Characterization of the Lysyl Adducts Formed from Prostaglandin H_2 via the Levuglandin Pathway†

Olivier Boutaud,*,‡ Cynthia J. Brame,‡ Robert G. Salomon,§ L. Jackson Roberts, II, and John A. Oates

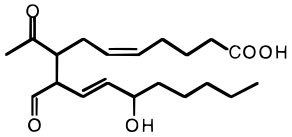
Departments of Pharmacology and Medicine, Vanderbilt University, Nashville, Tennessee 37232-6602, and Department of Chemistry, Case Western University, Cleveland, Ohio 44106-2699

Received March 1, 1999; Revised Manuscript Received May 17, 1999

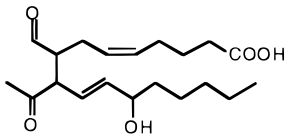
ABSTRACT: Prostaglandin H_2 has been demonstrated to rearrange to γ-ketoaldehyde prostanoids termed levuglandins E_2 and D_2. As γ-dicarbonyl molecules, the levuglandins react readily with amines. We sought to characterize the adducts formed by synthetic levuglandin E_2 and prostaglandin H_2-derived levuglandins with lysine. Using liquid chromatography/electrospray mass spectrometry, we found that the reaction predominantly produces lysyl-levuglandin Schiff base adducts that readily dehydrate to form lysyl-anhydrolevuglandin Schiff base adducts. These adducts were characterized by examination of their mass spectra, by analysis of the products of their reaction with sodium cyanide, sodium borohydride, and methoxylamine and by the mass spectra derived from collision-induced dissociation in tandem mass spectrometry. The Schiff base adducts also are formed on peptide-bound lysyl residues. In addition, synthetic levuglandin E_2 and prostaglandin H_2-derived levuglandins produced pyrrole-derived lactam and hydroxylactam adducts upon reaction with lysine as determined by tandem mass spectrometry. A marked time dependence in the formation of these adducts was observed: Schiff base adducts formed very rapidly and robustly, whereas the lactam and hydroxylactam adducts formed more slowly but accumulated throughout the time of the experiment. These findings provide a basis for investigating protein modification induced by oxygenation of arachidonic acid by the cyclooxygenases.

Salomon and colleagues have demonstrated that the endoperoxide intermediate prostaglandin H_2 (PGH_2)[1] can rearrange in an aqueous environment to seco-prostanes characterized by a γ-ketoaldehyde moiety (*1*). He termed these molecules levuglandins D_2 and E_2 (LGE_2 and LGD_2) because of their structural similarity to levulinaldehyde and their formal relationship to prostaglandins D_2 and E_2. The γ-keto aldehyde moiety of these molecules leads to their rapid reaction with amines, such as the ϵ-amino group of lysyl residues on proteins, and Salomon's group demonstrated that LGE_2 can avidly induce both protein−protein and protein-DNA cross-links (*2, 3*). Other γ-dicarbonyl compounds, notably the hexane metabolite 2,5-hexanedione, are known to induce well-characterized pathologies by similar reactions in vivo (*4*). Therefore, downstream effects of LGE_2/D_2 formation may be of great interest.

The oxygenation of radiolabeled arachidonic acid by cyclooxygenase-1 in vitro as well as in platelets leads to covalent adduction of radiolabeled molecules to the enzyme and to other proteins (*5*−*7*). As these adducts incorporate the carboxyl carbon, they cannot be accounted for by reactive fragmentation products of oxidized arachidonic acid such as malondialdehyde and 4-hydroxynonenal. As a basis for determining whether these protein adducts were derived from PGH_2 via the levuglandin pathway, the lysyl adducts formed from PGH_2 itself were characterized and compared with those formed from its rearrangement product, levuglandin E_2.



LGE_2



LGD_2

MATERIALS AND METHODS

Materials. PGH_2 was from Cayman Chemical Company (Ann Arbor, MI). Methoxylamine hydrochloride, diisopropyl ethylamine, pentaflurobenzyl bromide, undecane, lysine, sodium cyanide, sodium borohydride, and sodium borodeuteride were purchased from Sigma (St. Louis, MO). C_{18} Sep Pak cartridges were obtained from Waters Corp. (Milford, MA), and dimethyl formamide was from Aldrich Chemical Co. (Milwaukee, WI). Bis(trimethylsilyl)trifluoroacetamide is from Supelco (Bellefonte, PA).

PGH_2 Rearrangement Product Analysis by GC/MS. PGH_2 was incubated in phosphate-buffered saline for 2 h at room temperature. An equal volume of 6% methoxylamine hy-

† This work was supported in part by NIH Grants CA 68485, GM 15431, and GM 42056.

* To whom correspondence should be addressed. Phone: (615) 343-7398. Fax: (615) 322-4707. E-mail: oliver.boutaud@mcmail.vanderbilt.edu.

‡ These authors contributed equally to this work.

§ Case Western University.

[1] Abbreviations: LC/MS, liquid chromatography /mass spectrometry, LC, liquid chromatography, LG, levuglandin, LGD_2, levuglandin D_2, LGE_2, levuglandin E_2, $[MH]^+$, molecular ion, $NaBH_4$, sodium borohydride, PBS, phosphate-bufferd saline, PFB, pentafluorobenzyl, PGD_2, prostaglandin D_2, PGE_2, prostaglandin E_2, PGH_2, prostaglandin H_2, TMS, *O*-trimethylsilyl.

10.1021/bi990470+ CCC: $18.00
Published on Web 07/01/1999

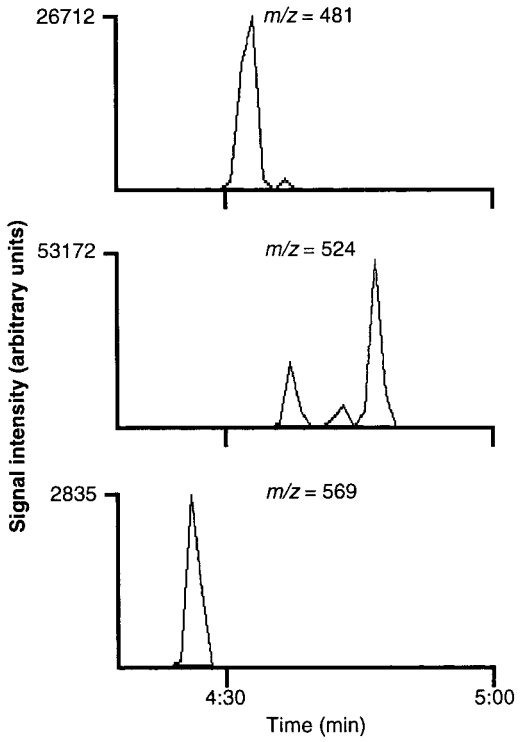


FIGURE 1: Rearrangement products of PGH_2 in aqueous solution. Following incubation of PGH_2 in 100 mM PBS, pH 7.5, at 37 °C for 2 h, the rearrangement products were converted to *O*-methyloxime, TMS ether, and PFB ester derivatives. The compounds were then analyzed by selected ion monitoring GC/MS for LGE_2/D_2 (*m*/*z* 481), PGE_2/D_2 (*m*/*z* 524), and $PGF_{2\alpha}$ (*m*/*z* 569).

drochloride was then added and allowed to incubate for 30 min at room temperature. The pH of the reaction mix was adjusted to 3, and the sample applied to a C_{18} Sep Pak that had been preconditioned with 5 mL methanol and 10 mL of pH 3 water. The sample was washed sequentially with 10 mL of pH 3 water and 10 mL of heptane before elution with 10 mL of heptane/ethyl acetate (1:1). The sample was dried under N_2 and the prostaglandins converted to pentafluorobenzyl esters by a 30 min, 37 °C incubation with 20 μL of 10% diisopropyl ethylamine and 40 μL of 10% pentaflurobenzyl bromide in acetonitrile. The sample was again dried under N_2 before conversion to O-trimethylsilyl ether derivatives by incubation with 10 μL of dimethyl formamide and 20 μL of bis(trimethylsilyl)trifluoroacetamide. The reagents were dried under a stream of N_2 and the sample resuspended in undecane. The rearrangement products were analyzed by gas chromatography/electron capture negative ionization mass spectrometry, scanning from 300 to 600 mass units.

LGE_2 Synthesis. LGE_2 was synthesized from a 15-R,S epimeric mixture of the methyl ester, O-*tert*-butyl-dimethylsilyl ether, isopropylidine precursor by published methods (*8*). To facilitate comparisons with PGH_2, other prostaglandins, and isoprostanes, we used prostaglandin numbering for this 10,11-seco-prostane. Synthesis by this method yields a diastereomeric mixture of 15-*R*- and 15-*S*-LGE_2.

Generation and Reactions of Lysine Adducts Formed from PGH_2 and LGE_2. PGH_2 or LGE_2 (1 mM) was incubated with 1 mM lysine or synthetic peptides in phosphate-buffered

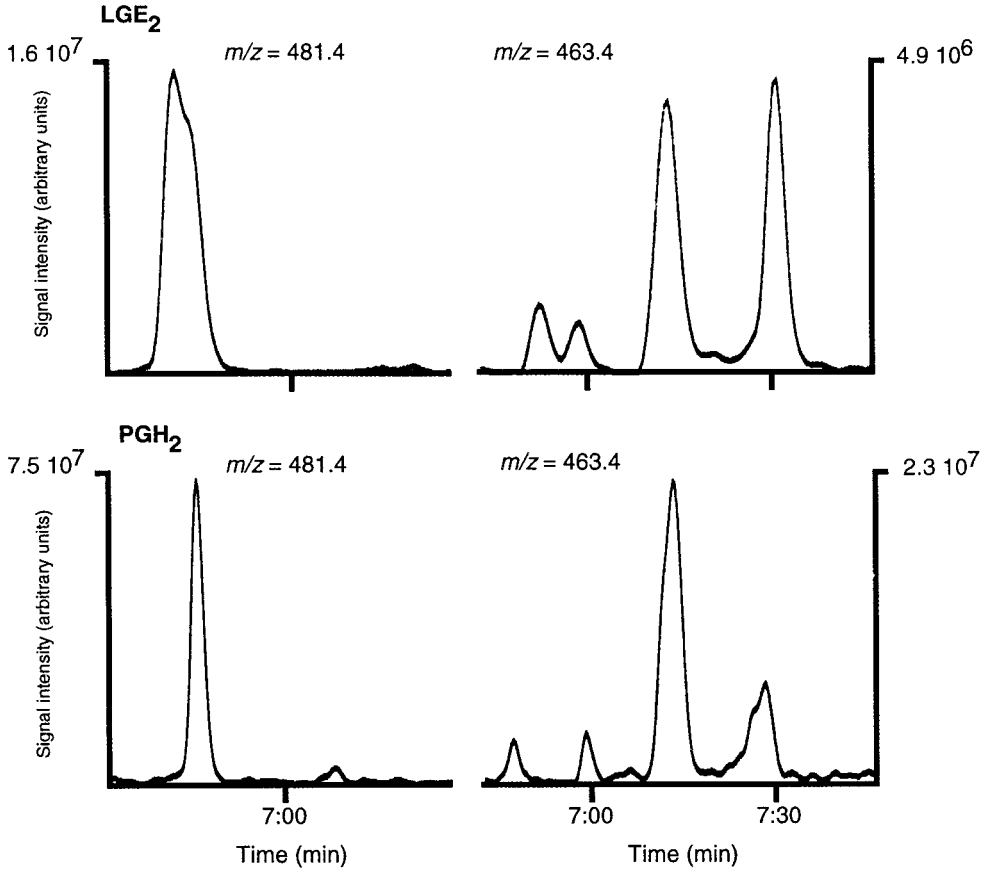


FIGURE 2: Lysyl adducts formed by PGH_2 and LGE_2. Lysine (1 mM in PBS) was incubated with 1 molar equivalent of PGH_2 or synthetic LGE_2 under argon at 37 °C for 2 h. The samples were then analyzed by LC/ESI MS scanning ions between *m*/*z* 450 and 520. Selected ion current chromatograms of the predominant products of the reaction are shown.

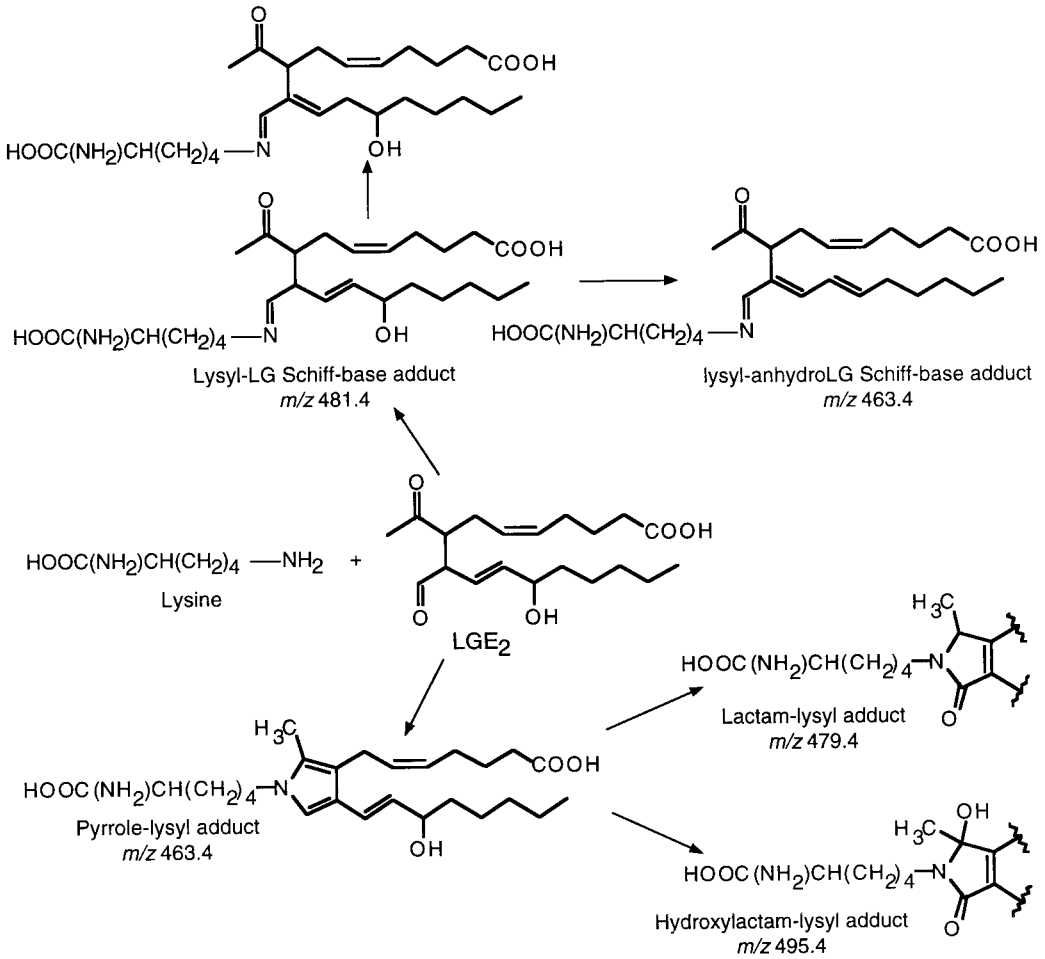


FIGURE 3: Structures of adducts hypothesized to be formed from the reaction of LGE_2 with the ϵ-amino group of lysine. Corresponding adducts are expected to be formed from LGD_2.

saline at room temperature for 2 h (unless otherwise noted). To generate cyanide adducts, 1/10 volume of 100 mM sodium cyanide in water was added and allowed to incubate for 30 min at room temperature before LC/MS analysis. To reduce adducts, 1/10 volume of 100 mM sodium borohydride in dimethylformamide was added and allowed to incubate for 30 min at 0 °C before analysis by LC/MS. To generate methoxylamine derivatives, an equal volume of 6% methoxylamine hydrochloride in phosphate-buffered saline at pH 6 was added and allowed to incubate for 30 min at room temperature before LC/MS analysis.

The Waters Accutag amino acid analysis of the peptidic-LG adducts was carried out by the Protein Chemistry Core Laboratory of Vanderbilt University.

LC/MS/MS Analysis. The adducts were chromatographed on a 2.1 × 15 mm XDB C8 column (MacMod Analytical) with a flow rate of 0.2 mL/min using an linear gradient of 10 to 90% acetonitrile in 5 mM ammonium acetate/0.1% acetic acid. Electrospray tandem mass spectrometric analysis was carried out on a Finnigan TSQ7000. Sheath gas pressure was held at 70 psi; auxiliary gas pressure at 10 psi. The spray voltage was held at 3.8 eV. Voltage on the capillary was 20 V and on the tube lens 90.3 V. Selected ion monitoring or scanning was carried out as indicated in figure legends. When needed, ions were subjected to collision-induced dissociation at −28 eV with 2.0 mTorr collision gas, scanning daughters between *m*/*z* 50 and 500.

RESULTS

Formation of Levuglandins from PGH_2. Initially, we sought to confirm that levuglandins were formed by the decomposition of PGH_2 in an aqueous environment. PGH_2 was allowed to rearrange in phosphate-buffered saline and the rearrangement products, after conversion to O-methyloxime, pentafluorobenzyl ester, O-trimethylsilyl ether derivatives, were analyzed by gas chromatography/electron capture negative ionization/mass spectrometry. Negative ions were scanned from 300 to 600 mass units. The main signals seen corresponded to the $[M\text{-}PFB]^-$ carboxylate anions of LGE_2/D_2 [mass-to-charge ratio (*m*/*z*) 481], PGE_2/D_2 (*m*/*z* 524), and prostaglandin $F_{2\alpha}$ (*m*/*z* 569) and are shown in Figure 1 as selected ion current chromatograms. Syn and anti methoxime isomers can be formed at each carbonyl group that is derivatized; signals for three of the four methoxime isomers predicted to be formed by PGE_2/D_2 are seen at *m*/*z* 524. The fourth methoxime isomer expected presumably coelutes with another isomer. The same is true for the LGE_2/D_2 signals at *m*/*z* 481, where a single broad peak is observed. These signals co-chromatographed with authentic deuterated standards for these compounds (data not shown). As expected, the largest products of this rearrangement were PGE_2/D_2 (81%), but substantial amounts of LGE_2/D_2 also were formed (18%), predominating over prostaglandin $F_{2\alpha}$ (0.4%).

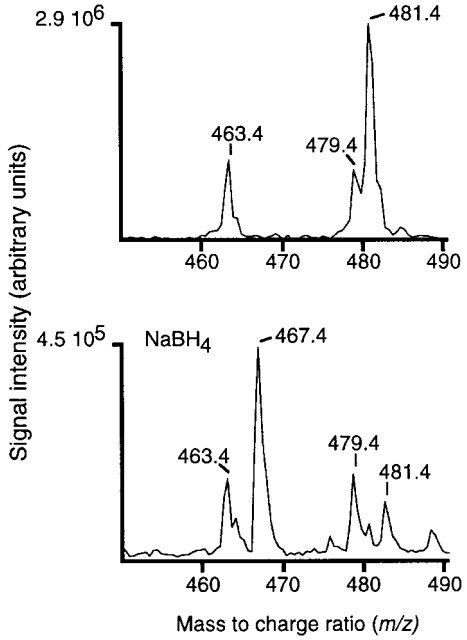


FIGURE 4: Reduction of Schiff base adducts (*m*/*z* 481.4). LGE_2-lysine adducts were reduced at 4 °C for 30 min by sodium borohydride ($NaBH_4$). The samples were then analyzed by LC/MS, scanning ions between *m*/*z* 450 and 520. The Schiff base adduct (*m*/*z* 481.4) was reduced and dehydrated by the reaction as indicated by the appearance of the ion at *m*/*z* 467.4. The lactam (*m*/*z* 479.4) and lysyl-anhydroLG Schiff base (*m*/*z* 463.4) adducts were not reduced under these conditions.

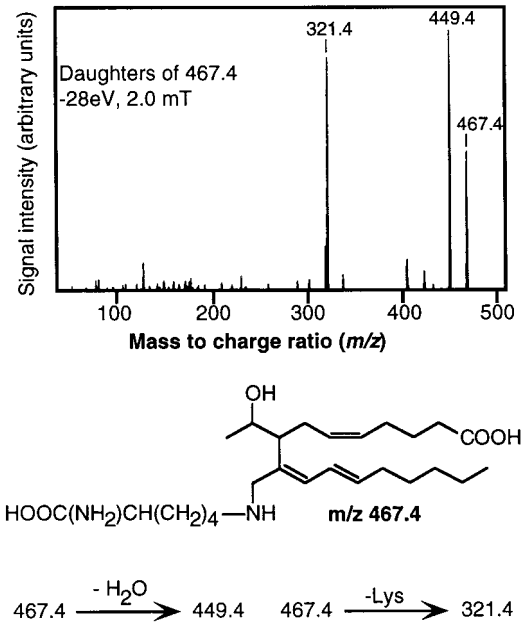


FIGURE 5: Collision-induced dissociation spectrum of the hypothetical reduced dehydrated lysyl-LG Schiff base at *m*/*z* 467.3. The reduced dehydrated Schiff base adduct (*m*/*z* 467.4) was subjected to collision-induced dissociation at −28 eV with 2.0 mTorr collision gas. Daughter ions were scanned between *m*/*z* 50 and 500. Interpretations of the fragmentations are shown and were supported by fragmenting corresponding adducts formed with [^{13}C]lysine.

Characterization of the Lysyl Adducts Formed from PGH_2 and LGE_2. To determine whether rearrangement of PGH_2 in the presence of lysine leads to formation of the same adducts seen during reaction of synthetic LGE_2 with lysine, LGE_2 or PGH_2 was incubated with lysine in PBS for 2 h. The reaction mixture was then analyzed by liquid chromatography/electrospray ionization/mass spectrometry, scanning for adducts between *m*/*z* 450 and 520. New signals at *m*/*z* 463.4, 479.4, 495.4, and 481.4, not present in any of the reactants alone, appear after reaction of LGE_2 or PGH_2 with lysine. The adducts at *m*/*z* 481.4 predominate in both cases; adducts at *m*/*z* 463.4 (481.4−18) are also present at significant levels (Figure 2).

There is precedent for formation of Schiff bases from the reaction of a γ-ketoaldehyde with amines (*9*), and *m*/*z* 481 is the expected molecular ion $[MH]^+$ of a lysyl-LG Schiff base (Figure 3). The *m*/*z* 463 (Figure 2) is consistent with loss of the 15-hydroxyl by dehydration, yielding a lysyl-anhydroLG Schiff base as depicted in Figure 3; such a dehydration has been shown to occur with LGE_2 (*10*).

Notably, the adducts at *m*/*z* 481.4 formed by LGE_2 have broader LC peaks, presumably due to the fact that it is a diastereomeric mixture of 15-*S*- and 15-*R*-LGE_2 (*8*). In contrast, LGE_2 and LGD_2 formed by PGH_2 rearrangement will have only the 15-S configuration, resulting in sharper peaks. Additionally, both the PGH_2 and LGE_2 reactions result in two predominant peaks at *m*/*z* 463.4 (Figure 3). Their ratio, however, is not constant and varies between experiments. The two peaks may correspond to the anti and syn isomers of a lysyl-anhydroLG Schiff base. These peaks are sharper than those observed for LGE_2 products at *m*/*z* 481.4. This observation supports a lysyl-anhydroLG Schiff base structure for the compounds at *m*/*z* 463.4, as such an adduct has lost the 15-hydroxyl group that gives the diastereomers discussed above. Thus, PGH_2 rearrangement in the presence of lysine leads to formation of adducts that have the same molecular weight and similar retention characteristics as adducts formed by the reaction of LGE_2 with lysine, consistent with their identity as LGE_2/D_2-lysine adducts.

Because the formation of the lysyl-LG and lysyl-anhydroLG Schiff bases from the reaction of LGE_2/D_2 with lysine had not been described previously, we sought to further characterize the putative lysyl-LG and lysyl-anhydroLG Schiff bases with molecular ions ($[MH]^+$) of *m*/*z* 481 and 463, respectively. We initially took advantage of the ability of cyanide to undergo nucleophilic addition to characterize these adducts. Addition of sodium cyanide to the mixture of LGE_2-lysine adducts led to formation of a predominant derivative (*m*/*z* 517.4) and two others in lesser quantities (*m*/*z* 490.4 and 535.4). All three of these species correspond to molecular ions of predicted cyano-lysyl-LG Schiff bases. Cyanide can add to the electrophilic imine (*11*) or carbonyl (*12*−*14*) carbon and by Michael addition to the β-carbon of carbonyl-conjugated carbon−carbon double bonds (*15*). As the lysyl-LG and lysyl-anhydroLG Schiff base adducts have all of these functional groups, it is difficult to be definitive about the structures of the cyanide reaction products. Nevertheless, the product at *m*/*z* 535.4 represents a dicyano adduct that has retained a hydroxyl group and, therefore, is strong evidence that the *m*/*z* 481.4 ion represents the lysyl-LG Schiff base. In contrast, the species at *m*/*z* 517.4 is a dicyano adduct that has lost its hydroxyl group. As this loss can occur before or during the treatment with cyanide, we do not know if this dicyano adduct is produced from the lysyl-anhydroLG Schiff base (*m*/*z* 463.4) or the lysyl-LG Schiff base (*m*/*z* 481.4). Similarly, the cyanide adduct at *m*/*z* 490.4 could result from reaction of cyanide with the lysyl-

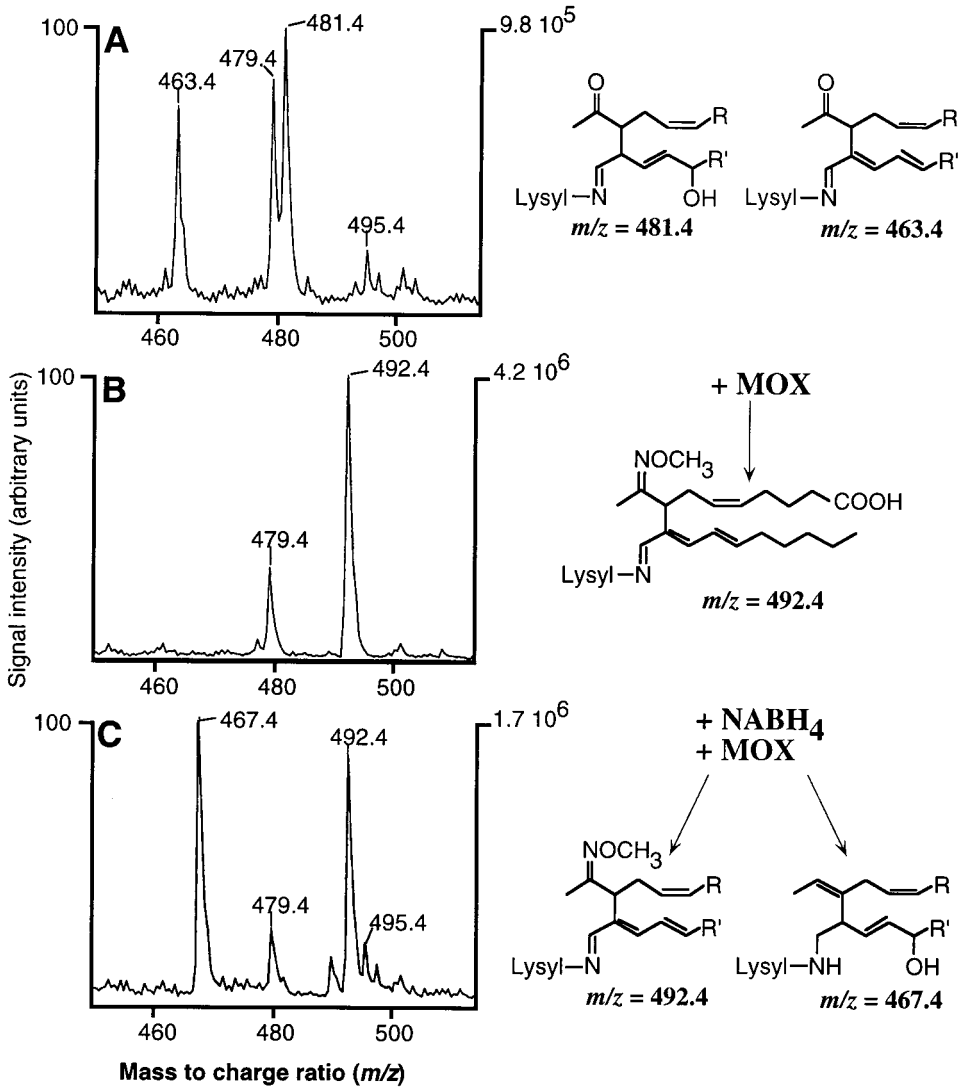


FIGURE 6: Derivatization of the adducts with methoxylamine. LGE$_2$-lysine adducts (A) were treated with methoxylamine at room temperature for 30 min (B) or similarly treated after reduction by sodium borohydride (C). The samples were then analyzed by LC/MS, scanning between *m*/*z* 450 and 520.

anhydroLG Schiff base (*m*/*z* 463.4) or with the lysyl-LG Schiff base (*m*/*z* 481.4), followed by dehydration. As the pH of this reaction is high, it could promote dehydration of the LGE$_2$ moiety on the lower side chain via the assistance of the carboxylic group (*10*). Moreover, the pH of the reaction could not be lowered, because we have found that the lysyl-LG Schiff bases are not stable under acidic conditions. To differentiate between these possibilities, we attempted to purify each of the cyanide adducts. We were unable to isolate the putative lysyl-anhydroLG Schiff base (*m*/*z* 463.4) by HPLC. However, we were able to isolate a small amount of lysyl-LG Schiff base (*m*/*z* 481.4). It was reduced with sodium cyanide under conditions identical to those used in the above experiment. We observed the disappearance of the Schiff base signal at *m*/*z* 481.4 and appearance of a signal at *m*/*z* 517.4 but not at *m*/*z* 490.4, suggesting that the cyanide adduct we observed at *m*/*z* 490.4 arises exclusively from an lysyl-anhydroLG Schiff base. The cyano adducts at *m*/*z* 517.4 may be a combination of cyanide addition products from both the lysyl-LG and the lysyl-anhydroLG Schiff bases. The cyanide reaction products at *m*/*z* 490.4 and 535.4 clearly demonstrate, however, that both the lysyl-LG and lysyl-anhydroLG Schiff bases are produced in the reaction of LGE$_2$ with lysine. These experiments were repeated with the adducts formed upon reaction of PGH$_2$ with lysine; the same results were obtained.

To further characterize the lysyl-LG Schiff base and its dehydration product, we exploited the ability of sodium borohydride to reduce Schiff bases (*11*). Following addition of sodium borohydride to a mixture of LGE$_2$-lysine adducts, we expected to see two new species: one at *m*/*z* 485.4 and one at *m*/*z* 467.4, resulting from the reduction of the imine and the carbonyl groups of the lysyl-LG Schiff base at *m*/*z* 481.4 and the lysyl-anhydroLG Schiff base at *m*/*z* 463.4, respectively. Moreover, it was anticipated that the appearance of those two reduced species would be paralleled with the disappearance of the two initial adducts. The putative lysyl-anhydroLG Schiff base (*m*/*z* 463.4) appeared to be relatively resistant to reduction (Figure 4), which might be explained by stabilization of the adduct by conjugation of the double bonds (*11*), such stabilization being stronger at the high pH of the reducing reaction mixture. In contrast, the putative lysyl-LG Schiff base (*m*/*z* 481.4) reacted with sodium borohydride to produce a product at *m*/*z* 467.4 (Figure 4),

consistent with dehydration in addition to the expected reduction of the imine and carbonyl groups. Dehydration could have deleted the hydroxyl group at the lower side chain or the hydroxyl group resulting from reduction of the carbonyl group. As the reduction is done at high pH, it is very likely that the dehydration of the LGE_2 moiety occurs on the lower side chain (*10*) leading to the dehydrated lysyl-LG Schiff base adduct (*m*/*z* 467.4).

The ion at *m*/*z* 467.4 was further characterized as the dehydrated reduced lysyl-LG Schiff base by analysis of the daughter ions produced upon collision-induced dissociation (Figure 5). The interpretation of the fragment ions is shown in the figure. Especially notable is the daughter ion at *m*/*z* 321.4, which is consistent with the eicosanoid moiety derived from fragmentation at the carbon−nitrogen bond of the dehydrated reduced lysyl-LG Schiff base. We demonstrated that the daughter ion is derived from loss of the lysyl moiety by performing the same experiment with the [^{13}C]lysine and observing the same daughter ion at *m*/*z* 321.4.

Because the lysyl-anhydroLG Schiff base was resistant to reduction, we sought to further characterize its functional groups. We derivatized the carbonyl groups of the adducts by reacting the mixture of LGE_2-lysine adducts (Figure 6A) with methoxylamine hydrochloride. The results, shown in Figure 6B, indicate the disappearance of the ion at *m*/*z* 463.4 and the appearance of a signal at *m*/*z* 492.4, corresponding to the molecular ion of the methoximated lysyl-anhydroLG Schiff base. In addition, the signal at *m*/*z* 481.4 disappeared, but its methoxylamine derivative at *m*/*z* 510.4 did not appear. To explain this, we hypothesized that the lysyl-LG Schiff base underwent dehydration during the methoximation reaction. To assess whether this was the case, we treated the lysyl adducts sequentially with sodium borohydride and methoxylamine. The basis for this strategy was as follows. The lysyl-LG Schiff base undergoes reduction of the carbonyl upon treatment with sodium borohydride and then dehydration. No further derivatization would occur upon treatment with methoxylamine due to the lack of a carbonyl group; the final product being the dehydrated reduced lysyl-LG Schiff base (*m*/*z* 467.4). On the other hand, the carbonyl in the lysyl-anhydroLG Schiff base is not reduced by sodium borohydride and, subsequently upon treatment with methoxylamine, is converted to a O-methoxime derivative (*m*/*z* 492.4). As noted in Figure 6C, following sequential treatment of the lysyl adducts with sodium borohydride and methoxylamine, both the *m*/*z* 467.4 and 492.4 products are formed in approximately equal amounts, supporting the stated hypothesis.

Characterization of Peptidyl-LG Adducts. The reaction of both LGE_2 and PGH_2 with an equimolar amount of the synthetic peptide Tyr-Pro-Lys-Gly (YPKG) led to the formation of the Schiff base adduct (Figure 7, panel A). The nonadducted peptide would generate an $[MH]^+$ ion at *m*/*z* 463 and the predicted ion for the LG Schiff base adduct is *m*/*z* 797.4. Again, a pair of unresolved *m*/*z* 797.4 peaks were seen following incubation with LGE_2, presumably because it is a mixture of lower side-chain hydroxyl epimers. This adduct was further characterized as the lysyl-LG Schiff base by analysis of the products of its reduction and derivatization with cyanide (data not shown). These data, however, did not allow the determination of whether the α-amine or the ϵ-amine of the peptide is adducted. Therefore, LGE_2 was

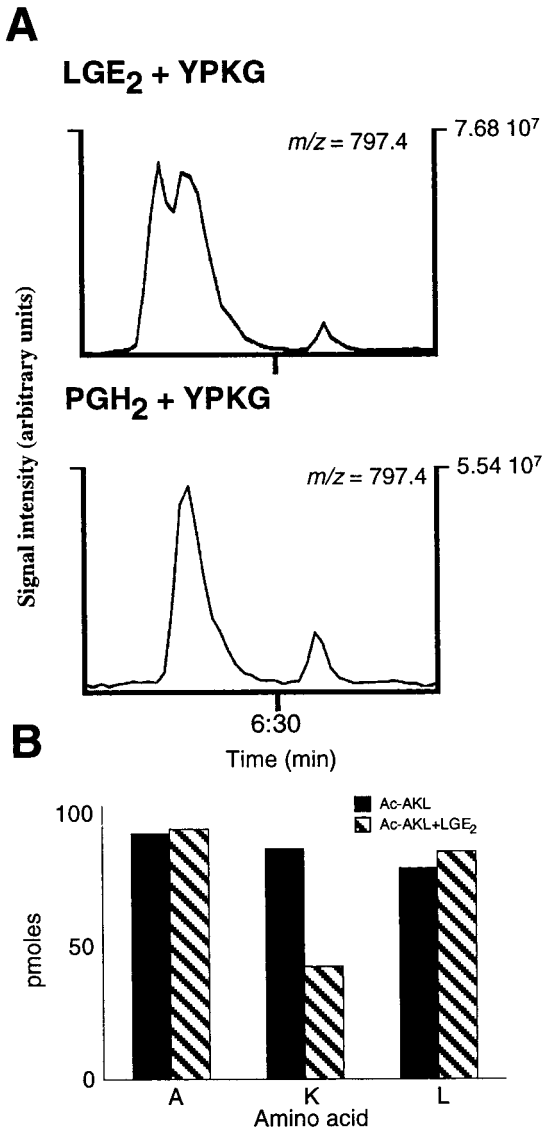


FIGURE 7: Formation of Schiff base adducts with ϵ-amino groups of peptidyl lysine residues. (A) LGE_2 or PGH_2 (1 mM) was reacted with the peptide Tyr-Pro-Lys-Gly (YPKG). Analysis of the products by LC/MS revealed strong signals consistent with the molecular ion of an LG Schiff base adduct; selected ion current chromatograms are shown. (B) LGE_2 or PGH_2 was reacted with the peptide acetyl-Ala-Lys-Leu (Ac-AKL) and the products subjected to Waters Accutag amino acid analysis. Reaction of the lysyl residue with LGE_2 results in a reduction in the amount of unmodified lysine.

reacted with the N-acetylated peptide acetyl-Ala-Lys-Leu (Ac-AKL). Analysis of the reaction products by LC/MS demonstrated a molecular ion of *m*/*z* 707.4 consistent with a Schiff base adduct. Amino acid analysis indicates that about 50% of the lysine residues in the N-acetylated peptide are adducted under these conditions (Figure 7, panel B).

Pyrrole-Derived Adducts Formed by LGE_2/PGH_2. LGE_2, as a γ-ketoaldehyde molecule, also forms pyrrole adducts upon reaction with amines (*16*), and previous work by Brame et al. has shown that pyrrole adducts formed by levuglandin-like compounds are unstable in the presence of oxygen and readily oxidize to lactam and hydroxylactam adducts (Figure 3) (*17*). Although we found that lysyl-LG pyrrole adducts ($[MH]^+ = 463$) were too unstable for analysis by LC/MS, we did detect adducts with the molecular ions of the lactam ($[MH]^+ = 479$) and hydroxylactam adducts ($[MH]^+ = 495$)

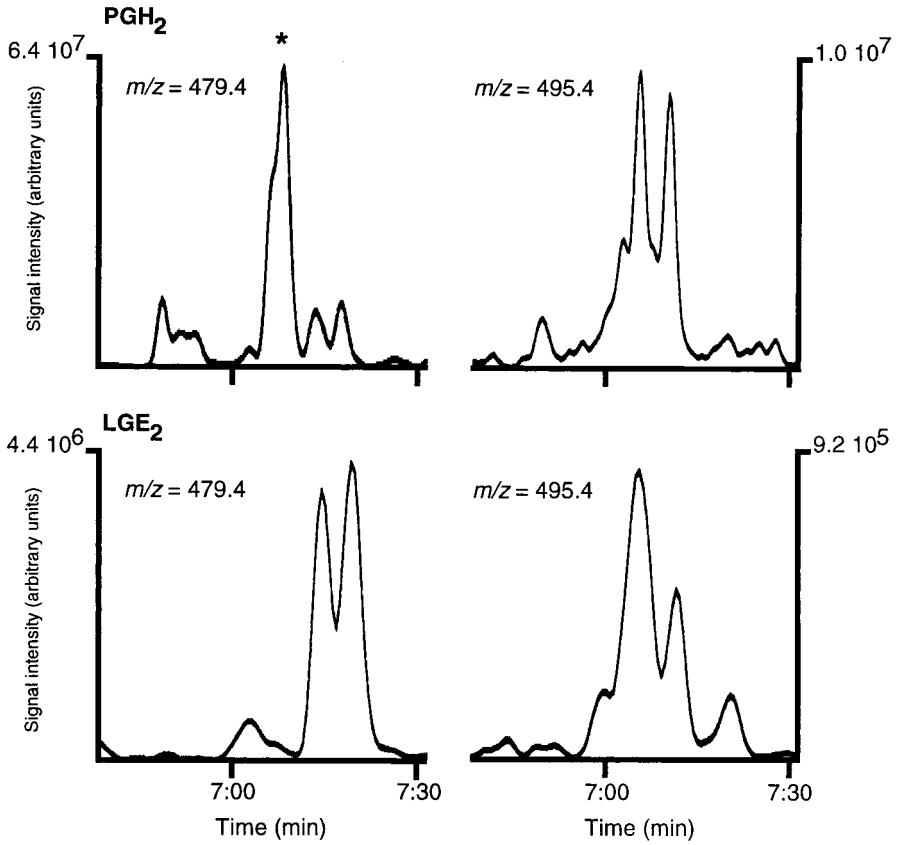


FIGURE 8: Pyrrole-derived lysyl adducts formed by PGH_2 and LGE_2. Lysine (1 mM in PBS) was incubated with one molar equivalent of PGH_2 or synthetic LGE_2 under argon at 37 °C for 2 h. The samples were then analyzed by LC/ESI MS scanning ions between *m*/*z* 450 and 520. Selected ion current chromatograms of the lysyl-LG lactam (*m*/*z* 479.4) and hydroxylactam (*m*/*z* 495.4) adducts are shown. The asterisk (*) shows the peak at *m*/*z* 479.4 that does not co-chromatograph with [^{13}C]lysyl-LGE_2 standards.

(Figures 4, 6, and 8). The multiple chromatographic peaks observed may arise from formation of isomers during insertion of oxygen (in the case of the hydroxylactam adduct) or hydrogen (in the case of the lactam adduct) in a free radical intermediate. These adducts co-chromatographed with [^{13}C]lysyl-LGE_2 standards with the exception of the early prominent peak seen at *m*/*z* 479.4 in the PGH_2 reaction that appears to be comprised of two unresolved components [denoted by an asterisk (*)]. We speculate that these peaks, which have variable abundance, are due to LG isomers which, in the case of PGH_2, could include one derived from LGD_2. Collision-induced dissociation of the putative lactam and hydroxylactam adducts resulted in fragment ions at *m*/*z* 461.4 (lactam), 459.4 (hydroxylactam), 332.4 (lactam), and 84.1 (both), confirming their identitiy as such (*17*).

Time Course of the Formation of the Adducts. To ascertain the rate of formation of the lysyl-levuglandin Schiff base relative to that of the lactam adducts, we determined the rate of LGE_2-lysine adducts formation in vitro. For this purpose, we mixed lysine and synthetic LGE_2 and analyzed aliquots by LC/MS every 18 min. Selected ion monitoring of molecular ions of the lysyl-LG Schiff base, lysyl-anhydro LG Schiff base, lactam, and hydroxylactam adducts revealed that the different species did not appear at the same time (Figure 9). The lysyl-LG Schiff bases both were detectable at the first time point, which corresponds to about 2 min of reaction. The lactam and hydroxylactam adducts formed more slowly. The quantities of lysyl-LG Schiff bases increased to a maximum at 162 min and subsequently decreased, whereas the levels of lactams continue to rise.

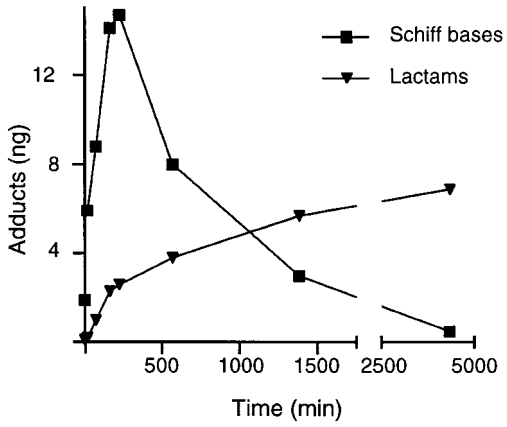


FIGURE 9: Time course of formation of the lysyl-LG adducts. Lysine was incubated with one molar equivalent of synthetic LGE_2. The samples were analyzed by LC/MS every 18 min, scanning between *m*/*z* 450 and 550. The peaks at *m*/*z* 463.4, 479.4, 481.4, and 495.4 were integrated and normalized against [^{13}C]lactam and [^{13}C]-reduced Schiff base (*m*/*z* 485.4 and 467.4, respectively) internal standards. Squares indicate the sum of lysyl-LG Schiff base (*m*/*z* 481.4) and lysyl-anhydroLG Schiff base adducts; triangles correspond to the sum of lysyl-LG lactam (*m*/*z* 479.4) and hydroxylactam (*m*/*z* 495.4) adducts.

DISCUSSION

The immediate product of the cyclooxygenases is PGH_2. This investigation affirms that the conversion of PGH_2 to the highly reactive levuglandins is a tangible, and indeed substantial, pathway derived from the nonenzymatic rearrangement of the endoperoxide. Salomon and colleagues (*18*)

estimated that 22% of PGH_2 was transformed in aqueous solution to levuglandins, based on analysis with nuclear magnetic resonance spectroscopy. We have confirmed that conclusion with direct physical evidence, utilizing analysis of LGE_2/D_2 with gas chromotography/mass spectrometry to demonstrate that these levuglandins constitute approximately 18% of the products formed from PGH_2 in 30 min. This is a minimal estimate as it reflects the net of both formation and degradation of the labile levuglandins within that period of time. The importance of this pathway of PGH_2 transformation is further emphasized by the evidence that PGH_2 itself will yield adducts of lysine that are the same as those derived from LGE_2.

Our findings demonstrate that the predominant adduct derived from the reaction of either PGH_2 or LGE_2 with lysine is a Schiff base that is formed from reaction of a lysyl amino group with levuglandin. Several lines of evidence support the structure of the Schiff base adduct. The molecular ion of 481.4 is consistent with that structure. The characteristics of cyanide derivatives provide strong support for the Schiff base structure. Reduction with sodium borohydride yields a reduced and dehydrated product that is consistent with its formation from the Schiff base structure, evidence that is further strengthened by the production of an daughter ion at *m*/*z* 321.4 upon collision-induced dissociation in tandem mass spectrometry. This ion is characteristic of the eicosanoid moiety of the adduct.

In addition to formation of adducts of free lysine, we find that both PGH_2 and LGE_2 react with peptides to yield a Schiff base adduct of the ϵ-amine of its lysyl residue. This evidence provides a basis for considering that the lysyl-LG Schiff base adduct could represent a novel mechanism for lipid modification of proteins by the cyclooxygenase.

Previous investigations (*16*) have demonstrated that a pyrrole adduct is formed from the reaction of levuglandin E_2 with protein-based lysyl ϵ-amino groups, and that oxidation of a similar pyrrole leads to formation of the more stable lactam and hydroxylactam structures (*17*). Both the Schiff base and the lactam adducts are identified in the present study. The Schiff base is the predominant adduct and is formed more rapidly than the lactam. The studies of Armanath et al. (*9*) with the model γ-ketoaldehyde, 4-oxohexanal, suggest that both the pyrrole and Schiff base are formed from a common hemiaminal intermediate with which the Schiff base is in equilibrium. Such an equilibrium would permit delayed formation of the pyrrole-derived lactam and hydroxylactam from the Schiff base, which would be consistent with our observation (Figure 9) that the lactam and hydroxylactam continued to accummulate concurrently with the decline in the levels of the Schiff bases. The loss of total lysyl-LG adducts over time is notable and among the possible explanations is intermolecular cross-linking to yield polymers. Aliphatic Schiff bases are relatively unstable and can undergo polymerization (*12*). Such cross-linking also has been shown to occur via pyrrole adducts (*19*) but is not thought to be due to reactions of lactam adducts. In addition, LGE_2-induced cross-linking of proteins and DNA (*3*) could be derived from LG adducts of these macromolecules.

In conclusion, PGH_2 can form both Schiff base and pyrrole-derived adducts of lysine via the levuglandin pathway. Characterization of these adducts provides a basis for evaluating their formation as a consequence of increased PGH_2 synthesis in cells and in vivo.

ACKNOWLEDGMENT

The authors gratefully acknowledge the valuable advice of Amit Kalgutkar and the capable technical assistance of Gwenn Sobo, Elizabeth Shipp, and Jiemin Wang.

REFERENCES

1. Salomon, R. G., and Miller, D. B. (1985) *Adv. Prostaglandin Thromboxane Leukot. Res. 15*, 323.
2. Iyer, R. S., Ghosh, S., and Salomon, R. G. (1989) *Prostaglandins 37*, 471−480.
3. Murthi, K. K., Friedman, L. R., Oleinick, N. L., and Salomon, R. G. (1993) *Biochemistry 32*, 4090−4097.
4. Graham, D. G., Amarnath, V., Valentine, W. M., Pyle, S. J., and Anthony, D. G. (1995) *Crit. Rev. Toxicol. 25*, 91−112.
5. Wilson, A. G., Kung, H. C., Anderson, M. W., and Eling, T. E. (1979) *Prostaglandins 18*, 409−422.
6. Kulmacz, R. J. (1987) *Biochem. Biophys. Res. Commun. 148*, 539−545.
7. Lecomte, M., Lecocq, R., Dumont, J. E., and Boeynaems, J. M. (1990) *J. Biol. Chem. 265*, 5178−5187.
8. Miller, D. B., Raychaudhuri, S. R., Avasthi, K., Lal, K., Levison, B., and Salomon, R. G. (1990) *J. Org. Chem. 55*, 3164−3175.
9. Amarnath, V., Amarnath, K., Valentine, W. M., Eng, M. A., and Graham, D. G. (1995) *Chem. Res. Toxicol. 8*, 234−238.
10. Iyer, R. S., Miller, D. B., and Salomon, R. G. (1990) *J. Org. Chem. 55*, 3175−3180.
11. Harada, K. (1970) in *The Chemistry of the Carbon−Nitrogen Double Bond* (Patai, S., Ed.) pp 255−298, Interscience Publishers, New York.
12. Ogata, Y., and Kawasaki, A. (1970) in *The Chemistry of the Carbonyl Group* (Zabicky, J., Ed.) pp 21−69, Interscience Publishers, New York.
13. Okano, V., do Amaral, L., and Cordes, E. H. (1976) *J. Am. Chem. Soc. 98*, 4201−4203.
14. Ching, W.-M., and Kallen, R. G. (1978) *J. Am. Chem. Soc. 100*, 6119−6124.
15. Patai, S., and Rappoport, Z. (1964) in *The Chemistry of Alkenes* (Patai, S., Ed.) pp 469−584, Interscience Publishers, New York.
16. Iyer, R. S., Kobierski, M. E., and Salomon, R. G. (1994) *J. Org. Chem. 59*, 6038−6043.
17. Brame, C. J., Salomon, R. G., Morrow, J. D., and Roberts, J. R., II (1999) *J. Biol. Chem. 274*, 13139−13146.
18. Salomon, R. G., Miller, D. B., Zagorski, M. G., and Coughlin, D. J. (1984) *J. Am. Chem. Soc. 106*, 6049−6060.
19. Amarnath, V., Valentine, W. M., Amarnath, K., Eng, M. A., and Graham, D. G. (1994) *Chem. Res. Toxicol. 7*, 56−61.

BI990470+