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Synthesis, in vitro percutaneous absorption and phototoxicity of new benzylidene derivatives of 1,3,3-trimethyl-2-oxabicyclo[2.2.2]octan-6-one as potential UV sunscreens

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Abstract

A series of 5-(alkoxyphenylmethylene)-1,3,3-trimethyl-2-oxabicyclo[2.2.2]octan-6-ones (**1a–i**) were prepared to evaluate their potential cosmetic application as UV sunscreens. All new synthesised compounds were studied for their UV absorption and the relative sunscreening action was determined by sunscreen index values obtained from the UV spectra: compound **1c** showed its maximum absorption in the UVB region, compounds **1a** and **1d** showed their maximum absorption in the UVA–UVB immediate zone, the others in the UVA sunscreen area. Since an ideal sunscreen should show a high extent of substantivity without penetrating the skin, on the basis of UV spectral data, four of them (**1a**, **1d**, **1f**, **1h**) were evaluated as regards their in vitro percutaneous absorption. Subsequently, the two less absorbed compounds, **1f** and **1h**, were also assayed for their potential in vitro phototoxicity, by photohemolysis and lipid peroxidation tests. The skin permeation and the phototoxicity data of the studied compounds were compared to the data of 3-(4-methylbenzyliden)bornanone (**2**), a commercially available UV filter having a chemical structure similar to the new derivatives. Compounds **1f** and **1h** were less absorbed than compound (**2**) and showed a photoinduced lytic activity which is comparable to that displayed by the marketed compound. © 1998 Elsevier Science B.V.

Keywords: Sunscreens; Cineole derivatives; In vitro percutaneous absorption; In vitro phototoxicity

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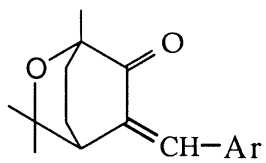
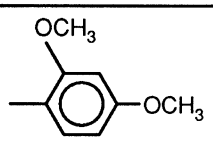
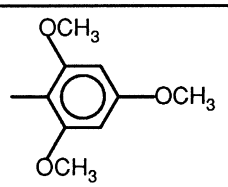
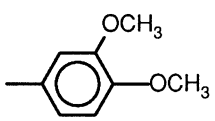
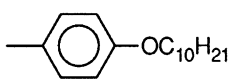
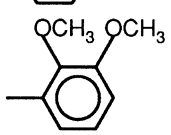
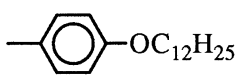
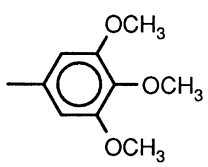
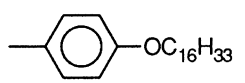
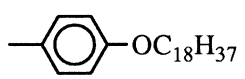
1. Introduction

It is a well-established finding that ultraviolet radiation causes damage to the skin, damage with results in both precancerous and cancerous skin lesions, as well as enhancement of normal skin ageing. These important findings prompted the cosmetic industry to create new sunscreen products which would afford the consumers more efficient protection.

Our interest in this field, and our research on new cineole derivatives led, in recent years, to the synthesis of some benzylidene derivatives of 1,3,3-trimethyl-2-oxabicyclo[2.2.2]octan-6-one (Mariani et al., 1993, 1994, 1996) which could be considered potential UVA and UVB sunscreens. These compounds are related to 3-benzylidene-camphor derivatives, a well-known class of UV sunscreens (Ger. Offen., 1974, 1990; US Patent, 1982; Shaats, 1987) taking into account the structural correlation between the bicyclic rings of bornane and cineole. In pursuing our research on cineole derivatives as UV filters we have now synthesised a series of 5-(alkoxyphenylmethylene)-1,3,3-trimethyl-2-oxabicyclo[2.2.2]octan-6-ones, compounds **1a–i**.



1 a-i

Comp.	Ar	Comp.	Ar
1a		**1 e**	
1b		**1 f**	
1 c		**1 g**	
1 d		**1 h**	
		1 i	

All new synthesised compounds were studied for their UV absorption and the sunscreen index values were determined from the UV spectra using Kumler's method. These values, which correspond to the absorbance of 0.1% solutions of the compounds at 308 nm, at a path length of 0.1 mm, may give an indication of the relative UV screening action (Kumler, 1952).

A sunscreen product, for its particular use, is generally applied to large areas of the skin and exposed to solar radiations for long periods of time. Therefore, besides good sunscreen power, a UV filter should have a high extent of substantivity without penetrating the skin (Watkinson et al., 1992; Hagedom-Leweke and Lippold, 1995) to avoid cutaneous or systemic toxic effects. Therefore, on the basis of UV spectral data and sunscreen index values, derivatives **1a**, **1d**, **1f**, and **1h**, were evaluated with regard to their in vitro percutaneous absorption through excised human skin from an O/W emulsion vehicle.

Another requirement for a safer UV absorber is a low degree of phototoxicity risk, which can be assessed through suitable techniques capable of providing a rapid and useful screening for the potential phototoxic activity of the tested compound; this phototoxicity can be of a different nature with regard to both its chemical structure and its utilisation. Several sunscreens have been assayed for their in vitro phototoxicity using photohemolysis and yeast growth inhibition tests (Sugiyama et al., 1994).

Taking into account the in vitro percutaneous absorption results, the less absorbed compounds, **1f** and **1h**, were evaluated to establish their potential phototoxicity.

This in vitro phototoxicity evaluation was carried out both by photoinduced lysis of red blood cells and a lipid peroxidation test.

In vitro percutaneous absorption and phototoxicity data were compared with the data of 3-(4-methylbenzyliden)bornanone (**2**); this compound is a commercially available UV filter having a chemical structure similar to the new derivatives.

2. Materials and methods

2.1. Apparatus

UV spectra were recorded on a Perkin-Elmer Lambda 3 spectrophotometer. IR spectra were obtained with a Perkin-Elmer 398 spectrophotometer. ^{1}H-NMR spectra were recorded on a Varian-Gemini 200 instrument (200 MHz) with TMS as internal standard; chemical shifts are reported as δ (ppm) relative to TMS as internal standard. Elemental analyses for C and H were performed on the Carlo Erba Elemental Analyser Model 1106. Melting point was determined on a Büchi 510 apparatus and is uncorrected. Analytical HPLC procedures were performed using a Hewlett-Packard 1100 liquid chromatograph equipped with a Hewlett-Packard diode array detector. The column used for the analysis was Lichrocart 250-4, Purospher RP-18 (5 μm), from Merck (Darmstadt, Germany). Emulsions were carried out using a Silverson apparatus equipped with a dispersing riddler from Crami (Milan, Italy). Irradiation was performed using a Rayonet photochemical reactor (The Southern New England Photochemical Company, Hamdem, CT, USA) equipped with eight 'black light' phosphor lamps with an emission in the UVA range (310–390 nm) with a maximum at 350 nm.

2.2. Chemicals

2,4-Dimethoxybenzaldehyde, 3,4-dimethoxybenzaldehyde, 2,3-dimethoxybenzaldehyde, 3,4,5-trimethoxybenzaldehyde, 2,4,6-trimethoxybenzaldehyde, 4-hydroxybenzaldehyde, 1-bromodecane, 1-iodododecane, 1-iodohexadecane, 1-bromooctadecane were obtained from Aldrich Chimica (Milan, Italy). Syntesqual (polyisoprene), Xalifin-15 (C_{12-20} acid peg-8 ester), Isoxal-12 (Ceteth-5, Steareth-5, Myreth-5, Laureth-5) were obtained from Vevy Europe (Genova, Italy). 3-(4-Methylbenzyliden)bornanone and HPLC-grade gradient acetonitrile were obtained from Merck (Darmstadt, Germany). Phosphatidylcholine from egg yolk (PC) was provided by Sigma (Milan, Italy).

2.3. Chemistry

The new compounds were prepared by reaction of (+)-1,3,3-trimethyl-2-oxabicyclo[2.2.2]octan-6-one with the appropriate alkoxy aromatic aldehydes in presence of sodium methoxide; the lipophilic aromatic aldehydes used for the synthesis of compounds **1f–i** were obtained by reacting 4-hydroxybenzaldehyde with appropriate alkyl halides to afford the 4-alkoxybenzaldehydes.

2.3.1. General procedures for the synthesis of 4-alkoxybenzaldehydes used for the preparation of compounds *1f–i*

The 4-alkoxybenzaldehydes were prepared starting from a solution of 4-hydroxybenzaldehyde (50 mmol) and the appropriate alkyl halide (50 mmol) in anhydrous DMF (20 ml), which was added to a solution of sodium methoxide, prepared from sodium (50 mmol) and anhydrous methanol (30 ml), followed by evaporation of excess methanol at reduced pressure and treatment of the residue with anhydrous DMF (20 ml); the resulting solution was refluxed for 6 h and left at room temperature for 12 h. DMF was evaporated under reduced pressure, the residue was treated with water (100 ml) and extracted thoroughly with dichloromethane. The extracts were dried ($MgSO_4$) and evaporated to give a residue that was employed as crude compound in the next step.

2.3.2. General procedures for the synthesis of 5-(alkoxyphenylmethylene)-1,3,3-trimethyl-2-oxabicyclo[2.2.2]octan-6- ones (*1a–i*)

(+)-1,3,3-Trimethyl-2-oxabicyclo[2.2.2]octan-6-one (50 mmol) and the appropriate alkoxybenzaldehyde (50 mmol) were added to a solution of sodium methoxide, prepared from sodium (50 mmol) and anhydrous methanol (30 ml); the resulting solution was refluxed for 12 h and left at room temperature for 12 h. The methanol was evaporated under reduced pressure, the residue was treated with water (100 ml) and extracted with diethyl ether. The extracts were dried ($MgSO_4$) and evaporated to give a residue that was purified by recrystallization from a suitable solvent. All compounds (**1a--i**) were analyzed for C and H, and elemental analyses were within $\pm 0.3\%$ of the theoretical values; the structure of these compounds was confirmed by IR and ^{1}H-NMR spectral data; as examples, the spectral data of compounds **1a** and **1f** are reported.

Compound **1a** (IR, cm^{-1} ($CHCl_3$): 1702 C=O, 1604 C=H): ^{1}H-NMR, δ ($CDCl_3$): 1.12 (s, 3H, CH_3), 1.25 (s, 3H, CH_3), 1.38 (s, 3H, CH_3), 1.5–2.4 (m, 4H, $2CH_2$), 3.10 (br s, 1H, H-4), 3.83 (m, 6H, CH_3O), 6.5–7.3 (m, 3H, C_6H_4), 7.83 (s, 1H, C=CH).

Compound **1f** (IR, cm^{-1} ($CHCl_3$): 1700 C=O, 1600 C=H): ^{1}H-NMR, δ ($CDCl_3$): 0.9–2.3 (mc, 32H, $4CH_3 + 10CH_2$), 3.25 (br s, 1H, H-4), 4.01 (m, 2H, CH_2O), 6.85–7.62 (m, 3H, C_6H_4), 7.80 (s, 1H, C=CH).

2.4. O/W emulsion preparation

A stable cosmetic O/W formulation was used as vehicle of compounds **1a**, **1d**, **1f**, and **1h**, and of 3-(4-methylbenzyliden)bornanone used as reference. The composition of the vehicle was Xalifin-15 (15%), Isoxal-12 (2%), Syntesqual (20%), filter (**2** = 2.0%, **1a** = 4.0%, **1d** = 4.0%, **1f** = 3.5%, **1h** = 4.5%), H_2O (71–68.5%).

The amount of the new compounds in the above emulsions were calculated, with approximation, on the basis of the sunscreen index (SI) values of all compounds (Table 1) and taking into account the percentage of compound **2** [$\lambda_{max}(\log \epsilon)$ in 95% EtOH = 300 (4.36); SI = 8.30] used in commercial products and needed to screen out 1% of rays in the sunburn region through a film 0.025 mm thick. The obtained values were doubled to allow for a safety factor (Kumler, 1952).

2.5. In vitro skin permeation experiments

The studies were carried out using samples of adult human skin mounted in Franz-type static diffusion cells (LGA, Berkeley, CA, USA). Samples of adult human skin (mean age 35 ± 6 years) were obtained from breast reduction operations. Stratum corneum and epidermis membranes

(SCE) were prepared, stored and assessed for barrier integrity as described in a previous paper (Bonina and Montenegro, 1992). Dried SCE samples were rehydrated by immersion in distilled water, at room temperature, for 1 h before being mounted in Franz-type diffusion cells. The skin surface available for absorption was 0.75 cm^2 and the receptor volume was 4.5 ml. The receiving compartment contained water/ethanol (50:50) to ensure sink conditions (Mueller, 1988; Touitou and Fabin, 1988). The receiving solution was stirred and maintained at 35 ± 1°C throughout the experiments. Twenty mg of O/W emulsion were placed on the skin surface in the donor compartment and the experiment was run for 23 h. Samples of the receiving solution (200 ml) were withdrawn at intervals and replaced with fresh water/ethanol solution. The samples were analysed for contents of compounds **1a**, **1d**, **1f**, **1h**, and **2** using high-performance liquid chromatography (HPLC).

2.6. HPLC analysis

The sample solutions were injected into a column through a 20 μl sample loop, the eluent was acetonitrile–water (90:10), the flow rate was 1.5 μl/min and the compounds were quantified using an external standard method.

The flux through the skin for **1a**, **1d**, **1f**, **1h** and **2** was calculated by plotting the cumulative amount of compound penetrating the skin against time, determining the slope of the linear portion of the curve and the x-intercept values (lag time) by linear regression analysis. Flow ($\mu g/cm^2\ h^{-1}$), at steady state, was calculated by dividing the slope of the linear portion of the curve by the area of the skin surface through which diffusion took place (Table 2).

The analysis of individual standards and analysed samples was performed by isocratic reversed-phase HPLC. The lipophilic indices of studied compounds are expressed by the logarithm of capacity factor, k, defined as $\mathrm{Log}\, k = \log[(t_r - t_o)/t_o]$, where t_r and t_o are the retention times of the compound and a non-retained compound, respectively.

2.7. Phototoxicity in vitro studies

2.7.1. Irradiation conditions

The fluence rate at the irradiation position was about 800 $\mu W/cm^2$ and the incident photon flux on 3 ml solution in quartz cuvettes (optical length, 1 cm), which is of the same order as the solar fluence incident on skin, was 6×10^{16} quantum s^{-1}. The experimental procedures for the irradiation and light intensity measurements have been described previously (Costanzo et al., 1989).

2.7.2. Photohemolysis assays

Red blood cells (RBC) were prepared by washing, in phosphate-buffered saline (PBS; consisting of 0.01 M phosphate buffer and 0.135 M NaCl solution), samples of out-of-date (not more than 15 days from the date stated) packed human erythrocytes, supplied by the local blood bank. Each series of tests was performed with aliquots from the same sample of blood.

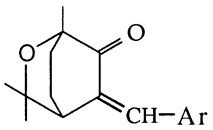
Photohemolysis experiments were carried out by irradiation of RBC suspensions in PBS buffer:ethanol (95:5, v/v) solutions of the investigated compounds. This medium was chosen to dissolve completely the investigated compounds of the sunscreen in polar solvents. The degree of photohemolysis was followed by measuring the decrease in absorbance at 650 nm (delayed hemolysis), since the optical density is linearly proportional to the number of intact RBC. Experiments were performed in saturated air solution. The results are expressed as a percentage of total hemolysis in comparison with a sample in which the cells had been completely hemolysed by brief sonication.

The delayed hemolysis (measured after 2 h of incubation in the dark) was reported as a function of the dose D. This value equals the product $F \times I \times t$, and has been specifically chosen to provide a reliable comparison between the sunscreens (Giuffrida et al., 1995).

These procedures aim to provide reliable data and a correct experimental approach towards phototoxicity.

The term I represents the light intensity at the irradiation wavelength (mol photons min^{-1}) and t is the irradiation time (min). The first parameter,

Table 1
5-(Alkoxyphenylmethylene)-1,3,3-trimethyl-2-oxabicyclo[2.2.2]octan-6-ones **1a–i**



Compoud	Yield (%)	M.p (°C)	λ_{max} (log ϵ) 95% EtOH	SI[c]
1a	59	95[a]	320 (4.19)	3.81
1b	57	98[a]	340 (4.09)	2.18
1c	74	88[b]	294 (4.22)	4.15
1d	70	74[b]	320 (4.19)	4.00
1e	58	153[a]	326 (4.10)	3.02
1f	42	58[a]	330 (4.37)	4.54
1g	86	90[a]	327 (4.24)	2.84
1h	79	63[a]	340 (4.34)	3.50
1i	53	71[a]	324 (4.35)	3.33

[a] From EtOH (95%).
[b] From EtOH/H_2O (1:1).
[c] Sunscreen index.

$F = 1 \times 10^{-A}$, represents the fraction of light absorbed by the sunscreen, and it is strictly related to its concentration. This value was calculated by considering an average molar extinction coefficient ϵ in the wavelength range of the overlap between the absorption spectrum of the sunscreen and the lamp emission band, which is located in the wavelength range 308–390 nm for **1f** ($\epsilon = 8500$), **1h** ($\epsilon = 8650$) and **2** ($\epsilon = 3500$), respectively. These values were calculated in PBS buffer:ethanol (95:5, v/v). No remarkable differences were noted in absolute alcohol.

Table 2
Fluxes through excised human skin, lag time values and lipophilic indices (Log K) of coumpounds **1a**, **1d**, **1f**, and **1h**, and of 3-(4-methylbezyliden)bornanone (**2**) from O/W emulsions

Compound	Flux ± S.D. (μg/cm^2 h^{-1})	Lag time (h)	Log K
1a	3.537 ± 0.624	1.56	−0.17
1d	3.135 ± 0.452	1.73	−0.26
1f	0	—	0.96
1h	0	—	1.59
2	0.726 ± 0.089	2.24	0.18

2.7.3. Lipid peroxidation assay

Lipid peroxidation was measured spectrophotometrically using the thiobarbituric acid (TBA) assay (Girotti et al., 1985) by testing ethanolic solutions of the investigated filters irradiated in the presence of phosphatidylcholine from egg yolk (PC) (1×10^{-3} M). The formation of TBA reactive substances (TBARS), detected at 532 nm by assuming an ϵ of 154 000 M^{-1} cm^{-1}, was assumed as the degree of peroxidation.

The data were corrected for the low amount of direct peroxidation of PC detected when irradiation was performed in the absence of the sunscreen.

3. Results and discussion

Table 1 reports the yields, the melting points, the UV absorption maxima (in 95% ethanol) and the SI values (Kumler, 1952) of compounds **1a–i**. The SI values may give an indication of the relative UV sunscreen action. It can be seen from Table 1 that compound **1c** has its maximum absorption in the UVB region, compounds **1a** and **1d** showed their maximum absorption in the

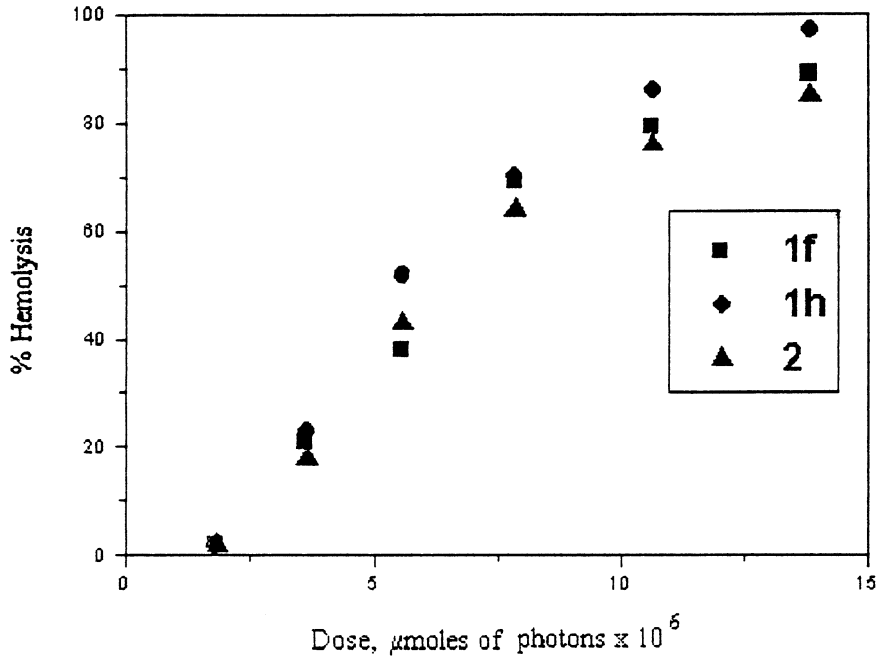


Fig. 1. Influence of irradiation dose D for compounds **1f**, **1h** and **2** (75, 47 and 71 μM, respectively) on sensitized photohemolysis under aerobic conditions: irradiation time, 5–35 min; [RBC] = 3.3×10^6 cells ml^{-1}; $T = 20°C$. Each point is the mean $\pm$ S.D. of triplicate experiments.

UVA–UVB immediate zone, and compounds **1e**, **1f**, **1g**, **1h** and **1i** have their maximum absorption in the UVA sunscreen area. In vitro skin permeation of compounds **1a**, **1d**, **1f**, **1h** and 3-(4-methylbezyliden)bornanone (**2**) was assessed using SCE membranes since some authors (Scheuplein and Blank, 1973; Bronaugh and Stewart, 1984) reported that the in vitro dermis can act as a significant additional barrier to the absorption of active lipophilic compounds. In vitro fluxes through human skin of compounds **1a**, **1d**, **1f**, **1h** and **2** from O/W emulsions are reported in Table 2.

3-(4-Methylbezyliden)bornanone (**2**), a commonly used UV filter having a chemical structure similar to new derivatives, was used as reference in order to evaluate skin permeation ability and phototoxicity risk of new synthesised UV filters. The in vitro flux through human skin, at steady state, of the reference compound was 0.726 ± 0.089 $\mu g/cm^2\ h^{-1}$. These flux values are of the same order of magnitude of those reported for in vivo skin permeation of 3-(4-methylbezyliden)bornanone in humans. The authors (Hagedom-Leweke and Lippold, 1995) reported that this compound, applied in vivo to the skin surface as a saturated solution, showed maximum fluxes ranging from 1.59 to 2.92 $\mu g/cm^2\ h^{-1}$; the lower skin permeation of compound **2** in our in vitro experiments could be attributed to the use of vehicles containing low percentages of this filter. As may be noted in Table 2, compounds **1f** and **1h** were not able to permeate the skin, since no detectable amount of these compounds was found in the receiving phase after 23 h. On the contrary, compounds **1a** and **1d** permeated the skin better than the reference compound. The different fluxes through human skin for compounds **1a**, **1d**, **1f**, **1h** and **2** could be explained taking into account their lipophilicity and molecular mass. Less lipophilic compounds, **1a** and **1d**, proved to permeate the skin better than very lipophilic compounds, **1f** and **1h**, thus suggesting that notable decrease of lipophilicity resulted in lower skin permeation. Similar findings have been reported by other authors studying in vitro and in vivo skin permeation of several active compounds (Yano et al., 1986; Sloan, 1989).

As compounds **1f** and **1h** resulted in the UVA filter not being absorbed through the human skin, they were assayed for their in vitro phototoxicity by photohemolysis and lipid peroxidation tests.

It must be outlined that a correct experimental approach in this screening requires the testing of these compounds at use levels which do not exceed common practice. Moreover, it is recommended to take into account the value of absorbed light dose in the irradiation 'window' used, which considers light intensity, absorbed light fraction, and irradiation time, providing that these latter parameters fall in the energy and intensity fields of solar radiation reaching the earth's surface.

The photohemolysis tests were performed using a RBC suspension containing **1f** and **1h**; these two potential sunscreens were thus tested for their ability to induce photohemolysis when irradiated with UVA light. For comparison, the marketed UVB–UVA filter was assayed under the same experimental conditions. As a marker of phototoxic activity, the percent of hemolysis of the irradiated samples, recorded after 2 h of incubation in the dark (delayed photohemolysis), was reported as a function of the dose *D*. The concentrations of the three sunscreens used were, respectively, 75, 47 and 71 μM (**1f, 1h, 2**), and the irradiation times used ranged between 5 and 35 min. Compound **1h** was used at a lower concentration due to its solubility limits; of course this parameter was compensated by irradiation time in order to homogenize the dose value. No hemolysis was detected when RBC were incubated in the dark in the presence of the sunscreens or were irradiated in their absence. A photoinduced hemolysis is indicative of membrane damage, which can proceed either via a type I mechanism, involving free radicals produced through the photosensitized (sunscreen) excitation, or via energy transfer (type II mechanism) from ground state oxygen to produce singlet oxygen (photodynamic damage). As reported in Fig. 1, the two investigated sunscreens show a photoinduced lytic activity, which is comparable to that displayed by the marketed compound (**2**). On the other hand, this compound does not display a remarkable phototoxicity, considering the doses used.

A further test of phototoxicity is provided by photoinduced lipid peroxidation performed in ethanolic solution of the sunscreens in the presence of phosphatidylcholine from egg yolk (PC) (1×10^{-3} M). In these experiments the amount of TBA-reactive species detected is indicative of a photoinduced peroxidation localised on the C=C double bonds of the phospholipidic acyl chain (Girotti et al., 1985). At **1f**, **1h** and **2** concentrations of 108, 115 and 136 μM (which correspond to doses of 1.58, 1.62 and 1.20 μmol of photons), TBARS concentrations of 0.65, 0.59 and 0.7×10^{-5} M, respectively (error $\pm$ 10%, mean of three experiments), for an irradiation time of 30 min with a black light phosphor lamp, were detected. Also, this experiment, which is indicative of the photosensitized damage directed to a main membrane component, points to a degree of phototoxicity which is of the same order as that displayed by the commercial sunscreen. This damaging activity is not as high when compared to that of other well-known photosensitizers, measured under the same experimental conditions (Costanzo et al., 1995).

In conclusion, considering that the introduction of parallel assays of phototoxicity and permeability into an in vitro test system can improve the affordability of an alternative test to in vivo experiments, the results obtained from these studies seem to indicate that the derivatives **1f** and **1h** may be considered promising and safe UVA absorbing agents for cosmetic and dermatological applications.

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