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# Isochromophilones from an endophytic fungus *Diaporthe* sp.

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**Abstract:** Three new azaphilone compounds, isochromophilones X–XII (**1**–**3**), together with two known ones sclerotioramine (**4**) and isochromophilone VI (**5**) were isolated from the cultures of an endophytic fungus *Diaporthe* sp. The structures were elucidated by extensive HRESIMS and NMR spectroscopic analyses. All compounds were tested for their cytotoxicities against five human cancer cell lines by MTT method, among which compound **1** showed moderate inhibitory effects on these cell lines. This was the first report of azaphilones isolated from *Diaporthe* sp.

*Keywords:* azaphilone, endophyte, *Diaporthe* sp., cytotoxicity

## Introduction

The azaphilones are a structurally diverse family of natural products containing a highly oxygenated, bicyclic core and a chiral quaternary center. The name of azaphilone arose as a result of their affinity for ammonia: the pigments react with amines, such as proteins, amino acids and nucleic acids, to form red and purple vinylogous γ-pyridones due to the exchange of pyrane oxygen for nitrogen[1–3]. Azaphilone compounds had a wide range of bioactivities including cytotoxic, antifungal, antimicrobial, antioxidant and anti-inflammatory activities,[4–8] and could be generally produced by *Penicillium*, *Chaetomium* and *Monascus*.[3] In the course of our search for novel and/or bioactive metabolites from microbes lived in special niche,[4,9,10] five azaphilones including three new isochromophilones X–XII (**1**–**3**) and two known compounds sclerotioramine (**4**)[11] and isochromophilone VI (**5**)[12] were isolated from the solid-substrate fermentation cultures of an endophytic fungus *Diaporthe* sp. The structures were identified on the basis of extensive spectroscopic methods. The cytotoxic activities of compounds (**1**–**5**) against human gastric cancer SGC-7901, colorectal carcinoma SW1116, breast adenocarcinoma MCF-7, lung adenocarcinoma epithelial A549 and melanoma A375 cells were evalutated by MTT method. Compound **1** exhibited moderate cytotoxic activities with its $IC_{50}$ values ranging from 14.90–35.75 μM.

## Results and Discussion

Compound **1** was obtained as red needles, the molecular formula was assigned as $C_{29}H_{32}ClNO_4$ evidenced by its HRESIMS spectrum at *m/z* 494.2080 $[M + H]^+$ (calcd. for $C_{29}H_{33}{}^{35}ClNO_4$, 494.2098), indicating 14 degrees of unsaturation. Compound **1** displayed similar $^1H$ NMR spectrum to that of known compound isochromophilone VI (**5**) except for a monosubstituted benzene ring signals [$\delta$ 7.26 (m, 1H), 7.32 (m, 2H), 7.10 (d, 7.0, 2H)] in **1**, which suggested that the 2'-OH in isochromophilone VI (**5**) could be replaced by a benzene ring in compound **1**. To confirm the proposed structure, extensive NMR spectra have been investigated (Figure 2). $^1H$-$^1H$ COSY correlations of H-10/H-11, and of Me-18/H-13/H-14/H-15(Me-17)/Me-16, together with the HMBC correlations from H-10 to C-12, H-11 to C-18, H-13 to C-11, C-15, C-17, C-18 indicated the presence of a 3,5-dimethyl-1,3-heptadiene residue. On the basis of the coupling constants of H-10, H-11, and the NOE correlation of H-11 with H-13, the configurations of these two double bonds were both determined to be *E*. The HMBC correlations from H-1' to

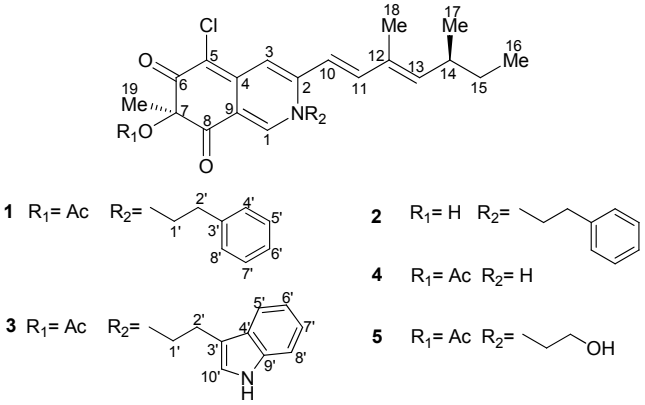


**Figure 1**. Compounds **1**–**5** from *Diaporthe* sp.

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**Table 1.** $^{1}$H (500MHz) and $^{13}$C NMR (125MHz) data for 1–3 (in $CDCl_3$)

| | 1 | | 2 | | 3 | |
|---|---|---|---|---|---|---|
| No. | $\delta_H$ (mult., *J*) | $\delta_C$ | $\delta_H$ (mult., *J*) | $\delta_C$ | $\delta_H$ (mult., *J*) | $\delta_C$ |
| 1 | 7.53 (s) | 140.8, CH | 7.49 (s) | 140.0, CH | 7.49 (s) | 141.0, CH |
| 2 | | 147.8, C | | 148.3, C | | 148.3, C |
| 3 | 6.96 (s) | 111.5, CH | 6.96 (s) | 111.0, CH | 6.94 (s) | 111.4, CH |
| 4 | | 144.3, C | | 145.7, C | | 144.6, C |
| 5 | | 103.1, C | | 100.0, C | | 102.1, C |
| 6 | | 184.3, C | | 187.6, C | | 207.3, C |
| 7 | | 84.8, C | | 83.4, C | | 84.8, C |
| 8 | | 193.7, C | | 196.3, C | | 193.8, C |
| 9 | | 114.6, C | | 115.5, C | | 114.6, C |
| 10 | 5.98 (d, 15.5) | 114.6, CH | 6.02 (d, 15.5) | 114.4, CH | 5.85 (d, 15.0) | 114.8, CH |
| 11 | 6.90 (d, 15.5) | 144.9, CH | 6.94 (d, 15.5) | 145.3, CH | 6.84 (d, 15.0) | 144.9, CH |
| 12 | | 131.5, C | | 131.5, C | | 131.7, C |
| 13 | 5.68 (d, 9.5) | 148.0, CH | 5.71 (d, 10.0) | 148.4, CH | 5.63 (d, 9.5) | 147.7, CH |
| 14 | 2.47 (m) | 35.0, CH | 2.49 (m) | 35.1, CH | 2.43 (m) | 35.0, CH |
| 15 | 1.44 (m); 1.35 (m) | 30.0, $CH_2$ | 1.44 (m); 1.34 (m) | 30.0, $CH_2$ | 1.42 (m); 1.33 (m) | 30.1, $CH_2$ |
| 16 | 0.89 (t, 7.5) | 11.9, $CH_3$ | 0.89 (t, 7.5) | 11.9, $CH_3$ | 0.87 (t, 7.5) | 12.0, $CH_3$ |
| 17 | 1.03 (d, 6.5) | 20.2, $CH_3$ | 1.03 (d, 6.6) | 20.2, $CH_3$ | 1.00 (d, 6.5) | 20.2, $CH_3$ |
| 18 | 1.81 (d, 1.0) | 12.6, $CH_3$ | 1.83 (s) | 12.6, $CH_3$ | 1.56 (s) | 12.1, $CH_3$ |
| 19 | 1.52 (s) | 23.2, $CH_3$ | 1.51 (s) | 29.5, $CH_3$ | 1.48 (s) | 23.2, $CH_3$ |
| 7-OAc | 2.16 (s) | 20.3, $CH_3$ | | | 2.17 (s) | 20.3, $CH_3$ |
| | | 170.0, C | | | | 170.1, C |
| 1' | 4.13 (m); 3.98 (m) | 55.4, $CH_2$ | 4.17 (m); 4.03 (m) | 55.6, $CH_2$ | 4.11 (m) | 54.5, $CH_2$ |
| 2' | 3.02 (m) | 36.6, $CH_2$ | 3.05 (m) | 36.4, $CH_2$ | 3.20 (m) | 26.5, $CH_2$ |
| 3' | | 135.8, C | | 135.6, C | | 109.6, C |
| 4' | 7.10 (d, 7.0) | 128.7, CH | 7.08 (d, 7.0) | 128.7, CH | | 126.7, C |
| 5' | 7.32 (m) | 129.2, CH | 7.32 (m) | 129.3, CH | 7.48 (d, 8.0) | 117.7, CH |
| 6' | 7.26 (m) | 127.7, CH | 7.26 (m) | 127.8, CH | 7.14 (t, 8.0) | 120.1, CH |
| 7' | 7.32 (m) | 129.2, CH | 7.32 (m) | 129.3, CH | 7.22 (t, 8.0) | 122.7, CH |
| 8' | 7.10 (d, 7.0) | 128.7, CH | 7.08 (d, 7.0) | 128.7, CH | 7.37 (d, 8.0) | 111.8, CH |
| 9' | | | | | | 136.5, C |
| 10' | | | | | 6.94 (s) | 123.2, CH |

C-3', H-4' to C-2', H-7' to C-3' confirmed the linkage of C-2' and the benzene ring. Moreover, the HMBC correlations of H-1 to C-1', C-2, C-4, C-5, C-8, C-9, of H-3 to C-5 and C-10, of Me-19 to C-6, C-8 indicated the presence of isoquinoline-6,8(2*H*,7*H*)-dione moiety, a typical group in azaphilone skeleton and the way the residues linked at. Thus, the planar structure of **1** was established.

Compound **2**, isolated as red needles, was determined to have the molecular formula $C_{27}H_{30}ClNO_3$ (13 degrees of unsaturation) based on an HRESIMS peak at *m/z* 452.1998 [M + H]$^+$ (calcd. for $C_{27}H_{31}{}^{35}ClNO_3$, 452.1992), 42 amu ($C_2H_2O$) less than compound **1**. Its $^1$H NMR spectrum revealed nearly all identical structural features to those found in compound **1**, except the absence of an acetyl signal in the $^1$H NMR spectrum (Table 1) and thus the structure of compound **2** could be readily determined as shown in Figure 1 which was supported by 2D NMR spectra.

Compound **3** was established the molecular formula as $C_{31}H_{33}ClN_2O_4$ by HRESIMS analysis (*m/z* 533.2203, calcd. for $C_{31}H_{34}{}^{35}ClN_2O_4$, 533.2207) with 16 degrees of unsaturation. The $^1$H NMR spectroscopic data of compound **3** were well comparable to those of **1**. Considering the molecular formula, an indole ring might substitute 2'-OH of isochromophilone VI in the compound **3**. The HMBC (Figure 2) correlations from H-10' to C-4' and C-9', from H-5' to C-7' and C-9', from H-8' to C-4' and C-6' confirmed the presence of indole ring.

The 7*R*-configurations of isochromophilones X–XII (**1**–**3**) were determined by circular-dichroism (CD) spectroscopy. The observed positive *Cotton* effect at the wavelength (370–390 nm) was well comparable to that of 7*R*-5-chloro-7,8-dihydro-3-(3-hydroxypropyl)-7-methyl-6,8-dioxo-6*H*-2-benzopyran-7-yl ester.[13,14] Furthermore, the 14*S*-configurations of **1**–**3** were determined by comparing their NMR data of the side chain moieties with those of similar azaphilone compounds, isochromophilone Ia, IIa and rotiorinol A.[15,16]

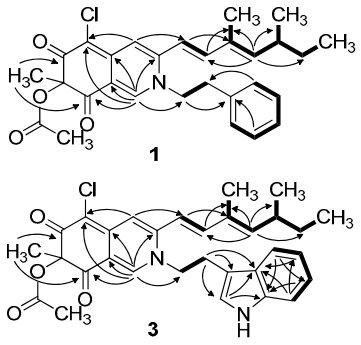
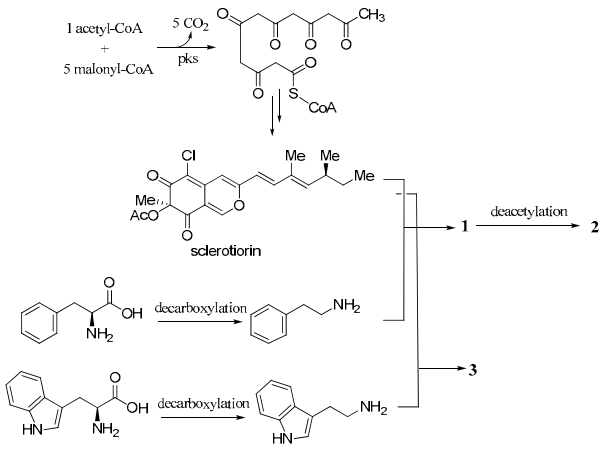



**Figure 2**. Key $^1$H-$^1$H COSY (bold), HMBC (arrow) correlations of **1** and **3**

Biosynthetically, azaphilone was derived from the polyketide pathway,[3,17] whose oxygen atom could be further substituted by primary amines or ammonia into *N*-containing compounds rapidly.[1] For example, sclerotiorin could be converted to sclerotioramine (**4**) with ammonia.[2] Thus, compounds **1** and **3** were deduced as consequences of sclerotiorin reacting with phenylethanamine and tryptamine, the decarboxylated product of phenylalanine and tryptophan, respectively (Scheme 1). The further deacetylation of compound **1** gave compound **2**. Also, we can deduce that compounds **1**–**3** possessed the same configurations as that of sclerotiorin (Figure 1) by their biosynthetic view.




**Scheme 1.** Proposed biogenetic pathway for isochromophilones X–XII (**1**–**3**)

All compounds were evaluated for their cytotoxicities against MCF-7, SGC-7901, SW1116, A549 and A375 cell lines by MTT method. Only, isochromophilone X (**1**) showed moderate cytotoxic activities with its $IC_{50}$ values of 14.90, 16.84, 24.15, 26.93 and 35.75 $\mu$M against MCF-7, SGC-7901, SW1116, A549 and A375 cell lines (Table 2), respectively, while the other compounds exhibited no activities with $IC_{50}$ values greater than 50 $\mu$M.

## Experimental Section

**General Experimental Procedures**. HRESIMS spectra were recorded on an Agilent 6210 TOF LC/MS. CD spectra were obtained on a JASCO J-810 spectrometer. IR spectra were obtained on a Nexus 870 FT-IR spectrometer. NMR spectra were recorded on Bruker AV-500, AV-300 or DRX500 NMR spectrometer. Optical rotations were recorded on a Rudolph Autopol III automatic polarimeter. UV spectra were recorded on a Hitachi U-3000 spectrophotometer. Silica gel (200–300 mesh; Qingdao Marine Chemical Factory, Qingdao, China) and Sephadex LH-20 gel (Pharmacia Biotech, Sweden) were used for column chromatography (CC). HPLC was performed with a Hitachi L-7110 pump, and UV detector L-7400 equipped with an Apollo C18 column (5 $\mu$m, 250 mm × 4.6 mm; Alltech Associates, Inc. Chicago, IL, USA).

**The Fungal Material and Cultivation Conditions.** The strain IFB-3lp-10 was isolated from the healthy leaves of *Rhizophora stylosa* collected in August 2010 from the mangrove forest of Hainan Province of China. The strain was identified as *Diaporthe* sp. by comparing the morphological character and 18S rDNA sequence with that of a standard record. A voucher specimen has been deposited in the Institute of Functional Biomolecules, State Key Laboratory of Pharmaceutical Biotechnology, Nanjing University. After growing on PDA medium at 28 °C for 5 d, the fungus *Diaporthe* sp. IFB-3lp-10 was inoculated into Erlenmeyer flasks (1000 mL) containing 400 mL of ME liquid medium. After incubation for 4 d at 28 °C on a rotary shaker at 150 rpm, 20 mL of culture liquid was transferred as the seed into 250 mL flasks, each preloaded with the evenly mingled medium (7.5 g of grain, 7.5 g of bran, 0.5 g of yeast extract, 0.1 g of sodium tartrate, 0.01 g of $FeSO_4 \cdot 7H_2O$, 0.1 g of sodium glutamate and 30 ml of $H_2O$). The fungus then grew for 30 d at 28 °C with the relative humidity in the range 60–70%.

**Extraction and Isolation.** The air-dried samples (30 kg) were extracted with 95% EtOH and the organic solvent was evaporated to dryness under a vacuum to afford a crude extract (1.2 kg) which gave six fractions (Fr.1, 21g; Fr.2, 75 g; Fr.3, 56 g; Fr.4, 25 g; Fr.5, 36 g; Fr.6, 158 g) upon column chromatography (10 × 120 cm) on silica gel (6000 g, 200–300 mesh) eluted with a gradient of $CH_2Cl_2$/MeOH (v/v 100:0, 100:1, 100:2, 100:4, 100:8, 100:20, 0:100, each 20L) based on TLC monitoring. Purification of the second subfraction ($CH_2Cl_2$/MeOH 100:1) by using column chromatography (petroleum ether/EtOAc 1:1) and Sephadex LH-20 (100% MeOH) followed by HLPC (90% MeOH) gave **1** (15.0 mg) and **2** (2.5 mg). Purification of the third subfraction ($CH_2Cl_2$/MeOH 100:2) in the same way mentioned above gave **3** (3.6 mg). The fourth fraction was subjected to reversed phase ODS column (4 cm × 40 cm) with a gradient of MeOH/$H_2O$ (v/v 20:80, 30:70, 40:60, 50:50, 60:40, 70:30, 80:20, 90:10, 100:0, each 3 L) to give nine subfractions, respectively. Purification of the fifth subfraction (MeOH/$H_2O$ 60:40) by using column chromatography (petroleum ether/EtOAc 1:2) and Sephadex LH-20 (100 % MeOH) gave **4** (2.8 g) and **5** (64 mg).

**Isochromophilones X (1):** red needles. $[\alpha]_D^{20}$ = +1116.4 (*c* = 0.028, MeOH). UV/Vis (MeOH): $\lambda_{max}$/nm (log $\varepsilon$) = 201 (4.5), 227 (4.4), 373 (4.5). CD (*c* 2.8 × $10^{-4}$ g $ml^{-1}$, MeOH) $\lambda_{max}$/nm ($\Delta\varepsilon$): 229.5 (+0.4), 246 (+6.2), 304 (−8.9), 381.5 (+7.9), 429 (+1.2). IR (KBr): $\nu_{max}$ = 3490.4, 2960.7, 2924.7, 1701.8, 1594.6, 1501.6, 1262.5, 1220.3, 1144.3, 1081.7, 1032.0, 801.9 $cm^{-1}$. HRESIMS: *m/z* 494.2080 ([M + H]$^+$, calcd. for $C_{29}H_{33}{}^{35}ClNO_4$, 494.2098). For $^1$H and $^{13}$C NMR spectroscopic data, see Table 1.

**Isochromophilones XI (2):** red needles. $[\alpha]_D^{20}$ = +596.0 (*c* = 0.08, MeOH). UV/ Vis (MeOH): $\lambda_{max}$/nm (log $\varepsilon$) = 206 (4.5), 228 (4.5), 350 (4.5). CD (*c* 8.0 × $10^{-4}$ g $ml^{-1}$, MeOH) $\lambda_{max}$/nm ($\Delta\varepsilon$): 209 (+5.6), 236 (+0.6), 249 (+1.4), 311 (−4.3), 375 (+3.6), 414.5 (+0.5). IR (KBr): $\nu_{max}$ = 3419.2, 2962.5, 1588.8, 1494.7, 1261.5, 1096.2, 1033.4, 801.5 $cm^{-1}$. HRESIMS: *m/z* 452.1998 ([M + H]$^+$, calcd. for $C_{27}H_{31}{}^{35}ClNO_3$, 452.1992). For $^1$H and $^{13}$C NMR spectroscopic data, see Table 1.

**Isochromophilones XII (3):** red needles. $[\alpha]_D^{20}$ = +936.0 (*c* = 0.025, MeOH). UV/ Vis (MeOH): $\lambda_{max}$/nm (log $\varepsilon$) = 201 (4.6), 221 (4.7), 375 (4.3). CD (*c* 2.5 × $10^{-4}$ g $ml^{-1}$, MeOH) $\lambda_{max}$/nm ($\Delta\varepsilon$): 229.5 (−1.6), 246.5 (+2.4), 304.5 (−4.5), 379

**Table 2.** ***In vitro*** **cytotoxicity of compound 1**

| $IC_{50}$ ($\mu$M) | MCF-7 | SGC-7901 | SW1116 | A549 | A375 |
|---|---|---|---|---|---|
| **1** | 14.90 ± 2.41 | 16.84 ± 1.06 | 24.15 ± 1.77 | 26.93 ± 1.17 | 35.75 ± 2.11 |
| Doxorubicin•HCl[a] | 0.55 ± 0.03 | 2.03 ± 0.05 | 5.09 ± 1.13 | 3.37 ± 1.66 | 1.75 ± 0.24 |

[a]used as a positive control

(+3.5), 419.5 (+0.3). IR (KBr): $\nu_{max}$ = 3440.3, 3237.5, 2958.9, 1736.0, 1574.3, 1486.1, 1219.3, 1145.0, 758.9 $cm^{-1}$. HRESIMS: *m/z* 533.2203 ([M + H]$^+$, calcd. for $C_{31}H_{34}{}^{35}ClN_2O_4$, 533.2207). For $^1H$ and $^{13}C$ NMR spectroscopic data, see Table 1.

**Cytotoxicity Assay.** The cytotoxic effects of **1**–**5** on the viability of the SGC-7901, SW1116, MCF-7, A549 and A375 cell lines were assayed with the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] colorimetric method. The data represent the mean of three experiments performed in triplicate and are expressed as means ± SD.

## Electronic Supplementary Material

Supplementary material is available in the online version of this article at http://dx.doi.org/ 10.1007/s13659-012-0023-2 and is accessible for authorized users.

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