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Generation of Fluorescent Adducts of Malondialdehyde and Amino Acids: Toward an Understanding of Lipofuscin¶

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ABSTRACT

Lipofuscin is a yellow-brown, highly fluorescent pigment that undergoes an age-related progressive accumulation in animal cells, mainly in postmitotic cells. It is a heterogeneous, high–molecular weight material associated with proteins, lipids and nucleic acids. Lipofuscin is implicated in many aspects of human health, including aging, oxidative stress, macular degeneration, lipid peroxidation, atherosclerosis, dementia (Alzheimer's Disease) and diseases associated with prions. Although the fluorescent properties of lipofuscin have long been recognized, neither histologists nor chemists have yet isolated the pigments themselves or characterized their optical properties. We have prepared lipofuscinlike species by reacting malondialdehyde (MDA) with cysteine (Cys). MDA:Cys adducts 3:2 and 2:2 are two of those that have been identified among the many that were present in the reaction. Whereas previous attempts to synthesize lipofuscinlike species resulted in compounds that were either nonfluorescent or emitted principally in the blue, the MDA:Cys adducts reported in this study are not only fluorescent but also emit over a broader range.

INTRODUCTION

As early as 1842 it had been recognized that aging process in humans and animals was accompanied by the progressive accumulation of yellow-brown intracellular pigments (1). These age-related fluorescent pigments are classically known as ''lipofuscin.'' Lipofuscin is a granular substance accumulating mainly in postmitotic cells, such as neurons, cardiac muscle and retinal epithelium. Lipofuscin is believed to be the product of ''oxidative stress.'' Many biomolecules, particularly polyunsaturated fatty acids, are highly susceptible to oxygen radicals. Free radical–induced lipid peroxidation processes have been suggested as an origin for the formation of lipofuscin; *i.e.* after the initiation of lipid peroxidation by reactive oxygen species, lipid hydroxyperoxides and cyclic peroxides are formed, which degrade into various carbonyl compounds, mainly aldehydes. Unsaturated aldehydes are often cytotoxic, and many of them are precursors of fluorescent products. The most extensively studied are malondialdehyde (MDA) and 4-hydroxynonenal (HNE) (2,3) (Table 1). Terman and Brunk proposed that the accumulation of nondegradable oxidized material (mainly from the autophagocytosis of mitochondria) in lysosomes may be essential for the generation of lipofuscin granules. Their proposal suggests that lipofuscin granules are residual bodies containing the products of previous autodigestive processes. The accumulation of these granules is most likely to be favored in long-lived postmitotic cells, such as neurons (1,4–10).

It is known that in humans with Creutzfeldt–Jakob's disease (CJD) and in experimentally induced instances of CJD, the amount of neuronal lipofuscin is increased. Boellaard *et al.* (4) have demonstrated a relationship among lipofuscin production, the presence of autophagocytosis and the experimental induction of CJD in mice (4). It has been argued that the accumulation of incompletely digested material, *e.g.* lipofuscin, interferes with cellular functions and increases the probability of cell death. Because cell division results in a dilution of nondegraded material, age-related cell damage and death is most likely to occur when cellular development is complete. Consequently, postmitotic cells, not proliferating cells, are most susceptible. This phenomenon has recently been referred to as the ''garbage catastrophe theory of aging'' (5,8).

In the past few years, attempts have been made to produce and to identify lipofuscinlike molecules *in vitro*. As discussed in great detail in the review by Yin (1), synthetic attempts to produce central nervous system (CNS) lipofuscin have resulted in blue-green emitters, and fluorometry of extracts has failed to detect yellow emission. Table 1 presents a summary of the emission maxima of lipofuscin pigments prepared in the laboratory—excluding those of the retina. It is very well established that a major component of lipofuscin in the eye is the ''orange''-emitting fluorophore, 2-[2,6-dimethyl-8-(2,6,6-trimethyl-1-cyclohexen-1-yl)-1E,3E,5E,7E-octatetraenyl]-1-(2-hydroxyethyl)-4-[4-methyl-6(2,6,6-trimethyl-1-cyclohexen-1-yl)-1E,3E,5E-hexatrienyl]-pyridinium (A2E) (11–14). Considerable effort has been devoted to

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Abbreviations: A2E, 2-[2,6-dimethyl-8-(2,6,6-trimethyl-1-cyclohexen-1-yl)-1E,3E,5E,7E-octatetraenyl]-1-(2-hydroxyethyl)-4-[4-methyl-6(2,6,6-trimethyl-1-cyclohexen-1-yl)-1E,3E,5E-hexatrienyl]-pyridinium; arbs, arbitrary units; CJD, Creutzfeldt–Jakob's disease; CNS, central nervous system; Cys, cysteine; HNE, 4-hydroxynonenal; HPLC, high-performance liquid chromatography; LC-MS, liquid chromatographic–mass spectrometric analysis; MDA, malondialdehyde; NMR, nuclear magnetic resonance.
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Table 1. Some previous attempts to prepare lipofuscinlike species

Oxidant	Reactant	λ_{max}^{em} (nm)	Ref.
HNE	Ribonuclease	435	(28)
HNE	Human low-density lipoprotein	440	(29)
HNE	Human low-density lipoprotein	445	(30)
HNE	N^{α}-Hyppuryllysine	430*	(31)
HNE	Lysine	430	(32)
MDA	Boc-Lys	Nonfluorescent†	(27)
MDA	Gly, Val, Leu	450	(26)
MDA	NAc-lysine	455	(33)
MDA	NAc-Gly-Lys-CO_2Me	450*‡	(3)
H_2O_2	Hexanal–hexylamine	430	(34)

*Cited detection wavelength for HPLC analysis.
†The product is $(Boc\text{-}Lys)_2MDA$.
‡The product is 1-[5-carboxymethyl-5-(*N*-acetylglycylamino)pentyl]-3-[1-(5-carboxymethyl-5-(*N*-acetylglycylamino)pentyl)-1,4-dihydropyridin-4-yl]pyridinium.

understanding the origin of lipofuscin generation in the eye, given its association with age-related macular degeneration (13,15–18). On the other hand, the identification of orange-emitting species from other tissues is incomplete. Retinoyl complexes have been found in diseased cerebral cortex (19), and retinoid-binding proteins have been isolated in adult CNS (20).

The methods most often used to characterize and to quantify lipofuscin substances in CNS tissue have been transmission electron microscopy of fixed cells or tissues, qualitative fluorometry based on observing emission as a function of cutoff filters and size-exclusion chromatography (with traditional Sephadex-type media) of tissue extracts (1). Although these approaches have been beneficial, lipofuscin is still not well understood in terms of its composition, properties, physicochemical behavior and origin. Clearly, more exacting and definitive experimental approaches and instrumental methodologies would be useful for our understanding of this enigmatic but important material. (We note that more quantitative studies have been conducted on the lipofuscin pigments found in the eye [13,15–18,21–23].) Ironically, analytical chemists seem to have regarded the CNS pigments as a nuisance rather than a researchable resource (24). This article presents an effort to isolate and characterize lipofuscin as well as to synthesize lipofuscinlike molecules as an approach to understand the formation of lipofuscin.

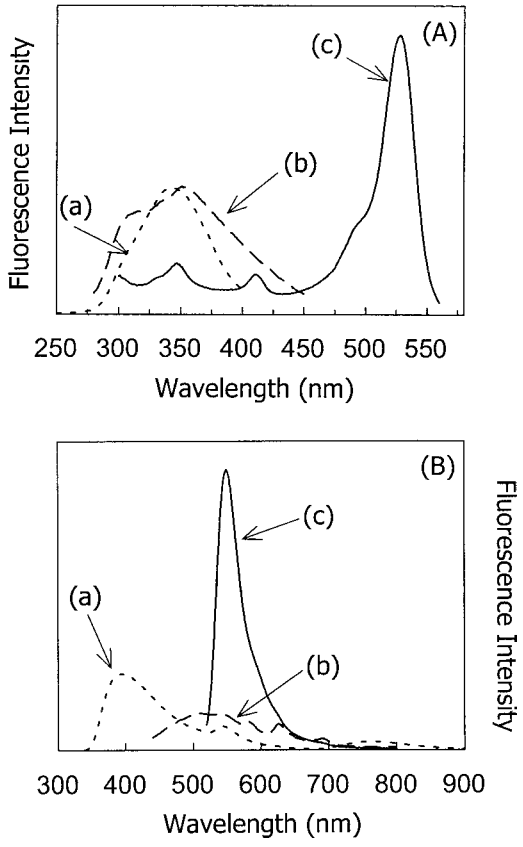


Figure 1. (A) Excitation spectra of spinal cord tissue extract in chloroform–methanol (2:1) mixture with emission monochromator at (a) 420 nm, (b) 480 nm and (c) 580 nm. Spectrum (a) has been scaled up by a factor of 20. (B) Emission spectra of the spinal cord tissue sample in chloroform–methanol (2:1) mixture: (a) λ_{ex} = 330 nm, (b) λ_{ex} = 420 nm and (c) λ_{ex} = 500 nm.

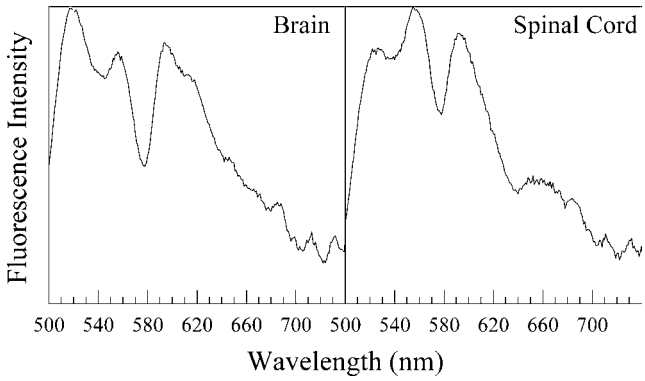


Figure 2. Front-faced fluorescence spectra from brain and spinal cord. Twelve samples of each type were analyzed. All samples were solid. The shape of the spectra in each case is highly conserved. λ_{ex} = 480 nm. A cutoff filter at 505 nm was placed before the detector to eliminate scattered light.

MATERIALS AND METHODS

Synthesis of MDA adducts. Preparation of cysteine (Cys) adducts of MDA was according to the detailed experimental conditions of Buttkus (25) at 25°C. The Cys solution was prepared by dissolving 5 g (0.028 moles) of Cys-HCl in 4 mL of water and adding 13.5 mL of 2 *N* KOH under an argon atmosphere. The pH was then adjusted to 6.5. MDA was prepared by

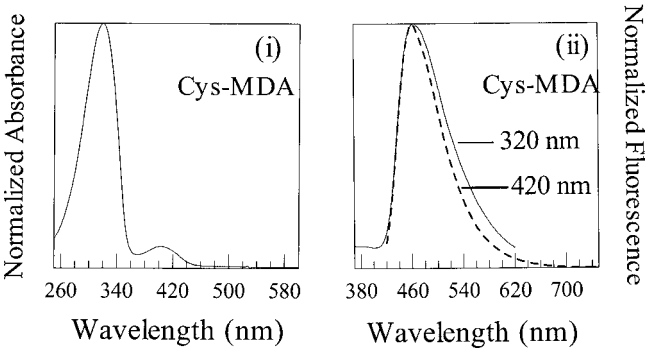


Figure 3. (i) Absorption spectrum of Cys-MDA adduct in water. (ii) Fluorescence spectra of Cys-MDA adduct in water: λ_{ex} = 320 nm (solid line) and λ_{ex} = 420 nm (dashed line).

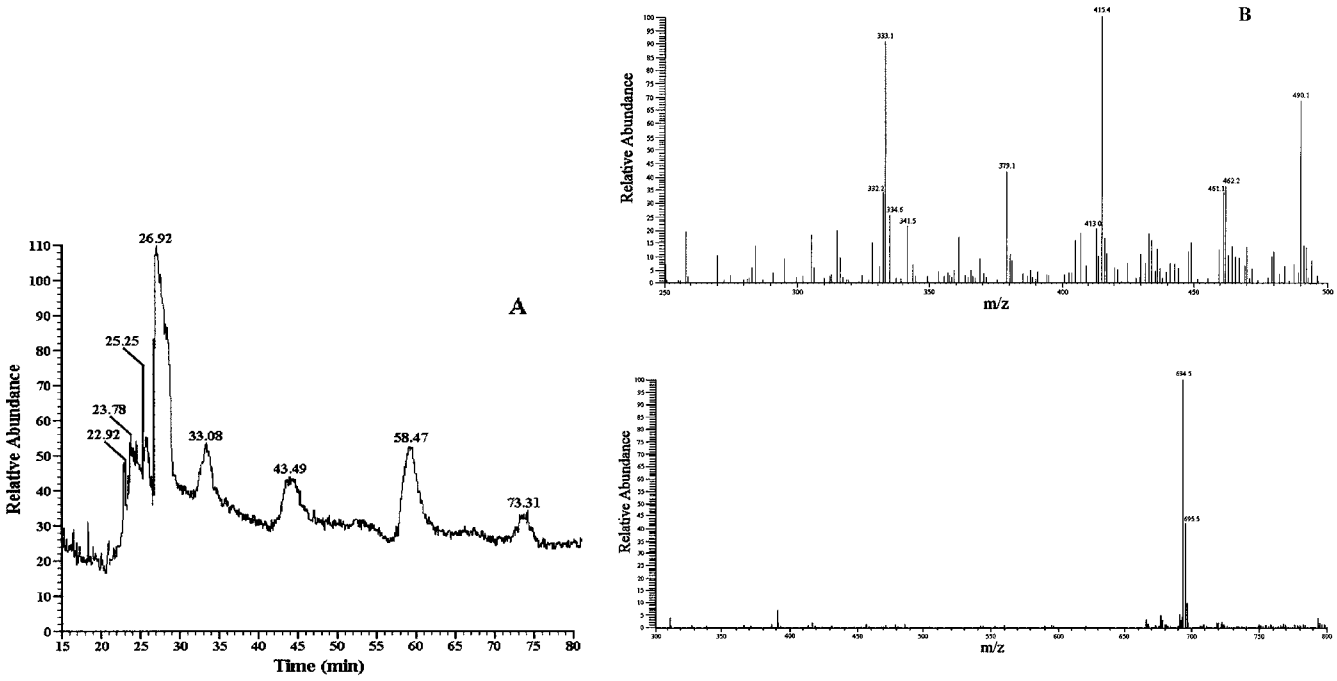


Figure 4. LC-MS analysis of MDA:Cys adducts (panel A). The HPLC mobile phase was 67:33 (water–ethanol), flow rate = 0.15 mL min^{-1}, column = 150 × 4.6 mm ID C-18 from Astec, Inc. (Whippany, NJ). The HPLC peak at 26.9 min may contain a protonated 2:2 (MDA:Cys) adduct at *m/z* 333 (upper portion of panel B). The HPLC peaks at 33.1, 43.5, 58.5 and 73.3 min are thought to be higher molecular weight adducts. For example, the second spectrum shown corresponds to the peak at 58.5 min and has a very strong MS response at 694.5 *m/z* (lower portion of panel B), corresponding to an unidentified higher molecular weight adduct from the Cys:MDA reaction. Such adducts most likely result from reaction products evolving over time, as indicated in the text.

hydrolyzing 8 mL (0.033 moles) of 1,1,3,3-tetraethoxypropane with 3 mL of 1 *N* HCl at 45°C until miscible and clear (20 min). After the addition of 2 mL of water, the solution was held for an additional 20 min at 45°C. The solution was cooled to room temperature and pH was adjusted to 6.5 with 10 mL of 2 *N* KOH. When the MDA solution was added to the Cys solution, the pH of the resulting reaction mixture dropped to 5.0. After 15 min, the pH of the solution was raised to 7.0 with 2 *N* KOH, and the solution was kept at pH 7.0 for another 15 min. The reaction mixture was then acidified slowly with 1 *N* HCl to pH 3.0. The product precipitated as a yellow colored material. The solid was washed with cold distilled water and then with acetone. It is important to note that the 3:2 and 2:2 adducts were not separable and appear to be mixtures of diastereomers. For the combined adducts—IR: 2360, 1734, 1594, 1419 and 1236 cm^{-1}; nuclear magnetic resonance (NMR): 2.15–2.80 (multiplet), 3.25–3.7 (multiplet), 4.4–5.3 (multiplet) and 7.8–8.1 (multiplet). The structures of certain adducts were determined by liquid chromatographic–mass spectrometric analysis (LC-MS) (below).

Preparation of extract. Bovine brain and spinal cord tissue (provided by the National Animal Disease Center, United Stated Department of Agriculture laboratory) was suspended in a 2:1 chloroform–methanol mixture and was sonicated for 20 min with the sample being immersed in an ice bath throughout to prevent excessive local heating. The resulting suspension was centrifuged at 20 000 ***g*** for 10 min. This was followed by careful decanting of the clear supernatant liquid, which was then used for the steady-state measurements.

Steady-state measurements. Steady-state emission and excitation spectra were obtained on a SPEX Fluoromax (Jobin-Yvon/Spex, Edison, NJ) with a 4 nm band-pass filter and corrected for detector response. A 1 cm path-length quartz cuvette was used for the measurements.

LC-MS analysis. LC-MS data were acquired using a Thermo Finnigan (San Jose, CA) Surveyor liquid chromatography system coupled to a Thermo Finnigan LCQ Advantage ion trap mass spectrometer with electrospray as the ion source. Spectra were collected in positive-ion mode with nitrogen used as both the auxiliary and sheath gas. The MS parameters were as follows: auxiliary gas, 10 arbitrary units (arbs); sheath gas, 35 arbs; source voltage, 4.50 kV; capillary voltage, 34.0 V; capillary temp, 200°C; tube lens, 30.0 V; microscans, 3 and injection time, 50.0 ms. Full-scan mass spectra were acquired over the mass range 150–800 *m/z*. MS infusion of samples was performed in positive-ion mode (full scan 150–2000). Auxiliary and sheath gases were 0 and 25 arbs, respectively. All other parameters remained the same as above.

RESULTS AND DISCUSSION

Fluorescence properties of naturally occurring lipofuscin

Figure 1 presents fluorescence excitation and emission spectra of lipofuscin extracted from bovine spinal cord. As expected, variation in the excitation spectra with emission wavelength and the variation in emission spectra with excitation wavelength indicate that the extract in Fig. 1 is very heterogeneous. A comparison with the spectra of specific MDA adducts (26) (Table 1) indicates that lipofuscin isolated from bovine tissues emits at very long wavelengths, much longer than had been suggested in the literature (1).

There are significant differences among the solution phase emission spectrum shown in Fig. 1B and the spectra of the tissue samples presented in Fig. 2. This is not surprising. The broad distribution of pigments may be inhomogeneous in the tissue. It is also likely that in the tissue there are significant self-quenching effects from closely packed pigments.

Although a rather clear picture of at least some of the lipofuscin pigments in the eye is emerging and quantitative studies are currently in progress (13,15–18,21–23), we believe that Figs. 1 and 2 (below) present the first optical spectra (emission intensity *vs* wavelength) recorded for CNS tissue. This is remarkable considering the importance of these pigments as an indicator of aging and disease.

Synthesis of lipofuscinlike pigments

A number of structures have been advanced for the chromophore responsible for lipofuscin fluorescence. Three of the most commonly proposed and studied structures are listed below. The first two might arise from the combination of MDA, a reactive dialdehyde produced from the oxidation of lipids, with amines from commonly available amino acids such as lysine. The third

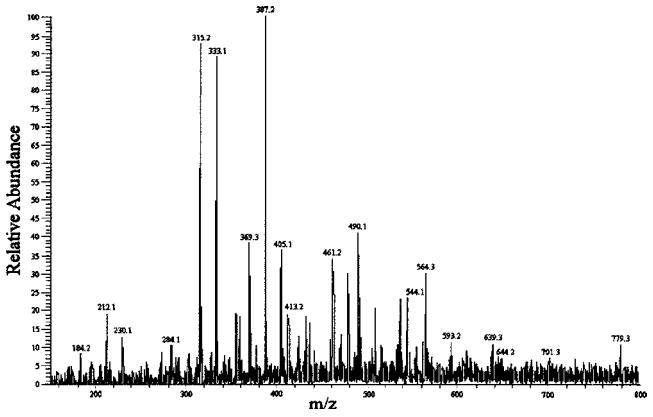
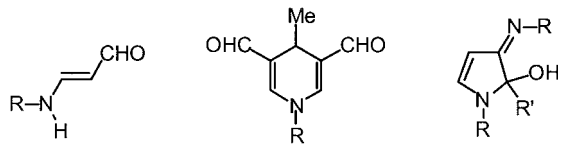


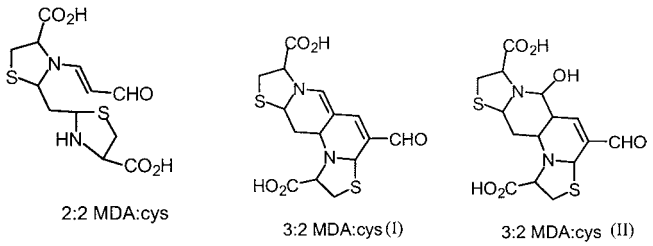
Figure 5. Direct infusion of MDA:Cys adducts into the mass spectrometer. The sample was dissolved in water and continuously introduced into the electrospray at 10 μL min^{-1}. The MS was operated in positive-ion mode full-scan from 150 to 2000 *m/z*. The *m/z* of 333.1 appears to be the protonated 2:2 (MDA:Cys) adduct. The peak at 369.3 corresponds to the first proposed structure of the 3:2 (MDA:Cys) adduct (I). The most abundant peak at 387.2 *m/z* corresponds to the second proposed structure of the 3:2 (MDA:Cys) adduct (II).

structure is proposed to come from 4-hydroxy-2-nonenal, an aldehyde that is produced by lipid peroxidation, plus an amine such as the ε-amino group in lysine. The amino acrolein structure suffers from the observation that simple 1:1 adducts such as **1** are not fluorescent (27).



1 **2** **3**

In 1969 Buttkus reported that Cys formed an unusual adduct with MDA (25). He noted that the molecular weight corresponded to a structure comprising two molecules of Cys and three molecules of MDA. The adduct was yellow and did not contain free SH groups. He observed that the molecular weight increased on storage at room temperature. Moreover, he found that methionine (which contains an SMe group) formed only a 1:1 adduct with MDA. (Buttkus did not report the fluorescence spectra of the adducts.) We reproduced these experiments with Cys and obtained a fluorescent yellow solid (Fig. 3). By NMR we determined that it was a mixture of isomers. On the basis of observations of Buttkus (no free SH groups) and our NMR, UV and mass spectra (Figs. 4 and 5), we assign the structures shown below to three adducts in the reaction mixture.



2:2 MDA:cys 3:2 MDA:cys (I) 3:2 MDA:cys (II)

NMR also shows small peaks that might be assigned as protons on a pyridinium salt. These resonances represent only a small fraction of the total material. The adducts shown above could be transformed into a pyridinium salt by cleavage of the C–S bond and double-bond migration.

Figures 4 and 5 show the LC-MS analysis of the mixture of the MDA:Cys adducts. The high-performance liquid chromatography (HPLC) peak at 26.9 min may contain a protonated 2:2 (MDA:Cys) adduct at *m/z* 333. We believe that the HPLC peaks at 33.1, 43.5, 58.5 and 73.3 min are higher molecular weight adducts. For example, the peak at 58.5 min has a very strong MS response at 694.5 *m/z*, corresponding to an unidentified higher molecular weight adduct from the Cys:MDA reaction. The most important feature of the data presented in these figures, at least in the context of this article, is to stress that the reaction of MDA and Cys produces a wide variety of products of higher and higher molecular weight. It is this feature of the reaction that we believe may be the most relevant from a physiological perspective.

It will be interesting to study the behavior of the MDA adducts over time at physiologically relevant temperature ranges. This may be significant for the production of lipofuscin because the molecular weight of the adduct of MDA with Cys increased over time at room temperature (data not shown). This suggests some dynamic evolution of structure that might mirror the production of the lipofuscins in cells. Importantly, mass spectral comparison of our freshly prepared MDA:Cys adduct with an adduct allowed to stand at room temperature for 1 week showed the presence of higher molecular weight fractions in the older material. Figure 3 presents the absorption and fluorescence spectra of the MDA:Cys adducts. It is significant, in that the adducts are yellowish, as opposed to those reported in Table 1, emitting farther to the red than those described by Chio and Tappel (26). In other words, they tend to exhibit characteristics more like those of natural lipofuscin.

CONCLUSIONS

In this study synthetic adducts of MDA with Cys have been subjected to various analyses. They emit over a broader range than other synthetically prepared lipofuscin. A better understanding of the structure of natural lipofuscin is important for the rational design of experimental systems that will mimic the synthesis of naturally produced lipofuscin. This will enable researchers to develop and test strategies that may prevent the accumulation of these pigments as cells age.

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