PART I: ISOLEVUGLANDIN-PROTEIN CROSS-LINKING: STRUCTURE AND MECHANISM

PART II: GENERATION AND CHARACTERIZATION OF A MONOCLONAL Isolevuglandin[4]-Protein Adduct Antibody

by

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Submitted in partial fulfillment of the requirements For the degree of Doctor of Philosophy

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Co-Advisor: Dr. John W. Crabb

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August 2014
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            Dr. Robert G. Salomon

(Date) 04/28/2014

*We also certify that written approval has been obtained for any proprietary material contained therein.
This thesis is dedicated to my parents and my husband.
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Part I: Isolevuglandin-protein Cross-linking: Structure and Mechanism


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## LIST OF ABBREVIATIONS AND ACRONYMS

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<tr>
<td>AA</td>
<td>arachidonic acid</td>
</tr>
<tr>
<td>AAA</td>
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<tr>
<td>AA-PC</td>
<td>2-arachidonyl-phosphatidylcholine</td>
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<tr>
<td>Aβ</td>
<td>amyloid β</td>
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<tr>
<td>ABTS</td>
<td>2,2-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid diammonium salt</td>
</tr>
<tr>
<td>AcOH</td>
<td>acetic acid</td>
</tr>
<tr>
<td>AMD</td>
<td>age-related macular degeneration</td>
</tr>
<tr>
<td>APS</td>
<td>ammonium persulfate</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
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<td>CDCl₃</td>
<td>deuterated chloroform</td>
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<tr>
<td>CEP</td>
<td>carboxy ethylpyrrole</td>
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<tr>
<td>¹³C-NMR</td>
<td>carbon magnetic resonance</td>
</tr>
<tr>
<td>CDI</td>
<td>1,1'-Carbonyldiimidazole</td>
</tr>
<tr>
<td>CID</td>
<td>collision-induced dissociation</td>
</tr>
<tr>
<td>COX</td>
<td>cyclooxygenase</td>
</tr>
<tr>
<td>Cys</td>
<td>cysteine</td>
</tr>
<tr>
<td>DBD-ED</td>
<td>4-(N,N-Dimethylaminosulfonil)-7-(2-aminoethylamino)-2,1,3-benzoxadiazole</td>
</tr>
<tr>
<td>kDa or kD</td>
<td>kilodalton</td>
</tr>
<tr>
<td>DCC</td>
<td>dicyclohexylcarbodiimide</td>
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<td>DMEM</td>
<td>dulbecco’s modified eagle’s medium</td>
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<td>ELISA</td>
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<td>FBS</td>
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<td>fourier transform ion cyclotron resonance</td>
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<td>Gly</td>
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<td>GSH</td>
<td>reduced L-glutathione</td>
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<td>HAT</td>
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<td>'H-NMR</td>
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<td>HPLC</td>
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<td>human serum albumin</td>
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<td>HUVEC</td>
<td>human umbilical vein endothelial cell</td>
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<td>lysine</td>
</tr>
<tr>
<td>mAb</td>
<td>monoclonal antibody</td>
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<td>MALDI-TOF</td>
<td>matrix assisted laser desorption ionization-time of flight</td>
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<tr>
<td>NAPE-PLD</td>
<td>N-acyl phosphatidylethanolamine-hydrolyzing phospholipase D</td>
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<td>MRM</td>
<td>multiple reaction monitoring</td>
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<td>MS/MS</td>
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<td>N-ethyl morpholine</td>
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<tr>
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Part I: Isolevuglandin-protein Cross-linking: Structure and Mechanism

Part II: Generation and Characterization of a Monoclonal

Abstract

By
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IsoLG modification of proteins is associated with loss-of-function, cross-linking and aggregation. The sites of 1:1 adduct formation and the types and extent of cross-linking between subunits of the multi subunit proteins calpain-1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) by iso[4]LGE2 were characterized mass spectroscopically and by SDS-PAGE. Modification of one or two lysyl residues calpain-1 suffices to abolish activity. LC-MS/MS analysis established that Lys280 is a target of isoLG adduction to calpain-1, Lys3 and Lys249 are targets of fluorescently labeled iso[4]LGE2 adduction to GAPDH. Hetero- and homo-oligomers of calpain-1 catalytic and regulatory subunits were detected by SDS-PAGE.

N-Acetyl-glycyl-lysine methyl ester and β-amyloid(11-17) peptide “EVHHQKL” were used as models for characterizing the cross-linking of protein lysyl residues resulting from adduction of iso[4]LGE2. Aminal, bispyrrole and trispyrrole cross-links of two model peptides, were identified and fully characterized by mass spectrometry, and a large variety of derivatives of the bispyrrole cross-link containing one or more additional atoms of oxygen were discovered. In sharp contrast with the dipeptide, the reaction of
“EVHHQKL” with iso[4]LGE₂ strongly favored bispyrrole versus aminal crosslink formation. It is tempting to speculate that the “EVHHQKL” peptide-pyrrole modification forms aggregates that favor the dimerization to produce bispyrrole since β-amyloid tends to spontaneously oligomerize.

I discovered that the pure LG/isoLG-pyrrole derivative of the N-acetyl-lys-gly methyl ester dipeptide could be produced in the presence of dithionite, an oxygen scavenger. The oxidation and dimerization of the pure pyrrole is promoted by oxygen but exhibits an induction period that can be eliminated by various catalysts, e.g., the stable free radical tetramethylpyrrolidine-N-oxide or by trimethylamine-N-oxide.

In the presence of N-acetylcysteine, incubation of iso[4]LGE₂-pyrrole at neutral pH under air showed no pyrrole-to-pyrrole cross-linking. Instead a series of new ions was produced corresponding to pyrrole cysteine adducts and a series derivatives incorporating one or more atoms of oxygen. It is suggested that the thiol inhibits the pyrrole dimerization by intercepting pyrrole cation radical generated by electron transfer to oxygen.

An anti-iso[4]LGE₂ mAb was prepared that exhibits high structural specificity for detecting iso[4]LGE₂-protein adduct epitope, and no significant cross-reactivity with analogs including carboxyethylpyrrole (CEP) or LGE₂-protein adduct epitopes.
CHAPTER 1

INTRODUCTION
1.1 Lipid Peroxidation

Lipids are essential and major components of cell membranes, and play important roles in signaling pathways,\textsuperscript{1-2} energy storage, and gene expression.\textsuperscript{3-4} Oxidative degradation of lipids occurs when there is an increase in the level of reactive oxygen species which damages the cell membrane and leads to subsequent cell death. The oxidation of polyunsaturated fatty acids (PUFAs) by enzymatic or non-enzymatic reactions with molecular oxygen is termed “lipid peroxidation”.

Lipid peroxidation products have been implicated in a number of disease conditions such as amyotrophic lateral sclerosis,\textsuperscript{5} age-related macular degeneration (AMD),\textsuperscript{6-7} end-stage renal disease (RD),\textsuperscript{8-9} Alexander’s,\textsuperscript{10} Alzheimer’s,\textsuperscript{11-13} and Parkinson’s\textsuperscript{14} diseases. It is also suspected to involve in the pathogenesis of atherosclerosis (AS),\textsuperscript{15-16} antiphospholipid antibody syndrome,\textsuperscript{17} rheumatoid arthritis,\textsuperscript{18} inflammatory bowel disease\textsuperscript{19} and multiple sclerosis.\textsuperscript{20} However, our understanding of these processes on a molecular level remains primitive. Research in Dr. Salomon’s group is making major strides to fill this gap. Our approach aims to understand the biologically important chemistry of oxidized lipids and to determine the extent and consequences of this chemistry in the pathogenesis of human diseases.

1.2 Enzymatic Pathways

Arachidonyl phospholipids, e.g., phosphatidyl choline with a palmityl or steryl ester on the C1 hydroxyl and a arachidonyl ester on the C2 hydroxyl (AA-PC), a linear twenty carbon methylene interrupted polyunsaturated fatty acid (C20:4\omega6) ester, is one of the most abundant polyunsaturated phospholipids in human low
density lipoprotein (LDL). It undergoes lipid peroxidation by two different pathways, enzymatic (cyclooxygenase or lipoxygenase) and free radical mediated processes, generating a vast array of biologically active oxidized products. The free fatty acid is the substrate for enzyme-mediated peroxidation, while the free radical pathway operates predominantly on esters.\(^\text{21}\)

\[
\begin{align*}
\text{AA} & \xrightarrow{2\text{O}_2} \text{cyclooxygenase} \\
\text{PGH}_2 & \xrightarrow{\text{rearrangement}} \text{PG}_2 \text{E}_2 \\
\text{PGD}_2 & \text{LGE}_2 \\
\text{LGD}_2 & 
\end{align*}
\]

\textbf{Scheme 1.1} Cyclooxygenase oxidation of AA generates PGs and LGs via rearrangement of PGH\(_2\).

In the cyclooxygenase pathway, arachidonic acid (AA) is oxidized in a stereospecific manner leading to the formation of the prostaglandin endoperoxide
PGH$_2$. Besides enzyme-mediated transformations, a nonenzymatic spontaneous rearrangement of PGH$_2$ generates prostaglandins PGD$_2$ and PGE$_2$ (scheme 1.1).

Dr. Salomon made the discovery$^{22-24}$ that PGH$_2$ undergoes rearrangement via an alternative pathway to form two $\gamma$-ketoaldehydes or levuglandins named LGE$_2$ and LGD$_2$ (Scheme 1.1). The word levuglandin comes from the combination of levulinaldehyde and prostaglandin because they are derivatives of levulinaldehyde with prostanoid side chains.$^{25}$ This rearrangement involves the intramolecular migration of a bridgehead hydride to an electron deficient incipient methyl group with concomitant cleavage of a C-C and O-O bond as shown in Scheme 1.2.$^{26-27}$

![Scheme 1.2 Schematic representation of rearrangement of PGH$_2$ to levuglandin.](image)

1.3 The Free Radical Pathway

Unlike the enzymatic pathway that converts free AA to an endoperoxide intermediate, the free radical pathway mainly oxidizes AA-PC directly, rather than free AA.$^{25}$ It produces stereo and structural isomers of prostaglandins$^{28}$ which were named isoprostanes.$^{29}$ Consequently, the free radical pathway is also called the isoprostane pathway. This pathway also generates racemic mixtures of 16 LG diastereomers, which a referred to collectively as isoLGE$_2$ and isoLGD$_2$ (Scheme 1.3). Free radical-induced cyclooxygenation also generates 8 diastereomers of each of 6
structurally isomeric analogues that we designate collectively as iso[n]LGs. They are structurally unique and give protein adducts that can only be products of free radical-induced lipid oxidation.

Scheme 1.3 Generation of isolevuglandins and isolevuglandin-protein adducts during free radical oxidation of AA-esters

The studies described in this thesis focused on iso[4]LGE2, an isomer formed exclusively through the free radical oxidation of AA-PC. LGs and isoLGs are highly reactive aldehydes and initially modify the ε-amino group of lysyl residues in proteins within seconds30 to form Schiff base intermediates that subsequently afford pyrroles
by dehydration.\textsuperscript{31} The pyrroles are readily oxidized producing lactams and hydroxylactams.\textsuperscript{32-34} LGs/isoLGs covalent binding with proteins is accompanied by cross-linking\textsuperscript{35} and hence polymerization of proteins orders of magnitude more rapidly than other reactive arachidonate metabolites such as malondialdehyde and 4-hydroxynon-2-enal. LGs/isoLGs also cause DNA-protein cross-links (DPCs) which are repair-resistant and were shown to be relevant to cell killing (Scheme 1.4).\textsuperscript{36}

![Scheme 1.4 Formation of LG-protein adducts, protein-protein and DNA-protein cross-links.](image-url)
LGs and isoLGs have since been implicated in a number of diseases such as age-related macular degeneration (AMD), atherosclerosis (AS) and cardiovascular disease (CVD). The possible pathological effects of LGs and isoLGs are summarized in Figure 1.1.

Figure 1.1 Pathological effects of LGs and isoLGs.

1.4 Biological Activities of Levuglandins and Isolevuglandins

1.4.1 The effects of LGs/isoLGs on protein activity

IsoLG-mediated loss of protein activity has been reported. IsoLG, generated in the epicardial border zone of the canine healing infarct, potentiated inactivation of cardiac Na⁺ channels in human embryonic kidney (HEK)-293 cells and cultured atrial (HL-1) myocytes, suggesting Na⁺ channel dysfunction evoked by lipid peroxidation was a candidate mechanism for ischemia-related conduction abnormalities and arrhythmias. In addition to the sodium channel, addition of synthetic isoLG/LG to an atrial tumor myocyte cell line, AT-1, led to a pronounced dose-dependent inhibition of the inward rectifying potassium current induced by a -40 mV voltage step (IC₅₀ = 2.2 μm). Oxidative stress reduces the activity of isolated Na⁺, K⁺-ATPase enzymes. In the pioneering study of Shattock et al., research was performed to determine the effects of oxidant stress on the Na(+)-K+ pump current with isolated rabbit ventricular myocytes using the whole-cell voltage-clamp technique. Through
photoactivation of rose bengal, singlet oxygen and superoxide were generated and isolated cells were exposed to the induced oxidant stress. Results showed that the Na\textsuperscript+-K\textsuperscript+ pump current was inhibited by oxidant stress at all voltages and this inhibition may contribute to ischemia/reperfusion injury.\textsuperscript{43} The enzyme activity of purified RNase A and glutathione reductase was found to decrease following a treatment with isoLGE\textsubscript{2} in vitro. An isoLG scavenger, pyridoxamine, protects enzymes against loss of activity caused adduction by isoLGE\textsubscript{2}.\textsuperscript{44}

The first direct evidence linking isoLG modification of a specific protein and loss of enzyme activity in vivo involved the protease activity of calpain-1. Elevated levels of the enzyme were detected in glaucomatous trabecular meshwork (TM)\textsuperscript{45} compared to nonglaucomatous control (calpain-1 extracted from normal eyes), but calpain-1 activity in the glaucomatous trabecular meshwork was greatly reduced, about 50% less activity than the same amount of protein from normal TM. Elevated levels of iso[4]LGE\textsubscript{2} protein adduct were also observed in glaucomatous astrocytes isolated from human optic nerve.\textsuperscript{46}

In an in vitro study, iso[4]LGE\textsubscript{2} modified-calpain-1 was ubiquitinated by a HeLa cell-derived system and the ubiquitinated calpain-1 could be efficiently recognized by HeLa cell-derived constituted proteasome 26S. Dynamic light scattering studies that were performed to monitor the size of aggregates confirmed the presence of large aggregates that correspond to ubiquitinated iso[4]LGE\textsubscript{2}-modified calpain-1. Furthermore, these aggregates were refractory toward degradation. Human eyes possibly lack the capacity to degrade iso[4]LGE\textsubscript{2}-modified proteins, resulting in their
accumulation. Glaucoma is thought to be caused in part by an increase in intraocular pressure (IOP), however normal IOP glaucoma is also common. Pressure increases either when too much aqueous humor fluid is produced or by decreased aqueous humor outflow. The trabecular meshwork is responsible for most of the outflow of aqueous humor. The inhibition of proteasome activity using epoxomicin on cultured human primary TM cells indicated increased calpain-1 accumulation, owing to protein modification by isoLGs. This could contribute to glaucoma pathophysiology by decreasing the ability of the TM to modulate outflow resistance. Given the importance of ubiquitination for proteasomal clearance of LG/isoLGs modified proteins, it is noteworthy that ubiquitin was shown to form adducts with LGE₂.

The mitochondrial enzyme cytochrome P450 27A1 (CYP27A1) plays a very important role in the maintenance of cholesterol homeostasis, bile acid biosynthesis, and activation of vitamin D₃ by catalyzing the C27-hydroxylation of cholesterol and other sterols. Recently CYP27A1 was found to be expressed in the retina and shown to be the major contributor to enzymatic degradation of cholesterol. Treatment of CYP27A1 with iso[4]LGE₂ in vitro diminished enzyme activity in a time- and phospholipid-dependent manner. To model membrane insertion, CYP27A1 was reconstituted into various PLs. It was found that the enzyme is modified even when other biological amines are present and iso[4]LGE₂ concentrations are relatively low (2-folder molar excess). Liposomes of various composition were as follows: bovine retinal mitochondrial PLs, a DLPC (1,2-dilauroyl-sn-glycero-3-phosphocholine) and DOPE (1,2-dioleoyl-sn-glycero-3-
phosphoethanolamine) mixture (4:3, w/w) that approximates the ratio of phosphatidylcholine to phosphatidylethanolamine found in human hepatic mitochondria, or DLPC that contains no free amines and is a standard model PL. In this set of experiments, the ratio of iso[4]LGE2 was a 2-fold molar excess. Enzyme activity was decreased sharply by 75% at 5 min and the P450 content decreased only by 25%, indicating that modification impaired activity without completely denaturing the protein. Most modification and loss of activity occurred within 15 min of incubation of a 2-fold molar excess of iso[4]LGE2 with the protein. In contrast, incorporation of the protein into liposomes containing ethanolamine phospholipids protected against modification owing to interception of isoLGs by the primary amino groups of the ethanolamine phospholipids.

Lys 358-isoLG adduct in CYP27A1 was found in human AMD retinal sample. To study the effect of Lys 358 modification on the enzyme activity, mutations were introduced to the wild-type (WT) CYP27A1. CYP27A1 Lys 358 was mutated to either hydrophobic Leu to mimic removal of the positive charge or to Arg to conserve the charge but abolish iso[4]LGE2 modification because Arg is at least 1,000-fold less susceptible than Lys to modification by isoLGs. The catalytic activity of the Lys 358 CYP27A1 mutant was characterized before and after isoLG treatment. It was confirmed that modification of Lys 358 by isoLG is the major contributor to isoLG-associated loss of CYP27A1 activity. This was first study to conduct the measurement of enzyme activity and simultaneously correlate the isoLG modification to specific locations in the primary sequence. The Lys 358-isoLG adduct in CYP27A1 was found
in human retina from individuals afflicted with age-related macular degeneration,\textsuperscript{52} providing direct evidence that isoLG adduction impairs enzyme activity and supporting the hypothesis that isoLG modification of CYP27A1, potentially impairs cholesterol homeostasis in the retina.\textsuperscript{52}

1.4.2 LGs/isoLGs effect on the interaction of histones with DNA

DNA-protein cross-linking by a levuglandin was first observed upon treatment of V79 Chinese hamster lung fibroblasts or nuclei with LGE.\textsuperscript{36} DNA in the cell lysate was retained on a nitrocellulose filter, presumably owing to covalent DNA-protein cross-linking. This was confirmed by proteolysis with proteinase K that allowed the DNA to pass through the filter. DNA tightly coils around histones in cells. The lysine-rich histones from calf thymus could form adducts with isoLG/LG. It was postulated that this protein is a likely candidate for DNA-isoLG-protein cross-linking.\textsuperscript{53} Recently, the formation of LG-lysine lactam adducts on histones were identified in RAW264.7 mouse macrophages, A549 cultured lung epithelial cells and rat liver, that is dependent on COX-2 activity or COX-2 expression. With an internal LG-lysyl standard, highest measurable amounts of adduct was detected on the H4 histone. Adduction was blocked by a \(\gamma\)-ketoaldehyde scavenger (4-ethylsalicylamine), which had no effect on COX-2 activity as measured by PGE\textsubscript{2} production. Formation of LG-histone adduct was associated with an increased histone solubility in aqueous NaCl and after LG-histone adduct formation, histone-DNA binding was disrupted with an increased DNA extraction in a salt solution. Chromatin access and transcription was regulated by a “histone code comprised with complex patterns of lysyl acetylation and
methylation.\textsuperscript{54} It is reasonable to conclude that irreversible adduction of lysyl residues could disrupt this code, or directly alter the access of DNA-interacting proteins. Changes in histone modifications are known to result in altered DNA methylation, deregulation of oncogenes, genomic instability, impaired DNA repair, and defects in cell cycle checkpoints.\textsuperscript{55-56} Changes in lysyl modifications of H4 in particular are a common hallmark of human cancers, and are associated with a global loss of DNA methylation.\textsuperscript{57} Further elucidation of the effects of LG-histone adduction on histone modification, DNA-histone interactions, and transcription are expected to increase the understanding of the molecular mechanisms whereby COX-2 contributes to cancer development and progression.

1.4.3 LGs/isoLGs covalently modify PE

Besides the ε-amino groups of protein lysyl residues, iso[4]LGE\textsubscript{2} covalently binds ethanolamine phospholipids \textit{in vitro} to form covalent pyrrole adducts that were oxidized in air to deliver stable lactam and hydroxylactam (HL) adducts.\textsuperscript{58} In lipid extracts from human plasma and mouse liver, isoLG-lysoPE-HL derivatives of isoLGs was detected through phospholipase A2 (PLA2)-catalyzed hydrolysis of isoLG-diacyl-PE precursors.\textsuperscript{58} The formation of isoLG/LG-PE is of interest since oxidatively modified phospholipids have been implicated in certain autoimmune diseases. Treatment both of human embryonic kidney (HEK293) and human umbilical vein endothelial cells (HUVEC) with isoLGE\textsubscript{2} (15-E\textsubscript{2}-isoketal, isoK) generated more modified PE (isoLGE\textsubscript{2}-PE) than modified protein. As internal standard, isoLGE\textsubscript{2}-[\textsuperscript{2}H\textsubscript{4}] ethanolamine was incubated with HEK293 cells and
isoLGE_2-PE was quantified by LC/MS/MS after hydrolysis to isoLGE_2-ethanolamine by *Streptomyces chromofuscus* phospholipase D. Analysis of isoLGE_2-protein adducts as isoLGE_2-lysyl-lactam adduct after protease digestion was performed on the protein pellet using isoLGE_2-[^{13}C_6,^{15}N_2]lysyl-lactam as the internal standard. Human umbilical vein endothelial cells (HUVEC) were treated the same way. IsoLGE_2-PE induced cytotoxicity towards human umbilical vein endothelial cells (HUVEC) with LC_50 as 2.2 µM. These observations indicate that cellular PE is a significant target of isoLGs.59

Another study showed that PE modified by isoLGE_2 (isoLGE_2-PE) induced THP-1 monocyte adhesion to human umbilical cord endothelial cells. IsoLGE_2-PE also induced expression of adhesion molecules and increased MCP-1 and IL-8 mRNA in human umbilical cord endothelial cells and this pyrrole-PE markedly altered membrane curvature.60 To test if cells could degrade isolevuglandin modified phosphatidylethanolamine (isoLG-PE), the stability of isoLG-PE in human embryonic kidney 293 (HEK293) cells and in human umbilical cord endothelial cells (HUVEC) was measured over time. HEK293 cells were incubated with isoLGE_2 to generate isoLG-PE and excess isoLG was removed by washing with DMEM. With isoLGE_2-[^{2}H_4] ethanolamine as internal standard, isoLGE_2-PE levels were measured by LC-MS after conversion to its phosphoglycero-ethanolamine derivative by base hydrolysis. The stability of isoLGE_2-PE in endothelial cells was assessed the same way. Cellular isoLGE_2-PE levels in HEK293 cells decreased more than 75% after 6 h. While in
HUVEC cells, the isoLGE₂-PE levels rapidly dropped with less than 5% of isoLGE₂-PE present after 24 h.⁶³

N-Acyl phosphatidylethanolamine-hydrolyzing phospholipase D (NAPE-PLD) is a key enzyme for the hydrolysis of N-acyl phosphatidylethanolamine (NAPE) including both NAPE and isoLGE₂-PE with large aliphatic headgroups. It hydrolyzes isoLGE₂-PE to isoLGE₂-ethanolamine. These results demonstrate that NAPE-PLD contributes to the degradation of isoLGE₂-PE and suggest that a major physiological role of NAPE-PLD may be to degrade aldehyde-modified PE, thereby preventing the accumulation of these harmful compounds.⁶¹

1.4.4 LG/isoLG scavengers

Pyridoxamine (PM) is a B6 vitamin which reacts with isoLGs 2000-fold faster than the ε-amino groups of protein lysyl residues, making it an efficient scavenger of this type of lipid peroxidation products.⁶² Therefore, PM and its analogues can be used as isoLG/LG scavengers to protect proteins from modification and inactivation by isoLGs/LGs and to determine the impact on pathophysiology of specifically reducing the levels of isoLG/LG protein adduction. Treatment with PM and its analogs, salicylamine and 5’-O-pentylypyridoxamine, had been shown to prevent H₂O₂-mediated cytotoxicity in HepG2 cell culture and decrease isoLG-protein adduct formation of ovalbumin exposed to isoLG. PM also prevented inhibition of RNase A and glutathione reductase activity by the γ-ketoaldehyde isoLGE₂. RNase activity and glutathione reductase (GR) activity were determined by measuring the formation of
Recent research showed that exposure of mice to bright light leads to isoLG-adduct formation in the retina. Pre-treatment of mice with PM greatly decreased retinal isoLG adduct levels resulting from light exposure, and morphological changes in photoreceptor mitochondria were not as pronounced as in untreated animals. This study demonstrated a novel concept in vision research, i.e., that preventing damage to biomolecules by lipid peroxidation products is a viable strategy to combat oxidative stress in the retina. This novel therapeutic activity of PM may be applicable to humans as PM has good drug-like properties and an excellent safety profile.

1.4.5 Detection of biological adducts of LGs/isoLGs

Detecting free LG/isoLG adducts in vivo is complicated by their proclivity to behave like superglue, adhering to normal physiological proteins within seconds. On the other hand, there are two complimentary methods for quantifying LG/isoLG adducts in vitro/vivo. One method utilizes antibody based approaches such as ELISA, Western blotting, or immunohistochemistry. The other uses mass spectroscopic detection of (1) LG/isoLG modified lysine or peptides derived from LG/isoLG modified proteins or of (2) LG/isoLG modified ethanolamine phospholipids. The first detection of LG/isoLG adducted proteins in vivo was in patients with atherosclerosis or end-stage renal disease using ELISA measurements. Immunoassays with antibodies raised against protein adducts of LGs generated by unambiguous chemical synthesis provided evidence for their presence in human and
mouse blood and tissues and enabled the discovery that free radical-induced oxidation of arachidonyl phospholipids in low-density lipoprotein generates LGE$_2^{25}$ as well as stereo and structural isomers referred to collectively as isolevuglandins.$^{38}$ A single-chain antibody from a phage displayed recombinant ScFv library that bound a model peptide adducted with synthetic isoLGE$_2$ was isolated and characterized. Recognition of isoLGE$_2$-protein adducts by this anti-isoLGE$_2$ adduct single-chain antibody is essentially independent of the amino acid sequence of the adducted peptides or proteins, and no cross-reactivity was detected with 4-hydroxynonenal or 4-oxononanal adducts or with 15-F$_2$t-isoprostane (8-iso-prostaglandin F2α).$^{67}$ However, cross reactivity with structurally isomeric isoLGs was not determined.

The second method for detection of isoLGE$_2$-modified proteins utilizes the sensitivity and specificity of liquid chromatography-mass spectrometry quantitation of the excised LG-modified lysine following exhaustive proteolysis of LG/isoLG-adducts in tissues. A heavy isotope labeled internal standard is added to the sample for quantification.$^{68}$ MS analysis revealed that the majority of LG/isoLG-protein modifications are lactams and hydroxylactams generated by oxidation of the initial pyrrole modifications.$^{32}$ LC–MS/MS analysis also detects isoLG-phosphatidylethanolamine adducts in human blood and mouse liver.$^{58}$ Site-specific post-translational protein modifications can be detected and quantified using mass spectrometry-based multiple reaction monitoring (MRM) assay.$^{69}$ With this method, the isoLG-modified tryptic peptide AVLKETLR in a mitochondrial protein, Cyp27A1,
extracted from human retina was detected and shown to be an adduct of the lysyl ε–amine group.51

1.5 Monoclonal Antibody Development

1.5.1 Antigens and antibodies

Substances that are recognized by B-cell receptors are termed antigens. Classically, an antigen is defined as any substance that elicits an immune response in a susceptible animal and is capable of binding with the specific antibodies generated. Antigens are usually of a high molecular weight and are commonly proteins or polysaccharides, although nucleic acids, lipids and peptides have also been reported to function as antigens.70 Antibodies are produced by plasma B-lymphocytes and function as a part of an immune system in mammals in the battle against disease. Antibodies have a common structure of four peptide chains, consisting of two identical light chains, of about 25 KDa and two identical heavy chains of about 50 KDa. Each light chain is bound to the heavy chain by a disulfide bond and a combination of hydrogen bonds, salt linkages and hydrophobic bonds. Similarly noncovalent interactions and disulfide bridges link two identical heavy and light chain combinations to each other to form a basic four chain immunoglobulin structure.71

Antibodies (Ab)s play an important role when foreign antigens (Ag)s, such as pathogens and toxins, are introduced into an organism and start a complex immune response. Antibodies label the foreign molecule or pathogen for destruction and removal in a lock-and-key type of mode. The basic principle of any immunological technique is that an antibody will combine with its specific antigen to give a unique
Ab/Ag complex. For an efficient Ag/Ab interaction to occur the epitope must be exposed and available for binding, alterations in the conformation of epitopes through tissue processing, fixation, reduction and pH changes may affect the binding. In modern medical diagnostics and basic research, antibodies have become crucial molecules since it is possible to develop specific antibodies against almost any component, from small drug molecules to intact cells.

1.5.2 Polyclonal antibodies

Prior to 1975, polyclonal serum was the only antibody option available. Polyclonal antibodies refer to antibodies present in the crude serum of an immunized animal, such as a mouse, rabbit, goat or hen, capable of recognizing one or several different immunogenic epitopes of the administered immunogen. These antibodies may be of different subclasses, but isolation of a single subclass, usually IgG, is readily achieved. Polyclonal antibodies are often used since they are easily purified from the blood of the mammal by chromatographic techniques. Their utilization is quick and inexpensive, requiring little skill or technical expertise. They are particularly useful when amplification of a signal from a target protein with low expression is required as they recognize multiple epitopes on one protein. However, despite these advantages a pAb raised against an antigen can bind several different epitopes on the target. This increases the likelihood that pAbs cross-react with biomolecules containing similar epitopes. Anomalous results and variability between batches often leads to inconsistencies in results. Furthermore, the supply of pAbs is limited for research purposes as the mammal is eventually killed.
1.5.3 Monoclonal antibodies and hybridoma technology

A major milestone in immunological research was the development of monoclonal antibodies by Köhler and Milstein in 1975. This Nobel Prize winning work (1984, physiology and medicine) revolutionized antibody production and today it forms the basis of many diagnostic applications, disease therapies and basic research. Production of monoclonal antibodies (mAbs), in contrast with pAbs, is more time consuming and expensive from pAb production and requires high technology and extensive technical skills. Monoclonal antibodies are only one subclass, usually IgG, allowing for selection of an appropriate secondary antibody for detection. Large quantities of specific antibodies can be produced and their specificity ensures that only one epitope is recognized on an antigen. This is extremely useful when studying subtle protein alterations, and the antibodies are less likely to cross react with other proteins and generally produce less background cross reaction.

Hybridoma technology involves fusion of a mouse myeloma cell line with mouse spleen cells from an immunized donor in the presence of polyethylene glycol (PEG). The resultant cells are grown in a selective medium in which only hybridoma cells can proliferate. The resultant hybridoma cell line possesses the immortal growth properties of the cancerous plasma cells and the antibody secreting properties of the normal B cells, thus creating an indefinitely growing cell line that produces large quantities of antibodies with identical specificity.

The fusion is random and fused hybridoma cells need to be selected and isolated from unfused B-lymphocytes and myeloma cells. The cells are cultured in
hypoxanthine-aminopterin-thymidine (HAT) medium. Aminopterin (A) blocks the de novo biosynthesis of purines and pyrimidines which are essential for DNA synthesis. When this pathway is blocked, cells use the salvage pathway utilizing hypoxanthine (H) and thymidine (T), and this requires the activity of the enzymes thymidine kinase (TK) and hypoxanthine-guanine phosphoribosyl transferase (HGPRT). The myelomas used for the fusion lack HGPRT, so that unfused myeloma cells and myeloma cells fused to other myeloma cells cannot proliferate in HAT medium. The unfused splenocytes do possess HGPRT but have a limited lifetime and will die within two weeks. The hybridoma cells grow effectively in the HAT medium. Many different hybridomas are created during the fusion and every cell type produces a specific antibody towards a wide range of antigens and not only the antigen used to immunize the mouse. To identify the hybridomas that are specific for the antigen, cells are distributed in 96-well plates and hybridoma supernatant is used in an enzyme-linked immunosorbent assay (ELISA) to detect the wells containing antibodies that bind with the antigen (positive wells) for subsequent cloning by limiting dilution. The method essentially consists of diluting the cells and growing them at very low densities, often in the presence of feeder cells, which supply growth factors. After screening, positive wells containing only one visible clone are selected for another round of cloning. After at least three rounds of cloning, the cell line is per definition monoclonal. Cloning continues until a stable hybridoma, i.e., that does not lose the mAb-producing ability, is achieved. The stable cloned hybridomas can then be expanded for large-scale mAb production. Hybridomas can be frozen for storage and in this way an
unlimited supply of mAb’s can be maintained (Figure 1.2). mAb may also be stored frozen but may be come nonspecific inactive with long term storage.

**Figure 1.2** Overview of monoclonal antibody production, see text for detailed description (Copyright from wikipedia commons).
1.6 References


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CHAPTER 2

ADDUCTION AND CROSS-LINKING OF CALPAIN-1 AND GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE (GAPDH) BY ISO[4]LG E₂
2.1 Background

2.1.1 Purpose

The first characterization of the site of in vivo isoLG post-translational modification was of the monomeric mitochondrial protein Cyp27A1. To elucidate isoLG-mediated protein-protein cross-linking, the reaction of iso[4]LGE₂ with multi subunit proteins, calpain-1 and GAPDH, was investigated. In this study, we characterized sites of modification and detected cross-linking between subunits of the multi subunit proteins calpain-1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

2.1.2 Isolevuglandin modification of proteins.

The eye and brain contain high levels of polyunsaturated fatty acids (PUFAs), of which arachidonic acid is one of the most abundant.¹ Arachidonyl phospholipids (PLs) undergo cyclooxygenase catalyzed oxidation to produce γ-ketoaldehyde levuglandins and free radical-induced oxidation in vivo to form levuglandins (LGs) and their stereo and structural isomers referred to collectively as isolevuglandins (isoLGs). LG and isoLG-protein adducts in human serum have been fully characterized.² Some isoLG isomers such as iso[4]levuglandin E₂ (iso[4]LGE₂) are not produced through the enzymatic cyclooxygenase pathway, but rather are only generated through free radical-mediated oxidation.³ On the other hand, a few diastereomers of two isoLG structural isomers, e.g., levuglandins E₂ and D₂, are not only generated through free radical-initiated lipid oxidation, but are also formed through the cyclooxygenase pathway.⁴ IsoLGs are a highly reactive family of γ-keto aldehydes⁵ that react with free primary amines such as the ε-amino group of lysine residues in proteins. They form covalent adducts with orders of magnitude greater avidity than most other lipid oxidation products such as 4-
hydroxynonenal.\textsuperscript{6-8} These adducts incorporate the primary amino group in a pyrrole ring that is readily oxidized to lactam and hydroxylactam derivatives. In the human eye, isoLG adducted calpain-1 accumulates in the trabecular meshwork, and isoLG adduction abolishes the enzyme’s activity.\textsuperscript{9} LG/isoLG-protein adduction has biological consequences. For example, preferential adduction of isoLG to a specific Lys in the mitochondrial enzyme Cyp27A1 abolishes its ability to metabolize cholesterol leading to cholesterol accumulation and retinal pathology.\textsuperscript{10-11} Lysyl-LG/isoLG-protein adduction also results in intermolecular protein-protein cross-linking. Pyrrole-pyrrole cross-link and aminal cross-link structures depicted in Scheme 2.1 have been postulated for isoLG-protein modifications, but direct molecular level evidence for the cross-link structure(s) is lacking.\textsuperscript{12}

2.1.3 Calpain-1.

Calpains are ubiquitous calcium-dependent cysteine proteases that mainly exist in two forms in all tissues, \( \mu \)-calpain and m-calpain. They belong to a superfamily of 14 cysteine proteases. Activation of calpains in vitro requires the presence of calcium at micromolar quantities for \( \mu \)-calpain and millimolar quantities for m-calpain at neutral pH.\(^{13-14}\) Both forms comprise a large subunit of about 80 kDa and a small subunit of about 30 kDa. Increased calpain activity has been discovered in several neurological disorders, such as spinal cord injury, ischemic brain injuries, stroke, and calpain activity has been implicated in several neurodegenerative disorders such as Alzheimer’s disease and multiple sclerosis.\(^{14}\) IsoLG-protein adducts accumulate in trabecular meshwork (TM) tissue in individuals with primary open angle glaucoma. Inactive calpain-1 accumulates in the TM in conjunction with massive levels of isoLG-modified protein. Treatment with isoLG inactivates calpain-1. There are elevated levels of the protease calpain-1 in glaucomatous TM based on western analysis. However, calpain-1 activity in glaucomatous TM is only about 50% of that in controls. This paradox is explicable by the fact that modification by isoLGs renders calpain-1 inactive. In addition, isoLG-modification of calpain-1 promotes its ubiquitination and the formation of aggregates that resist proteasomal processing\(^8, 15\), and presumably contribute to the impeded aqueous humor outflow through the TM that results in elevated intraocular pressure. It appears that calpain-1 is expressed to promote proteolysis of damaged proteins. However, this function is defeated by isoLG-modification that renders it inactive. In addition, isoLG modification can also make proteins resistant to proteolysis, e.g., by the 20S
proteasome. Thus, isoLG adduction presumably contributes to accumulation and inactivation of calpain in the TM owing to aggregation and/or resistance to proteolysis.

2.1.4 GAPDH

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) plays a key role in the glycolytic pathway. GAPDH catalyzes the oxidative phosphorylation of glyceraldehyde-3-phosphate (G3P) in the presence of NAD$^+$ and inorganic phosphate. GAPDH is well conserved during evolution, being a protein with a native molecular weight in the range of 140-150 kDa. It is composed of four identical of 35-37 kDa subunits. The ubiquity and evolutionary conservation of GAPDH indicate a highly important physiological function. Besides its traditional role in glycolysis, newly recognized activities of GAPDH include regulation of the cytoskeleton, membrane fusion and transport, glutamate accumulation into presynaptic vesicles, and activation of transcription in neurons. Elevated levels of nuclear GAPDH are observed in postmortem samples from patients with Parkinson's disease, Alzheimer's disease, Huntington's disease and glaucoma, among others. 4-Hydroxy-2-nonenal (HNE), a major lipid peroxidation-derived reactive aldehyde, exerts an inhibitory effect on GAPDH due to the modification of the cysteine residue (Cys-149) at the catalytic site where it forms an HNE-cysteine Michael addition type adduct. Elevated iso[4]LGE$_2$ protein adduct immunoreactivity is also found in glaucomatous astrocytes derived from the glaucomatous optic nerve head.
2.2 Results and discussion

2.2.1 In vitro modification of calpain-1 with iso[4]LGE₂ abolishes its protease activity.

The activity of calpain-1 extracted from glaucomatous trabeculectomy tissue is about 50% less than the same amount of protein extracted from normal trabecular meshwork tissue in spite of the fact that the levels of calpain-1 in glaucotomous TM are greatly elevated compared with those in normal TM.⁹ Previously, treatment of purified calpain-1 with isoLG \textit{in vitro} was shown to result in loss of catalytic activity but the time course of activity loss was not explored.⁹ To further characterize the relationship between modification of the protein by isoLG and decrease in activity, enzymatic activity was monitored after treatment of the protein in vitro with iso[4]LGE₂. Exposure of calpain-1 with iso[4]LGE₂ \textit{in vitro} diminishes enzyme activity in a time- (Figure 2.1) and ratio-dependent (Figure 2.2) manner.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure2_1.png}
\caption{Figure 2.1 Treatment of calpain-1 with 10 equivalents of iso[4]LGE₂ for various times. Left: SDS-PAGE of proteins showed 10 min, 30 min and 90 min modification. 90°C represented control within 90 min, while 90’M represented modification within 90 min,}
\end{figure}
same for 30 and 10 min. Right: calpain-1 activity assay with different modification time treatment.

Notably, the inhibitory effect was not linearly related to isoLG-protein ratio. Two equivalents of isoLG had little additional inhibitory effect over a 1:1 molar ratio and the inhibition caused by a 10 equivalents of isoLG to protein was less than twice that of 2 equivalents. This behavior suggests that a low level of modification by one or two molecules of isoLG has a profound effect on activity, and that additional modifications only provide minor additional inhibitory effects. The time dependence of the effect of exposure of the protein to 10 equivalents of iso[4]LGE2 was examined. Enzyme activity decreased by 50% by 30 min and gradually decreased to 10% by 90 min while the activity of the control, treated with buffer alone, was unchanged. Analysis by electrophoresis showed that within 10 min after treatment, both the calpain-1 catalytic and small subunits migrated at slightly higher mass than the untreated protein presumably because they had been covalently modified.

**Figure 2.2** Treatment of calpain-1 with various equivalents of iso[4]LGE2 for 30 min.

The ratio dependence of the effect of exposure of the protein to a 2, 5 10 equivalents of iso[4]LGE2 was also examined. Enzyme activity decreased sharply by 50% within 30 min. Analysis by electrophoresis showed that 30 min after treatment, both the calpain-1 catalytic and small subunits migrated slightly less than the untreated protein presumably because they had been covalently modified.

In the pioneering study of Govindarajan et al., isoLG modified calpain-1 was precipitated by cold acetone, and resuspended in PBS for enzyme activity test. Our study aims to find specific sites of modification by isoLG and to test enzyme activity at the same time, so calpain-1 modification was performed in sodium acetate solution. The reaction was quenched by addition of glycine and the mixture used directly for enzyme activity assay and MS analysis.

2.2.2 Calpain-1 Lys\textsuperscript{280} is modified upon exposure to isoLGs.

IsoLG modification of proteins, e.g., calpain-1, is associated with loss-of-function, cross-linking and aggregation. To elucidate isoLG-mediated protein-protein cross-linking, the reaction of iso[4]LGE\textsubscript{2} with calpain-1 was examined in detail. A site of isoLG modification of calpain-1 was identified by peptide mapping and sequencing. Using LC-MS and Mascot analysis one lysine residue in calpain-1 was identified that was converted by iso[4]LGE\textsubscript{2} into lactam derivatives. A full MS comparison of the iso[4]LGE\textsubscript{2} modified and unmodified calpain-1 chymotryptic peptide “SVTGAKQVNY” shows that K280 is the modification site (Figure 2.3).
Iso[4]LGE2-treated calpain-1 was proteolyzed in-gel with chymotrypsin followed by FTICR MS/MS. Peak lists from the acquired MS/MS spectra were submitted to the Mascot database search engine. Evaluation criteria for identification of the modified peptides were as follows: first, only hits with a significant peptide score as reported by Mascot’s scoring algorithm were considered. Second, the tandem MS spectra were manually inspected for the candidates to confirm the existence of y and b series ions upstream and downstream of the modified lysine. Third, the presence of multiple adduct dehydration and oxidation states was sought (Table 2.1S), e.g., observation of both the lactam and the monodehydrated lactam for a given residue. Mascot analysis of the spectra from the in-gel tryptic digest (31% sequence coverage, encompassing 13% of the Lys residues) did not identify any modified peptides with a significant score. In contrast, analysis of the in-gel chymotryptic digest (67% sequence, 28% Lys coverage) identified one distinct peptide, SVTGAK280QVNY, modified at Lys280 (Table 2.2S). In this peptide, the modified lysine residue was within the peptide and not at the C terminus, indicating that modification blocks tryptic cleavage. Thus, based on our evaluation criteria, we identified one lysine residue in calpain-1 as modified by iso[4]LGE2.

Figure 2.3 shows a full MS comparison of iso[4]LGE2 unmodified and modified calpain-1 peptide “SVTGAKQVNY” with modification at K280. The mass spectrum of the modified peptide revealed two forms of adducts, a lactam and a monodehydrated lactam, both as doubly charged ions (Table 2.1). In contrast, the spectrum of the unmodified peptide did not exhibit peaks for either of these modifications (Table 2.1). Due to their hydrophobicity, modified peptides eluted later than the unmodified peptide.
It is likely that the loss of water to form the monodehydrated lactam occurred in the mass spectrometer.

**Figure 2.3** MS of iso[4]LGE₂-modified and unmodified calpain-1 peptide “SVTGAKQVNY” with the modification site at K280. Panel A: Detection of the doubly charged ions m/z 699.88 and 690.88 corresponding to the lactam and monodehydrated lactam modifications of SVTGAKQVNY. Panel B: Detection of the doubly charged ion m/z 533.78 corresponding to the unmodified peptide. See table 2.1 for calculated molecular weights.

**Table 2.1** Molecular weight of iso[4]LGE₂-modified and unmodified calpain-1 peptide “SVTGAKQVNY”.

<table>
<thead>
<tr>
<th>Peptide Sequence</th>
<th>Formula of MW</th>
<th>of AMass</th>
<th>Found in Elution</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>SVTGAKQVNY</td>
<td>No Mod</td>
<td>1065.66 Da</td>
<td>Yes 33.18'</td>
<td>No Modification</td>
</tr>
<tr>
<td>SVTGAKQVNY</td>
<td>C₂₀H₂₈O₁</td>
<td>1381.20 Da</td>
<td>No ND</td>
<td>Pyrrole</td>
</tr>
<tr>
<td>SVTGAKQVNY</td>
<td>C₂₀H₂₆O₂</td>
<td>1363.86 Da</td>
<td>No ND</td>
<td>Pyrrole Monodehydrated</td>
</tr>
</tbody>
</table>
To unambiguously establish the site of modification, MS² analysis was performed to study the fragmentation patterns of the modified versus the unmodified chymotryptic peptide. A series of fragment y-ions and b-ions was detected that identify an iso[4]LGE₂ modification on the Lys residue of the calpain-1 peptide SVTGAKQVNY (Figure 2.4).

![Figure 2.4](image)

**Figure 2.4** Tandem MS characterization of the m/z 690.88 doubly charged ion from a MS scan of chymotrypsin digested iso[4]LGE₂-modified calpain-1 showed a series of fragment ions that unambiguously identify an iso[4]LGE₂ modification on the lysyl residue (K280). Asterisks denote fragment ions with a modified lysyl residue.

The iso[4]LGE₂-modified chymotryptic peptides that we identified enabled determination of the specific lysyl group that was altered in various samples of iso[4]LGE₂-modified calpain-1. MS/MS analysis consistently detected modification of the SVTGAKQVNY chymotryptic peptide from the catalytic subunit of iso[4]LGE₂-
calpain-1 in samples of iso[4]LG-modified calpain-1 that were generated with various iso[4]LGE₂ to protein ratios, showing that this sequence was readily accessed and covalently altered by this γ-ketoaldehyde electrophile. Figure 2.5 shows ball-and-stick and cartoon models that provide 3D visualization of calpain-1 using PyMOL v1.4.1. All lysines of calpain-1 are shown as space-filling models in the left panel while only the lysine that became incorporated into the iso[4]LGE₂-modification and three active site catalytic triad residues (Cys 115, His 272 and Asn 296) are shown in the right panel cartoon model. The 3D model shows that the modified lysine is located at and protrudes from the surface of calpain-1. The physical environment and chemical characteristics of this specific lysyl residue apparently facilitates access by iso[4]LGE₂ and covalent adduction through Schiff base formation with its aldehyde group followed by cyclization and pyrrole formation. In vivo, phospholipid derivatives containing the iso[4]LG esterified to the sn2-hydroxyl protrude like whiskers²⁸ from membrane bilayers and the surface of lipoprotein particles. These isoLGs esterified to phospholipids adduct to proteins to initially form Schiff basses and then cyclized to pyrroles. At some point, phospholipolysis releases the free carboxyl of the isoLG.
Figure 2.5 Ball-and-stick and cartoon 3D model models for the catalytic subunit of calpain-1 (pdb: 2ary). All the lysines were shown as space-filling models in the left panel. Only lysine (280) that was modified by iso[4]LGE2 and three active site amino acid residues (Cys 115, His 272 and Asn 296) were shown in the right panel.29-31

The architecture of calpain-1 consists of a short N-terminal extension (domain I),32 the catalytic core (dIIa and dIIb), the C2-like domain III,33 and a penta-EF hand domain IV,31 which interacts with the small subunit domains dV34 and dVI (Figure 2.1S, left panel).35-36 Subdomain Ila and domain Ilb, with a substrate-binding cleft in between, comprises domain II, also name as minicalpain. The catalytic triad residue Cys 115 is on subdomain Ila, whereas His 272 and Asn 296 are part of subdomain Ilb. In the absence of calcium, the distance between the catalytic Cys 115 and His 272 (10 Å) is too great to form a functional catalytic triad. It is suggested that calcium-induced conformational
changes draw subdomain IIa and IIb together\textsuperscript{29, 37-38, 37-38, 37-38} (Figure 2.1S, right panel).\textsuperscript{29, 37-38} As described above, calpain-1 loses protease activity when modified with iso[4]LGE\textsubscript{2}. The modified lysine 280 is located on β strand 11 (amino acid sequence 274-284) which is close to but not in the calpain-1 active site (His 272). The loss of activity consequent to modification of this lysine may result from a conformational change caused by the modification (Figure 2.6).

**Figure 2.6** The active sites of human minicalpain 1 are shown in stick representation and labeled (left panel).\textsuperscript{30} Modified lysine 280 and the active site (Cys 115, His 272 and Asn 296) and related β strands are shown in the cartoon 3D model for the catalytic subunit of calpain-1 (right panel).

### 2.2.3 In vitro modification of calpain-1 with iso[4]LGE\textsubscript{2} generates cross-links.

Human serum albumin (HSA) binds ten equivalents of LGE\textsubscript{2} within 1 minute.\textsuperscript{39} The initial lysyl-LG adducts with proteins are also reactive, eventually undergoing intermolecular protein-protein cross-linking, e.g., LGE\textsubscript{2} also causes extensive intermolecular cross-linking of ovalbumin.\textsuperscript{40}
The time course of intermolecular cross-linking of calpain-1 was monitored upon incubation with 2 to 460 equivalents of iso[4]LGE2. The oligomerizations were terminated after various time intervals by the addition of excess glycine, and the oligomers were fractionated by SDS-PAGE and Western analysis (see Figure 2.7 and appendix Figure 2.2S-2.6S). Dimers and higher oligomers were already prominent after 1 h. Primary antibodies against the catalytic and small regulatory subunits as well as anti-iso[4] monoclonal antibody were used for Western analysis to detect both calpain-1 and iso[4]LGE2 modified calpain-1. All the images of the gels include the loading well. Only the incubation with 460 equivalents of isoLG showed an appreciable accumulation of high molecular weight oligomer in the loading well.

**Figure 2.7** Western analysis of iso[4]LGE2 (50 equivalents) modified calpain-1 after reaction for 1h, 3h, 12h and 24h. Left panel: developed with anti-calpain-1 catalytic subunit primary antibody. Middle panel: developed with anti-calpain-1 small subunit primary antibody. Right panel: developed with anti-iso[4]LGE2 monoclonal primary antibody.
Notably, intense bands for the catalytic subunit, its dimer and trimer at 80, 160 and 240 kDa were detected with anti-calpain-1 catalytic subunit antibody but not with the regulatory subunit antibody. The dimer and trimer, in contrast with the monomer, were also not prominently stained by the anti-iso[4]LG antibody suggesting that the antibody does not detect isoLG involved in cross-linking and that the cross-linked catalytic subunits do not have extensive additional modification that is not involved in the cross-link. For example, with 50 equivalents of iso[4]LGE2 (Figure 2.7), Western analysis revealed that within in 1 h, a dimer, higher oligomers and even higher multimers appeared. By contrast, the control, calpain-1 in the absence of iso[4]LGE2, exhibited no oligomerization after incubation for 24 h. The anti-calpain-1 catalytic subunit (80 kDa) primary antibody detected a band at 110 kDa corresponding to a hetero dimer of one catalytic subunit with one regulatory small subunit (30 kDa). It also detected bands at 160 kDa, 240 kDa and 320 kDa corresponding to a dimer, trimer and tetramer of the catalytic subunit. The anti-calpain-1 small subunit primary antibody (30 kDa) also detected a band at 110 kDa corresponding to a hetero dimer of one catalytic subunit and one small regulatory subunit. It also revealed a band at 120 kDa corresponding to a tetramer of the small regulatory subunit, and a band at 140 kDa corresponding to a heterotrimer consisting of two small regulatory subunits cross-linked with one catalytic subunit. Both the anti-catalytic subunit and small subunit primary antibodies showed some, albeit low, cross-reactivity. The anti-iso[4]LGE2 monoclonal antibody detected virtually all oligomer bands resulting from modification most likely due to non-crosslinked modification. Although the molecular structure of the protein cross-link was unknown, the
oligomers are resistant to dissociation by SDS and heat under reducing conditions, indicating a high probability that the cross-link involves covalent bonding.

2.2.4 Preparation of fluorescently labeled iso[4]LGE₂.

The identification and quantitation of protein cross-linking have proved to be a formidable analytical challenge in proteomics. Fluorescence detection-HPLC is an important method for trace analysis and in combination with mass spectrometry may provide an approach to analyze isoLGE modified protein cross-linking. For this purpose, stable fluorescently labeled iso[4]LGE₂ was developed and tagged to protein through modification. After digestion, tagged samples were subjected to LC/MS with fluorescence detection for further analysis.

Preparation of fluorescently labeled iso[4]LGE₂ 2.4 was achieved in two steps (Scheme 2.2). Iso[4]LGE₂ precursor 2.1 was synthesized as described.³ (4-(N,N-Dimethylaminosulfonyl)-7-(2-aminoethylamino)-2,1,3-benzoxadiazole) (DBD-ED)⁴²-⁴⁴ is a widely used fluorescence labeling reagent which emits strong fluorescence at long wavelengths. Its 2-aminoethylamine group reacts in the presence of a condensing agent with carboxylic acids at room temperature to yield a stable amide. The EDCI-HoBt⁴⁵-⁴⁹ system was chosen as condensing agent after testing triphenylphosphine (TPP), 2,2'-Dipyridyl disulfide (DPDS)⁵⁰-⁵⁴ and 1,1'-Carbonyldiimidazole (CDI).⁵⁵ Fluorescently labeled iso[4]LGE₂ intermediate 2.3 was prepared by treating precursor 2.1 with EDCI-HoBt first and then adding DBD-ED at 25 °C in the dark. After overnight reaction, one product was formed and unreacted materials could be easily removed by flash silica gel chromatography. Stirring the acetal 2.3 in dilute acetic acid overnight followed by
evaporation of the solvent under high vacuum and flash chromatography accomplished hydrolysis of the dimethyl acetal to cleanly deliver pure 2.4. MS spectra were shown in appendix Figure 2.7S.

Scheme 2.2 Synthesis of a fluorescently labeled iso[4]LGE2 derivative.

2.2.5 GAPDH Lys<sup>3</sup> and Lys<sup>249</sup> are modified by fluorescently labeled isoLG.

Fluorescently labeled isoLG was first used to detect the sites of 1:1 lysine-isoLG adducts, e.g., pyrroles, lactams, hydroxylactams and their derivatives, on a homotetramer protein glyceraldehyde 3-phosphate dehydrogenase. Fluorescently labeled iso[4]LGE<sub>2</sub>-treated GAPDH was trypsinyzed in-solution followed by triple quadrupole MS and Q-Tof MS/MS analysis of tryptic peptides. Peak lists from the acquired MS/MS spectra were analyzed with the Mascot data base search engine. Evaluation criteria for identification of the modified peptides were the same as normal iso[4]LGE<sub>2</sub> modified peptides. Lysine modifications and chemical structures of lys-fluorescently labeled iso[4]LGE<sub>2</sub> adducts were presented in Table 2.4S and Scheme 2.1S. This analysis identified two lysine residues in GAPDH that were modified by fluorescently labeled iso[4]LGE<sub>2</sub> as a monodehydrated lactam and a bisdehydrated hydroxylactam. MS/MS characterization of
the doubly charged ion m/z 620.86 (LEKAAK) and triply charged ion m/z 537.96 (VKVGVNGFGR) showed a series of fragment ions sufficient to unambiguously identify a fluorescently labeled iso[4]LGE2 modification on the GAPDH lysyl residues K3 and K249 (Table 2.2 and Table 2.5S).

**Table 2.2** Tryptic peptides from GAPDH modified by fluorescently labeled iso[4]LGE2.

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Observed</th>
<th>Mr(expt)</th>
<th>Ion score</th>
<th>Variable Modification</th>
</tr>
</thead>
<tbody>
<tr>
<td>LEK((-C_{30}H_{39}N_{5}O_{5}S))AAK</td>
<td>620.8596(2+)</td>
<td>1239.7046</td>
<td>18</td>
<td>Lactam Monodehydrated</td>
</tr>
<tr>
<td>VK((-C_{30}H_{37}N_{5}O_{5}S))VGVNGFGR</td>
<td>537.9638(3+)</td>
<td>1610.8696</td>
<td>23</td>
<td>Hydroxylactam Bisdehydrated</td>
</tr>
</tbody>
</table>

MS/MS analysis consistently detected modification on the LEKAAK and VKVGVNGFGR sequences of fluorescently labeled iso[4]LGE2-GAPDH in preparations generated with various equivalents of fluorescently labeled iso[4]LGE2, implying that these sequences were readily accessed and covalently altered by this aldehyde electrophile. Figure 2.8 showed ball-and-stick and cartoon models providing 3D visualization of GAPDH using PyMOL v1.4.1. All lysines of GAPDH are shown as space-filling models in the left panel while only the lysines that becoming adducted by fluorescently labeled iso[4]LGE2 and active site are shown in the cartoon model in the right panel. The 3D model shows that these lysines are located at and protrude from the surface of GAPDH. The physical environment and chemical characteristics of these specific lysyl residues probably facilitated access by iso[4]LGE2 and covalent adduction through Schiff base formation with its terminal aldehyde group leading to pyrrole formation. This is similar to the preference noted above for isoLG modification of calpain-1.
Figure 2.8 A ball-and-stick and cartoon 3D model structure for catalytic subunit of GAPDH (pdb: 1j0x). All the lysines were shown as space-filling models in the left panel. Only lysines that became modified by fluorescently labeled iso[4]LGE2 and active site (Cys 149) were shown in the right panel.55-56

2.2.6 In vitro modification of GAPDH with fluorescently labeled iso[4]LGE2 forms cross-links.

The time course of intermolecular cross-linking of GAPDH upon incubation with 10 equivalents of fluorescently labeled iso[4]LGE2 was determined by quenching the oligomerization after various time intervals by the addition of excess glycine, and fractionating the oligomers by SDS-PAGE. Only the 24 h incubation showed an accumulation of high molecular weight oligomer in the loading well (Figure 2.9). Within 1 h, a dimer, trimer and even higher multimers appeared. By contrast, GAPDH in the absence of fluorescently labeled iso[4]LGE2 exhibited little or no oligomerization after incubation for 24 h. The traces of oligomers that were barely detectable in the control probably represent minor alternative oligomerization mechanisms.
2.2.7 Pilot studies on cross-linking during modification of GAPDH with fluorescently labeled iso[4]LGE₂.

2.2.7.1 Amino acid analysis GAPDH dimer, trimer and tetramer formation upon modification with fluorescently labeled iso[4]LGE₂.

Amino acid analysis was performed to quantify the percentage of modified GAPDH cross-linked as dimer, trimer and tetramer contained in a sample. The identity and quantity of each amino acid was determined by comparing retention time and chromatographic peak areas detected from the sample with those of a calibration file that was generated from an amino acid standard mixture (standard H, Pierce).

Dimer, trimer and tetramer fractions of fluorescently labeled iso[4]LGE₂ modified GAPDH were cut from a gel (Figure 2.9) and individually trypsin in gel-digested.

Figure 2.9 Coomassie blue staining of 10 equivalents fluorescently labeled iso[4]LGE₂ modified GAPDH after incubation for various times prior to addition of excess glycine.
AccQ·Tag\textsuperscript{TM} amino acid analysis was used to quantify each cross-linked fraction in a particular sample of GAPDH. First the tryptic peptides from the sample were hydrolyzed into free amino acids under HCl vapor. Then the amino acids were derivatized using a Waters AccQ·Tag\textsuperscript{TM} ultra derivatization kit. AccQ·Tag\textsuperscript{TM} ultra reagent reacts rapidly with primary amino groups to yield highly stable urea. The structure of the UV active derivatizing group is the same for all amino acids. Excess reagent hydrolyzes to yield 6-aminoquinoline (AMQ), a non-interfering by-product.

![Scheme 2.3 AccQ·Tag\textsuperscript{TM} derivatization.\textsuperscript{65-66}](image)

Finally, the derivatized hydrolysate was chromatographically analyzed. The identity and quantity of each amino acid was determined by comparing retention time and chromatographic peak areas with the peak area of the quantitative standard amino acids (Standard H, 50 pmol, appendix Figure 2.8S). The percent of total protein sample that corresponds to the fraction analyzed was determined by dividing the amount determined by the quantitative amino acid analysis of the fraction by the total amount of protein (GAPDH after 24 h modification and for SDS analysis) in the sample times 100. After 24 h incubation, about 2.5%, 2.5% and 1.5% of GAPDH was present in the sample in the form of dimer, trimer and tetramer, respectively (appendix Table 2.6S).
2.2.7.2 LC/MSMS analysis of fluorescently tagged tryptic peptides

A combination of fluorescence detection-HPLC and mass spectrometry was applied to analysis of tryptic peptides from an isoLG-modified protein (GAPDH) in an effort to identify isoLG-cross-linked peptides. To enable detection and facilitate isolation of isoLG-modified peptides, a fluorescent label was incorporated into the isoLG. Thus, homo-tetrameric protein GAPDH was modified with stable fluorescently labeled iso[4]LGE₂, trypsinized, and the tryptic peptides were first separated on a reverse phase column, fractions detected with an Acquity® fluorescence (FLR) detector were analyzed with a triple quadrupole electrospray mass spectrometer (Applied Biosystems Inc.). Fluorescence channels of tryptophan peptides (three digested peptides containing tryptophan) and iso[4]LGE₂ tag were both monitored. The fluorescence signal of tryptophan peptides (280 nm for excitation, 340 nm for emission) and mass spectrometry (ESI-triple quadrupole) were employed to evaluate the chromatographic separation of peptides on various LC columns. A column was identified that gave sharp peaks. Then using that column, the fluorescence signal of iso[4]LGE₂ tagged peptides (450 nm for excitation, 560 nm for emission) was monitored to detect the iso[4]LGE₂ modified peptides (see appendix Figure 2.9S and 2.10S). Peptides with iso[4]LGE₂ tag fluorescence signals were collected as one minute fractions and quickly dried using a high-speed vacuum evaporator and stored at -20 °C until used. These peptides were re-suspended in 0.1% formic acid/2% acetonitrile and further analyzed by LC-MS/MS using a quadrupole-time of flight (QTof2) mass spectrometer. To identify 1:1 isoLG-peptide adducts, peak lists from the acquired MS/MS spectra were analyzed with the Mascot database search engine.
To identify 2:1 isoLG-peptide aminal cross-links (Scheme 2.4), peptides with fluorescence signal were collected as one minute fractions that were analyzed by MS analysis on QTOF. The masses of all possible tryptic GAPDH peptides with one missing cleavage were calculated (Table 2.7S). The masses of cross-linked peptide [M] were then calculated by summing the mass of peptide #1 + mass of peptide #2 + 601.2934 (the mass increment for the cross-link, Scheme 2.4). The exact masses calculated for the [M+H]⁺, [M+2H]²⁺, [M+3H]³⁺, [M+4H]⁴⁺ ions are shown in Table 2.8S. Since the peptides “LEKAAK” and “MVKGVNGFGR” were found to form lysine 1:1 adducts, we postulated that these two peptides are particularly reactive toward iso[4]LGE₂. Consequently, we considered them to be high probability candidates for forming aminal cross-links. The molecular weights of aminal cross-links between “MVKGVNGFGR” + “MVKGVNGFGR”, “LEKAAK”+ “LEKAAK”, “LEKAAK”+ “MVKGVNGFGR” for ions from +1 to +4 charges were calculated and compared with all precursor ions exacted from QTOF MS of each HPLC fraction.

**Scheme 2.4** Chemical structure of peptides aminal cross-link. Mass of cross-linked peptide = mass of peptide #1 + mass of peptide #2 + 601.2935 (= 604.3169 – 3.0234).

An observed ion of mass 640.9626 corresponds approximately with the m/z 640.3732 expected for a +3 charged cross-link of the peptide “LEKAAK”+ “LEKAAK”. To
test the hypothesis that this ion corresponds to the above mentioned ion, MS2 daughter ions were examined for various expected fragmentation involving loss of b2 (243), a2 (215) or “AK” (199), “AAK” (270). However, none of the expected fragments were detected. Therefore, it is unlikely that the parent ion at 640.9626 corresponds to the proposed cross-link (Figure 2.11S).

To supplement the approach described above, in future studies, modification of proteins with a 1:1 mixture of d6-iso[4]LGE2 and d0-iso[4]LGE2, can provide additional evidence that will facilitate the detection and characterization of protein cross-links. If peptides were modified by both d6-iso[4]LGE2 and d0-iso[4]LGE2, the observation of characteristic pairs of peaks would provide convincing evidence for the presence of an isoLG in the ion being observed. The pair of peaks would be separated by +6 amu for singly charged ions, +3 amu for doubly charged ions, and +2 amu for triply charged ions.
2.3 Conclusions

IsoLG modification of proteins is associated with loss-of-function, cross-linking and aggregation. The sites of 1:1 adduct formation and the types and extent of cross-linking between subunits of the multi subunit proteins calpain-1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) by iso[4]LGE2 were characterized mass spectroscopically and by SDS-PAGE. Calpain-1 lost its protease activity with $t_{1/2} = \sim 40$ min upon incubation with 10 equivalents of iso[4]LGE2, and as little as 2 equivalents was almost as effective. This suggests that modification of one or two lysyl residues suffices to abolish activity. LC-MS/MS analysis established that Lys280 is a target of isoLG adduction to calpain-1. Since this lysine residue is not located in the active site, a conformational change induced by the modification may account for the loss of activity. Covalently cross-linked hetero and homo oligomers of calpain-1 catalytic and regulatory subunits were detected by SDS PAGE. The dimer and trimer, in contrast with the monomer, of the catalytic subunit were prominently stained by an antibody against the catalytic subunit but not by an antibody against the regulatory subunit or by the anti-iso[4]LG antibody suggesting that the iso[4]LG antibody does not detect isoLG involved in cross-linking and that the cross-linked catalytic subunits do not have extensive additional modification that is not involved in the cross-link.

The amounts of dimer, trimer and tetramer forms of GAPDH produced after 24 h incubation with 10 equivalents of fluorescently labeled iso[4]LGE2 was determined by amino acid analysis to be about 2.5%, 2.5% and 1.5% respectively. LC-MS/MS analysis established that Lys3 and Lys249 are targets of fluorescently labeled iso[4]LGE2 adduction to GAPDH. These lysine residues are located in the tryptic peptides.
“LEKAAK” and “VKGVNGFGR”. Since the peptides “LEKAAK” and “MVKVGVNGFGR” were found to form lysine 1:1 adducts, we postulated that these two peptides are presumably particularly reactive toward iso[4]LGE2. Consequently, we considered them to be high probability candidates for forming aminal cross-links. However, no ions were detected that could be confirmed by MS/MS analysis to correspond to these cross-linked peptides.
2.4 Experimental Procedures

2.4.1 General methods. All chemicals used were high purity analytical grade. The following commercially available materials were used as received: 4-((N,N-dimethylamino)sulfonyl)-7-(2-aminoethylamino)-2,1,3-benzoxadiazole (DBD-ED) and 2,2'-dipyridyldisulfide (DPDS) were from TCI America (Portland, OR). Calpain-1 (from human erythrocytes) was from Calbiochem (La Jolla, CA). 1-Hydroxybenzotriazole (HoBt), iodoacetamide, N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDCI), 1,1′-carbonyldiimidazole (CDI), and GAPDH (from rabbit muscle) were from Sigma (St. Louis, MO). Dithiothreitol was from Denville Scientific (South Plainfield NJ). Chymotrypsin and trypsin were from Promega (Madison, WI). Sodium acetate, ammonium bicarbonate, disodium hydrogen phosphate and monosodium phosphate were from Fisher Scientific Co. Iso[4]LGE2 was prepared by Dr. Jim Laird and iso[4]LGE2-FluoTag was prepared as discussed below.

Chromatography was performed with ACS grade solvent. Thin layer chromatography (TLC) was performed on glass plates precoated with silica gel (Kieselgel 60 F254, E. Merck, Darmstadt, West Germany). \( R_f \) values are quoted for plates of thickness 0.25 mm. The plates were visualized by viewing the developed plates under short or long wavelength UV light, iodine, or by heating the plates after dipping in 20% solution of phosphomolybdic acid in ethanol. Flash chromatography was performed according to established methods\(^{57} \) on ICN SiliTech 32-63 D 60A silica gel from ICN Biomedicals GmbH, Eschwege, Germany.
2.4.2 Synthesis of fluorescently labeled iso[4]LGE2. HOBt (1.48 mg, 11 µmol in 40 µl DMF), EDCI (2.12 mg, 11 µmol in 56 µl CH2Cl2), and precursor 2.1 (4 mg, 10 µmol in 50 µl CH2Cl2) were stirred at room temperature for 2.5 h. A solution of DBD-ED (11 µmol, 2.84 mg) dissolved in 140 µl of acetonitrile was added to the reaction mixture. After overnight reaction in the dark, TLC showed only one major product formed and complete disappearance of precursor 2.1. The reaction mixture was concentrated and the residue was purified by flash chromatography on a silica gel column (ethyl acetate: methanol=20:1, TLC: Rf = 0.7) to give the fluorescently tagged iso[4]LGE2 dimethylacetal derivative 2.3 (4.8 mg, 7 µmol, yellow oil). ESI-MS: m/z calcd for C32H51N5O8S (M), 665.35; found, 664.13 (M-H); 700.00 (M-H+Cl) for negative mode, and 688.33 (M+Na) for positive mode.

Hydrolysis of the dimethyl acetal in 2.3 was accomplished by dissolving it (4 mg, 6 µmol) in 3:1 acetic acid/water (1.3 mL) and incubating overnight at room temperature in the dark, followed by rotary evaporation of solvents. The residue was purified by flash chromatography on a silica gel column (ethyl acetate: methanol=40:1, TLC: Rf = 0.6) and placing under high vacuum through a dry ice-acetone cooled trap for 2 days to give compound 2.4. (2.3 mg, 3.8 µmol, yellow solid). ESI-MS: m/z calcd for C30H45N5O7S (M), 601.28; found, 600.13 (M-H), 635.87 (M-H+Cl) for negative mode, and 624.20 (M+Na) for positive mode.
2.4.3 In vitro modification of calpain-1 by iso[4]LGE$_2$. Calpain-1 was modified with 10 equivalents of iso[4]LGE$_2$. Calpain-1 (100 µg, 8.9 nmol) in 190 µl of a solution containing 20 mM imidazole, 5 mM β–mercaptoethanol, 1 mM EDTA, 1 mM EGTA, 30 % glycerol, pH 6.8 was mixed with 310 µl of sodium acetate solution (10 mM, pH 6.8). First 11 µl of iso[4]LGE$_2$ stock (1 µg/µl in methanol, 11 µg, 30 nmol) was added to calpain-1 and the mixture reacted on a shaker (IKA MTS 2/4 digital microtiter shaker, 300 rpm) for 40 min. Then another 11 µl of iso[4]LGE$_2$ stock (1 µg/µl, 11 µg, 30 nmol) was added and reacted for 40 min, then the last 11 µl of iso[4]LGE$_2$ stock (1 µg/µl, 11 µg, 30 nmol) was added and reacted for 40 min. The reaction mixture (total 533 µl) was quenched by addition of glycine (2 µl, 100 mM, 178 nmol, 2 molar equivalent to iso[4]LGE$_2$) with brief mixing. The mixture was then flash-frozen in liquid nitrogen and stored at -80 °C until analysis by MS.

Modified calpain-1 (20 µg) was subjected to 4-12% SDS-PAGE. The gel was stained with Coomassie blue, and a region corresponding to proteins with molecular mass between 80-90 and 30-40 kDa was excised respectively. Destained, reduced in 20 mM DTT for 30 min at room temperature, and alkylated in 100 mM iodoacetamide in the dark for 30 min at room temperature. For LC/MSMS analysis, the protein was first in gel digested with chymotrypsin. Chymotrypsin stock solution was prepared by dissolving chymotrypsin (25 µg) in NH$_4$HCO$_3$ (1000 µl 50 mM), to provide a final concentration of 25 µg/ml. Chymotrypsin solution (16 µl, 0.4 µg) was added to modified calpain (20 µg) for 22 h at 37 °C using a protease to calpain-1 ratio of 1:50 (w/w) in 50 mM NH$_4$HCO$_3$. Peptides were extracted with 50% acetonitrile and 5% formic acid, dried in a vacuum concentrator, and stored at -20 °C. Peptide separations were performed on an Ultimate
3000 LC system with a C18 Acclaim PepMap 100 column (0.075 x 150 mm, Dionex, Sunnyvale, CA). Peptides were eluted over a 50-min gradient from 0 to 80% acetonitrile in water, containing 0.1% formic acid, at a flow rate of 300 nl/min. The column effluent was continuously directed into the nanospray source of the mass spectrometer, a hybrid Fourier transform ion cyclotron resonance (FTICR)/linear ion trap mass spectrometer (LTQ FT Ultra, Thermo Scientific, West Palm Beach, FL). The following parameters were used for all acquisition methods on the LTQ FT Ultra MS: an ion spray voltage of 2400 V and an interface capillary heating temperature of 200 °C. Full mass spectra were acquired from the FTICR, and the tandem mass spectra (MS/MS) of the eight most intense ions were recorded by the linear ion trap in data-dependent mode with normalized collision energy of 35 eV, isolation width of 2.5 Da, and activation Q of 0.25.

**2.4.4 In vitro modification of GAPDH by fluorescently labeled iso[4]LGE2.** GAPDH was modified with 10 equivalents of fluorescently labeled iso[4]LGE2. Fluorescently labeled iso[4]LGE2 (168 µg, 280 nmol) (21 µl of 8 mg/ml in methanol) was added to GAPDH (28 nmol) (1 mg in 479 µl of 50 mM sodium phosphorus buffer, pH 7.0) and the mixture was under air on an IKA MTS 2/4 digital microtiter shaker at 300 rpm for a total 8 h at 25 °C. Then the reaction mixture (total 500 µl) was quenched by addition of glycine (6 µl, 100 mM, 560 nmol, 2 molar equivalent to fluorescently labeled iso[4]LGE2) with brief mixing. The mixture was flash-frozen in liquid nitrogen and stored at -80 °C for further analysis by MS and amino acid analysis.

For LC/MSMS analysis, in solution digestion was performed using trypsin. The protein was reduced with 20 mM DTT for 30 min at room temperature, and alkylated in
200 mM iodoacetamide in the dark for 1 h at room temperature. The protein was then digested by trypsin (20 µg) for 24 h at 37 °C with a protease to GAPDH ratio of 1:50 (w/w) in 50 mM of NEM·OAc (pH 8.6). Peptides were dried in a vacuum concentrator, and the residue stored at -20 °C.

The tryptic peptides were first separated on a reverse phase column (2.1x100 mm i.d. C18 1.8 µm column from Waters Acquity UPLC HSS). Chromatography was carried out by elution with a linear gradient (elute A, 0.1% formic acid/H₂O; elute B, 0.1% formic acid/ACN). The sample was loaded onto the column with 98% A and 2% B at a flow rate of 70 µl/min for 10 min. For the analysis, the gradient was as follows: 2-10% B over 5 min, to 70 % within 30 min at a flow rate of 70 µl/min, to 98 % within 0.1 min and hold for 5 min at a flow rate of 140 µl/min.

The analyses of digested peptides were performed by Acquity® fluorescence (FLR) detector and API-3000 triple quadrupole electrospray mass spectrometer (Applied Biosystems Inc.) operated in the positive ion mode and a MS scan of m/z 220-2000. Fluorescence channels of tryptophan peptides (three digested peptides containing tryptophan) and iso[4]LGE₂ tag were both monitored. The fluorescence of tryptophan peptides (280 nm for excitation, 340 nm for emission) and mass spectrometry were monitored to confirm the chromatographic separation. The fluorescence signal of iso[4]LGE₂ tag (450 nm for excitation, 560 nm for emission) was monitored to confirm the elution time of fluorescently labeled iso[4]LGE₂ modified peptides. Peptides with iso[4]LGE₂ tag fluorescence signals were collected as one minute fraction, quickly dried using a high-speed vacuum evaporator and then stored at -20 °C until for further analysis.
The tryptic peptides with fluorescence signals were re-suspended in 0.1% formic acid/2% acetonitrile and further analyzed by LC-MS/MS using a quadrupole-time of flight (QTof2) mass spectrometer equipped with a CapLC system (Waters, Millford, MA), ProteinLynx Global Server acquisition and processing software. Peptide digests were trapped on a precolumn (0.3 x 1mm, 5µC18, LC Packing) with 0.1% formic acid in 2% acetonitrile as loading solvent then eluted onto a capillary column (PicoFrit 0.050 x 75 mm, 15µ tip ID; New Objective Inc., Woburn, MA) over a 60-min gradient from 0 to 80% acetonitrile in water, containing 0.1% formic acid, at a flow rate of 250 nl/min. The column effluent was continuously directed into the nanospray source of the mass spectrometer which was operated in standard MS/MS switching mode with the three most intense ions in each survey scan subjected to MS/MS analysis. Protein identifications from MS/MS data utilized ProteinLynxTM Global Server (Waters Corporation) and Mascot (Matrix Science) search engines and the Swiss-Protein and NCBI protein sequence databases.

2.4.5 Electrophoresis. According to a published protocol, modified proteins (~ 5-10 µg) were suspended in Laemmli sample buffer, fractionated on a 4-12% SDS-PAGE, and then stained with coomassie blue (Bio-Rad coomassie stain #161-0786) or silver stain (GE Healthcare, PlusOne silver staining kit, protein, instructions 71-7177-00AL). The bands were quantified using a GS-710 scanner and QuantityOne® software from Biorad™ (Hercules, CA).
2.4.6 Western analysis. According to published protocols, modified proteins (~ 5-10 µg) were suspended Laemmli sample buffer, fractionated on a 4-12% SDS-PAGE, and electroblotted onto a polynivyl difluoride (PVDF) membrane (Millipore, Bedford, MA). The PVDF membranes were treated with Odyssey blocking buffer (Li-Cor Cat# 927-40000) and probed using various antibodies: anti-calpain-1 small subunit (Fisher Cat#01674303) mAb, anti-calpain-1 catalytic subunit (Fisher Cat#01674304) mAb, anti-iso[4]LGE2 mAb (see Chapter 5). Goat anti-mouse IRDye® 680 (Odyssey Cat#926-32220) was used as secondary antibody. The immunoreactive bands were detected and quantified using a Li-Cor infrared imaging system.

2.4.7 Calpain-1 activity assay. Calpain-1 assay was performed on aliquots of modified isoLG protein using a calpain activity assay kit (Biovision Inc., Mountainview, CA) following recommended protocols. The fluorometric assay was based on detecting the cleavage of a calpain substrate, Ac-LLY-AFC, that fluoresces at 505 nm, whereas the cleaved substrate fluoresces at 400 nm. Thus, calpain-1 (26 µl of 2.286 µM, 6.6 µg, 0.0595 nmol) was added to iso[4]LGE2 (0.21 µg 0.595 nmol) in 74 µl of aqueous sodium acetate (10 mM), mixed well and reacted under air on shaker (IKA MTS 2/4 digital microtiter shaker, 300 rpm) at 25 °C. At each time point (3, 10, 30 and 90 min), one aliquot (25 µl) was taken and quenched with 1.2 µl 1 mM glycine (1.2 nmol) before activity assay. The aliquot (26.2 µl) was added extraction buffer (58.8 µl), 10x reaction buffer (Ready-to-Use reagent from Biovision calpain activity assay kit) (10 µl) and calpain substrate (5 µl). Then the mixture was incubated at 37 °C for 1 hour in the dark and fluorescence was detected using Spectra Max Gemini spectrofluorometer (excitation
400 nm, emission 505 nm) (Molecular Devices, Sunnyvale, CA). Negative control was performed the same method with calpain inhibitor Z-LLY-FMK from Biovision calpain activity assay kit.

2.4.8 MS/MS identification of isoLG-modified sites in calpain-1 and GAPDH. Peptides were identified from LTQ FT Ultra MS/MS and quadrupole-time of flight (Q-Tof-2) experimental data by generating peak lists with Mascot Daemon and submitting these to the Mascot search engine, version 2.3.0 (Matrix Science, Boston). S-Carbamidomethylation of cysteine was set as a fixed modification, whereas oxidation of methionine (methionine sulfoxide) was set as a variable modification. Formulas corresponding to the various oxidation and dehydration states of iso[4]LGE2 adducts and fluorescently labeled iso[4]LGE2 adducts were manually entered into the Mascot search parameters and selected as variable modifications for lysine (appendix Table 2.1S and 2.4S, structures shown in Scheme 2.1S). Mass tolerances were ±15 ppm for precursor ions and ± 0.8 Da for fragment ions. One missed cleavage site was allowed for trypsin (cleaves at residues Lys and Arg but not at Pro) and three missed cleavage sites were allowed for chymotrypsin (cleaves at Phe, Leu, Trp, and Tyr but not at Pro). Searches were restricted to a sequence data base containing only human calpain-1 and rabbit GAPDH because purified protein was used. Only peptides with a significance score greater than 15 ($p<0.05$) according to Mascot’s scoring algorithm were considered.

2.4.9 Amino acid analysis. IsoLG modified GAPDH (400 µg) was subjected to 4-12% SDS-PAGE. The gel was stained with Coomassie blue, and a region corresponding to
proteins with molecular mass between 70-80, 110-120 and 140-160 kDa were excised. Each excised region was destained, reduced in 20 mM DTT for 30 min at room temperature, and then alkylated in 200 mM iodoacetamide in the dark for 1 h at room temperature. The protein was then digested in gel with trypsin (8 µg) for 24 h at 37 °C with a protease to GAPDH ratio of 1:50 (w/w) in 50 mM of NEM·OAc (pH 8.6). Peptides were extracted from the gel in 0.1%TFA/20%ACN, dried in a vacuum concentrator, and stored at -20 °C for later analysis. AccQ-Tag™ amino acid analysis was used to quantify cross-linked GAPDH. The trypsin digested peptides were hydrolyzed to amino acids with HCl, then with derivatized AccQ-Tag reagent and analyzed chromatographically as described below.

2.4.9.1 Vapor phase HCl Hydrolysis: Digested dimer, trimer and tetramer peptides were treated separately. Each sample was added 50 µl 0.1%TFA/20%ACN and then 150 µl 0.1%TFA. 20 µl aliquot was taken out from each tube and hydrolyzed to free amino acids under HCl vapor. Peptide solutions (20 µl) were transferred to 6×50 mm hydrolysis tubes and dried under vacuum. After drying, the glass tubes were placed into a screw-cap vial containing 300 µl of 6M HCl and a few crystals of phenol (~1-2 mg). The vial was evacuated briefly then flushed with argon and this alternating process was repeated three times. The vial was finally sealed under vacuum and was heated at 150 °C in an oven for 1 h. After hydrolysis, sample tubes were dried using a high-speed vacuum evaporator and stored at -20 °C until derivatization.

2.4.9.2 AccQ-Tag Derivatization and chromatography: AccQ-Tag™ ultra derivatization kit (Waters) was used. AccQ-Tag™ ultra reagent reacts rapidly with primary amino groups to yield highly stable urea. The structure of the derivatizing group
was the same for all amino acids.\textsuperscript{62-63} Peptide hydrolysates were transferred to reaction vials and AccQ-Tag\textsuperscript{TM} borate buffer (30 µl) was added to adjust the pH to above 9.5. After borate buffer was mixed well with the peptide hydrolysates by vortexing. To a 24 µl aliquot was added AccQ-Tag\textsuperscript{TM} reagent (6 µl, Ready-to-Use reagent from Waters AccQ-Tag\textsuperscript{TM} ultra derivatization kit). The contents of the vial were mixed by vortexing and then heated for 10 min at 55 °C. Then deionized water (30 µl) was added and the mixture was vortexed. All 60 µl of the sample solution was transferred to a UPLC sample vial for testing. The amino acid standard was prepared in the same manner.

The derivatized samples and H-standard were analyzed with a Waters Acquity ultra performance LC system using 2 µl injections for optimum resolution and sensitivity (100 x 2.1 mm column). For the separation, sodium acetate (140 mM) containing 17 mM triethylamine (pH 5) was used as buffer A and 60 % acetonitrile/H\textsubscript{2}O as buffer B. The gradient was as follows with a flow rate of 700 µl/min: time 0-0.54 min, 0.1% B; time 5.74 min, 9.1% B; time 7.74 min, 21.2 % B; time 8.04 min, 59.6% B and time 8.05 min, 90% B. AccQ-Tag amino acids were detected with the Acquity UPLC tunable UV detector at 260 nm.

\textbf{2.4.9.3 Amino acid analysis and data interpretation:} Amino acid analysis was performed to quantify the concentrations of digested dimer, trimer and tetramer peptides. The amount of dimer, trimer and tetramer were determined by (i) dividing the amount of each individual residue by the known residue value, (ii) calculating a mean amount analyzed, (iii) discarding all individual values that deviated more than 15% from the mean, and (iv) recalculating the mean amount analyzed from the remaining relevant residues.\textsuperscript{64} The identity and quantity of each amino acid was established by comparing retention time and
chromatographic peak areas with the peak area of a quantitative standard mixture of amino acids (Standard H, 50 pmol) that was derivatized and analyzed the same way as the digested peptides with the same amount of reagents and instruments.

Standard response (standard H) per pmol for each amino acid is equal to standard signal (HPLC peak area) divided by pmol amino acid for analysis. Sample signal (peak area of GAPDH derivatized dimer sample) divided by the standard response will get analyzed pmol of each amino acid. Then analyzed sample pmol of each amino acid is divided by the known number of amino acid per molecule protein (GAPDH) to obtain pmol protein. Then the mean pmol protein (within 15% deviation) is calculated and divided by sample dilution ratio to get pmol of the derivatized protein (GAPDH dimer).

Running gel with 400 µg (11200 pmol) of modified GAPDH (24 h)

Cut and accumulate all the dimer, trimer, tetramer bands for digestion

Speed vac the extraction buffer, each sample was dissolved in 50 µl 0.1% TFA, 20% ACN, then added 150 µl 0.1% TFA. Total volume of each sample was 200 µl

From each 200 µl, take out 20 µl for amino acid analysis

Dimer 1.34 pmol/µl (0.05 µg/µl)

1.34 pmol/µl × 200 µl = 268 pmol

modified percentage: 10 µg/400 µg = 2.5%

Trimer 1.5 pmol/µl (0.05 µg/µl)

1.5 pmol/µl × 200 µl = 300 pmol

percentage: 10 µg/400 µg = 2.5%

Tetramer 0.93 pmol/µl (0.03 µg/µl)

0.93 pmol/µl × 200 µl = 186 pmol

percentage: 6 µg/400 µg = 1.5%
2.5 References


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CHAPTER 3

3.1 Background

3.1.1 Purpose

Levuglandins (LGs) and isolevuglandins (isoLGs) react with protein lysyl residues to form covalent adducts that include pyrroles and their more oxidized derivatives, lactams and hydroxylactams, as well as protein-protein cross-links. Two alternative structures have been proposed for the protein-protein cross-links, a pyrrole-pyrrole dimer derived from two molecules of protein and two molecules of LG/isoLG or an aminal derived from two molecules of protein and one molecule of LG/isoLG. In the study, to obtain direct evidence for the molecular structure of lysyl-LG/isoLG cross-links, two simple model systems were investigated, the reaction iso[4]LGE$_2$ with N-acetylglycyllysine methyl ester and with the β-amyloid (11-17) peptide EVHHQKL.

3.1.2 Isolevuglandin modification of proteins

A variety of products are formed through oxidation of fatty acids by enzymatic or free radical induced oxidation pathways. The enzymatic oxidation of arachidonic acid (AA) by cyclooxygenase (COX) leads to the formation of levuglandins, whereas oxidation of phosphoesters of arachidonic acid leads to the production of isolevuglandins (isoLGs).$^{1-3}$

Iso[4]LGE$_2$, can only be generated through free radical oxidation of arachidonic acid phospholipids. Iso[4]LGE$_2$ is a highly reactive γ-ketoaldehyde which modifies the ε-amino groups of lysine residues forming protein-bound pyrroles lactams and hydroxylactams,$^4$ as well as protein-protein cross-links for which aminal$^5$ and pyrrole-pyrrole$^6-7$ structures have been postulated, as well as DNA-protein cross-links.$^8$ Incubation of salmon sperm DNA with LGE$_2$ causes DNA-protein cross-linking in cultured cells (10 mM MOPS buffer), suggesting that levuglandins can directly react with
DNA. Cyclopentenone PGs contain α,β-unsaturated carbonyl groups that readily adduct various biomolecules such as glutathione (GSH) \textit{in vitro} (Scheme 3.1).\textsuperscript{9}

\begin{align*}
\text{Cyclopentenone PGs} & \quad \text{GSH} \\
\text{PGJ}_2 & \quad \text{GSH transferase}
\end{align*}

\textbf{Scheme 3.1} Schematic representation of pyrrole cross-linking structure in previous study: adducts of PGJ\textsubscript{2} reaction with GSH in HCA-7 cells.\textsuperscript{9}

LGE\textsubscript{2} also reacts with nucleotides \textit{in vitro} to form covalent adducts with a dihydroxypryrrlolidine structure (Scheme 3.2).\textsuperscript{10}
Scheme 3.2 Schematic representation of pyrrole cross-linking structure in previous study: adducts of nucleosides and DNA formed by reaction with levuglandin.\textsuperscript{10}

PGH\textsubscript{2} reacts with arginine to yield covalent adducts (Scheme 3.3).\textsuperscript{11} These structures would be appropriate candidates for isoLG/LG-protein cross-link.
IsoLGs initially form reversible Schiff base adducts\textsuperscript{12-13} with proteins which can dehydrate to pyrroles. These adducts can be further oxidized to form stable lactams and hydroxylactams. A possible cross-linking mechanism identifies the LG-protein Schiff base adduct as the reactive electrophile that binds with a primary amino group in a second molecule of protein resulting in aminal formation.\textsuperscript{7} IsoLG/LG cross-link forms also include pyrrole further reaction with nucleophiles like unoxidized pyrroles (Scheme 3.4)\textsuperscript{4} or thiols (Scheme 3.5).\textsuperscript{14-15}

**Scheme 3.4** Schematic representation of pyrrole cross-linking structure in previous study: nucleophilic substitution of alkylpyrroles in the presence of oxygen.\textsuperscript{4}
Scheme 3.5 Schematic representation of pyrrole cross-linking structures in previous studies. (A) Pyrrole autoxidation products in 2,5-hexanedione-treated dipeptide. (B) Formation of thiol-pyrrole conjugates.

The different ways in which isoLGs have been shown or postulated to modify proteins is depicted in Scheme 3.6. IsoLGs modify proteins in a matter of seconds and cross-link them within minutes, which is much faster than the rates at which HNE or MDA form cross-links. Modification of the proteasome and other intracellular proteins targeted by isoLGs can lead to impaired degradation. For example, isoLG-adducted ovalbumin and Aβ1-40 aggregates significantly inhibit the proteasome activity of neuroglial cultured cells and induce cell death, suggesting that isoLG/LGs may have...
relevance to the pathogenesis of neurotoxicity\textsuperscript{19} and neurodegenerative diseases.\textsuperscript{17} Also these adducts have been shown to hinder the activity of the 20S proteasome, a component of cytosolic protein, degradation machinery in a dose dependent manner.\textsuperscript{17} Accumulation of iso[4]LGE\textsubscript{2}-modified proteins has been observed in many diseases such as arteriosclerosis\textsuperscript{20} and rheumatoid arthritis.\textsuperscript{21}

3.1.3 β-Amyloid and isolevuglandins

Alzheimer’s disease (AD) brain is under intense oxidative stress, manifested by increased protein oxidation, lipid peroxidation, free radical formation, DNA/RNA oxidation, nitrotyrosine levels, and advanced glycation end products.\(^{22-23}\) Cerebral accumulation of soluble oligomers of (Aβ) 1-42 that are cytotoxic to neurons is central to the pathogenesis of Alzheimer’s disease (AD).\(^{24-28}\) Addition of Aβ (1-42) to neurons leads to increased protein carbonyls and decreased cell survival compared to controls.\(^ {29-34}\) Protein oxidation is increased in AD brain in regions rich in Aβ (1-42). In parallel, evidence has indicated long-term use of inhibitors of the cyclooxygenases is associated with a decrease in the severity of Alzheimer’s disease. Meantime PGH\(_2\) accelerates the formation of oligomers of Aβ1-42 in conjunction with lipid modification of the protein. It provides a molecular basis for the hypothetical mechanism that links cyclooxygenase activity to the formation of oligomers of Aβ.\(^ {16}\) Aggregation of amyloid β (Aβ) 1-42 peptide forming neurotoxic oligomers may be a consequence of the LG-protein-protein cross-linking that was observed upon treatment of Aβ 1-42 with LGE\(_2\).\(^ {35}\)
3.2 Results and discussion


Iso[4]LGE$_2$ or iso[4]LGE$_2$-FluoTag was incubated with N-acetylglucyllysine methyl ester or β-amyloid (11-17) peptide “EVHHQKL” individually in NEM·OAc (pH 8.6) at 25°C for several days. Reactions were also conducted in both acidic (50 mM phosphate buffer, pH 5.8) and neutral (50 mM phosphate buffer, pH 7.0) solutions. The results were similar as in basic buffer. The reaction product profile exhibited no relationship with buffer pH (vide infra). The resulting mixtures were dried on a speed vacuum, and re-suspended in 10% acetonitrile/water with a trace of formic acid (0.1%) and analyzed by mass spectrometry.


N-Acetyl-glycyl-lysine methyl ester (N-acetyl-Gly-Lys-OMe) and β-amyloid(11-17) peptide “EVHHQKL” were used as models for characterizing the modification of protein lysyl residues resulting from adduction of iso[4]LGE$_2$. A preliminary analysis of the reaction product mixtures using MALDI-TOF mass spectrometry revealed the presence of numerous products of peptide modification and indicated that both β-amyloid(11-17) and N-acetylglucyllysine methyl ester showed similar products of adduction. Further analysis was performed using triple quadrupole by ESI-MS and Q-TOF by ESI-MS/MS to characterize the products.


MALDI-TOF MS analysis of the reaction product mixture from iso[4]LGE$_2$ with a large excess of N-acetylglucyllysine methyl ester showed major ions at m/z 576, 592,
608, 853, and 1149 corresponding to protonated pyrrole, lactam, hydroxylactam, aminal cross-link and bispyrrole in addition to ions resulting from the loss of one or two molecules of water from those adducts. Figure 3.1 shows a representative spectrum revealing the presence of for various adducts in the reaction product mixture (see appendix Table 3.1S for the molecular weights of various iso[4]LGE2-(N-acetyl-gly-lys-OMe) adducts).

The reaction products for various reactant molar ratios were recorded (see appendix Figure 3.1S (1) for N-acetyl-glycyl-lysine methyl ester: iso[4]LGE2 = 2000:1, (2) for 1000:1 and (3) for 200:1 in 1, 4, 7, 15, 21 and 50 day reactions). The initial concentration of N-acetyl-glycyl-lysine methyl ester was constant while various ratios of iso[4]LGE2 were added. At various incubation times, identical aliquots of reaction mixture were purified by Ziptip and then analyzed.

One-to-one adducts, i.e., pyrrole, lactam and hydroxylactam and their dehydration products were generated very quickly (within one day reaction) in the reaction of iso[4]LGE2 with N-acetyl-glycyl-lysine methyl ester (see appendix Figures 3.1S 1A).

Cross-link adducts included aminals, bispyrroles, trispyrroles and their dehydrated forms. With higher iso[4]LGE2 ratio and longer incubation times, a higher percentage (comparison of cross-link peak intensity with one-to-one adducts) of bispyrrole adducts was generated. Specifically, bispyrrole peak intensity was higher when the molar ratio of N-acetyl-glycyl-lysine methyl ester to iso[4]LGE2 was 200:1 than 2000:1 (compare appendix Figures 3.1S 3A-F). This suggests that the dipeptide interferes with pyrrole-pyrrole cross-link formation. This interpretation is also consistent with the results of later experiments (see Chapter 4, section 4.2.4) testing the effect of dipeptide on the oxygen-
promoted dimerization and adduct formation reactions of pure pyrrole. Bispyrrole peak intensity was also higher when N-acetyl-glycyl-lysine methyl ester reaction time with iso[4]LGE$_2$ was 15 days versus 1 day.

Derivatives of the polypyrroles, i.e., bispyrrole and trispyrrole showed a sequence of ions corresponding to further addition of oxygen atoms (+16). Each polypyrrole parent ion is accompanied by a series of ions corresponding to the addition of one or more atoms of oxygen to the polypyrrole. The details are discussed below.

**Figure 3.1** MALDI-TOF MS analysis of the reaction mixture from N-acetyl-glycyl-lysine methyl ester and iso[4]LGE$_2$ (molar ratio = 200:1) (50 mM NEM-OAc aqueous solution with 4% methanol, v/v, pH 8.6) at 25 °C for 4 days.
The product mixture from reaction of iso[4]LGE\textsubscript{2} with N-acetyl-gly-lys-OMe was separated by reverse phase HPLC using an acetonitrile/water gradient and monitored by LC-ESI extracted ion channels (Figure 3.2). The major products corresponding to lactam (trace 3.2C), aminal cross-link (trace 3.2B) and bispyrrole (trace 3.2D) eluted in order of decreasing polarity at about 12, 30, and 36 minutes.

**Figure 3.2:** LC-ESI for the product mixture from the reaction of N-acetylglycyllysin methyl ester with iso[4]LGE\textsubscript{2} (molar ratio = 200:1 at 25 °C for 4 days) with selected ion recording for aminal cross-link, lactam-H\textsubscript{2}O and bispyrrole. A) TIC, B) aminal cross-link \(m/z\) 854 (+ H\textsuperscript{+}), C) lactam-H\textsubscript{2}O \(m/z\) 574.7 (+ H\textsuperscript{+}), D) bispyrrole \(m/z\) 1150.5 (+ H\textsuperscript{+}).

MALDI-TOF MS analysis of the fractions eluting at about 12, 30 and 36 minutes confirmed the presence of the lactam, aminal and bispyrrole cross-links. Thus, MALDI-TOF MS analysis of the 30 minute elution showed major ions at \(m/z\) 853.5, 835.5 and 817.5 corresponding to protonated aminal cross-link in addition to ions resulting from the loss of one and two molecules of water. MALDI-TOF MS analysis of the 36 minute
elution showed major ions at m/z 1149.7, 1131.7 and 1113.7 corresponding to protonated bispyrrole in addition to ions resulting from the loss of one and two molecules of water. The MALDI-TOF mass spectra of these principle products are shown in Figure 3.3. A series of ions are present in the mass spectrum of the isoLG-N-acetylglylys-OMe bispyrrole cross-link chromatographic fraction corresponding to the addition of one or more atoms of oxygen to the bispyrrole, and the relative amount of these products of further oxidation is greater after incubation for 15 days compared to 4 days (Figure 3.3 panels A and B, respectively). The peak intensity of further oxidized bispyrrole (products with addition of oxygen atoms) was higher compared with bispyrrole and its dehydrated forms after 15 days modification than after 4 days. The pattern of further addition of oxygen atoms (+16) was also observed for the trispyrrole products. Thus, pyrrole oligomerizes to form polypyrroles, and all of these products undergo further oxidation. Possible structures for products of oxidized bispyrrole (+16, +32, +48, +64) were shown in Scheme 3.7.

Scheme 3.7 Possible structures for products of oxidized bispyrrole (+16, +32, +48, +64).

Previously, singlet oxygen, a major intermediate in the photodynamic effect, generated by the combined action of light, a photosensitizing dye and oxygen, was believed to insert oxygen atoms into biological substrates. For example, singlet oxygen
reacts with double bonds giving epoxides. The pyridinium bisretinoid A2E, a major hydrophobic fluorophore of retinal pigment epithelium lipofuscin undergoes such addition of multiple atoms of oxygen. Thus, a series of oxidized derivatives is generated upon 10 min of blue light irradiation, each of which represents the addition of an oxygen atom at a carbon-carbon double bond. A2E self-generates singlet oxygen with the singlet oxygen in turn reacting with A2E to generate epoxides at carbon-carbon double bonds (Figure 3.4). Epoxidation occurs at all nine double bonds in the hydrocarbon side chains without modifying the pyridinium ring.

Figure 3.3  MALDI-TOF MS spectra: panel A (4 days reaction) and B (15 days reaction) for 36 min HPLC fractions, panel C (15 days reaction) for 30 min HPLC fraction, panel
D (15 days reaction) for 41 min HPLC fraction of products from the reaction of N-acetylglycyllysine methyl ester with iso[4]LGE₂ (molar ratio = 200:1 at 25 °C for 4 and 15 days).

Figure 3.4 Top: Structures of A2E (1), the bisoxirane (2) and nonaoxirane (3). Bottom: ESI mass spectrum of A2E after illumination with blue light (430 nm; 10 min) to yield a mixture of various oxygenated derivatives resulting in a series of ions that differ in m/z by 16 owing to the addition of oxygen atoms to A2E. The addition of oxygen,
presumably to form epoxides, occurred at carbon-carbon double bonds of A2E, measured by Micromass ESI-Q-TOF.\textsuperscript{38-39}

To study their fragmentation patterns, tandem MS/MS analyses of pyrrole, lactam, hydroxylactam and aminal cross-link ions were carried by Q-TOF mass spectrometry. ESI-MS/MS analysis of Zip-Tip purified reaction mixture from N-acetylglycyllysine methyl ester and iso[4]LGE\textsubscript{2} (molar ratio = 200:1 at 25 °C for 4 days) provided additional support for the structure postulated for the aminal cross-link. Previously, it was postulated that an initial iso[4]LGE\textsubscript{2} Schiff base adduct with the lysine residue of N-acetyl-gly-lys-OMe is an electrophilic intermediate that undergoes aminal cross-linking with another nucleophilic side chain residue of a second molecule of N-acetylglycyllysine methyl ester.\textsuperscript{5} The MS/MS spectrum of the parent ion from the putative aminal cross-link (m/z 853.5) shows peaks at m/z 853.5 and 835.5 corresponding to the [M+H]\textsuperscript{+} ion from iso[4]LGE\textsubscript{2}-(N-acetyl-gly-lys-OMe) aminal cross-link and its dehydrated form (Figure 3.5B). The MS/MS spectrum of m/z 835.5 exhibits two diagnostic daughter ions at m/z 558.4 and 436.3. The ion at m/z 558.4, which is isobaric with protonated dehydrated pyrrole, corresponds to loss of one molecule of N-acetyl-gly-lys-OMe (-259) and one molecule of H\textsubscript{2}O (-18). The ion at m/z 436.3 corresponds to loss of non-3-en-1-yne (C\textsubscript{9}H\textsubscript{14}) (-122). The proposed structure of each daughter ion is shown in Figure 3.5 and Scheme 3.8. See appendix Figure 3.2S and Scheme 3.1S for spectra and fragmentation structures of the pyrrole, lactam and hydroxylactam one-to-one adducts.
Figure 3.5 ESI-MSMS of molecular ions at A) m/z 835 (+ H+), and B) m/z 853 (+ H+) corresponding to an iso[4]LGE2-(N-acetyl-gly-lys-OMe) aminal cross-link and its dehydrated form.

Scheme 3.8 Possible fragmentation of an iso[4]LGE2-(N-acetyl-gly-lys-OMe) aminal cross-link at m/z 853.5 (M + H+).

MALDI-TOF MS analysis of the reaction product mixture from the reaction of fluorescently labeled iso[4]LGE₂ (iso[4]LGE₂-FluoTag) with N-acetylglyclyllysine methyl ester showed major ions at m/z 843, 859, 875 and 1120 corresponding to protonated pyrrole, lactam, hydroxylactam and aminal cross-link, respectively, in addition to ions resulting from the loss of one or two molecules of water from those adducts. Figure 3.6 shows a representative spectrum showing the presence of various adducts (see appendix Table 3.1S for molecular weight of adducts of N-acetyl-gly-lys-OMe with iso[4]LGE₂-FluoTag).

The reaction products were recorded (see appendix Figure 3.3S (1) for two different reactant molar ratios of N-acetyl-glycyl-lysine methyl ester: iso[4]LGE₂-FluoTag = 2000:1 and (2) for 200:1 measured after incubation for 1, 4, 15, 21 and 50 days). The initial concentration of N-acetyl-glycyl-lysine methyl ester was constant while two different ratios of iso[4]LGE₂-FluoTag were added. At various incubation times, identical aliquots of reaction mixture were purified by Ziptip and analyzed.

The 1:1 adducts, i.e., pyrrole, lactam and hydroxylactam and their dehydrated forms were generated very quickly (within one day reaction) in the reaction of iso[4]LGE₂-FluoTag with N-acetyl-glycyl-lysine methyl ester (see appendix Figure 3.3S 1A). For the aminal cross-link and its dehydrated forms, with longer incubation times, a higher percentage of aminal cross-link adducts versus 1:1 adducts was generated. Specifically, the aminal cross-link peak intensity was higher when N-acetyl-glycyl-lysine methyl ester reaction time with iso[4]LGE₂-FluoTag was 15 days than just 1 day (see appendix Figures 3.3S 2A-E).
MALDI-TOF MS analysis was performed on 100 fmol of Zip-Tip purified reaction mixture. Bispyrrole couldn’t be detected. Higher loading amount (200-500 fmol) was also tested, but no bispyrrole was detected either. Thus, whereas bispyrrole is a minor but significant product from the reaction of iso[4]LGE$_2$ with N-acetylglysyllysine methyl ester, it is not even a detectable product from the reaction of iso[4]LGE$_2$-FluoTag with this peptide. The difference between these adducts is isoLG ester versus carboxylate. One possibility is that the carboxylate interacts with a putative cation radical intermediate and alters its proclivity for combination with $\text{O}_2^-$ versus a second molecule of pyrrole.

![MALDI-TOF MS analysis of the product mixture from the reaction of N-acetylglysyllysine methyl ester with iso[4]LGE$_2$-FluoTag (molar ratio = 200:1) (50 mM NEM-OAc aqueous solution with 6% methanol, v/v, pH 8.6) at 25 °C for 15 days.](image)

**Figure 3.6** MALDI-TOF MS analysis of the product mixture from the reaction of N-acetylglysyllysine methyl ester with iso[4]LGE$_2$-FluoTag (molar ratio = 200:1) (50 mM NEM-OAc aqueous solution with 6% methanol, v/v, pH 8.6) at 25 °C for 15 days.
An investigation of the fragmentation patterns from tandem MS/MS analysis of aminal cross-link ions by Q-TOF mass spectrometry provided additional support for the structure postulated for this type of cross-link. The initial Schiff base of iso[4]LGE2-FluoTag with the lysine residue of N-acetylglycyllysine methyl ester is an electrophilic intermediate that undergoes aminal cross-linking with another nucleophilic side chain residue of a second molecule of N-acetyl-gly-lys-OMe. The MS/MS spectrum of the parent ion from the putative aminal cross-link (m/z 1120.6) shows peaks at m/z 1120.6 and 1102.5 corresponding to the [M+H]^+ ion from a iso[4]LGE2-FluoTag-(N-acetyl-gly-lys-OMe) aminal cross-link and its dehydrated form (Figure 3.7B). The MS/MS spectrum of m/z 1102.5 exhibits two diagnostic daughter ions at m/z 825.5 and 703.4. The ion at m/z 825.5, which is isobaric with protonated dehydrated pyrrole, corresponds to loss of one molecule of N-acetyl-gly-lys-OMe (-259) and one molecule of H_2O (-18). The ion at m/z 703.4 corresponds to loss of non-3-en-1-yne (C_9H_14) (-122). The proposed structure of each daughter ion is shown in Figure 3.7 and Scheme 3.9. See appendix Figure 3.4S and Scheme 3.2S for spectra and fragmentation structures of one-to-one adducts: pyrrole, lactam and hydroxylactam.
Figure 3.7 ESI-MSMS of molecular ions at A) m/z 1102.5 and B) m/z 1120.6 corresponding to a iso[4]LGE2-FluoTag-(N-acetyl-gly-lys-OMe) aminal cross-link adduct and its dehydrated form.

Scheme 3.9 Possible fragmentation of iso[4]LGE2-FluoTag aminal cross-link with N-acetyl-gly-lys-OMe at m/z 1120.6 (M + H\(^+\)).

MALDI-TOF MS analysis of the reaction product mixture from iso[4]LGE$_2$ with $\beta$-amyloid(11-17) peptide “EVHHQKL” showed major ions at m/z 1206, 1222, 1238, 2114 and 2410 corresponding to protonated pyrrole, lactam, hydroxylactam, aminal cross-link and bispyrrole, respectively, in addition to ions resulting from the loss of one or two molecules of water at m/z 1188, 1204, 1220, 1202, 2096, 2078, 2392, 2374. Figure 3.8 shows a representative spectrum that shows ions corresponding to various adducts (see appendix Table 3.1S for the molecular weights of various iso[4]LGE$_2$-$\beta$-amyloid (EVHHQKL) peptide adducts).

![Figure 3.8](image)

**Figure 3.8** MALDI-TOF MS analysis of the product mixture from the reaction of $\beta$-amyloid(11-17) peptide “EVHHQKL” with iso[4]LGE$_2$ (molar ratio = 200:1) (50 mM NEM-OAc aqueous solution with 4% methanol, v/v, pH 8.6) 25 °C for 7 days.
Reaction products were recorded for two reactant molar ratios (see appendix Figure 3.5S (1) for β-amyloid (EVHHQKL) peptide: iso[4]LGE₂ = 2000:1 and (2) 200:1 measured after incubation for 1, 4, 7, 15, 21 and 50 days). The initial concentration of β-amyloid (EVHHQKL) peptide was the same for both reactions while various ratios of iso[4]LGE₂ were added. At various incubation times, identical aliquots of the reaction mixture were purified by Ziptip and then analyzed.

The 1:1 adducts, i.e., pyrrole, lactam and hydroxylactam and their dehydrated forms were generated very quickly (within one day) in the reaction of iso[4]LGE₂ with β-amyloid (EVHHQKL) peptide (see appendix Figure 3.5S 1A). Cross-link adducts included aminals, bispyrroles, trispyrroles and their dehydrated forms. With higher iso[4]LGE₂ ratio and longer incubation times, a higher percentage (comparison of cross-link peak intensity with one-to-one adducts) of bispyrrole adducts generated. Specificity, bispyrrole peak intensity was higher when the molar ratio of β-amyloid (EVHHQKL) peptide to iso[4]LGE₂ was 200:1 than 2000:1 (see appendix Figure 3.5S 2A-F). Bispyrrole peak intensity was also higher (5 % more) when β-amyloid (EVHHQKL) peptide reaction time with iso[4]LGE₂ was 15 days than just 1 day. A similar trend was observed with the dipeptide. It agrees with the conclusion the dipeptide or “EVHHQKL” peptide interferes with bispyrrole formation. Presumably for the same reason, i.e. that these peptides compete with a second molecule of pyrrole for a putative cation radical intermediate.

More cross-link product versus bispyrrole formed during dipeptide reaction with iso[4]LGE₂. In contrast, more bispyrrole versus aminal cross-link product formed during the reaction of “EVHHQKL” with iso[4]LGE₂. It is tempting to speculate that the
“EVHHQKL” peptide-pyrrole modification forms aggregates that favor the dimerization to produce bispyrrole since β-amyloid tends to spontaneously oligomerize. Future studies might seek evidence for noncovalent aggregation of “EVHHQKL” peptide-pyrroles under oxygen free conditions where oxidative transformations are precluded.

Polypyrroles, i.e., bispyrrole and trispyrrole showed a sequence of ions corresponding to the further addition of oxygen atoms (+16). A series of peaks corresponding ions for polypyrroles that had undergone the addition of one or more atoms of were observed. The details will be discussed below.

The product mixture from reaction of iso[4]LGE2 with β-amyloid(11-17) peptide “EVHHQKL” was separated by reverse phase HPLC using an acetonitrile/water gradient and monitored by LC-ESI extracted ion channels (Figure 3.9). The major products corresponding to aminal cross-link (trace 3.2C) and bispyrrole (trace 3.2B) eluted in order of decreasing polarity at about 22 and 32 minutes.

Figure 3.9 LC-ESI for the reaction mixture from β-amyloid(11-17) peptide and iso[4]LGE2 (molar ratio = 200:1 at 25 °C for 7 days) with selected ion recording for
bispyrrole and aminal cross-links. A) TIC, B) bispyrrole m/z 804.6 (+3), m/z 603.7 (+4), C) aminal cross-link m/z 1058.2 (+2).

MALDI-TOF MS analysis of the fractions eluted at 22 and 32 minutes confirmed the presence of the aminal and bispyrrole cross-links. MALDI-TOF MS analysis of the aminal fraction showed major ions at m/z 2114.2, 2096.2 and 2078.2 corresponding to protonated aminal cross-link in addition to ions resulting from the loss of one and two molecules of water. MALDI-TOF MS analysis of the bispyrrole fraction showed major ions at m/z 2410.3, 2392.3 and 2374.3 corresponding to protonated bispyrrole in addition to ions resulting from the loss of one and two molecules of water. The mass spectra of the principle products are shown in Figure 3.10. A series of peaks are present in the mass spectrum of the isoLG-β-amyloid(11-17) peptide chromatographic fraction corresponding to the addition of one or more atoms of oxygen to the bispyrrole, and the relative amount of these products of further oxidation as indicated by the relative peak intensity of further oxidized bispyrrole (products with addition of oxygen atoms) compared with bispyrrole and its dehydrated forms after 15 days modification than after 7 days (Figure 3.10 panels A and B, respectively).

A series of products of further addition of oxygen (multiple +16 m/z increments) pattern was also observed for the trispyrrole adduct. Thus, pyrrole oligomerizes form polypyrroles, and all of these products undergo further oxidation. Similar behavior was observed for the iso[4]LGE2-(N-acetyl-gly-lys-OMe) bispyrrole cross-link and discussed in section “(i) Iso[4]LGE2 modification of N-acetyl-gly-lys methyl ester”.
Figure 3.10  MALDI-TOF MS spectra: panel A (7 days reaction) and B (15 days reaction) for 32 min HPLC fractions, panel C and D (7 days reaction) for 22 min, 15 min HPLC fraction of products from reaction of the $\beta$-amyloid(11-17) peptide “EVHHQKL” with iso[4]LGE$_2$ reaction (molar ratio = 200:1 at 25 °C).


MALDI-TOF MS analysis of the reaction product mixture from iso[4]LGE$_2$-FluoTag with the $\beta$-amyloid (EVHHQKL) peptide showed major ions at m/z 1473, 1489 and 1505 corresponding to protonated pyrrole, lactam and hydroxylactam in addition to ions
resulting from the loss of one or two molecules of water from those adducts. Figure 3.11 is a representative spectrum that shows ions corresponding to various adducts (see appendix Table 3.1S for molecular weight of adducts of β-amyloid (EVHHQKL) peptide with iso[4]LGE2-FluoTag).

Reaction products were recorded for two reactant molar ratios (see appendix Figure 3.6S (1) for β-amyloid (EVHHQKL) peptide: iso[4]LGE2-FluoTag = 2000:1 and (2) 200:1 measured after incubation for 1, 4, 15, 21 and 50 days. The initial concentration of β-amyloid (EVHHQKL) peptide was the same for both reactions while various ratios of iso[4]LGE2-FluoTag were added. At various incubation times, identical aliquots of reaction mixture were purified by Ziptip and analyzed.

The 1:1 adducts, i.e., pyrrole, lactam and hydroxylactam and their dehydrated forms were generated very quickly (within one day) in the reaction of iso[4]LGE2-FluoTag with β-amyloid (EVHHQKL) peptide (appendix Figure 3.6S 1A). MALDI-TOF analysis was performed on 100 fmol of Zip-Tip purified reaction mixture. Compared with 1:1 adducts, aminal cross-link and bispyrrole were barely be detected.
Figure 3.11 MALDI-TOF MS analysis of the product mixture from the reaction β-amyloid (EVHHQKL) peptide with iso[4]LGE2-FluoTag (molar ratio = 200:1) (50 mM NEM·OAc aqueous solution with 6% methanol, v/v, pH 8.6) at 25 °C for 15 days.
3.3 Conclusions

IsoLG modification of proteins is associated with loss-of-function, cross-linking and aggregation. N-Acetyl-glycyl-lysine methyl ester (N-acetyl-Gly-Lys-OMe) and β-amyloid(11-17) peptide “EVHHQKL” were used as models for characterizing the cross-linking of protein lysyl residues resulting from adduction of iso[4]LGE2. Aminal, bispyrrole and trispyrrole cross-links of two model peptides, were identified and fully characterized by mass spectrometry, and a large variety of derivatives of the bispyrrole cross-link containing one or more additional atoms of oxygen were discovered. More aminal cross-link product versus bispyrrole is formed during dipeptide reaction with iso[4]LGE2, and bispyrrole is almost undetectable in the reaction of a fluorescently labeled iso[4]LGE2 with dipeptide. In contrast, more bispyrrole versus aminal cross-link product formed during the reaction of “EVHHQKL” with iso[4]LGE2. It is tempting to speculate that the “EVHHQKL” peptide-pyrrole modification forms aggregates that favor the dimerization to produce bispyrrole since β-amyloid tends to spontaneously oligomerize. Future studies might seek evidence for noncovalent aggregation of “EVHHQKL” peptide-pyrroles under oxygen free conditions where oxidative transformations are precluded.

With higher iso[4]LGE2 ratio:peptide, a higher percentage of bispyrrole versus 1:1 adducts was generated from both N-acetyl-glycyl-lysine methyl ester and β-amyloid (EVHHQKL) peptide. This agrees with the conclusion that these peptides interfere with bispyrrole formation. Presumably for the same reason, i.e. that these peptides compete with a second molecule of pyrrole for a cation radical intermediate that will be discussed in the following chapter.
3.4 Experimental Procedures

3.4.1 General methods. All chemicals used were high purity analytical grade. The following commercially available materials were used as received: N-acetylglycyllysine methyl ester was from Bachem (Torrance, CA), β-Amyloid (11-17) peptide “EVHHQKL” was from Peptide 2.0 Inc (Chantilly, VA), N-ethylmorpholine was from Aldrich (Milwaukee, WI), and all other chemicals were obtained from Fisher Scientific Co (Chicago, IL). Iso[4]LGE2 was prepared by Dr. Jim Laird and iso[4]LGE2-FluoTag was prepared as described in Chapter 2. Chromatography was performed with ACS grade solvents.

3.4.2 Peptide modification with iso[4]LGE2 or iso[4]LGE2-FluoTag. Reactant stocks were prepared as follows: 25 mM N-acetylglycyllysine methyl ester and β-amyloid (11-17) peptide “EVHHQKL” in NEM∙OAc (50 mM, pH 8.6), 1 mg/ml iso[4]LGE2 (without or with fluorescence tag) in MeOH.

4.4 µl of iso[4]LGE2 (12.5 nmol, 4.4 µg) or 7.7 µl of iso[4]LGE2-FluoTag (12.5 nmol, 7.7 µg) was added to N-acetylglycyllysine methyl ester (2.5 µmol, 647.5 µg) in 50 mM pH 8.6 NEM∙OAc (100 µl) (molar ratio of iso4: dipeptide = 1:200) and reacted under air on shaker (IKA MTS 2/4 digital microtiter shaker, 500 rpm) at 25 °C. At various times (1, 4, 7, 15, 21 and 50 days), an aliquot (~1.2 nmol, 10 µl) of reaction mixture was quickly dried using a high-speed vacuum evaporator and then stored at -20 °C until analysis. The aliquot was analyzed with a MALDI-TOF, triple quadrupole or Q-TOF mass spectrometer. Two different molar ratios (iso4: peptide = 1:2000 and 1:200) were studied. β-amyloid (11-17) peptide “EVHHQKL” was treated the same way.
3.4.3 Analysis of adducts using MALDI-TOF. Initial diagnostic analysis of the samples was performed by MALDI-TOF mass spectrometry on an AB Sciex4800 Plus MALDI TOF/TOF™ Analyzer equipped with a UV laser (355 nm) and reflector mode with a matrix of α-cyano-4-hydroxycinnamic acid (CHCA, 10 mg/ml in acetonitrile/water/3% formic acid, 5:4:1, v/v/v). A peptide mass standards kit for calibration of AB Sciex MALDI-TOFTM instruments was used to calibrate the plate. Each of the reaction aliquots was resuspended in 10% aqueous acetonitrile solution and vortexed to mix well before C18 Ziptip purification (Millipore, ZipTip with 0.6 µL C18 resin). To wet the C18 Ziptip pipettor plunger was depressed to a dead stop using the maximum volume setting of 20 µl. Acetonitrile (wetting solution) was aspirated into tip then dispensed to waste and the process repeated twice. The tip was equilibrated for binding by washing three times with the 0.1% formic acid/water (equilibration solution). Samples were bound to ZipTip by fully depressing the pipettor plunger to a dead stop then aspirating and dispense the sample 3 to 7 cycles. The tip was then washed with at least 3 cycles of 0.1% formic acid/water (wash solution) dispensing to waste using. The samples were eluted with 5 µl of 80% acetonitrile/0.1% formic acid (elution solution) into a clean vial to give a final concentration of 100 fmol/µl by aspirating and dispensing eluant through the ZipTip at least 3 times without introducing air. Two microliters of the sample was then mixed with 1 µl of matrix, and then 1.5 µl were applied to the plate spot and allowed to dry. The data was processed with Data Explorer 4.0 (AB Sciex) software.
Table 3.1: Optimized parameters for MALDI-TOF mass spectrometer.

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3.4.4 LC-MS (triple quadrupole) analysis. Iso[4]LGE2-modified β-amyloid(11-17) peptide was separated by HPLC with a 300 µm x 250 mm i.d. C18 5 µm column (DIONEX Acclaim™). Chromatography was carried out with linear elution gradient (elute A, 0.1% TFA/H₂O; elute B, 0.1% TFA/ACN) at a flow rate of 20 µl/min. The samples were loaded onto the column with 2% B for 10 min. The gradient was as follows: 2-12% B over 5 min, to 60 % over 48 min, to 98% B over 2 min, and hold for 10 min. Iso[4]LGE2 modified acetyl-gly-lys-O-methyl ester was seperated by HPLC with a 2.1 x 100 mm i.d. C18 1.8 µm column (Waters Acquity UPLC HSS). Chromatography was carried out with linear elution gradient (elute A, 0.1% FA/H₂O; elute B, 0.1% FA/ACN) at a flow rate of 70 µl/min. Sample was loaded onto the column with 2% B for 10 min. The gradient was as follows: 2-10% B over 2 min, to 98 % within 25 min, and hold for 18 min. The effluent was monitored by ESI-MS/MS with a API-3000 triple quadrupole electrospray mass spectrometer (Applied Biosystems Inc.) to determine the retention time of different oxidized adducts. The instrument was operated in the positive mode and high-pressure nitrogen was used as source gas and scanning from m/z 220-2000 using the parameters listed in table 3.2.
Table 3.2: Optimized parameters for triple quadrupole mass spectrometer.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Data</th>
</tr>
</thead>
<tbody>
<tr>
<td>Declustering Potential (DP)</td>
<td>30</td>
</tr>
<tr>
<td>Focus Potential (FP)</td>
<td>250</td>
</tr>
<tr>
<td>Entrance Potential (EP)</td>
<td>10</td>
</tr>
<tr>
<td>Nebulizer Gas (NEB)</td>
<td>10</td>
</tr>
<tr>
<td>Curtain Gas (CUR)</td>
<td>8</td>
</tr>
<tr>
<td>Ion Spray Voltage (IS)</td>
<td>4000</td>
</tr>
<tr>
<td>Temperature (TEM)</td>
<td>200</td>
</tr>
</tbody>
</table>

3.4.5 LC- MS/MS (Q-TOF) analysis. The samples were analyzed by a quadrupole-time of flight (Q-TOF-2) mass spectrometer (Micromass) equipped with MassLynx™ acquisition and processing software. 2 pmol iso[4]LGE₂ or iso[4]LGE₂-FluoTag modified N-acetylglucyllysine methyl ester (molar ratio of N-acetylglucyllysine methyl ester: iso[4]LGE₂ = 200:1, 25 °C for 4 days), prepared as described in Section 3.4.2, was analyzed by Q-TOF. For sample preparation, 62.5 pmol of reaction mixture from the reaction of iso[4]LGE₂ with N-acetylglucyllysine methyl ester was resuspended in 250 µl 10% acetonitrile. An aliquot (8 µl, 2 pmol) of this solution was purified with a Zip-Tip as described in section 3.4.3 and eluted in 10 µl of 80% acetonitrile/0.1% formic acid. This purified eluate was quickly dried using a high-speed vacuum evaporator and then dissolved in 15 µl of 5% acetonitrile/2% formic acid and injected into the Q-TOF. The samples were infused using a 100 µl Hamilton syringe onto a capillary column (PicoFrit™ 0.075 x 70 mm, 15 µ tip ID; New Objective Inc., Woburn, MA) at 0.5 µl/min using a syringe pump (Harvard apparatus, Pump 11) and ionized immediately using an electrospayer designed in-house. The mass spectrometer was operated in standard MS and MS/MS switching mode. The MS data was collected for the mass range of 50-1500 m/z and MS/MS data was analysed with Micromass software (MassLynx) Version 3.5.
The machine was calibrated using a solution of 2 fmol of [Glu1]-Fibrinopeptide B (Sigma) in 50% aqueous acetonitrile (+0.1% formic acid) infused using glass micropipette with 5 µ tip. The intensity of the peak from the MS/MS spectrum at 785.4 m/z was used as a reference for calibration. The final mass measurement accuracy of ≤ 10 ppm was achieved before the use of the machine.
3.5 References


CHAPTER 4

GENERATION OF ISOLG-PYRROLE BY THE REACTION OF ISOLG WITH N-ACETYL-GLY-LYS-OMe IN THE ABSENCE OF O$_2$ AND THE OXIDATION OF ISOLG-PYRROLE TO FORM PYRROLE-PYRROLE CROSS-LINKS AND AN ADDUCT WITH N-ACETYLCYSTEINE
4.1 Background

4.1.1 Purpose

Presumably, pyrrole-pyrrole cross-links are produced by oxidative coupling of LG/isoLG-pyrroles. To enable examination of the mechanism of isoLG-pyrrole-pyrrole cross-linking, it is desirable to synthesize pure isoLG-pyrroles. In this chapter, the preparation of pure isoLG-pyrroles and a study of their chemistry will be described. Evidence is presented that oxidation and cross-linking of isoLG-derived pyrroles is autocatalytic the initiation of which can be catalyzed by tetramethylpyrrolidine-N-oxide, a stable free radical.

4.1.2 LGs and isoLGs modification with proteins

Previously, we discovered that the prostaglandin endoperoxide intermediate of the cyclooxygenase pathway, and its stereo and structural isomers (isoprostane endoperoxides), which are generated through free radical-induced oxidation of arachidonyl phospholipids, undergo rearrangements that produce γ-ketoaldehydes, levuglandins (LGs) and isoLGs, respectively. Adduction of LGs/isoLGs to proteins initially produces pyrroles through hemiacetals within seconds. The pyrroles are readily oxidized to lactams and hydroxylactams that are the major lysyl derivatives present in proteins in vivo. LG/isoLG adduction also generates protein-protein cross-links within minutes.1 Pyrrole-pyrrole cross-link and aminal cross-link structures shown in Scheme 4.1 have been postulated, but no direct molecular level evidence for the cross-link structure(s) has been reported.
Scheme 4.1 LG/isoLG modification of proteins
4.2 Results and discussion


As reported in chapter 3, the formation of pyrrole-pyrrole cross-links was detected upon adduction of isoLG with N-acetyl-gly-lys methyl ester. Pyrrole-pyrrole dimer formation is an oxidative process. Therefore, cross-linking is not expected to occur under anerobic conditions. To test this hypothesis, the treatment of acetyl-gly-lys methyl ester was conducted in an oxygen-free aqueous solution containing sodium dithionite, an oxygen scavenger. As predicted, cross-linking was completely prevented. However, immediately after removing Na₂S₂O₄ (as describe below), the iso[4]LGE₂-pyrrole (Scheme 4.2) derivative underwent oxidation and dimerization that was extensive after the solution was aerated with oxygen overnight (Figure 4.1). It should be noted that this preparation contained a mixture of iso[4]LGE₂-pyrrole unreacted iso[4]LGE₂ and dipeptide as well as byproducts derived from them, possibly including peracid generated through oxidation of the aldehyde functional group in iso[4]LGE₂.

Incubation of N-acetyl-gly-lys methyl ester with iso[4]LGE₂ under an atmosphere of argon, including bubbling argon gas before and during reaction, produced iso[4]LGE₂-pyrrole (4.3) as well as products of further oxidation, i.e., lactam, hydroxylactam. Sodium dithionite (Na₂S₂O₄) is an oxygen scavenger that can maintain the oxygen concentration at extremely low levels.² Only iso[4]LGE₂-pyrrole (4.3) was produced if the adduction reaction was carried out in water solution in the presence sodium dithionite under an atmosphere of argon at 25 °C. An excess of acetyl-gly-lys methyl ester (20 equivalents) was incubated with iso[4]LGE₂ to favor complete consumption of the γ-ketoaldehyde. Since sodium dithionite prevented oxidative transformations of the pyrrole when exposed
to air, it was essential to completely remove dithionite from the sample in order to study further oxidative reactions. The reaction solution was concentrated under high vacuum to remove water and methanol was added to dissolve the organic product. Then the mixture was loaded onto a column of silica gel packed with a slurry in EtOAc and eluted with methanol to remove most of sodium dithionite. After concentration under reduced pressure, reagent grade chloroform that had been sparged with argon was added to dissolve iso[4]LGE2-pyrrole and the solution was filtered to remove sodium dithionite and any silical gel that had dissolved in the methanol. The crude iso[4]LGE2-pyrrole solution was concentrated by evaporation with argon gas and re-suspended in 10% acetonitrile/water for further use.


Before and immediately after removing the sodium dithionite, no oxidation products were detected by MALDI initially (Figures 4.1A and B). Upon exposure to air, within one hour, it formed various oxidized derivatives, e.g., lactam, hydroxylactam, and bispyrrole (Figure 4.1C). After bubbling O₂ overnight, more oxidized products had been generated, including aminal cross-link including bispyrrole as the major product (Figure 4.1D). Because this sample of iso[4]LGE2-pyrrole contained unreacted acetyl-gly-lys methyl ester, the formation of peptide-peptide aminal cross-link presumably was a consequence of the presence of excess N-acetyl-gly-lys methyl ester in the sample, perhaps involving a reaction between the pyrrole and the nucleophilic side chain residue of a second molecule of dipeptide.
Figure 4.1 MALDI-TOF MS analysis of iso[4]LGE₂-pyrrole and oxidation products generated upon short term exposure of the reaction mixture from iso[4]LGE₂ with N-acetylglycyllysine methyl ester in the presence of dithionite to air or longer term aeration with oxygen before or after removal of dithionite.

4.2.2 The oxidation of HPLC-purified iso[4]LGE$_2$-pyrrole with air exhibits an induction period.

A sample of iso[4]LGE$_2$-pyrrole contaminated with excess N-acetyl-gly-lys methyl ester and unreacted iso[4]LGE$_2$ was purified by HPLC using an acetonitrile/water gradient using LC-ESI to detect the components of interest by monitoring the appropriate molecular weights in the extracted ion channels (Figure 4.2). The fraction eluting at 12 min contained pure iso[4]LGE$_2$-pyrrole. The structural integrity of HPLC-purified iso[4]LGE$_2$-pyrrole was confirmed by MALDI-TOF MS (See appendix Figure 4.1S).

![Figure 4.2](image)

**Figure 4.2** LC-ESI for the purification of iso[4]LGE$_2$-pyrrole with selected ion recording for iso[4]LGE$_2$-pyrrole. A) TIC, B) LC monitoring and MS analysis for iso[4]LGE$_2$-pyrrole-H$_2$O ($m/z$ 558 (+ H$^+$)), C) LC monitoring for N-acetyl-gly-lys methyl ester ($m/z$ 260 (+ H$^+$)).

MALDI and triple quadrupole mass spectrometry was used to confirm that purified iso[4]LGE$_2$-pyrrole was not contaminated with N-acetyl-gly-lys methyl ester or...
iso[4]LGE2. The background of matrix for MALDI-TOF and solvent used in triple quadrupole mass spectrometry was determined to not exhibit any peaks corresponding to N-acetyl-gly-lys methyl ester or iso[4]LGE2 in the matrix or solvent (no m/z around 260 (+1 for N-acetyl-gly-lys methyl ester) or 353 (+1 for iso[4]LGE2)). Peaks not corresponding to the matrix or solvent peaks could be detected upon analysis by MALDI or triple quadrupole mass spectrometry of N-acetyl-gly-lys methyl ester or iso[4]LGE2 (see appendix Figure 4.2S).

The reaction time course (Figure 4.3) for incubation of pure iso[4]LGE2-pyrrole under air at 25 °C in aqueous acetonitrile solution (10% acetonitrile/H2O) was monitored for eight days (see appendix Figure 4.3S for details). MALDI-TOF mass spectra of pure iso[4]LGE2-pyrrole and the reaction product mixture after exposure to air for eight days are shown in Figure 4.4. Compared to iso[4]LGE2-pyrrole with un-reacted N-acetyl-gly-lys methyl ester and iso[4]LGE2, pure iso[4]LGE2-pyrrole was much more stable under air. During the first two days, almost no oxidized product was formed. At longer reaction times, 1:1 adducts, i.e., lactam, hydroxylactam and bispyrrole became prominent. But, as expected, no peptide-peptide aminal cross-link formed since excess N-acetyl-gly-lys methyl ester and iso[4]LGE2 were not present.

The pure pyrrole obtained by preparative HPLC (Figure 4.2: LC-ESI for the purification of iso[4]LGE2-pyrrole) was no longer prone toward oxidation or dimerization when exposed to oxygen. The product evolution time course for the reaction of this HPLC-purified iso[4]LGE2-pyrrole with oxygen exhibited an induction period followed by rapid increase in the generation of all products (Figure 4.3).
**Figure 4.3** Time course of the oxidative reactions of pure iso[4]LGE$_2$-pyrrole under air at 25 °C for 8 days. As shown in appendix Figure 4.3S, the observed peak intensity for pyrrole-H$_2$O is 100 (for each time point). Peak intensity of bispyrrole-H$_2$O, lactam-H$_2$O and hydroxylactam-2H$_2$O was zero for the first two days, and then gradually increased. The total intensity is the sum of pyrrole-H$_2$O, bispyrrole-H$_2$O, lactam-H$_2$O and hydroxylactam-2H$_2$O. The peak intensity of each product is divided by the total intensity to provide the percent of total shown in the figure.
4.2.3 Reaction of pure iso[4]LGE$_2$-pyrrole with N$^\alpha$-acetylcysteine.

The possible role of nucleophilic thiols and amines in pyrrole-mediated cross-linking of proteins was investigated in a model system. To test the possibility that nucleophilic thiols can participate in isoLG-derived pyrrole-mediated cross-linking, pure iso[4]LGE$_2$-pyrrole was incubated in the presence of N-acetylcysteine under air. N-acetylcysteine (4.4) was added to pure iso[4]LGE$_2$-pyrrole (4.3) and incubated under air at 25 °C for several days (Scheme 4.3).

Representative MALDI-TOF spectra of the reaction mixture are shown in Figure 4.5. In the presence of N-acetylcysteine, incubation of iso[4]LGE2-pyrrole at neutral pH under air showed no pyrrole-to-pyrrole cross-linking. Thus, the thiol-containing compound inhibited the cross-linking of iso[4]LGE2-pyrrole (Figure 4.6). Instead a series of new ions was produced. They corresponded to pyrrole cysteine adducts and a series derivatives produced from them by the addition of one or more atoms of oxygen (Figure 4.7). The amount of these products increased with longer incubation. A thiol-pyrrole conjugate was detected by MALDI-TOF MS that presumably incorporates a bond between the pyrrole ring at C-2 to the sulfur atom of the N-acetylcysteine. Thus, thiols inhibit the pyrrole dimerization by intercepting pyrrole cation radicals generated by electron transfer from the pyrrole to oxygen (Scheme 4.4).
Figure 4.7 MALDI-TOF MS of pure iso[4]LGE₂-pyrrole reaction with N-acetylcysteine under air at 25 °C for 8 days. A series of peaks that differ in m/z by 16 show the addition of oxygen atoms.
Scheme 4.4 Possible electron transfer mechanism for pure iso[4]LGE₂-pyrrole reaction with N-acetylcysteine.


Because the oxidative transformations of iso[4]LGE₂-pyrrole (from which excess dipeptide and isoLG had been removed by HPLC) exhibited a reaction time course consistent with an autocatalytic mechanism, a search was initiated to discover possible initiators. Pure iso[4]LGE₂-pyrrole was incubated under air in the presence of various
potential initiators. The mass spectra of the resulting reaction product mixtures are presented in the appendix.

\((i)\) Stability of iso[4]LGE2-pyrrole in air in the presence of Nε-acetyl-L-lysine, ethanolamine, L-lysine or β-amyloid peptide

Known components of the reaction mixture for the synthesis of isoLG-pyrroles were tested first as well as analogues including Nε-acetyl-L-lysine, ethanolamine, L-lysine and β-amyloid peptide. Pure iso[4]LGE2-pyrrole was comparably stable upon exposure to these compounds without decomposing or forming oxidized adduct. Thus, Nε-acetyl-L-lysine was added to pure iso[4]LGE2-pyrrole under air. After 2 days incubation, no reaction was detected either with one equivalent of Ac-lysine or with a large excess relative to pyrrole. Also the results were no different at pH=3 or 7. A representative mass spectrum of the reaction product mixture is shown in appendix Figure 4.4S. Similarly, after incubation for 2 days in the presence of 10 equivalents of L-lysine, pure iso[4]LGE2-pyrrole underwent no reaction. Additional incubation of iso[4]LGE2-pyrrole resulted in gradual consumption of the pyrrole, but no identifiable products were detected (see appendix Figure 4.5S). Similarly, no reaction was detected after 2 days incubation of pure iso[4]LGE2-pyrrole in the presence of 10 equivalents of β-amyloid peptide (see appendix Figure 4.6S).

Incubation in the presence of ethanolamine resulted in decomposition, especially with a large molar excess. Thus, a solution of 100 mM ethanolamine in sodium phosphate buffer was adjusted pH to 7. Incubation of iso[4]LGE2-pyrrole under air in the presence of one equivalent of ethanolamine resulted in gradual consumption of the pyrrole, but some remained un-decomposed after 2 days (Figure 4.7S left). However, in the presence
of 10 equivalents of ethanolamine, the pyrrole decomposed completely within 2 h (Figure 4.7S right).


Pure iso[4]LGE₂-pyrrole was incubated under air in the presence of N-acetyl-gly-lys methyl ester. Various conditions (N-acetyl-gly-lys methyl ester in H₂O, pH 7; N-acetyl-gly-lys methyl ester in phosphate buffer, pH 8.6; and N-acetyl-gly-lys methyl ester acetate salt in H₂O, pH 5.8; molar ratio from 0.1 to 10 equivalents to iso[4]LGE₂-pyrrole) were tested. The results were quite similar under all conditions. The oxidized pyrrole product dehydrated hydroxylactam formed within 1 h and percentage increased with continued incubation. But no aminal cross-link or bispyrrole was detected (see appendix Figure 4.8S). These results contrasted with the lack of reaction observed upon incubation of the pyrrole in the presence of Nε-acetyl-L-lysine, ethanolamine, L-lysine or β-amyloid peptide. It was considered that commercial N-acetyl-gly-lys methyl ester may contain some impurities that account for this discrepancy. To test this hypothesis, the commercial sample of N-acetyl-gly-lys methyl ester was purified by HPLC using an acetonitrile/water gradient. A fraction containing pure N-acetyl-gly-lys methyl ester was isolated and confirmed with MALDI-TOF MS. Purified iso[4]LGE₂-pyrrole was then incubated under air at 25 °C for 6 days in the presence of HPLC-purified N-acetyl-gly-lys methyl ester (Figure 4.9S). After incubation for several days, no oxidized products formed.

(iii) The effects of vitamin C, vitamin E, H atom donor ((R)-(−)-2-(2,5-dihydrophenyl)glycine), free radical initiator (2,2'-azobis(2-methylpropionamide)
dihydrochloride), UV irradiation, rose bengal, methylene blue, Co$_2$(CO)$_8$, H$_2$O$_2$/K$_2$CO$_3$, dimethyldioxirane, microwave irradiation, di-tert-butyl peroxide, riboflavin (VB2), K$_3$Fe(CN)$_6$, FeCl$_3$, K$_2$S$_2$O$_8$ (persulfate) on the air stability of pure iso[4]LGE$_2$-pyrrole

The purified isoLG-pyrrole is unchanged after incubation in aqueous solution under air for 2 days. In contrast, about half of the crude, unpurified pyrrole was transformed into lactam, hydroxylactam, and bispyrrole under these same conditions. Furthermore, the oxidative transformations of pure pyrrole exhibited a reaction time course suggestive of autocatalysis (Figure 4.1 above). Therefore, various free radical initiators, H atom donors and potential initiators of pyrrole oxidation were tested for their ability to trigger the oxidation of purified iso[4]LGE$_2$-pyrrole by air. In the presence of large amounts (0.1 equivalents) of vitamin C, vitamin E and 2,2'-azobis(2-methylpropionamidine)dihydrochloride, the purified pyrrole was completely consumed but no products could be detected in the mass range of m/z 400-2000 after incubation overnight. In the presence of smaller amounts (0.01 equivalents) of these additives, some pyrrole (about 20%) remained un-reacted, but no products could be detected in the mass range of m/z 400-2000 after incubation overnight (appendix Table 4.1S). Traces of metal ions are especially likely to be present in aqueous buffers. Therefore, catalysis by traces of redox active metal ions was investigated to test their ability to trigger the oxidation of purified iso[4]LGE$_2$-pyrrole by air. Fe(III) is known to promote polymerization of pyrroles through single electron transfer (SET) that generates a pyrrole radical cation. Reoxidation of the resulting Fe(II) by oxygen allows Fe(III) to function as a catalyst.$^{5-7}$ Similar reaction was performed by adding catalytic amount of metallic salt free radical initiators
to iso[4]LGE₂-pyrrole. All of these potential catalysts caused rapid consumption of the pyrrole but no traces of lactam, hydroxylactam, and bispyrrole were detected under the reaction tested. Reaction details were summarized in appendix Table 4.1S (e).

To test the hypothesis that the product distribution from oxidative transformations of LG/isoLG-derived pyrrole can be influenced by a hydrogen atom donor ((R)-(−)-2-(2,5-dihydrophenyl)glycine), the pyrrole was incubated for 2 h in the presence of 5 equivalents of this hydrogen atom donor. The pyrrole was completely consumed but no traces of lactam, hydroxylactam or bispyrrole were detected. With 1 equivalent, the pyrrole was partially consumed in 2 h, and completely consumed after incubation over night, but no traces of lactam, hydroxylactam or bispyrrole were detected. Incubation of the pyrrole for 2 days in the presence of 0.2 equivalents of this hydrogen atom donor resulted in no apparent reaction. The pyrrole was stable and no traces of lactam, hydroxylactam or bispyrrole were detected.

4.2.5 Autoxidation of iso[4]LGE₂-pyrrole promoted by APS/TEMED, TEMPO and TMAO.

Potential catalysts that could promote pyrrole-pyrrole oxidative coupling and oxidation to lactams and hydroxylactones were tested. Persulfate ion was reported to promote pyrrole polymerization by SET that generates a pyrrole radical cation. Incubation of the isoLG-pyrrole derivative of N-acetyl-gly-lys methyl ester with 1-2 mol% ammonium persulfate (APS) and 0.5-1 mol% tetramethylethylenediamine (TEMED) generated a product mixture containing small amounts of pyrole-pyrrole dimer
and much greater quantities of lactam and hydroxylactam (Figure 4.8), but these products were decomposed by treatment with more persulfate.

Since it was a free radical reaction, it was quite hard to control reaction to form one major product. If a little more of the APS (over 5 mol% equivalent) and TEMED (over 2 mol% equivalent) were added, the pyrrole was completely consumed but no lactam, hydroxylactam or bispyrrole were detected. The products were similar for reacting at 25 °C and 37 °C. Representative spectra are shown in appendix Figure 4.10-11S.

![MALDI-TOF MS of pure iso[4]LGE2-pyrrole and its oxidation products](image)

**Figure 4.8** MALDI-TOF MS of pure iso[4]LGE2-pyrrole and its oxidation products: lower trace = pure iso[4]LGE2-pyrrole, upper trace = after exposure to 2 mol% APS and 1 mol% TEMED at 37 °C for 2 h.
Nitroxide, also known as amino-N-oxide, is a functional group which contains an N-O bond and two C-N bonds. Nitroxide radicals, especially (2,2,6,6-tetramethylpiperidin-1-yl)oxyl, have been used\textsuperscript{7} as stable persistent free radical initiators. It is the most widely used nitroxide radical owing to its remarkable persistence.\textsuperscript{8-10}

Since tetramethylpyrrolidine-N-oxide (TEMPO) could function as a SET oxidant\textsuperscript{11}, we tested its ability to promote isoLG-pyrrole dimerization. Treatment of dimer-free pyrrole with 10 mol% of TEMPO at 37 °C for 3 h cleanly generated a product mixture containing similar amounts of lactam and pyrrole-pyrrole dimer, and the products were not degraded by longer incubation or use of larger mol% of TEMPO. Thus, pyrrole-pyrrole cross-linking of isoLG-pyrrole was also facilitated by the stable free radical tetramethylpyrrolidine-N-oxide (TEMPO) (Figure 4.9, see next page).

TEMPO hydroxylamine (TEMPOH) is formed by one electron reduction of TEMPO. In air, TEMPOH can be readily oxidized back to TEMPO. The applications of TEMPO in biochemistry often relate to its reduction to TEMPOH. In such systems, reduction of TEMPO usually involves metal ions as shown in Scheme 4.5.\textsuperscript{12}

\[
\text{N} \hspace{1cm} \overset{\phi}{\text{O}} \hspace{1cm} \text{+ Fe (II)} \quad \rightarrow \quad \text{N} \hspace{1cm} \overset{\phi}{\text{OH}} \hspace{1cm} \text{+ Fe (III)}
\]

**Scheme 4.5** Reduction of TEMPO by ferrous iron.

TEMPO can be reduced to TEMPOH by metal ions. To neutralize any traces of metal ions that might be present, EDTA was added to the reaction mixture. The same autoxidation of iso[4]LGE\textsubscript{2}-pyrrole occurred in the presence of EDTA (one equivalent relative to TEMPO) as in its absence. Apparently metal ion catalysis is not required for
TEMPO to promote oxidative reactions of iso[4]LGE\textsubscript{2}-pyrrole. Represented spectra are shown in appendix Figure 4.14-15S.

**Figure 4.9** MALDI-TOF MS of pure iso[4]LGE\textsubscript{2}-pyrrole and its oxidation products: lower trace = pure iso[4]LGE\textsubscript{2}-pyrrole, upper trace = after exposure to 10 mol\% TEMPO at 37 °C for 3 h.

Although trimethylamine-N-oxide (TMAO) is not a stable free radical analogue of TEMPO, it has a nitrogen bound oxygen atom and can act as a prooxidant in the autoxidation of methyl linoleate.\textsuperscript{12} TMAO can also react with Cu(I) and Cu(II) derivatives to generate transient oxo-Cu(III) (cupryl) intermediates in oxidations such as
ortho hydroxylation of N-benzoyl-2-methylanaline.\textsuperscript{12-13} TMAO decomposes to aminium radical TMA·\textsuperscript{+} then give the form of TMA (trimethylamine).\textsuperscript{14}

Levels of TMAO are strongly positively correlated with cardiovascular disease risk.\textsuperscript{15} Understanding the molecular mechanisms of its ability to promote oxidative transformations of biomolecules is likely to provide important insights into its pathological involvements.

Remarkably, TMAO proved similarly effective to TEMPO in promoting oxidation of the isoLG-pyrrole derivative of N-acetyl-gly-lys methyl ester to lactam and hydroxylactam and oxidative pyrrole-pyrrole coupling. It is especially interesting that TMAO can promote the oxidative transformations of isoLG-pyrroles. Treatment of dimer-free pyrrole with 10 mol\% of TMAO at 37 °C for 3 h cleanly generated a product mixture containing similar amounts of lactam and pyrrole-pyrrole dimer, and the products were not degraded by longer incubation or use of larger mol\% of TMAO. All reactions were done at 37 °C which was closed to human biological environment. We also characterized this reaction including examining the role of trace metal ions through adding EDTA (EDTA was equivalent to TMAO). Result indicated no direct effect to the pyrrole initial reaction. Represented spectrums were shown in appendix Figure 4.12-13S. It is conceivable that this chemistry contributes to the pathological consequences of TMAO biosynthesis that involves gut microbial metabolism of phosphatidylcholines,\textsuperscript{16-17} and L-carnitine.\textsuperscript{18} Furthermore, exploration of the mechanisms of LG/isoLG pyrrole oxidation reactions may provide insights into chemistry involved in pathologies associated with elevated levels of TMAO.
Scheme 4.6 Possible electron transfer mechanism for pyrrole oxidized reaction

The study results described above indicating the reaction of pure LG/isoLG-pyrroles with oxygen is slow in the absence of catalysts. Nevertheless, it is likely that the first step in the reaction of oxygen with these pyrroles generates a pyrrole cation radical-superoxide ion pair. Hypothetical mechanistic pathways are presented in Scheme 4.6 for the partitioning of this intermediate to generate pyrrole-pyrrole dimers, hydroxylactams and lactams. Dimer formation involves addition of the electrophilic cation radical to the electron rich pyrrole ring of a second molecule of pyrrole followed by further oxidation and proton loss. Hydrogen peroxide is a likely byproduct of oxidative pyrrole-pyrrole
coupling. The tendency of isoLG pyrroles to undergo spontaneous oxidation may be the result of a hydrogen peroxide promoted autocatalytic pyrrole oxidative coupling.

4.2.6 Pilot studies of bispyrrole synthesis.

Reaction conditions that promote bispyrrole formation were also probed with two simple model systems, 3-ethyl-2,4-dimethylpyrrole and 3-ethyl-1,2,4-trimethylpyrrole. Pilot studies showed that both of these compounds formed dimers and trimers when incubated under air in organic solvents. The details were discussed below and related mass spectra of the reaction product mixtures were presented in appendix Figure 4.16-18S.

(i) Initiate reactions of 3-ethyl-2,4-dimethylpyrrole with TEMPO and TMAO.

![Scheme 4.7](image)

Scheme 4.7 Initiate reactions of 3-ethyl-2,4-dimethylpyrrole

Commercial available 3-ethyl-2,4-dimethylpyrrole (4.6) was resuspended in organic solvent and tested its polymerization reactions in different conditions at 25 °C or 37 °C. Pyrrole self-reaction in acetonitrile was tested first and result showed 3-ethyl-2,4-dimethylpyrrole could form dimer and trimer spontaneously having no relationship with adding TEMPO or TMAO (TEMPO or TMAO was 1:1 equivalent to pyrrole) (related spectrums were shown in appendix Figure 4.16S). Methanol or acetone as solvents were
also tried and the results were quite similar. A possible way to avoid 3-ethyl-2,4-dimethylpyrrole polymerization itself was pyrrole methylation with CH₃I.

(ii) Preparation of 3-ethyl-1,2,4-trimethylpyrrole (MP)

![Scheme 4.8 Preparation of 3-ethyl-1,2,4-trimethylpyrrole (MP)](image)

Preparation of 3-ethyl-1,2,4-trimethylpyrrole 4.8 was carried out with the general method Heaney and Ley developed. After reaction, combined organic extracts removed solvent under reduced pressure to give pure 4.8 with silica gel column purification with ethyl acetate: hexane=1:8.

(iii) Initiate reactions of 3-ethyl-1,2,4-trimethylpyrrole with TEMPO and TMAO.

![Scheme 4.9 Initiate reactions of 3-ethyl-1,2,4-trimethylpyrrole with TEMPO and TMAO](image)

3-Ethyl-1,2,4-trimethylpyrrole (4.8) was incubated under air at 25 °C in the presence or absence of TEMPO (1 equivalent) in acetonitrile solution. 3-Ethyl-1,2,4-trimethylpyrrole underwent dimerization and trimerization under both conditions (spectra are shown in appendix Figure 4.18S), i.e, TEMPO had no significant effect on increasing
dimer formation. The relatively greater ease of autoxidation and pyrrole-pyrrole cross-linking of pyrrole 4.8 compared to iso[4]LGE2-pyrrole could simply be a consequence of the greater concentration of oxygen in organic versus aqueous medium. The concentration O₂ in air equilibrated H₂O (0.28 mM) is about 7 fold lower than in air equilibrated CHCl₃ (2.05 mM), acetone (2.4 mM), acetonitrile (2.42 mM), or ethanol (1.94 mM).²¹⁻²⁷ Because the autoxidation of isoLG pyrrole was performed in the presence of a lower concentration of O₂ than autoxidation of 4.8, initiators, e.g., TEMPO or TMAO exerted a greater influence on the autoxidation. Thus, autoxidation of isoLG pyrrole occurs less readily than oxidation of 3-ethyl-1,2,4-trimethylpyrrole in air saturated organic solvents in which the concentration of O₂ is much higher than in water.
4.3 Conclusions

We discovered that the pure LG/isoLG-pyrrole derivative of the N-acetyl-lys-gly methyl ester dipeptide can be produced in the presence of dithionite, an oxygen scavenger. The oxidation and dimerization of the pure pyrrole is promoted by oxygen and exhibits an induction period that can be eliminated by various catalysts, e.g., the stable free radical TEMPO as well as by TMAO. More vigorous oxidative decomposition was promoted by vitamin C, vitamin E and 2,2'-azobis(2-methylpropionamidine)dihydrochloride. Previously, the reaction of phosphatidylethanolamine (PE) with an isoLG was reported to produce a stable pyrrole derivative that resists further oxidation to lactams. This stability was ascribed to lower concentrations of O₂ in organic solvents such as CHCl₃ (in which the reaction of PE with isoLGs was conducted) than in H₂O (the solvent used for the reaction of proteins and the N-acetyl-lys-gly methyl ester dipeptide with isoLGs). However, the opposite is the case, the concentration of oxygen in CHCl₃ is seven times higher than in air saturated H₂O. Given the susceptibility of the autoxidation of the N-acetyl-lys-gly methyl ester dipeptide-derived isoLG pyrrole to catalysis, the stability of PE-isoLG pyrrole may be a consequence of its purity, i.e., the absence of catalytic impurities that promote its autoxidation.

In the presence of N-acetylcysteine, incubation of iso[4]LGE₂-pyrrole at neutral pH under air showed no pyrrole-to-pyrrole cross-linking. Instead a series of new ions was produced corresponding to pyrrole cysteine adducts and a series derivatives produced from them by the addition of one or more atoms of oxygen. It is suggested that the thiol inhibits the pyrrole dimerization by intercepting pyrrole cation radical generated by electron transfer to oxygen.
4.4 Experimental Procedures

4.4.1 General methods. All chemicals used were high purity analytical grade. The following commercially available materials were used as received: acetyl-gly-lys-O-methyl ester was from Bachem (Torrance, CA), 3-ethyl-2,4-dimethylpyrrole was from Aldrich (Milwaukee, WI), all other chemicals were obtained from Fisher Scientific Co (Chicago, IL). Iso[4]LGE₂ was prepared by Dr. Jim Laird. Chromatography was performed with ACS grade solvents.

4.4.2 Iso[4]LGE₂-pyrrole. N-Acetyl-gly-lys methyl ester (2 mg, 7.71 µmol) was added to 1 ml ddH₂O and the solution was sparged with bubbled argon for several minutes to remove air. Then Na₂S₂O₄ (1 mg, 5.75 µmol) was added. Iso[4]LGE₂ in methanol (136 µg, 0.386 µmol, 5 µg/µl) was added and the mixture was incubated overnight at room temperature. TLC showed the presence of unreacted dipeptide, only one major product and complete disappearance of iso[4]LGE₂. The solution was concentrated under high vacuum to remove water into a Dry Ice-acetone cooled trap, and then methanol (1 mL) was added to dissolve the organic product. Then the mixture was loaded onto a 6 cm column of silica gel packed with a slurry in EtOAc in a disposable pipette, and eluted with methanol (2 mL) (Rᵣ = 0.5) to remove most of sodium dithionite. After concentration under reduced pressure, reagent grade chloroform (1 mL) that had been sparged with argon was added to dissolve iso[4]LGE₂-pyrrole and the solution was filtered to remove sodium dithionite and any silical gel that had dissolved in the methanol. The crude iso[4]LGE₂-pyrrole (~222 µg, 0.39 µmol) solution was concentrated by evaporation with argon gas and re-suspended in 500 µl 10% acetonitrile/water.
Iso[4]LGE₂-pyrrole was purified by HPLC with a 2.1 x 100 mm i.d. C18 1.8 µm column (Waters Acquity UPLC HSS). Chromatography was carried out with linear elution gradient (elute A, 0.1% FA/H₂O; elute B, 0.1% FA/ACN) at a flow rate of 150 µl/min. For determining the retention time of iso[4]LGE₂-pyrrole (~222 µg, 0.39 µmol) that had been freed of Na₂S₂O₄ was dissolved in 10% acetonitrile (ACN) 0.1% formic acid (FA) (50 µl) and then loaded to the column with 20% B for 10 min, and eluted with the following gradient: 20-60% B over 20 min, to 98 % within 0.1 min and hold for 5 min. The effluent was monitored by ESI-MS/MS with an API-3000 triple quadrupole electrospray mass spectrometer (Applied Biosystems Inc.) The instrument was operated in the positive mode and high-pressure nitrogen was used as source gas and scanning from m/z 220-2000 using the parameters listed in Table 3.2. The pyrrole eluted between 12.8 and 14 minutes (Figure 4.2). Another aliquot of ~222 mg was injected and the eluent was collected in 1 minute fractions from 11 minutes to 31 minutes and the fractions were analyzed for purity by MALDI-TOF MS. Fractions containing the pure pyrrole were combined. Optimized parameters for triple quadrupole mass spectrometer were noted in section 3.4.4.

4.4.3 Autoxidation of crude iso[4]LGE₂-pyrrole. Crude iso[4]LGE₂-pyrrole that contained excess dipeptide (~222 µg, 0.39 µmol in 500 µl 10% acetonitrile/water) was incubated under air 1h on a shaker (IKA MTS 2/4 digital microtiter shaker, 500 rpm). Then 100 µl of the reaction mixture (0.077 µmol) was quickly dried using a high-speed vacuum evaporator and analyzed by MALDI-TOF MS. The remaining reaction mixture
(0.31 µmol) was aerated with oxygen overnight on a shaker (IKA MTS 2/4 digital microtiter shaker, 500 rpm) at room temperature, and then analyzed by MALDI-TOF MS.

4.4.4 Autoxidation of iso[4]LGE2-pyrrole. Purified iso[4]LGE2-pyrrole (44 µg, 0.077 µmol in 450 µl 10% acetonitrile/water) was incubated under air at 25 °C on shaker (IKA MTS 2/4 digital microtiter shaker, 500 rpm). At different time point (2, 6, and 8 days), same amount of reaction mixture (15 µg, 0.026 µmol in 150 µl 10% acetonitrile/water) was quickly dried using a high-speed vacuum evaporator and analyzed MALDI-TOF MS.

4.4.5 Autoxidation of iso[4]LGE2-pyrrole in the presence of Nα-acetylcysteine. 7.7 µl Nα-acetylcysteine (0.77 µmol, 126 µg) (100 mM in H2O, pH 3) was added to purified iso[4]LGE2-pyrrole (44.36 µg, 0.077 µmol in 500 µl 10% acetonitrile/water) and reacted under air at 25 °C on shaker (IKA MTS 2/4 digital microtiter shaker, 500 rpm). At different time point (2, 4, 6 and 8 days), same amount of reaction mixture (0.019 µmol, 125 µl) was quickly dried using a high-speed vacuum evaporator and analyzed by MALDI-TOF MS.

Control experiments were performed. 7.7 µl Nα-acetylcysteine (0.77 µmol, 126 µg) (100 mM in H2O, pH 3) was added to purified iso[4]LGE2-pyrrole (44.36 µg, 0.077 µmol in 500 µl 10% acetonitrile/water) and reacted under argon gas protection at 25 °C on shaker (IKA MTS 2/4 digital microtiter shaker, 500 rpm). At different time point (2, 4, 6 and 8 days), same amount of reaction mixture (0.019 µmol, 125 µl) was quickly dried using a high-speed vacuum evaporator and analyzed by MALDI-TOF MS.
7.7 µl N\textsuperscript{\alpha}-acetylcysteine (0.77 µmol, 126 µg) (100 mM in H\textsubscript{2}O, pH 3) was added to purified iso[4]LGE\textsubscript{2}-pyrrole (44.36 µg, 0.077 µmol in 500 µl 10% acetonitrile/water) and reacted under argon in the presence of dithionite at 25 °C on shaker (IKA MTS 2/4 digital microtiter shaker, 500 rpm). At different time point (2, 4, 6 and 8 days), same amount of reaction mixture (0.019 µmol, 125 µl) was quickly dried using a high-speed vacuum evaporator and analyzed by MALDI-TOF MS.

4.4.6 Autoxidation of iso[4]LGE\textsubscript{2}-pyrrole in the presence of acetyl-gly-lys methyl ester. 7.7 µl N-acetyl-gly-lys methyl ester (0.77 µmol, 200 µg) (100 mM in H\textsubscript{2}O, pH 7) was added to purified iso[4]LGE\textsubscript{2}-pyrrole (44 µg, 0.077 µmol in 500 µl 10% acetonitrile/water) and reacted under air on shaker (IKA MTS 2/4 digital microtiter shaker, 500 rpm). At different time point (1 h, 8 h, 1 and 2 days), same amount of reaction mixture (0.019 µmol, 125 µl) was quickly dried using a high-speed vacuum evaporator and analyzed by MALDI-TOF MS.

N-Acetyl-gly-lys methyl ester was purified by HPLC on a 2.1 x 100 mm i.d. C18 1.8 µm column (Waters Acquity UPLC HSS). N-Acetyl-gly-lys methyl ester (10 µl, 10 µmol, 259 µg) (100 mM in H\textsubscript{2}O, pH 7) was dissolved in 10 µl 2% ACN 0.1% FA. This solution (18 µl) was injected onto the column and eluted with a linear gradient (elute A, 0.1% FA/H\textsubscript{2}O; elute B, 0.1% FA/ACN) at a flow rate of 100 µl/min. The gradient was 2-8% B over 8 min. Fractions were collected every minute and analyzed for dipeptide by MALDI-TOF MS. The fraction eluting during the 4\textsuperscript{th} min contained purified N-acetyl-gly-lys methyl ester. It was quickly dried using a high-speed vacuum evaporator and resuspended in 10% acetonitrile/water.
A mixture of purified N-acetyl-gly-lys methyl ester (0.77 µmol, 200 µg) and purified iso[4]LGE2-pyrrole (44 µg, 0.077 µmol in 500 µl 10% acetonitrile/water) was incubated under air at 25 °C for several days on shaker (IKA MTS 2/4 digital microtiter shaker, 500 rpm). At various time points (2 h, 6 h, 2 and 6 days) aliquots of the reaction mixture (0.019 µmol, 125 µl) were quickly dried using a high-speed vacuum evaporator and analyzed by MALDI-TOF MS.

4.4.7 Autoxidation of iso[4]LGE2-pyrrole in the presence of free radical initiators or hydrogen atom donors. Vitamin C (7.7 µl of 1 mM in pH 7 H2O, 7.72 nmol, 1.36 µg) was added to the purified iso[4]LGE2-pyrrole (44.36 µg, 0.077 µmol in 450 µl 10% acetonitrile/water) and the mixture was incubated under air on shaker (IKA MTS 2/4 digital microtiter shaker, 500 rpm). At various time points (2 h, 24 h, 7 days), aliquots of the reaction mixture (150 µl) were quickly dried using a high-speed vacuum evaporator and analyzed by MALDI-TOF MS. Similar incubations were performed with 2,2'-azobis(2-methylpropionamidine) dihydrochloride (1 mM in H2O, pH 6) or vitamin E (5 mM in EtOH, pH 6) in place of vitamin C.

(R)-(−)-2-(2,5-Dihydrophenyl)glycine (7.7 µl of 10 mM in 200 mM (pH 2) HCl, 0.077 µmol, 12 µg) was added to purified iso[4]LGE2-pyrrole (44 µg, 0.077 µmol in 450 µl 10% acetonitrile/water) and the mixture was incubated under air on shaker (IKA MTS 2/4 digital microtiter shaker, 500 rpm). At various time points (2 h, 24 h, 7 days), aliquots of the reaction mixture (150 µl) were quickly dried using a high-speed vacuum evaporator and analyzed by MALDI-TOF MS.
4.4.8 Autoxidation of iso[4]LGE2-pyrrole in the presence of APS/TEMED, TMAO or TEMPO. Trimethylamine-N-oxide (TMAO, 7.7 µl of 1 mM in pH 7 H2O, 7.7 nmol, 5.8 µg) was added to purified iso[4]LGE2-pyrrole (44 µg, 0.077 µmol in 500 µl 10% acetonitrile/water) and mixture was incubated under air on a shaker (IKA MTS 2/4 digital microtiter shaker, 500 rpm). At various time points (1 h, 2 h, 3 h and 1 day), aliquots of the reaction mixture (0.019 µmol, 125 µl) were quickly dried using a high-speed vacuum evaporator and analyzed by MALDI-TOF MS. The same experiment was performed with (2,2,6,6-tetramethylpiperidin-1-yl)oxyl (TEMPO, 1 mM in 10% ACN, pH 6) in place of TMAO.

Ammonium persulfate (APS, 1.6 µl of 1 mM in H2O, 1.544 nmol, 352.4 ng) and tetramethylethylene diamine (TEMED, 0.7 µl of 1 mM in H2O, 0.772 nmol, 90 ng) were added to iso[4]LGE2-pyrrole (44 µg, 0.077 µmol in 500 µl 10% acetonitrile/water) and incubated under air on a shaker (IKA MTS 2/4 digital microtiter shaker, 500 rpm). At various time points (1 h, 2 h, 3 h and 1 day) an aliquot of the reaction mixture (0.019 µmol, 125 µl) was quickly dried using a high-speed vacuum evaporator and analyzed by MALDI-TOF MS.

4.4.9 3-Ethyl-1,2,4-trimethylpyrrole (4.8). Anhydrous DMSO (20 ml) was added to crushed KOH pellets (2.24 g, 0.04 mol) and the mixture was stirred for 5 min. 2,4-dimethyl-3-ethylpyrrole (4.6, 1.23 g, 0.01 mol) was added and the mixture was stirred for 45 min. The mixture was cooled in ice and CH3I (2.84 g, 0.02 mol) was added dropwise with stirring and the stirring was continued for 45 min at room temperature. H2O (20 ml) was added and the mixture was extracted with Et2O (3 × 50 ml). The combined organic
extracts were washed with H₂O (3 × 25 ml), dried (MgSO₄), and the solvent was removed under reduced pressure. The residue was purified by flash chromatography (ethyl acetate: hexane = 1:8, Rf = 0.8, yield 50%) to give 4.8 (0.68 g).²⁹

TEMPO (10 µl of 100 mM in acetonitrile, 1 µmol, 156 µg) was added to 10 µl 3-ethyl-1,2,4-trimethylpyrrole (1 µmol, 137 µg) (100 mM in acetonitrile) and the mixture was incubated under air on a shaker (IKA MTS 2/4 digital microtiter shaker, 500 rpm) for 3 h. The reaction mixture was quickly dried using a high-speed vacuum evaporator and analyzed by MALDI-TOF MS.

4.4.10 Analysis of adducts using MALDI-TOF MS. Initial diagnostic analysis of the samples was performed by MALDI-TOF mass spectrometry (as described in section 3.4.3).
4.5 References


CHAPTER 5

5.1 Background

IsoLGs are generated through free radical-induced oxidation of arachidonates (see chapter 1). They are extraordinarily reactive; forming covalent adducts incorporating protein lysyl ε-amino groups within seconds. Since proteins so efficiently trap them, also many plasma proteins have half-lives of several weeks, and modified proteins and protein-protein cross-links are resistant to proteolysis, isoLGE adducted plasma proteins are expected to accumulate in plasma which could provide a unique marker and effective dosimeter for the oxidative injuries in the brain or retina.

Development and validation of isolevuglandin (isoLG) biomarkers of oxidative stress could be used to assess the antioxidant effects of dietary supplements in vivo and to examine their mechanisms of action, efficacy and effectiveness with respect to human health. However, our understanding of these processes remains primitive, in part because of the lack of sensitive and specific tools for detecting cumulative damage resulting from oxidative stress. An enzyme-linked immunoassay (ELISA) with polyclonal antibodies against isoLG-protein adducts reveals disease-related elevations in these biomarkers in blood from patients with end-stage renal disease, cardiovascular disease, age-related macular degeneration, and children born prematurely, as well as in brain tissue from individuals with autism spectrum disease and liver from a murine model of alcoholic liver disease. Therefore, detection of blood plasma isoLGE may provide a convenient, minimally invasive method for monitoring such pathologies.

Previously, we generated anti-iso[4]LGE2 and anti-LGE2 polyclonal antibodies (pAb) that have been useful for ELISA or immunohistochemistry for detection of iso[4]LGE2- and LGE2-protein adducts. These antibodies are structurally specific showing low cross...
reactivity. They allow the confirmation that a variety of structural LG isomers are produced in vivo. Specially, isoLGE₂-KLH pAb bind isoLGE₂-human serum albumin (HSA) 200 times more strongly than they bind iso[4]LGE₂-HSA, while the iso[4]LGE₂-KLH pAb bind iso[4]LGE₂-human serum albumin (HSA) at least 2000 times more strongly than they bind LGE₂-HSA.⁴

However, pAbs have shortcomings that complicate standardization and accuracy. They vary from batch to batch, resulting in variable responses, background can be high and amounts are limited by the life-time of the animal source. In contrast, monoclonal antibodies can be generated in unlimited quantities, and their specificity ensures that only one epitope is recognized on an antigen. This is extremely useful when observing subtle protein alterations, and the antibodies can be selected to exhibit low background cross reaction, e.g., with abundant blood proteins such as serum albumin. Therefore, the generation of a highly specific monoclonal anti-iso[4]LGE₂ antibody was undertaken.
5.2 Results and discussion

5.2.1. Antibody production

Monoclonal anti-iso[4]LGE2 antibody was produced at the Hybridoma Cole Facility of Visual Sciences Research Center (VSRC), Case Western Reserve University. Five Balb/c mice were immunized with iso[4]LGE2-KLH. After 60 days, blood was draw from each mouse, and tested for immune response. High anti-iso[4]LGE2 responses were present in all 5 mice. Blood from mouse #972 showed high anti-iso[4]LGE2 response and the lowest cross-reactivity to LGE2-BSA (Figure 5.1). Competitive antibody binding inhibition studies with serum from all mice confirmed this conclusion (appendix Figure 5.1S). Therefore, mouse #972 was chosen for monoclonal antibody production.

![Figure 5.1](image_url)

Figure 5.1 Mouse serum immune responses (1:10,000 dilutions) comparison with pre-immune serum.

Splenocytes from mouse 972 were fused with myeloma cells and clone screening was done by both indirect and competitive ELISAs. Single cell clones from each round of
subcloning that produced the anti-iso[4]LGE2 monoclonal antibody were identified, and the clone exhibiting the strongest apparent immune-reactivity was grown in larger amounts. Anti-iso[4]LGE2 mAb from this clone was purified as described below using protein G column and the protein concentration of the purified mAbs was determined at OD 280 nm.

From fusion attempted on 1920 samples (wells on 20 plates) 725 wells were found to contain hybridoma cells (appendix Figure 5.2S). The antigens iso[4]LGE2-BSA and KLH were used for the first screening tests. Of 371 large and very large clones (clones with many cells), three showed strong response (clones number 9G8, 12G4, 18G7) against iso[4]LGE2-BSA, and two others showed lower response (clones number 2E8, 15A5). Of 354 small and medium clones (clones with less cells), six (clones number 4G5, 5B7, 8B3, 10C11, 16E4, 19A4) showed lower response against iso[4]LGE2-BSA compared to the above five clones from large clones (9G8, 12G4, 18G7, 2E8, 15A5). More antigens (iso[4]LGE2-BSA, iso[4]LGE2-HSA, BSA, HSA, LGE2-BSA, LGE2-KLH, CEP-HSA) were tested by indirect and competitive ELISAs of the supernatant of each positive clone (appendix Figure 5.3S). Clones 9G8, 12G4, 18G7 showed the best responses. Clones 9G8 and 12G4 were chosen for the first round of subcloning. The isotype of these was determined. Clone 9G8 was IgM and 12G4 was IgG1.

After the first screening, the chosen clones were selected by another two rounds of subcloning to test clone cells’ productivity and consistency, and to confirm that the clone was derived from a single cell. Since the isotype of 9G8 was IgM, we mainly focused on clone 12G4 that was IgG1. Clone 12G4 had 34 first-round subclones and all 34 clones showed strong response. It showed strong specificity to iso[4]LGE2-BSA and
iso[4]LGE\textsubscript{2}-HSA and almost no response to LGE\textsubscript{2}-BSA (appendix Figure 5.4S). To determine an optimal clone from 12G4 for the next round of subcloning, 10 clones were chosen from the 34 (first subclones) for test again. Both the original antibody supernatant concentration and a 10 fold dilution were tested. Both showed strong binding ability. With more cell supernatant, competitive ELISA of these ten 12G4 clones was tested, 12G4 F3 was chosen for next round subcloning (appendix Figure 5.5S).

The supernatant and a 10 fold dilution from each of 16 secondary subclones of 12G4 F3 was tested by indirect ELISA (appendix Figure 5.6S). The final three candidates from 12G4 F3--C1 D1 G4 were chosen and fully tested by competitive ELISA (appendix Figure 5.7S). Clone 12G4 F3 D1 was selected for adapting to serum free medium (appendix Figure 5.8S).

Anti-iso[4]LGE\textsubscript{2} mAb in serum free medium was diluted with binding buffer (20 mM sodium phosphate buffer, pH 7) and loaded onto a protein-G column using commercially available protein-G beads. Non-IgG proteins were washed away by eluting the column with neutral binding buffer (20 mM sodium phosphate buffer, pH 7) and the decomplexation is accomplished by changing the pH of the eluting buffer (glycine-HCl, pH 2.6). The purified mAb solution was neutralized and dialyzed immediately against 10 mM PBS (pH 7.4) and then lyophilized and stored at -80 °C. No non-specific binding was observed in the purified mAb sample analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) as shown in Figure 5.2. Purification of IgG was confirmed since only signal bands (heavy and light chain) were detected in SDS-PAGE.

To assess the structural specificity and selectivity of the monoclonal antibody, competitive inhibition of antibody binding to iso[4]LGE₂-modified proteins by various haptens was examined. For all cross-reactivity studies, iso[4]LGE₂-BSA was used as coating agent and iso[4]LGE₂-HSA was used as a standard. The concentration at 50% inhibition (IC₅₀) for iso[4]LGE₂-HSA was defined as 100% cross-reactivity. Duplicates of serial dilutions of all inhibitors were used and final curves were constructed using mean absorbance values (Figure 5.3).

The IC50 values refer to the concentration of antigen that is required to inhibit 50% binding of the antibody to the coating agent. The IC50 for [iso[4]LGE2]-HSA and [iso[4]LGE2]-KLH using [iso[4]LGE2]-BSA as coating agent are 88.2 µM and 106.6 µM, respectively. For a variety lysyl-ε-amino pyrrole protein modifications that have a carboxylic acid group and are derived from lipid oxidation products (LGE2-BSA, LGE2-KLH and CEP-BSA, CEP-HSA), anti-[iso[4]LGE2] mAb showed remarkable selectivity. All of them showed no significant cross-reactivity. Equally important, BSA and HSA were not recognized at all by this antibody.
5.2.3. Western analysis of anti-iso[4]LGE₂ mAb.

The anti-iso[4]LGE₂ mAb which exhibited high specificity towards the iso[4]LGE₂ epitope, allow the development of immunochemical assays for the detection and quantification of iso[4]LGE₂ adducts generated under oxidative stress. It was of special importance to employ these methods for diagnosing or screening for diseases in which elevated levels of iso[4]LGE₂ adducts were present at the disease site, or at remote sites, as in the blood.

We did pilot western blot analysis against iso[4]LGE₂-BSA, a standard protein containing the iso[4]LGE₂ modification plus BSA control. Different sample loading amounts and primary concentrations were tested (Figure 5.4). Anti-iso[4]LGE₂ mAb showed both high selectivity and sensitivity.


**Figure 5.4** Comparative Western analyses using anti-iso[4]LGE₂ mAb against iso[4]LGE₂-BSA, a standard protein containing the iso[4]LGE₂ modification. Left panel: developed with anti-iso[4]LGE₂ mAb concentration as 10 µg/ml in PBS. Middle panel: developed with anti-iso[4]LGE₂ mAb concentration as 5 µg/ml. Right panel: developed...
with anti-iso[4]LGE₂ mAb concentration as 1 µg/ml. Goat anti-mouse secondary antibody was diluted 2000 times for all panel treatments.

A single-chain antibody from a phage displayed recombinant ScFv library that bound a model peptide adducted with synthetic isoLGE₂ was developed by Roberts et al.⁵ Recognition of isoLGE₂-lysyl adducts isolated from oxidized arachidonic acid by this anti-isoLGE₂ adduct single-chain antibody was independent of the amino acid sequence of the adducted peptides or proteins, and no cross-reactivity was detected with 4-hydroxynonenal or 4-oxononanal adducts. But no test was performed to determine its cross-reactivity with iso[n]LGE₂-derived protein epitopes.⁵

The EC₅₀ for oxidized arachidonyl-lysine adduct and isoLGE₂-lysine adduct were 470 and 310 nM, respectively. HNE- or ONA-adducted peptides, and nonreactive F₂-isoprostane product, 15-F₂t-IsoP (8-iso-PGF₂a), showed no binding at peptide concentrations up to 5 µM. These results indicated that single-chain antibody would specifically recognize isoLGE₂-lysine adducts, but not those formed by other pyrrole-forming lipid aldehydes formed during free radical-mediated lipid peroxidation.⁵ Importantly, the binding of this antibody with iso[n]LGs was not determined.

Iso[4]LGE₂ is an isomer formed exclusively through the free radical oxidation of AA-PC, unlike isoLGE₂ which can also be produced through the COX pathway.⁶ The anti-iso[4]LGE₂ mAb produced in this thesis research allows unambiguous assessment of the formation of iso-LGs from the isoprostane pathway.
5.3 Conclusions

An anti-iso[4]LGE$_2$ mAb was prepared that exhibits high sensitivity and structural specificity for detecting iso[4]LGE$_2$–protein adduct epitope, and no significant cross-reactivity with analogs including carboxyethylpyrrole (CEP) or LGE$_2$-protein adduct epitope that have carboxyalkyl side chains. The detection range of anti-iso[4]LGE$_2$ mAb for ELISA and western analysis was 5 µg/ml. Pilot Western blot analysis against iso[4]LGE$_2$-BSA, a standard protein containing the iso[4]LGE$_2$ modification plus BSA control, showed high specificity and selectivity with our anti-iso[4]LGE$_2$ mAb.
5.4 Experimental Procedures

5.4.1 Materials. The following commercially available materials were used as received:
DMEM-high glucose (no pyruvate #10-017) was from Mediumtech. Oxaloacetic acid (#O7753), insulin (#I4011), Na pyruvate (#P3662), 8-azaguanine (#A5284), hybridoma growth supplement (NCTC-109) (#N1140), 50 x HAT (#H0262), 50 x HT (#H0137), naphthol blue black (N3393) were from Sigma (St. Louis, MO). PEG (#783641) was from Roche (Madison, WI). Fatal bovine serum (#16000-044), trypan blue (0.4%), penicillin/streptomycin (PS #12800) and gentamicin (#15750-060) were from Gibco (Grand Island, NY). Cell™ mAb medium, quantum yield and animal component free grade were from Becton&Dickinson (Franklin Lakes, NJ). HL-1™ chemically defined serum-free medium was from LONZA. Protein G resin was from GenScript (Piscataway, NJ). Glycine (161-0724) and Quick Start™ Bradford dye reagent (1x) were from Bio-Rad (Hercules, CA). ABTS Ready to USE substrate solution was from Invitrogen. Immunization stock iso[4]LGεKLH (3.604 μg/μl, pyrrole = 721.3 μM, ratio = 600.4) was prepared by Dr. Jim Laird.

General methods:

(1) Macrophage extraction:

Materials used for macrophage extraction included 10 mL sterile syringes (one for each mouse), 18 1x1/2 gauge needles (one for each mouse), 70% ethanol for spraying, 2 autoclaved surgical scissors, forceps, curled fine forceps, 50 mL sterilized conical tubes and an ice bucket. Macrophage medium, also called super HAT medium, was prepared by reconstituting the contents of a commercial vial (medium supplement (50×) Hybri-Max™ Sigma H0262, γ-irradiated, lyophilized powder) with
10 mL of sterile cell culture medium (DMEM). The final working concentration of each component was 100 μM hypoxanthine, 0.4 μM aminopterin and 16 μM thymidine.

(2) SP2/0 myeloma cells growth:

Preparation of 8-azaguanine was achieved by reconstituting a commercial vial with 10 mL DMEM, to make a 50x stock; that then aliquoted as 2 mL per tube and stored at -20 °C. The final concentration was 130 μM. 100x Solution I stock was prepared by adding 1320 mg oxaloacetic acid, 71 mg insulin (powder at 28 U/mg) and 550 mg Na pyruvate to 100 mL PBS, then aliquoting as 6 mL per tube and stored at -20 °C. The final concentration of each component was 0.2 Units/ml insulin, 0.5 mM Na pyruvate and 1 mM oxaloacetic acid. Solution I was a good supplement to DMEM medium to provide the proper pH and enhance growth of the hybridoma cells. SP2/0 passage medium contained 87 mL DMEM, 10 mL FBS, 1 mL 50x 8-azaguanine, 1 mL 100x Solution I, 1 mL L-glutamine and 100 μl gentamicin. SP2/0 growth medium was prepared by adding 20 mL FBS, 1 mL 100x Solution I, 2 mL NCTC-109, 1 mL L-glutamine and 100 μl gentamicin to 78 mL DMEM.

(3) Fusion:

Materials used for fusion included sterile scissors, forceps, scalpels and sterile glass slides with frosted end. Super DMEM medium was prepared by adding 120 mL FBS, 60 mL NCTC-109, 6 mL 100x Solution I, 12 mL 50x HAT or HT medium, 6 mL L-glutamine and 600 μl gentamicin to 400 mL DMEM. Hybridoma medium (20%) included 78 mL DMEM, 20 mL FBS, 6 mL NCTC-109, 1 mL 100x Solution I, 1 mL
L-glutamine and 100 µl gentamicin. Hybridoma medium (15%) contained 83 mL DMEM, 15 mL FBS, 2.5 mL NCTC-109, 1 mL 100x Solution I, 1 mL L-glutamine and 100 µl gentamicin.

(4) Antibody purification:

Binding and elution buffers were 20 mM sodium phosphate buffer (pH 7) and 0.1 M glycine, (pH 2.6) (375.35 mg/50 ml sterilized water, make fresh), respectively.

5.4.2 Immunization. Five ear tagged and numbered Balb/c mice were given intraperitoneal (IP) injections of the immunogen (iso[4]LGE2-KLH). For the first IP injection, 140 µl the antigenic stock solution (containing 500 µg of immunogen) was emulsified with 360 µl of PBS and 500 µl FCA. Each mouse received 0.2 ml of the emulsion utilizing a 25 gauge needle. The initial injection contained immunogen (100 µg per mouse) in PBS and an equivalent volume of Freund’s complete adjuvant (FCA, Sigma F-5881). For booster IP injections, 70 µl the antigenic stock (containing 250 µg of immunogen) solution was emulsified with 430 µl of PBS and 500 µl FIA. The animals received their second injection two weeks after the first injection. The second injections were given utilizing the same volume (0.2 ml) as the initial injection, but with half as much immunogen (50 µg per mouse). Freund’s incomplete adjuvant (FIA, Sigma, F-5506) was used for this and all subsequent injections. Three weeks latter a third injection was given and then the fourth injection. Ten to fourteen days following the fourth injection a blood sample was collected from each mouse. The mice were anesthetized with halothane at the Case animal facility. When the mice showed no response to the foot pinch test, they were restrained by hand and the retro orbital sinus was punctured with the
tip of a 250 μl non-heparinized capillary tube (Fisher cat# 02-668-10). Approximately 100 μl of blood was collected from each mouse. After the bleed, the mice were returned to their respective cages to recover. Serum was prepared and tested from each blood sample. Thus, collected blood was spun at 7000 rpm for 10 min. The serum was aspirated and diluted 1:10,000 for ELISA analysis. Once a satisfactory immune response was established in one of the mice, a fusion was initiated. Three days before the fusion, the mouse with the best immune response was injected intravenously (IV. injection into the tail vein) with 0.2 ml of the sterile antigenic solution (50 μg per mouse, 14 μl the antigenic stock solution mixed with 186 μl of PBS) without adjuvant utilizing a 27 gauge needle. The mouse was restrained with a small rodent restraint for this injection. For the fusion, the mouse was euthanized via rapid cervical dislocation and the spleen aseptically removed. The remaining four mice were kept until it was verified that antibody was obtained that exhibited high specificity to the immunogen.

5.4.3 Cell counting. To determine the cell density and viability, cell counting was performed using a hemocytometer. Thirty μl of the cell suspension was mixed with 30 μl (equal volume) of 0.4% trypan blue solution to create a dilution factor of 2 and then transferred into a fresh 1.5-mL tube followed by incubation for 5 min. With a cover-slip in place, 10 μl of the trypan blue-stained cell suspension was loaded into a chamber on the hemocytometer. All the cells were counted in the nine 1-mm wide center squares that hold up to a total volume of 0.9 μl. Viable cells remained opaque, whereas non-viable cells were stained in blue. The cell density and viability were calculated using the following equations.
Viable cell density (cells per mL) = (total number of viable cells/0.9μl) x dilution factor x 1000 μl/mL

Cell viability = (number of viable cells/number of viable and dead cells) x 100%

5.4.4 Macrophage intraperitoneal lavage exaction. Macrophage extraction from ten Balb/c mice was performed two weeks before fusion. Another five mice were used for each of two rounds of subcloning. After assembling all materials, autoclaving instruments and preparing super HAT medium and storing it at 37 °C, mice were euthanized by rapid cervical dislocation to avoid excessive bleeding into the peritoneal cavity. Thus, mice were firmly held in one hand by the nape of neck and the tail was pulled with other hand until a pop was felt owing to a separation between cranium and backbone. Mice were sterilized with 70% ethanol and immediately placed into tissue culture hood. A small incision was made for laparotomy in the abdominal skin of the mouse with forceps and scissor. Mice were opened by pulling the skin firmly with both hands to each side so that skin was completely pulled down. Then the peritoneum was sprayed with 70% ethanol. A needle was uncapped inside the hood and 5-6 ml 11.6% of sucrose in sterilized water (w/w) was squirted into the peritoneum. The needle was injected, hole-side down, into the mouse in its lower abdominal area, preferably in a region where there was fat. During the process, needle was inserted gently and not too deeply without perforating any organs and the needle was withdrawn slowly so that the fat didn’t clog the needle. The mouse skin was transparent after removing fur, so it could avoid puncture the organs when inserted needle. The mouse was massaged the abdominal area to promote the detachment of sucrose solution to tissues (15 seconds), and then repeated twice. For macrophage
extraction, the needle of the same syringe was inserted horizontally into the upper part of abdomen. Then about 4 ml sucrose solution containing macrophages was withdrawn. This procedure was repeated twice to provide a total of about 12 ml fluid. The cell suspension was poured into a 50 ml collection tube and the cells were centrifuged at 1000 rpm for 10 min at 20 °C. The cells were re-suspended using a 10 ml pipette and macrophage medium was added to the pellet (10 ml per plate at a concentration of $10^5$ cells/ml). The myeloma cells were fed with suspended macrophage cells, 100 µl per well ($10^4$ cells/well) and then incubated at 37 °C in 5% CO₂.

**5.4.5 Growth of SP2/0 myeloma cells.** The myeloma SP2/0 cells were grown in passage medium (15% FBS) for about 6 days then slowly (after making sure that the cell viability was 90% or higher before transfer to new medium, see p173 section 5.4.3) put into growth medium (20% FBS). After centrifugation at 1200 rpm for 5 min at 4 °C, the supernatant was removed and the pellet re-suspended in 10 ml of DMEM + penicillin/streptomycin (PS) + 2% FBS.

**5.4.6 Fusion Procedure.** All fusion work was done in a cell culture laminar flow hood under sterile conditions. The spleen was removed from the immunized mouse and placed in a petri dish containing 10 ml of DMEM, penicillin/streptomycin (100 µl) and 2% FBS (200 µl). The spleen was cut into 4 pieces. Each piece was gently ground between two frosted end slides to release all cells from the connective tissue. The spleen cells were collected in a 50 ml conical tube and put on ice. The SP2/0 myeloma cells were also collected in a 50 ml conical tube and chilled on ice. Both sets of cells were washed twice
with 20 ml of cold serum free-DMEM. Both pellets were re-suspended in 5 ml of cold serum free-DMEM and put on ice. Using a hemocytometer, the spleen cells were counted in a 20 µl aliquot and after adding 20 µl 4% acetic acid that would lyse any RBC’s. Fusion was done at a cell ratio of 3:1 (spleen cells: myeloma). 6.3 million spleen cells were used for this fusion, so the number of SP2/0 myeloma cells would be 2.1 million. Using a hemocytometer, the myeloma cells were also counted in a 20 µl aliquot. 6.3 million spleen cells with 2.1 million myeloma cells were mixed in one 50 ml conical tube and the mixed cells were spun down. All supernatant was removed and the pellet made as dry as possible. The pellet was loosened by briskly tapping the tube. It was important to break up the pellet so the PEG would contact as many cells as possible. The tube was placed in a 37 °C water bath and 1 ml of 37 °C PEG 1500 solution (Ready-to-Use PEG 1500 (Cat#783641, 50% PEG 1500 (w/v) in 75 mM Hepes (pH 8.0)) from Roche) was added dropwise directly onto the pellet over a full 1 minute period. The tube was rotated while adding the PEG and gently swirled to resuspend the cells allowing the mixture to sit in the water bath for 1 minute. While rotating the tube, 1 ml of 37 °C, serum free-DMEM was added over 1 minute. Then 20 ml more of 37 °C serum free-DMEM was added dropwise over 4 minutes. Then the cells were spun down at 1000 rpm for 5 minutes. It was not unusual to see two layers of cells at this stage. The supernatant was removed immediately and the cell pellet was re-suspended in 100 ml super HAT medium. Cell suspension (0.1 ml) was placed in each well of a twenty 96-well plate already that contained macrophages, and the medium was changed every 5 days. By 10-14 days, colonies of hybridoma cells were apparent. They usually grew on the periphery of each
well. Culture supernatant (50 μl) could be removed for screening when 1/4 to 1/3 of the well was covered with growing cells.

5.4.7 Clonal selection. At approximately 5 days post fusion the initial signs of viable hybridoma colonies were observable in the wells. Concomitantly, the wells containing unfused myeloma cells were dead or dying. By 12-14 days post fusion the hybridoma colonies were macroscopically visible and ready for assay by ELISA for antibody containing wells. After the first screening, viable clones were chosen and submitted to another two rounds of subcloning to test the clone cells’ productivity and consistency, and also to make sure that each clone was derived from a single cell.

5.4.8 Cultivation of hybridoma cells

The cultivation process was carried out inside a laminar flow hood. In order to prevent cells from any potential contamination, all tools and containers were disinfected with 70% ethanol before using in the hood. A cryogenic vial containing 1 mL of frozen (in liquid nitrogen) hybridoma cells (~10^7 cells) expressing anti-iso[4]LGE2 mAb was thawed at 37 °C in a water bath immediately after being taken out of the liquid nitrogen. The thawed cell suspension was transferred into 10 mL of cell culture medium DMEM in a fresh 15-mL conical tube and spun down at 1000 rpm for 5 min at room temperature. The supernatant was discarded after centrifugation. The cell pellet was resuspended gently in 10 mL hybridoma medium (DMEM 8.3 mL, FBS 1.5 mL, NCTC-109 250 μl, 100x Solution I 100 μl, L-glutamine 100 μl and gentamicin 10 μl). The 10-mL cell suspension was plated in a 100 mm cell culture dish with a seeding density of 10^6 viable
cells/mL and a cell viability of 90% or higher (see p173 section 5.4.3) and incubated at 37 °C in 5% CO₂. After 2-3 days, the cells were split into 3-4 fresh culture dishes with 10 mL of hybridoma medium for each. For freezing cell samples, about 10⁸ hybridoma cells (90% viable or higher) of each passage were harvested by centrifugation at 1000 rpm for 5 min and resuspended in 10 mL of freezing medium consisting of fresh DMEM (80%), FBS (10%) and DMSO (10%). The cell suspension was transferred in 1-mL aliquots into 1.7-mL cryogenic vials that were later placed into a foam box, and subsequently frozen at -80 °C for the first 24-48 hours, then placed into liquid nitrogen in a cryogenic tank for long-term storage.

5.4.9 Adaptation of hybridoma cells to serum free medium

The hybridoma cells cultivated in hybridoma medium (mentioned above) were sequentially adapted to HL-1™ chemically defined serum-free medium by following the phases as indicated in Table 5.1. To avoid decreases of cell viability, the cells were maintained under the same conditions in each phase for a few passages and then passaged to the next phase until the cell viability reached to 90% or higher (see p173 section 5.4.3).

Table 5.1 Sequential adaptation of hybridoma cells to serum free medium

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<tr>
<th>Phase</th>
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<tr>
<td>1</td>
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<td>2</td>
<td>75% hybridoma medium; 25% HL-1 serum-free medium</td>
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<td>3</td>
<td>50% hybridoma medium; 50% HL-1 serum-free medium</td>
</tr>
<tr>
<td>4</td>
<td>25% hybridoma medium; HL-1 serum-free medium</td>
</tr>
<tr>
<td>5</td>
<td>100% HL-1 serum-free medium</td>
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</tbody>
</table>
5.4.10 Expression of anti-iso[4]LGE2 mAb

The hybridoma cells were subcultured with a seeding density of about $10^6$ cells/mL in 200 mL of HL-1 serum-free medium with 2 mL of the penicillin and streptomycin solution in two 250-mL shaking flasks. To maximize the production of anti-iso[4]LGE2 mAb, each flask containing 100 mL of cell culture was incubated at 37 °C in 5% CO$_2$ with shaking at 60 rpm (Advanced digital orbital shaker from VWR) for 5-7 days without changing the medium. By the end of this period, most of the cells died. The cell suspension was transferred into six 50-mL conical tubes that were then centrifuged at 4 °C for 30 min at 10,000x g. The supernatant containing anti-iso[4]LGE2 mAb was filtered through a 0.45 μm filter membrane (MILLEX®-GP syringe driven filter unit from Millipore) with the aid of a water-driven aspirator, put into fresh tubes and kept at 4 °C.

5.4.11 Purification of the anti-iso[4]LGE2 mAb. A protein G column was packed with immunopure protein G beads (5.0 ml), and was then equilibrated with 10 column volumes of binding buffer (20 mM sodium phosphate buffer). Anti-iso[4]LGE2 monoclonal supernatant in animal component free medium (250 ml) was diluted 1:1 with binding buffer (250 ml). 2-5 ml of Tris-HCl (pH 8) was added to adjust the pH>7 (pH is important for IgG binding to protein G beads). The diluted supernatant was loaded onto the top of the column and allowed to flow through it with gravity. In order to increase the binding saturation of protein G, the eluate was recycled by loading it to columns three times. In the future, it would be advisable to re-load the eluate to a washed column to see if any mAb remained. Then binding buffer (50 ml) was passed through the column to
remove unbound non-IgG proteins. IgG was then eluted with elution buffer (100 mM glycine-HCl, pH 2.6) collected into 1.0 ml fractions containing 100 µl of 1 M Tris-HCl (pH 9.5) buffer to neutralize the pH. The protein level of each eluted fraction was qualitatively monitored using the Quick Start™ Bradford protein assay. Briefly, 100 µl of an eluted fraction was added to 100 µl of Bradford dye reagent in a 96-well plate followed by gently mixing with a pipette at room temperature. The color of the eluted protein-containing solution (blue) was compared to the color of the reference solution (bright green) containing a 1:1 mixture of elution buffer and Bradford dye reagent. The more protein present in the solution, the more blue the mixture appeared to be. Collection of fractions was terminated when the color of the dye mixture appeared nearly bright green. The higher absorbance fractions (2-3 ml) were combined and dialyzed against 10 mM PBS (pH 7.4) 2×2 L at 4 °C for 24 h. The final concentration (1-2 mg/ml) was measured at OD 280 nm. Several batches of mAb were purified, the concentrations varies. The concentrations were 1.1 mg/ml, 1.13 mg/ml and 2.2 mg/ml for three different batches. Binding buffer was used to re-equilibrate the column, then the column was washed with 20% ethanol (15 ml) for storage at 4 °C.

5.4.12 Indirect enzyme-linked immunosorbent assay (ELISA) for anti-iso[4]LGE2 mouse serum, clone screening and purified mAb. Antigen (iso[4]LGE2-BSA, iso[4]LGE2-HSA, BSA, HSA, LGE2-BSA, LGE2-KLH, CEP-HSA) solutions (1.2 µg in 10 mM PBS,100 µl/well, 0.012 µg/µl) was added to each well of a sterilized 96 well ELISA plate (Cat. 9018, Corning Inc. Corning, NY). The plate was incubated overnight at 4 °C. After the coating solution was discarded, each well was washed with PBST
Tween 0.1%) (4×300 µl) and tapped onto paper towels until dry, then filled with 2% BSA /PBS 300 µl, and incubated at 37 °C for 2 h to block the remaining active sites on the plastic solid phase. In a pilot study, 3% chicken egg ovalbuman/PBS was tested as blocking buffer. However, the blocking wasn’t efficient. The medium control also showed a weak signal. Using 2% BSA/PBS as blocking buffer, the medium control showed almost no signal, just like the untreated background. So 2% BSA/PBS was chosen as blocking buffer. Each well was then washed 4 times with PBST (Tween 0.1%) and tapped onto paper towels till dry. Mouse serum (100 µl diluted 10,000:1 with PBS) or clone supernatant or protein G purified anti-iso[4]LGE2 mAb from HL-1 serum free medium (5 µg/ml in PBS) was dispensed into the ELISA plate wells and the plate was incubated overnight at 4 °C. Pre-immune mouse serum (before injection with immunogen), diluted as above, was employed as a negative response control if necessary. After discarding the supernatant and washing with PBST (Tween 0.1%) (4×300 µl), 100 µl goat anti-mouse HRP-conjugated secondary antibody (IgG specific), diluted 1:1200 with PBST (Tween 0.1%), was added to each well. Then the plate was incubated with shaking at room temperature for 1 h using an orbital shaker (IKA MTS 2/4 digital microtiter shaker, 600 rpm). After discarding the supernatant, the wells were washed PBST (Tween 0.1%) (4×300 µl). To each well was then added 100 µl of ABTS Ready-to-USE substrate solution (from Invitrogen). The plate was allowed to develop at room temperature for 20 min at room temperature and all the absorbances were measured using a microplate reader with a test wavelength of 450 nm.

5.4.13 Enzyme-linked immunosorbent assay (ELISA) for competitive antibody
binding inhibition studies using the anti-iso[4]LGE₂ mAb. For antibody binding inhibition studies to measure cross-reactivities, iso[4]LGE₂-BSA was used as coating reagent and iso[4]LGE₂-HSA was used as a standard for purified anti-iso[4]LGE₂ mAb. LGE₂-BSA, CEP-BSA, CEP-HSA, HSA and LGE₂-KLH served as inhibitors. For each inhibitor, up to eleven serial dilutions of a standard, a blank, and a positive control, were run. Each well of a 96 well ELISA plate was coated with iso[4]LGE₂-BSA solution (100 μl), prepared by diluting a solution containing 1016 μM iso[4]LGE₂-BSA to 1.12 μM (0.3 μg/well) with pH 7.4 PBS (10 mM). The plate was incubated overnight at 4 °C, then washed with 10 mM PBST (Tween 0.1%) (4×300 μl) and then blocked by incubating 1 h at 37 °C with 300 μl of 2% BSA in 10 mM PBS. Then the plate was rinsed with 10 mM PBST (Tween 0.1%) (4×300 μl). Eleven serial dilutions of inhibitor or standard (120 μl each with a dilution factor of 0.2) were pre-incubated at 37 °C for 1 h with protein G purified anti-iso[4]LGE₂ monoclonal antibody from HL-1 serum free medium (120 μl, 5 μg/ml in 10 mM PBS). The initial inhibitor and standard concentrations were iso[4]LGE₂-HSA (446.5 μM), iso[4]LGE₂-KLH (446.5 μM), LGE₂-BSA (446.5 μM), CEP-BSA (446.5 μM), CEP-HSA (446.5 μM), BSA (446.5 μM) and HSA (446.5 μM) respectively. These were prepared by diluting a iso[4]LGE₂-HSA (893.3 μM), iso[4]LGE₂-KLH (721.3 μM), LGE₂-BSA (1974 μM), CEP-BSA (496 μM), CEP-HSA (623.6 μM), BSA (1500 μM) and HSA (1500 μM) with 10 mM PBS, respectively. Blank wells were filled with 2% BSA/PBS (100 μl). Positive control wells were filled with the diluted antibody solution (50 μl) and PBS (50 μl). The antibody antigen complex solutions (100 μl) were then added in duplicate to their respective halves of the plate, which was then incubated at room temperature with gentle agitation on an orbital shaker (IKA MTS 2/4 digital
microtiter shaker, 600 rpm) for 1 h. After discarding the supernatant and washing with PBST (Tween 0.1%) (4×300 µl), 100 µl goat anti-mouse HRP-conjugated secondary antibody (IgG specific), diluted 1:1200 with PBST (Tween 0.1%), was added to each well and then the plate was incubated with shaking at room temperature for 1 h on the orbital shaker. After discarding the supernatant, the wells were washed PBST (Tween 0.1%) (4×300 µl). To each well was then added 100 µl of Ready-to-Use substrate solution ABTS. The plate was allowed to develop at room temperature for 20 min at room temperature and all the absorbances were measured using a microplate reader with a test wavelength of 450 nm. The absorbance in each well was measured with a dual-wavelength Bio-Rad 450 microplate reader (Hercules, CA) with detection at 405 nm relative to 655 nm. Absorbance values for duplicate assays were averaged and scaled to make the maximum curve fit value close to 100 percent. The averaged scaled percent absorbance values were plotted against the log of concentration. Theoretical curves for each plot were fit to the absorbance data with a four parameter logistic function, f(x) = (a-d)/[1+(x/c)^b]+d using Origin 9.0 (OriginLab Corporation, Northampton, MA). Parameter a = the asymptotic maximum absorbance, b = slope at the inflection point, c = the inhibitor concentration at the 50 % absorbance value (IC50), and d = the asymptotic minimum absorbance.

5.4.14 Western analysis. It was carried out according to published protocols.7-8 Iso[4]LGE2-BSA (~ 10 ng -10 µg) was dissolved in Laemmli sample buffer,9 fractionated on a 4-12% SDS-PAGE, and electro blotted onto a polyvinyl difluoride (PVDF) membrane (Millipore, Bedford, MA). The PVDF membranes were then treated with
Odyssey blocking buffer (Li-Cor cat# 927-40000) and probed using anti-iso[4]LGE2 mAb at various dilutions. Goat anti-mouse IRDye® 680 (Odyssey cat# 926-32220) was used as secondary antibody. The immunoreactive bands were tested and quantified using a Li-Cor infrared imaging system.
5.5 References


Appendix
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<th>Adduct Form</th>
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Table 2.1S Lysine modifications with iso[4]LGE2

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Table 2.2S Peptide of calpain-1 modified by iso[4]LGE2, as identified by Mascot analysis

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Table 2.3S Mascot characterization of the doubly charged ion m/z 690.88 (SVTGAKQVNY) showed series of fragment ions sufficient to unambiguously identify a iso[4]LGE2 modification on the calpain-1 lysyl residue (K280).

Figure 2.1S Domain architecture of calpain-1 and three active sites (left panel). Minicalpain was shown as subdomain IIa and domain IIb, with catalytic triad residue Cys 115, His 272 and Asn 296 (right panel).
Figure 2.2S Coomassie blue staining image of calpain-1 after 2 h treatment with 460 equivalents of iso[4]LGE₂

Figure 2.3S Western blot images of calpain-1 after 30 min, 1 h and 3 h treatment with 460 equivalents of iso[4]LGE₂
Use Anti-Calpain 1 catalytic subunit primary antibody

Use Anti-Calpain 1 small subunit primary antibody

Use Anti-[Iso4]LGE2 monoclonal antibody

Figure 2.4S Western blot images of calpain-1 after 1 h, 3 h and overnight treatment with 50 and 100 equivalents of iso[4]LGE2
Figure 2.5S Western blot images of calpain-1 after 1 h, 3 h, 6 h and overnight treatment with 5 and 10 equivalents of iso[4]LGE₂

Figure 2.6S Western blot images of calpain-1 after 24 h and 48 h treatment with 2, 5, 10 and 20 equivalents of iso[4]LGE₂
Figure 2.7S ESI-MS of synthesis fluorescence labeled iso[4]LGE₂ intermediate (2.3, panel A1 and A2) and final product (2.4, panel A1 and A2) in both negative mode and positive mode.

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<tr>
<th>Adduct Form</th>
<th>Formula</th>
<th>Mass (Cal.)</th>
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</thead>
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<tr>
<td>Pyrrole monodehydrated</td>
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<tr>
<td>Hydroxylactam</td>
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<td>615.27</td>
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<td>Hydroxylactam monodehydrated</td>
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Table 2.4S Lysine modifications with fluorescence labeled iso[4]LGE₂.
Table 2.5S Mascot characterization of the doubly charged ion m/z 620.86 (LEKAAK) and triply charged ion m/z 537.96 (VKVGVNGFGR) showed series of fragment ions sufficient to unambiguously identify a fluorescence labeled iso[4]LGE₂ modification on the GAPDH lysyl residue.

**Table A**

<table>
<thead>
<tr>
<th>Seq.</th>
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<th>y*</th>
<th>#</th>
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<td>3</td>
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<td>476.7517</td>
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<td>998.5492</td>
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<tr>
<td>4</td>
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<td>512.2702</td>
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<td>289.1870</td>
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<tr>
<td>5</td>
<td>1094.5703</td>
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</tr>
<tr>
<td>6</td>
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**Table b**

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<tr>
<td>10</td>
<td>R</td>
<td>175.1190</td>
<td>88.0631</td>
<td>158.0924</td>
<td>79.5498</td>
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</table>

Table 2.5S Mascot characterization of the doubly charged ion m/z 620.86 (LEKAAK) and triply charged ion m/z 537.96 (VKVGVNGFGR) showed series of fragment ions sufficient to unambiguously identify a fluorescence labeled iso[4]LGE₂ modification on the GAPDH lysyl residue.
Figure 2.8S The peak area of the amino acids was compared with the peak area of the quantitative standard amino acids (Standard H). The detection limit was tested by 25, 50, 100, 250, 400 and 500 pmol of standard amino acids. Reliable relative standard deviation (< 10%) was achieved and the detection limit was determined with standard amino acids up to 50 pmol and was plotted with all amino acids in (B).
Table 2.6S (1) Amino acid analysis template.
<table>
<thead>
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<th>NAME/ID#</th>
<th>GAPDH(Rabbit) Dimer</th>
<th>Injection Id: 1489</th>
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<tr>
<td>VOL HYDROLYZED</td>
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<td>RATIO APPLIED</td>
<td>0.01600</td>
<td></td>
</tr>
<tr>
<td>MOLECULAR WEIGHT</td>
<td>35780.00</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>AMINO ACID</th>
<th>Known Composition pmol analyzed</th>
<th>pmol protein</th>
<th>over 15%</th>
<th>under 15%</th>
<th>exp comp</th>
<th>integer comp</th>
<th>% int err</th>
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<tr>
<td>His (H)</td>
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</tr>
<tr>
<td>Glu (Q)</td>
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<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Arg (R)</td>
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<td>0.31</td>
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**TOTAL #AA 326.00**

**ESTIMATED AMOUNT**

- **WITHIN 15% 0.43**
- **ERROR 26.78**

**PMOL PROTEIN 0.43**

- **15% OF PROTEIN 0.06**
- **-15% OF PROTEIN 0.36**
- **→15% OF PROTEIN 0.49**

**15% OF PROTEIN AMINO ACID 130.32**

**TOTAL PMOL HYDROL 27.00**

**TOTAL UGRAMS 0.97**

**CONC pmol/ul 1.35**

**CONC ug/ul 0.05**

Table 2.6S (2) Amino acid composition of dimer of modified GAPDH.
<table>
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<th>Injection Id: 1494</th>
</tr>
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<tbody>
<tr>
<td>VOL HYDROLYZED</td>
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<tr>
<td>RATIO APPLIED</td>
<td>0.01600</td>
<td></td>
</tr>
<tr>
<td>MOLECULAR WEIGHT</td>
<td>35780.00</td>
<td></td>
</tr>
<tr>
<td>AMINO ACID</td>
<td>Known Composition</td>
<td></td>
</tr>
<tr>
<td></td>
<td>pmol analyzed</td>
<td>pmol protein</td>
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<td>His (H)</td>
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<td>Asn (N)</td>
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<td>Ser (S)</td>
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<td>13.29</td>
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<tr>
<td>Gln (Q)</td>
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<td>0.00</td>
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<td>Arg (R)</td>
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<td>5.96</td>
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<td>Gly (G)</td>
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<td>Asp (D)</td>
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<td>Glu (E)</td>
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<td>Thr (T)</td>
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<td>Ala (A)</td>
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<td>+15% OF PROTEIN</td>
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Table 2.6S (3) Amino acid composition of trimer of modified GAPDH.
Table 2.6S (4) Amino acid composition of tetramer of modified GAPDH.

Table 2.6S Amino acid analysis template and amino acid composition of dimer, trimer and tetramer of modified GAPDH.
Figure 2.9S Solution trypsin digested unmodified GAPDH was monitored by iso4 fluorescence tag and tryptophan peptides fluorescence channels.

Figure 2.10S Solution trypsin digested fluorescently labeled iso[4]LGE2 modified GAPDH was monitored by iso4 fluorescence tag and tryptophan peptides fluorescence channels.
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<th>Peptides</th>
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</tr>
<tr>
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Table 2.7S Calculated tryptic digested GAPDH peptides with one miss cleavage. All cysteines have been treated with iodoacetamide to form carbamidomethyl-cysteine. Methionines have been oxidized to form methionine sulfoxide. Using monoisotopic masses of the occurring amino acid residues and giving peptide masses as [M].
\[ [M+H]^+ = \text{peptide }#1 + \text{peptide }#2 + 601.2934 + 1.0078 = \text{peptide }#1 + \text{peptide }#2 + 602.3012 \]

| A | B | C | D | E | F | G | H | I | J | K | L | M | N | O | P | Q | R | S | T | U | V | W | X | Y | Z |
| A | B | C | D | E | F | G | H | I | J | K | L | M | N | O | P | Q | R | S | T | U | V | W | X | Y | Z |

\[ [M+2H]^{2-} = (\text{peptide }#1 + \text{peptide }#2 + 601.2934 + 1.0078 \times 2)/2 = (\text{peptide }#1 + \text{peptide }#2 + 603.3091)/2 \]

| A | B | C | D | E | F | G | H | I | J | K | L | M | N | O | P | Q | R | S | T | U | V | W | X | Y | Z |
| A | B | C | D | E | F | G | H | I | J | K | L | M | N | O | P | Q | R | S | T | U | V | W | X | Y | Z |
Table 2.8S Mass of cross-linked peptides [M], including +1 to +4 charges

<table>
<thead>
<tr>
<th>Peptide #1</th>
<th>Peptide #2</th>
<th>Peptide #3</th>
<th>Peptide #4</th>
<th>Peptide #5</th>
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\[ M_{\text{prod}} = (\text{peptide } #1 + \text{peptide } #2 + 0.01234 + 1.0078 \times 4 + 4) = (\text{peptide } #1 + \text{peptide } #2 + 0.043169) \times 3 \]
Figure 2.11S TOF MS and MSMS of ion 640.9626.


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<th>Adducts—no fluorescence tag</th>
<th>Adduct Formula</th>
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Acetyl-Gly-Lys-O-Methyl Ester (Monoisotopic molecular weight): 259.1532
H:1.0078 H$_2$O:18.0106

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(3) Amyloid (11-17) “EVHHQKL” peptide reaction with iso[4]LGE₂

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<th>Adduct Formula</th>
<th>Adduct MW</th>
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Adducts--no fluorescence tag  Adduct Formula  Adduct MW  Product MW  Plus one charge (+1)  Plus two charges (+2)  Plus three charges (+3)  Plus four charges (+4)
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Pyrrole-H2O  C6H5NO.H2O  298.9133  1187.6703  1188.6781  594.8429  396.8979  297.9254
Lactam  C5H3N.O  332.1988  1221.6750  1222.6828  611.8453  408.2328  306.4266
Lactam-H2O  C5H3N.O.H2O  314.1882  1203.6652  1204.6730  602.8404  402.2295  301.9241
Hydroxylactam  C6H5NO.OH  348.1937  1237.6707  1238.6785  619.8431  413.5647  310.4255
Hydroxylactam-H2O  C6H5NO.OH.H2O  330.1831  1219.6601  1220.6679  610.8375  407.5612  305.9228
Hydroxylactam-2H2O  C25H42NO13  312.1725  1201.6495  1202.6573  601.8325  401.5576  301.4202
Cross-link  C30H51NO  337.2379  2113.1685  2114.1763  1057.5921  705.3973  529.2999
Cross-link-H2O  C30H51NO.H2O  319.2273  2095.1579  2096.1657  1048.5867  699.3938  524.7973
Cross-link-2H2O  C30H51NO2.H2O  301.2168  2077.1473  2078.1551  1039.5814  693.3902  520.2946
Bis-pyrrole  C30H51NO2  634.4233  2409.3461  2410.3539  1205.6808  804.1231  603.3443
Bis-pyrrole-H2O  C30H51NO2.H2O  616.4128  2391.3355  2392.3433  1196.6755  798.1193  598.8447
Bis-pyrrole-2H2O  C30H51NO22.H2O  598.4027  2373.3249  2374.3327  1187.6702  792.1161  594.3390
Tris-pyrrole  C40H61NO3S  952.6428  3617.9587  3618.9666  1809.9872  1206.9941  905.4975
Tris-pyrrole-H2O  C40H61NO3S.H2O  934.6322  3599.9482  3600.9560  1800.9819  1200.9905  900.9948
Tris-pyrrole-3H2O  C40H61NO3S2.H2O  916.6216  3581.9376  3582.9454  1791.9766  1194.9870  896.4922
Tris-pyrrole-3H2O  C40H61NO3S2.H2O  898.6110  3563.9270  3564.9348  1782.9713  1188.9835  891.9895
Pyrr+Lac-H2O  C30H51NO6S.H2O  632.4077  2407.3305  2408.3383  1204.6730  803.4513  602.8404
Pyrr+Lac-2H2O  C30H51NO6S2.H2O  614.3971  2389.3209  2390.3287  1195.6682  797.4481  598.3380

(4) Amyloid (11-17) “EVHHQKL” peptide reaction with iso[4]LGE2-Fluo tag
EVHHQKL (Monoisotopic molecular weight): 889.476977, (+1) 890.454253, (+2)
445.745765  H:1.0078  H2O:18.0106

Adducts--no fluorescence tag  Adduct Formula  Adduct MW  Product MW  Plus one charge (+1)  Plus two charges (+2)  Plus three charges (+3)  Plus four charges (+4)
Pyrrole  C6H5NoO  583.2828  1472.7598  1473.7676  737.3877  491.9277  369.1977
Pyrrole-H2O  C6H5NoO.H2O  565.2723  1454.7493  1455.7571  728.8245  485.9242  364.6951
Lactam  C5H3NO  599.2778  1488.7548  1489.7626  745.3852  497.2594  373.1965
Lactam-H2O  C5H3NO.H2O  581.2672  1470.7442  1471.7520  736.3799  491.2559  368.6935
Hydroxylactam  C6H5No.OH  615.2727  1504.7497  1505.7575  753.3826  502.5910  377.1925
Hydroxylactam-H2O  C6H5No.OH.H2O  597.2621  1486.7391  1487.7469  744.3773  496.5875  372.6925
Hydroxylactam-2H2O  C6H5No.OH2.2H2O  579.2515  1468.7285  1469.7363  735.3702  490.5840  368.1899
Cross-link  C30H51NO  604.3168  3266.6979  3281.6553  1281.1315  1089.9077  817.6827
Cross-link-H2O  C30H51NO.H2O  586.3062  3248.6891  3263.6474  1182.1262  1083.9042  813.1801
Cross-link-2H2O  C30H51NO2.H2O  568.2956  3230.6785  3245.6341  1173.1209  1077.9006  808.6774
Bis-pyrrole  C30H51NO2  1168.5813  3830.9642  3844.9119  1472.7598  1277.9959  958.7489
Bis-pyrrole-H2O  C30H51NO2.H2O  1150.5707  3812.9536  3926.5013  1463.7545  1271.9923  954.2462
Bis-pyrrole-2H2O  C30H51NO22.H2O  1132.5601  3794.9430  3908.8907  1454.7492  1265.9888  949.7436
Figure 3.1S Acetyl-Gly-Lys-O-Methyl ester reactions with iso[4]LGE$_2$ in different molar ratio and time point

1. Acetyl-Gly-Lys-O-Methyl ester reacts with iso[4]LGE$_2$ (Dipeptide: Iso4 = 2000:1 molar ratio) in 50 mM NEM OAc pH 8.6 for 1 day, 4 days, 8 days, 15 days, 21 days and 50 days.

Modified One Adducts

Bispyrrole Trispyrrole

- H₂O
- 2H₂O
- 3H₂O

D


Modified One Adducts

Bispyrrole Trispyrrole

+ 16

C

176
Modified One

Adducts


-2H2O

-3H2O

-2H2O

-3H2O

H2O

H2O

H2O

H2O

177
(2) Acetyl-Gly-Lys-O-Methyl ester reacts with iso[4]LGE₂ (Dipeptide: Iso4 = 1000:1 molar ratio) in 50 mM NEM OAc pH 8.6 for 1 day, 4 days, 8 days, 15 days, 21 days and 50 days.
Modified One Adducts

Acetyl-Gly-Lys-O-Methyl ester reacts with iso[4]LGE2 (1000:1) in 50 mM NEM OAc pH 8.6 for 8 days

-2H$_2$O

-H$_2$O

Trispyrrole

Crosslink

Bispyrrole

-3H$_2$O

Modified One Adducts

Acetyl-Gly-Lys-O-Methyl ester reacts with iso[4]LGE2 (1000:1) in 50 mM NEM OAc pH 8.6 for 15 days

-2H$_2$O

-H$_2$O

+16

Trispyrrole

Bispyrrole

+16

+16

+16
Acetyl-Gly-Lys-O-Methyl ester reacts with iso[4]LGE₂ (1000:1) in 50 mM NEM OAc pH 8.6 for 21 days.
(3) Acetyl-Gly-Lys-O-Methyl ester reacts with iso[4]LGE2 (Dipeptide: Iso4 = 200:1 molar ratio) in 50 mM NEM OAc pH 8.6 for 1 day, 4 days, 8 days, 15 days, 21 days and 50 days.

A

B

Modified One Adducts

Crosslink

Bispyrrole

Trispyrrole

Acetyl-Gly-Lys-O-Methyl ester reacts with iso[4]LGE2 (200:1) in 50 mM NEM OAc pH 8.6 for 1 day

-3H₂O

+16

+16

Bispyrrole

Trispyrrole

Acetyl-Gly-Lys-O-Methyl ester reacts with iso[4]LGE2 (200:1) in 50 mM NEM OAc pH 8.6 for 4 days

-2H₂O

+16

+16

+16
Acetyl-Gly-Lys-O-Methyl ester reacts with Iso[4] no fluorescence tag (200:1) in 50 mM NEM OAc pH 8.6 for 8 days

Modified One Adducts

Crosslink

Bispyrrole

Trispyrrole

-3H₂O

+16

-2H₂O

+16

+16

C

Acetyl-Gly-Lys-O-Methyl ester reacts with Iso[4] no fluorescence tag (200:1) in 50 mM NEM OAc pH 8.6 for 15 days

Modified One Adducts

Crosslink

Bispyrrole

Trispyrrole

-3H₂O

+16

-2H₂O

+16

+16

D
Acetyl-Gly-Lys-O-Methyl ester reacts with Iso[4] no fluorescent tag (200:1) in 50 mM NEM OAc pH 8.6 for 21 days

Modified One Adducts

Crosslink

Bispyrrole

-\(\text{H}_2\text{O}\)

-2\(\text{H}_2\text{O}\)

Trispyrrole

\(+16\)

\(+16\)

\(+16\)

\(+16\)

Acetyl-Gly-Lys-O-Methyl ester reacts with Iso[4] no fluorescent tag (200:1) in 50 mM NEM OAc pH 8.6 for 50 days

Modified One Adducts

Crosslink

Bispyrrole

-\(\text{H}_2\text{O}\)

-2\(\text{H}_2\text{O}\)

Trispyrrole

\(+16\)

\(+16\)

\(+16\)

\(+16\)
Figure 3.2S ESI-MSMS of molecular ions at A) aminal cross-link, B) pyrrole, C) lactam and D) hydroxylactam corresponding to a iso[4]LGE2–(N-acetyl-Gly-Lys-OMe) adduct.
Figure 3.3S Acetyl-Gly-Lys-O-Methyl ester reactions with iso[4]LGE2-Fluo tag in different molar ratio and time point

(1) Acetyl-Gly-Lys-O-Methyl ester reacts with iso[4]LGE2-Fluo tag (Dipeptide : Iso4 = 2000:1) in 50 mM NEM OAc pH 8.6 for 1 day, 4 days, 15 days, 21 days and 50 days.

Modified One Adducts


Modified One Adducts

Bispyrrole
(2) Acetyl-Gly-Lys-O-Methyl ester reacts with iso[4]LGE₂-Fluo tag (Dipeptide : Iso4 = 200:1) in 50 mM NEM OAc pH 8.6 for 1 day, 4 days, 15 days, 21 days and 50 days.
Acetyl-Gly-Lys-O-Methyl ester reacts with iso[4]LGE2-Fluo tag (200:1) in 50 mM NEM OAc pH 8.6 for 4 days

Modified One Adducts

Crosslink

Bispyrrole

Acetyl-Gly-Lys-O-Methyl ester reacts with iso[4]LGE2-Fluo tag (200:1) in 50 mM NEM OAc pH 8.6 for 15 days

Modified One Adducts

Crosslink

-H2O

-2H2O

Acetyl-Gly-Lys-O-Methyl ester reacts with iso[4]LGE2-Fluo tag (200:1) in 50 mM NEM OAc pH 8.6 for 15 days
Acetyl-Gly-Lys-O-Methyl ester reacts with iso[4]LGE2-Fluo tag (200:1) in 50 mM NEM OAc pH 8.6 for 21 days

Acetyl-Gly-Lys-O-Methyl ester reacts with iso[4]LGE2-Fluo tag (200:1) in 50 mM NEM OAc pH 8.6 for 50 days
Figure 3.4S: ESI-MSMS of molecular ions at A) animal cross-link, B) pyrrole, C) lactam and D) hydroxylactam corresponding to a Iso[4]LGE2-FluoTag - (N-acetyl-Gly-Lys-OMe) adduct.
Figure 3.5S Amyloid (11-17) “EVHHQKL” peptide reactions with iso[4]LGE2 in different molar ratio and time point

(1) Amyloid (11-17) “EVHHQKL” reacts with iso[4]LGE2 (Peptide: Iso4 = 2000:1) in 50 mM NEM OAc pH 8.6 for 1 day, 4 days, 15 days, 21 days and 50 days.

Modified One Adducts

Crosslink Bispyrrole Trispyrrole

Bispyrrole

+16

Amyloid (11-17) "EVHHQKL" reacts with iso[4]LGE2 (2000:1) in 50 mM NEM OAc pH 8.6 for 1 day

Bispyrrole

+16

Amyloid (11-17) "EVHHQKL" reacts with iso[4]LGE2 (2000:1) in 50 mM NEM OAc pH 8.6 for 4 days

Trispyrrole

193
Amyloid (11-17) "EVHHQKL" reacts with iso[4]LGE2 (2000:1) in 50 mM NEM OAc pH 8.6 for 15 days

Amyloid (11-17) "EVHHQKL" reacts with iso[4]LGE2 (2000:1) in 50 mM NEM OAc pH 8.6 for 21 days
Amyloid (11-17) "EVHHQKL" reacts with iso[4]LGE2 (200:1) in 50 mM NEM OAc pH 8.6 for 50 days.

Amyloid (11-17) "EVHHQKL" reacts with iso[4]LGE2 (Peptide: Iso4 = 200:1) in 50 mM NEM OAc pH 8.6 for 1, 4, 7, 15, 21, and 50 days.
Amyloid (11-17) "EVHHQKL" reacts with iso[4]LGE2 (200:1) in 50 mM NEM OAc pH 8.6 for 4 days

Amyloid (11-17) "EVHHQKL" reacts with iso[4]LGE2 (200:1) in 50 mM NEM OAc pH 8.6 for 7 days
Modified One Adducts

Amyloid (11-17) "EVHHQKL" reacts with iso[4]LGE2 (200:1) in 50 mM NEM OAc pH 8.6 for 15 days

D

Bispyrrole

-2H₂O

+16

Modified One Adducts

Amyloid (11-17) "EVHHQKL" reacts with iso[4]LGE2 (200:1) in 50 mM NEM OAc pH 8.6 for 21 days

E

-2H₂O

+16

+16

-197
Amyloid (11-17) “EVHHQKL” peptide reactions with iso[4]LGE2-Fluo tag in different molar ratio and time point

(1) Amyloid (11-17) “EVHHQKL” reacts with iso[4]LGE2-Fluo tag (Peptide: Iso4 = 200:1) in 50 mM NEM OAc pH 8.6 for 1 day, 4 days, 7 days, 15 days, 21 days and 50 days.

No Obvious crosslink or bispyrrole peak formed
Amyloid (11-17) "EVHHQKL" reacts with iso[LGE2-FluoTag (2000:1) in 50 mM NEM OAc pH 8.6 for 4 days

Modified One
Adducts

Crosslink

-H$_2$O

Amyloid (11-17) "EVHHQKL" reacts with iso[LGE2-FluoTag (2000:1) in 50 mM NEM OAc pH 8.6 for 7 days

Modified One
Adducts

Crosslink

-H$_2$O

Crosslink
Amyloid (11-17) "EVHHQKL" reacts with iso[4]LGE2-FluoTag (2000:1) in 50 mM NEM OAc pH 8.6 for 15 days.

Amyloid (11-17) "EVHHQKL" reacts with iso[4]LGE2-FluoTag (2000:1) in 50 mM NEM OAc pH 8.6 for 21 days.
Amyloid (11-17) "EVHHQKL" reacts with iso[4]LGE2-FluoTag (Peptide: Iso4 = 200:1) in 50 mM NEM OAc pH 8.6 for 50 days.

(2) Amyloid (11-17) “EVHHQKL” reacts with iso[4]LGE2-Fluo tag (Peptide: Iso4 = 200:1) in 50 mM NEM OAc pH 8.6 for 1 day, 4 days, 7 days, 15 days, 21 days and 50 days.
Amyloid (11-17) "EVHHQKL" reacts with iso[4]LGE2-FluoTag (200:1) in 50 mM NEM OAc pH 8.6 for 4 days.

Amyloid (11-17) "EVHHQKL" reacts with iso[4]LGE2-FluoTag (200:1) in 50 mM NEM OAc pH 8.6 for 7 days.
Amyloid (11-17) "EVHHQKL" reacts with iso[4]LGE2-FluoTag (200:1) in 50 mM NEM OAc pH 8.6 for 15 days.

Modified One Adducts

Crosslink - H₂O

Amyloid (11-17) "EVHHQKL" reacts with iso[4]LGE2-FluoTag (200:1) in 50 mM NEM OAc pH 8.6 for 21 days.

Modified One Adducts

Crosslink - H₂O

Crosslink - H₂O
Amyloid (11-17) "EVHHQKL" reacts with iso[4]LGE2-FluoTag (200:1) in 50 mM NEM OAc pH 8.6 for 50 days.

Modified One Adducts

Crosslink

-H$_2$O

Figure 4.1S MALDI-TOF spectrums of iso[4]LGE$_2$-pyrrole
Figure 4.2S MALDI-TOF spectrums of iso[4]LGE2 and Acetyl-Gly-Lys-O-methyl ester ---- indicating new peaks besides the matrix.

No oxidized crosslink and bispyrrole

No crosslink formed, a little bispyrrole formed

More than 8 days incubation

Figure 4.3S MALDI-TOF spectrums of HPLC-purified iso[4]LGE2-pyrrole reaction under air within 8 days.
Pure Iso[4]LGE2-pyrrole was added Ac-Lysine and reacted for 2 days. Iso[4]LGE2-pyrrole didn’t oxidize through this way. It didn’t relate with molar ratio and pH.

Figure 4.4S MALDI-TOF spectrums of purified iso[4]LGE2-pyrrole reaction with Nε-acetyl-L-lysine under air for 2 days.

Pure Iso[4]LGE2-pyrrole was added L-Lysine (1:10 molar ratio), reacted under air for 2 days.

Figure 4.5S MALDI-TOF spectrums of purified iso[4]LGE2-pyrrole reaction with L-lysine under air for 2 days.
Pure pyrrole was added Amyloid peptide in H2O (1:10 molar ratio), reacted under air for 6 h. No other oxidized peak formed No Lactam or Hydrolactam formed.

Pure pyrrole was added Amyloid peptide in H2O (1:10 molar ratio), reacted under air overnight. No other oxidized peak formed No Lactam or Hydrolactam formed.

Pure pyrrole was added Amyloid peptide in H2O (1:10 molar ratio), reacted under air for 2 days. No other oxidized peak formed No Lactam or Hydrolactam formed.

Figure 4.6S MALDI-TOF spectrums of purified iso[4]LGE2-pyrrole reaction with β-Amyloid peptide under air for 2 days.

The peak 544 was shown up after adding Ethanolamine.

Pure pyrrole was added Ethanolamine equal ratio and reacted for 2 days. Pyrrole decreased through this way. pH=7.

No pyrrole-H2O Pure pyrrole was added Ethanolamine much more excess and reacted for 2 h. Pyrrole couldn't be observed. pH=7.

Figure 4.7S MALDI-TOF spectrums of purified iso[4]LGE2-pyrrole reaction with ethanolamine under air.
Figure 4.8S MALDI-TOF spectrums of purified iso[4]LGE$_2$-pyrrole reaction with Acetyl-Gly-Lys-O-Methyl Ester under air.

Figure 4.9S MALDI-TOF spectrums of purified iso[4]LGE$_2$-pyrrole reaction with HPLC-purified Acetyl-Gly-Lys-O-Methyl Ester under air.
### Table 4.1S Summaries of HPLC-purified iso[4]LGE₂-pyrrole reaction with different chemicals.

(a) HPLC-purified iso[4]LGE₂-pyrrole reactions with Acetyl-Gly-Lys-O-Methyl Ester and Initiator (2,2’-Azobis(2-methylpropionamidine) dihydrochloride)

<table>
<thead>
<tr>
<th>Molar ratio</th>
<th>2 h reaction</th>
<th>Overnight reaction</th>
<th>A week reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyrrole: Acetyl-Gly-Lys-O-Methyl Ester =1:0.1</td>
<td>Pyrrole—no changes</td>
<td>Pyrrole—decreased little bit, very little oxidized product formed</td>
<td>Almost no pyrrole, little oxidized product formed</td>
</tr>
<tr>
<td>Pyrrole: Acetyl-Gly-Lys-O-Methyl Ester =1:1</td>
<td>Pyrrole—decreased little bit, little oxidized product formed</td>
<td>Pyrrole—decreased little bit, more oxidized product formed</td>
<td>Almost no pyrrole, much oxidized products formed</td>
</tr>
<tr>
<td>Pyrrole: Initiator=1:0.1</td>
<td>Pyrrole—decreased, no oxidized product formed</td>
<td>No pyrrole or oxidized product</td>
<td>No pyrrole or oxidized product</td>
</tr>
<tr>
<td>Pyrrole: Initiator=1:1</td>
<td>No pyrrole or oxidized product</td>
<td>No pyrrole or oxidized product</td>
<td>No pyrrole or oxidized product</td>
</tr>
</tbody>
</table>

(b) HPLC-purified iso[4]LGE₂-pyrrole reactions with Vitamin C, Acetyl-Gly-Lys-O-Methyl Ester and Initiator (2,2’-Azobis(2-methylpropionamidine) dihydrochloride)

<table>
<thead>
<tr>
<th>Molar ratio</th>
<th>2 h reaction</th>
<th>Overnight reaction</th>
<th>A week reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin C: Pyrrole=0.1:1</td>
<td>Pyrrole—no changes</td>
<td>No pyrrole</td>
<td>No pyrrole and formed a peak (+670), no other oxidized products detected</td>
</tr>
<tr>
<td>Vitamin C: Pyrrole: Acetyl-Gly-Lys-O-Methyl Ester =0.1:1:0.1</td>
<td>Pyrrole—no changes, No oxidized product formed</td>
<td>Pyrrole—decreased little bit, very little oxidized product formed</td>
<td>Pyrrole—decreased little bit, very little oxidized product formed</td>
</tr>
<tr>
<td>Vitamin C: Pyrrole: Acetyl-Gly-Lys-O-Methyl Ester =0.1:1:1</td>
<td>Pyrrole—decreased little bit, little oxidized product formed</td>
<td>Pyrrole—decreased little bit, more oxidized product formed</td>
<td>Pyrrole—decreased little bit, much more oxidized product formed. Also formed peak (+670)</td>
</tr>
<tr>
<td>Vitamin C: Pyrrole: Initiator=0.1:1:0.1</td>
<td>Pyrrole—no changes, No oxidized product formed</td>
<td>No pyrrole</td>
<td>No pyrrole, no oxidized product formed. Also formed peak (+670)</td>
</tr>
<tr>
<td>Vitamin C: Pyrrole: Initiator=0.1:1:1</td>
<td>Pyrrole—no changes, No oxidized product formed</td>
<td>No pyrrole</td>
<td>No pyrrole, no oxidized product formed. Also formed peak (+670), and some other unknown peaks</td>
</tr>
</tbody>
</table>

(c) HPLC-purified iso[4]LGE₂-pyrrole reactions with Vitamin E, Acetyl-Gly-Lys-O-Methyl Ester and Initiator (2,2’-Azobis(2-methylpropionamidine) dihydrochloride)

<table>
<thead>
<tr>
<th>Molar ratio</th>
<th>2 h reaction</th>
<th>Overnight reaction</th>
<th>A week reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin E: Pyrrole=0.1:1</td>
<td>Pyrrole—no changes</td>
<td>No pyrrole or oxidized product</td>
<td>No pyrrole or oxidized product</td>
</tr>
</tbody>
</table>

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Vitamin E: Pyrrole: Acetyl-Gly-Lys-O-Methyl Ester = 0.1:1:0.1

| Vitamin E: Pyrrole: Acetyl-Gly-Lys-O-Methyl Ester = 0.1:1:1 | Pyrrole—decreased little bit, little oxidized product formed | Pyrrole—decreased, little oxidized product formed | No pyrrole or oxidized product

No pyrrole formed

Vitamin E: Pyrrole: Initiator=0.1:1:1:1

| Vitamin E: Pyrrole: Initiator=0.1:1:1:1 | Pyrrole—decreased, No oxidized product formed | No pyrrole or oxidized product | No pyrrole or oxidized product

No pyrrole, some known peaks

Molar ratio

<table>
<thead>
<tr>
<th>2 h reaction</th>
<th>Overnight reaction</th>
<th>2 days reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyrrole: Vitamin E=1:0.01</td>
<td>Pyrrole—no changes. No oxidized product formed</td>
<td>Pyrrole—decreased. No oxidized product formed</td>
</tr>
</tbody>
</table>

Molar ratio

<table>
<thead>
<tr>
<th>4 h reaction</th>
<th>Overnight reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyrrole: Vitamin E:initiator =1:0.01:0.01</td>
<td>No pyrrole left and no oxidation</td>
</tr>
</tbody>
</table>

(d) HPLC-purified iso[4]LGE₂-pyrrole reactions with H atom donor ((R)-(−)-2-(2,5-Dihydrophenyl)glycine), Acetyl-Gly-Lys-O-Methyl Ester and Initiator (2,2'-Azobis(2-methylpropionamide) dihydrochloride)

<table>
<thead>
<tr>
<th>H atom Donor: Pyrrole=5:1</th>
<th>No pyrrole or oxidized product</th>
<th>No pyrrole or oxidized product</th>
<th>No pyrrole or oxidized product</th>
</tr>
</thead>
<tbody>
<tr>
<td>H atom Donor: Pyrrole: Acetyl-Gly-Lys-O-Methyl Ester =5:1:0.1</td>
<td>No pyrrole or oxidized product</td>
<td>No pyrrole or oxidized product</td>
<td>No pyrrole or oxidized product</td>
</tr>
<tr>
<td>H atom Donor: Pyrrole: Acetyl-Gly-Lys-O-Methyl Ester =2:1:1</td>
<td>No pyrrole, little oxidized products formed</td>
<td>No pyrrole, little oxidized products formed</td>
<td>No pyrrole, some known peaks</td>
</tr>
<tr>
<td>H atom Donor: Pyrrole: Initiator=5:1:0.1</td>
<td>No pyrrole or oxidized product</td>
<td>No pyrrole or oxidized product</td>
<td>No pyrrole or oxidized product</td>
</tr>
<tr>
<td>H atom Donor: Pyrrole: Initiator=5:1:1</td>
<td>No pyrrole or oxidized product</td>
<td>No pyrrole or oxidized product</td>
<td>No pyrrole or oxidized product</td>
</tr>
</tbody>
</table>

Molar ratio

<table>
<thead>
<tr>
<th>2 h reaction</th>
<th>Overnight reaction</th>
<th>2 days reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyrrole: H atom donor =5:1</td>
<td>Pyrrole—no changes. No oxidized product formed</td>
<td>Pyrrole—no changes. No oxidized product formed</td>
</tr>
</tbody>
</table>

Molar ratio

<table>
<thead>
<tr>
<th>4 h reaction</th>
<th>Overnight reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyrrole: H atom Donor =1:1</td>
<td>Very little pyrrole left and no oxidation</td>
</tr>
</tbody>
</table>

(e) HPLC-purified iso[4]LGE₂-pyrrole reactions with UV, rose bengal, methylene blue, Co₂(NO₃)₃, H₂O₂ K₂CO₃, dimethyldioirane, microwave, Di-tert-butyl peroxide, riboflavin (VB2), K₃Fe(CN)₆, FeCl₃, K₂S₂O₈.

<table>
<thead>
<tr>
<th>Molar ratio</th>
<th>30 min</th>
<th>1 h reaction</th>
<th>2 h reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyrrole: rose bengal=1:1, UV, air</td>
<td>No pyrrole or oxidized product</td>
<td>No pyrrole or oxidized product</td>
<td>No pyrrole or oxidized product</td>
</tr>
<tr>
<td>Pyrrole: methylene blue =1:1, UV, air</td>
<td>No pyrrole or oxidized product</td>
<td>No pyrrole or oxidized product</td>
<td>No pyrrole or oxidized product</td>
</tr>
<tr>
<td>UV light and air</td>
<td>No pyrrole or oxidized product</td>
<td>No pyrrole or oxidized product</td>
<td>No pyrrole or oxidized product</td>
</tr>
</tbody>
</table>

Condition

<table>
<thead>
<tr>
<th>2 min</th>
<th>8 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>UV light and air</td>
<td>Pyrrole—decreased. No oxidized product</td>
</tr>
<tr>
<td>Condition</td>
<td>formed</td>
</tr>
<tr>
<td>-----------</td>
<td>--------</td>
</tr>
<tr>
<td>Microwave and air</td>
<td>Pyrrole—decreased. No oxidized product formed</td>
</tr>
<tr>
<td>Condition</td>
<td>5 min</td>
</tr>
<tr>
<td>UV light and air, riboflavin (VB2)</td>
<td>No pyrrole or oxidized product</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Condition</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyrrole: Co$_2$(CO)$_8$=1:1, overnight reaction</td>
<td>No pyrrole or oxidized product</td>
</tr>
<tr>
<td>Pyrrole: H$_2$O$_2$K$_2$CO$_3$=1:1, overnight reaction</td>
<td>No pyrrole or oxidized product</td>
</tr>
<tr>
<td>Pyrrole: dimethyldioirane =1:1, 0.5 h reaction</td>
<td>No pyrrole or oxidized product</td>
</tr>
<tr>
<td>Pyrrole: Di-tert-butyl peroxide =1:1, 2 h reaction</td>
<td>No pyrrole or oxidized product</td>
</tr>
<tr>
<td>Catalytic amount of K$_3$Fe(CN)$_6$, 1 h reaction</td>
<td>No pyrrole or oxidized product</td>
</tr>
<tr>
<td>Catalytic amount of FeCl$_3$, 1 h reaction</td>
<td>No pyrrole or oxidized product</td>
</tr>
<tr>
<td>Catalytic amount of K$_2$S$_2$O$_8$, 1 h reaction</td>
<td>No pyrrole or oxidized product</td>
</tr>
</tbody>
</table>

Figure 4.10S MALDI-TOF spectrums of purified iso[4]LGE$_2$-pyrrole reaction with APS and TEMED at 25 °C under air.
Figure 4.11S MALDI-TOF spectrums of purified iso[4]LGE2-pyrrole reaction with APS and TEMED at 37 ºC under air.
Figure 4.12S MALDI-TOF spectrums of purified iso[4]LGE2-pyrrole reaction with 1 mM TMAO at 37 °C 3 h.

Figure 4.13S MALDI-TOF spectrums of purified iso[4]LGE2-pyrrole reaction with 1 mM TMAO/EDTA at 37 °C 3 h.
Pure pyrrole was added 1mM TEMPO in 10%ACN and reacted 37 degree 2 h. Ratio as Pyrrole:TEMPO=1:1

Figure 4.14S MALDI-TOF spectrums of purified iso[4]LGE2-pyrrole reaction with 1 mM TEMPO at 37 °C 2 h.

Pure pyrrole was added 1mM TEMPO in 10%ACN and reacted 37 degree 2 h. Ratio as Pyrrole:TEMPO:EDTA=1:1:1

Figure 4.15S MALDI-TOF spectrums of purified iso[4]LGE2-pyrrole reaction with 1 mM TEMPO/EDTA at 37 °C 2 h.
10 ul 100mM 3-Ethyl-2,4-dimethylpyrrole in ACN +10ul ACN 25 degree for 3h

Dimer

Trimer

10ul 100mM 3-Ethyl-2,4-dimethylpyrrole in ACN +10ul 100mM TEMPO in ACN 25 degree for 3h
10ul 100mM 3-Ethyl-2,4-dimethylpyrrole in ACN + 10ul 100mM TMAO in ACN.
25 degree for 3h

Figure 4.16S MALDI-TOF spectrums of 3-ethyl-2,4-dimethylpyrrole initiate reactions.
Figure 4.17S: NMR spectrums of 3-ethyl-2,4-dimethylpyrrole and 3-ethyl-1,2,4-trimethylpyrrole.
3-ethyl-1,2,4-trimethyl-1$^H$-pyrrole in ACN for 2 h at RT

Dimer

Trimer
3-ethyl-1,2,4-trimethyl-1$^H$pyrrole in ACN was added TEMPO for 2 h at RT

Figure 4.18S MALDI-TOF spectrums of 3-ethyl-1,2,4-trimethylpyrrole initiate reactions.
Figure 5.1S Cross-reactivity of iso[4]LGE2-HSA (■) with respect to LGE2-BSA (○) for immunized mice serum (10^4 dilution).
Figure 5.2S 20 plates (total 1920 wells) were done for the fusion step and 725 wells contained hybridoma cells.
<table>
<thead>
<tr>
<th></th>
<th>2E8 (VL)</th>
<th>9G8 (VL)</th>
<th>12G4 (L)</th>
<th>15A5 (L)</th>
<th>18G7 (XXL)</th>
<th>4G5 (S)</th>
<th>5B7 (M)</th>
<th>8B3 (S)</th>
<th>10C11 (M)</th>
<th>16E4 (M)</th>
<th>19A4 (M)</th>
<th>Mice Serum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iso4-BSA</td>
<td>0.336</td>
<td>2.826</td>
<td>2.557</td>
<td>0.271</td>
<td>2.528</td>
<td>0.123</td>
<td>0.12</td>
<td>0.112</td>
<td>0.136</td>
<td>0.12</td>
<td>0.119</td>
<td>2.825</td>
</tr>
<tr>
<td>Iso4-HSA</td>
<td>0.422</td>
<td>2.627</td>
<td>2.868</td>
<td>0.33</td>
<td>2.801</td>
<td>0.111</td>
<td>0.112</td>
<td>0.107</td>
<td>0.155</td>
<td>0.118</td>
<td>0.11</td>
<td>2.875</td>
</tr>
<tr>
<td>BSA</td>
<td>0.111</td>
<td>0.087</td>
<td>0.084</td>
<td>0.093</td>
<td>0.088</td>
<td>0.087</td>
<td>0.086</td>
<td>0.085</td>
<td>0.088</td>
<td>0.085</td>
<td>0.086</td>
<td>0.09</td>
</tr>
<tr>
<td>LGE2-BSA</td>
<td>0.238</td>
<td>0.105</td>
<td>0.106</td>
<td>0.199</td>
<td>0.835</td>
<td>0.087</td>
<td>0.085</td>
<td>0.084</td>
<td>0.103</td>
<td>0.087</td>
<td>0.088</td>
<td>0.193</td>
</tr>
<tr>
<td>LGE2-KLH</td>
<td>0.596</td>
<td>0.3</td>
<td>0.28</td>
<td>0.452</td>
<td>2.704</td>
<td>0.096</td>
<td>0.092</td>
<td>0.087</td>
<td>0.137</td>
<td>0.088</td>
<td>0.089</td>
<td>2.273</td>
</tr>
<tr>
<td>CEP-BSA</td>
<td>0.483</td>
<td>0.115</td>
<td>0.144</td>
<td>0.377</td>
<td>2.934</td>
<td>0.112</td>
<td>0.111</td>
<td>0.115</td>
<td>0.148</td>
<td>0.108</td>
<td>0.11</td>
<td>0.233</td>
</tr>
</tbody>
</table>

![Graph showing the results of experiments with different clones and proteins.](image-url)

<table>
<thead>
<tr>
<th>Clone 9G8(VL)</th>
<th>17.86 μM</th>
<th>3.572 μM</th>
<th>0.7144 μM</th>
<th>0.1429 μM</th>
<th>0.0286 μM</th>
<th>0.00572 μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iso4-HSA</td>
<td>0.101</td>
<td>0.139</td>
<td>0.341</td>
<td>1.721</td>
<td>2.901</td>
<td>2.866</td>
</tr>
<tr>
<td>LGE2-BSA</td>
<td>2.779</td>
<td>2.768</td>
<td>2.787</td>
<td>2.764</td>
<td>2.741</td>
<td>2.773</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Clone 12G4(L)</th>
<th>17.86 μM</th>
<th>3.572 μM</th>
<th>0.7144 μM</th>
<th>0.1429 μM</th>
<th>0.0286 μM</th>
<th>0.00572 μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iso4-HSA</td>
<td>0.162</td>
<td>0.282</td>
<td>1.077</td>
<td>1.581</td>
<td>1.908</td>
<td>2.161</td>
</tr>
<tr>
<td>LGE2-BSA</td>
<td>1.671</td>
<td>1.642</td>
<td>1.642</td>
<td>1.618</td>
<td>1.984</td>
<td>1.99</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Clone 18G7(XXL)</th>
<th>17.86 μM</th>
<th>3.572 μM</th>
<th>0.7144 μM</th>
<th>0.1429 μM</th>
<th>0.0286 μM</th>
<th>0.00572 μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iso4-HSA</td>
<td>0.158</td>
<td>0.28</td>
<td>0.796</td>
<td>1.039</td>
<td>1.908</td>
<td>1.23</td>
</tr>
<tr>
<td>LGE2-BSA</td>
<td>0.713</td>
<td>0.666</td>
<td>0.7</td>
<td>0.906</td>
<td>1.062</td>
<td>1.192</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Clone 2E8(VL)</th>
<th>17.86 μM</th>
<th>3.572 μM</th>
<th>0.7144 μM</th>
<th>0.1429 μM</th>
<th>0.0286 μM</th>
<th>0.00572 μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iso4-HSA</td>
<td>0.185</td>
<td>0.145</td>
<td>0.139</td>
<td>0.137</td>
<td>0.171</td>
<td>0.178</td>
</tr>
<tr>
<td>LGE2-BSA</td>
<td>0.141</td>
<td>0.132</td>
<td>0.135</td>
<td>0.134</td>
<td>0.165</td>
<td>0.183</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Clone 15A4(L)</th>
<th>17.86 μM</th>
<th>3.572 μM</th>
<th>0.7144 μM</th>
<th>0.1429 μM</th>
<th>0.0286 μM</th>
<th>0.00572 μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iso4-HSA</td>
<td>0.194</td>
<td>0.189</td>
<td>0.192</td>
<td>0.208</td>
<td>0.209</td>
<td>0.211</td>
</tr>
<tr>
<td>LGE2-BSA</td>
<td>0.195</td>
<td>0.210</td>
<td>0.208</td>
<td>0.214</td>
<td>0.205</td>
<td>0.216</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Clone 4G5(S)</th>
<th>17.86 μM</th>
<th>3.572 μM</th>
<th>0.7144 μM</th>
<th>0.1429 μM</th>
<th>0.0286 μM</th>
<th>0.00572 μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iso4-HSA</td>
<td>0.091</td>
<td>0.087</td>
<td>0.088</td>
<td>0.089</td>
<td>0.087</td>
<td>0.092</td>
</tr>
</tbody>
</table>
Figure 5.3S Indirect and competitive ELISA tests of first screening clones. 9G8, 12G4, 18G7 showed best responses. 9G8 and 12G4 were picked for first round of subclone.
<table>
<thead>
<tr>
<th>12G4</th>
<th>A2</th>
<th>A10</th>
<th>A11</th>
<th>A12</th>
<th>B2</th>
<th>B4</th>
<th>B5</th>
<th>B6</th>
<th>B8</th>
<th>B10</th>
<th>B12</th>
<th>C2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iso4-BSA</td>
<td>2.620</td>
<td>2.639</td>
<td>2.660</td>
<td>2.600</td>
<td>1.843</td>
<td>2.562</td>
<td>2.523</td>
<td>2.577</td>
<td>2.601</td>
<td>2.568</td>
<td>2.572</td>
<td>2.674</td>
</tr>
<tr>
<td>Iso4-HSA</td>
<td>2.879</td>
<td>2.788</td>
<td>2.841</td>
<td>2.785</td>
<td>2.305</td>
<td>2.809</td>
<td>2.863</td>
<td>2.857</td>
<td>2.884</td>
<td>2.775</td>
<td>2.825</td>
<td>2.833</td>
</tr>
<tr>
<td>LGE2-BSA</td>
<td>0.111</td>
<td>0.134</td>
<td>0.147</td>
<td>0.145</td>
<td>0.100</td>
<td>0.151</td>
<td>0.121</td>
<td>0.157</td>
<td>0.147</td>
<td>0.172</td>
<td>0.166</td>
<td>0.163</td>
</tr>
<tr>
<td>Control</td>
<td>0.038</td>
<td>0.037</td>
<td>0.035</td>
<td>0.034</td>
<td>0.037</td>
<td>0.037</td>
<td>0.036</td>
<td>0.035</td>
<td>0.035</td>
<td>0.035</td>
<td>0.037</td>
<td>0.039</td>
</tr>
<tr>
<td>12G4</td>
<td>C3</td>
<td>C4</td>
<td>C7</td>
<td>C11</td>
<td>D6</td>
<td>D7</td>
<td>D8</td>
<td>D10</td>
<td>D11</td>
<td>E3</td>
<td>E10</td>
<td>F3</td>
</tr>
<tr>
<td>Iso4-BSA</td>
<td>2.861</td>
<td>2.760</td>
<td>2.708</td>
<td>2.905</td>
<td>2.808</td>
<td>2.920</td>
<td>2.800</td>
<td>2.909</td>
<td>2.978</td>
<td>2.880</td>
<td>2.914</td>
<td>2.998</td>
</tr>
<tr>
<td>Iso4-HSA</td>
<td>2.817</td>
<td>2.842</td>
<td>2.876</td>
<td>2.809</td>
<td>2.848</td>
<td>2.873</td>
<td>2.813</td>
<td>2.828</td>
<td>2.828</td>
<td>2.860</td>
<td>2.863</td>
<td>2.966</td>
</tr>
<tr>
<td>LGE2-BSA</td>
<td>0.119</td>
<td>0.136</td>
<td>0.106</td>
<td>0.228</td>
<td>0.147</td>
<td>0.120</td>
<td>0.164</td>
<td>0.152</td>
<td>0.149</td>
<td>0.101</td>
<td>0.157</td>
<td>0.127</td>
</tr>
<tr>
<td>12G4</td>
<td>F4</td>
<td>F5</td>
<td>F10</td>
<td>F11</td>
<td>G1</td>
<td>G3</td>
<td>G4</td>
<td>G6</td>
<td>H2</td>
<td>H10</td>
<td>9G8</td>
<td>12G4</td>
</tr>
<tr>
<td>Iso4-BSA</td>
<td>2.773</td>
<td>2.676</td>
<td>2.793</td>
<td>2.812</td>
<td>2.805</td>
<td>2.857</td>
<td>2.790</td>
<td>2.904</td>
<td>2.688</td>
<td>2.795</td>
<td>2.877</td>
<td>2.802</td>
</tr>
<tr>
<td>Iso4-HSA</td>
<td>2.797</td>
<td>2.761</td>
<td>2.815</td>
<td>2.777</td>
<td>2.762</td>
<td>2.820</td>
<td>2.863</td>
<td>2.847</td>
<td>2.804</td>
<td>2.940</td>
<td>2.793</td>
<td>2.964</td>
</tr>
<tr>
<td>LGE2-BSA</td>
<td>0.117</td>
<td>0.134</td>
<td>0.150</td>
<td>0.132</td>
<td>0.157</td>
<td>0.117</td>
<td>0.149</td>
<td>0.152</td>
<td>0.129</td>
<td>0.163</td>
<td>0.126</td>
<td>0.169</td>
</tr>
<tr>
<td>Control</td>
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<td>0.036</td>
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<td>0.037</td>
<td>0.036</td>
<td>0.035</td>
<td>0.035</td>
<td>0.037</td>
<td>0.035</td>
<td>0.037</td>
<td>0.036</td>
</tr>
</tbody>
</table>

Figure 5.4S Indirect ELISA tests of first subclones of 12G4, and all 34 subclones showed strong specificity response.
### First subcloning of 12G4 (10 subclones)

<table>
<thead>
<tr>
<th>Clone F3</th>
<th>89.33 μM</th>
<th>17.86 μM</th>
<th>2.367 μM</th>
<th>0.1429 μM</th>
<th>2.86x10^-3 μM</th>
<th>5.72x10^-3 μM</th>
<th>1.14x10^-3 μM</th>
<th>2.28x10^-3 μM</th>
<th>4.58x10^-3 μM</th>
<th>9.15x10^-4 μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iso4-HSA</td>
<td>0.273</td>
<td>1.236</td>
<td>2.609</td>
<td>2.894</td>
<td>2.912</td>
<td>2.923</td>
<td>2.934</td>
<td>2.942</td>
<td>2.956</td>
<td>2.964</td>
</tr>
<tr>
<td>LGE2-BSA</td>
<td>2.583</td>
<td>2.691</td>
<td>2.828</td>
<td>2.924</td>
<td>2.964</td>
<td>2.969</td>
<td>2.972</td>
<td>2.961</td>
<td>2.962</td>
<td>2.920</td>
</tr>
</tbody>
</table>

### First SubClone F3 From 12G4 (IgG1)

- **Iso4-BSA**
- **Iso4-HSA**
- **LGE2-BSA**

---

### 12G4 Subclone supernatant A2 B2 B5 C3 C7 E3 F3 F4 G3 H2 12G4 control
<table>
<thead>
<tr>
<th>Iso4-BSA</th>
<th>2.909</th>
<th>2.124</th>
<th>2.866</th>
<th>2.850</th>
<th>2.837</th>
<th>2.904</th>
<th>2.946</th>
<th>2.946</th>
<th>2.877</th>
<th>0.095</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iso4-HSA</td>
<td>2.992</td>
<td>2.721</td>
<td>2.992</td>
<td>2.998</td>
<td>2.975</td>
<td>2.991</td>
<td>2.999</td>
<td>2.968</td>
<td>2.992</td>
<td>0.116</td>
</tr>
<tr>
<td>LGE2-BSA</td>
<td>0.139</td>
<td>0.098</td>
<td>0.136</td>
<td>0.121</td>
<td>0.119</td>
<td>0.114</td>
<td>0.156</td>
<td>0.129</td>
<td>0.117</td>
<td>0.162</td>
</tr>
</tbody>
</table>

### 12G4 Subclone supernatant 1 to 10 Dilution A2 B2 B5 C3 C7 E3 F3 F4 G3 H2 12G4 control
<table>
<thead>
<tr>
<th>Iso4-BSA</th>
<th>2.452</th>
<th>0.836</th>
<th>2.445</th>
<th>2.262</th>
<th>2.120</th>
<th>2.627</th>
<th>2.525</th>
<th>2.368</th>
<th>2.582</th>
<th>2.625</th>
<th>0.108</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iso4-HSA</td>
<td>2.801</td>
<td>1.336</td>
<td>2.881</td>
<td>2.711</td>
<td>2.799</td>
<td>2.753</td>
<td>2.884</td>
<td>2.852</td>
<td>2.966</td>
<td>2.869</td>
<td>0.094</td>
</tr>
<tr>
<td>LGE2-BSA</td>
<td>0.099</td>
<td>0.096</td>
<td>0.096</td>
<td>0.101</td>
<td>0.101</td>
<td>0.098</td>
<td>0.098</td>
<td>0.097</td>
<td>0.105</td>
<td>0.112</td>
<td>0.093</td>
</tr>
<tr>
<td>Clone F4</td>
<td>89.33 μM</td>
<td>17.86 μM</td>
<td>3.572 μM</td>
<td>0.7144 μM</td>
<td>0.1429 μM</td>
<td>2.86x10⁻² μM</td>
<td>5.72x10⁻³ μM</td>
<td>1.14x10⁻³ μM</td>
<td>2.28x10⁻⁴ μM</td>
<td>4.58x10⁻⁵ μM</td>
<td>9.15x10⁻⁶ μM</td>
</tr>
<tr>
<td>---------</td>
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<td>-------------</td>
<td>-------------</td>
<td>-------------</td>
<td>-------------</td>
</tr>
<tr>
<td>Iso4-HSA</td>
<td>0.103</td>
<td>0.123</td>
<td>0.372</td>
<td>1.81</td>
<td>2.411</td>
<td>2.672</td>
<td>2.867</td>
<td>2.701</td>
<td>2.483</td>
<td>2.567</td>
<td>2.828</td>
</tr>
<tr>
<td>LGE2-BSA</td>
<td>1.938</td>
<td>2.073</td>
<td>1.943</td>
<td>2.068</td>
<td>2.247</td>
<td>2.53</td>
<td>2.594</td>
<td>2.602</td>
<td>2.538</td>
<td>2.462</td>
<td>2.484</td>
</tr>
</tbody>
</table>

**Model Logistic Equation**

\( y = A_2 + (A_1 - A_2) / (1 + (x/x_0)^p) \)

**Reduced Chi-Sqr**

Iso4-HSA: 0.01975, 0.00443

Adj. R-Square

Iso4-HSA: 0.98458, 0.93767

<table>
<thead>
<tr>
<th>Value</th>
<th>Standard Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iso4-HSA</td>
<td>A1 2.66941</td>
</tr>
<tr>
<td></td>
<td>A2 0.07805</td>
</tr>
<tr>
<td></td>
<td>x0 1.06241</td>
</tr>
<tr>
<td></td>
<td>p 1.53969</td>
</tr>
<tr>
<td></td>
<td>EC20 0.43178</td>
</tr>
<tr>
<td></td>
<td>EC50 1.06241</td>
</tr>
<tr>
<td></td>
<td>EC80 2.61408</td>
</tr>
</tbody>
</table>

| LGE2-BSA | A1 2.53988    | 0.02970       |
|          | A2 1.9952     | 0.03776       |
|          | x0 0.13939    | 0.04200       |
|          | p 1.71788     | 1.08487       |
|          | EC20 0.0622   | --            |
|          | EC50 0.13939  | 0.04200       |
|          | EC80 0.31239  | --            |

<table>
<thead>
<tr>
<th>Clone H2</th>
<th>89.33 μM</th>
<th>17.86 μM</th>
<th>3.572 μM</th>
<th>0.7144 μM</th>
<th>0.1429 μM</th>
<th>2.86x10⁻² μM</th>
<th>5.72x10⁻³ μM</th>
<th>1.14x10⁻³ μM</th>
<th>2.28x10⁻⁴ μM</th>
<th>4.58x10⁻⁵ μM</th>
<th>9.15x10⁻⁶ μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iso4-HSA</td>
<td>0.152</td>
<td>0.528</td>
<td>1.923</td>
<td>2.821</td>
<td>2.823</td>
<td>2.935</td>
<td>2.978</td>
<td>2.894</td>
<td>2.799</td>
<td>2.908</td>
<td>2.97</td>
</tr>
<tr>
<td>LGE2-BSA</td>
<td>2.403</td>
<td>2.441</td>
<td>2.514</td>
<td>2.687</td>
<td>2.806</td>
<td>2.86</td>
<td>2.98</td>
<td>2.945</td>
<td>2.974</td>
<td>2.998</td>
<td>2.976</td>
</tr>
</tbody>
</table>

**Model Logistic Equation**

\( y = A_2 + (A_1 - A_2) / (1 + (x/x_0)^p) \)

**Reduced Chi-Sqr**

Iso4-HSA: 0.00413, 5.99668E-4

Adj. R-Square

Iso4-HSA: 0.99614, 0.98878

<table>
<thead>
<tr>
<th>Value</th>
<th>Standard Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iso4-HSA</td>
<td>A1 2.9075</td>
</tr>
<tr>
<td></td>
<td>A2 0.1184</td>
</tr>
<tr>
<td></td>
<td>x0 5.41547</td>
</tr>
<tr>
<td></td>
<td>p 1.50213</td>
</tr>
<tr>
<td></td>
<td>EC20 2.15194</td>
</tr>
<tr>
<td></td>
<td>EC50 5.41547</td>
</tr>
<tr>
<td></td>
<td>EC80 13.62829</td>
</tr>
</tbody>
</table>

| LGE2-BSA | A1 2.98294    | 0.01396       |
|          | A2 2.36034    | 0.03844       |
|          | x0 0.66943    | 0.22011       |
|          | p 0.57159     | 0.09008       |
|          | EC20 0.05921  | --            |
|          | EC50 0.66943  | 0.22011       |
|          | EC80 7.56842  | --            |

<table>
<thead>
<tr>
<th>Clone E3</th>
<th>17.86 μM</th>
<th>3.572 μM</th>
<th>0.7144 μM</th>
<th>0.1429 μM</th>
<th>2.86x10⁻² μM</th>
<th>5.72x10⁻³ μM</th>
<th>1.14x10⁻³ μM</th>
<th>2.28x10⁻⁴ μM</th>
<th>4.58x10⁻⁵ μM</th>
<th>9.15x10⁻⁶ μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iso4-HSA</td>
<td>0.112</td>
<td>0.405</td>
<td>1.466</td>
<td>1.721</td>
<td>1.614</td>
<td>1.572</td>
<td>1.651</td>
<td>1.617</td>
<td>1.733</td>
<td></td>
</tr>
<tr>
<td>LGE2-BSA</td>
<td>1.852</td>
<td>1.672</td>
<td>1.657</td>
<td>1.699</td>
<td>1.888</td>
<td>1.907</td>
<td>1.953</td>
<td>1.867</td>
<td>1.9</td>
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</tbody>
</table>

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First SubClone E3 From 12G4 (IgG1)

Clone 12G4 | 89.33 μM | 17.86 μM | 3.572 μM | 0.7144 μM | 0.1429 μM | 2.86x10^-3 μM | 5.72x10^-3 μM | 1.14x10^-3 μM | 2.28x10^-3 μM | 4.58x10^-3 μM | 9.15x10^-3 μM
Iso4-HSA   | 0.326    | 1.209    | 2.303    | 2.645     | 2.934     | 2.928       | 2.931       | 2.899       | 2.839       | 2.875       | 2.882       
LGE2-BSA   | 2.557    | 2.599    | 2.571    | 2.864     | 2.886     | 2.921       | 2.936       | 2.949       | 2.816       | 2.859       

Clone A2  
Iso4-HSA  | 0.408    | 1.937    | 2.398    | 2.436     | 2.417     | 2.380       | 2.598       | 2.436       | 2.379       
LGE2-BSA  | 2.383    | 2.304    | 2.324    | 2.368     | 2.365     | 2.288       | 2.372       | 2.415       | 2.362       

Clone B2  
Iso4-HSA  | 0.188    | 1.164    | 2.145    | 2.187     | 2.230     | 2.183       | 2.368       | 2.086       
LGE2-BSA  | 2.117    | 2.043    | 2.071    | 2.076     | 2.105     | 2.101       | 2.309       | 1.648       

Clone B5  
Iso4-HSA  | 0.178    | 0.972    | 1.992    | 2.039     | 2.116     | 2.018       | 2.208       | 2.223       | 2.023       
LGE2-BSA  | 2.131    | 1.998    | 1.995    | 1.969     | 2.077     | 2.055       | 2.123       | 2.231       | 1.983       

Clone C3  
Iso4-HSA  | 0.897    | 2.105    | 2.244    | 2.246     | 2.270     | 2.224       | 2.248       | 2.391       | 2.232       
LGE2-BSA  | 2.422    | 2.265    | 2.286    | 2.334     | 2.291     | 2.189       | 2.199       | 2.312       | 2.199       

Absorbance (405 nm)

Adj. R-Square 0.9886 0.56489

Figure 5.5S Indirect and competitive ELISA tests of 10 clones from 12G4, and 12G4 F3 was chose for next subclone.
Figure 5.6S Indirect ELISA tests of 16 secondary subclones from 12G4 F3.
C1 10time Dilution 2.157 2.635 1.777 0.102 0.104 0.105 0.107 0.103 0.102
D1 10time Dilution 2.370 2.839 2.057 0.096 0.098 0.094 0.106 0.102 0.105
G4 10time Dilution 1.576 2.422 1.338 0.100 0.106 0.100 0.103 0.099 0.103

Clone C1 446.65 89.33 μM 17.86 μM 3.572 μM 0.7144 μM 0.1429 μM 2.86x10^{-2} μM 5.72x10^{-3} μM 1.14x10^{-3} μM
Iso4-HSA 0.167 0.866 2.494 2.67 2.603 2.603 2.511 2.476 2.543
LGE2-BSA 2.725 2.727 2.725 2.662 2.679 2.629 2.573 2.545 2.569
Figure 5.7S Indirect and competitive ELISA tests of final three candidates from 12G4 F3--C1 D1 G4.
Figure 5.8S Indirect and competitive ELISA tests of final anti-iso[4]LGE2 clone 12G4 F3 D1 in serum free media.
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