Characterization of Bis(levuglandinyl) Urea Derivatives as Products of the Reaction between Prostaglandin H_2 and Arginine†

Irene Zagol-Ikapitte,‡ Nathalie Bernoud-Hubac,‡ Venkataraman Amarnath,+ L. Jackson Roberts, II,‡,§ Olivier Boutaud,*,‡ and John A. Oates‡,§

Departments of Pharmacology, Pathology, and Medicine, Vanderbilt University, Nashville, Tennessee 37232-6602

Received January 21, 2004; Revised Manuscript Received March 9, 2004

ABSTRACT: Levuglandins are γ-keto aldehydes formed by rearrangement of prostaglandin (PG) H_2 in aqueous solution. Levuglandins are highly reactive with primary amines. We had previously characterized adducts formed after reaction of levuglandin E_2 (LGE_2) or PGH_2 with lysine. In this study, we assessed whether reaction of PGH_2 with arginine yielded covalent adducts. Using N^{α}-acetylarginine and both PGH_2 and synthetic LGE_2, we discovered a novel series of levuglandinyl adducts derived from reaction of two levuglandin moieties with the guanidino group of arginine. Subsequent spontaneous hydrolysis of the adducted amino acid yields bis(levuglandinyl) urea and the corresponding ornithine residue. Using liquid chromatography tandem mass spectrometry, we characterized the molecular structure of these novel adducts and demonstrated their formation after coincubation of PGH_2 with synthetic peptides and proteins. The soluble characteristic of these molecules provides a potential strategy for development of biological markers of lipid modification of proteins following cyclooxygenase activity or lipid peroxidation.

The prostaglandin H synthases (PGHS) participate in a number of physiologic and pathologic functions and are targets of an array of pharmacologic inhibitors. The PGHSs catalyze the oxygenation of arachidonic acid to the endoperoxide, prostaglandin (PG) H_2. PGH_2 is further metabolized to the prostanoids PGD_2, PGE_2, $PGF_{2\alpha}$, thromboxane A_2, and prostacyclin by specific enzymes. Also, PGH_2 in aqueous solutions undergoes nonenzymatic rearrangement not only to PGE_2, and PGD_2, but also 20% of PGH_2 is converted to the highly reactive γ-keto aldehydes, levuglandins (LG) E_2 and D_2 (*1*, *2*). Levuglandins are known to react covalently with primary amines, such as the ϵ-amine of lysine on proteins and with DNA (*3*, *4*). We have characterized the adducts that are formed by the reaction of lysine with LGE_2 or PGH_2 (*2*, *5*), and knowledge of their structures has provided a basis for analysis of the adducts in protein digests utilizing liquid chromatography-tandem mass spectrometry (*6*−*8*). To further characterize the interaction of levuglandins with proteins, we examined the reaction of LG with arginine and found that two molecules of levuglandin react with the guanidino group. This labile product then cleaves to yield a bis(levuglandinyl) urea (BLU)[1] and ornithine.

EXPERIMENTAL PROCEDURES

Materials. L-Arginine, N^{α}-acetylarginine, human serum albumin (HSA), histone (fraction III), β-amyloid, methoxylamine hydrochloride, and sodium borohydride were purchased from Sigma (St. Louis, MO). N^{α}-acetylglycylglycylglycylglycylglycyllysylglycylglycylglycylarginine (N^{α}-AcetylGGGGKGGGR) was purchased from Research Genetics, Inc. (Huntsville, AL). Oasis Sep Pak Cartridges were obtained from Waters Corp. (Milford, MA). XDB C_8 column (2.1 × 15 mm) was from Mac Mod Analytical (Chadds Ford, PA). PGH_2 was synthesized as described previously (*9*). LGE_2 was synthesized by V. Amarnath (unpublished experiments).

LC/ESI/MS/MS Analysis. Aliquots of the incubation mixtures were analyzed by liquid chromatography (LC)/electrospray ionization (ESI)/tandem mass spectrometry (MS/MS) using a 2.1 × 15 mm XDB C_8 column with a flow rate of 0.2 mL/min. A linear gradient of 10−90% acetonitrile in 5 mM ammonium acetate/ 0.1% acetic acid was applied in 10 min. Electrospray tandem mass spectrometric analysis was carried out on a Finnigan TSQ 7000 system (Finnigan Corp., San Jose, CA). The mass spectrometer was operated in a positive ion mode with a potential of 4 kV applied to the electrospray needle. Nitrogen was used as the sheath gas (70 psi) and auxiliary gas (10 psi) to assist the nebulization. The heated capillary was operated at 200 °C and 20 V, and the tube lens voltage was set at 80 V. Spectra were displayed by averaging scans across the chromatographic peaks. Scanning was carried out as indicated in figure legends, or when needed, ions were subjected to collision-induced

† This work was supported in part by NIH Grants CA 68485, GM 15431, GM42056, and DK26657. J.A.O. is the Thomas F. Frist, Sr. Professor of Medicine.

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

‡ Department of Pharmacology.

+ Department of Pathology.

§ Department of Medicine.

[1] Abbreviations: BLU, bis(levuglandinyl) urea; CID, collision-induced dissociation; ESI, electrospray ionization; HSA, human serum albumin; HPLC, high performance liquid chromatography; LC/MS, liquid chromatography−mass spectrometry; $[MH]^+$, molecular ion; $NaBH_4$, sodium borohydride; LG, levuglandin; N^{α}-acetylGGGGKGGGR, peptide N^{α}-acetylGlycylGlycylGlycylGlycylLysylGlycylGlycylGlycylArginine; PBS, phosphate-buffered saline; SRM, selected reaction monitoring.

10.1021/bi049842r CCC: $27.50
Published on Web 04/16/2004

dissociation at −28 and −36 eV at an indicated argon pressure of 2.6 mTorr.

Formation of Arginyl-LG Adducts. LGE_2 (1 mM) was incubated with L-arginine (1 mM) in 10× phosphate-buffered saline (PBS) at room temperature, pH 7.4 for 6 or 24 h. To reduce and stabilize Schiff base adducts, 1:10 volume of 100 mM sodium borohydride in dimethylformamide was added and allowed to incubate for 30 min at 0 °C (final concentration 10 mM). The samples were extracted using Oasis cartridges preconditioned with methanol and water as described by the manufacturer. The reaction mixture was loaded, washed with water, and eluted with methanol. The samples were then analyzed by LC/ESI/MS/MS in the positive ion mode as described previously, scanning ions between *m*/*z* 400 and *m*/*z* 600.

Purification and Analysis of BLU Adducts Formed after Reaction of N^{α}-Acetylarginine with PGH_2 and LGE_2. PGH_2 or LGE_2 (1 mM) was incubated with N^{α}-acetylarginine (1 mM) in 10X PBS pH 7.4 at room temperature for 24 h. After incubation, the reaction mixture was immediately applied to an Oasis cartridge preconditioned with 10 mL of methanol and 10 mL of water. No reduction with sodium borohydride was performed in these experiments. The column was washed with water, and the adducts were eluted with methanol. The methanol was concentrated to 100 μL under a N_2 stream, and the products were analyzed by LC/ESI/MS/MS in the positive ion mode, scanning ions between *m*/*z* 450 and *m*/*z* 750. Collision induced dissociation (CID) of the putative BLU adducts was carried out at energies ranging from −10 to −45 eV with a collision gas pressure of 2.6 mTorr, scanning the daughter ions between *m*/*z* 50 and *m*/*z* 750.

Characterization of BLU Adducts Formed after Reaction of N^{α}-Acetylarginine with LGE_2. To confirm their structure, the BLU adducts were chemically derivatized and analyzed by LC/ESI/MS scanning ions between *m*/*z* 500 and *m*/*z* 900. After incubation of LGE_2 (1 mM) with N^{α}-acetylarginine (1 mM) in PBS pH 7.4 at room temperature for 24 h, the adducts were reduced with 1110 volume of 100 mM sodium borohydride in dimethylformamide for 30 min at 0 °C before analysis by LC/ESI/MS. To generate methoxylamine derivatives, an equal volume of 6% methoxylamine hydrochloride in PBS adjusted to pH 6 was added to the reaction mixture and allowed to incubate for 30 min at room temperature before LC/ESI/MS analysis. For catalytic hydrogenation, the adducts were first extracted on Oasis cartridge as described previously. The solvent was exchanged with 2 mL of ethanol, 75 mg of palladium on charcoal (10% w/w) was added, and hydrogen was bubbled for 10 min at room temperature (about one bubble per second). The palladium was then removed by centrifugation in a bench centrifuge, and the solvent was dried down to 100 μL under nitrogen before analysis by LC/ESI/MS/MS.

Comparative Analysis of the Rate of Formation of the Levuglandinyl Adducts with Lysine and Arginine. N^{α}-acetylGGGGKGGGR (1 mM) was incubated with 1 or 3 molar equivalents of LGE_2 in 10× PBS at room temperature, pH 7.4 for 6 h. The samples were extracted with an Oasis cartridge and analyzed by LC/ESI/MS/MS in the positive ion mode scanning ions between *m*/*z* 800 and *m*/*z* 1900.

Analysis of BLU Adducts Formed from Reaction of LGE_2 with Proteins. Human serum albumin **(**HSA) (1 mg/mL), histone (1 mg/mL), and β-amyloid (0.1 mg/mL) in 10× PBS (pH 7.4) were treated with 1 mM LGE_2 at room temperature for 24 h. After incubation, the reaction mixtures were applied to an Oasis cartridge, preconditioned with methanol and water. The Oasis was washed with water before elution with methanol. The eluate was dried, resuspended in methanol, and subjected to LC/ESI/MS/MS in the positive ion mode. The presence of BLU adducts was assessed by multiple reaction monitoring, following the transitions characteristic of fragments of anhydrolactam−anhydroSchiff base BLU adducts (*m*/*z* 691.4 to 375.1 and 691.4−357.4).

Analysis of Amino Acid Content of Amyloid-β after Reaction with LGE_2. $A\beta_{1-42}$ in sodium phosphate buffer 100 mM (pH 7.4) was incubated with 5 molar equivalents of synthetic LGE_2 at room temperature. After 24 h, the protein was hydrolyzed with 6 N HCl at 110 °C for 15 h. Then, the amino acid content was analyzed by ACCUTAG derivatization. The Waters Accutag amino acid analysis of the adducted amyloid was carried out by the Protein Chemistry Core Laboratory of Vanderbilt University.

RESULTS

Identification of Arginyl-LG Adducts. After incubation of arginine with synthetic LGE_2 followed by treatment with sodium borohydride, the analysis of the products of reaction by LC/ESI/MS (Figure 1) showed the presence of four different ions corresponding to the arginyl-LG anhydroSchiff base (anHSB, *m*/*z* 491.2), dehydrated reduced Schiff base (deHSB, *m*/*z* 495.2), lactam (Lact, *m*/*z* 507.2), and hydroxylactam adducts (OHLact, *m*/*z* 523.2). These structures were deduced by analogy from the published structures of levuglandinyl-lysine adducts (*2*, *5*). Because LGE_2 could react with the amine present at both the N^{α}- and the guanidino positions, we performed the same experiment using N^{α}-acetylarginine. In these conditions, there was formation of none of the previous ions, but three peaks were observed at higher *m*/*z* ratios (Figure 2). Because the products of reaction of LGE_2 with arginine were not present when the N^{α}-amine of arginine was acetylated, we concluded that the ions at *m*/*z* 491.2, 495.2, 507.2, and 523.2 represented levuglandin adducts formed at the N^{α}-amine of arginine. It also indicated that LGE_2 could react with the guanidino group of arginine to form what we hypothesized to be bis(levuglandinyl) urea (BLU) adducts with the following combination of structures: anhydrolactam-anhydroSchiff base (anHL-anHSB, *m*/*z* 691.8), lactam-Schiff base (L-SB, *m*/*z* 727.4), and hydroxylactam-Schiff base (OHL-SB, *m*/*z* 743.5) BLU adducts (Figure 2). We hypothesized that the formation of these BLU would follow the mechanism that we present in Figure 3: after both the amino groups of the guanidine function react with LGE_2 to form pyrroles, the bond between N^6 and C^7 becomes a localized double bond (*10*) and undergoes nonenzymatic hydrolysis (*11*) to yield the corresponding BLU and ornithine.

Characterization of Bis(levuglandinyl) Urea Adducts. We sought to further characterize the structure of the potential BLU adducts with three approaches: first, we performed LC/ESI/MS/MS analysis of three of the more prominent products of the reaction of LGE_2 with arginine and characterized the structures of the different daughter ions generated by collision-induced dissociation. Second, we confirmed the

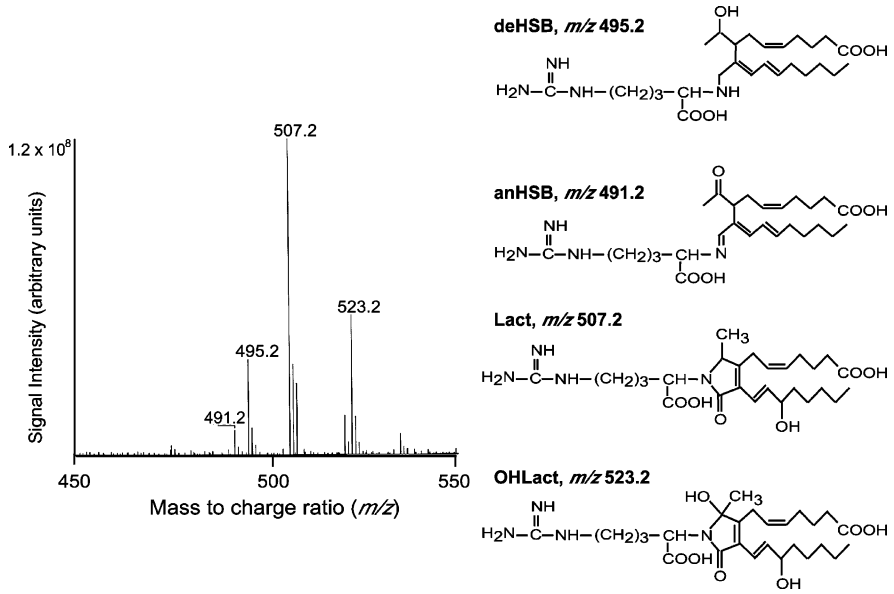

FIGURE 1: LC/ESI/MS spectrum of levuglandinyl-N^{α}-arginine adducts. Arginine was incubated with LGE_2 in PBS pH 7.4 at room temperature for 24 h. The Schiff base adducts were reduced with 10 mM sodium borohydride at 4 °C for 30 min. The samples were extracted with Oasis cartridge and analyzed by LC/ESI/MS as described in Experimental Procedures. Ions were scanned between *m*/*z* 400 and *m*/*z* 600. The structures of the lactam (Lact, *m*/*z* 507.2), hydroxylactam (OHLact, *m*/*z* 523.2), reduced Schiff base (DeHSB, *m*/*z* 495.2), and anhydroSchiff base (anHSB, *m*/*z* 491.2) adducts are shown.

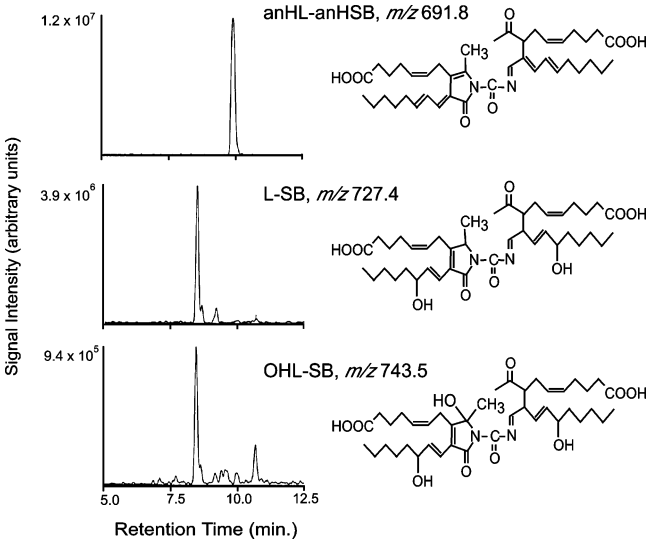

FIGURE 2: Selected ion current chromatograms of bis(levuglandinyl) urea (BLU) adducts. LGE_2 was incubated with N^{α}-acetylarginine, in PBS pH 7.4 at room temperature for 24 h. The samples were extracted using Oasis cartridges and analyzed by LC/ESI/MS. Selected ion monitoring chromatograms for anhydrolactam-anhydroSchiff base bis(levuglandinyl) urea (anHL-anHSB, *m*/*z* 691.8), Lactam-Schiff base bis(levuglandinyl) urea (L-SB, *m*/*z* 727.4), and hydroxylactam-Schiff base bis(levuglandinyl) urea (OHL-SB, *m*/*z* 743.5) adducts are shown. The structures of each adduct are represented.

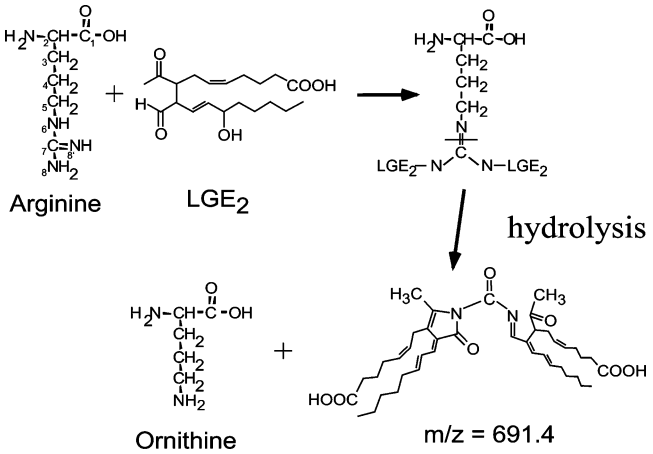

FIGURE 3: Formation of bis(levuglandinyl) urea. After formation of bis(levuglandinyl) adducts on the amines of the guanidino group of arginine, nonenzymatic hydrolysis of the guanidino group yields BLU and ornithine.

molecular features of the putative anhydrolactam-anhydroSchiff base BLU adduct (*m*/*z* 691.4) by LC/MS after chemical modification. Third, we confirmed the formation of the ornithine residue in a synthetic peptide following reaction with different molar equivalents of synthetic LGE_2.

To further substantiate the structural identity of these compounds as the anhydrolactam-anhydroSchiff base (*m*/*z* 691.4), lactam-Schiff base (*m*/*z* 727.4), and hydroxylactam-Schiff base (*m*/*z* 743.6) BLU adducts, collision induced dissociation of the molecular ions of BLU adducts was performed at energies ranging from −10 to −45 eV with 2.6 mTorr collision gas, scanning daughter ions from *m*/*z* 50 to 750. These ions and their corresponding fragments in the CID spectra obtained at −36 eV are shown in Figure 4. The ion shifts in the CID spectra of the adducts support the proposed structures of these species. CID of the ion at *m*/*z* 727.4 produced informative daughter ions at *m*/ *z* 375.1 and 357.4 (Figure 4B); these masses correspond to the lactam urea moiety, having lost one or two molecules of water, respectively (Figure 4B). CID of the ion at *m*/*z* 743.6 produced daughter ions at *m*/*z* 391.1 and 290.6 (Figure 4C). These ions correspond to the dehydrated hydroxylactam urea moiety and to the Schiff base moiety decarboxylated and dehydrated as represented in Figure 4C. The predominant compound was the anhydrolactam−anhydroSchiff base BLU adduct, which was characterized by the $[MH]^+$ ion at *m*/*z* 691.4. The daughter ions obtained by CID corresponded to the anhydrolactam moiety at *m*/*z* 375.1 and to its dehydrated form at *m*/*z* 357.4 (Figure 4A).

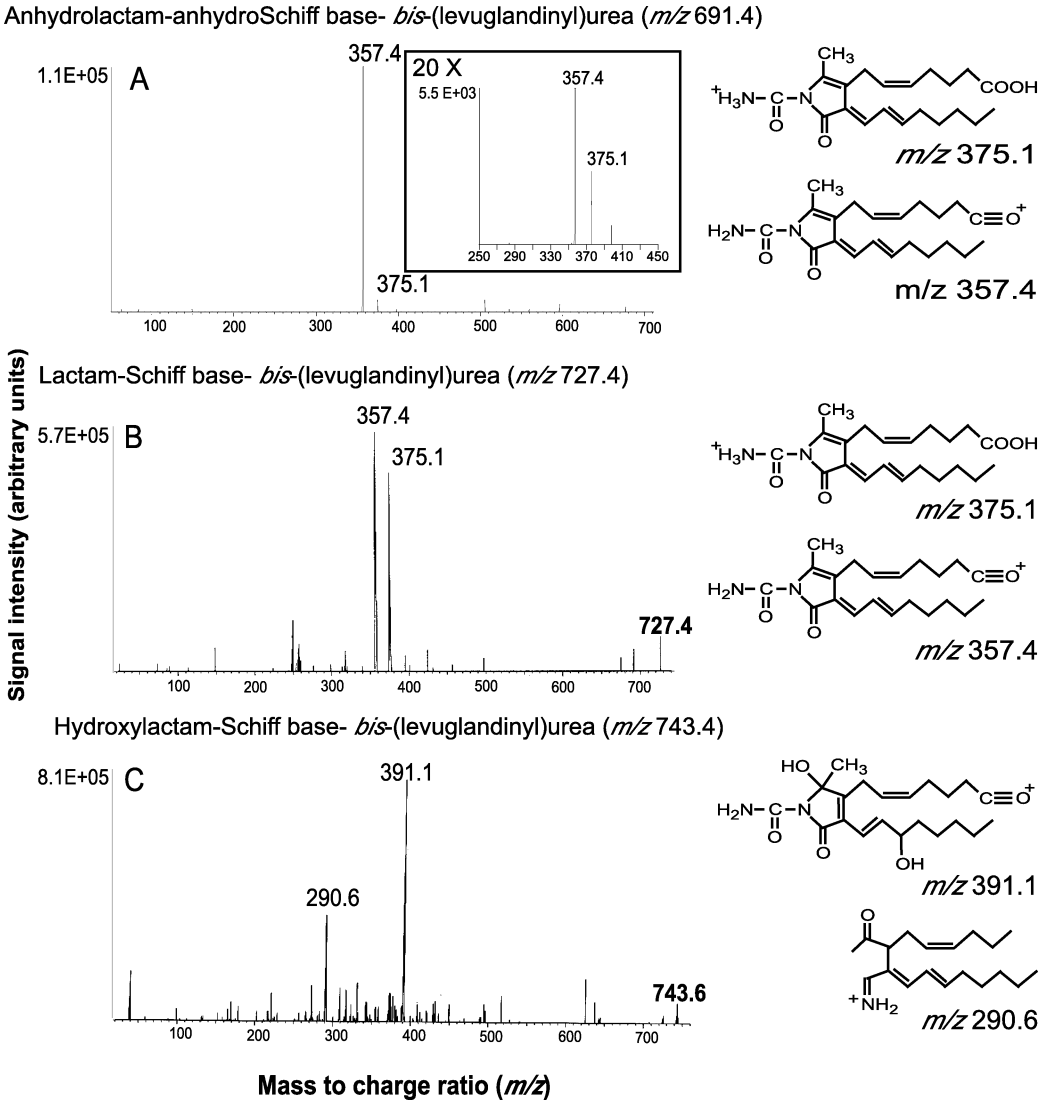

FIGURE 4: LC/ESI/MS/MS analysis of BLU adducts. After reaction of LGE_2 with N^{α}-acetylarginine, the adducts were purified by chromatography on Oasis cartridge and analyzed by LC/ESI/MS/MS. The $[MH]^+$ ions of the three major BLUs adducts were subjected to CID at −36 eV. The fragment ions were scanned from *m*/*z* 50 to 750. The proposed structures of the major fragment ions are indicated. (A**)** Anhydrolactam−anhydroSchiff base BLU (*m*/*z* 691.4). The inset depicts the spectrum was expanded 20 times to show the fragment ion at *m*/*z* = 375.1; the ion at *m*/*z* = 357.4 is off-scale in this expanded spectrum. (B) Lactam−Schiff base BLU (*m*/*z* 727.4). (C) Hydroxylactam−Schiff base BLU (*m*/*z* 743.6).

Because the species at *m*/*z* 691.8 was the most abundant, we sought to further characterize its chemical structure. We confirmed the presence of the Schiff base moiety by treating the sample with sodium borohydride (Figure 5, reduction). The carbonyl in the lactam ring is resistant to reduction by sodium borohydride (*5*); however, both the imine and the carbonyl groups in the Schiff base moiety can be reduced (*2*). After treatment with sodium borohydride, the ions at *m*/ *z* 691.8 disappeared concomitantly with the appearance of the species at *m*/*z* 677.8. By analogy with the levuglandinyl-lysine Schiff base adduct (*2*), we propose that the reduction of the carbonyl was followed by dehydration yielding a molecule 14 mass units less than the initial compound. The dehydration leads to the loss of chirality of carbon 8, yielding a single peak shape for the product at *m*/*z* 677.8.

We sought to further characterize the anhydrolactam moiety of the adduct by derivatizing its carbonyl groups with methoxylamine hydrochloride. The results, shown in Figure 5 (methoxylation), indicate the disappearance of the ion at *m*/*z* 691.8 and the appearance of a signal at *m*/*z* 749.8, corresponding to the molecular ion of the bismethoxime derivative. Although lactams usually do not form oximes, the delocalization of the electron pair of the nitrogen atom in the ring with the conjugated double bonds of the levuglandinyl moiety and the ketone in the urea group make the carbonyl more electrophilic, allowing its methoximation.

We determined the number of carbon−carbon double bonds by performing catalytic hydrogenation on palladium on carbon 10% (w/w) of the anhydrolactam- anhydroSchiff base BLU adduct (Figure 5, catalytic hydrogenation). We chose palladium on carbon because aliphatic aldehydes, ketones, and nitriles are hydrogenated very slowly over this catalyst (*12*, *13*). The ion at *m*/*z* 691.8 presents a double peak shape due to one chiral carbon on the anhydroSchiff base at position 8. Following hydrogenation, new peaks at *m*/*z* 705.8 (691.8 + 14) were observed, indicating the presence of seven double bounds. This reduction leads to generation of five chiral carbons yielding 10 stereoisomers that are separated during the chromatography in several

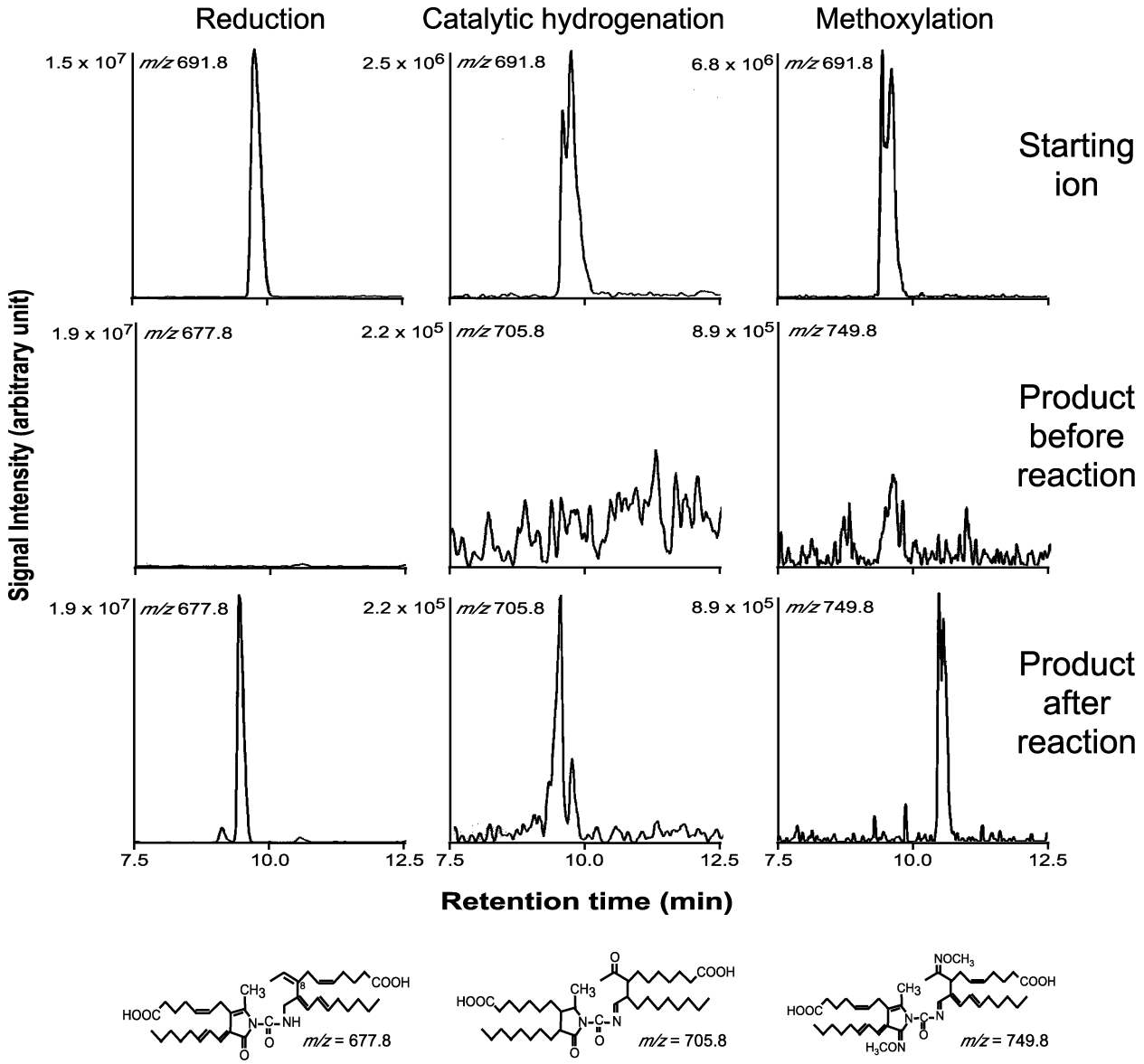

FIGURE 5: Analysis of BLU adducts before and after chemical treatment. After reaction of N^{α}-acetylarginine with LGE_2 for 24 h, the BLU adducts were reduced with sodium borohydride (reduction), derivatized with methoxyamine chloride (methoxylation), or hydrogenated with palladium 10% on carbon (catalytic hydrogenation) as described in Experimental Procedures. The chromatogram for the initial ion at *m*/*z* 691.8 is presented before each reaction. The selected ion current chromatograms corresponding to the products before and after each reaction are presented.

different peaks. Taken together, these results confirmed the proposed structure of the most abundant BLU adduct as the anhydrolactam-anhydroSchiff base (*m*/*z* 691.8).

Formation of BLU Adducts Derived from PGH_2. The anhydrolactam-anhydroSchiff base BLU was analyzed by selected reaction monitoring. The parent ion at *m*/*z* 691.4 was submitted to collision induced dissociation at −36 eV with a CID gas pressure of 2.6 mTorr, and the fragments at *m*/*z* 375.1 and 357.4 were monitored. We observed the appearance of the two ions after incubation of N^{α}-acetylarginine with synthetic LGE_2 or PGH_2 (Figure 6). This result demonstrates that BLU adducts are formed from PGH_2 through the levuglandin rearrangement pathway.

Characterization of Peptidyl-LG Adducts. To compare the relative reactivity of LG with lysine and arginine, we designed a synthetic peptide that had one lysine and one arginine separated by three glycines and that had the N^{α}-amine acetylated to prevent formation of LG adducts at that

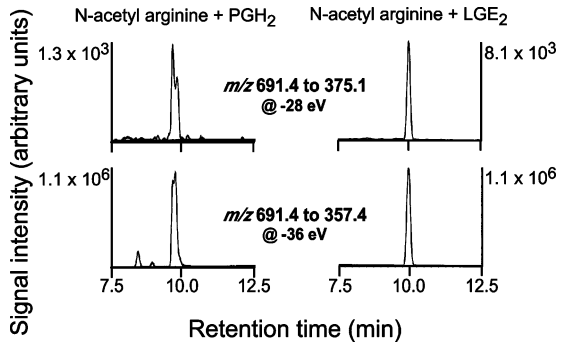

FIGURE 6: Comparison of anhydrolactam−anhydroSchiff base BLU formed after reaction of N^{α}-acetylarginine with LGE_2 or PGH_2. After reaction of LGE_2 or PGH_2 in the presence of N^{α}-acetylarginine for 24 h, the BLU adducts formed were purified by chromatography on Oasis cartridge and analyzed by LC/ESI/MS/MS. Selected reaction chromatograms for the daughter ions of the anhydrolactam−anhydroSchiff base BLU adduct (*m*/*z* 691.4−375.1 at −28 eV and *m*/*z* 691.4−357.4 at −36 eV) are shown.

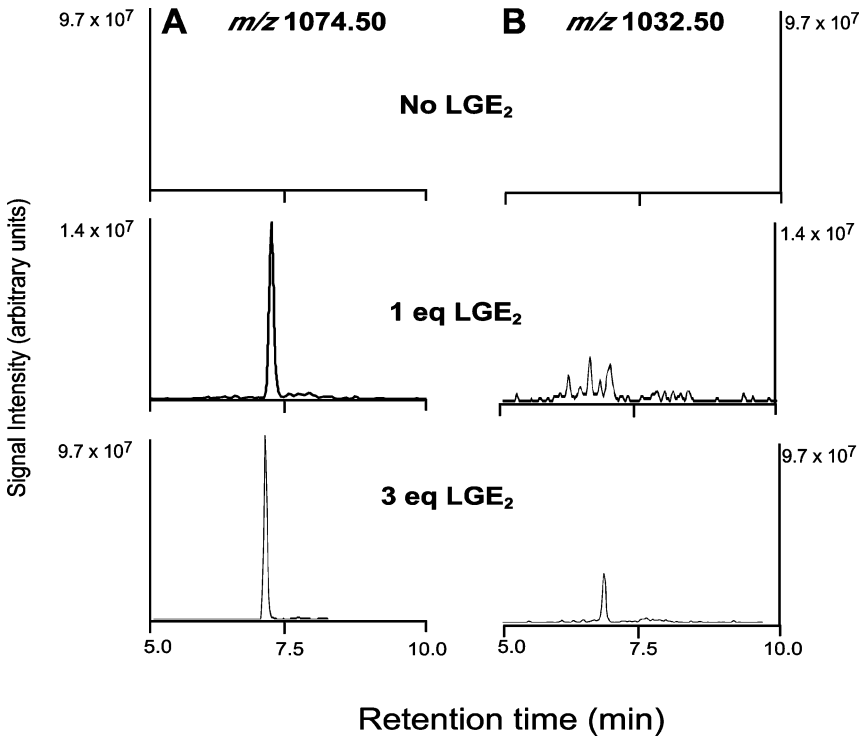

FIGURE 7: Formation of levuglandinyl adducts on synthetic N^{α}-acetylGGGGKGGGR leads to the modification of arginine to ornithine. N^{α}-acetylGGGGKGGGR was incubated with 1 or 3 molar equivalents of the synthetic LGE_2 at room temperature in PBS at pH 7.4 for 6 h. The samples were then analyzed by LC/ESI/MS scanning ions between *m*/*z* 800 and *m*/*z* 1900. Selected ion current chromatograms are shown for (A) N^{α}-acetylGGGGK-anhydroSchiff base GGG arginine (*m*/*z* 1074.5) and (B) N^{α}-acetylGGGGK-anhydroSchiff base GGG ornithine (*m*/*z* 1032.5).

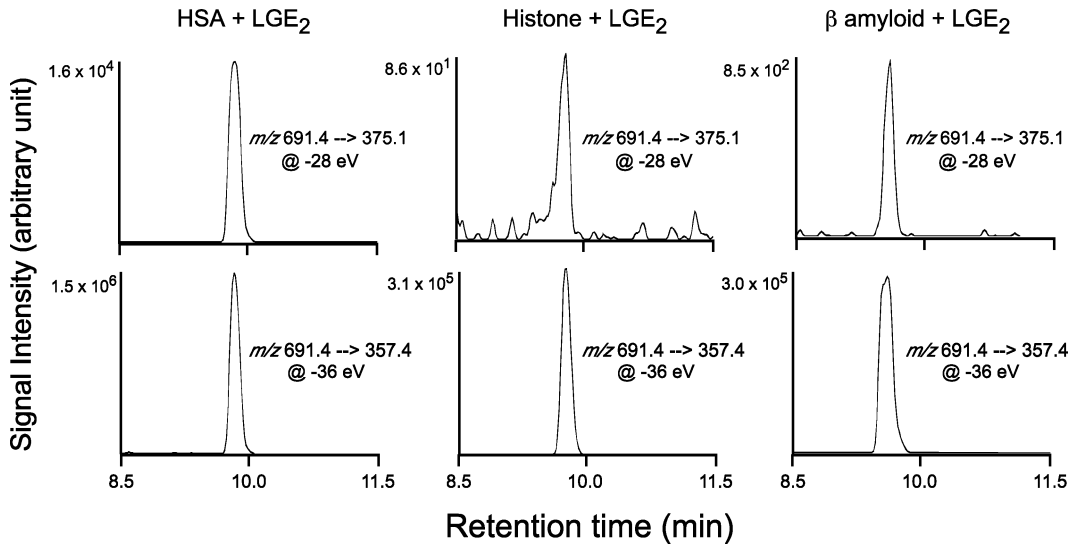

FIGURE 8: Protein-derived BLU adducts. HSA (1 mg/mL), histone (1 mg/mL), and β-amyloid (0.1 mg/mL) in 10× PBS (pH 7.4) were treated with 5 molar equivalents of LGE_2 at room temperature for 24 h. After incubation, the reaction mixtures were applied to an Oasis cartridge and subjected to LC/ESI/MS/MS. Selected reaction monitoring chromatograms for the daughter ions of the anhydrolactam−anhydroSchiff base BLU (*m*/*z* 691.4−375.1 at −28 eV and *m*/*z* 691.4−357.4 at −36 eV) are shown.

position. N^{α}-acetylGGGGKGGGR was incubated with 1 or 3 molar equivalents of synthetic LGE_2. After 6 h at room temperature, the samples were analyzed by LC/ESI/MS/MS scanning ions between *m*/*z* 800 and *m*/*z* 1900 (Figure 7). We predicted that formation of a lysyl-LG adduct would yield an ion at *m*/*z* 1074.5 and that formation of LG adducts at both the lysine and the arginine residues also would modify the arginine residue to an ornithine (Figure 3) yielding an ion at *m*/*z* 1032.5. As shown in Figure 7, the lysyl-LG species (*m*/*z* 1074.5) is the predominant species formed in the presence of 1 molar equivalent of LGE_2. At a higher molar ratio, the ornithine species is formed reflecting the formation of levuglandinyl adducts on the original arginine. These results indicate that lysine residues are more reactive than arginines and validate our hypothetical mechanism for the formation of BLU adducts depending on spontaneous hydrolysis of the bis(levuglandinyl) adduct on arginine.

Reaction of Levuglandin with Arginines of Proteins. To assess whether BLU adducts would form from proteins incubated in the presence of a ratio of LGE_2 to amine groups of less than one, we incubated β-amyloid ($A\beta_{1-42}$), histone, or human serum albumin (HSA) with 5 molar equivalents

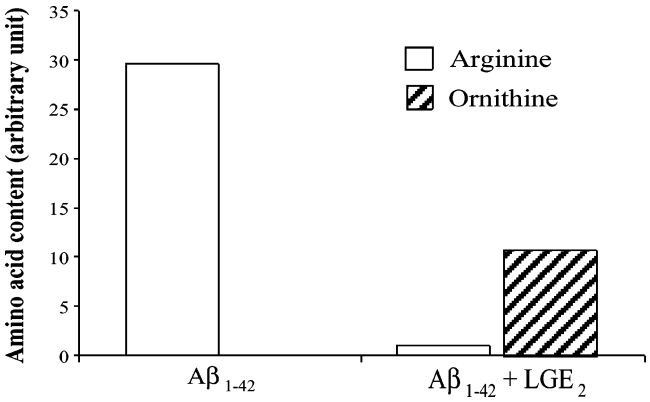

FIGURE 9: Arginine is modified into ornithine after reaction of $A\beta_{1-42}$ with LGE_2. After incubation of $A\beta_{1-42}$ with 5 molar equivalents of LGE_2 for 24 h, the protein was hydrolyzed with 6 N HCl at 110 °C for 15 h. The amino acid content was analyzed by ACCUTAG derivatization. The values for arginine and ornithine before and after reaction with LGE_2 are shown.

of synthetic levuglandin. After 24 h incubation, the reaction mixtures were subjected to LC/ESI/MS/MS. Selected reaction monitoring chromatograms for the daughter ions of anhydrolactam−anhydroSchiff base BLU adduct could be detected from HSA, histone, and β-amyloid, as shown in Figure 8. These results clearly indicated that BLU adducts were formed after incubation of proteins with LGE_2 in conditions that did not require levuglandin in excess of the lysine residues.

Generation of Ornithine Following Reaction of Protein with LGE_2. To demonstrate formation of ornithine as a result of reaction of LGE_2 with arginine residues in proteins, we incubated $A\beta_{1-42}$ with 5 molar equivalents of synthetic LGE_2 for 24 h. After acid hydrolysis, the amino acid content was analyzed by ACCUTAG derivatization. The results indicated that the arginine content was decreased after reaction with LGE_2 and that it was paralleled with the appearance of ornithine, which is not naturally present in $A\beta_{1-42}$ (Figure 9). These data identified a posttranslational modification resulting from covalent eicosanoylation of arginine residues, which is associated with a change in the peptide structure from a highly polar guanidino group to a primary amine.

DISCUSSION

Our studies have characterized a novel class of levuglandin adducts that are derived from reaction of LGE_2 with the guanidino group of arginine. Levuglandins are γ-keto aldehydes that form covalent adducts with free amines contained in proteins (*5*), DNA (*3*, *4*), and polyamines (*7*). They are formed from PGH_2 by nonenzymatic rearrangement (*1*, *2*). They also can be formed by lipid peroxidation, yielding 64 isomers called isoketals (isoK) (*5*). We have shown that LGE_2 reacts with arginine to form a series of bis-adducts on the guanidino group. These adducts are unstable in aqueous conditions and undergo hydrolytic cleavage to yield bis(levuglandinyl) urea and ornithine.

The main species generated in vitro by reaction of N^{α}-acetylarginine and LGE_2 is the anhydrolactam-anhydroSchiff base BLU adduct. Therefore, we developed a mass spectrometric assay to measure formation of this adduct as a result of reaction of LG with arginine residues in proteins. We have shown that ϵ-amine of lysine and α-amines of amino acids are more reactive than the guanidino group of arginine and that formation of BLU adducts using a synthetic peptide required a concentration of LGE_2 in excess of the concentration of lysine. However, when the reaction was carried on with proteins and 5 molar equivalents of LGE_2, we observed a strong signal corresponding to the BLU adducts at *m*/*z* 691.4. Human serum albumin contains 61 lysines and 28 arginines, which gives a molar ratio of LGE_2 to free amines of 0.04. This implies that the BLU adducts were formed even in the presence of excess lysine. These results are important because they suggest that protein microenvironment could modify the reactivity of certain arginines, leading to the formation of BLU adducts in conditions where levuglandins are generated in small amounts. Also, we have shown that BLU adducts were formed by reaction of PGH-derived LG with arginine residues in PGHS-1 following arachidonic acid oxygenation by the enzyme (data not shown).

The reaction of LG with arginine residues in proteins provided the basis for a hypothesis that substitution of an ornithine for arginine as the result of cyclooxygenase activity or lipid peroxidation would produce a novel posttranslational modification of proteins. Pursuing this hypothesis, we demonstrated that the arginine present in $A\beta_{1-42}$ was modified to ornithine following reaction of LGE_2. This finding provides a basis for determining the presence of this posttranslational modification in proteins in biological samples.

We have observed that the formation of BLU adducts also occurs when LGE_2 is incubated directly with urea in solution (data not shown). Free arginine is present at high concentration in biological systems such as plasma (76−81 μM), CSF (22 μM), and brain (0.1−0.2 μmol/g wet weight) (*14*−*17*). Therefore, reaction of LGE_2 with either free urea or free arginine could represent a hypothetical mechanism of detoxication of levuglandins by the body, leading to less reactive, soluble molecules that could be excreted more easily.

In summary, we have characterized the nature of the adducts formed by reaction of γ-keto aldehydes with arginine. We have shown that formation of the soluble BLU adducts from proteins leads to a novel posttranslational modification that yields ornithine in place of the original arginine. The fact that BLU adducts could be formed from both levuglandins derived from the PGHSs and isoketals formed by lipid peroxidation broadens the potential importance of these molecules in pathobiology to include both PGHS activity and oxidant injury. These findings provide a basis for the development of specific analytical tools for their detection and quantification in explorations of their formation in pathologic conditions and after pharmacologic intervention in vivo.

ACKNOWLEDGMENT

We thank Tina Masterson and Elizabeth Shipp for excellent technical assistance. We thank Eric F. Howard from the Vanderbilt Protein Chemistry Core Laboratory for the determination of the amino acid content.

REFERENCES

1. Salomon, R. G., Miller, D. B., Zagorski, M. G., and Coughlin, D. J. (1984) Solvent-Induced fragmentation of prostaglandin endoperoxides. New aldehyde products from PGH2 and a novel intramolecular 1,2-hydride shift during endoperoxide fragmentation in aqueous solution, *J. Am. Chem. Soc. 106*, 6049−6060.

2. Boutaud, O., Brame, C. J., Salomon, R. G., Roberts, L. J., II., and Oates, J. A. (1999) Characterization of the lysyl adducts formed from prostaglandin H2 via the levuglandin pathway, *Biochemistry 38*, 9389−9396.
3. Iyer, R. S., Ghosh, S., and Salomon, R. G. (1989) Levuglandin E2 cross-links proteins, *Prostaglandins 37*, 471−480.
4. Murthi, K. K., Friedman, L. R., Oleinick, N. L., and Salomon, R. G. (1993) Formation of DNA-protein cross-links in mammalian cells by levuglandin E2, *Biochemistry 32*, 4090−4097.
5. Brame, C. J., Salomon, R. G., Morrow, J. D., and Roberts, L. J., II. (1999) Identification of extremely reactive γ-keto aldehydes (isolevuglandins) as products of the isoprostane pathway and characterization of their lysyl protein adducts, *J. Biol. Chem. 274*, 13139−13146.
6. Wilson, A. G., Kung, H. C., Anderson, M. W., and Eling, T. E. (1979) Covalent binding of intermediates formed during the metabolism of arachidonic acid by human platelet subcellular fractions, *Prostaglandins 18*, 409−422.
7. Boutaud, O., Brame, C. J., Chaurand, P., Li, J., Rowlinson, S. W., Crews, B. C., Ji, C., Marnett, L. J., Caprioli, R. M., Roberts, L. J., II., and Oates, J. A. (2001) Characterization of the lysyl adducts of prostaglandin H-synthases that are derived from oxygenation of arachidonic acid, *Biochemistry 40*, 6948−6955.
8. Boutaud, O., Li, J., Zagol, I., Shipp, E. A., Davies, S. S., Roberts, L. J., II, and Oates, J. A. (2003) Levuglandinyl adducts of proteins are formed via a prostaglandin H2 synthase-dependent pathway after platelet activation, *J. Biol. Chem. 278*, 16926−16928.
9. Boutaud, O., Aronoff, D. M., Richardson, J. H., Marnett, L. J., and Oates, J. A. (2002) Determinants of the cellular specificity of acetaminophen as an inhibitor of prostaglandin H2 synthases, *Proc. Natl. Acad. Sci. U.S.A. 99*, 7130−7135.
10. Wang, N. C., and Anderson, H. J. (1977) Pyrrole chemistry. XVI. Acylation of the pyrrolyl ambident anion, *Can. J. Chem. 55*, 4103−4111.
11. Limatibul, S., and Watson, J. W. (1971) The mechanism of acid hydrolysis of guanidines, *J. Org. Chem. 36*, 3805−3807.
12. Kazmaier, U. (1995) in *Stereoselective Synthesis* (Helmchen, G., Hoffmann, R. W., Mulzer, J., and Schaumann, E., Eds.) pp 4248, Houben-Weyl, Stuttgart, Germany.
13. McMurry, J. (1984) in *Organic Chemistry* (Needham, M., Ed.) pp 172−174, Brook/Cole Publishing Company, Monterey, CA.
14. Perry, T. L. (1982) *Cerebral amino acid pools*, Vol. 1, 2nd ed., Plenum Press, New York.
15. Shih, V. E. (1978) in *Urea cycle disorders and other congenital hyperammonemic syndromes* (Stanbury, J. B., Wyngaarden, J. B., and Frederikson, D. S. Eds.) Plenum Press, McGraw-Hill, New York.
16. Wood, J. H. (1982) *Physiological neurochemistry of cerebrospinal fluid*, Vol. 1, 2nd ed., Plenum Press, New York.
17. Sadasivudu, B., and Rao, T. I. (1976) Studies on functional and metabolic role of urea cycle intermediates in brain, *J. Neurochem. 27*, 785−794.

BI049842R