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Alkaloids from *Galanthus nivalis*

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Abstract

Phytochemical studies on *Galanthus nivalis* of Bulgarian origin resulted in the isolation of five compounds: 11-*O*-(3′-hydroxybutanoyl)hamayne, 3,11-*O*-(3′,3″-dihydroxybutanoyl)hamayne, 3-*O*-(2″-butenoyl)-11-*O*-(3′-hydroxybutanoyl)hamayne, 3,11,3″-*O*-(3′,3″,3‴-trihydroxybutanoyl)hamayne, and 2-*O*-(3′-acetoxybutanoyl)lycorine, together with five known alkaloids: ungeremine, lycorine, tazettine, hamayne, and ismine. Their structures were determined by ^{1}H and ^{13}C NMR spectroscopy and two-dimensional ^{1}H–^{1}H and ^{1}H–^{13}C chemical shift correlation experiments.

Keywords: *Galanthus nivalis*; Amaryllidaceae; Alkaloids; 11-*O*-(3′-Hydroxybutanoyl)hamayne; 3,11-*O*-(3′,3″-Dihydroxybutanoyl)hamayne; 3-*O*-(2″-Butenoyl)-11-*O*-(3′-Hydroxybutanoyl)hamayne; 3,11,3″-*O*-(3′,3″,3‴-Trihydroxybutanoyl)hamayne; 2-*O*-(3′-Acetoxybutanoyl)lycorine

1. Introduction

Galanthus nivalis L. (snow drop) is an early-spring flowering bulbous plant species cultivated for its elegant flowers. Plants of the genus *Galanthus* are known for the bioactive alkaloids they contain, including galanthamine, an acetylcholinesterase inhibitor marketed as hydrobromide salt for the treatment of Alzheimer's disease (Maelicke et al., 2001).

The information on the alkaloids of *G. nivalis* is confusing owing to the taxonomical changes in this species over the years. Thus, until 1966, only one *Galanthus* species had been recognized in Bulgaria, namely *G. nivalis* L. (Jordanov, 1964), but this taxon was subsequently separated into *G. nivalis* L. and *G. elwesii* Hook. (Stojanov et al., 1966; Kozuharov, 1992). At present, due to the lack of voucher specimens, it is unclear which plant species the alkaloids isolated in the early sixties from *G. nivalis* collected in Bulgaria can be attributed to (Valkova, 1961; Bubeva-Ivanova and Pavlova, 1965). Kaya et al. (2004) have found five alkaloids for *G. nivalis* L. subsp. *silicicus* (Baker) Guttl.-Tann., a taxon that has been reported as a synonym of *Galanthus silicicus* Baker by other authors (Davis and Barnett, 1997; Davis, 1999). Kalashnikov (1970) also reported six alkaloids for *G. nivalis* L. (galanthamine, nivalidine, narwedine, lycorine, hippeastrine and tazettine). The structure of magnarine, another alkaloid isolated in the 1960s from *G. nivalis*, is unknown (DNP, 2004).

In our previous GC–MS study on *G. elwesii*, we detected unknown compounds with characteristic mass spectral fragmentation patterns of Amaryllidaceae alkaloids (Berkov et al., 2004). One of them was later found, together with other unknown compounds, when screening several *G. nivalis* populations. Plants from the population with the highest concentration of unknown compounds were collected and their alkaloids isolated. The structure determination of these alkaloids is reported in the present work.

2. Results and discussion

The EtOH extract of the fresh aerial parts and bulbs of *G. nivalis* was fractioned as described in Section 3. The alkaloid-containing fraction was separated by a

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combination of CC and preparative TLC and as a result, five novel Amaryllidaceae alkaloids (**1**–**5**) together with hamayne (**6**), lycorine (**7**), ungeremine (**8**), tazettine (**9**) and ismine (**10**) were isolated (Fig. 1). Their structures were determined by NMR, CD and MS spectroscopy. The 2D homo- and heteronuclear chemical shift correlation experiments (COSY, NOESY, HMBC and HMQC) were used to perform the complete assignment of the ^{1}H NMR spectra and the carbon chemical shifts.

Compounds **1**–**4** showed GC–MS, ^{1}H NMR and ^{13}C NMR data closely comparable to those of hamayne (**6**) (Viladomat et al., 1994). The HRMS of **1** suggested a molecular formula $C_{20}H_{23}NO_6$ with a parent ion at *m*/*z* 373.1514 (Calc. 373.1525). Its ^{1}H NMR spectrum (Table 1) exhibited: (1) three singlets at δ 6.46, 6.87 and 5.89 for the aromatic protons (H-7 and H-10) and the methylendioxy group, respectively. The aromatic proton at δ 6.46 was assigned to H-7 because of the spatial proximity with the protons H-6α and H-6 β and a three-bound HMBC correlation with C-6; (2) two signals at δ 6.15 (*dd*) and 5.96 (*dt*) for the olefinic protons H-1 and H-2, whose multiplicity is in agreement with the *cis* relationship between the substitu-

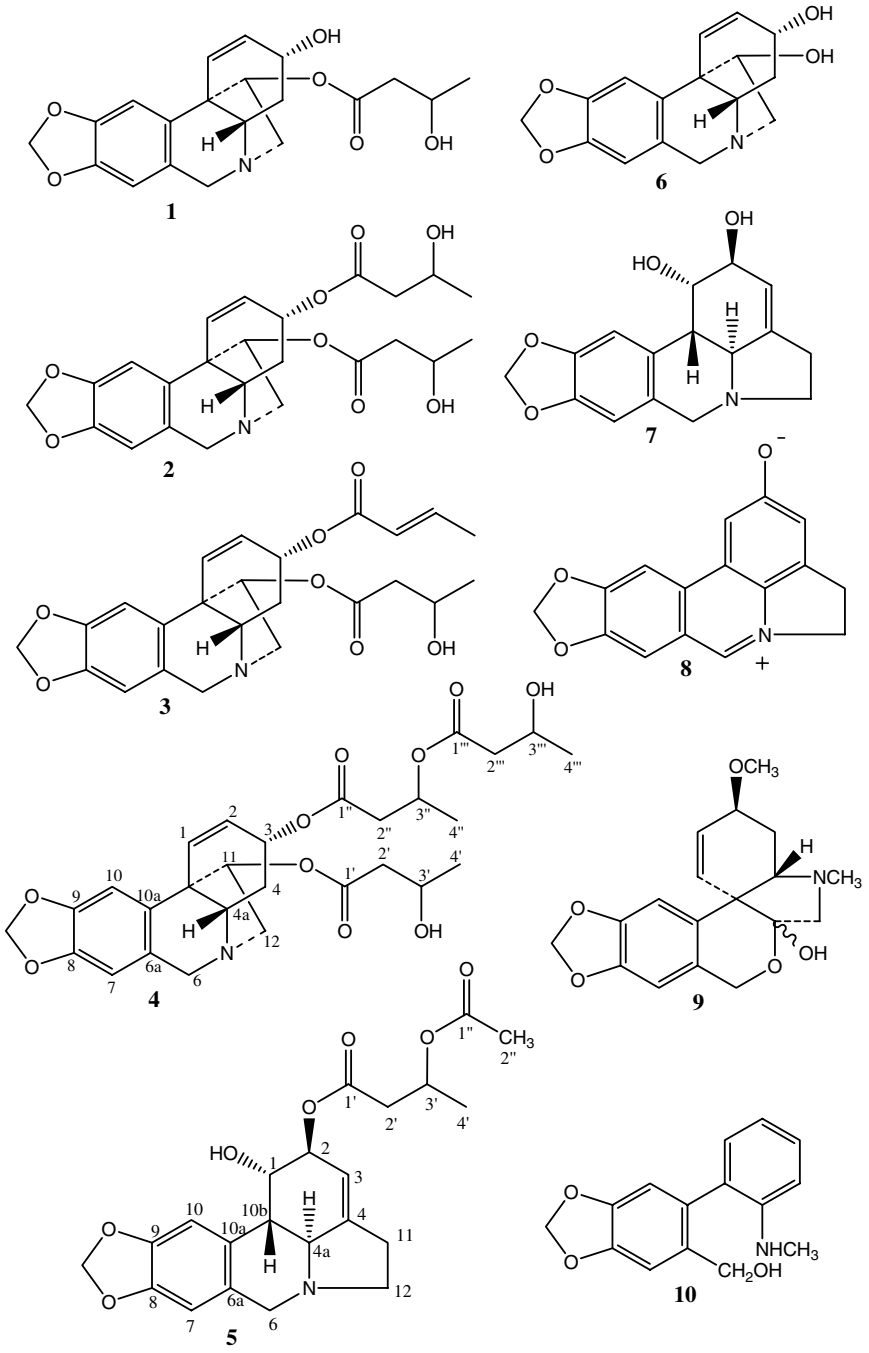

Fig. 1. Structures of the isolated alkaloids.

Table 1
^{1}H NMR, COSY, HMQC and HMBC data of **1**

Position	Hδ (*J* in Hz)	COSY	HMQC		HMBC
1	6.15 *dd* (10.2, 2.0)	H-2, H-3	123.9 *d*		C-4a, C-10a, C-10b
2	5.96 *dt* (10.5, 1.5)	H-1, H-3	135.9 *d*		C-4
3	4.37 *ddt* (10.2, 6.0, 2.0)	H-1, H-2, H-4α, H-4β	67.4 *d*		C-1, C-2
4α	1.90 *ddd* (13.5, 12.0, 10.0)	H-3, H-4β, H-4a	33.9 *t*		C-3, 10b
4β	2.11 *dddd* (12.0, 6.0, 4.0, 1.0)	H-3, H-4α, H-4a	33.9 *t*		C-2, C-3, 10b
4a	3.22 *dd* (13.5, 4.0)	H-4α, H-4β	66.5 *d*		C-3, C-4, C-6, C-10a, C-11, C-12
6α	3.69 *d* (17.0)	H-6β, H-7	61.1 *t*		C-4a, C-6a, C-7, C-8, C-10a, C-12
6β	4.32 *d* (16.5)	H-6α, H-7	61.1 *t*		C-6a, C-7, C-8, C-10, C-10a, C-11, C-12
			126.4 *s*	(C-6a)	
7	6.46 *s*	H-6α, H-6β	106.8 *d*		C-6, C-8, C-9, C-10, C-10a
			146.8 *s*	(C-8)	
			146.8 *s*	(C-9)	
10	6.87 *s*	–	104.0 *d*		C-6a, C-7, C-8, C-9, C-10b
			134.1 *s*	(C-10a)	
			49.0 *s*	(C-10b)	
11	4.95 *dt* (5.5, 1.0)	H-12	80.8 *d*		C-4a, C-10a, C-1′
12 (2H)	3.41 *d* (5.5)	H-11	60.6 *t*		C-4a, C-6, C-10b, C-11
OCH_2O	5.89 2*d* (1.0)	–	101.0 *t*		C-8, C-9
			171.7 *s*	(C-1′)	
$2'_A$	2.45 *dd* (16.0, 3.5)	H-$2'_B$, H-3′	43.8 *t*		C-1′, C-3′, C-4′
$2'_B$	2.39 *dd* (16.0, 8.5)	H-$2'_A$, H-3′	43.8 *t*		C-1′, C-3′, C-4′
3′	4.17 *ddq* (8.5, 6.2, 3.2)	H-$2'_A$, H-$2'_B$, H-4′	64.5 *d*		C-1′
4′	1.22 *d* (6.0)	H-3′	23.3 *q*		C-1′, C-2′, C-3′

ent at C-3 and the 5-10 ethano bridge; the magnitude of the coupling constants amongst H-3, H-2 and H-1 ($J_{1,3} = 2$; $J_{2,3} = 1.5$) as well as H-2 and H-4β ($J_{2,4a} = 1,5$ coupling by a W-mechanism) supported the *pseudoequatorial* disposition of the substituent at C-3 (Bastida et al., 2006); (3) two doublets at δ 3.69 and 4.32 for the protons H-6α and H-6β; H-6β was assigned to lower fields due to its *syn*-relation with the nitrogen lone pair; (4) one double doublet at δ 3.22 for H-4a; this proton has a large coupling with H-4α due to their *trans*-diaxial disposition; (5) a double triplet at δ 4.95 for H-11; this proton was deshielded in comparison to compound (**6**); (6) a signal δ 3.41 (*d*) integrating 2 protons, assigned to H-12 (2H); (7) four signals at δ 1.22 (*d*), 2.39 (*dd*), 2.45 (*dd*), and 4.17 (*ddq*) which were in agreement with a 3-hydroxybutanoyl group (Forgo and Hohmann, 2005). HMBC and HMQC experiments revealed 20 carbons for **1** (Table 1), 16 of which corresponded to those of hamayne (Viladomat et al., 1994). The lowfield ($\delta > 90$) signals were five singlets for the carbonyl group and the quaternary carbons of ring A, two doublets for the olefinic carbons C-1 and C-2, two doublets for the aromatic carbons C-7 and C-10, and one triplet for the methylendioxy group. The aliphatic shift range is characterized by one quaternary carbon (C-10b), four methine carbons (C-3, C-4a, C-11 and C-3′), four methylene carbons (C-4, C-6, C-12 and C-2′), and one methyl carbon (C-4′). The presence of a 3-hydroxybutanoyl group was confirmed by the carbon resonances at δ 171.7, 64.5, 43.8, and 23.3 (Forgo and Hohmann, 2005). The 3-hydroxybutanoyl group was assigned to the C-11 position due to the three-bound HMBC correlation between H-11 and C-1′ as well as its deshielding effect ($\Delta\delta + 0.95$) on H-11. On the basis of these spectral data, compound **1** was identified as 11-*O*-(3′-hydroxybutanoyl)hamayne.

Compound **2** exhibited a molecular ion (HRMS) at *m*/*z* 459.1888 suggesting a molecular formula $C_{24}H_{29}NO_8$ (Calc. 459.1893). Its ^{1}H NMR spectrum was similar to that of compound **1**, although the chemical shift of H-3 was considerably more deshielded ($\Delta\delta + 1.15$). Also, the integration and multiplicity of the signals in the ^{1}H NMR spectrum of **2**, corresponding to the 3-hydroxybutanoyl moiety, suggested the presence of an additional 3-hydroxybutanoyl group. This suggestion was confirmed by the ^{13}C NMR spectrum revealing two carbonyl groups (at δ 171.9 and 172.4) as well as signals at δ 43.4 and 43.6 corresponding to two methylene carbons, signals at δ 64.4 and 64.5 for two methine carbons as well as signals at δ 22.8 and 22.9 corresponding to the carbons of two methyl groups (Table 2). The three-bound HMBC correlation of H-11 with C-1′ indicated the presence of the first 3-hydroxybutanoyl group at C-11 and allowed the assignment of the chemical shift for C-1′ at δ 171.9 while the signal at δ 172.4 was assigned to C-1″. The presence of the second 3-hydroxybutanoyl group at C-3 was supported by the deshielded H-3 proton and the pronounced shifting effect of this group on the C-1, C-2, C-3 and C-4 ($\Delta\delta$ +2.3, −4.6, +3.0 and −3.9, respectively) in the ^{13}C NMR of **2** as compared to those of **1** and hamayne. Thus, compound **2** was identified as 3,11-*O*-(3′,3″-dihydroxybutanoyl)hamayne.

Compound **3** exhibited a molecular ion (HRMS) at *m*/*z* 441.1777 suggesting a molecular formula $C_{24}H_{27}NO_7$ (Calc. 441.1787). Its spectral data (Table 3) were very similar to those of compound **2**, although in contrast, the molecular ion of **3** was 18 mass units less than that of **2**,

Table 2
^{1}H NMR, COSY, ^{13}C NMR, HMQC and HMBC data of **2**

Position	H δ (*J* in Hz)	COSY	HMQC		HMBC
1	6.27 *dd* (10.4, 2.4)	H-2, H-3	126.2 *d*		C-3, C-4a, C-10a
2	5.92 overlapped	H-1, H-3	131.3 *d*		C-4, C-10b
3	5.52 *ddt* (10.0, 6.0, 2.0)	H-1, H-2, H-4α, H-4β	70.4 *d*		–
4α	2.01 *ddd* (13.6, 12.0, 10.4)	H-3, H-4β, H-4a	30.0 *t*		C-2, C-3, C-4a, 10b
4β	2.16 *dddd* (12,0, 6.4, 4.4, 1.5)	H-3, H-4α, H-4a	30.0 *t*		C-3, C-4a
4a	3.30 *dd* (13.6, 4.4)	H-4α, H-4β	66.2 *d*		C-6, C-11, C-12
6α	3.72 *d* (17.2)	H-6β	61.2 *t*		C-4a, C-6a, C-7, C-10a, C-12
6β	4.34 *d* (16.8)	H-6α, H-7	61.2 *t*		C-6a, C-7, C-8, C-10a, C-11, C-12
			126.5 *s*	(C-6a)	
7	6.49 *s*	H-6β	107.0 *d*		C-6, C-8, C-9, C-10a
			147.0 *s*	(C-8)	
			147.1 *s*	(C-9)	
10	6.87 *s*	–	103.9 *d*		C-6a, C-8, C-9, C-10b
			133.8 *s*	(C-10a)	
			49.3 *s*	(C-10b)	
11	5.01 *dd* (6.4, 4.0)	H-12 A, H-12 B	80.9 *d*		C-4a, C-10a, C-1′
12_A	3.39 *dd* (14.4, 4.4)	H-11, H-12 B	60.7 *t*		C-4a, C-6, C-10b
12_B	3.45 *dd* (14.4, 6,4)	H-11, H-12 A	60.7 *t*		C-4a, C-6, C-10b
OCH_2O	5.92 2*d* (3.6)	–	101.3 *t*		C-8, C-9
			171.9 *s*	(C-1′)	
$2'_A$	2.49 *dd* (16.4, 3.6)	H-$2'_B$, H-3′ H-4′	43.4 *t*		C-1′, C-3′, C-4′
$2'_B$	2.42 *dd* (16.0, 8.4)	H-$2'_A$, H-3′ H-4′	43.4 *t*		C-1′, C-3′, C-4′
3′	4.17 *m*	H-$2'_A$, H-$2'_B$, H-4′	64.4/64.5 *d* [a]		–
4′	1.24 *d* (6.0)	H-$2'_A$, H-$2'_B$, H-3′	22.8/22.9 *q*[a]		C-2′, C-3′
			172.4 *s*	(C-1″)	
$2''_A$	2.54 *dd* (16.4, 3.6)	H-$2''_B$, H-3″, H-4″	43.6 *t*		C-3, C-1″, C-3″, C-4″
$2''_B$	2.47 *dd* (16.0, 8.4)	H-$2''_A$, H-3″, H-4″	43.6 *t*		C-3, C-1″, C-3″, C-4″
3″	4.23 *m*	H-$2''_A$, H-$2''_B$, H-4″	64.4/64.5 *d* [a]		–
4″	1.26 *d* (6.0)	H-$2''_A$, H-$2''_B$, H-3″	22.8/22.9 *q*[a]		C-2″, C-3″

[a] The chemical shifts of C-3′ and C-3″ as well as of C-4′ and C-4″ can not be assigned.

Table 3
^{1}H NMR, COSY, HMQC and HMBC data of **3**

Position	H δ (*J* in Hz)	COSY	HMQC		HMBC
1	6.22 *dd* (10.0, 2.5)	H-2	125.6 *d*		C-3, C-4a, C-10a
2	5.89 overlapped	H-1	131.9 *d*		C-10b
3	5.51 *ddt* (10.0, 6.5, 2.5)	H-4α, H-4β	69.5 *d*		–
4α	1.99 *ddd* (13.7, 12.0, 10.5)	H-3, H-4β, H-4a	30.0 *t*		C-3, C-4a
4β	2.13 *dddd* (12.0, 6.0, 4.5, 1,5)	H-3, H-4α, H-4a	30.0 *t*		C-2, C-3, C-4a, 10b
4a	3.28 *dd* (13.5, 5.0)	H-4α, H-4β	66.3 *d*		C-4, C-6, C-10a, C-11
6α	3.69 *d* (17.0)	H-6β	61.2 *t*		C-4a, C-6a, C-7, C-10a, C-12
6β	4.32 *d* (17.0)	H-6α, H-7	61.2 *t*		C-6a, C-7, C-8, C-10a, C-11, C-12
			126.5 *s*	(C-6a)	
7	6.46 *s*	H-6β	106.8 *d*		C-6, C-8, C-9, C-10a
			146.9 *s*	(C-8)	
			146.9 *s*	(C-9)	
10	6.85 *s*	–	103.9 *d*		C-6a, C-8, C-9, C-10b
			133.9 *s*	(C-10a)	
			49.2 *s*	(C-10b)	
11	4.99 *dd* (6.5, 4.0)	H-12_A, 12_B	80.8 *d*		C-4a, C-10a, C-1′
12_A	3.37 *dd* (14.0, 3.5)	H-11, H-12_B	60.5 *t*		C-4a, C-6, C-11
12_B	3.41 *dd* (14.0, 6.5)	H-11, H-12_A	60.5 *t*		C-4a, C-6, C-11
OCH_2O	5.89 2*d* (1.5)	–	101.1 *t*		C-8, C-9
			172.1 *s*	(C-1′)	
$2'_A$	2.49 *dd* (16.5, 3.5)	H-$2'_B$, H-3′	43.3 *t*		C-1′ C-3′, C-4′
$2'_B$	2.41 *dd* (16.5, 9.0)	H-$2'_A$, H-3′	43.3 *t*		C-1′ C-3′, C-4′
3′	4.19 *m*	H-$2'_A$, H-$2'_B$, H-4′	64.2 *d*		–
4′	1.22 *d* (6.5)	H-3′	22.6 *q*		C-2′, C-3′
			166.1 *s*	(C-1″)	
2″	5.85 *dq*(15.5, 1.5)	H-3″, H-4″	122.8 *d*		–
3″	7.02 *dq* (15.5, 7.0)	H-2″, H-4″	145.4 *d*		C-1″
4″	1.88 *dd* (7.0, 1.5)	H-2″, H-3″	18.2 *q*		C-2″, C-3″

suggesting the loss of water. The signals of H-2″ and H-3″ ($\Delta\delta + 3.31$ and +2.79, respectively) as well as those of C-2″ and C-3″ ($\Delta\delta + 79.2$ and +81.0) were considerably deshielded and corresponded to two methine carbons. The large coupling constant $J_{2'',3''} = 15.5$ Hz indicated a *trans* relationship between H-2″ and H-3″. The ^{1}H NMR and ^{13}C NMR data as well as a fragment at *m/z* 69 (base ion) in the GC–MS spectrum of **3** indicated the presence of a 2-butenoyl group. The three-bound HMBC correlation of H-11 with C-1′ and of H-3″ with C-1″ (shielded in comparison to **2**, at δ 166.1) allowed us to assign the 3-hydroxybutanoyl group to C-11 and the 2-butenoyl group to C-3, respectively. Compound **3** was identified as 3-*O*-(2″-butenoyl)-11-*O*-(3′-hydroxybutanoyl)hamayne.

Compound **4** showed a mass spectral fragmentation pattern similar to that of **3** but it has no molecular ion under direct insertion probe and GC–MS conditions. CIMS (methane as reactant gas) of **4** showed a molecular ion at *m/z* 546 [$M^+ + 1$] indicating that the molecular weight of this compound is 545 mass units. The ^{1}H NMR spectrum of **4**, similar to that of **2**, showed signals for three methyl groups, six methylene and three methine protons corresponding to three 3-hydroxybutanoyl moieties (Table 4). The three-bound HMBC correlation between H-11 and the carbon at δ 172.1 (C-1′) allowed the attribution of the first 3-hydroxybutanoyl group at C-11. H-3 showed a three-bound HMBC correlation with a carbon at δ 169.8 (C-1″) allowing assignment of the second 3-hydroxybutyryl group at C-3. The proton at δ 5.34 assigned to H-3″ (deshielded in comparison to **2**, $\Delta\delta + 1.11$) due to its homonuclear correlations (2D COSY) with slightly deshielded protons at δ 2.65, 2.58 and 1.32 (H-2″$_A$, H-2″$_B$ and H-4″, respectively), showed a three-bound HMBC correlation with C-1″ (δ 169.8) and with a carbon at δ 171.9. As a result, the third 3-hydroxybutanoyl moiety was assigned to C-3″, and the signal at δ 171.9 to C-1‴. The chemical shifts of the deshielded protons H-2″$_A$, H-2″$_B$, H-3″ and H-4″ were in accordance with an ester substitution of the hydroxyl group in the 3-hydroxybutanoyl moiety (Forgo and Hohmann, 2005). Compound **4** was identified as 3,11,3″-*O*-(3′,3″,3‴-trihydroxybutanoyl)hamayne.

The absolute configuration of **3** and **4** was determined from the CD spectra, which were qualitatively similar to that of the known hamayne (with an α-ethano bridge), with a minimum of around 245 nm and maximum of around 290 (Viladomat et al., 1994). By analogy, we suggested

Table 4
^{1}H NMR, COSY, HMQC and HMBC data of **4**

Position	Hδ (*J* in Hz)	COSY	HMQC		HMBC
1	6.23 *dd* (10.5, 2.0)	H-2, H-3	126.7 *d*		C-3, C-4a, C-10a
2	5.84 *dt* (10.0, 1.5)	H-1, H-3	131.2 *d*		C-4, C-10b
3	5.47 *ddt* (10.5, 6.5, 2.0)	H-1, H-2, H-4α, H-4β	70.4 *d*		C-1″
4α	1.95 *ddd* (13.5, 12.0, 10.5)	H-3, H-4β, H-4a	29.7 *t*		C-3, C-4a, C-10b
4β	2.11 *ddd* (12.0, 6.0, 4.5)	H-3, H-4α, H-4a	29.7 *t*		C-1, C-3, C-4a, C-10b
4a	3.26 *dd* (13.5, 4.0)	H-4α, H-4β	66.1 *d*		C-4, C-6, C-10a, C-11
6α	3.70 *d* (17.0)	H-6β, H-7	61.1 *t*		C-4a, C-6a, C-7, C-8, C-10a, C-12
6β	4.32 *d* (17.0)	H-6α, H-7	61.1 *t*		C-6a, C-7, C-8, C-10a, C-11, C-12
			126.4 *s*	(C-6a)	
7	6.46 *s*	H-6α, H-6β	106.9 *d*		C-6, C-8, C-9, C-10a
			146.9 *s*	(C-8)	
			146.9 *s*	(C-9)	
10	6.84 *s*		103.9 *d*		C-6a, C-7, C-8, C-9, C-10b
			133.8 *s*	(C-10a)	
			49.1 *s*	(C-10b)	
11	4.99 *ddd* (6.5, 4.0, 1.0)	H-12$_A$, H-12$_B$	80.7 *d*		C-4a, C-10a, C-1′
12$_A$	3.37 *dd* (14.5, 4.5)	H-11, H-12$_B$	60.5 *t*		C-4a, C-6, C-10b
12$_B$	3.42 *dd* (14.5, 6.5)	H-11, H-12$_A$	60.5 *t*		C-4a, C-6, C-10b
OCH_2O	5.89 *d* (1.5)–5.90 *d* (1.5)	–	101.1 *t*		C-8, C-9
			172.1 *s*	(C-1′)	
2′$_A$	2.46 *dd* (15.5, 4.0)	H-2′$_B$, H-3′	43.4 *t*		C-1′ C-3′, C-4′
2′$_B$	2.39 *dd* (16.0, 8.5)	H-2′$_A$, H-3′	43.4 *t*		C-1′ C-3′, C-4′
3′	4.18 *m*	H-2′$_A$, H-2′$_B$, H-4′	64.3 *d*		C-1′
4′	1.21 *d* (6.0)	H-3′	22.6 *q*		C-1′, C-2′, C-3′
			169.8 *s*	(C-1″)	
2″$_A$	2.65 *dd* (15.5, 8.0)	H-2″$_B$, H-3″	41.1 *t*		C-1″ C-3″, C-4″
2″$_B$	2.58 *dd* (15.0, 5.0)	H-2″$_A$, H-3″	41.1 *t*		C-1″ C-3″, C-4″
3″	5.34 *m*	H-2″$_A$, H-2″$_B$, H-4″	67.7 *d*		C-1″, C-1‴
4″	1.32 *d* (6.5)	H-3″	20.0 *q*		C-1″, C-2″, C-3″
			171.9 *s*	(C-1‴)	
2‴$_A$	2.49 *dd* (16.0, 4.0)	H-2‴$_B$, H-3‴	43.4 *t*		C-1‴ C-3‴, C-4‴
2‴$_B$	2.42 *dd* (16.0, 8.5)	H-2‴$_A$, H-3‴	43.4 *t*		C-1‴ C-3‴, C-4‴
3‴	4.18 *m*	H-2‴$_A$, H-2‴$_B$, H-4‴	64.3 *d*		C-1‴
4‴	1.23 *d* (6.5)	H-3‴	22.6 *q*		C-1‴, C-2‴, C-3‴

the same α-position of the ethano bridge for **1** and **2**, which showed CD curves with rough shapes most probably due to the effect of the substitutents (Nair et al., 2005).

The HRMS of **5** suggested a molecular formula $C_{22}H_{25}NO_7$ with a parent ion at *m/z* 415.1649 (Calc. 415.1631). Its GC–MS spectrum showed a relatively intensive [M − 1] ion in comparison with the [M^+] as well as intensive ion pairs at *m/z* 269/268, 251/250 and 227/226 indicating a lycorine type skeleton. The ion fragment at *m/z* 354 [M − 1–60] suggests the loss of an acetyl group. The ^{1}H NMR spectrum (Table 5) was similar to that of lycorine (Likhitwitayawuid et al., 1993). The shifting of proton H-2 to a higher magnetic field than that of lycorine suggests a substitution of the hydroxyl group at C-2. The presence of a singlet at δ 2.00 indicated an acetyl group, whereas the signals at δ 2.51, 2.63, 5.25 and 1.29 were congruent with a substituted 3-hydroxybutanoyl group and were assigned to positions H-2′$_A$ and H-2′$_B$, H-3′ and H-4′ like those of compound **4**. These ^{1}H NMR data suggested a lycorine type skeleton with an acetyl and a 3-hydroxybutanoyl group. Heteronuclear (HMBC and HMQC) experiments showed a 22 carbon skeleton. The signals at δ 170 and 170.4 confirmed the presence of two carbonyl groups while the signals at δ 41.0 (C-2′), 67.5 (C-3′) and 20.0 (C-4′) were congruent with a substituted 3-hydroxybutanoyl group, like **4**. The signal at δ 21.3 was assigned to the acetoxy methyl carbon (C-2″). The HMBC experiment indicated an attachment of the 3-hydroxybutanoyl group at C-2 due to the correlation of the deshielded proton H-2 with C-1′. The attachment of the acetyl group was assigned at C-3′ due to the three-bound HMBC correlation of H-3′ with C-1″ and its deshielding effect on H-3′. On the basis of the spectral data, **5** was identified as 2-*O*-(3′-acetoxybutanoyl)lycorine.

The occurrence of several Amaryllidaceae alkaloids whose hydroxyl groups are substituted with one or more 3-hydroxybutanoyl groups in a single species is noteworthy, since this kind of compound is rarely reported. A 3-hydroxybutanoyl derivative of tazettine was found in *Galanthus plicatus* subsp. *bizanthus* (Ünver et al., 2001), while galanthamine and homolycorine type derivatives have been reported in species of the genera *Leucojum* (Kobayashi et al., 1985; Forgo and Hohmann, 2005) and *Narcissus* (Bastida et al., 1988). To our knowledge, this is the first report of the isolation of 3-hydroxybutanoyl derivatives of alkaloids with lycorine type skeletons. With the exception of **4**, the new compounds showed relatively intensive molecular ions under GC–MS conditions. Compound **4** showed the same retention time and GC–MS spectrum as **3**, indicating that it most probably degrades in the injection port of the GC. GC–MS has proved to be useful for the analysis of a wide range of Amaryllidaceae alkaloids in complex mixtures (Kreh et al., 1995) and for the search of novel compounds. Compound **1** was previously detected in alkaloid fractions of *G. elwesii*; its haemanthamine type structure and 3-hydroxybutanoyl group were predicted by CG–MS but the substitutent at C-3 was wrongly assigned (Berkov et al., 2004).

Table 5
^{1}H NMR, COSY, NOESY, HMQC and HMBC data of **5**

Position	H δ (*J* in Hz)	COSY	NOESY	HMQC		HMBC
1	4.51 *s*	H-2, 10b	H-2, H-10, H-10b	69.4 *d*		C-2, C-3, C-4a
2	5.31 *dt* (3.0, 1.5)	H-1, H-3, C-4a	H-1, H-3	73.9 *d*		–
3	5.44 *br t* (2.5)	H-2, H-4a	H-2, H-11	113.6 *d*		–
				146.17 *s*	(C-4)	–
4a	2.82 *br d* (10.5)	H-3, H-10b	H-6α, H-10b, H-12α	60.6 *d*		–
6α	3.53 *dd* (14.0, 1.0)	H-6β, H-10b	H-4a, H-6β, H-7, H-12α	56.8 *t*		C-6a, C-10a, C-12
6β	4.13 *d* (14.0)	H-6α	H-6α, H-7, H-12β	56.8 *t*		C-4a, C-6a, C-7, C-10a
				130.0 *s*	(C-6a)	–
7	6.58 *s*		H-6α, H-6β	107.9 *d*		C-6, C-8, C-9, C-10, C-10a
				146.7 *s*	(C-8)	–
				146.9 *s*	(C-9)	–
10	6.79 *s*		H-1, H-10b	104.8 *d*		C-6a, C-7, C-8, C-9, C-10b
				127.1 *s*	(C-10a)	–
10b	2.68 *br d* (10.5)	H-1, H-4a, H-6α	H-1, H-4a	41.7 *d*		C-4a
11 (2H)	2.63 *m*	H-12α, H-12β	H-3, H-12α, H-12β	29.7 *t*		–
12α	2.40 *dd* (9.0, 8.5)	H-11, H-12β	H-4a, H-6α, H-11α, H-11β, H-12β	53.7 *t*		C-4, C-6, C-11
12β	3.35 *ddd* (9.5, 9.2, 5.0)	H-11, H-12α	H-6β, H-11α, H-11β, H-12α	53.7 *t*		C-4, C-4a, C-11
OCH_2O	5.90 *d* (1.5)–5.92 *d* (1.5)			101.1 *t*		C-8, C-9
				170.0 *s*	(C-1′)	–
2′$_A$	2.51 *dd* (15.5, 5.5)	H-3′	H-3′	41.0 *t*		C-1′, C-3′, C-4′
2′$_B$	2.63 *dd* (15.5, 7.5)	H-3′	H-3′	41.0 *t*		C-1′, C-3′, C-4′
3′	5.25 *m*	H-2′$_A$, H-2′$_B$, H-4′	H-2′$_A$, H-2′$_B$, H-4′	67.5 *d*		C-1″
4′	1.29 *d* (6.0)	H-3′	H-2′$_A$, H-2′$_B$, H-3′	20.0 *q*		C-2′, C-3′
				170.4 *s*	(C-1″)	–
2″	2.00 *s*		H-4′	21.3 *q*		C-1′

3. Experimental

3.1. General experimental procedures

NMR spectra were recorded in a Mercury 400 MHz or a Varian VXR 500 MHz, using $CDCl_3$ (CD_3OD for **7**) as a solvent and TMS as the internal standard. Chemical shifts were reported in δ units (ppm) and coupling constants (J) in Hz. EIMS were obtained on a CG–MS Hewlett–Packard 6890+ MSD 5975 operating in EI mode at 70 eV. An HP-5 MS column (30 m × 0.25 mm × 0.25 μm) was used. The temperature program was: 100–180 °C at 15 °C min^{-1}, 1 min hold at 180 °C and 180–300 °C at 5 °C min^{-1} and 1 min hold at 300 °C. Injector temperature was 280 °C. The flow rate of carrier gas (Helium) was 0.8 ml min^{-1}. Split ratio was 1:20. A Jasco-J-810 Spectrophotometer was utilized to run CD spectra, all recorded in MeOH.

3.2. Plant material

Whole plants of *Galanthus nivalis* L. (Amaryllidaceae) were collected in March 2003 during the flowering period from a population near the village of Obrochishte, district of Varna, Bulgaria. A voucher specimen (SOM-162922) has been deposited in the Institute of Botany, Bulgarian Academy of Science, Sofia.

3.3. Extraction and isolation of alkaloids

Fresh whole plants (aerial parts and bulbs) of *G. nivalis* (3 kg) were crushed and extracted with 95% EtOH (5 × 10 l, 72 h each). The extract was evaporated under red. pres. and the residue dissolved in 300 ml of 2% H_2SO_4 and filtered after 12 h. After removing neutral material with Et_2O, the acidic solution, basified with 25% ammonia up to pH 9-10 and extracted with $CHCl_3$ (5 × 500) ml, gave extract A (1.2 g). Finally, the $CHCl_3$–MeOH (3:1) extraction of the basic solution gave extract B (0.3 g). After combining extracts A and B, the brown gummy residue (0.05% referred to the fresh weight) was dissolved in MeOH from which lycorine (**8**, 107 mg) crystallized directly. The solution was dried and subjected to CC (3 × 65 cm column) on Kiselgel (200 g – mesh 0.15–0.30). The alkaloids were eluted using $CHCl_3$ gradually enriched with MeOH (0–100%). Fractions of 30 ml were collected (175 in total) monitored by TLC (Dragendorff's reagent, UV light 254 nm) and combined according their TLC profiles. Fr. 1, subjected to CC and eluted with *n*-hexane–EtOAc (2:1) afforded ismine (6 mg). Tazettine (37 mg) crystallized from frs. 2–4 and 5–11. Compounds **5** (3 mg) and **4** (3 mg) were isolated from frs. 2–4 and 5–11, while **2** (8 mg) from frs. 5–11 by PTLC (20 cm × 20 cm × 0.25 mm, Silica gel F_{254}, EtOAc–MeOH–25% ammonia 5:1:0.01, v/v/v). Frs. 14–20 afforded **3** (2 mg) and more **4** by PTLC. Fr. 21 was subjected to CC (EtOAc–MeOH, 4:1) and afforded hamayne (30 mg). More lycorine crystallized from frs. 22–25. From these fractions **1** (3 mg) was isolated by CC (EtOAc–MeOH, 4:1). Ungeremine (2 mg) was eluted by CC (EtOAc–MeOH, 1:5) from frs. 125–175.

3.4. 11-O-(3′-Hydroxybutanoyl)hamayne (*1*)

HRMS m/z 373.1514 (Calc. 373.1525 for $C_{20}H_{23}NO_6$). CG–MS(EI) 70 eV (rel. int.): 373 $[M]^+$ (100), 344 (9), 286 (23), 269 (74), 252 (25), 240 (45), 224 (39), 210 (34), 181 (43), 128 (13), 115 (21), 87 (8). ^1H NMR (500 MHz, $CDCl_3$) and ^{13}C NMR (50 MHz, $CDCl_3$) see Table 1.

3.5. 3,11-O-(3′,3″-Dihydroxybutanoyl)hamayne (*2*)

HRMS m/z 459.1888 (Calc. 459.1893 for $C_{24}H_{29}NO_8$). CG–MS(EI) 70 eV (rel. int.): 459 $[M]^+$ (23), 356 (19), 286 (1), 269 (100), 252 (27), 240 (47), 224 (61), 210 (44), 181 (96), 128 (13), 115 (21), 87 (21). ^1H NMR (500 MHz, $CDCl_3$) and ^{13}C NMR (50 MHz, $CDCl_3$) see Table 2.

3.6. 3-O-(2″-Butenoyl)-11-O-(3′-hydroxybutanoyl)hamayne (*3*)

CD $[\Theta]_\lambda^{20}$: $[\Theta]_{251}$ −646, $[\Theta]_{290}$ +3031. HRMS m/z 441.1777 (Calc. 441.1787 for $C_{24}H_{27}NO_7$). CG–MS(EI) 70 eV (rel. int.): 441 $[M]^+$(29), 354 (7), 337 (15), 269 (84), 252 (26), 240 (47), 227 (60), 224 (57), 211 (33), 210 (32), 181 (86), 128 (13), 115 (24), 69 (100). ^1H NMR (500 MHz, $CDCl_3$) and ^{13}C NMR (50 MHz, $CDCl_3$) see Table 3.

3.7. 3,11,3″-O-(3′,3″,3‴-Trihydroxybutanoyl)hamayne (*4*)

CD $[\Theta]_\lambda^{20}$: $[\Theta]_{246}$ −3309, $[\Theta]_{285}$ +4379. CIMS (NH_3) 546 $[M^+ + 1]$. CG–MS(EI) 70 eV (rel. int.): 545 $[M]^+$ (−), 441 (22), 354 (8), 337 (14), 269 (70), 252 (31), 240 (41), 227 (50), 224 (59), 211 (34), 210 (33), 181 (87), 128 (16), 115 (23), 69 (100). ^1H NMR (500 MHz, $CDCl_3$) and ^{13}C NMR (50 MHz, $CDCl_3$) see Table 4.

3.8. 2-O-(3′-Acetoxyhydroxybutanoyl)lycorine (*5*)

HRMS m/z 415.1649 (Calc. 415.1631 for $C_{22}H_{25}NO_7$). CG–MS(EI) 70 eV (rel. int.): 415 $[M]^+$(8), 414 (5), 354 (3) 269 (44), 268 (58), 252 (35), 251 (39), 250 (100), 227 (16), 226 (26), 192 (14), 147 (11), 124 (13), 96 (16) 69 (10). ^1H NMR (500 MHz, $CDCl_3$) and ^{13}C NMR (50 MHz, $CDCl_3$) see Table 5.

Hamayne (Viladomat et al., 1994), ungeremine (Bastida et al., 1996), lycorine (Likhitwitayawuid et al., 1993; Bastida et al., 1995a) tazettine (Ghosal et al., 1984; Bastida et al., 1995b) and ismine (Viladomat et al., 1990) were identified by direct comparison of their chromatographic and spectroscopic properties (TLC, CG–MS, CD, MS, ^1H NMR) with those of authentic samples obtained in our laboratory from other plant sources.

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