Introduction

The genus *Veratrum* (Liliaceae) comprises about 40 species (Huang et al., 2008) which can be found in many areas of the temperate northern hemisphere. *V. lobelianum* Bernh. and *V. nigrum* L. are very important because of their wide-spread and usage in folk medicine (Chinese medicine “Li-lu”) (Zhao et al., 1991). Numerous phytochemical studies on the species have shown that the main active components are steroidal alkaloids (Zhao et al., 1991; Tezuka et al., 1998a; Cong et al., 2007). In an earlier paper concerning the alkaloid pattern of *V. nigrum* we described the structures of veramitaline and the new steroidal alkaloid veranigrine together with the assumption that they may possess cytotoxicity for the M-109 cell line (Christov et al., 2009).

Here we report the alkaloid pattern of *V. lobelianum* and *V. nigrum* from different populations as well as the antiproliferative activity of veranigrine, veralosinine, veratrolyzygadenine, neogermitrine, and verabenzoamine.

Material and Methods

General

Melting points were determined on a Kofler microscope (uncorrected). Optical rotation was defined with a Perkin-Elmer 241 polarimeter. The NMR experiments were recorded in CD$_3$OD with TMS as internal standard on a Bruker Avance II+ 600 NMR spectrometer using standard Bruker software. HRCIMS was done with a Waters QToF Premier instrument (Hannover, Germany) with an ESI-ion source equipped with an Acquity UPLC console. Column chromatography (CC) was carried out on neutral Al$_2$O$_3$ Brockmann II (1:100) and the mobile phase was a petroleum ether/Mc$_2$CO/MeOH gradient. TLC was performed on silica gel F$_{254}$ (Merck) plates with the mobile phase dichloroethane/Mc$_2$CO/EtOH (2:0.50:0.25) in vapours of NH$_3$, dichloroethane/MeOH/petroleum ether (2:0:5:0.2) in vapours of NH$_3$, and dichloroethane/Mc$_2$CO/MeOH/25% NH$_2$OH (2:0.50:0.50:0.02). Preparative TLC was run on 20 × 20 cm plates with silica gel GF$_{254}$ (1 mm thickness) Merck and above-mentioned mobile phases.
Plant materials

Roots and rhizomes of *V. lobelianum* Bernh. were picked up from Tsagaan-Uur sumon, Khubsugul province, Northwest Mongolia (N 195) at the end of August 2008 and from Southwestern Bulgaria (SOM-11407) at the beginning of December 2008. The samples from roots and rhizomes of *V. nigrum* L. were collected from Bayan-Dun province, Dornod, East Mongolia (N 39) at the end of August 2007 and from Northwestern Bulgaria (SOM-11156) in the middle of September 2007. Prof. E. Ganbold, Dr. D. Zumberelmaa, and Dr. D. Dimitrov identified the plant materials. A voucher specimen from each sample is deposited at the Herbarium of the Institute of Botany, Mongolian Academy of Sciences and Institute of Botany, Bulgarian Academy of Sciences.

Extraction and isolation

3.60 kg (Mongolian) and 0.75 kg (Bulgarian) dried and powdered plant materials of *V. lobelianum* and 6 kg (Mongolian) and 0.50 kg (Bulgarian) of *V. nigrum* were extracted exhaustively with EtOH. After evaporation to dryness the dried and powdered plant materials of *V. lobelianum* and Dr. D. Dimitrov identified the plant materials.

Isoveralosinine (2): Yield 1.2 mg solid phase from 3.6 kg *V. lobelianum* (as a mixture with 1 in the ratio 1:1.5 according to the 1H NMR spectrum). – 1H NMR: see Table I.

(−)-Veralosine (havanine, 3) (Khashimov et al., 1970; Basterechea et al., 1984): Yield 3.6 mg from 3.6 kg *V. lobelianum* (as a mixture with 4 in the ratio 2:1 according to the 1H NMR spectrum). – Pale yellow amorphous solid. – [α]D20 = −36.91° (c 0.0018, MeOH). – 1H and 13C NMR: see Table I.

Isoveralosine (4): Yield 3.6 mg from 3.6 kg *V. lobelianum* (as a mixture with 3 in the ratio 1:2). – 1H and 13C NMR: see Table I.


−1H NMR (600 MHz, CD3OD): δH = 5.3 Hz, H-8, J5,6 = 5.0 Hz, H-3, 3.04 (1H, overlapped, H-22, 2H-26), 2.23 (1H, dd, J5,6 = 13.0 Hz, J5,7 = 5.0 Hz, J6,7 = 2.1 Hz, H-4e), 2.21 (1H, m, H-4a), 2.07 (1H, overlapped, H-25), 2.01 (1H, dt, J = 12.5, 3.2 Hz, H-12), 2.0 (1H, overlapped, H-8), 1.95 (1H, overlapped, H-20), 1.9–1.5 (10H, H-19), 1.35 (1H, overlapped, H-17), 1.32 (1H, overlapped, H-12), 1.10 (2H, overlapped, H-9, H-11), 1.11 (3H, d, J = 7.0 Hz, CH3-27), 1.08 (3H, d, J = 7.4 Hz, CH3-21), 1.02 (3H, s, CH3-19), 0.77 (3H, s, CH3-18). – 13C NMR (150 MHz, CD3OD): δC = 142.6 (C-5), 122.4 (C-6), 77.3 (C-16), 72.8 (C-3), 64.0 (C-22), 62.3 (C-17), 55.6 (C-14), 52.0 (C-26), 51.8 (C-9), 45.7 (C-13), 43.3 (C-4), 41.3 (C-12), 39.3 (C-20), 38.7 (C-1), 37.9 (C-10), 36.8 (C-15), 33.1 (C-8, C-7), 32.5 (C-2), 30.2 (C-24), 27.6 (C-25), 24.5 (C-23), 22.1 (C-11), 20.1 (C-19), 17.1 (C-27), 16.6 (C-21), 13.8 (C-18).


−1H and 13C NMR spectra and comparisons with the data in literature: Zhao et al. (1991).

The known alkaloids veratroylzygadenine (6), 3-O-β-d-glucopyranosyl)etioline (7), (−)-jervine

Unauthenticated
rubijervine (9), (-)-neogermitrine (11),
(+)-germidine (12), (+)-verabenzoamine (13),
and zyadenine (14) were identified using 1H,
13C, DEPT, 2D-NMR spectra, HRCIMS, physical
constants like melting point and optical rotation,
and comparison with the literature data (Kadota
et al., 1995; Ripperger, 1996a; Tezuka et al., 1998a,
b; Sayed et al., 1995; Han et al., 1991). The alkaloids
6, 7 and 11, 12, 13 were from Mongolian
populations of V. lobelianum and V. nigrum, while
the alkaloids 8, 9 and 6, 14 were isolated from V.
lobelianum and V. nigrum from Bulgarian populations.

Cell cultures

L5178 mouse T-cell lymphoma cells (U.S. FDA,
USA) were transfected with pHa MDR1/A retro-

inactivation horse serum, L-glutamine, and

in McCoy's 5A medium supplemented with 10%

expression of the MDR phenotype. L5178 (paren-

heat-inactivated horse serum, L-glutamine, and

and comparison with the literature data (Kadota

and antibiotics. The mouse lymphoma cell line was

cells with 60 ng/ml colchicine to maintain the ex-

were selected by culturing the infected

alcoholase subline were cultured at 37 ºC

in 5A medium supplemented with 10% heat-inactivated horse serum, L-glutamine, and

lymphoma cell line was maintained in a 5% CO2 atmosphere.

Assay of antiproliferative effect

The effects of increasing concentrations of the

and their combinations with resistance modifiers on cell growth were tested in 96-

growth was determined as the percentage accord-

plates were further incubated at 37 °C overnight.

200 μl of sodium dodecyl sulfate (SDS) solution (10%) were added to each well, and the

plates were further incubated at 37 °C overnight. The cell growth was determined by measuring

the optical density (OD) at 550 nm (ref. 630 nm) with

a Multiscan EX ELISA reader (Thermo Lab-
systems, Cheshire, WA, USA). Inhibition of cell
growth was determined as the percentage according to the formula:

\[
\text{inhibition (\%) = 100 - } \frac{\text{OD(sample)} - \text{OD(medium control)}}{\text{OD(cell control)} - \text{OD(medium control)}} \times 100,
\]

where OD(sample) is the optical density of the sample, OD(medium control) is the optical density
of the medium control, and OD(cell control) is the optical density of the cell control. The ID50
value was defined as the concentration of compound, which inhibited 50% of cell proliferation.

Results and Discussion

Phytochemical studies

Twelve steroidal alkaloids have been isolated
from four populations of V. lobelianum and V.
nigrum. They are from verazine, cevamine, jervine,
and solanidine types.

In earlier papers 1 was identified mainly by
chemical transformations and CD (Khashimov
et al., 1971; Moiseeva et al., 1976). Here we report
for the first time full 1H and 13C NMR data of
1 (Table I). The assignment was made with the
aid of 1D- and 2D-NMR spectra and comparison
with the NMR data of etioline (Ripperger,
1996a). The NMR spectral data confirmed the
axial and \( \alpha \) orientation of H-3 at \( \delta 3.39 \) \( \text{tt (J = 11.4, 4.50 Hz) as well as the } \beta \text{ orientation of H-16 because of its NOESY correlation with CH}_3\text{-18.}

The \( S \) configuration at C-20 was determined by
comparison with the NMR data for solafloroi-

dine [(20S,25R)-22,26-epiminocholest-22(N)-ene-
3β,16α-diol] and 20-isosolafloridine [(20R,25R)-
22,26-epiminocholest-22(N)-ene-3β,16α-diol]. The
values of the coupling constants of H-20 at \( \delta 2.50 \)
dq \( (J = 11.4, 6.4 Hz, \) the NOE effect between
CH\text{-3} and CH\text{-3} are in agreement with the first structure (Ripperger, 1996b).

The 25S configuration was unambiguously de-
termined with the help of CD spectra (Moiseeva
et al., 1976) of veralosidine and its derivatives,
among them veralsosinine. The coupling constant
of H-26a in axial orientation at \( \delta 2.70 \) with H-25
at \( \delta 1.57 \) \( (J = 10.9 Hz \) indicated the axial position
of H-25 and that CH\text{-27 is in equatorial position. \text{This was also confirmed by the NOE effect of H-26a and CH}_3\text{-27.}

In the course of isolation of 1 from V. lobel-
ianum we obtained a fraction (1.2 mg) containing
two compounds. As it can be seen in Table I,
Table I. $^1$H and $^{13}$C NMR data of 1, 2, 3, and 4 in CD$_3$OD; $\delta$ in ppm ($J$ in Hz).

<table>
<thead>
<tr>
<th>No.</th>
<th>$^{13}$C NMR</th>
<th>$^1$H NMR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>1 CH$_2$</td>
<td>38.7</td>
<td>38.7</td>
</tr>
<tr>
<td>2 CH$_2$</td>
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<td>31.1</td>
</tr>
<tr>
<td>3 CH</td>
<td>72.6</td>
<td>79.8</td>
</tr>
<tr>
<td>4 CH$_2$</td>
<td>43.3</td>
<td>40.3</td>
</tr>
<tr>
<td>5 C</td>
<td>142.6</td>
<td>142.3</td>
</tr>
<tr>
<td>6 CH</td>
<td>122.4</td>
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</tr>
<tr>
<td>11 CH$_2$</td>
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</tr>
<tr>
<td>12 CH$_2$</td>
<td>41.3</td>
<td>41.2</td>
</tr>
<tr>
<td>13 C</td>
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</tr>
<tr>
<td>14 CH</td>
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</tr>
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</tr>
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<tr>
<td>Glc</td>
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</tr>
<tr>
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<tr>
<td>3' CH</td>
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</tr>
<tr>
<td>4' CH</td>
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<td></td>
</tr>
<tr>
<td>5' CH</td>
<td>78.2</td>
<td></td>
</tr>
<tr>
<td>6' CH$_2$</td>
<td>63.1</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ Interchangeable. $^b$ Overlapped signals. $^c$ Overlapped with the signal of the solvent.
the two compounds showed doubling of some signals, and most probably they are diastereomers. The \(^1\)H NMR spectrum of the first compound corresponded to \(1\). There were no differences in the signals for the rings A, B, and C. The coupling constants of H-20 were also the same for the two compounds. The main difference was in the signal of H-26\(\alpha\), \(\delta\) 2.70 dd (\(J = 16.3, 10.9\) Hz) and \(\delta\) 2.99 dd (\(J = 17.0, 9.8\) Hz) for \(1\) and \(2\), respectively. By analogy with etioline and isoetioline (Ripperger, 1990), a 25\(R\) configuration could be assigned to the second compound. That means most probably it is \((20S,25R)-16\alpha\)-acetoxy-22,26-epiminocholest-5,22(N)-dien-3\(\beta\)-ol (isoveralosinine, \(2\)) (Fig. 1.)

The \(^13\)C NMR spectrum of \(3\) showed differences in the chemical shifts for some of the carbon atoms, and doubling of the signals for the protons H-16, H-17, H-18, H-20, H-25, H-26\(\alpha\), H-26\(\alpha\), CH\(_3\), and CH\(_2\)CO in the \(^1\)H NMR spectrum. The main differences were in the chemical shifts of H-26\(\alpha\) at \(\delta\) 2.78 dd (\(J = 16.3, 10.8\) Hz) and \(\delta\) 3.01 dd (\(J = 16.5, 9.5\) Hz) for \(3\) and \(4\), respectively, or in other words there were again two isomers: \((20S,25S)-O(3\beta\delta\)-d-glucopyranosyl-16\(\alpha\)-acetoxy-22,26-epiminocholest-5,22(N)-dien-3\(\beta\)-ol (veralosine, \(3\)) and \((20S,25R)-O(3\beta\delta\)-d-glucopyranosyl-16\(\alpha\)-acetoxy-22,26-epiminocholest-5,22(N)-dien-3\(\beta\)-ol (isoveralosine, \(4\)) (Fig. 1) in approximately the ratio 1:1.

To our knowledge this is the first report for the presence of \(2\) and \(4\) in plants. The exhaustive \(^1\)H NMR data of \(3\) are presented here for the first time.

From the biogenetic point of view there is no inconsistency with the presence of \(2\) and \(4\) because the former is the aglycone of the latter one as well as \(1\) is the aglycone of \(3\). Teinemine (\(5\)) (Fig. 1) was identified by 1D- and 2D-NMR spectra and comparison with the data in the literature (Gaffield et al., 1982). Here we present exhaustive \(^1\)H NMR data of \(5\) for the first time. \((\pm\)-15\(\alpha\)-(2-Methylbutyroyl)germine (\(10\)) (Fig. 1) has not been isolated up to now.

### Biological assays

Among the mechanisms by which cancer cells evade chemotherapy multidrug resistance (MDR) is certainly the best known. MDR is characterized by cross-resistance between numerous natural products used in cancer treatment especially antibiotics and plant alkaloids (Robert, 1996; Lavie et al., 1996; Lavie et al., 1990). Here we present biological assays of the steroidal alkaloids veralosine (\(1\)), isoveralosine (\(2\)), veralosine (\(3\)), isoveralosine (\(4\)), teinemine (\(5\)), and 15\(\alpha\)-(2-methylbutyroyl)germine (\(10\)).

#### Table II. Antiproliferative effects of alkaloids on MDR cell line.

<table>
<thead>
<tr>
<th>Compound</th>
<th>ID(_{50}) [(\mu g/ml)](^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Veranigrine</td>
<td>20.76</td>
</tr>
<tr>
<td>Veralosinine ((1))</td>
<td>22.69</td>
</tr>
<tr>
<td>Veratroylzygadenine ((6))</td>
<td>24.86</td>
</tr>
<tr>
<td>Neogermitrine ((11))</td>
<td>21.76</td>
</tr>
<tr>
<td>Verabenzoamine ((13))</td>
<td>26.07</td>
</tr>
<tr>
<td>DMSO</td>
<td>25.95</td>
</tr>
</tbody>
</table>

\(^a\) ID\(_{50}\) concentration of the compound inhibiting 50\% of cell proliferation; each value is the mean from parallel experiments (\(n = 2\)).
etal., 2001). In this connection and with respect to our previous assumptions (Christov et al., 2009), we decided to examine some of the steroidal alkaloids for their antiproliferative activity. From the isolated alkaloids we have chosen veranigrine, veralosinine (1), veratroylzycadendeine (6), neoergemitrine (11), and verabenzoamine (13) as they are in pure form and in sufficient quantity. They were tested by the MTT test against the human MDR1 gene-transfected mouse lymphoma cells. All of them displayed close cell growth inhibitory potency. The preliminary investigations demonstrated that veranigrine had the lowest ID$_{50}$ value (20.76 μg/ml) while verabenzoamine showed the highest ID$_{50}$ value (26.07 μg/ml) (Table II).

These data are valuable for development of this kind of plant alkaloids as possible antiproliferative agents of cancer cells and for design and modification for new anticancer agents.

Acknowledgements

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