Substituted 2-Hydroxy-1,2-dihydropyrrol-3-ones: Fluorescent Markers Pertaining to Oxidative Stress and Aging

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Previous observations that the aging process correlates with occurrence of certain fluorescent biological pigments have led to numerous efforts in elucidating the chemical nature of the fluorophores generated through reactions of primary amines and various products of lipid peroxidation. In this study, model reactions of saturated aldehydes with aliphatic amines in the presence of peroxides were found to generate structurally unusual fluorescent compounds. Substitution of a lysine-containing peptide for simpler amines has also yielded similar fluorescence. The spectral excitation and emission maxima (around 360 and 430 nm, respectively) of these fluorophores match those widely reported in peroxidized biological objects. The fluorescent compounds in our model studies have been chromatographically isolated and their structures determined through mass spectrometry, NMR spectrometry, and Fourier-transform infrared spectroscopy. The spectrometric data indicate the fluorescent products to be alkylated 2-hydroxy-1,2-dihydropyrrol-3-ones, obtained by the action of 1,2,4-triketone intermediates upon the primary amines. Independent syntheses of several 1,2,4-triketones were carried out. One such triketone reacted with hexylamine to form a fluorescent compound spectroscopically identical to the fluorescent reaction product of hexanal, hydrogen peroxide, and hexylamine.

Introduction

It has been recognized since the early 1970s *(1−4)* that certain products of lipid peroxidation in biological systems may be responsible for the formation of fluorescent pigments. What has become gradually known as "lipofuscin pigments" appears to involve an ill-defined conglomerate of compounds which correlate with aging-related changes of mammalian tissues, oxidative stress, and a number of disease states *(5)*, all generally supporting the carbonyl toxification hypothesis of biological aging *(6)*. While the cascade of free radical-initiated lipid peroxidation processes can apparently produce a number of highly reactive species, much recent scientific effort has concentrated on reactive carbonyl compounds (malondialdehyde, 4-hydroxyalkenals, alkanals, alkenals, dienals, trienals, etc.) *(5)*, which, in turn, can modify the primary amino and sulfhydryl groups and other structures within the key biopolymers.

The lipofuscin pigments initially isolated from human brain tissues by Siakotos and Koppang *(3)* and by Taubold *(4)* exhibited two distinctly different excitation and emission ranges: one with λ_{ex} near 390 nm (λ_{em} ∼450−470 nm), and the other with λ_{ex} ∼360 nm (λ_{em} ∼430 nm). The initial assumption that most if not all fluorescent molecular entities are conjugated Schiff bases *(7)* has not been generally corroborated. Kikugawa and Ido *(8)* have later implicated 1,4-dihydropyridine-3,5-dicarbaldehydes, generated during the reaction of primary amines with malondialdehyde, as the fluorophores with characteristic excitation and emission maxima around 400 and 460 nm, respectively. However, neither conjugated Schiff base nor dihydropyridine derivative structures seem to fit the much-lower-wavelength excitation and emission maxima (typically, around 360 and 430 nm, respectively) of the fluorophores which are considerably more prevalent in peroxidized biological systems. The lower-wavelength fluorescent pigments have been widely documented in various materials: *in vitro* peroxidized microsomes *(9)*, mitochondria *(10)*, and lipoproteins *(11)*; reaction mixtures involving primary amines and oxidized lipids *(12)*; reaction of glycine with methyl linoleate hydroperoxide *(13)*; reaction of linoleic, linolenic, and arachidonic acids with methylamine, under aerobic conditions *(14)*, etc. Moreover, a similar type of fluorescence was demonstrated by Esterbauer *et al. (10)* in a highly relevant experiment with the cytotoxic 4-hydroxynonenal in microsomes, mitochondria, and phospholipid vesicles. To this date, the chemical nature of the lower-wavelength fluorophore has remained elusive.

The primary purpose of the experiments described here has been structural elucidation of the major fluorescent pigment generated in systems pertaining to the above-mentioned phenomena. Our strategy to counter the well-known complications of reaction complexity and product instability involved choosing (a) appropriate model reactions; (b) useful combinations of chromatographic isolation systems; and (c) prompt isolation and structural elucidation through spectroscopic techniques.

Fluorescence of around 360 nm excitation maximum and 430 nm emission maximum was generated by combining various enals, or conjugated unsaturated aldehydes (2-hexenal, 4-hydroxy-2-nonenal, and "malonaldehyde"—which actually exists mainly as tautomeric 3-hydroxy-2-propenal), with primary amines (or amino acids, peptides, and proteins) in the presence of hydrogen peroxide. Product mixtures in these cases proved difficult to resolve. Fluorescence of the same wavelength

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was seen when saturated aldehydes were substituted for the enals; now the fluorescent materials could be separated from other product components and purified. Though tending toward rapid decomposition, the fluorophore yielded a sufficient variety of spectral information in one instance to permit its identification as a 2-hydroxy-1,2-dihydropyrrol-3-one, or 2-hydroxy-3-pyrrolidone.

1,2,4-Triketones were postulated as precursors to the core fluorophoric structure and synthesized. One appropriately substituted triketone reacted with a primary amine to form a fluorescent compound spectroscopically identical to the fluorescent reaction product of hexanal, hydrogen peroxide, and hexylamine.

Chemical derivatization (acylation and trimethylsilylation) of the isolated core structure was also included for further clarification. Finally, kinetic data have been added to contrast different circumstances of fluorophore formation.

Experimental Section

Materials and General Procedures. Lithium diisopropylamide (2.0 M solution in heptane/THF/ethylbenzene) with magnesium bis(diisopropylamide) as a stabilizer was purchased from Aldrich (Milwaukee, WI). Ethyl ether, acetonitrile, and methanol (all HPLC grade) were purchased from EM Science (Cherry Hill, NJ). The peptide Ac-Gly-Lys-OMe was bought from BACHEM Bioscience, Inc. (Torrance, CA). THF was purified by distillation from lithium aluminum hydride.

GC-MS analyses were performed on a Finnigan MAT Magnum system and a Hewlett Packard 5971 mass selective detector with a 5890 Series II gas chromatograph. The Finnigan (ion trap) instrument usually gives molecular weight information as $[M + H]^+$, while the Hewlett Packard instrument provides $[M]^+$. High-resolution mass spectra were obtained on a Kratos MS80 RFAQ mass spectrometer. FTIR samples were prepared as thin films on a polished NaCl window purchased from Aldrich and measured on a Galaxy Series 4020 spectrophotometer from Mattson Instruments (Madison, WI). UV spectra were obtained on a Shimadzu UV 160U spectrophotometer. Fluorescence spectra were taken on a SLM $4800_{TM}C$ subnanosecond spectrofluorometer from SLM Instruments, Inc. (Urbana, IL), and on a Perkin-Elmer 650 spectrofluorometer equipped with a xenon arc lamp and a P-E 150 power supply. NMR spectra were recorded on a Bruker AM-500 spectrometer and reported in ppm relative to TMS. ^{13}C-NMR signals were assigned based on proton decoupling and off-resonance proton decoupling spectra, and by comparing with calculated values using C-13 NMR Module from SoftShell (Grand Junction, CO). HPLC analyses and purifications were done on a Perkin-Elmer Model Series 3 liquid chromatograph, equipped with a Linear UVIS-205 absorbance detector, and an on-line Schoeffel FS 970 LC fluorometer. The preparative C_{18} reversed-phase column used was a Bio-Rad Hi-Pore RP-318 (250 × 10 mm) with a Preparative Guard (80 × 10 mm). The analytical C_{18} reversed-phase column was a Biophase ODS C-18 (250 × 4.6 mm). A Bio-Sil SEC-250 (600 × 7.5 mm) column and a TOSOHAAS $G3000SW_{XL}$ (300 × 7.8 mm) column were used as the column substitutes during the normal-phase HPLC purifications. The weak cation-exchange column used was a TOSOHAAS TSK-Gel CM-3SW (150 × 7.5 mm).

Formation of Fluorescent Products. Fluorescence from the Reaction between 2-Hexenal, Butylamine, and *tert*-Butyl Hydroperoxide. To a stirred mixture of 29.6 μL of butylamine (0.30 mmol) and 100 μL of 3 M *tert*-butyl hydroperoxide solution in 2,2,4-trimethylpentane (0.30 mmol) was slowly added 139 μL of 2-hexenal (12.0 mmol). The mixture became brown but not fluorescent. In 2 min, however, some fluorescent aqueous drops were found at the bottom of the stationary flask. They were carefully removed with a pipette and washed 8 times with hexane. Chromatograms obtained from the aqueous phase are shown in Figure 1. The overall

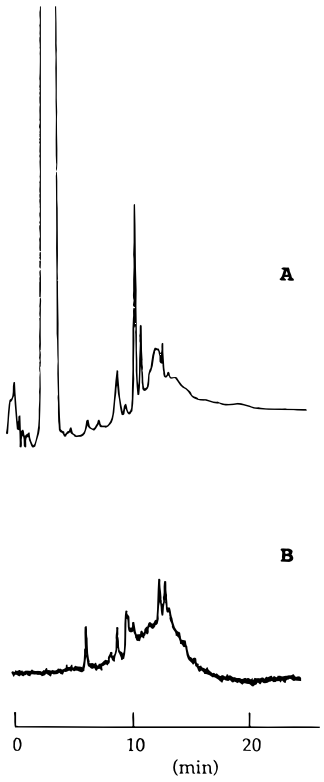


Figure 1. Chromatographic separation of the reaction products of *trans*-2-hexenal, butylamine, and *tert*-butyl hydroperoxide. Column: BIOPHASE ODS C-18 (250 × 4.6 mm). Elution: isocratic, 10% CH_3OH for 2 min, then linear gradient from 10% to 99% CH_3OH over 18 min, with isocratic 99% CH_3OH for the last 7 min. Flow rate: 1.0 mL/min. Detection: (A) UV, 254 nm; (B) fluorometric, with $\lambda_{ex} = 365$ nm, and a cutoff filter of 418 nm.

fluorescence showed excitation and emission maxima of 363 and 435 nm in methanol.

Fluorescence from the Reaction between Malonaldehyde, Butylamine or Glycine, and Hydrogen Peroxide. One milliliter of malonaldehyde bis(dimethyl acetal) (6 mmol) was added slowly to a mixture of 1 mL of hydrochloric acid and 5 mL of water. The solution was stirred at room temperature for 30 min and neutralized with $NaHCO_3$ solution. One half of the neutralized solution was mixed with 0.30 mL (3 mmol) of butylamine and 0.34 mL (3 mmol) of 30% hydrogen peroxide solution. The other half was treated identically, except that 0.21 g (3 mmol) of glycine was used instead of butylamine. It was found that fluorescence quickly developed in both reaction systems. Chromatographic analysis indicated complex, heterogeneous products. However, the fluorescent fractions collected showed excitation maxima in a range of 345−375 nm and emission maxima in a range of 415−440 nm. The fluorescence observed was found to feature different excitation and emission maxima from those collected from reactions in the absence of peroxide.

Fluorescence from the Reaction between Hexanal, Butylamine, and Hydrogen Peroxide (Formation of 1b). Hexanal (4 mL, 33 mmol) and 4 mL of 30% hydrogen peroxide solution (35 mmol) were combined in a flask and stirred for 2 min before slow addition of 4 mL of butylamine (40 mmol). Some heat was generated and the solution gradually turned yellow, but not brown. Some fluorescence was observed immediately, which became much stronger after overnight storage. The fluorescence was found to be present in both the aqueous and organic layers. The samples from both the organic and aqueous layers gave very similar chromatograms, with an example shown in Figure 2. The fluorescent compound was isolated through a series of micropreparative chromatographic steps: reversed-phase, normal-phase, and cation-exchange HPLC. In each purification step, the mobile-phase solvent, acetonitrile, was removed by aspirator at a temperature below 30 °C. Ethyl

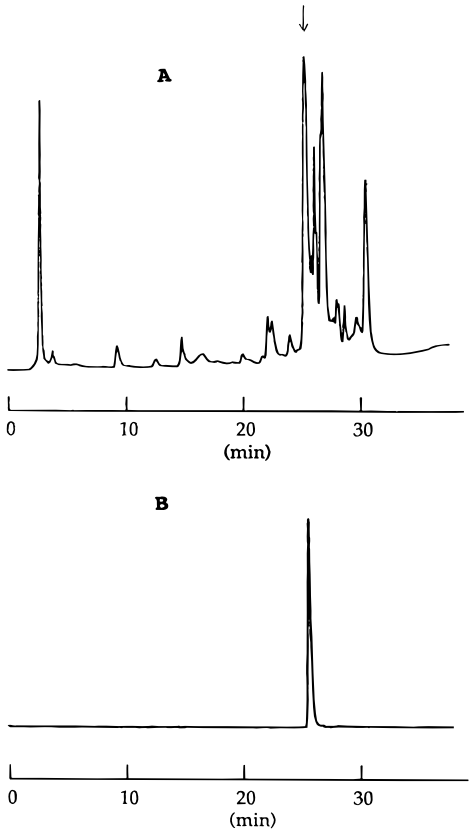


Figure 2. Chromatographic separation of the reaction products (aqueous layer) of butylamine, hexanal, and hydrogen peroxide. Column: BIOPHASE ODS C-18 (250 × 4.6 mm). Elution: a linear gradient from 10% to 70% CH_3OH over 24 min, then linear gradient from 70% to 99% CH_3OH over 5 min, and the final isocratic run, 99% CH_3OH for 8 min. Other conditions were the same as in Figure 1.

ether or benzene was used to extract the fluorescent compound out of the aqueous solution. Further evaporation reduced the solution volume before the next injection. A slightly yellowish precipitate (6 mg) was obtained in a pure form, indicated by single UV absorbance and fluorescent peaks in the reversed-phase, normal-phase, and cation-exchange HPLC analyses. The isolated compound had fluorescent excitation/emission maxima of 364/430 nm (in methanol). High-resolution electron impact MS data (HREIMS): *m/z* calculated for $C_{21}H_{39}NO_2$ 337.2981, found 337.2991; high-resolution chemical ionization MS data (HRCIMS): *(M + H)/e* calcd for $C_{21}H_{40}NO_2$ 338.3059, found 338.3055. The NMR measurement on this substance was not carried out successfully since the fluorescent compound changed color from slightly yellowish to bright yellow when deuterated chloroform was added, indicating some structural change. Worth mentioning is that the usage of *tert*-butyl hydroperoxide instead of hydrogen peroxide in the reaction system gave a fluorophore with identical excitation and emission characteristics.

Effect of Oxygen on the Reaction of Hexanal with Butylamine. Air was bubbled through 1 mL each of hexanal and butylamine, which were mixed and stirred overnight. A weak, blue fluorescence was observed under the UV light. After oxygen was bubbled through the sample overnight, increased fluorescence was observed. In another experiment, nitrogen was bubbled through hexanal and butylamine, individually, for 15 min before mixing. No fluorescence was observed after an overnight incubation. In another comparative experiment, oxygen was passed through hexanal and butylamine, individually, for 15 min before mixing. Some fluorescence was observed immediately after mixing.

Fluorescence from the Reaction of Hexanal, Hexylamine, and Hydrogen Peroxide (Formation of 1a). Under magnetic stirring, 10 mL hexanal (83 mmol) and 10 mL of 30% hydrogen peroxide solution (88 mmol) were combined in a flask and stirred magnetically for 5 min, before 3.5 mL of hexylamine (26 mmol) was slowly added. A water bath was used to cool the reaction flask. Some greenish-blue fluorescence was observed immediately under the UV light, and much stronger blue fluorescence was seen in approximately 1 h. A 5-mg amount of a slightly yellowish precipitate was isolated by preparative reversed-phase, normal-phase, and cation-exchange HPLC purification. The compound had fluorescent excitation/emission maxima of 364/430 nm in methanol, and 360/426 nm in acetonitrile. FTIR (thin film): 3600−3050 (br, m), 2953 (m), 2924 (s), 2857 (m), 1657 (m), 1543 (w), 1233 (m), 1098 (s), 1063 (vs) , and 733 (w) cm^{-1}; UV max (in CH_3OH): 361 (s), 280 (w) nm; HRCIMS: *(M + H)/e* calcd for $C_{23}H_{44}NO_2$ 366.3372, found 366.3260; ^{1}H-NMR (C_6D_6, 500 MHz): δ 0.81 (t, 3H), 0.83 (t, 3H), 0.87 (t, 3H), 0.97 (t, 3H), 1.10−1.30 (br m, app 16H), 1.33−1.45 (br m, app 4H), 1.50 (m, 1H), 1.68 (m, 2H), 1.79 (dt, 1H, -C*H*H- next to chiral C), ∼2.05−2.25 (m, 3H, allylic H), 2.39 (m, 1H, allylic H), 3.01 (m, 1H, -CH*H*N), 3.16 (m, 1H, -CH*H*N). Chromatographic analysis, repeated immediately after the NMR measurement, showed that the fluorescent compound was still pure.

Fluorescence from the Reaction of Pentanal, Butylamine, and Hydrogen Peroxide (Formation of 1c). The reaction was carried out using the same procedures as described above. This fluorescent compound was found to be unstable. During the purification processes, a small amount of it was finally recovered in aqueous acetonitrile solution. The solution gave excitation/emission maxima of 361/427 nm. FAB-MS indicated a $[M + H]^+$ of 296.

Fluorescence from the Reaction of Peptide Ac-Gly-Lys-OMe with Hexanal and Hydrogen Peroxide. After a 6 h reaction, one major fluorescent compound was isolated following the above-mentioned chromatographic separation steps. However, considerable losses occurred, and only a small quantity of pure material was recovered in aqueous acetonitrile solution. The solution gave excitation/emission maxima of 362/429 nm. FAB-MS indicated an expected $[M + H]^+$ of 524.

Reaction of 2,4-Pentanedione with Butylamine, in the Presence or Absence of Hydrogen Peroxide. 2,4-Pentanedione (1 mL, 9.7 mmol) was added to 0.48 mL of butylamine (4.9 mmol). During overnight storage, a yellowish color, but no fluorescence, was noted. The experiment was repeated, except that 1.1 mL of 30% hydrogen peroxide (9.7 mmol) was added. Many fluorescent compounds (as evidenced by a complex chromatogram) were generated and the overall excitation/emission maxima were around 360/430 nm, when measured in a methanol-diluted sample.

Reaction of 2,3-Butanedione with Butylamine, in the Presence or Absence of Hydrogen Peroxide. 2,3-Butanedione (0.85 mL, 9.7 mmol) was added to 0.48 mL of butylamine (4.9 mmol). Browning quickly developed, and no fluorescence was observed. When the same quantites of reactants were combined in the presence of 1.1 mL of 30% hydrogen peroxide (9.7 mmol), browning happened at a much slower rate and some fluorescence was observed.

Production of Fluorescent Pigments after Storage. Fluorescent products of one reaction system (hexanal/hexylamine/H_2O_2 in 2/1/2 molar ratio) were compared with another one (propanal/methylamine/H_2O_2 in 2/1/2 molar ratio). After overnight stirring, the major fluorescent compound generated in the first system seemed to have higher hydrophobicity, indicated by longer retention time in the reversed-phase chromatographic analysis. These two reaction systems were reanalyzed after 1-month storage. A great number of fluorescent peaks showed up in the chromatographic analyses for both systems. The total fluorescence in both systems was also quite intense. The overall excitation/emission spectral maxima for both systems were measured as 362/435 nm for methanol-diluted samples.

Independent Syntheses. 2-Methyl-[1,3]oxathiolane-2-carboxylic Acid Ethyl Ester (9). To a stirred solution of ethyl pyruvate (12.2 g, 105 mmol) and 2-mercaptoethanol (8.84 mL,

126 mmol) in 50 mL of anhydrous ether was added slowly, over a 10-min period, boron trifluoride etherate (26.9 mL, 105 mmol). After an additional hour of stirring at room temperature, the reaction was complete, as monitored by GC-MS. The solution was washed with 0.1 M $NaHCO_3$ solution (2 × 30 mL) and with 30 mL of saturated NaCl solution, dried over $MgSO_4$, and concentrated. Distillation yielded 17.5 g of compound **9**, 95%. GC-MS analysis indicated a purity of 96%. EIMS: 176 {1, M + H}, 116 {6}, 103 {100, $CH_3C(SCH_2CH_2O)$}, 61 {11, $HSCH_2CH_2$}, 59 {16}; CIMS: 177 {12, M + H}, 116 {100}, 103 {64, $CH_3C(SCH_2CH_2O)$}, 89 {23}, 61 {52, $HSCH_2CH_2$}; ^{1}H-NMR (CD_3Cl, 500 MHz): δ 1.22 (t, 3H, OCH_2CH_3), 1.73 {s, 3H, $CH_3C(SCH_2CH_2O)$}, 3.03 (m, 2H, SCH_2CH_2O), 4.11 (m, 2H, OCH_2CH_3), 4.18 (m, 1H, CH_2CHHO), 4.32 (m, 1H, CH_2CHHO); ^{13}C-NMR (CD_3Cl, 500 MHz): δ 14.0 (OCH_2CH_3), 26.2 (CH_3C), 33.7 (OCH_2CH_2S), 62.1 (OCH_2CH_3), 72.8 (OCH_2CH_2S), 89.1 (*C*COOEt), 172.2 (*C*OOEt).

1-(2-Methyl-[1,3]oxathiolan-2-yl)butane-1,3-dione (7b). 2.6 g of NaH (60% in oil, 66 mmol) was washed three times with dry hexane, and twice with dry THF. Subsequently, another 20 mL of THF was added, followed by 5.3 g of compound **9** (30 mmol). Acetone (4.4 mL, 60 mmol) was added dropwise into the stirred slurry. After additional stirring for 20 min, 20 mL of saturated $NaHCO_3$ was added. THF was removed on a rotary evaporator, and 20 mL of ethyl ether was added for extraction. The organic layer was washed with saturated $NaHCO_3$ (2 × 20 mL) and saturated NaCl solution (20 mL) and dried over $MgSO_4$. After concentration, the residue was distilled under vacuum to yield 4.7 g of compound **7b** (25 mmol, 83%). GC-MS analysis indicated a purity of 98%. EIMS: 189 {2, M + H}, 170 {1}, 129 {3}, 103 {100, $CH_3C(SCH_2CH_2O)$}, 85 {14}, 61 {13, $HSCH_2CH_2$}, 59 {14}; CIMS: 189 {100, M + H}, 171 {20}, 157 {16}, 129 {71}, 111 {47, 129 − H_2O}, 103 {61, $CH_3C(SCH_2CH_2O)$}, 89 {18}, 61 {52, $HSCH_2CH_2$}; ^{1}H-NMR (CD_3Cl, 500 MHz): δ 1.64 {s, 3H, $CH_3C(SCH_2CH_2O)$}, 1.97 (s, 3H, CH_3COH═), 2.95 (m, 1H, $SCHHCH_2O$), 3.02 (m, 1H, $SCHHCH_2O$), 4.05 (m, 1H, CH_2CHHO), 4.21 (m, 1H, SCH_2CHHO), 5.72 (s, 1H, $COCH$═COH); ^{13}C-NMR (CD_3Cl, 500 MHz): δ 24.2, 26.4, 33.8, 72.3, 93.0, 93.9, 189.4, 196.5.

2,3,5-Hexanetrione (6b). A solution of 1.0 g of compound **7b** (5.3 mmol) in aqueous 80% acetonitrile (20 mL) was added at room temperature to a vigorously stirred mixture of mercuric chloride (3.18 g, 11.7 mmol) and mercuric oxide (1.26 g, 5.8 mmol), in the same solvent (100 mL). The mixture was stirred overnight under nitrogen. The organic phase was decanted. The residue was washed with CH_2Cl_2/ether (1/1, 2 × 60 mL). The organic phases were combined, washed with saturated $NaHCO_3$ solution (2 × 60 mL), and dried over anhydrous Na_2SO_4. After removal of the solvents, a yellow oil remained along with some white precipitate. Acetonitrile (10 mL) was added and a clear solution obtained after filtration through a glass wool-fritted funnel. The yellow filtrate was dried over nitrogen flow. Finally, 0.57 g (4.5 mmol) of yellow compound **6b** was obtained in a yield of 84%. GC-MS analysis indicated purity of 96%. CIMS: 129 {7, M + H}, 111 {3, 129 − H_2O}, 99 {2}, 85 {100, $COCH_2COCH_3$}, 72 {9}, 69 {6}, 58 {4}; ^{1}H-NMR (DMSO-d_6, 500 MHz): δ 1.34 (s, 3H, ═$COHCH_3$), 2.19 (s, 3H, CH_3CO-), 5.42 (s, 1H, -CH═COH-); ^{1}H-NMR (CD_3CN, 500 MHz): 1st tautomer δ 1.41 (s, 3H, ═$COHCH_3$), 2.20 (s, 3H, CH_3CO), 5.33 (s, 1H,-CH═COH-), 2nd tautomer δ 2.21 (s, 3H, -$COCH_3$), 2.35 (s, 3H, -$COCH_3$); ^{13}C-NMR (DMSO-d_6, 500 MHz): δ 17.5 (CH_3CO-), 22.2 (CH_3COH═), 100.6 (-CH═COH-), 105.0 (-COH═), 188.7 (-CO-CH═), 201.6 (CH_3CO-).

2-Butyl-(2-methyl-[1,3]oxathiolan-2-yl)octane-1,3-dione (7c). NaH (0.13 g; 60% in oil, 3.3 mmol) was washed 3 times with dry hexane, and twice with THF. Another 5.0 mL of THF was added, followed by 0.53 g of compound **9** (3.0 mmol). 6-Undecanone (0.67 g, 4.0 mmol) was added dropwise into the resulting stirred slurry. After additional stirring for 20 min, 10 mL of saturated $NaHCO_3$ solution was added and the solution partially concentrated. Ethyl ether (10 mL) was added and the organic layer washed with saturated $NaHCO_3$ solution (2 × 10 mL) and saturated NaCl solution (10 mL), and dried over $MgSO_4$. GC-MS analysis indicated 79% conversion of **9** to **7c**. After concentration, the residue was purified through preparative reversed-phase HPLC, extraction and drying processes, to yield 0.54 g of compound **7c** (1.8 mmol, 64%). CIMS: 301 {6, M + H}, 283 {1, 301 − H_2O}, 223 {4}, 103 {100, $CH_3C(SCH_2CH_2O)$}, 61 {2, $HSCH_2CH_2$}.

4-Butyl-2,3,5-decanetrione (6c). Ten milliliters of 20% hydrochloric acid was added to 0.54 g of compound **7c**, and the mixture was stirred for 1 h. The ether-soluble portion was extracted, and after evaporation of the solvent, dilution with 1 mL of acetonitrile, and purification using preparative reversed-phase HPLC, 0.26 g (1.1 mmol, 61%) of compound **6c** was obtained. GC-MS analysis indicated purity of 88%. EIMS: 241 {4, M + H}, 223 {2, 241 − H_2O}, 197 {100, M − (CH_3CO)}, 99 {50, $COCH_2(CH_2)_3CH_3$}, 71 {30, CH_3COCO}; ^{1}H-NMR (CD_3Cl, 500 MHz): δ 0.86 (t, 3H), 0.88 (t, 3H), 1.2−1.4 (br m, 8H), 1.48 (s, 3H, CH_3CO), 1.62 (m, 2H), 2.05 (t, 2H), 2.47 (t, 2H); ^{13}C-NMR (DMSO-d_6, 500 MHz): δ 14.0 ($2CH_3$-), 21.3, 22.5, 22.6, 22.8, 26.0, 28.8, 31.5, 31.6, 103.0 (*C*═COH-), 112.2 (-*C*OH═), 187.2 (-*C*OC═), 203.4 (CH_3CO-).

Ethyl 2-Oxohexanoate (10). Oxalyl chloride (4.14 mL, 47.4 mmol) in 50 mL of methylene chloride in a 500 mL 3-neck flask was cooled in a dry ice/acetone bath; 8.06 g of DMSO (103.4 mmol) in 20 mL of CH_2Cl_2 was added over 5 min. Ten minutes later, 4.4 g (27.5 mmol) of ethyl-2-hydroxyhexanoate in 20 mL of CH_2Cl_2 was added over a 5 min period. The solution was allowed to warm to −60 °C and stirred for 15 min before 30 mL of triethylamine (215.2 mmol) was added. The solution was allowed to warm to room temperature. Water (60 mL) was added and the mixture was stirred for 10 min. The organic layer was separated and washed with 3 M HCl, excess water, and saturated $NaHCO_3$ solution. Concentration yielded compound **10** quantitatively (4.40 g, 101%). GC-MS analysis gave a purity of 94%. CIMS: 159 {7, M + H}, 85 {100, $COCH_2CH_2CH_2CH_3$}, 57 {3, $(CH_2)_3CH_3$}; ^{1}H-NMR (CD_3Cl, 500 MHz): δ 0.93 (t, 3H, $CH_3CH_2CH_2$-), 1.34 (m, 2H, $CH_3CH_3CH_2$-), 1.38 (t, 3H, CH_3CH_2O-), 1.62 (m, 2H, $CH_3CH_2CH_2$-), 2.84 (t, 2H, -CH_2CO-), 4.32 (q, 2H, CH_3CH_2O-); ^{13}C-NMR (CD_3Cl, 500 MHz): δ 14.0, 22.2, 25.2, 39.0, 62.3, 70.6, 161.5 (*C*OOEt), 194.7 (*C*OCOOEt).

2-Butyl-[1,3]dithiolane-2-carboxylic Acid Ethyl Ester (8). To a stirred solution of compound **10** (4.40 g, 27.8 mmol) and 1,2-ethanedithiol (4.20 mL, 50.0 mmol) in 20 mL of anhydrous ether was added slowly, over 20 min, boron trifluoride−etherate (12.8 mL, 50 mmol). After overnight stirring at room temperature, the solution was washed with saturated $NaHCO_3$ solution (2 × 20 mL) and once with 20 mL NaCl solution and dried over $MgSO_4$. After concentration, 10 mL of ethanol and 1.9 g (10 mmol) of *p*-toluenesulfonic acid were added and the mixture was stirred for 24 h at room temperature. Ethanol was removed under vacuum, and the residue taken up in ether was washed with saturated Na_2CO_3 solution and once with 20 mL of saturated NaCl solution and dried over $MgSO_4$. Ether was removed, and 6.7 g (28.6 mmol) of compound **8** was quantitatively obtained. CIMS 235 {4, M + H}, 161 {100, $CH_3(CH_2)_3C(SCH_2CH_2S)$}, 89 {16}.

2-Butyl-[1,3]dithiolane-2-carboxylic Acid (11). Compound **8** (1.94 g, 8.29 mmol) and $LiOH·H_2O$ (0.870 g, 20.7 mmol) were added into 20 mL of water. The solution was heated under reflux for 1 h, cooled, and washed with ethyl ether (2 × 20 mL). The pH of the aqueous layer was adjusted to 1−2 with 10% HCl. Ethyl ether (2 × 20 mL) was used for extraction. The solution was dried over $MgSO_4$, and the solvent was removed under vacuum. Product **11** (0.74 g, 3.6 mmol, 43%) was obtained. HRCIMS: *(M + H)/z* calcd for $C_8H_{15}O_2S_2$ 207.0513, found 207.0518. ^{1}H-NMR (CD_3Cl, 500 MHz): δ 0.81 (t, 3H), 1.25 (m, 2H), 1.36 (m, 2H), 1.61 (t, 2H), 3.29 (m, 2H), 3.35 (m, 2H).

2-Butyl-[1,3]dithiolane-2-carbonyl Chloride (12). Compound **11** (0.50 g, 2.4 mmol) was added to 5 mL of benzene, followed by 1.44 g (0.89 mL, 12.1 mmol) of thionyl chloride. The solution was stirred 0.5 h before removal of benzene and the excess of thionyl chloride on a water aspirator; the product was immediately used for the following reaction (see Scheme 4). EIMS: 226 {22, M + H}, 224 {48, M}, 161 {100, $CH_3(CH_2)_3$-

$C(SCH_2CH_2S)$}, 105 {81}.

2-Butyl-1-(2-butyl-[1,3]dithiolane-2-yl)octane-1,3-dione (7a). A mixture of 1.4 mL (2.80 mmol) of 2 M lithium diisopropylamide solution and 0.476 g (2.80 mmol) of 6-undecanone in 50 mL of dry benzene was stirred under nitrogen for 30 min at 0 °C. To this solution was added slowly, over 10 min, compound **12** (0.54 g, 2.43 mmol). After an additional hour of stirring at 0 °C, the solution was washed with saturated $NaHCO_3$ solution (2 × 30 mL) and once with 30 mL of saturated NaCl solution, and dried over $MgSO_4$. Removal of benzene under vacuum followed by preparative HPLC purification yielded 0.75 g compound **7a** (86%). EIMS: 359 {60, M + H}, 266 {97}, 244 {71}, 161 {100, $CH_3(CH_2)_3C(SCH_2CH_2S)$}, 105 {22}, 99 {29, $CO(CH_2)_4CH_3$}; ^{1}H-NMR (CD_3Cl, 500 MHz): δ 0.90 (br m, 9H), ∼1.2−1.5 (br m, app 12H), 1.60 (br m, 2H), 1.9 (br m, 2H), 2.2 (br m, 2H), 2.60 (br m, 2H), ∼3.3−3.5 (br m, 4H); ^{13}C-NMR (CD_3Cl, 500 MHz), downfield only: δ 128.3, 128.5, 200.3, 212.0, 214.5.

7-Butyl-5,6,8-tridecanetrione (6a). To a stirred solution of 3.0 g (16.8 mmol) of *N*-bromosuccinimide in 20 mL of 95% acetone was added 0.75 g (2.1 mmol) of compound **7a** in 20 mL of acetone. The solution was stirred 1 h and shaken with a mixture of saturated aqueous sodium sulfite and dichloromethane. The organic phase was washed with saturated aqueous $NaHCO_3$, water, and brine and dried ($MgSO_4$). Removal of solvent followed by pumping, followed by preparative HPLC, yielded 0.50 g of compound **6a** (1.8 mmol, 86%). FTIR (thin film): 3600−3100 (br, m), 2957 (vs), 2931 (vs), 2868 (s), 1707 (s), 1616 (m), 1462 (s), 1379 (s), 1047 (m) , and 731 (w) cm^{-1}; EIMS: 283 {3, M + H}, 265 {2, 283 − H_2O}, 197 {100, 283 − 85}, 99 {77, $CO(CH_2)_4CH_3$}, 85 {44, $CH_3(CH_2)_3CO$}, 71 {27, $CH_3(CH_2)_4$}, 57 {48, $CH_3(CH_2)_3$}; CIMS: 311 {7, M+29}, 283 {93, M + H}, 265 {100, 283 − H_2O}, 197 {8, (283 − 85)}, 185 {52}, 99 {44, $CO(CH_2)_4CH_3$}, 85 {4, $CH_3(CH_2)_3CO$}; ^{1}H-NMR (C_6D_6, 500 MHz): δ 0.79 (t, 3H), 0.83 (t, 3H), 0.90 (t, 3H), ∼1.05−1.25 (br m, appr 8H), 1.30 (br m, 2H), 1.52 (br m, 4H), 1.97 (br m, 2H), 2.11 (br m, 2H), 2.19 (t, 2H); ^{13}C-NMR (C_6D_6, 500 MHz): 14.0 (m), 21.3, 22.6, 22.8, 23.0, 25.1, 26.1, 28.8, 31.5, 31.6, 36.1, 104.0 (*C*═COH-), 113.2 (-*C*OH═), 187.0 (-*C*OC═), 202.9 (-CH_2-*C*OCO-).

2,4-Dibutyl-1-hexyl-2-hydroxy-5-pentyl-1,2-dihydropyrrol-3-one (1a). Compound **6a** (50 mg, 0.18 mmol) was added to a solution of 18 mg (0.18 mmol) hexylamine in 5 mL of acetonitrile. The solution was allowed to stand overnight, during which time strong fluorescence was developed. A chromatographic separation yielded a single fluorescent component. Seven milligrams of slightly yellowish solid (**1a**) was obtained after purification (yield, 11%). Excitation/emission spectral maxima of the purified fluorescent compound were 364/430 nm in methanol, and 360/426 nm in acetonitrile. FTIR (thin film): 3600−3050 (br, m), 2955 (s), 2925 (s), 2856 (m), 1660 (m), 1542 (w), 1253 (m), 1093 (vs), 1105 (vs) , and 732 (w) cm^{-1}; UV max (CH_3OH): 361 (s), 280 (w) nm; FAB-MS: 366 (M + H); HRCIMS: *(M + H)/e* calcd for $C_{23}H_{44}NO_2$ 366.3372, found 366.3372; ^{1}H-NMR (C_6D_6, 500 MHz): δ 0.81 (t, 3H), 0.83 (t, 3H), 0.87 (t, 3H), 0.97 (t, 3H), 1.10−1.30 (br m, app 16H), 1.41 (m, app 4H), 1.52 (m, 1H), 1.66 (m, app 2H), 1.77 (dt, 1H, -C*H*H- next to chiral C), 2.05 (dt, 1H, allylic H), ∼2.10−2.26 (m, 2H, allylic H), 2.39 (m, 1H, allylic H), 3.00 (m, 1H, -C*H*HN), 3.15 (m, 1H, -CH*H*N).

2,4-Dibutyl-1-hexyl-5-pentyl-2-[(trimethylsilanyl)oxy]-1,2-dihydropyrrol-3-one (13). Compound **1a** (1.5 mg) was dissolved in 0.5 mL of dry benzene, before addition of two drops of *N*-(trimethylsilyl)imidazole. The mixture was stirred at room temperature for 1 h. Benzene was removed by aspirator. Reversed-phase HPLC was then performed to collect the major fluorescent fraction (with a longer retention time than observed for compound **1a**). The collected solution was partially concentrated at room temperature before addition of 2 mL of ethyl ether for extraction. The organic layer was dried over $MgSO_4$. After concentration, the slightly yellowish solid (compound **13**) was used for spectroscopic measurements. FTIR (thin film): 3600−3150 (br, m), 2957 (s), 2928 (s), 2859 (m), 1714 (w), 1649 (m), 1555 (w), 1460 (w), 1094 (s), 1020 (w), 882 (w), 845 (w), and 799 (w) cm^{-1}; UV max (CH_3OH): 360 (s), 278 (w) nm; HRCIMS: *(M + H)/e* calcd for $C_{26}H_{52}NO_2Si$ 438.3767, found 438.3684; ^{1}H-NMR (C_6D_6, 500 MHz): δ 0.39 (s, 9H), 0.82 (t, 3H), 0.84 (t, 3H), 0.88 (t, 3H), 0.96 (t, 3H), 1.14−1.35 (br m, app 16H), 1.42 (m, app 4H), 1.54 (m, 1H), 1.70 (m, app 2H), 1.81 (dt, 1H, -C*H*H- next to chiral C), 2.19 (m, 2H, allytic H), 2.29 (m, 2H, allylic H), 3.04 (m, 1H, -C*H*HN), 3.17 (m, 1H, -CH*H*N).

4-Butyl-2-butylidene-1-hexyl-5-pentyl-1,2-dihydropyrrol-3-one (14). Compound **13** was found to turn yellow in deuterated chloroform. The yellow compound was collected during a reversed-phase chromatographic separation. The retention time of this yellow compound was shorter than that of the precursor **13**, but longer than that of compound **1a**. The collected solution was partially concentrated at room temperature before addition of ethyl ether for extraction. A small amount of yellow liquid (**14**) was recovered after drying over $MgSO_4$ and removal of ether. FTIR (thin film): 3500−3200 (br, w), 2957 (vs), 2928 (vs), 2861 (s), 1726 (m), 1665 (m), 1574 (m), 1462 (m), 1261 (w), and 1022 (w) nm^{-1}; UV max (CH_3OH): 265 (w), 220 (s) nm; HRCIMS: *(M + H)/e* calcd for $C_{23}H_{42}NO$ 348.3267, found 348.3269; ^{1}H-NMR (C_6D_6, 500 MHz): δ ∼0.82−1.00 (m, app 12H), ∼1.02−1.72 (m, app 22H), 2.21 (m, 2H, allylic H), 2.42 (m, 2H, allylic H), 3.09 (m, 1H, -C*H*HN), 3.18 (m, 1H, -CH*H*N), 5.36 (t, 1H, vinylic H).

Acetylation of 1a. Compound **1a** (4 mg) was added to a solution of 4 drops of acetic anhydride in 0.5 mL of benzene. After overnight stirring, the original fluorescence was lost. Reversed-phase chromatographic separation was carried out to isolate the major product whose retention time was longer than observed for its precusor **1a**. After removal of acetonitrile (mobile-phase component), this product was extracted into ethyl ether. The organic layer was dried over $MgSO_4$, and the ether was evaporated through an aspirator. The residue was dissolved in deuterated benzene for NMR and MS measurements. HRCIMS: only *(M − H_2O)/e* was found (389.3289), while calculated for $C_{25}H_{43}NO_2$ ($C_{25}H_{45}NO_3$ − H_2O) is 389.3294; ^{1}H-NMR (C_6D_6, 500 MHz): δ ∼0.81−0.97 (br m, app 12H), ∼1.05−1.40 (br m, app 14H), 1.46 (m, app 4H), 1.59 (m, app 4H), 1.77 (t, 2H), 1.95 (s, 3H), 2.16 (t, 2H), 2.51 (t, 2H), 2.70 (t, 2H). Structure **15** is suggested as consistent with the foregoing data.

Results and Discussion

Initial Observations. Various reactions between biological carbonyls and primary amines are generally characterized by formation of high-molecular-weight species which can further react with biomolecules and deposit on various biological entities (e.g., long-lived tissues and postmitotic cells *(5)*). These processes are usually associated with coloration. Development of the fluorescent lipofuscin pigments, which is considered to be a characteristic manifestation of aging, thus appears to be either competitive with or parallel to the above processes. In order to elucidate the chemistry behind lipofuscin formation, we had to first simplify our reaction system.

The first orientation experiments involved treatment of *trans*-2-hexenal, 4-hydroxy-2-nonenal, and malondialdehyde with the amino acids glycine and lysine and several small peptides. To obtain fluorescence with excitation maxima in the range of 345−375 nm (emission at 415−445 nm), the presence of H_2O_2, or a peroxide such as *tert*-butyl hydroperoxide, or at least exposure of the solution to air was essential. Attempts to separate chromatographically the constituents of these mixtures invariably resulted in complex substance profiles (viewed by UV absorbance and fluorescence detection). Fluorescent compounds formed from amino acids and small peptides were generally hard to extract into organic solvents. This complicated the isolation and character-

ization studies, since during the time of lyophilization, the samples gradually changed their composition (often in favor of coloration and loss of fluorescence). The much less polar aliphatic primary amines produced the same type of fluorophore, but being soluble in organic solvents, they could be more easily isolated chromatographically.

A clear case for different fluorophore formation under different conditions is the reaction between malondialdehyde and glycine or an aliphatic amine: with hydrogen peroxide present, characteristic fluorescence with excitation in a range of 350−370 nm readily appeared, but without peroxide, a higher-wavelength fluorophore (presumably a dihydropyridine derivative, as seen by Kikugawa and co-workers *(8, 15)*) was preferentially formed. It should be emphasized that our primary goal has been to identify the lower-wavelength fluorophore, the formation of which is shared with much larger molecules containing primary amino groups (human serum albumin, carbonic anhydrase, lysozyme, and cytochrome *c*) studied in our laboratory.[1]

Chromatographic Isolation and Spectroscopic Characterization. An example of how ubiquitous the fluorophore of our interest can be in even the simplest reaction mixture is seen in Figure 1 (reaction of *trans*-2-hexenal and butylamine with *tert*-butyl hydroperoxide present). The chromatogram obtained with a reversed-phase column shows the complexity of the general colored products (A: UV detection) and distribution of the fluorophore (B: fluorescence detection), indicating a daunting task of isolation and characterization. Fortunately, we observed that linear saturated aldehydes and primary amines can form identical fluorophores with considerably less product complexity. Figure 2 shows the chromatograms corresponding to the reaction products of hexanal with butylamine in the presence of hydrogen peroxide (after overnight treatment at room temperature). Among several UV-absorbing components (A), a single fluorescent peak is observed (B).

Purification of the fluorescent component seen in Figure 2B still had to involve additional chromatographic steps (results not shown): rechromatographing on a cation-exchange column and in a straight-phase separation system. In the early stage of reaction, the fluorescent fraction represented only a minor mixture component. The fluorescent compound was sensitive to heat and certain solvents. For example, solvent evaporation at about 30 °C during purification resulted in a loss of fluorescence and occurrence of extra UV absorbance peaks upon rechromatographing. The use of deuterated chloroform during NMR also caused a change in solution color and a drastic decrease of fluorescence. However, deuterated benzene was found to be an appropriate solvent for the NMR studies. High-resolution mass spectrometry indicated a formula $C_{21}H_{39}NO_2$ for the isolate.

A more stable fluorescent analog was subsequently formed using hexanal, hydrogen peroxide and hexylamine. The chromatographically isolated compound (results not shown, but separations similar to Figure 2) was subjected to a series of spectroscopic techniques. The fluorescence spectrum of this compound is shown in Figure 3. Analysis by Fourier-transform IR spectroscopy was indicative of C−N stretching at 1000−1200 cm^{-1}, C═C and C═O stretching at 1500−1700 cm^{-1}, C−H stretching at 2800−3000 cm^{-1}, and O−H and N−H at the 3000−3500 cm^{-1} region. The proton NMR result was particularly beneficial to the postulation of a structure in showing that any doubly-bonded carbons must be free of proton substituents. Four methyl groups could also be inferred through peak integration. The complexity of the proton signals between 1.1 and 3.2 ppm indicates possible chirality and/or tautomerism. High-resolution mass spectrometry indicates a molecular formula of $C_{23}H_{43}NO_2$, with a molecular weight of 365.

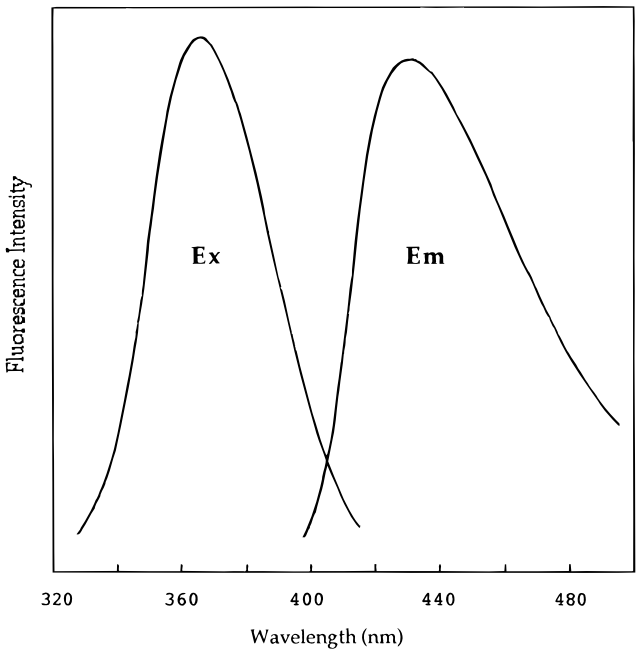


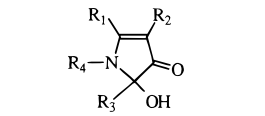
Figure 3. Excitation and emission spectra of the fluorescent compound isolated from the reaction products of hexanal, hexylamine, and hydrogen peroxide. Excitation was measured at 430 nm emission, while emission was measured at 364 nm excitation. Sample: 0.5 mg in 3 mL of methanol.

A comparison of the two molecular formulas mentioned above revealed that the second compound must have been produced by a combination of three hexanal molecules and one hexylamine molecule, with an inexplicable loss of one carbon atom. Further support for this conclusion can be found in yet another experiment where pentanal, butylamine, and hydrogen peroxide yielded a product whose $[M + H]^+$ is 296; the corresponding molecular formula of $C_{18}H_{33}NO_2$ (MW = 295) has three fewer CH_2 groups than $C_{21}H_{39}NO_2$ (MW = 337), generated from hexanal instead of pentanal. Furthermore, all three molecular formulas show that the compounds must have three units of unsaturation (such as two double bonds and one ring).

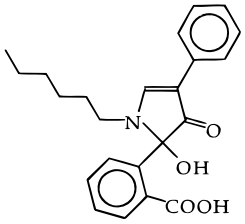
Structure **1a** (Chart 1) is proposed to be that of the fluorescent compound arising from the reaction of hexanal, hydrogen peroxide, and hexylamine. The cyclic and chiral nature of this compound was evidenced by its proton NMR spectrum which shows an AB pattern for the two methylene protons (3.01 and 3.16 ppm) next to the nitrogen atom. We could also observe multiple peaks for the four allylic protons and a doublet of triplets for one of the methylene protons (1.79 ppm) next to the chiral carbon (the other proton signal overlapped with adjacent peaks). Correspondingly, structures **1b** and **1c** have been assigned to the other fluorescent compounds mentioned above. The labile nature of such type of structure (**1c**, for example) can be attributed to its carbinolamine moieties, since tautomeric equilibrium between it and the

[1] Zidek, L., Chmelík, J., Baker, A., and Novotny, M. Modification of horse heart cytochrome *c* with *trans*-2-hexenal.

Chart 1



1a: R_1 = n-C_5H_{11}, R_2 = n-C_4H_9
R3 = n-C_4H_9, R_4 = n-C_6H_{13}
1b: R_1 = n-C_5H_{11}, R_2 = n-C_4H_9
R3 = n-C_4H_9, R_4 = n-C_4H_9
1c: R_1 = n-C_4H_9, R_2 = n-C_3H_7
R3 = n-C_3H_7, R_4 = n-C_4H_9



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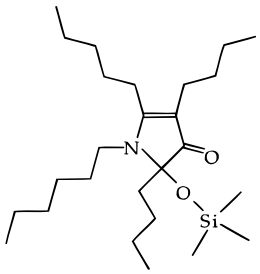

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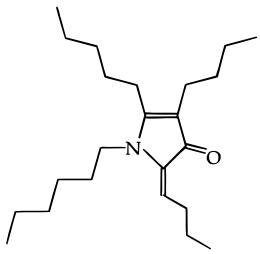

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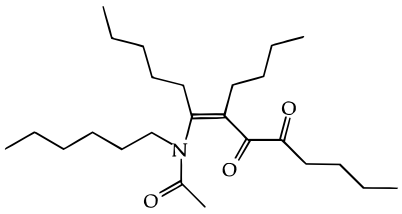
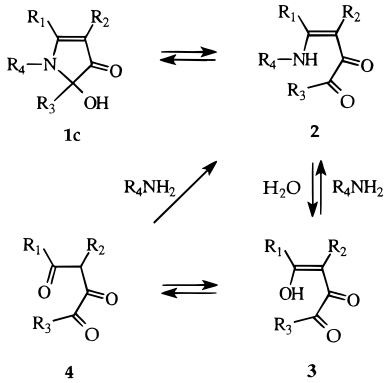


15

Scheme 1



1c **2** **4** **3**

enamino ketone structure (Scheme 1, **2**) is expected *(16)*. **2**, in turn, should undergo easy hydrolysis to triketone enol form **3**, which is tautomeric with triketone **4**. Neither **2**, **3**, nor **4** is expected to be fluorescent.

We have noticed that the smaller fluorescent molecules, such as **1c**, obtained from pentanal, butylamine, and hydrogen peroxide, are less stable than structure **1a**. It is conceivable that longer aliphatic chains contribute to the overall stability of the fluorophore through shielding the core structure from environmental influences.

It is obvious that fluorescence becomes stronger during storage of the isolated compounds in reaction flasks. In a month, many additional fluorescent compounds were generated, as evidenced by significantly enriched chromatographic profiles. Yet, it can be assumed from the unchanged spectral curves and the excitation/emission maxima around 360/430 nm that the additionally formed fluorescent substances carry the same fluorophore. The more structurally complicated products are likely to involve further oxidative modification, oligomerization and, possibly, cross-linking between the oligomeric molecules.

Table 1. Solvation Behavior of Compounds 1a and 5[a]

	max ex/em wavelength (nm)		rel ex spectral intensity[b]	
solvent	compd **1a**	compd **5**	compd **1a**	compd **5**
methanol	364/430	390/470	100	100
1-propanol	360/430		61	
dimethyl sulfoxide	361/426		42	
water/acetonitrile (1:1)	360/426		40	
hexane	361/428	388/470	32	25
acetonitrile	360/426	388/470	25	9
benzene	356/422	389/470	12	10
methylene chloride	360/428	391/474	9	13
chloroform	360/428	392/476	5	19

[a] Solute concentration: 0.12 mM. [b] Maximum intensity is normalized to 100% in each individual group; no comparison between two compounds was made.

Peptide Ac-Gly-Lys-OMe was combined with hexanal and hydrogen peroxide. The fluorescent compound generated gave an excitation maximum of 362 nm and an maximum emission at 429 nm in aqueous acetonitrile solution. This result indicates that primary amino groups can be expected to react in the manner described even when incorporated into larger substrate molecules.

Interestingly, structures **1a**−**c** strongly resemble the core structure of a seemingly unrelated molecule—the reaction product of fluorescamine (4-phenylspiro[furan-2(3H),1′-phthalan]-3,3′-dione) *(17*−*19)* with a primary amine.

Comparison with Fluorescamine Derivatives. Fluorescamine is a widely used reagent for determination of primary amines in various aspects of protein biochemistry *(17, 20)*. The reaction product of fluorescamine with hexylamine, given as **5**, is shown for the sake of reference. The phenyl group of the fluorescamine derivative is conjugated with the double bonds of the core structure, causing the derivative to have fluorescence excitation and emission maxima (390/470 nm) at higher wavelength than the isolated compounds (**1a**−**c**).

To compare further the fluorescamine derivatives with substituted 2-hydroxy-1,2-dihydropyrrol-3-ones generated in this work, we have studied the dependence of fluorescence maxima for structure **1a** and the fluorescamine derivative **5** on pH and the solvent environment. The pH dependencies are shown in Figure 4. The same trend has been found with both compounds: fluorescence increases gradually from the acidic (pH 2.3) to basic (pH 10.7) buffer, but the extremes of pH result in its loss. A similar observation for fluorescamine derivatives can be found in the literature *(20)*. Additional solvation properties of the compounds are compared in Table 1. Once again, similarities are observed. Higher fluorescence signals are favored in polar protic solvents such as methanol and water.

Triketone Precursors, Their Synthesis and Reaction. It is proposed that the precursor to a fluorophore such as **1a** is a 1,2,4-triketone such as **6a**. How such a triketone might be formed from hexanal is suggested in Scheme 2. Aldol condensation can impose oxygenated functionality upon alternate carbon sites, but for oxygen atoms to appear at adjacent sites, a different sort of reaction is necessary. Peroxides are known, through the Baeyer−Villiger oxidation mechanism, to effect oxidative cleavage of the carbon−carbon bond adjacent to a carbonyl group; if, at an appropriate point in the sequence,

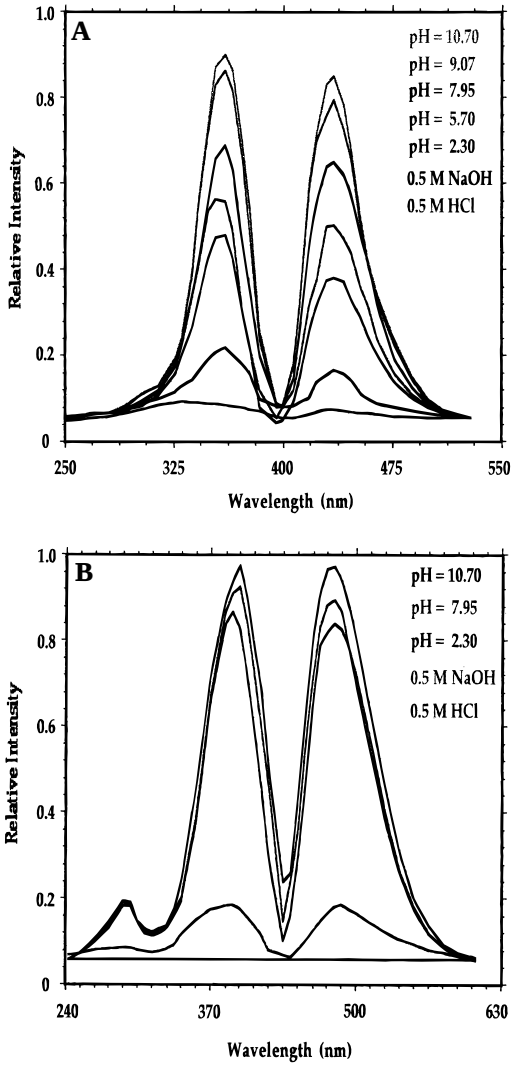


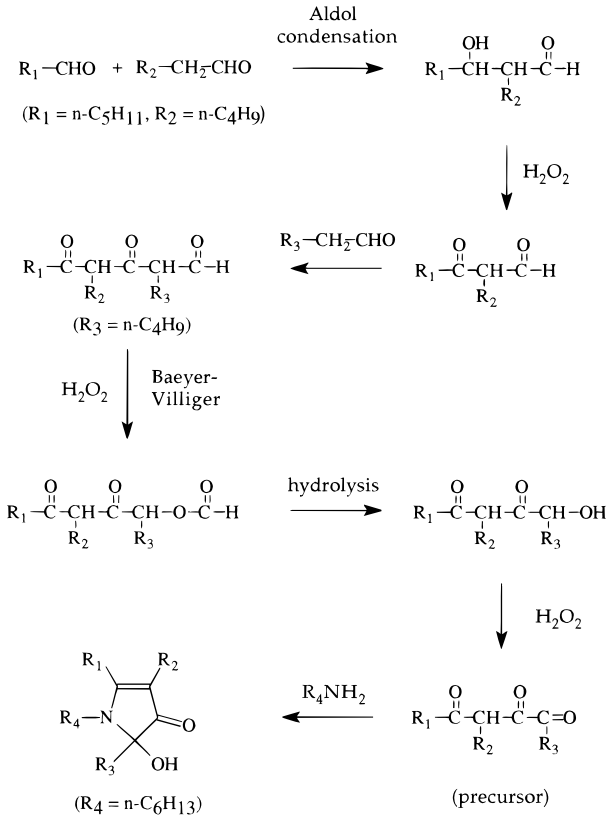
Figure 4. (A) Spectrofluorometric behavior of compound **1a** at different pH values. The buffer solutions were prepared from methanol and aqueous buffer solution (1:1), while the pH values of the mixed solutions were remeasured. The pH values at which the curves were measured are indicated in order in the legend. (B) Spectroscopic behavior of compound **5** under identical conditions.

an aldehyde group is thus converted to a formate ester group and the ester hydrolyzed, the necessary 1,2 oxygenated functionality is established, while at the same time the puzzling disappearance of the one carbon atom is accounted for.

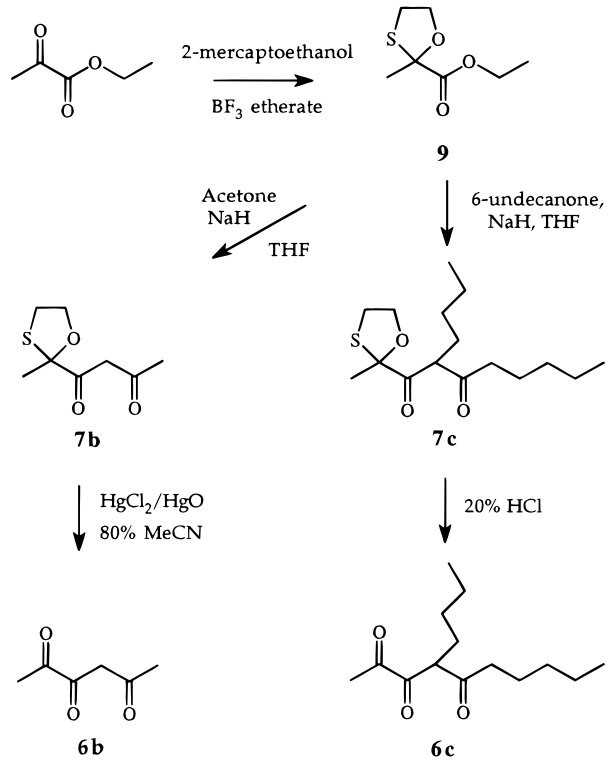
Ketone **6a**, believed to be the precursor of our most thoroughly characterized fluorophore, was selected as the primary target for synthesis. Because synthesis of **6a** was perceived as requiring some effort, known 2,3,5-hexanetrione *(21)* (**6b**) was also synthesized for the purpose of providing preliminary information (Scheme 3). Trione **6c** was synthesized in like manner.

A Claisen condensation of a suitable derivative of a 2-oxoalkanoic acid with a symmetrical ketone was regarded as the best route to **6a**−**c**. Stetter and von Praun *(21)* have indeed employed the Claisen condensation of the ethylene ketal of ethyl pyruvate with acetone in the synthesis of **6b**. We found oxathiolane **7b** (Scheme 3) to be more easily prepared, from the action of 2-ethanethiol on ethyl pyruvate, than the ethylene ketal, so the Stetter−von Praun synthesis was modified accordingly; deprotection using mercuric chloride and mercuric oxide completed our synthesis of **6b**. Synthesis of **6c** was

Scheme 2



Scheme 3



achieved similarly, 6-undecanone being substituted for acetone, except that the final deprotection was achieved using 20% hydrochloric acid after $HgCl_2$/HgO proved unsatisfactory.

During the synthesis of **6a** (Scheme 4), we found that dithiolane **8** was more easily prepared than oxathiolane. In agreement with Stetter's observation *(21)*, we found that carbonyl-protected ethyl 2-oxohexanoate **8** was much

Scheme 4

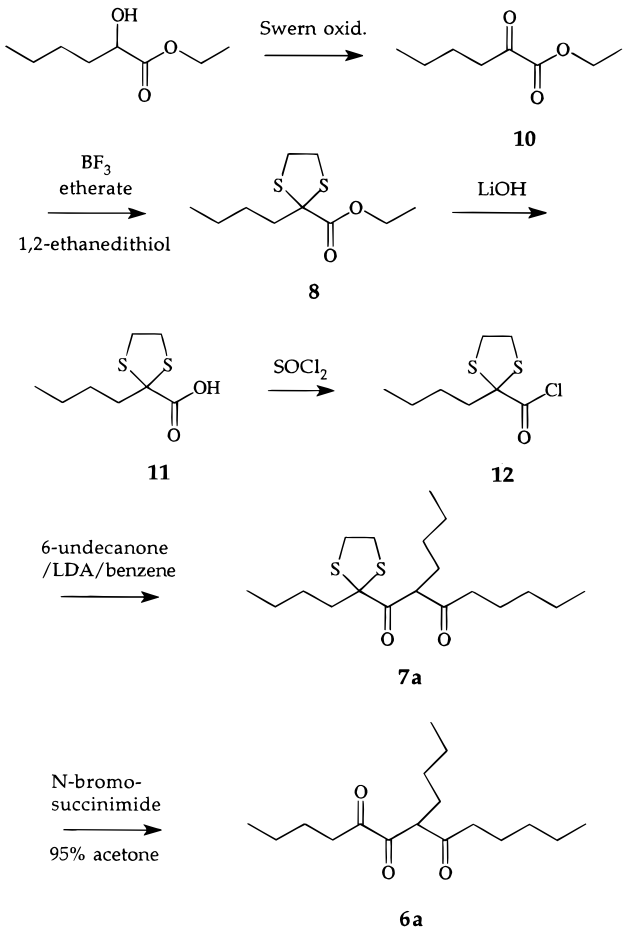


Table 2. Fluorescence Spectral Excitation/Emission Maxima[a]

	ex/em (nm)	
triketone	reaction product with butylamine	reaction product with glycine methyl ester
6a	364/432	
6b	362/432	355/420
6c	347/415	354/410

[a] All data measured in methanol.

less reactive in the Claisen condensation than pyruvate-derived **9**. It was necessary to use an acid chloride rather than an ester to effect a satisfactory condensation with 6-undecanone. Ethyl 2-oxohexanoate **10**, obtained from ethyl 2-hydroxyhexanoate by a Swern oxidation, was converted to the corresponding dithiolane **8**. Saponification followed by treatment with thionyl chloride yielded acid chloride **12** which was converted to **7a** by acylation of the lithium enolate of 6-undecanone. It was found that neither $HgCl_2$/HgO nor HCl could deprotect **7a** satisfactorily. Fortunately, deprotection of **7a** to **6a** was accomplished by *N*-bromosuccinimide *(22)*.

Triketones **6a**−**c**, upon treatment with butylamine or glycine, produced fluorescent solutions. Spectral maxima observed for these solutions compared favorably with those originating from aldehydes, primary amines, and hydrogen peroxide (Table 2). Finally, from a solution made by combining triketone **6a** with hexylamine was isolated a fluorescent compound identical in NMR, FTIR, fluorescence spectra, and mass spectra to the previously isolated **1a**.

Compound **1a** was silylated in benzene by (trimethylsilyl)imidazole. As expected, the product showed a higher hydrophobicity, as indicated by a longer retention time in our reversed-phase HPLC purification process. The assigned structure **13** is supported by FTIR spectroscopy, proton NMR, and high-resolution mass spectrometry. The silyl derivative **13** was also found to be fluorescent, featuring essentially the same UV absorbance and fluorescence maxima as its precursor. Desilylation of **13** was observed after overnight storage in deuterated chloroform, accompanied by a drastic color change from almost colorless to bright yellow. Structure **14** is assigned to the isolated yellow liquid based on its spectroscopic data. Compound **14** showed, as expected, less hydrophobicity than its precursor **13**, but greater hydrophobicity than structure **1a**, as indicated by their relative retention times during the reversed-phase HPLC separation. Interestingly, no distinct fluorescence was observed for structure **14**. Acylation with acetic anhydride was also carried out for structure **1a**. As a result, fluorescence was lost. Since acylation on the hydroxy group is expected to result in retention of fluorescence, the actual derivatization likely took place on the nitrogen atom of the open form of **1a** to yield structure **15** which is not expected to be fluorescent. The breakdown of the cyclic structure is further evidenced by our proton NMR measurement on structure **15**, which indicates the presence of several triplets, instead of the AB patterns and multiple signals previously observed in the cases of **1a** and **14**.

Two model reactions involving diketones (2,4-pentanedione and 2,3-butanedione) with butylamine were also investigated with regard to fluorescence formation. In both cases, weak fluorescence, but intense coloration (browning) was observed. In contrast, when hydrogen peroxide was added, fluorescence appeared in a short time, while coloration was restricted. This observation supports the notion that a 1,2,4-triketone is the precursor of our fluorescent molecules. Without oxidation steps caused by peroxides, the self-condensation products of 2,4-pentanedione and 2,3-butanedione could not easily change to the required 1,2,4-triketone forms, thus generating no fluorescence. The coloration process observed here may bear some similarities to the Maillard reaction (involving browning in various reactions of sugars and amines) *(6, 23)*, since multicarbonyl species are involved in both cases.

A kinetic study of the reaction between the suggested triketone precursor and Ac-Gly-Lys-OMe was carried out. Figure 5 shows that the reaction is first-order for both compounds.

Conclusions

Since the age-related fluorescent pigments (lipofuscins) were first discovered in the early 1970s *(1−4)*, many hypotheses have been advanced concerning their molecular structure. In this paper, we demonstrate that the 2-hydroxy-1,2-dihydropyrrol-3-one structure characteristic of certain minor products of the interaction between aldehydes and primary amines under oxidative conditions accounts to a significant degree for the commonly observed fluorescence having excitation/emission maxima at *ca.* 360/430 nm.

A complete structural elucidation of 2-hydroxy-1,2-dihydropyrrol-3-ones has been made possible by an integrated use of chromatographic isolation and spectral

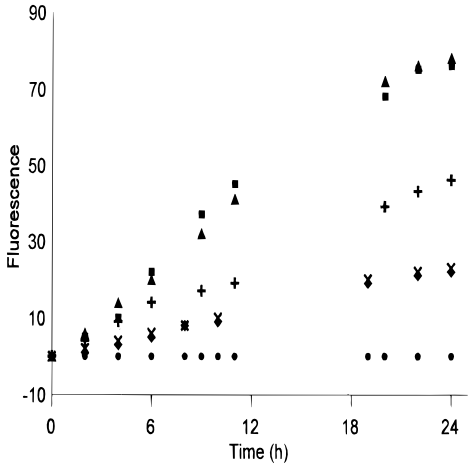


Figure 5. The time dependencies of fluorescence formation during the reaction of various concentrations of triketone **6c** (T) and peptide Ac-Gly-Lys-OMe (A) in 50% aqueous acetonitrile solution. Fluorescence was measured at λ_{ex} = 360 nm and λ_{em} = 430 nm. The reaction mixtures were incubated at room temperature in the dark. Reagent concentrations: (▲) 2 mM T and 1 mM A, (■) 1 mM T and 2 mM A, (+) 1 mM T and 1 mM A, (♦) 0.5 mM T and 1 mM A, (**x**) 1 mM T and 0.5 mM A, (●) 1 mM T (blank solution).

characterization techniques. The fluorescent properties of this simple, one-ring system are quite unusual and, to our knowledge, not previously implicated in a biochemical context. Yet, a strong similarity to a product of one of the most popular fluorogenic reagents, fluorescamine, appears obvious. A hypothesis that 2-hydroxy-1,2-dihydropyrrol-3-ones are formed from the corresponding aldehydes via a triketone precursor has been supported through the synthesis of authentic compounds and their reactions with primary amines.

Several 2-hydroxy-1,2-dihydropyrrol-3-ones were obtained from simple model systems favoring successful isolation. The use of alkanals in these systems is well justified by their natural occurrence as lipid peroxidation products *(5, 24−26)*. However, extending our findings to unsaturated aldehydes (e.g., 4-hydroxy-2-nonenal, various alkenals, dienals, trienals, etc.) remains a desirable goal. Moreover, the work reported here could provide valuable guidance in future efforts to isolate and characterize lipofuscins directly from biological matrices.

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