Formation of a Nonaoxirane from A2E, a Lipofuscin Fluorophore related to Macular Degeneration, and Evidence of Singlet Oxygen Involvement**

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The lipofuscin that accumulates in retinal pigment epithelium (RPE) cells may play a role in the deterioration and death of RPE cells which is typical of atrophic age-related macular degeneration (AMD). In human RPE cells, the deposition of this aging pigment is already apparent by age 20,[1] and while it continues to increase until approximately age 70, thereafter it declines.[2–4]

The major hydrophobic fluorophores of RPE lipofuscin are the pyridinium bisretinoids A2E (**1**) (Figure 1, top left) and iso-A2E, its 13-*Z* photo-isomer.[5–8] A2E is formed by hydrolytic cleavage of A2-PE, a precursor generated from the reaction between two molecules of *all-trans*-retinal and phosphatidylethanolamine (PE).[9] It has recently been shown that the accumulation of A2E by cultured RPE bestows a sensitivity to light-induced damage.[10, 11] Specifically, the blue region of the spectrum was found to induce the apoptotic death of A2E-containing cells, with a wavelength dependency that reflected the excitation spectrum of A2E.[11, 12] The propensity for A2E-laden RPE cells illuminated by blue light to undergo apoptosis is consistent with the known susceptibility of RPE cells to blue light damage in animal models.[13–15] Moreover, the ability of A2E to serve as an initiator of photodamage may be relevant to studies linking the incidence of advanced atrophic AMD with blue light exposure, particularly in later life.[16]

To investigate the blue-light-induced modification of A2E, solutions of it in phosphate-buffered saline (PBS) (200 μM; 200 μL) containing 0.1 % of DMSO for solubility purposes were irradiated with blue light ((430 ± 10) nm) delivered from a 150-W tungsten halogen lamp (radiant energy 0.19 mW mm^{-2}). Irradiation for 10 min generated a series of oxidative derivatives, each of which represented the addition of an oxygen atom at a carbon–carbon double bond (see Figure 3). Irradiation at longer wavelengths where A2E does not absorb gave no reactions. For further structural studies oxidation with *meta*-chloroperoxybenzoic acid (MCPBA) was performed. Purification of the reaction mixture by HPLC yielded **2** as the main component.

FAB (fast atom bombardment) high energy collision-induced dissociation (CID) mass spectra of **2** resulting from the photooxidation and/or MCPBA oxidation showed the same intense M^+ peak at *m*/*z* 624, and product ions at *m*/*z* 458 and 488 (Figure 1). Analysis of these ions led to the shown

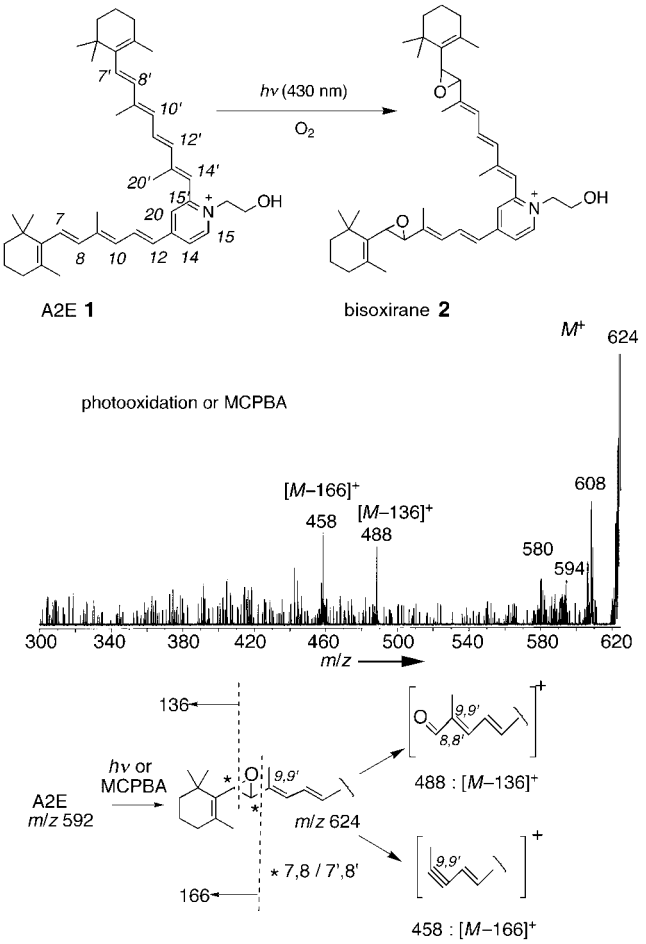

Figure 1. Top: Structures of A2E (**1**) and the bisoxirane **2**. Center: The FAB-CID mass spectra of **2** resulting from photooxidation and from chemical oxidation with MCPBA show the same intense product ions at *m*/*z* 488 and 458; spectrometer used: JMS HX 110A/110A MS/MS; accelaration voltage 10 kV, collision cell floating 8 kV, collision gas He. Bottom: probable fragment ions.

bisoxirane structure for **2**, a mixture of diastereomers as well as enantiomers, formed by epoxidation of the 7,8 and 7′,8′ double bonds. Opening of one epoxide ring at the C7/7′–O bond and MS cleavage of the C7/7′–C8/8′ bond gives rise to the fragment *m*/*z* 488 $[M-136]^+$ while cleavage of the C8/8′–C9/9′ bond gives rise to the fragment *m*/*z* 458 $[M-166]^+$ (Figure 1, bottom). Since the charge is localized on the pyridine ring, only product ions containing the pyridinium moiety are observed. Further corroboration for the structural

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assignment ($C_{42}H_{58}O_3N$) of **2** was provided by high-resolution mass spectrometry ($[M]^+$ obs. 624.4420, calc. 624.4417). The UV/Vis spectrum (EtOH) of **2** showed the expected hypsochromic shift relative to the absorption of A2E: $\lambda = 405$, 293 nm (**2**); 434 ($\varepsilon = 30\,000$), 335 nm (21 700) (A2E).[14]

The structure of **2** was corroborated by NMR spectroscopy (Figure 2). In comparison with A2E (Figure 2A), the signals of the protons at C7/8 and C7′/8′ were shifted upfield while those of the protons at C10/12 and C10′/12′ were unchanged (Figure 2B). ^{1}H-^{1}H COSY and ROESY correlations confirmed the above data and also verified that the pyridinium ring remained unchanged during the oxidation process (Figure 2, bottom).

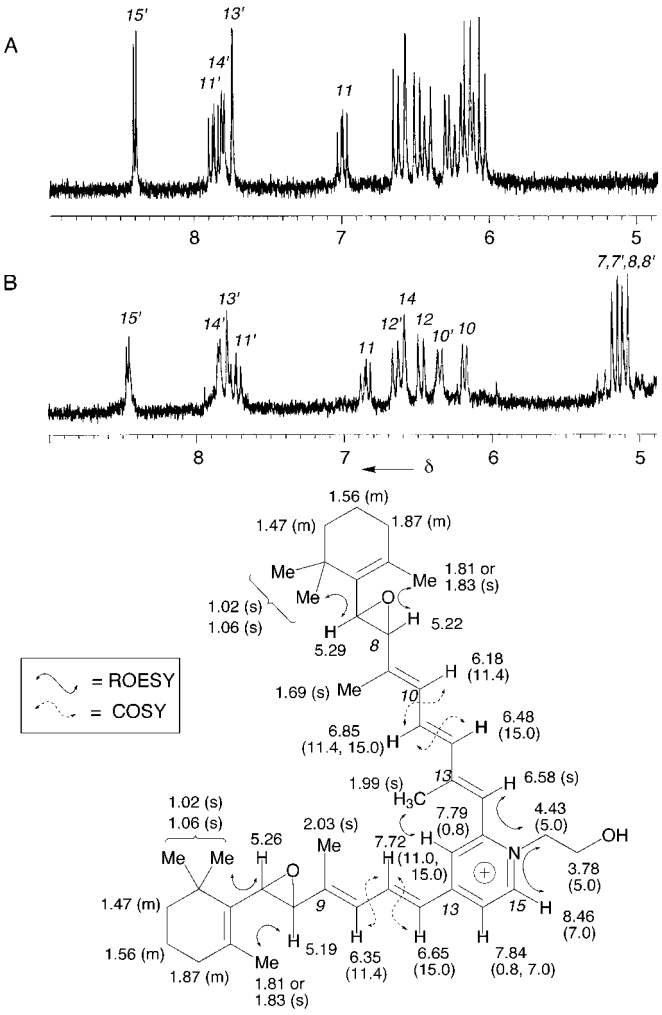

Figure 2. Top: ^{1}H NMR spectra of A2E (A) and the bisoxirane **2** (B), 400 MHz, CD_3OD. Bottom: assignment of the signals and observed COSY and ROESY peaks.

Subjecting the polyepoxide mixture to APCI (atmospheric pressure chemical ionization) mass spectrometry led to cleavage of the epoxide groups and decomposition of the fragments, giving the pyridine peak at *m/z* 79 (base peak). Continued irradiation of the polyepoxides led to further photodegradation, showing in all cases the *m/z* 79 base peak (APCI). The data support the conclusion that the photooxidation reaction starts at the 7,8 and 7′,8′ double bonds and then occurs at other double bonds. ESI (Figure 3) and FAB mass spectrometry show that epoxidation occurs at all nine double bonds in the hydrocarbon units without modifying the pyridinium ring. The *m/z* 736 peak and the series of *m/z* + 16 peaks starting from the M^+ peak of A2E demonstrate formation of an unprecedented nonaoxirane of A2E (**3**); no peak other than that of the pyridinium ion (*m/z* 79) was present below the *m/z* 592 peak.

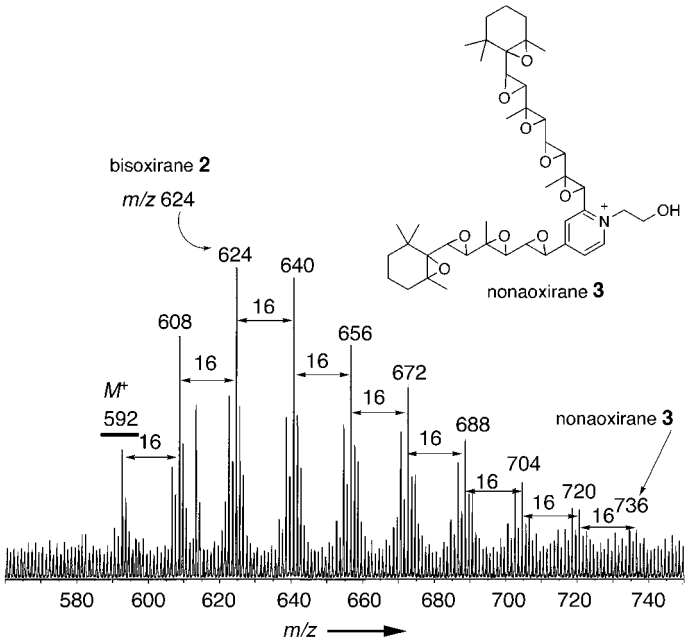

Figure 3. ESI mass spectrum of A2E after illumination with blue light (430 nm; 10 min) to yield **3**. A series of peaks that differ in *m/z* by 16 show the addition of oxygen atoms. The addition of oxygen occurred at carbon–carbon double bonds (epoxidation) of A2E, the initial oxidations taking place at positions 7,8 and 7′,8′. Measured by Micromass ESI-Q-TOF.

It is well known that singlet oxygen is a major intermediate in the photodynamic effect, the combined action of light, dye, and oxygen on a biological substrate.[17] Furthermore, singlet oxygen is known to add to double bonds giving epoxides.[18] Thus, to investigate the involvement of singlet oxygen in the epoxidation of A2E, the decrease in A2E levels during irradiation at 430 nm in H_2O and D_2O was determined by HPLC. Quantitative analysis showed two- to threefold greater decrease of A2E in D_2O than in H_2O. Due to the longer lifetime of singlet oxygen in D_2O (68 μs)[19] compared to H_2O (4.2 μs),[19] the epoxidation and the decrease in A2E levels should be faster in D_2O than in H_2O. Indeed, a strong isotope effect was observed. The rate of A2E decomposition increased by a factor of ten in CD_3OD ($\tau(^1O_2) = 270$ μs[20]) compared to CH_3OH ($\tau(^1O_2) = 9.5$ μs[19]) as determined by HPLC analysis.

This large deuterium isotope effect suggests the involvement of singlet oxygen, probably generated by photosensitization with excited states of A2E. Excited states of A2E are efficiently quenched by triplet oxygen present in the solution to produce singlet oxygen. A2E absorbs blue light more efficiently than any of the products. However, despite the fact that the higher epoxides, for example the octaoxirane, do not

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absorb blue light (430 nm), epoxidation continues to the nonaoxirane stage. Presumably the remaining A2E acts as a singlet oxygen sensitizer and the singlet oxygen then reacts with the polyoxiranes eventually leading to the unprecedented molecule **3** with nine epoxide rings.

Singlet oxygen can also be generated from the decomposition of aromatic endoperoxides.[21] The endoperoxide of 1,4-dimethylnaphthalene was selected for our experiments because of its convenient half-life of approximately 5 h at 25 °C, where it decomposes into 1,4-dimethylnaphthalene and singlet oxygen.[22] Solutions of A2E and the endoperoxide in methanol were stored in the dark for 10 h at room temperature. MS measurements of the reaction mixture revealed epoxidation of A2E and showed a spectrum similar to that of Figure 1.

Chemiluminescence studies confirmed the release of singlet oxygen from the endoperoxide. In CCl_4 it showed a strong chemiluminescence centered at 1270 nm, typical for singlet oxygen phosphorescence.[23] In the presence of A2E the luminescence intensity was reduced gradually with increasing A2E concentration, suggesting an efficient quenching reaction (Figure 4). The rate constant of the singlet oxygen

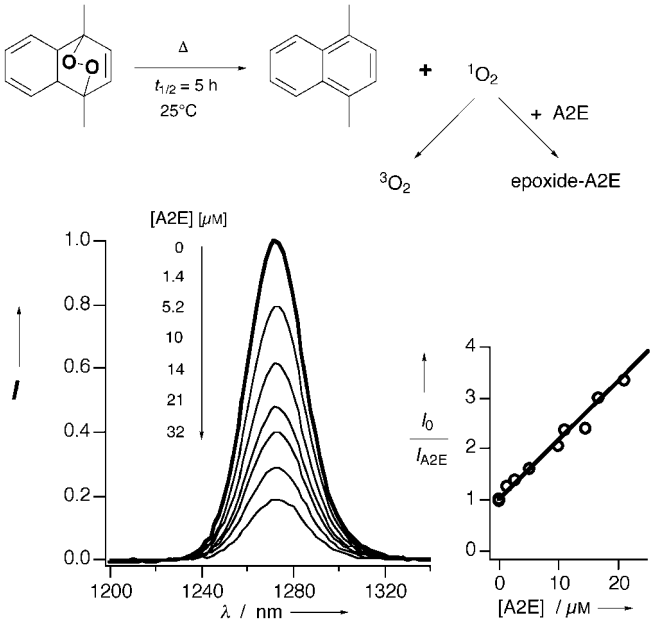

Figure 4. Chemiluminescence spectra (left) of singlet oxygen generated from the endoperoxide of 1,4-dimethylnaphthalene (2.0 mM) in the presence of A2E at different concentrations (0–32 μM) in CCl_4 at 25 °C. Right: the corresponding Stern–Volmer plot to determine the quenching rate constant, where I_0 is the intensity of the chemiluminescence at 1270 nm in the absence of A2E and I_{A2E} is the intensity in the presence of A2E.

quenching was determined by a Stern–Volmer treatment of the luminescence intensities.[24] The lifetime of singlet oxygen in CCl_4 under experimental conditions (that is at high concentrations of the endoperoxide) is expected to be significantly shorter than in pure CCl_4 (59 ms (photosensitization with 5,10,15,20-tetraphenylporphine)).[19] Therefore, the singlet oxygen lifetime ($\tau(^1O_2) = 3.4$ ms) was determined by luminescence quenching with 1-methyl-1-cyclohexene using its known quenching rate constant ($3.6 \times 10^5\,M^{-1}s^{-1}$).[25] This lifetime led to an estimation of the rate constant for the quenching of singlet oxygen by A2E to be $k_{A2E} \sim 3 \times 10^7\,M^{-1}s^{-1}$. Irradiation of A2E in CCl_4 with 430-nm light showed a luminescence around 1270 nm (see the Supporting Information), which is typical for the phosphorescence of singlet oxygen.[23]

In conclusion, it has been shown that A2E undergoes photooxidation during irradiation with blue light, producing epoxide rings initially at positions 7,8 and 7′,8′. The deuterium solvent-effect experiments utilizing endoperoxide, phosphorescence, and chemiluminescence quenching studies strongly support the involvement of singlet oxygen. It is proposed that upon irradiation, A2E acts as a sensitizer for generation of singlet oxygen from triplet oxygen present in the solution, and that subsequently the singlet oxygen reacts with A2E to produce epoxides, eventually the nonaoxirane **3**.

Experimental Section

Preparation of bisoxirane **2**: A solution of A2E (1.6 mg, 2.2 μmol) and MCPBA (0.39 mg, 2.2 μmol) in $CHCl_3$ (4.0 mL) was stirred at room temperature in a sealed vial overnight. The mixture was concentrated in vacuo and purified by reversed phase HPLC (Cosmosil C18, 250 × 4.6 mm) using acetonitrile in water (0.1 % TFA): 85–96 % (10 min), 96 % (5 min), 96–100 % (2 min), 100 % (13 min); monitored at 430 nm.

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Reversible Binding of C_{60} to an Anthracene Bearing a Dendritic Poly(amidoamine) Substituent to give a Water-Soluble Fullerodendrimer**

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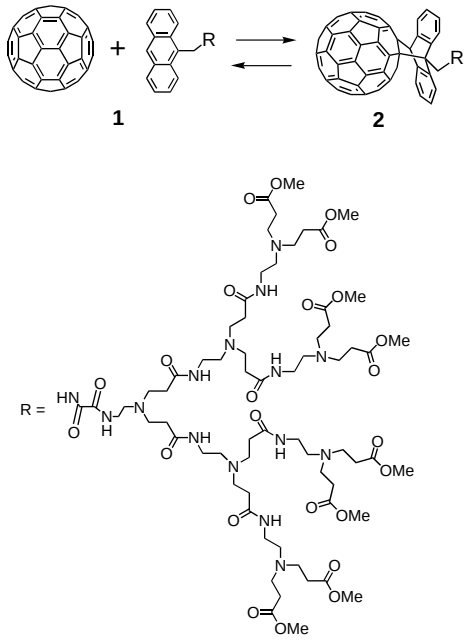
Fullerene-based compounds are attracting much interest in the fields of structural and synthetic organic chemistry.[1–3] In particular, there is an increasing focus on developing applications for fullerene-functionalized dendrimers (fullerodendrimers), because of a variety of interesting features in supramolecular chemistry and materials.[1, 2] Although a number of synthetic techniques have been devised, [4+2] cycloaddition proved to be one of the most expeditious methods for selective functionalization of [60]fullerene at the 6,6-ring junctions.[4] Several such reactions of C_{60} with anthracenes have been described.[5–7] However, the formation of fullerodendrimers by a Diels–Alder reaction of C_{60} with anthracene derivatives has never been reported. Recently, we described the synthesis, characterization, and photodimerization of a poly(amidoamine) dendron with an anthracene moiety at the focal point.[8] During our studies on the reactivity of this anthracene bearing a dendritic substituent, we found that it underwent reversible Diels–Alder reaction with C_{60}. Although a few examples of fullerodendrimers with amide dendrons have been reported, much less is known about the chemistry of poly(amidoamine) dendrimer grafted to C_{60}.[9] Here we describe the synthesis and characterization of a poly(amidoamine) dendron with C_{60} at the focal point. This fullerodendrimer also represents a new class of water-soluble fullerenes and acts as photosensitizer to generate singlet oxygen in water. To our knowledge, the only fullerodendrimer with reversible formation of bonds between C_{60} and the dendrimer is an iridium complex reported by Catalano et al.[10]

A mixture of C_{60} and dendron **1**[8] (1 equiv) in *o*-dichlorobenzene was irradiated with a high-pressure mercury lamp ($\lambda > 300$ nm) at room temperature under a nitrogen atmosphere for 1 h. The initial purple color of the solution became red as the reaction proceeded. After evaporation under reduced pressure below 60 °C, the products were separated by HPLC (LC 918, Japan Analytical Industry, Co. Ltd.) on gel-permeation columns (Jaigel 2H + 1H) with chloroform as eluent to give the fullerodendrimer **2** as a brown oil in 21% yield (Scheme 1). Notably, **2** is readily soluble in methanol, and hence unconsumed C_{60} was easily removed by filtration after the reaction. Fullerodendrimer **2** remained stable for several weeks when stored at −10 °C. The addition reaction was also conducted by heating at 45 °C for 4 d in *o*-dichlorobenzene in the dark to give **2** in 12% yield. When fewer equivalents of anthracenyl dendron **1** were used, the



Scheme 1. Reversible Diels–Alder reaction of C_{60} and **1**.

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