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Chemical Study of Secondary Metabolites from Selected Strains of the Actinomycete Clade MAR 4

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy

in

Oceanography

by

Ana Paula Domingues de Mello Espindola

Committee in charge:

William Fenical, Chair
Bianca Brahamsha
William Gerwick
Bradley Moore
Emmanuel Theodorakis

2008

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University of California, San Diego

2008

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LIST OF ABBREVIATIONS

ASP	Amnesic shellfish poisoning
DNA	deoxyribonucleic acid
ESI	electrospray ionization
FDA	food and drug administration
FT-IR	Fourier transform infrared
gCOSY	gradient correlation spectroscopy
gHMBC	gradient heteronuclear multiple bond correlation
gHMQC	gradient heteronuclear multiple quantum coherence
gHSQC	gradient heteronuclear single quantum coherence
HTS	high-throughput-screening
HR	high resolution
LC/MS	liquid chromatography / mass spectroscopy
MALDI	matrix-assisted laser desorption/ionization
MRSA	methicillin-resistant *Staphylococcus aureus*
MS	mass spectroscopy
NCI	National Cancer Institute
NOE	nuclear overhauser effect
NMR	nuclear magnetic resonance
ROESY	rotating frame overhauser effect spectroscopy
RNA	ribonucleic acid
THN	1,3,6,8 tetrahydroxynaphthalene

TOF	time of flight
UV	ultraviolet

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The text of chapter III, in full, is as will appear in Ana Paula Espindola, Hak Cheol Kwon, Alejandra Prieto-Davo, Paul R. Jensen, and William Fenical. Nitropyrrolins A-C, farnesyl-α-nitropyrroles from a marine-derived streptomycete.

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ABSTRACT OF THE DISSERTATION

Chemical Study of Secondary Metabolites from Selected Strains of the Actinomycete Clade MAR 4

by

Ana Paula Domingues de Mello Espindola

Doctor of Philosophy in Oceanography

University of California, San Diego, 2008

Professor William Fenical, Chair

Natural products (secondary metabolites) are one the most important sources of novel organic chemical scaffolds for drug discovery and for the synthetic chemists. However, over the past two decades, there has been a general consensus that the discovery of novel secondary metabolites requires new strategies as mostly known compounds are isolated today. This thesis discusses two different strategies for the discovery of natural products from a new group of actinomycetes. One is the phylogenetic approach to new marine taxa of actinomycetes of the clade MAR 4. The other is the use of new bioassays to isolate new secondary metabolites from microbial cultures.

Chapter I introduces the hypothesis in which this thesis research was performed. Chapter II is a review of terpenoids and meroterpenoids in actinomycetes. Chapters III, IV and V discuss the discovery of new secondary

metabolites from actinomycete clade MAR 4, based upon a phylogenetic approach to this new group. It includes the nitropyrrolins A-C (**97-99**), the marinophenazines A and B (**105**, **106**) and streptoquinone (**111**). Chapter VI provides an example of the utilization of a new chemopreventive bioassay, inhibition of NF-κB, which was used to obtain a new bioactive meroterpenoid, naphthomarine (**112**). Chapter VII provides concluding remarks about this thesis research.

I. Introduction to the thesis research: History and roles of natural products

Natural products, also known as secondary metabolites, are the end product of biosynthetic pathways not essential for the life of an organism. Usually, they are small molecules produced by a single species or by a cluster of related organisms.[1] Even though we believe that natural products confer a selective advantage to the producer, in great extent their function is still unknown as few studies on their roles in nature have been performed.[2]

Natural product chemistry is a discipline focused on the isolation and structure determination of new and known natural products. It pursues exploratory research, and like the great discoveries of the past, it unravels an incredible structural and stereochemical diversity.

I.1. History of natural products as a science

In a general sense, natural product science as we know it can be traced back to the nineteenth century with the isolation of clinically important substances like morphine,

and achieved its peak in the "golden era", the 1940's and 1950's, when most groups of antibacterial antibiotics were discovered.[1]

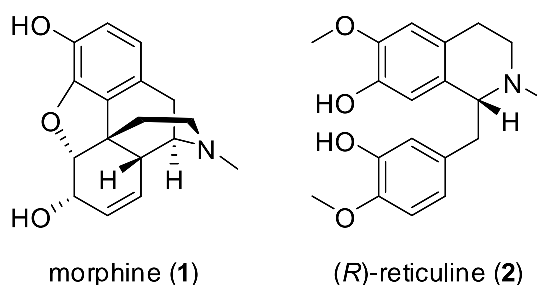
The use of Nature's products to improve quality of life dates back to the beginning of human society, and our history is full of examples of how plants and animals were used in every aspect of our civilization. For thousands of years, humans living in close connection with their environment tried to use everything that was around them, sometimes with a positive outcome, other times with a deadly one. Experimentation with virtually all life forms lead to their incorporation in medicine, cosmetics, economy, hunting and even war.

I.1.1. Secondary metabolites from plants

Classical examples of medicinal use of natural products from plants include morphine from opium (the latex from *Papaver somniferum*), salicilin from willow bark (*Salix alba*), digoxin from foxglove (*Digitalis purpurea*), quinine from cinchona (*Cinchona officinalis*) and more recently taxol from the Pacific yew (*Taxus brevifolia*).[2]

Opium has been used for at least 5000 years. It was known by the Sumerians as the joy plant and, as the name suggests, used for its euphoric effect.[3] Morphine (**1**), named "morphium" after Morpheus the greek god of dreams, is the major benzyltetrahydroisoquinoline alkaloid present in opium.[4] It was first isolated by the German pharmacist Friedrich Sertürner in 1804, thus beginning the field of alkaloid chemistry and according to Paul Blakemore and James White the beginning of Medicinal Chemistry.[5] This very complex natural product had its structure correctly determined only in 1925 by Sir Robert Robinson who studied the degradation products. Morphine's

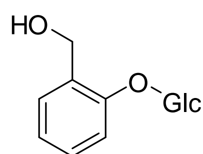
action is derived from its interaction with the opioid receptors. It is a powerful analgesic and narcotic drug with side-effects inducing addiction and severe respiratory depression.[6] Biosynthetically, morphine is derived from two molecules of tyrosine that, after biotransformation to dopamine and 4-hydroxyphenylacetaldehyde, are coupled through a Mannich-like reaction to form the alkaloid norcoclaurine. This alkaloid is subsequently modified to give (*R*)-reticuline (**2**), the precursor of the principal opium alkaloids. [4]

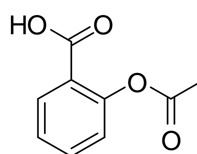


morphine (**1**) (*R*)-reticuline (**2**)

The use of the willow tree dates back to ancient Greece, where Hippocrates, “the father of medicine”, would prescribe the bark to be chew for extraction of its analgesic and antipyretic properties.[1] Later, in the Middle Ages, medicine followed concepts like the “doctrine of signatures”, by which people believed God had marked with a sign how a plant should be used. Following this belief, the flexible branches of the willow tree were used to reduce inflammation of joints, and as this tree grows in damp places where fever thrives, it was also used to reduce fever. Different species of willow were used through the centuries by Chinese and European healers. In 1845, the active compound salicin (**3**) was finally isolated and shown to be the *O*-glycoside of *o*-hydroxyl benzyl alcohol.[7] This compound had a very unpleasant taste and caused intense stomach irritation;

however, it served as lead to the synthesis of the widely used acetylsalicylic acid (**4**) (aspirin).

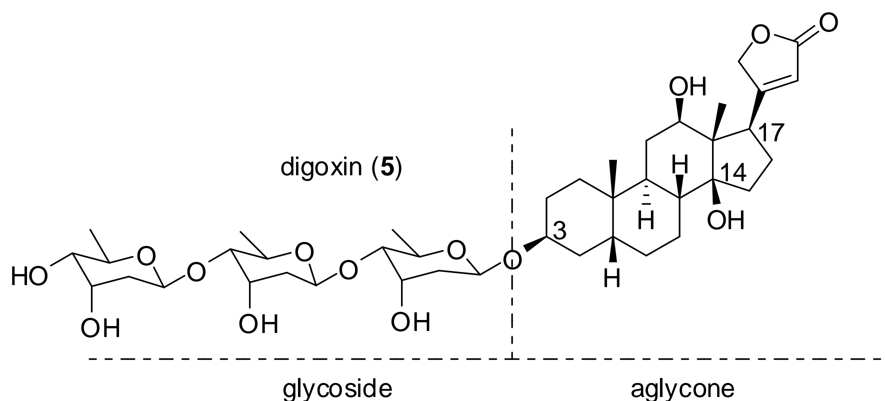




salicin (**3**) acetylsalicylic acid (**4**)

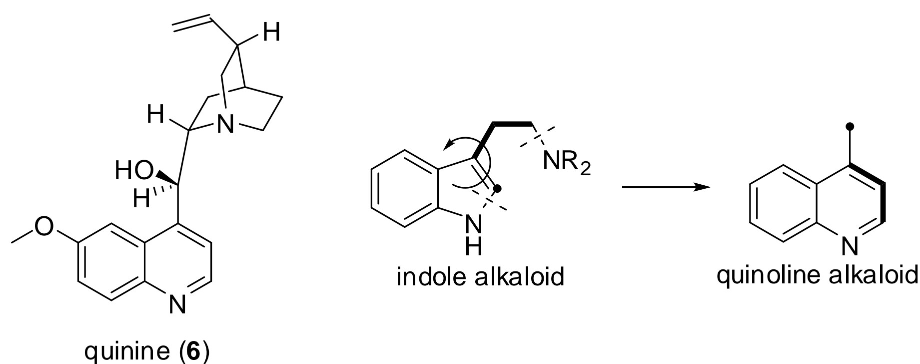
Relative to the thousands of years of use of opium and willow tree, the application of the herbal preparation foxglove, from the plant (*Digitalis purpurea*), is recent. The first description of its use was in a report "*An account of the foxglove and some of its medical uses"* by English physician William Withering.[8] Withering was able to recognize foxglove as the active ingredient in an herbal panacea used by a "wise woman" in Shropshire and to prepare a better form of administration (as dried powder) since the traditional use of boiling Foxglove destroyed some of the active compound. From herbal Foxglove, a series of cardiac glycosides were isolated by Sydney Smith of Burroughs Wellcome in 1930, with the most clinically important being digoxin (**5**), a molecule still in use today to treat congestive heart failure and cardiac atrial fibrillation. All cardiac glycosides share as common features *cis*-fused A-B and C-D rings that give the molecule a three dimensional "u" shape, a hydroxyl at the beta position of carbon 14, an unsaturated lactone at the beta position of carbon 17 and sugar residues at the beta position of carbon 3. Interestingly, cardiac glycosides are also present in some species of frogs. The frog-derived cardiac glycosides possess a six member lactone instead of

the five member lactone present in plants. The pharmacological activity is associated with the aglycone part of the molecule but the glycoside part is an important modulator of the activity by interfering with solubility and binding to the cardiac muscle. In digoxin (**5**), the sugar moiety is composed of three units of D-digitoxose.[4]



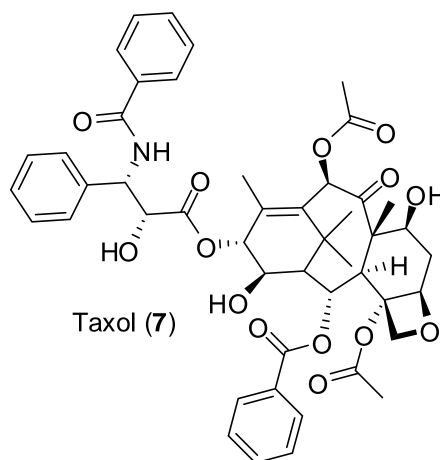
The native South Americans used the bark of the cinchona tree (*Cinchona officinalis*), an evergreen plant endemic to the eastern Andes from Venezuela to Bolivia, to treat malaria. The Jesuits are credited with bringing this traditional knowledge to Europe in the 17th century where it became widely used.[9] After many failed attempts at isolating the active component of cinchona, Pierre Joseph Pelletier from the Ecole de Pharmacie of Paris, in collaboration with Joseph Bienaim Caventou, a young student, employed the procedure used to successful isolate of morphine (that is partition with an acid solution) to isolate emetine (1817), strychnine (1818), brucine (1819), and veratrine (1819). In 1820, Pelletier and Caventou began to work with the cinchona extract and isolated the extremely bitter compound quinine (**6**). The terpenoid indole alkaloid quinine, together with some other alkaloids isolated from the genus *Cinchona,* is

considered an outstanding example of rearrangement.[4] Only subsequent biosynthetic work proved these to be originally indole alkaloids which were transformed to give the quinoline system. Quinine was heavily used during World War II for the treatment of malaria and today this "old" medicine is still used in critical situations. In his seminal paper on the formal synthesis of quinine Robert Woodward described quinine as "*the drug to have relieved more human suffering than any other in history*".[10]



The isolation of taxol (**7**), a complex oxygenated diterpenoid, is a more recent discovery. taxol was first isolated from the Pacific yew tree in 1967 as part of a National Cancer Institute (NCI) program to find new anticancer drugs. In 1958, the NCI contracted Department of Agriculture botanists to collect samples to be tested. More than 35,000 plants were gathered for this program. One of the botanists, Arthur S. Barclay, in 1962 collected plant material from the Pacific yew (*Taxus brevifolia*). Upon arrival at the NCI, the material was assigned to Monroe E. Wall to work with. Wall observed the intense antitumor qualities of the bark extract from the Pacific yew. Working with Mansukh C. Wani, Wall was able to isolate the compound responsible for

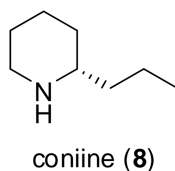
the activity. Finally in 1971, Wall and Wani published the chemical structure of taxol. The isolation of taxol raised serious concerns about the conservation aspect of its acquisition as the Pacific yew is a very slow-growing tree and the yield of taxol from its bark is approximately 0.004%.[11] The problem of obtaining sufficient taxol to allow clinical trials and subsequent clinical use was ultimately overcome by semi-synthesis. 10-Deacetylbaccatin, present in the needles of the English yew, is efficiently converted to taxol in a series of simple steps. taxol is used as a chemotherapy drug for the treatment of ovarian, breast and non-small lung cancer. Although taxol was first thought to have the same mechanism of action of many other anticancer compounds, Susan Horwitz and collaborators[12] demonstrated that taxol disrupts mitosis by stabilizing microtubules and inhibiting their depolymerization back to tubulin,[13] a completely new mechanism of action.



Taxol (**7**)

Over the centuries, humankind has also found sinister uses for natural products. The Greeks used extracts from the poison hemlock (*Conium maculatum*) to condemn

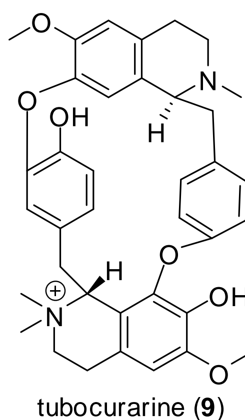
prisoners, including Socrates, to death. Coniine (**8**), the major alkaloid in hemlock consists of a piperidine ring with an alkyl side-chain at the 2 position. It causes a gradual paralysis of the muscles followed by convulsions and respiratory paralysis.[11] This neurotoxic alkaloid also has a teratogenic effect at low concentrations, and many cases of malformation in livestock have been described in the literature.[14]



coniine (**8**)

In isolated areas of the Amazonian forest, natives still use poisons derived from plants in hunting and warfare. Since Columbus' arrival on the new continent, accounts of deadly encounters with natives armed with a black pitch venom on their arrows started to come to Europe.[15] This poison was known as curare by the natives and its preparation was guarded as sacred. The plant wasn't identified until the 18th century when researchers such as Alexander Humboldt started to travel through South America. Curare is an extract of the vine *Chondrodendron tomentosum* that grows in the canopy of the Amazonian rainforest. Sir Arthur Conan Doyle was fascinated by this poison. He used curare in two of his Sherlock Holmes stories and aptly described its effect in the "Adventure of the Sussex Vampire".[16] The isolation of a quaternary alkaloid first from the poison and then from the plant was accomplished by Harold King in 1935, and finally the correct structure of d-tubocurarine (**9**) was assigned in 1974.[17] This compound is a competitive antagonist of nicotinic neuromuscular acetylcholine receptors. Long used in

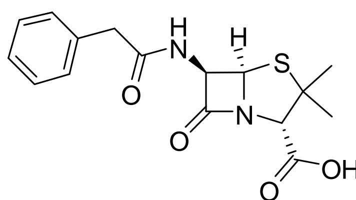
anesthesia prior to surgery to paralyze patients, tubocurarine has since been replaced by safer synthetic options.



tubocurarine (**9**)

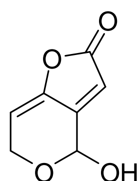
I.1.2. The "Golden Age" – Natural products from microorganisms

With the discovery of penicillin (**10**) by Alexander Fleming, a new chapter in the story of natural products began. Researchers until then had focused on compounds from plants, without realizing the vast potential that microorganisms could be. Since Fleming's discovery, microorganisms became the major source of antimicrobial drugs, producing between 60-80% of all anti-infectives used.[18]



penicillin (**10**)

The first scientist to write about the "bacteria killer molds" was French physician Ernest Duchesne. In his thesis, submitted in 1897 with the title "Contribution to the study of vital competition in micro-organisms: Antagonism between moulds and microbes," he explored the idea of survival of the fittest in the microbial world. Duchesne showed that co-injection of a mixture of the fatal bacterium *Salmonella typhi* and the fungus *Penicillium glaucum* in guinea pigs would result in the recovery of the animal. Although he was the first to recognize the competition between microorganisms, Duchesne failed to make the connection between chemical production by the fungus and death of the bacterium. He believed the nutritive aspects of the medium and the fast growing ability of the fungus gave it the edge to overpower bacteria. Although Duchesne did not continue this research, he argued in his thesis for the need to better understand the therapeutic use of microorganisms.[19] Almost a half century later, in 1942, Ernst Chain, Howard Florey and Margaret Jennings identified the antibiotic produced by *P. glaucum* as patulin (**11**).



patulin (**11**)

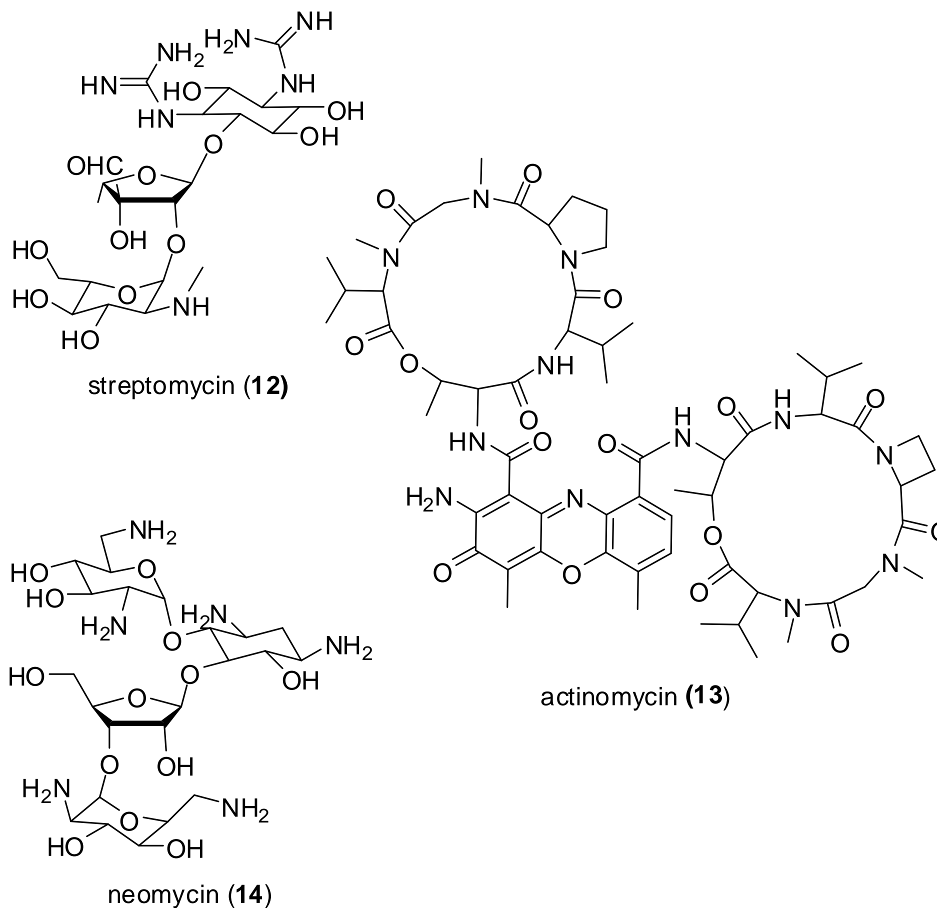
Humankind had a stroke of luck in the discovery of the antibiotic penicillin (**10**). The first natural product from a microorganism to have economic and clinical significance, penicillin from *Penicillium chrysogenum*, was discovered by Alexander

Fleming in 1928 in his laboratory in Oxford. Serendipity played a role in many phases of this discovery.[20] The story, as can be read in many reviews, is that Fleming was working with *Staphylococcus aureus* in his laboratory. When Fleming went for Summer vacation, petri dishes with *S. aureus* were left growing on his bench near the window. Mold spores from another laboratory infected his plates, producing the characteristic inhibition zone around the fungal growth. Many researchers tried to repeat Fleming's experiments without results. Ultimately, temperature was determined to be the critical variable. *Penicillium* grows at 20°C; *Staphylococcus* grows at 35°C. During Fleming's vacation a cold front hit Oxford allowing the fungi to grow. When Fleming returned from his vacation, he observed the plate with areas without growth of *S. aureus*. He analyzed what he was seeing, extracting the media and calling the unknown compound "penicillin". Importantly, Fleming recognized that the fungi were producing compounds that inhibited the bacterial growth.[21]

Penicillin (**10**) has a rare penam skeleton (a β-lactam thiazolidine) connected to a phenylacetic acid with an amide link at position 6.[4] The penam system has three chiral centers (3*S*, 5*R* and 6*R*) with all the natural and semisynthetic penicillins having the same stereochemistry.[22] Penicillin (**10**), as with all the β-lactams, is a bactericidal antibiotic that acts by inhibition of the cell wall formation. More specifically, it prevents the synthesis of the peptidoglycan layer by irreversibly binding to the transpeptidases. The tridimensional similarity of β-lactams to the D-alanyl-D-alanine allow them to acylate the transpeptidase enzymes, making them unable to form the crosslink between the peptidoglycan layers.[13]

Penicillin (**10**) proved to be a miracle drug useful not only against septicemia but also in the cure of other illnesses. Gonococcal infections could be treated with only one injection and syphilis with a ten-day treatment.[6] Despite warnings from Fleming,[23], the widespread belief that bacterial infections had been tamed led to an excessive and sometimes incorrect use of antibiotics resulting in resistance toward penicillin. In one example, the resistance takes the form of an enzyme, β-lactamase, present in many bacteria and able to be transferred to other diverse bacteria via lateral gene transfer on plasmids. This enzyme is able to open the β-lactam ring inactivating the β-lactam antibiotic. The scientific community responded by creating a series of semi-synthetic β-lactam antibiotics with different acids linked to the penam structure.

The term "Antibiotic" was coined by Selman Abraham Waksman, then the chair of the commission that developed guidelines to the naming of new antibiotics.[24] This biochemist and microbiologist was responsible for the discovery of more than eighteen antibiotics, with streptomycin (**12**) in 1944, actinomycin (**13**) in 1940 and neomycin (**14**) in 1949 as the most important in clinical applications. The 1952 Nobel Prize in Physiology and Medicine was awarded to Waksman for his discovery of streptomycin (**12**), the first drug active against *Mycobacterium tuberculosis*. His work with antibiotics started in 1939 as part of a systematic screening program to identify soil microorganisms to be used against infectious diseases. In the same way that Sertürner's protocol was the foundation for alkaloid chemistry, the processes for obtaining antibiotics developed in Waksman's laboratory lead to the isolation of a large number of antibiotic molecules.

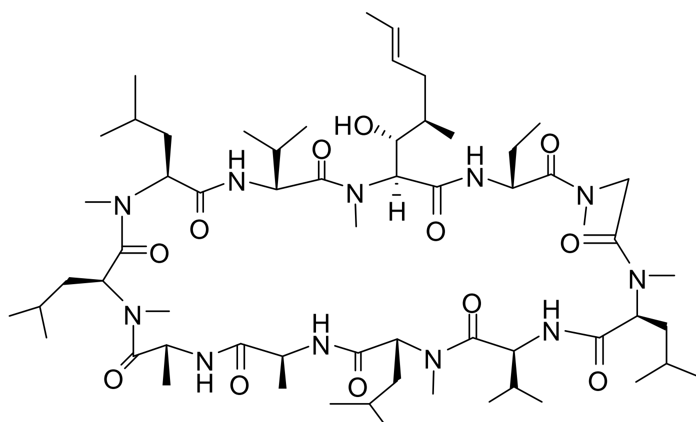


Streptomycin (**12**) and neomycin (**14**) are aminoglycoside antibiotics, that is, amino sugars connected through glycosidic bonds. The central ring, always a derivative of 1,3 diaminocyclohexane, is streptadine in streptomycin and deoxystreptamine in neomycin.[22] The activity of these compounds is due to their interaction with the 30S ribosomal subunit, where they inhibit protein synthesis and interfere with the accuracy of translation.[13]

Actinomycin (**13**), a member of the chromopeptides, was introduced as a chemoteraphic agent under the generic name "dactinomycin". While it is a potent antibiotic, it is mainly used today to treat Wilm's tumor and gestational choriocarcinoma

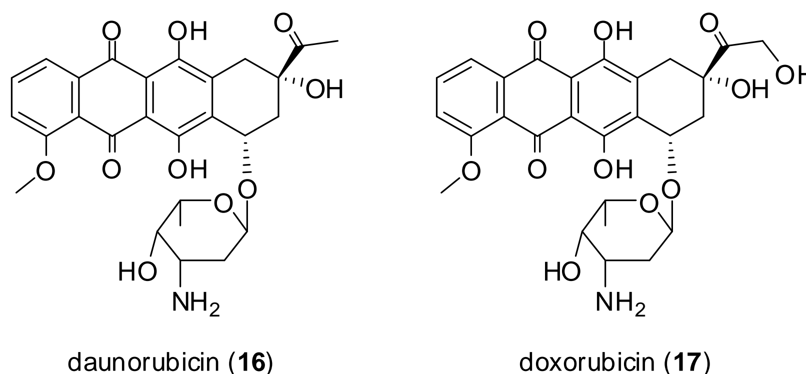
cancers. Actinomycin's (**13**) mechanism of action is thought to be a noncovalent interaction with guanine in DNA via a partial intercalation that distorts the DNA, inhibiting transcription.[13] This molecule is formed from a substituted 3-phenazone-1,9-dicarboxylic acid known as actinocin and two peptide lactones. Each of the carboxylic groups is bonded to a pentapeptide lactone through an L-threonine.

The discovery and development of the cyclic peptide immunosuppressant cyclosporine (**15**) allowed human organ transplant therapy to become a reality.[25] Cyclosporine (**15**), also used for the treatment of autoimmune diseases like rheumatoid arthritis,[13] is a non-ribosomal cyclic peptide composed of eleven amino acids (undecapeptide). Its producer, the fungus *Tolypocladium inflatum*, was isolated in early 1970 from Norwegian and Wisconsin soil samples as part of an antimicrobial program at the Sandoz Pharmaceutical Company. The compound showed poor antifungal activity, but when subjected to further testing showed strong immunosuppressive effects.[26]



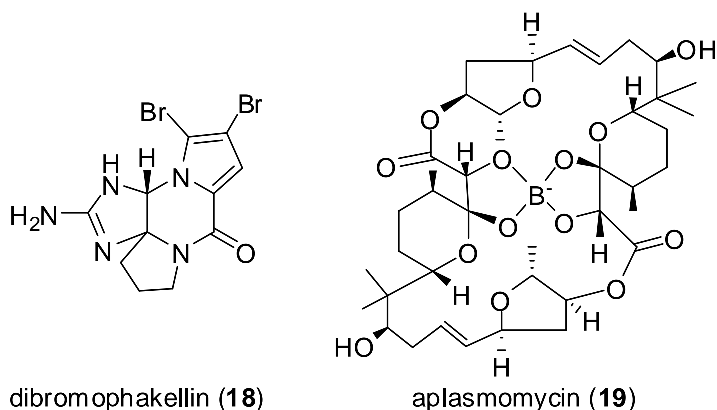
cyclosporine (**15**)

The anthracyclines are considered one of the most important classes of antibiotics ever discovered. These compounds, produced by various actinomycetes, are exemplified by daunorubicin (**16**) and its derivative doxorubicin (**17**).[27] Their activity is similar to actinomycin as it is believed that they intercalate with DNA inducing breakage of the DNA strand.[22]



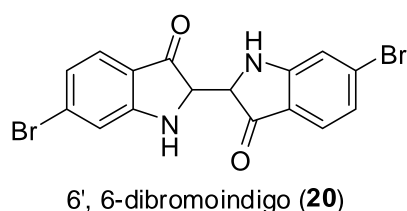
I.1.3. A new frontier – Marine natural products

Natural products from marine sources caught the eyes of scientists by virtue of their structural and biological uniqueness.[28] When scientists started to examine the marine environment as a source for new natural products, they were excited and surprised with the diversity of chemical structures. These new marine-derived structures, with features previously unknown from terrestrial sources, expanded and in some cases completely changed our perception of the biosynthetic potential of Nature. Intriguing characteristics such as polyhalogenation (e.g. dibromophakellin (**18**)[29]) or the presence of elements never seen before in a natural product like boron (for example aplasmomycin (**19**)[30] from a marine *Streptomyces sp.*), were commonplace in marine natural products.



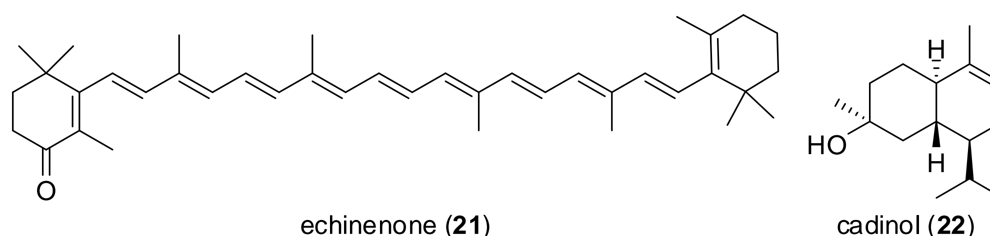
dibromophakellin (**18**) aplasmomycin (**19**)

The oldest example of human use of a marine natural product is the Phoenician dye royal Tyrian Purple. The production of this dye, from material isolated from *Murex brandaris*, is believed to be the first chemical industry in human civilization.[31] By today's prices, a silk scarf dyed in Tyrian Purple would cost around $900, making it the most expensive merchandise of ancient times. The intensely purple compound 6',6-dibromoindigo (**20**) is in reality an artifact, a dimer of the real secondary metabolite. To determine its structure in 1909, the German chemist Paul Friedländer isolated 1.4 g of the pure dye from 12,000 snails.[32]



6', 6-dibromoindigo (**20**)

In the first half of the 20th century, research in marine natural products was sparce. In 1935 Lederer wrote about the isolation of the carotenoid echinenone (**21**) from the sea urchin (*Strongylocentrotus lividus*). Japanese scientists were among the first to investigate the natural products of marine organisms. The isolation of (-) δ cadinol (**22**) from the brown alga (*Dictyopteris divaricata*) in 1951 by Takaoka and Ando was one of the first studies to be reported.[32]

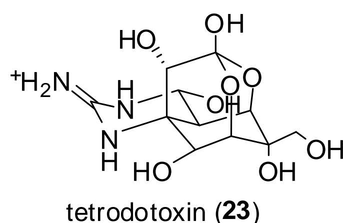


echinenone (**21**) cadinol (**22**)

The field of marine natural products started to blossom in the 1970's. During that time, the division of marine natural products chemistry into three areas: toxins, bioactive metabolites and chemical ecology became better defined.[28]

Both, toxins and venoms are poisoning compounds. Toxins are accumulated by organisms while venoms are actively produced and secreted through a bite or a sting.[33] Usually animals that produce venoms have a specialized gland to biosynthesize the poison and a specialized means of injecting them. An example of a marine venomous animal is the jellyfish. The production, storage and injection of jellyfish venom is accomplished within the nematocytes in specialized organelles that are ejected into the target.[34] Tetrodotoxin (**23**), domoic acid (**24**), ciguatoxin (**25**) and palytoxin (**26**) are a few examples of toxins found in the marine environment. Unlike their terrestrial

counterparts, marine toxins are not easily identified or avoided. They are usually produced by microorganisms and accumulated in the food chain. Hence, a mussel that today is safe to consume can in a few days become deadly. Most marine toxins are also heat resistant, so that preparation does not diminish the risk of poisoning. Accounts of seafood poisoning are common in the journals of explorers such as Captain Cook in 1772, Engelbert Kaempfer in 1727 and De Quiros in 1606.[35]

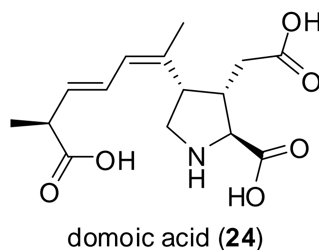


tetrodotoxin (**23**)

The potent neurotoxin tetrodotoxin (**23**) was first isolated from the puffer fish (*Tetraodon* sp.) in 1909 by the Japanese Scientist Y. Tawara. This complex structure was published independently by four groups (Tsuda at Tokyo, Hirata at Nagoya, Woodward at Harvard, and Mosher at Stanford) in 1964.[32] The wide distribution of tetrodotoxin - isolated from goblin fish, starfish, blue-ringed octopus, frogs, sediments and bacteria - led to an ongoing controversy over the true producers of this molecule,[36] and as consequence the biosynthetic pathway for this very unusual molecule is still unknown but hypothesized as being formed by arginine and a C_5 unit derived from either amino acids, isoprenoids, shikimates, or branched sugars.[37]

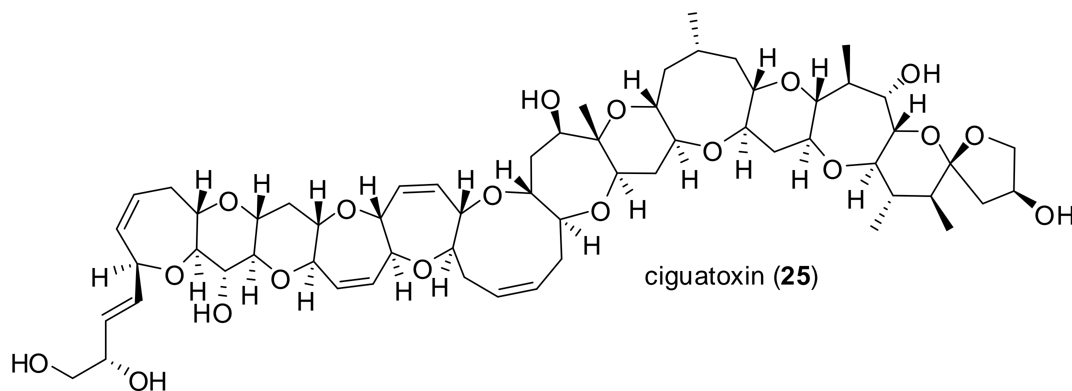
Tetrodotoxin (**23**) is a highly specific voltage-sensitive sodium channel blocker that binds to receptors near the extracellular surface of the channel blocking the

movement of sodium without affecting the movement of potassium.[6] The symptoms of tetrodotoxin poisoning include motor paralysis that can progress to respiratory failure.[33] Today, tetrodotoxin is mostly used as a pharmacological probe in the study of ion channels but in 1913 it was patented for the treatment of leprosy and other diseases.[38]

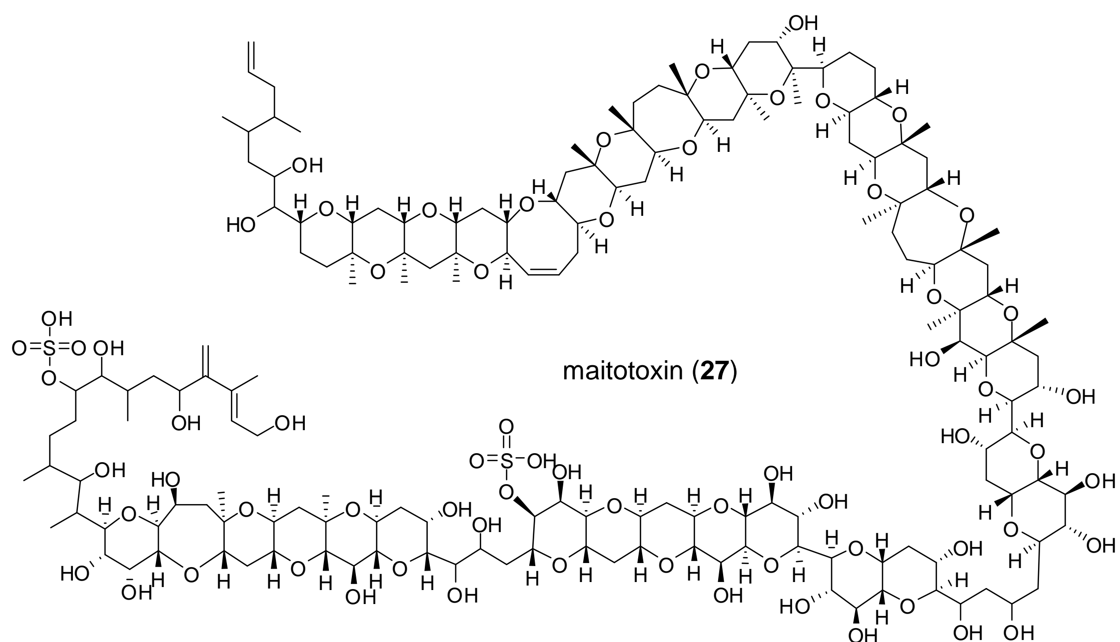


domoic acid (**24**)

Amnesic shellfish poisoning (ASP) is a type of cold water seafood poisoning caused by domoic acid (**24**). ASP is characterized by both gastrointestinal (including nausea vomiting, abdominal cramps, and diarrhea) and neurological symptoms (including dizziness, headache, seizures, disorientation, short-term memory loss and respiratory difficulty). In the most severe cases of contamination, ASP can be deadly.[39] Domoic acid (**24**) is produced by the benthic diatom *Pseudonitzschia* sp. Interestingly, this compound was first isolated from the benthic algae *Chondria armata* as an antihelmintic for *Oxyics* and *Ascaris*.[40] Domoic acid (**24**) is a member of the kainoids, a group of neurologically active amino acids that act by binding to a subtype of glutamate receptor of some neurons. This molecule is formed by condensation of a C-10 isoprenoid chain with a glutamate derivative.[41]

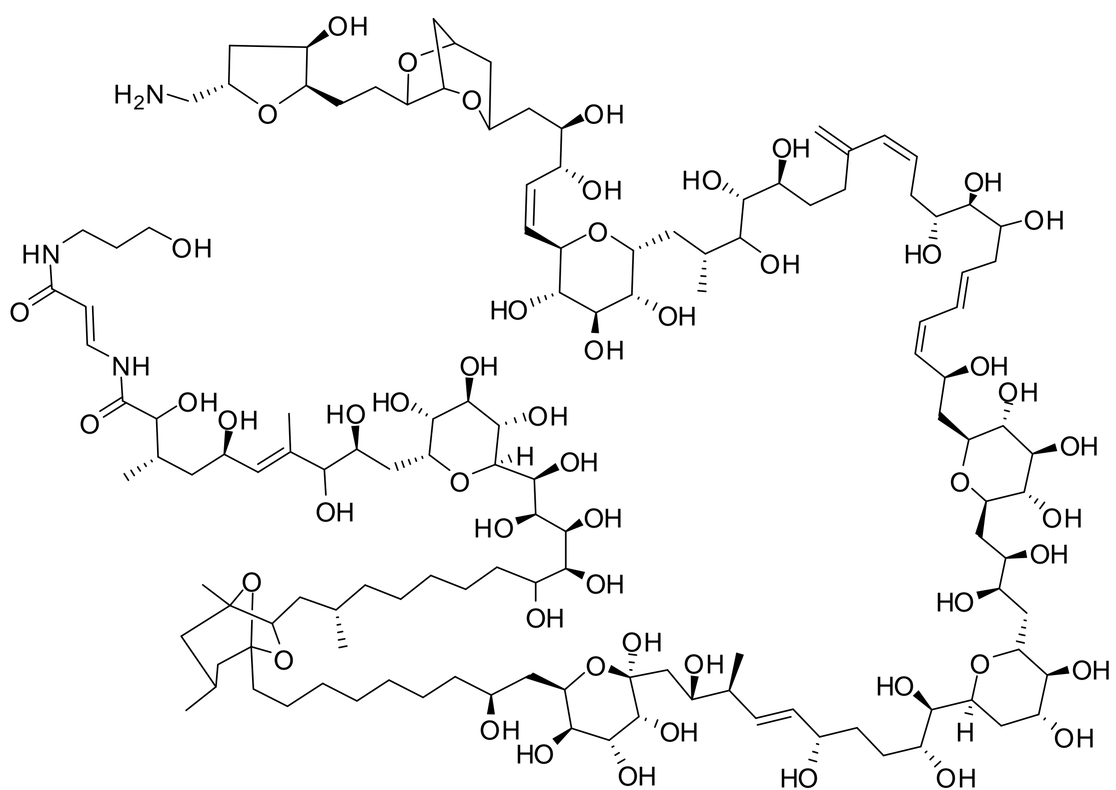


Ciguatoxin (**25**) is a polyketide toxin produced by the dinoflagellate *Gambierdiscus toxicus* that live in tropical waters, usually associated with coral reef algae.[39,42] Ciguatera poisoning is characterized by "typical" symptoms associated with food poisoning with moderate to severe gastrointestinal problems including vomiting, abdominal cramps and diarrhea along with some neurological effects such as muscle pain, itching, abnormal or impaired skin sensations, vertigo, and inability to coordinate muscular movement.[33] Some of the symptoms may recur for as long as six months. Even though ciguatera is the most common form of intoxication by marine food, fatality occurs only in 0.1% of a series of 3000 cases.[42] *Gambierdiscus toxicus* also produces another neurotoxic polyketide, maitotoxin (**27**), considered one of the most complex natural products ever isolated.



maitotoxin (**27**)

Another example of marine toxin, palytoxin (**26**), is a marine polyketide once employed to poison spears in Hawaii.[43,44] This molecule is another superb example of the complexity found in marine natural products. It was originally isolated from a zoanthid of the genus *Palythoa,* but now some argue that the true producer is a dinoflagellate related to *Ostreopis siamensis*.[45,46] Palytoxin (**26**) is a polyketide with 129 carbon atoms, 64 chiral centers, 8 double bonds and 8 rings. Palytoxin interacts with the sodium potassium pump causing a passive transit of ions through the pump, and ultimately unbalancing the ion gradient in the cells. This mechanism of action results in symptoms of poisoning: breathing difficulties, chest pains, tachycardia, unstable blood pressure and hemolysis.



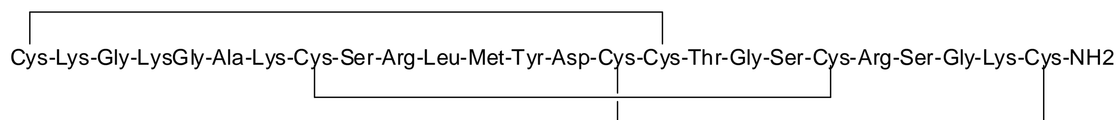
palytoxin (**26**)

The second focus of marine natural products is marine chemical ecology, a field dedicated to the study the chemical interactions between marine organisms. With the evolution of scientific diving, researchers were able to stay longer in the ocean and learn how organisms interact in that environment. As a result, scientists were able to observe that chemical interactions between organisms were a major component in the sea water ecology. Compounds produced by phytoplankton, algae and marine animals function as protection, reproduction and settlement signals. Researchers were also intrigued by compounds isolated from unrelated organisms such as dibromophakellin (**18**), a brominated pyrrole alkaloid first isolated from the marine sponge *Phakellia flabellata*[47] from Great Barrier Reef, and later from a red algae (*Laurencia majuscula*)[29] from the

South China Sea. Studies also focused on organisms like the sea hare and its phagomimicry defense mechanism[48] and the chemical cues used by invertebrate larvae to know where to settle.[49]

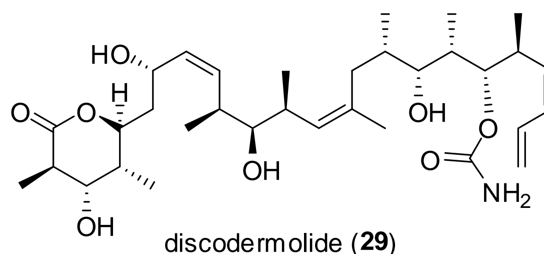
The interaction between the Ocean and humans is an old one. Sometimes seen as a capricious god, the ocean was a source of nourishment and mystery to population that lived in coastal areas. But interestingly enough, there is only one reported example of marine organisms' use in folk medicine in a small region in the South of China[50]

ω Conotoxin M-VII-A (**28**), produced by the cone snail *Conus geographus,* is a neuroactive peptide composed of 25 amino acids residues with three disulfide bonds.[51] The synthetic copy of conotoxin M-VII-A (**28**), Ziconotide®, is the first marine drug approved by the FDA under the name Prialt.[52] ω Conotoxin M-VII-A (**28**) is a potent analgesic prescribed for the management of severe chronic pain[53] which specifically blocks the presynaptic N-type calcium channel. The side effects of ziconotide include delirium, acute psychotic reactions, suicidal ideation and coma. Cone snails are venomous marine invertebrates found in tropical and subtropical oceans. The lethal blend of peptides produced by these snails includes α-conotoxin, a nicotinic receptor antagonist with activitiy similar to cobra venom and μ-conotoxin, a sodium channel blocker that acts like tetrodotoxin,[54] causing a instantaneous paralysis of the prey. To inject the venom, the snail fires a hollow, barbed harpoon on its prey. Because of the high mortality rate (70% if untreated) for humans stung by *C. geographus*, this genus intrigued many scientists, including Professor Baldomero Olivera from University of Utah, who has studied the venom of different *Conus* species for more than 25 years.[54]



ω conotoxin M-VII-A (**28**)

Discodermolide (**29**) is a polyketide isolated from the Caribbean deep sea sponge *Discodermia dissoluta* in a yield of around 10 mg/kg. This molecule is believed to be derived from four acetate and eight propionate units. With thirteen chiral centers and a tetrasubstituted δ lactone this structure frustrated early synthetic chemistry effort to obtain quantities needed for clinical trials.[55] Ultimately, 60 g of discodermolide (**29**) was synthesized by a group of scientists at Novartis[56] by modifying the synthesis done by Amos Smith and Paterson groups. Discodermolide (**29**), first isolated in 1990 by Sarath Gunasekera at Harbor Branch Oceanographic Institution, shows potent immunosuppressive and antitumor biological activity. Its mechanism of action is through the stabilization of microtubules causing cell cycle arrest in the G_2/M phase.[57] This compound is in clinical trials for the treatment of cancer resistant to taxol and epothilone.[58]

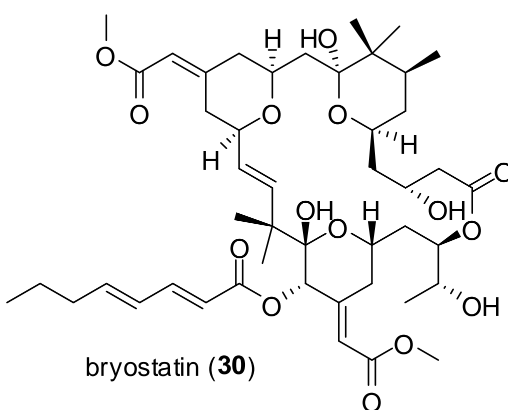


discodermolide (**29**)

Bryostatin-1 (**30**), a macrolide polyketide, is currently in clinical trials for use in combination with taxol in the treatment of esophageal cancer. Bryostatin-1 (**30**) was

classified as an orphan drug by the FDA in 2001. This status is granted to products that treat rare diseases or conditions. Bryostatin-1 was initially isolated from bryozoans (colonial filter feeder animals very similar to corals) by George Pettit in 1982,[59] however the true producer of bryostatim-1 (**30**) now appears to be the symbiont '*Candidatus* Endobugula sertula', an uncultivated γ-proteobacterium. [59,60]

Bryostatin-1 (**30**) acts on the protein serine/threonine kinase C. Unlike most drugs, bryostatin-1 does not inhibit the protein kinase but activates it. The activation of the protein leads to its down regulation by proteolysis.



I.2. History of natural products chemistry methodology

Before the Second World War, natural products chemistry was performed in a completely different way. None of the instruments used routinely in a laboratory today were available. Liquid-liquid extraction (also called partition) and chromatography (only as gravity column with normal phase) were the methods used for separating

complex mixtures. For structure elucidation, chemists used solubility schemes[60] and degradative chemistry[61] to propose structures.

In liquid-liquid extraction, a technique still in use, components of a mixture are partitioned between two immiscible liquid phases, usually, an aqueous solution and an organic solvent in a separation funnel. By virtue of their chemical and physical properties, compounds dissolve preferentially in one of the phases allowing their separation from the rest of the mixture. There are two major class of solubility: miscibility-driven and chemical reaction-driven solubility.[62]

In miscibility-driven solubility, the compounds being mixed do not change their chemical composition. Miscibility, the principle that “like dissolves like”, arises from intermolecular forces, ionic bonds, hydrogen bonds, dipole-dipole and Van der Waals interactions.

In chemical reaction-driven solubility, reactions of functional groups present in an organic molecule are used to change the nature of the type of interaction between that molecule and the solvent thereby changing the phase where the compound is most soluble. Acid-base reactions are frequently used in liquid-liquid extraction. (**Figure I.2.1**)

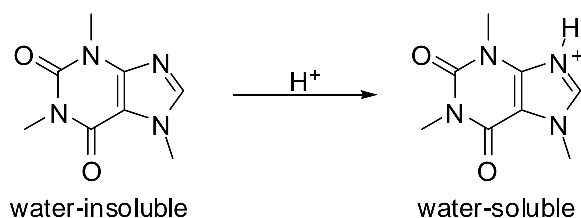


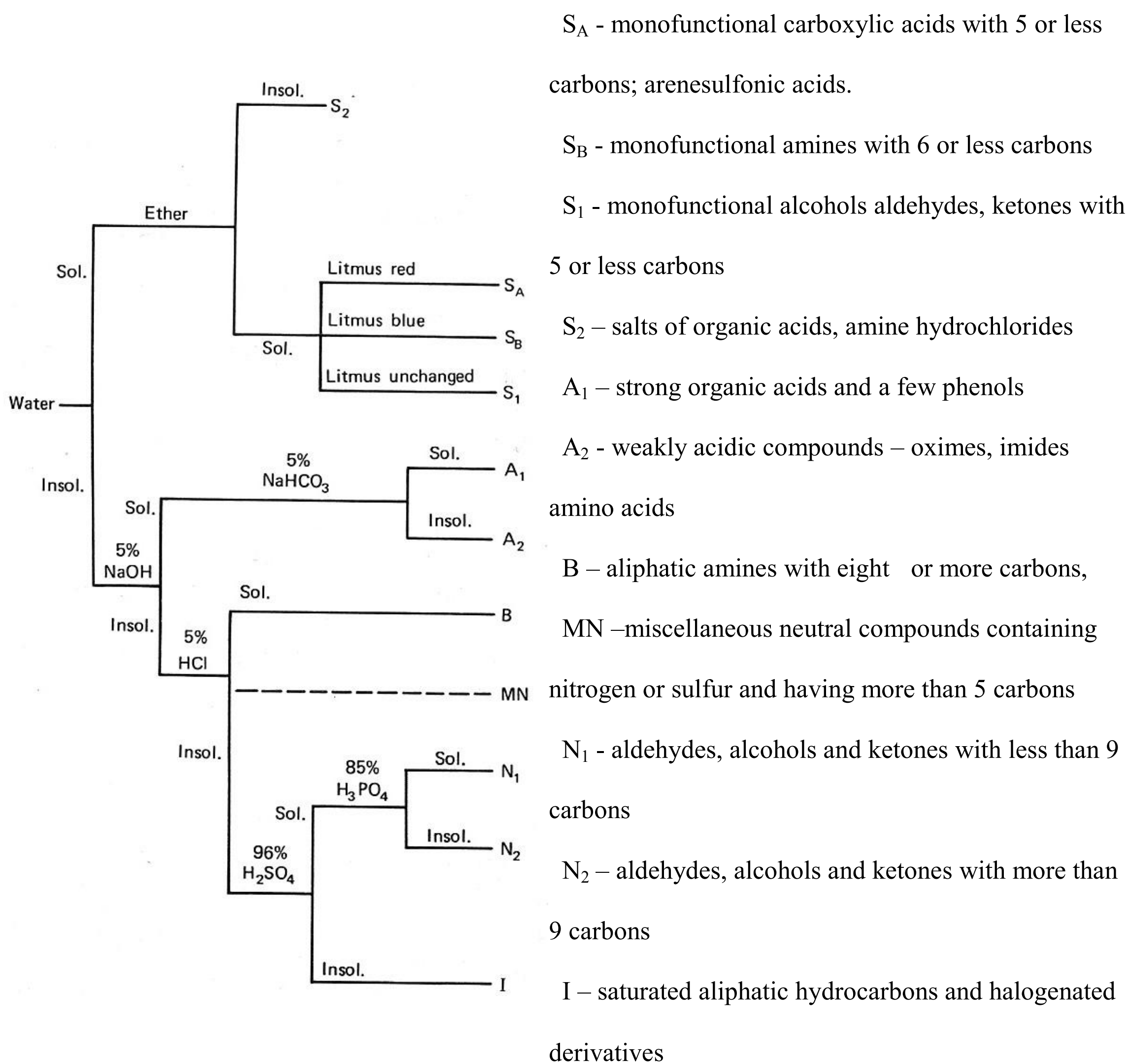
Figure I.2.1 - Acid-base reaction of caffeine and an acid aqueous solution resulting in change of solubility

Predicting the solubility of an organic molecule is also useful when trying to purify a molecule (e.g. recrystallisation).

Chromatography is a series of physical methods widely used to separate and analyze chemical components in complex mixtures. In a chromatographic process, the components to be separated are carried by a mobile phase which percolates through a stationary phase. Differences in the interaction of the sample components and the stationary phase lead to differences in the migration rates facilitating the separation of the mixture.[63] This technique was invented by the Russian Mikhail Tswett in the beginning of the 19th century to facilitate the separation of plant pigments such as chlorophylls and xanthophylls.[64]

In a solubility scheme (e.g. **scheme I.2.1**), the substance of interest is dissolved in a series of solvents and determined to be “soluble” or “insoluble” by visual analysis. The solubility characteristics of an organic compound provide evidence for the presence (or absence) of functional groups in the structure. Although solubility schemes have largely been replaced by better methods (e.g. IR spectroscopy) in the research laboratory, they are still taught in organic chemistry classes to help students to acquire a better

understanding of chemical and physical properties of each functional group in organic molecules.



Scheme 1.2.1 - Separation of organic compounds into solubility classes with some of the compounds divided.[62]

The most important structure elucidation technique to early natural products chemists was degradation chemistry. In this procedure, the unknown compound was subjected to a series of chemical reactions to obtain known smaller molecules. Structures were proposed based on the reactions results but unfortunately many times more than one structure was possible.[65]

After the middle of the 20th century, profound changes in the host of analytical tools available to scientists greatly improved their ability to study natural products. Important advances in spectroscopy in the 1940s included infrared (IR) spectroscopy which illustrates functional groups and visible/ultraviolet spectroscopy which supplies clues about chromophores. In the 1960s nuclear magnetic resonance (NMR) came on the scene to give information on the chemical environment of the atoms in the molecule.[17]

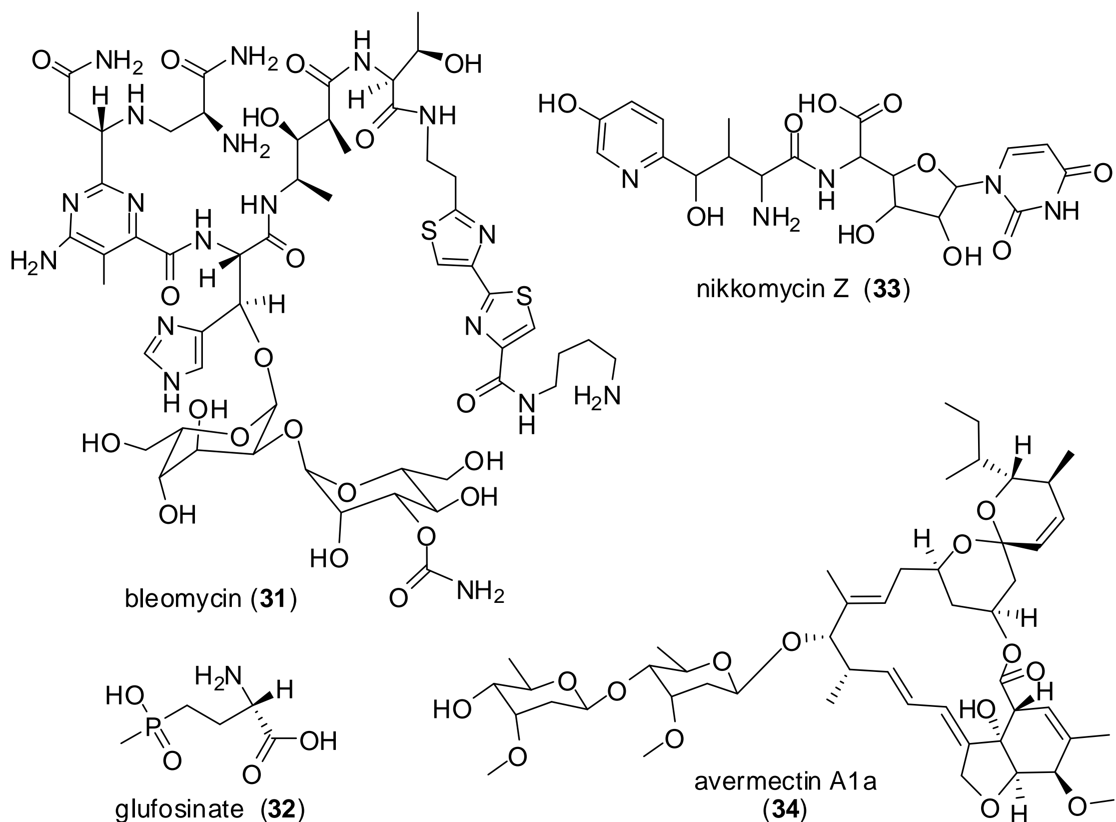
Interestingly, the improvement of separation techniques took longer. Flash column chromatography, a preparative technique that uses medium pressure to speed up the separation was only invented in 1978 by Clark Still.[66] Vacuum column chromatography, was first reported in 1985[67] and it was improved in 2001[68] to make it more practical for everyday laboratory use. High performance liquid chromatography HPLC), perhaps the greatest advancement in separation tools, was developed in the 1970's.

I.3. Rise and fall of natural products chemistry

As can be deduced from this glimpse of the history of natural products and from a search in the literature,[28] biological activity and pharmaceutical usefulness were the most important driving forces behind early studies of natural products. Over time however, a series of factors lead to a decreased investments in natural products. The shrinking rate of novelty with the re-isolation of known or derivatives of known compounds; the slowness of the isolation process; the complexity of the natural extracts; problems in material re-supply coupled to the increased demand of unique compounds for high-throughput-screening (HTS); the belief that an adequate therapeutic antimicrobial arsenal was available; and the per molecule expense of natural products relative to synthetic libraries altogether led to a gradual abandonment of natural product discovery programs by the pharmaceutical industry in favor of synthetic libraries of small molecules.[69] Since many chemists believe, incorrectly,[70] that natural products are only useful as anticancer and antimicrobial drugs, the decrease in antimicrobial research led to a decrease in natural products research.

Fortunately, the natural products scientific community is rising to meet these challenges. By the 1990's scientists recognized the need to overhaul the traditional approach of testing extracts of whole organisms for bioactivity.[71] The simple and easy-to-perform diffusion assay used for more than 50 years was returning a ratio of 1 new active compounds for every 500 tested[72] and the NCI screening had few positive results after testing for more than 20 years against mouse leukemia cell lines.[73] Dereplication, a process to rapidly identify known molecules in the early stages became a major focus.[74]

In 1970's Hamao Umezawa revived several natural products that failed development as antibiotics by testing microbial extracts in new enzyme-based assays.[75] A few examples are bleomycin (**31**) (antitumor), glufosinate (**32**) (herbicide), nikkomycin (**33**) (bioinsecticide) and avermectin (**34**).[76]



Until recently, natural products isolation and structure elucidation was very time consuming thus restricting the number of samples able to be processed. This long timeframe from detection of activity to pure compound is a hindrance in collaborations. The multi-step process of chromatographic separation followed by bioassay testing of each fraction is used to isolate the active compound. As soon as a pure compound is

obtained, a series of physical methods are used to obtain the structure. Nuclear magnetic resonance (NMR), infrared spectroscopy and mass spectroscopy are the preferred method utilized today. Improvements in these techniques have made the identification step more scientific friendly. Samples on the microgram range can be elucidated in a reasonable time.

However, bioassay-guided fractionation remains a major bottleneck in natural products screening. This concern is magnified by changes in the way pharmaceutical companies screen for new drugs. High-throughput screening (HTS) has made it possible to assay compounds in a order of hundreds of thousands per day.[77] As a result, a screen for molecules against a specific target may only run for a very short period of time, usually not long enough to do bioassay-guided fractionation of natural product extracts.

Natural products are also perceived to be inherently “dirty”. Usually they are part of a more complex matrix, the crude extract, which often contains interfering compounds. Those interfering compounds can show nonspecific activity in screens or can obscure the activity of interest exhibited by another compound.[78] Even after an active compound is successfully identified, supply issues may complicate further research, as subsequent collections of the organism may not produce the same natural product.[79-81]

The idea that new technologies would give access to a whole new reservoir of structurally diverse chemical scaffolds led to a heavier commitment to techniques like combinatorial chemistry, a very powerful tool in the stage of fine tuning the bioactivity but not capable of delivering the variety of chemical scaffolds that nature is able to.[82]

The widely accepted knowledge that natural products display an incredible chemical diversity in terms of structural complexity and biological activity led to papers analyzing the statistical aspect of this field.[1,83,84] Henkel and collaborators[85] investigated natural product and synthetic compound databases and compared to known drugs to address the relative diversity of natural products as compared with synthetic compounds. When compared to synthetic compounds, natural products show a higher complexity as measured by a number of factors. They have a higher oxygen content but a lower halogen, nitrogen and metals content. Characteristic like bridgeheads with three or four ring bonds and pharmacophoric groups are prevalent in natural products. The most interesting piece of data however is that 40% of the natural product scaffolds in the Dictionary of Natural Products (the major database of published natural product structures) did not have a counterpart in synthetic chemistry.

Evolution has conferred to natural products additional advantages for use in drug discovery. Usually natural products are biocompatible, with built in features that allow them to travel through the body to the appropriate organ or cell, penetrate cell membranes and reach their target.[70] Natural products also have evolved ways to evade metabolism in the target organism. Finally, as a consequence of their biosynthetic origins, bioactive natural products are often produced as a series of derivatives,[86] which can be isolated and used in structure-activity relationship (SAR) studies. Because of all of these factors, the wisdom of abandoning natural products research should be seriously questioned.[87,18]

I.4. A new source of natural products

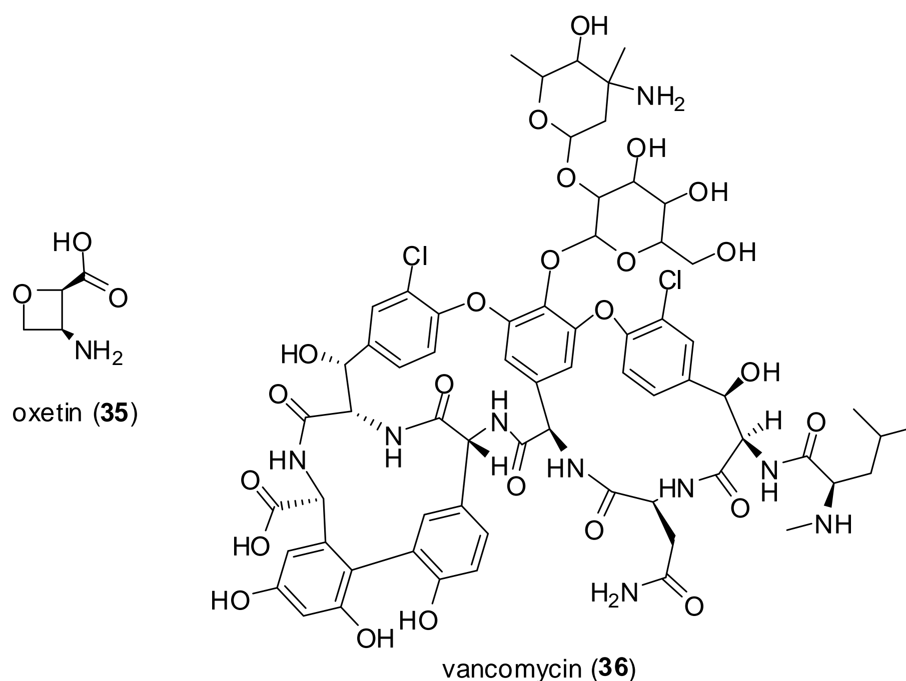
One way to avoid the re-isolation of known compounds is to study organisms from new ecological niches. Organisms in different environments presumably face different threats and consequently evolve a unique biochemical arsenal to deal with those threats. This trend, new biological sources – new structures, has been followed by natural products researchers. The source of organism of choice for natural products discovery has moved from terrestrial plants in the 19th century to soil microorganisms in the 1940s and 1950s, marine organisms and alga in the 1970s[61] and finally microorganisms in 2000. New sources led to new chemotypes, new biological activities, and new insights into the way nature constructs and utilizes small molecules.

The generic term microorganism includes different classes of organisms. The actinomycete bacteria have been the most explored microbial source of compounds.

Bacteria of the order Actinomycetales, the “actinomycetes,” are a group of Gram-positive eubacteria with a high G-C content. This group accommodates a large amount of morphological, physiological and chemical variety. They include groups that develop only in the mycelia state, groups where the mycelia is transitory appearing only during certain stages of development and some unicellular representatives of irregular shape.[88] In terms of physiology, the majority of them are strict aerobes with only a few described anaerobes (e.g. *Actinomyces israelii*, a gastrointestinal parasite) and microaerophiles (e.g. *Actinomyces bovis*[89] and facultative (e.g. *Cellulomonas xylanilytica*[91]).

Chemically, actinomycetes are prolific “biosynthetic machines” producing novel, bioactive molecules. They produce compounds belonging to all classes of secondary

metabolites in an astonishing variety of skeletons, from small structures such as the glutamine synthetase inhibitor oxetin (**35**),[90] to very complex structures like the antibiotic vancomycin (**36**). Their incredible ability to produce new natural products has made the actinomycetes the major source of new metabolites from microorganisms, and as a result substantial research has been invested in trying to mine out even more unique, bioactive compounds.[91] More than 10,000 new natural products, accounting for around 45% of all microbial products, have been isolated from this source.[1]



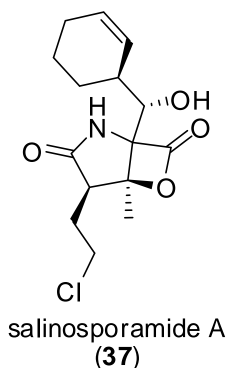
oxetin (**35**)

vancomycin (**36**)

As the chemically rich actinomycetes were believed to be present only in soil, species easily isolated from that environment became the standard source for natural products in industry. However, biodiversity studies show that this is not the case, and

actinomycetes have been found in many different environments[92] including inside plants[93] and insects,[94] acidic rice fields[95] and Antarctic soil.[96]

Over the past few years, the marine environment has proven to be an excellent place to look for new, biologically active molecules. Examples from algae and invertebrates like sponges abound, but only recently, with the discovery of obligate marine actinomycetes, efforts to find new actinomycetes have turned toward microbes living in marine sediments.[97,98] This source has already proved fruitful with the isolation of salinosporamide A (**37**), a new potent proteosome inhibitor isolated from the new marine actinomycetes genus, *Salinispora*.[101]



salinosporamide A
(**37**)

I.5. Marine Actinomycetes as a source of natural products

In 2000, the Fenical laboratory began studying marine microorganisms derived from ocean sediments. While exploring this formerly neglected habitat, the group discovered the obligate marine actinomycete genus, *Salinispora*,[97] the first marine actinomycete genus to be described, demonstrating the potential of the marine

environment as a source of new compounds. *Salinispora* turned out to be a prolific producer of novel secondary metabolites with diverse molecular skeletons. A notable example is salinosporamide A (**37**) a proteasome inhibitor that is currently in phase I clinical trials. Sporolide A (**38**), rifamycin (**39**) and cyanosporaside A (**40**) illustrate the chemical diversity found in *Salinispora* species (**figure I.5.1**). Since the discovery of *Salinispora*, approximately 15 new microbial groups, which appear to be new taxa of actinomycetes, were discovered using a variety of isolation techniques and growth media studies (**figure I.5.2**).[99] Because these organisms were discovered in a habitat not previously studied, it was important to understand their capacity to produce biologically active molecules and to investigate their underlying biosynthetic machinery.

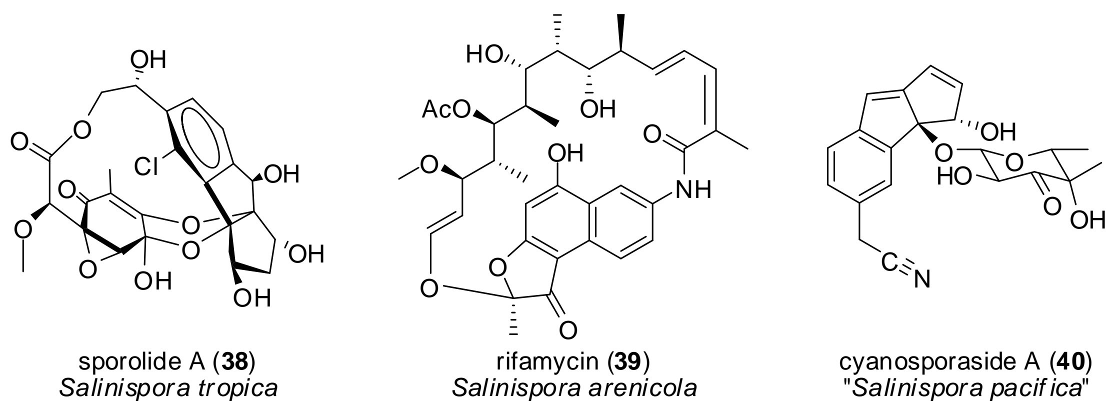


Figure I.5.1 - Example of compounds produced by the three *Salinispora* species

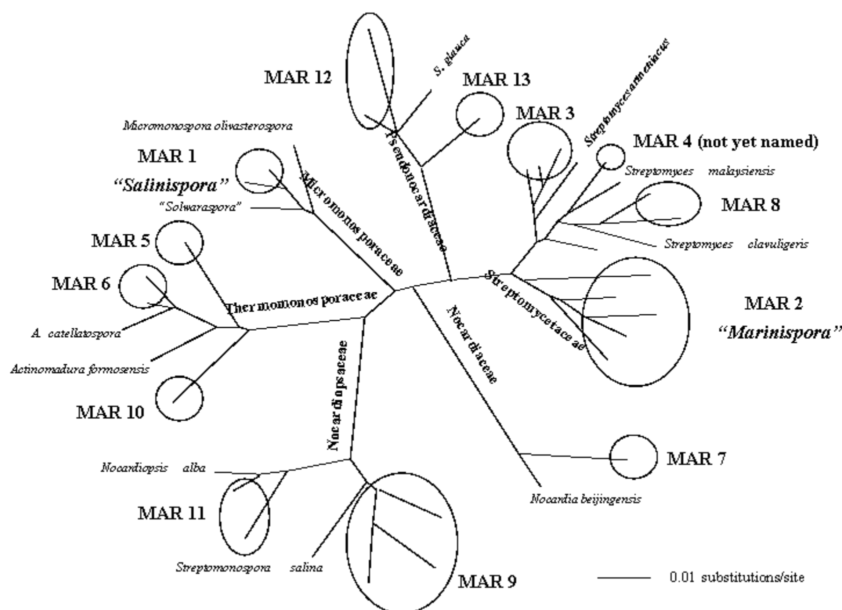
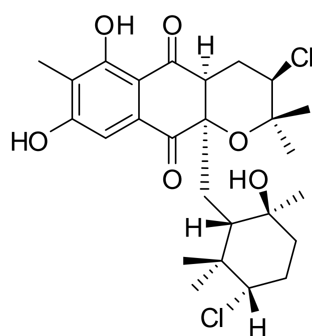


Figure I.5.2 - Phylogenetic diversity from cultured marine actinomycetes[99]

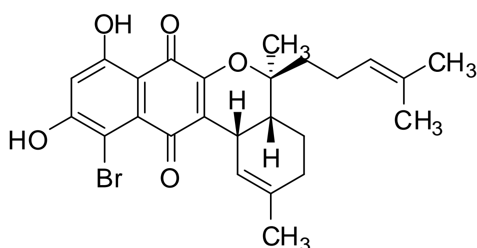
I.5.1. MAR 4 group

MAR 4 is a phylogenetically unusual group of marine actinomycetes related to those of the genus *Streptomyces*. (**Figure I.5.3**) The specimens in the Fenical collection came from marine sediments off the coast of San Diego, Baja California and Palau. Preliminary chemical studies indicated that this genus is capable produces a whole new set of related natural products. This group makes a series of molecules combining different biosynthetic pathways, including many naphthoquinones (e.g. napyradiomycin (**41**) and marinone (**42**)).[100,101] This combination of terpenoid biosynthesis with other

pathways present in all member of the group makes the MAR 4 group unusual among both marine and terrestrial actinomycetes. Clearly the structural details of this unusual metabolism were worthy of comprehensive study.



napyradiomycin (**41**)



marinone (**42**)

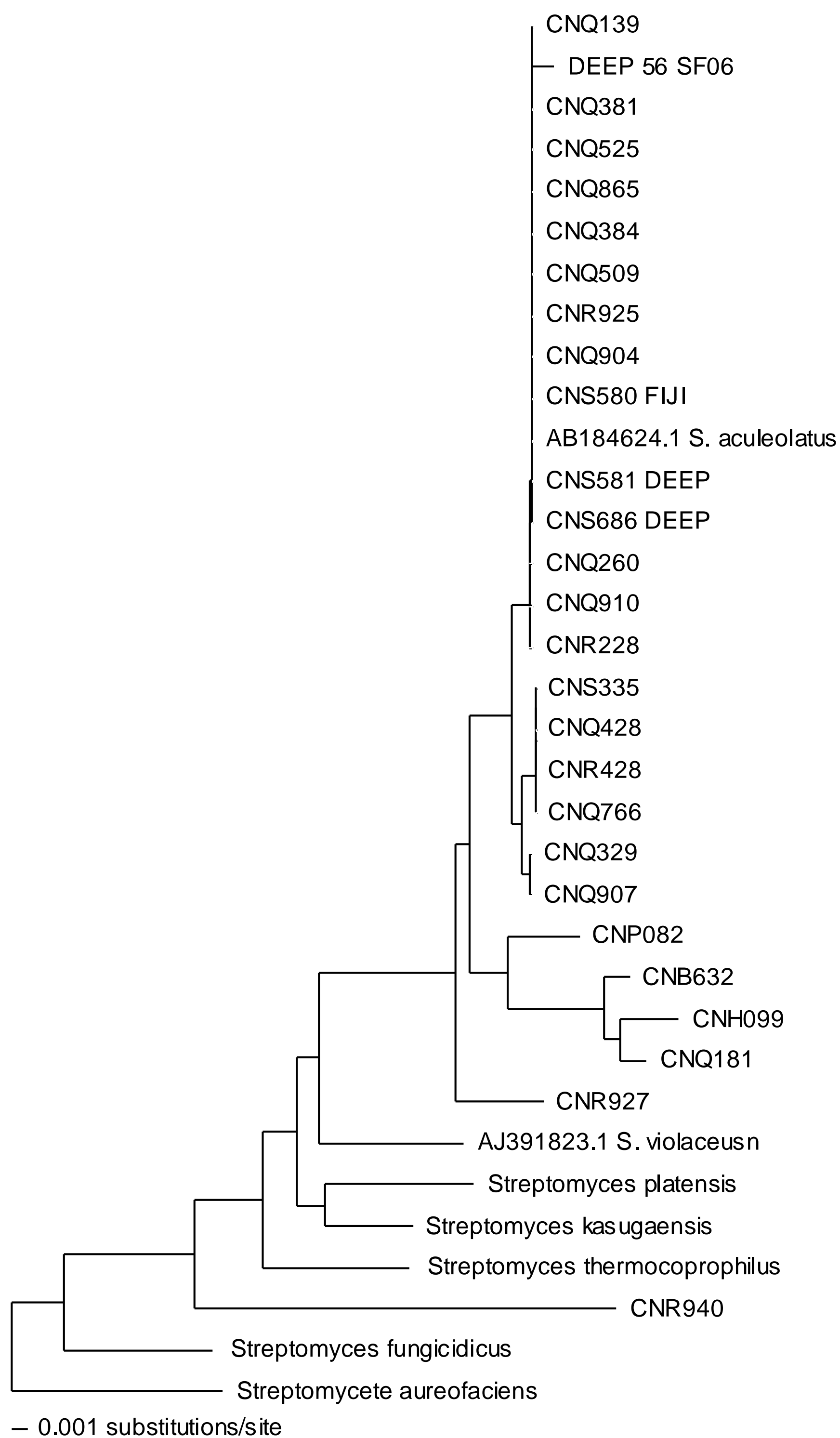


Figure I.5.3 - Phylogenetic tree of MAR 4 strains[102]

Phylogenetic analysis of the MAR 4 group, based on their full 16S rRNA sequence, situates them in the *Streptomycetaceae* family, the most chemically prolific of the actinomycetes. Members of this family alone are responsible for the production of around 7600 compounds (approximately 74% of all known compounds from actinomycetales) (**Figure I.5.4**).[1]

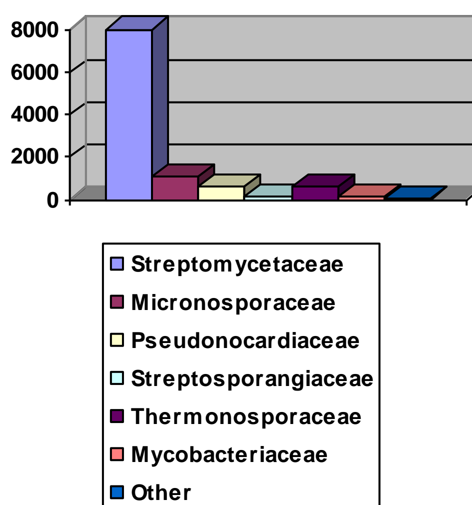


Figure I.5.4 - Approximate number of compounds produced by actinomycete families

The MAR 4 group showed a fascinating relationship between phylogeny and secondary metabolite production. All the isolated strains belonging to this group produced naphthoquinone type compounds, and exhibited a clear group relationship between the phylogeny of the group and the compounds its members produce.

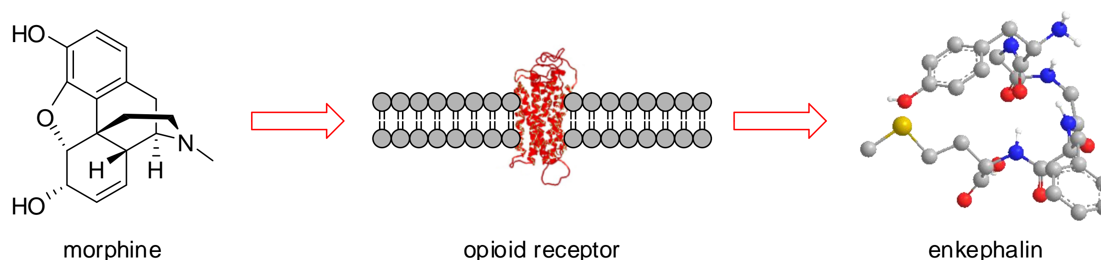
This observation indicated that phylogenetic analysis may permit the prediction of secondary metabolite production patterns, which contradicts the conventional wisdom that secondary metabolite production is strain-specific. This strain-specific paradigm

was a result of traditional bacterial taxonomic methods. Bacteria were, for a long time, classified only by their morphology and physiology. Even though those are very important features for taxonomy, the use of genetic material (16s RNA and DNA content) based classification showed that many unrelated organisms were considered as same species.

I.6. Importance of the study of natural products

The study of natural products brought an incredible expansion in the understanding of our organism and of our environment. Almost every field in biology, organic chemistry, health science and ecology has directly or indirectly profited from studies of natural products. Scientists working with natural products have found compounds to cure or prevent diseases. Synthetic chemists building natural products had to develop new methodologies to be able to construct more complex structures. Ecologists working with marine and terrestrial organisms broadened the understanding of how the biota is chemically regulated. The use of natural products in pharmacology allowed not only a quantitative evaluation of the effects of the drug but also the development of better clinical applications. Every new bioactive natural product has the potential to be a probe to a new aspect of our physiology. A good example of this "probe to a new world" is the series of discoveries that started with morphine (**Scheme I.6.1**). The study of morphine's mechanism of action led to the search for corresponding receptors in the human brain. In 1975, the first two natural agonists for these receptors

were described.[103] Pharmacologists are still finding new subsets of the opioid receptors and new agonists for them and as a consequence are learning more about the chemistry of the human brain.



Scheme I.6.1

Despite all the scientific knowledge brought directly and indirectly, natural products research remains far from its potential because of the relative small number of species that have been studied. If based on the number of living organisms estimated on the planet prior to the genomic era, approximately 1% of bacteria and 5% of fungi were studied for the production of natural products.[104] However, studies in metagenomics revealed that the number of organisms is higher than predictions, making the percentage of organisms researched smaller.[105,106] Unfortunately, the habitats where these organisms live suffer with anthropogenic interventions before there is an opportunity of thoroughly study them.

These facts - historical novelty, high diversity of producers and high human interventions - make extremely important the study of the natural products before their producers are lost.

I.7. Thesis

I hypothesized that a careful chemical investigation of a new marine group of actinomycetes and the use of an innovative chemoprevention screening platform would be effective approaches to the discovery of novel natural products. The objective of this dissertation research was to examine novel secondary metabolites from the marine microbial source MAR 4 and to isolate, purify and determine the structures of the new compounds.

In Chapter II, III and IV the isolation and structure elucidation of new natural products from the strain CNQ 509, belonging to the new clade MAR 4 is described. These studies include the isolation of nitropyrrolins A-C (**97-99**) and marinophenazines A (**105**) and B (**106**) and streptoquinone (**111**).

In Chapter V the isolation of a new naphthoquinone, naphtomarine (**112**) active in the NF-κB cancer chemoprevention screen is described. Comparison of the activities of the different structural derivatives revealed features that are important for the biological activity.

The final chapter will summarize my work and provide conclusions based upon this thesis research.

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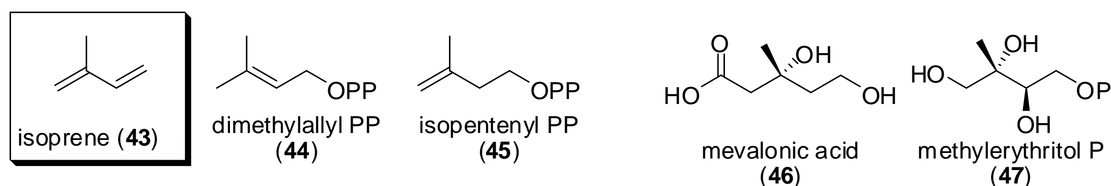
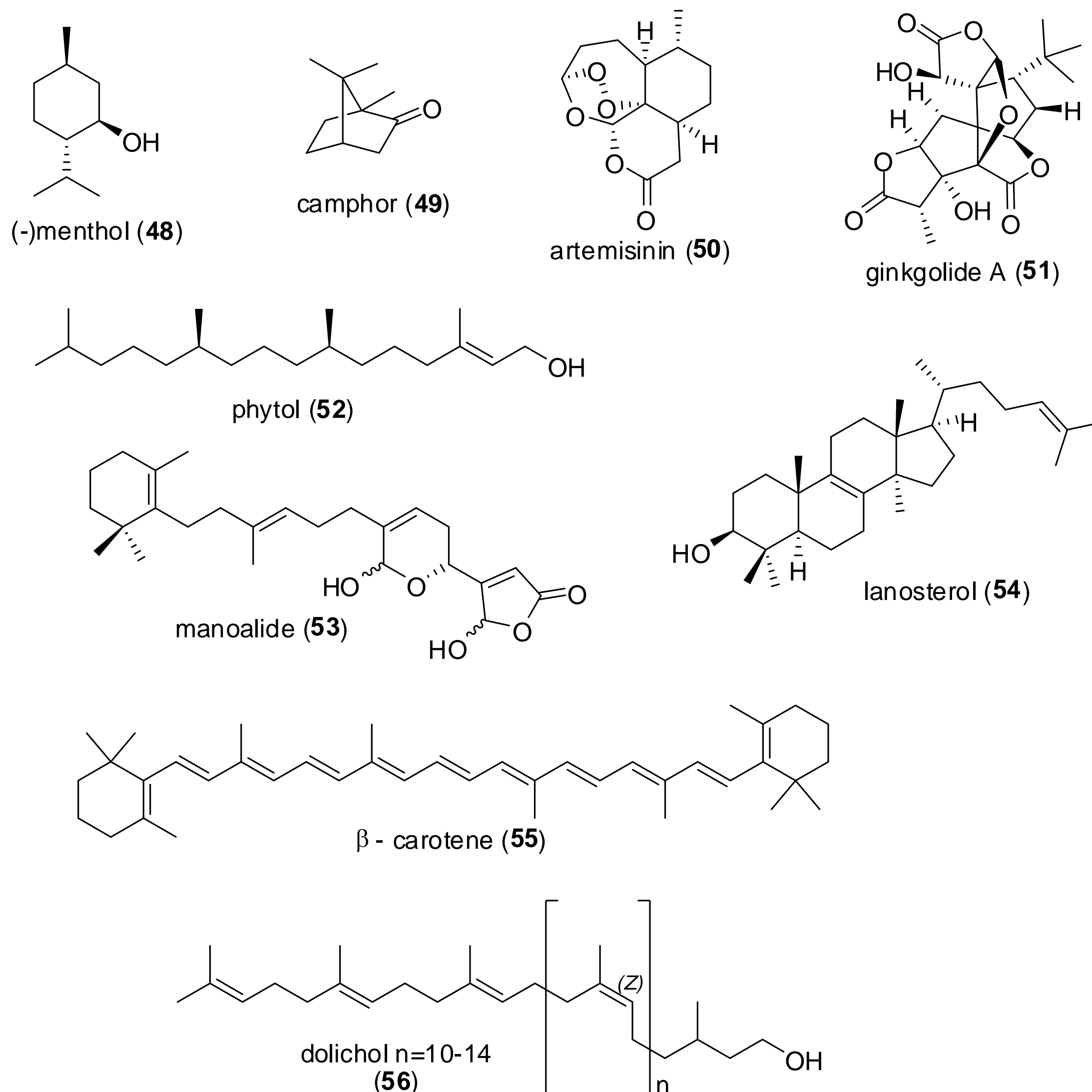
II. Review of terpenoids and meroterpenoids in actinomycetes

II.1.1. Terpenoids

Terpenoids are the largest and most diverse group of natural products. This group includes essential oils of plants, insect pheromones, plant hormones, auxiliary photosynthetic pigments and chemical defenses.[1] Vital for all organisms, terpenoids are didactically described as formed by isoprene units linked in a head to tail fashion. Even though isoprene (**43**) is produced by some plants, it is not an intermediate in terpenoid biosynthesis. Instead, dimethylallyl diphosphate (DMAPP) (**44**) and isopentenyl diphosphate (IPP) (**45**) are the activated building blocks incorporated in terpenoids. Until 1993, mevalonic acid (MVA) (**46**) was believed to be the only central precursor to DMAPP and IPP. Today, the methyl-erythritol phosphate (MEP) (**47**) pathway, first described in bacteria and later in plants, is also known.[2] Interestingly, there is no homogeneity in which pathway is used for natural product biosynthesis; examples can be found in which the same compound is produced by the MVA pathway in one species and by the MEP pathway in another.[3]



Terpenoids are classified based on the number of isoprene units they contain. Terpenoids formed of only one unit (C_5) are called hemiterpenoids. There are very few examples of hemiterpenoids such as the volatile isoprene (**43**). The components of the essential oils menthol (**48**) and camphor (**49**) are examples of monoterpenes, C_{10} molecules consisting of two isoprene units. C_{15} isoprenoids, or sesquiterpenes, are derived from three isoprene units. The sesquiterpene lactone artemisinin (**50**), used for centuries in China as an antimalarial drug has a unique peroxide bridge responsible for its action.[3] Artemisinin (**50**) activity as an antimalarial is outstanding; it was considered the best treatment for uncomplicated falciparum malaria recommended in the "2006 guidelines for treatment of malaria" from the World Health Organization (WHO).[4] The diverse group of diterpenes (C_{20}) includes simple molecules such as phytol (**51**) along with complex structures like ginkgolide A (**52**). Sesterterpenes (C_{25}) are rare in the terrestrial environment but not in marine organisms. Manoalide (**53**), a potent antiinflamatory drug, is a typical example of a sesterterpene isolated from sponges.[5] Triterpenes (C_{30}), such as lanosterol (**54**), are found in all eukaryotes. Carotenoids are the most common tetraterpenes (C_{40}). Carotenoids like β-carotene (**55**) are auxiliary photosynthetic pigments aiding in the harvesting of light during photosynthesis. Polyterpenes containing more than eight isoprene units include rubber and polyisoprenoid alcohols such as dolichol (**56**),[6] used by cells to prenylate proteins.



Each class of terpenoids is derived biosynthetically from a single parent molecule (**Figure II.1.1**). Most of these parent molecules are formed by head-to-tail elongation with C_5 isoprene units. The exceptions are triterpenes and tetraterpenes for which two C_{15} (farnesyl) and two C_{20} (geranylgeranyl) molecules, respectively, come together in a tail-to-tail fashion. Modifications to the basic carbon skeleton are common in terpenoids with changes such as ring formation, oxidation, rearrangements and hydrogenation

occurring in these molecules and sometimes making recognition of the terpenoid origin difficult.[3]

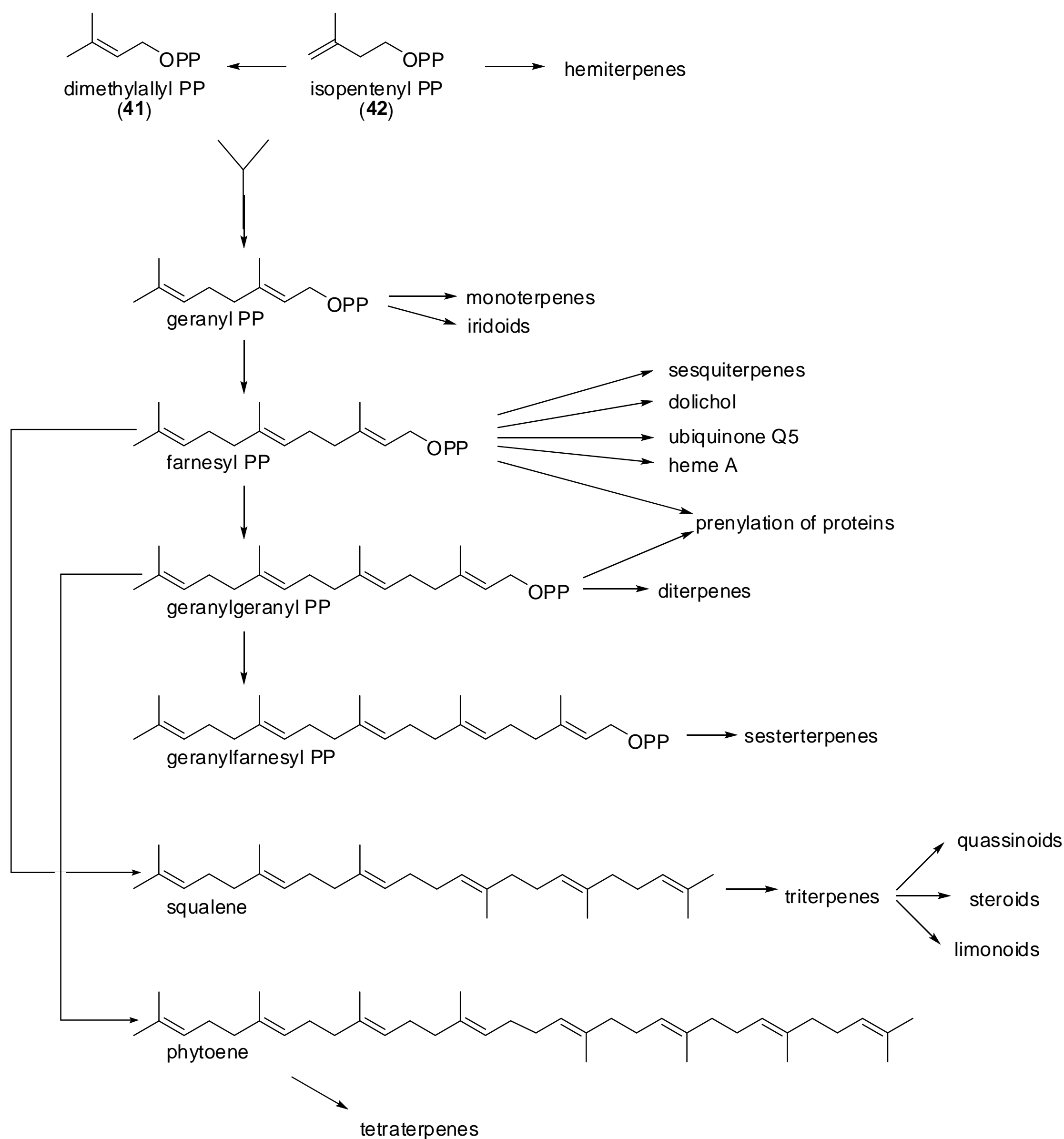
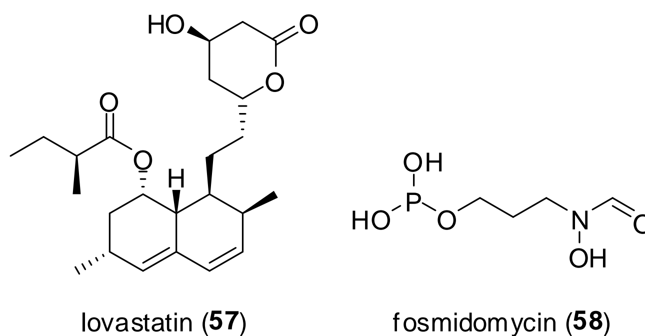


Figure II.1.1 – Terpenoid classes[3,6]

The ability to modulate terpenoid biosynthesis has very important medical applications; both the MVA and MEP pathways are currently being targeted for new drugs. Inhibition of the enzyme HMG-CoA reductase from the MVA pathway is a key step in cholesterol blood level control, a primary risk factor for cardiovascular disease.[1] Lovastatin (**57**), a HMG-CoA reductase inhibitor, is a hypolipidemic drug isolated in 1979 from the fungus *Monascus ruber* by Akira Endo.[7] As the MEP pathway is absent in humans, it is a very interesting target for antibiotic drug discovery. Fosmidomycin (**58**), an inhibitor of the MEP pathway, was first isolated in an antibiotic screening and subsequently abandoned because of low efficacy. Fortunately, this compound was later tested as an antimalarial, with positive results.[8]



lovastatin (**57**) fosmidomycin (**58**)

II.1.2. Meroterpenoids

Meroterpenoids are compounds with mixed biosynthesis consisting of both a terpene unit and a non isoprene unit. Examples of meroterpenoids include the rotenoids (**59**), indole alkaloids (**60**) and cannabinoids (**61**) (**Figure II.1.2**).[3]

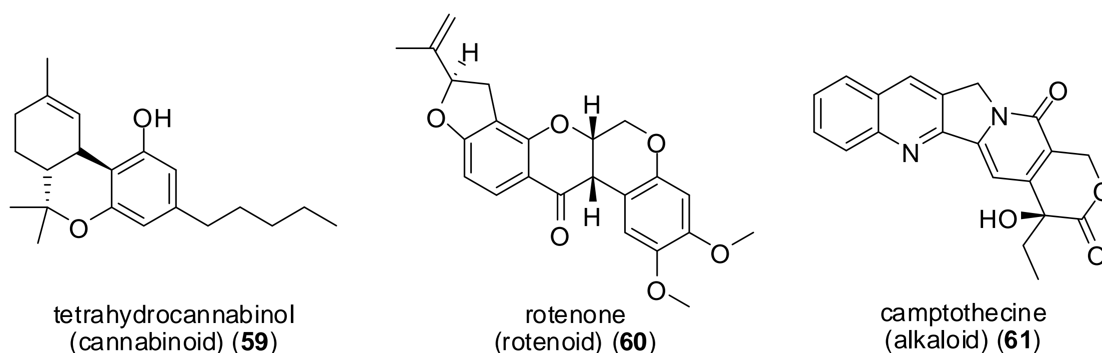


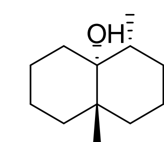
Figure II.1.2 – Examples of meroterpenoids.

II.2 Terpenoids in actinomycetes

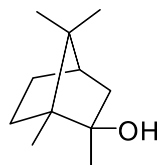
Actinomycetes are very prolific producers of secondary metabolites; they make astonishingly diverse collections of polyketides and peptides. However, terpenoids are not a major group of natural products produced by actinomycetes.[9] By 2002, around 40,000 compounds had been isolated from actinomycetes,[10] with only a couple hundred terpenoids.[11] Interestingly, both MEP and MVA pathways are used by actinomycetes in terpenoid biosynthesis. All the terpenoids involved in primary metabolism are biosynthesized using the MEP pathway, but the MVA pathway is used to some extent for secondary metabolism. [9] Terpenoids in actinomycetes have unique structures very different from ones found elsewhere.[12]

Monoterpenoids are in general associated with intensely pungent odor. They are responsible for most fragrances and flavors.[1] In actinomycetes, the characteristic earthy smell of several cultures is from a volatile, partly degraded monoterpenoid, geosmin

(**62**).[13,14] Geosmin (**62**) and 2-methylisoborneol (**63**), another monoterpenoid produced by actinomycetes, are associated with unpleasant odors and are considered pollutants of drinkable water. [13]

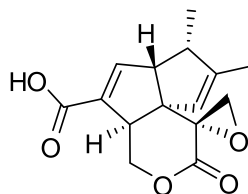


geosmin (**62**)



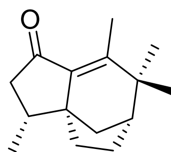
2-methylisoborneol (**63**)

Pentalenolactone (**64**) is an antibiotic isolated from *Streptomyces arenae* whose mechanism of action is a highly specific inhibition of the glyceraldehyde-3-phosphate dehydrogenase enzyme (GAPDH). [15] This compound also inhibits IL2 production and is being examined for its potential as an immunosuppressive agent. [16]



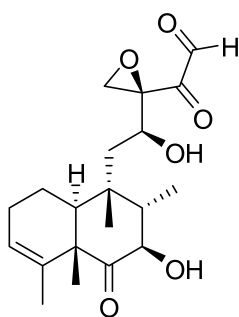
pentalenolactone (**64**)

Albaflavenone (**65**) is a sesquiterpene antibiotic with a rare zizaene skeleton. This compound is partly responsible for the odor of the producing *Streptomyces* species.[17]

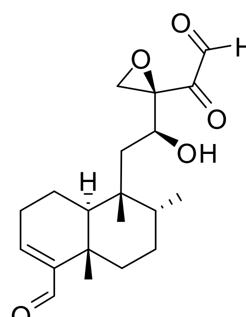


albaflavenone (**65**)

Terpentecin (**66**), a DNA synthesis inhibitor, was isolated in 1985 from a *Kitasatosporia* strain MF730-N6.[18,19] Terpentecin (**66**) shares the same skeleton with clerocidin, (**67**) an antibiotic isolated from the fungus *Oidiodendron truncatum*. It is believed that the fungus acquired the biosynthetic machinery for this molecule via lateral gene transfer, as no other *Oidiodendron* species demonstrates the ability to produce it.[18]



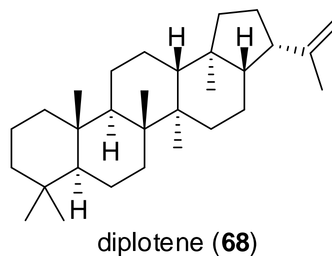
terpentecin (**66**)



clerocidin (**67**)

A major difference between eukaryotes and prokaryotes is the terpenoid content of membranes. Triterpenoids have the function of stabilizing and lowering the fluidity of cellular membranes. In eukaryotes, steroids like cholesterol have this function. However, steroids are absent from most prokaryotes, and it has been postulated that the widely distributed hopanoids serve the same function.[19,20] In *Streptomyces coelicolor*

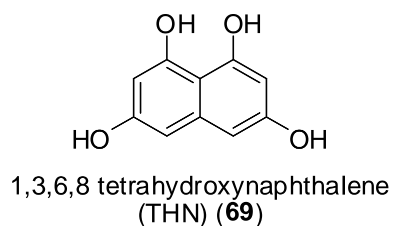
A3, hopanoids like diplotene (**68**) are produced in sporulating culture in solid media, presumably to alleviate stress in aerial mycelium.[21]



diplotene (**68**)

II.2.1. Meroterpenoids in actinomycetes

Most of the terpenoids found in actinomycetes are meroterpenoids and can be classified by the nonterpenoid part: polyketide, phenazines and miscellaneous.



1,3,6,8 tetrahydroxynaphthalene (THN) (**69**)

The symmetrical pentapolyketide 1,3,6,8 tetrahydroxynaphthalene (THN) (**69**) is an intermediate in the biosynthesis of many polyketide meroterpenoids including napyradiomycin A1 (**70**)[22], azamerone (**71**)[23], furaquinocin A (**72**)[24], naphthablin (**73**)[25] and naphterpin (**74**)[26] (**Figure II.2.1.1**).

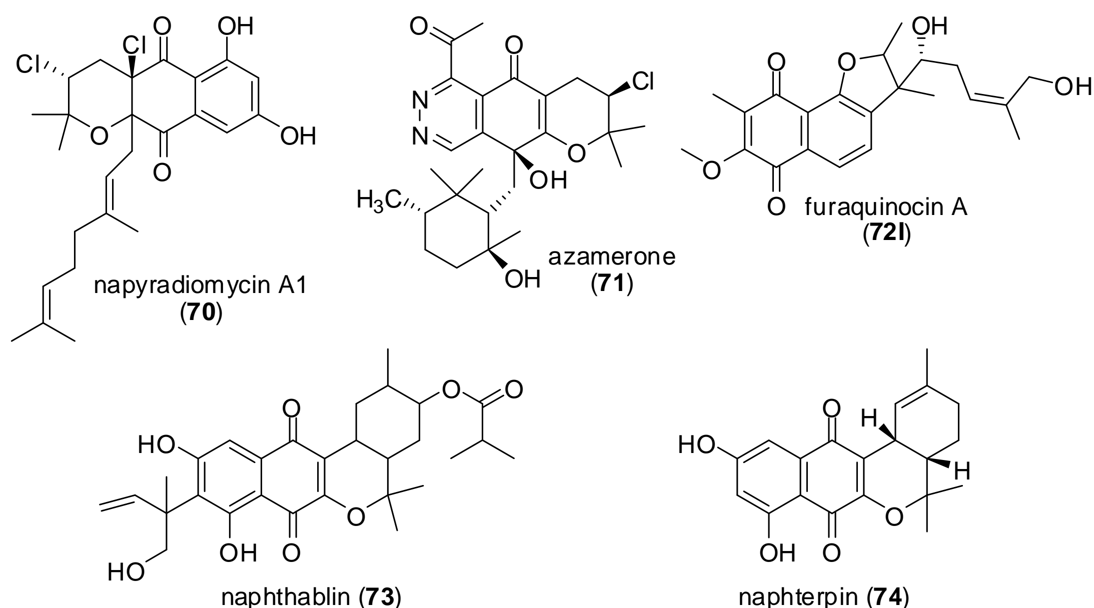
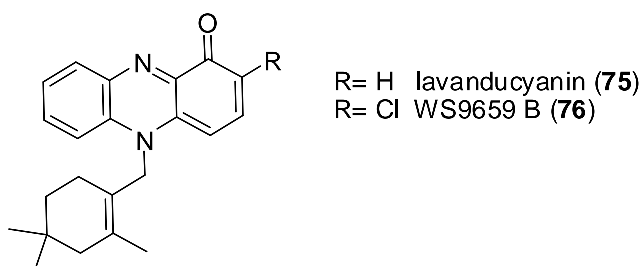


Figure II.2.1.1 – Examples of meroterpenoids derived from THN

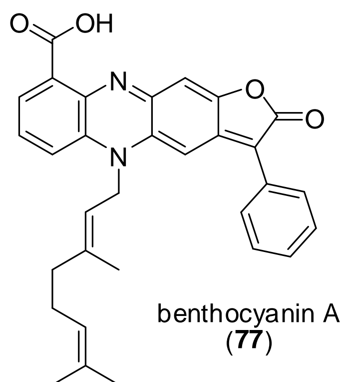
Napyradiomycin (**70**), furaquinocin A (**72**) and naphthablin (**73**) are cytotoxic naphthoquinones isolated from *Streptomyces* species. The napyradiomycin (**70**) group of natural products has more than 50 derivatives reported in the literature.[11] All napyradiomycins possess the naphthopyran chromophore and many of them also have halogens attached to the terpene portion.[27] The napyradiomycins (**70**) are very active natural products acting also as gram-positive antibiotics and estrogen-receptor antagonists. [24,30] The mechanism of naphthablin (**73**) cytotoxicity involves inhibition of RNA synthesis.[25] Naphterpins (**74**) are antioxidant compounds that act as free radical scavengers.[26,28] Azamerone (**71**) was the first natural product isolated with the pyranophthalazinone core.[23] The pyranophthalazinone ring system is formed by a rearrangement of the precursor THN (**69**).[29]

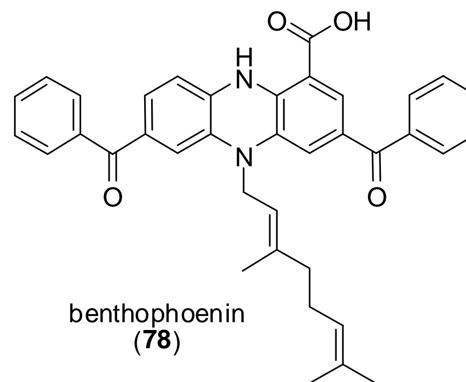
Streptomyces spp. and *Pseudomonas* spp. are the major producers of phenazine compounds in nature. *Streptomyces* and *Pseudomonas* each produce several simple phenazines. However, *Streptomyces* also have the ability to produce complex phenazine derivatives, with many of them having N- or C- prenylations.[30]

Lavanducyanin (**75**) is an antitumor natural product produced by *Streptomyces* spp., same producer of naphterpins (**74**), which is active against P388 and L1210 cell lines.[31] Together with WS-9659 A (**76**), a chlorinated derivative, lavanducyanin (**75**) is also active as an inhibitor of testosterone 5 alpha-reductase the primary target of drugs that treat male pattern baldness.[32]

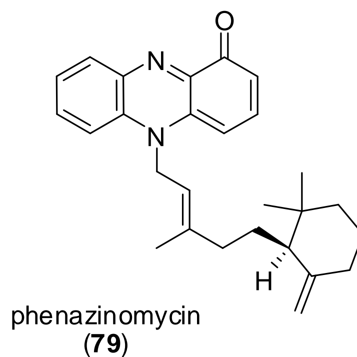


Benthocyanin A (**77**) and related benthophoenin (**78**) are compounds with powerful radical scavenger activity isolated from *Streptomyces prunicolor*.[33,34] Benthocyanin A (**77**) has a highly conjugated furophenazine ring and it is twice as potent as vitamin E.

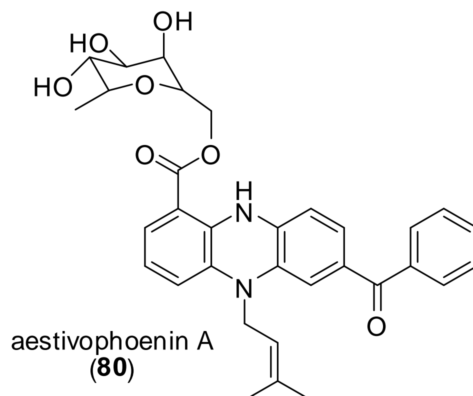




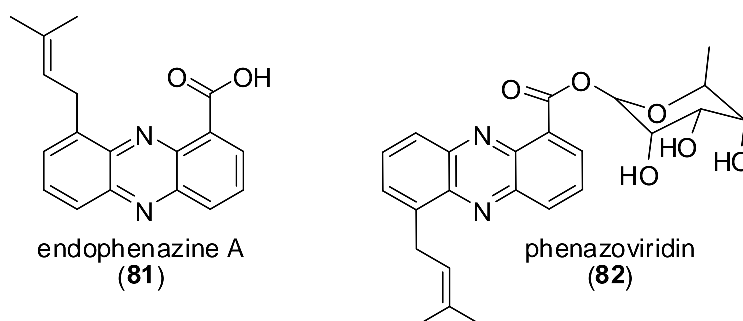
Phenazinomycin (**79**) is a cytotoxic phenazine isolated from a *Streptomyces sp*. This compound with the longest terpene chain between phenazines is active against leukemia cell lines.[38,39]



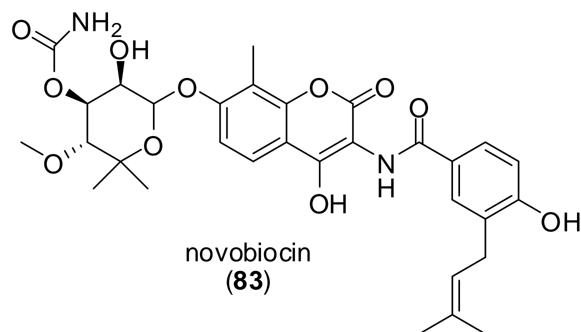
Aestivophoenins A-C (**80**) were isolated from *Streptomyces purpeofuscus*. These compounds are active as neuronal protectors, effectively suppressing the toxicity induced by L-glutamate.[39,40]



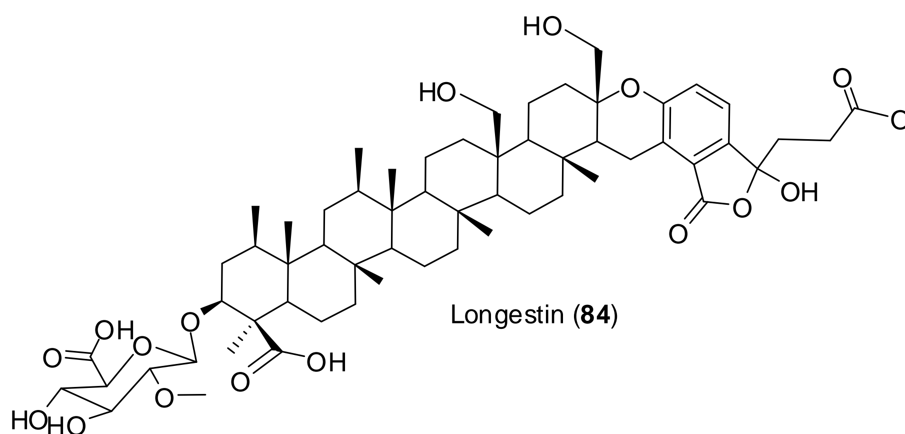
Endophenazines A-C (**81**) and phenazoviridine (**82**) are the only C-prenylated phenazines reported in the literature. Endophenazines were isolated from cultures of an arthropod symbiont, *Streptomyces anulatus*.[35] Endophenazine A (**81**) showed antimicrobial activity against gram-positive bacteria and some filamentous fungi.[42] Phenazoviridin (**82**), isolated in 1993, was the first glycosilated phenazine with antioxidant activity.[43]



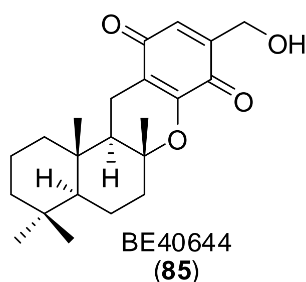
Novobiocin (**83**) was the first meroterpenoid isolated from an actinomycete in 1956.[9] The biosynthesis of this compound also utilizes THN (**69**) as an intermediate but in this case the isoprene unit is not linked directly to it.



Longestin (**84**), isolated from *Streptomyces argenteolus*, as the name implies, is probably the largest meroterpenoid isolated. This very complex natural product has a molecular formula of $C_{61}H_{88}O_{17}$, eleven rings, twenty chiral centers and a novel 2-O-methylated sugar.[36] The tetraterpenoid portion of longestin (**84**) presents all the terpene rings in a *trans* configuration hinting at a sequential addition of isoprene units and not the tail to tail fusion of two geranylgeranyl phosphate units typically observed in tetraterpenoids.[12]

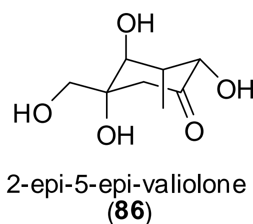


Longestin (**84**) is a good example of the change of paradigm in bioassays from antimicrobials to enzyme inhibitors. This compound was first isolated in a memory bioassay following increase in resistance to amnesia in rats.[37] Longestin inhibits brain Ca^{2+} and calmodulin-dependent cyclic-nucleotide phosphodiesterase[38] and is now used to stimulate oocyte production.[39]



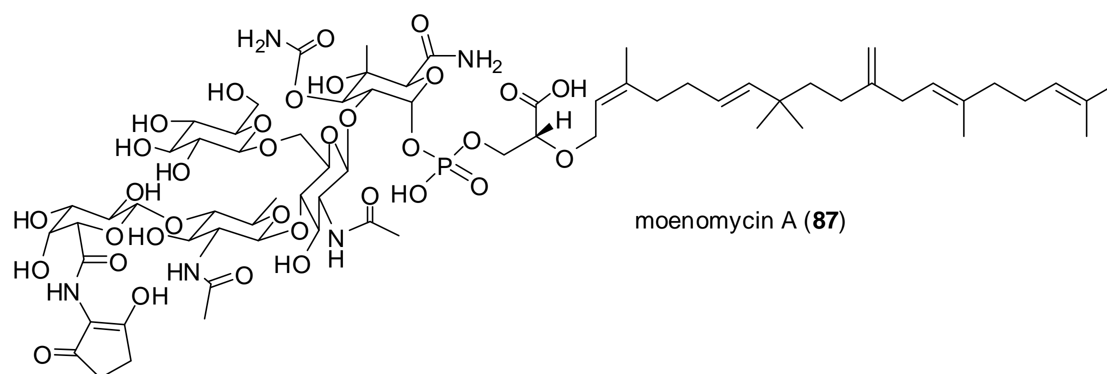
BE40644
(**85**)

BE40644 (**85**) was isolated from an *Actinoplane* sp. in a screen targeting human thioredoxin system.[40] BE40644 (**85**) is also active as an antitumor and an antiviral agent and is being examined for use in allergic processes associated with eosinophiles, immune system cells which combat parasite infections.[41,42]



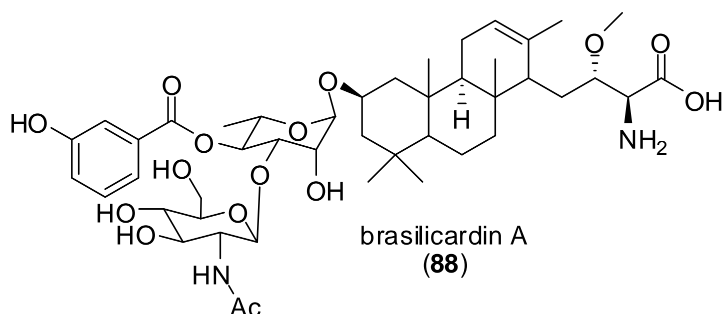
2-epi-5-epi-valiolone
(**86**)

Although longestin (**84**) and BE-40644 (**85**) resemble polyketides derived natural products, their precursor is 2-epi-5-epi-valiolone (**86**) which is biosynthesized from sedo-heptulose (an intermediate in the pentose phosphate pathway).



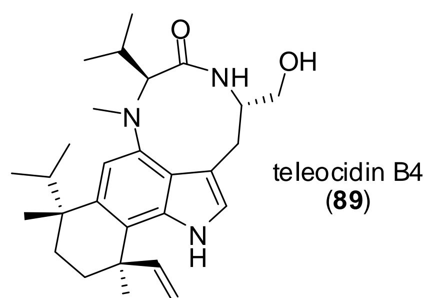
moenomycin A (**87**)

Moenomycin A (**87**) is a phosphoglycolipid antibiotic that inhibits extracellular peptidoglycan glycosyltransferases, also called transglycosylases.[43] Transglycosylases, catalyze the elongation of the peptidoglycan layer of bacterial cell walls.[44] Transglycosylases are also target by vancomycin (**36**).[45] However, moenomycin A (**87**) and vancomycin (**36**) do not have the same mechanism of action. Vancomycin binds to the peptidoglycan intermediates, the enzyme substrate, whereas moenomycin A (**87**) binds to the active site of the transglycosylases, a unique mechanism of action.[46]

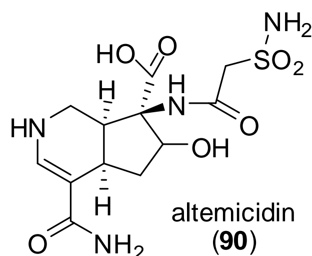


Brasilicardin A (**88**), an immunosuppressive compound, is one of the few meroterpenoids in actinomycetes which is not produced by *Streptomyces*. This diterpene meroterpenoid was isolated from a *Nocardia brasiliensis* cultured from an infected patient.[47]

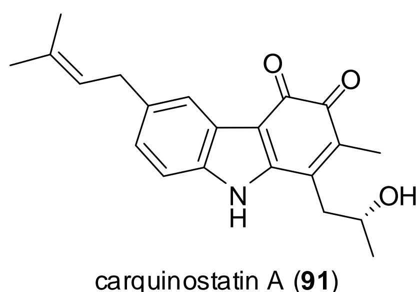
Teleocidins (**89**) are a family of toxic indole alkaloids produced by *Streptomyces* spp.[48] Interestingly, teleocidins are related to the Lyngbyatoxin, marine natural products isolated from the cyanobacteria *Lyngbia majusculata*.[49] First isolated in 1960, this group of compounds modulates protein kinase C resulting in strong tumor promoter activity.[50]



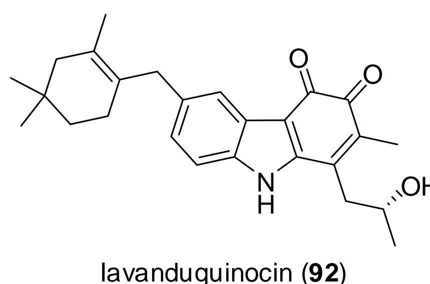
Altemicidin (**90**) is a unique monoterpene alkaloid with insecticidal, acaricidal and antitumor activity. This molecule with its unusual 6-azaindene skeleton was isolated from *Streptomyces syoyaensis* from Japanese sea mud.[51,52]



An *o*-quinone carbazole ring system is the core of carquinostatin A (**91**) and lavanduquinocin (**92**), two potent neuronal cell protecting agents that are also radical scavengers. [53,54]

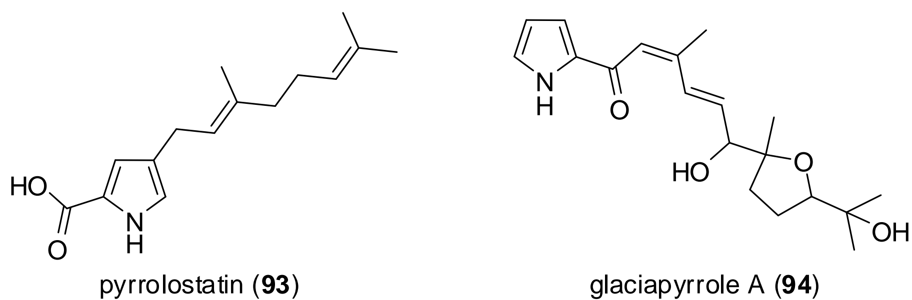


carquinostatin A (**91**)



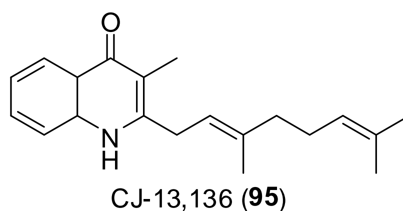
lavanduquinocin (**92**)

Pyrrolostatins (**93**) and glaciapyrroles (**94**) are the only pyrrole-containing meroterpenoids described to date. Pyrrolostatin (**93**) is an inhibitor of peroxylipid formation as it scavenges free radicals.[55] Glaciapyrroles (**94**) were isolated from *Streptomyces* sp. in marine sediments from Alaska.[56]



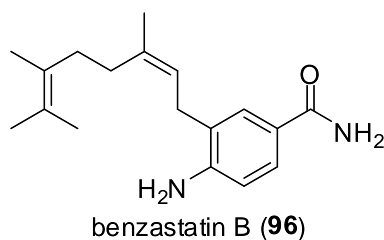
pyrrolostatin (**93**) glaciapyrrole A (**94**)

CJ-13,136 (**95**) is a quinolone antibiotic active against *Helicobacter pylori*, the causative agent of gastric and duodenal ulcers. This compound is part of a group of quinolones isolated from a *Pseudonocardia* sp. from Indian soil.[57]



CJ-13,136 (**95**)

Benzastatin B (**96**) has as its non-terpenoid moiety, the rare para-aminobenzamide unit. This meroterpenoid is part of a group of nine compounds with free radical scavenger activity.[58]



benzastatin B (**96**)

II.3. Importance of terpenoid production by actinomycetes

The research focus in meroterpenoids from actinomycetes is recent and has two driving-forces: bioactivity and biosynthesis. Meroterpenoids present a broad range of activity, but not usually as antimicrobials, which explains why they were largely overlooked during the "golden age" of natural products drug discovery.

Terpenoid biosynthesis is less thoroughly studied than the biosynthesis of other classes of secondary metabolites,[12] due to the technical challenges associated with this research. Plants are the major producers of terpenoids in nature, but the enzymes in their production are membrane bound, making it difficult to isolate or heterologously express their products. In addition, the genes for plant terpenoid biosynthesis are scattered throughout the genomic DNA. In *Streptomyces,* the presence of both terpenoid pathways, MEP and MVA is common.[9] As in the actinomycetes, the enzymes for terpenoid biosynthesis are not membrane-bound and the genes are clustered together, facilitating studies of these enzymes. [12]

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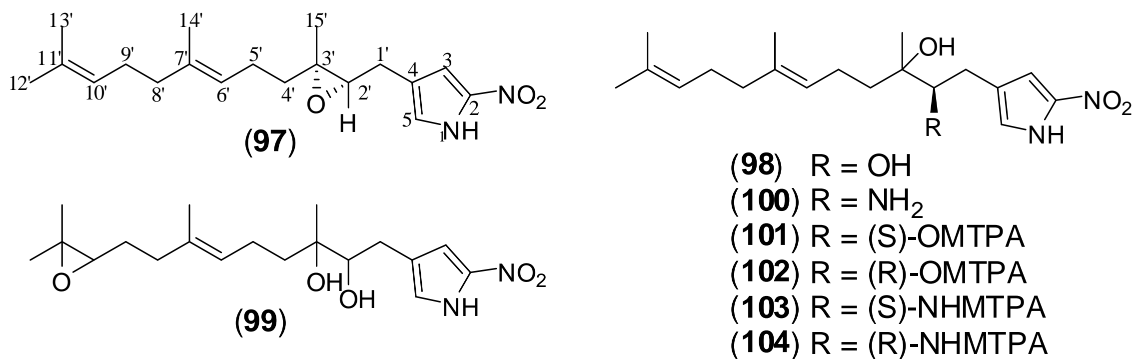
III. Nitropyrrolins A-C, farnesyl-α-nitropyrroles from the marine derived actinomycete CNQ 509.

III. 1. Introduction

As part of recent studies of actinomycete diversity in marine sediments, we have identified a group of actinomycetes, the MAR 4 clade, that is affiliated with the genus *Streptomyces* and can be cultivated from diverse marine sediment samples.[1] As a group, these bacteria possess the ability to produce compounds of mixed terpenoid biosynthesis (meroterpenoids).[2-5] Of the 19 MAR 4 strains that we have chemically examined to date, all have demonstrated the ability to produce this class of biosynthetically hybrid metabolites. One of these strains, CNQ509, was isolated from a sediment sample collected near La Jolla, California at a depth of 44 m in 2001. When cultivated under saline conditions, strain CNQ509 produced a series of unique terpenoid pyrroles, nitropyrrolins A-C (**97-99**), which possess rare α-nitropyrrole functionalities. To the best of our knowledge, these are the first naturally occurring α-nitropyrroles described.[6,7]



III. 2. Results and discussion

Nitropyrrolin A (**97**) was isolated as a light yellow oil, which analyzed for the molecular formula $C_{19}H_{28}N_2O_3$ ($[M+Na]^+$ at *m/z* 355.1979, $C_{19}H_{28}N_2O_3Na$) by HR ESI-TOF mass spectrometry. This formula, which was consistent with NMR spectroscopic data, indicated that nitropyrrolin A possessed 7 double bond equivalents. The UV spectrum of (**97**) showed significant absorption at 344 nm indicating the presence of an extended aromatic chromophore.

The ^{1}H NMR spectrum of nitropyrrolin A (**97**) (**Table III.1**) exhibited bands suggesting the presence of an aromatic ring and a terpene chain. Two downfield signals with small coupling constants [δ 6.86 (1H, d, 1.3 Hz) and δ 7.03 (1H, dd, 1.3 Hz)], plus the presence of nitrogen in the molecular formula, indicated that **97** contained a disubstituted pyrrole ring. Supporting this assignment were the presence, in the ^{13}C NMR spectrum, of four aromatic carbons at δ 137.7, 111.1, 123.1, and 122.0, respectively.

The remaining fifteen carbons were assigned to a farnesyl sesquiterpenoid side chain. The ^{1}H NMR spectrum contained one two proton olefinic signal (two protons δ 5.07 (2H, m)), which indicated the presence of two olefinic bonds. Three olefinic methyl groups were also observed at δ 1.59 (6H, bs) and 1.67 (3H, bs), indicating the presence of a terminal gem-dimethyl substituted olefin. Confirmation of this assignment was obtained by analysis of gHMBC NMR data, which showed correlations from these three methyl groups to their corresponding olefinic carbons. The presence of a trisubstituted epoxide ring was also clear based upon analysis of ^{1}H and ^{13}C NMR spectroscopic data. One of the epoxide substituents was found to be a methyl group (δ 1.37, s 3H) that showed gHMBC correlations to the quaternary epoxide carbon (δ 61.7). gHMBC correlations from the epoxide proton ($\delta_{H\text{-}2'}$ = 2.95; $\delta_{C\text{-}2'}$ = 63.4) to C-4 of the pyrrole ring clearly established the presence of the epoxide at C-2' –C-3'. In total, these components accounted for 6 of the 7 unsaturations present in **97**.

The remaining degree of unsaturation in **97** was assigned to the presence of a nitro group. The nitro functionality was suggested based upon the molecular formula, which required an additional nitrogen atom and two oxygen atoms to be assigned. In addition, mass spectral fragmentation showed a clear loss of 46 amu (M^{+}-NO_2, m/z = 309.2030).

After the three pieces of the molecule (nitro group, pyrrole ring and terpene chain) were established, how they connected had to be determined. The substitution pattern for the pyrrole ring was determined using the coupling constants between the two aromatic protons. The proton coupling constants in a pyrrole ring are very characteristic and can be used to determine the position of substitutes in the ring. (**Figure III.1**)[8] The value of 1.3 Hz between δ 6.86 and δ 7.03 peaks match the literature value for coupling constant

proton in 2, 5 positions. The chemical shift of the protons in the pyrrole ring was used to decide if the nitro group was in a α or β position. A nitro group in the β position would have a deshield effect in the proton at position 5, with a chemical shift of δ 7.67 for this proton.[9] In addition to the NMR data, **97** showed the characteristic UV absorption of an α-nitropyrrole (344 nm), substantiating the presence of the nitro group but also supporting the C-2 position on the pyrrole ring (3-nitropyrrole shows λ_{max} = 315 nm).[10] Strong absorptions for **97** in the IR spectrum at 1509 and 1370 cm^{-1}, associated with asymmetrical and symmetrical stretching of the nitrogen oxygen bond, are virtually identical to the literature values of 1510 and 1350 cm^{-1} observed for synthetic 2-nitro pyrrole.[9] These data allowed the structural fragments in **97** to be then confidently assembled by analysis of 2D NMR spectroscopic data.

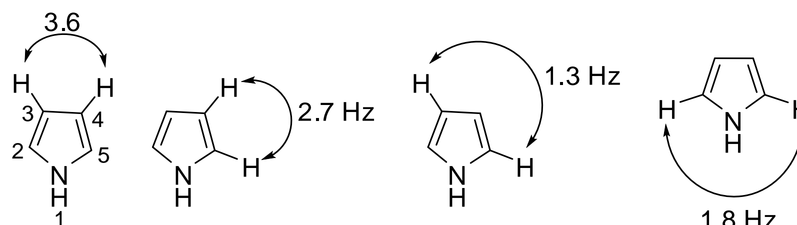


Figure III.1 – Literature values for coupling constants in pyrrole rings[8]

The relative stereochemistry of the epoxide ring was determined by interpretation of 1D NOE NMR data. Irradiation of H-2’ (δ 2.95) and H-1’ (δ 2.71) showed enhancement of H-4’ (δ 1.49), while irradiation of H-1’ enhanced the C-15’ methyl protons (δ 1.37). There was no NOE enhancement observed between the H-2’ and the C-15’ methyl protons, indicating a *trans* configuration of the epoxide ring (**Figure III.2**)

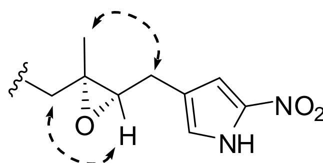


Figure III.2 - Proton NMR NOE enhancements for select protons in nitropyrrolin A (**97**), which established the relative configuration of the C-2' – C-3' trisubstituted epoxide.

Despite the fact that more than 6000 secondary metabolites containing epoxides have been reported in the literature, the determination of their absolute configuration is rare.[11] The most common approach is to obtain the absolute configuration of a chiral center close to the epoxide, and to then relate the configuration of the epoxide to this center. Due to the remoteness of the epoxide in **97** as part of a linear terpene chain, the absolute configuration had to be addressed in an independent manner. The problem is even more complex due to the quaternary carbon at C3', the steric bulk of which hinders epoxide derivatization. As a result, the absolute configuration of the epoxide was determined by epoxide conversion to the β-amino alcohol followed by modified Mosher amide analysis.[12,13] This route was selected because it leads cleanly to an S_{N2} product with retention of configuration at C-3'. Direct ammonolysis with 9N NH_4OH was carried out in a microwave oven (125° C for 3 min) to yield the β-amino alcohol (**100**) as the sole product (60% yield) in a recycled reaction. The β-amino alcohol was then converted to the two Mosher amides with *R*- and *S*-MTPA-Cl, and each was separately analyzed by ^{1}H NMR methods. Calculation of $\Delta\delta_{S-R}$ values indicated the absolute configuration of **97** is *S* at C-2', as shown in **Figure III.3.**

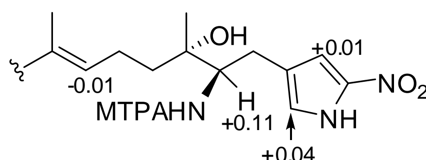


Figure III.3 - Absolute configuration of the amino alcohol **100** derived from **97**. Delta-delta values ($\Delta\delta_{S-R}$) in ppm as measured from the *S*- and *R*- NHMTPA amides (**103**, **104**) of nitropyrrolin A (**97**) in $CDCl_3$**.**

Nitropyrrolin B (**98**) was isolated as a viscous oil that analyzed by mass spectrometry for the molecular formula $C_{19}H_{30}N_2O_4$ [M+H$^+$ *m/z* = 351.2397 (calculated for $C_{19}H_{30}N_2O_4$+H$^+$ *m/z* = 351.2284)]. This formula indicated that **98** differed from **97** by the addition of water. The major difference in the ^{1}H NMR spectrum of **98** was the downfield shift of H-2' from δ 2.95 in **97** to δ 3.59 (**Table III.2**). The HMQC spectrum showed that H-2' was attached to a methine carbon at δ 78.6, demonstrating that the epoxide had been opened to yield a secondary alcohol at this position. Consistently, H-2' showed correlations to the quaternary carbon C-3' in the HMBC spectrum. Likewise, C-3' was assigned as a tertiary alcohol on the basis of its lowfield shift (δ 74.9). Subsequent comprehensive analysis of all NMR data allowed all protons and carbons to be fully assigned resulting in the assignment of the planar structure of **98**.

To assess the absolute configuration at C-2', *S*- and *R*-MTPA ester derivatives of **98** were prepared by standard methods. Interpretation of NMR data clearly showed that the configuration at C-2' is *S* in nitropyrrolin B (**98**) (**Figure III.4**).

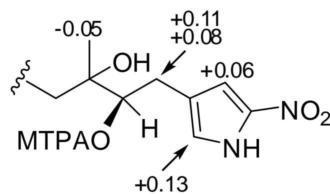


Figure III.4. Absolute configuration of C-2' in nitropyrrolin B (**98**) as defined by the Mosher ester NMR method. Shown are the delta-delta values ($\Delta\delta_{S\text{-}R}$, in ppm) for the *S*- and *R*-MTPA (**101**, **102**) esters measured from NMR data recorded in $CDCl_3$

Nitropyrrolin C (**99**), the most oxygenated in this series of compounds, analyzed for the molecular formula $C_{19}H_{30}N_2O_5$ by HR-ESI-MS ($[C_{19}H_{30}N_2O_5+H]^+$ *m/z* = 367.2274 (calc. 367.2233). This molecular formula showed the presence of three oxygen atoms in the farnesyl side chain. Analysis of NMR spectral data for **99** (**Table III.3**), which includes extensive analysis of HMBC data, clearly showed the presence of a trisubstituted epoxide at the chain terminus and the same diol constellation at C-2' – C-3' as observed in nitropyrrolin A. Comprehensive analysis of NMR data in comparison with **97** and **98** allowed this structure to be confidently assigned.

Although prevalent in nature as tetrapyrrole pigments such as chlorophyll and the heme group of proteins,[14] pyrroles are relatively uncommon as secondary metabolites. This scarcity is balanced by an astonishing chemical diversity, ranging from simple halogenated pyrrole rings to complex pyrrole-containing alkaloids. Excluding the simplest cases, pyrroles are usually part of complex structures displaying mixed biosynthetic origins. The common motif for these molecules is the combination of pyrrole rings within peptides or polyketides. Pyrrole metabolites of mixed biosynthetic origin represent more than 90% of all the compounds in this class reported in the literature. While rare, metabolites possessing a farnesyl chain attached to the pyrrole

ring are known. Only two other groups of molecules, illustrated by pyrrolostatin (**93**)[15] and the marine microbe-derived glaciapyrrole A (**94**),[16] are reported in the literature with these features. Both pyrrolostatin (**93**) and glaciapyrrole A (**94**) appear to be derived from a pyrrole α -carboxyl moiety[7] but in nitropyrrolins a nitro group is present as the α position electron rich group. Pyrrolostatin biosynthesis was proposed as an electrophilic aromatic substitution with the molecule still attached to a carrier protein. Further biosynthetic studies need to be done to elucidate if the biosynthesis of nitropyrrolins follow the same mechanism or if same unusual coupling is occurring. Nitropyrroles are even less known, with a small group of nitropyrroles such as pyrrolomycin B (**105**),[17] a highly chlorinated antibiotic, as one of the few natural nitropyrroles isolated (**Figure III.5**).

Interestingly, pyrrole containing secondary metabolites often have strong biological activity, mainly as antimicrobials. Pyrrolnitrin[18] is still in use as antifungal agent, while the bacterial pigment prodigiosin has been extensively studied because of its as antifungal, antibacterial, antimalarial, immunosuppressive and anticancer properties.[19]

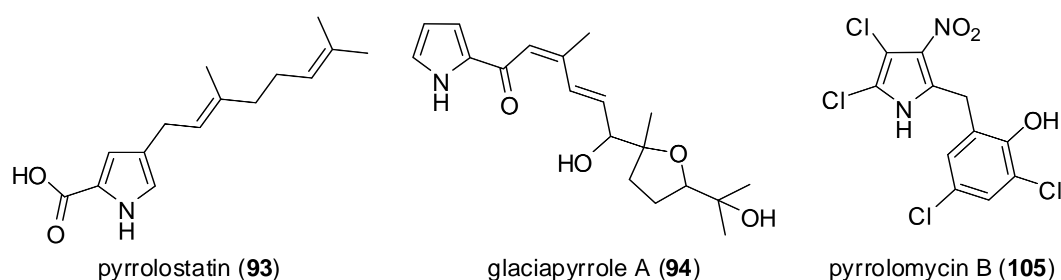


Figure III.5. Examples of naturally-occurring mono- and sesquiterpene-substituted pyrroles and a nitropyrrole.

Other examples of pyrrole containing secondary metabolites, streptopyrrole (XR587), verrucarin E and violacein were reported as antibacterial and antifungal agents.[20-22] In our hands, the nitropyrrolins failed to show meaningful cancer cell cytotoxicity towards the HCT-116 cell line or antibacterial activity towards methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant *Enterococcus faecium.*

III. 3. Experimental section

III.3.1. General experimental procedures

Optical rotations were measured on a Rudolph Research Autopol III polarimeter with a 10-cm cell. IR spectra were recorded using a Perkin-Elmer 1600 FT-IR spectrometer. ^{1}H, ^{13}C, and 2D NMR spectral data were recorded on Varian Inova 500 MHz and Varian Inova 300 MHz NMR spectrometers. High resolution mass spectral data were acquired on Agilent ESI-TOF mass spectrometer at The Scripps Research Institute, La Jolla. LC/MS data were obtained using a Hewlett-Packard series 1100 LC/MS system with a reversed-phase C18 column (Phenomenex Luna C-18(2), 4.6 mm × 100 mm, 5 μm) with CH_3CN/H_2O gradient solvent system. Ammonolysis was performed in a CEM discovery microwave reactor.

III.3.2. The marine actinomycete (CNQ 509)

Strain CNQ 509 is an actinomycete isolated from a marine sediment sample collected in 2001 at a 44 m depth west of the Scripps Institution of Oceanography in La

Jolla, California. The bacterium was isolated on medium TCG (3 g triptone, 5 g casitone, 4 g glucose, 2 g krill, 18 g agar, 1 L of seawater) and cryopreserved at -80°C prior to study. Analysis of the 16S rRNA gene sequence (1451 nucleotides) indicated that this strain belongs to the genus *Streptomyces* and that it groups with other identified marine derived strains previously characterized as the MAR 4 clade. The 16S sequence has been deposited under accession number EF581384. Strain CNQ 509 was cultured by shaking at 230 rpm in a total volume of 40 x 1 L in medium A1 (10 g of starch, 4 g of yeast extract, 2 g of bacto-peptone, and 1 L of seawater) at 28°C for 7 days, and then extracted by stirring for 2 h with Amberlite XAD-7 resin (20 - 30 g/L). The resin was collected by filtration, washed with de-ionized water, and extracted twice with 2 L of acetone, and the solvent removed under vacuum to generate the crude extract.

III.3.3. Compound isolation

The whole culture extract was subjected to a partition between H_2O and hexanes, chloroform, EtOAc, to generate four fractions. The hexane soluble component (1.732 g from 40 L) was subjected to silica normal-phase flash chromatography generating five fractions using a gradient of hexane and EtOAc (hexanes, 25% EtOAc, 50%EtOAc, 25% EtOAc, 100% EtOAc) (Fr. A: 350.1 mg, B: 364.7 mg, C: 235.8 mg, D: 321.7 mg, and E: 128.1 mg). Further purification of fraction A by normal phase HPLC, gradient (100% hexanes-50% EtOAc), gave nine fractions, A1 to A9. Final purification of A3 by Si gel HPLC (10%EtOAc) gave nitropyrrolin A (**97**) (14.3 mg) in pure form. Subsequent purification of fraction C by Si gel HPLC (gradient elution 10% hexanes-50% EtOAc

with ELSD detection) followed by an isocratic separation with 20% EtOAc in hexanes afforded nitropyrrolin B (**98**) (4.5 mg). Finally, nitropyrrolin C (**99**) (2.3 mg) was isolated from fraction D by isocratic Si gel HPLC using 50% EtOAc in hexanes.

Nitropyrrolin A (97), a light yellow oil; showed $[\alpha]^{D}_{20}$ +122 (*c* 0.05, CH_2Cl_2); IR ν_{max} (NaCl): 3231, 3117, 2978, 2917, 2847, 1509, 1455, 1395, 1377, 1303, 1272; ^{1}H and ^{13}C NMR: see Table 1; LRESI-MS (positive mode): $[M-H]^+$ m/z = 331; LRESI-MS (negative mode): $[M+^{35}Cl]^-$ m/z = 333; HR-ESI-TOF-MS: $[M+Na]^+$ m/z = 355.1979 (calcd for $[C_{19}H_{28}N_2O_3+Na]^+$ 355.1997).

Nitropyrrolin B (98), a light yellow oil; showed $[\alpha]^{D}_{20}$ -80 (*c* 0.05, CH_2Cl_2); IR ν_{max} (NaCl): 3403, 3127, 2968-2856, 1504, 1452, 1385, 1367, 1291, 1272 cm^{-1}; ^{1}H and ^{13}C NMR see Table 2; API-ES MS (negative mode): $[M-H]^-$ m/z = 349; (positive mode): $[M+Na]^+$ m/z = 372; HR-ESI-TOF-MS: $[M+H]^+$ m/z = 351.2387 (calcd for $[C_{19}H_{30}N_2O_4+H]^+$ 351.2284).

Nitropyrrolin C (99), a yellow oil; showed $[\alpha]^{D}_{20}$ +108 (*c* 0.0069, CH_2Cl_2); IR ν_{max} (NaCl): 3340, 3145, 2983-2921, 1780, 1736,1719, 1704, 1698 cm^{-1}; ^{1}H NMR, ^{13}C NMR, ^{1}H-^{1}H COSY, and HMBC data, see Table 3; HR-MALDI-MS $[M+H]^+$ m/z = 367.2274 (calculated for $[C_{19}H_{30}N_2O_5+H]^+$ 367.2233)

Ammonolysis of 97

To 2.0 mg of the nitropyrrolin A (**97**) was added 5.0 mL of NH_4OH. The mixture was heated in a microwave oven (CEM discovery) for 3 min at 125°C. After removal of solvents under reduced pressure, the residue was purified by reversed phase HPLC (Beckman System gold 126, Phenomenex ultracarb, 10.0 mm x 250 mm, 5 µm, 2.0 mL/min, UV detection at 350nm) isocratic (70% CH_3CN - 30% 0.02mM NH_4Ac) The

amino alcohol (**100**) eluted at 15 min. Compound (**100**) showed LRMS peaks $[M+H]^+$ *m/z* = 350, and $[M+Na]^+$; 372, corresponding to the molecular formula $C_{19}H_{31}N_3O_3$. ^1H NMR (CD_3CN, 500MHz) δ 7.00(1H), 6.93 (1H), 5.18 (2H), 5.11 (1H), 4.18 (1H), 2.80 (1H), 2.19 (2H), 2.06 (H), 1.80, 1.66 (3H), 1.62 (3H), 1.58 (3H), 1.47 , 1.40, 1.27 (3H);

Mosher derivatization of 100

To 1.0 mg (2.8 μmol) of amino alcohol (**100**) was added 500 μL of freshly distilled pyridine. A small crystal of dimethylaminopyridine was added to the solution and the mixture was stirred at room temperature for 1h. Twenty μL of (*R*)-methoxy-(trifluoromethyl) phenylacetyl chloride solution (5.36 μmol/mL) was added and the mixture was stirred at room temperature overnight. After drying the solution under vacuum, the residue was purified with reversed-phase HPLC (Beckman System gold 126, phenomenex ultracarb, 10.0 mm x 250 mm, 5 μm, 2.0 mL/min, UV detection at 350nm) using a gradient solvent system (0-10 min: 20% aqueous CH_3CN, 10-50 min: 20-100% aqueous CH_3CN, 50-60 min: 100% CH_3CN). The *S*-MTPA ester (**103**) was eluted at 49.3 min. The identical procedure was carried out to obtain the *R*-MTPA amide (**104**) with (*S*)-methoxy-(trifluoromethyl) phenylacetyl chloride. The unit masses of the (*R*) and (*S*)-MTPA amide ($[M+H]^+$; 566, $[M+Na]^+$; 588, molecular formula; $C_{29}H_{37}F_3N_3O_4$) (*S*)-NMTPA (**103**): ^1H NMR (CD_3CN, 500 MHz) δ 7.67, 7.54, 7.34, 7.02, 6.96, 5.10, 3.57, 2.94, 1.83, 1.69, 1.61, 1.24 (*R*)-NMTPA (**104**): ^1H NMR (CD_3CN, 500 MHz) δ 7.68, 7.56, 7.46, 7.01, 6.92, 5.12, 3.46, 2.99, 2.69, 2.82, 1.84, 1.69, 1.62, 1.46, 1.30.

Mosher derivatization of 98

To 1.0 mg (2.9 μmol) of nitropyrrolin B (**98**) was added 500 μL of freshly distilled pyridine. A small crystal of dimethylaminopyridine was added to the solution

and the mixture was stirred at room temperature for 1h. Twenty µL of (*R*)-methoxy-(trifluoromethyl) phenylacetyl chloride solution (5.36 µmol/mL) was added and the mixture was stirred at room temperature overnight. After drying the solution under vacuum, the residue was purified with reversed-phase HPLC (Beckman System gold 126, phenomenex ultracarb, 10.0 mm x 250 mm, 5 µm, 2.0 mL/min, UV detection at 350nm) using a gradient solvent system (0-10 min: 20% aqueous CH_3CN, 10-50 min: 20-100% aqueous CH_3CN, 50-60 min: 100% CH_3CN). The *S*-MTPA ester (**101**) was eluted at 52.5 min. The identical procedure was carried out to obtain the *R*-MTPA ester (**102**) with (*S*)-methoxy-(trifluoromethyl) phenylacetyl chloride. The unit masses of the (*R*) and (*S*)-MTPA esters ($[M+H]^+$; 567, $[M+Na]^+$; 589, molecular formula; $C_{29}H_{36}F_3N_2O_5$) were identified by LC/MS analysis (API-ES MS). (*S*)-OMTPA (**101**): ^{1}H NMR (CD_3CN, 500 MHz) δ 9.26, 7.58, 7.44, 7.30, 6.96, 6.73, 5.23, 3.42, 2.96, 2.72, 1.99, 1,66, 1.63, 1.61, 1.21; (*R*)-OMTPA (**102**): ^{1}H NMR (CD_3CN, 500 MHz) δ 9.13, 7.54, 7.38, 7.30, 6.90, 6.59, 5.18, 3.47, 2.99, 2.85, 2.67, 1.66, 1.63, 1.61, 1.26.

Table III.1 - NMR spectroscopic data for nitropyrrolin A (**97**) in CDCl3.

position	δ_H[a]	mult (*J* in Hz)	δ_C[b], #H	COSY	HMBC
1	9.82	bs			
2			137.7, qC		
3	7.02	d (1.3)	111.1, CH	4, 1'	2, 4, 5, 1'
4			123.1, qC		
5	6.86	d (1.3)	122.0, CH	2, 1'	2, 3, 4
1’	2.71	m	26.9, CH_2	2, 4, 2'	3, 4, 5, 2'
2’	2.95	m	63.4, CH	1'	4, 1', 3', 4', 15'
3’			61.7, qC		
4’	(a) 1.49	m	38.8, CH_2	4'(b), 5'	1', 2', 5', 6'
	(b) 1.71	m		4'(b)	1', 2', 5', 6'
5’	2.10	m	23.9, CH_2	4'(a), 6'	3', 4', 6', 7'
6’	5.07	m	123.5, CH	5', 14'	4', 5', 8', 14'
7’			136.0, qC		
8’	2.05	m	26.8, CH_2		7', 9', 10', 11'
9’	1.95	m	39.9, CH_2	10'	7', 8', 10', 11', 14'
10’	5.07	m	124.4, CH	9', 13'	8', 9', 11', 13'
11’			131.7, qC		
12’	1.67	bs	25.9, CH_3		10', 11', 13'
13’	1.59	bs	17.9, CH_3	10'	
14’	1.59	bs	16.2, CH_3		
15’	1.37	s	16.9, CH_3		2’, 3’, 4’

Assignment by gHSQC and DEPT NMR methods. [a]500 MHz, [b]125 MHz

Table III.2 - NMR spectroscopic data for nitropyrrolin B (**98**) in $CDCl_3$.

position	δ_H[a]	mult (*J* in Hz)	δ_C[b], #H	COSY	HMBC
1	9.58	bs		3, 5	
2			137.8, qC		
3	7.05	d (2.2)	111.5, CH	1, 5	2, 4, 5, 1'
4			124.6, qC		
5	6.89	d (2.2)	122.4, CH	1, 3	2, 3, 4
1’	(a) 2.73	dd (14.6, 2.2)	29.1, CH_2	1'(b), 2'	3, 4, 5, 2'
	(b) 2.53	dd (14.6, 10.7)			3, 4, 5, 2', 3'
2’	3.59	dd (10.7, 2.2)	78.6, CH	1(a)', 1(b)'	4, 1', 3', 4', 15'
3’			74.9, qC		
4’	(a) 1.49	m	36.5, CH_2	4'(b), 5(a)', 5(b)'	1', 2', 5', 6'
	(b) 1.73	m		4'(a),	1', 2', 5', 6'
5’	(a) 2.12	m	22.2, CH_2	4'(a), 6'	3', 4', 6', 7'
	(b) 2.19	m		4'(a), 6’	
6’	5.18	m	124.3, CH	5(b)', 14'	4', 5', 8', 14'
7’			136.4, qC		
8’	2.01	m	39.9, CH_2	9'	7', 9', 10', 11'
9’	2.08	m	26.9, CH_2	8'	7', 8', 10', 11', 14'
10’	5.09	m	124.4, CH	9', 12', 13'	8', 9', 11', 13'
11’			131.8, qC		
12’	1.69	d (1.6)	25.9, CH_3	10'	10', 11', 13'
13’	1.61	d (1.1)	17.9, CH_3	10'	
14’	1.65	bd (1.6)	16.2, CH_3	6'	
15’	1.27	s	23.7, CH_3		2’, 3’, 4’

Assignment by gHSQC and DEPT NMR methods. [a]500 MHz, [b]125 MHz

Table III.3 - NMR spectroscopic data for nitropyrrolin C (**99**) in CDCl3.

position	δ_H[a]	mult (*J* in Hz)	δ_C[b], #H	COSY	HMBC
1	9.56	bs			
2			137.5, qC		
3	7.03	d (1.3)	111.4, CH	1, 4, 1'	2, 4, 5, 1'
4			124.4, qC		
5	6.87	d (1.3)	122.4, CH	1, 2, 1'	2, 3, 4
1’	2.71	m	28.9, CH_2	2, 4, 2'	3, 4, 5, 2'
	2.49	m		4'a	3, 4, 5, 2'
2’	3.57	m	78.5, CH	1'	
3’			74.7, qC		
4’	(a) 1.46	m	36.4, CH_2	4'(b), 5'	5', 15'
	(b) 1.69	m		4'(a)	5', 15'
5’	2.13	m	22.2, CH_2	4'(a), 6'	3', 4', 6', 7'
6’	5.23	m	124.9, CH	5', 14'	4', 5', 8', 14'
7’			135.0, qC		
8’	2.04	m	36.5, CH_2		7', 9', 10', 11'
9’	1.62	m	27.5, CH_2	10'	7', 8', 10', 11', 14'
10’	2.69	m	64.5, CH	9'	8', 9', 11', 13'
11’			58.7, qC		
12’	1.28	bs	25.0, CH_3		10', 11', 13'
13’	1.25	bs	18.8, CH_3		
14’	1.58	s	16.2, CH_3		6’, 7’
15’	1.24	s	23.7, CH_3		2’, 3’, 4’

Assignment by gHSQC and DEPT NMR methods. [a]500 MHz, [b]125 MHz

III. 4. References

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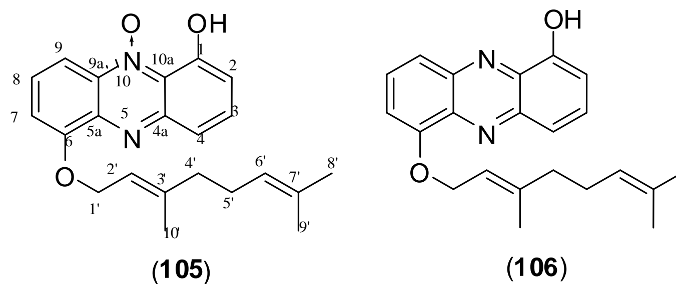
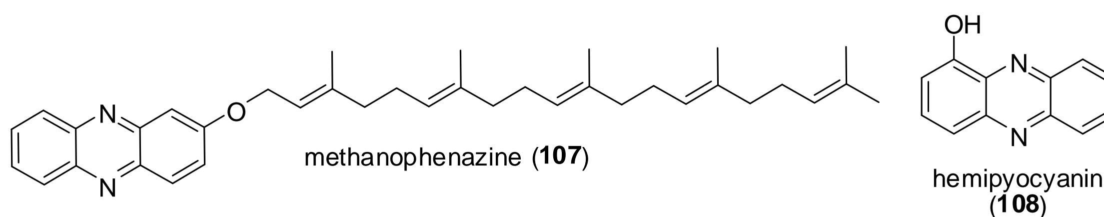
IV. Marinophenazines A and B, from the marine derived actinomycete CNQ 509

IV. 1. Introduction

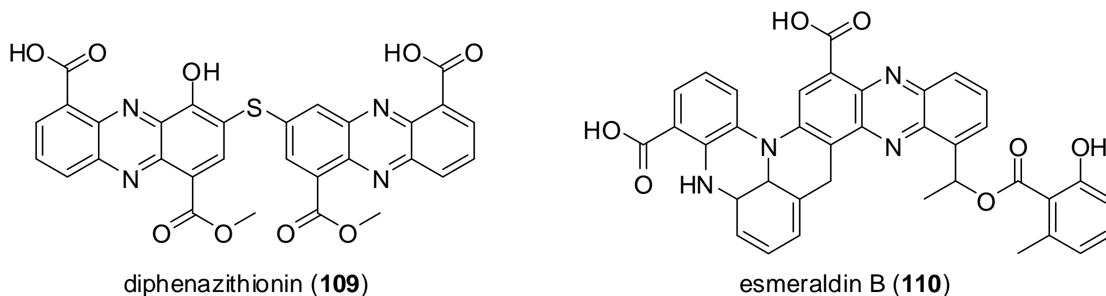
A new actinomycete clade, homogeneous in their unique ability to produce meroterpenoids, was isolated by Fenical and Jensen from marine sediments.[1] With 19 strains isolated off the coast of San Diego, Baja California, Palau and Fiji, this clade is phylogenetically placed within the genetically-diverse genus *Streptomyces*. The strain CNQ509, isolated from a sediment sample collected at a depth of 42.4 m near La Jolla in 2001 produced two unique geranyl-phenazines, marinophenazine A (**105**) and B (**106**). To the best of my knowledge, these compounds are the first examples of O-prenylated phenazines described from actinomycetes. The only other example of a phenazine with O-prenylation in the literature is methanophenazine (**107**), a membrane bound natural product produced by an anaerobic archaea (*Methanosarcina mazei Go1*).[2]



Phenazines have a long history in natural products chemistry. These heterocyclic ring systems were first observed in the 1860s as a blue pus in *Pseudomonas*-infected patients. Later, in the late 1890s, the first natural phenazine was isolated from extracts of a cultured *Pseudomonas* sp.[3] Today, more than 50 natural phenazine derivatives are reported in the literature[4] many possessing antimicrobial, antiparasitic, antimalarial and anticancer biological activities.[5-10]



Phenazines are only produced by prokaryotes, with *Pseudomonas* spp. and *Streptomycetes* spp. as the major producers. Both genera produce simple phenazines such as hemipyocyanin (**108**). However, only *Streptomyces* species produce complex phenazines like diphenazithionin (**109**) and esmeraldin B (**110**).



diphenazithionin (**109**) esmeraldin B (**110**)

For a long time, the ecological function of phenazine was unclear, but recently, a series of papers broadened the understanding of these molecules in producer organisms. Phenazines have multiple functions in the microorganism and, when present result in increased fitness of the producer.[11] The antibiotic activity of phenazines has been correlated to the ability of *Pseudomonas fluorescens* to colonize roots and to protect plants from fungal infections.[12] Phenazines act as electron shuttles in mineral reduction and scavenging, increasing iron uptake of the bacteria.[13] The oxidant effect of pyocyanin, a phenazine derivative produced by *Pseudomonas aeruginosa* is responsible for an increased pathogenicity in lung infections in patients with cystic fibrosis.[14] One remarkable discovery about phenazines was their role in quorum sensing in *Pseudomonas*.[15] In *Methanosarcina* archea, phenazines used as the electron transporter in anaerobic respiration.[2]

Phenazines are shikimic acid pathway-derived molecules formed when two molecules of chorismic acid are fused together in a diagonally-symmetrical fashion forming their azatricyclic structure. Biotransformations of this core ring system lead to all the known phenazines.

IV. 2. Results and discussion

Marinophenazine A (**105**) was isolated as part of a project to understand the secondary metabolites production capability of the strain CNQ 509. Marinophenazine A (**105**), a bright orange powder, showed a sharp and strong UV absorption at 280 nm, characteristic of this class. (**Figure IV.1.1**) Phenazines have very characteristic UV absorptions with one intense, sharp peak between 250 and 290 nm and a second weak absorption between 350 and 400. These metabolites range in color depending upon the pattern of substitution on the phenazine core.[16]

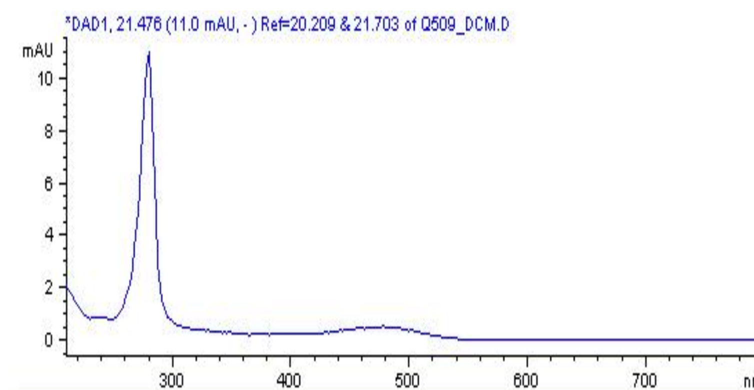


Figure IV.1.1 - UV absorption of marinophenazine A (**105**)

The low resolution ESI mass spectrum of marinophenazine A (**105**) showed an intense $[M+Na]^+$ ion at *m/z* 387.1 (positive mode). High resolution EI-MS mass spectral data showed a base ion at *m/z* 364.1774 in the positive mode, allowing the

molecular formula to be assigned as $C_{22}H_{24}N_2O_3$ (calculated as 364.1781), a molecular formular with thirteen double bond equivalents.

The ^{1}H-NMR spectrum for marinophenazine A (**105**) (**Table IV.1**) exhibited the presence of typical phenazine aromatic signals and a complex set of resonances attributed to a geranyl chain. The downfield signals, which integrated for six protons in two separated spin systems, suggested a mono substituted phenazine ring. (**Figure IV.1.2a**) Further, interpretation of their *J* values indicated a phenazine with 1,-6 di-substitution. All twelve carbons expected for the phenazine ring were represented in the ^{13}C NMR and the gHMBC spectrum confirming the 1,6 substitution pattern. (**Figure IV.1.2b**) The remaining oxygen was assigned as a N-oxide, a common group in phenazines. The UV profile, with the weak absorption at 480 nm and the fact that no other carbon showed a chemical shift compatible with an oxygen substitute confirmed this assertion. The hydroxyl group at δ 13.7 ppm was assigned as in position 1 due to its strong intramolecular hydrogen bond with the N-O. A literature search also showed that the ^{1}H and ^{13}C NMR data for **105** matched the literature values for phenazines.[17,18]

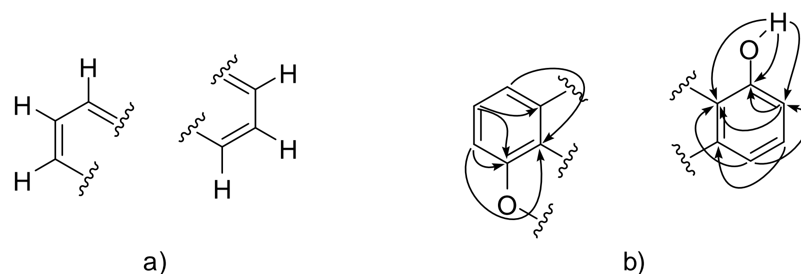


Figure IV.1.2 - a) spin systems in the phenazine core observed in the gCOSY spectrum of marinophenazine A (**105**). b) Important gHMBC correlations in the phenazine core of **105**.

The remaining ten carbons could be assembled to form a linear monoterpene unit. The olefinic region contained two signals (two protons δ 5.64 and 5.10), indicating that the geranyl chain was not oxidized. The gCOSY spectrum again showed two spin systems, one for each C_5 isoprene unit present in the chain. (**Figure IV.1.3a**). Two dimensional gHMBC correlations indicated that all methyls groups (δ 1.60, 1,65 and 1.82) were attached to olefinic carbons, thus confirming this assertion. The interpretation of gHMBC correlations also made it possible to establish the connectivity between the phenazine core and the geranyl unit. (**Figure IV.1.3b**)

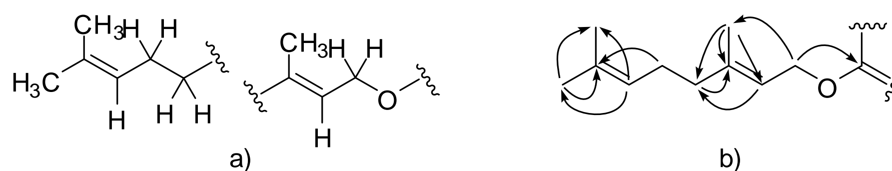


Figure IV.1.3 - a) spin systems in the geranyl chain observed in the gCOSY spectrum of marinophenazine A (**105**). b) Important gHMBC correlations in the geranyl chain of **105**

Marinophenazine B (**106**) NMR data resembled those of **105** (**Table IV.2**). The high resolution of *m/z* 348.1798 (calculated for $C_{22}H_{24}N_2O_2$), showed the difference between them being only an oxygen. The ^{1}H NMR spectrum of **106** was almost identical to that of **105** except for the change in the hydroxyl group, from a δ 13.7 ppm to 8.16 ppm, showing the difference between the chemical shift of a hydroxyl in a hydrogen bond and a free hydroxyl. The ^{13}C NMR spectrum of **106** also showed the same type of shift with carbon 1 in **105** being slightly more shielded than the same carbon in **106**.

IV. 3. Experimental section

IV.3.1. General experimental procedures

IR spectra were recorded using a Perkin-Elmer 1600 FT-IR spectrometer. ^{1}H, ^{13}C, and 2D NMR spectral data were recorded on Varian Inova 500 MHz and Varian Inova 300 MHz NMR spectrometers. High resolution mass spectral data were acquired on

Agilent ESI-TOF mass spectrometer at The Scripps Research Institute, La Jolla. LC/MS data were obtained using a Hewlett-Packard series 1100 LC/MS system with a reversed-phase C18 column (Phenomenex Luna C-18(2), 4.6 mm × 100 mm, 5 μm) with CH_3CN/H_2O gradient solvent system.

IV.3.2. The marine actinomycete (CNQ-509)

The strain isolation and cultivation was described in section III.3.2

IV.3.3. Compound isolation

The whole culture extract was subjected to a partition between H_2O and hexane, chloroform, EtOAc, to generate four fractions. The hexane soluble component (1.732g from 40L) was subjected to silica normal-phase flash chromatography generating five fractions using a gradient of hexanes and EtOAc (hexanes, 25% EtOAc, 50% EtOAc, 25% EtOAc, 100% EtOAc) (Fr. A: 350.1 mg, B: 364.7 mg, C: 235.8 mg, D: 321.7 mg, and E: 128.1mg). Further purification of fraction A by normal phase HPLC, using a gradient from 100% hexane to 50% EtOAc, gave nine fractions, A1 to A9. Final purification of A2 by Si gel HPLC (10%EtOAc) gave marinophenazine A (**105**) (9.2 mg) in pure form. Subsequent purification of fraction A4 by Si gel HPLC (gradient elution 10% hexane-50%EtOAc with ELSD detection) afforded marinophenazine B (**106**) (2.1mg).

Marinophenazine A (105) was obtained as a bright orange powder; IR ν_{max} in NaCl: 2926, 2855, 1720, 1718, 1464, 1379, 1254, 1088, 1029; ^{1}H NMR, ^{13}C NMR, ^{1}H-^{1}H COSY, and HMBC, see Table VI.1; HR-ESI-TOF-MS *m/z* 364.1774 (calculated for $[C_{22}H_{24}N_2O_3]^+$ 364.1781).

Marinophenazine B (106) was obtained as a bright yellow powder; IR ν_{max} in NaCl: 3406, 33074, 2969, 2917, 2855, 1633, 1530, 1485, 1377, 1303, 1272; ^{1}H NMR, ^{13}C NMR, ^{1}H-^{1}H COSY, and HMBC, see Table IV.2; HR-ESI-TOF-MS *m/z* 348.1798 (calculated for $[C_{22}H_{24}N_2O_2]^+$ 348.1838).

Table IV.1 - NMR spectroscopic data for marinophenazine A (**105**) in $CDCl_3$.

position	$\delta_C{}^b$	mult (*J* in Hz)	$\delta_H{}^a$	COSY	HMBC
1	155.1				
2	109.7	bd(7.8, 1)	7.07	3, 4	1, 4, 10a
3	131.5	dd(7.9, 7.8)	7.66	2, 4	1, 4a
4	109.5	bd(7.9, 1)	8.15	2, 3	2, 10a
4a	133.7				
5a	123.7				
6	152.3				
7	112.1	dd(7.8, 1)	7.08	8, 9	5a, 6, 9
8	132.3	dd(7.8, 7.4)	7.68	7, 9	6, 9a
9	120.5	dd(7.4, 1)	7.84	7, 8	5a, 7
9a	145.4				
10a	139.4				
1’	67.1	d(6.3)	4.96	12, 20	6, 2’, 10’
2’	119.1	t(6.3)	5.64	11, 20	1’, 4’, 10’
3’	141.7				
4’	39.7		2.13	20	2’, 3’, 5’
5’	26.4		2.15	16, 18, 19	4’, 7’
6’	123.9	m	5.10	15, 18, 19	4’, 5’, 8’, 9’
7’	132.2				
8’	25.9		1.65	15, 16	6’, 7’, 9’
9’	17.9		1.60	15, 16	6’, 7’, 8’
10’	17.1		1.82	12, 14	2’, 3’, 4’
-OH			13.70		

Assignment by gHSQC and DEPT NMR methods. [a]500 MHz, [b]125 MHz

Table IV.2 - NMR spectroscopic data for marinophenazine B (**106**) in $CDCl_3$.

position	δ_C[b]	mult (*J* in Hz)	δ_H[a]	COSY	HMBC
1	151.6				
2	108.8	dd	7.05	3, 4	1, 4, 10a
3	131.4	dd	7.66	2, 4	1, 4a
4	109.5	dd	7.97	2, 3	2, 10a
4a	133.7				
5a	141.0				
6	154.7				
7	112.1	dd	7.08	8, 9	5a, 6, 9
8	131.2	dd	7.68	7, 9	6, 9a
9	121.2	dd	7.94	7, 8	5a, 7
9a	145.4				
10a	139.4				
1’	66.8	d	4.99	12, 20	6, 2’, 10
2’	119.7	m	5.66	11, 20	4’, 10’
3’	140.9				
4’	40.1		2.12	20	2’, 3’, 5’
5’	26.6		2.20	16, 18, 19	4’, 7’
6’	123.9	m	5.10	15, 18, 19	8’, 9’
7’	132.0				
8’	26.1		1.67	15, 16	6’, 7’, 9’
9’	17.9		1.61	15, 16	6’, 7’, 8’
10’	17.3		1.83	12, 14	2’, 3’
-OH			8.16		

Assignment by gHSQC and DEPT NMR methods. [a]500 MHz, [b]125 MHz

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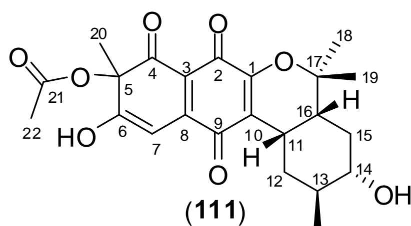
V. Streptoquinone, a new napthoquinone meroterpenoid from the marine derived actinomycete CNQ 509

V. 1. Introduction

The Fenical and Jensen laboratory, with the use of innovative isolation and cultivation techniques, was able to obtain 19 Mar 4 strains from the coastal sediments near San Diego, Baja California, Palau and Fiji. One of these strains, CNQ 509, isolated from a sediment sample collected at a depth of 42.4 m near La Jolla in 2001, was found to produced meroterpenoids with a very different aromatic moiety. In addition to the nitropyrrolins A-C discussed in chapter III and the marinophenazines A and B discussed in chapter IV, this organism was found to produce a new napthoquinone derivative named streptoquinone with the structure shown as **111**.



Quinones are a large and important group of naturally occurring pigments.[1] They are conjugated cyclic di-ketones that are stabilized by resonance involving charged aromatic contributing structures. Quinones are widely distributed in nature with few known organisms not producing them.[2] Naphtoquinones are bicyclic aromatic quinones. The 1,4 naphthoquinones are the most common type of naphthoquinone present in nature, with 1,2, 1,5 and 1,6 rarely occurring.[1] Isoprenylated quinones such as ubiquinone, menaquinone and phylloquinone are important metabolites involved in the electron transport through a reversible oxidation-reduction reaction.[3]

Naphtoquinones are known for their potent activity in a variety of biological targets such as cytotoxic, antimicrobial, antifungal, antiviral, and antiparasitic assays.[4-9] Naphthoquinones have also being studied as UV-absorbing ingredients in sunscreens. Interestingly, small variations in the naphthoquinone structure have a measurable impact on their biological activity. QSAR studies for antiplatelet and anticancer activities have shown that naphtoquinones activity is largely dependent of their hydrophobicity.[10,11] In the anti-platelet assay, the molar refractivity, or volume occupied by the molecules also influenced their activity.

1,4 Naphthoquinones derivatives are the main class of natural products known to be produced by members of the MAR 4 clade. The MAR 4 is a new clade related to the genus *Streptomyces*.[12] These microorganisms isolated from marine sediment have a unusual ability among actinomycetes: to produce meroterpenoids. Even though actinomycetes are extremely prolific in natural product production, terpenoids are not common in this group of microorganism, with only a few hundred reported in the literature from more than 40,000 compounds known.[13]

V. 2. Results and discussion

Streptoquinone (**111**) was isolated as a bright red powder, which showed strong UV absorption at 250, 350 and 550 nm. (**Figure V.1**)

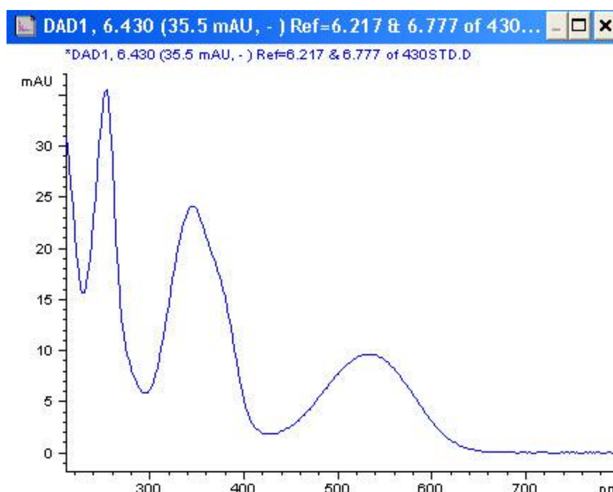


Figure V.1 – UV absorption spectrum of streptoquinone (**111**).

The low resolution ESI mass spectrum of streptoquinone (**111**) showed an intense $[M+Na]^+$ ion at *m/z* 453.1 (positive mode). The high resolution EI-MS mass spectrum of (**111**) showed a base ion at *m/z* 453.1550 (positive mode) corresponding to the molecular formula $C_{23}H_{26}O_8Na$ (calculated as 453.1525), a formula consisting of thirteen double bond equivalents.

The structure of streptoquinone (**111**) was assigned by a combination of spectral analyses mainly involving 2D NMR tecquiniques. The ^1H NMR spectrum for

streptoquinone (**111**) (**Table V.1**) exhibited the presence of a series of singlet resonances at δ 5.77 (one proton), 1.99 (three protons), 1.32 (three protons), 1.30 (three protons) and 1.19 (three protons). The ^{13}C NMR spectrum showed all of the 23 expected carbons. The ^{1}H-^{1}H gCOSY spectrum of **111** showed only one spin system composed of eleven protons in a tri-substituted cyclohexane ring. (**Figure V.2a**)

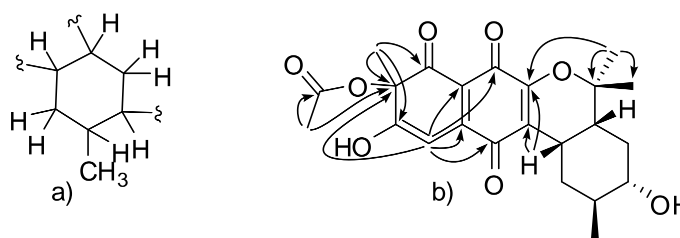


Figure V.2 - a) Spin system observed in the gCOSY spectrum of streptoquinone (**111**). b) Some of the key gHMBC correlations for **111**.

Usually, when dealing with a structure elucidation problem, the interpretation of the ^{1}H-^{1}H COSY spectrum is the most important step to propose the structure. In the case of streptoquinone (**111**), with most of the protons present in the molecule as singlets and with eleven quaternary carbons in the structure, interpretation of ^{1}H-^{13}C gHMBC spectral data became very important. The methine proton at δ 2.94 (H-11) correlated with the quaternary carbons at δ 160.2 (C-1) and δ 117.8 (C-10), tertiary carbon at δ 35.4 (C-16) and two secondary carbons at 27.5 (C-12) and δ 31.4 (C-15). The proton at 1.94 (H-16) correlated with carbons at δ 117.8 (C-10), δ 30.2 (C-11) and δ68.0 (C-14). Both

methyl protons at δ 1.32 (H-18) and δ 1.19 (H-19) showed gHMBC correlations with the quaternary carbons at δ 160.2 (C-1), δ 80.6 (C-17), the tertiary carbon at δ 35.4 (C-16) and with each other. The gHMBC correlations of H-11, H-16 H18 and H-19 established the dihydropyran ring and the connectivity between quinone and terpene unit. (**Figure V.2b**)

To obtain as much information as possible from the remaining proton signals, additional gHMBC spectra were acquired with the ^{1}H-^{13}C coupling constant set for 12 and 4 Hz, respectively. As one more measure to improve the spectroscopic data, the determination of T1 relaxation time for streptoquinone (**111**) protons were measured. T1, or longitudinal relaxation, is a measure of the relaxation time of nuclei to reestablish the original equilibrium along the Z axis. The T1 value will affect the intensity of the signal and sensitivity of the NMR experiment. In the case of nuclei with a spin of ½, the most important relaxation mechanism is the dipole-dipole interaction between spins. As a consequence, the absence of protons in the vicinity will result in longer relaxation times. The proton at δ 5.77 (H-7) was observed to possess the longest relaxation time, 7.1 s, which confirmed the information from the gCOSY spectrum that this proton was completely isolated. This proton (H-7) showed strong correlations with C-3 (δ 148.1), C-5 (δ 85.4) and C-9 (δ 186.0) in the gHMBC spectrum performed with an 8 Hz effective coupling constant. Two additional weak correlations at δ 103.5 (C-8) and δ 171.3 (C-2) were observed when the gHMBC spectrum was acquired with a 4 Hz coupling constant.

The two remaining fragments, a methyl (C-20) and an acetyl group (C-21 and C-22) were able to be connected to the quaternary carbon at δ 85.8 on the basis of gHMBC

correlations. The methyl protons at δ 1.30 (H-20) showed three strong correlations with the carbons at δ 196.4 (C-4), δ 85.8 (C-5) and δ 191.3 (C-6). The acetyl protons at δ 1.99 (H-22) showed correlations with the carbonyl at δ 169.2 (C-21) (in the 8 Hz gHMBC spectrum) and additionally with C-5 at δ 85.8 (in the 4 Hz gHMBC spectrum). These data allowed the planar structure of streptoquinone (**111**) to be assigned

To determine the relative stereochemistry of streptoquinone (**111**), a series of ROESY 1D NMR experiments were performed. Irradiation of the proton at δ 1.94 (C-16) led to an enhancement in the proton at δ 2.94 (H-11) and the two methyls at δ 1.32 (H-18) and δ 1.19 (H-19). When the proton at δ 2.94 (H-11) was irradiated, an enhancement occurred at δ 1.94 (C-16) and δ 1.19 (H-19). Irradiation on the proton at δ 3.55 (H-14) produced an enhancement in both methylene protons at δ 1.79 and δ 1.09 (H-15 a and b) and the methyl protons at δ 0.77 (H-23)

The information available by NMR experiments produced a solid but not unequivocal assignment of the structure of **111**. To determine a definitive assignment of **111** a series of experiments involving chemical derivatization and NMR were planned. The first step was to do the acetylation of **111**. This reaction would confirm the presence of the hydroxyl and enol in the molecule and potentially the four bonds ^{1}H-^{13}C correlations between the acetyl mehyl would determine the carbon in the molecule where it is attached to it. A second step would be the crystallization of the acetyl derivative of **111** as we could not get good crystal of this secondary metabolite. Unfortunately, my sample of streptoquinone (**111**) degraded during storage and it was not possible to isolate more of **111** as all subsequent culture of strain CNQ 509 failed to produce the compound.

Hence a structure of **111,** which fully satisfies all the 2D NMR data, is proposed but remains to be rigorously proven

V. 3. Experimental section

V.3.1. General experimental procedures

Optical rotations were measured on a Rudolph Research Autopol III polarimeter with a 10-cm cell. IR spectra were recorded using a Perkin-Elmer 1600 FT-IR spectrometer. ^{1}H, ^{13}C, and 2D NMR spectral data were recorded on Varian Inova 500 MHz and Varian Inova 300 MHz NMR spectrometers. High resolution mass spectral data were acquired on Agilent ESI-TOF mass spectrometer at The Scripps Research Institute, La Jolla. LC/MS data were obtained using a Hewlett-Packard series 1100 LC/MS system with a reversed-phase C18 column (Phenomenex Luna C-18(2), 4.6 mm × 100 mm, 5 μm) with CH_3CN/H_2O gradient solvent system.

V.3.2. The marine actinomycete (CNQ 509)

The isolation and cultivation of strain CNQ 509 was discussed on item III.3.2, chapter III.

V.3.3. Extraction and Isolation

The whole culture extract was subjected to a partition between H_2O and hexanes, chloroform, EtOAc, to generate four fractions. The $CHCl_3$ soluble component (1.52 g from 40 L) was subjected to reverse-phase preparative HPLC chromatography generating 12 fractions using a gradient from 15% CH_3CN to 70% CH_3CN in water for 50 min with UV detection at 250 nm. Further purification of fraction 7 (25 mg) by reverse-phase HPLC, isocratic (45% CH_3CN in water) with an ELSD detector, gave streptoquinone (**111**) (4.0 mg) in pure form.

Table V.1 - NMR spectroscopic data for streptoquinone (**111**) in DMSO-d_6.

position	δ_C[b], #H	mult (*J* in Hz)	δ_H[a]	COSY	HMBC
1	160.2				
2	171.3				
3	148.1				
4	196.4				
5	85.8				
6	191.3				
7	106.8	s	5.77		2, 3, 5, 8, 9
8	103.5				
9	186.0				
10	117.8				
11	30.2		2.94	12a, 12b, 16	1, 10, 12, 15, 16
		m	a) 2.44	11, 12b, 13	10, 13
12	27.5	m	b) 1.59	11, 12a, 13	13, 14, 15, 23
13	31.8		1.16	12b, 14, 23	
14	68.0		3.55	13, 15	12, 16
		m	a) 1.79	14, 15b	
15	31.4	m	b) 1.09	15a, 16	
16	35.4	m	1.94	11, 15b	10, 11, 14
17	80.6				
18	26.4	s	1.32		1, 16, 17, 19
19	25.7	s	1.19		1, 16, 17, 18
20	24.8	s	1.30		4, 5, 6
21	169.2				
22	21.0	s	1.99		5, 21
23	19.0	d	0.77	13	12, 13, 14

Assignment by gHSQC and DEPT NMR methods. [a]500 MHz, [b]125 MHz

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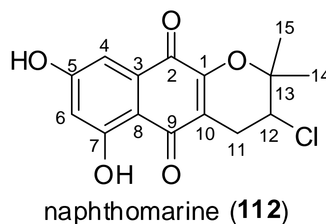
VI. Active secondary metabolites in a chemoprevention assay from the new marine derived actinomycete clade MAR 4 (CNR 925)

This chapter reports the isolation of the naphthoquinone, naphthomarine (**112**), a selective inhibitor of the NF-κB transcription factor, and QSAR studies of marine derived naphthoquinones in the NF-κB transcription factor. This work was performed in collaboration with Professor John Pezzutto, who is greatfully acknowledged for his assistance.



naphthomarine (**112**)

VI. 1. . Chemoprevention

Chemoprevention is defined by the National Institutes of Health as the use of natural or synthetic substances to reduce the risk of developing cancer, or to reduce the chance that cancer will recur.[1]

VI.1.1. Cancer

The term cancer describes a series of diseases where the main feature is an out of control cellular growth that can develop from any of the tissue or cell types in the body. In a healthy body, cells divide, grow and die as part of a natural script. A neoplastic cell however, will behave as a separate entity from the body and will not answer to chemical cues to stop growing and dividing.[2] Carcinogenesis is a multi step sequence of events with a timeframe of years, sometimes even decades, in which normal cells develop into neoplastic cells. The closely linked steps in the carcinogenic process are initiation, promotion and progression.[3] In the initiation stage, genotoxicity occurs when a normal cell exposed to a carcinogenic agent suffers DNA mutation. Initiation is considered to be a fast process, happening in a matter of one to two days. Promotion is a slow process where a selective proliferation of initiated cells with a high growth rate occurs. The last stage, progression, is where the pre-neoplastic cells receive additional aberrant changes. Those changes, which increase aggressiveness, can be additional DNA mutations or epigenetic changes in gene expression (**figure VI.1**).[2]

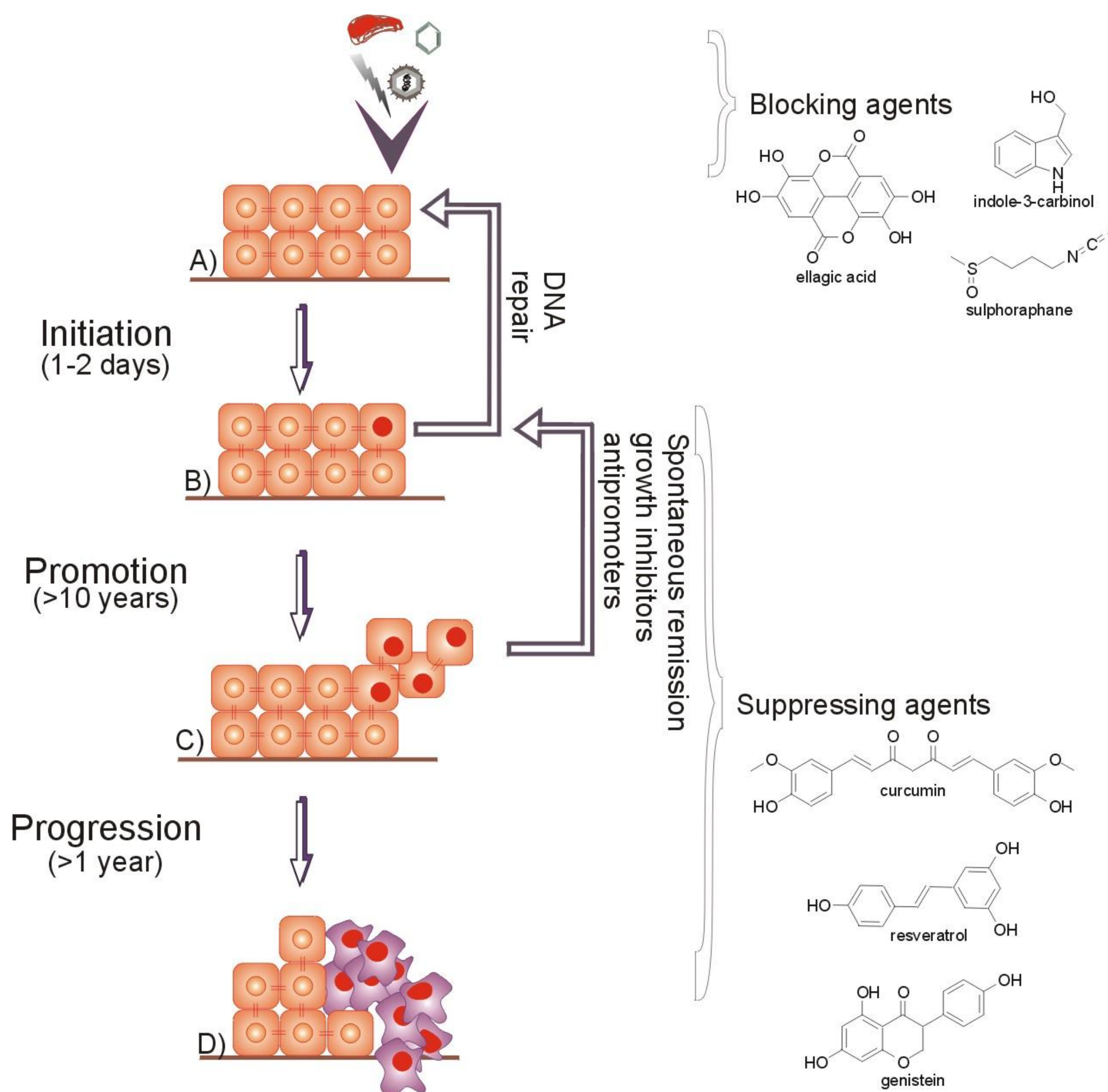


Figure VI.1.1 – Stages of development of cancer and chemopreventive agents – adapted from Kleinsmith[2]

The cumulative effect of cellular changes in the promotion and progression stages allows cancer cells to escape the organism biochemical control. Which gene is mutated is a very important factor. In some types of cancer a single mutation is sufficient to initiate the neoplastic process. For example, a single mutation in the *rb1* gene is responsible for the occurrence of retinoblastoma, a type of eye cancer in children.[4]

Another small change, with a disastrous effect, is a point mutation in the *Ha-ras* gene present in Harvey Sarcoma cells. The change of a guanine to thymine in this oncogene is sufficient for its activation.[5] In other types of cancer such as lung and breast, a series of alterations must occur before the cancer develops.

Genes involved in cancer development are called proto-oncogenes.[6] They can be broadly divided into two classes: genes that promote growth and multiplication (oncogenes) and genes that inhibit replication (tumor suppressor genes). Present in all cells, oncogenes can be activated by direct mutations or through mutations of their promoters. Tumor suppressors are the genes that inhibit cell division or induce apoptosis, a form of induced cellular suicide in response to DNA damage or cytokine stimulus.[3] Tumor suppressor genes are also responsible for DNA repair. Problems in DNA repair genes allow replication errors to propagate to daughter cells. Once cells lose their ability to repair DNA errors, mutations can accumulate in many genes, including tumor suppressors and oncogenes.[6]

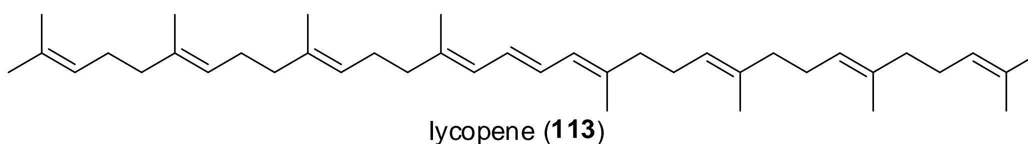
The stage of the cancer at diagnosis time is a major factor in the predicting the outcome. Early detection correlates with a high success rate for treatment. For example, melanoma, a very aggressive form of cancer, shows a survivor level of 96% if detected in the initial stage.[7] Unfortunately, as cancers develop from a single cell, few or no symptoms occur in the initiation stage.[2]

Nowadays, the treatment of cancer is based on a triad: surgery, radiation and chemotherapy.[2] The two physical methods, radiation and surgery, usually do not have the cellular effectiveness of chemotherapy. However, cancer chemotherapy has some drawbacks. There are a limited number of drugs available to treat the disease in an

advanced stage. In an established cancer, multiple biochemical alterations work together to maintain the neoplasia, making it difficult for only one medicine to reverse it. Most drugs generate, as a side effect, non-selective cytotoxicity because of the similarities between healthy and cancer cells.[8]

VI.1.2. Cancer chemoprevention

With a better understanding of cancer etiology, scientists are pursuing strategies for early intervention. Numerous studies linking diet to a lower level of cancer incidence in the population raised the question of whether compounds found in food could be used to prevent or delay the onset of cancer. Chemoprevention is closely related to diet intervention. Using lycopene (**113**) as an example, we can understand the difference between the two. Ingestion of tomatoes, a food with a high level of lycopene is considered a dietary intervention but taking a pill of lycopene is considered chemoprevention.[9]



lycopene (**113**)

Chemoprevention is especially suited to treat cells in the beginning of the cancer process because the compounds circulate through the bloodstream and can reach the cancer cells before they form visible tumors. Chemopreventive compounds are classified in two groups: blocking agents and suppressing agents. Blocking agents act

with carcinogens before they interact with their molecular target, while suppressing agents act in the cell already initiated thus avoiding the promotion and progression stages.[10]

VI. 2. NF- κB

NF-κB describes a family of transcription factors that regulate immune and inflammatory response and prevent cells from undergoing apoptosis.

The study of NF-κB transcription factors as a therapeutic target for the treatment of a series of diseases has received great attention. Two good examples are aspirin and glucocorticosteroids, whose anti-inflammatory activity is associated with the inhibition of the NF-κB response.[11] The mechanism of action of the plant metabolites resveratrol (**114**), quercetin (**115**) and myrcetin (**116**) is thought to be through inhibition of NF-κB and subsequent induction of apoptosis, potentiating the effects of chemotherapy and radiation therapy in the treatment of cancer. As NF-κB transcription factors are involved in a series of pathological processes such as asthma,[12] rheumatoid arthritis,[13] heart ischemia,[14] graft rejection[15] and spinal cord injury,[16] drugs that interfere with these transcription factors have potential beyond cancer (**figure VI.2.1**).[17]

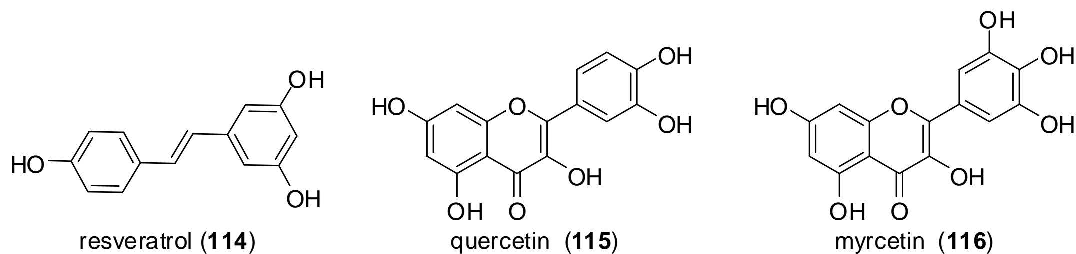


Figure VI.2.1 – Natural products that inhibit the NF-κB transcription factor

NF-κB transcription factors are activated by several signal transduction cascades. The production of NF-κB can be induced by external stimuli such as UV irradiation, viral infection, oxidants and stress, and by internal stimuli such as growth factors and cytokines (TNF, IL-1, LPS, or T-cell activators).[17] These signals activate IκB kinases, IKKα and IKKß, which phosphorylate the inhibitory proteins known as IκB resulting in their ubiquitination and degradation by the proteasome. The degradation of IκB results in the translocation of NF-κB from the cytoplasm to the nucleus where it activates the expression of genes for cytokines regulating immune recognition, neutrophil adhesion and migration, and cell proliferation, growth and differentiation.[18] NF-κB induced cytokines can also activate the NF-κB transcription factor, thus establishing a positive loop that can increase the inflammatory response and the duration of chronic inflammation (**figure VI.2.2**)

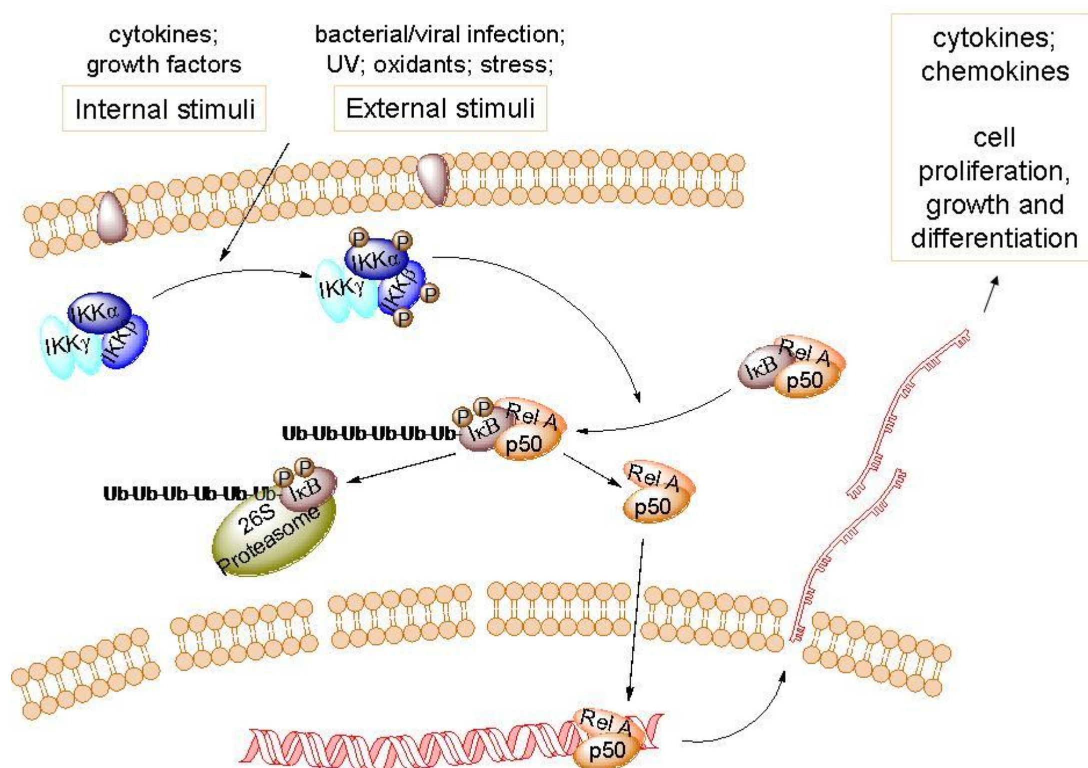


Figure VI.2.2 - NF-κB transcription factor - adapted from Yamamoto[11]

VI. 3. Results and discussion

The marine derived strain CNR 925 showed unique potent activity against NF-κB in a luciferase based assay. CNR 925 was identified by 16sRNA sequence as a *Streptomyces* species belonging to the new marine clade MAR 4.

In order to purify the NF-κB inhibitory compound, the bacterial culture was extracted exhaustively with EtOAc, and the resulting extract evaporated *in vacuo*. Isolation began with a normal phase flash chromatography. The extract was

incorporated onto celite. The celite was loaded onto the top of the column, and the column was eluted with a step gradient of hexane/EtOAc followed by a 10% MeOH wash yielding ten fractions. The 50% ethyl acetate fraction was further fractionated by reversed phase C-18 HPLC (CH_3CN/H_2O linear gradient) to give pure naphthomarine (**112**).

Naphthomarine (**112**) was obtained as a yellow glass. HR-ESI-MS revealed an [M+1]+ ion with exact mass 309.0231, corresponding to the molecular formula $C_{15}H_{13}ClO_5$ + H (calculated 309.0452) and nine degrees of unsaturation. The presence of a chlorine atom was clear by the mass spectrum due to the isotopic abundance of the chlorine isotopes ($^{35}Cl/^{37}Cl$). As naphthomarine (**112**) contains one chlorine atom, it mass spectrum presents two peaks, M+ and M+2, with a ratio of 3:1. This compound showed the UV absorption typical of the naphthoquinone chromophore present in marinone (**42**) and analogs (**figure III.3.1**). The combined NMR data allowed all protons to be correlated with their respective carbons (mainly using ^{1}H-^{13}C gHSQC data).

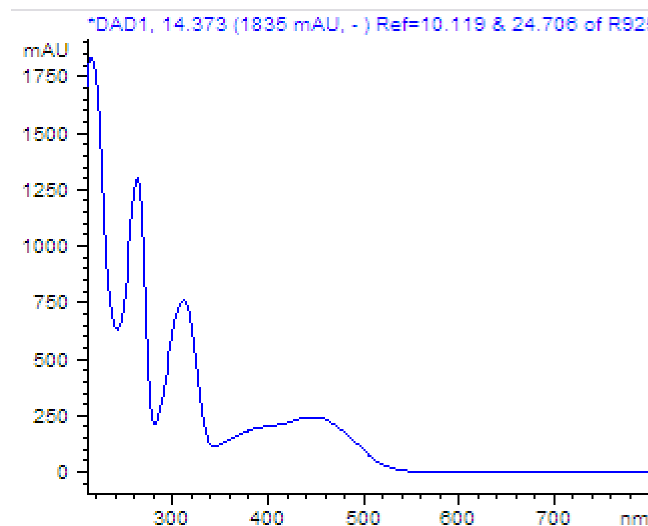


Figure VI.3.1 – UV chromophore present in naphthomarine (**112**)

Two spin systems were observed in the ^{1}H-^{1}H gCOSY NMR spectrum of **112** in DMSO-d_6 (**Figure VI.3.2a**). One spin system was composed of two mutually coupled aromatic protons (at δ 6.9 and 6.46), and the second spin system was composed of one methine proton at δ 4.55 and the two methylene protons at δ 3.02 and 2.74, respectively.

Correlations seen in the ^{1}H-^{13}C HMBC spectrum of **112** confirmed the naphtoquinone core skeleton shown in **figure VI.3.2b**. Both methyl protons at δ 1.45 and 1.38 showed strong correlations to the methine carbon at δ 58.9, and to the quaternary carbon at δ 80.4, thus establishing the connectivity of carbons C-11 through C-15. The methylene proton at δ 3.02 (H-11) showed ^{1}H-^{13}C HMBC correlations to carbons at δ 153.3 (C-1), 187.9 (C-9), 80.5 (C-13), 58.9 (C-12) and a weak correlation with the quaternary carbon at δ 117.8 (C-10).

The correlations observed for the two aromatic protons in the ^{1}H-^{13}C HMBC spectrum were important not only for the assignment of the carbons but also for

positioning the hydroxyl group with relation to the isoprene unit. The proton at δ 6.94 showed strong correlations with C-2 (δ 178.9), C-6 (108.6) and C-8 (107.4) and a weak correlation with C-3 (δ 156.4), C-5 (δ165.8). In addition, the δ 6.4 proton showed correlations to the C-7 carbon (δ 163.6).

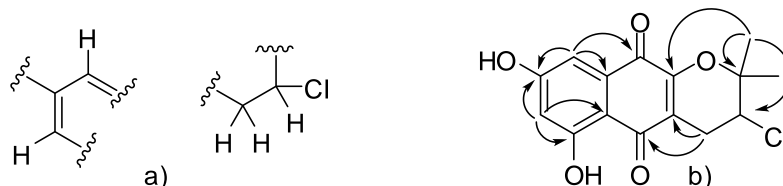


Figure VI.3.2 - a) Spin systems observed in the gCOSY spectrum of naphthomarine (**112**). b) key gHMBC correlations for **112**.

Naphthomarine (**112**) was active in the luciferase based NF-κB assay at a concentration of 0.86 μg/mL. The bioassays were performed at Professor John Pezzutto's laboratory at Purdue.

VI.3.1. QSAR study

With the positive result shown by naphthomarine (**112**) in the NF-κB assay, a fermentation effort was made to obtain analogs of **112**. To increase the possibility of secondary metabolite diversity, strain CNR 925 was grown in a series of different media. Strain CNR 925 shows the typical MAR 4 natural products production pattern with the observation of several known napyradiomycin- and marinone-class derivatives. To obtain analogs of **112** with different halogens at C-12, strain CNR 925 was grown in

medium M1, a very rich media known to enhance the production marinone derivatives. The cultivation was undertaken with artificial seawater and with added bromide and iodide. **Figure VI.3.3a** shows the typical chromatogram of the CNR 925 culture extract when grown in A1, the control media used. In **Figure VI.3.3b**, the strain was cultivated in M1 a very rich media containing cane molasses, glucose and peptone in tap water. Comparing the two figures, it is clear that there is a shift in the secondary metabolite production in this strain. Interestingly, the extract from the culture grown on artificial seawater with iodide showed a noticeable decrease in the production of all naphthoquinones and a shift to the production of lavanducyanin (**75**), the peak at 13.47 min in chromatogram d **(Figure VI.3.3.d**). Unfortunately, no brominated or iodinated analogs of **112** were detected by UV or LC-mass spectral data.

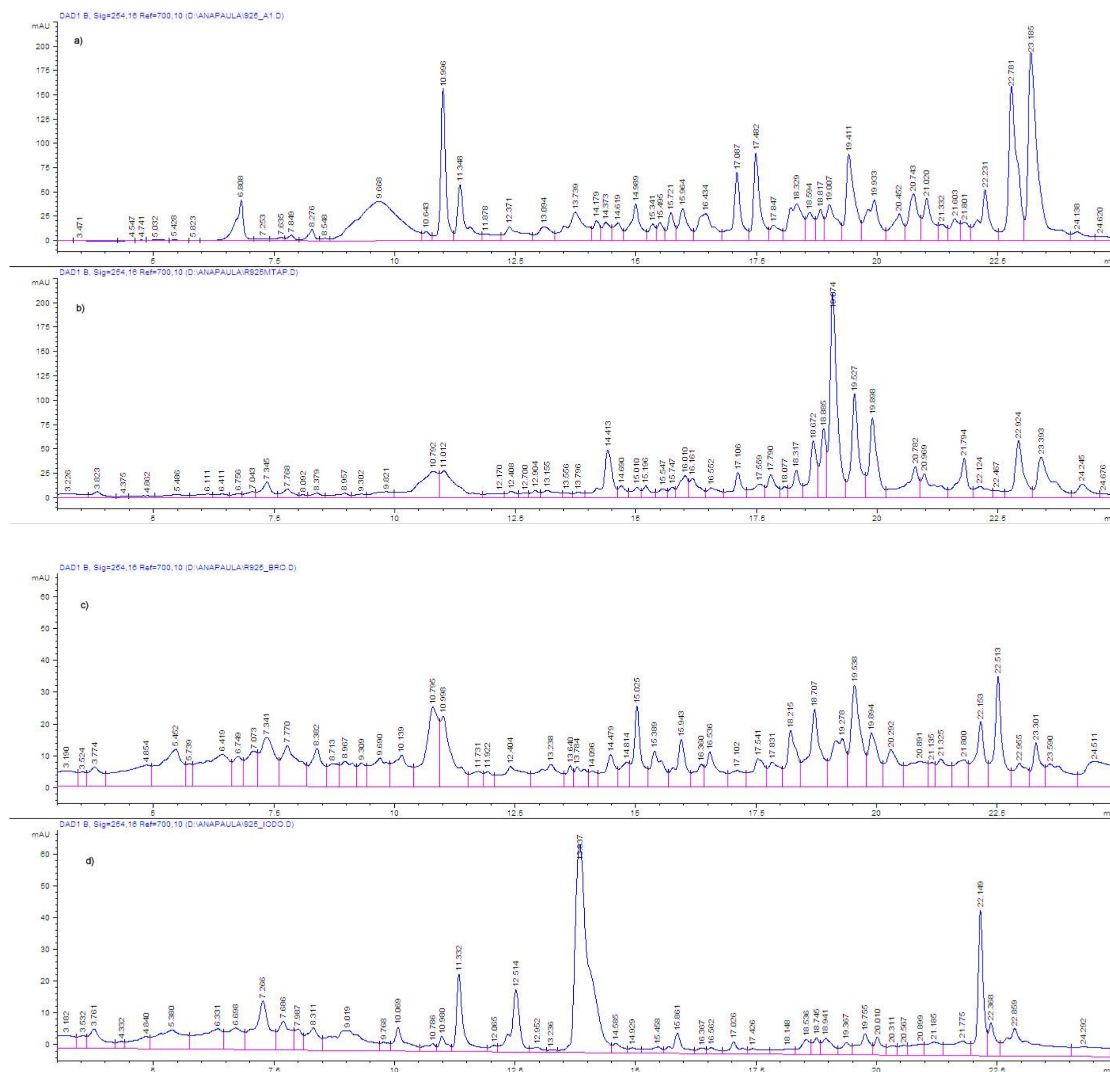


Figure VI.3.3 – Chromatogram of the extract of strain CNR 925 extract, with 254 nm detection, when growth in different media. a) A1 media (starch, yeast extract and peptone in seawater); b) M1 (glucose, cane molasses, peptone, $CaCO_3$ in tap water); c) M1 with artificial seawater without NaCl and enriched with NaBr; d) M1 with artificial seawater without NaCl and enriched with NaI

Strain CNR 925 also yielded a napyradiomycin derivative with a molecular weight of 498 (**117**) and the known metabolite debromomarinone (**118**). These compounds together with a few other naphthoquinones (**119**, **120**, **121**) available in the Fenical and Jensen laboratory were also submitted to the chemoprevention assay. The structures, and the biological activities obtained, are shown in **Figure VI.3.4**. Even though lavanducyanin (**75**) is a aromatic meroterpenoid, it did not show activity in the chemoprevention assay. In a first glance, the presence of a double bond connecting C-10 to C-1 or C-11 appears to have a significant impact in the biological activity but careful analysis of the structures showed that the size of the groups connected to the naphthoquinone core may be a major factor with the smaller **112** as the most potent compound on this series.

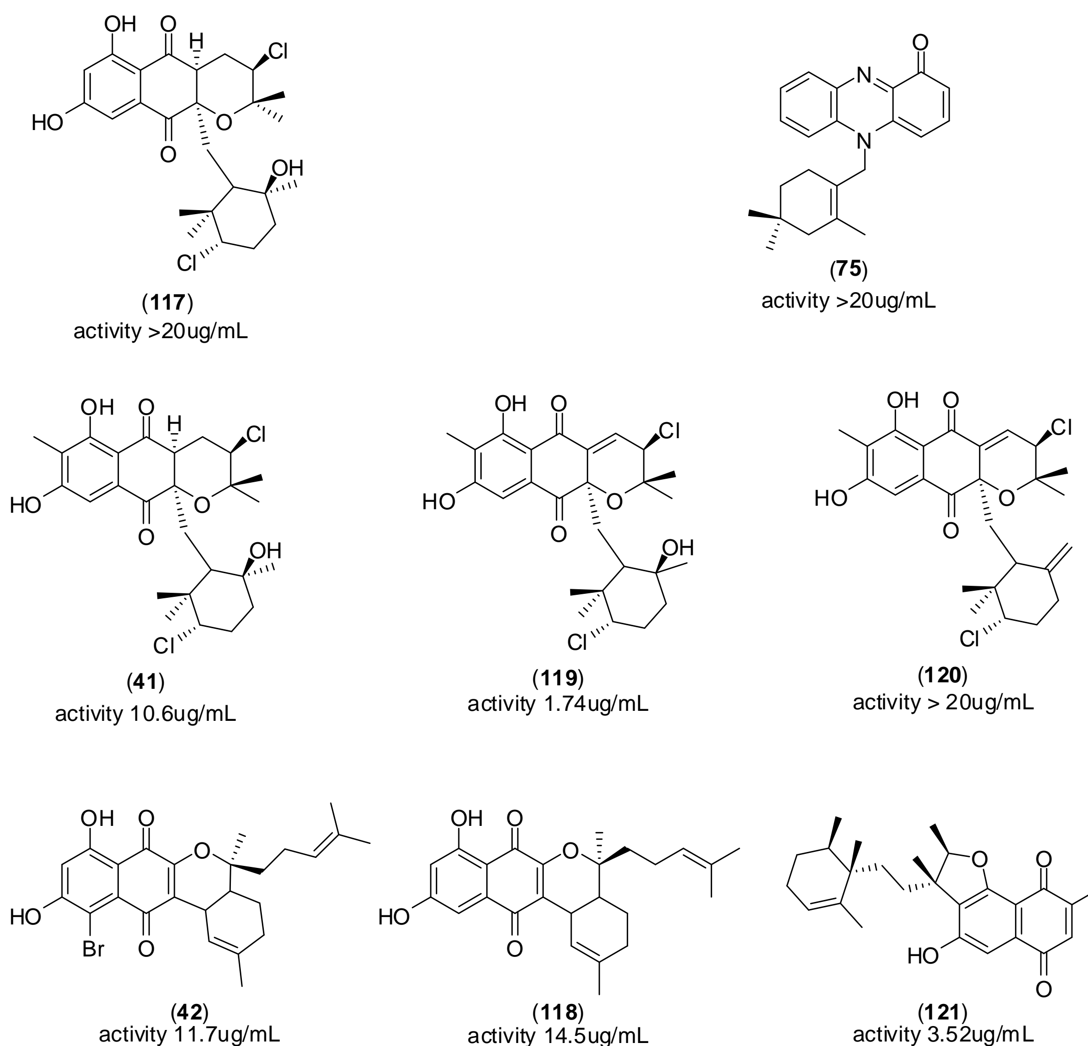


Figure VI.3.4 – Naphthoquinone derivatives and lavanducyanin tested in the NF-κB chemoprevention assay with biological activity. The bioassays were performed in Professor John Pezutto laboratory, Department of Pharmacology at the Purdue University.

VI. 4. Experimental Section

VI.4.1. General experimental procedures

IR spectra were recorded using a Perkin-Elmer 1600 FT-IR spectrometer. ^{1}H, ^{13}C, and 2D NMR spectral data were recorded on Varian Inova 500 MHz and Varian Inova 300 MHz NMR spectrometers. High resolution mass spectral data were acquired on Agilent ESI-TOF mass spectrometer at The Scripps Research Institute, La Jolla. LC/MS data were obtained using a Hewlett-Packard series 1100 LC/MS system with a reversed-phase C18 column (Phenomenex Luna C-18(2), 4.6 mm × 100 mm, 5 μm) with CH_3CN/H_2O gradient solvent system.

VI.4.2. The marine actinomycete (CNR-925)

Strain CNR 925 is an actinomycete isolated from a shallow (2 m depth) marine sediment sample collected in February 2004 off the coast of the Republic of Palau. The bacterium was isolated by molecular biologist Erin Gontang using a pre-selection protocol and ultimately growing on a solid agar medium (18 g agar in 1 L natural seawater and rifampicin (5 μg/ml)). Analysis of the full 16sRNA sequence (deposited with genbank under accession number DQ448742) indicated this strain as belonging to the newly identified marine clade MAR 4. The strain was cultured by shaking in A1 liquid medium (10 g of starch, 4 g of yeast extract, 2 g of bacto-peptone, and 1 L of seawater) at 28° C for 7 days, and then extracted by partition with EtOAc. The EtOAc extracte was dried with anhydrous sodium sulfate, filtered and, the solvent removed under vacuum to generate the crude extract (1.83 g).

VI.4.3. Compound isolation

The whole culture extract was subjected to silica normal-phase flash chromatography using a step gradient of hexane and EtOAc (Hexane, 12.5% EtOAc, 25% EtOAc, 37.5% EtOAc, 50% EtOAc, 62.5% EtOAc, 75% EtOAc, 87.5% EtOAc, 100%EtOAc, 10% MeOH in EtOAc) to generate ten fractions (Fr. 1: 1.9 mg, 2: 4.9 mg, 3: 7.7 mg, 4: 46.1 mg, 5: 58.2 mg, 6: 46.1 mg, 7: 23.6 mg, 8: 13.0 mg, 9: 47.7 mg and 10: 832.1 mg). Further purification of fraction 5 by C-18 reversed phase HPLC, gradient (40% CH_3CN to 100% CH_3CN), gave fifteen fractions, 5A to 5N. Final purification of fraction 5D by C-18 reversed phase HPLC (52% CH_3CN) gave naphthomarine (**112**) (1.1 mg) in pure form.

Table VI.1 - NMR spectroscopic data for naphthomarine (**112)** in $CDCl_3$.

position	$\delta_C{}^b$	mult (*J* in Hz)	$\delta_H{}^a$	COSY	HMBC
1	153.3				
2	178.9				
3	156.4				
4	109.1	d	6.94	6	2, 3, 5, 6, 8
5	165.8				
6	108.6	d	6.41	4	4, 5, 7, 8
7	163.6				
8	107.4				
9	187.9				
10	117.8				
11	27.3		2.74 3.02	12	1, 9, 10, 12, 13
12	58.9	dd	4.55	11	10, 14, 15
13	80.4				
14	24.3		1.45		12, 13, 15
15	25.2		1.38		12, 13, 14

Assignment by gHSQC NMR methods. [a]500 MHz, [b]125 MHz

VI. 5. Reference

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VII. Conclusion

The MAR 4 actinomycete clade, detected during the thesis work of molecular biologist Alejandra Prieto-Davo, has a new 16sRNA signature that group them phylogenetically in the *Streptomycetales* family. The members of this clade were isolated from the Pacific Ocean mainly in the Coast of United States and Mexico. The two strains used in this work, CNQ 509 and CNR 925, were more specifically isolated from near the coast of La Jolla for CNQ 509, and from Palau shallow sediments for CNR 925.

My thesis research has broadened the chemical knowledge of the marine actinomycete group MAR 4. My chemical studies of the MAR 4 clade revealed a unique set of compounds, all of them having as a major characteristic a mixed biosynthesis involving a terpenoid linked to an aromatic moiety. For a long time it was believed that the production of secondary metabolites by actinomycetes was strain rather than clade (species or genus) specific, with related strains producing completely unrelated compounds. Contrary to this old belief, all the MAR 4 clade strains produced only meroterpenoid compounds in our chemotyping analysis.

This thesis was based upon the assumption that a phylogenetically new group of microorganisms would produce new secondary metabolites. This theory proved correct with four different types of meroterpenoids isolated from the strains studied.

The structural features of the meroterpenoids isolated from MAR 4 actinomycetes are unique in numerous ways. The nitropyrrolins A-C (**97-99**), to the best of my knowledge, are the first α-nitro pyrroles isolated from a natural source. Marinophenazines A (**105**) and B (**106**) are the first *O*-prenylated phenazines produced by an actinomycete species, and naphthomarine (**112**) is a meroterpenoid which shows very potent inhibition of the NF-κB transcription factor. This latter biological activity may indicate potential to be used as a chemopreventive agent.