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Plasmodium Active Compounds from Endophytic Fungi

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Abstract

Malaria is a parasitic disease that involves high fevers, shaking chills, flu-like symptoms, and anemia. It is caused by a parasite *Plasmodium falciparum* that is passed from one human to another by the bite of infected *Anopheles* mosquitoes. Pregnant women, patients with HIV/AIDS, non-immune travelers, and in high transmission areas children under five years of age are in high risk of contracting malaria. One of the objectives of the Millennium Development Goals (MDGs) is eradicating malaria in Africa. Thus, a well-researched and documented work that may give rise to a new lead compound that can combat malaria effectively and at affordable rate is urgently desirable. Methanolic extract of the freeze-dried endophytic fungi coded MSX – 285138 from mangrove, subjected to a bioassay-guided fractionation yielded fractions that significantly inhibited the replication of malaria parasite. Some of these fractions demonstrated low cytotoxicity. The active fractions were purified using high performance liquid chromatography (HPLC) and were further examined and characterized by spectrometric techniques. Two cytochalasin derivatives, cytochalasin D and epoxycytochalasin D were identified from the active isolates which were found to inhibit *Plasmodium falciparum* (3d7) with IC50 of 10.58 and 2 9.77 nM respectively. However these compounds cannot yet be developed as antimalarial drug because they are also found to be cytostatic. They could also be useful in the management of cancer related ailments.

Keywords: Malaria, Endophytic fungi, Plasmodium, Cytotoxicity, Cytochalasin

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1. Introduction

The need for new and useful compounds to provide assistance and relief in all aspects of the human condition is ever growing. Drug resistance in bacteria, the appearance of life threatening viruses, the recurring problems with disease in persons with organ transplants, and the tremendous increase in the incidence of fungal infections in the world's population underscore our inadequacy to cope with these medical problems. Added to this are enormous difficulties in raising enough food in certain areas of the world to support local human populations. Environmental degradation, loss of biodiversity, and spoilage of land and water also add to problems facing mankind, (Strobel and Daisy, 2003).

Natural products are naturally derived metabolites and/or by-products from microorganisms, plants, or animals. These products have been exploited for human use for thousands of years, and plants have been the chief source of compounds used for medicine. Recently, it was found that the mangrove plants and its endophytes can produce lots of significant natural structures (Zhu *et al*., 2009). Endophytes are microorganisms that reside in the tissues of living plants, they are relatively unstudied potential sources of novel natural products for exploitation in medicine, agriculture, and industry. It is noteworthy that, of the nearly 300,000 plant species that exist on the earth, each individual plant is host to one or more endophytes. Only a few of these plants have ever been completely studied relative to their endophytic biology.

Consequently, the opportunity to find new and interesting endophytic microorganisms among myriads of plants in different settings and ecosystems is great (Strobel and Daisy, 2003; Strobel *et al*., 2008). Their biological diversity, especially in temperate and tropical rainforests, is enormous. Many are known to produce biologically active substances which have found application in pharmacology (e.g. the anticancer drug taxol) and agriculture (Strobel and Daisy, 2003; Strobel *et al*., 1996). For instance, the recovery of a volatile antibiotic producing fungus, *Muscodor albus*, from *Cinnamomum zeylanicum* demonstrated that novel micro-organisms in the world's rainforests can be put to useful purposes (Worapong *et al*., 2001; Strobel *et al*., 2001; Strobel, 2006; Mercier, and Jimenez 2007).

As part of the global effort in this research area, we embarked on searching for active antimalarials from terrestrial fungal samples supplied by the Mycosynthetix Inc., USA.

2. Materials and Methods

Bioassay-guided isolation of active anti-malarials

This part of the experiment was carried out in Dr. Baker's Lab at Department of Chemistry, University of South Florida (USF). Freeze-dried endophytic fungi samples supplied by the Mycosynthetix, Inc. were extracted with Methanol (MeOH). The crude extracts were analyzed for preliminary malaria assay. The active antimalarial crude extract was subjected to normal phase Medium Polar Liquid Chromatography (MPLC) by gradient elution (100% hexane to 100% ethyl acetate (EtOAc) to 100% MeOH) for the first round of fractionation. Teledyne Isco Combiflash Companion MPLC with Teledyne Isco silica gel cartridges was used for the fractionation. The MPLC-generated fractions were prepared for the antimalarial and cytotoxicity screening by plating 200 µl of 2 mg/mL of each fraction in 96-well plates.

Further chromatographic purification employing reverse phase High Performance Liquid Chromatography (HPLC) by sometimes gradient or isocratic elution (water: acetonitrile) separation was carried out on the fully (>67% inhibition, high priority) and partially active fractions (33-67% inhibition, low priority) that exhibit anti-malarial activity. Cytostatic property was observed in the cytotoxicity assay when the cells tested stopped dividing. The fractions were resubmitted for a second round of bioassay. Active fractions were further purified into pure compounds for the final bioassay.

Malaria Bioassay and Cytotoxicity Assay

The antimalarial and cytotoxicity bioaasay was carried out in Dr. Kyles laboratory at the Department of Public Health, USF. For the bioassay the method described by Franke-Fayard *et al*. (2008) was used.

Compounds Characterization

^{1}H, ^{13}C, Distortionless Enhancement by Polarization Transfer (DEPT) spectroscopy, ^{1}H-^{1}H Correlation (COSY), Heteronuclear single Quantum Coherence (HSQC), and Heteronuclear Multiple Bond Correlation (HMBC) spectra were obtained on a Varian Inova 500 instrument operating at 500 MHz for ^{1}H and 125 MHz for ^{13}C. HPLC was performed with a Shimadzu LC-8A multisolvent delivery system connected to a Shimadzu SPD-10A UV – VIS tunable absorbance detector using a YMC Pack ODS-AQ C-18 analytical column (250 × 10 mm) for reversed phase, Phenomenex Sphereclone (250 × 10 mm), Sun Fire Prep silica column (250 ×

10 mm) for normal phase, or YMC Pack CN preparative column (250 × 20 mm). Mass spectroscopic data were obtained from Agilent Technologies LC/MSD TOF.

Known compounds from marine and microbial source database, Antimarin, was used to help identify known compounds. Isolated pure compounds were subjected to search using the database using molecular mass data, ^{1}H or ^{13}C NMR chemical shift data. A complete NMR data set (^{1}H, ^{13}C, COSY, HMBC, HSQC, gHSQC) was obtained for potential new compounds whose structure was not found in the database.

3. Results and Discussion

Cytochalasin D and epoxy cytochalasin D were isolated from the MSX – 285138 – 3g fungal extract. High Resolution Electrospray Ionization Mass Spectrometry (HRESIMS) (Fig I and II) indicated the $[M + H]^+$ ion and $[M + Na]^+$ for MSX-285138-3g-M-14 at *m/z* 524.3 and 546.2 respectively, suggesting a molecular mass of 523 and molecular formula of $C_{30}H_{37}NO_7$ (calculated for $C_{30}H_{37}NO_7$, 523.62). Extensive NMR data (Table 1) revealed the planar structure of MSX-285138-3g-M-14 was identical to that of epoxycytochalasin D (Figure III) (Espada *et al*., 1997).

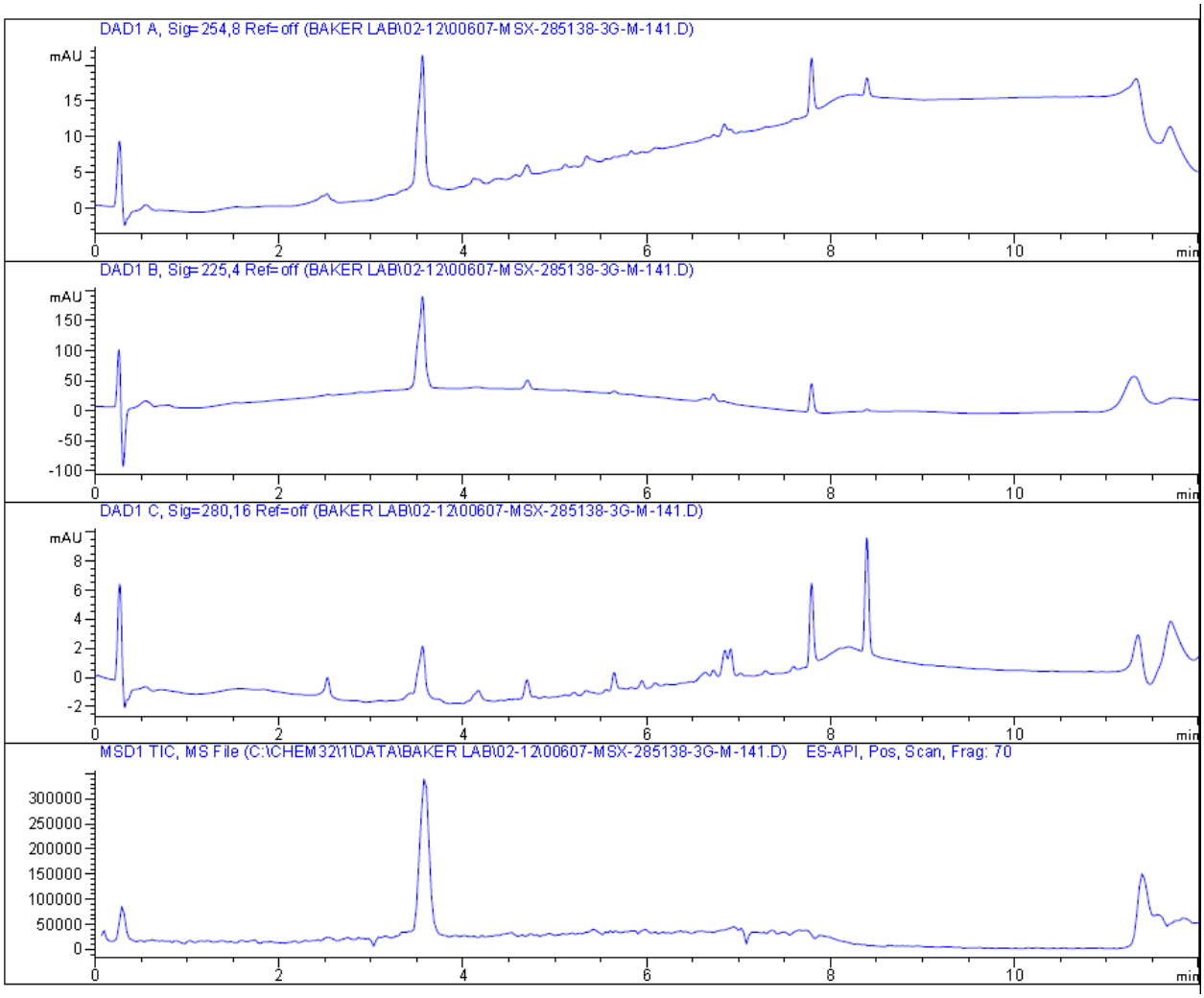

Figure I: LCMS Chromatogram for MSX-285138-M-14

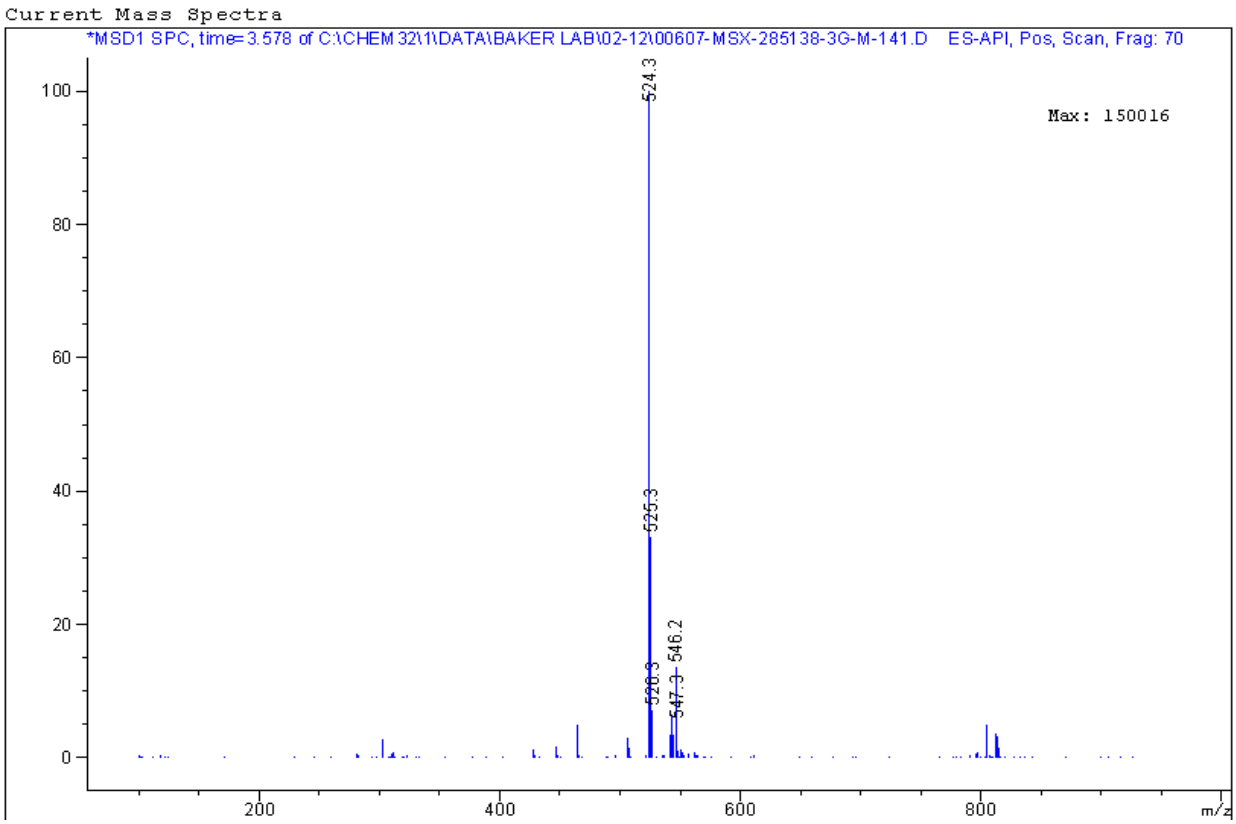

Figure II: TIC of the LCMS Chromatogram for MSX-285138-M-14 6

Table 1: ^{1}H and ^{13}C chemical shifts of MSX-285138-3g-M-14 and that reported for the epoxycytochalasin D.

Position	MSX-285138-3g-M-14		Epoxycytochalasin D [10]	
	$^{1}H^{a}$	$^{13}C^{b}$	$^{1}H^{c}$	$^{13}C^{d}$
NH	5.53-5.57 (m, 2H)	173.5	5.46 (br s)	173.43
3	3.20-3.29 (m 2H)	53.94	3.24 (m)	53.9
4	2.28 (dd, 4.88, 3.42 1H	50.68	2.25 (dd, 5.2, 3.3)	50.71
5	2.61-2.72 (m, 3H)	32.55	2.62 (m)	32.56
7	3.84 (d, 10.74, 1H)	147.34	3.81 (br d, 10.1)	147.35
8	2.61-2.72 (m, 3H)	69.97	2.62 (m)	69.98
10	2.88 (dd, 13.43, 5.13, 1H; 2.76 (dd, 13.67, 9.28, 1H)	46.53	2.85 (dd, 13.4, 5.0); 2.73 (dd, 13.4, 9.1)	46.54
11	0.88 (m, 3H)	52.48	0.89 (d, 6.9)	52.45
12	5.08 (s, 1H); 5.30 (s, 1H)	45.16	5.27 (br s); 5.06 (br s)	45.18
13	5.92 (dd, 15.62, 9.77, 1H)	13.49	5.89 (dd, 15.5, 9.8)	13.49
14	5.72 (ddd, 15.62, 9.77, 5.86, 1H)	114.44	5.69 (ddd, 15.5, 9.9, 5.8)	114.44
15	2.61-2.72 (m, 3H); 2.09-2.15 (m, 1H)	131.15	2.62 (m); 2.09 (m)	131.17
16	3.20-3.29 (m, 2H)	133.47	3.22 (m)	133.47
19	3.17 (d, 1.95, 1H)	37.41	3.14 (d, 1.9)	37.4
20	3.56 (s, 1H)	41.89	3.53 (dd, 1.9, 0.8)	41.89
21	5.53 5.57 (m, 2H)	215.28	5.51 (br s)	215.28
22	1.22 (d, 6.84, 3H)	76.33	1.18 (d, 6.6)	76.32
23	1.55 (s, 3H)	59.64	1.53 (s)	59.63
25	2.17 (s, 2H)	52.73	2.14 (s)	52.72
2', 6'	7.31-7.36 (m, 2H)	74.06	7.32 (m)	74.06
3', 5'	7.17 (d, 6.84, 2H)	19.18	7.15 (m)	19.17
4'		21.86	7.24 (m)	21.86

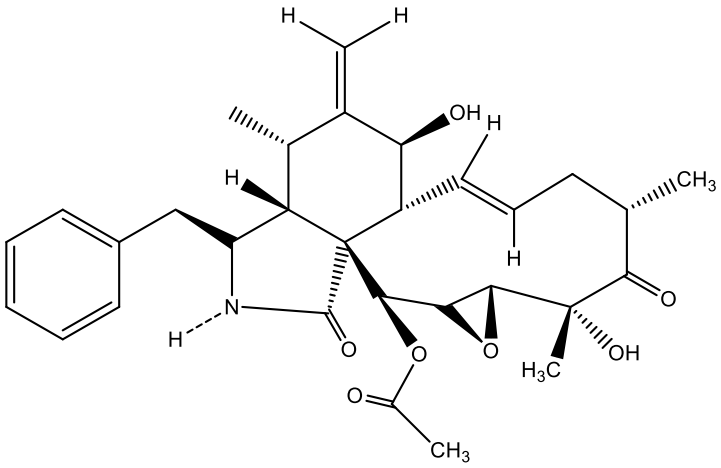

Figure III: Epoxycytochalasin D

On the other hand HRESIMI (Figure IV & V) indicated the $[M + H]^+$ ion and $[M + Na]^+$ for MSX-285138-3g-M-16-2 at *m/z* 508.1 and 530.2 respectively, suggesting a molecular mass of 507 and molecular formula of $C_{30}H_{37}NO_6$, in addition a peak corresponding to $[M-H_20+H]^+$ was observed at m/z at 490.2. ^{1}H NMR data (Table 2, 3) and 2D NMR data revealed the structure of MSX-285138-3g-M-16-2 to be that of cytochalasin D (Figure VI) (Xu *et al*., 2001).

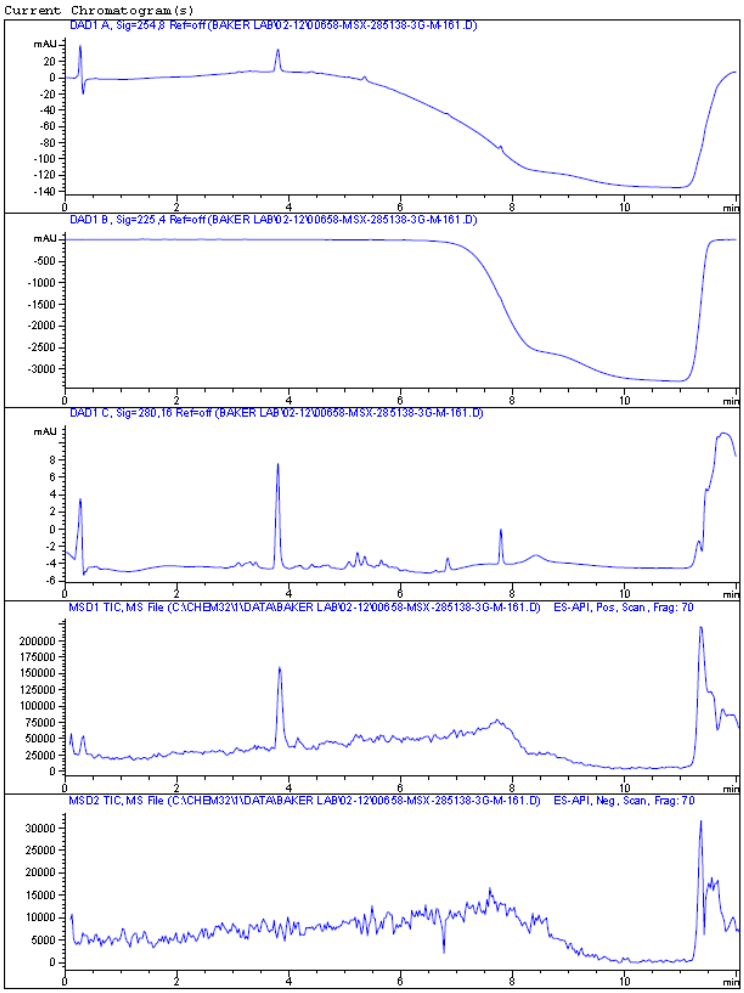

Figure IV: LCMS Chromatogram for MSX-285138-M-16-2

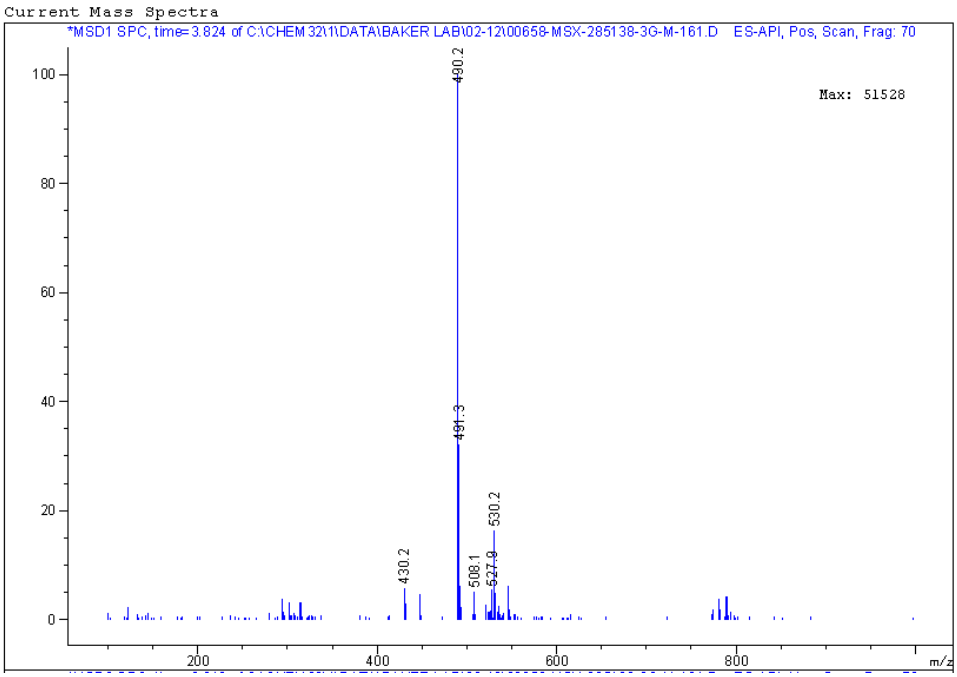

Figure V: TIC of the LCMS Chromatogram for MSX-285138-M-16-2 at 3.824 min

Table 2: [1]H chemical shifts of MSX-285138-3g-M-16-2 and that reported for the epoxycytochalasin D.

[1]H	**MSX-285138 - 3g – M – 16 – 2[a]**	**Cytochalasin D[a] [11]**
H-3 α	3.24 (1H, dt, 8.73, 4.04)	3.22 (m, 4.5, 4.0, 1H)
H-4		2.84 (m, 5, 2, 1H)
H-5 β	2.14 - 2.18 (1H, m)	2.14 (t, 5, 1H)
H-7 α	3.82 (1H, d, 10.99)	3.80 (d, 10.5, 1H)
H-8 β	2.80 - 2.89 (2H, m)	2.83 (m, 10.5, 5, 2, 1H)
H-10 α	2.66 (1H, s)	2.65 (dd, 13.5, 9, 5, 1H)
H-10 β		2.83 (m, 13.5, 10, 2.5, 1H)
H-11	0.96 (2H, d, 6.47)	0.95 (d, 7, 3H)
H-12	5.10 (1H, s)	5.09 (s); 5.29 (s), 2H
H-13	5.29 - 5.39 (2H, m)	5.35 (m, 15.5, 10, 5, 1H)
H-14	5.70 (1H, dd, 15.52, 9.70)	5.65 (dd, 15.5, 10, 1H)
H-15 α	2.03 (1H, dd, 2.93, 5.17)	2.02 (dd, 13.5, 5, 1H)
H-15 β	2.48 - 2.57 (1H, m)	2.51 (dd, 13.5, 11, 1H)
H-16 β	2.74 (2H, d, 6.47)	2.73 (m, 11, 4.5, 1H)
H-19	5.15 (1H, dd, 15.84, 2.26)	5.15 (dd, 16, 2.5, 1H)
H-20	6.12 (1H, dd, 15.52, 2.59)	6.11 (dd, 16, 2.5, 1H)
H-21 α	5.64 (1H, br s)	5.63 (dd, 2.5, 1H)
H-22	1.21 (2H, d, 7.11)	1.20 (d, 7, 3H)
H-23	1.52 (3H, s)	1.51 (s, 3H)
Ph-H	7.25 (1H, br s); 7.30 - 7.35 (2H, m)	7.25 (m, 15, 7, 1.5, 5H)
OAc	2.27 (2H, s)	2.26 (s, 3H)
NH	5.51 (1H, br s)	5.53 (bsr, 1H)
	1.26 (2H, br s)	
	2.63 (1H, s)	
	2.68 (1H, d, 3.88)	
	2.70 (1H, s)	
	7.14 (1H, d, 7.11)	

[a]Recorded at 500 MHz in CDCl3

Table 3: ^{13}C chemical shifts of MSX-285138-3g-M-16-2 and that reported for the epoxycytochalasin D.

Carbon	MSX – 285138 - 3g – M – 16 – 2[b]*	Cytochalasin D[b 11]
C-1		173.64
C-3	51.51	53.53
C-4	46.03	46.96
C-5	50.23	49.96
C-6	147.6	147.46
C-7	69.92	69.80
C-8	32.98	32.63
C-9	53.73	53.24
C-10	45.54	45.28
C-11	13.67	13.63
C-12		114.50
C-13	133.95	134.11
C-14	132.44	130.59
C-15	37.92	37.70
C-16	42.42	42.29
C-17	210.13	210.23
C-18	77.4	77.66
C-19	127.1	127.08
C-20	132.44	132.26
C-21	77.2	77.26
C-22	19.48	19.39
C-23	24.3	24.16
C-1’	137.24	137.21
C-2’-C6’	129.19	129.06
C-3’-C-5’	128.7	128.92
C-4’	127.74	127.57
O$\underline{C}$O$\underline{C}$H$_3$	169.57	169.69
OCO$\underline{C}$H$_3$	21	20.84

[b]Recorded at 125 MHz in CDCl3

*****The data for the ^{13}C NMR were deduced from the data generated for gHSQC and gHMBC

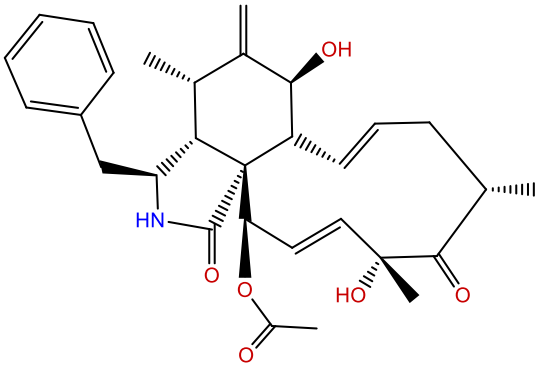

Figure 6: Cytochalasin D

These compounds have been previously isolated from *Engleromyces goetzii* (Jikai *et al*., 2002) and their activities against malarial parasite have been reported (Wai, 2010), the activities of the compounds and molecular structures are summarized in Table 4.

Table 4: The anti-malarial activity against *Plasmodium falciparum* and cytotoxicity of MSX-285138-3g-M-14 and MSX-285138-3g-M-16-2

Sample	**Compound Name**	**MW (g/mol)**	**IC50 (ng/ml)**	
			Antimalarial Activity [a]	**Cytotoxicity [b]**
MSX-285138-3g-M-14	Epoxycytochalasin D	523	9.77	150*
MSX-285138-3g-M-16-2	Cytochalasin D	507	10.58	625*

[a]3D7 *Plasmodium falciparum* [b]A549 Cytotoxicity

*compound is cytostatic until it reaches a concentration around 150/625 ng/ml then it begins to lose its cytostatic properties.

Table 4 reveals the very high antiplasmodic properties of the two compounds as well as their cytostatic nature. The cytotoxic activity against tumor cell line P-388 of epoxycytochalasin D has been previously reported (Miller *et al*., 1979). Cytochalasins are common fungal metabolite and bad drug lead, they are known to bind to actin filaments in cells and block polymerization and elongation of actin. The cytostatic nature of cytochalasin has prevented it from progressing onto the next stage of the study because the cytostatic activity is not

selective. Cytochalasin exerts its antimalarial activity by allowing the malaria parasite attached to the red blood cells but not being able to move or enter the red blood cells (Smythe *et al*., 2008).

In a more recent study, the mode of action of cytochalasin D was investigated. Since cytochalasin D is well-known to prevent actin elongation and promotes the dispersion of existing actin filaments, the study suggested that actin is required for endocytic trafficking in *Plasmodium falciparum*. Cytochalasin D promotes actin filament of the parasite to depolymereize and inhibits haemoglobin digestion as a result of disrupted endocytosis within the parasite (Shi and Zhan, 2007).

The cytotoxic and cytostatic properties of these compounds have been established and has been suggested that they may be useful as cancer treating agents (Parra-Delgado *et al*., 2005; Polkowskia *et al*., 2004; Poletto *et al*., 2008). Though these compounds are not yet suitable as candidate for malaria drug development, they may be developed after further testing as anticancer agents.

4. CONCLUSION

Two cytochalasin derivatives, cytochalasin D and epoxycytochalasin D were identified from the active isolates of the endophytic fungi which were found to inhibit *Plasmodium falciparum* (3d7) with IC_{50} of 10.58 and 9.77 nM respectively. However these compounds cannot yet be developed as antimalarial drug because they are also found to be cytostatic. They could be useful in the management of cancer related ailments.

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