Running title: Antioxidant functions of carnosic acid and carnosol

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Research area:
Signaling and Response
Carnosic acid and carnosol, two major antioxidants of rosemary, act through different mechanisms

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One-sentence summary:

Rosemary leaves contain two phenolic diterpenes, carnosic acid and carnosol, which provide protection against oxidative stress by distinct mechanisms involving ROS scavenging or inhibition of lipid oxidation.
M.L. was supported by a CIFRE studentship from the French national association for research and technology (ANRT).

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M.L., S.B. and M.H. designed the experiments; M.L. performed most experiments; A. K.-L. performed EPR analyses; L.S. performed LC-HRMS analyses; M.L., A.B., S.B. and M.H. interpreted the data; M.L. and M.H. wrote the article with some input from the other authors.
Abstract

Carnosic acid, a phenolic diterpene specific of the *Lamiaceae* family, is highly abundant in rosemary species. Despite numerous industrial and medicinal/pharmaceutical applications of its antioxidative features, this compound in planta and its antioxidant mechanism have received little attention, except a few studies of rosemary plants under natural conditions. In vitro analyses, using HPLC-UV and luminescence imaging, revealed that carnosic acid and its major oxidized derivative, carnosol, protect lipids from oxidation. Both compounds preserved linolenic acid and monogalactosyldiacylglycerol from singlet oxygen and from hydroxyl radical. When applied exogenously, they were both able to protect thylakoid membranes prepared from Arabidopsis leaves against lipid peroxidation. Different levels of carnosic acid and carnosol in two contrasted rosemary varieties correlated with tolerance to lipid peroxidation. Upon ROS oxidation of lipids, carnosic acid was consumed and oxidized into various derivatives, including into carnosol, while carnosol resisted, suggesting that carnosic acid is a chemical quencher of ROS. The antioxidative function of carnosol relies on another mechanism, occurring directly in lipid oxidation process. Under oxidative conditions that did not involve ROS generation, carnosol inhibited lipid peroxidation, contrary to carnosic acid. Using spin probes and EPR detection, we confirmed that carnosic acid, rather than carnosol, is a ROS quencher. Various oxidized derivatives of carnosic acid were detected in rosemary leaves in low light, indicating chronic oxidation of this compound, and accumulated in plants exposed to stress conditions, in parallel with a loss of carnosic acid, confirming that chemical quenching of ROS by carnosic acid takes place in planta.
Introduction

Carnosic acid is a labdane-type diterpene present in plant species of the *Lamiaceae* family, such as rosemary and common salvia (Hossain et al., 2010; Birtic et al., 2015). This lipid-soluble compound is recognized for its high antioxidative capacities which have led to many industrial applications in the fields of food and beverage, personal care, nutrition and health (Birtic et al., 2015). The antioxidant properties of carnosic acid, presumably due to the presence of a catechol moiety (Supplemental Fig. S1), were evaluated mainly in vitro in a large variety of artificial and/or model systems. For instance, when tested in bulk and emulsified lipid systems, carnosic acid was found to protect fatty acids and triglycerides against oxidation (Hopia et al., 1996). Carnosic acid was also observed to prevent low-density lipoprotein oxidation in human aortic endothelial cells (Pearson et al., 1997) and lipid hydroperoxide-mediated oxidative stress in Caco-2 cells (Wijeratne and Cuppett, 2007). Inhibition of lipid peroxidation by carnosic acid was reported in rat liver microsomes and ox brain phospholipid liposomes (Aruoma et al., 1992). Food material such as oil, raw and cooked meat, and cooked meat patties were protected from oxidation by carnosic acid, in most cases with a higher efficiency than synthetic antioxidants (Erkan et al., 2009; Zhang et al., 2010; Naveena et al., 2013; Jordan et al., 2014). Carnosic acid was also described as a scavenger of hydroxyl and DPPH (2,2-diphenyl-1-picrylhydrazyl) radicals (Aruoma et al., 1992; Luis and Johnson, 2005). While the potential antioxidative activity of carnosic acid is well documented, its exact mechanism of action has not been studied extensively. In particular, little is known on the interactions of carnosic acid with distinct reactive oxygen species (ROS) or lipid radicals. Moreover, in most studies, in vitro oxidation was generated by prolonged and artificial heating treatments so that it is difficult to extrapolate the results to the in vivo situation in plants. Surprisingly, the role of carnosic acid in plant leaves has received little attention, and the biological role of this compound in plants is not firmly established.

Carnosic acid is present at very high concentrations, up to several % of dry weight, in leaves of the Mediterranean half-shrub rosemary (*Rosmarinus officinalis* L.) (Luis and Johnson, 2005; Munné-Bosh and Alegre, 2001; Del Baño et al., 2003). Carnosic acid biosynthesis and accumulation take place exclusively in young rosemary leaves at the branch apexes, with the diterpene molecule being partially consumed during leaf development and...
aging (Hidalgo et al., 1998; Brückner et al., 2014; Božić et al., 2015). Beside carnosic acid, less abundant phenolic diterpenes can be measured in rosemary leaves including carnosol (Supplemental Fig. S1), the major oxidation product of carnosic acid. The antioxidative activity of the latter compound, produced spontaneously from carnosic acid by non-enzymatic reaction, has been seldom investigated (Aruoma et al., 1992; Zeng et al., 2001).

Diterpene levels in field-grown rosemary plants displayed seasonal changes, with a tendency for carnosic acid losses in response to environmental stress conditions (Luis and Johnson, 2005). In particular, carnosic acid concentration in rosemary leaves under natural conditions was found to decrease at high temperatures and low precipitation rates in summer with a concomitant increase in oxidized derivatives, suggesting that cellular oxidative stress is accompanied by consumption of carnosic acid (Munné-Bosch et al., 1999; Munné-Bosch and Alegre, 2003). Both carnosic acid and carnosol accumulate in photosynthetic green tissues only (leaves, sepals and petals) and have been localized in the chloroplasts (Munné-Bosch and Alegre, 2001), although synthesis of carnosic acid has also been reported in glandular trichomes (Brückner et al., 2014).

Because the functions of the major rosemary diterpenes in plant leaves is poorly understood, we have performed a comprehensive study of the antioxidant activity of carnosic acid and its oxidized derivative carnosol, both in vitro and in rosemary plants. This study reveals different modes of action for carnosic acid and carnosol against ROS and lipid radicals, which make this diterpenoid tandem a peculiar and efficient antioxidant system in planta. It is likely that this carnosic acid-based protection mechanism is an important component in the tolerance of rosemary to withstand harsh climatic conditions that can prevail in its natural Mediterranean habitat.
RESULTS

Lipid protection by carnosic acid and carnosol in vitro

The lipid monogalactosyldiacylglycerol (MGDG), solubilized in methanol/chloroform, was oxidized with $^3$O$_2$ generated by illuminating the photosensitizing agent methylene blue. As expected (Birtić et al., 2011), the MGDG solution became luminescent after this oxidation treatment as imaged with a high-sensitivity cooled CCD camera (Fig. 1A). This photon emission originates from lipid peroxides whose slow decomposition produces light-emitting species such as triplet carbonyls and singlet oxygen, with the intensity of this signal being correlated with the extent of lipid peroxidation in the sample (Birtić et al., 2011; Cifra and Pospisil, 2014). When MGDG was supplemented with carnosic acid during $^3$O$_2$ oxidation, the luminescence signal intensity was noticeably reduced (Figs. 1A and 1B), indicating lower levels of MGDG oxidation and of lipid peroxides. Protection of MGDG against oxidation was also observed with carnosol and tocopherol, the protective effect of the latter compound appearing however to be slightly lower than the protection provided by carnosol and carnosic acid. Lipid protection by carnosic acid and carnosol was also obtained when the experiments were done with linolenic acid (C18:3) instead of MGDG (supplemental Fig. S2). Those observations show that, similarly to tocopherols (Liebler et al., 1986), both carnosic acid and carnosol are lipid protectors against attack by $^3$O$_2$. These effects were confirmed by analyzing HOTE (hydroxyoctadecatrienoic acid, oxidation product of the main fatty acid in leaves, linolenic acid) and HODE (hydroxyoctadecadienoic acid, oxidation product of linoleic acid) in the MGDG solution after $^3$O$_2$ oxidation (Fig. 1C). Both HOTEs and HODEs were substantially reduced by carnosic acid and carnosol.

MGDG was also oxidized by hydroxyl radicals produced by H$_2$O$_2$ and iron (Fenton reaction), leading to luminescence emission (Figs. 2A and 2B). Addition of carnosol to MGDG protects the galactolipid solution against oxidation, as shown by the marked decrease in luminescence (Figs. 2B and 2D). Surprisingly, addition of carnosic acid did not reduce MGDG luminescence after oxidation by hydroxyl radicals (Figs. 2A and 2C). On the contrary, carnosic acid strongly increased luminescence, and this phenomenon was still observed when carnosic acid concentrations were increased up to 600 μM. Actually, this luminescence enhancement was observed to be due to carnosic acid itself which became highly
luminescent when incubated in the presence of free radicals (without lipid). We checked that the mixture H$_2$O$_2$ + iron or a solution of carnosic acid in the absence of any ROS were not luminescent. The data of Fig. 2A thus suggest that carnosic acid reacts with free radicals leading to its oxidation and to the formation of light-emitting derivatives. This is confirmed
in Supplemental Fig. S2: a drastic loss of carnosic acid occurred when lipids were oxidized by \( \text{O}_2 \) or free radicals whereas carnosol levels were less affected. As a consequence, the lipid protective action of carnosic acid cannot be assessed through autoluminescence measurements. HPLC analyses of HOTE and HODE levels can overcome this problem. The
data shown in Fig. 2 revealed that carnosic acid, similarly to carnosol, does protect MGDG 
from oxidation by free radicals: the HOTE and HODE levels were noticeably reduced in the 
presence of carnosic acid or carnosol.

Interactions of carnosol and carnosic acid with reactive oxygen species

Oxidative degradation of carnosic acid by the hydroxyl radical is confirmed in Fig. 3. When H$_2$O$_2$ and iron were added to a solution of carnosic acid, the diterpene concentration rapidly fell, and an accumulation of carnosol was observed in parallel (Fig. 3A). The same phenomena were found with $^1$O$_2$ although the rates of carnosic acid disappearance and carnosol accumulation were slower compared to the effect of hydroxyl radicals. In striking contrast, carnosol was resistant to this oxidation: the carnosol concentration remained stable in the presence of hydroxyl radical or $^1$O$_2$ (Fig. 3B). These findings indicate that carnosic acid has a high reactivity towards ROS and is easily oxidizable. It is therefore likely that the antioxidant activity of carnosic acid relies on chemical quenching of ROS.

In Fig. 4, $^1$O$_2$ was produced from rose bengal in the light and was quantified using TEMPD, a $^1$O$_2$-specific spin probe (Hideg et al., 2011). The amplitude of the EPR spectra of TEMPD was strongly reduced by carnosic acid, and this effect was visible even at a low concentration of 10 μM (Fig. 4A and 4B). As expected from the data of Fig. 3, EPR analyses showed that carnosol does not quench $^1$O$_2$: 60 μM carnosol had very little effect of the amplitude of the $^1$O$_2$ EPR spectrum (Fig. 4C). This result confirms that carnosol is not able to eliminate $^1$O$_2$ in the μM concentration range although it protects lipids against oxidation in this concentration range. We also examined the effects of α-tocopherol, a known quencher of $^1$O$_2$ (Foote et al., 1974; Di Mascio et al., 1990). The quenching effect of tocopherol was visible at concentrations in the mM range only (Fig. 4D), thus indicating that tocopherol is a less efficient $^1$O$_2$ quencher than carnosic acid.

The spin probe POBN was used to measure the hydroxyl radical by EPR spectroscopy (Hideg et al., 2011). Carnosic acid was able to quench this ROS (Fig. 5A and 5B) while carnosol had virtually no effect on ROS concentration (Fig. 5C). Taken together, the data of Figs. 4 and 5 confirm that carnosic acid and carnosol differ in their reaction with ROS although both can protect lipids against ROS-induced lipid peroxidation (Fig. 1).
Using liquid chromatography coupled with mass spectrometry (LC-HRMS), we characterized oxidized derivatives (besides carnosol) generated during in vitro oxidation of carnosic acid by $^{1}$O$_2$ or hydroxyl radical in solution (Supplemental Fig. S3). Authentic standards were used to determine the retention times, m/z, full mass spectra and MS/MS.

**Figure 3.** Time course of the changes in carnosic acid and carnosol concentrations upon exposure to $^{1}$O$_2$ or hydroxyl radicals. $^{1}$O$_2$ was produced by illumination of methylene blue, and hydroxyl radicals were produced by the Fenton reaction using H$_2$O$_2$ + Fe$^{2+}$. A and B) Carnosic acid; C and D) carnosol. Data are mean values of 3 separate experiments ± SD.
spectra of carnosic acid and carnosol (Supplemental Figs. S4 and S5), allowing unambiguous identification of those compounds in oxidized solutions and in leaf extracts. Oxidation of carnosic acid was confirmed by a decrease in the carnosic acid peak and a concomitant production of carnosol. A variety of compounds obtained by oxidation of carnosic acid by $^1\text{O}_2$
and by hydroxyl radical was detected. There was a strong overlap between the oxidation
profiles of carnosic acid induced by $^{1}$O$_{2}$ and by the hydroxyl radical. Structures of rosmanol,
isorosmanol and 12-o-methyl carnosic acid were confirmed by matching their retention
times and MS/MS spectra with those of the reference compounds (Supplemental Figs. S6 to
S8), while rosmaridiphenol, 11’12-o-methylrosmanol, 7-methylisorosmanol, rosmadial isomers and 5,6,7,10-tetrahydroxyrosmaniquinone were putatively identified by matching bibliography data with the retention times and MS/MS spectra obtained experimentally with oxidized carnosic acid solutions and with rosemary leaf extracts (Supplemental Figs. S9 to S13).

**Carnosic acid and carnosol levels in rosemary leaves**

Rosemary leaves are known to accumulate high amounts of carnosic acid (Birtić et al. 2015), as confirmed in Fig. 6A for young leaves of the Sudbury Blue variety in which carnosic acid represented up to 10% of leaf dry weight under control growth conditions (250 μmol photons m⁻² s⁻¹ and 25°C). The major oxidized derivative of carnosic acid, carnosol, was less abundant (~ 2 μg/mg dry leaf weight), nevertheless representing about 0.2% of dry weight. We also analyzed the prenyl lipids, tocopherols and plastochromanol, which are both ubiquitous plastid antioxidants (Kruk et al. 2014, Kruk et al. 2016). Both compounds were found in rosemary leaves at concentrations noticeably lower than carnosol: ca. 0.1 and 0.01 μg/mg, respectively.

Growing rosemary plants for 4 weeks under harsh conditions of light and temperature (1200 μmol photons m⁻² s⁻¹ and 35°C/5°C (day/night)) led to a strong decrease in carnosic acid compared to control conditions (Fig. 6A). Concomitantly, the loss of carnosic acid was associated with a marked increase (about x3) in carnosol levels, suggesting consumption of the former compound during its antioxidant activity under stress conditions with partial conversion to its oxidized metabolite carnosol. A strong accumulation of tocopherols and plastochromanol was also observed after exposure of rosemary plants to high light and heat, thus exhibiting a behavior that contrasts with that of carnosic acid. This contrasting response was previously observed for carnosic acid and α-tocopherol in sage and rosemary exposed to natural drought conditions (Munné-Bosch and Alegre, 2003).

Carnosic acid and carnosol are present in photosynthesizing green tissues only (Munné-Bosch and Alegre, 2001; Luis and Johnson, 2005) and, in leaves, they have been found in the chloroplasts (Munné-Bosch and Alegre, 2001). However, carnosic acid and carnosol have also been reported to partition between trichomes at the leaf surface and internal leaf tissues (Bozić et al., 2015). This partitioning was estimated by briefly washing...
rosemary leaves (for 30 s) with dichloromethane in order to extract hydrophobic compounds from the trichomes. As shown in Fig. 6C, this treatment caused a complete emptying of the glandular trichomes while epidermal cells remained unaltered. The solvent after leaf dipping was found to contain both carnosic acid and carnosol (Fig. 6B), indicating storage of those compounds.
compounds in the trichomes. However, the amounts of diterpenes present in this fraction were relatively small, representing less than 10% of total amounts. Thus, in the Sudbury Blue variety investigated here, carnosic acid and carnosol are stored mainly within the leaves. This partitioning of carnosic acid and carnosol between trichomes and internal leaf tissues was not significantly modified by growth in high light at high temperature (Fig. 6B).

Oxidized derivatives of carnosic acid in planta

Some of the compounds detected in vitro after ROS oxidation of carnosic acid (supplemental Fig. S3) were also found in rosemary leaves grown under control conditions: rosmanol, isorosmanol, rosmaridiphenol, 7-methyl-epirosmanol, 7-methyl-rosmanol, 12-o-methylcarnosic acid and 5,6,7,10-tetrahydroxyrosmariquinone (Supplemental Figs. 4-12, Fig. 7), indicating chronic oxidation of carnosic acid by ROS in leaves. In line with this conclusion, 30-h adaptation of rosemary plants to darkness brought about a strong decrease in those compounds (Supplemental Fig. S14), confirming the link with light and the associated ROS production in the chloroplasts.

Under stress conditions that caused a strong decrease in carnosic acid and a concomitant accumulation of carnosol (Fig. 6), the levels of several oxidation products of carnosic acid, including rosmanol, isorosmanol, 5,6,7,10-tetrahydroxyrosmariquinone, 7-methyl-epirosmanol and 7-methyl-rosmanol, strongly increased in rosemary leaves (Fig. 7). The concentration of other oxidized metabolites of carnosic acid, such as rosmaridiphenol and 12-o-methylcarnosic acid, did not increase with the stress conditions. Accumulation of rosmanol and isorosmanol, as well as of methylated isorosmanol, was previously reported in rosemary plants exposed to drought stress in the field (Munné-Bosch et al., 1999). Accumulation of oxidized derivatives in rosemary leaves exposed to high light and high temperature supports the idea that the loss of carnosic acid observed under those conditions (Fig. 6) resulted from its oxidative degradation by ROS.

Exogenous carnosic acid and carnosol protect thylakoid membranes

Supplementing chloroplast membranes with carnosic acid was previously shown to preserve α-tocopherol and to reduce oxidative damage in high light (Munné-Bosch and Alegre, 2003). Moreover, a marked consumption of the exogenously applied carnosic acid
was observed during the high light treatment. We have performed a similar experiment with thylakoid membranes prepared from leaves of *Arabidopsis thaliana*, a species that does not contain carnosic acid, supplemented with carnosol or carnosic acid. Thylakoid suspensions were exposed for 2 h to high light (3000 μmol photons m⁻² s⁻¹), causing photooxidative...
Figure 8. Effects of carnosic acid or carnosol on Arabidopsis thylakoid membranes exposed to high light. Thylakoid suspensions were exposed to white light of PFD 1500 μmol photons m⁻² s⁻¹ for 2 h. A) Decrease in chlorophyll content after light treatment; B) HOTE with or without addition of 50 μM carnosic acid or carnosol to the membrane suspensions. Data are mean values of 3 separate experiments ± SD. * and *** indicate significant differences from control at P < 0.05 and P < 0.005, respectively (Student’s t test).
confirm that both carnosic acid and carnosol can protect biomembranes and function as membrane lipid protectors in vivo.

**Direct interaction of carnosol with the lipid peroxidation process**

As shown above (Fig. 3), carnosol is resistant to direct oxidation by ROS. However, when exposure to ROS took place in a lipid environment (linolenic acid, Supplemental Fig. S2), some loss of carnosol was observed. This could suggest that carnosol has the capacity to interact directly with the lipid peroxidation mechanism itself and can be degraded by reactions with some lipid oxidation-derived products. It has been shown that lipid hydroperoxides are capable of inducing membrane damage and lipid peroxidation in cell cultures (Wijeratne and Cuppett, 2006). Based on this observation, in vitro oxidation of linolenic acid was triggered with a hydroxy fatty acid (15-HEDE, 15-hydroxyeicosadienoic acid) in darkness and in absence of ROS or ROS generator. Luminescence from linolenic acid was noticeably increased by adding 15-HEDE (Fig. 9A), indicating oxidation of the fatty acid molecule. The luminescence of HEDE was found to be higher than that of linolenic acid (but lower than the linolenic acid + 15-HEDE combination), probably due to the spontaneous decomposition of the hydroxy fatty acid and the generation of light-emitting species. Addition of 60 μM or 120 μM carnosol to the mixture of linolenic acid + 15-HEDE significantly decreased luminescence (by 30 or 40%, respectively, Fig. 9B). This indicates that carnosol has a direct inhibitory effect on the lipid peroxidation process. We checked that carnosol had no effect of the 15-HEDE intrinsic luminescence (data not shown), excluding an action of the diterpene on 15-HEDE decomposition products. In contrast with carnosol, carnosic acid had no significant effect on HEDE-induced oxidation of linolenic acid (Fig. 9B).

The effect of carnosic acid in ROS-independent lipid oxidation was also tested in vivo. Wounding is known to trigger lipoxygenase activity in leaves, causing enzymatic lipid peroxidation (e.g. Chauvin et al., 2013) and inducing the associated generation of photon emission (Birtic et al., 2011). In Fig. 9C, leaves were injured with a scalpel in darkness. As previously shown (Birtic et al., 2011), the wounds can be visualized by the lipid oxidation-related luminescence emission. The intensity of this luminescence signal was significantly decreased in leaves pre-infiltrated with carnosol compared with leaves pre-infiltrated with a buffer that did not contain carnosol. Then, in line with the in vitro data shown in Fig. 9B,
carnosol can reduce lipid peroxidation in planta through a mechanism different from ROS scavenging. Similarly to what we observed with linolenic acid oxidized by 15-HEDE, carnosic acid was unable to inhibit lipid peroxidation in wounded Arabidopsis leaves. Thus, taken together, our results show that the antioxidant activities of carnosic acid and carnosol rely
on distinct mechanisms, involving direct interactions with ROS or with the lipid oxidation process, respectively.

**Comparison of two rosemary varieties containing different concentrations of carnosic acid and carnosol**

As shown in Fig. 10A, leaves of the Barbecue variety contains substantially less carnosic acid and carnosol than Sudbury Blue. As expected from the data of Fig. 6, exposure to high light at high temperature conditions caused a drastic loss of carnosic acid in both varieties (Fig. 10A), which was accompanied by increased levels of carnosol (Fig. 10B). However, the latter effect was less pronounced in the Barbecue variety relative to Sudbury Blue. Photooxidative damage to lipids in plants grown in high light or in control conditions was visualized in both rosemary varieties by autoluminescence imaging (Fig. 10C). Interestingly, Barbecue plants exposed to stress conditions were noticeably more luminescent than Sudbury Blue plants, indicating more oxidative stress and lipid peroxidation in the former variety. The correlation found in the experiment of Fig. 10 between leaf content in carnosic acid and carnosol and tolerance of rosemary to photooxidative stress is consistent with the lipid protective functions of those diterpenes observed in vitro (Figs. 1 and 8). However, the differential tolerance of Sudbury Blue and Barbecue to photooxidative stress must be interpreted with caution because the involvement of other factors in the responses of the two rosemary varieties cannot be excluded.
DISCUSSION

This study has confirmed that the phenolic diterpene carnosic acid is a potent antioxidant and has shown that this compound can efficiently protect lipids from oxidation, both in vitro (lipid solutions) and in vivo (biomembranes). This study provides also some...
insights into the mechanism underlying the antioxidative activity of carnosic acid. This compound was found to have a very high reactivity towards ROS, being readily oxidized and converted into a variety of metabolites in this process. Thus, carnosic acid acts as a ROS scavenger that can eliminate toxic ROS through its oxidation. Both singlet oxygen, an excited form of oxygen, and free radicals can be scavenged by carnosic acid, giving rise to overlapping profiles of oxidized molecules. Oxidized derivatives of carnosic acid were observed in rosemary leaves, both under control and stress conditions, and prolonged adaptation of rosemary plants to darkness brought about a marked decrease in their concentrations. This indicates chronic oxidation of carnosic acid in plants in the light and suggests that carnosic acid plays a protective role, not only under excess light energy when ROS production is expected to be elevated, but also in low light. This is in agreement with previous observations showing the presence of $^{1}\text{O}_2$-specific degradation products of polyunsaturated fatty acids in plant leaves in low light, reflecting continuous generation of $^{1}\text{O}_2$ in illuminated chloroplasts (Triantaphylidès et al., 2008). This phenomenon has led to the concept of lipid membranes acting as supramolecular antioxidants that capture ROS (Schmid-Siegert et al., 2016). This concept could be extended to the carnosic acid pool in rosemary leaves.

Interestingly, carnosol, the major oxidized metabolite of carnosic acid, was found to be an antioxidant and lipid protector as efficient as carnosic acid. This result is in line with early works that showed a protective effect of carnosol against lipid peroxidation in microsomal and liposomal systems (Aruoma et al., 1992). In previous studies, other carnosic acid-derived metabolites, such as rosmanol, epirosmanol or rosmaridiphenol, were also found to possess some antioxidative capacities. For instance, carnosol, rosmanol and epirosmanol were able to inhibit oxidation of lipoproteins in vitro (Zeng et al., 2001). Methyl carnosate was reported to be even more active than carnosic acid in the protection of triglyceride emulsions at 60°C (Huang et al., 1996). Rosmanol and epirosmanol were reported to inhibit mitochondrial and microsomal lipid peroxidation (Haraguchi et al., 1995), and the antioxidative activity of rosmanol and 20-deoxocarnosol was observed using the DPPH antioxidant assay (Escuder et al., 2002). The in vitro antioxidant activity of rosmanol, epirosmanol and isorosmanol was found to be higher than that of α-tocopherol (Nakatani and Inatani, 1984). Thus, when scavenging ROS, carnosic acid can generate a variety of
secondary antioxidants. This cascade-type process is likely to amplify the antioxidative
power of carnosic acid and to constitute an effective defense mechanism. Moreover, ROS
scavenging by carnosic acid can be fueled by the very large pools of this compound
(representing several % of leaf dry weight) that rosemary plants are able to accumulate in
their leaves.

Carnosol was much more resistant to oxidation by ROS than carnosic acid although it
protected lipids from oxidation as efficiently as carnosic acid. Contrary to carnosic acid,
carnosol could not lower the concentration of singlet oxygen or hydroxyl radical in solution.
The chemical quenching capacities of carnosol thus appear to be weak compared to carnosic
acid, and therefore the antioxidative activity of carnosol relies on a different mechanism that
does not involve its direct oxidation by ROS. A possibility is that carnosol directly reacts with
lipid radicals and hence blocks the lipid peroxidation chain-process. This idea was supported
by the inhibitory effect of carnosol on lipid peroxidation induced in vitro by a lipid
hydroperoxide or in vivo by lipoxygenase. Since this effect was observed in darkness under
conditions where ROS production was not induced, carnosol can act by interfering with the
lipid peroxidation process playing a lipid oxidation-blocking role like tocopherols (Tavadyan
et al., 2007). This phenomenon was not observed with carnosic acid. In the case of
tocopherol, the tocopheroxyl radical and tocopherol quinone formed in this process are
recycled either by reductants such as ascorbate (Liebler et al., 1986, Szarka et al., 2012) or by
enzymatically catalyzed reactions (Eugeni Piller et al., 2014). A similar recycling mechanism
could take place for carnosol. Interestingly, it has been shown that carnosol quinone, an
oxidized form of carnosol, is converted into carnosol in water-containing solvent (Masuda et
al., 2005). Similarly, thermal treatments of carnosol quinone in lipids can reform carnosol
(Masuda et al., 2004). These results suggest the possibility of a recycling mechanism for
carnosol which promotes recovery of its antioxidant activity under oxidative conditions. Also,
it has been shown that the antioxidative efficiency of carnosol surpasses that of carnosic acid
when assayed in model membranes (Perez-Fons et al., 2010 and 2006). This effect was
attributed to the enhanced lipid order by carnosol at the hydrophobic core of the
membrane, presumably contributing to membrane stabilization and hindrance of radical
propagation. Independently of the exact mechanism underlying the antioxidative function of
carnosol, the fact that the modes of action of carnosic acid and carnosol differ widens the action spectrum of rosemary diterpenes in the defense of plants against oxidative stress.

Carnosic acid is present exclusively in some species of the *Lamiaceae* family, such as rosemary, sage or oregano (Hossain et al., 2010; Birtić et al., 2015). However, some *Lamiaceae* species, such as basil and thyme, accumulate carnosol rather than carnosic acid. Most carnosic acid/carnosol-containing species are Mediterranean plants which can adapt to harsh climatic conditions and therefore need to protect themselves from oxidative stress. Although the in vitro antioxidant properties of carnosic acid have led to numerous applications in food science and medicine (Birtić et al., 2015), evidence for an antioxidative role in plants is missing. The main source of information on this aspect is the pioneering work by Munné-Bosch and co-workers who showed interdependence between the concentrations of carnosic acid and other low-molecular antioxidant molecules in rosemary leaves (Munné-Bosch and Alegre, 2003) as well as the presence of oxidized abietane diterpenes in field-grown rosemary plants in the summer (Munné-Bosch et al., 1999). The present work extends those previous studies and provides several arguments supporting that carnosic acid does fulfill an antioxidant function in planta. First, both carnosic acid and carnosol can protect chloroplast membranes against high light-induced oxidation. Because biomembranes are targets of high light, drought and high temperatures (Schwab and Heber, 1984; Conde et al., 2011), accumulation of those antioxidants is beneficial in Mediterranean climatic conditions. In rosemary, there is a wide diversity of carnosic acid accumulation levels in leaves (Wellwood and Cole, 2004). In a preliminary experiment, we analyzed the carnosic acid concentration in leaves of a large range of rosemary varieties from various geographic origins (not shown). The Barbecue variety contained low levels of carnosic acid. When grown under control conditions in a phytotron, leaf concentration in carnosic acid was lower by 40% in Barbecue compared to the Sudbury Blue variety. Also, under stress conditions, Barbecue was found to contain less carnosol than Sudbury Blue. Interestingly, these lower concentrations of carnosic acid and carnosol were correlated with a lower resistance to photooxidative stress, in line with a role for those diterpenes in the resistance of rosemary plants to photooxidative stress. Moreover, considering that carnosic acid functions as a chemical quencher of ROS, the light-dependent presence of oxidized carnosic acid derivatives in rosemary leaves and their marked accumulation in plants exposed to...
stress conditions indicate that the ROS-scavenging antioxidative action of carnosic acid does operate in vivo.

The biosynthesis pathway of carnosic acid is currently being elucidated. In particular, the enzymatic activities responsible for the first three steps in the pathway have been identified, and synthesis of the carnosic acid precursor ferruginol was achieved using yeast and *Nicotiana benhamiana* expression systems (Bozić et al., 2015). Subsequently, four P450-cytochromes have been identified, the combined activities of which account for all of oxidation events leading to the biosynthesis of carnosic acid when expressed in yeast (Ignea et al., 2016). As a perspective, it could be envisaged from those results to introduce the whole carnosic acid biosynthetic pathway in model plants that are naturally deficient in carnosic acid, such as tobacco or Arabidopsis. It is clear that a successful transformation of a vascular plant to express the newly elucidated steps and hence to induce carnosic acid accumulation would provide a useful tool to confirm the antioxidative and lipid protective activities of carnosic acid and carnosol described here.

**MATERIALS AND METHODS**

**Plant material and growth conditions**

Rosemary cuttings (*Rosmarinus officinalis* L., variety Sudbury Blue and Barbecue) were obtained from the plant nursery ‘SARL du Tilleul’ at Chateaurenard (France). Plants were grown on a soil/sand mixture (70/30) in a phytotron under a photon flux density (PFD) of 250 μmol photons m⁻² s⁻¹, a photoperiod of 12 h and a day/night temperature of 25/19°C. Stress conditions were imposed by transferring plants to a high PFD of 1200 μmol photons m⁻² s⁻¹ (photoperiod, 12 h) at a high day-temperature of 35°C combined with a low night-temperature of 5°C for 4 weeks. Young leaves at the top of plants aged 2 months were collected, weighed, frozen in liquid nitrogen and stored at -80°C before analyses.

**In vitro oxidation of biological molecules**

Linolenic acid (1-3 mg/ml, obtained from Fluka), monogalactosyl diacylglycerol (MGDG, 1-2 mg/ml, from Larodan), α-tocopherol (Naturex), carnosic acid (Extrasynthèse) and carnosol (Sigma Aldrich) were supplemented with methylene blue (final concentration...
0.1 mM). Oxidation of these molecules by $^{1}\text{O}_2$ was induced by exposing the mixture to white light produced by HQI metal halide lamps (Osram) (PFD, 750 $\mu$mol photons m$^{-2}$ s$^{-1}$) at 7°C (except when specified otherwise). For oxidation of linolenic acid (5-8 mg/ml methanol), MGDG (5 mg/ml methanol/CHCl$_3$), carnosic acid and carnosol by hydroxyl radicals, H$_2$O$_2$ and iron chloride (Fenton reaction) were added to the solutions and left to react for 20 s. 15-HEDE (15-hydroxy-11,13(Z,E)-eicosadienoic acid) was also used to oxidize linolenic acid in vitro: 15-HEDE in methanol was incubated at 60°C for 10 s and then mixed with linolenic acid (5 mg/ml in methanol) at a final concentration of 10 $\mu$M. 15-HEDE was prepared from eicosadienoic acid and soybean lipoxygenase according to the procedure described in Martini et al. (1994).

**Preparation of thylakoid membranes**

7 g of leaves (fresh weight) were grinded for 2 s in 50 ml of extraction buffer (330 mM sorbitol, 50 mM Tricine, 2 mM EDTA(Na$_2$), 1 mM MgCl$_2$, 2 mM Ascorbate, pH 7.7) with 5 mM dithiothreitol (DTT) in a Warring blender at low speed. The liquid phase was removed and set aside, and 50 ml of extraction buffer was added for a second extraction. The extracts were filtered onto 4 Miracloth layers, and the filtrate was centrifuged for 4 min at 1500 g at 4°C. The pellet was washed twice with the extraction buffer and centrifuged for 4 min at 1500 g at 4°C. The washed pellet was resuspended in 21 ml of lysis buffer pH 7.8 (10 mM Tricine, 10 mM NaCl, 10 mM MgCl$_2$) with 1 mM PMSF (phenyl methylsulfonyl) with occasional stirring for 15 min. The sample was centrifuged at 48400 g for 15 min. The pellet was resuspended in 1.75 ml of storage buffer (100 mM Tricine, 10 mM NaCl, 10 mM MgCl$_2$, 400 mM sucrose, pH 7.8) and stored at -80°C before analyses.

**HPLC-UV determination of carnosic acid and carnosol**

5 ml methanol/H$_3$PO$_4$ (99.5/0.5, v/v) was added to 25 mg of leaves (fresh weight). The mix was ground for 1 min with an Ultra-Turrax T25 (IKA-Werke) at 24000 rotation per min. After centrifugation at 4500 g for 10 min at 4°C, the pellet was resuspended in 2.5 ml methanol/H$_3$PO$_4$ for a second extraction. After filtration through 0.45 $\mu$m PTFE Costar filter, the extract was analyzed by HPLC-UV with a reverse phase column (Waters NovaPak 4 $\mu$M, 39 x 300 mm), isocratic elution with 65/34.8/0.2 (v/v/v) acetonitrile/water/H$_3$PO$_4$ at a flow
rate of 1 ml min⁻¹ and UV detection at 230 nm. Quantification was done using authentic standards of carnosic acid and carnosol.

**Diterpene extraction from trichomes by leaf dipping in solvent**

Carnosic acid and carnosol extraction from leaf trichomes was performed by dipping detached rosemary leaves for 30 s in 1 ml dichloromethane. The solvent was then evaporated under nitrogen. 250 µl of methanol with 0.5% H₃PO₄ was then added and the solution was subsequently analyzed by HPLC-UV, as described above. Diterpenes were extracted from the solvent-dipped leaves as described above.

**Prenyl lipid determinations**

60 mg of leaves was ground for 1 min in 2 ml of 100% ethyl acetate with an Ultra-Turrax at 24 000 rpm. After centrifugation for 3 min at 16900 g, 600 µl of extract were filtered with 0.2 µm PTFE filter. The extract was evaporated under a stream of nitrogen, and 1 ml of methanol/hexane (17/1, v/v) was added to the tubes before analysis by HPLC-UV/fluorescence. The samples were submitted to reverse phase HPLC using a Phenomenex Kinetex 2.6 µm column (100 x 4.6 mm) operating in the isocratic mode with methanol/hexane (17/1, v/v) as a solvent system at a flow rate of 0.8 mL min⁻¹, as previously described (Ksas et al., 2015) Tocopherols and prenyl lipids, excepted oxidized plastoquinone-9, were detected by their fluorescence at 330 nm with an excitation at 290 nm. Plastoquinone-9 in the oxidized state was measured by its absorbance at 255 nm.

**Chlorophyll fluorometry**

Chlorophyll fluorescence emission from leaves attached to the plant was measured with a PAM-2000 modulated fluorometer (Walz), as previously described (Havaux et al., 2003). The maximal quantum yield of PSII photochemistry was measured in dark-adapted samples by \( \frac{F_m - F_o}{F_m} = \frac{F_v}{F_m} \) ratio, where \( F_o \) is the initial fluorescence level and \( F_m \) is the maximal fluorescence level. \( F_m \) was measured with a 800-ms pulse of intense white light, and \( F_o \) was measured with a 1-s pulse of far red light.
Lipid peroxidation imaging

Lipid peroxides were visualized by autoluminescence imaging (Havaux et al., 2006). Imaged autoluminescence signals are attributed to the spontaneous decomposition of lipid peroxides (Birtić et al., 2011). Spontaneous photon emission from whole rosemary plants was measured after 2.5 h dark adaptation using a liquid N\textsubscript{2} cooled charge-coupled device (CCD) camera, as detailed previously (Birtić et al., 2011). Acquisition time was 20 min and pixel binning was 2 x 2. In vitro oxidation of lipid solutions (MGDG) was also measured by this method without dark pre-adaption and with a pixel binning of 5 x 5. The luminescence signals were analyzed and quantified with Image J software.

Biochemical analysis of lipid peroxidation

In vitro oxidation solution with 30% (w/v) MGDG was ground with Ultraturax T25 (IKA-Werk) in CHCl\textsubscript{3}/methanol (50/50, v/v) containing 5mM triphenyl phosphine, 1mM butylated hydroxytoluene and citric acid (1M). 15-hydroxy-11,13(Z,E)-eicosadienoic acid was added as internal standard. After centrifugation at 700 g for 5 min at 4°C, the organic phase (CHCl\textsubscript{3}) was evaporated under a stream of N\textsubscript{2} at 40°C for 30 min. Then, the organic phase was re-solubilized in ethanol and NaOH (3.5M). The sample was hydrolyzed at 80 °C for 30 min. pH was adjusted between 4 and 5 by addition of citric acid (1M), and hydroxy fatty acids were then extracted with hexane/ether (50/50). HOTE isomers (hydroxy octadecatrienoic acid, produced by the oxidation of linolenic acid) and HODE isomers (hydroxyl octadecadienoic acid, produced by the oxidation of linoleic acid) were separated and quantified by straight phase HPLC-UV analysis, as described previously (Montillet et al., 2004).

\(^{1}\text{O}_2\) and \(\text{OH}^\ast\) detection by EPR spin trapping

Spin-trapping assays with 4-pyridyl-1-oxide-N-tert-butylnitrone (4-POBN) to detect the formation of hydroxyl radicals (\(\text{OH}^\ast\)) were carried out using H\textsubscript{2}O\textsubscript{2} solution (50 µM), 50 mM 4-POBN and 50 mM Fe-EDTA in presence of carnosic acid, carnosol or α-tocopherol. To detect singlet oxygen, the spin probe 2,2,6,6-tetramethyl-4-piperidone hydrochloride TEMPD (100mM) was illuminated for 2 min with red light (RG 630) (1000 µmol photons m\textsuperscript{-2} s \textsuperscript{-1}) with rose bengal (100 µM) in presence of carnosic acid, carnosol or α-tocopherol. EPR spectra were recorded at room temperature in a standard quartz flat cell using an ESP-300 X-
band spectrometer (Bruker, Rheinstetten, Germany). The following parameters were used:
microwave frequency 9.73 GHz, modulation frequency 100 kHz, modulation amplitude: 1 G,
microwave power: 63 mW in TEMPD assays, or 6.3 mW in 4-POBN assays, receiver gain:
2x104, time constant: 40.96 ms; number of scans: 16.

Mass spectrometric analysis of metabolites
Mass spectrometry analyses were performed at the CRIBIOM platform (CHU Timone, Marseille, France). The LC-MS method was developed from Zhang et al. (2012) and Song et al. (2014). Samples were diluted in acetonitrile/water (65/35) and then analyzed by UPLC-HRMS and MS/MS.

The chromatographic separation was carried out on a Dionex Ultimate 3000 (Thermo Fisher Scientific) consisted of a rapid separation pump (RS) (LGP-3400 RS), an autosampler (WPS-3000 TRS) and a column compartment (TCC-3000 RS) all operated by Chromeleon 6.8 software. A reverse phase column Hypersil Gold (100 nm x 2.1 mm x 1.9 µm) (Thermo Scientific, Les Ulis, France) was used for the compound separation. Accurate mass measurements were performed on the Q-Exactive Plus mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) with a Heated Electrospray Ionization (H-ESI II) probe. Thermo Xcalibur 3.0.63 software was used for the instrument setup, control of the LC-MS system during acquisition and data treatment. Tune Q Exactive Plus 2.5 application was used for the direct control of the mass spectrometer. The column oven was maintained at 40°C, while the sample chamber temperature was set at 4°C. The mobile phase was 0.1% formic acid aqueous solution (A) and acetonitrile containing 0.1% formic acid (B), eluting according to the following program: 0-10 min, 40-80% B, 10-12 min, 80% B, 12-12.1 min, 80-40% 12.1-18 min, 40% B. The flow rate was set at 0.4 mL/min and the injection volume was 5 µL.

LC-HRMS analyses were performed with external calibration in positive and negative ionization mode providing a mass precision lower than 3 ppm. The H-ESI probe and the transfer capillary temperature were kept at 310°C and 320°C, respectively. Spray voltage was set at 3500 V and the S-lens RF level at 55. Sheath and auxiliary gas were maintained at 30 and 8 arbitrary units. Mass resolving power was set to 70000 full width at half maximum (FWHM) for m/z 200, the maximum injection time to 250 ms and auto gain control (AGC) to 10e6. LC-MS spectra were acquired in the mass range from m/z 80 to m/z 700.
MS/MS analyses were performed on the Q-Exactive Plus mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) using parallel reaction monitoring (HCD) experiments. For this purpose, resolving power was set to 70000 for m/z 200, AGC target 1e6 and maximum injection time 250 ms. Precursor ions were isolated in the 2 m/z isolation window in the quadrupole and then fragmented in the higher collision energy (HCD) cell under normalized collision energies previously determined. Thermo Xcalibur 3.0.63 software was used for the instrument set-up and control of the LC-MS system during acquisition as well as for data treatment. Carnosic acid and isorosmanol were obtained from Sigma, carnosol was purchased from Extrasynthèse (Genay, France), and rosmanol and 12-o-methyl carnosic acid were obtained from Phytolab (Vestenbergsgreuth, Germany). Carnosic acid-derived metabolites for which standards are not available were quantified using the conversion factor of carnosic acid.

Environmental scanning electron microscope

Rosemary leaves were dipped in dichloromethane for 30 s and the leaf surface was examined with a FEI QUANTA 200 FEG environmental scanning electron microscope (ESEM) operating at 30 kV. The sample was placed in a 5-mm diameter platinum crucible inside the microscope analysis chamber at a water vapor pressure of 600 Pa at 2°C.

Statistical analyses

All experiments were done at least in triplicates. Statistical differences between measurements on different treatments were analyzed following the Student’s t test. Differences were considered significant at a probability level of \( P < 0.05 \). One, two or three asterisks was attributed to \( 0.01 < P < 0.05, 0.005 < P < 0.01 \) and \( P < 0.005 \), respectively.

Supplemental Data

The following materials are available in the online version of this article.

SUPPLEMENTAL DATA TITLES
Supplemental Figure S1. Chemical structures of carnosic acid, carnosol, rosmanol, isorosmanol, 7-methyl-epirosmanol, 7-methyl-rosmanol, 12-O-methylcarnosic acid, 5,6,7,10-tetrahydroxyrosmariquinone, 11-12-di-O-methylisorosmanol, rosmadal and rosmaridiphenol.

Supplemental Figure S2. In vitro oxidation of linolenic acid with \( ^1 \text{O}_2 \) or with hydroxyl radicals in the presence of carnosic acid or carnosol.

Supplemental Figure S3. List of carnosic acid derivatives measured after in vitro oxidation of carnosic acid by singlet oxygen or hydroxyl radical.

Supplemental Figure S4. LC-HRMS analysis of carnosic acid in a standard product solution, a solution of oxidized carnosic acid and in a rosmeray leaf extract.

Supplemental Figure S5. LC-HRMS analysis of carnosol in a standard product solution.

Supplemental Figure S6. LC-HRMS analysis of 12-o-methylcarnosic acid in a standard product solution.

Supplemental Figure S7. LC-HRMS analysis of isorosmanol in a standard product solution and a solution of oxidized carnosic acid.

Supplemental Figure S8. LC-HRMS analysis of rosmanol in a standard product solution and a solution of oxidized carnosic acid.

Supplemental Figure S9. LC-HRMS analysis of probable 7-methyl rosmanol and 7-methyl epirosmanol in a rosemary leaf extract and in a solution of oxidized carnosic acid.

Supplemental Figure S10. LC-HRMS analysis of probable rosmadial isomers in a rosemary leaf extract and in a solution of oxidized carnosic acid.

Supplemental Figure S11. LC-HRMS analysis of probable isomers of 5,6,7,10-tetrahydroxy rosmariquinone in a rosemary leaf extract and in a solution of oxidized carnosic acid.

Supplemental Figure S12. LC-HRMS analysis of probable rosmaridiphenol in a rosemary leaf extract and in a solution of oxidized carnosic acid.
Supplemental Figure S13. LC-HRMS analysis of probable 11,12-o-dimethylrosmanol in a rosemary leaf extract and in a solution of oxidized carnosic acid.

Supplemental Figure S14. Effects of growth under dark adaptation (30 h) on the levels of several oxidized metabolites of carnosic acid in rosemary leaves.

SUPPLEMENTAL DATA LEGENDS

Supplemental Figure S1. Chemical structures of carnosic acid, carnosol, rosmanol, isorosmanol, 7-methyl-epirosmanol, 7-methyl-rosmanol, 12-O-methylcarnosic acid, 5,6,7,10-tetrahydroxyrosmariquinone, 11-12-di-O-methylisorosmanol, rosmadinol and rosmaridiphenol.

Supplemental Figure S2. In vitro oxidation of linolenic acid (C18:3) with $^1$O$_2$ (produced by methylene blue in the light) or with hydroxyl radicals (Fenton reaction) in the presence of carnosic acid or carnosol. A) Lipid luminescence after $^1$O$_2$ oxidation of C18:3. B) Decrease in carnosic acid and carnosol concentrations (in % of initial concentration) after $^1$O$_2$ oxidation of C18:3. C) Decrease in carnosic acid and carnosol concentrations (in % of initial concentration) after hydroxyl oxidation of C18:3.

Supplemental Figure S3. List of carnosic acid derivatives measured after in vitro oxidation of carnosic acid by singlet oxygen or hydroxyl radical.

Supplemental Figure S4. LC-HRMS analysis of carnosic acid in a standard product solution, a solution of oxidized carnosic acid and in a rosemary leaf extract: extracted ion chromatogram XIC (m/z 331.19039), full mass spectra and MS/MS spectra.

Supplemental Figure S5. LC-HRMS analysis of carnosol in a standard product solution, a solution of oxidized carnosic acid and in a rosemary extract: extracted ion chromatogram XIC (m/z 329.17474), full mass spectra and MS/MS spectra.

Supplemental Figure S6. LC-HRMS analysis of 12-o-methylcarnosic acid in a standard product solution, a solution of oxidized carnosic acid and in a rosemary leaf extract: extracted ion chromatogram XIC (m/z 345.20603), full mass spectra and MS/MS spectra.
Supplemental Figure S7. LC-HRMS analysis of isorosmanol in a standard product solution and a solution of oxidized carnosic acid: extracted ion chromatogram (m/z 345.16965) and MS/MS spectra.

Supplemental Figure S8. LC-HRMS analysis of rosmanol in a standard product solution and a solution of oxidized carnosic acid: extracted ion chromatogram XIC (m/z 345.16965) and MS/MS spectra.

Supplemental Figure S9. LC-HRMS analysis of probable 7-methyl rosmanol and 7-methyl epirosmanol in a rosemary leaf extract and in a solution of oxidized carnosic acid: extracted ion chromatogram XIC (m/z 359.1853) and MS/MS spectra.

Supplemental Figure S10. LC-HRMS analysis of probable rosmadial isomers in a rosemary leaf extract and in a solution of oxidized carnosic acid: extracted ion chromatogram XIC (m/z 343.15400) and MS/MS spectra.

Supplemental Figure S11. LC-HRMS analysis of probable isomers of 5,6,7,10-tetrahydroxy rosmariquinone in a rosemary leaf extract and in a solution of oxidized carnosic acid: extract ion chromatogram XIC (m/z 301,17982) and MS/MS spectra.

Supplemental Figure S12. LC-HRMS analysis of probable rosmaridiphenol in a rosemary leaf extract and in a solution of oxidized carnosic acid: extract ion chromatogram XIC (m/z 315.19547) and MS/MS spectra.

Supplemental Figure S13. LC-HRMS analysis of probable 11,12-o-dimethylrosmanol in a rosemary leaf extract and in a solution of oxidized carnosic acid: extract ion chromatogram XIC (m/z 373.20095) and MS/MS spectra.

Supplemental Figure S14. Effects of growth under dark adaptation (30 h) on the levels of several oxidized metabolites of carnosic acid in rosemary leaves.

ACKNOWLEDGMENTS

We are grateful to the members of the Phytotec platform (CEA/Cadarache) for their help in growing plants under control and stress conditions, to Bernard Genty (CEA
Cadarache) for help with the autoluminescence imaging technique, to Brigitte Ksas (CEA Cadarache) for advices in HPLC analyses, Jerzy Kruk (Krakow, Poland) for the gift of plastochromanol standard, and Renaud Podor (CEA/Marcoule) for help with ESEM analyses. We also thank Alain Tissier (Halle, Germany) and Jean-Luc Montillet (CEA Cadarache) for useful discussions.

FIGURE LEGENDS

Figure 1. Effects of carnosic acid, carnosol and \( \alpha \)-tocopherol on \textit{in vitro} oxidation of lipids by \( ^1 \text{O}_2 \). \( ^1 \text{O}_2 \) was produced by 30-min illumination of the lipid solution (MGDG) in the presence of methylene blue. (A-C) \( ^1 \text{O}_2 \) oxidation of MGDG in the presence of 60 \( \mu \text{M} \) or 120 \( \mu \text{M} \) carnosic acid, carnosol or \( \alpha \)-tocopherol. A) Luminescence images of the oxidized solutions. The color palette indicates signal intensity from black (0) to white (highest values). B) Quantification of the luminescence signals. Data are normalized to the control signal values measured in the absence of antioxidant. C) Hydroxy fatty acid quantification (HOTE and HODE). Data are normalized to the control HOTE values measured in the absence of antioxidant. CA, carnosic acid; CARN, carnosol. *, ** and *** indicate significant differences from control (0 \( \mu \text{M} \)) at \( P < 0.05 \), \( P < 0.01 \) and \( P < 0.005 \), respectively (Student’s t test).

Figure 2. Effects of carnosic acid and carnosol on \textit{in vitro} oxidation of lipids by free radicals. Hydroxyl radicals were produced by the Fenton reaction using \( \text{H}_2\text{O}_2 + \text{Fe}^{2+} \) in the presence of 60 or 600 \( \mu \text{M} \) carnosic acid (CA) or carnosol (CARN). A) Luminescence imaging of MGDG oxidized by the hydroxyl radical in the presence or absence of carnosic acid (60 and 600 \( \mu \text{M} \)). The luminescence signal of the mixture \text{H}_2\text{O}_2 + \text{iron} (hydroxyl radicals) and of CAR in the presence or absence of hydroxyl radicals was also measured, as controls. B) Luminescence imaging of MGDG oxidized by hydroxyl radical in the presence of carnosol (60 and 600 \( \mu \text{M} \)). C) Quantification of the luminescence signals shown in panel A. Data are normalized to the signal values measured from oxidized MGDG in the absence of antioxidant. D) Quantification of the luminescence signals shown in panel B. Data are normalized to the signal values measured from oxidized MGDG in the absence of antioxidant. E) Hydroxy fatty acid quantification (HOTE and HODE). Data are normalized to the HOTE or HODE values.
measured in the absence of antioxidant. ** and *** indicate significant differences from control at $P < 0.01$ and $P < 0.005$, respectively (Student’s $t$ test).

Figure 3. Time course of the changes in carnosic acid and carnosol concentrations upon exposure to $^{1}O_{2}$ or hydroxyl radicals. $^{1}O_{2}$ was produced by illumination of methylene blue, and hydroxyl radicals were produced by the Fenton reaction using $H_{2}O_{2} + Fe^{2+}$.

A and B) Carnosic acid; C and D) carnosol.

Figure 4. $^{1}O_{2}$ quenching capacity of carnosic acid and carnosol. $^{1}O_{2}$ was generated by 5-min illumination of 100µM rose bengal in the presence of the spin probe TEMPD. A) Effect of different concentrations of carnosic acid (CA) on the EPR spectra of TEMPD. B) Quantification of the decrease in the EPR signal amplitude induced by increasing concentrations of carnosic acid. C) Effect of carnosol (CARN) on the EPR spectra of TEMPD. D) Effect of α-tocopherol (TOC) on the EPR spectra of TEMPD. E) Quantification of the decrease in the EPR spectra by increasing concentrations of α-tocopherol.

Figure 5. Quenching of hydroxyl radicals by carnosic acid and carnosol. Hydroxyl radicals were generated by the Fenton reaction using $H_{2}O_{2} + Fe^{2+}$ in the presence of the spin probe POBN. A) Effect of different concentrations of carnosic acid (CA) on the EPR spectra of POBN. B) Quantification of the decrease in the EPR signal amplitude induced by increasing concentrations of carnosic acid. C) Effect of carnosol (CARN) on the EPR spectra of POBN.

Figure 6. Carnosic acid and carnosol in leaves of rosemary plants (Sudbury blue variety) grown under two different conditions of light and temperature (250 µmol photons $m^{-2} s^{-1}$ at 25/15°C (day/night) [control] or 1200 µmol photons $m^{-2} s^{-1}$ at 35/5°C [stress]). A) Carnosic acid, carnosol, α-tocopherol and plastochromanol-8 concentrations in leaves. B) Carnosic acid and carnosol were measured in leaves after organic solvent dipping and in the organic solvent after leaf dipping (representing the compounds stored in the trichomes). C) Glandular trichomes before and after solvent dipping. * and *** indicate significant differences from control at $P < 0.05$ and $P < 0.005$, respectively (Student’s $t$ test).
Figure 7. Oxidation products of carnosic acid in planta. Rosemary plants were grown under two different conditions (control and stress, described in the legend of Fig. 6). The carnosic acid metabolites were measured in rosemary leaves by UPLC-MS. Quantification of the metabolites for which no standard is available was done using the conversion factor of carnosic acid. *, ** and *** indicate significant differences at P < 0.05, P < 0.01 and P < 0.005, respectively (Student’s t test).

Figure 8. Effects of carnosic acid or carnosol on Arabidopsis thylakoid membranes exposed to high light. Thylakoid suspensions were exposed to white light of PFD 1500 μmol photons m⁻² s⁻¹ for 2 h. A) Decrease in chlorophyll content after light treatment; B) HOTE with or without addition of 50 μM carnosic acid or carnosol to the membrane suspensions. * and *** indicate significant differences from control at P < 0.05 and P < 0.005, respectively (Student’s t test).

Figure 9. Inhibition of ROS-independent lipid peroxidation by carnosol. A) Induction of linolenic acid oxidation by addition of the hydroxy fatty acid 15-HEDE in the dark, as measured by luminescence emission. B) Effect of 60 μM and 120 μM carnosol or carnosic acid on in vitro oxidation of linolenic acid induced by addition of 15-HEDE. C) Effect of leaf infiltration with carnosol or carnosic acid on wounding-induced lipid peroxidation in Arabidopsis leaves in the dark, as measured by autoluminescence. Top panels: autoluminescence emission of Arabidopsis leaves wounded with a scalpel. Leaves were pre-infiltrated with a buffer (MES 10 mM, pH 5.6, MgSO4 10 mM, 1% DMSO) containing 0 or 60 μM carnosic acid or carnosol. Bottom panel: autoluminescence intensity of the wounds in leaves infiltrated with 0 or 60 μM carnosic acid or carnosol. * indicates significant differences from control (0 μM) at P < 0.05 (Student’s t test).

Figure 10. Comparison of two rosemary varieties (Sudbury Blue and Barbecue) containing different amounts of carnosic acid. Control, plants grown in low light; Stress, plants grown in high light at high temperature (see legends of Fig. 6). A) carnosic acid, B) carnosol, C) autoluminescence imaging of lipid peroxidation in rosemary plants. * and *** indicate significant differences at P < 0.05 and P < 0.005, respectively (Student’s t test).
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