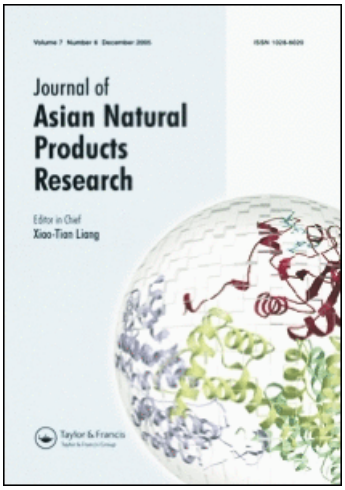

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A new diimide derivative from the co-culture broth of two mangrove fungi (strain no. E33 and K38)

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NOTE

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A new diimide derivative, named (−)-byssochlamic acid bisdiimide (**1**), was isolated from the mixed broth of two mangrove fungi (strain no. K38 and E33) from the South China sea coast. The structure of **1** was determined by comprehensive spectroscopic methods, including 1D and 2D NMR (COSY, HMQC, and HMBC) and semi-synthesis. Primary bioassays showed that **1** had weak cytotoxic activity against Hep-2 and HepG2 cells.

Keywords: mangrove fungi; mixed fermentation; (−)-byssochlamic acid bisdiimide; cytotoxic; semi-synthesis

1. Introduction

The word 'nonadride', which first occurred in 1962 [1], meant the substances, such as glaucanic acid, glauconic acid, and byssochlamic acid, that were biosynthesized by two C_9 units. Later, the name nonadride has evolved to mean the compounds that own the core structure of nonadrides, a C_9 ring with an affixed anhydride. Various pharmacological activities have been reported for compounds belonging to this class. Because of the specific structure and bioactivities, they have attracted many research groups to their synthesis [2]. As part of our studies on the metabolites of mangrove fungi from the South China sea coast, two nonadride derivatives, (−)-1-hydroxybyssochlamic acid and (−)-byssochlamic acid, have been isolated from the fungus (stain no. K38), and their relative configurations were confirmed by means of X-ray crystallographic analysis [3,4].

Mixed fermentation has been used in the food industry to enhance enzyme production. However, the method has been seldom used for secondary metabolite discovery [5]. To enhance metabolite production and search for new natural bioactive compounds, we have begun exploring the use of mixed fermentation in the fungi with potent metabolic ability. The fungi K38 and E33 were both isolated from the South China sea coast and their metabolites had been investigated by us previously [3,4,6]. They can grow well in a GYT culture medium by mixed fermentation. Primary bioassay showed that the ethyl acetate extract of their co-culture broth had higher cytotoxic activity against

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Hep-2 and HepG2 cells than that of K38 or E33. Therefore, the chemical constituents of the bioactive fraction were examined.

In this paper, we describe the isolation and identification of compound **1**, a new diimide derivative with weak inhibitory activity to Hep-2 and HepG2 cells. It was obtained from the mixed fermentation broth using GYT culture medium of two mangrove fungi (strain no. K38 and E33). In previous studies [3,4,6], compound **1** was not isolated from the fermentation broth when either fungus K38 or E33 was cultured individually. Since the compound is an analogue of (−)-byssochlamic acid, it is named (−)-byssochlamic acid bisdiimide (**1**). The relative configurations of **1** were determined by its semi-synthesis from (−)-byssochlamic acid. Its cytotoxic effects against Hep-2 and HepG2 cells were measured.

2. Results and discussion

(−)-Byssochlamic acid bisdiimide (**1**) was isolated as a colorless needle (15 mg) (Figure 1). Its EI-MS results showed that **1** had a molecular ion peak at *m/z* 330. The HR-EI-MS results (*m/z* 330.1573, calcd 330.1574) indicated that it had a molecular formula of $C_{18}H_{22}O_4N_2$, which suggested that compound **1** had nine degrees of unsaturation. The ^{13}C NMR spectrum of **1** (Table 1) revealed 18 carbon signals.

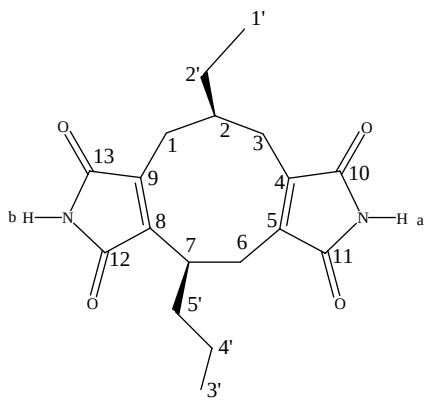


Figure 1. The structure of **1**.

Furthermore, the DEPT spectrum confirmed that this compound had two methyl, six methene, two methine, and eight quaternary carbon atoms. It was also shown that six degrees of unsaturation in **1** were due to the four carbonyl carbons (δ 170.6, 171.0, 171.1, and 171.3) and four olefinic carbon signals (δ 140.9, 141.2, 141.7, and 143.9). The other three degrees of unsaturation indicated that the molecule was a tricyclic compound. In the ^1H NMR spectrum, two signals of a three-proton triplet at δ 0.92 and 1.08 suggested the presence of two alkyl groups. Two vivid hydrogen signals at δ 8.19(s) and 8.24(s), corresponding to the absorption at 3268 cm^{-1} in the IR spectrum, are assigned to be —NH groups by analyzing the molecular formula and ^{13}C NMR spectrum. The NMR spectral data suggested that compound **1** was an analogue of (−)-byssochlamic acid.

The assignments of protons attached to their corresponding carbons were readily accomplished by the HMQC technique. The construction of the molecular framework was based on the analysis of ^1H–^1H COSY and HMBC experiments. An ethyl side chain was revealed by the contiguous ^1H–^1H COSY correlations from H-1′ to H-2′, H-2′ to H-2, H-2 to H-3 and H-1 and HMBC correlations from H-2′ to C-1, C-2 and C-1′, thereby placing the ethyl side chain at the C-2 position. The ^1H–^1H COSY correlations from H-3′ to H-4′ and H-4′ to H-5′, and HMBC correlations from H-5′ to C-7 and C-6 showed the presence of a propyl side chain connected to C-7. While the positions of two five-membered diimides were determined by the HMBC correlations from H-3 to C-4 and C-10, from H-6 to C-5 and C-11, from H-1 to C-9 and C-13, from H-2 to C-9, and from H-7 to C-8, C-9 and C-12, respectively. Additionally, the locations of two —NH groups were determined by the HMBC correlations from H-a to C-10 and C-11, and from H-b to C-12 and C-13. Thus, the planar structure of **1** was unambiguously established. Figure 2 shows the key HMBC correlations of **1**.

Table 1. ^{1}H and ^{13}C NMR spectral data of **1** (δ in ppm, J in Hz).

	^{1}H NMR	^{13}C NMR (DEPT)	^{1}H–^{1}H COSY
1′	1.08 (t, J = 7.2 Hz)	11.9 (CH_3)	H-2′
2′	1.59 (overlap)	33.8 (CH_2)	H-1′,2
3′	0.92 (t, J = 7.2 Hz)	14.2 (CH_3)	H-4′a,4′b
4′	a1.42 (qd, J = 7.2, 13.8 Hz), b1.32 (qd, J = 7.2, 13.8 Hz)	21.0 (CH_2)	4′aH-4′b,3′,5′, 4′bH-4′a,3′,5′
5′	1.67 (m)	35.4 (CH_2)	H-4′a,4′b,7
1	2.20 (d, J = 12.6 Hz)	28.8 (CH_2)	H-2
2	1.75 (overlap)	40.4 (CH)	H-3a,3b,2′
3	a2.60 (qd, J = 12.6, 1.5 Hz), b2.78 (bd, J = 12.6 Hz)	30.6 (CH_2)	3aH-3b,2, 3bH-3a,2
4	–	141.2 (C)	–
5	–	141.7 (C)	–
6	a2.65 (dd, J = 11.1, 3.6 Hz), b2.74 (bd, J = 11.1 Hz)	28.0 (CH_2)	6aH-6b,7, 6bH-6a,7
7	3.34 (m)	36.9 (CH)	H-5′,6a,6b
8	–	140.9 (C)	–
9	–	143.9 (C)	–
10	–	171.3 (C)	–
11	–	170.6 (C)	–
12	–	171.0 (C)	–
13	–	171.1 (C)	–
H-a	8.19 (s)	–	–
H-b	8.24 (s)	–	–

To determine the relative stereochemistry of **1**, the semi-synthesis method [7–10], which has been approved to involve no change in configuration of byssochlamic acid and other compounds belonging to nonadride, was adopted. When (−)-byssochlamic acid, isolated from the fungus (strain no. K38) as described in the previous paper [4], was treated with aqueous ammonia in toluene by heating for 6 h, a small amount of the product appeared. It showed a (−) peak on the optical rotation detector the same as for the (−)-byssochlamic acid. The values of the NMR spectral data and the specific optical rotation were the same as that for compound **1**, which suggested that the synthetic product has the same structure as compound **1**. Figure 3 shows the chemical reaction equation from (−)-byssochlamic acid to **1**. Therefore, the relative configuration of **1** should be consistent with that of (−)-byssochlamic acid, which is confirmed by X-ray crystallographic analysis reported in the previous paper [4]. From the above result, the relative stereochemistry of **1** has been determined. Two alkyl side chains were consequently oriented as the *cis*-configuration. Figure 1 shows the structure of **1**.

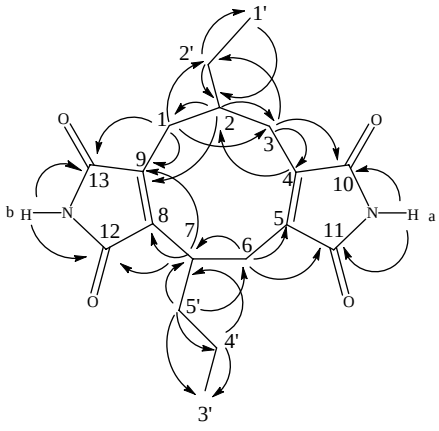
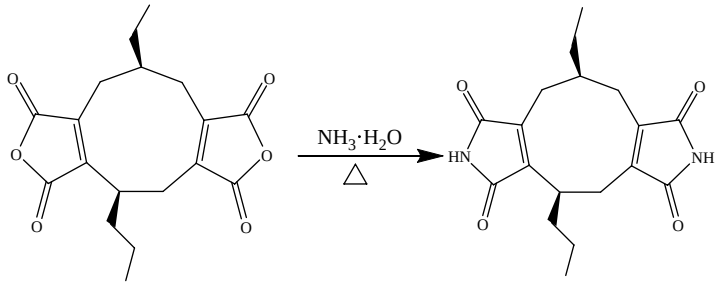


Figure 2. The key HMBC correlations of **1**.

Primary bioassays using MTT method [11] showed that **1** exhibited weak cytotoxic effects against Hep-2 and



(–)-byssochlamic acid **1**

Figure 3. The chemical reaction equation from (−)-byssochlamic acid to **1**.

HepG2 cells, with IC_{50} values of 45 and 51 μg/ml, respectively.

3. Experimental

3.1 General experimental procedures

Melting points were detected on a Fisher-Johns hot-stage apparatus and are uncorrected. IR spectra were obtained on a Nicolet 5DX-FTIR spectrophotometer. NMR data were recorded on a Varian Inova-500 NB spectrometer, using $CDCl_3$ as the solvent and TMS as the internal standard. Mass spectra were acquired on a VG-ZAB mass spectrometer. Optical rotation was determined in acetone at room temperature using a Horiba high sensitivity SEPA-300 polarimeter. Chromatography was carried out on a silica gel column (200–300 mesh; Qingdao Haiyang Chemicals, Qingdao, China).

3.2 Fungus material

The strains of the fungi K38 and E33 were isolated from the South China sea coast. Both are apospory and their general species have not been identified. They were maintained on cornmeal seawater agar. Plugs of agar supporting mycelial growth were cut and transferred aseptically to a 250 ml Erlenmeyer flask containing 100 ml of liquid medium (glucose 10 g/l, peptone 2 g/l, yeast extract 1 g/l, NaCl 30 g/l). The flask was incubated at 30°C on a rotary shaker for 5–7 days. The mycelium was aseptically transferred to 500 ml Erlenmeyer flasks containing culture liquid (200 ml). The flasks were then incubated at 30°C for 25 days.

3.3 Extraction and isolation

The cultures (100 liters) were filtered through cheesecloth. The filtrate was concentrated to 5 liters *in vacuo* below 50°C and extracted five times by shaking with an equal volume of ethyl acetate. The combined extracts were chromatographed repeatedly on a silica gel column using gradient elution from petroleum ether to ethyl acetate to obtain (−)-byssochlamic acid bisdiimide (**1**) from the ethyl acetate/petroleum ether (60/40) fraction.

(−)-Byssochlamic acid bisdiimide (**1**), colorless needles, mp 170–172°C, $[\alpha]_D^{20}$ −40.0 (*c*=0.07, acetone). IR (KBr) ν_{max} (cm^{-1}): 3268(—NH), 3061, 2961, 2931, 2873, 1774 (HN—C=O), 1710 (HN—C=O), 1459, 1384, 1077, 772. ^1H NMR (500 MHz, $CDCl_3$) and ^{13}C NMR (125 MHz, $CDCl_3$) spectral data: see Table 1. EI-MS: *m/z* 330, 301, 259, 207, 178, 165. HR-EI-MS: *m/z* 330.1573 (calcd for $C_{18}H_{22}O_4N_2$, 330.1574).

3.4 Synthesis of byssochlamic acid bisdiimide from (−)-byssochlamic acid

Aqueous ammonia (30%, 40 ml) was dropped slowly to a solution of (−)-byssochlamic acid (20 mg) in 8 ml toluene within 1 h and the mixture was stirred under reflux for 5 h. The reaction mixture

was acidified with 4 mol/l HCl and extracted with CH_2Cl_2. The extract was evaporated *in vacuo*. The residue was purified by VLC using hexane–acetone (3:2) to obtain (−)-byssochlamic acid (4 mg), along with its diimide derivative (6 mg). Figure 3 shows the chemical reaction equation from (−)-byssochlamic acid to **1**.

3.5 Bioassays for cytotoxic activity

The cytotoxic assays were performed using the MTT assay method [10]. The Hep-2 and HepG2 cells were cultured in RPMI-1640 medium (Nissui) supplemented with 10% heat-inactivated fetal bovine serum and kanamycin (100 mg/l) in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. The cell suspension (100 ml) was added to each well of a 96-microwell plate and incubated for 24 h. Test compounds were dissolved in DMSO in various concentrations (100, 50, 25, 12.5, and 6.25 mg/ml) and 10 μl of the test solution or DMSO (control) was added to each well. The plate was kept in an incubator for 48 h. After terminating the cell culture by adding 20 ml MTT (5% in PBS) to each well, the plate was further incubated for 4 h. To each well, 100 ml of 10% SDS–0.01 N HCl was added. The plate was read on a microplate reader (MPR A4i; Tosoh, Tokyo, Japan) at 492 nm. A dose-response curve was plotted for each compound and the concentrations giving 50% inhibition of cell growth (IC_{50}) were recorded. Primary bioassays showed that compound **1** exhibited weak cytotoxicity against Hep-2 and HepG2 cells.

Acknowledgements

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