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ORIGINAL PAPER

Accumulation of the labdane diterpene Marrubiin in glandular trichome cells along the ontogeny of *Marrubium vulgare* plants

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Abstract The content of the diterpene Marrubiin was assessed by GC-FID in leaves of *Marrubium vulgare* plants along their ontogeny. Maximum accumulation occurred just before flowering time and in fully expanded leaves. After feeding the plants with radio labeled [^{3}H]-geranyl geranyl diphosphate, up to 70% of the radioactivity was recovered in HPLC-Rt coincidental with authentic Marrubiin, which was also characterized by GC-EIMS, thus confirming that the biosynthesis of Marrubiin proceeds through the 1-deoxy-D-xylulose-5-phosphate pathway. The major accumulation of radioactivity occurred in glandular trichome cells, and the product remained stable throughout.

Keywords *Marrubium vulgare* · Diterpene · Marrubiin

Abbreviations

[^{3}H]-GGPP	Tritium-labeled geranyl geranyl di-phosphate
DOX/MEP	1-Deoxy-D-xylulose-5-phosphate/2-methyl-D-erythritol-4-phosphate pathway
GC-FID	Capillary gas chromatography-flame ionization detection
GC-EIM	Capillary gas chromatography-electron impact mass spectrometry

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Introduction

Marrubium vulgare Tourn. (Horehound) is an herbaceous perennial plant belonging to the Lamiaceae family, which has been used since ancient time to solve cough and respiratory problems worldwide. The furanic labdane diterpene Marrubiin (Fig. 1) is the major product responsible of therapeutic properties produced by *Marrubium* sp. (Karioti et al. 2005).

Terpene biosynthesis in plants goes through both the mevalonate pathway in the cytosol and the 1-deoxy-D-xylulose-5-phosphate/2-methyl-D-erythritol-4-phosphate (DOX/MEP) pathway in plastids (Lichtenthaler et al. 1997; Rohmer 1999). Terpenes include many metabolites like pigments such as carotenes (Van den Berg et al. 2000), phytohormones such as abscisic acid, gibberellins and brasinosteroides (Crozier et al. 2000), and phytoalexins (Lange and Ghassemian 2003; Kanno et al. 2006). The production and accumulation of Marrubiin in plants of *M. vulgare* may follow a non-mevalonate pathway like in Eubacteria and Gymnospermae (Knoss et al. 1997; Knoss and Zapp 1998; Paseshnichenko 1998; Cunningham et al. 2000). Studies by Kanno et al. (2006) showed that rice plants produce both

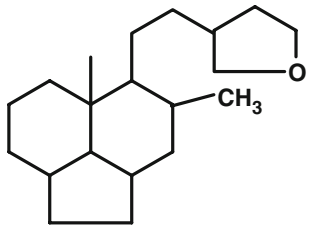

Fig. 1 Chemical structure of Marrubiin

enantiomeric forms of the diterpene copalyl diphosphate (*ent*-CDP and *syn*-CDP) via the DOX pathway, as the precursors for several classes of phytoalexins and the phytohormone gibberellins. Anatomical studies suggest that the synthesis of some diterpenes in Lamiaceae family is localized in the secretory cells of glandular trichomes, and the filling of the subcuticular space occurs only in actively growing protodermal regions on the leaf surface (Amelunxen 1965; Gershenzon et al. 1989; McCaskill et al. 1992; Werker et al. 1993). The occurrence of diterpene biosynthesis in other plant species is also associated with the metabolic activity of glandular trichomes which do not exhibit any apparent turnover (Kolb and Muller 2004).

In this work, Marrubiin production was assessed throughout the ontogeny of *M. vulgare* plants. We also show that feeding with an acyclic diterpene precursor (geranyl geranyl di-phosphate) labeled with tritium, [^{3}H]-GGPP, on the surface of protodermal leaves, had a major impact on the accumulation of Marrubiin in glandular trichome cells via the DOX/MEP pathway, and that the product remained stable.

Materials and methods

Seeds of *M. vulgare* collected from plants located at the foothills in the South of Córdoba, Argentina, were used. Seeds were surface-sterilized for 20 s with a mixture of Na hypochlorite-ethanol 70%, carefully washed with sterile-distilled water, and then germinated and initially grown in Petri dishes on semi-solid synthetic Murashige and Skoog (1962) quarter-strength middle containing 2% sucrose and 0.8% agar, pH 5.8. The Petri dishes were placed in a growth chamber at 25 ± 2°C with a 14 h photoperiod (fluorescent light, 10 W m^{-2}). Selected plantlets were transferred to plastic pots with a mixture of peat moss: pumice: sand and cultivated in greenhouse with 16 h photoperiod (350 μmol m^{-2} s^{-1} of photosynthetic active radiation), and day/night temperature cycles of 22°C/10°C. Plants were watered and fertilized daily with a complete fertilizer commercial solution (N:P:K, 20:20:20, plus micronutrients).

To study developmental changes of Marrubiin accumulation in *M. vulgare* a single cohort of leaves from the apex to the base was utilized. Plant samples were harvested at 7, 14, 21, 28, 33, 47, 54 and 61 days. Between 28 and 33 days, whitish flower buds appeared on the apices of the stem, which began to open when leaves were 33–47 days old. By 54–61 days, the plants had finished flowering and were senescing. Triplicate samples of leaves of the various ages were harvested for analysis, each sample consisting of 4–15 leaves (depending on the plant age), and weighed prior to immediately freezing in liquid N_2 and extracted or stored at −20°C. Leaves were separated into apex, middle, and basal and soaked in methylene chloride at 0°C. After concentrating under a N_2 stream at room temperature, the samples were treated with active charcoal, filtered, and evaporated to dryness again under N_2. The residue was re-dissolved in methanol-water-acetic acid (8.9:2:0.1, v/v) and the solution pre-purified on reverse-phase C_{18} cartridges (Waters Associates, Milford, MA) after pre-conditioned with methanol–water–acetic acid (8.9:2:0.1, v/v). Marrubiin was further purified by reverse phase HPLC (μ-Bondapack C_{18} 3.4 × 300 mm columns, Waters Associates) eluted at 1 ml min^{-1} The first 10 min with methanol–water–acetic acid (8.9:2:0.1; v/v), then from 10% to 73% methanol over 30 min followed by 10 min at 73% methanol, and finally 100% methanol for 10 min. Fifty-five 1 min HPLC fractions were collected. Standard Marrubiin (Sequoia Research Products Ltd., Oxford, UK) was found in fractions 24–28 min. The samples were dried under vacuum and resuspended with 100 μl hexane and diluted 1:200 for GC-FID analysis, or submitted to GC-EIMS analysis.

GC-FID analysis showed the highest concentration of Marrubiin in middle leaves of 28 days-old plants (Fig. 2). Therefore, the metabolic studied used middle-aged leaves of *M. vulgare* that were 28 days-old. In order to investigate the metabolism and the possible turnover of Marrubiin, one protodermal middle leaf of a 28 days-old plant was fed with 5.4 KBq (3.8 × 10^7 dpm) [^{3}H]-GGPP (J Chappell, University of Kentucky, KY) in 100 μl ethanol. After 0, 6, 12, 24,

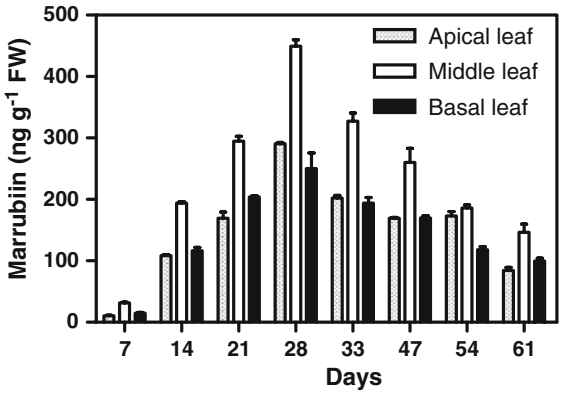

Fig. 2 Total concentration of Marrubiin in three levels of leaves (apical, middle, and basal) along the ontogeny of *M. vulgare* plants grown as stated in Material and Methods. HPLC fractions of methylene chloride extracts co-chromatographying with authentic Marrubiin standard were analyzed by GC-FID. The amount of Marrubiin was calculated by using hexadecane as reference compound, and from a calibration curve performed with authentic Marrubiin. Each value represents the mean of three biological replicates (different plants) with its corresponding SD

48, and 72 h of incubation, the plants were dissected and the middle aged leaves soaked twice for 30 min in methylene chloride at 4°C. The leaf extracts were then submitted to the same protocol described above for further analysis. Aliquots were removed from the HPLC fractions for label counting. Radioactivity was concentrated in fractions coincident with the Marrubium standard Rt's (fractions 24–26 min). The remaining HPLC fractions were analyzed by GC-FID and GC-EIMS.

Leaves of *M. vulgare* 28 days-old plants (15–20 g, ca. 10 mm in length) were excised and soaked in ice-cold 0.5 mM potassium phosphate buffer for 30 min. Glandular cells were then isolated according to Gershenzon et al. (1993) abrasion method using a cell disruptor (Bead beater, Biospec Products, Bartlesville, OK) with 120 g of 0.5 mm-diameter glass beads, 15 g XAD-4 polystyrene resin glass and an extraction buffer (pH 6.6, and consisting of 200 mM sorbitol, 10 mM sucrose, 25 mM Hepes pH 7.3, 10 mM KCl, 5 mM $MgCl_2$, 5 mM ditioeritritol, 0.5 mM potassium phosphate, 0.1 mM *PPi*, 0.6% [w/v] methyl cellulose, and 0.7% [w/v] polyvinylpyrrolidone [PVP_3]). Abrasion was carried out for three 1-min periods at 4°C using a rotor speed controlled with a rheostat set at 60 V. Following abrasion, the contents of the disruption chamber were filtered through a series of nylon filters of successively smaller mesh size (350, 150 and 20 μm) to separate the cell clusters from the glass beads, XAD-4 resin and residual plant material. Trichomes were collected on 20 μm mesh filter, resuspended, and then re-filtered several times in the 25 mM HEPES buffer without methyl cellulose, and PVP_3 to remove impurities (McConkey et al. 2000). To quantify the number of clusters obtained, a sample was removed for counting with a hemocytometer (1×10^6 cell trichomes g leaf^{-1} and ca. 50–70 μm in diameter).

Trichome cells were disrupted by grinding with liquid nitrogen in pre-chilled Eppendorfs. The ground material was suspended in methylene chloride for 30 min at 4°C and again, in a second portion of methylene chloride for 30 at 4°C. Then, the extract was concentrated under a stream of N_2 and processed as described above.

Capillary gas chromatography-flame ionization detection (GC-FID) was performed on a Hewlett-Packard model HP5890 gas chromatograph series II system (Foster City, CA) with a DB-5 column (30 m, 0.25 mm i.d. and 0.25 μm film thickness, J & W Scientific, Folsom, CA). The injector temperature was 250°C; the flow rate of gas carrier (N_2) was 1 ml min^{-1}; the oven temperature program was 2 min at 100°C, then 5°C min^{-1} up to 150°C, and 10°C min^{-1} to 250°C, finally 250°C for 2 min; flame ionization detector was set at 300°C. Marrubiin was identified by comparison of retention times with authentic standard (Sequoia Research Products Ltd.), and quantification was carried out based on a calibration curve performed with known amounts of standard and by comparison of the area peak of Marrubiin versus that of an injection of 1 μl hexadecane. After dissolving in 10 μl of hexane, 1 μl was injected split-splitless in a capillary gas chromatography-electron impact mass spectrometry (GC-EIMS) system (PerkinElmer Clarus 500, Chicago, IL). The GC column was a PerkinElmer Elite-5MS, cross-linked methyl silicone capillary column (0.25 mm internal diameter and 30 m long, 0.25 μm film thickness) eluted with He (1 ml min^{-1}). The oven temperature program was 2 min at 100°C, then 5°C min^{-1} up to 150°C, and 10°C min^{-1} to 250°C, finally 250°C for 2 min. The interface temperature was 280°C, electron impact was 70 eV.

Results and discussion

Marrubiin was characterized by capillary gas chromatography-electron impact mass spectrometry (GC-EIMS) from fractions 24–28 min of HPLC extracts of middle-aged leaves from 28 days-old *M. vulgare* plants. The mass spectrum data showed the characteristic ions with similar relative abundance as compared with authentic Marrubiin standard (Table 1).

Maximum Marrubiin concentration was obtained from middle-aged leaves after 28 days of plant growth (61 days in total for the experiment; Fig. 2).

Trichomes from leaves of 28 days-old plants accumulated up to 69% of the total radioactivity recovered after labeling with [^{3}H]-GGPP pulse (Fig. 3). The concentration of Marrubiin varied in trichome cells; after 6 h 40% of the total radioactivity was incorporated, this increased to approximately 69% after 24 h and then declined to 51% after 72 h. In leaves, the proportion of radioactivity recovered was similar to that in trichomes, although it only represented at maximum 12% of the total radioactivity after 12 h of incubation. Hence, the leaves of *M. vulgare* were able to incorporate the label from [^{3}H]-GGPP into Marrubiin in considerable amounts in trichomes but to a lesser extent in leaves. Also, by labeling with [^{3}H]-GGPP we were able to show that the high incorporation of labeled substrate into Marrubiin could be explained by the action of the DOX/MEP pathway in glandular trichome cells. As Marrubiin was identified by GC-EIMS as the main product of ^{3}H-GGPP metabolism in leaves of *M. vulgare* plants, the implications are that Marrubiin biosynthesis proceeds in plastids through terpene cyclization by cyclases-like Copalyl-diphosphate-synthase via the DOX/MEP pathway (Kanno et al. 2006). In fact, the proportion of GGPP radioactivity incorporated into Marrubiin was higher in glandular trichome cells compared to leaves (Fig. 3). Coincidentally, in leaves of 28 days-old *M. vulgare* plants, the higher concentration of Marrubiin was found in glandular trichome cells with respect to whole leaves (Fig. 4). Consequently these results suggest that the amphipolar molecule GGPP is the precursor in the cyclization of the labdane skeleton, and the high accumulation of the biosynthetic enzymes are located primarily in the peltate glandular trichome cells. In peppermint leaves, it has been demonstrated that diterpene synthesis is localized in the secretory cells of glandular trichomes (Gershenzon et al. 1989; McCaskill et al. 1992), and the diterpenes are discharged into a surmounting subcuticular storage compartment formed by expansion of the cuticle in protodermal regions of the leaf surface (Amelunxen 1965) only in actively growing tissues, i.e. middle-aged leaves (Werker et al. 1993). The occurrence of diterpene biosynthesis in other species is also associated with the metabolic activity of glandular trichomes in which diterpenes are synthesized.

These results correlate with the measurements of Marrubiin concentration in leaves versus trichomes as assessed by capillary gas chromatography-flame ionization detection (GC-FID) from samples of the same time-course experiment described before (Fig. 4), since the amount of Marrubiin found in trichomes exceeded that in leaves by an average factor of 10.

Interestingly, the Marrubiin accumulation showed a low turnover during these experiments. This suggests that Marrubiin is a stable product showing little catabolism, which is a useful characteristic for therapeutic compounds and nutraceuticals of economic importance. Our results support those of earlier studies where there were no significant losses of radio labeled diterpenes over time (Mihaliak et al. 1991). In the current study, *M. vulgare* leaves showed no significant losses of diterpenes over 72 h. It is however important to state that in these experiments we focused only on Marrubiin turnover in middle-aged leaves on 28 day-old plants; also we did not study the possible transport implications within the plant. What happens to the remaining radioactive product is unclear, but it is possible that as with peppermint, the catabolic pathway involving the sequential reduction and

Table 1 Mass Spectrum data of Marrubiin standard, and of Marrubiin identified in middle leaves of 28 days-old *M. vulgare* plants

Compound	M^+ and characteristic ions (relative abundance)
Marrubiin standard	332 (10), 207 (5), 191 (6), 163 (12), 152 (25), 135 (31), 123 (22), 109 (58), 95 (68), 81 (100), 67 (22), 55 (19)
Purported Marrubiin	332 (2), 207 (3), 191 (1), 163 (3), 152 (22), 135 (30), 123 (21), 109 (64), 95 (62), 81 (100), 67 (21), 55 (21)

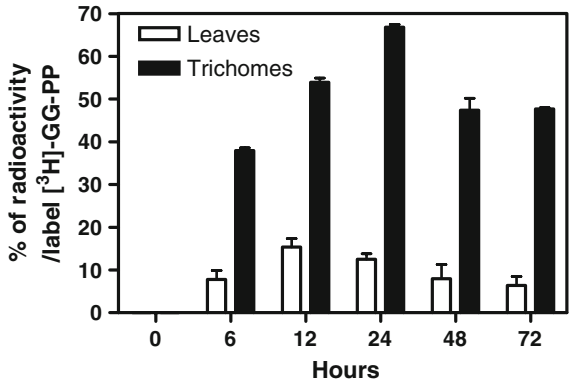

Fig. 3 Percentage of radioactivity recovered in a time-course experiment from leaves and trichomes of middle leaves of 28 days-old plants fed with [^{3}H]-GGPP. Radioactivity was assessed from HPLC fractions of methylene chloride extracts co-chromatographying with authentic Marrubiin standard. The values shown for leaves were obtained after substraction of trichome values to the total values for entire leaves (leaves with trichomes). Each value represents the mean of three biological replicates (different plants) with its corresponding SD

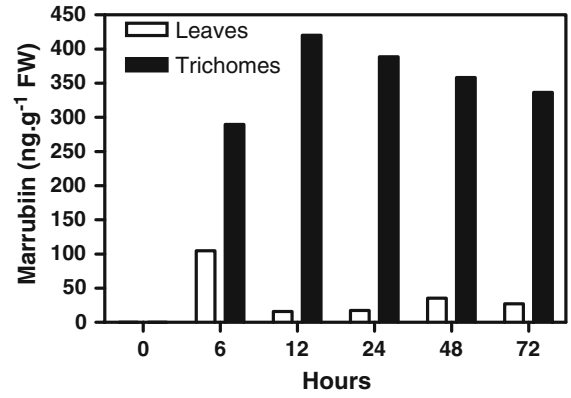

Fig. 4 Marrubiin concentration in a time-course experiment with leaves and trichomes of middle leaves of 28 days-old plants. HPLC fractions of methylene chloride extracts co-chromatographying with authentic Marrubiin standard were analyzed by GC-FID. The amount of Marrubiin was calculated by using hexadecane as reference compound, and from a calibration curve performed with authentic Marrubiin. The values shown for leaves were obtained after substraction of trichome values to the total values for entire leaves (leaves with trichomes). Each value represents the mean of three biological replicates (different plants) with its corresponding SD

glucosylation of menthone to neomenthol glycoside and its transport to the rhizome where it degrades may occur (Croteau and Martinkus 1979; Martinkus and Croteau 1981; Ringer et al. 2005). This may at least in part explain the fate of a small portion of Marrubiin in the rhizomes of *M. vulgare*.

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