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INCORPORATION OF PHENETHYLISOQUINOLINES INTO COLCHICINE IN ISOLATED SEEDS OF *COLCHICUM AUTUMNALE**

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Key Word Index—*Colchicum autumnale*; Liliaceae; autumnaline; biosynthesis; colchicine; isoandrocymbine; phenethylisoquinoline.

Abstract—The physiological parameters for application experiments using immature seeds of *Colchicum autumnale* were optimized, so that incorporation rates in the range of 10% and higher were consistently obtained with intermediate precursors. Application of tritium and ^{13}C-labelled phenethylisoquinolines showed that *N*-methylation occurs prior to the substitution of ring C and that the free hydroxy group in position 13 of autumnaline is necessary for the phenolic coupling reaction. Autumnaline gives rise to a vast array of metabolites in seeds of this plant. © 1997 Published by Elsevier Science Ltd. All rights reserved

INTRODUCTION

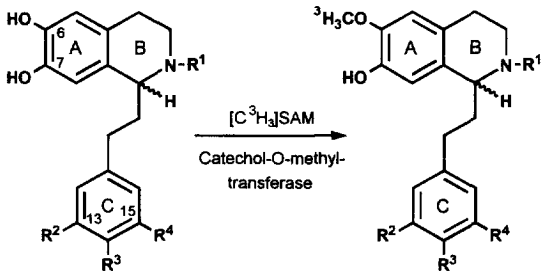
It has been known since ancient times that the poisonous *Colchicum* plants also serve useful medicinal purposes. Colchicine (**11**), the main alkaloid of *Colchicum* species, has antimitotic properties. Traditionally, this activity is pharmaceutically used for the treatment of gout and employed in plant breeding for the production of polyploid plants. Recently colchicine derivatives have shown promising anti-cancer and anti-inflammatory activities [1–3].

Despite considerable interest, the sequence of colchicine biosynthesis is not yet completely elucidated. Colchicine (**11**) possesses a unique tropolone ring and its structure originally seemed unrelated to any other alkaloid. The first biosynthetic experiments on *Colchicum* were conducted by Leete in 1960, demonstrating the incorporation of [3-^{14}C]phenylalanine into colchicine and summarizing previous biogenetic hypotheses [4]. These experiments were rapidly followed by Battersby demonstrating the incorporation of labelled tyrosine, methionine and acetate into this tropolonic alkaloid [5]. The major surprise arose, however, when it was shown that colchicine is a modified isoquinoline alkaloid [6, 7] and the major breakthrough in the biosynthesis of colchicine came from the demonstration that autumnaline [8] and *O*-methylandrocymbine [9] are precursors showing extraordinarily high incorporations into the target alkaloid. This led to the postulation that (*S*)-autumnaline (**12**) [10] undergoes enzyme-catalysed phenolic coupling yielding, after further modification, *O*-methylandrocymbine (**13**), which is then converted by homoallylic ring expansion to colchicine (**11**) [11]. The early and intermediate stages of colchicine biosynthesis were investigated by Herbert and his group [12] who demonstrated that the early stage of biosynthesis in the major pathway to colchicine involves the conversion of L-phenylalanine to cinnamic acid. This compound is in turn reduced to cinnamaldehyde, subsequently to dihydrocinnamaldehyde, which after hydroxylation in the 4 position yields the aldehyde (**14**) that condenses stereoselectively with dopamine (**15**) to a trihydroxylated phenethylisoquinoline (**16**). Compound (**16**) is the first alkaloidal precursor to colchicine. Further modification of this precursor by ring C modification (hydroxylations, methylations) would yield autumnaline (**6**).

The described pathway [12] leading to the early and intermediate stage precursors still leaves a number of unresolved questions, such as the exact sequence of the hydroxylation and methylation steps. It is also not exactly clear at what stage *N*-methylation occurs. It has been suggested that *N*-methylation need not precede aromatic oxygenation [12], but this is in conflict with earlier results [9]. Furthermore, the fate of carbon atoms of the dopamine side chain of autumnaline during colchicine biosynthesis should be reinvestigated [11]. Our aim with the present study was to shed light on some of these open questions by applying radioactive and ^{13}C-labelled precursors under optimized feeding conditions.

* Dedicated to Professor Peter Welzel, Leipzig, on the occasion of his 60th birthday.

† Author to whom correspondence should be addressed.



Compound	R¹	R²	R³	R⁴
1	H	H	OH	H
2	Me	H	OH	H
3	H	H	OH	OMe
4	Me	H	OH	OMe
5	H	OH	OMe	OMe
6	Me	OH	OMe	OMe
7	H	OMe	OH	OMe
8	Me	OMe	OH	OMe
9	H	OMe	OMe	OMe
10	Me	OMe	OMe	OMe

Fig. 1. Synthesis of tritium-labelled (*R,S*)-phenethylisoquinolines with different substitution patterns in ring C.

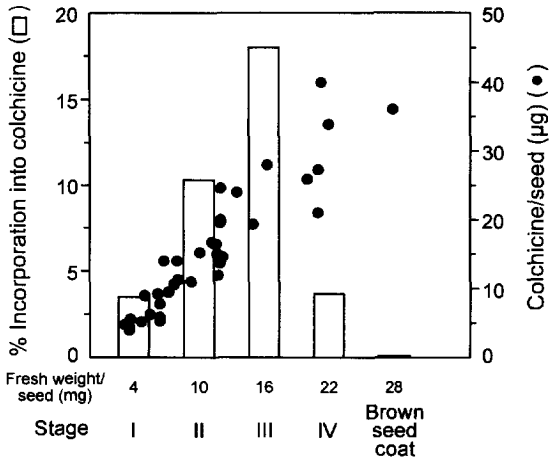


Fig. 2. Incorporation rates of (*R,S*)-[6-O-C^{3}H$_3$]autumnaline into colchicine in relation to fresh weight and colchicine content during seed development. Colchicine was quantified by HPLC, each point represents the mean value of four individual seeds. For the incorporation experiments four seeds of different stages of maturity were added to 0.5 ml of aqueous precursor solution (2 μCi, 80 mCi/μmol) and subsequently shaken for 48 hr.

RESULTS AND DISCUSSION

Optimization of the feeding conditions

Previous tracer experiments were carried out either on the intact plant by wick feeding or injection or by incubation of tissue slices with the labelled precursors. These methods gave only relatively low incorporation rates in the range of 0.01–1%. Using the intact plants also had the disadvantage of long application times lasting two–four weeks [e.g. 4, 11, 12].

All parts of the *Colchicum* plants contain colchicine, the corm, for instance, contains 0.3% of dry weight, but the highest accumulation with about 1% of dry weight is found in the fully ripe seeds [Poulev, A., personal communication], which in the case of *Gloriosa* is exploited as the commercial source of colchicine. If the biosynthetic pathway to colchicine is indeed present in the seeds, then the isolated maturing seed should give high rates of incorporation from distant and close precursors. With this reasoning we tried to feed the known precursor autumnaline to isolated, immature seeds.

Tritium-labelled autumnaline (**6**) was synthesized by an enzymatic methylation of the corresponding molecule with a free OH-group in position 6 using [C^{3}H$_3$]SAM (Fig. 1). The reaction is catalysed by catechol-*O*-methyltransferase isolated from pig liver [13], which is known to preferentially methylate the 6-OH group in isoquinoline alkaloids [14]. The 6-*O*-methyl and 7-*O*-methyl isomers can be cleanly separated by TLC.

According to their fresh weight, the seeds of *Colchicum autumnale* were divided into four developing stages. Stage I had an average fresh weight of 4 mg per seed, stage II had 10 mg, stage III 16 mg and stage IV 22 mg. HPLC quantifications showed that the colchicine content of the seeds rises continuously from an average of 5 μg per seed in stage I to an average of 30 μg per seed in stage IV within a period of four weeks. Completely mature seeds also contained about 30 μg colchicine per seed. Seeds of all four stages were examined by application experiments with [6-O-C^{3}H$_3$]autumnaline. Stage I gave a satisfactory incorporation rate into colchicine of 3.5%, while with stages II and III, high and reproducible incorporation rates of 10 and 18% were achieved. In the rather mature stage IV, the incorporation rate was reduced to 3.7%. Fully mature seeds showed no alkaloid biosynthesis. Figure 2 shows that the highest incorporation rates of (**6**) parallel the greatest increase in colchicine content. For all further experiments, seeds of stage II and III between 8 and 18 mg fresh weight per seed were used. The dependence of incorporation of labelled autumnaline (**6**) supplied in the aqueous incubation medium was next studied with respect to the concentration of the precursor supplied. As shown in Fig. 3, the rate of incorporation under standard conditions was almost constant in the range of 0.01–0.1 mM. However, if higher concentrations were used, the incorporation drops strongly to about 1/3 of the rate at 0.1 mM. As a consequence of this experiment, precursors were fed in 0.3 M concentrations which was a compromise to achieve sufficient incorporation with ^{13}C-labelled precursors.

The dependence on immature seeds limits the possibility of biosynthetic experiments to a short period of four–six weeks each year. We tried to feed corms, leaves and flowers of *C. autumnale* with labelled autumnaline (**6**) under differing feeding conditions,

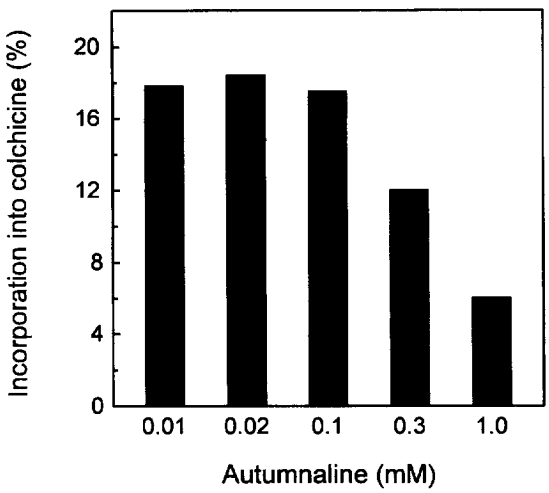


Fig. 3. Rates of incorporation of (*R,S*)-[6-O-C^{3}H$_3$]autumnaline in immature seeds of *C. autumnale*, stage III under standard conditions as a function of precursor concentration.

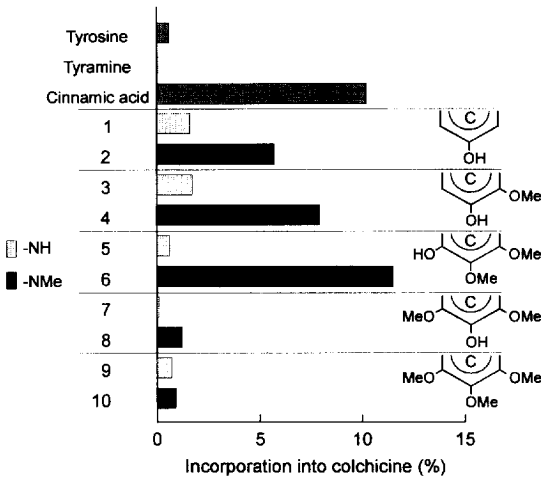


Fig. 4. Rates of incorporation of differently ring C substituted racemic [6-O-C^{3}H$_3$]phenethylisoquinolines into colchicine in immature seeds (stage II) of *C. autumnale*. Note the differences between nor- and *N*-methyl precursors with the same ring C substitution pattern. The numbers refer to the compounds shown in Fig. 1.

but the incorporation into colchicine was always 0.5% or less, which is about 1/20 of what can be achieved with immature seeds of stage II and III and which lies in the same range as previously described in the literature [12]. It is evident from previous work [12] that different parts of *C. autumnale*, such as corms or flowers, are able to synthesize colchicine, however, at relatively low rates. It was striking that the incubation medium of the seeds contained between 40 and 70% of the labelled colchicine formed. This may suggest that colchicine synthesized within the seeds might be excreted into the incubation medium, reflecting an active transport mechanism of this alkaloid, which normally supplies other parts of the *Colchicum* plant with this defense compound. We (A. Poulev) succeeded in this laboratory to establish a good growing cell suspension culture of *C. autumnale*; however, this culture was unable to synthesize tropolonic alkaloids, even in different media with or without elicitation. In our opinion, colchicine biosynthesis at the precursor feeding and enzyme level can presently only be solved by using this immature seed system.

Incorporation of potential precursors

To study the sequence of proposed intermediates, a set of different phenethylisoquinolines (**1–10**) shown in Fig. 1 was synthesized. They include all major substitution patterns of hydroxy- and methoxy-groups located in ring C of potential intermediates leading via autumnaline (**6**) the established precursor [8] to colchicine (**11**). All of these potential precursors (Fig. 1) were enzymatically labelled in position 6 of ring A with a tritiated methyl group, yielding precursors with a specific activity of 80 mCi/μmol. In order to get information as to the stage at which *N*-methylation occurs, the incorporation of *N*-nor compounds was compared with that of their *N*-methyl counterparts.

The precursors shown in Fig. 1 were fed to the same batch of *Colchicum* seeds, originating from one seed capsule. Early precursors like L-tyrosine, tyramine and *trans*-cinnamic acid were also included. The percentage of incorporation into colchicine is summarized in Fig. 4. As expected, cinnamic acid shows a high incorporation rate of about 10% and it was previously found to be one of the best precursors [1%, ref. 15]. Since ^{14}C-labelled cinnamic acid is easily accessible, this colchicine precursor was always included in each batch of feeding experiments as an internal standard for the physiological state of the seeds. Incubation experiments where cinnamic acid as an internal standard reached rates of incorporation of less than 7% were discarded. Tyrosine, the precursor of dopamine, gave only a 0.2% incorporation into colchicine. This could be explained by the multiple use of this amino acid in metabolic pathways, such as protein synthesis, in these developing seeds. For tyramine, which could also be regarded as a precursor of dopamine, no incorporation was detected. If this amine has reached the site of colchicine biosynthesis, this would suggest that the dopamine precursor for colchicine is synthesized via L-dopa and not via tyramine. Comparing the rate of incorporation of the phenethylisoquinoline alkaloids into colchicine, it was at once obvious that the potential nor precursors (**1**), (**3**) and (**5**) had a much lower incorporation rate compared to their *N*-methyl derivatives. This result is strong evidence that *N*-methylation already occurs at a very early stage, probably directly after the condensation of *p*-hydroxydihydrocinnamaldehyde [12] and dopamine and before the subsequent hydroxylation and methylation steps.

In order to check the incorporation capacity for labelled cinnamic acid into colchicine, immature seeds of different *Colchicum* species were supplied under standard conditions with this ^{14}C precursor acid. As

Table 1. Incorporation of [3-^{14}C]cinnamic acid (0.5 μCi, sp. act 55 μCi/μmol) fed to isolated immature seeds of different *Colchicum* species under standard conditions

Species of *Colchicum*	Incorporation into colchicine
C. agrippinum	3.2%
C. neapolitanum	5.0%
C. bornmuelleri	9.1%
C. speciosum	10.0%
C. autumnale	10.2%

shown in Table 1, there is a considerable difference in the alkaloid forming capacity of the different species tested. *Colchicum speciosum* and *C. autumnale* proved to be the best experimental materials for the incorporation experiments under the given conditions. Application of [6-O-C^{3}H$_3$]autumnaline (**6**) in *C. speciosum* also gave a good incorporation rate of about 16%.

Looking at the different substitution patterns in ring C, the incorporation rates of the *N*-methylated compounds reflect the sequence of the substitution reactions (Fig. 4). They rise continuously from the monohydroxy substituted compound (**2**) with 5.7% via (**4**) with 7.4% to autumnaline (**6**) with 11.5% incorporation in seed stage II. But as soon as the *meta*-hydroxy group in position 13 is methylated as in compounds (**8**) and (**10**), the incorporation rate drops significantly to only about 1%. This strengthens the hypothesis that the free hydroxy group in position 13 of autumnaline (**6**) is essential for the mechanism of the phenolic coupling reaction [16, 17]. The last methylation step to complete the trimethoxy substitution pattern found in colchicine occurs clearly after the phenoloxidative formation of (**6**) yielding isoandrocymbine (**17**), as shown by the presence of a specific enzyme [17], a fact which is supported by the lack of incorporation of compound (**8**). The small rate of incorporation (less than 1%) of the wrongly trisubstituted compounds (**7, 8**) and (**9, 10**) must, therefore, be a result of a de- and re-methylation occurring in the seed tissue [18].

Incorporation of ^{13}C-*labelled autumnaline*

The incorporation results based on the application of tritium-labelled *O*-methyl precursors give good evidence for the incorporation of these precursors into colchicine, but they cannot be taken as an absolute proof. For instance, an enzymatic transfer of the labelled methyl group must be taken into consideration, as shown previously during benzylisoquinoline alkaloid biosynthesis [18]. To confirm and extend the results obtained with the tritiated precursors, two differently ^{13}C-labelled autumnaline molecules were synthesized, (*R,S*)-[3,9-^{13}C]autumnaline and (*R,S*)-[N-^{13}C]autumnaline. Both were administered in a final

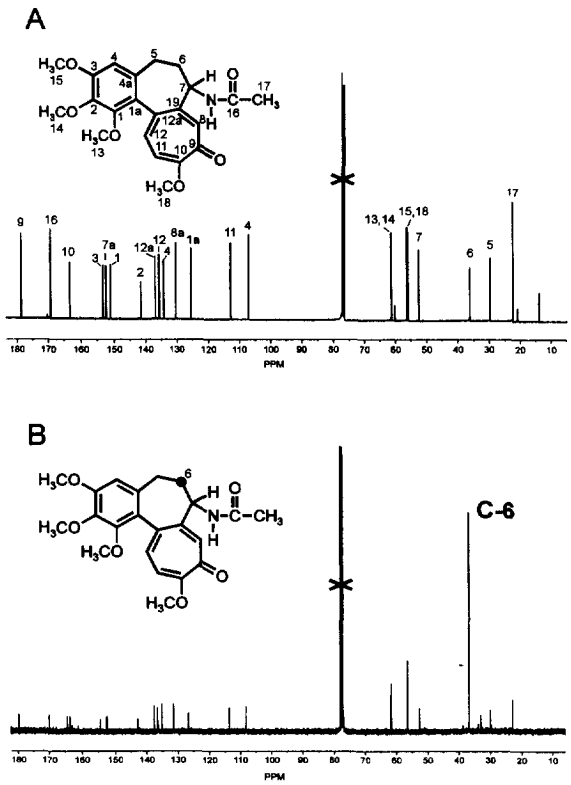


Fig. 5. ^{13}C NMR spectra of colchicine (A) showing the natural abundance of ^{13}C in comparison to C-6 enriched colchicine (B) isolated after application of (*R,S*)-[3,9-^{13}C] autumnaline. The doubly-labelled precursor gives rise to only one label in colchicine, which confirms [11] that C-3 of autumnaline is lost during the expansion of ring C (see Fig. 6).

concentration of 0.3 mM to the seeds of *C. autumnale*. After the usual 48 hr incubation period, colchicine was isolated from the crude methanolic extract and incubation medium by TLC, followed by HPLC. The ^{13}C NMR spectrum ($CDCl_3$) of the purified colchicine exhibited only one resonance of a ^{13}C enriched carbon atom at C-6 of colchicine (δ 6.2) (Fig. 5), whereas the applied (*R,S*)-[3,9-^{13}C]autumnaline has resonances at δ 48.7 (C-3) and 37.6 (C-6). This finding fits the proposed pathway, where C-9 of autumnaline is incorporated into C-6 of colchicine, whereas C-3 of autumnaline is lost [11] during the ring expansion reaction (Fig. 6). The colchicine isolated from the incubation with *N*-$^{13}CH_3$-labelled autumnaline gave no ^{13}C enriched signal. This proves that the required *N*-demethylation reaction in the transition from autumnaline to colchicine does not lead to C_1-compounds (CH_2O; Me–OH; HCOOH etc.) in a proximal C_1-pool which could potentially be metabolized and could reappear in the alkaloids under investigation. Furthermore, this experiment clearly confirms that the bond between N and Me is broken before the acetylation occurs [6]. In our experiment, however, no 'feed back' of label (10% as observed in [6] would have been detected) occurs.

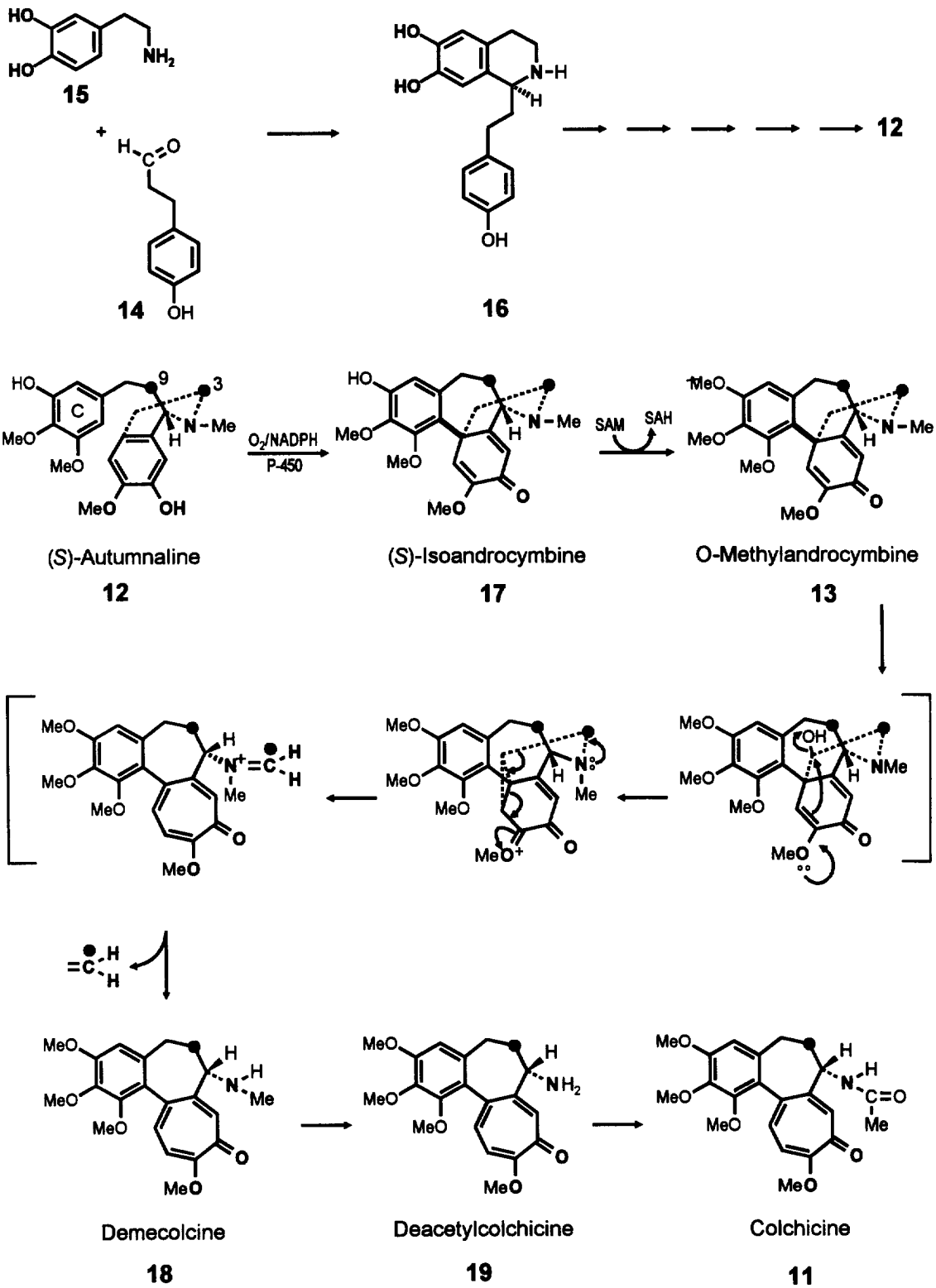


Fig. 6. Proposed pathway for the biosynthesis of colchicine summarizing results from refs [4–12, 15–17].

One application in 1995 gave an unexpected high incorporation of the trimethoxyphenethylisoquinoline (**10**) into colchicine. Since this result would be in contradiction to those mentioned above, we decided to check colchicine labelling with a trimethoxy compound, which has all three *O*-methyl groups and the *N*-methyl ^{13}C-labelled. The idea was that the methyl group in position 3 may be removed

enzymatically. The (*R,S*)-[13,14,15-O-$^{13}CH_3$, N-$^{13}CH_3$]trimethoxy derivative was administered at a concentration of 0.1 mM. The ^{13}C NMR spectrum of the crude extract showed that the administered compound remained unmodified. Colchicine was purified by TLC, the ^{13}C NMR spectrum in $CDCl_3$ did not contain any visible ^{13}C enriched resonances outside of the natural abundance. The lack of incorporation confirms again that the trimethoxy derivative cannot be converted by the oxidative phenolic coupling and there is no refixation of any potentially liberated labelled C_1 units in the seed tissue.

Further metabolites originating from tritium-labelled (R,S)-*autumnaline*

The crude methanolic seed extract obtained after application of racemic tritium-labelled autumnaline (**6**) was subjected to two-dimensional TLC. The autoradiogram of the TLC plate revealed in addition to unmetabolized autumnaline (**6**) (*R*-isomer?) and colchicine (**11**) a strikingly large number of unknown radiolabelled metabolites (Fig. 7). The TLC system used separates more than 20 distinct spots. Certainly not all of them are intermediates between autumnaline and colchicine, but may be side products of the main colchicine pathway. The structural identity of some of these labelled intermediates and end products and an explanation of how they fit into a general scheme of alkaloid biosynthesis in *C. autumnale* will be left to future work.

Taken together, the results obtained over the past 35 years [4–12, 15–18] on the biosynthesis of colchicine, provide the following picture (Fig. 6): *p*-hydroxydihydrocinnamaldehyde (**14**) condenses with dopamine (**15**) to yield a trihydroxylated phenethylisoquinoline alkaloid with (*S*)-configuration (**16**). Methylation both at the 6-OH group and the nitrogen atom as well as further modification of the C ring to yield a 3-OH, 4,5-OMe pattern gives (*S*)-autumnaline (**12**), which by action of a highly specific cytochrome P-450 enzyme undergoes an oxidative phenol coupling reaction to yield isoandrocymbine (**17**). This intermediate is further methylated to *O*-methylandrocymbine (**13**) by action of a specific SAM dependent *O*-methyltransferase. In the further transformation of (**13**), carbon 3 of this compound is lost, leading to ring expansion to create the tropolone ring system in (**18**), which subsequently leads to the loss of the *N*-methyl group of the precursor alkaloid. Acetylation of the deacetylcolchicine molecule (**19**) leads to colchicine (**11**), one of the most unusual alkaloids found in the plant kingdom. While the early and intermediate steps slowly become clear even at the enzyme level, the proximal steps of colchicine biosynthesis remain unelucidated and present an interesting task for the enzymologists as well as for molecular biology.

We assume that the knowledge taken from the optimization of the incorporation experiments and finding the right plant organ and time to conduct feeding and enzyme experiments will allow future structural and biochemical work to further unravel the complex pathway leading to one of nature's most unusual alkaloids.

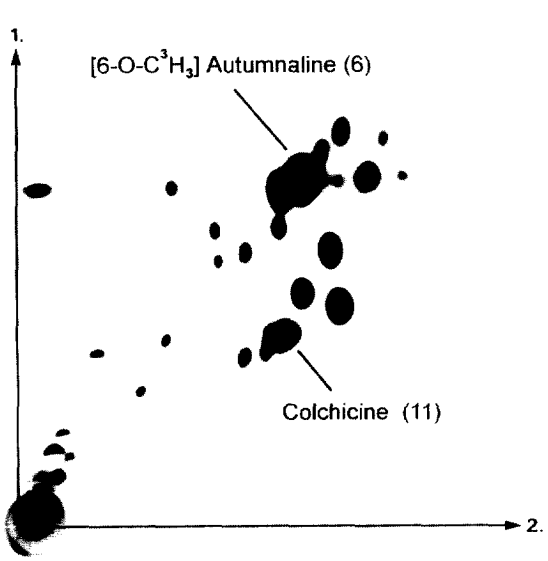


Fig. 7. Autoradiogram from a two-dimensional TLC of the crude methanolic seed extract. Solvent 1, Me_2CO–toluene–EtOH–NH_4OH (45:45:7:3); solvent 2, MeOH–$CHCl_3$ (1:9). The applied (*R,S*)-[6-O-C^3H_3]autumnaline is converted into colchicine and *ca* 20 other distinctly visible unidentified metabolites.

EXPERIMENTAL

General. NMR spectra recorded on a Bruker AM 360 at 360.166 MHz (^1H) and 90.56 MHz (^{13}C). [^{13}C]Formaldehyde and K^{13}CN from Cambridge Isotope Laboratories.

Application experiments, extraction and purification. Immature seed capsules of *C. autumnale* and other *Colchicum* species collected in the university or botanical gardens of Munich. For feeding experiments, immature seeds were carefully removed from the freshly harvested capsules, four seeds each were placed in 0.5 ml of an aqueous solution containing the radiolabelled precursor. ^{14}C-Labelled substances ([U-^{14}C]tyrosine, [U-^{14}C]tyramine, each 443 μCi/μmol, [3-^{14}C]cinnamic acid, 55 μCi/μmol) purchased from Amersham and applied in a concentration of 2 μCi/ml. The tritium labelled phenethylisoquinolines were fed with 4 μCi/ml. The seeds were incubated at 24° under agitation (150 strokes/min) and in the dark to avoid light induced degradation of colchicine. After 48 hr the seeds were extracted with MeOH for 1 hr at 60°. The concd seed extract and the freeze dried incubation medium were sepd by TLC. The labelled metabolites were quantified with a radioscanner. Colchicine was identified as a major radioactive product in two different solvent systems: Me_2CO–toluene–EtOH–NH_4OH (45:45:7:3) R_f 0.4 and Me_2CO–$CHCl_3$–Et_2NH (2:7:1) R_f 0.6 and further purified by HPLC

sepn on an RP C18 select B column (250 × 4 mm) with a 20 min linear gradient from 100% solvent A (98% water, 2% acetonitrile, 0.1% H_3PO_4) to 100% B (98% acetonitrile, 2% water, 0.1% H_3PO_4), 1 ml min^{-1}, UV detection at 350 nm. For ^{13}C experiments, 384 seeds (6 g fr. wt) were incubated in the same way as described above. TLC was performed on silica gel sheets (0.25 mm, F_{254}, Macherey-Nagel).

Synthesis of 2-(4-*benzyloxy*-3-*methoxyphenyl*)-[1-^{13}C]*ethylamine*. $NaBH_4$ (0.5 g, 13.2 mmol) was added to a solution of 4-benzyloxy-3-methoxybenzaldehyde (3 g, 12.3 mmol) in MeOH (50 ml). After stirring for 3 hr, HOAc (1 ml) was added and the mixture was concd *in vacuo*. The residual aqueous extract was neutralized with $NaHCO_3$ (20%) and extracted with EtOAc (50 ml). The organic layer was dried with Na_2SO_4 and evapd to dryness *in vacuo* (40°). The residue crystallized from MeOH–H_2O yielding the alcohol (2.9 g, 96.6%) as white needles.

Thionyl chloride (2.6 ml) was then added to a solution of the foregoing alcohol (2.9 g, 11.8 mmol) in dry Et_2O (15 ml) and stirred for 6 hr. The reaction mixture was extracted with H_2O, the organic layer was evapd to dryness and crystallization from C_6H_6–hexane furnished 4-benzyloxy-3-methoxybenzyl chloride (2.9 g, 93.5%): CIMS *m/z* (rel. int.): 263 $[M+H]^+$ (100), ^1H NMR ($CDCl_3$): δ 3.9 (3H, *s*, OMe), 4.6 (2H, *s*, $PhCH_2Cl$), 5.9 (2H, *s*, OCH_2Ph).

A mixt. of the foregoing chloride (1.6 g, 6.1 mmol), $K^{13}CN$ (0.4 g, 6.14 mmol) and dry DMF (8 ml) was stirred at room temp. for 7 days. The reaction mixt. was diluted with H_2O (20 ml) and extracted with EtOAc. The organic layer was dried giving 4-benzyloxy-3-methoxyphenyl-[1-^{13}C]acetonitrile (1 g, 64.9%) as white crystals.

Cobaltous chloride hexahydrate (2.9 g, 12.1 mmol) was added to a solution of the foregoing [1-^{13}C]acetonitrile (1 g, 3.9 mmol) in MeOH (35 ml) then $NaBH_4$ (2 g, 52.8 mmol) was added in portions with stirring at 20°. A black ppt appeared and stirring was continued for 1 hr at 20°. 3 N HCl (18 ml) was poured into the reaction mixt. and stirred until the black ppt was dissolved. After removal of MeOH, the residue was extracted with EtOAc, dried over Na_2SO_4 and evapd. The resulting amine was converted into its hydrochloride, which crystallizing from MeOH–EtOAc furnished 2-(4-benzyloxy-3-methoxyphenyl)-[1-^{13}C]ethylamine hydrochloride (0.5 g, 49%): CIMS *m/z* (rel. int.): 258 $[M+H]^+$ (100), mp 170°, ^1H NMR (CD_3OD): δ 2.64 (2H, *t*, H-1), 2.85 (2H, *t*, H-2), 3.67 (3H, *s*, OMe), 4.89 (2H, *s*, CH_2Ph), ^{13}C NMR (CD_3OD): δ 36.3 (C-2), 42.9 (C-1), 56.5 (OMe).

Formation of 3-(3-*hydroxy*-4,5-*dimethoxyphenyl*)[2-^{13}C]*propionic acid*. 3-Hydroxy-4,5-dimethoxybenzaldehyde (0.4 g, 10 mmol) was dissolved in pyridine (2 ml), piperidine (200 μl) and [2-^{13}C]malonic acid (200 mg, 11 mmol) were added then refluxed for 18 hr. The mixt. was poured onto ice-H_2O–HCl, extracted with EtOAc (50 ml) and concd *in vacuo* (40°) yielding amorphous solid acid (0.4 g, 81%), mp 146°, CIMS *m/z* (rel. int.): 226 $[M+H]^+$ (100), 196 (10). A soln of the corresponding cinnamic acid (0.4 g, 1.78 mmol) in EtOH was stirred with H_2 and 10% Pd-C (100 mg) until 1 mol equiv. of hydrogen had been absorbed. Evapn of the filtered soln and crystallization of the residue in EtOH–H_2O gave [2-^{13}C]propionic acid (0.39 g, 97.5%), CIMS *m/z* (rel. int.): 228 $[M+H]^+$ (100). The propionic acid was converted into 3-(3-benzyloxy-4,5-dimethoxyphenyl)-[2-^{13}C]propionic acid (0.16 g, 29.6%).

Synthesis of N-(3-*benzyloxy*-4,5-*dimethoxyphenyl*)-[2-^{13}C] *propionamide*. A solution of 3-(3-benzyloxy-4,5-dimethoxyphenyl)-[2-^{13}C]propionic acid (0.15 g, 0.6 mmol) in xylene (20 ml) was added to 2-(4-benzyloxy-3-methoxyphenyl)-[1-^{13}C]ethylamine (0.17 g, 0.6 mmol) and refluxed overnight. After cooling and washing the ppt with Et_2O, the doubly-labelled amide was achieved (0.1 g, 27%), mp 105°, CIMS *m/z* (rel. int.): 558 $[M+H]^+$ (100).

Synthesis of 1-(3-*benzyloxy*-4,5-*dimethoxyphenyl*-[2-^{13}C]*ethyl*)-1,2,3,4-*tetrahydro*-7-*benzyloxy*-6-*methoxy*-[3-^{13}C]*isoquinoline*. A mixt. of the above amide (0.1 g, 0.1 mmol), dry acetonitrile (20 ml) and $POCl_3$ (300 μl) was refluxed for 2 hr. The mixt. was concd *in vacuo* (40°). The residue was taken up in $CHCl_3$ and washed with H_2O. The organic layer was removed *in vacuo* (40°) yielding the imine hydrochloride (70 mg, 72.9%).

The above imine hydrochloride (70 mg, 1.3 mmol) was dissolved in EtOH (10 ml) and subsequently treated with $NaBH_4$ (200 mg, 5.1 mmol) in small portions at room temp. After stirring for 2 hr, 2 N HCl was added (pH 2), then basified with 2 M NaOH (pH 9–10), EtOH was removed and the reaction mixt. was extracted with $CHCl_3$. The organic layer was concd *in vacuo*. The resulting amine was converted into its hydrochloride, crystallized from MeOH–EtOAc affording the corresponding phenethylisoquinoline (46 mg, 65.7%), CIMS *m/z* (rel. int.): 542 $[M+H]^+$ (100), 452 (60).

Synthesis of 1-(3-*benzyloxy*-4,5-*dimethoxyphenyl*-[2-^{13}C]*ethyl*)-1,2,3,4-*tetrahydro*-7-*benzyloxy*-6-*methoxy*-2-*methyl*-[3-^{13}-C]*isoquinoline*. An excess of formaldehyde (1 ml) was added dropwise to a stirring solution of phenethylisoquinoline (46 mg, 0.08 mmol) in MeOH at room temp. over 0.5 hr. $NaBH_4$ (100 mg, 2.5 mmol) was then added carefully under ice-cooling and stirred at room temp. for another 2 hr. The solvent was removed after addition of H_2O (1 ml), and the aqueous phase was extracted with $CHCl_3$ and the organic layer was dried *in vacuo* (40°). The residue was crystallized from MeOH–EtOAc yielding (*R,S*)-*O,O*-dibenzyloxy-[3,9-^{13}C]autumnaline (30 mg, 63.8%) as white crystals, mp 88°, CIMS *m/z* (rel. int.): 556 $[M+H]^+$ (100), 466 (30), ^1H NMR (CD_3OD): δ 6.31 (2H, *s*, H-5 and H-8), 6.68 and 6.86 (each 1H, *s*, H-12 and H-16) 5.08 and 5.12 (each 2H, *s*, 2 × OCH_2Ph), 3.84, 3.86 and 3.88 (each 3H, *s*, 3 × OMe-14, 15, 6), 2.3 (3H, *s*, NMe), ^{13}C NMR (CD_3OD): δ 48.7 (C-9),

37.5 (C-3), 40.1 (N-Me), 56.0, 56.4, 56.6 (3 × OMe-14, 15, 6), 25.8 (C-10), 32.9 (C-4).

(R,S)-[3,9-^{13}C]*Autumnaline*. To a stirring solution of the preceding tetrahydroisoquinoline (30 mg, 0.05 mmol) in EtOH 10% Pd-C (15 mg) and three drops of concd HCl were added and the soln was allowed to stir at room temp. under H_2 for 5 hr. The filtered soln was evapd and then crystallized from MeOH–EtOAc affording (*R,S*)-[3,9-^{13}C]autumnaline (15 mg, 75%), mp 236°, CIMS *m/z* (rel. int.): 376 $[M+H]^+$ (100), 258 (10), ^{1}H NMR (CD_3OD): δ 3.13 (3H, *s*, NMe), 3.78, 3.83 and 3.90 (each 3H, *s*, 3 × OMe-14, 15, 6), 6.45 (2H, *s*, H-5 and H-8), 6.68, 6.86 (each 1H, *s*, H-12, H-16), 2.71 (2H, *t*, H-3), 3.30 (2H, *t*, H-9), 4.28 (1H, *t*, H-1), ^{13}C NMR (CD_3OD): δ 40.9 (N-Me), 56.4, 60.9, 62.2 (OMe-14, 15, 6), 37.6 (C-9), 48.7 (C-3), 32.7 (C-4), 22.7 (C-10).

Synthesis of 1-(3,4,5-[O^{13}CH$_3$]*trimethoxyphenethyl*)-1,2,3,4-*tetrahydro*-7-*benzyloxy*-6-*methoxy*-2-[^{13}CH$_3$]*methylisoquinoline*. A mixture of 3,4,5-trihydroxybenzaldehyde (0.5 g, 3.2 mmol), KI (2 mg, 12 μmol), K_2CO_3 (1 g, 7.2 mmol), DMSO (4 ml) and $^{13}CH_3I$ (600 μl) was stirred at room temp. under N_2 for 24 hr, diluted with H_2O (30 ml) and extracted with EtOAc. The organic layer was evaporated and crystallized in MeOH yielding 3,4,5-[O–^{13}CH$_3$]trimethoxy-benzaldehyde (0.3 g, 50%) as white crystals, CIMS *m/z* (rel. int.): 200 $[M+H]^+$ (100), ^{1}H NMR ($CDCl_3$): δ 3.90 (6H, *s*, OMe-4,6), 3.91 (3H, *s*, OMe-5), 7.09 (2H, *s*, ArH), 9.8 (1H, *s*, CHO), ^{13}C NMR ($CDCl_3$): δ 191.1 (CHO) 56.07, 61.01 (3 × OMe-4, 5, 6), 131.7 (C-2), 106.6 (C-3), 106.7 (C-7), 153 (C-4 and C-6). (*R,S*)-1-(3,4,5-[O-^{13}CH$_3$] trimethoxyphenethyl)-1,2,3,4-tetrahydro-7-benzyloxy-6-methoxyisoquinoline from 3,4,5-[O-^{13}CH$_3$]trimethoxybenzaldehyde (25 mg, 65%) was prepd in the same way as described above. CIMS *m/z* (rel. int.): 463 $[M+H]^+$ (100), ^{1}H NMR (CD_3OD): δ 3.71 (6H, *s*, OMe-13, 15), 3.69, 3.61 (3H, *s*, OMe-15, 6), 4.38 (1H, *t*, H-1), 4.97 (2H, *s*, OCH_2Ph), 6.73, 6.66 (both 1H, *s*, H-12, H-16), 6.47 (2H, *s*, H-5, H-8).

Synthesis of (R,S)-1-(3,4,5-[O-^{13}CH$_3$]*trimethoxyphenethyl*)-1,2,3,4-*tetrahydro*-7-*hydroxy*-6-*methoxy*-2-[^{13}CH$_3$]*methylisoquinoline*. [^{13}C]Formaldehyde (800 μl) was added to a solution of 1-(3,4,5-[O^{13}CH$_3$]trimethoxyphenethyl)-1,2,3,4-tetrahydro-7-benzyloxy-6-methoxyisoquinoline (25 mg, 0.05 mmol) in MeOH (3 ml), stirred 30 min at room temp. and then subjected to $NaBH_4$ (82 mg, 2.2 mmol) reduction for 1 hr. The reaction mixt. was evapd, the residue dissolved in $CHCl_3$ and this soln was concd *in vacuo* to yield the base as an amorphous solid (17 mg, 65.3%). To the resulting base (17 mg, 0.04 mmol) dissolved in EtOH, 10% Pd-C (8.5 mg) was added. The solution was allowed to stir at room temp. under H_2 for 4 hr. The filtered soln was evapd and then crystallized from EtOH–Et_2O affording (*R,S*)-1-(3,4,5-[O-^{13}CH$_3$]trimethoxyphenethyl)-1,2,3,4-tetrahydro-7-hydroxy-6-methoxy-2-[^{13}CH$_3$] methylisoquinoline (12 mg, 87.6%), CIMS *m/z* (rel. int.): 387 $[M+H]^+$ (100), 253 (20), ^{1}H NMR (360 MHz, CD_3OD): δ 3.21 (3H, *s*, N-Me) 3.48, 3.73, 3.75, 3.80 (each 3H, *s*, OMe-13, 14, 15, 6) 6.44 (2H, *s*, H-5, H-8), 6.53, 6.72 (each 1H, *s*, H-12, and H-16), 2.66 (2H, *t*, H-3), 4.10 (1H, *t*, H-1), ^{13}C NMR (CD_3OD): δ 41.1 (N–Me), 37.9 (C-9), 33.1 (C-4), 22.8 (C-10), 48.5 (C-3), 56.6, 56.7, 56.9, 64.5 (4 × OMe-13, 14, 15, 6).

(*R,S*)-[N-^{13}C]Autumnaline (20 mg, 76%) was synthesized from nor-autumnaline and [^{13}C]formaldehyde using the above described procedure.

Phenethylisoquinolines. Unlabelled phenethylisoquinolines were synthesized according to standard procedures [9].

Synthesis of tritium-labelled precursors. The incubation mixt. for enzymatic methylation contained: 10 μCi [C^3H_3]SAM (sp. act 80 mCi/μmol), 200 μl catechol-*O*-methyltransferase (1.8 mg protein, sp. act 0.02 pkat/mg) [13], 200 nmol phenethylisoquinolines (10 mM in 1% DMSO) and 100 μl/M tricine buffer, pH 7.5. After incubation for 2 hr at 30° the incubation mixt. was diluted with 760 μl H_2O and purified over an RP C18 column (Macherey-Nagel, C18f ec, 500 mg). The radiolabelled precursors were eluted with MeOH and identified by TLC, Me_2CO–toluene–EtOH–NH_4OH (45:45:7:3), with unlabelled 6-*O*-methylphenethylisoquinolines as references.

Autoradiography. For two-dimensional TLC, 2.5 μCi of the combined crude methanolic extract and medium received from an application experiment with (*R,S*)-[6-O-C^3H_3]autumnaline were used. After development on silica gel in (1) Me_2CO–toluene–EtOH–NH_4OH (45:45:7:3) and (2) MeOH–$CHCl_3$ (1:9), the plates were sprayed with an enhancer (Enhance, Dupont). X-ray film (Kodak, X-OMAT) was exposed to the plates for 6 months at −80°.

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