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A phenanthroindolizidine glycoside with HIF-1 inhibitory activity from *Tylophora atrofolliculata*

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ABSTRACT

A phenanthroindolizidine glycoside 6-O-β-D-glucopyranosyl-tylophorinidine (**1**), the first glycoside of phenanthroindolizidine alkaloid in nature, was isolated from whole plants of *Tylophora atrofolliculata* (Asclepiadaceae). Its structure was elucidated by means of spectral and chemical evidences. Compound **1** showed potent inhibitory effect (IC_{50}: 69 ± 13 nM) on hypoxia induced factor-1 (HIF-1) activation. It was discovered that glucosylation at C-6 of 1 could lead to reduction in cytotoxicity against normal cells and enhance its selectivity in tumor cells inhibition.

1. Introduction

Hypoxia induced factor 1 (HIF-1) is a transcription factor greatly affecting the survival of tumor cells under hypoxia microenvironment (Brown, 1999), which could bring about radiotherapy treatment failure and anticancer drugs resistance (Nagle and Zhou, 2006). HIF-1 inhibition has thus been recognized as an effective way in cancer therapy, raising numerous efforts that identify HIF-1 inhibitors from natural resource. Various natural products have been discovered to exhibit profound HIF-1 inhibitory effects (Nagle and Zhou, 2012; Du et al., 2013).

Tylophora atrofolliculata is a centuries-used folk medicine for the treatment of rheumatism in China (Jiangsu New Medical College, 1977). One group of its bioactive components is phenanthroindolizidine alkaloids, which have been demonstrated as lead compounds of anti-tumor agents in our previous studies (Chen et al., 2016a, b). Our further phytochemical exploration led to the isolation of a phenanthroindolizidine glycoside, 6-O-β-D-glucopyranosyl-tylophorinidine (**1**). Herein, we report the structure elucidation of compound **1** together with its capabilities in inhibition of HIF-1 activation and cancer cells growth.

2. Results and discussion

Compound **1** was obtained as a white amorphous powder. Its molecular formula was determined as $C_{28}H_{33}NO_9$ by molecular ion at *m/z* 528.2203 ($[M+H]^+$, calcd 528.2228). The ^1H NMR and ^{13}C data (Table 1) collectively indicated signals corresponding to one 1,2,5-tri-substituted benzene ring, one 1,2,4,5-tetra-substituted benzene ring, five alcoholic hydroxyl groups, two methoxyl groups, five methylene groups including two nitrogenated and one oxygenated ones, seven methine groups including six oxygenated and one nitrogenated. The anomeric proton [δ_H 5.29 (d, J = 7.8 Hz, 1 H)] and protons of four alcoholic hydroxyl groups [δ_H 5.37 (d, J = 4.8 Hz, 1 H), 5.19 (br, 1 H), 5.13 (d, J = 4.8 Hz, 1 H) and 4.74 (t, J = 5.4 Hz, 1 H)], together with the carbon signals (δ_C 61.4, 70.5, 73.7, 77.4, 77.7 and 100.8) (Table 1) were indicative of a β-glucopyranosyl moiety (Feng et al., 2016), whose absolute configuration was determined to be D- form based on the LC–MS analysis of derivatives of compound **1**'s glucose and standards (D-glucose, L-glucose) (Feng et al., 2016; Wang and Gao, 2013; Zhu et al., 2011). The remaining signals corresponding to the aglycone part suggested the presence of a phenanthroindolizidine moiety (Tanaka et al., 2007). Among them, the proton signals at δ_H 8.21 (d, J = 9.6 Hz, 1 H), 7.21 (dd, J = 2.4, 9.6 Hz, 1 H), 8.00 (d, J = 2.4 Hz, 1 H), 8.30 (s, 1 H) and 7.28 (s, 1 H) collectively suggested the presence of one 1,2,5-tri-substituted benzene and one 1,2,4,5-tetra-substituted benzene rings respectively. The two methoxyl groups were decided to be placed at C-3 and C-7 *via* the HMBC correlations between OCH_3-3 and C-3 and between OCH_3-7 and C-7. The alcoholic hydroxyl group on the aglycone was placed at C-14 through the HMBC correlation between OH-14 and

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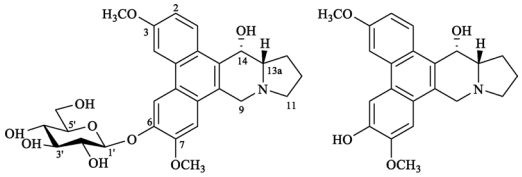
Table 1
NMR Spectroscopic Data (DMSO-d_6) for Compound **1**.

Position	δ_H (*J* in Hz)	δ_C
1	8.21, d (9.6)	126.9
2	7.21, dd (9.6, 2.4)	116.4
3		157.8
4	8.00, d (2.4)	103.9
5	8.30, s	108.7
6		146.9
7		149.7
8	7.28, s	104.6
9	4.60, d (15.6); 3.51, d (15.6)	54.0
11	3.34, m; 2.39, m	55.3
12	1.85, m	22.1
13	2.20, m; 1.86, m	24.4
13a	2.43, m	65.4
14	4.96, dd (1.8, 9.6)	64.0
4a		130.9
4b		123.9
8a		125.6
8b		126.6
14a		130.4
14b		125.3
OCH_3-3	3.98	56.0
OCH_3-7	3.97	55.7
14-OH	4.70, d (9.6)	
1'	5.29, d (7.8)	100.8
2'	3.38[a], m	73.7
3'	3.39[a], m	77.4
4'	3.19, m	70.5
5'	3.62, ddd (1.8, 6.6, 9.6)	77.7
6'	3.78, ddd (1.6, 5.4, 12.0); 3.49, m	61.4
2'-OH	5.37, d (4.8)	
3'-OH	5.19, br	
4'-OH	5.13, d (4.8)	
6'-OH	4.74, t (5.4)	

[a] Overlapped signals.



1 **2**

Fig. 1. Structure of 6-O-*β*-D-glucopyranosyl-tylophorinidine (**1**) and its aglycone tylophorinidine (**2**).

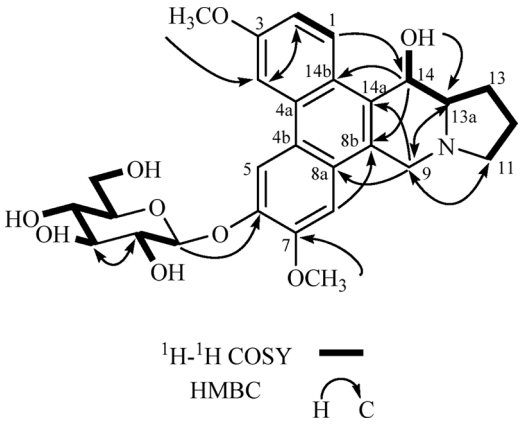


Fig. 2. Key correlations observed in ^{1}H–^{1}H COSY and HMBC (H→C) spectra of **1**.

C-14, as well as ^{1}H-^{1}H COSY correlation between OH-14 and H-14. The structure of **1** resembles that of tylophorinidine (**2**) (Tanaka et al., 2007; Huang et al., 2004) except for an additional glucose which was connected to C-6 by the HMBC correlation between H-1′ and C-6 (Fig. 1). The small coupling constant (J = 1.8 Hz) and strong NOE correlation between H-13a and H-14 led to the *cis* configuration of both hydrogens (Zhen et al., 2002; Stærk et al., 2000). The negative Cotton effect at 271 nm in the CD spectrum indicated the *S* configuration at C-13a (Zhen et al., 2002; Stærk et al., 2000; Damu et al., 2009), resulting in the *S* configuration at C-14 accordingly. Therefore, compound **1** was elucidated as 6-O-*β*-D-glucopyranosyl-tylophorinidine (Fig. 2). To the best of our knowledge, this is the first glucoside of phenanthroindolizidine type alkaloid isolated from nature.

Compound **1** showed potent inhibitory effect on HIF-1 activation induced by hypoxia in low nanomolar scale (IC_{50}: 69 ± 13 nM). The potency was even higher than the positive control digoxin (IC_{50}: 122 ± 3 nM) (Mi et al., 1992). As the aglycone of **1**, compound **2**(7) showed a lower IC_{50} value (5 ± 1 nM) than that of **1**, suggested that glucosylation at C-6 of **1** could decrease HIF-1 inhibitory effects.

Compounds **1** and **2** were further evaluated for their cytotoxic activities against a panel of cancer [human ovary (A2780) and liver (HepG2) carcinoma] and normal [human ovary (Hospeic) and liver (LO2)] cells. As shown in Table 2, compound **1** exhibited weaker cytotoxic effects against both cancer and normal cells than those of **2**. The IC_{50} values of **1** determined on A2780, HepG2, Hospeic and LO2 are 0.41, 4.58, 14.30 and 21.40 μM respectively, which is higher than those of **2** calculated on the four corresponding cell lines (A2780: 0.04 μM; HepG2: 1.49 μM; Hospeic: 0.46 μM; LO2: 2.06 μM). These data indicated that glucosylation at C-6, resulting in higher hydrophilicity, reduce the cytotoxicity against both normal and tumor cells.

The selectivity index (SI) was measured by the proportion of the IC_{50} values of normal cells relative to that of their tumor counterparts. As shown in Table 2, the SI values of compound **1** are significant higher than those of **2** for both A2780 (**1**: 34.9; **2**: 11.5) and HepG2 (**1**: 4.7; **2**: 1.4) cells, suggesting that **1** is more selective in inhibiting both cancer cell lines. Glucosylation strategy has been adopted in several cytotoxic drugs (including ifosfamide and taxol), resulting in lower toxicity and higher cancer cell inhibitory selectivity *in vitro* (Zhang et al., 2008; Calvaresi and Hergenrother, 2013; Pohl et al., 1995; He et al., 2016). Consistent with these previous results, our data suggested glucosylation at C-6 could significantly enhance tumor cell inhibitory selectivity.

3. Conclusion

This investigation led to the isolation of a new phenanthroindolizidine glycoside, named 6-O-*β*-D-glucopyranosyl-tylophorinidine (**1**), with glycosylation at C-6 of the aglycone part. Biological activity evaluation suggested that **1** demonstrated more potent HIF-1 inhibitory effect than digoxin. SAR analyses indicated that glycosylation at C-6 of **1** reduced its cytotoxicity against normal cells, but enhance cancer cell inhibitory selectivity *in vitro*. Above all, this is the first glucoside of phenanthroindolizidine type alkaloid from natural resource. Moreover, our results suggested that 6-O-*β*-D-glucopyranosyl-tylophorinidine (**1**) could be a promising lead compound in the development of anti-cancer drugs.

4. Material and methods

4.1. General experimental procedures

Optical rotations were determined using a Rudolph Research Analytical Autopol I Autometic polarimeter. CD spectra were measured on a Chirascan Circular Dichroism spectrometer. UV data were recorded using a Shimadzu UV-2700 UV/vis spectrophotometer. HRESIMS were recorded on an Agilent 6230 ESI-TOF mass spectrometer. NMR spectral data were obtained from Bruker Ascend™ 600

Table 2
Cytotoxicities of 1 and 2 against both cancer and normal cells.

Compounds	Cytotoxicity (μM)		SI[c]	Cytotoxicity (μM)		SI[c]
	Hospeic[a]	A2780[b]		LO2[a]	HepG2[b]	
1	14.30 ± 1.01	0.41 ± 0.09	34.9	21.40 ± 1.14	4.58 ± 0.47	4.7
2	0.46 ± 0.11	0.04 ± 0.01	11.5	2.06 ± 0.80	1.49 ± 0.31	1.4

Values are means ± SD, where SD = standard deviation; all experiments were independently performed at least three times.
[a] Normal cells, including ovary (Hospeic) and liver (LO2) cells.
[b] Cancer cells, including ovary (A2780) and liver (HepG2) carcinoma cells.
[c] Selectivity index (SI), referring to the IC_{50} value measured for the cytotoxicity of the alkaloids on normal cells divided by that measured for cancer cells.

spectrometer equipped with a cyro platform. Silica gel (Davisil®, 40˜63 μm, Germany) using solvent signals (DMSO-d_6: δ_H 3.41/δ_C 39.9) as references, ODS (Waters, Preparative C18 125 Å, 55˜105 μm, American) were used for column chromatography. Silica gel plates (Merck, DC Kieselgel 60 F_{254}, Germany) were used for TLC analyses. High-performance liquid chromatography (HPLC) was carried out on an Waters 1525–2489 apparatus with a semi-preparative column (Waters, XBridge® Prep C18, 5 μm, 250 × 10 mm, American). Solvents for HPLC separation are HPLC grade. Tylophorinidine (**2**) was isolated in our previous study (Chen et al., 2016b).

4.2. Plant material

Whole plants of *T. atrofolliculata* were collected at Wuping Village, Jingxi, Guangxi Province, China in 2012 and identified by Dr. Zhifeng Zhang (Faculty of Chinese Medicine, Macau University of Science and Technology). A voucher specimen (No. MUST-TA201302) was deposited at State Key Laboratory of Quality Research in Chinese Medicine, Macau University of Science and Technology.

4.3. Extraction and isolation

Whole plants of *T. atrofolliculata* (5.5 kg) were refluxed with MeOH for three times to afford a residue, which was suspended with water then adjusted to pH 1–2 by adding 12 M HCl. After extracted with EtOAc, the acidic aqueous phase was basified with 10% NaOH to pH 9–10, which was then partitioned with $CHCl_3$ and *n*-BuOH successively to obtain $CHCl_3$ extract and *n*-BuOH extract (75 g), respectively. The *n*-BuOH extract (10 g) was chromatographed on silica gel column eluting with $CHCl_3$-MeOH-HCOOH (10:0:0.05–0:10:0.05) to obtain 12 fractions (Fr. 1–12). Fr. 7 was subjected to semi-preparative HPLC (Waters XBridge Prep C_{18} 5 μm, 10 × 250 mm column, 33% ACN/0.1% Et_2N-H_2O, 2 mL/min, UV detection at 254 nm) to obtain compound **1** (2 mg).

6-O-*β*-D-glucopyranosyl-tylophorinidine (Brown, 1999): White powder; [*α*]21 D +129.0 (*c* 0.10, MeOH); UV (MeOH) λ_{max} (log *ε*) 258 (4.60), 286 (4.33), 339 (3.19) nm; ^1H and ^{13}C NMR data, see Table 1; HRESIMS *m/z* 528.2203 $[M + H]^+$ (calcd 528.2228 for $C_{28}H_{34}NO_9$).

4.4. Determination of the absolute configuration of glucose of compound 1

The absolute configuration of glucose in compound **1** was determined according to the reported protocol with minor modification (Feng et al., 2016; Wang and Gao, 2013; Zhu et al., 2011). Specifically, compound **1** (0.50 mg) was dissolved in MeOH (0.1 mL) and then added 1 N HCl (0.1 mL). The mixture was heated at 80 °C for 4 h and then dried up under N_2. The residue was re-dissolved with pyridine (0.1 mL) containing L-cysteine methyl ester hydrochloride (0.5 mg) then heated at 60 °C for 1 h. Subsequently, phenyl isothiocyanate (20 μL) was added to the mixture and heated at 60 °C for another 1 h. 20 μL of the reaction mixture was diluted to 500 μL in MeOH and then analyzed directly by UPLC-Q-TOF-MS [Eclipse XDB-C18 column (3.0 × 150 mm, Agilent), ACN: H_2O = 21 : 79, flow rate at 0.35 mL/min]. Standards D-glucose and L-glucose were processed in the same way. The retention time of glucose derivative from acid hydrolysates of compound **1** is 20.1 min. The retention time of the derivatives of D-glucose and L-glucose is 20.2 and 19.2 min, respectively.

4.5. Biological assay

HIF-1-mediated reporter gene assays in T47D cells were performed according to our previous protocol (Chen et al., 2016b). The cytotoxicity assays were carried out in accordance with previous literature protocol (Lin et al., 2008). Detail of each protocol could be seen in Supporting Information.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.phytol.2019.03.003.

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