

Ocular Age Pigment "A2-E": An Unprecedented Pyridinium Bisretinoid

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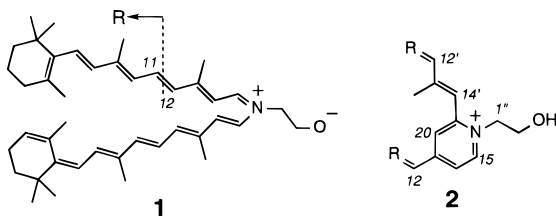
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With age, fluorescent granules called *lipofuscin* or age pigments accumulate in the retinal pigment epithelium (RPE).¹ These granules are believed to lead to cellular aging processes and related diseases, notably age-related macular degeneration (AMD),² the leading cause of blindness in elderly people for which no remedy exists. It is generally accepted that the pigments are formed as a consequence of accumulation of debris resulting from incomplete digestion of phagocytosed outer segment disks in lysosomes. Among the compounds that accumulate in lipofuscin, the orange fluorophores have attracted wide interest since they are considered to be the possible cause of age-related decline of cell functions. In this communication we report the structure of the major fluorophore.

This orange pigment was first isolated from >250 human donor eyes (age >40 years) by a series of silica gel column chromatography and preparative TLC.^{3,4a} Structural studies were mostly performed by analysis of the FAB-CAD MS/MS fragments of the hydrogenated pigment because of the limited amount, <100 μ g, and ill-defined ¹H NMR peaks. This led to structure **1** for the major orange fluorophore.^{4a} Since it consisted of two retinal moieties and one ethanolamine moiety, retinal and ethanolamine in a 2:1 molar ratio were reacted to give an orange pigment having the same TLC *R_f* values and UV and fluorescent spectra as the native fluorophore.^{4a,5} However, no further structural studies were performed due to the minuscule amount of material. The present studies were performed because of ambiguities^{4b} present in structure **1**; this has led to revised structure **2** for this fluorophore "A2-E" (2 mol of vitamin A aldehyde and 1 mol of ethanolamine).



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(5) The identity of natural and synthetic A2-E was further proven by the identical HPLC retention time and ¹H NMR spectra, although the natural fluorophore showed broader and extra signals.^{4a}

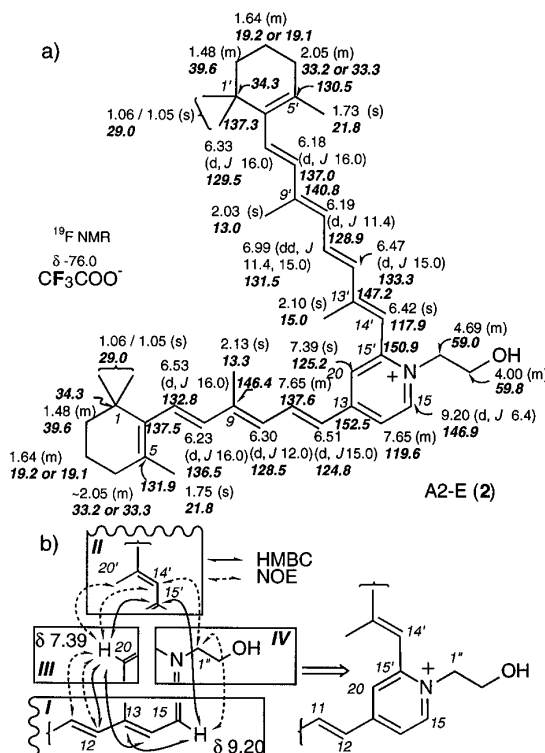


Figure 1. (a) ¹H (upper line, 500 MHz) and ¹³C (lower line, 125 MHz) NMR chemical shifts in ppm in CDCl₃. (b) Substructures I–IV and selected correlations of HMBC (solid arrows) and NOEs (dotted arrows).

Structural studies were performed with synthetic A2-E (5 mg)⁶ prepared from *all-trans*-retinal (1 g) and ethanolamine.^{4a} The FAB-MS *M*⁺ peak at *m/e* 592 agrees with the HR-MS derived C₄₂H₅₈NO formula reported for the native pigment,^{4a} which had two hydrogens fewer than expected from the earlier structure **1**; i.e., another degree of unsaturation should have been present. Two retinoid moieties up to C-12 could be readily discerned by comparison of its ¹H NMR spectrum with that of the quaternary iminium salt of retinal (Figure 1a).⁷ COSY, long-range COSY, NOEs, and coupling constants enabled one to extend these units to two retinoid moieties: substructure **I** lacking the 20-methyl and substructure **II** lacking the 15'-H (Figure 1b). The remaining signals were assigned to an sp² carbon (**III**) and an ethanolamine moiety (**IV**). HMBC correlations between the δ 7.39 proton signal in **III** and C-12, C-14, and C-15' indicated the presence of an sp² carbon at C-20 instead of methyl, thus leading to connections of moieties **I/III/II** through C-13/C-20/C-15'. Furthermore, HMBC between 15-H (δ 9.20) and C-15' disclosed the connectivity of **I/II** through C-15/C-15'. Their chemical shifts implied the presence of nitrogen between C-15 and C-15', thus forming a pyridine ring. The connectivities between **I**, **II**, and **IV** were clarified by the NOEs between 1''-H's and 14'-H/15-H, which revealed the attachment of the hydroxyethyl chain to the pyridinium nitrogen. The hydroxyl group was shown by the appearance of a hydroxyl proton (δ 5.20) in DMSO-*d*₆, while the presence of a trifluoroacetate counteranion⁸ revealed by ¹⁹F NMR (δ -76.0) and

(6) Synthetic A2-E (**2**): UV (EtOH) λ_{max} 434 nm (ϵ 30 000), 335 (21 700); IR (CCl₄) 1679 (CF₃COO⁻), 1630 (double bonds), 1580 (pyridinium) cm⁻¹. ¹H and ¹³C NMR data are shown in Figure 1.

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(8) TFA was used for the purification, from which this counterion originated. The counterion of natural A2-E is uncertain.

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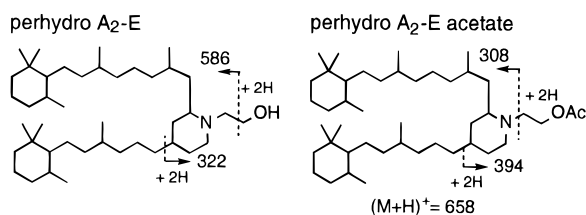


Figure 2. Major fragments observed^{14a} in FAB-CAD MS/MS of perhydro endogenous orange fluorophore and its acetate.

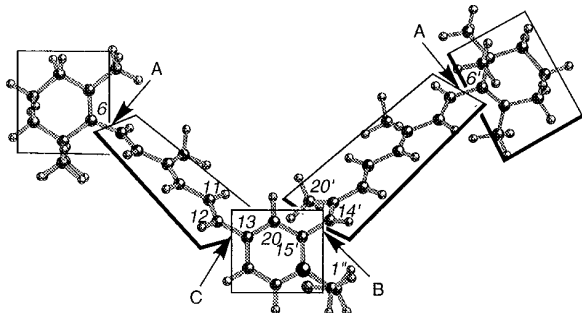


Figure 3. Energy-minimized conformation of A2-E in chloroform calculated using MacroModel V4.5.¹¹

IR (1679 cm^{-1}) indicated that A2-E is a salt and not a zwitterion as was proposed in structure **1**^{4a} and other pyridinium ethanolamines.⁹ This leads to structure **2** for A2-E. All major MS peaks in the FAB-CAD MS/MS of perhydro endogenous A2-E and its A2-E acetates, on which structure **1** was proposed,^{4a} are readily accountable (Figure 2). This provides further proof for structure **2** and its identity with native A2-E.

The conformations of the β -ionylidene ring/chain are considered to be similar to those of other retinoids, namely, a planar polyene moiety and a twisted 6-*s*-cis bond (Figure 3, arrow A). The observed NOEs between $14'\text{-H}/1''\text{-H}$ and $14'\text{-H}/20\text{-H}$ (Figure 1b) indicate that the $14'\text{-}15'$ bond is twisted (arrow B).¹⁰ Similarly, the twist around $12\text{-}13$ was shown by NOEs between $20\text{-H}/11\text{-H}$ and $20\text{-H}/12\text{-H}$ (arrow C). MacroModel calculations¹¹ also support the twisted orientations of polyene side chains relative to the pyridinium ring (Figure 3).

The formation of A2-E from retinal and ethanolamine *in vitro* suggests that A2-E **2** is also formed *in vivo* through a similar sequence of reactions of retinal and ethanolamine, both presumably arising from phagocytosed photoreceptor outer segments, *i.e.*, the former from rhodopsin and the latter from phosphatidylethanolamine, which comprises a high percentage of disk membranes.¹² The genesis of the A2-E *in vitro* at pH 5.2 and *in vivo* within the acidic environment of the RPE lysosome can be rationalized as summarized in Scheme 1. Thus: step 1, formation of protonated Schiff base **A** between retinal and ethanolamine; step 2, tautomerization to give enamine intermediate **B**; step 3, formation of iminium cation **C** between enamine and a second molecule of retinal; step 4, 6π -electrocyclization¹³ leading to intermediate **D**; and finally step 5, autooxidation leading to aromatization, *i.e.*, A2-E pigment **2**.

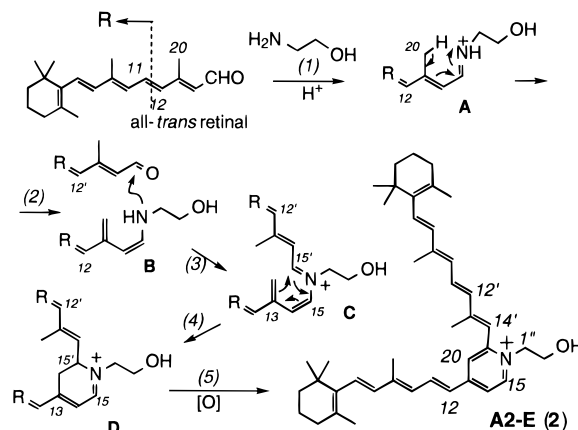
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Scheme 1. Proposed Formation Mechanism of A2-E (2)



In conclusion, the structure of the major human ocular age pigment has been determined as **2**, an unprecedented pyridinium bisretinoid. The structure accounts for its amphiphilic nature, which is responsible for the aggregation of A2-E as seen by the occasional broadening of ^1H NMR signals in CDCl_3 depending on the sample preparation, concentration, etc. Especially the extreme broadening of signals corresponding to the protons in the vicinity of the pyridinium moiety indicate the close intermolecular contact of the polar pyridinium portion in the micelle core formed by amphiphile **2** under conditions of NMR measurements. Aggregation of A2-E in various solvents with/without a lipid bilayer is corroborated by ongoing CD studies with a chiral $1''$ -methyl A2-E derivative.¹⁴ This aggregation may be responsible for the detergent-like activity of A2-E in biological systems, which was shown by vesicular shedding from red blood cell membranes.¹⁵ The extensively conjugated pyridinium structure is consistent with its photosensitizing activity to generate reactive oxygen species,¹⁶ which is harmful to biological molecules, such as lipids and proteins.¹⁷ These preliminary findings indicate the involvement of A2-E in the process of AMD, thus making it the potential target molecule for considering cures for this untreatable disease.

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Supporting Information Available: Experimental procedure for the preparation of A2-E, HPLC conditions, table of ^1H and ^{13}C NMR assignments with ROESY and HMBC correlations, and ^1H and ^{13}C NMR and 2D COSY, long-range COSY, HMQC, HMBC, and ROESY spectra of A2-E with partial assignment (12 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, can be ordered from the ACS, and can be downloaded from the Internet; see any current masthead page for ordering information and Internet access instructions.

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