Generation of Pyrroles in the Reaction of Levuglandin E_2 with Proteins[1]

Rajkumar S. Iyer, Michael E. Kobierski, and Robert G. Salomon*

Department of Chemistry, Case Western Reserve University, Cleveland, Ohio 44106-7078

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The reaction of levuglandin (LG) E_2 with proteins generates pyrrole derivatives that are detected with an Ehrlich assay. The pyrroles formed by the reaction of LGE_2 with simple amines are chemically sensitive, but a stable derivative is obtained by trifluoroacetylation.

Introduction

Levuglandin (LG) E_2 (**3**) is a fatty acid oxidative metabolite from arachidonic acid (AA, **1**). LGE_2 is coproduced[2] with, inter alia, prostaglandin (PG) E_2 (**4**) by rearrangements of the prostaglandin endoperoxide PGH_2 (**2**) that occur readily ($t_{1/2}$ = 5 min at 37 °C) in the aqueous environment of its biosynthesis (Scheme 1).[3] A remarkable characteristic of LGE_2 is its proclivity toward covalent binding with biological nucleophiles. Thus, we discovered that LGE_2 acts like molecular glue, sticking to proteins[4] and causing intermolecular crosslinking of proteins[5] as well as forming repair resistant DNA-protein cross-links in the nuclei of mammalian cells.[6]

Since binding of LGE_2 with protein is rapid relative to cross-linking,[4] we were able to use glycine as a model for the second protein molecule[7] to probe the reactivity of LGE_2-protein adducts toward primary amino groups of a second protein molecule. With ovalbumin (OA), addition of glycine to the reaction mixture after a 5 min incubation of LGE_2 with protein results in binding of 2 molecules of glycine per molecule of LGE_2. No glycine binds with OA that has not been preincubated with LGE_2. These observations can be accommodated by a mechanism involving formation of a dihydroxypyrrolidine (DHP) intermediate by reaction of LGE_2 with the ϵ-amino group of a lysyl residue in OA (Scheme 2). Cross-linking could occur by reaction of the ϵ-amino group of a lysyl residue in a second molecule of OA with the DHP intermediate forming an aminal from the hemiaminal in the DHP. Two molecules of glycine can react with the DHP to form a bisaminal. This also accounts for the ability of glycine to prevent cross-linking of protein by LGE_2.[5] Especially noteworthy was the fact that, upon aging, the ability of the LGE_2-protein adducts to bind glycine decreases. This loss of electrophilicity might be the consequence of conversion of the DHP intermediate into an aromatic pyrrole derivative (**5**).

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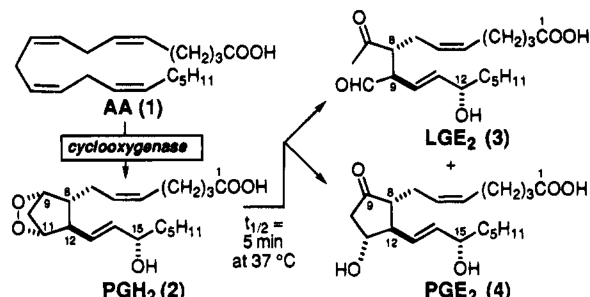
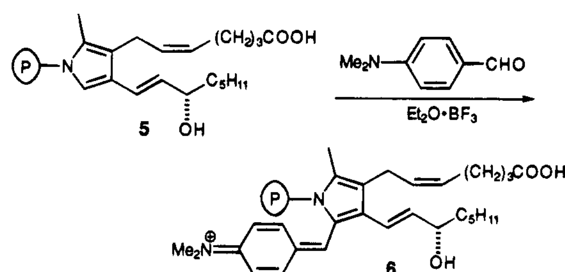
Scheme 1



We now report new evidence for pyrrole formation in the reaction of LGE_2 with proteins. Thus, the reaction of LGE_2 with simple primary amines gave pyrroles as major products. Also, the formation of pyrrole **5** in the reaction of proteins with LGE_2 was detected with the Ehrlich reagent[8] that produces a blue-green chromophore. The reagent, *p*-(dimethylamino)benzaldehyde-$BF_3{\cdot}OEt_2$ generates **6** by electrophilic aromatic substitution of the ring hydrogen in **5**.



Results and Discussion

Pyrrole Formation in the Reaction of LGE_2 with Simple Primary Amines. The condensation of 1,4-dicarbonyl compounds with primary amines is the Paal-Knorr synthesis of *N*-alkylpyrroles.[9] In pilot studies, a simple trisubstituted pyrrole (**12**) was prepared to provide some familiarity with the chemical reactivity of a 2,3,4-trialkylpyrrole (Scheme 3). A synthesis of the required γ-keto aldehyde **11** was accomplished by our general method that exploits selective tris(triphenylphosphine)-ruthenium(II) dichloride-catalyzed rearrangement of unsymmetrical diallyl ethers to γ,δ-unsaturated aldehydes.[10] Thus, O-alkylation of the allyl alcohol **7** afforded an unsymmetrical diallyl ether **8**. Ruthenium(II)-catalyzed rearrangement of **8** regioselectively afforded a mixture of diastereomeric aldehydes **10** in 77% isolated

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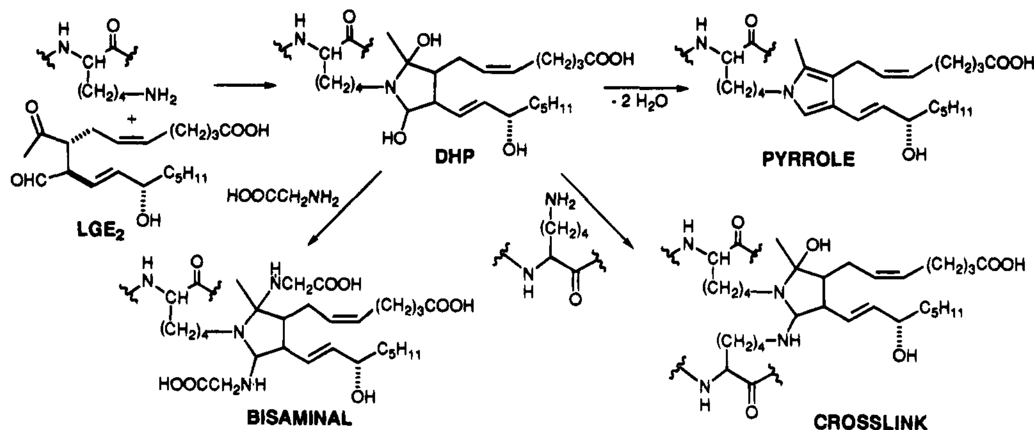
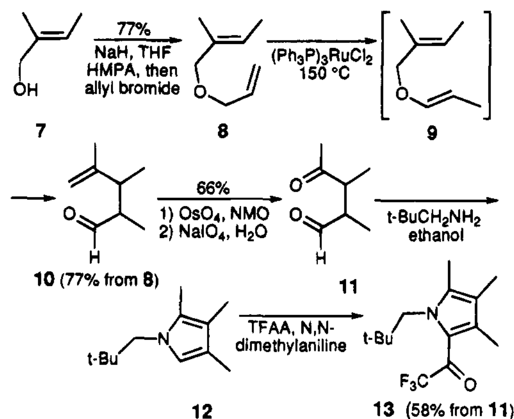
Scheme 2



Scheme 3



yield. As precedented,[10] allylic isomerization of the less substituted C=C bond in **8** occurs in preference to the more substituted C=C bond. Preliminary rearrangement to the allyl vinyl ether **9** followed by *in situ* Claisen rearrangement to **10** presumably is involved. Ozonolysis of the C=C bond in **10** followed by reductive workup did not give satisfactory yields of **11**. However, Os(IV)-catalyzed vicinal hydroxylation[11] to afford a vicinal diol followed by *in situ* oxidative cleavage of the vicinal diol with sodium periodate proceeded in 66% yield to give the γ-keto aldehyde **11**. ^{1}H NMR analysis of the flash chromatographically purified keto aldehyde **11** indicated two singlets at δ 2.21 and 2.16, each of which integrated to 1.5 H. Further, three sets of doublets are found in the methyl hydrogen region. This spectrum indicates that **11** is a 1:1 mixture of two diastereomers. As the two chiral centers in **11** would be lost upon conversion to a pyrrole, no attempt was made to separate this mixture of stereoisomers. Reaction of the γ-keto aldehyde **11** with neopentylamine in ethanol produced the pyrrole **12** as evidenced by ^{1}H NMR analysis of the crude reaction product mixture. However, attempted purification by flash chromatography over silica gel caused extensive decomposition. As the highly alkylated, electron rich pyrrole **12** was unstable, it was reasoned that an electron withdrawing trifluoroacetyl substituent on the ring might provide stabilization. Thus, *in situ* trifluoroacetylation of **12** with trifluoroacetic anhydride[12] in the presence of *N,N*-dimethylaniline provided **13** in 58% isolated overall yield from keto aldehyde **11**. The trifluoroacetylated pyrrole **13** is stable. No decomposition occurs over a period of several days at room temperature. The ^{1}H NMR spectrum of **13** ($CDCl_3$) shows two resonances for the two *N*-methylene hydrogens as two broad doublets at δ 5.04 and 3.64. Variable temperature ^{1}H NMR experiments in DMSO-d_6 suggested restricted rotation about the N–CH_2 bond or about the trifluoroacetyl to pyrrole C–C bond in this vinylogous amide. Thus, in DMSO-d_6, on increasing the temperature the two doublets at δ 3.8 and 4.8 gradually flattened out until they were barely visible at about 60 °C. At 70 °C, a broad singlet began to develop, and by 100 °C, a singlet had appeared at δ 4.3. Cooling the sample back to room temperature resulted in the reappearance of the two doublets at δ 3.8 and 4.8, respectively.

As chiral centers at C-8 and C-9 in LGE$_2$ methyl ester will be lost upon Paal–Knorr condensation with a primary amine, the more readily available 8,9-bisepi-LGE$_2$ methyl ester (**14**), derived from (*R*)-isopropylidene-D-glyceraldehyde as previously reported,[13] was used. Thus, condensation of neopentylamine with **14** proceeded readily in ethanol to afford pyrrole **15** in >90% yield. Pyrrole **15** was purified by HPLC, limiting exposure to air by constantly bubbling a stream of argon through both the sample and column eluates. By careful control of reaction conditions and rigorous exclusion of air, **15** was isolated in yields as high as 91%. Solutions of **15** in chlorinated solvents (CH_2Cl_2, $CDCl_3$) are especially unstable, decomposing on standing at room temperature. Thus, a $CDCl_3$ solution of **15** was monitored by ^{1}H NMR and TLC. On standing at room temperature, *under argon*, new spots appeared on TLC, and after 12 h, complete decomposition of **15** was evidenced by both TLC analysis and loss of characteristic pyrrole resonances in the ^{1}H NMR spectrum. However, solutions of **15** in benzene-d_6, CD_3CN, or pyridine-d_6 under argon were more stable. Characteristic in the ^{1}H NMR spectrum of **15** are the downfield aromatic and vinyl resonances. The sole ring hydrogen resonance appears as a singlet at δ 6.64. The doublet at δ 6.31 and the doublet of doublets at δ 5.68 are assigned to the two vinylic hydrogens in the octenyl side chain.

In analogy with the electron rich pyrrole **12**, a stable derivative was prepared from the levuglandin-derived pyrrole **15** by trifluoroacetylation. Thus, treatment of **15** with trifluoroacetic anhydride afforded a stable bistrifluoroacetylated derivative (**16**) in 40% overall yield from

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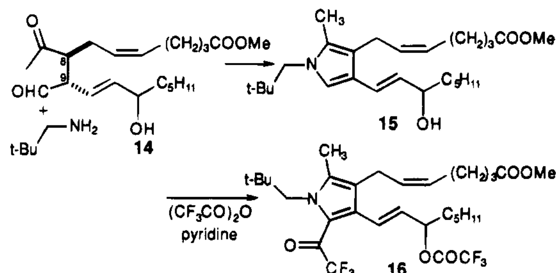
14. As seen for the simpler trifluoroacetyl pyrrole **13**, the ^1H NMR spectrum of **16** reveals restricted rotation around either the trifluoroacetyl to pyrrole bond or the $N-CH_2$ bond. The two *N*-methylene hydrogen resonances appear as broad doublets at δ 4.95 and δ 3.60, integrating to one proton each.



Pyrrole Formation in the Reaction of LGE$_2$ with Proteins. (a) Background. The purple-red color produced from the acid-catalyzed reactions of *p*-(dimethylamino)benzaldehyde (DMAB) with pyrroles and indoles which posses an unsubstituted site on the heterocyclic ring forms the basis of the classical Ehrlich test for π-electron-excessive heterocycles.[8,14] Protein-bound pyrroles, formed in the reaction of the neurotoxic diketone 2,5-hexanedione (HD) and various proteins, have been detected both *in vivo* and *in vitro* by reaction with DMAB. Thus, the Ehrlich assay was used to detect and quantitate HD derived pyrroles bound to bovine serum albumin (BSA), ovalbumin,[15] hemolysate proteins, axonal pad proteins,[16] and globin[17] isolated from rats exposed to HD.

In vitro mechanistic studies with various proteins demonstrated conversion of lysine ϵ-amino groups to pyrrole adducts by HD, with the degree of conversion directly proportional to the lysine content of the protein.[15] Model studies, conducted at near physiological conditions (pH 7.4, 37 °C), indicated that the ϵ-amino group of lysine was about 6 times more reactive toward various diketones than the α-amino group. Quantitative amino acid analysis of pyrrolylated BSA revealed molar decreases in lysine content equivalent to DMAB-detectable pyrrole adduct concentrations; no other amino acids were significantly altered.[15,18] Further, the rates of pyrrole formation in aqueous buffered solutions were shown to be pH dependent over a wide range, showing a progressive increase in the rate of heterocycle formation with increase in pH. Thus, over the pH range 5.5–8.0, the rate of pyrrole formation increased approximately 150-fold. This was interpreted largely as a reflection of the concentration of free base which increases 300-fold over this pH range.[16,18]

The reaction of γ-keto aldehydes such as LGE$_2$ (**3**) with proteins in aqueous solutions, while probably differing somewhat in mechanistic details, was expected to form pyrroles in a manner analogous to that seen for 2,5-hexanedione. Therefore, the Ehrlich assay was used to examine the formation of protein-bound levuglandin-derived pyrroles in the reaction of LGE$_2$ with serum albumins.

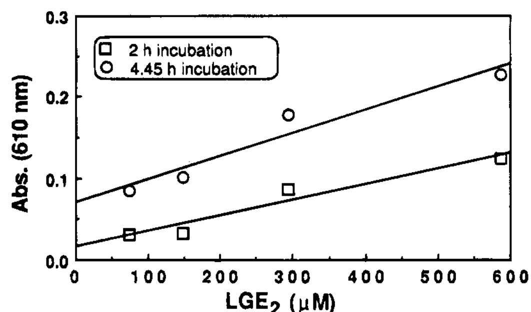


Figure 1. Ehrlich detection of LGE$_2$–BSA–pyrrole adducts. Time and concentration dependence of the extent of pyrrole formation. BSA (1.47×10^{-4} M) was incubated with LGE$_2$ (74–588 μM) for 2 or 4.45 h at 37 °C and pH 7, followed by addition of DMAB. Data are from Table 1 (see Experimental Section).

Table 1. Concentration and Time Dependence for the Appearance of Ehrlich Chromophore in the Reaction of BSA with LGE$_2$ at the Initial LGE$_2$ Concentrations and Reaction Times Indicated[a]

	absorbance (610 nm)	
LGE$_2$ (μM)	2 h	4.45 h
74	0.031	0.085
147	0.033	0.101
294	0.087	0.177
588	0.124	0.226

[a] Reaction conditions: 0.1 M pH 7 phosphate buffer, 37 °C (Data for Figure 1).

(b) DMAB Detection of Pyrroles Formed in the Reaction of LGE$_2$ with Bovine and Human Serum Albumins. Bovine serum albumin (BSA) was incubated with various initial concentrations of LGE$_2$ in pH 7, 0.1 M phosphate buffer at 37 °C. At time intervals of 2 and 4.45 h from the start of the reaction, aliquots were assayed for pyrrole content using the Ehrlich reagent (Figure 1). The chromophore (λ_{max} = 610 nm, blue-green colored) that developed increased in intensity with increasing initial LGE$_2$ concentration. While the formation of **6** is expected, the exact structure of the chromophore is not known. It is likely that under the highly acidic conditions of the Ehrlich test, acid-catalyzed dehydration of the allylic alcohol function in **6** would occur. This would produce **17**, which contains a highly conjugated chromophore.

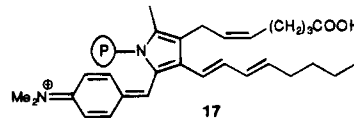


Figure 1 shows a linear correlation between the amount of pyrrole formed and the amount of LGE$_2$ present initially in the reaction mixture. At all initial concentrations of LGE$_2$ employed, more DMAB-detectable pyrrole is formed at longer incubation time (4.45 h compared to 2 h).

A modified Ehrlich assay procedure (method B) was used previously to detect pyrroles (1–3 nmol/mg protein) bound to globin[17] or tubulin.[19] These studies incorporated a peptic or tryptic digest of the protein samples prior to

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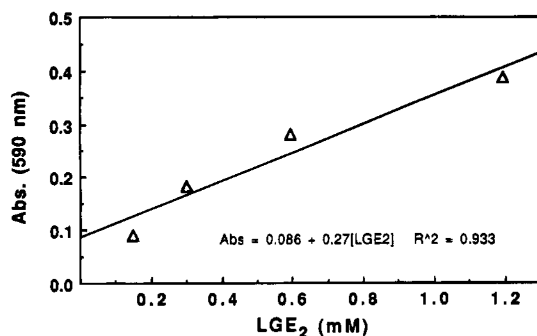

Figure 2. Effect of peptic digest on the development of Ehrlich chromophore in the reaction (2.5 h, pH 7.4, 37 °C) of BSA (7.35×10^{-5} M) with LGE_2 (150–1200 μM). Samples were digested with pepsin (pH 4, 2 h) prior to Ehrlich test. Three separate incubations were performed; the average of three measurements is shown. Data are from Table 2 (see Experimental Section).

Table 2. Incorporation of Peptic Digest in the Ehrlich Assay for Pyrroles Formed in the Reaction of BSA with LGE_2.[a] Three Separate Incubations Were Set Up and Assayed Identically (Data for Figure 2)

	absorbance (590 nm)			
LGE_2 (mM)	no. 1	no. 2	no. 3	average
0.15	0.096	0.117	0.059	0.090
0.30	0.189	0.202	0.157	0.183
0.60	0.290	0.270	0.288	0.283
1.20	0.382	0.370	0.407	0.386

[a] Reaction conditions: pH 7.4 0.1 M phosphate buffer, 2.5 h, 37 °C, followed by peptic digest (pH 4, 2 h, 37 °C); Ehrlich assay method B.

performing the Ehrlich test. In an attempt to improve the sensitivity of the assay for lower levels of LG-derived pyrroles, BSA was incubated with LGE_2 for 2.5 h at 37 °C in 0.1 M pH 6 phosphate buffer, and then the incubates were digested with pepsin (2 h, pH 4) prior to DMAB assay (method B). Figure 2 shows the results of three separate incubations. Interestingly, the chromophore shows λ_{max} = 590 nm instead of 610 nm. As shown in Figure 2, the concentration-dependent increase in the amount of chromophore produced is fairly reproducible but no significant improvement over method A was evident.

Using assay method A, the reaction of human serum albumin (HSA) with LGE_2 was examined. Thus, HSA was incubated with LGE_2 (2.5–45 μM) at pH 7.4 (phosphate-buffered saline solution) for 4 h at 37 °C. Protein was then precipitated with saturated ammonium sulfate solution and dialyzed extensively at 4 °C against buffer. The dialysate was analyzed for both pyrrole content (Ehrlich assay) and total protein concentration (biuret assay). A ratio (absorbance at 610 nm/mg of protein) was calculated and plotted against the initial concentration of LGE_2 in the reaction mixture (Figure 3). A linear relationship between the initial concentration of LGE_2 and the intensity of the chromophore produced is evident.

The results reported now support our earlier hypothesis that pyrroles are formed in the reaction of LGE_2 with proteins. The experiments using the Ehrlich reagent to detect pyrrole formation agree with the inference, from studies of the ability of LG–protein adducts to bind glycine, that pyrrole formation is a slow process, probably requiring many hours for completion (see Figure 1). Further, the present studies show that the Ehrlich assay can detect the micromolar levels of LGs involved in these

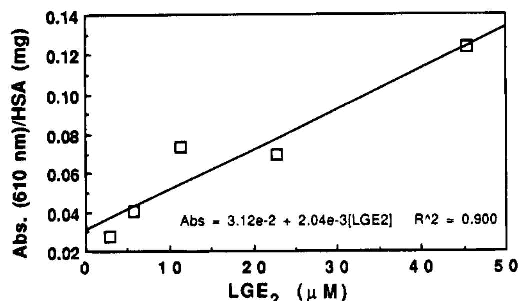

Figure 3. Detection of LGE_2-derived pyrrole bound to HSA. Effect of initial LGE_2 concentration on the extent of pyrrole formation (pH 7.4, 0.01 M PBS, 4 h, 37 °C). Total protein was determined (biuret assay), and pyrrole content determined by Ehrlich assay. Data are from Table 3 (see Experimental Section).

Table 3. Ehrlich Detection of Pyrroles in the Reaction of LGE_2 with HSA.[a] The Absorbance at 610 nm Was Divided by the Total Protein Concentration To Give the Ratio Absorbance at 610 nm/mg HSA (Data for Figure 3)

LGE_2 (μM)	absorbance (610 nm)	HSA[b] (mg)	absorbance (610 nm)/HSA (mg)
2.84	0.113	4.125	0.027
5.68	0.163	4.002	0.041
11.36	0.278	3.742	0.074
22.72	0.314	4.523	0.070
45.55	0.477	3.858	0.124

[a] Reaction conditions: 0.01 M pH 7.4 PBS, 4 h, 37 °C followed by ammonium sulfate precipitation and dialysis. [b] As determined by the biuret assay.

in vitro studies. However, at lower initial concentrations of LGE_2, Ehrlich colors were seen but the intensities were quantitatively irreproducible and good linear correlations were seldom obtained (results not shown). Thus, the Ehrlich assay would not be sensitive enough to detect levuglandin-derived protein-bound pyrroles formed *in vivo* if the levels present are well below micromolar concentrations.

Experimental Section

General. Proton nuclear magnetic resonance (NMR) spectra were recorded either at 200 or 400 MHz. Proton chemical shifts are reported in parts per million on the δ scale relative to tetramethylsilane (δ 0.00). Tetramethylsilane or residual chloroform (δ 7.24) was used as the internal standard. Significant ^{1}H NMR spectral data are tabulated in order: number of protons, multiplicity (s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; br, broad), and coupling constant(s) in hertz. The use of "apparent" in proton multiplicity descriptions implies a hyperfine splitting necessarily more complex than first order. All NMR samples were analyzed as solutions in $CDCl_3$. ^{13}C NMR spectra were recorded either at 50.3 or 100.6 MHz. ^{13}C NMR are reported in parts per million on the δ scale relative to chloroform-*d* (δ 77.0). Attached proton test (APT)[20] spectra for ^{13}C are designated as (+) to indicate a quaternary or methylene carbon or (−) to indicate a methine or methyl carbon, after the chemical shift position of the carbon resonance. High-resolution mass spectra were recorded on a dual beam, double focusing magnetic sector mass spectrometer. Samples were introduced to the ionization chamber by direct probe insertion. Thin layer chromatography (TLC) was performed on glass plates precoated with silica gel (kieselgel 60 F_{254}, E. Merck), R_f values are quoted for plates of thickness 0.25 mm. Visualization was done by viewing the developed plates under short-wavelength UV light and by heating the plates after spraying with vanillin–sulfuric acid. Flash

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column chromatography was performed on 230–400 mesh silica gel. For HPLC, the eluate was monitored with a differential refractometer or a UV absorbance detector. The pH of buffer solutions was measured at room temperature using a pH meter that was calibrated in the desired range using standard buffer solutions of pH = 7, and pH = 4 or pH = 10. UV spectra were recorded using matched (far UV) quartz cells of 10 mm path length. UV or visible spectra were recorded on a UV–visible–NIR spectrophotometer using a multiscan data acquisition program. For all experiments, the sample holder, as well as the sample, was kept at a constant temperature of 37 ± 0.5 °C using a cryostat. Disposable semimicro polystyrene cuvettes (1.6 mL capacity, 10 mm path length) were used. Homogenations were performed with a Brinkman kinematica Gmbh Polytron type Pt 10/35 using a PCU 1 power source. Smaller scale homogenations were carried out using a hand-held, Potter-Elvehjem tissue grinder fitted with a Teflon pestle. Standard cellulose dialysis tubing (M_r cut off 12 000–14 000) was used.

Microdetermination of total protein concentration was performed using a modified biuret reagent as described by Ohnishi and Barr.[21] A calibration curve was set up by monitoring the absorbance of protein standards at 725 nm.

Materials. All reactions were performed in an inert moisture free atmosphere under a positive pressure of nitrogen or argon except when working in aqueous media. Purification and handling of all solvents and reagents used in synthetic procedures were conducted under a nitrogen or argon atmosphere except for aqueous solutions. All solvents were reagent grade or purer. Ethyl acetate, hexane, and diethyl ether used for extractions or chromatography were distilled to remove nonvolatile impurities prior to use. Water used in reactions was purified by passage through a reverse osmosis membrane to remove organic and particulate matter followed by distillation under nitrogen with partial condensation. Monobasic sodium phosphate, dibasic sodium phosphate, and citric acid were ACS grade. Levuglandin E_2 (LGE_2) was synthesized as described previously.[13] Sodium phosphate pH = 5.7 to pH= 8.0 buffers were prepared according to published procedures.[22] Methanol and DMSO used were of spectrophotometric grade. Absolute (200 proof) ethanol was used without further purification.

(*E*)-2-Methyl-2-buten-1-ol (7). (*E*)-2-Methyl-2-butenal (10.0 g, 0.119 mol) was dissolved in methanolic $CeCl_3{\cdot}7H_2O$ (298 mL, 0.4 M, 44.4 g of $CeCl_3{\cdot}7H_2O$ in 298 mL of methanol), and sodium borohydride (4.5 g, 0.119 mol) was slowly added (2 min) with stirring. The reaction mixture was allowed to react at room temperature for 15 min and then treated with water (100 mL) and stirred for 5 min. Methanol was removed on the rotary evaporator, and the aqueous mixture was extracted with ether (4 × 100 mL). The combined ether extracts were dried over anhydrous magnesium sulfate and concentrated in vacuo to give an oil (7.6 g). Distillation gave (*E*)-2-methyl-2-buten-1-ol (**7**, 6.75 g, 78.4 mmol, 66% yield): bp 134–138 °C (760 mmHg); ^{1}H NMR (200 MHz, $CDCl_3$) δ 5.42 (1 H, m), 3.91 (2 H, s), 2.24 (1 H, br s), 1.60 (3 H, s), 1.57 (3 H, d, J = 6.85 Hz); lit.[23] ^{1}H NMR δ 5.5 (1 H, m), 3.98 (2 H, s), 1.62 (3 H, s), 1.68 (s H, d).

Allyl 2,3-Dimethylallyl Ether (8). NaH (8.36 g, 174 mmol, 50% oil dispersion) in a 500 mL three-necked flask equipped with reflux condenser, mechanical stirrer, and addition funnel was washed with pentane (2 × 10 mL). THF (300 mL) was then added followed by the portionwise addition of (*E*)-2-methyl-2-buten-1-ol (10.0 g, 116 mmol), and the resulting mixture was stirred 2 h under reflux. Then, while the mixture was still warm, HMPA (57 mL) was added followed by allyl bromide (21.05 g, 174 mmol) at such a rate as to maintain a gentle reflux. Upon addition, the mixture was again boiled 2 h under reflux. The reaction mixture was allowed to cool to room temperature, quenched with 10% aqueous HCl (100 mL), and extracted with pentane (3 × 75 mL). The combined organic fractions were washed with 10% aqueous HCl, saturated $NaHCO_3$, H_2O, and saturated NaCl and dried ($MgSO_4$). Solvent removal with rotary evaporation and distillation of the crude product yielded the title ether (11.2 g, 76.5%): bp 140–142 °C (760 mmHg); ^{1}H NMR (200 MHz, $CDCl_3$) δ 6.20–4.94 (4 H), 3.97–3.76 (4 H), 1.77–1.47 (6 H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 135.0, 132.8, 122.4, 116.6, 76.2, 70.4, 13.5, 13.1; mass spectrum m/z (M^+) calcd for $C_8H_{14}O$ 126.1044, found 126.1051. This diastereomeric mixture was not separated.

2,3,4-Trimethylpent-4-enal (10). Allyl 2,3-dimethylallyl ether (**8**) (8.7 g, 69 mmol) and tris(triphenylphosphine)ruthenium dichloride (66.2 mg, 0.07 mmol, 0.1 mol %) were mixed together and refluxed at 150 °C. The reaction was monitored for disappearance of starting material **8** by TLC in 20% ethyl acetate in hexanes (R_f values for **8** and **10** are 0.6 and 0.5, respectively). After 3 days, the reaction was essentially complete. The reaction mixture was allowed to cool to room temperature and filtered through a Celite bed to remove the catalyst. Distillation of the crude mixture afforded **10** (6.7g, 77%): bp 151–153 °C (760 mmHg); ^{1}H NMR (200 MHz, $CDCl_3$) δ 9.39–9.37 (1 H, m), 4.59–4.53 (2 H), 2.43–2.04 (2 H), 1.50 (1.5 H, s), 1.43 (1.5 H, s), 0.85–0.75 (6 H, 3 overlapping d, J = 6.51 Hz); ^{13}C NMR (50 MHz, $CDCl_3$) this diastereomeric mixture of aldehydes gives two peaks for each C; δ 205.1, 205.9, 147.0, 146.2, 112.2, 111.5, 49.5, 48.7, 42.3, 41.0, 20.47, 18.9, 17.6, 15.2, 12.3, 9.8; mass spectrum m/z (M^+ − 1) calcd for $C_8H_{13}O$ 125.0966; found 125.1101. Without separation of the diastereomers, this mixture was used for the preparation of 2,3-dimethyl-4-oxopentanal.

2,3-Dimethyl-4-oxopentanal (11). To a magnetically stirred solution of **10** (100 mg, 0.79 mmol) and *N*-methylmorpholine *N*-oxide (130.2 mg, 1.11 mmol) in 20.0 mL of 10% water in acetone (v/v) was added a solution of OsO_4 (600 μL, 0.012 mmol) in *tert*-butyl alcohol (0.02 M, 1.0 g of OsO_4 in 196 mL of *tert*-butyl alcohol containing 0.5% *tert*-butyl hydroperoxide). The reaction mixture was stirred overnite at room temperature, whereupon TLC analysis of the reaction mixture in 50% ethyl acetate in hexanes (v/v) indicated complete disappearance of starting material **10** (R_f = 0.52) and the appearance of a new, more polar spot (R_f = 0.14). A 1:1 (w/w) mixture of $NaHSO_3$ and Na_2SO_3 (600 mg) was then added to the reaction mixture and stirring continued for 5 min. Acetone was then removed by rotary evaporation, and the residue was taken up in ethyl acetate (3 × 15 mL) and filtered through a short silica gel column (5 mm × 50 mm). Removal of solvent in vacuo afforded crude diol that was used without further purification. To the crude diol was added a solution of $NaIO_4$ (267.0 mg, 1.25 mmol) in 5.0 mL of acetone–water (2:5 v/v), and the mixture was stirred at room temperature. The reaction was monitored by TLC in 50% ethyl acetate in hexanes (R_f values for the diol and **11** are 0.14 and 0.48, respectively). After 6 h, the reaction was essentially complete. The reaction mixture was diluted with water (20 mL) and extracted with ethyl acetate (3 × 25 mL). The organic extracts were dried ($MgSO_4$) and concentrated in vacuo. The residue was flash chromatographed over silica gel, eluting with ethyl acetate–hexanes (1:1) to afford the keto aldehyde **11** (66.7 mg, 65.5%): ^{1}H NMR (200 MHz, $CDCl_3$) δ 9.66 (0.5 H, s), 9.63 (0.5 H, s), 2.98–2.54 (2 H), 2.21 (1.5 H, s), 2.16 (1.5 H, s), 1.20–1.06 (6 H, three sets of doublets at δ 1.18, 1.09, 1.08; J = 6.9 Hz, 7.55 Hz, 7.0 Hz); mass spectrum m/z (M^+) calcd for $C_7H_{12}O_2$ 128.0837; found 128.0845. Although TLC analysis showed only a single spot (R_f = 0.46 in EtOAc-hexane 1:1), the ^{1}H NMR spectrum of this keto aldehyde clearly shows that it is a ~1:1 mixture of diastereomers. Without separation of the diastereomers, this mixture was used for the preparation of 1-neopentyl-2-(trifluoroacetyl)-3,4,5-trimethylpyrrole.

1-Neopentyl-2-(trifluoroacetyl)-3,4,5-trimethylpyrrole (13). Neopentylamine (12.4 mg, 0.143 mmol, 1.2 equiv) was added to a stirred solution of keto aldehyde **11** (15.2 mg, 0.119 mmol) in THF (1.0 mL) at room temperature under an atmosphere of argon. After the solution was stirred for 45 min at room temperature, TLC analysis (50% ethyl acetate in hexanes) of the reaction mixture indicated complete disappearance of starting material (R_f = 0.48) and the appearance

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of a new, UV-active spot (R_f = 0.72; yellow color on vanillin spray and purple on DMAB reagent). THF was evaporated under a stream of nitrogen. The crude pyrrole **12** was used immediately in the following step since attempts to purify it by flash chromatography resulted in extensive decomposition. The crude pyrrole was thus dissolved in ether (1.0 mL), and to the solution was added sequentially *N,N*-dimethylaniline (21.6 mg, 0.18 mmol, 1.5 equiv) and trifluoroacetic anhydride (30.0 mg, 0.143 mmol, 1.2 equiv) under an atmosphere of argon. The reaction was allowed to proceed at room temperature overnight. TLC analysis of the reaction mixture (2% ethyl acetate in hexanes) indicated the complete absence of starting material (R_f = 0.31) and the formation of a new spot (R_f = 0.21). The reaction mixture was extracted with ether (3 × 10 mL), washed with water (3 × 10 mL), and dried ($MgSO_4$), and solvents were removed by rotary evaporation. The residue thus obtained was flash chromatographed, eluting with 2% ethyl acetate in hexanes to afford 63.6 mg of product contaminated with *N,N*-dimethylaniline. The product was purified further by HPLC, eluting with 5% ethyl acetate in hexanes (flow rate = 1.0 mL/min, UV detection) to give **13** (18.9 mg, 57.9% overall yield for two steps) as a single symmetrical HPLC peak that was well resolved from a second fraction corresponding to *N,N*-dimethylaniline. **13:** ^{1}H NMR (200 MHz, $CDCl_3$) δ 5.04 (1 H, br d, *J* = 14.4 Hz), 3.64 (1 H, br d, *J* = 14.4 Hz), 2.22 (6 H, s), 1.94 (3 H, s), 0.84 (9 H, s); mass spectrum *m/z* (M^+) calcd for $C_{14}H_{20}NOF_3$ 275.1497, found 275.1488.

3-(6-Carbomethoxy-2(*Z*)-hexenyl)-4-(3-hydroxy-1(*E*)-octenyl)-2-methyl-1-neopentylpyrrole (15). Neopentylamine (32 mg, 0.375 mmol) was added to a stirring solution of 8,9-bisepi-LGE$_2$ methyl ester (**14**) (125 mg, 0.341 mmol) in deaerated, anhydrous ethanol (1.0 mL). The reaction mixture was stirred at room temperature for 45 min under a steady stream of argon. The resulting pale yellow solution was concentrated in vacuo and the residue purified by HPLC on a Whatmann preparative silica gel column (Whatmann Partisil, M9 10/50) using 18% ethyl acetate in hexanes as eluting solvent (flow rate 4 mL/min; UV detection 280 nm). The major, UV-active peak (retention time 24 min) was collected under argon and concentrated in vacuo to afford **15** (130 mg, 91% yield) as a pale yellow oil which darkens on standing (turns brown due to decomposition of the pyrrole). As **15** is extremely sensitive to air, all operations were carried out under a steady stream of argon. All solvents were degassed thoroughly prior to use. The title pyrrole **15** decomposes in chlorinated solvents and was hence stored at −78 °C as a solution in CD_3CN or C_6D_6. Pyrrole **15** stains orange with vanillin and is DMAB positive (R_f = 0.22; 20% ethyl acetate in hexanes): ^{1}H NMR (200 MHz, CD_3CN) δ 6.64 (1 H, s), 6.31 (1 H, d, *J* = 15.9 Hz), 5.68 (1 H, dd, *J* = 16.0 Hz, *J* = 7.1 Hz), 5.31 (2 H), 4.04−3.96 (1 H, m), 3.61 (3 H, s), 3.55 (2 H, s), 3.15 (2 H, d, *J* = 4.9 Hz), 2.32 (2 H, t, *J* = 7.5 Hz), 2.28−2.13 (2 H), 2.13 (3 H, s), 1.72−1.28 (10 H), 0.92−0.84 (12 H); ^{13}C NMR (50 MHz, CD_3CN) δ 174.6 (+, s), 131.5 (−, d), 129.2, (−, d), 128.2 (−, d), 126.9 (+, s), 123.7 (−, d), 119.6 (+, s), 119.5 (−, d), 117.2 (+, s), 73.9 (−, d), 57.9 (+, t), 51.8 (−, q), 38.5 (+, t), 38.1 (+, t), 34.0 (+, t), 32.5 (+, s), 28.1 (−, q, 3C), 27.3 (+, t), 26.0 (+, t), 25.7 (+, t), 23.7(+, t), 23.3 (+, t), 14.3 (−, q), 10.5 (−, q). Pyrrole **15** was further characterized as its stable trifluoroacetyl derivative **16** described below.

3-(6-Carbomethoxy-2(*Z*)-hexenyl)-2-methyl-1-neopentyl-4-(3-(triflouroacetoxy)-1(*E*)-octenyl)-5-(trifluoroacetyl)-pyrrole (16). To a stirred solution of 8,9-bisepi-LGE$_2$ methyl ester (**14**, 30 mg, 0.085 mmol) in anhydrous ethanol (2 mL) was added neopentylamine (9 mg, 0.101 mmol), and the mixture was stirred under argon for 2 h at room temperature. Ethanol was removed in vacuo, the residue was dissolved in ether (2 mL), and the resulting solution cooled was to −78 °C under argon. Pyridine (41 mg, 42 μL, 0.52 mmol) was added to the cooled mixture followed by dropwise addition of trifluoroacetic anhydride (72 mg, 0.34 mmol). The reaction mixture was allowed to warm to room temperature over 2 h and then allowed to continue overnight at room temperature. TLC analysis of reaction mixture in 20% ethyl acetate in hexanes showed at least six−seven spots. The reaction mixture was poured into a solution of sodium carbonate (15 mL) and extracted with ether (3 × 25 mL). The combined ether extracts were washed with water (2 × 10 mL) and dried with potassium carbonate. After removal of ether by rotary evaporation, the crude product was purified by flash chromatography over silica gel (10% ethyl acetate in hexanes). TLC analysis of the fraction containing **16** showed R_f = 0.31−0.45 (20% ethyl acetate in hexanes). This fraction was purified further by HPLC (10% ethyl acetate in hexanes) to give **16** (20.7 mg, 40% yield) as a single HPLC peak that showed a single spot on TLC (R_f = 0.38): ^{1}H NMR (200 MHz, $CDCl_3$) δ 6.73 (1 H, dd, *J* = 0.6 Hz, *J* = 15 Hz), 5.62 (1 H, dd, *J* = 7.4 Hz, *J* = 15 Hz), 5.56−5.17 (3 H), 4.95 (1 H, br d, *J* = 14 Hz), 3.66 (3 H, s), 3.60 (1 H, br d), 3.10 (2 H, d, *J* = 6.2 Hz), 2.33 (2 H, t, *J* = 7.5 Hz), 2.19 (3 H, s), 2.19−2.08 (2 H), 1.80−1.63 (4 H), 1.41−1.14 (6 H), 0.96−0.77 (12 H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 231.8, 173.9, 141.3, 131.8, 131.0, 129.1 (2 C), 128.8 (2 C), 126.7, 121.4, 120.5 (q, *J* = 296 Hz, CF_3), 116.6 (q, *J* = 290 Hz, CF_3), 79.5, 54.5, 51.5, 34.8, 33.9, 33.4, 31.3, 28.09 (3 C), 26.8, 24.6, 24.5, 22.8, 22.4, 13.9, 12.0; mass spectrum *m/z* (M^+) calcd for $C_{30}H_{41}F_6NO_5$ 609.2889, found 609.2892.

DMAB Detection of Protein-Bound Pyrroles. (a) Ehrlich Assay for the Detection of Pyrroles. Pyrrole assays were performed by either one of two methods. **Method A.** A 3% solution of *p*-(dimethylamino)benzaldehyde (DMAB) in methanol/boron trifluoride was prepared as previously described.[14a] Thus, DMAB (3 g) was dissolved in a mixture of methanol−boron trifluoride (40 mL) and absolute ethanol (60 mL). To 1.0 mL of the aqueous incubation mixture was added DMSO (500 μL), followed by addition of freshly prepared Ehrlich reagent (1.0 mL). Tubes were vortexed to ensure mixing, capped to minimize volume changes, and then heated at 85−90 °C for 15 min on a water bath unless otherwise indicated. After cooling to room temperature, visible spectra were recorded. For smaller sample volumes, proportionally less DMSO and Ehrlich reagent were added. **Method B.** A 80 mM DMAB reagent was prepared by dissolving DMAB (597 mg) in 50% aqueous methanol (50 mL) containing 1% concentrated HCl.[17] Reagent was freshly prepared prior to each use. Samples (1.0 mL) were assayed by addition of DMAB reagent (1.0 mL), followed by heating at 80−85 °C for 10 min. The samples were cooled to room temperature and centrifuged for 15 min at 5000 rpm to remove suspended material, and visible spectra were recorded. The control sample, prepared without added LGE$_2$, was used as the scan blank.

(b) Detection of Pyrroles Bound to BSA and HSA. To 2.0 mL of a solution of BSA (10 mg BSA/1.0 mL of pH 7 phosphate buffer; 1.47×10^{-4} M) were added varying amounts of a stock solution of LGE$_2$ (3.5 mg of LGE$_2$ /1.0 mL of EtOH) such that the concentration of LGE$_2$ in the tubes was 74, 147, 294, or 588 μM (i.e. 0.5, 1.0, 2.0, and 4.0 equiv with respect to BSA concentration). A control sample was prepared by adding just EtOH to BSA (no LGE$_2$). The tubes were equilibrated to 37 °C over 10 min in a water bath maintained at 37 °C. Incubation was continued at 37 °C with occasional stirring. At time intervals of 2 and 4.45 h, 500 μL aliquots were drawn from each tube and assayed for pyrrole content using method A described above. Tubes were cooled to room temperature and diluted 10× with EtOH. Visible spectra were recorded using the control sample as scan blank. Absorbance data are given in Table 1.

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Supplementary Material Available: ^{1}H NMR spectra of all new compounds (7 pages). This material is contained in libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.