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BIOSYNTHESIS OF THE AMARYLLIDACEAE ALKALOID GALANTHAMINE‡

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Key Word Index—*Leucojum aestivum*; Amaryllidaceae; biosynthesis; alkaloids; galanthamine; *N*-demethylgalanthamine.

Abstract—Application of radioactively as well as ^{13}C-labelled 4′-*O*-methylnorbelladine to organs of field grown *Leucojum aestivum* plants resulted in an optimal incorporation into galanthamine (27%), and *N*-demethylgalanthamine (31%), respectively. In contrast to expectations based on results from the literature, the *N*-methylated 4′-*O*-methylnorbelladine was metabolized into a minor extent in *L. aestivum* and was incorporated into galanthamine as well as into *N*-demethylgalanthamine to only about 1/3 of the rate of 4′-*O*-methylnorbelladine. Furthermore, it was shown that *N*-demethylgalanthamine is *N*-methylated to galanthamine in the final step of biosynthesis. A revised scheme for the biosynthesis of galanthamine is presented involving the phenol oxidative coupling of 4′-*O*-methylnorbelladine to a postulated dienone which undergoes spontaneous closure of the ether bridge to yield *N*-demethylnarwedine which upon stereoselective reduction and *N*-methylation yields the cholineesterase inhibitor galanthamine. © 1998 Elsevier Science Ltd. All right reserved

INTRODUCTION

The Amaryllidaceae alkaloid galanthamine is a dibenzofuran-type base that has been isolated from a number of species of this family [1]. The pharmacological potency of this substance was reported for instance by Irwin and Smith [2] who demonstrated the anticholineesterase activity of galanthamine. Furthermore, Dal-Bianco *et al*. [3] showed positive effects of the alkaloid for the treatment of Alzheimer's disease patients. Galanthamine raises the acetylcholine concentration in brain areas lacking cholinergic neurones, so that a future use as a palliative therapy of Alzheimer's disease is conceivable. In Eastern Europe, galanthamine is used as a reversal agent in anaesthetic practice [4] depending on its property to neutralize the neuromuscular blockade induced by tubocurarine [5, 6]. Besides this, galanthamine acts as a mild analeptic [7]; shows analgesic power as strong as morphine [8]; applied in eye drops, it reduces the intraoccular pressure [9] and it has been used for the treatment of several neurological disorders [4]. For medical application, galanthamine is isolated from plant material, especially from *Leucojum aestivum* L. [*e.g*. 10] since the total chemical synthesis of galanthamine on an industrial scale is presently not economical. The most difficult step in a biomimetic synthesis is the regiocontrolled intramolecular oxidative *para–ortho′* coupling of the phenolic rings which requires blocking groups to prevent the more facile *para–para′* coupling. The *para–ortho′* coupling can be achieved for example by using bromoamides as substrates [11]. Different oxidizing reagents such as potassium ferricyanide [11], manganese(III) acetylacetonate [12] or vanadium oxychloride [13] have been tested to effect the phenol-coupling reaction, the yields obtained, however, were generally low.

The biosynthesis of galanthamine is not only of scientific interest, but could also provide clues to the feasibility of a biotechnological production of this alkaloid. The knowledge of the biosynthetic pathway together with the present repertoire of gene technology should make it possible to clone and express the rate-limiting enzyme(s) responsible, for instance, for the phenol oxidative coupling reaction. In 1957, Barton and Cohen [14] postulated that all Amaryllidaceae alkaloids could be regarded as derivatives of the common precursor norbelladine via intra-

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‡ Dedicated to Prof. T. Hartmann, Braunschweig, on the occasion of his 60th birthday.

Abbreviations—COMT = Catechol-*O*-methyltransferase.

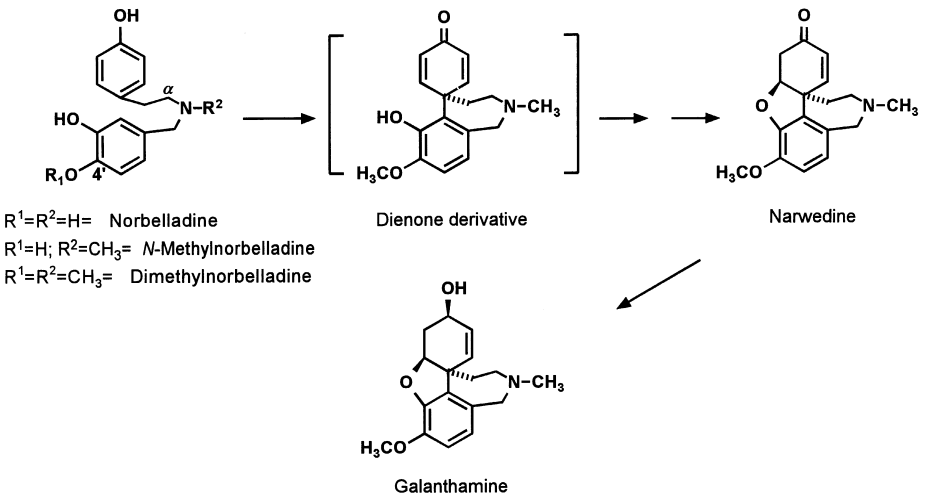

Fig 1. Previously postulated biosynthesis of galanthamine [15, 16].

molecular oxidative phenol-coupling. Feeding experiments using α-^{14}C-labelled norbelladine derivatives as precursors to study galanthamine biosynthesis in *Narcissus pseudonarcissus* cv. King Alfred, established experimentally norbelladine as the correct biogenetic precursor and led to the following biosynthetic sequence (Fig. 1) [15, 16]. During the process of phenol-coupling, a dienone was postulated as the first intermediate. Subsequently, the ether bridge of the galanthamine molecule is formed by an unknown mechanism. The enone alkaloid narwedine was postulated to be the direct precursor of galanthamine. Indeed, Bhandarkar and Kirby observed in 1970 an incorporation of [^{3}H]narwedine into galanthamine [17]. In addition, Fuganti reported in 1969 an incorporation of 4′-*O*-methylnorbelladine into galanthamine in *L. aestivum* [18].

The aim of our contribution was a closer examination of the postulated routes leading to galanthamine by the application of radioactive and heavy isotope-labelled potential precursors to parts of *L. aestivum* plants. A chemical model experiment is also presented, using hypervalent iodine reagents for the phenol-coupling reaction.

RESULTS

Synthesis of potential precursors

Using the reactions published by Battersby *et al.* [19], norbelladine HCl, 3′-*O*-methylnorbelladine HCl, 4′-*O*-methylnorbelladine HCl, *N*-methylnorbelladine HCl and 4′-*O*-methyl-*N*-methylnorbelladine HCl were synthesized and their identities confirmed by mp, MS and NMR spectroscopy. For the application experiments it was necessary to radiolabel these precursors with high specific activity. Already in 1963, Mann *et al.* [20] succeeded in the isolation of a catechol-*O*-methyltransferase from *Nerine bowdenii* bulbs that catalyzed the specific methylation of norbelladine at the *para* position to the alkyl substituent at a yield of more than 90%. This procedure seemed to be effective for the highly regiospecific introduction of a radiolabel into the precursor molecules.

Therefore, cell cultures of 17 species were screened for a similar enzyme that specifically methylates either norbelladine or its *N*-methyl derivative. However, the activity of all COMT enzymes detected was too low to be used for methylations at the μmole level. Subsequently, differentiated plants of six Amaryllidaceae species were investigated for their ability to methylate norbelladine. Leaves of *Clivia miniata* or *Leucojum vernum* proved to be the best source showing specific activities of 0.6 pkat mg^{-1} and 0.13 pkat mg^{-1} protein, respectively, with regiospecificities favouring the 4′ position over the 3′ position of 50:1 and 18:1, respectively.

After a simple purification step (DEAE or ACA 34), the *L. vernum* enzyme was used to label norbelladine in the presence of [C^{3}H$_3$]SAM (Fig. 2). Routinely, yields of 50% were obtained. By dilution analysis using synthetic unlabelled 4′-*O*-methylnorbelladine as well as 3′-*O*-methylnorbelladine as carrier, the labelled intermediate was unequivocally established to have the correct 4′-*O*-methyl configuration (Fig. 2). This highly radioactive (194 μCi μmol^{-1}) potential precursor was used for subsequent experiments.

The [OC^{3}H$_3$]3′-isomer was produced by catalysis of pig liver catechol-*O*-methyltransferase that favoured the 3′-*O*-position. Furthermore, 4′-*O*-methylnorbelladine ^{13}C-labelled at various positions was synthesized to check the intact incorporation of the precursors into the target molecules by NMR. [α-^{13}C]4′-*O*-Methylnorbelladine was produced from K^{13}CN and 4-benzyloxybenzyl chloride at an overall yield of 17% [16, 21]. [3,5-^{13}C$_2$]4′-*O*-Methylnorbelladine was obtained from L-[3,5-^{13}C$_2$]tyrosine that was decarboxylated by L-tyrosine decarboxylase to yield [3,5-^{13}C$_2$]tyramine which was in turned condensed with isovanillin at a yield of 70% [22].

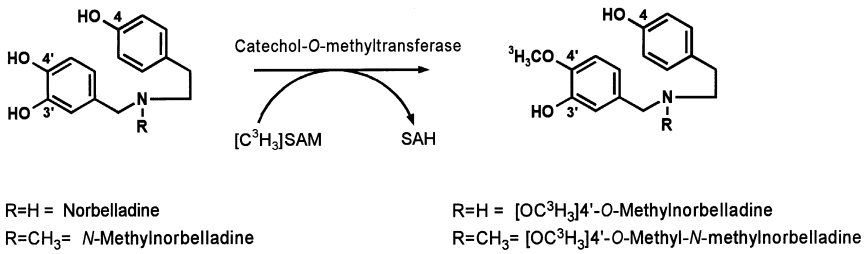

Fig 2. Enzymatic synthesis of radiolabelled norbelladine derivatives catalyzed by a regiospecific catechol-*O*-methyltransferase isolated from *Leucojum vernum* leaves.

Application of radiolabelled potential precursors

Administration of a labelled precursor to a given plant only gives unambiguous results if the substance penetrates to the site of synthesis and the enzyme systems are active at the time of application. In preliminary experiments, labelled 4′-*O*-methylnorbelladine and 4′-*O*-methyl-*N*-methylnorbelladine were administered in aqueous solution to adult flowering Amaryllidaceae species (*L. vernum*, *L. aestivum*, *Galanthus nivalis*, *Pancratium arabicum*) through the root system. After 48 h of application, the various organs were extracted, and the radioactivity of the extracts determined by scintillation counting as well as radioscanning after separation by TLC. Radioactivity was found exclusively in the roots, however, the precursors were not metabolized.

When the potential precursors were applied to isolated organs e.g. leaves of a flowering *G. nivalis* plant for 48 h, again no incorporation could be detected. In a further investigation, leaves were exposed to the radioactive precursor that was taken up by the transpiration stream. Subsequently, the leaves were sliced into 5 mm pieces which were macerated for 24 h in MeOH. Samples of the extracts were investigated by scintillation counting. The majority (87%) of the radioactivity was localized in that 5 mm piece at the bottom of the leaves that had been dipped into the application solution. Almost none of the radioactive precursor was transported further into the leaf tissue demonstrating that 4′-*O*-methylnorbelladine is not transported in this plant.

In order to support the precursor reaching the site of biosynthesis, the plant organ used was cut into 2 mm squares and floated on the substrate solution with agitation. After 48 h of shaking and subsequent extraction with hot MeOH–HCl, a satisfactory incorporation of [OC^3H_3]4′-*O*-methylnorbelladine into different alkaloids was observed. Experiments with *L. aestivum* and *G. nivalis* resulted in the desired incorporation into galanthamine in good yield. An autoradiogram of a methanolic extract obtained after feeding of [OC^3H_3]4′-*O*-methylnorbelladine to *L. aestivum* fruit walls for 48 h is shown in Fig. 3. The labelled compounds were purified, subjected to MS, compared with authentic material, and their structures identified to be *N*-demethylgalanthamine, *N*-demethylnarwedine, galanthamine, *N*-demethyl-7-dehydroxylycorenine, *O*-methyllycorenine, and 4′-*O*-methylnorbelladine. Similar experiments with various organs of flowering plants of the species *L. vernum*, *G. elwesii*, *P. arabicum*, *Chlidanthus fragrans*, *N. pseudonarcissus* cv. Golden Harvest and cv. King Alfred, all known to contain Amaryllidaceae alkaloids, resulted in no or extremely small incorporation. Therefore, *L. aestivum* was chosen as an experimental plant for further studies.

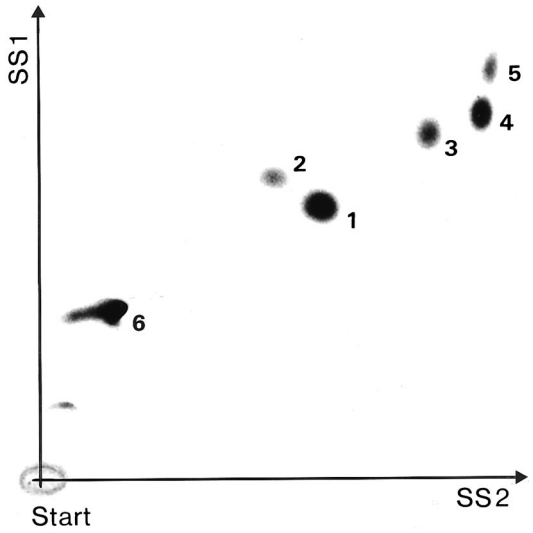

Fig 3. Autoradiogram of a methanolic extract obtained after feeding of [OC^3H_3]4′-*O*-methylnorbelladine to *Leucojum aestivum* fruit walls for 48 h. Compound **1**, *N*-demethylgalanthamine; **2**, *N*-demethylnarwedine; **3**, galanthamine; **4**, *N*-demethyl-7-dehydroxylycorenine; **5**, *O*-methyllycorenine; **6**, 4′-*O*-methylnorbelladine. Solvent 1, CH_2Cl_2–MeOH–NH_3 (90:9:1); solvent 2, $CHCl_3$–Me_2CO–Et_2NH (5:4:1).

To determine the plant organ with highest incorporation into galanthamine, [OC^3H_3]4′-*O*-methylnorbelladine was applied to various organs of flowering *L. aestivum* plants. In Table 1, the percentage of [OC^3H_3]4′-*O*-methylnorbelladine incorporation into galanthamine is presented. The best organs for this type of experiments were ovary walls and flower stalks showing the highest incorporation into galanthamine of 27 and 20%, respectively. These are the highest incorporation rates ever observed with an Amaryllidaceae plant. The other organs except petals and

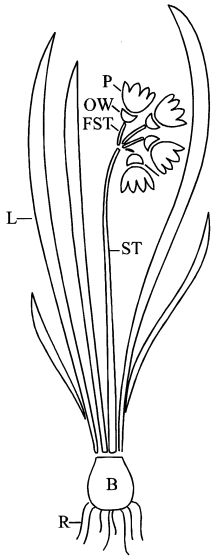

Table 1. Biotransformation of [OC^{3}H$_3$]4′-*O*-methylnorbelladine by different tissues of flowering *Leucojum aestivum*. Six main metabolites were isolated from *L. aestivum* and identified as galanthamine, *N*-demethylgalanthamine, *N*-demethylnarwedine, narwedine, *O*-methyllycorenine and *N*-demethyl-7-dehydroxylcorenine

Plant organ	Galanthamine (%)	*N*-Demethyl-galanthamine (%)	*N*-Demethyl-narwedine (%)	Narwedine (%)	*O*-Methyl-lycorenine (%)	*N*-Demethyl-7-dehydroxy-lycorenine (%)
Ovary wall (OW)	27.4	22.0	3.1	1.2	32.0	6.1
Flower stalk (FST)	19.8	31.4	1.4	0.3	1.8	0.7
Petal (P)	3.1	9.2	10.1	1.7	0	0.5
Stem (ST)	0	0	0	0	0	0
Leaf (L)	0	5.0	0	0	0	0
Bulb (B)	0	0	0	0	0	0
Root (R)	0	0	0	0	0	0

fruit walls did not show a comparable metabolism. The labelled alkaloids formed in the ovary walls after feeding of [OC^{3}H$_3$]4′-*O*-methylnorbelladine were identified by TLC, autoradiography, NMR and MS to be galanthamine (27% incorporation), *N*-demethylgalanthamine (22% incorporation), narwedine (1% incorporation) and *N*-demethylnarwedine (3% incorporation), all well labelled under these conditions. Parallel application experiments with [OC^{3}H$_3$]3′-*O*-methylnorbelladine showed absolutely no incorporation into any of these alkaloids, demonstrating an extremely high regiospecificity of the phenol-coupling enzyme which is in accordance with previous experiments [23].

According to the present biosynthetic hypothesis, [OC^{3}H$_3$]4′-*O*-methyl-*N*-methylnorbelladine was expected to be the closest precursor to galanthamine [15, 16]. However, in the application experiments presented here, comparing the incorporation of [OC^{3}H$_3$]4′-*O*-methyl-*N*-methylnorbelladine with that of the correspondingly labelled *N*-nor compound, only about 1/3 of the *N*-methyl derivative was incorporated into galanthamine. Surprisingly, in experiments using the labelled *N*-methyl derivative as a precursor, *N*-demethylgalanthamine (11% incorporation) and the demethylated 4′-*O*-methylnorbelladine (5% incorporation) could be detected. This clearly demonstrates that [OC^{3}H$_3$]4′-*O*-methyl-*N*-methylnorbelladine is at first *N*-demethylated to yield the *N*-nor intermediate which is then metabolized to galanthamine possibly via *N*-demethylnarwedine. Since both *N*-demethylnarwedine and *N*-demethylgalanthamine have been discovered heavily labelled in the previous experiments (Fig. 3; Table 1), obviously, *N*-demethylnarwedine is transformed *in vivo* first to *N*-demethylgalanthamine which then is *N*-methylated in the final biosynthetic step to yield the target compound galanthamine.

In order to prove this pathway, [OC^{3}H$_3$]4′-*O*-methylnorbelladine with high specific activity was fed to sliced ovary wall tissue. Subsequently, the potential intermediate *N*-demethylgalanthamine was isolated and purified and then fed again to ovary wall tissue. Figure 4 **A** demonstrates that *N*-demethyl-

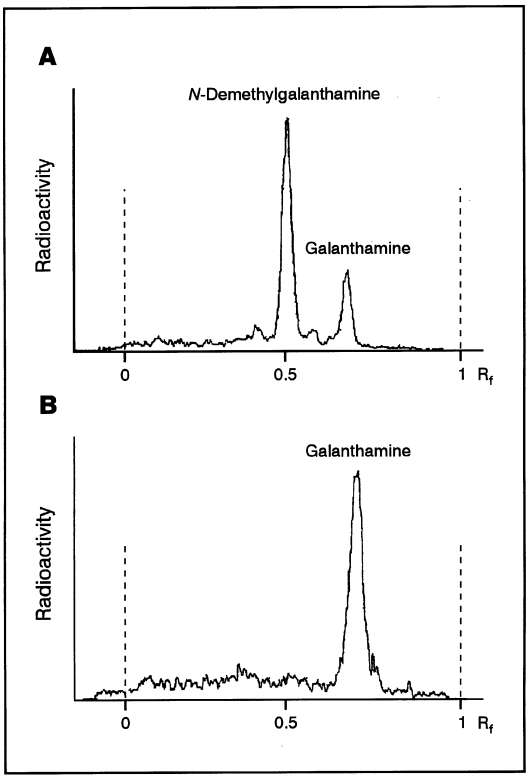

Fig 4. Radio-TLCs of methanolic extracts obtained after feeding biosynthetically radiolabelled *N*-demethylgalanthamine (**A**) and galanthamine (**B**) to *Leucojum aestivum* ovary wall tissue for 48 h. *N*-Demethylgalanthamine was *N*-methylated to yield galanthamine, while galanthamine was not demethylated.

galanthamine was clearly *N*-methylated to yield galanthamine (15% incorporation). The reciprocal experiment of feeding galanthamine to the ovary wall tissue gave no evidence for the demethylation of galanthamine (Fig. 4**B**).

Feeding experiments with ^{13}*C-labelled* 4′-O-*methylnorbelladine*

To confirm the intact incorporation of 4′-*O*-methylnorbelladine into the alkaloids under investigation, chemically synthesized ^{13}C-labelled potential precursor alkaloids were applied to sliced ovary walls of *L. aestivum* on large scale. The chosen precursor concentration was below 5×10^{-5} M to avoid the overloading of the cellular system and perturbing the normal metabolism. (Administration of concentrations higher than 5×10^{-5} M resulted in little or no incorporation into the target alkaloid!) After 48 h of incubation, the tissue was extracted and the crude alkaloids were obtained after acid-basic extraction with $CHCl_3$. The individual alkaloids were obtained by prep. TLC of the chloroform solution. ^{13}C NMR analysis (Fig. 5) showed clearly the intact incorporation of [3,5-$^{13}C_2$]4′-*O*-methylnorbelladine into galanthamine and [α-^{13}C]4′-*O*-methylnorbelladine into *N*-demethylgalanthamine. The rate of incorporation was determined by GC-MS to be 3 and 5% for galanthamine and *N*-demethylgalanthamine, respectively, thus verifying in principal the experiments with radioactively labelled precursors as presented above. The appreciably lower incorporation rate of heavy isotope-labelled precursors compared with the carrier free radioactive precursor of high specific activity is due to slight inhibition of galanthamine biosynthesis in the presence of an excess of precursor compounds, most likely by "feed forward" inhibition.

Generation of the ether bridge in galanthamine

The biochemical formation of the ether bridge in the galanthamine molecule was up to now obscure. Possibly, the transition of the hypothetical dienone (Fig. 1 or Fig. 7) to *N*-demethylnarwedine could either proceed analogously to the formation of salutaridinol-7-*O*-acetate to thebaine [24] or the ether bridge may be formed spontaneously.

This question should be answered with a chemical model system. Treating the norbelladine-derivative **7** (Fig. 6) with hypervalent iodoreagent phenyliodine(III)-bis(trifluoroacetate) (PIFA) at $-40°$ in trifluorethanol, two coupling products could be obtained in a ratio of 3.6 to 1 for the *para–para′*- (**8**) and *para–ortho′*-compound (**9**) at a total yield of 40%. Removal of the protecting group with *n*-tetrabutylammonium fluoride in THF yielded the expected *para–para′*-coupled dienone (**10**) and a narwedine derivative (**11**) in which the ether bridge was already closed [25]. The unprotected dienone of the *para–ortho′* coupling was unstable and spontaneously formed the ether bridge by Michael-type-cyclisation yielding the enone derivative (**11**).

This result is most likely an analogy to the *in vivo* galanthamine biosynthesis. The postulated dienone is generated by the enzyme catalyzed phenol-coupling reaction and spontaneously forms the ether bridge to yield *N*-demethylnarwedine. An enzymically generated *O*-acyl intermediate as in the case of thebaine formation [24] is not required.

Furthermore, with the help of a semi-empirical structure calculation program (AM1), the atomic distances in the postulated dienone (Fig. 7) between the oxygen atom of the phenolic hydroxyl group at C-14 and the carbon atom C-16 is 2.41 Å whereas the atomic distance between the nitrogen atom and the carbon atom C-4 was calculated to be 3.65 Å. Since the distance between the hydroxyl group and C-16 is much smaller than the distance between C-4 and the nitrogen, the nucleophilic attack by the oxygen atom is clearly preferred. This strengthens the postulated spontaneous formation of *N*-demethylnarwedine from the intermediate dienone which is generated

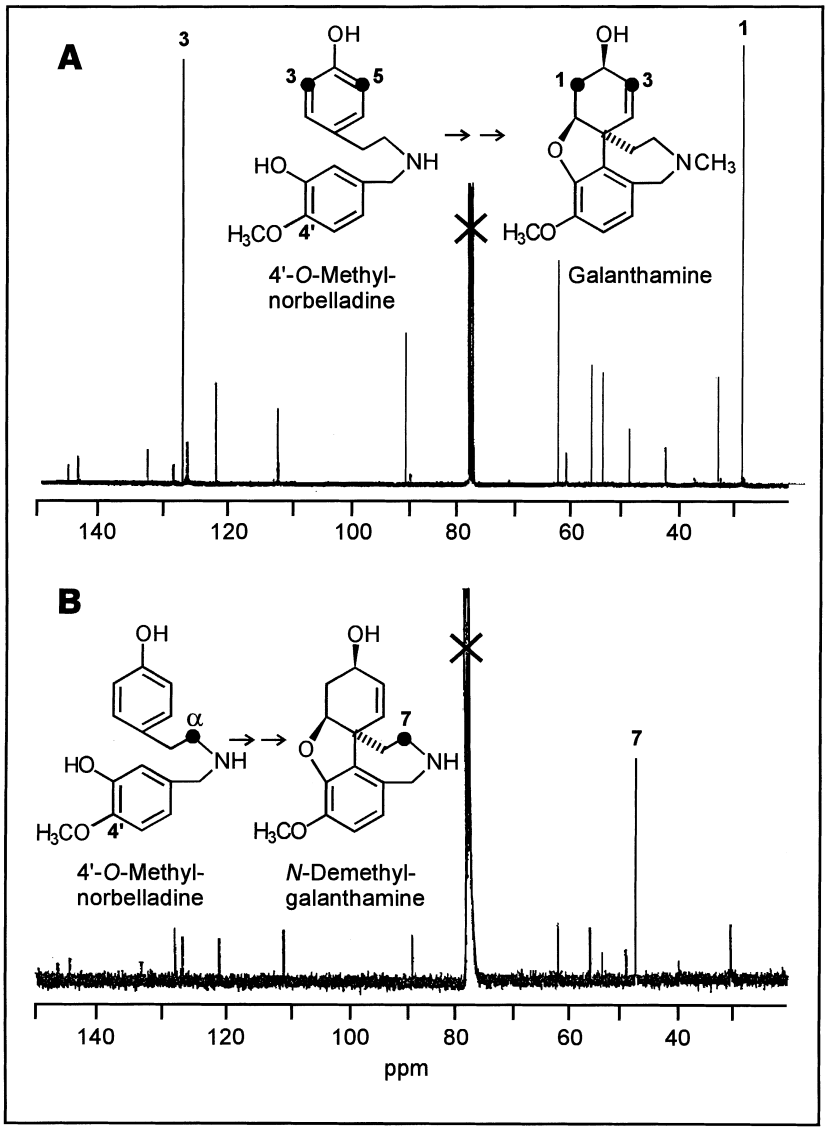

Fig 5. ^{13}C NMR analysis of galanthamine (**A**) and *N*-demethylgalanthamine (**B**) obtained after administering [3,5-$^{13}C_2$]4′-*O*-methylnorbelladine (**A**) and [α-^{13}C]4′-*O*-methylnorbelladine (**B**), respectively, to *Leucojum aestivum* ovary walls for 48 h. The ^{13}C-enrichment of the corresponding C-atoms in the phenol-coupled products demonstrates the intact incorporation of the precursor.

from norbelladine by action of a phenol-coupling enzyme.

DISCUSSION

The postulated biosynthesis of galanthamine published more than 30 years ago was based on rather low incorporation rates [16]. The results obtained here have confirmed these results only in part (Fig. 7). Clearly, 4′-*O*-methylnorbelladine is the universal precursor to Amaryllidaceae alkaloids and therefore also to galanthamine. Under optimized conditions using carrier free radioactively labelled precursor alkaloids, incorporation rates of up to 27% were obtained. The key step in the biosynthesis of galanthamine is the phenol oxidative *para–ortho′* coupling of 4′-*O*-methylnorbelladine, yielding a hypothetical dienone [14]. In the light of analogous reactions [26–28] it is very likely that this reaction might be catalyzed by a cytochrome P-450 enzyme. Three examples of phenol-coupling in alkaloid biosynthesis have so far been demonstrated to be catalyzed by highly specific cytochrome P-450 dependent oxidases without the introduction of oxygen into the final product [26–28]. In comparison to the galanthamine pathway, the intramolecular *para–ortho* coupling of (*R*)-reticuline to the dienone salutaridine in morphinan alkaloid biosynthesis [29] is quite similar to the reaction leading to galanthamine.

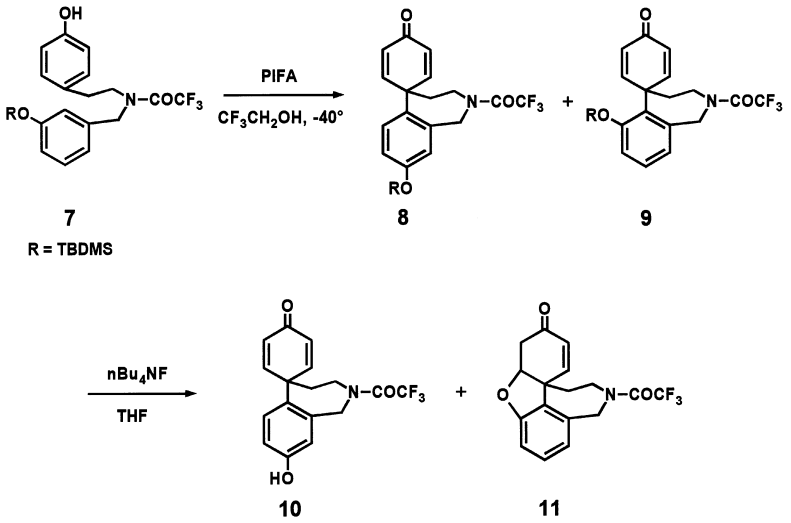

Fig 6. Oxidative phenol-coupling of norbelladine-derivative **7** with hypervalent iodine(III) reagent PIFA and subsequent deprotection of the phenolic hydroxy group. The unprotected dienone of the *para–ortho′* coupling product formed spontaneously the enone derivative **11**.

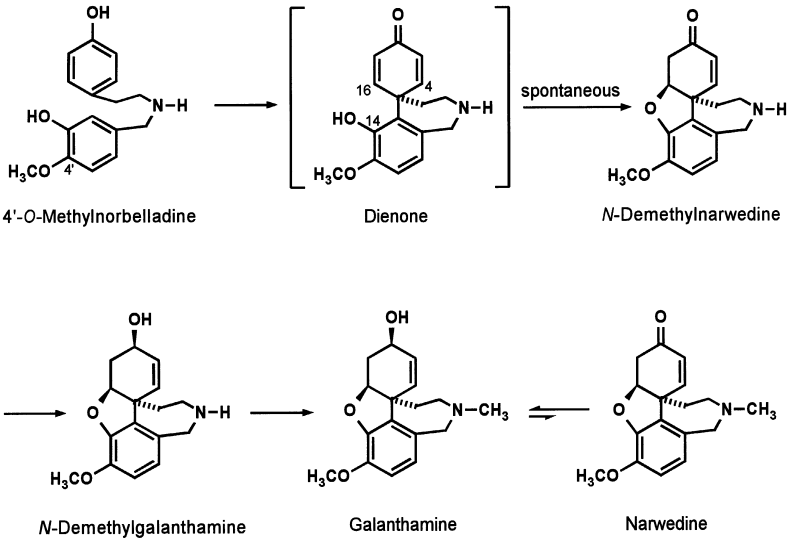

Fig 7. Postulated new pathway for galanthamine biosynthesis.

Furthermore, the *N*-methylated 4′-*O*-methylnorbelladine is also surprisingly incorporated into the *N*-demethylated intermediates of the galanthamine pathway allowing the conclusion that an early enzyme catalyzed *N*-demethylation of this precursor molecule may occur. It had been found already, that chlidanthine could be derived biosynthetically from galanthamine via methylation and demethylation [17] which would be in line with the observed phenomenon here. The main pathway, however, proceeds by phenol-coupling of 4′-*O*-methylnorbelladine to yield a dienone that is most likely unstable and rearranges spontaneously without enzyme catalysis forming the ether bridge to yield the alkaloid *N*-demethylnarwedine as illustrated also by chemical model experiments [25]. This alkaloid found to occur in *L. aestivum* must be subsequently enzymically and stereoselectively reduced to *N*-demethylgalanthamine and finally methylated to galanthamine as observed here by an incorporation of *N*-demethylgalanthamine into galanthamine *in vivo*. Narwedine, which is a natural product, is produced most likely by *N*-methylation of *N*-demethylnarwedine, possibly by the same type of enzyme system as galanthamine is formed from *N*-demethylgalanthamine. According to our experiments, narwedine is not the direct precursor of galanthamine. Narwedine could possibly exist in an equilibrium with galanthamine, a reaction catalyzed by a hypothetically reversible oxidoreductase. This might be one explanation for the observed incorporation of

narwedine into galanthamine [17]. On the basis of the experiments presented here a new pathway to galanthamine (Fig. 7) is proposed which deviates in several aspects from the previous assumptions (Fig. 1). The basis is now laid for enzymatic investigations on the biosynthesis of galanthamine which could be followed by the cloning of the genes encoding the individual enzymes and finally, a biotechnological process for galanthamine production might evolve as a result of this knowledge.

EXPERIMENTAL

General. All mps are uncorrected. NMR spectra were recorded at 360 MHz for ^{1}H or 90 MHz for ^{13}C, with TMS as int. standard. MS (CI) were measured at 120 eV, MS (EI) at 70 eV. Silica gel precoated plates were used for TLC (thickness: 0.25 mm). Relative protein values were determined according to ref. [30].

Plant material. Plant cell suspension cultures were provided by the cell culture laboratory of the Munich department. Bulbs of different Amaryllidaceae species were purchased and grown in pots in a greenhouse or outdoors.

Chemicals. The unlabelled precursors norbelladine HCl, 3′-*O*-methylnorbelladine HCl, 4′-*O*-methylnorbelladine HCl, *N*-methylnorbelladine HCl and 4′-*O*-methyl-*N*-methylnorbelladine HCl were synthesized according to ref. [19].

Norbelladine HCl. (yield 10.8%): mp 175–177°. ^{1}H NMR (360 MHz, CD_3OD): δ 6.84 (*d*, $J = 8.6$ Hz, 2H, H-3 and H-5), 6.71 (*s*, 1H, H-2′), 6.59 (*s*, 2H, H-5′ and H-6′), 6.52 (*d*, $J = 8.6$ Hz, 2H, H-2 and H-6), 3.80 (*s*, 2H, H-α′), 2.91 (*m*, 2H, H-α), 2.66 (*m*, 2H, H-*β*). ^{13}C NMR (90 MHz, CD_3OD): δ 157.6 (C-4), 147.8 (C-3′), 146.8 (C-4′), 130.8 (C-2 and C-6), 128.4 (C-1), 123.5 (C-1′), 122.9 (C-6′), 118.0 (C-5′), 116.7 (C-3 and C-5), 116.6 (C-2′), 52.2 (C-α′), 49.7 (C-α), 32.4 (C-*β*).

N-*Methylnorbelladine HCl*. (yield 7.9%): mp 208–210°. ^{1}H NMR (360 MHz, CD_3OD): δ 6.93 (*d*, $J = 8.6$ Hz, 2H, H-3 and H-5), 6.79 (*s*, 1H, H-2′), 6.70 (*d*, 2H, H-5′ and H-6′), 6.61 (*d*, $J = 8.6$ Hz, 2H, H-2 and H-6), 4.16 (*d*, $J = 13.1$ Hz, 1H, H-α′), 3.97 (*d*, $J = 13.1$ Hz, 1H, H-α′), 3.23–3.16 (*m*, 1H, H-α), 3.09–3.01 (*m*, 1H, H-α), 2.91–2.75 (*m*, 2H, H-*β*), 2.67 (*s*, 3H, NMe). ^{13}C NMR (90 MHz, CD_3OD): δ 157.8 (C-4), 148.4 (C-3′), 147.1 (C-4′), 130.8 (C-2 and C-6), 127.9 (C-1), 124.0 (C-1′), 121.6 (C-6′), 118.9 (C-5′), 116.7 (C-2′, C-3 and C-5), 61.0 (C-α′), 57.8 (C-α), 39.9 (NMe), 30.6 (C-*β*).

3′-O-*Methylnorbelladine HCl*. (yield 10.5%): mp 177–179°. ^{1}H NMR (360 MHz, CD_3OD): δ 6.95 (*d*, $J = 8.5$ Hz, 2H, H-3 and H-5), 6.76 (*m*, 3H, H-2′, H-5′ and H-6′), 6.62 (*d*, $J = 8.5$ Hz, 2H, H-2 and H-6), 3.79 (*s*, 2H, H-α′), 3.75 (*s*, 3H, OMe), 3.04 (*m*, 2H, H-α), 2.78 (*m*, 2H, H-*β*). ^{13}C NMR (90 MHz, CD_3OD): δ 157.7 (C-4), 149.4 (C-3′), 149.0 (C-4′), 130.8 (C-2 and C-6), 128.3 (C-1), 124.2 (C-1′), 123.5 (C-6′), 116.7 (C-3 and C-5), 116.6 (C-5′), 114.4 (C-2′), 56.5 (OMe), 52.3 (C-α′), 49.8 (C-α), 32.4 (C-*β*).

4′-O-*Methylnorbelladine HCl*. (yield 10.6%): mp 200–202°. ^{1}H NMR (360 MHz, CD_3OD): δ 6.91 (*d*, $J = 8.5$ Hz, 2H, H-3 and H-5), 6.79 (*m*, 3H, H-2′, H-5′ and H-6′), 6.59 (*d*, $J = 8.5$ Hz, 2H, H-2 and H-6), 3.91 (*s*, 2H, H-α′), 3.69 (*s*, 3H, OMe), 2.99 (*m*, 2H, H-α′), 2.74 (*m*, 2H, H-*β*). ^{13}C NMR (90 MHz, CD_3OD): δ 157.7 (C-4), 150.1 (C-4′), 148.1 (C-3′), 130.8 (C-2 and C-6), 128.3 (C-1), 124.9 (C-1′), 122.7 (C-6′), 117.7 (C-2′), 116.7 (C-3 and C-5), 112.8 (C-5′), 56.4 (OMe), 52.1 (C-α′), 49.7 (C-α), 32.4 (C-*β*).

4′-O-*Methyl*-N-*methylnorbelladine HCl*. (yield 8.5%): mp 229–231°. ^{1}H NMR (360 MHz, Cd_3OD): δ (ppm) = 6.95 (*d*, $J = 8.5$ Hz, 2H, H-3 and H-5), 6.89–6.81 (*m*, 3H, H-2′, H-5′ and H-6′), 6.62 (*d*, $J = 8.5$ Hz, 2H, H-2 and H-6), 4.22 (*d*, $J = 13.0$ Hz, 1H, H-α′), 4.02 (*d*, $J = 13.0$ Hz, 1H, H-α′), 3.76 (*s*, 3H, OMe), 3.27–3.17 (*m*, 1H, H-α), 3.12–3.04 (*m*, 1H, H-α), 2.93–2.77 (*m*, 2H, H-*β*), 2.69 (*s*, 3H, NMe). ^{13}C NMR (90 MHz, CD_3OD): δ 157.8 (C-4), 150.6 (C-4′), 148.3 (C-3′), 130.9 (C-2 and C-6), 127.9 (C-1), 123.9 (C-1′), 123.1 (C-6′), 118.6 (C-2′), 116.7 (C-3 and C-5), 112.8 (C-5′), 60.8 (C-α′), 57.9 (C-α), 56.4 (OMe), 40.0 (NMe), 30.6 (C-*β*).

Radiochemicals. [C^3H_3]SAM (sp. act 194 μCi μmol^{-1}) was provided by Dr M. Rueffer of the Munich laboratory.

Synthesis of [OC^3H_3]4′-O-*methylnorbelladine and* [OC^3H_3]4′-O-*methyl*-N-*methylnorbelladine*. Unlabelled norbelladine and *N*-methylnorbelladine were enzymically *O*-methylated in the presence of [C^3H_3]SAM and a specific catechol-*O*-methyltransferase isolated from leaves of *L. vernum*. Crude enzyme extracts were prepared by adding 50 g (fr. wt) of frozen crushed leaves to 75 ml buffer consisting of 0.02 M Tris-HCl, pH 7.5 and containing 0.01 M mercaptoethanol. The mixt. was stirred until completely thawed. The brei was pressed through cheesecloth and centrifuged at 10,000 *g*. The supernatant was applied to a DEAE-Sephacel column (2.3 × 13 cm) which was equilibrated with the above mentioned buffer. The column was washed with 100 ml buffer. Elution of the enzyme was achieved with a KCl step gradient at a flow rate of 60 ml h^{-1}. After eluting with 150 ml buffer containing 0.2 M KCl, the enzyme was desorbed with 150 ml 0.3 M KCl. The 5 ml-frs of major activity were collected (50 ml) and concd by ultrafiltration (10 kd). In a total volume of 350 μl (1 M Tris-HCl, pH 7.5), norbelladine (10 nmol) and [C^3H_3]SAM (62.7 μCi, 323 nmol) were incubated in the presence of the concd COMT (0.03 pkat) for 3 h at 30°. The reaction was terminated by the addition of 200 μl 1 M Na_2CO_3 buffer, pH 9.5. After total extraction with EtOAc, the extract was subjected to TLC ($CHCl_3$–MeOH–NH_3, 90:10:1) and after elution with 20 ml MeOH yielded [OC^3H_3]4′-*O*-methylnorbelladine ($R_f = 0.3$, 47.5%, 194 μCi μmol^{-1}). A portion of the product (7 nmol) was recrystallized four times from hot EtOH in the presence of unlabelled 4′-*O*-methylnorbelladine (81.4 μmol). In a separate experiment, the labelled product (3.7 nmol) was diluted with unlabelled 3′-*O*-methylnorbelladine

(149.9 μmol), respectively, and crystallized five times. Constant theoretical sp. act by the crystallisation experiments using the 4′-isomer corroborates the regiospecificity of the *Leucojum* COMT favouring the 4′ position. In contrast, all the radioactivity was lost when the labelled material was diluted and crystallized in the presence of 3′-*O*-methylnorbelladine. [OC^{3}H$_3$]4′-*O*-Methyl-*N*-methylnorbelladine was obtained from *N*-methylnorbelladine by the same labelling procedure at a yield of 44% (8.8 μCi, sp. act 194.4 μCi μmol^{-1}).

Synthesis of [*OC*^{3}*H*$_3$]3′-O-*methylnorbelladine*. In a total volume of 200 μl (1 M Tris-HCl, pH 7.5), norbelladine (10 nmol) and [C^{3}H$_3$]SAM (0.54 μCi, 2.8 nmol) were incubated in the presence of a COMT (0.13 pkat) isolated from pig liver according to ref. [31] for 12 h at 37°. The porcine COMT was obtained from Dr M. Rueffer of the Munich department. Extraction of 15 assays and purification by TLC as described above ($R_f = 0.35$) afforded [OC^{3}H$_3$]3′-*O*-methylnorbelladine. Yield 32%; sp. act 194 μCi μmol^{-1}.

Synthesis of [3,5-^{13}*C*$_2$]4′-O-*methylnorbelladine HCl*. In accordance with ref. [22] [3,5-^{13}C$_2$]4′-*O*-methylnorbelladine was obtained by stirring a mixt. of isovanillin (4.4 mg, 28.9 μmol) and 4 mg [3,5-^{13}C$_2$]tyramine (28.7 μmol) in 0.5 ml dry MeOH and one bead molecular sieve (4 Å) overnight at room temp. After removing the molecular sieve and diluting to 1 ml with MeOH, $NaBH_4$ (5 mg, 0.13 mmol) was added and the mixt. was stirred for additional 3 h at room temp. After purification by TLC as described above, evapn of the MeOH layer gave the oily amine that was dissolved in 600 μl EtOH containing 1% conc. HCl. After adding 5–10 ml EtOAc, the hydrochloride crystallized; overall yield 78% (22.5 μmol). The identity was determined via ^{1}H NMR and the ^{13}C NMR spectrum showed the enrichment at the expected positions C-3 and C-5 (116.7 ppm in CD_3OD). The [3,5-^{13}C$_2$]tyramine was obtained from L-[3,5-^{13}C$_2$]tyrosine after decarboxylation with L-tyrosine-decarboxylase (Fluka) and purification by PC using Whatman 3 paper (iso-PrOH–NH_3–H_2O, 8:1:1). L-[3,5-^{13}C$_2$]Tyrosine was a gift of Dr S. Sagner of the Munich department.

The synthesis of [α-^{13}C]4′-*O*-methylnorbelladine HCl proceeded from potassium-[^{13}C]cyanide (1 g, 15 mmol, Isotec Inc., > 99% ^{13}C) and 4-benzoyloxybenzyl chloride (3.5 g, 15 mmol) according to ref. [16, 21]; yield 17%. By ^{1}H NMR spectroscopy in CD_3OD, the identity of the precursor could be established and the ^{13}C NMR spectrum showed the desired enrichment at 49.7 ppm.

Standard assay of radiolabelled compounds. The application soln (5×10^5 cpm carrier free radiolabelled precursor in 0.5 ml H_2O) was pipetted in a cavity of a multidish (24 wells × 1 ml cavities, Nunclon). The plant organs were sliced into 1–2 mm squares and 20 pieces per cavity were floated in the substrate soln with agitation (200 strokes per min) at room temp. in daylight. After 48 h, the pieces were extracted for 20 min in 30 ml boiling MeOH containing 0.1% conc. HCl. After filtration and evapn, the extract was subjected to TLC (CH_2Cl_2–MeOH–NH_3, 90:9:1). The chromatograms were quantitated for radioactivity by a TLC linear analyser (Berthold) or by a Fujifilm BAS Phosphor Imager (Raytest). For determination of the total incorporation rate, a 20-μl-aliquot of the remaining feeding soln was removed for liquid scintillation counting.

^{13}*C assay*. Walls from ovaries of flowering *L. aestivum* plants were sliced into 2 mm squares. The fr. wt of one ovary was *ca*. 80 mg. Four hundred sliced ovaries were distributed to five 1 l Erlenmeyer flasks containing 40 ml of the tracer solution, 5×10^{-5} M of the ^{13}C-labelled precursor. Monitoring the incorporation rate, 1.5×10^6 cpm [OC^{3}H$_3$]4′-*O*-methylnorbelladine was added. The flasks were shaken for 48 h at room temp. in permanent incandescent light at 100 rpm. The tissue was harvested, washed and extracted with 75 ml and subsequently with 25 ml MeOH both containing 0.1% conc. HCl for 30 min each under reflux. The combined methanolic extracts were filtered and evapd to dryness. After redissolving with 100 ml H_2O, the acidic soln was extracted twice with 50 ml hexane and once with 50 ml $CHCl_3$ to remove neutral materials. The soln was basified with NH_3 to pH 8–9 and extracted five times with 50 ml $CHCl_3$ each and twice with 50 ml EtOAc each. The combined organic phases were washed with H_2O, dried over Na_2SO_4 and evapd to dryness yielding the crude alkaloids (ca 0.3% of fr. wt). The labelled alkaloids were purified by TLC with solvent systems CH_2Cl_2–MeOH–NH_3 (90:9:1) and $CHCl_3$–Me_2CO–Et_2NH (5:4:1). Structures of the metabolites were elucidated by MS and NMR spectroscopy.

N-*Demethylgalanthamine*. CIMS: ($M+H^+$) 274/256; EIMS 70 eV, *m/z* (rel. int): 273 (100, [M]$^+$), 272 (75), 230 (26), 202 (17), 174 (8). The fragments are in accordance with ref. [32]. ^{1}H NMR (360 MHz, $CDCl_3$): δ 6.66 (*d*, $J = 8.3$ Hz, 1H, H-12), 6.63 (*d*, $J = 8.3$ Hz, 1H, H-11), 6.06 (*d*, $J = 10.5$ Hz, 1H, H-4), 6.02 (*d*, $J = 10.5$ Hz, 1H, H-3), 4.62 (*br s*, 1H, H-16), 4.16 (*m*, 1H, H-2), 4.12 (*d*, $J = 15.1$ Hz, 1H, H-9), 3.84 (*s*, 3H, OMe), 3.65 (*d*, $J = 15.1$ Hz, 1H, H-9), 3.38 (*d*, $J = 14.5$ Hz, 1H, H-7), 3.23 (*d*, $J = 14.5$ Hz, 1H, H-7), 2.71 (*br d*, $J = 15.2$ Hz, 1H, H-1), 2.05–1.87 (*m*, 3H, H-1 and H-6). ^{13}C NMR (90 MHz, $CDCl_3$): δ 146.3 (C-13), 144.6 (C-14), 133.1 (C-15), 131.0 (C-10), 128.3 (C-3), 126.3 (C-4), 121.4 (C-11), 111.3 (C-12), 88.4 (C-16), 61.9 (C-2), 56.0 (OMe), 52.8 (C-9), 48.4 (C-5), 46.4 (C-7), 38.2 (C-6), 29.8 (C-1). This alkaloid was isolated first from bulbs of *Crinum asiaticum* [33].

N-*Demethylnarwedine*. CIMS: ($M+H^+$) 272; EIMS (70 eV), *m/z* (rel. int): 271 (100, [M]$^+$), 242 (15), 228 (14), 214 (31), 202 (20), 173 (34). ^{1}H NMR (360 MHz, $CDCl_3$): δ 6.96 (*d*, 1H, H-4), 6.69 (*d*, 1H, H-12), 6.67 (*d*, 1H, H-11), 6.06 (*d*, 1H, H-3), 4.69 (*br s*, 1H, H-16), 4.05 (*s*, 2H, H-9), 3.85 (*s*, 3H, OMe), 3.51–3.15

(*m*, 3H, H-7, H-1), 2.76 (*dd*, 1H, H-1), 2.15–1.87 (*m*, 2H, H-6). Because of the very low amount of substance, no ^{13}H NMR spectrum could be measured but the ^{1}H NMR spectrum was except for the missing NMe group in a good accordance with the narwedine spectrum.

Galanthamine was identified by comparing the spectral data obtained with those in refs. [34–36].

Narwedine. CIMS: $(M+H^+)$ 286; EIMS (70 eV), *m/z* (rel. int): 285 (100, $[M]^+$), 284 (98), 242 (22), 216 (15), 199 (17), 174 (28). ^{1}H NMR (360 MHz, $CDCl_3$): δ 6.96 (*d*, $J = 9.6$ Hz, 1H, H-4), 6.70 (*d*, $J = 8.1$ Hz, 1H, H-12), 6.66 (*d*, $J = 8.2$ Hz, 1H, H-11), 6.05 (*d*, $J = 9.7$ Hz, 1H, H-3), 4.74 (*br s*, 1H, H-16), 4.09 (*d*, $J = 15.4$ Hz, 1H, H-9β), 3.84 (*s*, 3H, OMe), 3.75 (*d*, $J = 15.4$ Hz, 1H, H-9α), 3.28–3.12 (*m*, 3H, H-7, H-1α), 2.75 (*dd*, 1H, H-1β), 2.45 (*s*, 3H, NMe), 2.28 (*t*, 1H, H-6α), 1.86 (*d*, $J = 14.3$ Hz, 1H, H-6β). ^{13}C NMR (90 MHz, $CDCl_3$): δ 194.4 (C-2), 147.0 (C-13), 144.3 (C-4), 144.0 (C-14), 130.5 (C-15), 129.4 (C-10), 127.1 (C-3), 122.0 (C-11), 111.8 (C-12), 88.0 (C-16), 60.7 (C-9), 56.0 (OMe), 54.1 (C-7), 49.0 (C-5), 42.4 (NMe), 37.3 (C-1), 33.2 (C-6).

Application assay of [*OC*^{3}*H*$_3$]*galanthamine vs.* [*OC*^{3}*H*$_3$]N-*demethylgalanthamine*. Radiolabelled galanthamine and *N*-demethylgalanthamine were biosynthetically prepared by feeding [OC^{3}H$_3$]4′-*O*-methylnorbelladine as described above to sliced ovary wall tissue of *L. aestivum*. After purification of the alkaloids by TLC (see above), [OC^{3}H$_3$]galanthamine (0.17 μCi) and [OC^{3}H$_3$]*N*-demethylgalanthamine (0.18 μCi) could be obtained. To determine the sp. act, samples of the alkaloid frs were subjected to HPLC analysis using a Knauer sepn column (Nucleosil 100 C18, 5 mm, 0.4×25 cm) coupled to a Macherey and Nagel precolumn (Vydac SC-201 RP, 0.4×5 cm) and performed as follows: component A: H_2O–MeCN (49:1) with 0.01% phosphoric acid; component B: MeCN–H_2O (99:1) with 0.01% phosphoric acid. Gradient: 100–0% A in 20 min, 0% A for 10 min, 0–100% A in 0.1 min, 100% A for 9.9 min; flow rate 1 ml min^{-1}; detection 275 nm. The isolated radiolabelled *N*-demethylgalanthamine (159,000 cpm, 0.14 μmol) and galanthamine (145,000 cpm, 0.12 μmol) were again fed to ovary wall tissue by the standard procedure.

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