THE ISOLATION AND CHARACTERIZATION OF NATURAL PRODUCTS FROM MARINE PLANTS AND MICROORGANSIMS

Amanda J. Krzysiak

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Approved by

Advisory Committee

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Chair

Accepted by

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Dean, Graduate School

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ABSTRACT

Many organisms, including plants, microbes and invertebrates produce compounds called as natural products. The production of natural products often confers some advantage to the producing organism such as chemical defense from predators. Other natural products can have a different role in the producing organism such as preventing or limiting ultraviolet light damage from the sun. Such sun screens are found in microalgae and vascular plants. These natural products have been shown to display cytotoxicity, anti-bacterial, anti-fungal, anti-viral, and anti-parasitic activity. Due to the biological activity shown by many natural products, these molecules have been used in our pharmacopia. Compounds like penicillin, isolated from a fungus, and non-pharmaceutical compounds such as ginseng from green tea are natural products used by humans to benefit human health. As the investigation of natural products has resulted in a remarkable number of compounds of benefit to humankind, the continued study of secondary metabolism and natural products has and continues to be of great importance. This thesis describes an investigation of the secondary metabolites of selected marine fungi to identify anti-microbial natural products as well as the UV protecting compounds of the endangered sea grass *Halophila johnsonii*. Although no novel compounds could be characterized from the marine fungi, a collection of flavonoid derivatives was identified in *Halophila johnsonii.*

ACKNOWLEDGEMENTS

I would like to thank my thesis committee, especially my advisor Dr. Jeffrey Wright, for guidance, instruction, and support. I am very grateful for your patience and generosity of time in allowing me the time I needed to complete this thesis.

Also, I would like to acknowledge Jan and Brigette Kohlmeyer for their donation of the collection of obligate marine fungi used in this study, and to David Padgett for assistance in the culture of the fungi used in this study. My thanks and appreciation also go to Dr. Michael Durako and Jennifer Kunzelman for their time and efforts in the collection of the *Halophila johnsonii* samples as well as a wonderful collaboration. I would also like to extend my appreciation to GlaxoSmithKline for the financial support.

DEDICATION

I dedicate this thesis to my parents. You never said that I couldn't do anything, always supported my decisions, and had faith in my abilities. I love you.

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CHAPTER 1: CHARACTERIZATION OF ULTRAVIOLET PROTECTING FLAVONOIDS FROM *HALOPHILA JOHNSONII*

Introduction

Halophila johnsonii is a submerged marine angiosperm located only in the coastal areas of south-eastern Florida. It has a very limited distribution within coastal lagoons and is found between Sebastian Inlet (27°51'N, 80°27'W) and Virginia Key (25°45' N, 80°07"W) at depths from 3 meters and less (*1*). It is the least abundant seagrass species within these areas (*2*). In 1998, due to its rare occurrence, *H. johnsonii* was listed as a threatened species on the Federal Register, the only marine angiosperm with that classification. In contrast, it has been suggested that *H. johnsonii* is a new and invading species to the lagoons of south Florida and may not, in fact, be a threatened species (*3*). In either case, *H. johnsonii* likely has an advantage for survival as it is the least abundant species within its area of population and has not been overgrown in the past eight years since its listing as a threatened species. *H. jonsonii*'s advantage may be due to its establishment and survival in the shallow, more demanding waters of its environment (*4*). Previous work has shown that *H. johnsonii* has a higher ultraviolet (UV) light tolerance compared to *Halophila decipiens*, a genus member co-existing at deeper depths in south Florida lagoons (*5*). Furthermore, acetone extracts of the leaves of both plants revealed a strong UV absorption at 345nm in *H. johnsonii* that was absent in *H. decipiens* (*5*). Additional studies showed that *H. johnsonii* is capable of rapidly adjusting to high levels of UVB radiation through changes in the production of UV protective compounds and pigments (*6*). It appears the production of these UV absorbing compounds gives *H. johnsonii* a competitive advantage over other species of seagrass, enabling it to survive the shallow, high UV areas of the intertidal zone.

The production of UV protecting compounds in plants and microalgae is not unusual, and indeed the presence of natural sunscreens in various organisms has been known for decades. The

sun emits three forms of UV radiation: UVA, UVB, and UVC. UVC (200-280 nm) radiation is completely absorbed by the ozone layer. However, UVA (320-400 nm) and UVB (280-320 nm) radiation penetrate the Earth's atmosphere and can cause damaging effects to humans and other living organisms, including plants (*7, 8*). For humans, sun damage from UV radiation manifests itself as burns and premature aging of the skin as well as increases the risk for skin cancer and cataracts. The use of sunscreens before UV exposure from the sun has become necessary for people of all ages and ethnicities to protect against UV damage.

In a parallel fashion to the application of sunscreens on human skin, a number of classes of secondary metabolites provide UV protection to a variety of organisms ranging from plants to marine invertebrates. The largest class of naturally occurring UV protecting compounds is found in plants and is composed of a group of compounds known as the flavonoids. Although there are more than five flavonoid structural types, the anthrocyanins and flavones represent the major compounds associated with UV protection due to their ability to absorb light in the 280-320nm (UVB) range (*9*). This ability of flavonoids to absorb UVB light can prevent the DNA damage and photosystem damage induced by ultraviolet light (*8*). Anthrocyanins can serve a dual purpose by providing not only UV protection to plants but also the pigments responsible for flower color. Such UV protecting compounds of the flavonoid type occur mainly as *O*- or *C*-linked glycosides and are located in the outer tissue layers (*10*). For instance, in the epidermal cells of the rice *Oryza sativa*, a series of *iso*-orientin-*O*-glucosides were identified as UV protecting compounds (*11*). In the frequently studied species *Arabidopsis thaliana*, kaempferol-3-gentiobioside-7-rhamnoside, and kaempferol-3,7-dirhamnoside were found to protect the plant from UV damage (*12*).

Carotenoid pigments have also been linked to UV protection in plants. In particular, it has been suggested that carotenoids play a role in photosynthetic protection from UVB damage in the Clark and Harosoy soybean (*Glycine max*) isolines (*13*). More recently, a unique group of amino acid-based compounds known as the mycosporines (MAAs) have been identified as UV protecting compounds in a number of marine organisms. MAAs absorb UV light in the 310-360 nm range and have been found in terrestrial fungi, marine microalgae, as well as symbiotic invertebrates and vertebrates (*14*). Interestingly it seems that MAAs are predominantly found in marine organisms and are not common in higher terrestrial plants that appear to rely on UV protection through flavonoid and carotenoid compounds. Examples of structures from these different classes of UV protecting compounds are shown in Figure 1.

Based on the observation that the deeper water seagrass variant *H. decipiens* does not appear to contain any photoprotecting compounds, whereas the shallow-water variant *H. johnsonii* can adapt to alterations in UV exposure (*5*), we reasoned that it may utilize natural sunscreens as a means of UV protection. Consequently, we embarked upon a chemical investigation of the secondary metabolites of *H. johnsonii* to determine if any metabolites may act as photoprotectants. This investigation has revealed a series of flavonoid derivatives produced by *H. johnsonii*, that likely play a UV protecting role.

Results and Discussion

A purified fraction of the methanol extract of *H. johnsonii* yielded a complex mixture of UVB absorbing compounds. Liquid chromatography-electrospray ionsization mass spectrometry (LC-ESIMS) analysis of the mixture resulted in the identification of five major compounds absorbing at 340 nm, and ranging in retention time between 19 and 25 minutes and a minor component eluting at 15.65 minutes (Figure 2a). The total ion chromatograms for each peak

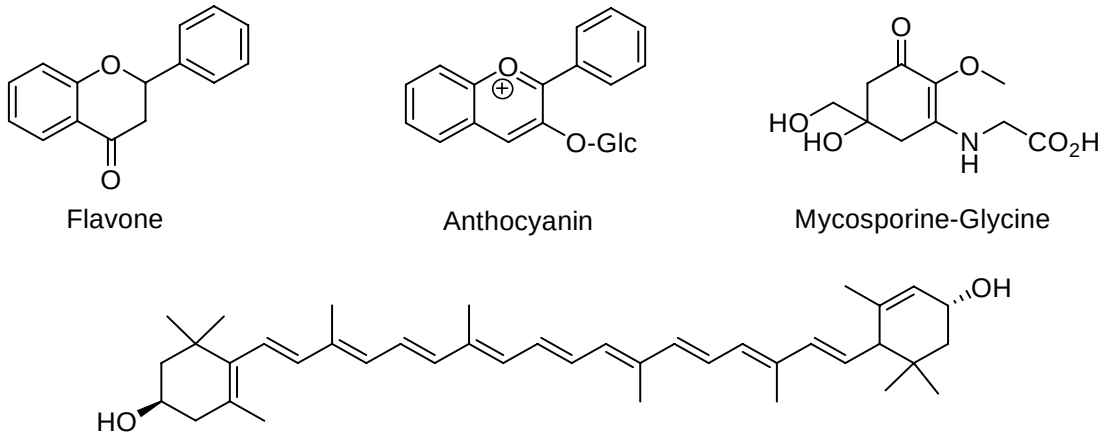
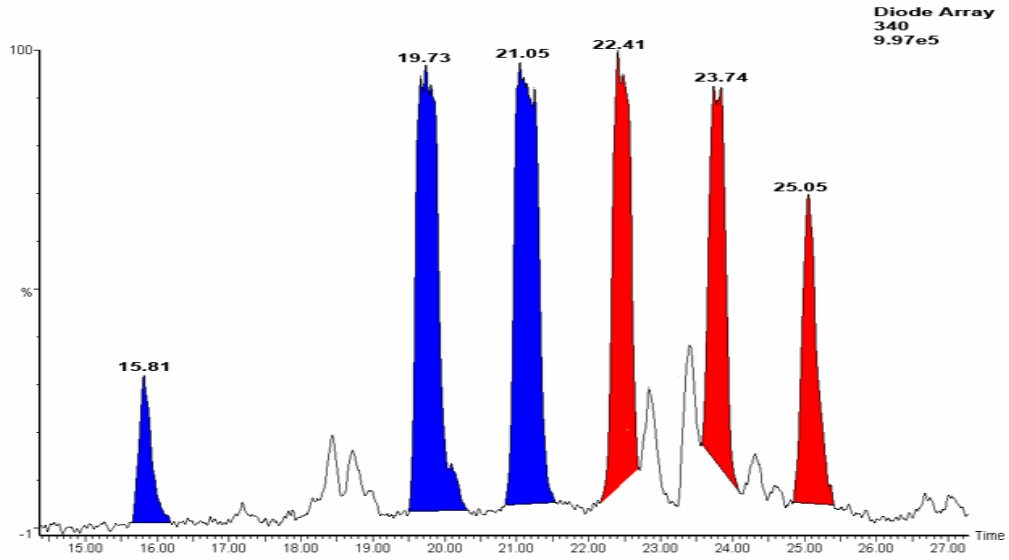


Figure 1. The structures of UV absorbing compounds.

were generated with ESI operating in the negative ion mode (Figure 2b). The ESI-MS spectra for peak 1 (R.T. 19.76 min) displayed ions at m/z 479 and 316. Peak 2 (R.T. 21.076 min) had ions at m/z 463 and 301. Peak 3 (R.T. 22.386 min) displayed ions at m/z 521, 316, and 284. Peak 4 (R.T. 23.70 min) showed ions at m/z 505, 301, and 284. Finally, peak 5 (R.T. 25.00 min) showed ions at m/z 489 and 285. The minor component had m/z ions at 957.5, 795.3, 633.4, and 323.1. The mass spectra of these six fractions obtained in negative ESI mode showed parent ion peaks as well as fragmentation peaks resulting from losses of 162 or 205 amu. The loss of such fragment ions is entirely consistent with the presence of glycosyl and acylglycosyl moieties, respectively, in these UV absorbing compounds, a common feature in many natural products (*15*).

Based on the observation that flavones are frequently glycosylated and absorb light in the UVB range, a flavone glycoside standard (leuteolin-7-glycoside) was purchased for comparison. The UV spectra of leuteolin-7-glycoside standard showed UV maximas at 215 nm, 250 nm, and 350 nm (Figure 3a). The UV spectra for each of the compounds of the *H. johnsonii* extract showed very similar maximas at 215, ~250, and 350 nm (Figure 3a). Furthermore, the leuteolin-7-glycoside standard eluted from the LC with similar retention time and displayed similar mass spectra and fragmentation ions as observed with the *H. johnsonii* compounds (Figure 3b). The parent ion for the intact compound is represented by the $[M-H]^-$ ion at m/z 445. Furthermore, in the electrospray ionization process, cleavage of the glycosyl bond results in a $[M-162]^-$ fragment ion at m/z 284 corresponding to the aglycone leuteolin. These data supported the idea that the protectant compounds found in *H. johnsonii* were a mixture of flavone glycosides and acetylglycosides.

a.



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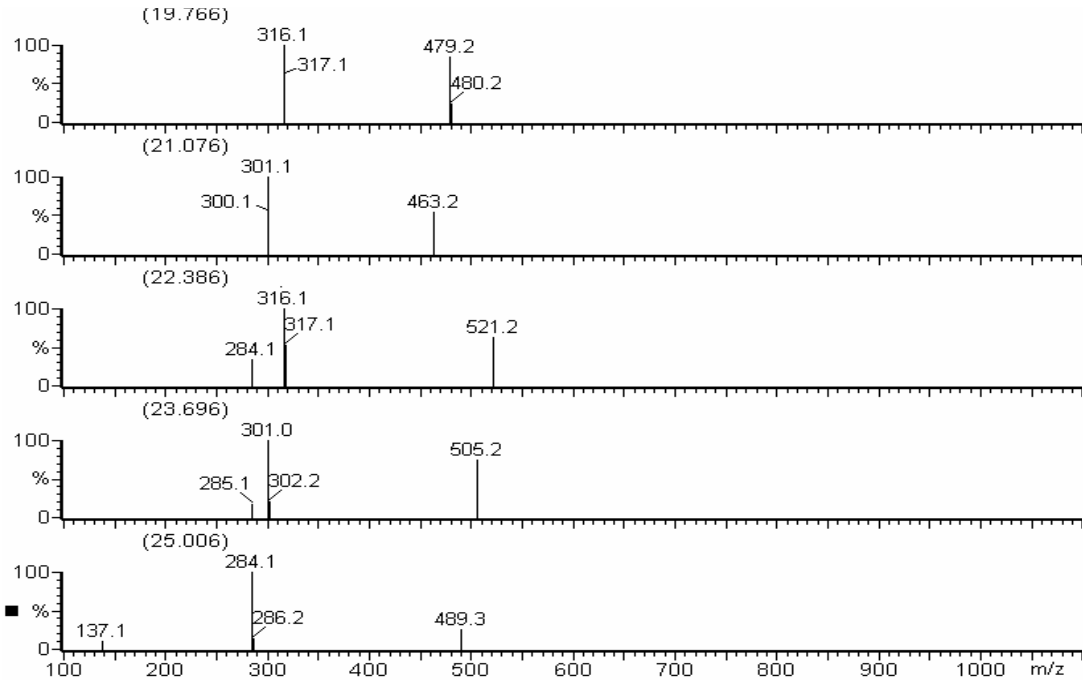
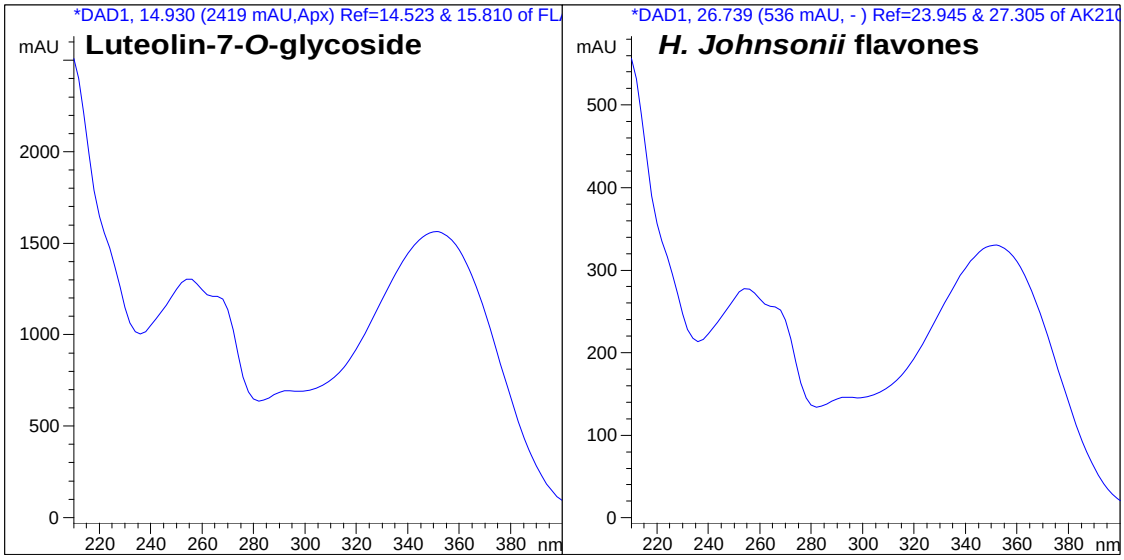


Figure 2. LC and MS chromatograms of *H.johnsonii* compounds
a. LC chromatogram of the *H. johnsonii* compounds with UV absorbance at 340 nm.
b. Mass spectra for the six UV active compounds in H. johnsonii. The compounds were analyzed in ESI- mode and represent $[M-H]^-$ ions.

a.



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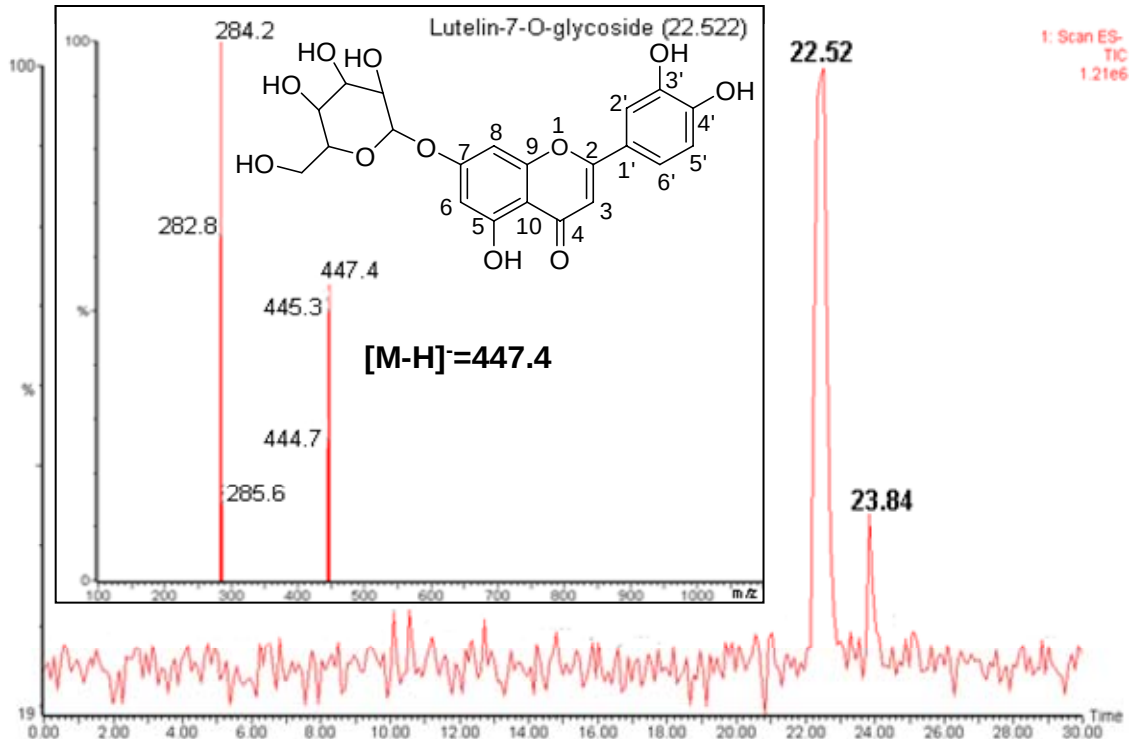
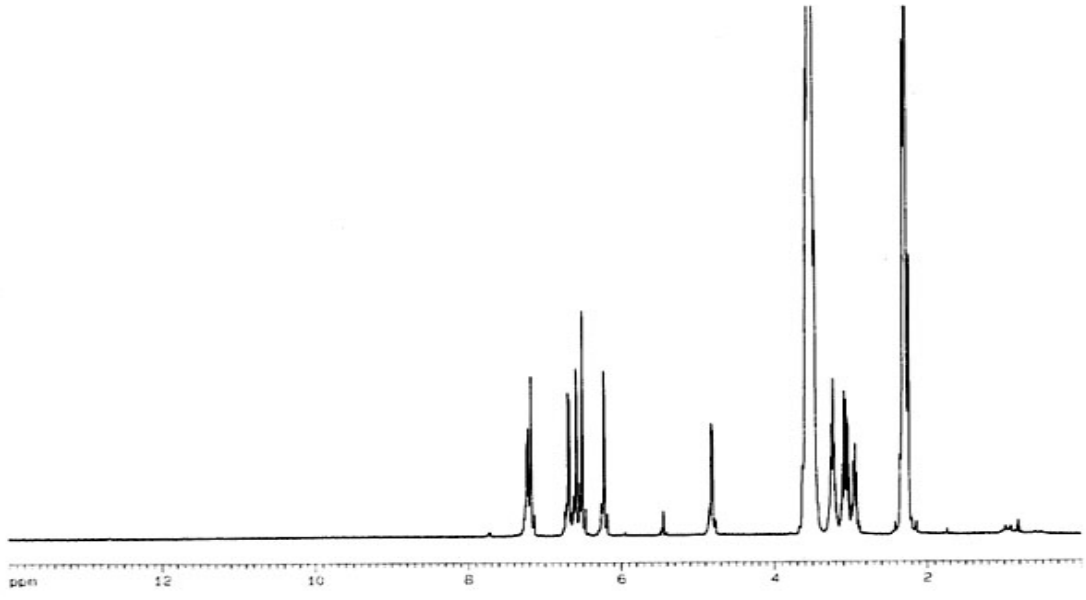


Figure 3. Luteolin-7-glycoside displays similar spectral properties to *H. johnsonii* compounds .
a. The UV spectra of leuteolin-7-glycoside and *H. johnsonii* compound.
b. LC chromatogram (back) and ESIMS spectra (inset) for leutolin-7 glycoside standard.

Comparision of the ^{1}H NMR spectra of the mixture and standard further supported the identification of the components as a series of glycosylated flavonoids. The ^{1}H NMR spectra of the mixture from *H. johnsonii* mixture displayed aromatic and hydroxyl resonances consistent with luteolin-7-glycoside. As further support, the ^{1}H NMR spectra also contained resonances around 5.0 ppm (Figure 4), consistent with a proton attached to an anomeric carbon of a sugar moiety such as glucose.

Identification of individual flavone components was made based on the mass spectral data obtained for each compound, and by comparison with literature information. The 5 major components of the *H.johnsonii* mixture retained the common flavone core and varied in the number on hydroxyl substituents attached to the core. Each was also modified either a glycoside or acetyl glycoside moiety. Using this analytical information, two flavone glycosides were identified. Peak 1 (19.73 min) was identified as a hexahydroxyflavone-glycoside (M.W. 480) as evidenced by the aglycone fragment peak at m/z 317.1 corresponding to the $[M-162]^-$ fragment ion and the parent $[M-H]^-$ ion at m/z 479.2. Peak 2 (21.05) was characterized as a pentahyroxyflavone-glycoside (M.W. 464) as suggested by the parent $[M-H]^-$ peak at m/z 463.2 and a fragment ion at m/z 301.1 corresponding to the aglycone and the loss of a sugar moiety. The final three components of the mixture contained the tetra-, penta-, and hexahydroxylated flavones modified with an acetylated glucose. Peak 3 (22.41 min) represented the hexahydroxylated-acetylglycoside (M.W. 522) and was identified by the molecular ion $[M-H]^-$ at m/z 521.2. The mass fragment ion at m/z 317.1 corresponds to the loss of the acetyl-glycoside of 204 amu. The pentahydroxyflavone acetylglycoside (M.W. 506), peak 4 (23.74 min), gave an $[M-H]^-$ ion peak at m/z 505.2 with a subsequent fragment ion at m/z 301.1 corresponding to the loss of the acetylglycoside moiety. Finally, the most nonpolar constituent of the mixture, peak 5

a.



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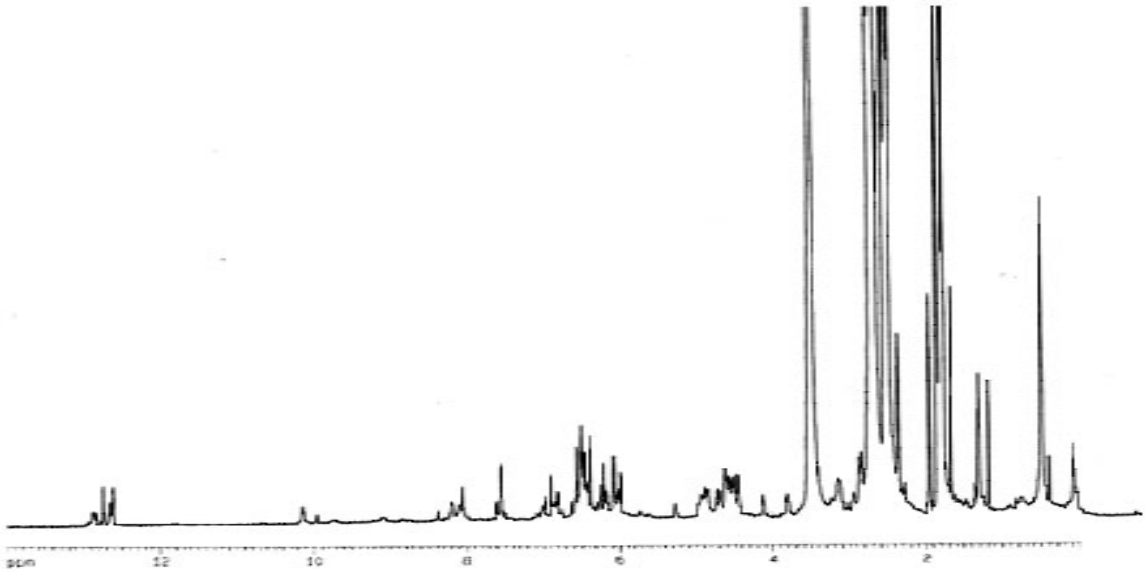


Figure 4. Comparision of ^{1}H NMR spectra of luteolin-7-*O*-glycoside standards and *H. johnsonii* compounds.

a. ^{1}HNMR spectra of luteolin-7-*O*-glycoside
b. ^{1}HNMR spectra of *H. johnsonii compounds*

(25.05 min) was identified as a tetrahydroxylated-monoacetylglycoside (M.W. 490) by the $[M-H]^-$ molecular ion at m/z 489.3. A fragment ion at m/z 447.3 corresponding to the loss of an acetyl group as well as a $[M-204]^-$ fragment peak at m/z 285.1 corresponding to the loss of the acetylglycoside from the parent ion also supported the identification. Through LC-MS analysis we identified two flavone glycosides and three flavone acetylglycosides in the *Halophila johnsonii* extract.

One of the minor components of the mixtures with a retention time of 15.81 minutes (Figure 2a) displayed an interesting mass spectral fragmentation pattern (Figure 5). Although we were unable to identify the flavone core of this molecule, we identified this constituent as a flavone tetraglycoside derivative. The parent ion at $[M-H]^-$ m/z 957.6 sequentially lost two glycoside units yielding fragment ions at $[M-162]^-$ and $[M-324]^-$m/z 795.5 and 633.2 respectively. Further fragmentation of 310 amu corresponding to the loss of another two sugars units resulted in an $[M-H]^-$ peak of the aglycone at m/z 323.2. Table 1 summarizes the spectral data, type of flavone, and identification of the *H. johnsonii* compounds.

LC-ESI MS was used to identify the UV absorbing constituents of *H. johnsonii* as a series of flavone glycosides. The general structures of these compounds are shown in Figure 6. These compounds are widely distributed among plants, and their biosyntheses and functions are well-studied (*10*). The extensive information and databases dealing with known flavonoid compounds assisted in the identification of these flavones by LC-MS methods, and these methods are now widely used as an analytical tool for this class of compound (*15-17*). In fact, electrospray mass spectrometry, has become the most prominent method for flavonoid identification (*18*), particularly in combination with known standards when it can be used to identify flavonoid compounds. However, NMR analysis is often still required for full structural

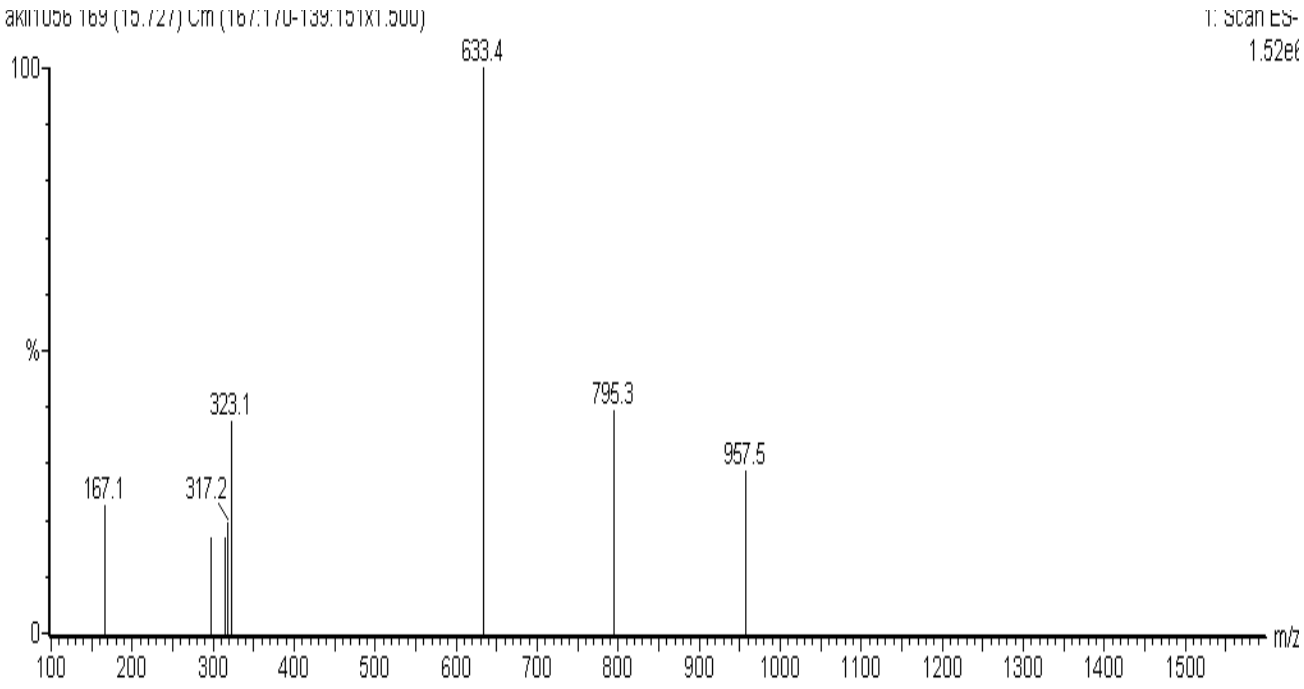
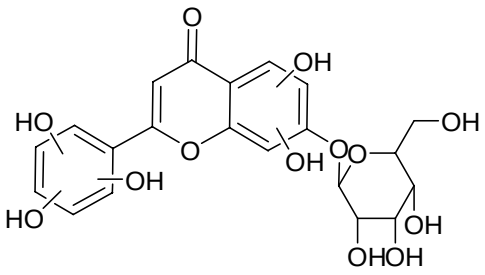


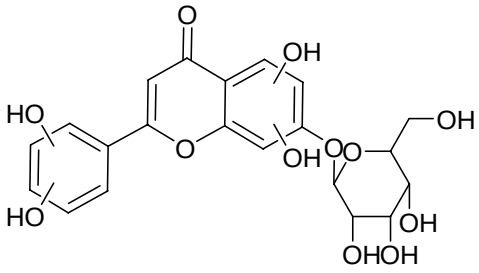
Figure 5. The ESI- mass spectral fragmentation pattern and proposed structure of the flavone-tetraglycoside

Table 1. The identification and spectral propteries of the *H.johnsonii* UV absorbing compounds.

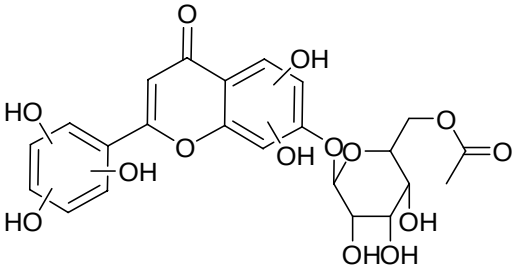
Peak	t_r (min)	UV l_{max} (nm)	[M-H]- (m/z)	[M-H]- fractions (m/z)	flavone
1	19.73	220,280,350	479	316	hexahydroxyflavone-*O*-glycoside
2	21.05	220,280,350	463	301	pentahydroxyflavone-*O*-glycoside
3	22.41	215,250sh,350	521	316	hexahydroxyflavone-*O*-acetylglycoside
4	23.74	220,280,350	505	301	pentahydroxyflavone-*O*-acetylglycoside
5	25.05	215,250sh,350	489	284	tetrahydroxyflavone-*O*-glycoside



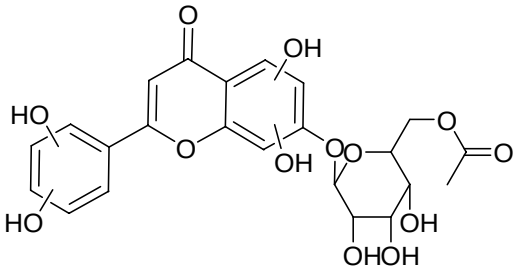
hexahydroxyflavone-*O*-glycoside



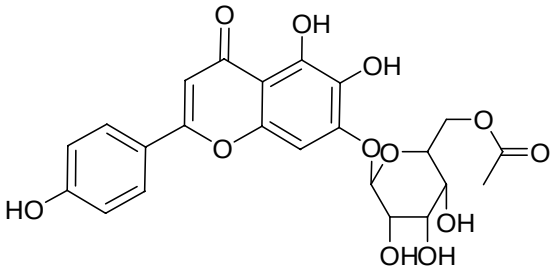
pentahydroxyflavone-*O*-glycoside



hexahydroxyflavone-*O*-acetylglycoside



pentahydroxyflavone-*O*-acetylglycoside



tetrahydroxyflavone-*O*-acetylglycoside

Figure 6. The general structures of the flavone glycosides in *H. johnsonii*

characterization and substitution pattern assignment. Tandem mass spectrometry (MS/MS), can provide even more detailed fragmentation data, and hence valuable structural information of flavonoids, than can be obtained using a single mass spectrometer alone (*18*). Regardless of the techniques used, the identification of flavonoids by mass spectrometry is frequently reported in the literature. Through the use of standards and MS, five flavone glycosides were identified from the extract of *Halophila* johnsonii.

The flavone compounds identified in the *Halophila johnsonii* extract may possess many biological properties, but most likely serve as UV photoprotectants for the seagrass, which survives in highly light exposed shallow areas of Biscayne Bay. Thus these secondary metabolites are used to filter UV light and protect the plant from UVB damage to DNA. It has been shown that the production of these compounds is upregulated with increased exposure to UV light (*6*). Two of the flavone compounds occur as glycosides, and three are modified with an acetylated glucose moiety making all of the flavone constituents hydrophilic. Such glycoside derivatives may be useful in the transportation of these compounds in the plant and may favor their location in the vacuoles of epidermal cells in the leaves (*9*). A further study of various plant components such as the leaf, rhizome, stem, etc., might provide additional information as to the production and storage centers for these compounds.

The production of flavones by angiosperms is not unusual. In fact, flavones are abundant in the plant kingdom, but the largest flavone containing taxon is the angiosperms (*10*). Futhermore, the presence of flavone compounds within the *Halophila* genus has previously been observed. Sulphated flavones have been noted in *H. ballonis, H. engelmanni, H. stipulacea,* and *H. ovalis* (*19*). In addition to *H. johnsonii*, glycosylated flavones have been observed in smaller leaved members of *Halophila* (*20*). Interestingly, and in support of their photoprotectant role,

the production of flavanoids appears to diminish in *H. johnsonii* when it is transplanted in deeper waters (*5*). A further striking feature is the apparent absence of flavones in the related species *H. decipiens* that grows in adjacent deep water areas, and thus may not require such photoprotectants. However, it is also possible that *H. decipiens* may produce different photoprotectant compounds. Regardless, a detailed investigation of the chemical constituents of *H. decipiens* seems warranted.

This study identified six flavone glycosides in *Halophila johnsonii* that we believe help protect this shallow dwelling seagrass from UV damage. This is the first characterization of secondary metabolites from this endangered species and sheds light on how this seagrass found its niche in the shallow waters of Biscayne Bay, FL. Our findings are consistent with the presence of flavones among most of the *Halophila sp.,* and the class and type of flavone may serve as a distinguishing characteristic between species and their geographic location. In addition to their biological functions, the distribution and type of flavones found among *Halophila sp*. May provide a useful chemotaxonomic tool in the classification of *Halophila* seagrasses.

Experimental

Plant material

Plant material was collected by Jennifer Kunzelman and provided courtesy of Dr. Michael Durako.

Isolation and Purification

The methanol extract from *Halophilia johnsonii* was initially fractionated by RP-C_{18} (ENVI Sep-Pak) chromatography using a 20-100% H_2O-MeOH gradient. The Sep-Pak column was first flushed with 100% MeOH to activate the column packing and flush any column bleed through. The column was then eluted with the methanol-water gradient. The 20 % H_2O-MeOH

fraction was then applied to a reversed-phase C_{18} column (Bakerbond 10 x 100 mm) and eluted with a methanol-water gradient. Combination of fractions yielded two samples, 2.0 mg and 0.5mg that absorbed light in the UVB range. These compounds were further purified with high performance liquid chromatography (HPLC) using a reversed-phase C_{18} column (Phenomenex Luna, 10 x 250 mm, 5μm) with a 5-80% H_2O-MeOH gradient containing 0.02% acetic acid (0.5 mL/min flow rate, 20 min) over 30 minutes. Both compounds were subjected to LC-ESIMS analysis (Waters Micromass ZQ) equipped with a reversed-phase C_{18} column (Phenomenex Luna, 2.5 x 150 mm, 5μm) and eluted with 5-80% H_2O-MeOH gradient containing 0.02% acetic acid (0.2 mL/min, 20 min) over 30 minutes.

Spectroscopy

^{1}H NMR and ^{13}C NMR were recorded using a 500MHz Brüker instrument at room temperature. All spectra were recorded in $CDCl_3$. Chemical shifts are presented as ppm (δ) with respect to the chemical shift of the solvent used. UV spectra were recorded with an Agilient 1100 UV detector in line with the HPLC system. ESI mass spectral data were obtained with a Waters Micromass ZQ linked to the Agilent HPLC instrument.

CHAPTER 2: ISOLATION OF ANTIMICROBIAL SECONDARY METABOLITES FROM MARINE FUNGI

Introduction

Infectious diseases were once considered curable illnesses, and if one antibiotic in the arsenal of drugs did not clear the infection, another would. However, that is no longer the case. Microbes resistant to not one but multiple therapies are common and represent a global problem (*21*). Ironically, hospitals are large reservoirs for breeding resistant bacterial strains. In fact, approximately 50% of hospital-acquired infections are methicillin resistant *Staphyloccocus aureus* (MRSA) and most are also resistant to other antibiotics (*22*). *M. tuberculosis,* responsible for TB infections, has numerous multi-drug resistant (MDR) strains for which six to seven drugs are needed for effective treatment (*23*). With drug resistance on the rise and the number of reported MDR infections increasing, there is a great need for new antibiotic therapies.

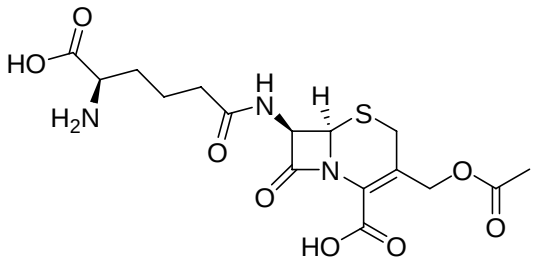
Clinically used antibiotics are compounds that selectively target a bacterial protein that is unlike a human protein in either function or structure. There are five main modes of action for antibacterial compounds: inhibition of cell wall biosynthesis, inhibition of protein synthesis, inhibition of RNA synthesis, inhibition of DNA synthesis, and inhibition of folic acid biosynthesis. The penicillins, cephalosporins, and glycopeptides (e.g. vancomycin) all inhibit cell wall biosynthesis. The beta-lactam antiobiotics (i.e. the penicillins and cephalosporins) inhibit of the cross-linking of the peptidoglycan layer of the bacterial cell wall. These compounds are generally selective for gram positive bacteria because gram positive bacteria have a largeer peptidoglycan layer than gram negative bacteria that have a very small peptdioglycan membrane layer. There are a number of antibacterial drug classes that inhibit the synthesis of bacterial proteins. The bacterial ribosome that is responsible for protein synthesis is

distinctly different in structure compared to the human or eukaryotic ribosome. Taking advantage of this, the aminoglycosides, macrolides, tetracyclines, and chloramphenical all bind to different portions of the bacterial ribosome halting protein synthesis. Because these drug classes inhibit protein synthesis they are largely bactericidal (causing cell death) agents rather than bacteriostatic agents (slowing cell gowth) like the drugs that inhibit cell wall biosynthesis. The quinolone antiobiotics inhibit the synthesis of bacterial DNA through the inhibition of DNA gyrase, an enzyme only contained in bacteria that is responsible for relaxing DNA before replication can occur. Finally, the trimethoprim and sulfonamide antibiotics inhibit bacterial synthesis of folic acid through inhibition of dihydrofolate reductase. There are a limited number of known selective targets for anti-bacterial agents and the number of novel drug targets has been declining (*24*). With antibiotic resistance posing one of the worlds most important public health issues, the discovery of novel antibacterial compounds is of utmost importance.

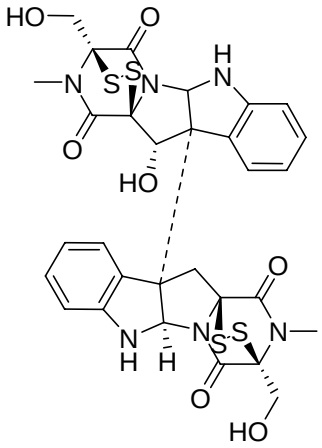
Natural products have been the greatest source of successful antibiotic compounds throughout history. The penicillins, cephalosporins, macrolides, tetracyclines are all antibacterial classes that were derived from natural product origins. Over the years, 80 % of the bioactive, natural products isolated have been antibiotic compounds, and this trend is continuing (*25*, *26*). Bacteria and fungi, in particular, have been a reliable source for the discovery of useful antibiotic compounds. For example, the penicillins and cephalosporins are both clinically used drug classes derived from fungal natural product structures. However, most of these leads have been derived from land-based or terrestrial organisms, and in recent times the discovery of totally new biologically active chemical compounds has diminished. In this respect, the oceans, which cover over 70% of the planet and are home to an enormous biodiversity of species, offer vast opportunities in the discovery of new bioactive natural products (*27*). Furthermore, there is

growing interest in exploring marine microbes such as fungi and bacteria as sources of new and unique secondary metabolites (*28-30*). Marine fungi are predicted to be a valuable resource in the search and discovery of novel natural products. It has been estimated that 90% of marine microorganisms, including fungi, have yet to be identified (*31*). For microorganisms, survival in the marine environment is highly competitive and comes with a number of environmental stresses that can stimulate the production of secondary metabolites (*32*). The isolation and chemical exploration of a number of marine fungal species have resulted in new structurally diverse natural products (*32, 33*), often displaying a variety of biological activities, such as anti-bacterial, anti-viral, and anti-protozoal activities.

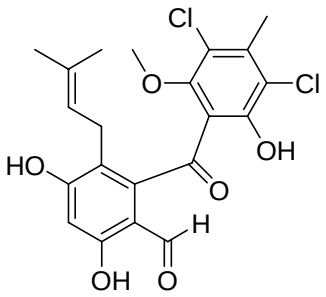
Some examples of obligate marine fungal products are shown in Figure 7. Cephalosporin C is the only marine fungal metabolite to have FDA approval as an antibiotic and falls into the beta-lactam class of drugs (*34*). Melinacidine III, one of the first marine fungal metabolites reported, is an antimicrobial compound isolated from *Corollospora puchella (33)*. Pestalone, a novel antimicrobial benzophenone, has been discovered in extracts of the marine fungus *Pestalotia sp*. and has shown activity against vancomycin resistant *enterococci* and MRSA (*35*). In addition to these examples of antibiotic marine fungal metabolites, other compounds have been identified with additional biological activities. Sansalvamide A is a depsipeptide compound with activity against the DNA topoisomerase of *Molluscam contagiosum* virus (MCV), a virus implicated in the effects of HIV (*36*). The macrolide aigialomycin D has been shown to be active against the malarial parasite *Plasmodiun falciparum* (*37*). Communesin A is an alkaloid isolated from a *Penicillin sp*. found to be associated with a marine alga (*38*). This compound and a derivative, communesin B, have shown activity against the P-388 human leukemia cell line. These are just a few examples of the novel, biologically active compounds discovered from



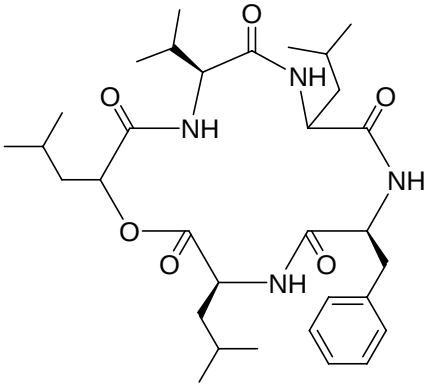
cephalosporin c



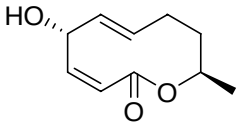
Melinacidine III



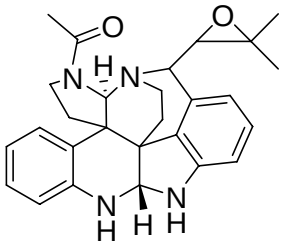
Pestalone



Sansalvamide a



Aigialomycin D



Communesin A

Figure 7. The structures of biologically active marine fungal natural products.

marine fungi that provide support for the investigation of marine fungi as sources of novel antibiotic compounds.

Although marine fungi appear to be an excellent biological source for the discovery of natural products, there are a number of challenges associated with the successful laboratory culture of these organisms. To begin with, not all fungi isolated in the field can be brought into culture in the laboratory. Even when culture is possible, marine fungi can be naturally slow growing, and many factors of the culturing process can alter the growth rate of the organism. Culture temperature, pO_2, pCO_2, pH, and nutrient variations have all been shown to affect the rate of fungal growth in culture (*39, 40*). Furthermore, even when laboratory cultures can be maintained, the production levels of secondary metabolites may be very low, necessitating large culture volumes in order to provide sufficient material for the complete structural characterization of a new compound. Many of these factors have been cited as problems in the development of marine pharmaceuticals (*41*). Even with the many challenges associated with the exploration of marine fungal natural products, these organisms still represent a source of natural product diversity with biological activity worth investigating. We have examined a number of obligate marine fungi for the production of antimicrobial compounds and identified organisms producing interesting natural products.

Results and Discussion

From organisms originally isolated and identified by the Kohlmeyers (*42*), a subset of 30 obligate marine fungi, maintained in culture at the Center for Marine Science, formed the basis for this study. Organic extracts of these fungi were screened for the presence of anti-microbial compounds using disc diffusion assays against two gram positive and two gram negative bacteria *Bacillus subtilis, Staphalococcus epidermis, Pseudomonas aeruginosa,* and *E. coli*, as well as for

the production of antifungal compounds using *Candida albicans*. Five of the marine fungal strains were found to produce anti-bacterial compounds in culture, but none of the fungi yielded extracts that displayed growth inhibition of *Candida albicans* (Table 2). Of those fungal extracts with detectable levels of anti-microbial activity, the extract of *Camarosporium I* show activity against gram-positive bacteria only. This suggests that the antimicrobial compound produced by *Camarosporium I* is likely to target the cell-wall biosynthesis of gram-positive bacteria in a manner such as the penicillins and cephalosporins. The extract from *Humicola alpallonella* showed biological activity against gram-negative bacteria only. Small ribosomal and non-ribosomal peptides have shown to be gram-negative selective agents, and the natural product produced by *H. alpallonella* could potentially act in that manner. Three extracts from *Sarcodella rhizophorae*, *Corollospora quinqueseptata*, and *Tiarosporella halymra* showed anti-bacterial activity against both gram-positive and gram-negative bacteria. The natural products being produced by these organisms are likely to target bacterial protein synthesis or one of the enzymes involving the replication and transcription of DNA as both of these mechanisms effect gram positive and gram negative organisms equally. These fungi could also be producing multiple anti-bacterial compounds with different bacterial targets.

The five fungi displaying anti-bacterial activity were grown in 6 L batches with the aim of producing enough biomass to yield sufficient material to permit the isolation and purification of the active agents. Unfortunately, these fungi grew very slowly in liquid culture, and provided only small amounts of a crude organic extract. Consequently, cycles of growth and extraction were repeated many times in order to obtain sufficient amounts of material with which to progress forward with chemical purification and analysis (Appendix A). This slowed progress and prevented the accumulation of sufficient material for chemical analysis. However, the

Table 2. Antimicrobial activity of marine fungal culture extracts. **X** indicates biological activity. **nd** indicates no activity detected.

Fungi	Gram-positive Inhibitic	Gram-negative Inhibiti	C. albicans Inhibition
Antennospora salina	nd	nd	nd
Aquamarina speciosa	nd	nd	nd
Camarosporium I	**x**	nd	nd
Ceriosporopsis halima	nd	nd	nd
Corollospora californica	nd	nd	nd
Corollospora californium filifo	nd	nd	nd
Corllospora californium mariti	nd	nd	nd
Corollospora colossa	nd	nd	nd
Corollospora novofusca	nd	nd	nd
Corollospora pulchella	nd	nd	nd
Corollospora quinqueseptata	**x**	**x**	nd
Gaeumannomyces medullaris	nd	nd	nd
Halosphaeriopsis mediosetiger	nd	nd	nd
Humicola alopallonella	nd	**x**	nd
Juncigena adarca	nd	nd	nd
Leptosphaeria typhicola	nd	nd	nd
Lignicola laevis	nd	nd	nd
Lineolata rhizophorae	nd	nd	nd
Lulworthia sp.	nd	nd	nd
Lulworthia cfr.grandispora	nd	nd	nd
Massarina ricifera	nd	nd	nd
Massariosphaeria erucacea	nd	nd	nd
Papulospora amerospora	nd	nd	nd
Passasiniella obiones	nd	nd	nd
Periconia prolifica	nd	nd	nd
Periconia cfr. Prolifica	nd	nd	nd
Phaeosphaeria halima	nd	nd	nd
Phomatospora bellaminuta	nd	nd	nd
Sarcodoella rhizophorae	**x**	**x**	nd
Scirrhia annulata	nd	nd	nd
Tiarosporella halmyra	**x**	**x**	nd

ultimate reason for the lack of success was that the fungal cultures were withdrawn before completion of this project.

As the project progressed, and before the cultures became unavailable, the number of fungi in large-scale culture was reduced to two, *Corollospora quinqueseptata* and *Tiarosporella halmyra.* This selection was based on the biological activity displayed by extracts of these organisms, and that this activity was reliably and consistently found. By focusing on only two cultures, more culture volume could be devoted to only these two species. By adopting this strategy, an increased amount of crude extract (~200mg) was obtained compared to prior cultures (~30mg) when all five were in larger scale culture. Using bioassay-guided fractionation (Figure 8) it was found that *Corollospora quinqueseptata* produced three anti-microbial compounds. The ESI mass spectral data (Figure 9) suggested the $[M+H]^+$ or $[M+Na]^+$ ions for these antimicrobial compounds were m/z 537, 625, and 713 respectively. Interestingly, the spectra of each compound contained a fragment peak corresponding to a loss of 44 amu from the parent ion, suggesting the loss of a carboxylic acid moiety. The UV spectra for the compounds also suggested the compounds contained interesting functionality, and displayed strong primary absorption at 220 nm and an additional absorption at 300 nm (Figure 9). However, as previously mentioned, the fungal cultures became unavailable to us before sufficient material was obtained for further structural characterization.

Tiarosporella halmyra was also cultured in four growth cycles from October 2002 to June 2003 and 72 L of culture material was generated for this fungi. Biologically active compounds were found in the ethyl acetate extract from the broth only. Although full structural characterization was not made, structural information for this compound was obtained by mass spectrometry and NMR. The ultraviolet spectrum of the *T. halmyra* compound showed an UV

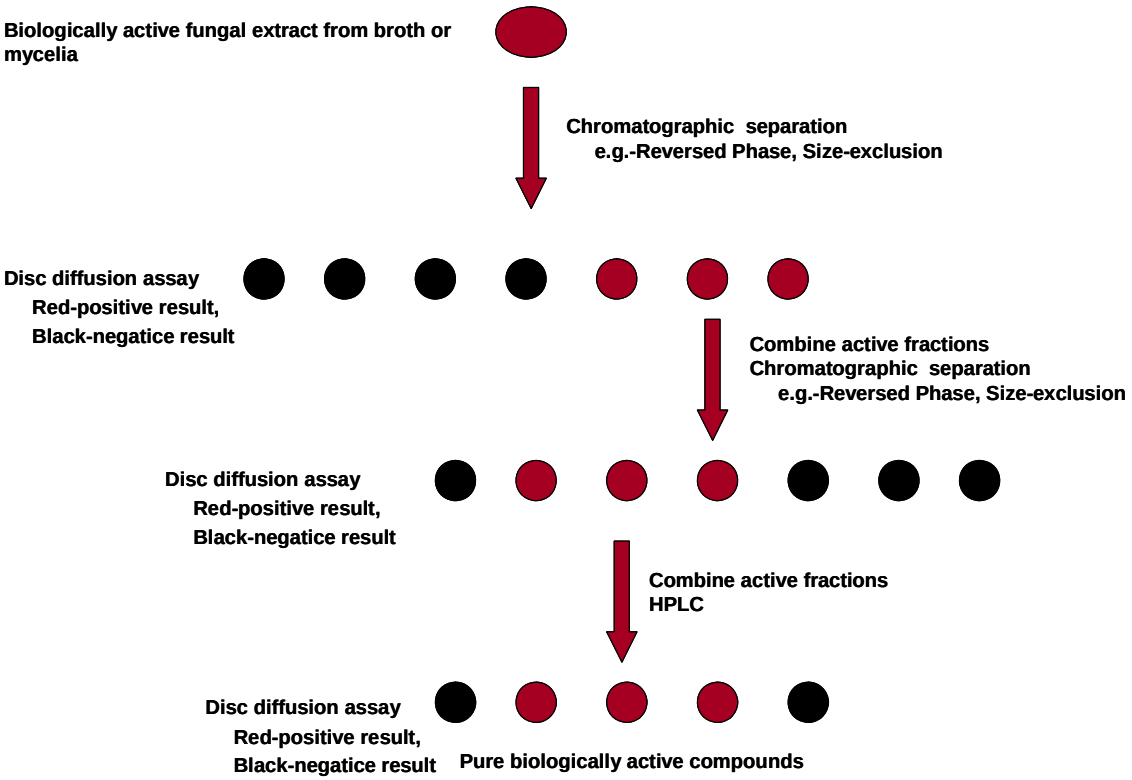


Figure 8. Schematic representing bioassay-guided fractionation of natural products.

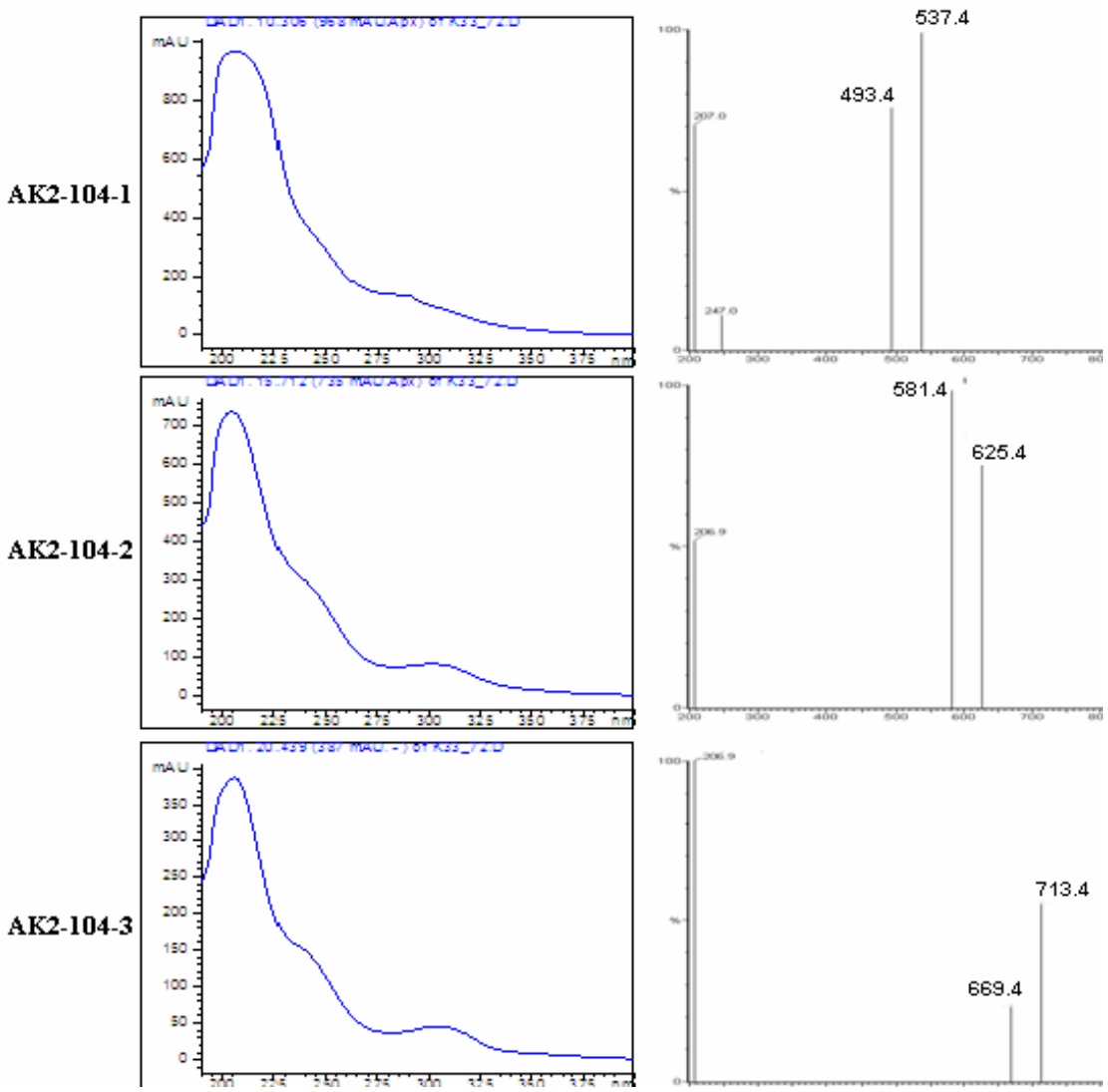
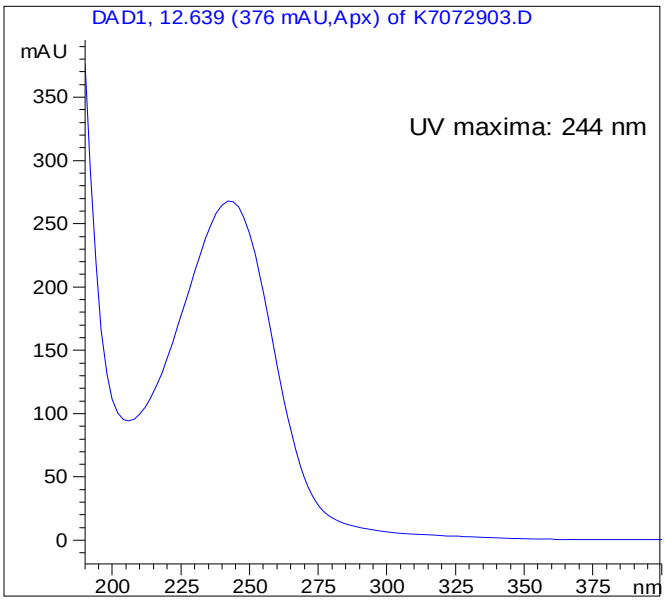


Figure 9. UV (left) and mass spectral data (right) of the anti-bacterial compounds isolated from *Corollospora quinqueseptata*.

maxima at 244 nm (Figure 10). This suggests the compound is composed of a simple conjugated diene system that would absorb around 215 nm and a few small modifications possibly an additional double bond or carbonyl that would extend that absorbance out to 244 nm. The most abundant ion from the mass spectral data suggested an $[M+H]^+$ at m/z 369 and an $[M+Na]^+$ at m/z 391 (Figure10). Two smaller peaks at m/z 337.2 and 305 are fragments ions with subsequent losses of 32 from the most abundant ion at m/z 369. An additional peak atm/z 441.3 was also present. The mass spectral data suggested a molecular weight of 368 Da or 440 Da for the *T. halmyra* metabolite. This datum suggested the compound cannot be an alkaloid because the molecular weight as determined by mass spectrometry appeared to be an even number. All alkaloids contain a nitrogen atom within a heterocyclic ring and possess an odd numbered molecular weight.

NMR data for the *T. halymra* compound was obtained and included ^{1}H, ^{13}C, DEPT135, HSQC and COSY. ^{13}C spectroscopy requires a greater sample volume than proton NMR spectroscopy due the lower abundance of ^{13}C in the environment. In order to obtain ^{13}C data, a small sample of <1 mg was used. The ^{13}C spectra suggested the sample was a mixture of two compounds, a major component with tall resonances containing ~12 carbons and a minor component containing ~ 30 resonances (Figure 11). The ^{1}H NMR spectrum for that sample (data not shown) also suggested the sample was composed of a major component and minor component. The chemical shift data in the ^{13}C spectrum suggested the mixture contained two natural products due to the interesting functionality of the compounds in the sample. The^{13}C spectrum contained resonances primarily in the aliphatic region from 10-30 ppm, but the spectrum also contained resonances indicative of carbons under oxygen or otherwise deshielded from an electronegative environment (40-80ppm), olefinic carbons between 100-150 ppm, and

a.



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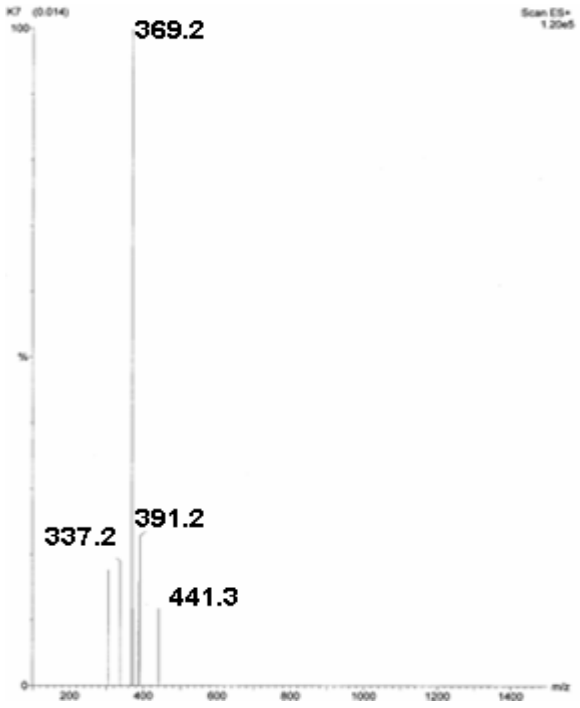
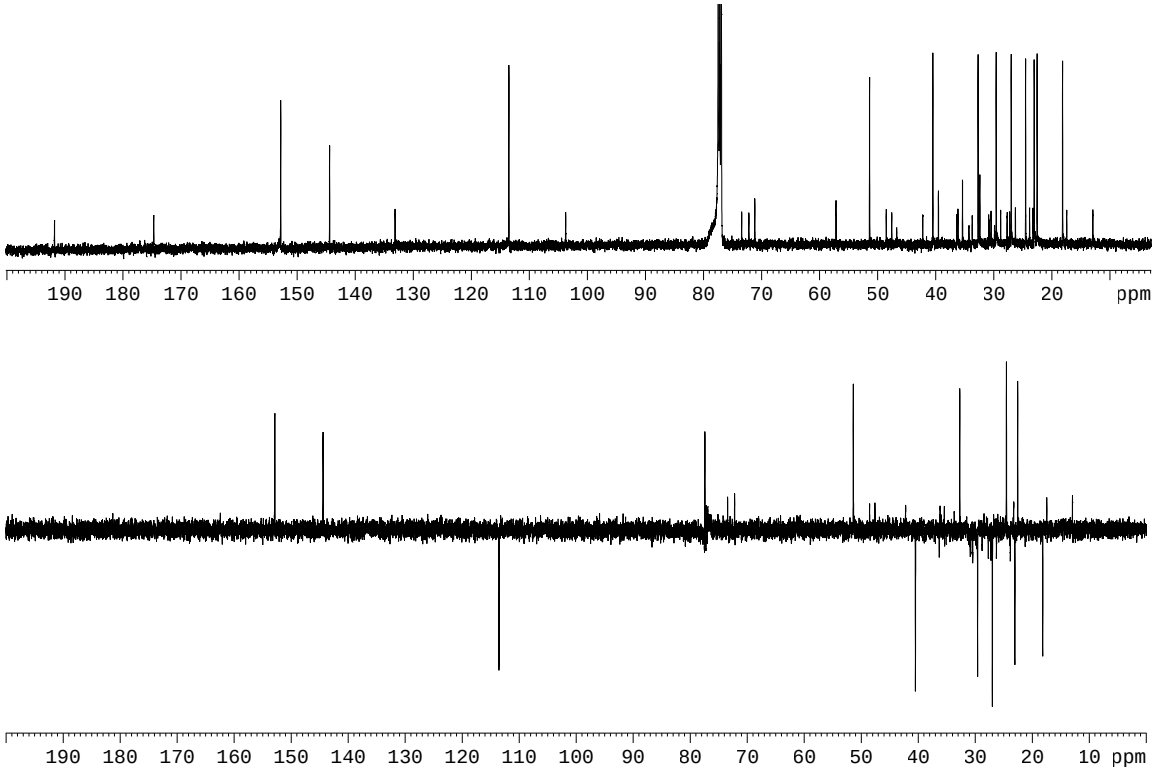


Figure 10. Spectral data for antimicrobial compound isolated from *T. halmyra*.
a. UV spectrum
b. ESI + Mass spectrum

a.



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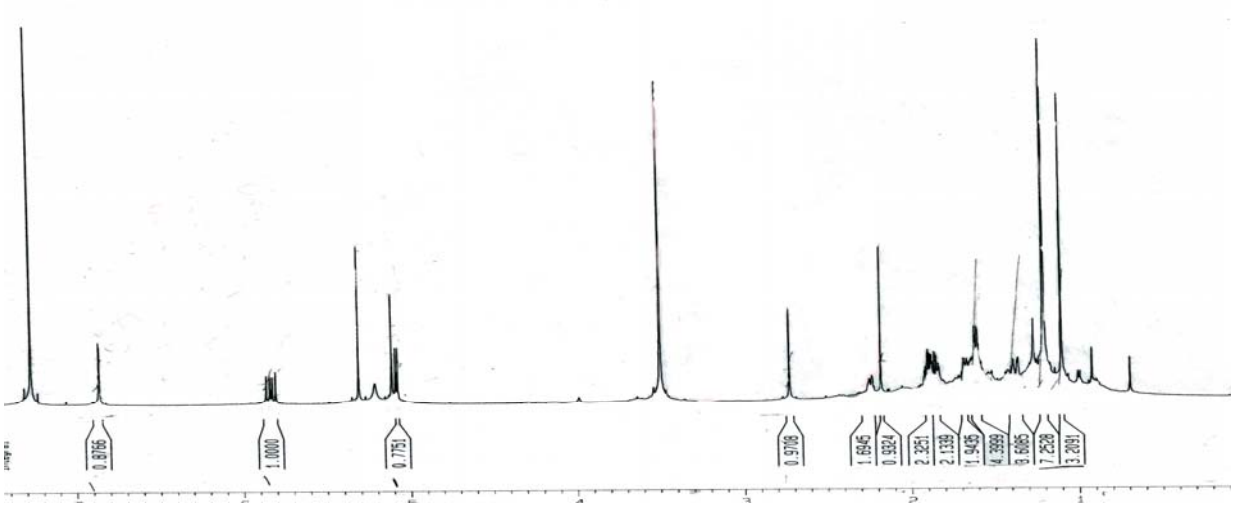

Figure 11. NMR spectral data for antimicrobial compound isolated from *Tiarosporella halmyra*.

a. ^{13}C NMR (top) and DEPT135 (bottom) spectra

b. ^{1}H NMR spectra

two carbonyl carbons around 170-200 ppm. DEPT 135 analysis of the sample revealed that the mixture contained approximately 13 quaternary centers (Figure 11). Because the resonances for quaternary centers are low in peak intensity, it was not possible to determine which compound in the mixture was associated with those quaternary resonances, or indeed how many quaternary centers the major component possessed.

^{1}H NMR and 2D spectroscopy of a more purified sample provided more structural information for the bioactive *T. halmyra* compound. The ^{1}H NMR spectrum displayed resonances in the aliphatic region (1-2 ppm), a deshielded resonance at 2.8 ppm, and olefinic resonances between 5-6 ppm (Figure 11). There were no aromatic resonances in the spectrum (7-8 ppm) or aldehyde resonances near 10 ppm. The 2D COSY (Figure 12) analysis for proton interactions of the sample revealed the olefinic doublet of doublets at 5.8 ppm interacts with a doublet 5.1 ppm. This suggested these two proton signals share a common double bond in the molecule. 2D HSQC spectroscopy for carbon-proton interactions showed that ^{1}H doublet at 5.1 ppm is attached to a carbon that resonates at 113 ppm (Figure 13). The carbon at 113 ppm was found to be a carbon bearing two hydrogen atoms from the DEPT 135 analysis (Figure 11). This suggested that the olefin bond between the two protons at 5.1 and 5.8 ppm is a terminal alkene. The splitting pattern on the ^{1}H spectrum for the doublet of doublets at 5.8 ppm had coupling constants with J= 7 Hz, and 12 Hz that are also consistent with a terminal alkene (*43*). Further analysis of the COSY data revealed that another olefinic proton at 6.8 ppm is not coupled with another olefinic proton suggesting this olefinic bond must contain a quaternary carbon. An olefinic bond containing a quaternary center is further supported by the presence of a quaternary carbon in the olefinic region of 100-150 ppm in the DEPT135 (Figure 11). The HSQC spectrum also revealed that ^{1}H signals at 5.15 and 5.3 ppm are not linked to any carbons in the molecule.

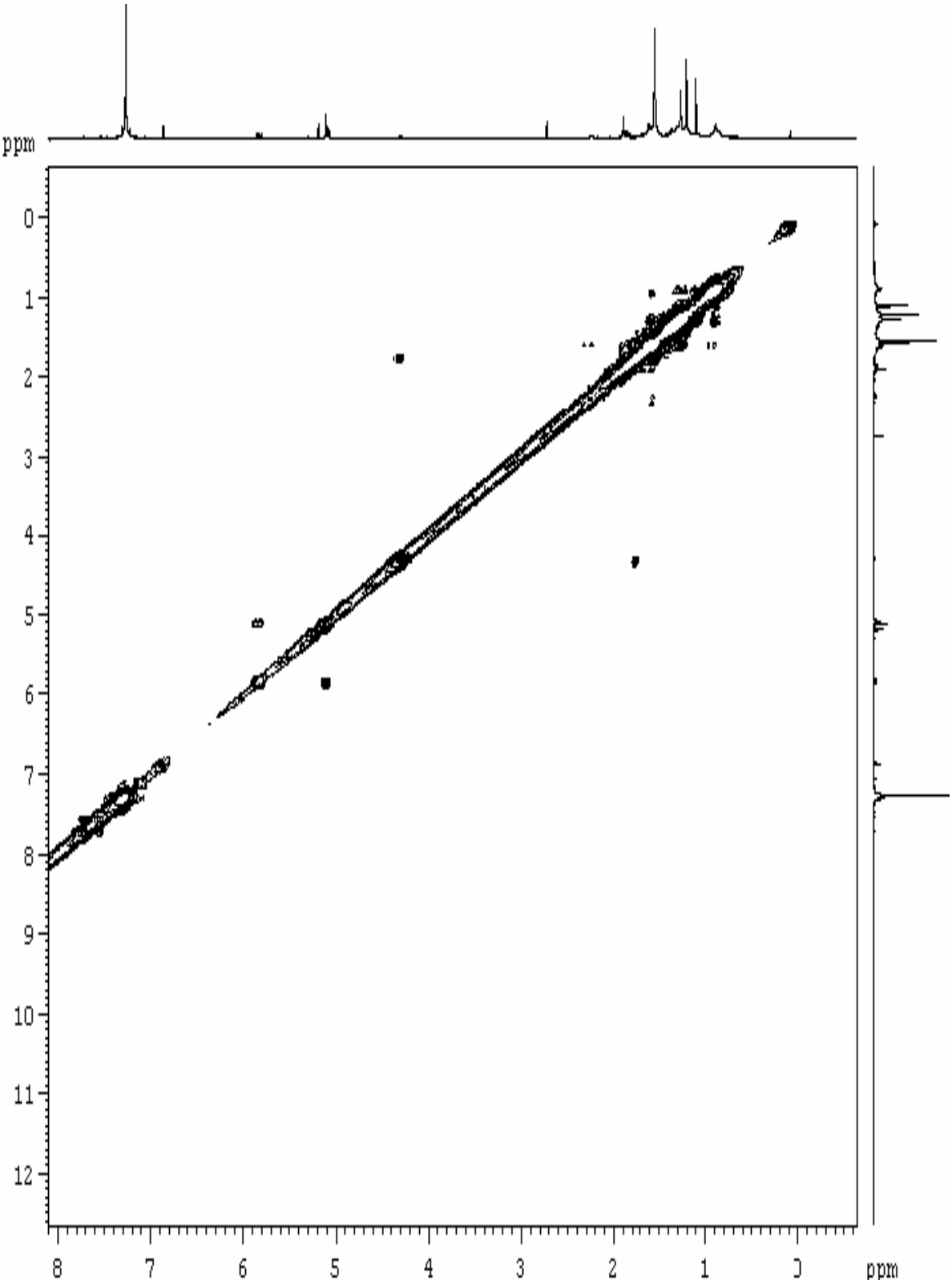

Figure 12. 2D COSY spectra for *Tiarosporella halmyra* compound

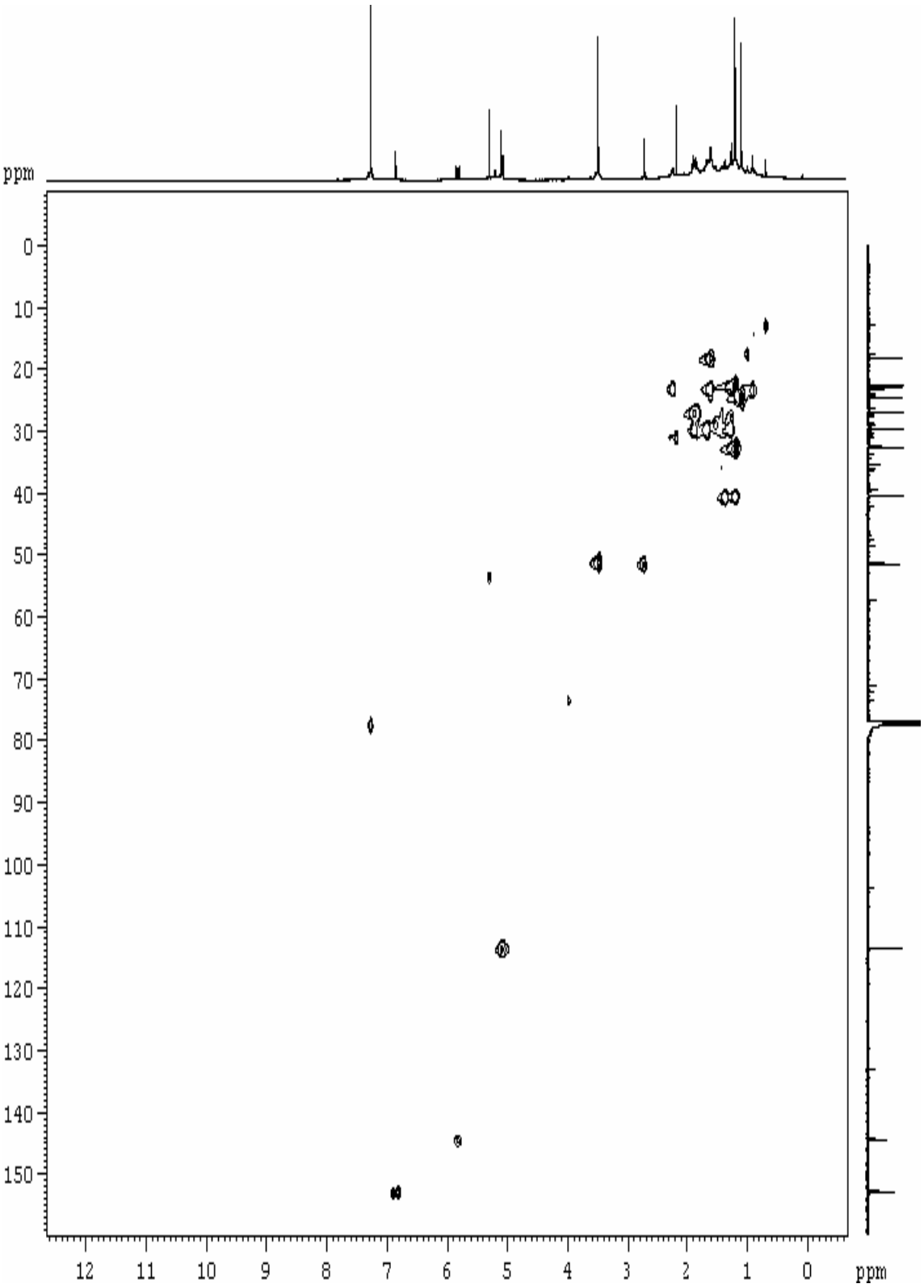

Figure 13. 2D HSQC spectrum for *T. halmyra* compound.

These singlet proton signals are hydroxyl proton signals in the molecule. The ^{13}C NMR and DEPT135 spectra revealed that there are two carbonyl carbons in the compound mixture that was analyzed. The proton singlet at 2.8 ppm that integrates to one proton in a deshielded signal that is consistent with a proton signal near an oxygen in the molecule. These data suggest that the *T. halymra* compounds contains at least one ketone moiety. The HSQC spectrum showed that the 2.8 ppm proton is linked to a carbon resonance at 51.4 ppm. DEPT135 analysis confirmed the 50.1 ppm carbon to be a CH bearing carbon. Together, the NMR data suggested that the *T. halymra* compound contains two olefinic bonds, a terminal bond as well as one involving a quaternary center, two hydroxyl substituents, a ketone carbonyl group, and a carbon bearing a single proton likely located next to the ketone moiety.

More structural detail of the *T. halymra* metabolite was mined from the aliphatic region of the spectroscopic data. The ^{1}H NMR spectrum contains two singlet resonances at 1.1 ppm and 1.2 ppm that integrated to 3H and 7H respectively. These resonances suggested the presence of three methyl group on the compound. The HSQC and DEPT135 spectra confirmed that the 1.1 ppm singlet was linked to a CH/CH_3 carbon at 24.6 ppm. The 7H singlet at 1.2 ppm was found to be linked to two CH/CH_3 carbons at 22.5 and 32.8 ppm. Because these three methyl groups were all singlets and not split by any other proton signals they all appeared to be attached to quaternary centers. Furthermore, the deshielded aliphatic carbon resonance at 32.8 ppm linked to a non-deshielded resonance at 1.2 ppm is characteristic. These resonances are consistent with reported values for gem-dimethyl groups attached to a quaternary center (*43*). The ^{1}H NMR spectrum also showed a number of signals with CH_2 splitting patterns between 1-2 ppm. Analyzing the HSQC spectrum, it revealed that in addition to the three methyl carbons on the major component of *T. halymra*, there were five additional carbons bearing hydrogens. All of

these carbons (18.1, 23.0, 26.9, 29.6, and 40.5 ppm) showed multiple ^{1}H interaction suggesting these carbons had at least two attached protons. The DEPT135 confirmed that these five carbon signals were CH_2 signals for the *T. halmyra* compound. The rigid and sharp shapes of the CH_2 peaks suggested that they were involved in a ring system. All of the structural information for the *T. halmyra* metabolite suggested that the compound has a ring system and is composed of at least 14 carbons: 3 -CH_3, 5 -CH_2, -CH, -C=CH, -HC=CH_2, and -C=O.

The many structural details for the *T. halmyra* compound obtained suggested that the metabolite was likely to be a terpenoid. This reasoning was based on the aliphatic nature of the compound, the non-aromatic ring system, the olefinic bonds, terminal methyl groups, as well as the carbonyl and hydroxyl moieties that are all common components of terpenoid compounds. Based on the number of identified carbons (14 carbons) in the major component it is likely that the compounds is a sesquiterpene composed of 15 carbons, the fourteen we identified and an additional quaternary center. It is also probable that the minor component of the *T. halymra* mixed sample is either a sester- or tri-terpene composed of 25 or 30 carbons respectively. Terpenoid antibiotics are not unusual and have been reported in the literature. In fact, in 2006, Rojas *et al* reported the characterization of aegicerin, a triterpene antibiotic showing biologically activity against resistant strains of *Mycobacterium tuberculosis* (*44*). Sesquiterpene antibiotics such as the series of lactones identified in *C. spinosa* are also found in the literature (*45*). Sesterterpene antibiotics have even been reported in marine fungi (*32*). Although full structural characterization of the *Tiarosporella halmyra* metabolite was not accomplished, it is likely that the metabolite is terpenoid in origin and appears to contain interesting functionality.

Experimental

Fungal material

The fungal samples were collected by Jan and Brigitte Kohlmeyer from a variety of marine environments. A subset of these fungal isolates were maintained in culture on agar plates at the mycology lab of the Center for Marine Science. Large-scale cultures, typically 6 L in volume, were grown in a liquid medium containing 38 ppt Instant Ocean, 0.5 g peptone, 1.0 g glucose, and 0.1 g yeast extract per liter of deionized water.

Fungal harvest and extraction

Sterile 1.5 L culture flasks containing growth medium were inoculated with a portion of fungal culture taken from stock cultures. The flasks were allowed to grow for a period of approximately eight weeks or until the top of the broth was covered with biomass. Fungi were harvested by separation of broth and mycelia. The spent culture broth was shaken against an equal volume of ethyl acetate (x 3) in a separatory funnel. The combined organic extract was dried ($MgSO_4$ anhydrous), filtered, and then concentrated *in vacuo* using a Buchi rotovap to yield the crude, organic broth extract.

The combined mycelia were frozen with liquid nitrogen then crushed in a mortar and pestle. The powdered cells were extracted with 80% aqueous methanol (100 mL/g cells), then filtered, and concentrated to dryness *in vacuo*. The extraction procedure was repeated twice, and the combined cell extracts were dried and weighed.

Biological Assay

Bacterial assay plates, culture slants, and broth culture media were prepared with Nutrient Agar (Bacto) and Luria Broth (Bacto). *Candida albicans* cultures and assay plates were prepared with potato dextrose agar (Bacto). Cultures of *E. coli* and *Pseudomonas aeruginosa* (gram negative) as well as *Staphylococcus epidermis, Bacillus subtilis* (gram positive), and *Candida albicans* were cultured for use in antimicrobial assays. Stock culture transfers were

performed regularly every two to four weeks. The slant cultures were transferred to broth for use in the antimicrobial assays.

Sterile assay plates were streaked with the test microorgamism. A 20 µL aliquot of each 1 mg/mL methanol-dissolved sample was placed on a disc (Schleicher and Schull Grade 740E-1/4in.). Erythromycin (15µg), Rifampin (25µg), Vancomycin (30µg), and methanol (20µL) were used for positive and negative controls. Plates were incubated at 37˚ C overnight and the results were taken the following day. A positive result was indicated by a zone of growth inhibition (mm) around the sample disc.

Isolation

Generally, the fungal broth and mycelia organic extracts were first fractionated by reversed-phase C_{18} chromatography using either a SepPak or open column and elution with H_2O-MeOH gradient. Each fraction was tested for biological activity using the suite of bioassays described above. After analysis, bioactive fractions were pooled and further separated by either size-exclusion (Sephadex LH-20: MeOH elution) or reversed-phase C_{18} open column chromatography (Bakerbond: elution with H_2O-MeOH mixtures). The resulting fractions were further tested for biological activity, and if still impure were further purified by C_{18} reversed-phase HPLC.

Spectroscopy

Mass spectral data were obtained on a Waters Micromass ZQ2000 instrument. Electrospray either in the positive and negative ionization mode was used with capillary 3.5V, variable cone voltage, extractor 5V, and RF lens 0.5V. ^{1}H and ^{13}C NMR data were obtained using a Brüker 500 MHz instrument. Spectra were recorded in $CDCl_3$ or d4-methanol.

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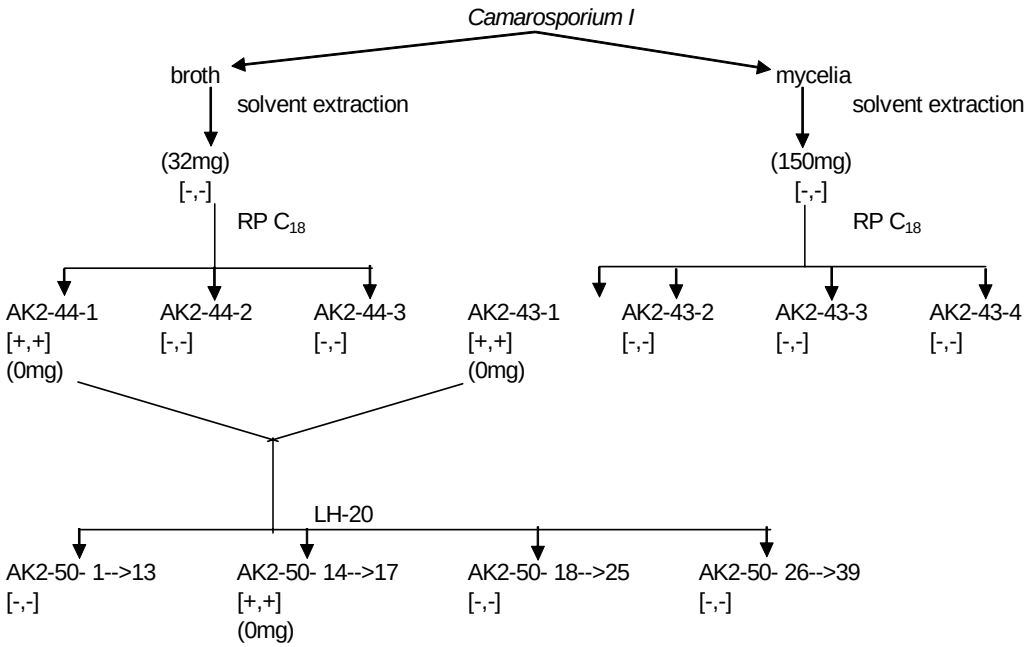
APPENDIX A

Appendix A: Detailed description of bioassay-guided fractionations of marine fungi.

Bioassay-guided fractionation of *Camarosporium I*

Approximately six liters of biomass of *Camarosporium I* (Fig. 1) was extracted (ethyl acetate) resulting in a crude, organic extract (32mg) and mycelia extract (190mg). The broth extract was applied to a reversed-phase C_{18} column (Bakerbond, 10 x 150 mm) and eluted with an aqueous methanol gradient (20, 30, 40, 50, 60, 70, 80, 100%) yielded twenty-seven fractions. The bioactive fractions were combined (2 mg). The mycelia extract was applied to a reversed-phase C_{18} (Bakerbond, 15 x 200 mm) column and eluted with a methanol-water gradient (20,30, 40, 50, 60, 70, 80, 100% meOH) resulting in twenty-six fractions. The bioactive fractions were combined (5mg) then added to the bioactive broth fractions. The bioactive material (7 mg) was applied to a LH-20 column (Sephadex, 10 x 150 mm). Elution with two column volumes of methanol gave thirty-nine fractions. The bioactive fractions were combined (<1 mg) and lack of sufficient material prevented further separation or spectral analysis.



*[Indicates gram-positive assay result, gram-negative assay result]

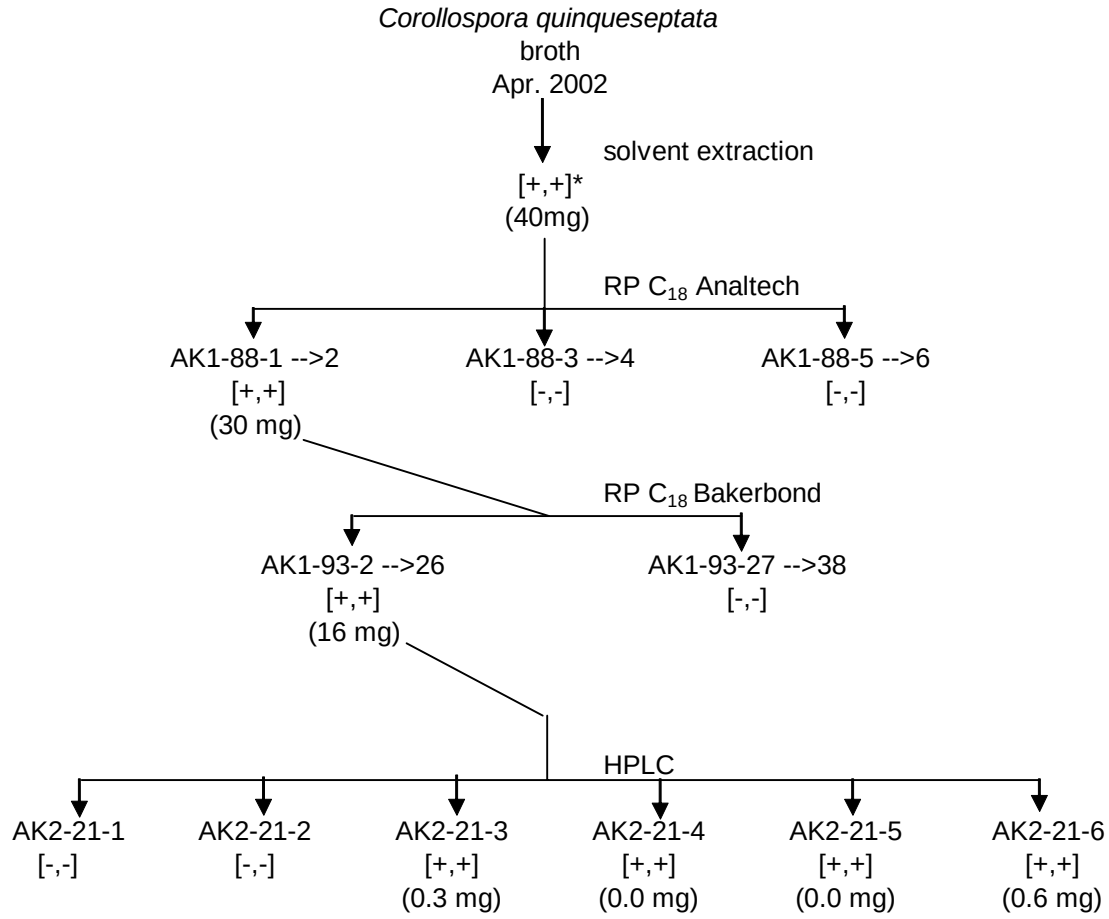
Figure 1. Bioassay guided fractionation of *Camarosporium I.*

Bioassay-guided fractionation of *Corollospora quinqeseptata*

In April 2002, 6 L of *Corollospora quinqeseptata* (Fig. 2) culture was harvested and a crude, organic broth extract (40 mg) and cellular extract (500 mg) was obtained. The broth extract was applied to reversed-phase C_{18} (Analtech 10 x 150 mm) column chromatography and eluted with a methanol-water gradient (20, 30, 40, 50, 60, 70, 80, 100%). The bioactive broth fractions (30 mg) were combined and applied to a reversed-phase C_{18} (Bakerbond, 10 x 100 mm) column. The column was eluted with 20, 30, 40, 50, 60, 70, 80, 100% methanol-water. The bioactive material was combined (5.8 mg) and subjected to further purification by HPLC on a Zorbax RP-C_{18} column (250 mm x 10 mm). Separation was achieved by a 20 minute gradient from 55-95 % meOH-water (2 mL/min). Four bioactive fractions were found: AK2-21-3 (0.3 mg), AK2-21-4 (0.0 mg), AK2-21-5 (0.0 mg), and AK2-21-7 (0.6 mg). The bioactive material was stored at -20˚ C.

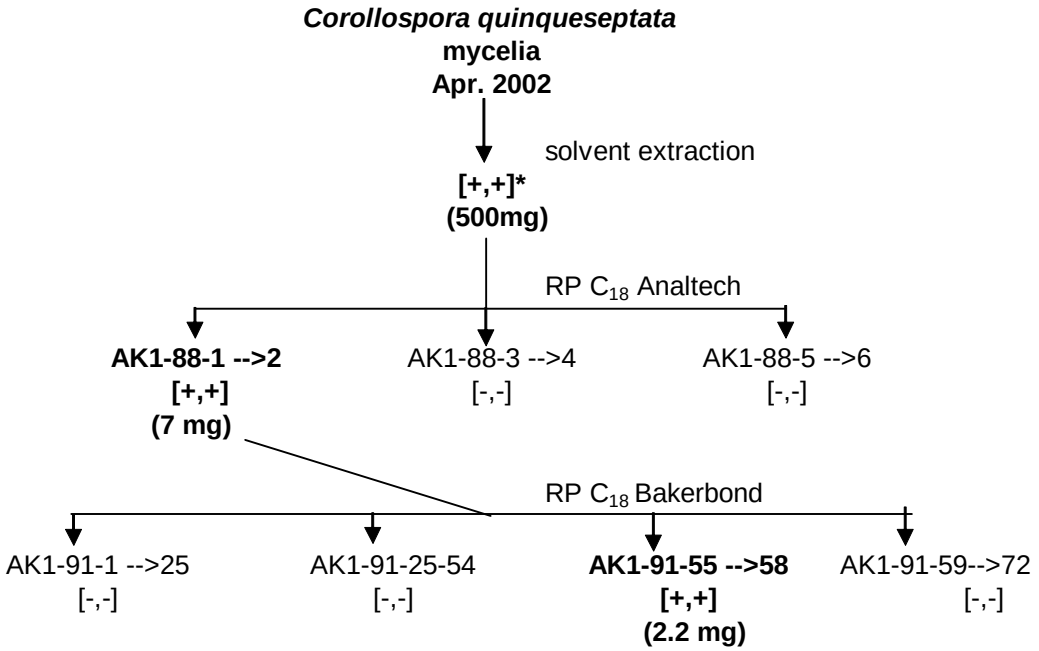
The mycelia extract (April 2002) (Fig. 3) was applied to a reversed-phase C_{18} column (Analtech, 15 x 200 mm) and eluted with a methanol-water gradient (20, 30, 40, 50, 60, 70, 80, 100%). The bioactive mycelia fractions (7 mg) were combined and applied to another reversed-phase C_{18} (Bakerbond, 10 x 100 mm) column. The column was eluted with 20, 30, 40, 50, 60, 70, 80, 100% methanol-water. The bioactive fractions were combined (2.2 mg) and held for more biomass.

In October 2002 (Fig. 4), 6L of culture was harvested. The organic extraction (ethyl acetate) of the 6 L of broth resulted in a crude, organic extract (52 mg). The extract was applied to a reversed- phase C_{18} (Analtech, 10 x 150 mm) column and eluted



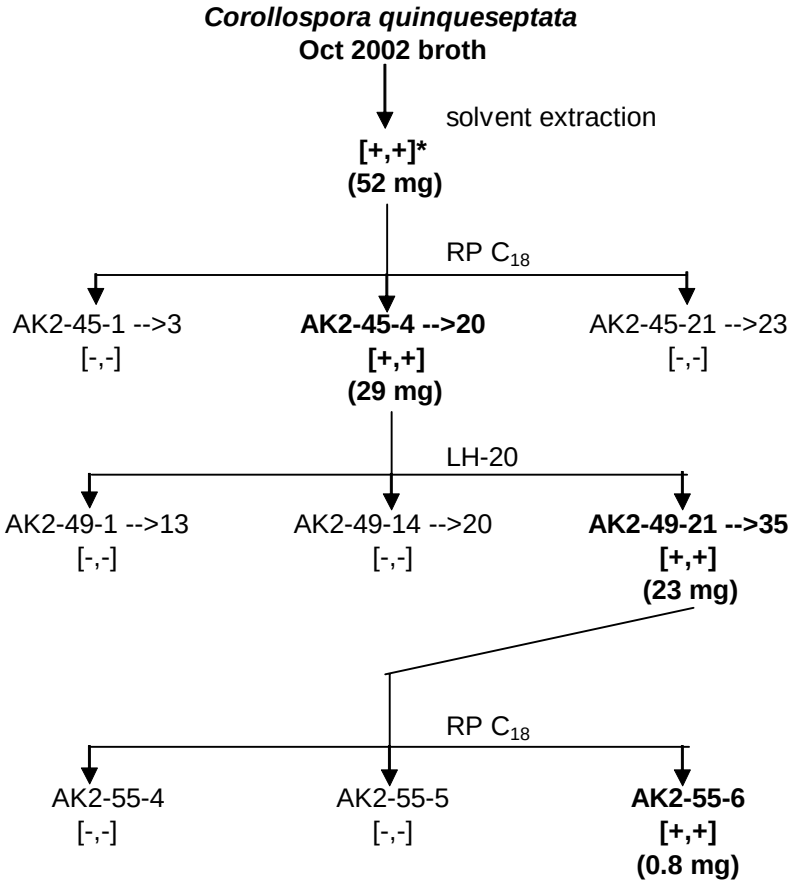
*[Indicates gram-positive assay result, gram-negative assay result]

Figure 2. Bioassay guided fractionation of *Corollospora quinqueseptata* broth in April 2002.



*[Indicates gram-positive assay result, gram-negative assay result]

Figure 3. Bioassay guided fractionation of *Corollospora quinqueseptata* mycelia in April 2002.



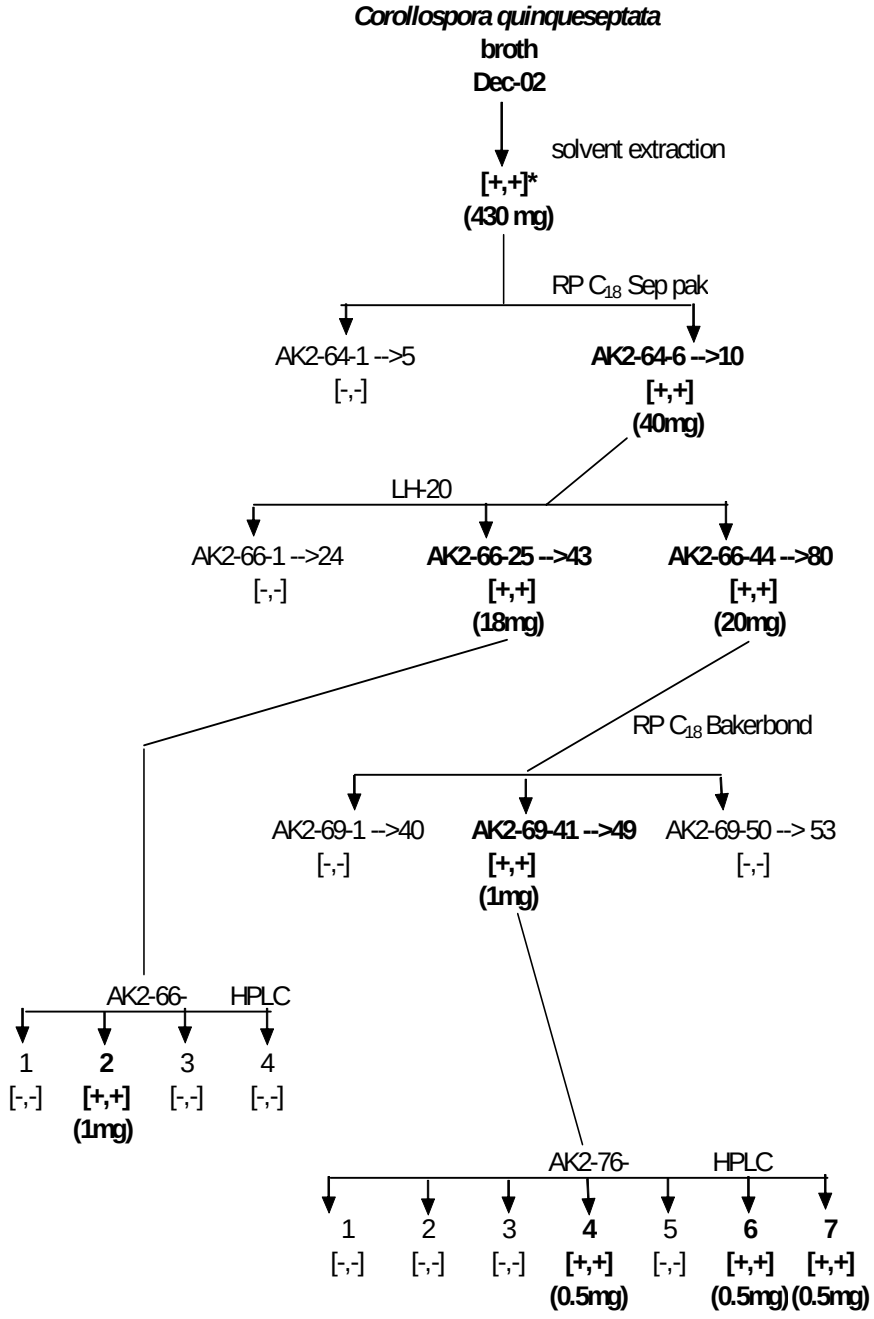
*[Indicates gram-positive assay result, gram-negative assay result]

Figure 4. Bioassay guided fractionation of *Corollospora quinqueseptata* broth in October 2002

with 20, 30, 40, 50, 60, 70, 80, 100% methanol-water. The bioactive fractions eluted in the latter part of the column and were combined (29 mg) and applied to a LH-20 column (Sephadex, 10 x 100 mm). The column was eluted with two column volumes of methanol. The bioactive fractions were combined (23 mg), applied to a reversed-phase C_{18} column (Bakerbond, 10 x 100 mm), and eluted with 20, 30, 40, 50, 60, 70, 80, 100% methanol-water. The bioactive fractions were combined as AK2-55-6 (0.8 mg) and used for LC-MS method development.

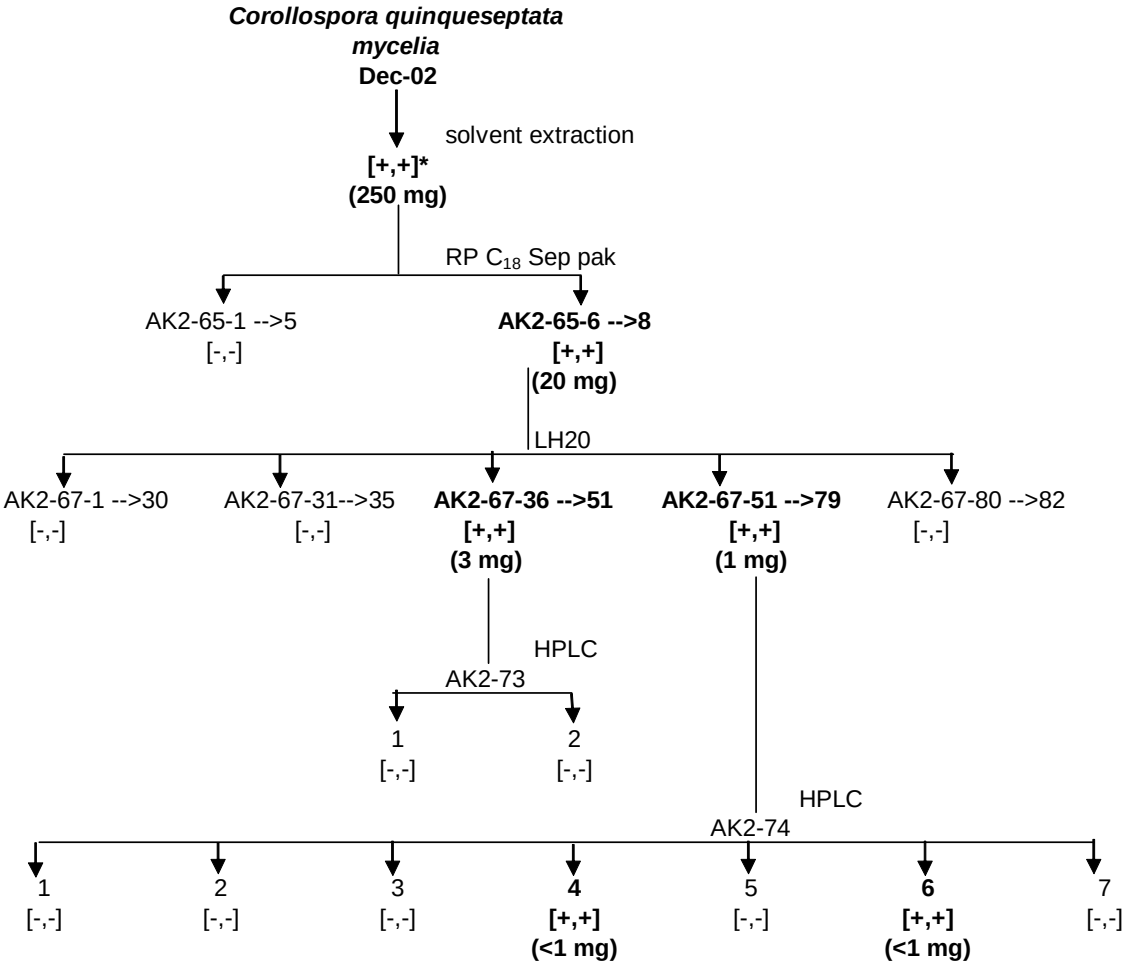
In December 2002, 16 L of *Corollospora quinqeseptata* (Fig. 5) was harvested. The organic extraction (ethyl acetate) of the 16 L of broth gave a crude, broth extract (430mg). The organic broth extract was applied to a reversed-phase ENVI-C_{18} Sep-Pak cartridge (Supelco, 5g) and eluted with 20, 30, 40, 50, 60, 70, 80, 100% methanol-water. The bioactive broth fractions were combined (40 mg), applied to a LH-20 column (Sephadex, 10 x 100 mm), and eluted with two column volumes of methanol. Based on differences in bioactivity, two bioactive fractions were formed (18 mg and 20mg). The 20 mg fraction was applied to a reversed-phase C_{18} (Bakerbond 10 x 75 mm) column and eluted with 20, 30, 40, 50, 60, 70, 80, 100% methanol-water. The bioactive fractions were combined (1 mg). The two bioactive fractions (18 mg and 1 mg) were subjected to further separation by HPLC on a Zorbax RP-C_{18} column (250mm x 10mm). Separation was achieved by a 20 minute gradient from 55-95 % meOH-water (2mL/min). Four bioactive fractions were found: AK2-66-2 (1 mg), AK2-76-4 (0.5 mg), AK2-76-6 (0.5 mg), AK2-76-7 (0.5 mg)

The mycelia (December 2002) (Fig. 6) was extracted (80% methanol-water) and gave a crude, organic extract (250mg). The crude extract was applied to a reversed-phase



*[Indicates gram-positive assay result, gram-negative assay result]

Figure 5. Bioassay guided fractionation of *Corollospora quinqueseptata* broth in December 2002.



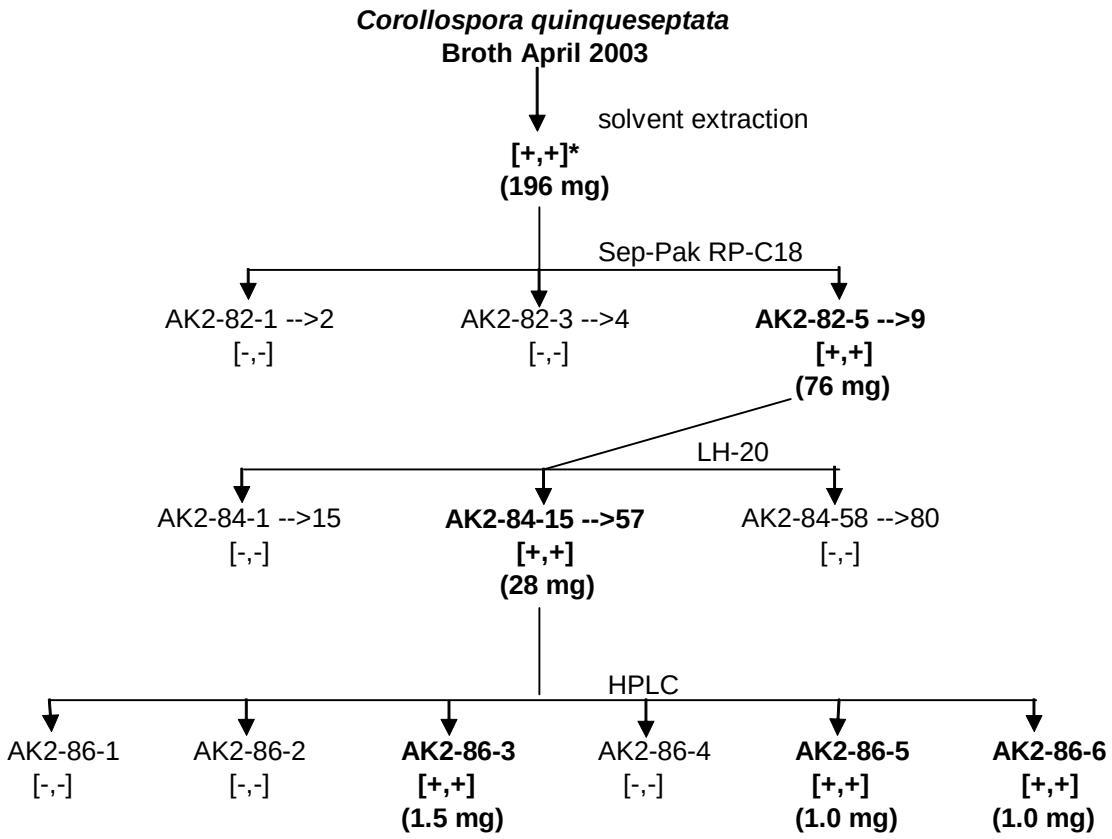
*[Indicates gram-positive assay result, gram-negative assay result]

Figure 6. Bioassay guided fractionation of *Corollospora quinqueseptata* mycelia in December 2002.

ENVI-C_{18} Sep-Pak cartridge (Supelco, 10g) and eluted with 20, 30, 40, 50, 60, 70, 80, 100% methanol-water. The bioactive fractions were combined (20 mg), applied to a LH-20 column (Sephadex, 10 x 100 mm), and eluted with two column volumes of methanol. Based on differences in bioactivity, two bioactive fractions were formed (1 mg and 3 mg). The two bioactive fractions were subjected further separation by HPLC on a Zorbax RP-C_{18} column (250mm x 10mm). Separation was achieved by a 20 minute gradient from 55-95 % meOH-water (2mL/min). Two bioactive fractions were formed: AK2-74-4 (0 mg) and AK2-74-6 (0 mg). All bioactive fractions were stored at -20˚C.

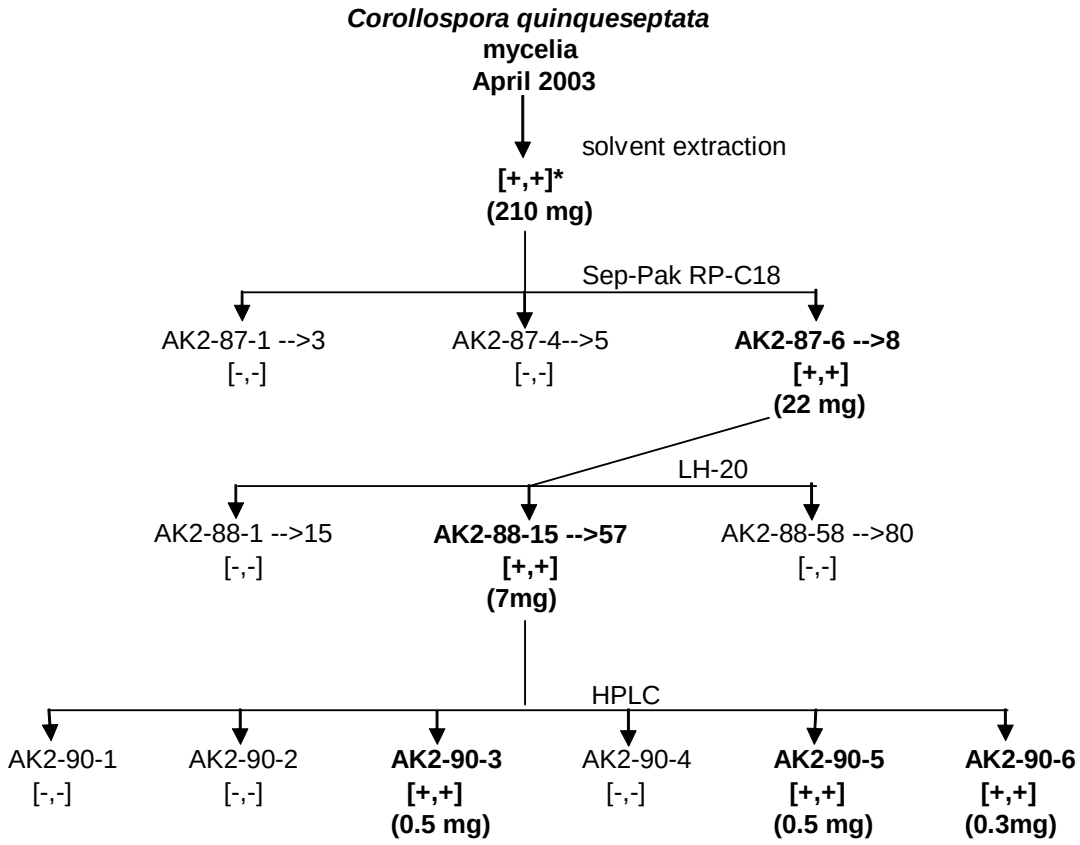
In April 2003, twenty-five liters of *Corollospora quinqueseptata* (Fig. 7) culture was harvested. Organic extraction (ethyl acetate) of the broth gave a crude, organic extract (196 mg). The crude broth extract was applied to an ENVI-C18 Sep-Pak (Supelco, 5 g, 20 mL) column and was eluted with 20, 30, 40, 50, 60, 70, 80, 100 % methanol-water. The bioactive fractions were combined (76 mg) and applied to a LH-20 (Sephadex, 10 x 150 mm) and eluted with two column volumes of methanol. The bioactive fractions were combined (28 mg) and purified by HPLC on a Zorbax RP-C_{18} column (250mm x 10mm). Separation was achieved by a 20 minute gradient from 55-95 % meOH-water (2mL/min). Four bioactive fractions were found: AK2-86-3 (1.5 mg), AK2-86-5 (1.0 mg), AK2-86-6 (1.0 mg).

The harvested mycelia (April 2003) (Fig. 8) was extracted (80% methanol-water) and gave a crude, organic extract (210 mg). The crude mycelia extract was applied to an ENVI-C18 Sep-Pak column (Supelco, 5 g, 20 mL) and eluted with 20, 30, 40, 50, 60, 70, 80, 100 % methanol-water. The bioactive fractions were combined (22 mg), applied to a LH-20 column (Sephadex, 10 x 100 mm), and eluted with two column volumes of



*[Indicates gram-positive assay result, gram-negative assay result]

Figure 7. Bioassay guided fractionation of *Corollospora quinqueseptata* broth in April 2003.



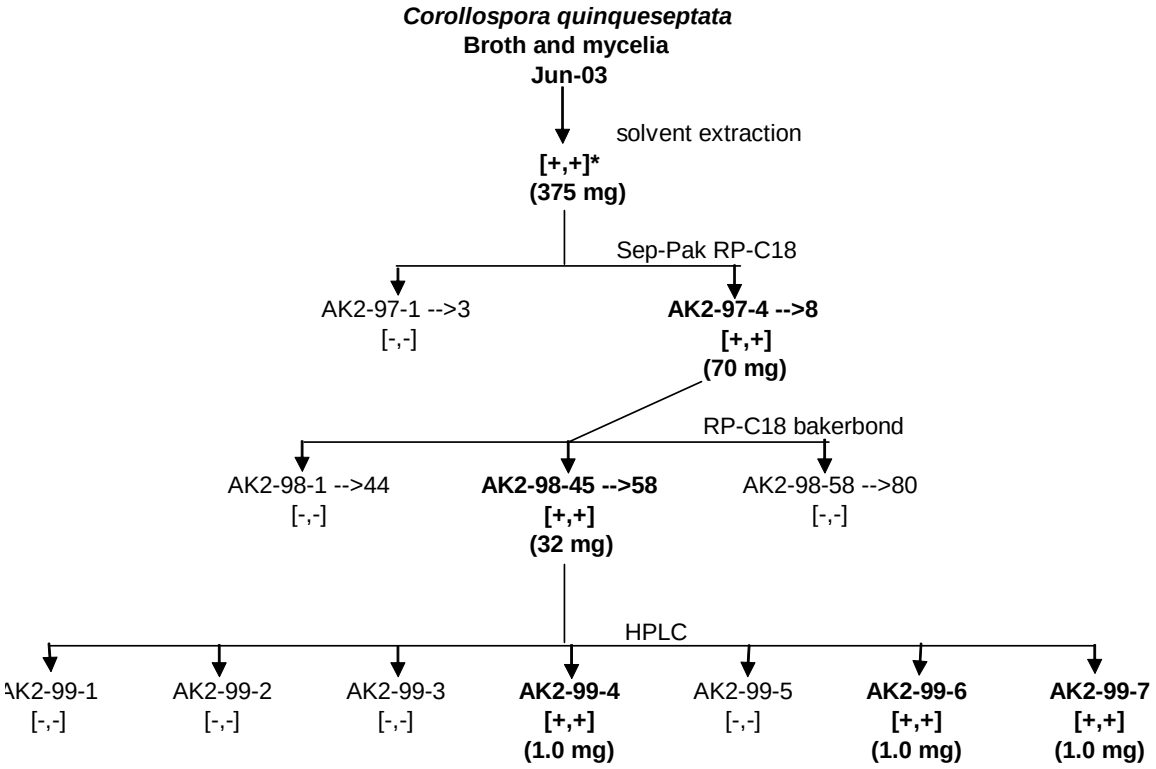
*[Indicates gram-positive assay result, gram-negative assay result]

Figure 8. Bioassay guided fractionation of *Corollospora quinqueseptata* mycelia in April 2003.

methanol. The bioactive fractions were combined (7 mg) and subjected to HPLC on a Zorbax SB-C18 column (55-95 % methanol-water, 2 mL/min, 20 min). Three bioactive fractions were identified: AK2-90-3 (0.5 mg), AK2-90-5 (0.5 mg), and AK2-90-6 (0.3 mg).

In June 2003, 25 L of *Corollospora quinqueseptata* (Fig. 9) culture was harvested. Organic extraction (ethyl acetate) of the broth gave a crude, organic extract (250 mg). Organic extraction of the mycelia (80% methanol-water) gave a crude, organic extract (125 mg). The crude broth and mycelia extracts were combined and applied to an ENVI-C18 Sep-Pak (Supelco, 10 g, 60 mL) column and was eluted with 20, 30, 40, 50, 60, 70, 80, 100 % methanol-water. The bioactive fractions were combined (70 mg), applied to a LH-20 (Sephadex, 10 x 150 mm), and eluted with two column volumes of methanol. The bioactive fractions were combined (32 mg) and purified by HPLC on a Zorbax SB-C18 column (72 % methanol-water, 3.36 mL/min, isocratic). Three bioactive fractions were identified AK2-99-4 (1.0mg), AK2-99-6 (1.0 mg), and AK2-99-7 (1.0mg)

All of the bioactive material purified since April 2002 was combined based on TLC, bioassay, and HPLC retention times. The fractions underwent final purification by HPLC on a Luna-C8 column (Phenomenex, 4.6 x 250 mm). Separation was achieved with a 12 minute gradient of 40-60% methanol-water (1.5 mL/min). Three bioactive fractions were isolated: AK2-104-1 (0.7 mg), AK2-104-2 (0.5mg), and AK2-104-3 (0.5 mg).



*[Indicates gram-positive assay result, gram-negative assay result]

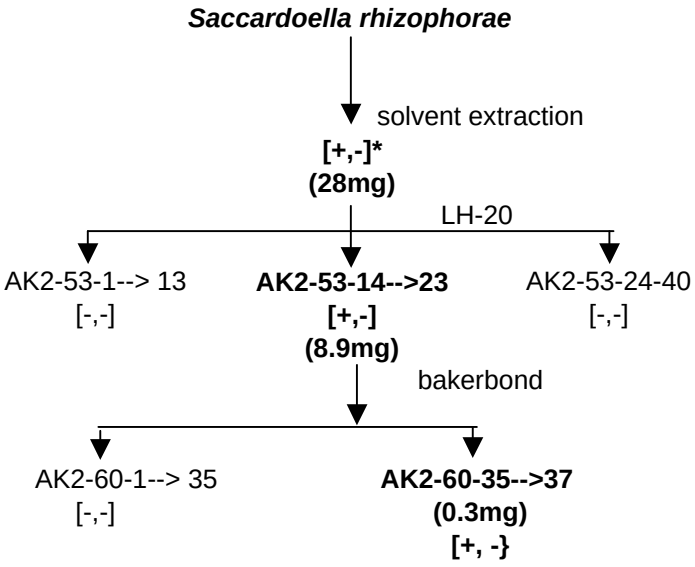
Figure 9. Bioassay guided fractionation of *Corollospora quinqueseptata* broth and mycelia in June 2003.

Bioassay-guided fractionation of *Saccardoella rhizophorae*

Organic extraction (ethyl acetate) of 5 L of *Saccardoella rhizophorae* (Fig. 10) culture was obtained in October 2002 gave a crude, organic extract (28mg). The extract was applied to a reversed-phase C_{18} (Analtech, 10 x 150 mm) column and eluted with 20, 30, 40, 50, 60, 70, 80, 100% methanol-water. After TLC and bioassay, the bioactive fractions were combined (8.9 mg). The bioactive fraction was applied to a LH-20 (Sephadex, 10 x 75 mm) and eluted with two column volumes of methanol, which were characterized by TLC and bioassay. The bioactive fractions were combined (0.3mg) and stored at -20°C.

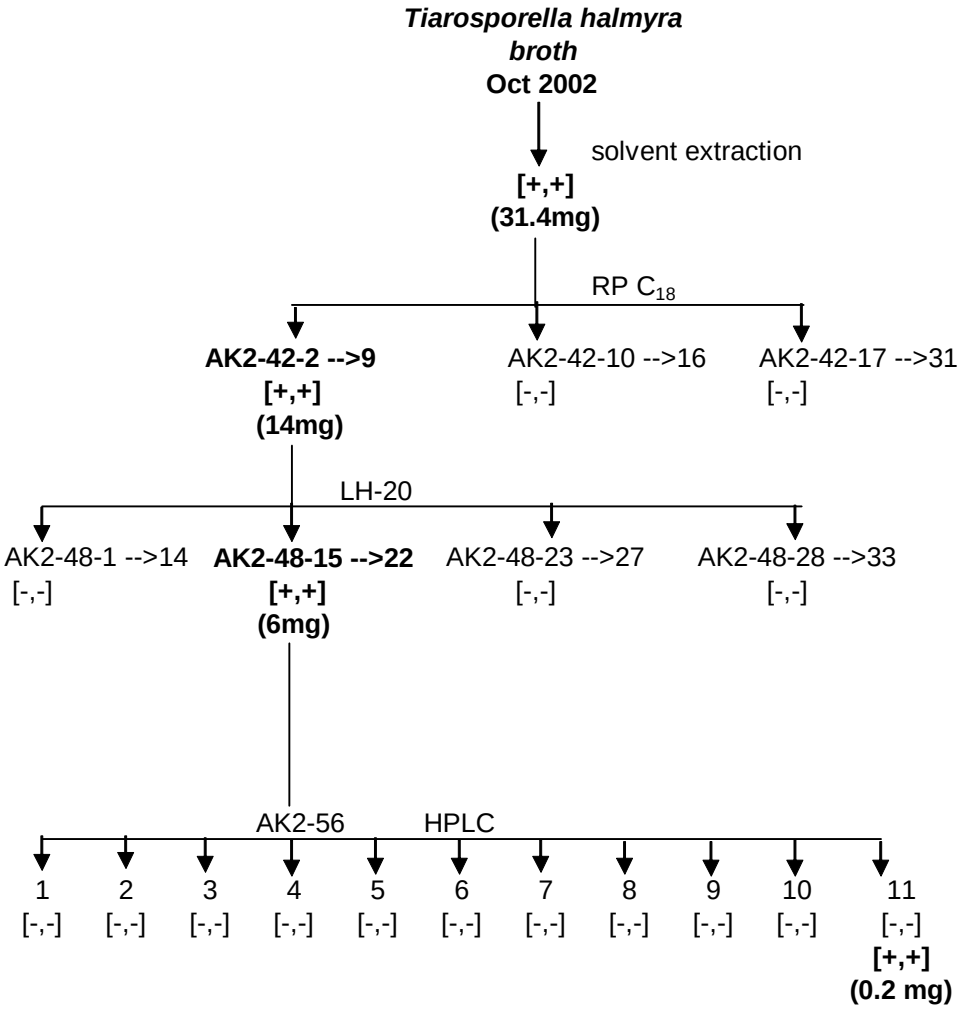
Bioassay-guided fractionation of *Tiarosporella halmyra*

In October 2002 (Fig. 11), 6.2 L of broth culture was harvested and extracted to yield a brown, oily crude extract (31.4 mg). The cellular material was held in a -20ºC freezer until the next harvest. The crude, organic, broth extract was subjected to a Reversed-phase C_{18} column (Bakerbond, 10 x 75 mm) and eluted with 20, 30, 40, 50, 60, 70, 80, 100 % methanol-water. The bioactive fractions (14 mg) were combined and applied to a LH-20 column (Sephadex, 10 x 75 mm). The column was eluted with two column volumes of methanol. The resulting bioactive fractions were combined (6 mg) and purified by HPLC on a Zorbax SB-C18 column (10 x 250 mm). Separation was



*[Indicates gram-positive result, gram-negative result]

Figure 10. Bioassay-guided fractionation of *Saccardoella rhizophorae*.



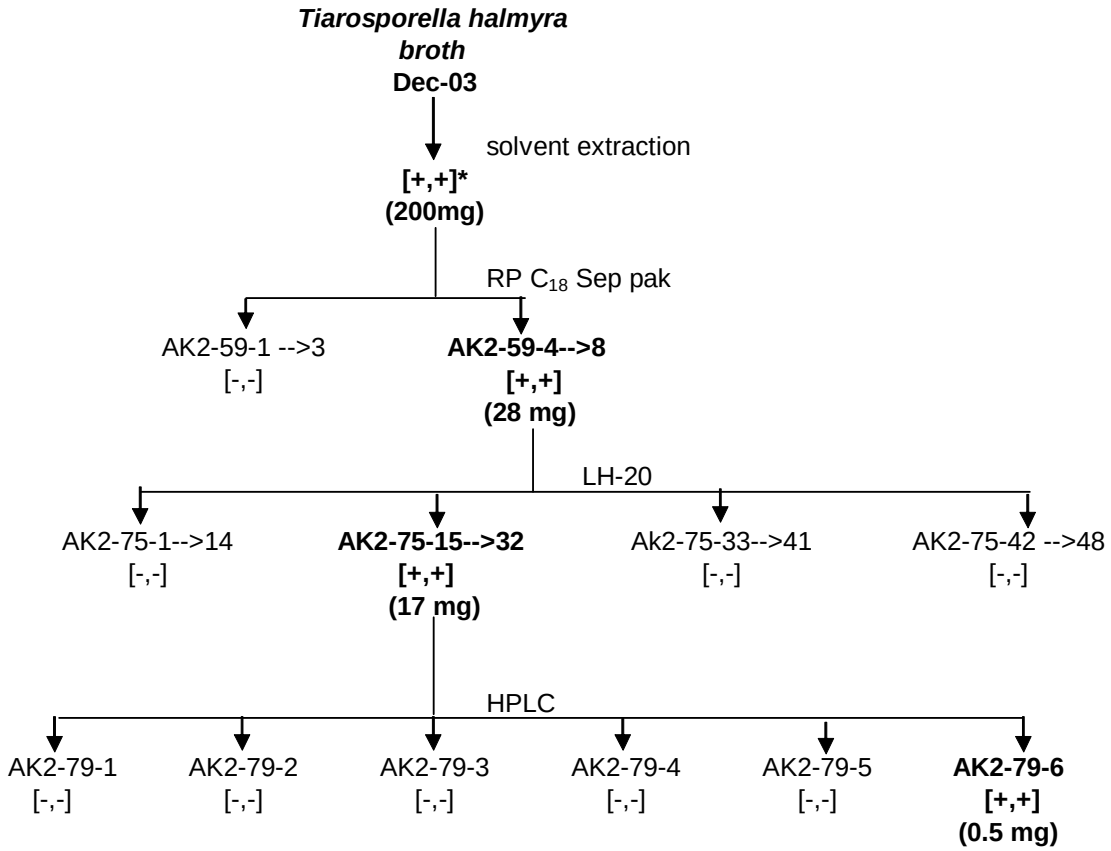
*[Indicates gram-positive assay result, gram-negative assay result]

Figure 11. Bioassay guided fractionation of *Tiarosporella halmyra* broth in October 2002.

achieved by an isocratic method of 21 % acetonitrile-water for twenty minutes (2 mL/min). AK2-56-11 (0.2 mg) was found to be bioactive.

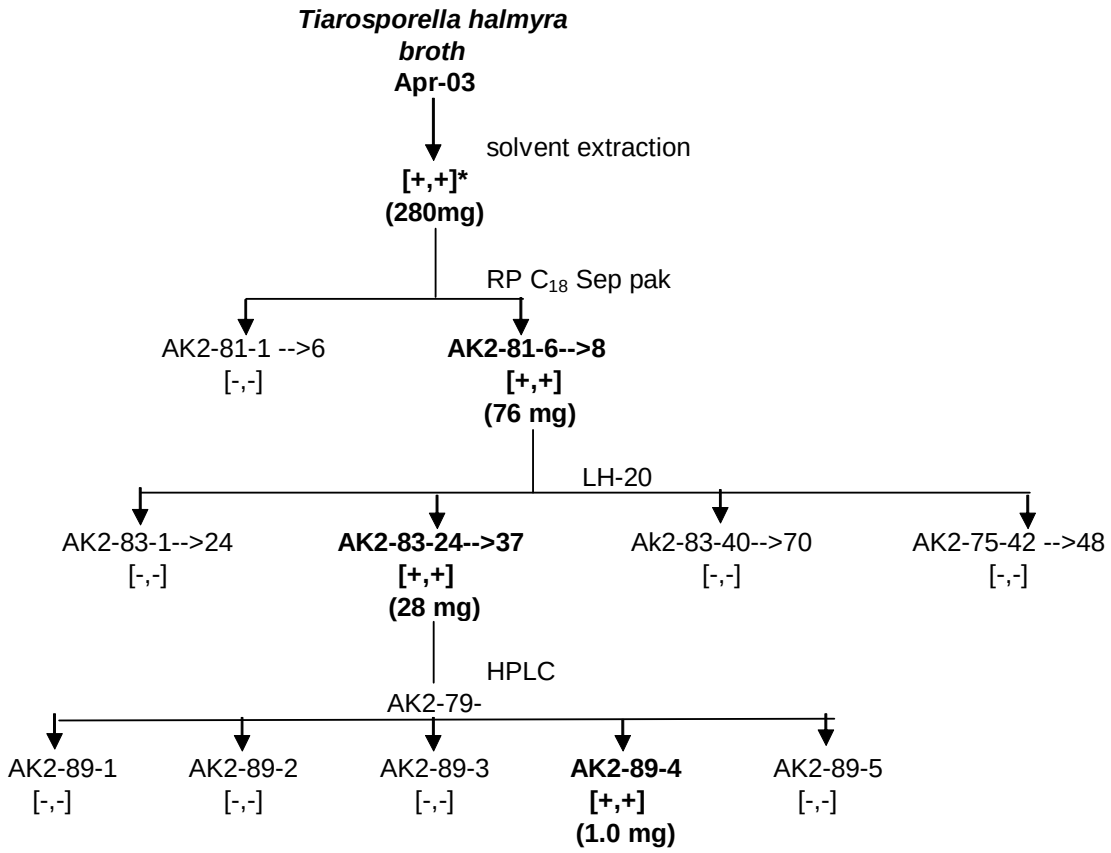
In December 2002 (Fig. 12), 16 L of broth and mycelia was harvested. Organic extraction (ethyl acetate) of the broth gave an oily, brown crude extract (200 mg). Organic extraction (80 % methanol-water) of the mycelia gave a crude organic extract (500 mg). The mycelia extract showed no bioactivity. The bioactive broth extract was applied to an ENVI-C18 Sep-Pak cartridge (Supelco, 5 g, 20 mL) and eluted with 20, 30, 40, 50, 60, 70, 80, 100 % methanol-water. The bioactive fractions were combined (28mg), applied to a LH-20 column (Sephadex, 10 x 150mm), and eluted with two column volumes of methanol. The resulting bioactive fractions were combined (17mg) and subjected to further separation by HPLC on a luna- C_{18} column (Phenomenex, 10 x 250 mm). Separation was achieved with 51% acetonitrile-water (20 min, isocratic 3mL/min) followed by 95 % acetonitrile –water (isocratic, 20 min, 3mL/min). A single bioactive fraction was found: AK2-79-6 (0.5 mg)

In April 2003 (Fig. 13), 25 L of culture was harvested. Organic extraction (ethyl acetate) of the broth gave an oily, brown crude extract (280 mg). Organic extraction (80 % methanol-water) gave a crude organic extract (550 mg). Again, the mycelia extract showed no bioactivity and was stored at -20°C. The bioactive broth extract was applied to an ENVI-C18 Sep-Pak cartridge (Supelco, 5 g, 20 mL) and was eluted with 20, 30, 40, 50, 60, 70, 80, 100 % methanol-water. The bioactive fractions were combined (76 mg), applied to a LH-20 column (Sephadex, 10 x 150 mm), and eluted with two column volumes of methanol. The resulting bioactive fractions were combined (28 mg) and subjected to further separation by HPLC on a luna- C_{18} column (Phenomenex, 10 x 250



*[Indicates gram-positive assay result, gram-negative assay result]

Figure 12. Bioassay guided fractionation of *Tiarosporella halmyra* broth in December 2002.



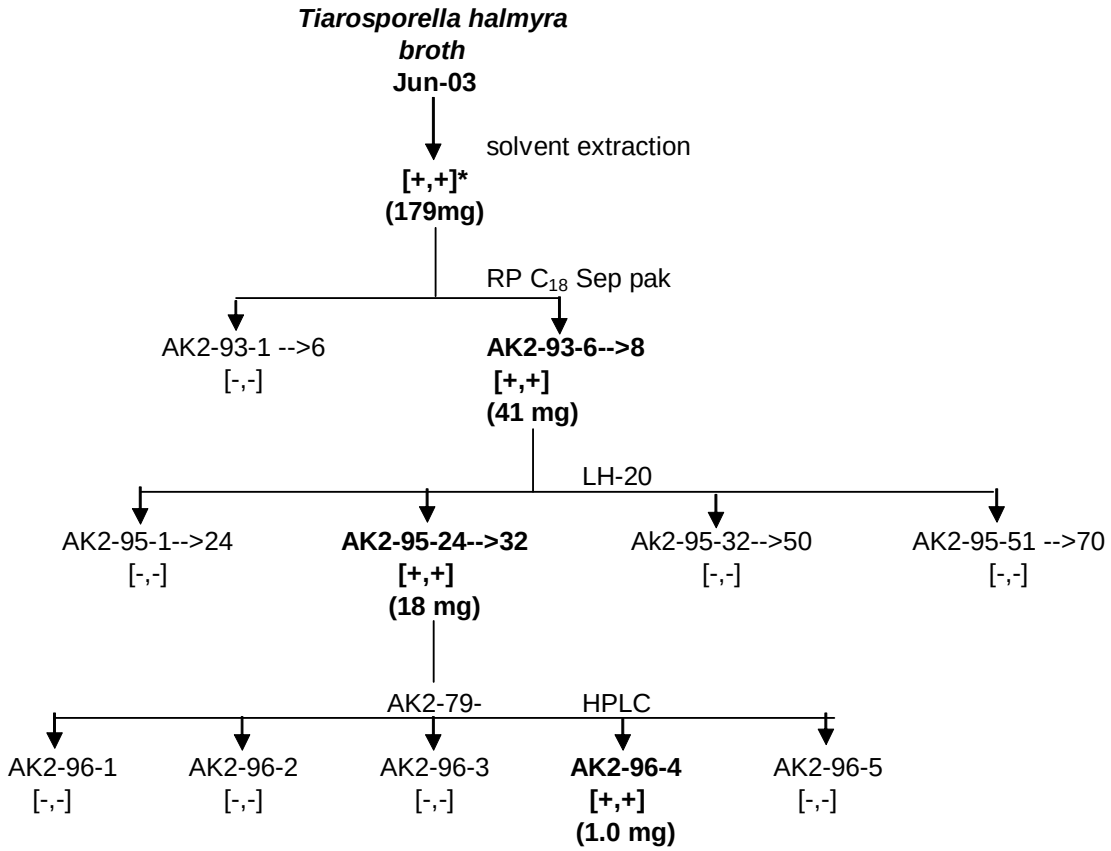
*[Indicates gram-positive assay result, gram-negative assay result]

Figure 13. Bioassay guided fractionation of *Tiarosporella halmyra* broth in April 2003.

mm). Separation was achieved with 51% acetonitrile-water (20 min, isocratic 3mL/min) followed by 95 % acetonitrile –water (isocratic, 20 min, 3mL/min). A single bioactive fraction was found: AK2-89-4 (1.0 mg)

In June 2003 (Fig. 14), 25 L of culture was harvested. Organic extraction (ethyl acetate) of the broth gave an oily, brown crude extract (179 mg). Organic extraction (80 % methanol-water) gave a crude organic extract (350 mg). The mycelia extract showed no bioactivity. The bioactive broth extract was applied to an ENVI-C18 Sep-Pak cartridge (Supelco, 5 g, 20 mL) and was eluted with 20, 30, 40, 50, 60, 70, 80, 100 % methanol-water. The bioactive fractions were combined (41 mg), applied to a LH-20 column (Sephadex, 10 x 150mm), and eluted with two column volumes of methanol. The resulting bioactive fractions were combined (18 mg) and subjected to further separation by HPLC on a luna- C_{18} column (Phenomenex, 10 x 250 mm). Separation was achieved with 51% acetonitrile-water (20 min, isocratic 3mL/min) followed by 95 % acetonitrile –water (isocratic, 20 min, 3mL/min). A single bioactive fraction was found: AK2-96-3 (1.0 mg).

The bioactive material from *Tiarosporella halmyra* was combined and applied to a Si-gel column (EM Science, 10 x 100 mm). The column was eluted with two volumes of 9:1 Chloroform:Methanol. The bioactive fractions were combined as AK2-101-1 (1.5 mg).



*[Indicates gram-positive assay result, gram-negative assay result]

Figure 14. Bioassay guided fractionation of *Tiarosporella halmyra* broth in April 2003.