METABOLISM & SIGNALING OF 4-HYDROXYACIDS:
NOVEL METABOLIC PATHWAYS AND INSIGHT INTO THE SIGNALING OF
LIPID PEROXIDATION PRODUCTS

By
SUSHABHAN SADHUHKAN

Submitted in partial fulfillment of the requirements for
the Degree of Doctor of Philosophy

Thesis Advisor: Gregory P Tochtrop, Ph.D.

Department of Chemistry
CASE WESTERN RESERVE UNIVERSITY
August 2012
We hereby approve the thesis/dissertation of

SUSHABHAN SADHUKHAN

candidate for the Doctor of Philosophy degree *

(signed) Michael G Zagorski, PhD
(Chair of the committee)

Gregory P Tochtrop, PhD

Robert G Salomon, PhD

Rajesh Viswanathan, PhD

Henri Brunengraber, PhD

(date) June 5, 2012

*We also certify that written approval has been obtained for any proprietary material contained therein.
This Thesis is Dedicated to my Parents

For their Love, Endless Support

and Encouragement
# TABLE OF CONTENTS

**TABLE OF CONTENTS** ........................................................................................................iv

**LIST OF SCHEMES, TABLES AND FIGURES** .............................................................xi

**ACKNOWLEDGEMENT** ................................................................................................xviii

**LIST OF ABBREVIATIONS** ...........................................................................................xxi

**ABSTRACT** ....................................................................................................................xxiv

**CHAPTER 1: INTRODUCTION: BACKGROUND AND SIGNIFICANCE** .......................1

1.1 Overview of 4-Hydroxyacids .....................................................................................2

1.2 Exogenous Source of 4-Hydroxyacids: Drugs of Abuse (GHB and GHP) ..........3

1.3 Endogenous Source of 4-Hydroxyacids: Lipid Peroxidation Products (4-HHE, 4-HNE and 4-ONE) ...............................................................7

1.4 Metabolomics ...........................................................................................................10

1.4.1 Non-targeted and Targeted Metabolomics .......................................................10

1.4.2 Stable Isotope-Labeled Compounds in Metabolomics ...................................12

1.5 Experimental Tools ................................................................................................13

1.5.1 Animal Experiments ......................................................................................13

1.5.2 Analytical Methods ......................................................................................15

1.6 Questions that have been Addressed ....................................................................16

1.7 4-HNE: A Xenobiotic or Very Carefully Regulated Small Molecule? ...............17
## Chapter 2: Elucidation of the Parallel Metabolic Pathways of 4-Hydroxyacids and 4-HNE via 4-Phosphoacyl-CoAs Using Isotopic Tools

### 2.1 Introduction

### 2.2 Results and Discussion

#### 2.2.1 Mass Isotopomer Analysis

#### 2.2.2 Identification of a New Class of CoA Esters: 4-Phosphoacyl-CoA

#### 2.2.3 Liver Perfusion with 4-Hydroxy-2-(E)-nonenal (4-HNE)

#### 2.2.4 Rat Liver Perfusion of 4-Hydroxyacids and their Metabolomics

#### 2.2.5 Measurement of Flux through the Two Pathways

#### 2.2.6 Transformation of 4-HNE to 4-Hydroxynonanoic Acid

### 2.3 Experimental Sections

#### 2.3.1 Materials and Methods

#### 2.3.2 Synthesis of 4-Hydroxy-\([3,4-^{13}C_2]\)-nonanoic Acid Lactone

#### 2.3.3 Synthesis of 4-Hydroxy-\([3-^{13}C]\)-nonanoic Acid Lactone

#### 2.3.4 Synthesis of 4-Hydroxy-(E)-Nonenoic Acid

#### 2.3.5 Synthesis of 4-Hydroxynonanal

#### 2.3.6 Animal Experiments: Liver Perfusions

#### 2.3.7 Analytical Procedures

#### 2.3.8 Acetyl-CoA Isotopomer Enrichment Calculation
CHAPTER 3: ISOTOPOMER SYNTHESIS OF γ-HYDROXYBUTYRATE (GHB) REVEALS CATABOLISM VIA α-OXIDATION

3.1 Introduction

3.2 Results and Discussion

3.2.1 Non-targeted Metabolomics of GHB in Rat Liver

3.2.2 Identification of α-Oxidation by Isotopic Analysis

3.2.3 Fate of 4-Phosphobutyryl-CoA

3.2.4 Construction of a Pathway for GHB Catabolism

3.2.5 Quantitation of the Rate of the β-Oxidation and α-Oxidation

3.3 Experimental Sections

3.3.1 Materials and Methods

3.3.2 Synthesis of Isotopically Labeled GHB Lactones

3.3.3 Liver Perfusions

3.3.4 LC-MS/MS Method for the Labeling Pattern and Concentration Measurement of Acyl-CoAs

3.3.5 GC-MS Assay of Formate and Acetate via PFBB Br Derivatization Reaction
CHAPTER 4: ENANTIOSELECTIVE GLUTATHIONYLATION OF 4-HYDROXY-2-(E)-NONENAL (4-HNE) & ITS DERIVATIVES IN RAT ORGANS ............................................. 134

4.1 Introduction ...................................................................................... 135

4.2 Results and Discussion .................................................................. 142
  4.2.1 Stereochemical Aspects of Glutathionylation of 4-HAEs ............ 142
  4.2.2 Method Validation .................................................................. 146
  4.2.3 Effect of IAA and BHT in the Sample Preparation ...................... 150
  4.2.4 4-HAE–GSH Conjugate Levels in Rat Liver, Brain and Heart Samples.. 153
  4.2.5 Liver Perfusion Experiment with $d_{11}$-4-HNE ......................... 158
  4.2.6 Liver Perfusion Experiment with [3,4-$^{13}$C$_2$]-4-ONE ............. 162

4.3 Experimental Sections .................................................................. 165
  4.3.1 Materials and Methods .......................................................... 165
  4.3.2 Synthesis of 4-Oxo-2-(E)-Nonenal (4-ONE) ............................... 166
  4.3.3 Synthesis of 4-Oxo-2-(E)-[3,4-$^{13}$C$_2$]Nonenal ([3,4-$^{13}$C$_2$]-4-ONE) ....... 166
  4.3.4 Synthesis of Standard 4-HAE Series ....................................... 168
  4.3.5 Preparation of 4-HAE–GSH Conjugates .................................. 168
4.3.6 Liver Perfusion Experiments.............................................................169
4.3.7 Sample Collections...........................................................................169
4.3.8 Sample Preparations........................................................................169
4.3.9 Method Validation.............................................................................170
4.3.10 LC-MS/MS for 4-HAE–GSH Conjugate Assay.................................171
4.3.11 Statistical Analysis..........................................................................172
4.4 Conclusions..........................................................................................172
4.5 Acknowledgement..................................................................................173
4.6 References............................................................................................174

CHAPTER 5: IDENTIFICATION OF A NEGATIVE FEEDBACK LOOP IN BIOLOGICAL
OXIDANT FORMATION REGULATED BY 4-HYDROXY-2-(E)-NONENAL (4-HNE)........182

5.1 Introduction...........................................................................................183
5.2 Results and Discussion...........................................................................190
5.2.1 Identification of a Negative Feedback Loop for NO Production.........190
5.2.2 Specificity of 4-Hydroxy-2-(E)-Alkenal Derivatives (C₅-C₁₂) for NO
Production....................................................................................................192
5.2.3 Mechanism of NO Production Inhibition by 4-HNE.........................195
5.3 Experimental Sections..........................................................................198
5.3.1 Materials and Methods.................................................................198
5.3.2 Synthesis of 4-Hydroxy-2-(E)-Alkenal Derivatives (C₅-C₁₂)..............199
CHAPTER 1: INTRODUCTION: BACKGROUND AND SIGNIFICANCE

**Figure 1.1** Chemical structures of (A) γ-hydroxybutyric acid (GHB); (B) γ-hydroxypentanoic acid (GHP); and (C) levulinic acid.

**Figure 1.2** Chemical structures of (A) 4-hydroxy-2-(E)-nonenal (4-HNE); (B) 4-hydroxy-2-(E)-hexenal (4-HHE); (C) malondialdehyde (MDA); and (D) 4-oxo-2-(E)-nonenal (4-ONE).

**Figure 1.3** Schematic representation of the rat liver perfusion setup.

CHAPTER 2: ELUCIDATION OF THE PARALLEL METABOLIC PATHWAYS OF 4-HYDROXYACIDS AND 4-HNE VIA 4-PHOSPHOACYL-COA USING ISOTOPIC TOOLS

**Table 2.1** Exact masses of 4-phospho-butyryl-CoA and 4-phospho-pentanoyl-CoA isolated from perfused rat livers.

**Figure 2.1** Fragmentation patterns of 4-phosphopentanoyl-CoA. Note the loss of phosphoric acid (98 Da) during two MS/MS/MS fragmentations (A → C and D → F). Fragments A and D are specific of 4-phospho-pentanoyl-CoA. Fragments B, C, E, F and G are common to 4-hydroxy-pentanoyl-CoA and 4-phospho-pentanoyl-CoA. Fragments B and E are common to all acyl-CoAs.

**Figure 2.2** $^{31}$P-NMR of (A) malonyl-CoA standard and of (B) 4-phosphopentanoyl-CoA isolated from a rat liver perfused with 4-hydroxypentanoate. The new peak at 86.6 ppm accounts for the additional phosphate group in 4-phosphopentanoyl-CoA.

**Figure 2.3** Accumulation of 4-phosphoacyl-CoAs derived from 4-hydroxyacids. Retention times and relative abundances of 4-phosphoacyl-CoAs assayed under identical LC-MS conditions in extracts of rat livers perfused with 2 mM of C$_4$ to C$_{11}$ 4-hydroxyacids (composite chromatogram). Note: Each peak denotes the 4-phosphoacyl-CoA derived from corresponding 4-hydroxyacid. For example, C$_5$ is the 4-phosphopentanoyl-CoA derived from...
4-hydroxypentanoic acid.

**Figure 2.4** (A) Structure of \(d_{11}\)-4-HNE; and (B) \(^2\text{H}\)-labeled metabolites identified from the liver perfused with \(d_{11}\)-4-HNE: 4-phosphononanoyl-CoA (I), propionyl-CoA (II), pentanoyl-CoA (III), hexanoyl-CoA (IV), heptanoyl-CoA (V), 4-hydroxynonanoate (VI), 4-hydroxynonenoate (VII), and 2-hydroxyheptanoate (VIII).

**Table 2.2** Mass isotopomer distribution of metabolites derived from \(d_{11}\)-4-HNE.

**Figure 2.5** (A) Two alternative metabolic pathways for 4-HNE: I. Glutathionylation via GSTA4 results in the 4-HNE-glutathione 1:1-conjugate; II. Reduction of 4-HNE to 1,4-dihydroxynonene by alcohol dehydrogenases or aldol reductase or aldehyde reductase; III. Oxidative process assisted by aldehyde dehydrogenases to give 4-hydroxyynonenoic acid; IV. Reduction of the \(\alpha,\beta\)-unsaturation catalyzed by NAD(P)H-dependent alkenal/one oxidoreductase. We identified another metabolite, namely 4-hydroxynonanoic acid from the metabolomics study of 4-HNE in perfused rat liver. Although it is not clear which enzymes are responsible for this particular biotransformation, the two probable pathways are shown by dotted lines. (B & C) Concentrations of 3-hydroxyacids with the same carbon number as substrate and 2-hydroxyacids with 2 carbons less than substrate in the different chain lengths of 4-hydroxyacids perfusates are shown in the panel B and C respectively.

**Figure 2.6** Proposed parallel pathways for the degradation of 4-hydroxyacids.

**Figure 2.7** Parallel pathways for the catabolism of 4-hydroxyacids using 4-hydroxynonanoate as an example. The initial experiment with \(d_{11}\)-4-HNE (see the grey highlights) enabled identification of the majority of the catabolic intermediates shown above. Singly labeled 4-hydroxynonanoate is highlighted in blue at C-3 and doubly labeled 4-hydroxynonanoate is highlighted in red at C-3 and C-4 to facilitate tracing their fates through pathways A and B. Note that the doubly labeled substrate forms acetyl-CoA, part of which is doubly labeled (M2) via pathway A and singly labeled (M1) via pathway B. Formate, derived from C-3 of the substrate, is formed via pathway B and delivers M1 formate from both labeled starting substrates. Note that for even-chain 4-hydroxyacids with at least six carbons, pathway A
leads to acetyl-CoA, whereas pathway B leads to acetyl-CoA, propionyl-CoA, and formate. The hypothesized mechanism of the isomerization of 4-hydroxyacyl-CoA to 3-hydroxyacyl-CoA is shown in the left-hand portion of the figure.

**Figure 2.8** Release of $[^{13}\text{C}]$formate by livers perfused with recirculating buffer containing 2 mm 4-hydroxy-$[^{3,13}\text{C}]$nonanoate (■), 4-hydroxy-$[^{3,4-13}\text{C}_2]$nonanoate (▲).

**Figure 2.9** Convergent synthetic routes for 4-hydroxy-$[^{3-13}\text{C}]$nonanoic acid lactone and 4-hydroxy-$[^{3,4-13}\text{C}_2]$nonanoic acid lactone.

**Table 2.3** Mass isotopomers of metabolites released by livers perfused with unlabeled and labeled 4-hydroxynonanoates. Metabolites were assayed by LC-MS/MS.

**Figure 2.10** The M1 and M2 acetyl-CoA enrichment from 0 to 1 mM of (A) 4-hydroxy-$[^{3,4-13}\text{C}_2]$nonanoate and (B) 4-hydroxy-$[^{3-13}\text{C}]$nonanoate non-recirculating perfusions. Ratio of M1 and M2 acetyl-CoA (from A) demonstrate the flux of the two pathways.

**Table 2.4** Identification of metabolites released by control liver, livers perfused with 4-hydroxynonanal (Compound A), and 4-hydroxynonenenoate (Compound B). Metabolites were assayed by LC-MS/MS.

**Figure 2.11** Alternative pathways for the transformation of 4-HNE to 4-hydroxynonanoyl-CoA.

**Chapter 3: Isotopomer Synthesis of γ-Hydroxybutyrate (GHB) Reveals Catabolism via α-Oxidation**

**Figure 3.1** Origin and fates of GHB in the physiology. 1,4 Butanediol, γ-butyraldehyde and γ-hydroxybutyraldehyde can act as pro-drugs for GHB.

**Figure 3.2** Identification of metabolites of GHB. (A) Mirror images depiction of total ion current chromatograms of trimethylsilyl derivatives of compounds released by a control liver and by a liver perfused with 2 mM unlabeled GHB. (B) Amplification of selected areas from the top
chromatograms. (C) Electron ionization spectra of peaks (1, 2 and 3) selected from the middle panels and their possible structures based on the NIST data base.

**Scheme 3.1** Proposed mechanism for the cleavage of the β-carbon of 3-hydroxypropionate to form formic acid.

**Figure 3.3** Release of (A) M1 formate and (B) M2 glycolate by livers perfused with 2 mM [1,2-13C2]GHB (♦) or [3,4-13C2]GHB (□). (C) M1 formate production in livers perfused with singly labeled GHB.

**Figure 3.4** The precursor-to-product relationship from (A) [1,2,3-13C3]GHB, (B) [1,2-13C2]GHB and (C) [2,3,4-13C3]GHB perfused liver.

**Figure 3.5** Pathway for metabolism of GHB illustrated with [1,2,3-13C3]GHB where 13C labeled atoms are highlighted in grey.

**Figure 3.6** Quantitation of the production of M1 3,4- and 2,4-dihydroxybutyryl-CoA from livers perfused with [3-13C]GHB. The ratio of the two is shown on the inset.

**Scheme 3.2** Synthesis route for the labeled GHB lactones.

**Scheme 3.3** (A) PFBBr derivatization of acids and (B) the corresponding mass ion in negative chemical ionization (NCI) mass spectrometry.

**Chapter 4: Enantioselective Glutathionylation of 4-Hydroxy-2-(E)-Nonenal (4-HNE) & Its Derivatives in Rat Organs**

**Figure 4.1** (A) Structure of 4-hydroxy-2-(E)-nonenal (4-HNE) and its electron density mapped by gaussian calculation, (B) Structure of 4-oxo-2-(E)-nonenal (4-ONE) and its electron density mapped by gaussian calculation, and (C) Michael addition of thiols to 4-HNE and 4-ONE. 4-HNE but not 4-ONE can form the hemiacetal of the Michael adduct. Note: In the electron density maps, red regions are high and blue regions are low density in electrons.

**Scheme 4.1** (A) Reduction of 4-ONE by carbonyl reductase in presence of NADPH resulting 1-hydroxy non-2-en-4-one, 4-oxononanal, and 4-HNE. (B)
Oxidation of 4-ONE by aldehyde dehydrogenase to form 4-oxo-2-nonenoic acid.

**Figure 4.2** (A) Structure of 4-hydroxy-2-(E)-alkenal (4-HAE) derivatives and their GSH conjugate where n=0-7 are represented as C5-C12 (corresponds to the number of carbon in the 4-HAE) respectively in the text, (B) Four different diastereomers of 4-HAE–GSH conjugates. (C) LC-MS trace of the different diastereomers of 4-HAE–GSH conjugates.

**Figure 4.3** LC-MS/MS trace of GSH conjugates of racemic, (R) and (S)-4-HNE.

**Figure 4.4** LC-MS/MS profile of 4-HAE–GSH conjugates. In the inset, the separation of GSH conjugate of (R) and (S)-4-HNE is shown.

**Figure 4.5** Product ion spectrum of 4-HNE–GSH conjugate and the structures of the major fragments.

**Table 4.1** LC-MS/MS method performance parameters for 4-HAE–GSH conjugates.

**Table 4.2** Recoveries of 4-HAE–GSH conjugates assayed by LC-MS/MS.

**Figure 4.6** (A) Enzymatic or non-enzymatic glutathionylation of 4-HNE. (B) IAA traps and masks the nucleophilicity of GSH.

**Figure 4.7** Effect of BHT on GSH conjugate of (A) (R)-4-HNE and (B) (S)-4-HNE.

**Figure 4.8** GSH conjugates of (R) and (S)-4-HAE (C5-C12) in liver, heart and brain tissues from rat.

**Figure 4.9** Diastereomeric contents of (R) and (S)-d11-4-HNE–GSH conjugates in the (A) perfused rat liver tissue, and (B) perfusate.

**Figure 4.10** (A) Uptake of d11-4-HNE by rat liver. (B) Acyl-CoA profile of d11-4-HNE catabolism in rat liver. (C) Mass isotopomer enrichment of propionyl-CoA on d11-4-HNE perfusion in rat liver.

**Figure 4.11** Concentrations of (A) [3,4-13C2]-4-ONE–GSH (B) [3,4-13C2]-4-
HNE–GSH and (C) [3,4-\textsuperscript{13}C_{2}]1-hydroxy-4-oxononene–GSH in the perfusate of rat livers perfused with various concentrations of [3,4-\textsuperscript{13}C_{2}]4-ONE.

**Figure 4.12** Diastereomeric contents of M2-(R) and -(S)-4-HNE–GSH conjugates in the perfusate.

**Chapter 5: Identification of a Negative Feedback Loop in Biological Oxidant Formation Regulated by 4-Hydroxy-2-(E)-Nonenal (4-HNE)**

**Scheme 5.1** Conversion of L-Arginine to Citrulline via N\textsuperscript{\omega}-hydroxy-L-arginine (NHA) to produce Nitric Oxide. In that reaction, 2 mole of O\textsubscript{2} and 1.5 mole of NADPH are consumed to produce one mole of NO.

**Figure 5.1** Molecular signaling pathways of macrophage activation through NF-κB, and the antagonistic signaling pathway Nrf2-Keap1.

**Figure 5.2** Nitrite levels from LPS-activated RAW 264.7 cells treated with various concentrations of 4-HNE for (A) 24 hours; (B) 30 minutes. Cells treated with 10 ng/mL LPS and 0.25% DMSO were used as control and (C) Viability measurements of LPS-activated RAW 264.7 cells treated with varying concentration of 4-HNE relative to DMSO treated LPS-activated control treated for 24 hours.

**Figure 5.3** (A) Synthesis of 4-hydroxy-2-(E)-alkenal derivatives C\textsubscript{5}-C\textsubscript{12}. Compound 3 is described as C\textsubscript{5} in the text and compounds 3a-3g are referred to as C\textsubscript{n} to C\textsubscript{12} where n = 0-6 respectively. (B) Nitrite levels from LPS-activated macrophage treated with 1 μM 4-hydroxy-2-(E)-alkenal derivatives C\textsubscript{5}-C\textsubscript{12} for 24 hours.

**Figure 5.4** (A) iNOS activity was measured via quantification of radiolabeled L-arginine to citrulline conversion. iNOS enzyme alone served as positive control while iNOS enzyme treated with N\textsuperscript{\omega}-nitro-L-arginine (L-NNA) served as negative control. (B) Immunoblot analysis of iNOS protein. Levels in 4-HNE treated LPS-activated macrophage treated for 18 hours. DMSO treated cells were used as negative control while DMSO treated LPS-activated cells were used as positive control. β-actin was used as protein loading control. (C) mRNA levels of 4-HNE treated LPS-activated macrophage treated for 18 hours. Fold change in expression were calculated relative to DMSO treated LPS-activated iNOS expression which was
normalized to 18s rRNA expression.

**Figure 5.5** A model for 4-HNE control of induced nitric oxide production. At low concentrations over long time periods, there is a negative feedback loop that maintains a constant level of production of nitric oxide with an inflection at approximately 1 µM. At higher concentrations, a positive feedback loop seems to be present whereby increasing concentrations of 4-HNE in turn elicits higher concentrations of nitric oxide.

**Chapter 6: Conclusions and Future Directions**

**Scheme 6.1** Proposed mechanism for the isomerization of 4-hydroxyacyl-CoA to 3-hydroxyacyl-CoA via 4-phosphoacyl-CoA.
ACKNOWLEDGEMENTS

Completing my PhD degree is probably the most challenging activity so far I have experienced in my life. The successful completion of this degree would have not been possible without the continuous efforts, direction, and support of my teachers, family, friends, and colleagues. There have been a lot of ups and downs during the last five years since I have come to United States for my doctoral degree. Initially I joined Dr. Lawrence Sayre’s group as a graduate student. Tragically, Dr. Sayre suffered a stroke in early 2008 which eventually led to his passing away. This was really sad and a great loss indeed to all of us who have had contact with him. I would like to give my sincere appreciation to Dr. Sayre. I was very lucky for the fact that Dr. Gregory Tochtrop was kind enough to take me into his group and hence providing me an opportunity to carry on my graduate work at Case Western Reserve University. Since then, Dr. Tochtrop has served as my mentor. As a research advisor, Dr. Tochtrop has always motivated me to perform to the best of my capability, and given me great freedom to pursue independent work. He patiently provided me the vision, encouragement and advice necessary for me to continue the doctoral program and complete my dissertation. I feel very fortunate to have had my doctoral training under the supervision of Dr. Tochtrop and will be indebted for the time he has spent mentoring me.

Secondly, I would like to thank Dr. Henri Brunengraber and Dr. Guo-fang Zhang for giving me the opportunity to carry on the biological studies on our synthesized compounds. I had many enlightening scientific discussions with Dr. Brunengraber and Dr. Zhang regarding our collaborative projects and furthermore, they spent their precious
times to read this thesis and gave their critical comments about it. I am grateful to Dr. Brunengraber and Dr. Zhang in every possible way and hope to keep up our collaboration in the future. I would also express my thanks to Dr. Stephanie Harris, Sophie Roussel-Kochheiser and John Koshy for their support in my research.

Most of the works described in this thesis have been conducted in collaboration with Dr. Brunengraber’s Lab and Dr. John Letterio’s Lab (Department of Pediatrics, Case Western Reserve University). I have acknowledged them in a separate section at the end of each chapter. I would like to thank Tonibelle and Dr. Zhang for helping me to gather the necessary data and information needed for this compilation.

Special thanks to my committee, Dr. Michael Zagorski, Dr. Robert Salomon, Dr. Rajesh Viswanathan, and Dr. Henri Brunengraber for their support, guidance and helpful suggestions. I also would like to thank Dr. Bholanath Mukherjee and Dr. Krishnendu Chakrabarty for inspiring and helping me to come here for the graduate studies. Thank you Emily Hamburg and Rebecca Skerrett for reading my thesis and providing with the corrections.

I would like to thank all the current and former lab members of Tochtrop lab, Sayre lab, and Brunengraber lab: Dr. Yong Han, Dr. Subrata Ghosh, Dr. Jianye Zhang, Dr. Shuang Deng, Tonibelle Gatbonton, Qingjiang Li, Brian Werry, Emily Barker, Roozbeh Eskandari, Vasily Ignatenko, Katie Doud, Mohsen Badiee, Anna Owensby, Chuan Shi, Detao Gao.

My most heartfelt thanks must go out to my family and friends. The two most important persons that I must thank foremost: my mom and dad. I love you. Thank you is not a big enough word to appreciate your support. It could have never been possible for
me to continue my graduate studies without your continuous support and love. I owe everything to you and wish I could show you just how much I love and appreciate you.

For the last seven years my best friend Mintu Porel (now my wife Mintu Porel-Sadhukhan) has helped me at every stage to override every hurdle that came in the way. Her love provided me inspiration and was my driving force. I would also like to thank all my friends in US, India and other parts of the World for their thoughtful supports. Special thanks go to my younger brother Jishu and little friend Sia (my friend’s three-year old daughter) who has been a source of laughter and joy for me.
<table>
<thead>
<tr>
<th>Abbreviation</th>
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<td>4-HAE</td>
<td>4-Hydroxy-2-(E)-alkenal</td>
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</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
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<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
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<tr>
<td>MRM</td>
<td>Multiple reaction monitoring</td>
</tr>
<tr>
<td>NHA</td>
<td>N$^\omega$-Hydroxy-L-arginine</td>
</tr>
<tr>
<td>NIST</td>
<td>National Institute of Standards and Technology</td>
</tr>
<tr>
<td>nNOS</td>
<td>Neuronal nitric oxide synthase</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NOS</td>
<td>Nitric oxide synthase</td>
</tr>
<tr>
<td>Nrf2</td>
<td>Nuclear factor (erythroid-derived 2)-like 2</td>
</tr>
<tr>
<td>$^1$O$_2$</td>
<td>Singlet oxygen</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>------------------------------------</td>
</tr>
<tr>
<td>PCC</td>
<td>Pyridinium chlorochromate</td>
</tr>
<tr>
<td>PUFA</td>
<td>Poly unsaturated fatty acid</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RNS</td>
<td>Reactive nitrogen species</td>
</tr>
<tr>
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</tr>
<tr>
<td>RT-PCR</td>
<td>Real time polymerase chain reaction</td>
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<td>SSA</td>
<td>Succinic semialdehyde</td>
</tr>
<tr>
<td>SSADH</td>
<td>Succinic semialdehyde dehydrogenase</td>
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<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
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Metabolism & Signaling of 4-Hydroxyacids: Novel Metabolic Pathways
and Insight into the Signaling of Lipid Peroxidation Products

Abstract

By

SUSHABHAN SADHUKHAN

The central goal of this thesis was to generate a better understanding of the role of 4-hydroxyacids in vivo. This work spans disciplines to gain a more fundamental understanding of the origins of endogenous 4-hydroxyacids, and the eventual metabolic fate(s) of this class of molecules. These molecules can be produced endogenously, such as with 4-hydroxy-2-(E)-nonenal (more commonly known as 4-HNE), which is derived from the peroxidation (LPO). Alternatively they can come from exogenous sources, such as drugs of abuse including γ-hydroxybutyric acid (GHB) and γ-hydroxypentanoic acid (GHP). Despite the ubiquitous nature of these molecules in vivo, we discovered that very little was known about their eventual metabolic fates.

Our main experimental tools for the majority of the studies detailed in this thesis were isotopically labeled 4-HNE and other 4-hydroxyacids, which were synthesized with ²H and ¹³C labels at strategic positions. These compounds were subsequently perfused in a live rat liver and led to our finding of a highly evolved catabolic pathway for 4-hydroxyacids. A number of analytical tools including LC-MS/MS, GC/MS and ³¹P NMR were used to characterize the metabolic intermediates. A key finding of this work was that 4-hydroxyacid catabolism can proceed via two parallel pathways that involve either a
phosphorylation and isomerization of the C-4 hydroxyl or a β-oxidation/α-oxidation sequence. This is the first report on the catabolism of this class of biological molecules. We were also able to quantify the differential catabolic flux of 4-HNE (via 4-hydroxyacids) down the two parallel pathways.

In addition to the aforementioned catabolism/recycling of 4-hydroxyacids, we also utilized many of our analytical methods to answer other questions important to LPO and general metabolism. For example, we were able to better define the enantioselectivity of glutathionylation of 4-HNE, 4-ONE, and other 4-HAE derivatives by developing a highly sensitive LC-MS/MS method. Using the isotopic chemistries developed, we were able to define new pathways for the catabolism of the smallest 4-hydroxyacid (which differs from longer-chain members), GHB. Finally, we were able to expand our understanding of 4-HNE, by studying how this molecule modulates oxidative stress in the macrophage. We identified a negative feedback loop in biological oxidant formation that is regulated by 4-HNE. This model stands in contrast to the accepted model in the field that views 4-HNE as a cytotoxic xenobiotic derived from physiology gone awry. This thesis is focused on defining a more comprehensive understanding of both the origins, and fates of 4-hydroxyacids and other LPO products. The signaling in conjunction with the metabolism presented in this thesis argues for a more fundamental role for the 4-HNE, and the formation of LPO products does not necessarily represent aberrant physiology in itself. Rather, the production and elimination of LPO products is a carefully controlled component of physiology, and the aforementioned disease states are indicative of a perturbation of this normal homeostasis.
“A man would do nothing if he waited until he could do it so well that no one could find fault with what he has done.”

Cardinal Newman
CHAPTER 1

Introduction:

Background and Significance
4-Hydroxyacids (γ-hydroxyacids) are ubiquitous in vivo and involved in different areas of mammalian metabolism. They are derived from both exogenous and endogenous sources. Exogenously these molecules are typically introduced through drugs of abuse including γ-hydroxybutyric acid (GHB, date rape drug) or its emerging alternative, γ-hydroxypentanoic acid (GHP). GHB is an inhibitory modulator of neurotransmission derived from γ-aminobutyric acid (GABA). It was synthesized for the first time in 1960 to mimic the ubiquitous inhibitory brain neurotransmitter GABA and would cross the blood-brain barrier. GHB impairs the capacity to exercise judgment for unknown reasons. Although GHP is not produced in normal physiology, it is an emerging alternative for GHB and is being used as a recreational drug. Endogenously, 4-hydroxyacids are derived from the oxidation and saturation of the ubiquitously occurring lipid peroxidation (LPO) products 4-hydroxy-2-(E)-hexenal (4-HHE), 4-hydroxy-2-(E)-nonenal (4-HNE) and 4-oxo-2-(E)-nonenal (4-ONE). Generally, 4-HNE is considered to be largely responsible for cytopathological effects observed during oxidative and nitrosative stress in vivo. It has achieved the status of one of the best recognized and most studied cytotoxic LPO products. Since its discovery in 1964, 4-HNE has been widely accepted as a modulator of numerous cellular systems and implicated in the pathogenesis of a number of degenerative diseases including Alzheimer’s disease, atherosclerosis, cataracts, and cancer. In spite of the clear connections with disease and the hypotheses that LPO products lead to disease progression, little is known about either the in vivo origins or catabolic fates of LPO products. This is particularly surprising when considering the prevalence of LPO products such as 4-HHE, 4-HNE, and 4-ONE in
living systems and the substantial nature of the field researching them. Before the work described in this thesis, the only substantive knowledge regarding the fate of 4-HNE dealt with the intact nine-carbon framework. In brief, the known major metabolic transformations involve either the oxidation or reduction of the aldehyde functionality, saturation of the carbon-carbon double bond, and/or conjugation to reduced glutathione (GSH). Glutathione (GSH) can form conjugates with 4-HNE and other LPO products via a Michael-type addition mediated by glutathione S-transferases (GSTs). The GSH conjugates can then be further metabolized in the liver and kidney to form mercapturic acid derivatives and subsequently excreted through the kidney. This renal excretion remains generally the accepted route for the elimination of this LPO product. Additionally, the formation of a γ-nonalactone has been reported. There is also evidence of hepatocytic metabolism of 4-HNE by the alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH), though little is known about the latter process.

A major accomplishment reported in this thesis is the discovery of a novel catabolic pathway that can effectively convert 4-HNE to acetyl-CoA, propionyl-CoA, and formate via 4-hydroxynonanoic acid. This catabolic process is so efficient that a perfused rat liver can use 4-HNE as its primary carbon source.

1.2 **Exogenous Source of 4-Hydroxyacids: Drugs of Abuse (GHB and GHP)**

GHB is a short-chain fatty acid (the smallest saturated 4-hydroxyacid, Fig 1.1A) that is primarily derived from GABA in mammalian brain at a concentration between 1-4 µM and possesses unique physiological and pharmacological properties. GHB has been clinically used as an anesthetic drug as well as for the treatment of alcohol and
opiate dependence and narcolepsy-associated cataplexy.\textsuperscript{15,16} On the other hand, due to its ability to induce short-term amnesia, GHB is used illicitly as a drug to facilitate acquaintance sexual assault and hence attained the reputation of a date rape drug.\textsuperscript{3} Following ingestion, GHB is rapidly transformed to succinic semialdehyde (SSA) and further oxidized to succinic acid, which then enters into the Krebs cycle. GHB accumulates at supraphysiological concentrations in succinic semialdehyde dehydrogenase (SSADH) deficiency, which is a rare inborn error of GABA metabolism.\textsuperscript{17} Despite being such an important small fatty acid with expanding clinical and illicit consumption, the catabolism of GHB remains elusive. A better understanding of the fates of GABA and/or GHB can provide important insights for treatment of SSADH deficiency.

In the United States, GHB was banned in 1990 as it was emerging as a major recreational drug and public health problem, then approved by the FDA in 2002 for the treatment of cataplexy and narcolepsy. GHB has a very narrow margin of safety: low doses of GHB at 10 mg per kilogram of body weight induce euphoria and passivity in humans, while doses over 50 mg per kilogram of body weight can result in coma, cardio-respiratory depression, and even death.\textsuperscript{18,19} In some cases, GHB was claimed to be the second most common drug detected in the serum of young people presenting with drug-induced coma, just behind cocaine.\textsuperscript{20} The toxic nature of GHB is amplified when co-ingested with alcoholic beverages, barbiturates, or salicylates, which have been observed to inhibit metabolism. The latter decreases the disposal of GHB by inhibiting its conversion to SSA, mainly via inhibition of GHB dehydrogenase.\textsuperscript{21}
GHB is believed to exhibit its pharmacological, behavioral and toxicological effects mainly through two distinct receptors: \( \gamma \)-aminobutyric acid type B (GABA\(_B\)) receptor and a GHB-specific receptor.\(^{22,23}\) At this point, it is still unclear whether GHB can function via \( \gamma \)-aminobutyric acid type A receptor (GABA\(_A\)) to exert some behavioral effects.\(^{24}\) GHB acts as a weak agonist of GABA\(_B\) receptor with an affinity in the mM range,\(^{25}\) which is well beyond the physiological concentration (1-4 \( \mu \)M) of GHB in the brain. Therefore, it is most likely that GHB works through GABA\(_B\) only when it is provided exogenously in an excessive amount or in individuals with SSADH deficiency. Alternatively, GHB can also activate the GABA\(_B\) receptor via its conversion to GABA.\(^{13}\) This conversion is achieved through the combined effect of GHB dehydrogenase and NADP to yield SSA, followed by the transamination of SSA into GABA via GABA-T (GABA transaminase) activity. This also explains the high dose dependence of GHB to act via GABA\(_B\), since a high concentration of GHB is required to produce enough GHB-derived GABA to activate the GABA\(_B\) receptor. It is not yet completely understood whether GABA\(_B\) activation by externally administered GHB is only due to GABA derived from GHB, or if GHB initiates secretion of additional GABA.\(^{26}\) However, there are several lines of evidence that GHB also functions through its own receptor, namely, the GHB receptor, which is separate and distinct from GABA\(_B\) receptor.

After GHB was banned in United States, addicts began alternatively using GHP, a five-carbon analogue of 4-hydroxyacids (Fig 1.1B) having the same alleged properties.\(^{4,27}\) Consequently, GHP is emerging as an alternative drug of abuse with similar properties to GHB such as sedation, catalepsy, and ataxia, although at reasonably higher dose. Its high availability makes GHP more popular in the drug community. Alkaline hydrolysis of \( \gamma \)-
valerolactone (a readily available industrial solvent) produces GHP readily. Very recently, we reported calcium or magnesium levulinate, an oral and parenteral source of respective minerals,\textsuperscript{28,29} as another pro-drug of GHP. Additionally, levulinic acid (4-oxopentanoic acid) (Fig 1.1C) is converted in the liver and other organs to GHP and this conversion is accelerated by ethanol oxidation.\textsuperscript{30} It is evident from the literature that GHP binds to the GHB receptor with 50\% lower affinity than GHB and might have some weak interaction with GABA\textsubscript{B} receptor as well.\textsuperscript{31,32} Levulinate is reduced to (R)-GHP by either cytosolic or mitochondrial dehydrogenases, which are NADPH- and NADH-dependent.\textsuperscript{30} A mitochondrial dehydrogenase or racemase also forms (S)-GHP. In our study, we have found that levulinate is catabolized by three parallel pathways in live rats, perfused rat livers, and liver subcellular preparations to propionyl-CoA, acetyl-CoA, and lactate.\textsuperscript{30}

\textbf{Figure 1.1} Chemical structures of (A) \(\gamma\)-hydroxybutyric acid (GHB); (B) \(\gamma\)-hydroxypentanoic acid (GHP); and (C) levulinic acid.
1.3 **Endogenous Source of 4-Hydroxyacids: Lipid Peroxidation Products**

(4-HHE, 4-HNE and 4-ONE)

Oxidative stress is the foremost source of damage to bio-macromolecules including proteins, DNA, lipids and sugars within cells. An imbalance between the production of reactive oxygen/nitrogen species (ROS/RNS) and their consumption in favor of production causes oxidative stress. As lipids serve as the primary barrier for free diffusion of ROS/RNS into the cell, they often are the primary target of these oxidative reactions in a process known as lipid peroxidation. ROS represents a class of highly reactive chemical species that are formed upon incomplete reduction of oxygen and includes the superoxide anion (O$_2^-$), hydrogen peroxide (H$_2$O$_2$), and the hydroxyl radical (HO'). They also function as intracellular signaling molecules. The role of RNS on lipid peroxidation is documented in the literature but still no clear evidences has been established. Peroxidation of lipids produces unsaturated lipid hydroperoxides that in turn undergo fragmentation. This gives rise to various aldehydes, including malondialdehyde (MDA), 4-ONE, 4-HHE, 4-HNE (Fig 1.2). 4-HNE is one of the major LPO products and has been widely accepted as an inducer of oxidative stress, as it is involved in the pathogenesis of a number of degenerative diseases such as Alzheimer’s disease, atherosclerosis, and cancer. This interesting pathogenesis is linked to the general abundance of this molecule. Under physiological conditions, typical concentrations of 4-HNE range from 0.1-1 μM in all tissues, but in response to severe oxidative insult this concentration can rise substantially in a localized manner to between 10 μM and 5 mM. 4-HNE has been mostly considered as a xenobiotic agent, and an example of physiology gone awry. However, recent findings from our work presented
in this thesis and others\textsuperscript{52,53} have uncovered a more fundamental role for 4-HNE as an evolved cytoplasmic signaling molecule. Increasing evidence suggests that 4-HNE at sublethal concentrations could act as a potential activator of NF-E2-related factor 2 (Nrf2) and induce the expression of phase II detoxification enzymes.

\textbf{Figure 1.2} Chemical structures of (A) 4-hydroxy-2-(\textit{E})-nonenal (4-HNE); (B) 4-hydroxy-2-(\textit{E})-hexenal (4-HHE); (C) malondialdehyde (MDA); and (D) 4-oxo-2-(\textit{E})-nonenal (4-ONE).
α,β-Unsaturated aldehydes are highly reactive small molecules formed during inflammation, normal biosynthesis, and metabolism of some drugs and/or drugs of abuse. They are well documented for their microbicidal, anti-viral, antitumoral and anti-mutagenic biological activities. These activities mainly arise from their ability to act as Michael acceptors. γ-Hydroxy-α,β-unsaturated aldehyde or 4-hydroxy-2-(E)-alkenal is one kind of α,β-unsaturated aldehyde which originates from a number of sources. For example, 4-HNE and 4-HHE are the lipid peroxidation end-products arising from (ω-6) and (ω-3) poly unsaturated fatty acids (PUFAs), respectively. 4-HNE is a strong electrophile which can react with the biological nucleophiles including protein side chains, DNA/RNA base pairs, lipids and sugars to modify or abrogate their ability to function normally. The electrophilic nature of 4-HNE is attributed to the conjugation of the C=C π-electrons with the aldehyde functionality. Due to resonance, it generates a regional electron deficiency at the β-carbon, and the γ-OH group further promotes electrophilicity via inductive effects. 4-ONE, which possesses the γ-oxo functionality, is even more reactive towards nucleophiles. In addition, the γ-oxo group of 4-ONE becomes another target for the nucleophilic side chains of proteins. At the same time, the hydration of 4-ONE at C-1 does not affect its ability to become a Michael acceptor, which is not the case for 4-HNE or 4-HHE.

GSTs play a key role in detoxifying the highly reactive aldehydes (4-HNE, 4-HHE, 4-ONE and MDA), that are formed during oxidative stress. GST mediated conjugation of 4-HNE to GSH, resulting in the formation of a GSH conjugate, is considered to be the major pathway for metabolism of 4-HNE in certain tissue types. GSH conjugates are known to be transported out of cells through an ATP-dependent
primary active efflux mechanism.\textsuperscript{63} GSTs also catalyze the conjugation of cholesterol-5,6-oxide, epoxyeicosatrienoic acid, and 9,10-epoxystearic acid with GSH. Thus GSTs provide the cell with protection against a range of harmful electrophiles produced during oxidative stress.\textsuperscript{64} The metabolism 4-HNE is known to be enantioselective in different organs. For example, (\textit{S})-4-HNE is preferentially detoxified by GST catalyzed glutathionylation\textsuperscript{65} while (\textit{R})-4-HNE is preferentially metabolized by aldehyde dehydrogenase (ALDH)-mediated NAD\textsuperscript{+}-dependent oxidation.\textsuperscript{66}

1.4 Metabolomics

The term ‘metabolome’ was first coined by Oliver \textit{et al.}\textsuperscript{67} to describe the metabolites synthesized by an organism. A broader definition was presented by Fiehn in 2001\textsuperscript{68} by introducing the term ‘metabolomics’ as a comprehensive analysis to identify and quantify the metabolites of an entire biological system. In the past decade, metabolomics has added an extra dimension to the ‘omics’ era by providing important insights of small molecule regulation as well as pathway discovery. Metabolomes are directly observable and thus can provide a wealth of information that can be correlated to various disease states.

1.4.1 Non-targeted and Targeted Metabolomics

Every metabolome is chemically diverse and comprised of numerous small molecules. So, different methods are required for the identification and quantification of all the metabolites of interest in a complex mixture. Regardless of the analytical platform, metabolomics can be divided into two different types: non-targeted and targeted approaches. Non-targeted metabolomics is used for overall metabolome analysis in a
comprehensive manner for the analysis of all the measurable entities. In a targeted metabolomics strategy, predefined metabolite-specific signals are often chosen to determine precise abundances and concentrations of a limited number of expected endogenous metabolites.

Non-targeted metabolomics is generally achieved by comparing the metabolic profile of a sample (or a sample treated with specific compounds) and the control to acquire the metabolic fingerprints. There are several ways to achieve this goal including comparing the nuclear magnetic resonance (NMR) or, gas chromatography with mass spectrometry (GC-MS) traces of the sample of interest to the appropriate control. NMR spectroscopy can be very useful for analysis of medium to large molecules, sugars, amines, or volatile liquids but has some disadvantages for analysis including its lack of sensitivity compared to mass spectrometry. Despite the potential applications of mass spectrometry for the analysis of small molecules, including the discovery of biomarkers, it has not been fully recognized in the field of metabolomics until recently. This is partly due to the huge number of metabolites (over 7000 molecular species in humans) typically found in biological samples.

Multiple reaction monitoring (MRM) using tandem quadrupole mass spectrometry is a standard technique in targeted metabolomics. MRM describes a subset of tandem mass spectrometry experiments that monitor both the precursor ion (molecule ion) and its consequent product ion (fragment of molecule). This approach effectively increases the sensitivity and reproducibility of tandem mass spectrometry under a broad dynamic range because double \( m/z \) selections reduce the interference and signal background of single selections.
1.4.2 **Stable Isotope-Labeled Compounds in Metabolomics**

Both radioisotopes and stable isotopes have been widely used in metabolomics. They differ in the analytical techniques that are used for their measurement. Quantification of radioisotopes is generally achieved by measuring the energy emitted during the loss of an electron from the nucleus, which is directly proportional to the number of radioactive atoms present in the sample. The three major disadvantages of radioisotope labeling are (i) they are hazardous and must be handled with extreme care, (ii) some radio isotopes (e.g. $^{32}$P and $^{125}$I) have very short half-life and hence, are not suitable for long experiments, and (iii) for $^{14}$C and $^{3}$H labeled molecules, total specific activity is measured, which does not account for the anaplerotic incorporations of the labeled atoms into the parent molecule and thus might be misleading. In contrast, measurements of stable isotopes are usually carried out by NMR, GC-MS, and LC-MS. Stable mass isotopomers are the molecules with 1 to n heavy atoms ($^2$H, $^{13}$C, $^{15}$N, $^{18}$O, etc.) and this mass difference is found in the mass spectrometer as unique mass-fingerprint. The strategic synthesis of stable isotopically labeled compounds enable metabolomics research for the identification of new pathways, measuring the flux of the different pathways, and turnover measurements of metabolites. This thesis work illustrates the application of isotopic tools with the combination of isotopomer analysis and metabolomics to uncover some previously unknown metabolic pathways and analytical techniques to measure the metabolites derived from lipid peroxidation and drugs of abuse.
1.5 Experimental Tools

1.5.1 Animal Experiments

The present metabolomics study relies upon a liver perfusion technique in which a live rat liver is perfused with the compound of interest under physiological conditions. This technique was developed by the Brunengraber Laboratory (Department of Nutrition, Case Western Reserve University), and they remain the premiere practitioner of the technique in the field. This method avoids anesthesia and anaerobic conditions. It yields rates of fatty acid synthesis comparable to rates observed in vivo and hence seems a suitable system for our metabolomics studies. A schematic diagram of our liver perfusion setup is shown in Fig 1.3. Briefly, overnight fasted male Sprague-Dawley rats (200-250 g) are concussively anesthetized and the liver is surgically removed immediately. The liver is then cannulated, through which the perfusate is pumped into the liver. The liver is perfused with Krebs-Ringer bicarbonate buffer containing 4% bovine serum albumin, glucose as indicated and the labeled or unlabeled compound(s) to be studied. The perfusate exits the liver through the inferior vena cava cannula and it is cannulated into the reservoir, collected at regular time intervals, quick-frozen, and stored at -80 °C for further analysis. The liver is also clamped at the end of the experiment under liquid nitrogen and stored at -80 °C for further analysis.
**Figure 1.3** Schematic representation of the rat liver perfusion setup.
1.5.2 Analytical Methods

The perfusates are analyzed via GC-MS to measure the concentrations and enrichment of different 4-hydroxyacids or their analogues. Briefly, 1 mL perfusate is treated with excess (5 or more equivalents) of acetonitrile to precipitate the proteins. After centrifuging, the supernatant is dried under N\textsubscript{2} gas and the dried residue is treated with trimethylsilyl or tert-butyldimethylsilyl chloride to derivatize and loaded for GC-MS analysis. The GC-MS trace is then compared to the control sample with the help of the NIST database and the differences in the chromatogram are searched for.\textsuperscript{79} The NIST search engine provides the plausible structure of the compound corresponding to the GC-MS peak. Based on these results, a non-targeted metabolomics study can be executed.

A wealth of information can be gathered through this type of GC-MS analysis, but less stable compounds require the use of an analytical technique with milder conditions, such as LC-MS. At the same time, not all compounds can be analyzed by GC-MS because of their low volatility. Free fatty acids are converted into unstable CoA esters before they are metabolized. Therefore, samples must be analyzed by LC-MS to obtain the acyl-CoA profile of the metabolome. The CoA esters remain inside the liver cells, so the frozen powdered liver tissues are used for acyl-CoA analysis by LC-MS/MS. Briefly, 200 mg powdered frozen liver spiked with the internal standard (1 nmol of \textit{d\textsubscript{5}-propionyl-CoA}) is extracted using a polytron homogenizer. The supernatant is eluted through a 3 mL ion exchange cartridge packed with 300 mg of 2-(2-pyridyl)ethyl-functionalized silica gel. The cartridge is preactivated with 3 mL of methanol. The acyl-CoAs trapped on the silica gel are released with (i) 3 mL of a 1:1 mixture (MeOH:H\textsubscript{2}O) including 50 mM ammonium formate, pH 6.3, and then (ii) 3 mL of a 3:1 mixture (MeOH:H\textsubscript{2}O) including
50 mM ammonium formate, pH 6.3 and (iii) 3 mL of methanol. The combined effluent is dried with N₂ gas and stored at -80 °C. The dried residue is redissolved in Milli-Q water and loaded for LC-MS/MS analysis.

The liver extracts are also subjected to the NMR (¹³C and ³¹P) analysis to obtain structural information of interesting metabolites. ¹³C NMR is a very efficient and versatile analytical tool when the rat livers are perfused with ¹³C-labeled compounds. Their higher isotopic enrichment helps to minimize the matrix effect. ³¹P NMR reveals information about the CoA esters.

1.6 QUESTIONS THAT HAVE BEEN ADDRESSED

As described before, 4-hydroxyacids are important physiological modulators that often play key roles in disease states. We demonstrated the catabolism of different 4-hydroxyacids (derived from LPO products and drugs of abuse) using metabolomics and mass isotopomer analysis. We also looked for the physiological sources of those 4-hydroxyacids and consequently their metabolomic profiles. These studies revealed the two carefully evolved catabolic pathways that efficiently convert 4-HNE to acetyl-CoA, propionyl-CoA, and formate via 4-hydroxynonanoic acid. This catabolic process is so efficient that a perfused rat liver can use 4-HNE as its primary carbon source. The stable isotope labeling strategy also allowed us to measure the flux of the newly discovered pathways.

In addition to the aforementioned catabolism/recycling of 4-HNE, we also utilized many of our analytical methods to answer other questions important to lipid peroxidation and general metabolism. For example, we were able to better define the enantioselectivity
of glutathionylation of 4-HNE, 4-ONE, and other 4-HAE derivatives by developing a highly sensitive LC-MS/MS method. Using the isotopic chemistries developed, we were able to define new pathways for the catabolism of the drugs of abuse, GHB and GHP. Finally, we were able to expand our understanding of 4-HNE, studying how this molecule modulates oxidative stress in the macrophage. We have identified a negative feedback loop in biological oxidant formation that is regulated by 4-HNE.

1.7 4-HNE: A Xenobiotic or Very Carefully Regulated Small Molecule?

Since its discovery in 1964, 4-HNE has been considered a xenobiotic compound that modulates numerous cellular systems and is implicated in the pathogenesis of a number of degenerative diseases including Alzheimer’s disease, atherosclerosis, cataracts, and cancer.\textsuperscript{8,9} Although a lot of research is being done focusing on the disease relevance of 4-HNE, there is new light being shed on the role of this reactive molecule in its physiological functions. In the last two decades, 4-HNE was found to regulate many of the physiological phenomena indicating that it is a cytoprotective agent.\textsuperscript{80} It was reported that a sublethal concentration of 4-HNE could function as a potential activator of Nrf2 and alter the expression profile of phase II detoxification enzymes.\textsuperscript{81-83} To better understand these signaling properties, and to explore whether 4-HNE could impact the formation of biological oxidants, we initiated a series of experiments examining the relationship between 4-HNE concentrations and the ability of activated cultured macrophages to produce nitric oxide (NO). We found that 4-HNE can control the expression of inducible nitric oxide synthase (iNOS) (detailed in \textbf{Chapter 5}). The significance of this observation is rooted in chemical processes that lead to the formation
of LPO products. As mentioned earlier, the first step in this process is hydrogen atom abstraction from PUFAs by ROS and RNS. We propose that this finding represents an evolved feedback pathway to control the concentration of 4-HNE (and other LPO products) in tissues which express inflammatory mediators (such as iNOS) via the production of RNS such as NO. This model stands in contrast to dogma in the field that views 4-HNE as a cytotoxic xenobiotic derived from physiology gone awry.
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CHAPTER 2

Elucidation of the Parallel Metabolic Pathways of 4-Hydroxyacids and 4-HNE via 4-Phosphoacyl-CoAs

Using Isotopic Tools
2.1 Introduction

Aliphatic 4-hydroxyacids are ubiquitous in human physiology and are involved in different areas of mammalian metabolism. They have different physiologic fates based on their structures and sites of origin. For example, the smallest saturated 4-hydroxyacid *i.e.* 4-hydroxybutyric acid (also known as γ-hydroxybutyric acid or GHB) acts as an inhibitory neurotransmitter derived from γ-aminobutyric acid (GABA) while its nine-carbon analogue (4-hydroxynonanoic acid) is a secondary metabolite of the lipid peroxidation end-product 4-hydroxy-2-(E)-nonenal or 4-HNE. GHB is also a drug of abuse that impairs the capacity to exercise judgment for unknown reasons. GHB has found very limited clinical use as an anesthetic agent and in the treatment of narcolepsy and alcoholism. Its known metabolism proceeds via (i) conversion to succinic semialdehyde by a cytosolic NADP⁺ dehydrogenase and by a mitochondrial pyridine nucleotide-independent enzyme system, and (ii) the oxidation of succinic semialdehyde to succinate, an intermediate of the Krebs cycle. The five-carbon analogue 4-hydroxypentanoic acid is also a newer drug of abuse. The calcium salt of a compound closely related to 4-hydroxypentanoate, levulinate (also known as 4-ketopentanoate or 4-ketovalerate), is used as an oral or intravenous source of calcium in humans. Levulinate is reduced to (R)-4-hydroxypentanoate by cytosolic and mitochondrial dehydrogenases, which are NADPH- and NADH-dependent, respectively. A mitochondrial dehydrogenase or racemase system is also known to form (S)-4-hydroxypentanoate. Thus, levulinate may act as a pro-drug of 4-hydroxypentanoate in a very efficient way.

This ubiquitous class of 4-hydroxyacids also comes from the highly reactive aldehyde molecules derived from the lipid peroxidation of polyunsaturated fatty acids
4-HNE is one of the major lipid peroxidation products and has been accepted as contributor to the pathogenesis of a number of degenerative diseases including Alzheimer’s disease, atherosclerosis, and cancer. At the time of writing this thesis there were more than 3400 peer reviewed research papers on this molecule. Since the seminal 1991 review on 4-HNE by Esterbauer et al., other very good reviews have been published. The largest portion of the literature on 4-HNE deals with how this molecule reacts and modulates the activity of various proteins/enzymes. Although 4-HNE is one of the main precursors for the production of 4-hydroxynonanoic acid in human physiology, the former lacks a clear downstream catabolic fate. Previous reports consisted of a pathway for elimination of 4-HNE via glutathionylation by glutathione S-transferase (GST) and subsequent excretion through the kidneys. There was also a report of hepatocyte metabolism through the alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH), though little was known about the processes. In the last two decades there were many efforts to uncover the final fate of 4-HNE. Before the work describing the catabolic fate of 4-HNE in this thesis, the only substantive knowledge regarding the fate of 4-HNE dealt with the in-tact nine carbon framework. A comprehensive summary of these transformations can be found in the review by Alary et al. To briefly summarize, the major transformations involve either the oxidation or reduction of the aldehyde functionality, saturation of the carbon-carbon double bond, and/or conjugation to glutathione. Additionally, the formation of a five-membered lactone was reported.

Specifically germane to this chapter, three studies examined the fate of radioactively labeled [4-\(^3\)H]-4-HNE or [2-\(^3\)H]-4-HНЕ in the rat model. In one
study, only 5.9% of the recovered radioactivity could be attributed to a nine-carbon skeleton, while two others found between 27% and 44% of 4-HNE was metabolized and excreted to a molecule that maintained its nine carbon framework. At the time of this work, it was suggested that degradation of the 4-HNE skeleton was a possibility, but there was no data to support it. A subsequent thorough review of the literature did not identify any report demonstrating the degradation of the carbon skeleton of 4-HNE. Consequently we began the studies that led to our discovery of a catabolic pathway for 4-HNE. We investigated the catabolism of different 4-hydroxyacids (C₄-C₁₁) as well as 4-HNE using a combination of metabolomics and mass isotopomer analysis. Live rat livers were perfused with various concentrations of unlabeled and labeled saturated 4-hydroxyacids and 4-HNE. Targeted and non-targeted metabolomics were performed on different 4-hydroxyacids in the context of perfused rat livers. This led us to identify a number of novel metabolites of 4-hydroxyacids.

The investigation on 4-hydroxyacid catabolism was initially started in the laboratory of Prof. Brunengraber in the Department of Nutrition, Case Western Reserve University. They were interested in exploring the metabolism of GHB, a drug of abuse. They took 4-hydroxyacids of different chain lengths (starting from C₄ to C₁₁) and perfused them into live rat organs. Then they used a combination of metabolomics and mass isotopomer analysis to get information about the metabolic intermediates. Prior to the work described here, there were almost no reports on the catabolism of 4-hydroxyacids with four or more carbons. Classical β-oxidation is considered to be the major carbon skeletal degradation pathway for the long chain fatty acids. It involves 3-ketoacyl-CoA and 3-hydroxyacyl-CoA intermediates before it cleaves off one molecule.
of acetyl-CoA which is a cellular fundamental building block. However, classical β-oxidation cannot use 4-hydroxyacyl-CoA as a substrate. Consequently, it was previously not clear how the carbon skeleton of a 4-hydroxyacids could be degraded. Those 4-hydroxyacids include the drugs of abuse GHB, GHP and also the secondary metabolites derived from the lipid peroxidation products 4-HNE and 4-hydroxy-2-(E)-hexenal (4-HHE, a lipid peroxidation product from ω-3 PUFAs). After a detailed targeted and non-targeted metabolomics study, many intermediates were identified during the course of 4-hydroxyacid catabolism. But, the major constraint remained that most of the intermediates are also present in vivo. Hence, it is very important to distinguish the intermediates of 4-hydroxyacid metabolism from endogenous ones that have some other origins. Here we decided to take advantage of isotopic tools to differentiate the metabolites present in vivo from those that are derived from 4-hydroxyacids.

We synthesized a number of stable isotopically labeled (13C and 2H) 4-hydroxyacids and related compounds (such as 4-HNE, 4-HHE, and levulinic acid) and perfused them into live rat organs for the metabolomics studies. Our metabolomics strategy focused on the identification of carboxylic acids and acyl-CoA esters derived from 4-hydroxyacids. The carboxylic acids were assayed by GC-MS of trimethylsilyl or tert-butyldimethylsilyl derivatives, using analog unlabeled or labeled compounds as internal standards and LC-MS/MS was used to profile different acyl-CoA esters from powdered frozen liver. The isotopic labeling of 4-hydroxyacids allowed us to perform mass isotopomer analysis on the substrates present in the perfusates and the tissue samples to acquire quantitative data. The use of isotopic tools along with metabolomics and mass isotopomer analysis led us to the discovery of two parallel metabolic pathways.
for the degradation of 4-hydroxyacids with five or more carbons. The first pathway (Pathway A) leads to the formation of 3-hydroxyacyl-CoAs, which are physiological β-oxidation intermediates, via 4-phosphoacyl-CoAs. The second pathway (Pathway B) is a sequence of β-oxidation, α-oxidation\textsuperscript{30,31}, and β-oxidation steps. 4-Hydroxyacids with five or more carbons are degraded to acetyl-CoA, propionyl-CoA, and formate via those two parallel pathways. This represented the first report on the catabolism of this ubiquitous class of biological molecules.\textsuperscript{1} The synthesis of specifically $^{13}$C labeled 4-hydroxyacids also enabled us to calculate the flux of the two pathways.\textsuperscript{29} A new class of acyl-CoA esters, \textit{i.e.} 4-phosphoacyl-CoAs, was identified in this study and is being subjected to further investigations for its physiological role.

2.2 RESULTS AND DISCUSSIONS

2.2.1 MASS ISOTOPOMER ANALYSIS

Mass isotopomers differ by the number of heavy atoms in their molecules, resulting in different molecular weights. For example, unlabeled glucose, [1-$^{13}$C]glucose, [1,2-$^{13}$C$_2$]glucose and $[^{13}$C$_6$]glucose are mass isotopomers. In mass isotopic terminology, they are referred to as M, M1, M2, and M6 glucose. A standard nomenclature for mass isotopomers utilizes M, M1, M2…Mn, where M is the base mass, and the number, n represents the isotopic shift in AMU. In experiments with $^{13}$C-enriched substrates, the measured mass isotopomer distribution of analytes includes a component resulting from the presence of 1.13% of $^{13}$C in natural carbon. This can be dealt with by either deducting the natural enrichment mass isotopomer distribution of the analyte from the raw data\textsuperscript{32,33} or using mathematical models that include the natural enrichment mass isotopomer distribution of the analyte in the computations.\textsuperscript{34} Mass isotopomers are used to study the
regulation of metabolic pathways and discover new pathways (for reviews, see Hellerstein et al.\textsuperscript{35} and Brunengraber et al.\textsuperscript{36}). Another important distinction is the difference between metabolism and catabolism. Metabolism is the set of chemical reactions that occur in the living system to carry out cellular processes, while in catabolism molecules are broken down into smaller units.

2.2.2 \textbf{Identification of a New Class of CoA Esters: 4-Phosphoacyl-CoA}

Carboxylic acids need to be activated to their corresponding CoA esters before they can be metabolized \textit{in vivo}. In the extracts from frozen rat livers perfused with C\textsubscript{4} to C\textsubscript{11} 4-hydroxyacids, LC-MS/MS analysis identified the expected CoA esters of the substrates. All identified CoA esters showed the typical signature fragments at \textit{m/z} 428 and 261 which correspond to the nucleoside and pantetheine fragments of CoA respectively (See \textbf{Fig 2.1}).\textsuperscript{37} In addition, an unexpected CoA ester was found that migrated faster than the expected esters on a C18 column. This implied that the new CoA ester is more polar than the expected 4-hydroxyacyl-CoA esters. In perfusions with different 4-hydroxyacids, the new CoA esters had an additional 80 Da mass increment than the expected 4-hydroxyacyl-CoA. The mass of the new CoA ester increased by 4 and 6 Da when the unlabeled GHB was replaced by the $^{13}$C\textsubscript{4} and $^2$H\textsubscript{6}-GHB respectively. This confirmed that (i) the new CoA esters had intact carbon skeletons as well as hydrogen atoms of the corresponding 4-hydroxyacids, (ii) their masses are 80 Da greater than the corresponding 4-hydroxyacyl-CoAs, and (iii) the new CoA esters contain an additional polar group compared to the usual CoA esters. Based on the LC-MS/MS fragmentation patterns of 4-hydroxypentanoyl-CoA and of the corresponding new CoA ester, Zhang \textit{et al.} hypothesized that the unknown ester was 4-phosphopentanoyl-CoA.
because the singly charged ion transitions $A \rightarrow C$ and $D \rightarrow F$ were accompanied by the loss of $m/z$ 98, which is equivalent to the loss of phosphoric acid (see Fig 2.1). Corresponding transitions were observed for the unknown CoA ester derived from 4-hydroxybutyrate, identified as 4-phosphobutyryl-CoA. Experiments with 4-hydroxy-$[^{13}\text{C}_4]\text{butyrate}$, 4-hydroxy-$[^2\text{H}_6]\text{butyrate}$, and 4-hydroxy-$[^2\text{H}_6]\text{pentanoate}$, also supported the assignment by exhibiting the equivalent mass increment in the fragments A, C, D, and F (Fig 2.1) as the substrates. The identity of the 4-phosphoacyl-CoAs was further supported by comparing the $^{31}\text{P}$ NMR spectra (see Fig 2.2) of the presumed 4-phosphopentanoyl-CoA and of a standard of malonyl-CoA, which showed that it has one extra phosphorous atom. The 4-phosphobutyryl-CoA and 4-phosphopentanoyl-CoA were also isolated from a series of rat liver perfusions with their corresponding substrates using semi-preparative high pressure liquid chromatography. The isolated CoA esters showed very good matches between the theoretical and measured masses (See Table 2.1). To test whether 4-phosphoacyl-CoAs are generated from other hydroxyacids, we perfused livers, each with 2 mM 2-hydroxyacid ($C_3$), 3-hydroxyacids ($C_3$ to $C_5$), or 5-hydroxyacid ($C_5$). None of these compounds formed a detectable 4-phosphoacyl-CoA. The amount of 4-phosphoacyl-CoA production varies considerably from one to another while rat livers were perfused with same concentration of different 4-hydroxyacids, $C_4$-$C_{11}$ (see Fig 2.3).
**Table 2.1** Exact masses of 4-phosphobutyryl-CoA and 4-phosphopentanoyl-CoA isolated from perfused rat livers.

<table>
<thead>
<tr>
<th>Formula</th>
<th>Measured mass</th>
<th>Theoretical mass</th>
<th>Deviation (Da)</th>
<th>Deviation (ppm)</th>
</tr>
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<tr>
<td>4-Phosphobutyryl-CoA</td>
<td>C_{25}H_{44}N_{7}O_{21}P_{4}S</td>
<td>934.12801</td>
<td>934.126153</td>
<td>-0.001857</td>
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<tr>
<td>4-Phosphopentanoyl-CoA</td>
<td>C_{26}H_{46}N_{7}O_{21}P_{4}S</td>
<td>948.14141</td>
<td>948.141803</td>
<td>0.000393</td>
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<tr>
<td>Fragments</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>C_{16}H_{30}N_{2}O_{8}PS</td>
<td>441.14612</td>
<td>441.146051</td>
<td>0.000069</td>
<td>0.15641</td>
</tr>
<tr>
<td>C_{10}H_{16}N_{5}O_{10}P_{2}</td>
<td>428.03732</td>
<td>428.037244</td>
<td>0.000076</td>
<td>0.17755</td>
</tr>
<tr>
<td>C_{16}H_{27}N_{2}O_{4}S</td>
<td>343.16905</td>
<td>343.169154</td>
<td>0.000104</td>
<td>0.30306</td>
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</table>
FIGURE 2.1 Fragmentation patterns of 4-phosphopentanoyl-CoA. Note the loss of phosphoric acid (98 Da) during two MS/MS/MS fragmentations (A → C and D → F). Fragments A and D are specific of 4-phosphopentanoyl-CoA. Fragments B, C, E, F and G are common to 4-hydroxypentanoyl-CoA and 4-phosphopentanoyl-CoA. Fragments B and E are common to all acyl-CoAs.
**Figure 2.2** $^{31}$P-NMR of (A) malonyl-CoA standard and of (B) 4-phosphopentanoyl-CoA isolated from a rat liver perfused with 4-hydroxypentanoate. The new peak at 86.6 ppm accounts for the additional phosphate group in 4-phosphopentanoyl-CoA.
Figure 2.3 Accumulation of 4-phosphoacyl-CoAs derived from 4-hydroxyacids. Retention times and relative abundances of 4-phosphoacyl-CoAs assayed under identical LC-MS conditions in extracts of rat livers perfused with 2 mM of C₄ to C₁₁ 4-hydroxyacids (composite chromatogram). Note: Each peak denotes the 4-phosphoacyl-CoA derived from corresponding 4-hydroxyacid. For example, C₅ is the 4-phosphopentanoyl-CoA derived from 4-hydroxypentanoic acid.
2.2.3 **Liver Perfusion with 4-Hydroxy-2-(E)-nonenal (4-HNE)**

Aldehyde dehydrogenase is known to oxidize 4-HNE to 4-hydroxynonenoic acid (see Fig 2.5A, Step III).\(^{20}\) We were interested to see whether 4-HNE could also transform into the saturated hydroxyacid (*i.e.* 4-hydroxynonanoic acid) and subsequently follow the 4-hydroxyacid metabolism. We perfused rat liver with re-circulating perfusate to which 2 mM [5,5,6,6,7,7,8,8,9,9,9-H\(^{11}\)]4-HNE or \(d_{11}\)-4-HNE (Fig 2.4A) was added at the very beginning. The \(d_{11}\)-4-HNE was employed to distinguish the metabolites from those of the unlabeled endogenous 4-HNE. The liver was quick-frozen after 2 h and analyzed by LC-MS/MS. The final perfusate was also analyzed by GC-MS. Fig 2.4B shows the eight \(^{2}\)H-labeled compounds: 4-phosphononanoyl-CoA, propionyl-CoA, pentanoyl-CoA, hexanoyl-CoA, heptanoyl-CoA, 4-hydroxynonanoate, 4-hydroxynonenoate, and 2-hydroxyheptanoate that we identified from that perfusion. The mass isotopomer distributions of these compounds are reported in Table 2.2. The same but unlabeled compounds were identified in one liver perfusion with unlabeled 4-HNE. The data of Table 2.2 show that the catabolism of 4-HNE leads to compounds containing seven, six, five, and three carbons. This suggests parallel metabolic pathways with more than one mechanism of degradation because β-oxidation of 4-hydroxynonanoate or 4-hydroxynonenoate alone would not form both hexanoyl-CoA and 2-hydroxyheptanoate.

The liver perfusion with \(d_{11}\)-4-HNE was a key experiment for the detection of its metabolites. The mass isotopomer distribution of metabolites of \(d_{11}\)-4-HNE with nine-carbon skeletons (4-phosphononanoyl-CoA, 4-hydroxynonanoate, and 4-hydroxynonenoate) is shown in Table 2.2. Interestingly there is a significant portion of unlabeled component (M) in 4-phosphononanoyl-CoA, 4-hydroxynonanoate, and 4-
hydroxynonenoate, which initially seemed somewhat paradoxical. Our first interpretation was that our synthetic $d_{11}$-4-HNE was contaminated with unlabeled 4-HNE. Careful analysis ruled out this possibility. Our subsequent interpretation pointed to the unlabeled metabolites with nine-carbon skeleton being from endogenous lipid peroxidation that occurs during the perfusion experiments. The unlabeled components were not found in the control livers perfused with only buffer (without 4-HNE) which further supported our interpretation. A similar finding was reported by Cadenas, et al. who observed stimulation of the production of ethane and $n$-pentane by hepatocytes incubated with 2 mM 4-HNE.\textsuperscript{38}
Figure 2.4 (A) Structure of $d_{11}$-4-HNE; and (B) $^2\text{H}$-labeled metabolites identified from the liver perfused with $d_{11}$-4-HNE: 4-phosphononanoyl-CoA (I), propionyl-CoA (II), pentanoyl-CoA (III), hexanoyl-CoA (IV), heptanoyl-CoA (V), 4-hydroxynonanoate (VI), 4-hydroxynonenoate (VII), and 2-hydroxyheptanoate (VIII).
**Table 2.2** Mass isotopomer distribution of metabolites derived from $d_{11}$-4-HNE.

<table>
<thead>
<tr>
<th></th>
<th>M</th>
<th>M1</th>
<th>M2</th>
<th>M3</th>
<th>M4</th>
<th>M5</th>
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<td>1</td>
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<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>18.7</td>
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<td>55.5</td>
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<td>II</td>
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<td>2.3</td>
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<tr>
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<td>0.4</td>
<td>0.0</td>
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<td>0.1</td>
<td>0.2</td>
<td>2.4</td>
<td>8.5</td>
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<td>1</td>
<td>1.9</td>
<td>5.1</td>
<td>3.1</td>
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<tr>
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<td>1.8</td>
<td>13.9</td>
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2.2.4 Rat Liver Perfusion of 4-Hydroxyacids and Their Metabolomics

We performed a series of liver perfusions with 4-hydroxyacids of different chain lengths (C5-C11). A pattern of metabolites derived from 4-hydroxyacids was identified which is shown in Fig 2.5B. With each of these 4-hydroxyacids, we observed the progressive accumulation of (i) a 3-hydroxyacid with the intact carbon skeleton, (ii) a 2-hydroxyacid with two carbons less than the starting substrate, and (iii) acids with two and three carbons less than the starting substrate. This again prompted us to hypothesize the existence of two mechanisms of degradation of 4-hydroxyacids with at least five carbons. These pathways are outlined in Fig 2.6 using 4-hydroxynonanoate (compound 1) as the starting substrate. The first mechanism (Fig 2.6, pathway A) is the isomerization of 4-hydroxyacyl-CoA (compound 2) to 3-hydroxyacyl-CoA (compound 4), the intermediate of β-oxidation, via 4-phosphoacyl-CoA (compound 3) and other CoA esters to be described below. This is followed by the well-known β-oxidation cycles producing (i) acetyl-CoA (compound 5) plus propionyl-CoA (compound 6), in the case of odd-chain 4-hydroxyacids or (ii) only acetyl-CoA (compound 5), in the case of even-chain 4-hydroxyacids. The second mechanism (Fig 2.6, pathway B), starting at 4-hydroxyacyl-CoA (compound 2), involves one cycle of β-oxidation, followed by one cycle of α-oxidation, and cycles of β-oxidation. Pathway B leads to formate (compound 9), via α-oxidation of 2-hydroxyacyl-CoA (compound 7) hydrolyzed from formyl-CoA,31,39 and either (i) acetyl-CoA plus propionyl-CoA, in the case of even-chain 4-hydroxyacids or (ii) only acetyl-CoA, in the case of odd-chain 4-hydroxyacids with at least five carbons. This parallel mechanism was confirmed by the mass isotopomer distribution of acyl-
CoAs and of carboxylic acids formed during the degradation of 4-hydroxyacids labeled with $^{13}$C or $^2$H as described below.

**Figure 2.5** (A) Two alternative metabolic pathways for 4-HNE: I. Glutathionylation via GSTA4 results in the 4-HNE-glutathione 1:1-conjugate; II. Reduction of 4-HNE to 1,4-dihydroxynonene by alcohol dehydrogenases or aldol reductase or aldehyde reductase;
III. Oxidative process assisted by aldehyde dehydrogenases to give 4-hydroxynonenenoic acid;\textsuperscript{20} IV. Reduction of the $\alpha,\beta$-unsaturation catalyzed by NAD(P)H-dependent alkenal/one oxidoreductase.\textsuperscript{40} We identified another metabolite, namely 4-hydroxynonanoic acid from the metabolomics study of 4-HNE in perfused rat liver.\textsuperscript{1} Although it is not clear which enzymes are responsible for this particular biotransformation, the two probable pathways are shown by dotted lines. (B & C) Concentrations of 3-hydroxyacids with the same carbon number as substrate and 2-hydroxyacids with 2 carbons less than substrate in the different chain lengths of 4-hydroxyacids perfusates are shown in the panel B and C respectively.
**Figure 2.6** Proposed parallel pathways for the degradation of 4-hydroxyacids (exemplified with 4-hydroxynonanoic acid, 1).
To secure evidence supporting the proposed pathways, we devised a labeling strategy that could incorporate either a single $^{13}$C label at position C-3 or a doubly labeled molecule with $^{13}$C at positions C-3 and C-4. We synthesized 4-hydroxy-$[3-^{13}$C$]$nonanoate and 4-hydroxy-$[3,4-^{13}$C$_2]$nonanoate to prove the hypothesized metabolic pathways outlined in Fig 2.6. The label at C-3 was used primarily to confirm that the $\alpha$-oxidation step in Pathway B leads to $[^{13}$C$]$formate. The C-3,4 labeled molecule allowed us to confirm both pathways and quantify the relative flux of each pathway. 4-Hydroxy-$[3-^{13}$C$]$nonanoate would lead to the formation of M1 acetyl-CoA via pathway B. 4-Hydroxy-$[3,4-^{13}$C$_2]$nonanoate would lead to the formation of M1 acetyl-CoA via pathway B and M2 acetyl-CoA via pathway A (Fig 2.7, follow the fates of C-3 and C-4 of 4-hydroxynonanoate, shown in red). In livers perfused with recirculating buffer containing 2 mM unlabeled, 3-$^{13}$C labeled or 3,4-$^{13}$C$_2$-labeled 4-hydroxynonanoate, we observed the M, M1, and / or M2 compounds listed in Table 2.3. The time dependent accumulation of $[^{13}$C$]$formate was also observed in perfusions with labeled substrates (Fig 2.8). The mass isotopomer distribution of these compounds is compatible with the scheme presented in Fig 2.7.
Figure 2.7 Parallel pathways for the catabolism of 4-hydroxyacids using 4-hydroxynonanoate as an example. The initial experiment with $d_{11}$-4-HNE (see the grey highlights) enabled identification of the majority of the catabolic intermediates shown above. Singly labeled 4-hydroxynonanoate is highlighted in blue at C-3 and doubly labeled 4-hydroxynonanoate is highlighted in red at C-3 and C-4 to facilitate tracing their fates through pathways A and B. Note that the doubly labeled substrate forms acetyl-CoA, part of which is doubly labeled (M2) via pathway A and singly labeled (M1) via pathway B. Formate, derived from C-3 of the substrate, is formed via pathway B and delivers M1 formate from both labeled starting substrates. Note that for even-chain 4-hydroxyacids with at least six carbons, pathway A leads to acetyl-CoA, whereas pathway B leads to acetyl-CoA, propionyl-CoA, and formate. The hypothesized mechanism of the
isomerization of 4-hydroxy acyl-CoA to 3-hydroxyacyl-CoA is shown in the left-hand portion of the figure.

**Figure 2.8** Release of $[^{13}\text{C}]$formate by livers perfused with recirculating buffer containing 2 mm 4-hydroxy-$[^{3}\text{C}]$nonanoate (■), 4-hydroxy-$[^{3,4}\text{C}_2]$nonanoate (▲).
The synthetic approach, illustrated in Fig 2.9 (details are provided in the experimental section), is based on a convergent route that minimizes the number of reactions that need to be optimized. For the C-3 labeled molecule we began with n-hexyl bromide and introduced carbon-13 via a Grignard reaction with labeled carbon dioxide. Esterification and reduction with diisobutylaluminium hydride (DIBAL-H) gave the common intermediate heptanal (circled in Fig 2.9). Introduction of the labeled carbon-13 for the C-3,4 molecule began with isotopically labeled ethyl bromoacetate, and built up the non-labeled carbon in the opposite direction (as compared to the C-3 labeled molecule) via a Wittig olefination and catalytic reduction. DIBAL-H reduction delivered the common intermediate heptanal. C-1 and C-2 are introduced via an additional Wittig olefination, and the C-4 hydroxyl group is introduced through the use of selenium dioxide under carefully optimized solvent conditions. Typically we converted this molecule to the saturated lactone by hydrogenation, and a two-step hydrolysis/acetic laconization sequence. For stability, we stored these molecules as the lactone and hydrolyze just prior to any animal experiments.
**FIGURE 2.9** Convergent synthetic routes for 4-hydroxy-[3-\(^{13}\)C]nonanoic acid lactone and 4-hydroxy-[3,4-\(^{13}\)C\(_2\)]nonanoic acid lactone.
Table 2.3 Mass isotopomers of metabolites released by livers perfused with unlabeled and labeled 4-hydroxynonanoates. Metabolites were assayed by LC-MS/MS.

<table>
<thead>
<tr>
<th>Intermediates</th>
<th>Intermediates of pathway</th>
<th>Retention time (min)</th>
<th>4-Hydroxy-nonanoate</th>
<th>4-Hydroxy-[3-13C]nonanoate</th>
<th>4-Hydroxy-[3,4-13C2]nonanoate</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-Hydroxynonanoyl-CoA</td>
<td>Before pathway bifurcation</td>
<td>20.2</td>
<td>M (m/z=924)</td>
<td>M1 (m/z=925)</td>
<td>M2 (m/z=926)</td>
</tr>
<tr>
<td>4-Ketononanoyl-CoA</td>
<td>Before pathway bifurcation</td>
<td>20.1</td>
<td>M (m/z=922)</td>
<td>M1 (m/z=923)</td>
<td>M2 (m/z=924)</td>
</tr>
<tr>
<td>4-Phosphononanoyl-CoA</td>
<td>A</td>
<td>17.3</td>
<td>M (m/z=1004)</td>
<td>M1 (m/z=1005)</td>
<td>M2 (m/z=1006)</td>
</tr>
<tr>
<td>3-Hydroxy-4-phosphononanoyl-CoA</td>
<td>A</td>
<td>16.4</td>
<td>M (m/z=1020)</td>
<td>M1 (m/z=1021)</td>
<td>M2 (m/z=1022)</td>
</tr>
<tr>
<td>3,4-Dihydroxynonanoyl-CoA</td>
<td>B</td>
<td>18.7</td>
<td>M (m/z=940)</td>
<td>M1 (m/z=941)</td>
<td>M2 (m/z = 942)</td>
</tr>
<tr>
<td>Diketononanoyl-CoA</td>
<td>B</td>
<td>17.2</td>
<td>M (m/z=936)</td>
<td>M1 (m/z=937)</td>
<td>M2 (m/z = 938)</td>
</tr>
<tr>
<td>2-Hydroxyheptanoyl-CoA</td>
<td>B</td>
<td>19.1</td>
<td>M (m/z=912)</td>
<td>M1 (m/z=913)</td>
<td>M2 (m/z = 914)</td>
</tr>
<tr>
<td>Heptanoyl-CoA</td>
<td>A</td>
<td>21.4</td>
<td>M (m/z=880)</td>
<td>M1 (m/z=881)</td>
<td>M2 (m/z = 882)</td>
</tr>
<tr>
<td>Hexanoyl-CoA</td>
<td>B</td>
<td>20.1</td>
<td>M (m/z=866)</td>
<td>M1 (m/z=866)</td>
<td>M2 (m/z = 867)</td>
</tr>
<tr>
<td>Acetyl-CoA</td>
<td>A &amp; B</td>
<td>13.9</td>
<td>M (m/z=810)</td>
<td>M1 (m/z=811)</td>
<td>M2 (m/z = 812) &amp; 811</td>
</tr>
</tbody>
</table>
2.2.5 Measurement of Flux through the Two Pathways

A key attribute of these labeling patterns is the ability to quantify the relative fluxes through both Pathway A and Pathway B (Fig 2.7). This could presumably be done with either the C-3 or C-3,4 labeled molecules. For the C-3 labeled molecule, it would be accomplished by quantitatively measuring the ratio of M1 isotopically enriched formate to that of M1 enriched acetyl-CoA. This would be difficult as formate is only produced in Pathway B, and there are other sources of formate in the liver. We chose, therefore, to utilize the 3,4 labeled molecule as we could quantify the relative fluxes by comparing M1 acetyl-CoA to M2 acetyl-CoA (rationalized in Fig 2.7). This approach has the distinct advantage of only monitoring acetyl-CoA. We therefore do not have to worry about variability in how the molecules will behave on LC/GC, or differences in ionization during the mass spectrometry. As shown in Fig 2.10A, the plateau of M2 acetyl-CoA is 15.9% (contribution from pathway A, Fig 2.8), and the plateau of M1 acetyl-CoA is 2.9% (contribution from pathway B, Fig 2.8). The other 81.2% acetyl-CoA is unlabeled (contribution from endogenous sources and the unlabeled carbons of the 4-hydroxyacids). The M2/M1 ratio of acetyl-CoA is therefore 5.5, which means the contribution of pathway A is 5.5 times that of pathway B. To further confirm that the source of M2 acetyl-CoA is from Pathway A and not from a reprocessing of labeled formate we performed an analogous series of perfusions utilizing the C-3 labeled molecule. As expected, we see a plateau of M1 acetyl-CoA derived from Pathway A, and no M2 acetyl-CoA as shown in Fig 2.10B.
**Figure 2.10** The M1 and M2 acetyl-CoA enrichment from 0 to 1 mM of (A) 4-hydroxy-[3,4-\(^{13}\)C\(_2\)]-nonanoate and (B) 4-hydroxy-[\(^{3}\)C]-nonanoate non-recirculating perfusions. Ratio of M1 and M2 acetyl-CoA (from A) demonstrate the flux of the two pathways.
2.2.6 TRANSFORMATION OF 4-HNE TO 4-HYDROXYNONANOIC ACID

We further investigated the mechanism of the formation of 4-hydroxynonanoic acid (more precisely 4-hydroxynonanoyl-CoA) from 4-HNE. From a mechanistic point of view, we considered two possible pathways for the formation of 4-hydroxynonanoyl-CoA. The first is that 4-HNE is transformed to its saturated version, i.e. 4-hydroxynonanal (Compound A, Fig 2.11) followed by oxidation of the latter to yield 4-hydroxynonanoyl-CoA, while a second is that the oxidation of 4-HNE to produce 4-hydroxynonenoic acid (Compound B, Fig 2.11) followed its saturation. To test whether the transformation goes through Compound A or Compound B, we synthesized both the compounds and perfused them into rat liver. Eventually, both the compounds were found to be catabolized through the metabolic pathways that we demonstrated for the degradation of 4-hydroxyacids (Fig 2.7). We identified the metabolites of both Pathway A and Pathway B (Fig 2.7) for the catabolism of 4-hydroxynonanal and 4-hydroxynonenoic acid as shown in Table 2.4. This confirmed that 4-HNE can be converted to 4-hydroxynonanoyl-CoA via both Compound A and Compound B. Next, we wanted to determine the main pathway for transformation of 4-HNE into 4-hydroxynonanoyl-CoA. The ratio of 4-phosphononenooyl-CoA (product of dehydrogenation of 4-phosphononanoyl-CoA) and 4-phosphononanoyl-CoA was calculated. For the perfusion with 4-hydroxynonanal the ratio was 7.5% and for 4-hydroxynonenoic acid it was 26.9%. The higher ratio for 4-hydroxynonenoic acid shows that the transformation of 4-HNE to 4-hydroxynonanoyl-CoA mainly proceeds via 4-hydroxynonenoic acid.
**Table 2.4** Identification of metabolites released by control liver, livers perfused with 4-hydroxynonanal (Compound A), and 4-hydroxynonenoate (Compound B). Metabolites were assayed by LC-MS/MS.

<table>
<thead>
<tr>
<th>Intermediates of pathway</th>
<th>Retention time (min)</th>
<th>Control liver</th>
<th>Livers perfused with 4-Hydroxynonenoate</th>
<th>Livers perfused with 4-Hydroxynonanal</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-Phosphononanoyl-CoA A</td>
<td>17.3</td>
<td>Not present</td>
<td>Present</td>
<td>Present</td>
</tr>
<tr>
<td>4-Phosphononenoyl-CoA A</td>
<td>16.4</td>
<td>Not present</td>
<td>Present</td>
<td>Present</td>
</tr>
<tr>
<td>Heptanoyl-CoA A</td>
<td>21.4</td>
<td>Not present</td>
<td>Present</td>
<td>Present</td>
</tr>
<tr>
<td>Propionyl-CoA A</td>
<td>14.6</td>
<td>Not present</td>
<td>Present</td>
<td>Present</td>
</tr>
<tr>
<td>Hexanoyl-CoA B</td>
<td>19.8</td>
<td>Not present</td>
<td>Present</td>
<td>Present</td>
</tr>
<tr>
<td>2-Hydroxyheptanoyl-CoA B</td>
<td>19.1</td>
<td>Not present</td>
<td>Present</td>
<td>Present</td>
</tr>
</tbody>
</table>
Figure 2.11 Alternative pathways for the transformation of 4-HNE to 4-hydroxynonanoyl-CoA.
2.3 Experimental Sections

2.3.1 Materials and Methods

Ethyl bromoacetate-$^{13}$C$_2$ (99 atom% $^{13}$C) and carbon-$^{13}$C dioxide (99.9 atom% $^{13}$C) were purchased from Isotec, Sigma-Aldrich. Triphenylphosphine (99%), valeraldehyde (97%), (ethoxycarbonylmethylene)triphenylphosphorane (+98%) were purchased from Acros Organics. Flash chromatography was performed on silica gel (230-400 mesh) purchased from Dynamic Adsorbents (Atlanta, GA). TLC was done on hard layer, organic binder TLC-plates with a fluorescent indicator, that allows visualization by UV light (254 nm), purchased from Dynamic Adsorbents (Atlanta, GA). Solvents and reagents were of commercially available analytical grade and used as received without further purification. $^1$H and $^{13}$C-NMR spectra were recorded on a Varian Inova spectrometer (at the Department of Chemistry, Case Western Reserve University) operating at 400 MHz and 100 MHz for the $^1$H and $^{13}$C-NMR spectra, respectively. The internal references were TMS (δ 0.00) and CDCl$_3$ (δ 77.2) for $^1$H and $^{13}$C spectra, respectively. NMR data are presented in the following order: chemical shift, peak multiplicity (b = broad, s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, dd = doublet of doublet, ddd = doublet of doublet of doublet, ddt = doublet of doublet of triplet, dq = doublet of quartet, dm = doublet of multiplet, br = broad), coupling constant, proton number. Mass spectra were obtained either on a Kratos MS 25 Mass Spectrometer (at the Department of Chemistry, Case Western Reserve University) using FAB ionization method in m-nitrobenzyl alcohol or glycerol matrices or on a Thermo Scientific LTQ-FT hybrid mass spectrometer (Rieveschl Laboratories for Mass Spectrometry, University of Cincinnati) using electrospray ionization (positive mode). IR
spectra were measured on a Midac M2000 as films on NaCl plates, the peaks being reported in reciprocal centimeters.

2.3.2 Synthesis of 4-Hydroxy-[3,4-\textsuperscript{13}C\textsubscript{2}]-nonanoic acid lactone

[1,2-\textsuperscript{13}C\textsubscript{2}](Ethoxycarbonylmethyl)triphenylphosphonium bromide (1)

\[
\begin{align*}
\text{Br} & \quad \text{O} & \quad \text{PPh\textsubscript{3}}, \text{EtOAc} & \quad \text{12 h, RT, 93.5\%} & \quad \text{Br} & \quad \text{PPh\textsubscript{3}} & \quad \text{O} \\
\text{1} & & & & & & & \\
\end{align*}
\]

Ethyl [1,2-\textsuperscript{13}C\textsubscript{2}]-2-bromoacetate (2.54 g, 15.03 mmol) in ethyl acetate (10 mL) was slowly added to a solution of triphenylphosphine (4.06 g, 15.48 mmol) in ethyl acetate (10 mL). The reaction mixture was then stirred at room temperature for overnight and the white precipitate filtered off, washed with diethyl ether (3 \times 10 mL), and dried under vacuum for 2 h to give 1 (6.06 g, 14.05 mmol, 93.5\% yield). \textsuperscript{1}H NMR (400 MHz, CDCl\textsubscript{3}): \( \delta \) 1.06 (t, 3H, \( J = 7.2 \) Hz), 4.03 (dq, 2H, \( J = 7.2 \) Hz, \( J = 3.2 \) Hz), 5.39 (ddd, 2H, \( J_{\text{H-C}} = 134.8 \) Hz, \( J_{\text{H-P}} = 14 \) Hz, \( J_{\text{H-C}} = 7.6 \) Hz), 7.66−7.93 (15H); \textsuperscript{13}C NMR (100 MHz, CDCl\textsubscript{3}): \( \delta \) 13.6, 33.4 (dd, \( J_{\text{C-P}} = 55.7 \) Hz, \( J_{\text{C-C}} = 58.7 \) Hz), 62.8, 117.8 (d, \( J_{\text{C-P}} = 88.9 \) Hz), 130.2 (d, \( J_{\text{C-P}} = 13.8 \) Hz), 133.8 (d, \( J_{\text{C-P}} = 10.7 \) Hz), 135.1 (d, \( J_{\text{C-P}} = 3.8 \) Hz), 164.7 (dd, \( J_{\text{C-C}} = 59.1 \) Hz, \( J_{\text{C-P}} = 3 \) Hz).

[1,2-\textsuperscript{13}C\textsubscript{2}](Ethoxycarbonylmethylene)triphenylphosphorane (2)

\[
\begin{align*}
\text{Br} & \quad \text{Ph\textsubscript{3}P} & \quad \text{O} & \quad \text{NaOH, CH\textsubscript{2}Cl\textsubscript{2}} & \quad \text{15 min, RT, 98.3\%} & \quad \text{Ph\textsubscript{3}P} & \quad \text{O} \\
\text{1} & & & & & & & \\
\text{2} & & & & & & & \\
\end{align*}
\]
A solution of sodium hydroxide (1.0 M, 50 mL) was added to a solution of [1,2-$^{13}$C$_2$](Ethoxycarbonylmethyl)triphenylphosphonium bromide 1 (6.0 g, 13.91 mmol) in dichloromethane (50 mL) and the reaction mixture stirred vigorously for 15 min. The organic layer was removed and the aqueous layer extracted with dichloromethane (3 × 15 mL). The combined organic layers were dried over Na$_2$SO$_4$ and concentrated under vacuum to give 2 (4.78 g, 13.64 mmol, 98.3% yield). $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ 1.06 (br, 3H), 2.9 (br, d, 1H, $J_{H-P} = 120$ Hz), 3.96 (br, 2H), 7.43–7.68 (15H); $^{13}$C NMR (100 MHz, CDCl$_3$): $\delta$ 15.0, 30.1 (dd, $J_{C-P} = 124.7$ Hz, $J_{C-C} = 87.3$ Hz), 58.1, 128.0 (d, $^1J_{C-P} = 90.6$ Hz), 128.9 (d, $^3J_{C-P} = 11.4$ Hz), 132.1, 133.2 (d, $^2J_{C-P} = 9.9$ Hz), 171.5 (dd, $J_{C-C} = 87.8$ Hz, $^2J_{C-P} = 11$ Hz).

Ethyl (E)-[1,2-$^{13}$C$_2$]hep-2-enoate (3)

To a solution of valeraldehyde (1.46 g, 16.95 mmol) in dichloromethane (25 mL) was added [1,2-$^{13}$C$_2$]-(ethoxycarbonylmethylene)triphenylphosphorane 2 (4.75 g, 13.56 mmol) in dichloromethane (25 mL) and the reaction mixture was stirred for 3 h. The solvent was evaporated in vacuo and the crude product purified by column chromatography over silica (hexane, 8% ethyl acetate) to give 3 (1.82 g, 11.50 mmol, 84.8% yield). $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ 0.91 (t, 3H, $J = 7.2$ Hz), 1.29 (t, 3H, $J = 7.2$ Hz), 1.31-1.46 (4H), 2.17–2.24 (2H), 4.18 (dq, 2H, $J = 7.2$ Hz, $J = 3.2$ Hz ), 5.80 (ddm, 1H, $^1J_{H-C} = 161.7$ Hz, $J = 15.6$ Hz, $J = 1.6$ Hz, CH=CH), 6.91-7.01 (1H); $^{13}$C NMR (100 MHz, CDCl$_3$): $\delta$ 14.0, 14.5, 22.6 (d, $J_{C-C} = 4.6$ Hz), 30.3 (d, $J_{C-C} = 3.4$ Hz), 32.1 (d, $J_{C-C} =$
6.6 Hz), 60.3, 121.4 (d, \(J_{C-C} = 75\) Hz), 149.6 (d, \(J_{C-C} = 70.0\) Hz), 167.0 (d, \(J_{C-C} = 74\) Hz).

IR \(\nu = 1681\) (C=O), 1622 (C=C). FAB-HRMS (positive mode): \(m/z\) Calcd. for \(C_{7}^{13}C_{2}H_{17}O_{2}\) [MH\(^+\)] 159.1150, found 159.1250.

**Ethyl (E)-[1,2-\(^{13}\)C\(_{2}\)]heptanoate (4)**

\[
\begin{align*}
\text{Ethyl (E)-[1,2-\(^{13}\)C\(_{2}\)]heptanoate (4)}
\end{align*}
\]

Ethyl (E)-[1,2-\(^{13}\)C\(_{2}\)]heptanoate (4, 1.80 g, 11.38 mmol) was dissolved in 25 mL ethanol and mixed with 0.18 g of 10\% Pt/C under 1 atm of H\(_{2}\). The mixture was subsequently stirred for 1 h at room temperature, filtered through a pad of celite, the filter cake was washed with 10 mL ethanol, and the solvent removed \textit{in vacuo} to afford 4 (1.81 g, 11.30 mmol, 99.3\%). This product was directly used for the next step without any further purification.

**[1,2-\(^{13}\)C\(_{2}\)]Heptanal (5)**

\[
\begin{align*}
\text{[1,2-\(^{13}\)C\(_{2}\)]Heptanal (5)}
\end{align*}
\]

Ethyl (E)-[1,2-\(^{13}\)C\(_{2}\)]heptanoate (4, 1.82 g, 11.36 mmol) was dissolved in 30 mL of dichloromethane and 9.5 mL of DIBAL (1.2 M solution in Toluene, 11.4 mmol) was added dropwise at -78 °C with continuous stirring. After 15 minutes the reaction mixture was allowed to warm to room temperature for 5 min and 1M HCl was carefully added until pH = 3. The aqueous phase was extracted with dichloromethane (2 \(\times\) 20 mL), the organic layers were combined and dried over Na\(_{2}\)SO\(_{4}\). The solvent was subsequently removed.
removed in vacuo and the product 5 (1.19 g, 10.24 mmol, 90.1%) obtained was used directly for the next step without any further purification.

**Ethyl (E)-[3,4-\textsuperscript{13}C\textsubscript{2}]non-2-enoate (6)**

To a solution of [1,2-\textsuperscript{13}C\textsubscript{2}]heptanal 5 (1.19 g, 10.24 mmol) in dichloromethane (25 mL) was added (ethoxycarbonylmethylene)triphenylphosphorane (4.46 g, 12.80 mmol) in dichloromethane (25 mL) and the reaction mixture was stirred for 3 h. The solvent was subsequently evaporated in vacuo and the crude product was purified by column chromatography over silica (hexane, 8% ethyl acetate) to give 6 (1.52 g, 8.16 mmol, 79.7% yield). \textsuperscript{1}H NMR (400 MHz, CDCl\textsubscript{3}): \(\delta\) 0.89 (t, 3H, \(J = 6.8\) Hz), 1.22-1.36 (9H), 1.40-1.49 (2H), 2.19 (dm, 2H, \(J_{H-C} = 126.4\) Hz), 4.18 (q, 2H, \(J = 7.2\) Hz), 5.81 (dd, 1H, \(J = 15.8\) Hz, 5.6 Hz), 6.97 (dm, 1H, \(J_{H-C} = 153.8\) Hz, \(J = 15.6\) Hz); \textsuperscript{13}C NMR (100 MHz, CDCl\textsubscript{3}): \(\delta\) 14.3, 14.5, 22.8, 28.7, 29.0 (d, \(J_{C-C} = 1.5\) Hz), 31.8, 32.4 (d, \(J_{C-C} = 41.2\) Hz), 60.3, 121.7, 149.7 (d, \(J_{C-C} = 41.2\) Hz), 167.0. IR \(\nu\) 1722(C=O), 1626(C=C). FAB-HRMS (positive mode): \textit{m/z} Calcd. for C\textsubscript{9}\textsuperscript{13}C\textsubscript{2}H\textsubscript{21}O\textsubscript{2} [MH\textsuperscript{+}] 187.1463, found 187.1638.

**Ethyl (E)-4-hydroxy-[3,4-\textsuperscript{13}C\textsubscript{2}]non-2-enoate (7)**

Ethyl (E)-[3,4-\textsuperscript{13}C\textsubscript{2}]non-2-enoate (6, 1.50 g, 8.05 mmol) was dissolved in a mixture of 9 mL dioxane and 1 mL water, to which 1.77 g (16 mmol) selenium dioxide was added and
then the mixture was heated under reflux overnight. The reaction mixture was cooled, 10 mL of water was added, and the suspension was extracted with dichloromethane (3 × 30 mL). The combined organic layers were dried over Na$_2$SO$_4$, concentrated in vacuo, and the crude product was purified by column chromatography over silica (hexane, 15% ethyl acetate) to give 7 (1.24 g, 6.13 mmol, 76.1% yield). $^1$H NMR (400 MHz, CDCl$_3$): δ 0.89 (t, 3H, $J$ = 6.8 Hz), 1.28-1.51 (9H), 1.54-1.61 (2H), 4.20 (q, 2H, $J$ = 7.2 Hz), 4.28-4.32 (1H), 6.03 (ddt, 1H, $J$ = 15.8 Hz, 5.6 Hz, 1.6 Hz), 6.95 (dt, 1H, $J_{H-C}$ = 156 Hz, $J$ = 15.6 Hz, 5.2 Hz); $^{13}$C NMR (100 MHz, CDCl$_3$): δ 14.2, 14.4, 22.8, 25.1, 31.9, 37.0, 60.7, 71.3 (d, $J_{C-C}$ = 45 Hz), 120.6, 150.5 (d, $J_{C-C}$ = 45 Hz), 166.9. IR ν 1703(C=O), 1629(C=C), 3450(OH). FAB-HRMS (positive mode): m/z Calcd. for C$_9$H$_{21}$O$_3$ [MH$^+$] 203.1412, found 203.1565.

Ethyl (E)-4-hydroxy-[3,4-$^{13}$C$_2$]nonanoate (8)

Ethyl (E)-4-hydroxy-[3,4-$^{13}$C$_2$]non-2-enoate (7, 0.9 g, 4.45 mmol) of was dissolved in 15 mL ethanol and mixed with 0.1 g of 10% Pt/C, and stirred for 1.5 h under 1 atm of H$_2$. The reaction was then filtered through a pad of celite, the filter cake was washed with 10 mL ethanol and the solvent removed in vacuo to afford the product 8 (0.895 g, 4.38 mmol, 98.4%), which was used directly in the next step without any further purification.
5-Pentyl-[3,4-\(^{13}\)C]dihydrofuran-2(3H)-one or 4-hydroxy-[3,4-\(^{13}\)C]nonanoic acid lactone (9)

At room temperature, ethyl (E)-4-hydroxy-[3,4-\(^{13}\)C]nonanoate (8, 0.895 g, 4.38 mmol) was dissolved in a mixture of THF (10 mL) and H\(_2\)O (5 mL), to which 0.46 g (19.21 mmol) LiOH was added. After stirring for 2 h, 1M HCl was added to the mixture until the pH was approximately 1-2. The mixture was then extracted with dichloromethane (3 \(\times\) 20 mL), the combined organic layers were dried over Na\(_2\)SO\(_4\), and the solvent was removed \textit{in vacuo}. The crude product was purified by column chromatography over silica (hexane, 7% ethyl acetate) to give 9 (0.53 g, 3.35 mmol, 76.5% yield).

\(^1\)H NMR (400 MHz, CDCl\(_3\)): \(\delta\) 0.90 (t, 3H, \(J = 6.8\) Hz), 1.30-1.79 (8H), 1.81-2.56 (4H), 4.49 (dm, 1H, \(J_{H-C} = 149.6\) Hz); \(^{13}\)C NMR (100 MHz, CDCl\(_3\)): \(\delta\) 14.2, 22.7, 25.1, 28.2 (d, \(J_{C-C} = 32\) Hz), 28.9, 31.7(d, \(J_{C-C} = 3.8\) Hz), 81.3 (d, \(J_{C-C} = 32\) Hz), 177.6. IR \(\nu\) 1775(C=O). FAB-HRMS (positive mode): \(m/z\) Calcd. for C\(_7^{13}\)C\(_2\)H\(_{17}\)O\(_2\) [MH\(^+\)] 159.1150, found 159.1296.

2.3.3 \textbf{SYNTHESIS OF 4-HYDROXY-[3-\(^{13}\)C]-NONANOIC ACID LACTONE}

[1-\(^{13}\)C]-Heptanoic acid (10)

Under a N\(_2\) atmosphere, 1.28 g (52.7 mmol) Mg was added to 200 mL diethyl ether and the reaction mixture was heated to reflux. 1-Bromohexane (8.25 g, 50 mmol) in 50 mL
diethyl ether was subsequently added maintaining reflux conditions. The reaction was kept at reflux for an hour, then cooled to 0 °C, sealed, and degassed under vacuum. $^{13}$CO$_2$ was bubbled in until the flask showed positive pressure as indicated by an attached balloon. The reaction was stirred for a further 3 h and then poured into 150 mL of 1M HCl. The aqueous phase was extracted with dichloromethane (2 × 100 mL), the combined organic layers were dried over Na$_2$SO$_4$, and the solvent removed in vacuo. The crude product was purified by column chromatography over silica (dichloromethane) to give 10 (5.4 g, 41.2 mmol, 82.5 % yield). $^1$H-NMR (400 MHz, CDCl$_3$): 0.89 (t, 3H, $J = 6.8$ Hz), 1.24-1.38 (6H), 1.61-1.67 (2H), 2.32-2.37 (2H). $^{13}$C-NMR(100 MHz, CDCl$_3$): 14.2, 22.9, 24.8, 28.9,29.0, 31.6, 180. IR: $\nu$ 1669 ($\text{C}=\text{O}$). ESI-HRMS (positive mode): $m/z$ Calcd. for $\text{C}_6\text{H}_{15}\text{O}_2$ [MH$^+$] 132.1100, found 132.1100.

**Ethyl (E)-[3-$^{13}$C]non-2-enoate (11)**

![Chemical structure diagram]

[1-$^{13}$C]Heptanoic acid (10, 0.33 g, 2.52 mmol) was dissolved in 10 mL diethyl ether. To which freshly prepared diazomethane (16 mmol in 50 mL diethyl ether) was added until a yellow color persisted for 2 min. Diethyl ether was subsequently removed by fractional distillation. The residue was then dissolved in 10 mL dichloromethane and 2.1 mL DIBAL-H (1.2 M solution in Toluene, 2.52 mmol) was added dropwise at -78 °C with continuous stirring. After 5 minutes the reaction mixture was allowed to warm to room
temperature for 5 min and 1M HCl was carefully added until the pH = 3. The aqueous phase was extracted with dichloromethane (2 × 20 mL), the organic layers were combined and dried over Na₂SO₄. The filtrate, without purification, was added directly to (ethoxycarbonylmethylene)triphenylphosphorane (0.88g, 2.53 mmol) in 50 mL dichloromethane at room temperature. After stirring for 1 hr, the solvent was removed in vacuo, and the residue was purified by column chromatography over silica (hexane, 10% ethyl acetate) to give 11 (0.41 g, 2.21 mmol, 87.7% yield). ¹H-NMR (400 MHz, CDCl₃): 0.89 (t, 3H, J = 6.8 Hz), 1.29 (t, 3H, J = 7.2 Hz), 1.29-1.32 (2H), 1.41-1.44 (2H), 2.17-2.21 (2H), 4.18 (q, 2H, J = 7.2 Hz.), 5.8 (d, 1H, J = 16 Hz), 6.97 (ddt, 1H, J = 146 Hz, 16 Hz, 6.8 Hz). ¹³C-NMR (100 MHz, CDCl₃): 14.2, 14.4, 22.7, 28.1, 29.0, 31.8, 32.3(d, J_C-C = 41 Hz), 60.3, 121 (d, J_C-C = 70 Hz), 149.7, 174.5. IR: ν 1720 (C=O), 1627 (C=C). ESI-HRMS (positive mode): m/z Calcd. for C₁₀H₂₁O₂ [MH⁺] 186.1569, found 186.1570.

**Ethyl (E)-4-hydroxy-[3-¹³C]non-2-enoate (12)**

![Chemical structure diagram](image)

Ethyl (E)-[3-¹³C]non-2-enoate (11, 0.56 g, 3.02 mmol) was dissolved in a mixture of 4.5 mL dioxane and 0.5 mL water, to which 0.66 g (6 mmol) selenium dioxide was added and then the mixture was heated under reflux for overnight. The reaction mixture was cooled, 10 mL of water was added, and the suspension was extracted with dichloromethane (3 × 20 mL). The combined organic layers were dried over Na₂SO₄, concentrated in vacuo, and the crude product purified by column chromatography over silica (hexane, 15% ethyl acetate) to give 12 (0.45 g, 2.24 mmol, 74.2% yield). ¹H-NMR
(400 MHz, CDCl₃): 0.89 (t, 3H, $J = 7.2$ Hz), 1.20-1.40 (6H), 1.29 (t, 3H, $J = 7.2$Hz),
1.40-1.44 (2H), 2.17-2.22 (2H), 4.19 (q, 2H, $J = 7.2$ Hz), 5.81 (dm, 1H, $J = 15.6$ Hz),
6.96 (ddt, 1H, $J_{C-H} = 156$ Hz, $J = 15.6$ Hz, 6.8 Hz). $^{13}$C-NMR (100 MHz, CDCl₃): 14.3,
14.5, 22.8, 28.2, 29.0, 31.8, 60.3, 64.6, 121.3 (d, $J_{C-C} = 69$ Hz), 149.7, 174.2.

**Ethyl (E)-4-hydroxy-[3-$^{13}$C]nonanoate (13)**

![Diagram of reaction](image)

Ethyl (E)-4-hydroxy-[3-$^{13}$C]non-2-enoate (12, 0.2 g, 0.99 mmol) was dissolved in 5 mL
of ethanol and mixed with 0.05 g 10% Pt/C, and stirred for 1.5 h under 1 atm of H₂. The
reaction mixture was then filtered through a pad of celite, the filter cake was washed with
10 mL ethanol and the solvent removed in vacuo to afford the product 13 (0.2 g, 0.98
mmol, 99%), which was used directly in the next step without any further purification.

**5-Pentyl-[3-$^{13}$C]dihydrofuran-2(3H)-one or 4-hydroxy-[3-$^{13}$C]-nonanoic acid lactone (14)**

![Diagram of reaction](image)

Ethyl (E)-4-hydroxy-[3-$^{13}$C]nonanoate (13, 0.2 g, 0.98 mmol) of was dissolved in THF (3
mL) and H₂O (1.5mL), to which 0.1 g (4.18 mmol) LiOH was added. After stirring for 2
h, 1M HCl was added to the mixture until the pH was approximately 1–2. The mixture
was then extracted with dichloromethane (3 × 15 mL), the combined organic layers were
dried over Na$_2$SO$_4$, and the solvent was removed in vacuo. The crude product was purified by column chromatography over silica (hexane, 7% ethyl acetate) to give 14 (0.12 g, 0.76 mmol, 77.6%). $^1$H-NMR (400 MHz, CDCl$_3$): 0.90 (t, 3H, $J = 6.8$ Hz), 1.25-2.20 (9H), 2.30-2.50 (3H), 4.47-4.52 (1H); $^{13}$C-NMR (100 MHz, CDCl$_3$): 14.1, 22.7, 25.1, 28.2, 29.1 (d, $J_{C-C} = 33$ Hz), 31.7, 35.7, 81.3 (d, $J_{C-C} = 32$ Hz), 177.5. IR: $\nu$ 1775 (C=O). ESI-HRMS (positive mode): $m/z$ Calcd. for C$_8$H$_{13}$O$_2$ [MH$^+$] 158.1256, found 158.1256.

### 2.3.4 SYNTHESIS OF 4-HYDROXY-(E)-NONENOIC ACID

![Reaction scheme]

Ethyl 4-hydroxy-(E)-non-2-enoate (15) was synthesized following the same procedure as described above for compound 7 starting from commercially available unlabeled ethyl (E)-non-2-enoate. An aqueous solution of LiOH (0.14 g, 6.0 mmol, 3 mL) was added to a solution of 15 (0.50 g, 2.5 mmol) in MeOH (10 mL). After stirring for 3 h at room temperature, the reaction mixture was acidified with 1M HCl. The mixture was then extracted with dichloromethane (3 x 15 mL), the combined organic layers were dried over Na$_2$SO$_4$, and the solvent was removed in vacuo. The crude product was purified by column chromatography over silica (hexane, 45% ethyl acetate) to give 16 (0.33 g, 1.9 mmol, 76%). The NMR was consistent with the literature.$^{42}$ $^1$H-NMR (400 MHz, CDCl$_3$): 0.89 (t, 3H, $J = 5.9$ Hz), 1.22-1.30 (6H), 1.54-1.59 (2H), 4.35 (dt, 1H, $J = 5.1, 5.9$ Hz), 6.00 (d, 1H, $J = 15.6$ Hz), 6.39 (br, 1H), 7.05 (dd, 1H, $J = 4.6, 15.6$ Hz); $^{13}$C-NMR (CDCl$_3$): 13.9, 22.7, 24.8, 31.6, 36.3, 71.1, 119.4, 152.7, 171.7.
2.3.5 **SYNTHESIS OF 4-HYDROXYLONANAL**

![Chemical Reaction Diagram]

4-Hydroxynonanal (18) was synthesized via partial reduction of γ-nonalactone (17). Lactone 17 (2.0 g, 12.8 mmol) was dissolved in 60 mL of dichloromethane and 16 mL of DIBAL (1.2 M solution in toluene, 19.2 mmol) was added dropwise at -78 °C with continuous stirring. After 3 h the reaction mixture was allowed to warm to room temperature for 5 min and 1 M HCl was carefully added until the pH = 3. The aqueous phase was extracted with dichloromethane (3 × 30 mL), the organic layers were combined and dried over Na$_2$SO$_4$. The solvent was subsequently removed *in vacuo* and the crude product purified by column chromatography over silica (hexane, 25% ethyl acetate) to give 18 (1.64 g, 10.4 mmol, 81.3%). The NMR was consistent with the literature.$^{43}$

$^1$H-NMR (400 MHz, CDCl$_3$): 0.89 (t, 3H, $J = 6.7$ Hz), 1.30-2.21 (12H), 2.96 (d, 1H, $J = 2.7$ Hz), 3.06 (d, 1H, $J = 2.7$ Hz), 3.90-4.01 (1H), 4.22-4.29 (1H), 5.40-5.49 (1H), 5.52-5.63 (1H). The NMR data suggests that 4-hydroxynonanal 18 is in equilibrium with its cyclic hemiacetal in the solution.

2.3.6 **ANIMAL EXPERIMENTS: LIVER PERFUSIONS**

The lactones were hydrolyzed with 1.1 equivalent of aqueous NaOH solution at 60 °C for 1 h to generate the sodium salt of the 4-hydroxyacids. These 4-hydroxyacid salts were then used for the liver perfusions. Livers from male Sprague-Dawley rats were perfused$^{44}$ with bicarbonate buffer containing 4 mM glucose and either 4% dialyzed, fatty acid-free, bovine serum albumin (recirculating perfusions) or no albumin (non-
recirculating perfusions). After equilibration, 0 to 1 mM or 2 mM sodium 4-hydroxynonanoate, labeled or unlabeled, was added to the perfusate. Livers were quick-frozen at the end of the experiments (2 h for recirculating perfusion and 30 min for non-recirculating perfusion).

2.3.7 **Analytical Procedures**

The concentration of M1 formic acid was assayed by gas chromatography-mass spectrometry (An Agilent 6890 gas chromatograph linked to a 5973 MSD mass spectrometer) of 2,4-difluoroaniline derivatives, using sodium [2,2,2-\textsuperscript{2}H\textsubscript{3}, 2-\textsuperscript{13}C]acetate as internal standards.\textsuperscript{45}

For the concentration and labeling pattern of acyl-CoA esters, powdered frozen liver (≈ 200 mg) was extracted for 1 min with 4 mL of (methanol/water 1:1 containing 5% acetic acid) using a Polytron homogenizer. The supernatant was added to a 3 mL ion exchange cartridge packed with 300 mg of 2-2(pyridyl)ethyl silica gel (Sigma). The cartridge had been pre-activated with 3 mL methanol, then with 3 mL of extraction buffer. The acyl-CoAs that were trapped on the silica gel cartridge were released with (i) 3 mL of a 1:1 mixture of ammonium formate 50 mM pH 6.3, and methanol (to release the short- and medium-chain acyl-CoAs), then (ii) 3 mL of a 1:3 mixture of ammonium formate 50 mM pH 6.3, and (iii) 3 mL of methanol (to release the medium- and long-chain acyl-CoAs).\textsuperscript{46} The combined effluent was dried under a stream of nitrogen gas and stored at -80 °C until LC-MS analysis.

After dissolving the acyl-CoAs in 100 μl of buffer A (5% acetonitrile in ammonium formate 100 mM, pH 5.0), 40 μl were injected on a Thermo Electron Hypersil
GOLD column (150 × 2.1 mm) protected by a guard column (Hypersil Gold 5 μm, 10 × 2.1 mm), in an Agilent 1100 liquid chromatograph. The chromatogram was developed at 0.2 mL/min (i) for 3 min with 98% buffer A and 2% buffer B (95% acetonitrile in ammonium formate 5 mM, pH 6.3), (ii) from 3 to 25 min with a 2 to 60% gradient of buffer B in buffer A, (iii) from 26 to 31 min with 10% buffer A/90% buffer B, (iv) from 32 to 41 min with a 90% to 2% gradient buffer B in buffer A, and (v) 10 min stabilization with 98% buffer A before the next injection.

The liquid chromatograph was coupled to an 4000 QTrap mass spectrometer (Applied Biosystems, Foster City, CA) operated under positive ionization mode with the following source settings: turbo-ion-spray source at 600 °C under N₂ nebulization at 65 psi, N₂ heater gas at 55 psi, curtain gas at 30 psi, collision-activated dissociation gas pressure was held at high, turbo ion-spray voltage at 5,500V, declustering potential at 90V, entrance potential at 10V, collision energy at 50V, collision cell exit potential at 10V. The Analyst software (version 1.4.2; Applied Biology) was used for data collection and processing.

2.3.8 Acetyl-CoA Isotopomer Enrichment Calculation

The labeling of acetyl-CoA from the samples of M1 or M2 4-hydroxynonanoate is corrected from natural isotopomer distribution of acetyl-CoA by the matrix correction method. 4-Hydroxy-[3,4-13C₂]nonanoate and 4-hydroxy-[3-13C]nonanoate at 6 different concentrations from 0.1 to 1.0 mM were used to perfuse the isolated rat livers, with each point representing an individual perfusion. The enrichments of M1 and M2 acetyl-CoA were plotted versus the increasing concentration of both labeled 4-hydroxynonanoates.
For the statistical analysis of the results, the data were fit to a monoexponential curve ($R^2 > 0.97$). When the substrate concentration is 0.1 mM or higher, M1 and M2 acetyl-CoA from 4-hydroxy-[$3,4^{13}$C$_2$]nonanoate reach the plateau at 2.9 and 15.9% with standard error of 0.11 and 0.43, respectively. M1 acetyl-CoA from 4-hydroxy-[$3^{13}$C]nonanoate reaches a plateau at 13.6% with a standard error of 0.41.

2.4 CONCLUSIONS

The existence of parallel metabolic pathways is typically indicative of fundamental pathways that are critical for normal physiology. For example, conversion of glucose to pyruvate is omnipresent across biology, but the eventual catabolism of pyruvate can proceed through different parallel pathways dictated by physiologic conditions. In aerobic organisms under normal conditions, pyruvate enters the citric acid cycle to be eventually catabolized to carbon dioxide. However, if that organism is transferred to anaerobic conditions, a parallel metabolic step is utilized whereby pyruvate is converted to lactate.$^{47}$ This example is by no means unique, with other prominent examples including bile acid biosynthesis occurring via either neutral (classic) or acidic (alternative) pathways$^{48}$ and β-oxidation occurring in either the peroxisome or mitochondria. These examples further illustrate that this parallelism may occur in the same compartment, or be separately compartmentalized within the cell.$^{49}$ We have uncovered the existence of another previously unknown parallel metabolic pathway using the combination of metabolomics and mass isotopomer analysis. This novel pathway involves a new phosphoacyl-CoAs, which appear to be derived only from 4-hydroxyacids. These 4-phosphoacyl-CoAs are intermediates in the catabolism of 4-hydroxyacids with at least four carbons.
The predominant pathway (Fig 2.7, pathway A) involves the isomerization of 4-hydroxyacyl-CoAs to 3-hydroxyacyl-CoAs. We propose that this isomerization proceeds via the scheme shown in Fig 2.7. According to this scheme, the phosphorylation of 4-hydroxyacyl-CoA is followed by dehydrogenation and hydration, forming a 3-hydroxy-4-phosphoacyl-CoA. The latter is dephosphorylated to the enol form of 3-ketoacyl-CoA, which undergoes cycles of β-oxidation yielding short chain acyl-CoAs. Pathway A (isomerization followed by β-oxidation) is supported by the finding that in perfusions with 4-hydroxy-[3,4-\textsuperscript{13}C\textsubscript{2}]nonanoate (Table 2.3), the following intermediates are doubly labeled: 4-phospho-nonanoyl-CoA, 3-hydroxy-4-phosphononanoyl-CoA, heptanoyl-CoA, and acetyl-CoA. Pathway B (β-oxidation followed by α-oxidation and β-oxidation) is supported by the finding that, in perfusions with 4-hydroxy-[3,4-\textsuperscript{13}C\textsubscript{2}]nonanoate (Table 2.3), (i) the following metabolites are doubly labeled: 3,4-dihydroxynonanoyl-CoA, 2-hydroxyheptanoyl-CoA, and acetyl-CoA whereas (ii) the subsequent metabolites are singly labeled: hexanoyl-CoA, and acetyl-CoA. Through our careful review of the literature, this seems to be the only example of a sequence of β-oxidation, α-oxidation, and β-oxidation steps. This is further confirmed by the release of \textsuperscript{[13]C}formate in livers perfused with 4-hydroxy-[3-\textsuperscript{13}C]nonanoate, and 4-hydroxy-[3,4-\textsuperscript{13}C\textsubscript{2}]nonanoate (Fig 2.8).

4-HNE is one of the major lipid peroxidation products and undergoes different cellular metabolism to form 4-hydroxynonanoate and related compounds with intact carbon skeletons.\textsuperscript{21} Since its discovery in 1964,\textsuperscript{50} 4-HNE has been generally accepted as a modulator of numerous cellular systems and implicated in the pathogenesis of a number of degenerative diseases including Alzheimer’s disease, atherosclerosis, cataracts, and cancer. The generally accepted pathogenesis of 4-HNE is linked to the γ-hydroxy-α,β-
unsaturated aldehyde acting as a strong electrophile, which can form adducts with a variety of cellular nucleophiles via Michael addition or Schiff base formation. This interesting pathogenesis is linked to the general abundance of this molecule. Under physiological conditions typical concentrations of 4-HNE range from 0.1-1 µM in all tissues, but under severe oxidative stress this concentration can rise substantially in a localized manner to between 10 µM and 5 mM. Our data demonstrate the complete oxidation of 4-HNE by following the label from \( d_{11}-4 \)-HNE down to propionyl-CoA (Table 2.2). Also, our experiments with 4-hydroxy-[3,4-\( ^{13} \)C\(_2\)]nonanoate (Fig 2.8) demonstrate the complete oxidation of the carbon skeleton of 4-HNE to acetyl-CoA, propionyl-CoA, and formate. This represented the first report on the catabolism of this ubiquitous class of biological molecules.

This catabolic pathway is impressively robust. In our experiments we observed that under high concentrations of 4-hydroxyacids the liver can utilize the liberated acetyl-CoA, propionyl-CoA, and formate as its primary carbon source. Given the robust nature and the parallelism, we concluded that this pathway represents a fundamental process in normal physiology. The implication of these observations and the role that this pathway may play in either normal physiology or disease pathogenesis is not yet known. However, the disease relevance of 4-HNE (and consequently 4-hydroxyacids) has been clearly delineated in the literature. For example, there is increasing evidence that 4-HNE is partially responsible for the development of alcoholic liver disease. A highly plausible hypothesis is that loss of the ability to catabolically process lipid peroxidation products precedes their accumulation and their contribution towards disease progression. It is possible that under different physiologic stresses, the predominant pathway may
switch from Pathway A to Pathway B. Alternatively, different pathways may predominate in different tissues.

Trapping of CoA esters by the metabolism of some drugs has been implicated in the deleterious effects of the drugs.\textsuperscript{59} Also, in some inborn errors of metabolism, the accumulation of CoA esters has been implicated in the physiopathology of the diseases.\textsuperscript{60} We have observed similar effects in the case degradation of 4-hydroxyacids where the trapping of CoA results in the decrease in the liver concentration of free CoA, acetyl-CoA, and malonyl-CoA.\textsuperscript{1} However, in the case of 4-hydroxynonanoate, this trapping of CoA does not prevent the substrate from (i) being taken up at a rate similar to that of a long-chain fatty acid like palmitate and (ii) contributing 55-60% of acetyl-CoA produced by the liver. However, in some situations, the trapping of CoA by the metabolism of 4-hydroxyacids could result in metabolic perturbations.

The present work illustrates the potential of the combination of metabolomics and mass isotopomer analysis for pathway discovery. This also indicates the need for studies that will identify and characterize the enzymes that catalyze the reactions involved in the isomerization of 4-hydroxyacyl-CoA to 3-hydroxyacyl-CoAs (Fig 2.8, pathway A). The physiological implication of the new CoA esters i.e. 4-phosphoacyl-CoA is also worthy of further investigation. It should be noted that unlike 4-hydroxyacids with five or more carbons, 4-hydroxybutyrate (GHB, a drug of abuse) is not degraded to acetyl-CoA, as confirmed by the absence of M2 acetyl-CoA and with 4-hydroxy-[\textsuperscript{13}C\textsubscript{4}]butyrate. The detailed catabolism of GHB will be discussed in Chapter 3. Stable-isotope labeling technology provides a powerful and sometimes unique tool for the metabolomics study at the whole-body level. With continuing advances in availability, instrumentation and
amount of research, it is very likely that the stable-isotopic tool will become increasingly important in the near future for diagnosing the disease states.

2.5 ACKNOWLEDGEMENT

This work would have not been possible without the support from Prof. Henri Brunengraber, Prof. Guo-fang Zhang, and John Koshi. All the animal experiments were performed in Prof. Brunengraber’s lab (Department of Nutrition, Case Western Reserve University). Some of the results presented in this chapter (identification of 4-phosphoacyl-CoA) were acquired by Prof. Guo-fang Zhang and are presented here with his prior permission. Dr. Yong Han (Department of Chemistry, Case Western Reserve University) synthesized the 4-hydroxy-[3-13C]nonanoic acid lactone.
2.6 REFERENCES


CHAPTER 3

Isotopomer Synthesis of γ-Hydroxybutyrate (GHB)

Reveals Catabolism via α-Oxidation
3.1 introduction

Gamma-Hydroxybutyric acid (GHB), the smallest saturated 4-hydroxyacid, primarily derived in vivo from gamma-aminobutyric acid (GABA) in brain, possesses a number of physiological and pharmacological properties.\(^1,2\) GHB has been clinically used as an anesthetic drug as well as for the treatment of alcohol and opiate dependence and narcolepsy-associated cataplexy.\(^3,4\) On the other hand, due to its ability to induce short-term amnesia, GHB (date rape drug) is being used illicitly as a drug to facilitate acquaintance sexual assault.\(^5\) Its known metabolism proceeds via (i) conversion to succinic semialdehyde (SSA) by a cytosolic NAD\(^+\) dehydrogenase\(^6\) and by a mitochondrial pyridine nucleotide-independent enzyme system,\(^7\) and (ii) the oxidation of SSA to succinate, an intermediate of the Krebs cycle. GHB accumulates to supraphysiological concentrations in succinic semialdehyde dehydrogenase (SSADH) deficiency, which is a rare inborn error of GABA metabolism caused by the deficit of the GABA degradative enzyme SSADH.\(^8\) Despite being such an important small fatty acid and expanding clinical and illicit consumption, the catabolism of GHB remains elusive. The SSADH deficiency is a rare but medically relevant disease and a better understanding of the fates of GABA and/or GHB can provide important insights for its therapy. Thus, we became interested in studying the complete carbon skeletal degradation of GHB using a combination of metabolomics and mass isotopomer analysis. We synthesized a number of stable isotopically (\(^{13}\text{C}\)) labeled GHB and perfused them into the isolated rat liver. Mass isotopomer analysis disclosed GHB catabolism via the Krebs cycle and other processes and revealed some key features including direct evidence for the existence of \(\alpha\)-oxidation and \(\beta\)-oxidation in the course of its catabolism.
**Figure 3.1** Origin and fates of GHB in the physiology. 1,4 Butanediol, γ-butyraldehyde and γ-hydroxybutyraldehyde can act as pro-drugs for GHB.
In the previous chapter, we reported a study of the catabolism of 4-hydroxyacids in perfused rat liver that used a combination of metabolomics and mass isotopomer analysis. A key finding of this work was that 4-hydroxyacid catabolism can proceed via two parallel pathways that involve either a phosphorylation and isomerization of the C-4 hydroxyl or a β-oxidation/α-oxidation sequence. We identified 4-phosphoacyl-CoAs, a new class of acyl-CoA ester generated from 4-hydroxyacids with more than four carbons. During that study, we found that GHB produces a very small (and in fact the least) amount of 4-phosphoacyl-CoA for the series of 4-hydroxyacids (C₅ to C₁₁). Pertinent to that, we also found the concentration of 4-phosphobutyryl-CoA to be 40 times greater in the brain and liver in SSADH deficient mice than their wild types. Those led us to further investigate the catabolism of GHB to know its final fate as well as the physiological implications of 4-phosphobutyryl-CoA. In the study of GHB metabolism, we found α-oxidation playing a key role and thus an efficient analytical technique was needed to study α-oxidation. The transient stability of formyl-CoA, the product of α-oxidation makes its detection difficult. Hence, we decided to incorporate heavy stable isotopes (¹³C) at specific positions of GHB to track isotopically labeled formate (¹³C-formate or M1 formate). But, the observation of M1 formate is not sufficient to conclude that the putative α-oxidation occurs because M1 formate may arise from oxidation of glycine, serine, glycolate and 3-phosphoglycerate, if these compounds become ¹³C-labeled from any labeled compounds. Thus, production of M1 formate is not a specific indication of α-oxidation. Therefore, a new index of α-oxidation is needed.

Despite being a potential drug of abuse, the catabolism of GHB has not been thoroughly explored. GHB is primarily eliminated by the liver, and less than 2-5% of it is
excreted unchanged in the urine for humans\textsuperscript{3,12}, suggesting a rapid metabolic process. Although the existence of β-oxidation in the metabolism of GHB was proposed, no direct evidence was observed. In this chapter we describe a metabolomic study on GHB using the combination of isotopic tools and mass isotopomer analysis.

3.2 RESULTS AND DISCUSSION

3.2.1 NON-TARGETED METABOLICOMICS OF GHB IN RAT LIVER

We conducted a non-targeted GC-MS analysis of the final perfusates from livers perfused with buffer containing 0 or 2 mM unlabeled GHB. The aim of this study was to obtain the mirrored GC-MS spectra of the metabolites from the perfused rat liver with and without GHB. The NIST database was then used to identify the possible chemical structures corresponding to the peaks that appeared or were enhanced in the GHB perfused samples. Fig 3.2 shows the analytical steps leading to the identification of GHB metabolites in the final perfusate of a liver that had been perfused for 120 min with recirculating buffer containing 2 mM GHB. A liver perfused without GHB served as control. The GC-MS data of trimethylsilyl derivatives is shown in Fig 3.2A for compounds released by a control liver and those (displayed as a mirror image) released by a liver perfused with GHB. High resolution plotting of the data in areas of the scan where the signal was very low identified peaks in the GHB perfusion that appeared absent or present in very low amounts in the control perfusion as shown in Fig 3.2B. Processing of the data using the NIST software identified 3-hydroxypropionic acid, 2,4-dihydroxybutyric acid, and 3,4-dihydroxybutyric acid as possible chemical structures corresponding to peaks 1, 2 and 3 respectively (Fig 3.2C).
To confirm our findings, livers were perfused with $^{13}$C$_4$GHB and similar analysis led to the identification of labeled 3-hydroxypropionic acid (M3), 2,4-dihydroxybutyric acid (M4), 3,4-dihydroxybutyric acid (M4 as expected, but also M2), 4-hydroxycrotonic acid (M4), oxalic acid (M2) and glycolic acid (M2). A very small amount of unlabeled 3,4-dihydroxybutyric acid, 3-hydroxypropionic acid, glycolic acid and oxalic acid were found in control experiments without GHB. The identities of the metabolites were further confirmed by perfusing the liver with various differently $^{13}$C labeled GHB (singly, doubly and triply labeled GHBs). Additional analyses demonstrated the formation of low concentrations of $^{13}$C]formate and $^{13}$C]acetate. These were assayed as pentafluorobenzyl derivatives under negative chemical ionization to minimize natural enrichment of the analytes. The very low labeling of free acetate (< 0.6%) reflects a very low labeling of acetyl-CoA which could not be detected in the LC-MS/MS assay of acetyl-CoA because of the high M1 and M2 natural abundance enrichments of acetyl-CoA (30% and 12%, respectively). Identification of glycolate is compatible with β-oxidation of GHB as hypothesized by Vamecq et al.$^{13}$ Formation of $^{13}$C]formate may be an indication of α-oxidation. But as discussed before, it might come from other physiological sources or anaplerotic pathways. Thus, additional experiments were needed to confirm the occurrence of both α- and β-oxidation.
**FIGURE 3.2** Identification of metabolites of GHB. (A) Mirror image depiction of total ion current chromatograms of trimethylsilyl derivatives of compounds released by a control liver and by a liver perfused with 2 mM unlabeled GHB. (B) Amplification of selected areas from the top chromatograms. (C) Electron ionization spectra of peaks (1, 2 and 3) selected from the middle panels and their possible structures based on the NIST data base.
3.2.2 Identification of α-Oxidation by Isotopic Analysis

To gain insight into the source of M1 formate (\(^{13}\text{C}\) labeled formate) and define α-oxidation, we performed liver perfusion experiments with various \(^{13}\text{C}\) labeled GHB isotopomers. We synthesized singly labeled GHBs ([1-\(^{13}\text{C}\)-, [2-\(^{13}\text{C}\)-, [3-\(^{13}\text{C}\)-, [4-\(^{13}\text{C}\)]-GHB), doubly labeled GHBs ([1,2-\(^{13}\text{C}\)_2]-, [3,4-\(^{13}\text{C}\)_2]-GHB), and triply labeled GHBs ([1,2,3-\(^{13}\text{C}\)_3]-, [2,3,4-\(^{13}\text{C}\)_3]-GHB) to identify the labeling pattern in the metabolites (particularly, formate and 3-hydroxypropionate). As shown in Fig 3.3A, M1 formate released by the liver perfused with [3,4-\(^{13}\text{C}\)_2]-GHB is significantly higher than [1,2-\(^{13}\text{C}\)_2]-GHB perfused liver. The perfusion experiments with doubly labeled GHB also confirm that glycolate comes only from C-3,4 of GHB as no labeled glycolate (Fig 3.3B) was detected from [1,2-\(^{13}\text{C}\)_2]-GHB perfused liver. To further explore the root of the difference in M1 formate production evident in Fig 3.3A, we perfused singly labeled GHB and measured the M1 formate production from each singly labeled GHB perfused liver. Fig 3.3C shows that the M1 formate mainly comes from C-1, C-3 and C-4 of GHB. That justifies the higher amount of M1 formate production from [3,4-\(^{13}\text{C}\)_2]-GHB perfused liver as both the labeled carbons can transform into formate. Further, glycolate or glycolyl-CoA can hydrolyze into formate and \(\text{CO}_2\) and the formate comes from the α-carbon.\(^{14}\) Consequently, formate from glycolate or glycolyl-CoA (labeled formate in case of [3,4-\(^{13}\text{C}\)_2]-GHB) also contributes for the higher M1 formate production from [3,4-\(^{13}\text{C}\)_2]-GHB perfused liver. The production of labeled formate from [3-\(^{13}\text{C}\)]-GHB results from the cleavage of the β-carbon of 3-hydroxypropionate and it may involve a retro-aldol type of reaction as shown in Scheme 3.1. Further experiments are needed to fully elucidate the source of formate from the β-carbon of GHB.
**SCHEME 3.1** Proposed mechanism for the cleavage of the β-carbon of 3-hydroxypropionate to form formic acid.
Fatty acid α-oxidation reactions are generally detected by the formation of labeled formate from a fatty acid labeled on C-1. However, labeled formate can also form from processes completely unrelated to α-oxidation. For example, part of the \(^{13}C\) formate from \([3,4-^{13}C_2]GHB\) (Fig 3.3A) results from the catabolism of glycolyl-CoA (Fig 3.3B, discussed in the following sections). Also, we previously showed that part of the label from anaplerotic compounds (such as \(^{13}C_3\)propionyl-CoA derived from \(^{13}C_5\)levulinate\(^{15}\)) is found in glycine and formate. Thus, the detection of M1 formate alone is not conclusive of α-oxidation in the case of GHB catabolism. However, we can conclude that α-oxidation processes are operative from specific precursor-to-product relationships between the mass isotopomer distributions of pairs of metabolites, besides formate. We employed the isotopic strategies to demonstrate the precursor-to-product relationships and hence α-oxidation. In the \([1,2,3-^{13}C_3]\)GHB perfused liver, we identified M3 GHB-CoA, M3 2,4-dihydroxybutyryl-CoA, M2 3-hydroxypropionyl-CoA, and M1 formate which are in clear precursor-to-product relationship (Fig 3.4A). In perfusions with \([1,2-^{13}C_2]\)GHB, M2 GHB-CoA, M2 2,4-dihydroxybutyryl-CoA, M1 3-hydroxypropionyl-CoA, and M1 formate also exhibit a precursor-to-product relationship (Fig 3.4B). The first step of the α-oxidation process involves the addition of a hydroxyl group to the α-carbon of GHB-CoA (GHB-CoA to 2,4-dihydroxybutyryl-CoA) followed by the cleavage of C-1 as formyl-CoA (or formate after hydrolysis). Thus the removal of C-1 of \([1,2,3-^{13}C_3]\)GHB or \([1,2-^{13}C_2]\)GHB as M1 formate in the sequence of compounds shown in the Fig 3.4A and B is clearly an α-oxidation process.

The above discussion demonstrates the final fate of C-1 of GHB which undergoes α-oxidation yielding formate. We also found M1 formate from \([4-^{13}C]\)GHB which
implies that C-4 (Fig 3.3C) might also undergo some sort of hydrolysis reaction. In perfusions with [2,3,4-\textsuperscript{13}C\textsubscript{3}]GHB, we identified M3 GHB-CoA, M3 3,4-dihydroxybutyryl-CoA, M2 3-hydroxypropionyl-CoA and M1 formate which are in clear precursor-to-product relationship (Fig 3.4C). But as it involves an intermediate of β-oxidation (3,4-dihydroxybutyryl-CoA), we are hesitant to designate it as α-oxidation. There is no literature precedence for a similar kind of reaction in physiology. The removal of C-4 probably involves the conversion of the primary alcohol function to a carboxyl or a carboxyl-CoA, followed by the removal of C-4 as formate or CO\textsubscript{2}. In the latter case, the product will be \textsuperscript{13}C labeled CO\textsubscript{2} but as we use a large amount of unlabeled CO\textsubscript{2} in our perfusion experiments,\textsuperscript{16} it is practically impossible to account for the extent of labeling in the released labeled CO\textsubscript{2}. Again, [2,3,4-\textsuperscript{13}C\textsubscript{3}]GHB can also form M1 formate via the hydrolysis of M2 glycolyl-CoA as discussed above. Hence, C-4 of GHB either cleaves as formate via α-oxidation or may undergo a decarboxylation reaction to produce CO\textsubscript{2}. 
**Figure 3.3** Release of (A) M1 formate and (B) M2 glycolate by livers perfused with 2 mM $[1,2^{-13}C_2]GHB$ (●) or $[3,4^{-13}C_2]GHB$ (□). (C) M1 formate production in livers perfused with singly labeled GHB.
**Figure 3.4** The precursor-to-product relationship from (A) [1,2,3-$^{13}$C$_3$]GHB, (B) [1,2-$^{13}$C$_2$]GHB and (C) [2,3,4-$^{13}$C$_3$]GHB perfused liver
3.2.3 Fate of 4-Phosphobutyryl-CoA

In the previous chapter, we showed that 4-phosphoacyl-CoAs are intermediates of the pathway A in the catabolism of 4-hydroxyacids with more than 4 carbons (Fig 2.8, Chapter 2). We also showed that the production of 4-phosphoacyl-CoA varies considerably for different 4-hydroxyacids, and GHB (C₄) produces the least amount among the series of 4-hydroxyacids (C₄-C₁₁) that were studied (Fig 2.3, Chapter 2). The 4-phosphoacyl-CoAs are involved in the isomerization of 4-hydroxyacyl-CoAs to 3-hydroxyacyl-CoAs or 3-ketoacyl-CoAs which are well-known substrates for further fatty acid β-oxidation. The liver perfusion of [¹³C₄]GHB yielded 4-phospho-[¹³C₄]butyryl-CoA. In the case of 4-phospho-[¹³C₄]butyryl-CoA, such isomerization would generate [¹³C]acetoacetyl-CoA which would (i) equilibrate with [¹³C₂]acetyl-CoA via thiolase, and (ii) label ketone bodies, i.e., acetoacetate and β-hydroxybutyrate via the 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) cycle. We could only detect traces of ¹³C in ketone bodies. These could also be explained by incorporation of label from acetyl-CoA derived from the β-oxidation of GHB to glycolyl-CoA and acetyl-CoA (via the reversal of thiolase and the HMG-CoA cycle). Thus, the experiments with [¹³C₄]GHB do not provide unequivocal information on the fate of 4-phosphobutyryl-CoA.

However, in perfusions with [²H₆]GHB, the M6 enrichments of GHB-CoA and 4-phosphobutyryl-CoA were 86.9 ± 0.7% and 79.0 ± 1.7%, respectively (p< 0.01), while the M5 enrichments of GHB-CoA and 4-phosphobutyryl-CoA were 13.1 ± 0.7% and 21.0 ± 1.7%, respectively (p< 0.01). The lower M6 and higher M5 enrichments of 4-phosphobutyryl-CoA compared to GHB-CoA reflect a partial loss of ²H in reversible reactions downstream from 4-phosphobutyryl-CoA. At this point, the observed
phenomenon resembles our previous finding that in perfusions with 4-hydroxy-[3,4-
$^{13}$C$_2$]nonanoate or $[^{13}$C$_5$]levulinate (reduced in the liver to 4-hydroxy-$[^{13}$C$_5$]pentanoate),
the corresponding 4-phosphoacyl-CoAs are degraded via $\beta$-oxidation after conversion to
3-ketoacyl-CoAs. Thus, it is likely that GHB is also $\beta$-oxidized to acetyl-CoA via 4-
phosphobutyryl-CoA.

3.2.4 CONSTRUCTION OF A PATHWAY FOR GHB CATABOLISM

In summary, we have identified a number of intermediates having different
carbon skeletons (e.g. 2,4-and 3,4-dihydroxybutyrate bearing all the four carbons of
GHB; 3-hydroxypropionate having three carbons form GHB; glycolate and formate
carrying two and one carbon respectively) in the catabolism of GHB. The present study
identified a number of processes through which GHB is catabolized in liver, in addition
to its known metabolism through the Krebs cycle via succinate. The identification of
processes was based on the combination of metabolomics and mass isotopomer analysis
of metabolites from isotopically labeled substrates.

From all the labeling data, especially those obtained with singly labeled GHB,
$[1,2,3-^{13}$C$_3]$GHB and $[2,3,4-^{13}$C$_3]$GHB, we can construct a scheme for GHB catabolism
via $\beta$-oxidation and two additional processes that remove C-1 or C-4 of GHB. This
scheme is presented in Fig 3.5 with $[1,2,3-^{13}$C$_3]$GHB as the substrate and the isotopically
labeled carbons are highlighted in grey. The $\alpha$-oxidation process as shown in Fig 3.5 goes
from GHB-CoA to 2,4-dihydroxybutyryl-CoA to 3-hydroxypropionyl-CoA plus formate.
Through this sequence, M3 $[1,2,3-^{13}$C$_3]$GHB forms M2 3-hydroxypropionyl-CoA plus
M1 formate, while [2,3,4-\textsuperscript{13}C\textsubscript{2}]GHB forms M3 3-hydroxypropionyl-CoA and unlabeled formate (not shown in Fig 3.5).

The β-oxidation process (Fig 3.5) goes from GHB-CoA to 3,4-dihydroxybutyryl-CoA to 4-hydroxy-3-ketobutyryl-CoA (putative) to glycolyl-CoA and acetyl-CoA. Through this sequence, M3 [1,2,3-\textsuperscript{13}C\textsubscript{3}]GHB forms M3 3,4-dihydroxybutyryl-CoA, and M2 acetyl-CoA plus M1 glycolyl-CoA. Through the same sequence, M3 [2,3,4-\textsuperscript{13}C\textsubscript{3}]GHB forms M3 3,4-dihydroxybutyryl-CoA, and M1 acetyl-CoA plus M2 glycolyl-CoA (not shown in Fig 3.5). M3 [2,3,4-\textsuperscript{13}C\textsubscript{3}]GHB also forms M1 formate from C-4, presumably via the catabolism of M2 glycolyl-CoA. Lastly, a process that removes C-4 of GHB branches off from the β-oxidation pathway: 3,4-dihydroxybutyryl-CoA to 3-hydroxypropionyl-CoA (Fig 3.5). Through this sequence, M3 [1,2,3-\textsuperscript{13}C\textsubscript{3}]GHB forms M3 3-hydroxypropionyl-CoA. Through the same sequence, M3 [2,3,4-\textsuperscript{13}C\textsubscript{3}]GHB forms M2 3-hydroxypropionyl-CoA.

3.2.5 Quantitation of the Rate of the β-Oxidation and α-Oxidation

In the previous chapter, we measured the flux of two parallel pathways in the metabolism of 4-HNE (via 4-hydroxynonanoic acid) by the labeling ratio (M2 acetyl-CoA)/(M1 acetyl-CoA) measured in the presence of 4-hydroxy-[3,4-\textsuperscript{13}C\textsubscript{2}]nonanoic acid. This type of calculation is not possible for GHB catabolism because it is catabolized by five pathways, none of which yields a common end-product that accumulates. But, we can quantitate the rate of β-oxidation and α-oxidation by measuring the steady state concentration of 3,4- and 2,4-dihydroxybutyryl-CoA. We measured the concentrations of 3,4- and 2,4-dihydroxybutyryl-CoA from the livers perfused with [3-\textsuperscript{13}C\textsubscript{1}]GHB and found
3.6-fold higher concentration of M1 3,4-dihydroxybutyryl-CoA than the corresponding 2,4-dihydroxybutyryl-CoA (Fig 3.6) which suggests that the β-oxidation of GHB (Fig 3.5) is its predominant catabolic pathway. This interpretation is further supported by the higher M1 formate production from [3,4-\textsuperscript{13}C\textsubscript{2}]GHB than [1,2-\textsuperscript{13}C\textsubscript{2}]GHB (Fig 3.3).
**Figure 3.5** Pathway for metabolism of GHB illustrated with \([1,2,3-^{13}C_3]\)GHB where \(^{13}\)C labeled atoms are highlighted in grey.
Figure 3.6 Quantitation of the production of M1 3,4- and 2,4-dihydroxybutyryl-CoA from livers perfused with [3-$^{13}$C]GHB. The ratio of the two is shown on the inset.
3.3 EXPERIMENTAL SECTIONS

3.3.1 MATERIALS AND METHODS

General chemicals, ethyl bromoacetate-1-$^{13}$C (99 atom % $^{13}$C), ethyl bromoacetate-2-$^{13}$C (99 atom % $^{13}$C), ethyl bromoacetate-$^{13}$C$_2$ (99 atom % $^{13}$C), [$^{13}$C$_4$]GHB lactone, and [$^2$H$_6$]GHB lactone were from Sigma-Aldrich-Isotec. Triphenylphosphine (99%) was purchased from Acros Organics. Glycolaldehyde dimer was purchased from Sigma-Aldrich. Flash chromatography was performed on Silicycle silica gel (230-400 mesh) at medium pressure (200 mbar). TLC was done on Macherey-Nagel pre-coated (0.25 mm) TLC-plates SIL G-25 UV$_{254}$ with a fluorescent indicator and visualized by UV light (254 nm). Solvents and reagents were of commercially available analytical grade quality and used as received without any further purification. $^1$H and $^{13}$C-NMR spectra were recorded on a Varian Inova spectrometer (at the Department of Chemistry, Case Western Reserve University) operating at 400 MHz and 100 MHz for the $^1$H and $^{13}$C-NMR spectra, respectively. The internal references were TMS (δ 0.00) and CDCl$_3$ (δ 77.2) for $^1$H and $^{13}$C spectra, respectively. NMR data are presented in the following order: chemical shift, peak multiplicity (b = broad, s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, dd = doublet of doublet, dm = doublet of multiplet), coupling constant, proton number. Mass spectra were obtained on a Kratos MS 25 Mass Spectrometer (at the Department of Chemistry, Case Western Reserve University) using FAB ionization method (positive mode) in $m$-nitrobenzyl alcohol or glycerol matrices.
3.3.2 SYNTHESIS OF ISOTOPICALLY LABELED GHB LACTONES

The synthesis routes for GHB lactones with $^{13}$C label at different positions has been presented in Scheme 3.2.

**Scheme 3.2** Synthesis route for the labeled GHB lactones
2-(Ethoxycarbonylmethyl)triphenylphosphonium bromide (2)

Ethyl bromoacetate (1) (2.54 g, 15.21 mmol) in ethyl acetate (10 mL) was slowly added to a solution of triphenylphosphine (4.06 g, 15.47 mmol) in ethyl acetate (10 mL). The reaction mixture was then stirred at room temperature for 12 h and the white precipitate filtered off, washed with ether (3 × 10 mL), and dried under vacuum for 5 h to give 2 (6.06 g, 14.12 mmol, 93% yield), mp 158-160 °C (lit. 159-161 °C): 1H NMR (400 MHz, CDCl3) δ 1.07 (t, 3H, J = 7.2 Hz), 4.03 (q, 2H, J = 7.2 Hz), 5.62 (d, JHP = 13.8 Hz), 7.67−7.94 (15H; 13C NMR (100 MHz, CDCl3) δ 13.6, 33.3 (d, JC-P = 55.7 Hz), 62.8, 117.8 (d, JC-P = 88.9 Hz), 130.2 (d, 3JC-P = 13.8 Hz), 133.8 (d, 2JC-P = 10.7 Hz), 135.1 (d, 4JC-P = 3.8 Hz), 164.7 (d, 2JC-P = 3 Hz).

2-[2-13C]-(Ethoxycarbonylmethyl)triphenylphosphonium bromide (2a)

The same procedure as for the synthesis of 2 was used starting from ethyl-[1-13C]-2-bromoacetate (1a) (2.54 g, 15.12 mmol) to give the phosphonium 2a (6.10 g, 14.18 mmol, 93.8% yield): 1H NMR (400 MHz, CDCl3) δ 1.06 (t, J = 7.1 Hz, 3H), 4.05 (dq, 3JHC = 3.2 Hz, J = 7.1 Hz, 2H), 5.59 (dd, JHP = 13.8 Hz, JHC = 7.3 Hz, 2H), 7.58−7.90 (15H). 13C NMR (100 MHz, CDCl3): δ 13.7, 33.3 (dd, JC-P = 55.7 Hz, JC-C = 58.7 Hz), 62.7, 117.8 (d, JC-P = 88.7 Hz), 130.3 (d, JC-P = 13.6 Hz), 134.2 (d, JC-P = 10.5 Hz), 135.1 (d, JC-P = 3.7 Hz), 164.8 (d, JC-P = 3.1 Hz).
2-[1-\textsuperscript{13}C]-(Ethoxycarbonylmethyl)triphenylphosphonium bromide (2b)

The same procedure as for the synthesis of 2 was used starting from ethyl-[2-\textsuperscript{13}C]-2-bromoacetate (1b) (2.54 g, 15.12 mmol) to give the phosphonium 2b (5.99 g, 13.92 mmol, 92.1% yield): \textsuperscript{1}H NMR (400 MHz, CDCl\textsubscript{3}) \(\delta\) 1.06 (t, \(J = 7.1\) Hz, 3H), 4.04 (q, \(J = 7.1\) Hz, 2H), 5.62 (dd, \(J_{H-C} = 134.6\) Hz, \(J_{H-P} = 13.6\) Hz, 2H), 7.66–7.93 (15H). \textsuperscript{13}C NMR (100 MHz, CDCl\textsubscript{3}) \(\delta\) 13.7, 33.2 (d, \(J_{C-P} = 55.7\) Hz), 62.7, 117.8 (d, \(J_{C-P} = 88.5\) Hz), 130.2 (d, \(J_{C-P} = 13.1\) Hz), 133.9 (d, \(J_{C-P} = 9.8\) Hz), 135.1 (d, \(J_{C-P} = 3.3\) Hz), 164.3 (dd, \(J_{C-P} = 4.9\) Hz, \(J_{CC} = 59.0\) Hz).

2-[1,2-\textsuperscript{13}C\textsubscript{2}](Ethoxycarbonylmethyl)triphenylphosphonium bromide (2c)

The same procedure as for the synthesis of 2 was used starting from ethyl-[1,2-\textsuperscript{13}C\textsubscript{2}]-2-bromoacetate (1c) (2.54 g, 15.03 mmol) to give the phosphonium 2c (6.06 g, 14.05 mmol, 93.5% yield). \textsuperscript{1}H NMR (400 MHz, CDCl\textsubscript{3}): \(\delta\) 1.06 (t, 3H, \(J = 7.2\) Hz), 4.03 (dq, 2H, \(J = 7.2\) Hz, \(J = 3.2\) Hz), 5.59 (ddd, 2H, \(J_{H-C} = 134.8\) Hz, \(J_{H-P} = 14\) Hz, \(J_{H-C} = 7.6\) Hz), 7.66–7.93 (15H); \textsuperscript{13}C NMR (100 MHz, CDCl\textsubscript{3}): \(\delta\) 13.6, 33.4 (dd, \(J_{C-P} = 55.7\) Hz, \(J_{C-C} = 58.7\) Hz), 62.8, 117.8 (d, \(J_{C-P} = 88.9\) Hz), 130.2 (d, \(J_{C-P} = 13.8\) Hz), 133.8 (d, \(J_{C-P} = 10.7\) Hz), 135.1 (d, \(J_{C-P} = 3.8\) Hz), 164.7 (dd, \(J_{C-C} = 59.1\) Hz, \(J_{C-P} = 3\) Hz).

2-(Ethoxycarbonylmethylene)triphenylphosphorane (3)

![Chemical structure of 2-(Ethoxycarbonylmethylene)triphenylphosphorane (3)]

A solution of sodium hydroxide (1.0 M, 50 mL) was added to a solution of 2-(ethoxycarbonylmethyl)triphenylphosphonium bromide (2) (6 g, 13.98 mmol) in
dichloromethane (50 mL) and the reaction mixture stirred vigorously for 15 min. The organic layer was removed and the aqueous layer extracted with dichloromethane (3 × 15 mL). The combined organic layers were dried over Na₂SO₄ and concentrated under vacuum to give phosphorane 3 (4.78 g, 13.72 mmol, 98.1% yield), mp 123-125 °C (lit. 120–122 °C): ¹H NMR (400 MHz, CDCl₃): δ 1.06 (br, 3H), 2.79 (br, 1H), 3.96 (br, 2H), 7.43–7.68 (15H); ¹³C NMR (100 MHz, CDCl₃): δ 15.0, 58.1, 128.0 (d, ¹J_C-P = 90.6 Hz), 128.9 (d, ³J_C-P = 11.4 Hz), 132.1, 133.2 (d, ²J_C-P = 9.9 Hz), 171.5 (d, ²J_C-P = 11 Hz).

2-[2-¹³C]-(Ethoxycarbonylmethylene)triphenylphosphorane (3a)

The same procedure as for the synthesis of 3 was used starting from 2a (6 g, 13.94 mmol) to give 3a (4.75 g, 13.72 mmol, 97.5% yield): ¹³C NMR (100 MHz, CDCl₃): δ 15.0, 58.0, 128.1 (d, ¹J_C-P = 90.6 Hz), 128.9 (d, ³J_C-P = 11.4 Hz), 132.1, 133.2 (d, ²J_C-P = 9.9 Hz), 171.5 (d, ²J_C-P = 11 Hz).

2-[1-¹³C]-(Ethoxycarbonylmethylene)triphenylphosphorane (3b)

The same procedure as for the synthesis of 3 was used starting from 2b (6 g, 13.94 mmol) to give 3b (4.80 g, 13.74 mmol, 98.6% yield): ¹³C NMR (100 MHz, CDCl₃): δ 15.0, 57.9, 128.1 (d, ¹J_C-P = 90.6 Hz), 128.9 (d, ³J_C-P = 11.4 Hz), 132.1, 133.1 (d, ²J_C-P = 9.9 Hz), 171.7 (dd, ²J_C-P = 11 Hz, ¹J_C-C = 87.3 Hz).

2-[1,2-¹³C₂]-(Ethoxycarbonylmethylene)triphenylphosphorane (3c)

The same procedure as for the synthesis of 3 was used starting from 2c (6.0 g, 13.91 mmol) to give 3c (4.78 g, 13.64 mmol, 98.3% yield): ¹³C NMR (100 MHz, CDCl₃): δ
15.0, 30.1 (dd, $J_{C-P} = 124.7$ Hz, $J_{C-C} = 87.3$ Hz), 58.1, 128.0 (d, $^1J_{C-P} = 90.6$ Hz), 128.9 (d, $^3J_{C-P} = 11.4$ Hz), 132.1, 133.2 (d, $^2J_{C-P} = 9.9$ Hz), 171.5 (dd, $J_{C-C} = 87.8$ Hz, $^2J_{C-P} = 11$ Hz).

**Ethyl (E)-4-hydroxy-but-2-enoate (5)**

To a solution of glycolaldehyde dimer (4) (0.66 g, 5.5 mmol) in absolute ethanol (10 mL) was added 2-(ethoxycarbonylmethylene)triphenylphosphorane (3) (3.38 g, 9.7 mmol) in absolute ethanol (15 mL) in presence of molecular sieves and the reaction mixture stirred for 3 h at RT. After removing the molecular sieves by filtration, the solvent was evaporated under reduced pressure and the crude product was purified by column chromatography over silica (hexane, 10% ethyl acetate) to give of 5 (1.08 g, 8.30 mmol, 85.6% yield): $^1$H NMR (400 MHz, CDCl$_3$) δ 1.30 (t, 3H, $J = 7.2$ Hz), 4.21 (q, 2H, $J = 7.2$ Hz), 4.36 (br, 2H), 6.11 (dt, 1H, $J = 15.6$ Hz, $J = 2$ Hz), 7.04 (dt, 1H, $J = 15.6$ Hz, $J = 4$ Hz); $^{13}$C NMR (100 MHz, CDCl$_3$) δ 14.4, 60.7, 62.1, 120.4, 147, 166.7.

**Ethyl (E)-[1-$^{13}$C]-4-hydroxy-but-2-enoate (5a)**

The same procedure as for the synthesis of 5 was used starting from 3a (3.35 g, 9.59 mmol) to give 5a (1.08 g, 8.24 mmol, 85.9% yield): $^1$H NMR (400 MHz, CDCl$_3$) δ 1.30 (t, 3H, $J = 7.2$ Hz), 4.21 (dq, 2H, $J = 7.2$ Hz, $J = 3.2$ Hz), 4.36 (br, 2H), 6.11 (ddt, 1H, $J = 16$ Hz, $J = 3.2$ Hz, $J = 2$ Hz), 7.04 (ddt, 1H, $J = 16$ Hz, $J = 6.8$ Hz, $J = 4$ Hz); $^{13}$C NMR
(100 MHz, CDCl$_3$) $\delta$ 14.4, 60.7, 62.1, 120.4, 147, 166.6. FAB-HRMS (positive mode): $m/z$ Calcd. for C$_5^{13}$C$_{11}$H$_{11}$O$_3$ [MH$^+$] 132.0742, found 132.0747.

**Ethyl (E)-[2-$^{13}$C]-4-hydroxy-but-2-enoate (5b)**

The same procedure as for the synthesis of 5 was used starting from 3b (3.4 g, 9.73 mmol) to give 5b (1.11 g, 8.46 mmol, 86.9% yield): $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 1.30 (t, 3H, $J$ = 7.2 Hz), 4.21 (q, 2H, $J$ = 7.2 Hz), 4.36 (m, 2H), 6.10 (ddt, 1H, $J$ = 163.8 Hz, $J$ = 15.6 Hz, $J$ = 2 Hz), 7.04 (dm, 1H, $J$ = 15.6 Hz); $^{13}$C NMR (100 MHz, CDCl$_3$) $\delta$ 14.4, 60.7, 62.1, 120.4, 147 (d, $J$ = 35.8 Hz), 166.7. FAB-HRMS (positive mode): $m/z$ Calcd. for C$_5^{13}$C$_{11}$H$_{11}$O$_3$ [MH$^+$] 132.0742, found 132.0730.

**Ethyl (E)-[1,2-$^{13}$C$_2$]-4-hydroxy-but-2-enoate (5c)**

The same procedure as for the synthesis of 5 was used starting from 3c (3.4 g, 9.70 mmol) to give 5c (1.12 g, 8.48 mmol, 87.4% yield): $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 1.30 (t, 3H, $J$ = 7.2 Hz), 4.18-4.22 (2H), 4.33-4.37 (2H), 6.10 (ddm, 1H, $J$ = 82 Hz, $J$ = 16 Hz), 7.04 (dm, 1H, $J$ = 16 Hz); $^{13}$C NMR (100 MHz, CDCl$_3$) $\delta$ 14.4, 60.7, 62.1, 120.4 (d, $J$ = 37.6 Hz), 147, 166.7 (d, $J$ = 37.6 Hz). FAB-HRMS (positive mode): $m/z$ Calcd. for C$_4^{13}$C$_2$H$_{11}$O$_3$ [MH$^+$] 133.0775, found 133.0779.

**Dihydrofuran-2-(3H)-one or γ-butyrolactone (6)**
Ethyl 4-hydroxy-but-2-enoate (5, 1.1 g, 8.45 mmol) was dissolved in 25 mL ethyl acetate and mixed with 0.12 g of 10 % Pd/C. Under 1 atm of H₂, the mixture was stirred for 2 h at RT. The reaction was filtered through a pad of celite, the solvent removed in vacuo and the product, ethyl 4-hydroxybutanoate (1.1 g, 8.32 mmol, 98.5%) obtained was directly used for the next step without any further purification.

A solution of ethyl 4-hydroxybutanoate (1.1 g, 8.32 mmol) in a mixture of TFA (40 ml) and water (20 mL) was stirred at RT for 5 h. The volatiles were removed by co-distillation with toluene and the residue purified by flash column chromatography over silica (hexane, 30% ethyl acetate) to give 6 (0.62 g, 7.20 mmol, 86.5% yield): ¹H NMR (400 MHz, CDCl₃) δ 2.25 (quint, 2H, J = 2.4 Hz), 2.49 (t, 2H, J = 7.6 Hz), 4.31 (t, 2H, J = 7.2 Hz); ¹³C NMR (100 MHz, CDCl₃) δ 22.3, 27.8, 68.6, 177.8.

[1-¹³C]-γ-Butyrolactone (6a)

The same procedure as for the synthesis of 6 was used starting from 5a (1.1 g, 8.39 mmol) to give 6a (0.61 g, 7.00 mmol, 83.4% yield): ¹H NMR (400 MHz, CDCl₃) δ 2.24 (m, 2H), 2.49 (dt, 2H, J = 8.6 Hz, J = 5.6 Hz), 4.32 (dt, 2H, J = 7.0 Hz, J = 3.6 Hz); ¹³C NMR (100 MHz, CDCl₃) δ 22.3, 27.8 (d, J = 44 Hz), 68.6 (d, J = 4 Hz), 177.8. FAB-HRMS (positive mode): m/z Calcd. for C₃¹³C₁H₇O₂ [MH⁺] 88.0479, found 88.0469.

[2-¹³C]-γ-Butyrolactone (6b)

The same procedure as for the synthesis of 6 was used starting from 5b (1.1 g, 8.39 mmol) to give 6b (0.63 g, 7.23 mmol, 86.2% yield): ¹H NMR (400 MHz, CDCl₃) δ 2.21-2.33 (2H), 2.49 (dm, 2H, J = 82 Hz), 4.32 (dt, 2H, J = 7.2 Hz, J = 2.6 Hz); ¹³C NMR (100
MHz, CDCl$_3$) δ 22.3 (d, $J = 32.5$ Hz), 27.9, 68.5 (d, $J = 2.1$ Hz), 177.7. FAB-HRMS (positive mode): $m/z$ Calcd. for C$_3$H$_7$O$_2$ [MH$^+$] 88.0479, found 88.0481.

[1,2-$^{13}$C$_2$]-$\gamma$-Butyrolactone (6c)

The same procedure as for the synthesis of 6 was used starting from 5c (1.1 g, 8.32 mmol) to give 6c (0.63 g, 7.15 mmol, 85.9% yield): $^1$H NMR (400 MHz, CDCl$_3$) δ 2.20-2.29 (2H), 2.49 (dm, 2H, $J = 35.1$ Hz), 4.30-4.35 (2H); $^{13}$C NMR (100 MHz, CDCl$_3$) δ 22.3 (d, $J = 33$ Hz), 28.0 (d, $J = 35.1$ Hz), 68.5 (d, $J = 8.4$ Hz), 177.9 (d, $J = 49.4$ Hz). FAB-HRMS (positive mode): $m/z$ Calcd. for C$_2$H$_7$O$_2$ [MH$^+$] 89.0513, found 89.0521.

Ethyl 2-(benzyloxy) acetate (8)

Sodium hydride (60% oil dispersion, 0.60 g, 15 mmol, pre-washed with petroleum ether) was suspended in dry THF (20 mL) under argon. A solution of benzyl alcohol (7) (1.51 g, 14 mmol) in 5 mL dry THF was added dropwise with stirring at room temperature until the evolution of H$_2$ ceased. Ethyl bromoacetate (1) (2.36 g, 14.1 mmol) was added dropwise at 0 °C. The reaction mixture was slowly brought to room temperature and stirred overnight and then the white precipitate was filtered off, washed with ethyl acetate (3 × 10 mL), and the filtrate was washed with brine (50 mL) and dried with Na$_2$SO$_4$. The solvent was removed in vacuo and the residual oil was purified by flash column chromatography over silica (hexane, 20% ethyl acetate) to give 8 (2.05 g, 10.6 mmol, 75.2% yield): $^1$H NMR (400 MHz, CDCl$_3$) δ 1.29 (t, 3H, $J = 7.2$ Hz), 4.09 (s, 2H), 4.23
(q, 2H, J = 7.2 Hz), 4.64 (s, 2H), 7.30-7.41 (5H); \(^{13}\)C NMR (100 MHz, CDCl\(_3\)) \(\delta\) 14.4, 61.1, 67.4, 73.6, 128.2, 128.3, 128.7, 137.3, 170.6.

**Ethyl 2-(benzyloxy) [1-\(^{13}\)C]acetate (8a)**

The same procedure as for the synthesis of 8 was used starting from 1a (2.35 g, 14.0 mmol) to give 8a (2.05 g, 10.5 mmol, 75% yield): \(^1\)H NMR (400 MHz, CDCl\(_3\)) \(\delta\) 1.29 (t, 3H, J = 7.2 Hz), 4.09 (d, 2H, J = 4.8 Hz), 4.23 (dq, 2H, J = 7.2 Hz, J = 3.2 Hz), 4.64 (s, 2H), 7.31-7.38 (5H); \(^{13}\)C NMR (100 MHz, CDCl\(_3\)) \(\delta\) 14.4 (d, J = 2.1 Hz), 61.1 (d, J = 2.5 Hz), 67.4 (d, J = 63 Hz), 73.6 (d, J = 2.3 Hz), 128.2, 128.3, 128.7, 137.3, 170.6. FAB-HRMS (positive mode): \(m/z\) Calcd. for C\(^{10}\)C\(^1\)H\(_{15}\)O\(_3\) [MH\(^+\)] 196.1055, found 196.1061.

**Ethyl 2-(benzyloxy) [2-\(^{13}\)C]acetate (8b)**

The same procedure as for the synthesis of 8 was used starting from 1b (2.35 g, 14.0 mmol) to give 8b (2.10 g, 10.8 mmol, 77.1% yield): \(^1\)H NMR (400 MHz, CDCl\(_3\)) \(\delta\) 1.29 (t, 3H, J = 7.2 Hz), 4.09 (d, 2H, J = 144 Hz), 4.23 (q, 2H, J = 7.2 Hz), 4.64 (d, J = 4.4 Hz, 2H), 7.30-7.38 (5H); \(^{13}\)C NMR (100 MHz, CDCl\(_3\)) \(\delta\) 14.4, 61.1, 67.4, 73.6, 128.2, 128.3, 128.7, 137.3, 170.6. FAB-HRMS (positive mode): \(m/z\) Calcd. for C\(^{10}\)C\(^1\)H\(_{15}\)O\(_3\) [MH\(^+\)] 196.1055, found 196.1053.

**Ethyl 2-(benzyloxy) [1,2-\(^{13}\)C\(_2\)]acetate (8c)**

The same procedure as for the synthesis of 8 was used starting from 1c (2.35 g, 13.9 mmol) to give 8c (2.10 g, 10.7 mmol, 77% yield): \(^1\)H NMR (400 MHz, CDCl\(_3\)) \(\delta\) 1.29 (t, 3H, J = 7.2 Hz), 4.09 (dd, 2H, J = 143.8 Hz, 4.4 Hz), 4.23 (dq, 2H, J = 7.2 Hz, 3.2 Hz), 4.64 (d, J = 4.4 Hz, 2H), 7.30-7.39 (5H); \(^{13}\)C NMR (100 MHz, CDCl\(_3\)) \(\delta\) 14.4, 61.1, 67.4
(d, \( J = 63 \) Hz), 73.6, 128.2, 128.3, 128.6, 137.3, 170.6 (d, \( J = 63 \) Hz). FAB-HRMS (positive mode): \( m/z \) Calcd. for C\(_{9}\)C\(_{2}\)H\(_{15}\)O\(_{3}\) [MH\(^+\)] 197.1088, found 197.1090.

**Ethyl 4-(benzyloxy)-but-2-enoate (10)**

![Chemical Structure](image)

Ethyl 2-(benzyloxy)acetate (8, 1.76 g, 9.1 mmol) was dissolved in 30 mL of dry dichloromethane and 9 mL of DIBAL (1.2 M solution in Toluene, 10.8 mmol) was added slowly into it dropwise at -78 °C with continuous stirring. The reaction was stirred for 1.15 h, then 1.5 mL H\(_2\)O was added to quench reaction, and 1M HCl was added until pH ~0. The solvent was removed \textit{in vacuo} and the product, 2-(benzyloxy)acetaldehyde (9) (1.35 g, 9.0 mmol, 98.9% yield) obtained was directly used for the next step without any further purification.

To a solution of 2-(benzyloxy)acetaldehyde (9, 1.35 g, 9.0 mmol) in 25 mL dry dichloromethane under argon atmosphere was added 2-(ethoxycarbonylmethylene)triphenylphosphorane (3.41 g, 9.41 mmol) in dry dichloromethane (25 mL) and the reaction mixture stirred for 5 h. The solvent was then evaporated under reduced pressure and the crude product was purified by column chromatography over silica (hexane, 20% ethyl acetate) to give 10 (1.75 g, 7.95 mmol, 88.3% yield), which comprised of a mixture of \textit{cis} and \textit{trans} isomers. \(^1\)H NMR (400 MHz, CDCl\(_3\)) \( \delta \) 1.29 (t, 3H, \( J = 7.2 \) Hz), 4.13-4.23 (4H), 4.57 (s, 2H), 5.82 (dt, 1H, \( J = 2.4 \) Hz, 11.6 Hz, H-3\textit{cis}), 6.13 (dt, 1H, \( J = 2.0 \) Hz, 15.6 Hz, H-3\textit{trans}), 6.43 (dt, 1H, \( J = 4.8 \) Hz, 15.6 Hz, H-3\textit{trans}).
Hz, 11.6 Hz, H-2\textsubscript{cis}), 6.98 (dt, 1H, $J = 4.4$ Hz, 15.6 Hz, H-3\textsubscript{trans}), 7.26-7.38 (5H); $^{13}$C NMR (100 MHz, CDCl\textsubscript{3}) $\delta$ 14.5, 60.6, 68.8, 73.1, 119.8 (CH=CH\textsubscript{cis}), 121.6 (CH=CH\textsubscript{trans}), 127.8, 128.0, 128.7, 137.9, 144.4 (CH=CH\textsubscript{trans}), 148.5 (CH=CH\textsubscript{cis}), 166.5.

FAB-HRMS (positive mode): $m/z$ Calcd. for C\textsubscript{13}H\textsubscript{17}O\textsubscript{3} $[\text{MH}^+]$ 221.1178, found 221.1169.

**Ethyl 4-(benzyloxy)-[3-$^{13}$C]-but-2-enoate (10a)**

The same procedure as for the synthesis of 10 was used starting from 8a (1.70 g, 8.7 mmol) to give 10a (1.64 g, 7.4 mmol, 85.1% overall yield): $^1$H NMR (400 MHz, CDCl\textsubscript{3}) $\delta$ 1.29 (t, 3H, $J = 7.2$ Hz), 4.09-4.23 (4H), 4.57 (s, 2H), 5.82 (dtd, 1H, $J = 11.8$ Hz, 2.4 Hz, 0.8 Hz, H-3\textsubscript{cis}), 6.13 (dm, 1H, $J = 16$ Hz, H-3\textsubscript{trans}), 6.43 (tdd, 1H, $J = 157.2$ Hz, 4.8 Hz, 11.6 Hz, H-2\textsubscript{cis}), 6.98 (tdd, 1H, $J = 157.6$ Hz, 4.4 Hz, 15.6 Hz, H-3\textsubscript{trans}), 7.28-7.38 (5H); $^{13}$C NMR (100 MHz, CDCl\textsubscript{3}) $\delta$ 14.5, 60.6, 68.7, 73.0, 119.5 (CH=CH\textsubscript{cis}), 121.9 (CH=CH\textsubscript{trans}), 127.8, 128.0, 128.7, 137.9, 144.4 (CH=CH\textsubscript{trans}), 148.5 (CH=CH\textsubscript{cis}), 166.5. FAB-HRMS (positive mode): $m/z$ Calcd. for C\textsubscript{12}$^{13}$C\textsubscript{1}H\textsubscript{17}O\textsubscript{3} $[\text{MH}^+]$ 222.1211, found 222.1219.

**Ethyl 4-(benzyloxy)-[4-$^{13}$C]-but-2-enoate (10b)**

The same procedure as for the synthesis of 10 was used starting from 8b (1.80 g, 9.2 mmol) to give 10b (1.68 g, 7.6 mmol, 82.6% overall yield): $^1$H NMR (400 MHz, CDCl\textsubscript{3}) $\delta$ 1.29 (t, 3H, $J = 7.2$ Hz), 4.21 (q, 2H, $J = 4.8$ Hz), 4.18 (ddd, 2H, $J = 141.6$ Hz, 4.4 Hz, 2.4 Hz), 4.58 (d, 2H, $J = 4.0$ Hz), 5.82 (dm, 1H, $J = 11.6$ Hz, H-3\textsubscript{cis}), 6.13 (dm, 1H, $J = 15.6$ Hz, H-3\textsubscript{trans}), 6.43 (dm, 1H, $J = 11.6$ Hz, H-2\textsubscript{cis}), 6.98 (dm, 1H, $J = 15.6$ Hz, H-3\textsubscript{trans}), 7.27-7.38 (5H); $^{13}$C NMR (100 MHz, CDCl\textsubscript{3}) $\delta$ 14.5, 60.6, 68.7, 73.0, 119.5 (CH=CH\textsubscript{cis}), 121.9 (CH=CH\textsubscript{trans}), 127.8, 128.0, 128.7, 137.9, 144.5 (CH=CH\textsubscript{trans}), 148.5
(CH=CH<sub>cis</sub>), 166.7. FAB-HRMS (positive mode): m/z Calcd. for C<sub>12</sub>\(^{13}\)C<sub>1</sub>H<sub>17</sub>O<sub>3</sub> [MH<sup>+</sup>] 222.1211, found 222.1213.

**Ethyl 4-(benzyloxy)-[3,4-\(^{13}\)C<sub>2</sub>]-but-2-enoate (10c)**

The same procedure as for the synthesis of 10 was used starting from 8c (1.75 g, 8.9 mmol) to give 10c (1.67 g, 7.5 mmol, 84.2% overall yield): \(^1\)H NMR (400 MHz, CDCl<sub>3</sub>) \(\delta\) 1.29 (t, 3H, \(J = 7.2\) Hz), 4.21 (q, 2H, \(J = 4.8\) Hz), 4.18 (dm, 2H, \(J = 141.6\) Hz), 4.57 (d, 2H, \(J = 4.4\) Hz), 5.82 (dm, 1H, \(J = 11.6\) Hz, H-3<sub>cis</sub>), 6.13 (dm, 1H, \(J = 15.6\) Hz, H-3<sub>trans</sub>), 6.43 (dtd, 1H, \(J = 157.6\) Hz, 11.6 Hz, 1.6 Hz, H-2<sub>cis</sub>), 6.98 (dmd, 1H, \(J = 157.6\) Hz, 15.6 Hz, H-3<sub>trans</sub>), 7.27-7.38 (5H); \(^{13}\)C NMR (100 MHz, CDCl<sub>3</sub>) \(\delta\) 14.5, 60.6, 68.8 (d, \(J = 46\) Hz), 73.1, 119.8 (CH=CH<sub>cis</sub>), 121.6 (CH=CH<sub>trans</sub>), 127.8, 128.0, 128.7, 137.9, 144.4 (d, \(J = 46.1\) Hz, CH=CH<sub>trans</sub>), 148.5 (d, \(J = 45.9\) Hz CH=CH<sub>cis</sub>), 166.5. FAB-HRMS (positive mode): m/z Calcd. for C<sub>11</sub>\(^{13}\)C<sub>2</sub>H<sub>17</sub>O<sub>3</sub> [MH<sup>+</sup>] 223.1245, found 223.1252.

**Ethyl [2,3,4-\(^{13}\)C<sub>3</sub>]-4-(benzyloxy)-but-2-enoate (10d)**

The same procedure as for the synthesis of 10 was used starting from 8c (1.70 g, 8.7 mmol) and 3b to give 10d (1.64 g, 7.4 mmol, 85.1% overall yield): \(^{13}\)C NMR (100 MHz, CDCl<sub>3</sub>) \(\delta\) 14.5, 60.6, 68.8 (d, \(J = 46\) Hz), 73.1, 119.7 (d, \(J = 69.3\) Hz, CH=CH<sub>cis</sub>), 121.6 (d, \(J = 71.2\) Hz, CH=CH<sub>trans</sub>), 127.8, 128.0, 128.7, 137.9, 144.4 (dd, \(J = 69.2\) Hz, 46 Hz, CH=CH<sub>trans</sub>), 148.5 (dd, \(J = 69.4\) Hz, 46.1 Hz, CH=CH<sub>cis</sub>), 166.6. FAB-HRMS (positive mode): m/z Calcd. for C<sub>10</sub>\(^{13}\)C<sub>3</sub>H<sub>17</sub>O<sub>3</sub> [MH<sup>+</sup>] 224.1278, found 224.1269.
**Ethyl 4-(benzyloxy)-[1,2,3-\textsuperscript{13}C\textsubscript{3}]-but-2-enoate (10e)**

The same procedure as for the synthesis of 10 was used starting from 8a (1.70 g, 8.7 mmol) and 3c to give 10e (1.64 g, 7.4 mmol, 85.1% overall yield): \textsuperscript{13}C NMR (100 MHz, CDCl\textsubscript{3}) \(\delta 14.5, 60.6, 68.7, 73.1, 119.7 \text{ (dd, } J = 73.5 \text{ Hz, 69.3 Hz, CH=CH\textsubscript{cis}}), 121.6 \text{ (dd, } J = 74.5 \text{ Hz, 71.4 Hz, CH=CH\textsubscript{trans}}), 127.8, 128.0, 128.7, 137.9, 144.4 \text{ (d, } J = 71.4 \text{ Hz, CH=CH\textsubscript{trans}}), 148.5 \text{ (d, } J = 71.4 \text{ Hz, CH=CH\textsubscript{cis}}), 166.6 \text{ (dd, } J = 74.5 \text{ Hz, 1.3 Hz}).\) FAB-HRMS (positive mode): m/z Calcd. for C\textsubscript{10}\textsuperscript{13}C\textsubscript{3}H\textsubscript{17}O\textsubscript{3} [MH\textsuperscript{+}] 224.1278, found 224.1281.

**\(\gamma\)-Butyrolactone (6)**

Ethyl 4-(benzyloxy)-but-2-enoate (10, 1.7 g, 7.72 mmol) was dissolved in 25 mL ethyl acetate and mixed with 0.2 g of 10 % Pd/C. Under 150 psi of H\textsubscript{2}, the mixture was stirred for 2 h at RT. The reaction was filtered through a pad of celite, the solvent removed \textit{in vacuo} and the product, ethyl 4-hydroxybutanoate (1.0 g, 7.58 mmol, 98.2%) obtained was directly used for the next step without any further purification.

A solution of 0.5 g (3.78 mmol) of ethyl 4-hydroxybutanoate in a mixture of TFA (20 mL) and water (10 mL) was stirred at RT for 5 h. The volatiles were removed by co-distillation with toluene and the residue purified by flash column chromatography over silica (hexane, 30% ethyl acetate) to give 0.27 g of 6 (3.13 mmol, 82.8% yield): \textsuperscript{1}H NMR (400 MHz, CDCl\textsubscript{3}) \(\delta 2.25 \text{ (quint, } 2\text{H, } J = 2.4 \text{ Hz}), 2.49 \text{ (t, } 2\text{H, } J = 7.6 \text{ Hz}), 4.31 \text{ (t, } 2\text{H, } J = 7.2 \text{ Hz}); \textsuperscript{13}C NMR (100 MHz, CDCl\textsubscript{3}) \(\delta 22.3, 27.8, 68.6, 177.8.\)

**[3-\textsuperscript{13}C]-\(\gamma\)-Butyrolactone (6d)**
The same procedure as for the synthesis of 6 was used starting from 10a (1.6 g, 7.23 mmol) to give 6d (0.53 g, 6.09 mmol, 84.2% yield): $^1$H NMR (400 MHz, CDCl$_3$) δ 2.24 (dquint, 2H, $J = 135.2$ Hz, 7.2 Hz), 2.45-2.64 (2H), 4.37 (dt, 2H, $J = 7.2$ Hz, 1.6 Hz); $^{13}$C NMR (100 MHz, CDCl$_3$) δ 22.3, 28.0, 68.5, 177.9. FAB-HRMS (positive mode): $m/z$ Calcd. for C$_3$H$_7$O$_2$ [MH$^+$] 88.0479, found 88.0484.

[4-$^{13}$C]-γ-Butyrolactone (6e)

The same procedure as for the synthesis of 6 was used starting from 10b (1.65 g, 7.46 mmol) to give 6e (0.56 g, 6.43 mmol, 86.2% yield): $^1$H NMR (400 MHz, CDCl$_3$) δ 2.24 (dquint, 2H, $J = 7.6$ Hz, 2.8 Hz), 2.58 (dt, 2H, $J = 3.6$ Hz, 8.4 Hz), 4.35 (dt, 2H, $J = 152.4$ Hz, 6.8 Hz); $^{13}$C NMR (100 MHz, CDCl$_3$) δ 22.2, 28.1, 68.7, 177.9. FAB-HRMS (positive mode): $m/z$ Calcd. for C$_3$H$_7$O$_2$ [MH$^+$] 88.0479, found 88.0481.

[3,4-$^{13}$C$_2$]-γ-Butyrolactone (6f)

The same procedure as for the synthesis of 6 was used starting from 10c (1.5 g, 6.75 mmol) to give 6f (0.50 g, 5.68 mmol, 84.1% yield): $^1$H NMR (400 MHz, CDCl$_3$) δ 2.25 (dm, 2H, $J = 135.2$ Hz), 2.50-2.58 (2H), 4.36 (dtd, 2H, $J = 151.6$ Hz, 6.8 Hz, 2.0 Hz); $^{13}$C NMR (100 MHz, CDCl$_3$) δ 22.3 (d, $J = 31.3$ Hz), 28.2, 68.7 (d, $J = 31.3$ Hz), 177.7. FAB-HRMS (positive mode): $m/z$ Calcd. for C$_2$H$_7$O$_2$ [MH$^+$] 89.0513, found 89.0517.

[1,2,3-$^{13}$C$_3$]-γ-Butyrolactone (6g)

The same procedure as for the synthesis of 6 was used starting from 10e (1.8 g, 8.06 mmol) to give 6g (0.59 g, 6.62 mmol, 82.1% yield): $^1$H NMR (400 MHz, CDCl$_3$) δ 2.26 (dm, 2H, $J = 135$ Hz), 2.53 (dm, 2H, $J = 135$ Hz), 4.35-4.40 (2H); $^{13}$C NMR (100 MHz,
CDCl$_3$) $\delta$ 22.2 (d, $J = 32.9$ Hz), 28.2 (dd, $J = 32.8$ Hz, 73.3 Hz), 68.7, 177.8 (d, $J = 48.8$ Hz). FAB-HRMS (positive mode): $m/z$ Calcd. for C$_1^{13}$C$_3$H$_7$O$_2$ [MH$^+$] 90.0547, found 90.0553.

**[2,3,4-$^{13}$C$_3$]-γ-Butyrolactone (6h)**

The same procedure as for the synthesis of 6 was used starting from 10d (1.8 g, 8.06 mmol) to give 6h (0.60 g, 6.73 mmol, 83.5% yield): $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 2.26 (dm, 2H, $J = 134.8$ Hz), 2.53 (dm, 2H, $J = 134.8$ Hz), 4.38 (dtt, 2H, $J = 154.8$ Hz, 7.2 Hz, 2.0 Hz); $^{13}$C NMR (100 MHz, CDCl$_3$) $\delta$ 22.2 (dd, $J = 32.9$ Hz, 30.6 Hz), 28.2 (dd, $J = 32.9$ Hz, 3.6 Hz), 68.8 (dd, $J = 30.6$ Hz, 2.2 Hz), 177.7. FAB-HRMS (positive mode): $m/z$ Calcd. for C$_1^{13}$C$_3$H$_7$O$_2$ [MH$^+$] 90.0547, found 90.0551.

### 3.3.3 Liver Perfusions

Livers from overnight-fasted Sprague-Dawley rats were perfused for 2 h with 150 mL of recirculating bicarbonate buffer containing 4% dialyzed bovine serum albumin (fraction V, fatty acid-free, Intergen), 4 mM glucose ± 2 mM GHB (unlabeled or labeled GHB). Livers were quick-frozen at the end of the perfusions and stored in liquid nitrogen for further analysis by LC-MS/MS.

### 3.3.4 LC-MS/MS Method for the Labeling Pattern and Concentration Measurement of Acyl-CoAs

The concentrations and $^{13}$C-mass isotopomer distributions of GHB, Krebs cycle intermediates, and acyl-CoAs were assayed by LC-MS/MS as previously described in Chapter 2.$^{10}$
3.3.5 GC-MS Assay of Formate and Acetate via PFBBr Derivatization Reaction

The concentrations and labeling of formate and acetate were assayed as the pentafluorobenzyl derivatives by NH$_3$-negative chemical ionization as described below. GC/MS is typically used for volatile compounds or compounds after derivatization. The common derivatizations for formic acid are methylation, ethylation, and 2,4-difluoroaniline. However, all of these derivatives dilute the isotopomer enrichment by introducing additional isotopomers from derivatizing reagents. Pentafluorobenzyl bromide (PFBBr), an alkylating reagent for derivatization of carboxylic acid, sulfonamides, mercaptans, and phenol compounds, meets the requirement of not diluting the isotopomer enrichment. PFBBr derivatization has been previously used for formic acid and acetic acid concentration quantification. In addition to better retention on the GC column and higher sensitivity, the PFBBr derivatives do not disrupt the natural isotopomer distribution in the negative chemical ionization (NCI) mode as only the acid moiety is measured in NCI (Scheme 3.3).

Sample preparation for short chain fatty acids with GC/MS assay is as follows. A 400 µl of 100 mM PFBBr in acetone solution was added into 200 µl of perfusate samples or standard aqueous solution without precipitating the protein. The sample was incubated at 60–70 °C for 1 h. 1 mL of hexane was added after the sample had cooled. The sample was then vortexed for 5 min followed by centrifugation at 300g for 1 min, and then 200 µl of upper phase (hexane phase) was transferred to GC vials and prepared for GC/MS injection. All the experiments were processed in a hood to avoid contamination. The distilled Milli-Q water was used for the preparation of all standard solutions.
Analyses were carried out on an Agilent 5973 mass spectrometer, linked to a Model 6890 gas chromatograph equipped with an auto-sampler, an Agilent OV-225 capillary column (30 m, 0.32 mm inner diameter). The carrier gas was helium (2 mL/min) with a pulse pressure of 40 psi. The injection volume was 1 μl with splitless. The injector temperature was set at 200 °C and the transfer line at 250 °C. The GC temperature program was as follows: start at 100 °C, hold for 1 min, increase by 3 °C/min to 145 °C, followed by 50 °C/min to 300 °C, and hold for 5 min. The ion source and the quadrupole were set at 150 °C. The ammonia pressure was adjusted to optimize peak areas. For each analyte, we monitored the signals at the nominal m/z (M) and at all detectable naturally labeled mass isotopomers with SIM mode. The m/z monitored are 45 (M) and 46 (M1) for formate; and 59 (M), 60 (M1), 61 (M2), and 63 (M4) for acetate.

Scheme 3.3 (A) PFBBR derivatization of acids and (B) the corresponding mass ion in negative chemical ionization (NCI) mass spectrometry
3.3.6 Calculations and Statistics

Correction of raw mass isotopomer profiles for natural enrichment at each mass was conducted with the CORMAT software. In perfusions with $[^{13}C_4]$GHB, relative anaplerosis was calculated as the enrichment ratio (M4 succinate)/(M4 GHB), because M4 succinate cannot be formed from recycling of label in the Krebs cycle. Statistical differences were tested using a paired t test (Graph Pad Prism Software, version 3).

3.4 Conclusions

In this chapter we showed that the catabolism of GHB ($C_4$) differs from that of longer chain 4-hydroxyacids ($C_5$-$C_{11}$) described in Chapter 2. For the longer chain 4-hydroxyacids (with more than four carbons) catabolism can proceed through two parallel pathways that involve either a phosphorylation and isomerization of 4-hydroxyacyl-CoAs to 3-hydroxyacyl-CoAs via 4-phosphoacyl-CoAs (Pathway A) or sequential β-oxidation, α-oxidation and β-oxidation steps (Pathway B). We were also able to measure the flux between the pathway A and B using the isotopic tools as both the pathways led the formation of acetyl-CoA, a common catabolite as an end-product. Our present study demonstrates a complete catabolic fate of GHB both by entering into the Krebs cycle via oxidation to a dicarboxylic acid, succinate, as shown by others and new pathways comprising of α-oxidation and β-oxidation and decarboxylation. Although we identified the 4-phosphobutyryl-CoA in the GHB metabolism, but no isomerization of 4-hydroxybutyryl-CoA to 3-hydroxybutyryl-CoA was observed.

At the same time, measurement of the flux of the different pathways was not possible for GHB catabolism as it is catabolized by five pathways, none of which yields
any common end-products or a product that accumulates. The six-fold higher concentration of M1 formate in perfusions with [3,4-\textsuperscript{13}C\textsubscript{2}]GHB compared to [1,2-\textsuperscript{13}C\textsubscript{2}]GHB (Fig 3.3A) suggests that the β-oxidation of GHB is its most abundant catabolic pathway (Fig 3.5). This is because M1 formate derives mostly from the oxidation of glycolate formed from C-3 and C-4 of GHB. Further, the higher concentration of 3,4-dihydroxybutyryl-CoA than the 2,4-dihydroxybutyryl-CoA (as shown in Fig 3.6) confirms β-oxidation as the predominant catabolic pathway of GHB.

Essential to this finding was the use of isotopically labeled GHB to define its catabolic pathway. We described a high-yielding method for the synthesis of \textsuperscript{13}C-labeled GHB. The previous synthetic route to \textsuperscript{13}C-labeled GHB, reported in the literature, uses potentially hazardous diazomethane or Dess-Martin periodinane and is also low-yielding (overall yield is 16.6% comprising 7 steps).\textsuperscript{23} Our synthetic routes are high-yielding (75% and 55% for Scheme 3.2A & B respectively) as well as involve less steps. At the same time, we avoided using the potentially hazardous diazomethane or Dess-Martin periodinane. We synthesized specifically \textsuperscript{13}C-labeled γ-butyrolactones, which were hydrolyzed with aqueous sodium hydroxide to yield labeled GHB. We evaluated a series of tools that were critical for this work and demonstrated how these tools can be used for the new biological pathway discovery as well as for identifying novel metabolites such as 4-phosphoacyl-CoAs. These newly identified pathways may play a role in the physiopathology of SSADH deficiency. Lastly, our study illustrates the potential of the combination of metabolomics and mass isotopomer analysis for pathway discovery.
3.5 ACKNOWLEDGEMENT

All the animal experiments were performed in Prof. Brunengraber’s lab (Department of Nutrition, Case Western Reserve University). The non-targeted metabolomics of GHB were conducted by Prof. Guo-fang Zhang and presented here with his permission.
3.6 REFERENCES


16. Brunengraber, H., Boutry, M. & Lowenstein, J.M. Fatty acid and 3-beta-hydroxysterol synthesis in the perfused rat liver. Measurements on the production


CHAPTER 4

Enantioselective Glutathionylation of 4-Hydroxy-2-(E)-Nonenal (4-HNE) & its Derivatives in Rat Organs
4.1 **INTRODUCTION**

Oxidative stress is a predominant source of damage to bio-macromolecules including proteins, DNA, lipids, and sugars within cells. The concentrations of reactive oxygen/nitrogen species (ROS/RNS) are elevated during oxidative stress. As lipids represent the primary barrier for free diffusion of ROS/RNS into the cell, they often become the primary target of oxidative reactions, which is commonly known as lipid peroxidation. Peroxidation of lipids results in unsaturated lipid hydroperoxides that undergo fragmentation, giving rise to variety of α,β-unsaturated carbonyl compounds such as 4-hydroxy-2-(E)-hexenal (4-HHE), 4-hydroxy-2-(E)-nonenal (4-HNE), acrolein, and 4-oxo-2-(E)-nonenal (4-ONE). 4-HNE is most studied among those lipid peroxidation products and has become accepted as a contributor to the pathogenesis of a number of degenerative diseases such as Alzheimer’s disease, atherosclerosis, and cancer.

A central tenet of the 4-HNE field is its highly electrophilic nature which can subsequently react with the biological nucleophiles including those in protein side chains, nucleic acid base pairs and lipids to modify or abrogate their ability to function normally. The electrophilic nature of 4-hydroxy-2-(E)-alkenal (4-HAE) is attributed to the conjugation of the C=C π-electrons with the aldehyde functionality. Due to resonance, there is a regional electron deficiency at β-carbon and in addition the presence of γ-OH group (see **Fig 4.1A**) further promotes its electrophilicity through its electron withdrawing effect. 4-ONE is even more reactive towards the nucleophiles because it possesses the γ-oxo functionality (see **Fig 4.1B**). In addition, the γ-oxo group of 4-ONE becomes another target for nucleophilic side chains of proteins. In addition, hydration of
4-ONE at C-1 does not affect on its ability to become a Michael acceptor, whereas hydration at C-1 completely abrogates Michael acceptor nature of 4-HNE or 4-HHE.

Specifically, 4-ONE was found to be 10- and 100-fold more reactive toward amino acid/peptide amines and thiols, respectively, as compared to 4-HNE. The 4-ONE Michael adducts can be more reactive than those of 4-HNE as the former cannot cyclize into hemiacetal form (Fig 4.1C). Thus, 4-ONE adducts may contain two free carbonyl groups, which are pre-disposed to react with the side chain amine residues of proteins. For the same reason, 4-ONE also becomes a potential protein cross linking reagent. This results from conjugate addition of side chain nucleophiles such as sulfhydryl or imidazole groups to the α,β-unsaturation of 4-ONE to give either a α- or β-substituted 4-ketoaldehyde, which then undergoes Paal−Knorr condensation with the primary amine of protein lysine side chains.
FIGURE 4.1 (A) Structure of 4-hydroxy-2-(E)-nonenal (4-HNE) and its electron density mapped by gaussian calculation, (B) Structure of 4-oxo-2-(E)-nonenal (4-ONE) and its electron density mapped by gaussian calculation, and (C) Michael addition of thiols to 4-HNE and 4-ONE. 4-HNE but not 4-ONE can form the hemiacetal of the Michael adduct. Note: In the electron density maps, red regions are high and blue regions are low density in electrons.
Severe oxidative stress has been implicated in several disease states such as Alzheimer’s disease,\textsuperscript{9,10} atherosclerosis,\textsuperscript{11-13} and cancer.\textsuperscript{14} High reactivity and transient stability of ROS and RNS make it difficult to directly measure the level of oxidative stress. Often to overcome this difficulty, secondary biomarkers are chosen to assess oxidative stress. But, frequent overestimation of background levels remains a major drawback of these techniques. For example, 3-nitrotyrosine has been widely used as a biomarker for oxidative stress but the reported level of 3-nitrotyrosine in normal human plasma varies 30-fold among different studies.\textsuperscript{21} Overestimated levels of aldehyde modified proteins are also reported in the literature.\textsuperscript{22} Other frequently used biomarkers include the lipid peroxidation end-products such as 4-HNE, 4-HHE or MDA but the high reactivity of those aldehydes often produces inconsistent values.

Glutathione S-transferases (GSTs) play a key role in metabolizing highly reactive electrophiles present in living systems including 4-HNE, 4-HHE and 4-ONE, formed during the oxidative insults.\textsuperscript{8} GST catalyzed conjugation of 4-HNE to glutathione (GSH), results in the formation of the 4-HNE–GSH diastereomers (see Fig 4.2A & B).\textsuperscript{23} GSH conjugates are known to be transported out of cells through an ATP-dependent primary active efflux mechanism.\textsuperscript{24} GSTs also catalyze the conjugation of cholesterol-5,6-oxide, epoxyeicosatrienoic acid, and 9,10-epoxystearic acid with GSH. Thus GSTs have been well accepted to provide the cell with protection against a range of harmful electrophiles generated during oxidative stress.\textsuperscript{25} The metabolism of 4-HAE at least for 4-HNE via glutathionylation is known to be enantioselective in living systems. For example, (S)-4-HNE is preferentially detoxified by GSTs catalyzed glutathionylation\textsuperscript{26} while (R)-4-HNE
is preferentially metabolized by NAD$^+$-dependent oxidation mediated by aldehyde dehydrogenase (ALDH).\textsuperscript{27}

In Chapter 2, we reported the catabolic fates of 4-HNE in perfused rat livers using a combination of metabolomics and mass isotopomer analysis.\textsuperscript{28,29} A key finding of this work was that 4-HNE catabolism can proceed \textit{via} two parallel pathways that involve (i) isomerization of 4-hydroxynonanoyl-CoA to 3-hydroxynonanoyl-CoA followed by 3 cycles of β-oxidation to form acetyl-CoA and propionyl-CoA; and (ii) β-oxidation/α-oxidation sequence of 4-hydroxynonanoyl-CoA resulting in the production of acetyl-CoA and formate. As β-oxidation is the major pathway for the catabolic degradation of 4-HNE, any intervention/interruption to this pathway can alter its metabolism resulting in the accumulation of free 4-HNE or other secondary metabolites. We became interested in understanding the interplay between the glutathionylation and catabolism of 4-HNE as well as the other 4-HAEs.

GSH can spontaneously react with 4-HNE, 4-HHE and possibly other 4-HAE even in absence of GSTs. Because of this, the GSH conjugates of 4-HNE and other 4-HAE are often overestimated as the non-enzymatic glutathionylation can continue between the released free GSH and free 4-HAE during the sample preparation. Previous GSH analyses showed that iodoacetic acid (IAA) can efficiently react with sulfhydryl group to stabilize GSH.\textsuperscript{30} Thus, IAA could be an option to prevent the reaction between free GSH and 4-HAE during sample processing, allowing an accurate estimate of the glutathionylation.
The purpose of this study was to develop a simple, rapid and sensitive analytical strategy to accurately measure endogenous GSH conjugates of 4-HAE enantiomers in rat organs. The effect of IAA was also investigated to prevent the artifactual data from the spontaneous chemical reaction between GSH and free 4-HAEs during sample preparation. The new method developed was validated and applied to measure and compare the endogenous level of GSH conjugates of 4-HAE enantiomers in various rat organs. Finally, rat livers were perfused with [5,5,6,6,7,7,8,8,9,9,9-\textsuperscript{2}H\textsubscript{11}]-4-HNE (d\textsubscript{11}-4-HNE) to investigate the extent of glutathionylation of exogenous 4-HNE, including its enantioselectivity and its release in the rat liver, by measuring the conjugates in the perfusate as well as rat liver tissues.

On the other hand, despite the physiologic importance 4-ONE, little is known about its catabolic fate.\textsuperscript{31,32} For example, carbonyl reductase has been reported to catalyze NADPH-dependent reduction of 4-ONE to 1-hydroxy non-2-en-4-one, 4-oxononanal, and 4-HNE (see Scheme 4.1).\textsuperscript{31} 4-ONE can also be oxidized by aldehyde dehydrogenase (human aldehyde dehydrogenase 2) to form 4-oxo-2-nonenoic acid (see Scheme 4.1). Again, GST can mediate conjugate formation, resulting in metabolism to the mercapturic acid conjugates. Given its physiologic consequences (e.g. protein polymerization, adduct formation etc.), 4-ONE should have an efficient detoxification/catabolism route to maintain its normal physiology. Consequently, understanding the mechanisms for elimination of 4-ONE is critical, as it may give key insights into the pathogenesis of the molecule. We synthesized stable isotopically (\textsuperscript{13}C) labeled 4-ONE to study the glutathionylation of 4-ONE in rat liver.
**SCHEME 4.1** (A) Reduction of 4-ONE by carbonyl reductase in presence of NADPH resulting 1-hydroxy non-2-en-4-one, 4-oxononanal, and 4-HNE. (B) Oxidation of 4-ONE by aldehyde dehydrogenase to form 4-oxo-2-nonenoic acid.
4.2 RESULTS AND DISCUSSION

4.2.1 STEREOCHEMICAL ASPECTS OF GLUTATHIONYLATION OF 4-HAEs

A simple and rapid LC-MS/MS method was developed to separate the GSH conjugates of 4-HNE enantiomers. The spontaneous reaction of racemic 4-HNE with free reduced GSH gives rise to four diastereomers as shown in Fig 4.2B. The LC-MS/MS chromatogram of 4-HNE–GSH conjugate contains three unresolved peaks. The (S)-4-HNE–GSH conjugate (peak III+IV in Fig 4.2C) comes in the middle of the two peaks that corresponds to the (R)-4-HNE–GSH conjugate (peak I and II in Fig 4.2C). These assignments were further confirmed by synthesizing GSH conjugates of the pure (R) and (S)-4-HNE and comparing the LC-MS/MS trace with that of the racemic 4-HNE as shown in Fig 4.3. All other 4-HAE–GSH conjugates showed the same pattern in the LC-MS/MS trace except for 4-hydroxy-2-(E)-pentenal which gave only two peaks (Fig 4.4).
Figure 4.2 (A) Structure of 4-hydroxy-2-(E)-alkenal (4-HAE) derivatives and their GSH conjugate where n = 0-7 are represented as C₅-C₁₂ (corresponds to the number of carbon in the 4-HAE) respectively in the text, (B) Four different diastereomers of 4-HAE–GSH conjugates. (C) LC-MS trace of the different diastereomers of 4-HAE–GSH conjugates.
FIGURE 4.3 LC-MS/MS trace of GSH conjugates of racemic, (R) and (S)-4-HNE.
**FIGURE 4.4** LC-MS/MS profile of 4-HAE–GSH conjugates. In the inset, the separation of GSH conjugate of (R) and (S)-4-HNE is shown.
4.2.2 Method Validation

An LC-MS/MS method using Multiple Reaction Monitoring (MRM) was developed for various 4-HAE–GSH isomers. Calibration curves were acquired in the MRM mode using the molecular weight of 4-HAE–GSH conjugate and the daughter ion of m/z 308 (protonated GSH). The product ion spectrum of 4-HNE–GSH that was in accord with literature\textsuperscript{23} is shown in Fig 4.5.

Calibration curves were plotted for the GSH conjugate of each individual 4-HAE enantiomer i.e. for (R)-4-HAE–GSH and (S)-4-HAE–GSH and were linear (R\textsuperscript{2} > 0.99) over the range from 1.28 pmol-1.25 nmol. LOQ and LOD data are enlisted in Table 4.1.

A recovery test was performed to assess the accuracy of the method. Three different concentrations (Low, Med and High) of 4-HAE–GSH conjugates were chosen for the recovery experiments. As summarized in Table 4.2, measured recoveries for different 4-HAE–GSH conjugates at different concentrations range from 81.8 % to 124.4 %.

The reproducibility of intra and inter-days measurements is indicated by standard deviations in Table 4.2. The relative standard deviations of all intra and inter-days assays are lower than 23% which demonstrates the fair precision of the present method for all 4-HAE–GSH at different concentrations.
Figure 4.5 Product ion spectrum of 4-HNE–GSH conjugate and the structures of the major fragments.23
Table 4.1 LC-MS/MS method performance parameters for 4-HAE–GSH conjugates.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Equation</th>
<th>Correlation coefficient</th>
<th>Correlation range</th>
<th>LOD (S/N&gt;3) (pmol)</th>
<th>LOQ (S/N&gt;10) (pmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C₅-R-GSH</td>
<td>$Y = 1.4X - 0.03$</td>
<td>0.9986</td>
<td>1.28 pmol-1.25 nmol</td>
<td>0.64</td>
<td>2.55</td>
</tr>
<tr>
<td>C₅-S-GSH</td>
<td>$Y = 1.4X + 0.01$</td>
<td>0.9977</td>
<td>1.28 pmol-1.25 nmol</td>
<td>0.64</td>
<td>2.55</td>
</tr>
<tr>
<td>C₆-R-GSH</td>
<td>$Y = 0.4X - 0.06$</td>
<td>0.9997</td>
<td>1.28 pmol-1.25 nmol</td>
<td>0.43</td>
<td>2.55</td>
</tr>
<tr>
<td>C₆-S-GSH</td>
<td>$Y = 0.6X - 0.02$</td>
<td>0.9936</td>
<td>1.28 pmol-1.25 nmol</td>
<td>0.43</td>
<td>2.55</td>
</tr>
<tr>
<td>C₇-R-GSH</td>
<td>$Y = 0.8X + 0.01$</td>
<td>0.9912</td>
<td>1.28 pmol-1.25 nmol</td>
<td>0.64</td>
<td>2.55</td>
</tr>
<tr>
<td>C₇-S-GSH</td>
<td>$Y = 1.8X - 0.02$</td>
<td>0.9996</td>
<td>1.28 pmol-1.25 nmol</td>
<td>0.64</td>
<td>2.55</td>
</tr>
<tr>
<td>C₈-R-GSH</td>
<td>$Y = 1.1X - 0.08$</td>
<td>0.9998</td>
<td>1.28 pmol-1.25 nmol</td>
<td>0.32</td>
<td>2.55</td>
</tr>
<tr>
<td>C₈-S-GSH</td>
<td>$Y = 1.6X - 0.04$</td>
<td>0.9939</td>
<td>1.28 pmol-1.25 nmol</td>
<td>0.32</td>
<td>2.55</td>
</tr>
<tr>
<td>C₉-R-GSH</td>
<td>$Y = 0.8X + 0.01$</td>
<td>0.9968</td>
<td>1.28 pmol-1.25 nmol</td>
<td>0.32</td>
<td>1.28</td>
</tr>
<tr>
<td>C₉-S-GSH</td>
<td>$Y = 1.5X + 0.02$</td>
<td>0.9981</td>
<td>1.28 pmol-1.25 nmol</td>
<td>0.32</td>
<td>1.28</td>
</tr>
<tr>
<td>C₁₀-R-GSH</td>
<td>$Y = 1.2X - 0.08$</td>
<td>0.9949</td>
<td>1.28 pmol-1.25 nmol</td>
<td>0.26</td>
<td>1.28</td>
</tr>
<tr>
<td>C₁₀-S-GSH</td>
<td>$Y = 2.0X - 0.05$</td>
<td>0.9975</td>
<td>1.28 pmol-1.25 nmol</td>
<td>0.26</td>
<td>1.28</td>
</tr>
<tr>
<td>C₁₁-R-GSH</td>
<td>$Y = 0.6X + 0.06$</td>
<td>0.9965</td>
<td>1.28 pmol-1.25 nmol</td>
<td>0.43</td>
<td>2.55</td>
</tr>
<tr>
<td>C₁₁-S-GSH</td>
<td>$Y = 0.9X + 0.05$</td>
<td>0.9969</td>
<td>1.28 pmol-1.25 nmol</td>
<td>0.43</td>
<td>2.55</td>
</tr>
<tr>
<td>C₁₂-R-GSH</td>
<td>$Y = 0.5X - 0.03$</td>
<td>0.9987</td>
<td>1.28 pmol-1.25 nmol</td>
<td>0.64</td>
<td>2.55</td>
</tr>
<tr>
<td>C₁₂-S-GSH</td>
<td>$Y = 0.6X - 0.02$</td>
<td>0.9973</td>
<td>1.28 pmol-1.25 nmol</td>
<td>0.64</td>
<td>2.55</td>
</tr>
</tbody>
</table>

Note: C₅-R-GSH and C₅-S-GSH represent the GSH conjugate of (R)-C₅ and (S)-C₅ respectively. Same nomenclature applies for all derivatives C₅-C₁₂. The calibration curves have been provided in the Appendix.
### Table 4.2 Recoveries of 4-HAE–GSH conjugates assayed by LC-MS/MS

<table>
<thead>
<tr>
<th>4-HAE–GSH (See Fig 4.2A)</th>
<th>Recovery (mean% ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1&lt;sup&gt;st&lt;/sup&gt; Day (n = 3)</td>
</tr>
<tr>
<td><strong>C&lt;sub&gt;5&lt;/sub&gt;-GSH</strong></td>
<td></td>
</tr>
<tr>
<td>Low (17 nM)</td>
<td>107.4 ± 7.8</td>
</tr>
<tr>
<td>Med (0.53 µM)</td>
<td>103.6 ± 19.5</td>
</tr>
<tr>
<td>High (4.17 µM)</td>
<td>100.6 ± 9.5</td>
</tr>
<tr>
<td>Low (8.5 nM)</td>
<td>104.0 ± 17.9</td>
</tr>
<tr>
<td><strong>C&lt;sub&gt;6&lt;/sub&gt;-GSH</strong></td>
<td></td>
</tr>
<tr>
<td>Med (0.53 µM)</td>
<td>115.4 ± 13.9</td>
</tr>
<tr>
<td>High (4.17 µM)</td>
<td>108.7 ± 3.2</td>
</tr>
<tr>
<td>Low (8.5 nM)</td>
<td>124.8 ± 22.9</td>
</tr>
<tr>
<td><strong>C&lt;sub&gt;7&lt;/sub&gt;-GSH</strong></td>
<td></td>
</tr>
<tr>
<td>Med (0.53 µM)</td>
<td>107.9 ± 14.5</td>
</tr>
<tr>
<td>High (4.17 µM)</td>
<td>112.0 ± 11.9</td>
</tr>
<tr>
<td>Low (17 nM)</td>
<td>116.8 ± 9.2</td>
</tr>
<tr>
<td><strong>C&lt;sub&gt;8&lt;/sub&gt;-GSH</strong></td>
<td></td>
</tr>
<tr>
<td>Med (0.53 µM)</td>
<td>109.9 ± 18.8</td>
</tr>
<tr>
<td>High (4.17 µM)</td>
<td>112.5 ± 6.7</td>
</tr>
<tr>
<td>Low (17 nM)</td>
<td>127.9 ± 21.6</td>
</tr>
<tr>
<td><strong>C&lt;sub&gt;9&lt;/sub&gt;-GSH</strong></td>
<td></td>
</tr>
<tr>
<td>Med (0.53 µM)</td>
<td>106.6 ± 14.8</td>
</tr>
<tr>
<td>High (4.17 µM)</td>
<td>86.5 ± 3.7</td>
</tr>
<tr>
<td>Low (8.5 nM)</td>
<td>84.7 ± 12.3</td>
</tr>
<tr>
<td><strong>C&lt;sub&gt;10&lt;/sub&gt;-GSH</strong></td>
<td></td>
</tr>
<tr>
<td>Med (0.53 µM)</td>
<td>97.9 ± 4.3</td>
</tr>
<tr>
<td>High (4.17 µM)</td>
<td>94.7 ± 3.1</td>
</tr>
<tr>
<td>Low (17 nM)</td>
<td>106.3 ± 5.8</td>
</tr>
<tr>
<td><strong>C&lt;sub&gt;11&lt;/sub&gt;-GSH</strong></td>
<td></td>
</tr>
<tr>
<td>Med (0.53 µM)</td>
<td>92.3 ± 11.1</td>
</tr>
<tr>
<td>High (4.17 µM)</td>
<td>94.1 ± 8.9</td>
</tr>
<tr>
<td>Low (17 nM)</td>
<td>91.9 ± 3.3</td>
</tr>
<tr>
<td><strong>C&lt;sub&gt;12&lt;/sub&gt;-GSH</strong></td>
<td></td>
</tr>
<tr>
<td>Med (0.53 µM)</td>
<td>84.8 ± 19.3</td>
</tr>
<tr>
<td>High (4.17 µM)</td>
<td>92.0 ± 14.9</td>
</tr>
</tbody>
</table>
4.2.3 **Effect of IAA and BHT in the Sample Preparation**

4-HNE can readily react with free reduced GSH to give the 4-HNE–GSH conjugate as a result of enzymatic or non-enzymatic (spontaneous) reaction which can produce erroneously elevated apparent values of the conjugates. In the sample preparation, we investigated the effect of iodoacetic acid (IAA) on the final assay results and found that the level of 4-HNE–GSH conjugate is much higher when IAA is not added prior to sample extraction. This higher amount of 4-HNE–GSH conjugate is attributed to the chemical reaction between free 4-HNE and reduced GSH released from tissues during the sample preparations. The presence of IAA traps and masks the free GSH and hence prevents any overestimated 4-HNE–GSH (as a result of *in vitro* chemical reaction) conjugates in the samples (Fig 4.6). Lipid peroxidation during the sample preparation was prevented by adding butylhydroxytoluene (BHT) as an antioxidant. In addition, it was verified that BHT has no effect on the stability of the conjugates (Fig 4.7).

One major concern about the IAA effect is whether 4-HAE–GSH conjugates are stable under the conditions of IAA reaction. We incubated 4-HAE–GSH conjugates with IAA under the same conditions (10 mM ammonium carbonate buffer, pH 9.8). No degradation of 4-HAE–GSH conjugates was observed. This excluded the possibility that the lower amount of 4-HAE–GSH conjugates detected when the isolation was performed in the presence of IAA compared to without IAA, comes from the degradation of GSH conjugates by IAA. The amounts of 4-HAE–GSH conjugates in various tissues were measured with and without IAA (Fig 4.8) and the results were analyzed as reported in the following sections.
**Figure 4.6** (A) Enzymatic or non-enzymatic glutathionylation of 4-HNE. (B) IAA traps and masks the nucleophilicity of GSH.

**Figure 4.7** Effect of BHT on GSH conjugate of (A) (R)-4-HNE and (B) (S)-4-HNE.
**Figure 4.8** GSH conjugates of (R) and (S)-4-HAE (C₅-C₁₂) in liver, heart and brain tissues from rat.
4.2.4 4-HAE–GSH CONJUGATE LEVELS IN RAT LIVER, BRAIN AND HEART SAMPLES

With this method, we profiled 4-HAE–GSH conjugates in rat liver, brain and heart for the first time. The data are shown in Fig 4.8. The following interesting findings were observed: (a) 4-HAEs (from C₅ to C₁₂) are present in all of these organs except the C₁₂ 4-HAE (n = 7, Fig 4.2) which is undetectable in the heart. Without IAA treatment, 4-HAE–GSH measured levels are more than 100 fold higher than those using isolation in the presence of IAA; (b) 4-HNE–GSH and 4-HHE–GSH are the two most abundant 4-HAE–GSH conjugates in all the organs under study with 4-HNE–GSH being the highest one. (c) There are no detectable 4-HAE–GSH conjugates (under IAA treatment) in any of the organs except the C₆, C₈ and C₉ 4-HAE–GSH conjugates. The distribution of C₆, C₈ and C₉ 4-HAE–GSH conjugate varies from organ to organ. Liver usually contains higher amounts of those three 4-HAE–GSH conjugates than heart or brain. (d) The effects of IAA on the measured 4-HNE–GSH diastereomers in various organs are also different. However, there is no difference between (R) and (S)-4-HNE–GSH levels when no IAA is added for sample isolation. The GSH conjugates of the (R) enantiomer of C₆ (4-HHE) and C₉ (4-HNE) are greater than their corresponding (S)- antipodes in the livers extracted in the presence of IAA (see Fig 4.8, C₆-GSH and C₉-GSH). There is no significant difference in the amounts of glutathionylated products derived from the enantiomers of C₆ and C₉ in the brain tissue with or without using IAA during sample isolation (see Fig 4.8, C₆-GSH and C₉-GSH). The actual 4-HAE–GSH concentrations for the derivatives other than C₆ and C₉ are too small to measure when IAA was used to prevent the generation of artifactual products (except for a trace amount of C₈-GSH).
4-HNE and 4-HHE are most abundant lipid peroxidation products, generated from (ω-6) and (ω-3) PUFA respectively. They have been correlated in many disease states. A number of bio-macromolecules such as proteins, enzymes, DNA and RNA base pairs are the prime targets of 4-HNE and 4-HHE. After modification they often partly or completely lose their activity. GSTs mediated conjugation of GSH with those reactive alkenals is considered to be their major metabolic pathway. Apart from GSTs, there are at least two other enzymes which play a key role in the 4-HNE metabolism, namely, aldehyde dehydrogenase (ALDH) and alcohol dehydrogenase (ADH). The aldehyde group of 4-HNE is oxidized by ALDH\textsuperscript{33} to 4-hydroxynonenoic acid (4-HNA), which is metabolized by hepatocytes through α-oxidation, or reduced by ADH\textsuperscript{34,35} resulting 1,4-dihydroxynonene (DHN).\textsuperscript{36,37} Both the DHN and 4-HNA eventually need to be converted into their corresponding mercapturic acid derivatives before they can be excreted into urine. In the previous chapters, we reported the catabolic fate of 4-HNE in perfused rat livers using a combination of metabolomics and mass isotopomer analysis. A key finding of that work was that 4-HNE catabolism can proceed via two parallel pathways that involve (i) isomerization of 4-hydroxynonanoyl-CoA to 3-hydroxynonanoyl-CoA followed by 3 cycles of β-oxidation to form acetyl-CoA and propionyl-CoA; and (ii) β-oxidation/α-oxidation sequence of 4-hydroxynonanoyl-CoA resulting in the production of acetyl-CoA and formate.

Hence, from the above discussions it is clear that there is more than one possibility to detoxify/metabolize 4-HNE or other 4-HAEs, and the contribution of each pathway varies depending on the tissue type. Glutathionylated 4-HNE has been considered as a stable biomarker for decades.\textsuperscript{23} Therefore; many methods were developed
for 4-HNE–GSH assay. Warnke et al. developed an LC-MS/MS method for 4-HNE–GSH, 4-HNE and 4-HNE analogues.\textsuperscript{38} Orioli et al. analyzed 4-HNE–GSH and other 4-HNE conjugates in skeletal muscles.\textsuperscript{39} Völkel et al. assessed the 4-HNE–GSH as a biomarker in oxidative stress in hepatic cells and liver tissues.\textsuperscript{23} However, the reported 4-HNE–GSH analyses overlooked the vital issue that free 4-HNE and GSH can spontaneously (non-enzymatically) react to form the conjugates during sample isolation if no measures are taken to impair their reactivity. We found that IAA prevents the spontaneous reaction of 4-HNE with GSH by trapping nucleophilic thiol of GSH (Fig 4.6B). The actual level of 4-HNE–GSH in rat liver tissue is a hundred fold less than that measured in the absence of IAA, if the spontaneous reaction of 4-HNE with free cellular GSH is prevented during the sample isolation as shown in the case of the C\textsubscript{9}-GSH conjugate (Fig 4.8). 4-HNE–GSH concentrations in liver tissues were reported to be 12 nmol/g by Warnke et al.\textsuperscript{38} and 20 nmol/g by Volkel and co-workers.\textsuperscript{23} Their measured 4-HNE–GSH concentrations obviously are in the same range of ours when no IAA was used during sample isolation (Fig 4.5). Thus, the net 4-HNE–GSH concentration is the combination of endogenous 4-HNE–GSH and artifactual 4-HNE–GSH (generated exogenously from free 4-HNE and cellular GSH during sample isolation) if no IAA treatment is carried out during the sample preparation. 4-HNE–GSH formed during the sample preparation was the majority detected in those studies (over 99%) according to our data. The measured 4-HNE–GSH levels could vary considerably with sample preparation time as the completion of the spontaneous reaction of 4-HNE and GSH is time dependent. Moreover, 4-HNE as a stable oxidative stress biomarker is not ideal for the following reasons: (i) 4-HNE is a reactive compound, and (ii) 4-HNE is in a steady-
state system and its level cannot be determined only by its production via oxidative stress induced lipid peroxidation but also by its metabolic downstream, such as detoxification and catabolism. However, at a minimum, the use of IAA during sample isolation excludes the interferences of free 4-HNE and provides an approach to determine an accurate endogenous 4-HNE–GSH concentration.

A general method to profile endogenous 4-HAE–GSH conjugate levels was presented in this work. 4-HHE (C₆) and 4-HNE (C₉) are well known reactive end-products derived from the peroxidation of (ω-3) and (ω-6) PUFAs. Other 4-HAE and their GSH conjugates are much less known in the literature. Our 4-HAE–GSH conjugate profiling studies without the presence of IAA during the sample isolation surprisingly seemed to show that all 4-HAE (C₅ to C₁₂) can be found in different rat organs (liver, heart and brain). 4-HNE is the most abundant 4-HAE followed by 4-HHE. Non-enzymatic 4-HAE–GSH formation is not an enantioselective reaction. This is indicated from our 4-HAE–GSH data for those samples which were isolated without IAA (Fig 4.8). In contrast, the GSH conjugates of (R) enantiomers of C₆ and C₉ (4-HHE and 4-HNE respectively) were found in higher amount than the corresponding antipode in the rat liver and heart when IAA was present during sample isolation. However, the glutathionylated adduct of (R) and (S) 4-HHE and 4-HNE are not significantly different in brain even with IAA present during sample isolation. A similar finding was also reported by Honzatko et al. stating that 4-HNE glutathionylation is not enantioselective in the brain.⁴⁰ Other 4-HAE–GSH conjugates in the samples were too low to allow conclusions in the present work. Two possible explanations for the different concentrations of 4-HNE–GSH and 4-HHE–GSH diastereomers we detected are: (i)
enzymatic glutathionylation is enantioselective, or (ii) the transporters of 4-HNE–GSH and 4-HHE–GSH are enantioselective. The 4-HNE enzymatic glutathionylation is catalyzed mainly by glutathione S-transferase A4-4 (GSTA4-4) which has negligible stereoselectivity toward 4-HNE enantiomers.\textsuperscript{41} On the other hand, while the 4-HNE–GSH conjugate transporter is known to be ATP dependent, nothing is known about its enantio or diastero-selectivity.\textsuperscript{42} Thus, the stereoselectivity of 4-HNE–GSH conjugates is not clearly understood and requires more study.
4.2.5 Liver Perfusion Experiment with $d_{11}$-4-HNE

Although lipid peroxidation generates both enantiomers of 4-HNE, GST A4-4 shows a substrate selectivity towards the (S)-4-HNE.\textsuperscript{26} In the perfused rat liver tissue we found a higher amount of (S)-4-HNE–GSH conjugate while the exact opposite was found in the perfusate where the (R)-4-HNE–GSH is predominant (Fig 4.9). The 4-HNE–GSH conjugate can form in different organs and tissues including liver. The further metabolism of the released 4-HNE–GSH conjugate occurs in the kidney where it can be oxidized to 4-HNE-mercapturic acid conjugates and secreted into urine. We found that the release of (S)-4-HNE–GSH into perfusate by liver is around 6 fold higher than (R)-4-HNE–GSH. This possibly is attributable to the selectivity of GSTs for (S)-4-HNE and/or the enantiomeric selectivity of the 4-HNE–GSH conjugate transporter for the (S)-4-HNE–GSH conjugate. Chemical reaction of GSH with 4-HNE is very fast but not as fast as the GSTs mediated enzymatic reaction. 4-HNE is detoxified (S)-preferentially by GSH conjugation mediated by GSTs\textsuperscript{26} and this will result in the accumulation of (R)-4-HNE. Non-enzymatic glutathionylation can form (R)-4-HNE–GSH conjugate in lower amounts. This explains the lower abundance of the (R)-4-HNE–GSH in biological samples. (R)-4-HNE–GSH was accumulated in the perfused rat liver tissue probably because of its lower efficiency of transport out of liver cells compared to (S)-4-HNE–GSH conjugate. (R)-4-HNE could be more harmful to the liver based on our $d_{11}$-4-HNE liver perfusion as we observed the greater accumulation of the (R)-4-HNE–GSH conjugate than the (S)-4-HNE–GSH conjugate.

In the effluent of perfusates with 1 to 50 $\mu$M $d_{11}$-4-HNE, we could not find any trace of free $d_{11}$-4-HNE. This suggested the fast and complete uptake of 1-50 $\mu$M $d_{11}$-4-
HNE by rat livers (see Fig 4.10A). Such fast uptake of \(d_{11}-4\)-HNE indicates the zonation of 4-HNE metabolism in rat liver.\(^{43}\) However, compared to uptake of \(d_{11}-4\)-HNE by the perfused liver, only 1.8 % of \(d_{11}-4\)-HNE formed the conjugate with GSH. This strongly suggests that catabolism of 4-HNE is the major metabolic pathway of 4-HNE in the liver.\(^{28,29}\) The catabolism of \(d_{11}-4\)-HNE can be demonstrated by acyl-CoA profiles (see Fig 4.10B).\(^{29}\) The level of \(d_{11}-4\)-phospho-nonanoyl-CoA, \(d_{11}-4\)-hydroxy-nonanoyl-CoA, \(d_{11}\)-heptanoyl-CoA and \(d_{11}-2\)-hydroxy-heptanoyl-CoA in the rat liver tissues increased with increasing amount of \(d_{11}-4\)-HNE (Fig 4.10B). Propionyl-CoA was substantially labeled at M3 (from 1.3 to 14%) and M5 (from 1 to 23%) from 1 to 50 \(\mu M\) \(d_{11}-4\)-HNE (Fig 4.10C). DHN that is reduced from 4-HNE by aldehyde reductase was even in less amount compared to the GSH conjugates.

4-HNE metabolism in liver has been extensively investigated in our lab and others.\(^{7,27-29,44}\) Our previous work revealed two parallel catabolic pathways of 4-HNE in rat livers.\(^{29}\) In the present work, we investigated the enantioselective formation of 4-HNE–GSH in the rat liver by perfusing with racemic \(d_{11}-4\)-HNE. Our results showed that (\(S\))-4-HNE–GSH released into perfusate was 6 fold higher than the (\(R\))-4-HNE–GSH. This is probably due to the diastereoselectivity of the 4-HNE–GSH transporter in rat liver. The presence of a higher amount (3 fold) of (\(S\))-4-HNE–GSH than (\(R\))-4-HNE–GSH in the rat liver tissue further confirms our proposed mechanism.
**Figure 4.9** Diastereomeric contents of (R) and (S)-d<sub>11</sub>-4-HNE–GSH conjugates in the (A) perfused rat liver tissue, and (B) perfusate.
**FIGURE 4.10** (A) Uptake of $d_{11}$-4-HNE by rat liver. (B) Acyl-CoA profile of $d_{11}$-4-HNE catabolism in rat liver. (C) Mass isotopomer enrichment of propionyl-CoA on $d_{11}$-4-HNE perfusion in rat liver.
4.2.6 Liver Perfusion Experiment with [3,4-13C₂]-4-ONE

We perfused rat livers with recirculating perfusate to which 0-100 µM [3,4-13C₂]-4-ONE was added at zero time. In the perfusate collected after 20 min, we identified the M2 4-ONE–GSH, M2 4-HNE–GSH and another new M2 labeled glutathionylated adduct with m/z of 464.2067. Additional tandem mass analysis reveals that the latter adduct corresponds to M2 1-hydroxy-4-oxononane–GSH conjugate. The concentrations of 4-ONE–GSH, 4-HNE–GSH and 1-hydroxy-4-oxononane–GSH get increased with increasing concentrations of 4-ONE (Fig 4.11). Among the three GSH conjugates, 1-hydroxy-4-oxononane-GSH is produced in the highest amount which suggests that the C-1 of 4-ONE first gets reduced by some alcohol dehydrogenase to form 1-hydroxy-4-oxononene (Fig 4.11C).

4-ONE also gets reduced to 4-HNE via the reduction of C-4. We were interested to explore the enantioselectivity of the glutathionylation of 4-HNE derived from 4-ONE. When livers were perfused with racemic $d_{11}$-4-HNE, (S)-4-HNE–GSH was preferentially released into perfusate over (R)-4-HNE–GSH as shown in Fig 4.9. But when [3,4-13C₂]-4-ONE was perfused in the liver, we found the opposite result. In case of 4-ONE perfused liver, we found a greater amount of (R)-4-HNE–GSH released into perfusate than (S)-4-HNE–GSH as shown in Fig 4.12. Taken together, these data suggest that compartmentalization of glutathionylated 4-HNE is enantioselective and this enantioselectivity also depends upon the source of 4-HNE.
**Figure 4.11** Concentrations of (A) [3,4-\(^{13}\)C\(_2\)]-4-ONE–GSH (B) [3,4-\(^{13}\)C\(_2\)]-4-HNE–GSH and (C) [3,4-\(^{13}\)C\(_2\)]-1-hydroxy-4-oxononene–GSH in the perfusate of rat livers perfused with various concentration of [3,4-\(^{13}\)C\(_2\)]-4-ONE.
**Figure 4.12** Diastereomeric contents of M2-(R) and -(S)-4-HNE–GSH conjugates in the perfusate.
4.3 EXPERIMENTAL SECTIONS

4.3.1 MATERIALS AND METHODS

General chemicals, solvents, reduced glutathione (GSH), iodoacetic acid (IAA), butylhydroxytoluene (BHT) were purchased from Sigma-Aldrich. All the standard solutions and buffers were prepared in freshly distilled Milli-Q water. Ethyl bromoacetate-$^{13}$C$_2$ was purchased from Isotec, Sigma-Aldrich. Triphenylphosphine (99%), valeraldehyde (97%), (ethoxycarbonylmethylene)triphenylphosphorane (+98%) were purchased from Acros Organics. 2-Pentylfuran was purchased from TCI America. Flash chromatography\footnote{Flash chromatography was performed on silica gel (230-400 mesh) purchased from Dynamics Adsorbents (Atlanta, GA). TLC was done on Hard Layer, Organic Binder TLC-plates with a fluorescent indicator and visualized by UV light (254 nm) purchased from Dynamics Adsorbents (Atlanta, GA). Solvents and reagents were of commercially available analytical grade quality and used as received without any further purification. 1H and 13C-NMR spectra were recorded on a Varian Inova spectrometer (at the Department of Chemistry, Case Western Reserve University) operating at 400 MHz and 100 MHz for the 1H and 13C-NMR spectra, respectively. The internal references were TMS (δ 0.00) and CDCl$_3$ (δ 77.2) for 1H and 13C spectra, respectively. NMR data are presented in the following order: chemical shift, peak multiplicity (b = broad, s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, dd = doublet of doublet, ddd = doublet of doublet of doublet, ddt = doublet of doublet of triplet, dq = doublet of quartet, dm = doublet of multiplet, br = broad), coupling constant, proton number. Mass spectra were obtained either on a Kratos MS 25 Mass Spectrometer (at the Department of Chemistry, Case Western Reserve University) using EI ionization method.} was performed on silica gel (230-400 mesh) purchased from Dynamics Adsorbents (Atlanta, GA). TLC was done on Hard Layer, Organic Binder TLC-plates with a fluorescent indicator and visualized by UV light (254 nm) purchased from Dynamics Adsorbents (Atlanta, GA). Solvents and reagents were of commercially available analytical grade quality and used as received without any further purification. 1H and 13C-NMR spectra were recorded on a Varian Inova spectrometer (at the Department of Chemistry, Case Western Reserve University) operating at 400 MHz and 100 MHz for the 1H and 13C-NMR spectra, respectively. The internal references were TMS (δ 0.00) and CDCl$_3$ (δ 77.2) for 1H and 13C spectra, respectively. NMR data are presented in the following order: chemical shift, peak multiplicity (b = broad, s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, dd = doublet of doublet, ddd = doublet of doublet of doublet, ddt = doublet of doublet of triplet, dq = doublet of quartet, dm = doublet of multiplet, br = broad), coupling constant, proton number. Mass spectra were obtained either on a Kratos MS 25 Mass Spectrometer (at the Department of Chemistry, Case Western Reserve University) using EI ionization method.
4.3.2 SYNTHESIS OF 4-OXO-2-(E)-NONENAL (4-ONE)

4-ONE was synthesized following the literature procedure. In brief, 2-pentylfuran (1, 2.5 g, 18 mmol) was dissolved in 150 mL dichloromethane. Then, pyridinium chlorochromate (20.7 g, 95 mmol) was added slowly with stirring. The reaction was continued overnight at room temperature. The mixture was then refluxed for 12 h. The reaction mixture was cooled and diluted with 150 mL ether and filtered through a celite bed. The combined organic layers were dried over Na₂SO₄, concentrated in vacuo, and the crude product purified by column chromatography over silica (hexane, 20% ethyl acetate) to give pure 4-ONE 2 as an oil (1.67 g, 10.8 mmol, 60% yield). ¹H NMR (400 MHz, CDCl₃): δ 0.89 (t, 3H, J = 6.8 Hz), 1.21-1.42 (4H), 1.54-1.71 (2H), 2.66 (t, 2H, J = 7.2 Hz), 6.79 (dd, 1H, J = 15.8 Hz, 6.6 Hz), 6.86 (d, 1H, J = 15.8 Hz), 9.79 (d, 1H, J = 6.6 Hz); ¹³C NMR (100 MHz, CDCl₃): δ 14.1, 22.6, 23.7, 31.5, 41.4, 137.5, 145.2, 193.7, 200.4. EI-HRMS (positive mode): m/z Calcd. for C₉H₁₅O₂ [MH⁺] 154.0994, found 154.0989.

4.3.3 SYNTHESIS OF 4-OXO-2-(E)-[3,4-¹³C₂]NONENAL ([3,4-¹³C₂]-4-ONE)

[3,4-¹³C₂]-(E)-Non-2-ene-1,4-diol (4)
The synthesis of $^{13}$C$_2$ labeled 4-ONE was started from ethyl (E)-4-hydroxy-[3,4-$^{13}$C$_2$]non-2-enoate (3). The synthesis of 3 was described in Chapter 2. Ethyl (E)-4-hydroxy-[3,4-$^{13}$C$_2$]non-2-enoate (3, 0.6 g, 2.96 mmol) was dissolved in 50 mL of dry dichloromethane and 7.5 mL of DIBAL (1.2 M solution in Toluene, 9 mmol) was added dropwise at 0 °C with continuous stirring. After 2 h the reaction mixture was allowed to warm to room temperature for 5 min and 1M HCl was carefully added until the pH = 3. The aqueous phase was extracted with dichloromethane (3 × 20 mL). The combined organic layers were dried over Na$_2$SO$_4$, concentrated in vacuo, and the crude product purified by column chromatography over silica (hexane, 50% ethyl acetate) to give 4 (0.43 g, 2.69 mmol, 90.9%). $^1$H NMR (400 MHz, CDCl$_3$): δ 0.89 (t, 3H, $J$ = 6.8 Hz), 1.28-1.82 (10H), 4.13 (dm, 1H, $J$ = 143.2 Hz), 4.15-4.19 (2H), 5.52-5.97 (2H); $^{13}$C NMR (100 MHz, CDCl$_3$): δ 14.1, 22.7, 25.2, 32.2, 37.9, 60.8, 72.6 (d, $J_{C-C}$ = 46.7 Hz), 130.2, 134.7 (d, $J_{C-C}$ = 46.7 Hz).

4-Oxo-2-(E)-[3,4-$^{13}$C$_2$]Nonenal (5)

At room temperature, 0.4 g (2.5 mmol) of [3,4-$^{13}$C$_2$]-(E)-non-2-ene-1,4-diol 4 was dissolved in 20 mL of dichloromethane and 21 mL of Dess-Martin periodinane (0.3 M solution in CH$_2$Cl$_2$, 6.3 mmol). The mixture was stirred for 2 h at room temperature and 45 mL of Et$_2$O and 24 mL of 1.3 M NaOH were added with vigorous stirring for 1 min. The aqueous NaOH layer was removed and an additional 30 mL of 1.3 M NaOH was added with vigorous stirring for 15 min. The aqueous layer was removed and the organic
phase washed with brine solution. The organic layer was dried over Na$_2$SO$_4$, and the solvent removed in vacuo to obtain the crude 4-oxo-2-(E)-[3,4-$^{13}$C$_2$]nonenal (5), and purified by column chromatography over silica (hexane, 20% ethyl acetate) to give pure 5 (0.27 g, 1.73 mmol, 69.2% yield). $^1$H NMR (400 MHz, CDCl$_3$): δ 0.90 (t, 3H, $J$ = 6.8 Hz), 1.20-1.44 (4H), 1.51-1.73 (2H), 2.69 (td, 2H, $J$ = 7.6, 5.6 Hz), 6.75-6.83 (1H), 6.87 (ddd, 1H, $J$ = 160.8, 16, 2.4 Hz), 9.79 (dd, 1H, $J$ = 7.6, 0.8 Hz); $^{13}$C NMR (100 MHz, CDCl$_3$): δ 14.1, 22.6, 23.7, 31.5, 41.4 (d, $J_{C-C}$ = 42 Hz), 137.4, 145.2 (d, $J_{C-C}$ = 49.8 Hz), 193.7, 200.4 (d, $J_{C-C}$ = 49.8 Hz). EI-HRMS (positive mode): $m/z$ Calcd. for C$_{7}^{13}$C$_2$H$_{15}$O$_2$ [MH$^+$] 157.1139, found 157.1140.

4.3.4 Synthesis of Standard 4-HAE Series

4-Hydroxy-2-(E)-alkenal (4-HAE) derivatives (Fig 4.2, n = 0-7; C$_5$-C$_{12}$) were prepared by the methods reported in Chapter 5. $d_{11}$-4-HNE was prepared following a procedure reported by our group$^{47}$ as for 4-HNE, replacing $n$-C$_5$H$_{11}$Br with its perdeuterated version. (R) and (S) 4-HNE were synthesized following the modified literature procedure starting from commercially available oct-2-(E)-en-1-ol.$^{48}$ The purity of the synthesized compounds was verified by gas chromatography mass spectrometry, high resolution mass spectroscopy and NMR.

4.3.5 Preparation of 4-HAE–GSH Conjugates

4-HAE–GSH conjugates were synthesized as described previously with minor modifications.$^{49}$ 4-HAE–GSH conjugates (100 µM) were prepared by incubating 4-HAE with 1.1 equivalent of reduced GSH in Milli-Q water at 4 °C overnight. The conjugates were then analyzed by GC-MS and LC-MS/MS to make sure that no free 4-HAE is
present. Similarly 2 µM internal standard ($d_{11}$-4-HNE–GSH conjugate) was prepared using the $d_{11}$-4-HNE.

### 4.3.6 Liver Perfusion Experiments

All rats were kept on a 12 h light/dark cycle with ad libitum access to food and water. All experiments were performed in accordance with the Institutional Animal Care and Use Committee (IACUC) at Case Western Reserve University. Livers from overnight fasted male Sprague-Dawley rats (160-180 g) were perfused with oxygenated Krebs-Ringer bicarbonate buffer containing 4 mM glucose in non-recirculating perfusions.\textsuperscript{29,50} The flow rate of perfusate was 30 mL/min. After equilibration, $d_{11}$-4-HNE was added to the perfusate to reach final concentration from 0 to 50 µM. Inflow perfusate and the perfusate coming out of the liver at 18-19 min were collected and quick frozen. Livers were quick-frozen in liquid nitrogen at the end of the experiments (20 minutes perfusion).

### 4.3.7 Sample Collections

Control liver, brain and heart were collected from male Sprague-Dawley rats (160-180 g) following the regular laboratory procedure and stored at −80 °C until the analysis.

### 4.3.8 Sample Preparations

Powdered frozen control organs (~250 mg), spiked with 0.1 nmol of $d_{11}$-4-HNE–GSH conjugate as internal standard, was homogenized for 2 min with 2 mL of 10 mM ammonium bicarbonate buffer pH 9.8 with or without IAA (10 mM), and 4 mL of acetonitrile and 2 mL of chloroform using a Polytron homogenizer. Then the homogenate was centrifuged for 30 min at 4 °C at 800 ×g and the aqueous part was dried with
nitrogen gas and stored at −80 °C until LC-MS/MS analysis. The dried residue was then dissolved in 100 µl Milli-Q water and analyzed by LC-MS/MS.

### 4.3.9 Method Validation

The biological matrix sample was prepared by homogenizing 100 mg of powdered frozen liver, followed by the extraction for 2 min with 2 mL of 10 mM ammonium bicarbonate buffer pH 9.8 with 10 mM IAA, 4 ml of acetonitrile and 2 mL of chloroform using a Polytron homogenizer. Then the homogenate was centrifuged for 30 min at 4 °C at 800 ×g and the supernatant was dried with nitrogen gas. The dried residue was dissolved in 500 µl Milli-Q-water and 2 mL of acetonitrile to precipitate any remaining protein. The sample was again centrifuged for 30 min at 4 °C at 800 ×g and the aqueous part was dried with nitrogen gas and redissolved in 4 mL of Milli-Q-water and stored as the biological matrix at −80 °C until further analysis. For the recovery test, 100 µl of the biological matrix was taken and spiked with 0.1 nmol of $d_{11}$-4-HNE–GSH conjugate as internal standard. Then various known amounts of the standard 4-HAE–GSH mixtures were added. Low concentrations of 4-HAE–GSH were in the range of 0.53 to 17 nM, 0.53 µM as middle concentrations and high concentrations were 4.17 µM. Recovery of various concentrations of 4-HAE–GSH was measured to validate the accuracy of the method. All these experiments were repeated 3 times within one day and 3 times on different days so that the precision of method can be assessed. A calibration curve was obtained by serial dilution of a working solution of standard 4-HAE–GSH. The limit of detection (LOD), and limit of quantitation (LOQ) for each 4-HAE–GSH were characterized based on the ratio of S/N of 3 and 10, respectively.
4.3.10 LC-MS/MS FOR 4-HAE–GSH CONJUGATE ASSAY

After dissolving the dried sample in 100 µl of Milli-Q water, 40 µl were injected on a Thermo Scientific Hypersil GOLD C18 column (150 × 2.1 mm), protected by a guard column (Hypersil GOLD C18 5 µm, 10 × 2.1 mm), in an Agilent 1100 liquid chromatograph. The chromatogram was developed at 0.2 ml/min (i) from 0 to 25 min with a 1-45% gradient of buffer B (95% acetonitrile, 2% water and 0.25% formic acid) in buffer A (98% water, 2% acetonitrile and 0.25% formic acid), (ii) from 25 to 26 min with a 45-90% gradient of buffer B in buffer A, (iii) from 26 to 31 min with 90% buffer B in buffer A, (iv) from 31 to 32 min with a 90-1% gradient of buffer B in buffer A and (v) for 10 min of stabilization with 99% buffer A before the next injection.

The liquid chromatograph was coupled to a 4000 QTrap mass spectrometer (Applied Biosystems, Foster City, CA) operated under positive ionization mode with the following source settings: turbo-ion-spray source at 600 °C under N₂ nebulization at 65 p.s.i., N₂ heater gas at 55 p.s.i., curtain gas at 30 p.s.i., collision-activated dissociation gas pressure held at high, turbo ion-spray voltage at 5,500 V, declustering potential at 90 V, entrance potential at 10 V, collision energy at 50 V, and collision cell exit potential at 10 V. The Analyst software (version 1.4.2; Applied Biosystems) was used for data collection and processing.

Data acquisition was performed in multiple reaction monitoring (MRM) mode monitoring the transition of [M+H]⁺ m/z 464 in Q1 to [MH₂−156]⁺ m/z 308 (protonated GSH) in Q3 and of [M+H]⁺ m/z 464 in Q1 to [MH₂−156−129]⁺ m/z 179 (protonated GSH–pyroglutamic acid) in Q3 as qualifier for 4-HNE–GSH conjugates. Similarly, the
data acquisition were performed for other 4-HAE–GSH conjugates by changing the corresponding [M+H]+ m/z values.

### 4.3.11 Statistical Analysis

The results are presented as mean values ± standard deviation from five samples. The statistical significance of difference between groups was determined by student’s t test. The level of significance was set at $p<0.05$.

### 4.4 Conclusions

The 4-HAE–GSH conjugates in rat liver, heart and brain were profiled with the present developed method. This work demonstrated the presence of all 4-HAE from C$_5$ to C$_{12}$ and several detectable 4-HAE–GSH conjugates (C$_6$, C$_8$ and C$_9$) in these organs. The C$_9$ and C$_6$ 4-HAE–GSH conjugates are the most abundant. In exogenous 4-HNE perfused rat liver, (S)-4-HNE–GSH was released from the liver; (R)-4-HNE–GSH was accumulated in the liver tissue. When the 4-HNE is derived from 4-ONE (in case of the 4-ONE perfused liver), we found a greater amount of (R)-4-HNE–GSH than (S)-4-HNE–GSH. This suggests that the enantioselectivity of glutathionylation depends on the source of 4-HNE. In our study, we found glutathionylation to be a very small portion of the disposal pathway for exogenous 4-HNE by rat liver, and the transporters of 4-HNE–GSH and 4-HHE–GSH are enantioselective. The liver perfusion experiment with $d_{11}$-4-HNE suggests that catabolism of 4-HNE forming acetyl-CoA and propionyl-CoA and entering into citric acid cycle is probably the major disposal pathway for 4-HNE at least in the rat liver.
4.5 ACKNOWLEDGEMENT

All the animal experiments were performed in Prof. Brunengraber’s lab (Department of Nutrition, Case Western Reserve University).
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CHAPTER 5

Identification of a Negative Feedback Loop in Biological Oxidant Formation Regulated by 4-Hydroxy-2-(E)-Nonenal (4-HNE)
5.1 Introduction

Polyunsaturated fatty acids (PUFAs) may undergo both enzymatic and non-enzymatic lipid peroxidation (LPO) leading to unsaturated lipid hydroperoxides. The enzymatic processes are typically mediated by members of the lipoxygenase family and lead to the formation of a family of physiologic mediators of inflammation, such as leukotrienes and prostaglandins.\textsuperscript{1,2} The non-enzymatic peroxidation is an altogether different and much less understood process whereby PUFAs, under conditions of oxidative stress, can spontaneously form lipid peroxides at the allylic (or doubly allylic) positions of a number of physiologic lipids.\textsuperscript{3,4} These lipid peroxides can subsequently undergo a variety of secondary reactions, some leading to stable oxygenated and polyoxygenated acyl chains, while others lead to chain cleavage and production of metabolites containing either the methyl or carboxy terminus. \textit{4-Hydroxy-2-\textsuperscript{(E)}-nonenal} (4-HNE) is the most studied lipid peroxidation product, and has generally become accepted as a contributor to the pathogenesis of multiple disease states, including Alzheimer’s disease,\textsuperscript{5,6} atherosclerosis,\textsuperscript{7,9} and cancer.\textsuperscript{10} Generally speaking, the pathogenicity of lipid peroxidation products has been rationalized through the formation adducts with nucleophilic sites on proteins and DNA.\textsuperscript{11,12} Recently, though, the viewpoint regarding 4-HNE has evolved to appreciate the complex physiology and signaling aspects of this LPO product.\textsuperscript{13-15}

Phagocytic monocytes, such as neutrophils and macrophages initiate a massive consumption of oxygen upon exposure to either microbial signals and/or inflammatory mediators such as platelet activating factor, tumor necrosis factor, and the interleukins. This process is typically referred to as macrophage activation (this process via the nuclear
The factor-kappaB (NF-κB) pathway is shown in Fig 5.1, and serves as one of the most prolific localized sources of reactive oxygen species (ROS) in vivo. This oxygen consumption is linked to the NADPH oxidase complex that is organized upon macrophage activation. Thus, superoxide and hydrogen peroxide (H$_2$O$_2$) are generated by a variety of oxidoreductases via a leak of electrons to molecular oxygen from the mitochondrial electron transport chain, redox cycling of quinones and other auto-oxidation reactions followed by the secondary generation of H$_2$O$_2$, hypochlorous acid (HOCl), hydroxyl radical (•OH) and singlet oxygen (¹O$_2$) via enzymes like myloperoxidase. Together, these ROS participate along with non-oxidative mechanisms in killing the invading pathogens.

In parallel, reactive nitrogen species (RNS) are a family of biological oxidants that derive from nitric oxide (NO). The latter is produced via the action of a family of heme containing proteins referred to as the nitric oxide synthases (NOSs). NOS mainly exists in three isoforms (inducible or iNOS, endothelial or eNOS, and neuronal or n-NOS) and is comprised of an N-terminal oxygenase domain containing cysteine-ligated heme and tetrahydrobiopterin cofactors, a multi-domain C-terminal reductase domain that binds flavin mononucleotide and flavin adenine dinucleotide, and an intervening calmodulin binding region. The mechanism of NOSs to form NO is shown in Scheme 5.1. NO is biosynthesized via the 5-electron oxidation of the amino acid L-arginine by those NOS isoforms. A mechanistic description of NO biosynthesis is beyond the scope of this thesis but can be found in the extensive literature on the subject.
SCHEME 5.1 Conversion of L-arginine to citrulline via $N^{\omega}$-hydroxy-L-arginine (NHA) to produce nitric oxide. In that reaction, 2 mole of $O_2$ and 1.5 mole of NADPH are consumed to produce one mole of NO.

Although it has been implied, there is no direct evidence that RNS have the ability to catalyze the formation of LPO products, two key publications from the Hazen laboratory make a very compelling argument though.$^{31,32}$ First, Schmitt et al. showed that activation of human neutrophils in media containing nitrite and myeloperoxidase generated an oxidant that was capable of initiating LPO.$^{31}$ Second, Abu-Soud et al. was able to demonstrate that the mammalian myeloperoxidase superfamily family is able to use nitric oxide directly as a substrate to generate a host of oxidants that could potentially initiate LPO using a nitric oxide selective electrode.$^{32}$ Taken together, these two reports suggest that phagocytic monocytes may utilize myeloperoxidase in conjunction with RNS as a physiological pathway for the initiation of LPO in vivo. An important argument is that the localized generation of ROS/RNS is higher in an activated macrophage than any
other physiologic tissue. Consequently, lipid peroxidation in the macrophage will be disproportionately higher as compared to other tissue types.

4-HNE has generally become accepted as a contributor to numerous cellular functions and the pathogenesis of a number of degenerative diseases including Alzheimer’s disease, atherosclerosis, cataracts, and cancer.\textsuperscript{33,34} The generally accepted pathogenesis of 4-HNE is linked to the γ-hydroxy-α,β-unsaturated aldehyde acting as a strong electrophile, which can form adducts with a variety of cellular nucleophiles via Michael addition or Schiff base formation. This interesting pathogenesis is linked to the broad abundance of this molecule. Under physiological conditions typical concentrations of 4-HNE range from 0.1-1 μM in all tissues,\textsuperscript{35,36} but under severe oxidative stress this concentration can rise substantially in a localized manner to between 10 μM and 5 mM.\textsuperscript{11,37,38} Recently, 4-HNE was also shown to be involved in signal transduction in a concentration-dependent manner,\textsuperscript{39,40} which implies that the regulation of its intracellular concentrations play a crucial role in normal physiology and disease states.

4-HNE is known to be reduced to its corresponding alcohol by alcohol dehydrogenase and aldose reductase,\textsuperscript{41} oxidized to acid by aldehyde dehydrogenase,\textsuperscript{41} or metabolized via its conjugation to glutathione catalyzed by glutathione S-transferases (GSTs) and subsequently excreted through the kidneys.\textsuperscript{42} However, in our recent studies as described in Chapter 2, we discovered a new pathway of 4-HNE degradation where 4-HNE first transforms into 4-hydroxynonanoic acid and then it follows a parallel metabolic pathway for the degradation of the 4-hydroxyacid. The first pathway (Pathway A) leads to the formation of 3-hydroxyacyl-CoAs, which are physiological β-oxidation intermediates, via 4-phosphoacyl-CoAs. The second pathway (Pathway B) is a sequence
of β-oxidation, α-oxidation, and β-oxidation steps. 4-HNE is thus degraded to acetyl-CoA, propionyl-CoA, and formate via those two parallel pathways.\textsuperscript{43,44}

The concept of 4-HNE as a signaling molecule was first suggested in 1982 by Curzio \textit{et al.} describing its chemotactic effects.\textsuperscript{45} At very low and nontoxic concentrations, 4-HNE can modulate several cell functions including signal transduction, gene expression, and cell proliferation.\textsuperscript{46} 4-HNE at sublethal concentration is also known to induce the expression of antioxidant enzymes through the activation of NF-E2-related transcription factor-2 (Nrf2) in PC12 cells.\textsuperscript{47-49} To better understand these signaling properties, and to explore whether 4-HNE could impact the formation of biological oxidants (nitric oxide in our case), we initiated a series of experiments examining the relationship between 4-HNE concentrations and the ability of activated cultured macrophages to produce nitric oxide (NO). We were particularly interested in NO as it plays a key role in mammals as a mechanism of cytotoxicity in macrophages. NO also serves as a signaling molecule involved in neurotransmission, and function of many organs and tissues. Our rationale behind these experiments was based on previous work showing that 4-HNE could modulate the Nrf2-Keap1 signaling pathway, and our own work showing that molecules which activate Nrf2 transcription also perturb iNOS expression.\textsuperscript{50} The human Keap1 protein contains 27 surface cysteine residues.\textsuperscript{51} 4-HNE is a strong Michael acceptor and hence can make Keap1 as a potential target for covalent modification which can be instrumental in the Nrf2-Keap1 signaling pathway.
**FIGURE 5.1** Molecular signaling pathways of macrophage activation through NF-κB, and the antagonistic signaling pathway Nrf2-Keap1. When considering the molecular mechanisms of macrophage activation, the most important pathway for pro-inflammatory transcription is the NF-κB family of transcription factors. Canonical NF-κB signaling is illustrated on the left portion of the figure whereby signaling is initiated by a ligand (such as the lipopolysaccharide (LPS) molecules of Gram-negative bacteria) through a Toll-like receptor (TLR, in this case likely TLR4) which activates IκB kinase (IKK). IKK phosphorylates I-κBα (inhibitor of NF-κB), which causes its dissociation from NF-κB, allowing the transcription factor to translocate to the nucleus and effect the transcription of a wide range of genes including key inflammatory regulators such as iNOS and cyclooxygenase 2 (COX-2). The signaling pathway that this work aims to study is Nrf2 (shown on the right portion of the figure). Not nearly as well defined as NF-κB, the evolving understanding of Nrf2-activation involves the interaction of a variety of
chemical effectors with Keap1 (Kelch-like ECH-associated protein 1), which is an endogenous suppressor of Nrf2. This interaction causes the dissociation of Nrf2 and Keap1, allowing the translocation of Nrf2 to the nucleus where it upregulates the expression of a host of phase 2 response enzymes and antioxidant stress proteins. Of central importance to this part is that, in addition to the phase 2 response, Nrf2 has the ability to suppress the expression of pro-inflammatory proteins through a yet-to-be defined mechanism.
5.2 RESULTS AND DISCUSSION

5.2.1 IDENTIFICATION OF A NEGATIVE FEEDBACK LOOP FOR NO PRODUCTION

Our experimental system for studying this signaling relies on a murine macrophage-like culture cell (RAW 264.7) that undergoes an inflammatory response when stimulated with interferon-gamma (IFN-γ) or lipopolysaccharide (LPS). This stimulation has the net effect of upregulating a host of inflammatory enzymes including iNOS, in addition to a host of other cytokines and chemokines. Our initial experiment aimed to examine whether 4-HNE could modulate inducible NO formation (presumably through perturbation of expression or activity of iNOS) in RAW 264.7 culture macrophages induced with LPS. Using the standard Griess assay, we measured NO production after a 24-hour exposure to LPS and various concentrations of 4-HNE to examine steady state levels of NO production. Surprisingly, we observed a dramatic decrease in NO production at 4-HNE concentrations greater than 1 µM (Fig 5.2A). We interpreted this result as significant because the basal concentrations of 4-HNE found ubiquitously throughout mammalian tissues range from 100 nM to 1 µM. Given that NO is a major biological oxidant, and the fact that the first chemical step in the production of LPO products (including 4-HNE) is radical abstraction of a doubly allylic hydrogen catalyzed by a biological oxidant, we hypothesized that this result could represent the discovery of a negative feedback loop for the production of 4-HNE through inhibition of oxidant formation. It is also worth noting that the observed decrease in NO production is not gradual. NO levels drop dramatically over concentration ranges as small as 2.5 fold (from 2.5 µM to 1 µM, see Fig 5.2A). Further, the observed effect cannot be attributed to toxicity (Fig 5.2C).
**FIGURE 5.2** Nitrite levels from LPS-activated RAW 264.7 cells treated with various concentrations of 4-HNE for (A) 24 hours; (B) 30 minutes. Cells treated with 10 ng/mL LPS and 0.25% DMSO were used as control and (C) Viability measurements of LPS-activated RAW 264.7 cells treated with varying concentration of 4-HNE relative to DMSO treated LPS-activated control treated for 24 hours.
To examine whether 4-HNE has an effect on the constitutively expressed NOS isoforms (neuronal nNOS and endothelial eNOS) we tested the activity of 4-HNE at various concentrations at earlier time-points before induction could occur. After 30 minutes of exposure to 4-HNE, no effect was seen in the nM to µM range, however at higher concentrations (5 mM to 10 mM, Fig 5.2B) a dramatic increase in NO production was observed, with nitrite concentrations reaching greater than 20 µM. It is difficult to gauge whether this effect is due to a specific activation of nNOS/eNOS, or a secondary effect that is related to the toxicity inherent in these high concentrations of 4-HNE. Regardless, given our laboratory’s interests in iNOS, we decided to further explore the mechanistic underpinnings of the observed negative feedback profile.

5.2.2 Specificity of 4-Hydroxy-2-(E)-Alkenal Derivatives (C₅-C₁₂) for NO Production

Our next experiment aimed to test whether the observed phenotype was specific for 4-HNE, or simply a non-specific effect derived from the electrophilic nature of the molecule. To test this, several 4-hydroxy-2-(E)-alkenal derivatives with various chain lengths (C₅-C₁₂) were synthesized and evaluated for induced NO production as above. 4-Hydroxy-2-(E)-pentenal (C₅) was synthesized via a minor modification of the reported procedure (Fig 5.3A). The other 4-hydroxy-2-(E)-alkenal derivatives (C₆-C₁₂) were synthesized using a strategy inspired by Gardner et al., which treats homo-allylic alcohols of appropriate chain length with m-CPBA to afford the 3,4-epoxyalcohols that are then oxidized with Dess-Martin periodinane. The resulting 3,4-epoxyaldehydes undergo an in situ α-hydrogen elimination and concomitant epoxide opening forming the desired 4-hydroxy-2-(E)-alkenals. Evaluation of NO production in activated macrophages
showed a clear pattern whereby the C₉ (4-HNE) and C₁₀ alkenals showed the most pronounced decrease in NO production (Fig 5.3B). This result was not due to toxicity (Fig 5.2C), and points to the observed negative feedback being specific for 4-HNE. The observation that C₁₀ is slightly more potent is clear and repeatable across multiple concentrations. The implications of this are unclear, but will be the subject of further evaluation in our laboratory. On this point, in our recent studies, we found the presence of glutathionylated C₁₀ in rat liver and heart tissues (shown in Chapter 4), and others have reported the presence of C₁₀ in tissues. It is plausible that the similar molecular volume of C₉ and C₁₀ makes them identical to the signal transduction pathways in the macrophage for NO production.\textsuperscript{55}
**Figure 5.3** (A) Synthesis of 4-hydroxy-2-(E)-alkenal derivatives C$_5$–C$_{12}$. Compound 3 is described as C$_5$ in the text and compounds 3a-3g are referred to as C$_6$ to C$_{12}$ where n = 0-6 respectively. (B) Nitrite levels from LPS-activated macrophage treated with 1 μM 4-hydroxy-2-(E)-alkenal derivatives C$_5$–C$_{12}$ for 24 hours.
5.2.3 MECHANISM OF NO PRODUCTION INHIBITION BY 4-HNE

From a mechanistic point of view, we proposed two possible explanations for the observed phenotypes. The first was that 4-HNE inhibits the enzymatic activity of iNOS resulting in decreased NO production, while a second explanation is rationalized as a consequence of decreased iNOS expression. To differentiate between these two possibilities we first investigated the effect of varying concentrations 4-HNE on recombinant murine iNOS. Activity was measured from the conversion of radiolabeled L-arginine to citrulline using a standard assay and quantified using scintillation counts per minute (CPM).\(^56\) We tested the recombinant iNOS at various concentrations, which correlated to the inhibitory concentrations observed in Fig 5.4A. Between 1 µM and 1 mM no appreciable perturbation to iNOS activity was seen. From this data, we consequently concluded that 4-HNE is not a direct enzymatic inhibitor of iNOS. To explore our alternate hypothesis, we examined iNOS expression utilizing immunoblot and RT-PCR analysis of 4-HNE treated LPS-activated RAW macrophages. We evaluated concentrations from 1 µM to 10 µM, and observed that at concentrations of 5 µM and above, 4-HNE iNOS protein levels are not detectable (Fig 5.4B). This correlated well with quantitative RT-PCR analysis (Fig 5.4C) that showed a dose dependent inhibition of oxidant formation is due to an inhibition of expression of iNOS. It is subsequently straightforward to postulate that this perturbation in iNOS expression is due to activation of Nrf2 signaling.
**Figure 5.4** (A) iNOS activity was measured via quantification of radiolabeled L-arginine to citrulline conversion. iNOS enzyme alone served as positive control while iNOS enzyme treated with N\(^\circ\)\-nitro-L-arginine (L-NNA) served as negative control. (B) Immunoblot analysis of iNOS protein levels in 4-HNE treated LPS-activated macrophage
treated for 18 hours. DMSO treated cells were used as negative control while DMSO treated LPS-activated cells were used as positive control. β-actin was used as protein loading control. (C) mRNA levels of 4-HNE treated LPS-activated macrophage treated for 18 hours. Fold change in expression were calculated relative to DMSO treated LPS-activated iNOS expression which was normalized to 18s rRNA expression.
5.3 **Experimental Sections**

5.3.1 **Materials and Methods**

Unless otherwise stated, the solvents and reagents were of commercially available analytical grade quality. Flash chromatography was performed on silica gel (230-400 mesh) purchased from Dynamics Adsorbents (Atlanta, GA). TLC was done on Hard Layer, Organic Binder TLC-plates with a fluorescent indicator and visualized by UV light (254 nm) purchased from Dynamics Adsorbents (Atlanta, GA). Solvents and reagents were of commercially available analytical grade quality and used as received without any further purification. $^1$H and $^{13}$C-NMR spectra were recorded on a Varian Inova spectrometer (at the Department of Chemistry, Case Western Reserve University) operating at 400 MHz and 100 MHz for the $^1$H and $^{13}$C-NMR spectra, respectively. The internal references were TMS ($\delta$ 0.00) and CDCl$_3$ ($\delta$ 77.2) for $^1$H and $^{13}$C spectra, respectively. NMR data are presented in the following order: chemical shift, peak multiplicity (b = broad, s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, dd = doublet of doublet), coupling constant, proton number.

The leukemic mouse macrophage cells (RAW 264.7) were obtained as a gift from Dr. Michael Sporn (Dartmouth College, NH). DMEM and RPMI media were purchased from GIBCO (Grand Island, NY) and supplemented with low endotoxin FBS (< 0.06 EU) from Thermo Scientific (Logan, UT). DMSO and lipopolysaccharide (LPS) from *E. coli* were purchased from Sigma (St. Louis, MO). The penicillin/streptomycin, Griess assay kit, RIPA buffer, 0.2 µm PVDF membrane, Novex® 4–20% tris-glycine gels, running and transfer buffers, PureLink™ RNA Mini Kit, Superscript® III Reverse Transcriptase and TaqMan® Fast Universal PCR Master Mix were all purchased from
Invitrogen (Grand Island, NY). All the primary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and secondary antibodies from Southern Biotech (Birmingham, AL). The Protease cocktail inhibitor tablets were purchased from Roche (Indianapolis, IN) and PBS from Cellgro by Mediatech, Inc. (Manassas, VA). The ECL plus was purchased from Amersham (Buckinghamshire, UK) and the autoradiography film was from MidSci (St. Louis, MO). The MTT cell proliferation assay kit was purchased from ATCC (Manassas, VA). The iNOS probe was purchased from Applied Biosystems (Carlsbad, California) and the 18s rRNA probe was purchased from IDT (Coralville, Iowa). The iNOS activity kit and the murine recombinant iNOS were purchased from Cayman Chemicals (Ann Arbor, MI) and the $[^3]H$ arginine monohydrochloride from Perkin Elmer (Waltham, MA).

5.3.2 Synthesis of 4-Hydroxy-2-(E)-Alkenal Derivatives (C$_5$-C$_{12}$)

4-Hydroxy-2-(E)-pentenal (3)

A solution of aldehyde, 2 (0.14 g, 1.076 mmol) in dry THF (6 mL) was cooled to -78 °C, and 0.8 mL of MeLi (1.6 M solution in Et$_2$O, 1.28 mmol) was added slowly. The solution was stirred for 30 min followed by work-up with saturated solution of NH$_4$Cl at -78 °C. The reaction mixture was then extracted with Et$_2$O (3 × 10 mL), the combined organic layers were dried over Na$_2$SO$_4$, and the solvent was removed in vacuo to obtain the crude product. The latter was then hydrolyzed with Amberlyst-15 as described for 2 to afford the crude 4-hydroxy-2-(E)-pentenal (3), which was then purified by column chromatography over silica (hexane, 30% ethyl acetate) to give 3 (0.083 g, 0.829 mmol, 77%). $^1$H-NMR (400 MHz, CDCl$_3$): 1.39 (d, 3H, $J = 6.8$ Hz), 4.59-4.62 (1H), 6.29 (ddd,
1H, J = 15.6 Hz, 7.6 Hz, 1.6 Hz), 6.84 (dd, 1H, J = 15.6 Hz, 4.8 Hz), 9.57 (d, 1H, J = 7.6 Hz); $^{13}$C-NMR (100 MHz, CDCl$_3$): 22.7, 67.3, 130.2, 160.1, 193.9.

**Fumaraldehyde dimethylacetal (2)**

Fumaraldehyde dimethylacetal, 2 was obtained by partial acid hydrolysis of fumaraldehyde bis(dimethylacetal), 1. $^{57}$ Amberlyst-15 catalyst in acid form (0.06 g) was added to the bisacetal 1 (0.2 g, 1.135 mmol) in acetone (6 mL) and water (0.08 mL) under magnetic stirring at room temperature. Stirring was continued for 10 min (longer time resulted the hydrolysis of the second acetal group), then the reaction mixture was filtered through a bed of anhydrous sodium carbonate and sodium sulfate 1:1 (w:w) followed by the solvent evaporation in vacuo to get 2 (0.14 g, 1.076 mmol, 95% yield). NMR data were in accord with the literature.$^{57}$

**General method for 4-hydroxy-2-(E)-alkenals (3a-3g)**

4-Hydroxy-2-(E)-alkenals, 3a-3g were synthesized using Gardner method.$^{54}$ To a stirring solution of 3-alkenol, 4a-4g (1 mmol) in 4 mL CH$_2$Cl$_2$ was added a 1.5 molar excess of 70% m-chloroperoxybenzoic acid (0.37 g). The solution was stirred at room temperature for 1.5 h after which 4 mL of 10% NaHCO$_3$ was added with vigorous stirring for 45 min. The reaction mixture was then extracted with CH$_2$Cl$_2$ (3 × 15 mL), the combined organic layers were dried over Na$_2$SO$_4$, and the solvent was removed in vacuo to obtain the crude product which was redissolved in 5 mL of CH$_2$Cl$_2$ and 6.6 mL of Dess-Martin periodinane (0.3 M solution in CH$_2$Cl$_2$, 2 mmol) was added. The mixture was stirred for 2 h at room temperature and then 15 mL of Et$_2$O and 8 mL of 1.3 M NaOH was added into that and vigorously stirred for 1 min. The aqueous NaOH layer was removed and an additional 10 mL of 1.3 M NaOH were added with vigorous stirring.
for 15 min. The aqueous layer was removed and the organic phase was washed with brine solution, the organic layer was dried over Na$_2$SO$_4$, and the solvent was removed in vacuo to obtain the crude 4-hydroxy-2-(E)-alkenal, which was then purified by column chromatography over silica (hexane, 30-40% ethyl acetate) to give pure 4-hydroxy-2-(E)-alkenal, 3a-3g.

### 4-Hydroxy-2-(E)-hexenal (3a)

Overall yield: 42.1% (0.048 g, 0.421 mmol); $^1$H-NMR (400 MHz, CDCl$_3$): 0.98 (t, 3H, $J$ = 7.6 Hz), 1.57-1.74 (2H), 4.36 (m, 1H), 6.29 (ddd, 1H, $J$ = 15.6 Hz, 8.0 Hz, 1.6 Hz), 6.82 (dd, 1H, $J$ = 15.6 Hz, 4.8 Hz), 9.55 (d, 1H, $J$ = 8.0 Hz); $^{13}$C-NMR (100 MHz, CDCl$_3$): 9.6, 29.5, 72.3, 130.9, 159.3, 193.9.

### 4-Hydroxy-2-(E)-heptenal (3b)

Overall yield: 40% (0.051 g, 0.40 mmol); $^1$H-NMR (400 MHz, CDCl$_3$): 0.92 (t, 3H, $J$ = 7.6 Hz), 1.32-1.61 (4H), 4.39-4.42 (1H), 6.27 (ddd, 1H, $J$ = 15.6 Hz, 8.0 Hz, 1.6 Hz), 6.82 (dd, 1H, $J$ = 15.6 Hz, 4.8 Hz), 9.52 (d, 1H, $J$ = 8.0 Hz); $^{13}$C-NMR (100 MHz, CDCl$_3$): 14.1, 18.7, 38.6, 70.9, 130.6, 160.1, 194.2.

### 4-Hydroxy-2-(E)-octenal (3c)

Overall yield: 43% (0.061 g, 0.43 mmol); $^1$H-NMR (400 MHz, CDCl$_3$): 0.89 (t, 3H, $J$ = 7.6 Hz), 1.28-1.66 (6H), 4.39-4.41 (1H), 6.28 (ddd, 1H, $J$ = 15.6 Hz, 8.0 Hz, 1.6 Hz), 6.82 (dd, 1H, $J$ = 15.6 Hz, 4.8 Hz), 9.53 (d, 1H, $J$ = 8.0 Hz); $^{13}$C-NMR (100 MHz, CDCl$_3$): 14.1, 22.7, 27.5, 36.3, 71.2, 130.7, 159.9, 194.1.

### 4-Hydroxy-2-(E)-nonenal (3d)

Overall yield: 46% (0.072 g, 0.46 mmol); $^1$H-NMR (400 MHz, CDCl$_3$): 0.94 (t, 3H, $J$ = 7.6 Hz), 1.29-1.68 (8H), 4.40-4.43 (1H), 6.27 (ddd, 1H, $J$ = 15.6 Hz, 8.0 Hz, 1.6 Hz),
6.82 (dd, 1H, J = 15.6 Hz, 4.8 Hz), 9.51 (d, 1H, J = 8.0 Hz); $^{13}$C-NMR (100 MHz, CDCl$_3$): 14.2, 22.7, 25.1, 31.8, 36.6, 71.3, 130.7, 159.6, 194.0.

4-Hydroxy-2-(E)-decenal (3e)

Overall yield: 39% (0.066 g, 0.39 mmol); $^1$H-NMR (400 MHz, CDCl$_3$): 0.90 (t, 3H, J = 7.6 Hz), 1.29-1.65 (10H), 4.42-4.45 (1H), 6.31 (ddd, 1H, J = 15.6 Hz, 8.0 Hz, 1.6 Hz), 6.84 (dd, 1H, J = 15.6 Hz, 4.8 Hz), 9.57 (d, 1H, J = 8.0 Hz); $^{13}$C-NMR (100 MHz, CDCl$_3$): 14.2, 22.7, 25.3, 29.2, 31.8, 36.6, 71.3, 130.7, 159.5, 194.0.

4-Hydroxy-2-(E)-undecenal (3f)

Overall yield: 41% (0.076 g, 0.41 mmol); $^1$H-NMR (400 MHz, CDCl$_3$): 0.90 (t, 3H, J = 7.6 Hz), 1.28-1.67 (12H), 4.42-4.44 (1H), 6.32 (ddd, 1H, J = 15.6 Hz, 8.0 Hz, 1.6 Hz), 6.83 (dd, 1H, J = 15.6 Hz, 4.8 Hz), 9.58 (d, 1H, J = 8.0 Hz); $^{13}$C-NMR (100 MHz, CDCl$_3$): 14.2, 22.8, 25.4, 29.3, 29.5, 31.9, 36.7, 71.3, 130.8, 159.4, 193.9.

4-Hydroxy-2-(E)-dodecenal (3g)

Overall yield: 44% (0.087 g, 0.44 mmol); $^1$H-NMR (400 MHz, CDCl$_3$): 0.92 (t, 3H, J = 7.6 Hz), 1.25-1.66 (14H), 4.42-4.45 (1H), 6.31 (ddd, 1H, J = 15.6 Hz, 8.0 Hz, 1.6 Hz), 6.84 (dd, 1H, J = 15.6 Hz, 4.8 Hz), 9.58 (d, 1H, J = 8.0 Hz); $^{13}$C-NMR (100 MHz, CDCl$_3$): 14.3, 22.8, 25.4, 29.4, 29.6, 29.7, 32.0, 36.7, 71.3, 130.8, 159.3, 193.8.

3-Alken-1-ol (4a-4g)

Alkenols 4a-4d were purchased from TCI America. The cis-3-decen-1-ol (4e) was obtained by catalytic semi hydrogenation (1 atm) of a solution of 3-decyn-1-ol. 3-decyn-1-ol (1 g, 6.483 mmol) was dissolved in dry Et$_2$O (25 mL) in a small round bottom flask. Lindlar catalyst (50 mg, Pd on CaCO$_3$) and 1 g of quinoline were added and hydrogen was supplied from balloons. After completion of the reaction (confirmed by
NMR) the mixture was filtered to remove the catalyst and the solvent was removed on a rotary evaporator. Dichloromethane was added to the residue and it was washed with 1M Acetic acid, brine, and water, and dried with Na$_2$SO$_4$. The crude was then purified by column chromatography over silica (dichloromethane, 1% methanol) to afford cis-3-decen-1-ol, 4e (0.932 g, 5.964 mmol, 92%). $^1$H-NMR (400 MHz, CDCl$_3$): 0.89 (t, 3H, $J$ = 6.8 Hz), 1.23-1.55 (8H), 2.08 (td, 2H, $J$ = 6.8 Hz, 6.6 Hz), 2.37 (td, 2H, $J$ = 6.6 Hz, 6.4 Hz), 3.63 (td, 2H, $J$ = 5.8 Hz, 5.6 Hz), 5.31-5.39 (1H), 5.51-5.63 (1H); $^{13}$C-NMR (100 MHz, CDCl$_3$): 14.1, 22.6, 27.3, 29.0, 29.6, 30.8, 31.8, 62.4, 124.8, 133.8.

The trans-3-undecen-1-ol (4f) and trans-3-dodecen-1-ol (4g) were synthesized using Knoevenagel condensation of nonanal or decanal and malonic acid$^{58}$ followed by the reduction by LiAlH$_4$. Malonic acid (2.03 g, 19.5 mmol) was dissolved in triethylamine (2.97 g, 29.27 mmol) in a 2-neck round-bottom flask fitted with a magnetic stirring bar, a reflux condenser. Nonanal (2.77 g, 19.5 mmol) was added very slowly under inert atmosphere at room temperature. The reaction mixture were then heated to 80 °C and maintained at this temperature for 3 h. The product was then acidified with 1M HC1 and extracted with Et$_2$O. The organic layers were thoroughly washed with brine and water and dried over Na$_2$SO$_4$, and the solvent was removed in vacuo to obtain trans-3-undecenoic acid (2.91 g, 15.8 mmol, 81%). The crude product was pure enough to do the next step. The LiAlH$_4$ (0.93 g, 24.5 mmol) was dissolved in 15 mL dry THF was stirred for 5 min under inert atmosphere and then the trans-3-undecenoic acid (1.5 g, 8.15 mmol) dissolved in 5 mL THF was added slowly. The reaction was allowed to continue for 4 h under inert atmosphere, then 2 mL H$_2$O was added to quench reaction, and 1M HCl was added until pH < 3. Most of the THF was removed in vacuo, and the residue
was diluted with 10 mL H$_2$O, and extracted with CH$_2$Cl$_2$ ($3 \times 30$ mL). The combined organic layers were dried with Na$_2$SO$_4$, and the solvent was removed in vacuo. The resulting residue was purified by column chromatography over silica (hexane, 30% ethyl acetate) to give pure trans-3-undecen-1-ol 4f (1.3 g, 7.7 mmol, 94%). The trans-3-dodecen-1-ol 4g was also synthesized following the same method as for 4f and the yield was comparable.

**trans-3-Undecen-1-ol (4f)**

$^1$H-NMR (400 MHz, CDCl$_3$): 0.88 (t, 3H, $J = 6.8$ Hz), 1.27-1.39 (10H), 2.01 (q, 2H, $J = 6.4$ Hz), 2.26 (q, 2H, $J = 6.4$ Hz), 3.61 (t, 2H, $J = 6.4$ Hz), 5.37 (dt, 1H, $J = 15.2$ Hz, 7.2 Hz), 5.55 (dt, 1H, $J = 15.2$ Hz, 6.8 Hz); $^{13}$C-NMR (100 MHz, CDCl$_3$): 14.2, 22.8, 29.3, 29.4, 29.6, 32.0, 32.8, 36.1, 62.2, 125.8, 134.4.

**trans-3-Dodecen-1-ol (4g)**

$^1$H-NMR (400 MHz, CDCl$_3$): 0.88 (t, 3H, $J = 7.2$ Hz), 1.22-1.37 (12H), 2.01 (q, 2H, $J = 6.4$ Hz), 2.26 (q, 2H, $J = 6.4$ Hz), 3.62 (t, 2H, $J = 6.4$ Hz), 5.37 (dt, 1H, $J = 15.2$ Hz, 7.2 Hz), 5.56 (dt, 1H, $J = 15.2$ Hz, 6.8 Hz); $^{13}$C-NMR (100 MHz, CDCl$_3$): 14.3, 22.8, 29.4, 29.5, 29.7, 29.7, 32.1, 32.8, 36.1, 62.2, 125.8, 134.6.

**5.3.3 CELL CULTURE**

RAW 264.7 cells were cultured in DMEM media supplemented with 10% FBS and 1% penicillin/streptomycin and were kept in culture at 37 °C in a 5% CO$_2$ environment. Cells were kept in culture for no longer than a month and routinely checked for LPS responsiveness every few passages via detection of nitrite production measured using the Griess Reagent kit for nitrite determination.
5.3.4 **Nitrite Level Measurements**

RAW 264.7 cells were plated at $1 \times 10^6$ cells/well in a 96-well plate and allowed to attach for 3 hours before activation with 10 ng/mL LPS and treated with 4-hydroxy-2-(E)-alkenal derivatives (C$_5$-C$_{12}$, C$_9$ being 4-HNE) or 0.25% DMSO control. 4-Hydroxy-2-(E)-alkenal derivatives’ stocks were freshly made in DMSO. LPS-activated cells were treated with varying concentrations of 4-HNE for either 30 minutes or 18 hours. For other 4-hydroxy-2-(E)-alkenal derivatives study, LPS-activated cells were treated with 1 $\mu$M of the derivatives for 18 hours. Nitrite levels were measured via Griess assay according to manufacturer’s specifications using 100 $\mu$L Griess reagent with 100 $\mu$L sample supernatant. Absorbance was read at 550 nm using the Sunrise$^{TM}$ plate reader by TECAN (Mannedorf, Switzerland).

5.3.5 **Viability Measurement**

Remaining cells from the Griess assay were used for viability measurement using the MTT cell proliferation assay kit from ATCC following manufacturer’s instruction. Absorbance was read at 550 nm using the Sunrise$^{TM}$ plate reader by TECAN. Percent viability of treated cells were calculated relative to the LPS-activated treated with DMSO control as 100% viability.

5.3.6 **Immunoblotting Analysis**

RAW 264.7 cells were plated at $4 \times 10^6$ cells/60 mm plate and allowed to attach for 3 hours. Cells were activated with 10 ng/mL LPS and treated with varying concentrations of 4-HNE, DMSO control, or LPS with DMSO control and harvested for immunoblotting analysis and quantitative RT-PCR after an 18-hour treatment. Proteins
were isolated via lysing the cells with RIPA buffer containing protease inhibitors and equal amounts of proteins were loaded into a Novex 4–20% tris-glycine gel and separated. Proteins were transferred to a PVDF membrane, blocked with 5% non-fat dry skim milk in 0.5% tris-buffered saline containing Tween-20 (TBST), and probed with antibodies. iNOS was used at 1:1000 primary antibody dilution and a 1:5000 dilution for the secondary horse-radish peroxidase antibody. Antibodies were detected using ECL plus with autoradiography.

5.3.7 QUANTITATIVE RT-PCR

Cells harvested for the immunoblot analysis were also harvested for quantitative RT-PCR. Total RNA was extracted using the PureLink™ RNA Mini Kit and reverse transcription reaction was performed using the Superscript® III Reverse Transcriptase. PCR was performed using TaqMan® Gene Expression Assay iNOS probe (Assay ID: Mm00440502_m1), and PrimeTime qPCR assay 18s rRNA control probe (Assay ID: Mm.PT.42.122532.g) with TaqMan® Fast Universal PCR Master Mix. Amplification was performed using the 7500 Fast Real-Time PCR system and the 7500 Fast System SDS Software-Sequence Detection Software version 1.3.1.21 by Applied Biosystems. The relative quantification assay (ΔΔCt) was used using the Run mode Fast 7500 profile (95 °C for 20 sec, followed by 40 cycles of 95 °C for 3 sec, and 60 °C for 30 sec).

5.3.8 iNOS ACTIVITY ASSAY

iNOS activity was measured using the iNOS activity kit via detection of radiolabeled arginine (arginine monohydrochloride L-[2,3,4-3H]) conversion to citrulline. The reactions were prepared according to manufacturer’s instructions using the iNOS (murine recombinant) enzyme. Each reaction was treated with various concentrations of
4-HNE and allowed to proceed at room temperature for 1 h. Radioactivity was quantified by transferring the sample eluted into scintillation vials containing scintillation fluid and the CPM measured with 1450 MicroBeta TriLux Microplate Scintillation Counter. The positive control contained iNOS enzyme alone, and the negative control contained the iNOS enzyme treated with a NOS competitive inhibitor L-N^ω-nitroarginine (L-NNA).

5.4 CONCLUSIONS

The significance of these observations is rooted chemical processes that lead to the formation of lipid peroxidation products. As mentioned earlier, the first step in this process is radical hydrogen abstraction of PUFA by ROS and RNS. We propose that these findings represent an evolved feedback pathway to control the concentration of 4-HNE (and other lipid peroxidation products) in tissues which express inflammatory mediators (such as iNOS) via the production of ROS and RNS such as NO. The fact that the observed inhibitory concentrations of 4-HNE correspond to the observed physiologic concentrations lends further credence to our hypothesis. Taken together we have devised a model of the expression control of 4-HNE as shown in Fig 5.5 that would argue for the production of 4-HNE as a carefully regulated process with an observed negative feedback loop. This model stands in contrast to dogma in the field that views 4-HNE as a cytotoxic xenobiotic derived from physiology gone awry.
FIGURE 5.5 A model for 4-HNE control of induced nitric oxide production. At low concentrations over long time periods, there is a negative feedback loop that maintains a constant level of production of nitric oxide with an inflection at approximately 1 µM. At higher concentrations, a positive feedback loop seems to be present whereby increasing concentrations of 4-HNE in turn elicits higher concentrations of nitric oxide.
5.5 ACKNOWLEDGEMENT

All the biological experiments were performed in Prof. John Letterio’s lab (Department of Pediatrics, Case Western Reserve University). Some data presented in this thesis have been recorded by Tonibelle Gatbonton.
5.6 **REFERENCES**


CHAPTER 6

Conclusions and Future Directions
6.1 Aim of the Thesis

Prior to this thesis work, there was no report on the complete carbon skeleton degradation of the 4-hydroxyacids with five or more carbons. They are products of ubiquitously occurring lipid peroxidation (4-HHE, 4-HNE) or drugs of abuse (GHB, GHP). The ultimate goal of this project was to uncover the final fate of the 4-hydroxyacids and 4-HNE, a lipid peroxidation product. As discussed before, 4-HNE is highly electrophilic and targets many biological molecules. The second-order rate constant for the reaction of 4-HNE and GSH has been reported to be 1.1-1.3 M$^{-1}$s$^{-1}$ at pH 7.4. Such a value would yield a half-life of only about 2 min for 4-HNE in the presence 5 mM GSH, as it occurs in physiology. The cellular concentration of 4-HNE at any given time ranges between 0.1-1 µM. So, consequently we can estimate that we produce about 10-20 g of 4-HNE on a daily basis. That enormous production of this cytotoxic product must have a very efficient degradation pathway to counter-balance its toxic nature and protect the survival of the cellular macromolecules. With this hypothesis, we started our metabolomics study on this ubiquitous class of molecules with the help of isotopic tools and a live rat organ perfusion experiment. In particular, we sought to define a thorough catabolic pathway for different 4-hydroxyacids that are either the drugs of abuse (GHB, GHP) or derived from lipid peroxidation products (4-HNE, 4-HHE or 4-ONE). Further, we were able to expand our studies with 4-HNE to a thorough understanding of how this molecule modulates oxidative stress in the macrophage.

Since high concentrations of these lipid peroxidation products or drugs of abuse have already been implicated in many disease states, it was necessary to get in depth information about their fates as well as regulatory pathways. Our strategy employed the
tools of synthetic organic chemistry to generate regiospecifically $^{13}$C- and $^2$H-enriched lipid peroxidation products or drugs of abuse that could be used in the respective metabolomics studies. This approach was not only useful for the elucidation of previously unknown parallel metabolic pathways, but also enabled us to determine the flux of different pathways. Therefore, a dominant theme of this dissertation is how isotopic tools can provide a wealth of information via a thorough metabolomics study. The studies presented in this thesis can be summarized in the following sections.

6.2  PARALLEL METABOLIC PATHWAYS OF 4-HYDROXYACIDS AND 4-HNE VIA 4-PHOSPHOACYL COA ESTERS

6.2.1  DISCUSSION AND CONCLUSIONS

A central focus of this thesis is an interesting lipid peroxidation product named 4-hydroxy-2-(E)-nonenal, more commonly known as 4-HNE. 4-HNE has become an interesting small molecule for researchers with its dose dependent nature in physiology. At sub-lethal concentrations it can act as a signaling molecule, while in higher concentrations it reacts with bio-macromolecules such as proteins, enzymes, lipids and DNA and abrogates their physiologic functions. The largest portion of the literature on 4-HNE deals with how this molecule reacts with and modulates the activity of various proteins and enzymes.

So, what are the unanswered questions in the 4-HNE field? Looking back into the literature, we found that researchers have explored the metabolism of 4-HNE but all of the studies dealt with the intact nine-carbon framework. We performed a thorough metabolomics study of 4-HNE (via 4-hydroxyacids) using isotopic tools as the final fate
of this ubiquitous lipid peroxidation product was not defined clearly in the previous reports. A key finding of this work was that all 4-hydroxyacids (with more than five carbons) as well as 4-HNE catabolism can proceed via two new parallel pathways. The first and major pathway, which involves 4-phosphoacyl-CoAs, leads in six steps to the isomerization of 4-hydroxyacyl-CoA to 3-hydroxyacyl-CoA. The second and minor pathway involves a sequence of β-oxidation, α-oxidation, and β-oxidation steps. We were also able to quantify the differential catabolic flux of 4-HNE (via 4-hydroxyacids) down the two parallel pathways. A new class of CoA esters, namely 4-phosphoacyl-CoAs was discovered that is only derived from 4-hydroxyacids (as described in Chapter 2) and is intermediate in the catabolism of 4-HNE and 4-hydroxyacids.

6.2.2 Future Directions

Although we have accomplished much on understanding the catabolism of 4-HNE, the story is not yet complete. We have yet to identify the various enzymes responsible for the transformation of 4-hydroxyacyl-CoA to 3-hydroxyacyl-CoA via 4-phosphoacyl-CoA. The proposed mechanism of the isomerization was shown in Scheme 6.1. According to this scheme, the phosphorylation of 4-hydroxyacyl-CoA (compound 1) is followed by dehydrogenation and hydration, forming a 3-hydroxy-4-phosphoacyl-CoA (compound 2). The latter would be dephosphorylated to the enol form of 3-ketoacyl-CoA (compound 3), which undergoes cycles of β-oxidation yielding short chain acyl-CoAs. Characterization of the series of enzymes involved will lead to a new direction towards the pathophysiological implication of 4-HNE catabolism.
We have outlined a strategy for the identification of the kinase(s) that is (are) responsible for the transformation of 4-hydroxyacyl-CoA to 4-phosphoacyl-CoA. To get adequate amounts of the crude enzyme, we are going to use pig liver instead of the rat liver. The purification will follow the order: ammonium sulfate precipitation > gel filtration with Sephadex G-25 > ion-exchange chromatography with SP Sepharose Fast Flow > affinity chromatography with Blue Sepharose 6 Fast Flow. Identification of the kinase responsible for the phosphorylation of 4-hydroxyacids will provide a better understanding on the implication of the described parallel pathways.
**Scheme 6.1** Proposed mechanism for the isomerization of 4-hydroxyacyl-CoA to 3-hydroxyacyl-CoA via 4-phosphoacyl-CoA.
6.3 Metabolomics of γ-Hydroxybutyrate (GHB) Using Isotopic Tools

6.3.1 Discussion and Conclusions

GHB is both a neurotransmitter and a drug of abuse which primarily derives from GABA in brain. The known metabolism of GHB proceeds via (i) conversion to succinic semialdehyde by a cytosolic NADP$^+$ dehydrogenase and by a mitochondrial pyridine nucleotide-independent enzyme system, and (ii) the oxidation of succinic semialdehyde to succinate, an intermediate of the Krebs cycle. GHB accumulates at supraphysiological concentrations in succinic semialdehyde dehydrogenase deficiency, which is a rare inborn error of GABA metabolism caused by the deficit of GABA degradative enzyme succinic semialdehyde dehydrogenase.

We synthesized a number of stable isotopically ($^{13}$C) labeled GHB isotopomers and perfused them in the isolated rat liver. Using a combination of metabolomics and mass isotopomer analysis, we showed that GHB is metabolized by multiple processes, in addition to its previously reported metabolism in the Krebs cycle via oxidation to succinate. Also, GHB undergoes (i) β-oxidation to glycolyl-CoA and acetyl-CoA, (ii) two parallel processes which remove C-1 or C-4 of GHB and form 3-hydroxypropionate, and (iii) degradation to acetyl-CoA via 4-phosphobutyryl-CoA. The triply labeled GHB isotopomers ([1,2,3-$^{13}$C$_3$]-GHB and [2,3,4-$^{13}$C$_3$]-GHB) allowed us to demonstrate a precursor-to-product relationship for the formate production (as a result of α-oxidation) from 2,4-dihydroxybutyryl-CoA. This study illustrates the potential of the combination of metabolomics and mass isotopomer analysis for pathway discovery. In this chapter we
also showed that the catabolism of GHB differs from that of longer chain 4-hydroxyacids (C5-C11) described in Chapter 2.

### 6.3.2 Future Directions

GHB at mM concentrations in body fluids causes amnesia and impairs the capacity of the subject to exercise judgment (date rape drug). The disposal of exogenous GHB *in vivo* is inhibited by ethanol. Glycolate, 2,4-dihydroxybutyrate and 3-hydroxypropionate have been identified in the urine of humans with succinic semialdehyde dehydrogenase deficiency. Our studies showed the origin of those compounds from GHB and hence the newly characterized pathways described here, which are probably peroxisomal, may play a role in the physiopathology of succinic semialdehyde dehydrogenase deficiency. Additional experiments will be conducted with succinic semialdehyde dehydrogenase deficient mice (SSADH\(^{-/-}\)) to get further insight into this argument.

Other questions abound. What is the implication of having parallel pathways? It is possible that under different physiologic stresses, the predominant pathway may switch from one to another (α-oxidation and β-oxidation pathways, as shown in Fig 3.5). Alternatively, different pathways (α-oxidation and β-oxidation) may predominate in different tissues. Regardless, with these tools we now have the ability to answer these questions about this and other fundamental physiologic processes. The implications of 4-phosphobutyryl-CoA also need to be addressed in disease states, particularly, in succinic semialdehyde dehydrogenase deficiency.
6.4 ENANTIOSELECTIVE GLUTATHIONYLATION OF 4-HNE & ITS DERIVATIVES IN RAT ORGANS

6.4.1 DISCUSSION AND CONCLUSIONS

Oxidative stress represents an elevated level of reactive oxygen and nitrogen species (ROS and RNS). High reactivity and transient stability of ROS and RNS make it difficult to directly measure the level of oxidative stress. Often to overcome this difficulty, secondary biomarkers are chosen to quantitate oxidative stress. Commonly employed methods for the measurement of lipid peroxidation-derived aldehydes or modified proteins suffer from the artifactual formation of 4-HNE or 4-HNE adducts to cellular macromolecules during sample isolation and derivatization, resulting in an overestimation of background levels. Glutathione S-transferases (GSTs) play a key role in metabolizing highly reactive electrophiles present in living systems including 4-HNE, formed during the oxidative insults.\(^1\) GSTs catalyzed conjugation of 4-HNE to glutathione (GSH) results in the formation of the 4-HNE–GSH adducts which are often used as a biomarker for the oxidative stress.\(^4\)

In this chapter we described a sensitive analytical strategy to accurately measure endogenous GSH conjugates of 4-HNE enantiomers in rat organs. Iodoacetic acid (IAA) was found to efficiently quench the \textit{ex vivo} nonenzymatic glutathionylation of 4-HNE. The new developed method was validated and applied to measure and compare the endogenous level of GSH conjugates of different 4-hydroxy-2-(\(E\))-alkenal (4-HAE) enantiomers in various rat organs. We profiled the various 4-HAE–GSH conjugates in rat liver, heart and brain with the newly developed method. This demonstrated the presence
of all 4-HAE from C\textsubscript{5} to C\textsubscript{12} and several detectable 4-HAE–GSH conjugates (C\textsubscript{6}, C\textsubscript{8} and C\textsubscript{9}) in these organs. The C\textsubscript{9} and C\textsubscript{6} 4-HAE–GSH conjugates are the most abundant among all. In exogenous 4-HNE perfused rat liver (\textit{S})-4-HNE–GSH was preferentially released from the liver, where (\textit{R})-4-HNE–GSH was accumulated. When the 4-HNE is derived from 4-ONE (in case of the 4-ONE perfused liver), we found greater accumulation of (\textit{R})-4-HNE–GSH than (\textit{S})-4-HNE–GSH in the liver. Thus, it suggests that the enantioselectivity of glutathionylation depends on the source of 4-HNE. In our study, we have found the glutathionylation as a very small portion of disposal pathway of exogenous 4-HNE by rat liver. We think that the transporters of 4-HNE–GSH and 4-HHE–GSH are enantioselective.

### 6.4.2 Future Directions

The newly developed analytical method for the detection of glutathionylation of different 4-HAE enantiomers will be a valuable tool to investigate the further implications of the presence of C\textsubscript{5} to C\textsubscript{12} 4-HAEs in the liver and other tissue types. We also would like to explore the role of various 4-HNE–GSH diastereomers in diseased states by perfusing liver with the labeled (\textit{R})-4-HNE and (\textit{S})-4-HNE. We are synthesizing \textsubscript{[5,6,7,8,9,\textsuperscript{13}C\textsubscript{5}]}-4-HNE to explore a full spectrum of the 4-HNE metabolomics. This labeling strategy will enable us to calculate the proportions of 4-HNE that go down the parallel catabolic and the glutathionylation pathways (as described in \textbf{Chapters 2} and \textbf{4} respectively). We have detected all the 4-HAEs from C\textsubscript{5} to C\textsubscript{12} in the rat organs but the source of C\textsubscript{6} to C\textsubscript{9} (lipid peroxidation products) are only established at present. Further experiments need to be done to figure out the origin and significance of the various 4-HAEs in the physiology.
6.5 IDENTIFICATION OF A NEGATIVE FEEDBACK LOOP IN BIOLOGICAL OXIDANT FORMATION REGULATED BY 4-HNE

6.5.1 DISCUSSIONS AND CONCLUSIONS

4-HNE is the most studied of the known lipid peroxidation products, and has generally become accepted as a modulator of multiple disease states. The majority of the research on 4-HNE deals with how this molecule reacts with and modulates the activity of various proteins/enzymes. Recently though, the viewpoint regarding 4-HNE has evolved to appreciate the complex physiology and signaling aspects of this LPO product.\textsuperscript{5-7} In this chapter, we showed that 4-HNE can control the expression of inducible nitric oxide synthase (iNOS). This is significant as iNOS produces a key reactive nitrogen species (NO) implicated in the formation of 4-HNE and other LPO products. As mentioned earlier, the first step in this process is radical hydrogen abstraction of PUFA by ROS and RNS. We propose that these findings represent an evolved feedback pathway to control the concentration of 4-HNE (and other lipid peroxidation products) in tissues which express inflammatory mediators (such as iNOS) via the production of ROS and RNS such as NO. We have devised a model (\textbf{Fig 5.5, Chapter 5}) of the expression control of 4-HNE that would argue for the production of 4-HNE as a carefully regulated process with an observed negative feedback loop.

6.5.2 FUTURE DIRECTIONS

The next step in this work is a thorough examination of the transcriptional and translational levels of several key proteins that can be controlled by the Nrf2-Keap1 transcriptional pathway. This will be done through a combination of Western analysis
and RT-PCR. We also would like to do the same set of experiments in the primary macrophages to translate similar findings *in vivo*. We have obtained the Nrf2\(^{(-/-)}\) mouse strain and are currently developing this colony such that we can harvest primary macrophages deficient in the signaling pathway we hypothesize to be controlling iNOS expression. After obtaining macrophages from Nrf2\(^{(-/-)}\) mice we will repeat the experiments described in *Chapter 5*.

We also hypothesize that Keap1 (with 27 surface cysteine residues) is the direct protein target of 4-HNE for Michael addition and Schiff base formation. Studies looking at how 4-HNE chemically modifies Keap 1 will give important insights on the mechanism of Nrf2 signaling activation.
6.6 REFERENCES


APPENDIX
Calibration curves for the 4-HAE-GSH conjugates described in Table 4.1 (Chapter 4, pp 148):

**C₅-R Calibration**

\[ y = 1.3556x - 0.0282 \]

\[ R^2 = 0.9986 \]

**C₅-S Calibration**

\[ y = 1.4212x + 0.0061 \]

\[ R^2 = 0.9977 \]
**C₆-R Calibration**

\[ y = 0.3836x - 0.0614 \]

\[ R^2 = 0.9997 \]

**C₆-S Calibration**

\[ y = 0.6359x - 0.0233 \]

\[ R^2 = 0.9936 \]
$y = 0.8326x + 0.0049$
$R^2 = 0.9912$

$y = 1.7643x - 0.0177$
$R^2 = 0.9996$
**C₈-R Calibration**

\[ y = 1.0732x - 0.0822 \]

\[ R^2 = 0.9998 \]

**C₈-S Calibration**

\[ y = 1.575x - 0.0432 \]

\[ R^2 = 0.9939 \]
C₉-R Calibration

\[ y = 0.7696x + 0.0144 \]
\[ R^2 = 0.9968 \]

C₉-S Calibration

\[ y = 1.4815x + 0.0155 \]
\[ R^2 = 0.9981 \]
\[ y = 1.1587x - 0.0757 \]
\[ R^2 = 0.995 \]

\[ y = 1.9548x - 0.0497 \]
\[ R^2 = 0.9975 \]
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