

## Investigation of Secondary Metabolites from Marine-Derived Fungi *Aspergillus*.

Supriya Tilvi,<sup>\*[a][d]</sup> Rajesh Parvatkar,<sup>[b]</sup> Avinash Awashank,<sup>[c][d]</sup> Safia Khan<sup>[a]</sup>

<sup>[a]</sup> Bio-organic Chemistry Laboratory, Chemical Oceanography Division,  
CSIR-National Institute of Oceanography, Donapaula Goa;  
E-mail:supriyatilvi@nio.org

<sup>[b]</sup> Department of Chemistry, Government College of Arts, Science and Commerce,  
Sankhali, Goa, India 403505

<sup>[c]</sup> CSIR-National Institute of Oceanography, Regional Centre, Four Bungalows,  
Andheri (West), Mumbai, Maharashtra, India-400053.

<sup>[d]</sup> Academy of Scientific and Innovative Research (AcSIR), Ghaziabad, Uttar Pradesh, India.-201002.

### Abstract:

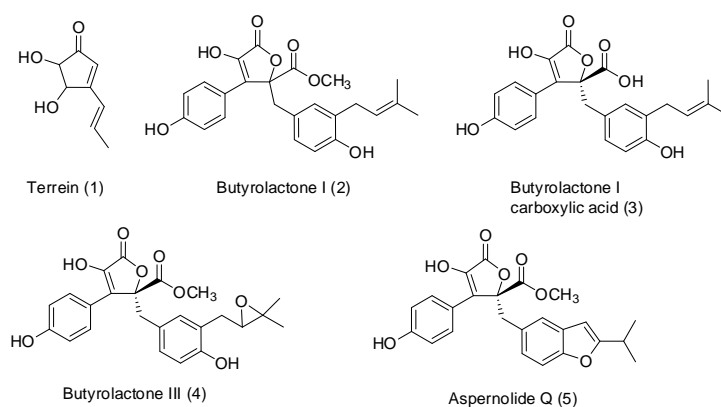
Marine fungi *Aspergillus flavipes* and *A. terreus* were investigated for secondary metabolites using electrospray ionization-quadrupole time-of-flight mass spectrometry (ESI-QTOF MS) analysis. The ESI-MS analysis showed that *A. flavipes* showed molecular ion peaks for compounds, terrein (**1**), butyrolactone I (**2**), butyrolactone I-carboxylic acid (**3**), butyrolactone III (**4**), while, *A. terreus* had additional presence of new compound aspernolide Q (**5**) with absence of compound (**3**). By using tandem mass spectrometry data, the prominent fragment ions helped in the establishing the structure of compounds (**2-5**). Further, chemical transformation of butyrolactone I to butyrolactone III and aspernolide Q were carried out followed by NMR analysis which strongly suggested their structures. The evaluation of salt stress experiment of *A. terreus* culture medium affected the production of major bioactive metabolites, terrein and butyrolactone I (**2**). The HPLC analysis showed that culture produces high amount of terrein at 35 ‰, while, maximum production of butyrolactone I was observed in fresh water i.e. 0‰. The study highlighted an importance of salinity in producing these metabolites where, terrein and butyrolactone I could be produced in equal ratio at 18‰. Importantly, we have demonstrated that using this fungal strain and by growing in medium at specific salinity could be way forward for commercial production of these bioactive compounds.

**Keywords:** *Aspergillus*. Butenolide. Fungi. Marine. Tandem Mass spectrometry. Terrein

## Introduction

Butenolides (butyrolactone derivatives) are a group of natural products consisting of three moieties: an  $\alpha,\beta$ -unsaturated- $\gamma$ -lactone and two phenyl moieties. Fungi belonging to genus *Aspergillus* both terrestrial and marine are found to be rich source of butenolides.<sup>[1-4]</sup> Butyrolactone-I shows a  $\alpha$ -glucosidase inhibitor activity for type 2 diabetes.<sup>[5]</sup> Butyrolactones I and V have shown antioxidant activity and also exhibited anti-cancer activity against the breast tumor cells MDA-MB-231 and MCF-7.<sup>[6]</sup> Asperbutenolide A, an unusual aromatic butenolide dimer derived from marine *A. terreus* exhibited strong cyclooxygenase-2 and  $\alpha$ -glucosidase inhibitory activities.<sup>[7]</sup> Similarly, versicolactone G<sup>[8]</sup> and aspernolides N-P<sup>[9]</sup> are reported to show potent  $\alpha$ -glucosidase inhibitory activity. So till date, there are several reports of bioactive butenolides and aromatic butenolides from *Aspergillus* covered in different reviews.<sup>[10-11]</sup>

In the recent years, liquid chromatography and tandem mass spectrometry (MS/MS) have been extensively used for quantitative and qualitative metabolomics studies.<sup>[12]</sup> With high sensitivity and selectivity, MS offers quantitative analysis and characterization of natural products.<sup>[13]</sup> Tandem or hybrid mass analyzers allows identification of compounds without prior purifications by generating highly resolved and accurate product ion spectra which can be achieved through ion fragmentation by collision-induced dissociation (CID).<sup>[14]</sup> In our earlier work, we have demonstrated use of electrospray ionization-tandem mass spectrometry for identification ceramides, kahalalides (cyclodepsipeptides), bromotyrosine alkaloids (purpurealidins A-K) from *Haliclona cribricutis*, *Elysia grandifolia* and *Psammaphysilla purpurea*, respectively. These work demonstrated the capabilities of tandem mass spectrometry in identification of related compounds which are produced in trace quantity by the organisms without any prior chromatographic purification. Earlier we have isolated and characterized various metabolites, aspernolides A-B<sup>[15]</sup>,  $4\beta$ -[(1*E*)-propen-1-yl]cyclopentane-1 $\beta$ ,2 $\beta$ -diol and its diastereoisomer,  $4\beta$ -[(1*E*)-propen-1-yl]cyclopentane-1 $\beta$ ,2 $\alpha$ -diol, (+)-terrein, butyrolactone I-II and physcion from *Aspergillus* fungi.<sup>[16]</sup> However, we may have missed identification of several metabolites from both the cultures and hence, we made an effort of identifying those molecules using ESI-MS and collision induced dissociation (CID)-MS/MS studies. In this study, we have successfully identified five compounds terrein (**1**), butyrolactone I (**2**), butyrolactone I-carboxylic acid (**3**), butyrolactone III (**4**) and new compound, aspernolide Q (**5**) (**Figure 1**). The chemical transformation studies strongly suggested the structures of compounds (**4**) and (**5**). In addition, how the variation in the salinity of culture medium, changes the proportion of the major compounds terrein (**1**) and butyrolactone I (**2**) has been described using reversed phase HPLC.



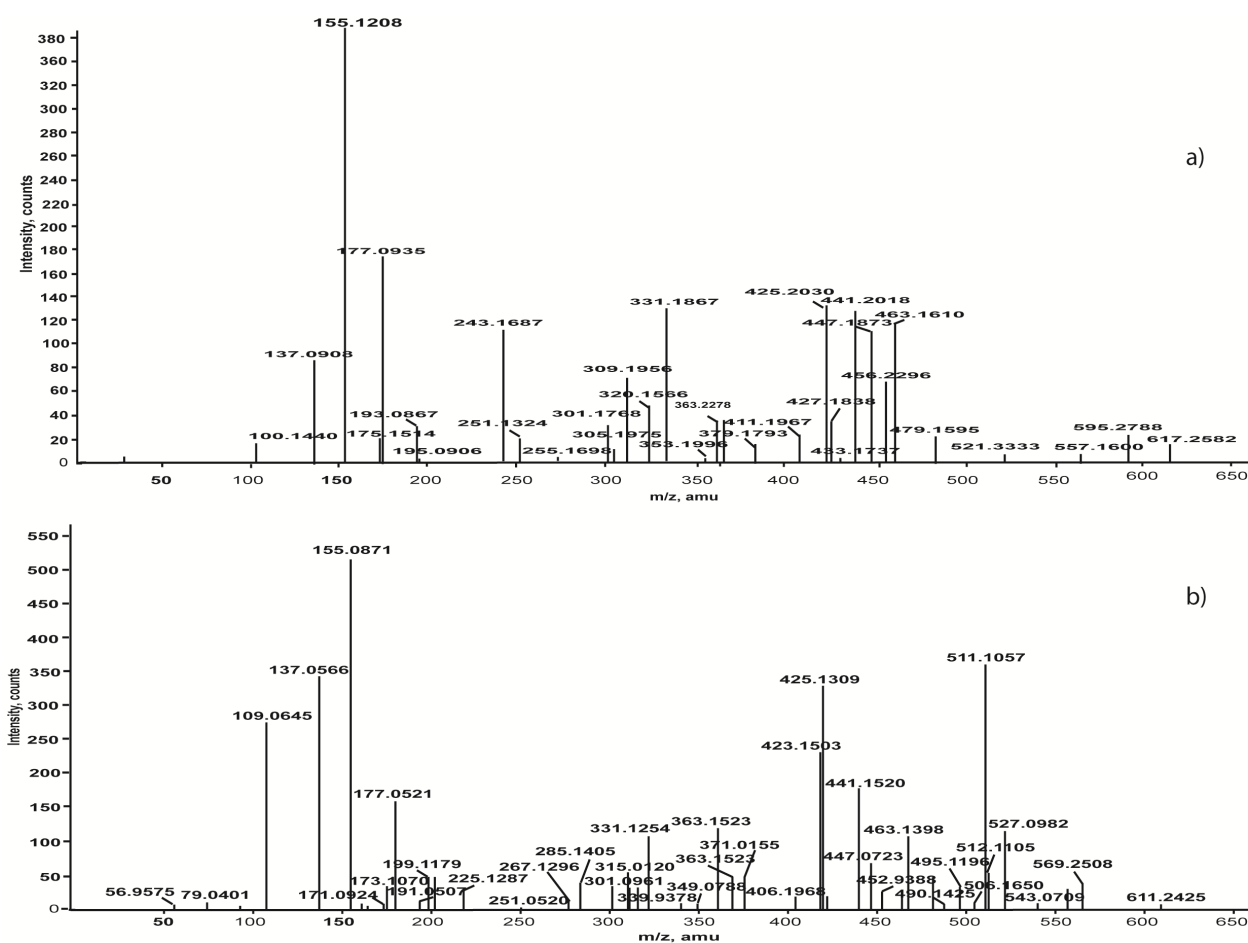
**Figure 1.** Structures of compounds (1-5) identified from marine fungi *Aspergillus*

## Results and discussion

*Aspergillus* fungi are known to produce several butenolides like butyrolactones I-VII,<sup>[17-19]</sup> aspulvinones,<sup>[20]</sup> aspernolides,<sup>[21]</sup> rubrolides etc.<sup>[22]</sup> having  $\gamma$ -butyrolactone moiety substituted with hydroxyl, carboxylate and prenyl substituted *p*-hydroxybenzenes groups. In the present study, ESI-MS/MS prominent sites of fragmentation based on which, it becomes possible to assign structures of the biosynthetically related metabolites (**Figure S1b**). The ethyl acetate extracts obtained from the culture media of two fungi *A. flavipes* and *A. terreus* were subjected to ESI-QTOF instrument by direct infusion (**Figures 2a-b**). The spectra showed several molecular ion peaks with  $m/z$  ranging 150-700 amu in the positive ion mode. On comparison, **Figures 2a** and **2b**, it was observed that some of the molecular ions peaks were not matching indicating few molecules to be different in both strains. Based on these observations and studying molecules reported in the literature, we have tentatively assigned some of the peaks to the known metabolites that may be present in the fungi (**Table S1**) which were suggested after detailed MS/MS analysis.

In the positive ion mode, the ESI-MS spectrum (**Figure 2a**) of *A. flavipes* showed molecular ion peaks of known compounds. The peaks at  $m/z$  155[M+H]<sup>+</sup>, 177[M+Na]<sup>+</sup>, and 193[M+K]<sup>+</sup> was observed for terrein (**1**) which could be confirmed from our previous study.<sup>[15]</sup> The cluster of peaks at  $m/z$  425[M+H]<sup>+</sup>, 447[M+Na]<sup>+</sup>, 463[M+K]<sup>+</sup> and  $m/z$  411[M+H]<sup>+</sup>, 433[M+Na]<sup>+</sup> were identified for butyrolactone I (**2**) and butyrolactone I-carboxylic acid (**3**), respectively. In addition, peaks at  $m/z$  441[M+H]<sup>+</sup>, 463[M+Na]<sup>+</sup> and 479[M+K]<sup>+</sup> were attributed for butyrolactone III (**4**). It was interesting to note that, the mass spectrum for *A. terreus* showed an additional molecular ion peak at  $m/z$  423[M+H]<sup>+</sup> for a new compound, aspernolide Q (**5**) while, there was absence of butyrolactone I-carboxylic acid (**3**) in it. The structures were identified after subjecting each molecular ion peaks to

the collision induced dissociation and by studying mass fragment ions which has been discussed in the detail. **Table S1** gives molecular ion peaks observed in ESI-MS of *A. flavipes* and *A. terreus*.



**Figure 2:** ESI-MS of EA extract of fungi in positive ion mode (a) *Aspergillus flavipes* and (b) *A. terreus*

**Identification of butyrolactone I (2) and butyrolactone I-carboxylic acid (3) using MS/MS analysis:**

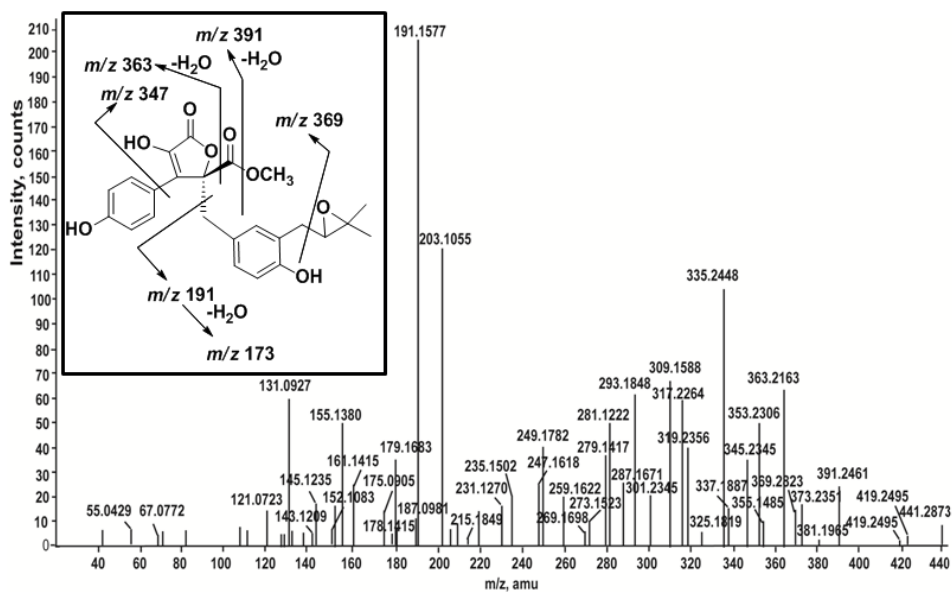
**MS/MS at  $m/z$  425 $[M+H]^+$  of butyrolactone I (2):** The product ion spectrum of butyrolactone I (2) at  $m/z$  425  $[M+H]^+$  gave several fragment ions (**Figure S2**). The cleavage at ‘T’ gave fragment  $m/z$  175 retaining the ring B carrying prenyl chain. The elimination of  $CH_3OH$  molecule gave fragment ion at  $m/z$  393. Further, elimination of  $-COOCH_3$  i.e cleavage at ‘Q’ gave fragment ion at  $m/z$  365. Fragment ion at  $m/z$  369 was formed by a benzylic cleavage ‘S’ of the prenyl group thereby eliminating  $C_4H_8$  moiety. Cleavage at ‘P’ displayed peak at  $m/z$  331 eliminating phenolic moiety.

**MS/MS at  $m/z$  411  $[M+H]^+$  ion of butyrolactone I carboxylic acid (3):** Another protonated ion analyzed was  $m/z$  411 which was 14 mass units less than butyrolactone I (2). Careful observation of

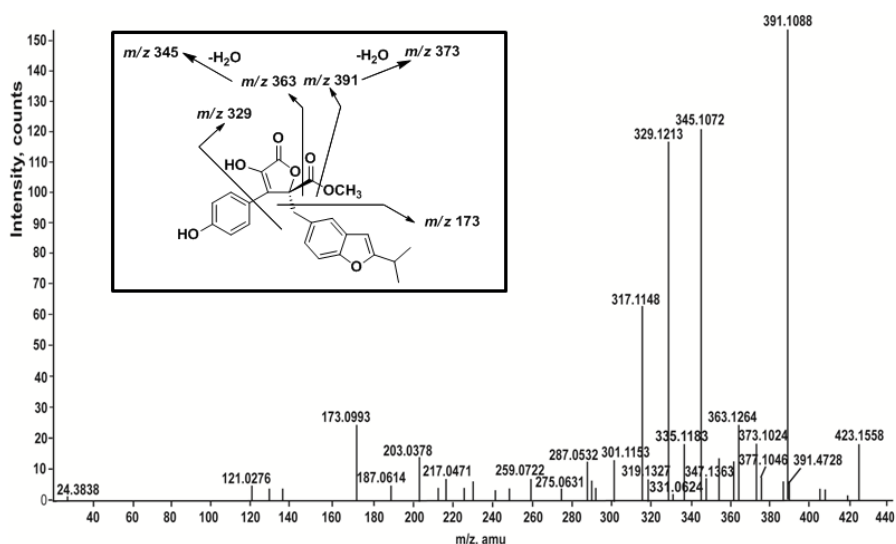
MS/MS data showed that some fragment ions  $m/z$  175, 393 and 365 for cleavage at 'U', 'R', 'Q', respectively were retained as shown in butyrolactone I (2) (**Figure S3**). This revealed that the modification has occurred in the carboxylate ester group which could be replaced with carboxylic acid and thus molecule has molecular weight 410 amu. This was further suggested by the presence of  $[M+Na]^+$  at  $m/z$  433 in ESI-MS. Cleavage 'P' of ring A phenol moiety with elimination of  $-CO_2$  gave  $m/z$  303. Fragment ion at  $m/z$  351 appeared for the benzylic cleavage 'S' on prenyl group by elimination of  $C_4H_8$  along with loss of water. The cleavage at ring B with prenyl moiety gave fragment ion  $m/z$  160. The elimination of ring B with prenyl moiety gave  $m/z$  240 which further on decarboxylation ( $-CO_2$ ) resulted in ion  $m/z$  221. Based on the fragment ions formed and their assignment strongly suggested the structure of the molecule as butyrolactone I-carboxylic acid (3).

**Identification of Butyrolactone III (4) using MS/MS analysis:** A molecular ion  $[M+H]^+$  at  $m/z$  441 was present in both the extracts indicating presence of molecule along with their corresponding sodiated and potassiated molecular ions at  $m/z$  463 $[M+Na]^+$  and  $m/z$  479 $[M+K]^+$ , respectively (**Figure 3**). The molecular ion suggests that, it is 16 units more as compared to butyrolactone I (2). From the literature reports, it was known that there are three butyrolactone derivatives, butyrolactone III, IV and V with molecular weight 440 amu majorly isolated from *A. terreus*. The product ion spectrum of a protonated molecular ion at  $m/z$  441 gave fragment ions that match very well with that of butyrolactone III (4). Fragment ion at  $m/z$  369 clearly indicates the presence of additional oxygen on the terminal C-4 unit of prenyl chain which would be possible only in the case of (4). Since, butyrolactone IV and V would give fragment ion at  $m/z$  368 as shown in **Figure S4**.

**Identification of aspernolide Q (5) using MS/MS analysis:** The molecular ion  $[M+H]^+$  at  $m/z$  423 is two mass units smaller than butyrolactone I (1) and the characteristic fragment ions by elimination of  $-CH_3OH$  and  $-COOCH_3$  are displayed at  $m/z$  391 and 363 with further, loss of  $-H_2O$  gave peaks at  $m/z$  373 and 345, respectively (**Figure 4**). The other fragment ion  $m/z$  173 indicated that the modification has occurred in the ring B side of the molecule while, substituted- $\gamma$ -butyrolactone moiety with ring A remained intact. The loss of phenol moiety gave fragment ion at  $m/z$  329. Cyclization at ring A with prenyl moiety removed two mass units that fitted the molecular ion at  $m/z$  423 which gave structure of new compound aspernolide Q (5).



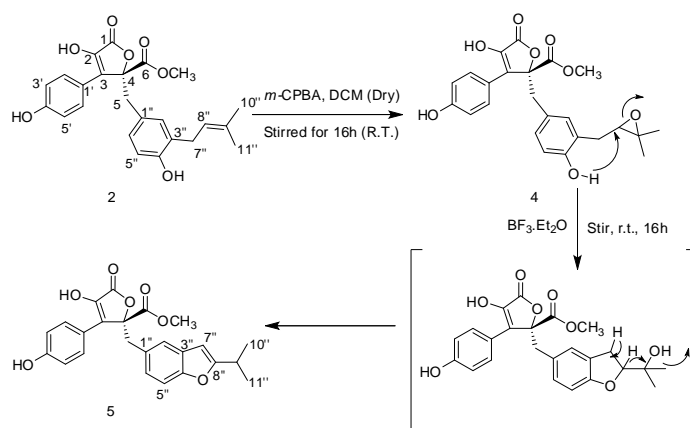
**Figure 3:** Product ion spectrum of butyrolactone III (**4**) at  $m/z$  441  $[M+H]^+$



**Figure 4:** Product ion spectrum of aspernolide Q (**5**) at  $m/z$  423  $[M+H]^+$

**Preparation of butyrolactone III (3) and aspernolide Q (5):** The major compound, butyrolactone I (**2**) was purified and obtained as colorless crystalline solid. Its structure was confirmed by NMR data and compared with our previous data published.<sup>[16]</sup> To confirm the structures of butyrolactone III (**4**) and aspernolide Q (**5**), both compounds were prepared using butyrolactone I (**2**) as starting material. The treatment of butyrolactone I (**2**) with mCPBA in anhydrous DCM yielded butyrolactone III (**4**) in 78% yield. This on further treatment with a Lewis acid,  $BF_3 \cdot Et_2O$  gave isomerized dehydrated product (**5**) (**Scheme 1**). The ESI-MS of both the products (**4**) and (**5**) were recorded. The molecular ion peaks appeared at  $m/z$  441  $[M+H]^+$ ,  $m/z$  463  $[M+Na]^+$  and  $m/z$  479  $[M+K]^+$  for product (**4**). While, product (**5**) showed peaks at  $m/z$  at 423  $[M+H]^+$ , 445  $[M+Na]^+$  and 461  $[M+K]^+$  as expected. On

comparison of MS/MS studies of synthetic products with natural ones gave similar results, strongly suggesting that the structures assignment of (4) and (5) are correct. Further, structure elucidation was carried out using NMR data. The detailed  $^1\text{H}$  and  $^{13}\text{C}$  NMR data analysis (Table 1, Figures S5-S6) accounted for all the hydrogen and carbons to the assigned structures. The proton signals for 1,4-disubstituted benzene moiety were observed at  $\delta_{\text{H}}$  7.64 (2H, d,  $J=8.1\text{Hz}$ ) and 6.97 (2H, d,  $J=8.7\text{Hz}$ ) while, signals  $\delta_{\text{H}}$  6.92 (s, 1H), 6.66 (1H, d,  $J=8.1\text{Hz}$ ), and 7.15 (1H, d,  $J=8.4\text{Hz}$ ) for a presence of 1,3,4-trisubstituted benzene moiety. A methylene proton  $\delta_{\text{H}}$  3.68 (2H, d,  $J=12.0\text{Hz}$ ) and methoxy protons 3.78 (3H, s) indicated molecule resembling butenolide scaffolds.



**Scheme 1:** Chemical transformation of butyrolactone I (2) to butyrolactone III (4) and aspernolide Q (5)

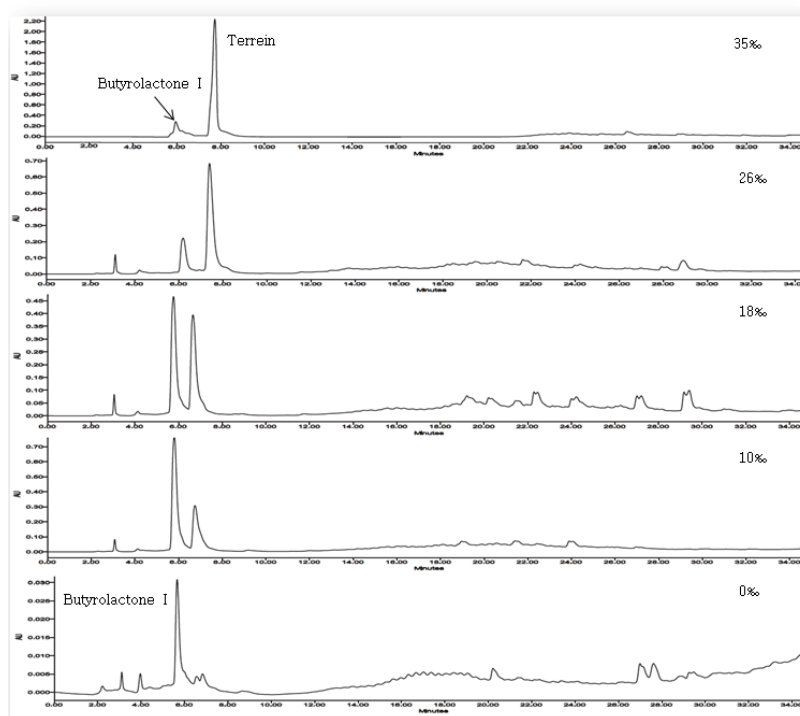
In addition, carbon signals at  $\delta_{\text{C}}$  168.9 (C-1), 137.0 (C-2), 128.7 (C-3), 86.0 (C-4), 39.2 (C-5) and 169.7 (C-6) were characteristic of butyrolactone III. On comparison the very visible changes were seen in the NMR data, as the result of epoxidation of the prenyl double bond in product (4). There was disappearance of vinylic proton (H-8'') and a new signal appeared for oxymethylene at  $\delta_{\text{H}}$  3.75 (m, H-8'') and  $\delta_{\text{C}}$  69.5 (C-8''). The carbon at C-9'' was observed at  $\delta_{\text{C}}$  76.7 and two methyls attached to it were observed at  $\delta_{\text{C}}$  22.2 ( $\delta_{\text{H}}$  1.24, s, H-10'') and 24.6 (1.30, s H-11''). In the case of product (5), the dimethyls were observed at  $\delta_{\text{C}}$  20.8 ( $\delta_{\text{H}}$  1.30, d, H-10'', 11''). The major changes were observed for C-7'', C-8'' and C-9'' which displayed signals at  $\delta_{\text{C}}$  99.7 ( $\delta_{\text{H}}$  6.19, s), 165.1 and, 28.1 ( $\delta_{\text{H}}$  3.00, m), respectively. The MS/MS was carried out for synthetic aspernolide Q (5) (Figure S7) in which all the peaks matched with that of natural compound.

<b>Table 1:</b> NMR data of butyrolactone III ( <b>4</b> ) and aspernolide Q ( <b>5</b> ) recorded in CDCl <sub>3</sub> (Bruker 300 MHz)				
<b>Butyrolactone III (4)</b>			<b>Aspernolide Q (5)</b>	
<b>Carbon No.</b>	<b>δ<sub>C</sub>, Mult.</b>	<b>δ<sub>H</sub>, mult., (J, Hz)</b>	<b>δ<sub>C</sub>, mult.</b>	<b>δ<sub>H</sub>, mult., (J, Hz)</b>
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	3.44, d, (15.3) 3.53, d, (15.3)	39.2 t	3.68, d, (12.0)
6	169.8s		169.7 s	
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	20.8 q	1.30, d, (6.6)
11''	24.6 q	1.30, s	20.8 q	1.30, d, (6.6)
6-OMe	53.5 q	3.78, s	53.5 q	3.79, s

**Salt stress response on the production of terrein and butyrolactone I in *A. terreus*:** Growth medium used for fermentation of microbes contains major carbon and nitrogen along with other inorganic components that support growth and production of natural products. However, other conditions such as pH, temperature, agitation speed, salinity etc. may play a very crucial role in fermentation of certain microorganisms. Aquatic fungi are a widespread and phylogenetically heterogeneous that occurs in marine, estuarine, and freshwater systems where salinity represents a frequently fluctuating environmental variable. They are known to adapt rapidly, colonize, grow and reproduce. However, effects of salinity remain poorly understood especially in the expression of secondary metabolite. Herein, we have investigated the effect of salt stress on for the variation in the production of important bioactive metabolites in the *A. terreus* and monitored using reverse phase HPLC. The HPLC showed that major metabolites produced are terrein (**1**) and butyrolactone I (**2**). The fungi were cultured in PDB at varying salinities 35, 26, 18, 10 and 0 ‰. The chromatographic analysis using RP-C18 showed overall variation in the metabolite profile at different salinities (**Figure 5, Table S1**). As expected, the major peaks were for butyrolactone I and terrein eluted at Rt 6.0 and 7.5 mins using isocratic mobile phase water:acetonitrile (56:44), respectively and area %



gave their abundance. It was observed that at higher salt stress i.e. 35‰, there was presence of terrein (92%) as major compound. As the salt stress was reduced, there was production of butyrolactone I. For instance, when fungus was grown at salinity 26‰, along with terrein production reduced to 79% and there was slight production of butyrolactone I with 21%.



**Figure 5:** HPLC Chromatograms showing metabolite profiling of *A. terreus* at different salinities.

It is interesting to note that, at 18‰ salinity both the compounds are produced in a close to equal ratio with butyrolactone I and terrein in 54 and 46 %, respectively. Further reduction in salinity 10 ‰ of culture medium, decreases the production in the terrein (33%) while, major metabolite production of butyrolactone I (95%) was obtained at 0 ‰ salinity media culture. Considering the production of other secondary metabolites, it was observed that maximum number of chromatographic peaks was observed when fungus was grown in 18‰ salinity culture medium as compared when grown at other salinities. Thus, high salt stress was seen promising to enhance the production of terrein, while, low salinity culture conditions were better for butyrolactone I production. According to various reports, these molecules exhibit promising biological activities. In our previous investigation, we have revealed an anti-inflammatory activities of these compounds.<sup>[16]</sup> (+)-Terrein displayed anti-inflammatory activity with  $IC_{50}$  8.5  $\mu$ M (IL-6) and 15.76  $\mu$ M (TNF- $\alpha$ ), While, butyrolactone I, exhibited  $IC_{50}$  12.03  $\mu$ M (IL-6) and 43.29  $\mu$ M (TNF- $\alpha$ ). It has been reported that (+)-Terrein displayed significant anticancer activities against different cancer lines.<sup>[23-29]</sup> Terrein has been found to be quorum sensing and c-di-GMP inhibitor in *P. aeruginosa*.<sup>[30]</sup> Besides being a selective cdk2 kinase inhibitor on human lung cancer cell lines,<sup>[31]</sup> butyrolactone I have been found to possess anti-

inflammation,<sup>[32]</sup> antioxidant activities<sup>[33]</sup> and anti-neuro-inflammatory effect on LPS-induced BV-2 cells.<sup>[34]</sup> In the recent report, it was revealed to show potent cytotoxicity towards ovarian adenocarcinoma cells (SKOV3) lines and human metastatic prostate cells (PC-3) with IC<sub>50</sub> of 0.6 and 4.5 µg/mL, respectively.<sup>[35]</sup> So, it becomes important to produce these molecules via commercially viable process and therefore, the simple method demonstrated here could give scope for its industrial production.

## Conclusion

Overall, ESI-MS/MS gave quick identifications of compounds (**1-4**) along with new compound, aspernolide Q (**5**) from marine fungi *A. flavipes* and *A. terreus* without subjecting them to chromatographic purifications. The preparation of butyrolactone III and aspernolide Q was achieved by utilizing butyrolactone I as starting material in just single step. Evaluation of different salinities in culture medium gave metabolite profiling of *A. terreus*. More importantly, the experiment and HPLC analysis showed that at what salinity conditions are required for maximum production of bioactive compounds, terrein and butyrolactone I which can be used for commercially.

**Supporting Information Summary:** This segment contains the spectral data and experimental Section.

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**Conflict of Interest:** The authors declare no conflict of interest.

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## Supporting Information

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### Experimental Section

#### *General experimental procedures*

Column chromatographies were carried out using Silica gel (60–120 mesh, Qualigens). Fractions were monitored on thin layer chromatography (TLC) using alumina-backed sheets (Si gel 60 F254, 0.25 mm thick) with visualization under UV (254 nm) and 30% methanolic sulphuric acid spray reagent. <sup>1</sup>H and <sup>13</sup>C NMR experiments were recorded in CDCl<sub>3</sub> on a Bruker 300MHz spectrometer with TMS as internal standard. Mass spectra were recorded on ESI-QTOF MS/MS (PE Sciex-QSTAR Applied Biosystems, Canada). The sample was prepared in methanol (MS grade) and directly infused in the MS at a constant flow rate of 5 µl/min into the ion spray source using an integrated syringe pump. The instrument was operated in positive ionization mode. The MS scans were performed in the mass range from  $m/z$  100 to  $m/z$  2000. The product ion spectra of selected precursor ions were obtained by collision-induced dissociation (CID) using collision energy ranging between 20-60V. The mass was analyzed using TOF analyzer of the instrument.

#### *Isolation and culturing of fungi*

Fungus *Aspergillus flavipes* was isolated from mangrove *Acanthus illicifolius* pneumatophores. The mangrove was collected Banastarim (Goa, India). Another fungi *A. terreus* was derived from soft coral collected from Mandapam (Tamil Nadu, India). Standard techniques were used for the isolation of fungi on potato dextrose agar (PDA) in sea water:distilled water (75:25). For the mass spectrometric analysis, both the fungi were grown in 50 ml PDB containing seawater:distilled water (75:25). The flasks were incubated for 21 days under static condition and ethyl acetate extract was obtained for MS analysis.

*Preparation of compounds (i) butyrolactone III and (ii) aspernolide Q (5)*

(i) 75 mg of butyrolactone I (**2**, 0.177 moles) was added to *m*CPBA (39.6 mg, 0.230 moles) along with 10 ml DCM (dry) and stirred under inert atmosphere for 16 hrs. The reaction was stopped by addition of Liq NH<sub>3</sub> after monitoring TLC. The resultant mixture was extracted with ethyl acetate. The extracted product was purified on silica gel column chromatography using gradient elution of 0-5% methanol:chloroform to obtain butyrolactone III (**4**, 68 mg) as a colourless solid.

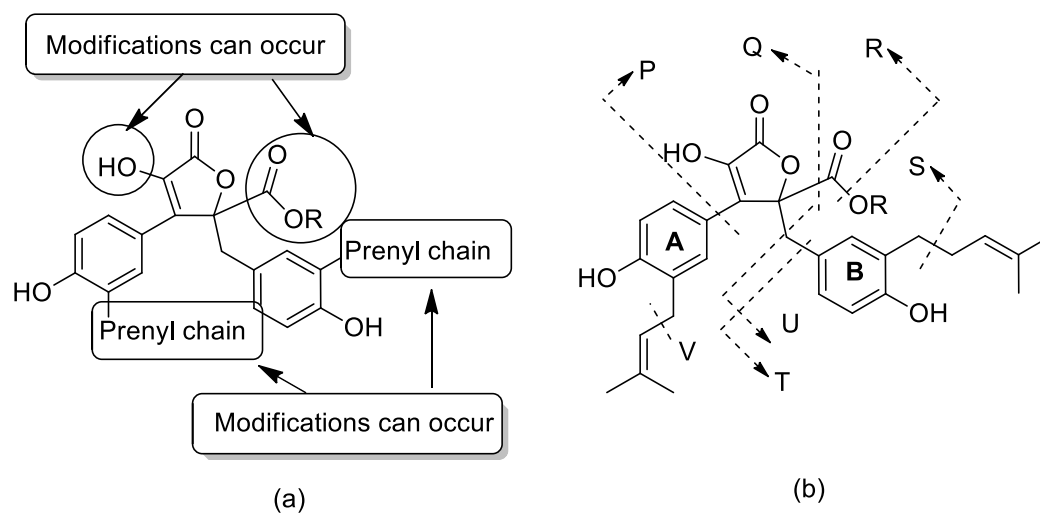
(ii) In the round bottom flask, 10 ml of BF<sub>3</sub>.Et<sub>2</sub>O and 30 mg of butyrolactone III (**4**) was mixed and stirred under anhydrous condition for 16 hrs. The reaction mixture was neutralized with aq NH<sub>3</sub> after checking thin layer chromatography (TLC) for complete conversion of (**4**) into a non polar compound. This was followed by extraction with ethyl acetate. After drying, the thick liquid compound was subjected to silica gel column chromatography using 0% to 5% methanol:chloroform gradient mobile phase which resulted in aspernolide Q (**5**) as sticky solid (10 mg).

*Production of terrein and butyrolactone I using HPLC*

Five flasks containing 100 ml PDB in each with salinities 35, 26, 18, 10 and 0 ‰ were prepared using sea water and distilled water (**Table S1**). After, sterilizing 0.5 ml of *A. terreus* culture inoculums were added to each flask. All the flasks were incubated for 21 days under static condition at 28°C. Each broth was extracted using ethyl acetate which was subjected to reverse phase HPLC analysis. 2.0 mg of ethyl acetate extracts were dissolved in 10 mL MeOH. These solutions were subjected to analytical HPLC system (Waters) using Sunfire 4.6 x 250 mm (id 5 µm) column at 254 nm wavelength. The mobile phase method used was isocratic with water and acetonitrile (56:44) for 30 min runtime and flow rate of 1.0 ml/min.

**Table S1: PDB media composition along with production of metabolites by HPLC, Area %**

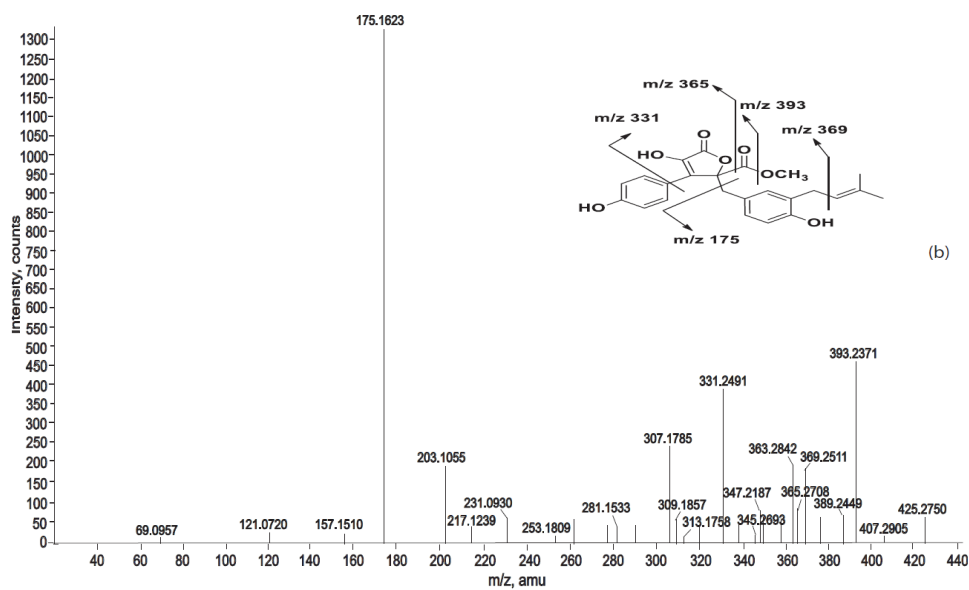
Flasks	Salinities (%)	Sea water (ml)	Distilled water (ml)	HPLC (Area %) Butyrolactone I (R <sub>t</sub> 6.0 min)	HPLC (Area %) Terrein (R <sub>t</sub> 7.5 min)
1	35	100	0	8	92
2	26	75	25	21	79
3	18	50	50	54	46
4	10	25	75	67	33
5	0	0	100	95	5



**Figure S1: a) Butenolide scaffold showing sites for biosynthetic modifications; b) Key MS fragment ions with labels**

**Table S2: Molecular ion peaks observed in ESI-MS of *A. flavipes* and *A. terreus***

Molecular Formula	Molecular ion peaks ( $m/z$ ) <i>A. flavipes</i>	Molecular ion peaks ( $m/z$ ) <i>A. terreus</i>	Compounds	Observed Mass for $[M+H]^+$ (amu)	Requires $[M+H]^+$ (amu)
$C_8H_{10}O_3$	155 $[M+H]^+$ 177 $[M+Na]^+$ 193 $[M+K]^+$	155 $[M+H]^+$ 177 $[M+Na]^+$ 193 $[M+K]^+$	Terrein ( <b>1</b> )	155.0871	155.0708
$C_{24}H_{24}O_7$	425 $[M+H]^+$ 447 $[M+Na]^+$ 463 $[M+K]^+$	425 $[M+H]^+$ 447 $[M+Na]^+$ 463 $[M+K]^+$	Butyrolactone I ( <b>2</b> )	425.1309	425.1600
$C_{23}H_{22}O_7$	411 $[M+H]^+$ 433 $[M+Na]^+$	Not observed	Butyrolactone I carboxylic acid ( <b>3</b> )	411.1967	411.1444
$C_{24}H_{24}O_8$	441 $[M+H]^+$ 463 $[M+Na]^+$ 479 $[M+K]^+$	441 $[M+H]^+$ 463 $[M+Na]^+$ 479 $[M+K]^+$	Butyrolactone III ( <b>4</b> )	441.1520	441.1549
$C_{24}H_{22}O_7$	Not observed	423 $[M+H]^+$	Aspernolide Q ( <b>5</b> )	423.1503	423.1444



**Figure S2: MS/MS at  $m/z$  425  $[M+H]^+$  of butyrolactone I (2).**



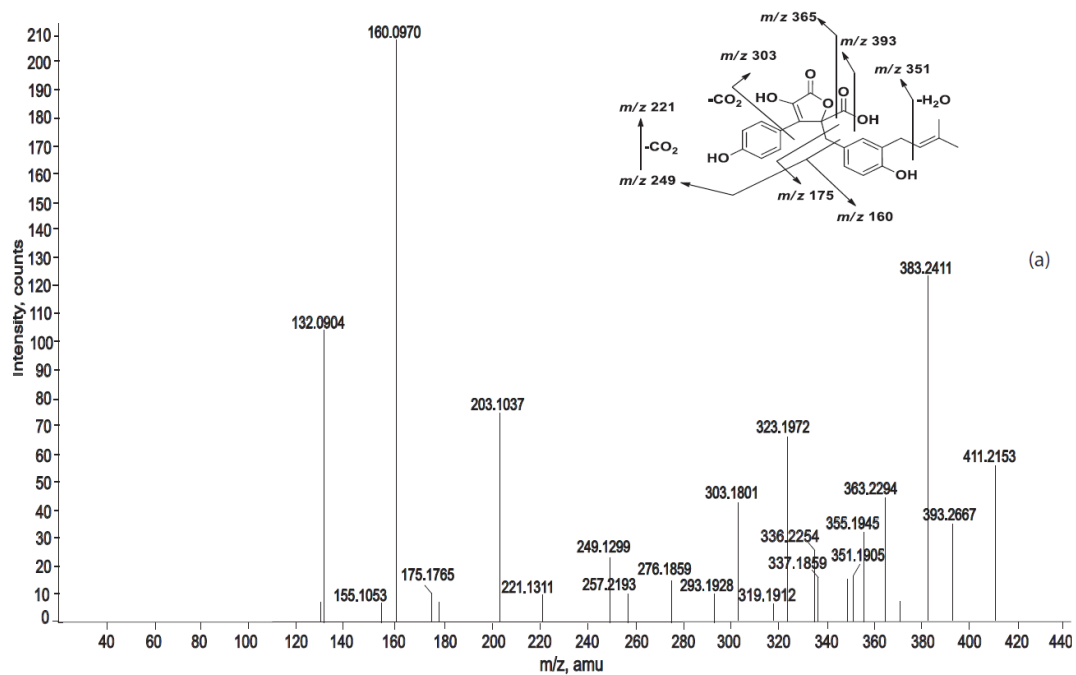


Figure S3: MS/MS at  $m/z$  411  $[M+H]^+$  ion of butyrolactone I-carboxylic acid (3)

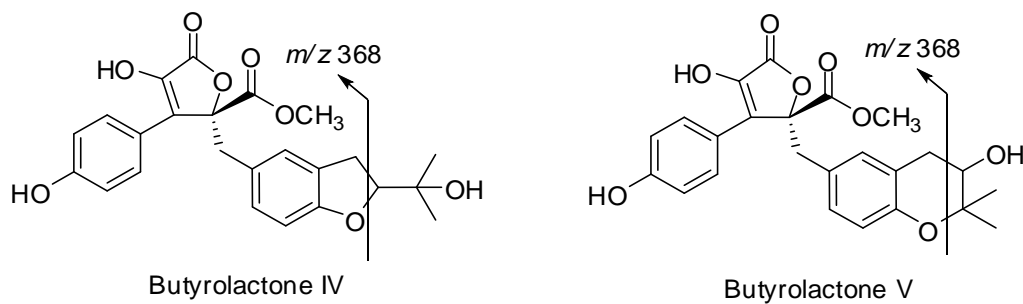


Figure S4: Possible fragment ion  $m/z$  368 in Butyrolactones IV and V.

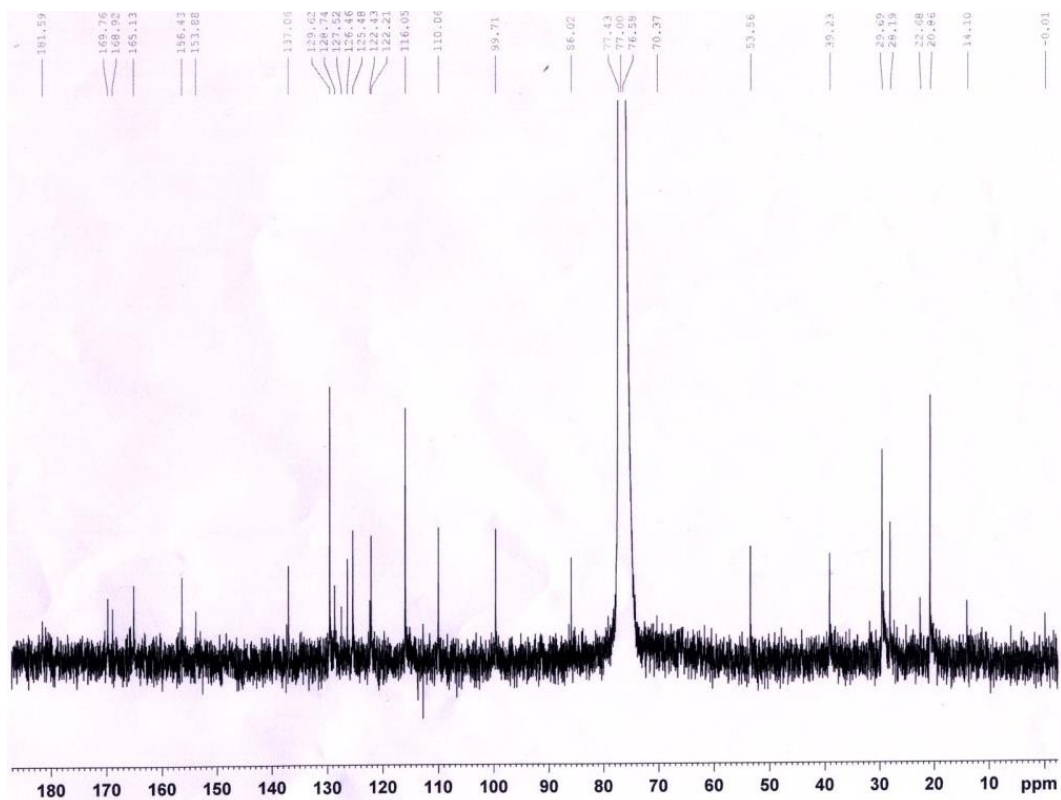


Figure S5:  $^{13}\text{C}$  NMR spectrum for aspernolie Q (5)

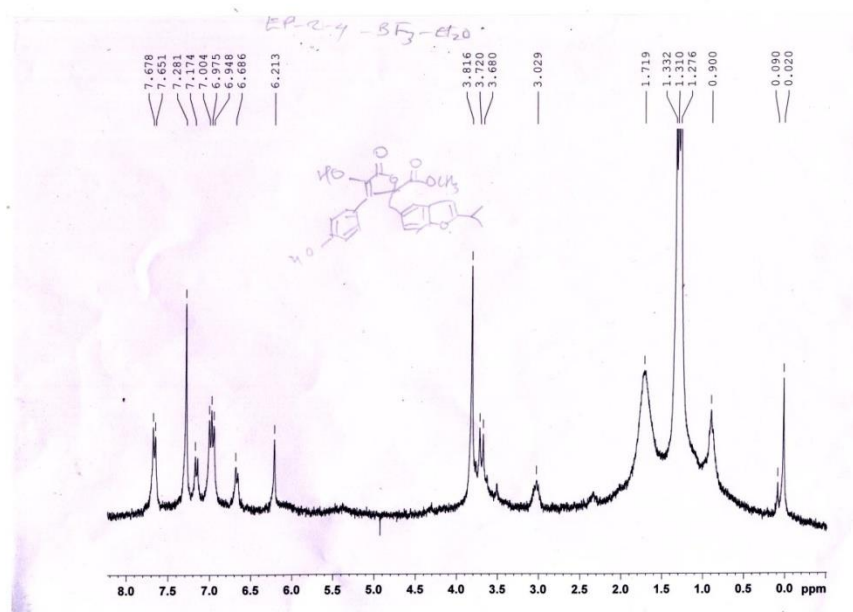
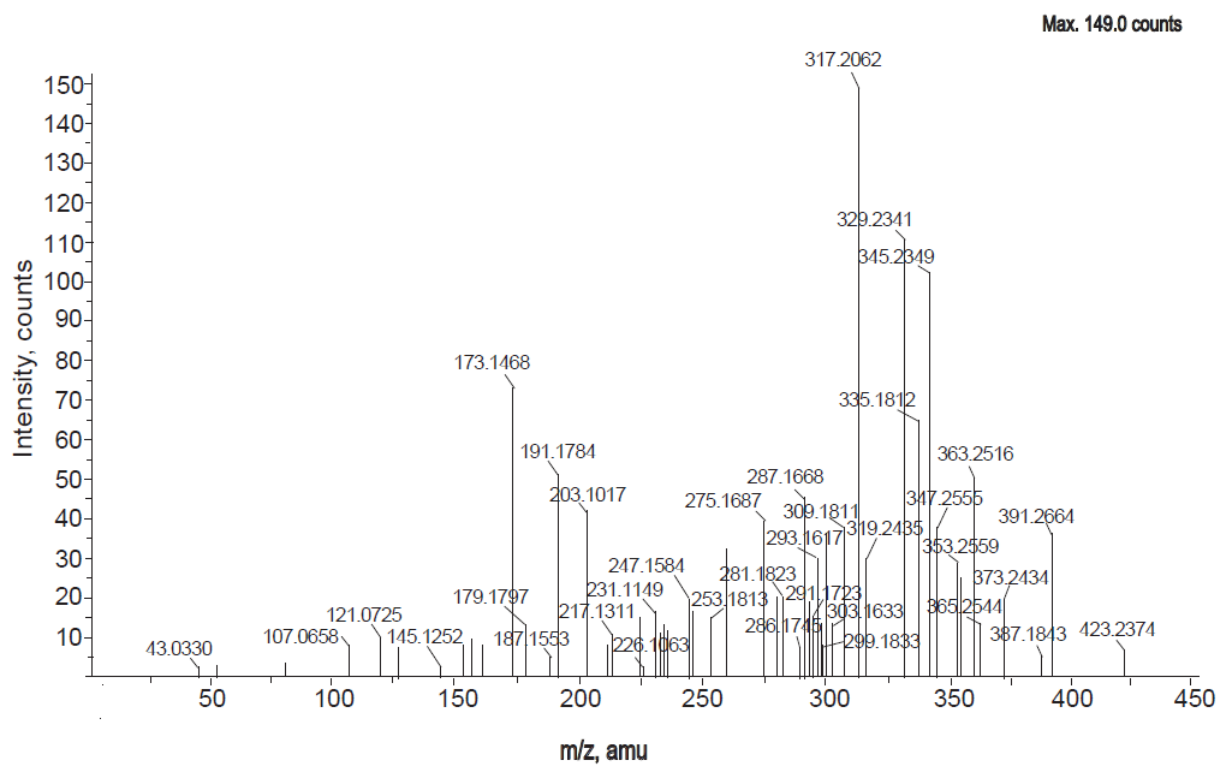


Figure S6:  $^1\text{H}$  NMR spectrum for aspernolide Q (5)



**Figure S7: MS/MS spectrum for Synthetic aspernolide Q (5)**