Leucovernine and Acetylleucovernine, Alkaloids from *Leucojum vernum*

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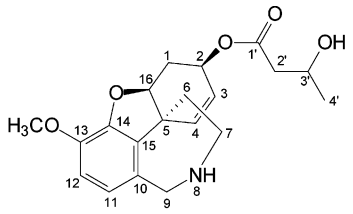
The fresh bulbs of *Leucojum vernum* provided seven tyrosine-derived alkaloids; two of them have not been reported before and are named leucovernine and acetylleucovernine. The five known alkaloids were *N*-demethylgalanthamine, hippeastrine, 9-*O*-demethylhomolycorine, 5α-hydroxyhomolycorine, and 11-hydroxyvittatine. These compounds have been isolated from this species for the first time. The structure determination was carried out by the combination of liquid-phase one- and two-dimensional NMR spectroscopy and high-resolution mass spectrometry.

The Amaryllidaceae family, including 65 genera and about 860 species, occur naturally throughout the tropics and warm temperate regions of the world. Major centers of diversity are South America and South Africa. In Europe only seven genera (*Amaryllis*, *Sternbergia*, *Leucojum*, *Galanthus*, *Lapiedra*, *Narcissus*, *Pancratium*), including 48 species, are native.[1] The medicinal value of the Amaryllidaceae species is attributed to the presence of tyrosine-derived alkaloids, which are produced exclusively by this family. These alkaloids possess cytotoxic and antiviral activity and a wide range of other physiological effects, such as acetylcholinesterase inhibitory, anti-inflammatory, and antibacterial.[2−5] Some members of this group of alkaloids (e.g., pancratistatin, narciclasine) have strong human cancer cell growth inhibitory activities and are promising candidates for drug development programs as antineoplastic agents.[6,7] Galanthamine, a centrally acting competitive inhibitor of acetylcholinesterase, is now introduced in clinical therapy for the treatment of Alzheimer's disease.[8]

As a part of our ongoing studies of Amaryllidaceae alkaloids, the bulbs of *Leucojum vernum* L. were investigated. In our previous paper the isolation and identification of lycorine, 2-*O*-acetyllycorine, and homolycorine were reported from *L. vernum*, together with the assay of the HIV-1 inhibitory activity of some Amaryllidaceae alkaloids.[9] The present paper deals with the isolation and structure elucidation of two new galanthamine-type alkaloids, named leucovernine (**1**) and acetylleucovernine (**2**), in addition to the known alkaloids *N*-demethylgalanthamine (**3**),[10] 9-*O*-demethylhomolycorine (**4**),[11] hippeastrine (**5**),[12] 5α-hydroxyhomolycorine (**6**),[13] and 11-hydroxyvittatine (**7**),[14] which are reported for the first time from this plant.

The methanolic extract of the fresh bulbs of *L. vernum* yielded seven alkaloids (**1**−**7**), which were isolated by means of pH-gradient extraction and multistep chromatographic purification, including vacuum liquid chromatography and preparative TLC.

The HREIMS data of compound **1** suggested the molecular formula $C_{20}H_{25}NO_5$ with a molecular ion at *m/z* 359.17295. The ^{1}H NMR spectrum of **1** afforded signals in an approximately 6 ppm range with the presence of aliphatic and unsaturated functional groups. The doublet at 1.18 ppm was assigned to a methyl group attached to a methine carbon, and the singlet at 3.83 ppm represents an *O*-methyl resonance. The coupling constant modulated ^{13}C spin−echo experiment (JMOD) revealed the two methyl



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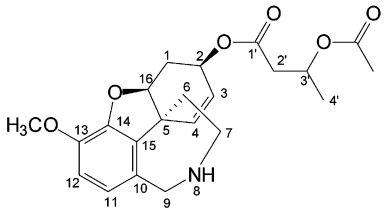

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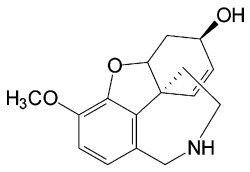

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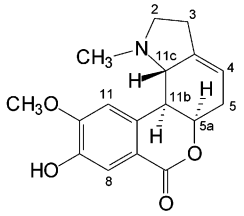

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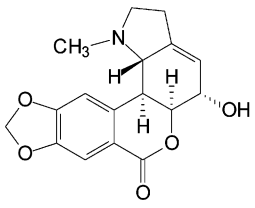

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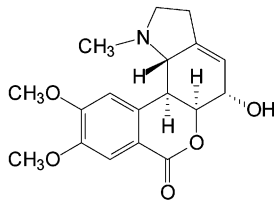

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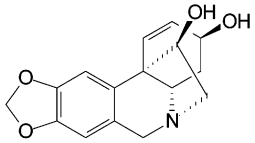

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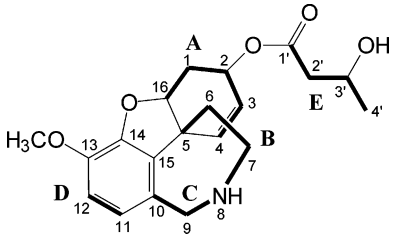

Figure 1. COSY correlations in **1**.

frequencies as 22.6 and 55.9 ppm along with seven methine resonances. The spectrum confirmed five methylene groups and six quaternary carbons. The signal assignment and the structure determination required the extensive use of two-dimensional NMR techniques; a homonuclear COSY experiment was applied to identify the scalar coupling pattern, and heteronuclear correlations were monitored by HSQC and HMBC experiments. Furthermore the solution conformation and relative configurations were obtained by the NOESY experiment. On the basis of the two-dimensional experiments it was found that the molecule has a galanthamine alkaloid skeleton containing a fused ring system.

The COSY experiment identified several scalarly coupled spin systems, a $-CH-CH_2-CH-CH=CH-$ moiety (A), a $-CH_2-CH_2-$ fragment (B), an isolated $-CH_2-$ group (C), an aromatic $-CH-CH-$ group (D), and a $-CH_2-CH-CH_3$ (E) functionality (Figure 1.). The assignment of the subunits (A–E) was carried out by analyzing the coupling pattern in the COSY spectrum. The largest spin system is A, in which there is a terminal olefinic CH (6.31 ppm) resonating as a doublet (10.3 Hz). Its partner has a chemical shift of 5.87 ppm, but it is also coupled (5.0 Hz) to an sp^3 carbon-attached proton at 5.38 ppm. This CH proton has a CH_2 neighbor, resonating as two nonequivalent protons at 2.70 ppm (16.2, 3.2, and 1.4 Hz) and 2.05 ppm (16.2, 4.8, and 3.2 Hz). This spin system terminates with an oxygen-attached sp^3 CH proton with a chemical shift of 4.56 ppm (small couplings to the previous CH_2 group). The B subunit has two methylene groups with four nonequivalent protons. The highest two chemical shifts (3.35 and 3.24 ppm) were assigned to the nitrogen-attached CH_2, and its coupling partner has two proton resonances at 1.85 and 1.79 ppm. The isolated CH_2 group (C) has a quartet-like appearance with 4.02 and 3.93 ppm chemical shifts (15.4 Hz). The aromatic system (D) contains two *o*-coupled doublets at 6.64 and 6.58 ppm (8.2 Hz). Subunit E has a doublet methyl at 1.18 ppm, which is coupled to a methine signal (4.19 ppm), and it is assumed to be attached to a hydroxyl group. Furthermore, the CH proton has couplings to two protons at 2.48 and 2.39 ppm, which are nonequivalent methylene protons. The ^1H NMR assignment of the subunits allowed the identification of the corresponding ^{13}C resonances through the JMOD and HSQC experiments. The HSQC experiment provided the ^{13}C chemical shift correlations, and the JMOD identified the functional groups by the phase of the detected signal. The connections between the five different subunits were determined by the proton-detected long-range correlation experiment (HMBC). The E subunit was determined to be a 3′-hydroxybutyryl group since its protons (2.48, 2.39, and 4.19 ppm) are coupled to a carbonyl signal at 171.9 ppm. Furthermore, this carbonyl group has a correlation to a proton (5.38 ppm), which proves the connection between subunits A and E. The protons (4.56 ppm and the olefinic proton at 6.31 ppm) at the two ends of group A have long-range correlations to the same sp^3 ^{13}C signal at 48.6 ppm,

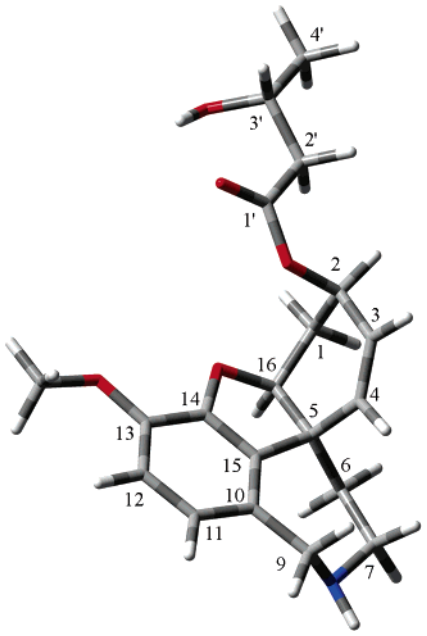

Figure 2. Calculated conformation of **1**.

indicating that group A forms a six-membered ring. The high-field component (1.79 ppm) of the B group also has long-range connection to the 48.6 ppm ^{13}C signal. Furthermore the low-field methylene protons (3.35 and 3.24 ppm) show correlation to the isolated methylene function (group C). The protons of the C moiety have additional correlations to the aromatic function (quaternary carbon at 133.3 ppm), and one of the aromatic protons (6.58 ppm) in the D system also has connections to group C protons. The methoxy group is also connected to the aromatic system, because an interaction can be observed between the methyl protons (3.83 ppm) and the ^{13}C signal at 144.0 ppm. This carbon signal has correlations to both protons of the D moiety. The aromatic ring has two more unidentified ^{13}C resonances (146.9 and 132.0 ppm); however the long-range correlations to the aromatic protons (6.64 and 6.58 ppm) establish their identity. Furthermore, the 132.0 ppm ^{13}C signal has a long-range interaction to the protons in the isolated CH_2 group (C), which implies that B and C form a heterocyclic ring that is fused with both ring A and the aromatic functionality. The HMBC experiment identified group A as $CH(16)-CH_2(1)-CH(2)-CH(3)=CH(4)$, group B as $CH_2(6)-CH_2(7)$, group C as $CH_2(9)$, group D as $CH(11)-CH(12)$, and group E as $CH_2(2')-CH(3')-CH_3(4')$. Furthermore, the quaternary carbons were also assigned and the constitution of the molecule was unambiguously deduced.

The solution conformation of the molecule and the relative configurations were obtained from the analysis of the scalar coupling constants and the NOESY spectrum and by performing molecular modeling calculations (Figure 2). The conformation of the six-membered unsaturated ring was first examined. The H-16 singlet shows two small couplings to the neighboring H-1 protons. The small scalar interaction (3.1 Hz) indicates that the dihedral angle between H-16 and the two H-1 protons is close to 90° (calculations showed 56.1° and −60.2°), and the relative orientation of these protons is staggered. This conformation is further supported by the NOESY spectrum, where H-16 shows correlations to both H-1 protons. A similar structural element can be deduced for the interactions between H-1 and H-2; the scalar coupling is weak (5.0 Hz), and cross-peaks between the neighboring protons are strong and in good agreement with the −69.0° and 48.2° dihedral angles.

Moreover, this geometrical arrangement proves the β-orientation of the substituent at C-2. The conformation of the unsaturated six-membered ring can be described as a twisted chair. A similar procedure can be applied to study the conformation of the seven-membered ring containing the nitrogen atom. The seven-membered ring has one conformation, as dynamic exchange by conformational change was not detected in the ^{1}H NMR spectrum. The ring properties were studied mainly with the scalar and dipolar interactions of 6-CH_2, 7-CH_2, and 9-CH_2. Each of the four nonequivalent protons of C-6 and C-7 has scalar interactions with the remaining three in the spin system. One large geminal coupling constant was measured for $^2J_{6\alpha,\beta}$ = 13.8 Hz and $^2J_{7\alpha,\beta}$ = 13.6 Hz. One of the 6-CH_2 protons (at 1.85 ppm), however, has two additional small scalar couplings to the 7-CH_2 protons, indicating a staggered orientation. The other 6-CH_2 proton (1.79 ppm) has a large vicinal coupling, similar in magnitude to the geminal coupling, and it can be attributed to the large dihedral angle (176.5°). The signal assignment of the diastereotopic methylene protons hence can be carried out by the analysis of the coupling constants and the NOESY enhancements and showed the following conclusions: the 1.85 ppm signal of the 6-CH_2 has an equatorial β orientation, and its geminal partner at 1.79 ppm has an axial α orientation; one of the 7-CH_2 protons at 3.35 ppm has an equatorial α position, while its geminal partner at 3.24 ppm has an axial β position. The 9-CH_2 moiety is an isolated group; however, the β proton at 4.02 ppm has a strong NOESY interaction with the olefinic H-4; the other H-9 proton (α orientation) at 3.93 ppm gives an intense cross-peak with H-11. The overall conformation of the seven-membered ring can be described as a strained chair. The relative configuration of C-3′ and the unambiguous assignment of the two diastereotopic H-2′ protons could not be established. The isolation and structure determination of this derivative have not been carried out before; we have given the name leucovernine to compound **1**.

The HREIMS data of compound **2** indicated the molecular formula $C_{22}H_{27}NO_6$ with a molecular ion at *m/z* 401.18260. The ^{1}H NMR spectrum of **2** shows a signal appearance similar to that in the case of **1**, and the galanthamine skeleton can be clearly identified. There are two aromatic doublets (H-11 and H-12) at higher chemical shifts (6.64 and 6.58 ppm) along with two olefinic protons (H-4 at 6.27 ppm and H-3 at 5.89 ppm). The protons of the unsaturated six-membered ring resonate at 5.33 ppm (H-2), 2.67 ppm (H-1β), and 2.09 ppm (H-1α). The last member of the unsaturated ring is H-16α, with a chemical shift of 4.55 ppm. The protons of the seven-membered heteroring (H-6, H-7, and H-9) show chemical shifts and fine structure similar to those in the case of **1** (H-7α: 3.36 ppm, H-7β: 3.24 ppm, H-6β: 1.86 ppm, H-6α: 1.80 ppm, H-9β: 4.04 ppm, and H-9α: 3.95 ppm). The presence of the 3′-hydroxybutyryl group was confirmed by the following proton resonances: H-2′a: 2.61 ppm, H-2′b: 2.49 ppm, H-3′: 5.24 ppm, and H-4′: 1.27 ppm. The significant difference of H-2′ and H-3′ chemical shifts in **1** and **2** indicated the presence of a substituent at the 3′ position. On the basis of the mass spectrometric data and the appearance of a singlet at 1.95 ppm 3′-OH is substituted with an acetyl group. Compound **2** was named acetylleucovernine.

HRESIMS data of compound **3** indicated the molecular formula $C_{16}H_{19}NO_3$ with a molecular ion at *m/z* 273.13623. The structure determination revealed the compound to be a galanthamine derivative. The proton chemical shifts and coupling constants supported the twisted chair conformation of the unsaturated six-membered ring and a chairlike conformation of the seven-membered ring. The resonances of the 3′-hydroxybutyryl moiety of compound **2** were missing from the spectrum, indicating the presence of a β-oriented hydroxyl function at the 2-position. NMR data were identical with those published for *N*-demethylgalanthamine (**3**).[10]

The chemical shifts and coupling constants in the ^{1}H NMR spectrum of **4** confirmed that the compound is 9-*O*-demethylhomolycorine.[11]

Compounds **5** and **6** were not separated and were identified as a mixture. The ^{1}H NMR spectrum of the mixture showed the presence of **5** and **6** in a ratio of 2.2:1. Both **5** and **6** have structures similar to **4** on the basis of the measured chemical shifts. However, a methylenedioxy moiety (−O−CH_2−O−) at 6.07 and 6.06 ppm was identified in **5**, while **6** showed two *O*-methyl groups (3.95 and 3.94 ppm). Furthermore, the deshielded H-5 (5.66 ppm) in both cases indicated the presence of a C-5 hydroxyl function. Comparison with literature data revealed that **5** is hippeastrine, while **6** is 5α-hydroxyhomolycorine.

The known alkaloid 11-hydroxyvittatine (**7**) was also isolated from *L. vernum* and identified by comparing its spectroscopic data with literature data.[14]

Experimental Section

General Experimental Procedures. Melting points are uncorrected. Optical rotations were measured on a Perkin-Elmer 341 polarimeter. NMR spectra were recorded in $CDCl_3$ on a Bruker Avance DRX 500 spectrometer at 11.7 T (500 MHz for ^{1}H and 125 MHz for ^{13}C); the signals of the deuterated solvent were taken as the reference (7.26 ppm in ^{1}H NMR and 77.0 ppm in ^{13}C NMR). Two-dimensional data were acquired and processed with standard Bruker software. In the ^{1}H,^{1}H-COSY, HSQC, and HMBC experiments, gradient-enhanced versions were used. HREIMS spectra were obtained on a Finnigan MAT 95 S spectrometer. For vacuum liquid chromatography, silica gel (Kieselgel GF_{254} 15 μm, Merck) was used. Preparative TLC was carried out on silica gel (Kieselgel $60F_{254}$, Merck). Chromatographic fractions were monitored by TLC on silica gel plates (Merck 5715), visualized by spraying with Dragendorff reagent or concentrated H_2SO_4, followed by heating. Molecular modeling calculations were carried out by the semiempirical PM3 package of the Gaussian03 software.[15]

Plant Material. The bulbs of *Leucojum vernum* (1500 g) were purchased from Spalax Ltd. (Ecser, Hungary) in September 2002. Voucher specimens (No. 604) have been preserved at the Department of Pharmacognosy, University of Szeged, Hungary.

Extraction and Isolation. The fresh bulbs of *L. vernum* were extracted at room temperature with 12 L of MeOH. The MeOH extract was concentrated to 300 mL, acidified with 2% HCl to pH 2.5, and exhaustively extracted with 6 × 500 mL of Et_2O in order to remove neutral lipophilic materials. The acidic solution was then adjusted to pH 8.5 with concentrated NH_3 and extracted with 10 × 500 mL of $CHCl_3$. The alkaloid-containing $CHCl_3$ fraction (7.2 g) was separated by vacuum liquid chromatography (VLC) on silica gel 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 were monitored by TLC and combined according to their composition. Combined fractions 28−39 afforded crystalline lycorine. The mother liquor of this fraction was subjected to preparative TLC on silica gel in $CHCl_3$−MeOH (49:1) under NH_3 vapor to give leucovernine (**1**) (9.0 mg), 9-*O*-demethylhomolycorine (**4**) (4.6 mg), and a mixture of hippeastrine (**5**) and 5α-hydroxyhomolycorine (**6**) (2.3 mg). Fractions 62−75 from the VLC separation were purified by preparative TLC on silica gel in two steps. First benzene−$CHCl_3$−MeOH (7:2:1) in NH_3 vapor was used as a

Table 1. ^{1}H and ^{13}C NMR Spectroscopic Data of **1**

#	^{1}H ppm (Hz)	^{13}C	COSY	NOESY	HMBC (C→H)
1β	2.70 ddd (16.2, 3.2, 1.4)	27.5	1α, 16α, 2α	1α, 16α, 2α	2α, 3
1α	2.05 ddd (16.2, 4.8, 3.2)		1β, 16α, 2α	1β, 16α, 2α, 6β	
2α	5.38 t (5.0, 4.8)	63.4	1α, 1β, 3	1α, 1β, 3	1β, 3, 4, 16α
3	5.87 ddd (10.3, 5.0, 1.2)	122.5	2, 4	2α, 4	1β, 2α
4	6.31 d (10.3)	131.6	3	3, 9β, 7β, 6β	2α, 2, 6α, 6β, 16α
5		48.6			1β, 3, 4, 6α, 16α
6β	1.85 dt (13.8, 3.5, 3.0)	40.8	6α, 7α, 7β	6α, 7α, 7β, 4, 1α	16α
6α	1.79 ddd (13.7, 12.0, 3.0)		6β, 7α, 7β	6β, 7α, 16α	
7α	3.35 dt (13.6, 3.5, 3.0)	47.0	6α, 6β, 7β	7β, 6α, 6β	6α, 9β
7β	3.24 ddd (13.5, 12.1, 3.0)		7α, 6α, 6β	7α, 4, 9β	
9β	4.02 d (15.4)	53.8	9α	7β, 4, 9α	11
9α	3.93 d (15.4)		9β	9β, 11	
10		133.3			12, 9β, 9α
11	6.58 d (8.2)	120.1	12	12, 9β	9β, 9α, 12
12	6.64 d (8.2)	111.3	11	11, OMe	11
13		144.0			11, 12, OCH_3
14		146.9			12
15		132.0			11, 4, 9β, 16α
16α	4.56 t (3.2, 3.2)	86.2	1α, 1β	6α, 1α, 1β,	1β, 2, 4, 6
OCH_3	3.83 s	55.9		12	
1′		171.9	2′a, 2′b		2α, 2′a, 2′b, 3′
2′a[a]	2.48 dd (16.3, 3.2)	43.9	1′, 2′b, 3′	2′b, 3′	4′
2′b[a]	2.39 dd (16.3. 9.4)		1′, 2′a, 3′	2′a, 3	
3′	4.19 ddq (9.4, 6.4, 3.2)	64.0	2′a, 2′b, 4′	2′a, 2′b, 4′	2′a, 2′b, 3′, 4′
4′	1.18 d (6.4)	22.6	3′	3′	2′a, 2′b, 3′

[a] Orientation of the diastereotopic protons cannot be determined.

solvent system. In the second step $CHCl_3$–MeOH (9:1)/NH_3 vapor was applied, affording acetylleucovernine (**2**) (2.5 mg), *N*-demethylgalanthamine (**3**) (74 mg), and 11-hydroxyvittatine (**7**) (10.4 mg).

Leucovernine (1): amorphous solid; $[\alpha]^{31}_D$ −42° (*c* 1, $CHCl_3$); UV (MeOH) λ_{max} (log ϵ) 209 (3.62), 232.6 (sh) (3.14), 290 (2.67) nm; IR (KBr) ν_{max} 2926, 1626, 1591, 1508, 1438, 1377, 1289, 1272, 1207, 1168, 1119, 1086, 1056, 1029, 754 cm^{-1}; ^{1}H NMR and ^{13}C NMR, see Table 1; EIMS *m/z* 359 $[M]^+$ (5), 322 (4), 272 (14), 256 (16), 202 (11), 191 (7), 149 (25), 125 (18), 111 (30), 97 (48), 85 (42), 71 (62), 57 (100), 43 (46); HREIMS *m/z* 359.17327 (calcd for $C_{20}H_{25}NO_5$ 359.17327).

Acetylleucovernine (2): amorphous solid; UV (MeOH) λ_{max} (log ϵ) 208 (3.67), 232.6 (sh) (3.21), 289 (2.69) nm; ^{1}H NMR ($CDCl_3$, 500 MHz) δ 1.27 (3H, d, J = 6.2 Hz, 4′-CH_3), 1.80 (1H, ddd, J = 13.5, 12.4, 3.2 Hz, H-6α), 1.86 (1H, dt, J = 13.5, 2.5; 2.5 Hz, H-6β), 1.95 (3H, s, Ac-CH_3), 2.09 (1H, ddd, J = 16.3, 5.5, 3.0 Hz, H-1α), 2.49 (1H, dd, J = 15.8, 6.0 Hz, H-2′); 2.61 (1H, dd, J = 15.7, 6.9 Hz, H-2′); 2.67 (1H, ddd, J = 16.7, 3.0, 1.2 Hz, H-1β), 3.24 (1H, ddd, J = 13.3, 13.4, 2.3 Hz, H-7β); 3.36 (1H, dt, J = 13.5, 3.6, 3.4 Hz, H-7α), 3.84 (3H, s, OCH_3); 3.95 (1H, d, J = 15.3 Hz, H-9α); 4.04 (1H, d, J = 15.5 Hz, H-9β); 4.55 (1H, t, J = 3.2, 3.1 Hz, H-16α), 5.24 (1H, ddq, J = 9.5, 6.9, 3.0 Hz, H-3′), 5.33 (1H, t, J = 5.0, 5.0 Hz, H-2α), 5.89 (1H, ddd, J = 10.1, 4.8, 1.0 Hz, H-3), 6.27 (1H, d, J = 10.3 Hz, H-4), 6.58 (1H, d, J = 8.0 Hz, H-11), 6.64 (1H, d, J = 8.0 Hz, H-12); EIMS *m/z* 401 $[M]^+$ (2), 341 (20), 256 (39), 226 (10), 211 (18), 202 (14), 181 (14), 165 (23), 149 (15), 125 (21), 111 (37), 97 (54), 83 (54), 71 (61), 57 (100), 41 (71); HREIMS *m/z* 401.18260 (calcd for $C_{22}H_{27}NO_6$ 401.18383).

***N*-Demethylgalanthamine (3):** colorless crystals ($CHCl_3$); mp 153–155 °C; $[\alpha]^{31}_D$ −72° (*c* 0.28, $CHCl_3$) [lit.[16] $[\alpha]^{21}_D$ −72.9° (*c* 0.29, $CHCl_3$)]; ^{1}H and ^{13}C NMR, identical with published data;[10] EIMS *m/z* 273 $[M]^+$ (100), 256 (6), 242 (4), 230 (37), 211 (10), 202 (26), 174 (11), 165 (10), 152 (8), 141 (6), 128 (10), 115 (13), 103 (4), 91 (6), 77 (6), 55 (3); HREIMS *m/z* 273.13623 $[M]^+$ (calcd for $C_{16}H_{19}NO_3$ 273.13649).

9-*O*-Demethylhomolycorine (4): amorphous solid; $[\alpha]^{31}_D$ +57° (*c* 0.28, $CHCl_3$) [lit.[17] $[\alpha]_D$ +89.6° (*c* 0.41, $CHCl_3$)]; ^{1}H and ^{13}C NMR, identical with published data.

Mixture of hippeastrine (5) and 5α-hydroxyhomolycorine (6): ^{1}H and ^{13}C NMR, identical with published data.[12,13]

11-Hydroxyvittatine (7): amorphous solid; $[\alpha]^{25}_D$ +10.0° (*c* 0.60, MeOH) [lit.[14] $[\alpha]^{25}_D$ +11.3° (*c* 0.88, MeOH)]; ^{1}H NMR and ^{13}C NMR, identical with published data;[14] EIMS *m/z* 287 $[M]^+$ (18), 272 (13), 243 (12), 227 (22), 211 (13), 181 (22), 149 (30), 125 (24), 111 (34), 97 (56), 85 (56), 71 (77), 57 (100), 43 (62); HREIMS *m/z* 287.11518 (calcd for $C_{16}H_{17}NO_4$ 287.11575).

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