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DYNAMICS OF THE BIOSYNTHESIS OF METHYLURSUBIN IN PLANT CELLS EMPLOYING *IN VIVO* ^{13}C NMR WITHOUT LABELLING*

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Key Word Index—*Rauwolfia serpentina*; Apocynaceae; cell suspension; *in vivo* NMR; ^{13}C natural abundance; biosynthesis; methylursubin.

Abstract—*In vivo* NMR experiments with a digital 600 MHz instrument, exploiting the natural abundance of ^{13}C, allowed us for the first time to follow the biosynthesis of the newly detected glycoside, methylursubin (4-methoxyphenyl-*O*-β-D-primeveroside), from 4-methoxyphenol through the intermediate methylarbutin in cell suspensions of the Indian medical plant, *Rauwolfia serpentina*. The metabolic dynamics indicate that, within 48 hr, 4-methoxyphenol is almost completely converted into the primeveroside, methylursubin. Because of the higher sensitivity at 150.9 MHz compared to that at 100.6 MHz, measuring times could be reduced to 1.5 hr. This allows detailed monitoring of the conversion of 4-methoxyphenol with an excellent signal-to-noise ratio.

INTRODUCTION

High density cell suspension cultures of *Rauwolfia serpentina* have previously been demonstrated not only to be an excellent tool for the study of biosynthetic sequences of alkaloids at the enzymatic level [1, 2], but also of *in vivo* biotransformations of exogenously-added compounds. When, for instance, one of the major endogenous alkaloids of these *Rauwolfia* cells, the antiarrhythmic ajmaline, is supplied exogenously to this system, the alkaloid is converted into a novel group of indole bases, named raumaclines [3 and refs cited therein]. A range of phenolic compounds added to the plant cell is, however, transformed into the corresponding glucosides as has been described frequently for various plant cell systems [e.g. 4–6].

Recently, we reported that our *Rauwolfia* cells are an efficient system for the detoxification of hydroquinone by glucosylation [7]. The observed transformation rate exceeds significantly that published for cell suspension cultures of *Catharanthus roseus* [8,9]. In contrast to the *Catharanthus* cells, hydroquinone is converted by the *Rauwolfia* system into a novel diglycoside, which was identified as 4-hydroxyphenyl-*O*-β-D-primeveroside [10]. We are now calling this new natural product ursubin. These results indicate a broader glycosylating capability for the *Rauwolfia* cells when compared to the *Catharanthus* system. Recent *in vivo* NMR experiments with those cultivated *Rauwolfia* cell suspensions, which had allowed the measurement of metabolic changes at the natural abundance of ^{13}C for the first time, clearly demonstrated that the primeveroside is formed in relatively low concentration after nearly complete conversion of hydroquinone into its monoglucoside [11]. Moreover, these studies allowed us also to follow the metabolism of sucrose, which is the carbon source for the *Rauwolfia* cells and which influences their glucosylating capacity.

Because of the low accumulation and slow biosynthesis of ursubin, it is difficult to monitor its formation *in vivo* by ^{13}C NMR routinely if unlabelled hydroquinone is supplied. Therefore, we investigated the metabolic flux of 4-methoxyphenol (**1**) in *Rauwolfia* cells by application of the methods established recently [11]. Herein we report on the structural determination of the novel primeveroside of **1**, named methylursubin (**3**), and the direct monitoring of the metabolic dynamics of **1**. These experiments were performed mainly with a 600 MHz instrument in order to obtain the best sensitivity for the detection of the unlabelled metabolites of **1** within the living plant cells.

RESULTS AND DISCUSSION

In continuation of our work on the *in vivo* formation of glycosides by cultivated plant cells on a preparative scale, we added **1** to high density cell suspensions of *R. serpentina* grown for four days in Linsmaier and Skoog medium [12]. At this time, the cells have taken up nearly all of the sucrose from the nutrition medium and exhibit good

*In honour of Professor Richard Neidlein's 65th birthday.
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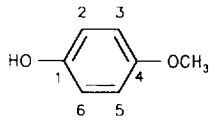
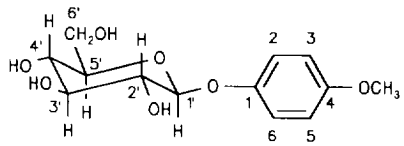
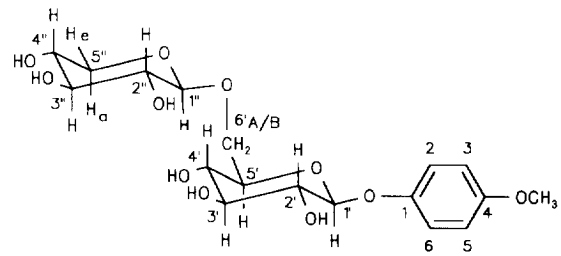


4-Methoxyphenol (1)



Methylarbutin (2)



Methylursubin (3)

transformation rates. From such a feeding experiment, we could isolate the *O*-glucoside of **1**, which is a known natural product, named methylarbutin (**2**). For the structural elucidation of this glucoside, which has been isolated from chemical varieties of *Arctostaphylos uva ursi* (Ericaceae) [13], the resonances of all carbon atoms were assigned in the ^{13}C NMR spectrum (see Experimental).

Compound **2** is one of the major components of the cell culture when **1** is allowed to be metabolized for one day. Further analysis of the cells indicated, however, the biosynthesis of a second derivative of **1**, which was more polar than **2** as shown by TLC analysis. Infrared spectroscopy revealed very similar data to those known for ursubin (4-hydroxyphenyl-*O*-β-D-primeveroside), which suggested the formation of 4-methoxyphenyl-*O*-β-D-primeveroside (**3**). This suggestion became evident when the compound was analysed by FD mass spectroscopy and NMR spectroscopy. In fact, the spectroscopic data obtained (see Experimental) were in complete agreement with the structure of **3**, especially when compared with ^{1}H and ^{13}C data published for ursubin, which we have recently identified [10]. This conversion product of **1**, which we now name methylursubin, has not yet been detected in differentiated plants including those which synthesize **2**.

Because the ^{13}C NMR resonances of C(3)/C(5) (115.3 ppm) and C(2)/C(6) (116.3 ppm) of **1** do not overlap with the appropriate signals of **2** or its primeveroside (**3**), the aromatic region should in principle allow tracking of the metabolism of **1** into **2** or **3** by *in vivo* NMR spectroscopy. In contrast, the corresponding pairs of signals of compounds **2** and **3** cannot be distinguished. Both structures show signals for C(3)/C(5) and C(2)/C(6) at 114.9 and 118.0 ppm, respectively. On the other hand, the analysis of resonances in the region of anomeric carbons indicated that the appropriate signals of the glycosides **2** and **3** are quite well separated. Whereas C(1′) of **2** is located at 101.0 ppm, C(1′) and C(1″) of **3** resonate at 100.7 and 103.0 ppm, respectively. On the basis of such ^{13}C data, it should be possible to monitor the metabolism of **1** in *Rauwolfia* cell suspensions under non-destructive conditions at the natural abundance of ^{13}C if high field *in vivo* NMR is applied.

The necessary high amounts of metabolites required when using unlabelled **1** can be obtained by application of high density cell suspensions, when about 250–300 g of fresh *Rauwolfia* cells are inoculated with 150 ml nutrition medium in one litre Erlenmeyer flasks. After the cells were allowed to adjust for four days at this high density stage, the fine suspension obtained was fed with relatively high amounts of **1** (300–400 mg), which was solubilized in sucrose-free LS medium. Under these feeding conditions, cells survived the high phenol concentrations and were not damaged. After 48 hr, **1** was almost completely transformed into its glycosides **2** (10.5%) and **3** (88.0%). Only small traces of **1** remained unchanged in the nutrition medium. Because some of our preliminary experiments, analysed by HPLC and ^{13}C NMR spectroscopy (100.6 MHz), indicated a rapid metabolism of **1** during the period between 4 and 20 hr after feeding, short measuring times were a prerequisite for the observation of the biosynthesis of **2** and of the diglycoside (**3**) under *in vivo* conditions in the viable plant cells. Taking advantage of the high sensitivity of a 600 MHz instrument, we measured the metabolic flux of **1** during that time by ^{13}C NMR at 150.9 MHz. In fact, measuring times could be adjusted to 1.5 hr, which are extremely short for *in vivo* NMR experiments, but still gave excellent signal-to-noise ratios (S/N) even when the precursor was not labelled (Fig. 1b–e). Because the entire feeding experiment was performed over a period of two days, cells were not cultivated directly in the NMR tube, but were periodically taken from the flask for each measurement.

As illustrated in Fig. 1a, which was recorded at 100.6 MHz 10 min after addition of **1**, only the starting material **1** was observed, based on the signals at 115.3 and 116.3 ppm. The small signal at 103.6 ppm belongs to C(2) of the fructose moiety of sucrose, which independently also allows observation of changes in the disaccharide concentration during the feeding experiment. In the following spectra measured at high field (150.9 MHz), the dynamics of the conversion of **1** can easily be observed. After only 4.5 hr, significant transformation of **1** into **2** had occurred (Fig. 1b). Estimated by the intensities of the signals at 114.9 and 118.0 ppm, which represent C(3)/C(5) and C(2)/C(6) of **2**, *ca* 35% of the intracellular **1** might have been transformed into its glucoside (**2**). However, because these carbon signals would not be separated from those of **3**, the spectra were searched for signals of the corresponding anomeric carbons of **2** and **3**, which can easily be distinguished. After 9 hr of feeding time, only the C(1′) resonance of **2** at 101.0 ppm could be

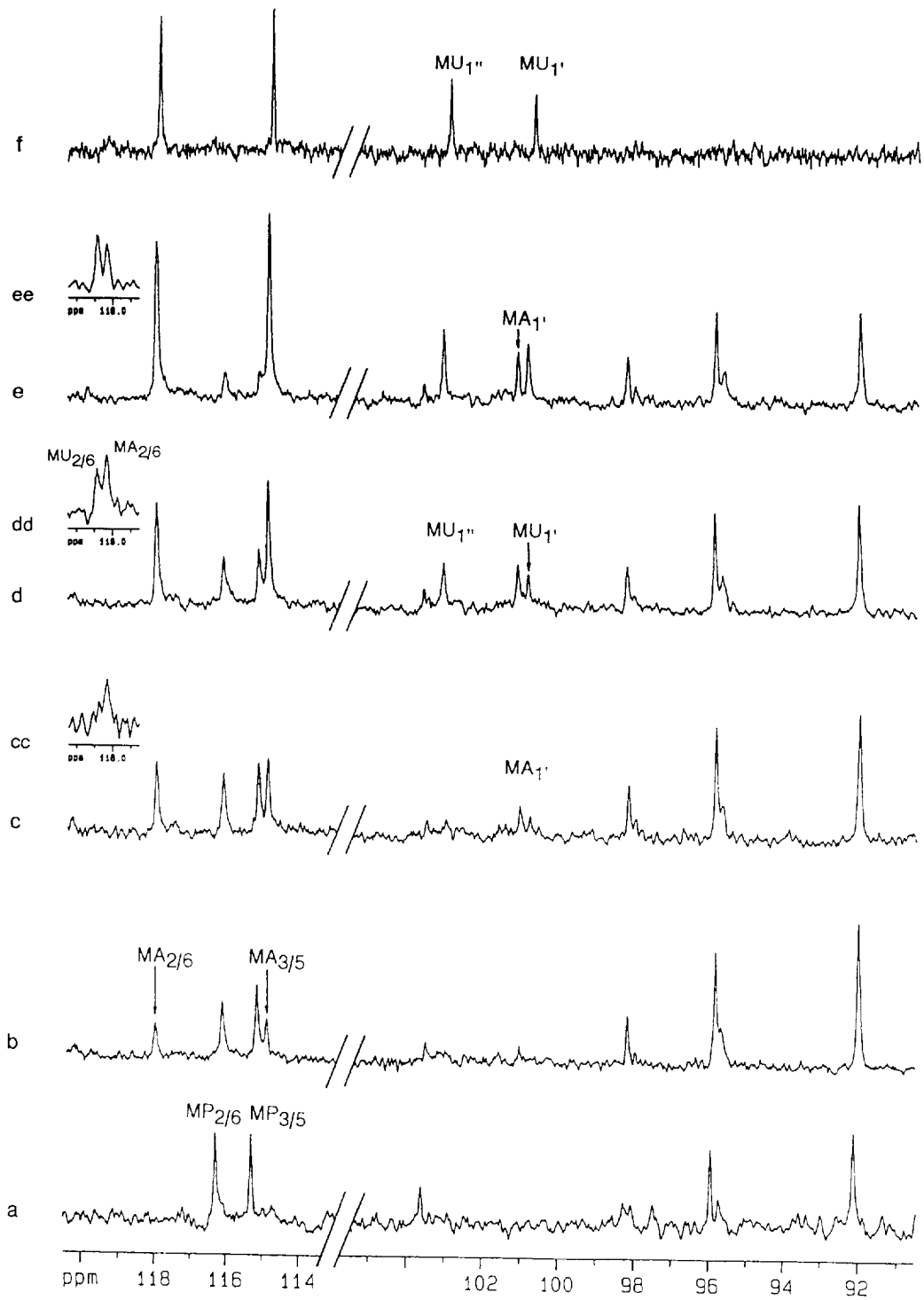


Fig. 1. Time-course of the biotransformation of 4-methoxyphenol (**1**) into methylursubin (**3**) through the intermediate methylarbutin (**2**), shown by 150.9 MHz *in vivo* ^{13}C NMR spectroscopy (spectra b–e) of cells from suspension cultures of *R. serpentina*. Spectra recorded sequentially starting (a) 10 min (time zero, 100.6 MHz ^{13}C data), (b) 4.5 hr, (c) 9 hr, (d) 13 hr, (e) 21 hr, and (f) 48 hr (100.6 MHz ^{13}C data) after addition of 3 mmol (372 mg) of **1** to a cell suspension of *ca* 250 g wet cells cultivated for 4 days in LS medium (450 ml total volume). All measured samples contained about 15% D_2O. Each spectrum represents 3700 accumulations (measuring time 1.5 hr) with a pulse repetition time of 1.3 sec. Digital resolution is 0.57 Hz per data point for spectra b–e and 0.76 Hz per data point for spectra a and f. Resolution-enhanced spectral parts shown in (cc), (dd) and (ee) were obtained by Gaussian-multiplication (GB, 0.15; LB, −6 Hz). Abbreviations used: $MP_{2/6}$, $MP_{3/5}$: aromatic C-atoms of **1**, $MA_{2/6}$, $MA_{3,5}$: aromatic carbons of **2**; $MA_{1'}$: C(1) of the glucose unit of **2**; $MU_{2/6}$: aromatic C-atoms of **3**; $MU_{1'}$; $MU_{1''}$: C(1) of the glucose unit and C(1) of the xylose unit of **3**.

recognized just at the limit of detection (S/N < 2), with low intensity (Fig. 1c). When, however, the signal resolution at the aromatic region was significantly enhanced by Gaussian-multiplication, broadening of the signal at 118.0 ppm (Fig. 1cc) pointed already at this early stage of feeding to the biosynthesis of traces of **3**. This effect was much more pronounced after 13 hr, as illustrated in Fig. 1dd. In fact, the beginning of the accumulation of **3** can also be observed at this point (Fig. 1d) by the appearance of resonances for the anomeric carbons C(1′) and C(1″) at 100.7 and 103.0 ppm, respectively. At this time, more than 60% of intracellular **1** had been metabolized. Based on the low field signal in Fig. 1dd, the ratio between **2** and the primeveroside (**3**) was *ca* 1.3:1. It should be noted that these data could not be obtained by 100.6 MHz NMR within a 1.5 hr measuring time, but only at 150.9 MHz, because the sensitivity at the lower field was not adequate.

After 21 hr of feeding, *ca* 85% of **1** was glycosylated. From Fig. 1ee one can roughly estimate that the mixture of glycosides consists of *ca* 45% of **2** and 55% of **3**. A similar ratio can be determined, using the signal intensities of C(1′) of **2**, compared with those of C(1′) and C(1″) of **3**. After an additional 14 hr, the ratio of **2** and **3** was almost unchanged (data not shown) and the feeding experiment was terminated because of limited access to the 600 MHz instrument. From comparable experiments on a 400 MHz instrument we knew, however, that the biosynthesis of **3** should be nearly completed after 48 hr, provided that optimum cell culture conditions are applied (Fig. 1f). The only four resonances observed in the discussed ppm region can be attributed to C(2)/C(6), C(3)/C(5), C(1′) and C(1″) of the target compound **3**. The ^{13}C spectrum of the *Rauwolfia* cells measured after 48 hr, including the higher field resonances, also showed almost exclusively signals for the primeveroside (**3**) (Fig. 2a).

The experiments performed at 150.9 MHz clearly indicated that the conditions of biosynthesis were not optimal. Indeed, the feeding experiments described were not performed in a plant cell culture unit, but in a standard chemistry laboratory. Temperatures at this 'application laboratory' where the cells were grown fluctuated between 27° and 19° during the day and night time, respectively which, of course, influenced *in vivo* biosynthesis. Under controlled cell culture conditions the metabolism of **1** was clearly enhanced. Whereas the appropriate signals between 105 and 60 ppm continuously decreased at the beginning of experiment (4.5–13 hr), they remained unchanged during the last 14 hr. In sharp contrast to this situation, were the experiments carried out under more controlled conditions (100.6 MHz), where complete sugar metabolism was recorded [11].

The current NMR studies illustrate the efficient dynamics of *in vivo* transformation of **1**, catalysed by *Rauwolfia* cell suspensions. These reactions take place within the plant cells and do not occur in the surrounding nutrition medium. When the supernatant of the cell suspension was analysed 35 hr after feeding, besides the internal standard (ethanol), only traces of the starting product **1** could be recognized (Fig. 2b). From these observations it can be concluded that the glycosides **2** and **3** are not excreted by the cells into the nutrition solution. In accordance with the NMR experiments, HPLC analyses from such feeding experiments indicated that less than 1% of **2** and **3** occur outside of the cells after two days of feeding.

From the data presented here one can assume that the biosynthesis of the diglycoside (**3**) proceeds at the expense of **2**. Compound **2** finally disappears within the analysed time-frame, whereas the concentration of **3** increases dramatically. Several biosynthetic mechanisms for the formation of **3** can be expected, e.g. a second glucosylation of **2** followed by fast oxidation and decarboxylation of the corresponding C(6″) or the transformation of glucose into arabinose, which then is converted into xylose followed by activation and transfer to the 6′-position of **2**. A further possibility, the initial formation of primeverose, which could be attached to the phenolic structure, can be excluded by our NMR results. Our original hope of solving this biosynthetic problem at 150.9 MHz by an *in vivo* study without ^{13}C-enriched precursors has not yet been fulfilled. Although we could demonstrate excellent sensitivity compared to that of 100.6 MHz and ease of handling of the 600 MHz instrument, which meant that for each *in vivo* measurement routinely performed only about 3 min were necessary for optimization of the instrument, the spectral resolution was not adequate for the assignment of each carbon in the 'sugar region' of the proton-decoupled ^{13}C NMR spectra. This, however, would be necessary if further metabolites involved in the biosynthesis of **3** were identified, e.g. UDP-xylose. One must, however, keep in mind that a minimum of nine monosaccharide units in quite different concentrations, like the remaining sucrose, α-, β-D-glucose, β-D-fructopyranose, β-D-fructofuranose and the three sugar residues of **2** and **3**, leads to a total of 53 ^{13}C NMR signals. Of these carbon signals 42 appear between 80 and 60 ppm and give a pattern which cannot be easily unravelled (Fig. 2c).

It would appear that more advanced NMR techniques than proton-decoupled ^{13}C NMR need to be applied in order to solve the biosynthesis of **3** unambiguously by *in vivo* NMR at the natural abundance of ^{13}C. On the other hand, the application of ^{13}C-labelled precursors (e.g. monosaccharides), which has been reported recently in the field of alkaloid biosynthesis [14–16], should simplify these *in vivo* investigations. The appropriate investigations are now in progress. The current study also indicates that high-field NMR might become a very powerful method for tracking anabolic and catabolic reactions under *in vivo* conditions. In the case when metabolically efficient plant cell systems are available and the most sensitive NMR instruments can be applied, the natural abundance of ^{13}C will be sufficient to permit monitoring of such processes directly in living cells, thus avoiding the complicated and time-consuming labelling of biosynthetic precursors.

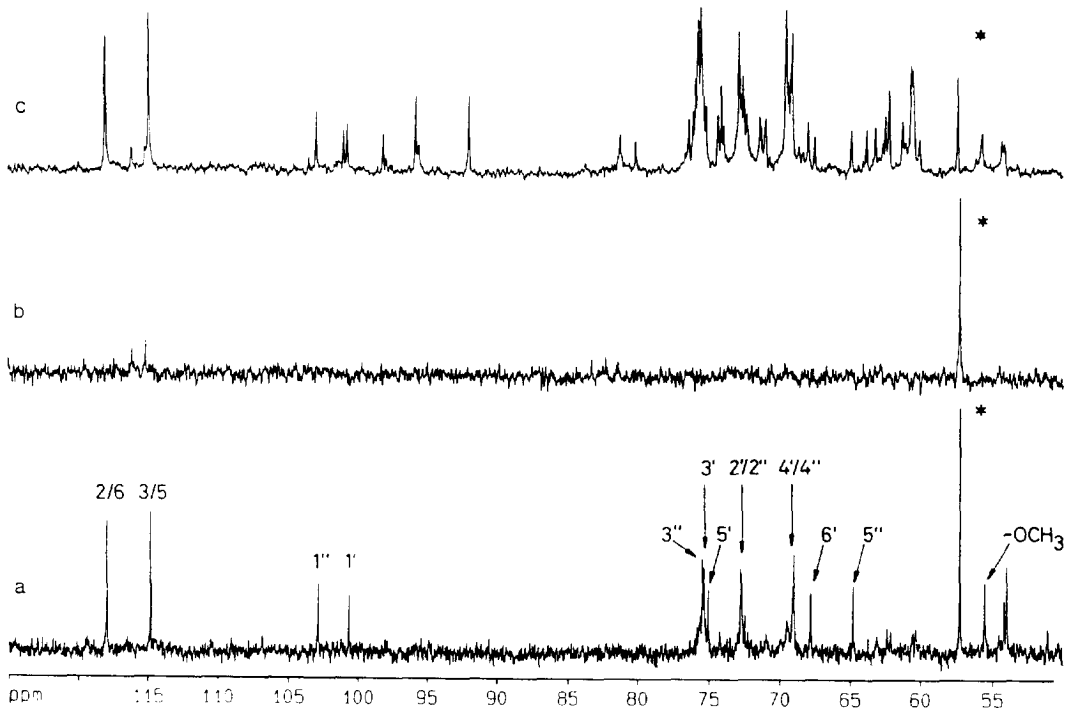


Fig. 2. (a) 100.6 MHz ^{13}C NMR spectrum (measuring time 2 hr) of densely packed cells from a suspension culture of *R. serpentina* after 48 hr incubation with 4-methoxyphenol (**1**) (3.5 mmol) under optimal growth conditions; constant temp. 25°, 100 rpm, 600 lx [peak assignments according to methylursubin (**3**); 2/6 and 3/5: aromatic carbons, $-OCH_3$: aromatic methoxy-group, 1′–6′: C-atoms of the glucose unit, 1″–5″: C-atoms of the xylose unit]. (b) 150.9 MHz ^{13}C NMR spectrum of the supernatant of a *R. serpentina* cell suspension 35 hr after the addition of 3 mmol of **1** (measuring time 1.5 hr). (c) 150.9 MHz ^{13}C NMR spectrum of *Rauwolfia* cells taken from a suspension culture incubated for 21 hr with 3 mmol of **1** (measuring time 1.5 hr). Signals labelled with an asterisk (*) represent the C(1) of EtOH used as internal standard.

EXPERIMENTAL

Plant cell culture. Cell suspension cultures of *R. serpentina* (L.) Benth. ex Kurz were cultivated under standard conditions in 1 l Erlenmeyer flasks at constant temp. (25°) on a gyratory shaker (100 rpm) under light (600 lx). Cell fr. wt was determined after filtration of cells through a nylon filter (mesh 125 μm); dry wt was measured after freeze-drying of cells.

Feeding experiments. Similar to previously published procedures [7], 250–300 g of wet cells (7.5–10 g dry wt) grown for 7 days in LS medium [12] were filtered off the nutrition soln under sterile conditions and then cultivated for 4 days in 150 ml fr. LS medium. A soln of 400 mg **1** in 50 ml sugar-free LS medium was added. After 2 days, cells were harvested by suction filtration.

Isolation and identification of **2** *and* **3**. After sepn of the cells from the nutrition medium, 50 g cells were extracted twice with 100 ml MeOH under reflux. Compounds **2** and **3** were isolated from the obtained extracts by TLC on Merck silica gel plates G/UV$_{254}$ with EtOAc–MeOH–H_2O (7:2:1). Quantitative HPLC analysis was carried out using a LiChroCart® 125-4 Supersphere 100 column (RP-18). The solvent system, applied with a flow rate of 1 ml min^{-1}, was MeOH–H_2O (1:4) (pH 2.5) adjusted with H_3PO_4 (detection at 285 nm). R_t for **2** 4.5, for **3** 5.9 and for **1** 8.9 min.

Compound **2**. ^{1}H NMR (400 MHz, D_2O): δ7.20 (*d*, $J_{H\text{-}2/6/H\text{-}3/5}$ = 9.0, 2H, H-2/6), 7.07 (*d*, $J_{H\text{-}3/5/H\text{-}2/6}$ = 9.0, 2H, H-3/5), 5.04 (*d*, $J_{H\text{-}1'/H\text{-}2'}$ = 7.6, 1H, H-1′), 3.97 (*dd*, $J_{H_A\text{-}6'/H_B\text{-}6'}$ = 12.9, $J_{H_A\text{-}6'/H\text{-}5'}$ = 1.7, 1H, H_A-6′), 3.89 (*s*, 3H, OCH_3), 3.80 (*dd*, $J_{H_B\text{-}6'/H_A\text{-}6'}$ = 12.9, $J_{H_B\text{-}6'/H\text{-}5'}$ = 5.3, 1H, H_B-6′), 3.64 (*ddd*, $J_{H\text{-}5'/H\text{-}4'}$ = 9.3, $J_{H\text{-}5'/H_B\text{-}6'}$ = 5.3, $J_{H\text{-}5'/H_A\text{-}6'}$ = 1.7, 1H, H-5′), 3.59 (*dd*, $J_{H\text{-}2'/H\text{-}3'}$ = 9.3, $J_{H\text{-}2'/H\text{-}1'}$ = 7.6, 1H, H-2′), 3.56 (*dd*, $J_{H\text{-}3'/H\text{-}2'}$ = 9.3, $J_{H\text{-}3'/H\text{-}4'}$ = 9.0, 1H, H-3′), 3.52 (*dd*, $J_{H\text{-}4'/H\text{-}5'}$ = 9.3, $J_{H\text{-}4'/H\text{-}3'}$ = 9.0, 1H, H-4′). ^{13}C NMR (100.6 MHz, D_2O): δ154.5 (C-4), 150.6 (C-1), 117.9 (C-2/6), 114.8 (C-3/5), 100.9 (C-1′), 75.8 (C-3′), 75.3 (C-5′), 72.7 (C-2′), 69.1 (C-4′), 60.2 (C-6′), 55.5 ($-OCH_3$). FDMS *m/z*: 286.3 $[M]^+$. IR ν (KBr) cm^{-1}: 3500–3200, 2947, 2898, 2862, 1519, 1466, 1449, 1416, 1237, 1112, 1079, 1055, 1031, 827.

Compound **3**. ^{1}H NMR (400 MHz, D_2O): δ7.17 (*d*, $J_{H\text{-}2/6/H\text{-}3/5}$ = 9.1, 2H, H-2/6), 7.03 (*d*, $J_{H\text{-}3/5/H\text{-}2/6}$ = 9.1, 2H, H-3/5), 5.08, (*d*, $J_{H\text{-}1'/H\text{-}2'}$ = 7.5, 1H, H-1′), 4.45 (*d*, $J_{H\text{-}1''/H\text{-}2''}$ = 7.7, 1H, H-1″), 4.18 (*dd*, $J_{H_A\text{-}6'/H_B\text{-}6'}$ = 11.8, $J_{H_A\text{-}6'/H\text{-}5'}$ = 1.4, 1H, H_A-6′), 3.94 (*dd*, $J_{H_B\text{-}6'/H_A\text{-}6'}$ = 11.8, $J_{H_B\text{-}6'/H\text{-}5'}$ = 5.5, 1H, H_B-6′), 3.91 (*dd*, $J_{He\text{-}5''/Ha\text{-}5''}$ = 11.4, $J_{He\text{-}5''/H\text{-}4''}$ = 6.0, 1H, He-5″), 3.86 (*s*, 3H, $-OCH_3$), 3.78

(*ddd*, $J_{H\text{-}5'/H\text{-}4'} = 9.3$, $J_{H\text{-}5'/H_B\text{-}6'} = 5.5$, $J_{H\text{-}5'/H_A\text{-}6'} = 1.4$, 1H, H-5'), 3.64 (*ddd*, $J_{H\text{-}4''/Ha\text{-}5''} = 10.7$, $J_{H\text{-}4''/H\text{-}3''} = 8.8$, $J_{H\text{-}4''/He\text{-}5''} = 6.0$, 1H, H-4''), 3.62 (*dd*, $J_{H\text{-}3'/H\text{-}2'} = 9.3$, $J_{H\text{-}3'/H\text{-}4'} = 8.8$, 1H, H-3'), 3.58 (*dd*, $J_{H\text{-}2''/H\text{-}3''} = 9.3$, $J_{H\text{-}2''/H\text{-}1''} = 7.5$, 1H, H-2''), 3.52 (*dd*, $J_{H\text{-}4'/H\text{-}5'} = 9.3$, $J_{H\text{-}4'/H\text{-}3'} = 8.8$, 1H, H-4'), 3.41 (*dd*, $J_{H\text{-}3''/H\text{-}2''} = J_{H\text{-}3''/H\text{-}4''} = 8.8$, 1H, H-3''), 3.30 (*dd*, $J_{H\text{-}2''/H\text{-}3''} = 8.8$, $J_{H\text{-}2''/H\text{-}1''} = 7.7$, 1H, H-2''), 3.21 (*dd*, $J_{Ha\text{-}5''/He\text{-}5''} = 11.4$, $J_{Ha\text{-}5''/H\text{-}4''} = 10.7$, 1H, Ha-5''). ^{13}C NMR (100.6 MHz, D_2O): δ154.4 (C-4), 150.6 (C-1), 118.0 (C-2/6), 114.8 (C-3/5), 102.8 (C-1''), 100.6 (C-1'), 75.3 (C-3''), 75.2 (C-3'), 75.1 (C-5'), 72.7 (C-2''), 72.6 (C-2'), 69.0, 68.9 (C-4') and (C-4''), 67.8 (C-6'), 64.8 (C-5''), 55.5 ($-OCH_3$). FDMS *m/z*: 418.3 $[M]^+$. IR ν (KBr) cm^{-1}: 3500–3200, 2952, 2908, 2825, 1531, 1505, 1452, 1380, 1222, 1162, 1101, 1070, 1040, 830.

In vivo *NMR measurements*. *Compound* **1**. 3 mmol (372 mg) was added to a cell suspension of *ca* 250 g wet cells (dry wt 7.5 g, 450 ml total vol.) grown for 4 days in 11 Erlenmeyer flasks (LS medium). To follow the biotransformation of **1**, *ca* 8 ml suspended cells were transferred periodically (after 0, 4.5, 9, 13, 21, 35 and 48 hr) to a 10 mm NMR tube, containing 1.4 ml of a soln of 0.5% EtOH (int. standard) in D_2O. Final D_2O concn was *ca* 15%. After cells had settled down (*ca* 10 min), measurements were started with 200 dummy scans to achieve stable temp. conditions (22 ± 0.5°). For each measurement, a new cell sample was taken from the flask. Spectra were obtained at 100.6 MHz (^{13}C) and 150.9 MHz (^{13}C) with standard Bruker software under the following conditions: Accumulation of 3700 FIDs (measuring time 1.5 hr), or 5600 FIDs (100.6 MHz, measuring time 2 hr), pulse repetition time of 1.3 sec (pulse angle 30°); spectrum width on the 150.9 MHz instrument was 37594 Hz and digital resolution 0.57 Hz per data point. Spectrum width on the 400 MHz instruments was 25000 Hz, with a digital resolution of 0.76 Hz per data point. Resolution enhancement was obtained by Gaussian-multiplication (GB: 0.15, LB: − 6 Hz). After each expt, cells were mixed in the NMR tube, the supernatant decanted and then analysed by ^{13}C NMR under the conditions described above.

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