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Biflavonoids from *Araucaria angustifolia* protect against DNA UV-induced damage

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ABSTRACT

Ultraviolet radiation is one of the most deleterious forms of radiation to terrestrial organisms and is involved in formation of mutagenic pyrimidine dimers and oxidized nucleotides. The biflavonoid fraction (BFF), extracted from needles of *Araucaria angustifolia* was capable of protecting calf thymus DNA from damage induced by UV radiation. This occurred through prevention of cyclobutane thymine dimer and 8-oxo-7,8-dihydro-2′-deoxyguanosine formation, this being quantified by high-performance liquid chromatography coupled to tandem mass spectrometry (HPLC–MS/MS) in a multiple reaction monitoring mode (MRM) and by HPLC-coulometric detection, respectively.

1. Introduction

The incidence of skin cancer is increasing steadily and this trend has often been attributed to increased levels of highly genotoxic solar UV radiation. It is widely accepted that mutations generated in critical growth-control genes by UV sunlight component are primary factors triggering multistage photocarcinogenesis (Sinha and Hader, 2002). The mutagenic effects of UV radiation are primarily caused by photoreactions of DNA bases (Cadet and Vigny, 1990; Cadet et al., 2005). Adjacent pyrimidines can be dimerized by [2+2] and Paternò-Büchi cycloaddition reactions yielding mutagenic cyclobutane pyrimidine dimers and pyrimidine (6–4) pyrimidinone photoproducts, such as, cyclobutane thymine dimer (**2**) and thymine (6–4) thyminone adduct (**3**). The latter product **3** can be converted into the related Dewar valence isomer (**4**) by UV-B radiation (Fig. 1) (Ravanat et al., 2001). Further consequences of UV radiation on the cell involve excitation of specific endogenous photosensitizers that ultimately photooxidize guanine residues leading to 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodGuo) (**7**) (Fig. 2). It appears that such a process is mediated by singlet molecular oxygen [$O_2(^1\Delta_g)$] and to a lesser extent by ·OH radicals as oxidation inducers (Zhang et al., 1997; Cadet et al., 1997, 2006). However, in the isolated DNA, 2′-deoxyguanosine (dGuo) (**6**) absorbs UV radiation generating triplet state of purine bases which may react with molecular oxygen ($^3O_2$) yielding $O_2(^1\Delta_g)$. This species oxidizes DNA giving rise mainly **7** (Bishop et al., 1994; Mohammad and Morrison, 1996).

Araucaria angustifolia (Bert.) O. Kuntze, an endemic conifer in southern and southeastern Brazil, is the main component of the forest named "Mata de Araucarias", an endangered bioma. Their needles contain several amentoflavone-type (C8″–C3′) biflavonoids, which differ from each other in number and position of methoxyl groups, including, amentoflavone, ginkgetin and tetra-*O*-methylamentoflavone (Fonseca et al., 2000; Yamaguchi et al., 2005). These biflavonoids have potent antioxidant capacity and are capable of quenching $O_2(^1\Delta_g)$ and suppress oxidation of DNA and lipoperoxidation promoted by this excited species (Yamaguchi et al., 2005).

The BFF, biflavonoids rich fraction, from *A. angustifolia* needles, was able to prevent formation of a **2** photoproduct and **6**, which were monitored as a marker of photodamage promoted by UV radiation with regards to photocarcinogenesis and photoaging. The products were quantified using sensitive and accurate high-performance liquid chromatography coupled to tandem mass spectrometry (HPLC–MS/MS) in a multiple reaction monitoring mode (MRM) and by HPLC-coulometric detection.

2. Results and discussion

2.1. BFF protection against UV-induced thymine dimers formation

Several attempts have been made to develop biochemical and chemical assays to quantify dimeric thymine photoproducts in

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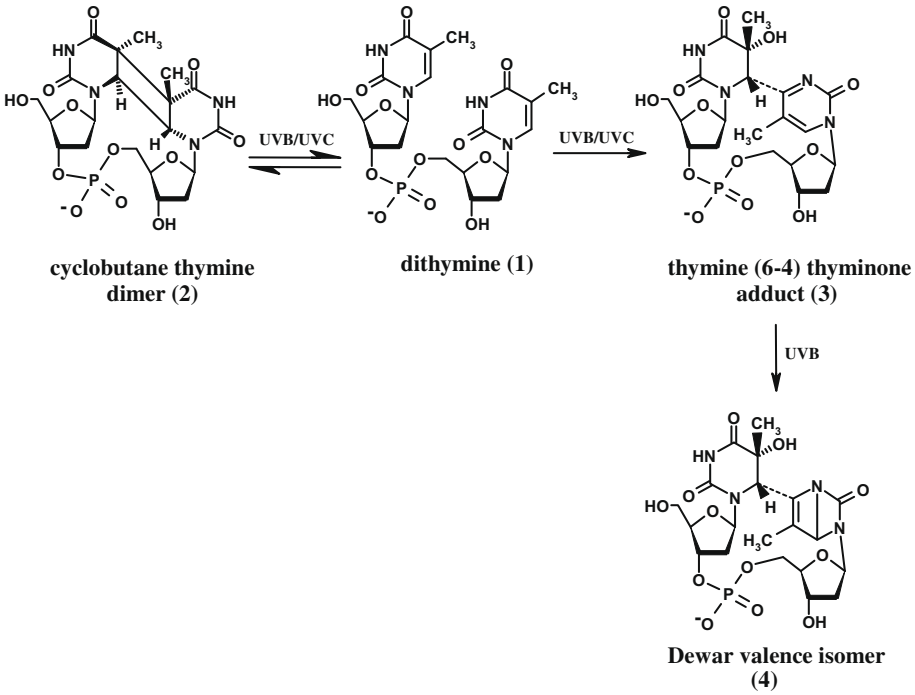


Fig. 1. Schematic representation of UV-radiation induced thymine dimeric photoproducts.

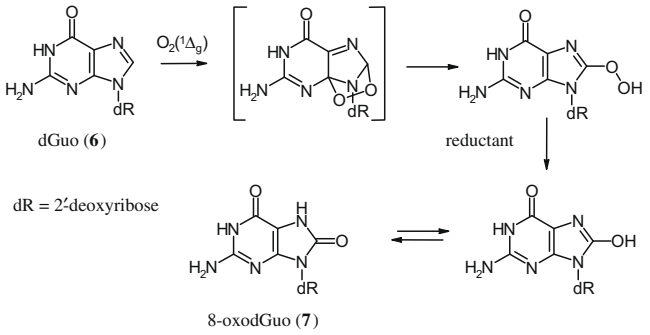


Fig. 2. Mechanism for reaction of **6** with $O_2(^1\Delta_g)$ leading formation of **7**.

DNA. The most common approach consists of immunological detection that involves use of poly- and mono-clonal antibodies raised against **2**, **3**, or **4**. However, this indirect approach has some drawbacks, including no accurate quantitative measurement due to lack of either calibration or individual measurement for each photoproduct (Ravanat et al., 2001).

Direct characterization and quantification of **2** and **3** was achieved by HPLC–MS/MS analysis. The method involved DNA digestion with 3′- and 5′-exonucleases and alkaline phosphate releasing **2** and **3** as dinucleoside monophosphates, and unmodified bases as nucleosides (Douki et al., 2000). The use of tandem mass spectrometry operating in the MRM mode combined with an automated switching valve increased significantly their sensitivity and detection specificity (Loureiro et al., 2002). A third quadrupole was set to detect only major daughter ions of investigated molecules. This technique allowed the simultaneous detection of **2** and **3** since they exhibit different fragmentation patterns even though they are constitutional isomers (Douki et al., 2000). The internal standard cytidine (**5**) was added to DNA samples prior to analysis. The full scan and ion mass spectra of **5**, **2** and **3** are presented in Fig. 3. The predominant ion in full scan mass spectra of both **2** and **3** was *m*/*z* 545, corresponding to $[M-H]^-$ ions. The major advantage of this method was quantification of different photoproducts having the same molecular mass based on their different fragmentation patterns. The switching valve was programmed to deliver only the photoproducts to the mass spectrometer avoiding contamination with compounds such as BFF from the sample.

The irradiation of calf thymus DNA with UV-B promoted formation of **2** as the major compound (0.156 μM) (Fig. 4A), while formation of **3** was detected under UV-C (250 nm) irradiation and not under UV-B radiation probably due to conversion to the **4** (Fig. 5A).

The linearity and precision of the method over the range of **2** content in the DNA samples were determined by calibration. The calibration curve was plotted using the chromatographic peak area ratios of **2**/**5** from each injection using four different concentrations *versus* amounts (micromoles) of **2**. The injected **5** internal standard was kept constant at 5 μM. The capacity of BFF to prevent **2** formation induced by UV-B radiation was evaluated using 0.5, 1.0 and 2.0 mg and was compared to that observed for commercial photoprotector octyl *p*-methoxycinnamate (Parsol MCX) (**8**) (Fig. 6). Formation of **3** was prevented down to 42% (0.091 μM) by 0.5 mg of BFF, twice as much **8** (20% at 0.12 μM). The use of 1 mg of compounds increased the protection to 50% for BFF, while **8** maintained the prevention (22%). These data demonstrated that the efficiency of BFF to reduce UV-B damage in DNA was twice as much than **8** which is widely used in cosmetics. Biflavonoids have characteristic absorptions in the UV range ($\lambda_{max.}$ = 272 and 338 nm) which includes UV-A and UV-B regions. The filtering effect of biflavonoids may contribute to the prevention of the UV-B induced **2** formation.

2.2. BFF protection against formation of 7 induced by UV radiation

The BFF prevented significantly **7** formation in UV irradiated calf thymus DNA (Fig. 7). The BFF concentration of 100 μM decreased UV-induced **7** formation by 90%, and reached a plateau, considering that at 500 μM the prevention was 91%. It has been demonstrated that UV radiation may generate reactive oxygen species (ROS), which consequently induce cell and DNA damage. Thus, the capacity of BFF to decrease oxidation of DNA can be associated

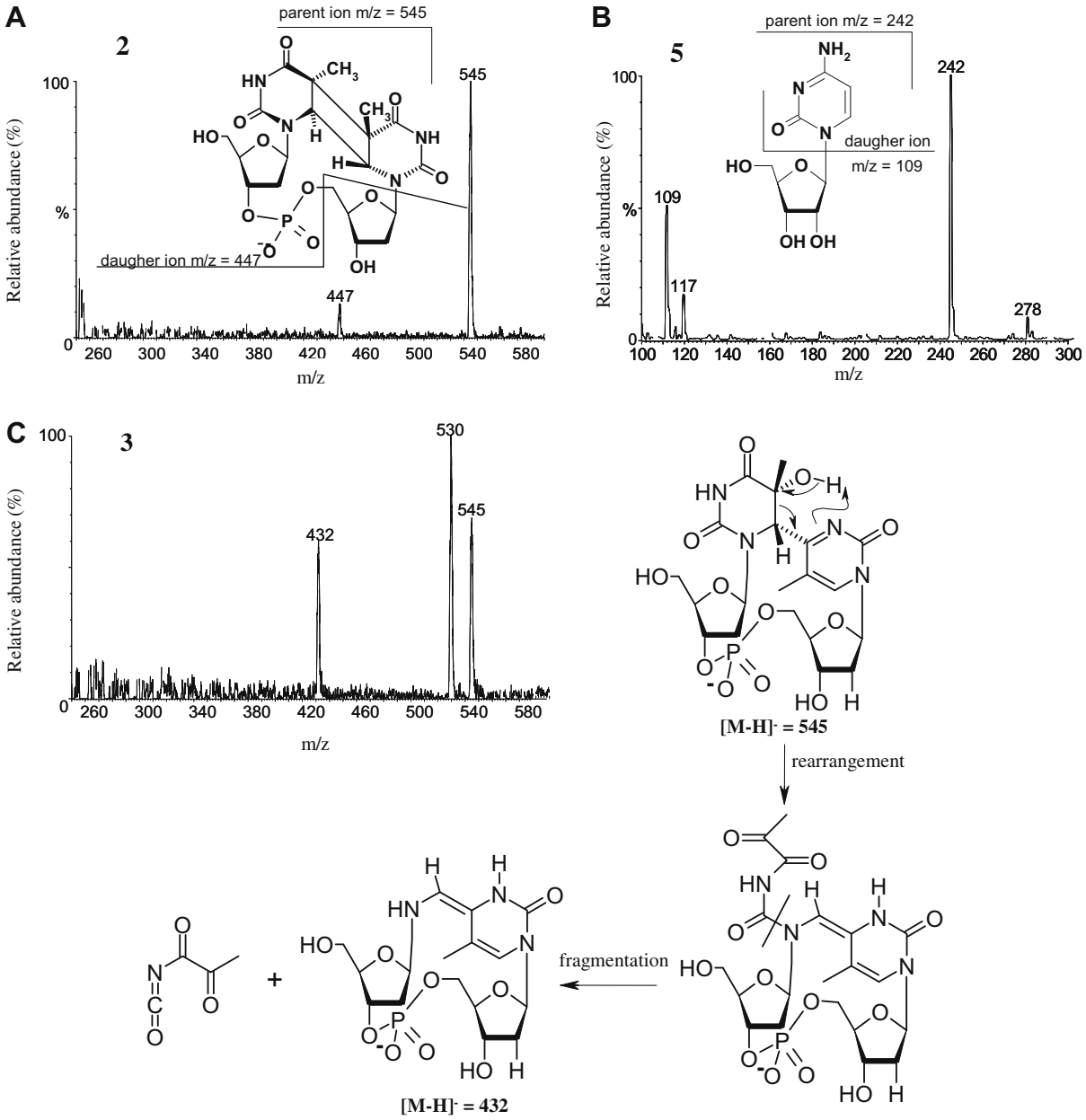


Fig. 3. Mass spectra of **2** (A), **5** (B) and **3** (C) and their correspondent fragmentation. The parent ions for **2** and **3** were observed at *m/z* 545, and were distinguished by their fragmentation profile.

to its ability to quench $O_2(^1\Delta_g)$ or other ROS as previously described (Yamaguchi et al., 2005).

3. Conclusions

The biflavonoids from *A. angustifolia* were able to prevent formation of cyclobutane thymine dimers under UV-B light acting more efficiently than **8**, widely used in cosmetics as UV-B screen, however, not efficient to protect against UV-A radiation. The biflavonoids maxima absorptions are 272 and 338 nm, a broad spectrum and strong enough to be effective as UV screens. Moreover, **7** formation by UV radiation was also prevented. These data pointed out to biflavonoids from *A. angustifolia* as promising UV-A and UV-B radiation protector. Besides, the use of HPLC–MS/MS coupled to a solvent delivery valve has allowed the measurement of thymine dimers directly with high specificity and accuracy.

4. Experimental

4.1. General experimental procedures

All chemicals were of the highest purity grade commercially available. Methanol, formic acid and hexane were purchased from Merck (Darmstadt, Germany), whereas, nuclease P1, sodium acetate, Tris, alkaline phosphatase, calf spleen phosphodiesterase, succinic acid, EDTA, snake venom phosphodiesterase, 2′-deoxyguanosine, $CaCl_2$, 8-oxodGuo and calf thymus DNA were from Sigma (St. Louis, MO). The standard cyclobutane thymine

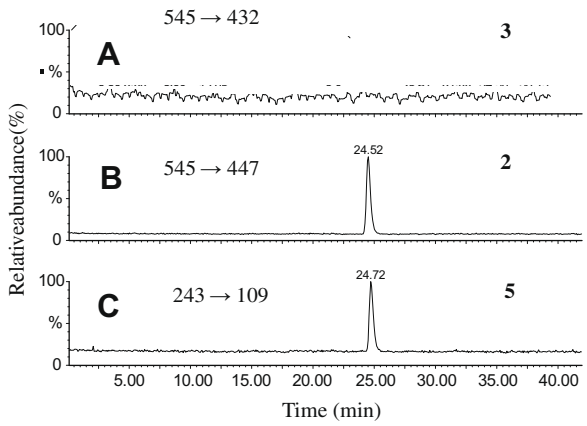


Fig. 4. Detection of **3** (A), **2** (B) and **5** (C) in calf thymus DNA after exposition to UV-B light by HPLC/ESI/MS–MS analysis in a MRM mode. Transitions 545 → 432, 545 → 447 and 243 → 109 were monitored to **3**, **2** and **5**, respectively. Detailed conditions see Section 4.

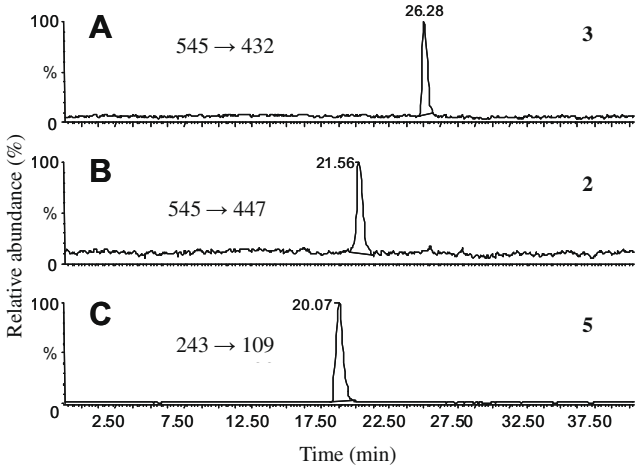


Fig. 5. Detection of **3** (A), **2** (B) and **5** (C) in calf thymus DNA after exposition to UV-C light by HPLC/ESI/MS–MS analysis in MRM mode. Transitions 545 → 432, 545 → 447 and 243 → 109 were monitored to **3**, **2** and **5**, respectively. See conditions in Section 4.

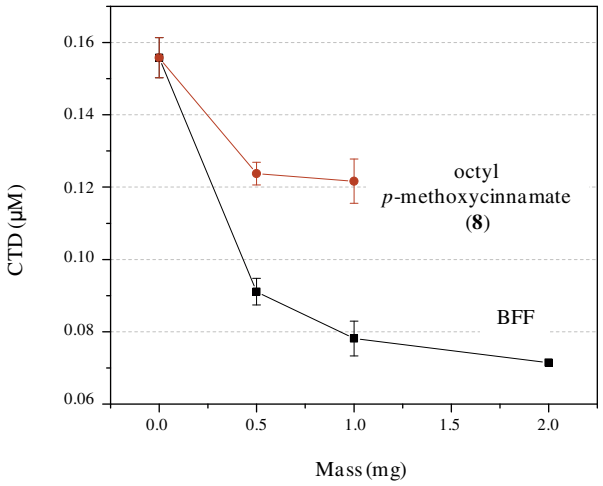


Fig. 6. Efficacy of BFF and **8** to prevent **2** formation in calf thymus DNA induced by UV-B.

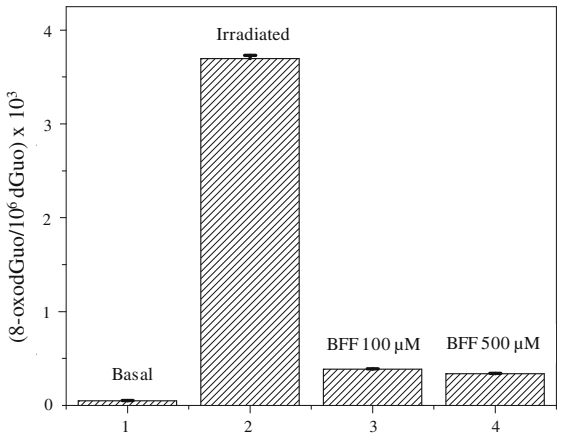


Fig. 7. Prevention of **7** formation in calf thymus DNA exposed to UV by BFF at concentrations of 100 and 500 μM (see Section 4).

dimer was kindly provided by Dr. Thierry Douki (CEA, Grenoble, France).

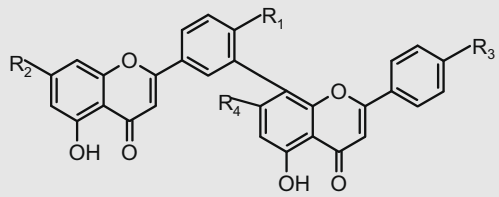
4.2. Plant material

Fresh needles of *A. angustifolia*, Araucariacea, were collected from a tree growing in the garden of Instituto de Química, Universidade de São Paulo, Brazil. The species was identified by Prof. Walter Handro of Instituto de Biosciências, Universidade de São Paulo and a voucher specimen (169963) has been deposited in the Herbarium of the Instituto de Biociencias, USP.

4.3. Extraction and isolation of biflavonoids fraction (BFF)

Dried and powdered *A. angustifolia* needles (500 g) were extracted exhaustively with MeOH (4 × 500 ml) at room temperature. The resulting crude extract was suspended in MeOH:H_2O (8:2) and extracted with hexane (3 × 500 ml). Addition of H_2O (20%) to the hydroalcoholic phase yielded an amorphous yellow precipitate, which was separated and dried, yielding a 0.5% fraction of biflavonoids (BFF). HPLC/MS analysis allowed the identification of six major biflavonoids, amentoflavone (2.4%), ginkgetin (27.1%), tetra-*O*-methylamentoflavone (16.4%), mono-*O*-methylamentoflavone (11.4%), di-*O*-methylamentoflavone (26.3%) and tri-*O*-methylamentoflavone (14.5%) (Table 1). The HPLC/MS analyses of the BFF were performed using a Shimadzu 10A*vp* chromatograph with a diode array detector, with detection at 270 and 330 nm. A Synergi 250 × 4.6 mm, 5 μm (Phenomenex, CA, USA), analytical column was used, with a mobile phase of MeOH:-H_2O + 1% formic acid (A:B), in a linear gradient mode. The gradient profile was 0–20 min: from A:B (3:1 → 9:1); 20–30 min: A:B (9:1 to 100% of B). The electrospray ionization (ESI) mass spectrometry measurement was achieved in the positive mode with in a Quattro II triple quadrupole MS/MS mass spectrometer (Micromass, Altricham, UK). The flow of 1 ml/min derived from HPLC was reduced using a splitter to deliver the mobile phase A:B at a final flow rate of 20 μl/min. Sample of BFF (1 mg/ml in MeOH) was injected through a 10 μl Rheodyne loop (Rheodyne, CA, USA). The source temperature was kept at 100 °C, and the flow-rates of the drying and nebulizing gas (N_2) were optimized to 250 and 30 l/h, respectively. The cone voltage was held at 70 V, and the capillary and HV-electrode potentials were kept at 3.00 and 0.69 kV, respectively. Full-scan data were acquired over a mass range of 100–800 Da. The data were processed and transformed into molecular masses on a mass scale, using version 3.2 of the MassLynx NT™ data system (Micromass, Altricham, UK) (Yamaguchi et al., 2005).

Table 1
Six major biflavonoids present in BFF.



Compounds	Presence in BFF (%) (w/w)
R_1=R_2=R_3=R_4=OH amentoflavone	2.4
Mono-*O*-methylamentoflavone	11.4
Di-*O*-methylamentoflavone	26.3
R_1=R_2=OMe, R_3=R_4=OH ginkgetin	27.1
Tri-*O*-methylamentoflavone	14.5
R_1=R_2=R_3=R_4=OMe tetra-*O*-methylamentoflavone	16.4

4.4. Irradiation of calf thymus DNA with UV radiation

Calf thymus DNA (250 μg) was exposed to UV light as a thin film in a flat surface after drying with N_2. The BFF (0.5, 1.0 and 2.0 mg) dissolved in MeOH (200 μl) was added to this film and dried. The **8** (0.5 and 1.0 mg) was used as a positive control in order to prevent **2** formation. The lamps UV-B (320 nm, Philips 40 W/TL 12 RS, 1.12×10^4 W/cm^2 s) and UV-C (250 nm) were used to produce cyclobutane thymine dimers while UV (UV-A 5.66×10^{-3} W/cm^2 min, UV-B 1.023×10^{-2} W/cm^2 s) was used to evaluate formation of **7**. The lamps were placed 10 cm above the DNA film. The irradiation was kept during 1 h and then the DNA was resuspended in H_2O (250 μl) and hydrolyzed.

4.5. Enzymatic hydrolysis of DNA

The hydrolysis of DNA towards the formation of **7** was performed using 1 unit of nuclease P1 in buffer (sodium acetate 1 M pH 5) incubated at 37 °C for 30 min, followed by addition of three units of alkaline phosphatase in buffer Tris–HCl 1 M pH 7.4 and incubation for 1 h at 37 °C. The final samples volume was adjusted to 100 μl with H_2O. The enzymes were precipitated by addition of $CHCl_3$ (100 μl) and, after centrifugation at 1000*g* for 5 min, the resulting aqueous layer was subjected to HPLC with coulometric detection.

The DNA digestion analysis of **2** and **3** formation was carried out as follows: 100 μg of DNA was hydrolyzed with 0.004 units of calf spleen phosphodiesterase and 10 units of nuclease P1 with 10 μl of succinic buffer (200 mM succinic acid, 100 mM $CaCl_2$, pH 6). This mixture was incubated for 2 h at 37 °C. Then, 0.003 units of snake venom phosphodiesterase and five units of alkaline phosphate were added followed by 10 μl of Tris–EDTA buffer (Tris 500 mM, EDTA 1 mM, pH 8). After 5 h of incubation at 37 °C the resulting sample was centrifuged (5000*g*) and analyzed by HPLC–MS/MS.

4.6. HPLC with coulometric detection of **7**

The separation of **7** by HPLC was carried out using a C18 Phenomenex column (250 × 4.6 mm, 5 μm) with a flow rate of 1 ml/min of KH_2PO_4 (50 mM pH 5.5 + 8% of MeOH). A Shimadzu HPLC system (Shimadzu, Kyoto, Japan) consisting of two pumps (Class LC 10AD), an SPD-10AV/VP UV at 260 nm and Coulochem II ESA, the guard cell potential set to 800 mV using the analytical cell to 130 and 280 mV detectors. The quantification was performed using **7** as the external standard.

4.7. Detection of **2** and **3** by HPLC coupled to mass spectrometry

The HPLC/MS/MS analyses of **2** and **3** were performed using a Shimadzu 10A*vp* (Kyoto, Japan) chromatograph consisting of two pumps (Class LC 10AD) with a UV detector at 260 nm (SPD-10AV/VP), an automated switching valve (FCV-12AH). A Synergi (250 × 4.6 mm, 5 μm, Phenomenex, Torrance, CA) analytical column was used, with ammonium formate (5 mM):MeOH (C:D) as the mobile phase in a linear gradient mode. The gradient profile was 0–8 min: from C (100%) to C:D (98:2); 8–30 min: C:D (98:2 → 1:1); 30–35 min: C:D (1:1 → 100% of D). The flow rate of 0.2 ml/min derived from HPLC column was discharged using the valve. A third HPLC pump (LC-10AD, Shimadzu) was used to keep a constant flow of the mobile phase to the mass spectrometer during the analysis, with an isocratic flow (0.05 ml/min) of a (1:1) solution of H_2O:MeOH. The valve was switched on between 15 and 30 min, in order to direct only the desired photoproducts to mass spectrometer avoiding contaminations. DNA hydrolyzed samples (10 μg) were injected through an autosampler (SIL-10AD/VP). The Quattro II mass spectrometer (Micromass, Manchester, UK) was operated in a negative mode and in a multiple reaction monitoring mode. The source temperature was kept at 100 °C, and flow-rates of drying and nebulizing gas (nitrogen) optimized to 250 and 30 l/h, respectively. The cone voltage was held at 60 V and the collision energy was set at 10 eV. The collision gas (Ar) pressure in gas cell was adjusted to 6.0×10^{-4} mbar and the capillary and HV-electrode potentials were kept at 3.5 and 0.69 kV, respectively. Full-scan data were acquired over a mass range of 100–800 Da. The data were processed and transformed into molecular masses on a mass scale, using version 3.2 of the MassLynx NT™ data system (Micromass, Altricham, UK). In order to quantify the **2**, an external standard (**2**) was used to plot a curve and **5** was used as internal standard. Three different transitions were monitored simultaneously: 545 → 447, corresponding to **2**, 545 → 532 to **3** and 242 → 109 to **5** (Douki et al., 2000).

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