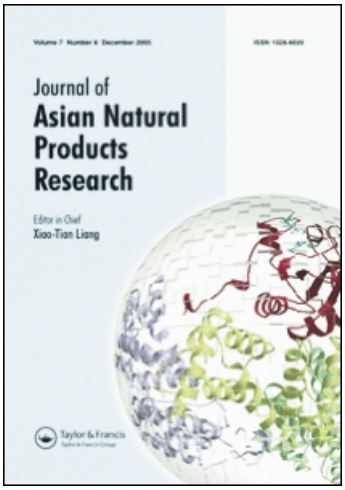

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A study on the chemical constituents of *Veratrum nigrum* L. processed by rice vinegar

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One new steroid alkaloid, 12β-hydroxylveratroylzygadenine (**1**) and four known compounds, verdine (**2**), jervine (**3**), veramarine (**4**), and veratroylzygadenine (**5**), have been isolated from the roots and rhizomes of *Veratrum nigrum* L. processed by rice vinegar. Their structures were established through a combined analysis of physicochemical properties and spectroscopic evidence. The assay results revealed that compounds **1**, **4**, and **5** exhibited cell toxicity against human HL-60 cells with IC_{50} values 52.67, 52.90, and 56.51 μmol/l, respectively.

Keywords: *Veratrum nigrum* L; steroid alkaloid; cytotoxicity; 12β-hydroxylveratroylzygadenine

1. Introduction

Veratrum nigrum L., belonging to the Liliaceae family, is a famous traditional medicinal plant in China. The plant has been reported to exhibit a variety of biological effects, such as hypotensive, emetic, and antifungal activities [1]. In China, it has been used for the treatment of hypertension, blood-stroke, excessive phlegm, and epilepsy [2]. However, its efficacy has been declined due to central nervous system toxicity of the plant. The toxicity can be reduced by roasting it with rice vinegar [3]. Herein, we describe the isolation and structural elucidation of one new steroid alkaloid – 12β-hydroxylveratroylzygadenine (**1**), as well as four known steroid alkaloids – verdine (**2**), whose NMR spectral data are completely assigned for the first time, jervine (**3**), veramarine (**4**), and veratroylzygadenine (**5**) (Figure 1) from the roots and rhizomes of *Veratrum nigrum* L. processed by rice vinegar. In addition, we also investigated the cytotoxicities of these compounds against human HL-60 cells.

2. Results and discussion

Compound **1**, white amorphous solid, mp 250–252°C, $[\alpha]_D^{20}$ + 16.0 [$c = 0.025$, MeOH, and $CHCl_3$ (2:1)], was assigned a molecular formula of $C_{36}H_{51}NO_{11}$ based on $[M + H]^+$ at m/z 674 in ESI-MS and 674.3554 $[M + H]^+$ in the HR-MS. In the IR spectrum, **1** showed absorptions at 3475, 2910, 2760 (*trans*-quinolizidine) [4], 1718, 1604, and 1517 cm^{-1}. The ^1H NMR spectrum of compound **1** exhibited three aromatic proton signals at δ_H 7.54 (1H, d, $J = 1.8$ Hz, H-3″), 7.01 (1H, d, $J = 8.4$ Hz, H-6″), 7.67 (1H, dd, $J = 8.4$, 1.8 Hz, H-7″), and two methoxyl signals at δ_H 3.89 (3H, s), 3.91 (3H, s). Together with ^{13}C NMR, HSQC, and HMBC spectra, we could make a conclusion of the presence of the veratroyl group. The HMBC

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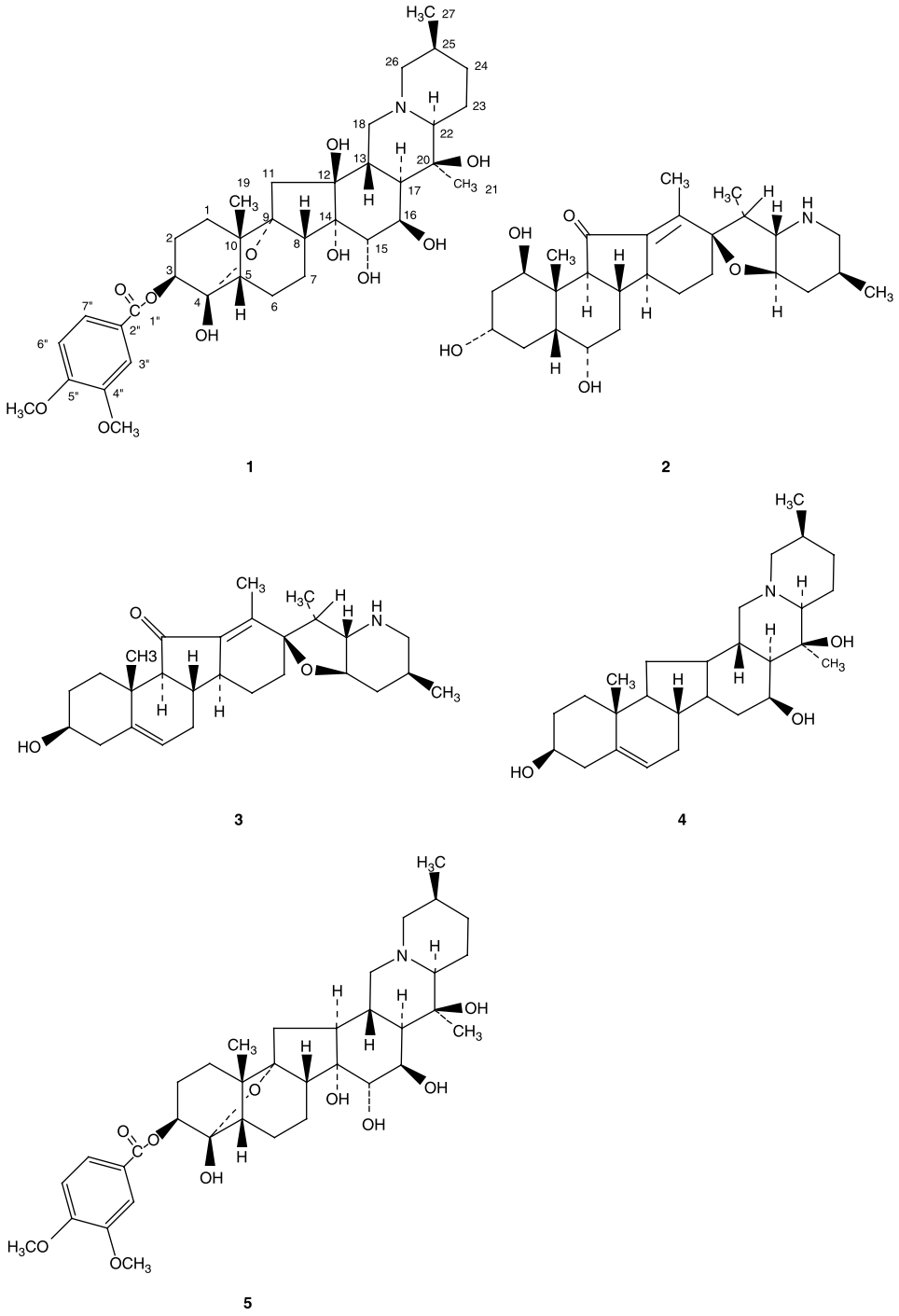

Figure 1. Structures of compounds **1**–**5**.

correlations between a carbonyl carbon signal at δ_C 167.2 (C-1″) and H-3 indicated the location of the veratroyl group at the C-3 position. The ^1H and ^{13}C NMR spectra of **1** were similar to those of compound **5**, except for an additional signal at δ_C 81.7 (C-12) in **1**, which could be ascribed to a quaternary carbon bearing a hydroxyl group according to the HMQC spectrum. Based on the HMBC and HMQC spectral data, the carbon signals at δ_C 81.7, 43.0, 36.9, 71.7, 80.9, and 57.5 were reasonably assigned to C-12, 11, 13, 15, 14, and 18, which were respectively shifted downfield by 37.6, 9.0, 3.5, 2.6 ppm and upfield by 2.1, 2.0 ppm, in comparison with those of compound **5**, due to the substituent effects of a hydroxyl group at C-12 [5]. The result was further confirmed by HMBC correlations between the quaternary carbon at δ_C 81.7 (C-12) and H-11, H-15, and H-17.

The relative stereochemistry of **1** was confirmed by the NOESY spectrum (Figure 2); Me-19 showed NOE correlations with H-5, H-8, and H-11β at δ_H 2.35 (d, $J = 15.6$ Hz), leading to the fusion of rings A, B, and C as follows: A/B *cis*, B/C *trans*, the same as those of compound **5**. The couplings between H-3 and H-2 [$J_{3,2\alpha}$ ($= J_{ea}$) = 4.2 Hz, $J_{3,2\beta}$ ($= J_{ee}$) ≈ 0 Hz] suggested that veratroyl group, connected to the position of C-3, should be β-orientated. The fusion of rings C and D in compound **5** is *cis*, which leads to a boat conformation of ring D [6]; there was a NOE correlation between H-13 and H-15 in the NOESY spectrum of compound **5**. However, there was no NOE correlation between H-13 and H-15 and a NOE correlation between H-7β at δ_H 1.58(m) and H-15 in the NOESY spectrum of compound **1**, which makes clear that the fusion of rings C, D of compound **1** is *trans*, leading to assignments of OH-12β. Moreover, several other NOE correlations were observed between H-11β and H-13, between H-8 and H-15, between Me-21 and H-16, H-17, H-22, as well as the absorption at 2760 cm^{-1} in the IR spectrum, indicating the fusion of rings D, E, and F as follows: D/E *trans*, E/F *trans*, and assignments of OH-15α, OH-16β, and Me-21α. H-25α was deduced by the couplings between H-25 and H-26 [$J_{25,26\alpha}$ ($= J_{ee}$) ≈ 0 Hz, $J_{25,26\beta}$ ($= J_{ea}$) = 4.0 Hz]. Based on the above information, a computer-generated plot for the 3D structure of **1** was obtained using the molecular modeling program Chemoffice 2002 CHEM 3D V7.0 by MM2 force-field calculations for energy minimization. The calculated distances between H—C(5)/3H—C(19) (2.863, 3.968, and 3.263 Å),

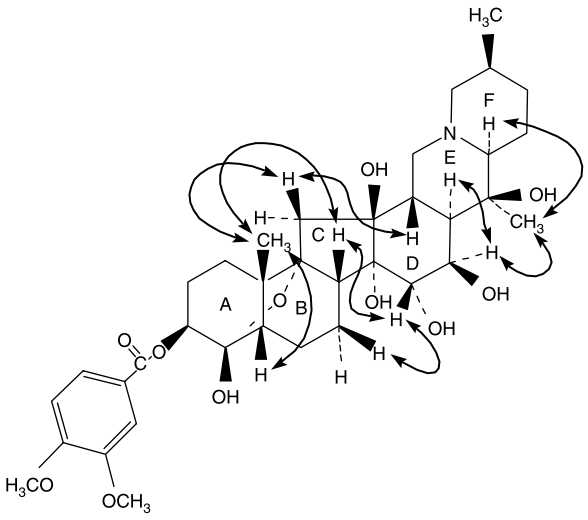

Figure 2. Key NOE correlations of **1**.

H—C(8)/3H—C(19) (3.604, 2.815, and 2.065 Å), Hβ—C(11)/2H—C(19) (2.607 and 3.819 Å), H—C(15)/H—C(8) (2.425 Å), H—C(15)/Hβ—C(7) (2.988 Å), H—C(13)/Hβ—C(11) (3.387 Å), H—C(16)/3H—C(21) (2.115, 2.982, and 3.720 Å), H—C(17)/3H—C(21) (3.121, 2.522, and 3.736 Å), and H—C(22)/3H—C(21) (3.205, 2.626, and 3.835 Å) are less than 4.00 Å, which are consistent with the well-defined NOEs observed for each of these proton pairs conforming the relative stereochemistry proposed above. Thus, compound **1** was determined to be 12β-hydroxylveratroylzygadenine.

Other four known compounds **2**–**5** were identified as verdine (**2**), jervine (**3**), veramarine (**4**), and veratroylzygadenine (**5**), respectively, by direct comparison of their spectral data with literature values [5,7–11].

Compounds **1**–**5** were tested for their cytotoxic activities *in vitro* against human HL-60 cells (from American Type Culture Collection) using the MTT method; adriamycin was used as a positive control with IC_{50} values of 0.018 μmol/l. Compounds **1**, **4**, and **5** exhibited a moderate activity with IC_{50} values of 52.67, 52.90, and 56.51 μmol/l, while compound **2** was inactive against human HL-60 cells and compound **3** was undissolved in the culture fluid. The result indicated that the carbon skeleton of the cevine group [12] was essential for the activity.

3. Experimental

3.1 General experimental procedures

The melting points were determined using MEL-TEMP capillary melting point apparatus and are uncorrected. ESI-MS was performed on a Finnigan LCQ mass spectrometer and HR-MS was performed on QSTAR LCQ mass spectrometer. NMR spectra were taken on a Bruker AV-600 and Bruker ARX-300 spectrometers using TMS as the internal standard. Optical rotations were measured on a Perkin-Elmer 241MC polarimeter. UV spectrum was recorded on a Shimadzu UV-2201 spectrometer and IR spectrum was obtained using IFS-55 spectrum instrument. Drum Type Herbal Medicine Roaster CYD500 was provided by Hangzhou Chunjiang Automation Research Institute. Silica gel for chromatography was provided by Qingdao Ocean Chemical Group Co. of China. All other chemicals and reagents were of analytical grade.

3.2 Plant material

The plant material of *Veratrum nigrum* L. was collected from Shenyang City, Liaoning Province, and was identified by Professor Qishi Sun (Shenyang Pharmaceutical University). A voucher specimen (No. 20040710) is deposited in Research Department of Natural Medicine, Shenyang Pharmaceutical University. The dried roots and rhizomes of *Veratrum nigrum* L. (10 kg) were mixed with a blend of rice vinegar (1.8 kg) and water (3.6 kg). When rice vinegar was absorbed completely, the mixture was roasted by Drum Type Herbal Medicine Roaster until dry [3,13].

3.3 Extraction and isolation

The dried roots and rhizomes of *Veratrum nigrum* L. (10 kg) processed by rice vinegar were extracted with 95% EtOH (50 l × 3) under reflux. The extract was concentrated and then acidified (pH = 3) with HCl followed by filtration. The filtrate was basified (pH = 10) with NH_4OH and extracted with $CHCl_3$ to get total alkaloids (50 g), which was subjected to column chromatographic separation on silica gel, gradiently eluting with petroleum ether (PE)–acetone to give five fractions (A–E). Fraction A [eluted with PE/acetone (100:10), 0.5 g] was rechromatographed on a silica gel column eluting with PE/acetone (100:8) to give **3** (30 mg). Fraction B [eluted with PE–acetone (100:18), 0.8 g] was rechromatographed over a silica gel column by a gradient elution with $CHCl_3$–acetone (5:1) to yield compound **4** (50 mg). Fraction C [eluted with PE–acetone (100:40), 0.5 g] was rechromatographed over a silica gel

column by a gradient elution with $CHCl_3$–acetone (1:1) to yield compound **5** (50 mg). Fraction D [eluted with PE–acetone (100:50), 3.4 g] was rechromatographed over a silica gel column with $CHCl_3$–MeOH (10:1) as the eluent to yield compound **1** (405 mg, mp 250–252°C). Fraction E [eluted with PE–acetone (20:80), 3.7 g] was rechromatographed over a macroporous resin column by elution with H_2O–MeOH (1:1) to yield fraction E-10 (0.4 g), which was further rechromatographed over a silica gel column by elution with $CHCl_3$–MeOH (10:2) to yield compound **2** (70 mg).

*3.3.1 Compound **1***

White amorphous solid; mp 250–252°C, $[\alpha]_D^{20}$ + 16.0 [c = 0.025, MeOH, and $CHCl_3$ (2:1)], IR (KBr) $\gamma^{max}cm^{-1}$: 3475, 2910, 2760, 1718, 1604, 1517, 1423, 1378, 1273, 1249, 1113, 1024, 950, 900, 769; UV λ_{max}^{MeOH} (nm):

Table 1. ^1H and ^{13}C NMR spectral data for compounds **1** (600 MHz, in $CDCl_3$ and CD_3OD), **2** (300 MHz, in CD_3OD), and **5** (300 MHz, in $CDCl_3$ and CD_3OD).

	1		**5**	**2**	
Position	H, m, (*J*)	C	C	H, m, (*J*)	C
1	1.68 m, 1.60 m	33.3	32.9	4.66 brs	72.5
2	2.16 m, 1.73 m	25.7	27.5	1.49 m, 1.83 m	38.4
3	5.09 brd (4.2)	76.6	76.2	3.88 m	66.6
4		105.5	105.7	1.24 quar (12.0), 1.88 m	30.2
5	2.26 m	47.4	47.1	1.90 m	43.9
6	1.98 m, 1.83 m	20.1	20.0	3.87 m	69.2
7	2.13 m, 1.58 m	18.2	18.2	1.46 m, 1.85 m	33.6
8	2.76 m	44.2	44.9	1.47 m	42.6
9		95.7	96.7	2.11 brd (12.3)	54.7
10		46.5	46.4		41.2
11	2.35 d (15.6), 1.88 d (15.6)	43.0	34.0		207.4
12		81.7	44.1		138.5
13	2.15 m	36.9	33.4		146.6
14		80.9	83.0	2.05 m	45.0
15	3.70 d (3.3)	71.7	69.1	1.17 m, 1.86 m	25.0
16	4.41 br t (2.7)	71.9	71.8	1.53 m, 1.84 m	31.6
17	1.76 m	40.8	39.7		87.2
18	2.77 m, 2.26 m	57.5	59.5	2.02 s	12.3
19	1.08 s	18.9	18.4	0.93 s	18.2
20		74.3	73.1	2.44 quin (7.8)	41.1
21	1.28 s	22.8	22.5	0.90 d (7.8)	11.0
22	1.67 m	72.0	70.7	2.63 t (9.0)	67.2
23	1.69 m, 1.60 m	19.6	17.9	3.29 td (10.2,3.8)	76.7
24	1.60 m, 1.58 m	30.5	27.5	1.09 quar (11.4), 2.06 m	39.6
25	1.90 m	28.9	27.2	1.60 m	31.7
26	2.74 brdd (11.4), 2.28 dd (11.4, 4.0)	63.3	60.5	2.22 t (12.0), 2.95 dd (12.0,4.2)	54.8
27	1.14 d (7.2)	17.9	16.4	0.87 d (7.2)	19.0
1″		167.2	166.7		
2″		123.8	123.4		
3″	7.54 d (1.8)	113.3	112.8		
4″		149.9	149.3		
5″		154.6	154.0		
6″	7.01 d (8.4)	111.6	111.2		
7″	7.67 dd (8.4, 1.8)	124.8	124.5		
4″-OCH_3	3.89	56.5	56.4		
5″-OCH_3	3.91	56.5	56.4		

292 (ε, 4.83×10^3), 261 (ε, 9.45×10^3), 221 (ε, 1.50×10^4); positive ESI-MS: *m/z* 674, HR-MS: *m/z* 674.3554 $[M + H]^+$ (calcd for $C_{36}H_{52}NO_{11}$, 674.3540); ^1H and ^{13}C NMR spectral data are listed in Table 1.

*3.3.2 Compound **2***

White amorphous solid; mp 200–202°C, $[\alpha]_D^{20}$ − 95.0 ($c = 0.02$, MeOH), positive ESI-MS: *m/z* 460. ^1H and ^{13}C NMR spectral data are listed in Table 1.

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