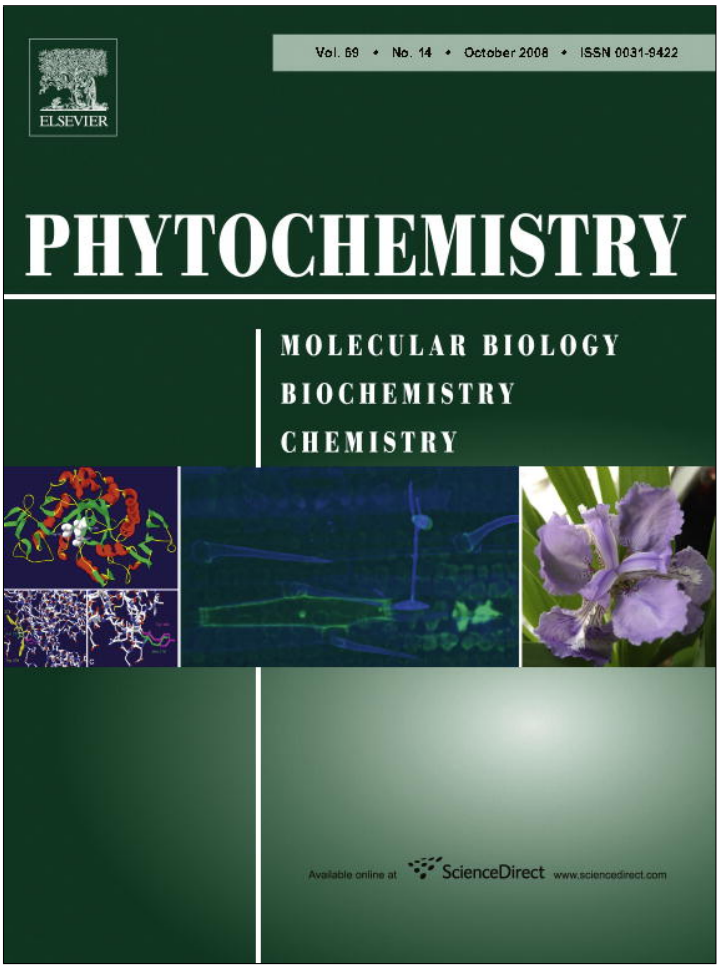

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# Cytotoxic and antiplasmodial substances from marine-derived fungi, *Nodulisporium* sp. and CRI247-01

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ABSTRACT

Nodulisporacid A (**1**) was isolated from a marine-derived fungus *Nodulisporium* sp. CRIF1, while vermelhotin (**5**) was obtained from an unidentified fungus CRI247-01 (a member of the Order Pleosporales). Both **1** and **5** occurred as equilibrium *E*/*Z* mixtures. Ester derivatives (**2** and **3**) and vermelhotin (**5**) showed cytotoxic activity against eleven cancer cell lines. Nodulisporacid A (**1**) and vermelhotin (**5**) exhibited moderate antiplasmodial activity.



## 1. Introduction

Marine natural products exhibit interesting biological activities and several compounds are in clinical and advanced preclinical trials (Newman and Cragg, 2004). Marine-derived fungi are rich sources of bioactive compounds, and thus, have received attention from chemists (Abdel-Wahab et al., 2007; Cueto et al., 2006; Kralj et al., 2007; Wright and Lang-Unnasch, 2005; Zhang et al., 2008). Fungi isolated from marine organisms are divided into two groups, obligatory marine fungi and marine-derived fungi; the first group grows only in salt containing media (sea water or artificial sea water), while the latter can be cultured either in salt- or in fresh water-containing media. However, marine-derived fungi normally grow rapidly in salt-containing media but hardly grow in media without salt supplement, and sometimes, from our experience, they even change their morphology when cultured in fresh water-containing media. Herein, we report the isolation of a new tetronic acid, nodulisporacid A (**1**), from a marine-derived fungus *Nodulisporium* sp. CRIF1, and a known tetramic acid, vermelhotin (**5**), from an unidentified fungus CRI247-01 (a member of the Order Pleosporales) (Fig. 1). The isolated compounds and derivatives are evaluated for their cytotoxic (against eleven cancer cell lines) and antiplasmodial activities. Nodulisporacid A (**1**) did not exhibit cytotoxic activity, however, its derivatives **2** and **3** obtained from methylation and benzylation showed cytotoxic activity. Nodulisporacid A (**1**) and vermelhotin (**5**) exhibited moderate antiplasmodial activity.

## 2. Results and discussion

### 2.1. Structural determination

Separation of a cell extract of the marine-derived fungus *Nodulisporium* sp. CRIF1 yielded a new tetronic acid, nodulisporacid A (**1**) (Fig. 1). APCI-TOF MS spectrum established the molecular formula $C_{16}H_{20}O_6$ for **1**. The $^1$H NMR (DMSO-$d_6$) technique indicated spontaneous inter-conversion between *E*- and *Z*-isomers, which finally equilibrated at the *E*/*Z* ratio of 1:1. Attempts to separate an equilibrium *E*/*Z* mixture by semi-preparative reversed-phase HPLC met with failure; therefore, structural analysis of nodulisporacid A (**1**) was carried out on the equilibrium *E*/*Z* mixture. The $^1$H and $^{13}$C NMR spectra of the equilibrium *E*/*Z* mixture of **1** showed two sets of resonances with a ratio of 1:1 for *E*- and *Z*-isomers. However, only the *Z*-isomer was structurally elucidated in detail here. The $^{13}$C NMR spectrum established the presence of an unsaturated ketone ($\delta_C$ 196.8, C-3) and an oxygenated sp$^3$ methine ($\delta_C$

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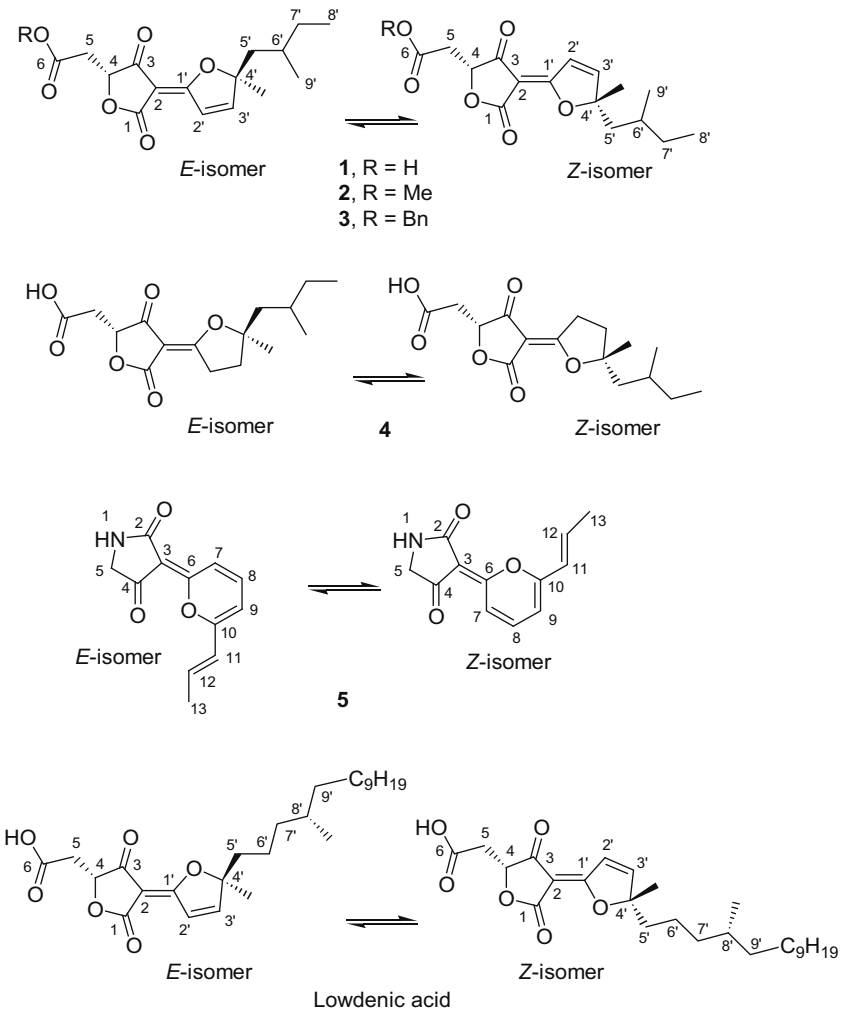


**Fig. 1.** Structures of nodulisporacid A (**1**) and derivatives **2**-**4**, vermelhotin (**5**), and lowdenic acid.

79.0, C-4). DEPT experiments indicated the presence of three methyls, three methylenes, two $sp^2$ methines, and two $sp^3$ methines. The $^1H$–$^1H$ COSY spectrum established the partial structure from H-5′ through H-9′, and showed couplings of H-4/H-5 and H-2′/H-3′. The HMBC spectrum showed the correlations from H-4 to C-1, C-3, C-5, and C-6; and H-5 to C-3, C-4, and C-6, establishing a structure of a derivative of tetrahydrofurandione. HMBC correlations from both H-2′ and H-3′ to C-1′ and C-4′; H-5′ to C-3′ and C-4′; and 4′-Me to C-3′, C-4′, and C-5′, suggesting the attachment of a furfurylidene to an aliphatic side chain. Unfortunately, there was no HMBC correlation to the $sp^2$ quaternary C-2 ($\delta_C$ 93.4). However, the information from the presence of an unsaturated ketone and the molecular formula implied the connection of C-2 to C-1′, establishing a gross structure of nodulisporacid A (**1**), which was a derivative of lowdenic acid (Angawi et al., 2003) (Fig. 1). The evidence from the similarity of $^{13}C$ NMR resonances (Table 1) of C-2 ($\delta_C$ 93.3) and C-1′ ($\delta_C$ 180.1) of lowdenic acid (Angawi et al., 2003) and those [C-2 ($\delta_C$ 93.4) and C-1′ ($\delta_C$ 179.4)] of nodulisporacid A (**1**) further confirmed the structure of nodulisporacid A (**1**). Assignment of $^1H$ and $^{13}C$ resonances for the *E*-isomer and *Z*-isomer of nodulisporacid A (**1**) was accomplished by correlation with *E* and *Z* isomers of lowdenic acid and carolic acid (Angawi et al., 2003; Jacobsen et al., 1978). Generally, $\delta_C$ of C-1 of the *E*-isomer was about 3 ppm lower field than that of the *Z*-isomer (Angawi et al., 2003; Jacobsen et al., 1978). Chemical transformations of nodulisporacid A (**1**) by methylation, benzylation, and hydrogenation yielded respective products **2**, **3**, and **4** (Fig. 1).

We further tried to clarify stereochemistry of **1** by a single crystal X-ray crystallography; however, several attempts to crystallize nodulisporacid A (**1**) and its derivatives **2**–**4** in various solvent conditions failed to obtain suitable crystals for an X-ray crystallographic technique. Spontaneous *E*/*Z* inter-conversion of compounds **1**–**4** may hinder uniform crystallization. The biosynthesis of nodulisporacid A (**1**) may be similar to that of lowdenic acid (Angawi et al., 2003), involving the condensation of a modified 12-carbon polyketide (decorating as a side chain, the furylidene ring, and C-1 and C-2 of the tetrahydrofurandione ring) with a four carbon dicarboxylic acid. Such tetronic acid biosynthesis had been experimentally proven, for examples, italicic acid (Arai et al., 1989), and carolic acid (Jacobsen et al., 1978; Simonsen et al., 1980). Lowdenic acid and nodulisporacid A (**1**) shared the same core structure (Fig. 1), but differed at the aliphatic side chain (C-5′ to C-9′). Lowdenic acid is biosynthesized from the condensation of a modified 20-carbon polyketide with a four carbon dicarboxylic acid unit (Angawi et al., 2003), which differs from that of nodulisporacid A (**1**) only at the polyketide intermediate. Therefore, it is more likely that the furylidene ring and tetrahydrofurandione in both **1** and lowdenic acid were biosynthesized in a similar manner. Based upon the biosynthetic analogy, it was proposed that the relative configurations at C-4 and C-4′ of nodulisporacid A (**1**) were the same as those of lowdenic acid (Angawi et al., 2003). The similarity between the $^{13}C$ NMR chemical shifts (Table 1) of the tetronic acid core structure (C-1 through C-6; and C-1′ through C-4′) of both *E*-and *Z*-isomers of nodulisporacid A (**1**) and those of *E*-and

**Table 1**
NMR spectroscopic data comparison of the core structure of nodulisporacid A (**1**) and lowdenic acid (Angawi et al., 2003)

| | Nodulisporacid A (1)[a] | | | | Lowdenic acid[b] | | | |
|---|---|---|---|---|---|---|---|---|
| | $\delta_H$ (mult., *J* in Hz) | | $\delta_C$ | | $\delta_H$ (mult., *J* in Hz) | | $\delta_C$ | |
| | *E* | *Z* | *E* | *Z* | *E* | *Z* | *E* | *Z* |
| 1 | | | 170.3 | 167.5 | | | 170.1 | 167.6 |
| 2 | | | 93.8 | 93.4 | | | 93.6 | 93.3 |
| 3 | | | 194.3 | 196.8 | | | 194.2 | 196.3 |
| 4 | 4.78 (*dd*, 6.2, 4.0) | 4.85 (*dd*, 6.2, 4.1) | 78.8 | 79.0 | 4.87 (*dd*, 7.2, 4.2) | 4.91 (*dd*, 7.2, 4.2) | 78.2 | 78.5 |
| 5a | 2.78 (*dd*, 17.0, 4.0) | 2.82 (*dd*, 17.0, 4.0) | 36.1 | 36.0 | 3.05 (*dd*, 17.0, 4.2) | 3.02 (*dd*, 17.0, 4.2) | 35.8 | 35.8 |
| 5b | 2.68 (*dd*, 17.0, 6.2) | 2.71 (*dd*, 17.0, 6.2) | | | 2.81 (*dd*, 17.0, 7.2) | 2.83 (*dd*, 17.0, 7.2) | | |
| 6 | | | 171.2 | 171.2 | | | 173.7 | 173.7 |
| 1′ | | | 178.4 | 179.4 | | | 179.3 | 180.1 |
| 2′ | 7.27 (*d*, 5.8) | 7.37 (*d*, 5.8) | 121.7 | 121.7 | 7.50 (*d*, 6.0) | 7.55 (*d*, 6.0) | 123.3 | 123.1 |
| 3′ | 7.98 (*d*, 5.8) | 7.98 (*d*, 5.8) | 163.4 | 163.3 | 7.44 (*d*, 6.0) | 7.45 (*d*, 6.0) | 160.3 | 160.4 |
| 4′ | | | 102.4 | 101.8 | | | 102.5 | 102.0 |
| 5′a | 1.98 (*m*) | 2.01 (*m*) | 43.7 | 43.7 | 1.92 (*m*) | 1.95 (*m*) | 38.3 | 38.2 |
| 5′b | 1.60 (*m*) | 1.64 (*m*) | | | | | | |
| 4′-Me | 1.50 (*s*) | 1.51 (*s*) | 23.7 | 23.6 | 1.62 (*s*) | 1.63 (*s*) | 23.2 | 23.3 |

[a] NMR spectra were acquired in DMSO-$d_6$, operating at 400 MHz for $^1$H and 100 MHz for $^{13}$C.
[b] NMR spectra were acquired in $CDCl_3$; the $^1$H NMR spectrum was operated at 600 MHz, however, the $^{13}$C NMR spectrum was recorded at 90 MHz (Angawi et al., 2003).

*Z*-isomers of lowdenic acid (Angawi et al., 2003) further supported that both shared the same relative configurations at C-4 and C-4′. In addition, both nodulisporacid A (**1**) and lowdenic acid exhibited negative specific optical rotation.

Separation of a crude broth extract of the fungus of Order Pleosporales CRI247-01 gave a known tetramic acid, vermelhotin (**5**) (Fig. 1), which was previously obtained as a single *E*-isomer from an unidentified fungus IFM 52672 (Hosoe et al., 2006). However, as indicated by the $^1$H spectrum, vermelhotin (**5**) from the fungus CRI247-01 spontaneously underwent inter-conversion between *E*- and *Z*-isomers, forming an equilibrium *E*/*Z* mixture with the ratio of 1:2. Unfortunately, this equilibrium *E*/*Z* mixture could not be separated by semi-preparative reversed-phase HPLC.

### 2.2. Biological activity

Fungal metabolites and derivatives were evaluated for their cytotoxic activity against eleven cancer cell lines. Nodulisporacid A (**1**) and its hydrogenated product **4** were inactive against all cell lines tested, while methyl ester **2** and benzyl ester **3** exhibited cytotoxic activity (Table 2). It should be noted that nodulisporacid A (**1**) and its hydrogenated product **4** possessed a polar carboxylic acid that may hinder compound permeation into lipophilic membrane of cancer cells. Reduction of polarity of nodulisporacid A (**1**) by methylation or benzylation (i.e. compounds **2** and **3**) significantly improved cytotoxic activity (Table 2). Nodulisporacid A (**1**) and its derivatives **2** and **4** exhibited moderate antiplasmodial activity with the $IC_{50}$ values of 1–10 μM (Table 3). Vermelhotin (**5**) was found to exhibit cytotoxic activity (Table 2), and also showed antiplasmodial activity with the $IC_{50}$ values of 1–10 μM (Table 3). Previously, vermelhotin (**5**) was tested against fungi and bacteria, however it showed no antifungal and antibacterial activities (Hosoe et al., 2006). Recently, tetronic and tetramic acids are of pharmaceutical interest due to their interesting biological activities, for examples, inhibition of aspartic protease β-secretase (Larbig and Schmidt, 2006), protein tyrosine phosphatases (Sodeoka et al., 2001), hepatitis C virus RNA-dependent RNA polymerase (Evans et al., 2006), and induction of cell cycle arrest and apoptosis (Choi et al., 2005).

### 2.3. Concluding remarks

A new tetronic acid, nodulisporacid A (**1**), was isolated from a marine-derived fungus *Nodulisporium* sp. CRIF1, while a tetramic acid, vermelhotin (**5**), was isolated from an unidentified fungus CRI247-01. Reduction of the polarity in **1** led to a significant improvement of cytotoxic activity; the mechanism of action of this tetronic acid is interesting to be pursued. Both nodulisporacid A (**1**) and vermelhotin (**5**) exhibited antiplasmodial activity. The presence of tetronic acid **1** and tetramic acid **5** in the fungal strains CRIF1 and CRI247-01 proves that marine-derived fungi are rich sources of biologically active compounds which may be pharmaceutically important.

**Table 2**
Cytotoxic activity of compounds **1**–**5** against eleven cancer cell lines

| Cell line[a] | Cytotoxic activity ($IC_{50}$, μg/mL); mean ± s.d., *n* = 3 | | | | | |
|---|---|---|---|---|---|---|
| | **1** | **2** | **3** | **4** | **5** | Etoposide[c]/ Doxorubicin[d] |
| HuCCA-1 | >50 | 2.30 ± 0.10 | 2.10 ± 0.21 | >50 | 2.90 ± 0.17 | 5.30 ± 1.53[c] |
| KB | >50 | 2.20 ± 0.30 | 3.20 ± 0.44 | >50 | 0.50 ± 0.00 | 0.46 ± 0.15[c] |
| HeLa | >50 | 2.70 ± 0.17 | 2.60 ± 0.12 | >50 | 0.33 ± 0.08 | 0.40 ± 0.12[c] |
| MDA-MB231 | >50 | 2.50 ± 0.46 | 0.38 ± 0.04 | >50 | 0.31 ± 0.08 | 0.40 ± 0.10[c] |
| T47D | >50 | 1.70 ± 0.25 | 0.14 ± 0.02 | >50 | 1.25 ± 0.35 | 0.04 ± 0.01[c] |
| H69AR | >50 | ND[b] | ND[b] | >50 | 2.50 ± 0.42 | 36.0 ± 1.41[c] |
| HepG2 | >50 | 2.30 ± 0.35 | 2.00 ± 0.42 | >50 | 2.50 ± 0.70 | 0.19 ± 0.02[d] |
| A549 | >50 | 7.50 ± 0.71 | 2.20 ± 0.28 | >50 | 8.20 ± 2.54 | 0.48 ± 0.03[d] |
| HCC-S102 | >50 | 6.00 ± 1.41 | 4.80 ± 0.14 | >50 | 13.5 ± 0.70 | 1.20 ± 0.14[d] |
| HL-60 | >50 | 1.01 ± 0.20 | 1.18 ± 0.14 | >50 | 1.60 ± 0.03 | 0.77 ± 0.35[c] |
| P388 | >50 | 0.77 ± 0.00 | 0.70 ± 0.06 | >50 | 1.23 ± 0.02 | 0.10 ± 0.01[c] |

[a] Cancer cell lines are: HuCCA-1 Human cholangiocarcinoma cancer cells; KB Human epidermoid carcinoma of the mouth; HeLa Cervical adenocarcinoma cell line; MDA-MB231 Hormone-independent breast cancer cell line; T47D Hormone-dependent breast cancer cell line; H69AR Multidrug-resistant small cell lung cancer cell line; HepG2 Human hepatocellular liver carcinoma cell line; A549 Human lung carcinoma cell line; HCC-S102 Hepatocellular carcinoma cell line; HL-60 Human promyelocytic leukemia cell line; and P388 Murine leukemia cell line.
[b] ND = Not determined.
[c] Etoposide was used as the reference drug.
[d] Doxorubicin was used as the reference compound.

## 3. Experimental

### 3.1. General experimental procedures

$^1$H and $^{13}$C NMR spectra were recorded on a Bruker AM 400 NMR instrument (operating at 400 MHz for $^1$H and 100 MHz for $^{13}$C) and a Bruker AVANCE 600 NMR spectrometer (operating at 600 MHz for $^1$H and 150 MHz for $^{13}$C). FTIR were obtained using

**Table 3**
Antiplasmodial activity of compounds **1–5**

| 1 | 2 | 3 | 4 | 5 | Chloroquine hydrochloride[a] |
|---|---|---|---|---|---|
| *Antiplasmodial activity (μM)* | | | | | |
| 1–10 | 1–10 | >10 | 1–10 | 1–10 | 0.29 |

[a] Chloroquine hydrochloride was a standard drug.

a universal attenuated total reflectance attached on Perkin–Elmer Spectrum One spectrometer. APCI-TOF MS were determined using a Bruker MicroTOF$_{LC}$ spectrometer. Optical rotations were measured with sodium D line (590 nm) on JASCO DIP-370 digital polarimeter.

### 3.2. Fungal materials and identification

*Nodulisporium* sp. CRIF1 was isolated from an unidentified soft coral CRI258 (collected from Surin Island, Phang-nga Province, Thailand), while the fungus CRI247-01 was isolated from an unidentified sponge CRI247 (collected from Surin Island, Phang-nga Province, Thailand). Voucher specimens of an unidentified soft coral CRI258 and an unidentified sponge CRI247 were deposited at Chulabhorn Research Institute, Bangkok, Thailand. Both unidentified soft coral CRI258 (dark brown body with finger-like lobes) and sponge CRI247 (orange body with wavy surface and small prickles) were collected by SCUBA diving at depths of approximately 35–40 feet, and they inhabited rock substrates. *Nodulisporium* sp. CRIF1 and the fungus CRI247-01 were deposited both at Chulabhorn Research Institute and at the MIM Laboratory, Department of Microbiology, Faculty of Science, Mahidol University, Thailand.

The CRIF1 and CRI247-01 fungi were cultured on different mycological media including potato dextrose agar, Sabouraud's dextrose agar, corn meal agar, and oat meal agar, with or without sea water supplemented. Incubation was at 25 °C with 12/12 h dark/light cycle and the cultures were examined daily for characteristic morphology. For molecular based identification, fungal mycelia from 7 days old cultures in potato dextrose broth were prepared for PCR using the FTA® Plant Kit according to the manufacturer's instructions. The ITS1-5.8S-ITS2 ribosomal RNA gene region of fungal genomic DNA was amplified using the ITS5 (GGAAGTAAAAGTCGTAACAAGG) and ITS4 (TCCTCCGCTTATTGATATGC) primers (White et al., 1990) as previously described (Prachya et al., 2007). PCR products were sequenced in both directions primed with either of the two primers used to originally amplify the fragment. DNA sequence of the 5.8S rRNA gene and the ITS1-5.8S-ITS2 were used to search for similar sequences in GenBank using BLASTN 2.2.18 (Altschul et al., 1997). DNA sequence similarity was determined by the ClustalW2 multiple sequence alignment program (Larkin et al., 2007). Alignments and boundary approximations were adjusted manually using BioEdit v7.0.0. Phylogenetic relationship was estimated using PAUP* (v 4.0 b10) (Swofford, 2003).

The CRI247-01 fungus grew as brown colony but did not produce conidia or sporulation structure. Its 5.8S rRNA gene sequence was novel, having 97–98% homology with known fungal species in the Order Pleosporales. Classification to Family level was not certain due to the uniqueness of this 5.8S sequence, the CRI247-01 was thus identified as mycelia sterilia fungus member of Pleosporales.

On sea water-based oat meal agar, the CRIF1 fungus grew rapidly as white colony producing conidiogenous structures and conidia characteristic for the form-genus *Nodulisporium* as described in the Ju and Rogers monograph (Ju and Rogers, 1996). DNA sequence of the 5.8S rRNA gene is perfectly matched (100% homology) with a group of ascomycetous fungi of the Family Xylariaceae. Phylogenetic analysis of the ITS1-5.8S-ITS2 sequence using maximum parsimony placed the CRIF1 in the same clade with known *Nodulisporium* sp. and *Hypoxylon* a genus of sexual fungi commonly produce *Nodulisporium* anamorph. Based on microscopic morphology and rRNA sequence, the CRIF1 was tentatively classified in the form-genus *Nodulisporium*, possibly an anamorph of a species of *Hypoxylon*. The ITS1-5.8S-ITS2 sequences of CRI247-01 and CRIF1 have been deposited in GenBank with accession EU445294 and EU365344, respectively.

### 3.3. Extraction and separation

Potato dextrose agar and potato dextrose broth used for fungal cultivation were prepared in sea water instead of distilled water. The fungus *Nodulisporium* sp. CRIF1 was maintained on potato dextrose agar medium at room temperature for 7 days, and then inoculated into 10 × 1 L Erlenmeyer flasks, each containing 250 mL of potato dextrose broth, and further incubated under static condition. The fermentation broth (2.5 L) of *Nodulisporium* sp. CRIF1 was filtered through a filter paper. The broth filtrate was partitioned with EtOAc 3 times. The EtOAc layers were combined, and evaporated under reduced pressure at temperature not exceeding 40 °C to yield a dark brown solid (4.95 g). Fungal mycelia were extracted twice with MeOH (each with 500 mL) and the fungal residue was subsequently extracted twice with $CH_2Cl_2$ (each with 500 mL). The MeOH and $CH_2Cl_2$ extracts were combined and extracted with EtOAc (3 × 500 mL) to give a mycelial crude extract (dark brown solid, 1.93 g). A crude cell extract was separated by Sephadex LH-20 column chromatography (CC) (eluted with MeOH) to give eighteen fractions (A1–A18). Fraction A9 (378.8 mg) was further purified by Sephadex LH-20 CC (eluted with MeOH), yielding nodulisporacid A (**1**) (173 mg). $^1$H NMR analysis of nodulisporacid A (**1**) revealed spontaneous inter-conversion between *E*- and *Z*-isomers, forming an equilibrium *E*/*Z* mixture with the ratio of 1:1. Unfortunately, this equilibrium *E*/*Z* mixture could not be isolated by semi-preparative reversed-phase HPLC. A crude broth extract was also separated by Sephadex LH-20 CC in the same manner as that of a cell extract, providing 19.4 mg of nodulisporacid A (**1**).

The fungus strain CRI247-01 was grown on potato dextrose agar at room temperature for 7 days, after which was transferred into 20 × 1 L Erlenmeyer flasks each containing 250 mL of potato dextrose broth. Fermentation was conducted under static conditions (without shaking) at room temperature for 24 days. The fungal culture was filtered to obtain the fermentation broth (3.75 L) and mycelia. Fermentation broth was extracted with an equal volume of EtOAc to obtain a dark brown solid (213.9 mg), while mycelia were extracted sequentially with MeOH (2 × 500 mL) and $CH_2Cl_2$ (2 × 500 mL). To the MeOH and $CH_2Cl_2$ extracts were added 200 mL of $H_2O$ and extracted with EtOAc (3 × 500 mL) to obtain a dark brown solid (2.55 g). A crude broth extract was separated by Sephadex LH-20 CC, eluted with MeOH, yielding ten fractions (B1-B10). Fractions B8-B10 were combined and separated by silica gel CC, sequentially eluted with hexane/EtOAc (80:20), $CH_2Cl_2$/EtOAc (80:20), and $CH_2Cl_2$/MeOH (95:5), to furnish vermelhotin (**5**) (23.2 mg). It was found that, as indicated by the $^1$H NMR spectrum, vermelhotin (**5**) underwent spontaneous inter-conversion between *E*- and *Z*-isomers, and finally equilibrated at the *E*/*Z* mixture of 1:2. Attempts to separate the equilibrium *E*/*Z* mixture of **5** by semi-preparative reversed-phase HPLC had met with failure. A crude cell extract of the fungus strain CRI247-01 was separated in a similar manner to that of a broth extract, yielding of vermelhotin (**5**) (21.3 mg).

### 3.4. Bioassays

#### 3.4.1. Cytotoxicity

The cytotoxic assay was performed as previously described by Tengchaisri and co-workers (1998). Briefly, cell lines suspended in RPMI 1640 containing 10% FBS were seeded at $1 \times 10^4$ cells (100 μL) per well in 96-well plate, and incubated in humidified atmosphere, 95% air, 5% $CO_2$ at 37 °C. After 24 h, additional medium (100 μL) containing the test compound and vehicle was added to a final concentration of 50 μg/mL, 0.2% DMSO, and further incubated for 3 days. After that, the cells were fixed with EtOH–$H_2O$ (95:5, v/v), stained with crystal violet solution, and lysed with a solution of 0.1 N HCl in MeOH, after which absorbance was measured at 550 nm. The number of surviving cells was determined from the absorbance. Etoposide and doxorubicin were used as the reference drugs (Table 2).

#### 3.4.2. Antiplasmodial activity

Human erythrocytes (type O) infected with *Plasmodium falciparum* strain 94, (Chloroquine resistant) was maintained in continuous culture, according to the method described by Trager and Jensen (1976). RPMI 1640 culture medium supplemented with 25 mM of HEPES, 40 mg/L gentamicin sulfate and 10 mL of human serum was used in continuous culture. Before starting the experiment, *P. falciparum* culture was synchronized by using sorbitol induced hemolysis according to the method of Lambros and Vanderberg (1979) to obtain only ring-infected cells and then incubated for 48 h prior to the drug testing to avoid effect of sorbitol. The experiments were started with synchronized suspension of 0.5–1% infected red blood cell during ring stage. Parasites were suspended with culture medium supplemented with 15% human serum to obtain 10% cell suspension. The parasite suspension was put into 96-well microculture plate; 50 μL in each well and then added 50 μL of various test drug concentrations. These parasite suspensions were incubated for 48 h in the atmosphere of 5% $CO_2$ at 37 °C. After 48 h incubation, parasite culture was fixed by adding 0.25% (v/v) glutaraldehyde in phosphate buffer saline (PBS) and these were kept for DNA staining. Before parasite DNA staining, $5 \times 10^6$ red blood cells from each glutaraldehyde-fixed sample were washed once with PBS and re-suspended in PBS containing propidium iodide (PI) at a final concentration at 10 μg/mL, and held for at least 1 h in dark. The PI stained cells were excited with 488 nm. Red fluorescence was detected at 585 nm. Red blood cells were gated on the basis of their forward scatter and side scatter. For each sample, 30,000 cells were required, stored and analyzed. Percent parasitemia, fluorescence intensity, and any abnormal fluorescence pattern were obtained from an integrated fluorescence histogram between the test and the control sample. Chloroquine hydrochloride was used as a standard drug, exhibiting the $IC_{50}$ value of 0.29 μM (Table 3).

### 3.5. Spectral data of compounds

#### 3.5.1. Nodulisporacid A (**1**)

(*E*:*Z* mixture); Yellow amorphous solid; $[\alpha]_D^{27}$ −20.6 (*c* 1.10, MeOH); UV (MeOH) $\lambda_{max}$ (log ε) 317.0 (4.28) nm; IR $\nu_{max}$ 3433, 2962, 2931, 2873, 1755, 1696, 1583, 1563, 1047, 1021, 992, 821 $cm^{-1}$; ***E*-isomer**: $^1$H NMR (DMSO-$d_6$, 400 MHz) δ 7.98 (1H, *d*, *J* = 5.8 Hz, H-3′), 7.27 (1H, *d*, *J* = 5.8 Hz, H-2′), 4.78 (1H, *dd*, *J* = 6.2, 4.0 Hz, H-4), 2.78 (1H, *dd*, *J* = 17.0, 4.0 Hz, H-5a), 2.68 (1H, *dd*, *J* = 17.0, 6.2 Hz, H-5b), 1.98 (1H, *m*, H-5′a), 1.60 (1H, *m*, H-5′b), 1.50 (3H, s, 4′-Me), 1.25−1.15 (2H, *m*, H-6′, H-7′a), 1.09 (1H, *m*, H-7′b), 0.83 (3H, *d*, *J* = 6.4 Hz, H-9′), 0.76 (3H, *t*, *J* = 7.2 Hz, H-8′); $^{13}$C NMR (DMSO-$d_6$, 100 MHz) δ 194.3 (C, C-3), 178.4 (C, C-1′), 171.2 (C, C-6), 170.3 (C, C-1), 163.4 (CH, C-3′), 121.7 (CH, C-2′), 102.4 (C, C-4′), 93.8 (C, C-2), 78.8 (CH, C-4), 43.7 ($CH_2$, C-5′), 36.1 ($CH_2$, C-5), 30.2 ($CH_2$, C-7′), 30.1 (CH, C-6′), 23.7 ($CH_3$, 4′-Me), 21.0 ($CH_3$, C-9′), 11.3 ($CH_3$, C-8′); EIMS *m/z* 308 [$M^+$] (88), 290 (99), 262 (42), 234 (51), 220 (95), 192 (75), 164 (100), 150 (52), 149 (50), 121 (66), 108 (63), 93 (47), 79 (42); ***Z*-isomer**: $^1$H NMR (DMSO-$d_6$, 400 MHz) δ 7.98 (1H, *d*, *J* = 5.8 Hz, H-3′), 7.37 (1H, *d*, *J* = 5.8 Hz, H-2′), 4.85 (1H, *dd*, *J* = 6.2, 4.1 Hz, H-4), 2.82 (1H, *dd*, *J* = 17.0, 4.1 Hz, H-5a), 2.71 (1H, *dd*, *J* = 17.0, 6.2 Hz, H-5b), 2.01 (1H, *m*, H-5′a), 1.64 (1H, *m*, H-5′b), 1.51 (3H, *s*, 4′-Me), 1.25−1.15 (2H, *m*, H-6′, H-7′a), 1.09 (1H, *m*, H-7′b), 0.83 (3H, *d*, *J* = 6.4 Hz, H-9′), 0.76 (3H, *t*, *J* = 7.2 Hz, H-8′); $^{13}$C NMR (DMSO-$d_6$, 100 MHz) δ 196.8 (C, C-3), 179.4 (C, C-1′), 171.2 (C, C-6), 167.5 (C, C-1), 163.3 (CH, C-3′), 121.7 (CH, C-2′), 101.8 (C, C-4′), 93.4 (C, C-2), 79.0 (CH, C-4), 43.7 ($CH_2$, C-5′), 36.0 ($CH_2$, C-5), 30.2 ($CH_2$, C-7′), 30.1 (CH, C-6′), 23.6 ($CH_3$, 4′-Me), 20.9 ($CH_3$, C-9′), 11.3 ($CH_3$, C-8′); EIMS *m/z* 308 [$M^+$] (88), 290 (99), 262 (42), 234 (51), 220 (95), 192 (75), 164 (100), 150 (52), 149 (50), 121 (66), 108 (63), 93 (47), 79 (42); APCI-TOF MS *m/z* 309.1331 (calcd for $[C_{16}H_{20}O_6+H]^+$, 309.1338).

#### 3.5.2. Vermelhotin (***5***)

(*E*:*Z* mixture); Red micro-needles (MeOH); m.p. 213.0-214.5 °C (decomposed); UV (MeOH) $\lambda_{max}$ (log ε) 222.5 (4.16), 276.0 (4.34), 319.0 (3.78), 438.5 (4.19) nm; IR $\nu_{max}$ 3173, 3042, 2919, 2853, 1698, 1650, 1610, 1552, 1489, 1460, 1430, 1349, 1258, 1151, 1076, 979, 961, 930, 815, 780, 730, 719, 676 $cm^{-1}$; ***E*-isomer**: [see Hosoe et al. (2006)]; ***Z*-isomer**: $^1$H NMR (DMSO-$d_6$, 600 MHz) δ 8.01 (1H, *d*, *J* = 9.3 Hz, H-7), 7.66 (1H, br *s*, N*H*), 7.62 (1H, *dd*, *J* = 9.3, 7.1 Hz, H-8), 7.16 (1H, *dq*, *J* = 15.4, 7.1 Hz, H-12), 6.62 (1H, *d*, *J* = 7.1 Hz, H-9), 6.37 (1H, *dq*, *J* = 15.4, 1.7 Hz, H-11), 3.57 (2H, *d*, *J* = 0.6 Hz, H-5), 1.95 (3H, *dd*, *J* = 1.6, 7.1 Hz, H-13); $^{13}$C NMR (DMSO-$d_6$, 150 MHz) δ 192.9 (C, C-4), 171.4 (C, C-2), 164.4 (C, C-6), 157.8 (C, C-10), 142.6 (CH, C-8), 136.6 (CH, C-12), 123.2 (CH, C-11), 115.5 (CH, C-7), 108.6 (CH, C-9), 98.4 (C, C-3), 50.4 ($CH_2$, C-5), 18.8 ($CH_3$, C-13); APCI-TOF MS *m/z* 218.0811 (calcd for $[C_{12}H_{11}NO_3+H]^+$ 218.0817).

#### 3.5.3. Preparation of methyl ester ***2***

Nodulisporacid A (**1**) (97.9 mg) was dissolved in anhydrous MeOH (25 mL). 2–3 Drops of conc. HCl were added, and the reaction mixture was refluxed for 5 h. Separation by silica gel CC gave ester **2** (52.9 mg). *E*:*Z* mixture; Yellow viscous oil; $[\alpha]_D^{27}$ −11.4 (*c* 1.00, $CHCl_3$); ***E*-isomer of 2**: $^1$H NMR (DMSO-$d_6$, 400 MHz) δ 8.01 (1H, *d*, *J* = 5.8 Hz, H-3′), 7.28 (1H, *d*, *J* = 5.8 Hz, H-2′), 4.84 (1 H, *dd*, *J* = 6.4, 4.2 Hz, H-4), 3.59 (3H, *s*, OMe), 2.91 (1H, *dd*, *J* = 17.0, 4.2 Hz, H-5a), 2.82 (1H, *dd*, *J* = 17.0, 6.4 Hz, H-5b), 1.99 (1 H, *m*, H-5′a), 1.62 (1H, *m*, H-5′b), 1.52 (3 H, *s*, 4′-Me), 1.26−1.16 (2H, *m*, H-6′, H-7′a), 1.10 (1 H, *m*, H-7′b), 0.84 (3H, *d*, *J* = 6.4 Hz, H-9′), 0.77 (3H, *t*, *J* = 7.2 Hz, H-8′); ***Z*-isomer of 2**: $^1$H NMR (DMSO-$d_6$, 400 MHz) δ 8.01 (1H, *d*, *J* = 5.8 Hz, H-3′), 7.38 (1H, *d*, *J* = 5.8 Hz, H-2′), 4.90 (1H, *dd*, *J* = 6.5, 4.1 Hz, H-4), 3.57 (3H, *s*, OMe), 2.95 (1 H, *dd*, *J* = 17.0, 4.1 Hz, H-5a), 2.86 (1H, *dd*, *J* = 17.0, 6.5 Hz, H-5b), 2.03 (1H, *m*, H-5′a), 1.66 (1H, *m*, H-5′b), 1.52 (3H, *s*, 4′-Me), 1.26−1.16 (2H, *m*, H-6′, H-7′a), 1.10 (1H, *m*, H-7′b), 0.84 (3H, *d*, *J* = 6.4 Hz, H-9′), 0.78 (3H, *t*, *J* = 7.2 Hz, H-8′); APCI-TOF MS *m/z* 323.1490 (calcd for $[C_{17}H_{22}O_6+H]^+$, 323.1495).

#### 3.5.4. Preparation of benzyl ester ***3***

A reaction mixture containing **1** (125.3 mg), benzyl alcohol (5 mL), and a catalytic amount of *p*-toluenesulfonic acid was refluxed in toluene (25 mL) using a Dean–Stark apparatus (3 h). Separation by Sephadex LH-20 CC yielded benzyl ester **3** (100.4 mg). *E*:*Z* mixture; Yellow viscous oil; $[\alpha]_D^{27}$ −17.3 (*c* 1.16, $CHCl_3$); ***E*-isomer of 3**: $^1$H NMR (DMSO-$d_6$, 400 MHz) δ 8.01 (1H, *d*, *J* = 5.8 Hz, H-3′), 7.37−7.31 (5H, *m*, Ar*H*), 7.29 (1H, *d*, *J* = 5.8 Hz, H-2′), 5.10 (1H, *d*, *J* = 2.3 Hz, ArC$H_2$-O), 5.09 (1H, *d*, *J* = 2.3 Hz, ArC$H_2$-O), 4.88 (1H, *dd*, *J* = 6.3, 4.3 Hz, H-4), 3.04 (1H, *dd*, *J* = 17.0, 4.3 Hz, H-5a), 2.96

(1H, *dd*, *J* = 17.0, 6.3 Hz, H-5b), 2.01 (1H, *m*, H-5′a), 1.61 (1H, *m*, H-5′b), 1.52 (3H, *s*, 4′-Me), 1.28−1.16 (2H, *m*, H-6′, H-7′a), 1.11 (1H, *m*, H-7′b), 0.85 (3H, *d*, *J* = 6.3 Hz, H-9′), 0.78 (3H, *t*, *J* = 7.6 Hz, H-8′); ***Z*-isomer of 3**: $^1$H NMR (DMSO-$d_6$, 400 MHz) $\delta$ 8.01 (1H, *d*, *J* = 5.8 Hz, H-3′), 7.39 (1H, *d*, *J* = 5.8 Hz, H-2′), 7.37−7.31 (5H, *m*, Ar*H*), 5.10 (1H, *d*, *J* = 2.4 Hz, ArC*H*$_2$-O), 5.09 (1H, *d*, *J* = 2.2 Hz, ArC*H*$_2$-O), 4.94 (1H, *dd*, *J* = 6.0, 4.4 Hz, H-4), 2.99 (1H, *dd*, *J* = 17.0, 4.4 Hz, H-5a), 2.92 (1H, *dd*, *J* = 17.0, 6.5 Hz, H-5b), 2.01 (1H, *m*, H-5′a), 1.65 (1H, *m*, H-5′b), 1.52 (3H, *s*, 4′-Me), 1.28−1.16 (2H, *m*, H-6′, H-7′a), 1.11 (1H, *m*, H-7′b), 0.85 (3H, *d*, *J* = 6.3 Hz, H-9′), 0.78 (3H, *t*, *J* = 7.6 Hz, H-8′); APCI-TOF MS *m/z* 399.1808 (calcd for $[C_{23}H_{26}O_6+H]^+$, 399.1808).

### 3.5.5. Preparation of *4*

Nodulisporacid A (**1**) (54.6 mg) was dissolved in anhydrous EtOH (15 mL), and 6.0 mg of Pd/C (10%) was added to the solution mixture. The reaction mixture was kept under $H_2$ for 8 h, evaporated, then separated by Sephadex LH-20 CC to give **4** (29.0 mg). *E*:*Z* mixture; Yellow viscous oil; $[\alpha]_D^{27}$ −15.9 (*c* 1.01, $CHCl_3$); ***E*-isomer of 4**: $^1$H NMR ($CDCl_3$, 400 MHz) $\delta$ 6.47 (1 H, br *s*, OH-6, carboxylic), 4.81 (1 H, *dd*, *J* = 7.7, 4.0 Hz, H-4), 3.55 (1 H, *m*, H-2′a), 3.44 (1 H, *m*, H-2′b), 3.01 (1 H, *dd*, *J* = 17.0, 4.0 Hz, H-5a), 2.81 (1H, *dd*, *J* = 17.0, 6.9 Hz, H-5b), 2.09 (2H, *m*, H-3′), 1.79 (1H, *t*, *J* = 4.0 Hz, H-5′a), 1.66 (1H, *m*, H-5′b), 1.60 (1H, *m*, H-6′), 1.53 (3H, *s*, 4′-Me), 1.34 (1H, *m*, H-7′a), 1.25 (1H, *m*, H-7′b), 1.02 (3H, *d*, *J* = 6.4 Hz, H-9′), 0.89 (3H, *t*, *J* = 7.4 Hz, H-8′); ***Z*-isomer of 4**: $^1$H NMR (DMSO-$d_6$, 400 MHz) $\delta$ 6.47 (1H, br *s*, OH-6, carboxylic), 4.84 (1H, *dd*, *J* = 7.1, 4.2 Hz, H-4), 3.55 (1H, *m*, H-2′a), 3.44 (1H, *m*, H-2′b), 3.01 (1H, *dd*, *J* = 9.2, 4.0 Hz, H-5a), 2.81 (1H, *dd*, *J* = 10.8, 6.9 Hz, H-5b), 2.09 (2H, *m*, H-3′), 1.81 (1H, *t*, *J* = 4.0 Hz, H-5′a), 1.70 (1H, *dd*, *J* = 7.7, 5.8 H-5′b), 1.60 (1H, *m*, H-6′), 1.55 (3H, *s*, 4′-Me), 1.34 (1H, *m*, H-7′a), 1.25 (1H, *m*, H-7′b), 1.02 (3H, *d*, *J* = 6.4 Hz, H-9′), 0.89 (3H, *t*, *J* = 7.4 Hz, H-8′); APCI-TOF MS *m/z* 309.1343 (calcd for $[C_{16}H_{20}O_6-H]^-$, 309.1338).

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