Supporting Information

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Alkaloid Profiling of *Hippeastrum* Cultivars by GC-MS, Isolation of Amaryllidaceae Alkaloids and Evaluation of Their Cytotoxicity

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Materials and Methods

General Experimental Procedures

All solvents were treated by using standard techniques before use. All reagents were purchased from commercial sources (Sigma Aldrich, Czech Republic) and used without purification. The NMR spectra were obtained in $CDCl_3$, CD_3OD and DMSO at ambient temperature on a VNMR S500 (Varian) spectrometer operating at 500 MHz for ^{1}H and 125.7 MHz for ^{13}C. Chemical shifts were recorded as δ values in parts per million (ppm) and were indirectly referenced to tetramethylsilane (TMS) via the solvent signal ($CDCl_3$ - 7.26 ppm for ^{1}H and 77.0 ppm for ^{13}C; CD_3OD – 3.30 ppm for ^{1}H and 49.0 ppm for ^{13}C; DMSO – 2.49 ppm for ^{1}H and 39.7 ppm for ^{13}C). Coupling constants (*J*) are given in Hz. For unambiguous assignment of ^{1}H and ^{13}C signals, 2D NMR experiments, namely gCOSY, gHSQC, gHMBC and NOESY, were conducted using standard parameter settings and standard pulse programs provided by the producer. The EI-MS of isolated alkaloids were obtained on an Agilent 7890A GC 5975 inert MSD operating in EI mode at 70 eV (Agilent Technologies, Santa Clara, CA, USA). A DB-5 column (30 m × 0.25 mm × 0.25 μm, Agilent Technologies, USA) was used. The temperature program was: 100-180°C at 15°C/min, 1 min hold at 180°C, and 180-300°C at 5°C /min and 5 min hold at 300°C; detection range *m/z* 40-600. The injector temperature was 280°C. The flow-rate of carrier gas (helium) was 0.8 mL/min. A split ratio of 1:15 was used. TLC was carried out on Merck precoated silica gel 60 F254 plates. Compounds on the plate were observed under UV light (254 and 366 nm) and visualized by spraying with Dragendorff´s reagent.

Plant Materials

The fresh bulbs of all *Hippeastrum* taxa (between 150 g - 250 g) were obtained from the herbal dealer Lukon Glads (Sadská, Czech Republic). The botanical identification was performed by Prof. L. Opletal, CSc. Voucher specimens are deposited in the herbarium of the Faculty of Pharmacy in Hradec Králové under the following numbers: *Hippeastrum* cv. Pretty Nymph CUFPH-16130/AL-569, *H.* cv. Artic Nymph CUFPH-16130/AL-574, *H.* cv. Daphne CUFPH-16130/AL-563, *H.* cv. Double King CUFPH-16130/AL-567, *H.* cv. Ferrari CUFPH-16130/AL-562, and *H.* cv. Spartacus CUFPH-16130/AL-570.

Preparation of Alkaloidal Extracts

Fresh bulbs (3 x 15 g) were extracted 3 times with EtOH (50 mL) at room temperature for 24 h. The solvent was evaporated under reduced pressure and the residue dissolved in 2% HCl (10 mL). After removal of neutral compounds with diethyl ether (3 x 15 mL), the extract was basified with 10% $NaHCO_3$ and the alkaloids extracted with EtOAc (3 x 15 mL). The organic solvent was removed by evaporation. The dry alkaloid fraction (5 mg) was dissolved in MeOH to a final concentration of 1 mg/mL for further analysis. The isolation of montanine (**1**), vittatine (**2**), 11-hydroxyvittatine (**3**), lycorine (**4**), and hippeastrine (**5**) is described in detail in Supplementary Material. The isolated alkaloids were characterized by comparison of their MS, NMR, and additional physical properties with literature data [1-3]. The purity of all the isolated compounds was ≥ 95 % (Supplementary Material).

GC-MS Analysis of Alkaloidal Extracts

GC-MS analysis was performed on an Agilent 890A GC 5975 inert MSD operating in EI mode at 70 eV (Agilent Technologies, Santa Clara, CA, USA). The separation was carried out on a DP-5 MS column (30 m × 0.25 mm × 0.25 μm, Agilent Technologies Santa Clara, CA, USA). The temperature program was from 100-150°C at 15°C/min, 1 min hold at 180°C and then 180°C-300°C at 5°C/min and a 35 min hold at 300°C. The injector temperature was 280°C. The flow rate of carrier gas (helium) was 0.8 mL/min. The detection range was *m/z* 35-600, and the detector temperature 200°C. An injection of 1 μL of alkaloid solution (1 mg/mL) was introduced in split mode (split ration 1:10). The individual alkaloids were identified based on comparison of their MS with those in the NIST library, with reported spectra in the literature, and finally with spectra of reference compounds isolated earlier in our laboratory. The confirmation of molecular weight was accomplished by a GCMS-QP2010 plus system with chemical ionization (Shimadzu, Japan). Isobutane (3.5; Linde Gas a.s. – Linde Technoplyn a.s., Czech Republic) was used as a reagent gas. The separation was carried out on a HP-5MS UI column (30 m × 0.25 mm × 0.25 μm, Agilent Technologies Santa Clara, CA, USA) and the temperature gradient described above was used. The injector temperature was maintained at 280°C. The carrier gas (helium) flow rate was set at 0.8 mL/min. An injection of 1 μL of alkaloid solution (1 mg/mL) was introduced in split mode (split 1:3) on the column. The samples were monitored over the full scan *m/z* 70-550. The detector temperature was kept at 200°C.

Isolation of Amaryllidaceae Alkaloids

*Isolation of Montanine (**1**)*

Montanine (**1**; 25 mg) was isolated from the alkaloidal extract of *Hippeastrum* cv. Pretty Nymph (265 g, 187 mg of extract) by preparative TLC (To:Et_2NH 9:1, three times). The structure was determined by comparison of its MS and NMR data, and additional physical properties with literature data [1]. The purity of the isolated compound was ≥ 95 %.

^{1}H NMR (500MHz, $CDCl_3$) δ: 6.56 (1H, s), 6.47 (1H, s), 5.91–5.90 (1H, m), 5.88–5.87 (1H, m), 5.60–5.58 (1H, m), 4.36 (1H, d, *J* = 16.6 Hz), 4.12–4.09 (1H, m), 3.84 (1H, d, *J* = 16.6 Hz), 3.50–3.48 (1H, m), 3.48–3.45 (2H, m, overlapped), 3.45 (3H, s, overlapped), 3.33–3.31 (1H, m), 3.11 (1H, dd,

J = 11.3 Hz, *J* = 2.4 Hz), 3.05 (1H, d, *J* = 11.3 Hz), 2.24–2.17 (1H, m), 1.59 (1H, td, *J* = 12.2 Hz, *J* = 3.5 Hz)

^{13}C NMR (125MHz, $CDCl_3$) δ: 153.5, 146.8, 146.1, 132.2, 124.2, 113.2, 107.3, 106.8, 100.8, 79.6, 68.8, 60.7, 58.8, 57.6, 55.3, 45.5, 32.5

*Isolation of Vittatine (**2**)*

Vittatine (**2**; 12 mg) was isolated from the alkaloidal extract of *Hippeastrum* cv. Double King (191 g, 120 mg of extract) by preparative TLC (To:Et_2NH 9:1, two times). The structure was determined by comparison of its MS and NMR data, and additional physical properties with literature data [1]. The purity of the isolated compound was ≥ 95 %.

^{1}H NMR (500MHz, $CDCl_3$) δ: 6.85 (1H, s), 6.59 (1H, d, *J* = 9.8 Hz), 6.47 (1H, s), 5.67 (1H, dd, *J* = 9.8 Hz, *J* = 5.3 Hz), 5.90 (1H, d, *J* = 8.3 Hz, overlapped), 5.89 (1H, d, *J* = 8.3 Hz, overlapped), 4.37 (1H, d, *J* = 17.0 Hz, overlapped), 4.36–4.33 (1H, m, overlapped), 3.76 (1H, d, *J* = 17.0 Hz), 3.41–3.31 (2H, m), 2.93–2.85 (1H, m), 2.18 (1H, ddd, *J* = 12.2 Hz, *J* = 9.3 Hz, *J* = 4.4 Hz), 2.02–1.96 (1H, m), 1.91 (1H, ddd, *J* = 12.2 Hz, *J* = 10.3 Hz, *J* = 5.8 Hz), 1.74 (1H, td, *J* = 13.7 Hz, *J* = 4.4 Hz)

^{13}C NMR (125MHz, $CDCl_3$) δ: 146.1, 145.7, 138.4, 132.2, 127.5, 126.4, 107.0, 102.8, 100.7, 64.0, 62.8, 62.4, 53.6, 44.25, 44.22, 32.8

*Isolation of 11-hydroxyvittatine (**3**)*

11-Hydroxyvittatine (**3**; 12 mg) was isolated from the alkaloidal extract of *Hippeastrum* cv. Ferrari (218 g, 120 mg of extract) by preparative TLC (To:cHx:Et_2NH 45:50:5, three times). The structure was determined by comparison of its MS and NMR data, and additional physical properties with literature data [1]. The purity of the isolated compound was ≥ 95 %.

^{1}H NMR (500MHz, $CDCl_3$) δ: 6.85 (1H, s), 6.48 (1H, s), 6.41 (1H, d, *J* = 10.3 Hz), 6.36 (1H, dd, *J* = 10.3 Hz, *J* = 4.9 Hz), 5.92–5.90 (2H, m), 4.42–4.38 (1H, m), 4.32 (1H, d, *J* = 17.1 Hz), 4.01–3.98 (1H, m), 3.69 (1H, d, *J* = 17.1 Hz), 3.43–3.36 (2H, m), 3.26 (1H, dd, *J* = 14.0 Hz, *J* = 2.9 Hz), 2.26 (1H, td, *J* = 14.0 Hz, *J* = 4.4 Hz), 1.95–1.90 (1H, m)

^{13}C NMR (125MHz, $CDCl_3$) δ: 146.5, 146.3, 135.1, 134.2, 126.9, 126.7, 106.9, 103.2, 100.9, 80.1, 64.2, 63.5, 62.3, 61.4, 50.1, 32.3

*Isolation of Lycorine (**4**)*

Lycorine (**4**, 35 mg) was isolated from the alkaloidal extract of *Hippeastrum* cv. Artic Nymph 256 g, 187 mg of extract) by preparative TLC (To:EtOH:Et_2NH 7:2:1, two times). The structure was determined by comparison of its MS and NMR data, and additional physical properties with literature data [2]. The purity of the isolated compound was ≥ 95 %.

^{1}H NMR (500MHz, DMSO) δ: 6.80 (1H, s), 6.67 (1H, s), 5.95–5.93 (2H, m), 5.38–5.35 (1H, m), 4.87 (1H, d, *J* = 5.3 Hz), 4.76 (1H, d, *J* = 3.8 Hz), 4.28–4.25 (1H, m), 4.01 (1H, d, *J* = 14.0 Hz), 3.99–3.95 (1H, m), 3.35–3.32 (1H, m, overlapped), 3.32 (1H, d, *J* = 14.0 Hz, overlapped), 3.21–3.16 (1H, m), 2.60 (1H, d, *J* = 10.5 Hz), 2.53–2.37 (1H, m), 2.20 (1H, dd, *J* = 17.3 Hz, *J* = 8.6 Hz)

^{13}C NMR (125MHz, DMSO) δ: 145.8, 145.4, 141.8, 129.9, 129.8, 118.7, 107.2, 105.2, 100.7, 71.9, 70.4, 61.0, 56.9, 53.5, 40.3, 28.3

*Isolation of Hippeastrine (**5**)*

Hippeastrine (**5**, 15 mg) was isolated from the alkaloidal extract of *Hippeastrum* cv. Daphne 175 g, 162 mg of extract) by preparative TLC (To:EtOH:Et_2NH 7:2:1, two times). The structure was determined by comparison of its MS and NMR data, and additional physical properties with literature data [3]. The purity of the isolated compound was ≥ 95 %.

^{1}H NMR (500MHz, CD_3OD) δ: 7.41 (1H, s), 7.05 (1H, s), 6.11 (2H, s), 5.69–5.66 (1H, m), 4.59–4.57 (1H, m), 4.28–4.25 (1H, m), 3.18 (1H, ddd, *J* = 9.8 Hz, *J* = 8.2 Hz, *J* = 2.3 Hz), 2.88 (1H, dd, *J* = 9.8 Hz, *J* = 2.3 Hz), 2.66–2.49 (3H, m), 2.36–2.30 (1H, m), 2.05 (3H, s)

^{13}C NMR (125MHz, CD_3OD) δ: 166.5, 153.8, 149.7, 145.3, 140.8, 120.2, 119.5, 110.2, 109.8, 103.9, 84.1, 68.3, 68.0, 57.1, 43.6, 40.8, 28.7

In Vitro Cytotoxicity Study

Cell Culture and Culture Conditions

Selected human tumor and non-tumor cell lines {Jurkat (acute T cell leukemia), MOLT-4 (acute lymphoblastic leukemia), A549 (lung carcinoma), HT-29 (colorectal adenocarcinoma), PANC-1 (pancreas epithelioid carcinoma), A2780 (ovarian carcinoma), HeLa (cervix adenocarcinoma), MCF-7 (breast adenocarcinoma), SAOS-2 (osteosarcoma) and MRC-5 (normal lung fibroblasts)} were purchased from either ATCC (Manassas, USA) or Sigma-Aldrich (St. Louis, USA) and cultured according to the provider´s culture method guidelines. All cell lines were maintained at 37 °C in a humidified 5% carbon dioxide and 95% air incubator. Cells in the maximum range of either 10 passages for the primary cell line (MRC-5), or 20 passages for the cancer cell lines (Jurkat, MOLT-4, A549, HT-29, PANC-1, A2780, HeLa, MCF-7 and SAOS-2) and in an exponential growth phase were used for this study.

Cell Treatment

All the alkaloids evaluated and doxorubicin, used as positive control, were dissolved in dimethyl sulfoxide – DMSO (Sigma-Aldrich, St. Louis, USA) to prepare stock solutions with a concentration of 10 - 50 mM based on their solubility. Stock solutions were freshly prepared before use in the experiments. For the experiments, the stock solutions were diluted with the appropriate culture medium to create final concentrations (10 µM for a single-dose alkaloid cytotoxicity screen and 1 µM for doxorubicin, used as a reference compound) making sure that the concentration of DMSO was < 0.1 % to avoid toxic effects on the cells. Control cells were sham-treated with a DMSO vehicle only (0.1 %; control).

WST-1 Cytotoxicity Assay

The WST-1 (Roche, Mannheim, Germany) reagent was used to determine the cytostatic effect of the test compounds. WST-1 is designed for the spectrophotometric quantification of cell proliferation, growth, viability and chemosensitivity in cell populations using a 96-well-plate format (Sigma, St.Louis, MO, USA). The principle of WST-1 is based on photometric detection of the

reduction of tetrazolium salt to a colored formazan product. The cells were seeded at a previously established optimal density (30000 Jurkat, 25000 MOLT-4, 500 A549, 1500 HT-29, 2000 PANC-1, 5000 A2780, 500 HeLa, 1500 MCF-7, 2000 SAOS-2 and 2000 MRC-5 cells/well) in 100 µL of culture medium, and adherent cells were allowed to reattach overnight. Thereafter, the cells were treated with 100 µL of either corresponding alkaloids or doxorubicin stock solutions to obtain the desired concentrations and incubated in 5% CO_2 at 37 °C. WST-1 reagent diluted 4-fold with PBS (50 µL) was added 48 hours after treatment. Absorbance was measured after 3 hours incubation with WST-1 at 440 nm. The measurements were performed in a Tecan Infinite M200 spectrometer (Tecan Group, Männedorf, Switzerland). All experiments were performed at least three times with triplicate measurements at each drug concentration per experiment. The viability was quantified according to the following formula: (%) viability = (Asample - Ablank) / (Acontrol - Ablank) x 100, where A is the absorbance of the employed WST-1 formazan measured at 440 nm. The viability of the treated cells was normalized to the viability of cells treated with 0.1 % DMSO (Sigma-Aldrich, St.Louis, MO, USA) as a vehicle control.

Statistical Analysis

The descriptive statistics of the results were calculated and the charts made in either Microsoft Office Excel 2010 (Microsoft, Redmond, WA, USA) or GraphPad Prism 5 biostatistics (GraphPad Software, La Jolla, CA, USA). In this study, all of the values were expressed as arithmetic means with SD of triplicates (n = 3), unless otherwise noted. The significant differences between the groups were analyzed using the Student's t-test and a P value ≤ 0.05 was considered statistically significant.

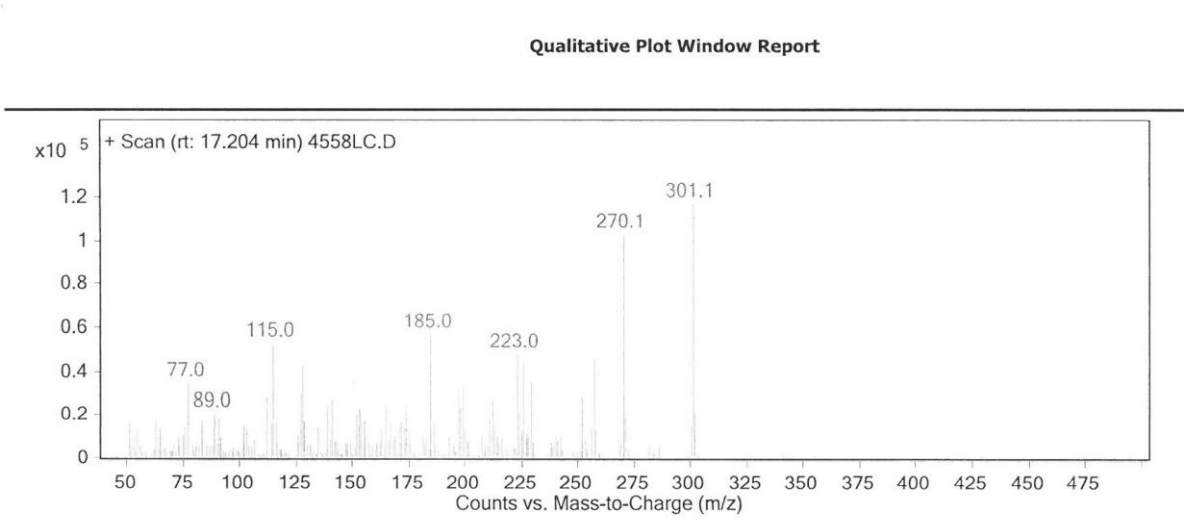

Figure S1: EI-MS Spectrum of Montanine (**1**)

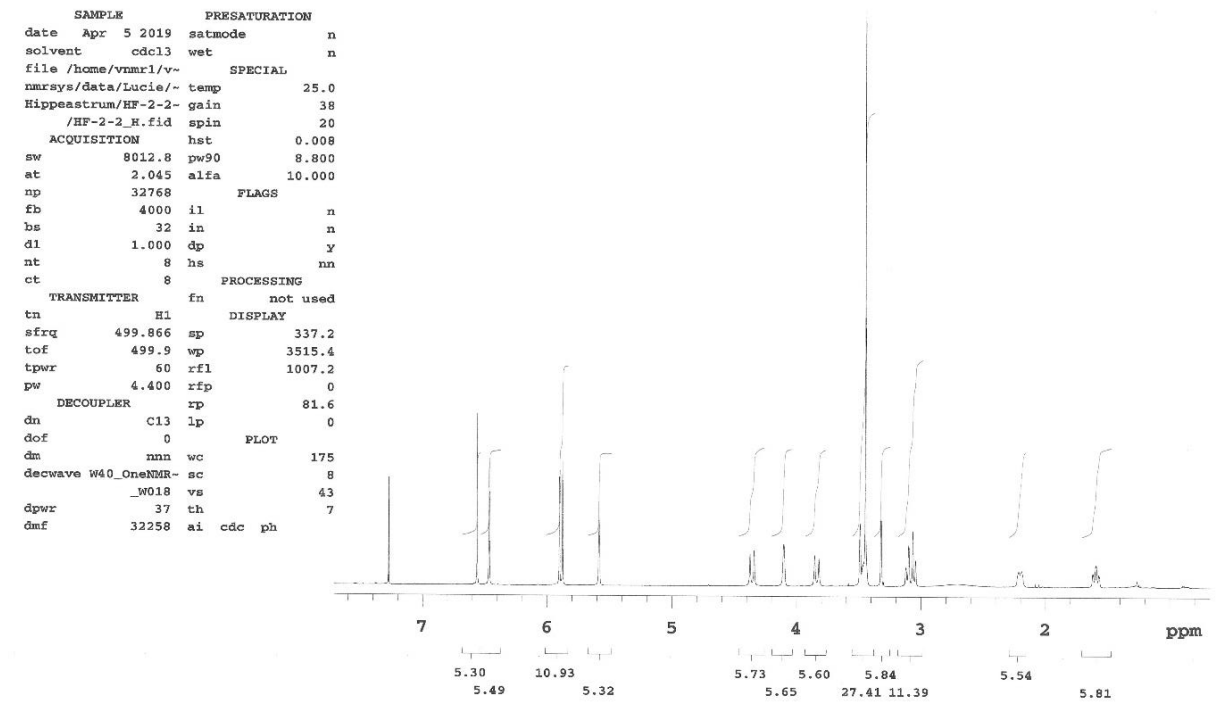

Figure S2: ^{1}H-NMR (500 MHz, $CDCl_3$) Spectrum of Montanine (**1**)

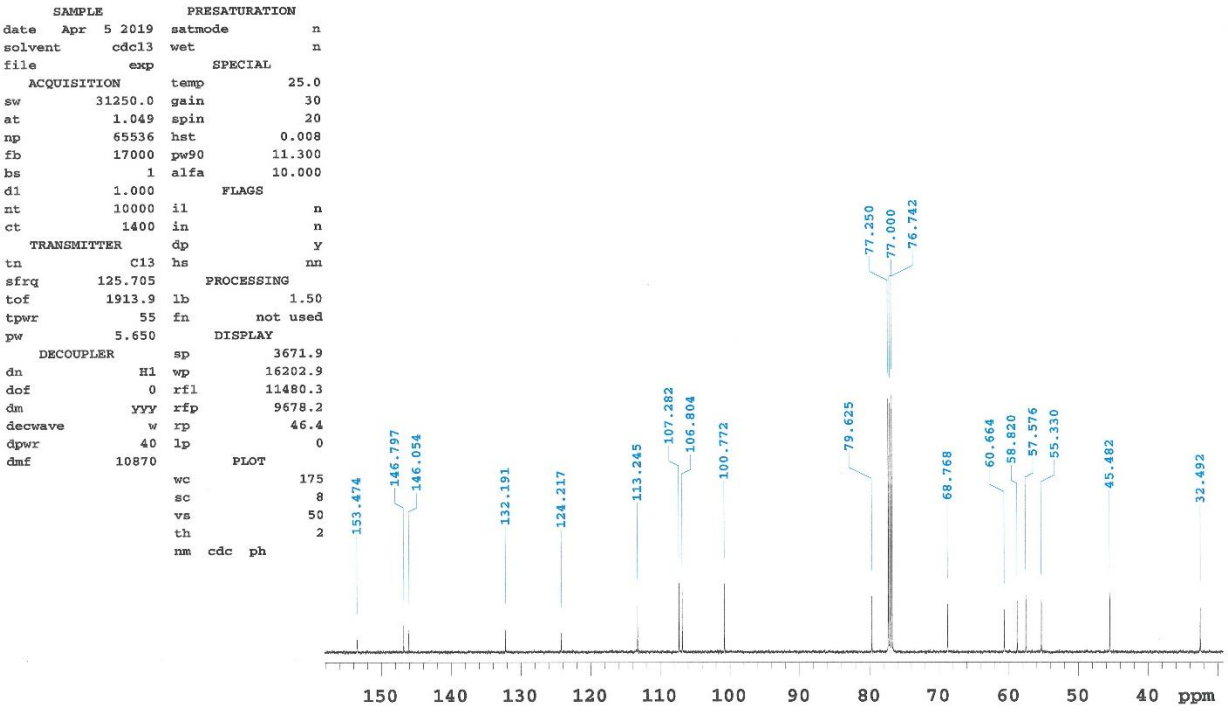

Figure S3: ^{13}C-NMR (125 MHz, $CDCl_3$) Spectrum of Montanine (**1**)

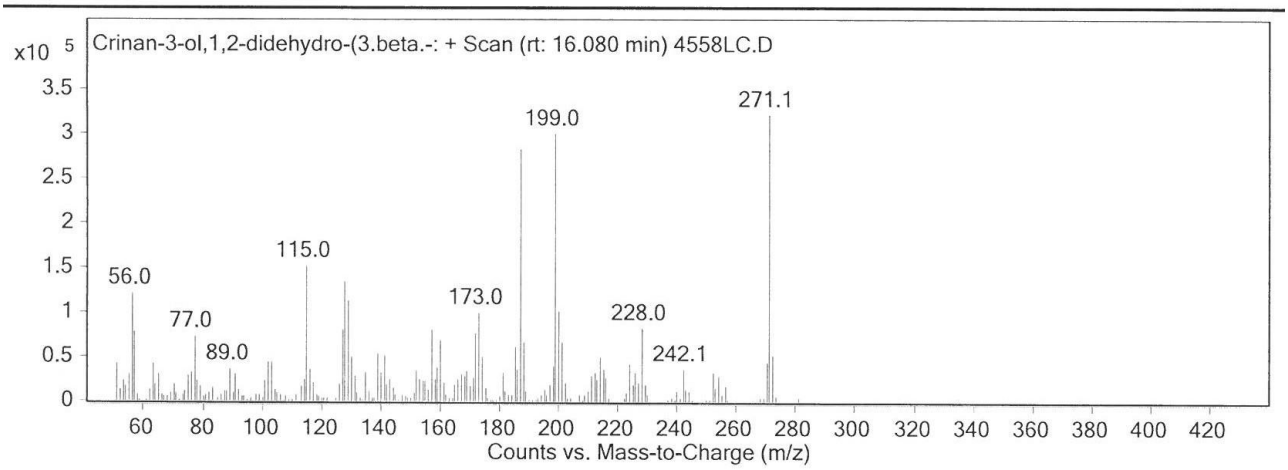

Figure S4: EI-MS Spectrum of Vittatine (**2**)

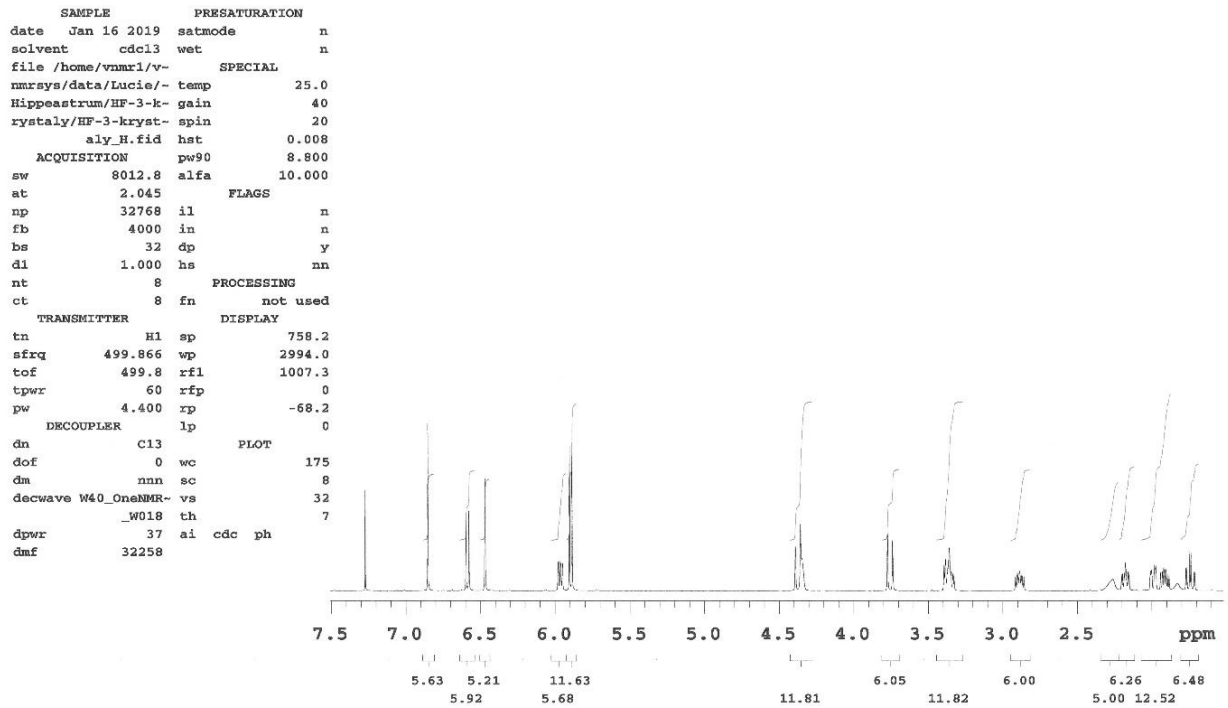

Figure S5: ^{1}H-NMR (500 MHz, $CDCl_3$) Spectrum of Vittatine (**2**)

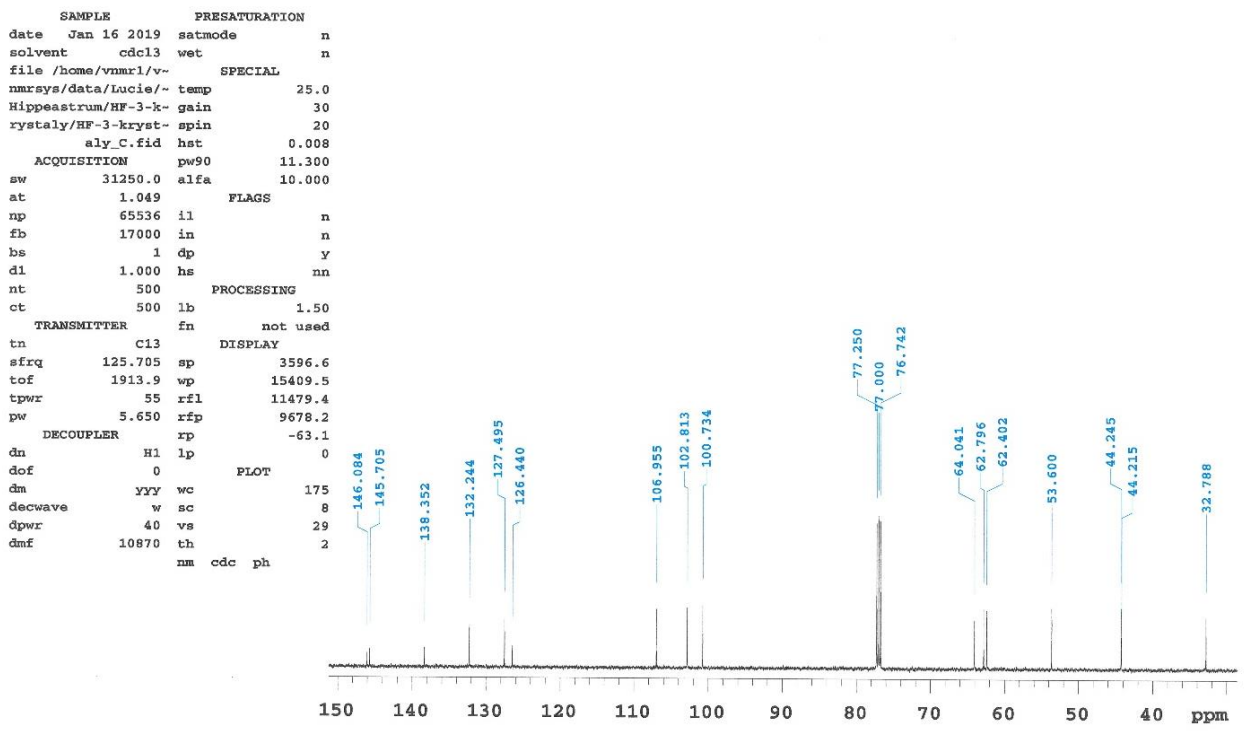

Figure S6: ^{13}C-NMR (125 MHz, $CDCl_3$) Spectrum of Vittatine (**2**)

1-HYDROXY

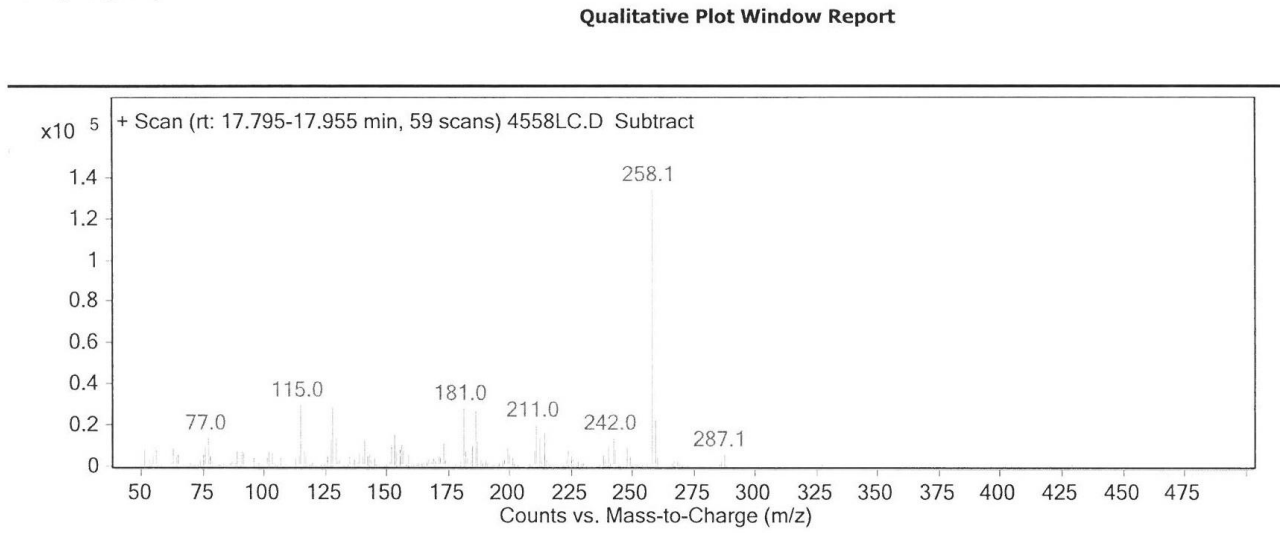

Figure S7: EI-MS Spectrum of 11-Hydroxyvittatine (**3**)

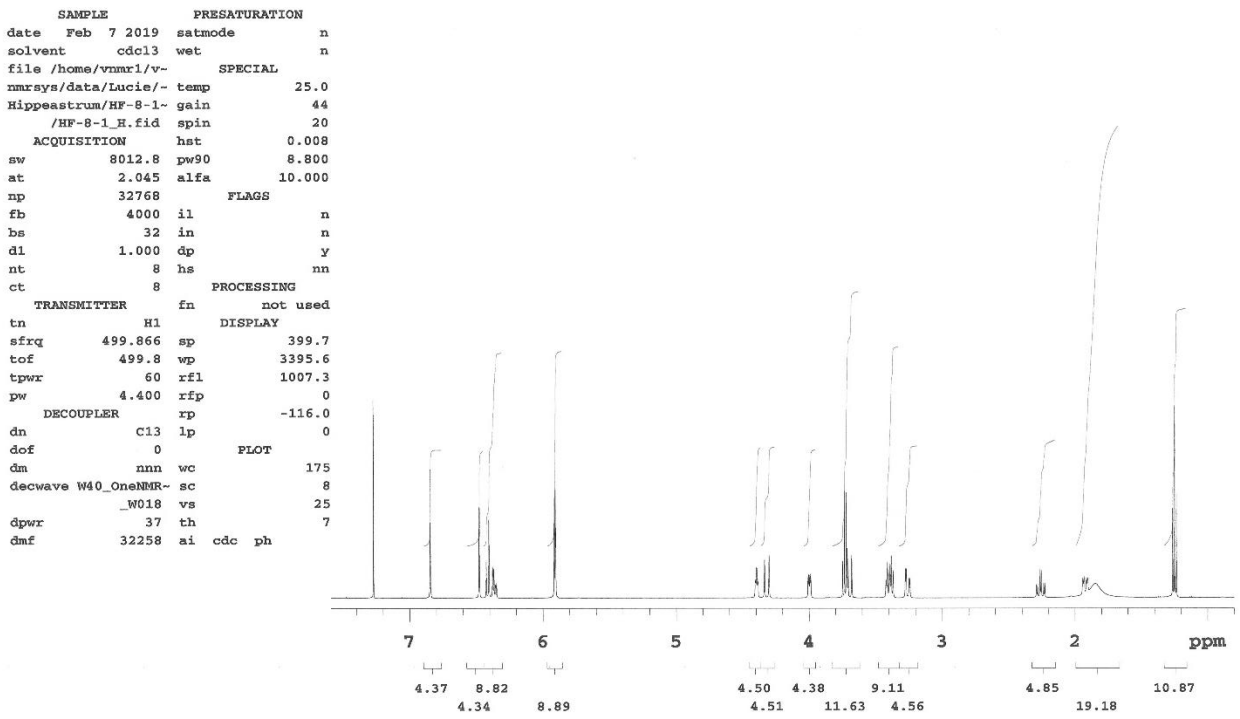

Figure S8: ^{1}H-NMR (500 MHz, $CDCl_3$) Spectrum of 11-Hydroxyvittatine (**3**)

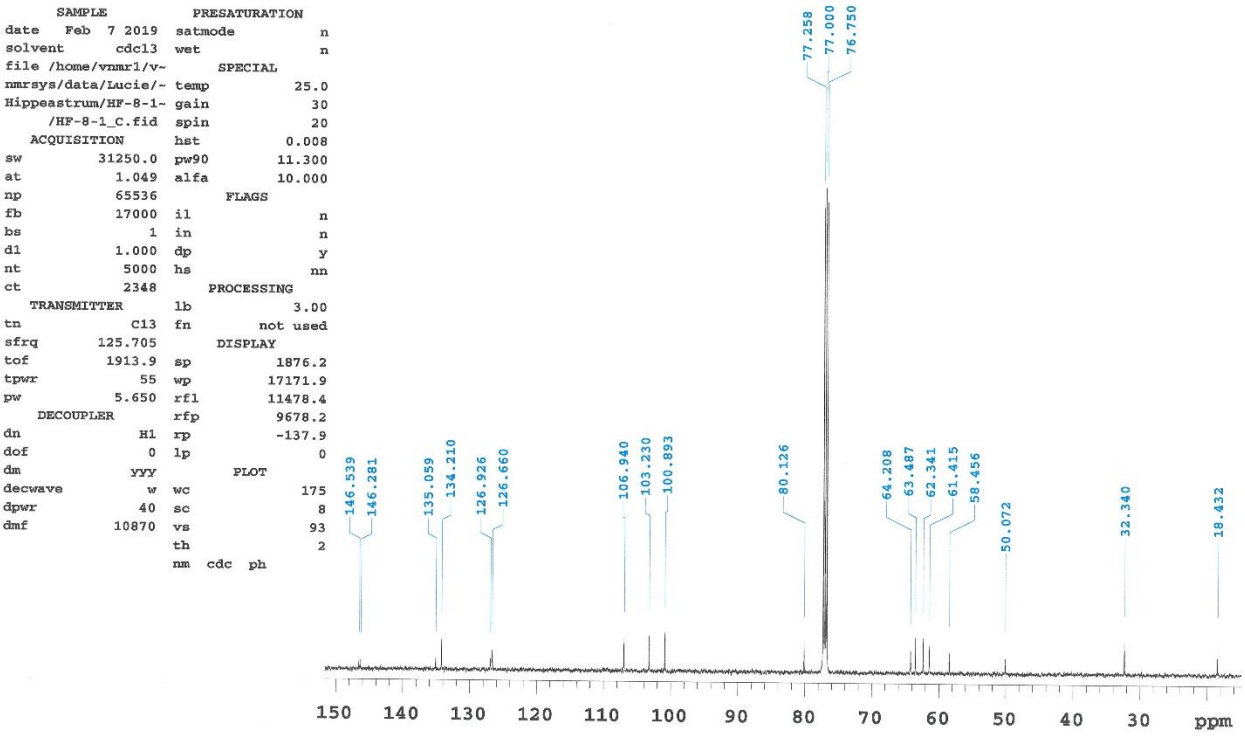

Figure S9: ^{13}C-NMR (125 MHz, $CDCl_3$) Spectrum of 11-Hydroxyvittatine (**3**)

LYC

Qualitative Plot Window Report

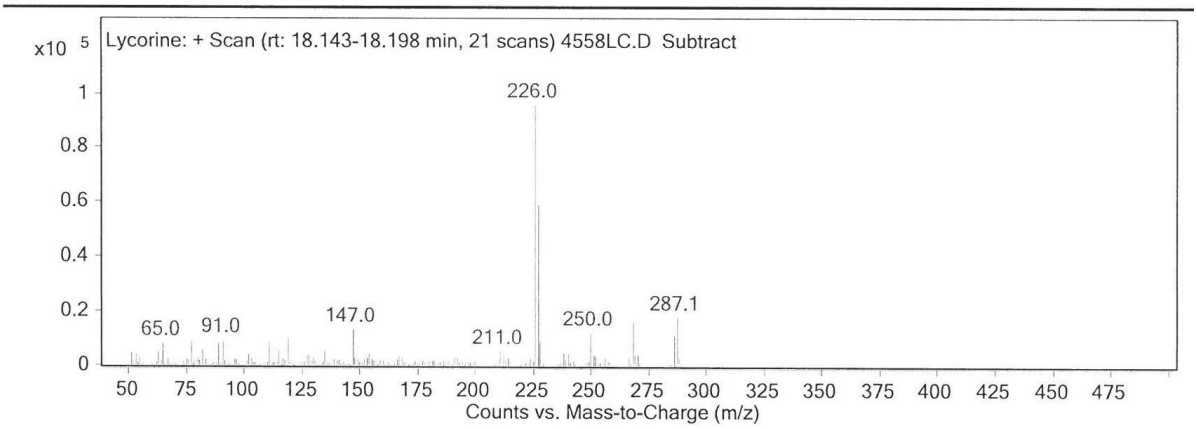

Figure S10: EI-MS spectrum of Lycorine (**4**)

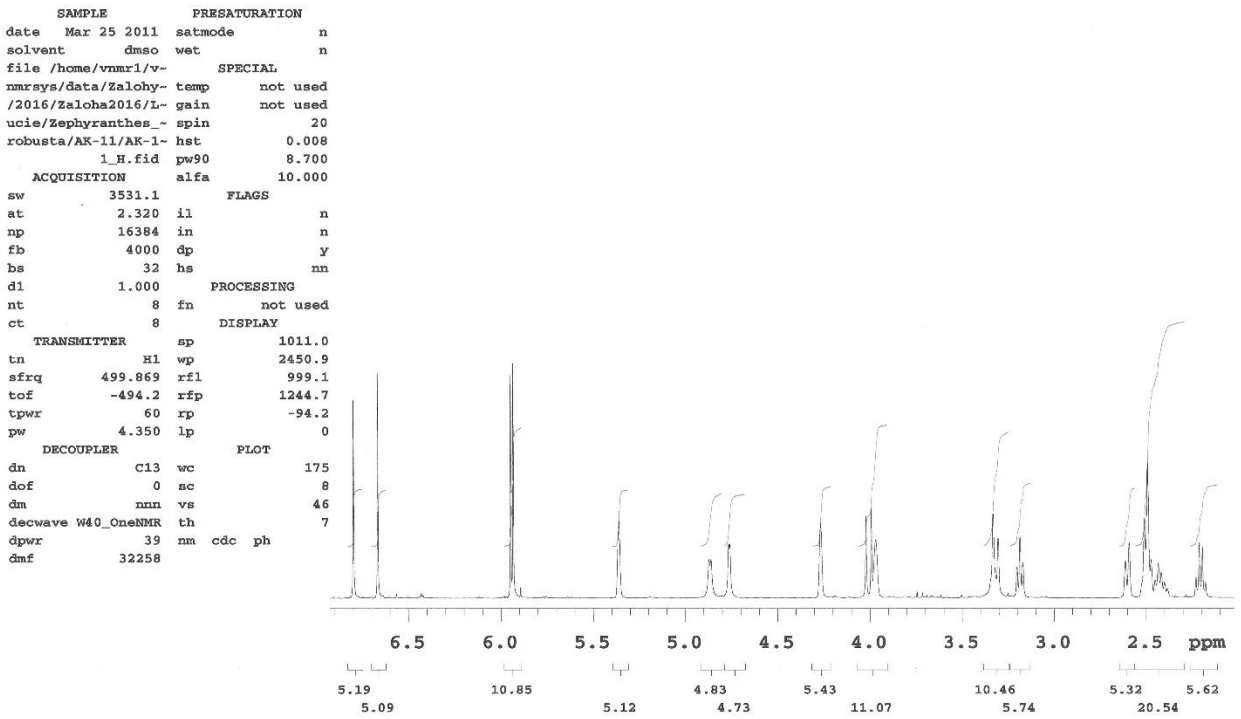

Figure S11: ^{1}H-NMR (500 MHz, $CDCl_3$) Spectrum of Lycorine (**4**)

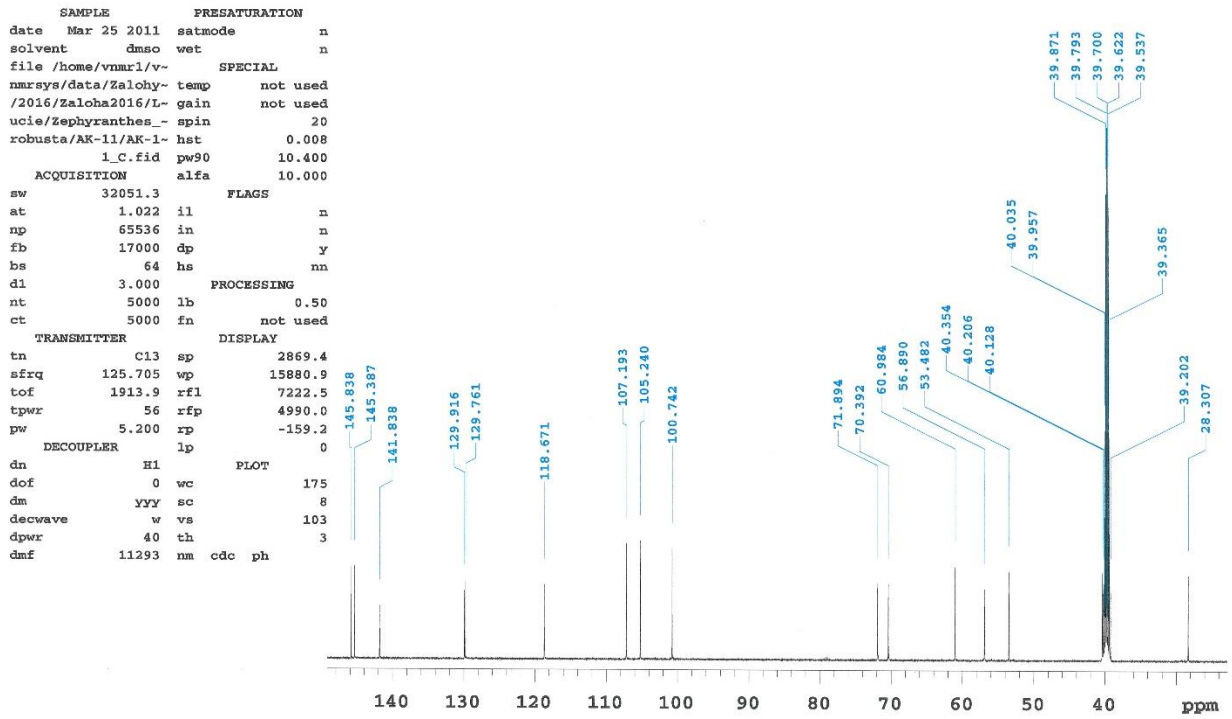

Figure S12: ^{13}C-NMR (125 MHz, $CDCl_3$) Spectrum of Lycorine (**4**)

HIPP

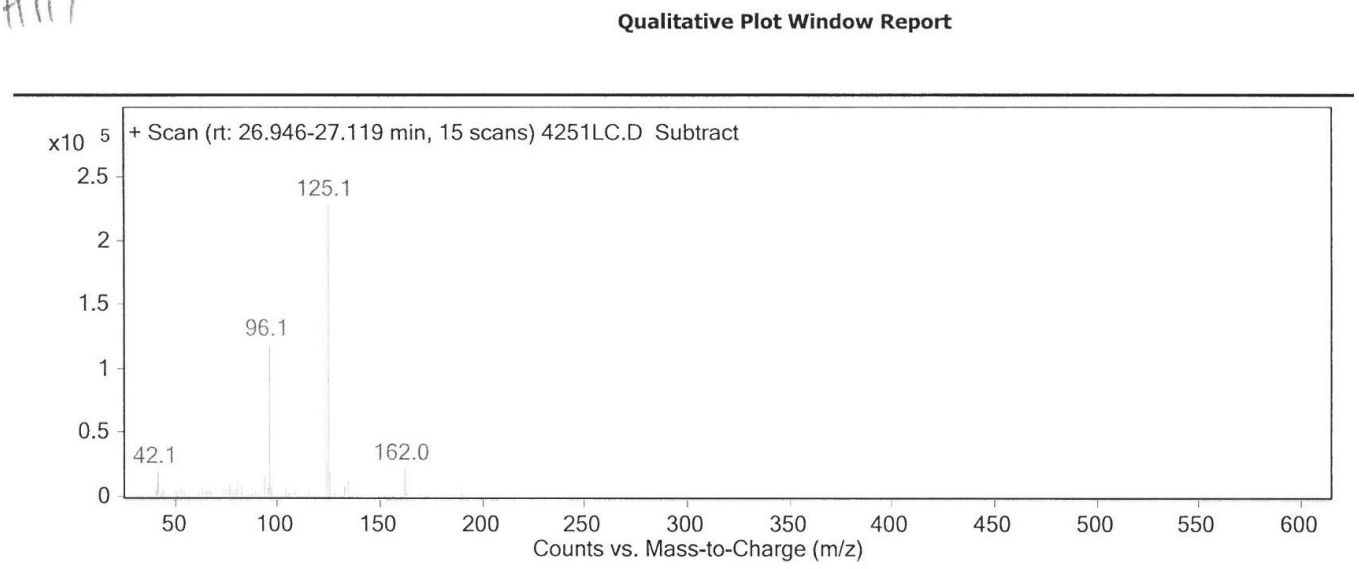

Figure S13: EI-MS spectrum of Hippeastrine (**5**)

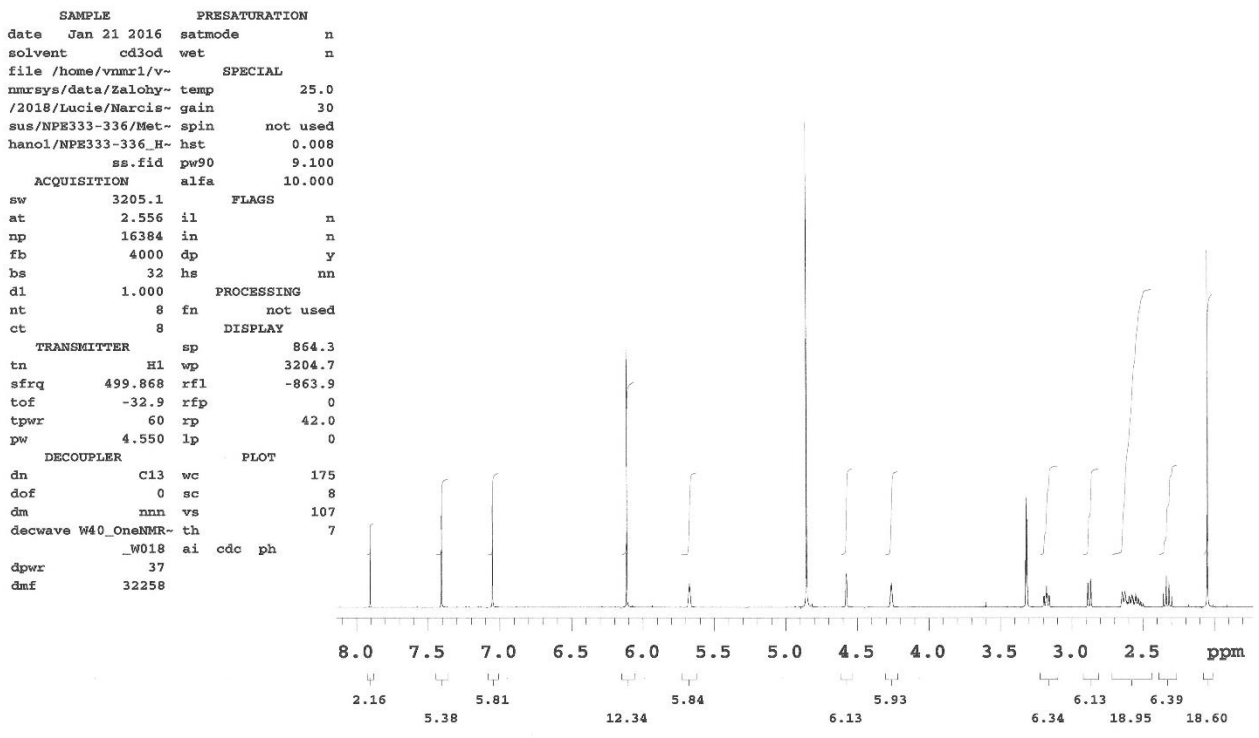

Figure S14: ^{1}H-NMR (500 MHz, $CDCl_3$) Spectrum of Hippeastrine (**5**)

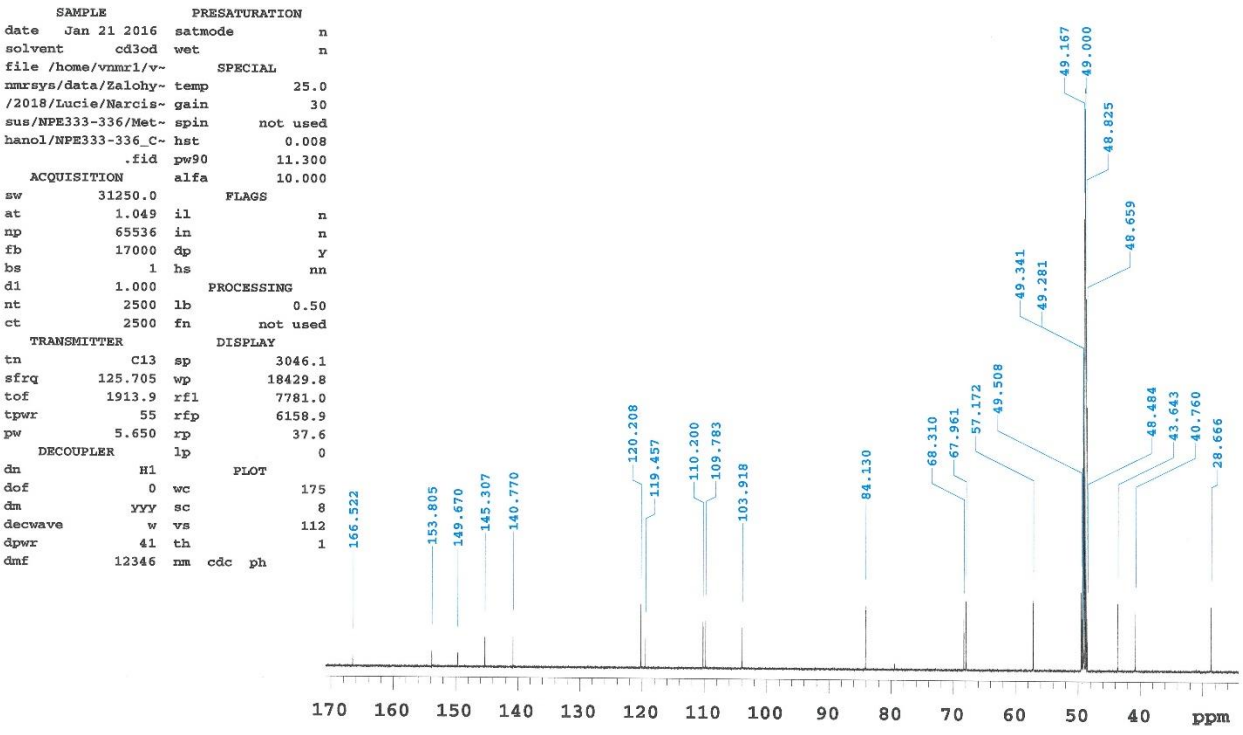

Figure S15: ^{13}C-NMR (125 MHz, $CDCl_3$) Spectrum of Hippeastrine (**5**)

Table S1. Cytotoxicity of montanine, vittatine and hippeastrine following a single-dose exposure at a concentration of 10 µM. Doxorubicin at 1 µM was used as a reference drug. Data are shown as mean values ± SD of at least three independent experiments and are expressed as percent of proliferation of 0.1% DMSO mock treated control cells (100 %)

Cell line/alkaloid	Montanine	Vittatine	Hippeastrine	Doxorubicin
Jurkat	4 ± 1	91 ± 3	40 ± 1	2 ± 0
MOLT-4	2 ± 1	92 ± 12	50 ± 20	0 ± 0
A549	23 ± 2	80 ± 4	69 ± 4	11 ± 5
HT-29	36 ± 3	84 ± 5	66 ± 5	47 ± 4
PANC-1	29 ± 5	84 ± 4	84 ± 5	78 ± 3
A2780	26 ± 7	98 ± 5	50 ± 10	5 ± 1
HeLa	18 ± 2	100 ± 5	86 ± 5	11 ± 6
MCF-7	12 ± 2	79 ± 2	70 ± 21	37 ± 3
SAOS-2	25 ± 4	79 ± 6	83 ± 2	17 ± 5
MRC-5	22 ± 11	83 ± 8	69 ± 10	29 ± 3

Table S2. Sensitivity to the antiproliferative activities of montanine, vittatine and hippeastrine following a single-dose exposure at a concentration of 10 µM. Doxorubicin at 1 µM was used as a reference drug[a,b].

Compound	Mean GP[a]	Range of GP[b]	Most sensitive cell lines	% inhibition
Montanine (**1**)	20	2 - 36	MOLT-4, Jurkat, MCF-7	2, 4, 12
Vittatine (**2**)	87	79 - 100	MCF-7, SAOS-2, A549	79, 79, 80
Hippeastrine (**5**)	67	40 - 86	Jurkat, MOLT-4, A2780	40, 50, 50
Doxorubicin	24	0 - 78	MOLT-4, Jurkat, A2780	0, 2, 5

[a]Mean growth percent (GP) value was calculated for each compound as an average of 9 cell lines proliferation in percent. [b]Range of growth percentage, as well as the three most sensitive cell lines with growth percentage values are indicated for each compound.

Table S3. MS spectra of identified Amaryllidaceae alkaloids

Alkaloid	RI[a]	[M^{+}] and characteristic ions *m/z*	Ref. for MS and RI data
Ismine	2278	257(28), 239(10), 238(100), 225(7), 211(7), 196(10), 180(8), 139(10)	[4]
Trisphaeridine	2284	223(100), 222(38), 193(4), 164(15), 138(28), 111(14)	[4]
Galanthamine	2408	287(90), 286(100), 270(20), 244(30), 230(5), 216(45), 174(30), 115(15)	c,d
Lycoramine	2442	289(60), 288(100), 232(10), 202(15), 187(15), 159(10), 115(20)	c,d
Vittatine/crinine*	2498	271(100), 228(25), 199(90), 187(80), 173(30), 128(30), 115(35), 56(20)	c,d
A1	2518	303(100), 288(15), 272(55), 260 (12), 242(23), 230 (20), 217(65), 202(25)	
9-*O*-Demethyllycosinine B	2575	283(100), 256(11), 255(70), 254(72), 240(30), 239(15), 223(10), 222(30), 210(10), 194(15),	[5]
11,12-Dehydroanhydrolycorine	2604	249(60), 248(100), 190(25), 163(10), 123(5), 95(15)	[4]
A2 Homolycorine type	2609	345(5), 286(4), 248(3), 177(5), 109(100), 108(21), 94(15), 43(15)	
Montanine	2615	301(100), 270(88), 257(35), 252(25), 229 (28), 226(30), 223(30), 199(20), 185(35), 115(20)	c,d
Haemanthamine	2640	301(15), 272(100), 240(15), 225(5), 211(15), 128(10)	c,d
Tazettine/Pretazzenine*	2655	331(20), 316(20), 298(25), 247(100), 230(10); 201(15); 181(10), 152(8)	c,d
Pancracine	2719	287(100), 286(23), 270(20), 243(26), 223(30), 214(25), 199(30), 185(41), 128(20), 115(25)	c,d
11-Hydroxyvittattine	2736	287(5), 258 (100), 211(15), 186(20), 181(23), 153(13), 128(25), 115(25)	c
Lycorine	2749	287(35), 286(30), 268(20), 250(15), 227(70), 226(100), 211(8), 147(15)	c,d
Homolycorine	2769	315(<1), 206(<1), 178(2), 109(100), 150/1), 108(23), 94(3), 82(3)	c
3-Epimacronine	2813	329(30), 314(25), 245(100), 225(15), 201(80), 139(10)	c,d
Pseudolycorine	2823	289(25), 270(21), 252(14), 228(100), 214(10), 147(20), 111(20), 82(10)	[6]
Hippeastrine	2918	315(-), 162(4), 134(4), 125(100), 96(36), 82(3)	c
A3	3012	331(19), 330(20), 271(89), 270(100), 254(60), 252(65), 242(22), 229(34), 228(69), 210(18), 147(19), 91(13)	

*Cannot be distinguished by GC-MS; [a]For GC conditions see Experimental section;

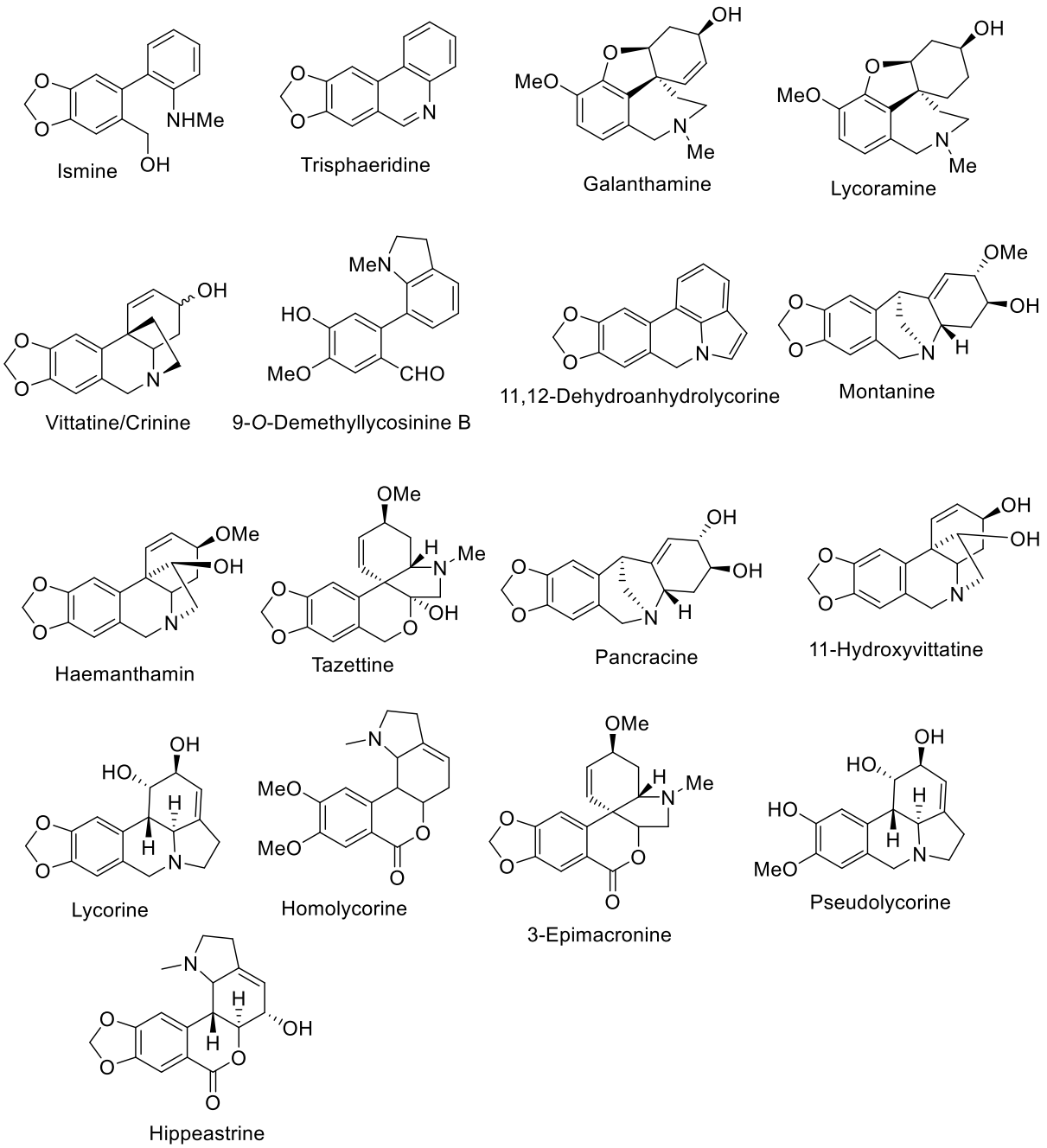

Figure S16: Structures of identified Amaryllidaceae alkaloids in fresh bulbs of *Hippeastrum* cultivars

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