Thymol Derivatives from a Root Culture of *Inula helenium*

Anna Stojakowska*, Janusz Malarz, and Wanda Kisiel

Department of Phytochemistry, Institute of Pharmacology, Polish Academy of Sciences, Smętna 12, PL 31-343, Kraków, Poland. Fax: +48 12 637 45 00. E-mail: stoja@if-pan.krakow.pl

* Author for correspondence and reprint requests

Z. Naturforsch. **59 c**, 606–608 (2004); received March 19/April 20, 2004

A root culture of *Inula helenium* L. was established from leaf explants of aseptic seedlings. An ethanol extract from the lyophilised roots was fractionated using different chromatographic techniques (CC, TLC). The main secondary metabolites found in the root culture were two thymol derivatives: 10-isobutyryloxy-8,9-epoxy-thymol isobutyrate (**1**) and 10-isobutyryloxy-6-methoxy-8,9-epoxy-thymol isobutyrate (**2**). The compounds were identified by spectral methods. Quantification of compound **1** in plant material was done by analytical RP-HPLC.

Key words: Inula helenium, Root Culture, Thymol Derivatives

Introduction

Elecampane (*Inula helenium* L., Asteraceae, tribe Inuleae) is a reputed medicinal plant native of Middle Asia. This perennial herb widely occurs in Asia, Europe and Northern America. Roots collected in the autumn from two- or three-year-old *I. helenium* plants (*Radix Inulae*, *Radix Helenii*) are officially listed in some European pharmacopoeias (*e.g.* PF X, Ned 5, HAB 34). The roots contain up to 5% of essential oil with eudesmane-type sesquiterpene lactones (mainly alantolactone and isoalantolactone), thymol derivatives, triterpenes, sterols and up to 44% of the polysaccharide inulin (Blaschek *et al.*, 1998). The sesquiterpene lactones of *I. helenium* show cytotoxic and antiproliferative activities against human cancer cell lines. Moreover, the compounds are plant growth inhibitors (Blaschek *et al.*, 1998; Lawrence *et al.*, 2001; Dirsch *et al.*, 2001; Konishi *et al.*, 2002). To our knowledge, no reports are available on tissue culture of the species. In the course of the present study a root culture of *I. helenium* was established and its capability to accumulate secondary metabolites characteristic of the intact plant was investigated.

Material and Methods

Plant material

Seeds of *I. helenium* were delivered by the Botanical Garden of All-Russian Research Institute of Medicinal and Aromatic Plants VILAR in Moscow. The seeds were aseptically germinated on a hormone free MS (Murashige and Skoog, 1962) medium, solidified with 0.8% agar, at 25 °C and continuous light (ca. 40 μmol m^{-2} s^{-1}, cool white fluorescent tubes). Regeneration of roots from leaf explants of aseptic seedlings was achieved by wounding of a main vein with a scalpel previously immersed in liquid nutrient medium. Root tips were excised and cultivated in liquid Gamborg B5 medium (Gamborg *et al.*, 1968) with macro- and micronutrients of ½ strength, containing 5% sucrose, on a gyrotory shaker (110 rpm) at 25 °C with a 16 h photoperiod (20 μmol m^{-2} s^{-1}, cool white fluorescent tubes). The roots were subcultured every four weeks by inoculating 0.3 g fresh weight of biomass in 30 ml of a fresh nutrient medium. Quantification of **1** was done in the cultured roots as well as in callus tissue of *I. helenium*, grown on solidified MS medium containing 1.0 mg l^{-1} 2,4-D and 0.3 mg l^{-1} of kinetin, in roots of *I. helenium* aseptic seedlings and in roots of field grown *I. helenium* plants, which were used as a reference material.

Isolation of thymol derivatives **1** *and* **2** *from in vitro cultured roots of I. helenium*

Lyophilised and pulverized roots (48 g) were extracted with EtOH (4 × 450 ml) at room temperature. The extract was evaporated *in vacuo* providing an oily residue (19 g) which was subjected to column chromatography on silica gel (Merck, Art. 7754) using hexane/EtOAc (up to 100% EtOAc) followed by a EtOAc/MeOH (up to 10% MeOH) gradient solvent systems. Fractions eluted with hexane/EtOAc (9:1) and hexane/EtOAc (4:1) were further separated by preparative TLC on precoated silica gel plates (Merck, Art. 5553) in solvent systems identical with those used in column

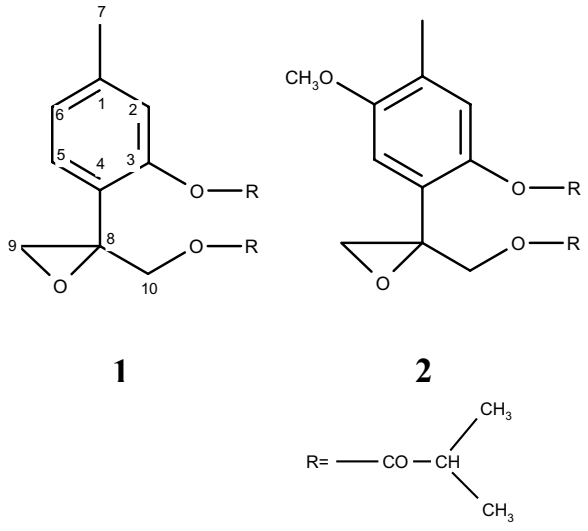

Fig. 1. Chemical structures of 10-isobutyryloxy-8,9-epoxy-thymol isobutyrate (**1**) and 10-isobutyryloxy-6-methoxy-8,9-epoxy-thymol isobutyrate (**2**) isolated from *I. helenium* root culture.

chromatography. The fractions yielded 11.3 mg of **1**, and an additional amount of **1** (1.0 mg) and 1.4 mg of **2**, respectively.

Identification of compounds **1** *and* **2**

Structures of the isolated compounds were determined by comparison of their spectral data (^{1}H NMR, 500.13 MHz; UV) with those found in the literature (Bohlmann *et al.*, 1969, 1976; Mossa *et al.*, 1997).

Quantification of compound **1** *by RP-HPLC*

A dry, pulverized plant material (0.1 g) was extracted twice with 10 ml of $CHCl_3$ at room temperature. The combined extracts were dried over anhydrous Na_2SO_4 and the solvent was removed under reduced pressure. The dry residue was redissolved in 0.5 ml of MeOH for HPLC analysis. The sample (20 μl) was injected into a Merck Purospher RP-18e cartridge column (3 × 125 mm) coupled with a dual wavelength UV/VIS absorbance detector. As mobile phase MeOH/H_2O (3:2) at a flow rate of 1 ml min^{-1} was employed. Peak areas were measured at 205 nm, with a reference to a standard curve derived from five concentrations of **1** ranging from 0.063 to 1.000 mg ml^{-1}. Five consecutive injections of **1** at a concentration of 62.5 μg ml^{-1} gave a deviation in area of 1.4% and in retention time of 0.6% (12.14–12.37 min).

Results and Discussion

Roots of *I. helenium*, excised from leaf explants of aseptic seedlings grew well in the hormone free nutrient medium. The growth index (G. I.) calculated as the ratio of the final weight of biomass, after four weeks of culture, to the weight of inoculum used was 16.7 ± 0.95.

Isoalantolactone and alantolactone, sesquiterpene lactones characteristic of roots of the intact plant, were not detected neither in calli nor in the analysed root culture. Their absence could be interpreted either as a lack of capability to synthe-

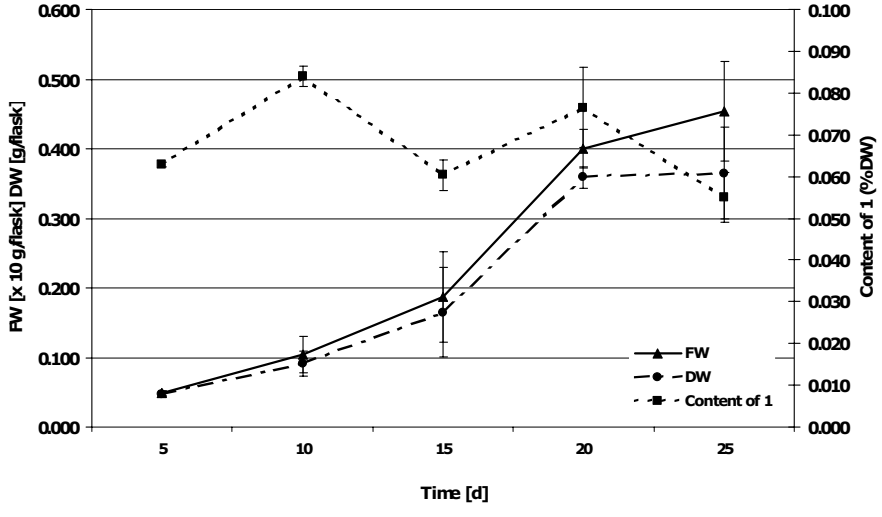

Fig. 2. Time course of biomass and 10-isobutyryloxy-8,9-epoxy-thymol isobutyrate (**1**) accumulation in root culture of *I. helenium*. Values are means of three measurements. Bars represent standard deviation. FW, fresh weight; DW, dry weight.

size the eudesmanolides or as a shortage of storage compartment. The alantolactone/isoalantolactone content in roots of *in vitro* cultivated aseptic seedlings of *I. helenium* was two orders of magnitude lower than that found in the roots of the intact plant, so no one of the explanations could be excluded.

Compounds **1** and **2** isolated from the root culture were identified as 10-isobutyryloxy-8,9-epoxy-thymol isobutyrate and 10-isobutyryloxy-6-methoxy-8,9-epoxy-thymol isobutyrate, respectively (Fig. 1). They were previously isolated from roots of some plants of *Inula* sp. (Bohlmann and Zdero, 1977; Bohlmann *et al.*, 1978). Their content in the analysed root culture was similar to that found in the roots of seedlings and remained relatively stable throughout all phases of the culture growth. A transient decrease in accumulation of **1** in the examined roots could be observed in the beginning of the logarithmic phase of growth and in the late stationary phase (Fig. 2). The thymol derivatives were absent from the callus tissue examined. Results of a quantitative analysis of **1** in plant material obtained from *in vitro* culture and in roots of field grown plants are summarized in Table I. A time course of both fresh and dry weight accumulation as well as of compound **1** content in *I. helenium* root culture are shown in Fig. 2.

Table I. Compound **1** content, estimated by RP-HPLC, in plant material derived from *in vitro* culture and field grown plants of *Inula helenium*.

Plant material	Content of **1** (% dry weight)
Roots of field grown two-year-old plants	0.200 ± 0.017
Roots of aseptic seedlings	0.084 ± 0.002
Root culture	0.071 ± 0.010
Callus culture	not detected

A thymol derivative (10-acetoxy-8,9-epoxy-thymol isobutyrate) isolated from *Arnica sachalinensis* showed moderate antifeedant activity (Passreiter and Isman, 1997). Whether or not thymol derivatives participate in chemoprotective and antibacterial (MIC $< 10\,\mu g\ ml^{-1}$ against *Staphylococcus aureus* FDA 209P) activity of *I. helenium* root extract (Gorecki, 2001) remains to be elucidated.

Blaschek W., Hänsel R., Keller K., Reichling J., Rimpler H., and Schneider G. (1998), Hagers Handbuch der Pharmazeutischen Praxis, Vol. 2: Drogen A–K. Springer Publ., Berlin–Heidelberg–NY, pp. 526–530.

Bohlmann F. and Zdero C. (1977), Neue Sesquiterpenlactone und Thymol-Derivate aus *Inula*-Arten. Phytochemistry **16**, 1243–1245.

Bohlmann F., Niedballa U., and Schulz J. (1969), Über einige Thymolderivate aus *Gaillardia*- und *Helenium*-Arten. Chem. Ber. **102**, 864–871.

Bohlmann F., Zdero C., and Grenz M. (1976), Inhaltsstoffe einiger Gattungen der Tribus Helenieae und Senecioneae. Phytochemistry **15**, 1309–1310.

Bohlmann F., Mahanta P. K., Jakupovic J., Rastogi R. C., and Natu A. A. (1978), New sesquiterpene lactones from *Inula* species. Phytochemistry **17**, 1165–1172.

Dirsch V. M., Stuppner H., and Vollmar A. M. (2001), Cytotoxic sesquiterpene lactones mediate their death-inducing effect in leukemia T cells by triggering apoptosis. Planta Med. **67**, 557–559.

Gamborg O. L., Miller R. A., and Ojima K. (1968), Nutrient requirements of suspension cultures of soybean root cells. Exp. Cell Res. **50**, 151–158.

Gorecki P. (2001), Attempt at estimation of therapeutic value of some medicinal plants including those, which are not documented. Herba Pol. **47**, 29–45.

Konishi T., Shimada Y., Nagao T., Okabe H., and Konoshima T. (2002), Antiproliferative sesquiterpene lactones from the roots of *Inula helenium*. Biol. Pharm. Bull. **25**, 1370–1372.

Lawrence N. J., McGown A. T., Nduka J., Hadfield J. A., and Pritchard R. G. (2001), Cytotoxic Michael-type amine adducts of α-methylene lactones alantolactone and isoalantolactone. Bioorg. Med. Chem. Lett. **11**, 429–431.

Mossa J. S., El-Feraly F. S., Muhammad I., Zaw K., Mbwambo Z. H., Pezzuto J. M., and Fong H. H. S. (1997), Sesquiterpene lactones and thymol esters from *Vicoa pentanema*. J. Nat. Prod. **60**, 550–555.

Murashige T. and Skoog F. (1962), A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. **15**, 473–497.

Passreiter C. M. and Isman M. B. (1997), Antifeedant bioactivity of sesquiterpene lactones from *Neurolaena lobata* and their antagonism by γ-aminobutyric acid. Biochem. Syst. Ecol. **25**, 371–377.