Reactions of *N*-Methyl-2-phenylindole with Malondialdehyde and 4-Hydroxyalkenals. Mechanistic Aspects of the Colorimetric Assay of Lipid Peroxidation

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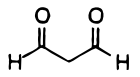
Under specific acidic conditions, both malondialdehyde (**1**, MDA) and 4-hydroxynonenal (**2**, 4-HNE) react with *N*-methyl-2-phenylindole (**3**) to give the same chromophoric cyanine **4** with maximal absorbance at 586 nm. Under such conditions, the reaction of **3** with 4-HNE (**2**) as well as with alkanals yields a second chromophoric cyanine **10** with maximal absorbance at 505 nm. The influence of different acids, iron(III), and oxygen on the reaction of **3** with such aldehydes was studied in detail. Under anaerobic conditions, the acid-induced reaction of 4-HNE with **3** afforded three rapidly interconverting intermediates, **5**−**7**. Their subsequent fragmentation to **4** and hexanal in the presence of iron(III) and oxygen is consistent with the tandem β-fragmentation of an indolyl radical cation. 1-Indolylalkenes were identified as essential intermediates in the acid-induced reaction of **3** with alkanals. A very mild iron(III)-catalyzed fragmentation of these intermediates afforded the corresponding 3-formylindole **11** as the direct precursor of the 505 nm chromophore **10**. Such reactions were markedly influenced by the nature of the acid. Contrary to the rapid chromogenic reaction of 4-HNE which was observed in the presence of methanesulfonic acid, the HCl-induced reaction of 4-HNE with **3** did not afford the 586 nm chromophore. Furthermore, hexanal did not yield the 505 nm chromophore **10** upon reaction with **3** in the presence of HCl, again in contrast with the rapid chromogenic reaction which was observed in the presence of methanesulfonic acid. Comparison of the reaction mixtures under the two assay conditions confirmed that the same intermediates were formed. We conclude that the nature of the acid plays a crucial role in the oxidative fragmentation of intermediates into chromophores, allowing the selective assay of MDA in the presence of 4-HNE, using HCl acidic conditions.

Introduction

In the preceding paper, we described a colorimetric assay for the cytotoxic aldehydes malondialdehyde (MDA,[1] **1**) and 4-hydroxynonenal (4-HNE, **2**) as byproducts of lipid peroxidation.



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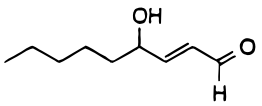

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The principle of this assay is the measurement of the absorbance at 586 nm, which is obtained with MDA as well as with 4-HNE upon reaction with *N*-methyl-2-phenylindole (**3**) under acidic conditions.

Moreover, the reaction of 4-HNE, which is accompanied by the formation of a second chromophore at 505 nm, can be suppressed completely by using hydrochloric instead of methanesulfonic acid, allowing the selective measurement of MDA in the presence of 4-HNE (*23*).

These experimental findings prompted us to study the reaction of MDA, 4-hydroxynonenal, and alkanals with *N*-methyl-2-phenylindole in the presence of either acid and with or without iron(III) salts.

Materials and Methods

Caution: *4-Hydroxynonenal (**2**) is reported to be cytotoxic (1). Pentanal, hexanal, heptanal, and octanal are irritants; hydrochloric and methanesulfonic acid are strong corrosive liquids. All these chemicals should be handled with protective gloves and the reactions described carried out in a hood.*

Chemicals. All reagents used were of the highest grade commercially available. 1,1,3,3-Tetramethoxypropane (TMP) was used as a source of MDA. 4-Hydroxynonenal diethyl acetal was prepared according to ref *2* and used for the in situ preparation of 4-HNE. 3-Formyl-*N*-methyl-2-phenylindole (**11**) was synthesized according to ref *3*. For the investigations of the influence of iron(III) salts, methanesulfonic acid of purissime grade (>99%) was purchased from Fluka (St. Quentin-Fallavier, France). Batches of methanesulfonic acid from other sources were in general contaminated with trace amounts of iron(III). Column chromatographic purifications were performed on silica Merck Si60 F_{254} or alumina Merck Al_2O_3 90 (activity II−III), and TLC plates were purchased from Macherey-Nagel (Polygram Sil G/UV_{254}, 0.25 mm). Melting points are uncorrected and were measured on a Gallenkamp apparatus.

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[1] Abbreviations: MDA, malondialdehyde; 4-HNE, 4-hydroxy-2(*E*)-nonenal; TMP, 1,1,3,3-tetramethoxypropane; TBME, *tert*-butyl methyl ether; TBA, 2-thiobarbituric acid; MPLC, medium-pressure liquid chromatography.

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Spectroscopy. ^{1}H (200 MHz) and ^{13}C NMR spectra (50 MHz) were recorded on a Varian Gemini-200 spectrometer and are reported in parts per million downfield from TMS. Fast atom bombardment mass spectrometry (FAB/MS), electron-impact mass spectrometry (EI-MS), and chemical-ionization mass spectrometry (CI-MS) were performed on a Nermag R10-10B apparatus. UV/visible spectra were recorded on a Uvikon 941 spectrophotometer from Kontron Instruments, in quartz microcuvettes with a 1 cm optical path length.

Gas Chromatography. GC analyses were conducted using a Varian 3400 gas chromatograph equipped with a DB-1 column of 30 m, with a 0.23 mm diameter (phase 0.25 μm), from J&W Scientific and FID detection (detector temperature of 280 °C and injector temperature of 250 °C). The vector gas was helium (10 psi); the split divisor was set at 20 mL/min. The temperature program was as follows: an initial temperature of 40 °C for 10 min and a final temperature of 150 °C (5 °C/min). The injection volume was 1−2 μL.

Isolation of 586 nm Chromophore 4a. TMP (164 mg, 164 μL, 1 mmol) was dissolved in 1 mL of acetonitrile and the mixture added to a solution of indole **3** (414 mg, 2 mmol) in 15 mL of hydrochloric acid (35%), 20 mL of water, and 65 mL of acetonitrile/methanol (3:1) at 40−45 °C. The mixture, which turned blue immediately, was initially stirred for 1 h at 40−45 °C and then for 1 h at 5 °C. After filtration, washing with 30 mL of TBME, and drying, 343 mg of **4a** (71%) was obtained as intensely green light crystals (*4*): mp 214 °C dec; ^{1}H NMR (CD_3OD) δ 8.20−7.40 (m, 21H), 3.74 (s, 6H, NCH_3); ^{13}C NMR (CD_3OD) δ 158.9, 141.4, 132.7, 131.9, 130.9, 127.7, 126.9, 122.8, 122.4, 120.4, 113.9, 33.1; FAB/MS (3-nitrobenzyl alcohol matrix) *m/z* (relative intensity) 451 (M − Cl^-, 60); UV (3:1 CH_3CN/MeOH) λ_{max} (log ϵ_{max}, M^{-1} cm^{-1}) 586 nm (5.04).

Isolation of 586 nm Chromophore 4b upon Reaction of MDA with Indole 3. TMP (164 mg, 164 μL, 1 mmol) was dissolved in 1 mL of acetonitrile and the mixture added to a solution of indole **3** (414 mg, 2 mmol) in 15 mL of methanesulfonic acid, 20 mL of water, and 65 mL of acetonitrile/methanol (3:1) at 40−45 °C. The mixture, which turned blue immediately, was stirred for 1 h at 40−45 °C. The organic solvents were removed under reduced pressure, and 150 mL of CH_2Cl_2 with 50 mL of water was added to the residue. The organic phase was decanted and washed three times with 50 mL of water. Evaporation of the solvents after drying ($MgSO_4$) and filtration afforded the crude product. After trituration with 20 mL of TBME and filtration, 360 mg of **4b** (67%) was obtained as green powder: mp 204 °C dec; ^{1}H NMR (CD_3OD) δ 8.30−7.40 (m, 21H), 3.78 (s, 6H, NCH_3), 2.67 (s, 3H, $CH_3SO_3^-$); ^{13}C NMR (CD_3OD) δ 158.7, 141.1, 132.8, 132.0, 131.0, 127.8, 127.0, 122.8, 122.4, 120.4, 114.0, 39.8, 33.2; FAB/HRMS (3-nitrobenzyl alcohol matrix) *m/z* (relative intensity) 451.2156 (M − $CH_3SO_3^-$, 100; $C_{33}H_{27}N_2$, calcd *m/z* 451.2174); UV (3:1 CH_3CN/MeOH) λ_{max} 586 nm.

Isolation of 586 nm Chromophore 4b upon Reaction of 4-HNE with Indole 3. 4-Hydroxynonenal diethyl acetal (230 mg, 1 mmol) was dissolved in 1 mL of acetonitrile and the mixture added to a solution of $FeCl_3$ (324 mg, 2 mmol) and indole **3** (414 mg, 2 mmol) in 15 mL of methanesulfonic acid, 20 mL of water, and 65 mL of acetonitrile/methanol (3:1) at 40−45 °C. The mixture, which turned blue immediately, was stirred for 1 h at 40−45 °C. Following the extraction procedure which was described above, 586 nm chromophore **4b** (350 mg, 64%) was obtained as a green powder. The same reaction in the presence of catalytic quantities of $FeCl_3$ (40 mg, 0.25 mmol) afforded 312 mg of **4b** (57%).

For GC analysis, 10 mL of the reaction mixture was quenched under cooling, with 25 mL of NaOH (2 N), and extracted with 4 mL of a solution of heptanal (25 mM) in TBME. The organic phase was dried (Na_2SO_4) and filtered. One to two microliters of this solution was injected and the peak of hexanal (t_R = 9.4 min) compared to the peak of heptanal (t_R = 15.8 min).

Isolation of Intermediates 5−7 from the Reaction of 4-HNE with 3 under N_2. 4-Hydroxynonenal diethyl acetal (230 mg, 1 mmol) was dissolved in 1 mL of acetonitrile and the mixture added under N_2 to a degassed solution of indole **3** (414 mg, 2 mmol) in 15 mL of methanesulfonic acid, 20 mL of water, and 65 mL of acetonitrile/methanol (3:1) at 40−45 °C. After being stirred for 10 min at 40−45 °C, the mixture was cooled to 5 °C. Concentrated NaOH (25 mL) was added, and then 50 mL of water and 50 mL of TBME. The organic phase was decanted, washed three times with 50 mL of saturated brine, and dried (K_2CO_3). TLC analysis of this solution (silica, 5:1 cyclohexane/ethyl acetate) revealed four main products with R_f values of 0.78 (**3**), 0.46 (**5**), 0.26 (**6**), and 0.20 (**7**). The spot corresponding to **7** turned blue immediately at ambient oxygen pressure. After removal of the solvents, intermediates **5**−**7** were isolated by MPLC (silica, 9:1 cyclohexane/ethyl acetate).

5: colorless oil; ^{1}H NMR ($CDCl_3$, mixture of two diasteroisomers, 70:30 A:B) δ 8.20−7.75 (m, 2H), 7.60−7.10 (m, 16H), 5.58 (t, 1H, J = 7 Hz, 1-H_B), 4.93 (dd, 1H, J = 11 Hz, J = 4.5 Hz, 1-H_A), 4.43 (m_{sym}, 1H, 4-H_B), 4.14 (td, 1H, J = 7 Hz, J = 3 Hz, 4-H_A), 3.95−3.65 (m, 1H), 3.59 (s, 3H, NCH_{3B}), 3.58 (s, 3H, NCH_{3A}), 3.57 (s, 3H, NCH_{3A}), 3.56 (s, 3H, NCH_{3B}), 3.07 (q, 1H_A, J = 11 Hz), 2.75 (ddd, 1H_B, J = 13 Hz, J = 8.5 Hz, J = 7 Hz), 2.51 (ddd, 1H_B, J = 13 Hz, J = 7.5 Hz, J = 5 Hz), 2.15 (m_{sym}, 1H_A), 1.90−0.75 (m, 11H); ^{13}C NMR[2] ($CDCl_3$) δ 83.6, 82.5, 76.1, 73.9, 41.5, 41.2, 39.8, 38.6, 33.7, 32.1, 31.5, 31.0, 27.2, 26.9, 22.9, 14.3, 14.2; CI-HRMS (isobutane) *m/z* (relative intensity) 553.3218 (MH^+, 35; $C_{39}H_{41}ON_2$, calcd *m/z* 553.3219).

6: white solid; mp 192−194 °C dec; ^{1}H NMR ($CDCl_3$) δ 7.70−6.46 (m, 27H), 4.54 (m, 1H, 1-H), 3.44−3.34 (m, 1H, 4-H), 3.38 (s, 3H, NCH_3), 3.30 (s, 3H, NCH_3), 3.27 (s, 3H, NCH_3), 3.04−2.60 (m, 3H), 1.24−0.72 (m, 11H); ^{13}C NMR ($CDCl_3$)[2] δ 74.8, 41.3, 36.0, 35.1, 32.5, 31.8, 30.5, 30.3, 30.1, 25.7, 22.5, 13.9; EI-HRMS (70 eV) *m/z* (relative intensity) 759.4191 (M^+, 100; $C_{54}H_{53}N_3O$, calcd *m/z* 759.4189).

7: colorless oil; ^{1}H NMR (CD_3CN) δ 7.83 (m, 2H), 7.48−7.04 (m, 16H), 6.62 (dd, 1H, J = 16 Hz, J = 8.5 Hz, 2-H), 6.22 (d, 1H, J = 16 Hz, 1-H), 4.10 (m, 1H, 4-H), 3.49 (s, 3H, NCH_3), 3.48 (s, 3H, NCH_3), 3.37 (t, 1H, J = 8.5 Hz, 3-H), 2.31 (d, 1H, J = 3.5 Hz, OH), 1.70−1.00 (m, 8H), 0.81 (t, 3H, J = 6.5 Hz); ^{13}C NMR (CD_3CN) δ 141.3, 141.1, 139.7, 139.5, 134.0, 133.2, 133.0, 132.8, 130.3, 130.2, 130.1, 129.9, 128.2, 127.4, 125.6, 123.9, 123.4, 122.6, 122.0, 121.8, 120.9, 114.7, 113.0, 111.8, 111.6, 74.7, 51.5, 36.9, 33.4, 32.1, 32.0, 26.7, 24.0, 15.0; EI-HRMS (70 eV) *m/z* (relative intensity) 552.3124 (M^+, 5; $C_{39}H_{40}N_2O$, calcd *m/z* 552.3141); EI-MS (70 eV) *m/z* (relative intensity) 552 (M^+, 3), 451 (100), 425 (45), 320 (35).

Synthesis of Tetrahydrofuran 5 from 6. 4-Hydroxynonenal diethyl acetal (153 mg, 0.66 mmol) and indole **3** (414 mg, 2 mmol) were dissolved in 100 mL of methanol. After the addition of 10 μL of methanesulfonic acid, the solution was stirred for 24 h at room temperature. A white precipitate formed, and the solution turned slightly blue. The precipitate was filtered and washed with 25 mL of TBME to afford **6** (292 mg, 58%) as a white solid. Two hundred fifty milligrams (0.33 mmol) of this crude product was dissolved in 25 mL of dichloromethane. After addition of 3 μL of methanesulfonic acid, the mixture was stirred for 5 min at room temperature. The solution was consecutively washed with 25 mL of saturated bicarbonate solution and brine (25 mL) and dried (Na_2SO_4). After removal of the solvent, the residue was purified by MPLC (silica, 2:1 to 1:1 cyclohexane/dichloromethane) to afford **5** (117 mg, 64%) as a colorless oil.

Isolation of 505 nm Chromophore 10a upon Reaction of Hexanal with Indole 3. Hexanal (100 mg, 120 μL, 1 mmol) was added to a solution of $FeCl_3$ (40 mg, 0.25 mmol) and indole **3** (414 mg, 2 mmol) in 15 mL of methanesulfonic acid, 20 mL of water, and 65 mL of acetonitrile/methanol (3:1) at 40−45 °C. Following the extraction procedure described for **4b**, **10a** [42 mg after 1 h (8%) and 172 mg after 14 h (33%)] was isolated as a red powder (*5*): ^{1}H NMR ($CDCl_3$) δ 7.82 (m, 2H), 7.79 (s, 1H), 7.67−7.27 (m, 16H), 4.05 (s, 6H, NCH_3), 2.73 (s, 3H, CH_3SO_3).

[2] Due to the complexity of the aromatic region and the superposition of most of the sp^2 carbons, only the aliphatic region is described.

The reaction of octanal (128 mg, 1 mmol) under the same conditions also afforded **10a** [42 mg after 1 h (8%) and 129 mg after 14 h (25%)].

Synthesis of 505 nm Chromophore 10b Starting from 3-Formylindole 11. 3-Formylindole **11** (*3*) (235 mg, 1 mmol) was added to a solution of indole **3** (414 mg, 2 mmol) in 15 mL of methanesulfonic acid, 20 mL of water, and 65 mL of acetonitrile/methanol (3:1) at 40−45 °C. After 3 h, the intensely red mixture was similarly extracted, affording **10a** (315 mg) as a red powder, which was dissolved in 5 mL of methanol. After being heated to reflux and addition of 6 mL of concentrated perchloric acid to eliminate traces of (1-methyl-2-phenylindol-3-yl)methane, the red solution was slowly cooled to room temperature. Red crystals precipitated, which were filtered and washed with 4 × 5 mL of TBME to afford **10b** (232 mg, 44%): mp 128−129 °C; ^{1}H NMR ($CDCl_3$) δ 7.82 (s, 1H), 7.76 (m, 2H), 7.68−7.30 (m, 16H), 4.04 (s, 6H); ^{13}C NMR ($CDCl_3$) δ 160.8, 152.2, 141.1, 132.4, 131.9, 129.6, 127.7, 126.8, 126.2, 125.2, 124.8, 120.3, 113.3, 34.0; FAB/MS (glycerol/3-nitrobenzyl alcohol matrix) *m*/*z* (relative intensity) 425 (M − ClO_4^-, 100), 185 (40), 93 (75); UV (3:1 CH_3CN/MeOH) λ_{max} 505 nm. **10** was described as bromide (*5*).

1,1-Bis(*N*-methyl-2′-phenylindol-3-yl)octane (12). Octanal (128 mg, 1 mmol) was added to a solution of indole **3** (1.03 g, 5 mmol) in 20 mL of acetonitrile. After the addition of 3 μL of methanesulfonic acid, the mixture was stirred for 48 h at room temperature. After neutralization with 5 mL of saturated bicarbonate solution and evaporation, the residue was dissolved in 50 mL of TBME and washed with 50 mL of water and saturated brine. Drying, filtration, and evaporation afforded 978 mg of a yellow oil. Purification by MPLC (silica, 4:1 to 1:1 cyclohexane/dichloromethane) yielded **12 (**380 mg, 72%) as a highly viscous colorless oil: ^{1}H NMR ($CDCl_3$) δ 7.57 (d, 2H, J = 8 Hz), 7.38−7.10 (m, 10H), 6.94 (m, 6H), 4.40 (t, 1H, J = 7 Hz, 1-H), 3.38 (s, 6H, NCH_3), 2.18 (q, 2H, J = 7 Hz, 2-H), 1.26−0.94 (m, 10H), 0.80 (t, 3H, J = 6.5 Hz, 8-H); ^{13}C NMR ($CDCl_3$) δ 137.9, 136.9, 132.9, 131.0, 127.9, 127.8, 127.4, 121.2, 120.8, 118.8, 116.5, 108.9, 35.5, 34.7, 31.6, 30.3, 29.0, 28.8, 27.9, 22.3, 13.8; EI-MS (70 eV) *m*/*z* (relative intensity) 524 (M^+, 20), 425 (100), 317 (17), 246 (17), 207 (18).

1,1-Bis(*N*-methyl-2′-phenylindol-3-yl)hexane (13). Hexanal (150 mg, 1.5 mmol) was added to indole **3** (621 mg, 3 mmol) in 15 mL of acetonitrile. After addition of 4 μL of methanesulfonic acid, the mixture was stirred for 4 h. The white precipitate was filtered and washed with 3 × 5 mL of cold acetonitrile to afford **13** (460 mg, 61%): mp 145 °C; ^{1}H NMR ($CDCl_3$) δ 7.60 (d, 2H, J = 8 Hz), 7.40−7.10 (m, 10H), 7.06−6.84 (m, 6H), 4.43 (t, 1H, J = 8 Hz, H-1), 3.39 (s, 6H, NCH_3), 2.21 (m, 2H, 2-H), 1.26−0.94 (m, 6H), 0.72 (t, 3H, J = 7 Hz, H-6); ^{13}C NMR ($CDCl_3$) δ 138.0, 136.9, 132.9, 131.1, 127.9, 127.8, 127.4, 121.2, 120.8, 118.8, 116.5, 108.9, 35.5, 34.9, 31.4, 30.3, 27.7, 22.2, 13.9; EI-MS (70 eV) *m*/*z* (relative intensity) 496 (M^+, 15), 425 (100), 218 (30).

1-(*N*-Methyl-2′-phenylindol-3-yl)oct-1-ene (14). Octanal (512 mg, 4 mmol) was added under N_2 to a solution of indole **3** (828 mg, 4 mmol) in 20 mL of TBME. After the addition of 3 μL of methanesulfonic acid, the mixture was stirred for 24 h at room temperature under N_2 and then washed with 2 × 20 mL of a saturated bicarbonate solution. The solvent was removed under reduced pressure after drying (Na_2SO_4) and filtration. Purification of the crude product by MPLC (silica, 8:1 cyclohexane/dichloromethane) afforded **14** (141 mg, 32%) as a pale yellow oil:[3] ^{1}H NMR ($CDCl_3$) δ 8.01 (m, 1H), 7.60−7.20 (m, 8H), 6.42 (d, 1H, J = 16 Hz, 1-H), 6.25 (td, 1H, J = 7 Hz, J = 16 Hz, 2-H), 3.61 (s, 3H, NCH_3), 2.20 (q, J = 7 Hz, 3-H), 1.56−1.20 (m, 8H), 0.92 (t, 3H, J = 6.5 Hz); ^{13}C NMR ($CDCl_3$) δ 138.9, 137.8, 131.8, 131.1, 128.9, 128.4, 128.2, 125.9, 122.8, 122.1, 120.5, 120.2, 112.0, 109.5, 33.8, 31.6, 30.7, 29.8, 28.7, 22.5, 13.9; EI-HRMS (70 eV) *m*/*z* (relative intensity) 317.2162 (M^+, 44; $C_{23}H_{27}N$, calcd *m*/*z* 317.2144). The spectrum shows the presence of an impurity: *m*/*z* (relative intensity) 333 ($C_{23}H_{27}NO$, 4).

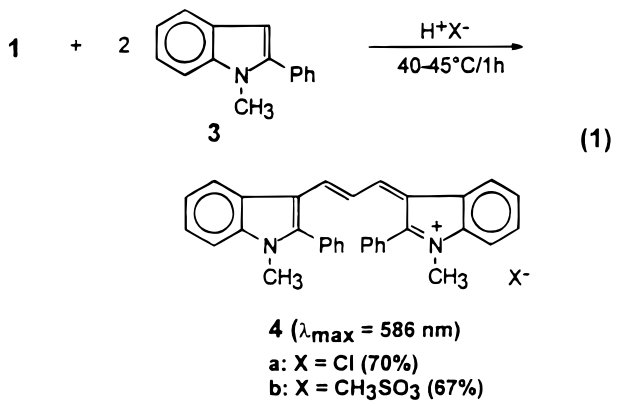
1-(*N*-Methyl-2′-phenylindol-3-yl)hex-1-ene (15). The reaction of hexanal (400 mg, 4 mmol) as described for **14** afforded **15** (380 mg, 33%) as a pale yellow oil:[3] ^{1}H NMR ($CDCl_3$) δ 7.98 (m, 1H), 7.56−7.16 (m, 8H), 6.38 (d, 1H, J = 16 Hz, 1-H), 6.21 (td, 1H, J = 6.5 Hz, J = 16 Hz, 2-H), 3.61 (s, 3H, NCH_3), 2.17 (q, J = 6.5 Hz, 3-H), 1.50−1.24 (m, 4H), 0.91 (t, 3H, J = 7 Hz); ^{13}C NMR ($CDCl_3$) δ 140.0, 138.4, 132.8, 132.0, 130.0, 129.4, 129.0, 126.4, 124.0, 123.0, 121.5, 121.0, 113.0, 110.4, 34.2, 32.8, 31.2, 22.5, 14.2; EI-HRMS (70 eV) *m*/*z* (relative intensity) 289.1840 (M^+, 82; $C_{21}H_{23}N$, calcd *m*/*z* 289.1830). The spectrum shows the presence of an impurity: *m*/*z* (relative intensity) 305 ($C_{21}H_{23}NO$, 4).

2-Pentylfuran. 4-Hydroxynonenal diethyl acetal (690 mg, 3 mmol) in 1 mL of acetonitrile was added to a solution of 7.5 mL of concentrated HCl, 10 mL of water, and 32.5 mL of acetonitrile/methanol (3:1) at 40−45 °C. After 10 min at 40−45 °C, the reaction mixture was neutralized with 40 mL of NaOH (1 N). Extraction with 10 mL of TBME, followed by drying (Na_2SO_4) and evaporation, afforded a yellow oil, which was identified as 2-pentylfuran (380 mg, 87%, purity of ∼95%): ^{1}H NMR (CD_3CN) δ 7.35 (m, 1H, 5-H), 6.30 (m, 1H, 4-H), 6.03 (m, 1H, 3-H), 2.61 (t, 2H, J = 7 Hz, 1′-H), 1.62 (quint, 2H, J = 7 Hz, 2′-H), 1.31 (m, 4H, 3′-H, 4′-H), 0.90 (t, 3H, J = 7 Hz, 5′-H); ^{13}C NMR (CD_3CN) δ 157.9, 142.2, 111.5, 105.9, 32.2, 28.6, 28.5, 23.2, 14.4; EI-MS (70 eV) *m*/*z* (relative intensity) 138.1041 (M^+, 100; $C_9H_{14}O$, calcd *m*/*z* 138.1045).

GC Analysis. 4-Hydroxynonenal diethyl acetal (115 mg, 0.5 mmol) in 0.5 mL of acetonitrile was added to a solution of concentrated acid (7.5 mL), 10 mL of water, and 32.5 mL of acetonitrile/methanol (3:1) at 40−45 °C. Ten milliliters of the reaction mixture was quenched while being cooled, with 25 mL of NaOH (2 N). Four milliliters of a solution of 2-ethylfuran (25 mM) in TBME was added, and the organic phase was decanted, dried, and filtered. One to two microliters of this solution was injected and the peak of 2-pentylfuran (t_R = 20.7 min) compared with the peak of 2-ethylfuran (t_R = 5.65 min).

Results

Acid-Induced Reaction of MDA (1) with *N*-Methyl-2-phenylindole (3). The acid-catalyzed reaction of MDA with indole compounds is known to generate intensely colored trimethincyanine dyes (*3*, *6*). Hence, we isolated as expected 586 nm chromophores **4a** (70%) and **4b** (67%) upon reaction of 2 equiv of indole **3** with 1 equiv of MDA, generated in situ from tetramethoxypropane (TMP), under the described assay conditions, e.g., in an aqueous solution of acetonitrile/methanol, in the presence of either hydrochloric or methanesulfonic acid at 40−45 °C for 1 h[4] (eq 1).



[3] **14** and **15** decomposed slowly in solution and have to be stored under nitrogen.

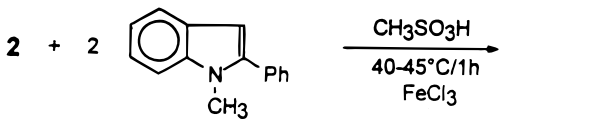
Identification of the Final Products of the Acid-Induced Reaction of 4-HNE (2) with Indole 3. 4-Hydroxynonenal reacts with an excess of indole **3** to yield a concentration-dependent absorbance at 586 nm when methanesulfonic acid is used in the presence of catalytic amounts of iron(III) salts. Surprisingly, when HCl was substituted for methanesulfonic acid, no significant production of chromophore was observed (see Figure 1B).

A closer investigation of the methanesulfonic acid-induced reaction showed that no chromophore was produced in the absence of contaminating or added iron(III) salts (Figure 1C). Under anaerobic conditions, complete transformation of 4-HNE to the chromophore was obtained upon addition of 2 equiv of iron(III) chloride to the reaction mixture (Figure 1D).

Similarly, a crystalline dye could be isolated upon stoichiometric reaction of 2 equiv of indole **3** with 4-hydroxynonenal, generated in situ from the diethyl acetal instead of MDA. However, this was only possible when methanesulfonic acid was used, in the presence of iron(III) salts (64% with 2 equiv of $FeCl_3$ and 57% with 0.25 equiv of $FeCl_3$).

The analytical characterization (^{1}H NMR, ^{13}C NMR, MS, mp, and UV/vis) of the isolated dye established complete structural identity with cyanine dye **4b**, the reaction product for reaction of indole **3** with MDA. The ^{1}H and ^{13}C NMR spectra especially showed the sole presence of sp^2 carbons in the molecule (besides the two methyl groups at 39.8 and 33.2 ppm), implying a fragmentation of the alkyl chain of starting aldehyde **2** during the reaction.

A GC analysis of TBME extracts of neutralized samples from the highly colored reaction mixture of eq 2, taken after 10−60 min, allowed us to identify hexanal as a second product besides 586 nm chromophore **4**, formed by the stoichiometric reaction of 4-HNE with indole **3** in about 45−60% yield (eq 2).



3 (2)

4b + $C_5H_{11}CHO$ (45-60%)

Identification of the Intermediates of the Acid-Induced Reaction of 4-HNE with Indole 3. Trying to gain more insight into the course of this reaction, we repeated the methanesulfonic acid-induced reaction of 4-HNE with indole **3**, but in the absence of both oxygen and iron(III) salts. Under these conditions, the homogeneous solution remained colorless upon addition of either stoichiometric (2 equiv) or excess quantities (10 equiv) of indole **3**. TLC examination of neutralized and extracted samples of this colorless mixture (with either 2 or 10 equiv of indole **3**) revealed the formation of

[4] Despite the rapid and irreversible polymerization of MDA under acidic conditions, a complete conversion of MDA to 586 nm chromophore **4** is possible using a large excess of indole **3**; see ref *23* for further details.

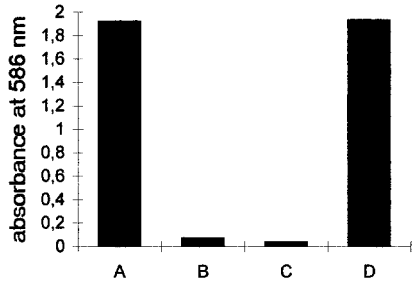
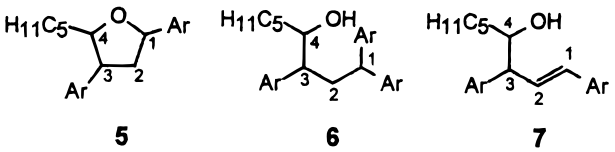
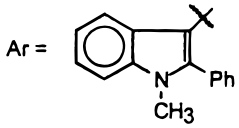


Figure 1. Effects of the acid type, iron salt, and oxygen on the formation of the 586 nm chromophore from the reaction of 4-HNE (20 μM) with indole **3** (5 mM) for 30 min at 45 °C in acetonitrile/methanol. Ten microliters of a solution of 4-HNE in acetonitrile/methanol (3:1, 8 mM) was added to 4 mL of a solution of indole **3** in acetonitrile/methanol/water/H^+X^-. The 586 nm absorbance was measured after 30 min at 45 °C: (A) methanesulfonic acid, $FeCl_3$ (0.2 equiv), and ambient oxygen pressure, (B) hydrochloric acid, $FeCl_3$ (0.2 equiv), and ambient oxygen pressure, (C) methanesulfonic acid and ambient oxygen pressure without $FeCl_3$, and (D) methanesulfonic acid, $FeCl_3$ (2 equiv), and an inert atmosphere.

essentially three products. Separation, isolation, and characterization of these products allowed the attribution of the structures **5**−**7** for these intermediates, confirming that all three products were still bearing the alkyl side chain of starting aldehyde **2**.





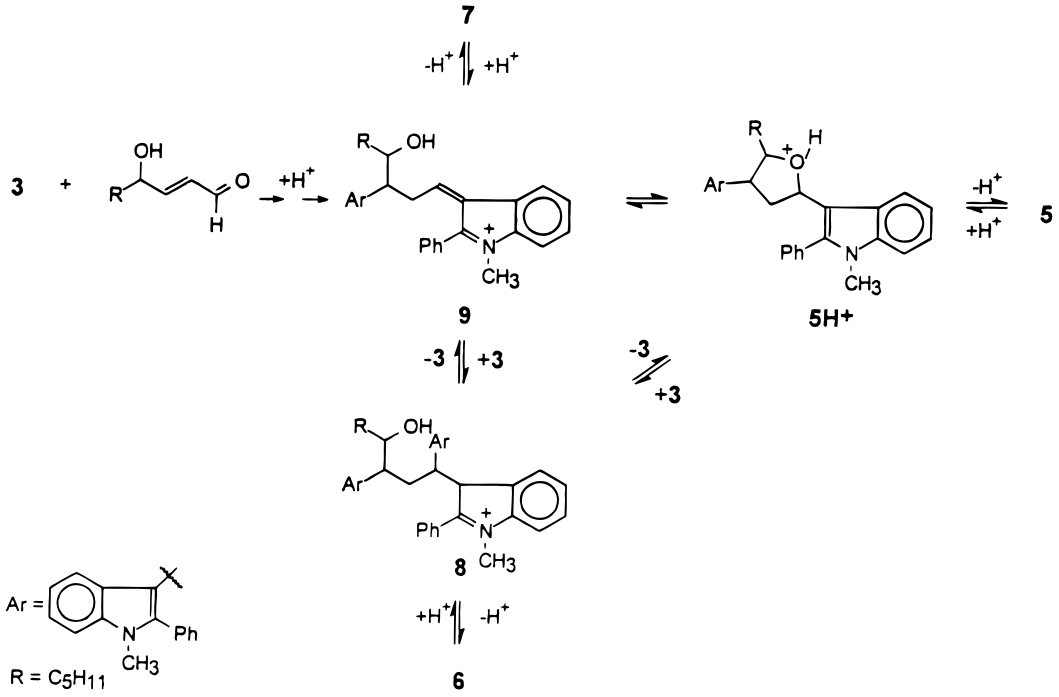
HRMS and ^{1}H NMR data were crucial for the assignment of structures **5**−**7** to the three reaction products. The isotopic patterns of the $M^{\bullet+}$ for **6** and **7** (after HR-EI) and MH^+ for **5** (following HR-DCI) were consistent with the molecular formulas $C_{39}H_{41}N_2O$, $C_{54}H_{53}N_3O$, and $C_{39}H_{40}N_2O$ for **5**−**7**, respectively (see Materials and Methods). This corresponds to the addition of two molecules of indole **3** to one molecule of 4-HNE for **5** and **7**, versus three molecules of indole for **6**.

The following key features in the ^{1}H NMR spectrum of **7** allowed the assignment of the olefinic structure: (i) two olefinic protons at 6.22 (d, 1H, J = 16 Hz) and 6.62 ppm (dd, 1H, J = 16 and 8.5 Hz) assigned to H-1 and H-2, (ii) a multiplet corresponding to one proton at 4.10 ppm, assigned to H-4, and (iii) finally two singlets (three equivalent hydrogens integrated) at 3.48 and 3.49 ppm, corresponding to the *N*-methyl groups.

The identification of compound **5** as a tetrahydrofuran-type addition product was based on the following. Its molecular formula is the same as that of **7**, but no olefinic proton shows up in the ^{1}H NMR spectrum. Two sets of diastereoisomers were observed with a ratio of 70:30 (**5A**:**5B**). Peaks observed at 5.58 ppm (0.3H, **5B**) and 4.93 ppm (0.7H, **5A**) were assigned to H-1 and those observed at 4.14 ppm (0.7H, **5A**) and 4.43 ppm (0.3H, **5B**) to H-4.[5]

The ^{1}H NMR spectrum of **6** displayed three singlets (three equivalent hydrogens integrated) for the three

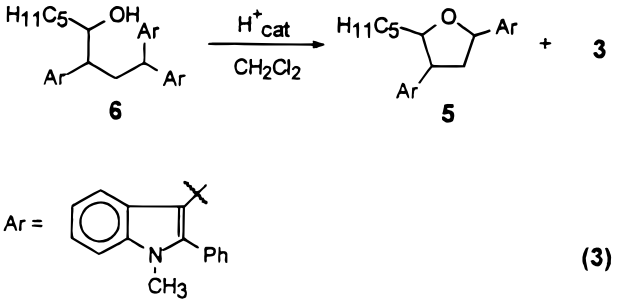
Scheme 1



distinct *N*-methyl groups, whereas one proton (t, $J = 7$ Hz) at 4.54 ppm was assigned to H-1. This chemical shift is typical of H-1 in 1,1-diindolyl-substituted alkyl derivatives. For example, it is observed in **12** at 4.40 ppm (t, $J = 7$ Hz) and in **13** at 4.43 ppm (t, $J = 7$ Hz).

Whereas compounds **6** and **7** are the expected addition products for addition of indole **3** to 4-HNE, an α,β-unsaturated aldehyde, the tetrahydrofuran derivative **5**, could be formed via various pathways (see Scheme 1), i.e., either by direct intramolecular addition to the indolenium cation fragment in **9** (**9** → **5H**$^+$) or by an addition−elimination sequence via intermediate **8** (**9** → **8** → **5H**$^+$). Actually, **9** is a direct intermediate of the acid-induced addition of indole **3** to 4-HNE, but could also be obtained by protonation of one indole nucleus in **6**, followed by elimination of indole **3** (see Scheme 1).

Interestingly, tetrahydrofuran derivative **5** is obtained as the major product (along with indole **3**) after addition of catalytic amounts of an acid to a CH_2Cl_2 solution of intermediate **6**, and could conveniently be isolated in 64% yield after purification (eq 3).



(3)

Separate dissolution of each purified intermediate in the acidic solvent mixture of the described (*23*) assay results in the immediate formation of the three intermediates **5**−**7** and the starting indole **3**, as shown by TLC analysis. This rapid equilibration of all intermediates excluded the identification of the direct precursor for the rapid and quantitative fragmentation to 586 nm chromophore **4** and hexanal under the assay conditions in the presence of iron(III) salts.

Reaction of Intermediates 5−7 with $FeCl_3$. To compare the reactivities of the three intermediates, we studied their chromogenic transformation in the presence of $FeCl_3$ (0.5 equiv) in acetonitrile/methanol.

Air-saturated solutions (0.1 mM) of intermediate **5**, **6**, or **7** in acetonitrile/methanol (3:1) were treated at 40−45 °C with 0.5 equiv of $FeCl_3$. The reactions were followed by monitoring the absorbance at 586 nm. As shown in Figure 2, the 586 nm chromophore was formed in all reaction mixtures, even though it was formed much more slowly than under the standardized assay conditions. Moreover, significant differences in the rates of chromophore formation could be observed in the first hours, with the solution of 1-indolylalkene **7** turning blue far more quickly than the solution of the tetrahydrofuran derivative **5** and that of intermediate **6** (Figure 2).

Interestingly, no significant absorbance at 505 nm could be detected in any of these reaction mixtures, whereas the transformation of 4-HNE to the 586 nm chromophore and hexanal is accompanied by the formation of a second chromophore at 505 nm.

Reactivity of 4-HNE with Indole 3 in Hydrochloric Acid Medium. As shown above (see Figure 1), the reaction of 4-HNE with indole **3** to form 586 nm chromophore **4** and the corresponding saturated aldehyde depends not only on the presence of iron salts but also on the nature of the acid used; while the reaction is fast and quantitative in the presence of methanesulfonic acid (1 h at 45 °C), no significant production of the 586 nm

[5] The formation of diastereomeric tetrahydofuran derivatives following Michael addition of nucleophiles such as *n*-butylamine and *N*-acetylhistamine to 4-HNE was previously reported (*7*). The corresponding chemical shifts for H-1 of 4.69−4.74 ppm for the former and 5.64−5.80 ppm for the latter are in good agreement with the figures which were obtained for **5**.

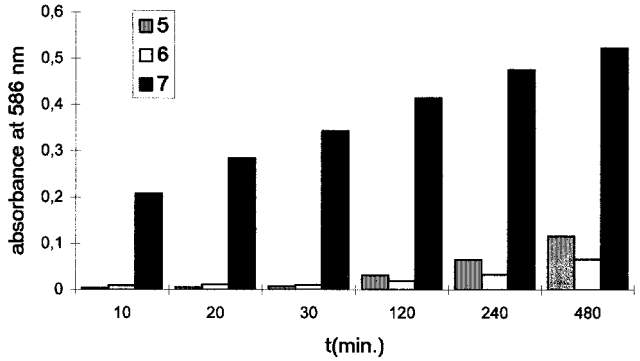
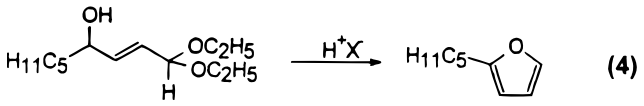


Figure 2. Formation of the 586 nm chromophore upon reaction of **5**, **6**, or **7** (0.1 mM) with $FeCl_3$ (50 μM) in acetonitrile/methanol (3:1) at 40−45 °C. Each aliquot of the reaction mixture was diluted with 4 volumes of acetonitrile/methanol (3:1) before measuring the 586 nm absorbance.

chromophore is observed when hydrochloric acid is used instead of methanesulfonic acid.

This result was unexpected given the complete reaction of MDA with indole **3** yielding 586 nm chromophore **4**, and therefore the stability of the chromophore under such conditions.

To assess the stability of the starting aldehyde under the assay conditions,[6] a solution of 0.1 mmol of 4-hydroxynonenal diethyl acetal in $CD_3CN/CD_3OD/D_2O$ (850 μL) was treated with deuterated HCl (150 μL). The NMR spectrum obtained after 5 min showed the rapid conversion of 4-HNE (obtained upon hydrolysis of the acetal) to a new product, which was identified as 2-pentylfuran (eq 4).



(4)

The formation of 2-pentylfuran was observed earlier by Sayre et al. (*10*) upon refluxing ethanolic solutions of 4-HNE in the presence of phenethylamine chlorohydrate. Interestingly, 2-pentylfuran was isolated by Chang et al. (*9*) as a component of autoxidized soybean oil.

A rough estimation of the formation rate of this product under the assay conditions in the absence of indole **3** was obtained by means of gas chromatography, using 2-ethylfuran as the internal standard. The results indicated a faster transformation in the presence of HCl than in the presence of methanesulfonic acid (see Table 1).

To test the hypothesis that rapid formation of pentylfuran in the presence of HCl competes efficiently with chromophore formation, we repeated such measurements in the presence of indole **3**. Even in the presence of only 2 equiv of indole **3**, essentially no pentylfuran was formed (see Table 1). TLC examination of neutralized and extracted samples indicated the same intermediates **5**−**7** as for the methanesulfonic acid-induced reaction.

Obviously, the difference between the two assay conditions, using either HCl or methanesulfonic acid, concerns the transformation of the intermediates to 586 nm chromophore **4**, which is practically inhibited in the presence of HCl, allowing the selective measurement of MDA under these conditions.

[6] Hydroxynonenal was reported to dehydrate under acidic conditions to *trans*,*trans*-2,4-nonadienal (*8*).

Table 1. Comparison of Hydrochloric and Methanesulfonic Acid-Induced Formation of 2-Pentylfuran from 4-Hydroxynonenal (Generated in Situ from the Diethyl Acetal) in the Presence or Absence of Indole 3

acid	*t*	equiv of **3**	% 2-pentylfuran[a]
HCl	30 s	0	61 ± 7
	60 s	0	87 ± 16
	2 min	0	114 ± 2
methanesulfonic acid	30 s	0	9 ± 2
	60 s	0	13 ± 2
	2 min	0	20 ± 3
	10 min	0	57 ± 9
HCl	2 min	2	3 ± 1
	10 min	2	2 ± 2

[a] GC analysis. percentage of the peak area of 2-ethylfuran as the internal standard, means of two experiments (see Materials and Methods).

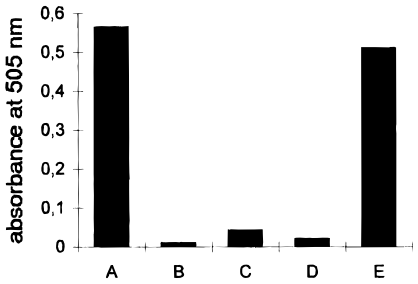


Figure 3. Effects of the acid type, iron salt, and oxygen on the formation of the 505 nm chromophore from the reaction of hexanal (A−D, 10 μM) or octanal (E, 10 μM) with indole **3** (5 mM) for 30 min at 45 °C. Four microliters of a solution of hexanal or octanal in acetonitrile/methanol (3:1, 10 mM) was added to 4 mL of a solution of indole **3** in acetonitrile/methanol/water/H^+X^-. The 505 nm absorbance was measured after 30 min at 45 °C: (A) methanesulfonic acid, $FeCl_3$ (5 μM), and ambient oxygen pressure, (B) hydrochloric acid, $FeCl_3$ (5 μM), and ambient oxygen pressure, (C) methanesulfonic acid, $FeCl_3$ (20 μM), and an inert atmosphere, (D) methanesulfonic acid and ambient oxygen pressure without $FeCl_3$, and (E) methanesulfonic acid, $FeCl_3$ (5 μM), and ambient oxygen pressure.

Interestingly, we observed that the transformation of alkanals into the 505 nm chromophore upon reaction with indole **3** was similarly affected by hydrochloric versus methanesulfonic acid, as described in the following section.

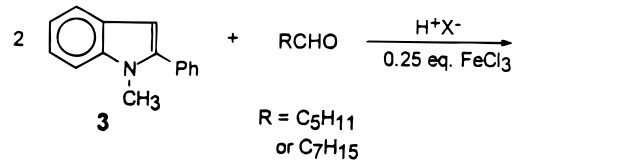
Identification of the Final Products of the Acid-Induced Reaction of Alkanals with Indole 3. As mentioned above, the acid-induced reaction of 4-HNE with indole **3** results in the formation of 586 nm chromophore **4**, accompanied by the formation of a second chromophore with a maximal absorbance wavelength of 505 nm. This second absorption was not a linear function of the concentration of 4-HNE, and the maximal absorbance was somewhat variable.

Since hexanal was one byproduct of the acid-induced reaction of 4-HNE with 2 equiv of indole **3** in the presence of iron salts (and also a nonspecific byproduct of lipid peroxidation), we compared the reaction of saturated aldehydes with **3** under the assay conditions.

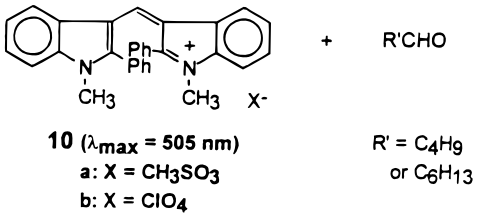
Interestingly, after the addition of hexanal or octanal (10 μM) to a large excess (5 mM) of indole **3** in aqueous acetonitrile/methanol containing methanesulfonic acid and catalytic amounts of $FeCl_3$ (5 μM), the reaction mixture turned red immediately. This is a result of the formation of a 505 nm chromophore (see Figure 3A,E), but apparently in a nonlinear manner. Under the same conditions, but using HCl instead of methanesulfonic

acid, no significant chromophore formation took place (Figure 3B). The same result was obtained either under anaerobic conditions or in the absence of iron(III) salts (Figure 3D). In the first case, even in the presence of 2 equiv of $FeCl_3$, no significant absorbance at 505 nm could be detected (Figure 3C), underlining the requirement for both iron(III) and oxygen in the formation of the 505 nm chromophore.

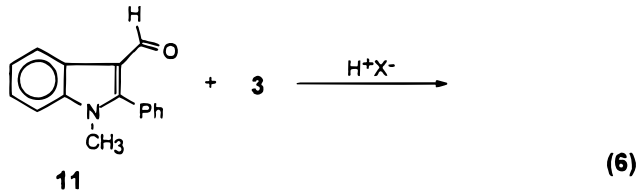
To isolate and characterize the corresponding dye compound, 1 equiv of hexanal was added to a solution of 2 equiv of indole **3** in methanesulfonic acid in the presence of 0.25 equiv $FeCl_3$. After 1 h at 45 °C, the intensely red reaction mixture yields a red precipitate. The ^1H NMR spectroscopic analysis of this compound (with a λ_{max} of 505 nm in acetonitrile/methanol) indicates the diindolylmethine structure **10a (**8% at 1 h and 33% at 14 h; eq 5).



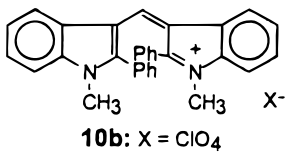
(5)



In a similar way, **10a** (8% at 1 h and 25% at 14 h) was isolated after treating octanal under the same conditions. This structure was further confirmed by the independent synthesis of **10b**, upon reaction of 3-formylindole **11** with indole **3** under acidic conditions and subsequent precipitation of **10b** as a perchlorate salt (eq 6).



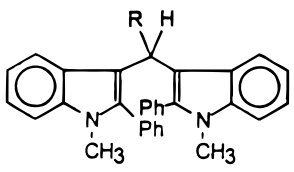
(6)



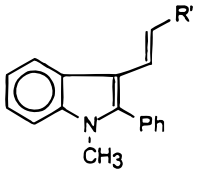
The fact that the same 505 nm-absorbing structure is obtained by the reaction of either hexanal, octanal, or 3-formylindole **11** indicates a one-carbon fragmentation of the starting alkanal during the methanesulfonic acid-induced reaction with indole **3** in the presence of iron(III) salts and oxygen.

Reaction of 1-Indolylalkenes 14 and 15 with $FeCl_3$. The acid-induced reaction of indole compounds with saturated aldehydes is known to yield essentially two products, 1,1-diindolylalkanes and 1-indolylalkenes (*11*, *12*).

The corresponding diindolyloctane **12** and -hexane **13** as well as the indolyloctene **14** and -hexene **15**, which we synthesized and characterized independently, were also identified by TLC analysis as major products of eq 5 in the absence of iron(III) salts.



12: R = C_7H_{15}
13: R = C_5H_{11}

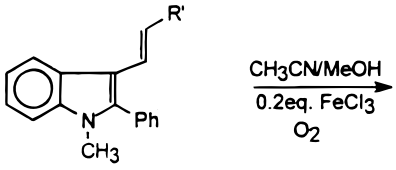


14: R' = C_6H_{13}
15: R' = C_4H_9

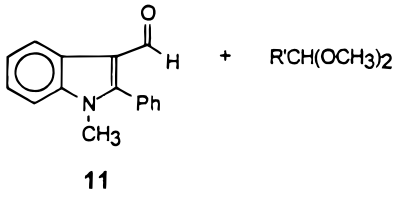
As already mentioned for addition products **5**−**7**, products **12** and **14** just like products **13** and **15** are in rapid equilibrium under acidic conditions, as shown by separate dissolution of each product in the acidic reaction medium and TLC analysis of the resulting mixture.

Accordingly, we studied the reaction of 1-indolylalkenes **14** and **15** in the presence of $FeCl_3$ in acetonitrile/methanol.

$FeCl_3$ (20 mol %) was added to a 1 mM solution of 1-indolyloctene **14** in acetonitrile/methanol at 40−45 °C under aerobic conditions. The TLC analysis of the reaction mixture at 10 min revealed the transformation of **14** to a new, more polar, UV-active product, the reaction going to completion within 90 min. This product was shown by ^1H and ^{13}C NMR analysis and comparison with an authentic sample to be 3-formylindole **11**, isolated in 72−78% yield after evaporation of the solvents and filtration of the residue over alumina. Using hexanal dimethylacetal[7] as the internal standard after completion of the transformation, GC analysis showed the presence of heptanal (as dimethylacetal) as the second major product besides 3-formylindole **11**, formed in 80−100% yield (eq 7).



(7)



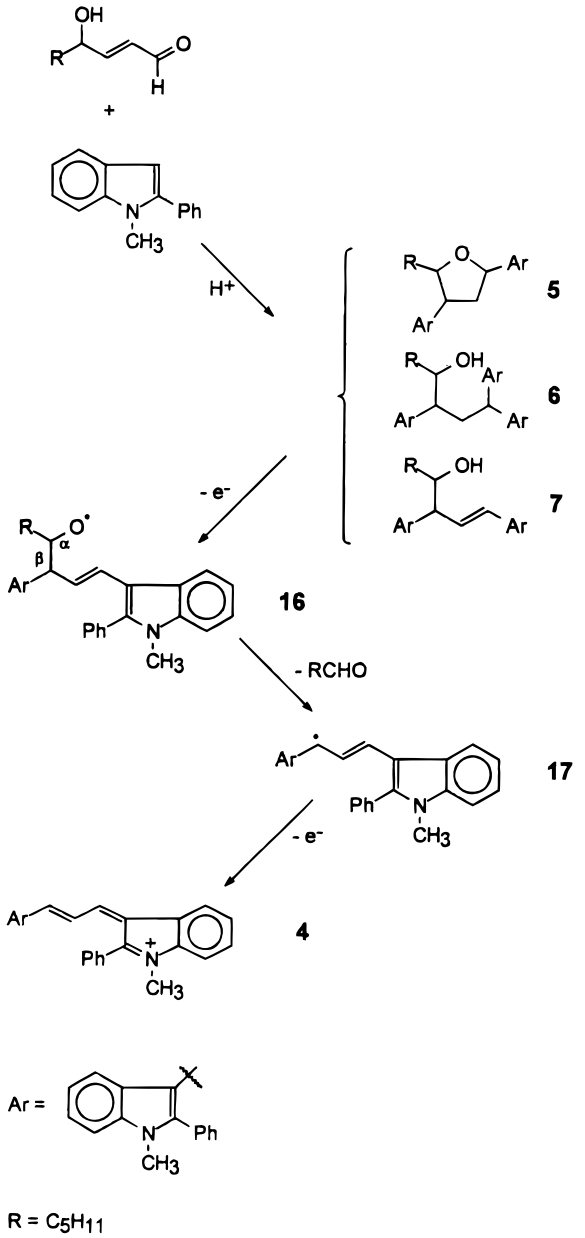
In a similar way, the solution of 1-indolylhexene **15** in acetonitrile/methanol reacted with $FeCl_3$ to give 3-formylindole **11** (78%) and pentanal (as dimethylacetal, 83%).

Again, 3-formylindole **11** is the direct precursor of 505 nm chromophore **10** via reaction with indole **3** under acidic conditions.

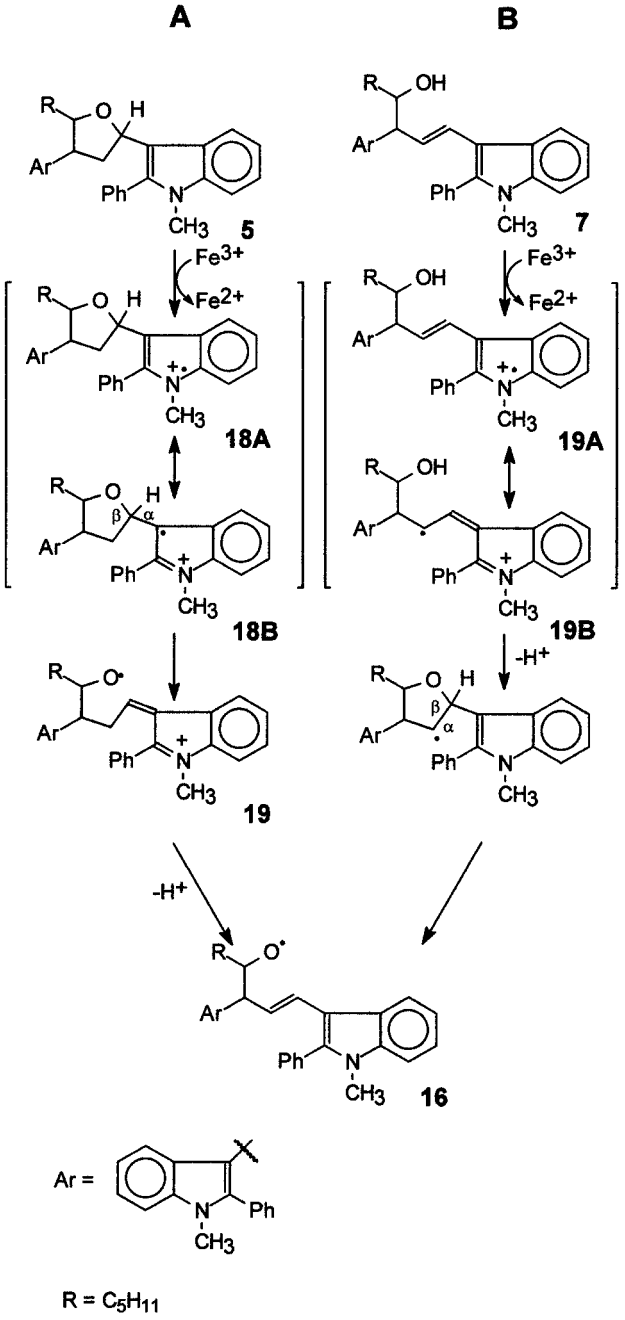
Reactivity of Alkanals with Indole 3 in Hydrochloric Acid Medium. As shown in Figure 3, 505 nm

[7] Formed in situ by adding 3 mg of $FeCl_3$ to 100 mL of a solution of hexanal (1 mM) in acetonitrile/methanol (3:1).

Scheme 2. Possible Mechanism for the Formation of 586 nm Chromophore 4 via *β*-Fragmentation of an Intermediate Alkoxyl Radical 16



Scheme 3. Hypothetical Pathways for the Formation of Alkoxyl Radical 16



chromophore **10** is not obtained by the reaction of hexanal or octanal with indole **3** in the presence of HCl and iron salts, whereas such a transformation is fast in the presence of methanesulfonic acid.

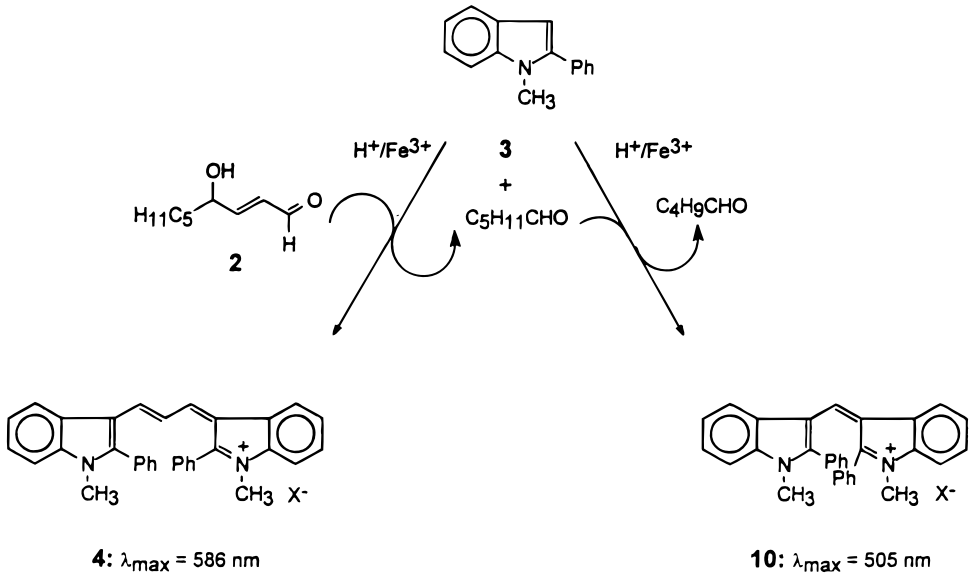
Nevertheless, in the presence of iron salts, the reaction of hexanal or octanal with indole **3** yields essentially the same products (**12** and **14**, and **13** and **15**) using HCl or methanesulfonic acid, as shown by TLC analysis of the reaction mixture. Moreover, the formation of 505 nm chromophore **10** via the reaction of the 3-formylindole **11** with indole **3** takes place in the presence of either HCl or methanesulfonic acid.

Like the transformation of intermediates **5**−**7** into 586 nm chromophore **4**, hydrochloric acid apparently inhibits or slows the transformation of the 1-indolylalkenes **14** and **15** to 3-formylindole **11** as a precursor of the chromophore **10**. Both transformations (**5**−**7** → **4** and **14** and **15** → **10**) are induced by iron(III) salts and lead to the fragmentation of the alkyl side chain.

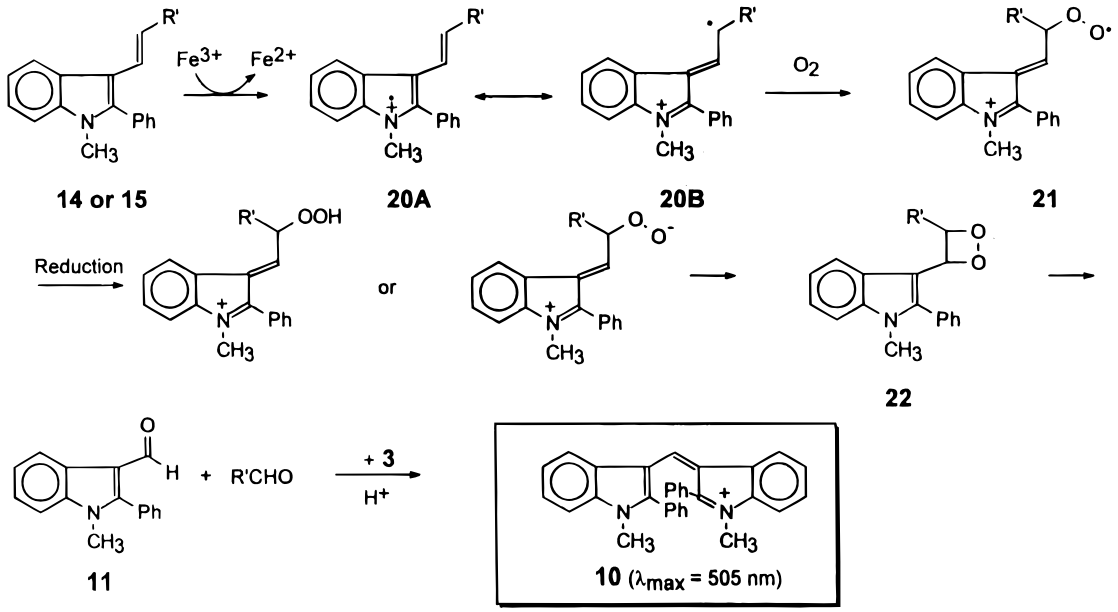
Discussion

An interesting feature of the colorimetric assay of MDA and 4-HNE as byproducts of lipid peroxidation is the formation of the same chromophore **4** at 586 nm. Starting from MDA, this is a well-known acid-induced condensation reaction with indoles (*6*, *11*, *12*). Starting from 4-HNE, the formation of the same chromophore **4** obviously requires an oxidative fragmentation of the alkyl side chain, as shown by the concomitant formation of hexanal in the stoichiometric reaction (see eq 2). From a mechanistic point of view, cleavage of the side chain could be explained by a classical *β*-fragmentation (*13*), if an alkoxyl radical **16** was formed by one-electron oxidation of the rapidly interconverting adducts **5**−**7**. This hypothetical alkoxyl radical **16** would then afford hexanal

Scheme 4



Scheme 5. Proposed Mechanism for the Iron(III)-Mediated Formation of Chromophore 10 (505 nm) via Oxidative Cleavage of 14 or 15 to 11



and an allyl radical **17**, which should be easily oxidized to the final chromophore **4** (see Scheme 2). Identification of the actual oxidant under the assay conditions would require further investigation. Keeping in mind that catalytic amounts of Fe(III) in the presence of oxygen as well as 2 equiv of $FeCl_3$ under anaerobic conditions are sufficient for the reaction to be complete (see Figure 1), we could envisage various mechanisms. One possibility would be the addition of O_2 to carbon-centered radical **17** followed by the extrusion of superoxide, most likely in the form of $HO_2^{\bullet}$ which would easily recycle iron(III) from iron(II).

The mechanism responsible for the formation of postulated alkoxyl radical **16** is less obvious. As described above, intermediates **5**−**7** are all possible precursors of chromophore **4**, with 1-indolylalkene **7** reacting by far more quickly in the presence of iron(III) chloride.

A one-electron oxidation (*14*) by iron(III) (*15*) would afford radical cations **18** and **19** from the tetrahydrofuran intermediate **5** and from 1-indolylalkene **7**, respectively (see Scheme 3). While the oxidation of **5** via β-fragmentation of the mesomeric form **18B** would lead to the alkoxyl radical **19**, which represents the protonated form of alkoxyl radical **16** (pathway A), an indirect pathway can be envisaged for the transformation of 1-indolylalkene **7** to alkoxyl radical **16** (see Scheme 3, pathway B). Despite the apparent greater complexity of the second pathway, it is in better agreement with the experimental facts.

Not only the greater reactivity of 1-indolylalkene **7** with $FeCl_3$ under model conditions but also the supposed lower oxidation potential of the 1-indolylalkene fragment in **7** compared to that of the nonconjugated system in **5** favors 1-indolylalkene **7** as the precursor of chromophore **4** via alkoxyl radical **16** (see Scheme 3).

The formation of a second chromophore at 505 nm is observed in the iron(III)-catalyzed reaction of indole **3** with 4-HNE, but also with alkanals. We therefore

assumed that hexanal produced upon β-fragmentation reacted immediately under the assay conditions with excess indole **3** to yield chromophore **10** and pentanal and so on (see Scheme 4).

When the implications of both oxygen and iron(III) are considered in the transformation of alkanals to 505 nm chromophore **10** (see Figure 3), we suggest that 1-indolylalkene **15** which is obtained from the reaction of hexanal with indole **3** is oxidized by iron(III) to radical cation **20A** (Scheme 5). The mesomeric form **20B** would be trapped by oxygen, yielding a peroxyl radical intermediate **21** which could then yield indolyl dioxetane **22** after reduction and nucleophilic intramolecular addition. Dioxetanes are known to yield carbonyl compounds by electrocyclic ring opening (*16*), e.g., in this case the 3-formylindole **11** and heptanal starting from **14** and **11** and pentanal from **15**. As shown before, 3-formylindole **11** reacts immediately with indole **3** to give 505 nm chromophore **10** (see Scheme 5). A similar indolyldioxetane is proposed (*17*) to explain the fragmentation of 1-methyl-3-styrylindole to 3-formylindole and benzaldehyde upon reaction with singlet oxygen.

Besides the role of this reaction in the colorimetric assay of byproducts of lipid peroxidation, the global reaction represents a one-carbon fragmentation of a saturated aldehyde under relatively mild conditions. This rather rare reaction type was observed earlier by Read et al. (*18*, *19*) during the reaction of alkanals with 2-thiobarbituric acid (TBA) and their oxo analogues in the presence of iron(III). Their proposed mechanism (*18*, *19*) included the oxidation of an allylanion by iron(III), followed by reaction of oxygen with the allyl radical and anionic fragmentation of the hydroperoxide intermediate to give the chromophore and an aldehyde which is one methylene group shorter than the starting aldehyde.

Even if our hypothetical pathway, including a dioxetane intermediate as proposed in Scheme 4, is not fully demonstrated, the anionic pathway proposed by Read et al. for the TBA reaction does not seem to be compatible with the strong acidic conditions of our indole-based assay.

A striking aspect of the colorimetric assay for 4-HNE is the influence of the acid used, with HCl suppressing the formation of both 586 nm chromophore **4** and 505 nm chromophore **10**, in contrast to the complete transformation observed in the presence of methanesulfonic acid. Our experiments have shown that the same intermediates **5**−**7** are produced from the reaction of 4-HNE with indole **3**, in the presence of either methanesulfonic or hydrochloric acid. Similarly, the reaction of hexanal and octanal with **3** afforded 1-indolylalkenes **14** and **15**, respectively, under both conditions as precursors for the formation of the 505 nm chromophore **10**.

Obviously, the influence of the acid has some bearing on the transformation of intermediates to final chromophores **4** and **10**. As proposed in Schemes 3 and 5, the formation of 586 nm chromophore **4** from **5** or **7** and that of 505 nm chromophore **10** from **14** or **15** require an oxidation step. A difference in the coordination sphere of the iron(III) complex in the reaction mixture could possibly account for the experimentally observed differences. While the weakly coordinating (*20*, *21*) and hence more flexible methanesulfonato ligands would enable the coordination of indole intermediates to the iron(III), the chloride counterions, being more nucleophilic (*22*), could inhibit or delay that coordination and therefore the oxidation and subsequent transformations to chromophores **4** and **10**. The difference in the coordination sphere of the iron(III) complex would then account for the selective assay of MDA in the presence of 4-HNE in hydrochloric acid medium.

In conclusion, this study outlines the chemical background of a new colorimetric assay of MDA and 4-HNE as an example for 4-hydroxyalkenals. A detailed investigation of the reaction of these aldehydes with indole **3** showed that the oxidation of 1-indolylalkene intermediates by iron(III) to the corresponding radical cations is a prerequisite of the formation of chromophores **4** and **10**.

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