Sesquiterpene Lactones in a Hairy Root Culture of *Cichorium intybus*

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A transformed root culture of *Cichorium intybus* L. (Asteraceae) was found to produce sesquiterpene lactones of guaiane and germacrane type. Lactucopicrin, 8-desoxylactucin and three sesquiterpene lactone glycosides: crepidiaside B, sonchuside A and ixerisoside D were isolated from the roots. The yield of 8-desoxylactucin reached 0.03 g l^{-1} at the early stationary phase of the culture.

Introduction

Chicory (*Cichorium intybus* L., Asteraceae), a medicinal plant used to promote appetite and digestion, contains bitter-tasting sesquiterpene lactones. The most abundant ones, lactucin, 8-desoxylactucin (**1**) and lactucopicrin (**2**), and their 11β,13-dihydroderivatives are based on a guaiane skeleton. Of these, lactucopicrin and dihydrolactucopicrin are more bitter than quinine hydrochloride (van Beek *et al.*, 1990). Roots of the plant elaborate eudesmanolides, germacranolides and guaianolides, accumulated mainly as glycosides (Blaschek *et al.*, 1998; Kisiel and Zielińska, 2001). Some of the guaianolides isolated from *C. intybus* play a role in chemical defence of chicory plant as antifeedants (Rees and Harborne, 1985) and phytoalexins (Monde *et al.*, 1990; Grayer and Harborne, 1994), and possess cytotoxic activity towards cultured cancer cells (Hładoń *et al.*, 1978; Seto *et al.*, 1988). Pharmacological studies of the root extracts from *C. intybus* have shown their anti-inflammatory and hepatoprotective actvities (Zafar and Ali, 1998; Ki *et al.*, 1999). Recently, a molecular mechanism of anti-inflammatory action of sesquiterpene lactones, *via* inhibition of transcription factor NF-κB, has been proposed (Rüngeler *et al.*, 1999; Han *et al.*, 2001).

Current status of biotechnological studies on the species has been already reviewed by Bais and Ravishankar (2001). Hairy root culture of witloof chicory (*C. intybus* L. cv. Lucknow local), obtained by transformation with *Agrobacterium rhizogenes* LMG 150, has been shown to produce coumarins, esculin and esculetin (Bais *et al.*, 1999). No data are available on sesquiterpene lactone accumulation in transformed root cultures of chicory.

Experimental

Transformed root culture

Aseptic seedlings of *Cichorium intybus* L. were obtained from seeds of known wild origin, delivered by the Botanical Garden of Free University in Berlin. *Agrobacterium rhizogenes* strain LBA 9402, containing agropine type Ri plasmid pRi 1855, was used in the experiment. The plasmid derived from a wild type *A. rhizogenes* 1855 was mapped by Pomponi *et al.* (1983). Hairy roots were induced on leaf explants excised from the seedlings, and their transformed nature was proved by opine assay and *rol B* gene detection in plant genomic DNA, as described elsewhere (Stojakowska and Malarz, 2000).

The transformed roots were cultivated on a gyrotory shaker (110 r.p.m.), at 25 °C, with a 16 h photoperiod (20 μE m^{-2} s^{-1}, cool white fluorescent tubes), in a modified liquid MS medium (Murashige and Skoog, 1962), containing ½ strength macronutrients and 3% sucrose. The medium was initially supplemented with 500 mg l^{-1} of cefotaxime to obtain bacteria-free culture, which was further sub-cultured every four weeks by inoculating 0.7 g fresh weight of roots in 250 ml Erlenmeyer flask with 30 ml of the nutrient medium.

A time course experiment was performed by harvesting roots every five days during 35 days of culture. The experiment was done in triplicate. A dry weight of roots, as well as 8-desoxylactucin content were estimated.

General chromatographic procedure

Conventional column chromatography: silica gel Merck Art. 7754; TLC: silica gel Merck Art. 5553; semipreparative and analytical HPLC: Waters Delta-Pak C-18 cartridge column (particle size 15 μm, 25 × 100 mm, flow rate of 3 ml min^{-1}) and Waters μ-Bondapak C-18 column (particle size 10 μm, 2 × 300 mm, flow rate of 0.5 ml min^{-1}), respectively, using MeOH-H_2O systems as mobile phase and monitoring with a Waters 991J UV photodiode array detector.

Extraction and isolation of compounds

The lyophilised roots (19.7 g) were ground and exhaustively extracted with methanol at room temperature. The extract was concentrated *in vacuo* providing a residue (7 g) which was subjected to column chromatography on silica gel using hexane-EtOAc (up to 100% EtOAc) followed by EtOAc-MeOH (up tp 100% MeOH) gradient solvent systems. Fractions from hexane-EtOAc (1:1, v/v) elution were further separated by preparative TLC ($CHCl_3$-MeOH, 19:1) to give **1** (1.4 mg) and a mixture (1.2 mg) containing **2** as a major constituent. Fractions eluted with EtOAc and EtOAc-MeOH (19:1) were purified by preparative TLC ($CHCl_3$-MeOH, 17:3) to afford sesquiterpene lactone glycoside mixtures. The mixtures were processed by semipreparative HPLC (MeOH:H_2O, 1:1) to give crepidiaside B (**3**, 0.5 mg), sonchuside A (**4**, 0.8 mg) and ixerisoside D (**5**, 0.8 mg). Compounds **1–5** were identified by direct comparison with compounds isolated previously in our laboratory.

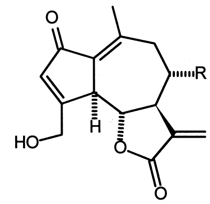
Quantification of 8-desoxylactucin

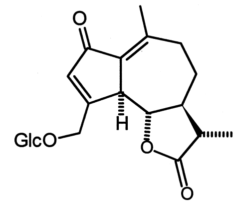
A quantitative analysis was performed according to the procedure described by Peters and van Amerongen (1997). Freshly harvested roots were frozen with solid CO_2, ground and stored at – 20 °C, until use. A frozen root powder (5 g) was suspended in 5 ml of water and incubated at 40 °C for 6 h. After incubation, the suspension was centrifuged at 12,000 × *g* for 10 min and the supernatant was extracted three times with 5 ml of ethyl acetate. The organic fractions were combined, evaporated under reduced pressure and the dry residue was redissolved in 0.5 ml of methanol for HPLC analysis. The sample (25 μl) was injected into a Waters μ-Bondapak C-18 column coupled with a photodiode array detector. Gradient elution was employed. Methanol content in the mobile phase changed linearly from 10% to 80%, throughout 90 min analysis. Peak areas were measured at 256 nm, with reference to a standard curve derived from four concentrations of 11β,13-dihydrolactucin ranging from 0.25 to 2.00 mg ml^{-1}.

Results and Discussion

An infection of *C. intybus* leaf explants with *A. rhizogenes* LBA 9402 resulted in formation of hairy roots in 30% of the explants. The roots were capable of growing in the nutrient medium without growth regulators and synthesized both agropine and mannopine. An analysis of amplification products obtained in PCR confirmed the presence of the *rol B* gene in their genomic DNA. The gene codes for a β-glucosidase capable to hydrolyse indole-β-glucosides and is partly responsible for altered phenotype of transformants (Estruch *et al.*, 1991). Root tips were individually inoculated in the modified MS medium containing cefotaxime. After 10 passages, the obtained bacteria-free clones were transferred to the medium without antibiotics. The aseptic roots of the clone which showed the most favourable growth characteristics were chosen for phytochemical investigation and the time course experiment.

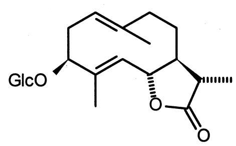
The methanol extract of the roots was repeatedly chromatographed on silica gel to afford **1** and several mixtures of compounds of almost the same polarity. The mixture of structurally closely related compounds, containing **2** as the main constituent was not further separated. The mixtures containing sesquiterpene lactone glycosides **3–5** were separated by RP-HPLC. All the compounds (Fig. 1) were identified by comparison of their spectra (500.13 MHz ^{1}H NMR, UV) and retention times with those of authentic samples and with literature data. Sesquiterpene lactones and their glycosides, isolated previously in our laboratory from *Cicho-*

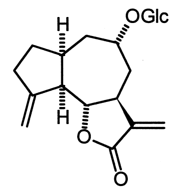




1 R=H 8-desoxylactucin
2 R=A lactucopicrin

3 crepidiaside B





4 sonchuside A

5 ixerisoside D

Fig. 1. Structures of sesquiterpene lactones isolated from *C. intybus* hairy root culture.

rium sp. and *Lactuca sp.* (Kisiel and Zielińska, 2001; Kisiel *et al.*, 1995) were used as authentic samples for identification of compounds **1–5**. The transformed roots of *C. intybus* synthesised sesquiterpene lactones characteristic of roots of the intact plant, except for eudesmanolides which were not detected in the analysed plant material.

Figure 2 shows the time course of growth and 8-desoxylactucin production in the hairy root culture of *C. intybus*. A dry weight of roots reached its maximum (0.38 g per flask) after 30 days of culture and was ca. 10 times higher than the respective weight of inoculum used. The biomass increase was the most abundant from 15 to 25 day of culture. The content of 8-desoxylactucin (**1**) ranged from 0.03 to 0.18 mg g^{-1} fresh weight, in 5 and 25 days old cultures, respectively. After 30 days of culture, accumulation of **1** in the roots decreased rapidly. Taking into consideration that a ratio of fresh weight to dry weight of the roots was ca. 20, the measured content of **1** was similar to that found by Rees and Harborne (1985) in roots of field grown chicory plants.

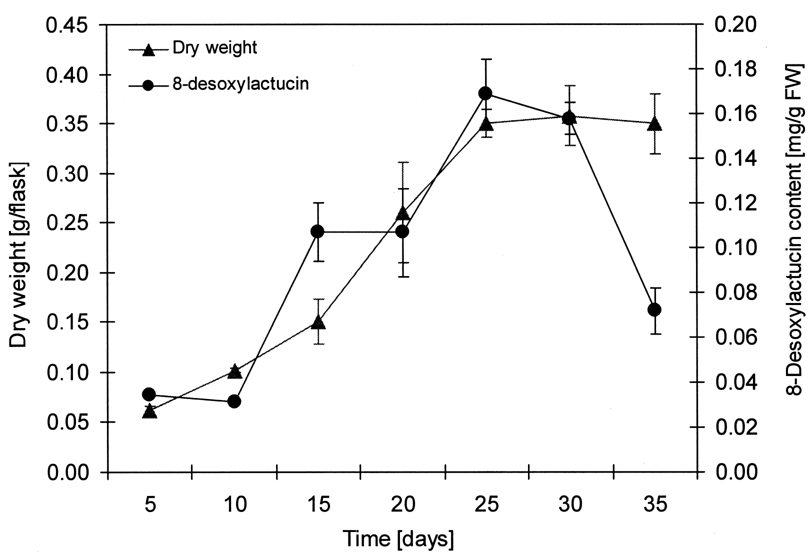


Fig. 2. Time course of biomass (dry weight) and 8-desoxylactucin production in transformed root culture of *C. intybus*. Values are means of three measurements. Bars represent standard deviation.

Lactucin-type sesquiterpene lactones, as well as some enzymes involved in their biosynthesis, could be readily obtained from roots of cultivated plants. The roots in the past were roasted and used as a coffee additive or substitute and at present, in Europe, are regarded as a waste product in the production of chicory heads (chicons) (de Kraker *et al.*, 1998, 2001). However, the hairy root culture offers an appropriate system to study factors influencing sesquiterpene lactone biosynthesis.

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