as kinase inhibitors rose notably after the disclosure of the tyrosine kinase inhibitory properties of semaxanib (SU-5416), sunitinib (SU-11248) and SU-9516 (Fig. 1.28). Sunitinib is a multi-targeted receptor tyrosine kinase inhibitor that was approved by FDA for the treatment of renal cell carcinoma in 2006. Semaxanib is a tyrosine kinase inhibitor and is
currently in the experimental stage of drug development. SU-5416 and SU-11248 were reported to exhibit protein tyrosine kinase inhibitory and antiangiogenic properties, while SU-9516 was found to be an inhibitor of CDK that induces apoptosis in colon carcinoma cells.\(^\text{152}\) Crystal structures of the tyrosine kinase domain of fibroblast growth factor receptor 1 (FGFR1) in complex with the two indolin-2-one compounds was analysed previously.\(^\text{153}\) The results indicated that two 2-oxindole compounds bind to the enzyme in the manner almost identical to ATP binding, the oxindole part of the molecule occupies the site in which the adenine of ATP binds. The C-3 oxindole part of the molecule takes place, that is occupied by the rest of the ATP structure, the moieties that extend from the oxindole extend into the hinge region between the two kinase lobes.\(^\text{153}\)

![Chemical structures of SU-11248, SU-5416, and SU-9516](image)

**Figure 1.28:** Structures of 2-indoline derivatives currently approved for use, or in clinical trials as anticancer agents.

A series of \(N\)-benzyl-indole-3-imine and 3-amine compounds were prepared and analysed by Kilic et al. for their kinase activity against the pp60c-Src tyrosine kinase, as potential new therapeutics for the treatment of hepatitis B and rheumatoid arthritis (e.g. \(\text{61, Fig. 1.29}\)).\(^\text{154}\) The authors found that bromination at the position 5 of the indoline core was important for the kinase inhibitory activity and also indole amine derivatives were more potent than their imine analogues.\(^\text{154}\) Src kinase activity of indolin-2-one analogues was also reported by Guan et al. along with the inhibitory activity against Yes kinase.\(^\text{155}\) The authors showed the importance of the sulfonyl group at the 5-position, the tetrahydroindole ring and the 3-aminopropyl side chain for inhibitory activity against Src (e.g. \(\text{62, Fig. 1.29}\)).\(^\text{155}\)

Indoles bearing a sugar moiety at the aromatic nitrogen of the indole molecule were screened for kinase inhibitory and antibacterial activity.\(^\text{156}\) The addition of the sugar residue on the indole ring improved solubility and slightly enhanced interaction with the enzyme active site, these analogues only exhibited low kinase inhibitory activity (e.g. \(\text{63, Fig. 1.29}\)).\(^\text{156}\) To further improve the activity of these compounds, isoindigo derivatives bearing a sugar moiety with benzyl groups at the \(N-1\)-position of the molecule were prepared and they displayed potent
activity against CDK2 (e.g. 64, Fig. 1.29).\textsuperscript{157} A range of compounds with an indolin-2-one core as CDK2 inhibitors were reported by Tymoshenko et al. who found the importance of the secondary amine group on the 4’-position of the phenyl group of indoline for incorporating at the ATP binding cleft (e.g. 65, Fig. 1.29).\textsuperscript{158} Sun et al. discovered a range of indolin-2-one derivatives as potential protein tyrosine kinase (PTK) inhibitors finding that the activity of analogues is dependent on the substituents at position 3 of the indoline molecule.\textsuperscript{159} PTK has a vital role in the cell controlling signal transduction processes (e.g. 66, Fig. 1.29).\textsuperscript{160} Li et al. investigated the indolin-1-one core further and developed novel potent PTK inhibitors by replacing the α-pyrrole ring with a β-ring and adding methyl group at the 5’-position of the pyrrole ring (e.g. 67, Fig. 1.29).\textsuperscript{160}

The activity of oxindole derivatives against janus kinase 3 (JAK 3) was demonstrated by Adams et al. (e.g. 68, Fig. 1.29).\textsuperscript{161} Compounds with an indoline core were also found to be potent inhibitors of Ser/Thr phosphoinositide-dependant kinase-1 (PDK1), which is the major activator of many protein kinases in the AGC kinase family (e.g. 69, Fig. 1.29).\textsuperscript{162} Indoline analogues are potent inhibitors of another Ser/Thr kinase, checkpoint kinase 1 (Chk1) which has a linkage role between DNA damage repair and cell cycle arrest at G2 (e.g. 70, Fig. 1.29).\textsuperscript{163} Among many tested inhibitors compound 70 was found to be the most active with an IC\textsubscript{50} value of 4 nM, showing the importance of the hydroxyl group at the C4’ position and the unsubstitution at the pyrrole ring.\textsuperscript{163} Indolin-2-ones were also found to be inhibitors of RET tyrosine kinase, an important tyrosine receptor involved in many tumours (e.g. 71, Fig. 1.29).\textsuperscript{164} Compounds with a pyrrole ring at position 3 displayed the highest activity, which is due to the Z configuration compared with the other analogues that usually have a predominant E configuration such as furan-substituted analogues.\textsuperscript{164} Also, substitution of the 5 position was important as bulky and hydrophobic groups exhibited low activity.\textsuperscript{164}
Synthetic isatin, firstly prepared by oxidation of indigo by Erdmann and Laurent more than a hundred years ago, was discovered to be a natural product also from plants of the *Isatis* genus, in fruits of the cannon ball tree *Couroupita guianensis* Aubl. and in secretions from the parotid gland of the *Bufo* frog.\textsuperscript{165-170} Afterwards isatin was also isolated from the marine bacterium, *Pseudomonas* C55a-2 strain, while various isatins derivatives have now been identified in plants, fungi, bryozoan and marine molluscs (Fig. 1.30).\textsuperscript{171-178}

Figure 1.29: Indole and isatin based kinase inhibitors.

Figure 1.30: Sources of isatins found in Nature a) *Isatis tinctoria*;\textsuperscript{179} b) the toad *Bufo gargarizans*;\textsuperscript{180} and c) the marine mollusc *Dicathais orbita*.\textsuperscript{181}
Isatin was also identified in humans, however it’s biosynthetic and metabolic pathways have not yet been discovered.\textsuperscript{182} It is assumed that isatin can be obtained through tryptophan-rich food, as tryptophan can potentially be converted to an indole by bacteria from the gastrointestinal tract and then be transported and oxidised in the liver.

1.9 Previous research in our group

A range of isatin derivatives were previously investigated in our research group for cytotoxicity against a range of human cancer cell lines and found to display potent activity, causing cell proliferation in all cancer cell lines studied (e.g. breast, colon, lung etc). Isatin derivatives have been found to inhibit cell proliferation and to promote apoptosis.\textsuperscript{183}

Investigation on isatin and its derivatives as potential cytotoxins started at the University of Wollongong in 2002 during the study of egg masses of the Australian mollusc \textit{Dichathais orbita} by Benkendorff \textit{et al.}\textsuperscript{184} The chloroform extract of the egg masses exhibited potent cytotoxicity and further fractionation of the extract yielded tyrindoleninone (72), tyriverdin (73) and 6-bromoisatin (74) (Fig. 1.31). Of these three compounds, tyrindoleninone was found to exhibit the highest cytotoxicity against human monocyte-like histiocytic lymphoma cells (U937). Tyriverdin exhibited weak activity and 6-bromoisatin moderate activity (Fig. 1.31). However, due to the reactivity of tyrindoleninone, 6-bromoisatin was used as the starting point for the further development and investigation of novel cytotoxin agents.

![Figure 1.31: Components isolated from the egg masses of the Australian mollusc \textit{Dichathais orbita} tested against human monocyte-like histiocytic lymphoma cells (U937).](image)

Development of more potent isatin analogues showed that electron withdrawing groups at position 5, 6 and 7 are important for increased cytotoxicity and in particular substitution at the 5 position was most significant.\textsuperscript{183} The results suggested that the cytotoxicity increased with halogenation (6.8, 7.8 and 10.5 \textmu{}M respectively) and was not related to their lipophilicity.\textsuperscript{183}
Chapter 1

Introduction

Figure 1.32: Cytotoxicity of halogenated isatin derivatives (against U937 human lymphoma cell lines).\(^{185}\)

\(N\)-alkylation of isatin analogues led to improved cytotoxicity against a variety of cancer cell lines. Furthermore, Vine et al. found that isatin derivatives cause G2/M cell cycle arrest and induce apoptosis via binding to the cytoskeletal protein tubulin (Fig. 1.32).\(^{186}\) The authors used the fluorescence emission to monitor the rate of polymerization. The \(N\)-alkylisatins are found to interrupt natural tubulin polymerisation by binding to microtubules and either stabilising or destabilising the dynamic assembly and disassembly required for cell division, similarly to known drugs vinblastine, taxol and colchicine (Fig. 1.33).\(^{188,189}\) Vinblastine has been found to be a potent microtubule destabiliser, while paclitaxel is a known stabiliser.

Figure 1.33: Effects of \(N\)-alkyl isatins on tubulin polymerisation. DMSO was used as a control, a shift of the curve to the left or right of the control is indicative of either an increase or a decrease, respectively, in the rate of tubulin polymerization.\(^{189}\)

The substituents on the isatin ring were also found to influence the mode of action of isatin derivatives. For example, the presence of a small electron-withdrawing group(s) on the aromatic (A) ring of the isatin core (Fig. 1.34) improves cytotoxicity.\(^{186}\) A one-carbon linker attaching an alkyl group to N1 leads to the potent cytotoxicity with nanomolar activity.\(^{189}\)
Substitution at position C2 gives compounds that inhibit CDK1, CDK2 and GSK kinase, while the substitution on the C3 carbonyl group provides inhibitors of VEGFR-2, PDGFR-β and epidermal growth receptor.

**Figure 1.34:** Position of different substituents on isatin ring and their influence on biological activities of molecule.

Furthermore, with over 150 cytotoxic isatin derivatives previously synthetised within our group, the next step was to conjugate these compounds to tumour target protein in pro-drug delivery. The conjugated isatin drugs (79-83, Fig. 1.35) were formed from potent N-alkylated isatin (78, 1.83 µM against U937 cells). Due to the high similarity of these compounds to the known kinase inhibitors sunitanib and semaxenib and many indole kinase inhibitors in literature (Fig. 1.29), these compounds were screened for protein kinase A inhibitory activity. The 3-iminoisatins exhibited potent PKA inhibitory activity (> 80% inhibition at 100 µg/mL), comparable to the inhibitory activity of the commercially available PKA standards used in the assay. These results showed a promising start for further exploration of these compounds, their modification and kinase testing.

**Figure 1.35:** Synthesis of isatin-imine analogues for conjugation to tumour targeting proteins.
1.9 Aims of Project

The research described herein was divided into two sections:

1. Isolation, purification, identification and biological evaluation of marine natural products from marine algae and deep-sea sponges; and
2. Structural modification of known isatin derivatives to develop novel PKA inhibitors.

In a two-pronged approach of discovering and developing new kinase inhibitors, the specific aims of the two sections, that comprised of both classical natural product chemistry and synthetic medicinal chemistry, were:

- **Section 1: Natural products:**
  - Collection, identification and extraction of:
    - Local, abundant but understudied marine algae and
    - Rare deep-sea sponges.
  - Biological screening of the extracts for protein kinase A (PKA) inhibition, brine shrimp cytotoxicity and haemolysis.
  - Fatty acid and sterol composition of the marine organism extracts and purification and structure elucidation of the metabolites responsible for the bioactivity observed.

- **Section 2: Synthetic medicinal chemistry:**
  - Synthesis of a wide variety of isatin-imine analogues with variations at positions N1, C3, C5 and C7 (Fig. 1.36).
  - Biological evaluation of the above derivatives as potential new PKA and PKB inhibitors along with their evaluation as cytotoxic agents.

![Figure 1.36: General scheme of the proposed isatin based kinase inhibitors.](attachment:image.png)
Natural structures represent the core foundation for the development of new drugs. Among the diverse range of organisms found in marine fauna, marine algae have an important role as a source of novel drugs. Herein, is described the PKA inhibitory, haemolytic and cytotoxic activities and chemical composition of extracts obtained from ten algae collected off the South Eastern Australian coast.

2.1 Metabolites from marine algae

Marine algae produce a variety of secondary metabolites and are an exceptionally rich source of pharmacologically active metabolites. About 6000 species of seaweeds have been identified so far and are grouped into two categories: macroalgae, which includes green (Chlorophytes), brown (Pheophytes) and red (Rhodophytes) algae; and microalgae, which contains bluegreen (Cyanobacteria) algae. Algal species are widespread and readily available for collection, however in many regions of the world they have not yet been explored in detail.

Every year a plethora of novel and bioactive compounds are isolated from marine algae, in particular from brown and red algae species, while less novel metabolites have been discovered from green algae, although they are broadly distributed. There are approximately 40 papers published annually about the isolation of bioactive constituents from green algae, which are typically found to be a source of non-polar metabolites such as the known linolenic [18:3 (n-3)] and linoleic [18:2 (n-6)] acids as potent peroxisome proliferator-activated receptor gamma (PPAR a/g) agonists from Chlorella sorokiniana and two unsaturated cytotoxic FAs and the triterpenoid disulfate isolated from Tydemania expeditions (Fig. 2.1).

Brown algae usually produce a wide range of novel terpenoids such as the brominated sesquiterpenoids from Dictyopteris divaricata and the meroditerpenoids from Cystoseira baccata. However a wide variety of other metabolites have also been isolated from brown algae including: the phlorotannin diphlorethohydroxycarmalol with radical-scavenging activity from the Japanese alga Ishige okamurae and six bromophenols (e.g) from Leathsia nana of which 94 displayed potent cytotoxicity against eight cancer cell lines with IC_{50} values below 10 μg/mL (Fig. 2.1). Reports on the isolation of novel compounds from red algae decreased in the last couple of years. However, research has returned to the previous prolific levels in the last two years and red algae have proven once again to be a productive source of novel bioactive metabolites. In particular, red algae are rich in terpenes and polyhalogenated metabolites along with compounds such as the bromoindole alkaloids from Laurencia similis and the halogenated chamigranes from L. saitoi.
2.2 Biological assays

2.2.1 Kinase inhibition assay

Natural product extracts are often screened for cytotoxicity in whole cell assays against both human and murine cancer cell lines. However, in recent years testing for inhibition against specific enzymes has become more widespread, as this provides greater information about a natural product’s mode of action. Herein, screening against the enzyme PKA was performed because c-AMP dependent protein kinase (PKA) is an important enzyme involved in the regulation of an increasing number of physiological processes including immune, cardiovascular and reproductive functions; steroid biosynthesis; adipocyte metabolism; and exocytotic processes. As such, the inhibition of PKA has lately become an attractive drug target in a number of areas, in particular in immune function and for memory disorders such as Alzheimer’s and Parkinson’s disease and schizophrenia. However, it only recently become studied as a drug target and not been validated for any particular disease state yet.\textsuperscript{121,122}
Protein kinase A inhibitory activity of extracts of different polarity was determined using a Promega luminescence kinase assay. For measuring luminescence this assay uses bioluminescence, which is one type of chemiluminescence found in living organisms. The assay involves two reaction steps (Fig. 2.2). In the first step, the reaction between the kinase enzyme, ATP, an appropriate protein substrate (kemptide) and sample extract as a potential kinase inhibitor occurs. The ability of the sample to inhibit the PKA enzyme is directly proportional to the amount of ATP left behind in the first reaction step, which is then quantified in the next step using the enzyme luciferase (step 2). Luciferase is an enzyme isolated from firefly. This enzyme uses ATP for catalysing beetle luciferin to form the intermediate luciferin-AMP. In the next step another intermediate is formed of oxygen and the first intermediate-oxyluciferin, which is in a high-energy state. Energy transition from the exited state to the ground state yields yellow-green light with a spectral maximum of 560 nm. For mono-oxygenation, luciferase produces one photon of light per reaction turnover.

The common detection method in many bioassays is either colorimetric or fluorescence. However, the luminescence method described herein, is particularly desirable for coloured products such as marine natural products. Marine algae usually provide very colourful extracts as a result of their high chlorophyll level, which can give false results when using absorbance as the end point. Therefore, utilization of luminescence, can be beneficial and more useful for biological screening as it can identify more hits with a lower number of false positives.

To-date a number of potent kinase inhibitors have been isolated from algae species including three cycloartanol sulfates from the green algae Tydemania expeditionis, which are found to be modest inhibitors of protein tyrosine kinase pp60v-src (99-101). Furthermore, the new terpenoid compound stypoquinonic acid (102) isolated from brown algae Stypopodium zonale exhibited inhibition of protein tyrosine kinase p56lck and also two bioactive glycoglycerolipids (103-104) that exhibited activity against the enzyme Myt1 kinase were isolated from an unidentified alga species (Fig. 2.3).
2.2.2 Haemolytic assay

Haemolysis is the release of haemoglobin and other internal components into the surrounding fluid caused by the breakage of red blood cell membranes.\textsuperscript{201} Haemolytic activity, prevalent among marine organisms, is a general indicator of bioactivity and membrane-directed cytotoxicity, which is desirable in the development of bactericidal molecules and targeted intracellular drug delivery.\textsuperscript{202-204} Herein, a haemolysis assay was used to determine whether samples could cause lysis of horse red blood cells, releasing haemoglobin, which is measured on a spectrophotometer at 550 nm. Its concentration is then determined relative to positive and negative controls giving readings of 0% and 100% haemolysis respectively. Haemolytic activity was previously found among a range of compounds isolated from marine algae including two novel diterpenes, amijiol acetate and amijiol-7,10-diacetate obtained from the brown alga \textit{Dictyota dichotoma} collected from the Red Sea and glycosidic toxins prymnesins isolated from the brown alga, \textit{Prymnesium parvum}.\textsuperscript{205,206}

2.2.3 Brine shrimp (cytotoxic) lethality assay

In addition to screening for specific enzyme inhibitors, the brine shrimp lethality assay is a simple and inexpensive way to screen natural product extracts for pharmacological activity.\textsuperscript{207} Brine shrimp lethality is one of the assays that has been used for many years in natural product discovery to determine the toxicity of both plant extracts and pure compounds. This cytotoxic assay has to its advantage that it is fast, reliable and an easy to perform bench assay for extracts of different polarity as well as pure compounds. Nowadays, natural product extracts are subjected to many different assays to test for numerous activities, which can be a
complicated and long process. Another benefit of this method is that the extracts are tested for a broad spectrum of activities, which are usually found in natural products and therefore can give preliminary results of the active sample. A strong correlation has also been found between brine shrimp toxicity and cytotoxicity in human cancer cell lines. Cytotoxicity is also the most commonly reported biological activity in marine fauna, in particular marine sponges.

2.3. Algal collection and identification

A variety of different seaweeds were collected by hand from four different locations in South-East Australia (Fig. 2.4; Table 2.1; Fig. 9.1). Collection places were chosen on the basis of reports identifying them as a biodiversity hotspots. Samples from Bellambi and Bass point were taken from, rocky platforms and rock pools, while samples in Austinmer where collected from the walls of the local pool. One alga species was collected from Jones beach.

![Figure 2.4: Four collection places of alga samples in South-East Australia: A) Bellambi Pt; B) Bass Pt; C) Austinmer; and D) Jones beach.](image)

<table>
<thead>
<tr>
<th>Entry</th>
<th>Phyla</th>
<th>Scientific Name</th>
<th>Location</th>
<th>Habitat</th>
<th>Weight*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Rhodophyta</td>
<td><em>Prionitis linearis</em></td>
<td>Austinmer</td>
<td>PS, P</td>
<td>45 g</td>
</tr>
<tr>
<td>2</td>
<td>Rhodophyta</td>
<td><em>Corallina vancouveriensis</em></td>
<td>Austinmer</td>
<td>PS, P</td>
<td>53 g</td>
</tr>
<tr>
<td>3</td>
<td>Chlorophyta</td>
<td><em>Codium fragile</em></td>
<td>Bellambi Pt</td>
<td>FS</td>
<td>129 g</td>
</tr>
<tr>
<td>4</td>
<td>Chlorophyta</td>
<td><em>Codium dimorphum</em></td>
<td>Bellambi Pt</td>
<td>PS, RP</td>
<td>90 g</td>
</tr>
<tr>
<td>5</td>
<td>Chlorophyta</td>
<td><em>Ulva lactuca</em></td>
<td>Austinmer</td>
<td>PS, P</td>
<td>65 g</td>
</tr>
<tr>
<td>6</td>
<td>Phaeophyta</td>
<td><em>Colpomenia sinuosa</em></td>
<td>Bass Pt</td>
<td>FS</td>
<td>230 g</td>
</tr>
<tr>
<td>7</td>
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<td><em>Phyllospora comosa</em></td>
<td>Bellambi Pt</td>
<td>FS</td>
<td>140 g</td>
</tr>
<tr>
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<td><em>Padina sp.</em></td>
<td>Austinmer</td>
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<td>195 g</td>
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<tr>
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<td><em>Sargassum vestitum</em></td>
<td>Jones Beach</td>
<td>PS, P</td>
<td>500 g</td>
</tr>
<tr>
<td>10</td>
<td>Phaeophyta</td>
<td><em>Sargassum sp.</em></td>
<td>Bellambi Pt</td>
<td>PS, RP</td>
<td>30 g</td>
</tr>
</tbody>
</table>

*Wet weight of the collected alga samples. PS = partial shade; P = pool; S = shade; FS = full sun; RP = rock pool; PW = pool walls.

2.4 Algal description and previous (reported) studies

*Prionitis linearis*: The seaweed *Prionitis linearis* is a red alga, representative of the family Halymeniaceae, belonging to the Order Halymeniales, Division Rodophyta. *Prionitis linearis* is up to 30 cm tall, dark red to brown in colour, growing in clusters. It is usually found near the shore, along the Pacific coast. To date, there have only been a few investigations on both bioactivity and chemical composition of this red alga.
**Corallina vancouveriensis**: The seaweed *Corallina vancouveriensis* is a red alga, representative of the family Corallinaceae, belonging to the order Corallinales, division Rodophyta. The thallus is calcareous, light pink to light purple, forming dense tufts to 6 or more cm tall from a basal crust. This species forms dense mats on emergent bedrock or in tide pools in the mid to low intertidal zones of exposed habitats. This species have been found to exhibit a variety of biological activities, while there have been only a few studies on its metabolites.

**Codium fragile**: The seaweed *Codium fragile* is a shallow water green alga, representative of the family Codiaceae, belonging to the order Bryopsidophyceae, division Chlorophyta. The species inhabits the middle and lower intertidal zone as well as subtidal regions of rocky shores. The common name for this alga is dead man’s finger, which comes from the shape of the species. It has a dark green colour and consists of branched segments that can be up to 40 cm long. The alga is distributed worldwide. Fatty acid composition was studied in detail of this green alga, while extracts have been found to display a range of biological properties.

**Codium dimorphum**: The seaweed *Codium dimorphum* is a shallow water green alga, representative of the family Codiaceae, belonging to the order Bryopsidophyceae, division Chlorophyta. *Codium dimorphum* is a blue, dark green alga, smooth and firm, hairless on the surface, forming rounded patches on the rock. It occurs in warm climates like in South America, Australia and Tasmania. Although there have been investigation in other *Codium* species, there are very few reports of bioactivity and chemical composition of *C. dimorphum*.

**Ulva lactuca**: The green seaweed *Ulva lactuca*, commonly known as sea lettuce, is a green alga, representative of the family Ulvaceae, belonging to the order Ulvales, division Chlorophyta (green algae). *Ulva lactuca* is commonly found on rocky shores distributed worldwide on all continents. Sterol composition and bioactivity of extracts isolated from the genus *Ulva* have been studied previously.
**Colpomenia sinuosa**: The seaweed *Colpomenia sinuosa* is a shallow water brown alga, representative of the family Scytosiphonaceae, belonging to the order Scytosiphonales, division Phaeophyta. *C. sinuosa* is a small, globular, hollow, and leathery alga of brown-olive colour. It is distributed in the intertidal zone at the water's edge and is widespread all over the world. The biological activities of this species have been studied, while there is little information on the chemical composition of this alga.

**Phyllospora comosa**: The seaweed *Phyllospora comosa* is a shallow water brown alga, which can be found up to 20 m depth, representative of the family Seirococcaceae, belonging to the order Fucales, division Phaeophyta. It is distributed in the intertidal zone at the water's edge and is widespread all over the world. *P. comosa* is a dark green/brown colour, up to 3 m long, branched with single and short stipes. *Phyllospora* is endemic in temperate regions of Australia. So far, only one report was published on the chemical constituents of this alga, while there is no biological data on this alga.

**Padina sp.**: The brown seaweed *Padina* sp., which is distributed worldwide, is a representative of the family Scarabaeoidea, belonging to the order Dictyotales, division Phaeophyta. It is golden-brown in colour and often found attached to a hard surface. It has a fan-like shape, consisting of a group of funnel shape fragments, and the common name is Mermaid’s ear or fan. Extracts from *Padina* sp. have showed an array of biological activities and the chemical composition concerning FA and sterol constituents of the genus *Padina* was also investigated earlier.

**Sargassum vestitum** (A) /**Sargassum** sp. (B): *Sargassum* is an olive green, brown colour alga, usually 1-2 m long and consists of perennial, dark brown basal axes, and lighter coloured annual primary laterals. This species is distributed in tropical and subtropical areas, representative of the family Sargassaceae, belonging to the order Fucales, division Phaeophyta. The genus *Sargassum* has been found to be a rich source of bioactive compounds, in particular of meroditerpenoids.
2.5 Biological screening of algal extracts

A total of ten alga samples collected from various Illawarra lower intertidal regions (Table 2.1) were extracted and their extracts screened for PKA inhibitory, haemolytic and cytotoxic activity (Fig. 2.5). The ten samples, comprising two red seaweeds, five brown seaweeds and three green seaweeds were divided into two groups. The first group (90-500 g each): *C. fragile*, *C. dimorphum*, *C. sinuosa*, *Padina* sp., *P. comosa* and *S. vestitum*, underwent a more detailed extraction protocol (method A, see experimental chapter 7 for further details) to give three extracts water (polar), butanol (intermediate polar) and dichloromethane (non-polar) that were each tested for biological activity. Of these six algal species, four dichloromethane extracts (*C. fragile*, *C. sinuosa*, *Padina* sp. and *S. vestitum*), which were obtained in the highest yields were further separated using column chromatography. The second group (30-65 g): *Prionitis linearis* and *Corallina vancouveriensis*, *Ulva lactuca* and *Sargassum* sp., underwent a simplified extraction protocol as they were collected in low amounts (method B, see experimental chapter 7 for further details). In this method only two extracts, an ethanol and dichloromethane extract were screened for activity.

![Diagram of extraction protocols](image)

*Extracts screened for activity

**Figure 2.5:** Two protocols used for extraction of seaweed samples.
2.5.1. Biological screening of six seaweeds of SE Australia – Method A

*Codium fragile*: The non-polar dichloromethane extract of *C. fragile* displayed significant haemolytic activity (67%, Fig. 2.6B) and moderate PKA inhibitory activity (26%, Fig. 2.6A). It exhibited low cytotoxic activity towards brine shrimp after 24 h but the activity increased significantly after 48 h (3%, 43% respectively, Fig. 2.6C). The activities of the other two polar extracts generated from *C. fragile* were less active than the non-polar extract in all three biological assays (Fig. 2.6 A-C). Only the non-polar extract, which displayed bioactivities in all three assays was further studied (see later section).

Extracts isolated from *C. fragile* alga have been found to exhibit antibacterial, cytotoxic, antiviral and anticoagulant properties. Polysaccharides biosynthesized by this green alga have been found to be responsible for the potent anticoagulant activity of the species. However, there are no reports on both the kinase inhibitory and haemolytic activities of either extracts or pure compounds isolated from *C. fragile*.

![Figure 2.6](image_url): Biological activity of dichloromethane (DCM), butanol and water extracts generated from the green alga *C. fragile*, where A is % PKA inhibitory activity; B is % haemolytic activity; C is % brine shrimp lethality (all at 100 µg/ml). Data are presented as the mean of triplicate measurements ± SEM.

*Codium dimorphum*: The greatest activity in the PKA inhibition assay was observed in the butanol extract of *C. dimorphum* (92%, Fig. 2.7A) with the dichloromethane extract displaying moderate activity of 28% and the water extract showing negligible activity. The dichloromethane and butanol extracts exhibited modest haemolytic activity toward equine erythrocytes (48% and 52%, respectively, Fig. 2.7B), while the water extract exhibited weak activity. All three extracts showed low cytotoxicity in the brine shrimp lethality assay, with the butanol extract displaying the highest activity of 23% after 48 h (Fig. 2.7C). The results described herein are the first report on biological activity of green algae *C. dimorphum*. 
**Figure 2.7:** Biological activities of dichloromethane (DCM), butanol and water extracts generated from the green alga *C. dimorphum*, where A is % PKA inhibitory activity; B is % haemolytic activity; C is % brine shrimp lethality (all at 100 µg/ml). Data are presented as the mean of triplicate measurements ± SEM.

**Colpomenia sinuosa:** PKA inhibitory activity was observed in the non polar dichloromethane extract (44%, Fig. 2.8A) of *C. sinuosa*, along with very low levels of activity in the haemolytic and brine shrimp assays (Fig. 2.8 B and C). Substantial haemolytic activity was observed in the intermediate polarity extract (75%), which displayed low PKA inhibitory and cytotoxic activity. The polar water extracts displayed modest haemolytic activity of 17% but negligible PKA inhibitory activity and cytotoxicity.

The PKA results described herein are the first report on the kinase inhibitory activity of this brown alga. The low brine shrimp cytotoxicity results found in extracts of *C. sinuosa* are in conclusion with previous studies of this alga from the Pakistani coast, which also exhibited moderate to low activity in the brine shrimp lethality assay.\(^21^\) Also, methanol extracts of *C. sinuosa* previously did not have any haemolytic activity against human erythrocytes.\(^21^\)

In addition, although there has been no investigation into the Australian species of *C. sinuosa*, antimicrobial, antiviral and antioxidant activities were detected in *Colpomenia* species collected overseas.\(^21^3\)-\(^21^7\) Furthermore, unusual bromophenols were isolated from this species collected in the Gulf of Eilat, Israel, which exhibited potent cytotoxicity.\(^21^8\) The phenolic and lipid composition including carotenoids and vitamins C and E of this species from India was studied and plant extracts proved to possess potent antioxidant activity.\(^21^9\) The ethyl acetate extract of *C. sinuosa* from Taiwan was found to be cytotoxic for human hepatoma HuH-7 cells and induced apoptosis in human leukemia U937 and HL-60 cells.\(^22^0\)

**Figure 2.8:** Biological activities of dichloromethane (DCM), butanol and water extracts generated from the brown alga *C. sinuosa*, where A is % PKA inhibitory activity; B is % haemolytic activity; C is % brine shrimp lethality (all at 100 µg/ml). Data are presented as the mean of triplicate measurements ± SEM.
**Phyllospora comosa**: The results presented in Fig. 2.12 indicate that biological activity was mainly observed in the non polar dichloromethane extract of *P. comosa*. This extract exhibited considerable activity in the kinase inhibition assay (55%) and moderate activity in the haemolytic and brine shrimp lethality assays (33% and 30% respectively) (Fig. 2.9). The polar extracts did not exhibit any activity as kinase inhibitors (Fig. 2.9A). The results described herein are the first report on biological activity of brown algae *P. comosa*.

![Figure 2.9](image-url)  
**Figure 2.9**: Biological activities of dichloromethane (DCM), butanol and water extracts generated from the brown alga *P. comosa*, where A is % PKA inhibitory activity; B is % haemolytic activity; C is % brine shrimp lethality (all at 100 µg/ml). Data are presented as the mean of triplicate measurements ± SEM.

**Padina sp.**: The non-polar dichloromethane extract of *Padina* sp. displayed potent activity of 93% in the kinase inhibition assay, strong haemolytic activity of 72% and low brine shrimp lethality (Fig. 2.10 A, B and C). The butanol extract showed much lower activity of 22% in the PKA inhibition assay, and negligible activity in the other two assays. The polar water extract possessed considerable haemolytic activity (39%), while in the kinase inhibition assay displayed only weak activity. The cytotoxic activity of this extract was equal after 24 and 48 h (23%).

Although herein dichloromethane and water extracts of *Padina* sp. displayed noticeable haemolytic activities, methanol extracts of *Padina* sp. analysed previously were not active. The ethanol extract of this brown alga has been found to display moderate brine shrimp cytotoxicity, while here all extracts were inactive. This is the first report on kinase inhibitory activity of *Padina* extracts.

The aqueous extract of *Padina* species have previously exhibited potent antioxidant and anti-inflammatory activity. Antimicrobial activity was observed in the methanol extract obtained from *P. pavonica* collected off the coast of Egypt, while cytotoxic diterpenes showed in vitro antitumor activities against lung carcinoma (H460) and liver carcinoma (HepG2) human cell lines. The dichloromethane extract of alga collected in Tunisia showed cytotoxic activity, where sterol compounds were found to be responsible for the potent activity.
Sargassum vestitum: The non-polar dichloromethane extract of \textit{S. vestitum} displayed potent PKA inhibitory activity (76%), higher than the other two polar extracts, following the same trend that was also observed with the other algal samples (Fig. 2.11A). Low haemolytic activity towards equine erythrocytes was observed in all three extracts of \textit{S. vestitum} (Fig. 2.11B). The dichloromethane extract displayed moderate brine shrimp toxicity after both 24 and 48 h (43% and 47% respectively), while the butanol and water extracts displayed only weak activity in this assay (Fig. 2.11C).

\textit{Sargassum swartzii} from Pakistan exhibited significant brine shrimp cytotoxicity while \textit{Sargassum miyabei} from Japan displayed low haemolytic activity.\textsuperscript{211} The genus Sargassum has been found to be a rich source of bioactive compounds including metabolites with antifouling, antibacterial, anticancer, antitumor and cytotoxic activity.\textsuperscript{227-229}
2.5.2. Biological screening of four seaweeds of SE Australia – Method B

Four alga samples: Prionitis linearis and Corallina vancouveriensis, Ulva lactuca and Sargassum sp. were collected in low amounts (< 50g of wet material, method B, Fig. 2.5) and an ethanol extract was generated using protocol C (see experimental chapter 7 for further details). To compare PKA inhibitory activity of these samples with the other six alga species, dichloromethane extracts were also prepared and screened in the assays. The combined biological results are presented in Fig. 2.12.

![Biological activities of dichloromethane (DCM) and ethanol extracts generated from Prionitis linearis, Corallina vancouveriensis, Ulva lactuca and Sargassum sp. (all at 100 µg/ml). Data are presented as the mean of triplicate measurements ± SEM.](image)

**Prionitis linearis**: The ethanol extract of the red seaweed *Prionitis linearis* displayed potent haemolytic activity towards equine erythrocytes of 55%, low PKA inhibition activity of 26% and slightly increased PKA inhibitory in the dichloromethane extract (37%) (Fig. 2.12). Although in previous analyses, potent brine shrimp cytotoxicity was found in extracts of this species, herein the ethanol extract exhibited only low toxicity towards *A. franciscana*.228
The results described herein are the first report on the PKA and haemolytic activity of the red alga *P. linearis*. Although low brine shrimp lethality was observed among extracts analysed herein, Bernart *et al.* detected brine shrimp cytotoxicity and antimicrobial activity in the organic crude extracts isolated from *P. lanceolata* and *P. linearis* from Cape Perpetua on the Oregon coast and isolated four known indoline derivatives (Fig. 2.13).  

**Corallina vancouveriensis**: The ethanol extract of the red seaweed *Corallina vancouveriensis* displayed moderate activity in the PKA inhibition and haemolytic assays (38% and 19% respectively) while only low cytotoxicity (10%). The PKA inhibitory activity of the dichloromethane extract was almost identical to the activity of the ethanol extract (36%, Fig. 2.12). There is no published literature on kinase inhibitors isolated from this species and no data on the potential activity of *C. vancouveriensis* in either haemolytic or brine shrimp lethality assays so far. However, there is evidence of antiprotozoal, antimycobacterial and cytotoxic activity of *C. officinalis*.  

**Ulva lactuca**: The ethanol extract of the green seaweed *U. lactuca* displayed low PKA inhibitory activity of 12.0% and brine shrimp lethality and modest activity in the haemolysis assay of 28% (Fig. 2.12). The dichloromethane extract of *U. lactuca* displayed very similar activity as the ethanol extract in the PKA inhibition assay. So far, there are no data reported on potential kinase inhibitors isolated from *U. lactuca* or kinase inhibitory activity obtained from extracts of this alga. Ethanol extracts generated from *U. fasciata* from the Karachi coast displayed cytotoxicity in the brine shrimp assay of LC$_{50}$ 724μg. Linolenic acid and linoleic acids isolated from *U. fasciata* and displayed low toxicity towards brine shrimp and mammalian cell lines (U937, HeLa, Vero, and CHO cells). Extracts isolated from the genus *Ulva* have also exhibited antioxidant, anti-inflammatory, antibacterial and antimicrobial activity in species collected worldwide. Sulfated polysaccharides extracted from the alga *U. pertusa* from China displayed excellent antioxidant activity. *U. lactuca* have been demonstrated to contain phenolic compounds, among them bromophenols, and the ability to produce bromoperoxidase enzymes capable of brominating organic substances in the presence of bromide and hydrogen peroxide that are frequently detected in marine algae. The crude methanol extract of this seaweed from Egypt and three new isolated FAs from this alga displayed weak activity in a brine shrimp assay, but the crude methanol extract exhibited potent activity when tested against a breast carcinoma cell line.
**Sargassum sp.:** Both, the ethanol and dichloromethane extracts of the brown seaweed \textit{Sargassum} sp. was tested in all three assays where it displayed significant activity in the PKA inhibition assay of 70% and 82% respectively, which is consistent with the potent activity of the dichloromethane extract of \textit{S. vestitum} from Jones beach which also displayed 76%. The ethanol extract displayed only low haemolytic and cytotoxic activity towards \textit{A. franciscana} (Fig. 2.12).

The results described herein are the first report on the PKA inhibitory activity of the ten algal species under investigation. Also, haemolytic activity and brine shrimp cytotoxicity of \textit{C. dimorphum}, \textit{P. comosa} and \textit{C. vancouveriensis} has not been detected before. In general, the non-polar algal extracts gave more potent biological results, in particular greater PKA inhibitory activity, compared with the polar extracts. The bioactivity profiles of the alga samples were strongly species dependent, with no apparent correlation between PKA inhibition, haemolysis and/or brine shrimp lethality.

### 2.6 Fatty acid and sterol composition of marine algae

An additional distinction of marine algae is their complex FA composition, which is greater than that found among terrestrial plants. Fatty acid composition, particularly the unsaturated FAs composition, is of great importance as these compounds often contribute to the biological activity of marine alga.\textsuperscript{239} Herein, a series of brown, red and green algae were investigated for both their FA and sterol composition as described in the following sections (Section 2.7). Each of the extracts was also screened for a PKA inhibitory activity.

#### 2.6.1 Algal fatty acid composition

Each phylum of marine algae has a characteristic FA composition, although some acids predominate in more than one phylum.\textsuperscript{240} Fatty acid composition also varies among different species within the same genus and also among the same species collected in different locations.\textsuperscript{241} Lang \textit{et al.} analysed more than 2000 strains in their work and determined that the difference between alga genera and species cannot be achieved by comparing their FA profile alone due to great FA variations.\textsuperscript{242} Fatty acid composition mainly depends on algae habitat conditions, temperature, nutrients and depth.\textsuperscript{243} Typically green algae have a high level of C16:0 and C18:0 polyunsaturated FAs (PUFAs).\textsuperscript{240} In brown algae, the two main saturated FAs are tetradecanoic and hexadecanoic acids, along with the monounsaturated oleic acid C18:1 (n-9) and C18 and C20 PUFAs.\textsuperscript{244} Red algae are characterised by a high content of arachidonic [C20:4 (n-6)] and eicosapentaenoic acids [C20:5 (n-3)], but hexadecanoic acid and oleic acid [C18:1 (n-9)] can also be abundant.\textsuperscript{245}
The antibiotic activity of some algae species has been attributed to the mixture of oleic, palmitic, linoleic and capric acids isolated from them. Hexadecanoic acid was previously found to have selective cytotoxicity to human leukemic cells and no toxicity towards human dermal fibroblast cells and moreover displayed potent antitumor activity. Bazes _et al._ suggested usage of hexadecanoic acid as an antifouling paint as it possessed potent antibacterial activity and didn't display any significant cytotoxicity to normal cell lines. N-3 Acids are also found to possess kinase inhibitory activity as these FAs interact with critical calcium regulatory enzymes, related to the activity of kinases. PUFAs, in particular eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are found to be a precursor of a range of bioactive metabolites that have an important role in the regulation of human physiology. EPA and DHA have been found to have an important role in protection against cancer, arthritis, psoriasis, lung disease and attention-deficit disorder. Humans obtain these FAs through food consumption because they are unable to synthesise them _de novo_. One of the main sources of EPA and DHA are algae, which possess the enzymes required for the synthesis of PUFAs, which starts from carboxylation of acetyl CoA as shown in Fig. 2.14.
2.6.2 Algal sterol composition

The sterol compositions of different classes of algae have also been well studied. Some classes of algae have a distinct sterol composition, however taxonomic classification based on the sterol composition is not always substantiated. In green (Chlorophyceae) and brown algae (Phaeophyceae) the main sterols are fucosterol and isofucosterol, which are the methylation products of 24-methylenecholesterol. In green algae the reduction of the 24(28) double bond also occurs and an accumulation of sitosterol can be found. Red algae (Rhodophyceae) mainly contain cholesterol and its precursor cholesta-5,24(25)-dien-3β-ol as the major sterols. Sterols with C27 are also predominant compounds in red algae although occurrence of C24 alkylation has also been reported previously, despite earlier suggestions that red algae are incapable of alkylation at the C24 position. While the main sterols of the Rhodophyceae are C27 sterols with cholesterol as the predominant
compound and the Phaeophyceae are rich in C29 sterols with the most abundant being fucosterol, the sterol composition of Chlorophyceae is not well studied.

Figure 2.15: Sterol compounds characteristic for specific alga divisions.

2.7. Investigation of the chemical composition of the ten algal samples

Of the ten alga samples collected from SE Australia, six alga species Prionitis linearis, Corallina vancouveriensis, Codium dimorphum, Ulva lactuca, Phyllospora comosa and Sargassum sp. underwent a simplified FA and sterol analysis, which included GC-MS analysis of the dichloromethane extract for each sample, while the remaining four seaweeds including Codium fragile, Colpomenia sinuosa, Sargassum vestitum and Padina sp. were studied in more detail (Section 2.6, Fig. 2.5).

2.7.1 GC-MS analysis of Prionitis linearis, Corallina vancouveriensis, Codium dimorphum, Ulva lactuca, Phyllospora comosa and Sargassum sp.

The dichloromethane extracts of six algal samples were collected in only low yields (30-65 g, Table 2.1) and therefore were analysed directly by GC-MS with the aim of identifying the chemical constituents in each sample and making a comparison with the activity results. The chemical composition of the six algal samples is presented in Table 2.2.

Prionitis linearis

In the dichloromethane extract of P. linearis obtained by extraction method B (Fig 2.5), 14 compounds were identified by GC-MS analysis (Table 2.2). The predominant compounds were FAs with the major component being hexadecanoic acid (32.1%) and octadecadienoic acid C18:1 (n-6) (26.7%). In addition to 11 FAs, one sterol was identified and two aldehydes. Aldehydes in marine algae have been found to be involved in attractant or repellent roles and to take an active part in higher plant-insect relationships. It is assumed that aldehydes present in
marine algae are possibly derived from the PUFAs. There are only a few published papers on the FA composition of *P. linearis* including work of Khotimchenko *et al.* in which *P. linearis* from California was analysed among seven other algae species and C16:0 and two PUFA acids C20:4 (n-6) and C20:5 (n-3) were identified as the dominant acids.

**Corallina vancouveriensis**

In the dichloromethane extract of *C. vancouveriensis* obtained by extraction method A (Fig 2.5), 26 compounds were identified by GC-MS analysis (Table 2.2) and their structural identification was based on comparisons to the NIST 08 mass spectral database. The dominant FA was hexadecanoic acid (24.9%) (Table 2.2), which is consistent with previous results. Aldehydes contribute to the pleasant taste of some algae species that have been traditionally used as a food source in Japan for many years. Three volatile alcohol compounds were identified in the dichloromethane extract (entry 10, 11 and 13). Alcohols in terrestrial plants have been found to serve as repellent or attractants but their role in marine algae is still unknown.

**Table 2.2:** Composition of the dichloromethane extracts from the SE Australian marine algae

<table>
<thead>
<tr>
<th>No</th>
<th>Fatty acids</th>
<th>Rhodophyta</th>
<th>Chlorophyta</th>
<th>Phaeophyta</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1 2 3 4 5 6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Tetradecanoic acid</td>
<td>14:0</td>
<td>4.00 5.8 2.1 6.0</td>
<td>5.4 12.8</td>
</tr>
<tr>
<td>2</td>
<td>12-Methyltetradecanoic acid</td>
<td>15:0</td>
<td>- 0.1 0.7 0.3</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>3,7,11,15-Tetramethyl-2-hexadecen-1-ol</td>
<td>- 0.6 0.7 0.9</td>
<td>0.4 0.4</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>Pentadecanal</td>
<td>0.8 0.7 0.9 0.5</td>
<td>0.6 -</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>7-Tetradecenal</td>
<td>-</td>
<td>5.6 0.6 0.7 2.3</td>
<td>0.3 -</td>
</tr>
<tr>
<td>6</td>
<td>Hexadecanoic acid</td>
<td>16:0</td>
<td>32.1 24.9 20.6 25.5</td>
<td>23.3 23.3</td>
</tr>
<tr>
<td>7</td>
<td>7-Hexadecenoic acid</td>
<td>16:1 n-9</td>
<td>- 0.2 - -</td>
<td>0.1 -</td>
</tr>
<tr>
<td>8</td>
<td>9-Hexadecenoic acid</td>
<td>16:1 n-7</td>
<td>6.3 2.3 15.4 3.5</td>
<td>4.4 3.6</td>
</tr>
<tr>
<td>9</td>
<td>2,5-Dimethyl-4-hexen-3-ol</td>
<td>-</td>
<td>0.4 1.9 0.8</td>
<td>0.5 0.3</td>
</tr>
<tr>
<td>10</td>
<td>1-Butylcyclohexanol</td>
<td>-</td>
<td>0.6 - 0.9</td>
<td>0.7 -</td>
</tr>
<tr>
<td>11</td>
<td>Isomethanol</td>
<td>-</td>
<td>0.3 0.7 2.9</td>
<td>0.2 0.9</td>
</tr>
<tr>
<td>12</td>
<td>Heptadecanoic acid</td>
<td>17:0</td>
<td>- 0.5 1.6 1.2</td>
<td>1.7 0.3</td>
</tr>
<tr>
<td>13</td>
<td>Citronellol</td>
<td>-</td>
<td>0.1 - -</td>
<td>3.9 2.9</td>
</tr>
<tr>
<td>14</td>
<td>Octadecanoic acid</td>
<td>18:0</td>
<td>2.3 12.2 2.3 0.4</td>
<td>0.5 1.9</td>
</tr>
<tr>
<td>15</td>
<td>9-Octadecenoic acid</td>
<td>18:1 n-9</td>
<td>2.2 8.8 7.2 3.1</td>
<td>4.5 4.4</td>
</tr>
<tr>
<td>16</td>
<td>9,12-Octadecadienoic acid</td>
<td>18:2 n-6</td>
<td>26.7 19.3 22.4 14.9</td>
<td>20.0 16.5</td>
</tr>
<tr>
<td>17</td>
<td>6,9,12-Octadecatrienoic acid</td>
<td>18:3 n-6</td>
<td>1.8 0.9 1.9 0.7</td>
<td>0.4 1.2</td>
</tr>
<tr>
<td>18</td>
<td>6,9,12,15-Octadecatetraenoic acid</td>
<td>18:4 n-3</td>
<td>- 0.1 - 0.3</td>
<td>0.4 -</td>
</tr>
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<td>19</td>
<td>Eicosanoic acid</td>
<td>20:0</td>
<td>- 0.4 - tr</td>
<td>0.1 -</td>
</tr>
<tr>
<td>20</td>
<td>11,14-Eicosadienoic acid</td>
<td>20:2 n-6</td>
<td>7.00 6.2 5.8 8.8</td>
<td>14.3 8.3</td>
</tr>
<tr>
<td>21</td>
<td>8,11,14-Eicosatrienoic acid</td>
<td>20:3 n-6</td>
<td>4.2 5.2 4.8 4.7</td>
<td>5.4 11.3</td>
</tr>
<tr>
<td>22</td>
<td>5,8,11,14-Eicosatetraenoic acid</td>
<td>20:4 n-6</td>
<td>0.9 0.5 - 0.9</td>
<td>1.4 0.5</td>
</tr>
<tr>
<td>23</td>
<td>5,8,11,14,17-Eicosapentaenoic acid</td>
<td>20:5 n-3</td>
<td>1.1 1.6 - 1.3</td>
<td>0.7 1.7</td>
</tr>
<tr>
<td>24</td>
<td>7,10,13,16,19-Docosapentaenoic acid</td>
<td>22:5 n-3</td>
<td>0.1 - -</td>
<td>0.1 -</td>
</tr>
<tr>
<td>25</td>
<td>Tricosanoic acid</td>
<td>23:0</td>
<td>- 0.3 - -</td>
<td>0.2 0.2</td>
</tr>
<tr>
<td>26</td>
<td>Cholesterol</td>
<td>27Δ3</td>
<td>0.9 5.6 0.1 0.2</td>
<td>0.6 0.9</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>% of total extract</th>
<th>Rhodophyta</th>
<th>Chlorophyta</th>
<th>Phaeophyta</th>
</tr>
</thead>
<tbody>
<tr>
<td>95.9</td>
<td>87.3</td>
<td>89.8 80.1</td>
<td>90.1 91.4</td>
</tr>
</tbody>
</table>

Six algae species analysed: 1: *Priotitis linearis*; 2: *Corallina vancouveriensis*; 3: *Codium dimorphum*; 4: *Ulva lactuca*; 5: *Phyllospora comosa*; 6: *Sargassum sp.*. tr – traces amounts. The relative intensity of each peak was calculated as a percentage of the summed total.

Red algae are characteristically dominated by PUFAs, in particular C20:4 (n-6) and C20:5 (n-3), which were found in the analysed species but in slightly lower amounts. Of the
FAs identified in three *Corallina* species, two unsaturated hexadecanoic acids C16:1 (n-9) and C16:2 (n-9) were the most abundant along with eicosapentadecenoic acid C20:5 (n-3).\(^{253}\)

Cholesterol was detected in three *Corallina* samples collected from the Black and Mediterranean seas where it was found to be the main sterol that was present at more than 87%, which is in accord with the results in this study where only cholesterol among sterol compounds was identified (5.6%).\(^{253}\) In the three previously analysed *Corallina* species four to five other sterols were detected but in considerably lower yields, which can be related to the results of this study where no sterols were detected, probably due to the small amount of samples for analysis.\(^{253}\)

The chemical compositions of the two red algae species *P. linearis* and *C. vancouveriensis* were very similar with the same dominant FAs. Moreover, biological activities of the two red algae species were similar, they both displayed PKA inhibitory activities in the range of 25-37% and both exhibited low cytotoxicity levels, while *P. linearis* showed significant activity in the haemolytic assay (Fig. 2.12).

**Codium dimorphum**

In the dichloromethane extract of *C. dimorphum* obtained by extraction method A (Fig. 2.5), 17 compounds were identified by GC-MS analysis (Table 2.2). Fatty acids were the predominant compounds in *C. dimorphum* with the main acid found to be octadecadienoic acid C18:2 (n-6) (22.4%). Hexadecanoic acid and hexadecadienoic acid (n-7) were also present in high amounts (20.6% and 15.4%, respectively). In *C. dimorphum* collected in Chile hexadecanoic acid was the main acid.\(^{207}\) Aldehydes, alcohols and cholesterol were also found in the dichloromethane extract but in considerably lower yields compared with the amount of FAs.

Two *Codium* species that were collected from the same location, had similar FA contents but the ratio of some FAs were different; in *C. fragile* the dominant FAs were hexadecanoic acid followed by octadecatrienoic acids [C18:3 (n-3) and (n-6)] and low amounts of linoleic acid 18:2 (n-6) were found. In contrast, linoleic acid was identified to be the major acid in *C. dimorphum* whereas hexadecanoic acid was the second main acid. While the PKA inhibitory and haemolytic activities of the dichloromethane extracts of the two *Codium* species analysed in this work were similar, the activities of the butanol extracts were considerably different. The butanol extract of *C. dimorphum* displayed more potent bioactivity (Fig. 2.12), while the polar water extracts showed a similar trend between the two algae with low activities.

**Ulva lactuca**

In the dichloromethane extract of *U. lactuca* obtained by extraction method B (Fig. 2.5), 22 compounds were identified by GC-MS analysis (Table 2.2). The main compounds in the
dichloromethane extract of the green alga *U. lactuca* were found to be FAs as previously described here for the two red alga samples (Table 2.2). Predominant were hexadecanoic (25.5%) octadecadienoic and octadecadienoic acid (18:2 n-6) (entry 6 and 16, Table 2.2). Hexadecanoic acid was also identified as the major acid in *U. lactuca* from the Bohai Sea in China and *U. fenestrate* from Japan.\(^2\) Most other FAs including unsaturated, monoenoic, dienoic, trienoic FAs and PUFAs were detected but in different ratios compared with the sample analysed in this work, which may be due to different environmental conditions.\(^2\) The only sterol found in this alga was cholesterol, which is consistent with previous results. The main sterol found in the genus *Ulva* is reported to be isofucosterol.\(^2\) However, in the species *U. lactuca* collected from the South Adriatic the principal sterols were cholesterol (34%) and isofucosterol (26%). The authors assume it could be due to specific ecological conditions.\(^2\) In the marine alga *U. fasciata* from Egypt the only sterol identified was cholesterol.\(^5\)

Compared with the chemical constituents of the other algae species analysed in this work and in particular with the other green alga *C. dimorphum*, *U. lactuca* had a similar FAs composition. The two green algae species (*U. lactuca* and *C. dimorphum*) were collected from different locations, but grew under similar environmental conditions and were collected during the same time of year.

**Phyllospora comosa**

In the dichloromethane extract of *P. comosa* obtained by extraction method B (Fig 2.5), 24 compounds were identified by GC-MS analysis (Table 2.2). Fatty acids were the most abundant compounds and cholesterol was the only sterol compound identified (Table 2.2). A range of aldehydes and alcohols were also detected. Hexadecanoic acid (23.3%) was the main acid followed by octadecadienoic acid 18:2 n-6 (20.0%) and eicosadienoic acid 20:2 n-6 (14.3%). So far, only one report was published FA composition of this alga, where hexadecanoic acid was found to be dominant followed by octadecenoic acid C18:1 (n-9) and PUFA C20:4 (n-6).\(^6\)

**Sargassum sp.**

In the dichloromethane extract of *Sargassum sp.* obtained by extraction method B (Fig 2.5), 26 compounds were identified by GC-MS analysis (Table 2.2). Hexadecanoic acid was the main acid identified followed by 9,12-octadecadienoic acid and 8,11,14-eicosatrienoic acid. The chemical composition was similar to the composition of *S. vestitum* collected at Jones Beach in which hexadecanoic acid was the dominant FA and the presence of linoleic acid was also observed. The activity of the two dichloromethane extracts of both *Sargassum* species displayed potent activity in the protein kinase A inhibition assay (Fig. 2.11 and 2.12).
In summary, among all six algal samples, hexadecanoic acid C16:0 was the most dominant acid in five species, ranging from 23.2% to 32.1% whereas only in *C. dimorphum* it was the second abundant acid (20.6%). In *C. dimorphum* the predominant acid was octadecadienoic acid 18:2 (n-6) (22.4%), while this acid was the second most abundant acid in the other five samples ranging from 14.9% to 26.7% consistent with literature reports. Only one sterol compound was observed in all six samples. Cholesterol was detected in the range of 0.08% to 1.64%. Although there was a difference in the PKA inhibitory activity among the samples, ranging from 12% to 73%, the chemical composition of the ten algal samples was fairly similar and consistent with that reported for each species. Notably, the algal samples all came from similar environments, and were collected from close locations around the same date, which may also contribute to the similar chemical profiles observed.

### 2.7.2 EI-MS and GC-MS analysis of *Codium fragile*, *Colpomenia sinuosa*, *Padina* sp. and *Sargassum vestitum*

Four seaweeds including *Codium fragile*, *Colpomenia sinuosa*, *Padina* sp. and *Sargassum vestitum* were collected in larger quantities (129-500 g, Fig. 2.5) and analysed in more detail, which included further separation of the more biologically active dichloromethane extract for each algae sample.

**Codium fragile**

The non-polar dichloromethane extract of *C. fragile* (1.24 g) obtained using extraction method A (Fig. 2.8) was further purified by column chromatography over silica gel using a gradient solvent system of Hex/EtOAc to give 27 fractions that were pooled into seven major fractions based on their metabolite compositions as judged by TLC (Fig. 2.16).

The pooled column fractions isolated from the non-polar dichloromethane extract were tested for their PKA inhibitory activity and the results are presented in Fig. 2.16. All extracts were tested at a concentration of 100 µg/ml. Three of the seven column fractions displayed activities of over 70% (fractions 1, 5 and 7), which were significantly higher than the original extract (26%, Fig. 2.16). The column fractions were analysed by TLC and the results indicated that each fraction was a mixture of more than two compounds. Attempts were made to further purify these fractions, but as they were mixtures of compounds of similar structure and obtained in low yields, it was difficult to achieve full and complete separation of the compounds. Therefore, chemical constituents of the most active column fractions (1, 2, 5 and 7) were analysed by GC-MS (Table 2.3). By comparison to data in the NIST mass spectral database, 11 compounds were identified in total, of which ten were FAs and one a sterol (Table 2.3).
Figure 2.16: A: Extraction protocol of the green alga *Codium fragile* and B: PKA inhibitory activity of the column fractions obtained from the dichloromethane extract.

GC-MS analysis determined that column fraction 1 consisted of FAs only (Table 2.3). In total, nine acids were identified, where the dominant FAs were octadecatrienoic acid (18:3 n-6) and hexadecanoic acid. Four FAs were saturated (C14:0, C16:0, C18:0 and C20:0), one monoenoic [C18:1 (n-9)], one dienoic [C18:2 (n-6)], two trienoic [C18:3 (n-6), C18:3 (n-3)], and one tetraenoic acid [20:4 n-6]. Interestingly, the chemical composition of column fraction 2 was similar to the composition of column fraction 1 but the PKA inhibitory activity results of these two fractions were notably different (Fig. 2.16). Column fraction 2 possessed only FAs with hexadecanoic acid as the main compound (26.9%), which was also found in fraction 1 in a similar amount. Even though the same compounds were identified in these two column fractions, the ratio of acids was different, which could account for the difference in the activity results (Fig. 2.16). Hexadecanoic acid was previously also found to be the dominant acid in *Codium* species collected in the Sea of Japan, South Eastern Australia, Tasmania and Chile.²⁴¹,²⁴⁵,²⁶²

Compared with the number of FAs identified in column fractions 1 and 2, the number of detected FAs in the column fractions 5 and 7 was lower (Table 2.3). Five FAs were identified in fraction 7 and two FAs in the fraction 5. Both column fractions had the same sterol compound (entry 18, Table 2.3) present in high amounts and as the dominant compound. These active column fractions isolated from the dichloromethane extract of *C. fragile* were rich in FAs, in particular with hexadecanoic and octadecatrienoic acids [C18:3 (n-3) and (n-6)] in accord with previous results.²⁶⁰

Three column fractions (3, 4 and 6, Fig. 2.16) exhibited lower activity than the other column fractions and lower than the original dichloromethane extract. These fractions were analysed by EI-MS (Table 2.4).
Fractions 3 and 4 were obtained as dark green oils, suggesting that they contained a high percentage of chlorophyll, which is common for green algae. Therefore, in order to remove it from these fractions, the method described by Sargenti et al. was used. TLC analysis indicated that these two fractions contained the same compounds. Three spots were detected on the TLC plate and they had identical Rf values. As a result, fractions 3 and 4 were combined giving 466 mg of the mixture. After the procedure, the fraction retained the green colour, but lighter than the original material. EI-MS analysis was performed before and after chlorophyll removal, giving three major compounds by comparison with library mass spectral data. The compounds were tentatively identified as hexadecanoic acid, octadecanoic acid and 24-ethylcholesta-7,24(28)-dien-3β-ol.

Table 2.3: GC-MS analysis of column fractions generated from *C. fragile*, *C. sinuosa* and Padina sp.

<table>
<thead>
<tr>
<th>No</th>
<th>Compound name</th>
<th>Type</th>
<th>C. fragile</th>
<th>C. sinuosa</th>
<th>Padina sp.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>#1#2#3#4#5#6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Dodecanal</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>2.1</td>
</tr>
<tr>
<td>2</td>
<td>Tetradecanoic acid</td>
<td>14:0</td>
<td>1.1</td>
<td>4.1</td>
<td>3.6</td>
</tr>
<tr>
<td>3</td>
<td>Pentadecanoic acid</td>
<td>15:0</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>Hexadecanoic acid</td>
<td>16:0</td>
<td>20.2</td>
<td>26.9</td>
<td>16.7</td>
</tr>
<tr>
<td>5</td>
<td>9-Hexadecenoic acid</td>
<td>16:1 n-7</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>Octadecanoic acid</td>
<td>18:0</td>
<td>0.1</td>
<td>8.3</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>9-Octadecenecio acid</td>
<td>18:1 n-9</td>
<td>15.1</td>
<td>10.1</td>
<td>-</td>
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<td>8</td>
<td>9,12-Octadecadienoic acid</td>
<td>18:2 n-6</td>
<td>0.1</td>
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<td>-</td>
</tr>
<tr>
<td>9</td>
<td>6,9,12-Octadecatrienoic acid</td>
<td>18:3 n-6</td>
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<td>12.0</td>
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<td>18:3 n-3</td>
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<td>Eicosanoic acid</td>
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<td>21.2</td>
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<tr>
<td>12</td>
<td>5,8,11,14-Eicosatetraenoic acid</td>
<td>20:4 n-6</td>
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<td>3.6</td>
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<td>13</td>
<td>Vitamin E</td>
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<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>14</td>
<td>24-Methylcholesta-5-en-3β-ol</td>
<td>28Δ⁵</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>15</td>
<td>24-Methylcholesta-5,22-dien-3β-ol</td>
<td>28Δ⁵,22</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>16</td>
<td>24-Methylcholesta-5,24(28)-dien-3β-ol</td>
<td>28Δ⁵,24(28)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>17</td>
<td>Cholesta-5-en-3β-ol</td>
<td>27 Δ⁵</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>18</td>
<td>24-Ethylcholesta-5,24(28)-dien-3β-ol</td>
<td>29Δ⁵,24(28)</td>
<td>- -</td>
<td>64.8</td>
<td>30.3</td>
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<tr>
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<td>24-Ethylcholesta-5,22-dien-3β-ol</td>
<td>29Δ⁵,22</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>20</td>
<td>24-Ethylcholesta-5-en-3β-ol</td>
<td>29Δ⁵</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

*Numbers #1, 2, 3 etc. refer to column fraction numbers. The relative intensity of each peak was calculated as a percentage of the summed total.
Table 2.4: EI-MS analysis of column fractions generated from *C. fragile*, *C. sinuosa* and *Padina* sp.

<table>
<thead>
<tr>
<th>Compounds identified</th>
<th>C. fragile</th>
<th>C. sinuosa</th>
<th>Padina sp.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>#3</td>
<td>#4</td>
<td>#6</td>
</tr>
<tr>
<td>Tetradecanoic acid</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>6,10,14-Trimethyl-2-pentadecanone</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3,7,11,15-Tetramethyl-2-hexadecen-1-ol</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>14-Methyl-pentadecanoic</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Hexadecanoic acid 16:0</td>
<td>√</td>
<td>-</td>
<td>√</td>
</tr>
<tr>
<td>9-Octadecenoic acid 18:1 n-9</td>
<td>√</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>9,12-Octadecadienoic acid 18:2 n-6</td>
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<td>-</td>
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<tr>
<td>1-Phenantherencarboxylic acid</td>
<td>-</td>
<td>√</td>
<td>-</td>
</tr>
<tr>
<td>Squalene</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Sandaracopimar-15-ene-6β, 8β-diol</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>Pregna-5-en-2β-ol</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>Pregna-5,17(20)-dien-2β-ol</td>
<td>-</td>
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<td>-</td>
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<td>Cholesta-7,24-dien-3β-ol</td>
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<td>-</td>
</tr>
<tr>
<td>Cholesta-5-en-3β-ol (Cholesterol)</td>
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<td>-</td>
<td>-</td>
</tr>
<tr>
<td>24-Methylcholesta-5,24(28)-dien-3β-ol</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>24-Ethylcholesta-7,24(28)-dien-3β-ol</td>
<td>√</td>
<td>√</td>
<td>-</td>
</tr>
<tr>
<td>24-Methyl-26,27-dinorcholesta-5,24(28)-dien-3β-ol</td>
<td>-</td>
<td>-</td>
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<tr>
<td>24-Ethylcholesta-5,22-dien-3β-ol</td>
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<td>-</td>
<td>-</td>
</tr>
<tr>
<td>24-Ethylcholesta-5,24(28)-dien-3β-ol</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

*Numbers #1, 2, 3 etc. refer to column fraction numbers.

Even though the genus *Codium* is a very common species, it has attracted much attention as a result of its interesting chemical composition. It was found that *C. fragile* contains unusual FAs, sterols, polysaccharides and especially essential C16 and C18 PUFAs.²⁶⁵ *Codium fragile*, collected from different locations, have been investigated regarding their FA composition and there were attempts to find a correlation with their collection place, however the results were quite contradictory. While some authors state that C16:0 FA is the dominant and characteristic acid for the genus *Codium*, other authors could not detect the presence of this particular FA, or any other C16:0 FAs even when the alga species were collected from the same area.²⁴⁰ Herein, the most dominant FAs among *C. fragile* column fractions were C16:0, 18:3 (n-6) and 18:3 (n-3). It has been suggested that FA composition is not a particularly good indicator for chemotaxonomy for alga species belonging to the genus *Codium* because the composition and level of FAs is different for different locations and under different environmental conditions.²⁴¹

**Colpomenia sinuosa**

The dichloromethane extract of *C. sinuosa* obtained using extraction method A (Fig. 2.5) was further partitioned by column chromatography, using a hexane/EtOAc mixture as the eluent to give 33 fractions that were pooled into six major fractions based on their metabolite compositions as judged by TLC (Fig. 2.17). These column fractions were tested for their PKA inhibitory activity and the results are presented in the Fig. 2.17. Three of the six column fractions (2, 4 and 5) were more potent than the original dichloromethane extract with 60%,
61% and 67% inhibition respectively, column fraction 3 displayed similar activity to the dichloromethane extract (45%), while column fractions 1 and 6 exhibited low PKA inhibitory activity.

Figure 2.17: A: Extraction protocol of the green alga Colpomenia sinuosa and B: PKA inhibitory activity of the column fractions obtained from the dichloromethane extract.

The column fractions were obtained in low yields and attempts to further analyse them and also to purify and isolate pure active compound were difficult. Column fractions 1, 3, 4 and 6 were analysed by EI-MS (Table 2.4), while the most active column fractions 2 and 5 underwent a more detailed investigation by GC-MS (Table 2.3). These results indicated that the main constituents in each fraction were non-polar lipid compounds including FAs and sterols with similar polarity and therefore difficult to separate and isolate.

Column fraction 1 (Fig. 2.17) was obtained as a yellow oil in low yield (32 mg). Only 14-methyl-pentadecanoic acid was identified by database matching, although the TLC analysis indicated more than one compound was present. In fractions 3 and 4, which exhibited moderate PKA inhibitory activities (Fig 2.17), only sterols were identified, two sterols in fraction 3 and four in fraction 4. In fraction 4 two known short side chain sterols were found with a pregnane structure, which was not previously detected in this species (Fig. 2.18). Short side chains sterols have been previously found in marine organisms, mainly in sponges and corals.\(^{264}\) They were also identified in some algal species such as Cladophora vagabond and Zanardinia prototypes, which suggested that short side chain sterols are derived from algae and through the food chains are transferred to invertebrates.\(^{265}\) In all three fractions fucosterol was identified, as is expected for brown algae.\(^{217}\) Column fraction 6 exhibited the lowest PKA inhibitory activity and was found to contain three sterols (Table 2.3).
Chapter 2

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Figure 2.18: Short side chain sterols identified in column fraction 4 of *C. sinuosa*.

Two saturated FAs were detected along with two sterol compounds, fucosterol (26.3%) and 24-methylcholesta-5-en-3β-ol in column fraction 2, which displayed moderate PKA inhibitory activity (Table 2.3). Column fraction 5, which displayed the most significant activity in the PKA inhibition assay (68%), had the highest number of identified compounds (Table 2.3). In total, 11 compounds were identified, among them six FAs and five sterols while one was unidentified. All five sterols identified have the Δ⁵ structure. The most active column fraction 5 was rich in Δ⁵ sterols in particular with fucosterol and cholesterol identified as the dominant sterols. Similar sterol compositions to those described here were observed in alga samples of *C. sinuosa* from Greece and *C. peregrine* from the Black sea. Hexadecanoic acid was the dominant FA (20.1%) in column fraction 5 followed by octadecanoic acid (16.5%). Of five FAs, four were saturated (C14:0, C16:0, C18:0, C20:0) and one tetraenoic acid [C20:4n-6].

In total, in the analysed species fucosterol was detected in three of the six column fractions (2, 5 and 6), proving that it was the main sterol of this algal species. Hexadecanoic acid was present in the two column fractions 2 and 6 in significant quantities. Interestingly, it was detected in a fraction which exhibited great PKA inhibitory activity. In marine algae *C. sinuosa* collected in Senegal the dominant sterol proved to be fucosterol and the main FAs were hexadecanoic acid (35.1%) and oleic acid (C22:0). Shaikh *et al.* also found hexadecanoic acid to be the most dominant in the *C. sinuosa*.

*Padina* sp.

The non-polar fraction of the dichloromethane extract of *Padina* sp. obtained using extraction method A (Fig. 2.5) was separated to give 29 fractions that were pooled into six major fractions based on their metabolite compositions as judged by TLC (Fig. 2.19). Only the first column fraction exhibited significant PKA activity of 92% inhibition, close to the activity of the original material, while other fractions displayed lower activity.

The two most active column fractions generated from the dichloromethane extract, fractions 1 and 6, underwent a more detailed analysis by GS-MS (Table 2.3) while all other column fractions were analysed by EI-MS mass spectrometry (Table 2.4). TLC analysis of all
column fractions indicated that none of the fractions were pure and that all compounds had similar polarity and Rf values.

Figure 2.19: A: Extraction protocol of the green alga 
*Padina* sp. and B: PKA inhibitory activity of the column fractions obtained from the dichloromethane extract.

In the first fraction, which displayed the greatest kinase inhibitory activity, GC-MS analysis identified eight compounds (Table 2.3) of which (α-tocopherol) vitamin E was the most abundant compound in the mixture (40.3%). Tocopherols have been found previously among marine algae.269,270 These compounds benefit to nutritional value and also are responsible for antioxidant activity of algal extracts. Furthermore, tocopherols have been found to inhibit activation of various kinases including PKC, Akt and ERK/MAP.271,272 The other compounds in alga extracts were identified to be FAs and one aldehyde. Of six identified FAs, three were saturated (C14:0, C16:0, C18:0), two monoenoic [C16:1, n-9)] and one trienoic acid [C18:3 (n-3)]. Hexadecanoic acid was determined as the main acid (20.6%) in this column fraction. That is consistent with the analysis of *Padina vickersiae* from Senegal of which the main compound was found to be hexadecanoic acid.242 Of six identified sterols in column fraction 6, all had Δ5 structure, five of them were C24-alkylated with the ratio of C27:C25:C29 of 1:3:2. Fucosterol (39.9%) was identified, as the main compound followed by 24-methylcholesta-5,22-dien (21.5%) and cholesterol (18.4%).

In column fraction 2, by EI-MS three FAs were identified, one saturated (C16:0) and two unsaturated [C18:1 (n-9); C18:2 (n-6). The usual predominant FAs found in *Padina* sp. are C14, C16 and C18 acids.244 In *P. gymnosophra* from Qatar, hexadecanoic acid was the main acid isolated followed by C18:1 and C20:2.271 Also, in some *Padina* sp., such as *P. vickersia* from Turkey, the presence of only saturated FAs was detected and none of the unsaturated acids were identified.61 So far, there are no data on the FA and sterol composition of this species from Australia. The other three column fractions isolated from the dichloromethane extracts of *Padina* sp. (3-5) analysed by EI-MS were found to contain only sterols (Table 2.4).
In total, in the *Padina* sp. column fractions, fucosterol was identified in three fractions, in fraction 6 by GC-MS analysis and in fractions 4 and 5 by EI-MS. The second most abundant sterol in the analysed species was cholesterol that was identified in three fractions (4, 5 and 6). The presence of fucosterol and cholesterol in many of the fractions confirms the dominance of these two sterol compounds in *Padina* sp. as observed previously for *P. pavonia* from Adriatic sea, *P. vickersiae* from Senegal, *P. gymnospora* from Qatar and in *P. pavonica* collected in Greece.\(^{274,275}\) Cholesterol was the main sterol in species *P. vickersia* from the Senegalese coast in the work of Aknie *et al.* and Combut *et al.*\(^{244,246,275}\) Fucosterol is usually a dominant sterol of *Padina* species while cholesterol is generally detected in *Padina* species but in lower amounts.\(^{246}\) However, the dominance of cholesterol in some *Padina* sp. was previously observed.\(^{244}\)

**Sargassum vestitum**

The non-polar dichloromethane fraction generated from *S. vestitum* obtained using extraction method A (Fig. 2.5) exhibited significant biological activity in the PKA inhibition assay (76%, Fig. 2.13 A) and it was further separated by column chromatography to yield four column fractions judged by TLC. The four column fractions were screened for PKA inhibitory activity and fraction 4 which displayed the highest activity was further separated (Fig. 2.20) using column chromatography to give one pure fraction and three partially purified fractions which were also tested for their PKA inhibitory activity.

![Figure 2.20](image)

**Figure 2.20:** A: Extraction protocol of the green alga *Sargassum vestitum* and B: PKA inhibitory activity of the column fractions obtained from the dichloromethane extract.

Further purification of column fraction 4 by preparative HPLC gave four fractions 4.1-4.4 (Fig 2.20). The compound in fraction 4.4 was identified as thunbergol A or 9-(3,4-dihydro-2,8-dimethyl-6-hydroxy-2H-1-benzopyran-2-yl)-6-methyl-2-(4-methyl-3-pentenyl)-(2E,6E)-
nonadienoic acid (Fig. 2.21). Thunbergol A was isolated previously from *Sargassum thunbergi*\(^{27}\). The structure of this compound was confirmed with HRESI-MS and 1D and 2D NMR data. Thunbergol A was screened in the PKA assay, however it did not exhibit potent inhibitory activity (7% PKA inhibition).

Attempts were performed to purify the dichloromethane fraction of *S. vestitum*, due to the complex mixture and low yield of the fraction, the non-polar fraction was analysed by GC-MS method and identified 18 compounds that are presented in Table 2.5. The predominant compounds were six FAs, three monosaturated (C14, C16 and C18), one monoenoic (C18, n-9), one dienoic (C18:2 n-9) and one tetraenoic acid (C20:4 n-5). Along with FAs, several types of other compounds including aldehydes and alcohols were also found to be present in this fraction. In previous analyses of FA composition of *Sargassum* sp. hexadecanoic acid was reported as the major acid along with C18, C18:1 (n-6), C20:4 (n-6) and C20:5 (n-3).\(^{61,239}\) It was earlier reported *Sargassum* species characteristically have a high content of meroditerpenoids, consisting of a polypropenyl chain attached to a hydroquinone ring moiety. The fact that these compounds were not observed herein could be a result of the database used (NIST 08) and may have been identified using alternative database such as MarinLit and Dictionary of Natural Compounds.\(^{277, 278}\)

### Table 2.5: GC-MS analysis of the dichloromethane extract of *S. vestitum*

<table>
<thead>
<tr>
<th>No</th>
<th>Compound</th>
<th>Rt (min)</th>
<th>% Area</th>
</tr>
</thead>
<tbody>
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<td>2.37</td>
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<tr>
<td>2</td>
<td>2,3,4-Trimethylhexane</td>
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<td>4.25</td>
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<td>3</td>
<td>3-Methylenedecan-4-ol</td>
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<td>6,10-Dimethyl-2-undecanone</td>
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<td>Pseudoiocone</td>
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<tr>
<td>8</td>
<td>3,7,11,15-Tetramethyl-2-hexadecen-1-ol</td>
<td>28.30</td>
<td>3.94</td>
</tr>
<tr>
<td>9</td>
<td>2,6,6-Trimethyl-2-cyclohexene-1-carboxaldehyde (cyclocitral)</td>
<td>28.40</td>
<td>2.84</td>
</tr>
<tr>
<td>10</td>
<td>1-Hexadecyne</td>
<td>28.64</td>
<td>2.02</td>
</tr>
<tr>
<td>11</td>
<td>1-Octadecyne</td>
<td>29.04</td>
<td>2.76</td>
</tr>
<tr>
<td>12</td>
<td>Hexadecanoic acid (16:0)</td>
<td>29.39</td>
<td>9.02</td>
</tr>
<tr>
<td>13</td>
<td>Isomenthol</td>
<td>30.90</td>
<td>2.01</td>
</tr>
<tr>
<td>14</td>
<td>Octadecanoic acid (18:0)</td>
<td>31.29</td>
<td>4.65</td>
</tr>
<tr>
<td>15</td>
<td>2-(Pentadec-12-en-1-yloxy)tetrahydro-2H-pyran</td>
<td>31.76</td>
<td>3.09</td>
</tr>
<tr>
<td>16</td>
<td>9,12-Octadecadienoic acid (18:2 n-6)</td>
<td>32.24</td>
<td>5.12</td>
</tr>
<tr>
<td>17</td>
<td>9-Octadecenoic acid (18:1 n-9)</td>
<td>32.33</td>
<td>4.99</td>
</tr>
<tr>
<td>18</td>
<td>5,8,11,14-Eicosatetraenoic acid (20:4, n-6)</td>
<td>34.85</td>
<td>6.03</td>
</tr>
</tbody>
</table>
2.8 PKA inhibitory activity of pure fatty acids and sterols

The main constituents of the non-polar fractions were identified as FAs and sterols mainly in all algae samples. The FAs and sterols that occurred in higher concentrations in the algae extracts were tested for their PKA inhibition activity with the aim of relating the activity of the sample with the concentration of the particular acid or sterol (Fig. 2.22). A series of commercially available FAs (C14:0, C16:0, C18:0, C18:1) and a FA mixture (C14-22, C18-22) were examined for their ability to inhibit PKA, at a concentration of 100 µg/ml. In column fraction 1 of the dichloromethane extract of Padina sp., α-tocopherol was identified as the main constituent (40.3%). This fraction exhibited high PKA inhibitory activity, hence pure α-tocopherol was also screened in the PKA assay. Results obtained from the PKA inhibition assay are presented in Fig. 2.22. α-Tocopherol exhibited 21% PKA inhibitory activity (data not in figure).

These results showed that FAs were more potent than sterols. C14 acid displayed the highest activity of (64%) while C16, C18 and C18:1 displayed moderate activity. These results are consistent with previous research on bioactive FAs from marine algae. The results have practical value as marine algae are rich in PUFAs of the n-3 and n-6 series, which are considered essential FAs for humans and animals. It is also reported in the literature that the antibiotic activity of some algae species could be attributed to the presence of a mixture of organic acids such as: capric, lauric, linoleic, myristic, oleic, palmitic, stearic.²⁴⁶ N-3 PUFAs have been used in the cancer prevention and treatment and for modulate the immune system.²⁷⁹ Linolenic acid has been found to be able to slow down cell growth in prostate, breast, and colon cancers, while arachidonic acid can inhibit the growth of human lung tumor A549 cells.²⁸⁰ Because of the huge and renewable biomass, seaweeds are a potential source of FAs for biotechnology and a dietary source of essential FAs.

![Figure 2.22: PKA inhibitory activity of fatty acid and sterols typically found in ten alga species collected from South Eastern Australia.](image-url)
In conclusion, 10 algae species from SE Australia were analysed and the non-polar fractions isolated from all alga samples were found to give potent biological results and displayed more activity compared with the activity of the more polar fractions. The collection place, environmental conditions and the date of sample collection was almost identical with all algal species collected herein. Therefore, the activity results were found to be mainly related to the different algae divisions. This is the first report of marine algal extracts exhibiting PKA inhibitory activity. The dichloromethane extracts isolated from brown algae were found to display the highest activity in the PKA inhibition assay except for *C. sinuosa*, which exhibited the lowest activity compared with the other algae dichloromethane extracts. There are no literature data so far on the kinase inhibitory of extracts isolated from these algae samples and for most of the samples haemolytic data are reported for the first time. Although, all compounds found in these algae were identified before, this work shows for the first time the chemical composition and activity of ten algal extracts of algae species from Australia. The FA composition of all ten algal species investigated was dominated by saturated FAs, and in particular hexadecanoic, 9,12-octadecadienoic acid and unsaturated C18 FAs. The dominance of cholesterol and fucosterol was detected in all algal non-polar extracts. The chemical composition of the algal species analysed herein was found to be both species related due to the similarity of our data to the literature data on fatty acid and sterol constituents of the corresponding algal species. However, environmental conditions, as suggested in the literature analysis, may also play a role in the similar chemical composition of algal species, as the samples were exposed to almost identical environmental conditions.
Sponges are the largest source of novel marine natural products published every year.\textsuperscript{31} Herein, the PKA inhibitory, haemolytic and cytotoxic activities and chemical composition of extracts obtained from three deep sea sponges collected from North Western Australia are described.

3.1 Marine sponges

Sponges are the most primitive multicellular animals, belonging to the phylum Porifera which comprise three groups: the Demospongiae, the Calcarea (calcareous sponges) and the Hexactinellida (glass sponges), where the class Demospongiae constitutes more than 90% of known living sponges (Fig. 3.1).\textsuperscript{281,251} Sponges belong to the group of sessile animals that also include mollusks, tunicates and byrozans and they are characterised by a lack of physical protection. Therefore, in order to protect themselves from predators, bacterial and fungal infection, growth of other fouling organisms and invasion by other marine organisms, sponges are found to produce a range of bioactive secondary metabolites that are used in ecological interactions.\textsuperscript{282} However, it is now believed that many of the bioactive compounds extracted from marine sponges are produced by the microbes and bacteria which are found in the sponge tissues.\textsuperscript{283} Only a few studies have shown whether the bioactive secondary metabolites were products of the sponge itself or the sponge symbiotic organism (e.g. bacteria).\textsuperscript{284-286}

![Figure 3.1: Sponges belonging to three different phyla (Demospongiae, Calcarea and Hexactinellida).](image)

3.2 Metabolites from marine sponges

Sponges are found to produce the highest variety of different natural products among Metazoa (animals) producing compounds with cytotoxic, antibacterial, haemolytic and other biological properties.\textsuperscript{288,289} As for shallow-water sponges, the deep-water organisms have also proven to be a productive source of bioactive metabolites with diverse structures.\textsuperscript{68} The research on deep-sea sponges was encouraged by the isolation of bioactive halichondrin B (30, page 17) from the deep sea marine sponge \textit{Lissodendoryx} sp., of which a synthetic analogue (10, page 9) was approved for breast cancer in 2010.\textsuperscript{52,88} The metabolites from deep-sea sponges are also of particular interest because they account for over 60% of the deep-sea natural products reported.\textsuperscript{68}
Some of the potent secondary metabolites with different scaffolds and biological activities isolated from deep-sea marine sponges include: antitumor compound dericitin (114) isolated from *Dericitus* sp.; sollasins A-F (115-117) with cytotoxic and antifungal activity obtained from *Poecillastra laminaris*; HIV-integrase and tyrosine protein kinase inhibitors ircinol sulfate (118) and 2-heptaprenyldihydroquinone (119) isolated from *Ircinia* sp.; dragmacidin (120), isolated from *Dragmasidon* sp. which displayed significant cytotoxicity towards a range of cancer cell lines and leiodolide A (121) with potent cytotoxic activity towards ovarian and leukemia cancer cell lines, isolated from *Leiodermatium* sp. In addition, a number of kinase inhibitors have also been isolated from both shallow- and deep-water sponges including the non-competitive inhibitor manzamine A of GSK-3β with an IC$_{50}$ value of to 10.2 μM (122) from *Hymeniacidon aldis*, and the EGFR inhibitors aeroplysinin-1 (123) from *Verongia aerophoba* and halenaquinone (124) from *Xestospongia exigua* (Fig. 3.2).

![Chemical structures of bioactive compounds isolated from marine sponges.](image)

**Figure 3.2**: Bioactive compounds isolated from marine sponges.

### 3.3 Deep-sea sponge collection and identification

Accessing deep-sea samples is prohibitively expensive and logistically difficult which has meant that much of the research to-date has been conducted through non-selective dredging and trawling operations. Selective sampling, performed with manned submersibles and remotely
operated vehicles (ROVs), has minimal environmental impact and allows for valuable *in situ* ecological observations, however, it is limited to a handful of scientific institutions with access to such equipment.\(^{68,296}\) In recent years, industrial collaborations have expanded access to deep-sea technologies such as ROVs to a wider number of researchers, facilitating further research into deep-sea fauna biology, physiology, and chemistry.\(^{89,296,297}\) Through such multidisciplinary collaboration between a consortium of Australian universities and the Australian Oil and Gas industry, a variety of deep-sea invertebrates were recently collected from subsea structures off the North West (NW) Shelf of Australia. The samples were collected as part of a decommissioning program conducted by Woodside Petroleum during Jun/July 2008. The deep-sea sample collection was performed by the SEA SERPENT (Scientific and Environmental ROV Partnership Using Existing Industrial Technology) group in June and August 2008. The decommissioning of oil rigs located off the NW Shelf of Western Australia from 1986 to 1992 resulted in a series of abandoned wellheads that had been on the ocean floor for 30 years. These six wellheads are at the Echo 1, Yodel 1, Coassack 2 & 3, Wanaea 5 and Goodwyn 9 oil fields. These structures act as artificial reefs and attract a diverse array of both vertebrate and invertebrate marine fauna including fishes, sponges, cnidarians, molluscs, echinoderms and crustaceans.\(^{298,299}\)

![Figure 3.3: Map showing the six North West Shelf wellhead removal sites from the 2008 collecting expedition.\(^{300}\)](image)
Three demosponge samples were obtained from two subsea structures/locations, with one sample found to comprise two species of sponge (Fig. 3.3). The three sponges were identified as: *Haliclona* sp. (Order Haplosclerida, Family Chalinidae); *Geodia* sp. (Order Astrophyorida, Family Geodiidae); and an intertwined sample of *Sarcotragus* sp. and *Ircinia* sp. (both belonging to the Order Dictyoceratida, Family Irciniidae) (Table 3.1).

**Table 3.1: Description of collection sites.**

<table>
<thead>
<tr>
<th>Sponge species</th>
<th>Well Site</th>
<th>Depth (m)*</th>
<th>Latitude</th>
<th>Longitude</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Haliclona</em> sp.</td>
<td>Yodel 1</td>
<td>135</td>
<td>19°44’40“S</td>
<td>115°43’12”E</td>
</tr>
<tr>
<td><em>Geodia</em> sp.</td>
<td>Yodel 1</td>
<td>135</td>
<td>19°44’40“S</td>
<td>115°43’12”E</td>
</tr>
<tr>
<td><em>Sarcotragus</em> sp. / <em>Ircinia</em> sp.</td>
<td>Wanaea 5</td>
<td>84</td>
<td>19°35’13“S</td>
<td>116°24’41”E</td>
</tr>
</tbody>
</table>

† All locations and depths from Geoscience Australia (http://dbforms.ga.gov.au/www/npm.well.search).
* Below mean sea level.

### 3.4 Sponge description and previous (reported) studies

**Figure 3.4:** Above water photographs of the deep-sea sponges *Haliclona* sp. (A), mixed *Ircinia* sp. / *Sarcotragus* sp. (B) and *Geodia* sp. (C).

#### 3.4.1 *Haliclona* sp.

Is a branching sponge with well spaced holes on its surface, which form an encrustation on rocks. It can be yellow, light blue, purple or sometimes green in colour (Fig. 3.4A). This sponge requires light conditions and therefore occurs usually in shallow-water, the higher intertidal zone area closer to the surface. Approximately 390 species have been found to belong to the genus *Haliclona* and the sample described herein is the first report of a *Haliclona* species from the deep-sea.

A diverse range of bioactive metabolites have been reported from *Haliclona* sp. collected worldwide from both warm and cold waters. In particular *Haliclona* species have been found to be rich in bioactive alkaloids including a range of polycyclic pyridinium alkaloids (125), and araguspongin C (126, 127), which displayed antibacterial, cytotoxic, antimicrobial, anti-dormant mycobacterial and antifungal activity (Fig. 3.5).³⁰¹-³⁰⁷
Additionally, other types of metabolites have been isolated from this sponge including: novel peptides with potent antifungal activity, the bioactive terpenes, haliclotroli A and B (128, 129), a macrocyclic diamide haliclonin A (130) with moderate cytotoxic and antibacterial activity against diverse microbial strains; cytotoxic glycoprotein lecithin; halipeptins A and B that exhibit potent anti-inflammatory activity in vivo and a novel ceramide (131) which acts as an antifouling substance against macroalgae (Fig. 3.6).

Not only polar constituents were isolated from Haliclona species but also sterols and FAs were obtained. In particular, these sponges are known as an excellent source of a high diversity of sterol metabolites. The lipid composition of various Haliclona sp. determined by GC and GC-MS analysis has been reported by several research groups.314-317 Cholesterol has been characterised as the main sterol in Haliclona sp., while among the FAs the dominant acid has been hexadecanoic acid.318 As a source of kinase inhibitors, species of Haliclona sponge have so far only yielded p-sulfoxyphenylpyruvic acid (132) that exhibits tyrosine kinase inhibitory activity (Fig. 3.6).319

3.4.2 Sarcotragus sp. can vary in size from a small sponge to a massive globular size. It is usually dark in colour with a horizontally flattened surface. This species is distributed
worldwide but some of the species are more common in the European zone. Although there have been reports on this species from Australia, this is the first report on a deep-sea Sarcotragus species from Australia.

Sponges of the genus Sarcotragus are reported to be a rich source of linear sesterterpenes. Liu et al. isolated five new furanosesterterpene tetronic acids (e.g. 133) with potent cytotoxicity against five human tumour cell lines from sponge material collected at the depth of 15-25 m in Korea. Moreover in their continuing studies on the genus Sarcotragus the authors isolated two new norsesterterpenoids, four new pyrroloterpenoids (e.g. 134) and known furanosesterterpenoid (Fig. 3.7). Also other compounds, such as alkaloids (135-137) and glycerolipids were isolated from this species and proven to be bioactive including two new compounds isolated from the deep-sea Sarcotragus sponge also collected from 25 m depth off the coast of Cheju Island in Korea. Barrow et al. isolated variabilin (138) from Sarcotragus sp., which was first isolated from the sponge I. variabilis, and found to display potent antiviral and antitumor activity (Fig. 3.7). Nortriterpenoids (e.g. 139) have also been reported from deep-sea species of Sarcotragus sponges.

![Images of compounds isolated from the sponge Sarcotragus sp.]

**Figure 3.7:** Compounds isolated from the sponge Sarcotragus sp.

**3.4.3 Ircinia sp.** is usually a large size sponge with widely spaced conules. The colour is creamy yellow or light brown. Oscules are dark and usually grouped on top of the animal. Characteristic of this sponge is an unpleasant sulfur/garlic smell. There are 79 species belonging to the genus Ircinia and 12 belonging to the genus Sarcotragus. Ircinia species have been found in shallow and deep waters worldwide, however this is the first report on a deep-sea example from Australia.

Sponges belonging to the genus Ircinia are among the most explored sponges in terms of marine natural compounds. The genus Ircinia has yielded a series of novel and bioactive compounds including: tedanolide C (140), a new 18-membered macrolide with potent
cytotoxicity against HCT-116 cells in vitro; ircinamine (141) showing moderate activity against the murine leukemia cell line P388; four cheilanthane sesterterpenoids (e.g. 142) as potent kinase inhibitors; palinurin and fasciculatin sulfates and novel 5,6-epoxystersols (143) with potent cytotoxic activity (Fig. 3.8).\textsuperscript{195,327-330} Seven new linear C-22-sterterpenoids (irciformonins E-K, e.g. 144), were isolated from sponge \textit{I. formosana}.\textsuperscript{331} Previously, the same authors isolated irciformonin A-D from the same species.\textsuperscript{332} All the above mentioned compounds including many others were isolated from shallow water sponges but there are also examples from deep-sea species such as sulfircin (145), isolated from an \textit{Ircinia} sp. collected at a depth of 119 m in the Bahamas.\textsuperscript{333}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{compounds.png}
\caption{Bioactive compounds isolated from the sponge \textit{Ircinia} sp.}
\end{figure}

\textbf{3.4.4 \textit{Geodia} sp.} is a large, globular sponge often found to have a white or brown colour (Fig. 3.4C). It has a rounded shape with an irregular, largely smooth, but often encrusted surface and it is common for it to form large colonies that resemble dots. So far approximately 115 species belonging to the genus \textit{Geodia} have been found.

There have also been several reports on bioactive compounds isolated from different species of \textit{Geodia} sponge, including a southern Australian sample collected from a depth of 51 m, which has yielded the potent nematocidal agent geodin A (146), a novel macrocyclic polyketide lactam tetramic acid (Fig. 3.9).\textsuperscript{334} Furthermore, water and petroleum ether extracts of the Swedish deep-water sponge \textit{G. barretti}, exhibiting antibacterial and antiviral activity, have yielded the novel diketopiperazine-containing indole alkaloid, barettin (147).\textsuperscript{335} Also, a novel dibrominated cyclopeptide, bromobenzisoxazolone barettin which showed significant antifouling activity, was isolated from the same species \textit{G. barretti} collected in Sweden at a depth of 50 m, by Hedner \textit{et al.}\textsuperscript{336}
Sponges collected from shallow-water were also found to produce bioactive secondary metabolites including two novel isomalabaricane-type nortriterpenoids, geoditins A and B (148, 149) and a novel sterol compound; and an unusual polyoxygenated sterol compound (150) (Fig. 3.9).\textsuperscript{337,338} Two new cyclodepsipeptdes, geodiamolide A and B, were isolated from a Geodia sponge collected at 25 m depth in Trinidad and Tobago followed by the isolation of two novel geodiamolide H and I a few years later from the same genus from Trinidad (20 m).\textsuperscript{335,336} Rangel \textit{et al.} found significant antibacterial, antifungal, cytotoxic, haemolytic and neurotoxic activities of the methanol extract generated from \textit{G. corticostylifera}.\textsuperscript{341} Furthermore they isolated cyclic peptides geodiamolides A, B, H and I with potent anti-proliferative effect against sea urchin eggs and human breast cancer cell lines.\textsuperscript{341}

3.5 Biological screening of sponge extracts

Extracts of the three seep-sea sponges collected from the NW Shelf were prepared according to a reported procedure by Thale \textit{et al.} as described in the experimental chapter 7 (method C) to give seven extracts of different polarity (hexane 1, hexane 2, dichloromethane, 10% methanol, 50% methanol, butanol and water extracts) (Fig. 3.10).\textsuperscript{257} The protocol was designed to separate the metabolites into groups of similar compounds. This partitioning step provided a preliminary separation of the metabolites based on their polarity, where the non-polar fractions would be expected to be dominated by lipids, sterols, and terpenes and the polar fractions by alkaloids, peptides, polyketides, and carbohydrates.
Extracts from marine organisms are widely screened for cytotoxicity against both human and murine cancer cell lines. However, in recent years testing for inhibition against specific enzymes has become more widespread, as this provides greater information about a natural product’s mode of action.

The deep sea is one of the most biodiverse and species-rich habitats on the planet, and deep-sea organisms are emerging as an important new source of unexplored chemical, genetic and biological diversity. In particular, sponges have yielded a variety of novel metabolites which display a range of biological properties. As previously discussed in Chapter 2, protein kinases are one of the most targeted enzymes and involved in the regulation of an increasing number of biological processes. Thus, the extracts of different polarity generated from the three deep-sea sponges were tested for their ability to inhibit PKA; to lyse equine erythrocytes and for their toxicity towards the brine shrimp *Artemia franciscana* (Fig. 3.11).

### 3.5.1 *Haliclona* sp.: The greatest activity (27%) in the PKA inhibition assay was found in the 50% methanol extracts obtained from *Haliclona* sp. (Fig. 3.11). The non-polar dichloromethane fraction and the polar butanol fraction exhibited the second and third highest activity in the PKA inhibition assay of 26% and 16% respectively, while the other four fractions had low to no activity in the PKA inhibition assay.

Interestingly, both polar and non-polar fractions displayed stronger potency in the haemolytic assay compared with the results from the PKA inhibition assay. Moderate to strong haemolytic activity towards equine erythrocytes was observed in both the polar and non-polar fractions of all extracts isolated from the sponge *Haliclona* sp., with the 50% methanol fraction
exhibiting the greatest activity (75%, Fig. 3.11). The two non-polar fractions, dichloromethane and the hexane 1 fraction, along with the more polar 10% aq. methanol fraction also displayed significant haemolytic activity (47%, 39%, and 50% respectively). The butanol fraction displayed moderate activity in the haemolytic assay of 38%, while the water and hexane 2 fractions displayed very low activity. Both polar and non-polar haemolytically active compounds have been found in various marine organisms, including cone snail peptides, dinoflagellate toxins, and echinoderm saponins. Extracts obtained from sponges *H. viridis* and *H. rubens* were previously found to display haemolytic activity.\(^{342-344}\)

Moderate lethality towards the brine shrimp *A. franciscana* was observed in the hexane 1 extract (33%, Fig. 3.11). The water extract, although displaying negligible PKA inhibitory activity, exhibited some activity in both the haemolytic (12%) and the brine shrimp lethality assay (13%). The 50% aq. methanol fraction and the hexane 2 extract exhibited some cytotoxic activity (18% and 8%, respectively) while other fractions exhibited low levels of brine shrimp lethality (< 4%). Based on these results found herein the three non-polar extracts (hexane 1, hexane 2 and dichloromethane extracts) and 10% and 50% methanol extracts would warrant further investigation in order to identify the metabolites responsible for the observed activity. Brine shrimp toxicity has been reported previously for eight new cerebrosides from *Haliclona* sp and three new alkaloids, isohalitulin, haliclorensin B, and haliclorensin C obtained from *H. tulearemsis*.\(^{345,346}\)

### 3.5.2 *Geodia* sp.

In the PKA inhibitory assay, the hexane 1 extract of *Geodia* species displayed the most potent activity of 97% followed by the non-polar hexane 2 extract (41%). The remaining five *Geodia* extracts exhibited lower activity in the PKA inhibition assay, where the water extract exhibited the least activity, which is consistent with the results of the other two deep-sea sponge samples where the water extracts also displayed low PKA inhibitory activity (Fig 3.11). Previous work of *Geodia* sponges resulted in the isolation and identification of receptor tyrosine kinases from *Geodia* species such as *G. cydonium* indicating that this sponge is also likely to produce endogenous kinase modulators.\(^{347}\) Furthermore, the hexane 1 extract of the *Geodia* sample also exhibited potent cytotoxicity in the brine shrimp assay (87%) and was more active than other extracts generated from the sponge *Geodia* sp (Fig. 3.11). The other six extracts displayed less than 10% lethality in this assay.
Figure 3.11: % PKA inhibition, haemolysis and brine shrimp (BS) lethality of deep-sea sponge extracts. Measurements were performed in triplicate at 100 μg/mL and data are presented as means ± S.E.M. DCM, dichloromethane.

Moderate haemolytic activity towards equine erythrocytes was observed in both the polar and non-polar fractions from the sponge *Geodia* sp. (Fig. 3.11). The greatest activity of 30-45% was found in four extracts, the hexane 1, dichloromethane, 50% methanol and water extracts (41%, 34%, 36% and 28%, respectively). Interestingly the water extract, although displaying negligible PKA inhibitory activity, showed some level of both haemolytic activity and brine shrimp lethality (28% and 9% respectively). Based on the above results the most active non-polar hexane 2 extract would warrant further investigation in order to identify the metabolites responsible for the observed activity.

The haemolytic activity of the extracts isolated from *Geodia* sp. herein is consistent with the results from Rangel et al. who demonstrated the ability of the same sponge species to lyse equine erythrocytes. Haemolytic activity has been also identified in the crude extracts from *G. corticostylifera*, which were found to contain antibacterial, cytotoxic and neurotoxic cyclic peptides geodiamolides A, B, H and I.

3.5.3 *Ircinia/Sarcotragus* sp.: Two non-polar fractions of *Ircinia/Sarcotragus* sp., the hexane 1 and dichloromethane extract, displayed significant PKA inhibitory activities of 100% and 84% respectively. These results are consistent with the large number of non-polar kinase inhibitors described from marine sponges, which include sesterterpenoid mitogen activated protein kinase
MSK1 and MAPKAPK-2 inhibitors from Ircinia sp. Furthermore prenylhydroquinones isolated from the sponges S. muscarum and I. fasciculate were found to inhibit various protein kinases including EGFR, Src tyrosine kinase, vascular endothelial growth factor receptor 3 and insulin-like growth factor I receptor. Unlike the other two non-polar fractions, the hexane 2 fraction exhibited low PKA inhibitory activity (8%). Similar PKA inhibitory activity was observed among the butanol, 10% and 50% methanol fractions (20%, 20% and 22%, respectively), while the water fraction displayed negligible PKA inhibitory activity.

When tested for haemolytic activity and brine shrimp toxicity, extracts generated from Ircinia/Sarcotragus sp. did not give potent results. Three extracts, the hexane 1, butanol and water extract, displayed moderate haemolytic activity towards equine erythrocytes (30%, 28%, and 22%, respectively) while the remaining four extracts exhibited lower activity levels (Fig. 3.1). However, previous analyses of the sponge I. felix collected in Australia and the Netherlands showed strong haemolytic activity in the organic extracts.

Only the hexane 2 and the 10% methanol extracts of the Ircinia/Sarcotragus sp. sponge displayed any activity towards the brine shrimp A. franciscana (10% and 9%, respectively), while the remaining extracts were inactive (Fig. 3.1). We assumed that the extracts in the brine shrimp lethality assay displayed very low cytotoxicity due to the fact that shrimps used the sponge extracts as food source. However, this might suggest that extracts are of very low cytotoxicity. Compounds with brine shrimp cytotoxicity toward A. salina were previously isolated from the sponge I. spinosula. Furthermore, the brine shrimp cytotoxicity of methanol extracts of Sarcotragus sp. from Korea led to isolation of the new cyclitol compound sarcotride D. The above results of the hexane 1 and dichloromethane extracts indicated that these two extracts should be further analysed in order to investigate the chemical constituents responsible for the bioactivity of extracts.

In summary, the PKA inhibitory activity was found to follow the order Ircinia/Sarcotragus sp. > Geodia sp. > Haliclona sp., while the haemolytic activity followed the reverse order and all samples were relatively low in brine shrimp lethality except the hexane 1 extract of Geodia sp. In general, the non-polar extracts (hexane 1, hexane 2 and dichloromethane) isolated from all sponge samples were found to give more potent bioactive results, in particular greater PKA inhibitory activity, compared to the polar fractions. The most active extracts were hexane 1 and dichloromethane extract from Ircinia/Sarcotragus sp. and the hexane 1 and hexane 2 from Geodia sp. in the PKA inhibition assay. The results described herein are the first report on the biological activity of deep-water sponges belonging to the genera Haliclona and first report on the bioactivity of an Australian deep-sea Sarcotragus sp.
3.6 Investigation of the chemical composition of three deep-sea sponges

Following from the bioactivity screening, all three sponges were further purified and analysed to determine their chemical composition.

3.6.1 Sponge fatty acid and sterol composition

Sponges also provide a mixture of unusual and diverse sterols with unique structures that are different from sterols found in terrestrial plants; in fact sponges are believed to be the richest source of the most diverse sterols in the animal kingdom. Compared with terrestrial plants, which usually contain a simple sterol composition of three to six compounds, marine sponges possess a rich mixture of sterols. Unusual sterols are usually present in low yields, appearing with the typical 3β-hydroxy sterols. Generally sterols are considered to have a 3β-hydroxy-Δ5-cholestane (or Δ5) structure where the number 5 indicates the number of the double bond position with a 8-10 carbon side chain.

![Figure 3.12: Typical sterols found in marine sponges.](image)

The differences in the sterol structure in sponges can include a modified base carbon skeleton, oxygenation, degradation and alkylation of the side-chain giving sterols with C0-C12 side chains where alkylation usually occurs at position C-24 (Fig. 3.12). The polar sterols that have sulfate or polyhydroxy groups are of particular interest as they have been reported to exhibit a range of biological activities. The diversity of sterol structures in the marine environment are likely due to the different environmental factors such as water temperature, salinity, pollution and diet. The main source of marine sponge sterols is food intake, but in general the origin of sterols in sponges comes through four pathways: de novo synthesis; through diet; dietary origin with modification and from associated organisms attached to the sponge surface. Δ5 Sterols have been found to be the dietary sterols which sponges take up from food. Furthermore, sponges have been found to be able to transform dietary sterols to stanols and to Δ7 sterols. Transformation of dietary Δ5 to Δ7 sterols goes through the thermodynamically more stable Δ5,7 sterols in sponges, but it has been found in starfishes that this transformation can also proceed through stanols. The source of Δ5,7 sterols can be also a diet as they were found to occur in marine yeasts.

Although sponges have been characterised as a rich source of novel and unique sterols, they are also known to be a rich source of a variety of fatty acid (FA) compounds including long
chain FAs, and FAs with odd, even and branched chains. Marine sponges usually contain long-chain Δ 5,9 FA with up to 34 carbon chain and some sponges have been found to contain significant amount of FA with up to 60 FAs.

3.6.2 *Haliclona* sp.

The hexane 1 fraction of the deep-water sponge *Haliclona* sp.: The total number of identified fatty acids by GC-MS analysis in this fraction was 14, while only three sterol compounds were found (Tables 3.2 and 3.3). Fatty acids make up 62.0% of the hexane 1 extract and sterols 13.9%. The polyunsaturated fatty acid C20:4 (n-6) is present in a large amount (21.2%) in this extract, while there was no detection of this FA in the other two non-polar fractions obtained from *Haliclona* sp. The other two acids which are present in high content are 9-octadecenoic acid (6.5%) and 9-hexadecenoic acid (5.9%), but in substantially smaller amounts when compared to the C20:4 (n-6) acid. A similar trend of FA constituents was observed in a *Haliclona* sp. from India, where the main FAs were C16, C16:1, C18 and C18:1 types. Of the 14 fatty acids identified, 10 were saturated (C10:0, C14:0, 12-methyl C14:0, C15:0, C16:0, C17:0, C18:0, C19:0, C20:0, C24:0), two monoenoic [C16:1, (n-9)], one dienoic C20:2 (n-6) and one tetraenoic acids C20:4 (n-6), of which two are substituted acids.

**Table 3.2:** Fatty acid composition of non-polar extracts from *Haliclona* sp., *Geodia* sp. and *Ircinia/Sarcotragus* sp.

<table>
<thead>
<tr>
<th>N</th>
<th>Fatty acids</th>
<th>Type</th>
<th><em>Haliclona</em> sp.</th>
<th><em>Geodia</em> sp.</th>
<th><em>Ircinia/Sarcotragus</em> sp.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>A</td>
<td>B</td>
<td>C</td>
<td>A</td>
</tr>
<tr>
<td>1</td>
<td>2-Methylbutanoic acid</td>
<td>5.0</td>
<td>-</td>
<td>2.4</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>3-Methylbutanoic acid</td>
<td>5.0</td>
<td>-</td>
<td>13.5</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>Pentanoic acid</td>
<td>5.0</td>
<td>-</td>
<td>5.3</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>Hexanoic acid</td>
<td>6.0</td>
<td>-</td>
<td>0.3</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>Decanoic acid</td>
<td>10.0</td>
<td>4.8</td>
<td>2.2</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>10-Methylundecanoic acid</td>
<td>12.0</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>Dodecanoic acid</td>
<td>12.0</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>Tridecanoic acid</td>
<td>13.0</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>9</td>
<td>Tetradecanoic acid</td>
<td>14.0</td>
<td>1.6</td>
<td>-</td>
<td>5.3</td>
</tr>
<tr>
<td>10</td>
<td>12-Methyltetradecanoic acid</td>
<td>15.0</td>
<td>3.8</td>
<td>6.4</td>
<td>5.7</td>
</tr>
<tr>
<td>11</td>
<td>5,9,13-Trimethyltetradecanoic acid</td>
<td>17.0</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>12</td>
<td>Pentadecanoic acid</td>
<td>15.0</td>
<td>1.5</td>
<td>0.5</td>
<td>1.3</td>
</tr>
<tr>
<td>13</td>
<td>Hexadecanoic acid</td>
<td>16.0</td>
<td>2.2</td>
<td>7.7</td>
<td>3.4</td>
</tr>
<tr>
<td>14</td>
<td>7-Methylhexadecanoic acid</td>
<td>17.0</td>
<td>1.3</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>15</td>
<td>9-Hexadecenoic acid</td>
<td>16.1</td>
<td>5.9</td>
<td>0.8</td>
<td>2.6</td>
</tr>
<tr>
<td>16</td>
<td>Heptadecanoic acid</td>
<td>17.0</td>
<td>-</td>
<td>-</td>
<td>3.3</td>
</tr>
<tr>
<td>17</td>
<td>10-Methylheptadecanoic acid</td>
<td>17.0</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>18</td>
<td>Octadecanoic acid</td>
<td>18.0</td>
<td>3.9</td>
<td>-</td>
<td>6.6</td>
</tr>
<tr>
<td>19</td>
<td>17-Methyloctadecanoic acid</td>
<td>19.0</td>
<td>-</td>
<td>0.2</td>
<td>-</td>
</tr>
<tr>
<td>20</td>
<td>9-Octadecenoic acid</td>
<td>18.1</td>
<td>6.5</td>
<td>0.4</td>
<td>2.3</td>
</tr>
<tr>
<td>21</td>
<td>11-Octadecenoic acid</td>
<td>18.1</td>
<td>1.7</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>22</td>
<td>9,12,15-Octadecatrienoic acid</td>
<td>18.3</td>
<td>-</td>
<td>1.2</td>
<td>-</td>
</tr>
<tr>
<td>23</td>
<td>Nonadecanoic acid</td>
<td>19.0</td>
<td>1.8</td>
<td>3.0</td>
<td>-</td>
</tr>
<tr>
<td>24</td>
<td>Eicosanoic acid</td>
<td>20.0</td>
<td>2.2</td>
<td>2.0</td>
<td>7.1</td>
</tr>
<tr>
<td>25</td>
<td>11,14-Eicosadienoic acid</td>
<td>20.2</td>
<td>3.5</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>26</td>
<td>5,8,11,14-Eicosatetraenoic acid</td>
<td>20.4</td>
<td>2.1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>27</td>
<td>Docosanoic acid</td>
<td>22.0</td>
<td>-</td>
<td>-</td>
<td>1.7</td>
</tr>
<tr>
<td>28</td>
<td>Tetracosanoic acid</td>
<td>24.0</td>
<td>-</td>
<td>0.2</td>
<td>3.7</td>
</tr>
<tr>
<td>29</td>
<td>Hexacosanoic acid</td>
<td>26.0</td>
<td>1.8</td>
<td>-</td>
<td>1.2</td>
</tr>
</tbody>
</table>

% of total extract: 62.0 23.2 67.1 3.9 13.2 23.8 65.8 41.7 83.0

A: Methanol-derived hexane extract (hexane 1); B: dichloromethane-derived hexane extract (hexane 2); C: dichloromethane extract.
Three sterols (Entry 3, 4 and 21, Table 3.3, 151-153) were identified in this extract, the short side chain sterol, pregn-5-en-3β-ol (151) is the major compound accounting for 12.7% of the total chemical composition (Fig. 3.13). Two sterol compounds 152 and 153 are present at lower levels. The two short side chain sterols have a Δ5 structure while the third sterol (Entry 21) has a C30Δ7 structure and has an alkylated side chain at position C24, which regularly occurs in sponges. The main pregnane sterol was not observed in the other two non-polar extracts from this sponge, which could contribute to the differences in the biological activities among the extracts. Three short side chain sterol compounds have been found previously in *Haliclona* sp., of which two (Entry 2 and 4) were the same as the pregnane sterols in the hexane 1 extract.364 Also, four sterols with a pregnane structure were found in *H. rubens* from Jamaica.365 They were identified as 20-keto and 20-hydroxy sterols with the usual 5-en-3β-ol structure and the authors suggested that the biosynthesis of short side-chain sterols might be achieved through oxidative cleavage of marine sterol with longer side chains.365 Moreover, sterols with both type of the short side chain sterol, with androstan and pregnane structures, were found in *H. flavescentis* from the Black Sea, and in other marine organisms such as algae and gorgonians.364

Firstly, it was assumed that short side chain sterols could be formed as artefacts through marine sample work-up and the second assumption was that they are products of a biological degradation process or biosynthesis from terpene precursors.366,367 Presently it is believed that precursors of short side-chain sterols are oxidized side-chain sterols that occur through an *in vivo* autoxidation process.364,366 The role of short side chain sterols in marine invertebrate is still unknown, however these sterols are important in the reproduction system of vertebrates.368

### Table 3.3: Sterol composition of non-polar extracts from *Haliclona* sp., *Geodia* sp., and *Ircinia/Sarcotragus* sp.

<table>
<thead>
<tr>
<th>N</th>
<th>Sterols</th>
<th>Type</th>
<th><em>Haliclona</em> sp.</th>
<th><em>Geodia</em> sp.</th>
<th><em>Ircinia/Sarcotragus</em> sp.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>A</td>
<td>B</td>
<td>C</td>
</tr>
<tr>
<td>1</td>
<td>Androst-5-en-3β-ol</td>
<td>Δ5</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>21-Norpregna-5-en-3β-ol</td>
<td>Δ5</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>Pregna-5-en-3β-ol</td>
<td>Δ5</td>
<td>12.7</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>Pregna-5,17(20)-dien-3β-ol</td>
<td>Δ5</td>
<td>0.4</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>Pregna-5-en-20-en-3β-ol</td>
<td>Δ5</td>
<td>-</td>
<td>3.5</td>
<td>6.8</td>
</tr>
<tr>
<td>6</td>
<td>24-Norcholesta-5,22-dien-3β-ol</td>
<td>Δ5,22</td>
<td>-</td>
<td>-</td>
<td>1.0</td>
</tr>
<tr>
<td>7</td>
<td>Cholesta-5,22-dien-3β-ol</td>
<td>Δ5</td>
<td>-</td>
<td>-</td>
<td>10.2</td>
</tr>
<tr>
<td>8</td>
<td>Cholesta-5-en-3β-ol</td>
<td>Δ5</td>
<td>-</td>
<td>tr.</td>
<td>1.5</td>
</tr>
<tr>
<td>9</td>
<td>Cholesta-7,22-dien-3β-ol</td>
<td>Δ5</td>
<td>-</td>
<td>6.8</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>Cholesta-5,7-dien-3β-ol</td>
<td>Δ5</td>
<td>0.5</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>11</td>
<td>24-Methylcholesta-5,22-dien-3β-ol</td>
<td>Δ5,22</td>
<td>-</td>
<td>-</td>
<td>1.3</td>
</tr>
<tr>
<td>12</td>
<td>Cholesta-7-en-3β-ol</td>
<td>Δ5</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>13</td>
<td>4α,5α-Cholestane-4,5-epoxy</td>
<td>Δ6</td>
<td>-</td>
<td>-</td>
<td>14.8</td>
</tr>
<tr>
<td>14</td>
<td>24-Methylcholesta-5,24(28)-3β-ol</td>
<td>Δ6</td>
<td>-</td>
<td>9.5</td>
<td>-</td>
</tr>
<tr>
<td>15</td>
<td>24-Methylcholesta-5-en-3β-ol</td>
<td>Δ6</td>
<td>-</td>
<td>4.0</td>
<td>-</td>
</tr>
<tr>
<td>16</td>
<td>24-Methylcholesta-7-en-3β-ol</td>
<td>Δ6</td>
<td>-</td>
<td>2.7</td>
<td>5.2</td>
</tr>
<tr>
<td>17</td>
<td>24-Methylcholesta-7,24(28)-3β-ol</td>
<td>Δ6</td>
<td>-</td>
<td>-</td>
<td>3.5</td>
</tr>
<tr>
<td>18</td>
<td>24-Ethylcholesta-5-en-3β-ol</td>
<td>Δ6</td>
<td>-</td>
<td>9.0</td>
<td>3.0</td>
</tr>
<tr>
<td>19</td>
<td>24-Ethylcholesta-5,24(28)-dien-3β-ol</td>
<td>Δ6</td>
<td>-</td>
<td>1.1</td>
<td>1.8</td>
</tr>
<tr>
<td>20</td>
<td>24-Ethylcholesta-7-en-3β-ol</td>
<td>Δ6</td>
<td>0.8</td>
<td>1.2</td>
<td>-</td>
</tr>
<tr>
<td>21</td>
<td>24-Propylcholesta-5,24(28)-dien-3β-ol</td>
<td>Δ6</td>
<td>-</td>
<td>0.2</td>
<td>7.8</td>
</tr>
<tr>
<td>22</td>
<td>24-Isopropylcholesta-5,24-dien-3β-ol</td>
<td>Δ6</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>23</td>
<td>22,23-Cyclopropylcholesta-5-en-3β-ol</td>
<td>Δ6</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

| % of total extract | 13.9 | 28.1 | 13.4 | 72.1 | 50.4 | 46.5 | 16.3 | 6.8 | 8.0 |

A: Methanol-derived hexane extract (hexane 1); B: dichloromethane-derived hexane extract (hexane 2); C: dichloromethane extract; tr. (trace amounts) < 0.1%.
In the *Haliclona* hexane 1 extract a range of aldehydes, hydrocarbons and alcohols were also identified (Table 3.4). Hydrocarbons occur in sponges regularly and often play an important role in the defence mechanisms. However there have also been sponges found that do not contain these compounds, indicating that they are not the only compounds responsible for the defence mechanisms of sponges. Aldehydes and alcohols characteristically appear in plants and algae where they serve as allelochemicals, protecting plants from other organisms. While they have also been found to occur among marine organisms as a protection agent, the complete role of volatile compounds and their composition in the sponges is still not completely understood.

### Table 3.4: Chemical composition of non-polar extracts from *Haliclona* sp., *Geodia* sp. and *Ircinia/Sarcotragus* sp.

<table>
<thead>
<tr>
<th>N</th>
<th>Compounds</th>
<th><em>Haliclona</em> sp.</th>
<th><em>Geodia</em> sp.</th>
<th><em>Ircinia/Sarcotragus</em> sp.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>A</td>
<td>B</td>
<td>C</td>
</tr>
<tr>
<td>1</td>
<td>4-Methyltridecane</td>
<td>-</td>
<td>3.7</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>1-Tetradecene</td>
<td>6.8</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>14-Noncosane</td>
<td>-</td>
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<td>-</td>
</tr>
<tr>
<td>4</td>
<td>3-Tridecene</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>1,3-Propanediol, 2-dodecyl</td>
<td>11.6</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>2-Propylheptanoic</td>
<td>3.9</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>1-Nonadecanone</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>1-Eicosanol</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>9</td>
<td>10-Pentadecanol</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>1,3-Propanediol, 2-dodecyl</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>11</td>
<td>Heptadecanol</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>12</td>
<td>2-Cyclohexyl-3-isopropyl-pent-4-en-2-ol</td>
<td>-</td>
<td>-</td>
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</tr>
<tr>
<td>13</td>
<td>2-Methyl-2-pentenal</td>
<td>-</td>
<td>21.3</td>
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<td>14</td>
<td>2-Octanal</td>
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<td>2.1</td>
<td>0.1</td>
</tr>
<tr>
<td>15</td>
<td>2-Nonanal</td>
<td>-</td>
<td>2.1</td>
<td>0.5</td>
</tr>
<tr>
<td>16</td>
<td>Octanal</td>
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<td>-</td>
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</tr>
<tr>
<td>17</td>
<td>Undecanal</td>
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<td>-</td>
</tr>
<tr>
<td>18</td>
<td>2-Tridecanol</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>19</td>
<td>6,10-Dimethyl-5,9-undecadien-2-one</td>
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<td>2.7</td>
<td>-</td>
</tr>
<tr>
<td>20</td>
<td>4-Bromo-2-methylpent-2-enoic acid</td>
<td>-</td>
<td>6.3</td>
<td>-</td>
</tr>
</tbody>
</table>

The hexane 2 extract of the deep-water sponge *Haliclona* sp.: Nine fatty acids, seven sterols and six other non-polar compounds were identified in the hexane 2 extract of the *Haliclona* sponge by GC-MS analysis (Tables 3.2 and 3.3 and 3.4). In this extract, in contrast to
the results from the hexane 1 extract (preceding section), the sterols comprised a higher percentage of the total chemical composition, 28.1%, compared to 23.2% for the FAs. The two main FAs detected were hexadecanoic acid (7.7%) and 12-methyltetradecanoic acid (6.4%), while other FAs were present in low percentages. The nine FAs identified include seven saturated (C10:0, C15:0, C16:0, C19:0, C20:0, C24:0) and two monoenoic acids [C16:1 (n-9), C18:1 (n-9)], of which one is a substituted acid (12-methyl C14:0). Interestingly, in some samples of *H. cinerea* more than 50 FAs were found, as well as being rich in PUFAs with C22- C34 carbon atoms.361

From the 13 sterols identified in the three non-polar extracts of the *Haliclona* sp. by GC-MS, seven were found to be present in the hexane 2 extract (Table 3.3). The main sterols were 24-methylcholesta-5,24(28)-3β-ol (9.5%) and 24-ethylcholesta-5-en-3β-ol (9.0%) (Fig. 3.14). Interestingly, cholesterol was only found in the hexane 2 extract of the three non-polar extracts of *Haliclona* sp. (Table 3.2) and only in a very low percentage in the 50% methanol extract. Previously, in shallow-water *Haliclona* sp., from Western Australia, Chile and India, cholesterol was reported to be the main sterol.292,318,370 Although, in *Haliclona* sp. from Antarctica cholesterol was observed in only minute amounts.371 The lack of cholesterol in the analysed sample could indicate the importance of the environmental factors of the sponge collection, as this *Haliclona* sp. was from 135 m depth in cold water, where the temperature and environmental conditions may be comparable to those of sponges collected from Antarctica.

![Sterol Structures](image)

**Figure 3.14:** The main sterols identified in the hexane 2 extract obtained from the deep-sea sponge *Haliclona* sp.

In this extract, sterols with $\Delta^5$, $\Delta^7$ and $\Delta^{5,7}$ structures were identified with the $\Delta^5$ structure dominant and no stanols were found. The ratio of $\Delta^5$: $\Delta^7$: $\Delta^{5,7}$ compounds in this sponge is 4:1:2 while the ratio of C27:C28:C29 sterols is found to be 3:1:3. Typically the family Halicloniidae, are rich in the dietary $\Delta^5$ sterols but some sponges belonging to this family have only 5% of this sterol and possess stanols and $\Delta^7$ sterols.364 In *Haliclona* sp. from the Black Sea, $\Delta^5$ sterols accounted for only 2% of the sterol mixture and the main sterols contained either a $\Delta^7$ or stanol structure, which indicates that transformation through stanols could also occur in sponges. Interestingly, Elenkov *et al.* recently found a mixture of 4-methyl stanols and $\Delta^7$ sterols in *H. cinerea* and *H. flavescens*.372 These unusual sterols are not regularly found among marine sponges and are believed to be synthesised *in vivo*.368 The hexane 2 extract was also rich in
aldehydes, with the 2-methyl-2-pentanal present in very high yields compared with the other compounds identified in this extract (Table 3.4).

The dichloromethane extract of the deep-water sponge *Haliclona* sp.: By GC-MS analysis, this extract had a very high percentage of FAs (67.1%) with 18 fatty acids identified in total. Of these, 10 were saturated acids (C5:0, C6:0, C10:0, C14:0, 12-methyl C14:0, C15:0, C16:0, C17:0, C18:0, 17-methyl C18:0, C20:0, C22:0, C24:0, C26:0), two monoenoic [C16:1 (n-9), C18:1 (n-9)] and one trienoic acid C18:3 (n-9), of which two were substituted acids (Table 3.2). The main FA was eicosanoic acid (7.1%) followed by octadecanoic acid (6.6%) and 12-methyltetradecanoic acid (5.7%). Short chain FAs, such as butanoic, pentanoic and hexanoic acids, were also identified in this extract. In the dichloromethane extract three saturated long chain acids were identified (C22, C24 and C26), while in the two other non-polar extracts (hexane 1 and hexane 2) only one was found. Characteristic for shallow-water sponges is that they contain long chain fatty acids which can make up to 85% of the fatty acid composition. However, in the dichloromethane extract of *Geodia* sp. analysed in this work the FA profile was dominated by saturated C14- C20 fatty acids, while only two long chain FA were detected (C24:0 and C26:0).

Five sterols were found in the dichloromethane extract, comprising 13.4% of the total extract determined by GC-MS (Table 3.3). Four of five sterols were identified in this extract only, while their presence was not detected among the other two non-polar extracts obtained from the *Haliclona* sample. The main sterol was 24-methylcholesta-5-en-3β-ol (Table 3.3, entry 15), although all sterol compounds were present in a similar ratio. In this extract none of the Δ^5,7 sterol was found, only Δ^5 and Δ^7 sterols in a ratio of 4:1.

Of the other compounds identified in the three non polar extracts only two aldehydes were detected in the dichloromethane extract and both were present in low amounts (Table 3.4).

The 10% methanol extract of the deep-water sponge *Haliclona* sp.: The 10% methanol extract (0.47 g), generated from the crude dichloromethane extract, was further separated by silica gel column chromatography using a mixture of hexane, EtOAc and MeOH, into the major six fractions as judged by TLC (Fig. 3.15). Although, TLC indicated that none of these fractions were pure. The six fractions obtained from column chromatography were analysed by EI-MS, the identified compounds are presented in Table 3.5. Even though, the particular separation method was chosen to separate lipid and other non-polar compounds, some non-polar compounds remained in this extract. The pooled column fractions isolated from the 10% methanol extract were tested for their PKA inhibitory activity and the results are presented in Fig. 3.15. Only two fractions displayed PKA inhibitory activity of 17 and 21% respectively,
while the other four fractions exhibited activity lower than 10%. Attempts were made to further purify these fractions, but as they were mixtures of compounds of similar structure and obtained in low yields, it was difficult to achieve full and complete separation of the compounds (chapter 2). The chemical composition of the six column fractions, analysed by EI-MS, is presented in Table 9.1 in the Appendix section.

**Figure 3.15**: Extraction protocol of 10% methanol extract of the deep-water sponge *Haliclona* sp. and biological activity of the column fractions obtained.

**The 50% methanol extract of the deep-water sponge *Haliclona* sp.:** The 50% methanol extract (0.187 g), obtained from the crude methanol extract, was further purified by silica gel column chromatography using a gradient solvent system with hexane, ethyl acetate and methanol, which gave six major fractions as judged by TLC. The pooled column fractions isolated from the 50% methanol extract were tested for their PKA inhibitory activity and the results are presented in Fig. 3.16. None of the six column fractions was more potent in the PKA inhibitory assay than the original 50% methanol extract. In this extract the main constituents were lipid compounds. The chemical composition of the six column fractions was analysed by EI-MS and the results obtained are presented in Table 9.2 (Appendix section).

**Figure 3.16**: Extraction protocol of 50% methanol extract of the deep-water sponge *Haliclona* sp. and biological activity of the column fractions obtained.
The butanol extracts of the deep-water sponge *Haliclona* sp.: The butanol extract was obtained in low yield and also did not exhibit any significant bioactivity in the three assays, hence this extract was not further studied.

### 3.6.3 *Geodia* sp.

**The hexane 1 extract of the deep-water sponge *Geodia* sp.:** Eight FAs were identified in the hexane 1 extract by GC-MS analysis and their percentage was low at yields of less than 1% each, while the sterol compounds were present in higher quantities (Table 3.2 and 3.3). Of the eight FAs in the hexane 1 fraction, seven were saturated (C10:0, C13:0, C14:0, 12-methyl C14:0, C15:0, C16:0, C17:0) and one was a monoenoic acid C18:1 (n-9).

Interestingly, the dominant sterol in the hexane 1 extract was an unusual short side-chain sterol 19-norpregna-5-en-3β-ol present in 23.9% and an epoxy sterol 4α,5α-cholestan-4,5-epoxy (14.8%). Previously, *Geodia* sp. was reported to produce unusual sterols such as 26-methylergosta-5,24(28)-dien-7-one-3α-ol along with the same sterols as identified in this species. Four short side chain sterols were identified in this extract (Table 3.4). Only one short side chain sterol was found in the hexane 2 extract and none were identified in the dichloromethane extract. The hexane 1 extract displayed potent activity in the PKA inhibition assay (97%) and the brine shrimp lethality assay (87%) which could be due to either the high level of sterols or the difference of the sterol composition compared with the other fractions obtained from this sponge. There are previously reported examples of bioactive short side-chain sterols from marine species, including the gorgonian *Muricea cf. austera*, which has yielded known sterols with a pregnane structure that displayed potent antibacterial activity and two novel pregnane glycosides with moderate cytotoxicity toward HCT-116 human colon carcinoma.

**The hexane 2 extract of the deep-water sponge *Geodia* sp.:** Six FAs were detected in the hexane 2 extract with the main FA identified as dodecanoic acid (3.7%, Table 3.2). Of the six FAs, five were saturated (C6:0, C12:0, C14:012-methyl C14:0, C15:0) and one was a monoenoic acid C18:1(n-7). The hexane 2 extract was the richest in compound yields compared to other non-polar extracts obtained from the same sponge.

Seven sterols were identified in the hexane 2 extract (Table 3.3). Fucosterol was determined as the dominant sterol (20.2%), and in higher yield compared to the other two non-polar extracts. 24-Methylcholesta-5,24(28)-dien-3β-ol was also identified in this fraction (7.0%) (Table 3.3), although not detected in the other two non-polar fractions of *Geodia* sp. extracts. These results are consistent with literature reports where this sterol was found to be the main sterol in the sponge *G. robusta* from the Sea of Okhotsk and also identified as the main sterol along with fucosterol in *Geodia* sp. from the Faroe Islands. The ratio of the Δ5:Δ7 sterols in
this extract was found to be 5:2. In the hexane 2 extract and in the other two non-polar extracts of the analysed Geoda sp. the predominant sterols also possessed a Δ^5 structure.

The dichloromethane extract of the deep-water sponge Geodia sp.: In total, 10 FAs were identified with 9-octadecenoic acid (5.2%) and octadecanoic acid (5.0%) as the dominant acids. In G. cydonium the predominant FA was hexadecanoic acid with 31.8% followed by octadecanoic acid with 12.3%. Of the 10 FAs identified in the dichloromethane extract, seven were saturated (C6:0, C14:0, C15:0, C17:0, C18:0, C20:0, C22:0), two were monoenoic [C16:1 (n-9), C18;1 (n-9)] and one dienoic acid C20:2 (n-6).

Seven sterols were identified in the dichloromethane fraction (Table 3.3), with 24-norcholesta-5,22-dien-3β-ol (13.4%) and cholesta-7-en-3β-ol (11.2%) as the principal sterols. The ratio of the Δ^5:Δ^7 sterols in this fraction was 5:3, and the ratio of C_{26}:C_{27}:C_{28}:C_{29} was found to be 1:2:2:1. The same norcholesta compound was previously identified in the G. robusta sponge collected from Sea of Okhotsk. Along with the FAs and sterols, two aldehydes and two alcohols were also identified in the dichloromethane fraction (Table 3.4).

The 10% methanol extract of the deep-water sponge Geodia sp.: The 10% methanol extract was analysed by EI-MS and two compounds were identified by comparing the mass spectra with data in the NIST 08 library, and tentatively characterised as a sterol cyclic ether compounds.

The 50% methanol extract of the deep-water sponge Geodia sp.: The 50% methanol extract was prepared from the crude methanol extract of Geodia sp. as shown in Fig. 3.11. The 50% methanol did not exhibit significant biological activity in all three assays. Due to this low biological activity and the small amount of sample, this extract was analysed only by EI-MS. Three sterol compounds were tentatively identified as cholesta-5-en-3β-ol, 24-methylcholesta-7-en-3β-ol and 24-methylcholesta-5,24-dien-3β-ol.

The butanol extract of the deep-water sponge Geodia sp.: Although this fraction did not display significant biological activities, it was further analysed in order to determine the type of metabolites that occur in the more polar extract. Thus, this extract was further purified by preparative HPLC using an isocratic mobile phase with H_2O as the eluent. Five fractions were isolated and analysed by EI-MS. The identified compounds (156-160) are presented in Fig. 3.17. Four known nucleoside compounds and one known alkaloid were identified. The structure of thymidine was also confirmed by NMR and HRMS. NMR data of other four fractions showed that they were not completely pure. These butanol fractions were screened for PKA...
inhibitory activity but they displayed low activity (< 11%). Nucleosides, such as thymidine, adenosine, were previously isolated from marine organisms, including marine sponges and the sponge Haliclona sp. Nucleosides isolated from marine organisms have been found to display antiviral and anticancer activities.

Figure 3.17: Compounds identified in the butanol fraction of the Geodia sp.

3.6.4 Ircinia/Sarcotragus sp.

The hexane 1 extract of the deep-water sponge Ircinia/Sarcotragus sp.: By GC-MS analysis, 10 FAs comprising 65.8% in this extract were identified (Table 3.2). The dominant acids were 7-methylhexadecanoic acid (9.7%) and eicosanoic acid (9.6%). Interestingly, of the 10 FAs identified, only one was an unsaturated tetraenoic acid C20:4 (n-6), while the other nine were saturated acids (Table 3.2). The hexane 1 extract lacked long chain fatty acids with only two C20 fatty acids identified, one saturated and the other an unsaturated FA. It has been reported that Ircinia species are particularly rich in C24-C25 FAs, however none were found in this extract.

The hexane 1 extract possessed more sterols and in higher quantities compared with the other two non-polar extracts obtained from the Ircinia/Sarcotragus sample (Table 3.3). Six sterols were identified, comprising 16.3% of the total extract. Five of the six sterols had a Δ5 structure and there was one short side-chain sterol compound. The main sterol was cholesta-5,24-dien-3β-ol. Previously, unusual sterols were isolated from I. aruensis from China, containing a 5,6-epoxy group and displaying significant cytotoxic activity.

The hexane 2 extract of the deep-water sponge Ircinia/Sarcotragus sp.: Twelve fatty acids were identified in this extract by GC-MS analysis which comprised 41.7% of the total extract (Table 3.2). Characteristic of the hexane 2 extract was the high content of aldehydes and alcohols which were detected in this extract as well (Table 3.4). Similar to the other two non-polar extracts obtained from this sponge, the 12 identified acids were dominated by saturated acids, with nine saturated FAs, two monoenoic and one tetraenoic acid. The main FA detected was 11-octadecenoic acid (9.5%). This extract contained long chain fatty acids that were in
similar ratios as found in the dichloromethane extract of *Ircinia/Sarcotragus* sp. Hence, as the hexane 2 extract had low PKA inhibitory activity, this could indicate that long chain FAs are not responsible for the potent PKA inhibitory activity of the dichloromethane extract. The activity could be a result of contribution of the main FA or other FAs detected in the dichloromethane extract.

The hexane 2 extract contained four sterol compounds (Table 3.3) with the dominant sterol being 24-isopropylcholesta-5,24-dien-3β-ol. The ratio of Δ⁵:Δ⁵,7 sterols in this extract is 3:1 while the ratio of C₂₇:C₂₉:C₃₀ is found to be 1:1:2. Cholesta-5,7-dien-3β-ol was only detected in this extract of the *Ircinia/Sarcotragus* sponge and represents the sole sterol with a Δ⁵,7 structure. Previously it was observed that some deep-sea sponge samples contain none or a very small number of sterols.³⁷¹ For example, it was reported that members of the order Dictyoceratida generally possess low sterol content such as in the sponge *S. spinulosus* where no sterols were found at all.³⁷¹,³⁷⁸,³⁷⁹ On the contrary, sterol composition in shallow water species is usually found to be very complex, which is likely to be due to the different ability of sponges to obtain sterols. However, some shallow water sponges do have only a simple sterol composition such as the sponge *Chondrosia reniformis*.²⁸⁶,³⁶²,³⁶⁴,³⁸⁰ It was found herein that the *Ircinia/Sarcotragus* sample contains a low diversity of sterols in low amounts.

In the *Ircinia/Sarcotragus* hexane 2 extract a number of aldehydes, ketones and alcohols were also identified (Table 3.6). Volatile low-molecular weight molecules have been found to be responsible for *Ircinia’s* characteristic unpleasant garlic odour and may also be used for chemical defence.³⁸¹

**The dichloromethane extract of the deep-water sponge *Ircinia/Sarcotragus* sp.** By GC-MS analysis, 14 FAs were identified in the dichloromethane extract obtained from *Ircinia/Sarcotragus* sp. and they comprised 83.0% of this extract. The 14 FAs identified included 10 saturated, two monoenoic and one trienoic acid, of which three were substituted acids. The dominant acid was octadecanoic acid (9.8%) but also seven other FAs were present in high levels. Fatty acids with long chains were identified in this extract (C₂₂–C₂₆, Table 3.2), which are common for sponge samples.³⁶⁹ Long chain acids with 26 carbon atoms are dominant fatty acids in the class Demospongiae whereas in contrast here, only one PUFA was identified in the dichloromethane extracts as a minor compound. However, in some sponge samples C₂₆ acids were either not identified or found in low percentage and fatty acids with C₂₅ and C₂₇ were the major compounds.³⁸²

In addition, Δ⁵ sterols were identified by GC-MS analysis of the dichloromethane extract and they comprised 8.0% of the total extract (Table 3.3). The dichloromethane extract contained Δ⁵ sterols exclusively with 24-methylcholesta-5-en-3β-ol sterol as the dominant sterol.
Sterols were less dominant in this extract in comparison with the percentage of the FAs identified in the same extract. In this extract short side chain sterol, 21-norpregna-5-en-3β-ol, was identified by GC-MS. It was the only sterol with a pregnane structure identified in the *Ircinia/Sarcotragus* sample. Androst-5-en-3β-ol was found in the hexane 1 extract and was the only other the short side chain sterol identified in *Ircinia/Sarcotragus* sp. Interestingly, cholesterol was only present in the dichloromethane extract and this was similar to the results obtained from *Haliclona* sponge where cholesterol was only found in the hexane 2 extract. Whilst it wasn’t detected in the other two non-polar extracts, it was identified in the more polar extract of the same sponge.

Compared with the other two sponges, *Haliclona* sp. and *Geodia* sp., the least number of sterols were found in this sponge. *Ircinia* sp. and *Sarcotragus* sp. belong to the order Dictyoceratida, which has been shown before to have low sterol content. In particular, these sponges are usually rich in terpenoid compounds which could indicate an alternative pathway of isoprenoid metabolism or the oxidation and other modification of sterols.\(^{220,371}\)

**The 10% methanol extract of the deep-water sponge *Ircinia/Sarcotragus* sp.:** The 10% methanol extract was generated from the crude dichloromethane extract of *Ircinia/Sarcotragus* sponge in low yield and it was further purified by column chromatography using a solvent mixture of hexane and EtOAc, to give six fractions. Column fractions were analysed by TLC, indicating that each fraction possessed more than one compound, which was also confirmed by EI-MS analysis. The pooled column fractions isolated from the 10% methanol extract were tested for their PKA inhibitory activity and the results are presented in Fig. 3.18. The actual yields of constituents were not recorded. The chemical composition of the six column fractions was analysed by EI-MS and the results showed that the main constituents were FAs and sterols (Table 9.3, Appendix section).
The 50% methanol extract of the deep-water sponge *Ircinia/Sarcotragus* sp.: The 50% methanol extract, which displayed low activities in all three assays (Fig. 3.11) was analysed by EI-MS and only 1,3,4,6-O-tetraacetyl-d-glucosamine was identified.

The butanol extract of the deep-water sponge *Ircinia/Sarcotragus* sp.: The butanol extract was further purified by preparative HPLC using an isocratic mobile phase with H$_2$O as the mobile phase. Four fractions were isolated. The HPLC butanol fractions were analysed by EI-MS. The structure of thymine identified in the column fraction 6 was confirmed by proton NMR spectroscopy. Low yields of purified compounds did not enable further analysis. The chemical constituents of the column fractions 1 and 2 were unidentified, while identified compounds in column fractions 3-6 were 5-methyl-5-docosane, 2-(hydroxyimino)indan-1-one, thymine and phenethylamine, respectively.

3.6.5 Investigation into the water extracts of the three deep-water sponges

The water extracts obtained from all the three deep-sea sponges were found to display low biological activities (Fig. 3.11). Chemical composition in water extracts was analysed by high resolution ESI-QToF-MS; the tentatively identified compounds are presented in Table 9.4 (Appendix). Compounds were identified by comparing HRMS data with those from the Dictionary of Natural Compounds. Compounds identified were of different structures with most of them being halogenated as is typical for marine natural products and containing heteroatoms, mainly nitrogen.
In summary, four deep-sea sponges from the NW Shelf in Australia were found to be a rich source of PKA inhibitory and haemolytically active substances. Using a modified Kupchan partitioning method, the following results were obtained: the hexane 1 extracts from the *Ircinia* sp./*Sarcotragus* sp. and *Geodia* sp. displayed potent PKA inhibitory activity of 100% and 97% respectively, with the *Geodia* sample also showing potent brine shrimp lethality. The 50% methanol extract from the *Haliclona* sp. exhibited potent haemolytic activities of 75%. In general, the non-polar fractions isolated from all three deep sea sponges were found to have more biological activity compared with the activity of the more polar fractions. As this is the first example of a *Haliclona* sp. of sponge obtained from the deep-sea sponge, the results described herein are also the first report on bioactivity of deep-sea *Haliclona* sp. Similarly, herein we have the first example on the deep-sea *Sarcotragus* and *Ircinia* species from Australia and the first description of kinase inhibitory activity from any of the sponges under investigation.

The main constituents of the non-polar fractions were identified as fatty acids and sterols with a total of 23 known sterols and 29 known fatty acids. Similar sterol and fatty acids were found among three sponges which could be a result of these being collected on the same date from the same or a similar collection place. These results indicate that the small differences that occurs among chemical composition of three sponge samples analysed is the result of the different biodiversity of the sponges as they were collected under same environmental conditions. The percentage of the main chemical classes in all non-polar fractions is shown in Table 3.5. These data show that *Ircinia/Sarcotragus* sp. was rich in FA, followed by *Haliclona* sp. and then *Geodia* sp., while sterols were the most abundant in *Geodia* sp.

**Table 3.5:** Summary of chemical constituents of the non-polar extracts in three deep-water sponges.

<table>
<thead>
<tr>
<th>Compound Type</th>
<th><em>Haliclona</em> sp.</th>
<th><em>Geodia</em> sp.</th>
<th><em>Ircinia/Sarcotragus</em> sp.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>B</td>
<td>C</td>
</tr>
<tr>
<td>Fatty acid (Table 3.2)</td>
<td>62.0</td>
<td>23.2</td>
<td>67.1</td>
</tr>
<tr>
<td>Sterols (Table 3.3)</td>
<td>13.9</td>
<td>28.1</td>
<td>13.4</td>
</tr>
<tr>
<td>Other (Table 3.4)</td>
<td>22.3</td>
<td>38.2</td>
<td>0.6</td>
</tr>
<tr>
<td>Unidentified</td>
<td>1.8</td>
<td>10.5</td>
<td>18.9</td>
</tr>
</tbody>
</table>

A: Methanol-derived hexane extract (hexane 1); B: dichloromethane-derived hexane extract (hexane 2); C: dichloromethane extract.
Isatin and 2-oxindole derivatives are widely found among marine organisms as bioactive agents. Herein, the synthesis of a series of isatin analogues based on a natural compound isolated from a marine mollusc is described, along with screening for their PKA inhibitory activity.

4.1 Previous research on isatin analogues

The bioactivity of egg masses of the Australian mollusc *Dicathais orbita* have been extensively studied by Benkendorf *et al.* at the University of Wollongong, based on the concept that they were likely to provide chemical defensive substances to promote survival. Indeed, the egg masses were found to contain a range of cytotoxic and antibacterial compounds. Among these, tyrindoleninone (6-bromo-2-methylthio-3H-indol-3-one, 72) (Fig. 1.33, Chapter 1) exhibited specific cytotoxicity towards cancer cell lines over human mononuclear cells of 4 µM and 195 µM respectively, giving a selectivity index of almost 50. Attempts to prepare this compound in the lab were unsuccessful due to the reactivity of the indoleninone nucleus. 6-Bromoisatin was also found as a decomposition product formed through the oxidation of tyriverdin and shown to exhibit modest cytotoxicity against a human lymphoma cell line (Fig. 1.32, Chapter 1). Based on the above, the development of a novel series of cytotoxic compounds was undertaken starting with the readily synthetically accessible 6-bromoisatin (74).

Further developments of 6-bromoisatin (74) derivatives as potent cytotoxins included modification of isatin (75) with a range of various substituents on the aromatic ring and nitrogen. These results showed that the presence of an electron-withdrawing group(s) on the aromatic ring of isatin were essential for low micromolar cytotoxicity. In particular 5,7-dibromoisatin (76) was more cytotoxic than the starting compound and the activity increased with N-alkylation of isatin, to give derivatives with submicromolar activity (Fig. 4.1). A one-carbon linker attaching an alkyl group to the aromatic nitrogen on isatin was found to be significant in the tubulin binding mechanism. Substitution at the C3 position was found to further increase cytotoxic activity and also to impart potent kinase inhibitory activity (Fig. 4.1). 189,182

![Figure 4.1: Isatin derivatives displaying significant cytotoxicity. *against U937 cells.](image_url)
4.2 Initial specific targets

Previous research of isatin and indole compounds proved that these scaffolds are important for kinase inhibitory activity, as previously discussed in Chapter 1 (Fig. 1.29). Figure 1.31 shows over 11 indole and isatin compounds that have been found to inhibit various kinases. This was confirmed with the indole based kinase inhibitor sunitinib and semaxanib (Fig. 1.28, Chapter 1). In particular, C3 substituted isatin derivatives have been found to display significant kinase inhibitory properties including inhibition of fibroblast growth factor receptor (VEGFR-2), epidermal growth receptor factor, PDGFR-β and RTKs. These known kinase inhibitors have a very similar core to the potent cytotoxic compounds previously analysed our group. Hence, it was of interest to further develop their structures and test them for potential protein kinase inhibitory activity. The aim was to determine the structural requirements of the different groups and their position on the isatin scaffold necessary for PKA inhibitory activity. The imino-isatin (83) was synthesised by Matesic as a part of a separate project involving conjugating isatins to tumour targeting proteins and it was found to display potent PKA inhibitory activity (IC$_{50}$ 20.0 µM). The activity of 83 was comparable to the activity of the commercially available standards (staurosporine, ellagic acid and H-89 (Chapter 1, Section 1.6.1) used in the kinase assay established within the group (Fig. 4.2). Compounds 76 and 78 were also previously screened for kinase inhibitory activity. N-alkylated isatin 78 was found to display lower activity than the imino-isatin 183 in the PKA inhibition assay, while 5,7-dibrominated isatin 76 exhibited low inhibitory activity against cyclin-dependent kinase 2 (CDK2). These results showed that the imino group on carbonyl C3 is important for kinase inhibition.

![Figure 4.2: Isatin derivative found to be a potent PKA inhibitor.](image)

Therefore, the first goal was to prepare a range of 5,7-dibromo-N-methoxybenzyl-3-imine isatin analogues with different substituents on the distal phenyl ring and test their activity (Fig. 4.3). Dibromination at position 5 and 7 and N-alkylation were found to be essential for the cytotoxicity of these compounds. To compare whether those groups are also required for the PKA inhibitory activity the next step was to change the groups on the aromatic isatin ring, as well as at the aromatic N and C3 position.
Although imines are not widely used in drug discovery due to their perceived instability, aromatic imines with extended π-π conjugation are found to be stable at physiological pH, while readily hydrolysing in mildly acidic solutions.\textsuperscript{182} Aryl imines have been used as pH-sensitive linkers in drug delivery as these compounds have been found to be stable in contrast to alkyl imine derivatives which are known to hydrolyse at physiological pH.\textsuperscript{182,185}

![Figure 4.3](image_url)

\textbf{Figure 4.3:} The aim of this project is the synthesis of a wide variety of isatin imine derivatives as potential PKA inhibitors. PMB = para-methoxybenzyl

### 4.3 Preparation of isatin imine derivatives

The first N-alkylated imino-isatin derivatives were prepared as shown in Scheme 4.1. The synthesis involved dibromination for the first step, then N-alkylation followed by preparation of the final imine isatin derivative.

![Scheme 4.1](image_url)

\textbf{Scheme 4.1:} Summary of the proposed synthetic strategies in the formation of 5,7-dibromo-1-(4-methoxybenzyl)-isatin imine derivatives. PMB = para-methoxybenzyl

#### 4.3.1. Bromination of isatin

In the preparation of the various isatin analogues, the first step involved electrophilic aromatic substitution of isatin. This dibromination of isatin was largely performed following the protocol reported by Lindwal \textit{et al.}\textsuperscript{385}

The 5,7-dibromination reaction was carried out in 95% ethanol using bromine, that was added dropwise while maintaining the temperature at 70-75 °C (Scheme 4.2). Three equivalents of bromine were used to obtain the desired 5,7-dibromoisatin compound. When lower amounts of bromine were used, 5-bromoisatin was the major product obtained. During the reaction, at the temperature of 75°C, evaporation of the bromine was noticed, which resulted in the formation of more 5-bromoisatin. The 5 position on the isatin ring is favourable for the electrophilic aromatic substitution as a result of the electro-donating effect of the nitrogen, which has both ortho and
para directing activity. Adding more bromine equivalents, bromination of the less active 7 position was achieved. $^1$H NMR spectral data, with two aromatic singlets for H4 and H6, and LREI-MS with m/z value of 303/305/307 ($^{79}$Br$^{81}$Br; $^{79}$Br$^{81}$Br; $^{81}$Br$^{81}$Br) [M$^+$] confirmed that the desired product, 5,7-dibromoisatin, was generated in 85% yield. An alternative strategy to prepare 5,7-dibromoisatin was developed by Semenov et al., which included addition of H$_2$SO$_4$ and acetic acid. Although 5,7-dibromoisatin was prepared in 95% yield, this method required a longer reaction time of 16 h.

![Scheme 4.2: 5,7-Dibromination of isatin.](image)

Previously, it was reported that the dibromination of isatin could be performed in ethanol, acetic acid or methanol. However, when attempted the reaction in methanol was not particularly successful and the product was mainly 5-bromoisatin. When the temperature of the reaction and the equivalents of bromines were increased the desired 5,7-dibromoisatin was generated. Unfortunately, the side product 5,7-dibromo-3,3-diethoxy-indoline was also obtained. The same acetal compound was obtained when using 100% ethanol instead of 95% ethanol. The acetal product occurs as a result of the formation of acidic hydrogen bromide, which further catalyses the formation of the acetal groups on the highly reactive carbonyl C3 position. Due to the adjacent amide group and aromatic ring, the C3 position of isatin has strong electrophilic affinity and is easily available for condensation and addition reactions. Acetal products are not stable and formation occurs as a reversible reaction. In the case where bromination was performed in 95% ethanol, the presence of water molecules hydrolyses the acetal and moves the reaction back to the starting ketone.

4.3.2 N-alkylation of isatin

The next step in preparing 5,7-dibromo-N-alkylated imine isatin compounds was N-alkylation step, which was performed according to the protocol of Vine et al. (Fig. 4.4). There are several different methods published in the literature that describe N-alkylation but, the method established by Vine et al. in the lab has given constantly good yields previously. N-Alkylation included formation of the dark violet isatin anion in situ with the presence of a base, followed by nucleophilic attack of the formed anion on the appropriate alkyl halide by an S$_2$2 mechanism. A range of bases such as NaH, CaH$_2$, K$_2$CO$_3$, NaOH and Cs$_2$CO$_3$ can be employed in preparation of the isatin anion and using various anhydrous solvents. Herein, the reaction was performed using either CaH$_2$ or NaH (as a stronger base) with DMF as a solvent, which allowed
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faster formation of the isatin anion compared with the weaker carbonate bases. The utilization of CaH₂ was preferable over NaH due to difficulties in working with sodium hydrides because of their reactivity. Seven dibrominated-N-alkylated isatin compounds (162-168) were prepared in this manner with yields ranging between 27 – 86% (Fig. 4.4). N-alkylated derivatives 162-165 were known compounds while 166-168 are novel N-alkylated isatins.

Reagents and conditions: a) CaH₂, DMF, rt, 20 min; b) RX, KI, 80 °C 18 h (28-90%, over 2 steps).

<table>
<thead>
<tr>
<th>Cpd. no</th>
<th>R</th>
<th>% Yield</th>
<th>Cpd. no</th>
<th>R</th>
<th>% Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>162</td>
<td>-OCH₃</td>
<td>86</td>
<td>165</td>
<td>-1</td>
<td>78</td>
</tr>
<tr>
<td>163</td>
<td>-CH₃</td>
<td>67</td>
<td>166</td>
<td>N-O</td>
<td>39</td>
</tr>
<tr>
<td>164</td>
<td>-Cl</td>
<td>69</td>
<td>167</td>
<td>N-O</td>
<td>27</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>168</td>
<td></td>
<td>78</td>
</tr>
</tbody>
</table>

Figure 4.4: Mechanism of formation and yields of the N-alkylated-5,7-dibromoisatins prepared.

Although the N-alkylation reaction gives good yields, there are some drawbacks to this method. DMF was used as a solvent in the generation of the N-alkylated compounds; it has the high boiling point so it is difficult to remove once the reaction is finished. The other disadvantage of this method is the utilization of hazardous metal hydrides. Microwave irradiation is one of the novel methods for the preparation of N-alkylated isatins. The advantages include the reduction of reaction time, usage of very small amounts of solvents and higher yields. The microwave method also has more benefits for the preparation using phenacyl compounds. With weaker base used in microwave reactions, there are fewer chances of the production of the halide anion and the occurrence of the competitive by-product and thus the use of microwave irradiation is suggested here as a possible method for future work.

Garden et al. observed that isatins with electron withdrawing groups on the 5 and 7 position react more easily with CaH₂ in DMF and that the reaction can be performed at room temperature. No dry conditions are required for the N-alkylation as the reaction works better with wet DMF, which indicates that hydroxide ion from the DMF acts as a base.
In addition to the seven dibrominated-N-alkylated isatins, three known compounds with an unsubstituted aromatic isatin ring and 5-nitro and 5-methoxy-N-alkylated isatins (169-171) were also prepared following the same procedure for N-alkylation (Fig. 4.5).

![Figure 4.5: N-alkylated isatin derivatives.](image)

The Sandmeyer method for the synthesis of the substituted isatins, including 5-nitroisatins, is one of the oldest and the most important and utilised isatin procedures. This method involves reaction of an appropriate aniline with chloral hydrate and hydroxylamine in water to yield the intermediate, which is then treated with conc. H$_2$SO$_4$ to give substituted isatin (Scheme 4.3). Other methods used for the preparation of isatins are the Stolle, Martinet and Gassman syntheses. However, nitration of isatin with KNO$_3$ and H$_2$SO$_4$ was shown to be successful previously in our lab; hence the same method was implemented here.

![Scheme 4.3: Sandmeyer procedure for the synthesis of isatins.](image)

The starting 5-methoxy isatin was commercially available while the requisite 5-nitro isatin (172) was prepared following a literature procedure. Nitration of isatin (75) at position 5 was performed according to the protocol of Vine et al., which involved slow addition of a solution of KNO$_3$ in H$_2$SO$_4$ to the solution of isatin in conc. H$_2$SO$_4$ over 1 h (Scheme 4.4). Even though the temperature was monitored between 0-4ºC, the occurrence of the 5,7-dinitro product (170 a) was still observed (12%). $^1$H NMR spectral analysis of the product indicated that the desired compound was made, with doublet of a doublet at $\delta$ 8.79 ($J = 3$ Hz) for H6 and doublets at $\delta$ 7.08 ($J = 8.5$ Hz) and 8.18 ($J = 2.0$ Hz) for H7 and H4 respectively.

![Scheme 4.4: Synthesis of 5-nitroisatin derivatives.](image)
Using 4-(chloroacetyl)-morpholine for the preparation of the N-alkylated isatin derivative (167) resulted in the lowest yield (27%). The possible explanation is the occurrence of a Darzens product in addition to the desired product. Exactly the same conditions were used for the preparation of all N-alkylated compounds; the only difference here was the starting alkyl halide, which had an additional carbonyl group on the carbon atom next to the carbon with the halide atom. The Darzen condition involves the reaction of the α carbon of a carbonyl compound with a halogen that forms an α,β-epoxy ester in the presence of a base (Scheme 4.5). The resonance stability of the anion that is formed on the carbon where the halogen atom is attached makes the reaction occur without difficulties. Therefore the formation of the anion of the alkyl compound is the competitive reaction for the formation of the N-isatin anion. Nucleophilic attack on the carbonyl compound is the next step to give formation of an epoxide.


The identification of the ten synthesised N-alkylated analogues (162-171) were validated by 1H and 13C NMR spectral data along with EI-MS data, which was in accordance with the literature data for known compounds. The structure of the novel derivatives (166-168) were additionally confirmed with HRESI-MS and 2D NMR data, using 1H NMR and gCOSY spectra to identify protons on the N-substituted ring and HSQC and HMBC spectra to assign carbon atoms in the 13C NMR. Compounds 162-171 were prepared in sufficient quantities, with yields which were consistent with the literature data, for the next steps and also for kinase inhibition screening and in vitro cytotoxicity testing.
4.3.3 Preparation of isatin imine derivatives

The carbonyl group at position 3 of isatin is reactive towards nucleophiles which has resulted in the generation of a series of biologically active 3-substituted isatins. Imine isatin derivatives were previously found to inhibit various kinases. 3-Phenylhydrazones (e.g. 172) and 3-benzothiazoles (e.g. 173) have been shown to inhibit CDK (Fig. 4.6). Furthermore, amino acid 3-substituted isatin derivatives (e.g. 174) were found to inhibit several kinases including CDK1/cyclin B, CDK5/p25 and GSK3α/β.

![Figure 4.6: Known 3-imine-2-oxindole kinase inhibitors.](image)

A diverse library of imine compounds were synthesized from N-alkylated isatins and an appropriate amine compound in ethanol with acetic acid as catalyst following the method reported by Bramson et al. (Scheme 4.6). Several methods describe formation of imines, which include utilization of microwave, traditional reflux and reactions in water as a solvent. Furthermore, a variety of solvents can be employed such as methanol, heptanes in the presence of acetic acid or ethanol.

Acidic catalysis is important for imine formation, otherwise the reaction proceeds very slowly or will not run to completion. The pH of the reaction should be adjusted between 4 and 6 with acetic acid. Acid plays two roles in the formation of an imine; one is protonation of the amine; and the second role, which is more important, is protonation of the hydroxyl group and elimination of water at the end of the reaction. If the pH value is lower than 4 the reaction proceeds slowly due to protonation of the amine. If it is higher than 6, the concentration of the protons will be insufficient for the protonation of the OH as a leaving group in the dehydration step.
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With the aim of broadening the range of compounds in the isatin series with potential biological activity, 29 imino-isatin derivatives were prepared. Table 4.1 summarizes the list of imine compounds prepared from the starting dibrominated isatin (including the different substitutions at the N-position of the starting isatin) and an appropriate amine. Most of the imine derivatives, after purification with column chromatography, were obtained in good to high yields, while lower yields were observed mainly for those analogues which were prepared from deactivated anilines with an electron withdrawing group at the para position (e.g. NO$_2$, COOH).

Table 4.1: Isatin imine derivatives 175-203 prepared in the project.

<table>
<thead>
<tr>
<th>Cpd. no</th>
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<th>$R_2$</th>
<th>% Yield</th>
</tr>
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<td>p-COOH</td>
<td>16</td>
</tr>
<tr>
<td>176</td>
<td>OCH$_3$</td>
<td>p-CH$_2$COOH</td>
<td>59</td>
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<td>p-OH</td>
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<tr>
<td>179</td>
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<td>80</td>
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<td>p-I</td>
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<tr>
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<td>I</td>
<td>m-Br</td>
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<tr>
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<td>I</td>
<td>p-Cl</td>
<td>15</td>
</tr>
<tr>
<td>203</td>
<td>I</td>
<td>p-F</td>
<td>41</td>
</tr>
</tbody>
</table>

Scheme 4.4: Mechanism of imine formation in isatins.
Recently, Khan et al. published the preparation of imines in water instead of using an organic solvent and acid catalyst (Scheme 4.7).\textsuperscript{397} Compared with our isatin derivatives, their compounds were not dibrominated or \textit{N}-alkylated. This method was attempted with our isatins however they did not dissolve in water. Other authors, such as Jarrahpour et al. used water as a solvent for imine preparation, but these methods are not applicable to our compounds.\textsuperscript{396}

\textbf{Scheme 4.5:} Reported formation of imine-isatin analogues in water.\textsuperscript{397}

Herein, the formation of imines was performed with molecular sieves and absolute ethanol. Imines are unstable compounds with the tendency to decompose to the starting material. Although, some imine compounds can be more stable, such as the compounds in this work where the stability of the imine compounds increases if there is a group present which can contribute to the delocalization and resonance stabilisation of the imine double bond.

In addition to 5,7-dibromo and \textit{N}-alkylated isatin derivatives, analogues that are not substituted at the phenyl ring and aromatic \textit{N} were also prepared (Scheme 4.8). As previously mentioned, although these substituents were required for cytotoxicity (Fig. 1.3, Chapter 1) it was of interest to probe their role in the PKA inhibitory activity of these compounds. Derivatives with an unsubstituted phenyl ring were synthesised from isatin after being \textit{N}-alkylated and coupled with an appropriate aniline to yield the desired novel imine analogue (204). Compounds with a free nitrogen (205, 206) were produced in good yield starting with dibromination of isatin followed by direct preparation of the imine (Scheme. 4.8).

\textbf{Scheme 4.6:} Preparation of a) \textit{N}-alkylated and b) 5,7-dibromo imine derivatives.

In addition to isatin imines with phenyl group on the nitrogen, three isatin imines with morpholino groups were prepared using \textit{N}-alkylated isatin 167 as a starting material and one imine using compound 166 (Fig. 4.7). These analogues were prepared using the same protocol for imine preparation as described previously. They were prepared in good yield (46-69%) and
characterised using MS and NMR spectral data. Imine-morpholino derivatives were obtained also as Z and E isomers and their ratios were identified using 1D NOE NMR analysis.

4.3.3.1 E and Z imine isomers

The 3-substituted isatin analogues can exist as a mixture of E and Z isomers depending on the substituents at the C3 position and on the characteristics of the substituent at the C4 and C5 position of the isatin core as well (Fig. 4.8). The isomers readily interconvert in solution and cannot be readily separated by chromatographic methods.

The ratio of E: Z isomers were determined by the integral height values of H2’ and H6’ chemical shifts of the protons on the external phenyl group and the H4 and H6 protons on the isatin core of the E and Z isomers. 1D NOE analysis was performed in order to identify E and Z isomers, where the E-configuration shows a NOE correlation between the H4 hydrogen atom of the isatin and C2’ or C6’ hydrogen of the phenyl ring on the 3-position of the isatin core, while the same NOE peak is not detected for the Z isomer due to the distance between the protons.

Another indication of the E or Z isomers is the signal of the H4 proton of the isatin molecule, which is shifted upfield (~6.2 ppm) for the E isomer compared with the starting material and Z isomer, which is the result of interaction with the ring current of the phenyl substituent at the position C3 on the isatin molecule that is shielding this proton. The signal for

Figure 4.7: N-Morpholino-imine isatin derivatives.

Figure 4.8: E and Z isomers of imine-2-oxindole.
the Z-isomer is close to the value of the chemical shift of the H4 of the starting isatin. Fig. 4.9 shows the \(^1\)H NMR spectrum of compound 183, where it can clearly be observed that \(4E\) H is further upfield at 6.88 ppm while the same H4 proton of the other Z isomer is at 7.69 ppm. Although there are many aromatic protons in these compounds, some were clearly resolved into doublets and singlets as seen in the figure below. However, other imino-isatin derivatives gave complex spectra with not properly resolved peaks. In addition, NOE experiments could be used to distinguish H2” and H6” protons in the phenyl ring at the C-3 position of the isatin core. The chemical shifts for the protons at the C2’ and C6’ positions were shifted downfield (~7.65 ppm) for the Z isomer, while the E isomer was found to be more upfield (~7.10 ppm). This has been found to be due to the deshielding effect of the C-2 carbonyl of isatin ring on the H2’ and H6’ protons in the Z isomer form.\(^{159}\) Only imino-isatin derivative 204, which has an unsubstituted aromatic isatin ring, was obtained as the pure E isomer.

![Figure 4.9: \(^1\)H NMR spectral data of the imino isatin derivative 183 (CDCl₃, 500 MHz).](image)

Along with the series of imine derivatives already described, two novel hydrazone compounds were also prepared following the same method as that for imine, only that an appropriate phenylhydrazine was used in place of an aniline in the reaction giving the final products (207 and 208) in high yields (Fig. 4.10). In contrast to imine derivatives that exist in E/Z isomers, hydrazones appears mainly in the Z (cis) conformation while the E isomers can be observed only as a trace in the \(^1\)H NMR spectra of 208 (Fig. 4.11). The major formation of only
one isomer is attributed to hydrogen bonding between the carbonyl group of the indoline core and NH group of the hydrazone linkage.

\[
\text{Figure 4.10: Two hydrazone derivatives 207 and 208 prepared showing intramolecular hydrogen bonding.}
\]

\[
\text{Figure 4.11: } ^1\text{H NMR spectral data of hydrazone isatin 208 (CDCl}_3, 500 \text{ MHz).}
\]

Together with imine analogues that have phenyl or phenyl hydrazine groups on position C3 on isatin, compounds with oxime group were also synthesised following the same procedure as already described (Fig. 4.12). $^1\text{H NMR}$ analysis of the desired products indicated that only the Z isomer was obtained after purification (Fig. 4.13). The formation of only one isomer is presumably due to hydrogen bonding between the carbonyl group of the isatin core and the OH group at the 3-position of the isatin core, as seen for the hydrazine analogues. Work up of the reactions of the oxime isatin products gave hydroxylimino-isatin compounds 209-211 in 57-92% yield.
4.3.4 Synthesis of 5-nitro and 5-methoxy isatin-imine derivatives

Along with 5,7-dibromoisatin and unsubstituted phenyl ring isatin derivatives, compounds with 5-nitro and 5-methoxy group were also synthesised. Furthermore, nitro and methoxy indole derivatives (e.g. SU-9516 (60) and 216, Fig. 4.14) were previously reported to inhibit different kinase enzymes.395,399

Figure 4.14: Known 5-substituted 2-oxindole kinase inhibitors.
Preparation of the 5-nitro and 5-methoxy-N-alkylated imine derivatives were achieved in good yield (67-79%) following N-alkylation and imine formation procedures previously described (section 4.3.2 and 4.3.3, Fig. 4.15). These groups were chosen with the aim to improve the lipophilicity of the isatin compounds. Figure 4.15 also shows clogP values of isatin derivatives bearing different groups on the aromatic ring.

![Figure 4.15: 5-Nitro and 5-methoxy isatin-imine derivatives.](image)

### 4.3.5 5-Sulfonyl imine isatin derivatives

Guan et al. found that substitution with a sulfonyl group at the position C5 of the indole core was essential for Src and Yes tyrosine kinase inhibitor activity (Fig. 4.16). These results were in conclusion with previous analyses where substitution at the 5-position increased activity, including isatins as cytotoxins and indirubins which are potent CDK and GSK inhibitors. Alkylaminosulfonylisatins are well studied as they have been previously identified as potent and selective inhibitors of caspases 3 and 7. Based on their discovery and to further investigate kinase inhibitory abilities of isatins, two isatin sulfonyl derivatives were synthesised.

![Figure 4.16: Structure of a 5-sulfonyl isatin derivative and its Src kinase inhibitory activity.](image)

The synthesis of the desired sulfonyl isatin derivative (224) required four steps: substitution at position five of isatin, N-alkylation of the newly formed sulfonyl group, N-alkylation of isatin and condensation of the prepared isatin with aniline. To access the desired sulfonyl products, isatin or indole ring should be substituted at position five with a sulfonyl group. The first step was adding chlorosulfonic acid dropwise to a solution of isatin in DCM and stirring it at room temperature for 2 h, as described by Bebernitz et al. (Scheme 4.9).
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Scheme 4.7: Synthesis of sulfonamide isatin derivatives.

The gem-dichloro isatin-5-sulfonyl chloride derivative was then coupled to an appropriate amine (4-aminomorpholine) in either THF or an ethanol/water mixture in a 1:1 ratio in an overnight reaction to yield the sulfonamide products. N-Alkylated sulfonamide isatin (221) was formed in good yield using both solvent systems (Scheme 4.9, THF (62%); EtOH/H₂O (88%)). 221 was hydrolysed before proceeding to next step to give 222. Sulphonamide (222) was then coupled with an aryl alkyl halide to give the N-alkylated product (223) obtained in 62% yield (Scheme 4.10). Compound 222 was also coupled with aniline give the 3-substituted isatin product (224), following procedures described previously (Section 4.3.3) in 59% yield.

Scheme 4.8: Synthesis of 5-sulfonyl isatin analogues.

4.3.6 5-Aryl isatin derivatives

Next, our interest turned towards the development of biaryl derivatives prepared by metal catalysed cross coupling reactions. GW-491619 (225), a potent kinase inhibitor, has a large group on position C-4 and C-5 (Fig. 4.17). Kilic et al. reported a series of 3-aryl substituted 2-oxindoles (e.g. 226, Fig. 4.17) as potent pp60c-src tyrosine kinase inhibitors. Therefore, substitution of the phenyl ring with 5-phenyl and 5-pyrole groups was also investigated, with the aim to identify any alterations in kinase inhibitory activity between varying hydrophobic substituents.
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Metal catalysed biaryl coupling reactions are used for formation of carbon-carbon single bonds. Palladium catalysts are widely used due to their ability to catalyse a variety of chemical reactions. Palladium catalysts are used for Suzuki coupling, which are reactions between organoboronic acid and halides (Scheme 4.11). Suzuki coupling requires the presence of a base that activates the arylboronic acid and further enhances transmetalation process. Suzuki coupling is one of the methods that can be employed in the preparation of these compounds and it is considered a ‘green’ reaction, compared with others such as the Stille coupling, which utilizes tin molecules that are toxic and have low solubility in water.

Scheme 4.9: Mechanism of Suzuki coupling.

Preparation of compounds 228-230 was carried out by a Suzuki cross-coupling reaction according to the method of Gerard et al. The Suzuki biaryl coupling between 227 and 162a and boronic acid was performed in dimethoxyethane with palladium (tetrakis(triphenylphosphine)palladium (0)) as the catalyst (Scheme 4.12). Target compounds were obtained in low yield (16-24%) and examination of the ¹H NMR and ¹³C NMR spectrum was correlated with the literature data for the known derivatives (228, 229), while the structure of 230 was confirmed by 2D NMR data. ¹H NMR and ¹³C NMR data revealed five aromatic
peaks in addition to the peaks that were attributed to starting material. Low resolution mass spectrometry (LREI-MS) also provided further evidence with a molecular ion at m/z 343. The yields of the products were only low, as was reported in the work of Gerard et al. due to the low solubility of the products in the reaction solvents.\textsuperscript{408}

\begin{align*}
\text{Scheme 4.10: Preparation of biaryl isatin derivatives. PMB} &= \text{para-methoxybenzyl; DME} = \text{dimethoxyethane.}
\end{align*}

### 4.3.7 Preparation of N-triazolo isatin imines

With the aim to further probe diversity, the library of compounds was extended to a number of analogues with triazolo groups on the aromatic nitrogen of isatin. Click chemistry, or the Huisgen 1,3-dipolar cycloaddition reaction between terminal alkynes and azides, has gained interest in medicinal chemistry recently.\textsuperscript{409} 1,2,3-Triazoles compounds can be used as mimics for amides and peptide bonds and in modifications of natural products.\textsuperscript{409}

#### 4.3.7.1 Click chemistry reaction

The Cu (I)-catalysed Huisgen reaction is a 1,3-dipolar [3+2] cycloaddition reaction between a terminal alkyne and an appropriate azide, developed by Medal et al. and further explored by Sharpless et al.\textsuperscript{410,411} As the final product, this method gives five membered 1,4-disubstituted 1,2,3-triazole heterocycles.\textsuperscript{412} One of the major issue with the click chemistry reaction is formation of 1,4 and 1,5 regioisomers (Scheme 4.13).\textsuperscript{410} However, regioselectivity can be improved with the employment of Cu (I) salts to give only the 1,4-triazole product.\textsuperscript{411} Rostovtsev et al. found that the Cu(II) salts are better for the reaction in terms of purity of the Cu salts and price and that they can be reduced in situ to Cu(I) salt by ascorbic acid or sodium ascorbate.\textsuperscript{410} Various solvents can be used, including water and the reaction proceeds to completion within 6-36 hours at room temperature giving good yields of products.\textsuperscript{410} Cu(I) salts can also be used in the click chemistry reactions without using the reducing reagent. Still Cu(I) salts require acetonitrile as a co-solvent and one equivalent of nitrogen base, usually 2,6-lutidine.
which enables preparation of high purity and better product yields. The utilization of nitrogen base in the reaction and exclusion of oxygen also improves the regioselectivity.

![Scheme 4.11: Regioselectivity of the click chemistry reaction.](image)

4.3.7.2 Synthesis of $N$-triazolo imines

The two novel triazolo imine derivatives 234-235 were readily prepared from $N$-triazoloisatin derivate 233 with hydroxyl amine and 4-aminophenol. The first step in the preparation of the triazolo imines was synthesis of compound 168. The $N$-propargylisatin derivate 168 was made following the previously described protocol of $N$-alkylation of isatin using 5,7-dibromoisatin and propargyl bromide in good yield (78%). $^1$H NMR spectrum confirmed the structure of the synthesised compound with additional peaks at 2.36 and 4.93 ppm compared to the starting 5,7-dibromoisatin and attributed to the propargyl hydrogen and two protons of CH$_2$ adjacent to nitrogen.

Derivatives with the triazolo group on the nitrogen position of the isatin core were prepared from $N$-alkylated isatin 168 and 3-azidopropanol using click chemistry conditions. 3-Azidopropanol was synthesised from 3-bromopropanol and sodium azide in DMF in 4 h according to the protocol of Riva et al. (Scheme 4.14). $^{413}$ EI-MS of the reaction mixture confirmed formation of the desired product with a m/z value of 101, comparing to a formula of C$_3$H$_7$N$_3$O. 3-Azidopropanol was used not purified and the actual yield not calculated because small molecule azides are known to be dangerous and explosive. The reaction mixture of 3-azidopropanol 232 was extracted with ether. Into the organic layer, water was added and then the organic solvent removed in vacuo. A solution of 232 in water was used for the next reaction step.
Three methods for the preparation of the triazole product were employed and all gave the target molecule, with differences in the product yields of 6-59%. The first method for the preparation of the N-triazoloisatin, involved utilisation of Cu(I) salt with 2,6-lutidine as a nitrogen base. For this reaction, compounds were dissolved in H$_2$O/ACN solvent (Scheme 4.15). After column chromatography the product was obtained in 59% yield. The $^1$H NMR spectrum of compound 233 in CDCl$_3$ revealed that the desired compound was formed with peaks for protons in the propyl part of the molecule at 1.91, 3.64 and 4.36 ppm and the proton peak of the triazolo ring at 8.12 ppm.

The second protocol to access triazolo derivatives involved Cu(II) salt as CuSO$_4$ in H$_2$O/tBuOH. Sodium ascorbate was used for the reduction of Cu(II) to Cu(I) *in situ*. The reaction was carried at overnight at room temperature (Scheme 4.15). The yield of this reaction after purification was 46%. $^1$H NMR and $^{13}$C NMR spectra confirmed formation of 233 as above.

In the last attempt, the click product was prepared in a one-pot synthesis. Low molecular weight organic azides can be dangerous. Therefore, Fokin *et al.* developed a method which included preparation of the 1,4-triazole product in a one pot, two step synthesis. 4-Alkylated isatin (168) was mixed with 3-bromopropanol, sodium-azole, copper salt and sodium ascorbate in the presence of Na$_2$CO$_3$ and in 9:1 DMSO/H$_2$O. The mixture was stirred at 65°C overnight, however after the purification of the product the yield of the reaction was only 6% (Scheme 4.16). Thus the original method was chosen for any future scale-up procedures.
The final step in the preparation of the two imine derivatives (234-235) was reaction of dibrominated triazolo product and an appropriate amine to give C3 substituted product following a previously described procedure (Section 4.3.3). The $^1$H NMR spectrum of the product revealed that 234 was prepared as a mixture of $E$ and $Z$ isomers, while 235 was obtained as the pure $Z$ isomer, presumably due to intramolecular H-bonding (Fig. 4.18).

![Figure 4.18: Triazolo isatin imines 234 and 235 and their yields.](image)

The $E$ stereochemistry of 234 was assigned as previously described to a particular isomer based on the signal from H4 (on the isatin core) (Section 4.3.3.1). The $E$ isomer in the $^1$H NMR spectrum was shifted upfield 1.2-1.4 ppm relative to the H4 signals of the $Z$ isomer which occurs as a result of 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 described earlier. As previously mentioned 3-substituted isatins exist as the $E$ and $Z$ isomers that interconvert rapidly in solution at room temperature, although some C3-substituted derivatives have been reported to appear as mainly $E$ isomers.\(^{159}\) In Fig. 4.19, the $^1$H NMR spectrum of compound 234 is presented where it is easily seen that the 4H proton is significantly moved upfield compared to the corresponding proton of the $Z$ stereoisomer.

![Figure 4.19: $^1$H NMR spectral data of triazolo-imine derivative 234 (CDCl$_3$, 500 MHz).](image)
4.4 Preparation of 3-substituted indolin-2-ones (alkenyl-isatins)

4.4.1 Synthetic targets

Along with the series of isatin imines, a variety of alkene analogues were also prepared, with the aim to test their activity and because these type of structures are similar to reported kinase inhibitors. For example, the alkenyl isatin 236 was found to be highly specific against vascular endothelial growth factor (VEGF), (Flk-1) RTK, and related compound 237 also displayed high selectivity towards epidermal growth factor (EGF) and Her-2 RTKs (Fig. 4.20). The alkenyl isatin 238 was prepared by Islam et al. and was found to be selective to PDK1 and cAKT2 over PKA. In modelling studies of 238 it was confirmed, as predicted earlier, that the indolinone core occupies the same site of the enzyme as the adenosine moiety of ATP and it makes three H-bonds with the hinge region.

![Figure 4.20: Known alkene 2-oxindole compounds as kinase inhibitors.](image)

4.4.2 Synthesis of alkenyl isatin derivatives

The step required for the preparation of 3-substituted alkenyl isatin derivatives are shown in Scheme 4.17. The first step in the synthesis of the desired alkenyl-isatin compounds was 5,7-dibromination of 2-oxindole (239), followed by N-alkylation and substitution at C3 (Scheme 4.17). A literature search provided two methods for the synthesis of 5,7-dibromo-2-oxindole. Sumpter et al. performed dibromination of the 2-oxindole ring using Br₂ and a KNO₃ solution. Crestini et al. published another procedure, where preparation of 5,7-dibromo-2-oxindole was carried out using 5,7-dibromoisatin, which was reduced using hydrazine hydrate.

![Scheme 4.15: Summary of the proposed synthetic strategies in the formation of 5,7-dibromo-1-(4-methoxybenzyl)-isatin alkenyl derivatives.](image)
Taking these literature examples into consideration, we first tried the method which utilizes 2-oxindole as the starting material described by Sumpter et al.\textsuperscript{415} Bromine and KNO\textsubscript{3} were dissolved in water and it was slowly added to the hot solution of oxindole in water. A light yellow crude product was crystallized from ethanol. EI-MS analysis of the product indicated the presence of 5,7-dibromooxindole (240), but also of 3,5,7-tribromooxindole (241) and 3,3,5,7-tetrabromooxindole (242) (Scheme 4.18). By column chromatography compounds were purified and the yield of the dibrominated product was very low (<15%), where the side products yielded more. Initially, two equivalents of bromine were used, as described in the protocol, but as we could not obtain the desired product in the good yield we optimised conditions. The bromine equivalents used were reduced to 1.5 and 1.2 but it did not result in improvement of the final yield. Instead of using water, isatin was dissolved in 95% ethanol but still the major occurrences of the polybrominated compounds were detected.

\begin{center}
\includegraphics[width=\textwidth]{scheme_4.16.png}
\end{center}

\textbf{Scheme 4.16:} Bromination of 2-oxindole.\textsuperscript{415}

\subsection*{4.4.3 Wolff-Kishner reduction of isatin derivatives}

Following on from the unsuccessful attempt to prepare dibrominated oxindole 240 by dibromination of 2-oxindole, a Wolff-Kishner reduction method described by Crestini et al. was used.\textsuperscript{416} N-Alkylated isatins (241 and 242) were reduced with hydrazine hydrate by a modified Wolff-Kishner method that reduces the C3 carbonyl group to an alkanes (Scheme 4.19). The successful mechanism of the reaction is due to the structure of the isatin molecule and the ability of the α-ketoamide to promote the stage of the decomposition of hydrazone. Instead of water molecules that drive the reaction forward, the possibility of intermolecular hydrogen bonding could lead to the formation of the intermediate and through the enol form the final reduced product can be formed.\textsuperscript{416} Crestini et al. employed the same method for the preparation of the similar compounds, except their N-alkyl substituent were hydrogen, methyl or methoxy group and they were using only 5-substituted isatins.\textsuperscript{416} The slight difference in the molecule structures could result in the reaction time required for the reaction. While Crestini et al. performed their reactions in 30 minutes, preparation of the compounds in this project needed a longer time of 2 h. 5-Bromo-1,3-dihydroindole was synthesized previously as well by Wilk et al., although they used ethylene glycol in addition and performed the reaction over 6 h.\textsuperscript{417}
The formation of the desired products was confirmed via $^1$H and $^{13}$C NMR spectral analysis. Compared to the $^1$H NMR spectra of $N$-alkylated isatin 162, proton NMR of the reduced isatin compound (241) was similar with the addition of a signal at 3.63 ppm, which belongs to the two protons at the 3 position of the isatin core. As a by-product in this reaction formation of a corresponding 5-bromo-2-oxindoles (241a and 242a) was also observed and confirmed by $^1$H NMR (Fig. 4.21 and 4.22). $N$-alkylated-2-oxindoles 241 and 242 was obtained in 63% and 57% yields, comparable to yields reported in the literature (76-92%).
4.4.4 Preparation of 3-substituted indolin-2-ones

Following on from the method of Sun et al., who made numerous C3-substituted indoles, reaction of N-alkylated indoles 241 and 242 with an appropriate aldehyde provided the desired product.\(^{159}\) The reaction was refluxed for 3-6 h in ethanol with piperidine base as the catalyst, giving good yields (>60%) of the desired product (Scheme 4.20).

The formation of 3-substituted products is performed by a Knoevenagel condensation mechanism, which includes nucleophilic attack of the active hydrogen compound (2-oxindole) to a carbonyl group (aldehyde) followed by elimination of water (Scheme 4.21).
Scheme 4.19: Knoevenagel condensation reaction of 5,7-dibromo-N-alkylated-isatina and an appropriate aldehyde.

3-Substituted indolin-2-ones may also exist as either Z or E isomers as previously reported for 3-substituted isatins. The isomer ratio is also found to depend on the substituents on the C4 and C5 positions on the aromatic ring of oxindole and characteristics of the substituents at the C3 position of the 3-substituted 2-oxindole. Although imino-isatins isomers cannot be separated, alkenyl isatin derivatives are able to be obtained as pure isomers using column chromatography. As for the imino-isatins, the two isomer forms could be distinguished by 1D NOE analysis, with additional correlation for the Z-configured alkenyl isatin isomers, which show a NOE effect between the proton at the C4 position of oxindole core and the vinyl proton. Also for these isomers, NOE experiments can be used to distinguish protons at the C2’ and C6’ positions in the phenyl ring at the C3 position of the 3-substituted-2-oxindoles with different chemical shifts for the Z and E isomers due to deshielding effect of the C-2 carbonyl of the 2-oxindole ring in the Z isomer form. Five of 10 alkenyl isatin derivatives were synthesised as the mixtures of both Z and E isomer forms (Table 4.2).

Table 4.2: Isatin alkene derivatives 243-252 prepared in the project.
In addition to ten isatin alkenes with substituted phenyl group on the C3-position, three compounds with a pyrrole group were synthesised (253-255) and they were obtained exclusively as Z isomers.

![Figure 4.23: Pyrrolo alkene isatin derivatives prepared in this work.](image)

### 4.4.5 Preparation of 2,4-dimethyl-1H-pyrrole-3-carbaldehyde

In the preparation of the alkene isatin derivatives, one of the aldehydes needed for the Knoevenagel reaction with isatin compound was prepared by a Vilsmeier reaction. The Vilsmeier reaction is used for formylation of electron-rich arenes. It is an alternative reaction to Friedel-Crafts acylation and avoids the use of strong Lewis acids such as AlCl$_3$.

The procedure for the preparation of 2,4-dimethyl-1H-pyrrole-3-carbaldehyde was based on the protocol described by Terenin et al. (Scheme 4.22). POCl$_3$ in DMF was added slowly to 2,4-dimethylpyrrole in DMF and stirred at 20°C for 24 h. TLC analysis of the reaction showed that the starting material was consumed and $^1$H NMR and MS were compared with the literature data and the formation of the desired product confirmed.

![Scheme 4.20: Formation of pyrrole-carbaldehyde 257 by the Vilsmeier reaction.](image)

### 4.4.6 Sulfonyl alkenyl-isatin derivatives

In addition to dibrominated alkene and sulfonyl imino-isatin derivatives, alkene isatin compound (260, Scheme 4.23) with a sulfonyl group at position five was prepared. Alkenyl sulfonyl compound 260 was synthesised in three steps, following a procedure for the synthesis of imine derivatives, discussed in section 4.3.5 including substitution at the position five of 2-oxindole, N-alkylation of the formed sulfonyl group and condensation of the prepared compounds with aldehyde (Scheme 4.23).
In summary, this chapter reported the successful synthesis of 41 novel imino-isatin and 14 novel alkenyl-isatin derivatives under various conditions. The main aim was to modify the isatin core in order to obtain new potent and selective kinase inhibitors.

Evaluation of protein kinase A and B inhibitory activity of the novel compounds and their precursors are presented in the Chapter 5. Chapter 5 also includes testing of 16 isatin compounds against MAPK. Isatin derivatives were also screened for their cytotoxicity to test how this modification affected their activity.

**Scheme 4.21:** Synthesis of sulfonamide alkene-isatin derivative.
The 77 isatin-based compounds synthesised in Chapter 4 were screened for their PKA inhibitory activity. In addition, selected isatin derivatives were also screened for Akt and MAPK kinase inhibitory activity. Isatins are known to display potent cytotoxicity, therefore the majority of the isatin analogues were also tested in an anti-proliferative assay using U937 lymphoma and MBA-MD-231 breast cancer cell lines.

5.1 Biological assays

Chapter 4 described the synthesis of 77 isatin derivatives, including 41 novel imino-isatin and 14 novel alkenyl isatin derivatives. These compounds were designed as potential kinase inhibitors and were tested for PKA inhibitory activity using a kinase assay developed in our laboratory. Previously, a number of indolin-2-one analogues have been found to display activity against both serine/threonine and tyrosine kinases. c-AMP Dependent protein kinase (protein kinase A, PKA) is an important serine/threonine kinase involved in the regulation of an increasing number of physiological processes including immune, cardiovascular and reproductive functions; steroid biosynthesis; adipocyte metabolism; and exocytic processes. As such, the inhibition of PKA has become an attractive drug target in a number of areas, in particular in immune function and for memory disorders such as Alzheimer’s and Parkinson’s disease and schizophrenia. In addition to PKA testing, compounds were also screened for inhibitory activity against Ser/Thr MAPK and Akt (PKB) kinases.

Within previous research in our group, isatin compounds were found to exhibit significant cytotoxicity. The characteristic feature of isatins to inhibit cell proliferation and hence cause apoptosis was suggested to be through intercalation with extracellular signal-related protein kinase enzyme (ERKs). Cytotoxic screening of a range of isatin derivatives was performed on several cell lines but isatins display the most potent activity when the tests were carried out on a U937 lymphoma cell line. This cell line has been used widely within our research group to test the cytotoxicity of isatin derivatives. Consequently, compounds prepared in Chapter 4 were also screened for their ability to cause cell death in U937 cells. Isatin derivatives that expressed the highest activity were also screened against MDA-MB-231 breast adenocarcinoma cell lines. The cytotoxicity results are presented in IC₅₀ values, the concentration which is required to inhibit 50% cell viability after a 24 h incubation period.

5.1.1 Protein kinase A inhibition testing

All synthesised compounds were tested for their potential PKA inhibition ability as described in Chapter 2 and experimental protocol (Chapter 6). Tests were carried out on the purified enzyme PKA, where all compounds were initially tested at a concentration of 100 µg/ml, finding a single point inhibitory concentration for each sample (Chapter 2). To further test the specificity of inhibitor to a specific enzyme, compounds were tested for inhibitory
activity against MAPK and Akt kinase. Kinase inhibitory activity results are expressed in percent inhibition for all isatin derivatives tested. IC\textsubscript{50} values were determined for those compounds that showed the highest percent inhibition (>70%). Standards used in the kinase assay included three known potent PKA inhibitors: staurosporine (IC\textsubscript{50} 23 nM), ellagic acid (IC\textsubscript{50} 2 µM)\textsuperscript{421} and H-89 (IC\textsubscript{50} 135 nM) (Chapter 1, Section 1.6.1).\textsuperscript{422}

5.1.2 Protein kinase B (Akt) inhibition testing

Protein kinase B belongs to the group of serine/threonine kinases and begins to attract more attention because of the important signalling role in cancer.\textsuperscript{423} The protein kinase B inhibition testing was performed at Illawarra Health and Medical Research Institute (IHMRI), in collaboration with Dr. Jiezhong Chen. All compounds were tested at concentration of 100 µM. Phosphor-Akt levels were determined by Western blotting to indicate Akt activity (Appendix, 9.5).

5.1.3 MAPK kinase inhibition testing

The MAPK enzyme belongs to the serine/threonine family of protein kinases, the same as PKA and Akt. MAPK signalling is involved in peripheral tissue inflammatory disorders and recognised as the drug target to treat Alzheimer's disease (Fig. 5.2).\textsuperscript{424} Sixteen isatin derivatives were screened for MAPK inhibitory activity at University of Sydney in collaboration with Dr. Lenka Munoz from the Faculty of Pharmacy at the University of Sydney.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{p38_MAPK.png}
\caption{p38 MAPK participates in different cell types and mechanisms in Alzheimer's disease.\textsuperscript{424}}
\end{figure}
5.1.4 The MTS cell proliferation assay

The MTS cell proliferation assay is an *in vitro* cytotoxicity assay that is found to be very accurate, reliable and a convenient method for determining cell viability with good sensitivity and specificity. This MTS assay gauges the interaction between potential drug and whole intact cells and allows detection of a variety of bioactive compounds. The MTS CellTiter 96® AQ®ueous assay utilizes 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt and an electron coupling reagent (phenazine ethosulfate) PES. The MTS cell proliferation assay determines the number of viable cells which is measured through colourimetry. This assay works on the principle that the mitochondrial dehydrogenase enzymes, bioreduces the colourless tetrazolium salt (MTS reagent) into a coloured aqueous soluble formazan product by the mitochondrial activity of viable cells at 37°C (Fig. 5.2). The reduction of MTS reagent is accompanied by production of NADH or NADPH by these enzymes. The amount of formazan produced by dehydrogenase enzymes is directly proportional to the number of living cells in culture and can be measured at 492 nm. The quantity of the coloured product is directly proportional to the number of live cells in the culture since MTS can only be reduced to formazan by metabolically active cells.

![Figure 5.2: The reduction of the MTS tetrazolium salt to the red formazan product by viable cells.](image)

5.2 Structure-activity relationship studies

5.2.1 Biological activities of N-alkylated isatin derivatives

*N*-Alkylated compounds were prepared as the starting material for the synthesis of the imine and alkene derivatives (Chapter 4). Eleven compounds were made including compounds with an unsubstituted aromatic isatin ring, 5,7-dibromo, 5-nitro and 5-methoxyisatin analogues, of which seven derivatives were novel. All *N*-alkylated isatins were screened for their PKA inhibitory activity and MTS cytotoxicity. In addition, five compounds were screened for Akt (PKB) and one compound was tested for MAPK kinase inhibitory activity (Table 5.1).

There are several conclusions that can be drawn from the testing results presented in Table 5.1. It can be observed that *N*-alkylated derivatives displayed weak to moderate activity in the PKA inhibition assay only giving a maximum of 23% inhibition. Only *para*-substituted...
phenyl derivatives were prepared as these compounds were previously found to display better cytotoxicity over ortho and meta analogues.\textsuperscript{183} The para-substituted phenyl derivatives were weakly active, displaying similar inhibitory activity. Changes of the different substitution of the phenyl ring did not change the biological results of these compounds (e.g. entries 162-165, Table 5.1). Both, electron withdrawing and donating groups on the phenyl ring did not significantly alter the PKA inhibitory activity.

Table 5.1: Biological results of $N$-alkylated isatin derivatives

<table>
<thead>
<tr>
<th>Cpd. no</th>
<th>$R_1$</th>
<th>$R_2$</th>
<th>$R_3$</th>
<th>clog P</th>
<th>% PKA Inhibition\textsuperscript{1}</th>
<th>% Akt inhibition\textsuperscript{2}</th>
<th>MTS IC\textsubscript{50} (µM)\textsuperscript{a}</th>
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<tr>
<td>233*</td>
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<td>Br</td>
<td>N\textsuperscript{2}$\equiv$N\textsuperscript{2}$\equiv$N\textsuperscript{2}OH</td>
<td>1.25</td>
<td>5</td>
<td>0</td>
<td>10.5</td>
</tr>
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</table>

\*Compounds screened for MAPK kinase inhibitory activity; \textsuperscript{1}at 100 µg/ml; \textsuperscript{2}at 100 µM; nt-compounds which were not tested. \textsuperscript{a}tested on U937 lymphoma cell lines.

Modification of the compounds which led to increased polarity of the molecules, using morpholino or triazolo groups instead of a phenyl group on the aromatic nitrogen of isatin, didn’t improve kinase inhibition. A comparison of the PKA inhibitory activity and their corresponding calculated logP (clogP) values found no correlation. The kinase assay results indicate that the activity of $N$-alkylated derivatives observed was not related to lipophilicity. The $N$-propargyl isatin derivative 168 was the only derivative that did not have a ring appended to the aromatic nitrogen of the isatin and its modification did not significantly affect the PKA inhibitory activity and cytotoxicity. It also appeared that the presence of bromine atoms on the aromatic isatin ring increased PKA inhibitory activity while a methoxy, nitro group or unsubstitution let to a decrease in activity.
Although these compounds were not found to be potent PKA inhibitors, 163 was shown to have good Akt (PKB) kinase inhibitory properties (Table 1). Akt results also showed that dibromination was important for activity. Compound 169 which has an unsubstituted aromatic isatin ring, displayed low Akt inhibitory activity, and the other three tested analogues exhibited no Akt inhibitory activity at all, although compounds 166, 167 and 233 were prepared with the aim to increase the polarity of the products. In addition, compounds 162 and 233 were screened for MAPK kinase inhibitory activity but were not found to be active.

The N-alkylated derivatives were previously found to exhibit potent cytotoxicity against a variety of tumour cell lines, and to cause G2/M cell cycle arrest, and inducing apoptosis via binding to the cytoskeletal protein tubulin. Cytotoxicity of isatin compounds 162-165 and 169 were determined in earlier work, while compounds 166-168 and 233 were tested here (Table 5.1). Cytotoxic data confirmed prior results that attachment of an aromatic ring via a 1-carbon linker on the aromatic nitrogen was required for the antiproliferative activity. Hence, compounds which did not have an aryl group that is attached to the nitrogen via one carbon linker and had either morpholino, triazolo or an alkene group on the isatin nitrogen resulted in lower activity (higher IC_{50} values) (166-168, 233).

The biological screening of a series of N-alkylated isatin derivatives for kinase inhibition indicated that these starting compounds are not particularly potent PKA inhibitors as was to be expected. However, isatin derivative 163 exhibited potent Akt kinase inhibitory activity. These results are significant because they indicate that compound 163 showed selectivity for Akt over PKA, which is significant as selectivity is one of the major issues in the development of novel kinase inhibitors.

5.2.2 Isatin imine derivatives

A range of C3-substituted analogues were prepared as described in Chapter 4, followed by the investigation of their activity as kinase inhibitors and also their cytotoxicity. The results of the kinase inhibition and MTS assays on these analogues are summarised in Table 5.2. The biological data of the isatin imine derivatives indicate varying degrees of kinase inhibitory activity and cytotoxicity.

Investigation on isatins as kinase inhibitors started with imino-isatin 83 (Fig. 4.2, Chapter 4), which was found to exhibit significant PKA inhibitory activity with a IC_{50} value of 20.0 µM. Firstly, compounds 175-177 with a carboxyl group attached to a different position of the distal phenyl ring on isatin and also via a varying length carbon linker were prepared and tested. To further probe the ability of isatins to inhibit PKA, the first objective was to change the para-carboxyl group on the distal phenyl ring with other functional groups. The synthesis included compounds with substitution at position 4 and also other positions on the distal phenyl ring on C3 position and multisubstitution of the phenyl ring at position C3 as well. Compounds
were analysed with a range of different substituents having either electron acceptor or donor properties (Table 5.2).

PKA inhibitory activities of three of the isatin derivatives with a carboxyl group (175-177) and PMB group on the aromatic isatin nitrogen were higher than 70%. However, the results indicated that when the carboxyl group was replaced, not all C3-substituted derivatives displayed as high PKA inhibitory activity (Table 5.2). Of the 29 isatin derivatives in Table 5.2, four compounds displayed activity higher that 70%. It is evident to observe that the most active compounds were carrying either a COOH or OH group on the phenyl ring, while all other derivatives where those groups were replaced by other moieties, exhibited lower activity. These results highlighted the impact of the carboxylic and hydroxyl function upon the inhibition of the PKA kinase. However, the analogue 200 with iodo-phenyl N-substituted isatin, which also had COOH group, did not exhibit potent kinase inhibitory activity, suggesting that PMB group also contributed to kinase inhibitory activity.

Table 5.2: Biological activity of isatin imine derivatives.

<table>
<thead>
<tr>
<th>Cpd. no</th>
<th>R₁</th>
<th>R₂</th>
<th>clogP</th>
<th>PKA % Inhibition</th>
<th>AKT % Inhibition</th>
<th>MTS IC₅₀ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>175*</td>
<td>OCH₃</td>
<td>4-COOH</td>
<td>6.68</td>
<td>78</td>
<td>72</td>
<td>1.95 (10²)</td>
</tr>
<tr>
<td>176</td>
<td>OCH₃</td>
<td>4-CH₃COOH</td>
<td>6.21</td>
<td>75</td>
<td>70</td>
<td>2.13 (10²)</td>
</tr>
<tr>
<td>177</td>
<td>OCH₃</td>
<td>3-CH₃COOH</td>
<td>6.21</td>
<td>91</td>
<td>84</td>
<td>0.46 (10²)</td>
</tr>
<tr>
<td>178*</td>
<td>OCH₃</td>
<td>4-OH</td>
<td>6.27</td>
<td>86</td>
<td>nt</td>
<td>3.8³</td>
</tr>
<tr>
<td>179</td>
<td>OCH₃</td>
<td>4-Br</td>
<td>7.80</td>
<td>9</td>
<td>nt</td>
<td>6.6</td>
</tr>
<tr>
<td>180</td>
<td>OCH₃</td>
<td>2-Br</td>
<td>7.80</td>
<td>28</td>
<td>nt</td>
<td>na</td>
</tr>
<tr>
<td>181</td>
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<td>3-Br</td>
<td>7.80</td>
<td>30</td>
<td>nt</td>
<td>2.8</td>
</tr>
<tr>
<td>182</td>
<td>OCH₃</td>
<td>2,4-di Br</td>
<td>8.66</td>
<td>4</td>
<td>nt</td>
<td>na</td>
</tr>
<tr>
<td>183*</td>
<td>OCH₃</td>
<td>4-Cl</td>
<td>7.65</td>
<td>12</td>
<td>24</td>
<td>2.1</td>
</tr>
<tr>
<td>184</td>
<td>OCH₃</td>
<td>4-I</td>
<td>8.06</td>
<td>21</td>
<td>17</td>
<td>9.2</td>
</tr>
<tr>
<td>185*</td>
<td>OCH₃</td>
<td>4-F</td>
<td>7.08</td>
<td>30</td>
<td>1</td>
<td>3.1</td>
</tr>
<tr>
<td>186</td>
<td>OCH₃</td>
<td>4-CF₃</td>
<td>7.82</td>
<td>16</td>
<td>nt</td>
<td>10.1</td>
</tr>
<tr>
<td>187</td>
<td>OCH₃</td>
<td>4-OCH₃</td>
<td>6.86</td>
<td>26</td>
<td>60</td>
<td>4.3</td>
</tr>
<tr>
<td>188</td>
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<td>3-OCH₃</td>
<td>6.86</td>
<td>19</td>
<td>nt</td>
<td>na</td>
</tr>
<tr>
<td>189</td>
<td>OCH₃</td>
<td>2,4-CH₃</td>
<td>7.94</td>
<td>12</td>
<td>nt</td>
<td>na</td>
</tr>
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<td>190</td>
<td>OCH₃</td>
<td>2,6-CH₃</td>
<td>7.94</td>
<td>14</td>
<td>nt</td>
<td>25.1</td>
</tr>
<tr>
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<td>4-NO₂</td>
<td>6.68</td>
<td>19</td>
<td>0</td>
<td>4.1</td>
</tr>
<tr>
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<td>OCH₃</td>
<td>4-OPh</td>
<td>9.04</td>
<td>27</td>
<td>14</td>
<td>5.9</td>
</tr>
<tr>
<td>193</td>
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<td>4-OCH₃</td>
<td>7.44</td>
<td>20</td>
<td>nt</td>
<td>na</td>
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<tr>
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<td>1.9</td>
</tr>
<tr>
<td>195</td>
<td>CH₃</td>
<td>4-CF₃</td>
<td>8.40</td>
<td>1</td>
<td>nt</td>
<td>na</td>
</tr>
<tr>
<td>196</td>
<td>Cl</td>
<td>3-Br</td>
<td>8.60</td>
<td>14</td>
<td>nt</td>
<td>2.9</td>
</tr>
<tr>
<td>197</td>
<td>Cl</td>
<td>4-OCH₃</td>
<td>7.65</td>
<td>24</td>
<td>nt</td>
<td>1.6</td>
</tr>
<tr>
<td>198</td>
<td>Cl</td>
<td>4-I</td>
<td>8.64</td>
<td>14</td>
<td>nt</td>
<td>na</td>
</tr>
<tr>
<td>199</td>
<td>I</td>
<td>4-OCH₃</td>
<td>8.06</td>
<td>6</td>
<td>nt</td>
<td>6.8</td>
</tr>
<tr>
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<td>I</td>
<td>3-CH₃COOH</td>
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<td>27</td>
<td>nt</td>
<td>1.4</td>
</tr>
<tr>
<td>201</td>
<td>I</td>
<td>4-Cl</td>
<td>8.64</td>
<td>12</td>
<td>nt</td>
<td>7.0</td>
</tr>
<tr>
<td>202</td>
<td>I</td>
<td>3-Br</td>
<td>9.01</td>
<td>2</td>
<td>nt</td>
<td>1.0</td>
</tr>
<tr>
<td>203</td>
<td>I</td>
<td>4-F</td>
<td>8.29</td>
<td>4</td>
<td>nt</td>
<td>na</td>
</tr>
</tbody>
</table>

*Compounds screened for MAPK kinase inhibitory activity; ¹ at 100 µg/ml; ² at 100 µM; nt-compounds which were not tested; ³ tested on U937 lymphoma cell lines; ⁴ tested on MDA MB 231 breast cell lines.
Three imine isatins (175-177) which displayed significant activity in the PKA inhibition assay were also found to exhibit potent Akt inhibitory activity. In addition, six more imine isatins were screened in the Akt assay but they showed low activities. Akt assay results also suggested that the carboxyl group was beneficial for potent activity (Table 5.2).

Introducing groups on the imino-phenyl ring of isatin gave little effect on the potency. There was no clear correlation between the activity of ortho, meta and para iminophenyl-isatin derivatives, as compounds with a COOH group at either the meta or para position displayed significant PKA inhibition activity, while derivatives with OCH₃ (187 and 188) or halogen groups (179-181) present at the different positions of the phenyl ring exhibited low PKA inhibitory activity with no apparent influence of their position (Fig. 5.3). Changing small groups on the phenyl ring at the C3 position of isatin to a bulkier phenyl group to make a phenoxy phenyl derivative (192) also had no effect on the activity.

![Figure 5.3](image)

Figure 5.3: PKA inhibitory activity of different phenyl substituted isatin analogues.

Compound 209 which had hydroxyl-amino group at the 3-position of isatin instead of the arylimino group also displayed strong PKA inhibitory activity (65%) indicating that the bulkiness of the C3 substituent is not important for activity (Fig. 5.4). This compound also exhibited 34% Akt inhibitory activity.

![Figure 5.4](image)

Figure 5.4: PKA and Akt inhibitory activity of hydroxylimino-isatin derivative (209).

Two analogues with a hydrazone group (Fig. 5.5) in the PKA inhibition assay showed that compounds with a carboxyl group on the distal phenyl ring displayed higher inhibitory activity (84%) than the imine analogues with a para-Cl group (6%). These results were similar to the results obtained from the imine analogues. The hydrazone imine derivative 208 was equally potent as imine derivatives in the PKA inhibition assay (175-177), while 207 was less active than the imino-isatin analogue 183 (Fig 5.5). These two compounds (207 and 208) were also screened in the Akt inhibition assay and compound 208 showed 57% inhibition, while 207 exhibited 42% inhibition, which was higher than the result obtained in PKA inhibition assay.
These two hydrazone containing isatins were additionally evaluated against MAPK, however they were found to be inactive.

Figure 5.5: PKA inhibitory activity of hydrazone and imine isatins.

To investigate the importance of the dibromination and N-alkylation of the isatin molecule for the PKA and Akt kinase inhibitory activity, two compounds that were not substituted at the aromatic isatin ring (204, 210) were also prepared. The activity of these compounds was compared with their analogues and the results indicate that removal of the dibromine as well as removing groups from the aromatic nitrogen led to a decrease in PKA inhibitory activity (Table 5.2, Fig. 5.6). Dibromination on the phenyl ring of isatin or substituents with electron withdrawing ability were found to be beneficial for cytotoxicity of these analogues. N-alkylation of isatin resulted also in compounds with more potent kinase inhibitory activity, thus confirming the positive effect of the N-alkylation upon potency (205, 211, Fig. 5.6). A comparison of the inhibitory activity and their corresponding, calculated logP (clogP) values, found no correlation. Although compounds with two bromine groups on the aromatic ring were less polar, they displayed higher PKA inhibitory activity. Compounds 209-211, screened for Akt inhibitory activity, showed the same trend as in the PKA assay with low inhibitory results when dibromination and N-alkylation was removed. In addition, imino-isatin derivative 206 exhibited 68% PKB inhibition while it was not active in the PKA inhibition assay (12%). Compounds with a 5-nitro group (217, 14% PKA inhibition) were also found to be inactive as well as compounds with a 5-methoxy group (218, 31% PKA inhibition) on the isatin benzene ring. These compounds were also made with the aim to reduce clogP values of the
isatin analogues and make them more polar. The results indicated that the kinase inhibitory activity of imine derivatives observed was not based on lipophilicity alone. In addition, four compounds (175, 178, 183, 185 and 209), were tested for p38 mitogen activated protein kinases (MAPK) activity, however none displayed any significant activity.

![Chemical Structures](image)

**Figure 5.6:** Influence of bromination and N-alkylation of isatins on PKA and Akt inhibitory activities.

All imine compounds prepared were also screened for cytotoxicity against U937 human lymphoma cell lines and the activities for most appear to be in the micromolar range (Table 5.2). Activity against U937 cells of the hydrazone derivatives was found to be lower than the cytotoxicity of imine analogues which was also observed earlier. Oxime isatins (209-211) did not display any significant cytotoxicity and their IC\textsubscript{50} values have not been determined. All imine compounds exhibited activity in low micromolar range regardless of the clogP value. Cytotoxicity of compounds 178, 205, 208 and 209 were additionally evaluated against MDA-MB-231 breast cancer cell lines (Table 9.7, Appendix). The IC\textsubscript{50} values of the three tested compounds showed that 178, 208 and 209 were more susceptible to U937 cell lines and were less potent against cancer breast cell lines.

### 5.2.3 More polar isatin analogues

It has often been noted in the literature that for isatin derivatives there was no significant correlation between clogP values and their bioactivity results. Solomon et al. studied the cytotoxicity of isatin derivatives and did not find any correlation between activity and logP values of all 30 compounds prepared. The authors concluded that differences in lipophilicity may not be a significant factor for the difference in cytotoxicity.

In order to improve polarity of the starting isatin compounds which had PMB group on the nitrogen of the isatin ring, more polar groups were introduced. Isatin was N-alkylated with
morpholino (212), acetomorpholino groups (213-215) and triazole ring (234, 235). Two compounds, using a Suzuki coupling to introduce an aromatic ring at the position 5, were also prepared and tested. All compounds were screened for PKA inhibitory activity and cytotoxicity and some of these were also tested for Akt and MAPK kinase as well as described below.

5.2.3.1 Morpholino-isatin derivatives

Analysis of the kinase inhibitory activity results of the isatin analogues 212-215 revealed that morpholino groups were deleterious for the kinase inhibitory activity of these compounds. Neither N-morpholino nor N-acetomorpholino isatin analogues exhibited any activity (Table 5.3). It appeared that the more polar substituents decreased the kinase inhibitory activity as they all displayed less than 5% inhibition. The target compound 212 was also screened against other two kinases, Akt and MAPK, but also did not display any positive results.

\[ N\text{-Alkylation of 5,7-dibromo derivatives of isatin with morpholino substituents also resulted in lower cytotoxicity against U937 human lymphoma cell lines compared with imine derivatives with higher clogP values. These cytotoxic results indicate the importance of the aromatic phenyl ring at the aromatic nitrogen for cytotoxicity as has been found previously.} \]

Table 5.3: Biological data of isatin morpholino derivatives.

<table>
<thead>
<tr>
<th>Cpd. no</th>
<th>R</th>
<th>clogP</th>
<th>PKA % Inhibition</th>
<th>Akt % Inhibition</th>
<th>Cytotoxicity IC_{50} (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>212*</td>
<td>OCH_{3}</td>
<td>5.55</td>
<td>3</td>
<td>0</td>
<td>15.1</td>
</tr>
<tr>
<td>213</td>
<td>F</td>
<td>5.21</td>
<td>0</td>
<td>nt</td>
<td>na</td>
</tr>
<tr>
<td>214</td>
<td>CF_{3}</td>
<td>5.55</td>
<td>1</td>
<td>nt</td>
<td>20.7</td>
</tr>
<tr>
<td>215*</td>
<td>OCH_{3}</td>
<td>4.59</td>
<td>0</td>
<td>0</td>
<td>19.4</td>
</tr>
</tbody>
</table>

*Compound screened for MAPK kinase inhibitory activity; † at 100 µg/ml; ‡ at 100 µM; nt-compounds which were not tested. * tested on U937 lymphoma cell lines.

5.2.3.3 5-Aryl isatin derivatives

Isatin derivatives with aryl groups on the 5-position, which were prepared using a Suzuki coupling, were screened for PKA inhibitory activity and cytotoxicity (Fig. 5.7). The three 5-substituted compounds 228 - 230 showed only weak PKA inhibitory activity of 16, 28 and 25%, respectively. Compound 228 was also screened for MAPK inhibitory activity but found to display low activity (<5%). Cytotoxicity against U937 cells of these three compounds
could not be determined as an IC\textsubscript{50} value was not reached within the concentration range tested. Based on these results it was concluded that substitution of the isatin nucleus at the 5-position was detrimental to both kinase inhibitory activity and cytotoxicity.

![Figure 5.7: PKA inhibitory results of aryl isatin derivatives](image1)

5.2.3.2 Triazole isatin compounds

Triazole compounds, that can be synthesised through a Huisgen 1,3-dipolar cycloaddition, also known as the click chemistry reaction between azides and terminal alkynes, are widely used in drug discovery projects. The interests of these compounds in medicinal chemistry are due to their ability to mimic amides and peptide bonds.\textsuperscript{409,427} N-triazolo isatin 233 was introduced in section 5.2.1. It was prepared as the starting point for the synthesis of imine and oxime analogues (234 and 235). The triazole isatin imine derivatives 234 and 235 displayed higher PKA inhibitory activity than the starting material (168 and 233) (Fig. 5.8). However, PKA testing results revealed that these compounds were less active than other imine derivatives with the phenyl group on the aromatic nitrogen of isatin. The triazolo derivative 234 was also screened for Akt kinase inhibitory activity but results indicate that this compound had also weak activity (15% inhibition). These two imines were also tested for cytotoxicity (U937 and MDA MB 231 cell lines) however they were found to exhibit low activity, again supporting the conclusion that a benzyl or phenyl group is required at this position for optimum activity.

![Figure 5.8: PKA inhibitory activity of triazolo isatin imine derivatives 234 and 235.](image2)
5.2.3.4 Sulfonyl isatin derivatives

A range of compounds that have the sulfonyl group on position 5 of the isatin ring were prepared as compounds bearing an indole ring were previously found to be inhibitors of Src and Yes kinase. Both imine and alkenyl derivatives of sulfonyl products were prepared along with compounds that are N-alkylated but unsubstituted on the C3 position. All compounds were tested for PKA inhibitory activity and cytotoxicity against U937 cell lines. In addition, three of seven compounds were screened for Akt and one for MAPK inhibitory activity (Table 5.4).

<table>
<thead>
<tr>
<th>Cpd. no</th>
<th>Structure</th>
<th>clogP</th>
<th>PKA % Inhibition†</th>
<th>Akt % Inhibition‡</th>
<th>Cytotoxicity IC₅₀ (μM)§</th>
</tr>
</thead>
<tbody>
<tr>
<td>220</td>
<td><img src="image" alt="Structure" /></td>
<td>0.07</td>
<td>17</td>
<td>0</td>
<td>na</td>
</tr>
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<td><img src="image" alt="Structure" /></td>
<td>-0.03</td>
<td>6</td>
<td>31</td>
<td>21.4</td>
</tr>
<tr>
<td>223*</td>
<td><img src="image" alt="Structure" /></td>
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<td>27</td>
<td>nt</td>
<td>28.3§</td>
</tr>
<tr>
<td>224*</td>
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<td>2.75</td>
<td>8</td>
<td>nt</td>
<td>18.8§</td>
</tr>
<tr>
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<td>14</td>
<td>nt</td>
<td>na</td>
</tr>
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<td>30</td>
<td>34</td>
<td>113.1</td>
</tr>
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</tr>
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<td>19</td>
<td>nt</td>
<td>49.4</td>
</tr>
</tbody>
</table>

*Compound screened for MAPK kinase inhibitory activity; † at 100 μg/ml; ‡ at 100 μM; nt-compounds which were not tested. * tested on U937 lymphoma cell lines; † tested on MDA MB 231 breast cell lines.

In the sulfonyl series it appeared that only one, the sulfonyl isatin derivative 260, exhibited significant PKA inhibitory activity of 57% while others displayed low to moderate activity. Replacement of two bromines at positions 5 and 7 of isatin with a sulfonyl group reduced activity. These derivatives possess better solubility than their bromine analogues and their clogP values are within the Lipinski rule range, but increased polarity and solubility did...
not improve their activity. Replacing the bromine atoms with SO\(_2\) did not result in improving cytotoxicity as well (Table 5.4). The IC\(_{50}\) values of these compounds were all higher compared to their bromine analogue. Replacing the bromine atoms with SO\(_2\) did not result in improving cytotoxicity as well (Table 5.4). The IC\(_{50}\) values of these compounds were all higher compared to their bromine analogues (i.e. less active). These results might be due to the size of the sulfonyl side group.

5.2.4 Alkenyl-isatin derivatives

In total, 13 alkenyl isatin derivatives were prepared and tested for PKA inhibitory activity and cytotoxicity, of which five were obtained as single isomers and five as mixtures of \(E\) and \(Z\) isomers (Table 5.5). PKA inhibitory results indicated that these compounds were less active than their imine analogues (Table 5.2 and 5.3). Similar trends to those observed for the imine derivatives were also noticed for the alkene analogues. The alkenyl-isatin 246, with the OH group on the distal phenyl ring, displayed the highest PKA inhibitory activity (61%). However, alkenyl-isatin derivative 246 was less potent than its imine-isatin analogue 178. Of those alkenyl isatin derivatives that were obtained as \(E\) and \(Z\) isomers, each isomer was tested separately in the PKA inhibition assay, whereas only the isomer obtained in greater yield was screened for cytotoxicity. \(E\) and \(Z\) isomers displayed a little difference in the kinase inhibitory activity. The MTS results show that the potency of alkenyl analogues was lower than that of the imine isatin compounds.

Table 5.5: Alkenyl-isatin derivatives and their biological activity results.

<table>
<thead>
<tr>
<th>Cpd. no</th>
<th>(R_1)</th>
<th>(R_2)</th>
<th>CLogP</th>
<th>%PKA Inhibition†</th>
<th>Cytotoxicity IC(_{50}) (µM)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>243</td>
<td>OCH(_3)</td>
<td>4-Cl</td>
<td>7.65</td>
<td>8 (E)</td>
<td>na</td>
</tr>
<tr>
<td>244</td>
<td>OCH(_3)</td>
<td>2-Cl</td>
<td>7.65</td>
<td>13 (Z)</td>
<td>na</td>
</tr>
<tr>
<td>245</td>
<td>OCH(_3)</td>
<td>4-NO(_2)</td>
<td>6.59</td>
<td>41 (E)</td>
<td>na</td>
</tr>
<tr>
<td>246*</td>
<td>OCH(_3)</td>
<td>4-OH</td>
<td>6.18</td>
<td>61 (E)</td>
<td>na</td>
</tr>
<tr>
<td>247</td>
<td>OCH(_3)</td>
<td>4-OCH(_3)</td>
<td>6.77</td>
<td>18 (E)</td>
<td>35.1</td>
</tr>
<tr>
<td>248</td>
<td>OCH(_3)</td>
<td>4-CH(_3)</td>
<td>7.35</td>
<td>12 (E)/9 (Z)</td>
<td>na</td>
</tr>
<tr>
<td>249</td>
<td>OCH(_3)</td>
<td>4-CH(_2)CH(_3)</td>
<td>7.88</td>
<td>3 (E)/4 (Z)</td>
<td>na</td>
</tr>
<tr>
<td>250</td>
<td>CH(_3)</td>
<td>4-Cl</td>
<td>8.14</td>
<td>9 (E)/6 (Z)</td>
<td>na</td>
</tr>
<tr>
<td>251</td>
<td>CH(_3)</td>
<td>4-CH(_3)</td>
<td>7.93</td>
<td>4 (E)/9 (Z)</td>
<td>22.3</td>
</tr>
<tr>
<td>252</td>
<td>CH(_3)</td>
<td>4-CH(_2)CH(_3)</td>
<td>8.46</td>
<td>14 (E)/7 (Z)</td>
<td>22.1</td>
</tr>
</tbody>
</table>

*Compound screened for MAPK kinase inhibitory activity, † at 100 µg/ml; ‡ at 100 µM; * tested on U937 lymphoma cell lines; na = not active.

2-Oxindole derivatives with a pyrrole group appended to the C3 atom were previously found to be potent kinase inhibitors. However, herein introducing a pyrrole group on the C3 position of isatin resulted in low PKA inhibitory activity (Table 5.6). The pyrrole isatin derivative 253 was also screened for MAPK and Akt kinase inhibitory activity and found to be
not particularly active. Cytotoxicity of these compounds was found to be in the micromolar range, but higher than the results obtained for imine derivatives.

Table 5.6: Alkene-isatin derivatives and their biological activity results

<table>
<thead>
<tr>
<th>Cpd. no</th>
<th>R₁</th>
<th>R₂</th>
<th>CLogP</th>
<th>%PKA Inhibition</th>
<th>Cytotoxicity IC₅₀ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>253*</td>
<td>PMB</td>
<td>2,4-CH₃</td>
<td>6.45</td>
<td>17</td>
<td>19.5</td>
</tr>
<tr>
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<td>PMB</td>
<td>2,4-H</td>
<td>5.45</td>
<td>7</td>
<td>27.2</td>
</tr>
<tr>
<td>255</td>
<td>Ph-CH₃</td>
<td>2,4-CH₃</td>
<td>7.03</td>
<td>6</td>
<td>29.2</td>
</tr>
</tbody>
</table>

*a Compound screened for MAPK kinase inhibitory activity; † at 100 µg/ml; ‡ at 100 µM; † tested on U937 lymphoma cell lines.

In summary 77 compounds were screened for PKA inhibitory activity and 62 for cytotoxicity against U937 cells, eight against MDA-MB-231 breast cancer cells and 33 for Akt and 16 for MAPK inhibitory activities. Compounds imino-isatin 178, hydrazine-isatin 208, hydroxylimino-isatin 209 and alkenyl-isatin derivative 246 displayed the highest PKA inhibitory activities and their IC₅₀ values were determined as 43.5, 51.8, 38.5, 52.2 µM respectively. Seven compounds exhibited potent Akt inhibitory activities, higher than 55% inhibition (163, 187, 175-177, 206, 208). Four isatin derivatives (175, 176, 177 and 208) were found to be active in both the PKA and PKB assay, which may be due to the fact that both PKA and PKB enzymes belong to the same group of Ser/Thr AGC kinase. However, three isatins (163, 187 and 206) displayed selective activity for PKB over PKA, while two compounds (178, 209) were more selective towards PKA. All 16 isatin derivatives tested in the MAPK assay were inactive. In addition the newly developed isatin derivatives also exhibited potent cytotoxicity against U937 human leukemic monocytic lymphoma cell lines in the micromolar range. This work suggests that isatin derivatives possess the potential to be used as PKA and PKB inhibitors in the future. The isatin derivatives warrant further investigation to improve both the potency and selectivity for PKA (or Akt) over other kinases and their drug like properties given they have high cLogP values. So far, potent and selective PKA inhibitor has not been found. Although, PKA inhibitors used as the standards in PKA inhibition assay herein display low micromolar to nanomolar inhibitory activity, they inhibit various other kinases and are not selective.
Conclusions

6.1. Isolation, purification, identification and biological evaluation of marine natural products

In this project significant progress was made towards the analysis of the bioactivity from marine natural products including common but under explored local algal species and rare and unique deep-sea sponge samples from the North West Shelf of Australia including the first example of a deep-sea Haliclona sponge and the first Australian deep-sea Ircinia and Sarcotragus sponges. Marine organisms have previously displayed a range of potent biological properties, which were also found in this study. The key findings from this study are described below.

6.1.1 Marine algae

Biological screening of extracts obtained from ten algal species including five brown, three green and two red algae collected from South Eastern Australia and their chemical constituents were investigated and described in Chapter 2. The bioactivity profiles of the algal samples were strongly species dependent, with no apparent correlation between PKA inhibition, haemolysis and/or brine shrimp lethality. Different biological results were obtained for samples collected from the same location even though they had been exposed to the same conditions (temperature, salinity) indicating that influence of the locations from which the samples were collected was not important for the bioactivity. Furthermore, there was no significant correlation in biological activities between two species that belong to the same genus Sargassum, but were collected from different locations. The results described herein are the first to show kinase inhibitory activity of South East Australian alga species and for most of the samples haemolytic data are reported for the first time.

In general, the non-polar algal extracts gave more potent biological results, in particular greater PKA inhibitory activity, compared to the polar extracts. The dichloromethane extracts isolated from brown algae were found to display the highest activity in the PKA inhibition assay. In summary, the PKA inhibitory activity of the more active non-polar extracts was found to follow the order brown algae > red algae > green algae. The most active extracts were dichloromethane extracts obtained from Padina sp., Sargassum sp., Sargassum vestitum and Phyllospora comosa (with 93%, 82%, 76% and 54% inhibition, respectively). Further purification of the dichloromethane extracts of C. fragile, C. sinuosa and S. vestitum yielded even more potent extracts than the original dichloromethane fraction.
6.1.2 Deep-sea sponges

In the second part of the study four deep-sea sponges assigned to the genera Haliclona, Sarcotragus, Ircinia and Geodia were investigated for their biological activities in a variety of assays. The PKA inhibition, haemolytic activity, and brine shrimp toxicity of 21 partitioned extracts obtained from four deep-water sponges were investigated. The sponges were collected...
from the North West Shelf of Australia from depths of 84-135 m using remotely operated vehicles.

Overall the sponge *Haliclona* sp. displayed greater haemolytic activity than PKA inhibitory activity, along with low levels of brine shrimp lethality with the bioactivity mainly found in the less polar fractions. The *Ircinia/Sarcotragus* sponge exhibited higher PKA inhibitory activity than the brine shrimp toxicity and haemolytic activity, with the two non-polar fractions displaying PKA inhibitory activity greater than 80%. The trend of the bioactive non-polar fractions continued for the *Geodia* sponge species where the non-polar hexane 1 extract showed both potent PKA inhibitory activity (97%) and brine shrimp lethality (88%), with modest haemolytic activity observed for the other fractions. The most active fractions isolated from the four sponges were the hexane 1 extracts from *Ircinia/Sarcotragus* sp. and *Geodia* sp. with PKA inhibitory activities of 100 and 97% respectively (at 100 μg/mL), the methanol extract from *Haliclona* sp. with haemolytic activity of 75% and the hexane extract from *Geodia* sp. with high toxicity of 88% to the brine shrimp *Artemia franciscana*.

As the three non-polar extracts (hexane 1, hexane 2 and dichloromethane extracts) from all samples gave the greatest PKA inhibition, these were further analysed by GC-MS. Most of these compounds were fatty acids and sterols and contributed to a partial fatty acid and sterol profile of these deep-sea demosponges. A total of 29 fatty acids were identified in the highest proportions according to the following order *Ircinia/Sarcotragus* sp. > *Haliclona* sp. > *Geodia* sp. In contrast to shallow-water sponges that are dominated by polyunsaturated fatty acids with a high percentage of long chain fatty acids, LCFAs (C24-C30), the deep-sea sponges investigated herein were all found to be rich in saturated fatty acids, in particular C14-C20 fatty acids, including odd and branched chain fatty acids, with only low levels (0-10%) of LCFAs, which may be a result of the different environmental parameters in the deep sea. In total 23 different sterols were identified by GG/MS analysis with the greatest sterol diversity found in *Geodia* sp.

In the *Haliclona* sponge the main sterol, which occurred in high yield in the hexane 1 fraction, was found to be pregna-5-en-3β-ol (Table 3.3) followed by sitosterol (24-ethylcholesta-5-en-3β-ol) in the hexane 2 extract. The predominant fatty acids were C16:0, C18:1 (n-9), C20:0 and C20:4 (n-5) FAs. The main FAs of the *Ircinia/Sarcotragus* sample were similar to the dominant acids found in the *Haliclona* sample including C20:0 and C20:4 (n-5) as well as C18:1 (n-9), 12-methyltetradecanoic and octadecanoic acids (Table 3.2). The main sterol in *Ircinia/Sarcotragus* sponge was found to be cholesta-7,22-dien-3β-ol identified in the hexane 1 extract, although overall sterols were identified in a much lower yield in this sample compared with the other two deep-sea sponges (Table 3.3). The *Geodia* sp. was rich in two C18 FAs including one saturated C18:0 and one monoenoic acid C18:1 (n-9) FA, while among the sterols
fucosterol and an unusual nor-pregna-5-en-3β-ol were detected as the major sterols (Table 3.2 and 3.3).

There was no apparent correlation amongst the sterol composition of the three deep-sea sponge samples. The sterol profile varied across the sponge species either in type of sterols or in relative percentages. Two of the samples (Haliclona and Geodia) that were obtained from the identical depth, location and on the same date, possessed different sterol profiles, indicating that sterol composition in the deep-sea sponges under investigation is largely species dependent. Of the 23 sterols identified amongst the three deep-sea sponges, 70% of the sterols possessed a Δ^5 structure including those with an additional double bound at positions C22 or C24. The ratio of Δ^5:Δ^7:Δ^5,7 sterols among the three deep sponge samples was 16:5:1, along with one Δ^0 sterol that was detected in the Geodia sp. consistent with literature reports for marine sterols.

The FAs and sterols that were found to be dominant in marine algae and deep sea sponges were also screened in the PKA inhibition assay. A series of commercially available FAs (C14:0, C16:0, C18:0, C18:1), fatty acid mixtures (C14-22, C18-22) and pure sterols (stigmasterol, campesterol, fucosterol, sitosterol and cholesterol) were examined for PKA inhibitory activity (Fig. 2.22, Chapter 2). These results showed that FAs were more potent than sterols at inhibiting PKA. The C14 FA displayed the highest activity of (64%) while C16, C18 and C18:1 displayed moderate activities. These results have practical value as marine algae are rich in PUFAs of the n-3 and n-6 series, which are considered essential fatty acids for humans and animals. Because of the huge and renewable biomass, seaweeds are a potential source of FAs for biotechnology and a dietary source of essential fatty acids. Our bioassay results showed that some of the dominant short side chain FAs such as C14:0 may be contributing to the PKA inhibitory activity observed in analysed marine extracts, which is consistent with other reports that have previously shown that FAs exhibit kinase inhibitory activity.

6.2. Structure modification of known isatin derivatives to develop potent kinase inhibitors

The third part of this project included synthesis of isatin derivatives as potential protein kinase A and B inhibitors. Numerous literature studies of isatin-indole compounds have proved that these scaffolds are important for kinase inhibitory activity, as evidenced by the fact that several indole based compounds have been approved as kinase inhibitor drugs. The successful synthesis of 77 novel derivatives under various conditions was achieved in good – excellent yields. The synthesis involved preparation of 41 imino and 14 alkenyl isatins.

Imino isatin derivative 83, with potent PKA inhibitory activity (20 µM), was used as a starting point for the preparation of isatin-based kinase inhibitors. Initially, the carboxyl group on the distal phenyl ring was replaced by a range of different substituents. Further modification
included replacement of bromine at position 5 and 7 of the aromatic ring with sulfonyl, aryl, nitro and methoxy groups. $N$-substitution of the parent compound with a PMB group was also changed and morpholino, triazolo, chloro, iodo and methyl substituted phenyl groups were incorporated into the isatin molecule. The biological results indicated that dibromination and $N$-alkylation are important both for cytotoxicity and for kinase inhibitory activity. When the dibrominated aromatic ring was replaced with other derivatives or the isatin aromatic ring was unsubstituted, the activity decreased in both the PKA and PKB inhibition assays (Fig. 6.2). It can be considered that a certain number of bromine atoms may be important to bring compounds into a suitable geometric orientation or favourable energetic states which alter the activity of enzyme. A similar trend was also observed when other groups were placed on the nitrogen instead of the PMB group. The PKA inhibition results showed that COOH, OH and $N$-OH groups contributed positively to the PKA inhibitory activity of the isatin derivatives. On the other hand, the position of the functional groups on the distal phenyl ring did not affect the activity. With respect to substitution on the C3 position of isatin, all imino isatin derivatives displayed more potent activities than their alkenyl analogues in the kinase inhibition assay.

![Figure 6.2: PKA inhibitory structure-activity relationships for the various isatin derivatives described herein.](image)

Of the 77 isatin derivatives synthesised, four novel compounds displayed potent PKA inhibitory activity ($>70\%$, at 100 $\mu$g/ml). The most active compounds were found to be the imino isatin 178, hydrazone isatin 208, oxime isatin 209 and alkenyl isatin 246 and their IC$_{50}$ values were determined as 43.5, 51.8, 38.5, 52.2 $\mu$M respectively (Fig. 6.3). A comparison of the inhibitory activity and their corresponding, calculated logP (clogP) values, found no correlation, indicating that the activity of the isatin derivatives was not based on lipophilicity alone.
Of the thirty-three compounds screened for Akt (PKB) inhibitory activity, three isatin derivatives displayed ≥70% inhibitory activity and another three ≥60% inhibition. Of these six most active compounds, imino isatin compounds \( \text{175, 176 and 177} \) were also found to display potent PKA inhibitory activity, while \( N \)-alkylated isatin \( \text{163} \), imino isatin derivates \( \text{187 and 206} \) exhibited low PKA inhibition (Fig. 6.4). These results show potential for further investigation especially because the active molecules have ready synthetic availability and structural simplicity. PKA and Akt (PKB) enzymes belong to the same group of ACG kinases and have similar homology. However, selective inhibitors for one enzyme over another have not been identified previously. Co-crystal structures of these two kinase enzymes show that PKA has a smaller and more lipophilic hinge region compared to Akt. Therefore, selective inhibitors can be designed considering the difference in the hinge region of the enzymes. Sixteen compounds were screened for MAPK inhibitory activity, however none of the isatins was found to be active in this assay. These results are significant and they can further contribute to the selectivity profile of the isatin derivatives synthesised herein.

All the compounds synthesised were evaluated for their cytotoxic effects on human leukemic monocyte lymphoma U937 cell line and eight compounds were screened for cytotoxicity on breast cancer MDA-MB-231 cell line. Cytotoxicity data confirms previous
results that the presence of an electron-withdrawing group(s) on the aromatic ring of the isatin core, as well as a one-carbon linker attaching an aryl group to N1, are required for potent cytotoxicity. Introduction of morpholino or triazolo groups on the nitrogen and aryl and sulfonyl groups on the aromatic isatin ring resulted in lower cytotoxic activity (Table 5.2 and 5.3; Fig. 5.8).

6.2 Future work

Both the various algal species and the deep-sea sponge extracts displayed significant PKA inhibitory activity along with haemolysis and brine shrimp cytotoxicity. Algal samples are widespread along the Australian coast and their advantage is being able to be hand collected easily. Therefore, future work should include the collection of a variety of algal species, preferably in higher quantities, followed by extraction and isolation of pure bioactive constituents. On the other hand, deep-sea samples are difficult to collect but they were found to be a prolific source of bioactive compounds. However, for their collection, continued collaboration with researchers and industry involved in the deep-sea work is essential.

Marine organisms are found to contain a large number of non-polar metabolites such as fatty acids and sterols. These compounds were found to display a range of biological activities. However, due to their dominance in marine extracts, other compounds were hard to detect. An extraction protocol that can separate polar and non-polar constituents is important, followed by extensive rounds of purification using HPLC or column chromatography.

To further improve PKA inhibitory activity of our compounds, future work of this project could involve the development of further novel analogues. The main problem with isatin derivatives synthesised in this work was their lipophilicity. To improve this, analogues with different groups on nitrogen can be made. Herein, we tried with triazolo and morpholino groups but found that activity decreased. However, compounds with a sugar moiety can be prepared as similar compounds have previously shown promising kinase inhibitory activity. Also, instead of an isatin core, other more polar starting cores can be used such as 7-azaindole (Fig. 6.5).

Figure 6.5: Structures of proposed analogues for future work.
The most active compounds in this work were those containing a carboxyl group. It is known that the presence of this acidic functional group is not particularly desirable for drug development. Therefore, the carboxyl functional group is often converted into another functional group that has similar physicochemical properties and will result in similar biological properties. Hence, the carboxyl acid moiety could be transformed into a tetrazole group (tetrazolic acids) and then those derivatives tested for PKA inhibitory activity (Fig. 6.5).

![Diagram of conversion of carboxyl group into the tetrazole group.](image)

**Figure 6.6:** Conversion of carboxyl group into the tetrazole group.

The isatin derivatives described in this project could also be tested against a wider range of other kinases to examine the selectivity of the compounds. In particular, several indole derivatives were found to be CDK inhibitors; hence these compounds should also be tested against that enzyme. Over 20 isatin analogues are currently being sent to Prof. Laurent Meijer, Centre National de la Recherche Scientifique, one of the world experts in kinase inhibition, for further kinase testing, however selectivity results have yet not been received at the time of writing. In addition, chemical genetic screening by A/Prof. Paul Teesdale Spittle, Victoria University of Wellington, is also under way to determine a complete bioactivity profile of selected isatin derivatives (175 and 204).

Future work could also include molecular modelling studies of the isatin derivatives that displayed the highest inhibitory activities in the PKA inhibition assay. In order to fully evaluate the SAR of these analogues computational docking studies can be performed with cAMP-dependent protein kinase catalytic subunit, for which a crystal structure is available from the protein data bank.
7.1 General experimental

All chemicals were purchased from Crown Scientific or Sigma Aldrich Chemical Co (St. Louis, MO, USA) and used as received. All solvents were either AR or HPLC grade for extractions and synthesis and HPLC grade for HPLC purification. Solvents were purified by short path distillation, while solvents for HPLC were filtered through membrane filters (Membrane nylon filters, #7404-009, Whatman®) and degassed in the sonicator. Sonication of samples was performed using a Soniclean 250HT ultrasonic bath (Soniclean, Thebarton, Australia). Solvents were mixed in volume to volume ratio. Evaporation of solvents was performed on a Büchi R-114 or R200 rotary evaporator, under vacuum and under 40 °C to avoid decomposition of samples. Water was removed from samples on a CHRIST® LDC-1M lyophiliser. Marine samples were homogenised using either an InvitroTM IKA® T10 basic Ultra-Turrax® homogeniser or a Waring® blender. Melting points were determined using a Reichert melting points apparatus and are uncorrected. The reaction yields were obtained after drying procedure, performed under high vacuum.

7.1.1 Chromatography

Thin layer and silica gel chromatography

Thin layer chromatography (TLC) was performed on Merck thin layer aluminium sheets or Macherey-Nagel Polygram® Sil G/UV254 thin layer pre-coated plastic TLC sheets both with fluorescent indicator UV254. Various staining reagents were used including cerium sulphate, dinitrophenylhydrazine (DNP), vanillin, and a natural product stain (polyethylene glycol (PEG) 4000 (5%w/v) solution in ethanol) for the natural product extracts while isatin compounds were coloured and directly visible on the TLC. Colourless compounds were visualised under UV light at λ 254 nm. For the natural product analysis three solvent systems were typically used to separate compounds on the TLC plates: a non-polar system (ethyl acetate – hexane, 1:1), an intermediate polarity (ethyl acetate – methanol, 19:1) and a polar solvent system (n-propanol - ethyl acetate – water, 7:2:1). Silica gel used for column chromatography was Merck silica gel 60 (230-400 mesh). Column chromatography was done according to the protocol described in Vogel et al. Preparative TLC was performed on silica or 20 x 20 glass plates of Merck silica Gel 60 F254 plates.

HPLC chromatography

Analytical HPLC was performed on a Shimadzu system with either a dual UV detector or a PDA detector. Columns used for analytical HPLC are Phenomenex Luna C18, Waters SunFire C18 and C8, all with the same size of 4.6 nm x 250 nm. Preparative HPLC was performed on a Waters system with a dual UV detector. Columns used for preparative HPLC were C18 Waters 19 x 150 mm for samples up to 100 mg and C18 Waters 30 x 100 mm for
samples up to 500 mg. Samples for HPLC analysis were removed by passing through a syringe filter (Filter device puradisc™ 25TF, # 6784-2504, Whatman®).

7.1.2 Nuclear magnetic resonance (NMR) spectroscopy

Nuclear magnetic resonance (NMR) spectra were obtained for all samples either on a Varian Unity 500 MHz Inova, where proton and carbon spectra were obtained at 500 MHz and 126 MHz respectively, or a Varian Unity 300 MHz spectrometer, where proton and carbon spectra were obtained at 300 MHz and 75 MHz respectively, Shigemi NMR tubes were used for samples less than 5 mg. The samples were dissolved in deuterated solvents as indicated in brackets. Chemical shifts are reported in parts per million (ppm) downfield from tetramethylsilane (TMS), which was used as internal standard. Coupling constants (J) are given in Hertz. Hydrogen and carbon assignments were performed using gradient correlation spectroscopy (gCOSY), gradient heteronuclear single quantum correlation (gHSQC) and gradient heteronuclear multiple bond correlation (gHMBC) spectroscopic techniques, nuclear Overhauser effect spectroscopy (NOESY). Multiplets were reported as singlet (s), doublet (d), doublet of doublets (dd), doublet of triplets (dt), triplet (t) and multiplet (m). Samples were subjected to high vacuum overnight before performing NMR. The superscript symbol * in the 13C NMR data denotes coincident peaks.

7.1.3 Mass spectrometry

Low resolution direct insertion electron impact mass spectrometry (EI-MS) was performed on a Shimadzu QP-5050A system. The probe was heated from 40 ºC to 200 ºC. Gas chromatography mass spectrometry (GCEI-MS) was performed on a Shimadzu QP-5050A GC-MS system equipped with a BP-5 fused silica Rxi-5ms capillary column (5% phenyl/95% dimethyl polysiloxane, 30 m x 0.25 mm, 0.25 μm film thickness, Resrek), using a helium carrier gas (1.0 mL/min) and samples of 1 μL over the temperature range 80-300 ºC. The relative intensity of each peak was calculated as a percentage of the summed total. Electrospray ionisation low resolution mass spectrometry (ESI-MS) was performed on Waters Platform LCZ spectrometer. High resolution electrospray ionisation mass spectra HRESI-MS was obtained using a Waters Q-Tof Ultima spectrometer using leucine enkephalin (LeuEnk) as the internal standard. All compounds were identified by comparing with compounds in the NIST 02 and NIST 08 spectral database, with identification based on matches of greater than 75%.

7.2 Biological assays

7.2.1 The kinase inhibition assay

Protein kinase inhibitor activity was determined using the Kinase Glo® Luminescent Kinase Assay (Promega Co, Madison, WI, USA). Purified PKA and specific kinase substrate
(kemptide) were purchased from Promega and used as supplied. The PKA inhibitory activity of the extracts was determined in opaque 96 white well plates (Corning) according to the manufacturer’s instructions. Each well contained 5 μl of sample (e.g. 1mg/ml in 20% dimethyl sulfoxide [DMSO] or ethanol), 25 μl of ATP (20 μM), and 20 μl of a mixture of kinase enzyme (0.1unit/μL) and kemptide substrate (140 μM) in reaction buffer (40 mM Tris, 20mM MgCl2, BSA 0.1mg/mL, pH 7.4). After 1 hour incubation at room temperature, 50 μl of the Kinase Glo® Reagent was added and the mixture incubated for a further 15 min, after which the luminescence was measured using a BMG Labtech FLUOstar Optima® luminometer (BMG Labtech Pty. Ltd., Mornington, Australia). The data were determined relative to the positive controls, which contained solvent (2% ethanol or 2% DMSO) in place of the sample. Negative controls contained no substrate. Staurosporine, elagic acid or H-89 were used as internal standards. All assays were conducted at 100 µg/ml (in 2% DMSO or 2% ethanol). Measurements were performed in triplicate and data are presented as means ± S.E.M.

7.2.2 Haemolytic assay

The haemolytic activity of the extracts was determined for samples prepared in either DMSO or ethanol as follows: 0.5% haemolytic solution was prepared using defibrinated horse blood (Oxoid Australia Pty Ltd). A suspension of horse blood erythrocytes in phosphate buffer saline (PBS) was centrifuged at 3000 rpm for 5 min at 6°C on a Sorvall SuperT21 Centrifuge and diluted with PBS to reach 0.5% erythrocyte suspension. The reaction mixture contained 100 μl of extracts (1mg/ml), 100 μl of PBS and 0.9 μl of erythrocyte suspension. The mixtures were incubated for 30 min at 37 °C, then 100 μl of reaction mixture was transferred to sterile clear 96 well microplates (Greiner Bio-one) and absorbance was measured at λ 550 nm on a Spectramax® 250 UV plate reader using Softmax Pro® program. All samples were done in triplicate and in at least two separate experiments.

7.2.3 Brine shrimp (cytotoxic) lethality assay

The brine shrimp lethality assay was performed as follows: brine shrimp eggs (Artemia franciscana) were hatched for 48 hours at 26-28°C in a light source in a 36 g/L salt solution (Coral Reef, Marine Reef Salt), prior to the tests being run. Each well of a sterile clear 96 well microplate (Greiner Bio-one) contained 10 animals, 20 µl of sample (1mg/ml in 20% DMSO or ethanol) and 180 ml of salt solution was added to each well to bring the volume to 200 µl. Each sample was done in triplicate in two separate experiments. The final concentration of the extracts was 100 μg/ml in 2% DMSO or ethanol. The incubation time was 48 hours under a light source and surviving animals were counted after 24 hours and percent mortality calculated.
7.2.4 MTS Cell proliferation assay

Cytotoxicity of the isatin derivatives was determined using the CellTiter 96® AQueous One Solution Cell Proliferation Assay, which employs [3-(4,5-dimethylthiazol-2- yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] (MTS) reagent (Promega Co., Madison, WI, USA). MTS assays were performed in clear 96-well microplates, including: 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). Plates were incubated for 24 h at 37 °C in a 5% CO₂ atmosphere, following the addition of the test compounds and then again incubated for another 24 h (2 x 24 h incubation). MTS reagent was added to each well and the assays were incubated at 37 °C for a further 3 h. Assays were analysed in a Spectromax 250 plate reader at 490 nm using Softmax Pro software (Molecular Devices, USA). The IC₅₀ values were determined from sigmoidal dose-response curves (variable slope) using GraphPad Prism 4.00 (GraphPad Software, Inc., San Diego, CA, USA) and are presented as the mean of triplicates ± SEM.

Cell lines and cell culture conditions

Human, leukemic, monocyte-like, histolytic lymphoma (U937) and human, metastatic breast adenocarcinoma (MDA-MB-231) 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) NaHCO₃ and 5% foetal calf serum. The cells were maintained in a Huracell incubator (Kendro Laboratory Products, Langenselbold, Germany) at 37 °C with a humidified atmosphere containing 5% CO₂. Viability of the cells was determined by the trypan blue exclusion method. The number of viable cells was counted with the aid of a haemocytometer.

7.3 Plant material

7.3.1 Shallow water algal species

Ten algal samples were collected from four locations along the New South Wales coast of Australia (Table 2.1, Chapter 2). The algae were collected from the intertidal zone by hand during the period March/April 2008 under Scientific Research Permit Number F95/269-4.0 issued by the New South Wales Department of Fisheries and stored frozen (-20 °C) until extraction. Voucher specimens are held at the University of Wollongong (AZ050, AZ56, AZ59, AZ061, AZ072, AZ073, AZ074, AZ076, AZ077 and AZ081). The collection of local marina algal species was fully documented (time, date, location, organism description, interaction with other organisms and habitat). Photographs of collected samples were taken at both the collection place and in the laboratory.
7.3.2 Deep-water sponge species

The deep-sea sponge samples were collected during a well head removal program conducted by Woodside Pty Ltd in conjunction with TS Marine and Well Ops. Three different subsea structures (well heads), which were abandoned on the ocean floor off the NW Australian coast at depths of 82-135 m during the period 1986-1992, were brought to the surface by remotely operated vehicle aboard the Havilla Harmony between June and August of 2008. Immediately upon being brought to the surface, the specimens were collected from the structure, frozen (-20 °C) and transported to the laboratory on dry ice. In situ video recording of the heavily encrusted well heads on the ocean floor was also performed using ROVs.

The deep-sea sponges were collected in the North West Shelf of Australia in June-August of 2008 and identified as: Haliclona sp., Geodia sp., and a mixed sample of intertwined Sarcotragus sp. and Ircinia sp. by Dr Jane Fromont, Western Australian Museum. The former two were obtained from the same location (depth 135 m), and the latter from a location 80 km away (depth 84 m) (Chapter 3, Table 3.1). Samples were collected under permit AU-COM2008032 issued by the Australian Government and voucher specimens (WAMZ45792-WAMZ45795) are held at the Western Australian Museum.

7.4 Extract preparation

Algal extracts were prepared using either extraction method A or B, while sponge extracts were prepared following method C, as described below.

7.4.1 Extraction method A

The frozen algal material was thawed and cut into small pieces using a scalpel or blender. Algae samples were extracted with methanol and left overnight in the fridge. The suspension was gravity filtered through a Whatman filter paper No. 1. The obtained tissue residue (marc) was re-extracted with methanol followed by two extractions with dichloromethane (DCM) where all extracts were left to stay for at least one hour on a magnetic stirrer. All organic extracts were combined, with a final solvent ratio 1:1 methanol-dichloromethane and solvents were evaporated in vacuo on a rotary evaporator to dryness to give a crude extract. The amount of solvent which were used in the above extraction 20-50 ml of solvent per one gram wet weight of sample.

Liquid-liquid partitioning of crude extract

The crude extracts were further separated into polar (water), intermediate polarity (butanol) and non-polar (dichloromethane, DCM) extracts. First, the crude extracts were dissolved in a small amount of DCM and then separated between DCM and water three times. The aqueous layer was further separated into water and butanol extracts. Solvents were removed
from all fractions in vacuo yielding different polarity fractions. Before removing the solvents, DCM and n-BuOH layers were dried over anhydrous MgSO₄.

7.4.2 Extraction method B

The DCM and water extracts were prepared as described in extraction method A (Fig. 2.5, Chapter 2). The frozen algal material was extracted with methanol and DCM, the solvents combined and evaporated to dryness, and the crude extract weighed and then partitioned between water and dichloromethane. The DCM layer was treated with anhydrous MgSO₄ and the solvent removed in vacuo to yield non-polar DCM extract. The stock solutions of 1mg/mL in DMSO of DCM extract were prepared and further diluted by 1:10 in the assay to give a final concentration of 100 µg/mL (in 2% DMSO or 2% ethanol).

The generation of ethanol or ‘quick” extracts was performed based upon the procedure of Wright et al. The ethanol extracts were obtained from approximately 8 g of frozen material cut into small pieces. Frozen samples were suspended in 20 ml of non-denatured 100% ethanol for 2 min, using a magnetic stirrer or INVITRO® homogeniser. Extracts were transferred into a scintillation screw cap glass vial and left overnight in the fridge. Extracts were gravity filtered and the filtrate used for biological testing. The concentration of prepared ethanol extract were approximately 9 mg/mL, but can range in value of 1 – 20 mg/mL for the reason that some organisms can give low yields of metabolites and concentration thus depends on the nature of the extracted sample.

7.4.3 Extraction method C

The extracts were prepared according to the method of Thale et al. (Fig 3.10, Chapter 3). The frozen sponge samples were cut into small pieces and suspended in 100% methanol (300 ml) for 24 hours at 4°C. The sample was filtered and the process repeated twice. The three methanol filtrates were combined and the solvent evaporated to give the crude methanol extract which was further partitioned between water and dichloromethane. The dichloromethane extract was then partitioned with 10% methanol and hexane. The 10% methanol extract was further partitioned between dichloromethane and 50% methanol. The water fraction was further partitioned between water and n-butanol to give another two extracts (Chapter 3, Fig. 3.10).

The solid residue, obtained from the original methanol was suspended in 100% dichloromethane for 24 hours at 4°C. The sample was filtered and the process repeated and the two dichloromethane extracts combined and the solvent removed in vacuo to give the crude dichloromethane extract. The dichloromethane was further partitioned between 10% methanol and hexane (Fig. 3.10, Chapter 3).

The organic fractions were treated with anhydrous MgSO₄ and the solvent removed in vacuo to yield dried extracts. The above procedure gave a total of seven extracts;
dichloromethane, hexane 1, hexane 2, 10% methanol, 50% methanol, butanol and a water extract.

7.5 Shallow water algal species (Chapter 2)

*Prionitis linearis*

Ethanol (0.09 g) and DCM extracts (0.31g) of the red algae *Prionitis linearis* (approximately 45 g of wet weight) were prepared according to method B (Fig. 2.5, Chapter 2) and tested in three biological assays (PKA inhibition, haemolytic and brine shrimp, Fig. 2.12). The DCM extract was subjected to GC-MS analysis.

*Corallina vancouveriensis*

Ethanol (0.071 g) and DCM (0.36 g) extracts of the red algae *Corallina vancouveriensis* (approximately 53 g of wet weight) were prepared according to method B (Fig 2.5, Chapter 2) and extracts were tested in three biological assays (PKA inhibition, haemolytic and brine shrimp, Fig. 2.12). The DCM extract was subjected to GC-MS analysis.

*Codium fragile*

The crude extract (4.12 g) of the green alga *Codium fragile* (approximately 129 g wet weight) was prepared according to method A and further partitioned to give a non-polar DCM extract (dark green oil, 1.24 g), an intermediate polarity butanol extract (dark yellow solid, 1.61 g) and a polar water extract (white solid, 1.42 g). Extracts were tested in three biological assays (PKA inhibition, haemolytic and brine shrimp, Fig. 2.6). The non-polar extract was further purified by column chromatography (Fig. 2.16). The intermediate and polar extracts didn’t show activity in any of the assays and were not further analysed.

Non-polar DCM extract of the green algae *Codium fragile*

The non-polar dichloromethane fraction (1.24 g) was chromatographed over silica gel using a gradient solvent system of 10:1 Hex/EtOAc through to 1:5 Hex/EtOAc to give seven fractions (Fig. 2.16). Column fractions 1, 2, 5 and 7 were subjected to GC-MS analysis and their chemical constituents are shown in Table 2.3 (Chapter 2). Column fractions 3, 4 and 6 were analysed by EI-MS and their constituents are shown in Table 2.4 (Chapter 2).

Removal of chlorophyll.

Chlorophyll was removed from the non-polar extracts 3 and 4 following procedure of Sargeneti *et al.*²⁶³ The extracts were mixed with silica gel and activated charcoal, in ratio of extract: silica: charcoal 1:3:1. The mixture was stirred for 15 minutes and than filtered. The final purified material was washed with hexane, dichloromethane and methanol.
**Codium dimorphum**

The crude extract (2.41 g) of the green algae *Codium dimorphum* (approximately 90 g wet weight) was prepared according to method A and further partitioned to give a non-polar dichloromethane extract (dark green oil 0.82 g), an intermediate polarity butanol extract (orange solid, 1.08 g) and a polar water extract (white solid, 2.21 g) with water fraction containing a small amount of residual water. Extracts were tested in three biological assays (PKA inhibition, haemolytic and brine shrimp, Fig. 2.7). The non-polar and intermediate polarity extracts were investigated by TLC and LREIMS but due to the complex nature of these extracts and small amount of material and low bioactivity they were not further purified. The polar extract was not studied further because it was not active in any assays. The DCM fraction was further analysed by GC-MS method (Chapter 2, Table 2.2).

**Ulva lactuca**

Ethanol (0.067 g) and DCM extracts (0.41 g) of the green algae *Ulva lactuca* (approximately 65 g of wet weight) were prepared according to method B and tested in the three biological assays (PKA inhibition, haemolytic and brine shrimp, Fig. 2.12). The DCM extract was subjected to GC-MS analysis (Table 2.2, Chapter 2).

**Colpomenia sinuosa**

The crude extract (5.83 g) of the brown algae *Colpomenia sinuosa* (approximately 230 g of wet weight) was prepared according to method A and further partitioned to give a non-polar DCM extract (dark green oil, 1.01 g), an intermediate polarity butanol extract (dark yellow solid, 1.82 g) and a polar water extract (white solid, 1.86 g) as described in method A (Fig 2.5, Chapter 2). Extracts were tested in three biological assays (PKA inhibition, haemolytic and brine shrimp, Fig. 2.8). The non-polar extract was further purified by column chromatography. The intermediate polarity and polar extracts didn’t show activity in the kinase assay and were not studied further.

Non-polar dichloromethane extract of *Colpomenia sinuosa*

The non-polar extract (1.01 g) was separated by silica gel column chromatography using a gradient solvent system of 100% hexane followed by Hex/EtOAc 4:1; 3:2; 1:1; 2:3; 100% EtOAc and than EtOAc/Methanol 2:3, which gave six major components as judged by TLC (Fig. 2.17). Column fractions 2 and 5 were subjected to GC-MS analysis and their chemical constituents are shown in Table 2.3 (Chapter 2). Column fractions 1, 3, 4 and 6 were analysed by EI-MS and their constituents are shown in Table 2.4 (Chapter 2).
**Phyllospora comosa**

The crude extract (4.6 g) of the brown alga *Phyllospora comosa* (approximately 140 g of wet weight) was prepared according to method A and further partitioned to give a non-polar dichloromethane extract (green oil, 1.40 g), an intermediate polarity butanol extract (orange solid, 2.54 g) and a polar water extract (light yellow solid, 3.23 g) as described in method A. Extracts were tested in three biological assays (PKA inhibition, haemolytic and brine shrimp, Fig. 2.9). The non-polar and intermediate polarity extracts were investigated by TLC and EIMS. All three extracts were not further purified due to low activity in the PKA assay. The chemical composition of the DCM extract was analysed by GC-MS method and results are shown in Table 2.2 (Chapter 2).

**Padina sp.**

The crude extract (2.34 g) of the brown algae *Padina sp.* (approximately 190 g of wet weight) was prepared according to method A and further partitioned to give a non-polar DCM extract (green oil, 1.04 g), an intermediate polarity butanol extract (orange solid, 1.17 g) and a polar water extract (light yellow solid, 2.26 g) as described in method A. Extracts were tested in three biological assays (PKA inhibition, haemolytic and brine shrimp, Fig. 2.10). The non-polar extract was further purified by column chromatography while butanol and water extracts were not further purified because they didn’t show any activity in the kinase assay.

**Non-polar dichloromethane extract of Padina sp.**

The non-polar extract (1.04 g) of *Padina sp.* was separated by silica gel column chromatography using a gradient solvent system of 100% Hex; Hex/EtOAc 9:1; 6:1; 1:1; 2:8; EtOAc/Methanol 95:5, to give six major partially purified fractions (Fig. 2.19). Column fractions 1 and 6 were analysed by GC-MS method (Table 2.3, Chapter 2) while fractions 2, 3, 4 and 5 were subjected to EI-MS analysis (Table 2.4, Chapter 2).

**Sargassum vestitum**

The crude extract of the brown alga *Sargassum vestitum* (5.21 g, approximately 500 g wet weight) was prepared according to method A. The crude extract yield three different polarity extracts as follows: water (3.81 g, brown solid), butanol (0.62 g, brown oil) and DCM (1.05 g, dark green oil). Extracts were tested in three biological assays (PKA inhibition, haemolytic and brine shrimp, Fig. 2.11). Only the non-polar extract was further purified by column chromatography.
Non-polar dichloromethane extract of *Sargassum vestitum*

Dichloromethane fraction was further separated using column chromatography with hexane, ethyl acetate and methanol as a gradient solvent system 10:1 Hex/EtOAc, 1:5 Hex/EtOAc. Monitored by TLC, four major fractions were isolated, where all were mixtures (Fig. 2.21). Further purification of column fraction 4 by preparative C18 HPLC (95% MeOH) gave compound 113.

Thunbergol A, colourless solid (0.009 g). **HREI-MS:** calcd for (M+H+) C27H38O5 442.2724 found 442.2719.

1H NMR (CDCl3, 500 MHz) δ 1.30 (s, 3H, H17'), 1.51-1.53 (m, 2H, H1'), 1.51-1.54 (m, 3H, H1'), 1.60-1.68 (m, 6H, H13', H14') 2.12-2.15 (m, 9H, H2', H5', H10', H18'), 2.18 (t, J = 7.0 Hz, 2H, H9'), 2.58-2.60 (m, 2H, H6'), 3.01 (dd, J = 16.0, 5.0 Hz, 1H, H4), 3.07 (dd, J = 16.0, 5.0 Hz, 1H, H4), 3.61 (dd, J = 4.0, 4.0 Hz, 1H, H3), 4.80 (t, J = 7.0 Hz 1H, H3'), 5.15-5.18 (m, 1H, H11'), 5.96 (t, J = 7.0 Hz, 1H, H7'), 6.4 (s, J = 2.0 Hz, 1H, H7), 6.51 (s, 1H, H5). 278

*Sargassum* sp.

The ethanol (0.059 g) and dichloromethane extracts (0.11 g) of the brown algae *Sargassum* sp. (approximately 30 g of wet weight) was prepared according to method B and tested in the three biological assays (PKA inhibition, haemolytic and brine shrimp, Fig. 2.12). The DCM extract was subjected to GC-MS analysis (Table 2.2, Chapter 2).

7.6 Deep-sea sponge samples (Chapter 3)

*Haliclona* sp.

The sponge *Haliclona* sp. (500 g wet weight) was separated according to extraction method C (section 7.4.3), to give seven different polarity extracts as follows: water (198 mg, a yellowish powder), butanol (156 mg, an orange gum), 10% methanol (117 mg, a pale yellow solid), dichloromethane (10.6 mg, yellow oil), hexane 1 (146 mg, orange oil), 50% methanol (102 mg, a pale yellow gum) and hexane 2 (174 mg, light orange oil) extract. The three non-polar extracts, dichloromethane, hexane 1 and hexane 2 were analysed by GC-MS (Tables 3.2, 3.3 and 3.4, Chapter 3) while the water extract was analysed by high resolution ESI-QToF-MS (Table 9.4, Appendix). Analyses of the remaining extracts are described below.

10% Methanol extract of the deep-sea sponge *Haliclona* sp.

The 10% methanol extract (97 mg) was separated by silica gel column chromatography using a gradient solvent system of 100% Hex; through to 100% EtOAc, and EtOAc/methanol...
1:4, which comprised six components by TLC. Six major column fractions were screened for kinase inhibitory activity and subjected to EI-MS analysis (Fig 3.15, Table 9.1).

50% Methanol extract of the deep-sea sponge *Haliclona* sp.

The 50% methanol extract (20.1 mg) was separated by silica gel column chromatography using a gradient solvent system of 100% Hex; Hex/EtOAc 3:1; 1:1; 2:3; 1:3; EtOAc 100%, EtOAc/Methanol 2:3; 1:4; 1:5, which gave six major fractions as judged by TLC, which were screened for kinase inhibitory activity and subjected to EI-MS analysis (Fig 3.16, Table 9.2).

*Geodia* sp.

The sponge *Geodia* sp. (500 g wet weight) was separated according to method C (7.4.3), to give seven different polarity extracts as follows: water (198 mg, a yellowish powder), butanol (156 mg, an orange gum), 10% methanol (97 mg, a pale yellow solid), dichloromethane (10.6 mg, yellow oil), hexane 1 (146 mg, orange oil), 50% methanol (20.1 mg, a pale yellow gum) and hexane 2 (174 mg, light orange oil) extract. The three non-polar extracts, dichloromethane, hexane 1 and hexane 2 were analysed by GC-MS (Tables 3.2, 3.3 and 3.4, Chapter 3), while the water extract was analysed by high resolution ESI-QToF-MS (Table 9.4, Appendix). Analyses of the remaining extracts are described below.

Butanol extract of the deep-sea sponge *Geodia* sp.

The butanol extract (BE, 156 mg) was purified by preparative RP-HPLC (isocratic mobile phase: H₂O, flow rate 43.5 ml/min). The collected fractions were tested on analytical HPLC (C18, mobile phase: H₂O), and of these fractions 6, 7/8 (these two fractions were identical), 9 and 11 appeared pure, while all others were mixtures and were combined. The four pure fractions were further analysed.

*Fraction 1-5 (0.4 mg, white powder)*

LREI-MS: m/z 152 (75) [M⁺], 109 (74), 54 (98). This data matches that of 1H-pyrolo[2,3-b]-pyridine. HREI-MS: calcd for C₇H₈N₂O₂ (M+H⁺) 153.0592 found 153.0586.

*Fraction 6 (0.9 mg, white solid)*

LREI-MS: m/z 326 [M⁺], 135 (100), 108 (35), 81 (28), 66 (20), 53 (42), 36 (49). This mass spectral data has a 68% match to that of septacidin aminonucleoside (C₁₂H₁₈N₆O₅) in the NIST 08 database.
Fraction 7 & 8 (1.0 mg, white solid)

**LREI-MS:** m/z 226 [M⁺], 117 (100), 96 (39), 73 (54), 69 (51), 54 (25), 40 (38). This mass spectral data has a 71% match to that of 2',5'-dideoxy-uridine \((\text{C}_9\text{H}_{12}\text{N}_2\text{O}_5)\) in the NIST database. **HREI-MS:** calcd for \(\text{C}_9\text{H}_{12}\text{N}_2\text{O}_5\) (M+H⁺) 227.0757, found 227.0746.

Fraction 9 (0.9 mg, white solid)

**LREI-MS:** m/z 242 (12) [M⁺], 136 (100), 108 (23), 97 (20), 81 (44), 63 (20), 54 (79), 53 (61), 44 (78), 36 (65). This mass spectral data has a 62% match to that of 2'-deoxy-inosine \((\text{C}_{10}\text{H}_{12}\text{N}_4\text{O}_4)\) in the NIST 08 database.

*Ircinia/Sarcotragus* sp.

The sponge *Ircinia/Sarcotragus* sp. (500 g wet weight) was separated according to method C (section 7.4.3), to give seven different polarity extracts as follows: water (126 mg, a yellowish powder), butanol (180 mg, an orange gum), 10% methanol (96 mg, an orange gum), dichloromethane (230 mg, yellow oil), hexane 1 (231 mg, orange oil), 50% methanol (374 mg, a pale yellow) and hexane 2 (261 mg, light orange oil) extract. The three non-polar extracts, dichloromethane, hexane 1 and hexane 2 were analysed by GC-MS (Tables 3.2, 3.3 and 3.4, Chapter 3), while the water extract was analysed by high resolution ESI-QTof-MS (Table 9.4, Appendix). Analyses of the remaining extracts are described below.

Butanol extract of the deep-sea sponge *Ircinia/Sarcotragus* sp.

The butanol extract (180 mg) was purified by preparative RP-HPLC (isocratic mobile phase: \(\text{H}_2\text{O}\), flow rate 43.5 ml/min). The thirty seven fractions collected were analysed on analytical HPLC to give 1 pure fraction and 3 fractions which were mixtures.

**Fraction 3.1.1 (1 mg, white solid)**

**LREI-MS:** m/z 44 (97), 136 (21), 176 (56). This data has a 82% match to that of 2-phenylethylamine in the NIST 08 database. **\(^1\text{H NMR}** \((\text{CDCl}_3, 500 \text{ HZ})\): δ 2.95 (t, 2H, \(J = 7.5\)), 3.16 (t, 2H, \(J = 7.5 \text{ Hz}\)), 7.26-7.28 (m, 2H), 7.35-7.37 (m, 3H).

**Fraction 3.1.2 (7.6 mg, white solid)**

**LREI-MS:** m/z 55 (80), 69 (100), 81 (41), 322 (56). This data has a 65% match to that of 5-methoxy-dokosane in the NIST 08 database.

**Fraction 3.1.3 (83.6 mg, white solid)**

**LREI-MS:** m/z 63 (21), 89 (43), 116 (58), 144 (97), 161 (35). This data has a 70% match to that of indanone-2-hydroxyimino in the NIST 08 database.
**Fraction 3.1.4** (23.0 mg, white solid)

**LREI-MS:** m/z 126 (89), 55 (100). This data has a 74% match to that of thymidine in the NIST 08 database.

10% Methanol extract of the deep-sea sponge *Ircinia/Sarcotragus* sp.

The 10% methanol extract (231 mg), was subjected to silica gel column chromatography, with increasing polarity of solvent, from 100% hexane to 100% EtOAc and then to 70/30 EtOAc/methanol. Thirty nine fractions were collected and combined according to TLC, to give a total number of six major fractions (3.2.1-3.2.6), all of which were obtained as mixtures and screened for kinase inhibitory activity and subjected to EI-MS analysis (Fig 3.18, Table 9.3).

### 7.7 Experimental for Chapter 4

The novel compounds synthesised in this work are assign with $^1$H NMR, $^{13}$C NMR and 2D NMR data. The structure of the known compounds was confirmed by 1H NMR and MS. Isatin derivatives that were obtained in low yield were only assigned by 1H NMR due to insufficient material for 2D NMR spectra.

**Synthesis of 5,7-dibromoisatin** (76)

The title compound (76) was synthesised according to the protocol of Vine *et al.*$^{183}$ Isatin (5 g, 0.034 mol) was warmed in ethanol (95%, 100 ml) with stirring until it dissolved. Bromine (5 ml, 102 mmol) was added dropwise to the stirred solution whilst maintaining the temperature between 70 - 75°C. The solution was cooled to room temperature and ice was placed into the solution. The precipitate was filtered, washed with water and cold ethanol and the product recrystallised from ethanol to yield an orange solid (7.8 mg, 84.9%), m.p. 260-261°C (lit. 261–263 °C),$^{431}$ R$_f$ 0.24 (DCM). **LREI-MS:** m/z 303; 305; 307 [M$^+$] $^{79}$Br$^{79}$Br; $^{79}$Br$^{81}$Br; $^{81}$Br$^{81}$Br. **$^1$H NMR** (CDCl$_3$, 500 MHz) δ 7.67 (s, 1H), 8.03 (s, 1H), 11.43 (s, 1H).

5,7-Dibromo-3,3-diethoxyindolin-2-one (161)

The title compound (161) was synthesised according to the protocol of Vine *et al.*$^{183}$ Isatin (5 g, 0.034 mol) was warmed in ethanol (100%, 100 ml) with stirring until it dissolved. Bromine (5 ml, 102 mmol) was added dropwise to the stirred solution whilst maintaining the temperature between 70 - 75°C. The solution was cooled to room temperature and ice was placed into the solution. The precipitate was filtered, washed with water and cold ethanol and the product recrystallised from ethanol to
yield an orange solid (6.3 mg, 79%), m.p. 267-269 °C, R_f 0.31 (DCM). \textbf{LREI-MS:} m/z 375; 377; 379 [M+Br]^79\text{Br}^{79}\text{Br}; 79\text{Br}^{81}\text{Br}; 81\text{Br}^{81}\text{Br}. \textbf{HRESI-MS:} C_{12}H_{13}Br_{2}NO_{3} 79\text{Br}^{81}\text{Br} \text{ (M+Li\textsuperscript{+})} 381.9422 \text{ found 381.9387.} \textbf{1H NMR (CDCl}_3\text{, 500 MHz) } \delta 1.11 \text{ (t, } J = 7 \text{ Hz, 6H, CH}_3\text{), 3.63 (q, } J = 7.5 \text{ Hz, 2H, CH}_2\text{), 3.82 (q, } J = 7.5 \text{ Hz, 2H, CH}_2\text{), 7.56 (s, 1H, H4), 7.78 (s, 1H, H6), 11 (s, 1H, NH).}

**Synthesis of 5-nitroisatin (170)**

5-nitroisatin was synthesized according to the protocol of Vine \textit{et al.}\textsuperscript{183} Isatin (500 mg, 0.0034 mol) was mixed with concentrated H\textsubscript{2}SO\textsubscript{4} (3.2 ml). A solution was added dropwise to the solution of KNO\textsubscript{3} (344 mg, 0.0034 mol) in conc. H\textsubscript{2}SO\textsubscript{4} (3.8 ml) over a period of 1h. Temperature was maintained between 0 and 4°C. The solution was poured into 25 ml of crushed ice and the precipitate filtered and washed with water. The product was purified by column chromatography with DCM/MeOH (98:2, 95:5) to yield a yellow/orange solid (0.26 g, 41%), m.p. 250-253 °C (lit. 252-254 °C), \textbf{R_f 0.48 (DCM/MeOH 98:2). \textbf{LREI-MS:} m/z 192 [M\textsuperscript{+}].} \textbf{1H NMR (DMSO-d_6, 500 MHz) } \delta 7.03 \text{ (d, } J = 8.5 \text{ Hz, 1H, H7), 8.16 \text{ (d, } J = 2.0 \text{ Hz, 1H, H4), 8.81 \text{ (dd, } J = 3, 8.5 \text{ Hz, 1H, H6), 10.80 (br s, 1H, NH).}

7.7.1 General method for 5,7-dibromo-\textit{N}-alkylation of 5,7-dibromoisatin (Method A)

Method A: \textit{N}-Alkylated isatin derivatives were synthesised according to the protocol of Vine \textit{et al.}\textsuperscript{183} and Garden \textit{et al.}\textsuperscript{392} The isatin derivative (1 equiv.) and calcium hydride (1.2 equiv.) were combined in DMF (8 ml per 100 mg of isatin derivative) and the solution stirred with gentle warming. After 45 minutes an appropriate benzyl halide (1.0 equiv) and potassium iodide (0.2 equiv.) were added to the reaction and reaction mixture was stirred at 80°C for 15-24 hour. Once the reaction had run to completion (as judged by TLC) the reaction mixture was poured into acidified aqueous solution (0.2 M HCl) and extracted with EtOAc. The extracts were combined and washed with a salt solution, dried over anhydrous MgSO\textsubscript{4} and the solvent removed under reduced pressure. The product was purified using column chromatography on silica gel.

5,7-Dibromo-\textit{N}(p-methoxybenzyl)isatin (162)

The title compound (162) was synthesised from \textit{76} and \textit{p}-methoxybenzylchloride according to Method A and purified by flash chromatography on silica gel (DCM) to give a red-orange solid (2.4 g, 86%), m.p. 165-166 °C (lit. 166–167 °C), \textbf{R_f 0.52 (DCM). \textbf{LREI-MS:} m/z 423; 425; 427 [M\textsuperscript{+}]}
5-Bromo-N-(p-methoxybenzyl)isatin (162a)

The title compound (162a) was obtained as a by-product in the above reaction and purified by column chromatography to yield a dark orange solid (0.045 g, 2%), m.p. 144–146 °C (lit. 144–146 °C), \( R_f \) 0.57 (DCM). LREI-MS: m/z 345; 347 [M+] \( ^{79}\text{Br}^{79}\text{Br}^{81}\text{Br}^{81}\text{Br} \). \( ^1\text{H NMR} \) (CDCl\(_3\), 500 MHz) \( \delta \) 3.81 (s, 3H, OCH\(_3\)), 5.38 (s, 2H, CH\(_2\)), 6.87 (d, \( J \) = 8.5 Hz, 2H, H3'/H5'), 7.22 (d, \( J \) = 8.0 Hz, 2H, H2'/H6'), 7.72 (d, \( J \) = 2.0 Hz, 1H, H4), 7.84 (d, \( J \) = 2.0 Hz, 1H, H6).

5,7-Dibromo-N-(p-methylbenzyl)isatin (163)

The title compound (163) was synthesised from 5,7-dibromoisatin and p-methylbenzylchloride according to Method A and purified by flash chromatography on silica gel (DCM) to yield an orange solid (0.89 g, 67%), m.p. 122–124 °C (lit. 121–123 °C), \( R_f \) 0.71 (DCM). LREI-MS: m/z 407; 409; 411 [M+] \( ^{79}\text{Br}^{79}\text{Br}^{81}\text{Br}^{81}\text{Br} \). \( ^1\text{H NMR} \) (CDCl\(_3\), 500 MHz) \( \delta \) 2.32 (s, 3H, CH\(_3\)), 5.36 (s, 2H, CH\(_2\)), 7.09 (d, \( J \) = 8.0 Hz, 2H, phenyl), 7.12 (d, \( J \) = 8.0 Hz, 2H, phenyl), 7.72 (d, \( J \) = 2.0 Hz, 1H, H4), 7.80 (d, \( J \) = 2.0 Hz, 1H, H6).

5,7-Dibromo-N-(p-chlorobenzyl)isatin (164)

The title compound (164) was synthesised from 5,7-dibromoisatin and p-chlorobenzylchloride according to Method A and purified by flash chromatography on silica gel (DCM) to yield an orange solid (0.97 mg, 69%), m.p. 158-161 °C (lit. 159-161 °C), \( R_f \) 0.89 (DCM). LREI-MS: m/z 427; 429; 431 [M+] \( ^{79}\text{Br}^{79}\text{Br}^{81}\text{Br}^{81}\text{Br} \). \( ^1\text{H NMR} \) (CDCl\(_3\), 500 MHz) \( \delta \) 5.38 (s, 2H, CH\(_2\)), 6.87 (d, \( J \) = 8.0 Hz, 2H, H2’/H6’), 7.22 (d, \( J \) = 8.0 Hz, 2H, H3’/H5’), 7.72 (d, \( J \) = 2.0 Hz, 1H, H4), 7.84 (d, \( J \) = 2.0 Hz, 1H, H6).
5,7-Dibromo-N-(p-iodobenzyl)isatin (165)
The title compound (165) was synthesised from 5,7-dibromoisatin and p-iodobenzylchloride according to Method A and purified by flash chromatography on silica gel (DCM) to yield an orange solid (0.66 mg, 78%), m.p. 139-142 °C (lit. 141-142 °C), Rf 0.89 (DCM). \textbf{LREI-MS:} m/z 519; 521; 523 [M+][\textsuperscript{79}Br][\textsuperscript{79}Br]; [\textsuperscript{79}Br][\textsuperscript{81}Br]; [\textsuperscript{81}Br][\textsuperscript{81}Br]. \textbf{1H NMR (CDCl\textsubscript{3}, 500 MHz)} δ 5.37 (s, 2H, CH\textsubscript{2}), 6.87 (d, J = 8.0 Hz, 2H, H2'/H6'), 7.22 (d, J = 8.0 Hz, 2H, H3'/H5'), 7.72 (d, J = 2.0 Hz, 1H, H4), 7.84 (d, J = 2.0 Hz, 1H, H6).

5,7-Dibromo-N-(2-morpholinoethy)isatin (166)
The title compound (166) was synthesised from 5,7-dibromoisatin and 4-(2-chloroethyl)morpholine hydrochloride according to Method A and purified by flash chromatography on silica gel (DCM/EtOAc 6:1) to yield a red solid (0.18 g, 27%), m.p. 124-126 °C, Rf 0.30 (DCM/EtOAc 4:1). \textbf{LREI-MS:} m/z 416; 418; 420 [M+] [\textsuperscript{79}Br][\textsuperscript{79}Br]; [\textsuperscript{79}Br][\textsuperscript{81}Br]; [\textsuperscript{81}Br][\textsuperscript{81}Br]. \textbf{HRESI-MS:} calcd for C\textsubscript{14}H\textsubscript{15}Br\textsubscript{2}N\textsubscript{2}O\textsubscript{3} (M+H\textsuperscript{+}) 416.9330 found 416.9324. \textbf{1H NMR (CDCl\textsubscript{3}, 500 MHz)} δ 2.47 (t, J = 4.5 Hz, 4H, H2''/H6''), 2.61 (t, J = 6.5Hz, 2H, H2'), 3.57 (t, J = 4.5 Hz, 4H, H3''/H5''), 4.28 (t, J = 6.5 Hz, 2H, H1'), 7.70 (s, 1H, H4'), 7.87 (s, 1H, H6'). \textbf{13C NMR (CDCl\textsubscript{3}, 126 MHz):} δ 39.4 (C1'), 53.8 (C2''/C6''), 42.7 (C2''/C6''), 45.4 (C1'), 66.5 (C3''/C5''), 56.6 (C2'), 67.2 (C3''/C5''), 106.3, 118.4, 121.5, 127.7 (C4), 145.1 (C6), 146.9 (C7a), 158.8 (C2), 181.8 (C3).

5,7-Dibromo-N-(2-morpholino-2-oxoethyl)isatin (167)
The title compound (167) was synthesised from 5,7-dibromoisatin and p-(chloroacetyl)morpholine according to Method A and purified by flash chromatography on silica gel (DCM/EtOAc 10:1, 6:1) to give a dark red solid (0.923 g, 39%), m.p. 164-167 °C, Rf 0.29 (DCM/EtOAc 4:1). \textbf{LREI-MS:} m/z 430; 432; 434 [M+] [\textsuperscript{79}Br][\textsuperscript{79}Br]; [\textsuperscript{79}Br][\textsuperscript{81}Br]; [\textsuperscript{81}Br][\textsuperscript{81}Br]. \textbf{HRESI-MS:} calcd for C\textsubscript{14}H\textsubscript{13}Br\textsubscript{2}N\textsubscript{2}O\textsubscript{4} (M+H\textsuperscript{+}) 430.9242 found 430.9258. \textbf{1H NMR (CDCl\textsubscript{3}, 500 MHz)} δ 3.71 (s, 2H, H1'), 3.72-3.76 (m, 8H, H2','H3''/H5'/H6''), 7.71 (s, 1H, H4), 7.81 (s, 1H, H6). \textbf{13C NMR (CDCl\textsubscript{3}, 126 MHz):} δ 42.7 (C2''/C6''), 43.2 (C2''/C6''), 45.4 (C1'), 66.5 (C3''/C5''), 66.3 (C3''/C5''), 105.6 (C3a), 117.2 (C5), 121.6 (C7a), 127.7 (C4), 144.9 (C6), 147.4 (C7), 158.5 (C2), 164.6 (C2'), 181.1 (C3).
Chapter 7

5,7-Dibromo-N-propargylisatin (168)
The title compound (168) was synthesised from 5,7-dibromoisatin and 3-bromo-1-propyne according to Method A and purified by flash chromatography on silica gel (DCM) to give a dark red solid (0.26 g, 78%), Rf 0.79 (DCM). LREI-MS: m/z 341; 343; 345 [M+][79]Br,[79]Br; [81]Br,[81]Br; [81]Br,[81]Br. HRESI-MS: calcd for C11H6Br2NO2 [M+H]+ 341.8765 found 341.8725.

1H NMR (CDCl3, 500 MHz) δ 2.36 (s, 1H, H3'), 4.93 (s, 2H, H1'), 7.01 (s, 1H, H4), 7.91 (s, 1H, H6).

13C NMR (CDCl3, 126 MHz): δ 32.1 (C1'), 74.1 (C2', C3'), 103.0 (Ar C), 118.7 (Ar C), 121.1 (Ar C), 128.2 (C4), 144.5 (C6), 144.9 (C7a), 158.3 (C2), 180.6 (C3).

N-(4-Methoxybenzyl)isatin (169)
The title compound (169) was synthesised from isatin and p-methoxybenzylchloride according to Method A and purified by flash chromatography on silica gel (DCM) to give a red solid (0.66 g, 83%), m.p. 170-172 ºC (lit. 169-171 ºC), Rf 0.62 (DCM). LREI-MS: m/z 267.28 [M+] 1H NMR (CDCl3, 500 MHz) δ 3.79 (s, 3H, OCH3), 4.87 (s, 2H, CH2), 6.80 (d, J = 8 Hz, 1H, H7), 6.87 (d, J = 8 Hz, 2H, H3'/H5'), 7.01 (t, J = 8 Hz, 1H, H5), 7.25 (d, J = 8 Hz, 2H, H2'/H6'), 7.51 (t, J = 1.8 Hz, 1H, H6), 7.61 (d, J = 7 Hz, 1H, H4).

5-Nitro-N-(4-chlorobenzyl)isatin (170)
The title compound (170) was synthesised from 5-nitroisatin and p-methoxybenzylchloride according to Method A and purified by flash chromatography on silica gel (DCM/MeOH 15:1, 10:1) to give a dark yellow solid (0.25 g, 79%), Rf 0.48 (DCM/EtOAc 5:1). LREI-MS: m/z 312 [M*]. 1H NMR (CDCl3, 500 MHz) δ 3.79 (s, 3H, OCH3), 4.95 (s, 2H, CH2), 6.88 (d, J = 8.5 Hz, 2H, H3'/H5'), 6.96 (d, J = 8.5 Hz, 1H, H7), 7.26 (d, J = 8.5 Hz, 2H, H2'/H6'), 8.45 (d, J = 9.0 Hz, 1H, H6), 8.45 (s, 1H, H4).

5-Methoxy-N-(4-methoxybenzyl)isatin (171)
The title compound (171) was synthesised from 5-methoxyisatin and p-methoxybenzylchloride according to Method A and purified by flash chromatography on silica gel (DCM/MeOH...
15:1, 10:1) to give a dark red solid (0.65 g, 74%), Rf 0.52 (DCM/EtOAc 5:1). LREI-MS: m/z 297 [M+].

**H NMR** (CDCl3, 500 MHz) δ 3.77 (s, 3H, OCH3), 3.79 (s, 3H, OCH3), 4.83 (s, 2H, CH2), 6.70 (d, J = 8.5 Hz, 1H, H6), 6.87 (d, J = 8.0 Hz, 2H, H3’/H5’), 7.08 (d, J = 8.0 Hz, 1H, H7), 7.13 (s, 1H, H4), 7.25 (d, J = 8.5 Hz, 2H, H2’/H6’).

### 7.7.2 General method for 3-imino-substituted-N-alkylated-5,7-dibromoisatin derivatives

Method B: The imine isatin derivatives were all synthesized using the same protocol with the only difference being the reaction time. N-alkylated-5,7-dibromoisatin (1 equiv. 0.2 g) was dissolved in ethanol (8 mL per 100 mg of isatin derivative) and mixture was sonicated for 5 min, until the isatin was totally dissolved. Molecular sieves (1g per 100 mg of compound) were added to the mixture, which was sonicated for another 5 min, followed by the addition of acetic acid (200 µL per 100 mg of compound) and the appropriate aniline (1 equiv.). The reaction mixture was refluxed for 2-48 h until the product was formed by TLC. The mixture was then poured into water and extracted with EtOAc, the solvent removed and the product purified by column chromatography.

#### Synthesis of (E and Z)-4-[5,7-dibromo-1-(4-methoxybenzyl)-2-oxoindolin-3-ylideneamino]benzoic acid (175)

The title compound (175) was synthesised from 1-(4-methoxybenzyl)-5,7-dibromoisatin (162) and p-aminobenzoic acid according to method B and purified by flash chromatography on silica gel (CHCl3/MeOH 95:5) to give a red powder (0.041 g, 16 %), m.p. 251-254 ºC (lit. 250-252 ºC), Rf 0.25 (DCM), mixture of isomers (E:Z 47:53). LREI-MS: m/z 542; 544; 546 [M+] [79]Br [79]Br; [79]Br [81]Br; [81]Br. **1H NMR** (DMSO-d6, 500 MHz) δ 3.69 (s, 3H, OCH3, Z), 3.72 (s, 3H, OCH3, E), 5.14 (s, 2H, CH2, E), 5.28 (s, 2H, CH2, Z), 6.42 (s, 1H, H4”, E) 6.86 (d, J = 8.0 Hz, 2H, H3’’/H5’’”, Z), 6.88 (d, J = 8.0 Hz, 2H, H3’’/H5’’”, E), 7.11 (d, J = 7.5 Hz, 4H, H2’/H6’, E + Z), 7.16 (d, J = 8 Hz, 2H, H2’’/H6’’”, Z), 7.22 (d, J = 8.0 Hz, 2H, H2’’/H6’’”, E), 7.84 (s, 1H, H6”, E), 7.83 (s, 1H, H4”, Z), 7.90 (d, J = 8.5 Hz, 2H, H3’/H5’, Z), 7.94 (s, 1H, H6”, Z), 8.09 (d, J = 8.0 Hz, 2H, H3’/H5’, E).
Synthesis of (E and Z)-4-[5,7-dibromo-1-(4-methoxybenzyl)-2-oxoindolin-3 -ylideneamino] benzoic acid (176)

The title compound (176) was synthesised from 1-(4-methoxybenzyl)-5,7-dibromoisoatin (162) and (p-aminophenyl)acetic acid according to Method B and purified by flash chromatography on silica gel (CHCl₃/MeOH 95:5) to give a red powder (0.15 g, 59 %), m.p. 153-156 ºC (lit. 151-153 ºC), Rₚ 0.25 (DCM), mixture of isomers (E:Z/58:42). LREI-MS: m/z 556; 558; 560 [M⁺] ⁷⁹Br ⁷⁹Br; ⁷⁹Br ⁸¹Br; ⁸¹Br ⁸¹Br. ¹H NMR (DMSO-d₆, 500 MHz) δ 3.55 (s, 2H, H₂, Z), 3.62 (s, 2H, H₂, E), 3.70 (s, 3H, OCH₃, Z), 3.74 (s, 3H, OCH₃, E), 5.14 (s, 2H, CH₂, Z), 5.28 (s, 2H, CH₂, E), 6.61 (d, J = 1.5 Hz, 1H, H₄''), E), 6.80 (d, J = 9 Hz, 2H, H₃''''/H₅''''), E), 7.05 (d, J = 8.5 Hz, 2H, H₂''''/H₆''''), E), 7.12 (d, J = 8 Hz, 2H, H₂''''/H₆''''), Z), 7.16 (d, J = 8 Hz, 2H, H₂''''/H₆''''), E), 7.20 (d, J = 7.5 Hz, 2H, H₂''''/H₆''''), Z), 7.45 (d, J = 8.5 Hz, 2H, H₃''''/H₅''''), E), 7.84 (d, J = 2 Hz, 2H, H₄''''/H₆''''), E), 7.91 (d, J = 2 Hz, 1H, H₆'', Z).

Synthesis of (E and Z)-2-[4-[5,7-Dibromo-1-(4-methoxybenzyl)-2-oxoindolin-3-ylideneamino]phenyl]acetic acid (177)

The title compound (177) was synthesised from 1-(4-methoxybenzyl)-5, 7-dibromo-indoline-2,3-dione and (m-aminophenyl)acetic acid according to Method B and purified by flash chromatography on silica gel (CHCl₃/MeOH 95:5) to give a red powder (0.13 g, 69 %), m.p. 181-184 ºC (lit.182-184 ºC), Rₚ 0.36 (DCM/MeOH 9:1), mixture of isomers (E:Z/57:43). LREI-MS: m/z 557; 559; 561 [M⁺] ⁷⁹Br ⁷⁹Br; ⁷⁹Br ⁸¹Br; ⁸¹Br ⁸¹Br. ¹H NMR (DMSO-d₆, 500 MHz) δ 3.54 (s, 2H, H₂, Z), 3.68 (s, 2H, H₂, E), 3.70 (s, 3H, OCH₃, Z), 3.73 (s, 3H, OCH₃, E), 5.16 (s, 2H, CH₂, Z), 5.32 (s, 2H, CH₂, E), 6.60 (d, 1H, H₄''', E), 6.90 (d, J = 8 Hz, 2H, H₃''''/H₅''''), Z), 6.93 (d, J = 8.5 Hz, 2H, H₃''''/H₅''''), E), 6.97 (d, J = 8.5 Hz, 1H, H₄''), E), 7.05 (s, 1H, H₂'', E), 7.09 (d, J = 8 Hz, 1H, H₂'', E), 7.17 (d, J = 8.5 Hz, 2H, H₂''''/H₆''''), Z), 7.21 (d, J = 8 Hz, 1H, H₆'', E), 7.25 (d, J = 8 Hz, 1H, H₆'', Z), 7.28 (d, J = 7.5 Hz, 2H, H₂''''/H₆''''), E), 7.31 (t, J = 7.5 Hz, 1H, H₅'', Z), 7.46 (t, J = 7.5 Hz, 1H, H₅'', E), 7.79 (s, 1H, H₆'', E), 7.82 (s, 1H, H₂'', Z), 7.91 (s, 1H, H₄'', Z), 8.02 (s, 1H, H₆'', Z), 12.19 (br s, 2H, OH, E + Z).
Synthesis of (\(E\) and \(Z\))-1-(4-methoxybenzyl)-3-(4-hydroxyphenylimino)-5,7-dibromoindolin-2-one (178)

The title compound (178) was synthesised from 5,7-dibromo-N-(4-methoxybenzyl)isatin (162) and 4-aminophenol according to method B and purified by flash chromatography on silica gel (DCM; DCM/EtOAC 4:1) to give an orange powder (0.071 g, 29% as a mixture of \(E\) and \(Z\) isomers (\(E:Z\) 43:57)), m.p. 214-216 °C, R\(_f\) 0.29 (DCM/MeOH 9:1). LREI-MS: m/z 514; 516; 528 [M+1] \(^{79}\)Br\(^{79}\)Br; \(^{79}\)Br\(^{81}\)Br; \(^{81}\)Br\(^{81}\)Br. HRESI-MS: calcd for C\(_{22}\)H\(_{17}\)N\(_2\)O\(_2\)Br\(_2\) \(^{79}\)Br\(^{79}\)Br (M+H\(^+\)) 514.9487 found 514.9524. \(^1\)H NMR (DMSO-\(d_6\), 500 MHz) \(\delta\) 3.69 (s, 3H, OCH\(_3\)), 3.70 (s, 3H, OCH\(_3\)), 5.18 (s, 2H, CH\(_2\)), 5.28 (s, 2H, CH\(_2\)), 6.79 (d, \(J = 8.5\) Hz, 2H, H\(_{3}/H_{5}\)), 6.85 (d, \(J = 8.0\) Hz, 2H, H\(_{3}/H_{5}\)), 6.87-6.89 (m, H\(_{3}/H_{5}\), E, H\(_{2}/H_{6}\)), 6.96 (s, 1H, H4, E) 6.97 (d, \(J = 8.5\) Hz, 2H, H\(_{3}/H_{5}\)), 7.14 (d, \(J = 8.5\) Hz, 2H, H\(_{2}/H_{6}\)), Z), 7.20 (d, \(J = 8.5\) Hz, 2H, H\(_{2}/H_{6}\)), E), 7.48 (d, \(J = 9\) Hz, 2H, H\(_{2}/H_{6}\)), Z), 7.69 (s, 1H, H6, E), 7.71 (s, 1H, H6, Z), 13C NMR: (DMSO-\(d_6\), 126 MHz): \(\delta\) 43.9 (CH\(_2\)), 44.4 (CH\(_2\)), 55.7 (OCH\(_3\)), 103.7 (ArC), 104.6 (ArC), 114.6 (C\(_3''/C5''\), E+Z), 114.8 (Arc), 115.6 (C\(_3''/C5''\), Z), 115.8, 116.8 (C2''/C6''), E), 120.7 (C3'''/C5'''), E), 124.1 (C6, E), 126.7 (C4, E), 127.1 (C2''/C6''), Z), 128.1 (C2'/C6', E+Z), 129.5 (C1', E + Z), 131.4, 139.2 (C4, Z), 139.3 (C1'''', Z), 140.5 (C6, Z), 141.4 (C4''', Z), 144.0 (ArC), 146.1 (ArC), 149.1, 149.9 (C3, Z), 151.4 (C3, E), 156.9 (C1'''', Z), 158.9 (C2), 159.0 (C4''', Z; C4', E+Z), 164.0 (C2).

Synthesis of (\(E\) and \(Z\))-1-(4-methoxybenzyl)-3-(4-bromophenylimino)-5,7-dibromoindolin-2-one (179)

The title compound (179) was synthesised from 5,7-dibromo-N-(4-methoxybenzyl)isatin and 4-bromoaniline according to method B and purified by flash chromatography on silica gel (DCM; DCM/EtOAC 9:1) to give an orange solid, m.p. 189-191°C, R\(_f\) 0.85 (DCM). LREI-MS: m/z 576; 578; 580 [M+1] \(^{79}\)Br\(^{79}\)Br; \(^{79}\)Br\(^{81}\)Br; \(^{81}\)Br\(^{81}\)Br. HRESI-MS: calcd for C\(_{22}\)H\(_{16}\)Br\(_3\)N\(_2\)O\(_2\) \(^{79}\)Br\(^{81}\)Br (M+H\(^+\)) 576.8729 found 576.8762. \(^1\)H NMR (CDCl\(_3\), 500 MHz) \(\delta\) 3.42 (s, 3H, OCH\(_3\)), 3.70 (s, 3H, OCH\(_3\)), 5.19 (s, 2H, CH\(_2\)), 5.37 (s, 2H, CH\(_2\)), 6.75 (d, \(J = 8.5\) Hz, 2H, H\(_{3}/H_{5}\)), 6.78 (d, \(J = 8.5\) Hz, 2H, H\(_{3}/H_{5}\)), 6.80 (s, 1H, H4, E), 6.82 (d, \(J = 8.5\) Hz, 2H, H\(_{2}/H_{6}\)), 7.09 (d, \(J = 7.5\) Hz, 2H,
H2'/H6', Z), 7.15 (d, J = 8.5 Hz, 2H, H2'/H6', E), 7.42 (d, J = 8.5 Hz, 2H, H3''/H5'', Z), 7.52 (d, J = 8.5 Hz, 2H, H3''/H5'', E), 7.55 (s, 1H, H6, E), 7.63 (s, 1H, H4, Z), 7.76 (s, 1H, H6, Z).

$^{13}$C NMR (CDCl$_3$, 126 MHz) $\delta$ 44.0 (CH$_3$, Z), 44.6 (CH$_2$, E), 55.5* (OCH$_3$, E + Z), 104.0 (ArC), 104.8 (ArC), 114.3* (C2'/C6', E + Z), 114.4 (ArC), 115.9 (ArC), 116.3 (ArC), 119.5* (C2''/C6'', E), 122.2 (C2''/C6'', Z), 125.6 (C6, Z), 126.1 (ArC), 128.2 (C3'/C5', E+Z)*, 128.7, 129.3, 131.9 (C3'/C5'', Z), 132.6, 133.1* (C3''/C5'', E), 136.7, 141.1 (C4, Z), 141.8, 142.0 (C6, E), 147.3 (C1''', Z), 148.5 (C1''', E), 149.4 (C3, Z), 151.8 (C3, E), 157.7* (C4', E+Z), 158.2* (C4'', E+Z), 164.4 (C2, Z), 172.3 (C2, E).

Synthesis of (E and Z)-1-(4-Methoxybenzyl)-3-(2-bromophenylimino)-5,7-dibromoindolin-2-one (180)

The title compound (180) was synthesised from 5,7-dibromo-N-(4-methoxybenzyl)isatin (162) and 2-bromoaniline according to method B and purified by flash chromatography on silica gel (DCM) to give an orange solid (0.032 g, 16%, as a mixture of E and Z isomers (E:Z/48:52)), m.p. 167-170 °C, R$_f$ 0.70 (DCM). LREI-MS: m/z 576; 578; 580; 582 [M$^+$] 79Br$^{79}$Br$^{79}$Br; 79Br$^{79}$Br$^{81}$Br; 79Br$^{81}$Br$^{81}$Br; 81Br$^{81}$Br$^{81}$Br. HRESI-MS: calcd for C$_{22}$H$_{16}$Br$_3$N$_2$O$_2$ 79Br$^{79}$Br$^{79}$Br (M+H$^+$) 576.8762 found 576.8749. $^1$H NMR (CDCl$_3$, 500 MHz) $\delta$ 3.77 (s, 3H, OCH$_3$, Z), 3.79 (s, 3H, OCH$_3$, E), 5.24 (s, 2H, CH$_2$, Z), 5.29 (s, 2H, CH$_2$, E), 6.58 (s, 1H, H4, E), 6.82 (d, J = 8.5 Hz, 2H, H3'/H5', Z), 6.83 (d, J = 8.5 Hz, 2H, H3'/H5', E), 6.95 (t, J = 8.5 Hz, 1H, ArH), 7.07-7.10 (m, 2H, ArH), 7.14 (d, J = 8.5 Hz, 2H, H2'/H6', Z), 7.23 (d, J = 9 Hz, 2H, H2'/H6', E), 7.30-7.32 (m, 2H), 7.41 (t, J = 9 Hz, 1H), 7.61 (s, 1H, H6, E), 7.61 (s, 1H, H3''', Z), 7.61 (s, 1H, H3''', E), 7.73 (d, 1H, J = 2.0 Hz, H4, Z), 7.91 (s, 1H, J = 2.0 Hz, H6, Z). $^{13}$C NMR (126 MHz, CDCl$_3$) $\delta$ 40.0* (CH$_3$, E+Z), 51*1 (OCH$_3$, E + Z), 98.3, 99.0, 99.6, 101.7, 102.7, 103.8, 110.2, 110.4* (C3'/C5', E+Z), 112.0, 112.9, 113.5, 114.8, 123.5, 123.7, 123.9, 124.5, 124.7, 125.0, 125.4, 128.7 (C2'/C6', E+Z), 131.0, 132.4, 133.3, 133.9, 137.4*, 137.8, 143.8, 144.1, 153.7, 155.6, 156.8, 175.6, 176.0.

Synthesis of (E and Z)-1-(4-Methoxybenzyl)-3-(3-bromophenylimino)-5,7-dibromoindolin-2-one (181)

The title compound (181) was synthesised from 5,7-dibromo-N-(4-methoxybenzyl)isatin (162) and 3-bromoaniline according to method B and purified by flash chromatography on silica gel (DCM) to give an orange solid (0.22 mg, 80%, as a mixture of E and Z isomers (E:Z/ 46:54))
as, m.p. 154-157 °C, Rf 0.67 (DCM). LREI-MS: m/z 576; 578; 580; 582 [M+]* 79Br79Br79Br; 79Br
79Br81Br; 79Br81Br81Br. HRESI-MS: calcld for C22H16Br3N2O2 79Br79Br79Br (M+H+) 576.8643 found 576.8639. 1H NMR (CDCl3, 500 MHz) δ 3.77 (s, 3H, OCH3, Z), 3.79 (s, 3H, OCH3, E), 5.25 (s, 2H, CH2, Z), 5.43 (s, 2H, CH2, E), 6.77 (s, 1H, 4E), 6.85 (d, J = 8.5 Hz, 2H, H3'/H5', E), 6.79 (d, J = 8.5 Hz, 2H, H3'/H5', Z), 6.90 (d, J = 8.5 Hz, 2H, H6'', E), 6.99 (d, J = 8.0 Hz, 1H, H6''), 7.14-7.16 (m, 3H, ArH), 7.20-7.24 (m, 4H, H2'/H6'', E+Z), 7.30-7.33 (m, 1H, ArH), 7.42 (s, 1H, H2'', Z), 7.61 (s, 1H, 4H, Z), 7.70 (s, 1H, 6H, Z), 7.82 (s, 1H, 6E).

13C NMR (126 MHz, CDCl3): δ 44.0 (CH2, Z), 44.6 (CH2, E), 55.4 (CH3, E+Z), 103.8, 104.6, 114.1 (C3'/C5', E+Z), 114.2, 115.7, 115.8, 116.6, 118.1, 119.3, 120.4, 122.4, 122.7, 123.4, 125.5 (C6, E), 128.1 (C2'/C6', E+Z, C1', E+Z), 128.3 (C4, E), 128.9, 129.0, 129.9, 131.1, 139.0, 141.2 (C6, Z), 141.9 (C4, Z), 142.3 (C7a, Z), 149.4, 150.3, 150.6, 151.8, 157.8 (C2, Z), 158.6 (C4', E+Z), 163.5 (C2, E).

Synthesis of (E and Z)-1-(4-methoxybenzyl)-3-(2,4-dibromophenylimino)-5,7-
dibromoindol-2-one (182)
The title compound (182) was synthesised from 5,7-dibromo-N-(4-methoxybenzyl)isatin (162) and
2,4-dibromoaniline according to method B and purified by flash chromatography on silica gel (100% DCM) to give an orange powder (0.011 g, 14%, as a mixture of E and Z
isomers (E/Z:27:73)) as, m.p. 194-196 °C, Rf 0.81 (DCM).

LREI-MS: m/z 654; 656; 658; 660; 662 [M+] 79Br79Br79Br; 79Br79Br81Br; 81Br81Br81Br. HRESI-MS: calcld for C22H16Br3N2O2 (M+H+) 658.7867 found 658.7876. 1H NMR (CDCl3, 500 MHz) δ 3.79 (s, 3H, OCH3, Z), 3.81 (s, 3H, OCH3, E), 5.27 (s, 2H, CH2, Z), 5.49 (s, 2H, CH2, E), 6.73 (s, 1H, H4, E), 6.84-6.87 (m, 5H, H4'' Z, H5'' E+Z, H3'/H5' Z), 7.17 (d, J = 8.5 Hz, 2H, H2'/H6', Z), 7.24 (d, J = 8.0 Hz, 2H, H3'/H5', E), 7.44 (d, J = 8.0 Hz, 2H, H2'/H6'', E), 7.56 (d, J = 8.5 Hz, 1H, H4''), 7.67 (s, 1H, H6, E), 7.75 (s, 1H, H4, Z), 7.79 (s, 1H, H2'', Z), 7.89 (s, 1H, H2'', E), 7.92 (s, 1H, H6, Z). 13C NMR (CDCl3, 126 MHz) δ 43.2 (CH2, E+Z), 56.1 (OCH3, E + Z), 110.2, 110.4 (C3'/C5', E+Z), 112.0, 112.9, 113.5, 114.8, 123.5, 123.7, 123.9, 124.5, 124.7, 125.0, 125.4, 127.2, 128.7 (C2'/C6', E+Z), 131.0, 132.4, 133.3, 133.9, 134.6, 135.2, 137.4, 137.8, 140.2, 140.9, 143.8, 144.1, 153.7, 155.6, 156.8, 175.6, 176.0.

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Synthesis of (E and Z)-1-(4-methoxybenzyl)-3-(4-chlorophenylimino)-5,7-dibromoindolin-2-one (183)

The title compound (183) was synthesised from 5,7-dibromo-N-(4-methoxybenzyl)isatin (162) and p-chloroaniline according to method B and purified by flash chromatography on silica gel (DCM) to give an orange solid (0.15 g, 65% as a mixture of E and Z isomers (E/Z=48:52)), m.p. 179-181 °C, Rf 0.87 (DCM). LREI-MS: m/z 532; 534; 536 [M'] 79Br79Br; 79Br81Br; 81Br81Br. HRESI-MS: calcd for C_{32}H_{28}Br_2ClN_2O_2 79Br81Br (M+H') 532.9243 found 532.9267. 1H NMR (CDCl_3, 500 MHz) δ 3.77 (s, 3H, OCH_3), 3.79 (s, 3H, OCH_3, E), 5.26 (s, 2H, CH_2, Z), 5.44 (s, 2H, CH_2, E), 6.82 (d, J = 8.5 Hz, 2H, H3'/H5'), 6.85 (d, J = 8.5 Hz, 2H, H3'/H5'), 6.89 (s, 1H, H4, E), 6.94 (d, J = 8.5 Hz, 2H, H2''/H6''), 7.08 (d, J = 8.5 Hz, 2H, H3'/H5'), 7.16 (d, J = 7.5 Hz, 2H, H2''/H6''), 7.21 (d, J = 8 Hz, 2H, H2''/H6', E), 7.34 (d, J = 8.5 Hz, 2H, H3'/H5'), 7.45 (d, J = 8.5 Hz, 2H, H3'/H5'), 7.61 (s, 1H, H6, E), 7.69 (s, 1H, H4, Z), 7.83 (s, 1H, H6, Z). 13C NMR (CDCl_3, 126 MHz) δ 44.2 (CH_2, Z), 44.8 (CH_2, E), 55.7 (OCH_3, E + Z), 105.1 (ArC), 109.4 (ArC), 114.5 (C4'/C6', E + Z), 116.0 (ArC), 116.9 (ArC), 119.4 (C2''/C6'', E), 122.2 (C2''/C6'', Z), 125.8 (C6, Z), 126.4 (ArC), 127.8, 128.3 (C6, Z), 128.5, (C2'/C6', E+Z), 129.2 (C3''/C5'', Z), 130.4 (C3''/C5'', E), 132.7, 133.2, 137.4, 139.0, 141.3 (C4, Z), 142.2 (C6, E), 148.4 (C1'', Z), 149.2 (C1'', E), 149.9 (ArC), 152.7 (ArC), 158.7 (C4', E+Z), 160.0 (C4'', E+Z), 164.6 (C2, Z), 172.9 (C2, E).

Synthesis of (E and Z)-1-1-(4-methoxybenzyl)-3-(4-iodophenylimino)-5,7-dibromoindolin-2-one (184)

The title compound (184) was synthesised from 5,7-dibromo-N-(4-methoxybenzyl)isatin (162) and 4-iodoaniline according to method B and purified by flash chromatography on silica gel (DCM) to give an orange powder (0.21 g, 71% mixture of E and Z isomers (E/Z=48:52)), m.p. 192-195 °C, Rf 0.85 (DCM). LREI-MS: m/z 624; 626, 628 [M'] 79Br79Br; 79Br81Br; 81Br81Br. HRESI-MS: calcd for C_{32}H_{28}Br_2I_2N_2O_2 79Br81Br (M+H') 624.8528 found 624.8525. 1H NMR (CDCl_3, 500 MHz) δ 3.77 (s, 3H, OCH_3), 3.79 (s, 3H, OCH_3, E), 5.26 (s, 2H, CH_2, E), 5.43 (s, 2H, CH_2, Z), 6.76 (d, J = 8.5 Hz, 2H, H2''/H6''), 6.82 (d, J = 8.5 Hz, 2H, H3'/H5'), 6.83 (s, 1H, H4, E), 6.87 (d, J = 8.0 Hz, 2H, H2''/H6''), 7.08 (d, J = 8.5 Hz, 2H, H3'/H5'), 7.57 (d, J = 8.0 Hz, 2H, H2''/H6', E), 7.21 (d, J = 8.0 Hz, 2H, H2''/H6', Z), 7.62 (s,
1H, H4, Z), 7.68 (d, J = 8.5 Hz, 2H, H3''/H5''), E), 7.69 (s, 1H, 6, Z), 7.79 (d, J = 8.5 Hz, 2H, H3''/H5''), Z), 7.83 (s, 1H, H6, E). 11C NMR (CDCl3, 126 MHz): δ 43.5 (OCH3, E), 43.6 (OCH3, Z), 57.1* (CH3, E+Z), 90.0 (C4'', E), 90.2 (C4'', Z), 106.0 (ArC), 106.7 (ArC), 114.0 (C3'/C5', E), 114.1 (C2''/C6'', Z), 116.3 (ArC), 116.9 (ArC), 119.5* (C2''/C6'', Z), 122.0* (C3'/C5', Z), 125.4 (C6, E), 125.8 (ArC), 126.2 (ArC), 127.3* (C2'/C6', E), 127.6* (C2''/C6'', Z), 127.9* (C2''/C6'', E), 128.2 (C4, E), 137.6* (C3''/C5'', E), 138.7* (C3''/C5''), Z), 140.6 (C6, Z), 141.1 (C4, Z), 142.0 (C7a, E), 142.4 (C7a, Z) 148.2 (C3, E), 148.9 (C3, Z) 149.6 (C1'', E), 152.2 (C1'', Z), 159.3 (C2, E), 159.2* (C4'', E; C4'', Z), 164.2 (C2, Z).

Synthesis of (E and Z)-1-1-(4-methoxybenzyl)-3-(4-fluorophenylimino)-5,7-dibromoindolin-2-one (185)

The title compound (185) was synthesised from 5,7-dibromo-N-(4-methoxybenzyl)isatin (162) and 4-fluoroaniline according to method B and purified by flash chromatography on silica gel (100% DCM) to give an orange powder (0.17 g, 72% as a mixture of Z and E isomers (E/Z=48:52)), m.p. 165-167 °C. Rf 0.59 (DCM). LREI-MS: m/z 516; 518; 520 [M+]* 79Br79Br; 79Br81Br; 81Br81Br. HRESI-MS: calcd for C22H16N2O2FBr2 79Br79Br (M+H+) 516.9545 found 516.9563. 1H NMR (CDCl3, 500 MHz) δ 3.77 (s, 3H, OCH3, E), 3.79 (s, 3H, OCH3, Z), 5.26 (s, 2H, CH2, E), 5.44 (s, 2H, CH2, Z), 6.82 (d, J = 8.5 Hz, 2H, H4'/H6', E), 6.85 (d, J = 8.5 Hz, 2H, H3'/H5', Z), 6.89 (s, 1H, H4, Z), 6.98 (dd, J = 5, 7.5 Hz, 2H, H3''/H5''), Z), 7.08 (t, J = 9 Hz, 2H, H3''/H5''), E), 7.16 (d, J = 9 Hz, 4H, H2'/H6', E+Z), 7.21 (d, J = 8.5 Hz, 4H, H2''/H6'', E+Z), 7.61 (s, 1H, H6, Z), 7.69 (s, 1H, H4, E), 7.82 (s, 1H, H6, E). 13C NMR: (CDCl3, 126 MHz): δ 44.0 (CH3, Z), 44.6 (CH3, E), 55.5 (OCH3, E+Z)*, 103.8, 104.8, 114.3* (C3'/C5', E+Z), 116.8* (C3''/C5'', E), 117.1, 119.6* (C2'/C6', Z), 119.7* (C3''/C5''), Z), 123.3* (C2''/C6'', Z), 125.4 (C6, E), 126.0, 126.4, 127.7* (C4, Z, C2'/C6', E), 127.9* (C2''/C6'', E), 127.6*, 128.1, 129.6, 131.1, 132.0, 140.8 (C4, E), 142.4, 142.9, 143.6 (C6, Z), 144.3 (ArC), 149.3 (ArC), 152.2 (ArC), 157.9 (C4, Z), 159.3 (C4, E), 164.3 (C2), 165.2 (C2).

Synthesis of (E and Z)-1-(4-methoxybenzyl)-3-(4-(trifluoromethyl)-phenylimino)-5,7-dibromo-indolin-2-one (186)

The title compound (186) was synthesised from 5,7-dibromo-N-(4-methoxybenzyl)isatin (162) and p-(trifluoromethyl)aniline according to method B and purified by flash chromatography on silica gel (DCM) to give a red solid (0.27 g, 65%, as a mixture of Z and E isomers (E/Z=44:56)), m.p. 147-149 °C. Rf 0.73 (DCM). LREI-MS: m/z 566; 568; 570 [M]+ 79Br79Br; 79Br81Br; 81Br81Br. HRESI-MS: calcd for C27H18N2O2F3Br2 79Br81Br (M+H+) 566.9495 found 566.9531.
\(^1\)H NMR (CDCl\(_3\), 500 MHz) \(\delta\) 3.74 (s, 3H, OCH\(_3\), Z), 3.77 (s, 3H, OCH\(_3\), E), 5.22 (s, 2H, CH\(_2\), Z), 5.42 (s, 2H, CH\(_2\), E), 6.60 (s, 1H, H4, E), 6.79 (d, \(J = 8.5\) Hz, 2H, H3’/H5’, Z), 6.83 (d, \(J = 8.5\) Hz, 2H, H3’/H5’, E), 7.04-7.07 (m, 4H, H2”/H6” E + Z), 7.12 (d, \(J = 8.5\) Hz, 2H, H2’/H6’, Z), 7.20 (d, \(J = 8.0\) Hz, 2H, H2’/H6’, E), 7.59 (d, \(J = 8.5\) Hz, 2H, H3’/H5’), Z), 7.61 (s, 1H, H6, E), 7.71 (d, \(J = 8.5\) Hz, 2H, H3’/H5’, E), 7.73 (s, 1H, H4, Z), 7.82 (s, 1H, H6, Z). \(^{13}\)C NMR (CDCl\(_3\), 126 MHz): \(\delta\) 43.8 (CH\(_2\), Z), 44.2 (CH\(_2\), E), 55.5 (OCH\(_3\), Z+E)*, 104.0, 104.8, 114.4* (C3’/C5’, E+Z), 115.8, 116.4, 117.5 (C2”/C6” E+Z), 119.3 (C2’/C6’, E+Z), 125.6, 126.2*, 126.4, 127.6, 128.3* (C3”/C5” E+Z), 128.6,* 129.9, 130.3, 140.1, 141.3, 142.8 (C4, Z), 143.6 (C6, E), 144.3, 151.2 (C1’, Z), 151.9 (C2’, Z), 152.4* (C4’, E+Z), 157.8 (C4”’, Z), 159.2 (C4”’, E), 159.3 (C2, Z), 161.8 (C2, E).

Synthesis of (E and Z)-1-(4-Methoxybenzyl)-3-(4-methoxyphenylimino)-5,7-dibromoindoindolin-2-one (187)

The title compound (187) was synthesised from 5,7-dibromo-N-(4-methoxybenzyl)isatin (162) and 4-methoxyaniline according to method B and purified by flash chromatography on silica gel (DCM) to give an orange solid (0.092 g, 37%, as a mixture of Z and E isomers (E/Z/ 48:52)), m.p. 143-145 °C, R\(_t\) 0.39 (DCM). LREI-MS: m/z 528; 530, 532 [M+] \(^{79}\)Br\(^{79}\)Br, \(^{79}\)Br\(^{81}\)Br; \(^{81}\)Br\(^{81}\)Br. HRESI-MS: calcd for C\(_{23}\)H\(_{19}\)Br\(_2\)N\(_2\)O\(_3\) \(^9\)Br\(^{79}\)Br (M+H\(^+\)) 528.9734, found 528.9742. \(^1\)H NMR (CDCl\(_3\), 500 MHz) \(\delta\) 3.76 (s, 3H, OCH\(_3\), Z), 3.78 (s, 3H, OCH\(_3\), E), 3.84 (s, 3H, OCH\(_3\), Z), 3.88 (s, 3H, OCH\(_3\), E), 5.31 (s, 2H, CH\(_2\), Z), 5.44 (s, 2H, CH\(_2\), E), 6.81 (d, \(J = 8.0\) Hz, 2H, H3’/H5’, E), 6.84 (d, \(J = 8.5\) Hz, 2H, H3’/H5’, Z), 6.91 (d, \(J = 8.5\) Hz, 2H, H3’/H5’, E), 6.99 (d, \(J = 8.5\) Hz, 2H, H3’/H5’, Z), 7.03 (d, \(J = 8.5\) Hz, 2H, H2’/H6’ E), 7.16 (d, \(J = 8.5\) Hz, 2H, H2’/H6’, Z), 7.18 (s, 1H, H4, E), 7.21 (d, \(J = 8.5\) Hz, 2H, H2’/H6’, Z), 7.49 (d, \(J = 8.5\) Hz, 2H, H2’/H6’, E), 7.58 (s, 1H, H6, E), 7.63 (s, 1H, H4, Z), 7.82 (s, 1H, H6, Z). \(^{13}\)C NMR (126 MHz, CDCl\(_3\)): \(\delta\) 43.7 (CH\(_2\), Z), 44.3 (CH\(_2\), E), 55.5* (OCH\(_3\), Z), 55.6* (OCH\(_3\), E), 103.3, 104.3, 113.7* (C3”/C5” E), 114.7* (C3”/C5” E+Z), 115.1* (C3”/C5” Z), 119.8, 120.3 (C2”/C6” Z), 124.6 (C6, Z), 125.6 (C2”/C6” E), 127.3 (C1”, E), 127.8 (C1”, Z), 128.0* (C4, E, C2”/C6”, E+Z), 130.3, 135.1, 139.6 (C4, Z), 140.1 (C6, E), 141.9, 143.5, 150.5, 158.1, 158.6, 158.9 (C4”), 159.3 (C4””), 160.5 (C4””), 161.9 (C4”), 162.2 (C4”, Z), 163.4 (C4”’, E), 165.9 (C2), 168.1 (C2).
Synthesis of (E and Z)-5,7-dibromo-1-(4-methoxybenzyl)-3-(3-methoxyphenyl)imino)indolin-2-one (188)

The title compound (188) was synthesised from 5,7-dibromo-N-(4-methylbenzyl)isatin (162) and 4-methoxyaniline according to method B and purified by flash chromatography on silica gel (DCM) to give a dark red solid (0.029 g, 12%, mixture of E and Z isomers E:Z/39:61), m.p. 152-154 °C. Rf 0.43 (DCM). **LREI-MS:** m/z 528; 530, 532 [M+] 79Br79Br; 79Br81Br; 81Br81Br. **HRESI:** calcd for C23H19Br2N2O5; Br (M+H+) 528.8925, found 528.9272. **1H NMR** (CDCl3, 500 MHz) δ 3.65 (s, 6H, OCH3, Z), 3.78 (s, 6H, OCH3, E), 5.28 (s, 2H, CH2, Z), 5.46 (s, 2H, CH2, E), 6.58 (s, 1H, H4, E), 6.82 (d, J = 7.0 Hz, 2H, H3'/H5', Z), 6.82 (d, J = 7.0 Hz, 1H, H4**, Z), 6.95 (m, 3H, H6**, E, H3'/H5'', E), 7.02 (t, J = 8.0 Hz, 1H, H5**, Z), 7.15 (d, J = 7.5 Hz, 2H, H2'/H6'', Z), 7.23 (d, J = 7.5 Hz, 2H, H2'/H6', Z), 7.30-7.35 (m, 2H, H6'', Z, H4**), E), 7.41 (t, 1H, J = 7.5 Hz, H5'', E), 7.61 (s, 1H, H4, Z), 7.63 (s, 1H, H6, E), 7.00 (s, 1H, H2'', E), 7.12 (s, 1H, H6, Z), 7.92 (s, 1H, H2'', Z). **13C NMR** (126 MHz, CDCl3): δ 44.7 (CH2, Z), 44.9 (CH2, E), 55.8* (OCH3, E+Z), 56.1* (OCH3, E+Z), 104.8, 113.9* (C3'/C5'', E), 114.2, 114.9* (C3'/C5', E+Z) 116.0* (C3'/C5'', Z), 119.6, 120.0, 120.9* (C2''/C6'', Z), 125.0 (C6, Z), 125.9 (C2''/C6'', E), 127.6 (C1', E), 128.4 (C1', Z), 128.5* (C2''/C6', E+Z), 129.6 (C4, E), 140.2 (C4, Z), 140.9 (C6, E), 142.3, 144.8, 146.9, 150.9, 156.2, 157.8 (C4'), 158.8, 160.0 (C4''), 160.9 (C4'''), 161.8 (C4''), 163.2 (C4'''), 163.9 (C4'''), 166.7 (C2), 168.2 (C2).

Synthesis of (E and Z)-1-(4-methoxybenzyl)-3-(2,4-dimethylphenylimino)-5,7-dibromoindolin-2-one (189)

The title compound (189) was synthesised from 5,7-dibromo-N-(4-methoxybenzyl)isatin (162) and 2,4-dimethylaniline according to method B and purified by flash chromatography on silica gel (DCM) to give a red solid (0.084 g, 34%, as a mixture of Z and E isomers E:Z/67:33), m.p. 149-151 °C, Rr 0.54 (DCM). **LREI-MS:** m/z 526; 528, 530 [M+] 79Br79Br; 79Br81Br; 81Br81Br. **HRESI-MS:** calcd for C23H21Br2N2O5; Br (M+H+) 526. 9907 found 526. 9885. **1H NMR** (CDCl3, 500 MHz): δ 2.13 (s, 3H, H7'', E), 2.23 (s, 3H, H7'', Z), 2.32 (s, 3H, H8'', Z), 2.38 (s, 3H, H8'', E), 3.76 (s, 3H, OCH3, Z), 3.78 (s, 3H, OCH3, E), 5.27 (s, 2H, CH2, Z), 5.44 (s, 2H, CH2, E), 6.68 (d, J = 8.5 Hz, 1H, H6'', E), 6.78 (s, 1H, H4, E), 6.81 (d, J = 8.5 Hz, 2H, H3'/H5'', Z), 6.85 (d, J = 8.5 Hz, 2H, H3'/H5', Z), 6.90 (d, J = 8.5 Hz, 1H, H6'').
Experimental

Synthesis of (E and Z)-1-(4-methoxybenzyl)-3-(2,6-dimethylphenylimino)-5,7-dibromoindolin-2-one (190)

The title compound (190) was synthesised from 5,7-dibromo-N-(4-methoxybenzyl)isatin (162) and 2,6-dimethylaniline according to method B and purified by flash chromatography on silica gel (DCM) to give a dark red solid (0.15 g, 61%, as a mixture of Z and E isomers (E:Z=80:20)), m.p. 143-146 °C, Rf 0.54 (DCM). **LREI-MS:** m/z 526; 528, 530 [M⁺] 79Br³Br, 79Br²Br, 81Br³Br, 81Br²Br. **HRESI-MS:** calcd for C₃₂H₂₁Br₂N₂O₂: 79Br³Br (M+H⁺) 526.9970 found 526.9971. **¹H NMR** (CDCl₃, 500 MHz) δ 2.04 (s, 6H, 2 x CH₃, E), 2.05 (s, 6H, 2 x CH₃, Z), δ 3.79 (s, 3H, OCH₃, Z), 3.80 (s, 3H, OCH₃, E), 5.25 (s, 2H, CH₂, E), 5.45 (s, 2H, CH₂, Z), 6.46 (s, 1H, H4, Z), 6.87 (d, J = 8.5 Hz, 2H, C3'/C5', E), 6.91 (d, J = 8.5 Hz, 2H, C3'/C5', Z), 7.05 (d, J = 8.5 Hz, 2H, C3'/C5', Z), 7.08-7.11 (m, 5H, E + Z), 7.18 (d, 3H), 7.26 (s, J = 8.5 Hz, 2H, C2'/C6', Z), 7.63 (s, 1H, C6, Z), 7.63 (s, 1H, C4, E), 7.99 (s, 1H, C6, Z). **¹³C NMR** (CDCl₃, 126 MHz): 17.3 (2 x CH₃, Z)*, 17.4 (2 x CH₃, E)*, 44.1 (CH₂, E), 44.8 (CH₂, Z), 55.4 (OCH₃, E), 55.6 (OCH₃, Z), 107.5 (ArC, Z), 112.9, 114.1 (C3'/C5', E)*, 114.4 (C3'/C5', Z)*, 116.2, 116.5, 116.9, 119.0, 120.3, 123.9 (C3'/C5', Z), 124.3 (ArC, E), 125.3 (C6, Z), 126.3, 127.1 (C4, Z), 128.0 (C2'/C6', Z)*, 128.1 (C1', Z), 128.9 (C3'/C5'), 129.0 (C1'), 130.4, 131.5, 136.2,* 138.1, 138.0,* 140.3 (C3, Z), 141.0 (C4, E), 141.6 (C6, Z), 142.6 (C7a, Z), 146.1, 151.4, 158.3 (C4', Z), 159.1 (C4', E), 164.0 (C2, Z), 164.5 (C2, Z).
Synthesis of (E and Z)-1-(4-methoxybenzyl)-3-(4-nitrophenylimino)-5,7-dibromoindolin-2-one (191)

The title compound (191) was synthesised from 5,7-dibromo-N-(4-methoxybenzyl)isatin (162) and 4-nitroaniline according to method B and purified by flash chromatography on silica gel (100% DCM, DCM/EtOAc 4:1) to give an orange/red solid (0.038 g, 3%, as a mixture of Z and E isomers (E:Z 27:73)), Rf 0.37 (DCM/EtOAc 5:1).

LREI-MS: m/z 543; 545; 547 [M⁺] 79Br⁺; 79Br⁺; 81Br⁺. HRESI-MS: calcd for C_{22}H_{16}Br_{3}O_{4} 543.9961 found 543.9997. ¹H NMR (CDCl₃, 500 MHz) δ 3.77 (s, 3H, OCH₃), 3.79 (s, 3H, OCH₃, Z), 5.23 (s, 2H, CH₂, Z), 5.65 (s, 1H, H4, E), 6.38 (d, J = 7.5 Hz, 2H, H3'/H5', Z), 6.87 (d, J = 8.0 Hz, 2H, H3'/H5', E), 7.04 (d, J = 8.0 Hz, 2H, H2''/H6''), 7.10 (d, J = 8.0 Hz, 2H, H2''/H6''), 7.15 (d, J = 8.5 Hz, 2H, H2'/H6', Z), 7.23 (d, J = 8.5 Hz, 2H, H2''/H6''), 7.53 (s, 1H, H6, E), 7.76 (s, 1H, H4, Z), 7.85 (s, 1H, H6, Z), 8.25 (d, J = 8.0 Hz, 2H, H3''/H5''), 8.37 (d, J = 8.0 Hz, 2H, H3''/H5''), 13C NMR (CDCl₃, 126 MHz) δ 43.2 (CH₂, Z), 43.9 (CH₂, E), 55.8* (OCH₃, E + Z), 106.9, 110.3, 116.0* (C4'/C6', E + Z), 116.9, 119.7* (C2''/C6''), 120.3, 122.3 (C2'/C6'), 124.5, 125.7 (C6, Z), 126.5, 128.5 (C6, Z), 128.7* (C2'/C6', E+Z), 129.6 (C3''/C5''), 130.2, 130.5 (C3''/C5''), 133.2, 137.3, 139.2, 141.9 (C4, Z), 143.2 (C6, E), 148.6 (C1''', Z), 149.5 (C1''', E), 150.2, 152.8, 158.6* (C4', E+Z), 160.1* (C4'', E+Z), 164.7 (C2, Z), 172.0 (C2, E).

Synthesis of (E and Z)-1-(4-methoxybenzyl)-3-(4-phenoxyphenylimino)-5,7-dibromoindolin-2-one (192)

The title compound (192) was synthesised from 5,7-dibromo-N-(4-methoxybenzyl)isatin (162) and 4-phenoxyaniline to method B and purified by flash chromatography on silica gel (DCM) to give a dark red solid (0.19 g, 70%, as a mixture of Z and E isoemrs (E:Z 48:52)), m.p. 209-211 °C, Rf 0.83 (DCM). LREI-MS: m/z 590; 592; 594 [M⁺] 79Br⁺; 79Br⁺; 81Br⁺; 81Br⁺. HRESI-MS: calcd for C_{30}H_{23}Br_{3}O_{5} (M+H⁺) 592.9820 found 592.9813. ¹H NMR (CDCl₃, 500 MHz) δ 3.74 (s, 3H, OCH₃), 3.76 (s, 3H, OCH₃), 5.30 (s, 2H, CH₂, Z), 5.45 (s, 2H, CH₂, E), 6.82-6.84 (m, 4H, H3'/H5', E+Z), 6.84-6.87 (m, 5H, H4, E; H2''/H6''; H2'''/H6''), 6.97-6.99 (m, 4H, H2''/H6'', H3''/H5''), 7.02-7.05 (m, 4H,
H2''/H6''; H2'/H6'), 7.22 (d, J = 8.5 Hz, 2H, H2'/H6'), 7.34-7.37 (m, 6H, H3''/H5''', E+Z, H4'''), 7.61 (s, 1H, H4, Z), 7.67 (s, 1H, H6, Z), 7.84 (s, 1H, H6, E). 13C NMR (CDCl3, 126 MHz): δ 43.7 (CH2, Z), 44.3 (CH2, E), 55.2 (OCH3, E+Z), 103.4, 104.2, 114.1* (C3'/C5', E+Z), 116.3, 118.1, 118.7, 119.4, 119.6, 120.1, 123.5, 123.7, 124.4, 124.8 (C6, E), 126.7* (C1', E+Z), 126.9* (C2'/C6', E+Z), 127.8, 128.3, 128.4, 129.3, 129.6*, 129.8*, 132.1, 132.8, 136.7, 138.5, 140.0 (C6, Z), 141.2 (C4, Z), 141.3 (ArC), 142.1 (ArC), 143.6 (ArC), 144.6 (ArC), 147.8 (ArC), 151.6 (ArC), 155.6 (C4''), 156.5 (C4''), 157.2* (C1''', E+Z), 157.8 (C2, Z), 158.9* (C4', E+Z), 159.1, 160.4, 163.7 (C2, E).

**Synthesis of (E and Z)-1-(4-methylbenzyl)-3 -(4-methoxyphenylimino)-5,7-dibromoindol-2-one (193)**

The title compound (193) was synthesised from 5,7-dibromo-N-(4-methylbenzyl)isatin (163) and 4-methoxyaniline according to method B and purified by flash chromatography on silica gel (DCM) to give a dark red solid (0.021 g, 35%, as a mixture of Z and E isomers (E:Z=48:52)), m.p. 137-139 °C, Rf 0.43 (DCM). LREI-MS: m/z 512; 514; 516 [M+] 79Br79Br; 79Br81Br; 81Br81Br. HRESI-MS: calced for C23H19Br3N2O2

**Synthesis of (E and Z)-1-(4-methylbenzyl)-3 -(3-bromophenylimino)-5,7-dibromoindol-2-one (194)**

The title compound (194) was synthesised from 5,7-dibromo-N-(4-methylbenzyl)isatin (163) and 3-bromoaniline according to method B and purified by flash chromatography on silica gel (DCM) to give an orange powder.
(0.090 g, 66%, as a mixture of Z and E isomers (E:Z/48:52)), m.p. 140-144 °C, Rf 0.83 (DCM).
LREI-MS: m/z 562; 564; 566 [M+] 79Br81Br, 79Br81Br, 79BrBr, 78Br81Br, 78BrBr81Br, 81Br81Br, 81BrBr81Br.
HRESI-MS: calcd for C23H16Br2N2O 79Br79Br (M+H') 562.8813 found 562.8808. 1H NMR (CDCl3, 500 MHz) δ 2.29 (s, 3H, CH3), 2.32 (s, 3H, CH3), 2.57 (s, 2H, CH2, E), 2.67 (s, 1H, H4, E), 6.91 (d, J = 8.5 Hz, 1H, H4', Z), 6.98 (d, J = 8.5 Hz, 1H, H4'', E), 7.13 (s, 4H, H2'/H3'/H5'/H6', Z), 7.16 (s, 4H, H2'/H3'/H5'/H6', E), 7.19 (bd, 1H, H6'', E), 7.21 (s, 1H, H2'', Z), 7.24 (t, J = 6.5 Hz, 1H, H5'', Z), 7.33-7.35 (m, 2H, H5'', E, H6'', Z), 7.41 (s, 1H, H2'', E), 7.59 (s, 1H, H4, Z), 7.61 (s, 1H, H6, E), 7.80 (s, 1H, H6, Z). 13C NMR (CDCl3, 126 MHz): δ 22.4 (CH3, E+Z), 44.3 (CH2, Z), 45.4 (CH2, E), 104.1, 104.8, 115.9, 116.1 (C4'', Z), 116.7, 118.4 (C4'', E), 120.6 (C6', Z), 122.6 (Arc), 122.9 (C4'', E), 124.3, 125.7 (C6, Z), 126.7 (C2'/C6', E+Z)*, 128.4 (C4, E), 129.1* (C2', E+Z), 129.2* (C6'', E+Z), 129.7* (C3'/C5', E+Z), 130.1 (C2', E), 131.3 (C5'', Z), 133.4* (C4', E+Z), 137.5* (C1', E+Z), 141.4 (C6, E), 142.1 (C4, E), 143.5 (C7a, E), 144.2 (C7a, Z), 149.7 (C1'', Z), 150.3 (Arc), 150.4 (C1'', E), 151.2, 157.8 (C2, E), 163.8 (C2, Z).

Synthesis of (E and Z)-1-(4-methylbenzyl)-3-(4-trifluoromethylphenylimino)-5,7-dibromomoiindol-2-one (195)

The title compound (195) was synthesised from 5,7-dibromo-N-(4-methylbenzyl)isatin (163) and 4-(trifluoromethyl)aniline according to method B and purified by flash chromatography on silica gel (DCM) to give a dark red solid (0.0012 g, 19%, as a mixture of Z and E isomers (E:Z/48:52)), m.p. 131-135 °C, Rf 0.87 (DCM). LREI-MS: m/z 550; 552; 554 [M+] 79Br81Br, 79Br81Br, 78Br81Br, 81Br81Br. HRESI-MS: calcd for C23H16Br2F2N2O 79Br79Br (M+H') 550.9577 found 550.9581. 1H NMR (CDCl3, 500 MHz) δ 2.30 (s, 3H, CH3, Z), 2.33 (s, 3H, CH3, E), 5.26 (s, 2H, CH2, Z), 5.45 (s, 2H, CH2, E), 6.62 (s, 1H, H4, E), 7.07-7.10 (m, 8H, H2'/H3'/H5'/H6', E, Z), 7.15-7.18 (m, 4H, H2''/H6'', E+Z), 7.60 (s, 1H, H6, E), 7.61 (d, J = 8.0 Hz, 2H, H3''/H5'', Z), 7.71 (s, 1H, H4, Z), 7.72 (d, J = 8.0 Hz, 2H, H3''/H5'', E), 7.84 (s, 1H, H6, Z). 13C NMR (CDCl3, 126 MHz): δ 21.3 (CH3, E+Z), 44.4 (CH2, E), 44.9 (CH2, Z), 108.3, 108.9, 114.7, 116.8, 117.6, 119.3, 125.4, 125.9 (C6, Z), 126.2 (C3''/C5'', Z), 126.3, 126.7 (C2'/C6', E+Z), 126.8 (C2''/C6'', E+Z), 127.3 (C4, Z), 127.4 (C3''/C5'', E), 128.0, 128.3 (C4, E), 129.0, 129.6, 129.7 (C3'/C5', E+Z), 131.0, 133.3, 137.6, 141.6, 142.2, 146.2, 151.6, 151.9, 158.7 (C1'', E), 159.1 (C1'', Z), 161.4 (C2, Z), 162.3 (C2, E).
Synthesis of (E and Z)-1-((4-chlorobenzyl)-3-(3-bromophenylimino)-5,7-dibromoindol-2-one (196)

The title compound (196) was synthesised from 5,7-dibromo-N-(4-chlorobenzyl)isatin (164) and 3-bromoaniline according to method B and purified by flash chromatography on silica gel (DCM) to give an orange powder (0.015 g, 23%), as a mixture of Z and E isomers (E:Z/44:56), m.p. 127-129 °C, Rf 0.49 (DCM/Hex 5:2). LREI-MS: m/z 580; 582; 584; 586 [M+H+] 79Br79Br79Br; 79Br79Br81Br; 79Br81Br81Br; 81Br81Br81Br. HRESI-MS: calcd for C21H15Br2Cl2N2O2 79Br79Br79Br (M+H+) 580.8266 found 580.8259. 1H NMR (CDCl3, 500 MHz) δ 5.28 (s, 2H, CH2), 5.46 (s, 2H, CH2, Z), 6.92 (d, J = 8.5 Hz, 1H, H6′), 6.97 (d, J = 8.5 Hz, 1H, H6′′, Z), 7.01-7.02 (m, 3H), 7.15 (d, J = 8.5 Hz, 1H, Z), 7.23-7.25 (m, 3H, ArH), 7.28-7.30 (m, 2H, ArH), 7.35-7.37 (m, 4H, ArH), 7.45 (d, J = 8.0 Hz, 1H), 7.62 (s, 1H, H6, E), 7.71 (s, 1H, H4, Z), 7.84 (s, 1H, H6, Z).

Synthesis of (E and Z)-1-((4-Chlorobenzyl)-3-(4-methoxyphenylimino)-5,7-dibromoindol-2-one (197)

The title compound (197) was synthesised from 5,7-dibromo-N-(4-chlorobenzyl)isatin (164) and 4-methoxyaniline according to method B and purified by flash chromatography on silica gel (DCM) to give a dark red solid (0.018 g, 30%), as mixture of E and Z isomers (E:Z/48:52), Rf 0.72 (DCM/Hex 2:1). LREI-MS: m/z 532; 534; 536 [M+H+] 79Br79Br; 79Br81Br; 81Br81Br. HRESI-MS: calcd for C22H16Br2Cl2N2O2 79Br79Br (M+H+) 532.9267 found 532.9244. 1H NMR (CDCl3, 500 MHz) δ 3.79 (s, 3H, OCH3, E), 3.86 (s, 3H, OCH3, Z), 5.37 (s, 2H, CH2, E), 5.43 (s, 2H, CH2, Z), 6.90 (d, J = 1.5 Hz, 1H, H4, E), 6.97 (d, J = 8.5 Hz, 2H, H3′′/H5′′, E), 7.07 (d, J = 8.5 Hz, 2H, H3′′/H5′′, Z), 7.19 (d, J = 8.5 Hz, 2H, H2′′/H6′′, E), 7.23 (d, J = 8.5 Hz, 2H), 7.32-7.36 (m, 6H), 7.54 (d, J = 8.5 Hz, 2H, H2′′/H6′′, Z), 7.59 (s, 1H, H6, E), 7.64 (s, 1H, H4, Z), 7.79 (s, 1H, H6, E). 13C NMR (CDCl3, 126 MHz): δ 44.0, 44.5, 55.7, 55.7, 103.4, 104.2, 113.9, 115.0, 115.9, 116.4, 119.9, 120.6, 125.0, 126.1, 127.5, 128.2, 128.3, 129.1, 130.2, 130.9, 132.4, 132.9, 133.5*, 133.7, 135.3, 135.4*, 139.7, 140.1, 140.6, 141.2, 141.9, 142.8, 144.1, 146.5, 150.3, 151.2, 158.3, 158.9, 160.4, 161.3, 162.8, 164.1.
Synthesis of (E and Z)-1-(4-Chlorobenzyl)-3-(4-iodophenylimino)-5,7-dibromoindol-2-one (198)

The title compound (198) was synthesised from 5,7-dibromo-N-(4-chlorobenzyl)isatin (164) and 4-chloroaniline according to method B and purified by flash chromatography on silica gel (DCM) to give an orange solid (0.12 g, 81% as a mixture of E and Z isomers (E:Z/42:58)), m.p. 180-182 °C, Rf 0.72 (DCM/Hex 1:1). LREI-MS: m/z 628; 630; 632 [M+] 79Br79Br; 79Br81Br; 81Br81Br. HRESI-MS: calcd for C31H13Br2ClIN2O 79Br79Br (M+H+)^+ 628.8128 found 628.8102. 1H NMR (CDCl3, 500 MHz): δ 5.23 (s, 2H, CH2, E), 5.43 (s, 2H, CH2, Z), 6.75 (d, J = 8.5 Hz, 2H, H3'/H5', E), 6.86 (d, J = 8.5 Hz, 2H, H3'/H5', Z), 6.89 (s, 1H, H4, E), 7.14 (d, J = 8.5 Hz, 2H, H2'/H6', Z), 7.18 (d, J = 8.5 Hz, 2H, H2'/H6', E), 7.25 (d, J = 8.5 Hz, 2H, H3'/H5', Z), 7.29 (d, J = 8.5 Hz, 2H, H2'/H6', Z), 7.60 (s, 1H, 6 E), 7.68 (d, J = 8.5 Hz, 2H, H3''/H5'', Z), 7.69 (s, 1H, H4, Z), 7.77 (d, J = 8.5 Hz, 2H, H3''/H5'', E), 7.83 (s, 1H, 6 Z). 13C NMR: (CDCl3, 126 MHz): δ 44.0 (CH2, E), 44.6 (CH2, Z), 90.4 (C4''', E), 91.6 (C4''', Z), 104.3 (ArC, E), 104.7 (ArC, Z), 116.1 (ArC, E), 116.9 (ArC, Z), 119.7* (C2'''/C6''', E), 122.3* (C2'''/C6''', Z), 125.8 (C6, Z), 126.4 (C6, E), 128.2* (C2''/C6' E; C2''/C6', Z; C4, E), 129.1* (C3''/C5', E; C3''/C5', Z), 130.1, 130.9, 132.4, 133.7* (C4', E+Z), 135.1* (C1', E+Z), 137.9 (C3''/C5'', Z), 138.4, 139.1 (C3''/C5'', E), 140.2, 141.1 (C4, Z), 141.9 (C6, E), 143.5 (C3, E), 147.6 (C1'', E), 148.6 (C1'', Z), 157.7 (C2, Z), 163.6 (C2, E).

Synthesis of (E and Z)-5,7-dibromo-1-(4-iodobenzyl)-3-(4-methoxyphenylimino)indolin-2-one (199)

The title compound (199) was synthesised from 5,7-dibromo-N-(4-iodobenzyl)isatin (165) and 4-methoxyaniline according to method B and purified by flash chromatography on silica gel (DCM) to give a dark red solid (0.011g, 28% as a mixture of E and Z isomer (E:Z 48:52)), Rf 0.55 (DCM). LREI-MS: m/z 624; 626; 628 [M+]

79Br79Br; 79Br81Br; 81Br81Br. HRESI-MS: calcd for C22H16Br2IN2O2 79Br79B (M+H+)^+ 624.8623 found 624.8607. 1H NMR (CDCl3, 500 MHz): δ 3.84 (s, 3H, OCH3, Z), 3.89 (s, 3H, OCH3, E), 5.29 (s, 2H, CH2, Z), 5.43 (s, 2H, CH2, E), 6.75 (s, 1H, H4, E), 6.90-6.93 (m, 4H, H3''/H5''; E+Z), 6.97 (d, J = 8.5 Hz, 2H, H2'/H6', Z), 7.05 (d, J = 8.5 Hz, 2H, H2'/H6', E), 7.22-7.25 (m, 4H, H2'/H6', E+Z), 7.52 (d, J = 7.5 Hz, 2H, H3'/H5', E), 7.58 (s, 1H, H6, E), 7.62 (d, J = 7.5 Hz, 2H, H3'/H5', Z), 7.65 (s, 1H, H4, Z), 7.84 (s, 1H, H6, Z).
Synthesis of (E and Z)-2-(3-((5,7-dibromo-1-(4-iodobenzyl)-2-oxoindolin-3-ylidene)amino)phenyl)acetic acid (200)

The title compound (200) was synthesised from 5,7-dibromo-N-(4-iodobenzyl)isatin (165) and (3-aminophenyl)acetic acid according to method B and purified by flash chromatography on silica gel (DCM, DCM:EtOAc 7:1) to give a dark red solid (0.042 g, 22%, mixture of E and Z isomers (E:Z/64:36)), m.p. 182-186 °C, Rf 0.49 (DCM). LREI-MS: m/z 652; 654; 656 [M+] 79Br2Br; 79Br81Br; 81Br81Br. HRESI-MS: calcd for C27H16N2O2Br2I 79Br81Br (M+H+) 652.9451 found 652.9509. 1H NMR (DMSO-d6, 500 MHz) δ 3.62 (s, 2H, CH3, Z), 3.65 (s, 2H, CH2, E), 5.25 (s, 2H, CH2, Z), 5.43 (s, 2H, CH2, E), 6.81 (d, J = 1.5 Hz, 1H, H4'), E), 6.89 (d, J = 8.0 Hz, 1H, H4', E), 6.93 (s, 1H, H2', E), 6.95 (d, J = 8.0 Hz, 1H, H4', Z), 7.02 (m, 3H, H2'''/H6''), E, H6', E) 7.08 (s, 1H, H2', Z), 7.15 (d, J = 8.0 Hz, 2H, H2'''/H6''), (Z), 7.21 (d, J = 7.5 Hz, 2H, H3'''/H5''', Z), 7.33 (t, J = 8.0 Hz, 1H, H5', Z), 7.43 (t, J = 7.5 Hz, 1H, H5', E), 7.59 (d, J = 2.0 Hz, 1H, H6'', E), 7.61 (d, J = 8.5 Hz, 2H, H3'''/H5''', E), 7.67 (d, J = 8.5 Hz, 1H, H6'', Z), 7.69 (d, J = 2.0 Hz, 1H, H4'', Z), 7.85 (d, J = 2.0 Hz, 1H, H6'', Z). 13C NMR (DMSO-d6, 126 MHz): δ 41.1 (CH3, E), 41.4 (CH3, Z), 44.1 (CH2, Z), 44.5 (CH2, E), 101.7 (C4'', E), 102.3 (C4'''', Z), 104.1, 104.7, 114.6, 114.6, 114.8, 116.0, 118.3, 120.7, 120.9, 122.0 (C2, E), 124.5, 125.2, 126.6, 127.5, 128.1 (C2''/C6''', E), 128.2 (C2''/C6''', Z), 129.3 (C2, Z), 129.4, 130.8 (C3'''/C5''', E), 132.1 (C3'''/C5''', Z), 140.8, 141.0, 143.1 (C7a'', Z), 144.5 (C7a'', E), 149.1, 150.4, 151.2, 153.0 (C3, E), 158.1 (C2'', Z), 159.0 (C4'', E +Z), 163.6 (C2'', E), 167.5 (C3'', Z), 168.0 (C3'', E), 169.8 (COOH, Z), 170.3 (COOH, E).

Synthesis of (E and Z)-1-(4-iodobenzyl)-3-(3-bromophenylimino)-5,7-dibromoindol-2-one (201)

The title compound (201) was synthesised from 1-(4-iodobenzyl)-5,7-dibromoisanilin (165) and 3-bromanilin according to method B and purified by flash chromatography on silica gel (DCM) to give a red solid (0.069 g, 54% as mixture of E and Z isomers (E:Z/42:58)), Rf 0.87 (DCM). LREI-MS: m/z 672; 674; 676; 678 [M+] 79Br2Br79Br; 79Br81Br; 81Br81Br. HRESI-MS: calcd for C23H19BrIN2O 79Br2Br79Br (M+H+) 672.7623 found 672.7584. 1H NMR (CDCl3, 500 MHz) δ 5.25 (s, 2H, CH3, Z), 5.43 (s, 2H, CH2, E), 6.81 (s, 1H, H4, E), 6.92 (d, J = 8.0 Hz, 1H, H6'', E), 6.97 (d, J = 8.0 Hz, 2H, H2'/H6', Z), 7.00-7.03 (m, 2H, H2'/H6', E), 7.18 (s, 1H, H2'', E), 7.21-7.24 (m, 2H, ArH), 7.31-7.33 (m, 2H, H2'', Z, H5'', E), 7.42-7.44 (m, 2H, ArH), 7.61-7.62
(m, 3H, H3’/H5’, H4, Z), 7.65 (d, J = 8.5 Hz, 2H, H3’/H5’), 7.71 (s, 1H, H6, Z), 7.84 (s, 1H, H6, E). \(^{13}\)C NMR (126 MHz, CDCl_3): 44.2 (CH_2, E), 44.8 (CH_2, Z), 93.2* (C4’, E+Z), 103.9, 104.7, 116.0, 116.2, 117.0, 118.4, 119.5, 120.6, 122.6, 122.9, 123.7, 125.8 (C6, E), 128.5 (C4, E), 128.7* (C2’/C6’, E+Z), 129.2, 129.3, 130.2 (C5’, Z), 131.1, 131.4, 136.3 (C1’, E+Z), 138.1 (C3’/C5’, E+Z), 141.4, 142.0, 142.1, 143.8, 149.4, 150.2 (C1’’, Z), 150.7 (C1’’, E), 152.2, 157.5 (C2, E), 163.5 (C2, Z).

**Synthesis of (E and Z)-1-(4-iodobenzyl)-3-(4-chlorophenylimino)-5,7-dibromoindol-2-one (202)**

The title compound (202) was synthesised from 5,7-dibromo-N-(4-iodobenzyl)isatin (165) and 4-chloroaniline according to method B and purified by flash chromatography on silica gel (DCM/Hex 7:1; 3:1) to give an orange solid (0.027 g, 15% mixture of E and Z isomers (E/Z:44:56)), Rf 0.72 (DCM/Hex 5:2). LREI-MS: m/z 628; 630; 632 [M+]^{79}Br^{79}Br; \(^{79}Br^{81}Br; \(^{81}Br^{81}Br. HRESI-MS: calcd for C_{21}H_{13}Br_{2}ClIN_{2}O \(^{79}Br^{79}Br (M+H^+)\) 628.8128 found 628.8124. \(^1\)H NMR (CDCl_3, 500 MHz) \(\delta\) 5.32 (s, 2H, CH_2, Z), 5.42 (s, 2H, CH_2, E), 6.87 (s, 1H, H4, E), 6.95-6.99 (m 4H, H2’/H6’’, E, H2’/H6’, E), 7.01 (d, J = 8.5 Hz, 2H, H2’/H6’’, Z), 7.08 (d, J = 8.5 Hz, 2H, H2’/H6’, Z), 7.32 (d, J = 8.5 Hz, 2H, H3’/H5’), 7.42 (d, J = 8.5 Hz, 2H, H3’/H5’, Z), 7.60 (d, J = 9 Hz, 2H, H3’/H5’, E), 7.62 (s, 1H, H6, E), 7.65 (d, J = 8.5 Hz, 2H, H3’/H5’, Z), 7.67 (s, 1H, H4, Z), 7.82 (s, 1H, H6, Z). \(^{13}\)C NMR (CDCl_3, 126 MHz): \(\delta\) 45.4 (CH_2, E), 45.7 (CH_2, Z), 92.3 (C4’, E), 93.2 (C4’, Z), 104.5, 104.9, 116.5, 117.0, 120.8* (C2’/C6’’, E), 122.5* (C2’/C6’’, Z), 126.3 (C6, Z), 127.6* (C2’/C6’ E+Z), 128.2 (C4, E), 129.1* (C3’/C5’, E+ Z), 130.3, 130.8, 132.4, 133.7 (C4’, E), 134.5 (C4’, Z), 135.1 (C1’, E), 136.2 (C1’, Z), 137.7* (C3’/C5’, Z), 138.8, 139.2* (C3’/C5’, E), 140.3, 141.4 (C4, Z), 141.6 (C6, E), 144.0 (C3, E), 147.5 (C1’’, E), 148.9 (C1’’, Z), 160.3 (C2, Z), 163.6 (C2, E).

**Synthesis of (E and Z)-1-(4-iodobenzyl)-3-(4-fluorophenylimino)-5,7-dibromoindol-2-one (203)**

The title compound (203) was synthesised from 5,7-dibromo-N-(4-iodobenzyl)isatin (165) and 4-fluoroaniline according to method B and purified by flash chromatography on silica gel (DCM/Hex 7:1; 3:1) to give an orange powder (0.073 g, 41%, as a mixture of E and Z isomers (E/Z:42:58)), Rf 0.49 (DCM). LREI-MS: m/z 612; 614; 616 [M+]^{79}Br^{79}Br; \(^{79}Br^{81}Br; \(^{81}Br^{81}Br. HRESI-MS: calcd for C_{21}H_{13}Br_{2}FIN_{2}O \(^{79}Br^{81}Br (M+H^+)\) 612.8423 found 612.8392.
**Synthesis of E-3-(4-hydroxyphenyl)imino)-N-(4-methoxybenzyl)isatin (204)**

The title compound (204) was synthesised from 4-aminophenol (169) and 1-(4-methoxybenzyl)isatin according to the method A and purified by flash chromatography on silica gel (DCM/EtOAc 5:1) to give a red solid (0.044 g, 65%), m.p. 207-209 °C, Rᵣ 0.57 (DCM/EtOAc 5:1). **LREI-MS:** m/z 358.39 [M⁺]. **HRESI-MS:** C₁₂H₁₉N₂O₃ (M+H⁺) 359.1396 found 359.1389. **¹H NMR** (CDCl₃, 500 MHz) δ 3.78 (s, 3H, OCH₃), 4.95 (s, 4H, CH₂), 6.73-6.78 (m, 4H, H₃''/H₅'', H₃'''/H₅'''), 6.84-6.87 (m, 4H, H₂''/H₆'', H₂'''/H₆'''). 6.91 (d, J = 9 Hz, 1H, H₄), 6.97 (t, J = 9 Hz, 1H, H₅), 7.24 (t, J = 9 Hz, 1H, H₆), 7.31 (d, J = 9 Hz, 1H, H₇). **¹³C NMR** (CDCl₃, 126 MHz): δ 34.9 (CH₂), 55.2 (OCH₃), 110.6 (C7), 114.5 (C3a), 114.6* (C3''/C5''), 116.5* (C3'''/C5'''), 120.7* (C2''/C6'''), 122.9 (C5), 122.0 (C6), 127.5 (C1'), 129.2* (C2'/C6'), 130.1 (C4), 143.1 (C1''), 147.3 (C7a), 154.0 (C3), 154.4 (C4''), 159.6 (C4'), 163.9 (C2). **Synthesis of (E and Z)-5,7-dibromo-3-(4-hydroxyphenyl)imino)indolin-2-one (205)**

The title compound (205) was synthesised from 5,7-dibromoindisatin (76) and 4-methoxyaniline according to method B and purified by flash chromatography on silica gel (DCM) to give a dark yellow powder (0.20 g, 63%), mixture of E and Z isomers (E/Z=64:36), m.p. 229-231 °C, Rᵣ 0.46 (DCM/EtOAc 4:1). **LREI-MS:** m/z 394; 396; 398 [M⁺]. **Br²⁷Br, Br²¹Br. HRESI-MS:** caled for C₁₄H₁₇Br₂N₂O₂ 79Br²⁷Br (M+H⁺) 394.9089 found 394.9104. **¹H NMR** (DMSO-d₆, 500 MHz) δ 6.72 (d, 2H, J = 9 Hz, H₃''/H₅'', Z), 6.86 (d, J = 1.5 Hz,1H, H₄, E), 6.89 (d, 2H, J = 8.5 Hz, H₃''/H₅'', E), 6.92 (d, 2H, J = 8.5 Hz, H₂''/H₆'', E), 7.36 (d, 2H, J = 8.5 Hz, H₂''/H₆'', Z), 7.58 (d, J = 2.0 Hz, 1H, 4Z), 7.74 (d, J = 2.0 Hz, H₆, Z), 7.81 (d, J = 1.5 Hz, H₆, E). **¹³C NMR** (126 MHz, DMSO): δ 104.7, 105.7, 113.8* (C3''/C5'').
Synthesis of (E and Z)-5,7-dibromo-3-((4-methoxyphenyl)imino)indolin-2-one (206)

The title compound (206) was synthesised from 5,7-dibromoisatin (76) and 4-methoxyaniline according to method B and purified by flash chromatography on silica gel (DCM) to give a dark yellow powder (0.174 g, 65%, mixture of E and Z isomers (E:Z/65:35)), m.p. 277-279 °C, Rf 0.53 (DCM/ EtOAc 4:1) LREI-MS: m/z 408; 410; 412 [M+] 79Br79Br; 79Br81Br; 81Br81Br. HRESI-MS: calcd for C15H11Br2N2O2 79Br79Br (M+H+) 408.9089 found 408.9104. 1H NMR (DMSO-d6, 500 MHz) δ 3.79 (s, 3H, OCH3), 4.04 (s, 2H, CH2), 6.65 (d, 1H, J = 9 Hz, H3'/H5'), 7.00 (d, 2H, J = 9 Hz, H2'/H6'), 7.06 (d, 2H, J = 9 Hz, H3'/H5'), 7.33 (d, 2H, J = 9 Hz, H2'/H6'), 7.60 (s, 1H, 4Cl). 13C NMR (126 MHz, DMSO): δ 56.0 (OCH3, Z), 56.1 (OCH3, E), 104.8, 106.1, 112.9, 114.2* (C3'/C5', Z), 115.4* (C2'/C6', E), 119.8, 120.4* (C3'/C5', E), 124.1 (C4, Z), 124.8* (C2'/C6', Z), 126.6 (C4, E), 126.9, 127.6, 128.3, 137.4 (C6, Z), 138.3 (C6, E), 138.6, 140.9 (C1', Z), 143.2 (C1', E), 154.8, 158.2 (C4', E), 159.1 (C4', Z), 160.1, 165.4 (C2, Z), 170.1 (C2, E).

Synthesis of 5,7-dibromo-3-(2-(4-chlorophenyl)hydrazono)-1-(4-methoxybenzyl)indolin-2-one (207)

The title compound (207) was synthesised from 5,7-dibromo-N-(4-methoxybenzyl)isatin (162) and 4-chlorophenylhydrazine hydrochloride according to method B and purified by flash chromatography on silica gel (DCM; DCM/EtOAc 7:1; 4:1) to give a yellow powder (0.09 g, 44%), m.p. 216-218 °C, Rf 0.63 (DCM/EtOAc 9:1). LREI-MS: m/z 547; 549; 551 [M+] 79Br79Br; 79Br81Br; 81Br81Br. HRESI-MS: calcd for C22H23Br2N3O4Cl 79Br79Br (M+H+) 547.9376 found 547.9384. 1H NMR (CDCl3): δ 3.76 (s, 3H, OCH3), 4.04 (s, 2H, CH2), 6.83 (d, 2H, J = 9 Hz, H2'/H6'), 7.15 (d, 2H, J = 9 Hz, H3'/H5'), 7.29-7.32 (m, 4H, H2''/H3''/H5''/H6''), 7.49 (d, 1H, J = 3.5 Hz, H4), 7.73 (d, 1H, J = 3.5 Hz, H6). 13C NMR (CDCl3, 126 MHz): δ 43.5 (CH2), 55.4 (OCH3), 103.8, 114.3* (C3'/C5'), 115.9, 116.1* (C2''/C6''), 120.9 (C6), 124.6 (C3), 126.1, 127.9* (C2'/C6'), 129.0 (C1'), 129.4 (C4'''), 129.8* (C3''/C5''), 135.2 (C4), 136.3 (C7a), 140.6 (C1'''), 158.4 (C4'), 162.4 (C2).
Synthesis of 4-(2-(5,7-dibromo-1-(4-methoxybenzyl)-2-oxoindolin-3-ylidene)hydrazinyl)benzoic acid (208)

The title compound (208) was synthesised from 5,7-dibromo-N-(4-methoxybenzyl)isatin (162) and 4-hydrazinobenzoic acid according to method B and purified by flash chromatography on silica gel (DCM; DCM/EtOAc 6:1; 3:1) to give a yellow powder (0.22 g, 85%), m.p. 248-250 ºC, Rf 0.55 (DCM/EtOAc 9:1). LREI-MS: m/z 557; 559; 561 [M+] 79Br79Br; 79Br81Br; 81Br81Br. HRESI-MS: calcd for C23H16Br2N3O4 79Br79Br (M+H) 556.9375 found 556.9383. 1H NMR (DMSO-d6, 500 MHz): δ 3.69 (s, 3H, OCH3), 5.33 (s, 2H, CH2), 6.81 (d, J = 8.5 Hz, 2H, H3'/H5'), 7.00 (d, J = 8.5 Hz, 2H, H2'/H6'); 7.03 (s, 1H, H4), 7.44 (d, J = 8.5 Hz, 2H, H2''/H6''); 7.83 (d, J = 8.5 Hz, 2H, H3''/H5''); 8.59 (s, 1H, C6).

13C NMR (126 MHz, DMSO-d6, 126 MHz): δ 43.6 (CH2), 55.8 (OCH3), 100.8, 113.2, 114.4* (C3'/C5'), 119.2* (C2''/C6''), 120.8 (C6), 125.6, 126.8, 128.0* (C2'/C6'), 129.8 (C1''), 130.8* (C3''/C5''), 132.1 (C1'), 132.5, 153.3, 158.6 (C4'), 159.0 (C4''), 166.8 (C2), 174.2 (COOH).

Synthesis of (Z)-5,7-dibromo-3-(hydroxyimino)-1-(4-methoxybenzyl)indolin-2-one (209)

The title compound (209) was synthesised from 5,7-dibromo-N-(4-methoxybenzyl)isatin (162) and hydroxylamine hydrochloride according to method B and purified by flash chromatography on silica gel (DCM/EtOAc 5:1) to give a yellow powder (0.19 g, 92%), Rf 0.67 (DCM/EtOAc 5:1). LREI-MS: m/z 438; 440; 442 [M+] 79Br79Br; 79Br81Br; 81Br81Br. HRESI-MS: calcd for C16H13N2O3Br2 79Br79Br (M+H) 438.9059 found 430.9089. 1H NMR (DMSO-d6, 500 MHz): δ 3.69 (s, 3H, OCH3), 5.21 (s, 2H, CH2), 6.83 (d, J = 8.5 Hz, 2H, H3'/H5'), 7.03 (s, 1H, H4), 7.44 (d, J = 8.5 Hz, 2H, H2''/H6''); 7.83 (d, J = 8.5 Hz, 2H, H3''/H5''); 8.59 (s, 1H, C6). 13C NMR (DMSO-d6, 126 MHz): δ 43.2 (CH2), 55.8 (OCH3), 100.8, 113.2, 114.4* (C3'/C5'), 119.2* (C2''/C6''), 120.8 (C6), 125.6, 126.8, 128.0* (C2'/C6'), 129.8 (C1''), 130.8* (C3''/C5''), 132.1 (C1'), 132.5, 153.3, 158.6 (C4'), 159.0 (C4''), 166.8 (C2), 174.2 (COOH).

Synthesis of (Z)-3-(hydroxyimino)-1-(4-methoxybenzyl)indolin-2-one (210)

The title compound (210) was synthesised from hydroxylamine hydrochloride (169) and 1-(4-methoxybenzyl)isatin according to method B and purified by flash chromatography on silica gel (DCM) to give an orange/red solid (0.23 g, 67%), Rf 0.62 (DCM). LREI-MS: m/z 282.29 [M+] 1H NMR (CDCl3, 500 MHz) δ 3.70
(s, 3H, OCH₃), 4.85 (s, 2H, CH₂), 6.86 (d, J = 8.0 Hz, 2H, H3'/'H5''), 7.00 (d, J = 8.0 Hz, 1H, H7), 7.06 (t, J = 8.0 Hz, 1H, H5), 7.26 (d, J = 8.0 Hz, 2H, H2'/'H6''), 7.34 (t, J = 1.8 Hz, 1H, H6), 7.98 (d, J = 7.0 Hz, 1H, H4).

Synthesis of (Z)-5,7-dibromo-3-(hydroxyimino)indolin-2-one (211)

The title compound (211) was synthesised from hydroxylamine hydrochloride and 5,7-dibromoisonicotinic acid (76) by Method A and purified by flash chromatography on silica gel (DCM/EtOAc 3:1; to 100% EtOAc) to give a red solid (0.09 g, 57%), Rf 0.62 (DCM/EtOAc 5:1). LREI-MS: m/z 318; 320; 322 [M+]. HRESI-MS: C₄H₂Br₂N₂O₃ 79Br81Br (M+H⁺) 381.8624 found 317.9301. ¹H NMR (DMSO-d₆, 500 MHz) δ 8.79 (s, 1H, H4), 8.03 (s, 1H, H6), 11.15 (s, 1H, NH). ¹³C NMR (DMSO-d₆, 126 MHz): δ 103.0 (C5), 118.6 (C3a), 121.1 (C7), 127.8 (C4), 145.2 (C6), 145.3 (C7a), 158.6 (C2), 180.1 (C3).

Synthesis of (E and Z)-5,7-dibromo-3-(4-methoxyphenylimino)-1-(2-morpholinoethyl)indolin-2-one (212)

The title compound (212) was synthesised 5,7-dibromo-N-(2-morpholinoethyl)isatin (166) and p-methoxyaniline according to Method B and purified by flash chromatography on silica gel (DCM/DCM/EtOAc 6:1; 4:1) to give a dark yellow powder (0.078 g, 52%, as a mixture of E and Z isomers (E:Z=42:58)), Rf 0.26 (DCM) LREI-MS: m/z 521; 523; 525 [M+] 79Br79Br; 79Br81Br; 81Br81Br. HRESI-MS: calcd for C₂₁H₂₃Br₂N₂O₃ 79Br79Br (M+H⁺) 521.9604 found 521.9758. ¹H NMR (CDCl₃, 500 MHz) δ 2.52-2.53 (m, 12H, H2', H2''/H6''), E,Z), 3.62-3.68 (m, 4H, H1', E,Z), 3.85 (s, 3H, OCH₃, Z), 3.88 (s, 3H, OCH₃, E), 4.21-4.24 (m, 8H, H3''/H5'', E,Z), 6.92 (d, 2H, J = 8.5, H3''/H5''), Z), 7.00-7.02 (m, 4H, H2''/H3''/H4''/H5''), E), 7.15 (d, 1H, J = 1.5, 4H, E), 7.43 (d, 2H, J = 9 Hz, H2''/H6'', Z), 7.64 (d, 1H, J = 1.5 Hz, H6, E), 7.68 (d, 1H, J = 1.5 Hz, H6, Z), 7.80 (d, 1H, J = 1.5 Hz, H4, Z). ¹³C NMR (CDCl₃, 126 MHz): δ 54.0 (C2''/C6'', E+Z), 55.7 (OCH₃, E+Z), 57.1 (C2', E+Z), 68.4* (C1', E+Z; C3''/C5'', E+Z), 114.0* (C3''/C5'''), Z), 115.0* (C3''/C5'''), E), 116.8, 117.2, 120.4* (C2''/C6'', E), 124.9 (C4, Z), 125.2* (C2''/C6'', Z), 127.4 (C4, E), 128.2, 128.8, 129.0 9 (ArC), 131.1 (ArC), 139.6 (C6, Z), 139.9 (C3a, Z), 140.9* (C6, E; C1''', E+Z), 141.3 (C3a, Z), 142.1, 142.9, 160.1* (C4''', E+Z), 167.5* (C2, E+Z).
Synthesis of (E and Z)-1-(4-morpholino-2-oxoethyl)-3-(4-fluorophenylimino)-5,7-dibromoindolin-2-one (213)

The title compound (213) was synthesised from 5,7-dibromo-N-(2-morpholino-2-oxoethyl)isatin (167) and 4-fluorolaniline according to method B and purified by flash chromatography on silica gel (DCM/EtOAc 6:1) to give a dark yellow powder (0.097 g, 52%, mixture of E and Z isomers (E:Z/54:46)), Rf 0.53 (DCM/EtOAc 6:1/4:1) LREI-MS: m/z 523; 525; 527 [M+] 79Br79Br; 79Br81Br; 81Br81Br. HRESI-MS: calcd for C20H17Br3F3N3O3 79Br79Br (M+H+) 523.9379 found 523.9396. 1H NMR (CDCl3, 500 MHz) δ 3.45-3.78 (m, 16H, H2', H3', H5', H6', E+Z), 4.94 (s, 2H, CH2, Z), 5.09 (s, 2H, CH2, E), 6.87 (d, J = 1.5 Hz, 1H, H4, E), 6.95-6.97 (m, 2H, ArH), 7.02-7.04 (m, 2H, ArH), 7.14-7.16 (m, 4H, ArH), 7.59 (d, J = 1.5 Hz, H6, E), 7.66 (d, J = 1.5 Hz, 1H,H6, Z), 7.81 (d, J = 1.5 Hz, 1H, H4, Z). 13C NMR (126 MHz, CDCl3): δ 42.8 (C1', Z), 43.5 (C1', E), 45.5* (C2''/C6''', E+Z), 66.6* (C3''/C5'''), 67.0* (C3''/C5'''), 105.0*, 115.5, 115.8*, 116.8, 117.0*, 119.7* (C2''''/C6''''', E), 123.1*, 125.6 (C4, Z), 128.0 (C4, E), 133.6*, 139.9*, 140.3 (C6, Z), 141.3 (C6, E), 145* (C1''', E+Z), 149.3, 156.1, 162.9 (C4''', E+Z), 179.5 (C2, Z), 181.4 (C2''), 201.4 (C2, E).

Synthesis of (E and Z)-1-(4-morpholino-2-oxoethyl)-3-(4-trifluoromethylphenylimino)-5,7-dibromoindolin-2-one (214)

The title compound (214) was synthesised from 5,7-dibromo-N-(2-morpholino-2-oxoethyl)isatin (167) and 4-(trifluoromethyl)aniline according to method B and purified by flash chromatography on silica gel (DCM/EtOAc 6:1) to give a dark red solid (0.131 g, 46%, mixture of E and Z isomers (E:Z/56:44)), Rf 0.68 (DCM/EtOAc 6:1) LREI-MS: m/z 573; 575; 577 [M+] 79Br79Br; 79Br81Br; 81Br81Br. HRESI-MS: calcd for C22H16Br3F4N3O3 79Br79Br (M+H+) 574.9470 found 574.9470. 1H NMR (CDCl3, 500 MHz) δ 3.46-3.79 (m, 16H, H2', H3', H5', H6', E+Z), 4.91 (s, 2H, CH2, Z), 5.11 (s, 2H, CH2, E), 6.64 (s, 1H, H4, E), 7.06 (d, J = 8.0 Hz, 2H, H2''/H6''', E), 7.10 (d, J = 8.0 Hz, 2H, H2''/H6''', Z), 7.60 (s, 1H, H4, Z), 7.62 (d, J = 8.5 Hz, 2H, H3''/H5''', Z), 7.72 (d, J = 8.5 Hz, 2H, H3''/H5''', E+Z), 7.83 (s, 1H, H6, Z), 7.85 (s, 1H, H6, E). 13C NMR (CDCl3, 126 MHz): δ 42.6 (C1', C3''/C5'''), 43.4 (C1'), 45.2* (C3''/C5'''), 66.3* (C2'', C6'''), 66.9* (C2'', C6''), 104.1, 104.9, 115.7, 116.6, 117.4* (C2''''/C6'''''), 119.0* (C2''''/C6'''''), 125.8 (C3''''/C5''''', Z),
125.9 (C6, E), 127.1* (C4''', E+Z), 127.3 (C6, Z), 128.2 (C4, E), 133.4, 139.6, 140.9* (C3''''/C5''''), 141.5* (C3''''/C5'''''), Z, C4, Z), 142.7, 144.3, 150.5 (C7a), 151.6 (C1''''), 152.2* (C1''''', C7a), 157.5 (C2'', E), 163.2 (C2'', Z), 164.6 (C2), 164.7 (C2).

Synthesis of (E and Z)-1-(4-morpholino-2-oxoethyl)-3-(4-methoxyphenylimino)-5,7-dibromoindolin-2-one (215)

The title compound (215) was synthesised from 5,7-dibromo-N-(2-morpholino-2-oxoethyl)isatin (167) and 4-methoxyaniline according to method B and purified by flash chromatography on silica gel (DCM/EtOAc 5:1; 3:1) to give a dark red solid (0.16 g, 69%, as a mixture of E and Z isomers (E/Z:42:58)), m.p. 121-124 °C, Rf 0.41 (DCM/EtOAc 4:1). LREI-MS: m/z 537; 537; 539 [M+] 79Br39Br; 79Br39Br; 81Br81Br. HRESI-MS: calcd for C32H28Br2N4O6 79Br 79Br (M+H+) 536.9821 found 536.9816. 1H NMR (CDCl3, 500 MHz) δ 3.48-3.59 (m, 16H, H2', H3', H5', H6'), 3.84 (s, 3H, OCH3, Z), 3.87 (s, 3H, OCH3, E), 4.91 (s, 2H, H1, Z), 5.06 (s, 2H, H1', E), 6.88 (d, J = 8.5 Hz, 2H, H3''/H5''), 6.99 (d, J = 8.5 Hz, 2H, H3''/H5''), 7.03 (d, J = 8.5 Hz, 2H, H2''/H6''), 7.12 (d, J = 1.5Hz, 1H, H4, E), 7.12 (d, J = 1.5 Hz, 1H, H4, Z), 7.21 (d, J = 1.5Hz, 1H, H6, Z), 7.24 (d, J = 1.5 Hz, 1H, H6, E), 7.24 (d, J = 1.5Hz, 1H, H6, E), 7.36 (d, J = 1.5Hz, 1H, H6, E), 7.62 (d, J = 1.5Hz, 1H, H4, Z), 7.62 (d, J = 1.5Hz, 1H, H4, Z), 7.62 (d, J = 1.5Hz, 1H, H6, Z), 7.62 (d, J = 1.5Hz, 1H, H6, E), 7.62 (d, J = 1.5Hz, 1H, H6, E). 13C NMR (CDCl3, 126 MHz): δ 42.1* (C2''/C6'', Z), 42.4* (C2''/C6'', E), 55.7* (CH2, E+Z), 55.8* (OCH3, E+Z), 62.3* (C3''/C5'', E), 62.5* (C3''/C5'', Z), 106.9, 107.1, 113.8* (C3''''/C5''''', Z), 114.9* (C3''''/C5'''''), E), 115.2, 115.6, 120.5* (C2''''/C6'''''), E), 125.2 (C6, E), 125.6* (C2''''/C6'''''), Z), 127.4, 127.8 (C4, E), 130.8, 138.5 (C6, Z), 140.2 (C1''''', Z), 141.5 (C4, Z), 141.8 (C7a, Z), 142.1 (C1'''', E), 144.1 (C2'', Z), 150.6, 158.0, 158.8 (C4''', E), 160.1 (C4'''', Z), 164.0 (C2, E), 165.7 (C2, Z), 165.6 (C2', E), 171.8 (C2', Z).

Synthesis of (E and Z)-2-(4-((1-(4-methoxybenzyl)-5-nitro-2-oxoindolin-3-ylidene)amino)phenyl)acetic acid (217)*

The title compound (217) was synthesised from 5-nitro-N-(4-chlorobenzyl)isatin (170) and (4-aminophenyl)acetic acid according to method B and purified by flash chromatography on silica gel (DCM/MeOH 15:1, 10:1) to give an orange powder (0.017 g, 24%, as a mixture of E and Z isomers (E/Z:64:36)), Rf 0.43 (DCM/RtOAc 4:1). LREI-MS: m/z 445 [M+]. HRESI-MS: calcd for C24H18N3O6 (M+H+) 445.8941 found 445.8964. 1H NMR (DMSO-d6, 500 MHz) δ 3.25 (s, 2H, H2, Z), 3.28 (s, 2H, H2, E), 3.90 (s,
Experimental

3H, OCH₃, Z), 3.94 (s, 3H, OCH₃, E), 5.01 (s, 2H, CH₂, Z), 5.18 (s, 2H, CH₂, E), 6.89 (d, J = 8.5 Hz, 2H, H3''/H5''', E), 6.91 (d, J = 8.5 Hz, 2H, H3''/H5''', Z), 7.02 (d, J = 8.5 Hz, 2H, H3''/H5'', E), 7.19 (d, J = 8.0 Hz, 1H, H2''/H6'', Z), 7.22 (d, J = 8.0 Hz, 1H, H2'/H6', Z), 7.35 (d, J = 8.0 Hz, 2H, H2''/H6'', E), 7.39 (d, J = 8.5 Hz, 2H, H3'/H5', Z), 7.46 (d, J = 8.0 Hz, 2H, H2'/H6', E), 7.51 (d, J = 2.0 Hz, 1H, H4'', E), 8.20 (d, J = 8.0 Hz, 1H, H7'', E), 8.32 (d, J = 8.0 Hz, 1H, H7'', Z), 7.48 (d, J = 8.5 Hz, 1H, H6'', E), 8.50 (d, J = 8.5 Hz, 1H, H6'', Z), 8.45 (s, 1H, H4'', Z).

Synthesis of (E and Z)-2-(4-((5-methoxy-1-(4-methoxybenzyl)-2-oxoindolin-3-ylidene)amino)phenyl)acetic acid (218)

The title compound (218) was synthesised from 5-nitro-N-(4-chlorobenzyl)isatin (171) and (4-aminophenyl)acetic acid according to method B and purified by flash chromatography on silica gel (DCM/MeOH 15:1, 10:1) to give an orange powder (0.027 g, 31%), to give an orange powder (0.017 g, 24%), as a mixture of E and Z isomers (E:Z=54:46). Rf 0.52 (DCM/RtOAc 4:1). LREI-MS: m/z 430 [M-H]. HRESI-MS: calcd for C₃₂H₂₄N₂O₃ (M-H⁺) (M+H⁺) 430.9021 found 430.9048. ¹H NMR (CDCl₃, 500 MHz) δ 3.25 (s, 2H, H2, Z), 3.28 (s, 2H, H2, E), 3.90 (s, 6H, OCH₃, Z+E), 3.94 (s, 6H, OCH₃, E+Z), 5.01 (s, 2H, CH₂, Z), 5.18 (s, 2H, CH₂, E), 6.01 (d, J = 2.0 Hz, 1H, H4'', E), 6.21 (d, J = 8.0 Hz, 2H, H3''/H5''', Z), 6.31 (d, J = 8.5 Hz, 2H, H3''/H5''', E), 6.45 (d, J = 8.5 Hz, 2H, H2''/H6', E), 6.62 (d, J = 8.0 Hz, 1H, H7'', Z), 6.71 (d, J = 8.0 Hz, 1H, H7'', E), 6.83 (d, J = 8.0 Hz, 2H, H2''/H6'', Z), 6.91 (d, J = 8.0 Hz, 2H, H2''/H6'', E), 7.01 (d, 2H, J = 8.0 Hz, H2''/H6'', Z), 7.10 (d, 2H, J = 8.0 Hz, H3'/H5', Z), 7.16 (d, J = 8.5 Hz, 2H, H3'/H5', E), 7.25 (d, J = 8.0 Hz, 1H, H6'', Z), 7.44 (d, J = 8.0 Hz, 1H, H6'', E), 7.49 (s, 1H, H4'', Z).

Synthesis of 3,3-dichloro-2-oxoindoline-5-sulfonyl chloride (220)

The title compound 220 was synthesised according to the protocol by Bebernitz et al.403 To the cooled solution of chlorosulfonic acid (150 ml) was added dropwise a solution of isatin (76) (150 mmol) in DCM (25 ml). Solution was stirred at rt for 2h, poured into ice and extracted with EtOAc. The organic layer was dried over MgSO₄, filtered and solvent removed in vacuo. Product was purified by column chromatography to yield an orange powder (0.23 g, 58%). LREI-MS: m/z 301 [M+] ¹H NMR (DMSO-d₆, 500 MHz) δ 7.46 (d, J = 8.5 Hz, 1H, H7), 8.20 (d, J = 2.0 Hz, 1H, H4), 8.61 (dd, J = 3 Hz, 1H, H6), 10.76 (br s, 1H, NH).
Synthesis of 3,3-dichloro-5-(morpholinosulfonyl)indolin-2-one (221)

The title compound 221 was synthesised according to the protocol by method of Ivachtchenko et al.\textsuperscript{432} 220 (1 equiv.) was dissolved in either THF (8 ml) or mixture EtOH/H\textsubscript{2}O 1:1 (8 ml) and morpholine (2 equiv.). Mixtures were stirred at the rt for 3h followed by the extraction with EtOAc. Organic layer was dried and solvent removed in vacuo. The product was purified by column chromatography using DCM/EtOAc. Both reactions gave the desired product EtOH/H\textsubscript{2}O (0.2805g, 88%), THF (0.1036g, 62%), m.p. 231-233 \degree C.\textsuperscript{432} LREI-MS: m/z 350 [M+]. \textsuperscript{1}H NMR (CDCl\textsubscript{3}, 500 MHz) δ 3.38 (t, J = 3.5 Hz, 4H, H\textsubscript{2}'/H\textsubscript{6}''), 3.77 (m, 4H, H\textsubscript{3}'/H\textsubscript{5}''), 6.79 (d, J = 9 Hz, 1H, H\textsubscript{6}), 7.62 (d, J = 8.5 Hz, 1H, H\textsubscript{7}), 7.93 (s, 1H, H\textsubscript{4}).

Synthesis of 1-(4-methoxybenzyl)-5-(morpholinosulfonyl)isatin (223)

The title compound (223) was synthesised from 5-(morpholinosulfonyl)indoline-2,3-dione (222) and p-methoxybenzylchloride according to Method A and purified by flash chromatography on silica gel (DCM/EtOAc 4:1/2:1) to give a red solid (0.087 g, 62%), m.p. 203-205 (lit 205.1-206.0 \degree C), R\textsubscript{f} 0.28 (DCM/EtOAc 3:4). LREI-MS: m/z 416 [M+]. HRESI-MS: calcd for C\textsubscript{20}H\textsubscript{21}N\textsubscript{2}O\textsubscript{6}S (M+H\textsuperscript{+}) 416.9622 found 416.9632. \textsuperscript{1}H NMR (DMSO-\textsubscript{d}\textsubscript{6}, 500 MHz) δ 2.85 (t, J = 4 Hz, 4H, H\textsubscript{2}'/H\textsubscript{6}''), 3.60 (t, J = 4 Hz,2H, H\textsubscript{3}'/H\textsubscript{5}''), 3.71 (s, 3H, OCH\textsubscript{3}), 4.88 (s, 2H, CH\textsubscript{2}), 6.89 (d, J = 9 Hz, 2H, H3''/H5''), 7.20 (d, J = 8.5 Hz, 2H, 1H, H7), 7.38 (d, J = 8.5 Hz, 2H, H2''/H6''), 7.73 (d, J = 1.5 Hz, 2H, 1H, H4), 7.91 (dd, J = 1.5 Hz, 8.5 Hz, 1H, H6).

Synthesis of 3-((4-methoxyphenyl)iminoo)-5-(morpholinosulfonyl)indolin-2-one (224)

The title compound (224) was synthesised from 5-(morpholinosulfonyl)indoline-2,3-dione (222) and 4-methoxyaniline according to method B and purified by flash chromatography on silica gel (DCM/EtOAc 3:1/2:1) to give an orange powder (0.068 g, 59%, mixture of E and Z isomers (E:Z/38:62), R\textsubscript{f} 0.28 (DCM/EtOAc 3:4). LREI-MS: m/z 401 [M+]. HRESI-MS: calcd for C\textsubscript{19}H\textsubscript{20}N\textsubscript{3}O\textsubscript{5}S (M+H\textsuperscript{+}) 401.9871 found 401.9882. \textsuperscript{1}H NMR (CDCl\textsubscript{3}, 500 MHz) δ 2.99-3.02 (m, 8H, H\textsubscript{2}'/H\textsubscript{6}'', E+Z), 3.75-3.78 (m, 8H, H3''/H5'', E+Z), 3.85 (s, 6H, OCH\textsubscript{3}, E+Z) 6.82 (d, J = 8.5 Hz, 1H, H7, Z), 6.92 (d, J = 8.0 Hz, 2H,
H3”/H5”, E), 6.98 (s, J = 7.5 Hz, 2H, H3”/H5”, Z), 7.11-7.15 (m, 3H, H7, E; H2”/H6”, Z), 7.36 (s, 1H, H4, E), 7.44 (d, J = 8.5 Hz, 2H, H2”/H6”, E), 7.60 (d, J = 9 Hz, 1H, H6, Z), 7.40 (d, J= 8.0 Hz, 1H, H6, E), 7.93 (s, 1H, H4, E), 7.36 (s, 1H, H4, E), 7.44 (d, J = 8.5 Hz, 2H, H2”/H6”, E), 7.60 (d, J = 9 Hz, 1H, H6, Z), 7.40 (d, J= 8.0 Hz, 1H, H6, E), 7.93 (s, 1H, H4, E), 7.36 (s, 1H, H4, E), 7.44 (d, J = 8.5 Hz, 2H, H2”/H6”, E), 7.60 (d, J = 9 Hz, 1H, H6, Z), 7.40 (d, J= 8.0 Hz, 1H, H6, E), 7.93 (s, 1H, H4, E).

13C NMR (CDCl3, 126 MHz): δ 46.3* (C2'/C6’, E+Z), 66.4* (C3'/C5’, E+Z), 67.0 (CH3, E+Z), 112.6 (C7, E), 113.3, 114.1* (C3”/C5”, E), 115.2 (C3”/C5”, Z), 116.7, 118.2 (C7, Z), 121.1 (C2”/C6”, Z), 122.3, 125.3* (C2”/C6”, E, C4, E), 126.4, 129.8, 139.1 (C6, E), 134.5 (C6, Z), 135.0 (C4, Z), 142.3* (C1”, E+Z), 149.6 (C7a, E), 152.6, 154.9 (C7a, Z), 159.3* (C4”, E+Z), 164.9 (C2), 165.2 (C2).

Synthesis of 5-(2-furyl)isatin (228)
The title compound (228) was synthesised according to the protocol of Gerard et al.408 To a solution of 5-bromoisatin (227) (1 equiv.) in DME under nitrogen Pd(PPh3)4 (0.05 equiv.) was added. After 5 min stirring at RT, arylboronic acid (3.6 mmol, 1.2 equiv.) and sodium hydrogen carbonate (2 equiv.) in H2O (10 mL) were added. Mixture was refluxed for 6 h. It was extracted with EtOAc, organic layer dried over MgSO4 and solvent removed in vacuo. The product was purified by column chromatography (DCM/EtOAC 4:1; 1:1) to yield purple solid (0.034 g, 19%), m.p. 211-214 ºC (lit 214 ºC), Rf 0.41 (DCM/EtOAc 4:1). LREI-MS: m/z [M+] 213.

1H NMR (DMSO-δ6, 500 MHz) δ 6.49 (s, 1H, Hfur), 6.63 (d, J = 1 Hz, 1H, Hfur), 6.93 (d, J = 7.5 Hz, 1H, H7), 7.48 (s, 1H, Hfur), 7.89 (m, 2H, H4 +H6), 11.1 (s, 1H, NH).

Synthesis of 5-phenylisatin (229)
The title compound (229) was synthesised according to the protocol of Gerard et al.408 To a solution of 5-bromoisatin (227) (1 equiv.) in DME under nitrogen Pd(PPh3)4 (0.05 equiv.) was added. After 5 min stirring at RT, arylboronic acid (3.6 mmol, 1.2 equiv.) and sodium hydrogen carbonate (2 equiv.) in H2O (10 mL) were added. Mixture was refluxed for 6 h. It was extracted with EtOAc, organic layer dried over MgSO4 and solvent removed in vacuo. The product was purified by column chromatography (DCM/EtOAc 4:1; 1:1), to give a red solid (0.021 g, 24%), m.p. 267-269 ºC (lit 260 ºC), Rf 0.64 (DCM/Hex/EtOAc 4:1:2). LREI-MS: m/z [M+] 223. 

1H NMR (DMSO-δ6, 500 MHz) δ 6.99 (d, 1H, J = 8, H6), 7.35 (m, 1H, phenyl), 7.45 (m, 2H, phenyl), 7.65 (d, J = 7.5 Hz, 2H, phenyl), 7.53 (s, 1H, H4), 7.89 (d, 1H, J = 8.0 Hz, H6), 11.16 (s, 1H, NH).

Synthesis of 5-phenyl-N-(p-methoxybenzyl)isatin (230)
The title compound (230) was synthesised according to the protocol of Gerard et al.404 To a solution of 5-bromo-1-(4-methoxybenzyl)isatin (162 a) (1 equiv.) in DME under nitrogen
Pd(PPh₃)₄ (0.05 equiv.) was added. After 5 min stirring at RT, arylboronic acid (3.6 mmol, 1.2 equiv.) and sodium hydrogen carbonate (2 equiv.) in H₂O (10 mL) were added. Mixture was refluxed for 6 h. It was extracted with EtOAc, organic layer dried over MgSO₄ and solvent removed in vacuo. The product was purified by column chromatography (DCM/EtOAc 4:1; 1:1), to give a red solid (0.016 g, 16%), m.p. 135-137 ºC, Rf 0.64 (DCM/Hex/EtOAc 4:1:2). LREI-MS: m/z [M+] 343. HRESI-MS: calcld for C₂₂H₁₈NO₃ (M+H⁺) 343.9772 found 343.9781. 

\[ ^1H \text{NMR (CDCl}_3, \text{500 MHz)} \delta 3.69 \text{ (s, 3H, OCH}_3 ) , 5.28 \text{ (s, 2H, CH}_2 ), 6.87 \text{ (d, } J = 8.5 \text{ Hz, 2H, H3'/H5'), 6.92 \text{ (q, } J = 8.0 \text{ Hz, 3H, phenyl Arc), 7.24 \text{ (d, } J = 6 \text{ Hz, 2H, H2'/H6'), 7.37 \text{ (d, } J = 2.0 \text{ Hz, 1H, H6), 7.49 \text{ (sept, } J = 8.5 \text{ Hz, 2H, phenyl Arc), 7.69 \text{ (d, } J = 2.0 \text{ Hz, 1H, H7), 7.82 \text{ (d, } J = \text{1Hz, 1H, H4).}} \]

\[ ^{13}C \text{NMR (CDCl}_3, \text{126 MHz)} \delta 42.4 \text{ (C} H_2 \text{), 54.1 (OCH}_3 ), 110.6 \text{ (ArC, isatin), 114.2* (C} 3'\text{/C5'), 117.9 (C1'), 123.4 (C7), 126.1* (C2'/C6''), 127.2 (C6), 128.7* (C3''/C5''), 130.1 (C1'), 130.8 (C2'/C6''), 136.3 (C4), 137.0 (C5), 138.7 (C1''), 149.2 (C7a), 158.3 (C4'), 156.0 (C2), 182.7 (C3).} \]

**Synthesis of 3-azidopropan-1-ol (232)**

The title compound 232 was synthesised according to the protocol of Engels et al. To the solution of bromopropanol (231) in anhydrous DMF (10 ml) sodium azide was added at rt. Mixture was stirred slowly and the temperature brought up to 90-95°C. After 4 h mixture was cooled and extracted with ether. Water was added into the organic layer and then solvent removed in vacuo. TLC and MS indicated the formation of the product. Without further purification product was used in the next step. LREI-MS: m/z 101 [M+].

**Synthesis of 5,7-dibromo-1-((1-(3-hydroxypropyl)-1H-1,2,3-triazol-4-yl)methyl)isatin (233)**

Method 1: The title compound 233 was synthesised according to the protocol by Chang et al. 5,7-dibromo-1-(prop-2-yn-1-yl)isatin (168) was added (1 equiv.) to the solution of azidopropanole (232) (1.2 equiv.) in H₂O/ACN followed by the addition of CuI (0.05 equiv.) and 2.6-lutidine (0.6 equiv.). After stirring on at rt, mixture was extracted with EtOAc, washed with brine, dried over MgSO₄, filtered and concentrated. Product was purified by column chromatography using DCM/EtOAc 1:1, and EtOAc to yield an orange solid (0.152 g, 59%).

TLC: Rf 0.41 (DCM/MeOH 9:1) LREI-MS: m/z 442; 444; 446 [M+] ⁷⁸Br⁷⁹Br; ⁷⁹Br⁸¹Br; ⁸¹Br⁸²Br. HRESI-MS: calcld for C₁₄H₁₂BrN₃O₃ ⁷⁹Br⁷⁹Br (M+H⁺) 442.9129 found 442.9211. 

\[ ^1H \text{NMR (DMSO-d}_6, \text{500MHz)} \delta 1.90-1.93 \text{ (m, 2H, H7'), 3.63-3.65 \text{ (m, 2H, H8'), 4.36 (t, } J = 7.0 \text{ Hz).}} \]
Chapter 7

Experimental

H 231
2
2
- OAc 2:1) to give a red solid
4
5
2
, sodium ascorbate (0.1 equiv.), Na
233
232
-
ed by flash chromatography on
2
21
89
freshly prepared 1
5
M
-(C2,
113x102](C2,
113x121]142.8, 143.5
124.3 (C
Z
H5
H5
J =
7
0.083 g, 67%, mixture of
551 [M+
550: m/z 547; 549;
51 |}

Method 2. 233 was also synthesised by method of Sharpless et al. and Chang et al.406,430 168 was added (1 equiv.) to the solution of 232 (1 equiv) in H2O/tBuOH. To the mixture, solution of CuSO4 in H2O and sodium ascorbate (freshly prepared 1M solution in H2O). The mixture was stirred at rt followed by the purification protocol as described above in Method 1 to yield 0.12 g, 46% of 233.

Method 3. 233 was also synthesised according to the protocol by Fokin et al.410 168 (1 equiv.) was added to the solution of bromopropanol (231) (1 equiv.) in DMF/H2O. To the mixture, sodium azide (1.2 equiv.) CuSO4.5 H2O (0.05 equiv.), sodium ascorbate (0.1 equiv.), Na2CO3 (0.2 equiv) and L-proline (0.2 equiv.) were added. Mixture was stirred at 60°C over-night. Product was purified by column chromatography to yield 0.0162 g, 6% of 233.

Synthesis of 5,7-dibromo-1-((1-(3-hydroxypropyl)-1H-1,2,3-triazol-4-yl)methyl)-3-((4-methoxyphenyl)imino)indolin-2-one (234)
The title compound (234) was synthesised from 5,7-dibromo-1-((1-(3-hydroxypropyl)-1H-1,2,3-triazol-4-yl)methyl)isatin (233) and p-methoxyaniline according to method B and purified by flash chromatography on silica gel (DCM; DCM:EtOAc 2:1) to give a red solid (0.083 g, 67%, mixture of E and Z isomers (E/Z/43:57)), Rf 0.31 (DCM/EtOAC 1:1). LREI-MS: m/z 547; 549; 551 [M+] 79Br2Br; 79Br81Br; 81Br81Br. HRESI-MS: calcd for C21H20Br2N2O2 79Br79Br (M+H+) 547.9834 found 547.9827. 1H NMR (DMSO-d6, 500 MHz) δ 1.89-1.92 (m, 4H, H7’, E+Z), 3.33-3.35 (m, 4H, H8’, E+Z), 3.81 (s, 2H, OCH3, E+Z), 4.36 (t, J = 7.5 Hz, 2H, H6’, E+Z); 5.27 (s, 2H, CH2, Z); 5.39 (s, 2H, CH2, E), 6.84 (s, 1H, H4, E), 6.96 (d, J = 9 Hz, 2H, H3’/H5’’, Z), 7.04 (d, J = 8.5 Hz, 2H, H3’/H5’’, E), 7.08 (d, J = 8.5 Hz, 2H, H2’/H6’’, E), 7.43 (d, J = 9 Hz, 2H, H2’/H6’’, Z), 7.75 (s, 1H, H4, Z), 7.83 (s, 1H, H6, E), 7.86 (s, 1H, H6, Z), 8.05 (s, 1H, H5’, Z), 8.09 (s, 1H, H5’, E). 13C NMR (DMSO-d6, 126 MHz): δ 33.5 (C7’, E+Z), 37.5 (C8’, E+Z), 47.2 (C6’, E+Z), 56.0 (OCH3, E+Z), 57.9 (CH2, E+Z), 104.0, 104.8, 114.2* (C3’/C5’’, Z), 114.8* (C3’/C5’’, E), 115.5*, 115.8* (C2’/C6’’, E), 120.1 (C4, Z), 120.5, 123.4 (C5’, Z), 124.3 (C5’, E), 125.5* (C2’/C6’’, Z), 127.6, 139.6, 140.6 (C1’’, Z), 140.7 (C1’’, E), 141.7, 142.8, 143.5, 144.0, 147.7, 152.1, 153.4, 158.0 (C4’’, E), 158.4 (C4’’, Z), 159.7 (C2, Z), 163.4 (C2, E).
Synthesis of (Z)-5,7-dibromo-3-(hydroxyimino)-1-((1-(3-hydroxypropyl)-1H-1,2,3-triazol-4-yl)methyl)indolin-2-one (235)

The title compound (235) was synthesised from 5,7-dibromo-1-((1-(3-hydroxypropyl)-1H-1,2,3-triazol-4-yl)methyl)isatin (233) and hydroxylamine hydrochloride according to method B and purified by flash chromato-ography on silica gel (DCM/MeOH 9:1) to give a red solid (0.048 g, 47%), Rf 0.52 (DCM/MeOH 9:1). **LREI-MS**: m/z 457; 459; 461 [M+] 79Br79Br; 79Br81Br; 81Br81Br. **HRESI-MS**: calcd for C14H14Br2N5O3 79Br79Br (M+H+) 457.9702 found 457.9719. **1H NMR** (DMSO-d6, 500 MHz) δ 1.90-1.93 (m, 2H, H7'), 3.31-3.33 (m, 2H, H8'), 4.33 (t, J = 7.5 Hz, 2H, H6'), 5.34 (s, 2H, CH2), 7.22 (s, 1H, H4), 7.99 (s, 1H, H5'), 8.25 (s, 1H, H6).

3,5,7-Tribromoxindole (240 a)

The title compound (240a) was isolated as a by-product of the above reaction (0.041 g, 17%), Rf 0.52 (DCM). **LREI-MS**: m/z 367; 369; 371; 373 [M+] 79Br79Br79Br; 79Br79Br81Br; 79Br81Br81Br; 81Br81Br81Br. **1H NMR** (DMSO-d6, 500 MHz) δ 5.78 (s, 1H, C3); 7.54 (s, 1H, C4); 7.73 (s, 1H, C6); 11.24 (s, 1H, NH).

Synthesis of 5,7-dibromo-2-oxindole (240)

The title compound (240) was synthesised according to the protocol of Sumpter et al. 2-oxindole (1 equiv.) was dissolved in water and into the hot mixture, solution containing bromine (2 equiv.) and potassium bromide in water was added slowly. The reaction mixture was filtered and the solid purified by column chromatography (DCM/EtOAc 9:1; 5:1) as a brown powder (0.040 g, 21%), m.p. 258-261 °C, Rf 0.19 (DCM). **LREI-MS**: m/z 289; 291; 293 [M+] 79Br79Br; 79Br81Br; 81Br81Br. **HRESI-MS**: calcd for C8H6Br2NO 79Br79Br (M+H') 289.8714 found 289.8717. **1H NMR** (DMSO-d6, 500 MHz) δ 3.29, (s, 2H); 7.36, (s, 1H); 7.56, (s, 1H); 10.75, (s, 1H). **13C NMR** (DMSO-d6, 126 MHz): δ 33.6 (C7'), 37.5 (C8'), 47.3 (C6'), 58.1 (CH2), 115.3, 120.5, 123.3 (C5'), 124.8 (C6), 131.2 (C4'), 137.6 (C4), 139.8 (C7a), 142.3, 144.1 (C3), 164.6 (C2).
3,3,5,7-Tetrabromooxindole (240b)

The title compound (240b) was isolated as a by-product of the above reaction (0.046 g, 19%) R, 0.47 (DCM). **LREI-MS:** m/z 367; 369; 371; 373. **1H NMR** (DMSO-\(d_6\), 500 MHz) δ 5.78 (s, 1H, C3); 7.54 (s, 1H, C4); 7.73 (s, 1H, C6); 11.24 (s, 1H, NH).

Synthesis of 5,7-dibromo-N-(4-methoxybenzyl)-indolin-2-one (241)

The compound 241 was synthesised according to the protocol of Crestini et al.\(^{41}\) 62 (2 g) was dissolved in hydrazine hydrate (15 ml) and refluxed for 4 h. The reaction mixture was extracted with EtOAc, dried over MgSO\(_4\) and solvent removed on the rotary evaporator. The product was purified by column chromatography using DCM/hexane mixture to yield brown powder (1.243 g, 63%), m.p. 116-118 °C, R\(_f\) 0.61 (DCM).

**LREI-MS:** m/z 409; 411; 413 [M+]+ \(^{79}\)Br\(^{79}\)Br; \(^{79}\)Br\(^{81}\)Br; \(^{81}\)Br\(^{81}\)Br. **HRESI-MS:** calcd for C\(_{16}\)H\(_{14}\)Br\(_2\)NO\(_2\) \(^{79}\)Br\(^{79}\)Br (M+H\(^+\)) 409.9391 found 409.9378.

**1H NMR** (CDCl\(_3\), 500 MHz) δ 3.63 (s, 2H, H3, CH\(_2\)), 3.77 (s, 3H, OCH\(_3\)), 5.30 (s, 2H, CH\(_2\)), 6.83 (d, J = 8.5 Hz, 2H, H3'/H5'), 7.15 (d, J = 8.5 Hz, 2H, H2'/H6'), 7.31 (s, 1H, H4), 7.51 (s, 1H, H6).

**13C NMR** (CDCl\(_3\), 126 MHz): δ 35.8 (C3), 45.1 (CH\(_2\)), 56.1 (OCH\(_3\)), 102.5 (C5), 114.8* (C3'/C5'), 116.7 (C7), 126.8 (C6), 129.4* (C2'/C6'), 130.0 (C1'), 131.7 (C3a), 135.6 (C4), 141.7 (C7a), 159.5 (C4'), 174.5 (C2).

1-(4-Methoxybenzyl)-5-bromooxindole (241a)

The title compound (241a) was isolated as a by-product of the above reaction (0.218 g, 14%), m.p. 151-154 °C, R, 0.53 (DCM). **LREI-MS:** m/z 331; 333; [M+] \(^{79}\)Br\(^{79}\)Br; \(^{79}\)Br\(^{81}\)Br; \(^{81}\)Br\(^{81}\)Br. **HRESI-MS:** calcd for C\(_{16}\)H\(_{15}\)Br\(_2\)NO\(_2\) \(^{79}\)Br\(^{79}\)Br (M+H\(^+\)) 332.0283 found 332.0286.

**1H NMR** (CDCl\(_3\), 500 MHz) δ 3.59 (s, 3H, OCH3), 3.77 (s, 2H, H3), 4.82 (s, 2H, CH\(_2\)), 6.60 (d, J = 8.5 Hz, 1H, H7), 6.84 (d, J = 8.5 Hz, 2H, H3'/H5'), 7.21 (d, J = 8.5 Hz, 2H, H2'/H6'), 7.28 (d, J = 8.5 Hz, 1H, H6), 7.35 (s, 1H, H4). **13C NMR** (CDCl\(_3\), 126 MHz): δ 31.8 (C3), 39.6 (CH\(_2\)), 51.6 (CH\(_3\)), 106.7 (C7), 110.5* (C3'/C5'), 122.8 (C5), 123.7 (C3a), 123.9 (C1'), 125.0* (C2'/C6'), 126.9 (C4), 127.2 (C6), 139.6 (C7a), 155.5 (C4'), 170.6 (C2).

1-(4-Methoxybenzyl)-3-hydrazyl-5,7-dibromo-indolin-2-one (241b)

The title compound (241b) was isolated as a by-product of
the above reaction as the orange powder (0.098 g, 5%), Rf 0.58 (DCM/EtOAc 1:1) LREI-MS: m/z 437; 439; 441 [M+] 79Br79Br; 79Br81Br; 81Br81Br. HRESI-MS: calcd for C16H14Br2N3O2 79Br79Br(M+H)+ 437.9354 found 437.9348. 1H NMR (CDCl3, 500 MHz) δ 3.76 (s, 3H, OCH3), 5.37 (s, 2H, CH2), 6.83 (d, J = 8.5 Hz, 2H, H3'/H5'), 7.13 (d, J = 8.5 Hz, 2H, H2'/H6'), 7.47 (s, 1H), 7.61 (s, 1H), 10.9 (NH2).

5,7-dibromo-1-(4-methylbenzyl)indolin-2-one (242)

The compound 242 was synthesised according to the protocol of Crestini et al.431 242 (0.5 g) was dissolved in hydrazine hydrate (8 ml) and refluxed for 4 h. The reaction mixture was extracted with EtOAc, dried over MgSO4 and solvent removed on the rotary evaporator. The product was purified by column chromatography using DCM/hexane mixture to yield brown powder (0.275 g, 57%), Rf 0.71 (DCM) LREI-MS: m/z 393; 395; 397 [M+] 79Br79Br; 79Br81Br; 81Br81Br. HRESI-MS: calcd for C16H14Br2NO 79Br79Br (M+H)+ 393.9008 found 393.9006. 1H NMR (CDCl3, 500 MHz) δ 2.38 (s, 2H, CH3), 3.64 (s, 2H, H3), 5.39 (s, 2H, CH2), 6.83 (d, J = 8.5 Hz, 2H, H3'/H5'), 7.15 (d, J = 8.5 Hz, 2H, H2'/H6'), 7.29 (s, 1H, H4), 7.47 (s, 1H, H6). 13C NMR (CDCl3, 126 MHz) δ 20.1 (CH3), 35.7 (C3), 44.5 (CH2), 101.8 (C5), 116.8 (C7), 116.7 (C6), 126.5* (C3'/C5'), 129.5* (C2'/C6'), 130.0 (C1'), 134.7 (C3a, C3)*, 141.7 (C7a), 156.2 (C4'), 176.5 (C2).

5-bromo-1-(4-methylbenzyl)indolin-2-one (242 a)

The title compound (242 a) was isolated as a by-product of the above reaction as the brown solid (0.046 g, 12%) Rf 0.38 (DCM/EtOAc 4:1) LREI-MS: m/z 315; 317; [M+] 79Br79Br; 81Br81Br. 1H NMR (CDCl3, 500 MHz) δ 2.31 (s, 3H, CH3), 3.60 (s, 2H, H3), 4.85 (s, 2H, CH2), 6.58 (d, J = 8.5 Hz, 1H, H7), 7.16 (m, 4H, H2'/H3'/H5'H6'), 7.26 (d, J = 8.5 Hz, 1H, H6), 7.38 (s, 1H, H4). 13C NMR (CDCl3, 126 MHz): δ 21.4 (CH3), 35.8 (C3), 43.8 (CH2), 110.7, 115.2, 126.7 (C3a), 127.5* (C2'/C6'), 127.8 (C4), 129.4* (C3'/C5'), 129.8, 132.6 (C1'), 137.8 (C4'), 143.6 (C3), 174.6 (C2).

7.7.3 Method for the preparation of 3-alkenyl-N-alkylated-5,7-dibromoisatin derivatives

General Method C: Alkene isatin derivatives were synthesised according to the protocol of Hung et al.432 The isatin derivative (1 equiv.) was dissolved in ethanol (10 ml per 0.15 g of isatin derivative) and to the stirred solution piperidine (0.1 equiv.) and an appropriate aldehyde (1equiv.) were added at 0°C. The reaction mixture was refluxed for 4h than extracted with
EtOAc, dried over MgSO₄ and the solvent removed on the rotary evaporator. The product was purified by column chromatography.

**Synthesis of 1–(4-methoxybenzyl)-3-(4-chlorophenylmethylidene)-5,7-dibromoindolin-2-one (243)**

The title compound (243) was synthesised from 5,7-dibromo-N-(4-methoxybenzyl)-indolin-2-one (241) and 4-chlorobenzaldehyde according to method C and purified by flash chromatography on silica gel (DCM/EtOAc 8:1) to give a yellow powder (0.021 g, 16% E isomer), m.p. 180-182 °C, Rₕ 0.71 (DCM/Hex 5:1). LREI-MS: m/z 531; 533; 535 [M+] 79Br²⁷Br; 79Br²⁷Br²⁹Br; ²⁹Br²⁷Br²⁹Br. HRESI-MS: calcd for C₂₃H₁₇Br²ClNO₂ 79Br²⁷Br²⁷Br (M+H⁺) 531.9243 found 531.9267. 

**Synthesis of 1–(4-methoxybenzyl)-3-(4-methylphenylmethylidene)-5,7-dibromo-indol-2-one (244 a and 244 b)**

The title compounds (244 a and 244 b) were synthesised from 5,7-dibromo-N-(4-methoxybenzyl)-indolin-2-one (241) and o-chlorobenzaldehyde according to method C and purified by flash chromatography on silica gel (DCM) to give two isomers (E, Z).

E isomer, a dark yellow solid (0.021 g, 31%), m.p. 173-175 °C/ LREI-MS: m/z 531; 533; 535 [M+] 79Br²⁷Br; 79Br²⁷Br²⁹Br; ²⁹Br²⁷Br²⁹Br. HRESI-MS: calcd for C₂₂H₁₇Br₂ClNO₂ 79Br²⁷Br²⁷Br (M+H⁺) 531.9323 found 531.9315. 

1H NMR (CDCl₃, 500 MHz) δ 3.71 (s, 3H, OCH₃), 5.31 (s, 2H, CH₂), 6.77 (d, J = 8.5 Hz, 2H, H3’/H5’), 7.10 (d, J = 8.5 Hz, 2H, H2’/H6’), 7.44-7.46 (m, 3H, H, H3’’/H5’’), 7.84 (d, J = 7.5 Hz, 2H, H2’’/H6’’), 7.67 (s, 1H, H-vinyl), 7.90 (s, 1H, H6). 1³C NMR (126 MHz, CDCl₃): δ 44.2 (CH₂), 57.6 (OCH₃), 114.1* (C3’/C5’), 115.1, 122.0, 125.0, 125.2, 127.9, 126.5 (C6), 128.2 (C4’), 130.8* (C2’/C6’), 133.8 (C4), 134.1 (C4’’), 136.5, 137.4, 139.1, 141.1, 159.8 (C4’), 167.0 (C2).
128.5* (C2’/C6’), 132.1 (C4), 133.1 (C1’), 135.8 (C4’’), 136.5, 137.2, 139.3, 141.0, 159.1 (C4’), 168.1 (C2).

Z isomer, a dark yellow solid (0.009 g, 14%). **LREI-MS:** m/z 531; 533; 535 [M+] \(^{79}\)Br\(^{81}\)Br; \(^{79}\)Br\(^{81}\)Br; \(^{81}\)Br\(^{81}\)Br. **\(^1\)H NMR** (CDCl\(_3\), 500 MHz) \(\delta\) 3.73 (s, 3H, OCH\(_3\)), 4.83 (s, 2H, CH\(_2\)), 6.79 (d, \(J = 8.5\) Hz, 2H, H3’/H5’), 7.15 (d, \(J = 9\) Hz, 2H, H2’/H6’), 7.38 (s, 1H, H4), 7.39-7.40 (m, H4’, H5’), 7.42 (s, 1H, H6), 7.51 (d, \(J = 8.5\) Hz, 1H, H3’), 7.58 (d, \(J = 8.5\) Hz, 1H, H6’), 7.98 (s, 1H, H-vinyl).

**Synthesis of 1–(4-Methoxybenzyl)-3-(4-nitrophosphylethylidenyl)-5,7-dibromoindolin-2-one (245) 71:29**

The title compound (245) was synthesised from 5,7-dibromo-N–(4-methoxybenzyl)-indolin-2-one (241) and \(p\)-nitrobenzaldehyde according to method C and purified by flash chromatography on silica gel (DCM/EtOAc 8:1) to give a yellow powder (0.034 g, 17%), \(R_f\) 0.87 (DCM), Z isomer. **LREI-MS:** m/z 542; 544; 546 [M+] \(^{79}\)Br\(^{81}\)Br; \(^{79}\)Br\(^{81}\)Br; \(^{81}\)Br\(^{81}\)Br. **HRESI-MS:** calcd for C\(_{23}\)H\(_{17}\)Br\(_2\)N\(_2\)O\(_4\) \(^{79}\)Br\(^{81}\)Br (M+H\(^+\)) 542.9555 found 542.9535. **\(^1\)H NMR** (CDCl\(_3\), 500 MHz) \(\delta\) 3.78 (s, 3H, OCH\(_3\)), 5.42 (s, 2H, CH\(_2\)), 6.84 (d, \(J = 8.5\) Hz, 2H, H3’/H5’), 7.18 (d, \(J = 8.5\) Hz, 2H, H2’/H6’), 7.48 (s, 1H, H4), 7.49 (s, 1H, H-vinyl), 7.51, (d, \(J = 7.5\) Hz, 2H, C3’/H5’), 7.94 (s, 1H, H6), 8.36 (d, \(J = 8.5\) Hz, 2H, H2’/H6’).

**Synthesis of (Z)-1–(4-Methoxybenzyl)-3-(4-hydroxyphenethylidenyl)-5,7-dibromoindolin-2-one (246) 73:27**

The title compound (246) was synthesised from 5,7-dibromo-N–(4-methoxybenzyl)-indolin-2-one (241) and \(p\)-hydroxybenzaldehyde according to method C and purified by flash chromatography on silica gel (DCM/EtOAc 6:1) to give a yellow powder (0.032 g, 26%), \(R_f\) 0.81 (DCM), Z isomer. **LREI-MS:** m/z 542; 544; 546 [M+] \(^{79}\)Br\(^{81}\)Br; \(^{79}\)Br\(^{81}\)Br; \(^{81}\)Br\(^{81}\)Br. **HRESI-MS:** calcd for C\(_{23}\)H\(_{18}\)Br\(_2\)NO \(^{79}\)Br\(^{79}\)Br (M+H\(^+\)) 542.9555 found 542.9535. **\(^1\)H NMR** (DMSO-\(d_6\), 500 MHz) \(\delta\) 3.67 (s, 3H, OCH\(_3\)), 3.68 (s, 2H, CH\(_2\)), 6.38 (d, \(J = 8.5\) Hz, 2H, H3’/H5’), 6.81 (d, \(J = 8.0\) Hz, 2H, H3’/H5’), 6.97 (d, \(J = 8.5\) Hz, 2H, H2’/H6’), 6.36 (s, 1H, H4), 7.86, (s, 1H, H-vinyl), 7.90 (s, 1H, H6), 8.41 (d, \(J = 8.5\) Hz, 2H, H2’/H6’). **\(^13\)C NMR**
(DMSO-$d_6$, 126 MHz): 43.4 (CH$_3$), 55.4 (OCH$_3$), 102.4, 114.3* (C3’/C5’), 114.6, 115.9* (C3’’/C5’’), 116.2, 121.3 (C6), 125.5 (C1’’), 127.7* (C2’/C6’), 130.2 (C1’), 132.1, 134.1 (C4), 136.4* (C2’’/C6’’), 136.6 (C7a), 142.0 (CH), 158.6 (C4’), 161.9 (C4’’), 166.3 (C2).

Synthesis of 1–(4-methoxybenzyl)-3-(4-methoxyphenylmethylidenyl)-5,7-dibromoindolin-2-one (247)

The title compound (247) was synthesised from 5,7-dibromo-N–(4-methoxybenzyl)-indolin-2-one (241) and p-methoxybenzaldehyde according to method C and purified by flash chromatography on silica gel (DCM) to give a dark yellow powder Z isomer (0.017 g, 26%), $R_f$ 84 (DCM). LREI-MS: m/z 527; 529; 531 [M$^+$ $^{79}$Br$^{79}$Br; $^{79}$Br$^{81}$Br; $^{81}$Br$^{81}$Br. HRESI-MS: calcd for C$_{24}$H$_{20}$Br$_2$NO$_3$ $^{79}$Br$^{79}$Br (M+H$^+$) 527.9810 found 527.9816.

$^1$H NMR (CDCl$_3$, 500 MHz) $\delta$ 3.74 (s, 3H, OCH$_3$), 3.78 (s, 3H, OCH$_3$), 5.39 (s, 2H, CH$_2$), 6.80 (d, $J = 8.5$ Hz, 2H, H3’/H5’), 6.94 (d, $J = 8.0$ Hz, 2H, H3’’/H5’’), 7.14 (d, $J = 8.5$ Hz, 2H, H2’/H6’), 7.44 (s, 1H, H4), 7.48 (s, 1H, H-vinyl), 7.55 (s, 1H, H6), 8.41 (d, $J = 8.0$ Hz, 2H, H2’’/H6’’). $^{13}$C NMR (CDCl$_3$, 126 MHz): $\delta$ 38.1 (CH$_3$), 50.0 (OCH$_3$), 50.1 (OCH$_3$), 108.9* (C3’/C5’), 109.0* (C3’’/C5’’), 118.0, 118.1 (C4), 121.0, 122.8* (C2’/C6’), 123.0* (C2’’/C6’’), 123.2, 124.2 (C6), 128.3 (C1’’), 130.3 (C1’), 130.4, 135.1, 138.0, 157.0 (C4’), 159.1 (C4’’), 161.0 (C2).

Synthesis of 1–(4-methoxybenzyl)-3-(4-methylphenylmethylidenyl)-5,7-dibromo-indolin-2-one (248)

The title compounds (248a and 248b) were synthesised from 5,7-dibromo-N–(4-methoxybenzyl)-indolin-2-one (241) and p-methoxybenzaldehyde according to method C and purified by flash chromatography on silica gel (DCM/EtOAc 8:1) to give two isomers.

Z isomer (0.020 g, 33%, a dark orange solid), $R_f$ 0.87 (DCM). LREI-MS: m/z 511; 513; 515 [M$^+$] $^{79}$Br$^{79}$Br; $^{79}$Br$^{81}$Br; $^{81}$Br$^{81}$Br. HRESI-MS: calcd for C$_{24}$H$_{20}$Br$_2$NO$_2$ $^{79}$Br$^{79}$Br (M+H$^+$) 511.9851 found 511.9861. $^1$H NMR (CDCl$_3$, 500 MHz) $\delta$ 2.41 (s, 3H, CH$_3$), 3.79 (s, 3H, OCH$_3$), 5.41 (s, 1H, CH$_2$), 6.81 (d, $J = 8.5$ Hz, 1H, H3’’/H5’’), 7.14 (d, $J = 8.5$ Hz, 1H, H3’/H5’), 7.26 (d, $J = 8.5$ Hz, 1H, H2’/H6’), 7.42 (s, 1H, H4), 7.54 (s, 1H, H-vinyl), 7.96 (s, 1H, H6), 8.25 (d, $J = 8.5$ Hz, 1H, H2’’/H6’’). $^{13}$C NMR (CDCl$_3$, 126 MHz): $\delta$ 22.0 (CH$_3$), 43.8 (CH$_2$), 55.4 (OCH$_3$), 113.0, 114.2* (C3’/C5’), 114.9, 121.1, 122.6* (C2’/C6’), 127.9, 129.0* (C2’’/C6’’), 129.5, 129.8, 130.1* (C3’’/C5’’), 131.1 (C1’’), 133.0, 135.8 (C4’’), 140.4 (CH), 142.8 (C4’’), 156.8 (C4’), 168.2 (C2).
Chapter 7

Experimental

_**E**_ isomer (0.014 g, 21%, a dark orange solid). **LREI-MS**: m/z 511; 513; 515 [M+] ⁷⁹Br⁷⁹Br; ⁷⁹Br³¹Br, ⁸¹Br⁸¹Br. **¹H NMR** (CDCl₃, 500 MHz) δ 2.43 (s, 3H, CH₃), 3.75 (s, 3H, OCH₃), 5.40 (s, 1H, CH₂), 6.82 (d, J = 8.5 Hz, 1H, H₃'/H₅''), 7.15 (d, J = 8.5 Hz, 1H, H₃'/H₅'), 7.28 (d, J = 8.5 Hz, 1H, H₂'/H₆'), 7.42 (s, 1H, H₆), 7.62 (d, J = 8.5 Hz, 1H, H₂''/H₆''), 7.81 (s, 1H, H₄), 7.96 (s, 1H, H-vinyl). **¹³C NMR** (CDCl₃, 126 MHz): δ 21.3 (CH₃), 44.3 (CH₂), 55.5 (OCH₃), 111.2, 114.2* (C₃'/C₅'), 123.3, 124.6* (C₂''/C₆''), 126.4* (C₃''/C₅''), 127.9, 128.0 (C1'), 129.7, 129.9* (C₃''/C₅''), 131.2, 134.3, 136.1, 138.9 (CH), 140.1 (C1''), 141.3 (C₄''), 158.2 (C₄'), 169.1 (C2).

Synthesis of 1–(4-methoxybenzyl)-3-(4-ethylphenylmethylidene)-5,7-dibromoindol-2-one (249a and 249b)

The title compound (249a and 249b) was synthesised from 5,7-dibromo-N-(4-methoxybenzyl)-indolin-2-one (241) and _p_-ethylbenzaldehyde according to method C and purified by flash chromatography on silica gel (DCM/EtOAc 8:1) to give _E_ and _Z_ isomer.

_Z_ isomer **249a** (0.035 g, 27%, a dark yellow solid), Rᵣ 0.90 (DCM). **LREI-MS**: m/z 525; 527; 529 [M+] ⁷⁹Br⁷⁹Br; ⁷⁹Br³¹Br, ⁸¹Br³¹Br. **HRESI-MS**: calcd for C₂₅H₂₂NO₂Br₂ ⁷⁹Br⁷⁹Br (M+H⁺) 526.0017 found 526.0005. **¹H NMR** (CDCl₃, 500 MHz) δ 1.20-1.22 (m, 3H, CH₃), 2.44-2.47 (m, 2H, CH₂), 3.71 (s, 3H, OCH₃), 5.35 (s, 2H, CH₂), 6.75 (d, J = 8.5 Hz, 2H, H₃''/H₅''), 7.11 (d, J = 8.0 Hz, 2H, H₃'/H₅'), 7.24 (d, J = 8.5 Hz, 2H, H₂'/H₆'), 7.43 (s, 1H, H₆), 7.50 (s, 1H, H-vinyl), 7.56 (s, 1H, H₄), 8.23 (d, J = 8.5 Hz, 1H, H₂''/H₆''). **¹³C NMR** (CDCl₃, 126 MHz): δ 15.4 (CH₃), 29.2 (CH₂), 44.3 (CH₂), 55.5 (OCH₃), 102.4, 114.2* (C₃'/C₅'), 123.7, 124.6* (C₃''/C₅''), 126.3, 128.0* (C₃''/C₆''), 128.7* (C₂'/C₆'), 129.8, 130.5, 132.6, 133.2, 136.0, 136.9, 139.9, 140.4, 141.3, 146.7, 148.6, 158.7, 164.9 (C2).

_E_ isomer **249b** (0.010 g, 8%, a dark yellow solid). **LREI-MS**: m/z 525; 527; 529 [M+] ⁷⁹Br⁷⁹Br; ⁷⁹Br³¹Br, ⁸¹Br³¹Br. **¹H NMR** (CDCl₃, 500 MHz) δ 1.31 (t, J = 7.5 Hz, 3H, CH₃-ethyl), 2.75 (q, J = 7.5 Hz, 2H, CH₂-ethyl), 3.77 (s, 3H, OCH₃), 5.42 (s, 2H, CH₂), 6.84 (d, J = 8.5 Hz, 1H, H₃''/H₅''), 7.17 (d, J = 9 Hz, 1H, H₃'/H₅'), 7.34 (d, J = 9 Hz, 1H, H₂'/H₆'), 7.48 (s, 1H, H₄), 7.55 (d, J = 8.5 Hz, 1H, H₂''/H₆''), 7.83 (s, 1H, H₆), 7.98 (s, 1H, H-vinyl). **¹³C NMR** (CDCl₃, 126 MHz): δ 18.3 (CH₃), 24.6 (CH₂), 44.3 (CH₂), 55.5 (OCH₃), 102.3, 114.2* (C₃'/C₅'), 123.7, 124.6* (C₃''/C₅''), 128.0* (C₂''/C₆''), 128.7, 129.8* (C₂'/C₆'), 130.1, 134.2, 137.0, 141.3, 148.6, 158.2 (C₄'), 162.5 (C2).
Synthesis of 1-(4-methylbenzyl)-3-(4-methylphenylmethylidene) 5,7-dibromoindolin-2-one (250a and 250b)

The title compounds (250a and 250b) were synthesised from 1-(4-methylbenzyl)-5,7-dibromo-indoline-2,3-dione (242) and \( \text{p}-\text{chlorobenzaldehyde} \) according to the method C and purified by flash chromatography on silica gel (DCM. EtOAc/DCM 9:1) to give two isomers (E:Z).

E isomer 250a (0.020 g, 33%), m.p. 180-182°C (dark yellow solid), \( R_f \) 0.63 (DCM/Hex 5:1). \( \text{LREI-MS: m/z} \ 515; 517; 519 \ [M+] \ 79^{79}\text{Br}^{79}\text{Br}, \ 79^{81}\text{Br}, \ 81^{81}\text{Br}. \ \text{HRESI-MS: calcd for C}_{23}\text{H}_{17}\text{Br}_2\text{ClNO} \ 79^{79}\text{Br}^{79}\text{Br} (\text{M}+\text{H})^+ \ 515.9278 \text{ found } 515.9385. \ \text{\textsuperscript{1}H NMR} \ 500 \text{ MHz} \ \delta \ 2.32 (s, 3H, CH$_3$), 5.44 (s, 2H, CH$_2$), 7.11 (s, 4H, H2'/H3'/H5'/H6'), 7.49 (d, \( J = 8.5 \) Hz, 2H, H2''/H6''), 7.50 (s, 1H, H6), 7.56 (d, \( J = 8.5 \) Hz, 2H, H4''/H6''), 7.68 (s, 1H, H4), 7.92 (s, 1H, H-vinyl). \ \text{\textsuperscript{13}C NMR} \ 126 \text{ MHz, CDCl}_3; \ \delta \ 21.3 \ (\text{CH}_3), 46.8 \ (\text{CH}_2), 109.4, 116.0, 126.8 \ (\text{C}6), 127.1^* \ (\text{C}2'/\text{C}6'), 127.8^* \ (\text{C}3'/\text{C}5'), 128.5^* \ (\text{C}3''/\text{C}5''), 129.3^* \ (\text{C}2''/\text{C}6''), 129.8, 132.5^* \ (\text{C}1''), 134.7 \ (\text{C}1'), 137.4 \ (\text{CH}), 142.2 \ (\text{C}6, E), 149.9, 152.7, 158.7 \ (\text{C}4'), 160.0 \ (\text{C}4''), 164.6 \ (\text{C}2).

Z isomer 250b (0.009 g, 10%, dark yellow solid). \( \text{LREI-MS: m/z} \ 515; 517; 519 \ [M+] \ 79^{79}\text{Br}^{79}\text{Br}, \ 79^{81}\text{Br}, \ 81^{81}\text{Br}. \ \text{HRESI-MS: calcd for C}_{23}\text{H}_{17}\text{Br}_2\text{ClNO} \ 79^{79}\text{Br}^{79}\text{Br} (\text{M}+\text{H})^+ \ 516.0879 \text{ found } 516.0899. \ \text{\textsuperscript{1}H NMR} \ 500 \text{ MHz} \ \delta \ 2.99 (s, 3H, CH$_3$), 5.41 (s, 2H, CH$_2$), 7.09 (s, 4H, H2'/H3'/H5'/H6'), 7.49 (d, \( J = 8.5 \) Hz, 2H, H3''/H5''), 7.49 (d, \( J = 8.5 \) Hz, 2H, H3'/H5'), 7.61 (s, 1H, H4), 7.68 (s, 1H, H6), 7.92 (s, 1H, H vinyl), 8.29 (d, \( J = 8.5 \) Hz, 2H, H2''/H6''). \ \text{\textsuperscript{13}C NMR} \ 126 \text{ MHz, CDCl}_3; \ \delta \ 21.3 \ (\text{CH}_3), 44.8 \ (\text{CH}_2), 113.8, 114.9, 121.5, 124.7, 126.5^* \ (\text{C}2'/\text{C}6'), 129.0^* \ (\text{C}3'/\text{C}5'), 129.5^* \ (\text{C}3'/\text{C}5''), 130.8, 134.1^* \ (\text{C}2''/\text{C}6''), 134.4, 136.4 \ (\text{C}4'), 137.5, 138.5, 139.2 \ (\text{CH-vinyl}), 140.1, 142.3, 168.6 \ (\text{C}2).

Synthesis of 1-(4-methylbenzyl)-3-(4-methylphenylmethylidene) 5,7-dibromoindolin-2-one (251a and 251b)

The title compounds (251a and 251b) were synthesised from 1-(4-methylbenzyl)-5,7-dibromo-indoline-2,3-dione (242) and 4-methylbenzaldehyde according to the method C and purified by flash chromatography on silica gel (DCM. EtOAc/DCM 9:1) to give two isomers (E:Z).
E isomer 251a (0.022 g, 36%, an orange solid), R, 0.85 (DCM). LREI-MS: m/z 495; 497; 499 [M+] 79Br79Br; 79Br81Br; 81Br81Br. HRESI-MS: calcd for C24H20Br2NO 79Br79Br (M+H') 495.9903 found 495.9912. 1H NMR (CDCl3, 500 MHz) δ 2.30 (s, 3H, CH3), 2.44 (s, 3H, CH3), 5.43 (s, 2H, CH2), 7.09 (s, 4H, H2'/H3'/H5'/H6'), 7.30 (d, J = 9 Hz, 2H, H2''/H6''), 7.46 (s, 1H, H4), 7.51 (d, J = 9 Hz, 2H, H3''/H5''), 7.82 (s, 1H, H6), 7.98 (s, 1H, H-vinyl). 13C NMR (CDCl3, 126 MHz): δ 20.1 (CH2), 20.3 (CH3), 44.1 (CH2), 114.8 (C5), 124.5 (C3), 124.8 (C7), 126.3 (C3a), 126.5 (C6), 126.8 (C2''/C6''), 129.1 (C4), 128.8* (C2''/C6''), 129.2* (C3'/C6'), 129.9* (C3''/C5''), 131.0 (C2'), 131.2 (C2''), 132.6, 134.5 (C5''), 137.0 (C5'), 141.3 (C7a, C1''), 170.2 (C2).

Z isomer 251b Yield 0.0081 g (14%, an orange solid). LREI-MS: m/z 495; 497; 499 [M+] 79Br79Br; 79Br81Br; 81Br81Br. HRESI-MS: calcd for C24H20Br2NO 79Br79Br (M+H') 495.9886 found 495.9899. 1H NMR (CDCl3, 500 MHz) δ 2.31 (s, 3H, CH3), 2.41 (s, 3H, CH3), 5.45 (s, 2H, CH2), 7.09 (s, 4H, H2'/H3'/H5'/H6'), 7.27 (d, J = 8.5 Hz, 2H, H3''/H5''), 7.49 (s, 1H, C4), 7.57 (s, 1H, H-vinyl), 7.62 (s, 1H, H6), 8.26 (d, J = 8.5 Hz, H2''/H6'').

Synthesis of 1-(4-methylbenzyl)-3-(4-methylphenylmethylidene) 5,7-dibromoindolin-2-one (252a and 252b)
The title compounds (252a and 252b) were synthesised from 1-(4-methylbenzyl)-5,7-dibromo-indoline-2,3-dione (242) and p-ethylbenzaldehyde according to the method C and purified by flash chromatography on silica gel (DCM. EtOAc/DCM 9:1) to give two isomers.

Z isomer 252a Yield 0.025 g (39%, a dark yellow solid), R, 0.91 (DCM). LREI-MS: m/z 509; 511; 513 [M+] 79Br79Br; 79Br81Br; 81Br81Br. HRESI-MS: calcd for C25H22Br2NO (M+H') 499.9912 found 495.9898. 1H NMR (CDCl3, 500 MHz) δ 1.23 (t, J = 7.5 Hz, 3H, CH3), 2.33 (s, 3H, CH3), 2.76 (q, J = 7.5 Hz, 2H, CH2), 5.46 (s, 2H, CH2), 7.12 (s, 4H, H2'/H3'/H5'/H6'), 7.35 (d, J = 8.5 Hz, 2H, H3''/H5''), 7.48 (s, 1H, H4), 7.85 (s, 1H, H6), 8.01 (s, 1H, H-vinyl), 8.25 (d, J = 8.5 Hz, 2H, H2''/H6''). 13C NMR (126 MHz, CDCl3): δ 19.3 (CH3), 20.5 (CH3), 29.1 (CH2), 43.6 (CH3), 103.1 (C5), 114.2 (C3), 124.1 (C3a), 124.5 (C7), 126.2 (C6), 126.6* (C3''/C5''), 128.7* (C2''/C6''), 129.5* (C2''/C6''), 129.9* (C3'/C5'), 130.6, 131.8 (C4), 133.5, 134.3 (C2''), 136.5 (C5''), 141.2 (C7a), 147.8 (C5''), 169.5 (C2).

E isomer 252b somer Yield 0.0103 g (16%, dark yellow solid). LREI-MS: m/z 509; 511; 513 [M+] 79Br79Br; 79Br81Br; 81Br81Br. 1H NMR (CDCl3, 500 MHz) δ 1.32 (t, J = 7.5 Hz, 3H, CH3),
2.32 (s, 3H, CH$_3$), 2.76-2.78 (m, 2H, CH$_2$), 5.39 (s, 2H, CH$_2$), 7.12 (s, 4H, H2'/H3'/H5'/H6'), 7.34 (d, $J = 8.5$ Hz, 2H, H2''/H6''), 7.45 (s, 1H, H4), 7.57 (d, $J = 8.5$ Hz, 2H, H3''/H5''), 7.85 (s, 1H, H6), 8.01 (s, 1H, H-vinyl).

**Synthesis of 3,5-dimethylpyrrole-2-carboxaldehyde (257)**

The title compound 257 was synthesised according to the protocol of Terenin et al. Phosphorus oxychloride (10 mmol) was added dropwise with stirring and cooling to dry DMF (25 ml). The product was stirred for 30 min at 0ºC and then a solution of 2,4-dimethylpyrrole (2 mmol) in DMF (3 ml) was added dropwise. The product was stirred for another 24 h at 20ºC and poured onto crushed ice. The aqueous solution was neutralized with sodium carbonate and the precipitate formed was filtered off, washed with warm water, dried, and purified by column chromatography to give 0.0369g, 67%. LREI-MS: m/z 123.15 [M+]. $^1$H NMR (DMSO) δ: 2.31 (s, 6H, CH$_3$), 5.85 (s, 1H, Arc), 9.46 (s, 1H, COOH).

**Synthesis of (Z)-1-(4-methoxybenzyl)-3-((3,5-dimethyl-1H-pyrrol-2-yl)methylene)-5,7-dibromoindolin-2-one (253)**

The title compound (253) was synthesised from 1-(4-methoxybenzyl)-5,7-dibromo-indoline-2,3-dione (241) and 3,5-dimethyl-1H-pyrrole-2-carbaldehyde according to the method C and purified by flash chromatography on silica gel (DCM) to give a dark yellow solid (0.1405 g, 57%, m.p. 205-207 ºC, R$_f$ 0.59 (DCM/Hex 5:2). LREI-MS: m/z 514; 516; 518 [M+] $^{79}$Br$^{79}$Br; $^{79}$Br$^{81}$Br; $^{81}$Br$^{81}$Br. HRESI-MS: calcd for C$_{23}$H$_{21}$Br$_2$N$_2$O$_2$ $^{79}$Br$^{79}$Br (M+H$^+$) 514.9933 found 514.9903. $^1$H NMR (CDCl$_3$, 500 MHz) δ 2.34 (s, 3H, CH$_3$), 2.36 (s, 3H, CH$_3$), 3.75 (s, 3H, OCH$_3$), 5.46 (s, 2H, CH$_2$), 6.01 (s, 1H, H4''), 6.82 (d, $J = 9$ Hz, 2H, H3'/H5''), 7.09 (d, $J = 8.5$ Hz, 2H, H2'/H6''), 7.31 (s, 1H, H-vinyl), 7.32 (s, 1H, H4), 7.51 (s, 1H, H6). $^{13}$C NMR (CDCl$_3$, 126 MHz): δ 11.9 (CH$_3$), 14.3 (CH$_3$), 43.9 (CH$_2$), 55.4 (OCH$_3$), 102.8, 108.4, 113.9 (C4''), 114.2* (C3'/C5''), 114.6, 118.8 (C6), 124.5 (C4), 127.5* (C2'/C6''), 127.7 (p), 130.1 (C1''), 130.5 (7a), 132.1 (C1''), 134.7, 135.3, 139.5, 158.8 (C4''), 168.6 (C2).

**Synthesis of 1-(4-methoxybenzyl)-3-((1H-pyrrol-2-yl)methylene-5,7-dibromoindolin-2-one (254)**

The title compound (2) was synthesised from 1-(4-methoxybenzyl)-5,7-dibromo-indoline-2,3-dione (241) and 1H-pyrrole-2-carbaldehyde according to the method C and purified by flash
chromatography on silica gel (DCM) to give a dark yellow solid (0.1013 g, 54%), m.p. 199-201 °C, Rf 0.57 (DCM/Hex 5:2). LREI-MS: m/z 486; 488; 490 [M+] \(^{79}\)Br\(^{79}\)Br; \(^{79}\)Br\(^{81}\)Br; \(^{81}\)Br\(^{81}\)Br. HRESI-MS: calcd for C\(_{21}\)H\(_{17}\)Br\(_2\)N\(_2\)O\(_2\) \(\text{Br}^{79}\)Br\(^{79}\)Br (M+H\(^+\)) 486.9594 found 486.9657. \(^1\)H NMR (CDCl\(_3\), 500 MHz) \(\delta\) 3.78 (s, 3H, OCH\(_3\)), 5.50 (s, 2H, CH\(_2\)), 6.43-6.45 (m, 1H, H5''), 6.85 (d, J = 9 Hz, 2H, H2'/H6'), 7.24 (broad d, 1H, H4''), 7.45-7.50 (1H, H5), 7.48 (s, 1H, H1-vinyl), 7.59 (s, 1H, H6).

\(^{13}\)C NMR (CDCl\(_3\), 126 MHz): \(\delta\) 43.9 (CH\(_2\)), 55.3 (CH\(_3\)), 103.0, 112.6 (C5''), 113.1, 114.1* (C3'/C5'), 115.0, 119.8 (C6), 122.7 (C6''), 127.2 (C4''), 127.6* (C2'/C6'), 128.2 (C1''), 129.6 (C3a), 129.9 (C1'), 133.6 (C4), 135.1 (7a), 158.2 (C2).

Synthesis of 1-(4-methylbenzyl)-3-((3,5-dimethyl-1H-pyrrol-2-yl)methylene)-5,7-dibromoindolin-2-one (255)

The title compound (255) was synthesised from 1-(4-methylbenzyl)-5,7-dibromo-indoline-2,3-dione (242) and 3,5-dimethyl-1H-pyrrole-2-carbaldehyde according to the method C and purified by flash chromatography on silica gel (DCM, DCM/EtOAc 5:1) to give a dark yellow solid (0.12 g, 68%), 186-188°C, Rf 0.70 (DCM/Hex 5:2). LREI-MS: m/z 498; 500; 502 [M+] \(^{79}\)Br\(^{79}\)Br; \(^{79}\)Br\(^{81}\)Br; \(^{81}\)Br\(^{81}\)Br. HRESI-MS: calcd for C\(_{23}\)H\(_{21}\)Br\(_2\)N\(_2\)O \(\text{Br}^{79}\)Br\(^{79}\)Br (M+H\(^+\)) 498.0009 found 498.0021. \(^1\)H NMR (CDCl\(_3\), 500 MHz) \(\delta\) 2.27 (s, 3H, CH\(_3\)), 2.32 (s, 3H, CH\(_3\)), 3.33 (s, 3H, CH\(_3\)), 5.46 (s, 2H, CH\(_2\)), 5.99 (s, 1H, H4''), 7.02 (d, J = 8.5 Hz, 2H, H2'/H6'), 7.06 (d, J = 8.5 Hz, 2H, H2'/H6'), 7.30 (s, 1H, H4), 7.48 (s, 1H, H6), 7.50 (s, 1H, H-vinyl). \(^{13}\)C NMR (CDCl\(_3\), 126 MHz): \(\delta\) 11.8 (C7''), 14.2 (C8''), 21.2 (CH\(_3\)), 44.2 (CH\(_2\)), 102.8, 108.3, 113.8 (C5''), 114.5, 118.7 (C6), 124.4 (C4), 126.1* (C2'/C6'), 127.7 (pyrrole), 129.4* (C3'/C5'), 130.4 (C3), 132.0 (C1''), 134.7 (C1'), 134.9 (C4'), 135.5, 136.6 (pyrrole), 139.3 (pyrrole), 168.5 (C2).

Synthesis of 2-oxoindoline-5-sulfonyl chloride (258)

The title compound 258 was synthesised according to the protocol by Liang et al.\(^{155}\) 2-Oxindole was added slowly to the cooled sulfonic acid and mixture stirred at rt for 4h. Mixture was poured into the crushed ice and then extracted with EtOAc. The organic layer was dried and solvent removed in vacuo. The product was purified by column chromatography using DCM/EtOAc to yield an orange solid (0.28 g, 89%). LREI-MS: m/z 231 [M+]. \(^1\)H NMR
Experimental

(DMSO-$d_6$, 500 MHz) $\delta$ 3.45 (s, 2H, C3), 6.97 (d, 1H, $J$ = 8.5 Hz, H5), 7.50 (d, 1H, $J$ = 7.5 Hz, H6), 7.55 (s, 1H, H4), 9.5 (s, 1H, NH).

Synthesis of $N$-(4-methoxyphenyl)-2-oxoindoline-5-sulfonamide (259)
The title compound 259 was synthesised according to the protocol by Ivachtchenko et al.432 2-Oxoindoline-5-sulfonyl chloride (258) (1 equiv.) was dissolved in EtOH/H$_2$O 1:1 mixture and to the stirred solution 4-methoxyaniline was added (2equiv.). After one hour stirring at the rt, mixture was extracted with EtOAc, organic layer washed with brine, dried over MgSO$_4$ and solvent removed in vacuo. The product was purified by column chromatography (DCM; DCM:EtOAc 5:1) to yield an orange powder (0.074 g, 54%). $R_f$ 0.32 (DCM/EtOAc 3:1)

LREI-MS: m/z 318 [M+]. HRESI-MS: calcd for C$_{15}$H$_{14}$N$_2$O$_4$S (M+H$^+$) 318.0674 found 318.0675.

$^1$H NMR (DMSO) $\delta$ 3.53 (s, 2H, H3), 3.65 (s, 3H, OCH$_3$), 6.78 (d, $J$ = 8.5 Hz, 2H, C2'/C6'), 6.94 (d, $J$ = 8.5 Hz, 1H, ArH isatin), 6.96 (d, $J$ = 8.5 Hz, 2H, C3'/C5'), 7.49-7.51 (m, 2H, ArH isatin).

Synthesis of (E and Z)-3-(4-hydroxybenzylidene)-$N$-(4-methoxyphenyl)-2-oxoindoline-5-sulfonamide (260)
The title compound (260) was synthesised from $N$-(4-methoxyphenyl)-2-oxoindoline-5-sulfonamide (259) and 4-aminophenol according to method B and purified by column chromatography (DCM/EtOAc 4:1; 1:2) to give a red solid (0.0159, 24%). $R_f$ 0.46 (DCM/EtOAc 4:1)

LREI-MS: m/z [M+] 422. HRESI-MS: calcd for C$_{22}$H$_{18}$N$_2$O$_5$S (M+H$^+$) 422.0941 found 422.0941.

$^1$H NMR (DMSO): $\delta$ 6.78 (d, $J$ = 9 Hz, 2H, H3''/H5'', E), 6.81 (d, $J$ = 9 Hz, 2H, H3'/H5', Z), 6.86 (m, 2H, H3''/H5'', E+Z), 6.91-6.94 (m, 3H, H2''/H6'', H7, Z), 6.97-6.99 (m, 2H, H2'/H6', E), 7.00 (d, $J$ = 7.5 Hz, H7, E), 7.44 (d, $J$ = 9 Hz, 2H, H2''/H6'', Z), 7.47 (d, $J$ = 7.5, 1H, H6, Z), 7.54 (d, $J$ = 8.5 Hz, 2H, H6, E), 7.61 (s, 1H, CH, Z), 7.79 (s, 1H, CH, E), 7.90 (s, 1H, C4, Z), 7.98 (s, 1H, C4, E), 8.43 (d, $J$ = 8.5 Hz, 2H, H2''/H6'', E). $^{13}$C NMR (126 MHz, DMSO): $\delta$ 58.1* (CH$_3$, E+Z), 109.7, 110.6, 114.9* (C3''/C5'', E+Z), 116.1* (C3''/C5'', E), 116.5* (C3''/C5'', Z), 118.3, 118.4, 121.1 (C4, E), 121.1 (C4, Z), 123.6, 124.1 (C2'/C6', E), 124.7* (C2'/C6', Z), 125.1, 125.9, 126.4, 127.8, 129.1, 129.3, 130.9, 131.1, 132.1, 132.6, 136.1, 139.6 (CH, Z), 141.1 (CH, E), 142.5, 143.7 (C7a, E), 146.4 (C7a, Z), 157.1 (C4', E), 157.3 (C4', Z), 160.6 (C4'', Z), 161.6 (C4'', E), 168.1 (C2, E), 169.7 (C2, Z).
Synthesis of 5-(morpholinosulfonyl)indolin-2-one (261)

The title compound (261) was synthesised from 2-oxoindoline-5-sulfonylchloride and morpholine by the method of Ivachtchenko et al. and purified by column chromatography (DCM/EtOAc 4:1; 1:2) to give a yellow powder (0.1295 g, 27%), Rf 0.21 (DCM/EtOAc 3:1).

**LREI-MS**: m/z [M+]+ 282. **HRESI-MS**: calcd for C_{12}H_{15}N_{2}O_{4}S (M+H') 282.0674 found 282.0678.

**{H NMR} (CDCl\textsubscript{3}, 500 MHz)** δ 3.51 (s, 2H, H3), 3.29 (t, J = 3.5 Hz, 4H, H2'/H6'), 3.73-3.75 (m, 4H, H3'/H5'), 6.76 (d, J = 9 Hz, 1H, H6), 7.61 (s, J = 8.5 Hz, 1H, H7), 7.92 (s, 1H, H4).
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Figure 9.1. Maps showing three algal collection sites, Picture 1: Red circle represents part of Australia where samples were collected⁴³⁸, Picture 2: Three collections spots are marked with a red flower.⁴³⁹
9.1 10% Methanol extract of Haliclona sp.

The chemical composition of the six column fractions of 10% methanol extract obtained from Haliclona sp. analysed by EI-MS is presented in the Table 9.1.

Table 9.1: EI-MS analysis of column chromatography fraction the 10% methanol extract obtained from Haliclona sp.

<table>
<thead>
<tr>
<th>No</th>
<th>Compounds identified</th>
<th>#1</th>
<th>#2</th>
<th>#3</th>
<th>#4</th>
<th>#5</th>
<th>#6</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10-Methylethapdecanoate</td>
<td>√</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>Androst-5-en-3β-ol</td>
<td>√</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>24-Methyl-26,27-dinorcholesta-5,23-dien-3β-ol</td>
<td>√</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>24-Ethylcholesta-5-en-3β-ol</td>
<td>√</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>Cholesta-5,20,24-trien-3β-ol</td>
<td>√</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>1-Phenanthreneacryloxylic acid</td>
<td>-</td>
<td>√</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>Cholesta-5-en-3β-ol</td>
<td>-</td>
<td>-</td>
<td>√</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>24-Methyl-3,25-bis(acetyloxy)-5-hydroxy-cholesta-6-one</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>√</td>
<td>-</td>
</tr>
<tr>
<td>9</td>
<td>1,3,4,6-Tetraacetyl-α-d-glucosamine</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>√</td>
</tr>
<tr>
<td>10</td>
<td>Pentaeacetylgalactose</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

*Fraction numbers refer to column chromatography combined fractions.

9.2 50% Methanol extract of Haliclona sp.

The chemical composition of the six column fractions of 50% methanol extract obtained from Haliclona sp. analysed by EI-MS is presented in the Table 9.2.

Table 9.2: EI-MS analysis of column chromatography fraction the 50% methanol extract obtained from Haliclona sp.

<table>
<thead>
<tr>
<th>No</th>
<th>Compounds identified</th>
<th>#1</th>
<th>#2</th>
<th>#3</th>
<th>#4</th>
<th>#5</th>
<th>#6</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3-Hydroxy-2(1H)-pyridinethionein</td>
<td>√</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>Hexadecanoic acid</td>
<td>√</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>Octadecanoic acid</td>
<td>√</td>
<td>-</td>
<td>-</td>
<td>√</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>2'-Deoxy-adenosine</td>
<td>-</td>
<td>√</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>1-Bromohexadecane</td>
<td>-</td>
<td>-</td>
<td>√</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>Cholesta-5,20,24-trien-3β-ol</td>
<td>-</td>
<td>-</td>
<td>√</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>14-Bromopentadecanoic acid</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>√</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>Eicosanoic acid</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>√</td>
</tr>
<tr>
<td>9</td>
<td>24-Ethylcholesta-5-en-3β-ol</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>√</td>
</tr>
<tr>
<td>10</td>
<td>24-Ethylcholesta-7-en-3β-ol</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>√</td>
</tr>
<tr>
<td>11</td>
<td>2-Hydrazino-8-hydroxy-4phenylquinoline</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>√</td>
</tr>
<tr>
<td>12</td>
<td>2-Methoxy-4-phenoxy-1,5-naphthyridine</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>√</td>
</tr>
<tr>
<td>13</td>
<td>Cholestan-3,5-diol</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>√</td>
</tr>
<tr>
<td>14</td>
<td>24-Methylcholesta-7,22-dien-3β-ol</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>√</td>
</tr>
<tr>
<td>15</td>
<td>22,23-Cyclopropyl-24-ethylcholesta-5-en-3β-ol</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>√</td>
</tr>
</tbody>
</table>

*Fraction numbers refer to column chromatography combined fractions.

9.3 10% Methanol column fractions of Ircinia/Sarcotragus sp.

The chemical composition of the six column fractions of 10% methanol extract obtained from Ircinia/Sarcotragus sp. analysed by EI-MS is presented in the Table 9.1.
9.4 Water extracts from *Haliclona* sp., *Ircina/Sarcotragus* sp. and *Geodia* sp.

Chemical composition of the three deep-sea sponge water extracts was analysed by high resolution ESI-QTof-MS; the tentatively identified compounds are presented in Table 9.4. Compounds were identified by comparing HRMS data with those from the Dictionary of Natural Compounds.

**Table 9.4:** EI-MS analysis of the water extracts from *Haliclona* sp., *Ircina/Sarcotragus* sp. and *Geodia* sp.

<table>
<thead>
<tr>
<th>Ret. time</th>
<th>Compound name</th>
<th>MW</th>
<th>A</th>
<th>B</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>16.5</td>
<td>1H-Indole-3-carboxaldehyde; N-Ac</td>
<td>187</td>
<td>√</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>18.0</td>
<td>Adenine; 6-N-Benzoyl</td>
<td>239</td>
<td>-</td>
<td></td>
<td>√</td>
</tr>
<tr>
<td>18.8</td>
<td>2-Debromo-dispacamide A9,10-dihydrokeramadine</td>
<td>326</td>
<td>-</td>
<td>√</td>
<td>-</td>
</tr>
<tr>
<td>19.8</td>
<td>5,6'-Dibromomindigotin; N,N'-Di-Ac</td>
<td>504</td>
<td>-</td>
<td>√</td>
<td>-</td>
</tr>
<tr>
<td>19.9</td>
<td>Pseudoceratinine B</td>
<td>504</td>
<td>√</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>21.4</td>
<td>2'-Deoxyinosine</td>
<td>256</td>
<td>-</td>
<td>√</td>
<td>-</td>
</tr>
<tr>
<td>24.1</td>
<td>Echinosulfone A</td>
<td>498</td>
<td>√</td>
<td>√</td>
<td>-</td>
</tr>
<tr>
<td>25.8</td>
<td>Purpuramine H</td>
<td>622</td>
<td>-</td>
<td>√</td>
<td>-</td>
</tr>
<tr>
<td>27.3</td>
<td>2'-Amino-16-methyl-1,3-octadecanediol</td>
<td>746</td>
<td>-</td>
<td>√</td>
<td>-</td>
</tr>
<tr>
<td>28.6</td>
<td>Easipentin A</td>
<td>870</td>
<td>√</td>
<td>√</td>
<td>-</td>
</tr>
<tr>
<td>30.2</td>
<td>2'-Deoxyctydine; β-D-form, 3',4N,5'-Tribenzoyl</td>
<td>539</td>
<td>-</td>
<td>√</td>
<td>-</td>
</tr>
<tr>
<td>31.2</td>
<td>Adenosine; N'-Benzyl</td>
<td>357</td>
<td>-</td>
<td></td>
<td>√</td>
</tr>
<tr>
<td>37.6</td>
<td>Hyrtiosin B</td>
<td>320</td>
<td>√</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>42.1</td>
<td>Ageliferin; 1'-N-Me</td>
<td>632</td>
<td>-</td>
<td>√</td>
<td>-</td>
</tr>
<tr>
<td>43.6</td>
<td>Didemnimide A</td>
<td>278</td>
<td>√</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>48.3</td>
<td>Pseudoceratinine B</td>
<td>502</td>
<td>-</td>
<td></td>
<td>√</td>
</tr>
</tbody>
</table>

A: *Haliclona* sp.; B: *Ircina/Sarcotragus* sp.; C: *Geodia* sp.

9.5 Protein kinase B inhibition testing

Phosphor-Akt levels were determined by the Western blotting to indicate Akt activity. Cells treated with isatin derivatives were lysed by NP40 lysis buffer containing protein inhibitor cocktail, beta-glycerophosphate and PMSF. Proteins were separated by Biorad sodium dodecyl sulfate-polyacrylamide gel electrophoresis. After electrophoresis, proteins were transferred onto nitrocellulose membranes using a Biorad transfer electrophoresis unit (100 V for 1 hour). The membranes were then washed in TBST and incubated with 5% BSA for 1 hour followed by overnight incubation (4°C) with anti-pAkt473 antibody. The membranes were washed by TBST for three times (5 minutes each time) and then incubated for 1 hour with a HRP-linked second antibody. After three times of wash with TBST, the membranes were incubated with ECL for 1 minute and exposure to x-ray film. The protein levels were determined by densities of bands showed in x-ray film and adjusted by beta-actin as loading control.
9.6 Setup of the 96-well microplates used in the MTS assay

![Diagram of microplates with color indications]

- Purple: 100 uL media
- Green: 90 uL cells + 10 uL test compound in 2.5% DMSO
- Yellow: 100 uL cells
- Red: 90 uL cells + 2.5% DMSO
- Blue: 90 uL media + 10 uL test compound in 2.5% DMSO

9.7 Table of IC\(_{50}\) values of the isatin compounds screened for cytotoxicity against MDA-MB-231 breast cancer cell lines

Table 9.7 shows IC\(_{50}\) values of the isatin compounds screened for cytotoxicity against MDA-MB-231 breast cancer cell lines in comparison with their values against U937 human lymphoma cancer cell lines.

<table>
<thead>
<tr>
<th>Cpd. no</th>
<th>Cytotoxicity IC(_{50}) (µM)</th>
<th>Cpd. no</th>
<th>Cytotoxicity IC(_{50}) (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MDA-MB-231</td>
<td>U937</td>
<td>MDA-MB-231</td>
</tr>
<tr>
<td>178</td>
<td>19.2</td>
<td>3.8</td>
<td>223</td>
</tr>
<tr>
<td>205</td>
<td>17.6</td>
<td>10.2</td>
<td>224</td>
</tr>
<tr>
<td>208</td>
<td>26.1</td>
<td>15.1</td>
<td>234</td>
</tr>
<tr>
<td>209</td>
<td>27.5</td>
<td>20.0</td>
<td>235</td>
</tr>
<tr>
<td></td>
<td>na</td>
<td>28.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>8.9</td>
<td></td>
</tr>
</tbody>
</table>
Publications
The chapters below contain work from the following published journal articles:

Chapter 1:

Chapter 2:
Ana Zivanovic, Danielle Skropeta, c-AMP dependent protein kinase A inhibitory activity of six algal extracts from South Eastern Australia and their fatty acid, *Natural Product Communications*, **2012**, 7(7), 923-927.

Chapter 3:

As the primary supervisor, I, Dr. Danielle Skropeta, declare that apart from the review in *Marine Drugs* where all authors contributed equally, for all other papers described above the greater part of the work is attributed to the candidate, Ana Zivanovic. In these articles, Ana 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 Ana 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. Ana 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.