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Isolation and identification of littorine from hairy roots of *Atropa belladonna*

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Abstract A hairy root clone (M8) of *Atropa belladonna*, producing high levels of tropane alkaloids, was established by transformation with *Agrobacterium rhizogenes* (MAFF 03-01724). Littorine, an intermediate of tropane alkaloids, was detected by high-performance liquid chromatography and gas chromatography-mass spectrometry in the alkaloid fraction of the hairy roots and identified by nuclear magnetic resonance analysis. Littorine was also detected in the non-transformed root culture of *A. belladonna*.

Key words *Atropa belladonna* · Hairy root · Littorine · Root culture · Tropane alkaloid

Abbreviations *COSY* Correlation spectroscopy · *DEPT* Distortionless enhancement by polarization transfer · *GC/MS* Gas chromatography-mass spectrometry · *HPLC* High-performance liquid chromatography · *MS medium* Murashige and Skoog medium · *NMR* Nuclear magnetic resonance

Introduction

Littorine, [(–)-3a-(2-hydroxy-3-phenylpropionyloxy)tropane], was simultaneously and independently isolated as a minor component of *Brugmansia (Datura) sanguinea* and as the major alkaloid of the indigenous Australian plant *Anthocercis littorea* (Evans and Major 1968; Cannon et al. 1969). It is now apparent that littorine and hyoscyamine are always found together in *Datura* and other tropane-alkaloid-producing solanaceous plants (Evans et al. 1972). Several experiments have indicated that littorine is a biosynthetic precursor of hyoscyamine (Leete and Kirven 1974; Sauerwein et al. 1993; Robins et al. 1994). *Atropa belladonna*, an important medicinal plant, produces high amounts of hyoscyamine. Alkaloid patterns of the various plant organs including transformed and non-transformed root cultures of *A. belladonna* have been investigated by several researchers (Kamada et al. 1986; Hartmann et al. 1986; Jung and Tepfer 1987; Ondrej and Protiva 1987; Sharp and Doran 1990; Walton et al. 1990; Kitamura et al. 1992; Hashimoto et al. 1993). During these studies, littorine was not detected. However, in our recent research into tropane alkaloid production in hairy roots of Solanaceae, littorine was detected in the hairy roots of *A. belladonna*, transformed with *Agrobacterium rhizogenes* ATCC 15834, by high-performance liquid chromatography (HPLC) and gas chromatography-mass spectrometry (GC-MS) (Aoki et al. 1997). Further confirmation of littorine production in *A. belladonna* is of interest in relation to alkaloid biosynthesis and metabolism. A hairy root clone of *A. belladonna* (M8), newly established by transformation with *A. rhizogenes* (MAFF 03-01724) in our laboratory, did produce littorine. We describe the isolation and nuclear magnetic resonance (NMR) analysis of littorine in this hairy root clone.

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Materials and methods

Plant material

Hairy roots of *A. belladonna* were induced by direct infection on stems of shoot cultures using *A. rhizogenes* (MAFF 03-01724). Hairy roots were excised and cultured on hormone-free half-strength Murashige and Skoog (1/2 MS) medium (1962) supplemented with 3% sucrose, 0.2% Gelrite (Kelco), and 0.5 g l^{-1} Claforan to eliminate the bacteria. The axenic hairy roots were subcultured at 4-week intervals on the same solid medium without antibiotics. Opine assay by

paper electrophoresis was performed to confirm the transformation as described by Petit et al. (1983). A hairy root clone (M8) producing high levels of tropane alkaloids was selected. It was subcultured in hormone-free 1/2 MS liquid medium with 3% sucrose in the dark at 25 °C at 2-month intervals. For isolation of alkaloids, about 0.1 or 0.2 g of the roots was inoculated into the same medium (50 ml/100-ml or 100 ml/200-ml Erlenmeyer flasks, respectively), and cultured in the dark at 25 °C on a rotary shaker at 100 rpm for 2 weeks. Non-transformed root cultures were established from the shoot culture of *A. belladonna*. Adventitious roots were excised from plantlets in vitro and transplanted into 1/2 MS liquid medium (3% sucrose) containing 0.5 ppm indole-3-acetic acid. One month later, five growing root tips (ca. 3 cm), equivalent to 0.1 g fresh weight, were excised and cultured in hormone-free 1/2 MS liquid medium (3% sucrose; 50 ml/100-ml Erlenmeyer flask). Other culture conditions were the same as those used for hairy root culture.

Sample preparation and HPLC analysis

About 50 mg of each lyophilized sample was extracted with 5 ml $CHCl_3$/MeOH/NH_4OH (15 : 5 : 1) using sonication (20 min). Further sample preparation was as described previously (Ishimaru and Shimomura 1989). The alkaloid extracts were dissolved in MeOH and analyzed by HPLC. A TOSOH ODS-120 A column (4.6 i. d.×250 mm) was used, kept at 40 °C and eluted isocratically with CH_3CN-10 mM SDS (pH 3.3, adjusted with 1% H_3PO_4) 2 : 3 (Shimomura et al. 1991). The flow rate was 1.1 ml/mim throughout. The effluent was monitored by a UV detector. 6β-Hydroxyhyoscyamine, scopolamine, hyoscyamine, and littorine were simultaneously quantified (retention time 14.3, 15.0, 19.0, and 22.0 min, respectively). For quantitative analysis, the system was calibrated with the authentic samples at 215 nm.

GC/MS analysis of tropane alkaloids

The dried alkaloid fraction prepared as described above was mixed with 10 ml bis (trimethylsilyl) trifluoroacetamide and heated at 60 °C for 1 h. The TMS-derivatized sample was dissolved in dichloromethane and applied to GC/MS equipped with a DB-1 column (0.25 mm×30 m). The column temperature was isothermal for 2 min at 70 °C, increased to 300 °C at 10 °C/min, and held at 300 °C for 5 min (Aoki et al. 1997). Littorine was identified by comparison with the authentic sample synthesized by Sauerwein et al. (1991).

NMR analysis

NMR was recorded in $CDCl_3$ on a JEOL GSX-270 spectrometer (270 MHz for ^{1}H-NMR and 67.5 MHz for ^{13}C-NMR). Chemical shifts are given on a δ scale (ppm) with $CHCl_3$ as an internal standard (7.27 ppm for ^{1}H or 77.0 ppm for ^{13}C). Assignments of the ^{1}H- and ^{13}C-NMR spectra were accomplished on the basis of distortionless enhancement by polarization transfer (DEPT), ^{1}H-^{1}H correlation spectroscopy (COSY) and ^{13}C-^{1}H COSY. ^{1}H-NMR: δ7.22–7.29 (5 H, multiplet, C_6H_5), 5.04 (1 H, multiplet, H-3), 4.37 (1 H, double doublet, J = 7.0, 5.0 Hz, H-2′), 3.11 (1 H, double doublet, J = 14.0, 5.0 Hz, H-3′a), 3.10 (2 H, multiplet, H-1 and H-5), 2.97 (1 H, double doublet, J = 14.0, 7.0 Hz, H-3′b), 2.25 (3 H, singlet, NCH_3), 2.13 (2 H, multiplet, J = 15.0, 5.0 Hz, H-2a and H-4a), 2.00 (2 H, multiplet, H-6a and H-7a), 1.76 (2 H, multiplet, H-6b and H-7b), 1.63 (2 H, multiplet, H-2b and H-4b). ^{13}C-NMR: δ 173.3 (C-1′), 136.4, 129.4, 128.4, 126.8 (C_6H_5), 71.5 (C-2′), 69.1 (C-3), 59.6 (C-1 and C-5), 40.6 (C-3′), 40.3 (NCH_3), 36.4 (C-4^{alt}), 36.3 (C-2^{alt}), 25.4 (C-6 and C-7).

Extraction and isolation

Lyophilized hairy roots (330 g) were extracted with 5.7 l of $CHCl_3$-MeOH-28% NH_4OH (15 : 5 : 1). The extract was concentrated in vacuo, 500 ml of 0.5 N H_2SO_4 was added and partitioned with (300, 200) ml of $CHCl_3$. The aqueous layer, 50 ml of 28% NH_4OH (pH ~ 10) and partitioned with (300, 150×4) ml of $CHCl_3$. The $CHCl_3$-soluble fraction was concentrated in vacuo to give a residue (3.44 g), which was fractionated on a silica gel column (175 g), eluting with $CHCl_3$-MeOH-28% NH_4OH (9 : 1 : 0.1–8 : 1 : 0.1). The eluates were combined on the basis of HPLC profiles to give a littorine-rich fraction (312 mg) and then subjected to ODS column (23 i. d.×220 mm) chromatography eluting with $CHCl_3$-MeOH (45 : 55–60 : 40) to afford an almost pure littorine fraction (160 mg). Half of the fraction obtained (80 mg) was applied to silica gel twice, eluting with $CHCl_3$-MeOH (4 : 1) to give 24 mg of littorine.

Table 1 Quantification of tropane alkaloids by HPLC. Hairy roots or non-transformed roots were cultured for 2 weeks or 4 weeks, respectively, in 50 ml 1/2 MS liquid medium (100-ml Erlenmeyer flask) at 25 °C in the dark on a rotary shaker at 100 rpm. About 50 mg of each lyophilized sample was extracted with 5 ml $CHCl_3$/MeOH/NH_4OH (15 : 5 : 1) and prepared for HPLC analysis [TOSOH ODS-120 A column [4.6 i. d.×250 mm], 40 °C CH_3CN/10 mM SDS (pH 3.3), 2 : 3, 1.1 ml/min, 215 nm]

Compound	Alkaloid content (% dry weight)	
	Hairy roots	Non-transformed roots
6β-Hydroxyhyoscyamine	0.026 ± 0.004	0.049 ± 0.015
Scopolamine	0.018 ± 0.004	0.011 ± 0.007
Hyoscyamine	0.177 ± 0.012	0.797 ± 0.065
Littorine	0.088 ± 0.002	0.043 ± 0.006

Results and discussion

Hairy root clone M8 was established by direct infection with *A. rhizogenes* MAFF 03–01724, and the transformation of the clone was confirmed by the detection of mikimopine production (data not shown). From this clone, littorine was identified by its retention time (20.48 min) and the mass spectrum (m/z 361, 124, 94, 82, 72, 41) by GC/MS. On HPLC analysis, a peak corresponding to littorine was detected in both the hairy roots and non-transformed root cultures and was present at levels of 0.088% and 0.043% of dry weight, respectively (Table 1).

To obtain sufficient amounts of littorine for NMR analysis, the hairy root clone was cultured repeatedly. First, we investigated the time course of growth and alkaloid production to determine the period suitable for isolation of littorine. The hairy roots grew rapidly until week 3 and the average dry weight in a 100-ml Erlenmeyer flask was about 300 mg. However, the littorine content reached a maximum level (0.088% dry weight, hairy roots: 187 mg dry weight) at week 2 when cultured in hormone-free 1/2 MS liquid medium. The littorine content then decreased after week 2. On the other hand, the hyoscyamine content was relatively low (0.18% dry weight) at week 2 and then rapidly increased (0.50% dry weight at week 4). For this reason, the hairy roots were harvested at week 2 in order to isolate littorine efficiently.

The lyophilized hairy roots (330 g dry weight) were extracted and the alkaloids were isolated by the procedures

reported previously (Ishimaru and Shimomura 1989). Based on the HPLC profiles, 24 mg of suspected littorine was purified by column chromatography and subjected to NMR analysis. The ^{13}C-NMR spectrum exhibited 13 resolved signals at 67.5 MHz, conprising $1{\times}CH_3$, $5{\times}CH_2$, $9{\times}CH$, and $2\times$quaternary carbons, as revealed by DEPT spectra and consideration of chemical shifts. Signals due to 22 protons were present in the ^{1}H-NMR spectrum at 270 MHz. These signals were identical with the published data (Cannon et al. 1969).

This is the first report of the isolation and the identification of littorine by NMR analysis from *A. belladonna* culture. Littorine was detected not only in hairy roots transformed with different strains of *A. rhizogenes* [ATCC 15834 (Aoki et al. 1997) or MAFF 03-01724] but also in non-transformed roots. These results indicated that *A. belladonna* in vitro can biosynthesize littorine. However, no littorine was described by Hartman et al. (1986) in a similar set of experiments. Indeed, in our preliminary experiments, littorine was not detectable in the roots and leaves of field-grown plants. Further investigations will reveal the relationship between physiological changes and littorine production in vitro and in field-grown plants.

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