Steroidal Alkaloids from *Veratrum maackii* REGEL with Genotoxicity on Brain-Cell DNA in Mice

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Two new steroidal alkaloids, 23-methoxycyclopamine 3-*O*-*β*-D-glucopyranoside (**1**) and isoecliptalbine (**2**), were isolated from the root and rhizoma of *Veratrum maackii* REGEL, together with five known compounds, *i.e.*, verussurine (**3**), verabenzoamine (**4**), verazine (**5**), isoverazine (**6**), and verazinine (**7**). Their structures were established by extensive analysis of spectroscopic data, as well as by comparison with literature data. Compounds **1**–**7** could cause DNA damage in the cerebellum and cerebral cortex of mice in a dose-dependent manner by using single-cell gel electrophoresis (comet assay).

Introduction. – In China, several species of *Veratrum* genus, such as *V. nigrum* L., *V. maackii* REGEL, *V. japonicum* (BAKER) LOES. f., *V. dahuricum* (TURCZ.) LOES. f., *etc.*, have been used for preparation of '*Li-Lu*' to treat blood-stroke, excessive phlegm, epilepsy, aphasia, and scabies [1][2]. Constituents of *Veratrum* species have been examined extensively, and more than 100 steroidal alkaloids have been isolated so far, but fewer studies have been carried out on geonotoxicity caused by steroidal alkaloids, which were established as the major bioactive and toxic ingredients with genotoxicity [3–5], reproductive toxicity [6], and neurotoxicity [3][5][7]. In continuation of our research on *Veratrum* alkaloids, we isolated seven compounds from the dried roots and rhizoma of *Veratrum maackii* REGEL, and identified two new compounds, 23-methoxycyclopamine 3-*O*-*β*-D-glucopyranoside (**1**) and isoecliptalbine (**2**), along with verussurine (**3**), verabenzoamine (**4**), verazine (**5**), isoverazine (**6**), and verazinine (**7**) (*Fig. 1*). Herein, we report the isolation and structure elucidation of the two new compounds **1** and **2**, as well as the neurotoxicities of the isolated compounds on brain-cell DNA of the cerebellum and cerebral cortex in mice by using single-cell gel electrophoresis (comet assay).

Results and Discussion. – Compound **1** was obtained as white amorphous powder. The molecular formula was determined as $C_{34}H_{53}NO_8$ on the basis of HR-ESI-MS (*m*/*z* 604.3847 ($[M+H]^+$)), which was supported by the ^{13}C-NMR data. The ^{1}H-NMR spectrum (*Table*) of compound **1** exhibited four Me signals (δ(H) 0.91 (*d*, *J* = 6.8, Me(27)), 0.89 (*d*, *J* = 8.0, Me(21)), 1.01 (*s*, Me(19)), and 1.80 (*s*, Me(18))), a signal of one O-bearing CH group (δ(H) 3.58–3.68 (*m*, H–C(3))), and signals of a glucose unit (δ(H) 4.42 (*d*, *J* = 7.6, H–C(1′)), 3.13–3.21 (*m*, H–C(2′)), 3.32–3.38 (*m*, H–C(3′)),

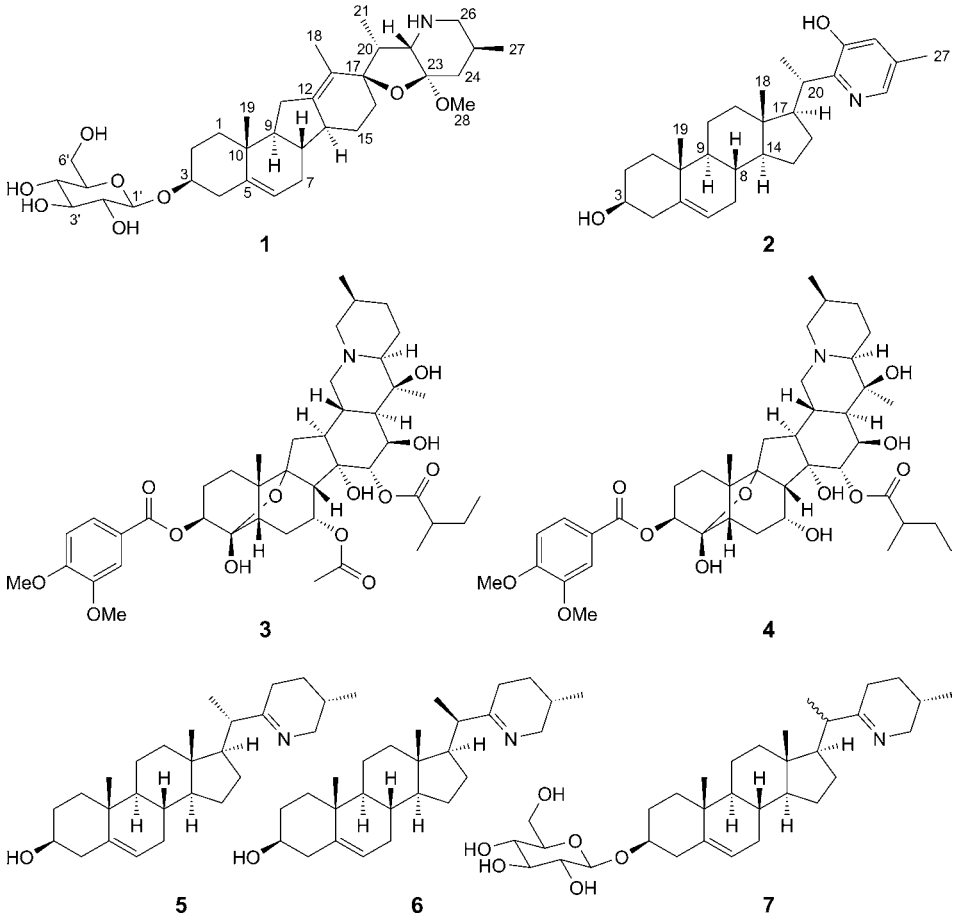

Fig. 1. *Structures of compounds* **1**–**7**

3.24–3.30 (*m*, H–C(4′)), 3.21–3.29 (*m*, H–C(5′)), 3.88 (*d*, $J = 11.6$, H_a–C(6′)), and 3.68 (*dd*, $J = 11.6$, 4.4, H_b–C(6′))). The ^{13}C-NMR spectrum displayed 34 C-atom signals, including those of four olefinic C-atoms (δ(C) 143.1, 123.1, 127.4, and 144.3), and of ten O-bearing C-atoms (δ(C) 79.0, 90.6, 106.5, 102.6, 75.0, 78.2, 71.8, 78.0, 49.1, and 62.9), suggesting that the aglycone of **1** was 23-methoxycyclopamine based on an analysis of HMBC and HMQC spectra [8]. The coupling constant ($J = 7.6$) of the glucose anomeric H-atom suggested a β-form. The position of the sugar moiety was confirmed by the HMBC spectrum (*Fig. 2*), which showed a correlation between δ(H) 4.42 (*d*, $J = 7.6$, H–C(1′)) and δ(C) 79.0 (C(3)), indicating that the glucose moiety was attached to C(3). In the NOESY spectrum (*Fig. 2*), cross-peaks were observed between Me(19) and H_a–C(1), H_a–C(4), and H–C(8), between H_b–C(4)) and H_b–C(1) and H–C(3), between H_b–C(1) and H–C(9), between Me(18) and Me(21) and H–C(8), between Me(27) and H_a–C(26), between H–C(25) and H_b–C(26), H_b–C(24), and Me(28)O, and between H_b–C(24) and Me(28)O, confirming the β-position of the glucose moiety.

Table. *^{1}H- and ^{13}C-NMR Data* (400 and 100 MHz, resp.) *of* **1** (in CD_3OD) *and* **2** (in CD_3OD and $CDCl_3$). δ in ppm, J in Hz.

Position	**1**		**2**	
	δ(H)	δ(C)	δ(H)	δ(C)
1	1.70–1.78 (*m*), 1.18–1.24 (*m*)	39.5	1.71–1.79 (*m*), 1.03–1.11 (*m*)	38.0
2	2.16–2.24 (*m*), 1.70–1.76 (*m*)	31.7	1.72–1.80 (*m*), 1.43–1.53 (*m*)	31.8
3	3.58–3.68 (*m*)	79.0	3.36–3.44 (*m*)	71.9
4	1.84–1.94 (*m*), 2.02–2.14 (*m*)	43.2	2.18–2.24(*m*), 2.20–2.28(*m*)	42.5
5		143.1		141.7
6	5.43 (br. *d*, *J* = 4.8)	123.1	5.32 (br. *d*, *J* = 4.8)	122.0
7	2.47–2.53 (*m*), 2.21–2.29 (*m*)	39.6	1.97–2.03 (*m*), 1.50–1.58 (*m*)	32.5
8	1.20–1.28 (*m*)	44.1	1.38–1.47 (*m*)	32.8
9	1.49–1.57 (*m*)	54.1	0.84 (*td*, *J* = 11.2, 4.8)	51.1
10		38.0		37.2
11	2.24–2.32 (*m*), 2.03–2.13 (*m*)	29.0	1.14–1.20 (*m*), 1.20–1.28 (*m*)	21.7
12		127.4	0.70–0.76(*m*), 0.38 (br. *d*, *J* = 12.4)	38.3
13		144.3		43.0
14	1.95 (*m*)	49.4	1.00–1.06 (*m*)	57.0
15	1.83–1.91 (*m*), 1.31 (br. *s*)	25.4	1.11–1.19 (*m*), 1.63–1.73 (*m*)	24.7
16	1.23–1.31 (*m*), 1.91–1.97 (*m*)	30.6	1.98–2.06(*m*), 1.41–1.49 (*m*)	28.4
17		90.6	1.98–2.04 (*m*)	54.0
18	1.80 (*s*)	16.1	0.70 (*s*)	12.8
19	1.01 (*s*)	18.9	0.94 (*s*)	19.6
20	2.65–2.71 (*m*)	48.6	3.25–3.31 (*m*)	42.7
21	0.89 (*d*, *J* = 8.0)	12.8	1.20 (*d*, *J* = 6.8)	19.6
22	3.04 (*d*, *J* = 6.4)	65.4		150.8
23		106.5		151.0
24	2.32–2.38 (*m*), 1.15–1.25 (*m*)	40.2	7.17 (br. *s*)	126.3
25	1.68–1.74 (*m*)	30.5		134.2
26	3.00 (br. *d*, *J* = 12.8), 2.19–2.27 (*m*)	52.9	7.82 (br. *s*)	137.5
27	0.91 (*d*, *J* = 6.8)	19.3	2.33 (*s*)	18.0
28	3.37 (*s*)	49.1		
Glc				
1′	4.42 (*d*, *J* = 7.6)	102.6		
2′	3.13–3.21 (*m*)	75.0		
3′	3.32–3.38 (*m*)	78.2		
4′	3.24–3.30 (*m*)	71.8		
5′	3.21–3.29 (*m*)	78.0		
6′	3.88 (*d*, *J* = 11.6), 3.68 (*dd*, *J* = 11.6, 4.4)	62.9		

Based on these data, the structure of compound **1** was identified and named 23-methoxycyclopamine 3-*O*-β-D-glucopyranoside.

Compound **2** was obtained as white amorphous powder. The molecular formula was deduced as $C_{27}H_{39}NO_2$ on the basis of HR-ESI-MS (*m*/*z* 410.3057 ($[M+H]^+$)), which was supported by the ^{13}C-NMR spectrum data. The ^{1}H-NMR spectrum (*Table*) of compound **2** exhibited four Me signals (δ(H) 2.33 (*s*, Me(27)), 1.20 (*d*, *J* = 6.8, Me(21)), 0.94 (*s*, Me(19)), and 0.70 (*s*, Me(18))), a signal of one O-bearing CH group (δ(H) 3.36–3.44 (*m*, H–C(3))), and three signals of olefinic H-atoms (δ(H) 5.32 (br. *d*, *J* =

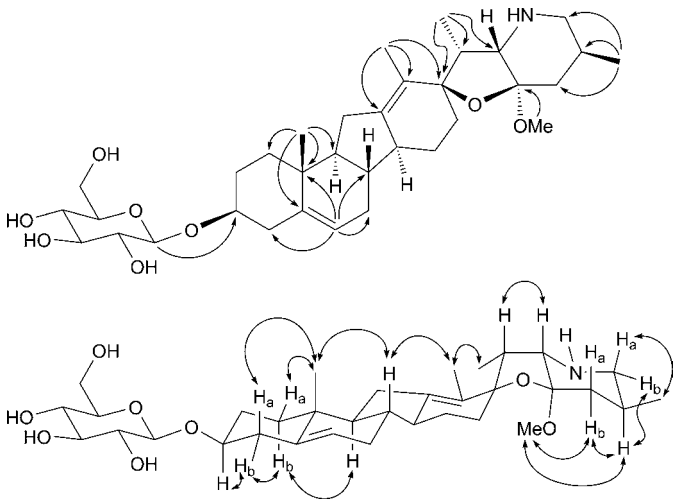

Fig. 2. *Key HMBCs* (H → C) *and NOESY* (H ↔ H) *correlations of* **1**

4.8, H–C(6)), 7.17 (br. *s*, H–C(24)), and 7.82 (br. *s*, H–C(26))). The ^{13}C-NMR spectrum displayed 27 C-atom signals, including those of four Me groups (δ(C) 19.6, 12.8, 19.6, and 18.0), of seven olefinic C-atoms (δ(C) 141.7, 122.0, 150.8, 151.0, 126.3, 134.2, 137.5), and of one O-bearing C-atom at δ(C) 71.9, evidencing the presence of an *O*-substituted pyridine ring, together with analyses of HMBC, HSQC, and ^{1}H,^{1}H-COSY spectra [9]. In the HMBC spectrum (*Fig. 3*), δ(H) 0.70 (Me(18)) showed cross-peaks with δ(C) 38.3 (C(12)), 43.0 (C(13)), 57.0 (C(14)), and 54.0 (C(17)); δ(H) 0.94 (Me(19)) correlated with δ(C) 38.0 (C(1)), 141.7 (C(5)), 51.1 (C(9)), and 37.2 (C(10)), δ(H) 1.20 (Me(21)) showed cross-peaks with δ(C) 54.0 (C(17)), 42.7 (C(20)), and 150.8 (C(22)), and δ(H) 2.33 (Me(27)) correlated with δ(C) 126.3 (C(24)), 134.2 (C(25)), and 137.5 (C(26)). The ^{1}H- and ^{13}C-NMR data of **2** were almost identical to those of ecliptalbine [9], except for three signals at δ(C) 54.0 (C(17)), 42.7 (C(20)), and 18.0 (C(27)) in **2**, which could be respectively ascribed to two CH groups at δ(H) 1.98–2.04 (*m*, H–C(17)), 3.25–3.31 (*m*, H–C(20)), and a Me group at δ(H) 2.33 (*s*, Me(27)), according to the HSQC and ^{1}H,^{1}H-COSY spectrum. In the NOESY spectrum (*Fig. 3*), the following cross-peaks were observed Me(19)/H$_a$–C(1), H$_a$–C(2), and H–C(8);

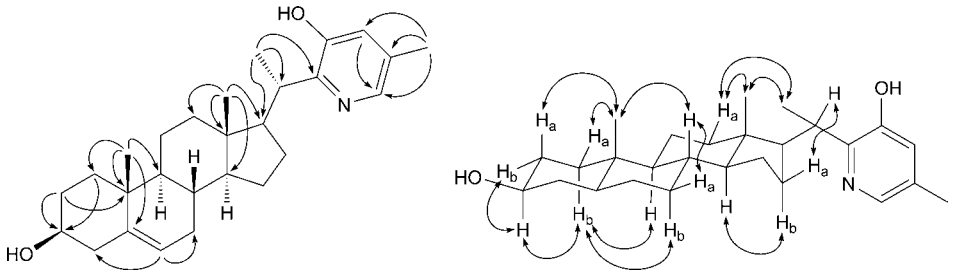

Fig. 3. *Key HMBCs* (H → C) *and NOESY* (H ↔ H) *correlations of* **2**

H–C(3)/H_b–C(1) and H_b–C(2); H_b–C(1)/H_b–C(7) and H–C(9); Me(18)/H_a–C(12) and Me(21); Me(21)/H_a–C(12)); H_a–C(16)/H–C(20), and H_b–C(16)/H–C(14), confirming β-positions of Me(19), H–C(8), and Me(18), α-orientations of H–C(3), H–C(9), and H–C(14), and the absolute configuration of **2** at C(20) was unequivocally determined as (*S*). Based on these data, compound **2** was the (20*S*)-epimer of ecliptalbine, and it was named isoecliptalbine.

Compounds **3**–**7** were identified as verussurine (**3**) [10], verabenzoamine (**4**) [10], verazine (**5**) [11], isoverazine (**6**) [11], and verazinine (**7**) [12], respectively, by various spectral analyses and comparison with literature data. Compound **3** and **4** were obtained from the title plant for the first time.

Genotoxicity. After the oral administration of compounds **1**–**7** at the doses of 0.25 and 2.5 μmol/kg, respectively, for 7 d, comet assays were performed in the cells obtained from the cerebellum and cerebral cortex region in mice. The extent of DNA damage was calculated from relative changes in tail moment. The obtained results showed that compounds **1**–**7** significantly increased the values of tail moment when compared to control groups (one-way ANOVA; $P < 0.001$; *Fig. 4*), suggesting that compounds **1**–**7** induced DNA damage to mouse cerebellum and cerebral cortex in a dose-dependent manner following a seven-day repetitive dose. Furthermore, compounds **1**–**3** and **7** caused more damage to brain cells of cerebellum and cerebral cortex of mice than compounds **4**–**6**, exhibiting different extents of DNA damage.

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Experimental Part

General. TLC: Precoated silica gel *GF*$_{254}$ (SiO_2; *Qingdao Haiyang Chemical Group Co.*) and SiO_2 *60 RP-18* $F_{254}S$ plates (*Merck*). Column chromatography (CC): SiO_2 (200–300 mesh; *Qingdao Haiyang Chemical Group Co.*). Optical rotations: *PerkinElmer 241 MC* polarimeter. UV Spectra: *Shimadzu UV-2201* spectrometer; λ_{max} (log ε) in nm. NMR Spectra: *Bruker AV-400* spectrometer; δ in ppm rel. to Me_4Si as internal standard, *J* in Hz. HR-ESI-MS: *Waters ESI-Q-TOF* mass spectrometer, in *m*/*z*.

Plant Material. The plant material of *Veratrum maackii* REGEL was collected in Henan Province of China in july 2012 and identified by Assoc. Prof. *Wang-Jun Yuan*, Henan University. A voucher specimen (2012007) was deposited with the Institute of Pharmacy, Pharmaceutical College, Henan University, P. R. China.

Animals. Male Swiss mice (22 ± 2 g) were obtained from the Experimental Animal Center of Zhengzhou University. All animal experiments were conducted according to guidelines established by the *NIH* Guide for the Care and Use of Laboratory Animals. The protocol was approved by an animal care and use committee of Henan University.

Extraction and Isolation. The dried roots and rhizomes of *Veratrum maackii* REGEL (6 kg) were extracted with 95% EtOH (3 × 42 l; 2 h each) under reflux. The extract was concentrated under reduced pressure, until the EtOH content was totally eliminated. The resulting aq. fraction was then partitioned successively with CH_2Cl_2 and AcOEt. The CH_2Cl_2 fraction (98 g) was subjected to CC (SiO_2; CH_2Cl_2/MeOH 100:1 → 1:1) to yield twelve fractions, *Frs. 1–12*. *Fr. 9* (0.7 g) was repeatedly separated by CC (SiO_2; CH_2Cl_2/MeOH 11:1) to yield **2** (25 mg). *Fr. 11* (0.6 g) was repeatedly submitted to CC (SiO_2; CH_2Cl_2/MeOH 8:1) to yield **7** (11 mg). The AcOEt extract (268 g) was subjected to CC (SiO_2; CH_2Cl_2/MeOH 100:1 → 0:100) to yield 15 fractions, *Frs. 1–15*. *Fr. 2* (2.8 g) was further subjected to CC (SiO_2; CH_2Cl_2/MeOH 100:4 → 0:100) to yield five fractions, *Frs. A1–A5*. *Fr. A2* (0.4 g) was further purified by

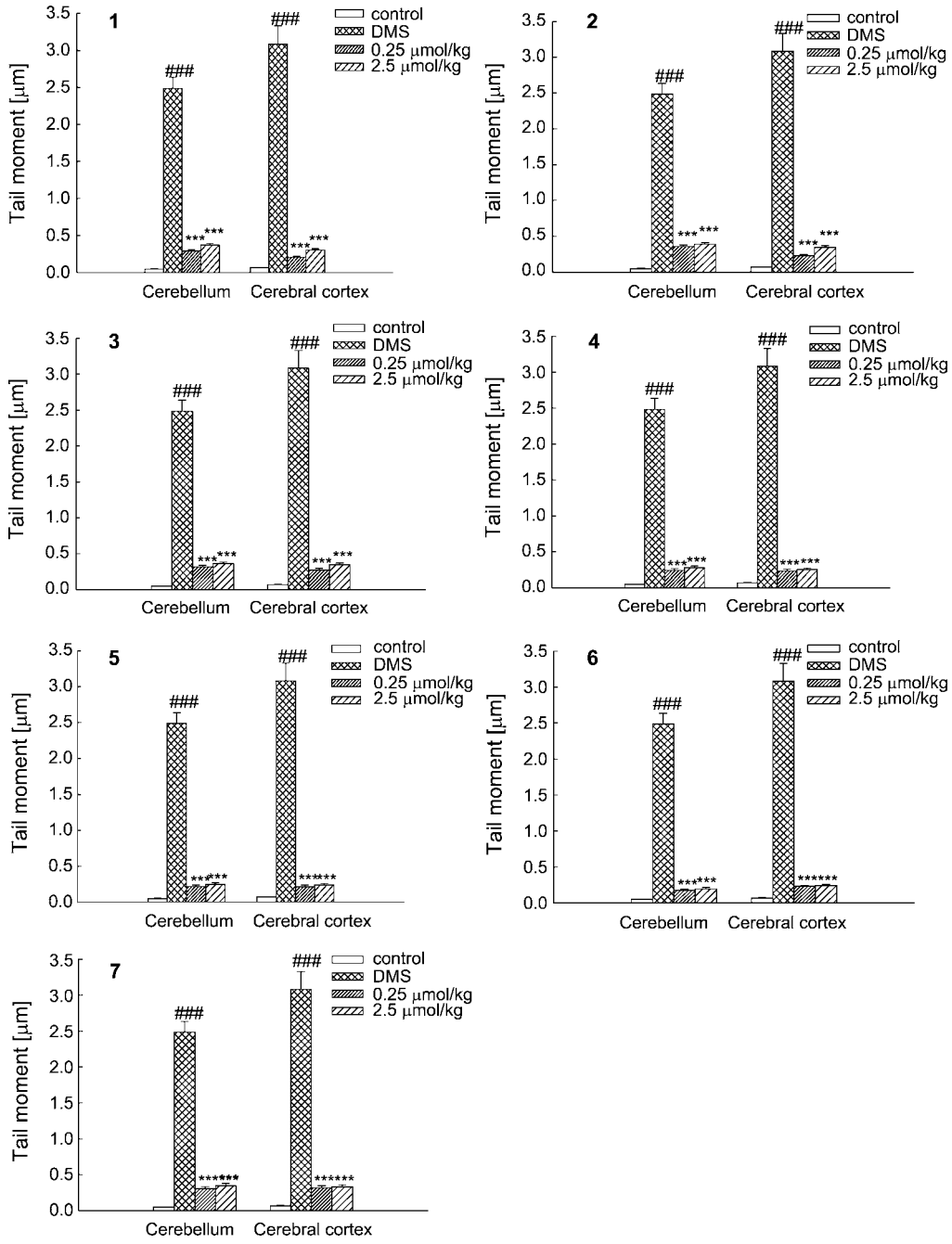

Fig. 4. *Effects of compounds* **1**–**7** *on brain-cell DNA strand breaks in the cerebellum and cerebral cortex of mice.* Two hundred cells were examined in duplicate for each condition, ***: $P < 0.001$; ###: $P < 0.001$.

CC (SiO_2; petroleum ether (PE)/AcOEt/MeOH 1:6:0.1) to furnish **1** (7 mg) and **3** (10 mg). *Fr. A3* (0.3 g) was further separated by CC (SiO_2; CH_2Cl_2/MeOH 12:1) to yield **4** (11 mg). *Fr. 3* (0.6 g) was further purified by CC (SiO_2; CH_2Cl_2/MeOH 100:4 → 0:100) to give four fractions, *Frs. B1–B4*. *Fr. B2* (0.1 g) was subjected to PTLC (SiO_2; PE/acetone 1:3) to yield **5** (6 mg) and **6** (4 mg).

*23-Methoxycyclopamine 3-*O*-β-*D*-Glucopyranoside* (*=(3β,22*S*,23*R*)-23-Methoxy-17,23-epoxyveratraman-3-yl β-*D*-Glucopyranoside = (3*S*,3′*R*,3a′*S*,6′*S*,6a*S*,6b*S*,7a′*R*,9*R*,11a*S*,11b*R*)-1,2,3,3′a,4,4′,5′,6,6′,6a,6b,7,7′,7′a,8,11,11a,11b-Octadecahydro-7′a-methoxy-3′,6′,10,11b-tetramethylspiro[9*H*-benzo[a]fluorene-9,2′(3′*H*)-furo[3,2-b]pyridin]-3-yl β-*D*-Glucopyranoside*; **1**). White powder. $[\alpha]_D^{20} = +4.7$ ($c =$ 0.01, MeOH). UV (MeOH): 218 (3.90), 255 (2.02). ^{1}H- and ^{13}C-NMR: see the *Table*. HR-ESI-MS: 604.3847 ($[M+H]^+$, $C_{34}H_{54}NO_8^+$; calc. 604.3849).

Isoecliptalbine (*=(3β,17β)-17-[(1*S*)-1-(3-Hydroxy-5-methylpyridin-2-yl)ethyl]androst-5-en-3-ol*; **2**). White powder. $[\alpha]_D^{20} = -2.8$ ($c = 0.01$, MeOH/CH_2Cl_2 1:1). UV (MeOH/CH_2Cl_2 1:1): 221 (3.96), 285 (4.16). ^{1}H- and ^{13}C-NMR: see the *Table*. HR-ESI-MS: 410.3057 ($[M+H]^+$, $C_{27}H_{40}NO_2^+$; calc. 410.3059).

Comet Assays. Mice were randomly divided into 16 groups of four mice in each group, which included control, positive, and compounds **1**–**7** at 0.25 and 2.5 μmol/kg, resp. The animals in the group of compounds **1**–**7** were treated by gavage with **1**–**7** at 0.25 and 2.5 μmol/kg every day for consecutive 7 d. The control group was treated with dist. H_2O for 7 d. At the last day, the positive group received an ip injection of dimethyl sulfate (DMS) at 20 mg/kg. The comet assay was performed according to the previous reports [3][5]. Tail moment was used as parameter to evaluate the DNA damage. Data were expressed as mean ± S.E.M. calculated from four mice of every group. Statistical comparisons were made by means of one-way analysis of variance (ANOVA), followed by the *Fisher*'s least-significant difference (LSD) test (SPSS 17.0 software, SPSS, USA).

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