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UNUSUAL METHYL TRANSFER IN THE BIOSYNTHESIS OF APORPHINE AND PROTOBERBERINE ALKALOIDS

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Key Word Index—*Peumus boldus*; *Berberis stolonifera*; Berberidaceae; cell suspension cultures; ^{13}C NMR; reticuline; aporphines; protoberberines; alkaloids; biosynthesis.

Abstract—(*S*)-Reticuline was triply-labelled with ^{13}C and administered to cell cultures of *Peumus boldus* and *Berberis stolonifera*, which are sources of aporphines and protoberberines, respectively. During incorporation of the labelled precursor into alkaloids of both groups, unexpected transmethylations of the methyl groups were observed by ^{13}C NMR spectroscopy which seem to proceed via demethylation, flux of ^{13}C through the C-1 pool, and remethylation.

INTRODUCTION

Aporphines and protoberberines are derived from (*S*)-reticuline (**1**) [1–4]. There are several intermediates of both groups of alkaloids with analogous methylation patterns in ring A (Fig. 1). The substitution of isoboldine and its congener columbamine (**2**) (Fig. 1: R_1 = Me, R_2 = H), is retained from reticuline. Incorporation into boldine, norboldine (**3**) and jatrorrhizine (**4**), respectively, implies a change to the reverse methylation pattern in ring A (Fig. 1: R_1 = H, R_2 = Me). Two possible biosynthetic sequences can be envisaged, either migration of a methyl group via a methylenedioxy bridge at ring A (Fig. 1: R_1, R_2 = –CH_2–, cassythicine and berberine, respectively) or methylation at the 2-hydroxyl yielding laurotetanine (**5**), and at the 3-hydroxyl yielding palmatine (Fig. 1: R_1 = Me, R_2 = Me), followed by demethylation of the alternative hydroxyl group.

In *Litsea glutinosa* for the biosynthesis of aporphines the loss of radioactivity of the methoxy group of ring A of reticuline was shown, indicating rather methylation/demethylation than the methylenedioxy mechanism [5]. In the biosynthesis of jatrorrhizine, the methyl migration via a methylenedioxy bridge seems to be well established in *Berberis* species [6, 7]. These studies were performed with radiolabelled precursors. This method affords laborious degradations of the intermediates and end products. Nevertheless, the results remained uncertain with regard to the true metabolic intermediates. Stable labelling with ^{13}C allows the rapid ^{13}C NMR analysis of the position of label in the intermediate without degradation. The (*S*)- and (*R*)-[1-^{13}C,6-O$^{13}CH_3$,N-$^{13}CH_3$]reticuline were synthesized to study possible transmethylations and other aspects of biosynthesis of aporphines and protoberberines by ^{13}C NMR.

RESULTS

(*S*)- and (*R*)-[1-^{13}C,6-O$^{13}CH_3$,N-$^{13}CH_3$]Reticuline and [N-$^{13}CH_3$]reticuline were synthesized and administered to cell suspension cultures of *Peumus boldus*. After 3 days incubation with the triply labelled (*S*)-enantiomer (final conc. 0.15 mM) the cells were extracted. The crude extract was purified by absorption chromatography and the methanol eluate was subjected to ^{13}C NMR analysis. The resulting spectrum (CD_3OD) showed no signals assigned to the labelled precursor which were expected at δ65.9 (C-1), δ56.3 (OMe-6) and δ42.3 (N-Me), indicating complete disappearance of (*S*)-reticuline from the cells. The aporphines, norboldine (laurolitsine) and laurotetanine, were isolated from the cells and purified by TLC. Identification was performed by CI mass spectrometry. The purified aporphines were analysed by ^{13}C NMR (assignments of signals were based on ref. [8]). The spectrum of norboldine exhibited three resonances of ^{13}C enriched carbon atoms (Fig. 2B; 2A natural abundance spectrum): δ54.9 (C-6a), δ56.6 (OMe-10) and δ60.3 (OMe-1). The enlarged resonances of C-6a and OMe-1 agreed with preliminary expectations that the OMe-6 of (*S*)-reticuline should migrate to the OMe-1 position via the methylenedioxy bridge. However, the resonances of OMe-1 represented only about half of the expected intensity (related to the C-6a). Surprisingly the signal of OMe-10 was increased. Unfortunately, estimation of ^{13}C label was not exactly possible because the CI mass spectrum exhibited no fragments which allowed the separate quantification of ^{13}C excess of each labelled carbon atom and, furthermore, because of the unequal distribution of label to 3 (norboldine) and 4 (laurotetra-

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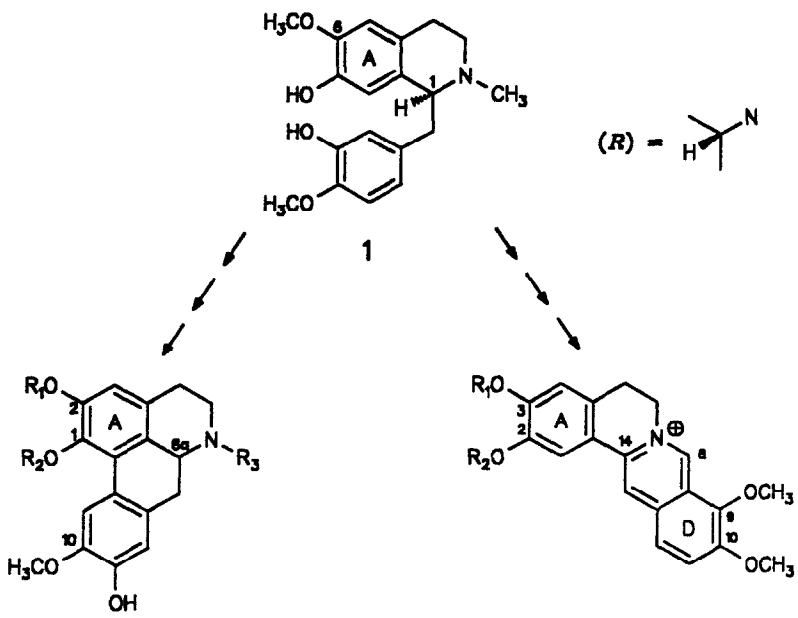


Fig. 1. Changes in the methylation pattern of ring A during the biosynthesis of aporphines and protoberberines starting from (*S*)-reticuline.

nine) carbon atoms, resp. The above results only could be explained by transfer of ^{13}C label either from $O^{13}CH_3$-6 or from $N\text{-}^{13}CH_3$ of reticuline.

To decide between these possibilities, (*S*)-[$N\text{-}^{13}CH_3$]-reticuline was supplied to cell cultures of *P. boldus*. The ^{13}C NMR spectrum of norboldine from this feeding indicated significant enlargement of the OMe-1 resonance at δ60.3 (Fig. 2C) indicating transfer of methyl from N-Me of reticuline to the OH-1 of norboldine. The ^{13}C atom excess of norboldine from this experiment was 2.1%.

For ^{13}C labelling experiments relatively high doses of the labelled precursor are necessary to obtain suitable ^{13}C NMR spectra, but high concentrations of precursor may increase the risk of artifacts. (*S*)-[$N\text{-}^{14}CH_3$]-Reticuline was, therefore, administered to *P. boldus* cell cultures to a final concentration of 1.5 μM. This corresponded to 1% of the final concentration of the ^{13}C labelled (*S*)-reticuline. Confirming the above results, 6.4% of the radioactivity of the fed (*S*)-[$N\text{-}^{14}CH_3$]reticuline were found in norboldine isolated from cells and medium of *P. boldus* cell cultures. The incorporation of the radiolabelled methyl from this low concentration of (*S*)-reticuline into norboldine substantiated that the methyl transfer was not an effect of substrate concentration.

Laurotetanine, bearing two methoxyl groups at ring A at C-1 and C-2, was also isolated from the above feeding experiment with triply labelled (*S*)-reticuline and studied by ^{13}C NMR. Both signals of OMe-1 (δ60.4) and OMe-2 (δ54.9) (Fig. 3) were enlarged but exhibited only about half of the expected intensity as compared with the resonance of C-6a (δ56.4) (formerly C-1 of reticuline) which was used as an internal standard. An explanation could be the splitting of the intermediate methylenedioxy bridge in two different directions forming about 50% $O^{13}CH_3$-1 and 50% $O^{13}CH_3$-2. Additionally, enlargement of OMe-10 (δ56.6) indicated methyl transfer from $N\text{-}^{13}CH_3$ or $O^{13}CH_3$ of reticuline.

To examine the stereospecificity of the incorporation of reticuline, triply labelled (*R*)-reticuline [(*R*)-**1**] was administered to cell cultures of *P. boldus*. The majority of the fed (*R*)-reticuline remained unmodified. The aporphines, norboldine and laurotetanine, were isolated in the same manner as after feeding of the (*S*)-enantiomer and were analysed by ^{13}C NMR spectrometry. The spectrum of laurotetanine did not contain any ^{13}C enriched resonances. However, norboldine showed a small but significant ^{13}C enriched resonance of C-6a (Fig. 2D). The methyl groups of norboldine were not labelled. This may be explained by demethylation/remethylation of a small amount of (*R*)-reticuline and intermediate racemization by oxidation/reduction in ring B via 1,2-dehydroreticuline as observed for the formation of morphine alkaloids [9, 10].

To verify these surprising results obtained with *Peumus boldus* with another cell culture, triply labelled (*S*)-reticuline was fed to cell suspension cultures of *Berberis stolonifera* (final concentration 150 μM) and incubated

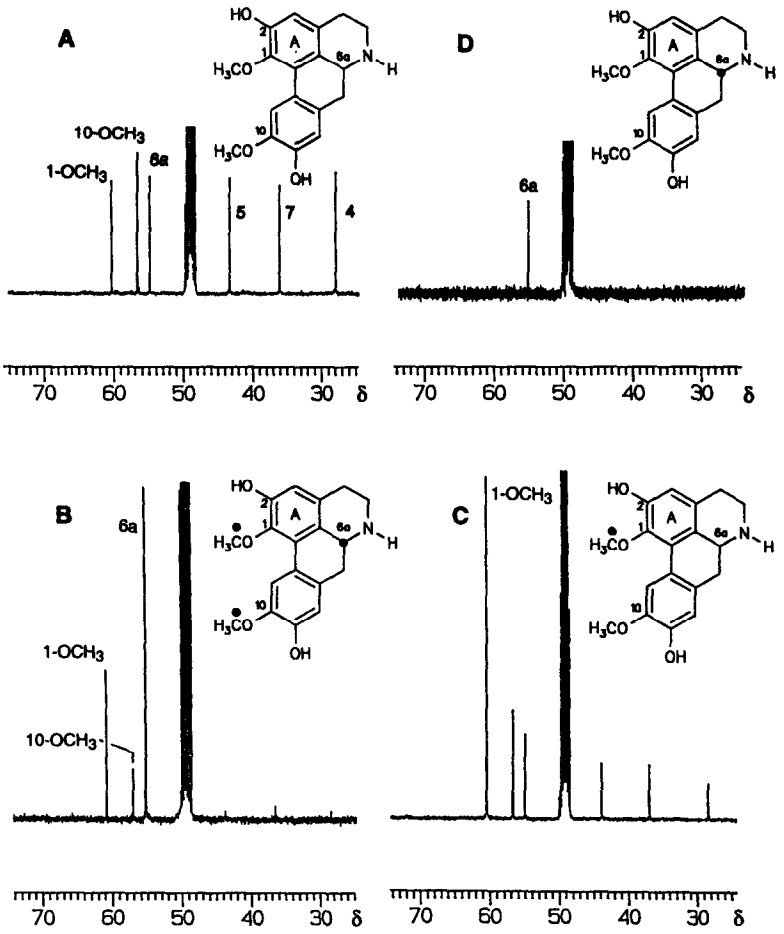


Fig. 2. Excerpts of ^{13}C NMR spectra of norboldine from *Peumus boldus* cell suspension cultures. A: Natural abundance spectrum. B: Biosynthesized from triply-labelled (*S*)-reticuline. C: Biosynthesized from (*S*)-[N-^{13}CH$_3$]reticuline. D: Biosynthesized from triply-labelled (*R*)-reticuline. ● = ^{13}C-label.

for 3 days. After extraction with methanol and absorption chromatography, columbamine and jatrorrhizine were separated, purified by TLC, and subjected to ^{13}C NMR analysis. The spectrum of columbamine (Fig. 4A; assigned according to ref. [11]) showed the expected results. The resonances of the ^{13}C labels were enlarged: δ139.8 (C-14), δ146.4 (C-8) and δ56.7 (OMe-3). The signals δ57.2 (OMe-9) and δ62.6 (OMe-10) were only slightly increased over the natural abundance level. The ^{13}C NMR spectrum of jatrorrhizine (Fig. 4B) also showed increased resonances δ139.9 (C-14), 146.4 (C-8). The methyl signal δ57.4 (OMe-2) was very small. However, δ58.3 (OMe-9) and δ63.0 (OMe-10) were significantly increased, indicating transfer of ^{13}CH$_3$ to both positions at ring D during the biosynthetic transformation from columbamine to jatrorrhizine.

The ^{13}C excess of columbamine and jatrorrhizine, measured by EI mass spectrometry after reduction to yield the tetrahydroprotoberberine type compounds, indicated the occurrence of singly, doubly, and triply labelled columbamine (Table 1A). The A/B-ring fragment (Table 1B) bears two labels (OMe-3 and C-14) and no specimen of this type with one label was present, indicating that no significant demethylation of OMe-3 occurred. About 20% of the D-ring fragment (Table 1C) is labelled with a single ^{13}C (C-8) and only a small amount is doubly labelled (C-8 and OMe-9 or OMe-10). The very small part of this doubly labelled specimen disappeared after demethylation of one methoxyl moiety (Table 1D). These findings confirm the results of ^{13}C NMR (Fig. 4A). The singly labelled portion of the D-ring fragment ion (Table 1C) can bear its label only at OMe-9 or OMe-10 because a singly labelled A/B-ring fragment is lacking and a specimen only labelled at C-8 is not explicable. Therefore, it follows that the doubly labelled specimen of the molecular ion can not be labelled at the C-8 and, consequently, the original N-Me of reticuline must have been lost during this biosynthetic sequence.

The label incorporated into jatrorrhizine was significantly smaller than the label of columbamine. A part of jatrorrhizine was singly labelled in the A/B-ring fragment (Table 1B; mainly C-14), confirming the results of ^{13}C NMR that the OMe-3 of columbamine did not migrate via a methylenedioxy group to OMe-2 of jatror-

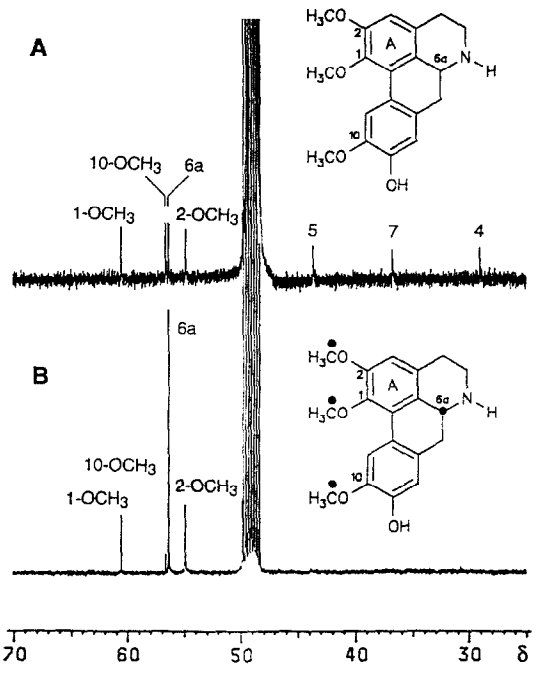


Fig. 3. Excerpts of ^{13}C NMR spectra of laurotetanine from *Peumus boldus* cell cultures. A: Natural abundance spectrum. B: Biosynthesized from triply-labelled (*S*)-reticuline. ● = ^{13}C-label.

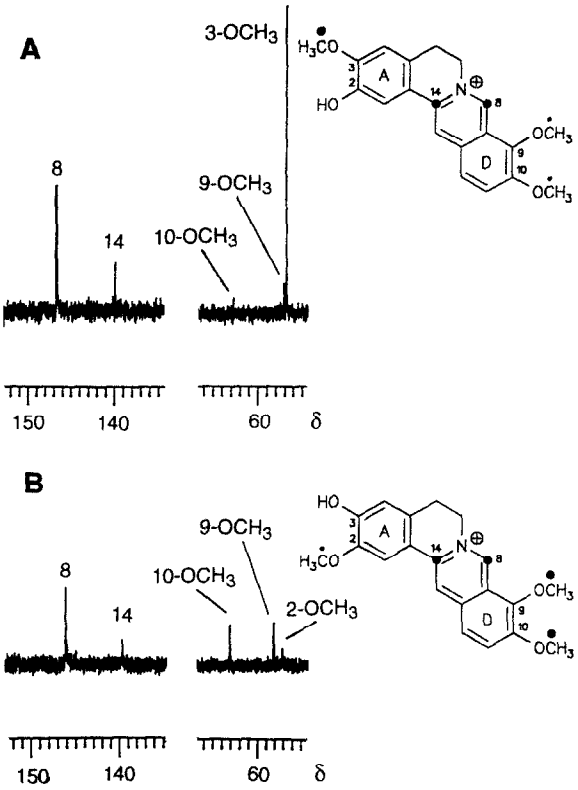


Fig. 4. Excerpts of ^{13}C NMR spectra of columbamine (A) and jatrorrhizine (B), biosynthesized from triply-labelled (*S*)-reticuline in *Berberis stolonifera* cell cultures. ● = High degree of ^{13}C labelling. • = Minor degree of ^{13}C labelling.

rhizine. Another part is singly labelled in the D-ring fragment (Table 1C and D; C-8 or OMe-9 or OMe-10). Consequently, the doubly labelled specimen of the jatrorrhizine molecule (Table 1A) must possess a label in each of its two main parts, A/B-ring fragment (Table 1B) and D-ring fragment (Table 1C and D).

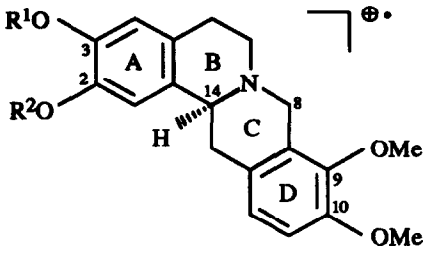
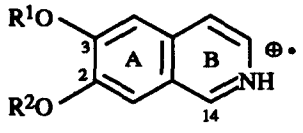
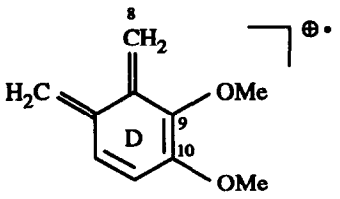
These results are not in contradiction with the isotopic ratio obtained in experiments with doubly labelled (*S*)-reticuline [7], because the radiolabel was not clearly assigned to OMe-2 but also may be situated at OMe-9 and OMe-10. We observed here two examples of such an unusual methyl transfer in the two different plant systems under study. Therefore, this general methyl transfer may also occur in other biosynthetic events or may even be a common phenomenon.

DISCUSSION

For the first time, methyl migration in the biosynthesis of alkaloids has been observed which are not explainable by intramolecular rearrangements. In the interpretation of these results, vigorous dynamics of methyl transfer, including demethylation of biosynthetic intermediates, flux of methyl through the C-1 pool, and remethylation, have to be recognized. This hypothetical mechanism should start with an oxidative attack on the methyl group yielding a hydroxymethyl moiety which is subsequently split off from the intermediate as, for instance, CH_2O by a hydroxymethylase. The CH_2O is transferred to tetrahydrofolate (THF) which, as an intermediate in the biosynthesis of methionine, reacts with homocysteine. Methionine is incorporated into *S*-adenosylmethionine (SAM) from which, in the final step of the flux, the methyl is transferred back to the alkaloid. An argument in support of this hypothesis is the occurrence of the signal δ 14.8 in the ^{13}C NMR spectrum of the crude extract from *Peumus boldus* assignable to methionine. There is, however, considerable need for the intermediates of the C-1 pool, methionine and SAM, in the general metabolism of the plant cell. Methionine is an important building block of proteins and other plant metabolites, while SAM is the general methylating agent in plants. Considering the high flux of the C-1 pool, it is very surprising that ^{13}C, after passing through this sequence, returns to the starting compound. With N-^{14}C labelled reticuline, it was shown that this phenomenon is independent of the concentration of the compound supplied. To account for this shuffling of methyl groups in alkaloid biosynthesis, it must be assumed that demethylation/remethylation proceeds within a subcellular compartment, may be even within a vesicle devoted to the formation of these alkaloids [12].

Results of this type have become possible only by the advances in ^{13}C NMR technology. Detection of methyl migration would not have been possible by standard radiolabelled precursor feeding techniques. Studies of the type presented here exemplify the power of NMR

Table 1. Incorporation of ^{13}C of triply labelled (*S*)-reticuline into columbamine and jatrorrhizine in cell suspension cultures of *B. stolonifera*, measured by EI mass spectrometry after reduction to the tetrahydroberberine type compounds

Fragment ion	*m/z*	Number of ^{13}C	Tetrahydro-columb-amine* (%)	Tetrahydro-jatrorrhizine† (%)
	341	0	65.7	85.3
	342	1	9.6	6.0
	343	2	16.2	8.4
	344	3	8.6	0.3
	345	4	0.0	0.1
A				
	176	0	78.1	85.9
	177	1	0.0	9.8
	178	2	17.1	0.0
	179	3	1.9	0.0
	180	4	2.0	2.8
B				
	164	0	74.2	88.1
	165	1	20.7	11.0
	166	2	2.8	0.0
	167	3	1.0	0.0
	168	4	0.4	0.6
C				
$[C-Me]^{\oplus\bullet}$	149	0	77.1	88.6
	150	1	18.7	10.3
	151	2	0.6	0.0
	152	3	0.1	0.0
	153	4	0.9	1.1
D				

* = Isocorypalmine: R^1 = Me, R^2 = H, obtained by reduction of columbamine with $NaBH_4$.

† = Corypalmine: R^1 = H, R^2 = Me, obtained by reduction of jatrorrhizine with $NaBH_4$.

spectroscopy in the elucidation of alkaloid biosynthetic pathways.

EXPERIMENTAL

General. ^{13}C NMR spectra were recorded at 90.6 MHz on a 360 MHz Bruker Aspect 3000 spectrometer. The solvents (as specified) were used as internal standards. Mass spectra (70 eV) were recorded in the CI mode (*iso*-butane), and in the EI mode. Radioactivity of solutions was measured by liquid scintillation counting (LSC) of aliquots (0.1 ml). TLC was performed on silica gel 60 F_{254} (0.5 mm layer for preparative mode) and silica gel sheets

for analytical mode. TLC plates were analysed in UV light at 254 nm. Distribution of radioactivity on TLC plates was recorded with a Berthold linear analyser LB 2821.

Plant material. Plant cell cultures of *Peumus boldus* and *Berberis stolonifera* were provided by our cell culture laboratory. The suspended cells were grown in Linsmaier–Skoog (LS) medium [13] at 23° on a gyratory shaker (100 rpm) under constant diffuse light (650 lux) in 300 ml flasks containing 150 ml cell suspension. Subculturing was performed every 8 days, using an inoculum of about 70 ml.

^{13}C-Labelling experiments. The filter-sterilized aq. solns of (*S*)- or (*R*)-[1-^{13}C,6-O^{13}CH$_3$,N-^{13}CH$_3$]reticuline or (*S*)-[N-^{13}CH$_3$]reticuline (both >99% ^{13}C-enriched, enantiomerically pure, final concn 150 μM) were administered to the cell cultures at day 3 of growth. The cell suspensions were maintained under identical conditions for another 3 days after which period the cells were harvested.

^{14}C-Labelling experiments. A filter-sterilized aq. soln (1 ml) of enantiomerically pure (S)-[N-^{14}CH$_3$]reticuline (20×10^3 Bq, final concn 1.5 μM) was added to the cell suspension cultures in the same manner as described for ^{13}C-labelling experiments.

Extraction of alkaloids. The cells were harvested by suction filtration through a nylon mesh, homogenized with an ultraturrax grinder at room temp. in MeOH (10 ml/g fr. wt), filtered through a sintered glass funnel, washed with MeOH and resuspended in the same vol. of fresh MeOH. The aq. soln remaining after concn of the combined filtrates *in vacuo* at less than 40° was purified by adsorption chromatography (15 × 50 mm DIAION HP 21).

Isolation and purification of alkaloids. The crude MeOH eluate from the adsorption chromatography column was used for recording ^{13}C NMR spectra. Afterwards, the alkaloids were sepd by TLC and identified by ^{13}C NMR, CIMS and EIMS, resp. The dehydroprotoberberines, columbamine and jatrorrhizine, before EIMS were reduced with $NaBH_4$ to yield the corresponding tetrahydroprotoberberines, tetrahydrocolumbamine (isocorypalmine) and tetrahydrojatrorrhizine (corypalmine) [14]. *Peumus boldus*: TLC: CH_2Cl_2–MeOH–NH_4OH (120:30:1); norboldine R_f 0.30; CIMS *m/z* (rel. int.): 314 $[M+H]^+$ (100), 312 (13); ^{13}C NMR (CD_3OD); δ27.8 (C-4), 35.8 (C-7), 43.2 (C-5), 54.7 (C-6a), 56.6 (OMe-10), 60.3 (OMe-1), 112.8 (C-11), 115.5 (C-3), 115.8 (C-8), 124.6 (C-11a), 126.8 (C-16), 127.9 (C-1a), 129.2 (C-3a), 129.4 (C-7a), 144.7 (C-1), 147.4 (C-9), 148.0 (C-10), 151.4 (C-2); laurotetanine R_f 0.45; CIMS *m/z* (rel. int.): 328 $[M+H]^+$ (100), 326 (8); ^{13}C NMR (CD_3OD): δ29.0 (C-4), 36.8 (C-7), 43.7 (C-5), 54.9 (OMe-2), 60.4 (OMe-1), 56.4 (C-6a), 56.6 (OMe-10). *Berberis stolonifera*: TLC: CH_2Cl_2–MeOH–NH_4OH (70:13:1), columbamine R_f 0.32; EIMS (after reduction with $NaBH_4$) *m/z* (rel. int.): 341 $[M]^+$ (88), 176 (24), 164 (100), 149 (86); ^{13}C NMR (CD_3OD + DMSO-d_6): δ56.7 (OMe-3), 57.2 (OMe-9), 62.6 (OMe-10), 139.8 (C-14), 146.4 (C-8); jatrorrhizine R_f 0.27; EIMS (after reduction with $NaBH_4$) *m/z* (rel. int.): 341 $[M]^+$ (100), 176 (15), 164 (20), 149 (36); ^{13}C NMR (CD_3OD + DMSO-d_6): δ57.4 (OMe-2), 58.30 (OMe-9), 63.00 (OMe-10), 139.9 (C-14), 146.4 (C-8). Detailed MS data on incorporation of ^{13}C into protoberberines are outlined in Table 1.

TLC R_f values and chemical shifts from ^{13}C NMR spectra of labelled compounds corresponded with nonlabelled standards. The ^{13}C NMR assignments were based on literature values [11, 8].

Synthesis of ^{13}C labelled (S)- *and* (R)-*reticulines.* 7-Benzyloxy-1-(3-benzyloxy-4-methoxy)-3,4-dihydro-6-methoxyisoquinoline was prepared by standard methods [15, 16] with slight modifications. The ^{13}C label of the OMe-6 was introduced by $^{13}CH_3I$ to the starting compound, 4-benzyloxy-3-hydroxybenzaldehyde. The ^{13}C label of C-1 originated from K^{13}CN which reacted with 3-benzyloxy-3-methoxybenzylchloride. The hydrochloride of the dihydroisoquinoline (800 mg, 1.5 mmol) was subjected to $NaBH_4$ reduction in MeOH. The resulting *O,O'*-dibenzylreticuline (660 mg, 1.3 mmol, 69%) was quantitatively derivatized with (*S*)-(−)-(α)-methylbenzylisocyanate (MBIC) (345 μl) in dry $CHCl_3$ by stirring 24 hr at room temp. to yield the diastereomeric ureas [17]. After $CHCl_3$–H_2O partition, the organic layer was concd *in vacuo*. Optical resolution of the MBIC derivative was achieved by MPLC on a silica gel column eluting with EtOAc–*n*-hexane (1:1). Successful separation of the diastereomers (each 307 mg, 478 μmol) was confirmed by TLC with toluene–Et_2O (1:1) with R_f 0.39 for the (*S,S*)-enantiomer and R_f 0.44 for the (*S,R*)-enantiomer.

The optically pure enantiomers were fragmented with NaOBu (2 M) in *n*-BuOH, 15 ml) refluxing for 1 hr, 20 ml H_2O were added and the *n*-BuOH was evapd *in vacuo* as an azeotropic mixture with H_2O. The remaining aq. phases were extracted with $CHCl_3$ and the organic layers were evapd again, each yielding 295 mg (593 μmol, 96%) *O,O'*-dibenzylnorreticuline. Methylation of both enantiomers with 150 μl of 20% aq. H^{13}CHO (99% ^{13}C excess) was carried out at room temp. for 2 hr. Afterwards 200 mg $NaBH_4$ were added in small portions and stirred for another 30 min. After $CHCl_3$–H_2O partition, 270 mg (530 μmol, 89%) of (*S*)- and (*R*)-*O,O'*-dibenzylreticuline, resp., were obtained from the organic phases. The hydrochlorides of both enantiomers were subjected to catalytic hydrogenation by Pd-C (10%) in EtOH (4 hr, room temp.). After final purification by preparative TLC with CH_2Cl_2–MeOH–NH_4OH (90:9:1), 160 mg (486 μmol, 96%) of triply-labelled (*S*)- and (*R*)-reticuline, resp., were obtained. The enantiomeric purity of the triply-labelled chiral reticulines was at least 99%. CIMS of both enantiomers revealed more than 99% ^{13}C excess for each of 1-^{13}C, 6-O^{13}CH$_3$ and N-^{13}CH$_3$: *m/z* (rel. int.): 333 $[M+H]^+$ (100), 195 (37); ^{13}C NMR: δ65.9 (C-1), 56.3 (OMe-6), 42.3 (N-Me).

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