

# Chapter 2

SECONDARY METABOLITES FROM  
THREE DIFFERENT MARINE DERIVED  
*ASPERGILLUS* SPECIES

## **I. Introduction**

### *i) General:*

Given the recent evidences, marine-derived fungi are proving to be excellent source of chemical entities with novel structures and biological potential (Ebel, 2008; Rateb and Ebel, 2011). A review of literature on fungal metabolites, (chapter 1) shows that even the marine derived *Aspergillus* spp. are one the richest producer of secondary metabolites. *Aspergillus* is one of the oldest named genera of fungi, received its name from Micheli in 1729. *Aspergilli* grow abundantly as saprophytes on decaying vegetation, but they can also live on substrates as diverse as dung, human tissues, and antique parchment. *Aspergillus* is ubiquitous genera of fungus commonly isolated from soil, plant debris, and indoor air environment and also from several marine habitats. It has ability to disperse globally in air currents and to grow almost anywhere when appropriate food, water are available and can even tolerate high salt concentration. As a result, isolates (facultative marine) similar to terrestrial ones have been obtained from marine habitats. Marine-derived strains may produce unusual secondary metabolites as an environmental adaptation. The genus *Aspergillus* includes over 250 species (Geiser, 2008) and around 20 species have so far been reported as causative agents of opportunistic infections in man.

### *ii) Role of Genus Aspergillus in Human Economy:*

*Aspergilli* are industrially important microorganisms due to their production of organic acids, statins and extracellular enzymes. Citric acid is a true 'bulk chemical' with an estimated production of more than 1.6 billion kg each year (Dodds and Gross, 2007) and gluconic acid, which is used for therapy for iron and calcium deficiency are obtained from *Aspergillus niger*. *Aspergillus terreus* is used for itaconic acid production, a synthetic polymer (Calam et al., 1939). *Aspergillus. oryzae* is fermented for kojic acid production which is used for skin whitening and as a precursor for synthesis of flavour enhancers (Ruijter et al., 2002). The first statin approved for

human use, was lovastatin, a secondary metabolite of *A. terreus* was developed by a group of scientists at Merck Inc. in the USA (Alberts et al., 1980). Lovastatin was sold under the brand named Mevacor<sup>TM</sup> and became a blockbuster drug for Merck. Other compounds with pharmacological activities include cholecystokinin and neurokinin antagonists, ion channel ligands, antifungal drugs and a host of other compounds. A useful comprehensive review of fungal drug discovery is provided by An (2005) in *Handbook of Industrial Mycology*.

Food fermentations are ancient technologies that harness microorganisms and their enzymes to improve the human diet. Hydrolytic enzymes from *A. oryzae* and *A. sojae* suit them for growth on starch and other carbohydrate-rich substrates, and are effective in production of rice wine. These enzymes are also used in legume fermentation to obtain miso and soy sauce. Although this genus produces many industrially useful enzymes, chemicals, and biomedical, they are also known as producer of deadly carcinogenic toxins such as aflatoxin from *A. flavus* (Reddy et al., 2005). There is probably no other genus of fungi so useful to humans that is also so harmful to humans.

### *iii) Scope of our study*

Fungi for many decades have played a pivotal role as a source of interesting new natural products, mainly due to their highly developed and diverse secondary metabolism. Thus, terrestrial fungi have been intensively studied over the three quarters of 20<sup>th</sup> century. The lack novelty in chemistry of the terrestrial fungi diverted the attention of mycochemists towards unexplored fungi from marine habitats as source of new chemical entities with potent bioactivity. The work described in this chapter is an effort to discover novel structures with potent biological activity from three marine-derived fungi, belonging to genus *Aspergillus*.

The chapter is divided into five sections. The first three sections, section 2.1, section 2.2 and section 2.3 deals with the purification, structure elucidation and biological activities of metabolites from *A. flavipes*, *A. terreus* and *A. aculateus* respectively. Section 2.4 deals with the detection of minor constituents and chemotaxonomic

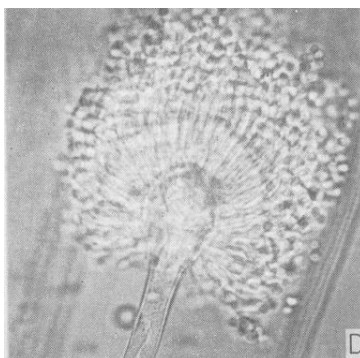
comparison of the two fungi, *A. flavipes* and *A. terreus* using electrospray ionization mass spectrometry. The last section *i.e* section 2.5 describes the effect of sea water on the production of butyrolactone I (**FM-1**) in *A. terreus*.

## **Section 2.1: Bioactive secondary metabolites from the mangrove-derived fungus *Aspergillus flavipes***

This section is further sub-divided into two subsections the subsection I gives the review of literature on *A. flavipes* and its metabolites and the subsection II describes the purification of fungal strains and structure elucidation of bioactive metabolites from *A. flavipes* isolated from pneumatophores of mangrove *Acanthus illicifolius*.

### **I. Review of Literature on metabolites from *Aspergillus flavipes***

*Aspergillus flavipes* produces colonies, which are white or silvery white, reverse the medium yellow to orange brown or reddish brown. The conidia are colourless, smooth globose 2-3  $\mu\text{m}$  in diameter (**Fig 2.1**).



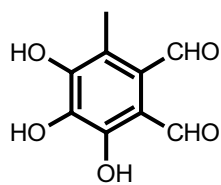
**Fig 2.1: Photomicrograph of typical head of *A. flavipes***

A review of literature indicates that over 15 novel and several known compounds have been identified from this species, most of them being soil-derived fungal strains and there being one a solitary reference on the metabolites from marine derived strain. The first metabolite with complete structure elucidation to be reported from this species was flavipin (**1**) having powerful antifungal property in 1956 (Raistrick and Rudman). It was identified as 1,2-diformyl-4,5,6-trihydroxy-3-methylbenzene, by using classical organic chemical methods (without the use of any available spectroscopic evidences). This was followed by the isolation of yet another new

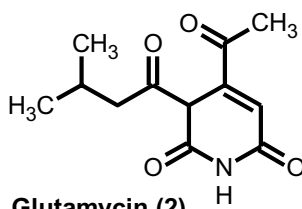
compound glutamycin (**2**), from *A. flavipes* by Casonovi et al., in 1968. Glutamycin (**2**) was highly potent against both gram +ve and -ve bacteria. In 1972, a pyridine base antibacterial, named flavipucine (**3**) was isolated from the same species by Findlay and Radices (1972) and identified as 3'-isovaleryl-6-methyl pyridine-3-spiro-2(1H),4(3H)-dione .

The first cytotoxic compound to be reported from *A. flavipes* was TMC-169 (**4**) (Kohno et al., 1999). Through modern spectroscopic analysis including 2D-NMR techniques such as HMBC and ROESY, **4** was identified as a new analogue of the aspochalasin group, represented by aspochalasins A-G (Fang et al., 1997) and phomactin C (Alvi et al., 1997). Aspochalasins consist of 11,13, or 14-membered carbocyclic (or oxygen-containing) rings connecting the C-8 and C-9 positions of a perhydroisoindol-1-one moiety bearing 2-methylpropyl group substituent at C-3. Later on, three new moderately cytotoxic aspochalasins I (**5**), J (**6**) and K (**7**) were isolated from *A. flavipes* obtained from rhizosphere of *Ericameria laricifolia* (Guang et al., 2004). Yet another aspochalasin L (**8**) having activity against HIV-1 integrase was isolated from the extract of a soil derived fungal culture of *A. flavipes* (Rochfort et al., 2005).

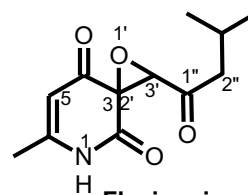
Barrow and Sun (1994) reports the isolation of spiroquinazoline (**9**), a novel P inhibitor with a new carbon skeleton. Structurally **9** is related to fumiquinazoline C (**10**), a metabolite of a marine fungus *A. fumigatus* (Numata et al., 1992),but differs from **10** in having a methyl and oxygen less in its structure. Additionally, a new but inactive metabolite, benzodiazepinedione (**11**) along with seven known diketopiperazine derivatives and known compounds acyl aszonalenin and N-benzoyl- L-phenylalaninol, were also isolated from this fungus. As mentioned earlier there is a single report on metabolites from marine derived strain of *A. flavipes*, namely flavicerebrosides A (**12**) and B (**13**), beside two known glycosphingolipids cerebrosides D and C (Jiang et al., 2004). The latest report on the metabolites from *A. flavipes* is the isolation of two highly hydroxylated 1,3 dihydrobenzofurans FR198248 (**14**) and FR202306 (**15**) from soil derived culture (Kwon et al., 2010).



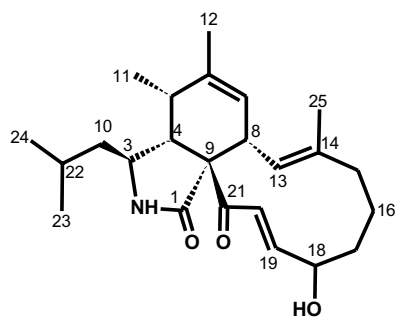
Flavipin (1)



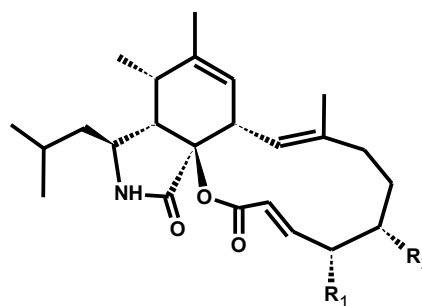
Glutamycin (2)



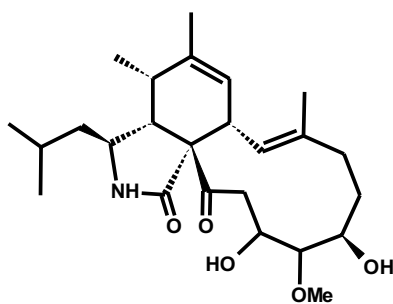
Flavipucine (3)



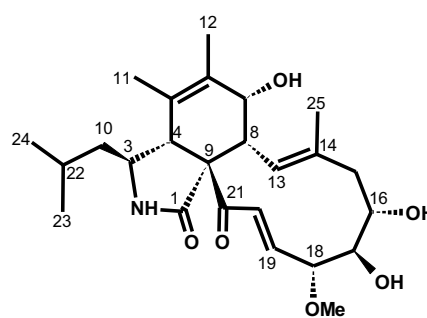
TMC-169 (4)



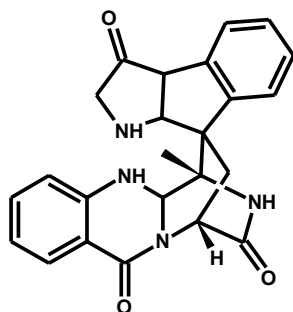
Aspochalasin I,  $R_1 = R_2 = \text{OH}$  (5)  
Aspochalasin J,  $R_1 = \text{OH}$   $R_2 = \text{H}$  (6)



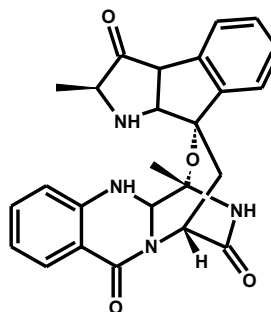
Aspochalasin K (7)



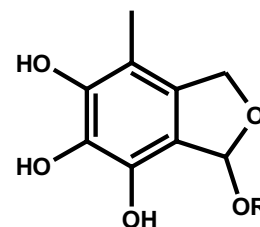
Aspochalasin L (8)



Spiroquinazoline (9)

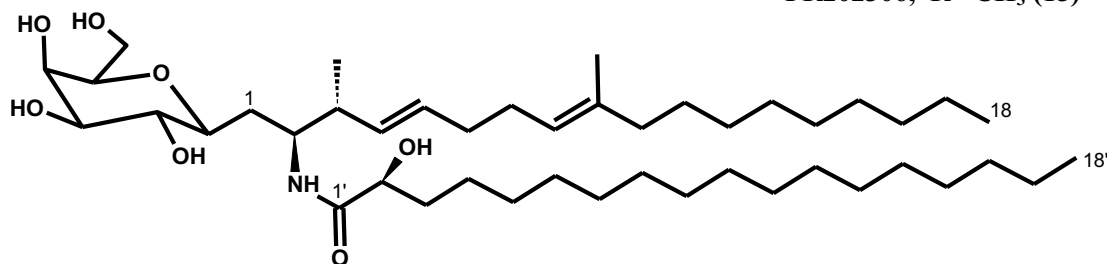


Fumiquinazoline C (10)

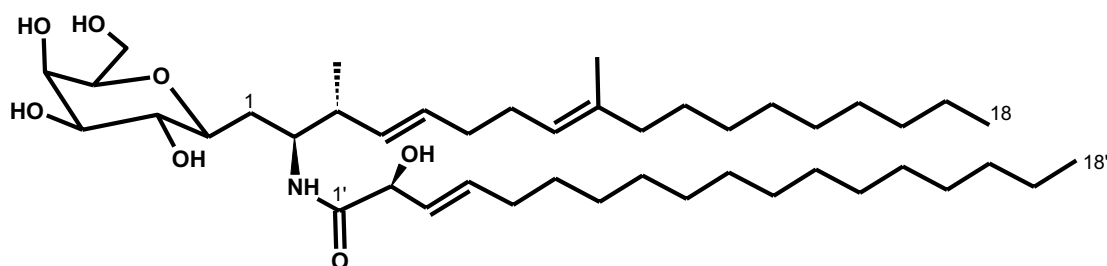


FR198248,  $R = \text{H}$  (14)

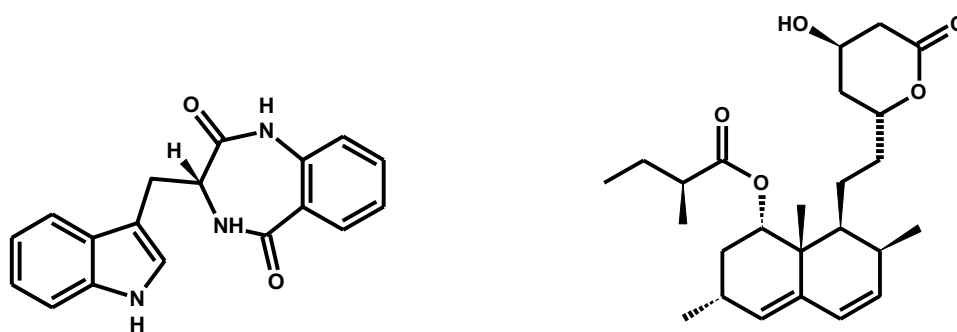
FR202306, R = CH<sub>3</sub> (15)



Flavicerebrosides A (12)



Flavicerebrosides B (13)



Benzodiazepinedione (11)

Lovastatin (16)

*A. flavipes* has resemblance to another well-known terrestrial fungus *A. terreus*. Morphologically, the young cultures of the two species are indistinguishable (Raistrick and Rudman, 1956). Genetically *A. terreus* is discriminated from *A. flavipes* only by presence pksM (6-methylsalicylic acid synthase) gene (Pazoutova et al., 1999). These similarities are also reflected in their metabolite chemistry. Lovastatin (16) (Valera et al., 2005; Alberts et al., 1980) and flavipin (1) are common metabolites to both species.



*A. flavipes* is also known to produce enzymes such as  $\alpha$ -galactosidase, L-Methioninase (Ashraf et al., 2009) and endo-1,4-beta-D-xylanase.

## II. Results and discussion:

### *i) Isolation and cultivation mangrove associated fungi*

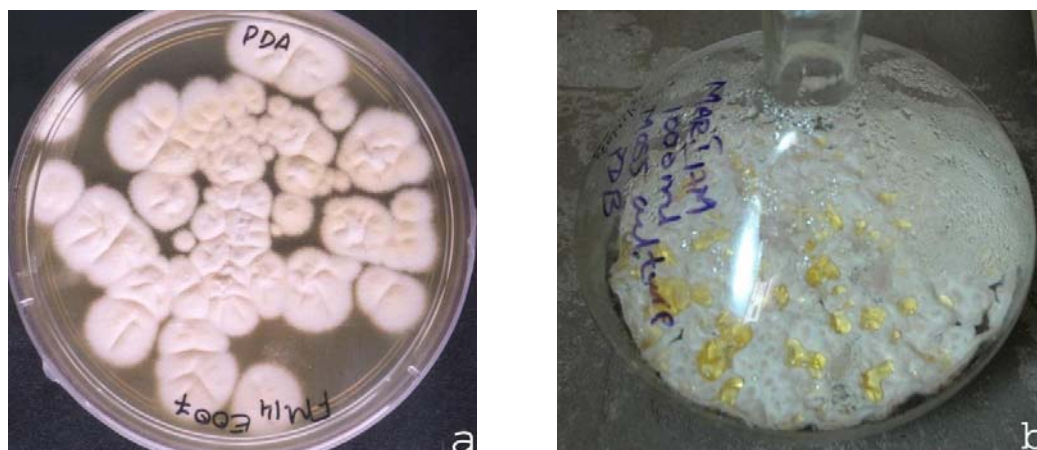
Mangroves are highly specialized ecosystem, which are characterized by salt resistant plants growing in the inter-tidal areas along sheltered seacoasts and estuaries in the tropical and subtropical regions. More than 80 species of mangroves have been recorded all over the world, out of which, 61 species are found in India. 19 of these species find their home in the saline waters of Goa

Pneumatophores of mangrove plant, *A. illicifolius* used for the isolation of associated fungi of the present study were collected from the bank of Cumbarjua canal, Banastari, Goa. After cleaning with sterile water pneumatophores were placed onto potato dextrose agar prepared in seawater-distilled water (75:25). Seven different fungi were purified by repetitive sub culturing. Morphological features visible with naked eyes of these fungi along with their identification and biological activities are tabulated below (Table 2.1).

**Table 2.1: Fungal epiphytes isolated from pneumatophores of *Acanthus illicifolius***

Culture No.	Description	Species	Biological activity
1.	Light green	<i>Aspergillus ornatus</i>	Anticancer
2.	Dark green	<i>Aspergillus cervinus</i>	Anticancer
3.	Brown		
4.	Dark green		
5.	Green with red base		
6.	Greenish brown		
7.	Off white pigmented yellow	<i>Aspergillus. flavipes</i>	Anti-inflammatory

All the fungi were grown on 50 ml scale and extracted with ethyl acetate, which was assessed for bioactivity in high throughput screening (HTS). Culture no.1 and 2 were found to be anticancer positive, whereas culture no. 7 exhibited anti-inflammatory activity (IL-6 and TNF- $\alpha$  inhibitor). Examination of morphological features of conidiophores of these fungi led to the identification of culture No. 1, 2 and 7 as *Aspergillus ornatus*, *Aspergillus cervinus* and *Aspergillus flavipes* respectively (**Fig 2.2a**). *A. ornatus* culture stored in 10% glycerol at -20°C for several days could not be re-grown or revived and *A. cervinus* did not produce sizeable quantity of the extract, therefore these two fungi could not be taken up for chemical studies. *A. flavipes* was grown on a potato dextrose broth (**Fig 2.2 b**) prepared in 1:1 seawater-distilled water under static condition for 21 days.

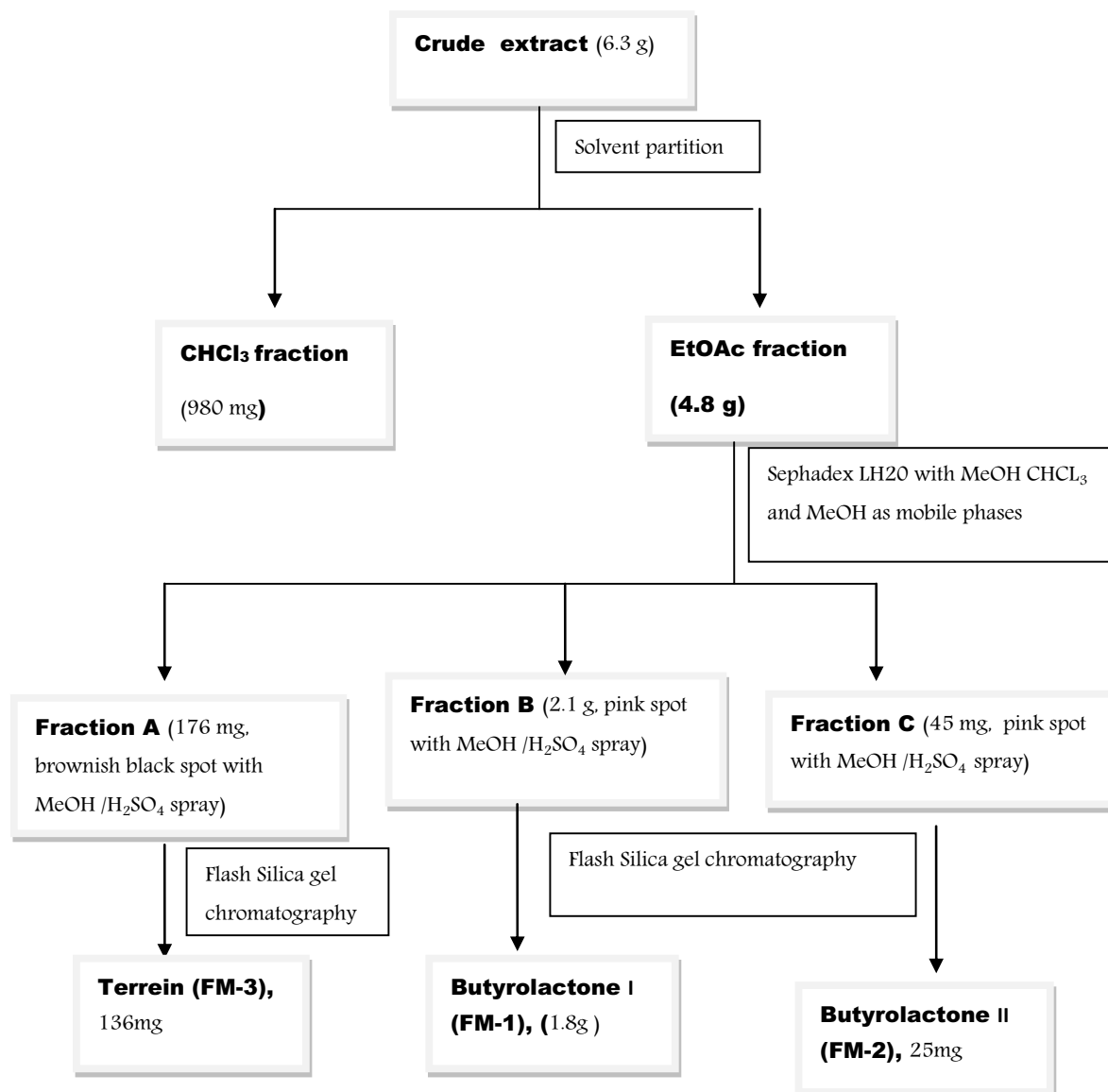


**Fig 2.2:** *A. flavipes* cultured on a) PDA, b) PDB

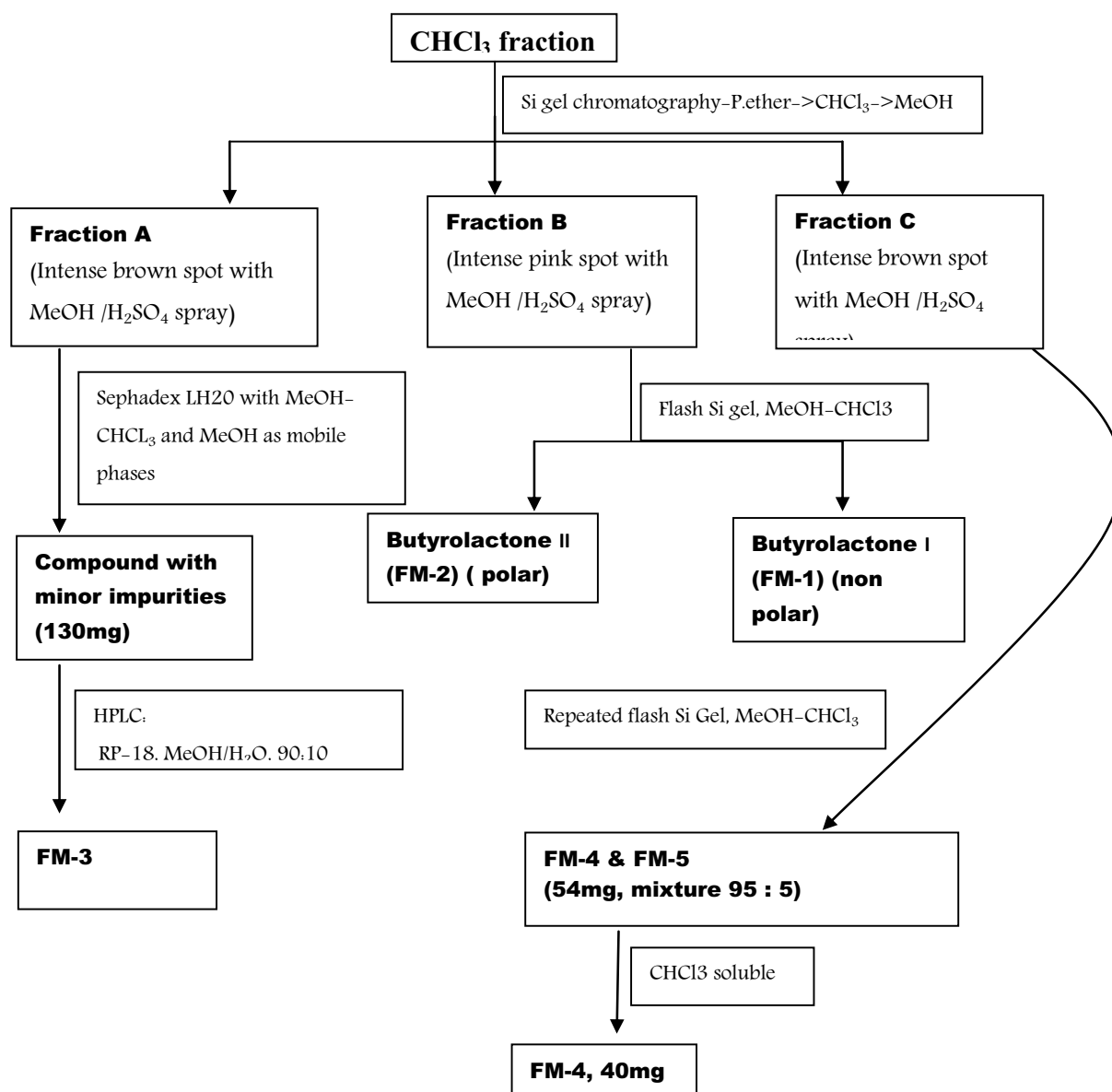
### *ii) Purification of metabolites*

after filtration, 21 days old culture broth yellow in colour was extracted with ethyl acetate and the mycelium was extracted with methanol. Both the extracts on TLC analysis showed similarities in metabolite profiles. Hence, these extracts were combined, and then fractionated between  $\text{CHCl}_3$  and EtOAc. Each organic extract was purified either with repeated Sephadex LH 20 gel filtration (MeOH/ $\text{CHCl}_3$ , 1:1, v/v and MeOH) or on silica gel column ( $\text{CHCl}_3$ /Petroleum ether, 0-100% to

MeOH/CHCl<sub>3</sub>, 0-30%) as depicted in flow diagram (Scheme 2.1 and 2.2). These chromatographic fractionation led to the isolation of five pure compounds designated as, butyrolactone I (FM-1), and butyrolactone II (FM-2) (+) terrein (FM-3), *meso*-4β-(1'-propenyl)cyclopentane-1β,2β-diol (FM-4) and (+) 4-(1'-propenyl) – *trans*-cyclopentane-1,2-diol (FM-5)



Scheme 2.1: Purification of metabolites from EtOAc Extract

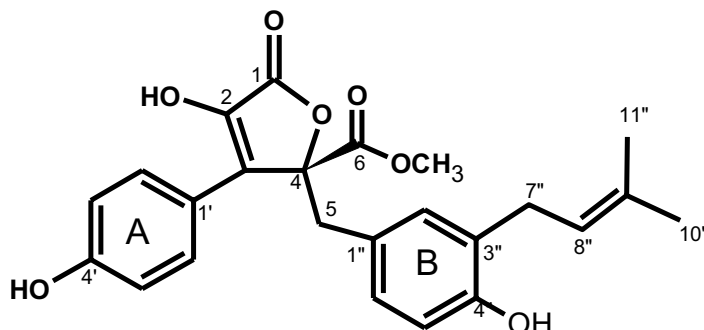


**Scheme 2.2 : Purification of metabolites from CHCl<sub>3</sub> fraction**

*iii) Structure elucidation of metabolites*

Butyrolactone I (**FM-1**) was obtained as colourless crystalline solid, which would eventually turn into a gummy material on storage for long time. **FM-1** showed

negative Lassigne's test for nitrogen, sulfur and halides (Cl, Br and I) indicating that molecule was mainly made up of C, H and O.



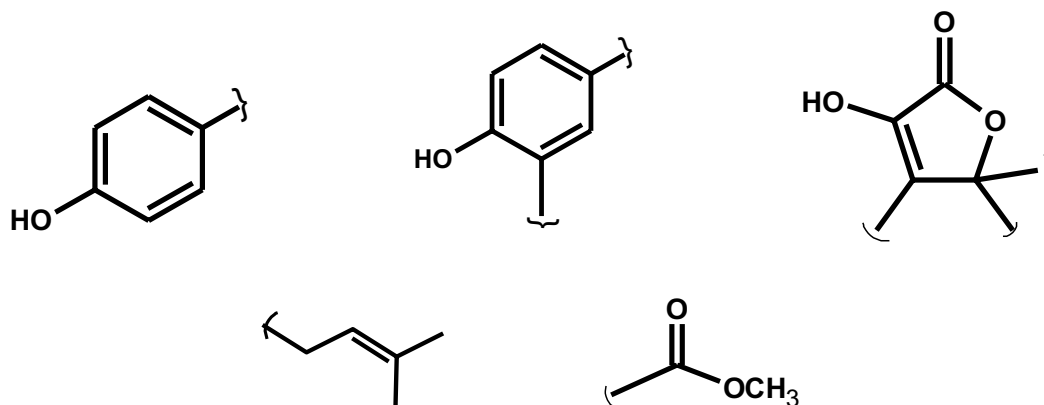
(+) Butyrolactone I (FM-1)

Molecular formula of the **FM-1** was determined to be  $C_{24}H_{24}O_7$  from its HRESITOFMS. It showed a pseudomolecular ion peak  $[M+H]^+$  at 425.1516 (calcd. 425.1522 for  $C_{24}H_{25}O_7$ ). IR spectrum (**Fig 2.3a**) showed presence of ester/lactone carbonyl  $1739\text{ cm}^{-1}$ , strong  $\text{OH}_{\text{str}}$  at  $3384\text{ cm}^{-1}$  and presence of absorption at  $1610\text{ cm}^{-1}$  was suggestive of aromaticity in the molecule.  $^1\text{H}$  NMR (**Fig 2.3b**) recorded in  $\text{CD}_3\text{OD}$  accounted for 21 hydrogens, therefore **FM-1** contained three hydroxyls. Analysis of  $^1\text{H}$ ,  $^{13}\text{C}$  and DEPT NMR data (**Table 2.2**) revealed the nature of all 24 carbons as three methyls of which one was methoxyl and two vinylic; two benzylic methylenes; eight methines of which three belonged to trisubstituted aromatic ring A and two equivalent pairs belonged to p-substituted aromatic ring B; and eleven quaternaries of which two were ester carbonyls, eight were olefenic/aromatic and only one was  $\text{sp}^3$  hybridized (C-5,  $\delta_{\text{c}}$  84.6), which was also the only chiral centre in the molecule.

In  $^1\text{H}$  NMR spectrum of **FM-1** exhibited aromatic  $\text{A}_2\text{B}_2$  system of ortho coupled doublets ( $J = 8.7\text{ Hz}$ ) attributed to two pairs of symmetric protons  $\delta_{\text{H}}$  7.54 and 6.82 ring A and another was ABM system of ring B  $\delta_{\text{H}}$  6.50, dd,  $J = 8.7, 1.8\text{ Hz}$ ; C-6'';  $\delta_{\text{H}}$  6.46, d,  $J = 8.7\text{ Hz}$ ; C-5'' and  $\delta_{\text{H}}$  6.36, d,  $J = 1.8\text{ Hz}$ ; C-2'' . In  $^{13}\text{C}$  NMR and DEPT (**Fig 2.3c**) two phenolic carbons were observed at  $\delta_{\text{C}}$  157.1, C-4'' (ring A) and 152.8, C-4' (ring B) and three alkylated aromatic carbons were evident from the signals at  $\delta_{\text{C}}$

120.9, C-1' (ring A), 122.8, C-1'' and 127.0, C-3'' (both for ring B). Therefore, one of the aromatic systems was p-substituted phenol; the other was unsymmetrical trisubstituted phenol.

NMR signals  $\delta_H$  3.02, d,  $J = 7.2$  Hz 2H;  $\delta_C$  26.46 C-7'' coupled to a vinylic proton  $\delta_H$  5.0, t,  $J = 7.2$  Hz;  $\delta_C$  122.8, C-8,'' and two vinylic methyls  $\delta_H$  1.52, s;  $\delta_C$  15.5 C-11'' and  $\delta_H$  1.61 s;  $\delta_C$  23.7, C-10'' both attached to quarternary olefinic carbon  $\delta_C$  130.7, C-9'' suggested presence of a prenyl group. The chemical shift of the methylene  $\delta_H$  3.02, indicated the prenyl to be attached to the aromatic ring. NMR signals  $\delta_H$  3.73, s;  $\delta_C$  51.6, C-5OMe and  $\delta_C$  169.4 s, C-5 indicated presence of carbomethoxy group. Two mutually coupling doublets of geminal protons  $\delta_H$  3.35, d,  $J = 13.0$  Hz;  $\delta_H$  3.41, d,  $J = 13.0$  Hz;  $\delta_C$  37.4, C-6 is indicative of another benzylic methylene probably attached to the chiral centre.

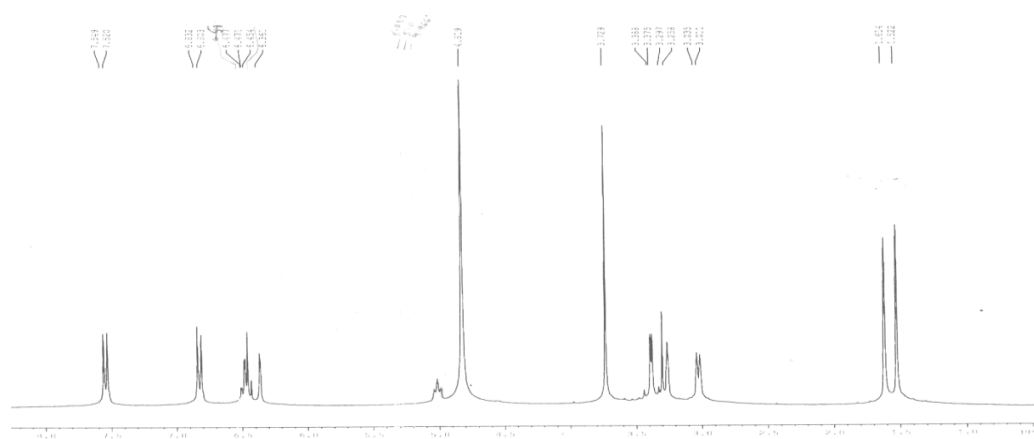


#### Substructures of FM-1 identified from spectroscopic data

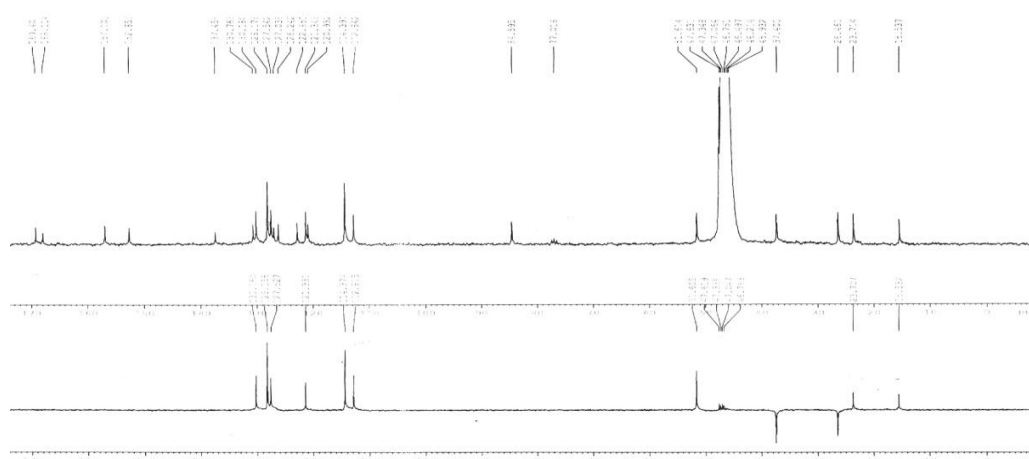
Presence of two aromatic rings, two ester carbonyls and two C-C double bonds accounted for 12 degrees of unsaturation out of 13, therefore presence of an alicyclic ring in the **FM-1** was evident. The above NMR data accounted for 20 carbons out of 24 carbons present in the molecule which meant three quarternary  $sp^2$  carbon signals  $\delta_C$  166.1, C-1; 137.4, C-2; 126.2, C-3 and only quaternary  $sp$  carbon signals 84.6, had to be accounted for. IR absorption of  $1739\text{ cm}^{-1}$  was strong evidence that the **FM-1** had be  $\alpha, \beta$  unsaturated  $\gamma$ -lactone ring comprising of four quarternary carbons (13<sup>th</sup> degree of unsaturation). Saturated  $\gamma$ -lactones are known absorb at  $1770\text{ cm}^{-1}$ ,

while  $\alpha$ ,  $\beta$  unsaturated  $\gamma$ -lactones absorb around  $1740\text{ cm}^{-1}$ . Out of the three -OHs two are accounted for phenolic; hence last one had to be enolic, as there was no evidence of alcoholic hydroxyl in the molecule.

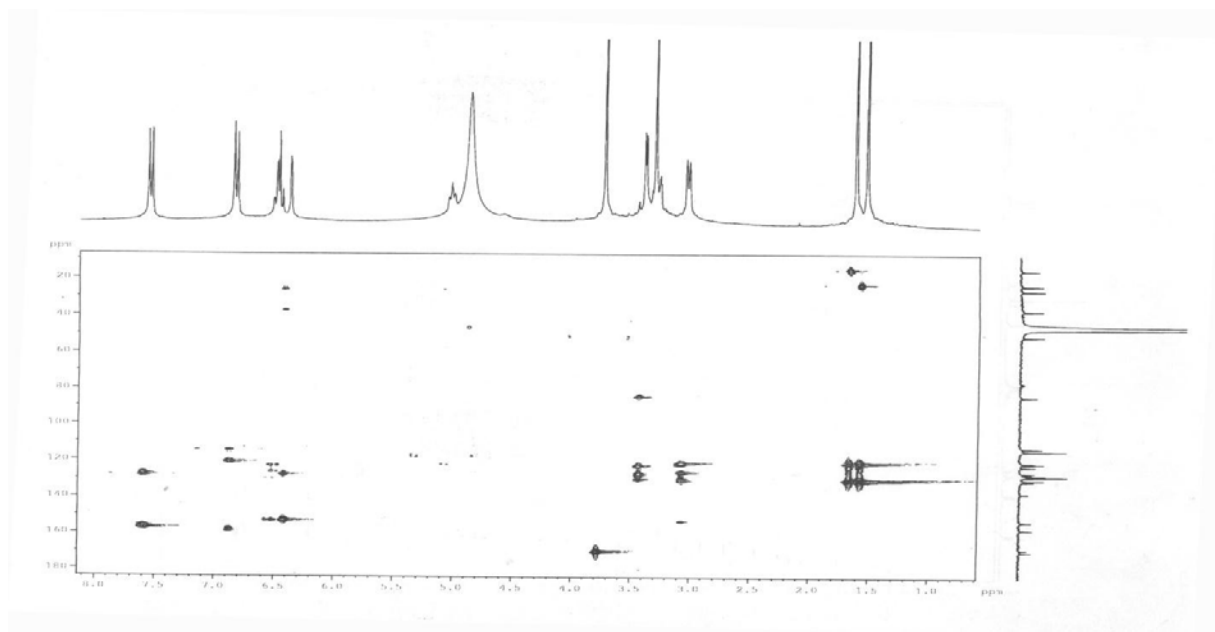
The comparison of the above data revealing the presence of above five substructures with a literature report (Rao et al., 2000) suggested that the molecule under investigation is no different from a butenolide named butyrolactone I (Kiryama et al., 1977), which was isolated for the first time in 1977 from a terrestrial isolate of *A. terreus*.



**Fig 2.3b:**  $^1\text{H NMR}$  of FM-1

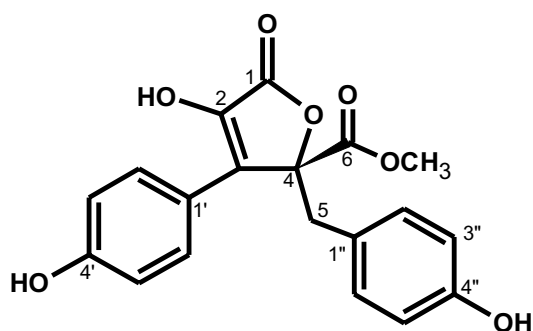


**Fig 2.3c:**  $^{13}\text{C NMR}$  and DEPT of FM-1



**Fig 2.3d: HMBC of FM-1**

**FM-2** was obtained as gummy solid $[\alpha]_{D}^{28.8} +85$  (c = 1, EtOH). It gave spot on TLC more polar, but having colour and appearance like **FM-1**, indicating it to have similar structure. IR spectrum showed presence of ester/lactone carbonyl  $1739\text{ cm}^{-1}$ , phenolic/enolic OHs at  $3360\text{ cm}^{-1}$  and presence of absorption at  $1608\text{ cm}^{-1}$  was suggestive of aromaticity in the molecule. The molecular formula  $C_{19}H_{16}O_7$  was revealed by ESI-MS, which showed  $[M+H]^+$  at m/z 357, and was 68 units lesser compared to **FM-1**, suggesting absence of prenyl chain.



**(+) Butyrolactone II (FM-2)**



The absence of prenyl chain was also evident from the absence of signals for vinylic methyls, allylic methylene, and olefinic methine in its NMR and complemented by the presence of signals for two pairs of *p*-disubstituted benzene rings in  $^1\text{H}$  NMR (Fig 2.4a) and 15 signal accounting for 19 carbons in  $^{13}\text{C}$  NMR (Fig 2.4b). Therefore, the FM-2 was identified as deprenyl derivative of FM-1 by comparison of its NMR data (Table 2.2) with FM-1. FM-2 is also a known terrestrial natural product.

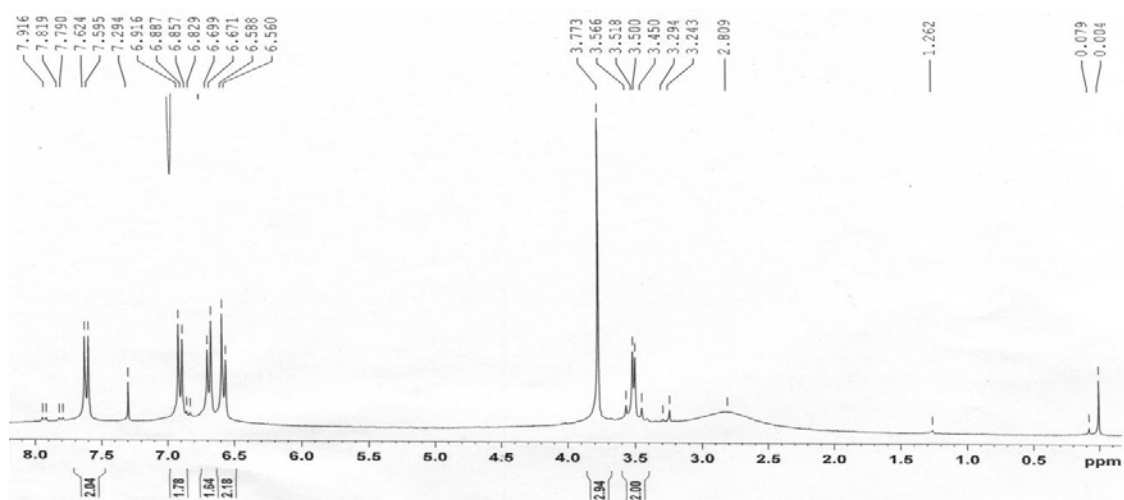


Fig 2.4a:  $^1\text{H}$ NMR of FM-2

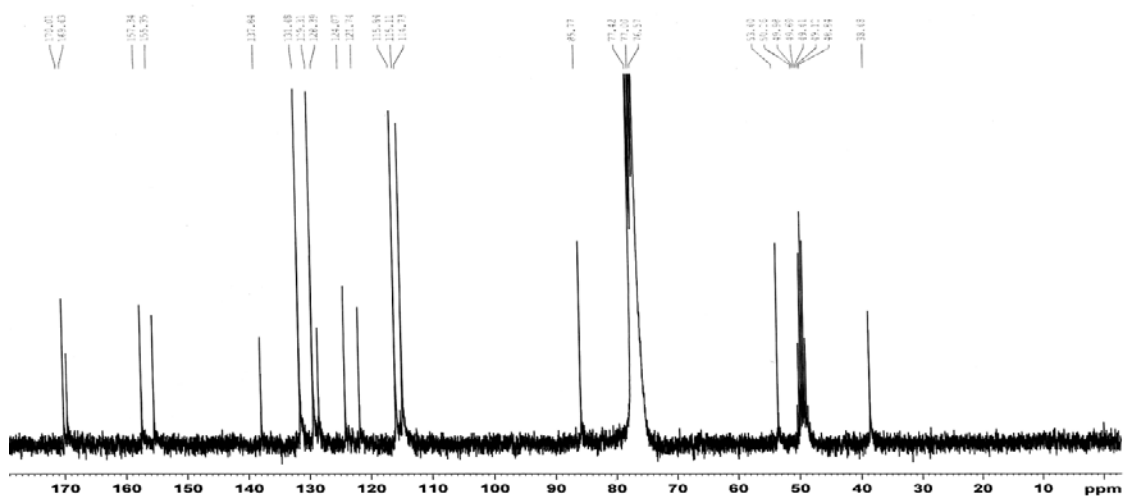
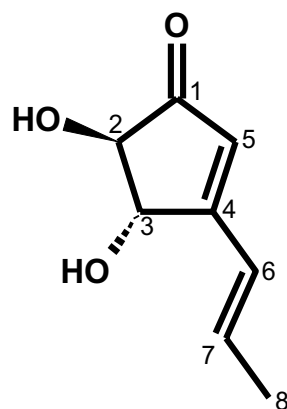


Fig 2.4b:  $^{13}\text{C}$ NMR of FM-2

**Table 2.2: NMR data of FM-1 and FM-2**

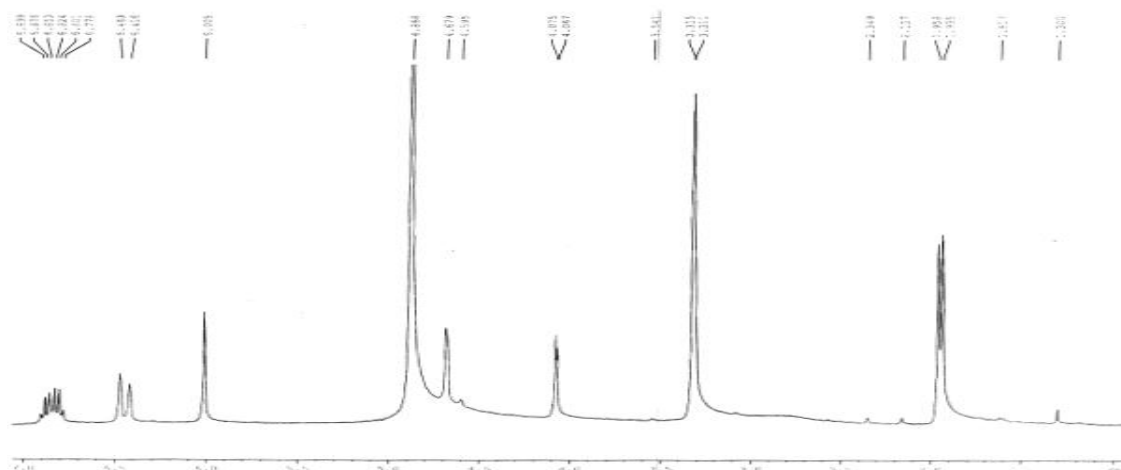
Position	Butyrolactone I (FM-1) (CD <sub>3</sub> OD)		Butyrolactone II (FM-2) (CDCl <sub>3</sub> )	
	$\delta_H$ , mult., J(Hz)	$\delta_C$ , mult.	$\delta_H$ , mult., J(Hz)	$\delta_C$ , mult.
1		166.1 s		169.4, s
2		137.4 s		137.8, s
3		126.2 s		128.3, s
4		84.6 s		85.7, s
5	3.35, d, 13.0 3.41, d, 13.0	37.4 t	3.47, d, 15.0 3.54, d, 14.4	38.4, t
6		169.4 s		170.0, s
1'		120.9 s		124.0, s
2' (6')	7.53, d, 8.7	128.1 d	7.61, d, 8.7	131.4, d
3'(5')	6.82, d, 8.7	114.3 d	6.90, d, 8.7	115.8, d
4'		157.1 s		157.3, s
1''		122.8 s		121.7, s
2''	6.36, d, 1.8	130.1 d	6.68, d, 8.4	129.3, d
3''		127.0 s	6.57, d, 8.4	114.7, d
4''		152.8 s		155.9, s
5''	6.44, d, 8.7	112.8 d	H-3'' eq.	C-3'' eq.
6''	6.48, d, 8.7, 1.8	127.5 d	H-2'' eq.	C-2'' eq.
7''	3.02, d, 7.2	26.4 t		
8''	5.0, t, 7.2	121.3 d		
9''		130.7 s		
10''	1.61, s	23.7 q		
11''	1.52, s	15.5 q		
6-OMe	3.72, s	51.6 q	3.77, s	53.4, q

**FM-3** (terrein) was obtained as colourless crystalline needles. It gave large intense brownish black spot with H<sub>2</sub>SO<sub>4</sub>-MeOH spray on TLC. Its molecular formula C<sub>8</sub>H<sub>10</sub>O<sub>3</sub> with four sites of unsaturation was deduced from HRESITOFMS, which showed pseudomolecular ion at [M+H]<sup>+</sup> at m/z 155.1312. IR spectra gave very sharp bands at 3396 and 3209 cm<sup>-1</sup> due to presence of hydroxyl groups, C-H str. at 3075, 2900 and 2858 cm<sup>-1</sup>, CH bending at 1444 cm<sup>-1</sup>, conjugated five membered ketonic C=O str. at 1693 cm<sup>-1</sup>, C=C str. 1633 cm<sup>-1</sup> and C-O str. 1083 cm<sup>-1</sup>.

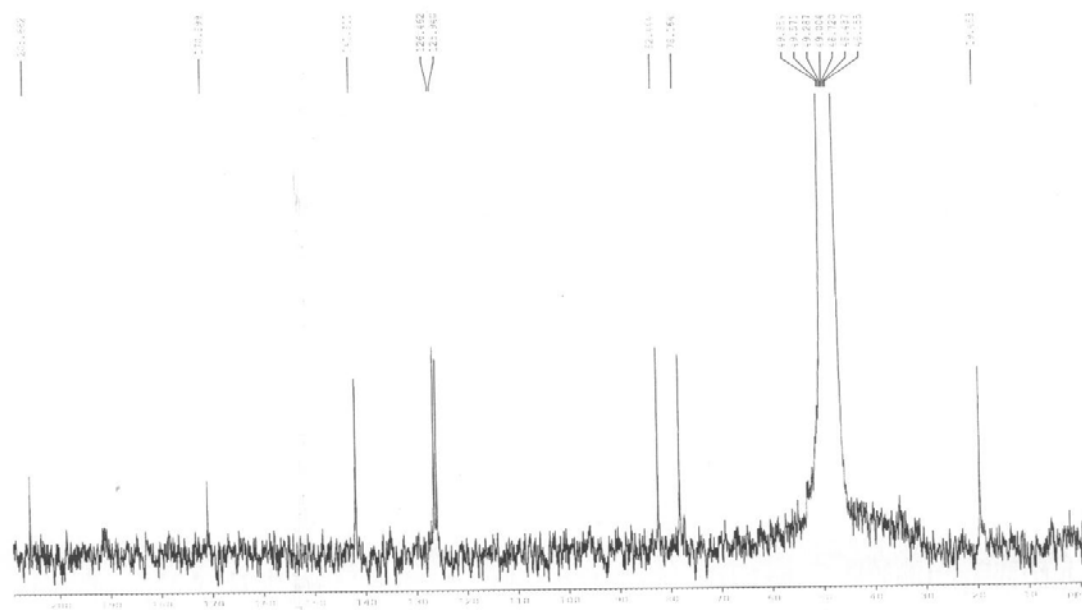


(+) Terrein (FM-3)

Its  $^{13}\text{C}$  NMR (**Fig 2.5b**) signal at  $\delta_{\text{c}}$  205.6 s suggested presence of a cyclic ketone functionality. Proton signals (**Fig 2.5a**) at  $\delta_{\text{H}}$  6.00 s, 1H, 6.44, d,  $J = 15.8$  Hz, 1H coupled to another sp<sup>2</sup> methine  $\delta_{\text{H}}$  6.92 dq,  $J = 15.8$  Hz, 6.9 Hz, which in turn showed coupling to a methyl at  $\delta_{\text{H}}$  1.96, d,  $J = 6.9$  Hz and the carbon signals  $\delta_{\text{c}}$  125.9 d, 170.8, s 126.4 d, 141.8 were indicative of two c-c double bonds in conjugation with carbonyl. The mutually coupling oxygenated methines at  $\delta_{\text{H}}$  4.07,  $\delta_{\text{c}}$  82.4 C-2  $\delta_{\text{H}}$  4.67,  $\delta_{\text{c}}$  78.1 with small coupling constant of 2.4 Hz was indicative trans stereochemistry of two hydroxyls. These data (**Table 2.3**) were found identical to that of a known metabolite terrein reported in the literature (Dunn et al., 1975).



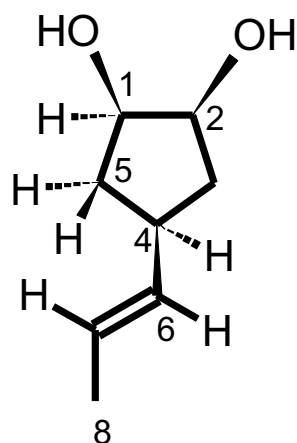
**Fig 2.5a:**  $^1\text{H}$ NMR of FM-3



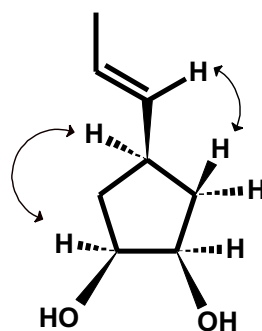
**Fig 2.5b**  $^1\text{H}$ NMR of FM-3

**FM-4** was obtained as optically inactive colourless liquid. It gave a large, intense brown spot with methanolic  $\text{H}_2\text{SO}_4$  acid spray on TLC. It showed  $\text{M}^+$  at  $m/z$  142 in EIMS. From its MS and NMR data molecular formula  $\text{C}_8\text{H}_{14}\text{O}_2$  was assigned to it. It showed absorption band in its IR spectrum at  $3360\text{ cm}^{-1}$  indicative of hydroxyl groups. The  $^{13}\text{C}$  NMR (**Fig 2.6b**) showed six carbons signals suggesting that compound had element of symmetry and revealed the constitution of all the carbons in the molecule. It included signals for 1-propenyl moiety, oxymethine carbons, methylene carbons and a methine carbon. The molecular formula requires 2 degrees of unsaturation. An alkene functionality of propenyl system accounts for one degree of unsaturation, which means other five carbons are involved in constructing a symmetric cyclopentane system. In the proton NMR (**Fig 2.6a**), the methyl of 1-propenyl resonated at  $\delta_{\text{H}}$  1.63, d  $J = 5\text{ Hz}$ . and the olefinic methines showed merged multiplet at  $\delta_{\text{H}}$  5.39, 2H. One of the olefinic proton was coupled to a methine  $\delta_{\text{H}}$  2.35 ddd. ( $J = 6, 8, 9.5\text{ Hz}$ ), (actually appeared as sextet due to merging of peaks) which in turn showed coupling of 9.5 to a pair of protons signal at  $\delta_{\text{H}}$  1.46 (ddd,  $J=5, 9.5, 13.5\text{ Hz}$ ) and smaller coupling of 8 Hz to a pair of protons at  $\delta_{\text{H}}$  2.09, (ddd,  $J = 5, 8, 13.5$

Hz). Each of the pairs of hydrogen showed correlation to the same carbon signal  $\delta_c$  38.5..indicating the pairs of hydrogen were stereochemically different ( $\alpha$  and  $\beta$  hydrogens of C-3 and C-5). Each of these sets of protons showed coupling to a signal at  $\delta_H$  3.98 (2H, br t,  $J = 5$  Hz) attributed to oxymethine protons. From these data, planar structure of **FM-4** was deduced. The *E* stereochemistry of double bond was rather easily assigned from the chemical shift of the vinylic methyl ( $\delta_c$  17.6) as was difficult to assign by using coupling constants of the merged olefinic hydrogens. The syn relationship of two hydroxyls was established from the observation that the compound was optically inactive and so the two hydroxyls had to be syn, so as to maintain the C-2 symmetry in the molecule for the compound to be meso. The 1-propenyl can either be syn or anti with respect to two hydroxyls. The same molecule with all three substituents with syn stereochemistry has been reported as natural product, wherein the stereochemistry of the propenyl unit has been assigned from NOESY data. Our IR and NMR spectroscopic data was in agreement with the reported (Ghisalberti et al., 1990), but in order to confirm their findings, we obtained the NOESY spectrum (**Fig 2.6c**) on our compound. The NOESY data (**Fig 2.6d**) established the propenyl group oriented syn with the two hydroxyls.



**FM-4**



**Fig 2.6d : NOESY correlation of FM-4**

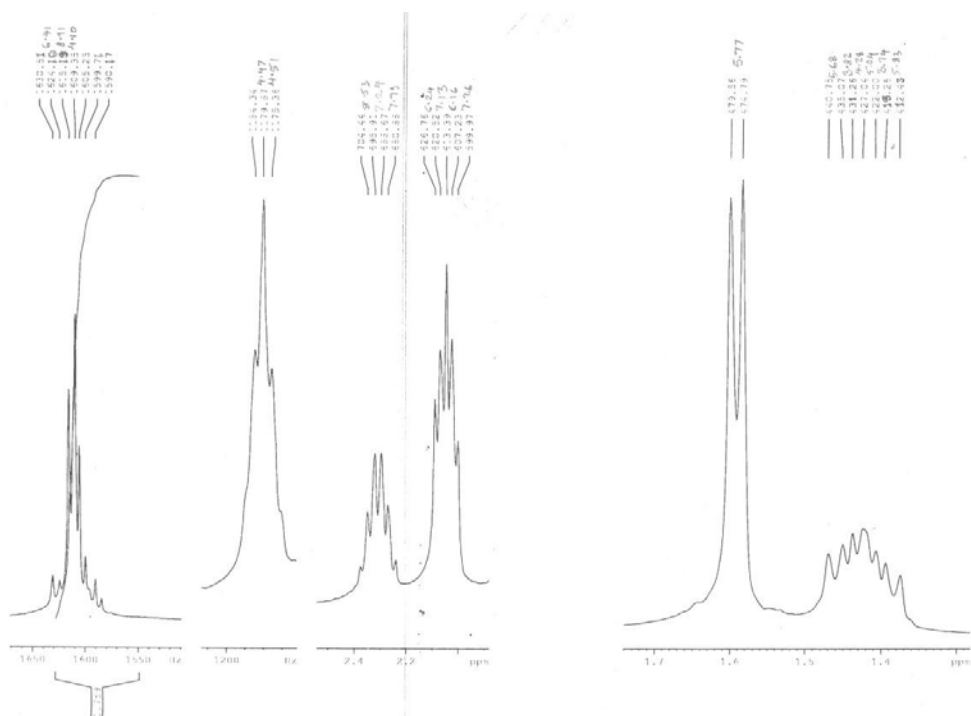


Fig 2.6a: <sup>1</sup>H NMR (expansion) of FM-4

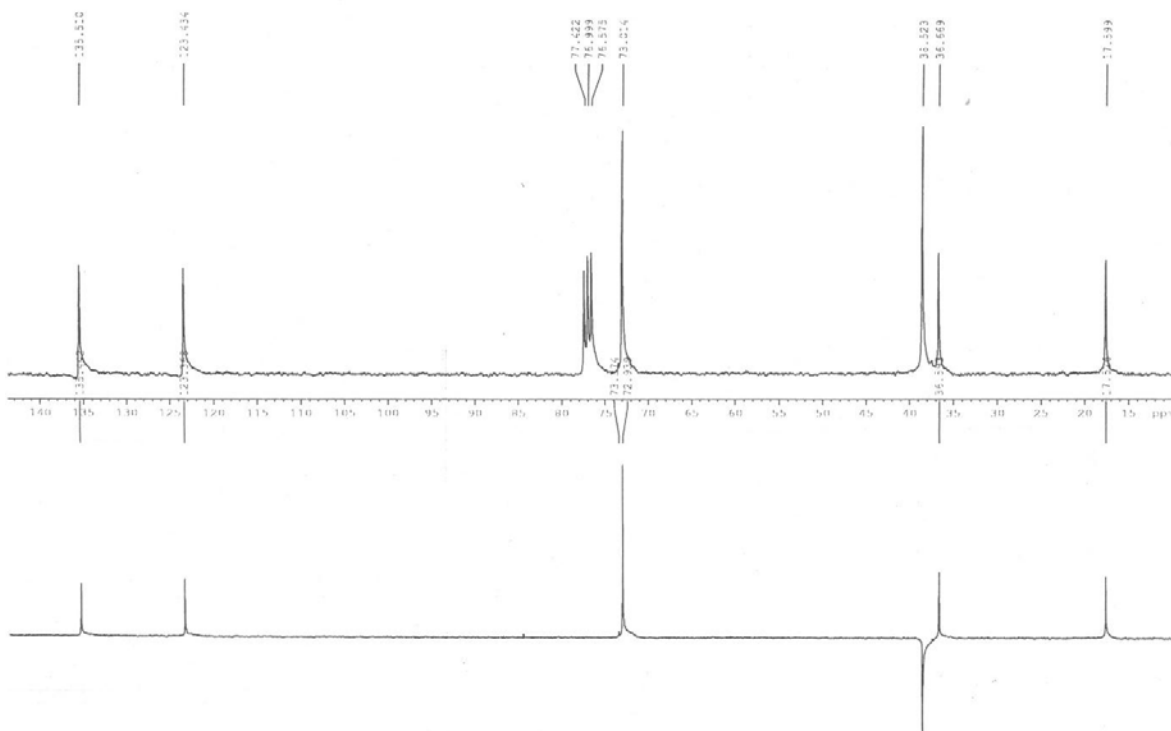
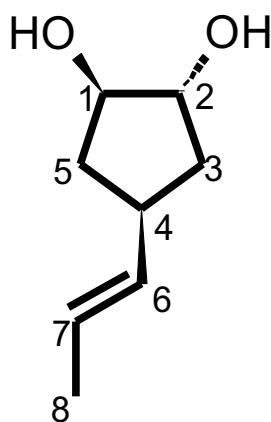


Fig 2.6b <sup>13</sup>C NMR and DEPT of FM-4

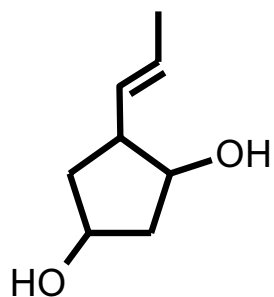
**FM-5** was obtained as optically active viscous liquid. It gave large intense bluish black spot with H<sub>2</sub>SO<sub>4</sub>-MeOH spray similar to compound **FM-3** and **FM-4** and also identical in retardation factor with **FM-3**. Unlike **FM-3**, **FM-5** was soluble in CHCl<sub>3</sub> and showed no fluorescence on TLC under UV light (254nm). Absence of fluorescence could be the indication that the **FM-5** lacked the conjugated carbonyl functionality in the molecule. As anticipated, IR spectrum of **FM-5** did not show any carbonyl absorption. It showed absorption bands at 3360 cm<sup>-1</sup> for hydroxyls like **FM-4**. EIMS gave M<sup>+</sup> at m/z 142 indicating molecular formula C<sub>8</sub>H<sub>14</sub>O<sub>2</sub> same as that of **FM-4**. The <sup>13</sup>C nmr (**Fig 2.7b**) showed eight carbons signals, which included signals for *E*-1-propenyl moiety, two oxymethine carbons, two methylenes and a methine. These <sup>13</sup>C nmr data when compared with **FM-4** suggested that **FM-5** had same constitution, but lacks C-2 symmetry due to which it shows eight carbons signals and also exhibited optical activity.



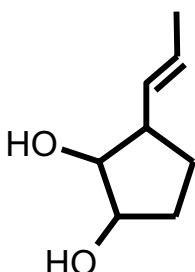
**FM-5**

The certain similarities between **FM-4** and **FM-5** were also seen in their <sup>1</sup>H NMR (**Fig 2.7a**). The <sup>1</sup>H NMR gave signals for 1-propenyl moiety ( $\delta_{\text{H}}$  1.62, d, 4.5 Hz, 3H  $\delta_{\text{C}}$  17.74 and  $\delta_{\text{H}}$  5.38, m, 2H  $\delta_{\text{C}}$  123.6 and 135.3). Additionally, there were five sets of proton multiplets. Their multiplicities and coupling constant could not be properly derived, as the splitting had complex pattern due to coupling with multiple protons.

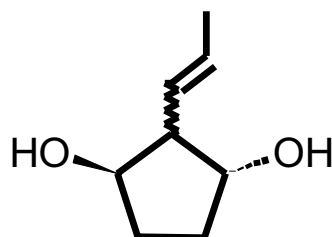
One of the olefinic hydrogen showed coupling to apparent sextet at  $\delta_H$  2.64, 1H, which showed coupling to two three sets of proton signals at  $\delta_H$  2.15 m 1H;  $\delta_H$  1.75, m, 2H and  $\delta_H$  1.29, m 1H. Out of the three sets of proton signals, two sets of proton signals  $\delta_H$  2.15, m, 1H and  $\delta_H$  1.29, m, 1H were due to the stereochemically non-equivalent hydrogens of a methylene (C-3).  $\beta$ -hydrogen of C-3 is shielded due to bulky groups ( $\beta$ -hydroxyl and a  $\beta$ -propenyl) and appears upfield, whereas  $\alpha$ -hydrogen of C-3 which is less shielded due to  $\alpha$ -hydrogen on adjacent carbons appears downfield. The third set of signal were due to two methylene protons of C-5, whose both  $\alpha$  and  $\beta$ -hydrogens experiences nearly similar electronic environment due to one bulky group on adjacent carbon (either C-1-OH or C-4-propenyl) on their face. The hydroxymethines were observed at  $\delta_H$  3.95, m and coupled with both the methylenes. Although, these data (**Table 2.3**) reveals the compound to have trans orientation of the two adjacent hydroxyls in **FM-5**, the fact that the asymmetry or chirality in the molecule is caused due to the geometry of the two hydroxyls (with 1,2,4 constitution of cyclopentane ring) and not due to the some other constitution (shown in **17-19**) is confirmed from the  $^1H$ - $^1H$ -COSY (**Fig 2.7c**).



17



18



19

Each sets of the three proton signal resulting due to the two methylene C-3 and C-5 showed correlation to a methine C-4 and also to oxymethines C-1 and C-2, while no correlations were observed between the two methylene protons and also between any of the oxymethines and C-3 methine (**Fig 2.7d**).



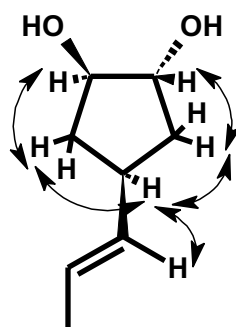


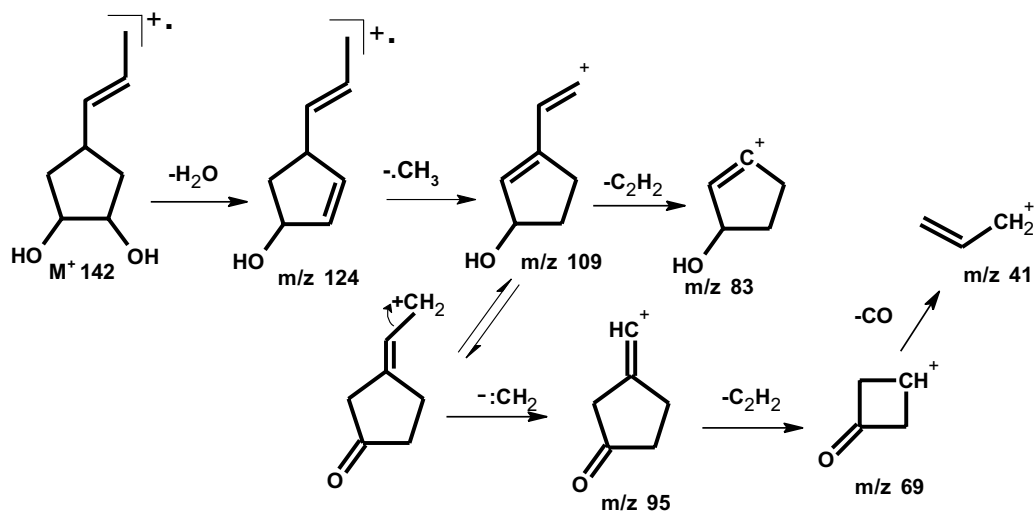
Fig 2.7d: H-H COSY of FM-5

Table 2.3 NMR data of FM-3, FM-4 and FM-5 at 300 MHz

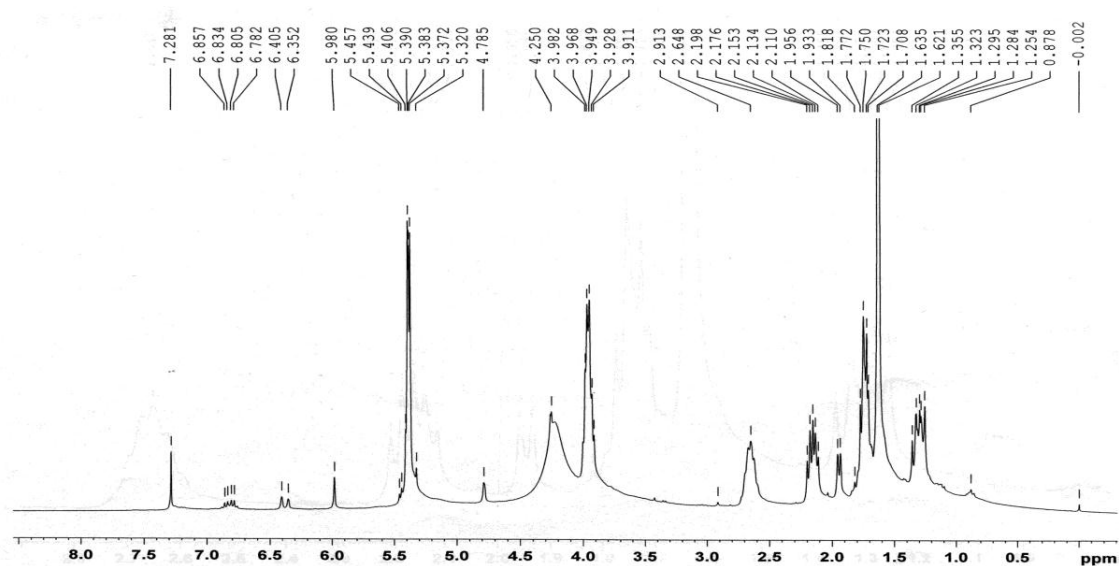
Carbon	(FM-3) (CD <sub>3</sub> OD)			(FM-4) (CDCl <sub>3</sub> )		(FM-5) (CDCl <sub>3</sub> )	
	$\delta_H$ , J(Hz)	mult.,	$\delta_C$ , mult.	$\delta_H$ , mult., J(Hz)	$\delta_C$ , mult.	$\delta_H$ , mult., J(Hz)	$\delta_C$ , mult.
1			208.6, s	3.93, br t, 5	73.0 d	3.91-3.98, m	79.4 d
2	4.59, d, 2.3		82.4, d	H-1 eq	C-1eq	3.91-3.98, m	78.5, d
3	4.06, d, 2.4		78.1, d	2.05, ddd, 13.5, 8.0, 5.0 (H-3 $\alpha$ ) & 1.43, ddd, 13.5, 9.5, 5.0 (H-3 $\beta$ )	38.5 t	2.15, ddd, H- $\alpha$ 1.29, ddd, H- $\beta$	39.1, t
4			170.8, s	2.30, ddd, 9.5, 8.0, 6.0	36.6 d	2.65, m	37.1, d
5	6.00, s		126.4, d	H-3 eq.	C-3 eq.	1.76, m	38.3, t
6	6.26 d, 15.9		125.9, d	5.29-5.43, m	135.5 d	5.37-5.40, m	135.0, d
7	6.84, dq, 15.9, 6.9		141.8, d	5.29-5.43, m	123.4 d	5.37-5.40, m	123.1, d
8	1.94, d, 6.9		19.4, q	1.59, d, 4.8	17.5 q	1.63, d, 4.8	17.7, t

The EI-MS spectrum of two epimers, FM-4 and FM-5 displayed the same fragmentation pattern. The molecular ion of both compounds initially eliminate a

molecule of water to yield fragment ion  $m/z$  124, and the smaller fragment ions  $m/z$  109, 95, 83, 69, 56 and 41 are then generated from this ion. The formation of this mass fragments are explained in the **scheme 2.3** below.



**Scheme 2.3: Mass fragmentation of FM-4/FM-5**



**Fig 2.7a :  $^1H$ NMR of FM-5**

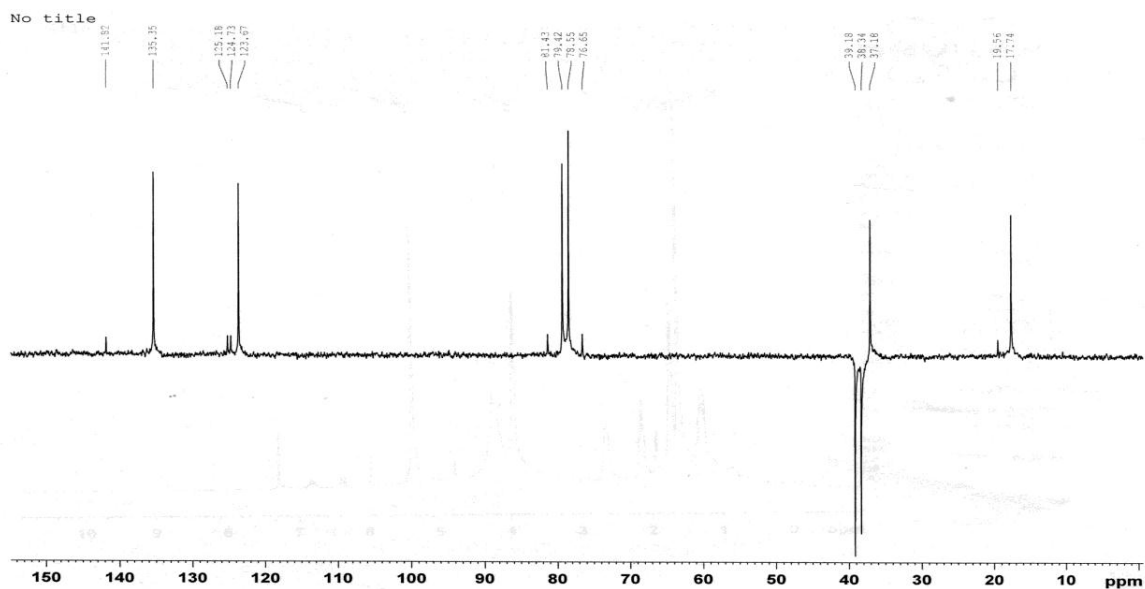


Fig 2.7b: DEPT showing signals for all the carbons of FM-5

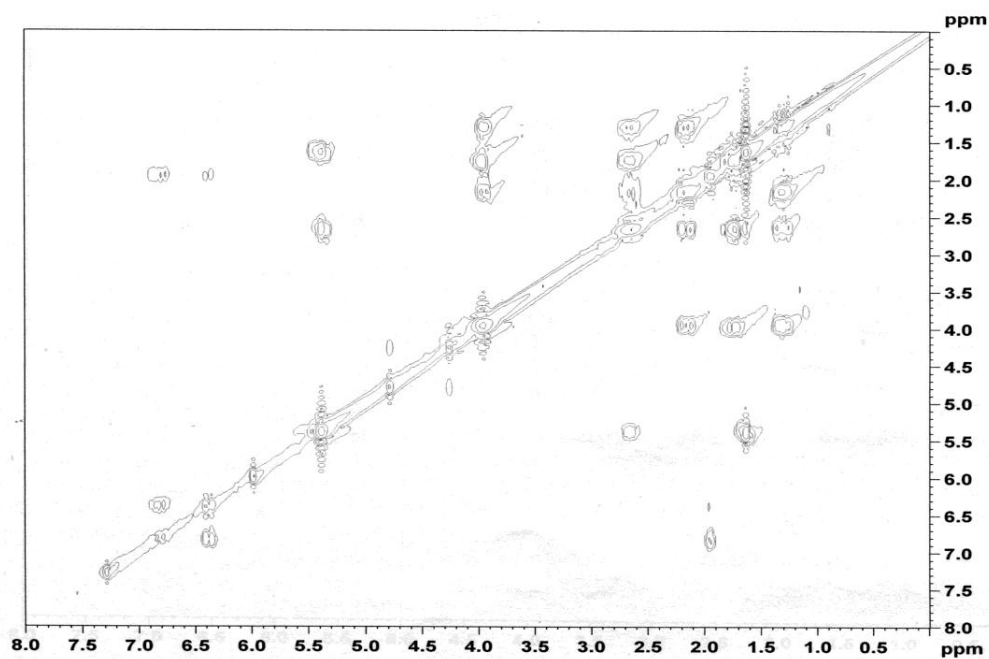


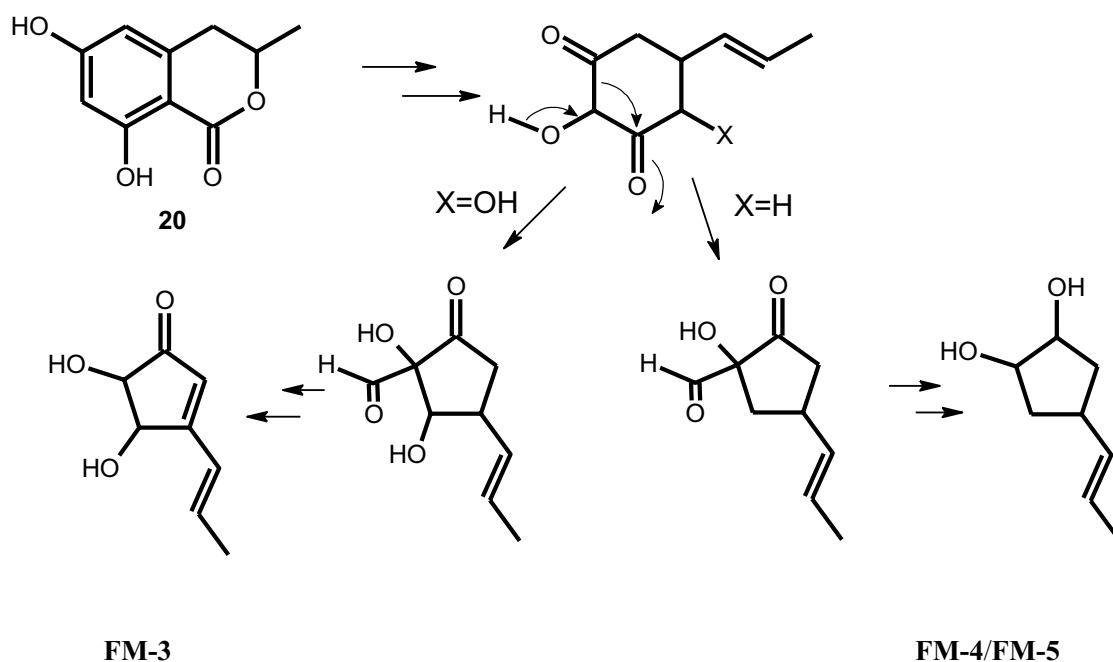
Fig 2.7c:  $^1\text{H}$ - $^1\text{H}$  COSY of FM-5

All the known compounds were first reported as metabolites of terrestrial isolates of *A. terreus*. **FM-3** is known from various terrestrial as well as marine-derived fungi, whereas **FM-4** *A. terreus* is the only source of **FM-4** (Ghisalberti et al., 1990). **FM-1**

is also known to be a metabolite of *A. fischeri* TISTR 3272 (Phattanawasin et al., 2007). The known metabolites are isolated for the first time from *A. flavipes*, a fungus, which is closely related to *A. terreus*.

*iv) Biogenesis of FM-1 to FM-5*

The biosynthesis of butenolides (FM-1 and FM-2) in *A. terreus* has been well known and the same has been discussed in **section 2.4, subsection iii** of this chapter. Among cyclopentanedioles, FM-3 is known to biosynthesize from a resorcylic acid lactone (20) (polyketide), whereas as proposed by Ghilsaberti et al. (1990) for FM-4, its epimer FM-5 must also be derived from the same common precursor of FM-3 as depicted in **Scheme 2.4**



**Scheme 2.4 : Biogenesis of cyclopentanedioles FM-3, FM-4 and FM-5**

v) *Biological properties of the metabolites: Interleukin-6 (IL-6) and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) activity*

The crude extract was able to inhibit IL-6 and TNF- $\alpha$  production from LPS stimulated THP-1 cells ( $IC_{50} = 2.69 \pm 0.5 \mu\text{g/ml}$  and  $6.26 \pm 0.4 \mu\text{g/ml}$  suggesting discovery of molecules that would be potent anti-inflammatory. Interleukins are a family of cytokines that govern leukocyte function in response to any challenge to the immune system. Over-expression of interleukins has been implicated in a variety of inflammatory diseases. One such interleukin-6 (IL-6) is an ubiquitous and multi-functional cytokine involved in the early steps of T-cell activation, B-cell differentiation, in the regulation of the acute phase response (which is characterized by the release of a set of serum proteins in response to injury) and in hematopoiesis (the maturation of blood cells from bone marrow). Consistently elevated levels of IL-6 are found in the synovial fluid associated with rheumatoid arthritis (Hossiau and Deuxchaisnes, 1990) and in multiple myeloma tumors (Malik, 1992). Their modulation, particularly via chemical entities, is currently of therapeutic interest.

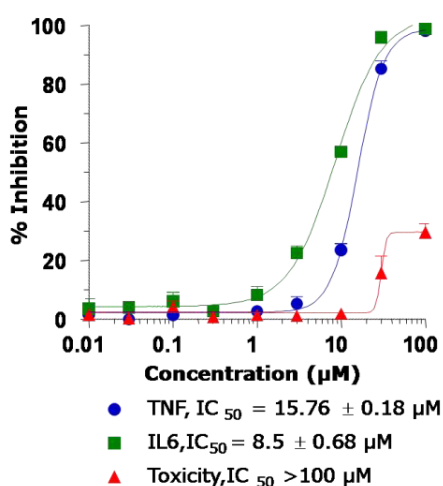


Fig 2.8: IL-6, TNF- $\alpha$  and toxicity of FM-3

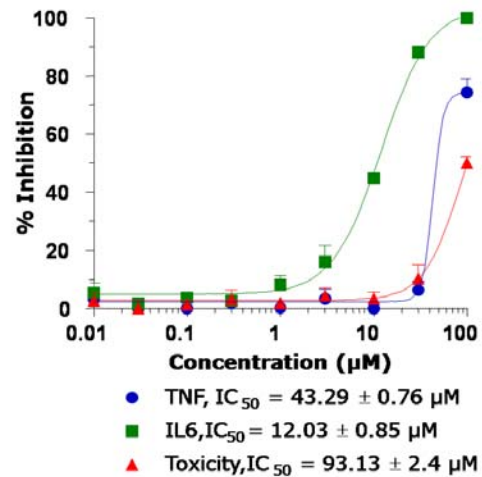


Fig 2.9: IL-6, TNF- $\alpha$  and toxicity of FM-1

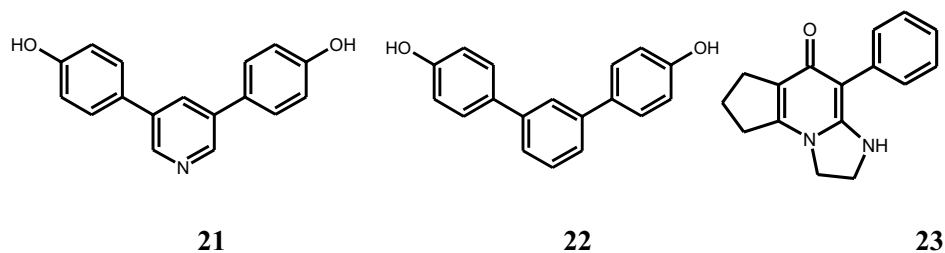
All the metabolites (**FM-1** to **FM-5**) were tested for inhibition of IL-6 and TNF- $\alpha$  in LPS stimulated THP-1 cells according to method described by Hwang and co-workers (Hwang et al., 1993). **FM-3** was found to be the most active. It showed IC<sub>50</sub> value of  $8.5 \pm 0.68 \mu\text{M}$  and  $15.76 \pm 0.18 \mu\text{M}$  for the inhibition of IL-6 and TNF- $\alpha$  respectively without affecting the host cells (**Fig 2.8**). The second best was compound **2**, which showed IC<sub>50</sub> of  $12.03 \pm 0.85 \mu\text{M}$  for the inhibition of IL-6, but inhibited TNF- $\alpha$  at comparatively higher concentration IC<sub>50</sub>  $43.29 \pm 0.76 \mu\text{M}$  (**Fig 2.9**). Other compounds did not show any significant activity below 30  $\mu\text{M}$ .

*vi) Structure-activity relationship*

The two inactive cyclopentane diols (**FM-4** and **FM-5**) differs from each other only by stereochemistry of two hydroxyls, whereas terrein (**FM-3**) (also a cyclopentane diol) has additional functionalities (a keto and endo double bond). These additional functionalities could be responsible for **FM-3** being highly active compared to **FM-4** and **FM-5**.

Among butenolides, the fact that **FM-1** is active and its precursor, **FM-2** is inactive could be attributed to the presence of prenyl chain in **FM-1**, because a similar trend has been also observed for these compounds towards the inhibition of cyclin dependant kinases CDK1 and CDK2. The importance of the prenyl group for maintaining CDK activities have been demonstrated by computer aided molecular modeling using automated docking methods and molecular dynamics simulations studies (Brana et al., 2004).

Till date synthetic derivatives of diarylpyridines (**21**), m-terphenyls (**22**), (Tagat et al., 1995a) and 4-aryl substituted cyclopentane and imidazole fused tricyclic pyridinones (**23**) (Tagat et al., 1995b) are the only compounds reported as inhibitors of IL-6. To best our knowledge, except the compound of present study no natural product possessing IL-6 activity has been reported so far.



### III. Experimental

#### *i) General experimental procedure*

Mixture of methanol and chloroform (10:90, v/v) was used as mobile phase for TLC analysis. Cyclopentanedioles and butenolides were visualized as blue-black and intense rose colour spots respectively on spraying with methanolic sulphuric acid (95:5, v/v) followed by heating at 120°C.

#### *ii) Fungal isolation and identification*

Pneumatophores of *Acanthus illicifolius* were collected from the bank of Mandovi River, Banastarim, Goa, India. Fungi were isolated using standard isolation technique as epiphytes on potato dextrose agar (PDA) from the pneumatophores of mangrove plant *Acanthus illicifolius*. PDA was prepared in 75% natural SW supplemented with penicillin benzyl sodium salt (0.02 g/l) to avoid any bacterial growth. After 6-7 days cultures subtending in medium were sub cultured repeatedly to obtain seven pure fungal cultures.

#### *iii) Fungal mass cultivation*

Fungi were grown in Erlenmeyer flasks containing 50 ml. PDB prepared in SW/DW-75:25 and incubated for 21 days under static condition and used for biological activity testing. Similarly 10 L culture of *A. flavipes* was made for isolation of metabolites.

#### *iv) Extraction of metabolites*

On 21<sup>st</sup> day mycelium was separated from the broth by filtration through cotton wool. Broth (50 ml) was extracted with ethyl acetate (30 ml x 3). This extract was used for biological activity screening.

*v) Biological activities*

Anticancer and anti-inflammatory results were obtained from other laboratories in India.

*vi) Identification of fungi*

Three active fungi were identified using molecular techniques. Additionally *A. flavipes* was identified from morphological characteristics of its conidiophores (Dr. Sanjay Singh, Agharkar Research Institute, Pune). A voucher specimen of *A. flavipes* with MTCC 5220 is deposited at IMTEC, Chandigarh

*vii) Purification of metabolites from A. flavipes*

Mycelium was filtered and extracted with methanol (300ml x 3). Culture filtrate (10 L) was concentrated on a rotary evaporator to a smaller volume (2.5 L) under reduced pressure and extracted with ethyl acetate (500ml x 4). Methanol and ethyl acetate extracts were concentrated under vacuum and mixed due to their similar TLC profile. The combined organic extract (6.3 g) was suspended in 10% aqueous MeOH (200ml) and partitioned between CHCl<sub>3</sub> (150ml x 4) and EtOAc (150ml x 4). The organic extracts were concentrated under vacuum to yield dry CHCl<sub>3</sub> extract (980mg) and EtOAc extract (4.8g). EtOAc extract was fractionated on Sephadex LH20 [gel permeation chromatography (GPC)] using MeOH-CHCl<sub>3</sub> (1:1) as mobile phase and common fractions were pooled to give three fractions A B and C. Fraction A, B and C were eluted with MeOH-CHCl<sub>3</sub> (3:97, v/v) (2:98, v/v) and (3:97, v/v) respectively on flash Si gel to yield pure compounds **FM-3** (136mg) **FM-1** (1.8g) and **FM-2** (28mg) respectively.

CHCl<sub>3</sub> extract was fractionated on Si gel by eluting with increasing concentration of petroleum ether in CHCl<sub>3</sub> (0 – 100%) to yield several fractions which were pooled



into fractions A B and C on the basis of their TLC profile. Fraction A was eluted with MeOH –CHCl<sub>3</sub> (1:1) on Sephadex LH20 (GPC) to obtain a **FM-4** (130mg) with closely resolved impurities. This compound (12mg) was purified by semipreparative HPLC (discovery® C18, 5μ, 1 x 25 cm, detection at λ 215nm) by eluting with MeOH-H<sub>2</sub>O (1:9) to give pure compound (6.4 mg) . Fraction B was flash Si gel chromatographed with increasing conc of MeOH in CHCl<sub>3</sub> (0-10, v/v) to serially give **FM-1** (43mg) and **FM-2** (19mg). Fraction C on repeated Si gel chromatography with MeOH-CHCl<sub>3</sub> (0:100 – 10:90, v/v) gave mixture (~5:95, 54mg) of **FM-3** and **FM-5**. **FM-5** was purified by repeatedly dissolving and filtering through Whatman 1 filter paper.

*viii) Butyrolactone I (FM-1)*

White powder;  $[\alpha]_{D}^{28.8} + 84.32$  (c 0.617, MeOH); and  $[\alpha]_{D}^{28.8} + 87.56$  (c 0.617, CHCl<sub>3</sub>); HRESITOFMS:  $[M+H]^+$   $m/z$  425.1516 (calcd. 425.1522 for C<sub>24</sub>H<sub>25</sub>O<sub>7</sub>). UV (MeOH)  $\lambda_{max}$  306, 230, 210 nm .; IR (KBr)  $\nu_{max}$  3271, 2927, 1741, 1703, 1609, 1516, 1436, 1361, 1222, 1180, 840 cm<sup>-1</sup>; <sup>1</sup>H NMR and <sup>13</sup>C NMR (300 MHz, CD<sub>3</sub>OD) see table 2.2

*ix) Butyrolactone II (FM-2)*

White sticky solid,  $[\alpha]_{D}^{25} = +85$  (c = 1, EtOH), ESITOFMS  $[M+H]^+$   $m/z$  357, UV (MeOH)  $\lambda_{max}$  303, 285 nm: IR (NaCl)  $\nu_{max}$  3290, 1740, 1730, 1660 cm<sup>-1</sup>; <sup>1</sup>H NMR and <sup>13</sup>C NMR (300 MHz, CDCl<sub>3</sub>) see table 2.2

*x) Terrein (FM-3)*

White crystalline solid; mp 121-122;  $[\alpha]_{D}^{28.8} + 144$  (C = 0.8 in MeOH); UV  $\lambda_{max}$  273 345 nm; IR (KBr)  $\nu_{max}$  3320, 3160, 1690, 1635, 1570 cm<sup>-1</sup>; EIMS,  $m/e$  154 (M), 139 (M-Me), 121 (M-Me-H<sub>2</sub>O); <sup>1</sup>H NMR and <sup>13</sup>C NMR (300 MHz, CD<sub>3</sub>OD) see table 2.3

***xii) 4β-(1'-Propenyl)cyclopentane-1β,2β-diol (FM-4)***

Colourless viscous liquid; optically inactive; IR (KBr)  $\nu_{\max}$  3400, 2968, 2931, 2856, 1660, 1436  $\text{cm}^{-1}$ ; EI-MS, m/e 142 (M), for fragments see scheme 1.4;  $^1\text{H}$  NMR and  $^{13}\text{C}$  NMR (300 MHz,  $\text{CDCl}_3$ ) see table 2.3

***xiii) 4-(1'-Propenyl) cyclopentane-1β,2α-diol (FM-5)***

Colourless viscous liquid;  $[\alpha]_D^{25} = +17.44$  ( $c = .0.043$ ,  $\text{CHCl}_3$ ); IR (KBr)  $\nu_{\max}$  3400, 2968, 2931, 2856, 1660, 1436  $\text{cm}^{-1}$ ; EI-MS, m/e 142 (M), for fragments see scheme 1.4;  $^1\text{H}$  NMR and  $^{13}\text{C}$  NMR (300 MHz,  $\text{CDCl}_3$ ) see table 2.3

***xiiii) Screening of compounds to identify inhibitors of TNF-α and IL-6 production: Screening in LPS stimulated THP-1 cells (Primary Screening)(as carried out by technicians at piramal life science, Mumbai)***

IL-6 and TNF- α production by lipopolysaccharide (LPS) in THP-1 cells was measured according to the method described by Hwang et al., (1993). Briefly, THP-1 cells were cultured in RPMI 1640 culture medium (Gibco BRL, Pasley, UK) containing 100 U/ml penicillin and 100mg/ml streptomycin, (100X solution, Sigma Chemical Co. St Louis, MO) containing 10% fetal bovine serum (FBS, JRH). The cells were differentiated with phorbol myristate acetate (PMA, Sigma) and treated with the test compounds or vehicle. The plate was incubated for 30 min at 37°C. Finally, LPS (Escherchia coli 0127:B8, Sigma Chemical Co., St. Louis, MO) was added at a final concentration of 1  $\mu\text{g}/\text{ml}$ . Plates were incubated at 37°C for 24h, 5%  $\text{CO}_2$ . Supernatants were harvested, and assayed for TNF-α and IL-6 by ELISA as described by the manufacturer (BD Biosciences). The cells were parallely evaluated for cytotoxicity using CCK-8. Percent inhibition of cytokine release compared to the control was calculated. The 50% inhibitory concentration ( $\text{IC}_{50}$ ) values were calculated by a nonlinear regression method.

## Section 2.2: Bioactive secondary metabolites from the soft coral associated fungus *Aspergillus terreus*

This section is further subdivided into Part A and Part B. Part A gives the review of literature on *A. terreus* and its metabolites. Part B describes the purification of fungal strains and structure elucidation of bioactive metabolites from *A. terreus* isolated from an octocoral, *Simularia kavarattiensis*

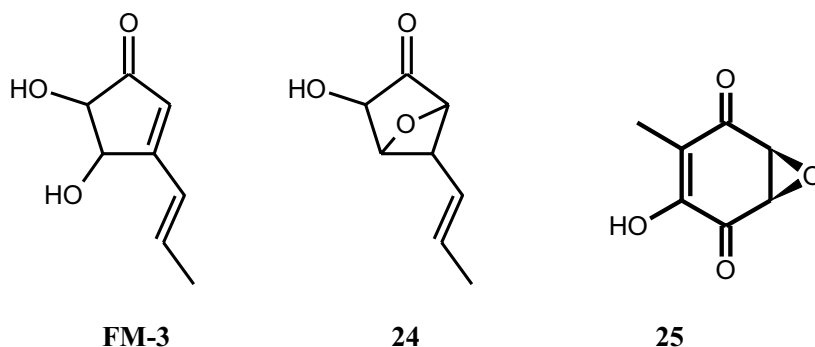
### I. Bioactive metabolites from *Aspergillus terreus*- a review



**Fig 2.10: Microscopic view *A. terreus* conidia**

*A. terreus* is a cosmopolitan fungus, which is primarily isolated from compost, plant material and from soil. It is more common in tropical or sub-tropical areas. It is an occasional causative agent of among immune-compromised patients. A few cases of cerebral infection have been reported. *A. terreus* is isolated occasionally from outer ear canal colonizations. The name *A. terreus* is used by Thom and Church (1918; 1926) to cover a series of strains, which have a number of well-marked morphological features in common, but which show a certain amount of variation in colour and type of growth. The fungus produces colonies which are velvety to floccose, buff, cinnamon to sand brown, velvety; reverse a medium yellow to deep dirty brown; heads columnar upto 500µm diameter; vesicles dome shaped, bearing closely packed metulae (**Fig 2.10**).

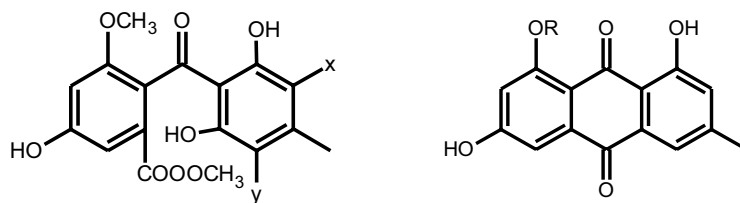
The earliest report on the chemical constituents of *A. terreus* appeared in 1934, when Raistrick and Smith, (1935) isolated a compound and named it as terreic acid. Subsequently, this substance was named terrein, as highly purified sample gave *pH* of 6.8 in 1% aq. solution, an indication of compound not having acidic proton. After studying the several reactions of terrein, the authors arrived at two possible structures for terrein (**FM-3** and **24**) and accepted compound to have most likely the structure **24**, on the basis of following two facts. Terrein gave only a mono-*p*-bromobenzoate and secondly it did not react with maleic anhydride to give Diels alder adduct. Eventually, **FM-1** was later proved to be the correct structure of terrein on the basis of spectroscopic evidences.



In 1949 (Abraham and Florey) an acidic metabolite having good antibiotic activity isolated from *A. terreus* was named terreic acid (**25**) and structure was assigned to be 5,6-epoxy-3-hydroxytoluquinone (Sheehan, 1958). It was later identified as potent inhibitor of Bruton's tyrosine kinase (Kawakami, 1999) and very recently as covalent inhibitor of the bacterial cell wall (Han, H. 2010).

*A. terreus* (Calam et al., 1939) was found to be a highly productive source of itaconic acid (**26**), a metabolite isolated first from *Aspergillus itaconicus* (Dwiarti, 2002). It is very useful industrial chemical and produced in bulk quantity from fermentation of *A. terreus*. (Petuchow et al., 1980). Geodin and erdin, the first chlorine containing fungal metabolites were isolated in 1936 from a strain of *A. terreus* Thom (Raistrick and Smith, 1936). After the initial efforts by Clutterback et al., (1937), the correct relative structures were assigned by Barton and Scott (1958). Some other chlorine containing metabolites include dihydrogeodin (**27**) sulochrin (**28**) and monochloro derivative of

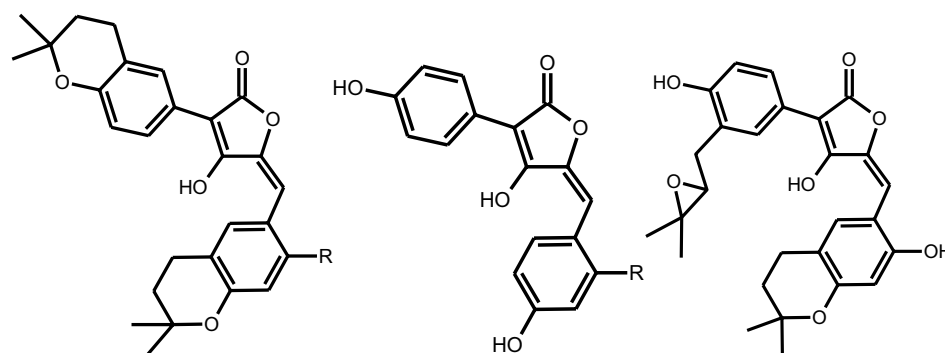
sulochrin (**29**) isolated as antimicrobial metabolites of *A. terreus* (Inamori et al., 1983). Biosynthetically related previously known metabolites, questin (**30**) and emodin (**31**) were also isolated from this fungus.



**Dihydrogeodin (27)** x = Cl, y = Cl  
**Sulochrin (28)** x = H, y = H  
**29** x = Cl, y = H

**emodin (30)** R = H  
**questin (31)** R = CH<sub>3</sub>

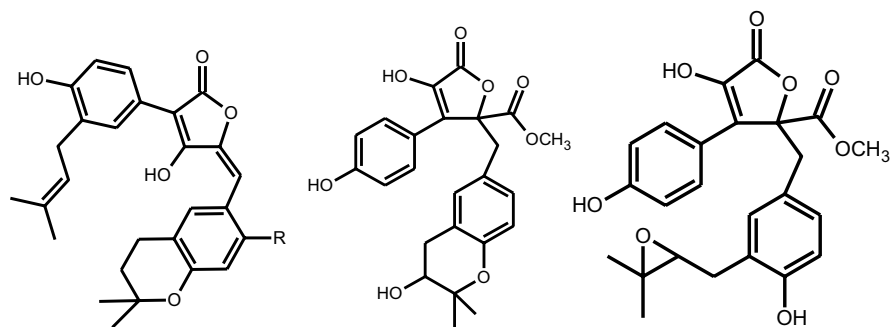
Aspulvinones were the first phenylpropanoid (C<sub>6</sub>-C<sub>3</sub>) derived metabolites obtained from strain of *A. terreus* IAM 2054 (Nobutoshi et al., 1973). This type of compounds were previously known synthetically (Claisen and Ewan, 1895) and also as natural products in higher fungi (Edwards and Gill, 1973). Biosynthesis of aspulvinones involves dimerization of hydroxylphenyl pyruvate derived from tyrosine/phenyl alanine. Additional feature of these aspulvinones isolated from *A. terreus* was that some have one or two C<sub>5</sub> units on aromatic rings (Nobutoshi et al., 1975). So far seven pulvinone derivatives, aspulvinones A-G (**32-38**) have been reported.



**Aspulvinone A (32)** R = H  
**Aspulvinone C (34)** R = OH

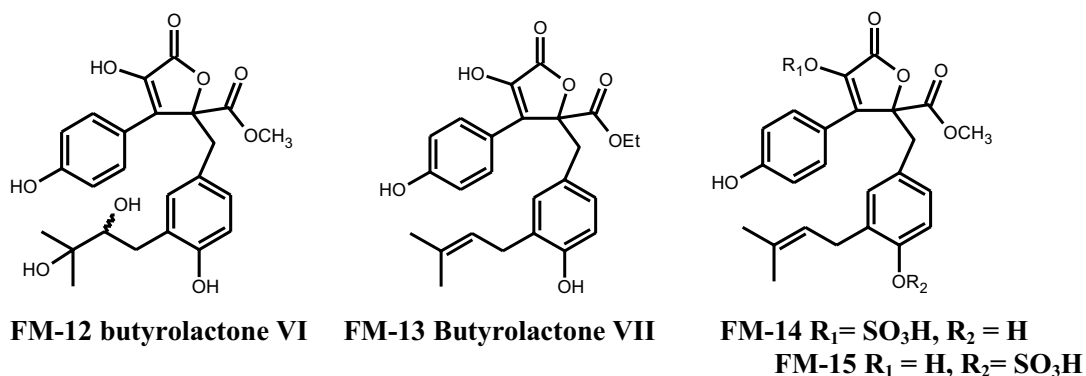
**Aspulvinone E (36)** R = H  
**Aspulvinone G (38)** R = OH

**Aspulvinone F (37)**

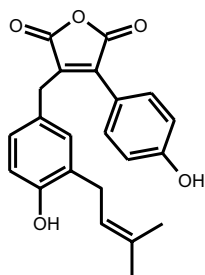


**Aspulvinone B (33) R = H Butyrolactone V (FM-11) Butyrolactone III (FM-9)**  
**Aspulvinone D (35) R = OH**

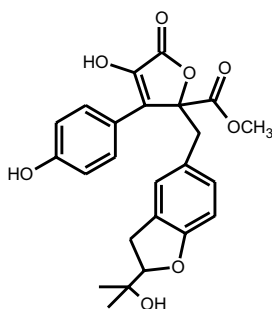
Since the isolation of aspulvinones, many phenyl propanoid derived metabolites, some of them also having C5 unit on aromatic ring, but differing in constitution of central furanone ring have been isolated from *A. terreus*. The first such compound to be isolated was butyrolactone I (**FM-1**) from strain of *A. terreus* IFO 8835 by Kiriya et al., (1977). This compound proved to be a rare potent metabolite that selectively inhibits CDK2 and CDK1 kinases, both of which play important roles in cell progression from G1 to S phase and from G2 phase to M phase, respectively, in mammalian cell. Later, the derivatives of **FM-1**, butyrolactones II-VII (**FM-2** and **FM-9 to FM-13**) (Rao et al., 2000; Haritakun et al., 2010) and two butyrolactone I sulfates (**FM-14** and **FM-15**) (Niu et al., 2008) were isolated from different strains of *A. terreus*. Incidentally, **FM-12** is identical to aspernolide D (Nuclear et al., 2010) reported in separate article during same time as a metabolite of *A. terreus*, along with a biosynthetically unusual six membered anhydride, asperterone (**39**). Some of these metabolites exhibit various activities those include anticancer, antiplasmodial and antioxidant.



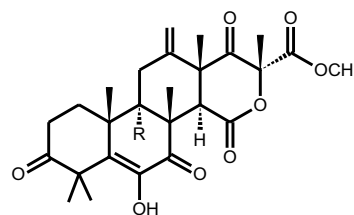
**FM-12 butyrolactone VI FM-13 Butyrolactone VII**  
**FM-14 R<sub>1</sub> = SO<sub>3</sub>H, R<sub>2</sub> = H**  
**FM-15 R<sub>1</sub> = H, R<sub>2</sub> = SO<sub>3</sub>H**



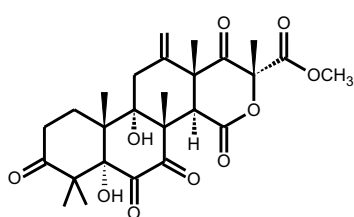
**Asperterone (39)**



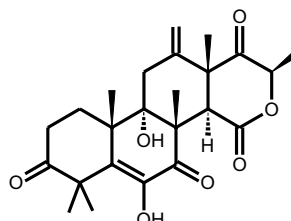
**Butyrolactone IV (FM-10)**



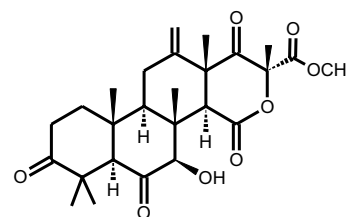
**Terrentonin (40) R= OH**  
**Terretonin A (41) R= H**



**Terretonin B (42)**



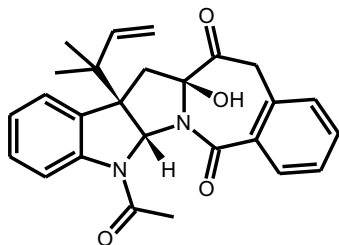
**Terretonin C (46)**



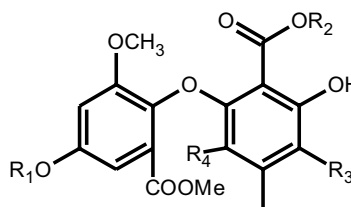
**Terretonin D (47)**

Some strains of *A. terreus* produces metabolites those are biosynthetically distinct. **FM-11** was previously isolated from an unidentified strain of *Aspergillus* sp. This strain produced metabolites those belong to five structure types namely, butenolides (**FM-1**, **FM-2** along with **FM-15**); seterterpenoids terrentonin (**40**) (Springer et al., 1979), terretonin A (**41**) and B (**42**) (Li et al., 2005), questin (**31**) (Mahmoodian and Stickings, 1964), dihydrogeodin (**27**) (Harold and George, 1936), asteric acid (**43**) (Hiroshi, et al., 1992) and four diphenyl ethers of which two were new (**44-45**) (Hargreaves et al., 2002). It appears that unidentified strain used to isolate these compounds must be *A. terreus*, because **FM-1** and **FM-2**, terrentonins (**40-42**) and compounds (**27**, **31** and **43**) have been previously isolated from *A. terreus*. Terretonin (**40**) (Springer et al., 1979) and terretonins A-D (**41-42** and **46-47**) (Li, et al., 2005), the only sesterpenoid class of metabolites were first obtained from *A. terreus*. The fungus that produced **41-42** and **46-47** also produced biosynthetically distinct metabolite, a novel alkaloid asterrelenin (**48**) (Li et al., 2005) None of these terrentonins were cytotoxic, but similar terretonins E and F metabolites of marine

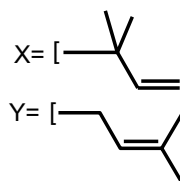
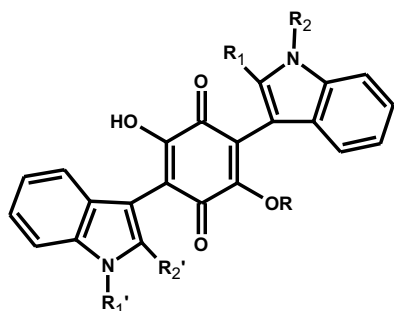
sponge-derived fungus *Aspergillus insuetus* have been shown to be inhibitors of the mammalian mitochondrial respiratory chain (Pilar et al., 2009).



**Asterlennin (48)**



**Asterric acid (43)**  $R_1=R_2=R_3=R_4=H$   
**44**  $R_1=H$   $R_2= n\text{-butyl}$   $R_3= R_4= Cl$   
**45**  $R_1= R_2= Me$   $R_3= R_4= Cl$



	$R_1$	$R_2$	$R_1'$	$R_2'$	$R$
<b>49</b>	X	H	X	H	H
<b>50</b>	X	H	X	H	Ac
<b>51</b>	X	H	H	Y	H
<b>52</b>	H	Y	H	Y	H

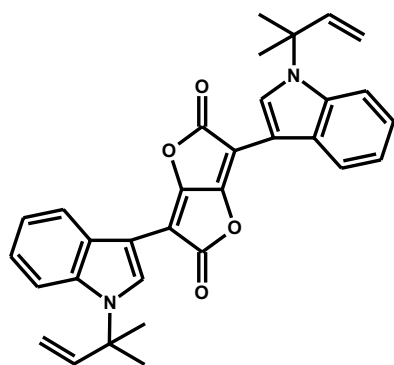
**Asterriquinone (49) and its derivatives (50-52)**

An investigation by Yamamoto et al., (1976) on *A. terreus* IFO 6123 led to the isolation of asterriquinone (**49**), a dimer biosynthesized by pyruvate derived from tryptophan. As in aspulvinones (**32-38**) and aspernolides (butyrolactone I derivatives) **49** also have C5 unit on aromatic rings. It showed good antitumor properties. Further investigation of the same strain by Kaji (2003) led to the isolation of three new derivatives of **49**, a monoacetate (**50**) and other two (**51-52**) differing by attachment of C5 unit on aromatic systems, beside a new congener having a different framework formed by oxidation and internal cyclization of quinone functionality of **49**. The new congener was named asterridinone (**53**).

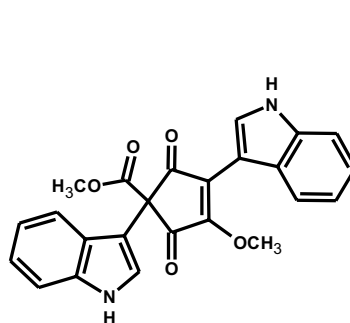
Asterridione (**54**) (not asterridinone, **55**) was cytotoxic metabolic product of *A. terreus* isolated from Sonoran desert (Wijeratne et al., 2003). Two new derivatives of



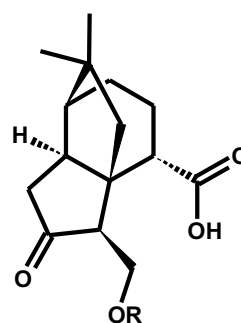
terricyclic acid (**56-57**) were also isolated from this source along with seven known molecules. **55** is supposed to be biosynthesized from asterriquinone D, which involves contraction of six membered ring to five membered ring.



**54 Asterridinone**  
**57 R = H**

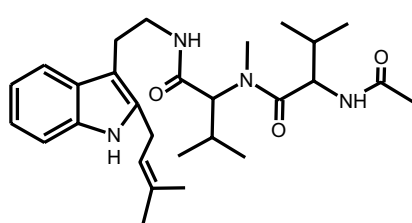


**55 asterridione**

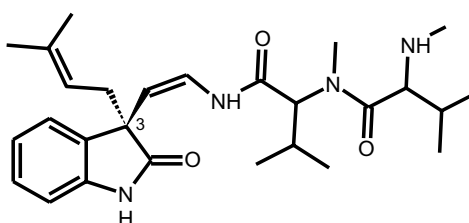


**56 R = CH<sub>3</sub>**

A new prenylated indolic enamide, terpeptin (**58**) was metabolic product of *Aspergillus terreus* 95F-1 (Kagamizono et al., 1997). It showed strong inhibition of cell cycle progression of mouse tsFT210 cells in the C2/M. A Chinese mangrove-derived unidentified strain of *Aspergillus* sp. also produced **58** along with two new derivatives, terpeptins A (**59**) and B (**60**) (Lin et al., 2008).



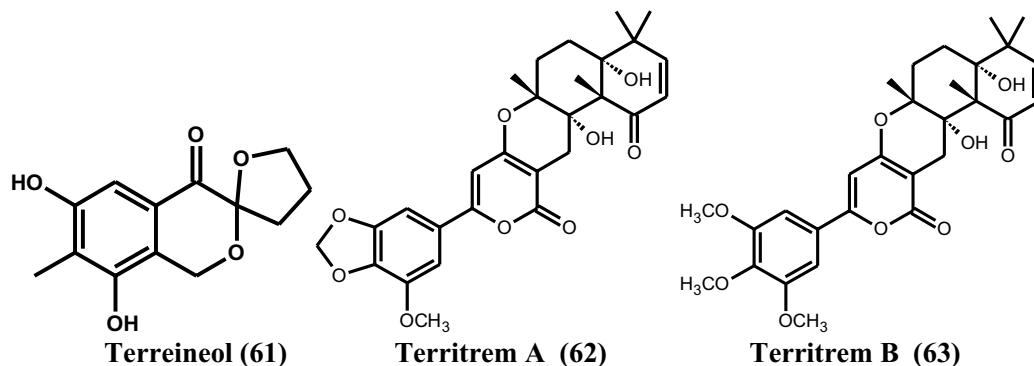
**58**



**59**  
**60 3- epimer**

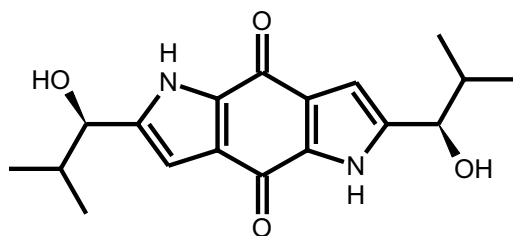
Terreineol (**61**), a novel polyketide, containing spiro furan isochromone moiety biosynthesized from six acetyl/malonyl units added by CoA was metabolic product of *A. terreus* (maceda et al., 2004). A metabolite, named aspergiketol was also obtained from *A. terreus* associate with *Opuntia ficusindica* Mill (Wu et al., 2008). Interestingly, this compound has been assigned the structure identical to terreineol

(61), but claimed it to be new metabolite. The spectroscopic data presented for terreineol and aspergiketol are identical.



Territrem A (62) and B (63) as the name indicates were isolated as tremorgenic metabolites from *A. terreus*. The tremorgenic effects of territrems arise from their being a potent inhibitor of acetylcholinesterase (AChE) (Arvanov et al., 1993). Analogous to territrems are arisugacins obtained from *penicillium* sp. (Omura et al., 1995) are also highly specific and potent AChE inhibitors as well, but less active than 63.

*A. terreus* have yielded large number of various types of secondary metabolites from terrestrial isolates. Some of these metabolites have been also obtained from marine sources, but there is only one report that exists on novel metabolite from marine derived *A. terreus*. A novel chiral dipyrrolobenzoquinone derivative, terreusinone (64), has been the only compound to be obtained from a marine-derived fungus (Lee et al., 2003). The fungus was isolated from a marine red alga *Halymenia acuminata* collected in the Bijin Island. It exhibited a UV-A absorbing activity with ED50 value of 70  $\mu\text{g/mL}$



**Terreusinone (64)**

## II. Results and discussion

### *i) Biological materials*

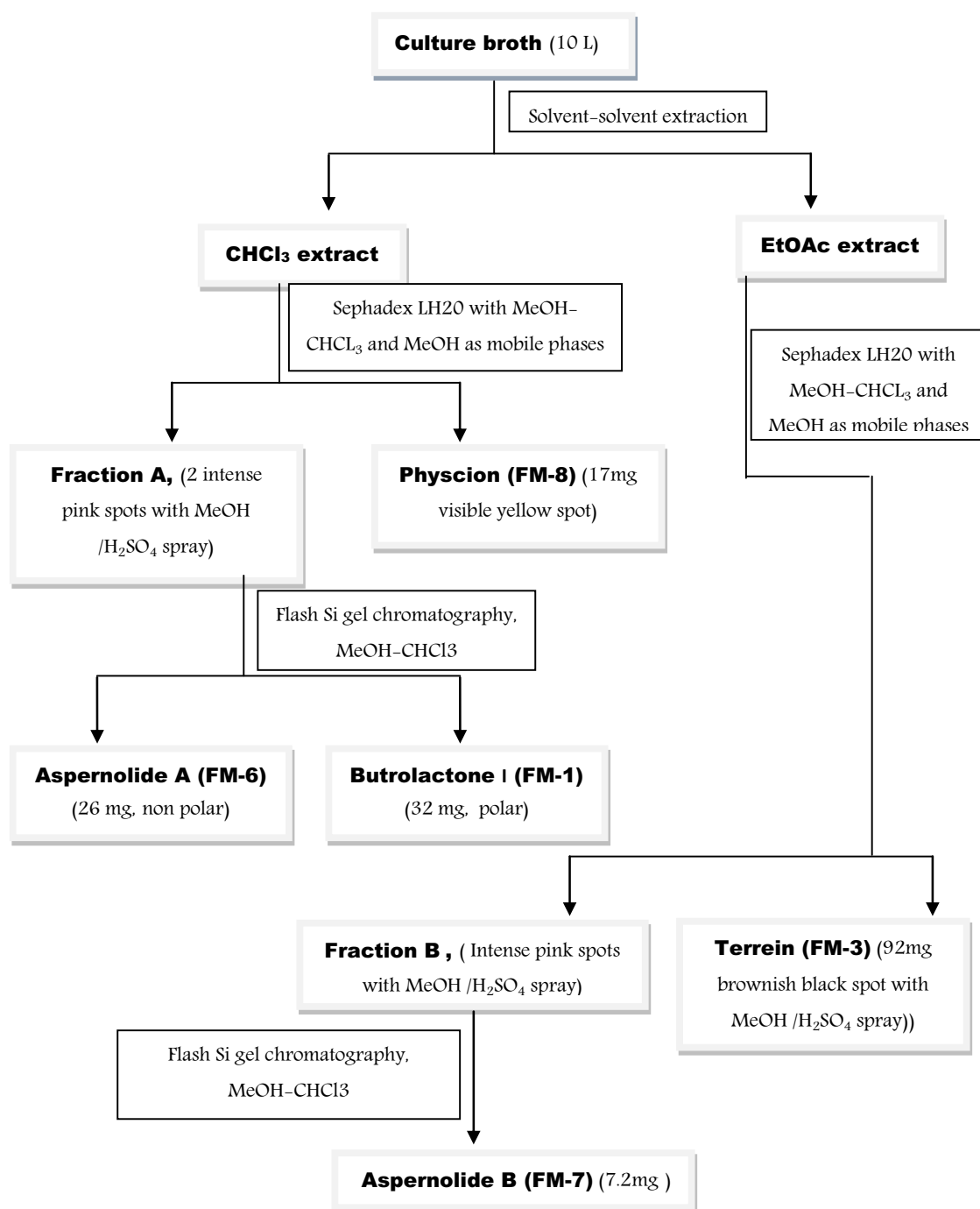
Indian coastline is potential source of marine organisms such as sponges soft corals, tunicates, algae etc and exhibits various biological activities. Each of these marine organisms harbours vast biodiversity of microorganisms. Numerous bioactive compound of marine origin are in fact microbial metabolites originating from dietary commensalic or endosymbiotic microorganisms.

One among these, a soft coral for our study was collected from Mandapam, Tamil Nadu and used for the isolation of associated fungi. Only one epiphyte grew on both PDA and Czapek Dox Agar, while on treatment of octocoral with hypochlorite no endophyte could be obtained. From the morphological features the epiphytic fungi was identified as *Aspergillus terreus*. Its 5.8SR gene sequences had 99% similarity to *Aspergillus terreus* isolates NRRL 4609, NRRL 260 NRRL 255 and NRRL 2399. The fungus was mass cultured on potato dextrose broth prepared in seawater and the ethyl acetate extract of cultured broth showed good anticancer activity.



**Fig 2.11: *Aspergillus terreus* cultured on PDA**

ii) Purification of metabolites



Scheme 2.5: Purification of metabolites from culture medium of *A. terreus*

After filtration the culture broth, was fractionated between chloroform and ethyl acetate. A new secondary metabolite aspernolide A (FM-6), along with a known

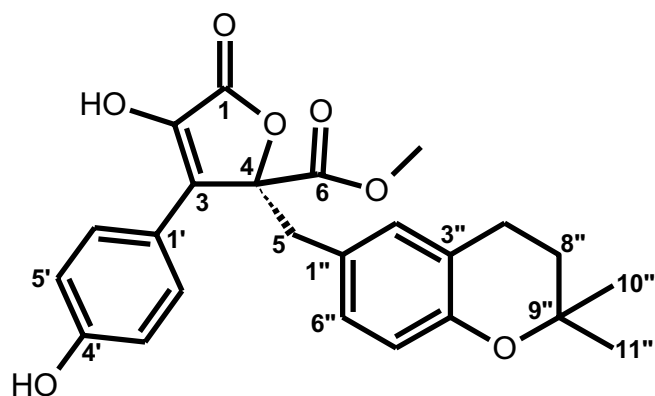
metabolite physcion (**FM-8**) were purified from the chloroform extract by successive fractionation with silica gel and Sephadex LH20 gel filtration chromatography. Repeated fractionation of the ethyl acetate extract by silica gel and Sephadex LH20 gel filtration chromatography yielded a new compound aspernolide B (**FM-7**) and two known compounds butyrolactone I (**FM-1**) and terrein (**FM-3**). The systematic scheme of isolation these metabolites is depicted in **Scheme 2.5**.

### *iii) Structure elucidation of metabolites*

**FM-1** was obtained as colourless crystalline solid, which would eventually turn into a gummy material on storage for long time, and **FM-3** was isolated as crystalline needles. On TLC **FM-1** and **FM-3** gave a spots identical in all respect to butyrolactone I (**FM-1**) and terrein (**FM-3**) respectively, isolated from *A. flavipes* (section 2.1). The spectroscopic data (UV-Vis, IR, NMR and MS) of **FM-1** and **FM-3** isolated from this fungus were well in agreement with the **FM-1** and **FM-3** respectively. The detail description of the structure and its spectroscopic data is discussed in preceding section of this chapter.

**FM-6** was obtained as white sticky solid. The molecular formula  $C_{24}H_{24}O_7$  of **FM-6** was determined by HRESITOFMS (**Fig 2.12a**), which showed pseudomolecular ion peaks  $[M+Na]^+$  at  $m/z$  447.1433 (calcd. 447.1420 for  $C_{24}H_{24}O_7Na$ ) and  $[2M+Na]^+$  at  $m/z$  871.2959 (calcd. 871.2942 for  $C_{48}H_{48}O_{14}Na$ ) and was isomeric with **FM-1**. IR spectrum (**Fig 2.12b**) showed presence of ester/lactone carbonyls at  $1731\text{ cm}^{-1}$  and  $1738\text{ cm}^{-1}$ , phenolic OHs were evident at  $3330\text{ cm}^{-1}$  and presence of absorption at  $1660\text{ cm}^{-1}$  was suggestive of aromaticity in the molecule.  $^1\text{H}$  NMR signals (**Fig 2.12c**) of the  $A_2B_2$  system at  $\delta_H$  7.56 (d, 2H,  $J = 8.7\text{ Hz}$ ) and 6.86 (d, 2H,  $J = 8.7\text{ Hz}$ ) revealed the presence of para substituted benzene moiety. Two aromatic signals 6.53 (s, 1H) and 6.47 (s, 2H) (two doublets merged into a singlet) were indicative of the presence of additional unsymmetrical trisubstituted benzene ring in the molecule. Its  $^{13}\text{C}$  NMR (**Fig 2.12d**) showed the presence of 10 aromatic signals for two aromatic rings, two ester carbonyls  $\delta_c$  169.3 (s), and 169.6 (s), olefinic carbon signals  $\delta_c$  137.2 (s) and 128.8 (s), three  $\text{sp}^3\text{ CH}_2\text{s}$   $\delta_c$  22.1 t, 32.5 t and 38.6 t, a carbomethoxy  $\delta_c$  53.4 q and

two oxygenated quaternaries  $\delta_c$  86.1 (s) and 74.2 (s). The molecular formula  $C_{24}H_{24}O_7$  requires 13 degrees of unsaturation. Presence of two aromatic rings accounts for eight while two carbonyls and two olefinic carbons accounts for another three, which makes total of eleven degrees of unsaturation. Therefore **FM-6** must possess two aliphatic rings in addition to two aromatic rings.



**Aspernolide A (FM-6)**

A detail comparison of NMR data (**Table 2.4**) of **FM-6** with that of **FM-1**, confirmed a common hydroxyphenyl pyruvate dimer type of network in the molecule. The significant difference observed in the NMR spectra of **FM-6** as compared to that of **FM-1** was the absence of a olefinic proton signals  $\delta_H$  5.0 (t, 1H), two olefinic carbon signals  $\delta_c$  121.0 (d) and 130.7 (s) and the presence of three methylenes and two oxygenated  $sp^3$  quaternaries compared to two methylenes and one oxygenated  $sp^3$  quaternary in **FM-1** in both  $^{13}C$  NMR spectra. These data was indicative of the presence of a dihydropyran ring fused to a trisubstituted benzene ring in place of the open prenyl chain present in **FM-1**.

HMBC (**Fig 2.12e**) was well in agreement to the given structure (**Fig 2.12f**). Key HMBC correlations from H-2'' to C-3'' and C-7'', from H-7'' to C-2'', C-3'' and C-4'' and from H-8'' to C-9'' and C-10''(11'') established dihydropyran ring fused through C3''-C4'' bond of benzene ring. HMBC correlations from H-2'' and H-6'' to C-6 and from H-6 to C-1'', C-6'', C-2'', C-4, C-5 and C-3 was evident of the benzodihydropyranmethylene moiety linked to lactone ring at C-4. Furthermore,

HMBC correlation from H-2'(6') to C-3 established p-substituted phenolic moiety at C-3. Out of two carbonyls, C-5 and C-1,  $\delta_c$  169.6 was assigned to C-5 on the basis of its HMBC correlation to the protons H-6 and H<sub>3</sub>-5OMe. **FM-6** is known previously as reaction product of **FM-1**, but was known as natural product.

**Table 2.4** NMR spectroscopic data of aspernolide A (FM-6) (500 MHz, CDCl<sub>3</sub>)

Position	$\delta_C$ , mult.	$\delta_H$ , mult., <i>J</i> (Hz)	HMBC
1	169.3 s		
2	137.2 s		
3	128.8 s		
4	86.1 s		
5	38.6 t	3.39, d, (15.0) 3.59, d, (15.0)	C4, C1'', C6'', C3, C2'', C5
6	169.6 s		
1'	122.2 s		
2'(6')	129.5 d	7.56, d, (8.7)	C4', C3
3'(5')	115.9 d	6.86, d, (8.7)	C1', C4'
4'	156.4 s		
1''	123.5 s		
2''	131.4 d	6.53, s	C7'', C6, C6'', C4''
3''	120.3 s		
4''	152.9 s		
5''	116.6 d	6.47, s	C1'', C3''
6''	129.0 d	6.47, s	C6, C2'', C4''
7''	22.1 t	2.53m	C8'', C9'', C2'', C3'', C4''
8''	32.5 t	1.66, t, (6.5)	C3'', C7'', C9'', C10''(11'')
9''	74.2 s		
10''(11'')	26.6 q	1.21, s	C7'', C8'', C9''
5-OMe	53.4 q	3.72, s	C5

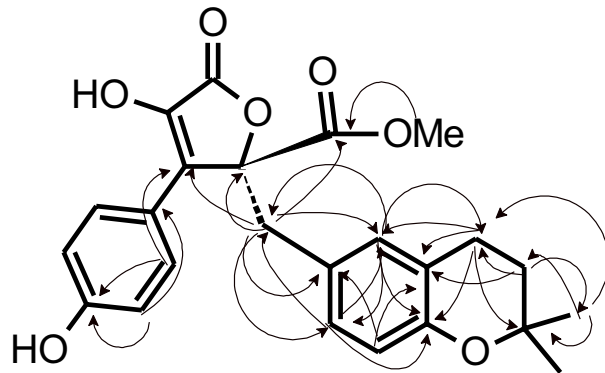


Fig 2.12f: HMBC correlation of FM-6

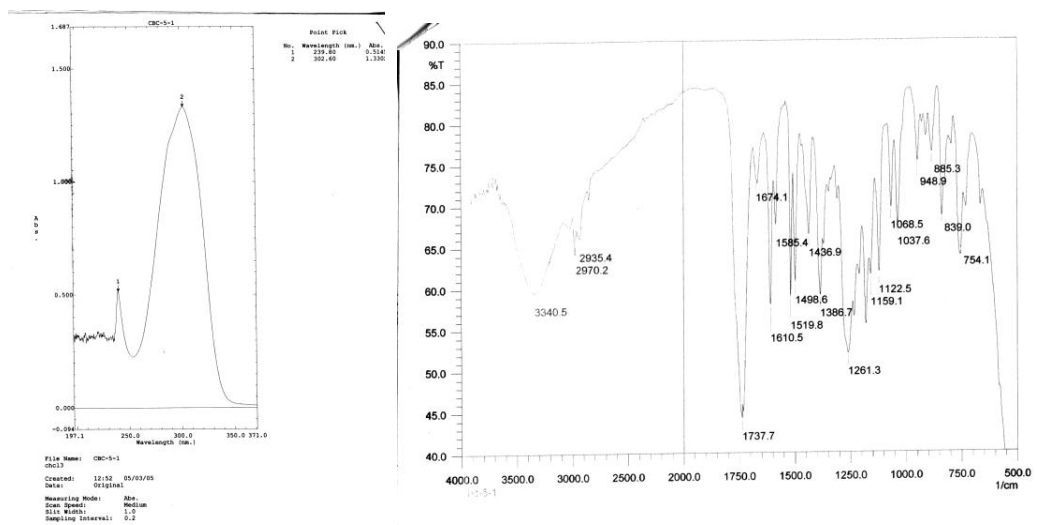


Fig 2.12g: UV-Vis spectrum of Aspernolide A Fig 2.12b: IR spectrum of Aspernolide A

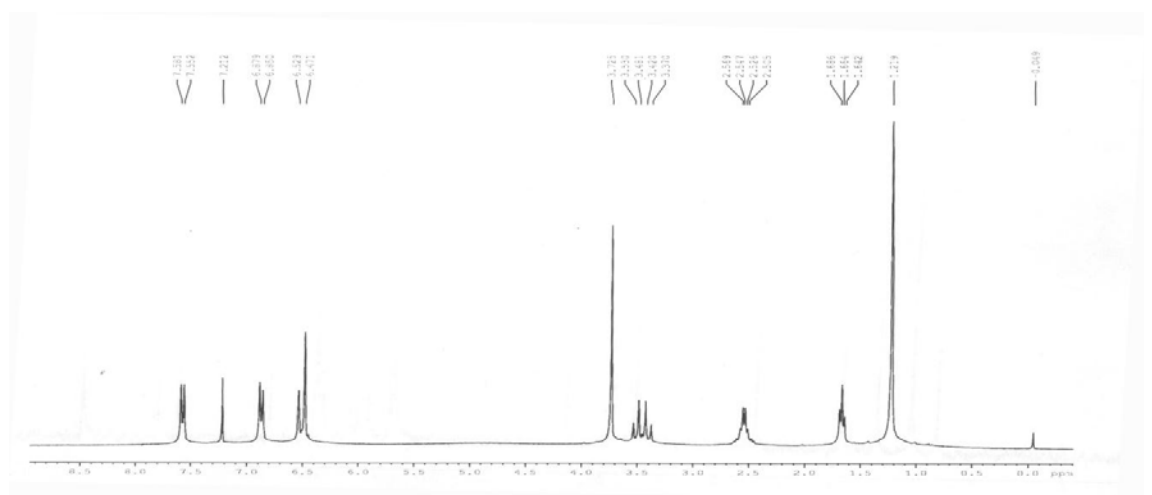


Fig 2.12c:  $^1\text{H}$  NMR of Aspernolide A



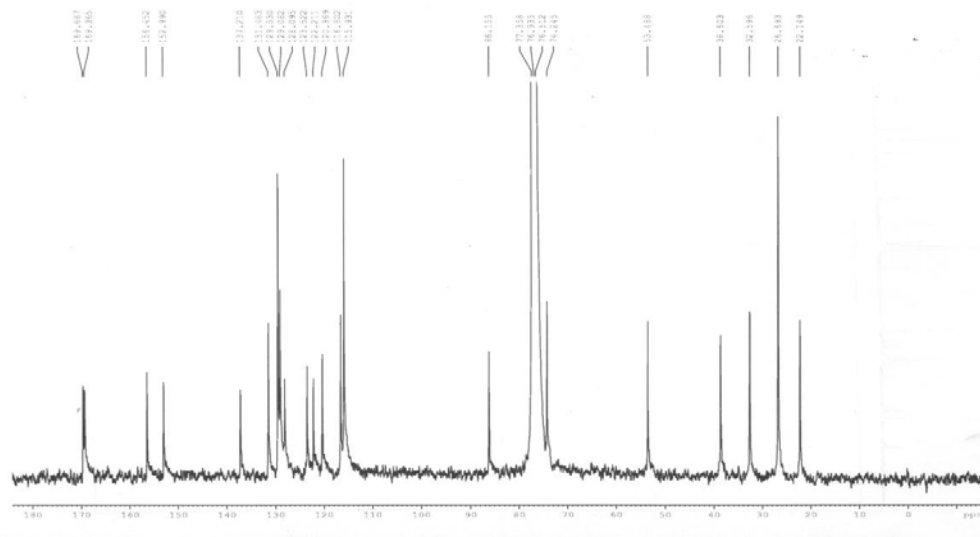


Fig 2.12d: <sup>13</sup>C NMR of Aspernolide A

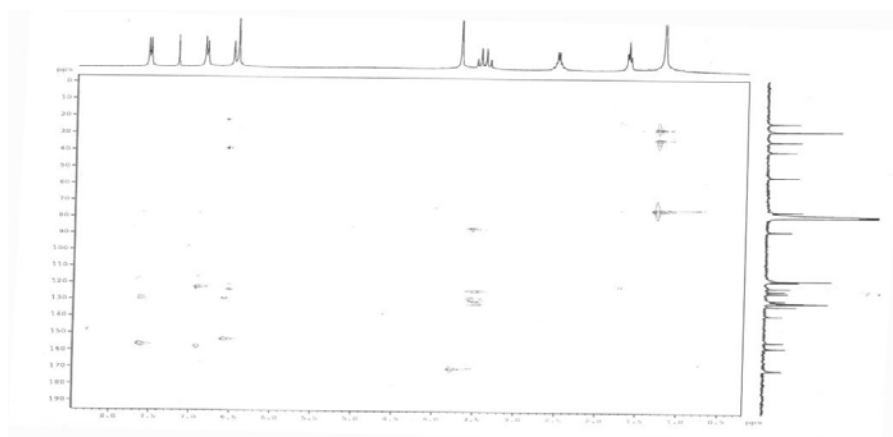


Fig 2.12e HMBC of Aspernolide A

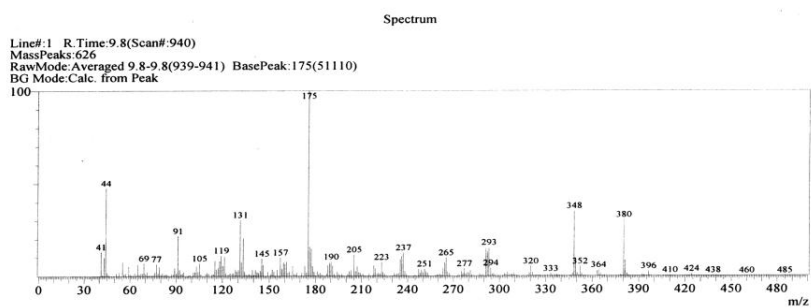
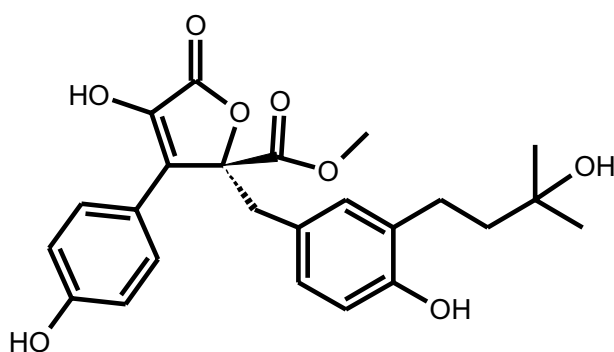


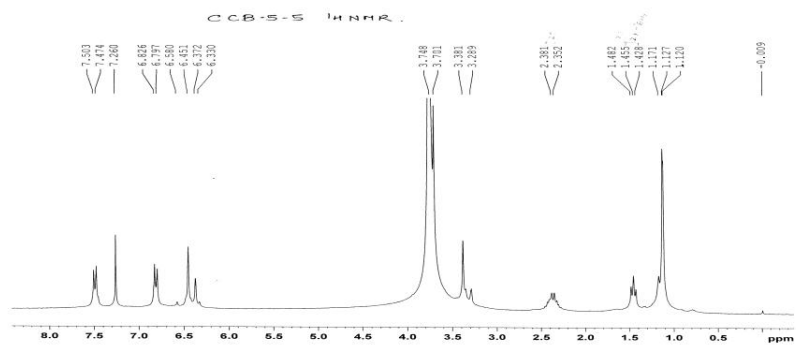
Fig 2.12f: EI-MS of Aspernolide A

Aspernolide B (**FM-7**) (Rf, 0.51), more polar than **FM-6** (Rf, 0.81) was obtained as light brown syrup  $[\alpha]_D + 48.27$  (c 0.29, MeOH). The IR spectra showed presence of OHs at  $3330\text{ cm}^{-1}$ , ester/lactone carbonyls overlapping peaks at  $1732$  and  $1747\text{ cm}^{-1}$  and  $1610$  and  $1519\text{ cm}^{-1}$  for aromatic rings very similar to **FM-6**. It gave  $^1\text{H}$  (**Fig 2.13a**) and  $^{13}\text{C}$  (**Fig 2.13b** and **Fig 2.13c**) NMR patterns very similar to **FM-6**. Although, spectral pattern matched with **FM-6** chemical shift variations were observed in the  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra. Significant variations in the chemical shifts were observed for ring carrying iso-pentyl chain wherein C-1'', C-3'', C-7'', C-8'' C-10'' and C-11'' were considerably deshielded to resonate at  $\delta_c$  128.1( $\Delta\delta$  4.6ppm), 124.0( $\Delta\delta$  3.7ppm), 24.2( $\Delta\delta$  2.1ppm), 43.2( $\Delta\delta$  10.7ppm), 28.4( $\Delta\delta$  1.8ppm) and 28.5( $\Delta\delta$  1.9ppm) while C-5'' and C-9'' were considerably shielded to resonate at  $\delta_c$  114.6 ( $\Delta\delta$  2.0ppm) and 70.8( $\Delta\delta$  3.4ppm) as compared to **FM-6**, suggesting a change on the aromatic ring carrying iso-pentyl chain (**Table 2.5**).

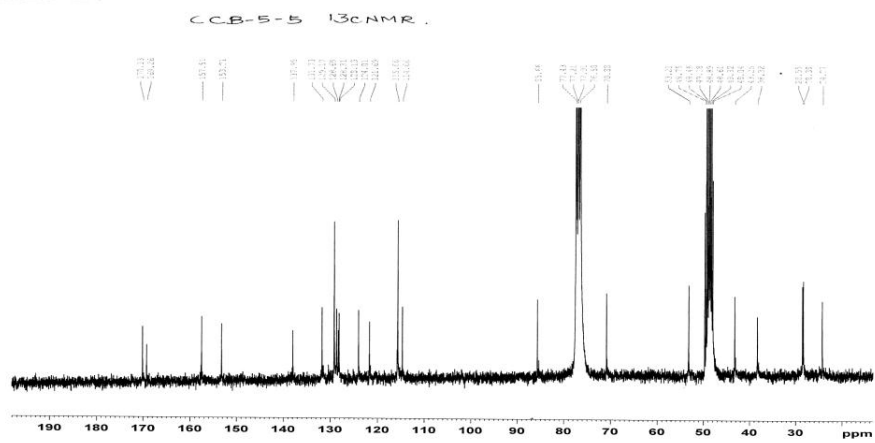


Aspernolide B (**FM-7**)

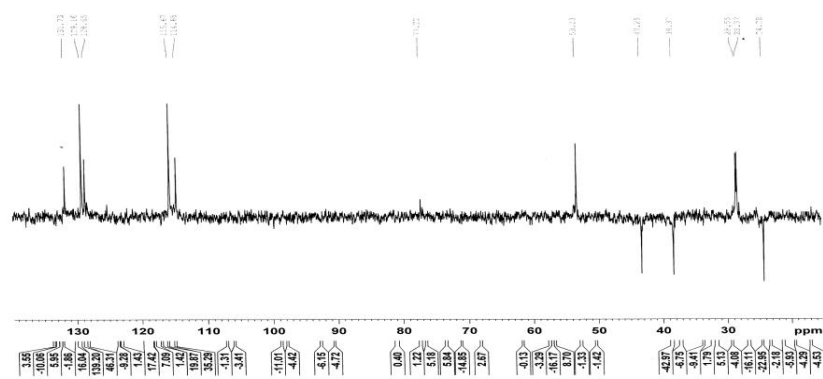
**FM-7** was well distinguished from ESI-MS spectrum (**2.13d**), which showed pseudomolecular ions  $[\text{M}+\text{H}]^+$  at  $m/z$  443.1699 (calcd. 443.1706 for  $\text{C}_{24}\text{H}_{27}\text{O}_8$ ) and  $[\text{M}+\text{Na}]^+$  at  $m/z$  465.1516 (calcd. 465.1525 for  $\text{C}_{24}\text{H}_{26}\text{O}_8\text{Na}$ ) suggesting molecular weight of 442 for the compound, which was 18 units more than **FM-6**. Based on the above observations it was evident that **FM-7** had open chain hydroxylated prenyl chain ortho to phenolic  $-\text{OH}(\text{C}-4'')$ . **FM-7** is a new natural product.



**Fig 2.13a: <sup>1</sup>H NMR of Aspernolide B**



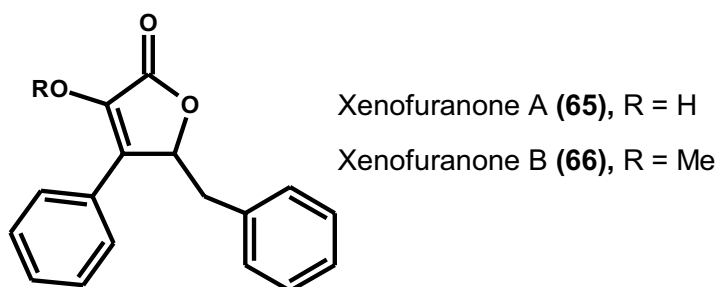
**Fig 2.13b: <sup>13</sup>C NMR of Aspernolide B**



**Fig 2.13c: <sup>13</sup>C NMR of Aspernolide B**

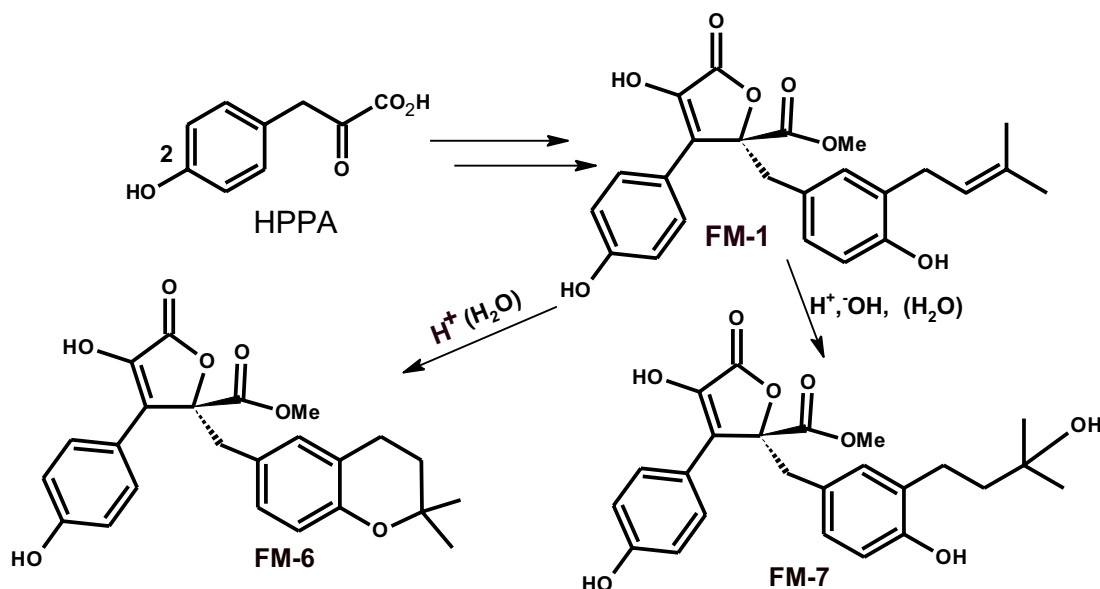
*iv) Biogenesis and stereochemistry of Aspernolide A (FM-6) and B (FM-7)*

To determine and confirm the stereochemistry of **FM-6** and **FM-7**, **FM-1** was converted to **FM-6** and **FM-7**. Based on the reported feeding experiments for establishing the biosynthesis of xenofuranones A (**65**) and B (**66**) together with compound **1** (Brachmann et al., 2006; Nitta et al., 1983) and isolation of **FM-6** and **FM-7** from *A. terreus*, it is apparent that the structures of **FM-6** and **FM-7** are extension of the biosynthesis of **FM-1**, which is derived from p-hydroxy phenylpyruvate. The enzyme catalyzed cyclization or addition of water across the double bond of the prenyl chain of **FM-1** results in the formation of **FM-6** and **FM-7** respectively (Scheme 2.6). The last step in the biogenetic scheme was mimicked using mild acid catalysis to confirm the absolute structure of **FM-6**.



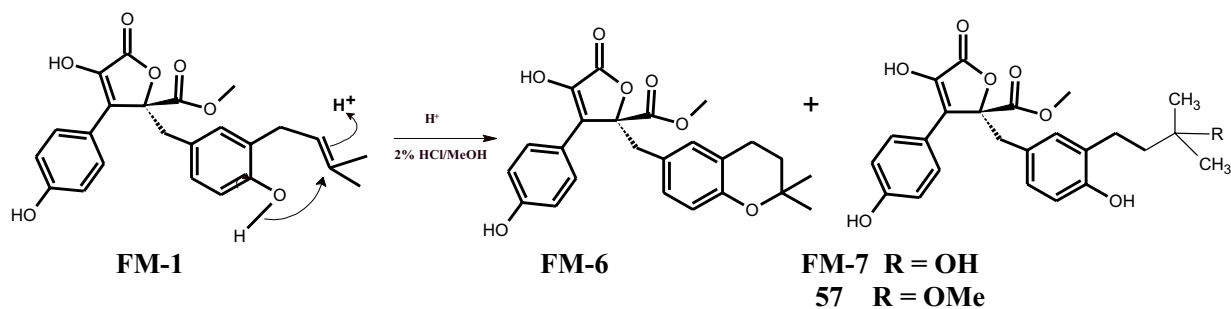
On heating with 1% aqueous sulphuric acid, **FM-1** was converted to **FM-6** as well as small amount of **FM-7** (Scheme 2.7), but only 50% conversion was observed. When the same reaction was carried out using 2% conc. HCl in methanol, complete conversion of **FM-1** was observed as indicated by TLC (90:10, CHCl<sub>3</sub>/MeOH) giving three products. On chromatographic separation, 75% yield of **FM-6** was obtained along with minor amounts of **FM-7** and a new product Aspernolide C (**67**). Compound **67** displayed an extra methoxy signal  $\delta_{\text{H}}$  3.15, s,  $\delta_{\text{C}}$  49.0 q in NMR spectra (Fig 2.14a and 2.14b) compared with **FM-7** (Table 2.5). In the rest of the NMR spectra it was seen that C-7'', C-8'', C-10'' and C-11'' were shielded and observed at  $\delta_{\text{C}}$  23.2( $\Delta\delta$  1.0), 40.0( $\Delta\delta$  3.2), 24.0 ( $\Delta\delta$  4.4), and 24.4( $\Delta\delta$  4.1), while only methoxylated quaternary carbon C-9'' was deshielded to 75.4( $\Delta\delta$  4.6) compared to **FM-7**. Pseudomolecular ion peaks  $[\text{M}+\text{H}]^+$  at  $m/z$  457.1851 and  $[\text{M}+\text{Na}]^+$  at  $m/z$  479.1669 observed in ESI-MS (Fig. 2.14c) indicated molecular weight of 456 for the

compound **67**. On the basis of these observations, the structure was assigned as shown in **67**. **FM-7** and **67** are formed by Markovnikoff's addition of water and methanol respectively, across the double bond of the prenyl chain.



**Scheme 2.6: Plausible biogenetic pathway for Aspernolide A (FM-6) and B (FM-7)**

During the structure elucidation of **FM-1**, **FM-7** has been reported as the product of its reaction with ethanolic HCl (Kiryama et al., 1977). In the present investigation, the same reaction in methanolic HCl yielded besides **FM-6**, two additional products **FM-7** and **57**. These butenolides **FM-1**, **FM-6** and **FM-7** have one chiral centre C-4. Co-metabolite **FM-1** used in the above reaction was determined to be 4*R* configured by comparison of specific rotation data  $[\alpha]_D + 84.32$  with the previously reported result (Kiryama et al., 1977). The absolute configurations of **FM-6** and **FM-7**, therefore could actually be deduced as 4*R* based on the biogenetic grounds and similarity of the specific rotations  $[\alpha]_D + 88.73$  for **FM-6** and  $+48.27$  for **FM-7** with **FM-1**. Further confirmation of the absolute stereochemistry of **FM-6** and **FM-7** results from the fact that, the natural **FM-6** and **FM-7** and those obtained as products of acid catalyzed reaction had identical spectral data and specific rotations. Therefore, we conclude that the compounds **FM-6** and **FM-7** also have 4*R*-configuration.

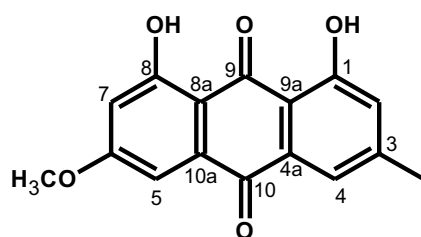


**Scheme 2.7: Conversion of butyrolactone I to Aspernolides A (FM-6), B (FM-7) and C (57)**

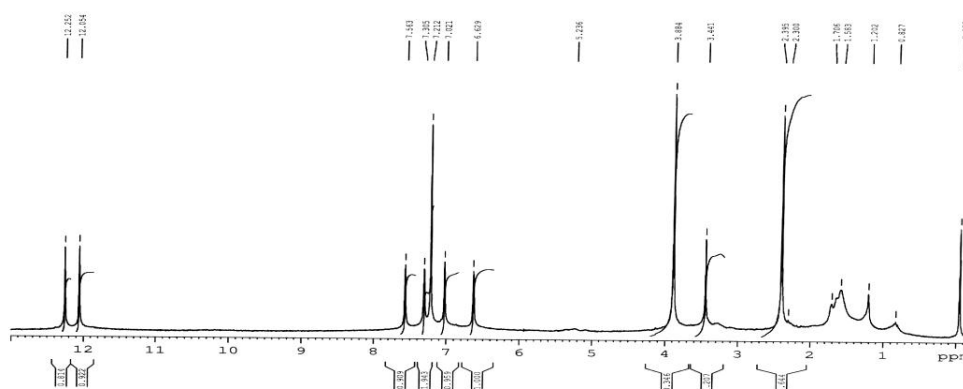
**Table 2.5:  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectroscopic data of aspernolides B (FM-7) and C (57) (300 MHz,  $\text{CDCl}_3$ + 2drops  $\text{CD}_3\text{OD}$ )**

Position	FM-7		57	
	$\delta_{\text{C}}$ , mult	$\delta_{\text{H}}$ , mult., J(Hz)	$\delta_{\text{C}}$ , mult	$\delta_{\text{H}}$ , mult., J(Hz)
1	169.2 s		169.3 s	
2	137.9 s		137.9 s	
3	128.3 s		128.2 s	
4	85.6 s		85.7 s	
5	170.1 s		170.1 s	
6	38.3 t	3.46, s	38.4 t	3.43, s
1'	121.6 s		121.8 s	
2'(6')	129.1 d	7.55, d, (8.7)	129.2 d	7.54, d, (8.7)
3'(5')	115.6 d	6.87, d, (8.7)	115.0 d	6.85, d, (8.7)
4'	157.5 s		157.5 s	
1''	128.1 s		128.1 s	
2''	131.7 d	6.41, d (1.8)	131.8 d	6.40, d (1.8)
3''	124.0 s		124.0 s	
4''	153.2 s		153.4 s	
5''	114.6 d	6.52, d, (8.7)	114.4 d	6.49, d, (8.1)
6''	128.6 d	6.55, dd, (8.7, 1.8)	128.9 d	6.54, dd, (8.1, 1.8)
7''	24.2 t	2.37, m	23.2 t	2.35, m
8''	43.2 t	1.53, t, (7.8)	40.0 t	1.54, t, (7.8)
9''	70.8 s		75.4 s	
10''	28.4 q	1.20, s	24.0 q	1.12, s
11''	28.5 q	1.19, s	24.4 q	1.12, s
9''-OMe	---		49.0 q	3.15, s
5-OMe	53.5 q	3.76, s	53.3 q	3.74, s

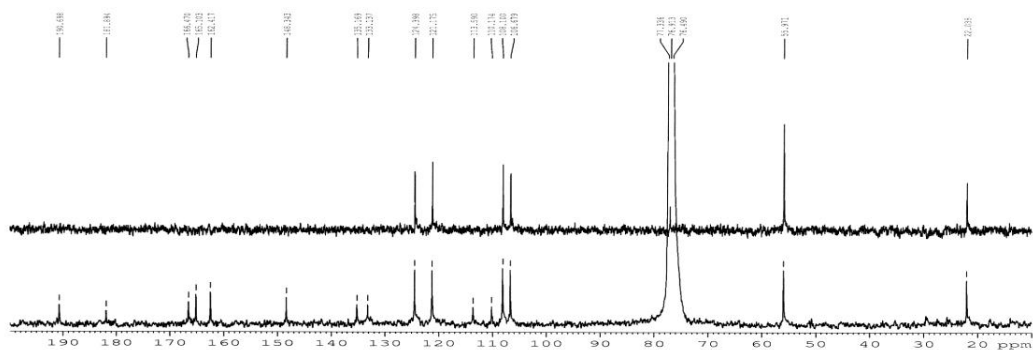
Physcion (**FM-8**) was obtained as a bright yellow solid. Pseudomolecular ion  $[M+H]^+$  at  $m/z$  285 indicated molecular formula to be  $C_{16}H_{12}O_5$ . The IR absorption band of  $1629\text{ cm}^{-1}$  and bright yellow colour of the compound was indication that compound had quinone functionality. Its  $^{13}\text{C}$  NMR along with DEPT 135 (**Fig 2.15b**) indicated presence of 16 carbon atoms distributed as 2 methyls, 4 methines and 10 quaternary carbons. The carbon signals at  $\delta$  181.8 and 190.6 were indicative of presence of quinone carbonyl groups. Presence of 14  $sp^2$  carbons including two carbonyls indicated it to be a anthraquinone derivative. The  $^1\text{H}$  (**Fig 2.15a**) and  $^{13}\text{C}$  spectra indicated presence of a methoxy ( $\delta_{\text{H}}$  3.88s,  $\delta_{\text{C}}$  55.9q), an aromatic methyl ( $\delta_{\text{H}}$  2.39 s,  $\delta_{\text{C}}$  22.04), four distinct aromatic methines ( $\delta_{\text{H}}$  7.02 brs,  $\delta_{\text{C}}$  124.3 d; 7.56 brs, 121.1d; 7.31 d, 2Hz, 108.1 d and 6.63 d, 2Hz, 106.7) and two chelated phenolic hydroxyl groups ( $\delta_{\text{H}}$  12.06 s and 12.27 s). Comparison of these NMR data with those reported in the literature (Bachmann et al., 1979; Parmeswaran et al., 2004) revealed the compound to be known anthraquinone derivative Physcion (**FM-8**).



Physcion (**FM-8**)



**Fig. 2.15a**  $^1\text{H}$  NMR spectra of Physcion



**Fig 2.15b**  $^{13}\text{C}$  NMR spectra of Physcion

*v) Biological activity*

Terrein (**FM-3**) and Physcion (**FM-8**) are well known metabolites and known mainly for their antimicrobial and antioxidant activities respectively. **FM-7** on standing in pure form or in the solution would change to **FM-6** indicating it to be unstable. Therefore, only **FM-6** along with **FM-1** was evaluated for anticancer, and anti-inflammatory activities.

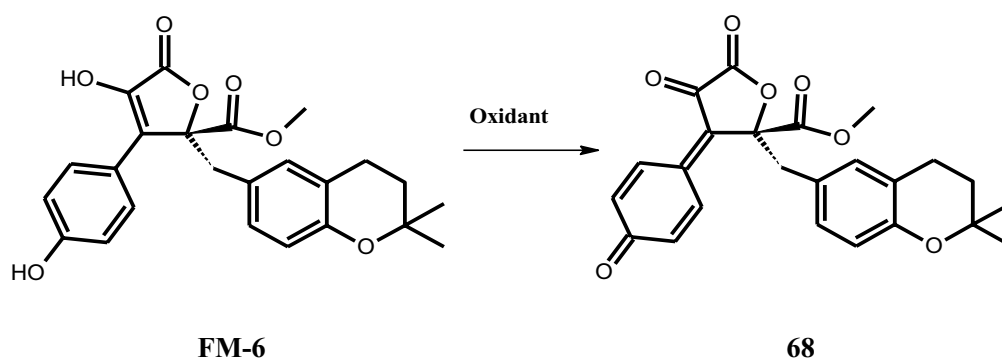
Butyrolactone I (**FM-1**) is a selective inhibitor of cyclin dependant kinases cdk1/cyclin B and cdk2/cyclin A (Fischer et al., 2000) and it is as potent as roscovitine, a drug currently undergoing phase IIb clinical evaluation ([www.cyclacel.com](http://www.cyclacel.com)). CDKs are involved in numerous diseases, among which are cancer, Alzheimer's disease, Parkinson's disease, stroke, diabetes, polycystic kidney disease, glomerulonephritis, inflammation, and AIDS (Knockaert, et al 2002.; Shapiro 2006). **FM-1** has been demonstrated to exhibit antiproliferative activity against colon and pancreatic carcinoma, human lung cancer (Nishio et al., 1996), and prostatic cancer cell lines (Suzuki et al., 1999).

In anticancer activity testing done by us, **FM-6** displayed inhibition against H460 (large cell lung cancer), ACHN (renal cell adenocarcinoma), Calu (epidermoid carcinoma), Panc1 (epithelioid carcinoma) and HCT116 (colorectal carcinoma) cell lines with  $\text{IC}_{50}$  88, 103, 147, 130, 121 $\mu\text{M}$  respectively. This activity was marginally better than observed for **FM-1**. It has been shown that elimination of hydroxyl groups



or changing them with other polar groups such as nitro, chloro, amino alkyl in **FM-1** does enhance the antiproliferative activity, but elimination of prenyl chain make the compound (**FM-2**) completely inactive (Brana et al., 2004). As the antiproliferative activity was maintained in the **FM-6** having prenyl chain, in the form of dimethyl pyran ring further provides the experimental proof for the conclusion derived by Brana et al (2004) that alkenyl chain (prenyl) is an essential component in maintaining antiproliferative activity.

There is a recent report on **FM-6** and its soybean lipoxygenase inhibitory activities. **FM-6** has been found to exhibit this activity with  $IC_{50}$  55.9  $\mu$ M. The inhibitory activity was much higher than that for 138.2  $\mu$ M, **FM-1**, 343.8  $\mu$ M for **FM-2** and 147.5  $\mu$ M for a standard compound kojic acid suggesting dimethyl pyran ring has role to play (Sugiyama et al 2010). The mechanism of lipoxygenase has been elucidated. **FM-6** is oxidized to a quinone methide (**68**) by loss of two hydrogens. It is difficult here to rationalize from the observed transformed product (**68**) scheme 2.7 as to why **FM-6** has higher activity than **FM-1** and **FM-2m**, as other two also would form similar products. From the observed activity trend it is apparent that **68** is more stable than the corresponding quinone methide of **FM-1** which in turn more stable than quinone methide of **FM-2**.



**Scheme 2.8: Lipoxygenase oxidation of FM-6**

### III. Experimentals

#### *i) General experimental procedures*

Mixture of methanol and chloroform (10:90, v/v) was used as mobile phase for TLC analysis. Compounds were visualized as intense rose colour spots on spraying with methanolic sulphuric acid (95:5, v/v) followed by heating at 120°C.

#### *ii) Fungal isolation, identification and cultivation*

Soft coral *Sinularia kavarattiensis* was collected by scuba diving at a depth of 8-10m from the coast of Mandapam, Tamil Nadu, India in May 2004. After washing the soft coral with sterile sea water, fungus *Aspergillus terreus* was isolated as an epiphyte using Czapek agar containing (g/l) NaNO<sub>3</sub> (2.0), MgSO<sub>4</sub>.7H<sub>2</sub>O (5.0) FeSO<sub>4</sub>.7H<sub>2</sub>O (0.1), KH<sub>2</sub>PO<sub>4</sub> (1.0), KCl (0.5), agar (3.0) sucrose (30.0) at pH 5.5 prepared in seawater supplemented with penicillin benzyl sodium salt (0.02) to avoid any bacterial growth. After 6-7 days sand brown, velvety, reverse the medium deep dirty brown colonies were observed. The strain was identified as *Aspergillus terreus* from the morphological features of conidiophores (by Dr. Sanjay K. Singh, mycologist, Agharkar Research Institute, Pune). Strain has a voucher specimen of the fungus is deposited at National Institute of Oceanography, Dona Paula, Goa, India. Stock cultures of the fungus, maintained at -20°C preserved with 20% glycerol was used to inoculate 500 ml of seed medium in Erlenmeyer flask (4 L) containing 24 g/l of potato dextrose broth in seawater. It was then cultured at 27±2°C on a rotary shaker at 200 rpm. The flask was incubated for 72 hr and used as a first stage inoculum. The same medium (1 L) was made in 10 Erlenmeyer flasks (4 L) and inoculated with 5% of first stage inoculum. The flasks were incubated for 21 days at 27±2°C on a rotary shaker at 200 rpm for 10 hr/day.

#### *iii) Extraction and isolation of metabolites*

21 days old fermentation broth (10 L) was separated from fungal mat and concentrated to a volume of 1 L under reduced pressure. The broth was extracted first with chloroform (200 ml X 4) followed by ethyl acetate (200 ml X 4). Chloroform

and ethyl acetate layers were separately concentrated under reduced pressure to yield chloroform extract (470 mg) and ethyl acetate extract (430 mg). The ethyl acetate extract was chromatographed over Sephadex LH-20 using MeOH-CHCl<sub>3</sub> (1:1) to yield pure crystalline compound, **FM-3** (92 mg) (Dunn et al., 1975) and a fraction containing an intense rose colour spot. The fraction containing intense rose colour spot was flash chromatographed over silica gel using gradient elution of MeOH-CHCl<sub>3</sub> (5:95 - 20:80) to yield **FM-7** (7.2 mg; R<sub>f</sub>, 0.51). The chloroform extract was repeatedly chromatographed over Sephadex LH-20 using MeOH-CHCl<sub>3</sub> (1:1) and 100% MeOH, which yielded a pure yellow coloured compound, **FM-8** (17 mg) Other fractions giving two prominent rose colour spots on TLC was further purified over silica gel column using gradient elution of MeOH-CHCl<sub>3</sub> (0:100- 20:80) to afford **FM-1** (32.0 mg; R<sub>f</sub>, 0.64) and another nearly pure compound which was fine purified over Sephadex LH-20 using MeOH/CHCl<sub>3</sub>/Pet. ether (40 :40 :20) to yield **FM-6** (26.3 mg; R<sub>f</sub>, 0.81).

*iv) Aspernolide A (FM-6)*

White sticky solid;  $[\alpha]_D^{28} +88.73$  (c 0.58, CHCl<sub>3</sub>); UV (MeOH)  $\lambda_{\max}$  nm : 303, 240; IR (NaCl)  $\nu_{\max}$  cm<sup>-1</sup> 3340, 3024, 2970, 2935, 1737, 1732, 1610, 1519, 1498, 1436, 1386, 1261, 1182, 1122, 1068, 1037, 948, 839, 754; For NMR data see Table 1; HRESITOFMS  $[M+Na]^+$   $m/z$  447.1433 (calcd. 447.1420 for C<sub>24</sub>H<sub>24</sub>O<sub>7</sub>Na),  $[2M+Na]^+$   $m/z$  871.2959 (calcd. 871.2942 for C<sub>48</sub>H<sub>48</sub>O<sub>14</sub>Na); EIMS  $m/z$ (%): M<sup>+</sup> 424(2.7), 380(37.8), 348(35.1), 320(6.8), 293(13.5), 265(8.1), 237(10.8), 218(5.4), 205(6.8) 189(6.8), 175(100), 157(8.1), 145(10.8), 131(16.2), 119(13.5), 107(5.4), 91(24.3), 77(3.5), 69(12.1), 44(37.8).

*v) Aspernolide B (FM-7)*

Light brown syrup;  $[\alpha]_D^{28} +48.27$  (c 0.29, MeOH); UV (MeOH)  $\lambda_{\max}$  nm : 303, 240; IR (NaCl)  $\nu_{\max}$  cm<sup>-1</sup> 3380, 3024, 2975, 2933, 1745, 1610, 1519, 1442, 1386, 1182, 1070, 1037, 838, 762; For <sup>1</sup>H and <sup>13</sup>C NMR spectroscopic data see Table 2; HRESITOFMS:  $[M+H]^+$   $m/z$  443.1699 (calcd. 443.1706 for C<sub>24</sub>H<sub>27</sub>O<sub>8</sub>),  $[M+Na]^+$   $m/z$  465.1516 (calcd. 465.1525 for C<sub>24</sub>H<sub>26</sub>O<sub>8</sub>Na); EIMS  $m/z$ (%):  $[M-CO_2]^+$  398(13),

380(70), 348(100), 333(18), 320(15), 293(40), 205(26), 249(10), 237(23), 218(17), 205(25), 188(16), 175(77), 145(13), 131(41), 119(23), 107(15), 91(35), 77(17), 69(20), 59(33), 43(23), 41(11).

*vi) physcion (FM-8)*

Yellow crystalline needles (EtOAc) mp 202-204 °C; UV (MeOH)  $\lambda_{\max}$  224, 255, 265, 287, 435 nm; IR (KBr)  $\nu_{\max}$  2940-3040, 2840, 1680, 1629, 1614, 1570, 1486, 1390, 1368, 1306, 1262, 1105, 1100, 1034, 992 cm<sup>-1</sup>; <sup>1</sup>H NMR (CDCl<sub>3</sub>)  $\delta_{\text{H}}$  2.39 (s, C-3-CH<sub>3</sub>), 3.90 (s, C-6-OCH<sub>3</sub>), 6.63 (d,  $J = 2.0$  Hz, H-7), 7.02 (brs H-2) 7.31 (d,  $J = 2.0$  Hz, H-5), 7.55 (brs, H-4), 12.06 (s, C-1-OH), 12.26 (s, C-8-OH); EI-MS, M<sup>+</sup> 284, 255 (M - CO - H), 241 (M - CO - CH<sub>3</sub> - CH<sub>2</sub>O), 185 (M - 3CO - CH<sub>3</sub>).

*v) Conversion of butyrolactone I (FM-1) to aspernolides A (FM-6), B (FM-7) and C (67)*

**FM-1** (76.9 mg) was dissolved in MeOH (10 ml) containing conc. HCl (0.2 ml). Mixture was stirred at r.t. approximately for 2 hr. or till complete conversion of **FM-1** as indicated from TLC. Solvent was removed under vacuum and resulting residue was separated on flash Si-gel column using gradient elution of MeOH/CHCl<sub>3</sub> (0:100 – 20:80, v/v) to yield in the order of increasing polarity **FM-6** (58.6 mg, 75 %), **57** (8.8 mg, 10.4%) and **FM-7** (6.9 mg, 8.3%).

*vi) Aspernolide C (57)*

Light brown syrup; <sup>1</sup>H and <sup>13</sup>C NMR spectroscopic data see Table 2; HRESITOFMS: [M+H]<sup>+</sup>  $m/z$  457.1851 (calcd. 457.1862 for C<sub>25</sub>H<sub>29</sub>O<sub>8</sub>) and [M+Na]<sup>+</sup>  $m/z$  479.1669 (calcd. 479.1682 for C<sub>25</sub>H<sub>28</sub>O<sub>8</sub>Na).

## **Section 2.3: Antifungal secalonic acid D from the marine sponge associated fungus *Aspergillus aculeatus***

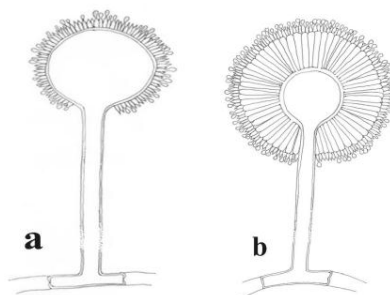
### **I. Introduction**

Marine animals, especially sponges, have been demonstrated to represent a rich source of fungal diversity (Proksch et al. 2008; Proksch et al. 2010). Of the total new metabolites discovered from marine derived fungi, 19% have been obtained from sponge associated fungi, which is second only to metabolites from fungi associated marine algae (Raftab and Ebel, 2011). Sponges are filter feeders; therefore the origin of fungal endophytes and the true nature of their interaction with their hosts remain largely unclear. The fact that many fungi so far isolated from sponges are well known from terrestrial habitats where they ubiquitously occur might suggest that fungal spores which are washed into the sea are sequestered by sponges through filter feeding and are retained in the inhalant canals from which they can be isolated and subsequently cultivated. Furthermore, there has been no evidence reported so far indicating that fungi actively grow inside sponges suggesting that their presence in these invertebrates is in fact limited to spores. On the other hand, compounds obtained from marine isolates of ubiquitously occurring fungal genera such as *Aspergillus* or *Penicillium* differ often significantly from those of terrestrial strains (Edrada et al., 2002; Lin et al., 2003) indicating that at least on a biochemical level many sponge derived fungi are distinguished from their terrestrial counterparts.

In an investigation on an endophytic fungus *Asprgillus aculateus* from a marine sponge led to the identification of secalonic acid D to be a potent antifungal against *Candida albicans*. The same along with short review on metabolites of *A. aculateus* and secalonic acids and its biosynthesis is presented in this section.

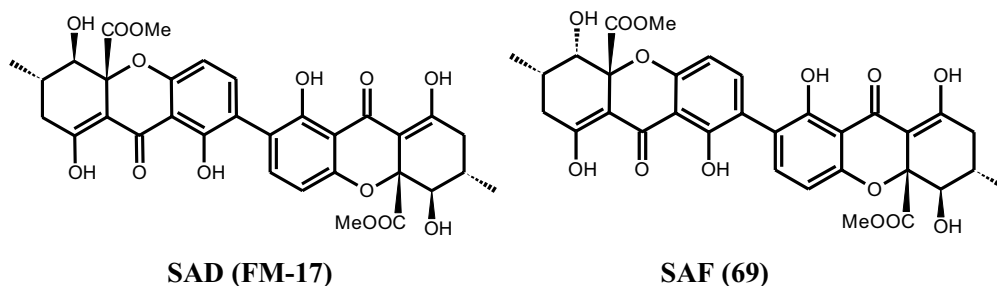
## II. Metabolites from *Aspergillus aculeatus*-literature review

*Aspergillus aculeatus* is member of *Aspergillus* section *Nigri* (formerly *A. niger* group). These fungi develop black colonies and therefore also called black *Aspergilli*. The morphological differences between the species are very subtle and many of them are sometime incorrectly identified as *Aspergillus niger*. Although, cladification of *Aspergillus* section *nigri* using the  $\beta$ -tubulin and calmodulin genes have been shown that three clades could be distinguished, it can be preliminary distinguished into two varieties by the uniseriate (single palisade of cells which give rise to conidia) or biseriate (two palisades of cells) structures of the conidiophores (**Fig 2.16**) the colour of the colony and the conidial heads, and the shape and ornamentation of the conidia (Abarca et al 2004). *A. aculeatus* which is very similar in morphology as well as on molecular level with *Aspergillus japonicus* are uniseriate species. Recently new species *Aspergillus aculeatinus* and *Aspergillus uvarum* two uniseriate black *Aspergillus* species have been described. (Samson et al 2007).

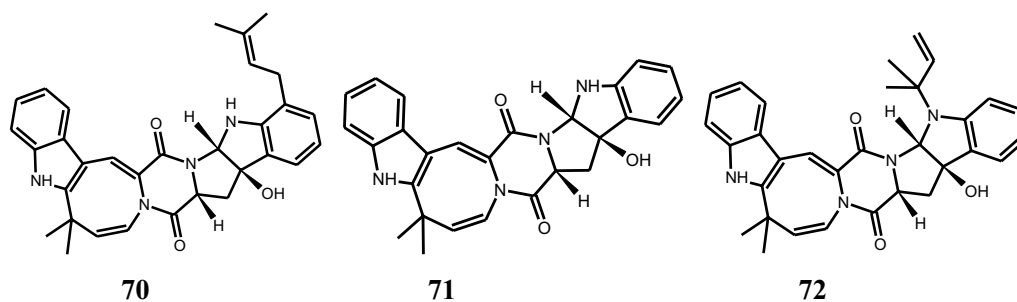


**Fig 2.16: Microscopic view of Black aspergilli conidiophores a uniseriate species; b biseriate species**

Only four reports have appeared so far on new metabolites of *A. aculeatus*, of which two are on marine derived strains. The first two metabolites to be unveiled from this fungus were secalonic acids D (SAD) (**FM-16**) and F (SAF) (**69**) (Andersen et al 1977). SAD was previously known, while SAF was new member of its group. SAD proved to be stronger antimicrobial than SAF against *Bacillus magaterium*.

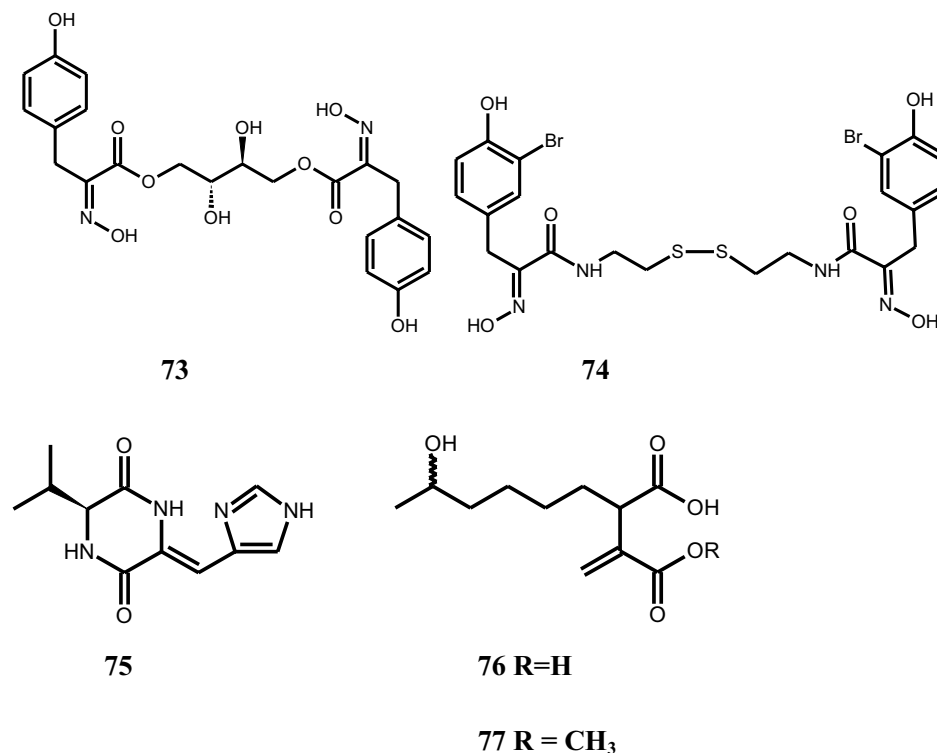


A soil derived isolate of *A. aculeatus* when cultured on okara (the water-insoluble residue of whole soybean) produced two new metabolites okaramines H (**70**) and I (**71**) along with known, okaramines A (**72**) and B (Hayashi et al., 1998). The known metabolite **72** showed much better insecticidal activity than its new congeners **70** and **71**. Okaramines A (**72**) B, D, E, F, and G were known from a strain of *Penicillium simplicissimum* ATCC 90288.



The Chemical exploration of the fungus *A. aculeatus* CRI323-04 derived from marine sponge *Xestospongia testudinaria* led to the isolation of one new and one known tyrosine-derived metabolites aspergillusol A (**73**) and methyl ester of 4-hydroxyphenylpyruvic acid oxime (Ingavat et al 2009). Aspergillusol A (**73**), has structure similar to that of a brominated tyrosine-derived metabolite, psammaphin A (**74**), a metabolite found in different types marine sponges. This is only instant wherein metabolites isolated from fungus derived from hydroxyphenylpyruvic acid have an oxime moiety in it resembling **74**. An investigation by same group researcher on metabolites from another marine sponge, *Stylissa flabelliformis* derived fungal strain of *A. aculeatus* resulted in isolation of three new metabolites, pre-aurantiamine (**75**), (-)-9-hydroxyhexylitaconic acid (**76**) and (-)-9-hydroxyhexylitaconicacid-4-

methyl ester (**77**), together with two known compounds, paraherquamide E and secalonic acid D (**FM-16**) (Antia et al., 2011).

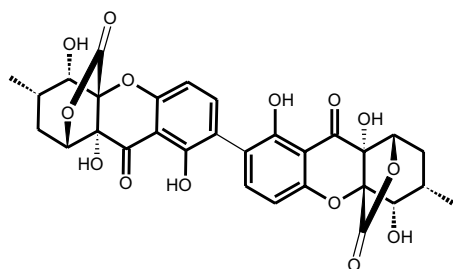


### III. Secalonic acids and their biosynthesis

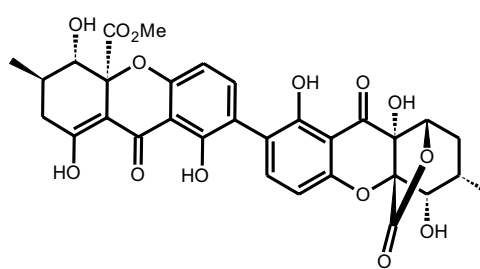
The secalonic acids along with ergoflavins [e.g. ergoflavin (**78**)] and ergochrysin [e.g. ergochrysin A (**79**)] are members of the ergochrome family. They represent important group of biologically highly active mycotoxins, produced by fungi and also from other microorganisms and plants (Franck, 1980). So far at least twenty-two members of the ergochrome family are known as natural products. They are dimers of six different monoxanthenes [hemisecalonic acids A–F (HAS A-F)], and ergochrome diversity is attributable to different homo- and heterodimers of these six monomeric units (Franck, 1980). The first of these type fungal metabolites, named ergoflavins, were isolated in pure form from the fungus *Claviceps purpurea* (ergot) in 1958 (Eglinton et al.). Secalonic acids have 2,2'-linkage, but secalonic acids A-D were incorrectly assigned to have 4,4'-linkage, eventually rectified by Hopper et al., (1971) to have 2,2'-linkage from transformational studies and their NMR data. The 4,2'-linkage and 4,4'-linkage in ergochrome dimers have also been reported. Isoergochrysin (**80**), and another class of dimers, the eumitrins are coupled through the 4,2'



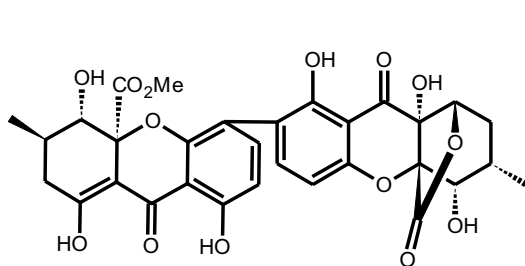
positions (Yang et al., 1973), while phomoxanthone A (**81**), a dimer isolated from *Phomopsis* sp. is coupled through 4,4'-position (Isaka et al., 2001).



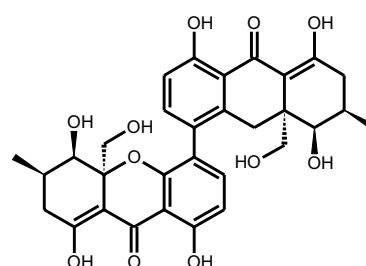
**Ergoflavin (78)**



**Ergochrysin A (79)**



**Isoergochrysin (80)**

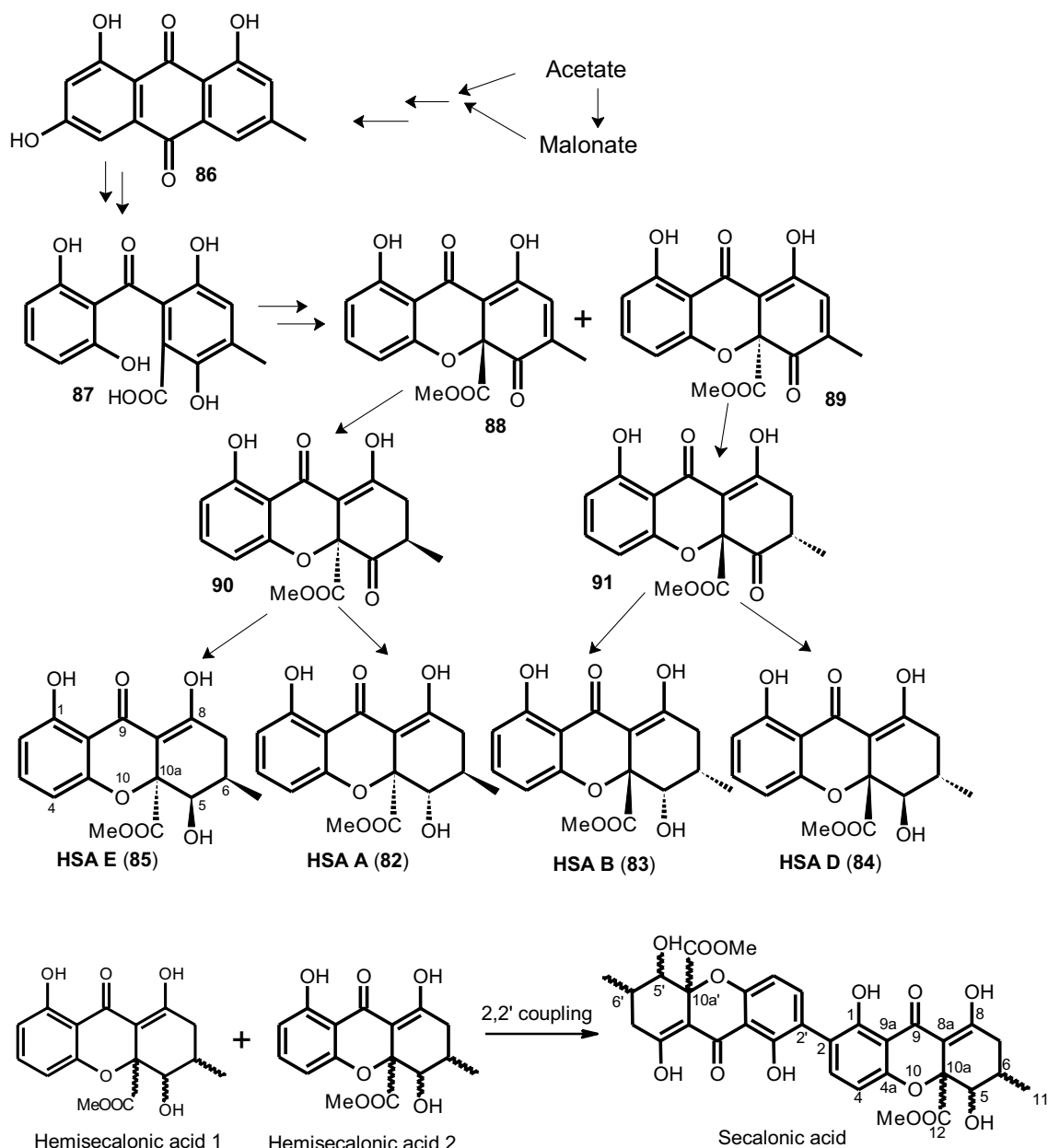


**Phomoxanthone (81)**

Secalonic acid have six chiral centres (three in each monomer) at 5, 6 and 10a in one half and 5', 6' and 10a' in another half of the molecule. Theoretically, large number of stereoisomers is possible for the secalonic acids, but the number is restricted to only ten isomers considering the specificity of the biosynthetic pathway, which can give only four stereoisomeric monomers of tetrahydroxanthones [hemisecalonic acids A (**82**), B (**83**), D (**84**) and E (**85**)]

The biosynthesis of the secalonic acid from emodin has been postulated (**Scheme 2.9**) (Kurobane and Vining, 1979). It is assumed that the emodin (**86**), which is a co metabolite in the culture producing secalonic acids is a intermediate precursor to these secalonic acids. Emodin (**86**) is first reduced at C-3 and oxidized at C-5, which is followed by Bayer-Villager type oxidation through C5-C10a bond giving rise to benzophenone carboxylic acid (**87**), which is methyl esterified, cyclized and reduced at C6-C7 double bond and C-5 carbonyl to HSAs. It is the final three steps involving a cyclization step and two reduction steps that govern the stereochemistry of **82-84** and thus stereochemistry of secalonic acids. The cyclization step followed by

reduction steps in principle should give rise to eight tetrahydroxanthones (HSAs), but the fact that no ergochromes with *cis* stereochemistry of substituents at C-6 and C-10a have been so far reported, restricts the formation of HSAs to only four. This implies that the reduction of C6-C7 double bond is catalysed by specific enzyme and configuration of C-10a directs the stereochemistry of reduction.



The different combinations (2,2'-linkage) of these four HSAs thus can give rise to only ten secalonic acids A-J. The self coupling of HSAs generates SAA (secalonic acid A), SAB, SAC and SAD, while cross coupling should give six hybrid secalonic acids of which four SAB (AB, coupling of hemisecalonic acid A and B), SAF (BD), SAG (AE), SAH (DE) should be chiral due to coupling of diastereomeric monomers, while two SAI (AD) and SAJ (BE) will be meso due to coupling of enantiomeric monomers.

Although four HSAs (**82-85**) can be formed during cyclization and reduction of benzophenone carboxylic ester intermediate, any organism that produces secalonic acid appears to make only two HSAs. The pair of HSAs produced either differ by position at C-5 or at the trans substituted C-6 and C10a. The pair of HSAs composing secalonic acids in fungus *Penicillium terrestris* differs only at C-5 which produces SAA, SAE and SAG. Same is also true in fungus *A. aculeatus* which produces SAB, SAD and SAF. These observations suggest that the cyclization of benzophenone methyl ester is very specific in these fungi giving only one isomer, while the reduction of double bond is specific in generating trans stereochemistry at C-6 and C10a the reduction of C-5 carbonyl is not specific leading to two HSAs (HSAs A and E in *P. terrestris*, whereas A and E in *A. aculeatus*) having opposite configuration at C-5.

On the other hand, fungus *Claviceps purpurea* produces SAA, SAB and SAC, whose pair of monomers had a same configuration C-5, but differed by having opposite configurations at trans substituted C-6 and C-10. This observation indicates that in this organism cyclization is not specific and produces both the enantiomers, but both reduction steps are specific giving rise to only two HSAs A (**82**) and B (**83**). A similar case can be formation of HSAs D (**84**) and E (**85**), which should lead to the formation of SAD, SAE and SAH, but likewise no reports have appeared yet.

The dimerization of HSAs is suggested to be the last step in the biosynthesis. The absence of any dimeric product in which two halves of the molecules are at different oxidation levels argues in favour of this.

In conclusion, the stereochemistry in the biosynthesis of secalonic acids is governed by three steps (a cyclization and two reductions) and the reduction of C6-C7 double is always specific. Of the other two steps at least one or both might be specific. Therefore, it appears any organism that will produce HSA A or B will not produce its enantiomer D or E respectively. The consequence is that it further reduces the number of secalonic acids that can be obtained naturally to eight as the meso secalonic acids SAI (AD) and SAJ (BE) cannot be formed.

#### **IV. Results and Discussion**

##### *i) Biological materials*

Marine sponge derived fungi represent a huge resource for discovery novel chemicals. South east coast of India harbours rich diversity of marine sponges. A marine sponge *Cinachyra cavernosa* was collected by scuba diving at a depth of 8 to 10 m from Mandapam, Tamil Nadu, India. Out of several fungi isolated from this sponge only one was identified initially as *Aspergillus* species. Initially it was identified to be *Aspergillus niger* (section nigri, also called black Aspergilli) from its morphological features. As our interest was to study metabolites from *Aspergillus* fungi, we took this fungus for chemical investigation, which yielded secalonic acids. The literature survey revealed *A. niger* is not known for production of secalonic acid but other species *A. aculeatus* and *A. aculeatinus* belonging to same section nigri are known to produce secalonic acids. Therefore, we got the phylogentic analysis done on this strain using molecular techniques, and as to our expectation the fungus was identified as *A. aculeatus*.

##### *ii) Purification of metabolites*

The organic constituents were adsorbed on HP 20 diaon resin from the culture medium and the resin was washed with MeOH and MeOH washings were dried to obtain yellow powder. This yellow powder on filtration through Sephadex LH20 (GPC) was collected in 45 fractions. Fractions 17-19 (**FM-16**) showed long yellow

bands in visible light on TLC (**Fig 2.17**), but at the centre of this band it had a bright spot visible in UV at 254 nm.



**Fig 2.17: TLC of chromatographic fractions of extract of *A. aculeatus***

Fractions 17-20 were pooled together and upon evaporation of solvent gave bright yellow solid (**FM-16**).

### *iii) Structure elucidation of FM-16*

**FM-16** was obtained as bright yellow solid  $[\alpha]_D^{28} = 0$  in  $\text{CHCl}_3$  or in MeOH. The molecular formula of the **FM-16** was determined to be  $\text{C}_{32}\text{H}_{30}\text{O}_{14}$  from its ESIMS (**Fig 2.18a**), which displayed a pseudomolecular ions at  $[\text{M}+\text{H}]^+$  at  $m/z$  639 and  $[\text{M}+\text{Na}]^+$  at  $m/z$  661. Its IR spectra (**Fig 2.18b**) showed absorption band at  $3552\text{ cm}^{-1}$  for hydroxyl. The  $^1\text{H}$  NMR spectra (**Fig 2.18c**) of **FM-16** showed every set of signal in duplicates. It showed signals for two methyl doublets  $\delta_{\text{H}}$  1.5 ( $J = 6\text{ Hz}$ ) and 1.7 ( $J = 6\text{ Hz}$ ); a set of signals in the region  $\delta_{\text{H}}$  2.25-2.77 Hz corresponding to pairs of methine, methylenes and hydroxyl; a signal at  $\delta_{\text{H}}$  3.73 (s) corresponding a pair of methoxyls; pair of hydroxymethines, a doublets at  $\delta_{\text{H}}$  4.02 ( $J = 10.4$ ) and broad singlet at  $\delta_{\text{H}}$  4.12; two pairs of aromatic ortho hydrogen doublets ( $J = 8.4\text{ Hz}$ ) at  $\delta_{\text{H}}$  6.56 and 7.42 and  $\delta_{\text{H}}$  6.62 and 7.44; and two chelated hydrogens at  $\delta_{\text{H}}$  11.74 and 1.88 Hz and another pair of chelated hydrogens at  $\delta_{\text{H}}$  13.77 and 13.96. As in  $^1\text{H}$  NMR in  $^{13}\text{C}$  NMR (**Fig 2.18d**) also signals were displayed in pairs. These NMR data accounts for all the 34 hydrogens in the compound and the signals obtained in pairs is suggestive of dimeric molecule like secalonic acid having one half of the molecule stereochemically different from the other half. If the two halves of the molecule are stereochemically different than the compound has to be optically active, which

contradicts with our earlier observation of optically inactivity of the compound, which suggest molecule to be achiral.

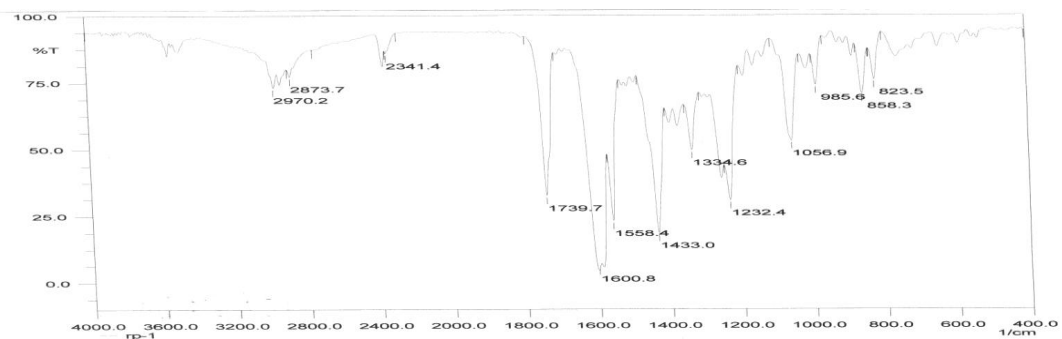


Fig 2.18b:  $^1\text{H}$  NMR spectra of FM-16

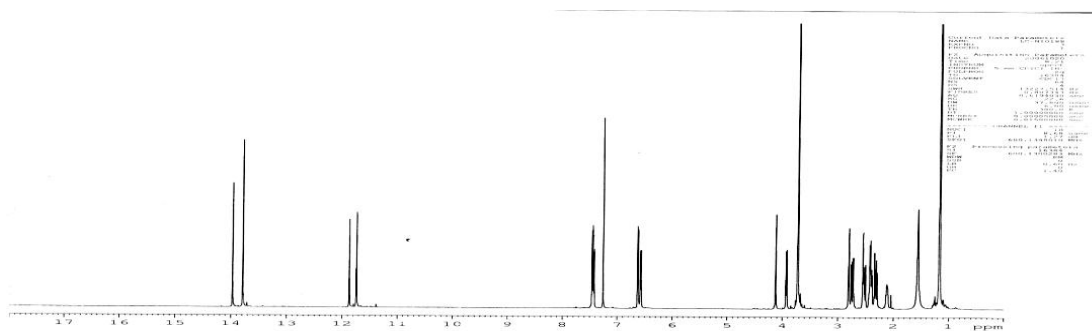


Fig 2.18c:  $^1\text{H}$  NMR spectra of FM-16

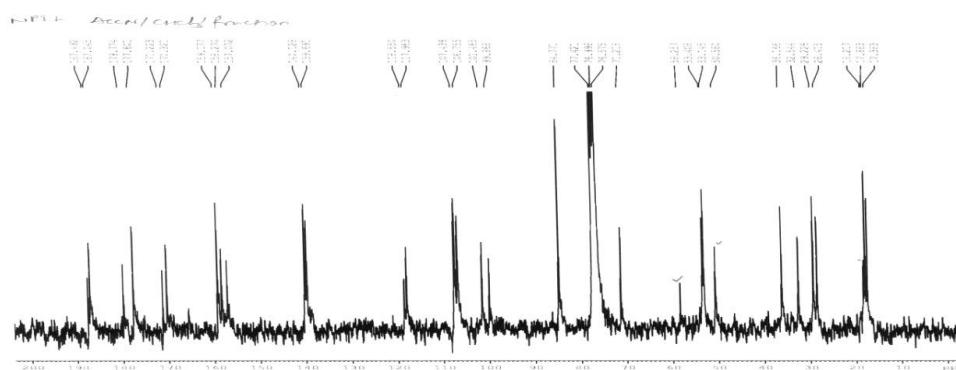


Fig 2.18d:  $^1\text{H}$  NMR spectra of FM-16

As discussed in **Part B** that in nature only eight types of secalonic acids can be formed by combinations of four monomeric HSAs, which are stereochemical variants

of each other. As per the spectroscopic data the **FM-16** has to be one or mixture of two secalonic acids from the possible ten.

The HSA A (**72**) is enantiomer of HSA D (**74**), while HSA B (**73**) is enantiomer of HSA E (**75**), hence SAA, SAB, SAC and SAF are indistinguishable from their enantiomers SAD, SAE, SAH and SAG respectively from their NMR data. While, the homo dimers SAA, SAB, SAD and SAE gives NMR signals for only one half of the molecule, the heterodimers SAC (AB), SAF (BD) and SAG (AE) gives separate signals for both the halves. The remaining three heterodimers, which are not reported yet, the two *meso* SAI (AD) and SAJ (BE) are expected to give signals for only one half, and the third one SAH (DE), which is chiral should give different signals for the monomeric units.

From the optical inactivity and NMR data for the compound (**FM-16**) under investigation following three cases can be considered.

1. **FM-16** can be mixture of two meso isomers SAI (AD) or SAJ (BE)
2. **FM-16** is a heterodimer SAC (AB) or SAH (DE)
3. **FM-16** might be mixture diastereomers SAA with SAB or SAD with SAE

Case 1. No *meso* secalonic acids have been yet reported as natural product, which suggests that any culture that produces the hemisecalonic acid A do not produces its enantiomer hemisecalonic acid D and culture that produces hemisecalonic acid B do not produces its enantiomer, hemisecalonic acid E. Therefore, it has been observed so far that SAA or SAB has never been isolated from the culture producing their enantiomer SAD or SAE respectively and vice versa. This goes to suggest that it is very unlikely even one of the *meso* secalonic acids will be obtained as natural product. Therefore the possibility of the **FM-16** as mixture of two meso secalonic acid is ruled out.

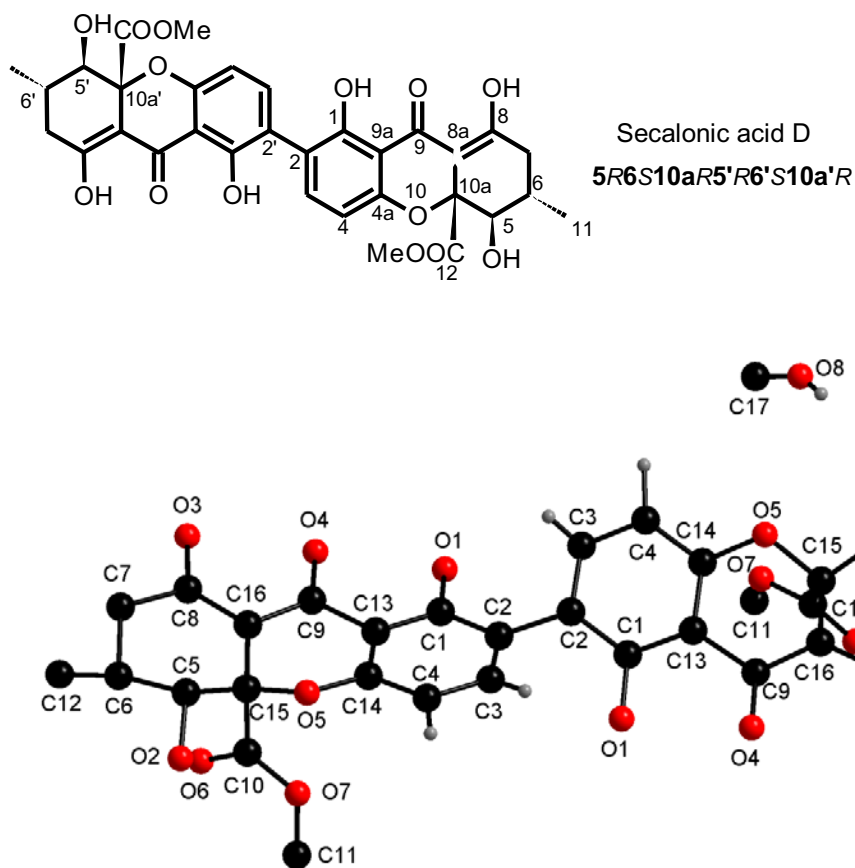
Case 2. SAC or SAH being heterodimers will give different NMR signals for both the half of the molecule. About optical rotation, SAC, has low optical rotation and SAH

should also have low rotation, the dimers made of monomeric units of opposite rotation should have a low optical rotation and, as we had low amount of the **FM-16** (~12 mg) the optical rotation recorded at very low concentration might not have been sensitive to the instrument we used.

Case 3. Another possibility is that the **FM-16** is mixture of two diastereomers such as SAA with SAB or SAD with SAE. These pairs diastereomers nearly have or expected to have same optical rotations and opposite sign as result their mixture in certain proportion would show very low or no optical rotation. Their NMR spectra would also show two sets of signals. As discussed in the biosynthesis of secalonic acids any organism to produce triplet of diastereomeric secalonic acids SAA, SAB and SAC or SAD, SAE and SAH the cyclization of benzophenone benzoic acid ester (**87**) should not be specific. Whereas the production of SAA, SAB and their hybrid dimer SAC has been observed in *C. purpurea* the production of triplet of secalonic acids SAD, SAE and SAH has not been observed in organisms so far. In contrary, previous study on *A. aculeatus* indicates that cyclization of benzophenone benzoic acid ester is specific and therefore cannot produce the triplet of secalonic acids SAD, SAE and SAH but known to produce triplet of SAD SAB and SAF indication the reduction of carbonyl step is no-specific.

So, to put an end to this ambiguity we crystallized the **FM-16** from a mixture of MeOH-CHCl<sub>3</sub> to obtain crystals for X-ray diffraction studies. On single X-ray diffraction studies on the sample it was revealed that the compound analyzed was SAD (**FM-17**). **Fig 2.19** shows the thermal ellipsoid representation of **FM-17**. The unit cell parameters of  $a = 17.647\text{\AA}$ ,  $b = 7.165\text{\AA}$ ,  $c = 13.127\text{\AA}$ ,  $\alpha = \gamma = 90^\circ$ , and  $\beta = 93.720$  were in agreement with the reported for SAD (Barnes et al., 1999). So, it was assumed that during crystallization fractional crystallization between the two isomers had taken place leading to isolation of crystals of SAD.





**Fig 2.19: Thermal ellipsoid representation of SAD**

The little crystalline sample of **FM-17** which was available with us, we recorded NMR on it, and to our expectations one set of signals were observed with larger intensity matching for SAD in its NMR spectra (**Fig 2.20a** and **2.20b**). The other set of signals were observed in very low intensity, indicating the crystallized sample still had the other isomer in minor amount in it, which we expect it to be SAE on the basis of optical inactivity of the mixture. Surprisingly the optical activity data taken on **FM-17** was still nearly zero. Therefore it cannot be conclusively stated, whether other isomer is SAE or SAB. The NMR data of SAD (**FM-17**) obtained on crystallized sample and NMR data of other stereoisomer obtained on diastomeric mixture with SAD (**FM-16**) are tabulated below (**Table 2.6**)

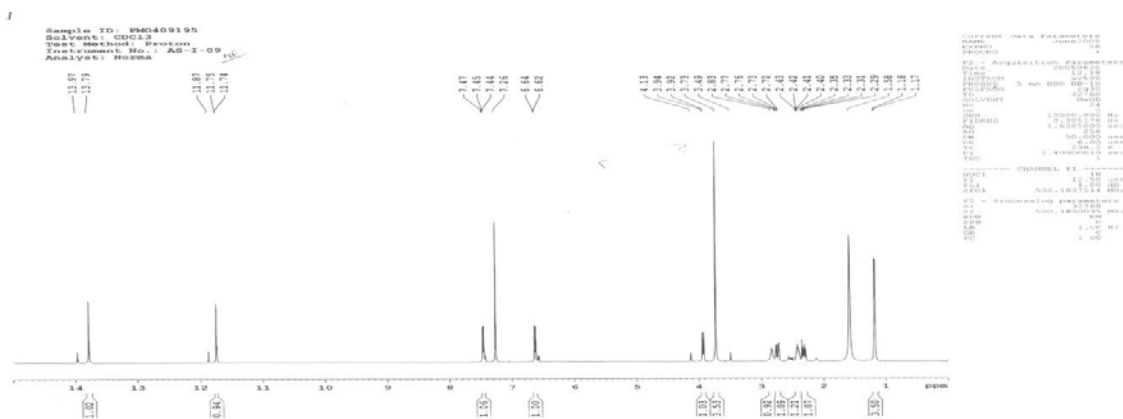


Fig 2.20a: <sup>1</sup>H NMR spectra of FM-17

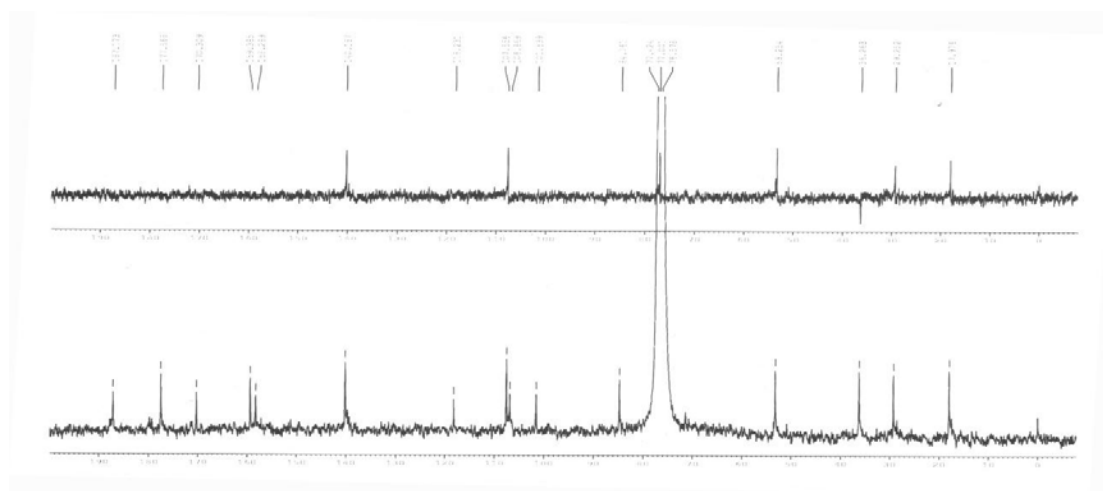


Fig 2.20b: <sup>13</sup>C NMR spectra of FM-17

Table 2.6: NMR data of FM-17 in CDCl<sub>3</sub>

Position	$\delta_H$ , mult., J(Hz)	$\delta_C$ , mult.	Position	$\delta_H$ , mult., J(Hz)	$\delta_C$ , mult.
1		158.3 s	11	1.17, d, 6	18.0 q
2		118.2 s	12		170.3
3	7.45, d, 8.4	140.2 d	13	3.73, s	53.2 q
4	6.20, d, 8.4	107.5 d	4a		159.4
5	3.91, d, 10.8	77.0 d	8a		101.5
6	2.24, m	28.4 d	9a		106.8
7	2.74, dd, 18.4, 5.4 2.31 dd, 18.4, 10.5	32.6 t	10a		84.7
8		177.5 s	1-OH	13.79	
9		187.1 s	8-OH	11.75, s	

#### iv) Antifungal activity

The strong antifungal activity of the extract against a human pathogen *Candida albicans* had prompted us to investigate its active principles. The purified metabolite **FM-17** also showed strong antifungal activity and therefore its minimum inhibition concentration on disc diffusion assay was determined. **FM-17** showed inhibition zone of 6 mm at 25 µg/disc and at conc. from 25 - 3µg/disc it consistently showed inhibition zone between 4-5 mm (**Fig 2.21**) while it showed inhibition zone of 2-3 mm at concentration as low as 0.5 µg/disc. A standard compound, flucanazole, a drug presently in the market showed inhibition zone of 2 mm at 25 µg/disc and at any concentration below this it was inactive (**Table 2.6**).

**Table 2.6: Antifungal activity of SAD and Flucanazole (std) against *Candida albicans***

Concentration(µg/disc)	Flucanazole (Std) Inhibition zone in mm	Secolanic Acid D Inhibition zone in mm
25	2	6
20	-	5
15	-	4-5
10	-	4-5
5	-	4-5
4	-	4-5
3	-	4
2	-	2-3
1	-	2-3
0.5	-	2-3



**Fig 2.21: Antifungal (*C. albicans*) assay plate at concentrations 4, 5, 10 and 15 µg/disc**

## V. Experimentals

### *i) Fungal isolation, identification and cultivation*

Marine sponge *Cinachyra cavernosa* was collected by scuba diving at a depth of 8 to 10 m from Mandapam, Tamil Nadu. The fungus *Aspergillus aculateus* was isolated as an endophyte using standard isolation techniques on PDA. It was identified as *A. niger* from its morphological characteristics (Dr. Sanjay Singh, ARI, Pune) and *A. aculateus* by molecular techniques. The pure isolate was inoculated from plates into flasks containing PDB (24g/l) in sea water (2 L x 3). The flasks were incubated at room temperature for 20 days on a rotary shaker at 200 rpm.

### *ii) Extraction and isolation of metabolites*

HP-20 diaon resin (250 g) column was prepared in distilled water. 20 days old fermentation broth (6 L) was filtered through cotton wool to remove mycelium and was passed through HP-20 diaon. Column was washed first with water (2 L) till the effluent was colourless and then with methanol (1 L). Methanol fraction was collected and evaporated under vacuum to yield yellow powder (0.785g). This bright yellow powder was loaded onto Sephadex LH 20 column and eluted with MeOH-CHCl<sub>3</sub> (1:1) and collected in 25 fractions. Fractions 16-22 were collected and evaporated to bright yellow solid (**FM-16**) (17 mg). This solid was recrystallized from CHCl<sub>3</sub>-MeOH to give thin yellow needles of secalonic acid D (**FM-17**).

### *iii) Secalonic acid D (FM-17)*

Yellow crystalline needles;  $[\alpha]_D^{28}$  0 (c 0.140, MeOH or CHCl<sub>3</sub>); HRESITOFMS:  $[M+H]^+$   $m/z$  639.1753; IR (KBr)  $\nu_{max}$  3552, 2968, 2931, 2873, 1739, 1604 1589 1433, 1232, 1058; <sup>1</sup>H NMR and <sup>13</sup>C NMR (500 MHz, CD<sub>3</sub>OD) see **Table 2.6**

### *iv) Antifungal activity*

Antifungal activity was using standard disk diffusion assay in which first an inoculum of pathogen was spread over sterile nutrient broth agar plates. In the center of the

plate a paper disk impregnated with the compound/extract was placed. The plates were incubated for 2-3 days at rt and inhibition zone was measured.

## Section 2.4 Comparison and Identification of metabolites from the fungi *Aspergillus flavipes* and *Aspergillus terreus* using tandem mass spectrometry

### I. Introduction

The chemical investigation of the marine-derived fungi *Aspergillus flavipes* (sections 2.1) and *Aspergillus terreus* (sections 2.2) led to the isolation of butyrolactone I (**FM-1**) and its derivatives, some of these were common to both the fungi. Additionally, terrein (**FM-3**), was a common metabolite of both the fungi, while a cyclopentandiol (**FM-4**) though reported earlier from *A. terreus* (Ghisalberti et al., 1990) was isolated only from *A. flavipes*. *A. terreus* is known to biosynthesize butenolides (hydroxyphenyl pyruvate dimers) through aryl pyruvic acids derived from amino acids. The same precursor with a different coupling pathway leads to other type of dimers called aspulvinones (Nobutoshi et al., 1973) whereas the biosynthetic pathway of aspulvinone, when followed for a pyruvate derived via tryptophan leads to asterriquinones (Yamamoto et al., 1976). Several **FM-1** derivatives have been reported from *A. terreus* which include sulfated derivatives and derivatives carrying a change on C5 unit.

Morphologically, the young cultures of both the fungi appears identical, but by molecular techniques it has been shown that *A. flavipes* unlike *A. terreus* do not contain pksM (6-methylsalicylic acid synthase) gene (Pazoutova et al., 1999). Although, there exist reports of isolation of metabolites like flavipin (Raistrick and Rudman, 1956) and lovastatin (Alberts et al., 1980) from both these fungi, which supports the closeness of these fungi, no **FM-1** derivatives or cyclopentanedioles (**FM-4** and **FM-5**) have been reported from *A. flavipes* prior to our study (section 2.1). A section of the present thesis uses tandem mass spectrometry (MS/MS) approach and molecular biology tools to study differences and similarities in two cultures. The following studies have been incorporated in this section.

1. Identification of new and known metabolites in *A. terreus* and *A. flavipes* using ESI-MS/MS and
2. Study of taxonomic relation between the two fungi using metabolite profile and molecular biology data.

Before going into the application of ESI-MS/MS, we thought it is desirable to give brief introduction to electrospray ionization tandem mass spectrometry (ESI-MS/MS) and also present a brief discussion on biogenesis of aryl pyruvate dimers. The section is initiated with a brief introduction to electrospray ionization tandem mass spectrometry (ESI-MS/MS) and discussion on biogenesis of aryl pyruvate dimers in *A. terreus*

## **II. Introduction to electrospray ionization tandem mass spectrometry (ESI-MS/MS)**

The interest in studying novel structures and functions of the molecules in the biological systems has created an impetus for the development of efficient, effective and information-rich methods and techniques of analysis. In this regard, mass spectrometry (MS) is gaining lot of attention for studying minor and unstable metabolites or their interactions in the system due to development of soft ionization methods such as electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI) and matrix assisted laser desorption ionization (MALDI).

MS is an important analytical tool in biological and biochemical research. The speed, accuracy and sensitivity is unmatched by conventional analytical techniques. For over ten decades, mass spectrometry has become a virtually ubiquitous research tool. Scientific breakthroughs made possible by MS have included the discovery of isotopes, the exact determination of atomic weights, characterization of new elements, quantitative gas analysis, stable isotope labeling, fast identification of trace pollutants and drugs and the characterization of molecular structure.

Soft ionization techniques like ESI are capable of producing ions of low energy, and hence have the power to generate ions from biological macromolecules of molecular masses in excess of 100,000, and when ESI is coupled with highly sensitive mass analyzers such as time of flight (TOF) in many cases these masses can be measured with accuracies better than 0.01%.

In early days of mass spectrometry two separate ionizations techniques had to be used for detection of molecular ions and fragment ions. Electron impact ionization (EI), the earliest technique of ionization produces molecular ion with high energy thereby quick disintegration of the molecular ion into fragment ions and hence in many cases molecular ions could not be detected. The chemical ionization (CI) method was required to serve the purpose as it is softer than EI. These techniques ionizes molecules in vapour state and hence are not suitable for non volatile and polar substances. In ESI-tandem mass spectrometry instruments, generally two mass analyzers are used, wherein the soft ionization techniques such as ESI or MALDI first produces molecular ions and then collision induced dissociation (CID) of the selected molecular ion leads to the fragmentation of the molecular ion. This method is suitable for wide range of substance from non polar lipids to high molecular weight polar compounds such as proteins and peptides.

In ESI, exact molecular ion ( $M^+$ ) of the compound is not produced, but multiple pseudomolecular ions of such as protonated  $[M+H]^+$ ; alkali metal adduct ions such as  $[M+Na]^+$  and  $[M+K]^+$  and polymeric ions such as  $[2M+H]^+$ ,  $[2M+Na]^+$  and  $[3M+H]^+$ , etc in positive ionization mode and  $[M-H]^-$ ,  $[M+X-H]^-$  in negative ionization mode where X is small molecules such as  $H_2O$ ,  $CH_3OH$ ,  $HCOOH$ , etc are produced. The multiple pseudomolecular ions thus give additional confirmation of molecular weight of the compound being investigated especially when analyzing mixture. Applications of ESI-MS/MS include the sequencing and analysis of peptides and proteins; studies of non-covalent complexes and immunological molecules; DNA sequencing; and the analysis of intact viruses (Watts, et al., 1997; Keki, et al 2003).



In our studies, we have used QSTAR XL-MS/MS Quadrupole-Time of Flight (QTOF) system in combination with electrospray ionization technique (ESI), which delivers sensitivity, selectivity and reproducibility while performing excellent performance for the characterization of proteins and peptides, analysis of drug metabolite information from small quantities of metabolites etc. The technique is well suited to mixture analysis because the characteristic product ion spectra can be obtained for each component in a mixture without interference from the other components, assuming that the product ions have unique  $m/z$  ratio. The sample introduction system manages the introduction of a sample into the QSTAR XL system and the conversion of the sample into ions, which can be analysed in the quadrupole. In a precursor ion scan, QSTAR XL detects precursor ions which generate a specific product ion. The instrument uses  $Q_1$  (Mass Filter Quadrupole) in mass resolving mode to scan over the mass range of interest, while the time of flight (TOF) section records product ion spectra for each precursor ion. The  $Q_1$  mass spectrum shows all precursor ions, which produce ion of interest. In a product ion scan,  $Q_1$  selects a precursor ion which fragments in  $Q_2$  (LINAC Collision Cell Quadrupole), generating product ions by a process termed 'collision-induced dissociation' (CID) which are detected in the time of flight (TOF) section. Product ions provide information on the molecular structure of the original (precursor) ions. This technique provides structural information by establishing relationships between precursor ions and their fragmentation products.

Recently, ESI-MS/MS technique have been successfully employed in identification of new cyclic depsipeptides, kahalalides R and S from the ethyl acetate fraction of marine mollusk *Elysia gradifolia* without any prior purification (Tilvi et al., 2007). The authors initially characterized the known anticancer agent cyclic depsipeptides, kahalalides F based on the mass fragmentation pattern. The two new homologous molecules, kahalalides R and S were successfully characterized by comparing the mass fragmentation pattern of new compounds with the known Kahalalide F. Kahalalide F was first isolated from *Elysia rufescens* collected from Black point, O'ah by Hamann et al. in May 1993. It showed anticancer activity *in vitro* and *in vivo*

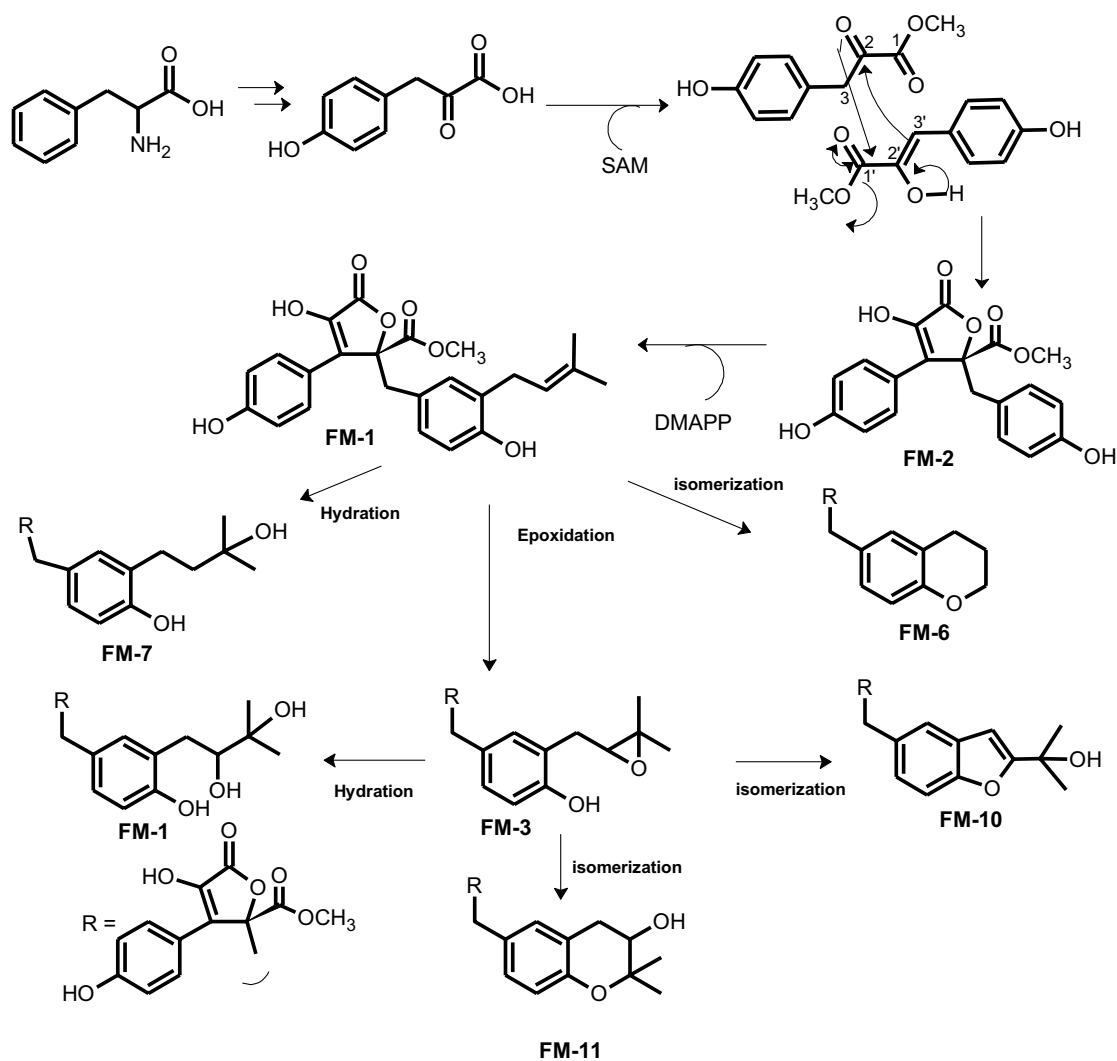
against breast cancer, colon cancer, non-small cell lung cancer (NSCLC), ovarian cancer and, in particular, against androgen-independent prostate cancer (Hamann et al.1993). PharmaMar has announced that kahalalide F has begun Phase II clinical trials for non small cell lung carcinoma (NSCLC) and melanoma (<http://www.cancersolution.tk/2011/05/kahalalide-f-phase-ii-trials-for-lung.html>).

We therefore, have made an attempt to characterize metabolites; especially butenolides derived from phenyl pyruvates from the crude extracts of fungi *A. flavipes* and *A. terreus* using ESI-MS/MS.

### III. Biosynthesis of aryl pyruvate dimers in *Aspergillus terreus*

Three distinct types of phenyl pyruvate dimers are produced by *A. terreus* from aryl amino acid. They are butyrolactones/aspernolides, aspulvinones and asterriquinones. Compounds of these classes are derived by homodimerization of pyruvate derived via an amino acid. In the biosynthesis of **FM-1** (Nitta et al, 1983), after the formation of p-hydroxyphenyl pyruvic acid from phenyl alanine the methyl esterification of the carboxyl group take place (**Scheme 2.10**).The methyl group in the ester is incorporated from *S*-adenosyl methionine (SAM). The esterification takes place before coupling, and has been proved by the observation that the parent carboxylic acid do not undergo condensation to give butenolide carboxylic acids. The intact condensation of two pyruvate ester molecules between 2 and 3' followed by lactonization gives butyrolactone II (**FM-2**). **FM-2** is enzymatically prenylated in presence of dimethylallyl pyrophosphate (DMAPP) to give **FM-1**. This biosynthetic pathway has been very well proved using tracer technique (Nitta et al, 1983; Brachmann et al., 2006). It is possible that other **FM-1** derivatives such as butyrolactone III-VII (**FM-9 to FM-13**) or aspernolides A (**FM-6**) and B (**FM-7**), which are obtained from long standing cultures (culturing time more than 15 days) might have been chemically derived from the **FM-1** and may not be enzymatically formed. It has also been observed that every time a derivative of **FM-1** has been isolated from the fungal culture **FM-1** has been always obtained and that too in major

amount, which suggest that other compounds might be chemically converted products of **FM-1** depending upon the pH and the aeration of the culture media.



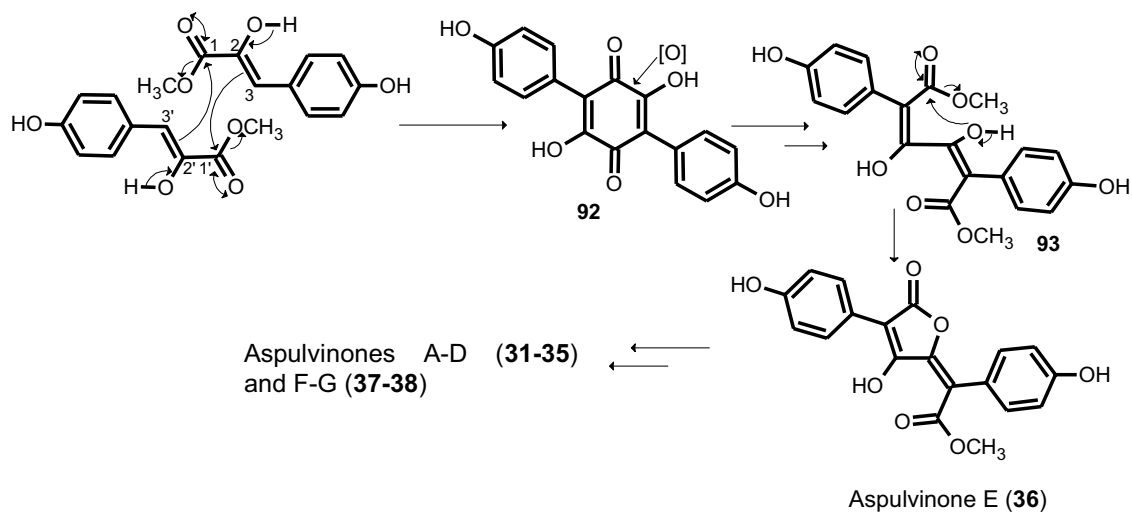
**Scheme 2.10: Biosynthesis of butyrolactone I (FM-1) and its derivatives**

Aspulvinones (**32-38**), another class of butenolides are also biosynthesized from the same precursor (phenyl pyruvate) involving a different pathway (**Scheme 2.11**). It involves condensation of phenyl pyruvate at two points between 1 and 3' and 3 and 1', to first give terphenyl benzoquinone (**92**). This terphenyl benzoquinone (**92**) is cleaved into a diketodicarboxylate (**79**) by Bayer-Villiger type oxidation followed by hydrolysis. Subsequent lactonisation and decarboxylation leads to butenolide aspulvinone E (**36**).

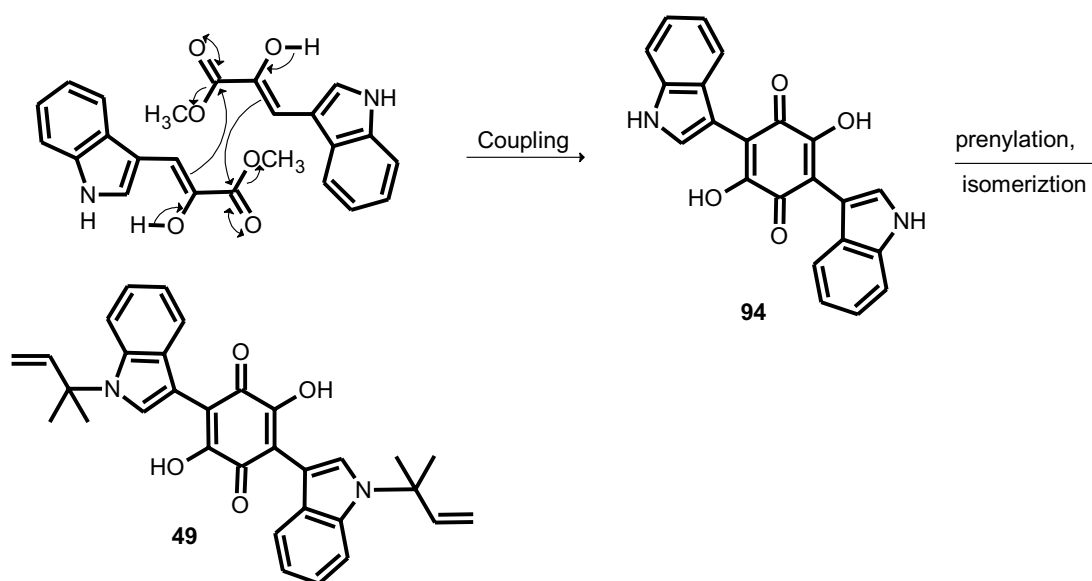
Aspulvinone E (**36**) is then hydroxylated, and prenylated at various positions on aromatic ring which gives series of aspulvinones (**32-35** and **37-38**). Interestingly, no derivatives of intermediate benzoquinone (**92**) derived from the phenyl pyruvate (derived via phenyl alanine) have been obtained as natural products so far from this fungus, but natural products called asterriquinones (**49-52**) derived from tryptophan via same pathway have been obtained from this fungus. More interestingly, butenolides, neither of aspulvinone type nor of butyrolactone I type derived via tryptophan have been obtained as natural products from these fungi. These observations leads to conclusion that the enzymes responsible for coupling of pyruvates and oxidation of benzoquinone derived from pyruvates are substrate specific beside its site specificity. The fungi produce two different enzymes responsible for coupling of pyruvates. Enzyme responsible for condensation between 2 and 3' acts only on *p*-hydroxylphenyl pyruvates leading to **FM-1** type of butenolides, whereas the other responsible for coupling in two points between 1 and 3' and 3 and 1' acts on pyruvates derived via phenyl alanine giving aspulvinones as well as tryptophan giving asterriquinones. A substrate specific enzyme oxidizes (Bayer-Villiger type oxidation) benzoquinone derived from phenyl alanine, but not benzoquinone derived from tryptophan to dienol dicarboxylates (**93**), which on series of transformations give aspulvinones. The activity of the oxidase is so strong in these fungal strains that the intermediate quinones (**92**) have never been isolated.

Interestingly, similar benzoquinones, but derived via tryptophan called asterriquinones (**49-52**) have been isolated as natural products from these fungal strain (**Scheme 2.12**) and the fact that no oxidation products of the intermediated benzoquinone (**94**) similar to aspulvinones are known as natural products is an indication that the oxidase is highly substrate specific. Nevertheless, a natural product called asterridinone (**54**) (Yamamoto et al., 1976) and asterridione (**55**) (Wijeratne et al 2003), which were metabolic products of *A. terreus* is proposed to be formed by oxidation of quinone moiety in asterriquinones. In this case another substrate specific enzyme must be responsible for this transformation as up to now no metabolites

similar to **54-55** derived from hydroxyphenyl pyruvates have been reported from *A. terreus*.



**Scheme 2.11: Biosynthesis of aspulvinones**



**Scheme 2.12: Biogenesis of asterriquinone**

#### IV. Results and discussion

##### i) Positive ESI-MS/MS analysis

Isolation and identification of the two fungi *A. flavipes* and *A. terreus* has been discussed in sections 2.1 and 2.2. These two fungi were cultured using identical conditions and the ESI-TOF-MS of EtOAc extracts of their culture media were recorded.

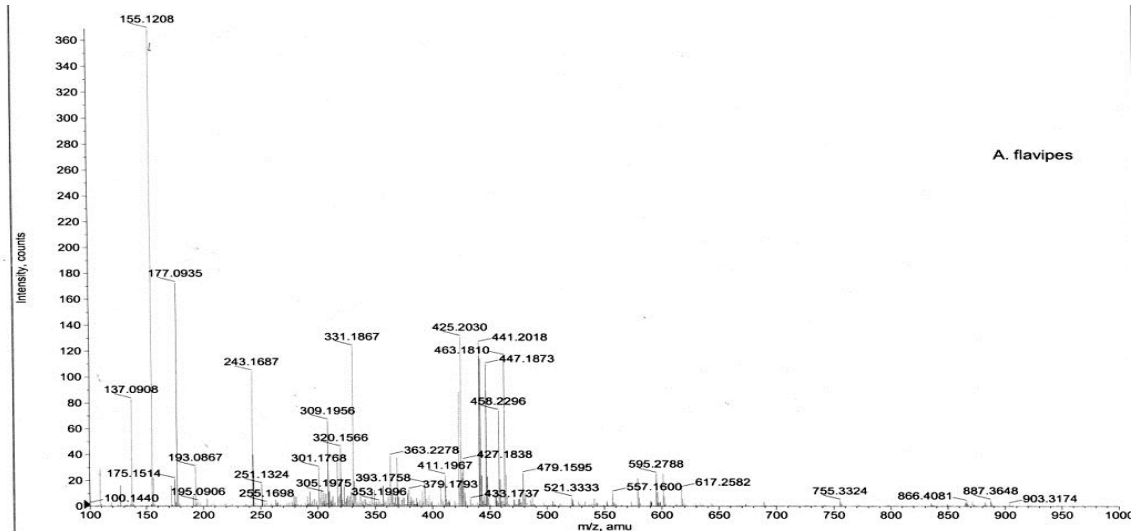


Fig 2.22: +ve ESI-TOF-MS of EtOAc extract of *A. flavipes*

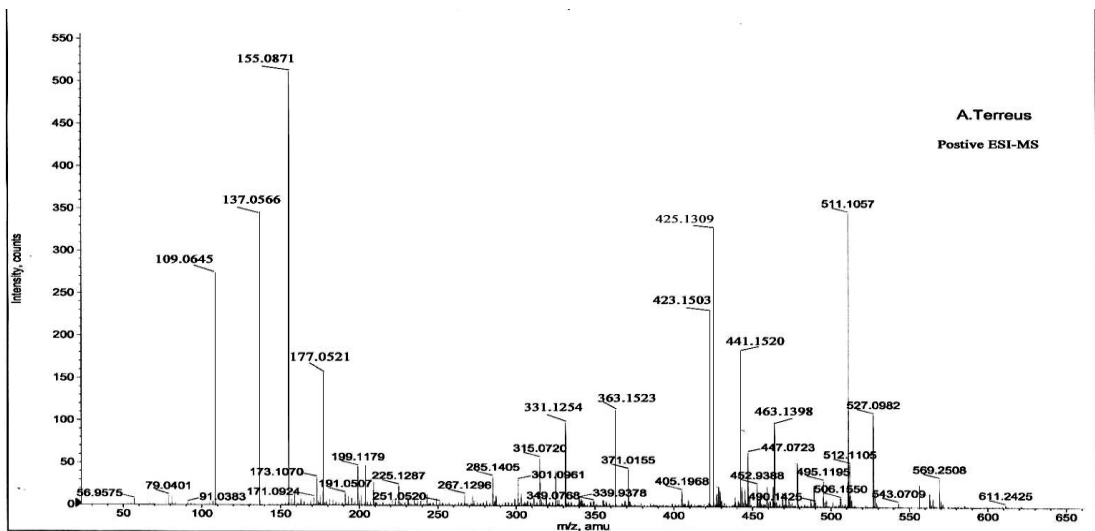


Fig 2.23: Fig 2.22: +ve ESI-TOF-MS of EtOAc extract of *A. terreus*

The positive ESI-MS Spectra (**Fig 2.22** and **2.23**) of the two fungi *A. flavipes* and *A. terreus* showed some identical peaks but several peaks were uncommon indicating the two fungi have different metabolism. The molecular masses obtained from the MS spectra were compared with molecular weights of known metabolites of *A. terreus* of the type class butyrolactone I, aspulvinones and asterriquinones those listed in the **Table 2.7**. No pseudomolecular ions of any of the aspulvinones or asterriquinone were observed, but pseudomolecular ions of certain butenolides corresponding to molecular formulae of butyrolactone I/aspernolide A and butyrolactones III-V were observed in both the fungi.

The important peaks which were common in both the spectra were  $[M+H]^+$  at  $m/z$  425 and  $[M+H]^+$  at  $m/z$  441 signifying the presence of butyrolactone I or Aspernolide A and probably butyrolactone III respectively, and also  $[M+H]^+$  at  $m/z$  155 was indicative of due to presence of terrein in both the fungal cultures. The  $[M+H]^+$  at  $m/z$  357 corresponding to the butyrolactone II (**FM-2**) was observed in *A. flavipes* but not in *A. terreus*.

**Table 2.7 Metabolites of *A. terreus*, their molecular formulae and molecular weights**

Metabolite	Molecular formula	Molecular weight	Metabolite	Molecular formula	Molecular weight
Butyrolactone I/Aspernolide A	C <sub>24</sub> H <sub>24</sub> O <sub>7</sub>	424	Butyrolactone I monosulphate	C <sub>24</sub> H <sub>23</sub> O <sub>10</sub> S	504
Butyrolactone II	C <sub>19</sub> H <sub>16</sub> O <sub>7</sub>	356	Aspulvinones A/B	C <sub>27</sub> H <sub>28</sub> O <sub>5</sub>	432
Butyrolactones III/IV/V	C <sub>24</sub> H <sub>24</sub> O <sub>8</sub>	440	Aspulvinones C/D	C <sub>27</sub> H <sub>28</sub> O <sub>6</sub>	448
Aspernolide D	C <sub>24</sub> H <sub>26</sub> O <sub>9</sub>	458	Aspulvinone E	C <sub>17</sub> H <sub>12</sub> O <sub>5</sub>	296
Butyrolactone VII	C <sub>25</sub> H <sub>26</sub> O <sub>7</sub>	438	Aspulvinone F	C <sub>27</sub> H <sub>28</sub> O <sub>7</sub>	464
Aspernolide B	C <sub>24</sub> H <sub>26</sub> O <sub>8</sub>	442	Aspulvinone G	C <sub>17</sub> H <sub>12</sub> O <sub>6</sub>	312
			Asterriquinone	C <sub>32</sub> H <sub>30</sub> N <sub>2</sub> O <sub>4</sub>	506

Besides, simple pseudomolecular ions such protonated, sodiated, potassiated and homodimeric pseudomolecular ions, the protonated or sodiated or potassiated adducts

of terrein (**FM-3**) with butyrolactone I (**FM-1**) and III (**FM-9**) were also observed. For e. g. terrein gave pseudomolecular ions  $[M+H]^+$  at m/z 155,  $[M+Na]^+$  at m/z 177,  $[M+K]^+$  at m/z 193,  $[2M+H]^+$  at m/z 309,  $[2M+Na]^+$  at m/z 331 and  $[2M+K]^+$  at m/z 347 and **FM-1** gave pseudomolecular ions at  $[M+H]^+$  at m/z 425,  $[M+Na]^+$  at m/z 447,  $[M+K]^+$  at m/z 463,  $[2M+H]^+$  at m/z 849,  $[2M+Na]^+$  at m/z 871 and  $[2M+K]^+$  at m/z 887, while a protonated adduct ion of **FM-3** (MW 154) and **FM-1** (MW 424) was observed at m/z 579.

To detect the presence of any new molecules (or known molecules, which were missed during purification) related to **FM-1**, some of the pseudomolecular ions between the mass range 400-500 amu were selected for fragmentation study. It is obvious that the prenyl chain in **FM-1** is most vulnerable to biogenetic alteration in the molecule, which is evident from the type of **FM-1** derivatives isolated from *A. terreus*. Initially, the mass fragmentation pattern of **FM-1** was studied using tandem mass spectrometry (MS/MS), in which the  $[M+H]^+$  ion at m/z 425 obtained from pure butyrolactone I was fragmented by collision induced dissociation (CID) by applying electrical potential between 20-40 ev. Similarly, the ions suspected to be originating from related molecules were fragmented by CID and their fragmentation was compared with **FM-1** fragments to deduce their structure.

#### *MS/MS spectra of butyrolactone I (FM-1)*

The CID (or MS/MS) of **FM-1**  $[M+H]^+$  at m/z 425 gave several fragment ions (**Fig 2.24**) which were m/z 407 ( $\Delta M = 18$ ), 393 ( $\Delta M = 32$ ), 389 ( $\Delta M = 36$ ), 369 ( $\Delta M = 56$ ), 363 ( $\Delta M = 62$ ), 347 ( $\Delta M = 78$ ), 331 ( $\Delta M = 94$ ), 307 ( $\Delta M = 118$ ), 231 ( $\Delta M = 194$ ), 217 ( $\Delta M = 208$ ), 203 ( $\Delta M = 222$ ), 175 ( $\Delta M = 250$ ), 157 ( $\Delta M = 268$ ), 121 ( $\Delta M = 304$ ), 69 ( $\Delta M = 356$ ). Out of these peaks four peaks at m/z 175, 331, 393 and 369 were assigned to the specific fragments and of diagnostic value considering the former three of them retains the ring B carrying prenyl chain. The mass difference of these ions with the corresponding fragment ion resulting from another pseudomolecular ion under investigation would give the information regarding the



change to be made in the **FM-1** structure to arrive at a structure of unknown molecule. The fragment at m/z 393 is formed by elimination of CH<sub>3</sub>OH molecule from protonated molecular ion. An m/z 331 fragment ion is supposed to be formed by elimination of phenol molecule and a fragment ion m/z is obtained due to formation of stable benzylic cation of ortho-prenylhydroxy benzyl by benzylic cleavage. A benzylic cleavage on prenyl group by elimination of C<sub>4</sub>H<sub>8</sub> leads to the formation of fragment ion at m/z 369.

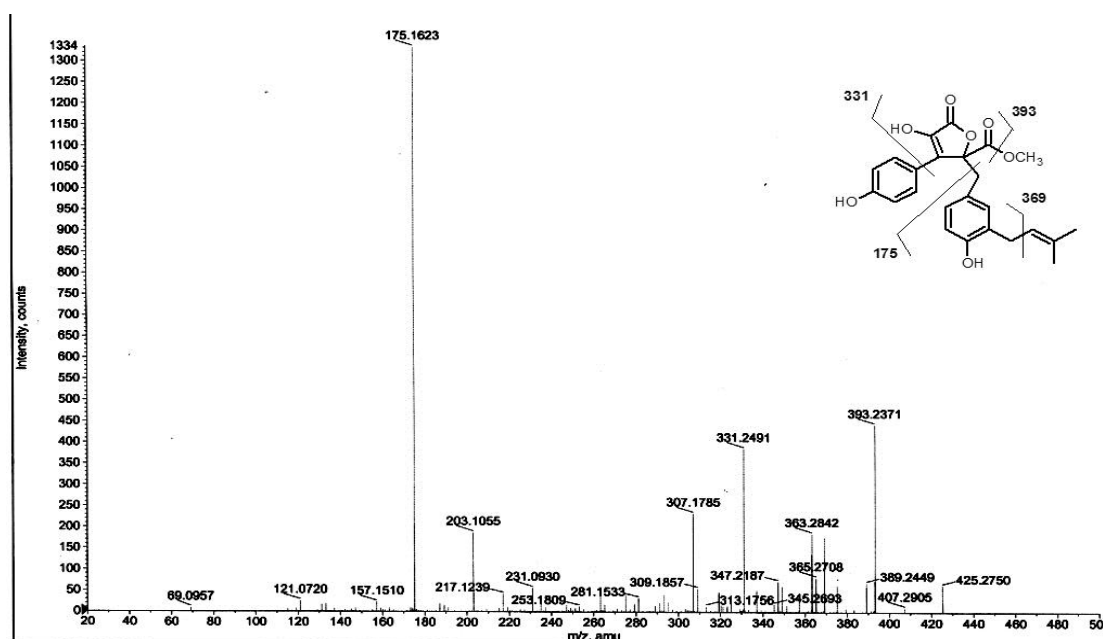
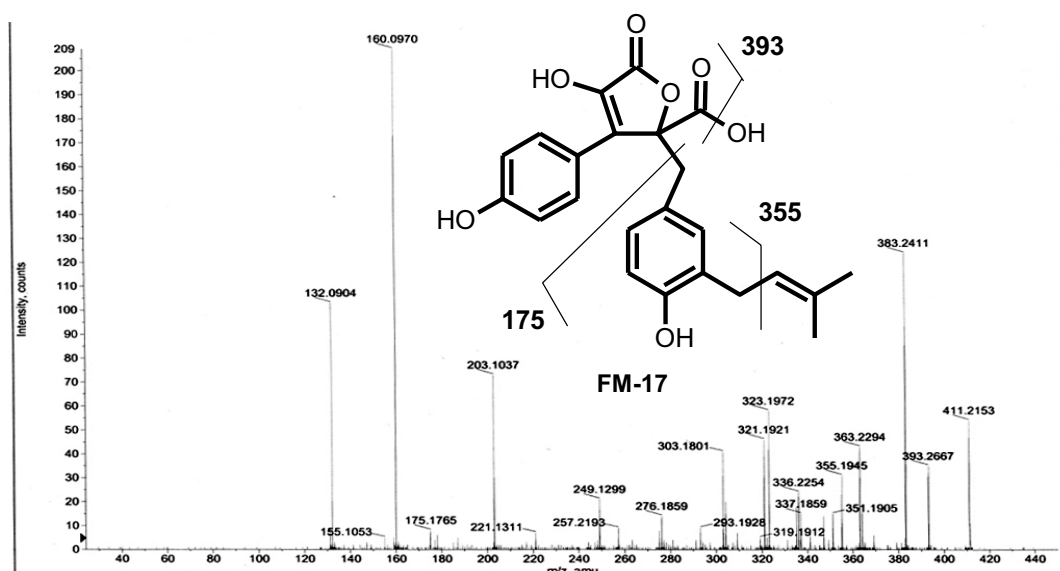


Fig 2.24: MS/MS of [M+H]<sup>+</sup> at m/z 425

*MS/MS of [M+H]<sup>+</sup> ion at m/z 411: Identification of FM-17*

One of the protonated ion that was analyzed by MS/MS (Fig 2.25) was m/z 411 assuming the existence of molecule (**FM-17**) with MW= 410 amu, which was further confirmed from the observation of the sodiated molecular ion [M+Na]<sup>+</sup> at m/z 433. These ion peaks were present only in spectra of *A. flavipes*. The molecular weight of 410 amu is less from **FM-1** by 14 amu suggesting a one methyl group is replaced by hydrogen atom in the molecule. One of the changes that can be done in **FM-1** is to replace one methyl group with hydrogen from the prenyl chain. In doing so the

fragment ions those are expected in MS/MS are  $m/z$  161, 317, 379. None of these peaks were observed in MS/MS spectra (**Fig 2.25**); instead the peaks at  $m/z$  175 and 393 along with the  $m/z$  369 were retained. This suggested that prenyl chain in **FM-17** is present without any change. The only other possibility is that molecule is the carboxylic acid of **FM-1**. In this case the methyl group of **FM-1** is replaced with hydrogen as that would display the fragment ions  $m/z$  175, 393 and 369. Therefore, most likely structure of the molecule yielding pseudomolecular ion at  $m/z$  411 is butyrolactone I carboxylic acid as shown in **FM-17**



**Fig 2.25: MS/MS of  $[M+H]^+$  at  $m/z$  411**

*MS/MS of  $[M+H]^+$  ion at  $m/z$  441: Identification of **FM-9***

A pseudomolecular ion  $[M+H]^+$  at  $m/z$  441 was present in both the extracts indicating presence of molecule with molecular weight 440 amu, which is 16 units more compared to **FM-1**. Corresponding pseudomolecular ions such as  $[M+Na]^+$  at  $m/z$  463,  $[M+K]^+$  at  $m/z$  479,  $[2M+H]^+$  at  $m/z$  849,  $[2M+Na]^+$  at  $m/z$  881 and  $[2M+K]^+$  at  $m/z$  903 and protonated adduct ion with terrein at  $m/z$  595 were also evident from

ESI-MS spectrum (Fig 2.22). Three **FM-1** derivatives, butyrolactone III (**FM-9**), IV (**FM-10**) and V (**FM-11**) of molecular weight 440 amu are known from *A. terreus*. The  $[M+H]^+$  at  $m/z$  441 in MS/MS (Fig 2.26) spectra displayed fragment peaks at  $m/z$  191, 347, which were also 16 units more than corresponding fragments of **FM-1**. The presence of fragment at  $m/z$  369 clearly suggests that the additional oxygen is present on terminal C-4 unit of prenyl chain and must be been derived from **FM-9** as other two should give corresponding fragment ion at  $m/z$  368 (Fig 2.27). The absence of  $m/z$  368 may not be enough evidence to suggest that the **FM-10** and **FM-11** are not present in the extract. It is also possible that the **FM-10** and **FM-11** are first converted into **FM-9** in ionization chamber then yielding fragment ion at  $m/z$  369. Therefore, the ion under investigation might have been produced due to any of the three known natural products (**FM-9**, **FM-10** and **FM-11**) or their mixture. As explained in proceeding biosynthetic section **FM-9** is precursor to **FM-10** and **FM-11**, and therefore, we decided to synthesize **FM-9** by simplest route and then **FM-10** and **FM-11** from it.

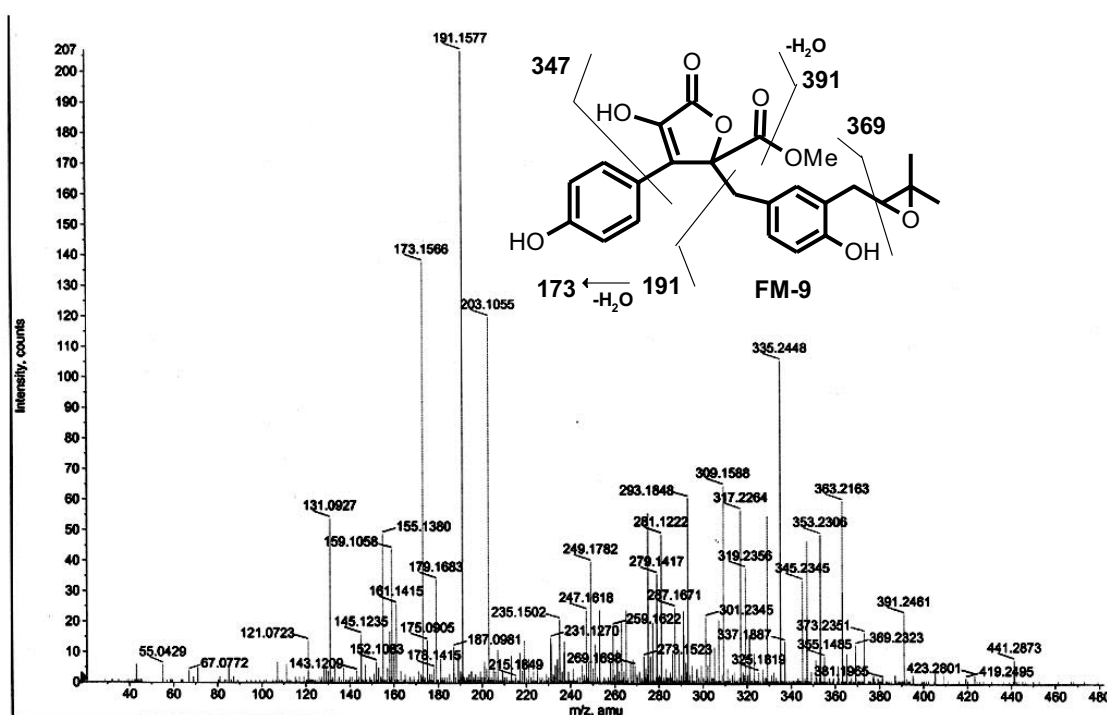
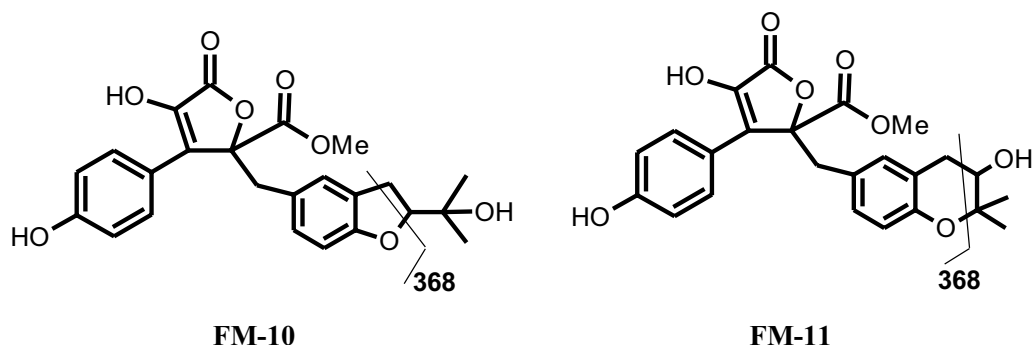
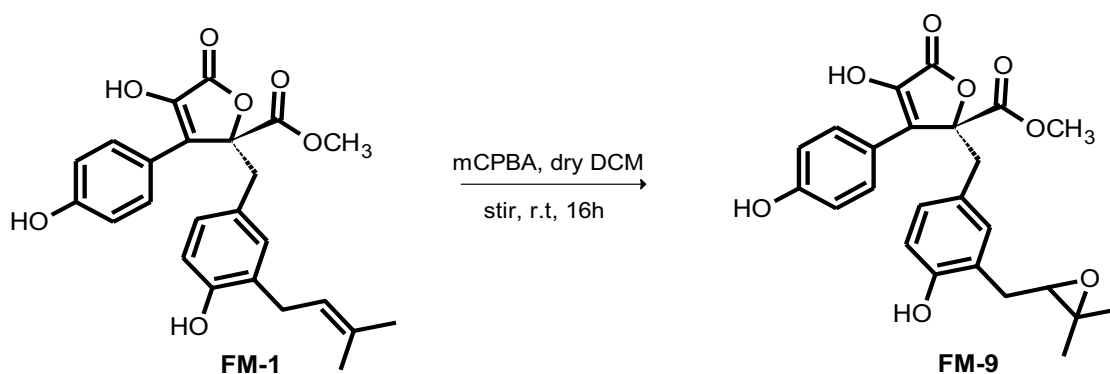


Fig 2.26: MS/MS of  $[M+H]^+$  at  $m/z$  441



**Fig 2.27: Structures of FM-10 and FM-11 showing expected mass fragment of 368 amu**

Thus, butyrolactone III (**FM-9**) was synthesized by treating **FM-1** with mCPBA in anhydrous DCM in 78% yield (**Scheme 2.13**). The compound was characterized using  $^1\text{H}$  and  $^{13}\text{C}$  NMR (**Figs 2.27a** and **2.27b**). Due to epoxidation of the prenyl double bond of **FM-1** the signal due vinylic proton at  $\delta_{\text{H}}$  disappeared and a new signal for oxymenthine appeared at  $\delta_{\text{H}}$  3.73, m. The proton signal for H-2' (6') was observed as quartet at  $\delta_{\text{H}}$  7.58 and one of the benzylic proton H-7'' showed two double doublets suggesting presence of C-8'' epimer in the compound. This is further supported from the observation that several carbons signals appeared in duplicates in  $^{13}\text{C}$  NMR (**Fig 2.27b**). The complete NMR data is listed in **table 2.8**. As expected, ESI-TOF-MS (**Fig 2.27c**) displayed a pseudomolecular ion at  $[\text{M}+\text{H}]^+$  at  $m/z$  441,  $[\text{M}+\text{Na}]^+$  at  $m/z$  463 and  $[\text{M}+\text{K}]^+$  at  $m/z$  479 and its MS/MS of  $m/z$  441 spectra (**Fig 2.27d**) was identical to MS/MS obtained on the  $[\text{M}+\text{H}]^+$  at  $m/z$  441 of the extract. Thus, it is confirmed that at least **FM-9** is present in the EtOAc extract of *A. flavipes* and *A. terreus*.



**Scheme 2.13: Preparation of FM-9 from FM-1**

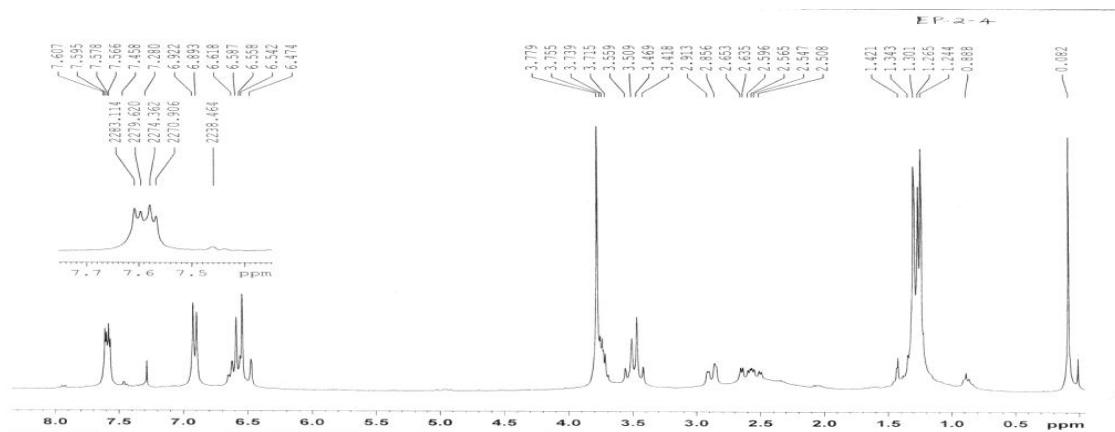


Fig 2.27a: <sup>1</sup>H NMR of FM-9 (synthetic)

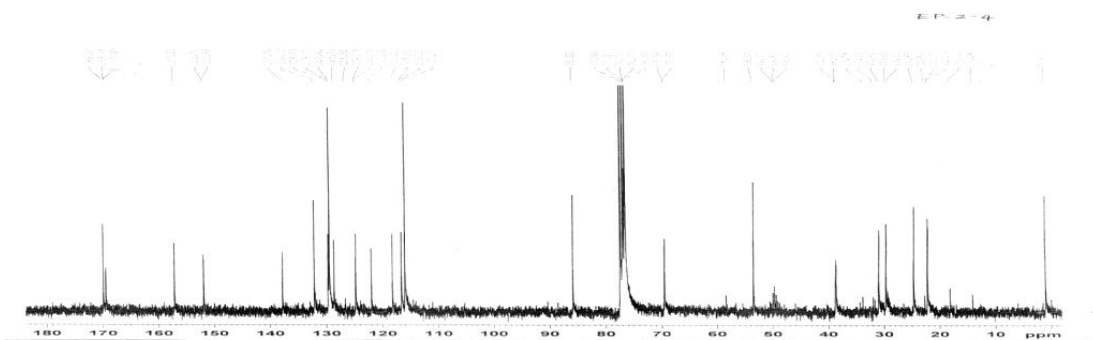


Fig 2.27b: <sup>13</sup>C NMR of FM-9 (synthetic)

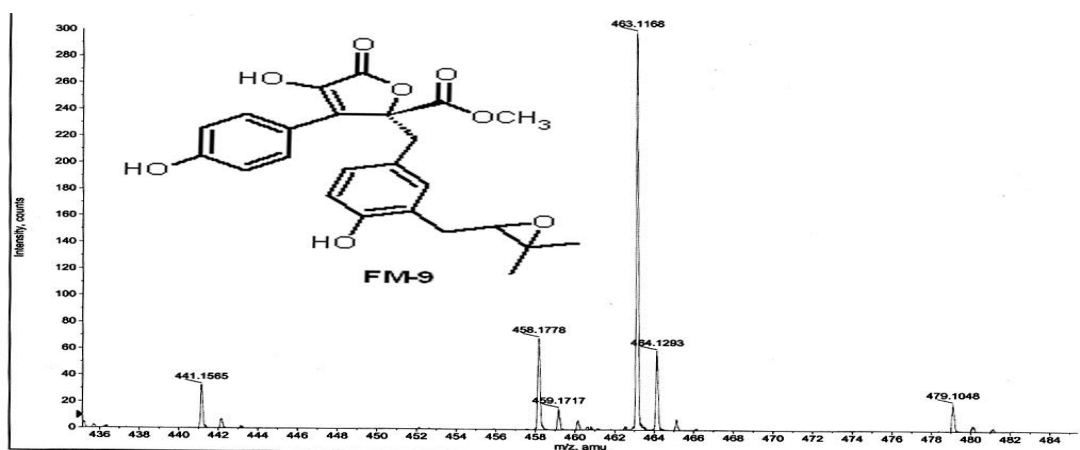


Fig 2.27c: <sup>1</sup>H ESI-TOF-MS of FM-9 (synthetic)

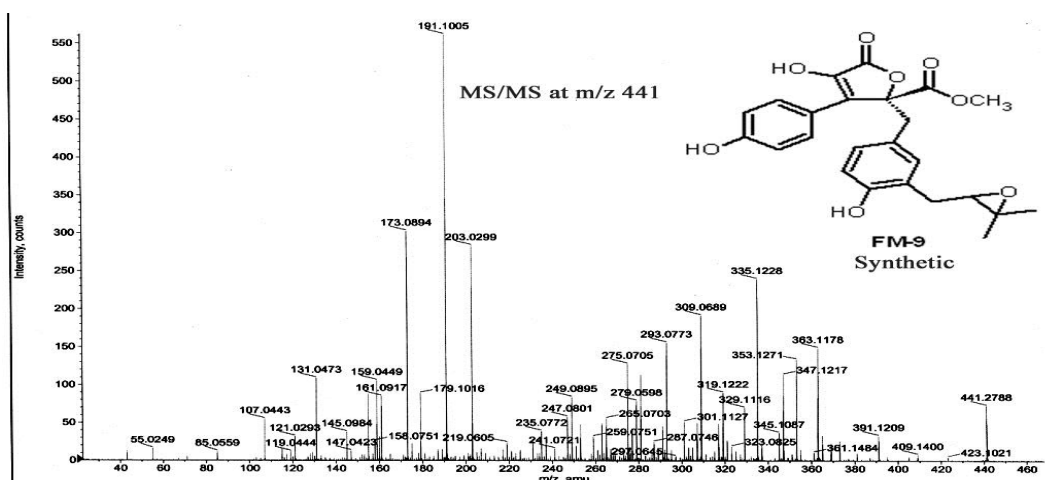
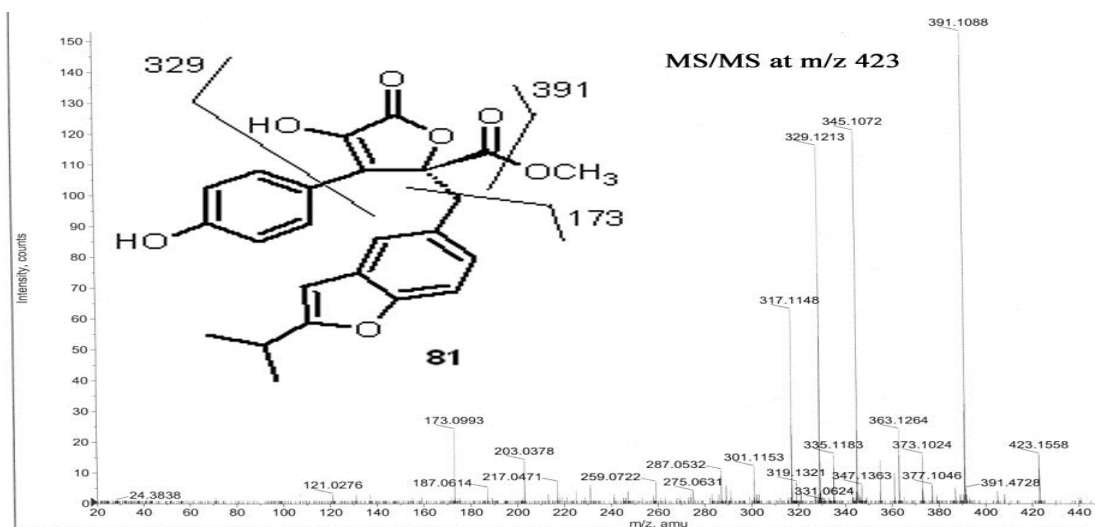


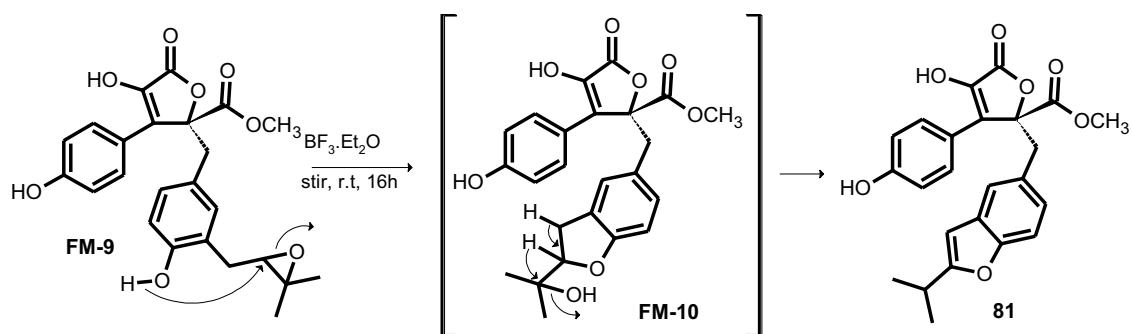
Fig 2.27d: MS/MS of FM-9 (synthetic)

Next, we treated the **FM-9** with a Lewis acid,  $\text{BF}_3 \cdot \text{Et}_2\text{O}$  in anticipation of obtaining **FM-10** or **FM-11**, but instead an isomerized dehydrated product (**81**) of **FM-10** was obtained (**Scheme 2.13**). Compound **81** has been reported as transformation product of **FM-9** obtained by treatment with pyridine/HCl during structure elucidation of **FM-9** (Nitta et al., 1983). Our product gave pseudomolecular ions  $[\text{M}+\text{H}]^+$  at m/z at 423,  $[\text{M}+\text{Na}]^+$  at m/z 445 and  $[\text{M}+\text{K}]^+$  at m/z 461. Its  $^1\text{H}$  NMR showed singlet at  $\delta_{\text{H}}$  6.91 for the methine of furan ring. The quartarnay carbon C-8'' was observed at  $\delta_{\text{C}}$  165.1 in  $^{13}\text{C}$  NMR. The complete 1D NMR data given in **table 2.8** accounts well for the given structure (**81**).

The MS/MS spectra (**Fig 2.28a**) of m/z 423 displayed fragment ions at m/z 391, 329 and 173, all of them as expected were two units smaller than corresponding fragment ions of **FM-1**.



**Fig 2.28a: MS/MS of 81**



**Scheme 2.13: Transformation of FM-9 into 81**

**Table 2.8: NMR data of FM-9 and 81 (CDCl<sub>3</sub>, 300 Mhz)**

Position	FM-9		81	
	$\delta_C$ , mult	$\delta_H$ , mult., J(Hz)	$\delta_C$ , mult	$\delta_H$ , mult., J(Hz)
1	169.4 s		168.9 s	
2	137.7 s		137.0 s	
3	128.5 s		128.7 s	
4	85.8 s		86.0 s	
5	38.6 t		169.7 s	
6	169.8s	3.44, d, (15.3) 3.53, d, (15.3)	39.2 t	3.68, d, (12.0)
1'	121.9 s		122.4 s	
2'(6')	129.6 d	7.59 d, (8.7)	129.6 d	7.64, d, (8.1)
3'(5')	115.9 d	6.91, d, (8.7)	116.0 d	6.97, d, (8.7)
4'	157.1 s		156.4 s	
1''	128.5 s		129.6 s	
2''	132.1 d	6.47, s	122.2 d	6.92, s
3''	124.7 s		127.5 s	
4''	151.9 s		153.9 s	
5''	116.6 d	6.59, d, (9.3)	110.0 d	6.66, d, (8.1)
6''	129.6 d	6.55, d, (9.3)	125.4 d	7.15, d, (8.4)
7''	30.9 t	2.52, dd (13.0, 5.4) 2.88, dd (13.0, 3.3)	99.7 d	6.19, s
8''	69.5 d	3.75, m	165.1 s	
9''	76.7 s		28.1 s	3.00, m
10''	22.2 q	1.24, s	14.1 q	1.30, s, (6.6)
11''	24.6 q	1.30, s	14.1 q	1.30, s, (6.6)
6-OMe	53.5 q	3.78, s	53.5 q	3.79, s

**ii) Negative ESI-MS/MS analysis**

A negative ESI-TOF-MS spectra (**Figs 2.29** and **2.30**) of ethyl acetate extracts of the both fungi showed some common peaks while several peaks were not common. Likewise, pseudomolecular ions for **FM-1** and **FM-9** [M-H]<sup>-</sup> at m/z at 423 and 439 respectively were present in both. Additionally, two pseudomolecular ions [M-H]<sup>-</sup> at m/z 503 and 519 were observed only in the extract of *A. flavipes*, which differed from **FM-1** and **FM-9** respectively by 80 units, an indication of the presence of molecules with molecular weight of 504 and 520 amu. The corresponding ions were not observed in positive ion mode. The mono sulfates (**FM-13** and **FM-14**) of **FM-1** has molecular weight of 504 and has been shown to give pseudomolecular ions [M-H]<sup>-</sup> in



negative mode (Niu et al., 2008). The presence of sulfate in the molecule was evidenced from the presence of signals at  $m/z$  423 and 439 of pseudomolecular ions  $[M-H]^-$  at  $m/z$  503 and 519 respectively formed by elimination of  $SO_3$ . The lower region of the MS/MS of the  $[M-H]^-$  at  $m/z$  503 (Fig 2.32) and 519 (Fig 2.354) had same ions as those observed for  $[M-H]^-$  at  $m/z$  423 (Fig 2.31) and 439 (Fig 2.33). It was not possible to predict the position of  $-SO_3H$  group, but it could be present on any of the three existing hydroxyl groups in the molecule. It is also possible that pseudomolecular ions might be resulting out of the mixture of two or three isomeric sulfates of FM-1 and FM-9. the possible structures of sulfated compounds are shown in FM-13, FM-14 and FM-18 to FM-21

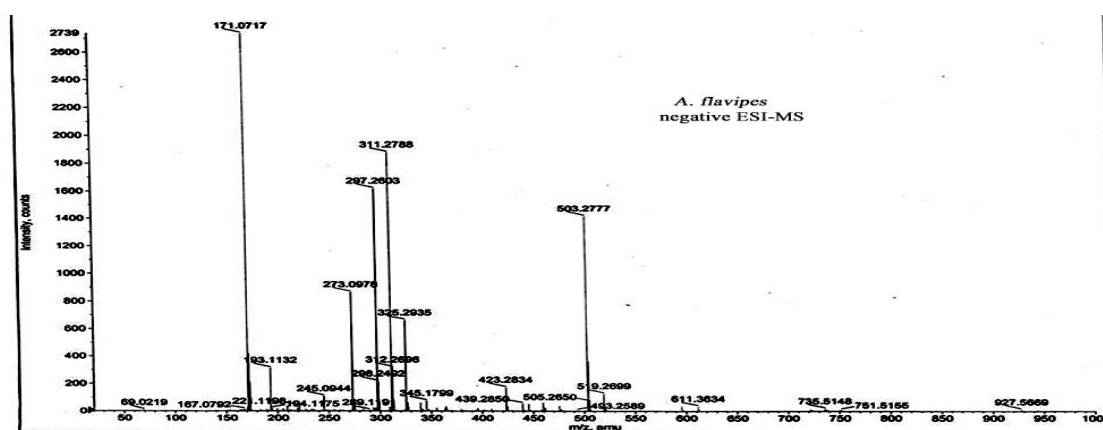


Fig 2.29: -ve ESI-TOF-MS of EtOAc extract of *A. flavipes*

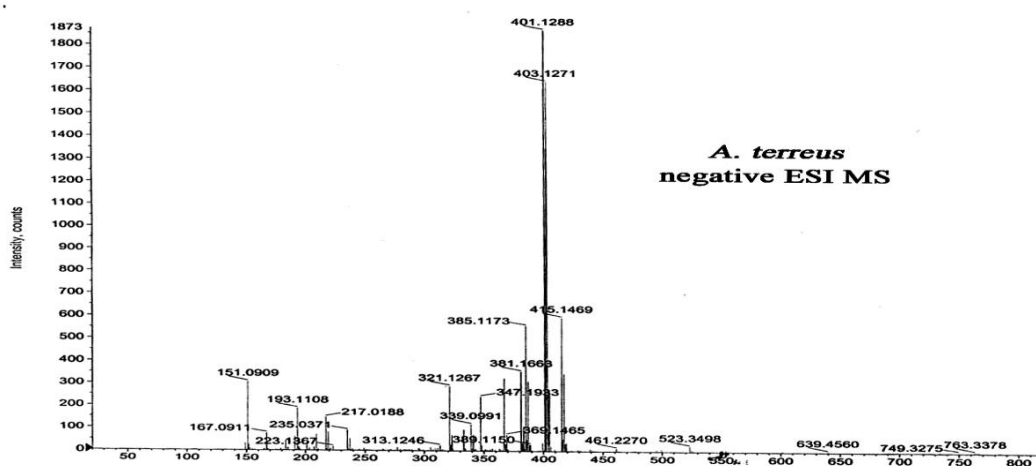


Fig 2.30: +ve ESI-TOF-MS of EtOAc extract of *A. terreus*

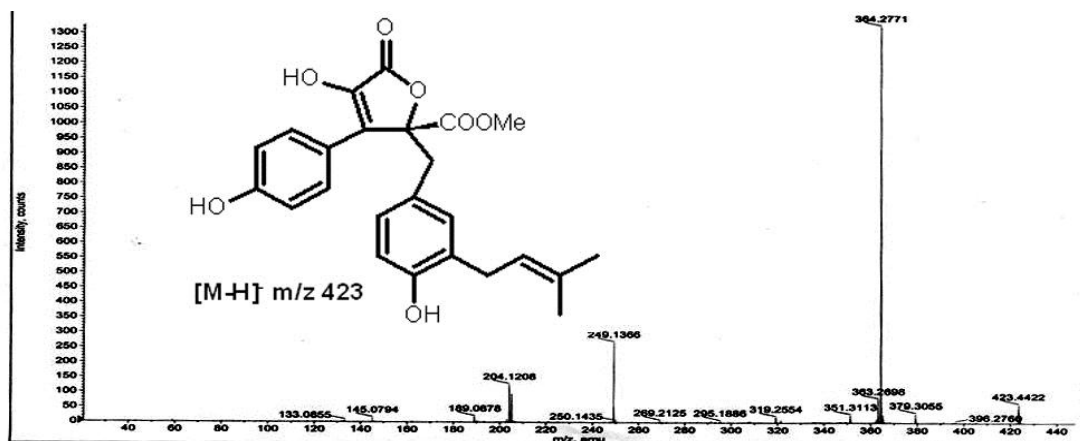


Fig 2.31: MS/MS of [M+H]. at m/z 423

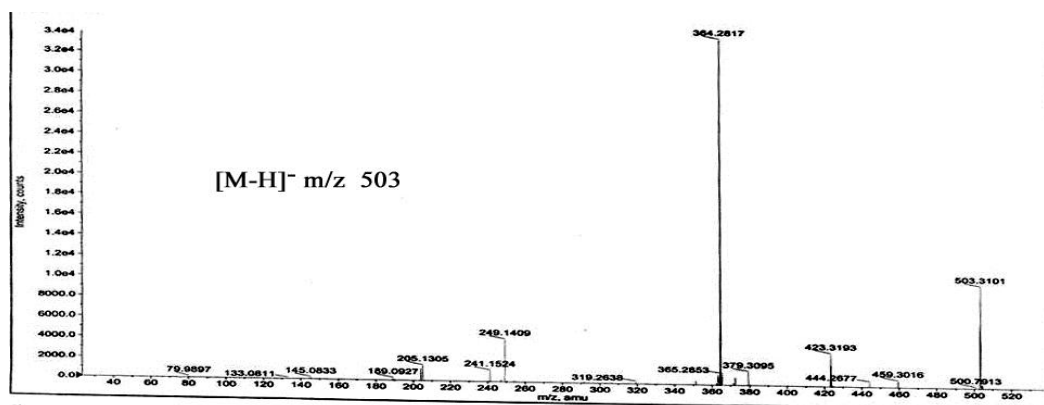


Fig 2.32: MS/MS of [M+H]. at m/z 503

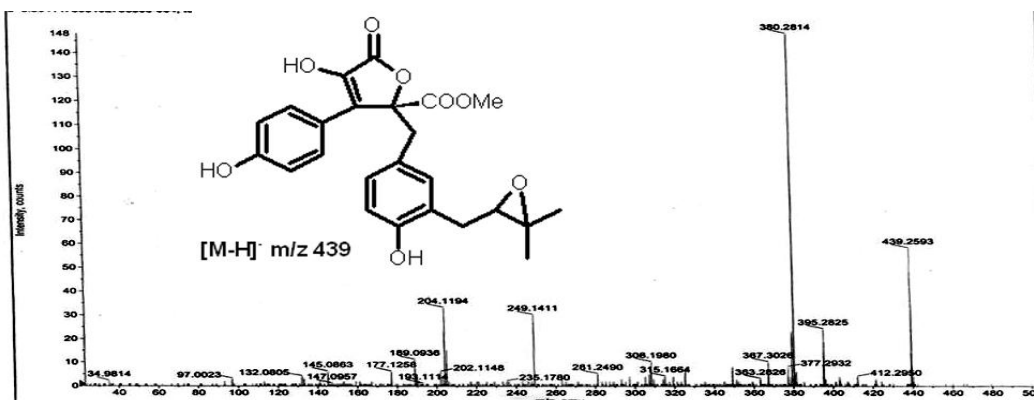


Fig 2.33: MS/MS of [M+H]. at m/z 439

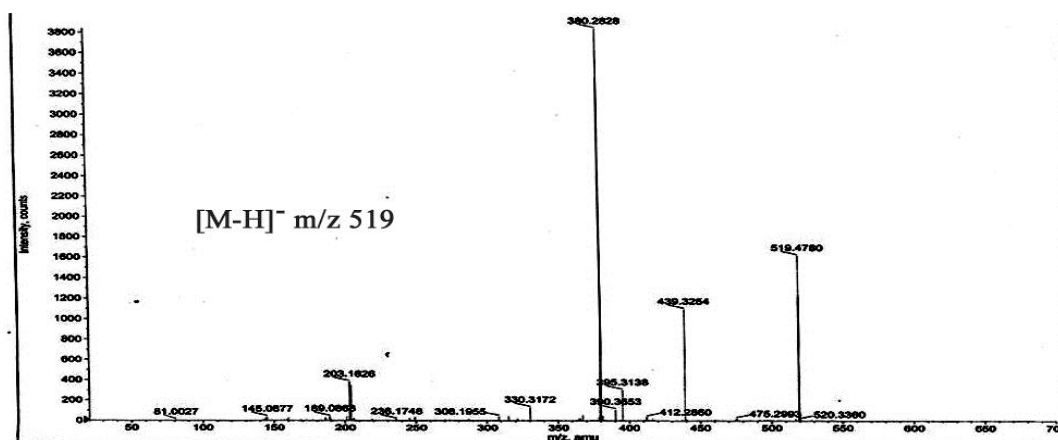
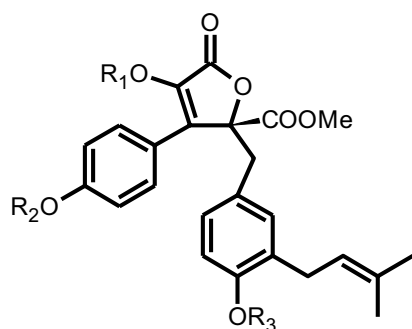
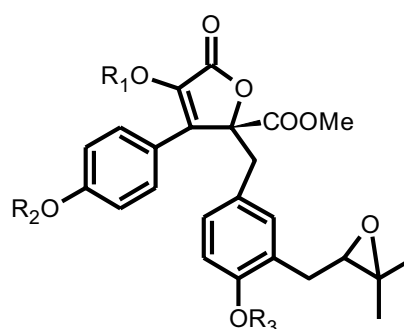


Fig 2.34: MS/MS of [M+H]. at m/z 519



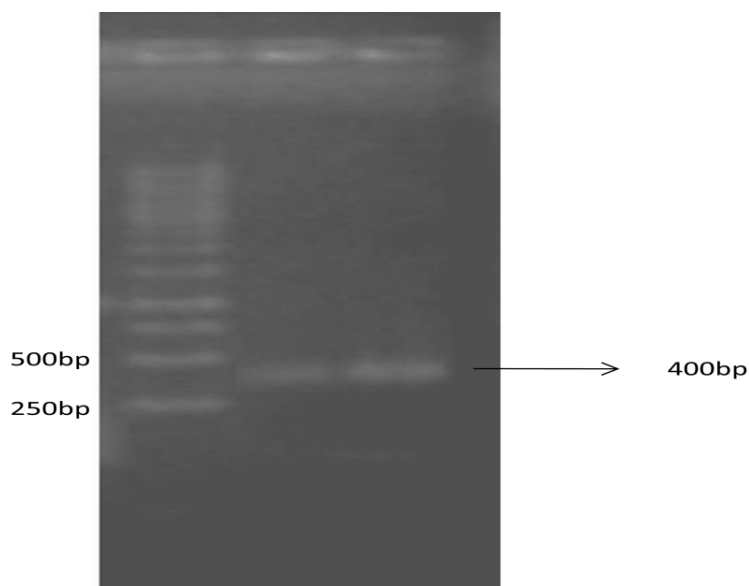
**FM-13** R1 = SO<sub>3</sub>H, R2 = R3 = H  
**FM-18** R1 = R3 = H, R2 = SO<sub>3</sub>H  
**FM-14** R1 = R2 = H, R3 = SO<sub>3</sub>H



**FM-19** R1 = SO<sub>3</sub>H, R2 = R3 = H  
**FM-20** R1 = R3 = H, R2 = SO<sub>3</sub>H  
**FM-21** R1 = R2 = H, R3 = SO<sub>3</sub>H

### iii) Phylogenetic analysis

The sequence of the ITS region gene has been widely used as a molecular marker to estimate relationships among fungi (phylogeny), but more recently it has also become important as a means to identify an unknown fungi to the genus or species level. It was recently reported that slight sequence differences in the ITS region gene could be used for species identification. Herein, to have comparison between the two fungi *A. flavipes* and *A. terreus* the two strains were identified using ITS region gene sequences.



**Fig 2.35: ITS gene amplification for fungi at approx. 400bp : Lane 1: 1Kb DNA ladder, Lane 2: Amplified product of *Aspergillus flavipes* and Lane 3: Amplified product of *Aspergillus terreus*.**

DNA of both the fungi was isolated, amplified on PCR (polymerase chain reactor) and the PCR product was further purified. The purified PCR product was sequenced using 5.8sr (5' TCGATGAAGAACGCAGCG 3') as sequencing primer. After amplification a PCR product of size 400 bp was obtained (**Fig 2.35**) and the sequences of PCR products bin FASTA format were analyzed by using Basic Local Alignment Search Tool (BLAST).

The 5.8SR gene sequence in FASTA format obtained for the two fungi were

*Aspergillus flavipes* Nucleotides sequenced: 329

```

AGGAAATGGTATTGCAGATTCAGTGATCATCGAGTCTTTGAACGCACATT
GCGCCCCCTGGTATTCCGGGGGGCATGCCTGTCCGAGCGTCATTGCTGCC
CTCAGCCCGGCTTGTGTGTTGGGCCCTCGTCCCCCGGCTCCCGGGGGACG
GGCCCGA
AAGGCAGCGGCGGCACCGCGTCCGGTCCTCGAGCGTATGGGGCTTCGTCT
TCCGCTCCGTAGGCCCGGCCGGCGCCCCGCCGACGCATTTATTTGCAACTT

```

GTTTTTTTCCAGGTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAA  
GCATATCAATAAGCGGAGGAA

*Aspergillus terreus* Nucleotides sequenced: 332

GGGAAAAAGTGAATTGCAGATTCAGTGAATCATCGAGTCTTTGAACGCAC  
ATTGCGCCCCCTGGTATTCCGGGGGGCATGCCTGTCCGAGCGTCATTGCT  
GCCCTCAGCCCGGCTTGTGTGTTGGGCCCTCGTCCCCCGGCTCCCGGGGG  
ACGGGCCCGAAAGGCAGCGGCGGCACCGCGTCCGGTCCCTCGAGCGTATG  
GGGCTTCGTCTTCCGCTCCGTAGGCCCGGCCGGCGCCCGCCGACGCATTT  
ATTTGCAACTTGTTTTTTTCCAGGTTGACCTCGGATCAGGTAGGGATACCC  
GCTGAACTTAAGCATATCAATAAGCGGAGGAA

**Table 2.9:**

Sr. No.	Gene Accession No.	Phylogenetic neighbours	% Similarity	
			<i>A. terreus</i>	<i>A. flavipes</i>
1	<i>EF669603</i>	<i>Aspergillus terreus</i> isolate NRRL 4609 internal transcribed spacer 1, 5.8S ribosomal RNA gene	99	98
2	<i>EF669587</i>	<i>Aspergillus terreus</i> isolate NRRL 260 internal transcribed spacer 1, 5.8S ribosomal RNA gene	99	98
3	<i>EF669586</i>	<i>Aspergillus terreus</i> isolate NRRL 255 internal transcribed spacer 1, 5.8S ribosomal RNA gene	99	98

The comparison of these fungi using basic local alignment search tool (BLAST) showed that our fungus *A. terreus* had 99% homology with several *A. terreus* isolates, three of which are listed below (**Table 2.9**) Interestingly, the fungus *A. flavipes* had 98% similarity to the same isolates indicating it is marginally different from our *A. terreus*. Phylogenetic analysis by Varga et al., (2005) have led to the

understanding that *A. flavipes* isolates are as closely related to *A. terreus* and have put the argument that these two fungi should be grouped in a same section separately as flavipedes and terri as presently done.

#### *iv) Concluding remarks*

By using positive and negative ESI-MS/MS we were able to detect presence of new compounds in *A. flavipes* **FM-17** to **FM-21** while known compounds such **FM-1** **FM-3** and **FM-9** in both fungi, *A. flavipes* and *A. terreus*. **FM-1** or its derivatives have not been previously reported to be metabolites of *A. flavipes*. The phylogentic anlalysis revealed that two fungi differed only marginally. Therefore, our study using metabolic profiling reveals that the two fungi, *A. flavipes* and *A. terreus* are very closely related. Phylogenetic analysis have led to the understanding that *A. flavipes* isolates are as closely related to *A. terreus*. The results obtained by us on these fungi showing high similarities are further proof of the earlier findings by Varga et al., (2005) that two fungi are phylogenetic neighbors.

## **V. Experimentals**

### *i) General experimental procedures*

The experiments carried for the phylogentic analysis were performed by trained molecular biologists and the procedures are not reported here. Cultivation of fungi and extraction of metabolites is given in sections 2.1 and 2.2

### *ii) Analysis on mass spectrometer*

Solutions (10 ppm approx.) of ethyl acetate extracts of the fungi *A. terreus* and *A. flavipes* were prepared. These solutions were injected in an ESI-QTOF MS/MS at 5ev to record ESI-TOF MS. Certain individual molecular ions were selected and

fragmented using collision induced dissociation (CID) by applying suitable potential between 20-40eV to obtain MS/MS spectra.

*iii) Preparation of butyrolactone III (FM-9)*

A mixture of **FM-1** (75 mg, 0.177 moles), mCPBA (55%) (39.6 mg, 0.230 moles) in dry DCM (10 ml) and stirred under N<sub>2</sub> atmosphere for 16 hrs. TLC (5% MeOH-CHCl<sub>3</sub>, v/v) indicated partial conversion of the **FM-1** to slightly polar compound upon which reaction was terminated by addition of liquor ammonia and the resultant mixture was extracted with EtOAc (20 ml x 3). Upon evaporation a sticky solid was obtained which was purified over flash Si gel by gradient elution of MeOH in CHCl<sub>3</sub> (0-5% v/v) to give **FM-9** as colourless solid (68 mg, 78%).

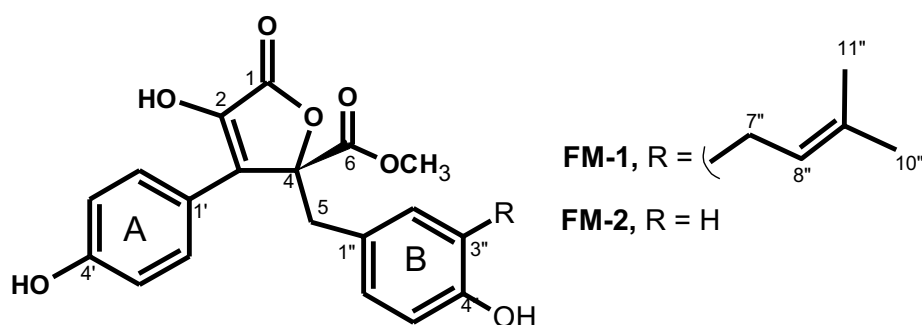
*iv) Conversion of butyrolactone III (FM-9) to 81*

BF<sub>3</sub>.Et<sub>2</sub>O (10 ml) was added to **FM-9** (30 mg) in RB flask and stirred under anhydrous condition for 16 hrs. TLC showed complete conversion of **FM-9** into a non polar compound. The reaction mixture was neutralized with aq NH<sub>3</sub> and extracted in EtOAc (20 ml x 3). Upon evaporation of the solvent a thick liquid compound was obtained which was purified on flash Si gel by gradient elution of MeOH in CHCl<sub>3</sub> (0-5% v/v) to give **81** as sticky solid (10 mg).

## Section 2.5 Effect of seawater concentration on the production of butyrolactone I in *Aspergillus terreus*

### I. Introduction

Butyrolactone I (**FM-1**) has been one of the most characteristic metabolite of the fungus *Aspergillus terreus*. Its unique structural properties and highly selective and potent cyclin dependent kinase (CDK) inhibition activity, makes it one of the most studied metabolite from this fungus. It has one chiral centre having 4*R* configuration and prenyl chain on ring B, and both the features are very important in maintaining high antitumor activity. **FM-1** selectively inhibit CDK2 and CDK1 kinases, both of which play important roles in cell progression from G1 to S phase and from G2 phase to M phase, respectively, in mammalian cells. Butyrolactone I has been shown to present antitumor effects on several lung cancer cell lines with IC<sub>50</sub> values in the order of 0.12 μM (Nishio et al., 1996) and prostatic cancer cell lines with IC<sub>50</sub> values in the order of 70 μM (Suzuki et al., 1999).



Although three decades have passed the isolation of **FM-1**, the synthesis of this molecule has not been reported. Butyrolactone II (**FM-2**), the immediate precursor of **FM-1**, has been synthesized, but it only in racemic form. Not only efficient chiral catalysis will be needed to produce optically pure **FM-2**, but selective prenylation of ring B in **FM-2** will also be problem to tackle as both the aromatic rings (rings A and B) are equally susceptible to prenylation. It is remarkable to know as of now no



synthetic route is available to access this molecule for biochemical studies; the alternative followed for its access is large scale fermentation. A procedure developed by Schimmel et al., (1999) yields 700 mg/l of **FM-1** from fermentation *A. terreus* ATCC 20542 using optimized basic medium of glucose peptone yeast extract.

Here in this section we wish to show that **FM-1** production in *A. terreus* can be elevated by altering the seawater concentration in the medium.

## **II. Results and discussion**

As discussed in section 2.2 the fungus *A. terreus* produced 3.2 mg/l of **FM-1** in PDB in seawater medium. Surprisingly, the re-culturing of this strain under same condition did not yield any **FM-1** and only a co-metabolite terrein (**FM-3**) was detected in major quantity. On the contrary, *A. flavipes* had produced over 200 mg/l of **FM-1** in PDB made in 75% SW medium (section 2.1). A careful observation of the culturing medium of the two fungi, goes to suggest that **FM-1** production in these fungi is function of seawater (SW) concentration in the culture medium. Natural SW composition changes with seasons and the change in SW composition of SW used for re-culturing might have been responsible for non production of **FM-1** in *A. terreus* in repeat cultures. To test the hypothesis that **FM-1** production is function of SW concentration, we carried experiments of growing the *A. terreus* in medium having different SW concentrations.

### *i) Cultivation of fungus*

The fungus *A. terreus* was cultured in PDB dextrose media containing different SW concentrations form 0-100% (**Table 2.10**). One more set of experiment was carried using medium additionally containing 4-hydroxyphenylpyruvic acid (HPPA), which is a precursor to **FM-1**. After the incubation of the fungus under static condition for 21 days broth was extracted in EtOAc, and the extract was quantitatively analyzed for **FM-1** on reverse phase ultra performance liquid chromatography (UPLC).

**Table 2.10: Culture media composition**

Experiment	DW (%)	SW (%)	Media components
Set I	100	0	PDB
Set II	50	50	PDB
Set III	0	100	PDB
Set IV	100	0	PDB + (..)

*ii) Analysis of the extracts on UPLC*

First a method to analyze butyrolactone I (**FM-1**) from the extract was developed by varying the gradient of AcCN and H<sub>2</sub>O on RP-18 column. The column used has AQUITY UPLC<sup>®</sup> BEH C18, 1.7 $\mu$ m, 2.1 x 100mm. The parameters used in the method is tabulated in **table 2.11**

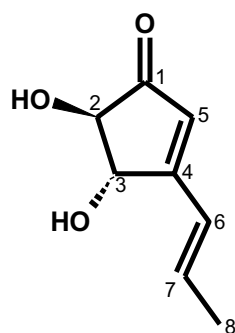
**Table 2.11: UPLC mobile phase parameters**

Time (min.)	Flow (ml/min)	H <sub>2</sub> O (%)	AcCN (%)
0	0.25	100	0.0
2.5	0.25	100	0.0
3.5	0.25	60	40
4.5	0.25	20	80
6.5	0.25	0	100
10	0.25	0	100

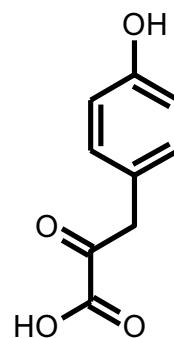
The wavelength of 254 nm was used on UV detector. First the standard methanolic solution of **FM-1** was analyzed, which gave a retention time (RT) of 5.57 min and area under the peak was recorded (**Fig 2.36**). Similarly, the EtOAc extracts of four experiments (**table 2.10**) were analyzed and the area under the peak at 5.57 was recorded (**Figs 2.37a -2.37d**). The amount of **FM-1** in the extracts was calculated by

correlating their area with area recorded for pure **FM-1**. The calculation of amount of **FM-1** present per milligram in the extracts is tabulated in **table 2.12** and the amount of **FM-1** present in the total EtOAc extract is given **table 2.12**.

As observed in one of the previous experiments hardly any amount (0.0067 mg) of **FM-1** was detected on UPLC in *A. terreus* culture in 100% SW. The change from 100% SW to 100% DW (0% SW) resulted in marginal decrease of EtOAc extract from 73 mg in 100% SW to 69 mg in 100% DW, but the amount of butyrolactone I in the extract was 19.87 mg which very high considering negligible amount in 100% SW. This indicates that the production of other components such as terrein (**FM-3**) (peak at RT 2.6 min) was getting reduced in 100% DW cultures. A change from 0% SW towards 50% SW saw increase in total extract (103 mg from 63 mg in 0% SW) as well increase in production of **FM-1** to 27.93 mg in 100ml culture. A peak at 2.60 min appears due to **FM-3**, the production of which also is affected by change in SW concentration of culture media. An experiment carried out with a 50 mg HPPA in 100 ml DW (Set IV) based medium as expected saw rise in production of **FM-1** from 19.87 mg in set I to 21.95 mg.



**Terrein (FM-3)**



**HPPA**

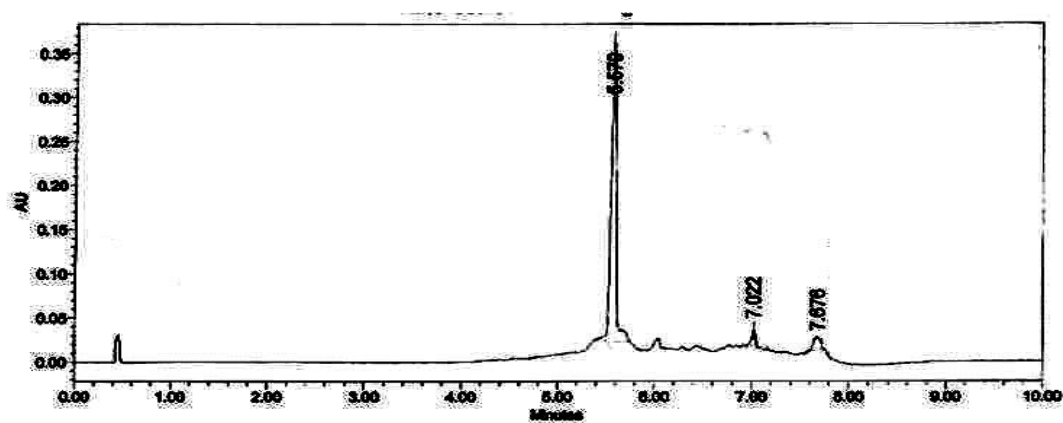


Fig 2.36: UPLC Chromatogram of Pure FM-1

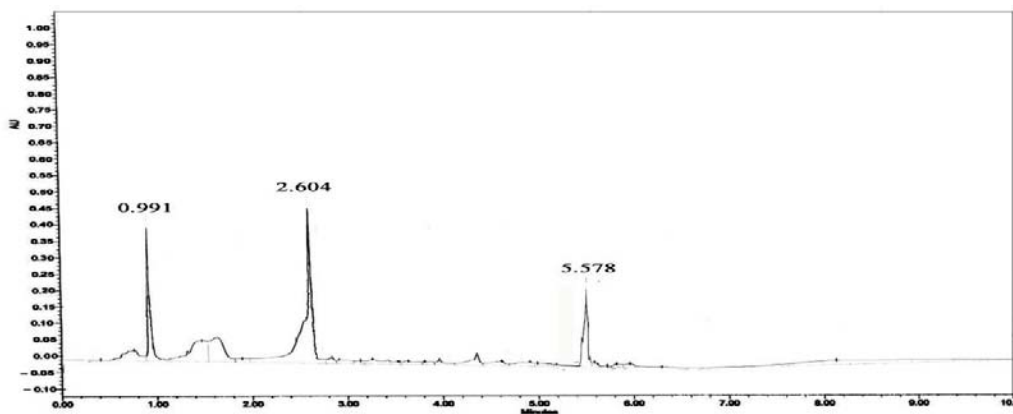


Fig 2.37a: UPLC Chromatogram of EtOAc of Set I

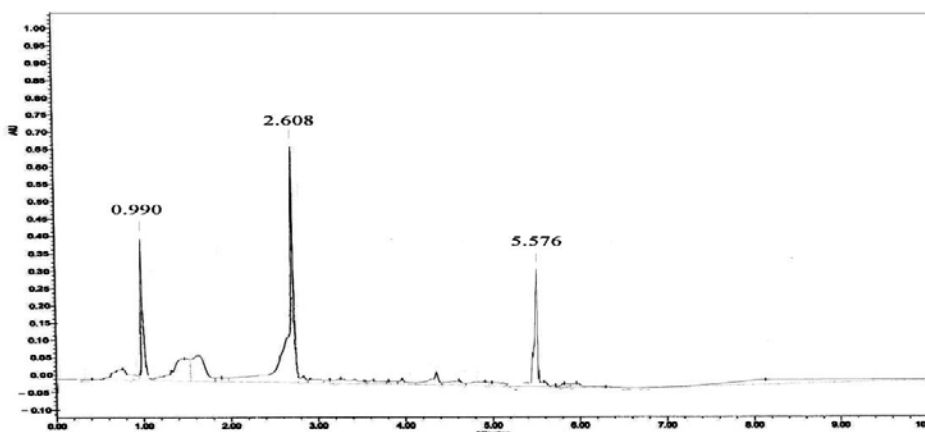


Fig 2.37b: UPLC Chromatogram of EtOAc of Set II

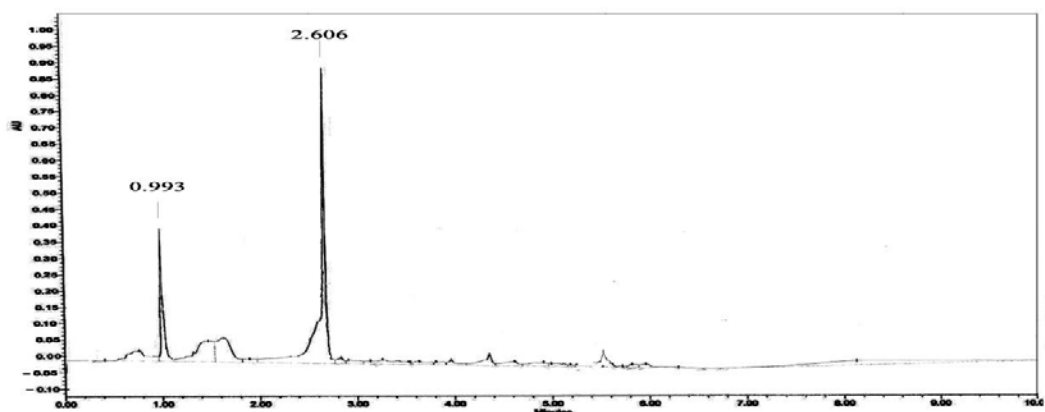


Fig 2.37c: UPLC Chromatogram of EtOAc of Set III

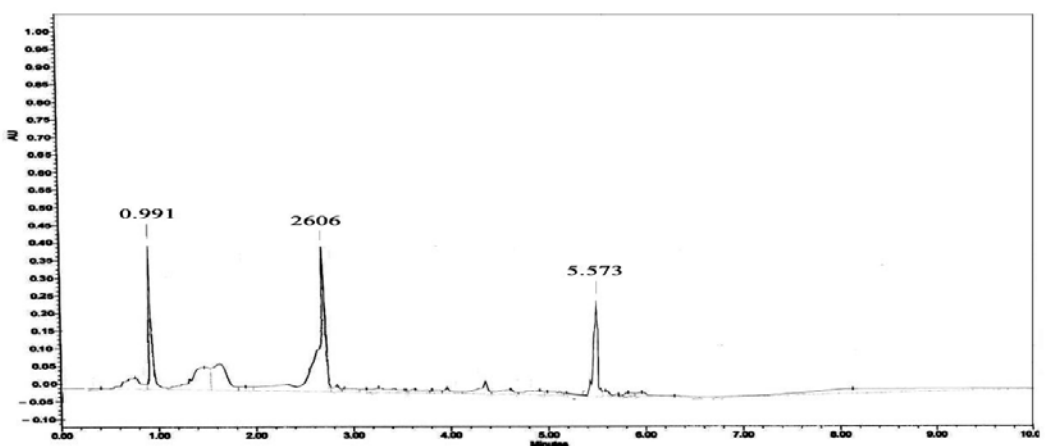


Fig 2.37d: UPLC Chromatogram of EtOAc of Set IV

Table 2.12 : Amount of FM-1 in per milligram of extract

Sample	Conc of Sample (mg/ml)	Peak area (units) (RT 5.57 min)	Area % w.r.t. FM-1	Conc of FM-1 sample solution (mg/ml)	Amount of FM-1 per mg of EA extract (X) (mg)
FM-1	1.3	19482003	100.00	1.30	1.000
Set I	1.7	7361024	37.78	0.49	0.288
Set II	1.4	5622312	28.86	0.38	0.271
Set III	1.5	114564	0.59	0.008	0.006
Set IV	1.4	5167613	26.53	0.34	0.257

**Table 2.12: Total amount of FM-1 produced in each set**

<b>Experiment</b>	<b>Total weight of EtOAc Extract (Y mg)</b>	<b>Total FM-1 In 100ml culture (X*Y mg)</b>
Set I	69.0	19.87
Set II	103.0	27.93
Set III	73.0	0.0067
Set IV	81.0	21.95

Our above results clearly indicate that the salinity of water has role to play in production of **FM-1** and other metabolites in *A. terreus* and the optimum production of **FM-1** occurs at intermediate concentration of SW. It is therefore suggested that an experiment designed by Schimmel et al., (1999) which produces 70 mg/l of FM-1 in DW glucose-peptone-yeast based medium would produce larger amount of FM-1, if carried with certain concentration of SW in the medium.

### **III. Experimentals**

#### *i) Cultivation of fungus*

Three media (100 ml each) containing PDB (24 g/l) having SW concentration 0%, 50% and 100% were prepared. Another PDB medium (2.4g in 100ml) containing 4-hydroxyphenylpyruvic acid (50mg) in DW (0% SW) was also prepared. All the media were inoculated with fungus *A. terreus* culture (0.5ml) grown on PDB. Flasks were incubated for 21 days under static condition at 27±2°C.

#### *ii) Preparation of extracts*

Each culture medium free of mycelium was extracted in ethyl acetate (40ml x 3). Organic extracts were washed with water, dried on anhyd. Na<sub>2</sub>SO<sub>4</sub>, concentrated under vacuum and weighed.

**iii) Analysis of Extracts on UPLC**

Accurately weighed amount between 1-2mg of EtOAc extracts were dissolved in MeOH and made up to the mark in std. flasks (10ml). These solutions were analyzed on UPLC as per the method parameters given in table No.

**References:**

- Abarca, M. L., Accensi, F., Cano, J., F., Cabañes, J., 2004. *Antonie van Leeuwenhoek.*, 86, 33-49.
- Abraham, E. P., Florey, H. W., 1949. *Antibiotics*, Flores.et al., eds. Oxford University Press, New York, Vol. I, 837.
- Alberts, A.W., Chen, J., Kuron, G., Hunt, V., Huff, J., Hoffman, C., 1980. *Proc. Natl. Acad. Sci. USA.* 77, 3957-3961.
- Alvi, K. A., Nair, B., Pu, H., Ursino, R., Gallo C., Mocek, U., 1997. *J. Org. Chem.*, 62, 2148-2151.
- An., 2005. *Handbook of Industrial Mycology* (New York: Marcel Dekker).
- Andersen, R., Buechi, G., Kobbe, B., Demain, A.L., 1977. *J. Org. Chem.*, 42, 352-353.
- Antia, B.S., Aree, T., Kasetrathat, C., Wiyakrutta, S., Ekpa, O.D., Ekpe, U.J., Mahidol, C., Ruchirawat, S., Kittakoop, 2011. *Phytochemistry.*, 72(8), 816 -820.
- Arvanov, V. L., Ling, K. H., Chen, R. C., Tsai, M. C., 1993. *Neurosci. Lett.* 152, 69-71.
- Bachmann, M., Luethy, J., Schlatter, C., 1979. *J. Agric. Food Chem.*, 27(6), 1342-1347.
- Barnes, C. L., Ubillas, R., Tempesta, M. S., 1999. *J. Chem. Crystallography*, 29(9), 1031-1035
- Barrow, C. J., Sun, H. H., 1994. *J. Nat. Prod.*, 57(4), 471-476.

- Barton, D. H. R., Scott, A. I., 1958. *J. Chem. Soc.*, 1767-1772.
- Brana, M. F., Garcia, M. L., Lopez, B., Pascual-Teresa, B., Ramos, A., Pozuelo, J. M., Dominguez, M. T., 2004. *Org. Biomol. Chem.*, 2, 1864-1871.
- Calam, C. T., Oxford, A. E., Raistrick, H., 1939. *Biochem. J.*, 33, 1488-1495.
- Casinovi, C. G., Grandolini, G., Mercantini, R., Oddo, N., Olivieri, R., Tonolo, A., 1969. *Ann Ist Super Sanita.*, 5(5), 523-535.
- Claisen, L., Ewan, Th., 1895. *Annalen*, 281, 235.
- Clutterbuck, P. W., Koerber, W., Raistrick, H., 1937. *Biochem. J.* 31, 1089–1092.
- Dodds, D.R., Gross, R.A., 2007. *Science*, 318, 1250–1251.
- Dunn, A.W., Ian, D.E., Johnstone, A.W., 1975. *Phytochemistry*, 14, 2081–2082.
- Edwards, R. L., Gill, M., 1973. *J. Chem. Soc., Perkin1* 1529.
- Eglinton, G., King, F. E., Lloyd, G., Loder, J. W., Marshall, J. R., Robertson, A., Whalley, W. B., 1958. *J. Chem. Soc.*, 1833–1842.
- El-Sayed, S. A., 2009. *J. Basic Microbiology*, 49, 331–341.
- Fang, F., Ui, H., Shiomi, K., Masuma, R., Yamaguchi, Y., Zhang, C. G., Zhang, X. W., Tanaka, Y., Omura S., 1997. *J. Antibiotics*, 50, 919-925.
- Findlay, J. A., Radics, L., 1972. *J. Chem. Soc., Perkin1*, 16, 2071-2074.
- Fischer, P. M., Lane, D. P., 2000. 7, 1213-1245.
- Franck, B., 1980. In *The Biosynthesis of Mycotoxins: A Study in Secondary Metabolism* (Ed.: P. S. Steyn), Academic Press, New York, 157 –191.
- Geiser, D.M., 2008. *Med. Mycol.* 46 (suppl. 1), S1-S6.
- Ghisalberti, E. L., Narbey, M. J., Rowland, C.Y., 1990. *J. Nat. Prod.*, 53(2), 520-522.
- Hamann M. T., Scheuer, P. J., 1993., *J. Am. Chem. Soc.*, 115, 5825.
- Hopper, J. W., Marlow, W., Whally, W. B., Borthwick, A. D., and Bowden, R., 1971. *Chem, Commn.*, 111-112.



- Hossiau, F. A., De Deuxchaisnes, C. N., 1990. *Clin. Exp. Rheumatology*, 8, 531.
- Han, H., 2010. *Biochemistry*, 49, 4276–4282.
- Haritakun, R., Rachtawee, P., Chanthaket, R., Boonyuen, N., Isaka M., 2010. *Chem. Pharm. Bull.*, 58(11), 1545-1548.
- Hwang, C., Catanaga, M., Gale, A., Gatanaga, T., 1993. *J. Immunology*, 151, 5631-5638.
- Inamori, Y., Kato, Y., Kubo, M., Takemoto, T., Nomoto, K.. 1983. *Chem. Pharm. Bull.*, (Tokyo) 31(12), 4543–4548.
- Isaka, M., Jaturapat, A., Rukseree, K., Danwisetkanjana, K., Tanticharoen, M., Thebtaranonth, Y., 2001. *J. Nat. Prod.*, 64, 1015-1018.
- Jiang, T., Li, T., Li, J., Fu, H., Peic, Y., Lin, W., 2004. *J. Asian Nat. Prod. Res.*, 6(4), 249-257.
- Kawakami, Y., 1999. *Proc. Natl. Acad. Sci.*, USA 96, 2227-2232.
- Keki, S., Sz. Szilagyi, L., Deak G., Zsuga, M., 2003. *Rapid Commun. Mass Spectrom.*, 17, 783.
- Kiriyama, N., Nitta, K., Sakaguchi, Y., Tagushi, Y., Yamamoto, Y., 1977. *Chem. Pharm. Bull.*, (Tokyo) 25(10), 2593-2601.
- Kohno, J., Nonaka, N., Nishio, M., Ohnuki, T., Kawano, Kimio., Okuda, Toru., Komatsubara, Saburo., 1999. *J. Antibiotics*, 52, 575-577.
- Knockaert, M., Greengard, P., Meijer, L., 2002. *Trends Pharmacol. Sci.*, 23, 417-425.
- Kurobane, I., Vining, L.C., McInnes, A.G., 1979. *J. Antibiot.*, 32, 1256–1266.
- Kwon, Y. J., Zheng, C.J., Kim, W.G., 2010. *Biosci Biotechnol Biochem.* 74(2), 390-393.
- Lee, S., M., Li, X. F., Jiang, H., Cheng, G. J., Seong, S., Choid, H. D., Sona, B. W., 2003. *Tetrahedron Lett.*, 44, 7707-7710.

- Li, G., Li, B., Yang, T., Yin, J., Qi, H., Liu, G., Zhang G., 2005. *J. Nat. Prod.* 68, 1243-1246.
- Lin, T., Lu, C., Shen, Y., 2009. *Nat.Prod. Res.*, 23(1), 77-85.
- Lin, Z. J., Zhu, T. J., Fang Y. C., Gu, Q. Q., 2008. *Magn. Reson. Chem.*, 46, 1212-1216.
- Malik, S., 1992. *British Med. Journal*, 305, 265.
- Nishio, K., Ishida, A., Arioka, H., Kurokawa, H., Fukuoka, K., Nomoto, T., Fukumoto, H., Yokote, H., Saijo, N., 1996. *Anticancer Res.*, 16, 3387-3395.
- Nitta, K., Fujita, N., Yoshimura, T., Arai, K., Yamamoto, U., 1983. *Chem. Pharm. Bull.*, (Tokyo) 31 (5), 1528-1533.
- Niu, X., Dahse, H., Menzel, K., Lozach, O., Walther, G., Meijer, L., Grabley, S., Sattler, I., 2008. *J. Natl. Prod.*, 71, 689–692.
- Nuclear, P., Sommit, D., Boonyuen, N., Pudhom, K., 2010. *Chem. Pharm. Bull.*, 58(9) 1221-1223.
- Numata, A., Takahashi, C., Matsushita, T., Miyamoto, T., Kawai, K., Usami, Y., Matsumura, E., Inoue, M., Ohishi, H., Shingu, T., 1992 *Tetrahedron Lett.*, 33, 1621.
- Ojima, N., Takenaka, S., Seto, S., 1973. *Phytochemistry*, 12, 2527-2529.
- Omura, S., Kuno, F., Otoguro, K., Sunazuka, T., Shiomi, K., Masuma, R., Iwai, Y. 1995. *J. Antibiot. (Tokyo)* 48, 745–746.
- Parneswaran, P. S., Gawas, D., Tilvi, S., Naik, C. G., 2004. *Proceedings of MBR: National Seminar On New Frontiers On Marine Bioscience Research*, 3-9.
- Pazoutova, S., Hamplova, I., Anderson, M., Denning, D. W., 1999. *Folia Microbiol (Praha)*, 44(5), 503-509.
- Petuchow, A. G., Tschernowa, N. G., Tischschow, W. A., Belenkaja, N. K., 1980. SU- Patent 732 379

- Phattanawasin, P., Pojchanakom, K., Sotanaphun, U., Piyapolrungrroj, N., Zungsontiporn, S., 2007. *Nat. Prod. Res.*, 21(14), 1286-1291.
- Proksch, P., Putz, A., Ortlepp, S., Kjer J., Bayer, M., 2010. *Phytochem. Rev.*, 9, 475-489.
- Proksch, P., Ebel, R., Edrada-Ebel, R. A., Riebe, F., Liu, H., Diesel, A., Bayer, M., Li, X., Lin, W.H., Grebenyuk, V., Muller, W. E. G., Draeger, S., Zuccaro, A., Schulz, B., 2008. *Botanica Marina*, 51, 209–218.
- Raistrick, H., Rudman, P., 1956. *Biochem. J.*, 63, 395-406.
- Raistrick, H., Smith, G., 1936. *Biochem. J.*, 30, 1315–1322.
- Rao, K.V., Sadhukhan, A.K., Veerender, M., Mohan, E.V.S., Dhanvantri, S.D., Sitaramkumar, S., Babu, M. J., Vyas, K., Reddy, O. G., 2000. *Chem. Pharm. Bull.*, (Tokyo) 48 (4), 559–562.
- Reddy, K. R. N., Reddy, C.S., Muralidharan, K., 2005. *J. Mycol. Pl. Pathol.*, 35(3), 470-474.
- Rochfort, S., Ford, J., Ovenden, S., Wan, S. S., George, S., Wildman, H., Tait, R. M., Meurer-Grimes, B., Cox, S., Coates, J., Rhodes, D., 2005. *J. Antibiot.*, 58(4), 279–283.
- Ruijter, G. J. G., Kubicek, C. P., Vissler, J., 2002. In: *The Mycota Vol. X. Industrial Applications*, Osiewacz, H.D., ed. (Heidelberg: Springer-Verlag), 213–230.
- Samson, R. A., Noonim, P., Meijer, M., Houbrakenm, J., Frisvad, J. C., Varga, J., 2007. *Studies in Mycology* 59, 129–145.
- Shapiro, G. I. J., 2006. *Clin. Oncol.*, 24, 1770–83.
- Sheehan, J. C., Lawson W. B., Gaul R. J., 1958, *J. Am. Chem. Soc.*, 80 (20), 5536-5538
- Sugiyama, Y., Yoshida, K., Abe, Ni., Hirota A., 2010. *Biosci. Biotechnol. Biochem.*, 74 (4), 881-883.

- Suzuki, M., Hosaka, Y., Matsushima, H., Goto, T., Kitamura, T., Kawabe, K., 1999. *Cancer Lett.*, 138, 121–130.
- Tilvi, S., Naik, C. G., 2007. *J. Mass. Spectrom.*, 42(1), 70-80.
- Valera, H.R., Gomesa, J. Lakshmi, S., Gururaja, R., Suryanarayan, S., Kumar, D., 2005. *Enzyme and Microbial Technology*. 37, 521–526.
- Watts, J. D., Gu, M., Polverino, A. J., Patterson S. D., Aebersold, R., 1997. *Proc. Natl. Acad. Sci., USA*, 94, 7292.
- Wijeratne, E. M. K., Turbyville, T. J., Zhang, Z., Bigelow, D., Pierson, L. S., VanEtten, H. D., Whitesell, L., Canfield, L. M., Gunatilaka, A., A. L., 2003. *J. Nat. Prod.*, 66, 1567-1573.
- Wu, S.-H., Chen, Y.-W., Qin, S., Huang, R., 2008. *J. Basic Microbio.*, 48, 140-142.
- Yamamoto, Y., Nishimura, K., Kiriya, N., 1976. *Chem. Pharm. Bull.*, 24(8), 1853-1859.
- Yang, D.-M., Takeda, N., Iitaka, Y., Sankawa, U., Shibata, S., *Tetrahedron*, 1973, 29, 519– 528.
- Zhou, G., Wijeratne, E. M. K., Bigelow, D. Pierson, L. S., VanEtten, H. D., Gunatilaka A. A. L., 2004 *J. Nat. Prod.*, 67, 328-332.