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Elfita

Department of Chemistry, Faculty of Mathematics and Natural Sciences, Universitas Sriwijaya, Indralaya, Kabupaten Ogan Ilir 30662, Indonesia

Munawar

Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Sriwijaya, Indralaya, Kabupaten Ogan Ilir 30662, Indonesia

Muharni

Department of Chemistry, Faculty of Mathematics and Natural Sciences, Universitas Sriwijaya, Indralaya, Kabupaten Ogan Ilir 30662, Indonesia

Gusti Pratiwi

Department of Chemistry, Faculty of Mathematics and Natural Sciences, Universitas Sriwijaya, Indralaya, Kabupaten Ogan Ilir 30662, Indonesia

Rahmadania

Department of Chemistry, Faculty of Mathematics and Natural Sciences, Universitas Sriwijaya, Indralaya, Kabupaten Ogan Ilir 30662, Indonesia

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Cover Page Footnote

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A New Benzoyl Compound Isolated from the Endophytic Fungi of Kandis Gajah (*Garcinia griffithii*) and Asam Kandis (*Garcinia cowa*)

Elfita^{1*}, Munawar², Muharni¹, Gusti Pratiwi¹, and Rahmadania¹

1. Department of Chemistry, Faculty of Mathematics and Natural Sciences, Universitas Sriwijaya, Indralaya, Kabupaten Ogan Ilir 30662, Indonesia

2. Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Sriwijaya, Indralaya, Kabupaten Ogan Ilir 30662, Indonesia

*E-mail: elfita_kimia@unsri.ac.id

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Abstract

Garcinia griffithii and *Garcinia cowa* belong to the genus *Garcinia*. The genus *Garcinia* has been known to be a rich source of secondary metabolites, such as xanthenes, benzophenones, flavonoids, steroids, terpenoids, and other phenolic derivatives. Previous investigations of endophytic fungi from *G. griffithii* revealed the presence of three compounds not found in the host. In order to continue the phytochemical work on endophytic fungi of *G. griffithii*, the constituent of the endophytic fungi of *G. griffithii* was re-examined. In this study, a benzoyl compound similar to that found in the endophytic fungus of *G. cowa* was observed. The same benzoyl compound was also isolated from the endophytic fungus *Acremonium sp* of *G. griffithii* and *Aspergillus sp* of *G. cowa* with cultivation of eight weeks in static conditions at room temperature. The culture medium was partitioned using ethyl acetate and evaporated to obtain the concentrated extract. Isolation of compounds was performed using the chromatography method. The chemical structure was proposed on the basis of spectroscopic data, including ultraviolet (UV), infrared (IR), mass spectrometry (MS), proton nuclear magnetic resonance (¹H-NMR), carbon nuclear magnetic resonance (¹³C-NMR), heteronuclear single-quantum correlation spectroscopy (HSQC), heteronuclear multiple-bond correlation spectroscopy (HMBC), and correlation spectroscopy (COSY).

Abstrak

Suatu Senyawa Benzoil Baru Hasil Isolasi dari Jamur Endofit Tumbuhan Kandis Gajah (*Garcinia griffithii*) dan Asam Kandis (*Garcinia cowa*). Tumbuhan *Garcinia griffithii* dan *Garcinia cowa* termasuk ke dalam genus *Garcinia*. Genus *Garcinia* dikenal kaya dengan kandungan metabolit sekunder, seperti santon, benzofenon, flavonoid, steroid, terpenoid, dan turunan fenolik lainnya. Penelitian sebelumnya terhadap jamur endofit dari *G. griffithii* telah menemukan tiga senyawa yang tidak ditemukan dalam tumbuhan inangnya. Sebagai lanjutan dari penelitian fitokimia mengenai jamur endofitik tumbuhan *G. griffithii*, kandungan kimia dari jamur endofitik ini kembali diteliti. Dalam studi ini, juga diperoleh senyawa benzoil yang sama dengan yang ditemukan dalam jamur endofit tumbuhan *G. Cowa*. Senyawa benzoil yang sama juga dapat diisolasi dari jamur *Acremonium sp* tumbuhan *G. griffithii* dan dari jamur *Aspergillus sp* tumbuhan *G. cowa* dengan masa kultivasi delapan minggu dalam kondisi statis pada suhu kamar. Medium kultur dipartisi menggunakan etil asetat dan dievaporasi untuk mendapatkan ekstrak pekatnya. Isolasi senyawa dilakukan dengan metode kromatografi. Struktur kimia diusulkan berdasarkan data spektroskopi yang meliputi ultraviolet (UV), infrared (IR), *mass spectrometry* (MS), *proton nuclear magnetic resonance* (¹H-NMR), *carbon nuclear magnetic resonance* (¹³C-NMR), *heteronuclear single-quantum correlation spectroscopy* (HSQC), *heteronuclear multiple-bond correlation spectroscopy* (HMBC), dan *correlation spectroscopy* (COSY).

Keywords: benzoyl, endophytic fungi, Garcinia griffithii, Garcinia cowa

Introduction

Endophytic fungi are a new source of bioactive compounds being explored recently. They live in plant tissue in a certain period of time, can form colonies in plant tissues

without harming the host, and usually provide symbiotic mutualism [1-3]. One of the interesting facts about endophytic fungi is their ability to produce bioactive compounds that can be similar or different with those produced by the host [4-5].

Plants of the genus *Garcinia* are known as a rich source of diversity for secondary metabolites including xanthenes, benzophenone, flavonoids, steroids, terpenoids, and other phenolic derivatives [6-8]. The extract of natural products have been used as drugs and important sources of traditional medicines, such as cytotoxic, antibacterial, antiviral, antifungal, antioxidant, anti-inflammatory, antidepressant, and anti-HIV [9-11].

Phongpaichit *et al.* (2007) and Ruma *et al.* (2013) reported endophytic fungal isolated from the *Garcinia* genus, including *Garcinia atroviridis*, *Garcinia dulcis*, *Garcinia mangostana*, *Garcinia nigrolineata*, *Garcinia Scortechinii*, *Garcinia gummigutta*, *Garcinia indica*, *Garcinia morella*, and *Garcinia xanthochymus*. The endophytic fungi are shown to have antimycobacterial, antimalarial, antiviral, antioxidant, anti-inflammatory, and cytotoxicity activities [12-13]. Several strategies used to isolate endophytic fungi have been reported recently to search for potential bioactive compounds, and one of them is based on ethnobotanical history specifically used by indigenous peoples [14]. *Garcinia griffithii* and *Garcinia cowa* are traditionally used by the local communities of Sarasah Bonta, Lembah Arau, and West Sumatra to treat various diseases, including gout, diarrhea, and malaria.

In previous work, we reported three compounds from endophytic fungi of *G. griffithii*, namely, 4,6-dihydroxy, 3,8a-dimethyl-1-oxo-5-(3'-oxobutan-2'-yl)-1,4, 4a, 5, 6, 8a-hexahydronaphthalen-2-yl-1'', 2''-di-methyl- 5''-(2'''-methyl-prop-1'''-enyl)-cyclopentane-carboxylate from *Aspergillus fumigatus* [15]; 3,5-dihydroxy-2,5-dimethyl-trideca-2,9,11-triene-4,8-dione from *Acremonium* sp [16]; and 10, 12-trihydroxy-9-methoxy-7a-methyl-7, 7a, 12a, 13-tetrahydrobenzo-cyclohepta-oxocin-6-one from *Aspergillus niger* [17].

In the current study, we reported another secondary metabolite called benzoyl compound, which is extracted from *Acremonium* sp of *G. griffithii* and found in the endophytic fungus *Aspergillus* sp of *G. cowa*.

Materials and Methods

Source of endophytic fungi. *Acremonium* sp of *G. griffithii* obtained from stock fungus was stored in the Microbiology Laboratory, Department of Biology, Faculty of Mathematics and Natural Sciences of Sriwijaya University. The fruit of *G. cowa* was collected in May 2012 from Lembah Arau, West Sumatra.

Isolation and identification of endophytic fungi. The isolation of *Acremonium* sp from twigs of *G. griffithii* was reported previously by Elfita *et al.* 2012 [16] and Debbab *et al.* [18]. The isolation method of *Aspergillus* sp from the fruit of *G. cowa* grown in Sarasah Bonta, Lembah Arau, Kabupaten Lima Puluh Kota, and West Sumatra was conducted according to the procedure by

Elfita *et al.* 2011 [15]. The fungal strain was identified on the basis of the morphological method by the School of Hayati Science and Engineering, Bandung Institute of Technology, Indonesia. The voucher of specimen was stored in the Microbiology Laboratory, Department of Biology, Faculty of Mathematics and Natural Sciences of Sriwijaya University.

Cultivation of endophytic fungi. Potato dextrose broth (PDB) medium of 300 mL was used for the cultivation of endophytic fungi and was placed into 30 flasks (1 L each). Fungal suspension containing 10^6 spores/mL was inoculated under sterile conditions to each 300 mL PDB medium (ratio 1:10). The cultures were incubated for eight weeks in static conditions at room temperature [19-20].

Extraction, exploration, and structure elucidation. Mycelia were removed from the endophytic fungus culture after eight weeks of incubation and the medium was filtered. The medium was extracted three times using ethyl acetate (1:1) followed by evaporation under vacuum to obtain the concentrated extract. The concentrated extract was separated by column chromatography over silica gel 60 (70–230 mesh) at the stationary phase (1:30) and eluent, which was previously determined by thin-layer chromatography silica gel 60 F₂₅₄. The chosen eluent with increased polarity was *n*-hexane:EtOAc at a ratio of 10:0 to 0:10 (v/v). An eluate was collected and then combined using thin-layer chromatography into column fractions. Each fraction was evaporated and purified using the chromatography technique to obtain the purified compound.

The ethyl acetate extract from *Acremonium* sp of *G. griffithii* (3.16 g) was subjected to column chromatography over silica gel (60 x 1.5 cm) and was eluted with *n*-hexane-ethyl acetate gradient (10:0–0:10) and collected in 60 vials each containing 10 mL. The thin layer chromatography (TLC) analysis showed the presence of five column fractions (F1–F5): F1 (724 mg), F2 (433 mg), F3 (406 mg), F4 (821 mg), and F5 (891 mg). Fraction F3 showed a major compound. It was further separated by column chromatography over silica gel (30 x 0.7 cm) and eluted with *n*-hexane-ethyl acetate (5:5–0:10) to give 37 vials. The fractions, which gave the same *R_f* on TLC, were combined and yielded four column fractions (F3.1–F3.4): F3.1 (81 mg), F3.2 (52 mg), F3.3 (143 mg), and F3.4 (105 mg). Fraction F3.3 was purified by rechromatography to yield compound **1** as a white crystal (87 mg). Its melting point is 109 °C–111 °C. Further separation and purification of fraction F4 by column chromatography yielded compound **2** as previously reported by Elfita *et al.* 2012 [16].

The ethyl acetate extract from *Aspergillus* sp of *G. cowa* (3.02 g) was subjected to column chromatography on silica gel (60 x 1.5 cm), eluted with *n*-hexane-ethyl acetate gradient (10:0–0:10), and collected in 50 vials each containing 10 mL. The TLC analysis showed the

presence of three column fractions (F1–F3): F1 (778 mg), F2 (653 mg), and F3 (906 mg). Fraction F2 showed a major compound. Thus, it was further separated by column chromatography over silica gel (30 × 0,7 cm) and eluted with *n*-hexane- ethyl acetate (5:5 – 0:10) to yield compound **1*** as a white crystal (228 mg). Its melting point is 109 °C–111 °C.

The chemical structure of the compound was determined using the following spectroscopy methods: UV, IR, 1D-NMR (¹H-NMR and ¹³C-NMR), 2D-NMR (HMOC, HMBC and COSY), NMR spectra (recorded at 500 MHz (¹H) and 125 MHz (¹³C) on JEOL JNM ECA-500 spectrometer), and high resolution electrospray ionization mass spectrometry (HRESI-MS).

3. Result and Discussion

In this study, *Acremonium* sp was cultivated on 9 L of PDB medium for eight weeks at room temperature. The culture broth was extracted by solvent partition with EtOAc (1:1), followed by evaporation. The extract showed two major spots on TLC.

The fungal strain from the fruit of *G. cowa* was identified as *Aspergillus* sp by the School of Hayati Science and Engineering, Bandung Institute of Technology, Indonesia. *Aspergillus* sp was cultivated on 9 L of PDB medium for eight weeks at room temperature. The culture broth was extracted by solvent partition with EtOAc (1:1),

followed by evaporation. The extract showed one major spot on TLC. Figure 1 briefly illustrates the procedure for isolating the pure compound from the endophytic fungi *Acremonium* sp from the twigs of *G. griffithii* and *Aspergillus* sp from the fruit of *G. cowa*.

Elucidation of compound 1 from *Acremonium* sp of *G. griffithii*. Compound **1** exhibited UV absorbance at λ_{\max} (MeOH) 258 nm (log ϵ = 4.54), which indicates the presence of an aromatic chromophore. These bands did not show a bathochromic shift with NaOH, thus indicating the absence of a hydroxyl group in the aromatic ring. The IR spectrum of **1** showed intense carbonyl absorption bands at 1701.1 and 1732.0 cm^{-1} because of the two ketone groups. The broad spectrum at 3436 cm^{-1} indicated the presence of a hydroxyl group. The characteristic vibrations at 3030.1, 1627.0, and 1598.9 caused by the aromatic group and stretching vibration at 2873.7–2964.4 cm^{-1} indicated the presence of C-H aliphatic. The HRESI-MS exhibited the $[\text{M}+\text{H}]^+$ ion peak at m/z 419.280 (calc. 419,1628), which is consistent with the molecular formula $\text{C}_{22}\text{H}_{26}\text{O}_8$ and implies 10 DoU (degree of unsaturation).

The ¹H-NMR spectrum of compound **1** recorded in CDCl_3 (¹H-500 MHz) revealed the presence of three proton sp^3 signals and two methyl proton signals at δ_{H} 0.97 ppm (3H, t, $J=7.2$) and 1.66 ppm (3H, s), respectively, and one methylene signal at δ_{H} 2.11 ppm (2H, m).

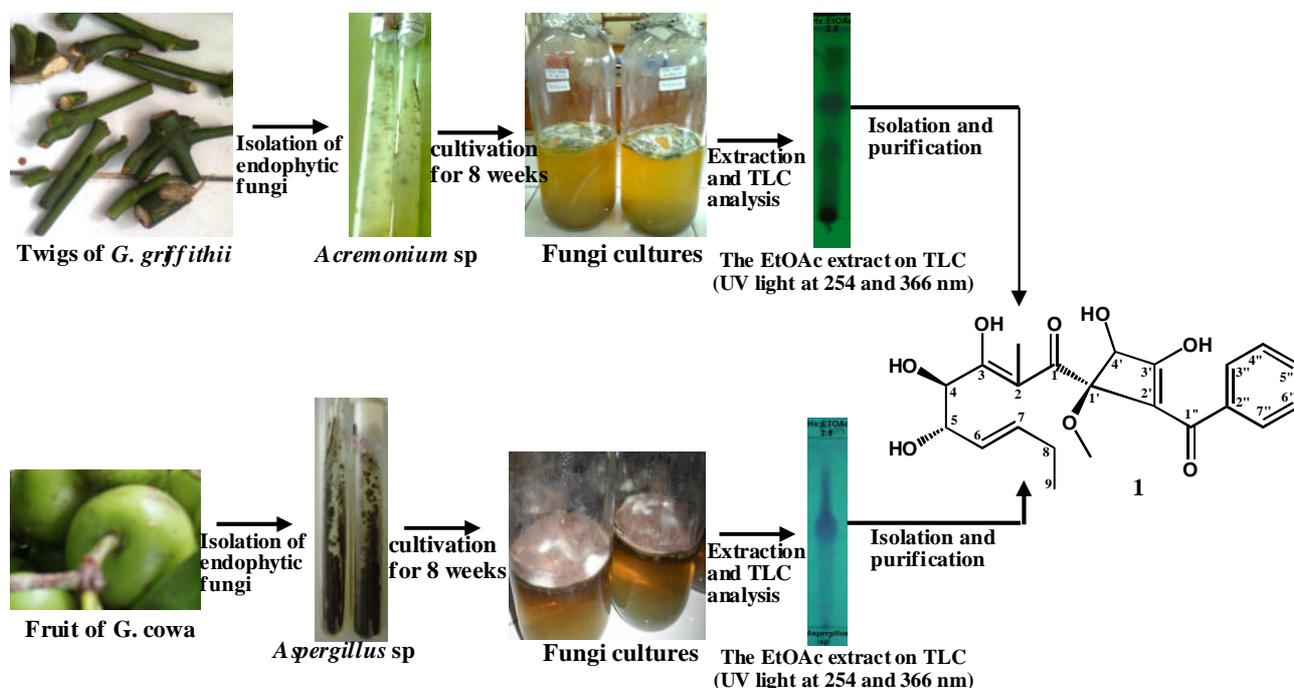


Figure 1. Brief Procedure for Isolating the Pure Compound from the Endophytic Fungi *Acremonium* sp from Twigs *G. griffithii* and *Aspergillus* sp from the Fruit of *G. cowa*

The presence of five aromatic protons showed the signal at δ_H 7.48 ppm (2H, d, $J=7.8$), 8.32 ppm (2H, d, $J=7.8$), and 7.64 ppm (1H, t, $J=7.5$). A signal was also found for methoxy proton at δ_H 3.45 ppm (3H, s) and two vinylic protons at δ_H 5.56 ppm (1H, m) and 5.23 ppm (1H, m). The $^1\text{H-NMR}$ revealed the presence of signals to three methyne protons sp^3 as oxygenated carbon at δ_H 4.69 ppm (1H, s), 4.74 ppm (1H, dd, $J=4.5$; 3.9), and 4.59 ppm (1H, d, $J=3.9$).

The $^{13}\text{C-NMR}$ spectrum showed 22 signals, including 8 carbon sp^3 and 14 carbon sp^2 . The $^{13}\text{C-NMR}$ and DEPT 135 spectrum, which were supported by HSQC spectrum, showed eight carbon sp^3 signals consisting of two methyl, one methylene, three methine, one methoxy, and one quaternary carbons.

Three signals of methine at δ_C 70.8, 71.3, and 73.1 ppm were assigned to carbon atoms bearing hydroxyl groups and one quaternary carbon bearing a methoxyl group. Furthermore, 14 carbon sp^2 signals included 6 aromatic carbons at δ_C 128.9 and 135.0, and 2 carbons with the same chemical shift at 131.0 ppm and 132.6 ppm. Two of these signals were assigned to the ketone carbons at δ_C 195.6 ppm and 196.5 ppm. Six other sp^2 carbon signals

were present at δ_C 93.1, 113.3, 126.7, 136.7, 166.7, and 186.2 ppm.

In the HMBC spectrum (Table 1.), correlations from H-3'' (δ_H 8.32) to C-1'' (δ_C 195.6), C-4'' (δ_C 128.9), and C-5'' (δ_C 135.0) suggested that C-1'' was attached to the aromatic ring as a benzoyl group. Correlations from the hydroxyl proton at C-3' (δ_H 8.46) to C-2' (δ_C 93.1) and C-4' (δ_C 73.1) and from 1'-OCH₃ (δ_H 3.45) to C-1' (δ_C 90.6) demonstrated the presence of a cyclobutene ring bearing methoxyl and hydroxyl groups. The methoxyl resonance connecting to C-1' in HMBC indicated C-1' with a high chemical shift (δ_C 90.6). Moreover, oxygenated carbon was attached to C-1 (carbonyl carbon). Correlations from the hydroxyl proton at C-3' (δ_H 8.46) to C-2' with a low chemical shift as sp^2 C=C (δ_C 93.1) showed an anisotropy effect of carbonyl groups (C-1''). HMBC correlations from 2-CH₃ (δ_H 1.66) to C-1 (δ_C 196.5), C-2 (δ_C 113.3), and C-3 (δ_C 186.2) indicated that C-1 and C-3 were located at the carbonyl group and hydroxyl group, respectively. Correlations from H-4 (δ_H 4.59) to C-5 (δ_C 71.3) and H-5 (δ_H 4.74) to C-4 (δ_C 70.8) revealed that C-4 and C-5 were attached to the hydroxyl groups. Figure 2 illustrates the HMBC correlation and δ -assignment of compound **1**.

Table 1. NMR Data of Compound **1** recorded at $^1\text{H-500MHz}$, $^{13}\text{C-125 MHz}$ in CDCl_3

Position of C	δ_C ppm		δ_H ppm, ΣH , multiplicity, J (Hz)		HMBC		COSY	
	1	1*	1	1*	1	1*	1	1*
1	196.5	196.5						
2	113.3	113.4						
3	186.2	186.1						
4	70.8	70.8	4.59 (1H;d; 3.9)	4.58 (1H;d; 3.9)	C-5	C-5	H-5	H-5
5	71.3	71.2	4.74 (1H;dd;4.5;3.9)	4.74 (1H;dd;4.5;3.9)	C-4; C-6; C-7; C-4; C-6; C-7; C-3	C-3	H-4; H-6	H-4
6	126.7	126.7	5.23 (1H;m)	5.25 (1H;m)	C-8	C-8	H-7; H-5	H-7
7	136.7	136.8	5.56 (1H;m)	5.56 (1H;m)	C-9; C-8; C-8	C-8	H-6	H-6; H-8
8	21.6	21.6	2.11 (2H;m)	2.12 (2H;m)	C-6; C-7; C-9	C-6; C-7; C-9	H-9	H-9
9	14.3	14.3	0.97 (3H;t;7.2)	0.97 (3H;t;7.2)	C-7; C-8	C-7; C-8	H-8	H-8
1'	90.6	90.6						
2'	93.1	93.0						
3'	166.7	166.9						
4'	73.1	73.2	4.69 (1H;s)	4.69 (1H;s)				
1''	195.6	195.5						
2''	132.6	132.5						
3''	131.0	131.0	8.32 (2H;d;7.8)	8.31 (2H;d;7.8)	C-7'' C-5''; C-1''	C-4''; C-5''; C-1''	H-4''	H-4''
4''	128.9	128.9	7.48 (2H;m)	7.48 (2H;t;7.8)	C-6''; C-2''; C-5''	C-2''	H-5''; H-3''	H-5''; H-3''
5''	135.0	135.0	7.64 (1H;t;7.5)	7.63 (1H;t;7.5)	C-3''; C-7''	C-3''; C-7''	H-4''; H-6''	H-4''; H-6''
6''	128.9	128.9	7.48 (2H;m)	7.48 (2H;t;7.8)	C-4''; C-2''; C-5''	C-2''	H-5''; H-7''	H-5''; H-7''
7''	131.0	131.0	8.32 (2H;d;7.8)	8.31 (2H;d;7.8)	C-3''; C-5''; C-1''	C-4''; C-5''; C-1''	H-6''	H-6''
2-CH ₃	6.3	6.3	1.66 (3H;s)	1.66 (3H;s)	C-2; C-3; C-1	C-2; C-3; C-1		
1'-OCH ₃	51.9	51.9	3.45 (3H;s)	3.43 (3H;s)	C-1'	C-1'		
3'-OH			8.46 (1H;s)	8.46 (1H;br)	C-4'; C-2'	C-4'; C-2'		

1 : Compound **1** from *Acremonium* sp from the twigs of *G. griffithii*

1*: Compound **1** from *Aspergillus* sp from the fruit of *G. cowa*

In the ^1H - ^1H COSY spectrum, the correlations from H-4 to H-5 indicated the presence of vicinal hydroxyl groups. The next correlations from H-6 to H-5, H-7 and from H-8 to H-9 revealed the presence of long-chain aliphatic subunits. The correlation of ^1H - ^1H aromatic indicated a mono-substituted aromatic ring. The COSY correlation of compound **1** is illustrated in Figure 2.

Carbon C-1' of compound **1** had a similar position and chemical environment to C-1' of talaroflavone [21]. Carbon C-1' of the two compounds is C sp³ (quarternar), which binds two C sp², one oxygenated C sp³, and one oxygen atom. Each carbon appeared at δ_{C} 90.6 and 91.8 ppm. Furthermore, carbon C-2' of compound **1** had a position and chemical environment similar to those of C-3 of methyltriacetic lactone [21], each of which appeared on δ_{C} 93.1 and 95.0 ppm. The chemical shift of C-1' (δ_{C} 90.6) was shown to approach the calculated data using Chem Draw Ultra 10 (δ_{C} 94.8)

Combining the results obtained by the spectrometric methods (UV, IR, MS, ^1H -NMR, ^{13}C -NMR, HSQC, HMBC, and COSY) and comparing the results of the literature data [21] with the calculated data of Chem Draw Ultra 10, we determined C₂₂H₂₆O₈ as the molecular formula for compound **1** with a molecular weight of 418. The structure of compound **1** is 1-(2'-benzoyl-3,4-dihydroxy-1'-methoxycyclobut-2'-enyl)-3,4,5-trihydroxy-2-methylnona-2,6-dien-1-one. The confirmation structure of **1** will be followed by an X-ray single crystal, and this work is still in progress.

Elucidation of compound 1* from *Aspergillus* sp of *G. cowa*. The ^1H -NMR spectra recorded in CDCl₃ (^1H -500 MHz) indicated that the spectroscopy data of **1** and **1*** are identical (see Table 1).

Based on the Dictionary Natural Products database (December 18, 2015), the benzoyl derivative could be a new compound. However, the identical benzoyl compounds (**1** and **1***) were produced by two different endophyte fungi could further raise the question of whether it is related to the host of the same genus or assumed as a secondary metabolite typically produced by the fungi. Based on these phytochemical studies, further studies on the metabolite profiling of these endophytic fungi and plants should be performed for confirmation.

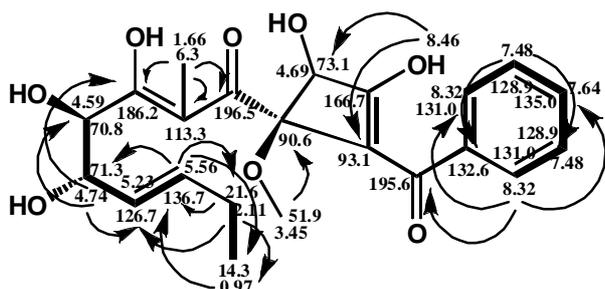


Figure 2. The HMBC Correlation

Conclusions

The endophytic fungi *Acremonium* sp from the twigs of *G. griffithii* produced the newly proposed benzoyl compound, which is identical to that found from the endophytic fungus *Aspergillus* sp from the fruit of *G. cowa*.

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