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Full Proceeding Paper

BIOACTIVE FUNGAL METABOLITES OF 9PR2 ISOLATED FROM ROOTS OF *CALLOPHYLLUM FERRUGINEUM*

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ABSTRACT

Bioactivity-guided isolation provided seven known polyesters, 15G256V **(1),** (+)-6-hydroxymellein **(2),** 15G256α-2 **(3),** 15G256π **(4),** 15G256α **(5),** 15G256α-1 **(6),** and 15G256β **(7).** These polyesters were isolated for the first time from unidentified endophytic fungi 9PR2 based on dereplication process using HPLC. These compounds were chemically characterized by mass spectroscopy, CapNMR and with AntiMarin database.

Keywords: Polyesters, CapNMR, Endophytes and dereplecation.

INTRODUCTION

It appears that all higher plants are hosts to one or more endophytic microbes but one of the least studied biochemical-chemical systems in nature is the relationship between organisms and their plant hosts. Endophytes are microorganisms that include fungi, bacteria and actinomycetes, which primarily reside in the plant tissues beneath the epidermal cell layers and often the host tissues are transiently symptomless[1]. Endophytic fungi are known to have mutualistic relations with their hosts, protecting plants against herbivores, insect attack or tissue invading pathogens[2-4] and in some instances endophyte may survive as a latent pathogen, causing or quiescent infections for a long period and symptoms only when physiological or ecological conditions favors virulence[5,6]. Since Malaysia is considered as one of the 12 mega biodiversity nation, we began a concerted search for endophytic microbes that are able to produce novel bioactive compounds. Such efforts are greatly facilitated by the development of a fast and efficient dereplication methodology that combines the highly sensitive NMR probes and systematic resource to spectroscopic database. The details of the procedure used has been published recently.[7,8] These methods have been applied as a key strategy in continuing search for new, bioactive metabolites from Malaysian fungi. In our present study, the isolation of seven known polyesters **1**-**7** (Figure-1) were isolated for the first time from a plant endophytic fungus.

MATERIALS AND METHODS

General Experimental Procedures

NMR spectra were recorded on a Varian INOVA AS-500 spectrometer (500 and 125 MHz for ^{1}H and ^{13}C NMR, respectively), using the signals of the residual solvent protons and the solvent carbons as internal references (δ_H 3.3 and δ_C 49.3 ppm for CD_3OD). A Protasis CapNMR capillary probe was used for the micro plate dereplication studies. ESIMS were acquired using a Micro mass LCT TOF mass spectrometer. Solvents used for extraction and isolation were distilled prior to use. Bioactivity assays were measured using standard protocols[9, 10].

Isolation and Extraction of Fungus (9PR2)

An endophytic fungus (9PR2) was isolated from the internal root tissue of *Callophyllum ferrugineum* (Guttiferae) using surface sterilization technique. The collection was done in August 2006 from Pangkor Island (Perak) according to the method detailed elsewhere[11]. 9PR2 was grown in 4.9 cm sterilized petri plates containing potato dextrose agar (PDA), were incubated for 30 days at 28° C. The agar medium was extracted three times with ethyl acetate. The EtOAc extract was evaporated to dryness resulting in a crude extract (12.5 mg) that was subjected to cytotoxicity and characterization for the isolation of pure compounds.

Cytotoxicity Test

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}).

Purification and Capillary probe NMR (CapNMR technique)

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. Once the active wells were detected (OD at 540 nm), the chromophore for active compounds could be identified from the HPLC chromatogram. The compounds were analyzed using LC-UV-MS-MS and CapNMR. Fractions were analyzed by positive electrospray mass spectrometry to establish molecular weight and molecular formula. The data were matched with available databases and determined to be members of known polyesters[13].

RESULTS AND DISCUSSION

Fungal code 9PR2 showed excellent cytotoxicity in the P388 assay with an IC_{50} value of 42.8 µg/mL. An aliquot of 9PR2 was analyzed by reverse phase C_{18} HPLC using a 25-85% linear gradient (see Experimental). The HPLC analysis revealed that the extract contained seven compounds. From the similarity of their UV profiles 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 and each examined by CapNMR to obtain their ^{1}H NMR spectra. Compound **1** displayed three doublet methyl and two aromatic proton signals in the ^{1}H 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. 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[13]. The literature NMR data for this compound also matched those obtained for compound **1**. Therefore, **1** was quickly identified as a known compound. 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[13] 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-tetra substituted. These features, together with the supposed mass of 680 Da, were used to initiate a search in AntiMarin. These searches found the polyester 15G256α[13] with matching data for compound **3** and have been in 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[13], matched the data for compound **4**. Therefore, compound **4** was also identified as known polyester.

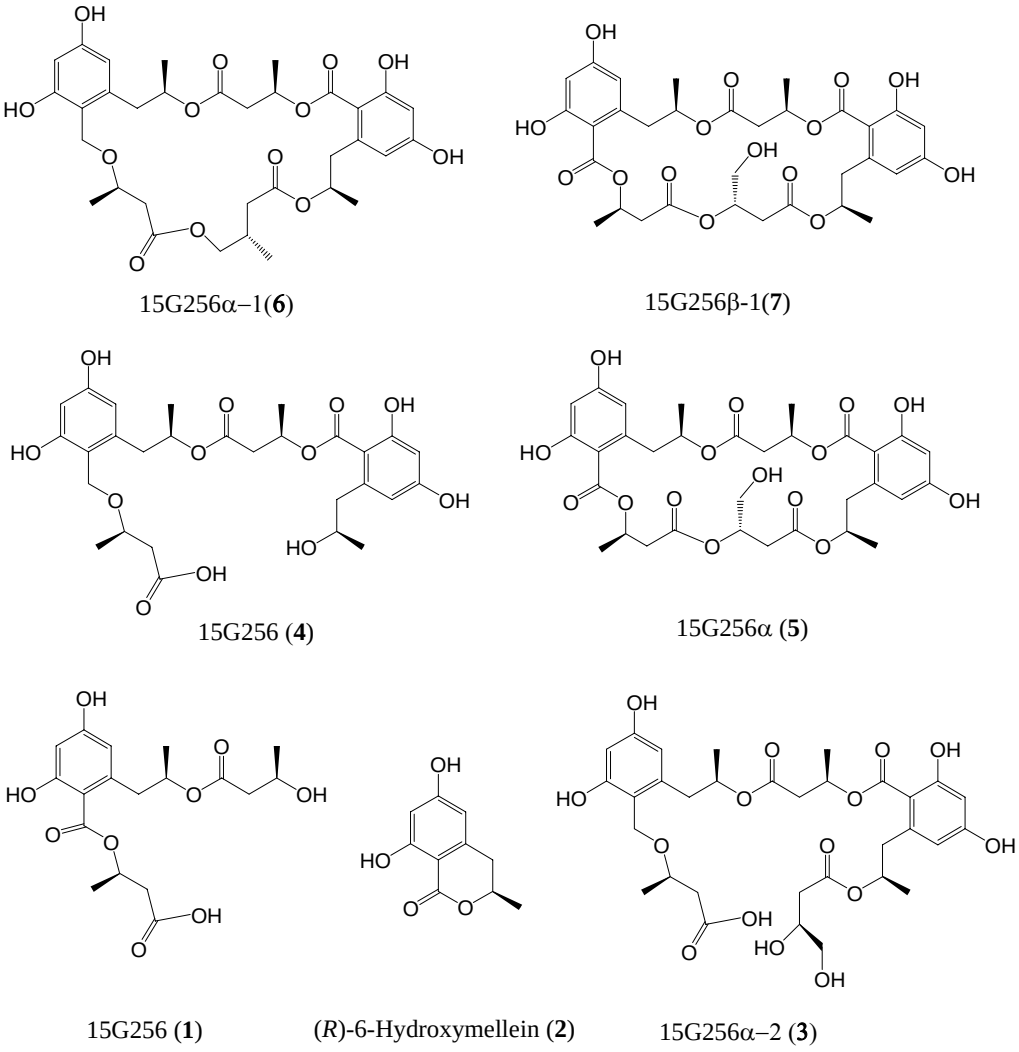

Fig. 1: Structures of polyesters

Table 1: Physical data of polyesters (1-7)

Compound name	Physical state	% Yield	UV λmax (nm)	NMR data	ESIMS (m/z)
15G256V (1)	White solid	3	216, 264, 301	NMR data reported previously[13]	407 $[M+Na]^{+\cdot}$
(+)-6-hydroxymellein (2)	White solid	1.2	216, 267, 301	NMR data reported previously[13]	193 $[M-H]^{-\cdot}$
15G256α-2 (3)	White solid	3.2	216, 265, 301	NMR data reported previously[13]	681 $[M+H]^+$, 703 $[M+Na]^+$
15G256π (4)	White solid	1.6	216, 265, 301	NMR data reported previously[13]	579 $[M+H]^+$, 601 $[M+Na]^+$
15G256α (5)	White solid	4.4	216, 265, 301	NMR data reported previously[13]	663 $[M+H]^+$, 685 $[M+Na]^+$
15G256α-1 (6)	White solid	4.4	216, 265, 301	NMR data reported previously[13]	663 $[M+H]^+$, 685 $[M+Na]^+$
15G256β (7)	White solid	4.6	216, 265, 301	NMR data reported previously[13]	647 $[M+H]^+$, 669 $[M+Na]^+$

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*.[13] 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 F8095-7 (Figure-1). These structural features, together with the mass 646 Da, were included in an AntiMarin database search. A macrocyclic polyester called 15G256β[12] 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[12]. This study demonstrates the dereplication method, including the use of CapNMR and database search for isolation and identification of compounds.

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