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N-Alkylated galanthamine derivatives: Potent acetylcholinesterase inhibitors from *Leucojum aestivum*

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Abstract—*N*-(14-Methylallyl)norgalanthamine, a new natural compound, together with five known alkaloids: *N*-allylnorgalanthamine, galanthamine, epinorgalanthamine, narwedine, and lycorine were isolated from mother liquors (waste material) obtained after industrial production of galanthamine hydrobromide from *Leucojum aestivum* leaves. The structures of *N*-allylnorgalanthamine and *N*-(14-methylallyl)norgalanthamine were completely determined by ^{1}H and ^{13}C NMR spectroscopy and two-dimensional experiments. *N*-allylnorgalanthamine (IC_{50} = 0.18 μM) and *N*-(14-methylallyl)norgalanthamine (IC_{50} = 0.16 μM) inhibit AChE considerably more than the approved drug galanthamine (IC_{50} = 1.82 μM).

Cholinesterase inhibitors are approved drugs for the treatment of a limited number of patients suffering from Alzheimer's disease, mainly those with mild to moderately severe degeneration of memory and cognitive function. Galanthamine (**3**), an Amaryllidaceae alkaloid, is a long-acting, selective, reversible, and competitive AChE inhibitor,[1] which produces beneficial effects even after the drug treatment has been terminated.[2] This product is marketed as a hydrobromide salt under the name Razadyne®, formerly Reminyl®, for the treatment of Alzheimer's disease. Galanthamine has been produced from *Leucojum aestivum* in Bulgaria since the 1960s and recently also by chemical synthesis.[3] The search for other natural AChE inhibitors has resulted in the identification of other active compounds.[4]

About 20 alkaloids have been isolated from *L. aestivum*.[5–7] The present paper reports the isolation of two potent AChE inhibitors: *N*-allylnorgalanthamine (**1**) and *N*-(14-methylallyl)norgalanthamine (**2**) as well as four other compounds from the mother liquors obtained after industrial production of galanthamine (Fig. 1). NMR data of compound **1**, an alkaloid isolated only from *Lycoris guangxiensis*,[8] were completely assigned. Compound **2** is reported here for the first time as a natural product.

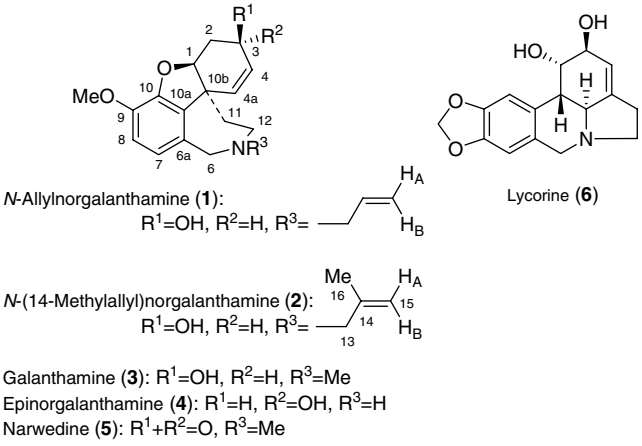

Figure 1. Chemical structures of compounds **1**–**6**.

Aerial parts of *L. aestivum* L., collected in the spring of 2005 from different populations in the south of Bulgaria, were used for industrial extraction of galanthamine. A voucher specimen (SOM 1134) of the plant species, authenticated by Dr. Ljuba Evstatieva, was deposited at the herbarium of the Institute of Botany, Sofia, Bulgaria.

A GC–MS study of mother liquors obtained after precrystallization of galanthamine hydrobromide (from *L.*

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aestivum leaves) showed the presence of *N*-allylnorgalanthamine (**1**) (1.67% of the total ion current, TIC), compound (**2**) (0.15% of TIC) with an MS fragmentation pattern characteristic of galanthamine type compounds, and other alkaloids. Twenty grams of alkaloid salts from the mother liquors was supplied by Galen-N Ltd (Bulgaria). The alkaloid salts were dissolved in distilled water, basified with 25% ammonia to pH 9–10 and the free bases were extracted with EtOAc. The alkaloid mixture (ca. 15 g) was subjected to column chromatography (silica gel, 40–60 mesh, 250 g, SDS-France) using EtOAc gradually enriched with MeOH. The fractions were monitored by TLC (Dragendorff's reagent) on silica gel plates (60F254, 0.2 mm thickness of the layer, SDS-France) developed with a mobile phase containing EtOAc–MeOH–25% ammonia (3:1:0.1, v/v/v). Compounds **1** (15 mg) and **2** were isolated from fractions 2 to 5 (eluted with EtOAc–MeOH, 3:1) by re-chromatography on silica gel column eluted with EtOAc–MeOH (9:1). Compound **2** (2 mg) was purified by silica gel TLC using hexane–EtOAc–MeOH–25% ammonia (10:5:1:0.1) as a mobile phase. Compounds **3** (50 mg) and **5** (5 mg) were pre-crystallized from fractions 5 to 20 eluted with EtOAc–MeOH (5:5). A mixture of **3**:**5** (880 mg) in a ratio of about 5:1 was obtained from these fractions. Compound **4** (2.12 g) was crystallized from fractions 20 to 41 eluted with EtOAc–MeOH (3:7). Compound **6** (17 mg) was crystallized directly from fractions 6 and 7.

The structures of the isolated compounds were assigned by NMR, MS, UV, and CD spectroscopy.[9] Compound **1**, isolated as a light-brown solid, was characterized as *N*-allylnorgalanthamine by its MS and ^{1}H NMR spectral data.[8,10] Its absolute configuration was confirmed by the CD curve.[11] A complete assignment of the proton signals (Table 1), hitherto unreported, was afforded by 1D- and 2D-NMR experiments (COSY, HMBC, and HMQC). Compound **1** exhibited signals whose chemical shifts and multiplicity are similar to those reported for **3**.[12] In addition, one d at δ 3.16 integrating 2H, one ddt at δ 5.87 and two dd at δ 5.14, and 5.12 for the protons at positions 13, 14, and 15, respectively, were congruent with a *N*-allyl group.[8] The ^{13}C NMR data of **1**, reported for the first time (Table 1), are in agreement with the proposed structure as well as with compound **3**.[13] Heteronuclear chemical shift correlation experiments (HMBC and HMQC) were performed in order to assign all the signals of the ^{13}C NMR spectrum and to confirm the assignments made for the ^{1}H NMR spectrum.

Compound **2** showed GC–MS, ^{1}H NMR, and ^{13}C NMR data closely comparable to those of **1** (Table 2). Its HR-ESI-MS suggested a molecular formula $C_{20}H_{25}NO_3$ with a parent ion $[M+H]^+$ at *m*/*z* 328.1901 (calcd 328.1913), which is with 14.0153 mass units (CH_2, calcd 14.0157) more than **1**. In contrast to compound **1**, the ^{1}H NMR spectrum of **2** showed (1) a signal (singlet) at δ 1.76, integrating three protons, corresponding to a methyl group in the α position with respect to an aliphatic double bond; (2) a shifting of the protons H-15_A and H-15_B to a higher field; and (3) a change in the multiplicity of the protons at positions 13 and 15 ($J_{13\alpha,13\beta}$ = 2.8 Hz, H-15_A and H-15_B were singlets). These data, together with the lack of a signal in the ^{1}H NMR spectrum corresponding to the H-14 of compound **1**, indicated the presence of a methyl group at C-14. The chemical shift of quaternary carbon C-14 at δ 142.7 was assigned due to its HMBC correlations with the protons at positions 13 and 16. The signals of C-13, C-14, and C-15 were shifted, with $\Delta\delta$ + 2.5, +6.8, and

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

Position	Hδ (*J*in Hz)	COSY	HMQC	HMBC
1	4.62 br s	H-2α, H-2β	89.1 d	C-3, C-4a, C-11
2α	2.00 ddd (15.6, 5.2, 2.4)	H-1, H-2β, H-3	30.3 t	C-4a, C-10b
2β	2.69 ddt (15.6, 3.6, 1.6)	H-1, H-2α	30.3 t	C-1, C-3, C-4, C-10b
3	4.14 t (4.8)	H-2α, H-4	62.4 d	C-1, C-2, C-4, C-4a
4	6.01 dd (10.4, 5.2)	H-3, H-4a	127.9 d	C-2, C-10b
4a	6.09 d (10.4)	H-4	127.3 d	C-1, C-3, C-10a, C-10b
6	3.82 d (15.2)	H-6′	58.2 t	C-6a, C-7, C-10a, C-12, C-13
6′	4.07 d (15.2)	H-6	58.2 t	C-6a, C-7, C-10a, C-12, C-13
			129.6 s (C-6a)	
7	6.59 d (8.0)	H-8	122.4 d	C-6, C-8, C-9, C-10a
8	6.65 d (8.0)	H-7	111.5 d	C-6a, C-7, C-10
			144.4 s (C-9)	
			146.2 s (C-10)	
			133.6 s (C-10a)	
			48.7s (C-10b)	
11α	2.03 ddd (13.6, 12.8, 3.2)	H-11 β, H-12α, H-12β	34.0 t	C-1, C-4a, C-12
11β	1.55 ddd (13.6, 3.6, 1.6)	H-11α, H-12α, H-12β	34.0 t	C-10a, C-10b
12α	3.18 dt (14.4, 3.2)	H-11α, H-11β, H-12β	52.0 t	C-10b
12β	3.29 ddd (14.4, 12.8, 1.6)	H-11α, H-11β, H-12α	52.0 t	C-6, C-11, C-10b, C-13
13 (2*H*)	3.16 d (6.8)	H-14, H-15_A, H-15_B	56.5 t	C-6, C-12, C-14, C-15
14	5.87 ddt (16.8, 10.4, 6.8)	H-13, H-15_A, H-15_B	135.9 d	C-13
15$_A$	5.14 dd (10.4, 1.6)	H-13, H-14, H-15_B	118.1 t	C-13, C-14
15$_B$	5.12 dd (16.8, 1.6)	H-13, H-14, H-15_A	118.1 t	C-13, C-14
OCH$_3$	3.84 s		56.3 q	C-9, C-10

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

Position	Hδ (J in Hz)	COSY	HMQC	HMBC
1	4.62 br s	H-2α, H-2β, H-4a	88.5 d	
2α	2.01 ddd (16.0, 5.2, 2.4)	H-1, H-2 β, H-3	29.8 t	
2β	2.69 ddt (16.0, 3.6, 1.6)	H-1, H-2α, H-4	29.8 t	C-1, C-3, C-10b
3	4.14 br s	H-2α, H-4	61.8 d	
4	6.01 dd (9.6, 5.2, 1.2)	H-2β, H-3, H-4a	127.3 d	C-2, C-3, C-10b
4a	6.11 d (9.6)	H-1, H-4	126.9 d	C-1, C-3, C-10b
6	3.74 d (15.2)	H-6′	57.4 t	C-6a, C-7, C-10a, C-12
6′	4.07 d (15.2)	H-6, H-7	57.4 t	C-7, C-12, C-13
			129.7 s (C-6a)	
7	6.58 d (8.0)	H-6′, H-8	122.1 d	C-6, C-9, C-10a
8	6.65 d (8.0)	H-7	111.1 d	C-6a, C-10
			144.0 s (C-9)	
			145.7 s (C-10)	
			133.3 s (C-10a)	
			48.3 s (C-10b)	
11α	2.05 ddd (14.0, 12.8, 3.2)	H-11β, H-12α, H-12β	33.4 t	
11β	1.53 d (14.0)	H-11α, H-12α, H-12β	33.4 t	
12α	3.05–3.12 m	H-11α, H-11 β, H-12β	51.3it t	
12β	3.32 t (13.6)	H-11α, H-11 β, H-12α	51.3 *t*	
13 (2*H*)	3.05 *d* (2.8)	H-15$_A$, H-15$_B$, H-16	59.0 t	C-6, C-12, C-14, C-15, C-16
14			142.8 s (C-14)	
15$_A$	4.86 br s	H-13, H-15$_B$, H-16	113.1 t	C-13, C-16
15$_B$	4.80 br s	H-13, H-15$_A$, H-16	113.1 t	C-13, C-16
16	1.76 s (3H)	H-13, H-15$_A$, H-15$_B$	20.4 q	C-13, C-14, C-15
OCH$_3$	3.84 s		55.8 q	C-9

−4.9, respectively, in comparison to **1**. Thus, the structure of **2** was determined as *N*-(14-methylallyl)norgalanthamine.[14] The chemical syntheses of the salts of both compounds were patented recently as potential drugs against Alzheimer's disease.[15]

Galanthamine (**3**), epinorgalanthamine (**4**), narwedine (**5**), and lycorine (**6**) were also isolated and identified by comparison of their chromatographic and spectroscopic properties (CD, MS, ^{1}H NMR),[8,12] with those of authentic samples obtained from other plant sources.

Among the Amaryllidaceae alkaloids, the AChE activity is associated mainly with the galanthamine structural type.[4] Until now, one natural galanthamine type alkaloid, sanguinine (9-*O*-demethylgalanthamine), has been reported to be a more active AChE inhibitor than galanthamine.[16] The AChE inhibitory assay of **1** and **2**, performed after López et al.,[16] demonstrated that they are considerably more potent AChE inhibitors (ca. 10 times) than galanthamine (IC_{50} = 1.82 ± 0.40 μM), showing an IC_{50} at 0.18 ± 0.01 and 0.16 ± 0.01 μM, respectively. Synthetic derivatives, which are quickly converted into sanguinine after administration, show low levels of brain bioavailability due to the phenolic group of sanguinine. This group increases the hydrophilicity and reduces the ability of the molecule to cross the blood–brain barrier.[17] Compounds **1** and **2** have a methoxyl group at C-9 and an alkyl group at the *N*-atom, suggesting higher lipophilicity as compared to sanguinine and galanthamine. It was confirmed by their higher R_f values on silica gel TLC plates (EtOAc–MeOH–25% ammonia, 3:1:0.1)—0.91 for **1** and 0.78 for **2**, as compared to galanthamine—0.33.

The crystal structure of galanthamine in the active site gorge of *Torpedo californica* acetylcholinesterase (*Tc*AChE) showed that this relatively tight binding comes from a number of moderate to weak interactions with the protein, including classical and non-classical hydrogen bonds.[18] The substituent at the N atom modifies the AChE inhibitory activity of the galanthamine derivatives. The tertiary amine appears to make a non-classical hydrogen bond, via its *N*-methyl group, to a Asp-72 of *Tc*AChE.[18] The loss of the *N*-methyl group, as in epinorgalanthamine, is associated with a decrease of AChE inhibitory activity.[16] On the other hand, some synthetic *N*-alkylated derivatives of galanthamine showed remarkable AChE inhibitory activity.[19] Apparently, the increased activity of **1** and **2** is due to the substitution of the *N*-methyl group with an allyl or 14-methylallyl group.

In summary, the isolation of natural *N*-alkyl derivatives of galanthamine type alkaloids is very rare due to their extremely low concentration in plants. GC–MS can be successfully applied for the search of new or rare alkaloids, especially when they are at trace amounts. The greater AChE inhibitory activity of **1** and **2** and their lipophilic properties, when compared to the closely related and approved drug galanthamine, indicates that they may have a therapeutic potential.

Acknowledgments

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9. ^{1}H, ^{13}C NMR, COSY, HMQC, and HMBC spectra were recorded on a Mercury 400F (400 MHz/100 MHz) spectrometer in $CDCl_3$ (CD_3OD for **6**) with TMS as internal standard. Chemical shifts were reported in δ units (ppm) and coupling constants (J) are expressed in Hz. Optical rotations were carried out on a Perkin–Elmer 241 Polarimeter. UV spectra were recorded on a Hitachi U-2000 spectrophotometer. The GC–MS spectra were recorded on a Hewlett–Packard 6890 equipped with MSD 5975, (Hewlett–Packard, Palo Alto, CA, USA) 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 the carrier gas (Helium) was 0.8 mL min^{-1}. HR-ESI-MS spectra were obtained on an LC/MSD-TOF (2006) Mass spectrometer (Agilent technologies). A Jasco-J-810 Spectropolarimeter was utilized to run the CD spectra, all recorded in MeOH.
10. *N*-Allylnorgalanthamine (**1**): light-brown solid; UV (MeOH): λ_{max} (log ε) = 295 (3.13) nm; $[\alpha]_D^{20}: -97^\circ$ (c0.31, MeOH); CD $[\Theta]_\lambda^{20}: [\Theta]_{242} + 4350, [\Theta]_{279} + 3951, [\Theta]_{247} - 2460, [\Theta]_{236} + 3317$. HR-ESI-MS $[M+H]^+$ m/z 314.1747 (calcd 314.1756). GC–MS(EI) 70 eV (rel. int.): $[M]^+$ 313(44), 312(100), 296(13), 286(9), 285(19), 270(20), 256(12), 242(25), 230(8), 226(11), 211(9), 174(30), 165(10), 128(13), 115(18), 103(7), 91(9), 77(9), 55(10). ^{1}H NMR and ^{13}C NMR (400 MHz/100 MHz, $CDCl_3$) spectral data see Table 1. TLC: R_f 0.78.
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14. *N-(14-Methylallyl)norgalanthamine* (**2**)*:* light-brown solid; UV (MeOH): λ_{max} (log ε) = 295 (2.65) nm; CD $[\Theta]_\lambda^{20}: [\Theta]_{284} + 4129, [\Theta]_{249} - 3398, [\Theta]_{236} + 3659$. HR-ESI-MS $[M+H]^+$ m/z 328.1901 (calcd 328.1913). GC—MS(EI) 70 eV (rel. int.): $[M]^+$ 327(63), 326(100), 310(11), 299(23), 298(16), 286(54), 271(58), 270(20), 256(29), 230(11), 226(12), 213(22), 211(16), 174(60), 165(24), 141(21), 128(31), 115(43), 103(13), 91(18), 77(19), 55(51). ^{1}H NMR and ^{13}C NMR (400 MHz/100 MHz, $CDCl_3$) spectral data see Table 2. TLC: R_f 0.91.
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