Alkaloids from *Leucojum vernum* and Antiretroviral Activity of Amaryllidaceae Alkaloids

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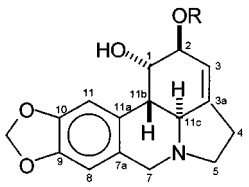
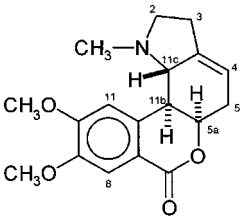
Abstract

Three alkaloids, lycorine, homolycorine and 2-*O*-acetyllycorine, were isolated from the bulbs of *Leucojum vernum* (Amaryllidaceae) and identified by means of NMR analysis. The alkaloids obtained from *L. vernum* and from other Amaryllidaceae species were studied *in vitro* for HIV-1 replication inhibitory activity on MT4 cells. The cytotoxicity of the compounds in uninfected cells was evaluated by using the MTT assay and the [^3H]thymidine incorporation test. The antiviral activities were determined by means of the p24 antigen assay and solid-phase reverse transcriptase testing. The results demonstrate that trisphaeridine, lycorine, homolycorine, and haemanthamine possess high antiretroviral activities (IC_{50} = 0.4 – 7.3 μg/mL), accompanied by low therapeutic indices (TI_{50} = 1.3 – 1.9).

The Amaryllidaceae species have provided many new promising antiviral alkaloids. Narciclasine, pancratistatine and lycorine have been found to exert pronounced inhibitory effects against Flaviviruses and Bunyaviruses [1]. Lycorine, the most intensively investigated compound of this group, displayed strong antiviral effects against poliovirus, Coxsackie, Semliki forest, measles and *Herpes simplex* type I viruses [2], [3], and pretazettine proved active against various oncogenic viruses [4]. Moreover, the DNA- and RNA-binding activities of Amaryllidaceae alkaloids have also been confirmed, suggesting that the antiviral effects are consequence of complex formation of these alkaloids with DNA and/or RNA [5], [6]. These results prompted us to investigate the alkaloids of *Leucojum vernum* L., which have not been investigated earlier, and to assay the HIV-1 inhibitory activities of the alkaloids obtained here and earlier from Amaryllidaceae species [7].

Fractionation of a MeOH extract of the bulbs of *L. vernum* with pH gradient solvent-solvent partition afforded an alkaloid fraction. When subjected to multiple chromatographic purifications this fraction yielded compounds **1 – 3**. By means of NMR spectral analysis, compound **1** was identified as lycorine, and **2** as homolycorine [7], [8], [9]. Its ^{1}H-NMR and JMOD spectra indicated that compound **3** was a monoacetyl derivative of lycorine. All carbon and proton signals were assigned through ^{1}H-^{1}H COSY, HSQC and HMBC experiments. The ^{1}H-^{1}H COSY spectrum demonstrated an -CH-CH-CH(OH)-CH(OAc)-CH = (C-11c–C-11b–C-1–C-2–C-3) structural fragment in the molecule (correlative signals between δ_H = 2.80/2.71, 2.71/4.52, 4.52/5.32 and 5.32/5.47), indicating the structure 2-*O*-acetyllycorine (**3**). This compound was previously isolated from *Hippeastrum aulicum* var. *robustum*, and also prepared as a semisynthetic derivative [10], [11]. As a result of extensive NMR studies previously unpublished ^{1}H and ^{13}C chemical shift assignments can now be reported.





	R
1	H
3	$COCH_3$

2

Compounds **1 – 3** and alkaloids isolated from other Amaryllidaceae species [7] were tested *in vitro* for HIV-1 growth inhibitory activity on the MT4 human T cell line. Cytotoxicity was controlled in uninfected cells by means of MTT and [^3H]thymidine incorporation assays (Table **1**). The TC_{50} values for lycorine (**1**), haemanthidine and haemanthamine were 0.75, 1.0, and 1.0 μg/mL, respectively. The MT4 cells tolerated trisphaeridine, 3-epimacronine, tazettine and homolycorine in higher concentrations (TC_{50} = 7.5 – 12.8 μg/mL). The $TCID_{50}$/mL of the HIV-1 used was characteristically 95. The antiviral activities were determined by p24 antigen assay and solid-phase reverse transcriptase (RT) assay on the supernatants of the cells on the 7th day after infection and treatment with alkaloids and with the positive control AZT. For homolycorine, the MTT assay was used [12]. Trisphaeri-

Table **1** Cytotoxicity and antiretroviral effects of Amaryllidaceae alkaloids[a]

Alkaloids	*TC_{50} (μg/mL)[b]*	*ID_{50} (μg/mL)[c]*	*ID_{50} (μg/mL)[d]*	*TI_{50}*
Lycorine (**1**)	0.75	0.4	0.4	1.9
Homolycorine (**2**)	12.8	7.3	–	1.8
Trisphaeridine	7.50	5.0	5.0	1.5
Haemanthamine	1.00	0.8	–	1.3
Haemanthidine	1.00	not effective	–	–
3-Epimacronine	7.50	not effective	–	–
Tazettine	7.50	not effective	–	–

[a] All values are averages of the results of five independent experiments.
[b] TC_{50} was determined by means of the MTT and [^3H]thymidine incorporation assays.
[c] Determined by p24 antigen assay, with the exception of homolycorine, where the MTT assay was used.
[d] Determined by RT assay.
[e] TI_{50} = ID_{50}/TC_{50}.

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dine, lycorine and haemanthamine exhibited pronounced antiviral activity in the p24 test and the RT assay (examples are presented in Fig. **1**), whereas haemanthidine, tazettine and 3-epimacronine were not effective (Table **1**). The MTT test revealed that homolycorine inhibited HIV-1 replication. The active compounds displayed low therapeutic indices (TI_{50}). None of the alkaloids directly inhibited the activities of AMV (avian myeloblastosis virus) RT and HIV RT (data not shown), suggesting that their targets are different. Our investigations permitted the conclusion that lycorine (**1**), homolycorine (**2**), haemanthamine and trisphaeridine inhibit HIV-1 replication effectively *in vitro*.

Materials and Methods

NMR spectra were recorded on a Bruker Avance DRX 500 spectrometer at 500 MHz (^{1}H) and 125 MHz (^{13}C). Optical rotations were determined with a Perkin-Elmer 341 polarimeter. Silica gel (Merck 5715) was used for preparative TLC, and silica gel (Kieselgel 60 G, 15 μm, Merck 11677) for vacuum liquid chromatography (VLC). The VLC column was developed under gentle vacuum (about 20 – 70 mm Hg), provided by a water aspirator. The degree of purity of the tested compounds was over 95% as documented by the ^{1}H NMR spectra.

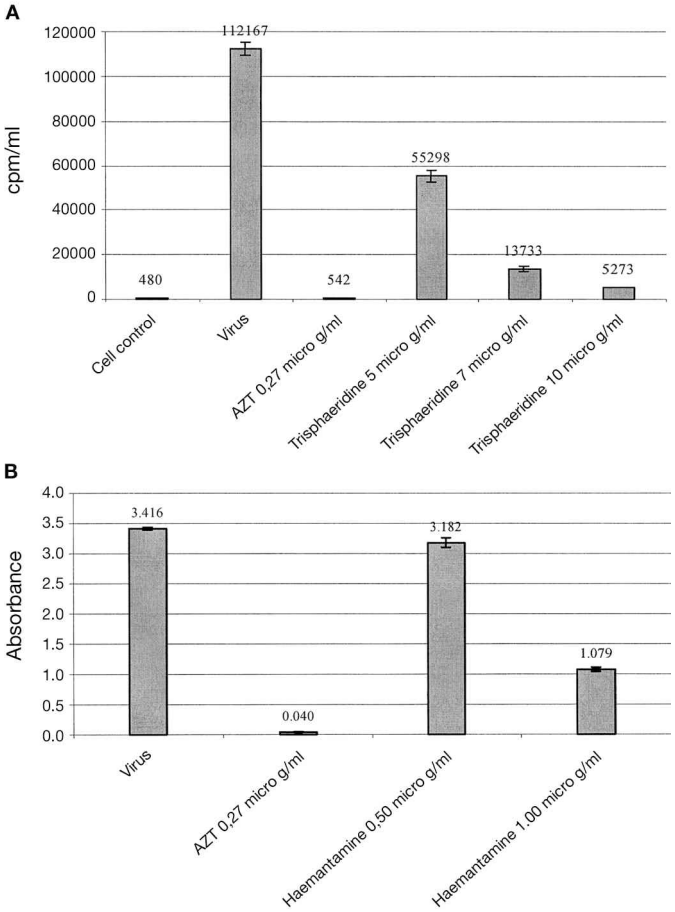

Fig. **1** Effects of Amaryllidaceae alkaloids on HIV-1 replication *in vitro*. **A** Reverse transcriptase test. **B:** p24 test.

The bulbs of *Leucojum vernum* were purchased from Spalax Ltd. (Ecser, Hungary) in September 2002. A voucher specimen (No. 604) has been preserved in the Herbarium of the Department of Pharmacognosy, University of Szeged, Hungary.

The external coloured leaves of the fresh bulbs (1500 g) were removed, and the bulbs were then exhaustively extracted with MeOH (12 L). The MeOH extract was concentrated (300 mL) and acidified with 2% HCl to pH 2.5. After extraction with 6×500 mL Et_2O, the acidic solution was adjusted to pH 8.5 with concentrated NH_3. The solution was extracted with 10×500 mL $CHCl_3$ to yield the crude alkaloid fraction (7.2 g). The $CHCl_3$ extract was fractionated by VLC using a gradient system of cyclohexane-$CHCl_3$-MeOH (1:1:0, 25:25:1, 25:25:2, 25:25:3, 25:25:5, 10:10:3, 10:10:6, and 1:1:1, each 150 mL) collecting fractions of 15 mL. Fractions 20 – 22 were chromatographed by preparative TLC, first with cyclohexane-$CHCl_3$-MeOH (50:30:7) (system A), and then with benzene-$CHCl_3$-MeOH (7:2:1) in NH_3 vapour (system B) with detection at UV 254 nm, affording compound **2** (R_f: system A 0.36; system B 0.74). After preparative TLC purification using system B, fractions 23 – 27 furnished **3** (R_f: system A 0.36; system B 0.56). Upon standing, fractions 36 – 39 yielded lycorine (**1**) as crystalline material (m.p. 248 – 249 °C, lit. [13] m.p. 248 – 249 °C).

Homolycorine (**2**): pale-yellow crystals; m.p. 177 – 178 °C. $[\alpha]_D^{28}$: +98 (*c* 0.1, EtOH); {lit. [14] m.p. 175 °C; $[\alpha]_D$: +85 (95% EtOH)}; ^{1}H-NMR ($CDCl_3$, 500 MHz): δ = 2.00 (3H, s, *N*-Me), 2.23 (1H, q, *J* = 9.8 Hz, H-2), 2.50 (2H, m, H-3), 2.63 (3H, m, H-5, H-11b), 2.71 (1H, brm, H-11c), 3.12 (1H, ddd, *J* = 9.8, 3.3, 7.2 Hz, H-2), 3.94 (3H, s, OCH_3), 3.95 (3H, s, OCH_3), 4.80 (1H, t, *J* = 2.3, H-5a), 5.50 (1H, brd, *J* = 2.3, H-4), 6.96 (1H, s, H-11), 7.56 (1H, s, H-8); ^{13}C-NMR: identical with published data [8].

2-O-Acetyllycorine (**3**): pale-yellow crystals; m.p. 224 – 225 °C; $[\alpha]_D^{28}$: +22 (*c* 0.1, EtOH); {lit. [10] m.p. 230 – 231 °C; $[\alpha]_D$: +20 (*c* 0.2, EtOH)}; ^{1}H-NMR ($CDCl_3$, 500 MHz): δ = 2.08 (3H, s, OAc), 2.39 (1H, brq, *J* = 8.9 Hz, H-5), 2.65 (2H, m, H-4), 2.71 (1H, d, *J* = 10.4 Hz, H-11b), 2.80 (1H, d, *J* = 10.4 Hz, H-11c), 3.36 (1H, ddd, *J* = 8.9, 4.6, 4.6 Hz, H-5), 3.53 (1H, d, *J* = 14.0 Hz, H-7), 4.15 (1H, d, *J* = 14.0 Hz, H-7), 4.52 (1H, s, H-1), 5.32 (1H, brs, H-2), 5.47 (1H, brs, H-3), 5.91 and 5.94 (each 1H, each d, *J* = 1.2 Hz, OCH_2O), 6.59 (1H, s, H-8) 6.81 (1H, s, H-11); ^{13}C-NMR ($CDCl_3$, 125 MHz): δ = 21.2 (CH_3CO), 28.8 (C-4), 41.9 (C-11b), 53.7 (C-5), 57.0 (C-7), 60.7 (C-11c), 69.4 (C-1), 73.8 (C-2), 101.0 (OCH_2O), 104.7 (C-11), 107.7 (C-8), 113.7 (C-3), 127.2 (C-11a), 130.1 (C-7a), 146.0 (C-3a), 146.4 (C-10), 146.7 (C-9), 170.5 (CH_3CO). Copies of the original spectra are obtainable from the author of correspondence.

Bioassays: Cells: The human T cell line MT4 (described in ref. [15]) originated from cord blood lymphocytes cocultured with leukaemic cells of a patient with adult T cell leukaemia. The cells were maintained in RPMI 1640 medium (SIGMA) supplemented with 10% foetal bovine serum (SIGMA), 100 IU/mL of penicillin and 100 μg/mL of streptomycin (SIGMA). For cytotoxicity and antiviral assays, the alkaloids were dissolved in DMSO and diluted with RPMI medium. The final concentration of DMSO used was between 0.05 and 1.0%. In the concentrations used, DMSO did not influence the cell viability.

Virus: The clarified cell-free supernatant of HIV-1 (HTLV-IIIB)-infected MT4 cells was used as virus source. The amount of HIV-1 ($TCID_{50}$/mL) in the supernatant was determined by a virus yield assay [16].

MTT assay: The modified MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay was performed as in ref. [17]. The 50% cytotoxic concentrations (TC_{50}) were determined.

[^{3}H]Thymidine incorporation assay: MT4 cells were incubated with virus and/or alkaloids. After incubation for 6 days, 5 μL of [^{3}*H*]TTP (2 μCi/mmol) was added to the wells. The incubation was continued for a further 24 h, the cells were then suspended, and aliquots were dropped onto filter paper squares. The radioactivities were measured in a scintillation cocktail, using a Packard 1600 CA liquid scintillation analyser.

P24 antigen assay: The INNOTEST™ HIV Antigen mAb test (INNOGENETICS®) was performed on 100 μL of the supernatant of the treated and control cells according to the instructions of the manufacturer. AZT (Retrovir, Glaxo Wellcome) was used as a positive control (data shown on Fig. **1**). The concentration of the drug was 0.27 μg/mL = 1 μM, chosen according to the literature data [18].

Direct reverse transcriptase (RT) assay: The modified microassay was carried out as described in [19].

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