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## New $\alpha$ -pyrones from an endophytic fungus, *Hypoxylon investiens* J2†

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Four new  $\alpha$ -pyrones, hypotiens A–D (1–4), were isolated from a fungal endophyte, *Hypoxylon investiens* J2, harbored in the medicinal plant *Blumea balsamifera*. Their structures were determined through detailed HRMS and NMR spectroscopic data. Compounds 1–4 are new  $\alpha$ -pyrone derivatives containing an unusual dimethyl substitution in the highly unsaturated side chain. Their plausible biosynthetic pathway was discussed. Biological assay indicated that compounds 1–4 showed no antimicrobial, quorum sensing inhibitory, and cytotoxic activities. The specific side chain in  $\alpha$ -pyrone derivatives 1–4 might be responsible for the weak pharmacological activities.

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## Introduction

Fungal endophytes asymptotically colonize living tissues of healthy plants.<sup>1–3</sup> They are now recognized as an invaluable source of structurally diverse and biologically active natural products.<sup>4</sup> More than one hundred endophytic fungi-derived secondary metabolites with new carbon skeletons, rare ring systems, or unusual structural units have been reported.<sup>5</sup> Exploration of these novel and bioactive secondary metabolites greatly facilitates the discovery of lead compounds.

From the endophytic fungus *Chaetomium* sp. IFB-E015 living in the leaves of *Adenophora axilliflora*, an unprecedented alkaloid, chaetominine containing an unusual alanine-derived  $\delta$ -lactam ring, was isolated and structurally elucidated.<sup>6</sup> It exhibited more potent cytotoxicity to the human colon cancer SW1116 and leukemia K562 cell lines than the positive drug 5-fluorouracil, and has received considerable attention from chemists and biologists in the field of total synthesis and biological investigations.<sup>6–8</sup> Papeo and co-workers reported a total synthesis of chaetominine based on a straightforward (nine steps) sequence and found that this compound exhibited negligible cytotoxic activities on several cancer cell lines.<sup>9</sup> *Rhizoctonia solani*, an endophyte isolated from the medical plant *Cyperus rotundus*, was discovered to biosynthesize

a degraded and rearranged steroid, solanioic acid with an unprecedented carbon skeleton.<sup>10,11</sup> It showed significant antibacterial activities against Gram-positive bacteria, especially the problematic human pathogen methicillin-resistant *Staphylococcus aureus* with an MIC of 1  $\mu\text{g mL}^{-1}$ .<sup>10</sup> The healthy plant *Paris polyphylla* contained an endophytic fungus *Aspergillus versicolor*.<sup>12</sup> Its chemical investigation resulted in the isolation and purification of a highly oxygenated cyclopiazonic acid-derived alkaloid, aspergilline E.<sup>12</sup> This compound has a new hexacyclic 6/5/6/5/5/5 scaffold and displayed significant biological activities, including anti-virus and cytotoxicity.<sup>12</sup>

As part of an ongoing program aimed at finding biologically active natural products from endophytic fungi,<sup>13,14</sup> *Hypoxylon investiens* J2 as a fungal endophyte, was isolated from the medicinal plant *Blumea balsamifera*. Chemical investigation on its rice cultures led to the isolation of four new  $\alpha$ -pyrone derivatives, hypotiens A–D (1–4). Compounds 1–4 possess a highly unsaturated side chain containing an unusual dimethyl substitution, which is similar to that of oxazolomycins with potent antibacterial, antiviral and cytotoxic activities.<sup>15</sup> Details of the isolation, structure elucidation, and biological activity, together with a proposed biosynthesis of compounds 1–4 are reported here.

## Results and discussion

Compound 1 (Fig. 1), yellow powder, has a molecular formula  $\text{C}_{18}\text{H}_{22}\text{O}_4$  as established by ESI-HRMS. The  $^1\text{H}$  NMR spectrum (Table 1, and Fig. S1†) showed the presence of five singlet methyls ( $\delta_{\text{H}}$  1.27, 1.27, 1.95, 2.04, and 2.14). In addition, six olefinic protons associated with three double bonds were also suggested in the  $^1\text{H}$  NMR spectrum ( $\delta_{\text{H}}$  6.56, d,  $J = 15.0$  Hz;  $\delta_{\text{H}}$  7.07, dd,  $J = 15.0, 11.0$  Hz;  $\delta_{\text{H}}$  6.47, dd,  $J = 15.0, 11.0$  Hz;  $\delta_{\text{H}}$  6.55, dd,  $J = 15.0, 10.0$  Hz;  $\delta_{\text{H}}$  6.31, dd,  $J = 15.5, 10.0$  Hz;  $\delta_{\text{H}}$  5.98, d,  $J =$

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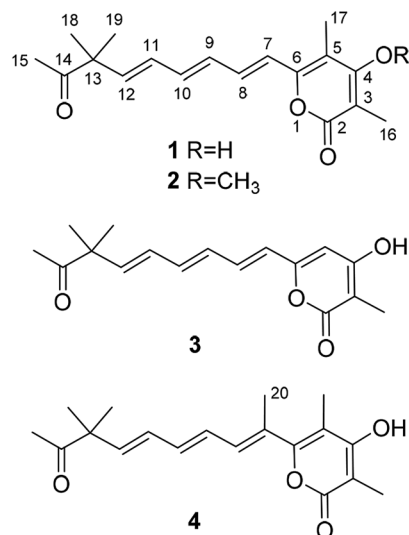


Fig. 1 Chemical structures of compounds 1–4.

15.5 Hz). The  $^{13}\text{C}$  NMR spectrum (Table 1, and Fig. S2†) indicated the presence of 18 carbon signals, including a ketone group at  $\delta_{\text{C}}$  213.0 and five methyls at  $\delta_{\text{C}}$  9.2, 9.6, 24.4, 24.4, and 25.9. The 1D NMR data in combination with the HSQC spectrum (Fig. S3†) revealed five methyls, three *trans*-disubstituted double bonds, a ketone unit, and the remaining six quaternary carbons including a saturated carbon at  $\delta_{\text{C}}$  51.8.

The planar structure of compound 1 was further constructed through the detailed analysis of the HMBC spectrum (Fig. 2, and S4†). The key HMBC correlations (Fig. 2) from H<sub>3</sub>-15 to C-13 and C-14 coupled with the requirement of chemical shifts of C-14 ( $\delta_{\text{C}}$  213.0) and H<sub>3</sub>-15 ( $\delta_{\text{H}}$  2.14, s) confirmed the connection

from C-13 to C-15. Two singlet methyls (C-18 and C-19) were further located at the C-13, which was confirmed by the HMBC correlations of H<sub>3</sub>-18 and H<sub>3</sub>-19 with C-13 and C-14. Based on the key HMBC correlations from H-12 to C-10, C-11, C-13, and C-14, from H-11 to C-9 and C-10, from H-9 to C-7 and C-8, and from H-7 and H-8 to C-6, a side chain from C-6 to C-15 was tentatively deduced. It contained three *trans*-disubstituted double bonds at C-7(8), C-9(10), and C-11(12), which was strongly supported by their chemical shifts and relatively large coupling constants.

Further analysis of the HMBC cross-peaks of H<sub>3</sub>-16/C-2, H<sub>3</sub>-16/C-3, H<sub>3</sub>-16/C-4, H<sub>3</sub>-17/C-4, H<sub>3</sub>-17/C-5, and H<sub>3</sub>-17/C-6 verified the connections from C-2 to C-6 (Fig. 2). A hydroxyl group was placed at C-4 based on its chemical shift ( $\delta_{\text{C}}$  167.5). The remaining one degree of unsaturation and the chemical shifts of C-2 ( $\delta_{\text{C}}$  167.4) and C-6 ( $\delta_{\text{C}}$  153.7) suggested that C-2 and C-6 in compound 1 were both linked to the same oxygen atom to form a  $\alpha$ -pyrone ring, which was consistent with its molecular formula. In the NOESY spectrum of compound 1, a key correlation between olefinic H-7 and aliphatic CH<sub>3</sub>-17 was observed (Fig. S5†), indicating these protons were close in space. Accordingly, the structure of compound 1 was established as depicted and it was named hypotien A.

Compound 2 (Fig. 1) was also obtained as a yellow powder and named as hypotien B. Based on the ESI-HRMS data, it was assigned the molecular formula C<sub>19</sub>H<sub>24</sub>O<sub>4</sub>, corresponding to one CH<sub>2</sub> group more than 1. Analysis of its  $^1\text{H}$ ,  $^{13}\text{C}$ , and HSQC NMR spectra (Table 1, and Fig. S7–S9†) indicated similar structural features to those of compound 1, except for the presence of a methoxy group ( $\delta_{\text{H}}$  3.85;  $\delta_{\text{C}}$  61.2) in 2 and the significant downfield shifts of C-3 and C-4. The above analysis revealed that a methoxy moiety in 2 instead of a hydroxyl group

Table 1  $^1\text{H}$  (500 MHz) and  $^{13}\text{C}$  (125 MHz) NMR data of compounds 1–4 in CD<sub>3</sub>OD ( $\delta$  in ppm)

No.	1	2	3	4
	$\delta_{\text{H}}$ , mult. ( <i>J</i> in Hz)	$\delta_{\text{C}}$ , mult.	$\delta_{\text{H}}$ , mult. ( <i>J</i> in Hz)	$\delta_{\text{C}}$ , mult.
2		167.4, C		168.0, C
3		100.6, C		99.6, C
4		167.5, C		168.8, C
5		110.5, C		109.8, C
6		153.7, C		159.4, C
7	6.56, d (15.0)	120.9, CH	6.54, d (15.0)	123.2, CH
8	7.07, dd (15.0, 11.0)	135.6, CH	7.07, dd (15.0, 11.0)	135.9, CH
9	6.47, dd (15.0, 11.0)	133.0, CH	6.48, dd (15.0, 11.0)	132.3, CH
10	6.55, dd (15.0, 10.0)	138.2, CH	6.56, dd (15.0, 10.0)	138.8, CH
11	6.31, dd (15.5, 10.0)	131.1, CH	6.31, dd (15.5, 10.0)	130.9, CH
12	5.98, d (15.5)	141.6, CH	6.00, d (15.5)	141.9, CH
13		51.8, C		51.8, C
14		213.0, C		213.8, C
15	2.14, s	25.9, CH <sub>3</sub>	2.14, s	26.0, CH <sub>3</sub>
16	1.95, s	9.2, CH <sub>3</sub>	2.02, s	8.7, CH <sub>3</sub>
17	2.04, s	9.6, CH <sub>3</sub>	2.04, s	2.04, s
18	1.27, s	24.4, CH <sub>3</sub>	1.27, s	24.3, CH <sub>3</sub>
19	1.27, s	24.4, CH <sub>3</sub>	1.27, s	24.3, CH <sub>3</sub>
20				2.05, d (1.0)
4-OMe		3.85, s	61.2, CH <sub>3</sub>	



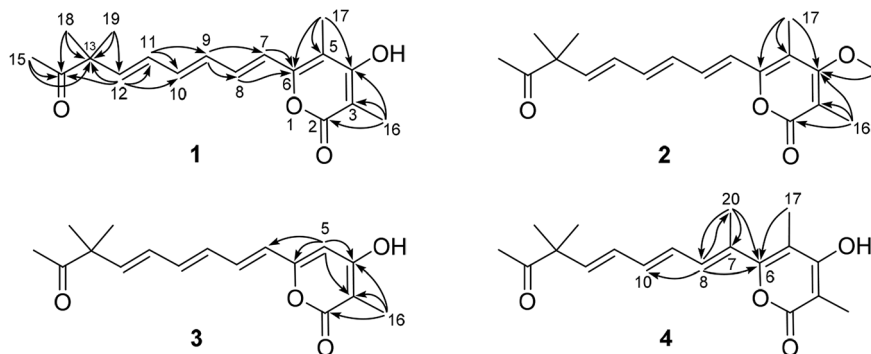


Fig. 2 Key HMBC correlations of compounds 1–4.

in **1** was linked to C-4, which was further supported by detailed analysis of the HMBC spectrum of compound **2** (Fig. 2).

For compound **3** (Fig. 1), its molecular formula  $C_{17}H_{20}O_4$  was determined by the same strategy as above and corresponded to one  $CH_2$  group less than compound **1**. The  $^1H$  NMR spectrum of **3** (Table 1 and Fig. S12†) was also close to that of **1** except for the presence of an olefinic proton (H-5,  $\delta_H$  6.13) in **3** and the absence of a methyl signal at C-5 in **1**. Key HMBC correlations (Fig. 2) from H-5 to C-3, C-4, C-6, and C-7 indicated the location of H-5 and assigned the structure of compound **3** as shown. Compound **3** was named hypotien C.

Hypotien D (**4**, Fig. 1) was a yellow powder. ESI-HRMS spectrum determined its molecular formula as  $C_{19}H_{24}O_4$ . Detailed analysis of the  $^1H$ ,  $^{13}C$ , and HSQC data of **4** (Table 1, and Fig. S17–S19†) suggested that compound **4** has similar structural characteristics to compound **1** and indicated a  $\alpha$ -pyrone derivative. By comparing the 1D NMR data of **4** with that of **1**, in addition to the absence of an olefinic proton signal in compound **4**, one more methyl group ( $\delta_H$  2.05;  $\delta_C$  15.2) was observed in compound **4**. The above methyl group was located at the olefinic C-7 based on the HMBC correlations of  $H_3$ -20 with C-6, C-7, and C-8 (Fig. 2). Further analysis of key HMBC correlations confirmed the structure of compound **4**, which was in accordance with the requirement of its molecular formula.

$\alpha$ -Pyrone, a six-membered lactone, is frequently discovered in microorganisms, plants, and animals, and is often substituted with a side chain.<sup>16</sup> The diverse substitutions of the six-membered lactone, as well as the variations in length and substitutions of the side chain, greatly contribute to the structural diversity and complexity of  $\alpha$ -pyrone derivatives.<sup>17–21</sup> Compounds **1–4** are new  $\alpha$ -pyrone derivatives containing an unusual dimethyl substitution in the highly unsaturated side chain (Fig. 1). Their plausible biosynthetic pathway was proposed through a polyketide synthase.<sup>16</sup> A linear polyketide chain was first constructed from an acetyl coenzyme A (CoA) and six malonyl-CoA followed by reduction, dehydration, methylation, oxidation, or cyclization to generate the  $\alpha$ -pyrone derivatives.

Natural products containing a  $\alpha$ -pyrone have exhibited diverse biological activities, such as the mostly reported antimicrobial efficacy,<sup>17–19</sup> quorum sensing (QS) inhibitory activity,<sup>22</sup> and cytotoxicity.<sup>19,21</sup> In this work, the antibacterial activities of

new  $\alpha$ -pyrones **1–4** were evaluated against four bacteria *Staphylococcus aureus* (ATCC 6538), *Bacillus subtilis* (ATCC 9372), *Pseudomonas aeruginosa* (ATCC 27853), and *Escherichia coli* (ATCC 25922), and their antifungal efficacies were tested against three agricultural pathogens *Colletotrichum musae* (ACCC 31244), *Colletotrichum coccodes* (ACCC 36067), and *Colletotrichum orbiculare* (ACCC 36095). Furthermore, the QS inhibitory activity against *Chromobacterium violaceum* and the cytotoxic assay against three human cancer cell lines A549, CT-26, and MCF-7 were also applied for compounds **1–4**. Unfortunately, in contrast to the positive controls, none of them at the given concentrations (Experimental section) were effective against the tested microorganisms or cancer cells. The specific side chain in new  $\alpha$ -pyrones **1–4** might be responsible for the weak pharmacological activities.

## Experimental section

### General experimental procedures

Mass spectra were measured on an LTQ-Orbitrap spectrometer equipped with an ESI source. 1D and 2D NMR spectra were recorded on a Bruker 500 MHz spectrometer. The semi-preparative HPLC was performed on an Agilent 1260 system (Agilent technologies, Germany) equipped with an RP-18 column (250 × 10 mm, YMC Park, 5  $\mu$ M). Silica gel GF<sub>254</sub> plates (Qingdao Haiyang Chemical Co., Ltd., China) was applied for thin-layer chromatography (TLC). Silica gel (200–300 mesh, Qingdao Haiyang Chemical Co., Ltd., China) and Sephadex LH-20 (25–100  $\mu$ m; Pharmacia, Uppsala, Sweden) were used for column chromatography and size exclusion chromatography, respectively.

### Fungal material

The fungal strain *Hypoxyylon investiens* J2 was isolated from the medicinal plant *Blumea balsamifera* collected from Danzhou, Hainan Province, People's Republic of China. It was identified based on its internal transcribed spacer sequence (Genbank no. MK757895). The fungus was deposited at the Tropical Crops Genetic Resources Institute, Chinese Academy of Tropical Agricultural Sciences CATAS, Hainan, People's Republic of China, and was maintained at  $-80\text{ }^\circ\text{C}$ . For the large-scale



fermentation, the fungus *H. investiens* J2 was cultured in rice culture (20 flasks each containing 80 g rice and 120 mL water) in an incubator at  $28 \pm 2$  °C for one month.

### Extraction and isolation

The fermented material was extracted by ethyl acetate for three times. The organic solvent was evaporated to give a crude extract (15 g), which was then fractionated into six fractions (Fr.1–Fr.6) by column chromatography on silica gel. Fr.1 was separated by a Sephadex LH-20 column eluting with MeOH, following by HPLC (MeOH/H<sub>2</sub>O, 77 : 23, 2 mL min<sup>−1</sup>) to afford compound 2 (15.0 mg,  $t_R$  = 29.6 min). Fr.2 was directly purified by HPLC (MeOH/H<sub>2</sub>O, 77 : 23, 2 mL min<sup>−1</sup>) to give compounds 1 (20.0 mg,  $t_R$  = 26.8 min), 3 (15.0 mg,  $t_R$  = 24.0 min), and 4 (10.0 mg,  $t_R$  = 27.3 min).

**Hypotien A (1).** Yellow powder; UV  $\lambda_{\max}$  222, 362; <sup>1</sup>H NMR (CD<sub>3</sub>OD, 500 MHz) and <sup>13</sup>C NMR (CD<sub>3</sub>OD, 125 MHz), see Table 1; (+)-ESI-HRMS  $m/z$ : 303.1589 [M + H]<sup>+</sup> (calcd for C<sub>18</sub>H<sub>23</sub>O<sub>4</sub>, 303.1591).

**Hypotien B (2).** Yellow powder; UV  $\lambda_{\max}$  218, 358; <sup>1</sup>H NMR (CD<sub>3</sub>OD, 500 MHz) and <sup>13</sup>C NMR (CD<sub>3</sub>OD, 125 MHz), see Table 1; (+)-ESI-HRMS  $m/z$ : 317.1750 [M + H]<sup>+</sup> (calcd for C<sub>19</sub>H<sub>25</sub>O<sub>4</sub>, 317.1747).

**Hypotien C (3).** Yellow powder; UV  $\lambda_{\max}$  220, 364; <sup>1</sup>H NMR (CD<sub>3</sub>OD, 500 MHz) and <sup>13</sup>C NMR (CD<sub>3</sub>OD, 125 MHz), see Table 1; (+)-ESI-HRMS  $m/z$ : 289.1433 [M + H]<sup>+</sup> (calcd for C<sub>17</sub>H<sub>21</sub>O<sub>4</sub>, 289.1434).

**Hypotien D (4).** Yellow powder; UV  $\lambda_{\max}$  218, 368; <sup>1</sup>H NMR (CD<sub>3</sub>OD, 500 MHz) and <sup>13</sup>C NMR (CD<sub>3</sub>OD, 125 MHz), see Table 1; (+)-ESI-HRMS  $m/z$ : 317.1750 [M + H]<sup>+</sup> (calcd for C<sub>19</sub>H<sub>25</sub>O<sub>4</sub>, 317.1747).

### Antimicrobial assay

The disk diffusion method was applied to evaluate the antibacterial and antifungal activities of compounds 1–4.<sup>23</sup> For bacteria, 200  $\mu$ L inoculum suspension was spread on the nutrient agar plates. For fungi, the mycelia were first macerated with mortar and pestle to generate a homogeneous inoculum. In antibacterial assay, sterile paper disks containing 40  $\mu$ L of the compounds with different concentrations (10, 5, 2, 1, 0.1  $\mu$ g mL<sup>−1</sup> in MeOH) were air-dried and then placed on inoculated plates. In antifungal test, paper disks were impregnated with 50  $\mu$ g of the samples. The plates were incubated at 37 °C for 24 h for bacteria or at 28 °C for 48 h for fungi. Streptomycin was used as the positive control for antibacterial evaluation, while actidione was employed as reference for antifungal efficacy.

### QS inhibitory activity

The strain *Chromobacterium violaceum* CV026 was inoculated in a 20 mL LB broth media overnight to afford seed culture.<sup>24</sup> 0.2 mL of seed broth was mixed with 15 mL of molten LB agar media. Kanamycin (0.72 mg) and *N*-hexanoyl-L-homoserine-lactone (C6-HSL, 1.5  $\mu$ g) were further added to the culture. Then, the agar was poured into a sterile Petri dish and then punched with a sterile cork borer. Compound at 40  $\mu$ g mL<sup>−1</sup> in MeOH was pipetted into each well. The positive control is

furanone C30 at 10  $\mu$ g mL<sup>−1</sup>. Finally, the Petri dish was incubated overnight at 37 °C.

### Cytotoxicity assay

The *in vitro* cytotoxic activities of compounds 1–4 were evaluated using the MTT method.<sup>25</sup> The cancer cells were properly seeded in 96-well culture plates and then treated with different concentrations of compounds (40, 20, 10, 5, 2, 1  $\mu$ M) for 24 h. After treatment, cells were incubated with MTT for 4 h. The plates were recorded at 570 nm by a plate reader. Adriamycin was applied as the positive control in the cytotoxicity assay.

## Conclusions

In summary, we isolated and characterized four new  $\alpha$ -pyrones, hypotiens A–D (1–4), from a fungal endophyte *Hypoxylon investiens* J2 living in the medicinal plant *Blumea balsamifera*. Their structures were determined by extensive spectroscopic analyses. Compounds 1–4, as  $\alpha$ -pyrone derivatives, possess an unusual dimethyl substitution in the highly unsaturated side chain. All compounds were measured for their antimicrobial, quorum sensing inhibitory, and cytotoxic activities but proved to be inactive. These results indicated that the specific side chain in compounds 1–4 might be responsible for the weak pharmacological activities.

## Conflicts of interest

There are no conflicts to declare.

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