Increase of Caryophyllene Oxide in Ageing Lemon Balm Leaves (*Melissa officinalis L.*) – A Consequence of Lipid Peroxidation?

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Oxidative processes, especially lipid peroxidation (LPO), are assumed to increase during ageing. In an attempt to provide experimental evidence for this assumption lemon balm plants (*Melissa officinalis* L.) of different age and location were analyzed for oxidatively caused changes in the etheric oil composition. The investigation revealed that the caryophyllene oxide (CarO) content of lemon balm plants – a main constituent – depends on two factors: age and habitat. The content of CarO increased with age continuously up to a factor of 3. Poor nutritive conditions, as growth on unfertilized soil, also caused an increase in CarO content. Addition of Fe^{2+}/ascorbate – enhancing oxidative processes – promoted the formation of CarO.

Hydroperoxides of unsaturated fatty acids (LOOHs) in the lipid extract of lemon balm leaves were converted to corresponding hydroxy acids (LOHs) by sodium borohydride reduction. These were hydrogenated and subjected to GC/MS analysis after derivatisation. A surplus of 9-hydroxy-octadecanoic acid over the 13 isomer indicated at least in part enzymatic lipid peroxidation. Polarographic determination of the oxygen consumption revealed a generally low but in ageing plants increased lipoxygenase activity. This indicates a contribution of lipid peroxidation in the epoxidation process of caryophyllene.

Introduction

Peel oil of oranges contains coumarins with isopentenyl side chains. These are accompanied by corresponding epoxidation products and dihydroxy derivatives (Ziegler and Spiteller, 1992). Similar observations are reported from other unsaturated terpenes e.g. linalool (Winterhalter *et al.*, 1986). Obviously the unsaturated terpenes are oxidized to epoxides and these are hydrolyzed to 1,2-diols.

Epoxidation processes were reported to be initiated by action of enzymes e.g. monooxygenases like cytochrome P-450 or epoxygenases (Ross *et al.*, 1978, Panthananickal *et al.*, 1983). Otherwise non enzymatic epoxidation of cholesterol by reaction with microsomal generated lipid hydroperoxides was demonstrated (Watabe *et al.*, 1984). We confirmed this observation: Hydroperoxides of linoleic acid are able to transform unsaturated terpenes, e.g. caryophyllene, to corresponding epoxides under physiological conditions (Meyer and Spiteller, 1993).

The terpene epoxide caryophyllene oxide (CarO) is reported to be one of the main constituents of lemon balm oil (*Melissa officinalis* L.) (Nykaenen 1985), but cell cultures of lemon balm did not produce caryophyllene oxide (CarO), although they contained caryophyllene (Car) (Schultze *et al.*, 1983). Because of the lack of an active enzyme system for the generation of CarO Berry *et al.* (1985) proposed a non-enzymatic epoxidation reaction (Fig. 1).

Production of hydroperoxides is increased by ageing (Dhindsa *et al.*, 1982). Since LOOH is able to produce epoxides, we suspected that caryophyllene epoxidation might be caused by ageing connected with an increased hydroperoxide production.

Abbreviations: BHT, 2,6-di-*tert*-butylhydroxytoluene; Car, caryophyllene; CarO, caryophyllene oxide; CH, cyclohexane; EA, ethylacetate; HODE, hydroxy-octadecadienoic acid; LOOH, lipid hydroperoxide; LOX, lipoxygenase; LPO, lipid peroxidation; MSTFA, N-methyl-N-trimethylsilyltrifluoroacetamide.

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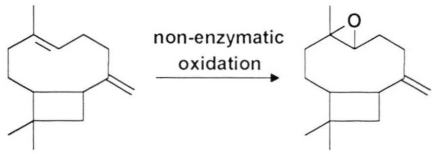


Fig. 1. Epoxide derived from the sesquiterpene caryophyllene.

In this communication we report on experiments to clarify this hypothesis.

Results and Discussion

Leaves of lemon balm plants (*Melissa officinalis* L.) from different sites (compost, unfertilized soil) and of different age (young and old leaves, see Experimental: plant material) were harvested and 50 g fresh intact leaves were subjected to continuous steam distillation. The volatile compounds were separated from the condensed steam by cyclohexane extraction. After drying and removal of solvent 32 ± 11 mg etheric oil (0.086 - 0.042 % relative to weight of fresh plant material) were obtained. The etheric oil was analysed without derivatisation by GC/MS.

Caryophyllene turned out to be one of the major components of lemon balm oil (13 ± 6% by GC peak area measurement) and the main constituent of the sesquiterpenes (37 ± 17%). Caryophyllene oxide represented about 1.7% of the total content of lemon balm oil. It was the main sesquiterpene oxidation product.

The caryophyllene (Car)/caryophyllene oxide (CarO) system turned out to be well suited for quantitative determination of terpene/terpene epoxide ratio during ageing for several reasons:

1) Car and CarO are both main constituents of lemon balm;

2) both compounds show baseline separation by GC analysis; Therefore quantification of caryophyllene and its epoxide could be achieved by peak area comparison;

3) caryophyllene oxide (CarO) is the stable epoxidation product of caryophyllene.

Thus the CarO/Car-ratio [%] is a characteristic index for the epoxidation process.

Plants from a habitat with sufficient supply of nutritive compounds (raised on compost) contained only small amounts of caryophyllene oxide in relation to caryophyllene (7.8% ± 0.9%). Plants of same age but grown on poor (unfertilized) soil contained considerably more caryophyllene oxide (22.2% ± 3.4%) related to the content of Car. The CarO/Car ratio increased with age and developed continuously from 4.6% ± 0.4% in young leaves (harvested in first half of May) of plants grown on compost to 7.8% ± 0.9% in leaves of blooming plants (harvested at the end of July) up to 11.0% ± 1.5% in seed-producing plants (harvested at the end of September).

Steam distillation of pure (-)-caryophyllene in water or Fe^{2+}/ascorbate solution did not cause formation of CarO, excluding its artificial genesis during steam distillation. If steam distillation of lemon balm leaves was carried out in Fe^{2+}/ascorbate solution – a typical lipid peroxidation inducing system – the CarO amount raised from 4.6% ± 0.4% to 12.6% ± 1.0% in young leaves (Fig. 2).

This indicated an autocatalytic transformation of plant linoleic acid to its hydroperoxides (9-LOOH and 13-LOOH) which in turn are able to epoxidize caryophyllene (Car), as shown recently (Meyer and Spiteller, 1993). Otherwise LOOH are produced physiologically in plants by action of lipoxygenases.

A distinction whether LOOH is generated autocatalytically or enzymatically was made by determination of the ratio of LOOH isomers. In a non-enzymatic lipid peroxidation the ratio of LOOH

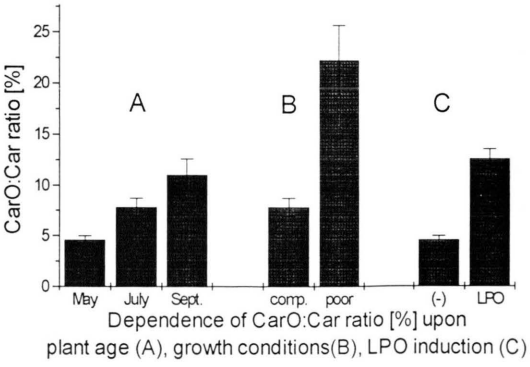
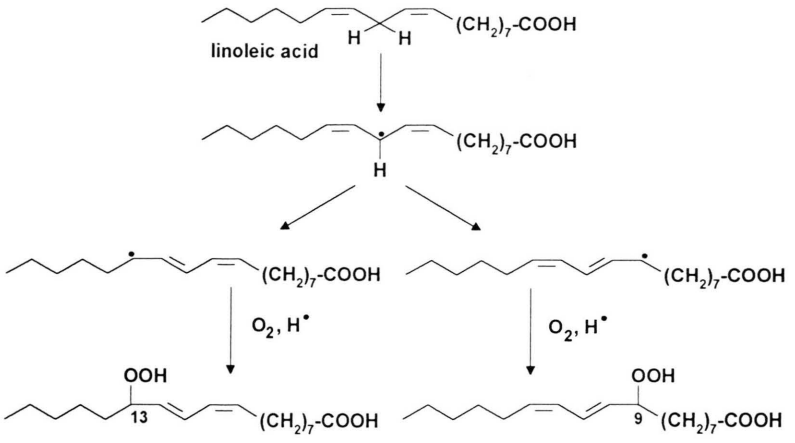


Fig. 2. Content of caryophyllene (Car) and caryophyllene oxide (CarO) of lemon balm oil from plant leaves harvested at different age [A: young leaves (harvested in May), leaves of blooming plants (July) and seed-producing plants (September)], at different location [B: compost and unfertilized (poor) soil] and in dependence of induced lipid peroxidation by addition of Fe^{2+}/ ascorbic acid [C: LPO in relation to untreated young leaves (-)].



13-LOOH : 9-LOOH ratio:		
autocatalytic:	1 : 1	*)
enzymatic: tomato:	5 : 95	**)
soybean:	95 : 5	**)
lemon balm leaves:	2 : 1	***)

Fig. 3. Distinction between autocatalytic or enzymatic oxidation of linoleic acid by determination of the 9-LOOH to 13-LOOH ratio (data presented according to *)Mlakar and Spiteller, 1996; **)Hamberg and Samuelsson, 1967; Gardner, 1991; ***)own measurement).

isomers produced from linoleic acid (9- and 13-LOOH) is nearly equal (Mlakar and Spiteller, 1996). A shift of this ratio to one of the isomers indicates a enzymatic – lipoxygenase catalyzed – reaction (Hamberg and Samuelsson, 1967; Gardner, 1991) (Fig. 3).

The 9-LOOH to 13-LOOH ratio was determined by the following method: Lipid extracts of homogenates of lemon balm leaves were obtained by the procedure of Bligh and Dyer (1959). Since a direct analysis of LOOHs by GC/MS is impossible, the LOOHs were treated with sodium borohydride to reduce them to corresponding hydroxy fatty acids (HODE). The double bonds were saturated by catalytic hydrogenation. The saturated fatty acids were transformed to their methyl esters by reaction with diazomethane and these were analyzed by GC/MS after trimethylsilylation.

The predominance of the 9-LOOH isomer over 13-LOOH (2.0±0.3 : 1) in lemon balm leaves indicated an at least partly enzymatic origin of LOOHs.

The rate of LOX involvement in this process could not be determined because of the lack of knowledge of the specificity of *Melissa officinalis* LOX.

Lipoxygenase activity was determined by measuring the oxygen consumption of lemon balm leave homogenates polarographically (Zhang *et al.*, 1991) at different pH, without and with addition of linoleic acid. 1.5 g fresh lemon balm leaves were homogenized in 15 ml buffer solution. After centrifugation 3 ml of sample solution were transferred to each of the two Clark oxygen electrode vials. To one vial linoleic acid substrate was added (see Experimental). From the difference of O_2-consumption of the two electrodes the LOX activity was determined.

The measurement revealed a maximum oxygen consumption at pH 5.5–7.0 in leave homogenates of lemon balm. The oxygen consumption of lemon balm was at pH 5.8 between $1.5 \pm 0.1 \times 10^{-6}$ [mol O_2 x g(sample)$^{-1}$ x min^{-1}] in young leaves and $0.8 \pm 0.2 \times 10^{-6}$ [mol O_2 x g(sample)$^{-1}$ x min^{-1}] in old leaves from plants harvested in September. Addition of the LOX substrate linoleic acid raised the oxygen consumption for 5% (young) or 27% (old) which can be assigned to LOX activity. Thus a continuous increase of LOX activity with age was detected by comparison of young ($0.07 \pm 0.01 \times 10^{-6}$ [mol O_2 x g(sample)$^{-1}$ x min^{-1}]) and old leaves of lemon balm ($0.29 \pm 0.01 \times 10^{-6}$ [mol O_2 x g(sample)$^{-1}$ x min^{-1}]) (Fig. 4).

This together with the finding that a surplus of 9-LOOH is produced is an indication for the increasing action of LOX in ageing lemon balm plants.

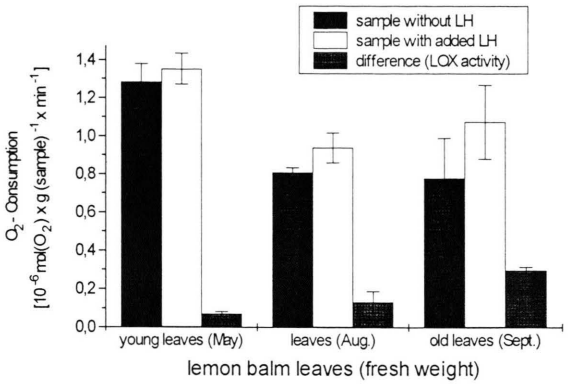


Fig. 4. Measurement of O_2-consumption in lemon balm leaves in dependence of age at pH 5.8 and determination of LOX activity by addition of linoleic acid (LH) as substrate.

Therefore we propose that at least in part enzymatically produced LOOH can be transformed – induced by Fe^{2+} – in LOO· respectively LO·. These species are able to epoxidize caryophyllene non-enzymatically (Fig. 5).

Independent from these considerations regarding the mechanism of epoxidation – the presented data reveal that ageing of lemon balm leaves is connected with an increase of epoxidation products, which can be used as markers for ageing.

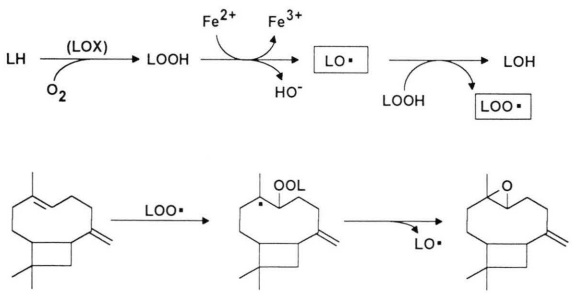


Fig. 5. Proposed mechanism of non-enzymatic epoxidation of caryophyllene by LOOH.

Experimental

Materials

N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) was obtained from Machery & Nagel (Düren). All other chemicals were purchased from Fluka (Neu Ulm). Solvents, obtained from Merck (Darmstadt), were distilled before use. TLC was performed on home made 0.75 mm PF_{254} silica gel 60 (Merck, Darmstadt) plates.

Plant material

Lemon balm (*Melissa officinalis* L.) plants were grown in the Botanical garden of the University of Bayreuth, Germany, on poor (unfertilized) soil (bad nutritious conditions) or compost (good nutritious conditions). Young leaves were harvested in the first half of May down to the fifth leave from the top of non-flowering plants. Old leaves were collected from the middle region of the stem of blooming (end of July) or seed-producing plants (end of September).

Steam distillation

Steam distillation was carried out on a modified apparatus for continuous steam distillation (Sprecher, 1963) as described in DAB 9 (Hartke and Mutschler, 1986). 50 -100 g of fresh plant material were added to 2 l of distilled water and heated for 4 hours. The volatiles were continuously extracted with 1 ml cyclohexane, concentrated by a nitrogen stream and subjected without derivatization to GC and GC/MS analysis.

Lipid peroxides were proven to initiate caryophyllene epoxidation by steam distillation of homogenized plant material treated with (2 l) 20 mM sodium-ascorbate/0.8 mM Fe_2SO_4 solution (Miller *et al.*, 1990).

To decide whether CarO is an artefact, steam distillation of pure Car was carried out in distilled water respectively ascorbate/Fe^{2+} solution.

All workup procedures were carried out twice, GC injection in triplicate to determine the experimental error.

GC- and GC/MS analysis

Gas-liquid chromatography (GC) was carried out with a United Technologies Packard Model 438S chromatograph equipped with a flame ionization detector and a Shimadzu C-R3A integrator.

GC conditions: fused silica DB-1 capillary column (30 m x 0.32 mm i. d., film thickness 0.1 µm, J&W Scientific, Mainz-Kastel); carrier gas: hydrogen; splitting ratio: 1:10; injector temperature:

270°C; detector temperature: 290°C; temperature program: 60°C isotherm for 3 min, heating rate from 60 to 280°C, 3°C min^{-1}, 280°C isotherm for 15 min;

Determination of linear retention indices was achieved by coinjection of a mixture of n-alkanes (C_{10} – C_{30}) (Van den Dool and Kratz, 1963).

Quantification of caryophyllene (Car) and caryophyllene oxide (CarO) was done by GC peak area comparison. The response factor of the CarO/Car system was determined to be 1.24 ± 0.15.

The correctness of peak assignment and compound identification was achieved by GC/MS: Gas chromatograph HP 5890 series II, fused silica DB-1 capillary column (30 m x 0.32 mm i. d., film thickness 0.1 μm, J&W Scientific, Mainz-Kastel; all other conditions were the same as reported above for GC). The GC was coupled to a Finnigan MAT 95 mass spectrometer, data system MAT ICIS (DECstation 5000/120); ionization energy: 70 eV.

Caryophyllene oxide: GC (DB-1): RI = 1505. GC-EIMS (70 eV): m/z (rel. Int.) = 41(100), 93(87), 91(81), 79(69), 105(66), 133(60), 69(52), 161(35), 187(34), 202(33), 220[M^+](3).

Extraction of fatty acids

The lipid fraction was extracted from the plant material by a modified procedure of *Bligh and Dyer* (Bligh and Dyer, 1959; Kates, 1986): 100 g plant material was homogenized in 500 ml methanol/dichloromethane (2:1, v/v). BHT (0.2 %) was added to avoid autoxidation (Esterbauer and Cheeseman, 1990). After stirring under argon atmosphere for 1 hr the homogenate was filtered. The aqueous phase was removed and the organic phase was brought to dryness in vacuum. About 2 · g crude lipid extract was gained which was resuspended in 50 ml methanol/dichloromethane (2:1, v/v). By treatment with 0.2 g sodium borohydride LOOHs were reduced to hydroxy fatty acids. After stirring for 2 hr at 25°C the surplus of sodium borohydride was hydrolized by addition of 20 ml of water. After extraction with dichloromethane the lipids were hydrolyzed by stirring with THF/sodium methanolate (1:2, v/v) for 3 hours. The solution was acidified by addition of 1N HCl and water. The dichloromethane extract was dried in vacuum and the residue (about 500 mg) was methylated by addition of an etheric diazomethane solution. The oxidized fatty acids methyl esters were separated from non-oxidized ones by preparative thin layer chromatography (CH:EA 8:2 v/v, R_f=0.5–0.25). Unsaturated hydroxy fatty acids methyl esters (about 10 mg) thus obtained were hydrogenated with Pd/C in methanol to obtain saturated hydroxy fatty acid methyl esters. A 0.3 mg sample was trimethylsilylated with 10 μl MSTFA and subjected to GC/MS analysis.

9-HODE (hydrogenated, TMS derivate, methyl ester, lit.: Graveland, 1970): GC (DB-1): RI = 2325. GC-EIMS (70 eV): *m/z* (rel. Int.) = 73(40), 155(19), 229(100), 259(81), 339(6), 355(6), 371(5), 386([M^+]4).

13-HODE (hydrogenated, TMS derivate, methyl ester, lit.: Terao and Matsushita, 1975): GC (DB-1): RI = 2352. GC-EIMS (70 eV): *m/z* (rel.Int.) = 73(17), 173(100), 211(16), 243(12), 286(22), 315(84), 339(10), 355(8), 371(7), 386([M^+]2).

Polarographic determination of lipoxygenase activity

The polarographic assay for measurement of lipoxygenase activity in homogenized plant tissue was carried out as described by Zhang *et al.* (1991) using a YSI Model 5300 Biological Oxygen Monitor equipped with two Clark style oxygen electrodes (YSI Inc., Yellow Springs, USA), a temperature controlled stirring unit (Schlag, Bergisch-Gladbach, Germany) and a LKB Bromma 2210 recorder.

The electrodes were calibrated before the assay by stirring with air saturated distilled water at 30°C. Linoleic acid substrate was prepared by dispersing 15 mg linoleic acid in 5 ml of borate buffer at pH 8.9. For the assay 1,5 g of fresh plant material was homogenized in 15 ml buffer solution. After centrifugation (1000 rpm, 1 min) 3 ml of clear sample solution was transferred to each of the two electrode vials. To one of the vial 200 μl of linoleic acid substrate was added. The vials were equipped with the electrodes and the O_2-consumption was measured at 30°C for 10 min. If no linear decrease of O_2 in relation to time was observed, the solution was diluted with buffer. LOX activity was calculated according to Zhang *et al.* (1991).

Determination of pH optimum of buffer solutions was carried out starting at pH 3.0 in steps of 0.5 units to pH 9.5 using citric acid/sodium hydrogenphosphate buffer from pH 3.0 to 7.0 and borate buffer from pH 7.6 to 9.5. Measurement of tomato and soy bean homogenates were carried out at pH 5.8 and 8.9, those of lemon balm at pH 5.8. Measurements were performed twice for each sample.

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