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Novel analogs and a protein target for the napyradiomycins

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Novel Analogs and a Protein Target for the Napyradiomycins

A Dissertation submitted in partial satisfaction of the
Requirements for the Degree of Doctor of Philosophy

in
Oceanography

by
Lauge Luster Lindgren Farnaes

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2009
The Dissertation of Lauge Luster Lindgren Farnaes is approved, and it is acceptable in quality and form for publication on microfilm:

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Chair

University of California, San Diego

2009
DEDICATION

To my wife
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<tr>
<td>COSY</td>
<td>Correlation spectroscopy</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>ESI</td>
<td>electrospray ionization</td>
</tr>
<tr>
<td>FT-IR</td>
<td>Fourier transform infrared</td>
</tr>
<tr>
<td>HMBC</td>
<td>heteronuclear multiple bond correlation</td>
</tr>
<tr>
<td>HR</td>
<td>high resolution</td>
</tr>
<tr>
<td>HSP</td>
<td>heat shock protein</td>
</tr>
<tr>
<td>HSQC</td>
<td>heteronuclear single quantum coherence</td>
</tr>
<tr>
<td>LC/MS</td>
<td>liquid chromatography / mass spectroscopy</td>
</tr>
<tr>
<td>MALDI</td>
<td>matrix-assisted laser desorption/ionization</td>
</tr>
<tr>
<td>MTT</td>
<td>3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide</td>
</tr>
<tr>
<td>MS</td>
<td>mass spectroscopy</td>
</tr>
<tr>
<td>NCI</td>
<td>National Cancer Institute</td>
</tr>
<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>TOF</td>
<td>time of flight</td>
</tr>
<tr>
<td>XTT</td>
<td>2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide</td>
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ABSTRACT OF THE DISSERTATION

Novel Analogs and a Protein Target for the Napyradiomycins

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Professor William Fenical, Chair

The discovery of novel compounds to fight cancer has been a major goal of the National Cancer Institute since its inception. In accordance with this national goal we are searching for compounds that may not only be effective in fighting cancers directly, but also may shed light on new biological targets that can be used to combat the disease. The napyradiomycins are unique actinomycete metabolites produced by a mixed polyketide and terpenoid biosynthesis. Previous work by the Fenical and Jensen groups has found these molecules are produced only by a probable actinomycete genus designated MAR4. These molecules were briefly explored in the early 1990’s for possible use as antibiotics but no comprehensive study of their full biological potential has been undertaken. In our studies we have built a library of known and previously undescribed members of this family and used them to elucidate the likely mechanism of action of the napyradiomycins
Chapter 1 of the thesis is an introduction to chemotherapeutic drug development including many of the highlights of the last 70 years. Chapter 2 examines the contributions of marine natural products to cancer drug development and examines some of the unique problems in developing marine compounds. Chapter 3 introduces five novel members of the napyradiomycin family (24-28) and examines the effects of structural variations within the class on cytotoxicity measured in HCT-116 colon cancer cells. Chapter three concludes with demonstrating the ability of the napyradiomycins to induce apoptosis in HCT-116 cells using a flow cytometry based assay. Chapter 4 examines the mechanism by which apoptosis is induced in cells treated with napyradiomycins. Although, both caspase-8 and caspase-3 were activated in napyradiomycin induced apoptosis it was found that neither of these caspases were necessary for napyradiomycin induced apoptosis. In Chapter 5 two napyradiomycins CNQ525.510B (27) and A80915C (34) are used to construct coumarin linked fluorescent probes. These probes are used to demonstrate the sub-cellular location of the compounds in the endoplasmic reticulum. Using anti-coumarin antibodies the GRP94, the resident HSP90 of the endoplasmic reticulum, was co-immuno-precipitated with the probes suggesting this is a likely target of the napyradiomycins.
Chapter 1

The history of cancer chemotherapeutic drug discovery

Abstract

Modern cancer chemotherapeutic drug development can trace its origins to chemical weapons research during World Wars I and II. Since then a large number of compounds with distinct mechanisms of action have been approved for the treatment of various cancers. Each of these agents has a unique story of discovery, development and the researchers who made it happen. Modern cancer chemotherapeutic drug development stands on the shoulders of all these researchers. In order to understand the developments and contributions that others have made this chapter is a brief overview of the history of drug development for the treatment of cancer. This chapter will provide reference for the rest of the work contained in this thesis.
Cancer, a disease manifest by uncontrolled cell growth, has affected people from the earliest identifiable human remains. In the quest to control and treat cancer few pharmacological agents have proved more powerful than agents isolated from natural sources. Traditionally, natural products isolation has focused on terrestrial sources. This is most likely due to the ease of collecting terrestrial plants (ethnobotany) and microbes as opposed to marine organisms. With increased awareness of the oceans and the development of new technical skill and sophistication, the marine world has now begun to yield its pharmacological treasure trove. To fully understand the unique nature of these marine compounds and their potential pharmaceutical importance it is necessary to first look back at the history of chemotherapeutic drug development and the pharmacological foundation which marine compounds will supplement.

Early records of cancer

Excavations of the necropolis at West Thebes, Egypt, allowed modern pathological techniques to be applied to the examination of disease occurrence in an ancient population. Thorough examination of human remains at the site dated between 3200 BC and 500 BC demonstrated that cancer is a disease that has been present in the human population since the earliest identifiable remains.\(^1\),\(^2\) Although there was loss of significant amounts of tissue, the mummification process saved material which has shown that cancer was a disease burden on ancient populations just as it is a burden on our current population. Looking beyond human remains to the entirety of the chordates one can trace cancer further back in the fossil record. It has been shown that a dinosaur
bone, found in Colorado, and originating from the late Jurassic exhibited the characteristic lytic lesions that are often associated with metastatic cancer.\textsuperscript{3} Cancer has been found in organisms broadly covering the Phylum Chordata and appears to be an inadvertent byproduct of complex multi-cellular life. Since this inevitable disease has been present throughout human history, efforts to understand it can also be traced back to some of the earliest identified writings.

Early writings about cancer often focused on surgery for the treatment of cancerous growths. The earliest written description of cancer is from the Edwin Smith papyrus currently residing at the New York Academy of Medicine. Dating from approximately 1600 BC the papyrus is essentially a trauma surgery casebook describing 48 distinct medical cases. Case 39 describes an ulcerating lesion of the chest which could be an ulcerating tumor. Case 45 covers a bulging mass of the chest, likely a cancerous growth, for which, according to these ancient Egyptian writings, there is no ready cure.\textsuperscript{4} In the 5\textsuperscript{th} century BC Hippocrates described growths that had projections coming out of them with a crab-like appearance. From the appearance of the tumor growth, Hippocrates dubbed these lesions crab lesions or “karkinoma” in Greek. Although the term carcinoma today refers specifically to cancers of epithelial origin, Hippocrates used karkinoma to refer broadly to large, bulging, non-healing masses. References to karkinoma are found throughout his complete works in various parts of the body such as skin, the breasts and the larynx. The only treatments for these conditions were excision, cauterization and application of ointments.\textsuperscript{5} The term metastasis is also derived from a Greek word, methistemi, translated as ‘to be set free.’"
“metastatic affections [are] those which travel from one to another part of the body.” This is remarkably close to our modern understanding of cancer pathogenesis, although the ancient physicians lacked our current diagnostic techniques and our understanding of cancer biology.

Galenic medicine, the major medical theory of medieval Europe, was based on the initial teaching of Hippocrates, focusing on the balancing of humors in the body. The humors of the body were believed to be four essential fluids, blood, phlegm, yellow bile, and black bile, that had to be kept in balance for a patient to stay healthy. Cancer was believed to be caused by an excess of the melancholic humor, corrupted black bile. Treatments to rebalance the humors were ineffective in the treatment of cancer, leaving only surgical treatment as a viable option. There was no significant improvement in either the diagnosis or treatment of cancer from ancient times to medieval Europe. Although it has been suggested that cancer is a modern disease, the incidence of cancer today appears to be similar to that of ancient and medieval times. Examination of the remains of the population of a small city in Germany dating between 1400AD and 1800AD show that the incidence rate of cancer in this population was similar to that expected of a modern day English population. Although, human cancer has been with us from our earliest dated remains, any treatment option for cancer other than surgical resection is a historically recent advancement.

The initial of the ‘modern’ treatments for cancer was radiation therapy which can trace its roots to the discovery of X-rays on November 8th 1895 when Wilhelm Conrad Rontgen passed electricity through a vacuum tube and noted fluorescence on a piece of
paper painted with barium platinocyanide. Soon after the learning of the effects of radiation on the body, Marie Curie and others led physicians at St. Louis Hospital in Paris to begin treating cancer patients with radiation. Although a variety of side effects and significant recurrence of cancer were noted, careful work by physician scientists developed radiation oncology into a viable treatment for many cancerous growths. By the 1940’s many larger hospitals had medical linear accelerators for radiation oncology. The development of chemical entities to specifically kill cancer cells came in the 1940’s and has given us the current triad of treatment for cancer, surgery, radiation, and chemotherapy.

History of Cancer Chemotherapy

The development of drugs to fight cancer has brought hope to patients who would otherwise have quickly died. Initial chemotherapeutic discoveries came from research on chemical weapons; the field has since expanded to include molecules from plant, microbial, synthetic, terrestrial and marine sources. The research efforts of companies and academics, encouraged and guided by the National Cancer Institute, have given us a pharmacopeia of drugs for treating cancers with varied toxicities, efficacies, and mechanisms of action. In order to understand any new potential cancer treatment it is important to understand how many of the drugs we have were discovered.

Alkylating agents
The roots of cancer chemotherapy lie in the horrendous use of chemical weapons in World War I. Many poisonous gases, including the notorious mustard gases, were developed by the Allies and Central powers with little understanding of how these gases worked on a molecular level. After World War I it was noted that mustard gases such as bis (2-chloroethyl) sulfane (1) had a horrendous effect on certain tissues such as hematopoietic tissue and the gastro-intestinal tract. However, with the ending of the war scientific interest in the mustards was relatively quiescent until the eve of World War II. Although chemical weapons were never used in World War II by the allies, there was a resumption of research into offensive chemicals which confirmed that the toxicity of these agents seemed to be especially pronounced on tissues that were compromised of rapidly dividing cells.\(^7\) In order to more effectively treat potential wartime chemical casualties the mechanism of action of the mustards was studied in research programs funded by the United States government. Mustards undergo an intramolecular cyclization in polar solvents that liberates a chlorine anion and creates a cyclic onium cation. This cyclic onium ion (S or N) alkylates nucleophiles such as DNA interfering with the ability of proteins to interact with the DNA (see scheme 1). Subsequent animal work in both murine and rabbit models encouraged the researchers to advance mustards and derivatives to human clinical trials. The initial trials with bis(2-chloroethyl)methylamine (2) were haphazard by today’s standard of patient care. In his recollection of the original clinical trials during the war, Gilman commented, “The selection of a proper
dose of a highly toxic chemical warfare agent for administration to man for the first time was made with unwarranted confidence.” A patient in the terminal stages of lymphosarcoma who was refractory to X-ray treatment was selected as the first patient. The dosage was based solely on the animal studies with no slow titration up to the maximum tolerated dose. Since these trials were performed during wartime, the research was still considered top secret. As a result, the details were concealed and the patient charts indicated that treatment was with “compound X.” Although there was initial improvement, the patient subsequently relapsed and died of lymphosarcoma. Hundreds of patients were treated with compound X and when this research was published after the war it provided impetus for the further study of chemotherapeutic agents. Although many patients eventually relapsed, the initial work with the mustards, referred to as alkylating agents, found that cancer could be treated by chemotherapeutic means.

Anti-metabolites

Several years later Sidney Farber, a pathologist at Harvard Medical School, observed that folic acid (3) appeared to stimulate the growth of acute lymphoblastic leukemia (ALL) cells. Dr. Farber theorized that the folic acid antagonist, 4-
aminopteroylaspartic acid (4), which was previously synthesized by the biochemists SubbaRow and Hutchings at Harvard, could be used to treat acute lymphocytic leukemia (ALL) in pediatric patients. Dr. Farber was able to produce a temporary remission using folic acid antagonist therapy. The compounds aminopterin and its analog amethopterin (methotrexate) (5) are antagonistic to the enzyme dihydrofolate reductase which is essential for DNA synthesis. Although it wasn’t known at the time, folate-requiring enzymes are key for the synthesis of DNA. Faber’s 1948 paper describing these results, published in the New England Journal of Medicine, was not widely accepted. At the time even temporary tumor remission was unheard of and thus his report was met with wide disbelief. Pediatric cancer was perceived as entirely incurable and treatments were palliative. Although the publication was a source of controversy when it was first published, it has been sited over 558 times in other scientific articles and was the oldest article listed in the 1984 listing of classics in the New England Journal of Medicine. Sidney Farber went on to work with other anticancer compounds including methotrexate, which is still a mainstay of treatment for a wide variety of cancers.
Before the scientific community realized the true importance of DNA many initial studies were based on a biological response of the cancer to the drugs being tested at the time, without an understanding of the mechanism of action on a molecular level. However, many of the initial cancer chemotherapeutic agents that were found, such as methotrexate (5) and 6-mercaptopurine (6), were effective due to their targeting of DNA replication, which is increased in rapidly dividing cells such as cancer cells. Concurrent with Dr. Farber’s research, other drugs that interfered with the synthesis of DNA were being developed. In the early 1950’s Joseph Burchenal, a physician at the Memorial Sloan-Kettering Cancer Center who had worked with Farber on the initial studies of methotrexate attempted to develop other anti-metabolites. With the help of George Hitchings and Gertrude Elion, two chemists at the Burroughs Wellcome pharmaceutical company, Burchenal developed multiple purine analogues including 6-mercaptopurine (6), a highly active antileukemic drug.\textsuperscript{12} Since the development of 6-mercaptopurine, numerous other purine and pyrimidine analogues have been developed for the treatment of cancer. For example, the pyrimidine analog 5-fluorouracil (7), is a mainstay of cancer chemotherapeutic treatment, currently being used in colorectal and pancreatic cancers.
Vinca Alkaloids

In early 1952, as synthetic chemists were working to synthesize antimetabolites based on the successes of dihydrofolate reeducates inhibitors and purine analogs, the Canadian physician Edward Clark Noble received an envelope containing 25 leaves from the Madagascar periwinkle plant (*Vinca rosea*) from a patient traveling abroad. The patient described how diabetics in Jamaica were treated using a therapeutic tea made from the leaves. Noble sent the leaves to his brother Robert Laning Noble at the University of Western Ontario, who found that the extracts from the periwinkle plant had very little effect on blood glucose levels but that they had a profound myelosuppressive effect and could cause severe leukopenia, a depletion of white blood cells. In 1957, the extracts were tested in the p1534 leukemic mouse model and the results were striking. The treated mice exhibited a 123%-238% prolongation of lifespan, and the mice receiving 3 mg/kg/day showed 100% indefinite survival. The structure of vindoline (8), the simplest of the vinca alkaloids, was solved in 1962. Followed shortly after by the structures of the most active of these alkaloids, vincristine (9) and vinblastine (10), which are still used clinically today. The structure elucidation of these complex alkaloids was a daunting task requiring extensive degradation studies of the molecule, which finally led to the complete structure. Although the vinca alkaloids were approved by the FDA for the treatment of cancer in 1963, it was not until 1991 that the mechanism of action of these compounds was fully established by Mary Ann Jordan. She convincingly demonstrated that the potent anti-proliferative effect of the vinca alkaloids was a result of cell cycle arrest during cell division. The vinca alkaloids were found to bind to the
intracellular protein tubulin, and in the process prevent the formation of tubulin-based microtubules. With microtubules in disarray, the cell cannot complete mitosis and will eventually undergo apoptosis (pre-programmed cell death). Like the alkylating agents (mustards), and anti-metabolites (methotrexate), the vinca alkaloids are able to preferentially destroy cancer cells by targeting those cells which are dividing the most quickly.

Developments at NCI

With the understating that cancer is a treatable disease using chemotherapy, the National Cancer Institute started the National Chemotherapy Program in 1955 with a $5 million authorization from the Congress. This new program was created in order to consolidate the development of anti-cancer compounds in the United States. The
program investigated compounds which arose both from industry and academia. A linear array system was developed to explore potential drugs which included screening new compounds, production and formulation of candidates, toxicology, and clinical trials.¹⁸ The linear array was designed to remove weak drug candidates early at each development step and advance the good candidate drugs. The development of new chemotherapeutic compounds became a major focus of the National Cancer Institute (NCI) and NCI funding a major resource for researchers endeavoring to develop new chemotherapeutic agents.

At same time, research at the NCI to develop new chemotherapy treatments was progressing rapidly. Roy Hertz and Min C. Li were working on an 18 patient inpatient ward in which they studied choriocarcinoma, a malignant placental tumor with a near 100% fatality rate. Using the anti-folate drug methotrexate, they were able for the first time to cure a malignant tumor.¹⁹ This advance encouraged the clinicians at NCI to continue work on effective chemotherapeutic regimes. In addition to creating novel chemical entities, an important aspect of clinical cancer research has been to discover which chemotherapeutic agents work best synergistically. In 1970 three NCI physicians, Vincent DeVita, Stanley Lowenbraun and Arthur Serpick, were working on developing a chemotherapy program to treat Hodgkin's disease and diffuse large cell lymphoma. By combining a nitrogen mustard, vincristine, prednisone, and procarbazine the team achieved regression of all lesions in 70% of patients with systemic disease.²⁰ This was a groundbreaking finding, as previous patients with Hodgkin’s disease had a near zero percent survival. The combination drug therapy that was developed, named MOPP (an
acronym from the commercial names of the drugs, Mustragen, Oncovin, Procarbazine, and Prednisone), is still currently employed for some patients who are refractory to current treatment or unable to tolerate the current treatment regime. Cancer therapy became more complex as more pharmacological agents were added with or without radiation treatment. In 1972, it was found that chemotherapy with the newly discovered drug doxorubicin (11) administered after surgical removal of osteosarcoma increased cure rates. The three modalities of cancer treatment, surgical, chemical, and radiological were being used more effectively together as clinical cancer researchers determined the most effective regimes for patients.

Anthracyclines

Doxorubicin, the compound used in the osteosarcoma adjuvant studies, traced its beginning to the 1950’s. The Italian company, Farmitalia, developed a program to identify compounds from soil bacteria that could be used in the treatment of cancer. In this study, a bright red pigment produced by Streptomyces peucetius was isolated and found to possess potent activity against murine tumors. The new compound was named daunorubicin (12) by combining the name Dauni, an ancient tribe that occupied the area where the original bacterial strain was found, and rubis, the French word for ruby. Although the drug exhibited great promise, its use was severely limited by its potent cardiotoxicity. However, using mutagenesis researchers at Farmitalia developed a clonal bacterial line of the original strain that produced a 14-hydroxy derivative of daunorubicin. The new compound, Doxorubicin(11) also possessed a degree of
cardiotoxicity but exhibited a much larger therapeutic window than daunorubicin. These two compounds became the prototype drugs for the now more expansive anthracycline class of chemotherapeutic drugs. The mechanism of action of the anthracyclines is to intercalate between strands of DNA, thereby inhibiting DNA replication.

Topoisomerase I inhibitors

The National Chemotherapy Program, which was initiated at NCI in 1955 to help discover drug candidates, identified numerous novel chemical entities for cancer treatment in collaboration with many different organizations such as the U.S. Department of Agriculture (DOA). Monroe Wall, who was working at the DOA to identify plant sterols that could be converted to cortisol, extracted material from the Chinese tree *Camptotheca acuminata* and sent it to the National Chemotherapy Program to be screened.\(^{25}\) At NCI, Jonathan Hartwell observed potent anticancer activity. Subsequently, the biologically active component was isolated and the structure of camptothecin (13) was elucidated.\(^{26}\) Unfortunately, it was found that camptothecin was extremely toxic in humans. Mechanism of action studies on camptothecin revealed that camptothecin acts as an inhibitor of topoisomerase I,\(^{27}\) a protein essential for the
uncoiling of DNA during DNA replication. This novel mechanism ultimately resulted in the development of the less toxic analogues topotecan (14), and irinotecan (15), that exhibited higher therapeutic indices.
The development of drugs, which exhibit entirely new mechanisms of action, such as the anthracyclines, encouraged the scientific community to look very broadly at potential drug candidates. A surprising start for one of the most effective anti-tumor agents can be traced to the biophysics department at Michigan State in the early 1960’s. Barnett Rosenberg was investigating the effect of electrical current on bacteria. The experiment indicated that bacteria in solution subjected to electric current began to elongate, suggesting an inhibition of cell division. It was found that the inhibition was not due to the electrical current directly, but rather to a compound formed at the electrodes in the chamber. The researchers discovered that a soluble platinum salt, \((\text{NH}_4)_2\text{PtCl}_6\), was being formed at the electrode. This compound appeared to interfere with cell division in bacteria. The compound was also tested on tumors and found to exhibit a potent cytotoxic effect. This was the first instance of an inorganic molecule being used as an anti-cancer agent. In 1972, researchers at the Institute for Cancer Research in the UK revealed that the mechanisms of action of the platinum compounds was to cross link complimentary strands of DNA. In 1978, cisplatin (16) was approved as a drug for the treatment of ovarian cancer.

The NCI-60 cell panel
It had been readily apparent since the earliest days of chemotherapy development that many chemotherapeutic compounds are more efficient at fighting some types of cancer than other types. In order to identify and develop specific chemotherapeutic agents for the optimal treatment of cancer, it was deemed important to be able to test lead compounds rapidly versus cancer lines derived from many tissue types. The NCI developed the NCI 60 cell panel to be a moderately high throughput screen against 60 cell lines representing cancers originating from nine distinct tissues types (breast, prostate, renal, ovarian, epithelial, nervous, lung, colon and lymphoid). The screen was designed to broadly test over 3,000 compounds submitted to the NCI per year and has currently tested over 70,000 compounds including all of the currently used chemotherapeutic drugs. Subsequently, NCI created a program called COMPARE to analyze the data from the NCI 60 cell panel and help to elucidate possible mechanisms of action of compounds; COMPARE uses the cytotoxicity profiles relative to the mean seeking selective potency of compounds in the 60 cell panel and compares that pattern to the activity patterns of compounds with known mechanisms of action. If a newly submitted compound has a pattern of inhibition that is similar to that of a known compound, it is likely that the two compounds may have similar mechanisms of action. By enabling a broad analysis against many tumor types, the 60 cell line panel at the NCI has become one of the most important resources in cancer drug development.
Taxanes

One of the most important drugs that might have potentially been missed due to insensitivity in certain tumor types is taxol (17), a compound that was approved for clinical use in 1992. Taxol was isolated from the Pacific yew tree *Taxus brevifolia* in 1966 and shown to have moderate activity toward P-388 murine leukemia, the then cell line of choice for overall screening at the NCI. Subsequently, interest in taxol at the NCI waned as numerous problems were realized including only moderate P-388 activity, insolubility, and the natural product supply problem. The Pacific yew tree produces taxol in small amounts, and is itself quite rare. Therefore, harvesting sufficient quantities of plant tissue would require extensive government support, which was not forthcoming based on the moderate activity of the compound. Interest in taxol grew when Susan Horwitz, at the Albert Einstein Medical Center, found that taxol was a potent inhibitor of cell division in HeLa cells at concentrations that exhibited no significant effect on DNA, RNA or protein synthesis. Taxol’s mechanism of action was subsequently found to be distinct from that of other molecules that affect microtubules (e.g. vincristine); instead of
inhibiting the assembly of the microtubule, taxol works by stabilizing the microtubule thus eliminating its reversible formation. Since microtubule disassembly is key to the separation of chromosomes during mitotic division, microtubule stability causes cell arrest in M-phase.

Renewed interest in taxol forced a reassessment of the supply problem and it was found that the needles of the more abundant European yew tree produced 10-deacetylbaccatin (18), a closely related natural product that is easily converted by semi-synthesis into taxol, thereby solving the supply problem. The solubility problem was solved at Bristol-Myers Squibb, which ultimately brought the drug to market. The company found that a mixture of polyethoxylated castor oil and ethanol was an efficient delivery vehicle for the compound. Taxol is currently one of the most important drugs in the treatment of ovarian, lung and breast cancer and might not have been been if it had not been for Susan Horwitz who looked beyond the leukemia based cell assays in which it was first screened. The search for natural products to fight cancer has been historically successful due to the combination of novel sources of molecules, efficient bioassays and the dedication of pharmacologists who decipher the molecular targets of these compounds.

Strain CNQ525

The development of cancer chemotherapeutic compounds has been greatly aided by natural products research. Many of the top drugs in cancer treatment including vincristine, taxol and doxorubicin are all derived from natural sources. Research at the
Scripps Institution of Oceanography in the Fenical lab has focused on one of the traditionally most prolific sources of terrestrial natural products, bacteria of the order Actinomycetales. Many of the most common drugs in use today, including doxorubicin and erythromycin, are secondary metabolites produced by terrestrial actinomycetes. With the discovery of penicillin by Alexander Fleming in 1929, academics and pharmaceutical companies alike have recognized this resource. Hence, from the early 1940s to the early 1990s, industry examined the secondary metabolites of terrestrial actinomycetes. During this period, the ocean was not recognized as a source for novel actinomycetes producing novel chemical entities. In recent times, explorations of marine microorganisms have led to the discovery of exciting new drugs for cancer such as the proteosome inhibitor salinosporamide A in clinical trials for multiple myeloma.

With this background, I initiated a project to search for novel marine compounds that might be used in the battle against cancer. In this work I have focused on developing the resources of the marine world using novel and previously described molecules. In chapter 2 I review some of the successes and unique challenges that are associated with developing anticancer compounds from marine sources. I have focused my studies on a strain of a marine-derived actinomycete, CNQ525, which produces the napyradiomycin family of compounds. Chapter 3 describes the isolation and structural characterization of five new members of the napyradiomycins, including never before seen structural variations. The structure activity relationship of 14 members of the family is also explored to show which common variations in the class have the largest effect on the potency of the compounds when compared in a human colon cancer cell line study.
Chapter 3 concludes with demonstrating the ability of the compounds to induce apoptosis. The caspase activation pattern during napyradiomycin induced apoptosis is examined in Chapter 4 suggesting that the apoptosis induced is neither classical intrinsic pathway nor extrinsic pathway. Finally, Chapter 5 includes the synthesis of novel coumarin-based fluorescent probes of two napyradiomycin derivatives. These new molecular probes are rapidly taken-up by cancer cells demonstrating the sub-cellular localization of the compound, and hence its protein target in the endoplasmic reticulum. Using Immuno-precipitation and protein mass spectrometry the likely protein target of the napyradiomycins was identified as the Heat Shock Protein Chaperone GRP94. This work builds on the work of many other scientists resulting on the identification of a novel, potentially important target for the treatment of cancer.
References


Chapter 2

Marine natural products in the development of cancer chemotherapy

Abstract

Marine natural products have proved to be a valuable resource in the battle against cancer. Numerous drug candidates from marine invertebrates and bacteria have entered clinical trials but drug development from marine natural products also presents a unique set of problems, mostly related to collection of samples, culture of isolated organisms, and acquisition of sufficient material for clinical trials. In this chapter, I discuss a few examples of marine cancer chemotherapeutic compounds and how research into these products has overcome the problems associated with identification, collection, and supply.
Introduction

The marine environment has been gaining recognition as a viable source of drug discovery lead compounds for the last 20 years. While terrestrial sources have been intensely examined for hundreds of years and have led to the discovery of compounds to treat human disease, the oceans, an environmentally more difficult resource to access, remain largely unexplored. Since the 1980s, the oceans have become an important source for new anticancer compounds with novel structures and novel activities.

The foundations of marine pharmacology can be found in the field of marine chemical ecology. Early on, it was noted that sponges appear to have no active defense mechanism against predators; they generally evade predation and grow to extraordinary sizes. In the early 1950’s Bergman and colleagues found that marine sponges produced a number of novel metabolites with biological activity.\textsuperscript{1-4} Since then, the field of marine anticancer drug discovery has expanded to explore diverse sources from the world’s oceans. G. R. Pettit, of Arizona State University, began to work with the National Cancer Institute (NCI) in the early years of marine drug discovery to test extracts from marine vertebrates and invertebrates, and reported that many extracts provided significant life extension in mouse based anti-cancer assays.\textsuperscript{5} His work, and the work of many other noteworthy organic chemists, helped to firmly establish the marine environment as an abundant source of new compounds with significant potential to treat cancer.

The first marine-derived drug to gain FDA approval was not a chemotherapy drug but rather a drug for the treatment of chronic pain. Ziconotide\textsuperscript{™} is a synthetic form of a peptide, \(\omega\)-conotoxin M-VII-A, originally isolated from the tropical marine cone snail
Conus magus. Ziconotide™ acts neither as an opioid, nor as a non-steroidal anti-inflammatory drug for chronic neurogenic pain. The peptide is an inhibitor of N-type calcium channels in the spinal cord and thereby reduces the sensation of pain in patients. Although Ziconotide™ can only be given by direct injection into the spine, it provides tremendous relief for patients with chronic pain that is refractory to other treatments. As more research and preclinical evaluation is accomplished on the many natural products isolated from the ocean, it is becoming readily apparent how valuable the marine world is as a potential source of new drugs.

Cancer chemotherapeutic compounds

The first cancer drug from a marine source to gain FDA approval was ecteinascidin-743 (19), isolated by Ken Rinehart at the University of Illinois, Urbana-Champaign, from an extract of the tunicate Ecteinascidia turbinata (Caribbean sea squirt) in 1990. It had been clear since 1969 that the extracts of the sea squirt had potent anti-cancer activity but the vanishingly small amounts of the compound, and the
primitive isolation methods available at the time, made identification of the compound impossible for over twenty years. Ecteinascidin-743 preferentially alkylates GC-rich regions of DNA.\textsuperscript{42} The reversible alkylation of guanine residues in the minor groove of DNA has the unique effect of poisoning transcription-coupled excision repair. The unique mechanism of ecteinascidin-743 may account for its potency against certain cancers refractory to other treatment courses. Ecteinascidin-743, now used in Europe under the name Yondelis\textsuperscript{TM} was developed by Pharmamar who subsequently licensed the drug to Johnson and Johnson. Pharmamar was responsible for developing one of the breakthroughs that allowed clinical trials of this compound to happen. The compound is made in very minute amounts (less than 10 parts per million) so collection of enough sample for human trials was impossible. Pharmamar developed a semi-synthetic method to create ecteinascidin-743 from cyanosafracin B (2) a compound which can be obtained in multi-kilogram quantities from fermentation of \textit{Pseudomonas fluorescens}.\textsuperscript{6}

Supply has been a constant problem with marine natural products. Although many marine invertebrates produce a multitude of fascinating compounds, our understanding of their biosynthetic pathways is still very limited. This has restricted the use of many compounds to what can be collected naturally, which is often a very small quantity. Biological evidence has to be exceptionally convincing before most regulatory agencies will allow large collections of marine invertebrates.
One such large collection of marine invertebrates was undertaken for the isolation of the bryostatins from the bryozoan *Bugula neritina*. The NCI commissioned large scale collections of the bryozoans for clinical trials and more than 13 metric tons of bryozoans were necessary for the purification of sufficient material for clinical trials. The clinical trials required large scale purification techniques to isolate minor compounds and it was only through heroic work by Pettit, and collaborators at the NCI, that enough material was collected by 1990 for clinical trials. The bryostatins activate phosphokinase C (PKC) isozymes and as a result, cancerous cells suffer from inhibited growth, alteration of differentiation, and likely death. Bryostatin-1 (21) has been in over 58 phase I and phase II trials and research into their clinical importance is still ongoing. Research into their origin is also still proceeding. Marine invertebrates are known for having complex symbiotic relationships with microorganisms. Margo Haygood, then of the Scripps Institution of Oceanography, demonstrated that the source of the natural product is likely an “un-culturable” symbiont. This has made collection difficult, as not all *B. neritina* samples contain the symbiont. An exciting area of current research concerns the
determination of which natural products, originally thought to be synthesized by marine invertebrates, are actually the result of symbiotic relationships with marine microorganisms.⁸

It is not always possible to purify enough material from natural sources, as was the case with halichondrin B (22). Halichondrin B and a number of analogues were first isolated and described in 1985 by Uemura and colleagues.⁹ The compound was initially isolated from the Japanese sponge *Halichondria okadai* but was subsequently found in numerous sponges from the Pacific and Indian Ocean. Initially, enough compound was isolated to confirm that halichondrin B destabilized tubulin at a site proximal to, but distinct from, the vinca alkaloid binding site. In collaboration with the NCI, and the University of Canterbury, NZ, the National Institute for Water and Atmospheric Research (NIWA) undertook a large-scale isolation program which yielded 300 mg of compound from sponges collected by trawling at 100 m depths. During the quest for more material, scientists were also able to show that the compounds could be harvested from sponges grown in aquaculture at depths as shallow as 10 m. Ultimately though, total synthesis was needed in order to acquire the amount of material necessary for clinical trials. The
total synthesis of halichondrin B and analogs was reported by Kishi’s group at Harvard in 2001. The synthetic route allowed a derivative, E7389, to be synthesized, thus facilitating the entry into phase I clinical trials in 2001. This derivative is currently involved in one phase III, eight phase II trials, and three phase I trials for the treatment of various cancers including breast, ovarian, and prostate cancers. Although total synthesis is often the only path to acquire enough material for human subjects work, it can be very time consuming and expensive. However, isolation of secondary metabolites from culturable marine bacteria has the potential to produce large amounts of material in a cost effective way.

Fenical and Jensen at the Scripps Institution of Oceanography have pioneered the study of marine actinomycete bacteria. In the course of studying marine actinomycetes, a genus of saltwater-requiring actinomycetes was discovered. This genus, the *Salinispora*, was the first obligate marine actinomycete taxon to be described. One *Salinispora* strain, *S. tropica*, strain CNB440, showed very potent activity in an HCT-116 colon cancer cell cytotoxicity screen. Bioactivity guided fractionation of a 20 L culture
led to the isolation of the novel compound salinosporamide A (23). The compound had an IC50 of less than 2 ng/mL against HCT-116 cells. Analysis by the NCI in their 60-cell panel, showed that the activity of salinosporamide was specific and warranted further study. It has since been determined that salinosporamide A is a potent inhibitor of the 20s core particle of the proteasome. Proteasome inhibitors have anti-tumor activity by inhibiting the degradation of pro growth proteins and leading to apoptosis. Previously, only one compound, bortezomib (Velcade), which specifically targets the proteasome, had been approved by the FDA. Velcade is the first treatment approved by the FDA for multiple myeloma in over 10 years. The prospect that salinosporamide A could also be used to treat multiple myeloma caused a great deal of interest in the compound and it was licensed by UC-San Diego to Nereus Pharmaceuticals in 2001. The ability to produce large amounts of the compound through fermentation of the actinomycete strain, and the specific action of the compound against a validated cancer target, greatly accelerated the advancement of salinosporamide A into clinical trials. Currently there are four clinical trials for salinosporamide for multiple myeloma, solid tumor malignancies, lymphoma, non-small cell lung cancer, pancreatic cancer, and melanoma. Because of its favorable pharmacological properties, and the ability to readily produce large amounts of material, salinosporamide A entered human trials much quicker than most other marine compounds.

This brief overview of marine natural products is by no means exhaustive, there have been over 40 distinct marine natural products brought to clinical trials. The process of identifying these compounds with vanishingly small quantities, the difficulty in
obtaining samples, and the supply problems associated with acquisition of sufficient quantities of compounds to use in human trials are hurdles for scientists working in this field. However, these hurdles can be overcome if there is sufficient motivation. In my studies I have focused on marine actinomycetes because of the plethora of biologically active compounds produced and the ability to acquire more compounds through large scale fermentation of the microorganisms. This has allowed us to explore known and unknown families of compounds which may possess unique pharmacological properties. In these studies the MAR 4 actinomycete strain CNQ525 was identified as a prolific producer of napyradiomycin compounds, a family of halogenated napthoquinone secondary metabolites. Through chemical and biological studies, I have identified five new members of this family and elucidated the likely biological action of these compounds. This is one small piece of the ever expanding field of marine pharmacology.
References


Chapter 3

Five novel napyradiomycins and the effects of structural variations within the family on cytotoxicity and apoptosis induction

Abstract

In the course of my exploration of the marine environment for new therapeutic agents, I have revisited the napyradiomycin class of secondary metabolites. This expanding family of compounds has been shown to be produced by a unique marine biosynthetic pathway yet very little pharmacological work has been undertaken to explore the target of these molecules. I report here the isolation and structural elucidation of five new members of the napyradiomycin class (24-28). In the course of isolation of these molecules I also isolated nine previously described napyradiomycins (29-37). Structure activity relationship studies (SARs) suggest that minor variations in the structure of these compounds may have significant effects on their activity. Through the use of flow cytometry analysis, I was also able to show that the cytotoxicity induced by the napyradiomycins is the result of the induction of apoptosis. The results of the structure activity relationship analysis (SAR), and the observed induction of apoptosis, suggests a specific protein target for these molecules and encourages further work to elucidate their mechanism of action and protein target.
Introduction

Marine natural products are receiving increased attention as a vast untapped resource in the quest for novel therapeutic agents.¹ Recent successes in the development of marine compounds such as Ecteinascidin 743 (Yondelis™) into viable clinical entities, have validated the ocean as a valuable source for novel therapeutic agents. In the course of exploring this vast resource, I have searched not only for compounds with novel carbon skeletons but also for known classes of compounds that have not been fully biologically explored. During this study, I identified an actinomycete strain, CNQ525, that is a prolific producer of secondary metabolites of the napyradiomycin class. The napyradiomycins were first discovered as microbial secondary metabolites of the cultured actinomycete Chainia rubra in 1986,²,³ a strain isolated from coastal sediments. Since the initial discovery of the napyradiomycins, these compounds have also been found to be produced by several other strains isolated from beach sand and ocean sediments.⁴,⁵ Fenical and Jensen have described a novel actinomycete taxon tentatively designated MAR4 (family Streptomycetaceae) which is the only known, genetically characterized taxon capable of producing these molecules.⁵ Work in the Moore group at the Scripps Institution of Oceanography has recently shown that all of the known napyradiomycin-producing isolates, which number more than 20, produce the napyradiomycins by the same biosynthetic pathway. These compounds are biosynthesized by a mixed biosynthesis involving terpenoid and polyketide pathways, a process which is exceedingly rare in Nature. The Moore group showed that the terpene rings in the
Napyradiomycins are likely produced by two chloronium ion-induced cyclizations involving vanadium-dependent chloroperoxidases, a biosynthetic mechanism which had not been seen in any prokaryotic system before it was reported by Winter et al. in 2007. A prenyl unit is cyclized forming an ether ring attached to C8 and C12 of the naphthoquinone core. The geranyl substituent, attached at C8, is cyclized to form a six-membered ring characteristic of one subset of the napyradiomycins, called the B class. Bromonium ion-induced terpene cyclization is very common in marine algae, but a similar system has never been observed in any terrestrial organism. A biosynthesis that is dependent on halides, coupled with the high halide concentration in the ocean, suggests that these organisms are of true marine origin. The napyradiomycins were initially characterized for their anti-microbial activity, but have since been further characterized for inhibiting gastric (H⁺-K⁺) ATPases, and being estrogen receptor antagonists. However, a thorough examination of the biological potential of these molecules has never been undertaken, and specific information defining their biological roles in cells has never been elucidated. The reported bioactivity, as well as the unique structure and biosynthesis of the napyradiomycins, suggested these compounds should be evaluated more completely.

Results and Discussion

Actinomycete strain CNQ525 was cultured at 30 °C for 7 days by rotary shaking in 20 × 1 liter cultures in two different seawater based media, A1bFe + C culture medium, or A1bFe + C medium with low NaCl and 200 mM NaBr. The whole cultures
were extracted using Amberlite XAD-7 resin for 6 hours. The resin was subsequently removed from the broth by filtering through cheese cloth and next extracted using acetone. The subsequent extract was loaded onto 20 grams of silica gel and dry-packed onto a 200 gram silica flash column for initial separation. Flash chromatography was performed using ethyl acetate and iso-octane to produce 10 fractions of differing polarity. These fractions were then analyzed by LCMS to assess the presence of members of the napyradiomycin family. Molecules with molecular weights that had not been observed in this series were prioritized for isolation and subsequent structure elucidation.
Fractionation resulted in the isolation of metabolites 24-28, which are new members of the napyradiomycin family.

Compound CNQ525.522 (24) was identified as a napyradiomycin with a novel mass by LCMS analysis. The pseudomolecular ion of this compound showed the characteristic isotope pattern indicating it contained one chlorine and one bromine atom. High resolution mass spectral analysis provided a measured mass of 545.0709 amu (Fourier transform mass spectroscopy, [M+Na]^+) which provided a chemical formula of C_{25}H_{28}O_{79}Br_{35}Cl (theoretical mass 522.0809, Δ 0.0003) for this new molecule. Analysis of combined spectral data from 1D proton, 2D HSQC, and HMBC NMR experiments showed the presence of two methyl groups (δ_\text{C} 16.6, C-23, δ_\text{H} 0.66, s, H-23; δ_\text{C} 27.3, C-25, δ_\text{H} 1.54, s, H-25) attached to sp^3 hybridized carbons, two aromatic protons (δ_\text{H} 6.72, d, H-16; δ_\text{H} 7.14, s, H-18), and the characteristic peri-proton from the –OH at C15 (δ_\text{H} 12.59.). HSQC analysis showed two distinct aliphatic methyl groups with a similar chemical shift (δ_\text{C} 28.5, C-22, δ_\text{H} 1.08, s, H-22; δ_\text{C} 20.4, C-24, δ_\text{H} 1.08, s, H-24). Two singlet resonances in the ^1H NMR spectrum (δ_\text{H} 4.31, s, H-21a; δ_\text{H} 4.83, s, H-21b), which exhibited HSQC correlations to a deshielded carbon (δ_\text{C} 109.6, C-21), were characteristic of the exocyclic methylene structural feature in the known napyradiomycin A80915A (32). The presence of four aliphatic methyl groups, two aromatic protons, an exocyclic methylene, and two halogens suggested that the structure of the compound may be similar to the published compound napyradiomycin B2. Comparison of the ^1H NMR spectrum of compound CNQ525.522 and the published data from napyradiomycin B2 was nearly identical including similar chemical shifts for C10 (napyradiomycin B2 = δ_\text{H}
Table 3.1: NMR Spectral Data for CNQ525.522<sup>a,b</sup> (<sup>24</sup>)

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<th>δ&lt;sub&gt;H&lt;/sub&gt; ppm (&lt;sup&gt;J&lt;/sup&gt;, Hz)</th>
<th>COSY</th>
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*<sup>a</sup> Spectra were recorded in CDCl<sub>3</sub> at 600 MHz (<sup>1</sup>H).  
*<sup>b</sup> Assignments were made based on interpretation of COSY, HMBC and HSQC experiments.*
4.46, CNQ525.522 = $\delta_H$ 4.47), although a $\delta_H$ shift downfield for C4 (napyradiomycin B2 = $\delta_H$ 3.82, CNQ525.522 = $\delta_H$ 4.06) was observed indicating a structural difference between the two molecules. This suggested that the bromine atom in CNQ525.522 is attached at C4 in the geranyl ring. One known analog, napyradiomycin B3 (30), containing bromine at this position (napyradiomycin B3 = $\delta_H$ 4.01), has been previously published.10 The full structure of was confirmed by complete two-dimensional NMR analysis (see Table 1).

In the napyradiomycins, bromine substitution on the monoterpene ring at C4 had been reported, yet bromine substitution at C12 or C10, the other reported sites of chlorination, had never been observed. The novel chloronium ion based biosynthetic mechanism of terpene cyclization that had been suggested by Winter et. al.6 encouraged us to examine whether the vanadium dependent chloroperoxidases active at C10 and the FADH$_2$-dependant halogenase active at C12 could also use bromine in the cyclization. To explore this, strain CNQ525 was cultivated with added sodium bromide. Examination of the extract from the bromide enriched cultivation yielded a new napyradiomycin, CNQ525.538 (25) that contained two halogens. The molecular ionization pattern observed in the mass spectrum suggested the presence of one chlorine and one bromine substituent. Analysis of HRTOF negative ion mass spectral data illustrated a pseudomolecular ion with a mass of 537.1042 amu for the [M-H]$^-$ peak. This corresponded with the molecular formula C$_{26}$H$_{32}$O$_5^{79}$Br$^{35}$Cl (theoretical mass 538.1122, $\Delta$ 0.0001) for the parent compound. Analysis of the HSQC, HMBC and $^1$H NMR spectra
Table 3.2: NMR Spectral Data for CNQ525.538\textsuperscript{a,b} (25)

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<tr>
<td>14</td>
<td>109.7 (C)</td>
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<td>120.6 (C)</td>
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<td>131.8 (C)</td>
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<tr>
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<td>196.4 (C)</td>
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<tr>
<td>21</td>
<td>16.2 (CH\textsubscript{3})</td>
<td>1.28 s</td>
<td></td>
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<td>1.58 s</td>
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\textsuperscript{a) Spectra were recorded in CDCl\textsubscript{3} at 600 MHz ($^{1}H$). \textsuperscript{b) Assignments were made based on analysis of COSY, HMBC and HSQC data.}
revealed the presence of one methyl group (δ_C 8.1, C-26, δ_H 2.22, s, H-26) attached to an aromatic carbon, three methyl groups (δ_C 16.2, C-21, δ_H 1.28, s, H-21; δ_C 25.5, C-22, δ_H 1.58, s, H-22; δ_C 17.4, C-23, δ_H 1.44, s, H-23) attached to non-aromatic sp^2 hybridized carbons, and two methyl groups (δ_C 23.2, C-24, δ_H 1.23, s, H-24; δ_C 1.49, C-25, δ_H 29.1, s, H-25) attached to sp^3 hybridized carbons. The presence of three methyl groups attached to sp^2 hybridized carbons suggested that the geranyl group in this molecule had not been cyclized. The combination of an aromatic methyl and linear terpenoid substituent suggested that this compound was similar to the published napyradiomycin SF2415B3,\textsuperscript{11,12} with the substitution of bromine for one of the chlorine atoms. Comparison of the published spectral data for compound SF2415B3 with those obtained for compound CNQ525.538 (25) showed the molecules were almost identical. However, there were variations in both the proton and carbon shifts at C10 (SF2415B3 – δ_C 58.8, C-10, δ_H 4.43, dd, J = 4.9, 11.3, H-10; CNQ525.538 – δ_C 50.7, C-10, δ_H 4.52, dd, J = 6.3, 9.6, H-10), suggesting that the substitution of bromine for chlorine occurred at C10. This was the first napyradiomycin to be isolated that contained a bromine at a position other than C4. The overall structure was subsequently confirmed by comprehensive two-dimensional NMR analysis (see Table 2). While it appears that the enzyme responsible for halogenation at C4 is more permissive in the incorporation of bromine than the enzyme responsible halogenations at C10, both enzymes are apparently able to accommodate bromine.
Isolation of a napyradiomycin with a bromine on the tetrahydropyran ring was an exciting new development. In addition, until now only napyradiomycins that were monobrominated had been isolated. Therefore, I began to look for molecules with an ionization pattern that suggested the presence of two or more bromine atoms. Compound CNQ525.600 (26) was isolated from a culture of CNQ525 grown in A1C+Fe medium with lowered sodium chloride and 200 mM added sodium bromide. LCMS analysis of the compound showed a pseudomolecular ion illustrating the isotope pattern for a molecule containing two bromine atoms. Negative mode high resolution MALDI TOF MS showed a pseudomolecular ion at $m/z$ 599.0649 for the [$M-H$]$^-$ peak yielding a molecular formula of C$_{26}$H$_{34}$O$_6$Br$_2$ (theoretical mass 600.0722, $\Delta$ 0.0005) for the parent compound. The NMR spectral data for compound CNQ525.600 (26) showed five methyl groups ($\delta_C$ 24.1, C-21, $\delta_H$ 1.21, s, H-21; $\delta_C$ 30.0, C-22, $\delta_H$ 0.41, s, H-22; $\delta_C$ 17.5, C-23, $\delta_H$ 0.72, s, H-23; $\delta_C$ 21.0, C-24, $\delta_H$ 1.36, s, H-24; $\delta_C$ 30.0, C-25, $\delta_H$ 1.50, s, H-25) attached to sp$^3$ hybridized carbons, one methyl group ($\delta_C$ 8.4, C-26, $\delta_H$ 2.18, s, H-26) attached to an aromatic carbon, one aromatic proton ($\delta_H$ 7.52, s, H-18), and the characteristic peri-proton resonance from the –OH at C15 ($\delta_H$ 12.47). The monoterpenoid ring of this compound was found to contain a hydroxyl moiety attached to C1 and a bromine atom attached at C4. This is similar to the monoterpenoid ring seen in napyradiomycin A80915C (34), which has a chlorine atom attached at C4.$^4$ Comparison of the $^1$H NMR spectrum of 26 with the published data from A80915C (34) showed a downfield chemical shift for C4 (A80915C = $\delta_H$ 3.47, dd, $J=3.7$, 12.1, H-4; CNQ525.600 = $\delta_H$ 3.71, dd, $J=3.9$, 12.5, H-4). There are no examples of a napyradiomycin with a hydroxy group and
### Table 3.3: NMR Spectral Data for CNQ525.600\(^{a,b}\) (26)

<table>
<thead>
<tr>
<th>Carbon #</th>
<th>(\delta_C) ppm</th>
<th>(\delta_H) ppm ((J, Hz))</th>
<th>COSY</th>
<th>(^1H - ^1{C}) HMBC</th>
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<tr>
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<td>1.44 dd (3.5, 13.8)</td>
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<tr>
<td></td>
<td></td>
<td>1.86 m</td>
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<td>2.04 dd (3.5, 13.9)</td>
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<td>4</td>
<td>66.3 (CH)</td>
<td>3.71 dd (3.9, 12.5)</td>
<td>H-3a, H-3b</td>
<td>C-2, C-3, C-5, C-22, C-23</td>
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</tr>
<tr>
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<td>51.0 (CH)</td>
<td>1.52 d (7.7)</td>
<td></td>
<td>C-1, C-2, C-3, C-4, C-5, C-21</td>
</tr>
<tr>
<td>7</td>
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<td>2.02 m</td>
<td></td>
<td></td>
</tr>
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<td></td>
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<td></td>
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</tr>
<tr>
<td>9</td>
<td>80.5 (C)</td>
<td>4.02 dd (3.4, 12.5)</td>
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<tr>
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<td>2.24 q (13.2)</td>
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<tr>
<td></td>
<td></td>
<td>2.56 dt (3.8, 13.5)</td>
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<td>13</td>
<td>199.3 (C)</td>
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<td>14</td>
<td>108.9 (C)</td>
<td></td>
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<tr>
<td>15</td>
<td>163.0 (C)</td>
<td></td>
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<tr>
<td>16</td>
<td>119.5 (C)</td>
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<td>162.9 (C)</td>
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<td>108.1 (CH)</td>
<td>1.21 s</td>
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<tr>
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<tr>
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<tr>
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<td>1.50 s</td>
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<td>1.36 s</td>
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<tr>
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<td>30.0 (CH(_3))</td>
<td>2.18 s</td>
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<tr>
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<td>8.1 (CH(_3))</td>
<td>12.47 s</td>
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</tr>
<tr>
<td>OH (15)</td>
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</tbody>
</table>

\(^{a}\) Spectra were recorded in CDCl\(_3\) at 600 MHz \(^1H\). \(^{b}\) Assignments were made based on interpretation of COSY, HMBC and HSQC experiments.
a bromine atom on the geranyl ring. Analysis of COSY, HMBC and HSQC NMR spectral data revealed that 26 contains a hydrogen atom at C12. Only one other napyradiomycin, CNQ525.512 (37), contains a hydrogen atom at C12 with all other napyradiomycins containing either a chlorine or hydroxyl moiety at C12 or a double bond at the C11 and C12 position. Comparison of the $^1$H NMR spectral data from compound 26 with that from CNQ525.512 showed a downfield chemical shift for C10 (CNQ525.512 = $\delta_H$ 3.92, dd, $J$ = 3.9, 12.3, H-10; CNQ525.600 = $\delta_H$ 4.02, dd, $J$ = 3.4, 12.5, H-10) suggesting the presence of the second bromine at C10. The hydroxylated and brominated geranyl ring, the presence of a bromine atom at C10, and the presence of a hydrogen atom at C12 makes 26 an exciting new compound to be added to the napyradiomycin library. The structure of CNQ525.600 was confirmed by comprehensive two dimensional NMR analysis (see Table 3).

The basic structural nature of the napyradiomycins, consisting of a napthoquinone core, a prenyl tetrahydropyran and a monoterpenoid substituent made it likely that there would be multiple compounds with the same chemical formula. Since these two compounds would likely be difficult to distinguish by LCMS, comparison of $^1$H NMR spectral data was essential for the identification of these compounds. One such compound that was isolated was CNQ525.510B (27), which appeared by low resolution mass spectrometry to have the same mass as compound CNQ525.510A (36) reported by Soria-Mercado et al. Initial examination of the NMR spectral data revealed that 27 lacked the double bond at C11 - C12 that was present in compound CNQ525.510A (27) (For 36 – $\delta_H$ 4.56, d, $J$ = 1.9, H = 11; For 27 – $\delta_{Ha}$ 2.37, m, H = 11a; $\delta_{Hb}$ 2.54, dd, $J$ = 3.8, ...
In order to confirm that the molecular formula of these two compounds was the same, the sample was analyzed by high resolution time of flight mass spectrometry (TOFMS). A pseudomolecular ion with a mass of 511.1652 amu was observed for the [M+H]$^+$ peak corresponding to a molecular formula of C$_{26}$H$_{32}^{35}$Cl$_2$O$_6$ (theoretical mass 510.1576, Δ 0.0002). This chemical formula matched that of compound CNQ525.510A but the lack of a double bond between C11 and C12 suggested another location for the unsaturation in the new compound. Analysis of HMBC, HSQC, and $^1$H NMR spectral data revealed the presence of one methyl group (δ$_C$ 8.6, C-26, δ$_H$ 2.21, s, H-26) attached to an aromatic carbon, five methyl groups (δ$_C$ 21.1, C-21, δ$_H$ 1.48, s, H-21; δ$_C$ 21.4, C-22, δ$_H$ 0.29, s, H-22; δ$_C$ 31.7, C-23, δ$_H$ 0.89, s, H-23; δ$_C$ 22.8, C-24, δ$_H$ 1.31, s, H-24; δ$_C$ 29.5, C-25, δ$_H$ 1.49, s, H-25) attached to sp$^3$ carbons and one aromatic proton (δ$_H$ 7.26, s, H18). The molecular formula of compound CNQ525.510B (27) could be derived from compound A80915C (34) with the loss of HCl.$^4$ In the biosynthesis of the napyradiomycins, there are three potential locations for halogenation, at C4, C10, and C12. The absence of double bonds adjacent to any potential sites of halogenation suggested that the loss of HCl must have occurred through the formation of a new ring in the structure. The most likely reactions for the formation of a new ring in the compound was the displacement of a chlorine through an Sn$^2$ attack from the hydroxyl attached to C1. Defining this ring by NMR methods was ambiguous due to the difficulty of detecting HMBC correlations through a quaternary carbon and an ether. Therefore, this compound was crystallized by slow evaporation from a 50:50 hexane:ethyl acetate mixture. X-Ray analysis, provided by the UCSD X-ray Facility, was
Table 3.4: NMR Spectral Data for CNQ525.510B$^{a,b}$ (27)

<table>
<thead>
<tr>
<th>Carbon #</th>
<th>$\delta_C$ ppm</th>
<th>$\delta_H$ ppm (J, Hz)</th>
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<th>$^1H$ - $^{13}C$ HMBC</th>
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<td>C-1, C-3, C-6</td>
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<td>H-3a, H-3b</td>
<td>C-1, C-3, C-6</td>
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<td>2.54 (3.8, 14.0)</td>
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<td>131.8 (C)</td>
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<td>192.9 (C)</td>
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<td>0.89 s</td>
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a) Spectra were recorded in CDCl$_3$ at 500 MHz ($^1$H). b) Assignments were made based on interpretation of COSY, HMBC and HSQC experiments.
successful and provided the structure in Figure 1. In this structure, it is apparent that the bicyclic ether was formed by tranannular displacement of chloride. Attempts to synthesize the ether from the parent compound (34), under base catalysis were, however, unsuccessful. It is possible that the precursor of the compound was not A80915C but instead a bis-chlorinated mono-brominated napyradiomycin with bromine at C4 acting as the leaving group. The structure of 27 was confirmed by comprehensive two dimensional NMR analysis (see Table 4).
Further purification of the extract yielded another compound that had a similar mass as the known napyradiomycin A80915B (33), but clearly lacked the UV profile of a napyradiomycin with the diazo moiety attached to the napthoquinone core that is present in A80915B.4 Examination of the isotope ionization pattern of the compound suggested that it was not tri-chlorinated as in compound A80915B but was instead mono-chlorinated and mono-brominated. This new compound CNQ525.554B (28) was analyzed by high resolution positive ion FTMS yielding a measured mass of 555.1150 amu for the [M+H]+ pseudomolecular ion. This mass corresponds to the molecular formula C_{26}H_{32}O_{6}^{79}Br_{35}Cl (theoretical mass 554.1071, Δ 0.0001). This compound had a similar molecular formula to CNQ525.510B, but chlorine was replaced with bromine. Since both of these compounds behaved similarly chromatographically, it appeared likely that they had the same basic structure. The 1H NMR spectral data for both compounds were virtually identical. HSQC NMR analysis showed a significant upfield shift for C10 (CNQ525.510B = δC 59.0, C10; CNQ525.554B = δC 50.9, C-10) suggesting this as the site of bromination. Subsequent comprehensive analysis of HSQC and HMBC NMR data demonstrated that compound CNQ525.554B (28) was a brominated analog of compound CNQ525.510B (27) with the bromine positioned at C10 (see Table 5).

Stereochemistry of the compounds was determined using NOESY correlations where possible and x-ray crystallography for compound CNQ525.510B. For compounds that were not crystallized stereochemistry was assumed based on the stereo-specific biosynthesis of the compounds. Chemical shift data suggested that all of these compounds had classical napyradiomycin stereochemistry.
Table 3.5: NMR Spectral Data for CNQ525.554B<sup>a,b</sup> (28)

<table>
<thead>
<tr>
<th>Carbon #</th>
<th>$\delta_C$ ppm</th>
<th>$\delta_H$ ppm ($J$, Hz)</th>
<th>COSY</th>
<th>$^1$H - $^{13}$C HMBC</th>
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<tr>
<td>1</td>
<td>90.6 (C)</td>
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<tr>
<td>2</td>
<td>29.4 (CH&lt;sub&gt;2&lt;/sub&gt;)</td>
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<td>C-1, C-3, C-6</td>
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<td></td>
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<td>1.20 m</td>
<td>C-1, C-3, C-6</td>
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<tr>
<td>3</td>
<td>26.7 (CH&lt;sub&gt;2&lt;/sub&gt;)</td>
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<td></td>
<td>1.62 m</td>
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<tr>
<td>4</td>
<td>87.2(CH)</td>
<td>3.77 d (4.8)</td>
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<td>6</td>
<td>48.6 (CH)</td>
<td>1.78 bs</td>
<td></td>
<td>C-1, C-2, C-7, C-8, C-21, C-22, C-23</td>
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<tr>
<td>7</td>
<td>36.7 (CH&lt;sub&gt;2&lt;/sub&gt;)</td>
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<td>H6</td>
<td>C-1, C-5, C-6, C-8, C-20</td>
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<td></td>
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<td>H6</td>
<td>C-1, C-5, C-6, C-8, C-20</td>
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<tr>
<td>8</td>
<td>83.6 (C)</td>
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<td></td>
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<td>9</td>
<td>78.3 (C)</td>
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<td>10</td>
<td>51.0 (CH)</td>
<td>4.58 dd (3.8, 12.2)</td>
<td>H11a, H-11b</td>
<td>C-9, C-11, C-12, C-24, C-25</td>
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<tr>
<td>11</td>
<td>43.6 (CH&lt;sub&gt;2&lt;/sub&gt;)</td>
<td>2.49 dd (12.4, 13.9)</td>
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<td>C-8, C-9, C-10, C-12, C-13,</td>
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<td>163.1 (C)</td>
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<td>131.6 (C)</td>
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<td>20</td>
<td>192.9 (C)</td>
<td></td>
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<td></td>
<td>C-4, C-5, C-22</td>
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<tr>
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<tr>
<td>26</td>
<td>8.5 (CH&lt;sub&gt;3&lt;/sub&gt;)</td>
<td>2.16 s</td>
<td></td>
<td>C-14, C-15, C-16, C-17, C-18</td>
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<td>OH (15)</td>
<td></td>
<td>12.36 s</td>
<td></td>
<td>C-14, C-15, C-16</td>
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</tbody>
</table>

a) Spectra were recorded in CDCl<sub>3</sub> at 600 MHz ($^1$H). b) Assignments were made based on COSY, HMBC and HSQC experiments.
Table 3.6: HCT-116 IC\textsubscript{50} results of napyradiomycins

<table>
<thead>
<tr>
<th>Compound</th>
<th>Structure</th>
<th>IC\textsubscript{50}</th>
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<tbody>
<tr>
<td>CNQ525.522</td>
<td>24</td>
<td>&gt;100 µM</td>
</tr>
<tr>
<td>CNQ525.538</td>
<td>25</td>
<td>6 µM</td>
</tr>
<tr>
<td>CNQ525.600</td>
<td>26</td>
<td>49 µM</td>
</tr>
<tr>
<td>CNQ525.510B</td>
<td>27</td>
<td>17 µM</td>
</tr>
<tr>
<td>CNQ525.554B</td>
<td>28</td>
<td>&gt;100 µM</td>
</tr>
<tr>
<td>Napyradiomycin B1</td>
<td>29</td>
<td>2 µM</td>
</tr>
<tr>
<td>Napyradiomycin B3</td>
<td>30</td>
<td>3 µM</td>
</tr>
<tr>
<td>Napyradiomycin B4</td>
<td>31</td>
<td>10 µM</td>
</tr>
<tr>
<td>A80915A</td>
<td>32</td>
<td>3 µM</td>
</tr>
<tr>
<td>A80915B</td>
<td>33</td>
<td>&lt;1 µM</td>
</tr>
<tr>
<td>A80915C</td>
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<td>15 µM</td>
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<tr>
<td>A80915D</td>
<td>35</td>
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<tr>
<td>CNQ525.510A</td>
<td>36</td>
<td>&gt;100 µM</td>
</tr>
<tr>
<td>CNQ525.512</td>
<td>37</td>
<td>&gt;100 µM</td>
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</table>

In the course of isolating these five novel napyradiomycins I was also able to isolate a number of known compounds 29-37 (see Table 6). Having a series of compounds allowed me to undertake an investigation comparing the cytotoxicity of these diverse napyradiomycins. Previously, the napyradiomycins had been shown to be antibacterial but also active against some cancer cell lines. Due to the high level of conjugation in the molecules, it seemed possible that the biological actions of these molecules could be the result of nucleophilic addition (acting as Michael acceptors) thus causing broad, non-specific cellular damage. On the other hand, if the mechanism of action was binding to a specific protein target then structural variations in the molecules should have a significant effect on the potency of the molecules by influencing how effectively they bind. Napyradiomycins (24-37) dissolved in DMSO, were added to HCT-116 colon cancer cells and incubated for 3 days after which cell death was
measured using an MTT assay. In interpreting the results, five structural features of the molecules were evaluated: 1) the presence of a methyl moiety on C16; 2) the presence of a hydroxyl or exocyclic methylene at C1; 3) the presence of a chlorine, unsaturation, or proton at C12; 4) the substitution of bromine for chlorine, and 5) the presence of a diazo moiety at C18.

The presence or absence of a methyl moiety at C16 is one of the most common variations seen in the napyradiomycins. The original napyradiomycins isolated by Shiomi et al. lacked this methyl moiety,\textsuperscript{10} however the compounds of the A80915 class published by Fukuda contained this structural feature.\textsuperscript{4} We found that the presence of the methyl group very slightly reduced the activity of the compounds in our cytotoxicity
assay (see Figure 2A). Napyradiomycin B1 (29) was found to be slightly more potent than compound A80915A (32), and napyradiomycin B4 (31) was found to be slightly more potent than A80915C (34). Although the differences between the compounds in both cases were not statistically significant, the reproducibility in two sets of compounds suggested that the methyl moiety may have a slightly negative effect on the potency of the molecules. Further examination of Figure 2A reveals that napyradiomycin B1 (29) is more potent than napyradiomycin B4 (31) and that A80915A (32) is more potent than A80915C (34). Napyradiomycin B1 and A80915A contain an exocyclic methylene at C1 while napyradiomycin B4 (31) and A80915C (34) contain an alcohol and a methyl moiety attached to C1. It appears from the results that the presence of the alcohol at C1 has a significant negative impact on the potency of the molecules in this assay. During the purification process, A80915C (34) and A80915A (32) were the major compounds purified from the extract. Compound A80915C (34) accounted for nearly 70% by weight of the napyradiomycins purified.

The addition of the diazo moiety at C18 in the A80915 compounds (33 and 35) is perhaps one of the most structurally interesting variations seen in the class. The diazo containing compounds are significantly more potent (see Figure 2B) than their non diazo containing analogues. It is not clear if this increased activity in A80915B (33) and A80915D (35) compared to A80915A (32) and A80915C (34) is due to greater activity against the target(s) of the napyradiomycins, or if in addition to the core activity of the napyradiomycins the diazo containing compounds are more potent by virtue of a reactive functionality. It is possible that the unique characteristics of the diazo-containing
compounds act *in vivo* by a mechanism quite distinct from that of the other napyradiomycins.

The halogenation pattern of the napyradiomycins is the greatest source of variation in the class. This variation is limited to chlorine and bromine however since iodine and fluorine are not seen in these compounds in natures. Notable variations in the halogenation patterns are found in the presence or absence of the chlorine atom at C12 and in the presence of either bromine or chlorine at C4. Although derivatives were evaluated that contained a hydrogen atom at C12, these molecules were either too inactive (CNQ525.512 (37)) to be meaningfully compared to their analogue (CNQ525.510A (36)) or did not possess a structural match for comparison (CNQ525.600
(26)). We were able to identify two sets of molecules that varied in either possessing a
double bond between C11 and C12, or chlorine on C12 and saturation at C11. The
previously identified compound CNQ525.510A (36) is an oxidized analogue of A80915C

The loss of the chlorine and formation of the double bond significantly lowers
the activity of the molecules (see Figure 2C). This loss of activity was also seen with
CNQ525.522 (24), which is the oxidized analogue of napyradiomycin B3 (30).

It appears that the presence of chlorine at C12 is important for the interaction of
the molecules with its target protein. There is variation in which halogens are used by the
vanadium dependent chloroperoxidases that halogenate at C4 and C10. The novel
compound CNQ525.554B (28) is a brominated analogue of CNQ525.510B (27). The
presence of bromine at C10 significantly reduced the activity of the compound. The

negative effect of bromine on the activity of the compound was significantly larger than the very slight difference seen between the brominated napyradiomycin B3 (30) and the chlorinated B1 (29) in which the variation occurs at C4. Since the increased size of the bromine atom appears to negatively affect the activity much more at C10 than at C4, it is possible that C10 sits deeper in a putative binding pocket than C4 does. The results of this structure activity relationship study were somewhat limited by the small amount of material that we had of many of the compounds (less than 1 mg of CNQ525.522 for
example). However, the consistent effects of the structural variations on the activity of the compounds suggested that there was a specific protein target for the napyradiomycins.

The indication that the mechanism of action of the napyradiomycins might be protein-specific led us to examine whether the compounds induced apoptosis, a regulated cell death, or necrosis, a non-regulated cell death. In healthy, multi-cellular organisms, a key part of controlled cell growth is apoptosis. Cancerous cells often become resistant to apoptotic signals and therefore continue to grow in environments in which normal cells would undergo regulated cell death. Compounds that induce

Figure 3.2: Inhibition concentration (IC\textsubscript{50}) values were deduced from the bioreduction of MTT/PMS by living cells into a formazan product measured at OD 492. Compound concentration is represented as 10\textsuperscript{5} nM.
apoptosis have a greater potential to become therapeutic agents than compounds which induce necrosis due to broad unregulated cell death being likely to cause significant undesirable side-effects. Four napyradiomycins were selected to be examined for the ability to induce apoptosis: one novel ether, CNQ525.510B (27), one of the potent exocyclic methylene compounds, A80915A (32), the major compound produced by the strain CNQ525, compound A80915C (34), and the diazo analogue of the exocyclic methylene compound tested, compound A80915B (33). HCT116 colon cancer cells were incubated in triplicate with compound for 24 hours then detached with trypsin and washed. Cells were stained with propidium iodide and Yo-pro® apoptosis detecting dye from Invitrogen. Samples were analyzed by FACS with gating determined by camptothecin control. All compounds were found to induce apoptosis in a dose dependent manner (See Figure 3). Due to the gating out of cell particles from the flow cytometry analysis, this assay is not able to assess total cell death but can only analyze the state of the intact cells that are still present. Therefore, reliable IC$_{50}$ values cannot be obtained from this assay but we can see at what point apoptosis begins to be induced. Napyriomycin CNQ525.510B (27) began to induce apoptosis at 4 µM, which is similar to compound A80915A. However the tertiary alcohol-containing compound A8015C (34) did not begin to induce significant apoptosis at less than 8 µM. The biomodal effect seen in the diazo-containing compound (see Figure 3D) may be due to two mechanisms taking place. At 1 µM and 2 µM there is significant cell death and apoptosis, which is lower at 4 µM and then is present again at 8 µM and 16 µM. We were not able to obtain the 50,000 events necessary for our analysis for the A808915B (33) sample at 32 µM due
Figure 3.2. Apoptosis induction assay. Four compounds A) CNQ525.510B (27), B) A80915 A (32), C) A80915C (34), and D) A80915B (33) were incubated with HCT-116 cells at various concentrations for 24 hours. Samples were subsequently stained with propidium iodide, a marker of cell death, and Yo-Pro™, a marker of apoptosis before FACS analysis.
to complete cell death. The initial activity may be due to activity associated with the loss of the diazo group, while the secondary activity seen at higher concentrations may be due to the napyradiomycin acting at the core target. The finding of apoptosis induction seen in four distinct compounds of the napyradiomycin class suggests that the class as a whole may be an inducer of apoptosis. This reinforces the suggestion that there is a specific protein and biochemical pathway with which these molecules are interfering.

It is important to remember that the work of natural products chemists has over the course of the last hundred years yielded a vast array of molecules that could be potential therapeutics. The vast majority of these molecules have never been thoroughly examined for their biological potential. Recently, pentabromopseudilin (38), which was the first bacterial marine natural product isolated, has been shown to have the ability to inhibit myosin motor activity, a quite unexpected mechanism. The molecule was first isolated in 1966 and briefly described as an *in vitro* antibiotic. For over four decades, very little work was done on this compound. However, it is now revealed that this known compound has an exciting and new mechanism of action which needs to be explored.

![Chemical Structure of 38](image-url)
further. Another example is the cancer drug taxol, which was discovered in the 1970s but revitalized in the 1990s based upon new pharmacology data. If the goal of academic and pharmaceutical research is to cure the diseases from which many are suffering, it would be negligent not to explore this vast array of potential therapeutic molecules simply because the carbon skeleton was discovered elsewhere first.

In the course of this chapter I have described the isolation and structure elucidation of five new napyradiomycins. By comparing 14 napyradiomycins in a structure activity relationship (SAR) study, and by the demonstration of induction of apoptosis, I provided evidence for a specific protein target for these molecules. From these initial studies it was not yet clear if the mechanism of action of the napyradiomycins was unique, however it did warrant further examination. In order to further explore the biological activity of the napyradiomycins, the next two chapters will explore the nature of the apoptotic response from select napyradiomycins and studies defining the likely protein target of this class of molecules will be presented.
Methods

General Experimental Procedures - Optical rotations were measured on a JASCO P-2000 polarimeter. UV spectra were measured on a Beckman Coulter DU800 spectrophotometer. CD spectra were recorded on an AVIV model 215 spectrometer. IR spectra were recorded on a Perkin-Elmer 1600 FTIR spectrometer. NMR spectra were obtained on Varion Inova 500 and 300 MHz spectrometers, and a Bruker 600 MHz DRX-600 equipped with a 1.7 mm cryoprobe and Avance III console. Chemical shifts (δ) are given in ppm, and coupling constants (J) are reported in Hz. High-resolution mass spectra were obtained on an Agilent ESI-TOF at the Scripps Center for Mass Spectrometry. Low-resolution LC/MS data were acquired using a Hewlett-Packard series 1100 system equipped with a reversed-phase C18 column (Phenomenex Luna, 4.6 × 100 mm, 5 μm) at a flow rate of 0.7 mL/min. HPLC separations were performed using a Waters 600E system controller and pumps with a Model 480 spectrophotometer. Separation was achieved using Phenomenex Luna semi-preparative C18 (250 × 10 mm, 5 μm) and Phenomenex Luna semi-preparative Si (250 × 10 mm, 5 μm) columns at flow rates of 5.0 and 3.0 mL/min, respectively.

Isolation of compounds 24-28 – The strain CNQ525 was cultured in A1bFe + C media (10 g starch, 4 g yeast extract, 2 g peptone, 1 g CaCO3, 5 mL of a 2% (w/v) KBr stock solution, and 5 mL of a 0.8% (w/v) Fe2(SO4)•4H2O stock solution in 1 L seawater) for compounds 27 and 28. A1bFe + C high bromine media made with high bromine
artificial seawater (20 g NaBr, 0.6 g KBr, 9.3 g MgCl₂•6H₂O, 1.3 g CaCl₂•2H₂O, 3.4 g Na₂SO₄, 1.7 g NaHCO₃, 0.002 g Na₂HPO₄, 1 liter distilled water) for compounds 24-26. Cultures were on a rotary shaker, at 30 °C for 9 days, and then extracted by stirring for 6 h with Amberlite XAD-7 resin (20–30 gm/L). The resin was filtered and extracted with acetone, and the solvent removed under vacuum to generate the crude extract. The subsequent extract was loaded onto 20 grams of silica gel and dry-packed onto a 200 gram silica flash column for initial separation. Flash chromatography was performed using ethyl acetate and isoctane to produce 10 fractions of differing polarity. These fractions were then analyzed by LCMS to assess the presence of members of the napyradiomycin family. Compound 24 was isolated from the 75% hexane 25% ethyl acetate flash column fraction using semi-preparative C18 HPLC run isocratically at 80% ACN 20% H₂O. Compound 25 was isolated from the 85% hexane 15% ethyl acetate flash column fraction using semi-preparative C8 HPLC run isocratically at 80% ACN 20% H₂O. Following this separation the major peak eluting at 30 minutes was separated using semi-preparative Silica HPLC run isocratically at 50% isoctane 50% ethyl acetate. Compound 26 was isolated from the 65% hexane 35% ethyl acetate flash column fraction with semi-preparative C8 HPLC using a 70:30 to 100:0 (ACN:H₂O) over 40 minutes. The 13-18 minute fraction was further purified with semi-preparative Silica HPLC run isocratically using a 20:80 to 40:60 (ethyl acetate:isoctane) gradient over 30 minutes. Compound 27 was isolated from the 65% hexane 35% ethyl acetate flash column fraction using semi-preparative C8 HPLC run isocratically at 80% ACN 20% H₂O. Compound 28
was isolated from the 65% hexane 35% ethyl acetate flash column fraction with semi-preparative C8 HPLC using a 70:30 to 100:0 (ACN:H₂O) over 40 minutes.

**X-Ray** - A colorless block 0.15 x 0.15 x 0.15 mm in size was mounted on a Cryoloop with Paratone oil. X-ray data were collected in a nitrogen gas stream at 100(2) K using phi and omega scans. Crystal-to-detector distance was 60 mm and exposure time was 5 seconds per frame using a scan width of 0.5°. Data collection was 98.2% complete to 67.00° in θ. A total of 7887 reflections were collected covering the indices, -11≤h≤12, -18≤k≤18, -12≤l≤12. 4187 reflections were found to be symmetry independent, with an R_int of 0.0191. Indexing and unit cell refinement indicated a primitive, monoclinic lattice. The space group was found to be P2(1) (No. 4). The data were integrated using the Bruker SAINT software program and scaled using the SADABS software program. Solution by direct methods (SIR-2004) produced a complete heavy-atom phasing model consistent with the proposed structure. All non-hydrogen atoms were refined anisotropically by full-matrix least-squares (SHELXL-97). All hydrogen atoms were placed using a riding model. Their positions were constrained relative to their parent atom using the appropriate HFIX command in SHELXL-97.

**Antiproliferative Bioassay** - Aliquot samples of HCT-116 human colon adenocarcinoma cells were transferred to 96-well plates and incubated overnight at 37 °C in 5% CO₂/air. Test compounds were added to the plates in DMSO and serially diluted. The plates were then further incubated for another 72 h, and at the end of this period, a CellTiter 96
Aqueous non-radioactive cell proliferation assay (Promega) was used to assess cell viability. Inhibition concentration (IC$_{50}$) values were deduced from the bioreduction of MTS/PMS by living cells into a formazan product. MTS/PMS was first applied to the sample wells, followed by incubation for 3 h. Etoposide (Sigma; IC$_{50}$ = 1.5–4.9 μM) and DMSO (solvent) were used as the positive and negative controls in this assay. The quantity of the formazan product (in proportion to the number of living cells) in each well was determined by the Molecular Devices Emax microplate reader set to 490 nm wavelength. IC$_{50}$ values were calculated using the analysis program Prism.

**Compound 24 – CNQ525.538** pale yellow oil, $[\alpha]_D$ -3.7 (CHCl$_3$); IR (film) max 2922, 2854, 1602, 1451, 1346, 1281, 1114, 1072 cm$^{-1}$. NMR see table 1. FTMS + c ESI, [M+Na]$^+$ m/z 545.0709.

**Compound 25 - CNQ525.522** pale yellow oil, $[\alpha]_D$ -1.1 (CHCl$_3$); IR (film) max 2921, 1713, 1457, 1375, 1267, 1177, 1148,1051, 1014, 951 cm$^{-1}$. NMR see table 2. HRMALDITOFMS, [M-H]$^-$ m/z 537.1042.

**Compound 26 – CNQ525.600** pale red orange oil, $[\alpha]_D$ -20 (CHCl$_3$); IR (film) max 2921, 1697, 1622, 1455, 1309, 1115, 1055 cm$^{-1}$. NMR see table 3. HRMALDITOFMS, [M-H]$^-$ m/z 599.0649.
Compound 27 – CNQ525.510B pale colorless oil, [α]D -9 (CHCl₃); IR (film) max 3399, 2920, 2851, 1711, 1608, 1457, 1286, 1163, 1079, 1012, 909 cm⁻¹. NMR see table 4. HRMALDITOFMS, [M+H]⁺ m/z 511.1652

Compound 28 – CNQ525.554B pale red oil, [α]D -42 (CHCl₃); IR (film) max 2922, 1701, 1604, 1454, 1285, 1197, 1118, 1081, 1007 cm⁻¹. NMR see table 5. FTMS + c ESI, [M+H]⁺ m/z 555.1150.
References


Chapter 4

Mechanism of apoptosis induction by the napyradiomycins

Abstract

Many chemotherapeutic agents have been shown to act by affecting apoptotic pathways. The biological mechanism by which these apoptotic effects are achieved can be quite variable. In this study I examine the induction of apoptosis by the napyradiomycins, a group of unique compound isolated from marine actinomycetes. I hypothesize that the napyradiomycins might induce apoptosis either by inducing cell cycle arrest, a mechanism similar to the action of etoposide, or by affecting the intrinsic or extrinsic pathways of apoptosis, mechanisms similar to doxorubicin\(^1\) and the marine-derived somocystinamide\(^2\), respectively. My findings indicate that the induction of apoptosis resulting from exposure to the napyradiomycins is not due to cell cycle arrest, nor is it likely due to an activation of the classical intrinsic or extrinsic pathways of apoptosis. My results suggest that the mechanism for apoptosis induction may be more convoluted than first suggested and reinforce the need to identify the protein target(s) of these compounds.
Introduction

Apoptosis is a key regulator of many cellular processes, such as removing karyotypically abnormal cells and in embryonic organogenesis. In healthy multi-cellular organisms, a key component of controlled cell growth is regulated cell death, also known as apoptosis. Cancerous cells often become resistant to apoptotic signals and therefore continue to grow in environments in which normal cells would sacrifice themselves for the good of the organism as a whole. It has been shown that a hallmark of cancer is the ability to evade apoptotic signals or to induce anti-apoptotic proteins. Defective apoptotic mechanisms have been implicated in cancer cell survival, not only against the host’s own defenses but also against chemotherapeutic agents. As a result, targeting apoptosis is a key area of current cancer chemotherapeutic drug research. The ubiquitous nature of apoptotic pathways and their role in multiple essential cellular processes raises the possibility that agents that target apoptosis might exhibit a low therapeutic index. However, this can likely be overcome by specifically targeting the mechanism of apoptosis resistance that is over expressed in a given cancer type. It appears that any agent that specifically targets an apoptotic mechanism will likely be part of a multiple drug regime to 1) re-sensitize the cancer to apoptosis, and then 2) kill the cancer with known chemotherapeutic drugs that induce apoptosis.

Apoptosis is generally understood as being mediated by two caspase dependent pathways, the intrinsic and the extrinsic pathway. Elements of the intrinsic pathway of apoptosis detect intracellular stress and initiate apoptosis by releasing cytochrome C from the mitochondria. The extrinsic pathway is activated by an extracellular signal that
activates apoptosis through a surface receptor. However, to simply explain apoptosis as a two pathways process is a simplification of the biological reality. These two pathways may interact with each other and there are non-classical pathways for the induction of regulated cell death that don’t conform strictly to the extrinsic/intrinsic pathway doctrine.

For instance, it has been found that apoptosis can also be regulated by steroid-hormone receptors acting on phosphoinositide 3-kinase (PI3K). Ongoing research in apoptosis is continually bringing to light new apoptotic mechanism and interactions. In order to understand the therapeutic value of any potential apoptosis inducing agent, it is of vital importance to understand the mechanism by which the compound induces apoptosis. An understanding of the mechanism of apoptosis induction will allow researchers and medical professionals to target the cancers that are most likely to respond to such a treatment.

Results and Discussion

A common mechanism by which cancer chemotherapeutic drugs induce apoptosis is through cell cycle arrest. Compounds that act on microtubules or enzymes necessary
for DNA replication, in addition to compounds that mimic DNA bases all ultimately inhibit the cell cycle. The cell cycle is an excellent target for chemotherapeutic agents because cancerous cells generally replicate with greater frequency than the majority of other cells in the body. Therefore, compounds that target the cell cycle will generally show a preference for the destruction of cancerous cells when compared to healthy non-cancerous cells. Many of the side effects often seen with chemotherapy result from effects on other normally proliferating cell types, rather than other off-target effects. For example, the nausea that is associated with chemotherapeutic treatment is associated with the destruction of the rapidly dividing cells of the gastrointestinal tract.
The cell cycle is divided into four main parts G1, S, G2, and M phases. In G1 the cell grows larger and prepares to replicate its DNA. In S phase the cell replicates its DNA. In G2 phase the cell continues to grow and prepare for division. M phase, or mitosis, is a very quick phase in which the cell divides into two daughter cells. There are multiple checkpoints along the way and numerous chemotherapeutic agents that arrest the cell cycle at distinct stages. Methotrexate and 6-mercaptopurine both arrest the cell in S phase by interfering with the ability to replicate DNA; whereas drugs like vincristine and taxol interfere with the mitotic spindle and arrest the cell in M phase. However, there are also compounds that are non phase specific, such as alkylating drugs and the cisplatinum compounds. In order to determine if the napyradiomycins affect the cell cycle we examined the effects of compound A80915C (34) on the DNA replication patterns of HCT-116 colon cancer cells.

HCT-116 cells were incubated with various concentrations of the napyradiomycin A80915C (34), etoposide (39), or DMSO alone. After incubations of various set time points, cells were detached from growth dishes, washed and permeabilized. The cells
were stained with propidium iodide, a fluorescent DNA stain, and the samples were analyzed by flow cytometry. Flow cytometry is able to quantify the fluorescence in a single cell by only passing one cell past the detector. The DNA quantity in the cell is directly proportional to the amount of fluorescence, as a result of staining the cell with propidium iodide. Therefore, there will be three distinct populations in a cell. The population of cells in G1 will have $1\times$ DNA and the cells in G2/M will have $2\times$ DNA. The cells that are in S-phase will have a DNA content that is between $1\times$ and $2\times$ because those cells are in the process of replicating their DNA. HCT-116 cells treated with DMSO alone for 36 hours (see figure 1A) demonstrate these three populations very clearly. The first large peak at fluorescence $= 90$ measured units corresponds to the G1 population of cells. The second, smaller, peak at fluorescence $= 180$ represents the G2/M-phase cells. The small ridge of cells seen from fluorescence 100 to 150 is the S-phase cells. Cells treated for 36 hours with 4 µM etoposide (see figure 1B) showed a substantial increase in the percentage of cells in G2/M-phase as a result of cell cycle arrest associated with the inhibition of topoisomerase II. Treatment of cells with 16 µM A80915C (34) for 36 hours (see figure 1C) did not show a significant cell cycle effect. This suggested that the mechanism of action of the napyradiomycins was not associated with the cell cycle and that apoptosis induction was not due to cell cycle arrest.

Having elucidated that the induction of apoptosis in HCT-116 cells upon treatment with napyradiomycins was not accomplished through cell cycle arrest, it became necessary to examine if the induction of apoptosis was accomplished through the intrinsic, extrinsic, or a non-classical pathway of apoptosis. In order to explore the myriad of possibilities it was necessary to develop a coherent scheme to explore
Figure 4.2. Research plan for the determination of the pathways involved in induction of apoptosis due to treatment with napyradiomycins A80915A (32) and A80915C (34). Pathway inclusion or exclusion was based upon Western Blot analysis of activation of specific caspases and survival of cells with controlled caspase levels.
apoptosis inductions. The scheme developed (see figure 2) was based on exclusion of pathways through testing whether or not the caspases involved in a given pathway were: 1) activated in response to the compounds, and 2) necessary for the cytotoxic action of the compounds. Caspases (cysteine-aspartic acid proteases) are the key enzymes of apoptosis. The caspases are roughly divided into two groups, the initiator caspases and the effector caspases. The initiator caspases (caspase-2, caspase-8, caspase-9 and caspase-10) cleave and activate the pro-forms of the effectors caspases (caspase-3, caspase-6, caspase-7), which degrade cellular proteins during apoptosis. Specific caspases act on specific pathways of apoptosis, thereby allowing us to determine which pathway of apoptosis is activated based the pattern of caspase activation. Initially I examined the role of caspase-8, the initiator caspase of the intrinsic pathway. An extracellular death signal, such as FAS ligand, results in the formation of the death inducing signaling complex (DISC) which subsequently activates caspase-8. While small molecules generally do not activate the extrinsic pathway of apoptosis, the marine natural product somocystinamide A (40) was recently shown to act by this pathway.² Therefore, we found it necessary to eliminate the extrinsic pathway as a potential source for the apoptosis observed in response to the napyradiomycins. Examination of cellular extract from HCT-116 cells treated with varying doses of A80915A (32) and A80915C (34) for 24 hours showed a dose dependant cleavage of pro-caspase-8 (see figure 3A). This suggested that caspase 8 was being activated in response to the treatment with these napyradiomycins. It was unclear however if the activation of caspase-8 was the initiating event of apoptosis or if caspase-8 was being activated as a result of a broader apoptotic event. In order to examine if caspase-8 was essential for the action of the
Figure 4.3. Examination of the effects of napyradiomycins on caspase-8. Western blot analysis of cell extract from HCT-116 cells treated with varying doses showed the degradation of pro-caspase-8 (A). Examination of the cytotoxicity of B) A80915A (32), C) A80915C (34) and, D) etoposide (39) showed no significant difference in the IC₅₀ values between NB7 cells (C8-) and NB7 cell with caspase-8 reintroduced (C8+). Cell survival is measured through an XTT assay with optical density at 492 nm correlating with cell survival. Compound concentration is displayed in 10⁶ nM.

Napyradiomycins, cytotoxicity was examined in two neuroblastoma cell lines. The neuroblastoma cell line NB7 lacks caspase-8. A comparable NB7 cell line, in which caspase 8 was reintroduced by stable transfection, has previously been described by the Stupack group.² Examination of the cytotoxicity of A80915A (32) and A80915C (34) showed no significant differences in the IC₅₀ values of the compounds tested. Although upon initial examination of the results it appears that there is a shift leftward for the caspase-8+ curve in both experiments, with the napyradiomycins this is an artifact of the lower maximum optical density obtained in these cultures than in the caspase 8- cultures.
Therefore, we conclude that although caspase-8 is activated in response to the napyradiomycins this activation is most likely an after-effect of a broader apoptotic cascade rather than representing the initiation of apoptosis.

The next step in the experimental design was to determine if caspase 3 is essential for induction of apoptosis by the napyradiomycins. Caspase-3 is an effector caspase and upon activation is responsible for targeting and destroying a large number of proteins. Utilizing the same experimental design as was applied for caspase 8, I began by examining whether or not pro-caspase-3 was activated in response to treatment with napyradiomycins for 24 hours. Pro-caspase-3 showed a dose dependent breakdown in response to treatment with both A80915A (32) and A80915C (34)(see figure 4A).

Subsequently, I examined the cytotoxic response in cells with controlled expression of caspase-3. Again, the Stupack lab had previously described two cell lines to use for this study. Both cell lines were based on the NB7 C8+ cells. In one cell line (C3-) a short hairpin RNA (shRNA) had been introduced which interfered with the ability of the cell line to express caspase 3.10 In the other cell line a scrambled shRNA had been introduced as a control. This allowed me to determine whether the effect I observed was due to the

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Figure 4.4. Examination of the effects of napyradiomycins on caspase 3. Western Blot analysis of cell extract from HCT-116 cells treated with varying doses of napyradiomycins showed the dose dependent degradation of pro-caspase 3 (A). Examination of the cytotoxicity of B) A80915A (32), C) A80915C (34) and D) etoposide (39), showed no significant difference in the IC50 values between caspase 3 negative (C3-) and caspase 3 positive (C3+). Cell survival is measured through an XTT assay with optical density at 492 nm correlating with cell survival. Compound concentration is displayed in 10X nM.

presence or absence of caspase-3 and not an artifact of the transfection process. Two napyradiomycins A80195A (32) (figure 3B) and A80915C (34) (figure 3B) were tested in both cell lines. There was a slight decrease in the efficacy of the napyradiomycins in the C3- cells. However, this drop was not large enough to suggest that caspase-3 was essential for the induction of apoptosis seen in the napyradiomycins. Examination of the original research scheme suggests that if caspase-3 is non-essential it is likely that the induction of apoptosis seen in the napyradiomycins is through a non-traditional pathway.
Non traditional pathways of apoptosis are an ever expanding field of research. In addition to the non-traditional pathways such as paroptosis, an alternative form of cell suicide, and autophagic cell death, there are also mechanisms by which apoptosis can be activated outside of our original research scheme. The most likely way to determine the mechanism by which apoptosis is being activated is to determine the specific protein target of the napyradiomycins. Identification of the specific target will allow us to understand if the napyradiomycins are activating apoptosis through a recognized mechanism or through a novel pathway. The lack of effects on the cell cycle and the activation of both caspase-8 and caspase-3 in the response to the napyradiomycin compounds suggests that the protein target may be novel and warrants further examination. The non-essential nature of caspase-3 and caspase-8 in napyradiomycin induced apoptosis can best be understood in the context of a protein target. In the following chapter we identify a major protein target of the napyradiomycins using two fluorescently compounds in a procedure pioneered by La Clair.11
Methods

Cell cycle analysis- HCT116 cells were cultured in DMEM/Glutamax media (Invitrogen) supplemented with 10% fetal bovine serum and A80915C or etoposide for 24 hours. Cells were incubated on ice in a final concentration of 3µg/mL propidium iodide for 30 minutes prior to flow analysis. Flow cytometric analysis was performed on a Becton-Dickinson LSR1. Cells were gated to exclude debris.

Western blot: samples of precipitated proteins were collected. Protein content was determined using bicinchoninic acid assay (BCA) assay (Pierce # 23235) according to manufacturer’s instructions. Protein (20 µg) was loaded per well on a 10% tris PAG polyacrylamide gel. Gels were wet transferred onto a polyvinylidene fluoride (PVDF) membrane and blocked in 5% dry milk. After blocking, the membrane was incubated overnight at 4º C with primary antibody (Caspase-8 antibody BD Biosciences #551242; Caspase-3 antibody BD Biosciences #611048) at 1:500 in 5% milk in Tris buffered saline with 0.01% tween (TBST). The membrane was washed 4× in TBST. Blot was incubated for 1 hour in TBST with anti-mouse HRP antibody (1:5000). Detection was performed using SuperSignal West Pico Chemiluminescent Substrate (Pierce # 34078) according to manufacturer’s instructions.

Cytotoxicity assays: Cell lines were maintained in RPMI media supplemented with 10% FCS. Cells were plated at 5000 per well in 96 well plates. Compounds A80915A (32)
and A80915C (34) were added to a final concentration of 20µM to the first row of cells and serially diluted 1:2 to a final concentration of 19 nM in the last row of cells. Cells were incubated with compounds at 37º C, 5% CO₂ for 72 hours. Viable cell survival was measured by the ability of cells to reduce the tetrazolium salt XTT to orange colored compounds of formazan according to XTT assay kit instructions (Biological industries cat# 20-300-1000).
References


Chapter 5

Identification of a major protein target of the napyradiomycins

Abstract

Marine natural products are proving to be a valuable resource for discovering new lead compounds in the pursuit of cancer chemotherapeutic agents. The napyradiomycins, an expanding family of marine microbial compounds, have been shown to induce apoptosis in HCT-116 cells through an undetermined pathway. Using two fluorescent coumarin-conjugated napyradiomycins, we have found that the likely target for the napyradiomycins are HSP90 proteins, possibly preferentially targeting the endoplasmic reticulum HSP90 chaperone GRP94. GRP94 is upregulated in numerous cancer types and is now considered a new target for drug discovery.1 The napyradiomycins may provide an exciting new way to study and combat various cancers.
Introduction

Cancer, a disease which manifests as uncontrolled cell growth, has affected people as evidenced from the earliest identifiable human remains. \textsuperscript{2, 3} In the quest to control and treat cancer, few pharmacological agents have proven more powerful than agents isolated from natural sources. Traditionally, natural products drug discovery has focused on terrestrial sources. This is most likely due to the ease of collecting terrestrial plants and microbes as opposed to the difficulty of collecting marine organisms. With increased awareness of the oceans and the development of new technologies and sophisticated isolation and analysis techniques, the marine world has now begun to yield its pharmacological treasure trove.\textsuperscript{4-6} Actinomycetes have long been recognized as the most prolific producers of secondary metabolites. Recent research at the Scripps Institution of Oceanography has focused on the discovery and development of new compounds from marine actinomycetes into clinically relevant drug candidates. In the course of my investigations in association with this program, I have isolated five new and nine previously described metabolites of the napyradiomycin class. The napyradiomycin compounds designated A80915A-D (\textbf{32-35}), which contain a methyl group on the aromatic ring of the naphthoquinone core, were explored by the pharmaceutical company Eli Lilly as potential anti-microbials but further development as antibiotics ceased in the 1990’s. Although a large number of these molecules has been isolated and described, their mechanism of action has yet to be elucidated. Using two napyradiomycins, CNQ525.510B (\textbf{27}) and A80915C (\textbf{2}), which were chemically modified to contain a
coumarin fluorescent tag, I was able to identify a major target of the napyradiomycins as the HSP90 proteins, and what appears to be the specific resident HSP90 of the endoplasmic reticulum, called GRP94.

Results and Discussion

The MAR4 strain CNQ525 was isolated by Alejandra Prieto-Davo and Paul Jensen from sediment sample taken off the coast of La Jolla, CA in 152 m of water. Strain CNQ525 was determined to be a prolific producer of many known and many previously unknown napyradiomycin compounds. Pure napyradiomycin analogs were isolated from organic extracts of 20 L cultures by solvent extraction and fractionation
using a combination of normal phase and reverse phase HPLC. In the course of isolation, a novel napyradiomycin containing a new terpenoid oxabicycle was identified. The compound, designated CNQ525.510B (27), was cytotoxicly active napyradiomycins when tested against the human colon cancer line HCT-116. Using the fluorescent dye Yo-Pro™ (Invitrogen), which has selective permeability to the plasma membranes of apoptotic cells, it became apparent that compound 27, as well as other napyradiomycins, induced apoptosis in a dose dependent manner. Confirmation of the apoptotic effect was achieved through western blot analysis for caspase 3 activation in an extract from cells incubated with increasing doses of the napyradiomycins (See figure 1). Previous work had shown that structural variations among the napyradiomycin class resulted in significant reproducible potency of the compounds. This structure activity pattern suggested that there likely was a specific protein target of the napyradiomycins.

In order to determine the protein target, I worked with Dr. Jim La Clair to synthesize two molecular probes using compound CNQ525.510B (27) and the known napyradiomycin A80915C (34) by conjugation to a highly fluorescent coumarin derivative. Initial probe construction using compound CNQ525.510B was complicated by the limited amount of material isolated from the extract (less than 8 mg). Construction of the probe was accomplished through a three step process (see scheme 1). The goal of the primary step of the synthesis was to attach a tert-butyl 2-bromoacetate to the hydroxyl at C17. It was believed that the carbonyl group beta to the hydroxyl at C15 would
stabilize this hydroxyl and would facilitate a mono-tagged product. Unfortunately the reaction was not clean and the material was largely bis-acylated (43). However,
Scheme 5.1: Synthetic pathway for construction of probes.
subsequent cleavage of the tert-butyl group with TFA and conjugation of the coumarin using HATU resulted in a low yield of the mono-coumarin compound 41. In order to confirm the results obtained from the use of probe 41, a second probe was constructed from napyradiomycin A80915C (34). Although this compound was less potent when tested in whole cell cytotox assays and the flow cytometry based apoptosis induction assays, it was chosen due to the large amount of available material. Having isolated over 800 mg of the compound, I had enough material to completely optimize the reaction conditions and was able to obtain 2 mg of the mono-substituted fluorescent probe 42.

The fluorescent probes were incubated with HCT-116 cells for 24 hrs and viewed on a fluorescent microscope to confirm uptake of the compounds. In order to ensure that the probe maintained the activity of the parent compound, the probe was assessed for the ability to induce apoptosis. To confirm that the probe had a similar biological action to the parent compound, caspase 3 activation was examined by Western Blot (figure 1B). The parent compounds 27 and 34 showed a significant activation of pro-caspase 3 in response to increasing dose. The same pattern was seen with the probes, comparing 10 µM and 100 µM of the compound. Although the potency of the probes 41 and 42 was significantly less than the parent compounds, the core biological activity was maintained.

The synthesis of the fluorescent coumarin probes allowed me to directly visualize the location of the compound in HCT-116 and Hela cells. Hela cells were chosen for much of the visualization work due to their larger cytoplasmic area and ease of visualization. Cells were grown directly on glass coverslips and incubated for 24 hours with 10 µM of the probe. All cell imaging was performed on live cells. Uptake of the
Figure 5.1: A) Apoptosis induction measured by flow cytometry in HCT-116 cells incubated for 24 hours with CNQ525.510B (27). B) Degradation of pro-caspase 3 in response to incubation with A80915C probe (42) (lanes 1-2), A80915C untagged (34) (lanes 3-5), CNQ525.510B probe (41) (lanes 6-7), DMSO control (8) CNQ525.510B untagged (27) (lanes 9-10).

probe became apparent after 8 hours (data not shown). The probe appeared to be unequally distributed predominantly adjacent to one side of the nucleus versus the other. The probes had architecture to their staining pattern suggesting an interaction with a subcellular organelle (see figure 2). In order to determine the sub-cellular localization of the molecule, untreated Hela and HCT cells were co-labeled with standard dyes specific for the endoplasmic reticulum and the golgi apparatus. The ER-tracker™ red stain exhibited a strong correlation to the localization of the fluorescent probe, suggesting that the target of the molecule is predominantly in the endoplasmic reticulum. Utilizing a ceramide-fluorescein stain (ceramide will selectively localize to the golgi apparatus and the fluorescein fluorophore will fluoresce in the green channel), I was able to visualize the golgi apparatus adjacent to the endoplasmic reticulum (see figure 2). Although there was some overlap of the golgi stain with the probes, it is believed that this is likely due
Figure 5.2: Spinning disc microscopy demonstrating sub-cellular localization of napyradiomycin compounds. Compounds were incubated with coumarin tagged probes (blue channel) for 24 hours and co-stained with ER-tracker (red channel), and the golgi stain ceramide-FITC (green channel) for 30 minutes. Cells were imaged live with a 100x objective.
subcellular localization of the organelles in which the golgi is often adjacent to the ER. There was no point of concentration of the probe correlating with the golgi. The ceramide stain also revealed faint staining of the plasma membrane of the cells. Together, these data indicate that the napyradiomycin fluorescent probes 41 and 42 were not predominantly in the cytoplasm but were contained within the endoplasmic reticulum. Confirmation of the sub-cellular localization was established with the A80915C-fluorescent probe 42, which showed a similar staining pattern to the CNQ525.510B-coumarin probe (41) (see figure 2). A fluorescent coumarin control consisting of the coumarin bound to an amino acid showed no significant staining (see figure 3).

The fluorescent probes not only allowed for visualization of the sub-cellular localization of the compound, but also allowed for direct immunoprecipitation of the probe and any proteins bound to it. An HCT-116 cell extract was incubated with either the CNQ525.510B probe 41 or the A80915C probe 42 at 4º C with gentle shaking. After 2 hours an anti-coumarin antibody bound resin, which had been prepared by Dr. La Clair through the immunization of rabbits with coumarin and conjugation of the serum onto the resin, was added to the extract and the mixture was incubated for another hour. After incubation, the resin was washed gently two times with phosphate buffered saline (PBS). Bound protein was then eluted using the unconjugated coumarin reagent and samples were analyzed on a 4-12% polyacrylamide gel. Proteins were visualized with a coomassie stain that revealed a protein at roughly 95 kd (see figure 3A). The protein band was excised and analyzed by mass spectrometry in the the Dorrestein Lab, which
led to the assignment of the protein band as an HSP90 protein (16 peptides from HSP90 seen with $p = 0.1651 - 0.00001$). The sub-cellular localization in the endoplasmic reticulum led us to believe that the protein identified might be GRP94, the resident HSP90 of the endoplasmic reticulum. Western Blot analysis of the eluted protein from the immunoprecipitation assay confirmed that GRP94 was the co-immunoprecipitated protein obtained with both coumarin tagged probes. The highly conserved nature of the

Figure 5.3: Immuno-precipitation of HCT-116 cell lysate using A80915C probe (42).

Cell lysate was incubated with Bovine Serum Albumin (BSA) tagged with coumarin (L1) or A80915C probe (42) (L2) with cell lysate (L3) stained as a control. Western Blot analysis of the target protein revealed it to be GRP94 (L4).

HSP90’s makes it probable that the compounds bind to many HSP90’s but may exhibit a preference for the GRP94 isoform. Isoform preference has been noted before with radicicol, an HSP90 inhibitor, which has a five times greater affinity for HSP90$\alpha$ or HSP90$\beta$ than it does for GRP94. This makes the napyradiomycins a potentially valuable
resource to probe the chemical biology of the HSP90 proteins. If the specific target of the napyradiomycins is GRP94 this would explain the pattern of apoptosis induction seen in response to the napyradiomycins. The targeting of GRP94 would result in apoptosis induction through the unfolded protein response. This mechanism of apoptosis induction is often referred to as ER stress. Although this mechanism of apoptosis will induce many caspases, it is known to strongly activate the effector caspase 7, which would explain the results seen in chapter 4. Since caspase 7 is the major effector caspase in ER stress induced apoptosis, caspase 3, which is usually the major effector caspase was not necessary for the induction of apoptosis.

Heat shock proteins are molecular chaperones that refold damaged proteins or mark them for degradation when repair is impossible. It has been found that the HSP90’s, a subfamily of the heat shock proteins, constitute 1-2% of total cellular protein content.\textsuperscript{9} In cancerous cells, HSP90’s often represent 4-6% of total protein.\textsuperscript{10} Several compounds have been identified which inhibit the activity of HSP90’s. These compounds, including geldanamycin (47), 17-AAG (48) and DMAG-17 (49), bind to HSP90’s ATP binding site and may therefore account for the tumor selectivity often noted with HSP90 inhibitors. HSP90 in tumors often exists in multi-chaperone complexes which have an increased affinity for ATP. Since the initial understanding that geldanamycin (47) exhibited its potent anti-cancer effects through the inhibition of HSP90’s, these proteins have been recognized as a potentially valuable target in cancer chemotherapy.\textsuperscript{11,12} Previous studies have found that HSP90’s are involved in the stabilization and maturation of oncogenic proteins associated with malignant tumor
In addition, the HSP90 proteins act to mediate the harsh environment often found in cancerous growths such as low pH, hypoxia, and nutrient starvation. Since the HSP90’s are an attractive target for cancer chemotherapy, the identification of natural and synthetic compounds that target these proteins has been an exciting area of ongoing pharmacological research.

From the fluorescent probe binding studies, it appears that the napyradiomycins may preferentially target GRP94, the resident HSP90 protein of the endoplasmic reticulum. It has been noted that this protein is upregulated in certain cancer cell lines refractory to chemotherapeutic treatment. In a study by Zhang and co-workers, it was found that ovarian cancer cell lines with increased expression of GRP94 had an increased resistance to adriamycin. Further development of the napyradiomycins is necessary in order to determine if this family of unique marine compounds could potentially act as an adjuvant treatment for refractory cancer chemotherapy. Currently, the HSP90 inhibitor, 17-AAG (48) is in phase II trials for Von Hippel-Lindau Disease and renal tumors. Additionally, the HSP90 inhibitor STA-9090 is in phase I trials for hematological cancers. The addition of the napyradiomycins to the growing family of HSP90 inhibitors
is an exciting development in this new avenue of treatment. In order to further explore the potential of these compounds, studies to define the specific binding site is essential. Clearly, examination of the effect of the napyradiomycins in more complex systems, such as animal models, will need to be undertaken.

In the course of these studies I have identified five new members of the napyradiomycins. Building upon this, I determined that there likely was a specific protein target for the napyradiomycins. It was determined that the compounds induced apoptosis by a pathway that activated both caspase 8 and caspase 3. However it was also determined that neither of these caspases were necessary for the activation of the napyradiomycin induced apoptosis. In order to determine the mechanism of apoptosis induction it was determined that the identification of the napyradiomycins’ protein target was necessary. Working in collaboration with Dr. Jim La Clair, two fluorescent probes were constructed using one new napyradiomycin and one previously described napyradiomycin derivative. The probes showed the sub-cellular localization of the napyradiomycins to be in the endoplasmic reticulum. Use of antibodies that respond to the fluorescent coumarin allowed me to immunoprecipitate an HSP90 protein that bound to the napyradiomycin based probes. The sub-cellular localization of the probe and the targeting of an HSP90 suggested that the specific target of the napyradiomycins might be GRP94, the resident HSP90 of the endoplasmic reticulum. In order to confirm that the napyradiomycins specifically target GRP94 rather than other HSP90 proteins, binding studies on various HSP90’s will be necessary. If it is determined that the
napyradiomycins specifically target GRP94 they will be the first natural products to specifically target this HSP90 ER analog.

The relevance of compounds from families that were discovered in the past has never been more important than today. As the scientific community develops the ability to more carefully examine the mechanism of action of compounds and as our understanding of the biology of disease processes increases, it is important to revisit some of these molecules. It can be argued that a secondary metabolite that is produced by an organism must have a biological function to warrant the energy that the organism has invested in it. Therefore, if in the past the biological function of a molecule has been deemed uninteresting or unknown, it is likely that the right bioassay has not been identified. This unfortunate scenario almost doomed taxol, were it not for the impressive work of Susan Horwitz. It is therefore of paramount importance that when searching for agents that can treat human diseases we not simply look for what is new, synthetic, and unpublished, but instead focus our energy on those compounds most likely to produce an effective treatment.
Methods

Synthesis of fluorescent probes 39 and 40. Construction of the probe was accomplished through a three step process. Parent compound CNQ525.510B, 4 mg, (27) was dissolved in 400 µL of DMF. Next, 8 mg of K2CO3 as added and 1 drop of 1:10 dilution tert-butyl 2-bromoacetate in DMF was added. The reaction was left at room temperature for 1 hour with constant stirring. After stirring, the sample was dried with air. The bis substituted probe 41 was purified using Si HPLC eluting with mixtures of isoctane in ethyl acetate. The bis substituted compound 41 was dissolved in 1 mL DCM with 1 drop of TFA added. The reaction mixture was stirred for 2 hours, then dried yielding compound 42. Compound 42 was dissolved in 400 µL DMF with the addition of 2 drops diisopropyl ethyl amine. HATU (5 mg) was added and the reaction was left at room temperature over night. The reaction mixture was dried and redissolved in 500 µL ACN. Mono coumarin substituted probe 39 was isolated using reversed phase C18 HPLC.

Compound A80915C (15 mg, 34) was dissolved in 400 µL of DMF. Dry K2CO3 (30 mg) as added along with 3 drops of 1:10 dilution tert-butyl 2-bromoacetate in DMF. The reaction was left at room temperature for 1 hour with constant stirring, after which the sample was dried with a stream of air. The compound 43 was purified using Si HPLC with mixtures of isoctane in ethyl acetate The mono substituted compound 43 was dissolved in 1 mL DCM with 3 drop of TFA added. The reaction mixture was stirred for
2 hours, then dried yielding compound 44. Compound 44 was dissolved in 400 µL DMF with the addition of 4 drops diisopropyl ethyl amine. HATU (10 mg) was added and the reaction was left at room temperature over night. The reaction mixture was dried and redissolved in 500 µL ACN. Mono coumarin substituted probe 40 was isolated using reversed phase C18 HPLC.

**Tissue culture protocols.** HeLa and HCT116 cells were cultured in DMEM/Glutamax media (Invitrogen) supplemented with 10% fetal bovine serum. Cells were cultured in T75 flasks at 37º C, 5% CO2. Cells for imaging were grown uncoated 35mm glass bottom microwell dishes (MatTek Cultureware #P35G-010-C).

**Apoptosis Assay.** Cells were incubated in 6 well plates with varying concentrations of compound for 24 hours. Cells were detached from plates using trypsin. Cells were spun down and washed 2× with PBS. Apoptotic analysis was performed using Yo-Pro 1 (Invitrogen) according to manufacturer’s instructions. Cells were incubated on ice in a final concentration of 0.1µM Yo-Pro1 and 3 µg/mL propidium iodide for 30 min prior to flow analysis. Flow cytometric analysis was performed on a Becton-Dickinson LSR1 flow cytometer. Cell gating was performed by analysis of both a negative control (DMSO) and a positive control (24 hours of 10 µM camptothecin). Cells were gated to exclude debris. 10,000 events were recorded on the flow cytometer.
Cell uptake and sub-cellular localization studies  Cells for imaging were grown uncoated 35mm glass bottom microwell dishes (MatTek Cultureware #P35G-010-C). Live cell staining was performed essentially following manufacturer’s direction (Invitrogen). Briefly, HCT-116 and Hela cells were incubated for 24 hours in a 20 nM solution of the fluorescent coumarin probe (39 and 40) in DMEM/Glutamax media (Invitrogen) supplemented with 10% fetal bovine serum. Subsequently, an equal volume 5μM BODIPY FL ceramide (cat#D3521) and 1μM ER-tracker Red (BODIPY TR glibenclamide cat#E34250) in HBSS (Hanks Balanced Salt Solution) was added, and cells were incubated for 45 min at 37 ºC. Cells were washed once in PBS and then imaged live. Imaging was performed on a CARVII spinning disk confocal microscope (Becton Dickinson Biosciences).

Immunoprecipitation assay: The cell lysis extract was incubated with either the CNQ525.510B probe (39) or the A80915C probe (40) at 4 ºC with gentle shaking. After 2 hours, the anti-coumarin antibody bound resin (prepared by Dr. La Clair through the immunization of rabbits with coumarin and conjugation of the serum onto the resin) was added to the extract and the mixture was incubated for another hour. After incubation the resin was washed gently two times with PBS what is this?. Bound protein was eluted using unconjugated coumarin and protein was analyzed on a 4-12% polyacrylamide gel. The gel was stained with a comasie protein stain.
Western blot: samples of precipitated proteins were collected. Protein content was determined using bicinchoninic acid assay (BCA) assay (Pierce # 23235) according to manufacturer’s instructions. Protein (20 µg) was loaded per well on a 10% tris PAG polyacrylamide gel. Gels were wet transferred onto a polyvinylidene fluoride (PVDF) membrane and blocked in 5% dry milk. After blocking, the membrane was incubated overnight at 4º C with primary antibody (sigma mouse anti-GRP94 cat# G4545) at 1:500 in 5% milk in Tris buffered saline with 0.01% tween (TBST). The membrane was washed 4× in TBST. Blot was incubated for 1 hour in TBST with anti-mouse HRP antibody (1:5000). Detection was performed using SuperSignal West Pico Chemiluminescent Substrate (Pierce # 34078) according to manufacturer’s instructions.
References


Spectral data for compounds 24-28
CNQ525.538 (24) pale yellow oil,

$[\alpha]_D -3.7$ (CHCl$_3$)

IR (film) $\text{max}$ 2922, 2854, 1602, 1451, 1346, 1281, 1114, 1072 cm$^{-1}$. 
CNQ525.538 (24) $^1$H spectrum in CDCl₃.
CNQ525.538 (24) COSY spectrum in CDCl₃.
CNQ525.538 (24) HMBC spectrum in CDCl₃.
CNQ525.538 (24) HSQC spectrum in CDCl₃.
CNQ525.522 (25) pale yellow oil

$[\alpha]_D$ -1.1 (CHCl$_3$)

IR (film) $\text{max} 2921, 1713, 1457, 1375, 1267, 1177, 1148, 1051, 1014, 951 \text{ cm}^{-1}$
CNQ525.522 (25) $^1$H spectrum in CDCl$_3$. 
CNQ525.522 (25) COSY spectrum in CDCl₃.
CNQ525.522 (25) HMBC spectrum in CDCl₃.
CNQ525.522 (25) HSQC spectrum in CDCl₃.
CNQ525.600 (26) pale red orange oil

$[\alpha]_D -20$ (CHCl$_3$)

IR (film) $\text{max } 2921, 1697, 1622, 1455, 1309, 1115, 1055 \text{ cm}^{-1}$. 

![Chemical structure image]
CNQ25.600 (26) ¹H spectrum in CDCl₃.
CNQ525.600 (26) COSY spectrum in CDCl₃.
CNQ25,600 (26) HMBC spectrum in CDCl3.
CNQ525.600 (26) HSQC spectrum in CDCl$_3$. 
CNQ525.510B (27) pale colorless oil

$[\alpha]_D^{\circ} -9$ (CHCl$_3$)

IR (film) $\max$ 3399, 2920, 2851, 1711, 1608, 1457, 1286, 1163, 1079, 1012, 909 cm$^{-1}$. 
CNQ525.510B (27) $^1$H spectrum in CDCl$_3$. 
CNQ525.510B (27) $^{13}$C spectrum in CDCl$_3$. 
CNQ525.510B (27) COSY spectrum in CDCl₃.
CNQ525.510B (27) HMBC spectrum in CDCl₃.
CNQ525.510B (27) HSQC spectrum in CDCl₃.
CNQ525.554B (28) pale red oil

$[\alpha]_D$ -42 (CHCl$_3$)

IR (film) $\text{max}$ 2922, 1701, 1604, 1454, 1285, 1197, 1118, 1081, 1007 cm$^{-1}$.
CNQ525.554B (28) $^1$H spectrum in CDCl$_3$. 
CNQ525.554B (28) COSY spectrum in CDCl₃.
CNQ525.554B (28) HMBC spectrum in CDCl$_3$. 
CNQ525.554B (28) HSQC spectrum in CDCl₃.