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Kinetic Study of the Rearrangement of Deuterium-Labeled 4′-*O*-Methylnorbelladine in *Leucojum aestivum* Shoot Cultures by Mass Spectrometry. Influence of Precursor Feeding on Amaryllidaceae Alkaloid Accumulation

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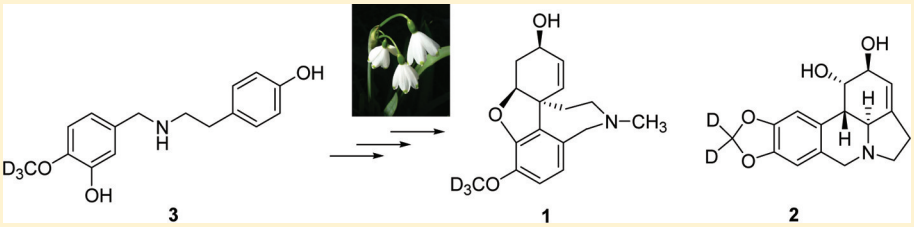
Supporting Information



ABSTRACT: Alkaloids from plants of the family Amaryllidaceae have important pharmacological properties and can be regarded as derivatives of the common precursor 4′-*O*-methylnorbelladine (**6**) via intramolecular oxidative phenol coupling. Their biosynthetic pathway, particularly in *Leucojum aestivum*, has not yet been totally elucidated. Therefore, shoot cultures of this plant were subcultured in medium containing the labeled precursor 4′-*O*-methyl-d_3-norbelladine (**3**) at various concentrations (0.05, 0.10, and 0.20 g/L) and were incubated for various periods of time (15, 30, and 40 days). The aim of this work was to study the influence of this precursor on both labeled and native alkaloid accumulation. Biotransformation into galanthamine (**1**) and lycorine (**2**) in shoot cultures was demonstrated using HPLC coupled to mass spectrometry. A maximal amount of 0.16% of **1** referred to the dry weight was obtained at day 15 in shoots fed with 0.10 g/L of precursor. In addition, a 20.5% dry weight of **2** was reached after 40 days of feeding with 0.20 g/L of precursor.

To date, over 300 alkaloids from various plants in the family Amaryllidaceae have been isolated,[1−3] and these are known to possess a wide variety of biological activities including antitumor, cytotoxic, antiviral, antinociceptive, anticholinergic, and anti-inflammatory effects. The alkaloid galanthamine (**1**) (Figure 1), for example, is used worldwide as an anticholinesterase drug for the treatment of Alzheimer's disease and is isolated currently from *Leucojum aestivum*. Lycorine (**2**) has shown antimalarial activities and may be isolated from a number of species of Amaryllidaceae such as *Pancratium maritimum*, *Narcissus* spp., and *L. aestivum*. A study of the biosynthetic pathway of **1** may provide clues to the feasibility of the biotechnological production of this alkaloid,[4] for which the total synthesis is low-yielding and complex.[5−7] In 1957, Barton and Cohen postulated that all alkaloids from the Amaryllidaceae could be regarded as derivatives of the common precursor norbelladine via intramolecular oxidative phenol coupling.[8] Feeding experiments using ^{14}C- or ^{3}H-labeled precursors like the norbelladine derivative caranine, and tyrosine and phenylalanine, have been reported in order to study the biosynthesis of **1** and **2** in *Narcissus pseudonarcissus*,[9] *Zephyranthes candida*,[10] and *Leucojum aestivum*.[4]

Deuterium, discovered in 1932, has been used with conspicuous success in research on enzyme-mediated reactions.[11] Deuterium labeling has its own particular advantages and, for example, is easily and abundantly used as a nonradioactive stable isotopic tracer incorporated into chemicals, biochemicals, and environmental studies. Its use can be revealed by mass spectrometric methods. Recently, we reported a novel procedure for the study of Amaryllidaceae alkaloid biosynthesis using the biotransformation of a deuterium-labeled precursor in *Leucojum aestivum* L. shoot cultures.[12] Altogether six labeled alkaloids were identified, indicating that 4′-*O*-methyl-d_3-norbelladine (**3**) is taken up by three different groups of Amaryllidaceae alkaloids. The deuterium labeling technique

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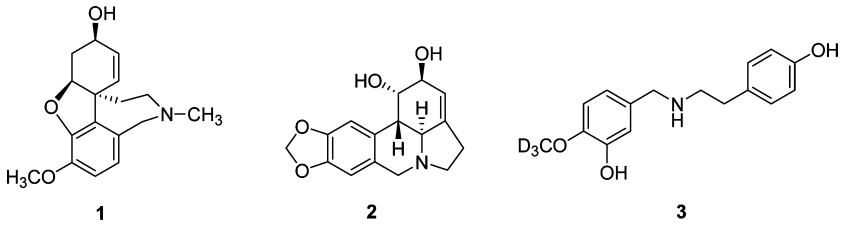

Figure 1. Structures of galanthamine (**1**), lycorine (**2**), and 4′-*O*-methyl-d_3-norbelladine (**3**).

confirmed a previously reported intramolecular rearrangement of this common precursor into these three types of alkaloids.

Eichhorn et al. showed that a ^{13}C-labeled precursor fed to *L. aestivum* shoot cultures influenced labeled alkaloid accumulation.[4] However, the present work has shown the influence of feeding with deuterium-labeled **3** not only on labeled alkaloid accumulation but also on the native alkaloids. This study was conducted through a kinetic approach, used for the first time, in the context of Amaryllidaceae alkaloids. LC-MS was used for the quantification of native and deuterated **1** and **2** both in shoot cultures and in the liquid medium.

RESULTS AND DISCUSSION

Synthesis of (−)-6-[$^2H_3$]Methoxygalanthamine (5). Authentic deuterated **1** was prepared in order to validate the method for quantitative determination of the labeled alkaloids biosynthesized in shoot cultures. This method of quantification of deuterated **1** could also be applied to the quantification of deuterated **2** (see Experimental Section).

The new compound **5** was prepared in one step from sanguinine[13] (**4**) by methylation with CD_3OD under Mitsunobu conditions (Figure 2).

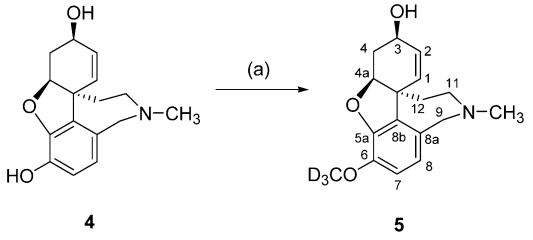

Figure 2. Synthesis of (−)-6-[$^2H_3$]methoxygalanthamine (**5**) from sanguinine (**4**). (a) Supported triphenylphosphine, CD_3OD, diisopropyl azodicarboxylate, THF, 18 h, 20 °C (39%).

Kinetic Growth of the Shoot Cultures. Control shoot cultures of *L. aestivum* were grown in Murashige and Skoog (MS)[14] liquid medium containing α-naphthalenacetic acid (10 μM) and benzylaminopurine (5 μM). In parallel, the labeled precursor **3** was added to experimental shoot cultures at various concentrations (0.05, 0.10, and 0.20 g/L). The shoot culture growth was evaluated after 15, 30, and 40 days of incubation with the labeled precursor. The growth rate of the control shoot cultures [(final shoot culture fresh weight − inoculum fresh weight)/inoculum fresh weight] was about 0.45 ± 0.19 after 30 days of culture and was much higher (up to 1 ± 0.15) after 40 days of culture. The growth rates of the treated shoot cultures were lower than those of the control cultures, especially at 15 days of culturing (Figure 3). It is worth noting that the growth rate values for the shoot cultures cultivated in the presence of 0.1 g/L of **3** were quite similar to the values of the control growth rate at 30 and 40 days of culture. In

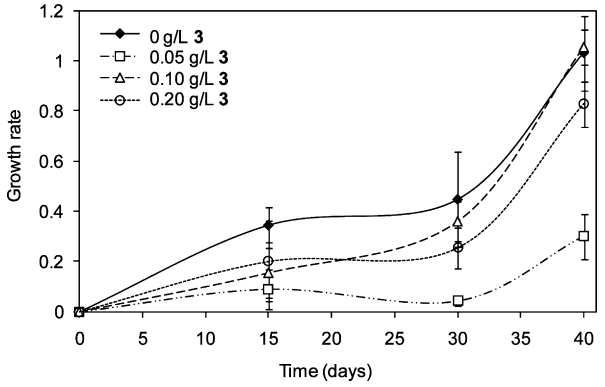

Figure 3. Time-course of *Leucojum aestivum* shoot culture growth in medium enriched with **3** at various concentrations (0, 0.05, 0.10, 0.20 g/L). The data represent the average of four replications with standard deviations.

addition, shoots incubated with 0.2 g/L of deuterated precursor led to a slow growth at 30 days (0.25), comparable to the control shoot growth at 40 days (0.83 ± 0.09). Surprisingly, shoot cultures grown in the presence of the lowest amount of deuterated precursor showed the slowest growth rate throughout the 40 days of incubation (0.09 ± 0.01 at 15 days to 0.3 ± 0.09 at 40 days). At 40 days of culture, the growth rate tended to be similar to the controls in all culture conditions. The kinetics of the production of deuterated and native **1** and **2** were then studied in order to establish a correlation between the shoot culture growth rates and the alkaloid accumulation.

Alkaloid Accumulations. After harvesting the shoot cultures grown for 15, 30, or 40 days in the MS liquid medium supplemented with or devoid of the labeled precursor, the native and deuterated alkaloids were extracted both from shoot cultures and from the liquid medium. To obtain quantitative data, a LC-MS quantification method was used to measure the amounts of native and deuterated **1** and **2**, as previously described.[15]

The incorporation of **3** was just monitored by comparing its amount remaining in the liquid medium to the amount present in the shoots. After the addition of 0.05, 0.1, and 0.2 g/L of labeled precursor, the content in the medium decreased from day 0 to day 15 (Figure 4a). After 30 days of feeding, only traces of the precursor were detected in the liquid medium. During the same period, the content of **3** increased in the shoots, with maximal absorption reached at day 15 (Figure 4b). In *Datura innoxia* hairy roots, Lanoue et al. have reported the absorption of phenylalanine within just five days.[16] These differences can be explained by a smaller growth rate of *L. aestivum* (0.45 after 30 days of culture) when compared with the growth rate of *D. innoxia* hairy root cultures.[17,18] Compound **3** efficiently diffused from the liquid medium into

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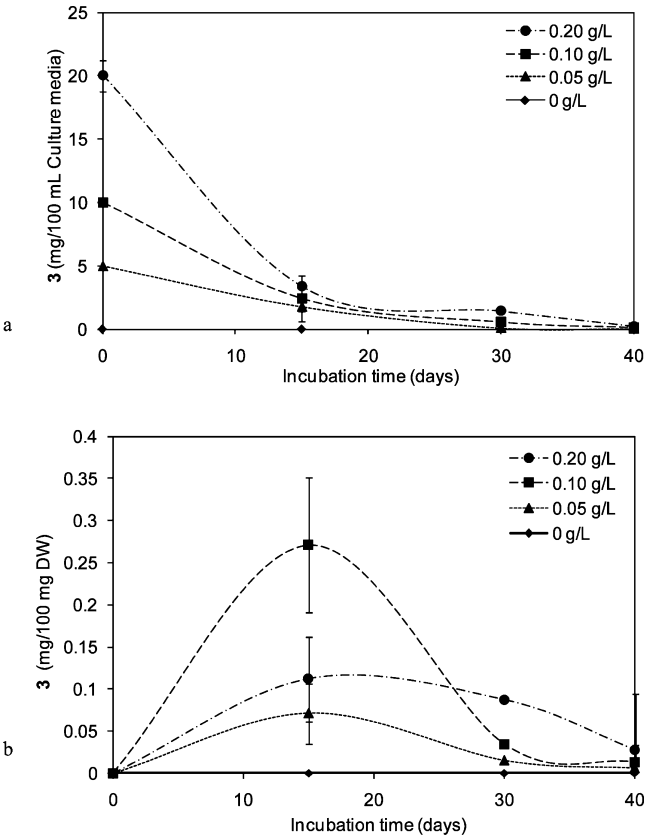

Figure 4. Profile of **3** (a) in the liquid medium and (b) in shoot cultures of *Leucojum aestivum* during feeding with various concentrations of deuterated precursor (0, 0.05, 0.10, 0.20 g/L).

L. aestivum shoot cultures within 15 days of feeding. From 15 to 30 days of feeding, the precursor content decreased in the shoots. The decrease of precursor content in both the liquid medium and in the shoots, after 15 days of feeding, suggested its metabolism to derivatives, namely, the labeled alkaloids. Surprisingly at day 15, the content of the precursor detected in the shoots fed with 0.1 g/L of **3** was two times higher than the content detected in the shoots fed with 0.2 g/L of this compound. This fact can be explained by metabolism occurring faster in the case of the 0.2 g/L concentration level of the precursor. El Tahchy et al. showed in a previous study that labeled precursor was incorporated in the alkaloid biosynthesis pathway and metabolized into three types of labeled Amaryllidaceae alkaloids.[12] Labeled **1** and **2**, crinine, *N*-demethylnarwedine, demethylgalanthamine, narwedine, *N*-formylgalanthamine, anhydrolycorine, trisphaeridine, and demethylmaritidine were found in shoot cultures of *L. aestivum* fed with the deuterated precursor.

Furthermore, the kinetics of the accumulation of both native and labeled **1** and **2** were measured over a 40-day period of the shoot culture fed with various concentrations of the labeled precursor (Figure 5).

Initially, native **1** and **2** accumulations were measured by LC-MS.[15] In control cultures, the accumulation of **1** was low both in the shoots (0.003% referred to the dry weight) and in the liquid medium (0.005 mg/100 mL), for the first 30 days of culturing. Ultimately, this reached 0.07% dry weight at day 40 in the shoots [Figure 5A(a)] and 0.12 mg/100 mL in the liquid medium [Figure 5A(b)], showing **1** to be released spontaneously from shoot cultures into the liquid medium. These results are in contrast with studies that have shown other alkaloid types such as tropane alkaloids to be poorly released into the culture medium.[19] It is worth noting that after the addition of 0.05 and 0.20 g/L of **3** only traces of native **1** were detected in the shoots (0.0003−0.003% dry weight) throughout the feeding period. However, feeding with **3** at 0.1 g/L induced a significant increase of the accumulation of native **1** both in the shoots (0.16% dry weight) and in the liquid medium (0.16 mg/100 mL) at day 15 (Figure 5A). The amounts of native **1** decreased after 15 days in the shoots and after 30 days in the liquid medium. These results showed that, at 0.1 g/L, **3** enhanced the native **1** accumulation in the shoot cultures, with the latter alkaloid then released into the liquid medium. The highest concentration of native **1** in the shoots was obtained at day 15; at that time the highest concentration of the deuterated precursor was observed in the shoots (Figure 4). These results suggest that the biotransformation of the natural precursor was enhanced after the addition of the deuterated precursor. From day 30 in the liquid medium, the concentration of **1** decreased, suggesting that this alkaloid is metabolized to other alkaloids or was decomposed. Native **2** was detected in control shoots (3.72 ± 1.9% dry weight), and only traces were detected in the liquid medium (Figure 5B). The synthesis of **2** in shoot cultures was enhanced by the addition of 0.05 or 0.10 g/L of **3** to the liquid medium. At day 15, a maximal accumulation of **2** was 9.4% and 18.7% dry weight in the shoots grown with 0.05 and 0.10 g/L of **3**, respectively. In the control, a trace amount of **2** was detected at this time. At day 30, the accumulation of **2** in the control shoots reached a constant level with an amount of 4% dry weight occurring between 30 and 40 days [Figure 5B(a)]. After day 15 in the presence of **3**, the accumulation of **2** started to decrease and reached, at day 30, a level similar to the controls (from 18% to 4% dry weight). At day 40, the accumulation of **2** increased again up to 16% dry weight and 19.8% dry weight in the presence of 0.10 and 0.20 g/L of **3**, respectively. Accordingly, adding 0.05 or 0.10 g/L of the deuterated precursor not only enhanced the production of **2** but also induced its accumulation 15 days earlier than in the controls. In contrast to **1**, native **2** was poorly released into the culture medium [Figure 5B(b)]. In the liquid medium treated with 0.05 g/L of **3**, the highest amount of **2** (5 mg/100 mL) was observed at day 30, while the concentration of **2** was the lowest in shoot cultures. Therefore, these results demonstrate that **3** has a double effect on the accumulation of native **1** and **2**, by enhancing their biosynthesis with yields of 0.16 for **1** and up to 19 for **2**. Additionally, **3** led to earlier alkaloid accumulation and release when compared to the controls.

Deuterated **1** and **2** were also detected in the shoot culture and in liquid medium samples (Figure 6). Both 0.05 and 0.2 g/L concentrations of **3** showed no significant effects on the accumulation of deuterated **1**. The latter was detected starting from day 15 to day 40, and its accumulation reached a maximal amount (0.005% dry weight) at day 30 in the presence of 0.1 g/L of **3** [Figure 6A(a)]. This maximum appeared 15 days later than the maximum of native **1** observed under the same conditions [Figure 5A(a)]. A correlation between the total consumption of the precursor at day 30 (Figure 4), the decrease of the content of native **1**, and the occurrence of deuterated **1** was evident. The uptake of **3** by the plant enabled the shoots to first use their natural 4′-*O*-methylnorbelladine (**6**) and to transform it into native **1** during the first 15 days. Thus, native **6** was not detected on day 15 in both the shoots and liquid medium, proving its metabolism into native alkaloids.

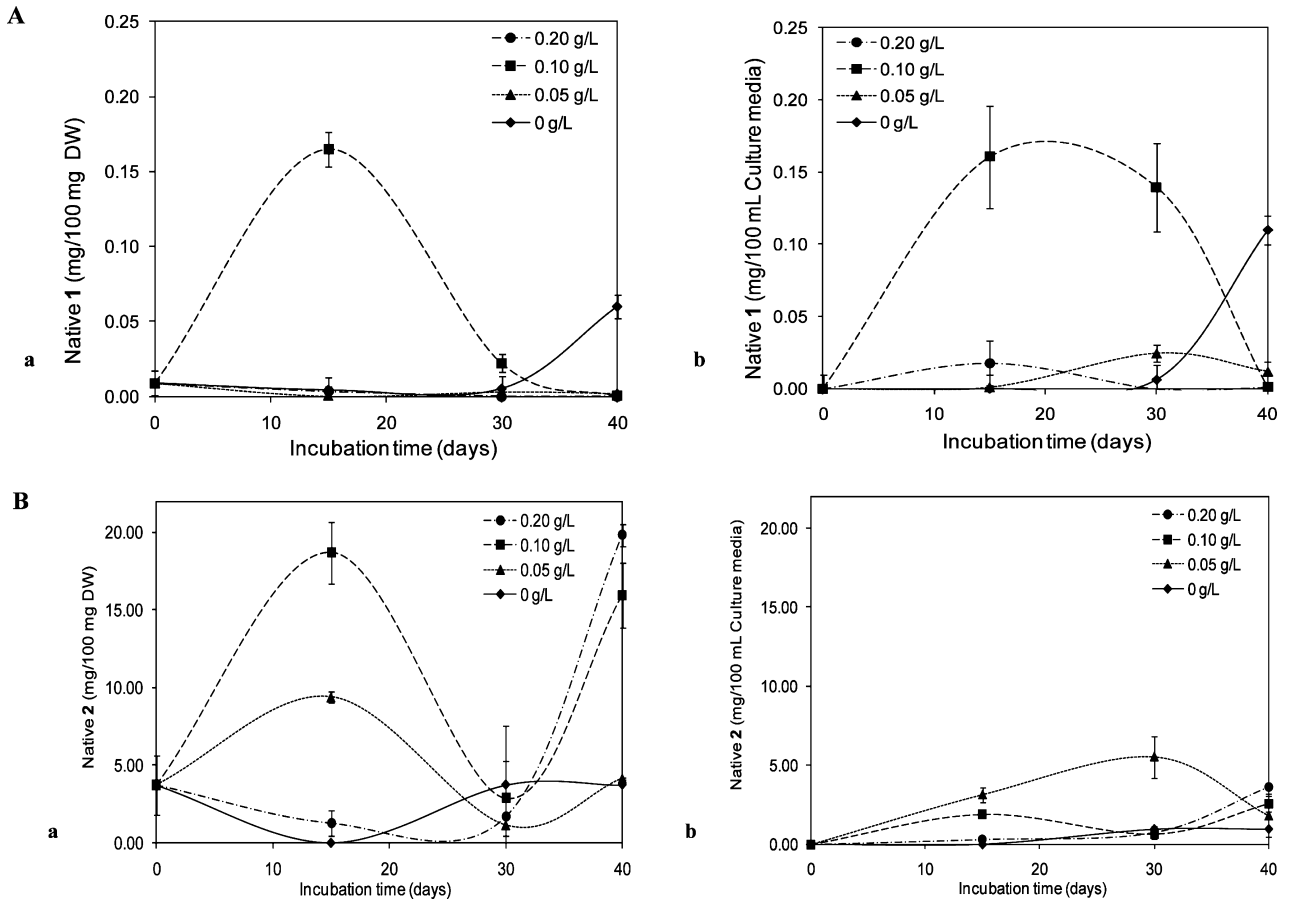

Figure 5. Profiles of native **1** (A) and **2** (B) contained (a) in shoot cultures and (b) in culture medium during feeding with various concentrations of **3** (0, 0.05, 0.10, 0.20 g/L).

From 15 to 30 days of feeding, when the uptake of **3** was almost completed and the native **6** was totally transformed into native alkaloids, deuterated **1** was then biosynthesized from the incorporated precursor. In the same way, it appeared in the liquid medium at day 15 and reached a maximal level at day 30, with 0.075 mg/100 mL and 0.017 mg/100 mL in the presence of 0.10 and 0.05 g/L of **3**, respectively [Figure 6A(b)]. These results showed that deuterated **1** was directly released into the medium after its biosynthesis in shoot cultures. Surprisingly, the highest amount of precursor afforded the lowest amount of deuterated **1**. Compound **2** was deuterated with two atoms of deuterium.[12] A low accumulation of deuterated **2** was observed in the shoots after feeding with 0.10 g/L of **3**, with two maxima of concentrations observed at day 15 (0.05% dry weight) and at day 40 (0.14% dry weight). In shoots fed with 0.20 g/L of **3**, an enhancement leading to the highest accumulation (0.50% dry weight) of deuterated **2** was observed at day 40. At that time, the biosynthesis of deuterated **2** in shoot cultures was followed by its release into the liquid medium, in which 0.32 mg/100 mL of deuterated **2** was observed. Feeding shoot cultures with 0.2 g/L of **3** did not lead to deuterated **1**, suggesting that the precursor at the highest concentration used could only furnish deuterated **2** in the present investigation. After 40 days, the stationary phase and the maximal amounts of deuterated **2** were not reached. After this period of culturing, shoot necrosis was observed, which could be explained by the continuous high release of native alkaloids in the culture medium.

In conclusion, combining the total production of both native and deuterated alkaloids, a significant elicitation effect of the precursor **3** on the accumulation of both **1** and **2** was observed. Feeding *L. aestivum* shoots with a common alkaloid precursor stimulated alkaloid biosynthesis using first the natural precursor and then the deuterated one. Therefore, the deuterium labeling of the precursor combined with the kinetic study of deuterated and native alkaloid accumulation enabled metabolism and elicitation to be distinguished. Previous studies have reported several optimizing experiments for the accumulation of **1** in *N. confusus* and *L. aestivum* shoot cultures; however higher amounts of these alkaloids were not obtained.[20−25] In the present study, the maximal amount of 0.16% dry weight of **1** was obtained at day 15 in shoots fed with 0.10 g/L of **3**. In addition, a very high concentration of **2** (20.5% dry weight) was reached after 40 days of feeding with 0.20 g/L of **3**. These results highlight enhanced alkaloid release in large amounts in the culture medium after feeding with the precursor. Accordingly, this technique can be considered for the harvesting of both these high-value-added alkaloids from the culture medium on a commercial scale.

EXPERIMENTAL SECTION

General Experimental Procedures. The melting point was measured in a capillary tube and is uncorrected. The infrared spectrum was recorded on a Fourier Perkin-Elmer Spectrum BX FT-IR. The ^{1}H NMR spectrum was recorded on a Bruker Avance-500 NMR spectrometer and is reported in parts per million (δ) relative to residual solvent peak. The ^{13}C NMR spectrum was recorded on a Bruker Avance-300 NMR spectrometer. ESIMS and HRESIMS were obtained on an ESI TOF Thermoquest AQA Navigator spectrometer.

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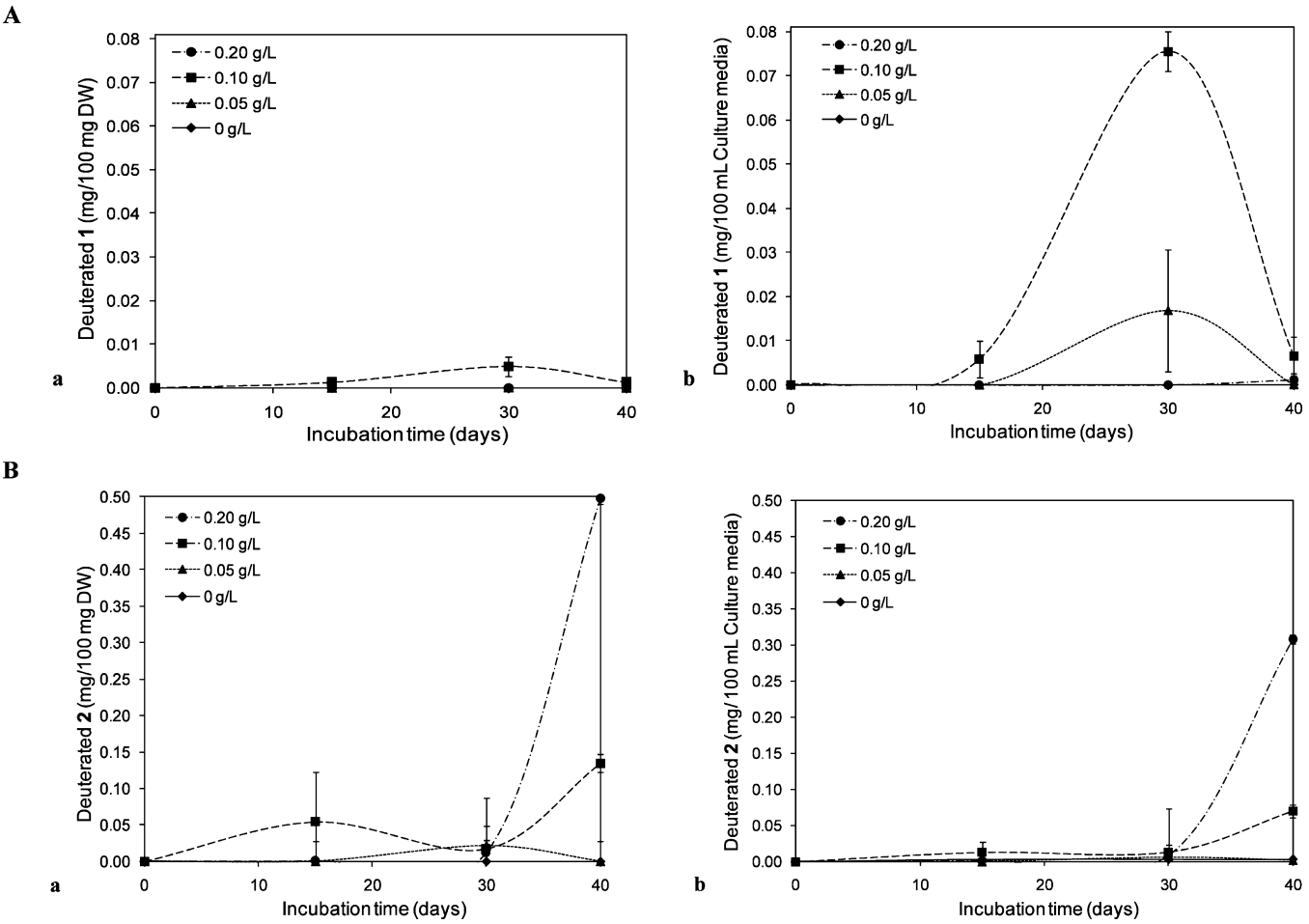

Figure 6. Profiles of deuterated **1** (A) and deuterated **2** (B) contained (a) in shoot cultures and (b) in culture medium during feeding with various concentrations of **3** (0, 0.05, 0.10, 0.20 g/L).

For LC-MS, HRESIMS were obtained on a micrOTOF$_Q$ (Bruker Daltonics) apparatus. Reactions were monitored by thin-layer chromatography on Merck silica gel plates ($60F_{254}$) with a fluorescent indicator. Separations were carried out under flash chromatographic conditions on a prepacked Redi Sep (230−400 mesh) silica gel column at medium pressure (20 psi) using a CombiFlash Companion apparatus.

The synthesis of **5** was carried out under argon with dry solvent. All commercially available reagents were used without further purification. THF, CD_3OD, diisopropyl azodicarboxylate, supported triphenylphosphine, triethylammonium acetate buffer, **1**, **2**, 6-benzylaminopurine, inositol, agar−agar, magnesium sulfate heptahydrate (98%), manganese sulfate, potassium nitrate, and ethylenediaminetetraacetic acid iron(III) sodium salt were purchased from Sigma Aldrich Chemie GmbH (Stennheim, Germany). Ammonium bicarbonate, iron sulfate, zinc sulfate, copper(II) sulfate pentahydrate (99%), thiamine hydrochloride, 2,4-dichlorophenoxyacetic acid, 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 (SDS).

Plant Material. Scales obtained from *Leucojum aestivum* bulbs (purchased at French local markets in September 2009) were chilled for 12 weeks at 5 °C and 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 scales were cut into thin slices (about 2−3 mm in length) and plated in culture. Professor Max Henry (UHP, Nancy, France) identified the plant, and a voucher specimen (NCY013687) is deposited at the herbarium of the Jardin Botanique, Nancy, France.

Shoot Cultures. Initial explants were cultivated on Murashige and Skoog (MS) medium[14] supplemented with auxin-α-naphthalene acetic acid (10 μM) and cytokinin-benzylaminopurine (5 μM) (control). The medium was supplemented with 3% sucrose, adjusted to pH 5.5 before autoclaving. The cultures were maintained at 25 ± 2 °C in the dark and subcultured every 4 weeks. Twelve-month-old shoot cultures (2 g) were submerged in a new liquid-shake medium (20 mL) containing the labeled precursor **3** at various concentrations (0.05, 0.10, and 0.20 g/L) and incubated for various periods of time (15, 30, and 40 days). A stock solution for feeding experiments was prepared from 50 mg of **3** solubilized in 10 mL of H_2O. Solutions at appropriate concentrations of the labeled precursor were filter-sterilized directly into the flasks.

Synthesis of (−)-6-[$^2H_3$]methoxygalanthamine (5). To a solution of sanguinine[13] (53.8 mg; 0.20 mmol) in anhydrous tetrahydrofuran (4 mL) were added supported triphenylphosphine (155.1 mg; 0.59 mmol), CD_3OD (100 μL; 2.47 mmol), and diisopropyl azodicarboxylate dropwise (120 μL; 0.57 mmol). The reaction mixture was stirred at 20 °C for 18 h under an inert atmosphere and then filtered. The insoluble material was washed successively with tetrahydrofuran (10 mL), methanol (50 mL), and a 1/1 mixture of dichloromethane−methanol (5 mL). The solvents were removed in vacuo. Silica gel flash-column chromatography (CH_2Cl_2−MeOH, 90/10) afforded **5** as a white solid (22.2 mg, 39%).

(−)-6-[$^2H_3$]Methoxygalanthamine (5): mp 127 °C; IR (neat) ν_{max} 3252, 1620, 1506, 1283, 1051 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.66 (1H, d, J = 8.0 Hz, H-7), 6.62 (1H, d, J = 8.0 Hz, H-8),

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6.07 (1H, d, J = 10,3 Hz, H-1), 6.00 (1H, dd, J_1 = 10.3 Hz, J_2 = 4.7 Hz, H-2), 4.61 (1H, brs, H-4a), 4.14 (1H, brt, J = 5.0 Hz, H-3), 4.08 (1H, d, J = 15.0 Hz, H-9α), 3.68 (1H, d, J = 15.5 Hz, H-9β), 3.27 (1H, dt, J_1 = 14.6 Hz, J_2 = 12.0 Hz, H-11α), 3.05 (1H, dm, J = 15 Hz, H-11β), 2.68 (1H, dm, J = 15.5 Hz, H-4α), 2.40 (3H, s, NCH_3), 2.09 (1H, ddd, J_1 = 13.5 Hz, J_2 = 13.0 Hz, J_3 = 2.5 Hz, H-4β), 2.01 (1H, dm, J = 15.5 Hz, H-12α), 1.58 (1H, ddd, J_1 = 14.0 Hz, J_2 = 2.0 Hz, J_3 = 2.0 Hz, H-12β); ^{13}C NMR (75 MHz, $CDCl_3$) δ 145.9 (C-6), 144.1 (C-5a), 133.0 (C-8b), 129.3 (C-8a), 127.6 (C-2), 126.9 (C-1), 122.0 (C-8), 111.2 (C-7), 88.7 (C-4a), 62.1 (C-3), 60.6 (C-9), 53.8 (C-11), 48.2 (C-4b), 42.1 (NCH_3), 33.8 (C-12), 29.9 (C-4); ESIMS m/z 291 $[M + H]^+$, 313 $[M + Na]^+$; HRESIMS m/z 291.1777 (calcd for $C_{17}H_{19}D_3NO_3$ $[M + H]^+$, 291.1788).

Alkaloid Extraction. Shoots were rinsed three times in new liquid culture medium before the alkaloid extraction step, in order to eliminate any precursor trace that could be fixed on their external membrane. The plant material was lyophilized and powdered, with 150 mg of powder macerated in methanol (10 mL) for 24 h, with sonication for 90 min in an ultrasonic bath (Transsonic 460/H Elma) at room temperature.[26] 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.

LC-MS Analysis. The LC system consisted of a U3000-Dionex, an injector with a 1 μL loop, and a UV detector at 280 nm. The analytical column used was an Acclain Pepmap C_{18} 1 mm i.d. column (150 mm × 3 μm × 100 μm), 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 ammonium bicarbonate pH 7.8 with 2.5% methanol, and solvent B consisted of 97.5% methanol and 2.5% 10 mM ammonium bicarbonate pH 7.8.

A HPLC coupled with high-resolution mass spectrometry was used in order to confirm the identity of **2** (22.4 ± 0.2 min) and **1** (33 ± 0.2 min) in sample extracts. This system was able to detect the compounds in amounts lower than 0.02 μg/mL. As shown in Figures S1 and S2 (Supporting Information), **1** and **2** displayed $[M + H]^+$ at m/z of 288.1609 and 288.1235, respectively.

Quantitative analytical procedures of native alkaloids were performed after a calibration method with standards of native **1** and **2**. In turn, quantitative analysis of deuterated compounds was carried out by measuring the intensity of their extracted ion chromatogram (EIC). To validate the method for quantitative determination, calibration graphs were prepared with the authentic deuterated **1**, i.e., compound **5** [(−)-6-[$^2H_3$]methoxygalanthamine]. The adducts and fragments of both standards of **1** and **5** had the same intensity ratio in each individual scan of the LC-MS measurements. Therefore, the EIC of native **1** could be used to estimate the amounts of the deuterated molecule **5** in plant extracts. The latter displayed a $[MD3 + H]^+$ peak at m/z 291.1788 (Figure S1, Supporting Information). Thus, quantitative measurements of deuterated **2** were also performed by reference to native **2** calibration graphs. In addition, the ion ($[MD2 + H]^+$) appeared in the mass spectrum of native **2** at an intensity of 2% (Figure S2, Supporting Information). Quantitative measurements of deuterated **2** in extracts were calculated after the subtraction of 2% from the theoretical concentration of the $[MD2 + H]^+$ ion, as provided by the LC-MS method.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Mass spectra of native and deuterated **1** and **2**. This information is available free of charge via the Internet at http://pubs.acs.org.

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