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FURTHER BISINDOLE ALKALOIDS FROM *CATHARANTHUS ROSEUS* AND THEIR CYTOTOXICITY

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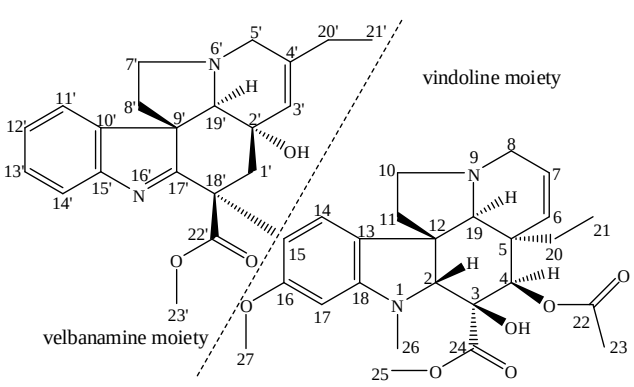
Abstract − Two novel bisindole alkaloids, cyclovinblastine A-B (**1-2**), together with ten known alkaloids 4-deacetoxycyclovinblastine (**3**), cycloleurosine (**4**), leurosine (**5**), vinblastine (**6**), leurosidine (**7**), vinblastine *N*'$_b$-oxide (**8**), isoleurosine (**9**), 4'-deoxyleurosidine (**10**), 4'-deoxyleurosidine *N*'$_b$-oxide (**11**) and 4-desacetoxyvinblastine (**12**) were isolated from the leaves of *Catharanthus roseus*. The structures of **1** and **2** were established by analysis of their NMR and HR-ESI-MS spectroscopic data. All alkaloids (**1-12**) were evaluated for their cytotoxic activities against the human hepatocellular carcinoma (HepG2) cell line, human colorectal carcinoma (Lovo) cell line, and human breast carcinoma (MCF-7) cell line by the MTT method *in vitro*, respectively. The results indicated that cytotoxic activities of alkaloids **9**, **10** and **12** were much more potent than those of the positive control vinblastine (**6**). In addition, the structure-activity relationships (SAR) were conducted on the basis of the cytotoxicity of these isolated alkaloids.

INTRODUCTION

Catharanthus roseus (L.) G. Don (Apocynaceae), the Madagascan periwinkle, has been regarded as a rich source of bisindole alkaloids.[1] Nearly 30 different bisindole alkaloids had been isolated from the whole plant of *Catharanthus roseus*.[2] Owing to their potential therapeutic benefits in cancer chemotherapy, bisindole alkaloids have been a focus of much research over last five decades. Many such alkaloids exhibited exceptional antitumor activity, and among these, vinblastine and vincristine are widely used in the treatment of Hodgkin's disease, non-Hodgkin lymphomas, testiscarcinomas, chorio-carcinomas, acute leukemia, rhabdomyosarcomas, Wilm's tumors in children and breast cancer.[2-8] In order to further search for the bioctive bisindole alkaloids of this plant, continuous research has been carried out in recent years.[2,9,10] In this paper, the isolation and structural elucidation of two new bisindole alkaloids, cyclovinblastine A-B (**1-2**) are described, together with ten known alkaloids 4-deacetoxycyclovinblastine (**3**),[9] cycloleurosine (**4**),[11] leurosine (**5**),[12] vinblastine (**6**),[13] leurosidine (**7**), vinblastine *N*'$_b$-oxide (**8**),[14] isoleurosine (**9**), 4'-deoxyleurosidine (**10**),[15] 4'-deoxyleurosidine *N*'$_b$-oxide (**11**),[16] and 4-desacetoxyvinblastine (**12**)[17] (Figure 1) from the leaves of *Catharanthus roseus*. The cytotoxicity of all these alkaloids against three human cancer cell lines (HepG2, Lovo, and MCF-7) evaluated by the MTT method show that cytotoxic activities of alkaloids **9**, **10** and **12** were much more potent than those of the positive control vinblastine (**6**). Subsequently, the structure-activity relationships (SAR) are presented on the basis of the cytotoxicity of the isolated alkaloids.



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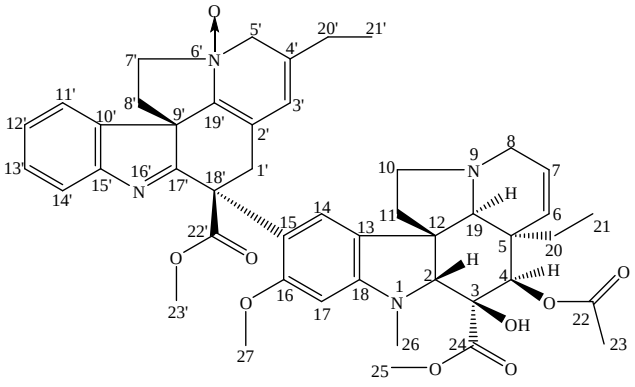

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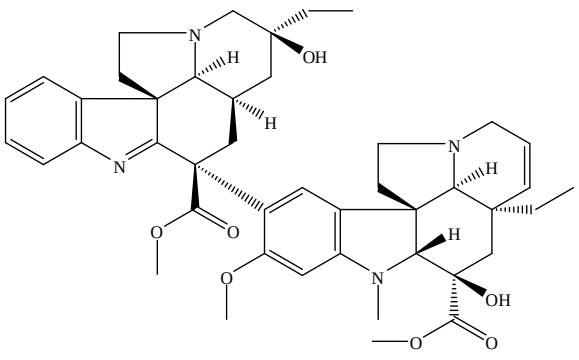

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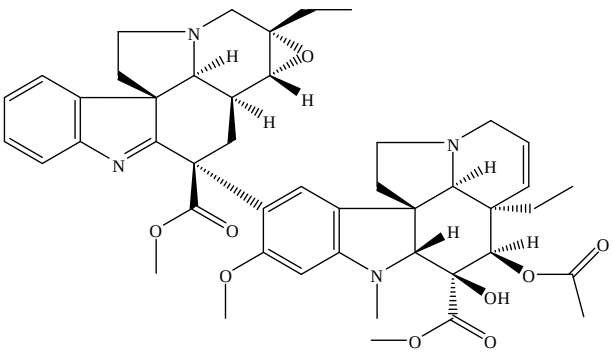

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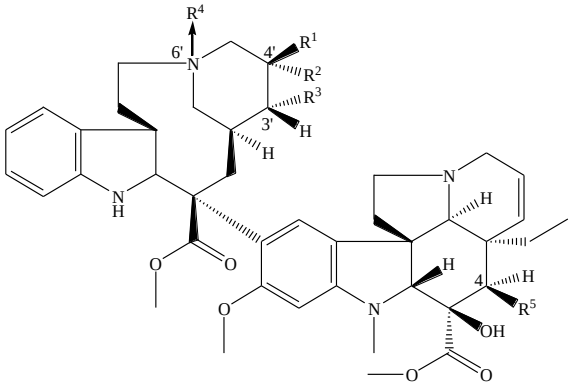

	R_1	R_2	R_3	R_4	R_5
5	Et	epoxy bond		lone pair	OCOMe
6	OH	Et	H	lone pair	OCOMe
7	Et	OH	H	lone pair	OCOMe
8	OH	Et	H	O	OCOMe
9	H	Et	H	lone pair	OCOMe
10	Et	H	H	lone pair	OCOMe
11	Et	H	H	O	OCOMe
12	OH	Et	H	lone pair	H

'epoxy bond' indicates that an epoxy group lies in between $C_{3'}$ and $C_{4'}$

Figure 1. Chemical structures of alkaloids **1-12**

RESULTS AND DISCUSSION

The MeOH extract of the dried and powdered leaves of *Catharanthus roseus* collected in Qionghai, Hainan Province, P.R. China was dissolved in 2.0% citric acid and the acid solution was subsequently basified using concentrated NH_4OH to pH 6-7. The basic solution was partitioned with $CHCl_3$ to afford a residue of crude alkaloids. From the crude alkaloids, two novel bisindole alkaloids, cyclovinblastine A-B (**1-2**), together with ten known alkaloids (**3-12**) were isolated and obtained by silica gel, C_{18} reversed phase, and Sephadex LH-20 column chromatography and preparative TLC method. The structures of **1-12** were established by analysis of their NMR and MS spectroscopic data.

Cyclovinblastine A (**1**) was isolated as a yellow amorphous powder. The molecular formula of **1** was revealed as $C_{46}H_{54}N_4O_9$ by its HR-ESI-MS data (found $[M+H]^+$ 807.3955, calcd. for 807.3969), corresponding to 22 degrees of unsaturation in the molecule. The IR spectrum showed absorptions at 3446, 1737, 1618 and 1504 cm^{-1}, indicating the presence of hydroxy, carbonyl and aromatic ring. The ^{1}H- and ^{13}C-NMR (including DEPT) spectra (Table 1) disclosed the presence of seven methyls, nine methylenes, thirteen methines, fourteen quaternary carbons, and three carbonyl carbons. Among them, the chemical shifts of the protons [δ_H 3.80 (3H, s), 3.79 (3H, s), 3.64 (3H, s), 2.70 (3H, s), 2.08 (3H, s), 1.02

(3H, t, J = 7.6 Hz), and 0.64 (3H, t, J = 7.2 Hz)] and the carbons [δ_C 55.8, 52.2, 52.8, 38.5, 21.9, 12.1, and 7.7] indicated the existence of three -OMe groups, one –NMe group, and three -Me groups in the molecule. Olefinic protons [δ_H 5.84 (1H, dd, J = 10.0, 4.0 Hz) and 5.26 (1H, d, J = 10.0 Hz)] and corresponding carbons [δ_C 124.0 and 130.8] were attributable to a *Z*-disubstituted double bond. The ^{1}H- and ^{13}C-NMR spectra also exhibited characteristic signals for a 1,2-disubstituted aromatic ring [δ_H 7.48 (1H, d, J = 7.2 Hz), 7.39 (1H, d, J = 7.2 Hz), 7.28 (1H, m), and 7.19 (1H, m); δ_C 153.7, 148.2, 127.3, 125.9, 124.3, and 120.6] and a 1,2,4,5-tetrasubstituted aromatic ring [δ_H 7.49 (1H, s) and 6.10 (1H, s); δ_C 157.8, 152.1, 124.5, 123.3, 120.8, and 93.8]. These data were compared with those of the previously isolated alkaloids from the title plant.[2] The moieties of vindoline and velbanamine in a bisindole skeleton were then considered for **1**.

Table 1. ^{1}H-NMR ($CDCl_3$, 400 MHz) and ^{13}C-NMR ($CDCl_3$, 100 MHz) data of alkaloids **1-2** (δ in ppm, J in Hz)*

position	**1**		**2**	
	δ_H	δ_C	δ_H	δ_C
2	3.72 s	83.6	3.63 s	83.7
3	—	79.7	—	79.3
4	5.50 s	76.8	5.46 s	76.7
5	—	43.0	—	42.9
6	5.26 d (10.0)	130.8	5.13 d (10.0)	130.2
7	5.84 dd (10.0, 4.0)	124.0	5.76 dd (10.0, 4.0)	124.0
8a	3.45 dd (16.0, 4.8)	51.1	3.33 dd (16.4, 4.6)	50.9
8b	2.80 m	—	2.50 d (16.4)	—
10a	3.34 m	51.8	3.07 m	52.0
10b	2.45 m	—	1.66 m	—
11a	2.24 m	44.1	2.22 m	43.9
11b	2.13 m	—	1.99 m	—
12	—	53.2	—	52.8
13	—	123.3	—	119.1
14	7.49 s	120.8	7.22 s	122.1
15	—	124.5	—	124.0
16	—	157.8	—	158.8
17	6.10 s	93.8	6.04 s	94.0
18	—	152.1	—	152.4
19	2.71 s	66.9	1.92 s	67.8
20a	1.67 m	31.1	1.67 m	30.9
20b	1.27 m	—	1.01 m	—
21-Me	0.64 t (7.2)	7.7	0.44 t (7.6)	7.6
22	—	170.8	—	170.9
23-Me	2.08 s	21.9	2.05 s	21.0
24	—	172.1	—	172.3

25-OMe	3.79 s	52.2	3.76 s	52.2
26-NMe	2.70 s	38.5	2.60 s	38.4
27-OMe	3.80 s	55.8	3.73 s	55.5
1'a	2.23 m	42.6	2.02 m	29.4
1'b	2.19 m	—	1.19 m	—
2'	—	71.0	—	116.5
3'	5.24 s	125.7	6.27 s	119.1
4'	—	140.3	—	126.3
5'a	3.38 d (16.0)	52.5	3.48 d (17.5)	45.1
5'b	3.22 d (16.0)	—	2.96 d (17.5)	—
7'a	3.27 m	52.1	3.67 m	40.9
7'b	3.01 m	—	3.45 m	—
8'a	2.86 m	36.1	2.22 m	29.6
8'b	1.73 m	—	2.02 m	—
9'	—	62.4	—	65.4
10'	—	148.2	—	129.9
11'	7.48 d (7.2)	124.3	7.10 d (7.6)	124.4
12'	7.19 m	125.9	6.81 m	119.9
13'	7.28 m	127.3	7.12 m	128.8
14'	7.39 d (7.2)	120.6	6.58 d (7.6)	110.4
15'	—	153.7	—	148.8
17'	—	182.2	—	191.9
18'	—	54.2	—	59.7
19'	2.84 br s	73.8	—	140.4
20'	2.02 m	27.1	2.77 m	19.4
21'-Me	1.02 t (7.6)	12.1	1.19 t (7.6)	14.4
22'	—	174.0	—	174.5
23'-OMe	3.64 s	52.8	3.47 s	51.8

* Assignments were established by interpretation of the ^{1}H-^{1}H COSY, HSQC and HMBC spectra.

In the HMBC spectrum (Figure 2), the proton signal at δ_H 7.49 (H-14) of the vindoline moiety was correlated with the quaternary carbon signal at δ_C 54.2 (C-18') of the velbanamine moiety, and the proton signals at δ_H 2.23, 2.19 (H-1') of the velbanamine moiety showed cross-peaks with carbon signal at δ_C 124.5 (C-15) of the vindoline moiety, which further confirmed that the vindoline and velbenamine moieties were connected via a C-15 to C-18' bond. In addition, the HMBC correlation between δ_H 2.84 (H-19') and δ_C 182.2 (C-17') and δ_C 148.2 (C-10') indicated that a C-C bond was present between C-9' and C-19', forming 16',17'-dehydro-ψ-Aspidosperma-Aspidosperma[11] skeletal structure. Detailed examination of the 1D and 2D NMR spectra of **1** and comparison with those of cycloleurosine (**4**)[11] revealed their considerable structural similarity. The main differences were that the hydrogen atom in **4** was substituted by a hydroxy group at C-2' of **1** and the epoxy group in **4** was cleaved to form a double bond at between C-3' and C-4' of **1**. By comparison of the NMR data with those of the known alkaloids, **3** and **4**,[9,11] the structure of **1** was determined as shown in Figure 1.

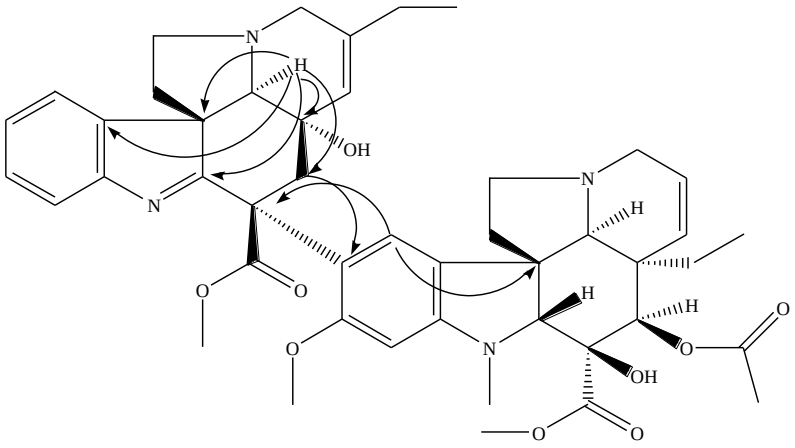

Figure 2. The key HMBC correlations of alkaloid **1** (H→C)

Cyclovinblastine B (**2**) was assigned the molecular formula $C_{46}H_{52}N_4O_9$ (23 degrees of unsaturations) on the basis of HR-ESI-MS data (found $[M+H]^+$ 805.3778, calcd. for 805.3807). Comparison of the ^{1}H- and ^{13}C-NMR (including DEPT) data (Table 1) of **2** with those of **1** revealed that the signals were similar, except for the formation of a double bond between C-19' (δ_C 140.4) and C-2' (δ_C 116.5) instead of a hydroxyl group at C-2' (δ_C 71.0) of **1**, as well as an *N*-oxide group was found at the N-6' position in **2**, which is deduced by biogenetic pathway of the bisindole alkaloids[2] and the comparison of the carbon signals at δ_C 45.1 (C-5') and δ_C 40.9 (C-7') and the carbon signals at δ_C 50.9 (C-8) and δ_C 52.0 (C-10). The HMBC correlations between H-3' (δ_H 6.27) and C-19' (δ_C 140.4), C-2' (δ_C 116.5), C-4' (δ_C 126.3), and C-20' (δ_C 19.4) further supported the above conclusion. According to these data, the planar structure of cyclovinblastine B was established to be **2**.

The ten known alkaloids including 4-deacetoxycyclovinblastine (**3**),[9] cycloleurosine (**4**),[11] leurosine (**5**),[12] vinblastine (**6**),[13] leurosidine (**7**), vinblastine *N*'$_b$-oxide (**8**),[14] isoleurosine (**9**), 4'-deoxyleurosidine (**10**),[15] 4'-deoxyleurosidine *N*'$_b$-oxide (**11**),[16] and 4-desacetoxyvinblastine (**12**)[17] from the leaves of *Catharanthus roseus* were also isolated and identified on the basis of their physical and spectroscopic data.

Alkaloids **1**-**12** were investigated for *in vitro* against the three human cancer cell lines (HepG2, Lovo, and MCF-7), and their IC_{50} values are summarized in Table 2. The tested alkaloids showed different cytotoxicity, with higher cytotoxicity against HepG2 than Lovo and MCF-7. Among them, alkaloids **9**, **10**, and **12** against HepG2, Lovo, and MCF-7 were much more potent than the positive control vinblastine (**6**). The cytotoxic activities of the new alkaloids **1**-**2** were weaker than those of vinblastine (**6**). In addition, compared to vinblastine (**6**), the cytotoxicity of alkaloids **3**, **4**, **5**, **7**, **8**, and **11** against HepG2, Lovo, and MCF-7 were also lower.

Table 2. IC_{50} values of inhibitory effects of alkaloids **1-12** against HepG2, Lovo, and MCF-7 cell lines[a,b,c]

	HepG2	Lovo	MCF-7
1	>100	>100	>100
2	72.25±1.53	>100	>100
3	39.32±1.21	—	>100
4	85.92±2.12	—	>100
5	>100	>100	>100
6	24.64±0.54	61.61±1.01	34.75±0.68
7	54.80±1.12	>100	57.26±0.98
8	32.43±0.75	>100	62.39±1.16
9	8.52±0.50	26.63±0.79	17.36±0.66
10	14.46±0.42	14.83±0.36	15.18±0.56
11	70.12±2.56	>100	>100
12	3.68±0.21	3.82±0.52	4.07±0.43

[a] All alkaloids were examined in a set of experiments repeated three times.
[b] '—' means that IC_{50} value of the alkaloid was not determined.
[c] Purity (%) of tested alkaloids was ≥98%.

Based on the analysis of the cytotoxicity and the structural characteristics of these tested alkaloids from *Catharanthus roseus*, it was found that the alkaloids' molecules which are missing the hydroxyl group at C-4' (**9** and **10**) or the acetoxy group at C-4 (**12**) could significantly increase the growth inhibition effects against the tested cell lines in comparison with vinblastine (**6**). On the other hand, comparison of the cytotoxic activities of alkaloids **1**-**5**, **7**, **8**, and **11** with those of **6** clearly indicated that formation of a skeleton of 16',17'-dehydro-ψ-Aspidosperma-Aspidosperma, the presence of an epoxy group between C-3' and C-4', incorporation of an *N*-oxide group at N-6' position, and epimerization of the hydroxy group at C-4' position could decrease the cytotoxic activities against the HepG2, Lovo, and MCF-7.

EXPERIMENTAL

General procedures. Optical rotations were determined on a Jasco P-1020 polarimeter (Jasco, Tokyo, Japan) at room temperature. Infrared radiation (IR) spectra were recorded on a Jasco FT/IR-480 Plus fourier transform infrared spectrometer (Jasco, Tokyo, Japan) using KBr pellets. Electrospray ionization mass spectra (ESI-MS) were carried out on a Finnigan LCQ Deca mass spectrometer. High-resolution

electron impact mass spectra (HR-ESI-MS) were obtained on a Thermo Finnigan MAT95XP mass spectrometer. Nuclear magnetic resonance (NMR) spectra were measured on a Bruker AV-400 spectometer with tetramethyl-silane (TMS) as an internal standard. Precoated silica gel GF_{254} plates (Qingdao Marine Chemical Plant, Qingdao, P. R. China) were used for TLC which visualized by spraying potassium hepta-iodobismuthate. Silica gel (200-300 mesh, Qingdao Marine Chemical Plant, Qingdao, P. R. China), C_{18} reverse-phase (Merck, Darmstadt, Germany) and Sephadex LH-20 (Pharmacia Biotec AB, Uppsala, Sweden) were used for column chromatography. Solvents used in column and HPLC chromatography were of analytical grade (Shanghai Chemical Plant, Shanghai, P. R. China) and HPLC grade (Fisher Scientific, New Jersey, U. S. A). Three human cancer cell lines (HepG2, Lovo, and MCF-7) were purchased from Key Laboratory for New Drugs Research of Traditional Chinese Medicine in Shenzhen, Tsinghua University (Shenzhen, P. R. China).

Plant material. The leaves of *Catharanthus roseus* (L.) G. Don (Apocynaceae) was collected in Qionghai, Hainan Province, P.R. China, in October of 2005 and authenticated by Prof. Guang-Xiong Zhou (Jinan University). A voucher specimen (No#CPU051024) was deposited in the Herbarium of China Pharmaceutical University.

Extraction and isolation. The dried and powdered leaves of *Catharanthus roseus* (15 kg) were extracted with MeOH (3×150 L) at room temperature. After the solvent was evaporated *in vacuo*, the residue was dissolved in 2.0% citric acid and the acid solution was subsequently basified using concentrated NH_4OH to pH 6-7. The basic solution was partitioned with $CHCl_3$ (4 × 2 L, room temperature) to afford the aqueous phase and $CHCl_3$ phase. The $CHCl_3$ phase was concentrated under reduced pressure (60 °C) to afford a residue of crude alkaloids (125 g).

The crude alkaloids was subjected to silica gel column chromatography (600 g, Φ 7×42 cm) eluting with a gradient of $CHCl_3$-MeOH [100:0, 90:10, 80:20, 70:30, 50:50 (8 L of each mixture)] to provide five fractions (A-E). Fraction B (12 g) was separated repeatedly by silica gel column chromatography (300 g, Φ 5×78 cm) using $CHCl_3$-MeOH (100:0, 99:1, 98:2, 97:3, 95:5, each 2 L) as eluent and further purified by Sephadex LH-20 ($CHCl_3$-MeOH, 1:1) to afford **9** (5 mg), **10** (23 mg) and **11** (7 mg). Fraction C (20 g) was resubjected to silica gel column chromatography (400 g, Φ 5×100 cm) eluting with $CHCl_3$-MeOH (100:0, 98:2, 95:5, 90:10, 80:20, each 3 L) and was further separated by preparative reversed-phase HPLC with MeOH-H_2O-Et_3N (62:38:0.01) to afford **1** (45 mg), **2** (3.0 mg), **3** (16 mg), and **4** (49 mg). Fraction D (35 g) was applied to a C_{18} reverse-phase column (350 g, Φ 5×88 cm) eluted with MeOH-H_2O (10:90, 30:70, 50:50, 80:20, 100:0, each 3 L) to give subfractions 1-5. Subfractions 1-5 were separated by preparative reversed-phase HPLC, eluting with MeOH-H_2O-Et_3N (62:38:0.01), in combination with preparative TLC with $CHCl_3$-MeOH (95:5) to afford **5** (24 mg), **6** (52 mg), **7** (30 mg), **8** (21 mg) and **12** (22 mg).

Cyclovinblastine A (1). Yellow amorphous powder; $[\alpha]_D^{21}$ -53.8 (*c* 0.1, $CHCl_3$); IR (KBr) ν_{max} = 3446, 2962, 1737, 1618, 1504, 1457, 1246, 1041, 752 cm^{-1}; HR-ESI-MS *m*/*z* = 807.3955 $[M+H]^+$ (calcd. for $C_{46}H_{55}N_4O_9$: 807.3969); ^1H- and ^{13}C-NMR data, see Table 1.

Cyclovinblastine B (2). Yellow amorphous powder; $[\alpha]_D^{21}$ -408 (*c* 0.025, $CHCl_3$); IR (KBr) ν_{max} = 3446, 2924, 1737, 1617, 1507, 1457, 1242, 1041, 745 cm^{-1}; HR-ESI-MS *m*/*z* = 805.3778 $[M+H]^+$ (calcd. for $C_{46}H_{53}N_4O_9$: 805.3807); ^1H- and ^{13}C-NMR data, see Table 1.

Cytotoxicity assay. The cytotoxicity assay was carried out according to the procedure described in the literature.[18] In brief, test cell lines (HepG 2, Lovo, and MCF-7) were maintained in RPMI 1640 medium (ICN) supplemented with 10% fetal bovine serum (Biological Industries Inc.), and were seeded in 96 well plates and incubated in a CO_2 incubator at 37 °C for 24 h. The seeding numbers were 10000 per well. The cells were treated with five different concentrations of test alkaloids in a CO_2 incubator for 72 h. The number of viable cells was estimated using the tetrazolium dye reduction assay (MTT assay), and the experiment was performed as the manufacturer recommended (Promega, Madison, WI). The absorbance was measured at 570 nm on a Wallac 1420 VICTOR2 Multilabel Counter (Perkin-Elmer, Boston, MA). The results of these assays were used to obtain the dose–response curves from which the IC_{50} values were determined. An IC_{50} value represents the concentration (μM) of the test alkaloid at which a 50% cell growth inhibition after 3 days of incubation is produced. The values represent averages of three independent experiments, each with duplicate samples.

SUPPLEMENTARY MATERIAL

HR-ESI-MS and NMR spectra of alkaloids **1**-**2** are available as supplementary material.

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Conflict of Interest

All authors have no conflicts of interest to declare.

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