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Photoirradiation of azulene and guaiazulene—Formation of reactive oxygen species and induction of lipid peroxidation☆

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ABSTRACT

Azulene and guaiazulene are popular ingredients in beauty, cosmetic, skin, and body care products. We previously determined that these chemicals are photomutagenic in *Salmonella* and phototoxic, causing DNA damage in human Jurkat T-cells. In this study we report that photoirradiation of azulene and guaiazulene, respectively, by UVA light at 0–70 J/cm^2 in the presence of a lipid, methyl linoleate, resulted in lipid peroxidation in a light dose–responsive manner. When irradiated in the presence of sodium azide or superoxide dismutase, the level of lipid peroxidation decreased, indicating that lipid peroxidation is mediated by free radical and superoxide in particular. In contrast, lipid peroxidation was not enhanced when deuterated methanol was incorporated to the system, which suggests that singlet oxygen is not a predominant photo-induced product. Electron spin resonance (ESR) spin trapping study confirmed that photoirradiation of azulene predominantly generated superoxide and none or very low quantity of singlet oxygen was produced. These results indicate that photoirradiation of azulene and guaiazulene by UVA light generates reactive oxygen species (ROS), and induces lipid peroxidation when irradiation in the presence of a lipid. These results implicate that azulene and guaiazulene are phototoxic when exposed to sunlight.

1. Introduction

Azulene (Fig. 1) is an essential oil derived from the German chamomile plant *Matricaria recutita*. In addition to its wonderful aroma, this oil is an anti-inflammatory, anti-spasmodic, and anti-microbial agent [1,2], and has been used as a folk medicine to treat inflammatory diseases. Azulene is clinically used for the medical treatment of pharyngitis, gastric ulcer, gastritis, conjunctivitis, adenoiditis and stomatitis. In addition, azulene is a popular ingredient for various products for human use [3–5]. Its alkyl derivative guaiazulene, 1,4-dimethyl-7-isopropylazulene (Fig. 1), is a constituent of guaiac wood oil and has been reported to exhibit antioxidative activity [6] and anti-inflammatory effect [7]. Furthermore, guaiazulene has been used as an antiulcer drug.

Azulene was not mutagenic in *Salmonella typhimurium* bacteria strains TA98, TA100, TA1535, and TA1537 with and without an S9 activation enzyme system [1]. In clinical studies, some patients showed allergic reactions to azulene [8].

Both azulene and guaiazulene are popular ingredients in beauty, cosmetic, skin, and body care products. While people using these cosmetic products are unavoidably exposed to sunlight, it is not known whether use of cosmetics containing azulene and guaiazulene with concomitant exposure to sunlight results in any deleterious effects. It had been reported that azulene and its derivatives exhibit photochemical reactivity [9]. We previously reported that concomitantly exposed to UVA/visible light, azulene and guaiazulene were photomutagenic in *Salmonella* TA102 and in human skin Jurkat T-cells [3]. Azulene can cause DNA strand cleavage in T-cell nucleus or pure ΦX174 plasmid DNA in solution [4]. In this paper, we report that photoirradiation of azulene and guaiazulene by UVA light produces reactive oxygen species (ROS) and induces lipid peroxidation.

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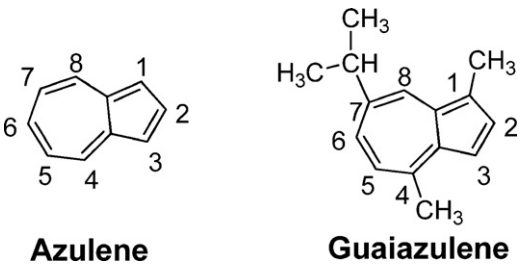

Fig. 1. Chemical structures of azulene and guaiazulene.

2. Materials and methods

2.1. Materials

Azulene, guaiazulene, methyl linoleate, sodium azide (NaN_3), superoxide dismutase (SOD), diethylene-triaminepentaacetic acid (DTPA), and 2,2,6,6-tetramethyl-piperidine (TEMP) were purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO). The nitrone spin trap, 5-*tert*-butoxycarbonyl 5-methyl-1-pyrroline *N*-oxide (BMPO), was a gift from Professor Kalyanaraman (Medical College of Wisconsin). All other reagents were obtained through commercial sources. All solvents were HPLC grade.

2.2. Light sources

The UVA light box was custom made using 4 UVA lamps (National Biologics, Twinsburg, OH) [10]. The irradiance of the light box was determined using an Optronics OL754 Spectroradiometer (Optronics Laboratories, Orlando, FL), and the light dose was routinely measured using a Solar Light PMA-2110 UVA detector (Solar Light Inc., Philadelphia, PA). The maximum emission of the UVA light box was determined to be between 340 and 355 nm. The light intensities at wavelengths below 320 nm (UVB light) and above 400 nm (visible light) are approximately two orders of magnitude lower than the maximum in the 340–355 nm spectral region.

In this study, the UVA-irradiation dose was from 7 to 70 J/cm^2, approximately 23–230 min exposure at the dose rate of 5 mW/cm^2. 10 J/cm^2 of UVA, equates to about 2 h exposure at the noontime of sunny days during the summer around world, based upon observations of UVA intensity of 2.1 mW/cm^2 in Okayama, Japan in September [11], 3.6 mW/cm^2 in Jackson, MS, USA in August [12], 5.4 mW/cm^2 in Paris, France in July [13], 6.6 mW/cm^2 in Coimbatore, India in July [14].

2.3. Peroxidation of methyl linoleate initiated by photoirradiation of azulene and guaiazulene

Experiments were conducted using a solution of 100 mM methyl linoleate and 1.0 mM azulene in methanol. Samples were placed in a UV-transparent cuvette and irradiated with 0, 14, 35, 56 or 70 J/cm^2 of UVA light. After irradiation, the levels of lipid peroxidation were expressed as the amount of methyl linoleate hydroperoxides determined by HPLC peak area by monitoring the elution at 235 nm [10,15]. Methyl linoleate hydroperoxides and the recovered substrate (azulene or guaiazulene) were separated by HPLC using a Prodigy 5 μm ODS column (4.6 mm × 250 mm, Phenomenex, Torrance, CA) eluted isocratically with 10% water in methanol (v/v) at 1 mL/min.

2.4. Peroxidation of methyl linoleate initiated by photoirradiation of azulene and guaiazulene in the presence of a free radical scavenger or enhancer

Experiments were conducted using a solution of 100 mM methyl linoleate and 1.0 mM azulene in methanol in the presence and absence of NaN_3 or SOD. All experiments were carried out as described in the previous paragraph. The concentration of SOD was 200 U/mL and NaN_3 was 20 mM.

It has been established that the lifetime of singlet oxygen is longer in deuterated solvent, such as water or methanol, than in protic solvent [16]. The effect on the levels of lipid peroxide formation induced from UVA photoirradiation of azulene and guaiazulene with CH_3OH and CH_3OD was conducted similarly.

2.5. ESR spectral measurements

The UVA light photoirradiation of azulene dissolved in 60% (for super oxide anion) and 90% (for singlet oxygen) CH_3CN in water was performed *in situ* at room temperature. A 50 μL quartz capillary tube was used. The UVA light was provided by a Schoeffel 500 W Xenon lamp coupled with a Schoeffel broad band (300–360 nm) photochromator. All experiments were performed in duplicate. The data were obtained with error of less than 10%.

Conventional ESR spectra were obtained with a Bruker EMX spectrometer (Bruker Instruments Inc., MA, USA). ESR signals were recorded at 20 mW incident microwave and 100 kHz field modulation of 1 G. The scan width was 100 G for both BMPO and TEMP experiments. All measurements were performed at room temperature.

2.5.1. Generation of singlet oxygen

Singlet oxygen generated from the photoirradiation of azulene with UVA light was detected by the ERS spin trapping method using the spin trap TEMP as described by Rinalducci et al. [17]. Samples were mixtures of ethanol (15 μL ethanol), 5 mg/mL azulene in ethanol (80 μL) and 100 mM TEMP in water (5 μL). The ESR spectra were recorded at room temperature at different times after exposure to UVA light (320 nm).

2.5.2. Detection of superoxide radical anion generation

The ESR-spin trapping method for superoxide radical detection using the spin trap BMPO is essentially the same as described by us previously [18], with the exception that all samples were dissolved in an ethanol/PBS buffer (70/30, v/v). For detection of superoxide radical anion formed from photoirradiation of azulene with UVA light, samples contained BMPO and azulene at final concentrations of 100 mM and 0.35 mg/mL, respectively.

3. Results

3.1. Photoirradiation of azulene and guaiazulene in the presence of methyl linoleate

Photoirradiation of azulene in methanol with UVA light in the presence of methyl linoleate was studied to determine whether photoirradiation of azulene can initiate lipid peroxidation. Photoirradiation of azulene, methyl linoleate, and a mixture of methyl linoleate and azulene with 0, 14, 35, 56, and 70 J/cm^2 of UVA light were conducted in parallel. The extent of lipid peroxide formation following irradiation was measured by calculation of the amount of methyl linoleate hydroperoxides based on the HPLC peak area detected at 235 nm (Fig. 2) [15]. As expected, without light photoirradiation, azulene did not produce any methyl linoleate hydroperoxides (Fig. 2). When irradiated at 14 J/cm^2 in the presence methyl linoleate, photoirradiation of azulene at concentrations of 0.002, 0.02, and 0.2 mM did not generate lipid