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Regular paper

New method for the study of Amaryllidaceae alkaloid biosynthesis using biotransformation of deuterium-labeled precursor in tissue cultures

Anna El Tahchy[1], Michel Boisbrun[1], Agata Ptak[2], François Dupire[3], Françoise Chrétien[1], Max Henry[1], Yves Chapleur[1] and Dominique Laurain-Mattar[1]✉

[1]Groupe S.U.C.R.E.S., UMR 7565 CNRS-Nancy-Université, Nancy-Vandoeuvre, France; [2]Department of Plant Breeding and Seed Science, Agricultural University, Kraków, Poland; [3]Service Commun de Spectrométrie de Masse, UHP-Institut Jean Barriol, Nancy-Vandoeuvre, France

Biotransformation of deuterated-4′-*O*-methylnorbelladine into alkaloids galanthamine and lycorine in tissue cultures of Leucojum aestivum was demonstrated using HPLC coupled to mass spectrometry. GC-MS screening was also carried to investigate other native and deuterated alkaloids. A total of six labeled alkaloids were identified indicating that 4′-*O*-methyl-d_3-norbelladine is incorporated into three different groups of Amaryllidaceae alkaloids that are biosynthesized by three modes of intramolecular oxidative phenol coupling.

Keywords: alkaloids, Leucojum aestivum, Amaryllidaceae, deuterated precursor, biotransformation

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INTRODUCTION

Amaryllidaceae alkaloids have important pharmacological properties such as acetylcholinesterase inhibitory activity, cytotoxicity and antitumoral activity (Bastida *et al*., 2006). Galanthamine, an isoquinoline alkaloid, is obtained on a commercial scale for its pharmacological interest from *Narcissus* spp. and *Leucojum aestivum* as well as synthetically (Guillou *et al*., 2001; Marco-Contelles *et al*., 2006). Lycorine, a pyrrolophenanthridine alkaloid, displays a strong antiviral effect against poliovirus, measles and *Herpes simplex* type 1 viruses, as well as high antiretroviral (Szlávik *et al*., 2004) and strong antimitotic activities (Kukhanova *et al*., 1983).

Several studies on the biosynthesis of Amaryllidaceae alkaloids belonging to different ring type subgroups have been reported (Barton & Cohen, 1957; Barton *et al*., 1963; Bernfeld, 1963; Eichhorn *et al*., 1998; Herbert, 2001; Kornienko & Evidente, 2008). All Amaryllidaceae alkaloids can be regarded as derivatives of the common precursor 4'-*O*-methylnorbelladine *via* intramolecular oxidative phenol-coupling (Barton & Cohen, 1957; Barton *et al*., 1963). There are three different groups of Amaryllidaceae alkaloids that are biosynthesized by three modes of intramolecular oxidative phenol coupling of the common precursor (Fig. 1) (Herbert, 2001; Kornienko & Evidente, 2008). The *para-ortho'* oxidative coupling leads to a dienone which spontaneously cyclizes to demethylnarwedine which upon stereoselective reduction leads to demethylgalanthamine. Subsequent N-methylation of this compound gives galanthamine. Narwedine exists in equilibrium with galanthamine, a reaction catalyzed by a hypothetically reversible oxidoreductase (Eichhorn *et al*., 1998). *para-para′* coupling gives another dienone which, depending on the nitrogen nucleophilic addition site, gives rise to marithidine or crinine derivatives. Finally, *ortho-para'* coupling yields lycorine.

Nevertheless, the biosynthetic pathway of Amaryllidaceae alkaloids, particularly in *Leucojum aestivum*, has not been totally elucidated yet. Eichhorn *et al.* (1998) have established a revised scheme for the biosynthesis of galanthamine (Fig. 1, *para-ortho′* oxidative coupling of 4'-*O*-methylnorbelladine).

The investigations on the biosynthesis of Amaryllidaceae alkaloids used ^{14}C-labeled 4′-*O*-methylnorbelladine injected into organs of daffodil plants or ^{13}C or $^{3}H_3$C-labeled 4'-*O*-methylnorbelladine applied to organs of field grown *L. aestivum* plants (Barton *et al*., 1963; Eichhorn *et al*., 1998).

Here we report the development of a rapid and convenient method for the study of the biosynthetic pathway of Amaryllidaceae alkaloids using a method based on deuterium labeled precursor fed to *in vitro* cultures of *L. aestivum*. This paper reports for the first time the biotransformation of the common precursor 4′-*O*-methyl-d_3-norbelladine in shoot cultures of *L. aestivum*. Mass spectrometry was used for the identification of the labeled alkaloids. HPLCMS was used for the analysis of deuterated galanthamine and lycorine in tissue cultures and in the liquid medium. GC-MS analyses are also reported for the screening and identification of other deuterated alkaloids.

MATERIALS AND METHODS

Plant material. Leaves isolated from *Leucojum aestivum* L. bulbs (from French local markets) chilled for 12 weeks at 5 °C were surface-sterilized in 70% ethanol (1 min), then shaken for 15 min in 15% Domestos (with sodium hypochlorite and sodium hydroxide content below 5%; Unilever, Hungary) and rinsed three times with sterile water. Sterilized leaves were cut into thin slices (about 2 to 3 mm in length) and plated on culture medium.

✉e-mail: dominique.laurain-mattar@pharma.uhp-nancy.fr

Abbreviations: AnhydroLYC, anhydrolycorine; BAP, 6-benzylaminopurine; CRI, crinine; DemethylGAL, demethylgalanthamine; DemethylMAR, demethylmarithidine; D3MN, 4′-*O*-methyl-d_3-norbelladine; GAL, galanthamine; NAA, naphthalene-1-acetic acid; *N*-FGAL, *N*-formylnorgalanthamine; LYC, lycorine; NAR, narwedine; *N*-demethylNAR, *N*-demethylnarwedine; TRIS, trisphaeridine.

Shoot cultures. Initial explants were cultivated on Murashige and Skoog (1962) medium (Murashige & Skoog, 1962) supplemented with auxin α-naphthalene acetic acid (NAA) (10 μM) and cytokinin benzylaminopurine (BAP) (5 μM) (control). The medium was supplemented with 3% sucrose, adjusted to pH 5.5 before autoclaving and gelled with 0.8% purified agar (Difco). The cultures were maintained at 25 ± 2 °C in the darkness and subcultured every 4 weeks. Twelve month old shoot cultures (2 g) were submerged in liquid-shake medium (20 mL) containing labeled precursor 4′-*O*-methyl-d_3-norbelladine at various concentrations (0.05, 0.10 and 0.20 g/L) and incubated at 25 ± 2°C in the darkness for various periods of time (15, 30 and 40 days). A stock solution for feeding experiments was prepared from 50 mg of 4′-*O*-methyl-d_3-norbelladine dissolved in 10 mL of H_2O. Solutions at appropriate concentrations of the labeled precursor were filter-sterilized directly into the flasks.

Chemicals and reagents. Triethylammonium acetate buffer, galanthamine, lycorine, 6-benzylaminopurine, inositol, agar-agar, magnesium sulphate heptahydrate (98%), manganese sulphate, potassium nitrate, and ethylenediaminetetraacetic acid iron (III) sodium salt were all from Sigma-Aldrich Chemie GmbH (Stennheim, Germany). Ammonium bicarbonate, iron sulphate, zinc sulphate, copper(II)sulphate pentahydrate (99%), thiamine hydrochloride, α-naphthalene acetic acid, absolute ethanol, and methanol (Hipersolv Chromanorum for HPLC — Isocratic Grade) were purchased from Prolabo VWR international bvba/sprl. Pyridoxol hydrochloride, nicotinic acid, potassium dihydrogenophosphate and calcium chloride dehydrate were obtained from Merck AG (Darmstadt, Germany). Acetonitrile was acquired from Carlo Erba Reagenti.

Synthesis of the labeled precursor. General experimental procedures: solvents were purified and dried according to recommended procedures. Melting points were measured on a Reichert Kofler apparatus and are uncorrected. Infra-red spectra were recorded on a Perkin-Elmer Spectrum 1000 FT-IR spectrometer. ^1H and ^{13}C NMR spectra were recorded on an AC 250 Bruker spectrometer (250 Mhz and 62.9 MHz, respectively). MS analysis of 3-hydroxy-4-methoxy-d_3-benzaldehyde was

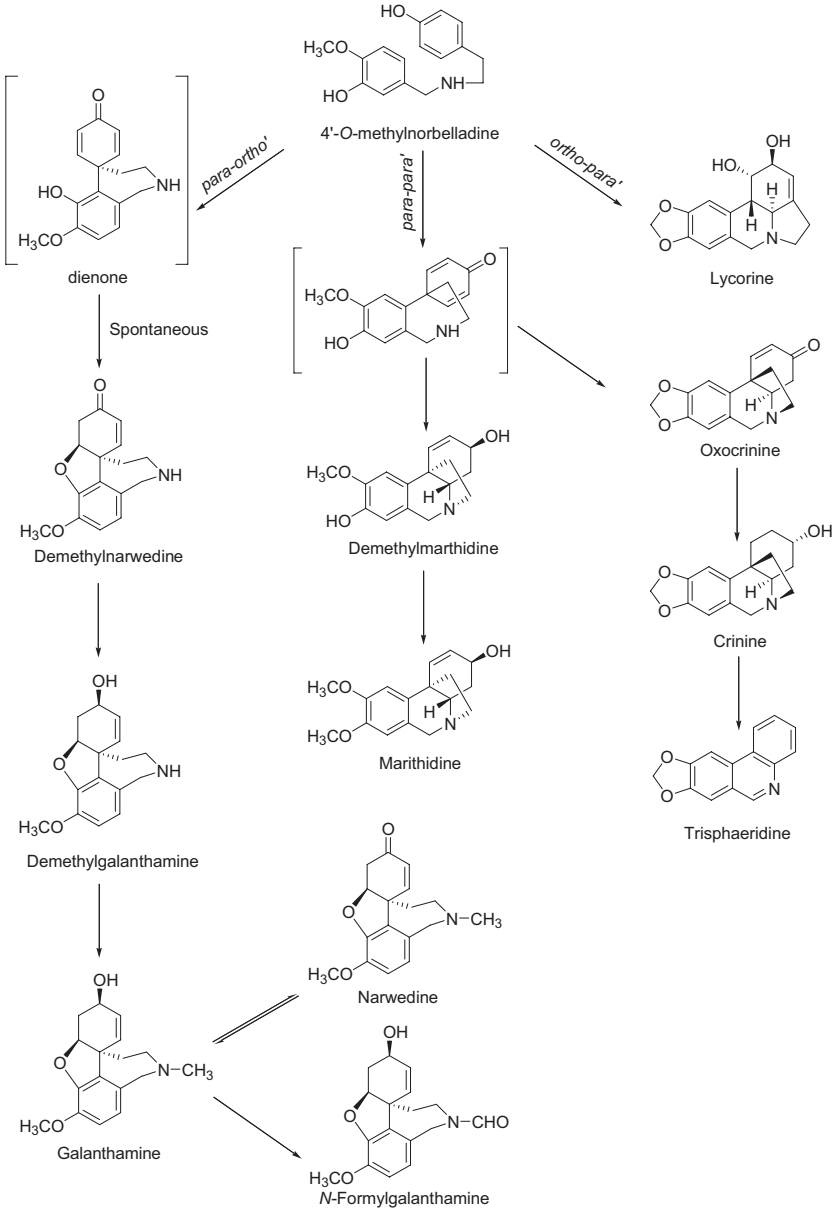

Figure 1. Postulated pathway for Amaryllidaceae alkaloid biosynthesis
After (Eichhorn *et al.*, 1998; Herbert, 2001; Kornienko & Evidente, 2008).

performed using QP2010 Shimadzu equipment operating in the CI mode. MS analysis of 4′-*O*-methyl-d_3-norbelladine was performed using a micrOTOF$_Q$™ (Bruker Daltonics) apparatus operating in the ES mode. Elemental analyses were performed on a Thermofinnigan FlashEA 1112 apparatus, at the Service Commun de Microanalyse (Nancy, France). Column chromatography was performed on silica gel SI 60 (63–200 μm) (Merck).

3-Hydroxy-4-methoxy-d_3-benzaldehyde: to a suspension of finely powdered potassium carbonate (1.901 g, 13.76 mmol) in acetone (70 mL) was added 3,4-dihydroxybenzaldehyde (1.900 g, 13.76 mmol) and methyl-d_3-iodide (856 μL, 13.76 mmol). The mixture was refluxed under inert atmosphere for 8 h, cooled and filtered. The solvent was evaporated and the residue was purified by column chromatography (eluent: ethyl acetate/hexane, 25:75, v/v) to give 3-methoxy-d_3-4-hydroxy benzaldehyde (0.300 g, 1.93 mmol), a mixture of the latter compound and the title compound (0.612 g, 3.94 mmol), and the title compound (0.628 g, 4.05 mmol). The mixture of the two isomers was re-purified the same way to give 3-methoxy-d_3-4-hydroxy benzaldehyde (0.107 g, 0.69 mmol) and the title compound (0.427 g, 2.75 mmol). Total yield: 1.055 g (6.80 mmol, 49% yield) of white crystals.

M.p. 111°C (lit.: 111°C) (Markey *et al.*, 1980) IR (KBr) ν_{max} 3225, 2865, 1670, 1605, 1579, 1508, 1291, 1251, 1121, 1101, 991, 827, 632 cm^{-1}; ^{1}H NMR ($CDCl_3$): δ 5.79 (s, 1 H, OH), 6.98 (d, J = 8.8 Hz, 1 H, H_{arom}), 7.44 (m, 2 H, H_{arom}), 9.86 (s, 1 H, CHO); ^{13}C NMR ($CDCl_3$): δ 110.4, 114.3, 124.8, 130.9, 146.3, 152.0, 191.3; CI-MS (pos. mode): m/z 156 [M + H]$^+$ (100), 311 [2M + H]$^+$ (25); Anal. calc. for $C_8H_5D_3O_3$: C 61.93; H+(D/2) 5.19; found: C 61.93; H 5.16.

4′-*O*-methyl-d_3-norbelladine: to a solution of 3-hydroxy-4-methoxy-d_3-benzaldehyde (979 mg, 6.31 mmol) and tyramine (866 mg, 6.31 mmol) in methanol (35 mL) was added 4 Å molecular sieves and the suspension was stirred overnight at room temperature under inert atmosphere. The mixture was filtered, cooled (0°C), then $NaBH_4$ (466 mg, 12.30 mmol) was added portionwise with stirring. The white suspension was stirred at room temp. for 3 h. The solvent was evaporated and to the residue were added succesively water (20 mL), brine (30 mL) and 3 M HCl until pH = 8. The suspension was extracted with EtOAc (3 × 50 mL). A dark insoluble material appeared, which was kept with the organic phases and the resulting suspension was extracted with 3 M HCl (2 × 20 mL) and water (1 × 20 mL). The aqueous phases were gathered, basified with 5% Na_2CO_3 until pH = 8 and the resulting suspension was extracted with EtOAc (4 × 50 mL). The solution was dried (Na_2SO_4) and the solvent evaporated. The residue was dissolved in hot ethanol (150 mL), the solution was filtered and, after cooling, concentrated HCl (6 mL) was added. The solution was concentrated to dryness under vacuum and ethanol was added to the residue (10 mL). The suspension was kept overnight at 4°C, then filtered and the solid was washed with cooled ethanol and dried to give 656 mg (2.10 mmol, 33% yield) of white crystals.

M.p. 208°C (lit.: 195°C for the undeuterated), (Szewczyk *et al.*, 1988) IR (KBr): ν_{max} 3454, 3265, 2967, 2799, 1614, 1596, 1515, 1435, 1280, 1260, 1223, 1134, 996, 832 cm^{-1}; ^{1}H NMR (DMSO-d_6): δ 2.90 (m, 2 H, CH_2), 2.99 (m, 2 H, CH_2), 4.01 (s, 2 H, CH_2), 6.75 (d, J = 8.3 Hz, 2 H, H_{arom}), 6.92–7.10 (m, 5 H, H_{arom}), 9.22 (s, 1 H, OH), 9.30 (br s, 2 H, NH_2^+), 9.43 (s, 1 H, OH); ^{13}C NMR (DMSO-d_6): δ 30.6, 47.6, 49.6, 112.0, 115.4, 117.3, 121.2, 124.2, 127.2, 129.5, 146.4, 148.2, 156.2; ESI-MS (pos. mode): m/z 140 [M — tyramine]$^+$ (100), 277 [M + H]$^+$ (65); Anal. calc. for $C_{16}H_{17}D_3NO_3Cl$: C 58.08; H+(D/2) 6.10; N 4.23; found: C 58.30; H 6.28; N 4.37.

Alkaloid extraction. Plant material was lyophilized, powdered and 150 mg of powder was macerated in methanol (10 mL) for 24 h, with sonication for 90 min in an ultrasonic bath (Transsonic 460/H Elma) at room temp. After centrifugation at 4000 rpm for 20 min, the mixture was filtered through 0.2 μm filters, and the total methanol extract was analyzed using LC-MS and GC-MS.

LC-MS analysis. The LC consisted of a U3000-Dionex system, an injector with a 1 μl loop and a UV detector at 280 nm. The analytical column used was an Acclain PepMap C18 ID 1 mm column (150 mm × 3 μm × 100 μm) and was eluted at a flow rate of 40 μl/min using a gradient ranging from 0% solvent B to 100% solvent B in a time span of 36 min. Solvent A consisted of 97.5% 10 mM ammoniumbicarbonate pH 7.8 with 2.5% methanol and solvent B consisted of 97.5% methanol and 2.5% 10 mM ammoniumbicarbonate pH 7.8. The ESi-HRMS was a micrOTOF$_Q$™ (Bruker Daltonics) apparatus.

GC-MS identification. Analyses were performed using QP2010 Shimadzu equipment operating in the EI mode at 70 eV. An AT-1 column (25 m × 0.32 mm × 0.30 μm) was employed with a 33 min temperature program of 80–280°C at 10°C/min followed by a 10 min hold at 280°C. The injector temperature was 280°C, the flow rate of the carrier gas (helium) was 0.8 ml/min, the split ratio was 1:50. Identification of the alkaloids was performed by comparing the measured data with those of authentic compounds (galanthamine, lycorine) or with literature data as specified in the text.

RESULTS AND DISCUSSION

Synthesis of the deuterated precursor of galanthamine biosynthesis

The synthesis of 4′-*O*-methyl-d_3-norbelladine was achieved in two steps, as depicted in Fig. 2. First, 3-hydroxy-4-methoxy-d_3-benzaldehyde (isovanillin-d_3) was obtained from 3,4-dihydroxybenzaldehyde, methyl-d_3 iodide and potassium carbonate using a modification of a published procedure (Markey *et al.*, 1980). Thus, crystallization was replaced by two successive column chromatographies of the crude mixture yielding pure isovanillin-d_3 in 49% yield, along with 19% of the undesired isomer (vanillin-d_3).

Then, as previously described (Szewczyk *et al.*, 1988) to get the non-labeled molecule, reductive amination with tyramine and $NaBH_4$ afforded the target molecule, which was isolated as a hydrochloride in 33% yield.

LC-MS analysis

Shoot cultures of *Leucojum aestivum* grown on Murashige and Skoog medium containing α-naphthalene

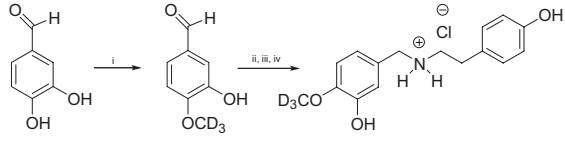

Figure 2. Steps of 4′-*O*-methyl-d_3-norbelladine synthesis
Reagents and conditions: (i) CD_3I, K_2CO_3; (ii) tyramine, molecular sieves; (iii) $NaBH_4$; (iv) HCl.

acetic acid (NAA) (10 μM) and benzylaminopurine (BAP) (5 μM) were subcultured in medium containing labeled precursor 4′-*O*-methyl-d_3-norbelladine at various concentrations (0.05, 0.10 and 0.20 g/L) and incubated for various periods of time (15, 30 and 40 days). Whatever the concentration of the deuterated precursor used, the growth kinetics of the shoot cultures were similar, although the growth rates (final shoot culture fresh weight — inoculum fresh weight/inoculum fresh weight) of the treated shoot cultures were lower than those of the control cultures (Table 1). These results indicated that the deuterated precursor added in the culture medium could be toxic for the shoot cultures. The lowest concentration (0.05 g/L) of 4′-*O*-methyl-d_3-norbelladine led, unexpectedly, to the slowest growth of the cultures. The medium concentration (0.10 g/L) also inhibited the growth, but the plant cultures showed the same growth rate as the control cultures after 40 days of incubation. This can be due to a correlation between shoot culture growth and the alkaloid synthesis in these tissues incubated with the labeled precursor.

After harvesting the shoot cultures, the precursor and the other alkaloids were extracted from the tissues as well as from the medium and the compounds were identified using mass spectrometry. HPLC coupled with high-resolution mass spectrometry (ESI/QqTOF) was used in order to identify native galanthamine and lycorine, by comparison with authentic compounds (Figs. 3a and 4a) as previously described (Ptak *et al.*, 2009), and also deuterated galanthamine and lycorine (Figs. 3b and 4b). These labeled alkaloids showed the same retention time as the native alkaloids, i.e. 33.4 min for galanthamine and 25.3 min for lycorine. Native galanthamine and deuterated galanthamine displayed $[M + H]^+$ at a m/z in accordance with the calculated values (288.1594 and 291.1783, respectively) (Fig. 3). Native lycorine and deuterated lycorine dis-

Table 1. Growth rate of *Leucojum aestivum* shoot culture in medium enriched with 4′-*O*-methyl-d_3-norbelladine (D3MN) at various concentrations
Data represent average of four replications with standard deviation.

	Growth rate		
[D3MN] g/L	Day 15	Day 30	Day 40
0.00	0.34 ± 0.07	0.45 ± 0.19	1.03 ± 0.15
0.05	0.09 ± 0.01	0.04 ± 0.03	0.30 ± 0.09
0.10	0.16 ± 0.05	0.36 ± 0.08	1.05 ± 0.07
0.20	0.20 ± 0.07	0.25 ± 0.07	0.83 ± 0.09

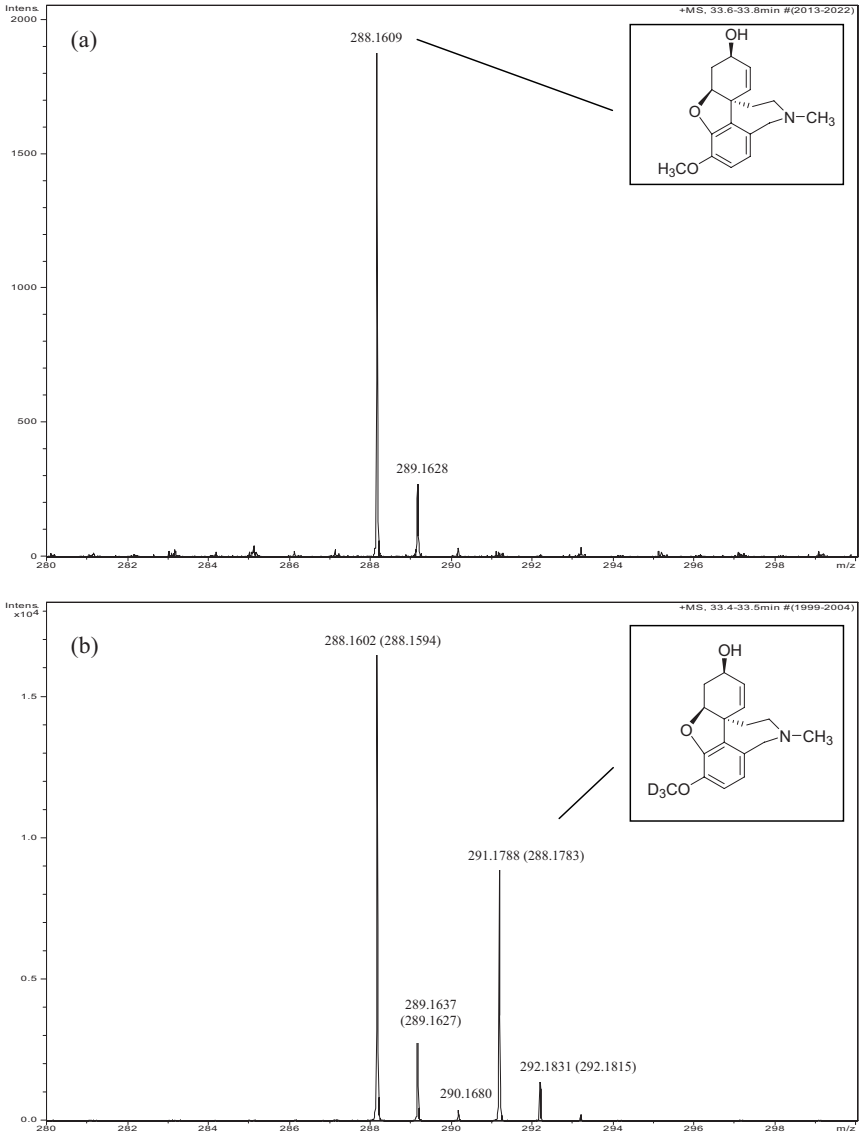

Figure 3. LC-MS mass spectra of galanthamine.
Spectra were acquired in the positive-ion mode. **(a)** Mass spectrum of authentic galanthamine; **(b)** mass spectrum of native and deuterated galanthamine from an extract of shoot cultures of *Leucojum aestivum*. Error < 4 ppm in both cases, theoretical masses are given in parentheses.

Table 2. Galanthamine and lycorine identified by LC-MS in shoot cultures of *Leucojum aestivum* and in liquid medium
Cultures were grown for 15, 30 and 40 days with 4′-*O*-methyl-d_3-norbelladine (D3MN) at various concentrations (0.05, 0.10, 0.20 g/L).

	GAL			LYC		
Molecular mass and Formula	C17H21NO3			C16H17NO4		
[M+H]+	288.1594			288.1230		
[MD2+H]+	-			290.1356		
[MD3+H]+	291.1783			-		
	15 Days	30 Days	40 Days	15 Days	30 Days	40 Days
Control shoot cultures	+	+	+	+	+	+
Culture Medium	+	+	+	+	+	+
Shoot cultures + 0.05 g/L MB*	-	+, D3	+, D3	+	+	+
Culture Medium	-	+, D3	+, D3	+	+	+
Shoot cultures + 0.1 g/L MB*	+, D3	+, D3	+, D3	+	+	+
Culture Medium	+, D3	+, D3	+, D3	+	+	+, D2
Shoot cultures + 0.2 g/L MB*	+	+, D3	-	+	+	+, D2
Culture Medium	+	+, D3	-	+	+	+, D2

GAL, galanthamine; LYC, lycorine; +, native alkaloid detected; -, no alkaloid detected; D3 or D2, deuterium-labeled alkaloid detected.

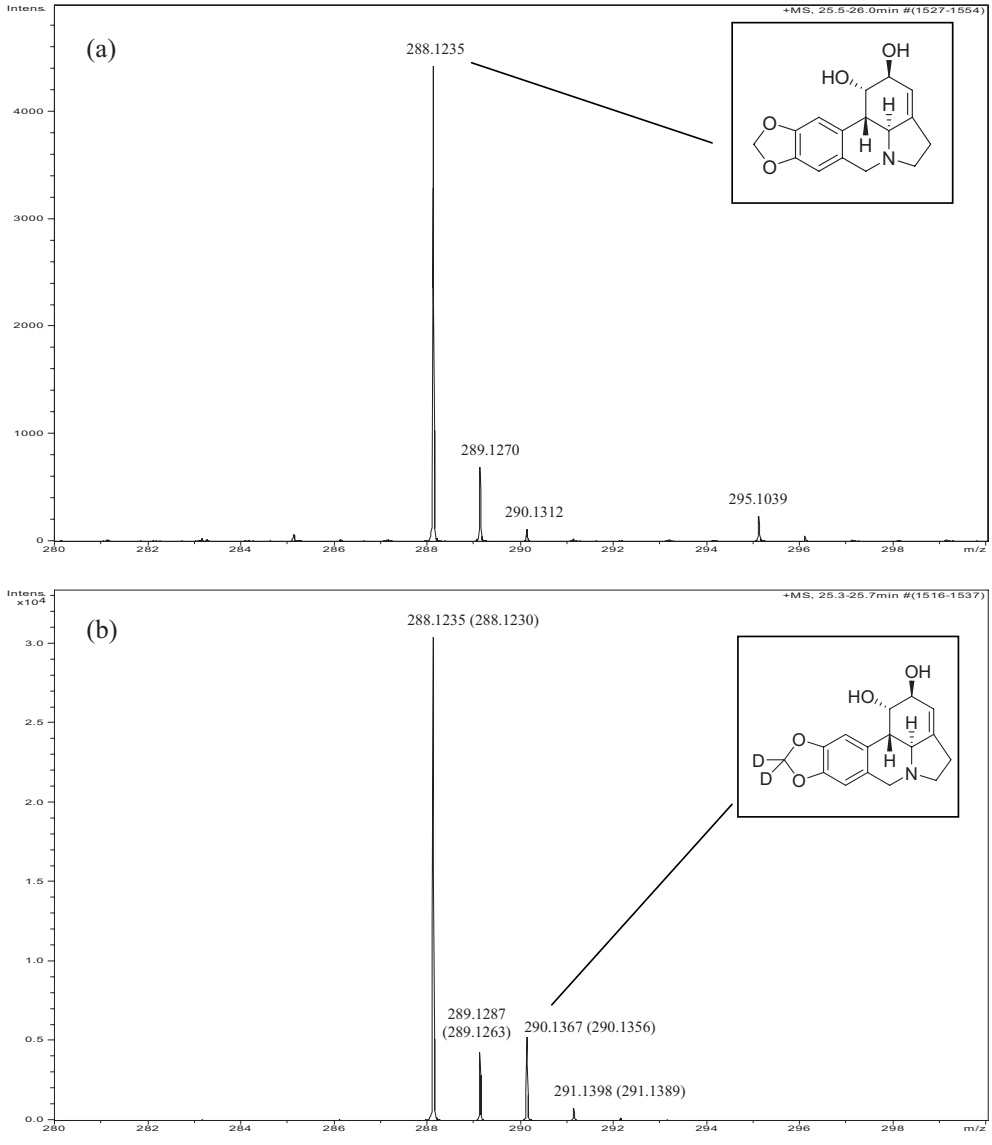

Figure 4. LC-MS mass spectra of lycorine.
Spectra were acquired in the positive-ion mode. **(a)** Mass spectrum of authentic lycorine; **(b)** mass spectrum of native and deuterated lycorine from an extract of shoot cultures of *Leucojum aestivum*. Error < 4 ppm in both cases, theoretical masses are given in parentheses.

Table 3. Alkaloids identified by GC-MS in shoot cultures of *Leucojum aestivum* and in liquid medium

Cultures were grown for 15, 30 and 40 days with 4′-O-methyl-d_3-norbelladine (D3MN) at various concentrations (0.05, 0.10, 0.20 g/L).

	D3MN	*N*-demethylNAR	DemethylGAL	GAL	NAR	*N*-formylGAL	LYC	AnhydroLYC	CRI	TRIS	DemethylMAR
Molecular mass and Formula	C16H19NO3, HCl	C16H17NO3	C16H19NO3	C17H21NO3	C17H19NO3	C17H19NO4	C16H17NO4	C16H13NO2	C16H17NO3	C14H9NO2	C16H19NO3
[M+]	-	271	273	287	285	301	287	251	271	223	273
[MD2+]	-	-	-	-	-	-	289	253	273	225	-
[MD3+]	312	274	276	290	288	304	-	-	-	-	276
15 Days											
Control shoot cultures	-	-	-	+	-	-	+	-	-	-	+
Culture Medium	-	-	-	+	-	+	+	-	+	-	+
Shoot cultures + 0.05 g/L D3MN	+	-	-	-	-	+	+	-	+	-	+, D3
Culture Medium	+	-	+	-	-	+	+	-	+	-	+, D3
Shoot cultures + 0.10 g/L D3MN	+	-	+, D3	+, D3	-	-	+	-	+, D2	-	+, D3
Culture Medium	+	D3	-	+, D3	-	-	+	-	+	-	+, D3
Shoot cultures + 0.20 g/L D3MN	+	-	-	+	-	+	+	-	-	-	-
Culture Medium	+	D3	-	+	-	-	+	-	-	-	-
30 Days											
Control shoot cultures	-	-	-	+	-	-	+	-	-	-	+
Culture Medium	-	-	-	+	-	+	+	-	+	-	+
Shoot cultures + 0.05 g/L D3MN	+	-	+, D3	+, D3	-	-	+	-	D2	-	+, D3
Culture Medium	+	-	+	+, D3	-	-	+	-	D2	-	D3
Shoot cultures + 0.10 g/L D3MN	+	-	+, D3	+, D3	-	-	+	-	+, D2	-	+, D3
Culture Medium	+	-	+, D3	+, D3	-	-	+	-	+, D2	-	+, D3
Shoot cultures + 0.20 g/L D3MN	+	-	+, D3	+, D3	-	+	+	-	+, D2	-	+, D3
Culture Medium	+	-	+, D3	+, D3	-	-	+	-	+, D2	-	D3
40 Days											
Control shoot cultures	-	-	-	+	-	-	+	-	-	-	+
Culture Medium	-	-	-	+	-	+	+	-	+	-	+
Shoot cultures + 0.05 g/L D3MN	+	-	+	+, D3	-	+	+	-	-	-	+ , D3
Culture Medium	+	D3	-	+, D3	-	-	+	-	-	-	-
Shoot cultures + 0.10 g/L D3MN	+	-	+, D3	+, D3	-	+	+	-	+, D2	-	+, D3
Culture Medium	+	D3	+, D3	+, D3	-	-	+, D2	-	+, D2	-	+, D3

D3MN: 4′-*O*-methyl-d_3-norbelladine; *N*-demethylNAR: *N*-demethylnarwedine; DemethylGAL: demethylgalanthamine; GAL: galanthamine; NAR: narwedine; *N*-FGAL: *N*-formylnorgalanthamine; LYC: lycorine; AnhydroLYC: anhydrolycorine; CRI: crinine; TRIS: trisphaeridine; DemethylMAR: demethylmarithidine; +: native alkaloid detected; –: no alkaloid detected; D3 or D2: deuterium-labeled alkaloid detected.

played $[M + H]^+$ at a m/z in accordance with the calculated values (288.1230 and 290.1356, respectively) (Fig. 4). In control shoot cultures, native galanthamine and lycorine that exhibited only the pseudo molecular ion characteristic of standard compounds were detected both in plant tissues and in liquid medium indicating that these alkaloids were able to diffuse in the culture medium. Isoquinoline alkaloids from *Papaver somniferum* tissue cultures were also found in liquid-medium culture (Le Flem-Bonhomme *et al.*, 2004). The excretion of a secondary metabolite in the medium is a characteristic leading to an improved productivity in bioreactor cultures (Bourgaud *et al.*, 2001). Regardless of the incubation duration and the concentration of precursor, the latter was detected both in shoot cultures and culture media indicating that it was transported into the plant tissue (Table 2). However, the uptake of this compound by plant tissue was not complete, as 4'-*O*-methyl-d_3-norbelladine was still detected in culture media after 15, 30 or 40 days of incubation.

Incorporation of 4′-*O*-methyl-d_3-norbelladine into both alkaloids was observed in different conditions tested. The labeled galanthamine and lycorine contained respectively 3 and 2 atoms of deuterium. Incorporation of two deuterium atoms in lycorine might result from an intramolecular cyclisation of the *ortho*-methoxyphenol yielding the methylenedioxy bridge. This is in accordance with what was previously proposed (Barton *et al.*, 1963) for haemanthamine, another Amaryllidaceae alkaloid, and for benzophenanthridine (Ikezawa *et al.*, 2007) or berberine-like alkaloids (Iwasa & Kim, 1997; Cui *et al.*, 2007). These results appear to constitute a new experimental proof of the proposed cyclisation mechanism.

Labeled galanthamine was observed after 15 days of incubation with 0.10 g/L of the precursor up to 40 days of incubation. Nevertheless, at this time 0.20 g/L concentration of precursor did not lead to galanthamine identification. On the other hand, labeled lycorine was observed only after 40 days of incubation with 0.20 g/L of this precursor. These interesting results demonstrate that the biosynthetic pathways of these two alkaloids are in competition, 4′-*O*-methylnorbelladine being the common precursor. Nevertheless, a domination of the *para-para'* oxidative coupling on the norbelladine, leading to marithidine and crinine derivatives, has been observed in various populations of *Galanthus elwesii* (Berkov *et al.*, 2004).

As for the native alkaloids, also deuterated galanthamine and lycorine diffused in the liquid culture medium.

GC-MS analysis

Capillary GC-MS was used in order to identify the various alkaloids, labeled or not, present in the complex fractions of *L. aestivum* shoot cultures. Derivatization was not required, since the Amaryllidaceae alkaloids retain their characteristic EI/MS fragmentation patterns when employing GC conditions, as reported by Kreh *et al.* (1995) and Tram *et al.* (2002). As the mass spectrometer coupled with GC works in low-resolution mode (see experimental part), some alkaloids show the same molecular mass, particularly galanthamine and lycorine [M^+ = 287], demethylnarwedine and crinine [M^+ = 271], and demethylgalanthamine and demethylmaritidine [M^+ = 273]. However, their fragmentation patterns and their retention times are different. The same GC-MS protocol as previously reported for *L. aestivum* alkaloids was used (Ptak *et al.*, 2009). Seven compounds showed MS fragmentation patterns characteristic of the Amaryllidaceae alkaloids (Table 3) (Fig. 5). The identification of these alkaloids was performed by comparing the measured data with previously published results (Ptak *et al.*, 2009) and with literature data (Berkov *et al.*, 2005). Similar retention times and similar fragmentation pattern permitted us to detect unambiguously deuterated alkaloids among native ones. This way, six labeled alkaloids were identified: d_3-demethylnarwedine, d_3-demethylgalanthamine, d_3-galanthamine, d_2-lycorine, d_2-crinine and d_3-demethylmarithidine (Fig. 5, Table 3). As was shown for lycorine, two deuterium atoms were incorporated in the crinine structure, bearing the same methylenedioxy bridge. These results show that 4′-*O*-methyl-d_3-norbelladine was incorporated into the three different groups of Amaryllidaceae alkaloids that are biosynthesized by three modes of phenol coupling on the common precursor (Fig. 1). All the labeled alkaloids were observed after 15 days of incubation with the labeled precursor except d_2-lycorine that was detected only after 40 days of incubation as shown above by the LC-MS analysis. Concerning the alkaloids biosynthesized by the *para-ortho'* oxidative coupling of 4′-*O*-methyl-norbelladine, labeled demethylnarwedine, demethylgalanthamine and galanthamine were detected while no labeled *N*-formylgalanthamine was observed. However, this alkaloid was detected in the extracts as a native compound. This could be due to a too short incubation time with the labeled precursor. Surprisingly, d_3-demethylnarwedine was detected only in the liquid culture medium while d_3-demethylgalanthamine was detected, initially, in the plant tissue only. All these results could be explained by the compartmentation in connection with enzymes that could be part of multienzyme complexes (Verpoorte *et al.*, 1999). It has been reported that compartmentation plays a major role in the regulation of secondary metabolite pathways. For example, the biosynthesis of terpenoid indole alkaloids requires at least three compartments, the plastids, the cytosol and the vacuole (Verpoorte *et al.*, 1997). The absence of native or labeled narwedine was noted. Concerning the alkaloids biosynthesized by the *para-para'* oxidative coupling, labeled crinine and demethylmarithidine were detected. Both alkaloids were first observed after 15 days of incubation with the labeled precursor respectively at 0.10 and 0.05 g/L. It is noteworthy that these labeled compounds appeared simultaneously with d_3-galanthamine suggesting that the competition between the three modes of phenol coupling on the common precursor *O*-methyl-norbella-

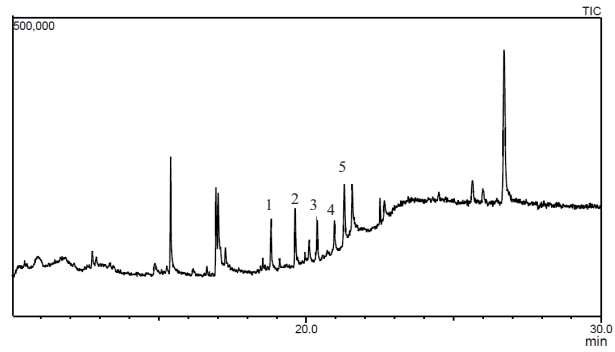

Figure 5. GC-MS chromatogram (total ion current) of the alkaloid fractions from shoot cultures of *Leucojum aestivum*.
Cultures were incubated 30 days with 0.10 g/L of 4′-*O*-methyl-d_3–norbelladine. Numbered peaks correspond to the following compounds: 1: galanthamine, 2: demethylmarithidine, 3: 4′-*O*-methyl-d_3-norbelladine, 4: crinine, 5: lycorine.

dine was in favour of the *para-ortho'* (galanthamine synthesis) and the *para-para'* (crinine and demethylmarithidine synthesis) oxidative couplings. No native nor labeled trisphaeridine was detected in the extracts. Further investigations with the labeled precursor are still required to better understand Amaryllidaceae alkaloid metabolism in tissue cultures of *L. aestivum*, in particular the flux rates between the biosynthetic intermediates. In conclusion, the common precursor 4′-*O*-methyl-d_3-norbelladine is biotransformed in shoot cultures of *L. aestivum* and gives labeled alkaloids previously reported in the literature but using ^{13}C or ^{14}C-labeled precursor.

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