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•Original article•

β-Carboline alkaloids from the roots of *Peganum harmala* L.

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[ABSTRACT] This study reports the isolation of four new *β*-carboline alkaloids (**1**−**4**) and six previously identified alkaloids (**5**−**10**) from the roots of *Peganum harmala* L. Among these compounds, **1** and **2** were characterized as rare *β*-carboline-quinazoline dimers exhibiting axial chirality. Compound **3** possessed a unique 6/5/6/7 tetracyclic ring system with an azepine ring, and compound **4** was a novel annomontine *β*-carboline. The structures of these compounds were elucidated by spectroscopic data and quantum mechanical calculations. The biosynthetic pathways of **1**−**3** were proposed. Additionally, the cytotoxicity of some isolates against four cancer cell lines (HL-60, A549, MDA-MB-231, and DU145) was evaluated. Notably, compound **4** exhibited significant cytotoxicity against HL-60, A549, and DU145 cells with IC_{50} values of 12.39, 12.80, and 30.65 μmol·L^{-1}, respectively. Furthermore, compound **2** demonstrated selective cytotoxicity against HL-60 cells with an IC_{50} value of 17.32 μmol·L^{-1}.

[KEY WORDS] *β*-Carboline alkaloids; *Peganum harmala*; Structure elucidation; Cytotoxic activity
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Introduction

β-Carboline alkaloids have been the focus of research in organic chemistry, medicinal chemistry, and pharmacology due to their distinctive structures and fascinating bioactivities [1-7]. *Peganum harmala* L. (*P. harmala* L.), belonging to the family Zygophyllaceae, is a perennial herb predominantly found in the semiarid regions of northern and northwestern China. Traditionally, it has been used for treating various conditions such as cancer, rheumatoid arthritis, asthma, swelling pain, and other ailments [8]. Recent phytochemical studies have identified numerous *β*-carboline alkaloids featuring unprecedented scaffolds and potent bioactivities in the seeds and roots of *P. harmala* L. [5, 6, 9-13]. In this study, the chemical constituents of the roots of the plant were further investigated, resulting in the discovery of four previously undescribed *β*-carboline alkaloids, namely pegaharmols C−E and 7-hydroxyannomontine (**1**−**4**), as well as six known alkaloids (**5**−**10**) (Fig. 1). Compounds **1** and **2** characterized as a *β*-carboline-quinazoline hybrid with an unusual chiral axis connecting the sp^2 C-8 of *β*-carboline with the sp^2 C-4 of quinazoline. Compound **3** represented a unique tetracyclic *β*-carboline alkaloid with a typical tricyclic *β*-carboline fused with an azepine ring. Compound **4** was a new annomontine *β*-carboline. Herein, the isolation, structural identification, plausible biosynthetic pathways, and cytotoxic activities of these strains were reported.

Results and Discussion

Pegaharmol C (**1**), a pale-yellow powder, possessed a molecular formula $C_{20}H_{14}N_4O$, implying 16 degrees of unsaturation, as determined by high-resolution electrospray ionization mass spectrometry (HR-ESI-MS) at *m/z* 327.1252 [M + H]$^+$ (Calcd. for $C_{20}H_{15}N_4O$, 327.1240). ^{1}H NMR data (Table 1) revealed characteristic signals corresponding to an *ortho*-tetrasubstituted benzene ring [δ_H 8.17 (d, J = 8.5 Hz, H-5) and 6.99 (d, J = 8.5 Hz, H-6)], a pair of *ortho*-coupled olefin-

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These authors have no conflict of interest to declare.

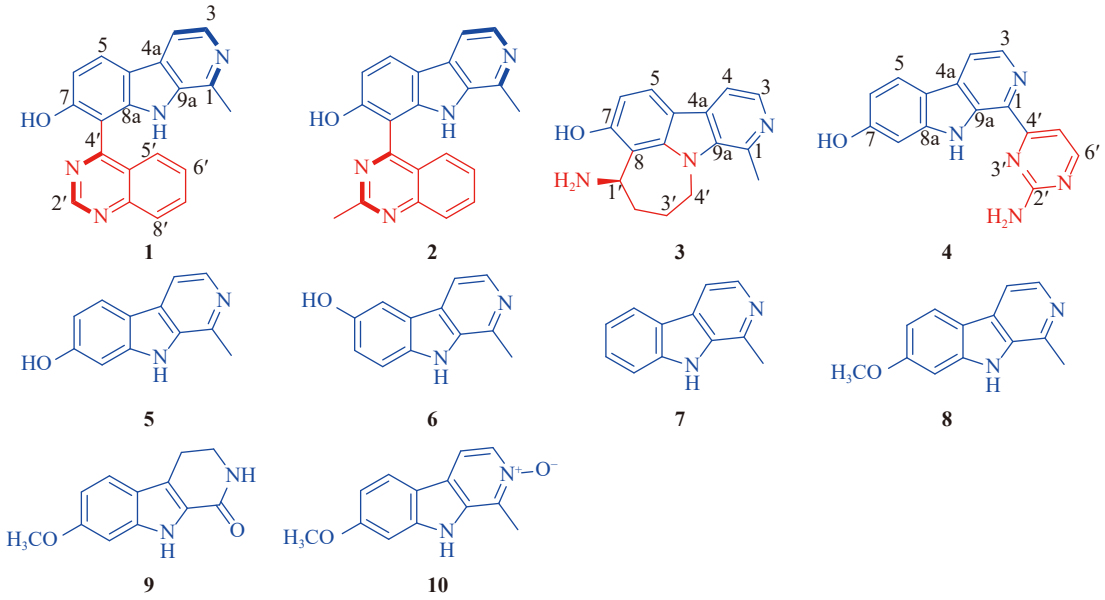


Fig. 1 Structures of compounds 1−10.

ic hydrogens [δ_H 8.15 (d, J = 5.4 Hz, H-3) and 7.84 (d, J = 5.4 Hz, H-4)], an *ortho*-disubstituted benzene ring [δ_H 8.13 (d, J = 8.5 Hz, H-8'), 8.02 (m, H-7'), 7.72 (d, J = 8.5 Hz, H-5'), and 7.64 (m, H-6')], and an NH unit [δ_H 10.60 (br s, NH-9)]. ^{13}C NMR data (Table 1) revealed 20 resonances assigned to a 1,7,8-trisubstituted β-carboline structure [14] with 7-hydroxy and 1-methyl groups [δ_C 155.2 (C-7), 141.4 (C-1), 140.5 (C-8a), 138.0 (C-3), 134.6 (C-9a), 127.7 (C-4a), 123.6 (C-5), 114.5 (C-4b), 111.7 (C-4), 109.5 (C-6), 107.1 (C-8), and 20.6 (1-CH_3)] and a 4-substituted quinazoline fragment [δ_C 165.1 (C-4'), 155.2 (C-2'), 150.0 (C-8'a), 134.1 (C-7'), 128.1 (C-8'), 127.7 (C-6'), 127.5 (C-5'), and 124.7 (C-4'a)]. The correlation spectroscopy (COSY), heteronuclear single quantum coherence spectroscopy (HSQC), and heteronuclear multiple bond correlation (HMBC) data (Fig. 2) confirmed the structures of the two proposed parts, implying that the sole linkage of the carboline and quinazoline sections occurred through the C-8/C-4' single bond. Additionally, the planar structure of **1** was elucidated through the molecular formula deduced by HR-ESI-MS.

The structure of **1**, with substantial substituent groups flanking the C-8/C-4' single bond, likely exhibited axial chirality. The atropisomerism of **1** was verified by an energy scan as a function of the dihedral angle C_7-C_8-$C_{4'}$-$N_{3'}$. This scan, conducted at the B3LYP/6-31G(d) level, exhibited two energy minima and an energy barrier to rotation above about 20 kcal·mol^{-1} [15] and a specific optical rotation $[\alpha]_D^{20}$ of −24.0° (c 0.3, MeOH). The absolute configuration of **1** was determined to be (*M*)-**1** based on a comparison between the experimental and calculated electronic circular dichroism (ECD) data (Fig. 3) at the B3LYP/6-31G + (d, p)//B3LYP/6-31G(d, p) level.

Pegaharmol D (**2**) was obtained as a pale yellow powder. Its molecular formula, $C_{21}H_{16}N_4O$, was assigned by a pseudomolecular ion peak at m/z 341.1399 $[M + H]^+$ (Calcd. for $C_{21}H_{17}N_4O$, 341.1397) observed in HR-ESI-MS, unveiling 16 degrees of unsaturation. A comparison of ^{1}H NMR and ^{13}C NMR data (Table 1) of **2** with those of **1** revealed high similarities. However, **2** exhibited an additional methyl signal [δ_H 2.84 (3H, s, 2'-CH_3), δ_C 26.3] and lacked a hydrogen signal present in **1**. These observations led to the conclusion that **2** shared the same framework as **1**, with the notable exception that the H-2' in **1** was displaced by a methyl group, which was further confirmed by HSQC, HMBC, and COSY data (Fig. 2) of **2**. Biogenetically, like **1**, **2** exhibited axial chirality and showed the same levorotation behavior with a specific optical rotation $[\alpha]_D^{20}$ of −18.0° (c 0.3, MeOH). Consequently, the absolute configuration of **2** was identified as (*M*)-**2**.

Pegaharmol E (**3**) was isolated as a white solid with a specific optical rotation $[\alpha]_D^{20}$ of −29.0° (c 0.1, MeOH). Its molecular formula, $C_{16}H_{17}N_3O$, requiring ten degrees of unsaturation, was established by HR-ESI-MS [m/z 268.1446 $[M + H]^+$ (Calcd. for $C_{16}H_{18}N_3O$, 268.1450)]. ^{1}H NMR spectra (Table 1) revealed a pair of *ortho*-coupled olefinic hydrogens [δ_H 8.09 (d, J = 5.4 Hz, H-3) and 7.70 (d, J = 5.4 Hz, H-4)], an *ortho*-tetrasubstituted benzene ring [δ_H 7.79 (d, J = 8.5 Hz, H-5) and 6.55 (d, J = 8.5 Hz, H-6)], a methine group [δ_H 4.90 (dd, J = 9.4, 7.4 Hz, H-1')], three methylene units [δ_H 3.06 (t, J = 7.4 Hz, H-4'), δ_H 2.50 (m, H-2'), δ_H 1.96−1.83 (m, H-3'), and 1.54 (m, H-2')], together with a methyl unit [δ_H 2.74 (s, 1-CH_3)]. ^{13}C NMR spectroscopic data (Table 1) revealed 16 carbons, comprising 11 sp^2 carbons and 5 sp^3 carbons, ascribed to a 1,7,8,9-tetrasubstituted β-carboline skeleton [14] with 7-hydroxy and 1-methyl groups [δ_C 159.3 (C-7), 141.1 (C-1), 139.8 (C-8a), 137.7 (C-3), 134.4 (C-9a), 127.9 (C-4a), 120.4 (C-5), 113.2 (C-4b), 111.3 (C-4), 111.2 (C-6), 108.3 (C-8), and 20.7 (1-CH_3)]. Moreover, HMBC and COSY correlations (Fig. 2) confirmed the proposed tetrasubstituted β-carboline structure. With the assistance of the COSY spectrum (Fig. 2), correlations from δ_H 4.90 (H-1') to

Table 1 ^{1}H (600 MHz) and ^{13}C NMR (150 MHz) data of **1**−**4** in DMSO-d_6.

No.	1		2		3		4	
	δ_H mult, *J* in Hz	δ_C	δ_H mult, *J* in Hz	δ_C	δ_H mult, *J* in Hz	δ_C	δ_H mult, *J* in Hz	δ_C
1		141.4		141.4		141.1		135.2
3	8.15 d, 5.4	138.0	8.15 d, 5.4	138.0	8.09 d, 5.4	137.7	8.40 d, 4.9	138.0
4	7.84 d, 5.4	111.7	7.84 d, 5.4	111.6	7.70 d, 5.4	111.3	8.09 d, 4.9	115.6
4a		127.7		127.4		127.9		130.9
4b		114.5		114.4		113.2		112.8
5	8.17 d, 8.5	123.6	8.16 d, 8.5	123.4	7.79 d, 8.5	120.4	8.05 d, 8.5	122.8
6	6.99 d, 8.5	109.5	6.98 d, 8.5	109.5	6.55 d, 8.5	111.2	6.77 dd, 8.5, 2.1	110.3
7		155.2		155.4		159.3		159.3
8		107.1		107.2		108.3	7.12 d, 2.1	97.3
8a		140.5		140.5		139.8		142.9
9	10.60 br s		10.61 br s				11.61 br s	
9a		134.6		134.5		134.4		134.5
1-CH_3	2.56 s	20.6	2.56 s	20.6	2.74 s	20.7		
7-OH							10.22 br s	
1'					4.90 dd, 9.4, 7.4	57.2		
2'	9.44 s	155.2		163.8	2.50 m, 1.54 m	32.6		164.7
3′					1.96−1.83 m	25.3		
4'		165.1		165.1	3.06 t, 7.4	44.8		163.4
4'a		124.7		122.6				
5′	7.72 d, 8.5	127.5	7.64 d, 8.0	127.1			7.65 d, 5.1	105.5
6'	7.64 m	127.7	7.54 m	126.7			8.41 d, 5.1	158.9
7'	8.02 m	134.1	7.95 m	133.8				
8′	8.13 d, 8.5	128.1	8.02 d, 8.3	127.5				
8'a		150.0		150.4				
2'-CH_3			2.84 s	26.3				

δ_H 1.54 (H-2'), from δ_H 1.54 (H-2') to δ_H 1.96−1.83 (H-3'), and from δ_H 1.96−1.83 (H-3') to δ_H 3.06 (H-4') suggested the presence of the H-1'/H-2'/H-3'/H-4' spin-spin system. Key HMBC correlations (Fig. 2) from δ_H 4.90 (H-1') to δ_C 159.3 (C-7), 139.8 (C-8a), and 108.3 (C-8) indicated the connection between C-1' and C-8. Hitherto, nine degrees of unsaturation were accounted for in the introduced structure, and the remaining one could be assigned, corresponding to an additional cyclic structure. The absence of hydrogen at the N-9 position, as indicated by the ^{1}H NMR data, suggested the formation of a C-4'/N-9 bond. An NH_2 motif was inferred to be attached to C-1' by virtue of the chemical shifts δ_H 4.90 and δ_C 57.2 (C-1') and the molecular formula. Thus, the planar structure of **3** was determined. To determine the absolute configuration of **3**, we calculated the ECD spectra of two possible stereoisomers of (*R*)-**3a** and (*S*)-**3b** at the B3LYP/6-311G + (2d, p)//B3LYP/6-31G(d) level. The experimental ECD spectrum of **3** closely matched the calculated spectrum of **3a** (Fig. 3). Therefore, the absolute configuration of **3** was recognized as (*R*)-**3**.

7-Hydroxyannomontine (**4**), an orange powder, had a molecular formula of $C_{15}H_{11}N_5O$, indicating 13 degrees of unsaturation, as determined by the HR-ESI-MS spectrum at *m/z* 278.1014 $[M + H]^+$ (Calcd. for $C_{15}H_{12}N_5O$, 278.1036). ^{1}H NMR data (Table 1) of **4** displayed two pairs of *ortho*-coupled olefinic hydrogens [δ_H 8.41 (d, *J* = 5.1 Hz, H-6') and 7.65 (d, *J* = 5.1 Hz, H-5'), and δ_H 8.40 (d, *J* = 4.9 Hz, H-3) and 8.09 (d, *J* = 4.9 Hz, H-4)], an aromatic AMX spin system [δ_H 8.05 (d, *J* = 8.5 Hz, H-5), 7.12 (d, *J* = 2.1 Hz, H-8), and 6.77 (dd, *J* = 8.5, 2.1 Hz, H-6)], a NH unit [δ_H 11.61 (br s, NH-9)], and a phenolic hydroxy group [δ_H 10.22 (br s, 7-OH)]. ^{13}C NMR data (Table 1) of **4** showed 15 sp^2 resonances, which were attributed to a 1,7-disubstituted *β*-carboline framework [14] with a hydroxy group at C-7 [δ_C 159.3 (C-7), 142.9 (C-8a), 138.0 (C-3), 135.2 (C-1), 134.5 (C-9a), 130.9 (C-4a), 122.8 (C-5), 115.6 (C-4), 112.8 (C-4b), 110.3 (C-6), and 97.3 (C-8)], and a 2-aminopyrimidine fragment [δ_C 164.7 (C-2'), 163.4 (C-4'), 158.9 (C-6'), 105.5 (C-5')]. HMBC and COSY correlations (Fig. 2) confirmed the proposed 1,7-disubstituted *β*-carboline and 2-aminopyrimidine structures. Additional HMBC cross-peaks from δ_H 8.40 (H-3) to δ_C 163.4 (C-4') and from δ_H 7.65 (H-5') to δ_C 135.2 (C-1)

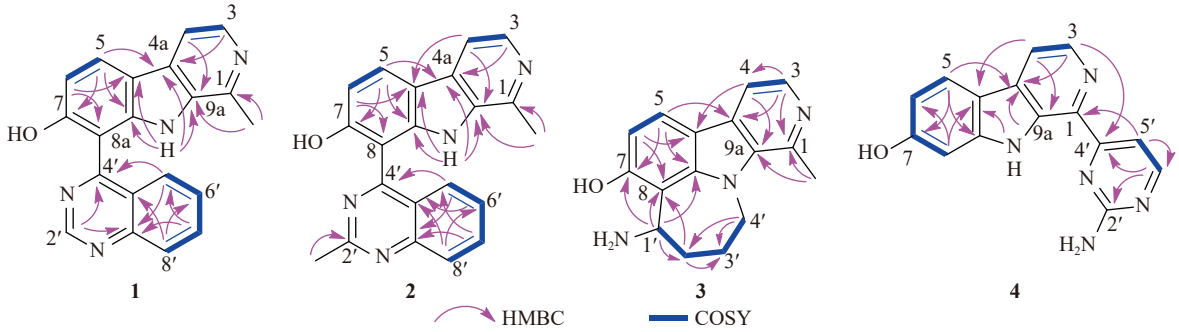


Fig. 2 Key COSY and HMBC interactions of 1−4.

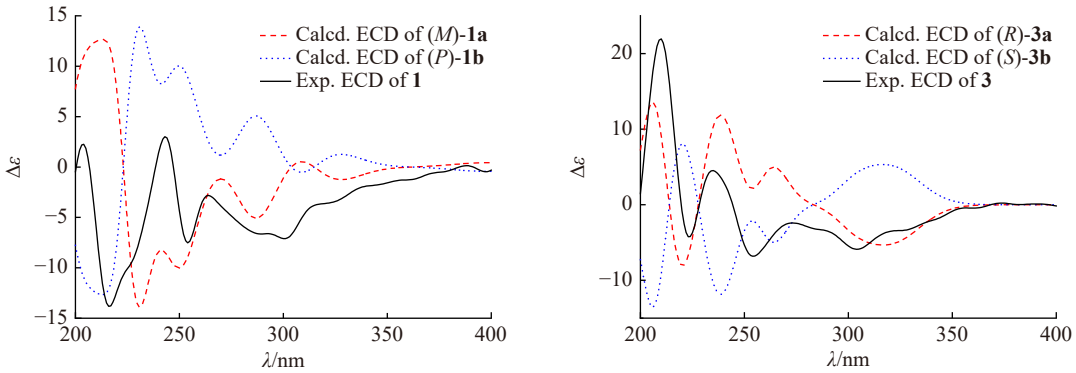


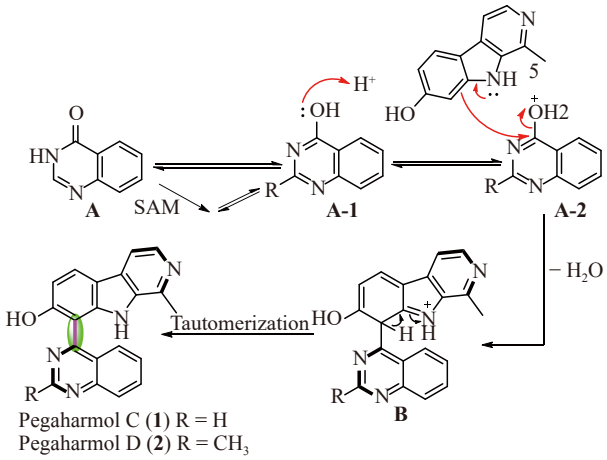
Fig. 3 Calculated and experimental ECD spectra of 1 and 3.

implied the connection between C-4' and C-1. Therefore, the structure of **4** was determined (Fig. 1).

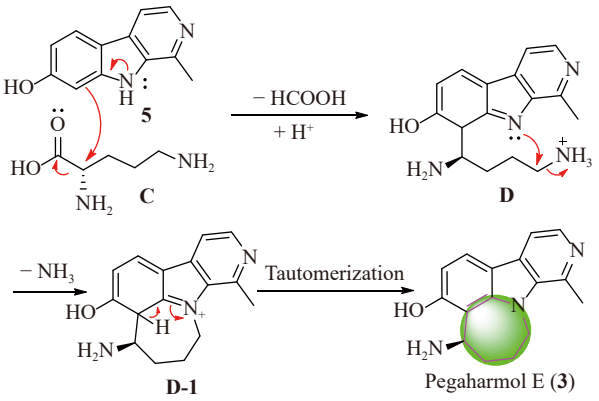
The identification of six known *β*-carboline alkaloids–harmol (**5**) [16, 17], 6-hydroxyharman (**6**) [18], harmane (**7**) [19], harmine (**8**) [14, 20], harmalacidine (**9**) [21], and harmine *N*-oxide (**10**) [22]–was achieved individually by comparing their spectroscopic data with documented ones in the literature.

1−**3** represent three rare alkaloids with unusual architectures. **1**−**2** are novel *β*-carboline-quinazoline dimers coupled by the Csp^2-8 of *β*-carbolines and the Csp^2-4 of quinazolines, featuring axial chirality between two aryl units. This structural motif is distinct from that of pegaharmols A−B [6], which are connected by the Csp^2-8 of *β*-carbolines and the Csp^3-4 of quinazolines and possess axial chirality between an aryl unit and a nonaryl unit, suggesting that the two classes of alkaloids may follow different biosynthetic pathways. **3** is a unique *β*-carboline natural product characterized by a classic tricyclic *β*-carboline core fused with an additional azepine ring. Herein, the proposed biosynthesis pathways for **1**−**3** are as follows: For **1** and **2** (Scheme 1), quinazoline-4(3*H*)-one (**A**) and harmol (**5**) isolated from *Peganum harmala* were defined as biogenetic precursors. **A** directly tautomerized to intermediate **A-1** or underwent methylation first, followed by tautomerization to compound **A-1**. Intermediate **A-1**, upon protonation, formed **A-2** under acidic conditions. A nucleophilic substitution between intermediate **A-2** and harmol (**5**) yielded intermediate **B**, which lost water. Finally, through a tautomerization process, pegaharmols C−D (**1**−**2**) were obtained. With respect to **3** (Scheme 2), harmol (**5**) and *L*-ornithine (**C**) were recognized as biogenetic precursors. A decarboxylic reaction and subsequent protonation of free amino groups could produce intermediate **D**. Afterwards, an intramolecular nucleophilic substitution reaction, followed by the release of ammonia, led to the formation of intermediate **D-1**, which then tautomerized to yield pegaharmol E (**3**).

Compounds **1**, **2**, **4**, **5**, **7**, and **8** were tested for their cytotoxic activities against four human cancer cell lines: HL-60, A549, MDA-MB-231, and DU145 (Table 2). Remarkably, **2** exhibited significantly enhanced cytotoxic activities against all tested cell lines compared with **1**, indicating that the



Scheme 1 Hypothetical biosynthetic pathway for pegaharmols C−D (1−2).



Scheme 2 Hypothetical biosynthetic pathway for pegaharmol E (3).

methyl group at C-2' of the skeleton is crucial for cytotoxicity. Additionally, **2** displayed selective effects on HL-60 cells, with an IC_{50} value of 17.32 μmol·L^{-1}. **4** showed potent cytotoxic activities against HL-60 and A549 cells, with IC_{50} values of 12.39 and 12.80 μmol·L^{-1}, respectively. The other compounds exhibited either moderate or weak activities against these cell lines. **2**, **4**, and **8** showed mild cytotoxic activities against MDA-MB-231 cells, with IC_{50} values of 43.98, 59.30, and 45.60 μmol·L^{-1}, separately, while the remaining isolates displayed no activities (IC_{50} > 100 μmol·L^{-1}). **4** and **8** showed moderate cytotoxic activities against DU145 cells, with IC_{50} values of 30.65 and 34.66 μmol·L^{-1}, respectively.

In summary, two novel axially chiral alkaloids (**1**−**2**), a unique fused tetracyclic *β*-carboline alkaloid (**3**), a new annomontine alkaloid (**4**), and six known alkaloids (**5**−**10**) were isolated from the roots of *P. harmala* L.. Their structures were elucidated through comprehensive spectroscopic analysis and theoretical calculations. The biosynthetic pathways for the novel skeletons (**1**−**3**) were hypothesized, providing valuable insights for organic chemists. Among these isolates, a subset was subjected to cytotoxicity evaluations. Notably, compounds **2**, **4** and **8** demonstrated potent or moderate cytotoxic activities, suggesting their potential as lead compounds in antitumor drug development.

Experimental

General experimental procedures

1D and 2D NMR spectra were obtained using Bruker AV-600 NMR spectrometers (Bruker BioSpin Group, Faellanden, Switzerland) using TMS as an internal standard. Mass spectra were recorded on a Varian QFT-ESI (Varian, Palo Alto, USA) and a Bruker micro-TOF-Q mass spectrometer (Bruker, Karlsruhe, Germany) for HR-ESI-MS. Optical rotations were measured using a Jasco P-2000 polarimeter (Jasco International Co., Ltd., Tokyo, Japan). UV spectra were obtained using a Shimadzu UV-2201 spectrometer (Shimadzu Corporation, Tokyo, Japan), and ECD spectra were measured on a Bio-logic MOS 450 spectropolarimeter (Bio-logic Corporation, Seyssinet-Pariset, France). Column chromatography (CC) was performed using silica gel (Qingdao Marine Chemical Co., Ltd., Qingdao, China), ODS (50 μm, YMC Co., Ltd., Kyoto, Japan), Sephadex LH-20 (GE Healthcare, Sweden), and microporous resin D101 (Shandong Yuwang reagent Co., Ltd., Dezhou, China). Preparative thin layer chromatography (TLC) was conducted using glass plates precoated with silica gel (GF_{254}, Qingdao Marine Chemical Co., Ltd., Qingdao, China). Preparative HPLC was performed on a YMC-Triart C_{18} column (250 mm × 10 mm, 5 μm, YMC Co., Ltd., Kyoto, Japan) equipped with an LC-6AD pump and a Shimadzu SPD-20A UV-Vis detector (Shimadzu Co., Ltd., Japan).

Plant materials

Air-dried roots of *P. harmala* L. were harvested in September 2015 from Anjihai Town, Shawan County, Xinjiang Uygur Autonomous Region, China, and were authenticated by Prof. LU Jincai of the School of Traditional Chinese Medicine, Shenyang Pharmaceutical University, Shenyang, China. A voucher sample (No. PH201509) was deposited in the Department of Natural Products Chemistry, Shenyang Pharmaceutical University.

Extraction and isolation

The roots of *P. harmala* L. (13.0 kg) were extracted with

Table 2 *In vitro* cytotoxic activities (IC_{50} ± SD[a], μmol·L^{-1}) against HL-60, A549, MDA-MB-231, and DU145 cell lines (means ± SD, *n* = 3).

Compounds	HL-60	A549	MDA-MB-231	DU145
1	34.80[b]	53.67 ± 7.27	> 100	> 100
2	17.32[b]	41.90 ± 1.26	43.98 ± 7.94	40.96 ± 0.64
4	12.39[b]	12.80 ± 0.35	59.30 ± 2.90	30.65 ± 0.86
5	> 40	66.52 ± 5.10	> 100	97.18[b]
7	/	64.25 ± 4.53	> 100	93.25[b]
8	/	29.08 ± 4.32	45.60 ± 4.05	34.66 ± 1.19
Etoposide[c]	/	7.25 ± 0.02	3.90 ± 0.35	1.64 ± 0.34
5-FU[c]	1.84 ± 0.29	2.05 ± 0.11	/	/

[a] IC_{50} is the concentration that inhibited 50% of cell growth; [b] The small amount of compound could not test many times; [c] Positive control.

95% ethanol (3 × 2 h × 100 L) under reflux *via* a water bath on the induction cooker. The ethanol extract was concentrated in a vacuum to obtain the concentrated solution (13 L) without ethanol. This solution was acidified to pH 2 using a 5% HCl solution. The acidic mixture was partitioned with CH_2Cl_2 (4 × 13 L) to obtain CH_2Cl_2-soluble fraction A (200.0 g). The aqueous portion was then basified to pH 9 using a 3 mol·L^{-1} NaOH solution and extracted with CH_2Cl_2 (4 × 13 L) to obtain CH_2Cl_2-soluble fraction B (90.0 g). The CH_2Cl_2-soluble fraction B was crystallized to obtain **8** (80.0 mg), and the mother liquor was subjected to liquid CC over silica gel using a CH_2Cl_2–MeOH gradient (100 ∶ 0→0 ∶ 100, *V*/*V*) to yield ten fractions (Frs. A–J).

Fr. F (26.0 g) was further separated by octadecylsilane (ODS) CC using a MeOH–H_2O gradient (20 ∶ 80→100 ∶ 0, *V*/*V*), resulting in five subfractions (Fr. F1–Fr. F5). Fr. F2 (5.0 g) underwent additional ODS CC with a MeOH–H_2O gradient (15 ∶ 85→100 ∶ 0, *V*/*V*) to yield eight subfractions (Fr. F2.1–Fr. F2.8). Fr. F2.6 was further purified using silica gel CC with a CH_2Cl_2–acetone gradient (100 ∶ 0→0 ∶ 100, *V*/*V*) and preparative TLC (CH_2Cl_2–MeOH–acetone 10 ∶ 2 ∶ 1, *V*/*V*/*V*) to yield **6** (1.0 mg). Fr. F3 (1.0 g) was loaded onto an ODS CC system with a MeOH–H_2O (15 ∶ 85→100 ∶ 0, *V*/*V*) as the mobile phase to yield thirteen subfractions (Fr. F3.1–Fr. F3.13). Fr. F3.10 was isolated by preparative HPLC with a MeOH–H_2O gradient (50 ∶ 50→70 ∶ 30, *V*/*V*) and subsequently purified with Sephadex LH20 CC using MeOH as the eluent and preparative TLC (CH_2Cl_2–MeOH, 10 ∶ 1, *V*/*V*) to yield **7** (2.4 mg).

Fr. G (2.5 g) was subjected to ODS CC using a MeOH–H_2O gradient (15 ∶ 85→100 ∶ 0, *V*/*V*) to yield seven subfractions (Fr. G1–Fr. G7). Fr. G7 (2.0 g) was rechromatographed using ODS CC with a MeOH–H_2O gradient (30 ∶ 70→ 100 ∶ 0, *V*/*V*) to obtain five subfractions (Fr. G7.1–Fr. G7.5). Fr. G7.4 was purified by preparative HPLC (MeOH–H_2O–DEA, 46 ∶ 54 ∶ 0.03, 3 mL·min^{-1}) to obtain **10** (1.1 mg, t_R 43.9 min). Fr. G7.5 was isolated by Sephadex LH-20 CC using MeOH as eluent, yielding seven subfractions (Fr. G7.5.1–Fr. G7.5.7). Fr. G7.5.3 was processed through ODS CC with a MeOH–H_2O gradient (20 ∶ 80→100 ∶ 0, *V*/*V*), followed by purification with preparative TLC (CH_2Cl_2–MeOH, 5 ∶ 1, *V*/*V*) to yield **5** (10.2 mg). Further purification using preparative HPLC (MeOH–H_2O–DEA, 43 ∶ 57 ∶ 0.03, 2 mL·min^{-1}) yielded **1** (1.5 mg, t_R 56.0 min) and **2** (2.3 mg, t_R 66.0 min). Fr. G7.5.7 was purified with preparative TLC (CH_2Cl_2–MeOH, 10 ∶ 1, *V*/*V*) to yield **4** (10.5 mg).

Fr. J (5.0 g) was subjected to D101 CC with a MeOH–H_2O gradient (0 ∶ 100→80 ∶ 20, *V*/*V*) as the mobile phase, yielding three subfractions (Fr. J1–Fr. J3). Fr. J1 (1.5 g) was loaded onto an ODS CC system using MeOH–H_2O (10 ∶ 90→100 ∶ 0, *V*/*V*) as the eluent, producing nine subfractions (Fr. J1.1–Fr. J1.9). Fr. J1.5 was further purified using preparative HPLC (MeOH–H_2O–DEA, 55 ∶ 45 ∶ 0.03, 2 mL·min^{-1}), yielding **3** (1.0 mg, t_R 57.0 min). Fr. J2 (0.6 g) was then subjected to ODS CC using a MeOH–H_2O gradient (20 ∶ 80→100 ∶ 0, *V*/*V*), which afforded four subfractions (Fr. J2.1–Fr. J2.4). Fr. J2.2 was purified with preparative HPLC (MeOH–H_2O–DEA, 80 ∶ 20 ∶ 0.03, 2 mL·min^{-1}), yielding **9** (1.5 mg, t_R 62.0 min).

Identification of new compounds

Pegaharmol C (**1**): a pale-yellow powder (MeOH); UV (MeOH) λ_{max} (log ε): 227 (3.6), 302 (3.1), 366 (2.7) nm; (+) HR-ESI-MS *m/z* 327.1252 $[M + H]^+$ (Calcd. for $C_{20}H_{15}N_4O$, 327.1240); $[\alpha]_D^{20}$ −24.0 (*c* 0.3 MeOH). ^{1}H NMR and ^{13}C NMR spectroscopic data are shown in Table 1.

Pegaharmol D (**2**): a pale-yellow powder (MeOH); UV (MeOH) λ_{max} (log ε): 227 (3.6), 302 (3.1), 366 (2.7) nm; (+) HR-ESI-MS *m/z* 341.1399 $[M + H]^+$ (Calcd. for $C_{21}H_{17}N_4O$, 341.1397); $[\alpha]_D^{20}$ −18.0 (*c* 0.3 MeOH). ^{1}H NMR and ^{13}C NMR spectroscopic data are shown in Table 1.

Pegaharmol E (**3**): a white solid (MeOH); UV (MeOH) λ_{max} (log ε): 303 (3.0) nm; (+) HR-ESI-MS *m/z* 268.1446 $[M + H]^+$ (Calcd. for $C_{16}H_{18}N_3O$, 268.1450); $[\alpha]_D^{20}$ −29.0 (*c* 0.1, MeOH). ^{1}H NMR and ^{13}C NMR spectroscopic data are shown in Table 1.

7-Hydroxyannomontine (**4**): an orange powder (MeOH); UV (MeOH) λ_{max} (log ε): 223 (3.5), 292 (3.2), 322 (3.0), 380 (3.1) nm; (+) HR-ESI-MS *m/z* 278.1014 $[M + H]^+$ (Calcd. for $C_{15}H_{12}N_5O$, 278.1036). ^{1}H NMR and ^{13}C NMR spectroscopic data are shown in Table 1.

Computational methods

Conformation analysis of compound **1** was conducted using a grid search in Gaussian 09 software (Gaussian, Inc., Wallingford CT, USA) [5, 6, 11-13]. The torsion angle on C_7-C_8-$C_{4'}$-$N_{3'}$ was altered from −105 to 255° in 10° increments, covering the complete range of conformers around this torsion bond.

Conformational searches for compounds **1a** and **3a** were performed using CONFLEX software (Conflex Corporation, Tokyo, Japan) [11] with the MMFF94s force field. All the conformers with a Boltzmann distribution greater than 1% were subsequently optimized at the B3LYP/6-31G(d) level using the polarizable continuum model (PCM) in MeOH solvent with the Gaussian 09 program package [5, 6, 11-13]. ECD calculations for all the conformers of **1a** and **3a** were conducted at suitable levels using the time-dependent density functional theory (TDDFT) method, incorporating the PCM in the methanol solvent. Finally, the overall calculated ECD spectra were generated by Boltzmann weighting using SpecDis 1.51 [6, 11-13].

Cytotoxic activity assay

The antiproliferative activities of isolates **1**, **2**, **4**, **5**, **7**, and **8** were evaluated using the trypan blue exclusion test [11, 23] on the human leukemia cell line (HL-60) and the MTT assay [24] on the human lung cancer cell line (A549), human breast cancer cell line (MDA-MB-231), and human prostate cancer cell line (DU145). These cell lines were purchased from America Type Culture Collection (ATCC, Rockville, MD, USA) and cultured in RPMI-1640 medium (Gibco, New York, NY, USA) supplemented with 100 U·mL^{-1} penicillin, 100 μg·mL^{-1} streptomycin, 1 mmol·L^{-1} glutamine, and

10% heat-inactivated fetal bovine serum (Gibco).

In the trypan blue exclusion test, cells in the logarithmic growth phase were seeded at a density of 4×10^4 cells/mL in 24-well microplates and incubated with various concentrations of the compounds, dissolved in DMSO and diluted appropriately, under a humidified atmosphere with 5% CO_2 and 95% air at 37 °C for three days. Post-incubation, cells were stained with trypan blue, and viable cells were counted using a hemocytometer. 5-Fluorouracil (5-Fu) served as a positive control.

In the MTT assay, 200 μL of cell suspensions at a density of 5×10^4 cells/mL were plated in 96-well plates and incubated for 24 h at 37 °C under 5% CO_2 and 95% air. Then 2 μL of different concentrations of test compounds dissolved in DMSO were placed into each well and further incubated for 72 h. Thereafter, 50 μL of a 0.4% MTT solution was added to each well, followed by a 4 h incubation. Post-incubation, MTT was removed from the wells, and formazan crystals were dissolved in DMSO (200 μL). The plates were agitated for 10 min, after which the absorbance was measured on a microplate reader (Bio-RAD) at the wavelength of 570 nm. Etoposide (VP16) was used as a positive control.

Supporting Information

HR-ESI-MS, 1D and 2D NMR, and UV spectra of compounds **1**−**4** can be requested by sending E-mails to the corresponding authors.

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