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Preparation of a Thiolated Derivative of A2E, a Pigment of the Lipofuscin of Retinal Pigment Epithelial Cells

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Retinal age pigments, or lipofuscin granula, contain a number of fluorophores that accumulate as a consequence of light-related vitamin A recycling in association with aging and with some retinal disorders in retinal pigment epithelial (RPE) cells.[1] Lipofuscin is a mixture of incompletely degraded materials trapped in lysosomes of RPE cells and the fluorophores of lipofuscin granular are thought to represent a biomarker for cellular aging, since the byproducts in RPE cells are considered to be a key contributor in the etiology of age-related macular degradation (AMD), a common cause of blindness in elderly people.[2,3] Although AMD is reported to be a leading cause of legal blindness in developed countries and several treatment options have been recently developed,[4-6] treatment of AMD still remains difficult because the molecular composition and pathogenic mechanisms of RPE lipofuscin have been poorly studied. This is mainly attributed to the heterogeneous nature of the most isolated lipofuscin preparations.

Numerous fluorophores have been identified so far in RPE lipofuscin such as the pyridinium bisretinoid isomers A2E (*N*-retinyledin-*N*-retinylethanolamin), isoA2E, oxidized derivatives of A2E, and several conjugates (Fig. 1).[7] Among these fluorophores, A2E is known to play an important role in causing membrane permeabilization,[8] inhibition of cytochrome *c* oxygenase,[9] inhibition of the ATP-driven proton pump,[10] and partial mediation of light damage by acting as a photosensitizer.[11] A2E is also reported to perturb cholesterol metabolism in RPE cells without light exposure.[12] Of particular importance is the finding that the photo-damage to cells induced by A2E involves the formation of A2E photo-oxidation products.[1] The oxygen-containing moieties formed by the photoexcitation of A2E include epoxides, furanoid oxide structures and cyclic peroxides that are expected to be reactive.[13-15] Such bioactivities support a possible role for lipofuscin in AMD progression, followed by choroidal neovascularization (CNV) development, but the molecular mechanisms remain to be determined.

Toward a better understanding of the pathogenesis caused by these potentially toxic lipofuscin fluorophores, we prepared a thiolated A2E derivative (A2S) in order to employ it for affinity chromatography as a first step to identify the target protein(s) of A2E in retina.

As described in Experimental Section and shown in Scheme 1, the reaction time for A2S synthesis was increased to 72 h, compared to the reaction conditions for A2E synthesis.[11] A2S purification was performed by reverse-phase HPLC. The retention time of A2E as a control was found to be approximately 10 min under the HPLC conditions employed, and it was anticipated that the retention time of A2S would be delayed by the slightly higher hydrophobicity of the thiol functional group caused by the lower electronegativity of the sulfur atom. The fractionated solution at ~15 min was analyzed *via* UV spectroscopy and MS for assigning the obtained fraction to A2S (Fig. 2). Existence of the terminal sulfhydryl group in the fractionated solution was confirmed by using thiol and sulfide quantitation kit (Molecular Probes, OR) according to the manufacturer's manual (data not shown).

UV data show λ_{max} at two regions indicating that A2S is present in the fractionated solution. According to the previous reports, however, the absorbance intensity at 438 nm of A2E is approximately 2 times larger than that at 337 nm. Therefore, the inconsistency of the relative intensities in the 337 nm and 438 nm regions in Figure 2(a) might result from a mixture of unknown compounds presumably including all *trans*-retinal, the thiolated derivatives of dihydro-A2E, or isoA2E. The presence of A2S in the fraction was also determined using mass spectrometry, as shown in Figure 2(b). Since possible fragments during mass spectroscopy could be expected and molecular structure of A2S was confirmed considering the cleavable sites in the molecule, our obtained mass signals were accordingly assigned [*m/z* 608.43 (calculated), 608.46 (observed), for example].

Importantly, UV analysis implies that there are no trisretinoids or monoretinoids in the 15-min fraction and MS analysis confirmed the absence of the thiolated derivative of dihydro-A2E (*i.e.* no 606 *m/z* signal), which suggests that the 15-min fraction may be a mixture of A2S, retinal (showing a single λ_{max} at the 337 nm region), and/or the thiolated derivative of isoA2E (or isoA2S that is interchangeable with

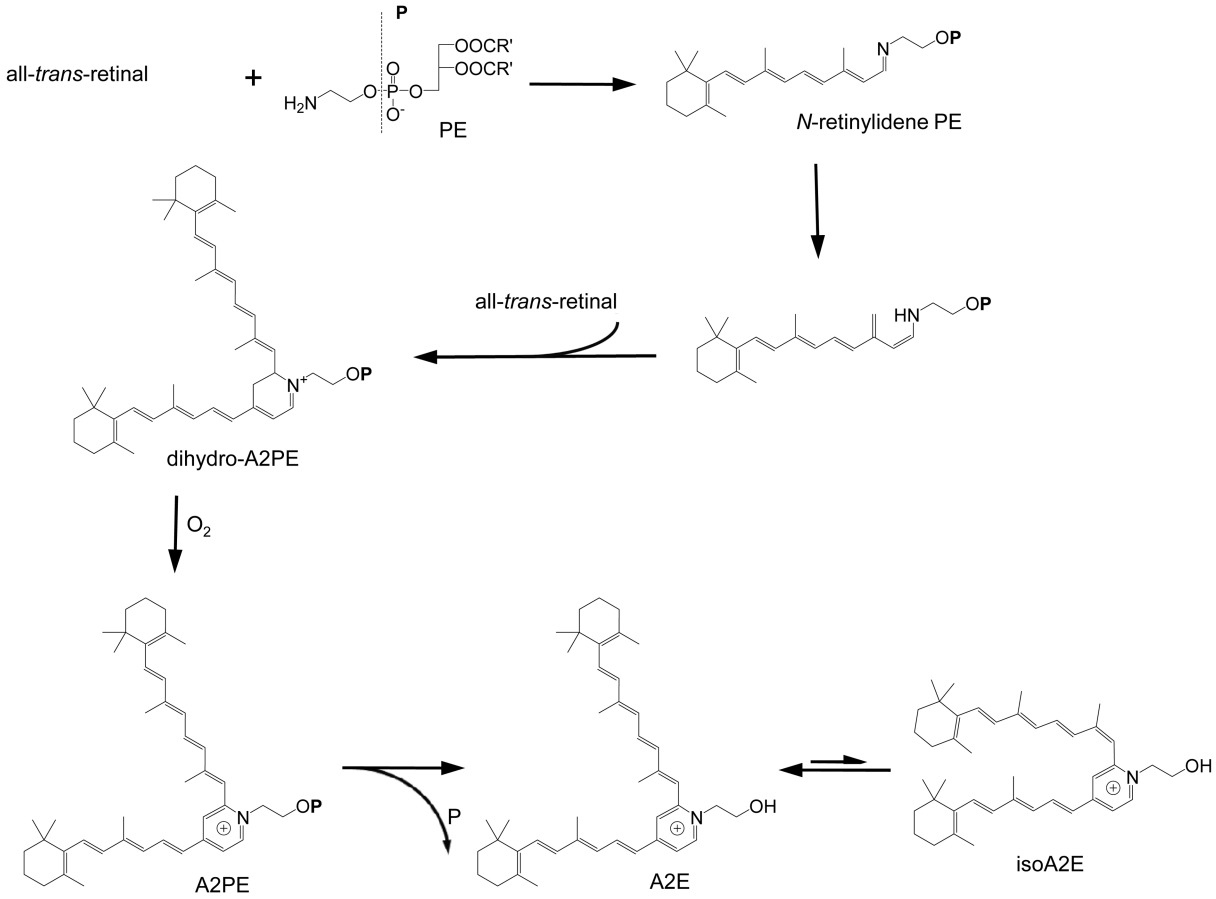
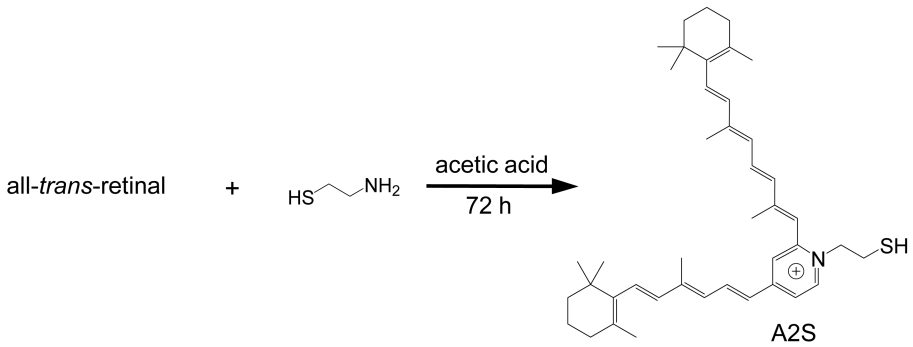


Figure 1. Plausible biosynthetic pathway for A2E (*N*-retinyledin-*N*-retinylethanolamin) formation in retina. All-*trans*-retinal released from opsin after photoisomerization of ground state 11-*cis*-retinal reacts with PE (phosphatidylethanolamine) in the disk membrane, followed by conjugation with a second molecule of all-*trans*-retinal to generate A2PE, a phosphatidyl-pyridium *bis*-retinoid. Dephosphorylation of A2PE yields A2E. IsoA2E stands for *Z*-isomer of A2E.



Scheme 1. One-pot synthetic scheme for A2S, a thiolated A2E derivative (refer to Experimental Section).

A2S). The present study was designed to make use of A2S for preparation of A2S-conjugated beads of affinity column chromatography, with an ultimate goal to better understand the molecular mechanism of CNV development due to AMD. In this regard, it should be noted that A2S is the thiolated compound in the 15-min fraction, and the fraction is expected to be used for affinity column chromatography and further protein identification methods development.

To summarize, we have prepared a thiolated derivative of A2E, a major lipofuscin component in RPE cells, for future identification of A2E-binding protein(s). Although our data accounted for a mixture of A2S, A2S was found to be the only molecule having the terminal sulfhydryl group in the obtained fraction. We will in the near future employ the thiolated molecule for affinity chromatography as a first step to better understand the molecular mechanism of CNV development due to AMD.

Experimental Section

General Methods. Unless otherwise noted, reagents were obtained from commercial suppliers and were used without

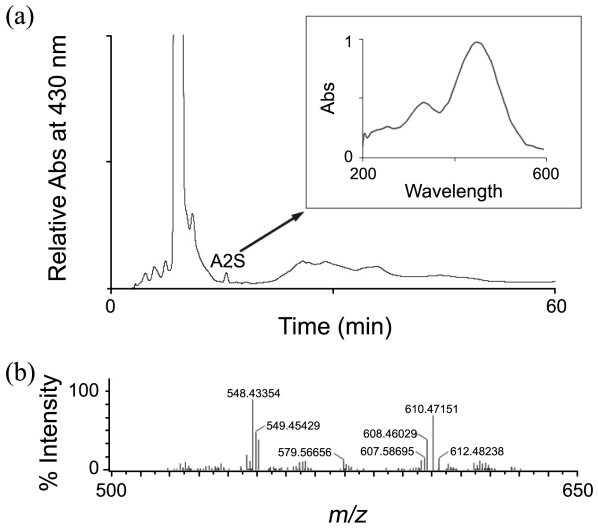


Figure 2. (a) HPLC chromatogram before purification of A2S. The insect is UV/vis spectrum for the purified A2S, demonstrating that A2S is present in the separated fraction based on λ_{max} at two regions. (b) Mass spectrum of the obtained fraction indicating the presence of A2S.

further purification. UV absorbance was measured using Agilent 8453 UV-Visible spectrophotometer. MS experiments were performed using an AccuTOF-TLC single-reflectron time-of-flight mass spectrometer (Jeol Ltd, Tokyo, Japan) equipped with a DART ion source (IonSense, Saugus, MA, USA).

A2E Synthesis. A2E was prepared from all-*trans*-retinal and ethanolamine according to the literature.[11] In brief, a mixture of all-*trans*-retinal (50 mg, 176 μmol) and ethanolamine (4.6 mg, 78 mmol) in ethanol (1.5 mL) were stirred in the presence of acetic acid (4.7 μL, 78 μmol) at room temperature under dark conditions for 2 days. The reaction mixture was evaporated and then purified by reverse-phase HPLC.

Synthesis of A2S, a Thiolated Derivative of A2E. A2S synthesis was slightly modified from the literature procedures.[11] Briefly, 2 eq. of all-*trans*-retinal (32.99 mg) and 1 eq. of 2-aminoethanethiol (4.45 mg) was dissolved in ethanol with 1 eq. of acetic acid in the dark, and stirred for 72 h. The mixture was then purified by reverse-phase HPLC using an analytical 250 mm × 4.6 mm Hypersil ODS column (Thermo Electron Corp., Bellefonte, PA). HPLC gradients were A: water 0.1% TFA, B: 0.1% methanol, of which the flow rate was 1.0 mL/min with a gradient of B from 85% to 100% over 10 min (detection of products at 430 nm).

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