Study on the secondary metabolites of grasshopper-derived fungi *Arthrinium* sp. NF2410

LI Wei¹, WEI Jing¹, CHEN Dao-Ying¹, WANG Mei-Jing², SUN Yang², JIAO Fang-Wen¹, JIAO Rui-Hua¹, TAN Ren-Xiang¹,³*, GE Hui-Ming¹*

¹State Key Laboratory of Pharmaceutical Biotechnology, Institute of Functional Biomolecules, School of Life Sciences, Nanjing University, Nanjing 210023, China;  
²State Key Laboratory of Pharmaceutical Biotechnology, Department of Biotechnology and Pharmaceutical Sciences, School of Life Science, Nanjing University, Nanjing 210023, China;  
³State Key Laboratory of Pharmaceutical Cultivation Base for TCM Quality and Efficacy, Nanjing University of Chinese Medicine, Nanjing 210023, China.

Available online 20 Dec., 2020

[ABSTRACT] Two new 2-carboxymethyl-3-hexyl-maleic anhydride derivatives, arthrianhydride A (1) and B (2), along with three known compounds 3–5, were isolated from the fermentation broth of a grasshopper-associated fungus *Arthrinium* sp. NF2410. The structures of new compounds 1 and 2 were determined based on the analysis of the HR-ESI-MS and NMR spectroscopic data. Furthermore, compounds 1 and 2 were evaluated on inhibitory activity against the enzyme SHP2 and both of them showed moderate inhibitory activity against SHP2.

[KEY WORDS] Grasshopper-associated fungi; Secondary metabolites; Structure elucidation; SHP2; Inhibitory activity


Introduction

Fungi are attractive producers for diverse natural products with significant bioactivities, which dramatically contribute to the new drugs discovery [1–2]. Notably, a number of bioactive natural compounds containing dialkylsubstituted maleic anhydride moiety were isolated and characterized from different kinds of fungi [3–5], such as 2-carboxymethyl-3-hexyl-maleic anhydride showing inhibitory activity against gram positive bacteria, originally from *Aspergillus* FH-X-213 [6]. FR222398, a maleic anhydride derivative isolated from the fungus *Talaromyces* sp. No.10092, exhibited a dispersant activity towards kaolin suspension [7]. Also, corydyanhydrides A and B from the insect pathogenic fungus *Cordyceps pseudomilitaris* BCC 1620 were reported by Masahiko Isaka, et al [8]. During our continuing search for novel bioactive natural products from fungi derived from unique ecological niches [9–14], we obtained a fungus *Arthrinium* sp. NF2410 from a grasshopper. Large scale fermentation and chromatographic separation afforded two new 2-carboxymethyl-3-hexyl-maleic anhydride derivatives, arthrianhydride A (1) and B (2), and three known compounds 3 [6], 4 [13] and 5 [15–16] (Fig. 1). Herein, we report the structure elucidation and biological activity of these compounds.

Results and Discussion

Arthrianhydride A (1) was isolated as a yellow oil. The molecular formula of 1 was assigned as C$_{20}$H$_{24}$O$_{5}$ with nine degrees of unsaturation based on its HR-ESI-MS spectroscopic data ([M + H]$^+$, m/z 345.1709, Calcd. for [C$_{20}$H$_{24}$O$_{5}$H]$^+$, 345.1702). Based on the $^1$H NMR data obtained in CDCl$_3$ (Table 1), five aromatic protons at δ$_H$ 7.30 (t, $J$ = 7.2 Hz, 2H), 7.25 (t, $J$ = 7.2 Hz, 1H) and 7.17 (d, $J$ = 7.2 Hz, 2H) and the $^3$H-H COSY from H-2' (H-6') to H-3' (H-5') and from H-3' (H-5') to H-4' indicated the presence of a mono-substituted aromatic ring. Meanwhile, the $^1$H NMR data exhibited one aliphatic methyl group at δ$_H$ 0.88 (t, $J$ = 6.0 Hz, 3H), eight aliphatic methylene groups at δ$_H$ 4.36 (t,
The 1H (400 MHz) and 13C NMR (100 MHz) spectroscopic data for compounds 1 and 2 in CDCl3 (δ in ppm)

<table>
<thead>
<tr>
<th>position</th>
<th>1</th>
<th>2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>δH (mult., J in Hz)</td>
<td>δC</td>
</tr>
<tr>
<td>2</td>
<td>165.2</td>
<td>165.2</td>
</tr>
<tr>
<td>3</td>
<td>147.5</td>
<td>147.7</td>
</tr>
<tr>
<td>4</td>
<td>136.1</td>
<td>136.1</td>
</tr>
<tr>
<td>5</td>
<td>165.3</td>
<td>165.3</td>
</tr>
<tr>
<td>6</td>
<td>2.38 (t, 7.7)</td>
<td>24.8</td>
</tr>
<tr>
<td>7</td>
<td>1.48 (m)</td>
<td>27.4</td>
</tr>
<tr>
<td>8</td>
<td>1.28 (m)</td>
<td>29.1</td>
</tr>
<tr>
<td>9</td>
<td>1.27 (m)</td>
<td>31.3</td>
</tr>
<tr>
<td>10</td>
<td>1.29 (m)</td>
<td>22.4</td>
</tr>
<tr>
<td>11</td>
<td>0.88 (t, 6.0)</td>
<td>14.0</td>
</tr>
<tr>
<td>12</td>
<td>3.47 (s)</td>
<td>29.5</td>
</tr>
<tr>
<td>13</td>
<td>167.1</td>
<td>167.7</td>
</tr>
<tr>
<td>14</td>
<td>4.36 (t, 6.8)</td>
<td>66.3</td>
</tr>
<tr>
<td>15</td>
<td>2.94 (t, 6.8)</td>
<td>34.8</td>
</tr>
<tr>
<td>1'</td>
<td>7.17 (d, 7.2)</td>
<td>128.8</td>
</tr>
<tr>
<td>2'</td>
<td>7.25 (t, 7.2)</td>
<td>126.8</td>
</tr>
<tr>
<td>3'</td>
<td>7.17 (d, 7.2)</td>
<td>128.8</td>
</tr>
</tbody>
</table>

J = 6.8 Hz, 2H), 3.47 (s, 2H), 2.94 (t, J = 6.8 Hz, 2H), 2.38 (t, J = 7.7 Hz, 2H), 1.45−1.52 (m, 2H) and 1.28 (m, overlap 6H), respectively. In the 13C spectrum (Table 1), three ester carbonyls (δC 167.1 (C-13), 165.2 (C-2) and 165.3 (C-5)), six aromatic (δC 137.2 (C-1′), 128.8 (2 × C-2′), 128.4 (2 × C-3′) and 126.2 (4-C′)), two olefinic (δC 147.5 (C-3) and 136.1 (C-4)) and nine aliphatic carbon signals were observed. The ester carbonyls (δC 165.2 and 165.3) and olefinic carbons (δC 147.5 and 136.1) indicated the presence of a maleic anhydride group. The DEPT and HSQC spectra further supported the presence of methyl and methylene groups. The complete structure elucidation was accomplished by detailed analysis of 2D NMR data including 1H-1H COSY, HSQC and HMBC spectra. As depicted in Fig. 2, the COSY correlations of H-2′ (6′) with H-3′ (5′) and of H-3′ (5′) with H-4′ displayed the fragment a. The COSY correlation of H-14 with H-15 indicated the presence of fragment b. Fragment d was determined by COSY correlations of H-6 with H-7, of H-7 with H-8 and of H-10 with H-11 and HMBC correlations from H-11 to C-9, from H-10 to C-8 and from H-9 to C-7. The connectivity of fragments a and b was revealed by HMBC correlations from H-14 to C-1′ and from H-15 to C-2′ (6′). The connection of partial structure b and c was confirmed by HMBC correlation from H-14 to C-13. The HMBC correlations from H-12 to C-3, C-4, C-5 and C-13 indicated that the carbon C-12 was connected with C-4 on the maleic anhydride group. The connectivity between d and maleic anhydride was confirmed by the HMBC correlations from H-7 to C-3 and H-6 to C-2, C-3 and C-4. Finally, the structure of compound 1 was confirmed.

Arthrianhydride B (2) was also isolated as a yellow oil, possessing a molecular formula of C18H18O7 with five degrees of unsaturation deduced from HR-ESI-MS data ([M − H]+, m/z 253.1085, Calcd. for [C16H17O3]−, 253.1076). The NMR spectra of 2 are similar to those of 1, and detailed comparisons of NMR spectroscopic data of 2 to those of 1 suggested the absence of the mono-substituted phenyl ring and two methylene groups, but the presence of one new oxygenated methyl group. On the basis of the 1H (δH 3.75), 13C (δC 52.9) NMR data and key HMBC correlations from H-14 to C-13, the connectivity of C-14 and C-13 via an ester bond was validated. The fragments c and d were all bonded to maleic anhydride moiety, which was confirmed by the analysis of 2D NMR data (Fig. 2). Thus, the structure of 2 was elucidated as shown in Fig. 1.

Arthrianhydride A (1) and B (2) were tested for their inhibitory activity against SHP2 (Scr homology-2-containing-protein tyrosine phosphatase 2), which is an important non-receptor phosphatase and involved in several cellular pro-
Fig. 2  Key HMBC and $^1$H-$^1$H COSY correlations of compounds 1 and 2

Fig. 3  The inhibitory activity of compounds 1 and 2 against SHP2 enzyme

In conclusion, we have isolated five compounds produced by a grasshopper-associated fungus NF2410, including two new 2-carboxymethyl-3-hexyl-maleic anhydride derivatives, arthrianhydride A (1) and B (2) and three known compounds (3–5). Additionally, new compounds 1 and 2 displayed moderate inhibitory activity against SHP2. The inhibitory activity of arthrianhydride A was better than that of arthrianhydride B, which was likely attributed to the 14-CH$_3$ of 2 instead of phenylethyl group of 1. Our work enriched the chemistry of natural products obtained from insect-derived fungi and the secondary metabolites of insect-derived fungi could be potential inhibitors of SHP2 enzyme.

**Experimental**

**General experimental procedures**

All NMR experiments were carried out on Bruker Avance III 400 spectrometer at 400 MHz for $^1$H and 100 MHz for $^{13}$C nuclei. Chemical shifts were given in ppm using TMS as internal standard. UV spectrum was obtained on a Nanodrop2000 spectrometer. IR spectrum was measured with a Nexus 870 FT-IR using KBr pellets. High resolution electrospray ionization mass spectroscopy (HR-ESI-MS) was performed on Agilent 1290 Infinity II HPLC/6530 Q-TOF MS for all compounds. Semi-preparative HPLC (Agilent Technologies 1260 Infinity II) equipped with an Agilent Eclipse XDB-C$_{18}$ column (5 μm, 250 mm × 9.4 mm) was used to purify and prepare samples. Sephadex LH-20 (purchased from GE Biotech, USA) and silica gel (200–300 mesh, obtained from Qingdao Marine Chemical Company, China) were employed for column chromatography (CC). Thin layer chromatography (TLC, GF254, 10–20 μm) was used for sample analysis.

**Fungus material**

The fungus NF2410 was isolated from the grasshopper caught from Qixia Mountain, Nanjing, Jiangsu Province, China, in September, 2018. The strain was identified as *Arthrinium* sp. according to the morphological features and sequence analysis of 18S rRNA (NCBI, GenBank accession number KM096274.1).

**Cultivation, extraction and isolation**

The fungus was grown on potato dextrose agar (PDA) at 30 °C for 8 days. Then the agar plates were cut into small pieces and inoculated into 1 L Erlenmeyer flasks containing 400 mL sterilized MD medium (composed of 0.5% peptone, 0.2% yeast extract, 2% sucrose, 0.1% KH$_2$PO$_4$, 0.05% MgSO$_4$·7H$_2$O and 2.7% sea salt), which were cultured for 14 days at 30°C with 160 r min$^{-1}$. After filtration by carbasus, the final fermentation broth (20 L) was obtained and subsequently extracted with ethyl acetate for 3 times. The extraction solvent was combined and evaporated under reduced pressure to yield 11.7 g crude extract. Using gradient solvents from CH$_3$Cl$_2$ to CH$_3$OH (V/V, 100 : 0, 100 : 1, 100 : 2, 100 : 4, 100 : 8, 100 : 16, 100 : 32 and 0 : 100) as mobile phase, the crude extract was applied to silica gel CC to furnish 15 fractions (Fr. A-O) based on the TLC results. Eight subfractions (Fr. C1-Fr. C8) were acquired after separation of Fr. C (1.1 g) by Sephadex LH-20 CC eluted with methanol and Fr. C3 (0.47 g) was further separated by semi-preparative HPLC to afford compounds 1 ($t_R$ = 20.5 min, 11.4 mg), 2 ($t_R$ = 19.2 min, 8.2 mg) and 3 ($t_R$ = 14.7 min, 6.0 mg) eluted with a linear gradient method of 35%–90% CH$_3$CN/H$_2$O added 0.1% trifluoroacetic acid over a period of 24.5 min (2.5 mL·min$^{-1}$). Using a stepwise gradient method of 10%–35% CH$_3$CN/H$_2$O from 0 to 15 min and 85% CH$_3$CN from 15 to 28 min (2.5 ml·min$^{-1}$), Fr. J was further fractioned by semi-preparative HPLC to yield compounds 4 ($t_R$ = 15.2 min, 6.2 mg) and 5 ($t_R$ = 19.3 min, 5.0 mg).

**Compound 1**: yellow oil; UV(MeOH) $\lambda_{max}$(logε) 211 (5.04) nm, 214 (4.99) nm, 248 (4.71) nm; IR (KBr)$_{max}$ 2957.2, 2929.6, 2858.3, 1771.4, 1741.8, 1455.6, 1276.5, 1174.4, 923.1, 751.6, 701.0 cm$^{-1}$; $^1$H and $^{13}$C NMR data, see Table 1; HR-ESI-MS data [M + H]$^+$ m/z 345.1709 (Calcd. for [C$_{20}$H$_{25}$O$_3$]$^+$ 345.1702).
Compound 2: yellow oil; UV(MeOH) \( \lambda_{\text{max}} \) (log e) 214 (4.80) nm, 249 (4.71) nm; IR (KBr) \( \nu_{\text{max}} \) 2957.2, 2932.3, 2860.2, 1771.2, 1744.0, 1437.5, 1276.4, 1174.3, 923.4, 765.0 cm\(^{-1}\); \( ^{1}\)H and \( ^{13}\)C NMR data, see Table 1; HR-ESI-MS data \([M – H]^{+} , m/z\) 253.1085 (Calcd. for \(\text{C}_{17}\text{H}_{15}\text{O}_{3}\)) 253.1076.

The assay for catalytic activity of SHP2

The catalytic activity of SHP2 was monitored using the surrogate substrate DiFMUP in a prompt fluorescence assay format \([18]\). The phosphatase reactions were conducted at room temperature in 96-well black polystyrene plate, flat bottom, low flange, nonbinding surface (Corning, Cat. No. 3575) using a final reaction volume of 100 μL and the following assay buffer conditions: 60 mmol·L\(^{-1}\) HEPES, pH 7.2, 75 mmol·L\(^{-1}\) NaCl, 75 mmol·L\(^{-1}\) KCl, 1 mmol·L\(^{-1}\) EDTA, 0.05% P-20, 5 mmol·L\(^{-1}\) DTT. 1 mmol·L\(^{-1}\) of SHP2\(^{\text{wt}}\) (residues 1–525) was co-incubated with 0.1 mmol·L\(^{-1}\) of bi-phosphorylated IRS1 peptide (sequence: H2N-LN (pY) IDLDLV (dPEG8) LST (pY) ASINFKQ-amide) and 30 μmol·L\(^{-1}\) of tested compounds. 30 μmol·L\(^{-1}\) SHP099 was used as positive control. After 30–60 min incubation at 25 °C, the surrogate substrate DiF MU P (Invitrogen, Cat. no. D6567, 100 μmol·L\(^{-1}\)) was added into the reaction and incubated at 25 °C for 30 min. The reaction was then quenched by the addition of 20 μL of a 160 mmol·L\(^{-1}\) solution of bpV (Phen) (Enzo Life Sciences Cat. No. ALX-270-204). The fluorescence signal was monitored using a microplate reader (TECAN, M200PRO) under excitation and emission wavelengths of 340 and 450 nm, respectively.

References

[16] Ström K, Sjögren J, Broberg A, et al. Lactobacillus plantarum MiLAB 393 produces the antifungal cyclic dipeptides cyclo (L-Phe–trans-4-OH-L-Pro) and 3-cyclo (L-Phe–L-Pro) and cyclo (L-Phe–L-Pro) [J]. Appl Environ Microbiol, 2002, 68(9): 4322-4327.