Figure 1. Chemical structures of compounds under study.

Table 1. Lycorine, Lycorine Analogues, and Their *in Vitro* Growth Inhibitory Activity in Cancer Cell Lines

lycorine analogue	no.	cell lines: *in vitro* IC_{50} growth inhibitory values (μM)[a]					
		A549	OE21	Hs683	U373	SKMEL	B16F10
lycorine	**1**	4.3 ± 0.3	5.1 ± 0.4	6.7 ± 0.3	7.6 ± 0.2	8.5 ± 0.3	6.3 ± 0.4
1-*O*-acetyllycorine	**2**	>10	>10	>10	>10	>10	>10
1,2-*O,O'*-diacetyllycorine	**3**	>10	>10	>10	>10	>10	>10
2-*epi*-lycorine	**4**	>10	>10	>10	>10	>10	>10
2-*O*-acetyllycorine	**5**	>10	>10	>10	>10	>10	>10
lycorine chlorohydrin	**6**	3.8 ± 0.2	9.6 ± 0.7	3.1 ± 0.3	2.3 ± 0.1	>10	6.9 ± 0.5
lycorin-2-one	**7**	9.9 ± 0.5	>10	>10	>10	>10	>10
α-dihydrolycorine	**8**	>10	>10	>10	>10	>10	>10
1,2-*O,O'*-diacetyl-α-dihydrolycorine	**9**	>10	>10	>10	>10	>10	>10
β-dihydrolycorine	**10**	>10	>10	>10	>10	>10	>10
1,2-*O,O'*-diacetyl-β-dihydrolycorine	**11**	>10	>10	>10	>10	>10	>10
α-dihydrolycorine lactam	**12**	>10	>10	>10	>10	>10	>10
lycorine *N*-methyl iodide	**13**	>10	>10	>10	>10	>10	>10
1,2-α-epoxylycorine	**14**	3.4 ± 0.1	8.5 ± 0.5	3.3 ± 0.2	2.4 ± 0.1	9.5 ± 0.4	4.6 ± 0.2
lycorine hydrochloride	**15**	4.3 ± 0.2	4.6 ± 0.1	6.5 ± 0.2	8.6 ± 0.3	8.3 ± 0.3	5.5 ± 0.2
lycorene	**16**	>10	>10	>10	>10	>10	>10
caranine	**17**	>10[b]	>10	>10	>10	>10	>10
pseudolycorine	**18**	7.5 ± 0.4	7.7 ± 0.3	7.9 ± 0.2	7.8 ± 0.3	>10	7.5 ± 0.3
norpluvine	**19**	>10	>10	>10	>10	>10	>10
anhydrolycorine	**20**	4.5 ± 0.1	8.8 ± 0.2	7.1 ± 0.3	5.1 ± 0.1	>10	>10
ungeremine	**21**	>10	>10	>10	8.3 ± 0.2	>10	>10
amarbellisine	**22**	7.2 ± 0.3	6.7 ± 0.2	8.3 ± 0.3	7.3 ± 0.2	8.3 ± 0.2	6.7 ± 0.3

[a] U373 (ECACC code 89081403) cell line was cultured in MEM medium supplemented with 5% heat-inactivated fetal bovine serum; Hs683 (ATCC code HTB-138), SKMEL-28 (ATCC code HTB-72), A549 (DSMZ code ACC107), and B16F10 (ATCC code CRL-6475) cell lines were cultured in RPMI medium supplemented with 10% heat-inactivated fetal bovine serum; MEM and RPMI cell culture media were supplemented with 4 mM glutamine, 100 μg/mL gentamicin, and penicillin–streptomycin (200 units/mL and 200 μg/mL). [b] The mean IC_{50} value could not be determined as one or more of the corresponding data points were higher than the threshold value.

(GBM, from astroglial origin[21]),[15,22] human A549 non-small-cell-lung cancer (NSCLC),[15,23] human OE21 esophageal cancer[24], and human SKMEL-28 melanoma[25] models. The two cancer cell lines that are sensitive to proapoptotic stimuli include human Hs683 anaplastic oligodendroglioma[15,22] and mouse B16F10 melanoma[25,26] models. Lycorine derivatives that displayed the most marked antitumor activities were also evaluated for their *in vitro* growth inhibitory effects in normal fibroblasts. We utilized the MTT colorimetric assay[14–16,25] in order to determine the IC_{50} *in vitro* growth inhibitory activity of each compound under study. Lycorine-induced changes in cell proliferation and cell migration were monitored by means of computer-assisted phase-contrast microscopy,[15,16] whereas modifications in actin cytoskeleton organization were observed

with computer-assisted fluorescence microscopy.[15,16] Apoptosis levels were quantified by means of flow cytometry.[15,23] Finally, we evaluated lycorine's *in vivo* toxicity in healthy mice and its *in vivo* antitumor activity in the B16F10 mouse melanoma model.[25,26]

Results

Previously, we developed a new purification method to extract lycorine in large amounts from bulbs of *Sternbergia lutea* Ker Gawl.[27] We also reported a procedure for lycorine analysis in crude extracts by HPLC[28] and an NMR study aimed at the assignment of all the chemical shifts to all protons and carbons in **1**.[29] Following these previous efforts, a significant quantity of lycorine was obtained and used for the

Table 2. Lycorine, Lycorine Analogues, and Their *in Vitro* Growth Inhibitory Activity in Cancer versus Normal Cell Lines

| | | | IC$_{50}$ *in vitro* growth inhibitory values (μM)^a | | | | | | | | | | |
| | | | cancer cell lines | | | | | | | normal cell lines | | | |
lycorine analogue	no.	therapeutic ratio	A549	OE21	Hs683	U373	SKMEL	B16F10	mean ± SEM	WI38	WS1	NHDF	mean ± SEM
lycorine	**1**	>15	4.3 ± 0.3	5.1 ± 0.4	6.7 ± 0.3	7.6 ± 0.2	8.5 ± 0.3	6.3 ± 0.4	6.4 ± 0.6	>100	>100	>100	>100
2-*O*-acetyllycorine	**5**	>2	47.6 ± 2.3	77.4 ± 3.1	43.0 ± 2.0	63.1 ± 4.1	87.8 ± 3.9	37.1 ± 2.8	59.3 ± 8.2	88.5	>100	>100	>96.2 ± 2.7
lycorine chlorohydrin	**6**	>11	3.8 ± 0.2	9.6 ± 0.7	3.1 ± 0.3	2.3 ± 0.1	14.8 ± 1.1	6.9 ± 0.5	6.8 ± 2.0	46.0	>100	72.1	>72.7 ± 11.0
1,2-α-epoxylycorine	**14**	5	3.4 ± 0.1	8.5 ± 0.5	3.3 ± 0.2	2.4 ± 0.1	9.5 ± 0.4	4.6 ± 0.2	5.3 ± 1.2	28.6	36.8	9.3	24.9 ± 8.2
lycorine hydrochloride	**15**	>15	4.3 ± 0.2	4.6 ± 0.1	6.5 ± 0.2	8.6 ± 0.3	8.3 ± 0.3	5.5 ± 0.2	6.3 ± 0.8	>100	>100	>100	>100
pseudolycorine	**18**	7	7.5 ± 0.4	7.7 ± 0.3	7.9 ± 0.2	7.8 ± 0.3	15.5 ± 1.1	7.5 ± 0.3	9.0 ± 1.3	44.0	79.1	66.9	63.3 ± 7.3
anhydrolycorine	**20**	2	4.5 ± 0.1	8.8 ± 0.2	7.1 ± 0.3	5.1 ± 0.1	20.9 ± 1.2	23.3 ± 1.2	11.6 ± 3.4	30.1	23.7	26.6	26.8 ± 1.3
amarbellisine	**22**	>13	7.2 ± 0.3	6.7 ± 0.2	8.3 ± 0.3	7.3 ± 0.2	8.3 ± 0.2	6.7 ± 0.3	7.4 ± 0.3	>100	97.6	>100	>99.2 ± 0.6

^{a} Cancer cell lines were cultured as specified in Table 1. WI-38 fibroblasts (ATCC code CCL-75; see Figure 2) were cultured in MEM medium supplemented with 10% heat-inactivated fetal bovine serum and 100 μM nonessential amino acids; WS-1 (ECACC code 88021104) fibroblasts were cultured in MEM medium supplemented with 10% heat-inactivated fetal bovine serum; NADH (PromoCell code c-12300) was cultured in MEM medium supplemented with 10% heat-inactivated fetal bovine serum and 100 μM nonessential amino acids.

preparation of its derivatives **2**−**12**, **14**−**17**, and **20** according to the previously reported procedures.[4,5,27,30] In addition, lycorine derivative **13** as well as the minor Amaryllidaceae alkaloids pseudolycorine (**18**), norpluvine (**19**), ungeremine (**21**), and amarbellisine (**22**) was obtained as described in the Experimental Section.

Determination of the IC$_{50}$ *in Vitro* Growth Inhibitory Activities of Lycorine and Its Analogues: Structure−Activity Relationship Analysis. In this first set of experiments (Table 1), we analyzed the IC$_{50}$ *in vitro* growth inhibitory activities of the 22 compounds under study at concentrations of up to 10 μM (testing at higher concentrations was not feasible due to insufficient quantity of material available for some of the analogues). The data indicate that lycorine exhibits a marked growth inhibitory activity toward all the cancer cell lines tested regardless of whether the cancer cells are resistant (A549, U373, OE21, and SKMEL-28 models) or sensitive (Hs683 and B16F10 models) to proapoptotic stimuli. However, the activity was significantly affected by structural modifications within the phenanthridine skeleton as revealed by the IC$_{50}$ values of lycorine analogues. Thus, the presence of the unaltered diol functionality in the C-ring in its original configuration, stereochemistry of the C/D-ring junction, and conformational freedom of the C-ring appear to be critical for anticancer activity. In fact, while activity of the hydrochloride salt of lycorine (**15**) was unaltered compared with the parent neutral alkaloid, analogues incorporating changes of the hydroxyl groups at C-1 and C-2 of the C-ring, as well as their stereochemistry, lacked antitumor activity at concentrations of up to 10 μM. This was observed for 2-*epi*-lycorine (**4**), lycorin-2-one (**7**), lycorene (**16**), and caranine (**17**). In contrast, good activity of lycorine chlorohydrin (**6**) and 1,2-α-epoxylycorine (**14**) should likely be attributed to the intracellular conversion of these derivatives to **1** by nucleophilic substitution with water. The lack of activity of 1-*O*- (**2**), 2-*O*- (**5**), and 1,2-*O,O'*-diacetyllycorine (**3**) is likely due to significant steric bulk introduced into the C-ring and inability of cancer cells to completely hydrolyze the ester groups and convert these derivatives to lycorine within the treatment time period. Further inspection of the SAR data reveals that stereochemistry and conformational freedom of the C-ring are additional important features for the antitumor activity. Thus, α- and β-dihydro derivatives (**8** and **10**), obtained by catalytic hydrogenation at C(3)−C(3a), proved to be inactive. The similar result was obtained with their 1,2-*O,O'*-diacetyl derivatives (**9** and **11**). The same

modification may serve as an explanation for the absence of antitumor activity of α-dihydrolycorine lactam (**12**). This analogue additionally incorporates a modification of the B-ring resulting in nonbasic character of the nitrogen atom, providing another point of difference from lycorine. Further, quaternization of the nitrogen atom of the B-ring, as found in *N*-methyllycorine iodide (**13**), also resulted in a loss of activity. Interestingly, ring C aromatized lycorine analogues ungeremine (**21**) and anhydrolycorine (**20**) are active against some of the cancer cell lines, but not all. It is noteworthy, however, that the responsive cells include the human U373 GBM model, which is resistant to proapoptotic stimuli.[15,22] Pseudolycorine (**18**) and amarbellisine (**22**), two alkaloids closely related to lycorine, exhibit growth inhibitory potencies that are very similar to those of lycorine in all of the cancer cell lines studied. These results show that the *trans*-position of the double bond from C(3)−C(3a) to C(2)−C(3) and the presence of the methoxy group at C-2, as found in amarbellisine (**22**), do not constitute structural changes that abolish antitumor activity. Similarly, good activity of pseudolycorine (**18**), which incorporates an open dioxole ring A, suggests that this structural feature is not essential for the antitumor activity. Indeed, the lack of anticancer activity of norpluvine (**19**), which also contains an open A-ring, should be due to the absence of the hydroxyl group at C-2, as is found in caranine (**17**). Finally, the analysis of the data in Table 1 would be incomplete without an important observation that all active analogues display similar antitumor activities in resistant as well as cancer cells sensitive to proapoptotic stimuli.

Lycorine Displays Higher *in Vitro* Antiproliferative Effects in Cancer than in Normal Cells. Liu et al.[11] demonstrated that lycorine displays higher *in vitro* growth inhibitory activity in human leukemic cells than in normal white blood cells. Leukemic cells normally grow in suspension, while solid cancer cells are usually adherent. There are, however, significant differences in biological properties of cells growing in suspension compared to adherent ones, especially with respect to their sensitivity to apoptosis. In the second set of experiments (Table 2), we analyzed the IC$_{50}$ *in vitro* growth inhibitory activity (using the MTT colorimetric assay) of eight of the most active compounds (as detailed in Table 1) at concentrations of up to 100 μM in six cancer and three normal cell lines. The results illustrated in Table 2 attest to a high potential (*in vitro*) therapeutic ratio for lycorine because its *in vitro* antigrowth activity is 15 times higher in cancer
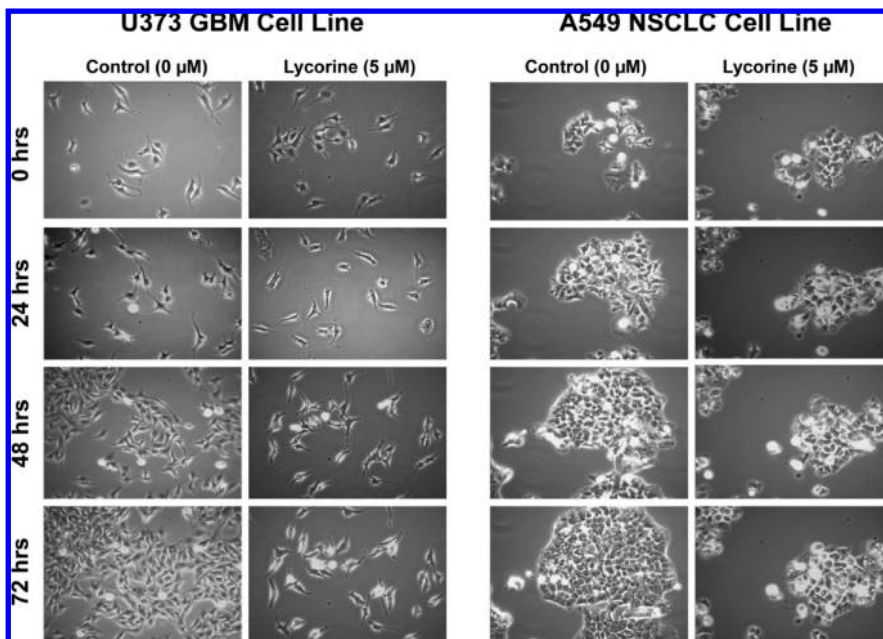
Figure 2. Morphological illustrations of U373 GBM and A549 NSCLC cell populations left untreated (control) or treated for 72 h with 5 μM lycorine. The illustrations reveal cytostatic effects for lycorine, rather than cytotoxic ones. The 5 μM concentration used here approximately corresponds to the IC$_{50}$ *in vitro* growth inhibitory value obtained in the MTT colorimetric assay (see Table 1).

than in normal cells. Amarbellisine (**22**) displays similar features. In contrast, anhydrolycorine (**20**), which exhibits antitumor activity similar to that of lycorine, displays a weaker potential therapeutic ratio than lycorine (Table 2). Thus, the analysis of the combined data from Tables 1 and 2 leads us to conclude that lycorine displays the highest antitumor activity and the highest potential (*in vitro*) therapeutic ratio in comparison to the other investigated compounds. We thus decided to pursue our investigation with lycorine as a potential anticancer lead.

Lycorine Is a Cytostatic Compound in U373 GBM and A549 NSCLC Cancer Cells That Are Resistant to Various Proapoptotic Stimuli. We made use of computer-assisted phase-contrast microscopy (quantitative videomicroscopy) to analyze the principal mechanism of action associated with lycorine's *in vitro* growth inhibitory effects as first revealed by the MTT colorimetric assay (Tables 1 and 2). Figure 2 shows that lycorine inhibits cancer cell proliferation without inducing cell death when assayed at its IC$_{50}$ *in vitro* growth inhibitory value (ca. 5 μM, Table 1) in U373 GBM[15,22,31] and A549 NSCLC[15,23] cells that are resistant to various proapoptotic stimuli. Based on the phase-contrast pictures obtained by means of quantitative videomicroscopy (Figure 2), for each cell line and each set of experimental conditions we calculated the global growth rate (GGR), which corresponds to the ratio of the mean number of cells present in the last image captured in the experiment (conducted at 72 h) to the number of cells present in the first image (at 0 h). We divided this ratio obtained in the lycorine-treated experiment by the ratio obtained in the control (Figure 3A). A GGR value of 0.2 in Figure 3A means that 20% of cells grew in the lycorine-treated set of conditions as compared to the control experiment over a 72 h observation period. Figure 3A, based on GGR calculations, thus confirms the MTT colorimetric assay-related data detailed in Tables 1 and 2; e.g., 5 μM lycorine displays marked *in vitro* growth inhibitory activity in both U373 GBM and A549

NSCLC cells, which display resistance to various proapoptotic stimuli. The quantitative determination of mitosis occurrence (using software that was developed in our laboratory[32]) made it possible to demonstrate the cytostatic effects associated with lycorine's antitumor properties (Figure 3B). Indeed, lycorine decreased by $\geq$90% the mitosis occurrence in U373 GBM as well as in A549 NSCLC cells at 5 μM for 72 h (Figure 3B). The white arrow in Figure 3Ca points to an individual A549 NSCLC cell belonging to an A549 NSCLC colony treated with lycorine at 5 μM and analyzed for 72 h. The use of computer-assisted phase-contrast microscopy[31,33] made it possible to analyze the behavior of this cell at high magnifications over the 72 h period of treatment with 5 μM lycorine as illustrated in Figure 3Cb. The NSCLC cell (Figure 3Cb; 0 h) entered mitosis 7 h after 5 μM lycorine was added to the culture medium, and this cell almost succeeded in accomplishing its mitosis at the 21st hour post-lycorine addition into the culture medium (Figure 3Cb). However, 3 h later this cell failed in completing its mitosis, and the two daughter cells merged at the 24th hour (Figure 3Cb; 24 h). At the 41st hour post-lycorine addition, this cell once more tried to enter mitosis and once more almost succeeded in completing its second mitotic attempt (see Figure 3Cb; 43 and 48 h), but last failed to achieve it (Figure 3Cb; 72 h). This feature is typical of what we observed on almost all U373 GBM and A549 NSCLC cells we analyzed, and it strongly suggests that the lycorine-induced cytostatic effects on cancer cells mainly occur during cytokinesis. Lycorine-induced impairment of cytokinesis in cancer cells could occur, at least partly, through a lycorine-mediated increase in rigidity of actin cytoskeleton as revealed by fluorescence microscopy analyses (Figure 3D). Indeed, 5 μM lycorine markedly increased the levels of polymerized actin in U373 GBM cells (green fluorescence in Figure 3Db) when compared to control (Figure 3Da). Similar features were observed with respect to A549 NSCLC cells (data not shown). These lycorine-induced cytostatic effects,
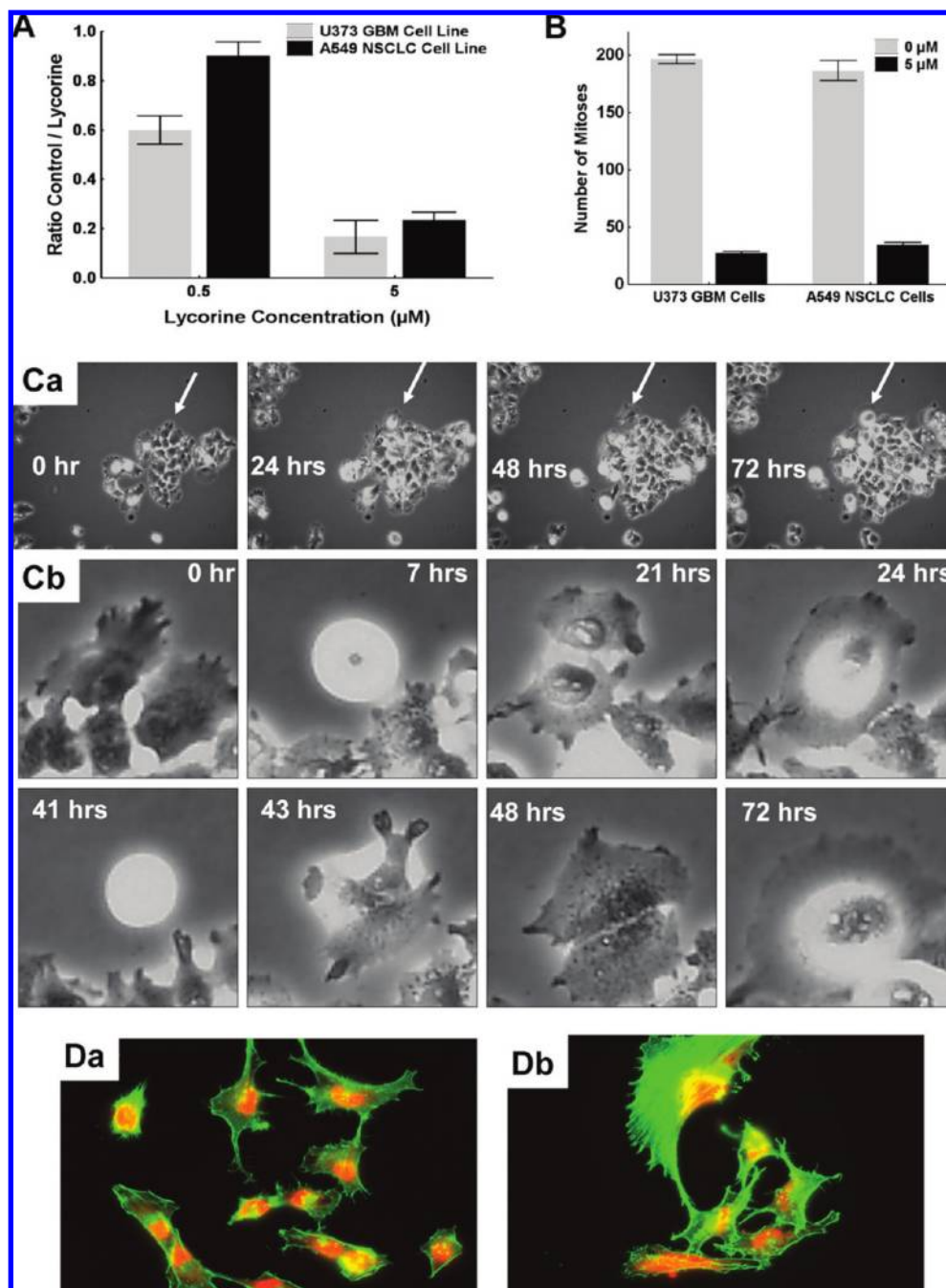
Figure 3. (A) In each (control or treated) set of conditions, the cell growth levels after 72 h of culture were evaluated by the ratio of the number of cells counted in the last and first frames of the image sequence. The global growth ratio (GGR) for a given set of experimental conditions was defined by the ratio of the two growth levels obtained in the control and the treated conditions (control/treated). All of the cell counts were performed in triplicate using an interactive computer tool. A GGR value of 0.6 in panel A means that 60% of cells grew in the treated as compared to the control set of conditions. (B) The number of mitoses occurring in a 2 mm^2 frame during 72 h of observation was quantitatively determined with computer-assisted phase-contrast microscopy (quantitative videomicroscopy) utilizing software packages developed in our laboratory.[32] (Ca) An A549 NSCLC colony was treated with 5 μM lycorine and then observed by means of quantitative videomicroscopy with the focus on the single cell indicated with the white arrow. (Cb) Behavior of the single cell indicated with a white arrow in Ca was monitored for 72 h. (D) Immunofluorescence analyses were performed to reveal in green fluorescence the fibrilar (polymerized) actin and in red fluorescence the globular (nonpolymerized) actin in U373 GBM cells (Da, control; Db, 5 μM lycorine for 6 h).

occurring partly through an increase in rigidity of actin cytoskeleton, closely resemble those of narciclasine, as we recently reported.[16]

Lycorine Impairs *in Vitro* U373 GBM and A549 NSCLC Migration. A common property that can impair both cell proliferation and migration, with no evidence of cell death, is associated with disorganization of actin cytoskeleton.[15,16,34−36]

A lycorine-induced increase in rigidity of actin cytoskeleton through the elevated polymerized actin prompted us to investigate whether lycorine could also impair cancer cell migration in addition to its cytostatic effects. We utilized computer-assisted phase-contrast microscopy (quantitative videomicroscopy) to calculate the maximum relative distance to the origin (the MRDO variable in Figure 4A) in A549
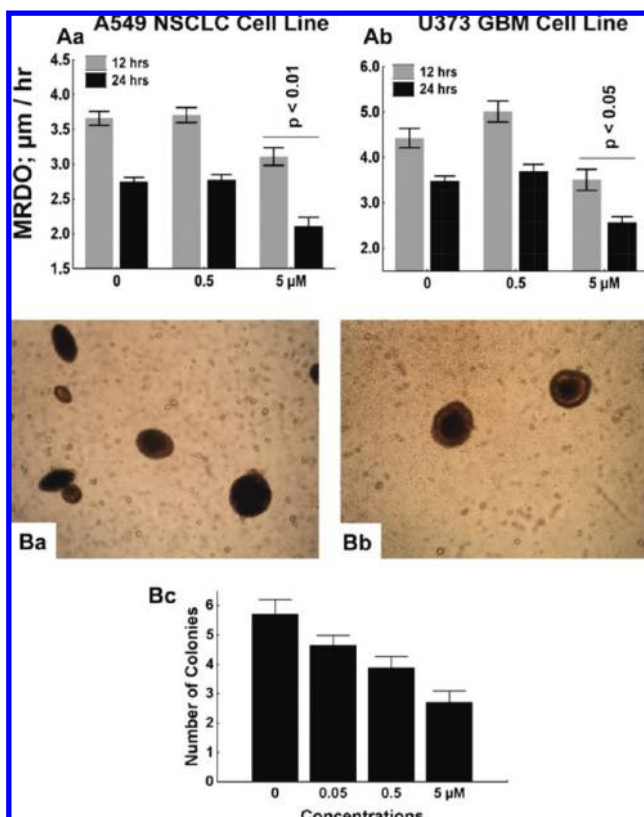
Figure 4. (A) Characterization of the effect of 0.5 and 5 μM lycorine (0 μM = control) on migration levels of A549 NSCLC and U373 GBM cells. Migration levels were determined by computing the MRDO variable (by means of quantitative videomicroscopy), which represents the maximal relative distance to the origin (the largest linear distance) traveled by each single cell during 12 (gray bars) or 24 (black bars) h of observation. Data are presented as means ± SEMs. Morphological illustrations of MRDO variable computation are provided in ref 50. (B) Morphological illustrations of A549 NSCLC cell colonies developing on soft agar for 38 days after cell plating procedure without (control; Ba) or with 5 μM lycorine (Bb). The number of colonies was determined in three 0.4 mm^2 areas for each set of experimental conditions, which was repeated as hexaplicate. Data are presented as means ± SEMs.

NSCLC (Figure 4Aa) and U373 GBM (Figure 4Ab) cells left untreated (control; "0 μM" in Figure 4A) or treated with 0.5 or 5 μM lycorine. The MRDO variable represents the greatest linear distance traveled by each single cell from the moment it was caught by the quantitative videomicroscopy system through the end of the experiment, as detailed elsewhere.[16,21,33] The data illustrated in Figure 4Aa (A549 NSCLC cells) and Figure 4Ab (U373 GBM cells) show that 5 μM lycorine significantly impaired migration processes in cancer cell populations that display resistance to various proapoptotic stimuli.

Lycorine Reduces Colony Formation of Undifferentiated A549 NSCLC Cells Growing under Anchorage-Independent Culture Conditions. Undifferentiated cancer cells, including cancer stem cells, are able to develop as colonies when cultured under anchorage-independent culture conditions (as on soft agar), while differentiated cancer cells necessitate anchorage-dependent culture conditions (as on solid supports when using the MTT colorimetric assay).[37] Panels Ba and Bb of Figure 4 illustrate A549 NSCLC colony formation after 38 days when the culture is under control and 5 μM lycorine-treated set of conditions, respectively. Although undifferentiated cancer cells are biologically more aggressive

than differentiated ones and more prone to metastasize (as evidenced in any pathology grading system of human cancers), the data illustrated in Figure 4Bc reveal that lycorine significantly impairs colony formation in the A549 NSCLC model when assayed between 0.5 ($p < 0.05$) and 5 ($p < 0.01$) μM. We obtained similar data with respect to the U373 GBM model (data not shown).

Lycorine Does Not Induce Apoptosis in U373 GBM Cells That Display Resistance to Various Proapoptotic Stimuli. Flow cytometry analyses based on double annexin V (AV)/propidium iodide (PI) staining revealed that narciclasine (positive control ("+control" in Figure 5A) used in the present study) induced significant proapoptotic effects in the MCF-7 breast cancer cell line (Figure 5A), as was shown previously.[14] Early apoptosis relates to AV^+/PI^- cells, while late apoptosis relates to AV^+/PI^+ cells.[14,15] Lycorine did not induce proapoptotic effects in the U373 GBM cell line at 5 μM or even at 50 μM (Figure 5A). Rather, at 50 μM, lycorine induced nonapoptotic cell death (AV^-/PI^+ cells) in U373 GBM cells (Figure 5A). This process is unrelated to necrotic cell death because it is known that when dying cells still stain heavily with PI, necrotic processes are unlikely to occur.[23] Quantitative videomicroscopy, which allows the monitoring of morphological changes induced by lycorine, revealed that U373 (Figure 5B) and A549 (data not shown) cell death occurred when actin cytoskeleton collapsed as illustrated for U373 GBM cells in Figure 5B.

Lycorine-Induced Toxicity in Mice: Determination of the Chronic Maximal Tolerated Dose. Lycorine was administered iv at 5, 10, 20, 40, and 80 mg/kg, chronically, i.e., once a day during five consecutive days, to healthy mice whose body weights were being recorded over 28 days. The data obtained revealed no gross toxicity displayed by lycorine at these doses (Figure 6A). The MTD for lycorine under iv chronic administrations is therefore higher than 80 mg/kg.

Demonstration of *in Vivo* Antitumor Activity of Lycorine in the Mouse B16F10 Melanoma Model. We determined the *in vivo* antitumor activity of lycorine in the B16F10 mouse melanoma model,[26] orthotopically grafted into the brains of mice in order to mimic melanoma brain metastases, a feature that occurs in a large proportion of melanoma patients[38] and contributes to an exceedingly poor prognosis.[39] The data we obtained when assaying lycorine iv at 40 mg/kg three times a week (Monday, Wednesday, Friday) during three consecutive weeks (with treatment starting at the fifth day post-tumor grafting) revealed that lycorine significantly ($p = 0.006$) increased the survival of B16F10 melanoma-bearing mice, while cisplatin (chosen as a reference compound for melanoma treatment[40,41]) failed to provide significant therapeutic benefit (Figure 6B). The active dose for lycorine in terms of *in vivo* antitumor activity identified in the current experiment, i.e., 40 mg/kg, is thus significantly lower than its maximal tolerated dose (> 80 mg/kg; Figure 6A). It must be additionally emphasized that lycorine succeeded in passing the blood brain barrier because while administered iv it significantly increased the survival of mice bearing melanoma brain-related metastases (Figure 6B).

Discussion

The increase in incidence of various types of cancers associated with dismal prognoses, such as gliomas, melanomas, esophageal cancers, and NSCLCs, among others, has not been paralleled by improved therapeutic options over the
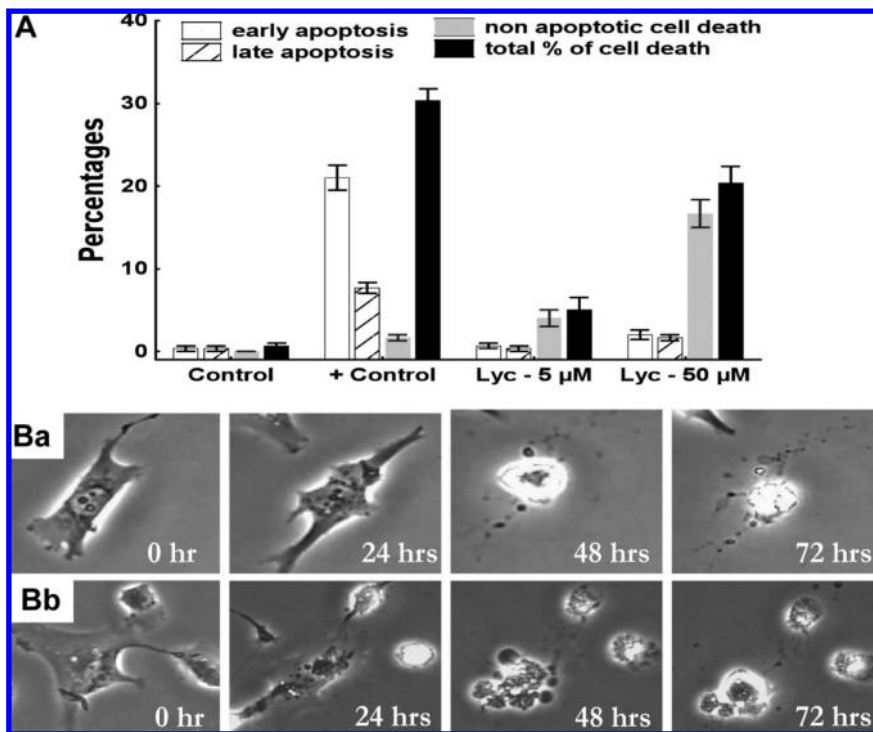
Figure 5. (A) Determination of apoptotic and nonapoptotic cell death levels in U373 GBM cells after 72 h in the presence of 5 (Lyc, 5 μM) and 50 (Lyc, 50 μM) μM lycorine as compared to control (absence of lycorine). Human MCF-7 breast cancer cells treated with 1 μM narciclasine for 72 h used as positive control (+control). U373 cells were stained with propidium iodide (PI) and annexin V (AV). Early apoptosis corresponds to AV$^+$/PI$^-$ U373 cells, while late apoptosis corresponds to AV$^+$/PI$^+$ U373 cells. Nonapoptotic cell death corresponds to AV$^-$/PI$^+$ cells. Data are presented as means ± SEMs. (Ba, Bb) The behavior of two individual U373 GBM cells cultured in the presence of 50 μM lycorine was monitored for 72 h.

years. More than 90% of cancer patients die from their metastases, which are naturally resistant to apoptosis. Nonetheless, a large majority of therapeutic agents used by oncologists to treat cancer patients are proapoptotic.[42,43] Glioma,[42] melanoma,[43] esophageal cancer,[44] non-small-cell-lung cancers,[45] and a number of others are also resistant to apoptosis. New types of drugs are, therefore, urgently needed to combat cancers that are resistant to proapoptotic stimuli and that are therefore associated with dismal prognoses. Lycorine could be a potential candidate to combat cancer cells that display resistance to proapoptotic stimuli as demonstrated in the current study. While lycorine has been suggested to exert its antitumor activity in leukemia cells through proapoptotic effects,[9–11] our current working hypothesis indeed relates to the fact that apoptosis induction by lycorine is not the principal mechanism of action by which this compound exerts its antitumor effects in solid cancers. In addition, we observed significant similarities between the anticancer properties of lycorine and narciclasine. We previously demonstrated that narciclasine is unable to induce apoptosis at a pharmacological concentration of 1 μM in glioma cells, which display resistance to proapoptotic stimuli,[15,16] while the IC$_{50}$ *in vitro* growth inhibitory values (i.e., the "physiological" values) for narciclasine are around 40 nM in carcinoma cells that are sensitive to proapoptotic stimuli as well as in glioma cells that display resistance to proapoptotic stimuli.[15,16] The same observations were made in the current study with respect to lycorine (Figure 5). Furthermore, we showed that narciclasine impairs both cell proliferation and cell migration in a large panel of cancer cell lines[15] by disorganizing actin cytoskeleton.[16] We report here that lycorine also impairs actin cytoskeleton organization (Figure 3D), a feature which in turn

impairs both cell migration and cell proliferation in differentiated as well as in undifferentiated cancer cell populations (Figures 2–4). The current study reveals that lycorine increases actin cytoskeleton rigidity, as we recently demonstrated with respect to narciclasine.[16] Indeed, we observed that narciclasine modulates the Rho/Rho kinase/LIM kinase/cofilin signaling pathway, greatly increasing GTPase RhoA activity as well as inducing actin stress fiber formation in a RhoA-dependent manner.[16] The possibility remains that lycorine also induces higher rigidity in actin cytoskeleton of cancer cells by modulating the Rho/Rho kinase/LIM kinase/cofilin signaling pathway.

Liu et al.[9] also observed a cytostatic effect of lycorine in leukemic cells when the alkaloid was assayed at its IC$_{50}$ *in vitro* growth inhibitory concentration (i.e., at physiological concentration), and it resulted in the increased population of cells in G2/M phase. Only after the concentration of lycorine was increased by 5–10 times above the IC$_{50}$ *in vitro* growth inhibitory values in leukemic cells were the actual apoptotic features observed.[9–11]

The current study demonstrates that lycorine is able to cross the blood brain barrier (Figure 6), as we showed earlier with respect to narciclasine.[15,16] However, the *in vivo* therapeutic ratio of narciclasine appears inferior[15] to that of lycorine (Figure 6). Thus, lycorine seems to be less toxic than narciclasine, and therefore, it should be more facile to manage lycorine clinically. Additionally, any modifications we attempted to bring on narciclasine led to a loss of its antitumor activity.[15] A final point giving advantage to lycorine over narciclasine as a potential candidate for clinical development in oncology relates to the fact that lycorine induces no CYP3A4 inhibitory activity, while narciclasine does.[46]
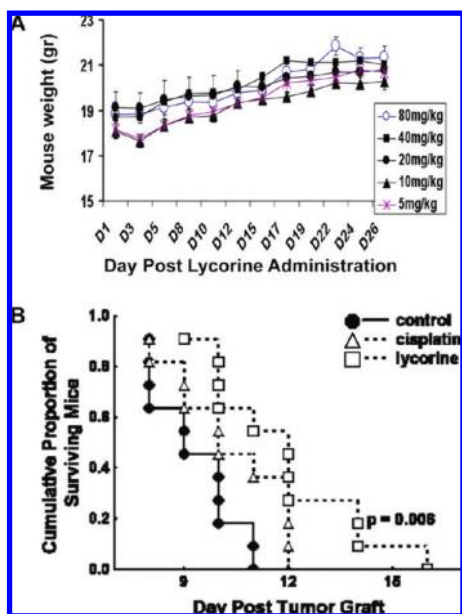
Figure 6. (A) Body weight measurements of mice having received iv administrations (tail vein; three mice per group) of 5, 10, 20, 40, and 80 mg/kg lycorine during five consecutive days. Data are presented as means ± SEMs. No single mouse died over a 28-day period of observation, and the mice did not lose weights, emphasizing the absence of lycorine-induced toxicity. (B) Survival analyses of mice orthotopically grafted into their brains with the mouse melanoma B16F10 which have been left untreated (control; black dots) or treated three times a week (Monday, Wednesday, Friday) during three consecutive weeks (treatment starting the fifth day post-tumor graft) with 5 mg/kg (the maximal tolerated dose) cisplatin (intraperitoneal administrations) or 40 mg/kg lycorine (iv administrations into the tail vein). There were 11 mice per experimental group.

Altogether, the current investigation provides significant impetus for the development of lycorine analogues possessing enhanced antitumor effects without concomitant elevation of toxicity levels.

The current study provides important preliminary SAR data that could be used for the design of novel lycorine derivatives to be used in oncology. Thus, 1-*O*-acetyllycorine (**2**), 1,2-*O*,*O*′-diacetyllycorine (**3**), 2-*epi*-lycorine (**4**), 2-*O*-acetyllycorine (**5**), lycorine chlorohydrin (**6**), lycorin-2-one (**7**), 1,2-α-epoxylycorine (**14**), lycorene (**16**), and caranine (**17**) incorporate modifications of one or both hydroxyl groups located at C-1 and C-2 of the C-ring. It is noteworthy that **6** and **14** are likely converted *in vivo* into lycorine by nucleophilic substitution with water, and therefore, their contribution to the SAR data should be treated with caution. Furthermore, **4** possesses altered stereochemistry of the C-ring, while **14** incorporates a fused three-membered ring bringing about significant conformational constraints. The conformational freedom of the C-ring is raised in α- and β-dihydrolycorines (**8** and **10**), which also contain modified stereochemistry of the C/D-ring junction. The same stereochemistry is present in the corresponding diacetyl derivatives (**9** and **11**) that additionally incorporate the altered diol system at C-1 and C-2. α-Dihydrolycorine lactam (**12**) contains a modified B-ring and nonbasic nitrogen atom. Both the diol system and the double bond of the C-ring are modified in amarbellisine (**22**). The basic nature of nitrogen and stereochemistry of the B/D-ring junction are altered in *N*-methyllycorine iodide (**13**), which is a salt containing the quaternized nitrogen atom. A similar

modification is present in lycorine hydrochloride (**15**), a salt reversibly converted to **1** *in vivo*. Anhydrolycorine and ungeremine (**20** and **21**) contain the aromatized C-ring. In addition, the latter is a betaine compound incorporating the quaternized nitrogen atom. Finally, pseudolycorine and norpluvine (**18** and **19**) possess an open dioxole ring with the inverted methoxy and hydroxyl groups at C-9 and C-10, respectively. The latter alkaloid also lacks the hydroxyl group at C-2 of the C-ring.

Conclusions

In conclusion, the current study reveals that lycorine modifies the organization of actin cytoskeleton in cancer cells and, in so doing, markedly impairs both cancer cell proliferation and migration. Lycorine is at least 15 times more active as a cytostatic compound in cancer than in normal cells. This alkaloid displays antitumor activity against cancer cells regardless of whether the cells display resistance or sensitivity to apoptosis, and it is also active against undifferentiated cancer cells growing under anchorage-independent conditions; thus potentially it will be active against cancer stem cells. It is able to provide significant therapeutic benefit in aggressive brain melanoma models with iv administrations and at doses that are nontoxic. Altogether, these data should encourage synthetic organic chemists to further explore chemistry associated with lycorine and related phenanthridine alkaloids. Such efforts are expected to identify compounds capable of combating cancers associated with dismal prognoses irrespective of whether they are resistant to proapoptotic stimuli or include significant proportions of cancer stem cells.

Experimental Section

In Vitro **Pharmacology: Cell Lines.** Human cancer cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA), the European Collection of Cell Culture (ECACC, Salisbury, U.K.), and the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany). The code numbers and histological types of all the cell lines used in the current study are detailed in Table 1.

Biology and Biochemistry Related Experiments. The overall growth level of human cancer cell lines was determined using the colorimetric MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma, Belgium) assay.[14−16,25] Briefly, the cell lines were incubated for 24 h in 96-microwell plates (at a concentration of 10000−40000 cells/mL of culture medium depending on the cell type) to ensure adequate plating prior to cell growth determination. The assessment of cell population growth by means of the MTT colorimetric assay is based on the capability of living cells to reduce the yellow product MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to a blue product, formazan, by a reduction reaction occurring in the mitochondria. The number of living cells after 72 h of culture in the presence (or absence: control) of the various compounds is directly proportional to the intensity of the blue, which is quantitatively measured by spectrophotometry, in our case using a Bio-Rad Model 680XR (Bio-Rad, Nazareth, Belgium) at a 570 nm wavelength (with a reference of 630 nm). Each experiment was carried out in sextuplicate.

Cell proliferation and cell migration in differentiated cancer cell populations were determined by means of computer-assisted phase-contrast microscopy.[15,16,32,33] Cell proliferation in undifferentiated cancer cell populations was determined by means of the soft-agar assay.[37] The actin cytoskeleton organization was determined by means of computer-assisted fluorescence

microscopy.[15,16] Phallacidin conjugated with the green fluorescent Alexa Fluor488 (Molecular Probes Inc., Eugene, OR) was used to label the fibrillar actin and Alexa Fluor594 conjugated DNase I (Molecular Probes Inc.) to label the globular actin.[15,16] Apoptotic features were determined by means of flow cytometry using double iodide propidium and annexin V staining, as detailed elsewhere.[15,23]

***In Vivo* Testing.** All of the *in vivo* experiments described in the present study were performed on the basis of authorization No. LA1230568 of the Animal Ethics Committee of the Belgian Federal Department of Health, Nutritional Safety and the Environment.

***In Vivo* Toxicology Study of Lycorine in Mice.** The chronic treatment (one iv (tail vein) administration (50 μL in saline) per day during five consecutive days (Monday−Friday)) of healthy mice with lycorine (doses ranging from 5 to 80 mg/kg) enabled the maximal tolerated dose (the MTD index) to be determined as detailed elsewhere.[15,60] The MTD index is defined as the dose just below the lowest dose level that kills at least one mouse in a treatment group of three mice after a maximum of 28 days.[15,60]

***In Vivo* Activity of Lycorine in the Mouse B16F10 Metastatic Melanoma.** *In vivo* orthotopic grafts of mouse B16F10 melanoma cells into the brains of B6D2F1 mice (female mice; 17−19 g; Iffa Credo, Charles Rivers, Arbresle, France) were obtained as described previously by our group for other tumor types.[15,16] Briefly, all mice in a given experimental group had B16F10 melanoma cells stereotactically implanted into their brains on the same day. Mice in the control group received an iv (tail vein) injection of 50 μL of saline. Cisplatin (5 mg/kg; intraperitoneal administrations in 50 μL volume; three times (Monday, Wednesday, Friday) a week during three consecutive weeks) was used as a reference compound for melanoma treatment. Lycorine has been administered iv at 40 mg/kg in 50 μL according to an experimental schedule identical to the one described above for cisplatin. All treatments began on the fifth day post-tumor graft. There were 11 mice per experimental group.

Statistical Analyses. Statistical comparison of control and treated groups was initially undertaken with the Kruskal−Wallis test (a nonparametric one-way analysis of variance). Where this revealed significant differences, the Dunn multiple comparison procedure (two-sided test) was applied. However, this was adapted to the special case of comparing treatment and control groups in which only $(k − 1)$ comparisons were undertaken among the k groups tested by the Kruskal−Wallis test (instead of the possible $k(k − 1)/2$ comparisons considered in the general procedure). The levels of statistical significance associated with survival indices were determined by using Gehan's generalized Wilcoxon test. All of these statistical analyses were carried out using Statistica (Statsoft, Tulsa, OK).

Plant Material. Bulbs of *Sternbergia lutea* Ker Gawl were collected near Bari, Italy, and identified by Prof. O. Arrigoni, Dipartimento di Botanica e Patologia Vegetale, Università di Bari, Italy, where a voucher sample was deposited. Bulbs and whole plants of *Amaryllis belladonna* L. and *Pancratium maritimum* L. were collected from flowering plants cultivated in Alexandria, Egypt, and sandy hills on the northern coast of Egypt (Baltim) and were identified by Prof. Alam El-Din Negm, University of Alexandria, and Prof. N. El Hadidy, University of Cairo, Egypt, respectively. The voucher samples of the two plants were deposited in the Collection of Department of Pharmacognosy, Faculty of Pharmacy, University of Alexandria, Egypt.

Alkaloids and Their Synthetic Derivatives. Lycorine (**1**) was isolated from dried bulbs of *S. lutea* using a published procedure.[27] Ungeremine (**21**) and amarbellisine (**22**) were isolated from *P. maritimun* L. and *A. belladonna* L., respectively, as previously reported.[47,48] 1-*O*-Acetyllycorine (**2**), 1,2-*O,O'*-diacetyllycorine (**3**), 2-*epi*-lycorine (**4**), 2-*O*-acetyllycorine (**5**), lycorine chlorohydrin (**6**), lycorine-2-one (**7**), α-dihydrolycorine (**8**), 1,2-*O,O'*-diacetyl-α-dihydrolycorine (**9**), β-dihydrolycorine (**10**),

1,2-*O,O'*-diacetyl-β-dihydrolycorine (**11**), α-dihydrolycorine lactam (**12**), 1,2-α-epoxylycorine (**14**), lycorine hydrochloride (**15**), lycorene (**16**), caranine (**17**), and anhydrolycorine (**20**) were prepared from lycorine according to the previously reported procedures.[4,5,27,30] *N*-Methyllycorine iodide (**13**), pseudolycorine (**18**), and norpluvine (**19**) were generously supplied by Prof. H. M. Fales, Department of Health, Education, and Welfare, Bethesda, MD, and Prof. C. Fuganti, Istituto di Chimica, Politecnico di Milano, Italy, respectively.

General Methods. General laboratory chemicals and solvents were purchased from commercial sources and used without purification. Reactions were performed in a reaction vessel open to the atmosphere and monitored by thin-layer chromatography (TLC). Melting points were determined with an Axioskop Zeiss microscope, and optical rotations were measured on a Jasco P-1010 digital polarimeter. IR spectra were recorded on a Perkin-Elmer Spectrum One FT-IR spectrometer, and UV spectra were taken on a Perkin-Elmer Lambda 25 UV−vis spectrophotometer. ^{1}H and ^{13}C NMR spectra were recorded at 600, 400, and 75 MHz on Bruker spectrometers. EI MS were taken at 70 ev on a QP 5050 Shimadzu spectrometer. ESI MS spectra were recorded on Waters Micromass Q-TOF Micro and Agilent 1100 coupled to a JEOL AccuTOF (JMS-T100LC) spectrometers. Analytical and preparative TLCs were performed on silica gel (Merck; Kieselgel 60 F$_{254}$, 0.25 and 0.50 mm, respectively) plates; the spots were visualized by exposure to UV light and/or I$_2$ vapor. The >95% purity of all compounds was ascertained by elemental analyses, performed on an EA 1108 Elemental Analyzer, Fisons.

Lycorine or (1*S*,2*S*,12b*S*,12c*S*)-1,2-Diol-2,4,5,7,12b,12c-hexahydro-1*H*-[1,3]dioxolo[4,5-*j*]pyrrolo[3,2,1-*de*]phenanthridine (1).[1,27,29] ^{1}H NMR (400 MHz, CDCl$_3$−CD$_3$COOD, 3:1 v/v): δ 6.98 (s, H-12), 6.80 (s, H-8), 5.95 (s, H$_2$-11), 5.77 (br s, H-3), 4.58 (br s, H-1), 4.48 and 4.19 (1H each, d, $J = 14.0$ Hz, H$_2$-7), 4.26 (m, H-2), 3.95 (d, $J = 11.8$ Hz, H-12c), 3.75 and 3.49 (1H each, m, H$_2$-5), 2.99 (d, $J = 11.8$ Hz, H-12b), 2.88 (m, H$_2$-4). ^{13}C NMR (75 MHz, CDCl$_3$−CD$_3$COOD, 3:1 v/v): δ 149.6 (C-9), 148.1 (C-10), 137.9 (C-3a), 130.7 (C-7a), 125.7 (C-12a), 122.9 (C-3), 108.8 (C-8), 106.4 (C-12), 102.8 (C-11), 71.9 (C-2), 70.1 (C-1), 61.8 (C-12c), 55.1 (C-5), 54.2 (C-7), 38.2 (C-12b), 30.3 (C-4). Mp: 276−277 °C (lit.[1] mp 277 °C). [α]^{25}$_D$ +1.40 (*c* 3, 1% H$_2$SO$_4$). UV (1% H$_2$SO$_4$) nm (log ε): 288 (3.55), 238 (3.45). IR (nujol) 3320 cm^{-1}. EI MS, *m/z*: 287 [M]$^+$, 286, 268, 252, 250, 227, 226. Elemental analysis: C, 66.94; H, 5.95; N, 4.84; O, 22.23 (calcd for C$_{16}$H$_{17}$NO$_4$, C, 66.89; H, 5.90; N, 4.88; O, 22.27).

1-*O*-Acetyllycorine or (1*S*,2*S*,12b*S*,12c*S*)-1,2-Diol-2,4,5,7,12b,12c-hexahydro-1*H*-[1,3]dioxolo[4,5-*j*]pyrrolo[3,2,1-*de*]phenanthridine-1-acetate (2).[4,49,50] ^{1}H NMR (400 MHz, CDCl$_3$): δ 6.70 (s, H-8), 6.44 (H-12), 5.89 (s, H$_2$-11), 5.56 (m, H-3), 5.50 (m, H-1), 4.14 and 3.48 (1H each, d, $J = 14.0$ Hz, H$_2$-7), 4.13 (m, H-2), 3.32 and 2.35 (br dd, $J = 8.5$ and 8.5 Hz, and br q, $J = 8.5$ and 5.2 Hz, H$_2$-5), 2.84 (d, $J = 10.3$ Hz, H-12c), 2.84 (dd, $J = 10.3$ and 2.2 Hz, H-12b), 2.59 (1H each, m, H$_2$-4). ^{13}C NMR (75 MHz, CDCl$_3$): δ 171.8 (Me$\underline{C}$O), 146.5 (C-9), 143.7 (C-10), 136.1 (C-3a), 129.3 (C-7a), 127.2 (C-12a), 117.4 (C-3), 107.3 (C-8), 104.9 (C-12), 100.9 (C-11), 72.7 (C-1), 69.4 (C-2), 61.5 (C-12c), 56.8 (C-5), 53.6 (C-7), 39.2 (C-12b), 28.5 C-4), 20.9 ($\underline{Me}$CO). Mp: 215−216 °C (lit.[49] mp 215−216 °C). [α]^{25}$_D$ −64.8 (*c* 0.9, CHCl$_3$). UV (CHCl$_3$) nm (log ε): 290 (3.70), 240 (3.60). IR (CHCl$_3$) 3610, 1733 cm^{-1}. EI MS, *m/z*: 329 [M]$^+$, 328, 269 252, 250, 227, 226. Elemental analysis: C, 65.66; H, 5.86; N, 4.28; O, 24.25 (calcd for C$_{18}$H$_{19}$NO$_5$, C, 65.64; H, 5.81; N, 4.25; O, 24.29).

1,2-*O,O'*-Diacetyllycorine or (1*S*,2*S*,12b*S*,12c*S*)-1,2-Diol-2,4,5,7,12b,12c-hexahydro-1*H*-[1,3]dioxolo[4,5-*j*]pyrrolo[3,2,1-*de*]phenanthridine-1,2-diacetate (3).[4,49] ^{1}H NMR (600 MHz, CDCl$_3$): δ 6.76 (s, H-8), 6.59 (s, H-12), 5.94 (s, H$_2$-11), 5.75 (s, H-1), 5.45 (s, H-3), 5.27 (s, H-2), 4.18 and 3.55 (1H each, d, $J = 13.9$ Hz, H$_2$-7), 2.89 (d, $J = 10.3$ Hz, H-12c), 3.38 and 2.42 (1H each, m, H$_2$-5), 2.79 (d, $J = 10.3$ Hz, H-12b), 2.67 (m, H$_2$-4), 2.09

and 1.97 (3H, each, 2 × MeCO). ^{13}C NMR (75 MHz, CDCl$_3$): δ 171.8 and 172.3 (2 × MeCO), 146.5 (C-3a), 146.4 (C-9), 146.2 (C-10), 129.5 (C-7a), 126.6 (C-12a), 113.9 (C-3), 107.4 (C-12), 105.1 (C-8), 101.0 (C-11), 71.0 (C-2), 69.3 (C-1), 61.3 (C-12c), 58.9 (C-7), 53.9 (C-5), 40.6 (C-12b), 28.7 (C-4), 21.3 and 20.9 (2 × MeCO). Mp: 216−217 °C (lit.[49] mp 215−216 °C). [α]$^{25}_D$ −31.1 (*c* 1.1, CHCl$_3$). UV (CHCl$_3$) nm (log ε): 290 (3.70), 240 (3.59). IR (CHCl$_3$) 1740 cm^{-1}. EI MS, *m/z*: 371 [M]$^+$, 370, 328, 311, 252, 250, 227, 226. Elemental analysis: C, 64.72; H, 5.67; N, 3.80; O, 25.81 (calcd for C$_{20}$H$_{21}$NO$_6$, C, 64.68; H, 5.70; N, 3.77; O, 25.85).

2-*epi*-Lycorine or (1*S*,2*R*,12b*S*,12c*S*)-1,2-Diol-2,4,5,7,12b, 12c-hexahydro-1*H*-[1,3]dioxolo[4,5-*j*]pyrrolo[3,2,1-*de*]phenanthridine (4).[4,49] Mp: 167−168 °C (lit.[50] mp 167−168 °C). [α]$^{25}_D$ −217 (*c* 1.1, CHCl$_3$). UV (MeOH) nm (log ε): 290 (3.67). IR (nujol) 3472, 3413 cm^{-1}. Elemental analysis: C, 66.85; H, 5.93; N, 4.82; O, 22.24 (calcd for C$_{16}$H$_{17}$NO$_4$, C, 66.89; H, 5.90; N, 4.88; O, 22.27).

2-*O*-Acetyllycorine or (1*S*,2*S*,12b*S*,12c*S*)-1,2-Diol-2,4,5,7,- 12b,12c-hexahydro-1*H*-[1,3]dioxolo[4,5-*j*]pyrrolo[3,2,1-*de*]phenanthridine-2-acetate (5).[1,4] ^{1}H NMR (400 MHz, CDCl$_3$−CD$_3$COOD, 3:1 v/v): δ 6.94 (s, H-12), 6.78 (s, H-8), 5.96 (s, H$_2$-11), 5.69 (s, H-3), 5.36 (s, H-2), 4.67 (s, H-1), 4.60 and 3.98 (1H each, d, *J* = 13.6 Hz, H$_2$-7), 4.13 (d, *J* = 12.1 Hz, H-12c), 3.87 and 3.32 (1H each, m, H$_2$-5), 2.90 (*J* = 12.1 Hz, H-12b), 2.80 (m, H$_2$-4), 2.04 (MeCO). Mp: 231−232 °C (lit.[1] mp 230−232 °C). [α]$^{25}_D$ +22.4 (*c* 0.2, CHCl$_3$). UV (CHCl$_3$) nm (log ε): 291 (3.67), 240 (3.60). IR (CHCl$_3$) 3580, 1718 cm^{-1}. EI MS, *m/z*: 329 [M]$^+$, 328, 269, 252, 250, 227, 226. Elemental analysis: C, 65.67; H, 5.84; N, 4.21; O, 24.25 (calcd for C$_{18}$H$_{19}$NO$_5$, C, 65.64; H, 5.81; N, 4.25; O, 24.29).

Lycorine Chlorohydrin or (1*S*,2*S*,12b*S*,12c*S*)-1-Ol-2-chloro- 2,4,5,7,12b,12c-hexahydro-1*H*-[1,3]dioxolo[4,5-*j*]pyrrolo[3,2,1- *de*]phenanthridine (6).[5,51] ^{1}H NMR (400 MHz, CDCl$_3$): δ 6.79 (s, H-12), 6.59 (s, H-8), 5.93 (d, *J* = 1.5 Hz, H-11A), 5.91 (d, *J* = 1.5 Hz, H-11B), 5.56 (m, H-3), 4.78 (dd, *J* = 2.2 and 2.0 Hz, H-1), 4.65 (m, H-2), 4.13 and 3.51 (1H each, d, *J* = 14.0 Hz, H$_2$-7), 3.35 and 2.37 (1H each, m, H$_2$-5), 2.99 (d, *J* = 10.7 Hz, H-12c), 2.85 (dd, *J* = 10.7 and 2.0 Hz, H-12b), 2.63 (m, H$_2$-4). UV (EtOH) nm (log ε): 292 (3.68), 280 (3.60). IR (CHCl$_3$) 3520, 1625, 1505, 1485 cm^{-1}. EI MS, *m/z*: 307 [M + 2]$^+$, 305 [M]$^+$, 270, 250, 227, 226. Elemental analysis: C, 62.88; H, 5.23; N, 4.61 (calcd for C$_{16}$H$_{16}$NO$_3$, C, 62.85; H, 5.27; N, 4.58).

Lycorin-2-one or (1α)-3,12-Didehydro-1-hydroxy-9,10-[meth- ylenebis(oxy)]galanthan-2-one (7).[4] ^{1}H NMR (400 MHz, CDCl$_3$−CD$_3$COOD, 3:1 v/v): δ 6.82 (s, H-12), 6.65 (s, H-8), 6.05 (s, H-3), 5.92 (s, H$_2$-11), 4.73 (d, *J* = 2.6 Hz, H-1), 4.36 (d, *J* = 14.7 Hz, H-7A), 4.29 (1H, d, *J* = 10.7 Hz, H-12c; 1H, d, *J* = 14.7 Hz, H-7B; by NMDR), 3.74 and 3.56 (1H each m, H$_2$-5), 3.49 (dd, *J* = 10.7 and 2.6 Hz, H-12b), 3.17 (m, H$_2$-4). Mp: 143−145 °C (lit.[4] mp 143−145 °C). [α]$^{25}_D$ −99.7 (*c* 2.5, 1% H$_2$SO$_4$). UV (CHCl$_3$) nm (log ε): 292 (3.47), 245 (3.50). IR (nujol) 3150, 1655 cm^{-1}. EI MS, *m/z*: 285 [M]$^+$, 284, 267, 226. Elemental analysis: C, 67.40; H, 5.26; N, 4.95; O, 22.48 (calcd for C$_{16}$H$_{15}$NO$_4$, C, 67.36; H, 5.30; N, 4.91; O, 22.43).

α-Dihydrolycorine or (1*S*,2*S*,3a*R*,12b*S*,12c*R*)-1,2-Diol-2,3, 3a,4,5,7,12b,12c-octahydro-1*H*-[1,3]dioxolo[4,5-*j*]pyrrolo[3,2,1- *de*]phenanthridine (8).[4,29,49] ^{1}H NMR (400 MHz, CD$_3$OD−CD$_3$-COOD, 3:1 v/v): δ 7.08 (s, H-12), 6.85 (s, H-8), 5.98 (s, H$_2$-11), 4.60 and 4.0 (1H each, d, *J* = 14.0 Hz, H$_2$-7), 4.53 (dd, *J* = 2.0 and 2.0 Hz, H-1), 4.07 (m, H-2), 3.65 (d, *J* = 11.7 Hz, 12c), 3.24 (dd, *J* = 11.7 and 2.0 Hz, 12b), 3.63 and 3.40 (1H each, m, H$_2$-5), 2.59 (m, H-3a), 2.59 and 2.10 (1H each, m, H$_2$-4), 2.59 and 2.10 (1H each, m, H$_2$-3). ^{13}C NMR (75 MHz, CD$_3$OD−CD$_3$COOD, 3:1 v/v): δ 149.7 (C-9), 147.7 (C-10), 133.0 (C-7a), 124.5 (C-12a), 108.6 (C-8), 106.9 (C-12), 102.6 (C-11), 70.5 (C-2), 69.6 (C-1), 64.3 (C-12c), 56.5 (C-5), 53.8 (C-7), 36.6 (C-3a), 33.2 (C-12b), 30.5 (C-4), 27.8 (C-3). Mp: 247 °C. [α]$^{25}_D$ +99.5 (*c* 3, 1% H$_2$SO$_4$). UV (1% H$_2$SO$_4$) nm (log ε): 287 (3.66), 238 (3.58). IR (nujol) 3410 cm^{-1}. EI MS, *m/z*: 289 [M]$^+$, 288, 270, 254.

Elemental analysis: C, 66.46; H, 6.65; N, 4.80; O, 22.16 (calcd for C$_{16}$H$_{19}$NO$_4$, C, 66.42; H, 6.62; N, 4.84; O, 22.12).

1,2-*O*,*O'*-Diacetyl-α-dihydrolycorine or (1*S*,2*S*,3a*R*,12b*S*, 12c*R*)-1,2-Diol-2,3,3a,4,5,7,12b,12c-octahydro-1*H*-[1,3]diox- olo[4,5-*j*]pyrrolo[3,2,1-*de*]phenanthridine-diacetate (9).[4,52] This compound was prepared as described in the literature[4] from α-dihydrolycorine (8). The physical and spectral properties were identical to those previously reported.[52] More specifically the ^{1}H NMR spectrum (400 MHz, CD$_3$OD−CD$_3$COOD, 3:1 v/v) differed from that of 8 by the downfield shift of H-1 and H-2 (Δδ 1.42 and 1.34) and the two singlets due to the acetyl groups (δ 2.04 and 1.97). Mp: 178−180 °C (lit.[52] 179−180 °C). Elemental analysis: C, 64.30; H, 6.25; N, 3.79; O, 25.84 (calcd for C$_{20}$H$_{23}$NO$_6$, C, 64.33; H, 6.21; N, 3.75; O, 25.71).

β-Dihydrolycorine or (1*S*,2*S*,3a*S*,12b*S*,12c*R*)-1,2-Diol-2,3,3a, 4,5,7,12b,12c-octahydro-1*H*-[1,3]dioxolo[4,5-*j*]pyrrolo[3,2,1-*de*]- phenanthridine (10).[4] ^{1}H NMR (400 MHz, CDCl$_3$−CD$_3$COOD, 3:1 v/v): δ 6.91 (s, H-12), 6.70 (s, H-8), 5.96 (s, H$_2$-11), 4.52 (1H, d, *J* = 14.0 Hz H-7A; 1H, t, *J* = 1.9 Hz, H-1; by NMDR), 4.27 (d, *J* = 14.0 Hz, H-7b), 4.09 (m, H-2), 3.82 and 3.45 (1H each, m, H$_2$-5), 3.31 (dd, *J* = 12.1 and 1.9 Hz, H-12B), 3.14 (t, *J* = 12.1 Hz, H-12c), 2.36 (m, H-3a), 2.23 (m, H-4A), 2.11 (dt, *J* = 13.7 and 3.8 Hz, H-3A), 1.81 (1H, dd, *J* = 13.7 and 2.3 Hz H-3b; 1H, m, H-4B; by NMDR). Mp: 120−123 °C (lit.[4] mp 120−123 °C). [α]$^{25}_D$ −85.0 (*c* 0.5, 1% H$_2$SO$_4$). UV (1% H$_2$SO$_4$) nm (log ε): 289 (3.37), 236 (3.29). IR (nujol) 3400 cm^{-1}. EI MS, *m/z*: 289 [M]$^+$, 288, 270. Elemental analysis: C, 66.45; H, 6.59; N, 4.87; O, 22.16 (calcd for C$_{16}$H$_{19}$NO$_4$, C, 64.42; H, 6.62; N, 4.84; O, 22.12).

1,2-*O*,*O'*-Diacetyl-β-dihydrolycorine or (1α,2β,12β)-1,2-Diol- 9,10-[methylenebis(oxy)]galanthan-diacetate (11).[4] This compound was prepared from lycorine as white prisms and as described in the literature.[4] The ^{1}H NMR spectrum (400 MHz, CDCl$_3$−CD$_3$COOD, 3:1 v/v) differed from that of the β-dihydrolycorine by the downfield shift of H-1 and H-2 (Δδ 1.03 and 1.24) and the two singlets due to the acetyl groups (δ 2.02 and 1.98). Elemental analysis: C, 64.38; H, 6.25; N, 3.71; O, 25.74 (calcd for C$_{20}$H$_{23}$NO$_6$, C, 64.33; H, 6.21; N, 3.75; O, 25.71).

α-Dihydrolycorine Lactam or (1α,2β)-1,2-Dihydroxy-9,10- [methylenebis(oxy)]galanthan-7-one (12).[4,29,52] This compound was prepared from lycorine as white prisms and as described in the literature.[4] The ^{1}H NMR spectrum (400 MHz, CDCl$_3$−CD$_3$COOD, 3:1 v/v) differed from that of the α-dihydrolycorine by the absence of the AB system of H$_2$-7. ^{13}C NMR (75 MHz, CD$_3$OD−CD$_3$COOD, 3:1 v/v): δ 165.1 (C-7), 152.3 (C-10), 147.8 (C-9), 137.3 (C-12a), 126.0 (C-7a), 108.9 (C-8), 106.4 (C-12), 103.0 (C-11), 73.9 (C-2), 70.4 (C-1), 57.3 (C-12c), 46.8 (C-5), 39.4 (C-3a), 36.1 (C-12b), 32.9 (C-4), 30.8 (C-3). Mp: 268−270 °C (lit.[52] mp 270 °C). Elemental analysis: C, 63.32; H, 5.68; N, 4.66; O, 26.33 (calcd for C$_{16}$H$_{17}$NO$_5$, C, 63.36; H, 5.65; N, 4.62; O, 26.37).

N-Methyllycorine Iodide or (1α,2β)-3,12-Didehydro-1,2-dihy- droxy-6-methyl-9,10-[methylenebis(oxy)]galanthanium Iodide (13).[53] The compound was generously supplied by Prof. H. M. Fales, Department of Health Education and Welfare, Bethesda, MD. The ^{1}H NMR spectrum (400 MHz, CDCl$_3$−CD$_3$COOD, 3:1 v/v) differed from that of the lycorine for the presence of the singlet at δ 7.90 due to the N-methyl. Elemental analysis: C, 47.60; H, 4.64; N, 3.29; O, 26.33 (calcd for C$_{17}$H$_{20}$NO$_4$, C, 47.57; H, 4.70; N, 3.26).

1,2-α-Epoxylycorine or (1α,2α)-3,12-Didehydro-1,2-epoxy- 9,10-[methylenebis(oxy)]galanthan (14).[5,53] ^{1}H NMR (400 MHz, CDCl$_3$): δ 6.79 (s, H-12), 6.56 (s, H-8), 5.93 (d, *J* = 1.5 Hz, H-11A), 5.90 (d, *J* = 1.5 Hz, H-11B), 5.45 (m, H-3), 5.01 (m, H-2), 4.64 (dd, *J* = 2.2 and 2.0 Hz, H-1), 4.12 and 3.52 (1H each, d, *J* = 14.0 Hz, H$_2$-7), 3.36 and 2.40 (1H each, m, H$_2$-5), 3.11 (dd, *J* = 10.7, 2.0 Hz, H-12b), 2.82 (d, *J* = 10.7 Hz, H-12c), 2.63 (m, H$_2$-4). [α]$^{25}_D$ −194.4 (*c* 0.1, CHCl$_3$). UV (EtOH) nm (log ε): 290 (3.74), 235 (sh). IR (CHCl$_3$) 1620, 1505, 1485 cm^{-1}. EI MS, *m/z*: 269 [M]$^+$, 265, 250, 227, 226. Elemental analysis: C, 71.32; H, 5.64; N,

5.23; O, 17.79 (calcd for $C_{16}H_{15}NO_3$, C, 71.36; H, 5.61; N, 5.20; O, 17.82).

Lycorine Hydrochloride or (1S,2S,12bS,12cS)-1,2-Diol-2,4, 5,7,12b,12c-hexahydro-1H-[1,3]dioxolo[4,5-j]pyrrolo[3,2,1-de]phenanthridine Hydrochloride (15).[27] This compound was prepared as described in the literature[27] from lycorine. The spectroscopic data (¹H and ¹³C NMR) were identical to those of lycorine. Elemental analysis: C, 59.32; H, 5.64; N, 4.37 (calcd for $C_{16}H_{18}NO_4$, C, 59.35; H, 5.60; N, 4.33).

Lycorene or (12bS,12cS)-2,4,5,7,12b,12c-Hexahydro-1H-[1, 3]dioxolo[4,5-j]pyrrolo[3,2,1-de]phenanthridine (16).[5,55] ¹H NMR (400 MHz, CDCl₃): δ 6.77 (s, H-12), 6.58 (s, H-8), 5.92 (d, J = 1.5 Hz, H-11A), 5.90 (d, J = 1.5 Hz, H-11B), 5.44 (m, H-3), 4.11 (d, J = 14.0 Hz, H-7A), 3.54 (dd J = 14.0 and 2.2 Hz, H-7B), 3.23 and 2.32 (m, H₂-5), 2.54 and 2.37 (1H each, m, H₂-1), 2.54 and 2.30 (1H each, m, H₂-2), 2.45 (ddd, J = 10.7, 2.2, and 2.0 Hz, H-12b), 2.43 (dd, J = 10.7 and 2.0 Hz, H-12c), 2.35 (m, H₂-4). Mp: 112−116 °C (lit.[55] mp 120−121). UV (EtOH) nm (log ε): 290 (3.67), 237 (sh). IR (CHCl₃) 1605, 1505, 1485 cm⁻¹. EI MS, m/z: 255 [M]⁺, 254, 252, 227, 226. Elemental analysis: C, 75.30; H, 6.75; N, 5.44; O, 12.58 (calcd for $C_{16}H_{17}NO_2$, C, 75.27; H, 6.71; N, 5.49; O, 12.53).

Caranine or (1R,12bS,12cS)-1-Ol-2,4,5,7,12b,12c-hexahydro-1H-[1,3]dioxolo[4,5-j]pyrrolo[3,2,1-de]phenanthridine (17).[5,55] ¹H NMR (400 MHz, CDCl₃): δ 6.82 (s, H-12), 6.58 (s, H-8), 5.92 (d, J = 1.5 Hz, H-11A), 5.90 (d, J = 1.5 Hz, H-11B), 5.41 (m, H-3), 4.70 (m, H-1), 4.13 (d, J = 14.0 Hz, H-7A), 3.52 (dd, J = 14.0 and 2.2 Hz, H-7B), 3.32 and 2.33 (m, H₂-5), 2.59 (m, H-2), 2.78 (dd, J = 10.7 and 2.6 Hz, H-12c), 2.41 (ddd, J = 10.7, 2.2, and 2.0 Hz, H-12b), 2.59 (m, H₂-4). Mp: 176−180 °C (lit.[55] mp 178−181 °C). UV (EtOH) nm (log ε): 291 (3.44), 280 (3.49), 237 (3.49). IR (CHCl₃) 3680, 1605, 1505, 1485 cm⁻¹. EI MS, m/z: 271 [M]⁺, 270, 252, 227, 226. Elemental analysis: C, 70.86; H, 6.36, N, 5.13; O, 17.70 (calcd for $C_{16}H_{17}NO_3$, C, 70.83; H, 6.32; N, 5.16; O, 17.69).

Pseudolycorine or (1S,2S,11bS,11cS)-1,2,10-Triol-2,4,5,7, 11b,11c-hexahydro-9-methoxy-1H-pyrrolo[3,2,1-de]phenanthridine (18).[56] The compound was generously supplied by Prof. H. M. Fales, Department of Health Education and Welfare, Bethesda, MD. ¹H NMR (400 MHz, CDCl₃−DMSO-d₆): δ 6.78 (s, H-11), 6.62 (br s, H-8), 5.41 (m, H-3), 4.5−2.0 (10H). Mp: 247−249 °C (lit.[56] mp 247−249 °C). [α]²⁵_D −57.8 (c 0.3 EtOH). EI MS, m/z: 289 [M]⁺, 274, 270, 258, 229, 228. Elemental analysis: C, 66.46; H, 6.67; N, 4.81; O, 22.16 (calcd for $C_{16}H_{19}NO_4$, C, 66.42; H, 6.62; N, 4.84; O, 22.12).

Norpluvine or (1R,11bS,11cS)-1,9-Diol-10-methoxy-2,4,5,7, 11b,11c-hexahydro-1H-pyrrolo[3,2,1-de]phenanthridine (19).[57] The compound was generously supplied by Prof. C. Fuganti, Istituto di Chimica, Politecnico di Milano, Italy. ¹H NMR (400 MHz, CDCl₃): δ 6.74 (s, H-11), 6.64 (s H-8), 5.97 (dd, J = 5.9, 1.0, Hz, H-1), 5.39 (d, J = 2.2 Hz, H-3), 4.26 (dd, J = 5.9, 1.0, Hz, H-1), 4.13 and 3.50 (1H each, d, J = 14.5 Hz, H₂-7), 3.34 and 2.37 (1H, each, m, H₂-5), 2.76 (d, J = 9.9 Hz, H-11c), 2.66 (d, J = 9.9 Hz, H-11b), 2.62 and 2.33 (m, H₂-2), 2.59 (m, H₂-4), 114.5−2.0 (10H). Mp: 274−275 °C (lit.[57] 274−275 °C). [α]²⁵_D −160 (c 0.15 MeOH). EI MS, m/z: 272 [M]⁺, 273, 252, 253, 244, 229, 228. Elemental analysis: C, 70.28; H, 7.06; N, 5.17; O, 17.53 (calcd for $C_{16}H_{19}NO_3$, C, 70.31; H, 7.01; N, 5.12; O, 17.56).

Anhydrolycorine or 4,5-Dihydro-7H-[1,3]dioxolo[4,5-j]pyrrolo[3,2,1-de]phenanthridine (20).[30,58] ¹H NMR (400 MHz, CDCl₃): δ 7.28 (d, J = 8.1 Hz, H-1), 7.16 (s, H-11), 7.00 (dt, J = 8.1 and 1.5 Hz, H-3), 6.75 (dd, J = 8.1 and 8.1 Hz, H-2), 6.63 (s, H-8), 5.97 (s, H₂-11), 4.06 (m, H₂-7), 3. 32 (t, J = 7.4, H₂-5), 3.02 (td, J = 7.4 and 1.5 Hz, H₂-4). Mp: 115−120 °C (lit.[58] mp 122−123 °C). UV (EtOH) nm (log ε): 342 (4.02), 287 (3.82), 280 (3.85), 249 (4.32). IR (CHCl₃) 1630 cm⁻¹; EI MS, m/z: 251 [M]⁺, 250. Elemental analysis: C, 76.44; H, 5.25; N, 5.54; O, 12.70 (calcd for $C_{16}H_{13}NO_2$, C, 76.48; H, 5.21; N, 5.57; O, 12.73).

Ungeremine or 2-Hydroxy-4,5-dihydro-[1,3]dioxolo[4,5-j]pyrrolo[3,2,1-de]phenanthridinium (21).[30,47,59] This compound was isolated from whole plant of *P. maritimum* L. as previously

reported in the literature.[47] ¹H NMR (400 MHz, CD₃OD−CD₃. COOD, 3:1 v/v): δ 9.32 (s, H-7), 7.96 (s, H-11), 7.66 (s, H-8), 7.33 (dt, J = 1.8 and 1.8 Hz, H-3), 6.36 (s, H₂-11), 5.22 (t, J = 7.0 Hz, H₂-5), 3.73 (td, J = 7.0, 1.8 Hz, H₂-4). ¹³C NMR (CD₃O-D−CD₃COOD, 3:1 v/v): δ 162.6 (C-2), 157.3 (C-9), 151.8 (C-10), 141.9 (C7), 139.8 (C-3a), 132.9 (C-12c), 132.0 (C-12b), 126.2 (C-7a), 124.0 (C-12a), 118.1 (C-3), 108.2 (C-8), 105.4 (C-11), 104.1 (C-1), 102.0 (C-12), 57.0 (C-5), 28.3 (C-4). Mp: 268−270 °C (lit.[59] mp 260−270 dec). UV (0.1 N NaOH) nm (log ε): 405 (3.90), 272 (4.57). IR (nujol) 1615 cm⁻¹. EI MS, m/z: 265 [M]⁺, 264. Elemental analysis: C, 72.43; H, 4.14; N, 5.31; O, 18.12 (calcd for $C_{16}H_{11}NO_3$, C, 72.45; H, 4.18; N, 5.28; O, 18.09).

Amarbellisine or (1S,3aS,12bS,12cS)-1-Ol-2-methoxy-3a,4,5, 7,12b,12c-hexahydro-1H-[1,3]dioxolo[4,5-j]pyrrolo[3,2,1-de]phenanthridine (22).[48] This compound was isolated from bulbs of *A. belladonna* L. as previously reported in the literature.[48] ¹H NMR (400 MHz, CDCl₃): δ 6.54 (s, H-12), 6.45 (s, H-8), 5.88 and 5.86 (1H each, d, J = 1.1 Hz, H₂-11), 5.56 (br s, H-3), 4.33 and 3.79 (1H each, d, J = 16.7 Hz, H₂-7), 4.08 (br s, H-12c), 3.48 (br s, H-1), 3.43 (s, OMe), 3.41 (br ddd, J = 11.8, 5.4, and 2.3 Hz, H-3a), 3.28 (br s, H-12b), 3.07 and 3.02 (dd, J = 11.2 and = 2.2 Hz, and d, J = 11.2 Hz, H₂-5), 2.14 (ddd, J = 12.9, 5.4, and 3.4 Hz, H2−4). ¹³C NMR (75 MHz, CDCl₃): δ 154.2 (C-2), 146.7 (C-10), 146.0 (C-9), 132.5 (C-7a), 124.6 (C-12a), 112.9 (C-3), 107.3 (C-8), 106.8 (C-12), 100.7 (C-11), 79.8 (C-1), 69.1 (C-12c), 60.9 (C-7), 58.6 (C-3a), 57.6 (OMe), 55.4 (C-5), 45.6 (C-12b), 32.7 (C-4). Mp: < 300 °C (lit.[48] mp < 300 °C). [α]²⁵_D (c 0.7, CHCl₃) −39.2. UV (MeOH) nm (log ε): 293 (2.90), 244 (2.90). IR (KBr) 3439, 1645 cm⁻¹. ESI MS, m/z: 340 [M + K]⁺, 324 [M + Na]⁺, 302 [M + H]⁺. Elemental analysis: C, 67.80; H, 6.33; N, 4.69; O, 21.22 (calcd for $C_{17}H_{19}NO_4$, C, 67.76; H, 6.36; N, 4.65; O, 21.24).

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