PERGAMON

Phytochemistry 62 (2003) 325–332

PHYTOCHEMISTRY

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Biosynthesis of calystegines: ^{15}N NMR and kinetics of formation in root cultures of *Calystegia sepium*

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Received 8 July 2002; received in revised form 27 August 2002

Dedicated to Professor Meinhard H. Zenk on the occasion of his 70th birthday

Abstract

Calystegines are nortropane alkaloids bearing between three and five hydroxyl groups in various positions. [^{15}N]Tropinone was administered to root cultures of *Calystegia sepium* and the incorporation into calystegines was followed. Increase of label in calystegines was measured by one-dimensional ^{15}N NMR and inverse-detected 2D NMR techniques. The results show that tropinone and pseudotropine are metabolites in the biosynthetic pathway of calystegines. The velocity of calystegine accumulation was followed kinetically by transfer of root cultures from ^{15}N-enriched medium to ^{14}N-medium and analysis by GC–MS. A constant calystegine formation with no interference by excretion or degradation was observed. A biosynthetic rate for individual calystegines at each time point was calculated, the maximum was 0.4 mg/day/g of biomass. This allowed the velocity of individual biosynthetic steps to be estimated.

Keywords: *Calystegia sepium*; Convolvulaceae; Biosynthesis; Tropane alkaloid; Calystegine; Root culture; ^{15}N NMR; [^{15}N]Tropinone; Kinetic

1. Introduction

Calystegines are glycosidase inhibitors with a high therapeutic potential (Asano et al., 2000). They are nortropane alkaloids bearing between three and five hydroxyl groups at various positions and orientations. The core skeleton of calystegines, including the nitrogen atom, is already preformed in tropinone, the key intermediate in tropane alkaloid and calystegine biosynthesis.

The biosynthetic pathways of tropane alkaloids and calystegines both use tropinone as a substrate. Tropinone reduction by TR II (pseudotropine forming tropinone reductase) is the first committed calystegine-specific step in the biosynthetic pathway. The next steps in the pathway are uncertain. Presumably *N*-demethylation takes place first followed by hydroxylations in various positions. This hypothesis is supported by the finding that *N*-methyl calystegines are present, but only in trace levels. In addition, the sequence of hydroxylations represents one of the most compelling questions that needs to be answered. Calystegines were first detected in *Calystegia sepium*, later in several Solanaceae and Convolvulaceae (Tepfer et al., 1988; Dräger et al., 1994; Schimming et al., 1998).

2,7-Dihydroxynortropane (2,7-DHN) (Fig. 1), identified in *Calystegia sepium* (Asano et al., 2001) resembles calystegines, but does not fit into the biosynthetic scheme due to the aberrant hydroxylation pattern.

Stable isotopes, such as ^{2}H, ^{13}C, and ^{15}N, which are detectable by NMR spectroscopy have been used extensively in plant biosynthetic and physiological studies (Martin, 1985). The choice of the nucleus for labelling depends on the availability of suitable precursors and on the specific aim of the investigation. ^{15}Nitrogen, although of low sensitivity in NMR analysis, has been successfully used in a number of investigations. One-dimensional ^{15}N NMR measurements of ^{15}N enriched isolated amino acids were used, for example, to detect nitrogen recycling in phenylpropanoid metabolism (Razal et al., 1996) and to monitor metabolic processes in vivo without isolation of the metabolites (Ford et al., 1994). Sig-

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0031-9422/03/$ - see front matter
PII: S0031-9422(02)00544-7

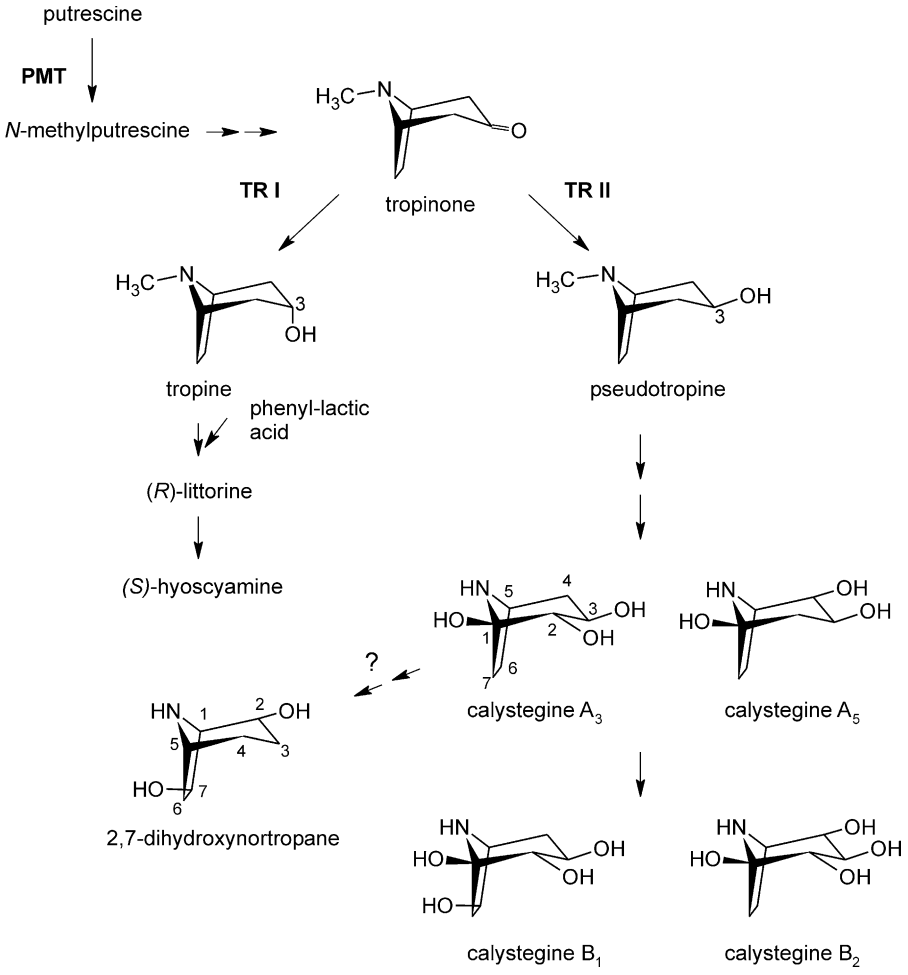

Fig. 1. Tropane and nortropane biosynthesis. **PMT**, putrescine *N*-methyl transferase, **TR I**, tropine forming tropinone reductase, **TR II**, pseudotropine forming tropinone reductase.

nificant enhancement of sensitivity and resolution was achieved by means of inverse-detected heteronuclear correlation experiments, which were used to detect metabolites in extracts of *Nicotiana* cell cultures (Mesnard et al., 2000). Special submicro NMR probes enabled further enhancement of sensitivity even without ^{15}N enrichment (Hadden and Martin, 1998). Tropane alkaloid metabolism has been studied by in vivo NMR of *Datura stramonium* root cultures (Ford et al., 1996), monitoring conversion of [^{15}N]tropinone to [^{15}N]tropine and [^{15}N]tropine esters.

In order to elucidate the biosynthetic pathway of calystegines, [^{15}N]tropinone was administered to root cultures of *Calystegia sepium*. The increase of ^{15}N in calystegines was monitored by GC–MS, and 40–98% of the typical fragments of the calystegines A_3, B_1 and B_2 appeared as M + 1 peaks over an incubation period of 6 days. The high incorporation rate enabled investigation on the calystegine biosynthesis by 1D ^{15}N NMR and inverse-detected 2D NMR techniques to gain insights into the biosynthetic sequence and velocity of calystegine accumulation.

2. Results and discussions

2.1. ^{15}N NMR of calystegines

Labelled tropinone or pseudotropine, bearing an isotopic label in the ring system, represent ideal precursors for the present studies. [^{15}N]Tropinone was chosen because it was available from Henry D. Boswell and David J. Robins, Glasgow. Since there is no information on the ^{15}N chemical shift of calystegines and their biosynthetic intermediates, these values had to be determined prior to feeding experiments. The chemical shifts of calystegine B_2 (δ −298.6) and pseudotropine (δ −304.6) were obtained from one-dimensional ^{15}N NMR spectra referenced to internal $K^{15}NO_3$ (δ 0.0) and γ-aminobutyric acid (δ −343.2) as an additional internal standard. The data were confirmed by means of ^{15}N HMBC experiments using [^{15}N]γ-aminobutyric acid for referencing. The intrinsically low sensitivity of ^{15}N NMR spectroscopy at natural abundance of ^{15}N of 0.37% required relatively large amounts of (unlabelled) standards, which unfortunately were not available for

other calystegines of interest in the present studies. Thus, the ^{15}N chemical shift of calystegine A_3 (δ −294.3) was determined from a ^{15}N enriched sample isolated from *C. sepium* root cultures grown on ^{15}N-nitrate as the only nitrogen source. The resulting [^{15}N]calystegine A_3 was almost completely labelled as determined by GC–MS (see, Fig. 6, 0.5 days).

The ^{15}N resonance of calystegines was highly pH-dependent above pH 5, and further influenced in plant samples by variable contents of calystegines and matrix. Thus, variable ^{15}N chemical shift values were found in D_2O under normal conditions but were stable below pH 5 when the nitrogen of calystegines is protonated. Accordingly, extracts from biosynthetic experiments were measured in 1 M phosphate buffer (H_2O/D_2O 90:10) at pH 4.0, which gave consistent chemical shifts. After the ^{15}N chemical shifts of calystegines and pseudotropine were assigned, *C. sepium* root cultures were administered [^{15}N]tropinone in the nutrient medium and extracted after 2, 4 and 6 days. The extracts were examined for ^{15}N-containing compounds by one-dimensional ^{15}N NMR (Fig. 2).

Three prominent signals appeared in the relevant region of the ^{15}N spectrum of root extracts after 2 days of [^{15}N]tropinone application (Fig. 2D), which could be assigned to calystegine A_3 (δ −294.3), B_2 (δ −298.6) and pseudotropine (δ −304.6) by means of reference spectra (Fig. 2A–C). Closer consideration of the signal of calystegine B_2 revealed another resonance (δ −298.7; expanded spectral regions in Fig. 2), which was provisionally assigned to calystegine B_1. This assignment was based on the chemical shift value and the ratios of the peak areas of calystegines, which showed similar values in GC–MS analyses of the same plant system (Scholl et al., 2001), and was later confirmed by ^{15}N gsHMBC experiments. The results confirmed the hypothesis that the specific calystegine branch of the tropane biosynthesis starts from tropinone and includes pseudotropine as metabolite, as shown in Fig. 1. Measurements of extracts obtained after 4 and 6 days indicated enhancement of these resonance signals in comparison to the internal standard ([^{15}N]γ-aminobutyric acid, δ −243.2). However, the ratios between the signals did not allow conclusions about the kinetics of the hypothetical biosynthetic sequence from pseudotropine to calystegine A_3 to calystegines B to be drawn. All extracts showed an additional small signal at δ −313.5 and further minor resonances that were not seen in every sample. These signals presumably are due to biosynthetic intermediates or side products. The signal of starting material, tropinone (broad signal of standard [^{15}N]tropinone, δ −311.7), was not detectable in these spectra.

^{15}N NMR spectra of extracts of *C. sepium* cultures, grown on ^{15}N nitrate as the only nitrogen source, also showed signals of calystegines A_3, B_1, and B_2 (Fig. 3). However, the signal of pseudotropine was very small, indicating accumulation of this intermediate at low concentrations under normal growth conditions. Due to the level of labelling evenly distributed among all nitrogen compounds derived from ^{15}N nitrate, these spectra may reflect endogenous pool sizes more realistically than those obtained after feeding of tropinone as an advanced precursor. In the pathway described here, pseudotropine and even more intermediates before calystegine A_3 are thought to be rapidly processed with little accumulation.

Further ^{15}N signals upfield of δ −333 were due to α-amino groups of amino acids and the calibration standard [^{15}N]γ-aminobutyric acid. This is in accordance with data from *Datura stramonium* root cultures (Ford et al., 1994). After growth on ^{15}N nitrate, an additional signal at δ −313.5 was again detected in each extract which was tentatively assigned to 2,7-dihydroxynortropane, and found by GC–MS to be labelled after feeding of [^{15}N]tropinone (Scholl et al., 2001).

For further investigations, a root extract from $^{15}NO_3$-grown roots was partially purified by ion exchange chromatography, followed by lyophilisation. The dry matter was taken up in phosphate buffer pH 4.0, sonicated, and centrifuged. The sediment was discarded and the supernatant subjected to ^{15}N gsHMBC experiments. Published ^1H NMR data of calystegines and other nortropane metabolites (Asano et al., 1995, 2001) were employed to assign ^{15}N resonances in the 2D spectra as shown, for example, in Fig. 4. Owing to the large dihedral angles in the 1β-amino cycloheptane skeleton, H-6*endo* and H-7*endo* should show strong long-range correlations through three bonds in the ^{15}N gsHMBC spectrum and, therefore, are potential diagnostic signals to identify calystegines in mixtures or extracts. The ^1H–^{15}N correlations with δ_{1H} 4.45 (H-7) and 2.25 (H-6*endo*) confirmed this suggestion and enabled identification of the signal at δ_{15N} −313.5 as resulting from 2,7-dihydroxynortropane (δ 4.45 and δ 2.15 in Asano et al., 2001). Further cross signals of H-6 (δ 4.18) and H-7*endo* (δ 2.62) (δ 4.11 and δ 2.52 in Asano et al., 2001) with δ_{15N} −298.7 confirmed that this signal is due to calystegine B_1 (Fig. 4). Small differences in chemical shift values ($\Delta\delta$ up to 0.1 ppm) between published data and those found in our measurements are considered to be pH and solvent effects. Assignment of calystegines A_3 and B_2, which was performed by means of 1D ^{15}N NMR (Fig. 2), was confirmed by ^1H–^{15}N cross signals in the same ^{15}N HMBC spectrum.

Additional signals were also apparent. The signal close to that of calystegine A_3 at δ −294.3 may be derived from calystegine A_5 (1α,3α,4β-trihydroxy nortropane), which was detected by GC–MS in all extracts from *Calystegia sepium*. However, cross peaks were too weak for unambiguous assignment.

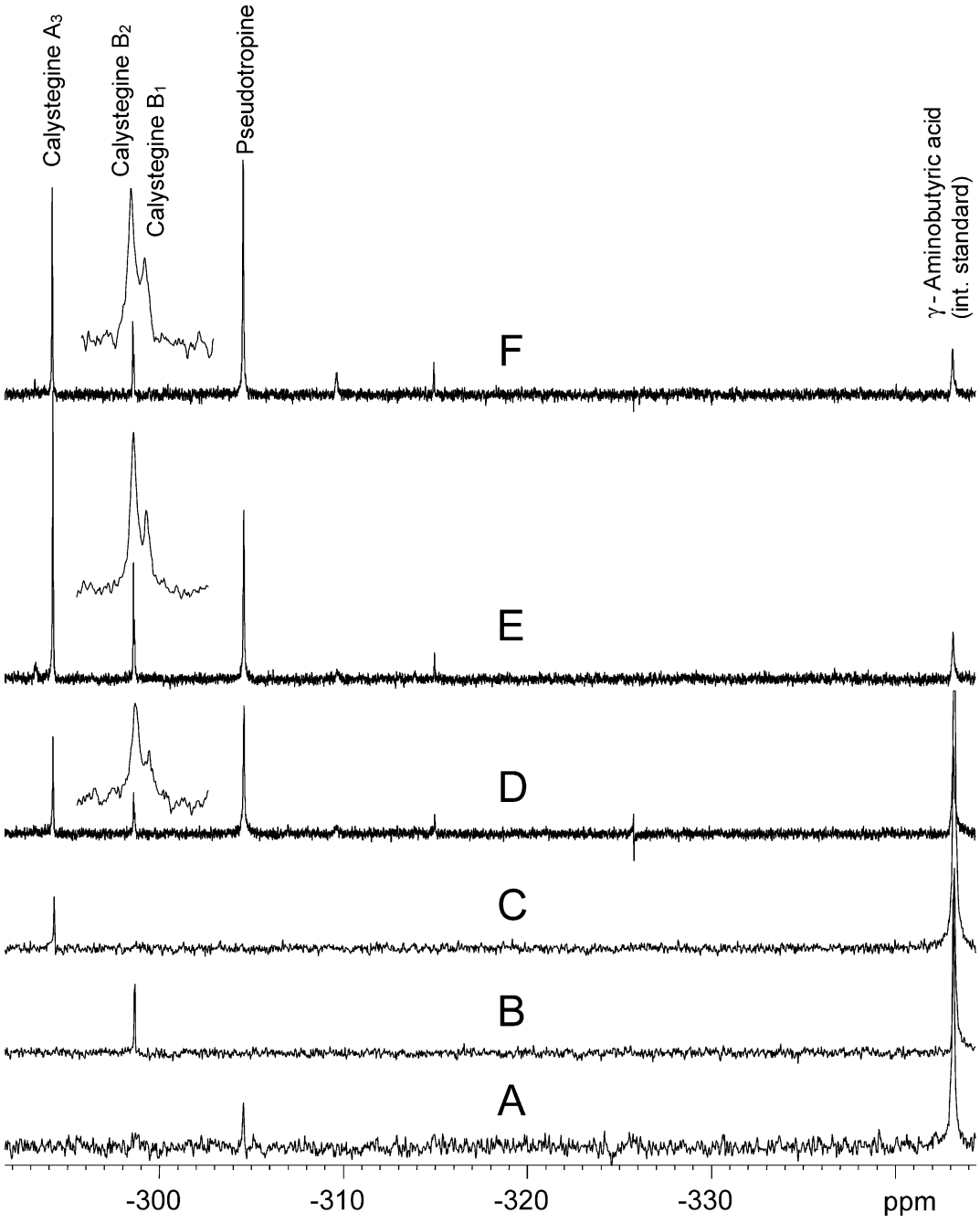

Fig. 2. ^{15}N NMR partial spectra of extracts from root cultures of *Calystegia sepium* obtained (D) 2, (E) 4, and (F) 6 days after administration of [^{15}N]tropinone. Spectra A (pseudotropine), B (calystegine B_2), and C (calystegine A_3) were recorded from authentic standards. The expanded region shows partially resolved signals of calystegines B_1 and B_2.

The experiments demonstrate that for nitrogen containing compounds, metabolites of the biosynthetic pathway can be identified and traced using ^{15}N enrichment and ^{15}N NMR. The results reinforce the conclusion from the GC–MS investigation of root extracts after [^{15}N]tropinone application. Tropinone and pseudotropine are metabolites in the biosynthetic pathway of calystegines, which starts from putrescine as a common precursor of tropane and nortropane alkaloids.

2.2. Kinetics of calystegine formation in root cultures

When labelled ^{15}N was taken up by the roots, irrespective of whether it was applied in tropinone or in nitrate, four intensively labelled nortropanes were detected after 24 h: the calystegines A_3, B_1, B_2 and 2,7-dihydroxynortropane. The high and reproducible incorporation of ^{15}N allowed the metabolism of the individual calystegines to be followed kinetically. Roots were cultivated on B5 medium containing

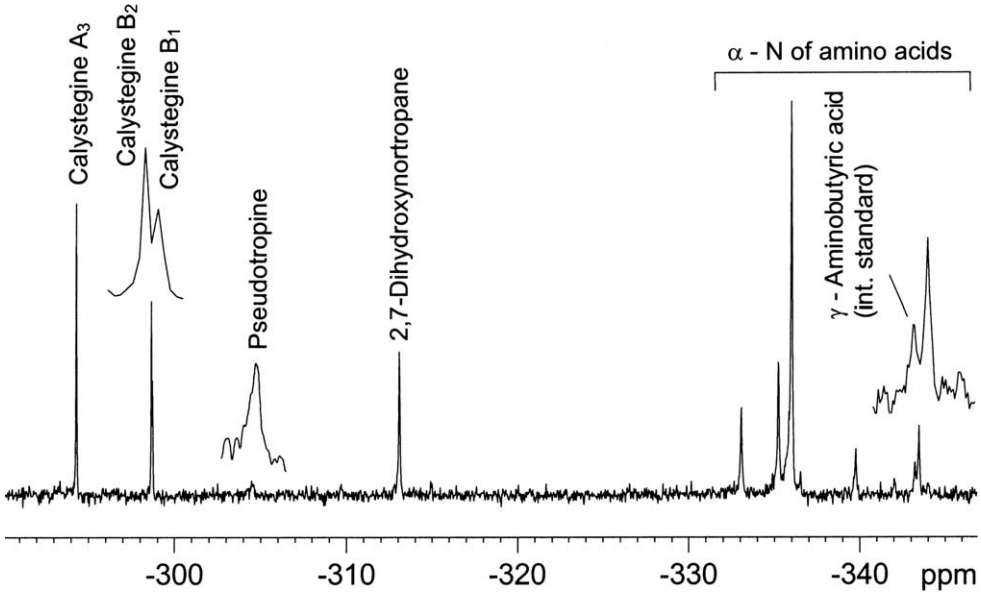

Fig. 3. ^{15}N NMR partial spectrum of an extract from root cultures of *Calystegia sepium* grown on ^{15}N nitrate. Expanded regions show signals of calystegines B_1 and B_2, pseudotropine (line broadening 5 Hz), and [^{15}N]γ-aminobutyric acid, which was used as internal standard.

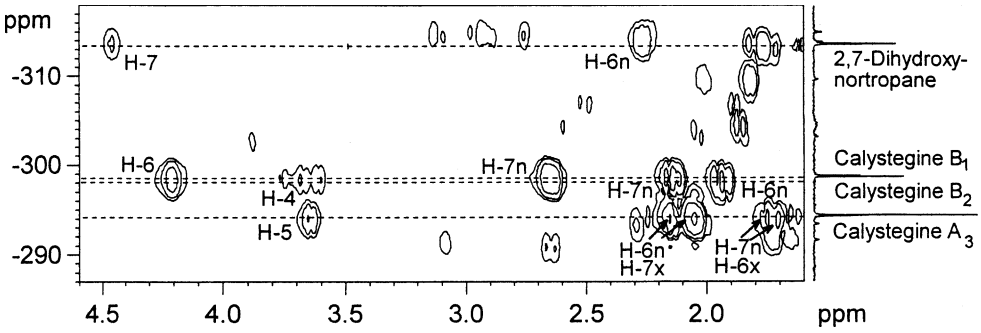

Fig. 4. ^{15}N gsHMBC partial spectrum of an extract from roots of *Calystegia sepium* grown on ^{15}N nitrate. Calystegines A_3, B_1, B_2, and 2,7-dihydroxynortropane were identified by means of ^{1}H–^{15}N long-range correlations. Stereochemical descriptors *endo* and *exo* have been abbreviated using "n" and "x", respectively.

^{15}N-nitrate for 14 days to ensure that growth was normal and calystegine metabolism was not altered due to stress. Thereafter, roots were transferred on to medium with $^{14}NO_3$ and cultivated for 0.5, 1, 2, 3, 6, and 9 days before harvesting. The calystegine levels increased in total, owing to an increase in root total biomass of approximately fourfold within 9 days (Fig. 5). The levels remained constant in relation to gram root mass (data not shown) over the period of 9 days.

For each calystegine at each time point, the ratio of labelled to non-labelled portion was determined by GC–MS. Typical nitrogen-containing MS-fragments were followed that were identified in former experiments as representative for the respective compounds (Scholl et al., 2001). Based on the ratio of labelled to non-labelled nitrogen atoms, the proportion of calystegines synthesized after transfer to fresh medium could be calculated (Fig. 6). Twelve hours after transfer, non-labelled calystegines could not be detected. A possible reason could be that precursors for calystegines, i.e. ornithine or putrescine, were provided by internal pools that were still fully labelled. Another explanation is a short cessation of calystegine formation after transfer to fresh medium. After a customisation phase, growth and calystegine biosynthesis are resumed. The accumulation curve for calystegines (Fig. 5) supports the second interpretation. The subsequent elevation in non-labelled fragments is quite uniform for all calystegines. The total portion of non-labelled calystegines never exceeded 75%, indicating that the labelled calystegines contained in the roots in the beginning were still present. This shows that calystegines are not excreted or degraded during a growth cycle, unless parts of the roots decay due to old age. In the exhausted growth medium calystegines were consistently found in trace amounts only (data not shown).

A constant calystegine formation with no interference by excretion or degradation can be presumed and thus, a biosynthetic rate for individual calystegines at each

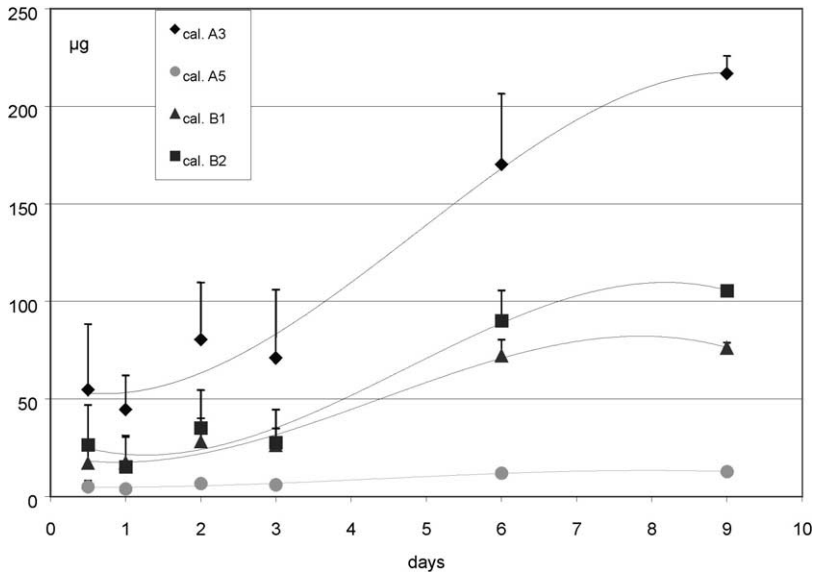

Fig. 5. Total calystegines per flask. Measurements from 5 individual flasks, bars represent standard deviation. Fresh mass increased from 0.5 to 2 g within 9 days. 2,7-DHN could not be quantified correctly due to small concentrations. It was estimated in comparison to calystegine A_5 to be approximately 5–10 µg per flask.

time point can be calculated (Fig. 7). For calystegine A_5 and for 2,7-dihydroxynortropane, the pool sizes are too small to result in statistically significant data. Transfer to fresh medium initiates high calystegine biosynthesis after a short lag phase, the total calystegine formation on day 2 is 0.4 mg g^{-1}. In later phases of the culture period, the specific biosynthetic rate per gram of biomass slows down, although total calystegine accumulation continues to increase (Fig. 5) due to increasing biomass per flask.

It is tempting to speculate on the sequence of the biosynthetic steps in calystegine formation starting from pseudotropine (Fig. 1). Demethylation is anticipated to be the first step as all further downstream metabolites are nortropanes. The subsequent hydroxylating steps are unlikely to be rate-limiting, because a dihydroxy metabolite with a matching pattern of hydroxyl groups does not accumulate. Considering the structures, calystegines A_3 and A_5 with three hydroxyl groups may be the precursors for the calystegines B_1 and B_2. Comparison of Figs. 6 and 7 leads to the conclusion that, if calystegine A_3 is an intermediate for the calystegines of the B-group, the last hydroxylation step must also be quite fast, because with ongoing accumulation, dilution of label in the calystegine A pool is simultaneous with the calystegines B. In spite of a fast introduction of the fourth hydroxyl group, approximately half of the calystegine A_3 is not metabolized, i.e. is not hydroxylated to the tetrahydroxyl stage. The reason may be a compartmentation procedure of calystegines starting from the trihydroxylated status. The calystegines A and B must be likewise withdrawn from further derivatisation by transport, e.g. into the vacuole. Although the localization of calystegines in the vacuole awaits conclusive experimental proof, this model of regulated hydroxylation and selective transport would explain how each

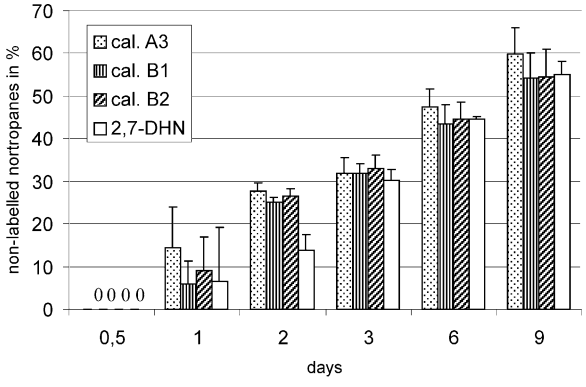

Fig. 6. Proportion of calystegines synthesised on new medium. Measurements from 5 individual flasks, bars represent standard deviation.

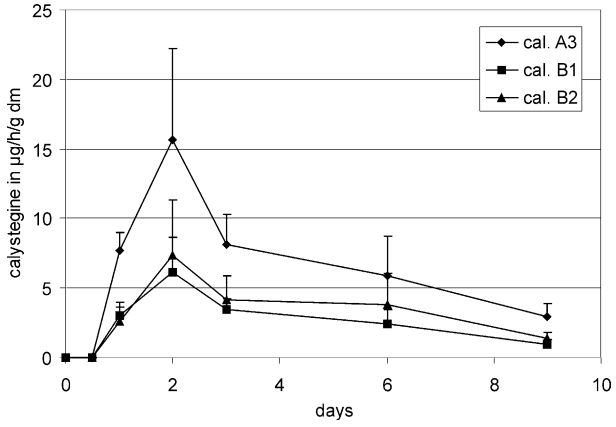

Fig. 7. Biosynthetic rate for the major calystegines. Measurements from 5 individual flasks, bars represent standard deviation.

plant and each tissue of the Convolvulaceae and Solanaceae can maintain its typical pattern of calystegines. The pattern of the individual calystegine mixtures is too constant to assume random hydroxylation. 2,7-DHN does not fit into this model of biosynthesis. Label in this compound rises or decreases uniformly with the other calystegines, which suggests that 2,7-DHN is a by-product of calystegine biosynthesis, and experiments to elucidate this are ongoing.

3. Experimental

3.1. Root cultures

Cultured roots of *Calystegia sepium*, established by transformation with *Agrobacterium rhizogenes* 15834 by the Institute of Pharmaceutical Biology, TU Braunschweig, Germany, were grown in B5 medium (25 ml in 100 ml flasks) that contained $K^{15}NO_3$ as the only nitrogen source. For kinetic measurements, roots were transferred into new B5 medium with $K^{14}NO_3$ as the only nitrogen source and harvested 0.5, 1, 2, 3, 6, and 9 days after transfer. Biomass was measured as fresh and dry weight.

Before feeding [^{15}N]tropinone, roots were maintained on standard B5 medium for 16 days. For extraction and purification of labelled calystegine A_3, root cultures were grown for 21 days in B5 medium that contained $K^{15}NO_3$ as the only nitrogen source.

3.2. Extraction of calystegines

Fresh or freeze-dried tissues (typical 1–3 g fresh mass) were homogenized twice with an Ultra Turrax in 20 ml 50% MeOH and centrifuged at 4500 *g*. The combined supernatants were reduced under vacuum to 1.5–2.0 ml aq. soln. in a rotary vaporizer. This extract was purified by strong acidic cation exchange resin I LAB (Merck, Darmstadt, Germany) as described (Keiner and Dräger, 2000).

3.3. Purification of calystegine A_3

A total fresh mass of 16.7 g roots cultured on B5 medium with $K^{15}NO_3$ was extracted and purified by acidic cation exchange chromatography as described above. The aqueous extract was lyophilized and dissolved in 1 ml water and applied to a chromatotron silica gel plate of 2 mm thickness. By chromatotron 8924 (Harrison Research, Palo Alto, USA), calystegines A were separated from the group of the B-calystegines with a solvent system of methanol–chloroform–ammonia (50 : 50 : 0.01), flow rate 2 ml min^{-1}. The fractions enriched in calystegine A were combined and further purified by a preparative TLC, plate thickness 2 mm (Merck, Darmstadt, Germany), with a solvent system methanol–water–chloroform–ammonia (22:7:4:0.3). The lyophilised extract was analysed by GC and was found to be 44% pure. B-calystegines were not detectable.

3.4. NMR

NMR analyses were performed on a Bruker AVANCE DRX 500 NMR spectrometer operating at 500.13 MHz (^{1}H) and 50.66 MHz (^{15}N) in 1 M phosphate buffer (H_2O/D_2O 9:1) at pH 4.0 or D_2O at 310 K. ^{15}N chemical shifts are quoted relative to $K^{15}NO_3$ (δ 0.0) as internal standard. [^{15}N]γ-Aminobutyric acid (δ -343.2; 50% ^{15}N) was used as an additional internal standard in 2D experiments, and TMSP-d_4 (δ 0.0) for ^{1}H NMR spectra of isolated calystegins. One dimensional ^{15}N NMR spectra were run in a 2.5 mm broadband microprobe using the following parameters: 25 kHz sweep width, 32 K data points, proton decoupling by WALTZ-16 composite pulse sequence, 3600 scans, relaxation delay 2.0 s. ^{1}H NMR and gradient-selected heteronuclear multiple bond correlation NMR (^{15}N gsHMBC) experiments were recorded in a 2.5 mm inverse-detection gradient microprobe. ^{15}N gsHMBC spectra were run without decoupling during acquisition, 1 K data points with 256 scans per increment and 80 experiments, spectral width of 1750 Hz in F2 and 6540 Hz in F1, refocusing delay 5.55 ms, delay for evolution of long-range coupling 100 ms. Data were filtered using a shifted sine-bell square function in both dimensions before Fourier transformation.

3.5. Calystegine measurements

A gas chromatograph HP6890 with simultaneous FID and PND detection was calibrated with original compounds for each individual calystegine. Azobenzene was used as internal standard, column HP5 (30 m×0.25 mm i.d., 0.25 μm film thickness), carrier gas helium, 1 ml/min. For each measurement five individual flasks were used.

3.6. GC–MS

3.6.1. Derivatization

The aq. root extract (0.5 or 1 ml) was lyophilized and the residue was derivatized by silylation with 30 μl pyridine, 40 μl hexamethyldisilazane and 10 μl trichlorosilane at 70 °C for 30 min. Azobenzene (1 mg ml^{-1} in *n*-hexane) was added as internal standard.

3.6.2. Instrumentation

Gas chromatograph HP 5890, detector HP 5972, column DB5 (30 m×0.32 mm i.d., 0.25 μm film thickness), splitless injection, carrier gas helium (1 ml min^{-1} constant flow), injection temp. 250 °C, detector temp. 250 °C, EI-MS 70 eV, temp. program 100 °C, 2 min hold, 5 min^{-1} up to 240 °C.

3.6.3. Evaluation

The area of the main fragments of calystegine A_3, B_1, B_2 and molecular mass of 2,7-DHN were calculated by GC–MS software (Scholl et al., 2001). Results were corrected considering the natural abundance of 1.1% ^{13}C and 4.7% ^{29}Si.

Acknowledgements

We thank Henry D. Boswell and David J. Robins, Glasgow, UK, for the synthesis of [^{15}N]tropinone. Isolated 2,7-dihydroxynortropane was a generous gift from Naoki Asano, Kanazawa, Japan. Dr. J. Kalbitz and A.-K. Schunack, BioService GmbH Halle, are thanked for access and introduction to the chromatotron. We thank Mrs. U. Ködel and Mrs. B. Marx, Institute of Pharmaceutical Biology, Halle, for technical help and Dr. Daniel J. Fowler, Jena, for linguistic support in the preparation of this manuscript.

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