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Compounds from *Polyphaga plancyi* and their inhibitory activities against JAK3 and DDR1 kinases

Hong-Jie Zhu [a,b,1], Yong-Ming Yan [b,1], Zheng-Chao Tu [c], Jin-Feng Luo [c], Rui Liang [c], Tong-Hua Yang [d], Yong-Xian Cheng [b,*], Shu-Mei Wang [a,*]

[a] Guangdong Pharmaceutical University, Guangzhou 510006, PR China
[b] State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Sciences, Kunming 650201, PR China
[c] Guangzhou Institutes of Biomedicine and Health, Chinese Academy of Sciences, Guangzhou 510530, PR China
[d] Department of Hematology, First People's Hospital of Yunnan Province, Kunming 650032, PR China

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ABSTRACT

Plancyamides A (**1**) and B (**3**), plancypyrazine A (**2**), and plancyols A (**4**) and B (**5**), five new compounds (**1**–**5**), and three known ones (**6**–**8**), were isolated from the whole bodies of *Polyphaga plancyi* Bolivar. Their structures were elucidated by a combination of spectroscopic analyses including 1D and 2D NMR, and HRESIMS. Among them, compound **3** is racemic, chiral HPLC separation afforded its respective enantiomers. The absolute configuration of **1** was assigned by computational methods. Biological evaluation of all the compounds with exception of **7** and **8** discloses that compounds **2** and **4** could inhibit JAK3 kinase with IC_{50} values of 12.6 and 5.0 μM, respectively. In addition, compound **4** exhibit inhibitory activity towards DDR1 kinase with IC_{50} value of 4.87 μM.

1. Introduction

Insects dominate on earth, it is estimated that more than one million insects have been taxonomically identified. Previous investigations indicate that structurally novel and biological non-peptide small molecules (NPSMs) are present in insects [1,2]. However, small molecules from insects and their roles in Nature, unlike plant or microorganism metabolites, are largely unknown. We became interested in insect metabolites and characterized a series of NPSMs from several insects [3–7]. *Polyphaga plancyi* Bolivar, a widely distributed insect species in China with blood stasis removing properties, has been used as a traditional Chinese medicine to treat a wide range of diseases such as amenorrhea and fracture. Modern pharmacological studies show that its extract possesses wide effects such as anticancer and anti-inflammatory properties [8]. In the course of our continuing study on insect NPSMs, the title insect were investigated, which led to the isolation of 8 NPSMs including five ones are new compounds (Fig. 1). Herein, we report their isolation, structure characterization and biological evaluation.

2. Experimental

2.1. General

Optical rotations were recorded on a JASCO P-1020 digital polarimeter. UV spectra were measured on a Shimadzu UV-2401PC spectrometer. CD spectra were determined on a Chirascan instrument. NMR spectra were recorded on a Bruker Avance III 600 MHz spectrometer, with TMS as an internal standard. ESIMS and HRESIMS were collected on an API QSTAR Pulsar 1 spectrometer. C-18 silica gel (40–60 μm; Daiso Co., Japan), MCI gel CHP 20P (75–150 μm, Mitsubishi Chemical Industries, Tokyo, Japan) and Sephadex LH-20 (Amersham Pharmacia, Uppsala, Sweden) were employed for column chromatography. Semi-preparative HPLC was underwent on an Agilent 1200 liquid chromatograph with an YMC-Pack ODS-A column (250 × 10 mm, i.d., 5 μm) and a Daicel Chiralpak (IC, 250 mm × 10 mm, i.d., 5 μm).

2.2. Insect material

The specimen of *Polyphaga plancyi* was purchased from Henan Province, China, in November 2014, and identified by Prof. Da-Rong Yang at Kunming Institute of Zoology, Chinese Academy of Sciences. A voucher specimen (CHYX-0593) was deposited at the State Key Laboratory of Photochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Sciences, PR China.

* Corresponding authors.
E-mail addresses: yxcheng@mail.kib.ac.cn (Y.-X. Cheng), shmwang@sina.com (S.-M. Wang).
[1] These authors contributed equally to this paper.

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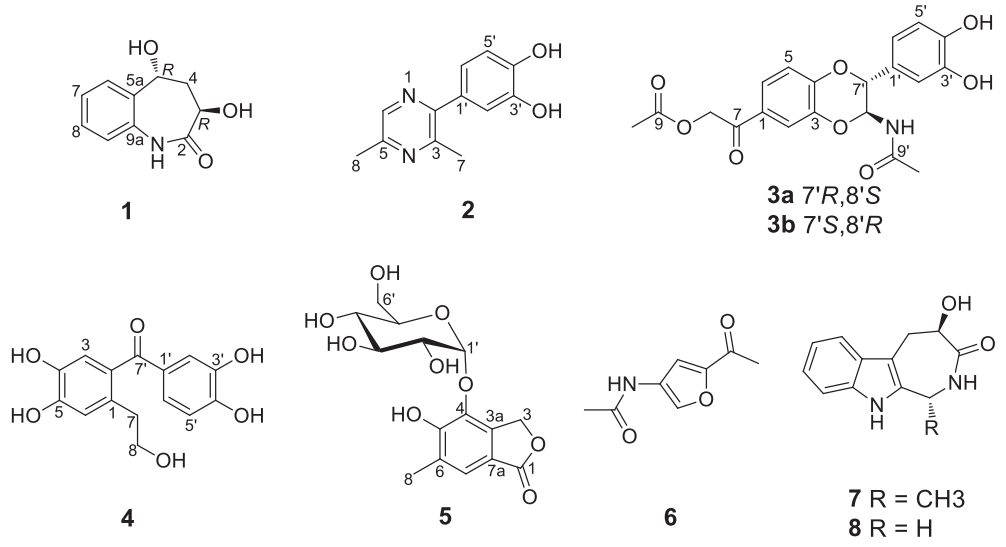

Fig. 1. Chemical structures of **1**–**8**.

2.3. Extraction and isolation

The whole bodies of *P. plancyi* (50 kg) were extracted under reflux with 70% EtOH (300 L, 4 h, 3 h, 3 h) to give a crude extract (6.32 kg), which was suspended in water followed by successive partition with petroleum ether and EtOAc to afford an EtOAc soluble extract. This extract (127 g) was divided into six parts (Fr. A–F) by using a MCI gel CHP 20P column eluted with gradient aqueous MeOH (10%–100%). Fr. A (8.6 g) was further separated via RP-18 column washed with gradient aqueous MeOH (0%–20%) to yield 3 fractions (Fr. A1-A3). Among them, Fr. A3 (2.1 g) was gel filtrated over Sephadex LH-20 (aqueous MeOH, 80%) followed by RP-18 column (aqueous MeOH, 5%–20%) to give Fr. A3.2 (320 mg), which was further purified by semi-preparative HPLC (MeCN/H_2O, 5%) to yield **1** (10.0 mg, t_R = 32 min). Fr. B (8.1 g) was gel filtrated over Sephadex LH-20 (aqueous MeOH, 80%) to afford 3 parts (Fr. B1–B3). Fr. B1 (4.34 g) was submitted to a RP-18 column eluted with gradient aqueous MeOH (10%–40%) to yield 4 subfractions (Fr. B1.1–B1.4). Of which, Fr. B1.3 (1.03 g) was purified by semi-preparative HPLC (MeCN/H_2O, 15%) to yield **6** (2.0 mg, t_R = 19 min), **7** (3.2 mg, t_R = 25 min), and **8** (1.5 mg, t_R = 20 min). Compound **4** (2.6 mg, t_R = 19 min) was obtained from Fr. B3 (400 mg) by semi-preparative HPLC (MeCN/H_2O, 13%). Fr. D (8.7 g) was gel filtrated over Sephadex LH-20 (aqueous MeOH, 80%) to afford 6 parts (Fr. D1–D6). Further, Fr. C4 (2.39 g) was gel filtrated over Sephadex LH-20 (MeOH) followed by semi-preparative HPLC (MeCN/H_2O, 25%) to produce **2** (1.0 mg, t_R = 23 min), **3** (1.8 mg, t_R = 27 min), and **5** (3.7 mg, t_R = 10 min). Notably, compound **3** was isolated as a racemic mixture, successive chiral HPLC separation afforded **3a** (0.8 mg) and **3b** (0.9 mg) (*n*-hexane/ethanol, 50:50).

Plancyamide A (**1**): yellowish gum; $[\alpha]_D^{25}$ +172.9 (*c* 0.61, MeOH); CD (MeOH) $\Delta\varepsilon_{215}$ −0.57, $\Delta\varepsilon_{241}$ +25.43; UV (MeOH) λ_{max} (log ε) 239 (3.86), 205 (4.29); ^{1}H and ^{13}C NMR data, see Table 1; HRESIMS (positive) *m/z* 216.0630 $[M + Na]^+$ (calcd for $C_{10}H_{11}NO_3Na$, 216.0631).

Plancypyrazine A (**2**): yellowish solid; UV (MeOH) λ_{max} (log ε) 328 (3.74), 291 (3.72), 212 (4.01); ^{1}H and ^{13}C NMR data, see Table 1; HRESIMS (positive) *m/z* 217.0973 $[M + H]^+$ (calcd for $C_{12}H_{13}N_2O_2$, 217.0972).

Plancyamide B (**3**): white solid; {$[\alpha]_D^{25}$ +13.7 (*c* 0.08, MeOH); CD (MeOH) $\Delta\varepsilon_{206}$ −32.06, $\Delta\varepsilon_{231}$ +12.11; **3a}**; {$[\alpha]_D^{25}$ −23.3 (*c* 0.09, MeOH); CD (MeOH) $\Delta\varepsilon_{207}$ +25.37, $\Delta\varepsilon_{230}$ −7.62; **3b}**; UV (MeOH) λ_{max} (log ε) 301 (3.68), 275 (3.92), 228 (4.15), 205 (4.37); ^{1}H and ^{13}C NMR data, see Table 1; HRESIMS (positive) *m/z* 402.1182 $[M + H]^+$ (calcd for $C_{20}H_{20}NO_8$, 402.1183).

Plancyol A (**4**): brownish red solid; UV (MeOH) λ_{max} (log ε) 532 (3.19), 320 (3.84), 207 (4.41); ^{1}H and ^{13}C NMR data, see Table 2; HRESIMS (negative) *m/z* 289.0713 $[M - H]^-$ (calcd for $C_{15}H_{13}O_6$, 289.0718).

Plancyol B (**5**): white solid; UV (MeOH) λ_{max} (log ε) 289 (3.90), 210 (4.39); ^{1}H and ^{13}C NMR data, see Table 2; HRESIMS (negative) *m/z* 341.0877 $[M - H]^-$ (calcd for $C_{15}H_{17}O_9$, 341.0878).

2.4. Assay for in vitro Janus kinase 3 (JAK3) kinase inhibition activity

The inhibitory activity of compounds **1**–**6** against JAK3 kinase was performed using the FRET-based Z'-Lyte assay system in line with the manufacturer's instructions (Invitrogen, Carlsbad, USA) and previously described methods [9]. In this study, staurosporine was used as a positive control with IC_{50} value of 0.59 nM.

Table 1
^{1}H (600 MHz) and ^{13}C NMR (150 MHz) data of **1**–**3** (δ in ppm, *J* in Hz).

Position	**1**[a]		**2**[b]		**3**[b]	
	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
1	9.78, s					129.5
2		174.1		151.4	7.57, overlap	123.5
3	3.72, t, 8.9	65.7		153.3		149.1
4	2.22, m	47.2				144.6
5	4.70, t, 9.2	64.8		150.3	7.01, d, 8.9	118.4
5a		137.5				
6	7.50, d, 7.5	124.5	8.58, s	138.1	7.57, overlap	118.0
7	7.18, t, 7.3	124.8	2.57, s	22.0		193.0
8	7.22, t, 7.0	127.2	2.53, s	21.2	5.35, s	67.3
9	6.93, d, 7.6	121.3				172.2
9a		134.4				
10					2.16, s	20.4
1′				129.5		128.1
2′			7.47, d, 1.9	114.8	6.85, brs	115.6
3′				146.9		146.6
4′				148.4		147.4
5′			6.86, d, 8.3	116.7	6.76, overlap	116.2
6′			7.35, dd, 8.3, 1.9	119.7	6.76, overlap	120.7
7′					4.76, d, 7.2	78.2
8′					5.81, d, 7.2	78.8
9′						173.3
10′					1.88, s	22.6
3-OH	4.86, brs					
5-OH	5.54, brs					

[a] In DMSO-d_6.
[b] In methanol-d_4.

Table 2
^{1}H (600 MHz) and ^{13}C NMR (150 MHz) data of **4** and **5** (δ in ppm, J in Hz, methanol-d_4).

Position	**4**		**5**	
	δ_H	δ_C	δ_H	δ_C
1		131.7		173.5
2		131.5		
3	6.74, s	117.9	5.52, d, 15.7 5.33, d, 15.7	69.5
3a				140.0
4		143.7		140.2
5		148.7		155.5
6	6.79, s	118.8		129.7
7	2.75, t, 7.1	36.9	7.42, s	124.4
7a				117.3
8	3.63, t, 7.1	64.5	2.27, s	16.3
1′		131.7	5.06, d, 3.7	106.0
2′	7.27, d, 1.9	118.0	3.60, dd, 9.6, 3.7	73.0
3′		146.3	3.84, t-like, 9.4	74.5
4′		152.3	3.34, overlap	71.3
5′	6.81, d, 8.3	115.7	3.96, overlap	75.9
6′	7.15, dd, 8.3, 1.9	125.7	3.95, overlap 3.72,dd, 12.0, 6.3	62.8
7′		199.5		

2.5. Assay for in vitro discoidin domain receptor 1(DDR1) kinase inhibition activity

Compounds **1**–**6** were evaluated for their inhibitory activity towards DDR1 kinase using a LanthaScreen Eu kinase activity assay technology (Invitrogen, USA). Kinase reactions were carried out in a 10 μL volume in 384-well plates. The kinase in reaction buffer includes 50 mM HEPES pH 7.5, 0.01% BRIJ-35, 10 mM MgCl2, and 1 mM EGTA. 100 nM Fluorescein-Poly GAT substrate (Invitrogen, USA) was used in this assay. Kinase reactions started from addition of 100 nM ATP and serials of dilutions of the isolates. The reactions proceeded for 1 h at room temperature before a 10 μL preparation of EDTA (20 mM) and Eu-labeled antibody (4 nM) in TR-FRET dilution buffer were added. The final concentration in this assay well is 2 nM for antibody and 10 mM for EDTA. The plate was incubated at room temperature for one more hour before the TR-FRET emission ratios of 665 nm/340 nm were acquired on a PerkinElmer EnVision multilabel reader (Perkin-Elmer, Inc.). Dasatinib was used as a positive control with IC_{50} value of 2.2 nM. Data analysis and curve fitting were performed using GraphPad Prism4 software.

3. Results and discussion

Compound **1** has the molecular formula $C_{10}H_{11}NO_3$ deduced from analysis of its positive HRESIMS ($[M + Na]^+$, m/z 216.0630, calcd 216.0631), ^{13}C NMR and DEPT spectra, indicating 6 degrees of unsaturation. The ^{1}H NMR spectrum indicates the presence of four aromatic/olefinic protons at δ_H 7.50 (d, J = 7.5 Hz, H-6), 7.18 (t, J = 7.3 Hz, H-7), 7.22 (t, J = 7.0 Hz, H-8) and 6.93 (d, J = 7.6 Hz, H-9), indicating the presence of a 1,2-disubstituted benzene ring. The ^{13}C NMR and DEPT spectra (Table 1) exhibit 10 carbon resonances ascribe to one methylene, six methine (four sp^2 and two sp^3 both oxygenated), and three quaternary carbons (a carbonyl). The structure construction of **1** was performed by 2D NMR experiments (Fig. 2). The ^{1}H-^{1}H COSY spectrum shows correlations of 3-OH (δ_H 4.86)/H-3/H-4/H-5/5-OH (δ_H 5.54). The HMBC correlations of *N*H (δ_H 9.78)/C-2, C-3, C-5a, C-9 and C-9a, H-3, H-4/C-2, H-5/C-6, C-5a and C-9a suggest the presence of a seven-membered lactam ring as shown. With these in hand, the planar structure of **1** was readily determined. There are two chiral centers in **1**, ROESY correlations of H-3/5-OH and H-5/3-OH evidently imply the relative configuration of **1**. To clarify the absolute configuration of **1**, computational methods were utilized. DFT and TD-DFT calculations were carried out at 298 K in the gas phase with Gaussian 09 [10]. Conformational search were completed by using the Conflex 7 software, then optimized at the B3LYP/6-311G(d,p) level in the gas phase by using the Gaussian 09 software package. Based on the result of ECD calculations which were conducted at the B3LYP/6-31G//B3LYP/6-31G level in MeOH (Fig. 3), the absolute configuration of **1** was assigned as 3*R*,5*R*. Therefore, the structure of **1** was established and named as plancyamide A.

The molecular formula of **2** was assigned as $C_{12}H_{12}N_2O_2$ by the positive HRESIMS at m/z 217.0973 $[M + H]^+$ (calcd for 217.0972), ^{13}C NMR and DEPT spectra, having 8 degrees of unsaturation. The ^{1}H NMR spectrum contains an ABX spin system comprised of resonances at δ_H 7.47 (d, J = 1.9 Hz, H-2′), 6.86 (d, J = 8.3 Hz, H-5′) and 7.35 (dd, J = 8.3, 1.9 Hz, H-6′), indicating the presence of a 1,3,4-trisubstituted benzene ring. In addition, there exists a signal at δ_H 8.58 (s, H-6) and two methyl singlets respectively at δ_H 2.57 (s, H-7) and δ_H 2.53 (s, H-8). The ^{13}C NMR and DEPT spectra (Table 1) demonstrate 12 signals ascribe to two methyl, four olefinic methine, and six olefinic quaternary carbons. The HMBC correlations of H-6, H-8/C-5 and ROESY correlation of H-6/H-8 indicate the substructure of C-6–C-5–C-8 (A). HMBC correlations of H-2′, H-6′/C-2 and H-7/C-2, C-3 allow establishing structure fragment

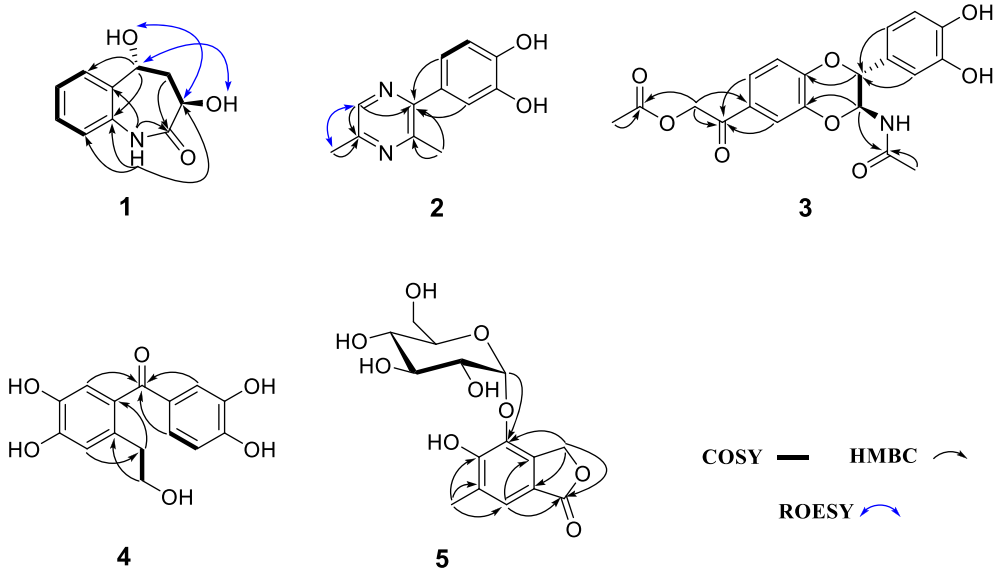

Fig. 2. ^{1}H–^{1}H COSY, Key HMBC and ROESY correlations of **1**–**5**.

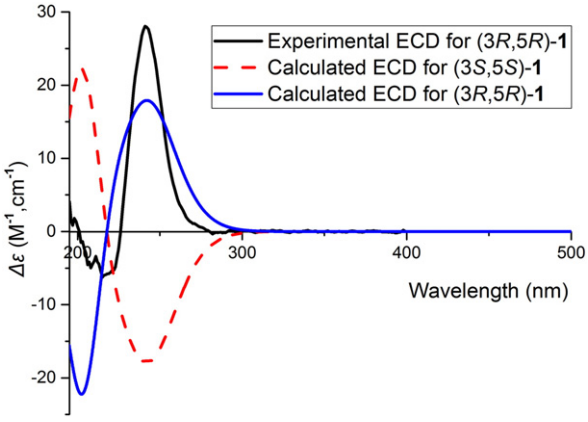

Fig. 3. Calculated and experimental ECD spectra of **1**.

of C-1′–C-2–C-3–C-7 (B). A pivotal HMBC correlation of H-6/C-2 in consideration of the chemical shifts of C-2, C-3, C-5, and C-6 suggest fragments A and B are connected via two *N*-atom bridges, in accordance with the requirement of degrees of unsaturation. Therefore, the structure of **2** was established and named as plancypyrazine A.

The molecular formula of **3** was determined to be $C_{20}H_{19}NO_8$ (12 degrees of unsaturation) deduced from its positive HRESIMS at *m/z* 402.1182 $[M + H]^+$ (calcd for 402.1183), ^{13}C NMR and DEPT spectra. The ^1H NMR spectrum of **3** (Table 1) reveals the presence of two methyl [δ_H 2.16 (s, H-10); δ_H 1.88 (s, H-10′)], and six olefinic protons. The ^{13}C NMR and DEPT spectra (Table 1) give 20 carbon signals classified into two methyl, one oxygenated methylene, eight methine (six olefinic and two oxygenated), and nine quaternary carbons. Inspection of these NMR data discloses that the structure of **3** extremely resembles that of molossusamide A [11], differing in that C-7 of **3** is oxidized into a ketone gaining support from HMBC correlations of H-6, H-8/C-7 (δ_C 193.0). Another difference between **3** and molossusamide A is that the side chain is positioned at C-1 in **3** instead of C-6 in molossusamide A evident from HMBC interactions of H-6, H-7′/C-4. Thus far, the planar structure of **3** was constructed. As far as the relative configuration of **3** is concerned, it is unambiguously assigned as *trans*-form by a coupling constant between H-7′/H-8′ ($J = 7.2$ Hz). The fact that compound **3** is optically inactive. Separation of **3** on chiral HPLC afforded **3a** and **3b**, whose absolute configuration was further determined by ECD comparison with those of previously reported data [12], allowing **3a** to be 7′*R*,8′*S*. Therefore, the structure of **3** was finally deduced and named as plancyamide B.

Compound **4** possesses a molecular formula $C_{15}H_{14}O_6$ as deduced from its negative HRESIMS and NMR data, indicating 9 degrees of unsaturation. The ^1H NMR spectrum of **4** shows an ABX system characteristic of signals at δ_H 7.27 (d, $J = 1.9$ Hz, H-2′), 6.81 (d, $J = 8.3$ Hz, H-5′) and 7.15 (dd, $J = 8.3$, 1.9 Hz, H-6′). Two aromatic/olefinic singlets at δ_H 6.74 (s, H-3) and δ_H 6.79 (s, H-6) are indicative of a 1,2,4,5-tetrasubstituted benzene ring. The ^{13}C NMR and DEPT spectra display 15 carbons, of which twelve are olefinic carbons belonging to two phenyl groups. Besides, the residue signals accounting for two methylene (one oxygenated) and one ketone are also observed. The ^1H-^1H COSY spectrum gives cross peaks of H-7/H-8 (δ_H 3.63), in conjunct with HMBC correlations of H-7/C-2, C-6 and H-8/C-1, and chemical shifts of C-4 (δ_C 143.7), C-5 (δ_C 148.7), revealing the west part of **4**. The east part of **4** is a 1,2,4-trisubstituted benzene with 1,2-dihydroxyl groups supported by the above mentioned an ABX spin system. Two parts are connected via a ketone (C-7′) aided by HMBC correlations of H-2′, H-6′, H-3/C-7′. The structure of **4** was therefore identified and named as plancyol A.

Compound **5** was found to have the molecular formula $C_{15}H_{18}O_9$ (7 degrees of unsaturation) derived by analysis of its negative HREIMS ($[M - H]^-$, *m/z* 341.0877, calcd 341.0878), ^{13}C NMR and DEPT spectra. The ^1H NMR spectrum of **5** shows a signal at δ_H 5.06 (d, $J = 3.7$ Hz) typical for the presence of an anomeric proton. The ^{13}C NMR and DEPT spectra exhibit signals for five oxygenated methine (one ketal at δ_C 106.0) and one oxygenated methylene, strongly supporting the presence of an α-glucose moiety. In addition to this moiety, the ^1H NMR spectrum also shows one aromatic proton at δ_H 7.42 (s, H-7) and one methyl at δ_H 2.27 (s, H-8), and the ^{13}C NMR and DEPT spectra also give nine carbons including six olefinic carbon signals, one methyl, one oxygenated methylene and one carbonyl. To assemble the structure of the aglycone, the data from HMBC experiment were completely analyzed, which shows correlations of H-7 (δ_H 7.42, s)/C-1, C-3a; H-3/C-1, C-3a, C-4, C-7a, indicating the presence of a five-membered lactone ring as shown. In addition, HMBC correlations of H-8/C-5 (δ_C 155.5), C-6, C-7 indicate the substituted pattern in the benzene ring. Finally, HMBC correlation of H-1′ (δ_H 5.06)/C-4 evidently implies the position of glucose moiety at the benzene ring.

There exists a glucose residue in the structure of **5**. Acid hydrolysis (Supplementary data) followed by TLC comparison with the reference compound as well as GC–MS analysis (L-cysteine methyl ester hydrochloride derivative: tR = 22.22 min for glucose residue of **5** and 22.46 min for the reference compound) confirmed the presence of an α-D-glucose, in accordance the observed *J* value of H-1′ (3.7 Hz). Taken together, the structure of **5** was identified and named as plancyol B.

It is notable that, apart from compounds **4** and **5**, all the other compounds are *N*-containing substances. Compound **2** is a pyrazine, similar compounds have been characterized from the defensive spray of *Phyllium westwoodii* [13]. Compounds **3a** and **3b** are *N*-acetyldopamine analogues which were reported to take participation in insect cuticle sclerotization and have been also isolated by us from several insects such as *Aspongopus chinensis* and *Blaps japanensis* [14–16]. As far as plancyol B (**5**) is concerned, it is a phtalide derivative. Such type of compounds was normally found in plants or microorganisms [17,18]. In this circumstance, whether compound **5** is inherent in the insects or from their dietary remains unclear.

Three known compounds were respectively identified as 3-acetamido-5-acetylfuran (**6**) [19], ginsenine (**7**) [20], and anoectochine (**8**) [20] by comparing their NMR data with those in the literatures.

Compounds **1**–**6** were evaluated for their inhibitory activities against JAK3 and DDR1 kinases. It was found that compound **4** exhibits potent inhibitory activities towards these kinases with IC_{50} values of 5.0 μM for JAK3 and 4.87 μM for DDR1. In contrast, compound **2** also exhibits inhibitory activity against JAK3 kinase (IC_{50} value: 12.6 μM), less than that of **4**. In conclusion, these results suggest the potential of compounds **2** and **4** in JAK3 or DDR1 associated disorders.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.fitote.2016.09.005.

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