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Chapter 3

Bioactive Polyesters in Marine and Plant Endophytic Fungi

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Additional information is available at the end of the chapter

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1. Introduction

Fungi are the second largest group of organisms in the world after the insects. It is estimated that there are one and a half million types of fungi in existence. However, with just 5% of this total having being described [1], a huge, still unknown and untapped microbial pool remains, which promise the discovery of novel, useful and economically profitable compounds. Organisms such as fungi, which generally living in highly competitive environments, are considered as major producers of secondary metabolites. Fungal secondary metabolites are characterized, not only by their structural diversity [2], but also by diversity of biological activity.

Endophytic microorganisms are to be found in virtually every plant on earth [3]. The most widely accepted definition of an endophyte is given by Bacon *et al*; "microbes that colonize living, internal tissues of plants without causing any immediate, overt negative effects" [4]. The most frequently encountered endophytes are representatives of the fungi [5]. In the past few decades however, it has been realized that plants may contain countless, previously undetected, numbers of these microorganisms known as endophytes. This has prompted a worldwide scientific effort to isolate endophytes and to study their natural products. Scientists have since discovered that endophytes may represent as potential sources of novel natural products for exploitation in medicine, agriculture, and industry. A few examples are presented in this chapter, with the focus on bioactive polyesters isolated from fungi coupled with the dereprilication of extract.

2. New methodology for rapid isolation and identification of known bioactive polyesters

Polyester is a widely used and useful material. Its usefulness extends into many fields including the medical field, industry, textiles and as bioactive natural and synthetic compounds.

2.1. Dereplication

Dereplication is a process for the rapid identification of already known natural products. This is strategically important for scientists when screening crude extracts from natural sources for novel bioactive compounds. The continued demand to get new drugs to the market more quickly and more cheaply, requires that the analytical technologies that support this work keep up with, for example, the rate at which new chemical entities (NCEs) are synthesized for high-throughput screening programmes [6]. There are numerous approaches to dereplication based on hyphenated techniques, and each has its own advantages, be they sensitivity, resolution, or scale (mg vs μg) [7]. The most common approaches are LC-UV, LC-MS, LC-MS/MS and LC-NMR, or combinations thereof, and the increasing use of capillary and cryo-NMR probes [8-16]. Of course, any technique involving mass spectrometry will always potentially suffer from problems associated with ionization (or lack thereof) of the compounds being studied. Despite this, in the pharmaceutical industry these hyphenated techniques are very powerful for the monitoring, characterization and identification of impurities [17]. Take LC-NMR as an example. There are three main coupling technologies, on flow, stopped-flow and loop-storage. However, they all have disadvantages. On flow results in poor signal-to-noise (S/N) ratio for the NMR spectra unless a reduced flow rate is used. However, reduced flow can then reduce the effectiveness of the chromatographic separation which makes this method only suitable for the more intense signals arising from the major constituents. Stopped-flow has the advantage that a number of chromatographic peaks can be studied, but the frequent stops then necessary for data acquisition can disturb the quality of separation, and concentrated samples from the major components can contaminate the NMR detection cell. Therefore, this approach is most suitable for mixtures having only a small number of constituents. In the loop-storage mode, the chromatographic run is not interrupted; instead each analyte is stored in a separate capillary loop in order for NMR acquisition to be carried out at a later stage. A prerequisite for this technique however, is that the analyte must be stable during the long NMR analysis time [18]. Also, it is very reliant on the sensitivity of the NMR instrument. For assisting with the sensitivity problem, the use of a cryoprobe has been a recent advancement in LC-NMR. In NMR cryoprobes, the electronic components are cryogenically cooled to ~20K while the sample remains at ambient temperature which reduces the electronic noise [19] thus gaining a better S/N. Cryoprobes provide quite significant sensitivity gains.

2.2. Advantages of dereplication with CapNMR

In the past, without CapNMR, the dereplication process was based mainly on data from the mass and UV spectra. However, both techniques have disadvantages and are not totally reliable. The mass spectrum can contain impurities which makes it difficult to assign the correct molecular mass. Furthermore, the spectrum may be dominated by the preferential ionization of a minor component, again making it difficult to assign a correct molecular mass to the major component. The UV spectrum only gives definitive structure information for those compounds with strong chromophores. The UV library of the Marine Group

within the Dionex analytical HPLC system only contains the known compounds which the group has worked on, and to date this is not yet a comprehensive collection. Furthermore, many entries in the AntiMarin database do not have reported UV data for the compound listed. However, even typical UV profiles may also give wrong answers since similar chromophore may give the same UV spectra. It is for these reasons the NMR data is so important because it can deliver definitive structural information. The AntiMarin database includes information on, for example, the number and type of methyl groups, that can be recognized from ^{1}H NMR spectra. With the introduction of CapNMR ^{1}H NMR spectra can be obtained from a single HPLC-MT plate collection derived from only 200-500 μg of crude extract. The ^{1}H NMR spectrum can then provide specific structural information for searching the AntiMarin database. The most readily interpreted information available is that for methyl groups which can be described as singlets, doublets or triplets depending on their environment. Other features, such as the type of substituted benzene rings, are also easily recognized. From the HPLC separation of 200-500 μg of crude extract, a master MT plate can be prepared for the CapNMR experiments and daughter plates made for P388 bioactivity tests to locate the bioactive components and ESMS (Electrospray mass spectra) measurements. The UV profiles are obtained while collecting the MT plate. Therefore, it is now possible to determine from ~ 500 μg if a crude extract contains bioactive new compounds, or known compounds. This task can be easily completed in one or two days. Small volume NMR flow probes were first constructed in the laboratories of Sweedler and Albert in the 1990s [20-22]. These were developed for coupling to various chromatographic methods, resulting in so-called "hyphenated" techniques [23]. They were designed especially for coupling to capillary electrophoresis (CE) and capillary HPLC (CapLC) for the detection of the small volumes associated with these techniques.

A diagrammatic view and photograph of the Protasis capillary probe NMR (CapNMR) system is presented in **Figure 1**

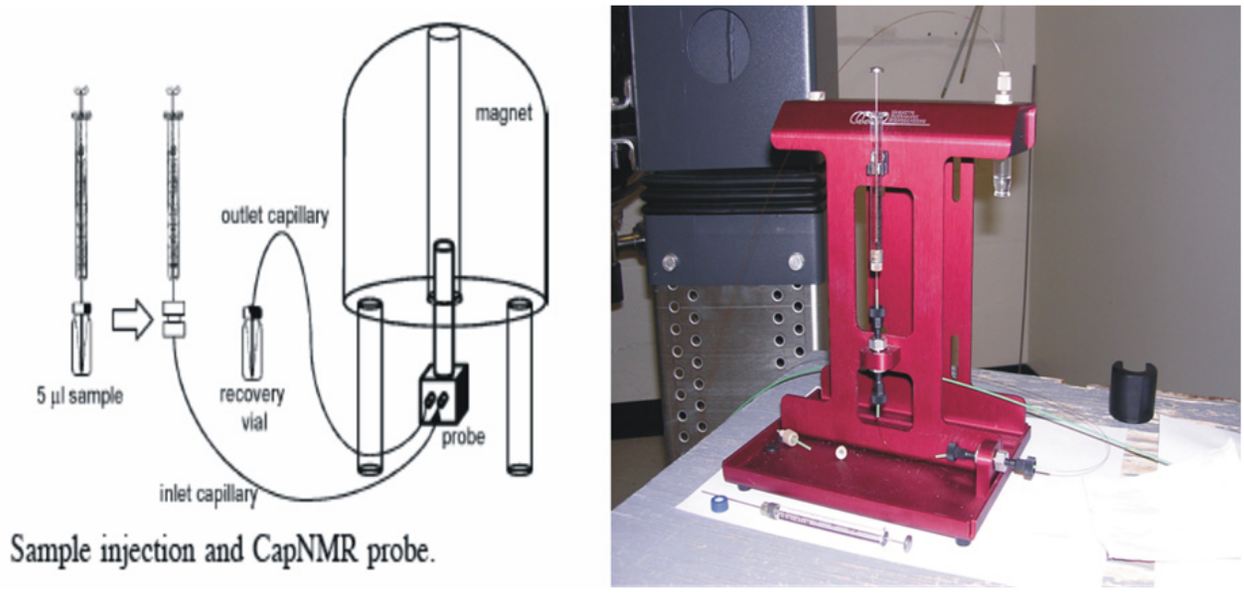


Figure 1. Capillary probe set-up and the Protasis capillary probe injection module.

2.3. Isolation of known bioactive polyesters using dereplication strategies

The natural products chemistry group at the University of Canterbury has been focused on natural products of fungal and marine origin. As well as searching for new bioactive natural products, the group has also focused on the development of new methodologies for dereplication.

Fungal extract 9PR2 which was isolated from the internal root tissue of *Callophyllum ferrugineum* (Guttiferae) [24] has provided a good example with which to demonstrate the new dereplication method, including the use of CapNMR and database searching.

Endophytic extract was diluted at 1 mgL^{-1} with methanol. 30 μL of the diluted extract was injected into HPLC. 10 % standard gradient programme was chosen with 80 % of 0.5 % formic acid plus distilled water and 20 % acetonitrile of HPLC grade.

The HPLC analysis revealed that the extract contained seven compounds. From the similarity of their UV profiles (Fig 2) the seven compounds were related. Based on the HPLC-UV profiles the assumption was made that the compounds contained a highly conjugated or aromatic system. These seven compounds were isolated from the appropriate wells in the MT plate (Fig 3) and each examined by CapNMR to obtain their ^1H NMR spectra. Compound **1** displayed three doublet methyl and two aromatic proton signals in the ^1H NMR spectrum. Because the compound had the same UV profile as the recorded for the lasiodiplodins, it was considered highly likely to also contain a 1,2,3,5-tetrasubstituted benzene system.

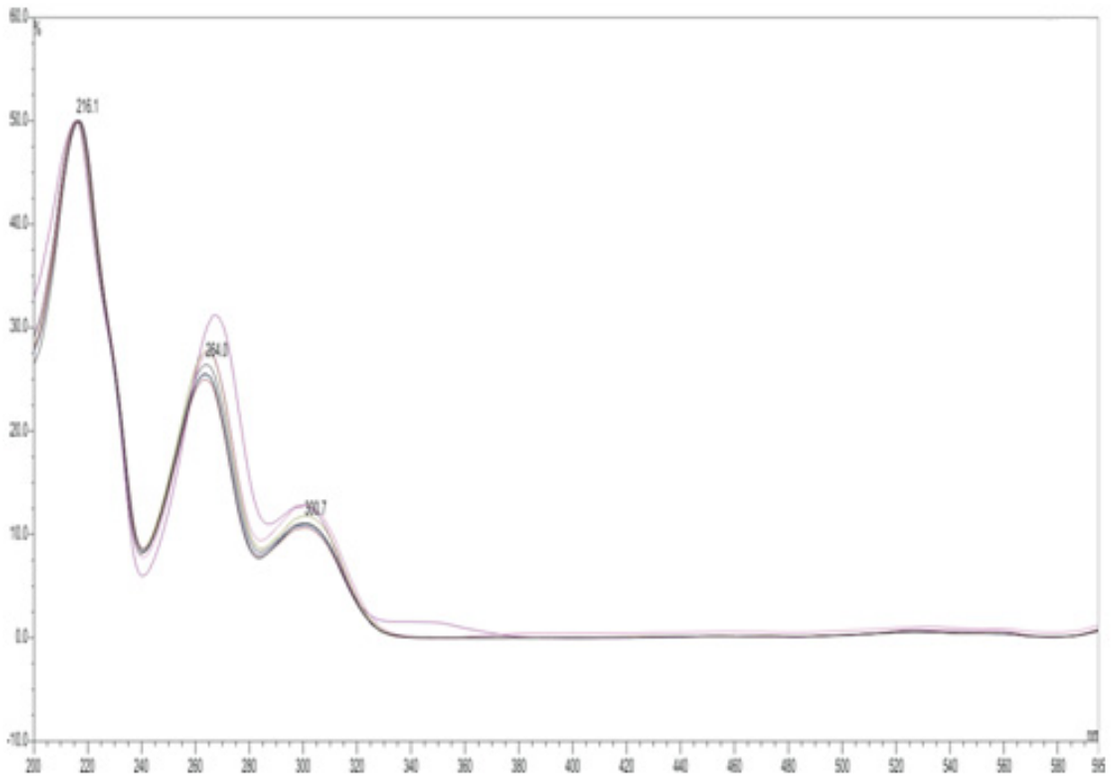


Figure 2. UV profile of the major peak for polyesters **1** to **7**

Figure 3. HPLC with DAD and ELCD detectors with fraction collector and microtitre plate.

These features were entered into the AntiMarin database, together with the supposed mass of 384 Da Two matches were found with the same structure, a polyester named 15G256V [25]. The literature NMR data for this compound also matched those obtained for compound **1**. Therefore, **1** was quickly identified as a known compound.

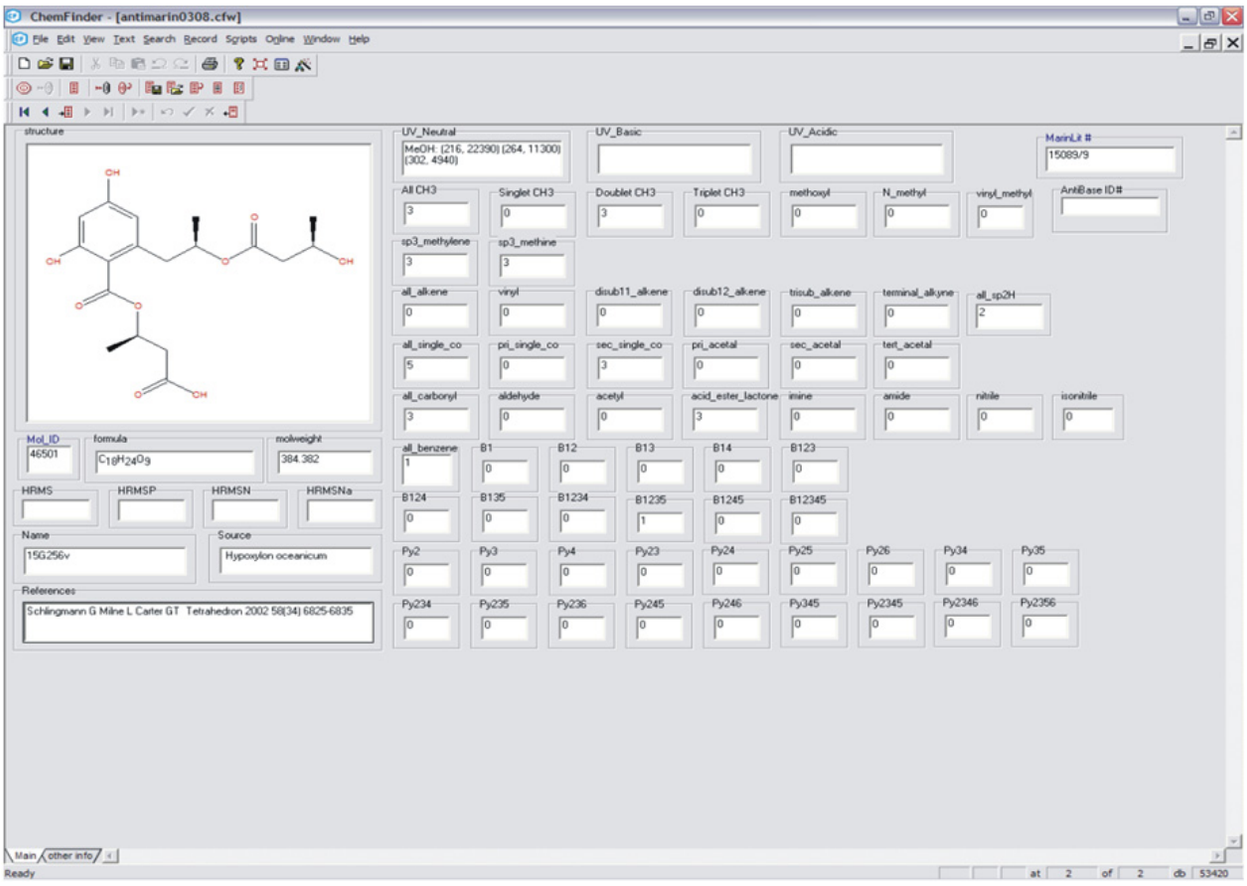


Figure 4. AntiMarin search result for **1**

The next compound examined, had signals for a doublet methyl group and for a 1,2,3,5-tetrasubstituted benzene ring in its ^{1}H NMR spectrum. These features, together with the supposed mass 194 Da, were used in an AntiMarin search. The NMR data for a (+)-6-hydroxymellein [25] matched with the data for Compound **2**. Therefore, **2** was also shown to be a known compound.

The ^{1}H spectrum of **3** was more complex, containing signals for four doublet methyl groups and four aromatic protons. These four aromatic protons were considered to come from two individual aromatic rings, and based on the UV profile, the two aromatic rings were both 1,2,3,5-tetrasubstituted. These features, together with the supposed mass of 680 Da, were used to initiate a search in AntiMarin. These searches found the polyester 15G256α [25] with matching data for compound **3** and have been previously reported. Therefore, compound **3** was identified as a known. Compound **4** had similar features to those for **3** in its ^{1}H NMR spectrum. It displayed signals for four doublet methyl groups as well as for two 1, 2, 3, 5-tetrasubstituted benzene rings. The mass, 578 Da, was different from **3**, and was put into an AntiMarin search. Again the polyester, 15G256π [25], matched the data for compound **4**. Therefore, compound **4** was also identified as known polyester.

The next compounds **5** and **6** had the same mass (662 Da), and both contained signals for four doublet methyl groups and four aromatic protons which could also be considered as 1,2,3,5-tetrasubstituted benzene rings from their ^{1}H NMR spectra. These features formed part of the AntiMarin search. Two similar macro cyclic polyesters were found from this search 15G256α and 15G256α-1, described by Schlingmann *et al* [25]. The literature data suggested that compound **5** was 15G256α and compound **6** was 15G256α-1. Both compounds (**5** and **6**) were thus readily identified as known Five doublet methyl groups together with two 1,2,3,5-tetrasubstituted benzene rings features were noted in the ^{1}H NMR spectrum of compound **7** (Figure-5). These structural features, together with the mass 646 Da, were included in an AntiMarin database search. Macrocyclic polyester called 15G256β [25] was found as a match, also from the previous literature by Schlingmann *et al*. The literature data were consistent with those observed for compound **7**, thus identifying it as a known compound [25]. This study demonstrates the dereplication method, including the use of CapNMR and database search for isolation and identification of compounds.

2.4. Cytotoxicity test

Fungal code 9PR2 showed excellent cytotoxicity in the P388 assay with an IC_{50} value 42.8 μg/mL [24]. Extract 9PR2 was tested for cytotoxic effect against a murine leukaemic cell line P388 and incubated for 72 h after which the MTT [3-(4, 5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide] assay was carried out as described in the literature [12], but with minor modifications. The cytotoxic activity was expressed as the mean concentration of extract required to kill 50% of the cell population (IC_{50}).

2.5. Antifungal activity

Polyesters **5**-**7** were also isolated previously from marine fungus Hypoxylon aceanicum (LL-15G256) [25] and were assayed for antifungal activity. In vitro tests using *Neurospora crassa* OS-1 (nikkomycin (MIC=2 μg/mL) as positive control) have demonstrated that compound **7** to be most potent with MIC=0.5 μg/mL while compound **5** and **6** with MIC = 2μg/mL.

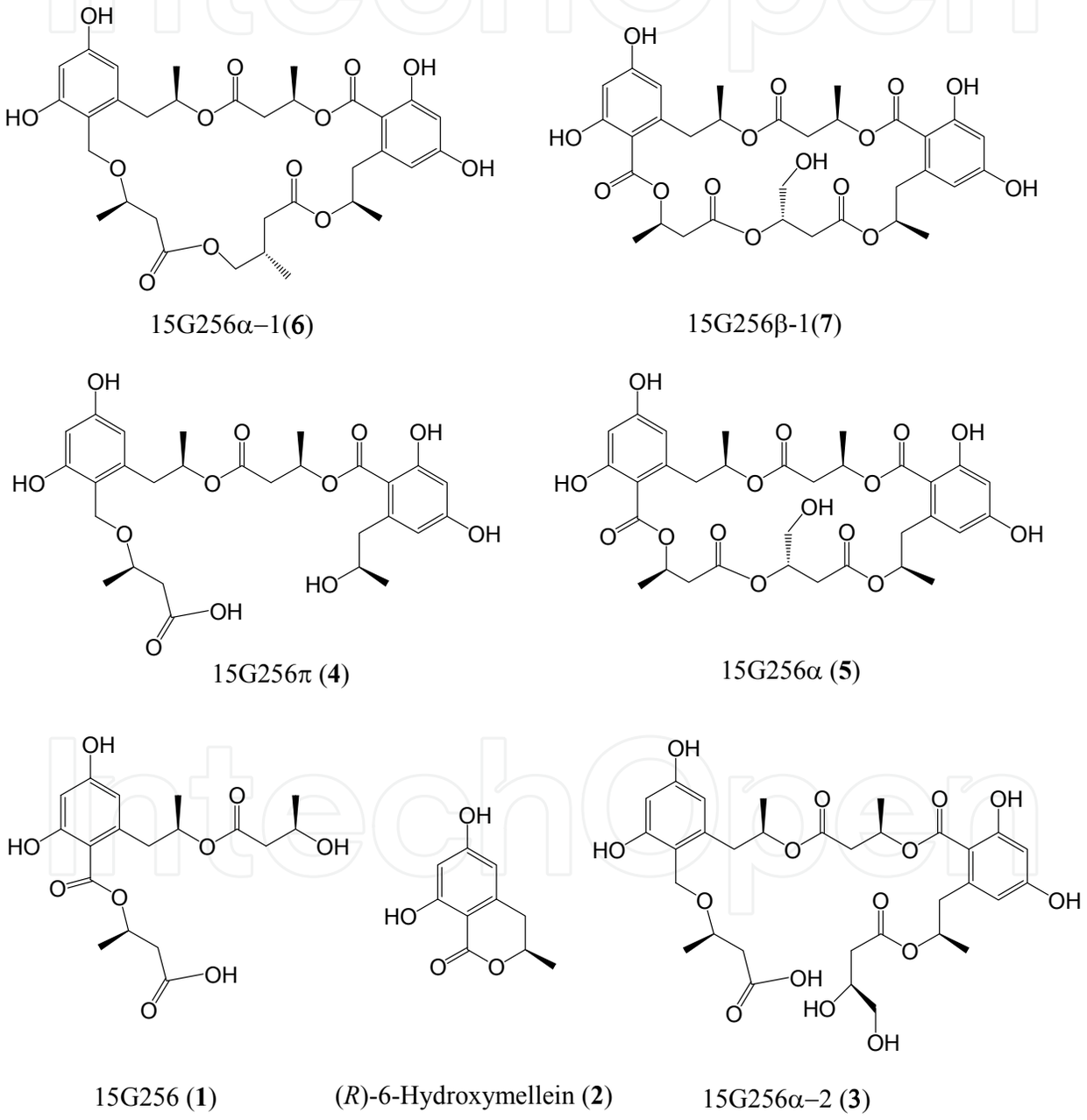


Figure 5. Structures of Polyesters

3. Novel macrocyclic polyesters from fungus menisporopsis theobromae

In a study to uncover the chemical diversity of plant associated microorganisms, the influence of culture conditions on metabolite production of the fungal strain *Menisporopsis theobromae* BCC 4162 has investigated. (Figure-6). Studies on optimization of culture conditions led to the isolation of new linear polyesters menisporopsin B [26], along with the known macrocylic polyester, menisporopsin, from the seed fungus *Menisporopsis theobromae* BCC 4162.

The fungus was collected from Khao Yai National Park, Thailand and was fermented in peptone-yeast extract-glucose medium (PYGM) under static condition. Chemical investigation led to the isolation of a novel macrocyclic polylatone menisporopsin A (**8**) which possesses an unusual 2,4-dihydroxy-6-(2,4-dihydroxy-*n*-pentyl)benzoic acid residue [27].

A recent study showed that fructose instead of glucose in PYGM is more suitable as carbon source. Shaking not only enhance the production of menisporopsin but also reduced the incubation time [26]. On the basis of these two factors fermentation of BCC 4162 was further studied which led to the isolation of new analogue, menisporopsin B (**9**) in the time profile studies.

Compound **8** exhibited antimicrobial activity, with an IC_{50} value of 4.0μg mL^{-1}, and antimycobacterial activity (MIC value of 50μg mL^{-1}. While compound **9** exhibited antimalarial activity with an IC_{50} value of 1.0μg mL^{-1}

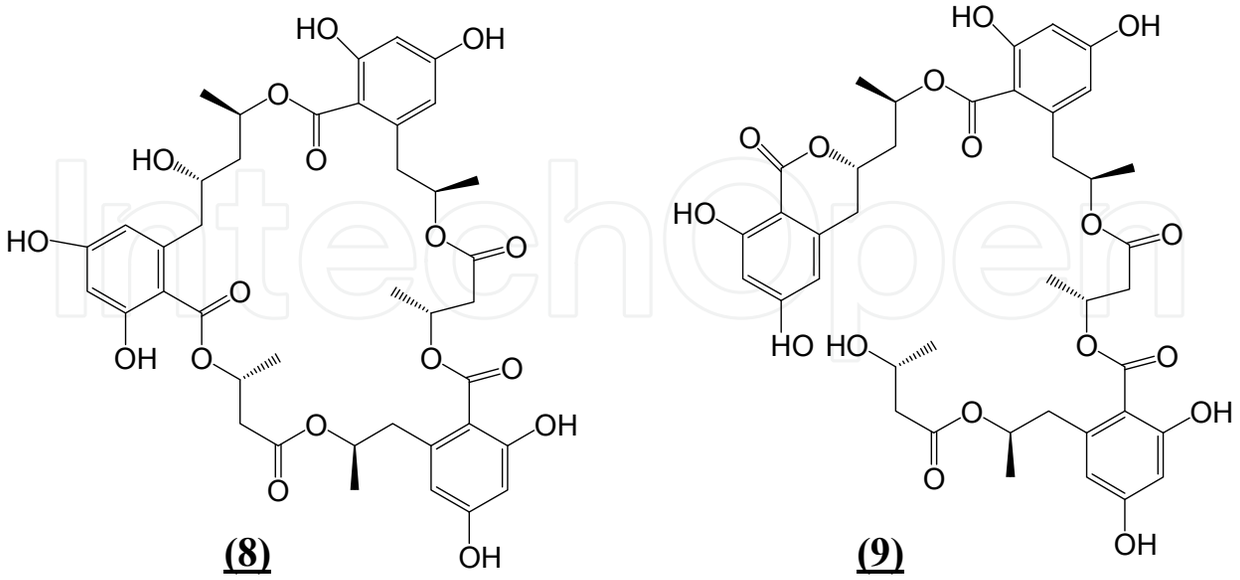


Figure 6. Structures of Menisporopsin A (**8**) and B (**9**)

4. Macrosphelides A and B, novel inhibitors of cell-cell adhesion molecule from microsphaeropsis sp. FO-5050

Cell adhesion molecules play important roles in various types of pathological conditions such as tumor, allergy and inflammatory diseases in the course of a screening program aimed at cell adhesion inhibitors obtained from microorganisms; macrosphelide was discovered in the fermentation broth of *Microsphaeropsis* sp. FO-5050 [28], which was isolated from a soil sample.

Macrosphelide is a newly discovered anti-cell adhesion substance, which is, a low molecular weight, unique 16-membered macrolide antibiotics possessing three ester bonds.

Macrosphelide **A** (**10**) and **B** (**11**) (Figure-7)were isolated by Satoshi et al in 1995 from culture broth of *Microsphaeropsis* sp. FO-5050 which had been isolated from a sample collected in Shizuoka prefecture and their structures were elucidated by spectroscopic methods and by chemical transformations [28]. Macrosphelides **A** and **B** with three esters in their molecules were classified as 6-membered macrocyclic compounds. Macrosphelide **B** was found to be a corresponding oxidative product of Macrosphelide A at C-14 position these Macrosphelides were classified as the first natural products bearing three lactones group in the molecule among the 16-membered macrocyclic antibiotics.

4.1. Biological activities

Anti-adherent activity: Mcrosphelides were assayed in an adhesion assay system using HL-60 cells and HUVEC cells both Macrosphelide **A** and **B** dose-dependently inhibited the adhesion of HL-60 cell to HUVEC stimulated with LPS. Macrosphelide **A** inhibited the adhesion of HL-60 cells to LPS-activated HUVEC monolayer (IC_{50}, 3.5 μM); Macrosphelide **B** also inhibited HL-60 adhesion but to a lesser extent (IC_{50}, 36 μM) in cell adhesion assay.

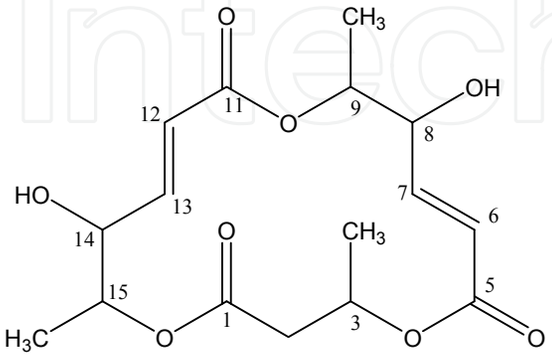
Antimicrobial activity: The antimicrobial activity of Macrosphelides was determined by the agar dilution method using paper disks. Macrosphelides **B** was active against bacteria, yeast and fungi, whereas Macrosphelide **A** showed no activities at a concentration of 1000 μg/ml against the microorganism tested [28].

5. Macrosphelides C and D, novel inhibitors of cell adhesion

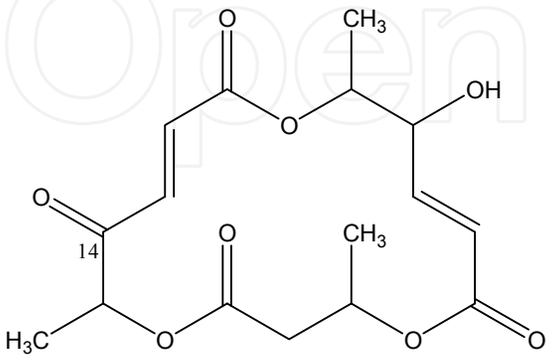
For further research the fermentation of strain *Microsphaeropsis* sp. FO-5050 was carried out in the same way as reported [28] two new 16-membered macrocyclic compounds macrosphelids C (**12**) and D (**13**) were discovered. Compound **12** and **13** (Figure-7) were obtained in the yield of 1.8 mg and 8.0 mg, respectively, together with Macrosphelide A (580 mg) and B (16.1 mg) [29-30].

The molecular formula of **13** as determined as $C_{16}H_{22}O_7$ by HR-FAB-MS. The IR absorptions at 3462 cm^{-1} and 1732 cm^{-1} of **12** showed the presence of hydroxyl group and ester functions, respectively. In the ^1H NMR spectrum the signals at δ 2.36 (dddd, J = 13.9, 10.1, 9.5, 1.5 Hz,

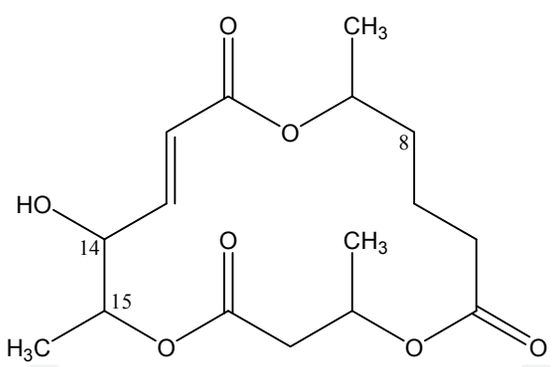
H-8a) and δ 2.55 (m, H-8b) were newly observed compared with those of Macrosphelide A (**10**). The ^{13}C NMR spectrum of **12** showed methylene carbon signal at δ 38.8, for C-8 compared with that of **8** which was appeared at δ 74.7. These signals suggest the presence of methylene carbon instead of methane carbon bearing OH group. On the basis of this compound **12** was assumed to be 8-deoxy derivative of **10**. Further on the basis of HMBC correlations the structure of **12** was confirmed 8-deoxymacrosphelide A.



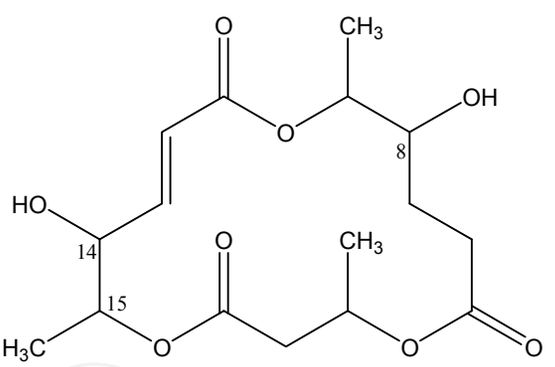
Macrosphelide A **(10)**



Macrosphelide B **(11)**



Macrosphelide C **(12)**



Macrosphelide D **(13)**

Figure 7. Structures of Macrosphelides **10-13**

Compound **13** gave the same molecular formula ($C_{16}H_{22}O_8$) as that of **8** based on the HR-FAB-MS but showed different on ^1H NMR spectra. The proton signals at H-14 (δ 5.05) shifted downfield of 0.92 ppm compared with that of **10**. On the other hand, the proton signals adjacent to the methyl carbon at H-15 (δ 4.06) shifted up field of 0.8 ppm. In the ^{13}C NMR spectrum of **13**, the chemical shifts of carbon signals showed similarly to those of **10**, except for signals of C-12, C-13, C-14 and C-15. In the ^1H NMR spectrum of compound **13** the coupling constants between δ 5.96 (d, *J* = 15.8, H-12) and δ 6.59, (d, *J* = 15.8, 8.6 Hz, H-13) showed the same *trans* configuration as that of **10**. On the basis of these Macrosphelide D (**13**) is presumed to be a stereoisomer of Macrosphelide A (**10**) at C-14 or C-15 positions.

5.1. Biological activity

Biological activities of Macrosphelides **C** and **D** were assayed according to the previous methods [28] in an adhesion assay system using HL-60 cells and HUVEC cells. The IC_{50} values of **12** and **13** were 67.5 μM and 25.0 μM, respectively.

6. Macrosphelides J (14) and K (15)

To clarify the structure-activity relationship of the Macrosphelides and to apply the results to the design of more potent inhibitors of cell-cell adhesion, more attention was devoted to the purification of other Macrosphelide derivatives from the broth of strain FO-5050 which resulted in the isolation of two new members of Macrosphelides J (**14**) and K (**15**) (Figure-8) [31].

The molecular formula of J (**14**)was determined to be $C_{16}H_{24}O_9$ by HR-FAB-MS. The IR absorbance at 3442 cm $^{-1}$ and 1730 cm $^{-1}$ of **14** indicated the presence of hydroxyl group and ester carbonyl group, respectively. From its ^{1}H NMR spectrum **14** was assumed to be a derivative of Macrosphelide B. The signals of δ 4.27 (dd, *J* = 18.9, 2.7 Hz, H-12) δ 2.66 ~ 2.81 (m, H-13) and δ 3.40 (s, 12-OMe) were newly observed when compared with those of Macrosphelide B. In addition, two olefinic protons of δ 6.73 and δ 7.03 which had been detected in Macrosphelide B were absent. The ^{13}C NMR spectrum of **15** showed one methine (δ 74.70, d, C-12), one methylene (δ 42.01, t, C-13) and one methyl (δ 58.79, q, 12-OMe) but the two olefinic carbons (C-12 and C-13 in Macrosphelide B) were absent. The low-field methyl signal showed the presence of a methoxy group. Thus, **14** was elucidated to be a 12, 13-hydro-12-methoxy-macrosphelide **B**.

The molecular formula ($C_{18}H_{26}O_9$) of **15** was assigned based on the HR-FAB-MS. The IR absorptions at 3442 cm^{-1} and 1730 cm^{-1} most likely correspond to a hydroxyl and an ester group. In the ^{1}H and ^{13}C NMR spectra, an additional methylene (δ_H 3.65, 3 47, δ_C 66.85, t, 12-CH_2) was observed compared with those of **15**. In addition the, low-field methyl proton of 5 at δ 3.40 (s, 12-Me) was shifted to high-field (δ 1.18) in **15**. From ^{1}H-^{1}H COSY, the direct connection between 12-CH_2 and 12-CH_3 was observed. By the PFG-HMBC experiment, the structure of **K** (**15)** was determined as shown below.

Biological Activities : These compounds were evaluated in an adhesion assay system using human leukemia cells (HL-60 cells) and HUVECS (human umbilical vein endothelial cells). The IC_{50} values of all compounds tested were greater than 100 μg/ml and did not indicate any effects on the cell growth of HL-60 and B16/BL6 melanoma when tested at 50 μg/ml.

SAR study of Macrosphelides: Macrosphelides J and K are compared to other neither showed inhibitory activity against HL-60 cells and HUVEC cells, the primary structural differences between the Macrosphelides J and K and Macrosphelides B was a double bond at C-12.

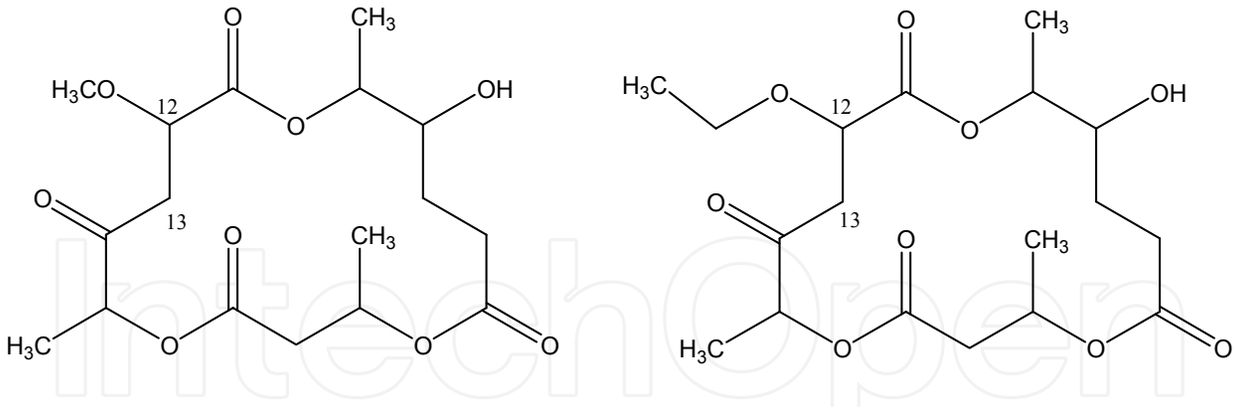


Figure 8. Structures of Macrosphelides **14**-**15**

On the other hand, Macrosphelides J and K were not artifacts of Macrosphelide B, because of their stability in MeOH or EtOH solution. These results show the double bond at C-12 to be important for the inhibitory activity and the analysis can help in the development of an inhibitor of the cell-adhesion molecule.

7. Conclusion

The Marine Group's dereplication method is a fast and reliable method for obtaining the information on compounds. An effort that would previously have taken possibly months of work, often with the unfortunate outcome of finding a known compound. This example using the 9PR2 extract illustrates just how quickly and efficiently the use of LC-UV-MS-NMR, through the intermediary of a microtitre plate and in combination with appropriate databases, can be used to identify the presence of known compounds starting from a very small amount of extract. The dereplication method developed by the Marine Group greatly saves the time taken to determine whether the compounds are known or unknown. Firstly, the sample collection amounts required are reduced from grams or kilograms down to milligrams only, which can provide a reduction in time from months to less than a week for working on the extracts. Furthermore, in the purification step, only several μg of compounds are required instead of the mg of compounds previously required, again reducing the time from weeks of effort to only one or two days using the MT plate HPLC method. The compounds are isolated from HPLC then can be analyzed by CapNMR and mass spectrometry to provide useful information for subsequent AntiMarin database searching to determine the novelty or otherwise of the compounds.

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