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Structure Elucidation of Phlegmacin Ether from Culture Broth of the Fungus, *Lambertella brunneola*, Y. Harada

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Abstract

A pigment known as Phlegmacin A 8,8'-di-O-methyl ether was isolated and reported for the first time from the fungus, *Lambertella brunneola*, Y Harada which was cultured in a potato-sucrose medium. The compound was isolated and purified using Si02 column chromatography while the structure elucidation was based on Nuclear Magnetic Resonance (NMR) spectroscopic data and Mass Spectroscopy (MS) analyses. Inital analyses suggested that Phlegmacin Ether has a 7, 10' biaryl bridge. Apparently, it has an anti-cancer activity against HeLa and HepG2 cancer cells.

Keywords: Lambertella brunneola, chromatography, Nuclear Magnetic Resonance (NMR), Phlegmacin A 8,8'-di-O-methyl ether, structure elucidation

Introduction

Pigments from fungi have been studied and extensively reviewed worldwide (Duran and others 2002, p 53; Martinkova and others 1995, p 609; Velisek and Cejpek 2011, p 87; Zhoua and Liu 2010, p 1531). Although fungi are generally recognized as a promising source of novel biologically active metabolites (Fujimoto and others 2004, p 98; Leeder and others 2011, p 440; Quang and others 2002, p 1869), there are still novel compounds that remain to be isolated. Natural products have always been the key source of leads forthe discovery and development of new drugs. Several naturalproduct-derived compounds are currently undergoing preclinical development and clinical trials (Harvey 2008, p 894). Most of these compounds are derived from plants and microbial sources and are predominantly being studied in anticancer areas. As part of the author's continuing studies directed at discovery of active metabolites from fungi (Tayone and others 2009, p 7464; Tayone and others 2011, p 425; Tayone and others 2011, p 2390; Tayone and others 2012, p 9), he investigated the chemical constituents of Lambertella brunneola species. This paper reports the isolation and planar structure elucidation of Phlegmacin A 8,8'di-O-methyl ethef as shown in Figure 1. Detailed structural analyses disclosed that this molecule has unique

polycyclic moiety, which was verified by Nuclear Magnetic Resonance (NMR) and Mass Spectroscopy (MS) data. The compound was first isolated by Wang's group (2005, p 333) from the fruit bodies of fungus ascomycete, Xylaria euglossa. Although the compound is already known, none has been published yet about the isolation of Phlegmacin A 8,8'di-O-methyI ether from the culture broth of L. brunneola. The objective therefore of this research was to establish and elucidate the two-dimensional structure of Phlegmacin A 8,8'-di-O-methyl ether.

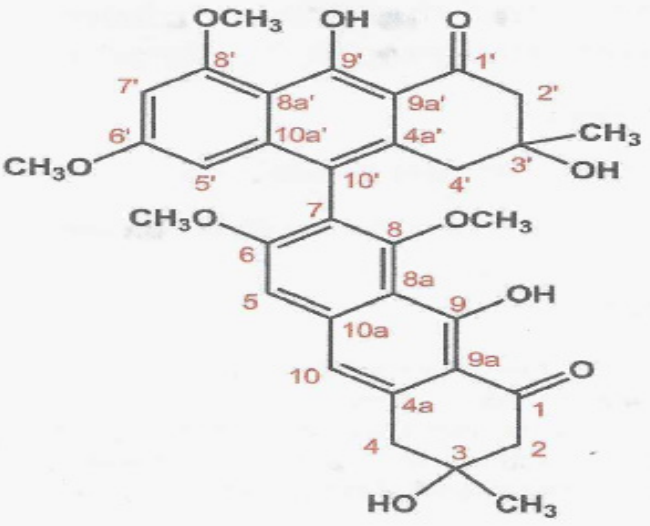

Figure 1 Planar structure of phlegmacin A 8,8'-di-O-methyl ether.

Materials and Methods

Fungus

TheAscomycetefungus, *Lambenella brunneola* (Yukio Harada) was collected from Tsugaru region which is part of Honshu, Japan's largest island. The species was identified by Professor Yukio Harada and the fungal isolate was deposited at the mycology laboratory of Professors Harada and Tanaka, Faculty of Agriculture and Life Sciences, Hirosaki University, Japan.

Fermentation and Isolation

The fungus was cultured in potatosucrose medium (200 mL in 500 mL Erlenmeyer flask 5) in stationary condition at 25.0^{0}C for 4 months. After the media were filtered by suction, the filtrate was extracted with about 200 mL (X 5) methanol (MeOH). The extracts were then partitioned with ethyl acetate (EtOAc) (1.0 L x 3) and the organic

layer was concentrated in vacuo followed by series of silica gel column chromatography using hexane/ethyl acetate solvent system to afford 5.6 mg of colorless Phlegmacin A 8,8'-di-O-methyl ether.

Structure Elucidation

The ^{1}H (500 MHz), ^{13}C (125 MHz), Correlation Spectroscopy (COSY), Heteronuclear Multiple Quantum Coherence (HMQC), Heteronuclear Multiple Bond Coherence (HMBC) and Nuclear Overhauser Effect (NOE) NMR spectra were recorded on a JEOL JNMECA500 spectrometer. In $CDCI_3$, the signal due to 7.24 ppm was used as the standard. Electrospray ionization (ESI) MS spectra were obtained from a HITACHI NanoFrontier LD spectrometer. Measurements of IR spectra were performed with a HORIBA FT 720 spectrometer on KBr cell. Chemicals used in these experiments were obtained from Wako Pure Chemical Industries Ltd. and Nacalai Tesque Inc.

Results and Discussion

Phlegmacin A 8,8'-di-O-methyl ether was isolated as colorless oil from the EtOAc extract of *L. brunneola* and has an Rf value of 0.2 (hexane/EtOAc 1:2). The Electrospray Ionization Mass Spectroscopy (ESIMS) suggested its molecular formula as C_{34} H_{34} O_{10} based on the observation of its protonated molecular ion at m/z = 603.2203. The ^{1}H-NMR spectrum shown in Figure 2 indicated the presence of four singlet methoxy groups (3.42, 3.58, 3.76 and 4.00 ppm), four aromatic protons (6.18, 6.41, 6.87 and 7.10 ppm) and two singlet methyl groups (1.25 and 1.42 ppm). The signals of eight methylene protons are found at 2.50 - 3.18 ppm.

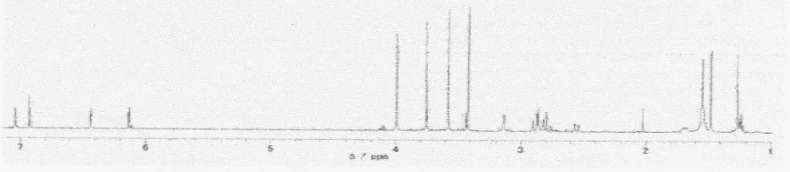

Figure 2 1H-NMR Spectrum of Phlegmacin A 8,8'-di-O-methyl ether.

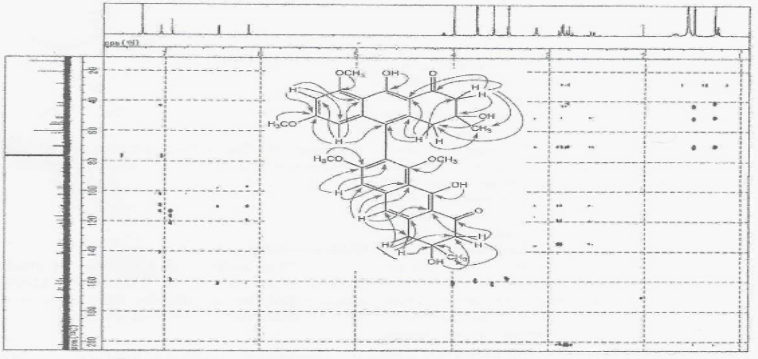

Figure 3 HMBC spectrum and representative HMBC correlations of phlegmacin A 8,8'-di-O-methyl ether.

This molecule showed 34 resonances in the ^{13}C NMR spectrum which accorded the molecular formula based on the mass spectrum. The two ^{13}C signals at approximately 200 ppm indicated the presence of a ketone functionality. The HMQC spectrum classified them as CH_30 4, CH_3 x 4, CH_2 x 4, and C x. 20, which also revealed 30 carbon bonded protons. In order to satisfy the molecular formula suggested by the mass spectroscopy, there must be four more hydrogens attached to hetero atoms. Thus, these were assigned to be alcoholic protons based on the suggested molecular formula. The above findings were further confirmed by an Infrared (IR) absorption at 3435 cm-I suggesting the presence of a hydroxy group. The connectivity was addressed by COSY, HMQC and HMBC (Figure 3) spectroscopic data. The absence of similar or double signal means that the dimer is not symmetrical or not a 10, 10' linked. The up-field shift of the proton signals assigned to CH_3 at C3', C4', H_2 at C5' and CH_30 at C6' was due to anisotropic shielding. These findings suggest a 7, 10' biaryl coupling or a Phlegmacin type linkage. These were corroborated by the NOE correlations detected for C8-methoxyto C3'-methyl, C4'-H_b and C5'-H as shown in figure 4. The I H NMR data were also identical to those of the literature values reported by Wang's group (2005, p 333 - 336). The Index of Hydrogen Deficiency (IHD) was 18, which means six rings and 12 double bonds. This number supported the planar structure in figure 1. Thus, the isolated compound was identified as Phlegmacin A 8,8'-di-O-methyl ether. The sample was sent to Graduate School of Medicine (Hirosaki University) for further anti-cancer tests against HeLa and HepG2 cancer cells. Initial test of Phlegmacin A 8,8'-di-O-methyl ether showed cytotoxicity against HeLa and HepG2 cancer cells at IC50 33 nM. However, confirmatory tests were not done since further attempts to culture *L. brunneola* in order to isolate more of the compound failed. It has been assumed that the conditions from the culture to incubation period could not be exactly duplicated to afford the Phlegmacin compound. As described, Phlegmacin A 8,8'-di-O-methyl ether was isolated from the culture broth of L. brunneola. The planar structure was established through NMR spectral data and MS analyses.

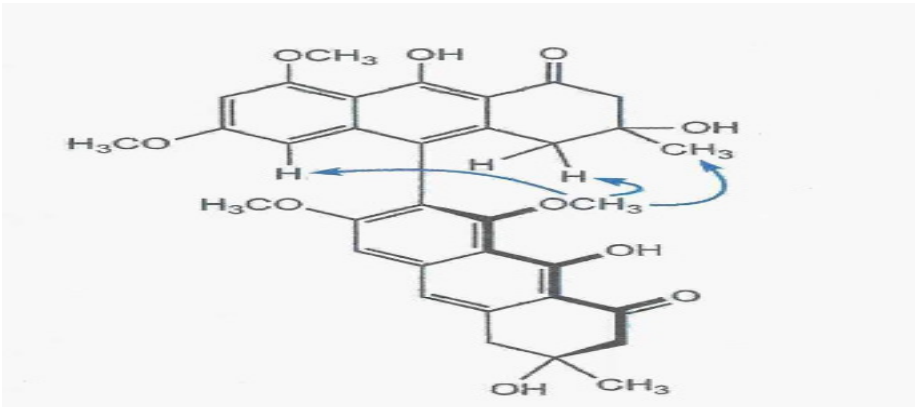

Figure 4 Selected NOEs observed for phlegmacin A 8,8'-di-O-methyl ether.

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