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ORIGINAL ARTICLE

Three New Pyridine Alkaloids from *Vinca major* Cultivated in Pakistan

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Abstract Three new pyridine type alkaloids, (−)-vinmajpyridines A–C (**1**–**3**), along with two known alkaloids, have been isolated from the aerial parts of *Vinca major* cultivated in Pakistan. Their structures have been elucidated by means of NMR and HRESIMS spectroscopic data. The new alkaloids were evaluated for their cytotoxicity against glioma initiating cell lines (GITC-3$^{\#}$ and GITC-18$^{\#}$), glioblastoma cell lines (U-87MG and T98G), and lung cancer cell line A-549, but none of them was active at 20 μg/mL concentration.

Keywords *Vinca major* · Apocynaceae · Pyridine alkaloids · Cytotoxicity

1 Introduction

The genus *Vinca* (Apocynaceae), distributed through Europe, Northwest Africa, and South-west Asia, represents a group of species which are rich in indole alkaloids of diverse structural patterns, many of which are of considerable therapeutic value [1–4]. *Vinca major* has been used for centuries as a folk remedy in the treatment of menorrhagia and diabetes, and as an abortifacient and vulnerary [5]. Delphinidin glycosides have been isolated from the flowers of *V. major* [6] while chlorogenic acid, robinin, and flavonol triglycoside are extracted from its leaves [7]. In addition, a number of indole alkaloids are also reported from this plant [8–13]. Previously, we isolated non-alkaloid

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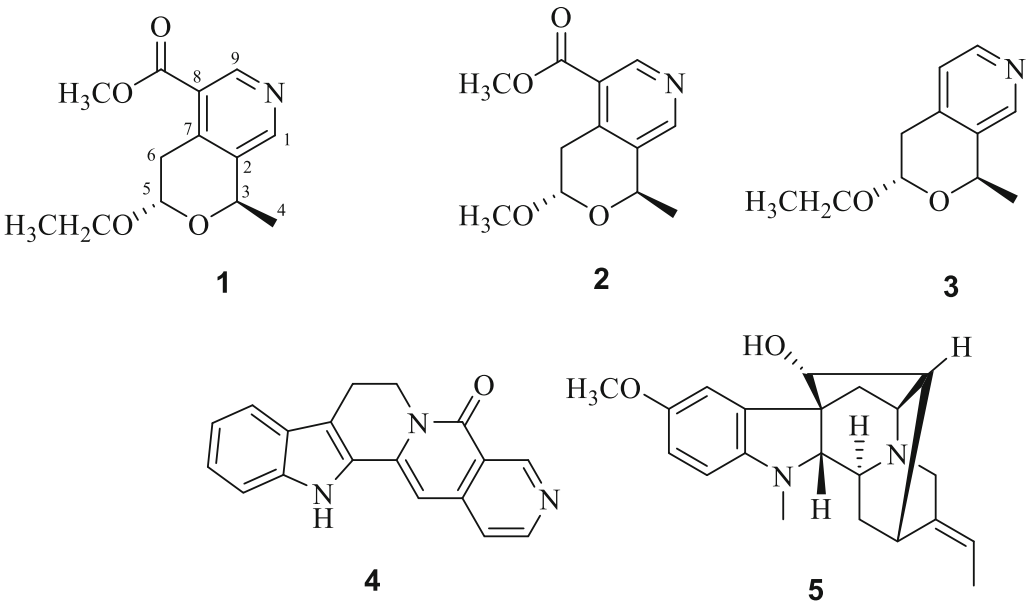

Fig. 1 Structures of compounds **1**–**5**

constituents [14] as well as indole alkaloids [15] from *V. major* cultivated in Kunming. In our ongoing search for alkaloids from this plant growing in different habitats, we carried out the phytochemical investigation of the aerial parts of *V. major* in Pakistan, and then isolated three new pyridine alkaloids, named as (–)-vincapyridines A–C (**1**–**3**) trivially (Fig. 1). The structures of new alkaloids were elucidated by means of spectroscopic methods. The new alkaloids were evaluated for their cytotoxicity against human glioma initiating cell lines (GITC-3$^{\#}$ and GITC-18$^{\#}$), glioblastoma cell lines (U-87MG and T98G), and lung cancer cell line A-549 using the reported MTS assay with DMSO as the control group. Unfortunately, none of these compounds exhibited significant cytotoxicity at 20 μg/mL concentration. Herein, we report the isolation, structural elucidation of these compounds.

2 Results and Discussion

The molecular formula of **1** was determined to be $C_{13}H_{17}NO_4$, by a quasi-molecular ion peak at *m/z* 252.1222 $[M+H]^+$ (calcd for $C_{13}H_{18}NO_4$, 252.1230) in the positive HRESIMS. Its IR spectrum revealed a characteristic absorption band at 1724 (C=O). The ^{1}H and ^{13}C NMR spectral data of **1** (Table 1), assumed that **1** was a tri-substituents pyridine derivative with two typical downfield protons [δ_H 8.89 (s) and 8.53 (s)] assigned to be pyridine aromatic protons [16]. In HMBC spectrum of **1**, correlations of pyridine protons δ_C 8.53 (s) with substituents aromatic carbons at δ_C 146.3 (C-7), 137.6 (C-2), and oxymethine at δ_C 71.3 (C-3), indicated substituents pattern (Fig. 2). Moreover, HMBC correlations of δ_H 4.88 (H-5) with δ_C 71.3 (C-3) and δ_C 146.3 (C-7) established a substituents pyranose ring fused to the pyridine ring (Fig. 2). Besides, HMBC correlations of δ_H 1.64 (3H, d, –CH_3) with δ_C 71.3 (C-3), and of δ_H 3.99 and 3.62 (2H, –O$\underline{CH_2}$$CH_3$) with δ_C 99.6 (C-5), placed a methyl at C-6 and oxyethyl group at C-5, respectively, which was further supported by the correlations of δ_H 3.37/4.88, 3.99/1.24, and 5.02/1.64, in its ^{1}H–^{1}H COSY spectrum (Fig. 2). Finally, the HMBC correlations of δ_H 8.89 (s) and 3.92 (–O$\underline{CH_3}$) with δ_C 167.2 (–$\underline{C}$O$\underline{C}$O$\underline{CH_3}$) positioned the methyl formate group at C-8. In ROESY spectrum of **1**, NOE correlation between δ_H 4.88 (H-5) and 1.64 (H-4) revealed two protons to be co-facial (Fig. 2), which indicated its relative configuration.

The molecular formula of **2** was established as $C_{12}H_{15}NO_4$ by a quasi-molecular ion peak in the HRESIMS at *m/z* 238.1072 $[M+H]^+$ (calcd for $C_{12}H_{16}NO_4$, 238.1074). The ^{1}H and ^{13}C NMR spectral data of **2** were similar to those of **1**, except for a methoxyl group connected to C-5 in **2** instead of the ethoxyl group in **1**, which was consistent with its molecular formula, and further supported by HMBC correlation. Compound **2** shared same relative configurations with **1**, supported by NOE correlation between H-5 and H-4 (Fig. 2).

Its molecular formula of **3** was deduced to be $C_{11}H_{15}NO_2$ on the basis of HRESIMS quasi-molecular ion peak at *m/z* 194.1172 $[M+H]^+$ (calcd for $C_{11}H_{16}NO_2$, 194.1176) and ^{13}C NMR data (Table 1). Signals for methyl formate group at C-8 in the ^{1}H and ^{13}C NMR spectra of **1** were absent in those of **3**,and corresponding δ_H 7.18 (d, 5.2, H-8) and δ_C 125.5 (d, C-8) were appeared in the ^{1}H and ^{13}C NMR spectra of **3**. Other parts of **3** were identical

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

Position	**1**		**2**		**3**	
	δ_H^a	δ_C^b	δ_H^a	δ_C^b	δ_H^a	δ_C^b
1	8.53 (s)	149.8	8.51 (s)	149.5	8.33 (br. s)	146.0
2		137.6		137.5		137.2
3	5.02 (q, 6.4)	71.5	4.98 (q, 6.6)	64.5	4.94 (q, 6.6)	64.7
4	1.64 (d, 6.4)	21.9	1.58 (d, 6.6)	21.4	1.57 (d, 6.6)	21.0
5	4.88 (dd, 3.2, 8.2)	99.6	5.07 (dd, 1.2, 4.6)	97.7	5.17 (dd, 1.4, 4.6)	96.7
6	3.37 (dd, 3.2, 18.2)	35.0	3.35 (dd, 4.6, 18.6)	33.2	3.14 (dd, 4.6, 17.3)	34.3
	3.07 (dd, 8.2, 18.2)		3.21 (br. d, 18.6)		2.75 (d, 17.3)	
7		146.3		144.7		143.5
8		126.9		126.6	7.18 (d, 5.2)	125.5
9	8.89 (s)	150.2	8.88 (s)	150.1	8.29 (br. d, 5.2)	147.8
$-O\underline{CH_2}CH_3$	3.99 (dq, 7.1, 9.4)	65.4			3.85 (dq, 7.1, 9.7)	64.3
	3.62 (dq, 7.1, 9.4)				3.58 (dq, 7.1, 9.7)	
$-OCH_2\underline{CH_3}$	1.24 (t, 7.1)	15.7			1.19 (t, 7.1)	15.5
$-\underline{C}OOCH_3$		167.2		167.3		
$-COO\underline{CH_3}$	3.92 (s)	52.9	3.92 (s)	52.9		
$-OCH_3$			3.44 (s)	55.6		

[a] Recorded at 600 MHz in CD_3OD

[b] Recorded at 150 MHz in CD_3OD

to those of **1**, supported by its HMBC and ROSEY spectra (Fig. 2).

The optical rotation of **1–3** with same negative sign, supposed their same absolute configuration. However, the specific rotation values of **1–3** were obviously different, which indicated that substituents at C-8 and 5 contributed to specific rotation significantly. In compound **1**, to avoid steric hindrance between $-COOCH_3$ and $-OCH_2CH_3$ (Fig. 3), the dihedral angle between H-6 and H-5 were changed, resulting in a large coupling constants ($J = 8.4$ Hz) for H-6/H-5 in its ^{1}H NMR spectrum, which were different from those of two other compounds. Meanwhile, the deformed substituent pyran forward the hemiacetal proton (H-5) to the shield area of pyridine ring (Fig. 3), which caused the up-field chemical shift of H-5 (δ_H 4.88) in **1,** comparison of those in **2** (δ_H 5.07) and **3** (δ_H 5.17). It is the first report of pyridine type alkaloids from genus *Vinca*. Besides, known indole alkaloids nauclefine (**4**) [17, 18] and vincamajoreine (**5**) [19] were also isolated.

3 Experimental Section

3.1 Plant Material

The aerial parts of *V. major* were collected from Oghi, Mansehra, KPK, Pakistan in June, 2015 and identified by one of us (A. Khan). A voucher specimen (No. Khan_20150601) has been deposited in the State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Sciences, China.

3.2 General Experimental Procedures

Optical rotations were performed on a P-1020 polarimeter. IR spectra were measured on a Bruker FT-IR Tensor 27 spectrometer with KBr pellets. UV spectra were obtained on Shimadzu UV-2401A spectrometer. 1D and 2D-NMR spectra were recorded on Bruker AV-600 MHz spectrometer. Coupling constants were expressed in Hertz and chemical shifts were given on a ppm scale with tetramethylsilane as an internal standard. HRESIMS were recorded on an API QSTAR Pulsar 1 spectrometer. Column chromatography (CC) was performed on silica gel (200–300 mesh, Qingdao Marine Chemical Ltd., Qingdao, People's Republic of China), Sephadex LH-20 (Pharmacia Fine Chemical Co., Ltd., Sweden), and MCI-gel CHP 20P (75–100 μm, Mitsubishi Chemical Co., Ltd). Thin-layer chromatography (TLC) was carried out on pre-coated silica gel plates (Qingdao Marine Chemical Co., Ltd.) with $CHCl_3$/MeOH (15:1, 4:1, v/v) as developing solvents and spots were visualized by Dragendorff's reagent. High performance liquid chromatography (HPLC) was performed using Waters 600 pump with semi-preparative C_{18} columns (150 × 9.4).

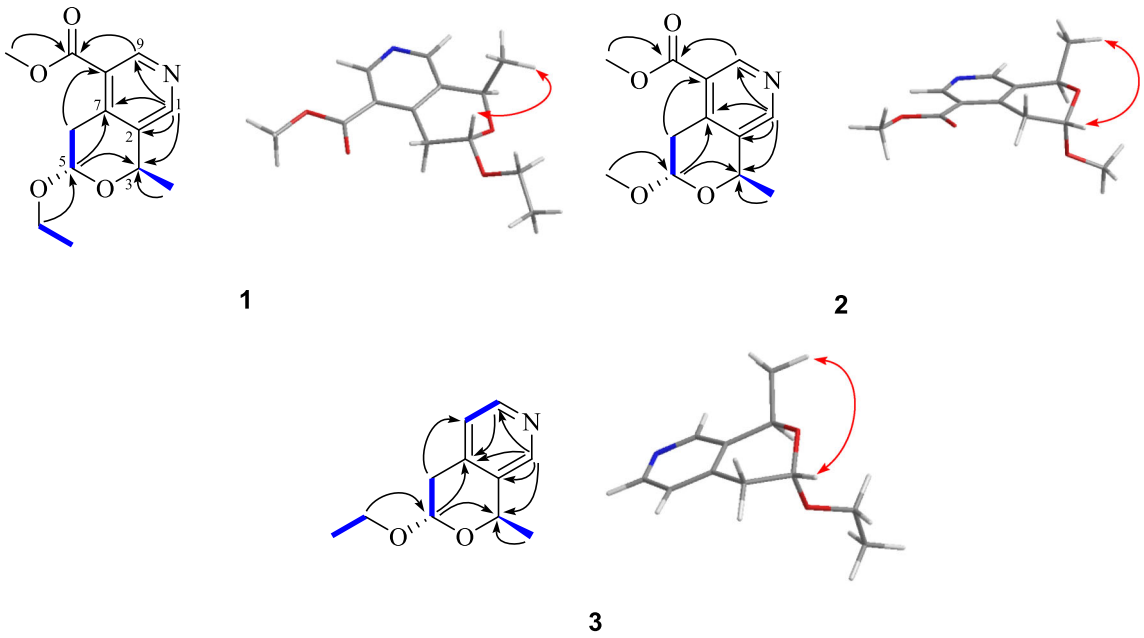

Fig. 2 Selective HMBC (→), ^{1}H–^{1}H COSY (−), and ROESY (↔) correlations for **1**–**3**

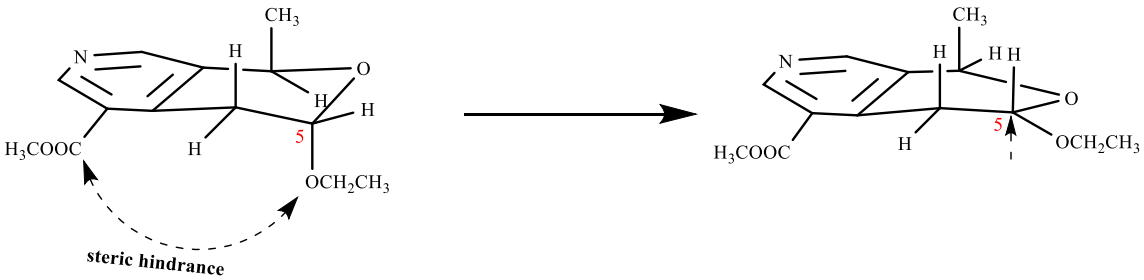

Fig. 3 Conformation change of compound **1** indicated by molecular model

3.3 Extraction and Isolation

The air-dried and powdered aerial parts of *V. major* (8 kg) were extracted with 80% aqueous MeOH (80 L × 3) at room temperature. After removal of the organic solvent under reduced pressure, the residue was dissolved in 0.3% aqueous hydrochloric acid (v/v). The solution was subsequently basified to pH 9–10 using aqueous ammonia, and then extracted with EtOAc (3 L × 4) to give an alkaloidal extract (34.3 g). The extract was applied to a silica gel column ($CHCl_3$/MeOH, 1:0–0:1) to afford nine fractions (Fr. A-I). Fr. B (2.5 g) was subjected to silica gel column chromatography (CC) using a petroleum ether/acetone gradient eluent (10:1–9:1) to afford sub-fractions (Fr. 1–5). Fr. 4 (200 mg) was further purified on MCI-gel CHP 20P column using a MeOH/H_2O gradient eluent (1:4–1:0) and on a semi-preparative C_{18} HPLC column with a gradient of MeOH/H_2O (50:50–90:10) to yield vincamajoreine (**5**) (6 mg). Fr. H (5 g) was subjected to silica gel CC ($CHCl_3$/MeOH, 9:1–0:1) to afford sub-fractions (Fr. 5–10). Fr. 5 (1200 mg) was further separated on MCI-gel CHP 20P column to yield nauclefine (**4**) (15 mg). Fr. 8 (800 mg) was subjected to Sephadex LH-20 CC using MeOH under isocratic conditions and was further separated on a semi-preparative C_{18} HPLC column with a gradient of MeOH/H_2O (30:70–70:30) to produce (−)-vincapyridine A (**1**) (3 mg), (−)-vincapyridine B (**2**) (2 mg), and (−)-vincapyridine C (**3**) (1.2 mg).

(−)-vincapyridine A (**1**): $C_{13}H_{17}NO_4$. white amorphous powder; $[\alpha]_D^{26}$ −26.6 (*c* 0.07, MeOH); UV (MeOH) λ_{max} (log ε): 203 (4.22), 220 (3.92), 270 (3.38); IR (KBr) ν_{max} 3418, 2929, 1724, 1572, 1381, 1295, 1079 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 1; HRESIMS *m/z* 252.1222 $[M+H]^+$ (calcd for $C_{13}H_{18}NO_4$, 252.1230).

(−)-vincapyridine B (**2**): $C_{12}H_{15}NO_4$. white amorphous powder; $[\alpha]_D^{26}$ −190.0 (*c* 0.04, MeOH); UV (MeOH) λ_{max}

(log ε): 204 (4.44), 219 (4.13), 269 (3.57); IR (KBr) ν_{max} 3419, 2926, 1724, 1606, 1383, 1042 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 1; HRESIMS *m/z* 238.1072 $[M+H]^+$ (calcd for $C_{12}H_{16}NO_4$, 238.1074).

(−)-vincapyridine C (**3**): $C_{11}H_{15}NO_2$. white amorphous powder; $[\alpha]_D^{26}$ −359.8 (*c* 0.02, MeOH); UV (MeOH) λ_{max} (log ε): 204 (3.35), 279 (2.64); IR (KBr) ν_{max} 3425, 2926, 1720, 1623, 1383, 1035 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 1; HRESIMS *m/z* 194.1172 $[M+H]^+$ (calcd for $C_{11}H_{16}NO_2$, 194.1176).

3.4 Cytotoxicity Assay

GITC-3# and GITC-18# (glioma initiating cell lines) were established previously in Kunming Institute of Zoology from three different human glioblastoma multiform samples. These cell lines were cultured in serum-free medium supplemented with 1XB27 (Life 12587-010), 50 ng/mL EGF (PeproTech AF-100-15), and bFGF (PeproTech AF-100-18B). GITCs were cultured in laminin (Gibco 1725712) pre-mdish. The cells could adhere and normally grow without differentiation. Culture dishes were pre-coated with laminin for 4-6 h at 10 mg/mL concentration. The T98G, U-87MG, and A549 cell lines were purchased from the American Type Culture Collection (ATCC), the cells were cultured in DMEM basic medium supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin in a humidified incubator at 37 °C and an atmosphere of 5% CO_2. Cells were digested with TryplE express (Gibco 12604-021) for 3–5 min at 37 °C in cell incubator and centrifuged at 1000 rpm/min for 3 min.

Cell viability analysis was performed by MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, Promega #G3581] assay [20, 21]. The cells were digested and seeded on a 96-well plate with 20000 cells/well. Each tumor cell line was exposed to the test compound dissolved in DMSO at concentrations of 20 μg/mL and kept in cell incubator for 72 h. MTS reagent was diluted 1:5 with fresh medium and mixed well. The old medium was removed and subsequently the fresh medium was added with 100 μL/well. The cells were incubated for 1 h. Absorbance was measured by Hybrid Reader (BioTek Synergy H1) at 490 nm. The cell viability was evaluated by percentage compared with DMSO as a control group.

4 Supplementary Information

1D and 2D NMR spectra, HRESIMS, and UV spectra of compounds **1–3** are available as Supplementary Information.

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Compliance with Ethical Standards

Conflict of interest The authors declare no conflict of interest.

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