

## Identification of blapsins A and B as potent small-molecule 14-3-3 inhibitors from the insect *Blaps japonensis*

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### ABSTRACT

In this study, we report three novel naturally occurring compounds, blapsins A (**1**) and B (**2**), and blapsamide (**3**) from the ethanol extract of the stink beetle, *Blaps japonensis*. The structures of these compounds were determined using spectroscopic methods. Compound **3** is a phenolic compound bearing a formamido group in the structure. Functional studies revealed that compounds **1** and **2** potently inhibited 14-3-3 protein–protein interactions (PPIs) with IC<sub>50</sub> values of 9.2 and 10.0 μM as determined by an ELISA assay, and 2.0 and 2.5 μM in an FP assay, respectively. These compounds represent the first example of natural small-molecule 14-3-3 inhibitors.

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Insects represent a megadiverse group of animals and have been shown to possess efficient chemical defense systems. Since ancient times, mankind has utilized insects and their by-products for medicinal purposes. Extracts from insects have been found to contain potential immunological, analgesic, antibacterial, diuretic, anesthetic, and antirheumatic properties.<sup>1,2</sup> Several unique compounds have been characterized from diverse insects.<sup>2–7</sup> From the information now at hand, insects appear to be a new and intriguing source of potentially useful drugs.

*Blaps japonensis* (Tenebrionidae), also known as the stink beetle, is a famous medicinal insect of Yi-nationality medicine of Yunnan province, (People's Republic of China) that has been used to treat inflammation, infection, and cancer.<sup>8</sup> Previous reports demonstrated that quinone and quinol derivatives from the extract or defensive sprays of *Blaps* species exhibit significant antibacterial activities against several microbial strains.<sup>9–12</sup> However, further investigation into the chemical components and biological properties of *B. japonensis* needs to be conducted to better understand the therapeutic potential of compounds from these insects.

The 14-3-3 proteins are a family of highly conserved acidic polypeptides that are expressed in all eukaryotic species.<sup>13</sup> Since their identification in brain tissue in 1967,<sup>14</sup> significant progress has been made on the research of 14-3-3 proteins. To date, seven 14-3-3 isoforms and more than 200 14-3-3 target proteins have been identified.<sup>15</sup> These target proteins are known to play critical roles in the regulation of diverse physiological and pathophysiological events, including cell differentiation, proliferation, and transformation. Importantly, the ability of 14-3-3 proteins to bind and regulate a wide range of oncogenes and tumor suppressor genes, such as Raf, Bad, p53, and Cdc25, highlights their roles in human cancer treatment.<sup>15</sup> Thus, compounds targeting the 14-3-3 PPIs have the potential to be promising therapeutics for the treatment of human cancers.<sup>16–18</sup>

In an effort to identify novel active compounds from *B. japonensis*, we have isolated and determined the structure of three new compounds from these insects (Fig. 1). Furthermore, to determine whether these compounds may have potential therapeutic benefits, we examined the ability of compounds **1** and **2** to inhibit 14-3-3 PPIs. In this Letter, we describe the isolation, structural elucidation and biological evaluation of the new compounds isolated from *B. japonensis*.

Compound **1**<sup>19</sup> was isolated<sup>20</sup> as a gum. Its molecular formula was determined to be C<sub>16</sub>H<sub>16</sub>O<sub>6</sub> by analysis of its HR-ESI-MS, <sup>13</sup>C NMR, and DEPT spectra. The <sup>13</sup>C NMR and DEPT spectra exhibited

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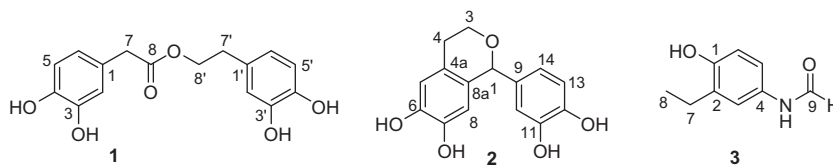


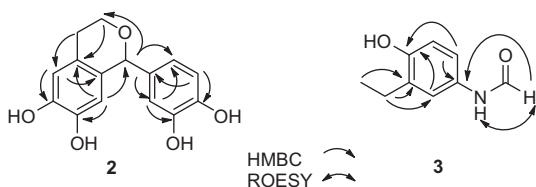
Figure 1. The structures of compounds 1–3.

Table 1  
NMR spectroscopic data for **1**<sup>a</sup> and **2**<sup>a</sup>

Position		1		Position		2	
		$\delta_{\text{H}}^b$ (J in Hz)	$\delta_{\text{C}}^b$			$\delta_{\text{H}}^b$ (J in Hz)	$\delta_{\text{C}}^b$
1			126.9 qC	1		5.47, s	80.7 CH
2		6.67, d, 1.8	117.4 CH	2			
3			146.2 qC	3 $\alpha$		4.08, m	64.9 CH <sub>2</sub>
4			144.9 qC	3 $\beta$		3.82, m	
5		6.67, d, 8.0	116.4 CH	4 $\alpha$		2.92, m	29.1 CH <sub>2</sub>
6		6.52, dd, 8.0, 1.8	121.7 CH	4 $\beta$		2.62, m	
7		3.42, s	41.5 CH <sub>2</sub>	4a			126.1 qC
8			174.1 qC	5		6.57, s	115.8 CH
1'			130.6 qC	6			144.5 qC
2'		6.63, d, 1.8	116.9 CH	7			145.3 qC
3'			146.2 qC	8		6.18, s	114.6 CH
4'			145.2 qC	8a			129.9 qC
5'		6.66, d, 8.0	116.3 CH	9			135.4 qC
6'		6.47, dd, 8.0, 1.8	121.3 CH	10		6.69, d, 1.8	117.1 CH
7'		2.73, t, 7.0	35.4 CH <sub>2</sub>	11			146.1 qC
8'		4.17, t, 7.0	66.9 CH <sub>2</sub>	12			146.4 qC
				13		6.75, d, 8.0	115.8 CH
				14		6.65, dd, 8.0, 1.8	121.9 CH

<sup>a</sup> 500 MHz for <sup>1</sup>H, 100 MHz for <sup>13</sup>C.<sup>b</sup> In methanol-*d*<sub>4</sub>.Table 2  
NMR spectroscopic data for **3**<sup>a</sup>

Position		3	
		$\delta_{\text{H}}^b$ (J in Hz)	$\delta_{\text{C}}^b$
1			152.1 qC
2			131.6 qC
3		7.41, d, 2.3	121.5 CH
4			131.4 qC
5		7.37, dd, 8.5, 2.3	118.9 CH
6		6.78, d, 8.5	115.6 CH
7		2.62, q, 7.5	23.9 CH <sub>3</sub>
8		1.18, t, 7.5	14.5 CH <sub>3</sub>
9		8.28, s	159.3 qC
NH		9.01, br s	
OH		8.16, s	

<sup>a</sup> 600 MHz for <sup>1</sup>H, 100 MHz for <sup>13</sup>C.<sup>b</sup> In acetone-*d*<sub>6</sub>.Figure 2. HMBC and ROESY correlations of compounds **2** and **3**.

resonances (Table 1) for three methylene (including one oxymethylene), six olefinic methine, and seven quaternary carbons (including four oxygen-bearing olefinic carbons, two of which overlap, and one carboxylic carbon). The <sup>1</sup>H NMR spectrum indicated two ABX coupling systems that resonated at  $\delta$  6.52 (H-6, dd,  $J$  = 8.0, 1.8 Hz), 6.67 (H-5, d,  $J$  = 8.0 Hz) and 6.67 (H-2, d,  $J$  = 1.8 Hz), and  $\delta$  6.47 (H-6', dd,  $J$  = 8.0, 1.8 Hz), 6.66 (H-5', d,  $J$  = 8.0 Hz), and 6.63 (H-2', d,  $J$  = 1.8 Hz), respectively. The <sup>1</sup>H–<sup>1</sup>H COSY spectrum showed cross peaks of H-5/H-6, H-5'/H-6', and H-7'/H-8'. The above data together with the HMBC correlations of H-8'/C-1', H-7'/C-2', C-6', and H-7/C-6, C-8 suggested the presence of one 2-(3,4-dihydroxyphenyl)acetic acid residue and one 4-(2-hydroxyethyl)benzene-1,2-diol residue. Their linkage through an ester bond was supported by the HMBC correlations of H-8'/C-8. Thus, the structure of **1** was established as 3,4-dihydroxyphenethyl 2-(3,4-dihydroxyphenyl)acetate, and the compound was given the name blapsin A.

Compound **2**<sup>21</sup> was obtained<sup>20</sup> as a yellowish solid. The molecular formula was determined to be C<sub>15</sub>H<sub>14</sub>O<sub>5</sub> from the HR-ESI-MS, <sup>13</sup>C NMR, and DEPT spectra, indicating nine degrees of unsaturation. The <sup>13</sup>C NMR and DEPT spectra indicated fifteen carbons, of which twelve were olefinic carbons belonging to two phenyl groups. In addition, one oxymethylene, one oxymethine, and one

methylene were observed. The <sup>1</sup>H NMR spectrum showed one ABX system resonated at  $\delta$  6.65 (H-14, dd,  $J$  = 8.0, 1.8 Hz), 6.75 (H-13, d,  $J$  = 8.0 Hz), and 6.69 (H-10, d,  $J$  = 1.8 Hz). Two aromatic protons resonated at  $\delta$  6.18 (H-8, s) and 6.57 (H-5, s) suggesting a 1,2,4,5-tetrasubstituted benzene ring. The <sup>1</sup>H–<sup>1</sup>H COSY spectrum showed cross peaks of H-3/H-4 and H-13/H-14. The HMBC correlations of H-1/C-4a, C-8, C-8a, C-10, and C-14 indicated that two phenyl groups were connected via C-1 (Fig. 2). The HMBC correlations of H-3/C-4a, H-4/C-5, C-8a, and H-1/C-3 suggested the structural motif of an isochroman. Thus, the structure of **2** was determined to be 1-(3,4-dihydroxyphenyl)isochroman-6,7-diol, and the compound was given the name blapsin B. Notably, compound **2** has been previously synthesized as a potent antioxidant.<sup>22</sup> Importantly, as a new naturally occurring compound, this was the first time that this compound was isolated from this species as racemic mixtures.

The molecular formula of **3**<sup>23</sup> was found to be C<sub>9</sub>H<sub>11</sub>NO<sub>2</sub>, as deduced from its HR-ESI-MS, <sup>13</sup>C NMR, and DEPT spectra (Table 2). The <sup>1</sup>H NMR spectrum showed one ABX system [ $\delta$  7.37 (H-5, dd,  $J$  = 8.5, 2.3 Hz), 6.78 (H-6, d,  $J$  = 8.5 Hz), and 7.41 (H-3, d,  $J$  = 2.3 Hz), two active protons at  $\delta$  9.01 (NH) and 8.16 (OH), and one proton at 8.28 (CHO). The <sup>13</sup>C NMR and DEPT spectra revealed six olefinic carbons belonging to a benzene ring, one methyl, one methylene, and one CHO. The <sup>1</sup>H–<sup>1</sup>H COSY correlation of H-7/H-8 and HMBC correlations of H-13/H-14 are shown in Figure 2. The HMBC correlations of H-7/C-1, C-2, C-3, and H-9/C-4 established the structure of **3** as shown. The presence of a formamide group was also supported by the distinct upfield shift of C-9 ( $\delta$  159.3) and ROESY correlations of CHO/NH recorded in acetone-*d*<sub>6</sub> (Fig. 2). The structure of **3** was thus determined to be *N*-(3-ethyl-4-hydroxyphenyl)formamide, and given the name blapsamide.

In order to determine whether these compounds have an effect on 14-3-3 PPIs, compounds **1** and **2** were evaluated for their 14-3-3 inhibitory activity. The results from these experiments demonstrated that compounds **1** and **2** exhibited potent inhibitory effects on 14-3-3 $\gamma$  with IC<sub>50</sub> values of 9.2 and 10.0  $\mu$ M obtained from ELISA assays (Fig. 3),<sup>24</sup> and 2.0 and 2.5  $\mu$ M in FP assays,<sup>25</sup> respectively. Thus, compounds **1** and **2** represent the first examples of

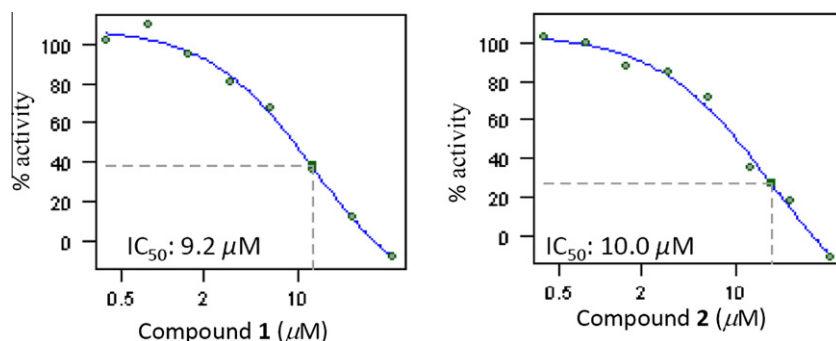


Figure 3. Inhibitory effect of compounds 1 and 2 on 14-3-3 PPIs in ELISA assay.

natural small-molecule 14-3-3 inhibitors. Considering that the 14-3-3 family regulates a wide range of pathophysiological processes and alteration of the expression of 14-3-3 proteins has been demonstrated in several human cancers. Therefore, the discovery and identification of these two natural 14-3-3 inhibitors may not only clarify traditional medical applications in part, but also provide chemical probes for further advance chemical biology investigations in the field of 14-3-3. Compounds 1 and 2 could be served as potential leads for the further development of anticancer drugs.

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- Blapsin A (1): Pale yellow gum; UV (MeOH)  $\lambda_{\max}$  (log  $\epsilon$ ) 284 (3.73), 207 (4.37) nm; IR (KBr)  $\nu_{\max}$  3424, 2959, 2931, 2873, 1727, 1625, 1519, 1450, 1383, 1287, 1122, 1075, 745, 705  $\text{cm}^{-1}$ ;  $^1\text{H}$  and  $^{13}\text{C}$  NMR data, see Table 1; FAB-MS (negative)  $m/z$  303  $[\text{M}-\text{H}]^-$ ; HRESIMS (negative)  $m/z$  303.0877  $[\text{M}-\text{H}]^-$  (calcd for  $\text{C}_{16}\text{H}_{15}\text{O}_6$ , 303.0868).
- Isolation: The bodies of *B. japonensis* were collected from Honghe County, Yunnan Province, PR China in September 2008, and authenticated by Professor Dazhi Dong at the Kunming Institute of Zoology, Chinese Academy of Sciences, PR China. A voucher specimen (CHYX0469) was deposited at the State Key Laboratory of Phytochemistry and Plant Resources in West China at our institute. The air-dried powder of *B. japonensis* bodies (5 kg) was extracted with 50% aqueous EtOH ( $3 \times 30\text{ L}$ ) at room temperature. The combined extracts were concentrated to obtain a crude extract (500 g), which was partitioned with BuOH in  $\text{H}_2\text{O}$  to obtain BuOH extract (150 g). The BuOH extract was separated by silica gel column chromatography (CC) using gradient  $\text{CHCl}_3/\text{MeOH}$  (1:0 to 0:1) to produce fractions A–F. Fraction B (5.7 g) was divided into fractions B1–B6 by MCI gel CHP 20P CC eluting with aq MeOH (20%–100%). Fraction B5 (140 mg) was passed through Sephadex LH-20 (MeOH) CC to afford fractions B5.1–B5.6. Of which, fraction B5.4 (30 mg) was purified by prep. TLC developed with  $\text{CH}_2\text{Cl}_2/\text{MeOH}$  (8:1) to give compound 3 (4.6 mg). Fraction D (8 g) was separated into fractions D1–D11 by MCI gel CHP 20P washing with MeOH/ $\text{H}_2\text{O}$  (5–80%). Fraction D8 (270 mg) was divided into fractions D8.1–D8.3 by Sephadex LH-20 (MeOH) CC. Fraction D8.3 (50 mg) was submitted to prep. TLC ( $\text{CH}_2\text{Cl}_2/\text{MeOH}/\text{AcOH}$ , 8:1:0.05) to give 2 (5 mg). Fraction D11 (90 mg) was separated by Sephadex LH-20 (MeOH) CC followed by prep. TLC ( $\text{CH}_2\text{Cl}_2/\text{MeOH}/\text{AcOH}$ , 8:1:0.05) to give 1 (7 mg).
- Blapsin B (2): Yellowish solid;  $[\alpha]_D^{25}$  0 (c 0.05, MeOH); UV (MeOH)  $\lambda_{\max}$  (log  $\epsilon$ ) 286 (3.81), 208 (4.60) nm; IR (KBr)  $\nu_{\max}$  3422, 2961, 2927, 1613, 1521, 1447, 1369, 1279, 1190, 1111, 1078, 1049, 870, 822, 785, 767  $\text{cm}^{-1}$ ;  $^1\text{H}$  and  $^{13}\text{C}$  NMR data, see Table 1; FAB-MS (positive)  $m/z$  275  $[\text{M}+\text{H}]^+$ ; HR-ESI-MS (positive)  $m/z$  274.0849  $[\text{M}]^+$  (calcd for  $\text{C}_{15}\text{H}_{14}\text{O}_5$ , 274.0841).
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- Blapsamide (3): White powder; UV (MeOH)  $\lambda_{\max}$  (log  $\epsilon$ ) 253 (4.05), 207 (4.31) nm; IR (KBr)  $\nu_{\max}$  3421, 2962, 2933, 2874, 1724, 1671, 1511, 1438, 1273, 1120, 1076, 817, 746  $\text{cm}^{-1}$ ;  $^1\text{H}$  and  $^{13}\text{C}$  NMR data, see Table 2; ESI-MS (positive)  $m/z$  166  $[\text{M}+\text{H}]^+$ ; HR-ESI-MS (positive)  $m/z$  166.0867  $[\text{M}+\text{H}]^+$  (calcd for  $\text{C}_9\text{H}_{12}\text{NO}_2$ , 166.0868).
- Enzyme-linked immunosorbent (ELISA) assay: A 14-3-3 ELISA assay was used to monitor the interaction of recombinant GST-tagged 14-3-3 proteins with endogenous client proteins, such as PRAS40, in COS-7 cell lysate. Briefly, GST-14-3-3 protein (1  $\mu\text{M}$ ) immobilized on an anti-GST plate was incubated with a test compound before adding COS7 cell lysate in 1% NP-40 lysis buffer.<sup>26</sup> After incubation and washing, antibodies specific to PRAS40 along with peroxidase-labeled anti-rabbit IgG (50  $\mu\text{L}$ ; 1:1000 dilution) were added. After washing, 100  $\mu\text{L}$  of tetramethylbenzidine was added. The reaction was stopped by the addition of sulfuric acid. Signals were recorded at 450 nm on an EnVision reader (PerkinElmer) and  $\text{IC}_{50}$  values were calculated using GraphPad software.
- Fluorescence polarization (FP) assay: The 14-3-3 FP assay was carried out in black 384-well microplates in a total volume of 50  $\mu\text{L}$ .<sup>27</sup> The reaction buffer used in the FP assay throughout this study contained HEPES (10 mM, pH 7.4), NaCl (150 mM), Tween-20 (0.05%), 1  $\mu\text{M}$  GST-14-3-3, 2 nM TMR-pS259-Raf peptide, and DTT (0.5 mM). One microliter of the test compound (2 mM stock in DMSO) was added to 49  $\mu\text{L}$  of reaction buffer. The plates were incubated at room temperature for 30 min and the FP value in millipolarization (mP) units was recorded using an EnVision 2103 multilabel reader (PerkinElmer). An excitation filter at 545 nm and an emission filter at 610–675 nm were used with a dichroic mirror at 565 nm. Compounds with recorded mP values greater than 3 SD from the negative control (1  $\mu\text{L}$  DMSO) were considered positive hits. Dose-response curves were obtained and  $\text{IC}_{50}$  values were calculated using GraphPad software.
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