Phenylisobenzofuranones from Fungicolous *Nodulisporium* sp. SH-1

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Two new phenylisobenzofuranone nodulisporones A (**1**) and B (**2**) were isolated from the fungicolous *Nodulisporium* sp. Their structures were determined by spectroscopic methods. Compound **1** exhibited antimicrobial activity against *Candida albicans* and *Trichoderma harzianum*.

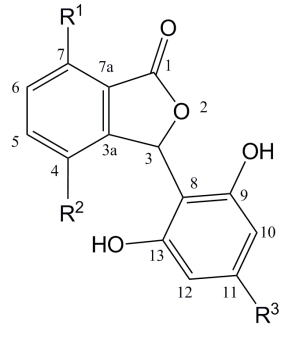
Key words: Phenylisobenzofuranone, Nodulisporones A and B, Fungicolous Fungus, *Nodulisporium* sp. SH-1, Antimicrobial Activity

Introduction

Fungicolous fungi are microorganisms that colonize other fungi [1]. Mycoparasitic fungi are also included in this ecological niche group, which are widespread and cause negative effects on the host fungi. Actually, some mycoparasitic fungi are recognized as potential biological control agents of economically important plant-pathogenic fungi [2]. These mycoparasitic fungi are known to produce both extracellular hydrolytic enzymes and antifungal compounds in the host plants [3]. Chemical studies of fungicolous fungi have led to the discovery of a variety of new bioactive metabolites, suggesting significant untapped potential among these microorganisms [4]. In connection with our ongoing search for biologically active compounds from fungi, we investigated the chemical materials produced by *Nodulisporium* sp. SH-1 which was isolated from the surface of a fruiting body of *Xylaria polymorpha* collected in the forest. *Xylaria* sp. belongs to the Xylariaceae, which comprise around 40 genera. *Xylaria* sp. are more frequently found as endophytic fungi which have been a rich source of structurally diverse natural products [5 – 7]. From the culture broth of the strain SH-1, we isolated two new compounds **1** and **2** exhibiting an isobenzofuranone skeleton, which we named nodulisporones A and B. Several members of isobenzofuran antibiotics have been reported so far. In particular, 3-substituted isobenzofuranones have attracted considerable attention due to their biological activities [8 – 11]. Compounds **1** and **2** are additional members of this group.

Results and Discussion

Nodulisporium sp. SH-1 was cultured and the cultured filtrate was extracted with EtOAc. Concentration of the EtOAc extract yielded a crude material which was purified by a combination of silica gel and ODS column chromatography to afford nodulisporones A (**1**) and B (**2**) (Fig. 1).



1: R^1 = H, R^2 = OH, R^3 = CH_3
2: R^1 = OH, R^2 = H, R^3 = COOH

Fig. 1. Structures of **1** and **2**.

The molecular formula of nodulisporone A (**1**) is $C_{15}H_{12}O_5$, as determined by HRMS (ESI-TOF), requiring 10 degrees of unsaturation. Compound **1**

Table 1. NMR data of compounds **1** and **2**[a].

	1			**2**		
No.	δ_C	δ_H	HMBC	δ_C	δ_H	HMBC
1	173.7 s			173.5 s		
3	77.4 d	6.82 (1H, s)	1, 7a, 9, 13	76.7 d	6.94 (1H, s)	4, 9, 13
3a	114.0 s			153.8 s		
4	154.7 s			116.0 d	6.68 (1H, d, 7.3)	3, 6, 7a
5	113.6 d	6.70 (1H, d, 7.4)	3a, 7	137.1 d	7.39 (1H, t, 7.3)	3a, 7
6	136.9 d	7.32 (1H, t, 7.4)	4, 7a	113.9 d	6.66 (1H, d, 7.3)	4, 7a
7	115.6 d	6.58 (1H, d, 7.4)	1, 5, 3a	157.7 s		
7a	157.6 s			113.9 s		
8	107.1 s			114.7 s		
9, 13	158.7 s			158.7 s		
10, 12	108.5 d	6.01 (2H, s)	8, 14	108.9 d	6.92 (2H, s)	8, 14
11	142.1 s			133.8 s		
14	21.5 q	2.05 (3H, s)	10, 11, 12	169.5 s		

[a] Measured in CD_3OD; values in parentheses are coupling constants in Hz.

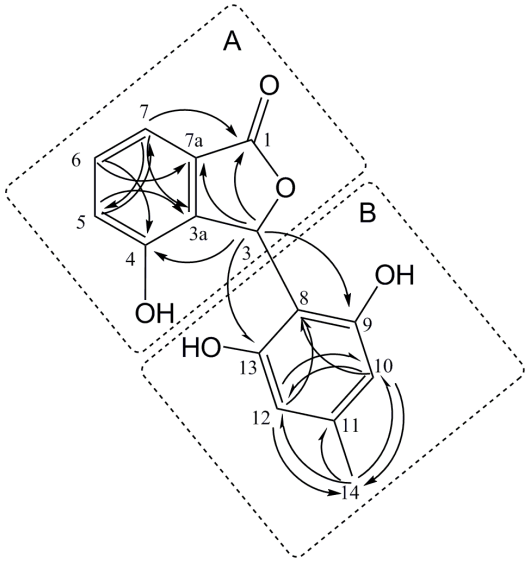


Fig. 2. Important HMBC correlations observed for **1**.

showed bands for hydroxy (3326 cm^{-1}), conjugated ester (1714 cm^{-1}) and benzene (1625, 1469 cm^{-1}) groups in the IR spectrum. The UV spectrum of **1** displayed absorptions at 219, 289 and 300 nm. The ^{13}C NMR and DEPT spectra indicated one methyl, one sp^3 methine, four sp^2 methines, six sp^2 quaternary carbons and one carbonyl carbon. The ^1H NMR spectrum of **1** showed an olefinic methyl singlet [δ_H = 2.05 (s, 3H, 14-H)], an oxygenated methine [δ_H = 6.82 (s, 1H, 3-H)], a 1,2,3-trisubsutituted benzene ring [δ_H = 6.58 (d, J = 7.4 Hz, 1H, 7-H), 6.70 (d, J = 7.4 Hz, 1H, 5-H), 7.32 (t, J = 7.4 Hz, 1H, 6-H)] and a double intensity singlet aromatic proton [δ_H = 6.01 (s, 2H, 10, 12-H)]. The COSY, HSQC and HMBC spectra of **1** revealed the presence of the two partial structures A and B (Fig. 2), a 1(3H)-isobenzofuranone unit and a 2,6-dihydroxy-4-methylphenyl moiety, respectively. For partial structure A, the 1(3H)-isobenzofuranone moiety was established by HMBC correlations from 3-H and 7-H to C-1, from 3-H and 6-H to C-7a and from 5-H to C-3a (Fig. 2). The location of 4-OH was assigned from HMBC correlations from 3-H and H-6 to C-4. Furthermore, the appearance of HMBC correlations from 10-H and 12-H to C-8 and from 14-H to C-10, C-11 and C-12 indicated the presence of the partial structure B. The manner in which the above partial structures are connected to each other was determined based on HMBC correlations from H-3 to C-9 and C-13. Therefore, **1** was assigned as 4-hydroxy-3-(2,6-dihydroxy-4-methylphenyl)-1(3H)-isobenzofuranone (Fig. 1). In addition, although there is a single stereogenic center at C-3, **1** was found to be a racemic mixture because no specific rotation could be observed.

The molecular formula of nodulisporone B (**2**) has been determined by HRMS (ESI-TOF) and elemental analysis to be $C_{15}H_{10}O_7$. Compound **2** is also racemic with no optical activity and exhibits spectral data similar to those of **1** (Table 1). However, it lacks a signal for a methyl group at C-11 which had been observed in **1**, and has a new carbonyl group at δ_C = 169.5 in the ^{13}C NMR spectrum. HMBC correlations (Table 1) between H-5 and C-7 suggested that the hydroxy group was situated at C-7. The methyl group at C-2 of **1** is replaced by a carboxyl group in **2** based on HMBC correlations from H-10 and H-14 to C-14. Therefore, **2** was assigned as 7-hydroxy-3-(2,6-dihydroxy-4-carboxy -phenyl)-1(3H)-isobenzofuranone (Fig. 1).

The structurally closest microbial metabolites with an isobenzofuran partial structure are pestacin [12] and isopestacin [13] isolated from *Pestalotiopsi microspora*, and cryphonectric acid [14] isolated from *Chryphonectria parasitica*. Pestacin is a first member of the isobenzofuran family of naturally occurring compounds containing a substituted benzene ring at C-3 of the benzofuran ring. Pestacin and isopestacin showed antifungal activity against *Pythium ultimum* and antioxidant activity, while cryphonectric acid is reported to have a moderate root growth inhibitory activity. Compounds **1** and **2** were evaluated by the agar diffusion method against Gram-positive and -negative bacteria, yeast, and fungus strains. Against *Candida albicans* and *Trichoderma harzianum*, **1** showed activities with observed zones of inhibition of 10 mm and 13 mm in diameter, respectively, at 10 μg per disk. On the other hand, **2** did not show antimicrobial activity (100 μg per disk). Further pharmacological studies of **1** and **2** are currently in progress.

Experimental Section

General experimental procedures

IR spectra were recorded with a JASCO J-20A spectrophotometer, and UV spectra were recorded with a Shimadzu UV mini-1240 instrument. Mass spectra were obtained with a Waters-Synapt G2 instrument, and ^{1}H and ^{13}C NMR spectra with a Jeol EX-400 spectrometer. Chemical shifts are given on a δ (ppm) scale with TMS as internal standard. Column chromatography was conducted on silica gel 60 (Kanto Chemical Co., Inc.). TLC was performed on a precoated silica gel plate (Merck), and spots were detected by spraying 10 % vanillin in H_2SO_4 followed by heating.

Biological material

Nodulisporium sp. SH-1 was isolated from the surface of a fruiting body of *Xylaria polymorpha* collected in October, 2009 from Mt. Gassan, Yamagata, Japan. The strain was identified by BEX Co. Ltd., Japan, using a DNA analysis of the 18S rDNA regions and has been deposited at our laboratory in the Faculty of Agriculture of Yamagata University.

Isolation procedure

Nodulisporium sp. SH-1 was cultivated on sterilized unpolished rice (1000 g) at 25 °C for 4 weeks. The moldy unpolished rice was extracted with MeOH, and the extract was concentrated. The resulting aqueous concentrate was partitioned into *n*-hexane and EtOAc layers. The purification of the EtOAc layer was guided by the characteristic intense blue coloration with vanillin-sulfuric acid solution on TLC plates. The EtOAc layer (10.5 g) was chromatographed on a silica gel column using first *n*-hexane-EtOAc (100 : 0 – 0 : 100) and then EtOAc-MeOH (100 : 0 – 0 : 100) as eluting solvents to give fractions Fr. 1-1 to 1-13. Fr. 1-4 (*n*-hexane-EtOAc, 50 : 50, 980 mg) was subjected to silica gel flash column chromatography using a mixture of $CHCl_3$-MeOH (90 : 10) to afford nodulisporone A (**1**, 15.1 mg). Fr. 1-6 (*n*-hexane-EtOAc, 30 : 70, 1010 mg) was crystallized from EtOAc to yield nodulisporone B (**2**, 350 mg).

*Nodulisporone A, (4-hydroxy-3-(2,6-dihydroxy-3-methylphenyl)-1(3H)-isobenzofuranone) (**1**)*

Pale-yellow amorphous solid. – UV/Vis (MeOH): λ_{max} ($\lg \varepsilon_{max}$) = 219 (4.1), 289 (3.6), 300 (sh, 3.6) nm. – IR (KBr): ν = 3326 (OH), 1714 (C=O), 1625, 1469, 1095 cm^{-1}. – ^{1}H NMR (400 MHz, CD_3OD) and ^{13}C NMR (100 MHz, CD_3OD) data: see Table 1. – HRMS ((+)-ESI-TOF): m/z = 273.0764 (calcd. 273.0763 for $C_{15}H_{13}O_5$, $[M+H]^+$).

*Nodulisporone B, (7-hydroxy-3-(2,6-dihydroxy-3-carboxyphenyl)-1(3H)-isobenzo-furanone) (**2**)*

Pale-yellow amorphous solid. – UV/Vis (MeOH): λ_{max} ($\lg \varepsilon_{max}$) = 221 (4.2), 253 (sh, 3.9), 301 (3.7) nm. – IR (KBr): ν = 3424 (OH), 3336, 3072, 1716 (C=O), 1594, 1469, 1091 cm^{-1}. – ^{1}H NMR (400 MHz, CD_3OD) and ^{13}C NMR (100 MHz, CD_3OD) data: see Table 1. – HRMS ((+)-ESI-TOF): m/z = 303.0500 (calcd. 303.0505 for $C_{15}H_{11}O_7$, $[M+H]^+$).

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