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Synthesis and application of a novel sunscreen-antioxidant

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Abstract
Background information on the inefficacy of sunscreens to provide free radical protection in skin, despite their usefulness in preventing sunburn/erythema, prompted us to synthesize a compound which would display in the same molecule both UV-absorbing and antioxidant capacities. For this purpose, the UVB absorber, 2-ethylhexyl-4-methoxycinnamate (OMC) was combined with the piperidine nitroxide TEMPOL, which has antioxidant properties. The spectral properties of the new nitroxide-based sunscreen (MC–NO) as well as its efficacy to prevent photo-oxidative damage to lipids induced by UVA, natural sunlight and 4-*tert*-butyl-4-methoxydibenzoylmethane (BMDBM), a photo-unstable sunscreen which generates free radicals upon UV radiation, was studied. The results obtained demonstrate that MC–NO: (a) absorbs in the UVB region even after UVA irradiation; (b) acts as free radical scavenger as demonstrated by EPR experiments; (c) strongly reduces both UVA-, sunlight- and BMDBM-induced lipid peroxidation in liposomes, measured as reduced TBARS levels; and (d) has comparable antioxidant activity to that of commonly used vitamin E and BHT in skin care formulations. These results suggest that the use of the novel sunscreen-antioxidant or of other nitroxide-based sunscreens in formulations aimed at reducing photoinduced skin damage may be envisaged.

Keywords: *Sunscreen, nitroxide, antioxidant, UVA, 2-ethylhexyl-4-methoxycinnamate, 4-tert-butyl-4′-methoxydibenzoylmethane, lipid peroxidation*

Introduction

It is now well recognized that ultraviolet (UV) radiation emitted by the sun extending from the UVA band (315–400 nm) through to the UVB (280–315 nm) is harmful to human skin. Erythema, sunburn, photodamage (photoageing), photocarcincogenesis, eye damage and alteration of the immune system, to name a few, are some of the consequences of acute and chronic UV exposure [1–5]. While the carcinogenicity of UVB radiation is well established and, to a large extent, understood as a process of direct photochemical damage to DNA from which gene mutations arise [6], UVA radiation is hardly absorbed by DNA. Instead, UVA radiation which penetrates deeper into the dermal layers of the skin, appears to act in an indirect way by photosensitizing cellular chomophores thus creating free radicals and reactive oxygen species (ROS) which can alter the structure and/or the function of DNA, membranes and other cellular constituents [7–9]. This UV-related damage may be minimized by proper protection and shielding, mainly through the use of sun protective agents which have been on the market for more than 60 years. They were first developed to protect the skin primarily against sunburn/erythema caused by UVB and to permit tanning by UVA; in fact the conventional sun protection factor (SPF) still relates to the protection from UVB-induced erythema [10,11]. However, because of the increasing concern on the harmful effects of UVA, compounds that absorb in this

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UV range have also been developed. Therefore, nowadays an efficient sunscreen should provide screening throughout the entire UVA/B range in order to not only prevent sunburn, but also to minimize the accumulation of all radiation induced damage in the skin which could eventually lead to irreversible alterations. Recently in fact, a new SPF based on the measurement of free radicals generated by UV irradiation inside a skin biopsy, using ESR spectroscopy, has been proposed which could be employed to complement the present SPF [12]. This is particularly relevant since a recent study by Haywood et al. has shown that sunscreens inadequately protect against UVA-induced free radicals in skin and this could have implications for skin ageing and melanoma [13]. Therefore, both UVA/B protection and scavenging of free radicals should be associated in modern sunscreen products. Indeed, antioxidants such as vitamins C and E are now being added to cosmetic formulations and others such as green and black tea polyphenols are being proposed to deactivate the levels of ROS produced during UV irradiation [14–16]. In the recent past, we also demonstrated that nitroxides, an interesting class of persistent organic free radicals, were most efficient at inhibiting UVA-mediated *in vitro* plasmid DNA, lipid and protein oxidation [17–19]. In addition, the nitroxide TEMPOL (4-hydroxy TEMPO) has recently been shown to afford protection against UV radiation in a transgenic murine fibroblast culture model of cutaneous photoaging [20] and in human dermal fibroblasts [21]. Based on these findings, we thought it of interest to synthesize a compound which would display in the same molecule, both UV-absorbing and antioxidant capacities. For this purpose, we combined the most common UVB absorber incorporated into over 75% of sunscreen-containing formulations, 2-ethylhexyl-4-methoxycinnamate (OMC) with one of the most common and exhaustively studied nitroxides, the piperidine nitroxide TEMPOL. The new compound obtained retains the 4-methoxycinnamate group responsible for the UVB absorbing capacity while the 2-ethylhexyl group has been replaced with the piperidine nitroxide function which has radical scavenging properties (Figure 1).

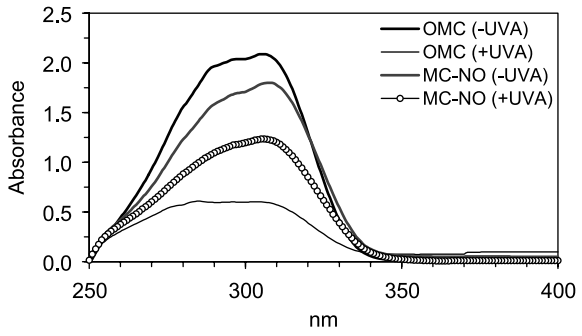


Figure 1. UV absorbance spectra of 100 μM compounds before (−UVA) and after (+UVA) irradiation with 275 kJ/m^2 UVA. See Materials and Methods for experimental details.

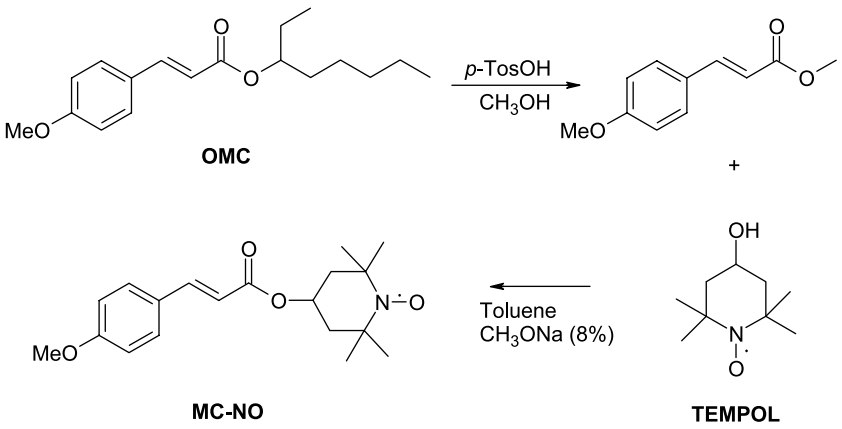
This new compound may be regarded as an UVB-absorbing nitroxide, i.e. a nitroxide-based sunscreen, which to the best of our knowledge is the first of its kind. Here, we report on the synthesis and the photochemical behaviour of this new compound as well as on its efficacy to prevent photo-oxidative damage to lipids induced by UVA and by 4-*tert*-butyl-4′-methoxydibenzoylmethane (BMDBM), a photo-unstable sunscreen which generates free radicals upon UVA illumination [18,22,23]. For comparison, vitamin E and the synthetic antioxidant butylated hydroxy-toluene (BHT) were also included in this study.

Material and methods

Materials

L-α-Phosphatidylcholine (P2772: Type XI-E), 4-hydroxy-2,2,6,6,-tetramethyl-piperidine-1-oxyl (TEMPOL), OMC (octylmethoxycinnamate), vitamin E, BHT as well as all other reagents and solvents were purchased from Sigma-Aldrich Chemical Co. (Milan, Italy). BMDBM was obtained in the form of Eusolex 9020 from Merck (Darmstadt, Germany) and its identity was confirmed by NMR. TEMP8 was synthesized according to the procedure described in reference [17]. 2,2,6,6-Tetramethyl-piperidin-4-yl-methoxy cinnamoyl-1-oxyl (MC–NO) was synthesized from TEMPOL and the methyl ester of *p*-methoxy cinnamic acid. The typical procedure (Scheme 1) requires the reactants to be refluxed in toluene for 4–5 h in the presence of sodium methoxide as catalyst, as reported in the reference [24]. 2,2′-Azobis(2,4-di-methylvaleronitrile) (AMVN) was kindly prepared and donated by Prof. R. Leardini from the University of Bologna (Italy) according to the method described in the reference [25].

^{1}H NMR spectra were recorded at room temperature in $CDCl_3$ solution on a Varian Gemini 200 spectrometer (δ in ppm referred to tetramethylsilane). EPR spectra were recorded on a Bruker EMX EPR spectrometer (Bruker, Karlsruhe, Germany) equipped with an XL microwave frequency counter, Model 3120 for the determination of *g* factors. Mass spectra were recorded on a Carlo Erba QMD 100 spectrometer (Milan, Italy) in EI + mode while FT-IR spectra were recorded in the solid state on a Nicolet Fourier Transform Infrared 20-SX spectrophotometer equipped with a Spectra Tech. As UVA irradiating source, a commercial sun lamp, Philips Original Home Solarium (model HB 406/A; Philips, Groningen, Holland) equipped with a 400 W ozone-free Philips HPA lamp, UV type 3, delivering a flux of 23 mW/cm^2 between 300 and 400 nm, at a distance of 20 cm was used. It was always pre-run for 15 min to allow the output to stabilize. The dose of UVA was measured with a UV Power Puck Radiometer (EIT Inc., USA).



Scheme 1. Synthesis of 2,2,6,6-tetramethyl-piperidin-4-yl-methoxy cinnamoyl-1-oxyl (MC–NO) from TEMPOL and 2-ethylhexyl 4-methoxycinnamate (OMC).

Synthesis of p-methoxy methyl cinnamate

OMC (4 mmol, 1.161 g) was dissolved in 10 ml of methanol in the presence of a catalytic amount of *p*-toluenesulfonic acid monohydrate. The reaction mixture was refluxed for 9 h, neutralised with $NaHCO_3$ 0.5 M, washed with water and extracted with ethyl acetate. The organic layer was dried over Na_2SO_4 anhydrous and the solvent was removed under reduced pressure. A white oil (0.5 g, 2.5 mmol) crystallizing on standing was obtained.

Synthesis of 2,2,6,6-tetramethyl-piperidin-4-yl-methoxy cinnamoyl-1-oxyl (MC–NO)

Stoichiometric amounts of TEMPOL (1 mmol, 172 mg) and *p*-methoxy methyl cinnamate (1 mmol, 204 mg) were dissolved in 5 ml of toluene together with 0.1 ml of a methanolic solution (8%) of sodium methoxide. The reaction mixture was refluxed for 5 h and repeatedly washed with water in order to remove the unreacted TEMPOL partially soluble in water. Purification by chromatography (SiO_2, 80:20 petroleum ether/diethyl ether) afforded 230 mg (70%) of MC–NO.

^{1}H NMR (200 MHz, $CDCl_3$, 25°C, after reduction with phenylhydrazine): $\delta = 1.34$ (s, 6H), 1.41 (s, 6H), 1.75–1.91 (m, 2H), 2.00–2.12 (m, 2H), 5.14–5.28 (m, 1H), 6.27 (d, 1H, $\mathcal{J} = 16$ Hz), 6.91 (d, 2H, $\mathcal{J} = 8.8$ Hz), 7.48 (d, 2H, $\mathcal{J} = 8.8$ Hz), 7.63 (d, 1H, $\mathcal{J} = 16$ Hz). MS (EI +): $m/z = 332(7)$; 302(2); 178(61); 161(100). FT-IR: 1707; 1631; 1604; 1515 cm^{-1}. ESR spectrum recorded in ethyl acetate (Figure 2): triplet with $a_N = 15.47$ G, $g = 2.0062_8$.

Optical absorption spectra

Stock solutions of about 10 mM of all compounds to be tested were prepared in acetonitrile. Appropriate amounts were then added to 5 mM phosphate buffer, 0.9% NaCl, 0.1 mM EDTA, pH 7.4 (acetonitrile $<$ 2% v/v) and mixed thoroughly to reach final concentrations of 100 μM in a final volume of 3 ml. The solutions were then transferred to a 24 multi-well plate for cell cultures (Orange Scientific, Cambrex BioScience, Walkerville, Inc.) which was placed on a brass block embedded on ice at a distance of 20 cm from the light source. The multi-well plate was covered with a 2 mm thick quartz slab to prevent any evaporation. The incident dose of UVA received from above by the samples was 275 kJ/m^2. After illumination, 2.4 ml of sample were collected from each well and extracted with the same volume of ethyl acetate. The organic phase was separated and its absorption spectrum was then run on a UV Kontron 941 spectrophotometer. For the non-illuminated samples, the same procedure was followed for the same length of time except that the samples were exposed to direct artificial laboratory working light.

Peroxidation of multilamellar phosphatidylcholine (PC) liposomes induced by UVA

PC multilamellar liposomes were prepared as follows. The desired amount of egg PC in chloroform was added to a glass test-tube kept in an ice bath and the

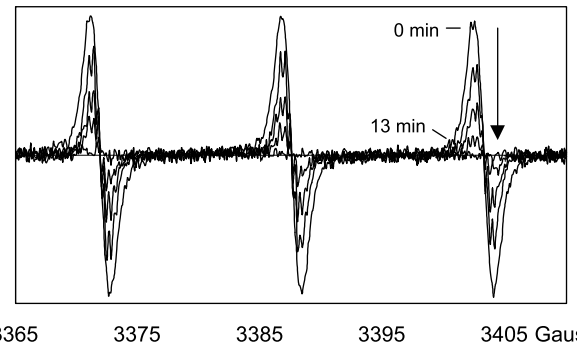


Figure 2. EPR spectral changes of MC–NO in the presence of AMVN in anoxic conditions. MC–NO (200 μM) was incubated with 20 mM AMVN in acetonitrile at 40°C and recordings were continuously taken until signal disappearance.

solvent was thoroughly removed under a stream of nitrogen. When compounds were to be tested, either alone or in combination, the desired amount of an acetonitrile solution of the compound/s was introduced into another glass test tube and, after solvent evaporation, egg PC was added and subjected to the same procedure as described above. The lipid films prepared were each dispersed in 1.5 ml of 5 mM phosphate buffer, 0.9% NaCl, 0.1 mM EDTA, pH 7.4 and vortexed for 2 min until a white, homogeneous, opalescent suspension was obtained. The final concentration of PC in the resulting multilamellar liposomal dispersion was 3.5 mM. Each sample was then aliquoted into two parts (700 μl each) and transferred into a multi-well plate, covered with a 2 mm thick quartz slab to prevent any evaporation and exposed to UVA as described above. The incident dose of UVA received from above by the samples was 275 kJ/m^2. At the end of UVA exposure, the extent of lipid peroxidation was assessed using a modified method of the thiobarbituric acid (TBA) assay [26]. In this procedure, 2 ml of TBA–TCA–HCl (0.375% w/v TBA, 15% w/v TCA, 0.2 M HCl) was added to 600 μl of sample containing BHT 0.3 mM to prevent possible peroxidation of liposomes during the TBA assay. The samples were heated for 15 min at 95°C followed by cooling and centrifugation. The absorbance of the pink chromophore of the supernatant developed upon heating was measured at 535 nm.

When natural sunlight was used as illumination source, the same procedure as above was followed except that the samples were exposed to direct natural sunlight for 30 min between 13.00 and 13.30, at sea level (Ancona, Italy), during the summer period 20–28 June 2005. The dose corresponding to 30 min of sunlight in Ancona situated at 43° latitude has been estimated to be around 90–100 kJ/m^2 based on the fact that 180 kJ/m^2 of UVA are equal to about 1 h of sunshine at the French Riviera (Nice) at noon which is also situated at 43° latitude [27].

EPR spectroscopy experiments

The scavenging ability of MC–NO towards alkyl radicals generated from the lipophilic azo-initiator AMVN was determined by EPR spectroscopy. Two hundred micro molar MC–NO and 20 mM AMVN in acetonitrile in a final volume of 1 ml were transferred to glass capillary tubes (1 mm i.d.), thoroughly degassed with argon and then placed in the EPR cavity equipped with a Stelar VTC87 temperature controller for spectral measurements at 40°C. Recordings were taken until complete disappearance of the EPR signal. The spectra were recorded with the following instrumental settings: 5 mM microwave power, 0.5 G modulation amplitude and 100 kHz field modulation.

Appropriate controls were carried out throughout all the experiments described above and the results reported are an average of at least three independent experiments each performed in duplicate.

Results

The main aim of this study was to examine the photochemical behaviour of the new sunscreen-antioxidant, MC–NO, and its efficacy to prevent photo-oxidative damage to lipids induced by UVA. Although MC–NO and OMC are UVB absorbing compounds, they were exposed to UVA radiation principally for two reasons; (a) in the pioneering study of Maier et al., all sunscreens were shown to be highly photostable, including OMC, at all UVB wavelengths tested while considerable loss of photostability was found in the UVA range for most sunscreens [28], and (b) UVA radiation which contributes to 95% of sunlight radiation, is mainly responsible for the indirect production of free radicals and ROS in skin [7–9].

Figure 1 shows the spectral profile of 100 μM OMC and MC–NO before and after exposure to 275 kJ/m^2 UVA in buffer and after solvent extraction. The reason for using buffer was to maintain the same medium as the peroxidation experiments so that comparisons between the optical absorption experiments and lipid peroxidation could be meaningful. As can be observed, the new compound has practically the same spectral profile and extinction coefficient as OMC. But, as previously observed by us and by others [29–31], there is a significant loss of absorption for OMC after UV exposure. This loss is without doubt the result of *trans*/*cis* photoisomerization and possibly (2 + 2) cyloaddition that occur at wavelengths above 300 nm [32–34]. The *cis*-isomer absorbs at the same wavelength but has a reduced extinction, thus accounting for the result here observed. Non-absorbing degradation products may also be the cause for the loss in absorbance. However, as can be observed in Figure 1, the loss of absorption of MC–NO is not as remarkable as that of its parent compound OMC, since it still retains ~50% of its original absorbance even after UVA irradiation. At present we have no explanation for this effect but it appears that the replacement of the ethylhexyl group with the nitroxide group might contribute to increasing the photostability of OMC in some way.

The radical scavenging activity of MC–NO was demonstrated by EPR spectroscopy as shown in Figure 2. The characteristic three-line signal of the nitroxide moiety of MC–NO progressively decreases in intensity until complete disappearance after 13 min in the presence of the alkyl radicals generated by AMVN. This decrease in intensity is unequivocally due to scavenging of the alkyl radicals at the nitroxide function to give the alkylated hydroxylamine, which is an EPR-silent species [35]. This is based on the

well-known fact that nitroxides react at an almost diffusion controlled rate with alkyl radicals ($\sim 10^9$/M/s) to give this non-paramagnetic species [36].

It is known that, compared with UVB, UVA generates more ROS and, at levels found in sunlight, it is 10 times more efficient than UVB at causing lipid peroxidation leading to plasma membrane damage [37,38]. Therefore, the effects on this oxidative event in the presence of MC–NO, OMC, TEMPOL and the latter two combined were studied *in vitro* using liposomes as membrane models. The extent of oxidative damage was determined through the popular method of evaluating the aldehydic breakdown products (TBARS) produced during lipid peroxidation which absorb at 535 nm, using the TBA assay [39]. Figure 3 shows the level of TBARS measured in liposomal suspensions before and after exposure to 275 kJ/m^2 UVA in the absence and presence of different concentrations of MC–NO. UVA induces a threefold increase in lipid peroxidation which is inhibited by increasing concentrations of MC–NO. Almost 50% inhibition is achieved at concentrations as low as 5 μM while almost 90% inhibition is observed at concentrations above 40 μM. These results demonstrate that inhibition of UVA-induced lipid peroxidation by MC–NO is concentration-dependent.

In order to exclude the possibility that lipid peroxidation was not caused by any possible thermal-related effects caused by the emitted heat from the lamp (the temperature of the samples after 20 min of irradiation reached 47 ± 3°C), experiments were performed by incubating the liposomes in the absence of MC–NO in a thermostatted water bath at 50°C. The results obtained after determination of TBARS were the same as those of the non-illuminated control. This proved that lipid peroxidation is due solely to exposure of the liposomal suspension to UVA illumination.

Figure 4 shows the effects of 50 μM MC–NO in comparison with its two parent compounds, OMC and TEMPOL alone and in combination, all at the same concentration. OMC alone protects UVA-induced lipid peroxidation to some extent even if it does not absorb UVA radiation, except for a very small fraction between 320 and 340 nm. This could account for the slight protection observed since a fraction of UVA energy is attenuated by OMC, so less lipids are oxidized as has already been observed by us [29]. TEMPOL alone also inhibits lipid peroxidation to the same extent as OMC. This is not due to its filtering capacity since it does not absorb in the UVA range but rather to its ability to react with ROS induced by UVA exposure as previously reported [17,20,21]. The combination OMC/TEMPOL led to no further increase in protection hence no additive effect was observed. However, noteworthy is the fact that 50 μM MC–NO is more effective than the combination of 50 μM OMC with 50 μM TEMPOL since the level of lipid peroxidation is greatly reduced to almost control levels (non-illuminated control). This may in part be due to the fact that MC–NO is more photostable than OMC, as observed in Figure 1; hence the fraction of UVA absorbed may be greater. However, the main reason for this protective capacity is most likely due to its ability to scavenge UVA-induced free radicals imparted by the nitroxide function present in the sunscreen compound. Although TEMPOL also bears a nitroxide function, therefore, it should protect similarly as MC–NO, it is not as lipid soluble as MC–NO. This was confirmed by experiments performed in the presence of TEMP8, an analogue of TEMPOL which bears an octyl side chain (Figure 5) and the result has been included in Figure 4. One can observe that TEMP8 inhibits lipid peroxidation to the same extent as MC–NO. This result corroborates our earlier findings where lipid soluble analogues of TEMPOL were shown to be more effective antioxi-

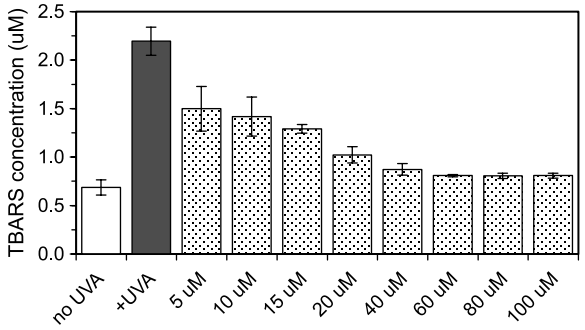


Figure 3. Concentration of TBARS determined in PC multilamellar liposomes (3.5 mM) in PBS, 0.1 mM EDTA, after irradiation with 275 kJ/m^2 UVA. White bar, no UVA exposure; black bar, UVA exposure; remaining bars, UVA exposure in the presence of increasing final concentrations of MC–NO (error bars represent SD).

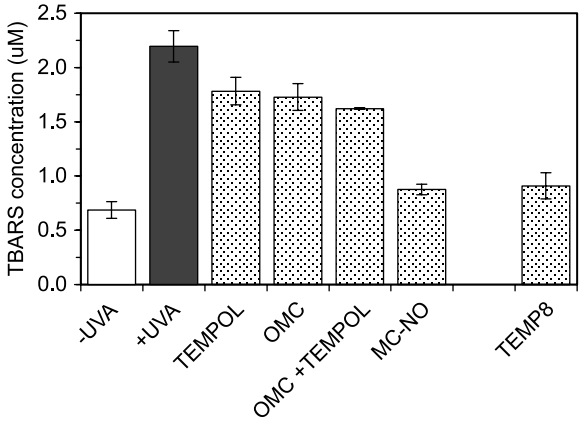


Figure 4. Concentration of TBARS determined in PC multilamellar liposomes (3.5 mM) in PBS, 0.1 mM EDTA, after irradiation with 275 kJ/m^2 UVA. White bar, no UVA exposure; black bar, UVA exposure; remaining bars, UVA exposure in the presence of 50 μM compounds and the various combinations at the aforementioned concentration (error bars represent SD).

dants against UVA-induced lipid peroxidation [17]. In fact, in multilamellar liposomes, UVA-induced oxidative damage occurs at sites less accessible to the aqueous phase [40]. Hence to efficiently protect against lipid peroxidation, the antioxidant has to reside in close proximity to the membrane interior region where lipid peroxidation chain propagation occurs.

The antioxidant activity of the new sunscreen was compared with that of commonly used antioxidants incorporated in cosmetics and suntan creams, namely vitamin E and BHT (Figure 5). The comparisons were made using low concentrations of tested compounds so that differences in protection, if any, could be easily noted. Figure 6 shows that at the lowest concentration used (2.5 μM), MC–NO protects slightly more (21% inhibition) than BHT (13% inhibition) and vitamin E (8% inhibition), while at the highest concentration (5 μM) no significant differences were observed among the three compounds. This suggests that the antioxidant activity of this new sunscreen-antioxidant, in terms of inhibition of oxidation of lipid-based substrates induced by UVA radiation, is comparable to that of common, natural and synthetic antioxidants present on the market.

Since the UV-protection spectrum of a sunscreen formulation is usually attained by a mixture of UV-filters, investigating the compatibility between different UV-filters is of utmost importance. Therefore, the photochemical behaviour and photo-antioxidant activity of MC–NO was tested in the presence of the most common UVA-absorbing sunscreen BMDBM. Figure 7 shows the UV absorbance spectra of BMDBM and its combination with OMC and MC–NO. One can observe that the strong absorption between 320 and 400 nm of BMDBM is greatly reduced after exposure to UVA confirming our previous findings and those of Tarras-Wahlberg et al. [29,31]. This loss in absorbance is due to keto/enol tautomerization, typical of dibenozylmethane derivatives, as well as to degradation products of BMDBM generated after UVA exposure [22,41,42]. When

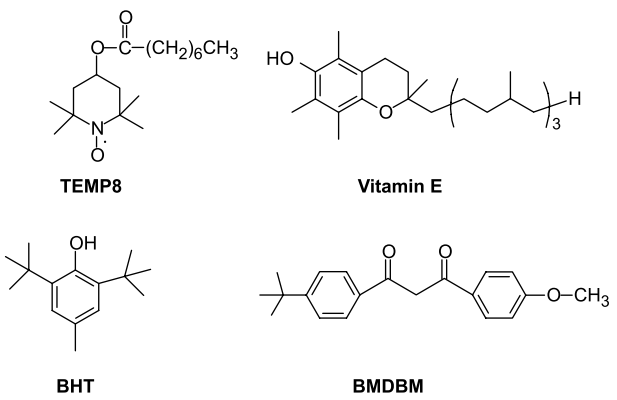


Figure 5. Chemical structures of compounds used.

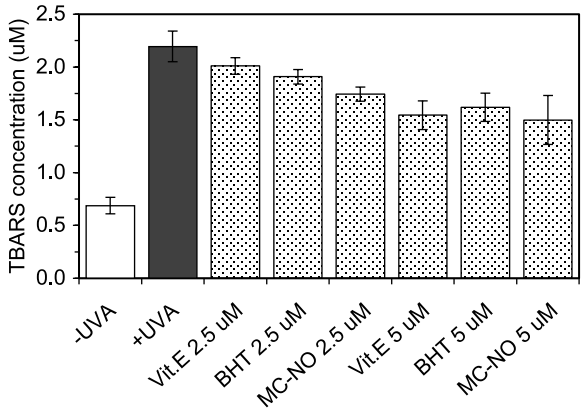


Figure 6. Concentration of TBARS determined in PC multilamellar liposomes (3.5 mM) in PBS, 0.1 mM EDTA, after irradiation with 275 kJ/m^2 UVA. White bar, no UVA exposure; black bar, UVA exposure; remaining bars, UVA exposure in the presence of different final concentrations of vitamin E (Vit. E), BHT and MC–NO (error bars represent SD).

BMDBM was co-irradiated with OMC or MC–NO, the strong absorbance observed over the whole UVA/B region prior to UVA irradiation is remarkably lost with a concomitant change in shape. This result confirms the recommendation that OMC and BMDBM should not be used together in formulations because of its photoinstability. Photoadducts have been reported between OMC and photogenerated fragments of BMDBM [43] and more recently, Sayre et al. showed that OMC was unexpectedly photolysed in the presence of BMDBM [44]. These events may all contribute to the overall decrease in absorbance observed here even when MC–NO is co-irradiated with BMDBM, despite the presence of the nitroxide moiety.

Figure 8 shows the effects of OMC, TEMPOL and MC–NO in the presence of BMDBM on UVA-induced lipid peroxidation. BMDBM leads to a significant increase in the level of TBARS compared to the illuminated control, confirming once again that free

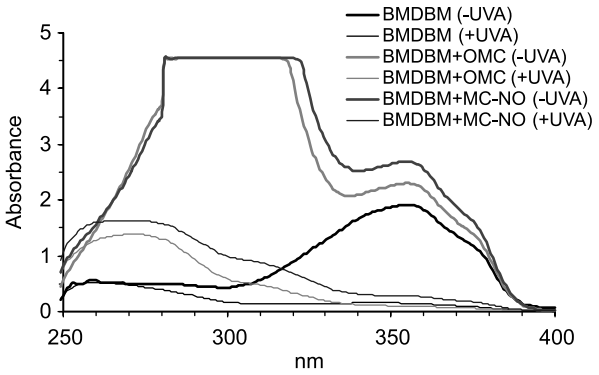


Figure 7. UV absorbance spectra of 100 μM compounds alone and in combination before (thick line) and after (thin line) irradiation with 275 kJ/m^2 UVA. See Materials and Methods for experimental details.

radicals are generated during UVA illumination of this filter [18,22] that contribute to exacerbating the free radical chain reaction of lipid peroxidation. With the combination BMDBM/OMC there was no significant difference in TBARS levels with respect to BMDBM alone, confirming the results previously reported by us [29]. With the combination BMDBM/TEMPOL the level of liposomal peroxidation compared to BMDBM alone is slightly reduced although it is still well above the levels of the illuminated control. The addition of OMC to this combination has no effect. However, the most striking result is the one obtained with the combination BMDBM/MC–NO. The presence of 100 μM of the new compound is able to greatly reduce lipid peroxidation to almost control levels when 100 μM of the UVA filter is present. This result is most likely due to the scavenging activity conferred by the nitroxide moiety present in the new sunscreen.

In order to investigate how the effects seen using a UVA lamp correlate with those observed in the sun, since sunlight is comprised of 95% UVA, lipid peroxidation experiments were also performed under a more realistic situation, i.e. natural sunlight. Thirty minute sunlight exposure lead to a twofold increase in the level of TBARS and the same pattern of results as shown in Figure 4 was obtained (results not shown) using 50 μM TEMPOL, OMC, MC–NO and the combination OMC/TEMPOL. Figure 9 shows that MC–NO, vitamin E and BHT at both concentrations tested (2.5 and 5 μM) reduce sunlight-induced lipid peroxidation by almost 100% and that all three compounds are equally effective. In this case, the low concentrations used were sufficient to confer considerable protection, most probably because the level of TBARS generated during 30 min sunlight exposure are less compared to those generated under

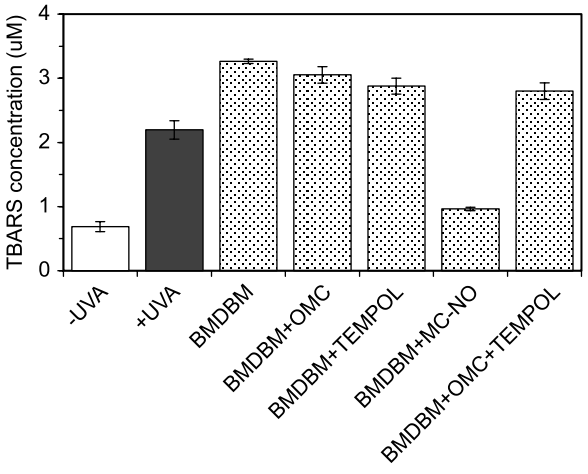


Figure 8. Concentration of TBARS determined in PC multilamellar liposomes (3.5 mM) in PBS, 0.1 mM EDTA, after irradiation with 275 kJ/m^2 UVA. White bar, no UVA exposure; black bar, UVA exposure; remaining bars, UVA exposure in the presence of 100 μM BMDBM alone and in combination with 100 μM compounds (error bars represent SD).

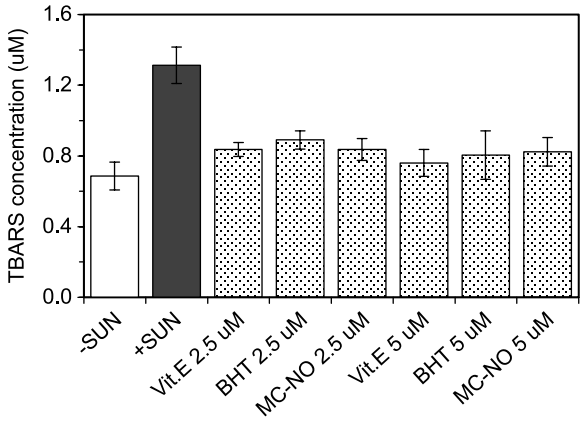


Figure 9. Concentration of TBARS determined in PC multilamellar liposomes (3.5 mM) in PBS, 0.1 mM EDTA, after exposure to 30 min of natural sunlight. White bar, no sunlight exposure; black bar, sunlight exposure; remaining bars, sunlight exposure in the presence of different final concentrations of vitamin E (Vit. E), BHT and MC–NO (error bars represent SD).

20 min UVA irradiation, therefore, lower antioxidant concentrations are required to fully inhibit this free radical process. The effects of sunlight exposure to liposomal suspensions containing BMDBM alone and in the presence of different concentrations of MC–NO and vitamin E are shown in Figure 10. Similarly to the results shown in Figure 8, the presence of BMDBM exacerbates sunlight-induced lipid peroxidation. The graph also shows that both 100 and 20 μM MC–NO or vitamin E are capable of totally inhibiting lipid peroxidation, thereby reducing the photo-oxidative effect induced in lipid systems by the UVA-absorber under natural sunlight.

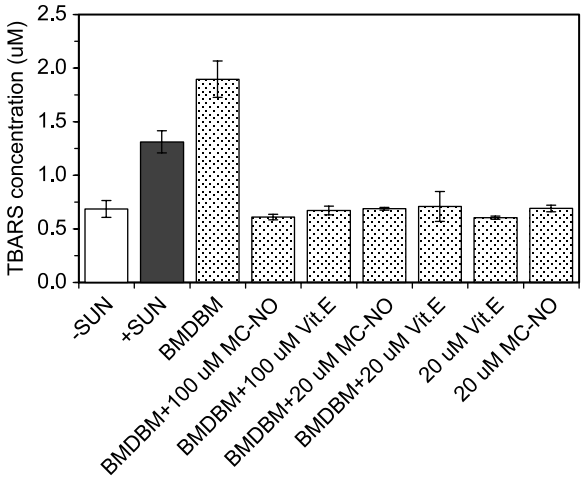


Figure 10. Concentration of TBARS determined in PC multilamellar liposomes (3.5 mM) in PBS, 0.1 mM EDTA, after exposure to 30 min of natural sunlight. White bar, no sunlight exposure; black bar, sunlight exposure; remaining bars, UVA exposure in the presence of 100 μM BMDBM alone and in combination with 100 μM or 20 μM compounds (error bars represent SD).

Discussion

Free radicals and other oxidative species are unequivocally known to contribute significantly to the environmental or exogenous ageing of skin, particularly UV-mediated damage [7–9]. Although the human skin possesses various enzymatic and non-enzymatic antioxidant defence systems which are intricately regulated to mitigate oxidative damage, both excessive and chronic exposure to free radicals can debilitate the skin's defensive barrier [45–47]. For example, it has been demonstrated *in vivo* [48] that depleted antioxidant enzyme expression, namely Cu–Zn/Mn superoxide dismutase and catalase, in photodamaged skin is associated with higher levels of protein oxidation. Photolipid peroxidation of the lipid environment of viable cell layers is another event related with free radicals, which leads to the depletion of vitamin E, the major lipophilic antioxidant of human skin [37,38,45,49]. Although solar radiation is the major factor responsible for the formation of free radicals and ROS in skin during exposure, it is not the only one. Paradoxically, sunscreens, regarded as first-line photoprotective measure, can also behave as photooxidants generating free radicals and ROS under UV radiation. The physical sunscreen titanium dioxide, although it attenuates light considerably in the UVA/B regions, also photocatalyses free radical formation causing single- and double-strand breaks in plasmid DNA [50]. The chemical sunscreen BMDBM, the most widely used UVA-absorber, generates free radicals when activated by UVA, that have the potential to damage important biological targets, such as lipids, proteins, DNA, and cultured keratinocytes [18,19,22,23,51]. 2-Hydroxy-4-methoxy-benzophenone (Oxybenzone), another UVA sun blocker, was shown to inactivate important antioxidant systems in skin following solar irradiation suggesting that it may be rather harmful to the homeostasis of the epidermis [52]. Other chemical sunscreens, such as paraminobenzoic acid (PABA) and 2-phenylbenzimidazole-5-sulfonic acid (PBSA) also photogenerate ROS including free radicals [53–55]. Because of their photoinstability, sunscreens may, therefore, provide less protection than expected since their photochemistry or absorbance characteristics are significantly altered during UV exposure [29–31]. This results in an increase in direct UVA-induced skin damage aside from the potential damage inflicted by the breakdown products of sunscreens. These findings thus provide a rationale for the development of efficient antioxidant strategies to prevent photoaging and acute photodamage in skin caused by free radicals and ROS.

The strategy pursued in this work was to synthesize a sunscreen exhibiting antioxidant properties and to test its ability to prevent UVA-induced ROS generation measured as reduced photo-oxidative damage to lipids. The results obtained demonstrate that the novel nitroxide-based sunscreen, MC–NO, strongly absorbs in the UVB region just as its parent compound OMC, and that following UVA irradiation at least half of its original absorbance is retained. Using liposomes as model membrane systems, we show that MC–NO leads to a significant reduction in UVA-induced lipid peroxidation and that its antioxidant activity is comparable to that of vitamin E and BHT, which are common antioxidants, used in skin care formulations. In addition, since sunscreen lotions contain more than one active agent, the new UVB sunscreen-antioxidant was studied in combination with the UVA absorber, BMDBM. Although photostabilization of BMDBM with MC–NO was not achieved (Figure 7) remarkable reduction to almost control levels of UVA- and BMDBM-induced lipid peroxidation was observed when MC–NO was present (Figure 8) contrarily to the lack of inhibition detected using OMC. These results suggest that although the combination of MC–NO with BMDBM may not effectively prevent sunburn as there is loss in overall absorbance in the UVA/B range, oxidative damage of the skin surface lipid fractions, which represent the first target of UV irradiation of the skin, may nevertheless be minimized when MC–NO is present.

The protective effect observed is primarily due to the many versatile antioxidant properties of nitroxide radicals on which this sunscreen is based. These include SOD-mimic activity, oxidation of metals since metals in their lower oxidation state have the potential to generate site-specific hydroxyl radicals, and inhibition/termination of free radical chain reactions by scavenging alkyl radicals, as also confirmed by the EPR experiments here described (Figure 2) [35,36,56,57]. These interesting features are most likely responsible for the protection of oxidative damage reported in a wealth of *in vitro* and *in vivo* biological systems investigated using nitroxides. Other features that contribute to make them suitable candidates, as biological antioxidants are that they are cell permeable, have low immunogenicity, no mutogenicity and have good cutaneous tolerance [58,59]. It is worth recalling that this unique class of compounds more commonly known for their applications as biophysical probes and labels as well as contrast agents in NMR and EPR imaging [58], have been used as antioxidants in several other fields too, such as in rubbers [60], polymers [61], paints [62] and in the paper industry [63].

In conclusion, in view of recent reports indicating that sunscreens inadequately protect against UVA-induced free radicals in skin and that the usefulness of adding free-radical scavengers to sunscreens should be seriously considered, we believe that combining an antioxidant moiety within a UV-absorber may be a useful strategy for addressing this issue. The preliminary results on the novel sunscreen-antioxidant

presented in this work suggest that this compound or other nitroxide-based sunscreens may in the future be relevant for improving the efficacy of skin care cosmetic formulations against photocarcinogenesis and photoaging in skin. The nitroxide moiety present in the sunscreen could act as a reservoir that deactivates ROS generated in the stratum corneum by: (a) the UV photons that sunscreens do not block, and/or (b) by photolabile UV-absorbers. However, it is important to bear in mind that this is an *in vitro* investigation; hence before the results here reported can be extrapolated to human use of sunscreens further *in vivo* studies are mandatory.

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