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Natural products have played a significant role in the drug discovery especially in the area of cancer. Over the period of 1940-2010, 50% of the small molecules introduced as anticancer agents were either natural products or directly derived therefrom. In one of the ongoing collaborative project to identify anticancer leads, the Mycosynthetix library, representing over 55,000 accessions of filamentous fungi, is being examined systematically. The bioactive-guided fractionation methodology is adopted for the isolation and purification of cytotoxic lead compounds. The first chapter describes the isolation of cytotoxic secondary metabolites using bio-activity guided fractionation scheme.

As part of ongoing investigations of filamentous fungi for anticancer drug leads, a bioactivity-guided fractionation methodology utilizes chloroform in the initial extraction and fractionation processes. Due to the concerns regarding human health and halogenated waste associated with chloroform, an attempt was made to replace it with more environmentally benign ester-based solvents. In this project, ethyl acetate, methyl acetate and ethyl formate were used in the initial stages of extractions and processing. The extraction efficiency of these was compared versus chloroform using two well-studied fungi.

BIOACTIVE NATURAL PRODUCTS: ISOLATION OF FUNGAL SECONDARY METABOLITES AND APPLICATIONS OF GREEN CHEMISTRY

by

Rabia M. Bukhari

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Committee Chair

APPROVAL PAGE

This thesis written by RABIA BUKHARI has been approved by the following committee of the Faculty of The Graduate School at The University of North Carolina at Greensboro.

Committee Chair _____

Committee Members _____

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Date of Acceptance by Committee

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Date of Final Oral Examination

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CHAPTER I

MINIMIZING THE ENVIRONMENTAL FOOTPRINT OF NATURAL PRODUCTS*

*This chapter will be submitted to the Journal of Green Chemistry.

Introduction

Since the introduction of the term “green chemistry” 20 years ago by Drs. Paul T. Anastas and John C. Warner, extensive research has been conducted in all fields of chemistry worldwide. One of the 12 principles proposed by Anastas and Warner was “to use safer solvents and auxiliaries”,[1] thereby raising awareness of toxic reagents and/or organic solvents in chemistry research and their impact on humans and the environment. Thus, the use of more green solvents is one means to minimize the environmental impact of chemistry research.[2]

Recently, Taygerly et al.[3] proposed a solvent selection guide for choosing solvent alternatives to dichloromethane (CH_2Cl_2) in chromatography procedures used frequently in medicinal chemistry. While the scale of medicinal chemistry may be smaller than that of process chemistry, a large amount of waste can be generated across disparate labs. Inspired by their work, we embarked on a similar journey to identify solvent alternatives greener than chloroform for the extraction of natural products. However, there is a fundamental difference between medicinal and natural products chemistry research.

In the former the structures of starting materials and products are known in advance. In fact, Taygerly et al.[3] use this to their advantage when predicting what solvents could be used to replace CH_2Cl_2 in chromatographic purifications. In contrast, in the early stages of natural products research, the structures are not known. Hence, if $CHCl_3$ were to be replaced in initial processing steps, a solvent that was "general" enough to dissolve a broad and often unknown series of structures was desired. While there have been countless advances in the separation techniques utilized for isolating natural products, there has been less emphasis placed on strategies to minimize or find alternatives to solvents used most frequently.

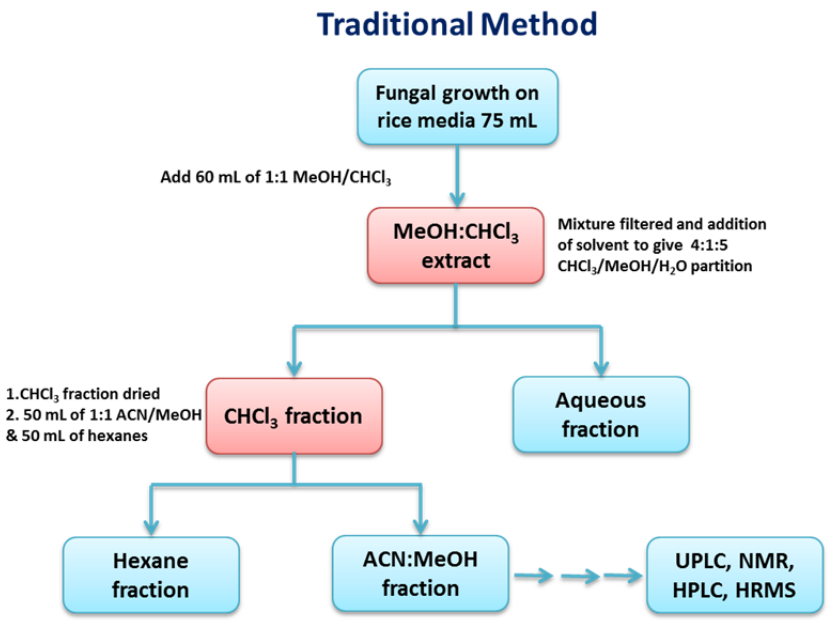
Previous Jessop[4] presented four grand challenges scientists face when introducing green solvents in to procedures: 1) finding an appropriate green solvent; 2) being able to identify whether a solvent is in fact green; 3) finding polar aprotic solvents that are easily removed; and 4) finding ways to eliminate distillation. These challenges, particularly 1 through 3, were considered while striving to minimize the halogenated waste associated with natural product extraction procedures.

In ongoing studies to explore nature for anticancer drug leads,[5,6] extracts of filamentous fungi from the Mycosynthetix library, representing over 55,000 accessions, are being investigated systematically for bioactive secondary metabolites. In the initial stages of extraction and fractionation, a suite of solvents are used, particularly chloroform (Figure 1). Its volatility makes it easy to evaporate. Its polarity, being between the extremes of hexane and H_2O, impart it with an ability to dissolve a diverse array of compounds.[7] Moreover, its specific gravity is greater than that of H_2O, making it

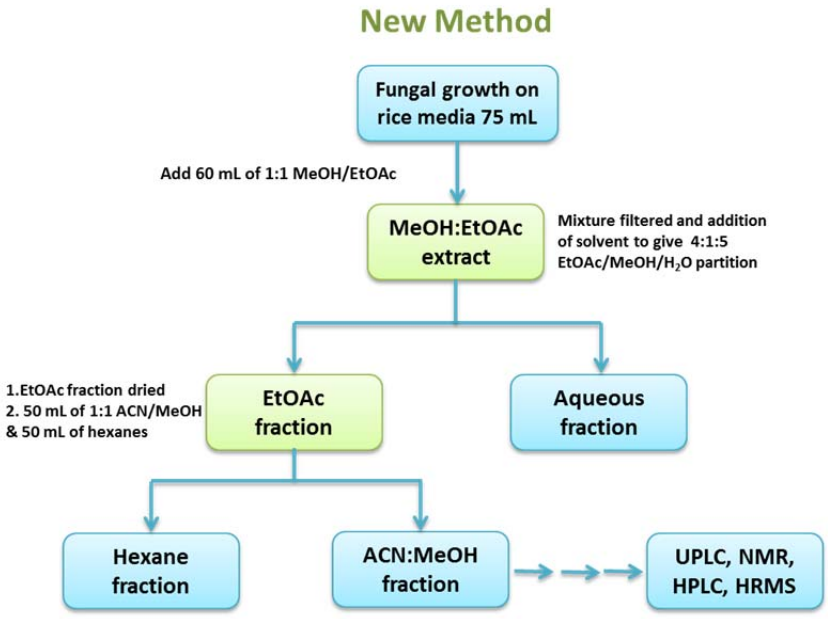
convenient for use in simple apparatus, like separatory funnels. Hence, chloroform is used frequently for extracting, bioactive secondary metabolites from fungal cultures.

Figure 1. Extraction and partition scheme for filamentous fungi on rice. a) $CHCl_3$ used as an extraction solvent in the initial two stages and b) EtOAc used in place of $CHCl_3$ in the initial two stages.

a)



b)

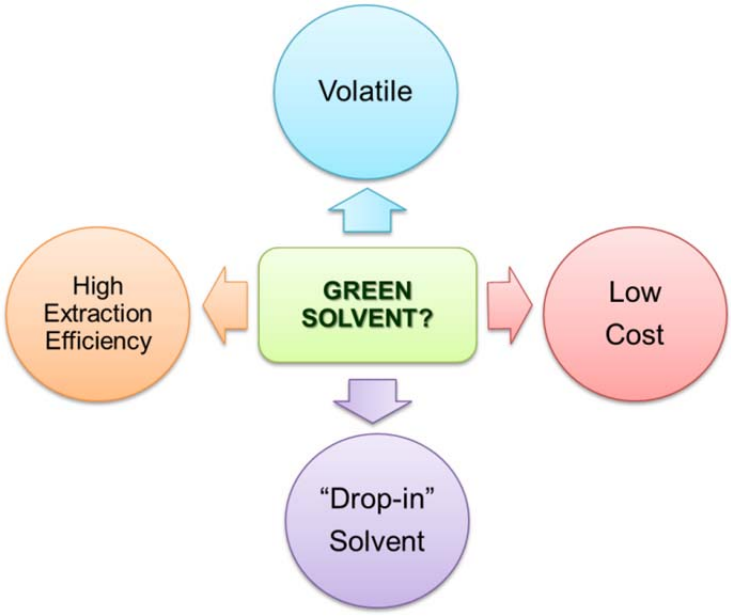


Despite those conveniences, several research studies that applied environmental, health and safety assessment and life-cycle assessment ranked chloroform unfavorable.[8] The costs associated with halogenated waste are high and improper handling may have carcinogenic effect.[9] For example, the US Environmental Protection Agency (EPA), following the Pollution Prevention Act of 1990, compiled a list of 17 priority pollutants known as EPA 33/50. Chloroform is the third solvent on the list, predominantly due to its anticipated human carcinogenicity.[10]

The UNCG Department of Environmental Health and Safety reported close to 20,000 pounds of waste generated at UNCG in last fiscal year out of which 1730 containers were marked as hazardous waste. UNCG spends close to $20,000 per year on waste management, and the Department of Chemistry and Biochemistry's' hazardous waste disposal costs somewhere from $1000 up to $5000 per year. In the light of these statistics, and the UNCG waste minimization policy, it would be beneficial to reduce the amount of hazardous waste generated on campus.

The goal of the present study was to replace chloroform with an alternative solvent that was low in toxicity, environmentally benign, and inexpensive, all without introducing significant changes to the well-established procedures or, most importantly, compromising extraction efficiency (Figure 2). Various 'green solvent' options listed in the literature were investigated, and their extraction efficiencies versus chloroform, were evaluated using two well-studied fungal cultures, MSX63935 and MSX45109. To do so, the yields and chromatographic profiles of key compounds were compared using Ultra Performance Liquid Chromatography (UPLC).

Figure 2. Criteria for choosing an alternative green solvent.



Alternative Solvent Selection Strategy

Substantial research effort is directed towards searching for alternative green solvents. As reported by Jessop[4], approximately 41% of the papers published in *Green Chemistry* in 2010 were on ionic liquids, 28% on water, 11% on processes using no solvent, and a relatively small number on the use of carbon dioxide. In this research project, these particular replacements were considered as possible green alternatives to chloroform.

Ideally, the best option available in terms of reducing waste would be the use of no solvent. Contrary to this, most of the 'solvent free' procedures reported in the literature used solvents at some stage. The amount of solvent used in these procedures were reduced compared to the conventional approaches, and therefore the methods were considered green.[11] For the extraction of secondary metabolites from filamentous fungi, a solvent of some kind was required.

Water maybe the second best candidate for a green solvent as it is plentiful and inexpensive. However, most secondary metabolites have limited solubility in H_2O, eliminate this option.

Ionic Liquids are considered designer solvents specific for a process or reaction. Which are salts with low melting points, and they are popular as an alternative to volatile organic solvents (VOC), due to low vapor pressures. However, due to the limited data available on toxicity and environmental impact, they cannot be considered green.[2] Also, their cost is prohibitive.[12]

Carbon dioxide exists as supercritical CO_2 ($scCO_2$) above a critical pressure and temperature. Due to its volatile nature, $scCO_2$ is a well-established extraction solvent for natural products. By simply releasing the pressure, "solvent" is completely removed.[12] It has very little environmental impact. However, the biggest disadvantage of $scCO_2$ is the high equipment cost, limiting its usage in research community.

Another interesting class of solvents is esters. Esters are generally considered environmentally friendly solvents, and they have relatively low health hazards. For example, ethyl lactate has become widely available as a bio-sourced and biodegradable cleaning fluid[11]. Ethyl lactate is prepared by esterification of lactic acid and ethanol; both of them are renewable sources and maybe obtained from fermenting corn starches. It is readily purified as it is prepared from natural resources and it is completely biodegradable to carbon dioxide and water. Methyl acetate (MeOAc) is useful as a fast-evaporating solvent in applications like coating and ink resins where fast solvent releases are needed. Ethyl acetate (EtOAc) is normally produced by esterification of ethanol and acetic acid. Currently, it is used in industrial lacquers and surface coating resins.[13] Furthermore, it is also used as an extraction solvent in the production of pharmaceuticals and food, especially in decaffeination of green tea.[7]

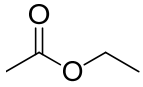
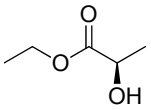
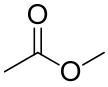
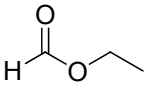
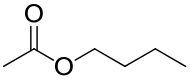
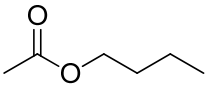
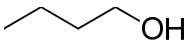
Solvent Selection

For a solvent to be considered as an alternative to chloroform in our lab set up, it should meet certain conditions. With this in mind, the following green solvent selection criterion was developed; (1) it needs to be volatile – easily evaporated; (2) extraction efficiency (product yield) has to be equal or higher than chloroform; (3) it must be low in cost and easily available for purchase; (4) extraction and partition procedure shouldn't be changed significantly (Figure 2).

With these criteria in mind, esters proved to be a better alternative solvent. But, which ester solvent is an optimal solvent for extraction of secondary metabolites from filamentous fungi? Ideally, the aim was to find an ester that had similar properties to chloroform; immiscible or has low solubility in water to give high product yield and having a boiling point lower than n-butanol (preferably lower than 80°C). In general, solvents with high boiling points are avoided due to longer evaporation time and high energy cost associated with separation by distillation.[14]

To assist with the solvent selection, in total six esters were evaluated. Esters were systematically listed according to their increasing carbon-chain lengths and properties like boiling point, cost per liter (US $), polarity index, density and solubility in water were compared to that of chloroform (Table 1).

Table 1. Esters and their properties compared to solvents presently used for extraction.

Solvent	Structure	BP (°C)	Cost (per L in US $)	Polarity Index	Density (g/cm^3)	Solubility in H_2O (g/L)
Ethyl acetate		70	4	4.3	0.90	80
Ethyl lactate		154	29	7.6	1.03	Miscible
Methyl acetate		56	33	4.4	0.93	250
Ethyl formate		54	37	4.3	0.92	110
n-Propyl acetate		102	5	4.2	0.89	20
n-Butyl acetate		126	27	4.0	0.88	7
Chloroform	$CHCl_3$	61	11	4.1	1.48	8
n-Butanol		118	15	4.0	0.81	80
Methanol	CH_3OH	65	0.95	5.1	0.79	Miscible

Ethyl lactate, n-propyl acetate and n-butyl acetate were eliminated due to their higher boiling points in comparison with n-butanol. Additionally, ethyl lactates' miscibility in water made it an unfavorable extraction solvent. Although MeOAc had a higher solubility in water than preferred, the low boiling point made it an eligible candidate for analysis.

EtOAc, MeOAc and ethyl formate were further investigated in an attempt to replace $CHCl_3$ as an extraction solvent for filamentous fungi. In order to assess the extraction efficiency of these solvents versus $CHCl_3$, they were tested against well-studied filamentous fungi, MSX63935 and MSX45109.

Method

General experimental information:

The fresh cultures of MSX63935 and MSX45109 on rice medium, from Mycosynthetix library were grown in parallel. To check solvents' extraction reproducibility, three separate rounds of extractions were done for each solvent. All solvents used for analysis were obtained from Fischer Scientific and were used without further purification. The solvent prices were obtained from PHARMO-AAPER at UNCG contracted price. UPLC was carried out on Waters Acquity system with data collected and analyzed using Empower software.

Producing Organisms And Fermentation:

Mycosynthetix fungal strain MSX63935 was isolated in 1992 from a leaf litter and the fungal strain MSX45109 was isolated in 1989 from a stump of banana tree in a

mangrove area, by Dr. Barry Katz of MYCOsearch. The cultures were stored on a malt extract slants and were transferred periodically. Fresh cultures were grown on a similar slant, and a piece was transferred to a medium containing 2% soy peptone, 2% dextrose, and 1% yeast extract (YESD media). Following incubation (7 days) at 22°C with agitation, the cultures were used to inoculate 50 mL of a rice medium, prepared using rice to which was added a vitamin solution and twice the volume of rice with H_2O, in a 250 mL Erlenmeyer flask. This was incubated at 22°C until the cultures showed good growth (approximately 14 days) to generate the screening cultures.[14, 15]

Extraction And Fractionation:

To each flask of solid fermentation culture, a mixture of 60 mL of 1:1 methanol/*solvent* was added (*solvent* = either $CHCl_3$, EtOAc, MeOAc, or Ethyl formate). Then, the samples were chopped with a spatula and stirred overnight (16 h) in a shaker at 100 rpm at room temperature. The samples were filtered by vacuum filtration into 250 mL sidearm flasks. The remaining residues were washed with small volumes of MeOH. To the filtrate, 90 mL of the investigated solvent and 150 mL of H_2O were added. The biphasic solutions were stirred on a stir plate for 30 min, and then transferred to separatory funnels.

The bottom layers for $CHCl_3$ were drawn off into round-bottom flasks and evaporated to dryness. For EtOAc, MeOAc and ethyl formate, top layers were drawn off into round-bottom flasks and evaporated to dryness. The organic extracts were then brought up in 50 mL of 1:1 ACN/MeOH and 50 mL of hexanes. The biphasic solutions were mixed for

an hour and transferred to separatory funnels. The ACN:MeOH layer were evaporated to dryness under vacuum and yields were taken.

UPLC Analysis:

Chromatography was performed using an Acquity UPLC BEH C18 (2.1 × 50 mm, 1.7 μm) column (Waters Corp., Milford, MA, USA) equilibrated at 30 °C. A mobile phase consisting of ACN and H_2O (acidified with 0.1% formic acid) was used, starting with 40:60 ACN/H_2O, going to 55:45 over 5 min, then re-equilibrating at 40:60 for 1 min. Chromatograms were collected at 233 nm.

Results

EtOAc, MeOAc and ethyl formate were used as solvents to extract secondary metabolites from well-studied fungi, designated by MSX63935 and MSX45109. $CHCl_3$ was used as a control to compare the extraction efficiencies. Analyses were carried out in five replicates to test the reproducibility of the solvents.

In our research lab, investigation of filamentous fungi for anti-cancer leads is the largest project. The group continually searches fungal extracts from the Mycosynthetix library throughout the year. On average, some 400 small-scale solid culture samples are being processed every year. In the light of that, it is imperative for us to compare the costs of EtOAc, MeOAc, ethyl formate against $CHCl_3$ (Table 2).

Table 2. Cost comparison of alternative solvents to $CHCl_3$.

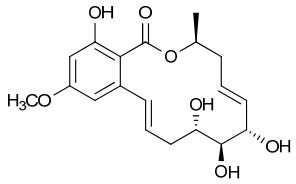
	1 fungus sample extraction (L)	400 samples per year (L)	Cost per liter (US $)	Total cost (US $ per year)
Chloroform	0.15	60	11	660
Ethyl acetate	0.15	60	4	240
Methyl acetate	0.30	120	33	3960
Ethyl formate	0.15	60	37	2220

Method Validation:

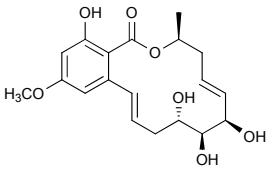
MSX63935: Previous research on an extract of filamentous fungi, MSX63935, showed promising cytotoxic activity against a human tumor panel. These results commenced a bioactivity-directed fractionation study and a series of resorcylic acid lactones (zeanols) were isolated and characterized (Figure 3).[14]

Figure 3. Structures of resorcylic acid lactone (zeaenols) in MSX63935 and secalonic acid in MSX45109.

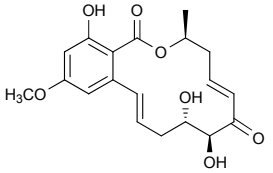
MSX63935



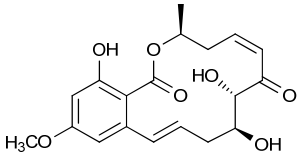
Zeaenol (**1**)



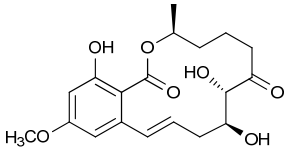
7-*epi*-zeaenol (**2**)



5E-7-oxozeaenol (**3**)

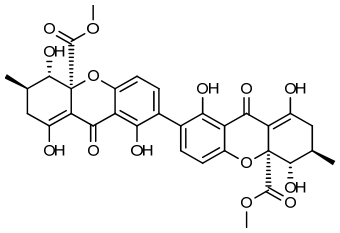


5Z-7-oxozeaenol (**4**)

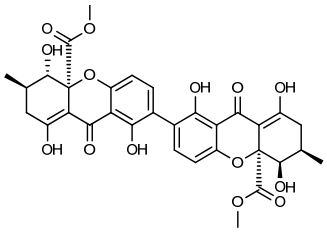


LL-Z1640-1 (**5**)

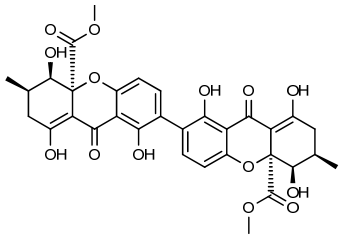
MSX45109



Secalonic acid A (**6**)



Secalonic acid G (**7**)



Secalonic acid E (**8**)

MeOAc fractions had relatively high yields (375 mg) compared to the other solvents (Table 3). The two solvents, MeOAc and H_2O, were miscible in each other at the ratio that was used in the standard procedure. Hence, additional MeOAc was added to get the phases separated, thus increasing costs, time and higher yields.

Table 3. Average of extract yields (mg) of MSX63935 for multiple rounds of extraction in solvents.

	Yield mg ± SD(%RSD)
Chloroform	322 ± 73(23)
Ethyl acetate	348 ± 88(25)
Methyl acetate	375 ± 105(28)
Ethyl formate	313 ± 52(17)

The extraction yield of EtOAc (348 mg) was analogous to that of $CHCl_3$ (322 mg) (Table 3). Ethyl formate also had a yield (313 mg) comparable to $CHCl_3$ extracts but with a much higher solvent cost; it does not make for a cost effective alternative solvent.

The yields and chromatographic profiles of key compounds in MSX63935 were compared with EtOAc, MeOAc and ethyl formate against $CHCl_3$ using UPLC. The stack plot of extract of MSX63935 in $CHCl_3$ and EtOAc were compared (Figure 4). The percent peak area was calculated by averaging the percent peak area for the replicates done in particular solvent. EtOAc and ethyl formate had similar peak areas to $CHCl_3$ for most of the compounds. Due to the high cost associated with ethyl formate it was eliminated as not being very economical and MeOAc miscibility in H_2O caused excess use of the solvent, therefore cannot be considered very green.

MSX45109: To check the efficiency of EtOAc as an extraction solvent, another well-studied fungus MSX45109 was extracted with $CHCl_3$ and EtOAc. This fungus was found to produce dimeric ergochromes, also known as secalonic acids, a biologically important class of mycotoxins (Figure 3).

There are 3 distinct peaks in the UPLC chromatogram (Figure 5). The yields and chromatographic profiles were compared for $CHCl_3$ and EtOAc. The average yield of 5 rounds of extraction in $CHCl_3$ was 149 mg ± 21 and in EtOAc it was 144 mg ± 9. Secalonic acid A, secalonic acid G and secalonic acid E were distinct in both chromatograms. The total percent peak area for all the secalonic acids extracted in EtOAc were very similar to the $CHCl_3$extract.

Figure 4. UPLC chromatogram stack plot of extract of fungus MSX63935 in $CHCl_3$ versu EtOAc extract.

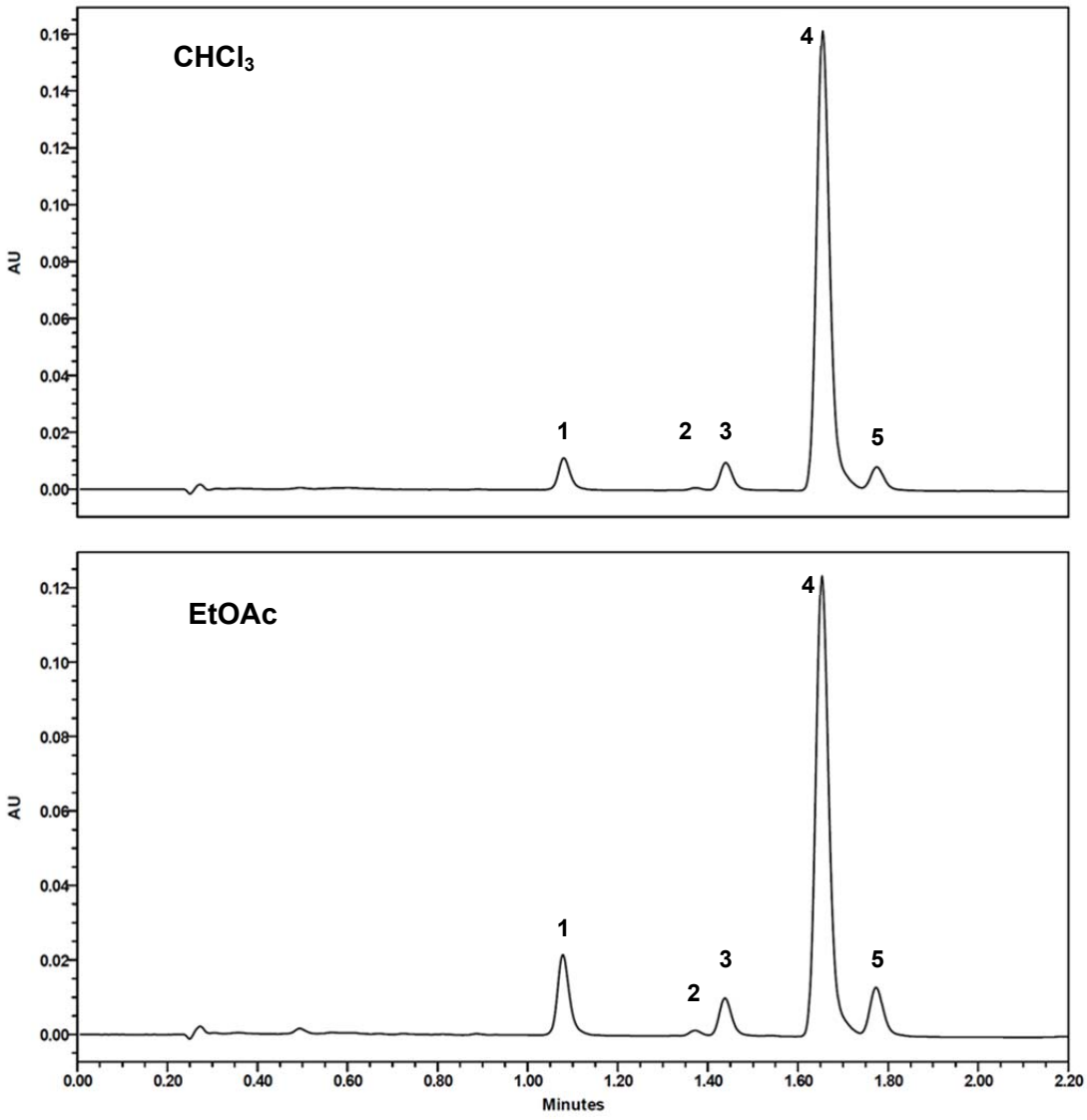
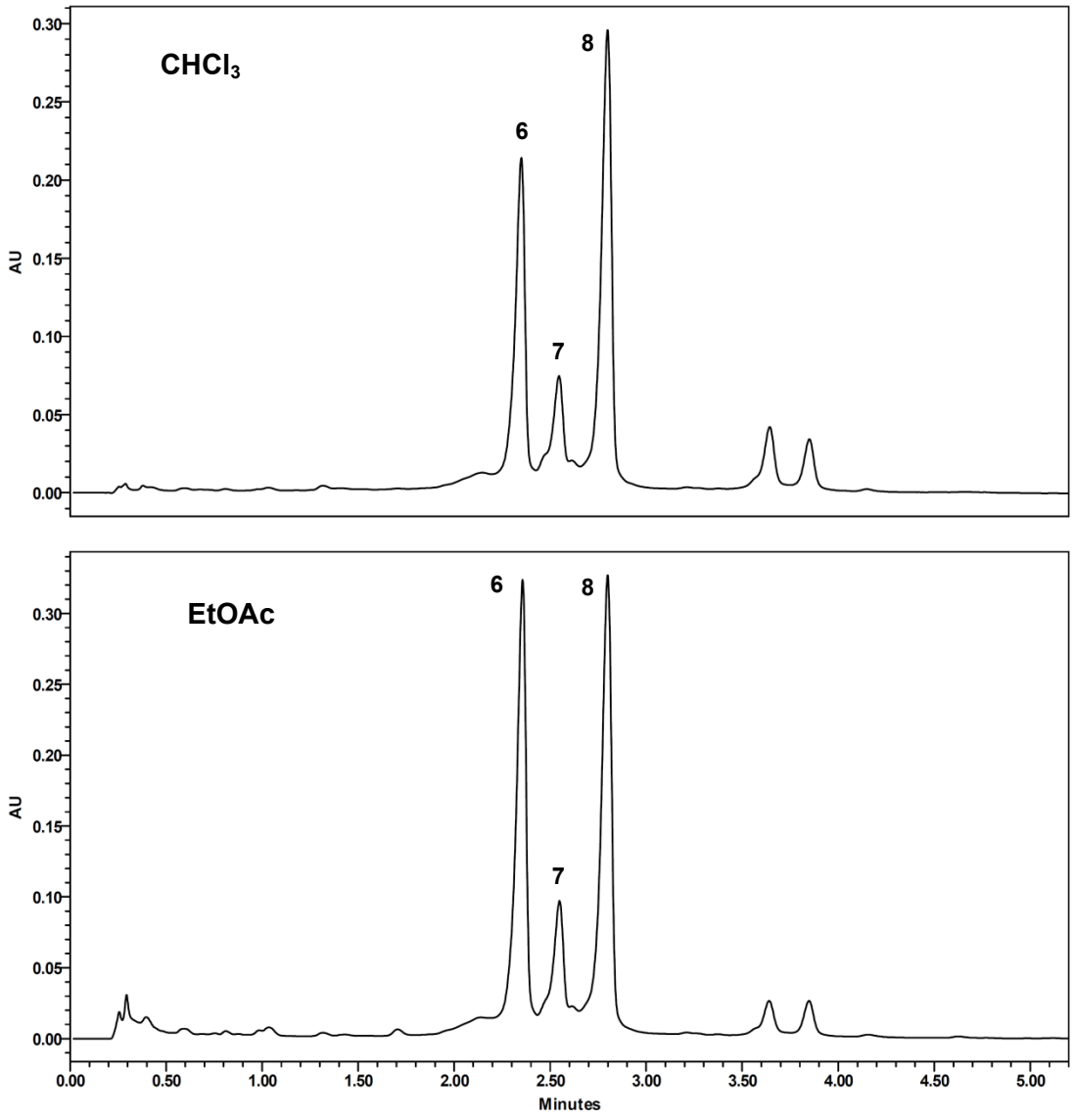


Figure 5. UPLC chromatogram stack plot of extract of fungus MSX45109 in $CHCl_3$ versus EtOAc extract.



Conclusion

The extraction with EtOAc yielded the best results based on UPLC chromatogram and the peak areas per milligram were very similar between $CHCl_3$ and EtOAc. Additionally, EtOAc is confirmed to be a good alternative solvent for $CHCl_3$ as it gave similar yields to $CHCl_3$ and it is much less expensive. EtOAc is also considered a greener solvent, as it has a much lower environmental impact and a relatively low health hazard. Based on the extract yield and other properties, EtOAc is considered as a promising alternative solvent to replace traditional $CHCl_3$ extractions in our lab.

CHAPTER II

ISOLATION AND STRUCTURE ELUCIDATION OF CYTOTOXIC SECONDARY METABOLITES FROM FILAMENTOUS FUNGI

In the history of pharmaceutical industry, natural products have always played an important role. Over the period of 30 years from 1981-2010, approximately 50% of new approved drugs were either naturally derived or natural product inspired.[16] Newman and Cragg reported that of the 175 small molecules, which were approved as the anticancer drugs between 1940 and 2010, 74.8% - were other than synthetic, with 48.6% were either natural products or directly derived a natural product.[16] Today, natural products are widely accepted for their chemical diversity and well suited to provide good scaffolds for the drug design.

Cancer is the fastest growing disease worldwide and leading cause of death in the US; one out of four deaths in US is due to cancer. In 2008, about 12.7 million people were diagnosed with cancer and 7.6 million deaths were caused by cancer worldwide.[17] It is estimated that over 1.6 million Americans will be diagnosed with cancer this year while the estimated number of deaths is 577, 190.[18] Every day, over 1500 Americans are dying of cancer. Breast cancer was the leading cause of death in women while the lung cancer was the leading cause of death in males in 2008.[17] In the light of these statistics, it is necessary to continue search for anti-cancer agents to inhibit cell survival

pathways in tumor cells and increase the survival rates of cancer patients.The natural product-based drugs cover variety of therapeutic indications including anti-infective, anti-diabetic and anti-cancer. Since the discovery of Penicillin G[19] in 1940, the first natural antibiotic isolated from *Penicillium chrysogenum* , interest in screening the micro-organisms like bacteria and fungi for drug discovery has grown significantly.

The role fungi have played in drug discovery cannot be ignored. For example, recently U.S. Food and Drug Administration (FDA) approved a natural product analogue fingolimod for the treatment of multiple sclerosis (MS);[20] a synthetic compound based on fungal secondary metabolite, which clearly demonstrates the importance of fungi as a valuable resource for bioactive secondary metabolites. Earlier publications have pointed out that very little of the world's microbial biodiversity has been available for screening.[21] Hence, targeting fungi may provide isolation of many new cytotoxic compounds for use in anticancer drug discovery assays.

One of the ongoing collaborative projects of our group is to examine the extracts of filamentous fungi from the Mycosynthetix library, representing over 55,000 accessions, for anti-cancer leads. Mycosynthetix library has been screened for many biological assays but not for the anticancer leads. Hence, it is a unique and underexplored resource for anticancer drug discovery lead.

This part of the research is focused on using bioactivity-guided fractionation methodology for the isolation and purification of bioactive compounds from the filamentous fungi, in which purification is guided by the bioassay results. The primary goal was to isolate and structurally elucidate cytotoxic fungal metabolites. Based on the

bioactivity for the crude extract, fungus (MSX47782, Mycosynthetix Inc.) was selected for further study.

The Mycosynthetix fungal strains were stored on malt extract slants and a fresh culture was grown on a screener scale on solid media, extracted and evaluated for cytotoxicity activity.

The cytotoxicity measurements against the NCI-H460 human large cell lung carcinoma (HTB-177, American Type Culture Collection (ATCC)),[22] the MDA-MB-435 human melanoma (HTB-38, ATCC) and the SW-620 human colon (ATCC) cell lines were performed on crude extracts and resulting moderately active fungal samples were further analyzed.

The screener cultures were dereplicated against an in-house library of known secondary metabolites in crude culture extracts to avoid re-isolation and re-characterization of previously known compounds. Dereplicated samples were fractionated via silica gel flash chromatography. The resulting fractions were submitted for bioassay testing. The active ones were purified via preparative and semi preparative HPLC yielding three fungal derived compounds (one new and two known) that displayed moderate cytotoxicity against the cancer cell lines.

Method

Producing Organisms And Fermentation:

The Mycosynthetix fungal strain (MSX47782) were stored on malt extract slant and transferred periodically. For the analysis of this fungal strain, fresh cultures were grown on a similar slant, and a piece was transferred to a medium containing 2% soy peptone,

2% dextrose, and 1% yeast extract (YESD media). Following incubation (7 days) at 22°C with agitation, the cultures were used to inoculate 50 mL of a rice medium, prepared using rice to which was added a vitamin solution and twice the volume of rice with H_2O, in a 250 mL Erlenmeyer flask. This was incubated at 22°C until the cultures showed good growth (approximately 14 days) to generate the screening cultures.[14,15]

Bioactivity-Guided Fraction:

To each flask of solid fermentation culture, a mixture of 60 mL of 1:1 MeOH/$CHCl_3$, Then, the sample was cut into small pieces with a spatula and stirred overnight (16 h) in a shaker at 100 rpm at room temperature. The sample was filtered by vacuum filtration into 250 mL sidearm flasks. The remaining residues were washed with small volumes of MeOH. To the filtrate, 90 mL of $CHCl_3$ and 150 mL of H_2O were added. The biphasic solutions were stirred on a stir plate for 30 min, and then transferred to separatory funnels.

The bottom layers for were drawn off into round-bottom flasks and evaporated to dryness. The organic extracts were then brought up in 50 mL of 1:1 ACN/MeOH and 50 mL of hexanes. The biphasic solutions were mixed for an hour and transferred to separatory funnels. The ACN:MeOH layer were evaporated to dryness under vacuum and then submitted for bioassay. This crude extract was tested in the cancer bioassay for activity and active ones were dereplicated via methodology developed in Oberlies lab using HRESIMS/MS, and samples with no hits proceeded to the fractionation step.

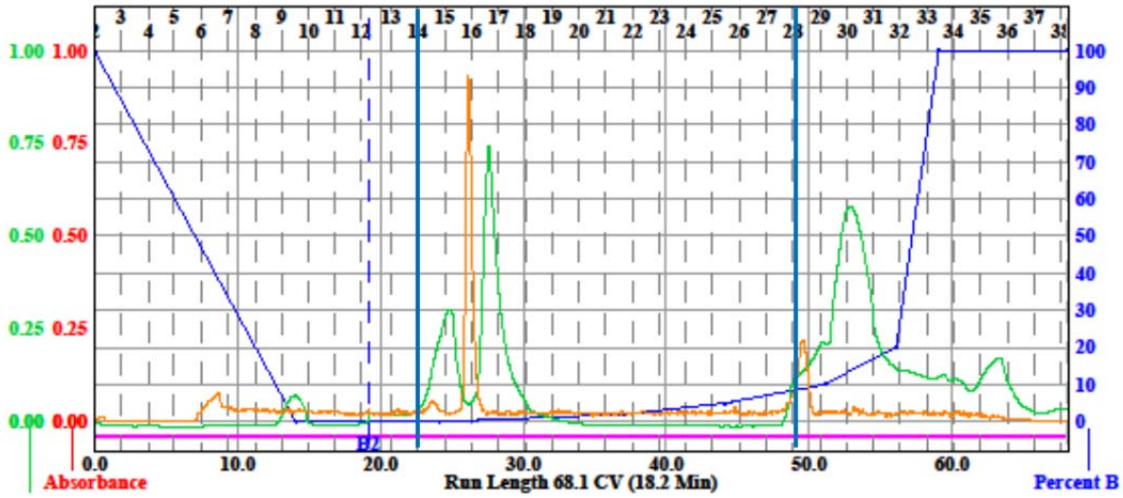
The dereplicaiton methodology was developed to avoid re-isolation of known fungal secondary metabolites in crude extracts. An in house database was constructed by

recording HRMS and MS/MS spectra of compounds isolated to date in the Oberlies lab, utilizing both positive and negative ionizations modes, additional information like UV-absorption maxima and retention times were recorded. This methodology was developed to focus resources on those cultures that are most likely to yield new bioactive compounds.

Fractionation:

The dereplicated sample was fractionated by normal phase flash silica gel chromatography. (Figure 6) The dereplicated sample was prepared by dissolving it in 1:1 $CHCl_3$/MeOH and adsorbed onto Celite 545 (Sigma-Aldrich). Solvent conditions were 100% hexane to 100% $CHCl_3$ followed by increasing amounts of MeOH and then 100% MeOH for the remainder of the run. There were 3 fractions collected. The fractions collected were pooled based on their UV/ELSD profiles, dried down and weighed. These fractions were screened against, the MDA-MB-435 human melanoma (HTB-38, ATCC) and the SW-620 human colon (ATCC) cancer cell lines (Table 4).

Figure 6. ISCO flash chromatography of organic extract from MSX47782.*



CombiFlash Rf system; Rf Gold silica gel column; Gradient Hex/$CHCl_3$/MeOH

*Blue line is the gradient. Orange line is all wavelength UV. Green line is ELS detection.

Pool	Fraction	Sample Code	Weight (mg)
1	01-12	01020-37-1	1.6
2	14-27	01020-37-2	9.1
3	28-End	01020-37-3	37.1
		Total recovery	47.7
		% Recovery	70%

Human Cancer Cell Panel:

The human cancer cell panel growth and testing were conducted at the University of Illinois at Chicago, College of Pharmacy in the laboratory of Steven M. Swanson. Initial crude extract and the ISCO fractionation bioassays were at concentrations of 20 µg/mL and 2 µg/mL. All samples were dissolved in DMSO and the final concentration was ≤0.5%.

The human cancer cell panel consisted of MDA-MB-435 human melanoma cancer cells and SW-620 human cancer cells, both purchased from the American Type Culture collection (Manassas, VA). All cell lines were propagated at 37°C in 5% CO_2 in RPMI 1640 medium supplemented with fetal bovine serum (10%) penicillin (100 units/mL) and streptomycin (100 µg/mL). All cells used were in log phase growth. The positive control was vinblastine tested at 2ng/mL and 1ng/mL: MDA-MB 435 cells had 30% and 46% viable cells, and SW-620 cells had 75% and 74% viable cells, respectively.

Table 4. Bioassay data obtained for flash chromatography fractions of MSX47782. All data is percent survival of cell line (sm=starting material prior to fractionation).

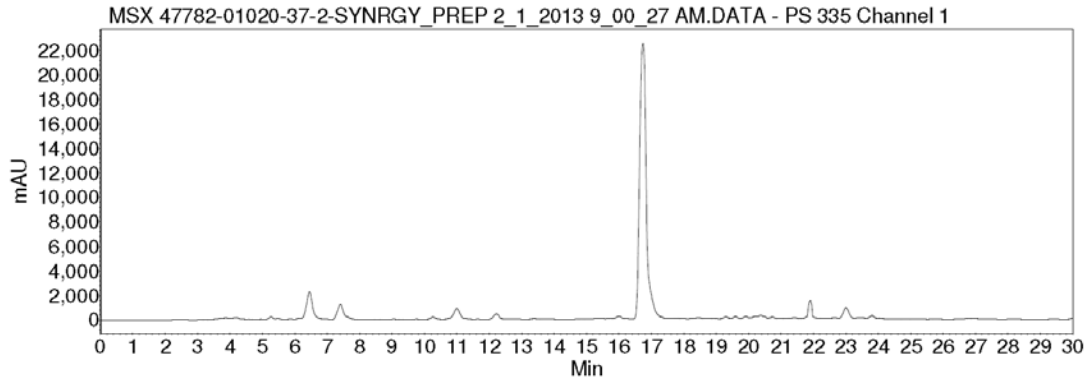
Sample ID	% survival (MDA-MB-435)	% survival (SW-620)
	20 µg/mL	20 µg/mL
01020-37-sm	59	39
01020-37-1	96	92
01020-37-2	49	33
01020-37-3	77	57

Purification:

Active fractions were purified and isolated via preparative and semi preparative HPLC. The preparative HPLC using a gradient that initiated with 40:80 consisting of MeCN:H_2O (0.1% formic acid) and increased linearly to 40:80 MeCN/H_2O within 30 minutes, at a flow rate of 21 mL/min. The column used was Phenomenex Synergy C_{12}, 4µm, 250 x 21.20mm. Spectra were collected at 204 nm (Figure 7). In total 9 fractions were collected, dried down and yields taken (Table 5). They were pooled based on the UV chromatogram and PDA profiles; the vertical black bars represent fractions. Based on the yields and analytical HPLC profiles, purified fractions 01021-86-2, 01021-86-4 and 01021-86-5 were further analyzed by NMR and HRMS for chemical structure elucidation.

Figure 7. a) Preparative HPLC chromatogram of 01020-37-2 @ 335 nm and b) the chromatogram showing fractions.

a)



b)

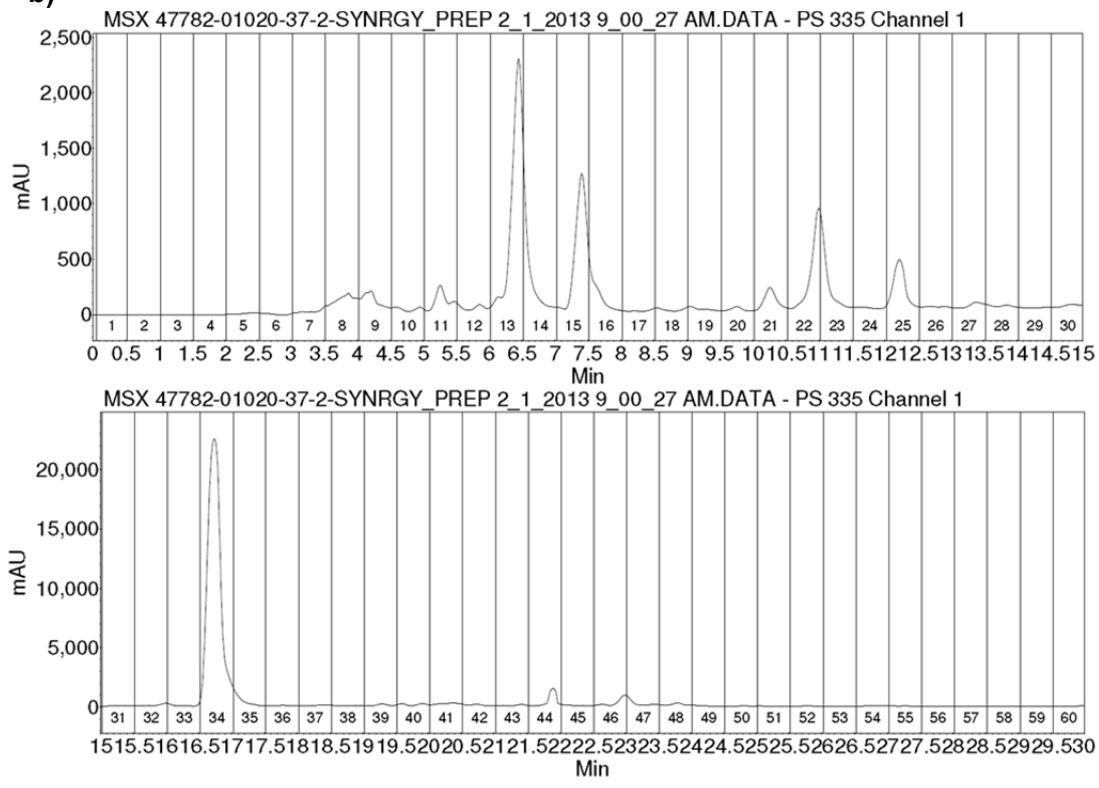


Table 5. Preparative HPLC fractions (01020-37-2) pooled based on the chromatogram.

Pool	Fraction	Sample Code	Weight (mg)	
1	13-14	01021-86-1	0.65	
2	**15-16**	**01021-86-2**	**1.18**	**Compound 1**
3	22-23	01021-86-3	0.72	
4	**25**	**01021-86-4**	**7.19**	**Compound 2**
5	**34-35**	**01021-86-5**	**1.87**	**Compound 3**
6	44	01021-86-6	0.65	
7	45	01021-86-7	0.22	
8	46-47	01021-86-8	0.49	
9	All Others	01021-86-9	5.46	

Structure Elucidation Of Active Compounds:

Isolated fractions, 01021-86-2, 01021-86-4 and 01021-86-5 will be further analyzed by 1D-NMR (^{1}H, ^{13}C, DEPT-135), , 2D-NMR (edited-HSQC, HMBC, COSY and NOESY) and HRMS for chemical structure elucidation.

Results And Conclusion

Throughout the ongoing exploration of extracts of filamentous fungi for cytotoxicity, the crude extract of the small scale fermentation of MSX47782 showed moderate activity in the human cancer cell panel, 21% cell survival of H460 cells when tested at 20µg/mL. This moderately active screener culture was chosen for further analysis due to the belief that the cytotoxicity activity of some low concentration compound is masked by other high yield non-bioactive compounds. The crude extract was then fractionated via ISCO normal phase chromatography to yield 3 fractions. These fractions were tested against

the MDA-MB-435 and SW-620 human cancer cell lines and fraction 2 showed marginal cytotoxic activities on the two cancer cell lines. Fraction 2 was subjected to further purification via preparative HPLC to yield three compounds (**1**-**3**).

Structure Elucidation Of 01021-86-2 (Compound 1):

01021-86-2 was isolated after one round of prep-HPLC (Figure 7). Dereplication of the compound was attempted by inputting the HRMS (Figure 9) into the Dictionary of Natural Products database (DNP ver. 20.1); no matches were found.

Compound **1** was a white powder (1.18 mg). The HRMS data was *m/z* = 213.11232 $[M + H]^+$ with a second mass peak at *m/z* = 425.21777 $[2M + H]^+$. Using the HRMS data, a chemical formula of $C_{11}H_{16}O_4$ was calculated, corresponding to an Index of Hydrogen Deficiency (IHD) of 4. The ^{13}C NMR (Figure 10) data showed 11 peaks in total, with 4 of those being quaternary carbons [δ_C 165.9 (C-1), δ_C 111.1 (C-2), δ_C 166.6 (C-3), and δ_C 120.7 (C-4)], with a peak at δ_C 61.4 denoting a methoxy group.

These results were combined with ^1H NMR data (Figure 11), the substituents were determined as terminal ethyl [δ_H 0.96 (CH_3) and δ_H 1.70 (CH_2)], methoxy (δ_H 3.95), oxymethine (δ_H 4.55), olifinic (δ_H 7.35) and methyl (δ_H 2.09) groups. The complete ^1H and ^{13}C NMR data are shown in Table 6.

The positions of the substituents and aromatic protons were determined by 2D-NMR. The three 2D-NMR experiments utilized here were: COSY (Correlation spectroscopy, ^1H-^1H correlations of adjacent protons, Figure 12) , HMBC (Heteronuclear multiple bond correlation, long range (2-4 bonds, 3 typically) ^1H-^{13}C correlations, Figure 13) and

HSQC-edited (Heteronuclear single quantum coherence, direct ^{1}H-^{13}C connectivity phase edited giving results similar to DEPT-135 ^{13}C NMR, Figure 14).

Only one spin system was observed in the COSY spectrum (Figure 12) for correlations between H-7 (δ_H 1.70), H-8 (δ_H 1.49), and H-9 (δ_H 0.96). In the HMBC experiment (Figure 13), the methyl group at δ_C 14.0 (C-9) correlated to the adjacent methalene carbon at δ_C 19.3 (C-8) and second correlation observed for the carbon at δ_C 39.1 (C-7). The two methylene groups at δ_C 39.1 (C-7) and δ_C 19.3 (C-8) respectively, showed correlations to oxymethine at δ_C 67.9 (C-6) in the HMBC spectrum. A broad singlet corresponding to one of the protons at H-6 (δ_H 4.55) showed no HSQC correlations (Figure 14) to any of the carbons, but useful HMBC correlations were observed which helped assign the proton to Hydroxyl group. Some relevant correlations from the olifinic proton helped assemble this unit and carbon NMR shifts justify the substitution patterns. At this point only 2 carbons were left to account for; chemical shift of this methyl group is consistent with the presence of this subunit to complete the structure. The structure of compound **1** was established as depicted in Figure 8.

Figure 8. Structure of New Compound 1 (01021-86-2).

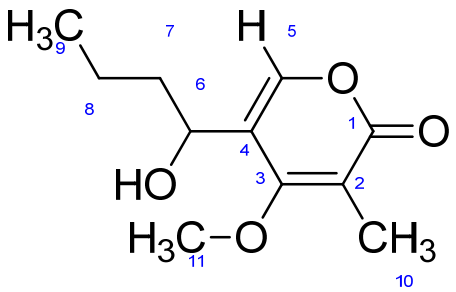


Figure 9. HRMS spectrum for Compound 1 (01021-86-2).

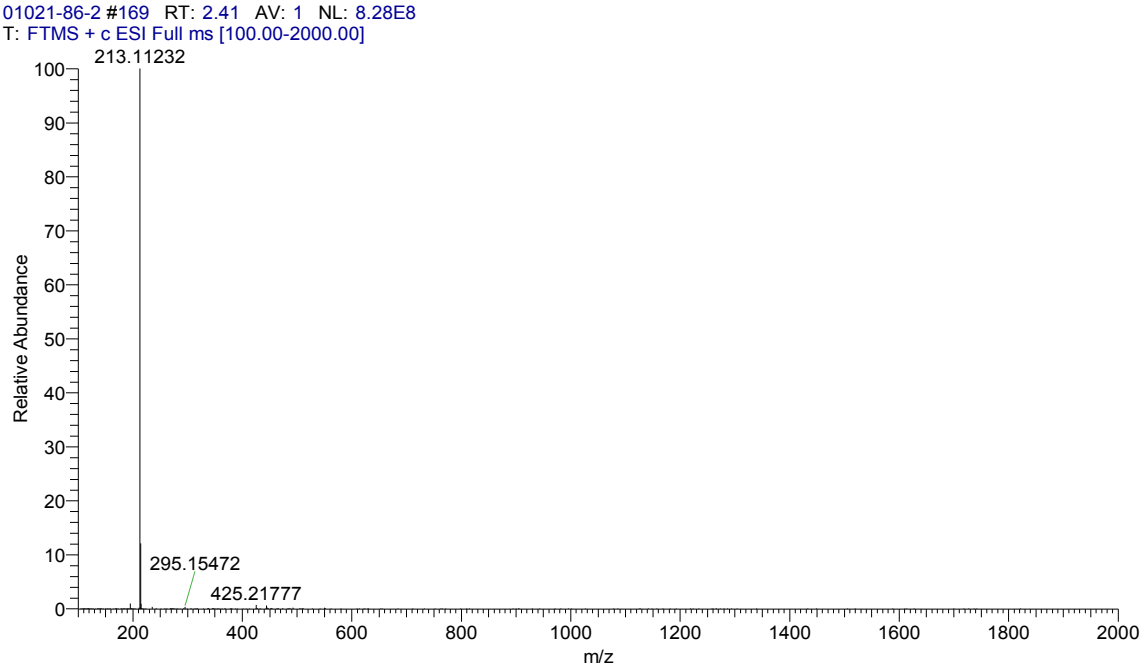


Figure 10. ^{13}C NMR of Compound 1 ($CDCl_3$).

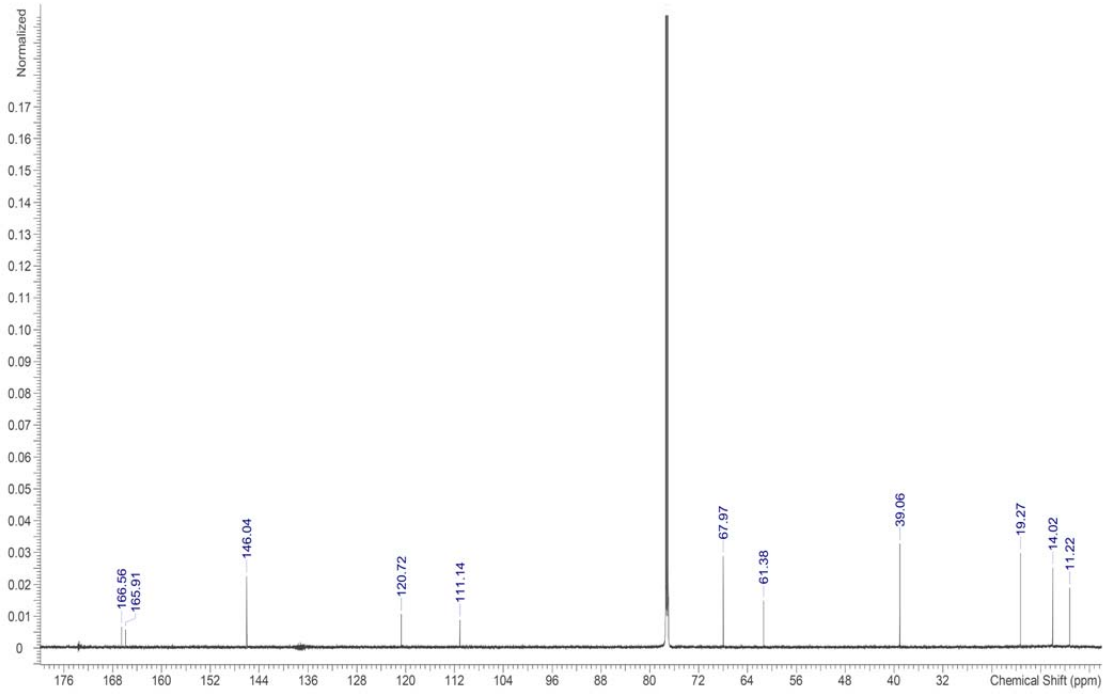


Figure 11. ^{1}H NMR spectrum of Compound 1 (01021-86-2).

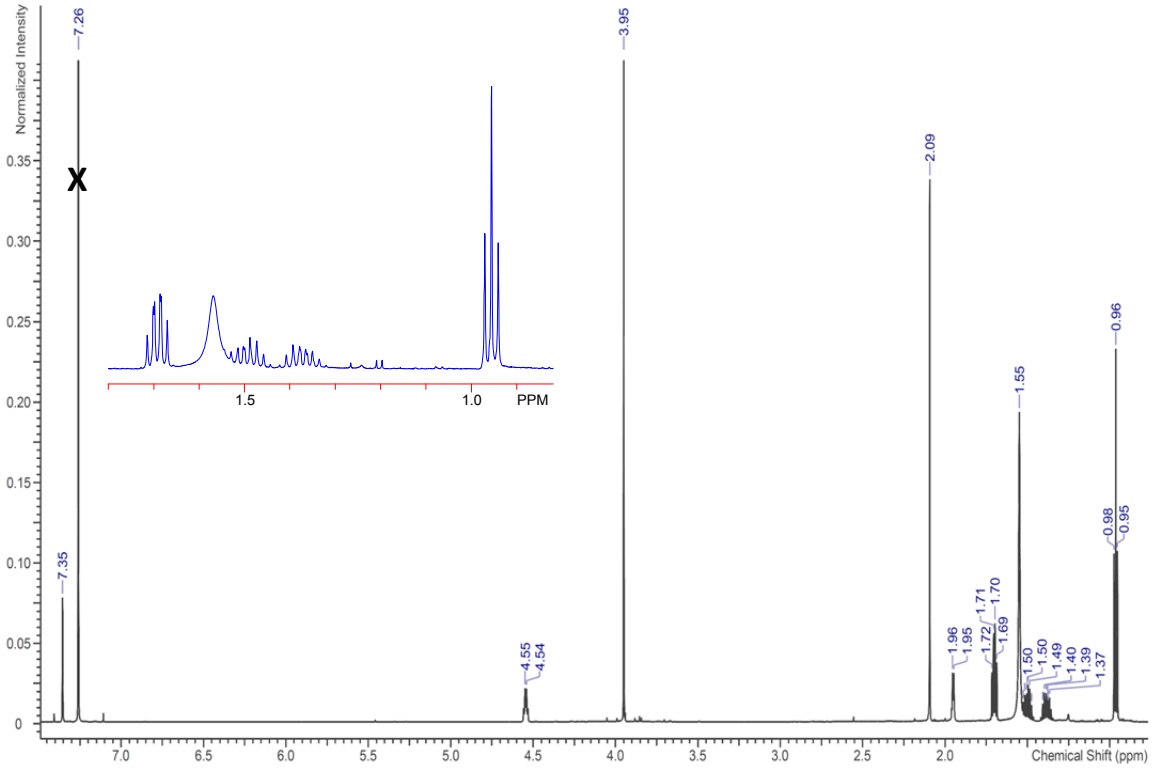


Figure 12. COSY Spectrum of Compound 1 (01021-86-2).

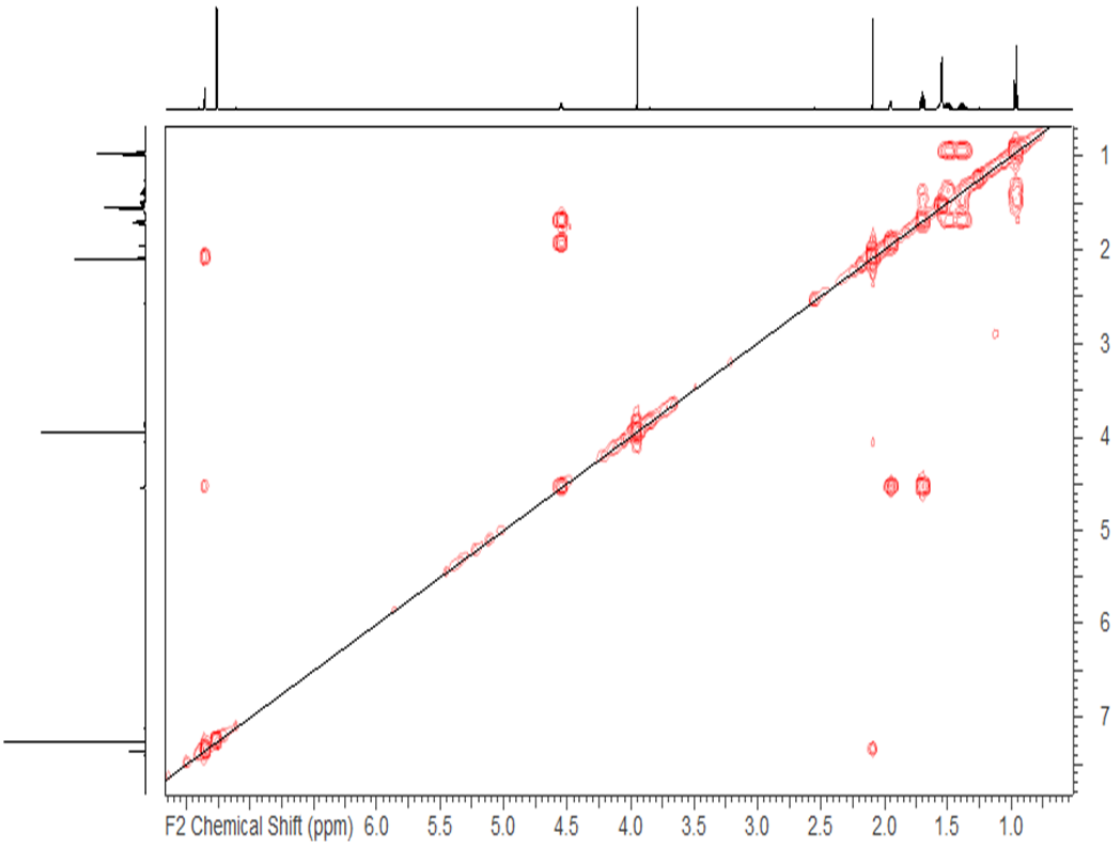


Figure 13. HMBC spectrum of Compound 1 (01021-86-2).

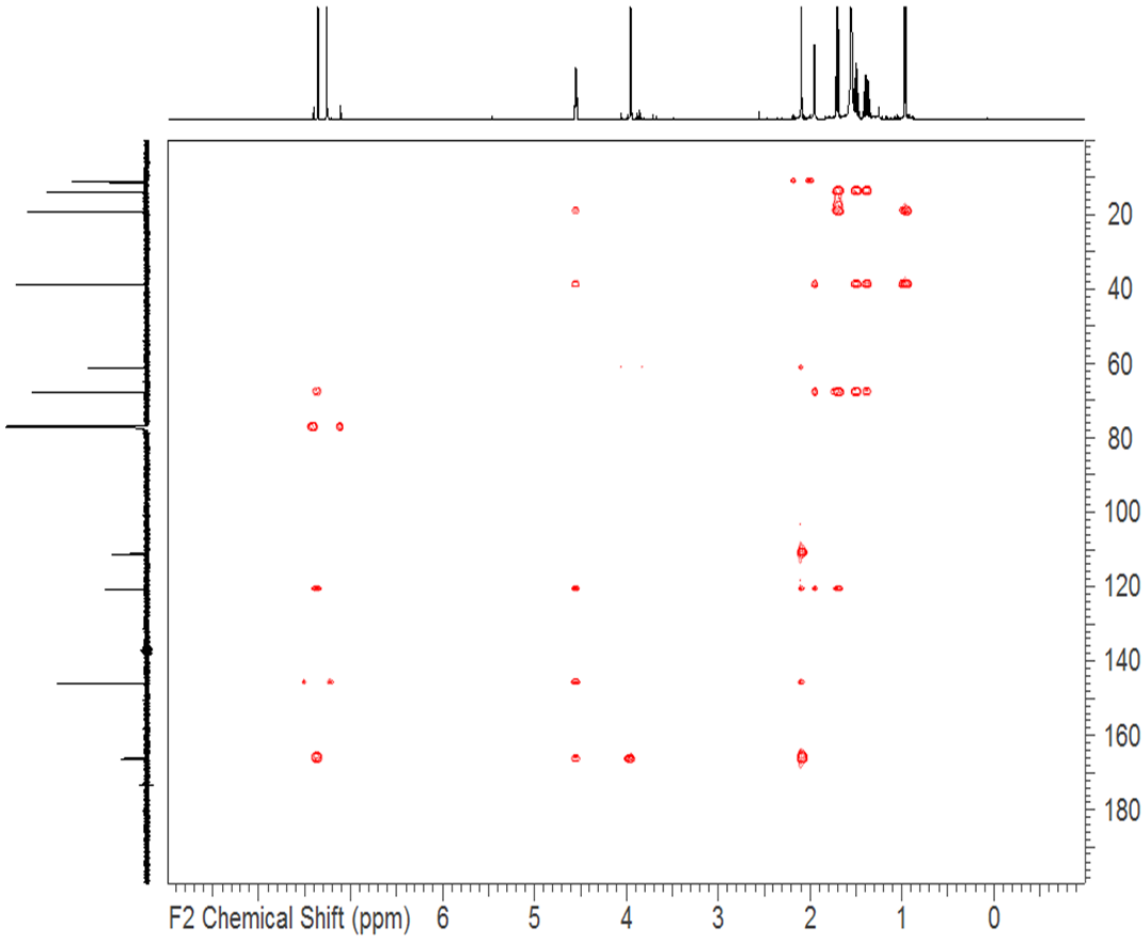


Figure 14. HSQC-edited NMR spectrum of Compound 1 (01021-86-2).

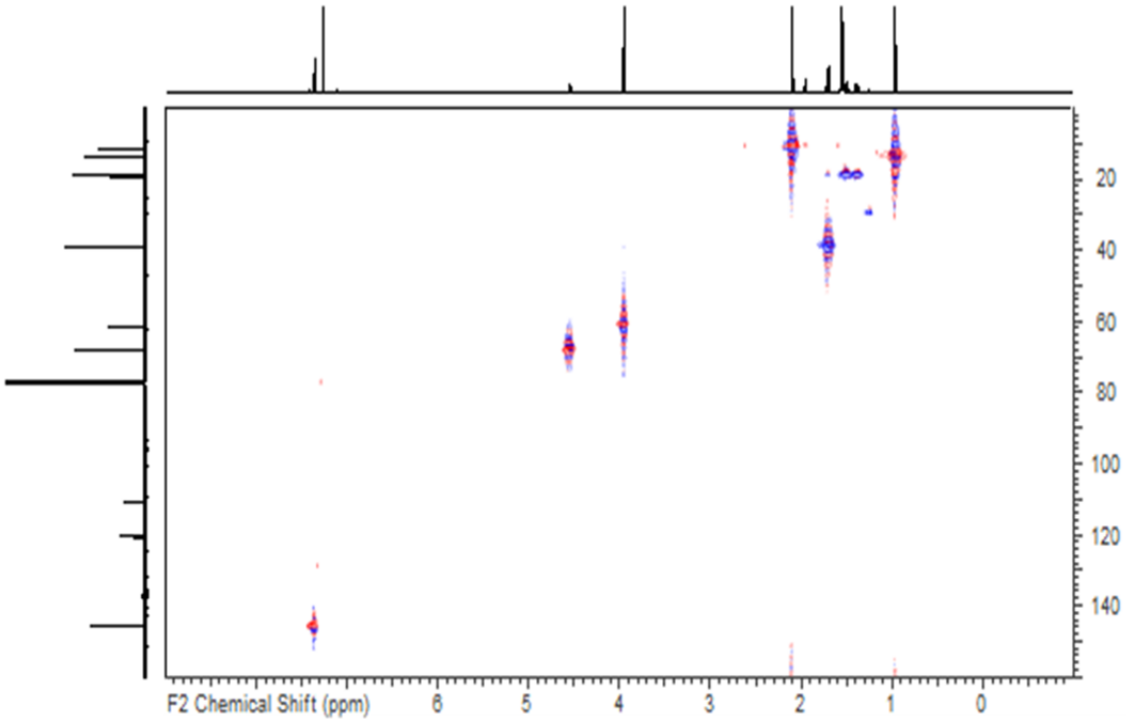


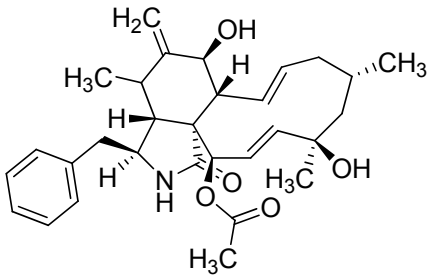
Table 6. NMR Data for Compound 1 (700 MHZ for ^{1}H, 176 MHz for ^{13}C; Chemical Shifts in δ, $CDCL_3$).

Position	δ_C	δ_H Mult
1	165.9	
2	111.1	
3	166.6	
4	120.7	
5	146.0	7.35 (s)
6	67.9	4.55 (t)
7	39.1	1.70 (m)
8	19.3	1.49 (m)
9	14.0	0.96 (s)
10	11.2	2.09 (s)
11	61.4	3.95 (s)

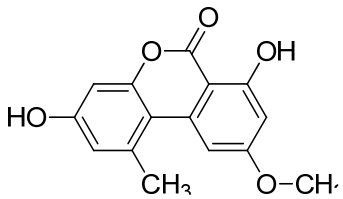
Compound **2** (01021-86-4, 7.19mg) was obtained as an oily sample. The molecular formula was determined as $C_{30}H_{39}NO_5$ by HRMS. The NMR data, in conjunction with HRMS data, identified **2** to be the known compound Cytochalasin H., reported by Tao et al in 2007 from the mangrove endophytic fungus *Phomopsis sp* (ZZF08) obtained from the South China Sea coast (Figure 15).[24]

Compound **3** (01021-86-5, 1.87mg) was obtained as a yellowy powder. The HRMS data suggested a molecular formula of $C_{15}H_{12}O_5$. The compound showed distinctive UV maxima at 222, 256, 287, 300 and 340 nm, having the typical pattern of alternariol derivatives. Positive ESI MS showed molecular ion peak at *m/z* 273.0761$[M+H]^+$. The spectral data of compound **3** were found in full agreement with those reported for Alternariol monomethyl ether, a compound reported in 1983 by Holker et al from the *Penicillium diversum,* (Figure 15).[25]

Figure 15. Chemical structures of compounds 2-3.



Cytochalasin H. (**2**)



Alternariol monomethyl ether (**3**)

Table 7. Comparison of ^{1}H and ^{13}C NMR data of Compound 2 (δ in ppm) with literature Cytochalasin H., recorded in $CDCl_3$ at 17°C at 500 MHz for ^{1}H and 125 MHz for ^{13}C.

	Cytochalasin H.[23]		Compound **2**	
Position	^{13}C	^{1}H	^{13}C	^{1}H
1	174.3	-	174.4	-
2	-	5.6	-	5.5
3	53.8	3.23	53.9	3.2
4	50.3	2.12	50.5	2.11
5	32.8	2.77	32.9	2.78
6	148.0	-	147.9	-
7	69.7	3.82	69.8	3.82
8	47.2	2.93	47.3	2.93
9	51.5	-	51.8	-
10	45.6	2.85	45.7	2.85
		2.64		2.64
11	14.1	1.0	14.2	0.98
12	114.1	5.15	114.3	5.11
		5.35		5.34
13	125.9	5.75	127.2	5.72
14	138.1	5.40	138.8	5.39
15	42.8	1.77	42.8	1.79
		2.0		2.03
16	31.1	1.8	31.2	1.34
17	53.8	1.56	53.8	1.57
		1.86		1.88
18	74.3	-	74.5	-
19	127.0	5.56	126.0	5.84
20	138.6	5.86	138.2	5.52
21	77.5	5.55	77.6	5.55
22	26.5	1.05	26.6	1.04
23	28.4	1.32	28.5	1.78
24	170.1		170.3	-
25	20.9	2.23	21.0	2.23
26	137.4	-	137.5	-
27,31	128.9	7.14	129.0	7.14
28,30	128.9	7.30	129.1	7.31
29	127.0	7.25	127.2	7.24

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