Pergamon

SCIENCE DIRECT®

Bioorganic & Medicinal Chemistry 11 (2003) 3913–3919

BIOORGANIC & MEDICINAL CHEMISTRY

In Vivo Monitoring of Alkaloid Metabolism in Hybrid Plant Cell Cultures by 2D Cryo-NMR without Labelling

Christiane Hinse,[a] Christian Richter,[b,†] Alessandro Provenzani[c] and Joachim Stöckigt[a,*]

[a]*Lehrstuhl für Pharmazeutische Biologie, Institut für Pharmazie, Johannes Gutenberg-Universität, Staudinger Weg 5, D-55099 Mainz, Germany*

[b]*Bruker BioSpin AG, NMR Division, Industriestrasse 26, CH-8117 Fällanden, Switzerland*

[c]*Centro di Risonanze Magnetiche, Universita degli Studi di Firenze, Via L. Sacconi 6, I-50019 Sesto Fiorentino, Firenze, Italy*

Received 28 March 2003; accepted 20 June 2003

Abstract—Non-invasive measurements of alkaloid metabolism in plant cell suspension cultures of a somatic hybrid from *Rauvolfia serpentina* Benth. ex Kurz and *Rhazya stricta* Decaisne were carried out. When cell samples were taken sequentially from a stock feeding experiment, measuring times for in vivo NMR of 40 min were sufficient for following conversions of alkaloids at the natural abundance of ^{13}C. Degradation of ajmaline added to the cells at 1.6 mM concentration to raumacline could be monitored after 96 h on a standard 800 MHz NMR instrument (Avance 800). Feeding vinorine an intermediate of ajmaline biosynthesis at 1.8 mM showed with a 500 MHz CryoProbe™ that the alkaloid enters two metabolic routes. Vinorine is intracellularly transformed on route I through vellosimine and 10-deoxysarpagine into sarpagine. On route II, the alkaloid is converted by hydroxylation through vomilenine into the glucoside raucaffricine. Intracellular alkaloid concentrations of ~500 μM are measurable in vivo with cryogenic NMR technology.

Introduction

Biosynthetic pathways in higher plants are elucidated by various approaches. In the past, the leading techniques consisted of feeding experiments with single and multiple radioactive or stable isotope labelled precursors. With the development of plant cell culture methodology it became feasible to reveal biosynthetic sequences by isolating and characterising each participating enzyme. Both of these are, however, invasive approaches that are not only extremely time consuming and tedious, but also might not necessarily reflect the metabolic in vivo situation of the plant cell. Nuclear magnetic resonance spectroscopy (NMR) is therefore frequently applied to study metabolic events non-invasively.[1–3] The major drawback of in vivo NMR is, however, its extremely low sensitivity, which, in principle, can be circumvented by the use of stable isotopes. There are, however, at least two disadvantages to this method: on the one hand it involves a complicated labelling procedure in the case of structural complex natural products and, on the other hand, there are difficulties of product identification, which is usually based on the chemical shift of one labelled nucleus (^{13}C, ^{15}N, ^{31}P) in 1-D NMR or on a pair of ^{1}H and ^{13}C in 2D NMR. The most convenient alternative would, of course, be the use of ^{13}C NMR at its natural abundance, allowing also more rigorous identification of intermediates by a whole set of signals.

Only a few examples of such an approach are known so far. They include some investigations on sucrose metabolism in cell cultures of *Rauvolfia*,[4] which were performed with low field NMR instruments (400 MHz). However, the sugar concentrations were remarkably high, exceeding the range of ~200 mM. But in this case each carbon signal of sucrose and its metabolites was easily detectable with excellent signal to noise ratio. Other examples are related to the glucosylation of phenolic compounds, such as the intracellular formation of arbutin or ursubin and glucosylation of methyl arbutin.[4,5] Again, precursor concentrations were high (~7–9 mM) and 10 mm probe heads made measurements easy,

Abbreviations: HSQC, heteronuclear single-quantum coherence; HMQC, heteronuclear multiple-quantum coherence.

*Corresponding author. Tel.: +49-6131-39-25751; fax: +49-6131-39-23752; e-mail: stoeckig@mail.uni-mainz.de

†Current address: Center for Biomolecular Magnetic Resonance, Institut für Organische Chemie, Goethe Universität Frankfurt, Marie-Curie-Str. 11, N160 D-60439 Frankfurt, Germany.

0968-0896/03/$ - see front matter © 2003 Elsevier Ltd. All rights reserved.
doi:10.1016/S0968-0896(03)00430-9

taking about 90–120 min only. A breakthrough in in vivo NMR occurred when Shachar-Hill et al.[6] and Tse et al.[7] established gradient-assisted inverse correlated ^{1}H–^{13}C 2D NMR spectroscopy to monitor the metabolism of ^{13}C labelled glucose in corn root tips and to measure the concentration of sucrose in plants. When we applied this method to the investigation of the metabolism of unlabelled vanillin in cultivated tobacco cells at 600 MHz, several metabolites could be identified.[8] For instance, vanillylalcohol deriving by reduction of vanillin, followed by its glucosylation or the direct glucosylation of vanillin (final vanillin concentration 6 mM) could be monitored. Measuring times of 38 min were still efficient enough in these experiments.[8] With an 800 MHz instrument, excellent spectra were obtained within 20 min when isatin-3-oxime ($\sim$5.8 mM) was fed to *Rauvolfia* cell suspensions indicating its intracellular transformation into isatin-3-oxime-glucoside.[9] The same methodology recently allowed the monitoring of a short metabolic sequence leading to the alkaloid sarpagine, when the alkaloid vellosimine was added to the cells.[10] This was the first example of successful application of in vivo NMR for elucidating a part of indole alkaloid biosynthesis. Since the accumulation of secondary metabolites is usually low in a biosynthetic pathway, observation of the short biosynthetic route to sarpagine at the natural ^{13}C level clearly demonstrated the general power of high field in vivo NMR with cultivated plant cells. If intermediates and products of the observed biosynthetic sequence differ in their chemical shifts of aromatic signals, product identification becomes easy since the aromatic region is almost free of background signals originating from carbohydrates and amino acids.

In this paper, we are presenting new examples of in vivo NMR investigations without tracer labelling on alkaloid biosynthesis by using hybrid cell cultures and 2D NMR with a standard 800 MHz instrument and cryogenic probe at 500 MHz, with the result that final precursor concentration can now be used between 1.6 and 1.8 mM routinously for such experiments.

Results and Discussion

Plant cell suspension cultures are apparently well suited for the study of metabolic events of primary and secondary products non-invasively by NMR as demonstrated by several examples.[4,5,11–13] But the resolution of spectra cannot be expected to be high because of pronounced sample inhomogeneity. However, thin cell walls, large vacuoles and relatively even cell sizes provide much better homogeneity after settling of cells compared to that reached with plant organs such as leaves, roots, seeds or stem segments. NMR with suspended cells is obviously more related to the liquid state NMR and therefore allows normal high field NMR measurements with satisfying resolution and sensitivity. Application of solid-state NMR (magic angle spinning) might be an interesting method too. With this technique the line width of peaks can be decreased and sensitivity will be improved. But it is not known until now, whether plant cell cultures which are more sensitive compared to microbial cells resist the rigorous conditions of HR-MAS[3] without affecting the cell metabolism.

Sufficient in vivo NMR sensitivity can be reached even at the natural abundance of ^{13}C, when 8, 10 or even 15 mm NMR tubes are used[5] or if high field instruments are applied in combination with inverse correlated gradient assisted ^{1}H–^{13}C spectroscopy [heteronuclear multiple-quantum coherence (HMQC), heteronuclear single-quantum coherence (HSQC].[8] With the technical development of 700–900 MHz machines over the past decade, the sensitivity and the in vivo application of NMR spectroscopy has increased remarkably. However, with the introduction of the cryogenic NMR the signal-to-noise ratio can be increased more than 3-fold.[14,15] This technical improvement is expected to extend broadly NMR applications for analyses of macromolecules,[16] for example, proteins[14,17,18] and nucleic acids.[19] But cryo-NMR has also already been applied recently in a biosynthetic study.[20] We have therefore chosen for our biosynthetic experiments an 800 and 500 MHz NMR instrument, the latter with cryogenic probe, for investigation of the alkaloid metabolism in a hybrid cell suspension culture. These cells originating from somatic hybridisation of *Rauvolfia serpentina* Benth. ex Kurz and *Rhazya stricta* Decaisne cells[21] have been sub-cultured for more than 15 years in liquid nutrition medium. Extensive phytochemical characterisation of this cell system performed several times in our laboratory[22–24] indicated that the cells in principle retained the capability of alkaloid biosynthesis of both parent plants, *Rauvolfia* and *Rhazya*. The overall production of alkaloids in these hybrid cells was in general lower compared to *R. serpentina* culture. However, a low alkaloid content could probably be advantageous, since NMR background signals would be reduced, which could in turn interfere with the signals of the different conversion products. In addition, the hybrid cells are growing as a fine suspension, allowing an even cell packing in the NMR tube for optimum measurements. These conditions therefore basically fulfilled all requirements for use of our previous approach of in vivo NMR employing feeding of unlabelled precursors under normal cell growth conditions in Erlenmeyer flasks, followed by measuring cell samples for 40 min only. Samples were taken sequentially from the stock feeding. Under these experimental conditions, cells retain their metabolic efficiency and metabolic changes can be followed over days if metabolites accumulate and show slow conversions.

We have here performed several sets of in vivo NMR studies in order to evaluate the alkaloid metabolism by these hybrid cells. The first experiments were run on a normal 800 MHz instrument to observe in vivo the metabolism of ajmaline within several days.

Ajmaline was applied to cell cultures of $R\times R$ so that a concentration of 1.6 mM was achieved. In the first spectrum (Fig. 1A, 24 h after addition of the precursor) only ajmaline can be identified by four aromatic signals (A-9, A-10, A-11 and A-12, for assignment see Fig. 1 and Scheme 1) at 7.39/124, 6.79/121, 7.13/128 and 6.72/112 ppm. A second cell sample was taken 96 h after

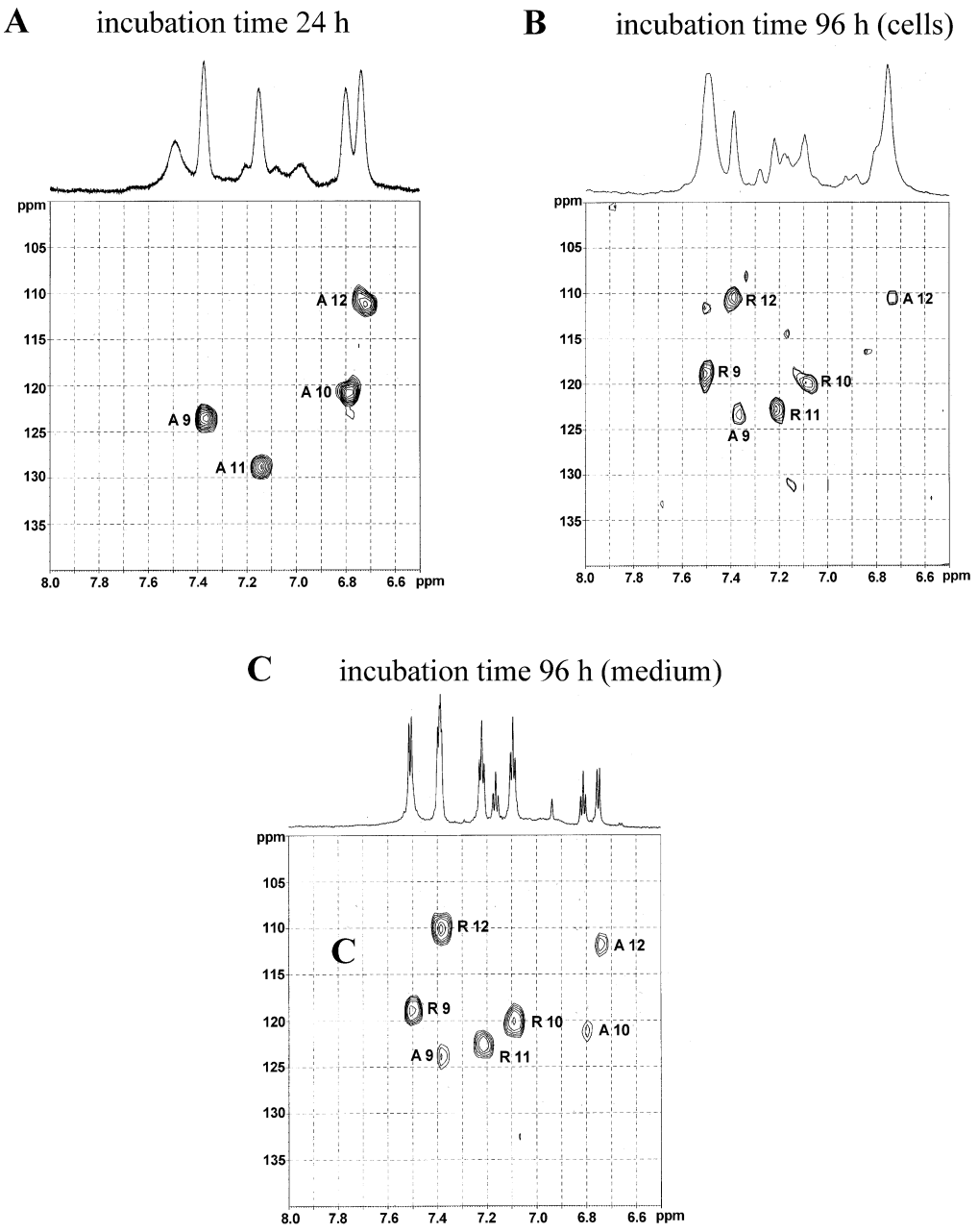
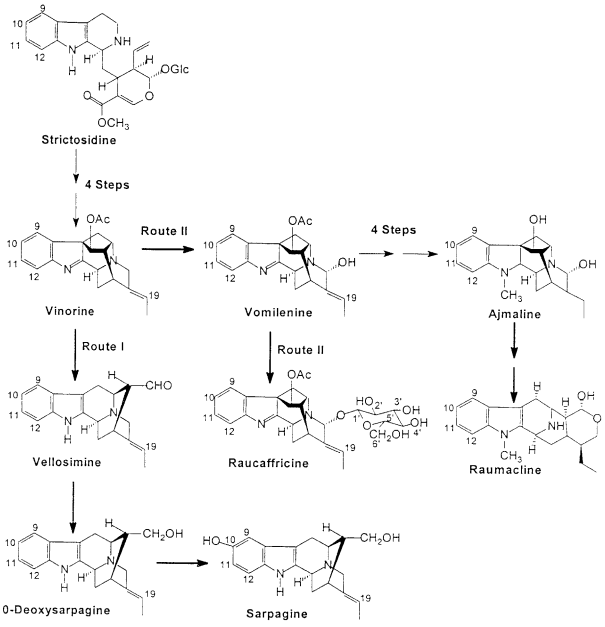


Figure 1. Time course of ajmaline metabolism in hybrid cell suspension of *Rauvolfia*×*Rhazya* shown by 2D NMR spectroscopy. HMQC spectra were recorded: with an Avance 800 Bruker instrument; (A) cell suspension 24 h after addition of ajmaline (scans 16; measuring time 40 min); (B) cell suspension 96 h after addition of ajmaline (scans 32, measuring time 80 min); (C) nutrition medium of cells 96 h after addition of ajmaline (scans 32, measuring time 80 min). The following abbreviations were used: A-9, A-10, A-11, A-12 for the C–H signals of ajmaline; R-9, R-10, R-11, R-12 for those of raumacline (see Scheme 1).

addition of ajmaline. In this case, cells and nutrition medium were separately measured. The spectra indicate that ajmaline is converted into a raumacline type alkaloid (Fig. 1B and C). Former feeding experiments with invasive analysis gave the same results. Thus chemical shifts of the substances were known from literature.[25] The signals of ajmaline are very weak after 96 h whereas four new aromatic signals at 7.50/118, 7.10/120, 7.22/124 and 7.39/111 ppm appear (R-9, R-10, R-11, R-12, for assignment see Fig. 1 and Scheme 1), corresponding to those of raumacline. When raumacline was measured in D_2O and 10% MeOH-d_4 as a standard substance it showed the identical aromatic shifts. It is also interesting to see that, in the case of ajmaline metabolism, the alkaloids can be found both inside the cells and in the nutrition medium. We also have confirmed independently this result by HPLC analyses. At this point it remains, however, to investigate the details of conversion such as uptake of the precursor, localisation of metabolism, excretion of metabolites by the cells, and so on. Applying the in vivo technique described here, such questions can be easily investigated in the near future and it should now be a routine procedure to record natural product metabolism non-invasively under the conditions we mention here.



Scheme 1. Partial illustration of metabolism of vinorine (route I and II), biosynthesis of ajmaline and degradation of ajmaline to raumacline by hybrid cell suspension cultures of *Rauvolfia* and *Rhazya*. The metabolic steps which could be followed by in vivo 2D NMR are marked with bold arrows.

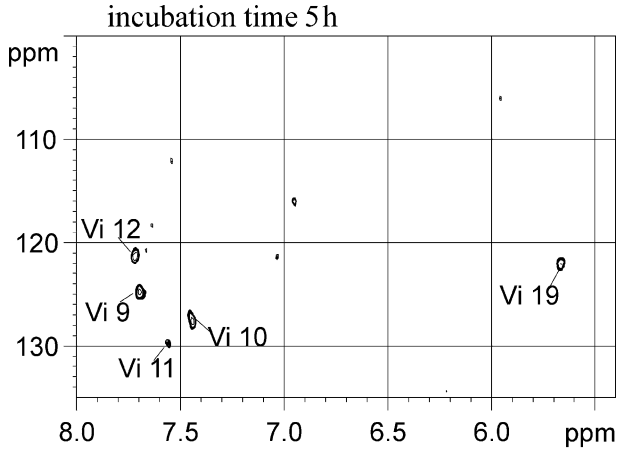


Figure 2. Hybrid cells of $R \times R$ suspension culture measured with a 500 MHz CryoProbe™ instrument (Bruker) 5 h after feeding of vinorine. The HSQC spectrum was obtained after 16 scans and a measuring time of 40 min. The following abbreviations were used: Vi-9, Vi-10, Vi-11, Vi-12 and Vi-19 for the C-atoms of vinorine (see Scheme 1).

In the second series of experiments a central biogenetic intermediate of ajmaline formation in *Rauvolfia*, the indolenine alkaloid vinorine was added to the cells and its metabolic fate was monitored by 500 MHz CryoProbe™ over a period of 96 h.

In this case, vinorine metabolism could also be easily followed after feeding it at 1.8 mM final concentration to the suspended cells. The results indicate an excellent sensitivity because measuring times of usually 40 min were applied. They also showed that vinorine is metabolically rather unstable. The alkaloid is detectable 5 h after feeding by five characteristic cross peaks, Vi-9, Vi-10, Vi-11, Vi-12, Vi-19 (for assignment see Scheme 1), in

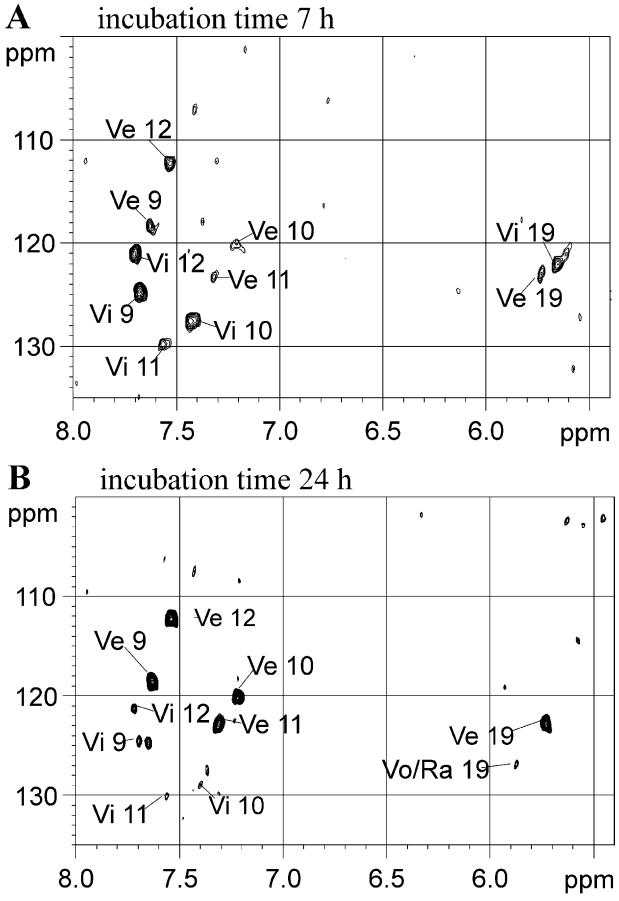


Figure 3. Time course of the first vinorine feeding experiment. HSQC spectra were measured on a 500 MHz CryoProbe™ instrument with 16 scans in 40 min; (A) 7 h and (B) 24 h after the feeding of vinorine to $R \times R$ suspension cultures. The abbreviation Ve-9, Ve-10, Ve-11, Ve-12 and Ve-19 stand for the corresponding C-H signals of vellosimine and/or 10-deoxysarpagine; Vo/Ra-9, Vo/Ra-10, Vo/Ra-11, Vo/Ra-12 und Vo/Ra-19 for C-atoms of vomilenine and/or raucaffricine (see Scheme 1).

the region between 5.5 and 7.7 ppm and 120 and 130 ppm (Fig. 2). In one set of this series, we monitored cell samples 7 and 24 h (Fig. 3A and B) after the addition of vinorine. After only a very short time, vinorine is transformed into an alkaloid of the sarpagan class (Fig. 3A). Vellosimine is obviously the first conversion product, which is reduced later to 10-deoxysarpagine.[10] The cross peaks of vellosimine/10-deoxysarpagine (Ve-9, Ve-10, Ve-11, Ve-12, Ve-19, see Scheme 1) are well separated from those of the applied precursor, vinorine. Figure 3B clearly illustrates an increase in the signals corresponding to the conversion product vellosimine/10-deoxysarpagine after 24 h of feeding. The experiment demonstrates that the hybrid cells deacetylate vinorine and that the deacetylated indolenine structure (not depicted) in turn converts, most probably spontaneously, into the aldehyde vellosimine (Scheme 1). However, it remains unclarified whether this deacetylation process is catalysed by a specific acetylesterase or an unspecific enzyme, a question which cannot be answered by in vivo NMR experiments. As we have recently demonstrated, *Rauvolfia* cells are able to reduce the aldehyde vellosimine very efficiently leading to the alkaloid 10-deoxysarpagine.[10,26] Here, we observe in

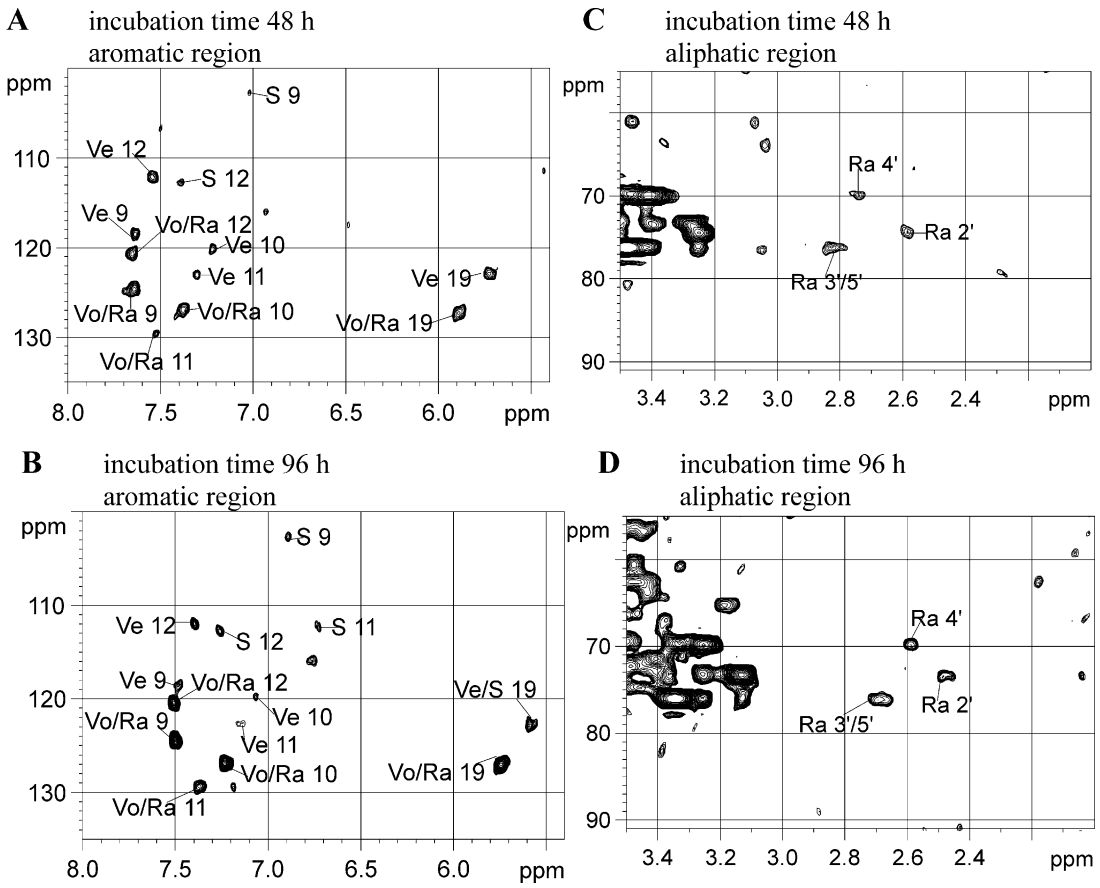


Figure 4. HSQC spectra of the second feeding experiment with vinorine (500 MHz CryoProbe™). Spectra were recorded (A) and (C) 48 h (16 scans, measuring time 40 min) and (B) and (D) 96 h (32 scans, measuring time 80 min) after addition of vinorine. (A) and (B) show the region between 5.4–8.0 and 100–135 ppm. (C) and (D) show the aliphatic region between 2.0–3.5 and 55–90 ppm. Sample of spectra (B) and (D) contained except MeOH-d_4 also 10% D_2O. Abbreviations used: Ve-9, Ve-10, Ve-11, Ve-12 and Ve-19 for the corresponding C–H signals of vellosimine and/or 10-deoxysarpagine; Vo/Ra-9, Vo/Ra-10, Vo/Ra-11, Vo/Ra-12, Vo/Ra-19, Ra-2′, Ra-3′/5′ and Ra 4′ for C–H signals of vomilenine and/or raucaffricine (see Scheme 1).

vivo the same metabolic event catalysed by the hybrid cells.

Apparently, vinorine enters after feeding to the hybrid cells two metabolic routes, since at the same time a signal pattern corresponding to vomilenine or raucaffricine appears (Fig. 3B). This assumption is based on the fact that the signal of the proton of carbon 19 is shifted to lower field. The aromatic signals of vinorine, vomilenine and raucaffricine (Vo/Ra-9, Vo/Ra-10, Vo/Ra-11, Vo/Ra-12, for assignment see Scheme 1 and Fig. 3) overlap under these conditions completely and cannot be resolved at 500 MHz.

The dual role of vinorine becomes, indeed, more pronounced in the second and third experiment. In the second experiment cells were measured after 48 and 96 h (Fig. 4). At this stage of feeding signal intensities fluctuate very strongly and several novel cross peaks can be detected. In Figure 4A, the five signals of 10-deoxysarpagine and five signals corresponding to vomilenine and/or raucaffricine demonstrate increasing alkaloid metabolism. Although the aromatic signals and the proton signal of carbon 19 of vomilenine and raucaffricine are not resolved from each other, identification of raucaffricine is undoubtedly accomplished by three other cross peaks. These signals belong to three cross peaks in the aliphatic region (Ra-2′, Ra-3′/5′, Ra-4′) located between 2.5–2.7 and 70–80 ppm (Fig. 4C). In addition, two very weak novel cross peaks marked with S-9 and S-12 in Figure 4A provide evidence for further alkaloid conversion by hydroxylation of 10-deoxysarpagine. The formed hydroxylated alkaloid is named sarpagine. This hydroxylation process could also be followed recently for the first time by 2D in vivo NMR with a *Rauvolfia* cell suspension[10] and by cell-free experiments indicating that hydroxylation is catalysed by a cytochrome P450 enzyme.[27]

The measurement made in this series confirmed after 96 h of feeding, on the one hand, the high metabolic activity of the hybrid cells. On the other hand, it also demonstrates the useful application of the described in vivo NMR method. The 10-deoxysarpagine cross peaks became after 96 h weaker while those of sarpagine increased, indicating further hydroxylation followed by sarpagine accumulation. Sarpagine is obviously the end product of the route I of vinorine conversion as depicted in Scheme 1. The major component of vinorine metabolism became at this time, however, the glucosylated vomilenine known as raucaffricine (Fig. 4B). The signals belonging to the three cross peaks in the aliphatic region

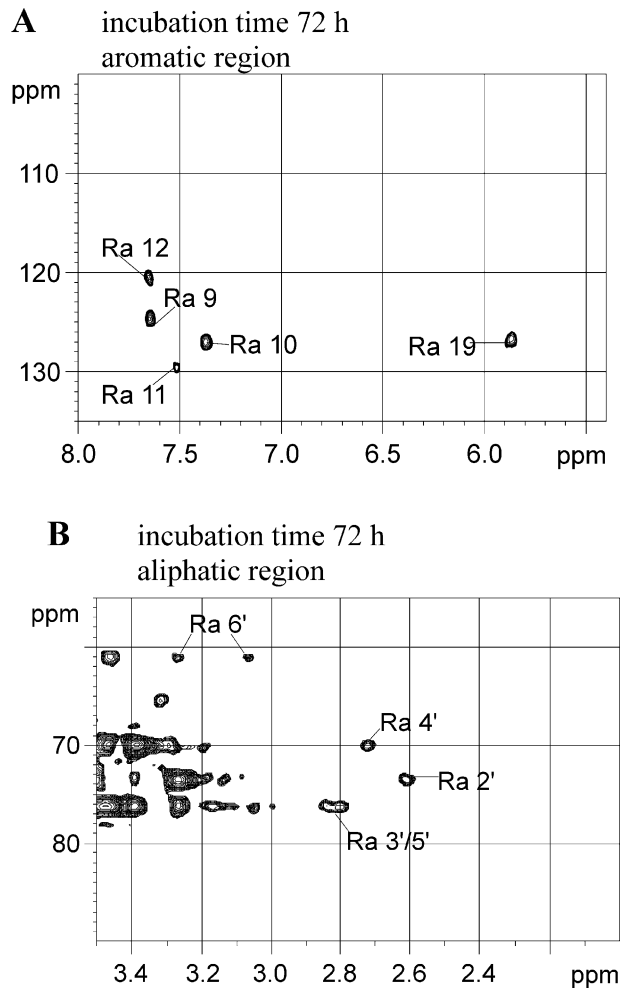


Figure 5. The HSQC spectrum was acquired on a 500 MHz CryoProbe™ instrument with 16 scans in 40 min, after 72 h of addition of vinorine to hybrid plant cell suspension of *Rauvolfia*×*Rhazya*. The abbreviation Ra was used for the corresponding C-atoms of raucaffricine. (A) shows the region between 5.4–8.0 and 100–135 ppm; (B) shows the aliphatic region between 2.0–3.5 and 55–90 ppm.

(Ra-2′, Ra-3′/5′, Ra-4′) showed lower intensities in the spectrum after 48 h compared to 96 h (Fig. 4C and D). From these data, one can conclude that raucaffricine is accumulated at the end of the feeding period and that vinorine also enters the metabolic route II (Scheme 1). A final evidence for two metabolic roles of vinorine is the last feeding experiment of vinorine. In this case, vinorine enters exclusively the second metabolic route, which is demonstrated clearly in Figure 5, in which 10 signals (Ra-9, Ra-10, Ra-11, Ra-12, Ra-19, Ra-2′, Ra-3′/5′, Ra-4′, Ra,-6′) can be observed with about the same intensities. The described in vivo NMR experiments were independently confirmed by HPLC analyses performed after the NMR measurements. For peak assignment reference spectra and literature data were used.[10]

From these in vivo results it can be deduced that the hybrid cells generated from *Rauvolfia* and *Rhazya* express the typical *Rauvolfia* enzymes such as vellosimine reductase, 10-deoxysarpagine hydroxylase, vinorine hydroxylase and raucaffricine synthase. All these proteins are specifically responsible for the biosynthesis of alkaloids of the sarpagan and ajmalan groups. The results obtained also agree with the described alkaloid pattern of the hybrid cells.[21] The pattern consists of sarpagine and vomilenine as trace alkaloids. As a major component, raucaffricine is produced in non-treated cells, which is more pronounced in methyljasmonate induced hybrid cells, when induction is performed exactly as reported by Gundlach et al.[28]

The results now conclusively indicate that during recent years significant progress has been made in the application of 2D NMR for the non-invasive study of biotransformations and biosynthesis of secondary metabolites, especially alkaloids at the natural abundance of ^{13}C. This methodology can obviously be used successfully in a broader context for investigation of intracellular secondary metabolism in plant cell suspension cultures. In this case, precursor concentrations between 1.6 and 1.8 mM routinously allow in vivo monitoring with reasonable measuring times of 40 min. Since under these circumstances at least three alkaloidal intermediates are detectable at the same time, a concentration of already 500 μM provides sufficient signal intensities for monitoring. Future work in this field must concentrate on reducing sugar and amino acid background signals in the aliphatic region in order to use this region for additional structural identification as it has been shown here for intracellular detection of raucaffricine. Together with the dramatic improvement in sensitivity achieved by cryogenic NMR probes, plant cell suspensions combined with the described in vivo approach may open new avenues for future metabolic research. After acquiring more NMR data of in-cell conversion of alkaloids, a complete NMR mapping of alkaloid biosynthesis could probably become possible with the applied *Rauvolfia* cell suspension. With a 'tricky' ^{13}C labelling, such multiple-step sequences starting with stricosidine and ending with raumacline, as depicted in the scheme, may soon become measurable without destruction of their natural environment.

Experimental

Plant material, growth conditions and feeding experiments

Cell suspension cultures of somatic hybrids of *Rauvolfia serpentina* Benth. ex Kurz and *Rhazya stricta* Decaisne (*R*×*R* cells) were routinously grown for 7–9 days at 24 °C in 300 mL Erlenmeyer flasks at 100 rpm in diffuse light (600 lux) containing a nutrition medium developed by Lindsmaier and Skoog.[29] The ajmaline feeding experiments were performed with 4–5-day-old *R*×*R* cell suspensions cultures under the same conditions. Around 80 mg ajmaline were suspended in MeOH-d_4 and D_2O (1:1) and added to 150 mL cell suspension; final concentration was 1.6 mM. Cell samples were sequentially taken 24 and 96 h after the addition of ajmaline. After NMR measurements the nutrition medium was separated from cells and cells were brought to 2 mL ethanol. Cell extracts and nutrition media were analysed by HPLC as an independent proof of alkaloid transformation.

For vinorine feeding experiments 10–15 mL cell suspension were transferred into 50 mL Erlenmeyer flasks 1 day before feeding. Vinorine was dissolved in MeOH-d_4 and added to 4–5-day-old cells so that a concentration of 1.8 mM was achieved. After 5, 7, 24, 48, 72 and 96 h of the addition of vinorine about 2.5 mL aliquots of suspended cells were sequentially taken, transferred to 5 mm NMR tubes and spectra were obtained. For HPLC analyses, samples were taken from the flask 48, 96 and 120 h after addition of vinorine, cells and nutrition media were separated and cells were extracted with methanol. Nutrition media and methanol extracts were analysed by HPLC.

In vivo NMR measurements

Plant cells were settled by gravity after transfer into the 5 mm NMR tubes for about 30 min before measurements. Spectra of ajmaline metabolism were recorded on an 800 MHz NMR instrument (AVANCE 800, Bruker, Karlsruhe, Germany) equipped with a TXI 1H{13C/15N} XYZ-GRD probe head. The inv4gp pulse sequence was applied. HMQC experiments were acquired with 128 increments in f1 and 16–32 scans in 40–80 min using an acquisition time of 103 ms and a relaxation delay of 1 s.

For monitoring in vivo feeding with vinorine a Bruker 500 MHz UltraShield™ instrument equipped with a 5 mm 1H {13C/15N} Z-Grad CryoProbe™ head was used. HSQC spectra (pulse sequence invietgpsi) were obtained under the following conditions: 128 increments in f1, 16–32 scans in 40–80 min, acquisition time 170 ms, relaxation delay of 1 s.

Chromatographic methods

HPLC analyses were performed on a Merck/Hitachi System (D 2500 Integrator, AS 2000 Autosampler, UV/VIS Detector L 4250, L 6200 Gradient Pump) and a Merck LiChrospher® RP select B (125×4 mm) column with a 4×4 mm pre-column of the same type. For analyses of ajmaline metabolism experiments, the flow rate was 1 mL/min and the elution was carried out by using a mixture of acetonitrile (A) and potassium-phosphate-buffer [6.66 mg KH_2PO_4/2.8 mL H_3PO_4 (85%)] (B). The gradient used was 10% A at the start of each run up to 45% A within 30 min to 10% A after 31 min. UV detection was at 284 nm.

The vinorine metabolism was observed by using the solvent system acetonitrile (A) and ammonium-carbonate-buffer (10 mM) with a gradient set initially for the first 6 min at 32% A (flow rate 1.25 mL/min) and changed to 60% A within 0.1 min for the next 5.9 min (flow rate 1.5 mL/min). At 12.1 min the gradient run back to 32% A (flow rate 1.25 mL/min) for 8 min again. UV detection was at 280 nm.

Acknowledgements

The assistance of Massimo Lucci and Fabio Calogiuri (Florence, Italy) is specially acknowledged. We thank the Fonds der Chemischen Industrie (Frankfurt/Main, Germany), the EU (Access to Research Infrastructures Action of Improving Human Potential Programme, Contract No. HPRI-CT-1999-0009) and Johannes Gutenberg-Universität, Mainz (Support for 'Introduction of novel Methodologies', Programme B5) for providing financial support.

References and Notes

1. Schneider, B. *Planta* **1997**, *203*, 1.
2. Roberts, J. K. M. *Trends Plant Sci*. **2000**, *5*, 30.
3. Ratcliffe, R. G.; Shachar-Hill, Y. *Annu. Rev. Plant Physiol. Plant Mol. Biol*. **2001**, *52*, 499.
4. Lutterbach, R.; Stöckigt, J. *Helv. Chim. Acta* **1994**, *77*, 2153.
5. Lutterbach, R.; Stöckigt, J. *Phytochemistry* **1995**, *40*, 801.
6. Shachar Hill, Y.; Pfeffer, P. E.; Germann, M. W. *Anal. Biochem*. **1996**, *243*, 110.
7. Tse, T. Y.; Spanswick, R. M.; Jelinski *Protoplasma* **1996**, *194*, 54.
8. Schroeder, C.; Sommer, J.; Humpfer, E.; Stöckigt, J. *Tetrahedron* **1997**, *53*, 927.
9. Hinse, C.; Unger, M.; Provenzani, A.; Stöckigt, J. *Nat. Prod. Lett*. **2001**, *15*, 119.
10. Hinse, C.; Sheludko, Y.; Provenzani, A.; Stöckigt, J. *J. Am. Chem. Soc*. **2001**, *123*, 5118.
11. Jendrzejewski, S. *Phytochemistry* **1990**, *29*, 135.
12. Schneider, B.; Zenk, M. H. In: *Plant Tissue Culture and Gene Manipulation for Breeding and Formation of Phytochemicals*; Oono, K., Hirabayashi, T., Kikuchi, S., Handa, H., Kajiwara, K., Eds.; Sato: National Institute of Agrobiological Resources, Tsukuba, 1992; p 323.
13. Schroeder, C. PhD Thesis, Johannes Gutenberg-Universität Mainz, 1998.
14. Hajduk, P. J.; Gerfin, T.; Boehlen, J. M.; Häberli, M.; Marek, D.; Fesik, S. W. *J. Med. Chem*. **1999**, *42*, 2315.
15. Serber, Z.; Dötsch, V. *Biochemistry* **2001**, *40*, 14317.
16. Serber, Z.; Richter, C.; Dötsch, V. *ChemBioChem* **2001**, *2*, 247.
17. Serber, Z.; Richter, C.; Moskau, D.; Boehlen, J. M.; Gerfin, T.; Marek, D.; Häberli, M.; Baselgia, L.; Laukien, F.; Stern, A. S.; Hoch, J. C.; Dötsch, V. *J. Am. Chem. Soc*. **2000**, *112*, 3554.
18. Banci, L.; Felli, I. C.; Kummerle, R. *Biochemistry* **2002**, *41*, 2913.
19. Sashital, D. G.; Allmann, A. M.; Van Doren, S. R.; Butcher, S. E. *Biochemistry* **2003**, *42*, 1470.
20. Bringmann, G.; Wohlfarth, M; Rischer, H.; Grüne, M.; Schlauer, J. *Angew. Chem. Int. Ed*. **2000**, *39*, 1464.
21. Kostenyuk, I. A.; Lubaretz, O.; Borisyuk, N.; Voronin, V.; Stöckigt, J.; Gleba, Y. *Theor. Appl. Genet*. **1991**, *82*, 713.
22. Kostenyuk, I. A.; Lubaretz, O. F.; Endreß, S.; Gleba, Y. Y.; Stöckigt, J. *Nat. Prod. Lett*. **1995**, *5*, 303.
23. Sheludko, Y.; Gerasimenko, I.; Unger, M.; Kostenyuk, I.; Stöckigt, J. *Plant Cell Rep*. **1999**, *18*, 911.
24. Gerasimenko, I.; Sheludko, Y.; Stöckigt, J. *J. Nat. Prod*. **2001**, *64*, 114.
25. Polz, L.; Stöckigt, J.; Takayama, H.; Uchida, N.; Aimi, N.; Sakai, S.-I. *Tetrahedron Lett*. **1990**, *31*, 6693.
26. Pfitzner, A.; Krausch, B.; Stöckigt, J. *Tetrahedron* **1984**, *40*, 1691.
27. Yu, B.; Ruppert, M.; Stöckigt, J. *Bioorg. Med. Chem*. **2002**, *10*, 2479.
28. Gundlach, B.; Mueller, M. J.; Kutchan, T. M.; Zenk, M. H. *Proc. Natl. Acad. Sci. U.S.A*. **1992**, *89*, 2389.
29. Linsmaier, E. M.; Skoog, F. *Physiol. Plant* **1965**, *18*, 100.