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Anti-Malarial Activity Exhibited by Florida Mangrove Endophytes

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Contents

Abstract

Many organisms around the world including plants, marine organisms, and microbes release chemicals used for things such as pigment or defense against other organisms. These chemicals are known as secondary metabolites and have been found to exhibit promising activity against many infectious diseases and cancer. Natural products chemistry is the study of these secondary metabolites with one of the purposes to discover new small organic molecules for potential pharmaceutical use in the future. With parasitic diseases, such as malaria, prominent throughout the poorest regions of the world, there are great opportunities for discovering anti-malarial drugs from natural products. Approximately 250 million new malaria cases are discovered each year, and of these cases one to three million will not survive the disease. Anti-malarial drugs, such as chloroquine and quinine, have decreasing effectiveness, because the parasites have already formed strong resistance. For this reason, new pharmaceutical drugs are needed to combat this growing global catastrophe. This project focuses on finding new anti-malarial drugs from secondary metabolites produced by endophytic fungi and bacteria. Approximately, 900 filamentous fungi and bacteria have been isolated from Florida mangroves for this study. Their compounds were extracted in methanol and sent off for bioassay against *Plasmodium falciparum*. The active and partially active compounds were then scaled up and fractionated via Medium and High Pressure Liquid Chromatography and re-submitted for bioassay. Active fractions were studied with Nuclear Magnetic Resonance Spectroscopy.

I. Introduction

Malaria is a vector borne disease caused by *Plasmodium* parasites, with *Plasmodium falciparum* being the most infectious. Being a vector borne illness, pharmaceuticals for human ingestion in conjunction with mosquito pesticides must be used in order to obliterate the virus completely. Malaria primarily affects poor populations in tropical and subtropical areas, where the temperature and rainfall are most suitable for the development of the malaria-causing *Plasmodium* parasites in *Anopheles* mosquitoes (Greenwood et al. 2008). Malaria has not had the same disastrous effects on highly developed nations due to the financial capabilities of those governments, therefore making it possible to fund large anti-malarial operations. With funding at a minimal, malaria plays a major role in the fatality rates of developing nations. Childhood deaths in Africa due to malaria climbed relentlessly as chloroquine-resistant *Plasmodium* parasites spread across the continent (Greenwood et al. 2008). With resistance occurring more rapidly, an opening has formed for those capable to develop new anti-malarial drugs to replace the current and ineffective pharmaceuticals. When there is more diversity between the anti-malarial drugs, there is less of a chance for resistance to develop, therefore the more compounds capable of killing the parasite, the better the results.

The major force behind pharmaceuticals today is the development of synthetic derivatives to the already established cures. Penicillin is a classic example of utilizing the compound's skeleton to create similar synthetic derivatives to fight the bacterial cell walls. After many attempts have been made with resistance showing up faster with each derivative or combination treatment created, other sources must be found in order to decrease the chance of resistance in bacteria, parasites, and the like (Greenwood et al. 2008). Malaria is already showing signs of rapid resistance to new drugs, therefore more diversity is necessary to help those is dire need of a cure (Greenwood et al. 2008).

Natural products chemistry presents an alternative route to discovering new classes of compounds to endure greater diversity between drugs. Instead of just modifying compounds discovered from nature, natural products chemistry searches for completely new small molecules being produced naturally all over the world. Pharmaceuticals around the world have been derived from natural products, and without these original discoveries, their synthetic counterparts might not exist today (Baker 2007). Examples of these naturally occurring ailments are penicillin, aspirin, taxol, and artemisinin. Artemisinin in particular is a molecule discovered to treat malaria, and it was found in the plant *Artemisia annua,* a plant commonly used in Chinese Medicine practices (Vroman et al, 1999). Although artemisinin was an effective antimalarial drug temporarily, drug combinations with an artemisinin derivative and anti-vector measures are already underway around the world as the global community recognizes the need for more effective interventions (Greenwood et al. 2008). It is obvious that derivatives are not enough as resistance continues to prevail, and although three of the fore said pharmaceuticals were isolated from trees and plants, there is always the possibility that the compounds they are producing are the direct result of a symbiosis with microorganisms. Regardless of the producer, these are all examples of organisms producing chemicals that are not directly related to their primary metabolic processes. Rather, they are dubbed secondary metabolites, and can be used for chemical defense against predation, chemical signaling, pigmentation, and anti-fouling or other surface phenomena (McClintock & Baker, 2001). Especially in the case of an organism fearing predation, it can produce these secondary metabolites to deter being devoured by the predator. They can produce compounds that are toxic to their predators as well as produce the exotic pigmentations to forewarn them of these toxic secondary metabolites. For marine life in particular, overgrowth of biofouling organisms can have fatal consequences; therefore anti-fouling compounds are extremely useful secondary metabolites to produce and many organisms have done just that.

Since marine and coastal environments have added reasons to produce secondary metabolites, while also being greatly diversified, they are great candidates for drug discovery and chemical ecology. Mangroves inhabit both marine and terrestrial ecosystems, therefore one might assume they would produce outstanding natural products on their own, such as the mangrove Avicennia marin (white mangrove), which has been used in Chinese Medicine for the treatment of diabetes (Li et al 2004). Although macroorganisms have been found to be very chemically defended, as stated above, there are sometimes other organisms deserving recognition for their relationship to these macroorganisms (McClintock & Baker 2001). As alluded to before when speaking of penicillin, artemisinin, etc., there is the possibility of microorganisms producing these potent secondary metabolites. Many of the coastal plants or marine algae have been found to contain microorganisms living in symbiosis with their plant or algal host. These organisms [endophytes] reside in the living tissues of the host plant and do so in a variety of relationships, ranging from symbiotic to slightly pathogenic (Strobel & Daisy, 2003). These endophytes can be defined as microorganisms colonizing healthy plant tissue without causing overt symptoms in or apparent injury to the host (Li et al 2004). The endophytes living within the mangrove habitat have proved to be a rich source of new fungal species, and now form the second largest ecological sub-group of marine fungi (Li et al 2004). As predicted these mangrove endophytes contain a plethora of secondary metabolites of their own waiting to be discovered. Castillo et al recovered six novel bioactive streptomycetes from *Notofagus spp.* growing in Patagonian Chile. The isolates were characterized by molecular and morphological features and found to be active against such plant pathogens as *Phytophthora cinnamomi* and *Pythium ultimum* (2007). With mangrove endophytes as the target, the Florida coast was an obvious candidate for the growth of diverse microorganisms.

II. Method

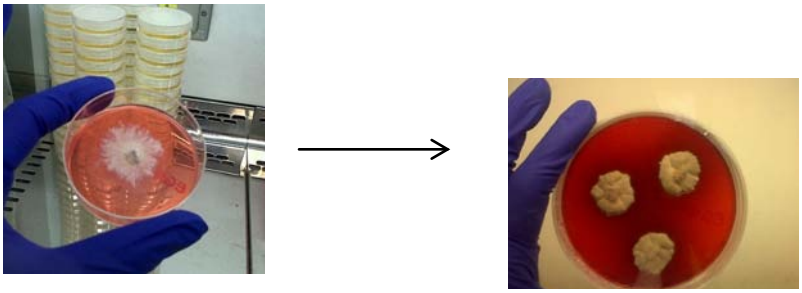
1. Collection

The greater the diversity of endophytes the better, therefore the mangrove samples taken were from different sections of branches and leaves of black, red, and white mangroves around Coquina Beach, Florida and the Everglades. Collections for Coquina Beach were done by walking

through the mangrove patches and cutting off the desired sections of mangroves greater than 5 feet tall. In the everglades, Jeremy Beau and his undergraduates canoed around specific areas of the everglades with exceptional mangrove growth. Of the samples collected, each branch was cut into five sections and surface sterilized. All surface sterilization and microbial work was done in a BSL-2 biosafety cabinet with tools flame sterilized after an isopropanol rinse. The surface sterilization consisted of a thirty second soak in bleach, followed by water, then an isopropyl alcohol rinse, and a final water rinse. The pieces were then divided into equal parts of four pieces per type of autoclave sterilized media. The types of media used were sabouraud dextrose agar (SDA), SDA containing the antibiotic p-chloramphenicol, Actinomyces media (1 L seawater, 10 g starch, 4 g yeast extract, 2 g peptone, 15 g/L SDA), potato dextrose agar, and "saw dust" agar, which was created from blended and freeze dried mangrove pieces. All of these different media help to produce the most diverse collection of bacterial and fungal endophytes. Actinomyces media was used to selectively culture those bacteria that are in the class actinobacteria. They look filamentous like fungi, however they have the properties of bacteria, therefore special media was used in the initial isolation to ensure actinomyces growth over other microbial endophytes.

2. Growth, Extraction, and Separation

As soon as growth was noticeable on the original cultures, individual isolates were precisely separated and placed on individual plates of SDA for fungi and trypticase soy agar (TSA) for bacteria. They were then incubated at 37°C for 18-21 days. If more than one isolate remained after the incubation time, those were further separated until only one isolate remained per petri dish of agar. In order to test these samples against *Plasmodium falciparum,* the sample size needed to be larger therefore increasing the concentration of the extract. Each individual bacterial or fungal isolate was scaled up into two larger plates of TSA or SDA, respectively and re-incubated for another 18-21 days.



The actinomyces samples were grown on actinomyces media for the first isolation step; however the following isolates were grown on TSA along with the other bacteria. All isolates were archived in test tubes with slants of their respective agar kept at 4°C and mycelia was placed in cryogenic vials with 20% Glycerol/Water kept at -80 °C. The incubated samples in petri dishes were then freeze dried and packed into 20 mL scintillation vials and extracted with methanol. Methanol was used because of its desirable broad range of polarity for extracting organic molecules. The methanol extract was then dried down and dissolved at 30mg/mL in dimethyl sulfoxide and submitted in 96 well plates for bioassay.

All samples collected from Coquina Beach, Florida were chemically investigated in addition to screening via bioassay guided fractionation, however the Everglades, Florida endophytes were only partially submitted for screening against Malaria due to time constraints. Samples showing activity from Coquina Beach had to be scaled up larger than the two plate sizes used for the bioassay in order to be chemically investigated. Therefore, some of those endophytes that were scaled up, were regrown from their archive in a two liter flask containing one liter of agar for 18 days. After following the same extraction process described above, they were then fractionated using normal and reversed phase medium pressure liquid chromatography (MPLC) and high pressure liquid chromatography (HPLC). These separations were done on a CombiFlaskRf Teledyne ISCO MPLC and a Shimadzu HPLC. The solvent systems used for the MPLC separations were hexane to ethyl acetate to methanol for normal phase and water to methanol for reversed phase. Only reversed phase HPLC was used with a 30 minute gradient from 100% water to 100% acetonitrile as a solvent system. Active fractions were analyzed via a 400 MHz Varian nuclear magnetic resonance spectrometer (NMR).

3. Bioassay

All bioassays were done in Dr. Dennis Kyle's lab with the University of South Florida's College of Public Health. The transgenic P. falciparum clone 3D7 expressing luciferase was grown in continuous culture using the Trager and Jenson method (1978). Chloroquine, dihydroartemisinin and atovaquone were used as the control drugs added to each set of assay plates. Positive and negative controls were included in each microtiter plate. Drug-free parasitized erythrocytes were the positive controls, while parasitized erythrocytes dosed with a high concentration of chloroquine or dihydroartemisinin were the negative controls. Assay plates were processed using Luciferase Assay System reagents purchased from Promega, data analysis was performed using Dataspects, Inc, and the EC50 was calculated using a non-linear regression analysis. Bioassay results displayed both active and partially active extracts and their cytotoxicity.

III. Results

Since the focus of this project was to find novel compounds to combat the Malarial causing vector *Plasmodium falciparum*, the microbial samples examined were all done via bioassay guided fractionation. Only the four fungal endophyte samples from the Coquina Beach collection were chemically investigated, whereas the Everglades samples only have bioassay results. The bioassay results for the Everglades microbes are shown in Table 1. The four samples shown in red have greater than 66% inhibition towards the parasite, and the remaining 10 samples in black show 33-66% inhibition. Further investigation into the activity of these samples is needed. Of all Everglades samples cultured and tested to date, there were a total of 14 active and partially active compounds out of 259 fungal and bacterial endophytes.

The samples from Coquina Beach resulted in four promising fungal isolates showing initial activity against Malaria out of 357 total isolates, as seen in Table 1. Of these four fungal endophytes, one continued to show greater than 66% inhibition at 5 and 50 μg/mL after the initial MPLC run. The other three samples all lost activity after the first separation, which could be due to synergistic effects between compounds or inconsistent bioassay results. A separation scheme can be seen in the flow chart of figure 1 showing all of the fractions collected and their respective masses. The initial MPLC separation of the fungal extract CQ10-20A-4 is shown in figure 2. The fractions eluted in approximately 100% ethyl acetate, D, E, and F as shown on the MPLC chromatogram for sample CQ10-20A-4, still exhibited greater than 66% inhibition at 5 and 50 μg/mL after the initial separation. These three fractions totaling 42 milligrams were combined and further separated using reversed phase HPLC. As seen in figure 3, there were 22 fractions separated from D, E, and F. Fractions 1-7 were not particularly UV active, so they were combined, while all other fractions were

analyzed via proton NMR spectroscopy. All 22 fractions were resubmitted for bioassay. Fractions 8 and 17 continue to show partial activity against the parasite, while fractions 13 and 14 still show greater than 50-90% inhibition at the IC50 values against *Plasmodium falciparum*. The ^{1}H NMR spectra of these four fractions are shown in figures 4-7. Of these four fractions, 13 and 14 hold the cytotoxicity of the initial crude submitted, however there is greater than a tenfold difference between the IC50 values for the therapeutic index and cytotoxicity, meaning these fractions remain plausible cures for Malaria.

In order to find what compound is responsible for this activity, further fractionation with HPLC must be done in conjunction with liquid chromatography-mass spectrometry (LC-MS) and carbon NMR. Although further detail is needed to identify the compound responsible for the promising anti-malarial activity in fractions 13 and 14, the ^{1}H NMR spectrum in figures 6 and 7 give some insight into the proton interactions. For example, at about δ_H = 0.75-0.85 in figure 6 there is a characteristic doublet peak of an isopropyl hydrogen. There are also multiple peaks of amines and olefins in both figures 6 and 7 at δ_H = 3.5-5.5.

IV. Tables and Figures

Table 1 – Bioassay Results for both Collections:

Coquina Beach Collection	Everglades Collection	
CQ10-20A-4	EG10-46C-3	EG10-66E-3
CQ10-36C-3	EG10-65C-3B	EG10-66B-3
CQ10-14C-1	EG10-65C-1	EG10-66C-4B
CQ10-18A-1	EG10-52C-2	EG10-47C-1
	EG10-30A-3	EG10-28B-4
	EG10-33B-1	EG10-30C-1
	EG10-33B-2	EG10-30D-3

Figure 1 – Separation Flow Chart of Active Sample CQ10-20A-4

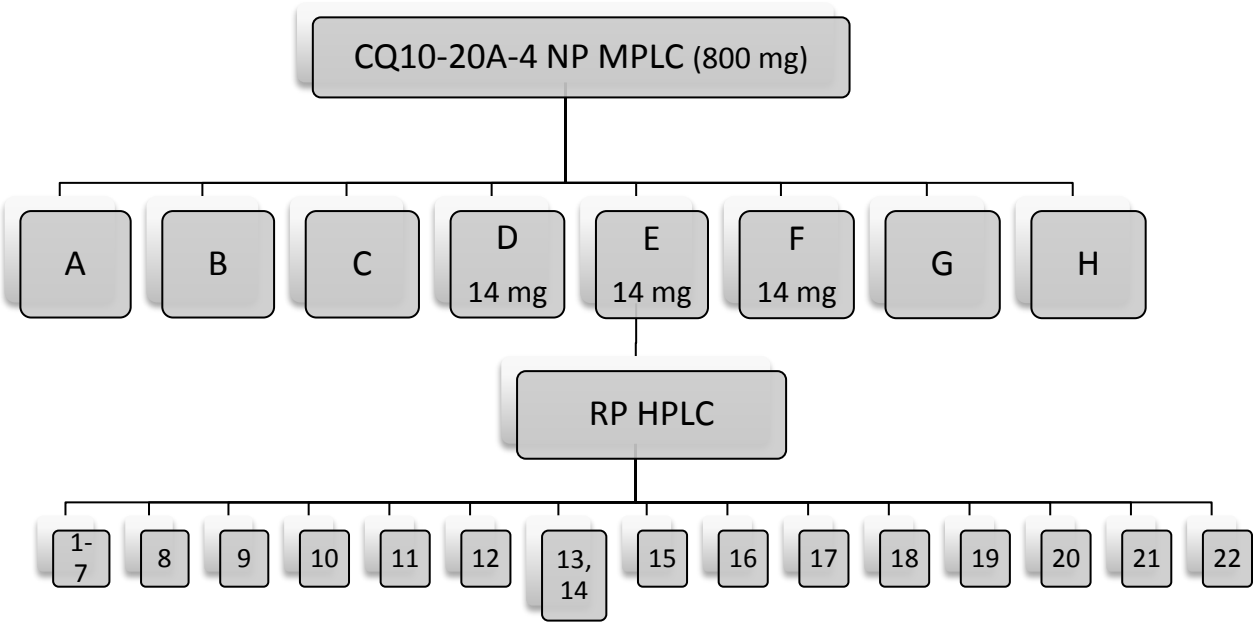


Figure 2 – CQ10-20A-4: Normal Phase MPLC Chromatogram

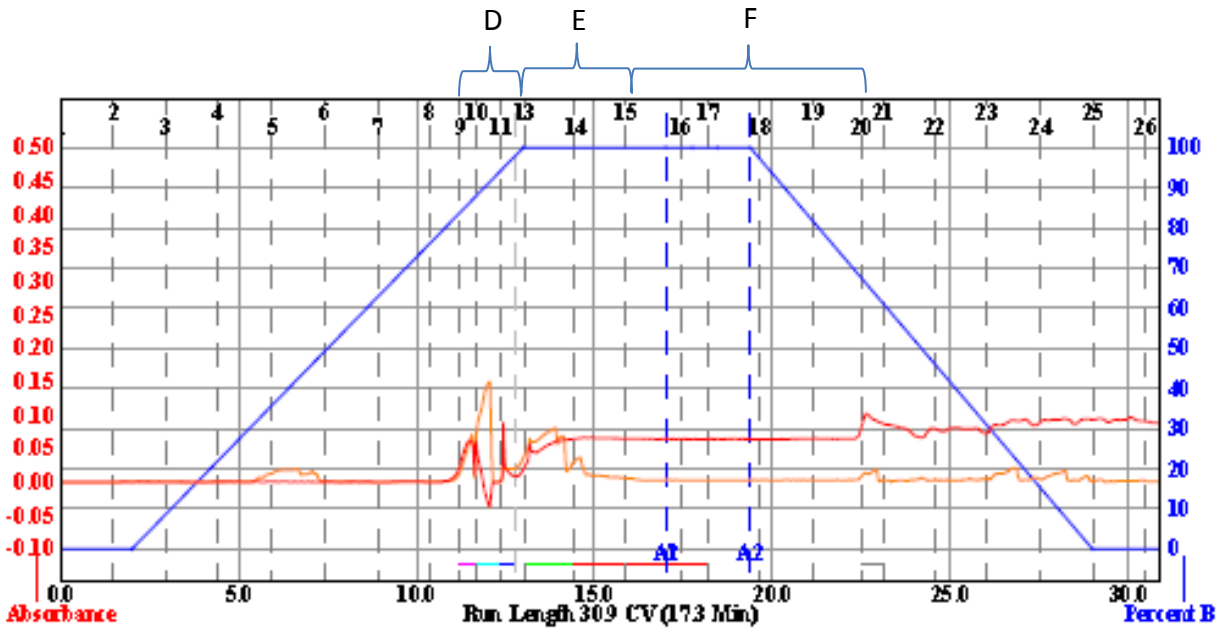


Figure 3 – CQ10-20A-4-DEF: Reversed Phase HPLC Chromatogram

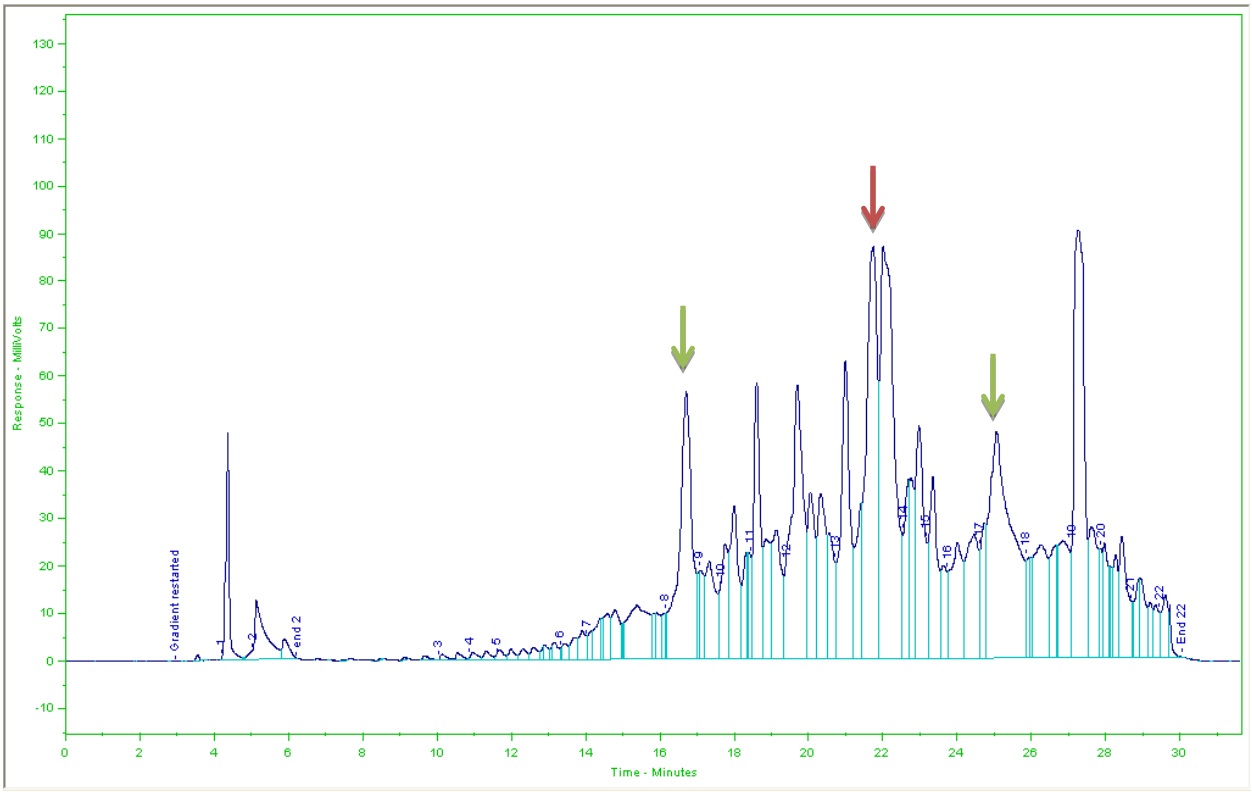


Figure 4 – CQ10-20A-4-DEF-8: ^{1}H NMR Spectrum

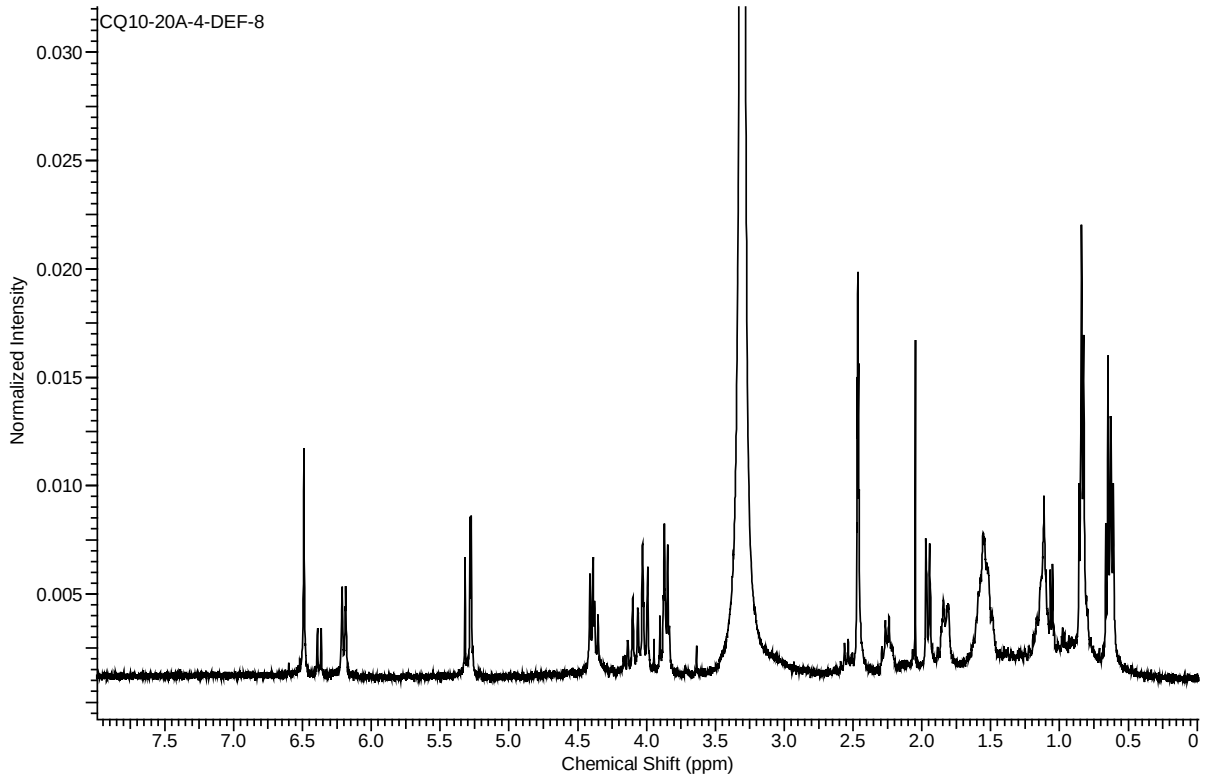


Figure 5 – CQ10-20A-4-DEF-17: ^{1}H NMR Spectrum

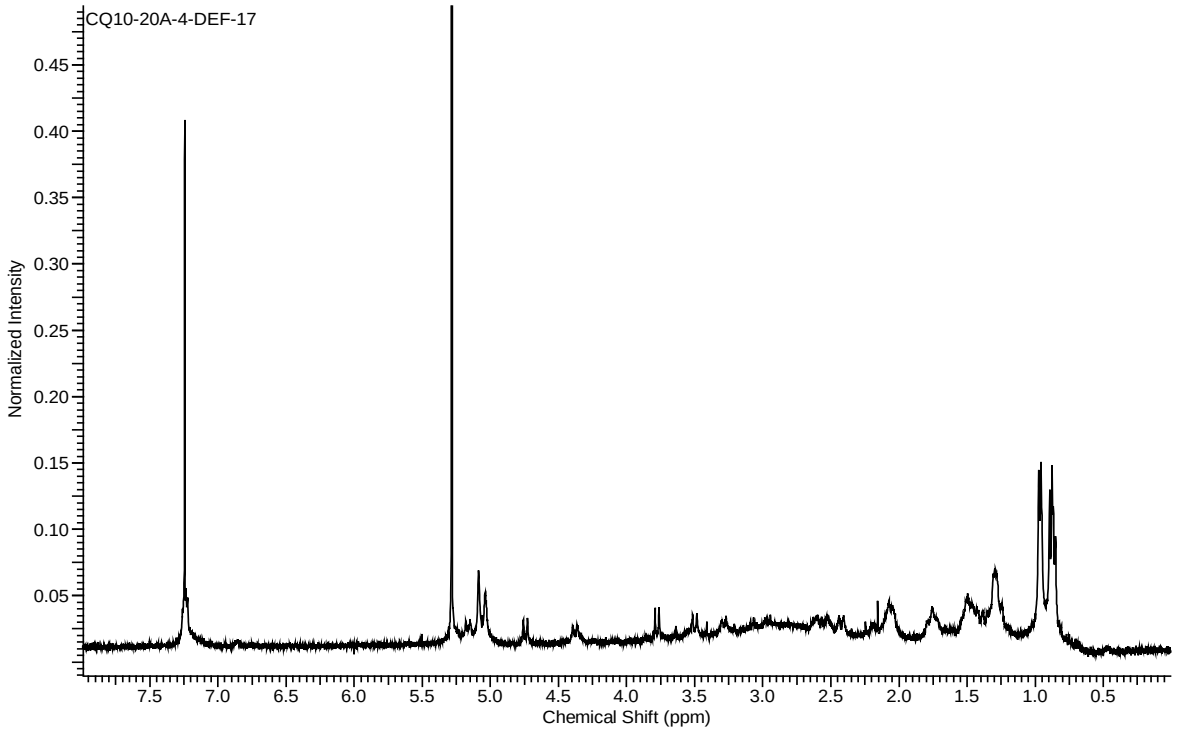


Figure 6 – CQ10-20A-4-DEF-13: ^{1}H NMR Spectrum

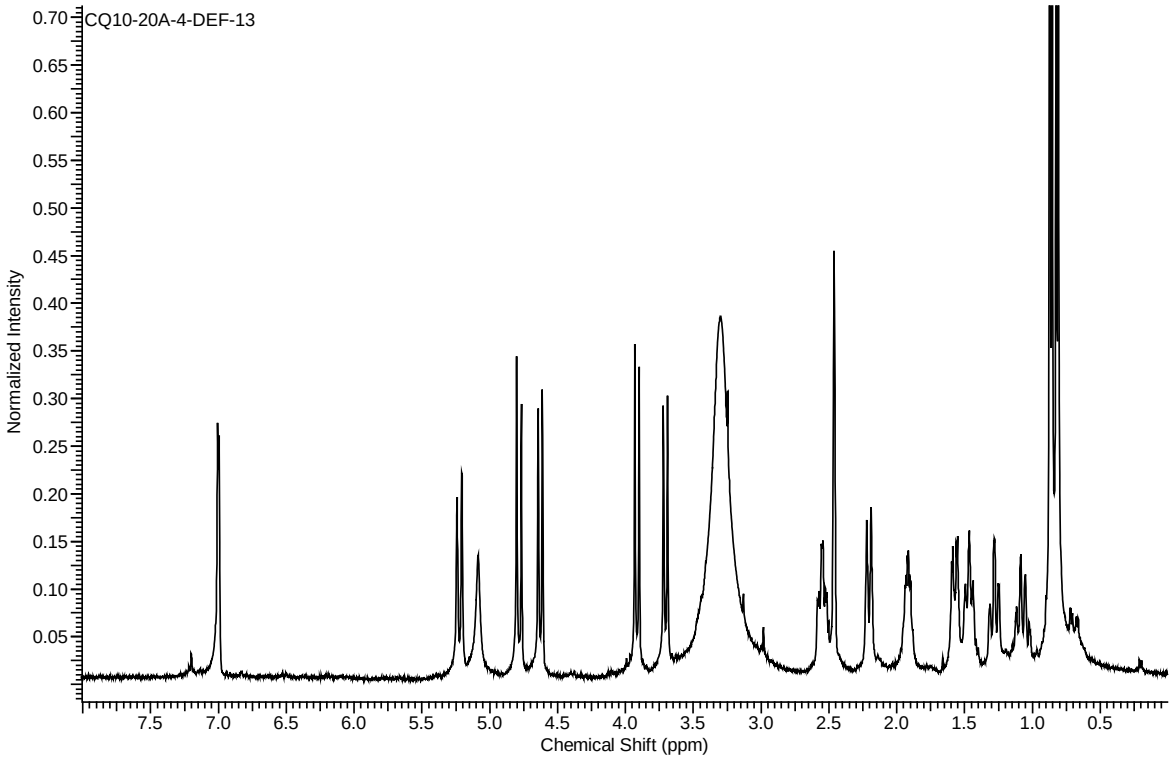
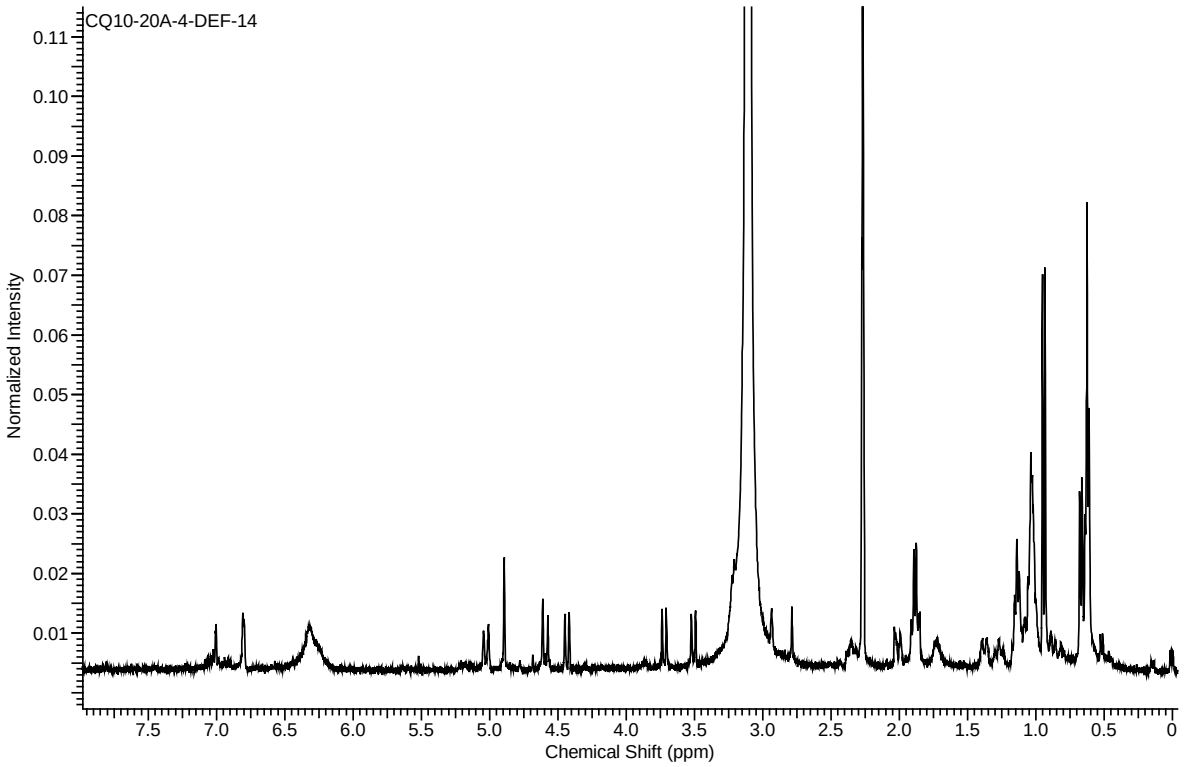


Figure 7 – CQ10-20A-4-DEF-14: ^{1}H NMR Spectrum



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