ENDOCANNABINOID ENZYMES MONOACYLGLYCEROL LIPASE AND DIACYLGLYCEROL LIPASE: BIOCHEMICAL STUDIES AND NOVEL LIGANDS

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by

Meghan Ryan Johnston

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ABSTRACT OF DISSERTATION

Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Chemistry in the Graduate School of Northeastern University August 2012
Abstract

The endocannabinoid system includes signaling ligands for the cannabinoid receptors, (CB1, CB2) as well as the enzymes that are responsible for their biosynthesis and degradation. The two primary endogenous ligands N-arachidonoyl ethanolamine (AEA) and (2-AG) interact with the CB receptors similar to ∆8-tetrahydrocannabinol (THC), a classical cannabinoid isolated from the Cannabis sativa plant. The endocannabinoids AEA and 2-AG are released “on demand” by neurons in the brain as well as in peripheral tissues. Manipulating the concentration of these signaling molecules is an important area of investigation as significant therapeutic benefits are anticipated.

Diacylglycerol lipase (DGL) is a lipid metabolic and endocannabinoid regulatory enzyme that hydrolyzes 1, 2-diacyl-sn-glycerol to endocannabinoid signaling ligand 2-arachidonoylglycerol (2-AG). DGL enzyme inhibitors are of pharmacological importance for the down-regulation of the endocannabinoid system. This has the therapeutic potential of attenuating Parkinson’s symptoms, inflammation, obesity, and the reward system for substance abuse. A series of lipid analogs structurally related to diacylglycerol were synthesized with functional groups in the
sn-1 position such as amides, carbamates, retrocarbamates and alkyloximinocarbamates targeting the catalytic site of diacylglycerol lipase (DGL). Another class of compounds structurally related to moderate DGL inhibitor tetrahydrolipstatin (THL, Orlistat™, Alli™), were also synthesized. These compounds incorporate the reactive β-lactone functionality as well as variations in the amino acid moiety. Aliphatic, constrained as well as heterocyclic analogs were investigated.

Monoacylglycerol lipase (MGL) is an endogenous serine protease that efficiently degrades endocannabinoid 2-AG to arachidonic acid and glycerol. It has recently been recognized as a potential target of drug development for the up-regulation of the endocannabinoid system to treat pain, and was recently identified as an important marker for aggressive cancers. The development of MGL-selective inhibitors is an active area of medicinal chemistry research. In order to better understand the structure and function of this enzyme, sufficient quantities of pure, well-characterized MGL were needed to enable further molecular and structural profiling. MGL was cloned and over-expressed in E. coli to meet that need. The hMGL membrane preparation was subsequently purified using IMAC and the protein was characterized by MALDI TOF mass spectrometry. Full sequence coverage was determined with additional MS/MS analysis. The hMGL protein was then used to elucidate the active site of hMGL, demonstrating the role in catalysis of residue Ser\textsuperscript{122}. 
For Mom, Dad, Caitlin and Kali
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<tr>
<td>ABPP</td>
<td>activity based protein profiling</td>
</tr>
<tr>
<td>AHMMCE</td>
<td>7-hydroxy-6-methoxy-4-methylcoumarin ester</td>
</tr>
<tr>
<td>AA</td>
<td>arachidonic acid</td>
</tr>
<tr>
<td>AEA</td>
<td>arachidonoyl-ethanolamine (i.e. anandamide)</td>
</tr>
<tr>
<td>2-AG</td>
<td>2-arachidonoyl-glycerol</td>
</tr>
<tr>
<td>CB</td>
<td>cannabinoid</td>
</tr>
<tr>
<td>Cbz</td>
<td>carboxybenzyloxy</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>DAG</td>
<td>diacyl glycerides</td>
</tr>
<tr>
<td>DCM</td>
<td>dichloromethane</td>
</tr>
<tr>
<td>DGL</td>
<td>diacylglycerol lipase</td>
</tr>
<tr>
<td>DiFMU</td>
<td>(6, 8-difluoro-4-methylcoumarin-octanoate)</td>
</tr>
<tr>
<td>DMAP</td>
<td>dimethylaminopyridine</td>
</tr>
<tr>
<td>DMF</td>
<td>dimethyl formamide</td>
</tr>
<tr>
<td>DOXYL</td>
<td>dimethyloxazolinyloxy</td>
</tr>
<tr>
<td>EDCI</td>
<td>1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride</td>
</tr>
<tr>
<td>FAAH</td>
<td>fatty acid amide hydrolase</td>
</tr>
<tr>
<td>FRET</td>
<td>Förster resonance energy transfer</td>
</tr>
<tr>
<td>GPCR</td>
<td>G-protein coupled receptor</td>
</tr>
<tr>
<td>HEK293T</td>
<td>Human embryonic kidney 293 cells</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
</tr>
<tr>
<td>HT</td>
<td>high throughput</td>
</tr>
<tr>
<td>IC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>50% inhibitory concentration</td>
</tr>
<tr>
<td>IMAC</td>
<td>immobilized metal affinity chromatography</td>
</tr>
<tr>
<td>K&lt;sub&gt;m&lt;/sub&gt;</td>
<td>Michaelis Menton constant</td>
</tr>
<tr>
<td>LC/MS</td>
<td>liquid chromatography mass spectrometry</td>
</tr>
<tr>
<td>MALDI-TOF</td>
<td>matrix assisted laser desorption ionization time of flight</td>
</tr>
<tr>
<td>MGL</td>
<td>monoacyl-glycerol-lipase</td>
</tr>
<tr>
<td>NAM</td>
<td>N-arachidonyl-maleimide</td>
</tr>
<tr>
<td>NBD</td>
<td>nitrobenzoxadiazole</td>
</tr>
<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>SAG</td>
<td>1-stearoyl-2-arachidonoyl-&lt;i&gt;sn&lt;/i&gt;-glycerol</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>&lt;i&gt;sn&lt;/i&gt;</td>
<td>stereo-specific numbering</td>
</tr>
<tr>
<td>TBAF</td>
<td>tetrabutylammonium fluoride</td>
</tr>
<tr>
<td>TBS</td>
<td>tert-butyldimethyl silyl</td>
</tr>
<tr>
<td>TBDPS</td>
<td>tert-butyldiphenyl silyl</td>
</tr>
<tr>
<td>TEA</td>
<td>triethylamine</td>
</tr>
<tr>
<td>TEA (HF)&lt;sub&gt;3&lt;/sub&gt;</td>
<td>triethylamine trihydro fluoride</td>
</tr>
<tr>
<td>THF</td>
<td>tetrahydrofuran</td>
</tr>
<tr>
<td>THC</td>
<td>tetrahydrocannabinol</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
<td>------------------------------</td>
</tr>
<tr>
<td>THL</td>
<td>tetrahydrolipstatin</td>
</tr>
<tr>
<td>TLC</td>
<td>thin-layer chromatography</td>
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</table>
CHAPTER 1

INTRODUCTION
1.1 Cannabinoid Discovery and Background

*Cannabis sativa* has been used by humans for thousands of years as nourishment, medicine, as well as for social and religious rituals. The earliest record of its medical use appeared nearly five thousand years ago in China. The Western world became interested in the plant’s therapeutic value during the early 19th century for use in treating ailments such as cough, fatigue, headache, asthma and rheumatism. In the 1920’s, medical use of marijuana gradually declined due to uncontrolled potency of the herbal prep, unpredictable response to oral administration and the introduction of alternative analgesics and hypnotics. It was ultimately banned in 1937 under the Marijuana Tax Act. It would take nearly sixty years for interest in the plant’s therapeutic value to be reinstated.

In 1964, Mechoulam et al. identified *Cannabis sativa’s* most active phytochemical to be ∆9-tetrahydrocannabinol (THC). They also defined the term “cannabinoid” (CB) to be a group of terpenoids responsible for the pharmacological effects of the plant. Although over four hundred cannabinoids have been identified, most of the biological effects of the plant, including psychotropic activity, are attributed to ∆9 and ∆8-tetrahydrocannabinol. Cannabinol and cannabidiol also possess a variety of pharmacological properties, but display fewer central nervous system (CNS) effects than the aforementioned cannabinoids. The identification of ∆9-THC, as well as other cannabinoids, led to questions regarding their interaction in vivo. Subsequent research led to the discovery of cannabinoid receptors and the endocannabinoid system.
Figure 1.1: Structural examples of cannabinoids found in *Cannabis sativa*. (A) cannabinol, (B) (-)-Δ⁹-tetrahydrocannabinol, (C) (-)-Δ⁸-tetrahydrocannabinol, (D) cannabidiol.

1.2 Cannabinoid Receptors

Two main cannabinoid receptors, termed CB₁⁰,¹¹,¹² and CB₂¹²,¹³ were discovered in the 1990’s and shown to become activated upon interaction with both exogenous and endogenous ligands. Currently, these are the only two cannabinoid receptor subtypes to have been fully characterized. They are considered members of the G-protein coupled receptor (GPCR) super family, which is characterized by seven trans-membrane helices.¹⁴ Evidence also suggests the presence of non-CB₁/CB₂ receptors such as the orphan G-protein coupled receptor GPR55.¹⁵ CB₁ is mainly expressed in the CNS and is the most abundant GPCR in the brain. The highest density of CB₁ receptors is found in cells of the basal ganglia, cerebellum, hippocampus and cerebral cortex. They are also minimally found in the brain stem, spinal cord, hypothalamus and amygdala.¹⁶,¹⁷,¹⁸,¹⁹,²⁰ The CB₂ receptor is generally undetectable in the CNS¹³,²¹ and is predominantly expressed in periphery such as the
immune system.\textsuperscript{22,23,24,25} It has also been detected in spleen, bone marrow, pancreas, uterus and lung.\textsuperscript{22} Direct information regarding the 3-dimensional structure of CB\textsubscript{1}/CB\textsubscript{2} receptors is currently lacking, as it is extremely difficult to obtain pure and active membrane-bound protein for X-ray crystallographic analysis. Structural templates for CB\textsubscript{1}/CB\textsubscript{2} receptors have been inferred from computational models using bacteriorhodopsin and bovine rhodopsin respectively.\textsuperscript{26,27}

Upon interaction with ligands, the GPCR conformationally changes in order to signal the associated hetero-dimeric G-protein. The stimulated receptor facilitates the exchange of GDP for GTP at the \( \alpha \)-subunit of the G-protein, leading to its dissociation from the receptor. Subsequently, the \( \alpha \)-subunit and \( \beta\gamma \)-heterodimer are released and ultimately act to regulate various downstream effectors. Receptor activation can lead to up- or down-regulation of adenylate cyclase\textsuperscript{28} and inhibition of mitogen-activated protein kinase. In addition to these well-established G-protein coupled signaling cascades; research has also shown that cannabinoid receptors are responsible for the generation of secondary messenger ceramide.\textsuperscript{29,30} Ceramide acts to mediate cannabinoid-induced metabolic regulation and apoptosis.\textsuperscript{31,32,33,34}

### 1.3 Endocannabinoid System

The endocannabinoid system was found to possess the essential components that constitute a signaling system. Firstly, enzymes are in cellular locations to produce and release the signaling molecules.\textsuperscript{35,36} Secondly, the various signaling molecules diffuse or are transported to other locations to deliver the signaling message.\textsuperscript{37,38,39} Thirdly,
the signaling molecules bind to a class of receptors that are members of the G-protein coupled receptors. Finally, following delivery of the signaling message, the signaling molecules are metabolically degraded. These properties distinguish endocannabinoids from classical peptide neurotransmitters, which are released from storage vesicles.\textsuperscript{40,41,42}

Endocannabinoid signaling is most clearly understood in the context of the biosyntheses and ultimately, degradations of endocannabinoid signaling lipids such as \textit{N}-arachidonoyl ethanolamine (anandamide, AEA)\textsuperscript{37,38} and 2-arachidonoyl glycerol (2-AG).\textsuperscript{39,40} In addition to these primary endocannabinoid signaling molecules, there exist a variety of molecules that interact with the cannabinoid receptors, such as virodhamine,\textsuperscript{43} meadethanolamide\textsuperscript{44} and noladin ether.\textsuperscript{45} Once endocannabinoids are formed post synaptically from stimulated neurons,\textsuperscript{35,36} they generate a retrograde signal as a result of binding to the cannabinoid receptors. Activation of these receptors leads to inhibition of \textit{Ca}^{2+} channels and stimulation of \textit{K}^+ channels, attenuating presynaptic activity. Anandamide and 2-arachidonoyl glycerol are subsequently rapidly degraded to terminate their biological actions.\textsuperscript{40,41,42}
Figure 1.2: Structures of lipid messengers in the endocannabinoid system. (A) anandamide, (B) 2-arachidonoyl glycerol, (C) nolandin ether, (D), virodhamine.

1.3.1 Endocannabinoid Signaling via Anandamide (AEA)

Anandamide was the first endogenous cannabinoid to be isolated and characterized as an agonist acting on the same receptors as THC. Anandamide is predominantly generated in vivo from phospholipid precursor, N-arachidonoyl phosphatidylethanolamine (NAPE) via phosphodiesterase-D (PLD) catalyzed hydrolysis. It is subsequently degraded intracellularly by fatty acid amide hydrolase (FAAH).

1.3.2 Endocannabinoid Signaling via 2-arachidonoylglycerol (2-AG)

2-arachidonoylglycerol (2-AG) is an endogenous cannabinoid receptor ligand, which binds to both central and peripheral cannabinoid receptors, eliciting a variety of cannabinergic responses in vitro and in vivo. 2-AG functions as a retrograde
synaptic neurotransmitter modulating both inhibitory GABAergic and excitatory glutamatergic signaling, such as at postsynaptic hippocampal CA1 pyramidal cells. 2-AG is more abundant in the brain than AEA and is considered the main endocannabinoid signaling molecule. Biosynthesis of 2-arachidonoyl-glycerol (2-AG) primarily occurs by two different pathways, both of which involve hydrolysis of lipids with an arachidonate at the sn-2 position. One pathway involves rapid hydrolysis of diacylglycerols by diacylglycerol lipase (DGL). The most common 2-AG precursor is 1-stearoyl-2-arachidonoyl-sn-glycerol. The other pathway involves hydrolysis of phospholipids by phospholipase A₁ and subsequent hydrolysis of the resulting lysophospholipid by a lysophospholipid specific phospholipase-C. Precursors other than membrane lipids have also been reported to generate 2-AG, such as the conversion of 2-arachidonyl lysophatidic acid to 2-AG and the transformation of 2-arachidonoyl phosphatidic acid to 2-AG.

1.4 Endocannabinoid Degradation

There is debate over the mechanism responsible for 2-AG and AEA transport across the plasma membrane for degradation. Some research suggests that 2-AG crosses the membrane via simple diffusion, while other research proposes that the uptake process is facilitated by various transmembrane carrier proteins. Regardless of active or passive transportation, anandamide and 2-arachidonoylglycerol are readily taken up by cells, where they are subjected to hydrolysis by catabolic enzymes.
1.4.1 Fatty Acid Amide Hydrolase (FAAH)

FAAH was first molecularly characterized by Cravatt in 1996.\textsuperscript{60,61} This catabolic enzyme belongs to the amidase signature (AS) family, which is defined by a conserved and linear sequence of approximately 130 amino acids. FAAH is the only characterized mammalian member of the AS super-family of serine hydrolases.\textsuperscript{60,62} It is distinguished from other amidase signature enzymes by its integration into membranes and its strong preference for hydrophobic substrates.\textsuperscript{60} It has been purified from rat tissue using mechanism-based affinity chromatography, as well as cloned from rat liver cDNA and expressed in COS-7 cells.\textsuperscript{63}

FAAH has been shown to be primarily responsible for intracellularly degrading endogenous ligand N-arachidonoylethanolamine. Increased levels of AEA in tissues have been linked with therapeutic effects, most notably, nociception. Cravatt has shown significantly elevated AEA levels in tissues of FAAH knockout mice. As a result of increased endocannabinoid levels, mice in this study exhibited a reduced response to pain.\textsuperscript{64,65}

In 2002, a crystal structure for FAAH revealed that its catalytic core contains a serine-serine-lysine catalytic triad (Ser\textsuperscript{241}-Ser\textsuperscript{217}-Lys\textsuperscript{142}), as opposed to the serine-histidine-aspartate triad seen in typical serine hydrolases.\textsuperscript{62,63} It also indicated that FAAH possesses a collection of channels that may allow simultaneous access to both the membrane and cytoplasmic compartments of the cell.\textsuperscript{60,66} These unusual structural features offer new opportunities for the design of inhibitors highly selective to FAAH over the hundreds of serine hydrolases present in the human proteome.
Many different classes of FAAH inhibitors have been investigated (e.g. trifluoromethyl ketones\textsuperscript{67-69}, fluorophosphonates\textsuperscript{70}, aryl carbamates\textsuperscript{71}, \(\alpha\)-ketoheterocycles\textsuperscript{69,72,73}, ureas\textsuperscript{74}) for potency and selectivity. Several potent as well as selective inhibitors of FAAH such as PF-3845\textsuperscript{74} have been reported. PF-3845, a piperidine urea developed by Pfizer and Cravat \textit{et al}, was determined by activity based protein profiling (ABPP) to selectively inhibit FAAH. It was found to raise AEA levels for 24 hours after administration leading to a marked reduction in inflammatory pain.\textsuperscript{74}

1.4.2 Monoacylglycerol Lipase (MGL)

Termination of 2-AG signaling occurs intracellularly by a degradative enzyme monoacylglycerol lipase (MGL) after transport from the extracellular space.\textsuperscript{53,75} Inhibition of this enzyme leads to higher 2-AG levels in the brain and therefore holds significant therapeutic potential. Interestingly, MGL has no effect on anandamide hydrolysis, while FAAH is able to catalyze the breakdown of 2-AG.\textsuperscript{76} Further discussion of MGL is in chapter two.

In addition to hydrolytic metabolisms, 2-AG and AEA are also consumed in oxidative metabolic pathways by several fatty acid oxygenases, such as cyclooxygenases (COX-2),\textsuperscript{77,78} lipoxygenases (12-LOX and 15-LOX)\textsuperscript{79,80} and cytochrome P450s.\textsuperscript{81,82} Interestingly, metabolites of 2-AG hydrolysis may themselves have therapeutic potential.\textsuperscript{83}
1.5 References:


(33) Guzman, M.; Sanchez, C.; Galve-Roperh, I. Pharmacology and Therapeutics 2002, 175.


(64) Cravatt, B. F. *Proc Natl Acad Sci USA* **2001**, *98*, 9371.


CHAPTER 2

BIOCHEMICAL STUDIES OF MONOACYLGLYCEROL LIPASE
2.1 Introduction

MGL is the main enzyme responsible for inactivating endocannabinoid agonist 2-AG for central CB\textsubscript{1} and peripheral CB\textsubscript{2} receptors.\textsuperscript{1,2} The highest concentrations of MGL are found in regions of cytosol and membrane where cannabinoid receptors are expressed.\textsuperscript{1,3} The physiological roles of MGL appear to be tissue specific. In adipose tissue, monoglycerides formed from stored triglycerides (via diglycerides) are hydrolyzed by MGL to fatty acid and glycerol. The released free fatty acids are then used as a source of energy \textit{in vivo}.\textsuperscript{4} In the central nervous system, MGL is the main enzyme modulating endocannabinoid signaling via hydrolysis of 2-AG to arachidonic acid and glycerol.\textsuperscript{5}

MGL, found to have a molecular weight of 33 kDA, was first purified from rat adipose tissue in 1975.\textsuperscript{6} In 1997 Karlsson \textit{et al.} reported the first cloning of MGL from a mouse-adipocyte cDNA library. MGL has also been cloned from human adipocytes and rat brain.\textsuperscript{1,7}

Homology modeling studies determined that MGL belongs to the serine hydrolase family of proteins. These enzymes are characterized by a catalytic site consisting of Ser, His and Asp residues located in the $\alpha/\beta$ hydrolase fold motif ($\beta$ sheets surrounded by $\alpha$ helices). Like most lipases, MGL is thought to have an alpha helical domain or lid covering the active site.\textsuperscript{8,9}

MGL displays catalytic specificity for monoacyl glycerols with medium to long-chain lipophylic moieties such as stearoyl, palmitoyl, oleoyl and especially
arachidonoyl acyl groups. MGL also demonstrates a preference for the hydrolysis of 2-acyl glycerols over their 1(3)-regioisomers.\textsuperscript{5,6,10,11}

There are four general types of small molecules which act to inhibit MGL: non-specific serine hydrolase inhibitors, 2-AG/1-AG substrate analogs, \textit{de novo} compounds (such as LY2183240 and URB602) in addition to molecules that target cysteine residues.\textsuperscript{12} Serine hydrolase inhibitors such as methyl arachidonyl fluorophosphonate (MAFP), arachidonoyl trifluoromethyl ketone (ATFMK) and arachidonoyl methlysulfonyl fluoride (PMSF) covalently bind to the catalytic site, thus rendering the enzyme inactive. These compounds ultimately did not exhibit inhibitory selectivity for MGL over other serine hydrolases, most notably FAAH. Inhibitors based on 1-AG and 2-AG homologues also showed a weak effect upon MGL activity. Inhibitors targeting the sulfhydryl groups of MGL such as \textit{N}-ethylnaleimide (NEM) and \textit{N}-arachidonoylnaleimide (NAM) bind irreversibly to the enzyme indicating the proximity of cysteine residues to MGL’s active site.\textsuperscript{9}

LY218240\textsuperscript{13} and URB602\textsuperscript{14} are characterized as \textit{de novo} compounds, a type of inhibitor generating a significant amount of interest. LY2183240 was initially characterized as an anandamide transport inhibitor demonstrating analgesic activity.\textsuperscript{15} It was subsequently shown by Alexander and Cravatt to exhibit potency for inactivating FAAH and MGL. This urea based compound was determined to have an inhibitory concentration (IC$_{50}$) value of 5.3 nM with MGL over-expressed in COS-7 cells. However, this potent inhibitor lacks the necessary selectivity over other enzymes.\textsuperscript{16}
URB602 is a non-competitive and partially reversible MGL inhibitor that is able to block 2-AG hydrolysis in rat brain. It was shown to selectively increase 2-AG levels when injected into the brain, enhancing stress-induced analgesia. This N-biphenyl substituted compound has been reported as a moderately active MGL inhibitor (IC$_{50}$ = 75 µM or 28 µM, depending on the MGL source)$^{14,17}$ as well as a poorly active one (IC$_{50}$ = 200 µM).$^{18}$ URB602’s ability to discriminate between 2-AG and AEA degradative pathways has also been a matter for debate. Hohmann et al. claim that FAAH is not inhibited by URB602$^{14}$ whereas, Vandevoorde et al reported that FAAH is in fact inhibited (IC$_{50}$ = 17 µM) by URB602.$^{18}$

Inhibition of MGL may offer more selectivity and less risk of psychotropic side effects in achieving therapeutic upregulation of the endocannabinoid signaling system as compared with cannabinoid receptor agonists.$^{19}$ Specifically, targeted inhibition of 2-AG deactivation is considered an attractive therapeutic approach against inflammation, pain, neurodegenerative and immune disorders.$^{20-23}$ Potent and selective high-affinity MGL inhibitors were lacking at the time when this research project was undertaken. The mechanism of known MGL inhibition was speculative, having been mainly inferred from MGL analogy modeling studies. Homology to other lipases and site-directed mutagenesis experiments$^8$ were used to predict the putative catalytic triad, consisting of Ser$^{122}$, Asp$^{239}$ and His$^{269}$. However, there was no direct evidence confirming the involvement of these or other amino acid residues in enzyme inactivation by the irreversible inhibitors. Increased knowledge of the enzyme’s catalytic site as well as mechanism of action will prove to be crucial in designing
potent and selective inhibitors. Regulation of endocannabinoid metabolism may provide a more selective therapeutic response than direct application of drug targeting cannabinoid receptors.

Recent MGL publications provide evidence contributing to the elucidation of MGL’s catalytic mechanism of action and therefore, are significant. Great strides have been made in investigating the role of cysteine residues in MGL’s catalytic activity. Zvonok et al. reported a sulfhydryl-based mechanism of MGL inhibition based on site-directed mutagenesis and mass spectral analysis. NAM is shown to compete in formation of a Michael addition product with Cys\(^{242}\) and Cys\(^{208}\) close to the catalytic site.\(^{24}\) King et al. reported a new class of compounds possessing the ability to interact with MGL cysteine residues. Through site-directed mutagenesis, isothiazolinone-based inhibitors were shown to interact with Cys\(^{208}\) with nanomolar potency \textit{in vivo}.\(^{25}\)

In 2009, Long et al. reported the first potent and selective MGL inhibitor, JZL184.\(^{26,27}\) This piperidine carbamate acts to irreversibly inhibit MGL via carbamylation of the enzyme’s catalytic serine. Use of activity-based protein profiling (ABPP) studies helped to identify JZL184’s potency to be in the low nanomolar range, with an IC\(_{50}\) of 6 nM. \textit{In vivo} studies have indicated that 2-AG levels in mouse brain are elevated eight to ten-fold upon administration of the compound. This was shown to lead to various CB\(_1\)-dependant behavioral effects including hypomotility, analgesia and hypothermia.

A hydrogen-bond network in hMGL’s active site was identified by Karageoros et al. using NMR studies.\(^{28}\) This strong hydrogen bonding network involving catalytic
and non-catalytic residues Asp$^{239}$, His$^{269}$, Leu$^{241}$ and Cys$^{242}$ was shown to be integral in hMGL’s catalytic activity. Experiments were also performed incorporating inhibitors AM6701 and NAM in order to further characterize hydrogen bonding patterns. Inhibition of hMGL by AM6701 was shown to subtly alter this network, while NAM’s interaction was shown to decrease the population of active-site hydrogen bonding. AM6701’s minimal alteration allows for the active site’s activity to be re-established after slow hydrolysis of the carbamoyl group suggesting reversible inhibition. NAM, however, prevents reformation of this network and therefore, inhibits hMGL irreversibly.$^{28}$

In 2010, Bertrand$^{29}$ and Labor$^{30}$ published hMGL crystal structures in its apo form as well as in complex with covalent inhibitor SAR629. These crystal structures confirmed predictions that MGL possesses the $\alpha/\beta$-hydrolase fold characteristic of serine hydrolases. In 2011, Schalk-Hihi et al.$^{31}$ also reported a crystal structure of hMGL in complex with inhibitor MAFP. The structure demonstrated a novel conformation of the regulatory lid-domain characteristic of the lipase family. The MGL structure revealed a closed confirmation of the lid-domain, enclosing the bound inhibitor, suggesting that MGL undergoes conformational and electrostatic changes leading to its dissociation from the membrane during the catalytic cycle. MGL research in the CDD was initiated in 2007, prior to the aforementioned advances.
2.2 Results and Discussion

2.2.1 hMGL large-scale expression, purification and characterization

There is great interest in potent and selective MGL inhibitors as potential therapeutics, especially for the suppression of inflammatory pain through a peripheral analgesic mechanism devoid of unwanted central nervous system psychoactivity.\textsuperscript{20,21,32} However, inhibitors of this caliber are currently lacking. Production of pure, active hMGL in substantial quantities was a prerequisite for addressing this therapeutic need and satisfying the protein requirements for crystallization and nuclear magnetic resonance (NMR) spectroscopy studies. These considerations led us to pursue human MGL overexpression in a prokaryotic system by incorporating a His\textsubscript{6}-tag into the N-terminus of the protein to facilitate its isolation using immobilized metal affinity chromatography (IMAC)\textsuperscript{33} and its subsequent immunodetection. The BL21 (DE3) \textit{E.coli} expression strain was chosen for this large-scale protein production based on evidence showing BL21 to have a two-fold greater yield of functional enzyme as compared to the Origami (DE3) \textit{E.coli} expression strain. The optimal expression period for active enzyme was considered to be 4-5 h as increasing amounts of viable protein was intracellularly lost to inclusion bodies if time was extended further. Although 0.5\% Triton X-100, a detergent inhibited hMGL by \approx 50\%, the presence of Triton during cell lysis and enzyme purification was deemed necessary to eliminate non-specific hMGL adhesion to glass- and plastic-ware and the resulting, extensive hMGL loss. A satisfactory yield of 30 mg of functional hMGL from 1 L was obtained using this optimized large-scale expression and purification protocol.
Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) analysis of various purification subfractions followed by Coomassie staining or western-blotting indicating that the single-step, IMAC-based purification protocol affords a single protein band suggesting acceptable purity (Figure 4.2 A-B). The protein’s molecular mass, ≈ 35 kDa as estimated relative to SDS PAGE standards, corresponds well to the reported molecular masses of purified, His-tagged recombinant mouse MGL (≈ 36.9 kDa), recombinant rat MGL (33.4 kDa), and a monoacylglycerol-hydrolyzing enzyme from rat adipose tissue (≈ 33 kDa).

Figure 2.1 MGL activity and purity data A-D: (A) Coomassie-stained SDS PAGE profiling (B) and western blot immunodetection of hMGL purification by IMAC. Total (lane 1) and soluble (lane 2) protein from E. coli cells expressing MGL; proteins unbound to IMAC resin (lane 3); proteins washed from IMAC resin by lysis (lane 4) and lysis-10 mM imidazole (lane 5) buffers; final elution from IMAC resin using lysis-200 mM imidazole buffer (lane 6). Detection was performed according to the procedures described in the Experimental Section. (C) Concentration-dependent inhibition of the hMGL to hydrolyze AHMMCE by AM6701. (D) IC₅₀ values for inhibition of rat brain MGL and expressed hMGL by AM6701.
MGL’s kinetic characteristics were characterized with native substrate 2-AG. Recombinant hMGL hydrolyzed 2-AG with a $K_m$ of 19.7 μM and $V_{max}$ of 25.1 μmol/μg/min. The $K_m$ of recombinant hMGL is comparable to crude rMGL (33.6 μM) and with recombinant rat MGL (10 μM) as reported. However, crude rMGL evidences a considerably lower $V_{max}$ (0.37 μmol/mg/min) than that of purified hMGL. This difference likely reflects the high content of extraneous tissue protein in the crude rMGL preparation. hMGL showed good affinity for fluorogenic reporter 7-hydroxy-6-methoxy-4-methylcoumarin ester (AHMMCE) with a $K_m$ of 8.8 μM (Figure 2.2). The hMGL $V_{max}$ of 550 μmol/mg/min for AHMMCE is, however, markedly (∼45-fold) lower than that for 2-AG. The comparative, substrate-related differences between 2-AG and AHMMCE are similar to other successful applications of model fluorogenic substrates, including those used to monitor FAAH activity. Fluorogenic reporters may have turnover rates orders of magnitude below those of the corresponding natural substrates, even if both have comparable enzyme affinities. Nonetheless, the inherently high sensitivity of fluorometric analysis enables facile monitoring of catalysis.
Figure 2.2  Kinetic parameters of crude rat-brain MGL (rMGL) and human recombinant His6-tagged MGL over-expressed in *E. coli* (hMGL) with either the natural substrate 2-AG or the model fluorogenic reporter substrate, AHMMCE.

<table>
<thead>
<tr>
<th>substrate</th>
<th><em>K_m</em>&lt;sup&gt; *&lt;/sup&gt; (μM)</th>
<th><em>V_max</em>&lt;sup&gt; *&lt;/sup&gt; (μmol/mg/min)</th>
</tr>
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<tbody>
<tr>
<td>2-AG</td>
<td>33.6</td>
<td>0.37</td>
</tr>
<tr>
<td>AHMMCE</td>
<td>n.d.&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8.8</td>
</tr>
</tbody>
</table>

2.2.2 Complete hMGL Characterization by MALDI-TOF-MS

After in-solution trypic digestion, full proteomic characterization of hMGL was carried out. Results indicated an absence of intramolecular disulfide bridges in the functional recombinant enzyme and the post-translational removal of the enzyme’s N-terminal methionine by methionyl aminopeptidase. Matrix assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF-MS) demonstrated the mass (34,126 Da) and validated the purity of the hMGL preparation as shown in Figure 2.3. Further MS/MS analysis determined full sequence coverage (Figure 2.4).
Figure 2.3 MALDI-TOF analysis of hMGL.

Figure 2.4 MS/MS analysis of trypsin digest.
In order to elucidate the amino acid residues responsible for hMGL’s catalytic properties, assays incorporating a known serine hydrolase inhibitor were designed. LY2183240, a previously described carbamoyl tetrazole, was synthesized in-house for use in such assays as a potent irreversible inhibitor. During the synthesis of LY2183240, the existence of two isomers was discovered, with the less polar isomer 5-[(biphenyl-4-yl)methyl]-N,N-dimethyl-2H-tetrazole-2-carboxamide, AM6701, showing distinct selectivity towards MGL. AM6701 was therefore utilized in MALDI-TOF-MS studies in order to determine the mechanism of hMGL inhibition. Spectra of digested free MGL and MGL/AM6701 mixtures were compared in order to identify a 71 Da addition to peptides as a result of carbamylation. As shown in Figure 2.5, the serine hydroxyl group attacks the carbonyl group of AM6701 forming carbamylation product with addition of 71 Da to the mass of the peptide. Spectra analysis revealed that only one peptide from the entire protein was modified by carbamylation.
Figure 2.5: Interaction of hMGL with AM6701 resulting in serine carbamylation.

2.3 Materials and Methods

Standard laboratory chemicals were purchased from Fisher Chemical (Pittsburgh, PA) and Sigma Chemical Co (St. Louis, MO) if not otherwise specified. BioSafe™ Proteins on membrane were visualized using the ECL Western blotting analysis system (GE Healthcare, Piscataway, NJ). PVDF membrane, Commmassie stain solution, Laemmli electrophoresis sample buffer, molecular weight markers, Micro Bio Spin™ P-6 columns and 1-D SDS–PAGE gels were from Bio-Rad (Hercules, CA). pET45b and E. coli BL21 (DE3) were from Novagen (Madison, WI). Luria Broth (LB) and Luria Agar medias for the growth of E. coli, isopropyl-β-D-thiogalactopyranoside (IPTG), lysozyme, DNase I were from Sigma Chemical Co. (St. Louis, MO). Mass spectrometry grade trypsin (Trypsin Gold) was obtained from Promega (Madison, WI). The synthesis of inhibitors, 5-((biphenyl-4-yl)methyl)-N,N-
dimethyl-2H-tetrazole-2-carboxamide (AM6701), the fluorogenic substrates, arachidonoyl, AHMMCE and arachidonoyl, 7-hydroxy-4-methylcoumarin ester (AHMCE), and the inhibition fluorescence assay using this compound will be described elsewhere. The ultrasonic homogenizer Sonics Vibra-Cell, 500W model was from Sonics (Newtown, CT). The electrophoresis apparatus Mini-Protean II, Trans-Blot Semi-Dry Electrophoretic Transfer Cell were from Bio-Rad (Hercules, CA). Alpha Innotech FluorChem Imaging System (Alpha Innotech Corporation, San Leandro, CA) was used to photograph DNA-agarose and Protein-PAGE gels, ECL Western blot detected proteins and in assays to monitor of hMGL inactivation by inhibitors. The protein concentrations at 600 nm and fluorescence reading at 360/460 nm were performed using BioTek Synergy HT Plate Reader (Bio-Tek Instruments, Inc., Winooski, VT). The hMGL trypsin digests were analyzed by a 4800 Maldi TOF/TOF™ mass spectrometer (Applied Biosystems, Foster City, CA)

2.3.1 Vector Construction

A full-length cDNA clone of the human MGL transcript variant 1 (gi: 51242951) was obtained from OriGene Technologies (Rockville, MD). The coding part of the MGL sequence (except the translation initiation codon ATG) was amplified by a polymerase chain reaction (PCR) using AACACGTGCCAGAGGA AAGTTCCC (forward) and  AAGAGCTCAGGGTGGGGACGCAG (reverse) primers containing PmlI and SacI restriction enzyme recognition sites, respectively. iProof high-fidelity DNA polymerase (Bio-Rad, Hercules, CA) was used in the PCR amplification with 33
cycles, each consisting of denaturation at 94 °C for 10 s, annealing at 55 °C for 33 s, and extension at 72 °C for 1 min. The PCR product and pET45b vector were digested with \textit{PmlI} and \textit{SacI} restriction enzymes, and the vector was dephosphorylated with CIP followed by in-gel purification using the MinElute Gel Extraction Kit (Qiagen Corp., Valencia, CA). The fragment was inserted into the vector directly downstream after the 6 histidine and valine codons, generating the construct pET45His6hMGL for expression of hMGL containing an N-terminal His-tag. GC10 \textit{E. coli} cells were used for DNA transformation and plasmid propagation. Mini and midi-scale plasmid DNA preparations were performed using a GenElute Plasmid Miniprep Kit (Sigma) and PureYield Plasmid Midiprep System (Promega, Madison, WI), respectively. In-frame vector-fragment junctions and the coding sequence of the recombinant gene were confirmed by sequencing. The pET45His6hMGL construct was transformed into BL21 (DE3) and Origami (DE3) \textit{E. coli} expression strains (Novagen, Madison, WI).

### 2.3.2 Culture growth

A single colony of \textit{E. coli} BL21 (DE3) cells containing the plasmid pET45His6hMGL was inoculated in 12 mL of LB media supplemented with ampicilin (100 µg/ml) and grown with 250 rpm shaking overnight at 37 °C. Subsequently, 10 mL of this culture was added into 1 L of the same LB medium and growth was facilitated by 250 rpm shaking at 37 °C. When culture turbidity reached an OD\textsubscript{600} of 0.6-0.8, expression from the T7 promoter was induced by adding IPTG to a final concentration of 1 mM. After 5 h induction the cells were harvested by
centrifugation at 7,000 rpm for 10 min at 4 °C, washed with PBS buffer and kept at -80 °C.

2.3.3 *E.coli* cell lysis and protein purification using Immobilized Metal Affinity Chromatography (IMAC)

Five grams of wet weight cells was resuspended in 50 mL of Lysis Buffer (100 mM NaCl, 50 mM Tris; pH 8.0, 0.5% Triton X-100) supplemented with lysozyme (0.2 mg/mL) and DNase I (25 µg/ml) then disrupted by sonication on ice three times for 1 minute (1 s on with 2 s off intervals, 50 W) each time. The cell lysate was subsequently centrifuged at 10,000 g for 30 minutes at 4 °C. The supernatant was incubated with 0.5 mL (bed volume) pre-equilibrated BD Talon™ metal affinity resin (Takara) for one hour at room temperature in a rotator in order for the His-tagged protein to coordinate with the cobalt in the resin. The suspension was transferred to a gravity-flow column and allowed to settle. The resin was washed with 15 mL of Lysis Buffer followed by 15 mL Lysis Buffer (0.1% Triton X-100) containing 10 mM imidazole. The His6-tagged protein was eluted with Lysis Buffer (0.1% Triton X-100) containing 200 mM imidazole; a concentration high enough to disrupt the chelation between resin and protein. The eluate was collected in 500 µL fractions and analyzed by SDS/PAGE.
2.3.4  SDS PAGE and Western Blot Analysis

Samples were denatured at 70 °C for 5 minutes in Laemmli sample buffer containing 5% β-mercaptoethanol and subsequently resolved on 10% PAGE SDS gels. The gels were stained using BioSafeTM Commassie solution or transferred to PVDF membranes. The membranes were prepared for immunodetection following the procedures outlined in the QIAexpress Detection and Assay Handbook (Qiagen, Valencia, CA). Membranes were incubated with a 1:10,000 dilution of anti-5His-HRP antibody (Qiagen). Protein bands were visualized using the ECL Western blotting analysis system.

2.3.5  Assay of hMGL Hydrolytic Activity using HPLC

Prior to the enzyme assay, the purified hMGL was desalted with a Zeba spin-column (Pierce) and 25 mM Tris-HCl; pH 7.4, containing 5 mM MgCl₂ and 2 mM EDTA (TME buffer). The hydrolysis of 2-AG to arachidonic acid (AA) was quantitatively measured using HPLC. Varying concentrations of 2-AG from 12.7 µM to 400 µM and hMGL (0.5-2.0 ng) in TME buffer (150 µL) were incubated together at 37 °C. Reaction samples (50 µL) were taken immediately at the start of the incubation and after 20 min, diluted 1:4 with acetonitrile, and centrifuged at 20,000g for 5 min at room temperature. A 10-µL aliquot of supernatant was injected onto the HPLC. In an 8-min HPLC run, 2-AG eluted at 3.0 min and AA at 6.0 min, allowing the reaction to be followed by either substrate (2-AG) turnover or product (AA) formation (Figure 2.6). Analytes were quantified with external standards.⁴⁰
Figure 2.6 A-B: HPLC-based hMGL activity assessment based on 2-AG hydrolysis yielding product AA. At T=0, 2-AG is the dominant peak eluting at 3.0 min (A) At T=20, the majority of 2-AG was hydrolyzed by hMGL to yield AA, eluting at 6 min (B).
2.3.6 Fluorescence based Inhibition Assays using AHMMCE

A fluorometric assay was developed in a 96-well plate (Costar 3650) format by which MGL activity was quantified based on the hydrolysis of substrate 7-hydroxy-6-methoxy-4-methylcoumarin ester (AHMMCE) to fluorophore 7-hydroxy-4-methylcoumarin (HMC). In brief, known amounts of hMGL were incubated with various concentrations of AHMMCE for up to 120 min at room temperature (≈ 22 °C). During this time, fluorescence readings at 360 nm/460 nm ($\lambda_{\text{excitation}}/\lambda_{\text{emission}}$) were taken every 15 min using a Synergy HT Plate Reader (Bio-Tek Instruments, Winooski, VT). The coumarin HMC formed after 2 h (i.e., within the linear assay response) was plotted against inhibitor AM6701 concentration and a nonlinear regression equation was used to determine IC$_{50}$ values. (Prism software, Version 4; GraphPad, San Diego, CA, USA). All MGL assays were performed in triplicate for each substrate concentration.

2.3.7 Inhibition of hMGL and sample preparation for MALDI TOF MS analysis

Purified enzyme in 10 mM Tris-HCl, pH 8.0 buffer (30 μL, 3.1 μg, 3 μM) with varying amounts of inhibitor AM6701 (3, 10 and 25 μM) were allowed to interact for 1 hour at room temperature. Inhibition was terminated by desalting using Bio-Spin 6 Column with 50 mM ammonium bicarbonate buffer containing 0.02% CYMAL, pH 8.0. The protein was digested with trypsin (200 ng) overnight either directly or after
reduction and alkylation under safe condition using DTT (17 mM at room temperature for 30min) and IAM (55 mM at room temperature for 1 hour in the dark).

2.4 Conclusions

His6-tagged hMGL was expressed in *E.coli* and subsequently purified in a single step using IMAC yielding substantial amounts of viable protein. Functional characterization of the recombinant hMGL enzyme demonstrated that its affinities ($K_m$) for both native substrate (2-AG) and a novel fluorogenic reporter, AHMMCE, were high with acceptable turnover rates ($V_{\text{max}}$). The affinity of hMGL for 2-AG was similar to that of the crude rat-brain enzyme.

Proteomic data obtained through a full MS characterization determined that pure hMGL was generated using the established large-scale expression and isolation protocol. The aforementioned results represent the first report on the functional characteristics and proteomics of purified human MGL. The carbamidomethylation patterns of cysteine-containing peptides identified in trypsin digests hMGL indicated that functional enzyme does not contain disulfide bonds, suggesting the importance of free sulfhydryls to functional hMGL. MALDI TOF-MS analysis of hMGL tryptic peptides also demonstrated the removal of N-terminal methionine as a post-translational modification. AM6701, a highly potent irreversible inhibitor was utilized for elucidation of enzyme inactivation using MS studies. Spectral analysis demonstrated that Ser$^{129}$ (Ser$^{122}$ in untagged protein) was a component in hMGL’s catalytic site and therefore, plays a vital role in enzyme activity. Our *E. coli*
expression system offers a high-yield source of functional hMGL whose purity makes it a unique asset for crystallization, structural analyses, and identifying potential pharmacotherapeutics that modulate hMGL activity.

The success of this project lies with the significant contributions made by several members of the CDD group. Dr. Pathi Pandarinathan synthesized AM6701 in addition to developing the fluorescent inhibition assay with Dr. Nikolai Zvonok. Dr. Zvonok and I were responsible for the expression and purification of human MGL. The mass spectrometric analysis was performed by Dr. John Williams. This work was supported by grants from the National Institute on Drug Abuse, National Institutes of Health: DA09158, DA00493, DA03801, DA07215, and DA07312 (AM).
2.5 References


(12) Viso, A.; Cisneros, J. A.; Ortega-Gutierrez, S. Topics in Medicinal Chemistry 2008, 8, 231.


CHAPTER 3

DIACYLGLYCEROL LIPASE ASSAY DEVELOPMENT
3.1 Diacylglycerol Lipase (DGL) Introduction:

Diacylglycerol lipases (DGLs) are metabolic enzymes that are involved in mediating neuronal growth during development and as a retrograde messenger.1-3 DGL was shown to exist in two isoforms α (120 kDa) and β (70 kDa). It was first cloned and characterized the enzyme in 2003. DGL has four transmembrane domains with both the C and N terminus located inside the membrane.4 The enzyme belongs to the serine hydrolase family of enzymes consistent with findings that characterize its catalytic site to include Ser472, Asp524 and His650.5

DGL-β is predominantly expressed in the developing brain, while the α-isoform is mainly found in adult cells. During brain development, DGL is located on the axonal tract producing 2-AG, which mediates axonal growth.2,3 In adults, DGL is located post synaptically to produce and release 2-AG from diacylglycerols in retrograde signaling.3,4 Diacylglycerides (DAGs) are mainly produced from hydrolysis of either phosphoinositides or phosphatidic acid containing an arachidonate group (Scheme 5.1). DAGs can also be produced by hydrolysis of triglycerides. 1-Stearoyl-2-arachidonoyl-sn-glycerol is the principal 1,2-diacyl-sn-glycerol component of the brain and nerves.6-11 The catalytic site of DGL is unique among the lipases as it selectively hydrolyses the sn-1 position of glycerides.4 DGL-α knockout mice showed an absence of 2-AG signaling, while in DGL-β knockout mice, 2-AG production was largely unaffected.1 It was further shown that DGL-α is more prevalent1 and more abundant1 than the β-isozyme in the central nervous system.
Scheme 3.1: Biosynthesis of 2-AG.

3.2 DGL Therapeutic Value

Pharmacological manipulation of 2-AG metabolism greatly impacts endocannabinoid signaling. Inhibition of DGL is expected to produce a blockade of 2-AG signaling with therapeutic application similar to those of cannabinoid antagonists. Known CB antagonists such have been shown to aid in weight loss.
inflammation\textsuperscript{17,18} and Parkinson’s symptoms,\textsuperscript{19,20} in addition to attenuating the reward system for substance abuse.\textsuperscript{21-27} The attenuation of signaling by the constitutive cannabinoid receptors would be distinct from the effects of inverse-agonist drugs such as rimonabant.\textsuperscript{28}

3.3 Previous DGL Assays

Various assays such as those incorporating radiolabeled substrates, mass spectrometry as well as fluorescent substrates have been reported.

a) Radiolabeled Substrates

Radiolabeled substrates containing carbon (\textit{sn}-1-[\textsuperscript{14}C]-oleoyl-2-arachidonoyl glycerol, \textit{sn}-1-stearoyl-2-[\textsuperscript{14}C]-arachidonoyl glycerol, \textit{sn}_1-2-dipalmitoyl-1-[\textsuperscript{14}C]-glycerol) and hydrogen (\textit{sn}-1-stearoyl-2-[\textsuperscript{3}H]-arachidonoyl glycerol, \textit{sn}-1-arachidonoyl-2-[\textsuperscript{3}H]-arachidonoyl glycerol) isotopes were utilized in thin layer chromatography (TLC)-based assays. In each of these assays, increasing concentrations of inhibitor compound were added to enzyme and substrate mixture before 10-20 minute incubation. After solvent extraction and lyophilization under vacuum, the resulting extracts were fractioned by TLC. The radioactive substrate and product spots were either quantified with a \(\beta\)-counter or phosphoimager.\textsuperscript{4,29-32}

b) Mass Spectrometry

Mass spectrometry has been utilized in many applications in order to characterize DGL’s enzymatic activity. Assays include incubation of enzyme with substrate and potentially inhibitors followed by spectral analysis of product via liquid chromatography mass spectrometry (LC/MS) or isotope-dilution LC/MS. Common
substrates include 1-stearoyl-2-arachidonoyl-\(sn\)-glycerol, 1,3-diheptadecanoy-\(sn\)-glycerol (DHDG), \(sn\)-1-[\(^{14}\)C]oleoyl-2-arachidonoyl glycerol, and triolein (triglyceride formed from oleic acid).\(^{4,33,34}\)

c) Fluorescence Assay

Utilizing fluorescent assays is considered a relatively fast and sensitive method, making it ideal for high throughput applications. Fluorescent substrate DiFMU-octanoate (6, 8-difluoro-4-methylcoumarin-octanoate) was used by Bristol Myers Squibb in their characterization of DGL activity. When incubated with DGL-\(\alpha\), enzymatic hydrolysis of the substrate releases the fluorescent methylcoumerin moiety, thus exhibiting an increase in fluorescence. Presence of inhibitors attenuates the output of fluorescence. Changes in DiFMU fluorescence were converted to specific activity.\(^5\)

3.4: Förster Resonance Energy Transfer (FRET)-based DGL Assay

3.4.1 Introduction

Inhibitor compound activity was previously tested using a TLC-based assay with radio-labeled substrate \([1^{-14}\text{C}]\) 1-stearoyl-2-arachidonoyl-\(sn\)-glycerol (\([1^{-14}\text{C}]\) SAG). However, it is time consuming and can’t be applied in a high throughput manner. We then envisioned developing a Förster resonance energy transfer (FRET) assay that can be utilized as a high through-put screening method.

The mechanism of FRET can be described by the energy transfer between two chromophores: a fluorophore (donor) and a quencher (acceptor). The donor, in an excited state, transfers energy to the acceptor through radiationless dipole-dipole coupling; limiting detectable fluorescence.\(^{35}\) When either the donor or quencher is
dissociated from the molecule, their interaction is disrupted (via hydrolysis), thus increasing the amount of detectable fluorescence (donor emission upon donor excitation). The efficiency ($E$, the percentage of energy transferred per excitation) of FRET pairings is demonstrated by the equation in Figure 3.1 where $K_{ET}$ represents the rate of energy transfer, $K_f$ equals radiative decay rate and $K_i$ equals the rate constants of additional de-excitation pathways. Efficiency of FRET also depends on many parameters including the distance between donor and acceptor which ideally should be between 10–100 Å, the necessary spectral overlap of donor and acceptor emission spectra (Figure 3.2), as well as the relative orientation of donor and acceptor absorption dipole moments (they should be approximately parallel). The following parameters describing efficiency is demonstrated by the equation in Figure 3.3 whereby $r$ equals the distance between fluorophore and quencher while $R_0$ represents the Förster distance: the distance at which energy transfer is 50% efficient.\(^{36-38}\)

\[
E = \frac{k_{ET}}{k_f + k_{ET} + \sum k_i}
\]

**Figure 3.1:** FRET efficiency equation

\[
E = \frac{1}{1 + (r/R_0)^6}
\]

**Figure 3.3:** FRET efficiency equation

**Figure 3.2:** spectral overlap of FRET pairs (Invitrogen.com).
3.4.2 Substrate Design and Synthesis

FRET substrate design (Figure 3.5) was based on DGL’s natural substrate; 1-stearoyl-2-arachidonoyl-sn-glycerol. These lipid molecules (1-6, Figure 3.5) were synthesized with \(sn-1\) and \(sn-2\) acyl groups containing terminal functionality as well as biomimetic stereochemistry at \(sn-2\) position. The FRET pairs studied were the dinitrophenyl or nitroxyl group quenchers with either the nitrobenzoxazole (NBD) or pyrene fluorophors. Excitation of the pyrene or NBD fluorophore results in non-radiative energy transfer to the quenchers when distance and orientation are sufficient.

![FRET substrate design](image)

**Figure 3.4:** FRET substrate design.

Scheme 3.2 outlines the synthetic route utilized in preparation of these fluorescent reporter compounds. Regio-selective opening of the epoxide of \((R)-(-)\)-glycidyl methyl ether 7 with benzyl alcohol and sodium hydride provided the secondary alcohol 8 which was treated with imidazole and tetrabutyldimethylsilyl chloride (TBS-Cl) in dichloromethane to provide the protected \(sn-2\) derivative 9. Before derivitizing the \(sn-1\) position, the benzyl protecting group was removed via...
hydrogenolysis (H₂, Pd/C, 40 psi, EtOH/EtOAc/CH₃COOH 1:1:0.2). The resulting free hydroxyl 10 was further esterified with acid in the presence of ethyldimethylamino carbodiimide (EDCI) and dimethylamino pyridine (DMAP).

Various derivatives (11, 13, 15, 17, 19) at this position were prepared by utilizing various carboxylic acids such as dinitrophenyl-ε-amino-n-caproic acid, 1-pyrene butyric acid, 1-pyrene decanoic acid and 5-dimethyloxazolinyloxy (DOXYL)-stearic acid, 16-DOXYL-stearic acid. The resulting compounds were then treated with triethylamine trihydrofluoride to deprotect the secondary hydroxyl group. Only 5% impurity was observed due to acyl migration.

Subsequently, the free sn-2 position was esterified with various acids such as dinitrophenyl-ε-amino-n-caproic acid, 1-pyrene butyric acid and (6-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino) hexanoic acid. Synthesizing a series of compounds 1-6 allowed for an exploration of optimum fluorphore and quencher moiety placement and pairings.

Scheme 3.2: FRET substrate synthesis.
3.4.3 FRET Results and Discussion

Pyrene donor and dinitrophenyl acceptor 1 and 2 FRET pairings were deemed satisfactory, although the 2-pyrenyl analog 1 was used for all high-throughput assays. Fluorescence data (Figure 3.6 and Figure 3.7) indicated that compounds 1 and 2 respectively demonstrate a good response to enzymatic hydrolysis (evident by marked increase in fluorescence) as shown in Figure 3.6.
**Figure 3.6**: Fluorescence data for reporter compounds 1, 2, 3, 5, 6 (EX $\lambda=485$ nM, EM $\lambda=535$ nm).

**Figure 3.7**: Fluorescence data for compound 4 (EX $\lambda=485$ nM, EM $\lambda=535$ nm).
The fully extended distances between the fluorophors and quenchers were estimated (Schrödinger Suite 2010, in an aqueous environment with a dielectric constant of 80) to be 18 Å for 1, 2, and the NBD analog; and, to be 24 Å for the pyrenedecanoyl analog. The assays with NBD had too much baseline instability as this fluorescent group is quite sensitive to the polarity of its environment (Figure 3.7). The nitroxyl groups did not quench sufficiently at the distances estimated to be 15 Å and 24 Å for the 5-DOXYL-stearoyl analog 5 and the 16-DOXYL-stearoyl analog, respectively, to show a good response to enzymatic hydrolysis (Figure 3.6). The pyrene-dinitrophenyl FRET pairings 1 and 2 were stable to uncatalyzed hydrolysis at neutral pH and were readily adapted to a 96-well format. Initial qualitative assay results utilizing substrate 1 and select hDGLα inhibitors were encouraging. THL, used as a control was shown to inhibit hDGLα 99% at 10 µM. Lipoprotein lipase standard (0.23 µg) was used as a positive control. THL analogs (L-isoleucyl, L-allo-isoleucyl, D-isoleucyl, D-allo-isoleucyl, α-aminobutryl tetrahydrolipstatin) discussed in chapter five, inhibited hDGLα with similar values.

Preliminary studies using ether lipid substrate 1 in the high-throughput FRET-based assay demonstrated inhibition of commercially available homogeneous bacterial lipoprotein lipase and commercially available homogeneous porcine triacylglycerol lipase, confirming the potency of THL for the inhibition of these closely related lipases (Figure 3.8).
A convenient high-throughput (HT) fluorometric esterase assay utilizing the BioTek Instruments Synergy™ HT Multi-Mode Microplate 96-well reader was developed that measured nanomolar concentrations of fluorescent reaction product. Cell lysate or membrane preparations containing over-expressed DGL catalyzed the hydrolysis of the reporter substrate, and a fluorescence response increased at a nearly linear rate for over two hours. Wells that had a 15 minute pre-incubation with DGL inhibitors and showed a concentration dependent attenuation of fluorescence response were identified as “hits.”

**Figure 3.8:** Fluorescence data for THL inhibition of DGL activity in presence of 1.
3.4.4 Experimental Section

3.4.4a FRET Substrates

(S)-3-methoxy-2-((4-(pyren-1-yl)butanoyl)oxy)propyl-6-((2,4-dinitrophenyl)amino)hexanoate

1: 1-pyrene butyric acid (31.4 mg, 0.108 mmol) was added to a stirred solution of alcohol 12 (35.0 mg, 0.0908 mmol) in anhydrous DCM under N₂ atmosphere. The reaction mixture was cooled to 0 °C and EDCI (60.9 mg, 0.317 mmol) and DMAP (22.0 mg, 0.181 mmol) were added. The reaction was stirred and monitored by TLC (1:1 ethyl acetate/hexane: starting material Rₖ 0.40, product Rₖ 0.55). After consumption of starting material, the reaction mixture was subjected to an aqueous workup and extraction with DCM. The organic layer was dried with MgSO₄ and concentrated under reduced pressure. The crude product was purified by column chromatography (silica gel 4:6 ethyl acetate/hexane) to yield a yellow semi-solid (44% yield). ¹H NMR (500 MHz, chloroform-d) δ ppm 1.20 - 1.32 (m, 2 H), 1.46 (quintet, J = 7.5 Hz, 2 H), 1.59 (quint, J = 7.6 Hz, 2 H), 2.14 - 2.26 (m, 2 H), 2.30 (t, J = 7.3 Hz, 2 H), 2.54 (t, J = 7.1 Hz, 2 H), 2.89 (apparent q, J = 7.3 Hz, 2 H), 3.39 (s, 3 H), 3.34 - 3.44 (m, 2 H), 3.55 (dd, J = 10.7, 5.1 Hz, 1 H), 3.58 (dd, J = 10.7, 5.1 Hz, 1 H), 4.22 (dd, J = 12.2, 7.3 Hz, 1 H), 4.44 (dd, J=12.0, 3.2 Hz, 1 H), 5.31-5.38 (m, 1
H), 6.37 (d, \( J = 9.8 \) Hz, 1 H), 7.85 (d, \( J = 7.8 \) Hz, 1 H), 7.90 - 8.01 (m, 4 H), 8.05 - 8.18 (m, 5 H), 8.28 (d, \( J = 9.3 \) Hz, 1 H), 8.89 (d, \( J = 2.9 \) Hz, 1 H).

(S)-1-methoxy-3-((4-(pyren-1-yl)butanoyl)oxy)propan-2-yl-6-((2,4-dinitrophenyl)amino)hexanoate

was added to a stirred solution of alcohol 16 (76.0 mg, 0.2 mmol) in anhydrous DCM under N\(_2\) atmosphere. The reaction mixture was cooled to 0 °C and EDCI (134.0 mg, 0.7 mmol) and DMAP (48.8 mg, 0.4 mmol) were added. The reaction was stirred and monitored by TLC (1:1 ethyl acetate/hexane: starting material R\(_f\) 0.40, product R\(_f\) 0.55). After consumption of starting material, the reaction mixture was subjected to an aqueous workup and extraction with DCM. The organic layer was dried with MgSO\(_4\) and concentrated under reduced pressure. The crude product was purified by column chromatography (silica gel 4:6 ethyl acetate/hexane) to provide a yellow semi-solid (50%). \(^1\)H NMR (500 MHz, chloroform-\( d \)) \( \delta \) ppm 1.21-1.30 (m, 2 H), 1.44 (quintet, \( J = 7.4 \) Hz, 2 H), 1.58 (quintet, \( J = 7.6 \) Hz, 2 H), 2.18 (quintet, \( J = 7.4 \) Hz, 2 H), 2.32 (t, \( J = 7.3 \) Hz, 2 H), 2.48 (t, \( J = 7.3 \) Hz, 2 H), 2.83 (dd, \( J = 13.2 \), 7.3 Hz, 2 H), 3.36 (s, 3 H), 3.32 - 3.38 (m, 2 H), 3.52 (dd, \( J = 10.7 \), 5.4 Hz, 2 H), 3.54 (dd, \( J = 10.7 \), 5.4 Hz, 2 H), 4.21 (dd, \( J = 12.0 \), 6.6 Hz, 1 H), 4.43 (dd, \( J = 12.2 \), 3.4 Hz, 1 H), 5.23-5.30 (m, 1 H), 6.28 (d, \( J = 9.3 \) Hz, 1 H), 7.82 (d, \( J = 7.3 \) Hz, 1 H), 7.85 (dd, \( J = 9.5 \), 2.7 Hz, 1 H),
7.90 - 7.97 (m, 3 H), 8.02 - 8.12 (m, 5 H), 8.23 (d, J = 9.3 Hz, 1 H), 8.83 (d, J = 2.4 Hz, 1 H). HRMS for C_{36}H_{37}N_{3}O_{9} [MH^+] calc'd, 655.2518; found, 655.2529.

(S)-2-((6-((2,4-dinitrophenyl)amino)hexanoyl)oxy)-3-methoxypropyl-10-(pyren-1-yl)decanoate

3: DNP-ε-amino-η-caproic acid (23.1 mg, 0.078 mmol) was added to a stirred solution of alcohol 14 (30 mg, 0.06 mmol) in anhydrous DCM under N$_2$ atmosphere. The reaction mixture was cooled to 0 °C and EDCI (40 mg, 0.21 mmol) and DMAP (14.6 mg, 0.12 mmol) were added. The reaction was stirred and monitored by TLC (1:1 ethyl acetate/hexane: starting material R$_f$ 0.50, product R$_f$ 0.7). After consumption of starting material, the reaction mixture was subjected to an aqueous workup and extraction with DCM. The organic layer was dried with MgSO$_4$ and concentrated under reduced pressure. The crude product was purified by column chromatography (silica gel 3:7 ethyl acetate/hexane) to provide an oil (46%). $^1$H NMR (500 MHz, chloroform-$d$) δ ppm 1.31 (br. s, 6 H) 1.35 - 1.43 (m, 2 H) 1.42-1.55 (m, 2 H) 1.54 - 1.65 (m, 4 H) 1.64 - 1.75 (m, 4 H) 1.86 (quin, J=7.57 Hz, 2 H) 2.31 (t, J=7.32 Hz, 2 H) 2.38 (t, J=7.32 Hz, 2 H) 3.21 (dd or q, J= 12.21 Hz, 6.84 Hz, 2 H) 3.33 (t, 2 H) 3.37 (s, 3 H) 3.53 (dd, J=4.88, 1.95 Hz, 2 H) 4.15 (dd, J=11.72, 6.35 Hz, 1 H) 4.36 (dd, J=12.21, 3.91 Hz, 1 H) 5.23 (dt, J=10.25 Hz, 5.37 Hz, 1 H) 6.67 (d, J=9.28 Hz, 1 H) 7.86 (d, J=7.81 Hz, 1 H) 7.94 - 8.04 (m, 4
H) 8.06 - 8.18 (m, 4 H) 8.26 (d, J=9.28 Hz, 1 H) 8.39 (m, 1 H) 9.03 (d, J=2.44 Hz, 1 H) HRMS for C₄₂H₄₉N₃O₉ [MH⁺] calc'd, 739.34683; found, 739.34693.

(S)-1-((6-((2,4-dinitrophenyl)amino)hexanoyl)oxy)-3-methoxypropan-2-yl-6-((7-nitrobenzol[c][1,2,5]oxadiazol-4-yl)amino)hexanoate

4: 6-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino hexanoic acid (36.6 mg, 0.12 mmol) was added to a stirred solution of alcohol 12 (40 mg, 0.10 mmol) in anhydrous DCM under N₂ atmosphere. The reaction mixture was cooled to 0 °C and EDCI (69.0 mg, 0.36 mmol) and DMAP (25.16 mg, 0.20 mmol) were added. The solution was stirred and monitored by TLC (1:1 ethyl acetate/hexane: starting material R₉ 0.40, product R₉ 0.55). After consumption of starting material, the reaction mixture was subjected to an aqueous workup and extraction with DCM. The organic layer was dried with MgSO₄ and concentrated under reduced pressure. The crude product was purified by column chromatography (silica gel 4:6 ethyl acetate/hexane) to provide a yellow semi-solid (70 %).

H NMR (500 MHz, chloroform-d) δ ppm 1.52 (m, 4 H) 1.65-1.77 (m, 4H) 1.75-1.88 (m, 4H) 2.38 (dt, J=15.02, 7.39 Hz, 4 H) 3.36 (s, 3 H) 3.44 (dd, J=12.21 Hz, 6.84 Hz, 2 H) 3.48 - 3.59 (m, 4 H) 4.14 (dd, J=11.96, 6.10 Hz, 1 H) 4.38 (dd, J=12.21, 3.91 Hz, 1 H) 5.22 (ddd, J=10.25 Hz, 5.37 Hz, 1 H) 6.18 (d, J=8.30 Hz, 1 H) 6.57 (t, J=5.13 Hz, 1 H) 6.92 (d, J=9.28 Hz, 1 H) 8.26 (dd, J=9.52, 2.69 Hz, 1 H) 8.47 (d, J=8.79 Hz, 1 H) 8.52-8.60
(m., 2 H) 9.10 (d, J=2.44 Hz, 1 H) HRMS for C_{28}H_{35}N_{7}O_{12} [MH\textsuperscript{+}] calc'd, 661.23303; found, 661.23433.

(S)-2-pyrenebueryl-3-methoxypropyl 5-(dimethyloxazolinyloxy)stearoate

5: Pyrene butyric acid (16.1 mg, 0.06 mmol) was added to a stirred solution of alcohol 18 (22 mg, 0.046 mmol) in anhydrous DCM under N\textsubscript{2} atmosphere. The reaction mixture was cooled to 0 °C and EDCI (31 mg, 0.16 mmol) and DMAP (11.4 mg, 0.09 mmol) were added. The solution was stirred and monitored by TLC (1:4 ethyl acetate/hexane: starting material R\textsubscript{f} 0.15, product R\textsubscript{f} 0.4). After consumption of starting material, the reaction mixture was subjected to an aqueous workup and extraction with DCM. The organic layer was dried with MgSO\textsubscript{4} and concentrated under reduced pressure. Impurities were remove The crude product was purified by column chromatography (silica gel 1:5 ethyl acetate/hexane) to provide an oil (66%). Product was confirmed by mass spectrometry. HRMS for C_{46}H_{66}N_{7}O_{7} [MH\textsuperscript{+}] calc'd, 744.4484; found, 744.483.

(S)-2-pyrenebueryl-3-methoxypropyl 16-(dimethyloxazolinyloxy)stearoate

6: Pyrene butyric acid (6.58 mg, 0.02
mmol) was added to a stirred solution of alcohol 20 (9.0 mg, 0.019 mmol) in anhydrous DCM under N₂ atmosphere. The reaction mixture was cooled to 0 °C and EDCI (12.7 mg, 0.06 mmol) and DMAP (4.6 mg, 0.03 mmol) were added. The solution was stirred and monitored by TLC (1:4 ethyl acetate/hexane: starting material R_f 0.15, product R_f 0.4). After consumption of starting material, the reaction mixture was subjected to an aqueous workup and extraction with DCM. The organic layer was dried with MgSO₄ and concentrated under reduced pressure. The crude product was purified by column chromatography (silica gel 1:5 ethyl acetate/hexane) to provide an oil (40%). Product was confirmed by mass spectrometry. HRMS for C₄₆H₆₆NO₇ [MH⁺] calc'd, 744.4839; found, 744.4844.

(S)-1-(benzyloxy)-3-methoxypropan-2-ol

8: Benzyl alcohol (2.69 g, 2.5 mL) was added dropwise to a stirred solution of NaH (0.59 g, 24.9 mmol) in anhydrous THF at room temperature under N₂ atmosphere. The solution was warmed to 0 °C and (R)-(−)-Glycidyl methyl ether 7 (2.0 g, 22.6 mmol) was added dropwise. The mixture was stirred at room temperature overnight while being monitored by TLC (3:7 ethyl acetate/hexane: starting material R_f 0.1, product R_f 0.3). Upon completion, the mixture was concentrated and re-dissolved in DCM before being acidified using 1 M HCl. After a series of aqueous extractions was performed, the organic layer was dried with MgSO₄, filtered and concentrated. The crude product was purified by column chromatography (silica gel, 3:7 ethyl acetate/hexane). A 22% yield was obtained.
NMR (500 MHz, chloroform-\textit{d}) \(\delta\) ppm 3.39 (s, 3 H) 3.42 - 3.59 (m, 4 H) 3.96 - 4.05 (m, 1 H) 4.57 (s, 2 H) 7.28 - 7.53 (m, 5 H).

**(S)-((1-(benzyloxy)-3-methoxypropan-2-yl)oxy)(tert-butyl)dimethylsilane**

9: tertbutyldimethyamo n chloride (TBS-Cl) (0.168 g, 2.48 mmol) was added to a stirred solution of secondary alcohol 8 (0.243 g, 1.24 mmol) and imidazole (0.168 g, 2.48 mmol) in anhydrous dimethylformamide (DMF) under nitrogen. The reaction was allowed to stir at room temperature while being monitored by TLC (5:95 ethyl acetate/hexane: starting material \(R_f\) 0.2, product \(R_f\) 0.85). Upon completion, an aqueous workup was performed. The resulting organic layers were dried with MgSO\(_4\) and concentrated. The crude product was purified by column chromatography (silica gel, 5:95 ethyl acetate/hexane). A 66% yield was obtained. \(^1\)H NMR (500 MHz, chloroform-\textit{d}) \(\delta\) ppm 0.08 (d, \(J=2.93\) Hz, 6 H) 0.89 (s, 9 H) 3.36 (s, 3 H) 3.38 (dd, \(J=10.01, 5.62\) Hz, 1 H) 3.45 (m, \(J=8.79\) Hz, 2 H) 3.52 (m, \(J=5.37\) Hz, 1 H) 3.97 (quin, \(J=5.37\) Hz, 1 H) 4.55 (s, 2 H) 7.28 - 7.42 (m, 5 H).

**(R)-2-((tert-butyldimethylsilyl)oxy)-3-methoxypropan-1-ol**

10: Benzyl-protected molecule 9 (0.73 g, 2.36 mmol) was dissolved in a solution of ethanol, ethyl acetate and acetic acid (1:1:0.2). After the solution was degassed, 10% Pd/C (150 mg) was added. The solution was stirred overnight under hydrogen (40.0 psi). The reaction was monitored
by TLC (1:4 ethyl acetate/hexane). Upon completion, the solution was filtered over a short pad of celite. The crude product was purified by column chromatography (silica gel, 1:4 ethyl acetate/hexane: product Rf 0.3). A quantitative yield was obtained. ¹H NMR (500 MHz, chloroform-d d) δ ppm 0.10 (s, 6 H) 0.90 (s, 9 H) 3.36 (s, 3 H) 3.40 (m, J=5.86, 3.91 Hz, 2 H) 3.58 (m, J=4.39 Hz, 1 H) 3.64 (m, J=4.39 Hz, 1 H) 3.83 - 3.92 (m, 1 H).

11: Primary alcohol 10 (0.39 g, 1.77 mmol) was added to a stirred solution of DNP-ε-amino-n-caproic acid (0.578 g, 1.945 mmol) in anhydrous DCM under nitrogen. The mixture was cooled to 0 °C and EDCI (0.848 g, 4.42 mmol) and DMAP (0.43 g, 3.54 mmol) were added. The solution was stirred and monitored by TLC (1:4 ethyl acetate/hexane). Upon completion, an aqueous workup was performed and the organic layers were dried with MgSO₄ and concentrated under reduced pressure. The crude product was purified by column chromatography (silica gel, 1:4 ethyl acetate/hexane: product Rf 0.2). An 80% yield was obtained. ¹H NMR (500 MHz, chloroform-d d) δ ppm 0.08 (d, J=2.44 Hz, 6 H) 0.88 (s, 9 H) 1.26 (t, J=7.32 Hz, 2 H) 1.50 (m, J=7.32 Hz, 2 H) 1.72 (quin, J=7.57 Hz, 2 H) 1.81 (quin, J=7.45 Hz, 2 H) 2.37 (t, J=7.32 Hz, 2 H) 3.35 (s, 3 H) 3.42 (m, J=5.37 Hz, 2 H) 3.93 - 4.05 (m, 2 H) 4.08 - 4.24 (m, 1 H) 6.91 (d, J=9.77
Hz, 1 H) 8.28 (dd, J=9.28, 2.44 Hz, 1 H) 8.50 - 8.62 (m, 1 H) 9.16 (d, J=2.44 Hz, 1 H).

(S)-2-hydroxy-3-methoxypropyl-6-((2,4-dinitrophenyl)amino)hexanoate

12: Tetrabutylammonium fluoride (1.69 mmol, 1.69 mL) was added dropwise to a stirred solution of silyl protected 11 (0.73 g, 1.45 mmol) in anhydrous THF under nitrogen. The solution was stirred and monitored by TLC (1:1 ethyl acetate/hexane). Following concentration, the crude product was purified by column chromatography (silica gel, 1:1 ethyl acetate/hexane: product R_f 0.15). A 79% yield was obtained. ¹H NMR (500 MHz, chloroform-d) δ ppm 1.48 - 1.61 (m, 4 H) 1.70 - 1.78 (m, 2 H) 1.82 (quin, J=7.32 Hz, 2 H) 2.39 - 2.47 (m, 2 H) 3.41 (s, 3 H) 3.42 - 3.51 (m, 2 H) 3.97 - 4.05 (m, 0 H) 4.13 (m, J=9.77, 6.84 Hz, 1 H) 4.17 - 4.23 (m, 1 H) 6.92 (d, J=9.28 Hz, 1 H) 8.29 (dd, J=9.28, 2.44 Hz, 1 H) 8.56 (br. s, 1 H) 9.16 (d, J=2.44 Hz, 1 H).

(S)-2-((tert-butyldimethylsilyl)oxy)-3-methoxypropyl 10-(pyren-2-yl)decanoate

13: Primary alcohol 10 (13.4 mg, 0.06 mmol) was added to a stirred solution of 1-pyrenedecanoic acid (25.0 mg, 1.27 mmol) in anhydrous DCM under N₂ atmosphere. The mixture was
cooled to 0 °C and EDCI (30.0 mg, 2.89 mmol) and DMAP (14.9 mg, 0.12 mmol) were added. The solution was stirred at room temperature while being monitored by TLC (1:9 ethyl acetate/hexane). Upon completion, an aqueous workup was performed and the organic layer was dried with MgSO₄ and concentrated under reduced pressure. The crude product was purified by column chromatography (silica gel, 1:9 ethyl acetate/hexane). A quantitative yield was obtained. ¹H NMR (500 MHz, chloroform-d) δ ppm 0.10 (s, 6 H) 0.90 (s, 9 H) 1.32 (br. s, 8 H) 1.35 - 1.43 (m, 2 H) 1.49 (quin, J=7.32 Hz, 2 H) 1.63 (m, J=6.84 Hz, 2 H) 1.86 (quin, J=7.69 Hz, 2 H) 2.32 (t, J=7.57 Hz, 2 H) 3.32 - 3.35 (m, 1 H) 3.36 (s, 3 H) 3.37 - 3.40 (m, 1 H) 4.01 (d, J=6.35 Hz, 2 H) 4.19 (q, J=6.84 Hz, 1 H) 7.88 (d, J=7.81 Hz, 1 H) 7.96 - 8.07 (m, 3 H) 8.12 (dd, J=8.55, 4.15 Hz, 2 H) 8.17 (m, J=5.37 Hz, 2 H) 8.29 (d, J=9.28 Hz, 1 H).

(S)-2-hydroxy-3-methoxypropyl 10-(pyren-2-yl)decanoate

14: Triethylamine trihydrofluoride (0.152 g, 0.95 mmol) was added to a stirred solution of TBS-protected molecule 13 (36.4 mg, 0.063 mmol) in anhydrous DCM under N₂ atmosphere. The solution was stirred while being monitored by TLC (1:1 ethyl acetate/hexane). Upon completion, the reaction mixture was concentrated and utilized directly in the next step.
(S)-2-((tert-butyldimethylsilyl)oxy)-3-methoxypropyl 4-(pyren-2-yl)butanoate

15: Primary alcohol 10 (67.6 mg, 0.3 mmol) was added to a stirred solution of with 1-pyrenebutyric acid (0.106 g, 0.37 mmol) in anhydrous DCM under nitrogen.

The mixture was cooled to 0 °C and EDCI (14.6 mg, 0.77 mmol) and DMAP (7.4 mg, 0.61 mmol) were added. The solution was stirred at room temperature while being monitored by TLC (3:7 ethyl acetate/hexane). Upon completion, an aqueous workup was performed and the organic layers were dried with MgSO$_4$ and concentrated under reduced pressure. The crude product was purified by column chromatography (silica gel, 3:7 ethyl acetate/hexane: product R$_f$ 0.75). A 66% yield was obtained. $^1$H NMR (500 MHz, chloroform-d) δ ppm 0.05 (d, $J$=6.35 Hz, 6 H) 0.85 (s, 9 H) 2.19 (quin, $J$=7.45 Hz, 4H) 2.46 (t, $J$=7.32 Hz, 2 H) 3.33 (s, 3 H) 3.33 - 3.42 (m, 2 H) 3.93 - 4.00 (m, 1 H) 4.02 (m, $J$=6.35 Hz, 1 H) 4.20 (dd, $J$=11.23, 3.91 Hz, 1 H) 7.84 (d, $J$=7.81 Hz, 1 H) 7.93 - 8.04 (m, 3 H) 8.05 - 8.11 (m, 2 H) 8.14 (t, $J$=6.59 Hz, 2 H) 8.28 (d, $J$=9.28 Hz, 1 H).

(S)-2-hydroxy-3-methoxypropyl 4-(pyren-2-yl)butanoate

16: Triethylamine trihydrofluoride (48.6 mg, 3.0 mmol) was added to a stirred solution of TBS-protected molecule 15 (99.5 mg, 0.2 mmol) in anhydrous DCM under nitrogen. The solution was
stirred while being monitored by TLC (1:1 ethyl acetate/hexane). Upon completion, the reaction mixture was concentrated and utilized directly in the next step.

(S)-2-tertbutyldimethylsilyl-3-methoxypropyl 5-(dimethyloxazolinyloxy)stearoate

17: Primary alcohol 10 (6.0 mg, 0.027 mmol) was added to a stirred solution of 5-DOXYL-stearic acid (10.5 mg, 0.027 mmol) in anhydrous DCM under nitrogen. The mixture was cooled to 0 °C and EDCI (13.0 mg, 0.07 mmol) and DMAP (6.6 mg, 0.054 mmol) were added. The solution was stirred at room temperature while being monitored by TLC (2:8 ethyl acetate/hexane). Upon completion, an aqueous workup was performed and the organic layer was dried with MgSO₄ and concentrated under reduced pressure. The crude product was purified by column chromatography (silica gel, 2:8 ethyl acetate/hexane: product Rf 0.7). Product was confirmed by mass spectroscopy. A 68% yield was obtained.

(S)-2-hydroxyl-3-methoxypropyl 5-(dimethyloxazolinyloxy)stearoate

18: Triethylamine trihydrofluoride (28.3 mg, 0.175 mmol) was added to a stirred solution of TBS-protected molecule 17 (10.3 mg, 0.0175 mmol) in anhydrous DCM under nitrogen. The solution
was stirred while being monitored by TLC (3:7 ethyl acetate/hexane: product $R_f$ 0.15). After completion of the reaction was verified by TLC, the mixture was concentrated and utilized directly in the next step.

**S-2-tertbutyldimethylsilyl-3-methoxypropyl 16-(dimethyloxazolinyloxyl)stearoate**

Primary alcohol **10** (13.0 mg, 0.059 mmol) was added to a stirred solution of 16-DOXYL-stearic acid (25.0 mg, 0.06 mmol) in anhydrous DCM under nitrogen. The mixture was cooled to 0 °C and EDCI (28.2 mg, 0.147 mmol) and DMAP (17.9 mg, 0.147 mmol) were added. The solution was stirred while being monitored by TLC (2:8 ethyl acetate/hexane: product $R_f$ 0.7). Upon completion, an aqueous workup was performed and the organic layers were dried with MgSO$_4$ and concentrated under reduced pressure. The crude product was purified by column chromatography (silica gel, 5:95 ethyl acetate/hexane). Product mass was confirmed by mass spectroscopy. A 33% yield was obtained.

**S-2-hydroxyl-3-methoxypropyl 16-(dimethyloxazolinyloxyl)stearoate**

Triethylamine trihydrofluoride (31.6 mg, 0.195 mmol) was added to a stirred solution of TBS-protected molecule **19** (11.5 mg, 0.0195 mmol) in anhydrous DCM under N$_2$ atmosphere. The
solution was stirred while being monitored by TLC (3:7 ethyl acetate/hexane: product \(R_f\) 0.15). Upon completion, the reaction mixture was concentrated and utilized directly in the next step.

3.4.4b High Throughput FRET-based DGL Assay

The high-throughput FRET assays were run in Tris-buffer with calcium (final volume 200 µL). The lipoprotein lipase standard (0.23 µg) was again used for a positive control. The freshly sonicated (100 µg total) protein containing DGL was used as a suspension for each assay. A 15 min period of gentle shaking at ambient temperature was used following the addition of 10 µL of pure DMSO (for the control) or the 10 µL dimethylsulfoxide (DMSO) solutions of inhibitors. The lipid substrate (25 µM final concentration) was then added in DMSO (10 µL) to all wells, and after 2 min of shaking at 37 °C, an initial reading was taken with excitation at 320 nm and emission obtained at 400 nm. Every 14 min, another 1 min of shaking would precede the fluorescence readings. The readings were followed over 2 h, but the time point of 1.5 h was used to calculate percent inhibitions. The inhibition of hydrolysis of the lipid reporter compound 1 by the DGL-containing protein preparations with the compounds under investigation screened at 10 µM was then compared with 10 µM THL control, which gives complete inhibition of all human and murine DGLs tested with the radio labeled endogenous substrate \(^{14}\text{C}\) SAG. Using the pyrene-dinitrophenyl reporter compound 1, the \(\text{IC}_{50}\) of THL was always approximately 10 nM with hDGL-α lipase using this high-throughput FRET-based assay.
Diacylglycerol lipase (DGL) proteins were either cell lysates or membrane preparations that were prepared as previously.\textsuperscript{4,39} The Cravatt group at Scripps provided hDGL\textsubscript{\(\alpha\)}, mDAGL\textsubscript{\(\alpha\)}, and mDGL\textsubscript{\(\beta\)} from over expression by transient infection of HEK293T cultures in addition to cell lysate of the empty vector HEK293T control. Some experiments used a second commercially prepared plasmid to provide additional human \(\alpha\)-isoform overexpressed in HEK293T using the same methodology. Human DGL\textsubscript{\(\alpha\)} was poorly overexpressed in all attempts from the transient infection method. The specific activities of hDGL\textsubscript{\(\alpha\)} were 1.5 to 3 times the activities of mock infections with empty plasmid. The DGL activity of hDAGL\textsubscript{\(\alpha\)} expressed in the human cell line was still used for assay of newly synthesized inhibitors, however. At least 100 \(\mu\)g of total protein from crude cell lysates was required per well. The proteins were utilized such that the substrate hydrolysis would proceed to the extent of about 5\% in 20 min. The more readily expressed mDGL\textsubscript{\(\beta\)} isoform (that has a 79\% homology with the human isoform) or mDGL\textsubscript{\(\alpha\)} isoform (that has a 97\% homology with the human isoform) were also used to confirm inhibition of DGL activities.\textsuperscript{4} The specific activities of the hDGL-\(\alpha\) were in the range of 0.003 to 0.01 nmol/mg-min for the cell lysates (specific activity was 0.06 in the presence of 0.05\% Triton x-100) and up to 0.1 nmol/min-mg for membrane (10,000 x g fraction) preparations. DGL activities of protein from empty plasmid transfections were 0.003 to 0.01 nmol/min-mg. The DGL analyses with mDGL-\(\alpha\) used 20 \(\mu\)g of protein from cell lysate with a specific activity above 0.1 nmol/min-mg. The DGL analyses with mDGL-\(\alpha\) used 8.8 \(\mu\)g of protein
from a membrane preparation with a specific activity above 0.3 nmol/min-mg. The specific activity of the lipoprotein lipase under the assay conditions was over 400 nmol/mg-min.

### 3.4.5 FRET Assay Conclusions

New lipid reporter compounds 1-6 were developed for a high throughput assay to screen inhibitors of 1,2-diacyl-sn-glycerol hydrolysis and related lipase activities using fluorescence resonance energy transfer (FRET). Compounds 1 and 2 were deemed the most active, especially 1, based on fluorescence spectroscopy data. False positives can occur due to inhibition of other hydrolytic enzymes present in the cell lysate preparations used. However, the assay can be utilized for preliminary screening of compound libraries. Thus, this new FRET-based methodology can be applied in the future to the assay of new molecules for the inhibition of human recombinant proteins including lipoprotein lipase, triacylglycerol lipase, and other related hydrolases to determine DGL selectivity in vitro.

### 3.5 Studies Towards Selective DGL Substrates

#### 3.5.1 Introduction

Currently, expression methods are unable to produce DGL devoid of superfluous esterases and amidases. These unwanted proteins negatively affect the ability to specifically assay the activity of DGL itself. FAAH and MGL are considered the biggest threats to specificity. To this end, design and synthesis of a selective
substrate was attempted. In order to obtain the most accurate DGL activity and inhibition it was important that the substrate not be active towards any other enzyme except DGL.

### 3.5.2 Substrate Design and Synthesis

The synthesized compounds were based on the DGL natural substrate 1-stearoyl-2-arachidonoyl-sn-glycerol (Figure 3.9) It has been shown that DGL prefers arachidonates at the sn-2 position while saturated chains are preferred in the sn-1 position. The arachidonyl moiety is essential for enzyme recognition. The sn-2 position was therefore, functionalized with different groups (e.g. carbamate, carbonate, retro-carbamate, amide, ester) of arachidonate.

The 2-acyl-sn-glycerol molecules 19, 20, 21 and 22 were first assayed for FAAH and MGL activity. Once certain functional groups susceptible to superfluous enzyme activity were ruled out (Table 3.1), the sn-1 position of the remaining molecules could then be functionalized. Different esters such as biphenyl butyrate, oleate, pyrene butyrate and phenyl caproate were incorporated and the molecules were subsequently tested for DGL recognition and energy transfer potential (Table 3.2).

The concept was to create a pseudo FRET environment where the arachidonyl moiety “quenches” the fluorescence or ultra violet activity of the sn-1 substituent. Upon enzymatic hydrolysis at the sn-1 position, the relationship between “quencher” and “fluorphore” would be disrupted and therefore an increase in energy could be detected and utilized in an assay.
3.5.2a Carbonate

In order to synthesize 2-arachidonyl carbonate-\textit{sn}-glycerol 19 (Scheme 3.3), the primary hydroxyl groups of glycerol were protected using TBS-chloride and DMAP in anhydrous pyridine 24. The secondary alcohol was then activated with \textit{p}-nitrophenyl chloroformate in the presence of DMAP and pyridine 25. An arachidonyl carbamate moiety was subsequently introduced after reaction of the \textit{sn}-2 position with arachidonyl alcohol 26. HF-pyridine was then used to deprotect the primary hydroxyls in order to yield arachidonyl carbonate glycerol 19.
Scheme 3.3: Synthesis of carbonates.

3.5.2b Carbamate

The initial synthesis of the arachidonyl carbamate moiety was similar to that of the carbonate as the primary alcohols of glycerol were TBS-protected while the secondary alcohol was activated with p-nitrophenyl chloroformate. The molecule was then treated with arachidonyl amine in order to yield the required carbamate. Subsequently, the TBS groups were deprotected with tetraethylammonium fluoride. The amine was synthesized from arachidonyl azide which in turn, was synthesized from arachidonyl alcohol. The sn-1 position was then either derivitized with acids in an ester coupling reaction or with coumarin chloroformate to produce a more stable carbonate linkage (Scheme 3.4).
Scheme 3.4: Synthesis of carbamates.

3.5.2c Esters

In order to generate arachidonoyl substrates, synthesis was initiated with methoxy propane diol 35 (Scheme 3.5). The primary alcohol was protected as its trityl derivative 36. The secondary hydroxyl was next protected with a tert-butyl dimethyl silyl group 37. Once the trityl group was removed with camphor sulphonic acid, the primary hydroxyl 38 could be derivitized with acids in an ester coupling reaction 39, 40. After the TBS group was removed with tetrabutyl ammonium fluoride, the sn-2
position was treated with either arachidonic acid or biphenyl butyric acid in EDCI coupling reactions 43, 44.

![Scheme 3.5: Synthesis of esters.](image)

3.5.2c Amides

The amide analog was synthesized by 2-amino-1,3-propanediol 45 with arachidonic acid in an ester coupling reaction 21. The sn-1 position was subsequently functionalized by further ester couplings with oleic acid to obtain 46 (Scheme 3.6).
Scheme 3.6: Synthesis of amides.

3.5.2d Retrocarbamates

A retro-carbamate analog 48 was synthesized by reaction of 2-amino-1,3-propanediol 45 with $p$-nitrophenyl arachidonyl carbonate 22 and subsequent ester couplings at the $sn$-1 position 48 (Scheme 3.7).

Scheme 3.7: Synthesis of retrocarbamates.

3.5.3 Results and Discussion

When assayed using HPLC, 19 and 21 demonstrated FAAH and MGL activity, while 22 and 20 did not (Table 3.1). This indicated that the arachidonyl amide and arachidonyl carbonate moieties were susceptible to hydrolysis and therefore would not
be useful in further development of selective substrates. The carbamate and retro-
carbamate moieties were deemed stable to superfluous hydrolysis via HPLC analysis
and were therefore, utilized in further studies. Activity of the substrate was
represented in specific activity (nmol/min-µg) using a substrate concentration of 100
µM and monitored over 20 min.

Compounds (46, 48, 33, 34, 31, 32) containing functionalized sn-1 positions of
arachidonyl carbamate-sn-glycerol and arachidonyl retrocarbamate-sn-glycerol were
then assayed for DGL activity and assay potential. Initial data indicated that DGLα
was not active for these substrates as hydrolysis was not observed.

<table>
<thead>
<tr>
<th>Compound</th>
<th>FAAH Specific Activity</th>
<th>MGL Specific Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nmol/min-µg</td>
<td>nmol/min-µg</td>
</tr>
<tr>
<td>21</td>
<td>2.2 E-06</td>
<td>1.10 E-04</td>
</tr>
<tr>
<td>22</td>
<td>No activity</td>
<td>No activity</td>
</tr>
<tr>
<td>19</td>
<td>0.015</td>
<td>2.25</td>
</tr>
<tr>
<td>20</td>
<td>No activity</td>
<td>No activity</td>
</tr>
</tbody>
</table>

**Table 3.1:** HPLC selectivity data for 2-acyl glycerols.

Compound 33, (having a pyrene butyrate moiety at the sn-1 position) was tested for activity and
selectivity on a fluorescence spectrometer. In an ideal situation, the fluorphore would be quenched
by the arachidonate moiety and the net fluorescence for the molecule would be nearly zero. When
hydrolyzed by DGL, the free fluorophore would exhibit a detectable amount of fluorescence that could be quantified. Figure 3.10 showed the differential between substrate and hydrolysis product. Although, a significant quenching of the intact substrate was not observed, the small differential could be applicable.

An assay performed with HEK293 cell lysate was next performed in order to make sure that background enzymes in the prep did not elicit the same response as the DGL. Although the same pattern of fluorescence was observed the magnitude of the responses were different (Figure 3.11).

Figure 3.10 Fluorescence differential between AM7843 (33) and hydrolysis product.
In order to obtain evidence of substrate selectivity, MGL (Figure 3.12) and FAAH (Figure 3.13) activity were tested against substrate 33. Hydrolysis was observed but only using significant quantities of enzyme. Regular concentrations of these enzymes in the membrane prep would produce minimal hydrolysis. Based on this evidence, substrate 33 was deemed selective for DGL.

Now that selectivity of the compound was observed, inhibition with known DGL inhibitor THL would be assessed. In assays where 1 µM and 1 nM of inhibitor were used, 75% inhibition of DGL activity was observed (Figure 3.14, Figure 3.15). The inhibition was evident by the reduction in fluorescence for the hydrolysis product, pyrene butyric acid. In order to apply this substrate for future DGL inhibition assays, conditions would have to be painstakingly optimized. Optimization of 33 is currently underway.
Figure 3.12: Hydrolysis of AM7843 (33) with 100 ng purified hMGL.

Figure 3.13: Hydrolysis of 33 with 15 µg purified rFAAH lysate.
Figure 3.14: THL (1µM) inhibition of DGLα for compound 33.

Figure 3.15: THL (1 nM) inhibition of DGLα for compound 33.
3.5.4 Experimental Section

3.5.4a Substrate Molecules

1,3-dihydroxypropan-2-yl (5Z,8Z,11Z,14Z)-icosa-5,8,11,14-tetraen-1-yl carbonate 19: A stirred solution of silyl-protected 26 (32.8 mg, 0.05 mmol) in anhydrous DCM was treated with hydrogen fluoride pyridine (0.013 mL, 0.147 mmol) at 0 °C under nitrogen. The resulting mixture was stirred while being monitored by TLC (3:7 ethyl acetate/hexane: product Rf 0.1). Upon completion of the reaction, the crude mixture was concentrated and purified by column chromatography (silica gel, 1:9 ethyl acetate/hexane) to provide an oil (65%).

$^1$H NMR (500 MHz, chloroform-$d$) δ ppm 0.89 (t, $J$=6.84 Hz, 3 H), 1.10 - 1.40 (m, 10 H), 1.45 (quin, $J$=7.57 Hz, 2 H), 1.55 (s, 2 H), 1.56 - 1.64 (m, 2 H), 2.00 - 2.09 (m, 3 H), 2.11 (q, $J$=7.00 Hz, 2 H), 2.73 - 2.92 (m, 6 H), 3.60 - 3.72 (m, 2 H), 5.23 - 5.52 (m, 8 H). A catalytic amount

1,3-dihydroxypropan-2-yl (5Z,8Z,11Z,14Z)-icosa-5,8,11,14-tetraen-1-ylcarbamate 20: A stirred solution of silyl-protected glycerol 27 (60.0 mg, 0.094 mmol) in anhydrous DCM was treated with hydrogen fluoride pyridine (55 mL, 0.188 mmol) at 0 °C under nitrogen. The resulting mixture was stirred at reduced temperature for 40 minutes and monitored by TLC (3:7 ethyl acetate/hexane: product Rf 0.3). Upon completion of the reaction, the crude mixture
was concentrated and purified by column chromatography (silica gel, 3:7 ethyl acetate/hexane) to provide an oil (75%). $^1$H NMR (500 MHz, chloroform-$d$) δ ppm 0.89 (t, $J=6.84$ Hz, 3 H) 1.16 - 1.46 (m, 8 H) 1.47 - 1.66 (m, 2 H) 1.98 - 2.16 (m, 4 H) 2.32 (br. s, 2 H) 2.72 - 2.90 (m, 6 H) 3.20 (q, $J=6.84$ Hz, 2 H) 3.83 (br. s, 4 H) 4.74 - 4.83 (m, 1 H) 4.85 (br. s, 1 H) 5.21 - 5.47 (m, 8 H).

(5Z,8Z,11Z,14Z)-N-(1,3-dihydroxypropan-2-yl)icos-5,8,11,14-tetraenamide

$^{21}$: A stirred solution of serinol $^{45}$ (100.0 mg, 1.09 mmol) and arachidonic acid (66.4 mg, 0.22 mmol) in anhydrous pyridine at 0 °C was treated with EDCI (125.0 mg, 0.65 mmol) and a catalytic amount of DMAP. The resulting mixture was stirred while being monitored by TLC (3:7 acetone/hexane: product R$_f$ 0.1). Upon completion of the reaction, the mixture was concentrated and the crude product was purified by column chromatography (silica gel, 3:7 acetone/hexane) to provide a clear oil (88%). $^1$H NMR (500 MHz, chloroform-$d$) δ ppm 0.89 (t, $J=6.84$ Hz, 3 H) 1.21 - 1.43 (m, 6 H) 1.73 (quin, $J=7.57$ Hz, 2 H) 2.06 (q, $J=7.32$ Hz, 2 H) 2.09 - 2.17 (m, 2 H) 2.24 (t, $J=7.57$ Hz, 2 H) 2.74 - 2.92 (m, 6 H) 3.71 - 3.80 (m, 2 H) 3.81 - 3.88 (m, 2 H) 3.96 (dt, $J=7.57$, 4.03 Hz, 1 H) 5.11 - 5.59 (m, 8 H) 6.23 (d, $J=6.35$ Hz, 1 H).
(5Z,8Z,11Z,14Z)-icosa-5,8,11,14-tetraen-1-yl-(1,3-dihydroxypropan-2-yl)carbamate

22: A stirred solution of 2-amino-1,3-propanediol 45 (50.0 mg, 0.55 mmol) in anhydrous pyridine at 0 °C was treated with arachidonoyl chloroformate 47 (290 mg, 0.66 mmol) and a catalytic amount of DMAP. The resulting mixture was stirred and monitored by TLC (3:7 ethyl acetate/hexane: product R$_f$ 0.3). Upon completion of the reaction, the mixture was concentrated and the crude product was purified by column chromatography (silica gel, 3:7 ethyl acetate/hexane) to provide a clear oil (81%). $^1$H NMR (500 MHz, chloroform-$d$) δ ppm 0.89 (t, $J$=6.84 Hz, 3 H) 1.19 - 1.52 (m, 8 H) 1.63 (quin, $J$=6.96 Hz, 2 H) 1.94 - 2.21 (m, 4 H) 2.72 - 2.90 (m, 6 H) 3.40 (br. s, 2 H) 3.64 - 3.76 (m, 3 H) 3.77 - 3.88 (m, 2 H) 3.97 - 4.13 (m, 2 H) 5.25 - 5.44 (m, 8 H) 5.52 (br. s, 1 H).

2,2,3,3,9,9,10,10-octamethyl-4,8-dioxa-3,9-disilaundecan-6-ol

24: A stirred solution of glycerol 23 (580.0 mg, 6.3 mmol) and TBS-Cl (1.99 g, 13.2 mmol) in anhydrous pyridine at 0 °C was treated with a catalytic amount of DMAP. The resulting mixture was stirred at reduced temperature for 1.5 hours and monitored by TLC (3:7 ethyl acetate/hexane: product R$_f$ 0.8). Upon completion of the reaction, the mixture was concentrated, subjected to an aqueous workup and subsequently purified by column chromatography (silica gel, 3:7 ethyl acetate/hexane) to provide a clear oil (87%). $^1$H NMR (500 MHz, chloroform-$d$) δ ppm 0.10 (s, 12 H) 0.92 (s, 18 H) 3.39 - 3.89 (m, 5 H).
4-nitrophenyl-(2,2,3,3,9,9,10,10-octamethyl-4,8-dioxo-3,9-disilaundecan-6-yl) carbonate

25: A stirred solution of silyl protected glycerol 24 (143.4 mg, 0.447 mmol) in anhydrous pyridine at 0 °C was treated with p-nitrophenyl chloroformate (270 mg, 1.34 mmol) and a catalytic amount of DMAP. The resulting mixture was stirred while being monitored by TLC (1:9 ethyl acetate/hexane: product Rf 0.5). Upon completion of the reaction, the mixture was subjected to an aqueous workup, extracted with ethyl acetate and subsequently concentrated. The crude product was purified by column chromatography (silica gel, 5:95 acetone/hexane) to yield a white solid (63%).

1H NMR (500 MHz, chloroform-d) δ ppm 0.10 (s, 12 H) 0.91 (s, 18 H) 3.78 - 3.85 (m, 2 H) 3.85 - 3.94 (m, 2 H) 4.79 - 4.94 (m, 1 H) 7.32 - 7.44 (m, 2 H) 8.21 - 8.35 (m, 2 H).

(5Z,8Z,11Z,14Z)-icosa-5,8,11,14-tetraen-1-yl-(2,2,3,3,9,9,10,10-octamethyl-4,8-dioxo-3,9-disilaundecan-6-yl) carbonate

26: A stirred solution of arachidonic alcohol 28 (108 mg, 0.373 mmol) in anhydrous pyridine at 0 °C was treated with chloroformate 25 (130 mg, 0.267 mmol) and a catalytic amount of DMAP. The resulting solution stirred for 12 hours and was monitored by TLC (1:9 ethyl acetate/hexane, product Rf 0.8). Upon completion of the reaction, the crude mixture
was concentrated and purified by column chromatography (silica gel, 1:9 ethyl acetate/hexane) to yield an oil (79%). $^1$H NMR (500 MHz, chloroform-$d$) $\delta$ ppm 0.06 (s, 12 H) 0.89 (s, 18 H) 1.18 - 1.41 (m, 6 H) 1.41 - 1.52 (m, 2 H) 1.61 - 1.77 (m, 2 H) 1.97 - 2.19 (m, 4 H) 2.73 - 2.93 (m, 6 H) 3.68 - 3.77 (m, 2 H) 3.77 - 3.88 (m, 2H) 4.13 (s, 2 H) 4.67 - 4.81 (m, 1 H) 5.31 - 5.46 (m, 8 H).

2,2,3,3,9,9,10,10-octamethyl-4,8-dioxa-3,9-disilaundecan-6-yl-(5Z,8Z,11Z,14Z)-icosa-5,8,11,14-tetraen-1-ylcarbamate

27: To stirred solution of amine 30 (155 mg, 0.54 mmol) in anhydrous pyridine, chloroformate 25 (338 mg, 0.69 mmol) and a catalytic amount of DMAP were added. The reaction was monitored by TLC (1:9 ethyl acetate/hexane: product $R_f$ 0.35) and subsequently subjected to an aqueous workup. The crude product was purified by column chromatography (silica gel, 1:90 ethyl acetate/hexane). An 80% yield was observed. $^1$H NMR (500 MHz, chloroform-$d$) $\delta$ ppm 0.06 (s, 12 H) 0.87 (t, $J=6.84$ Hz, 3 H) 0.89 (s, 18 H) 1.16 - 1.46 (m, 8 H) 1.47 - 1.66 (m, 2 H) 1.98 - 2.16 (m, 4 H) 2.32 (br. s, 2 H) 2.72 - 2.90 (m, 6 H) 3.20 (q, $J=6.84$ Hz, 2 H) 3.83 (br. s, 4 H) 4.74 - 4.83 (m, 1 H) 4.85 (br. s, 1 H) 5.21 - 5.47 (m, 8 H).
(5Z,8Z,11Z,14Z)-icosa-5,8,11,14-tetraen-1-ol

28: A solution of arachidonic acid (643.6 mg, 2.11 mmol) was added dropwise to a stirred solution of LiAlH₄ (80.0 mg, 2.11 mmol) in anhydrous THF. The resulting mixture was stirred at 0 °C for 1 hour and was monitored by TLC (3:7 ethyl acetate/hexane: product Rf 0.45). Upon completion of the reaction, the mixture was washed with aqueous NH₄Cl and extracted with ether. The organic layers were dried with MgSO₄, filtered and concentrated. The crude product was purified by column chromatography (silica gel, 3:7 ethyl acetate/hexane) to provide a clear oil (90%). ¹H NMR (500 MHz, chloroform-_-d) δ ppm 0.89 (t, J=6.84 Hz, 3 H) 1.21 (br. s, 1 H) 1.24 - 1.41 (m, 6 H) 1.45 (quin, J=7.45 Hz, 2 H) 1.51 - 1.65 (m, 2 H) 2.00 - 2.15 (m, 4 H) 2.67 - 2.95 (m, 6 H) 3.56 - 3.77 (m, 2 H) 5.23 - 5.50 (m, 8 H).

(5Z,8Z,11Z,14Z)-1-azidoicosa-5,8,11,14-tetraene

29: Arachidonic acid 28 (1720.0 mg, 5.92 mmol) was stirred in 10 mL of anhydrous DMF at 110°C. To this, 1,8-diazabicycloundec-7-ene (DBU) (1320 mg, 8.88 mmol) and diphenylphosphoryl azide (DPPA) (1910 mg, 8.88 mmol) were added. The reaction mixture was allowed to stir under nitrogen until consumption of starting material was observed via TLC (35:65 ethyl acetate/hexane). Upon completion of the reaction, the mixture was diluted with diethyl ether and quenched with water. The organic layer was collected and concentrated. The crude product was chromagrapghed (silica gel, 35:65 ethyl
acetate/hexane) to yield clear oil (52%). $^1$H NMR (500 MHz, chloroform-$d$) δ ppm 0.89 (t, $J$=6.87 Hz, 3 H) 1.20 - 1.40 (m, 6 H) 1.41 - 1.51 (m, 2 H) 1.56 - 1.68 (m, 2 H) 2.00 - 2.15 (m, 4 H) 2.77 - 2.88 (m, 6 H) 3.27 (t, $J$=6.87 Hz, 2 H) 5.19 - 5.57 (m, 8 H).

(5Z,8Z,11Z,14Z)-icosa-5,8,11,14-tetraen-1-amine

30: A 1.0 M solution of LiAlH$_4$ in THF (4.91 mL, 4.91 mmol) was added to a stirred solution of arachidonyl azide 29 (969.0 mg, 3.07 mmol) in 40 mL of anhydrous Et$_2$O at 0°C. The reaction mixture was allowed to come to room temperature and was stirred for an hour under nitrogen before being refluxed for an additional 3 hours. The reaction was cooled back down to 0 °C and subsequently quenched with a saturated solution of NH$_4$Cl. The reaction was monitored by TLC (1:9 TEA/MeOH). The product was extracted with Et$_2$O before the aqueous layer was washed with Et$_2$O three additional times. The combined layers were dried with MgSO$_4$, and the solvent was evaporated. The crude product was chromatographed on silica gel to provide a clear oil (72%). $^1$H NMR (500 MHz, chloroform-$d$) δ ppm 0.89 (t, $J$=6.84 Hz, 3 H) 1.21 - 1.47 (m, 8 H) 1.56 (quin, $J$=7.45 Hz, 2 H) 1.99 - 2.16 (m, 4 H) 2.68 - 2.94 (m, 8 H) 3.53 - 4.46 (m, 2 H) 5.04 - 5.67 (m, 8 H).
31: A stirred solution of 20 (15.9 mg, 0.04 mmol and oleic acid (0.0123 mL, 0.04 mmol) in anhydrous DCM at 0 °C was treated with EDCI (22.4 mg, 0.117 mmol) and a catalytic amount of DMAP. The resulting mixture was stirred monitored by TLC (3:7 acetone/hexane: product Rf 0.55). Upon completion of the reaction, the mixture was subjected to an aqueous work-up, dried with MgSO₄ and concentrated. Both mono and di-acylation were observed but the desired product was easily separated during purification (silica gel, 3:7 acetone/hexane). A 30% yield was observed. ¹H NMR (500 MHz, chloroform- d) δ ppm 0.79 - 0.95 (m, 6 H) 1.18 - 1.47 (m, 28 H) 1.53 (d, J=7.81 Hz, 2 H) 1.59 - 1.69 (m, 2 H) 1.93 - 2.16 (m, 8 H) 2.34 (t, J=7.57 Hz, 2 H) 2.72 - 2.92 (m, 6 H) 3.20 (d, J=6.84 Hz, 2 H) 3.74 (d, J=6.35 Hz, 2 H) 4.27 (d, J=4.88 Hz, 2 H) 4.65 - 4.83 (m, 1 H) 4.88 - 5.03 (m, 1 H) 5.31 - 5.49 (m, 10 H).
(S)-3-hydroxy-2-(((5Z,8Z,11Z,14Z)-icosa-5,8,11,14-tetraen-1-ylcarbamoyl)oxy)propyl 4-([1,1'-biphenyl]-4-yl)butanoate

32: A stirred solution of 20 (7.1 mg, 0.017 mmol) and biphenyl butyric acid (4.18 mg, 0.017 mmol) in dry DCM at 0 °C was treated with EDCI (10.0 mg, 0.052 mmol) and a catalytic amount of DMAP. The resulting mixture was stirred while being monitored by TLC (3:7 acetone/hexane: product R$_f$ 0.3). Upon completion of the reaction, the mixture was subjected to an aqueous work-up, dried with MgSO$_4$ and concentrated. Both mono and di-acylation were observed but the desired product was easily separated during purification (silica gel, 3:7 acetone/hexane). A 40% yield was observed.

$^1$H NMR (500 MHz, chloroform-$d$) δ ppm 0.84 (d, $J$=3.42 Hz, 2 H) 0.89 (t, $J$=6.84 Hz, 3 H) 1.18 - 1.43 (m, 8 H) 1.45 - 1.53 (m, 2 H) 1.91 - 2.16 (m, 4 H) 2.28 - 2.36 (m, 1 H) 2.39 (t, $J$=7.57 Hz, 2 H) 2.70 (t, $J$=7.57 Hz, 2 H) 2.74 - 2.90 (m, 6 H) 3.04 - 3.25 (m, 2 H) 3.74 (d, $J$=5.37 Hz, 2 H) 4.20 - 4.35 (m, 2 H) 4.67 - 4.81 (m, 1 H) 4.88 - 5.03 (m, 1 H) 5.25 - 5.48 (m, 8 H) 7.24 (s, 1 H) 7.33 (t, 1 H) 7.43 (t, $J$=7.57 Hz, 2 H) 7.52 (d, $J$=8.30 Hz, 2 H) 7.58 (d, $J$=7.32 Hz, 2 H).
(S)-3-hydroxy-2-(((5Z,8Z,11Z,14Z)-icosa-5,8,11,14-tetraen-1-
ylcarbamoyl)oxy)propyl 4-(pyren-2-yl)butanoate

33: A stirred solution of 20 (13.1 mg, 0.032 mmol and pyrene butyric acid (9.26 mg, 0.0.032 mmol) in anhydrous DCM at 0 °C was treated with EDCI (18.48 mg, 0.096 mmol) and a catalytic amount of DMAP. The resulting mixture was stirred at room temperature while being monitored by TLC (3:7 acetone/hexane: product Rf 0.5, starting material Rf 0.3). Upon completion of the reaction, the mixture was subjected to an aqueous work-up, dried with MgSO4 and concentrated. Both mono and di-acylation were observed but the desired product was easily separated during purification (silica gel, 3:7 acetone/hexane). A 20% yield was observed. 1H NMR (500 MHz, chloroform-d) δ ppm 0.88 (t, J=6.84 Hz, 3 H) 1.18 - 1.47 (m, 12 H) 1.92 - 2.11 (m, 2 H) 2.12 - 2.26 (m, 2 H) 2.27 - 2.37 (m, 1 H) 2.42 - 2.55 (m, 2 H) 2.72 - 2.89 (m, 6 H) 3.10 (dd, J=10.25, 6.35 Hz, 2 H) 3.40 (t, J=7.81 Hz, 2 H) 3.63 - 3.82 (m, 2 H) 4.29 (d, J=4.88 Hz, 2 H) 4.62 - 4.76 (m, 1 H) 4.96 (br. s, 1 H) 5.23 - 5.46 (m, 8 H) 7.86 (d, J=7.81 Hz, 1 H) 7.97 - 8.02 (m, 1 H) 8.03 (s, 2 H) 8.09 - 8.15 (m, 2 H) 8.17 (dd, J=7.57, 4.64 Hz, 2 H) 8.30 (d, J=9.28 Hz, 1 H).
(S)-3-hydroxy-2-(((5Z,8Z,11Z,14Z)-icosa-5,8,11,14-tetraen-1-ylcarbamoyl)oxy)propyl 5-phenylpentanoate

**34:** A stirred solution of 20 (7.9 mg, 0.019 mmol) and phenyl valeric acid (3.4 mg, 0.019 mmol) in anhydrous DCM at 0 °C was treated with EDCI (11.1 mg, 0.05 mmol) and a catalytic amount of DMAP. The resulting mixture was stirred while being monitored by TLC (3:7 acetone/hexane: product R_f 0.4). Upon completion of the reaction, the mixture was subjected to an aqueous work-up, dried with MgSO_4 and concentrated. Both mono and di-acylation were observed but the desired product was easily separated during purification (silica gel, 3:7 acetone/hexane). A 35% yield was observed. 

\[ ^1H \text{ NMR (500 MHz, chloroform-}d) \delta \text{ ppm } 0.89 (t, J=6.84 \text{ Hz, 3 } H) 1.19 - 1.45 (m, 6 \text{ H}) 1.45 - 1.60 (m, 4 \text{ H}) 1.60 - 1.75 (m, 4 \text{ H}) 2.07 (dd, J=14.89, 7.08 \text{ Hz, 4 H}) 2.20 - 2.26 (m, 1 \text{ H}) 2.28 - 2.42 (m, 2 \text{ H}) 2.63 (t, J=7.08 \text{ Hz, 2 H}) 2.73 - 2.94 (m, 6 \text{ H}) 3.17 (dd, J=6.35, 2.93 \text{ Hz, 2 H}) 3.72 (br. s, 2 \text{ H}) 4.26 (d, J=5.37 \text{ Hz, 2 H}) 4.64 - 4.79 (m, 1 \text{ H}) 4.94 (br. s, 1 \text{ H}) 5.26 - 5.48 (m, 8 \text{ H}) 7.14 - 7.22 (m, 3 \text{ H}) 7.27 - 7.31 (m, 2 \text{ H}).\]

**1-methoxy-3-(trityloxy)propan-2-ol**

**36:** Triethyl amine (1.67 g, 2.5 mL) was added dropwise to a stirred solution of 35 methoxy propane diol (2.0 g, 18.8 mmol) 21 and a catalytic amount of DMAP in anhydrous DCM under nitrogen. Trityl chloride was
then slowly added (5.25 g, 18.8 mg) to the reaction mixture before it was stirred at room temperature. The reaction was monitored by TLC (3:7 ethyl acetate/hexane: product R$_f$ 0.45). After an aqueous workup, the resulting organic layers were dried with MgSO$_4$, filtered and concentrated under reduced pressure. The crude product was purified by column chromatography (silica gel, 1:4 ethyl acetate/hexane). An 87% yield was observed. $^1$H NMR (500 MHz, chloroform-\textit{d}) $\delta$ ppm 2.41 (d, $J=4.88$ Hz, 1 H) 3.15 - 3.24 (m, 1 H) 3.36 (s, 3 H) 3.40 - 3.52 (m, 2 H) 3.91 - 3.99 (m, 1 H) 7.20 - 7.27 (m, 3 H) 7.30 (t, 6 H) 7.43 (d, $J=7.32$ Hz, 6 H).

tert-butyl((1-methoxy-3-(trityloxy)propan-2-yl)oxy)diphenylsilane

37: TBDPS-Cl (2.729 g, 2.58 mL) was added dropwise to a stirred solution of alcohol 36 (3.0 g, 8.27 mmol) and imidazole (1.127 g, 16.5 mmol) in anhydrous DCM under nitrogen. Completion of the reaction was determined by TLC (2:8 ethyl acetate/hexane: starting material R$_f$ 0.2; product R$_f$ 0.75). After an aqueous workup, the resulting organic layers were dried with MgSO$_4$, filtered and concentrated under reduced pressure. The crude product was purified by column chromatography (silica gel, 1:9 ethyl acetate/hexane). A 62% yield was obtained. $^1$H NMR (500 MHz, chloroform-\textit{d}) $\delta$ ppm 1.02 (s, 9 H) 3.12 (s, 3 H) 3.15 (d, $J=4.88$ Hz, 2 H) 3.33 (dd, $J=10.01$, 5.13 Hz, 1 H) 3.46 (dd, $J=9.77$, 4.88 Hz, 1 H) 3.92 - 4.00 (m, 1 H) 7.13 - 7.46 (m, 21 H) 7.61 (d, $J=6.84$ Hz, 2 H) 7.65 (d, $J=6.84$ Hz, 2 H).
**R)-2-((tert-butylidimethylsilyl)oxy)-3-methoxypropan-1-ol**

38: Camphor sulfonic acid (110.0 mg, 0.47 mg) was added to a stirred solution of tritylated compound 37 in 9:1 DCM/EtOH. The solution was vigorously stirred at room temperature for 3 days under nitrogen. Completion of the reaction was determined by TLC (2:8 ethyl acetate/hexane: product R_f 0.30). After a basic workup, the resulting organic layers were dried with MgSO_4, filtered and concentrated under reduced pressure. The crude product was purified by column chromatography (silica gel, 2:7 ethyl acetate/hexane). A 62% yield was obtained. 

^{1}H NMR (500 MHz, chloroform-d) δ ppm 1.07 (s, 9 H) 2.04 (t, 1 H) 3.19 (s, 3 H) 3.30 - 3.38 (m, 1 H) 3.38 - 3.49 (m, 1 H) 3.62 (br. s, 2 H) 3.79 - 3.97 (m, 1 H) 7.39 (d, J=6.84 Hz, 4 H) 7.41 - 7.48 (m, 2 H) 7.68 (dd, J=6.59, 4.6 Hz, 4 H).

**S)-2-((tert-butylidiphenylsilyl)oxy)-3-methoxypropyl-4-(5a1,10-dihydropyren-1-yl)butanoate**

39: A stirred solution of primary alcohol 38 (27.6 mg, 0.08 mmol and pyrene butyric acid (23.0 mg, 0.08 mmol) in anhydrous DCM at 0 °C was treated with EDCI (46.0 mg, 0.24 mmol) and a catalytic amount of DMAP. The resulting mixture monitored by TLC (2:8 ethyl acetate/hexane: product R_f 0.55, starting material R_f 0.45). Upon completion of the reaction, the crude mixture was concentrated and
purified by column chromatography (silica gel, 2:8 ethyl acetate/hexane). A 66% yield was obtained. $^1$H NMR (500 MHz, chloroform-$d$) $\delta$ ppm 0.79 - 0.93 (m, 9 H) 1.02 (s, 9 H) 1.25 (br. s, 1 H) 2.00 - 2.14 (m, 2 H) 2.17 - 2.36 (m, 2 H) 3.18 (s, 3 H) 3.26 - 3.39 (m, 2 H) 3.93 - 4.04 (m, 1 H) 4.06 - 4.13 (m, 1 H) 4.14 - 4.21 (m, 1 H) 7.21 - 7.35 (m, 6 H) 7.34 - 7.42 (m, 1 H) 7.65 (dd, $J$=16.36, 6.59 Hz, 3 H) 7.82 (d, $J$=7.81 Hz, 1 H) 7.94 - 8.05 (m, 3 H) 8.09 (dd, $J$=8.55, 4.15 Hz, 2 H) 8.16 (d, $J$=7.32 Hz, 2 H) 8.26 (d, $J$=9.28 Hz, 1 H).

$(S)$-2-((tert-butyldiphenylsilyl)oxy)-3-methoxypropyl-4-([1,1'-biphenyl]-4-yl)butanoate

$^1$H NMR (500 MHz, chloroform-$d$) $\delta$ ppm 1.05 (s, 9 H) 1.82 - 1.96 (m, 2 H) 2.09 - 2.27 (m, 2 H) 2.63 (t, $J$=7.57 Hz, 2 H) 3.19 (s, 3 H) 3.33 (dd, $J$=7.81, 5.86 Hz, 2 H) 3.95 - 4.04 (m, 1 H) 4.05 - 4.11 (m, 1 H) 4.13 - 4.20 (m, 1 H) 7.22 (d, $J$=8.30 Hz, 3 H) 7.29 - 7.39 (m, 4 H)

$^1$H NMR (500 MHz, chloroform-$d$) $\delta$ ppm 1.82 - 1.96 (m, 2 H) 2.09 - 2.27 (m, 2 H) 2.36 (t, $J$=7.57 Hz, 2 H) 2.63 (t, $J$=7.57 Hz, 2 H) 3.19 (s, 3 H) 3.33 (dd, $J$=7.81, 5.86 Hz, 2 H) 3.95 - 4.04 (m, 1 H) 4.05 - 4.11 (m, 1 H) 4.13 - 4.20 (m, 1 H) 7.22 (d, $J$=8.30 Hz, 3 H) 7.29 - 7.39 (m, 4 H)

$^1$H NMR (500 MHz, chloroform-$d$) $\delta$ ppm 1.05 (s, 9 H) 1.82 - 1.96 (m, 2 H) 2.09 - 2.27 (m, 2 H) 2.63 (t, $J$=7.57 Hz, 2 H) 3.19 (s, 3 H) 3.33 (dd, $J$=7.81, 5.86 Hz, 2 H) 3.95 - 4.04 (m, 1 H) 4.05 - 4.11 (m, 1 H) 4.13 - 4.20 (m, 1 H) 7.22 (d, $J$=8.30 Hz, 3 H) 7.29 - 7.39 (m, 4 H)

$^1$H NMR (500 MHz, chloroform-$d$) $\delta$ ppm 1.05 (s, 9 H) 1.82 - 1.96 (m, 2 H) 2.09 - 2.27 (m, 2 H) 2.63 (t, $J$=7.57 Hz, 2 H) 3.19 (s, 3 H) 3.33 (dd, $J$=7.81, 5.86 Hz, 2 H) 3.95 - 4.04 (m, 1 H) 4.05 - 4.11 (m, 1 H) 4.13 - 4.20 (m, 1 H) 7.22 (d, $J$=8.30 Hz, 3 H) 7.29 - 7.39 (m, 4 H)

A stirred solution of primary alcohol 38 (24.1 mg, 0.07 mmol and biphenyl butyric acid (18.5 mg, 0.077 mmol) in anhydrous DCM at 0 °C was treated with EDCI (40.0 mg, 0.2 mmol) and a catalytic amount of DMAP. The resulting mixture was monitored by TLC (2:8 ethyl acetate/hexane: product R$_f$ 0.7, starting material R$_f$ 0.45). Upon completion of the reaction, the crude mixture was concentrated and purified by column chromatography (silica gel, 2:8 ethyl acetate/hexane). An 85% yield was obtained. $^1$H NMR (500 MHz, chloroform-$d$) $\delta$ ppm 1.05 (s, 9 H) 1.82 - 1.96 (m, 2 H) 2.09 - 2.27 (m, 2 H) 2.63 (t, $J$=7.57 Hz, 2 H) 3.19 (s, 3 H) 3.33 (dd, $J$=7.81, 5.86 Hz, 2 H) 3.95 - 4.04 (m, 1 H) 4.05 - 4.11 (m, 1 H) 4.13 - 4.20 (m, 1 H) 7.22 (d, $J$=8.30 Hz, 3 H) 7.29 - 7.39 (m, 4 H)
7.39 - 7.46 (m, 4 H) 7.51 (d, $J=7.81$ Hz, 2 H) 7.58 (d, $J=7.32$ Hz, 2 H) 7.63 - 7.72 (m, 4 H).

(S)-2-hydroxy-3-methoxypropyl 4-(5a1,10-dihydropyren-1-yl)butanoate

41: Triethylamine trihydrogen fluoride (TEA-(HF)$_3$) (0.047 mL, 0.3 mmol) was added dropwise to a stirred solution of silyl-protected 39 (18 mg, 0.03 mmol) in anhydrous DCM. The reaction was monitored by TLC (3:7 ethyl acetate/hexane: product $R_f$ 0.3). Even though only fifty percent complete, the reaction mixture was concentrated before direct use in the next step.

(S)-2-hydroxy-3-methoxypropyl 4-([1,1'-biphenyl]-4-yl)butanoate

42: TEA-(HF)$_3$ (0.114 mL, 0.7 mmol) was added dropwise to a stirred solution of silyl-protected 40 (40.0 mg, 0.07 mmol) The reaction was monitored by TLC (3:7 ethyl acetate/hexane: product $R_f$ 0.3). Even though only fifty percent complete, the reaction mixture was concentrated before direct use in next step.
(5Z,8Z,11Z,14Z)-(2S)-1-((4-(5a1,10-dihydropyren-1-yl)butanoyl)oxy)-3-methoxypropan-2-yl icosa-5,8,11,14-tetraenoate

43: A stirred solution of secondary alcohol 41 (11.0 mg, 0.03 mmol) and arachidonic acid (10.6 mg, 0.035 mmol) in anhydrous DCM at 0 °C was treated with EDCI (11.1 mg, 0.06 mmol) and a catalytic amount of DMAP. The resulting mixture was monitored by TLC (2:8 acetone/hexane: product Rf 0.5). Upon completion of the reaction, the mixture was subjected to an aqueous workup, dried with MgSO₄ and concentrated. The crude product was purified by column chromatography (silica gel, 2:8 acetone/hexane). A 10% yield was obtained. ¹H NMR (500 MHz, chloroform-d) δ ppm 0.90 (t, J=6.84 Hz, 3 H) 1.18 - 1.47 (m, 8 H) 1.64 - 1.76 (m, 2 H) 1.95 - 2.14 (m, 4 H) 2.16 - 2.28 (m, 2 H) 2.28 - 2.43 (m, 2 H) 2.46 - 2.52 (m, 2 H) 2.75 - 2.88 (m, 6 H) 3.37 (s, 3 H) 3.48 - 3.60 (m, 2 H) 4.17 - 4.26 (m, 1 H) 4.32 - 4.44 (m, 1 H) 5.14 - 5.28 (m, 1 H) 5.28 - 5.49 (m, 8 H) 7.83 - 7.92 (m, 1 H) 7.97 - 8.04 (m, 1 H) 8.06 (s, 2 H) 8.11 - 8.17 (m, 2 H) 8.17 - 8.23 (m, 2 H) 8.28 - 8.36 (m, 1 H).

(5Z,8Z,11Z,14Z)-(S)-1-((4-(1,1'-biphenyl)-4-yl)butanoyl)oxy)-3-methoxypropan-2-yl icosa-5,8,11,14-tetraenoate

44: A stirred solution of secondary alcohol 42 (22.5 mg, 0.07 mmol) and arachidonic acid (25.6 mg, 0.084 mmol) in anhydrous
DCM at 0 °C was treated with EDCI (27.0 mg, 0.14 mmol) and a catalytic amount of DMAP. The resulting mixture was monitored by TLC (2:8 acetone/hexane: product R<sub>f</sub> 0.55). Upon completion of the reaction, the mixture was subjected to an aqueous work-up, dried with MgSO<sub>4</sub> and concentrated. The crude product was purified by column chromatography (silica gel, 2:8 acetone/hexane). A 38% yield was obtained.

1H NMR (500 MHz, chloroform-<em>d</em>) δ ppm 0.89 (t, <em>J</em>=6.84 Hz, 3 H) 1.18 - 1.44 (m, 6 H) 1.70 (t, <em>J</em>=7.57 Hz, 2 H) 1.89 - 2.18 (m, 6 H) 2.36 (dt, <em>J</em>=10.25, 7.57 Hz, 4 H) 2.69 (t, <em>J</em>=7.81 Hz, 2 H) 2.81 (dq, <em>J</em>=11.90, 5.96 Hz, 6 H) 3.36 (s, 3 H) 3.52 (dd, <em>J</em>=5.13, 2.20 Hz, 2 H) 4.16 (dd, <em>J</em>=11.96, 6.59 Hz, 1 H) 4.35 (dd, <em>J</em>=11.96, 3.66 Hz, 1 H) 5.14 - 5.27 (m, 1 H) 5.27 - 5.47 (m, 8 H) 7.24 (s, 2 H) 7.34 (d, <em>J</em>=7.32 Hz, 1 H) 7.43 (t, <em>J</em>=7.57 Hz, 2 H) 7.52 (d, <em>J</em>=8.30 Hz, 2 H) 7.58 (d, <em>J</em>=7.32 Hz, 2 H).

3-hydroxy-2-((5Z,8Z,11Z,14Z)-icosa-5,8,11,14-tetraenamido)propyl oleate

46: A stirred solution of arachidonoyl serinol 21 (42.6 mg, 0.11 mmol and oleic acid (0.035 mL, 0.11 mmol) in dry DCM at 0 °C was treated with EDCI (64.8 mg, 0.34 mmol) and a catalytic amount of DMAP. The resulting mixture was monitored by TLC (3:7 acetone/hexane: product R<sub>f</sub> 0.5). Upon completion of the reaction, the crude mixture was concentrated and purified by column chromatography (silica gel, 3:7 acetone/hexane). A 74% yield was obtained.

1H NMR (500 MHz, chloroform-<em>d</em>) δ ppm 0.84 - 0.93 (m, 6 H) 1.10 - 1.43 (m, 26 H) 1.62 (t, <em>J</em>=7.08 Hz, 2 H) 1.67 - 1.80
(m, 2 H) 1.95 - 2.04 (m, 4 H) 2.04 - 2.09 (m, 2 H) 2.12 (q, J=7.16 Hz, 2 H) 2.21 (t, J=7.81 Hz, 2 H) 2.34 (t, J=7.57 Hz, 2 H) 2.65 (br. s, 1 H) 2.74 - 2.90 (m, 6 H) 3.62 (dd, J=6.84, 3.91 Hz, 1 H) 3.67 (d, J=3.91 Hz, 1 H) 4.13 - 4.21 (m, 2 H) 4.21 - 4.29 (m, 1 H) 5.13 - 5.49 (m, 10 H) 5.92 (d, J=6.84 Hz, 1 H).

(5Z,8Z,11Z,14Z)-icosa-5,8,11,14-tetraen-1-yl (4-nitrophenyl) carbonate

Triethylamine (0.1 mL, 0.768 mmol) was added to a stirred solution of arachidonyl alcohol 28 (106.2 mg, 0.384 mmol) in anhydrous DCM under nitrogen. The reaction mixture was cooled to 0 °C and p-nitrophenyl chloroformate (93.0 mg, 0.46 mmol) and a catalytic amount of DMAP were added. The reaction was monitored by TLC (1:4 ethyl acetate/hexane: product Rf 0.8). Upon completion, an aqueous work-up was performed and the organic layer was subsequently dried with MgSO₄. The crude product was purified by column chromatography (silica gel, 1:9 ethyl acetate/hexane). A 70% yield was reported. ¹H NMR (500 MHz, chloroform-d) δ ppm 0.89 (t, J=7.08 Hz, 3 H) 1.19 - 1.43 (m, 6 H) 1.43 - 1.61 (m, 2 H) 1.69 - 1.86 (m, 2 H) 2.06 (q, J=7.16 Hz, 2 H) 2.15 (q, J=6.84 Hz, 2 H) 2.71 - 2.93 (m, 6 H) 4.30 (t, J=6.59 Hz, 2 H) 5.31 - 5.50 (m, 8 H) 7.38 (d, J=8.79 Hz, 2 H) 8.28 (d, J=9.28 Hz, 2 H).
(S)-3-hydroxy-2-(((5Z,8Z,11Z,14Z)-icosa-5,8,11,14-tetraen-1-yloxy)carbonyl)amino)propyl oleate

48: A stirred solution of 22 (9.5 mg, 0.023 mmol and oleic acid (0.007 mL, 0.023 mmol) in anhydrous DCM at 0 °C was treated with EDCI (13.4 mg, 0.07 mmol) and a catalytic amount of DMAP. The resulting mixture was monitored by TLC (3:7 acetone/hexane: product Rf 0.6). Upon completion of the reaction, the mixture was subjected to an aqueous work-up, dried with MgSO4 and concentrated. Both mono and di-acylation were observed but the desired product was easily separated during purification (silica gel, 3:7 acetone/hexane). A 30% yield was obtained. 1H NMR (500 MHz, chloroform-d) δ ppm 0.81 - 0.96 (m, 6 H) 1.19 - 1.40 (m, 26 H) 1.40 - 1.49 (m, 2 H) 1.59 - 1.70 (m, 4 H) 1.93 - 2.16 (m, 8 H) 2.35 (t, J=7.57 Hz, 2 H) 2.72 - 2.92 (m, 6 H) 3.54 - 3.66 (m, 1 H) 3.66 - 3.76 (m, 1 H) 3.85 - 3.99 (m, 1 H) 4.08 (t, J=6.10 Hz, 2 H) 4.23 (d, J=5.37 Hz, 2 H) 5.02 - 5.13 (m, 1 H) 5.31- 5.46 (m, 10 H).

3.5.4b Enzyme Preparation

Human DGLα was generated by transient transfection of HEK293T cells using Lipofectamine (following manufacturer’s standard procedure) and pcDNA 3.1/myc-His vector containing the human DGLα gene. Two days after transfection, the cells were harvested from the flask by scraping and the cell pellets were subsequently
frozen at -80 °C for later use. When needed, the cell pellets were re-suspended in lysis buffer containing 20 mM HEPES pH 7.0, 2 mM DTT and 0.25 M sucrose. The cells were then lysed on ice by sonication (3 cycles of 7 sec) and spun down via ultracentrifugation (30,000 rpm for 30 min at 4 °C). The membrane prep (pellet) was then re-suspended in sucrose-free lysis buffer. The protein concentration was determined by the Bradford dye-binding microassay (Bio-Rad) using BSA as a standard, and the protein was portioned into small aliquots (100 µg per tube) and stored frozen at -80 °C for future use.

3.6.4c Assay Conditions for HPLC Analysis

For the reaction mixtures, samples were made up to 297 µL total volume in 20 mM Tris buffer, pH 7.2. For FAAH testing, 15 µg total protein *E. coli* lysate over-expressing the rat enzyme was used, for MGL, 100 ng of purified human enzyme, and for DGLα, 30 µg total protein membrane prep (and 5 mM CaCl$_2$ final concentration added to buffer). The reaction mixtures were incubated at 37 °C for 5 minutes before addition of 3µL 10 mM compound (final concentration 100 µM) and vortexing. Immediately after addition of the compound a 100 µL aliquot was removed and added to 400 µL acetonitrile (t=0 sample). The reaction was allowed to proceed at 37 °C for 30 min, then another 100 µL aliquot was removed and added to 400 µL acetonitrile (t=30 sample). The samples were centrifuged at 13k for 5 minutes and the supernatants loaded into vials. A standard curve was prepared using 1, 5, 25, and 50 µM of the compound in acetonitrile in vials. The samples were run on an HPLC
(Waters model 515 pumps, 717 auto-sampler, 2487 detector, Agilent 4.6 x 50mm and 4.6 x 150 mm columns, (C18, sorbax, 5 uM beads) with 100% acetonitrile for the A solvent 60% water (with 8.5% phosphoric acid) and 40% acetonitrile for the B solvent. The gradient was 100% B solvent to 95% A solvent 5% B solvent over 2 minutes, then an isocratic flow of 95% A solvent 5% B solvent for 5 minutes followed by 100% B for 1 minute. The concentrations of product were quantified by determining the decrease in intensity in the sample peaks as quantified using the standard curve.

3.6.4d Assay Conditions for Fluorometer Analysis

Reactions with fluorescent substrate 33 were performed using a fluorescence spectrometer (Varian: Cory Eclipse, 100 µL cuvette). A solution of 33 (final concentration 100 µM, made in 100% DMSO stock, final concentration adjusted to 10%) was prepared with buffer (50 mM MES, pH 6.5, 5 mM CaCl₂). Reactions (100 µL) were initiated with DGLα (30 µg total protein of membrane prep) and the fluorescence differentials resulting from hydrolysis of the substrate were monitored and quantified.

3.6.5 Studies Towards Selective Substrate Conclusion:

In an effort to develop a selective DGL assay, substrates with arachidonyl analogs at the sn-2 position, that were demonstrated to be non-hydrolysable by FAAH and MGL, and with oleic acid at the sn-1 position, were synthesized. The very low
solubility of these compounds, in addition to the low turnover rate of DGLα, compromised efforts to detect the products of the reaction by HPLC. Even when using the highly non-specific lipoprotein lipase, that also has a very high turnover rate, we were unable to detect any products of the enzymatic reaction.

Substrates were also made with fluorogenic reporters at the sn-1 position. One particular compound, 33, showed promise as a compound selectively hydrolyzed by DGL. 33 has pyrene butyric acid at the sn-1 position, leading to an increase in fluorescence at 380 nm (when excited at 340 nm) when it is hydrolyzed to form free pyrene butyric acid and 2-AG. Neither the non-transfected HEK293 cells, nor buffer alone, hydrolyzed 33 at the sn-1 position. In the presence of DGLα transfected cells there was measurable hydrolysis at the sn-1 position, as detected by a modest increase in fluorescence 380 nm. However, the signal to noise is not great enough to obtain highly reliable inhibition curves with this particular compound. This compound demonstrates promise with the concept of arachidonyl analogs at the sn-2 position and a fluorogenic reporter at the sn-1 position as a substrate for high-throughput assays selectively hydrolyzed by DGLα.
3.6 References


(5) Pedicord, D. L.; Flynn, M. J.; Fanslau, C.; Miranda, M.; Hunihan, L.; Robertson, B. J.; Pearce, B. C.; Xuan-Chuan, Y.; Westphal, R. S.; Blat, Y. Biochemical and Biophysical Research Communications 2011, 411, 809.


(32) Bisogno, T. Biochimica et Biophysica Acta 2006, 1761, 205.


CHAPTER 4

PREPARATION OF RADIO-LABELED MONO AND DIACYL GLYCEROLS


CHAPTER 5

SYNTHESIS OF DIACYLGLYCEROL LIPASE INHIBITORS
5.1 Significance

Pharmacological manipulation of 2-AG metabolism via small molecule inhibition of DGL would greatly affect endocannabinoid signaling. Inhibition of DGL is expected to produce a blockade of 2-AG signaling with therapeutic application similar to those of cannabinoid antagonists.\(^1\) CB antagonists have been thought to aid in weight loss,\(^2\) inflammation\(^6,7\) and Parkinson’s symptoms,\(^8,9\) in addition to attenuating the reward system for substance abuse.\(^10\)\(^-\)\(^16\) The attenuation of signaling by the constitutive cannabinoid receptors would be distinct from the effects of inverse-agonist drugs such as Rimonabant.\(^17\)

5.2 Known DGL inhibitors

The lead inhibitors of DGL activity include bis-oximinocarbamate RHC80267\(^1\)\(^18,19\) fluorophosphonates O-3640\(^2\)\(^20\), O-3841\(^3\)\(^20\) and O-5596\(^4\)\(^21\) as well as β-lactone-based compounds tetrahydrolipstatin (THL)\(^5\)\(^18,22\) and OMDM-188\(^6\)\(^23\) (Figure 5.1)

5.2.1 bis-oximinocarbamate

RHC80267 1 is a weak inhibitor with an \(IC_{50}\) value in the range of 4-15 \(\mu\)M.\(^24\)\(^-\)\(^26\) This compound does not demonstrate selectivity over MGL;\(^26\) however, it does show some selectivity as a DGL inhibitor.\(^27\) RHC80267 has been shown to block 2-AG formation in intact cells.\(^19,28,29\)
5.2.2 Fluorophosphonates

This class of compounds, found to have good selectivity and activity, are derived from oleic acid and fluoro-phosphoyle esters. 2 has an IC\textsubscript{50} value of 500 nM while 3 has an IC\textsubscript{50} value of 160 nM for DGL\alpha. The more potent 3, does however, lack stability and bioavailability, both necessary for \textit{in vivo} application.\textsuperscript{20,21} By converting the \textit{sn}-3 methoxy group to a more sterically hindered tert-butoxy group, the stability of the compound 4 was significantly improved. The stability of the two compounds in DMSO was compared at varying temperatures. Degradation was only observed for 3 at -20 °C. 4 was shown to inhibit the biosynthesis of 2-AG in intact cells (whereas 3 did not), demonstrating its superior stability. An IC\textsubscript{50} value of 100 nM was determined using a radio-labeled assay with substrate 1-[\textsuperscript{14}C]-oleoyl-2-arachidonoyl-\textit{sn}-glycerol.\textsuperscript{21} Although potent and selective for DGL, fluorophosphonates are toxic and therefore, not deemed suitable for drug development.\textsuperscript{30}

5.2.3 \(\beta\)-lactone-based Inhibitors

THL (5) is the saturated version of lipstatin which was isolated from \textit{Streptomyces toxytricini} in 1987 by Hoffman-LaRoche. THL and lipstatin were found to be irreversible inhibitors of pancreatic lipase which is responsible for absorption of dietary fat.\textsuperscript{31,32} THL, the more stable derivative was identified by Bisogno in 2003 to inhibit DGL\alpha/\beta with IC\textsubscript{50} values of 60 nM and 100 nM respectively.\textsuperscript{18} THL, is also known as Orlistat and is available as weight loss aids Xenical (Roche) and Alli
(Glasko Smith Kline).\textsuperscript{33} Although potent, THL lacks selectivity and oral absorption.\textsuperscript{20,22}

Di Marzo \textit{et al} introduced OMDM0-188 6, a modified version of THL. This compound was synthesized by substituting an isoleucyl group for the leucyl group of THL. This compound is promising as it is significantly more potent (IC\textsubscript{50}=16 nM) than THL.\textsuperscript{23} Structure activity relationship (SAR) studies determined that THL analogs incorporating an N-formyl group in the S-configuration as well as a small amino acid moiety were more potent than analogs lacking these structural features.

\begin{figure*}[h]
\centering
\includegraphics[width=\textwidth]{known_dgl_inhibitors.png}
\caption{Known DGL Inhibitors}
\end{figure*}
5.3 DGL Inhibitor Design, Synthesis, Results and Discussion

As diacylglycerol lipase is a fairly recent therapeutic focus, there is ample room for research and exploration. Current DGL inhibitors can be described as having shortcomings such as toxicity (O-5586) and bioavailability (THL, OMDM1-88). The goal of our work was to explore the relationship between DGL inhibitor SAR and inhibitor selectivity.

5.3.1 Natural Substrate-based Compounds

A new series of racemic ether lipids 7-20 structurally related to diacyl-sn-glycerols as well as lead compounds 1 and 3 were prepared from either 3-O-methylglycerol 21 or the corresponding amine 1-amino-3-methoxy-2-

![Figure 5.2: Natural substrate-based analog design](image)
propanol 27. These compounds included derivatives having ester, amide, carbamate and cyclohexyloximinocarbamate functional groups in the \textit{sn}-1 position. The \textit{sn}-2 positions were generally ester groups. As DGL selectively hydrolyzes the \textit{sn}-1 position of glycerides, it was important to include reactive substituents at this position (Figure 5.2). The synthetic approaches for preparing esters and carbamates are outlined in Schemes 5.1-5.3. A common feature of these schemes is the use of methoxy propane diol. Methoxy propane diol 21 was utilized as a starting point instead of glycerol as it was reported that the methoxy group was comparably potent.\textsuperscript{20} Assay of the natural substrate compounds indicated that lipid analogs 7-20 were not inhibitors of hDGL\textalpha{} or mDGL\textbeta{} at 10 µM. Compounds 7-20 each had a Ki above 1 µM in competition binding assays for CB1 (rat brain preparation) and for CB2 (mouse or human receptor expressed in HEK293). They also did not inhibit rFAAH or hMGL.

\textbf{5.3.1a Esters}

The symmetrical ester derivatives 7 and 8 were synthesized by introducing acid (biphenyl butyric and octanoic), EDCI and a catalytic amount of DMAP to a solution of methoxy propane diol 21, in DCM (Scheme 5.1).
Scheme 5.1: Synthesis of esters.

5.3.1b Carbamates

Ether lipid analogs of lead compound O-3841 2 were synthesized, including reactive carbamate groups at the \( sn-1 \) position. Carbamate groups, have been used quite successfully with other serine hydrolases (URB59713 for selective inhibition of fatty acid amide hydrolase, and JZL18414 for selective inhibition of monoacylglycerol lipase)\(^{21,34}\) The 3-\( O \)-methyl glycerol derivatives 9 to 11 were synthesized utilizing a protecting group scheme developed in the Roush laboratory\(^{35,36}\) (Scheme 5.2).

Methoxy propane 21 diol was treated with trityl chloride, TEA and DMAP in DCM. Once the primary alcohol 22 was protected, the secondary alcohol was silylated with tertbutyldiphenyl silyl chloride 23. Camphor sulfonic acid provided deprotection 24, followed by the treatment of octyl isocyanate 25, yielding the carbamate functionality of 25. This compound was then treated with tetrabutylammonium fluoride in order to remove the tertbutyldiphenylsilyl (TBDPS) protecting group 26. The resulting free hydroxy group 26 was esterified with acid, EDCI and DMAP in
anhydrous DCM. This series of compounds having a carbamate group in the <i>sn</i>-1 position was functionalized with various esters [provided the octyl analog 9, the oleoyl analog 10, and the arachidonoyl analog 11] at the <i>sn</i>-2 position (Scheme 5.2).

Scheme 5.2: Synthesis of carbamates.

In order to synthesize a symmetrical carbamate molecule 12, methoxy propane diol 21 was treated with multiple equivalents of octyl isocyanate under reflux (Scheme 5.3).
Scheme 5.3: Synthesis of symmetrical carbamates.

5.3.1c Retrocarbamates

Analogs 13-17 incorporating retrocarbamate functionality at the \( sn \)-1 position, were synthesized from the corresponding amine 1-amino-3-methoxy-2-propanol 27. The amine was treated with chloroformate (octyl, oximinal) and TEA in dry DCM. Subsequently, the \( sn \)-2 position was functionalized with various esters (oleoyl 13, 15, octyl 14, 16, biphenyl butyrate 17) via EDCI coupling (Scheme 5.4).

Scheme 5.4: Synthesis of retrocarbamates.
5.3.1d Amides

Analogs 18-20, incorporating amide at the sn-1 position (octyl 30, biphenyl butyrl 31), were synthesized from the corresponding amine 1-amino-3-methoxy-2-propanol 27. The sn-2 position was subsequently functionalized with various esters (octyl 18, oleyl 19, biphenyl butyrate 20) via EDCI coupling (Scheme 5.5).

![Scheme 5.5: Synthesis of amides.]

5.3.2 Simple β-Lactone Compounds

The β-lactone analog 33 completely lacking both the N-formyl-α-amino and the n-hexyl groups was prepared by treating racemic 2-hydroxy 38 palmitic acid with N-phenyl-bis(trifluoromethanesulfonimide). The shorter chain separable trans-34 and cis-35 β-lactones were prepared according to the reported method for this structure-
activity relationship study (Scheme 5.6). The β-lactones 33-35 were not deemed active or selective as DGL inhibitors via radiolabeled TLC assays.

![Scheme 5.6: Synthesis of simple β-lactones.](image)

**5.3.3 THL-based Compounds**

Analogs 39-50, structurally related to the moderate irreversible inhibitor THL and OMDM-188 were synthesized (Figure 5.3). Variations of the N-formyl-α-amino acid pharmacophore for highly selective binding at the DGL active site were first investigated. We re-synthesized the known N-formyl-L-isoleucyl ester 42 (OMDM-188) analog of tetrahydrolipstatin 5 (THL is an N-formyl-L-leucyl ester) as well as its three other novel isoleucine diasteromers (40, 41, 43). Di Marzo reported that smaller amino acid groups showed the most activity towards DGL. This was a main factor in choice of amino acid and compound design. Other alaphatic (39), constrained (44-48), and heterocyclic analogs (49, 50) were prepared from the corresponding benzylxycarbonyl protected α-amino acids via the reported method. As only SAR...
of the amino acid moiety was being investigated, there was no need to painstakingly synthesize THL \textit{de novo} when starting material (in the form of Alli) is commercially available. This established synthesis is relatively straightforward and therefore, was utilized in the synthesis of THL analogs described here.

![Diagram of THL-based analogs](image)

**Figure 5.3:** THL-based analogs

THL 5 was extracted from Alli pills by first dissolving the contents in chloroform and refluxing. After filtering and purifying the product with column chromatography, synthesis began with the KOH hydrolysis of THL (Figure 5.9). The resulting potassium salt 51 was treated with benzyl bromide in DMF in order to protect the free acid 52. The $\delta$ hydroxy group was protected by treating the molecule
with triisopropylsilyl triflate (TIPS-OTf), base, in anhydrous DCM at reduced temperature 53. Subsequently, the benzyl group was deprotected by hydrogenolysis 54. The free acid was cyclized with N-phenyl bis-trifluoromethane sulfonamide and base in DCM to give 55. Epimerization of the lactone was not observed as chemical shifts and coupling constants corresponding to trans-lactone were exclusively observed in the proton spectrum. After preparation of the lactone, the TIPS protecting group was removed with TBAF to yield hydroxy lactone 56 (Scheme 5.7).

Scheme 5.7: Synthesis of β-lactones: hydroxyl lactone from THL.

Carboxybenzyloxy (Cbz)-protected amino acids 57-64 (Figure 5.4) were then coupled to the hydroxy lactone with EDCI and a catalytic amount of DMAP in anhydrous DCM. The Cbz groups were subsequently removed via hydrogenolysis and the resulting
free amines 77-88 were formylated using formic acid and DCC to yield compounds 39-50. Epimerization of the amino group was not observed (Scheme 5.8). Standard hydrogenation conditions are not effective in Cbz deprotection for compounds 75 and 76 due to the presence of the sulfur-containing thienyl group. Dimethyl sulfide and boron trifluoride diethyl etherate were utilized instead.

Figure 5.4: Cbz-protected compounds 57-64

Scheme 5.8: Synthesis of THL analogs.
In initial qualitative radiolabeled TLC assays, THL analogs 39-50 were shown to inhibit DGL with activity and selectivity comparable to literature values. Additional 8-pt data will be performed for compounds 39-50 in order to determine accurate IC\textsubscript{50} values. THL analogs were also assayed for binding to the CB1 (rat brain preparation) and CB2 (mouse or human receptor expressed in HEK293), and none had a Ki below 1 µM in these competition binding experiments.

5.4 Experimental

5.4.1 Natural Substrate-based Compounds

3-methoxypropane-1,2-diyl bis(4-([1,1'-biphenyl]-4-yl)butanoate)

7: Biphenyl butyric acid (4.0 mmol, 964 mg) was added to a stirred solution of 21 methoxy propane diol (1.33 mmol, 141.8 mg) in anhydrous DCM under nitrogen. The reaction mixture was brought to 0° C and EDCI (6.65 mmol, 1.27 g) and DMAP (3.32 mmol, 406 mg) were added. The reaction mixture was allowed to warm to room temperature and was monitored by TLC (1:1 ethyl acetate/hexane: 21 methoxy propane diol R\textsubscript{f} 0.5, product R\textsubscript{f} 0.75). Workup included addition of 1 M HCl, extraction of the organic layer with DCM and drying with MgSO\textsubscript{4}. The resulting solution was filtered and concentrated under reduced pressure. The crude product was purified by column
chromatography (silica gel, 1:4 ethyl acetate/hexane) to provide a clear oil (20\textsuperscript{1}H NMR (500 MHz, chloroform-\textit{d}) $\delta$ ppm 1.93 - 2.04 (m, 4 H), 2.36 (t, $J = 7.3$ Hz, 2 H), 2.39 (t, $J = 7.3$ Hz, 2 H), 2.67 (t, $J = 7.3$ Hz, 2 H), 2.68 (t, $J = 7.3$ Hz, 2 H), 3.36 (s, 3 H), 3.52 (dd, $J = 12.7$, 4.9 Hz, 1 H), 3.54 (dd, $J = 12.7$, 4.9 Hz, 1 H), 4.18 (dd, $J = 11.7$, 6.4 Hz, 1 H), 4.37 (dd, $J = 11.7$, 3.9 Hz, 1 H), 5.21 - 5.28 (m, 1 H), 7.23 (br d, $J = 6.8$ Hz, 4 H), 7.33 (d, $J = 7.3$ Hz, 2 H), 7.42 (t, $J = 7.6$ Hz, 4 H), 7.50 (d, $J = 8.3$ Hz, 4 H), 7.56 (br d, $J = 7.3$ Hz, 4 H). HRMS for C\textsubscript{36}H\textsubscript{38}O\textsubscript{5} [MH\textsuperscript{+}] calcd, 550.271; found, 550.272. Calcd for C\textsubscript{36}H\textsubscript{38}O\textsubscript{5}: C, 78.52; H, 6.96. Found: C, 78.45; H, 6.74.

**3-methoxypropane-1,2-diyl dioctanoate**

\[\text{8: Triethyl amine (5.72 mmol, 0.5 mL) was added dropwise to a stirred solution of 21 methoxypropane diol (2.86 mmol, 303.5 mg) and octanoyl chloride (5.72 mmol, 0.98 mL) in 2 mL of anhydrous THF. Progression of the reaction was monitored by TLC (1:9 acetone/hexane: product $R_f$ 0.5). An aqueous workup was utilized, followed by extraction of the organic layer with DCM. The resulting solution was dried with MgSO\textsubscript{4}, filtered and concentrated under reduced pressure. The crude product was purified by column chromatography (silica gel, 1:9 acetone/hexane) to provide a clear oil (16%).} \]

$\text{^1H NMR (500 MHz, chloroform-\textit{d}) $\delta$ ppm 0.86 (t, $J = 6.8$ Hz, 6 H), 1.14 - 1.39 (m, 18 H) 1.49 - 1.67 (m, 2 H), 2.29 (t, $J = 7.8$ Hz, 2 H), 2.32 (t, $J = 7.8$ Hz, 2 H), 3.35 (s, 3 H), 3.50 (dd, $J = 4.9$, 2.9 Hz, 2 H), 4.14 (dd, $J = 11.8$, 6.4 Hz, 1 H), 4.31 (dd, $J =}
11.8, 3.7 Hz, 1 H), 5.09 - 5.27 (m, 1 H). HRMS for C_{20}H_{39}O_{5} [MH^+] calcd, 359.2797; found, 359.3.

**1-methoxy-3-((octylcarbamoyl)oxy)propan-2-yl octanoate**

![Chemical Structure]

9: Octanoic acid (0.062 mmol, 9.8 µL) was added to a stirred solution of 26 (0.052 mmol, 13.6 mg) in anhydrous DCM under nitrogen. The reaction mixture was brought to 0° C and EDCI (0.10 mmol, 19.9 mg) and DMAP (0.062 mmol, 7.62 mg) were added. The reaction mixture was allowed to warm to room temperature and was monitored by TLC. Workup included 1 M HCl wash, saturated NaHCO_{3} wash with dichloromethane extraction of the organic layer. To remove excess acid and chloride, 50 mg of MP-Trisamine was added and stirred for 1 hour at room temperature. The resulting solution was filtered, dried with MgSO_{4} and concentrated under reduced pressure. A yellow oil in 89% yield was obtained. \(^1\)H NMR (500 MHz, chloroform-d) δ ppm 0.88 (t, \(J = 6.8\) Hz, 6 H), 1.20 -1.40 (m, 18 H), 1.45 -1.55 (m, 2 H) 1.55 - 1.70 (m, 2 H), 2.34 (t, \(J = 7.6\) Hz, 2 H), 3.10 -3.20 (m, 2 H), 3.36 (s, 3 H), 3.51 (d, \(J = 4.4\) Hz, 2 H), 4.17 (dd, \(J = 11.7, 6.4\) Hz, 1 H), 4.28 (dd, \(J = 11.7, 2.9\) Hz, 1 H), 4.61 - 4.73 (m, 1 H) 5.14 -5.25 (m, 1 H).HRMS for C_{21}H_{41}O_{5}N [MH^+] calcd, 387.298 found; 387.298 Calcd for C_{21}H_{41}O_{5}N: C, 65.08; H, 10.66; N, 3.61. Found: C, 65.08; H, 10.55; N, 3.41.
1-methoxy-3-((octylcarbamoyl)oxy)propan-2-yl oleate

10: 26 (0.190 mmol, 49.8 mg) was added to a stirred solution of oleic acid (0.247 mmol, 69.9 mg) in anhydrous DCM under nitrogen. The reaction mixture was brought to 0° C and EDCI (0.475 mmol, 91.0 mg) and DMAP (0.228 mmol, 27.8 mg) were added. The reaction mixture was allowed to warm to room temperature and was monitored by TLC (3:7 acetone/hexane: product Rf 0.5). Workup included addition of 1 M HCl, extraction of the organic layer with dichloromethane and drying with MgSO₄. The resulting solution was filtered and concentrated under reduced pressure. The crude product was purified by column chromatography (silica gel, 3:7 acetone/hexane) to provide a yellow solid (71.5%).

1H NMR (500 MHz, chloroform-d) δ ppm 0.89 (t, J=6.6 Hz, 6 H), 1.17 - 1.41 (m, 28 H), 1.45 -1.55 (m, 2 H), 1.60 – 1.70 (m, 2 H), 1.98 - 2.05 (m, 4 H), 2.34 (t, J = 7.6 Hz, 2 H), 3.12 -3.20 (m, 2 H), 3.37 (s, 3 H), 3.52 (d, J = 4.4 Hz, 2 H), 4.18 (dd, J = 11.7, 6.4 Hz, 2 H), 4.27 (dd, J = 11.7, 3.4 Hz, 2 H), 4.62 - 4.70 (m, 1 H), 5.16 - 5.23 (m, 1 H), 5.30 - 5.39 (m, 2 H). HRMS for C₃₁H₅₉O₅N [MH⁺] calcd, 525.43932; found, 525.43925.
(5Z,8Z,11Z,14Z)-1-methoxy-3-((octylcarbamoyl)oxy)propan-2-yl-icosa-5,8,11,14-tetraenoate

11: Arachidonic acid (0.277 mmol, 84.4 mg) was added to a stirred solution of 26 (0.184 mmol, 48.3 mg) in anhydrous DCM under nitrogen. The reaction mixture was brought to 0° C and EDCI (0.369 mmol, 70.8 mg) and DMAP (0.221 mmol, 27.0 mg) were added. The reaction mixture was allowed to warm to room temperature and was monitored by TLC (4:6 acetone/hexane: product Rf 0.6). Workup included addition of 1 M HCl, extraction of the organic layer with DCM and drying with MgSO₄. The resulting solution was filtered and concentrated under reduced pressure. The crude product was then purified by column chromatography (silica gel, 3:7 acetone/hexane) to provide a light yellow oil was obtained (87%). ¹H NMR (500 MHz, chloroform-d) δ ppm 0.89 (t, J = 6.7 Hz, 3 H), 0.90 (t, J = 6.7 Hz, 3 H), 1.21 - 1.41 (m, 16 H), 1.44 - 1.53 (m, 2 H), 1.72 (apparent quintet, J = 7.8 Hz, 2 H), 2.07 (dt, J = 6.8, 6.8 Hz, 2 H), 2.13 (dt, J = 6.8, 6.8 Hz, 2 H), 2.37 (t, J = 7.6 Hz, 2 H), 2.78 - 2.88 (m, 6 H), 3.12 - 3.20 (m, 2 H), 3.36 (s, 3 H), 3.52 (d, J = 3.9 Hz, 2 H), 4.17 (dd, J = 11.7, 6.3 Hz, 1 H), 4.29 (dd, J = 11.7, 2.9 Hz, 1 H), 4.64 -4.70 (m, 1 H), 5.17 – 5.24 (m, 1 H), 5.31 - 5.43 (m, 8 H). HRMS for C₃₃H₅₇O₅N [MH⁺] calcd, 547.423; found, 547.423. Calcd for C₃₃H₅₇O₅N: C, 72.35; H, 10.49; N, 2.56. Found: C, 72.61; H, 10.49; N, 2.41.
3-methoxypropane-1,2-diy bis(octylcarbamate)

12: Reaction of 21 methoxy-propane diol (0.66 mmol, 69.9 mg) and octyl isocyanate (1.97 mmol, 0.35 mL) was performed neat at 50 °C overnight. The reaction was monitored by LCMS. Additional equivalents of isocyanate were added to push the reaction to completion. Kugelrohr distillation and column chromatography (silica gel, 2:8 ethyl acetate/hexane: product R_f 0.3) were utilized in purifying the crude product to provide a white solid in (36%) yield. ^1H NMR (500 MHz, chloroform-d) δ ppm 0.90 (t, J=6.8 Hz, 6 H), 1.23 - 1.39 (m, 20 H), 1.44 - 1.55 (m, 4 H), 3.12 - 3.25 (m, 4 H), 3.39 (s, 3 H), 3.55 (d, J = 4.5 Hz, 2 H), 4.22 (dd, J = 11.5, 6.5 Hz, 1 H), 4.28 (br dd, J = 11.5, 2.5 Hz, 1 H), 4.68 - 4.81 (m, 2 H), 5.03 - 5.17 (m, 1 H). HRMS for C_{22}H_{44}O_2N_3 [MH^+] calcd, 416.325 found; 416.325. MP: 59°-62°C.

1-methoxy-3-(((octyloxy)carbonyl)amino)propan-2-yl oleate

13: Oleic acid (0.17 mmol, 40 µL) was added dropwise to a solution stirred of secondary alcohol 28 (0.09 mmol, 23.4 mg) in anhydrous DCM. The reaction mixture was brought to 0 °C and EDCI (0.22 mmol, 42.9 mg) and DMAP (0.1 mmol, 13 mg) were added. The reaction mixture was allowed to warm to room temperature and was monitored by TLC (3:7 acetone/hexane: starting material R_f 0.45,
product $R_f$ 0.7). Workup included addition of 1 M HCl, extraction of the organic layer with DCM and drying with MgSO$_4$. The resulting solution was filtered and concentrated under reduced pressure. The crude product was purified by column chromatography (silica gel, 1:4 acetone/hexane) to provide a clear oil (71%). $^1$H NMR (500 MHz, chloroform-$d$) $\delta$ ppm 0.89 (t, $J=6.8$ Hz, 6 H), 1.23 - 1.39 (m, 30 H), 1.56 - 1.67 (m, 4 H), 1.98 - 2.07 (m, 4 H), 2.34 (t, $J=7.6$ Hz, 2 H), 3.37 (s, 3 H), 3.35 - 3.43 (m, 1 H), 3.47 - 3.57 (m, 3 H), 4.05 (t, $J=6.35$ Hz, 2 H), 4.87 - 4.94 (m, 1 H), 5.01 - 5.08 (m, 1 H), 5.31 - 5.40 (m, 2 H). HRMS for C$_{31}$H$_{59}$O$_5$N [MH$^+$] calcd, 525.439; found, 525.44.

1-methoxy-3-(((octyloxy)carbonyl)amino)propan-2-yl octanoate

14: Octanoic acid (0.095 mmol, 15 $\mu$L) was added dropwise to a stirred solution of retro-carbamate 28 (0.08 mmol, 20.8 mg) in anhydrous DCM under nitrogen. The reaction mixture was brought to 0 $^\circ$C and EDCI (0.19 mmol, 38.1 mg) and DMAP (0.095 mmol, 11.6 mg) were added. The reaction mixture was allowed to warm to room temperature and was monitored by TLC (3:7 acetone/hexane: starting material $R_f$ 0.45, product $R_f$ 0.7). Workup included addition of 1 M HCl, extraction of the organic layer with DCM and drying with MgSO$_4$. The resulting solution was filtered and concentrated under reduced pressure. The crude product was purified by column chromatography (silica gel, 1:4 acetone/hexane) to provide a white solid (93.7%). $^1$H
NMR (500 MHz, chloroform-d) δ ppm 0.89 (t, J = 6.8 Hz, 6 H), 1.20 -1.40 (m, 18 H), 1.57 - 1.67 (m, 4 H), 2.34 (t, J = 7.6 Hz, 2 H), 3.37 (s, 3 H), 3.35 - 3.42 (m, 1 H), 3.47 - 3.56 (m, 3 H), 4.05 (t, J =6.6 Hz, 2 H), 4.88 - 4.95 (m, 1 H), 5.01 – 5.08 (m, 1 H).

HRMS for C_{21}H_{42}O_5N [MH^+] calcd, 388.31; found, 388.31. Calcd for C_{21}H_{41}NO_5: C, 65.08; H, 10.66; N, 3.61. Found: C, 65.35; H, 10.47; N, 3.85.

**1-(((cyclohexylideneamino)oxy)carbonyl)amino)-3-methoxypropan-2-yl oleate**

15: Oleic acid (0.14 mmol, 45 µL) was added dropwise to a stirred solution of alcohol 29 (0.0.10 mmol, 26.8 mg) in anhydrous DCM under nitrogen. The reaction mixture was brought to 0 °C and EDCI (0.27 mmol, 52.2 mg) and DMAP (0.13 mmol, 15.9 mg) were added. The reaction mixture was allowed to warm to room temperature and was monitored by TLC (4:6 acetone/hexane: starting material R_f 0.3, product R_f 0.5). Workup included addition of 1 M HCl, extraction of the organic layer with dichloromethane and drying with MgSO_4. The resulting solution was filtered and concentrated under reduced pressure. The crude product was purified by column chromatography (silica gel, 4:6 acetone/hexane) to provide a clear oil (71%). ¹H NMR (500 MHz, chloroform-d) δ ppm 0.89 (t, J = 7.1 Hz, 3 H), 1.20 - 1.40 (m, 22 H), 1.58 - 1.72 (m, 8 H), 1.71 - 1.80 (m, 2 H), 2.02 (dt, J =6.7, 6.7 Hz, 4 H), 2.28 (t, J = 6.4 Hz, 2 H), 2.35 (t, J = 7.6 Hz, 2 H), 2.60 (t, J = 6.4 Hz, 2 H), 3.38 (s, 3 H), 3.49 (ddd, J = 14.2, 12.2, 5.9 Hz, 1 H), 3.56 (d, J =4.9 Hz, 2 H), 3.63 (ddd, J = 14.1, 6.4, 4.9 Hz, 1
1-((((cyclohexylideneamino)oxy)carbonyl)amino)-3-methoxypropan-2-yl
octanoate

16: Octanoic acid (0.12 mmol, 20 µL) was added dropwise to a stirred solution of alcohol 29 (0.09 mmol, 23.8 mg) in anhydrous DCM. The reaction mixture was brought to 0 °C and EDCI (0.24 mmol, 46.5 mg) and DMAP (0.116 mmol, 14.3 mg) were added. The reaction mixture was allowed to warm to room temperature and was monitored by TLC (4:6 acetone/hexane: starting material Rf 0.3, product Rf 0.45). Workup included addition of 1 M HCl, extraction of the organic layer with dichloromethane and drying with MgSO₄. The resulting solution was filtered and concentrated under reduced pressure. The crude product was purified by column chromatography (silica gel, 4:6 acetone/hexane) to provide a clear oil (43%). ¹H NMR (500 MHz, chloroform-d) δ ppm 0.88 (br t, J = 6.8 Hz, 3 H), 1.22 - 1.39 (m, 10 H), 1.58 - 1.71 (m, 4 H), 1.70 - 1.80 (m, 2 H), 2.28 (t, J = 6.4 Hz, 2 H), 2.35 (t, J = 7.8 Hz, 2 H), 2.59 (t, J = 6.4 Hz, 2 H), 3.38 (s, 3 H), 3.51 (ddd, J = 14.6, 11.7, 5.9 Hz, 1 H), 3.56 (d, J = 4.4 Hz, 2 H), 3.63 (ddd, J = 14.6, 5.9, 5.8 Hz, 1 H), 5.07 - 5.16 (m, 1 H), 6.56 – 6.67 (m, 1 H). HRMS for [MH⁺] calcd, 371.254; found,
371.254. Calcd for C\(_{19}\)H\(_{34}\)N\(_2\)O\(_5\): C, 61.60; H, 9.25; N, 7.56. Found: C, 61.88; H, 9.21; N, 7.47.

1-(((cyclohexylideneamino)oxy)carbonyl)amino)-3-methoxypropan-2-yl-4-([1,1'-biphenyl]-4-yl)butanoate

17: Biphenyl butyric acid (0.21 mmol, 517.9 mg) was added dropwise to a stirred solution of alcohol 29 (0.16 mmol, 40.5 mg) in anhydrous DCM. The reaction mixture was brought to 0 °C and EDCI (0.41 mmol, 80 mg) and DMAP (0.19 mmol, 24.1 mg) were added. The reaction mixture was allowed to warm to room temperature and was monitored by TLC (4:6 acetone/hexane: starting material R\(_f\) 0.3, product R\(_f\) 0.50). Workup included addition of 1 M HCl, extraction of the organic layer with DCM and drying with MgSO\(_4\). The resulting solution was filtered and concentrated under reduced pressure. The crude product was purified by column chromatography (silica gel, 4:6 acetone/hexane) to provide a clear oil (31%). \(^1\)H NMR (500 MHz, chloroform-\(d\)) \(\delta\) ppm 1.52 - 1.72 (m, 6 H), 2.00 (quintet, \(J = 7.8\) Hz, 2 H), 2.21 (t, \(J = 6.4\) Hz, 2 H), 2.41 (t, \(J = 7.8\) Hz, 2 H), 2.55 (t, \(J = 6.4\) Hz, 2 H), 2.69 (t, \(J = 7.6\) Hz, 2 H), 3.37 (s, 3 H), 3.50 (dd, \(J = 14.2, 6.4, 6.3\) Hz, 1 H), 3.56 (d, \(J = 4.9\) Hz, 2 H), 3.63 (ddd, \(J = 14.2, 5.4, 5.4\) Hz, 1 H), 5.10 - 5.17 (m, 1 H), 6.56 – 6.64 (m, 1 H), 7.22 - 7.28 (m, 2 H), 7.30 - 7.36 (m, 1 H), 7.43 (t, \(J = 7.6\) Hz, 2 H), 7.51 (d, \(J = 8.3\) Hz, 2 H), 7.57 (d, \(J = 7.3\) Hz, 2H). HRMS for
1-methoxy-3-octanamidopropan-2-yl octanoate

Octanoyl chloride (0.6 mmol, 0.102 mL) was added dropwise to a stirred solution of 1-amino-3-methoxypropane-2-ol 27 (0.3 mmol, 31.6 mg) in anhydrous DCM under nitrogen. Triethyl amine (0.45 mmol, 0.04 mL) and a catalytic amount of DMAP were subsequently added to the reaction. The reaction was monitored by TLC (3:7 acetone/hexane). Workup included both HCl and NaHCO₃ washes, preceding extraction with DCM. The reaction was dried with MgSO₄, filtered and concentrated under reduced pressure. The crude product was purified by column chromatography (silica gel, 3:7 acetone/hexane) to provide a white solid (40%). ¹H NMR (500 MHz, chloroform-"d") δ ppm 0.88 (br t, J = 6.7 Hz, 6 H), 1.23 - 1.37 (m, 16 H), 1.58 - 1.66 (m, 4 H), 2.17 (t, J = 7.6 Hz, 2 H), 2.35 (t, J = 7.6 Hz, 2 H), 3.37 (s, 3 H), 3.44 (ddd, J = 14.2, 6.3, 5.9 Hz, 1 H), 3.51 (dd, J = 10.7, 4.3 Hz, 1 H), 3.54 (dd, J = 10.7, 4.3 Hz, 1 H), 3.61 (ddd, J = 14.2, 5.8, 4.4 Hz, 1 H), 5.02 -5.08 (m, 1 H), 5.79 -5.83 (m, 1 H).

1-methoxy-3-octanamidopropan-2-yl oleate

19: Oleic acid (0.053 mmol, 17 µL) was added to a stirred solution of 30 (0.048 mmol, 11.2 mg) in anhydrous DCM under nitrogen. The reaction mixture was brought to 0°C and EDCI (0.12 mmol, 23.1 mg) and DMAP (0.058 mmol, 7.0 mg) were added. The reaction mixture was allowed to warm to room temperature and was monitored by TLC (4:6 acetone/hexane: starting material R:\ 0.4, product R:\ 0.7). Workup included addition of 1 M HCl, extraction of the organic layer with DCM and drying with MgSO₄. The resulting solution was filtered and concentrated under reduced pressure. The crude product was purified by column chromatography (silica gel, 3:7 acetone/hexane) to provide a clear oil (61.3%). ¹H NMR (500 MHz, chloroform-d) δ ppm 0.89 (br t, J = 6.7 Hz, 6 H), 1.20 - 1.40 (m, 28 H), 1.57 - 1.66 (m, 4 H), 2.02 (apparent q, J = 6.4, 4 H), 2.16 (t, J = 7.8 Hz, 2 H), 2.34 (t, J = 7.6 Hz, 2 H), 3.37 (s, 3 H), 3.44 (ddd, J = 14.2, 6.1, 6.1 Hz, 1 H), 3.50 (dd, J = 10.2, 4.9 Hz, 1 H), 3.54 (dd, J = 10.2, 4.9 Hz, 1 H), 3.61 (ddd, J = 14.2, 5.9, 4.4 Hz, 1 H), 5.02 – 5.08 (m, 1 H), 5.30 - 5.39 (m, 2 H), 5.79 – 5.85 (m 1 H).HRMS for C₃₀H₅₇O₄N [MH⁺] calcd, 495.428; found, 495.428.
1-(4-[(1,1'-biphenyl)-4-yl]butanamido)-3-methoxypropan-2-yl-4-[(1,1'-biphenyl)-4-yl]butanoate

20: Biphenyl butyric acid (1.51 mmol, 364.0 mg) was added to a stirred solution of 1-amino-3-methoxypropane-2-ol 27 (0.505 mmol, 53.1 mg) in anhydrous DCM under nitrogen. The reaction mixture was brought to 0 °C and EDCI (2.52 mmol, 484 mg) and DMAP (1.26 mmol, 154 mg) were added. The reaction mixture was allowed to warm to room temperature and was monitored by TLC (1:1 ethyl acetate/hexane: product Rf 0.3, amine Rf 0.5). Workup included addition of 1 M HCl, extraction of the organic layer with dichloromethane and drying with MgSO4. The resulting solution was filtered and concentrated under reduced pressure. The crude product was purified by column chromatography (silica gel, 1:1 acetone/hexane: product Rf 0.5) to produce a white solid (19%). 1H NMR (500 MHz, chloroform-d) δ ppm 1.96 - 2.05 (m, 4 H), 2.22 (t, J = 7.6 Hz, 2 H), 2.42 (br t, J = 7.3 Hz, 2H), 2.70 (t, J = 7.1 Hz, 4 H), 3.38 (s, 3 H), 3.46 (ddd, J = 14.4, 6.1, 6.1 Hz, 1 H), 3.55 (d, J = 5.4 Hz, 2 H), 3.66 (ddd, J = 14.4, 5.9, 4.4 Hz, 1 H), 5.06 - 5.13 (m, 1 H), 5.80 – 5.88 (m, 1 H), 7.23 - 7.28 (m, 4 H), 7.35 (br t, J = 7.3 Hz, 2 H), 7.45 (t, J = 7.6 Hz, 4 H), 7.53 (d, J = 8.3 Hz, 4 H), 7.59 (d, J = 7.8 Hz, 4 H). HRMS for C36H39NO4 [MH+] calcd, 549.287; found, 549.288. Calcd for C36H39NO4: C, 78.66; H, 7.15; N, 2.55. Found: C, 78.41; H, 7.08; N, 2.46
1-methoxy-3-(trityloxy)propan-2-ol

22): Triethyl amine (2.5 mL, 28.3 mmol) was added dropwise to a stirred solution of 21 methoxy propane diol (2.0 g, 18.8 mmol) and a catalytic amount of DMAP in anhydrous DCM. The mixture was stirred for 15 minutes before trityl chloride was slowly added (5.25 g, 18.8 mg). Completion of the reaction was determined by TLC (3:7 ethyl acetate/hexane: product R_f 0.45). After an aqueous workup, the resulting organic layers were dried with MgSO_4, filtered and concentrated under reduced pressure. The crude product was purified by column chromatography (silica gel, 1:4 ethyl acetate/hexane). An 87% yield was obtained. ^1^H NMR (500 MHz, chloroform-d) δ ppm 2.41 (d, J=4.88 Hz, 1 H) 3.15 - 3.24 (m, 1 H) 3.36 (s, 3 H) 3.40 - 3.52 (m, 2 H) 3.91 - 3.99 (m, 1 H) 7.20 - 7.27 (m, 3 H) 7.30 (t, 6 H) 7.43 (d, J=7.32 Hz, 6 H).

tert-butyl((1-methoxy-3-(trityloxy)propan-2-yl)oxy)diphenylsilane

23: TBDPS-Cl (2.58 mL, 9.93 mmol) was added dropwise to a stirred solution of alcohol 22 (3.0 g, 8.27 mmol) and imidazole (1.127 g, 16.5 mmol) in anhydrous DCM under nitrogen. Completion of the reaction was determined by TLC (2:8 ethyl acetate/hexane: starting material R_f 0.2; product R_f 0.75). After an aqueous workup, the resulting organic layers were dried with MgSO_4, filtered and concentrated under reduced pressure. The crude product was purified by column chromatography (silica gel, 1:9 ethyl acetate/hexane) to provide a white solid. A 62%
yield was obtained. $^1$H NMR (500 MHz, chloroform-$d$) $\delta$ ppm 1.02 (s, 9 H) 3.12 (s, 3 H) 3.15 (d, $J$=4.88 Hz, 2 H) 3.33 (dd, $J$=10.01, 5.13 Hz, 1 H) 3.46 (dd, $J$=9.77, 4.88 Hz, 1 H) 3.92 - 4.00 (m, 1 H) 7.13 - 7.46 (m, 21 H) 7.61 (d, $J$=6.84 Hz, 2 H) 7.65 (d, $J$=6.84 Hz, 2 H).

2-((tert-butyldiphenylsilyl)oxy)-3-methoxypropan-1-ol

24: Camphor sulfonic acid (110.0 mg, 0.47 mg) was added to a stirred solution of tritylated compound 23 in 9:1 DCM/EtOH. The solution was vigorously stirred at room temperature for 3 days. Completion of the reaction was determined by TLC (2:8 ethyl acetate/hexane: product R$_f$ 0.30). After a basic workup, the resulting organic layers were dried with MgSO$_4$, filtered and concentrated under reduced pressure. The crude product was purified by column chromatography (silica gel, 2:7 ethyl acetate/hexane). A 62% yield was obtained. $^1$H NMR (500 MHz, chloroform-$d$) $\delta$ ppm 1.07 (s, 9 H) 2.04 (t, 1 H) 3.19 (s, 3 H) 3.30 - 3.38 (m, 1 H) 3.38 - 3.49 (m, 1 H) 3.62 (br. s, 2 H) 3.79 - 3.97 (m, 1 H) 7.39 (d, $J$=6.84 Hz, 4 H) 7.41 - 7.48 (m, 2 H) 7.68 (dd, $J$=6.59, 4.64 Hz, 4 H).

2-((tert-butyldiphenylsilyl)oxy)-3-methoxypropyl octylcarbamate

25: Alcohol 24 (0.78g, 2.26 mmol) was reacted neat with octyl isocyanate (0.7g, 4.53 mmol) at 60 °C for 12 hours. The
reaction was monitored by TLC (2:8 ethyl acetate/hexane: product R<sub>f</sub> 0.5). The crude product was purified by column chromatography (silica gel, 15:85 ethyl acetate/hexane). A quantitative yield was obtained. \(^1\)H NMR (500 MHz, chloroform-\(d\)) \(\delta\) ppm 0.88 (t, \(J=6.84\) Hz, 3 H) 1.06 (s, 9 H) 1.27 (br. s, 8 H) 1.35 - 1.46 (m, 2 H) 2.97 - 3.12 (m, 2 H) 3.20 (s, 3 H) 3.34 (d, \(J=3.91\) Hz, 2 H) 3.90 - 4.06 (m, 2 H) 4.08 - 4.20 (m, 1H) 4.27 (br. s, 1 H) 7.30 - 7.47 (m, 6 H) 7.68 (d, \(J=7.32\) Hz, 4 H).

2-hydroxy-3-methoxypropyl octylcarbamate

26: TBAF (2.83 mmol, 2.83 mL) was added dropwise to a stirred solution of TBDPS protected 25 (1.182 g, 2.36 mmol) in anhydrous THF under nitrogen. The reaction was deemed complete by TLC analysis (3:7 acetone/hexane: product R<sub>f</sub> 0.45). After concentration, the crude product was purified by column chromatography (silica gel, 1:4 acetone/hexane) to yield a white solid (83%). \(^1\)H NMR (500 MHz, chloroform-\(d\)) \(\delta\) ppm 0.88 (t, \(J=6.59\) Hz, 3 H) 1.18 - 1.38 (m, 10 H) 1.50 (t, \(J=6.84\) Hz, 2 H) 2.85 (br. s, 1 H) 3.18 (q, \(J=6.84\) Hz, 2 H) 3.40 (s, 3 H) 3.41 - 3.53 (m, 2 H) 4.00 (br. s, 1 H) 4.12 (d, \(J=6.35\) Hz, 1 H) 4.19 (dd, \(J=11.72, 3.42\) Hz, 1 H) 4.78 (br. s, 1 H)

octyl (2-hydroxy-3-methoxypropyl)carbamate

28: Octyl chloroformate (94.0 mg, 0.488 mmol) was added dropwise to a cooled (0 °C)
solution of 1-amino-3-methoxypropane-2-ol 27 (51.3 mg, 0.488 mmol) and TEA (43.3 mg, 0.732 mmol) in anhydrous DCM. The reaction was monitored by TLC (4:6 acetone/hexane: product R$_f$ 0.6). After concentration, the crude product was purified by column chromatography (silica gel, 4:6 acetone/hexane). An 80% yield was obtained. $^1$H NMR (500 MHz, chloroform-$d$) $\delta$ ppm 0.89 (t, $J=6.59$ Hz, 3 H) 1.13 - 1.46 (m, 10 H) 1.61 (d, $J=6.84$ Hz, 2 H) 2.78 (br. s, 0 H) 3.03 - 3.28 (m, 0 H) 3.32 - 3.38 (m, 1 H) 3.39 (s, 3 H) 3.41 - 3.48 (m, 1 H) 3.89 (br.s, 0 H) 4.06 (t, $J=6.59$ Hz, 1 H) 5.08 (br. s, 1 H).

cyclohexanone-O-((2-hydroxy-3-methoxypropyl)carbamoyl) oxime

29: A solution of 27 1-amino-3-methoxypropane-2-ol (89.0 mg, 0.848 mmol) in anhydrous DCM was added to prepared oximinal chloroformate 32 (0.164 g, 0.933 mmol). Progress of the reaction was monitored using TLC (1:1 acetone/hexane). After concentration, the crude product was purified by column chromatography (silica gel, 1:1 acetone/hexane). A 60% yield was obtained. $^1$H NMR (500 MHz, chloroform-$d$) $\delta$ ppm 1.48 - 1.80 (m, 6 H) 2.25 (t, $J=6.10$ Hz, 2 H) 2.55 (t, $J=6.35$ Hz, 2 H) 3.12 (d, $J=3.91$ Hz, 1 H) 3.23 - 3.32 (m, 1 H) 3.34 (d, $J=2.44$ Hz, 1 H) 3.36 (s, 3 H) 3.40 - 3.53 (m, 2 H) 3.77 - 4.06 (m, 1 H) 6.67 (br. s, 1 H).
**N-(2-hydroxy-3-methoxypropyl)octanamide**

30: Octanoyl chloride (78.8 mg, 0.485 mmol) was added to a stirred solution of 27 1-amino-3-methoxypropane-2-ol (51.0 mg, 0.485 mmol) and TEA (43.0 mg, 0.727 mmol) in anhydrous DCM. The reaction was monitored by TLC (4:6 acetone/hexane: product $R_f$ 0.4). Workup included both NaHCO$_3$ and HCl washes. The resulting organic layers were dried with MgSO$_4$ and concentrated under reduced pressure. The crude product was purified by column chromatography (silica gel, 4:6 acetone/hexane) to provide a white solid (72%). $^1$H NMR (500 MHz, chloroform-$d$) $\delta$ ppm 0.89 (t, $J=6.84$ Hz, 3 H) 1.21 - 1.39 (m, 8 H) 1.64 (t, $J=7.32$ Hz, 2 H) 2.21 (t, $J=7.57$ Hz, 2 H) 3.07 (d, $J=3.91$ Hz, 1 H) 3.25 (m, $J=6.35$ Hz, 1 H) 3.34 (dd, $J=9.52$, 6.59 Hz, 1 H) 3.39 (s, 3 H) 3.41 - 3.46 (m, 1 H) 3.48 - 3.60 (m, 1 H) 3.82 - 3.95 (m, 1 H) 5.89 (br. s, 1 H).

**Cyclohexanone-O-chlorocarbonyl oxime**

32: Phosgene was added dropwise to a cooled (-10 °C) solution of oxime (0.106 g, 0.936 mmol) in carbon tetrachloride. The solution was subsequently stirred at room temperature for 18 hours. Excess phosgene was removed by purging the solution with argon. The resulting clear oil was used directly in the next step.
5.4.2 Simple β-lactone Compounds

4-tridecyloxetan-2-one

33: A stirred solution of D,L-β-hydroxypalmitic acid 38 (20.0 mg, 0.0734 mmol) and TEA (14.8 mg, 0.020 mL) in anhydrous DCM was treated with N-phenyl-bis(trifluoromethanesulfonimide (39.34 mg, 0.11 mmol) at 0 °C. The resulting mixture was monitored by TLC (1:9 ethyl acetate/hexane: product Rf 4.75). Upon completion of the reaction, the crude mixture was concentrated and purified by column chromatography (silica gel, 1:9 ethyl acetate/hexane) to yield a white solid (51%); mp 39-40 °C. 1H NMR (500 MHz, chloroform-d) δ ppm 0.88 (t, J = 6.8 Hz, 3 H), 1.21 - 1.51 (m, 22 H), 1.69 - 1.79 (m, 1 H), 1.82 - 1.92 (m, 1 H), 3.06 (dd, J =16.4, 4.2 Hz, 1 H), 3.50 (dd, J=16.1, 5.9 Hz, 1 H), 4.50 (ddd, J=11.5, 6.0, 6.0 Hz, 1 H).

(3R,4S)-3,4-dihexyloxetan-2-one

34: Benzene sulfonyl chloride (13.6 mmol, 1.742 ml) was added dropwise to a stirred solution of 37 (6.8 mmol, 1.85 g) in 28 ml of anhydrous pyridine at 0 °C. The solution was subsequently shaken, sealed and stored in the refrigerator overnight. Work-up included pouring the reaction mixture over 3 volumes of crushed ice before extraction with several volumes of Et2O. The combined ether layers were washed with saturated NaHCO3 and water. The organic layer was dried with MgSO4, filtered and concentrated under reduced pressure. The crude product was purified by column chromatography (silica gel, 1:1 DCM/hexane:
product $R_f$ 0.5) to provide a clear colorless liquid (21%). $^1$H NMR (500 MHz, chloroform-\textit{d}) $\delta$ ppm 0.89 (t, $J$ = 6.8 Hz, 6 H), 1.20 - 1.47 (m, 16 H), 1.45 - 1.58 (m, 2 H), 1.59 - 1.71 (m, 2 H), 1.71 - 1.85 (m, 2 H), 3.60 (ddd, $J$ = 8.9, 6.8, 6.8 Hz, 1 H), 4.54 (ddd, $J$ = 9.7, 6.1, 4.2 Hz, 1 H).

### (3S,4S)-3,4-dihexyloxetan-2-one

35: Benzene sulfonyl chloride (13.6 mmol, 1.742 ml) was added dropwise to a stirred solution of $\beta$-hydroxy acid 37 (6.8 mmol, 1.85 g) in 28 ml of anhydrous pyridine at 0 °C. The solution was subsequently shaken, sealed and stored in the refrigerator overnight. Work-up included pouring the reaction mixture over 3 volumes of crushed ice before extraction with several volumes of Et$_2$O. The combined ether layers were washed with saturated NaHCO$_3$ and water. The organic layer was dried with MgSO$_4$, filtered and concentrated under reduced pressure. The crude product was purified by column chromatography (silica gel, 1:1 DCM/hexane: product $R_f$ 0.5) to yield a clear colorless liquid (30%). $^1$H NMR (500 MHz, chloroform-\textit{d}) $\delta$ ppm 0.89 (t, $J$ = 6.8 Hz, 6 H), 1.20 - 1.50 (m, 18 H), 1.66 - 1.77 (m, 2 H), 1.77 - 1.95 (m, 2 H), 3.16 (ddd, $J$ = 8.7, 6.4, 4.1 Hz, 1 H), 4.21 (ddd, $J$ = 6.7, 6.7, 4.1 Hz, 1 H).
5.4.3 THL-based Compounds

(S)-(S)-1-((2S,3S)-3-hexyl-4-oxooxetan-2-yl)tridecan-2-yl 2-formamido-4-methylpentanoate

5: THL mp 41-42 °C 'H NMR (500 MHz, chloroform-\textit{d})
\[ \delta \text{ ppm } 0.88 \text{ (t, } J = 6.4 \text{ Hz, 3 H)}, 0.89 \text{ (t, } J = 6.4 \text{ Hz, 3 H)}, 0.97 \text{ (d, } J = 5.9 \text{ Hz, 3 H)}, 0.98 \text{ (d, } J = 5.9 \text{ Hz, 3 H)}, 1.22 - 1.50 \text{ (m, 27 H)}, 1.50 - 1.87 \text{ (m, 6 H)}, 2.00 \text{ (ddd, } J = 15.1, 4.9, 4.9 \text{ Hz, 1 H}), 2.17 \text{ (ddd, } J = 15.2, 7.8, 7.8 \text{ Hz, 1 H}), 3.22 \text{ (ddd, } J = 7.3, 7.3, 3.9 \text{ Hz, 1 H}), 4.29 \text{ (ddd, } J = 7.8, 4.9, 3.9 \text{ Hz, 1 H}), 4.70 \text{ (ddd, } J = 9.0, 8.6, 4.9 \text{ Hz, 1 H}), 5.00 - 5.06 \text{ (m, 1 H)}, 5.89 \text{ (d, } J = 8.4 \text{ Hz, 1 H)}, 8.22 \text{ (s, 1 H)}.

(S)-(S)-1-((2S,3S)-3-hexyl-4-oxooxetan-2-yl)tridecan-2-yl 2-formamidobutanoate

39: Cooled formic anhydride (95.5 mg, 1.29 mmol) was added to a stirred solution of amine 79 (28.5 mg, 0.068 mmol) in anhydrous DCM. The solution was kept at reduced temperatures between -5 °C and -10 °C for 45 minutes. At this time diisopropylethylamine (8.37 mg, 0.011 mL) was added before being stirred at room temperature for 1 hour. The reaction mixture was monitored by TLC (1:9 ethyl acetate/DCM: product R$_f$ 0.3). After consumption of starting material, the reaction mixture was concentrated. The crude product was purified by column chromatography (silica gel 1:9 ethyl acetate/DCM) to yield a white solid (27 %); mp 43-44 °C. 'H NMR (500 MHz, chloroform-\textit{d}) \[ \delta \text{ ppm } 0.88 \text{ (t, } J = 6.7 \text{ Hz, 3 H)}, 0.89 \text{ (t,
\( J = 6.7 \text{ Hz, 3 H}, 0.96 \text{ (t, } J = 7.6 \text{ Hz, 3 H), 1.20 - 1.54 \text{ (m, 26 H), 1.54 - 1.90 \text{ (m, 5 H), 1.92 - 2.01 \text{ (m, 1 H), 2.02 (ddd, } J = 15.1, 4.4, 4.4 \text{ Hz, 1 H), 2.17 (ddd, } J = 15.1, 7.9, 7.8 \text{ Hz, 1 H), 3.23 (ddd, } J = 7.4, 7.4, 4.2 \text{ Hz, 1 H), 4.30 (ddd, } J = 8.0, 4.5, 4.4 \text{ Hz, 1 H), 4.64 (apparent q, } J = 7.3 \text{ Hz, 1 H), 5.02 - 5.09 \text{ (m, 1 H), 6.10 (d, } J = 7.3 \text{ Hz, 1 H), 8.25 (s, 1 H).}\)

\( (2S,3R)-(S)-1-((2S,3S)-3-hexyl-4-oxooxetan-2-yl)tridecan-2-yl-2-formamido-3-methylpentanoate \)

40: Cooled formic anhydride (148.8 mg, 2.0 mmol) was added to a stirred solution of amine 78 (47.0 mg, 0.10 mmol) in anhydrous DCM under nitrogen. The solution was kept at reduced temperatures between \(-5^\circ \text{C and -10 }^\circ \text{C for two hours. The reaction mixture was monitored by TLC (1:9 ethyl acetate/DCM: product } R_f 0.3).\) After consumption of starting material, the reaction mixture was concentrated. The crude product was purified by column chromatography (silica gel 1:9 ethyl acetate/DCM) to provide a white solid (55.47 \%); mp 49-50 \(^\circ\text{C.}\)

\(^1\text{H NMR (500 MHz, chloroform-}d\text{) } \delta \text{ ppm 0.87 (t, } J = 7.3 \text{ Hz, 3 H), 0.88 (t, } J = 7.3 \text{ Hz, 3 H), 0.96 (t, } J = 7.6 \text{ Hz, 3 H), 0.97 (d, } J = 7.6 \text{ Hz, 3 H), 1.14 - 1.52 \text{ (m, 28 H), 1.54 - 1.88 (m, 4 H), 1.92 - 2.00 (m, 1 H), 2.02 (ddd, } J = 15.1, 5.4, 5.4 \text{ Hz, 1 H), 2.19 (ddd, } J = 15.1, 7.7, 7.5 \text{ Hz, 1 H), 3.23 (ddd, } J = 7.6, 7.6, 3.9 \text{ Hz, 1 H), 4.29 (ddd, } J = 7.6, 5.4, 3.9 \text{ Hz, 1 H), 4.77 (dd, } J = 9.0, 3.7 \text{ Hz, 1 H), 4.96- 5.05 \text{ (m, 1 H), 6.10 (d, } J = 8.8 \text{ Hz, 1 H), 8.26 (s, 1 H).}\)
(2R,3S)-(S)-1-((2S,3S)-3-hexyl-4-oxooxetan-2-yl)tridecan-2-yl-2-formamido-3-methylpentanoate

41: Cooled formic anhydride (142.45 mg, 1.92 mmol) was added to a stirred solution of amine 81 (45.0 mg, 0.096 mmol) in anhydrous DCM under nitrogen. The solution was kept at reduced temperatures between -5 °C and -10 °C for 45 minutes. At this time diisopropylethylamine (12.4 mg, 0.016 mL) was added before being stirred at room temperature for 1 hour. The reaction mixture was monitored by TLC (1:9 ethyl acetate/DCM: product Rf 0.3). After consumption of starting material, the reaction mixture was concentrated. The crude product was purified by column chromatography (silica gel 1:9 ethyl acetate/DCM) to provide a clear colorless liquid (12 %). 1H NMR (500 MHz, chloroform-d) δ ppm 0.85 - 0.90 (m, 9 H), 0.97 (t, J = 7.3 Hz, 3 H), 1.16 - 1.51 (m, 28 H), 1.52 - 1.87 (m, 4 H), 1.91 - 2.01 (m, 1 H), 2.02 (ddd, J = 15.0, 5.0, 4.9 Hz, 1 H), 2.19 (ddd, J = 15.0, 7.7, 7.1 Hz, 1 H), 3.23 (ddd, J = 7.4, 7.3, 3.9 Hz, 1 H), 4.33 (ddd, J = 7.7, 5.0, 3.9 Hz, 1 H), 4.77 (dd, J = 9.0, 3.7 Hz, 1 H), 5.00 - 5.08 (m, 1 H), 5.96 (d, J = 8.8 Hz, 1 H), 8.27 (s, 1 H).

(2S,3S)-(S)-1-((2S,3S)-3-hexyl-4-oxooxetan-2-yl)tridecan-2-yl-2-formamido-3-methylpentanoate

42: Cooled formic anhydride (79.3 mg, 1.07 mmol) was added to a stirred solution of amine 77 (50.1 mg, 0.107 mmol) in anhydrous DCM under nitrogen. The solution
was kept at reduced temperatures between -5 °C and -10 °C for two hours. The reaction mixture was monitored by TLC (1:9 ethyl acetate/DCM: product R<sub>f</sub> 0.3). After consumption of starting material, the reaction mixture was subjected to an aqueous workup and extraction with DCM. The organic layer was dried with MgSO<sub>4</sub> and concentrated under reduced pressure. The crude product was purified by column chromatography (silica gel 1:9 ethyl acetate/DCM) to yield a white solid (7.16 %); mp 59-60 °C. <sup>1</sup>H NMR (500 MHz, chloroform-<sup>d</sup>) δ ppm 0.88 (t, <i>J</i> = 7.3 Hz, 3 H), 0.89 (t, <i>J</i> = 7.3 Hz, 3 H), 0.96 (t, <i>J</i> = 7.8 Hz, 3 H), 0.97 (d, <i>J</i> = 7.8 Hz, 3 H), 1.10 - 1.60 (m, 28 H), 1.58 - 1.84 (m, 4 H), 1.85-2.00 (m, 1 H), 2.02 (ddd, <i>J</i> = 15.0, 4.5, 4.5 Hz, 1 H), 2.19 (ddd, <i>J</i> = 14.9, 7.7, 7.7 Hz, 1 H), 3.24 (ddd, <i>J</i> = 7.4, 7.4, 4.2 Hz, 1 H), 4.29 (ddd, <i>J</i> = 7.7, 4.5, 4.2 Hz, 1 H), 4.68 (dd, <i>J</i> = 8.8, 4.9 Hz, 1 H), 5.00 - 5.07 (m, 1 H), 6.05 (d, <i>J</i> = 8.8 Hz, 1 H), 8.26 (s, 1 H).

**43:** Cooled formic anhydride (85.6 mg, 1.15 mmol) was added to a stirred solution of **80** (15.46 mg, 0.033 mmol) in anhydrous DCM under nitrogen. The solution was kept at reduced temperatures between -5 °C and -10 °C for two hours. The reaction mixture was monitored by TLC (1:9 ethyl acetate/DCM: product R<sub>f</sub> 0.3). After consumption of starting material, the reaction mixture was concentrated. The crude product was purified by column chromatography (silica gel
1:9 ethyl acetate/DCM) to yield a clear and colorless liquid (15.9%). $^1$H NMR (500 MHz, chloroform-$d$) δ ppm 0.89 (t, $J = 6.6$ Hz, 6 H), 0.95 (t, $J = 7.3$ Hz, 3 H), 0.97 (d, $J = 7.3$ Hz, 3 H), 1.14 - 1.52 (m, 28 H), 1.54 - 1.88 (m, 4 H), 1.92 - 2.00 (m, 1 H), 2.03 (ddd, $J = 14.9$, 4.5, 4.5 Hz, 1 H), 2.19 (ddd, $J = 14.9$, 7.9, 6.5 Hz, 1 H), 3.24 (ddd, $J = 7.4$, 7.4, 4.2 Hz, 1 H), 4.34 (ddd, $J = 7.9$, 4.5, 4.2 Hz, 1 H), 4.66 (dd, $J = 8.6$, 4.6 Hz, 1 H), 5.01 - 5.08 (m, 1 H), 6.03 (d, $J = 8.8$ Hz, 1 H), 8.25 (s, 1 H). isomer 3.99 (dd, $J = 10.3$, 4.3 Hz, 0.05 H), 5.09 - 5.17 (m, 0.05 H), 5.98 (dd, $J = 11.7$, 10.3 Hz, 0.05 H), 8.02 (d, $J = 11.7$ Hz, 0.05 H).

(S)-(S)-1-((2S,3S)-3-hexyl-4-oxooxetan-2-yl)tridecan-2-yl-2-cyclopropyl-2-formamidoacetate

$^44$: Formic anhydride (288 mg, 3.9 mmol) was added to a stirred solution of amine $^84$ (88.0 mg, 0.19 mmol) in anhydrous DCM under nitrogen at -5 ºC. The resulting mixture was stirred at reduced temperature for 40 minutes and subsequently, 80 minutes at room temperature. The reaction was monitored by TLC (5:95 ethyl acetate/DCM: product R$_f$ 0.4). Upon completion of the reaction, the crude mixture was concentrated and purified by column chromatography (silica gel, 5:95 ethyl acetate/DCM) to yield a white solid (20%). $^1$H NMR (500 MHz, chloroform-$d$) δ ppm 0.39 - 0.51 (m, 1 H) 0.50 - 0.60 (m, 2 H) 0.61 - 0.71 (m, 1 H) 0.80 - 0.96 (m, 6 H) 1.02 - 1.16 (m, 1 H) 1.19 - 1.39 (m, 24 H) 1.40 - 1.51 (m, 1 H) 1.59 - 1.64 (m, 1 H) 1.63 - 1.71 (m, 1 H) 1.71 - 1.76 (m, 1 H) 1.76 - 1.88 (m, 1 H) 1.94
(S)-(S)-1-((2S,3S)-3-hexyl-4-oxooxetan-2-yl)tridecan-2-yl-2-cyclopentyl-2-formamidoacetate

45: Formic anhydride (154 mg, 2.0 mmol) was added to a stirred solution of amine 85 (50.0 mg, 0.1 mmol) in anhydrous DCM at -5 °C. The resulting mixture was stirred at reduced temperature for 40 minutes and subsequently, 80 minutes at room temperature. The reaction was monitored by TLC (5:95 ethyl acetate/DCM: product R$_f$ 0.5). Upon completion of the reaction, the crude mixture was concentrated and purified by column chromatography (silica gel, 5:95 ethyl acetate/DCM) to yield a white solid (15%). $^1$H NMR (500 MHz, chloroform-$d$) δ ppm 0.85 - 0.92 (m, 6 H) 1.18 - 1.51 (m, 27 H) 1.59 - 1.71 (m, 6 H) 1.71 - 1.89 (m, 4 H) 1.99 (t, $J$=4.64 Hz, 1 H) 2.02 (t, $J$=4.64 Hz, 1 H) 2.18 (dt, $J$=14.89, 7.69 Hz, 1 H) 2.23 - 2.38 (m, 1 H) 3.14 - 3.32 (m, 1 H) 4.22 - 4.36 (m, 1 H) 4.56 - 4.70 (m, 1 H) 4.93 - 5.09 (m, 1 H) 6.02 (d, $J$=8.30 Hz, 1 H) 8.24 (s, 1 H).
(S)-(S)-1-((2S,3S)-3-hexyl-4-oxooxetan-2-yl)tridecan-2-yl-2-cyclohexyl-2-formamidoacetate

46: Formic anhydride (54 mg, 0.73 mmol) was added to a stirred solution of amine 86 (18.0 mg, 0.036 mmol) in anhydrous DCM at -5 °C. The resulting mixture was stirred at reduced temperature for 40 minutes and subsequently, 80 minutes at room temperature. The reaction was monitored by TLC (5:95 ethyl acetate/DCM: product R$_f$ 0.5). Upon completion of the reaction, the crude mixture was concentrated and purified by column chromatography (silica gel, 5:95 ethyl acetate/DCM) to provide a white solid (16%). $^1$H NMR (500 MHz, chloroform-$d$) δ ppm 0.85 - 0.95 (m, 6 H) 0.98 - 1.21 (m, 3 H) 1.21 - 1.53 (m, 26 H) 1.59 - 1.94 (m, 12 H) 2.02 (t, J=4.39 Hz, 1 H) 2.05 (t, J=4.64 Hz, 1 H) 2.20 (m, J=14.89, 7.69, 7.69 Hz, 1 H) 3.18 - 3.33 (m, 1 H) 4.22 - 4.37 (m, 1 H) 4.57 - 4.69 (m, 1 H) 4.97 - 5.13 (m, 1 H) 6.05 (d, J=8.79 Hz, 1 H) 8.28 (s, 1 H).

(S)-1-((2S,3S)-3-hexyl-4-oxooxetan-2-yl)tridecan-2-yl-1-formamidocyclobutanearboxylate

47: Formic anhydride (38.4 mg, 0.52 mmol) was added to a stirred solution of amine 82 (11.7 mg, 0.026 mmol) in anhydrous DCM at -5 °C. The resulting mixture was stirred at reduced temperature for 40 minutes and subsequently, 80 minutes at room temperature. The reaction was monitored by TLC (15:85 ethyl acetate/DCM: product R$_f$ 0.4). Upon completion of the reaction, the crude
mixture was concentrated and purified by column chromatography (silica gel, 15:85 ethyl acetate/DCM) to provide a white solid (7%). $^1$H NMR (500 MHz, chloroform-$d$) $\delta$ ppm 0.91 (t, J=6.84 Hz, 6 H) 1.06 - 1.42 (m, 21 H) 1.42 - 1.52 (m, 1 H) 1.59 - 1.94 (m, 6 H) 2.07 (s, 4 H) 2.15 - 2.26 (m, 1 H) 2.27 - 2.35 (m, 1 H) 2.35 - 2.42 (m, 1 H) 2.49 - 2.61 (m, 2 H) 2.61 - 2.73 (m, 2 H) 3.13 - 3.32 (m, 1 H) 4.36 - 4.47 (m, 1 H) 5.05 - 5.13 (m, 1 H) 6.11 - 6.28 (m, 1 H) 8.18 (s, 1 H).

**(S)-1-((2S,3S)-3-hexyl-4-oxooxetan-2-yl)tridecan-2-yl-1-formamidocyclohexanecarboxylate**

48: Formic anhydride (92.6.6 mg, 1.3 mmol) was added to a stirred solution of amine 83 (30.0 mg, 0.06 mmol) in anhydrous DCM at -5 °C. The resulting mixture was stirred at reduced temperature for 40 minutes and subsequently, 80 minutes at room temperature. The reaction was monitored by TLC (3:7 ethyl acetate/hexane: product R$_f$ 0.30). Upon completion of the reaction, the crude mixture was concentrated and purified by column chromatography (silica gel, 3:7 ethyl acetate/hexane) to yield a white solid (10%). $^1$H NMR (500 MHz, chloroform-$d$) $\delta$ ppm 0.88 (t, J=6.84 Hz, 6 H) 1.16 - 1.51 (m, 23 H) 1.56 (s, 5 H) 1.60 - 1.73 (m, 6 H) 1.73 - 1.81 (m, 4 H) 1.84 (m, J=11.72 Hz, 4 H) 2.04 (m, J=3.91 Hz, 4 H) 2.10 - 2.23 (m, 2 H) 3.13 - 3.30 (m, 1 H) 4.34 - 4.45 (m, 1 H) 4.91 - 5.03 (m, 1 H) 5.62 (s, 1H) 8.14 (s, 1 H).
(R)-(S)-1-((2S,3S)-3-hexyl-4-oxooxetan-2-yl)tridecan-2-yl-2-formamido-2-(thiophen-2-yl)acetate

49: Formic anhydride (177 mg, 2.38 mmol) was added to a stirred solution of amine 87 (59.0 mg, 0.119 mmol) in anhydrous DCM at -5 °C. The resulting mixture was stirred at reduced temperature for 40 minutes and subsequently, 80 minutes at room temperature. The reaction was monitored by TLC (5:95 ethyl acetate/DCM: product Rf 0.5). Upon completion of the reaction, the crude mixture was concentrated and purified by column chromatography (silica gel, 5:95 ethyl acetate/DCM) to yield a white solid (10%). ¹H NMR (500 MHz, chloroform-d) δ ppm 0.88 (t, J=6.84 Hz, 4 H) 0.99 - 1.10 (m, 2 H) 1.09 - 1.40 (m, 14 H) 1.39 - 1.47 (m, 2 H) 1.47 - 1.68 (m, 6 H) 1.68 - 1.87 (m, 4 H) 1.96 - 2.07 (m, 2 H) 2.17 - 2.26 (m, 2 H) 2.35 (t, J=7.57 Hz, 2 H) 3.15 - 3.27 (m, 1 H) 4.32 - 4.40 (m, 1 H) 4.98 - 5.09 (m, 1 H) 5.80 - 5.92 (m, 1 H) 6.53 (d, J=6.84 Hz, 1 H) 6.94 - 7.02 (m, 1 H) 7.10 (d, J=3.42 Hz, 1 H) 7.28 (d, J=4.88 Hz, 1 H) 8.25 (s, 1 H) (m, 1 H) 7.12 (d, J=3.42 Hz, 1 H) 7.28 (d, J=4.88 Hz, 1 H) 8.26 (s, 1 H).

(S)-(S)-1-((2S,3S)-3-hexyl-4-oxooxetan-2-yl)tridecan-2-yl-2-formamido-2-(thiophen-2-yl)acetate

50: Formic anhydride (94.0 mg, 1.27 mmol) was added to a stirred solution of amine 88 (31.5 mg, 0.064 mmol) in anhydrous DCM at -5 °C. The resulting mixture was
stirred at reduced temperature for 40 minutes and subsequently, 80 minutes at room temperature. The reaction was monitored by TLC (5:95 ethyl acetate/DCM: product R<sub>f</sub> 0.5). Upon completion of the reaction, the crude mixture was concentrated and purified by column chromatography (silica gel, 5:95 ethyl acetate/DCM) to yield a white solid (9%). <sup>1</sup>H NMR (500 MHz, chloroform-<sup>d</sup>) δ ppm 0.89 (q, <i>J</i>=7.16 Hz, 6 H) 1.10 - 1.42 (m, 20 H) 1.45 - 1.78 (m, 6 H) 1.86 - 1.96 (m, 2 H) 2.09 - 2.18 (m, 2 H) 2.35 (t, <i>J</i>=7.32 Hz, 2 H) 2.98 - 3.08 (m, 1 H) 3.94 - 4.03 (m, 1 H) 4.95 - 5.08 (m, 1 H) 5.88 - 5.94 (m, 1 H) 6.60 (d, <i>J</i>=6.84 Hz, 1 H) 6.98 - 7.01).

(2S,3S,5S)-2-hexyl-3,5-dihydroxyhexadecanoic acid and (S)-2-formamido-4-methylpentanoic acid

51: A cooled solution of 5 THL (12.9g, 26.0 mg) in MeOH (300 mL) was treated with 1M KOH (57.3 mmol). The resulting solution was mechanically stirred for 2 hours at room temperature under nitrogen. The reaction was monitored by TLC (3:7 ethyl acetate/hexane: starting material R<sub>f</sub> 0.5, product R<sub>f</sub> .07, 0.1) to yield an oil. Upon completion, the mixture was evaporated under vacuum before being dissolved in toluene and re-concentrated. The saponification products were used directly in the next step.
(2S,3S,5S)-benzyl 2-hexyl-3,5-dihydroxyhexadecanoate

52: The hydrolysis product 51 was dissolved in warm DMF (100 mL) and subsequently treated with benzyl bromide (9.6 g, 6.68 mL). The resulting solution was monitored by TLC (3:7 ethyl acetate/hexane: product Rf 0.4). Upon completion, the mixture was diluted with water and extracted with ethyl acetate. The organic layers were then dried with MgSO₄ and concentrated under reduced pressure. The crude product was purified by column chromatography (silica gel, 3:7 ethyl acetate/hexane). A 40% yield was obtained. ¹H NMR (500 MHz, chloroform-d) δ ppm 0.82 - 0.93 (m, 6 H) 1.14 - 1.34 (m, 18 H) 1.34 - 1.52 (m, 6 H) 1.53 - 1.65 (m, 6 H) 1.65 - 1.81 (m, 2 H) 2.42 - 2.57 (m, 1 H) 3.02 - 3.26 (m, 1 H) 3.33 - 3.56 (m, 1 H) 3.73 - 3.90 (m, 1 H) 3.92 - 4.06 (m, 1 H) 5.18 (s, 2 H) 7.29 - 7.56 (m, 5 H).

(2S,3S,5S)-benzyl 2-hexyl-3-hydroxy-5-((triisopropylsilyl)oxy)hexadecanoate

53: Sym-collidine (2.27 g, 18.8 mmol) and triisopropylsilyl trifluoromethanesulfonate (3.45 g, 11.3 mmol) were added to a stirred solution of 52 (3.48 g, 7.52 mmol) in anhydrous DCM at -78 °C. The solution was monitored by TLC (2:8 ethyl acetate/hexane: product Rf 0.75). After consumption of starting material, the reaction was quenched with H₂O. The organic layer was subsequently extracted with DCM, dried with MgSO₄, filtered and concentrated under reduced pressure. The crude product was purified by column chromatography (silica gel, 2:8 ethyl acetate/hexane).
A 71% yield was obtained. $^1$H NMR (500 MHz, chloroform-$d$) δ ppm 1.17 - 1.43 (m, 26 H) 1.41 - 1.65 (m, 4 H) 1.64 - 1.81 (m, 2 H) 2.51 (dt, $J$=9.89, 5.07 Hz, 1 H) 3.24 (d, $J$=5.37 Hz, 1 H) 3.86 - 3.98 (m, 1 H) 4.00 - 4.12 (m, 1 H) 5.17 (m, $J$=8.30 Hz, 2 H) 7.28 - 7.46 (m, 5 H).

$(2S,3S,5S)$-2-hexyl-3-hydroxy-5-((triisopropylsilyl)oxy)hexadecanoic acid

54: A stirred solution of 53 in EtOAc (18 mL) was hydrogenated in the presence of 10% Pd/C (460 mg) at room temperature and atmospheric pressure for 3 hours. The reaction was monitored by TLC (2:8 ethyl acetate/hexane: product $R_f$ 0.3, starting material $R_f$ 0.55). The suspension was filtered through a short pad of celite. The resulting filtrate was concentrated to yield hydroxy acid in quantitative yields. $^1$H NMR (500 MHz, chloroform-$d$) δ ppm 0.76 - 0.95 (m, 6 H) 1.08 (s, 21 H) 1.17 - 1.45 (m, 26 H) 1.46 - 1.69 (m, 4 H) 1.68 - 1.84 (m, 2 H) 2.35 - 2.48 (m, 1 H) 3.89 - 4.01 (m, 1 H) 4.04 - 4.17 (m, 1H).

$(3S,4S)$-3-hexyl-4-((S)-2-((triisopropylsilyl)oxy)tridecyl)oxetan-2-one

55: $N$-phenyl-bis(trifluoromethanesulfonimide (1.25 g, 3.5 mmol) was added to a stirred solution of 54 (1.24 g, 2.3 mmol) and TEA (473.0 mg, 4.67 mmol) in anhydrous DCM at 0 °C. The resulting mixture was stirred at room temperature overnight and monitored by TLC (15:85 DCM/hexane: product $R_f$ 4.75). Upon completion of the reaction, the crude mixture was concentrated and purified by column chromatography
(silica gel, 15:85 DCM/hexane) to yield a clear oil (80%). $^1$H NMR (500 MHz, chloroform-\textit{d}) $\delta$ ppm 0.89 (t, $J$=6.84 Hz, 6 H) 1.07 (s, 21 H) 1.17 - 1.43 (m, 27 H) 1.46 (m, $J$=5.86 Hz, 1 H) 1.68 - 1.89 (m, 2 H) 1.97 (m, $J$=5.13, 5.13 Hz, 1 H) 1.94 - 1.94 (m, 1 H) 2.08 (m, $J$=8.30, 3.91 Hz, 1 H) 3.28 (m, $J$=3.91 Hz, 1 H) 3.93 - 4.04 (m, 1 H) 4.49 (dt, $J$=8.06, 4.27 Hz, 1 H).

(3S,4S)-3-hexyl-4-((S)-2-hydroxytridecyl)oxetan-2-one

56: An aqueous solution of 48% HF (1.14 mL) was added to a stirred solution of 55 (189.5 mg, 0.37 mmol) in acetonitrile (56 mL). The solution was stirred at room temperature for 2 hours. The reaction was monitored by TLC (1:9 ethyl acetate/hexane: product $R_f$ 0.2, starting material $R_f$ 0.85). Once complete, the solution was concentrated under vacuum. The resulting residue was diluted in diethyl ether, transferred to a separatory funnel and extracted with aqueous NaHCO$_3$. The organic layers were dried with MgSO$_4$, concentrated and purified by column chromatography (silica gel, 1:9 ethyl acetate/hexane) to provide a white solid. A quantitative yield was obtained. $^1$H NMR (500 MHz, chloroform-\textit{d}) $\delta$ ppm 0.85 - 0.93 (m, 6 H) 1.18 - 1.38 (m, 22 H) 1.38 - 1.49 (m, 4 H) 1.51 (t, $J$=7.08 Hz, 2 H) 1.61 (d, $J$=4.39 Hz, 1 H) 1.68 - 1.80 (m, 1 H) 1.91 (m, $J$=5.86, 3.42 Hz, 1 H) 1.98 - 2.06 (m, 1 H) 3.32 (ddd, $J$=8.67, 6.71, 4.15 Hz, 1 H) 3.71 - 3.86 (m, 1 H) 4.48 (m, $J$=3.91 Hz, 1 H).
(2S,3R)-2-(((benzyloxy)carbonyl)amino)-3-methylpentanoic acid

57: One equivalent of 1 M NaOH (2 mL) was added dropwise to a room temperature solution of L-allo-isoleucine (0.20 g, 1.52 mmol) in dioxane (2 mL). An additional equivalent of NaOH (2 mL) was added alternately with dibenzyl dicarbonate (0.43 g, 1.52 mmol) over thirty minutes. The reaction was stirred for 2 hours and monitored by TLC (1:9 methanol/CH$_2$Cl$_2$: product R$_f$ 0.3). Upon completion, the solution was acidified using 1 M HCl. After an ethyl acetate extraction, the organic layers were dried with MgSO$_4$, filtered and concentrated. The crude product was purified by column chromatography (silica gel, 1:9 methanol/DCM). A 50% yield was obtained. $^1$H NMR (500 MHz, chloroform-d) $\delta$ ppm 0.90 (d, $J=6.84$ Hz, 3 H) 0.98 (t, $J=7.32$ Hz, 3 H) 1.17 - 1.34 (m, 2 H) 1.47 (dd, $J=13.67$, 6.84 Hz, 1 H) 4.52 (dd, $J=9.03$, 3.17 Hz, 1 H) 5.13 (s, 2 H) 5.18 (d, $J=8.79$ Hz, 1H) 7.29 - 7.46 (m, 5 H).

(S)-2-(((benzyloxy)carbonyl)amino)butanoic acid

58: One equivalent of 1 M NaOH (2 mL) was added dropwise to a room temperature solution of S-$\alpha$-aminobutyric acid (0.20 g, 1.94 mmol) in dioxane (2 mL). An additional equivalent of NaOH (2 mL) was added alternately with dibenzyl dicarbonate (0.55 g, 1.94 mmol) over thirty minutes. The reaction was stirred for 2 hours and monitored by TLC (1:9 methanol/DCM: product R$_f$ 0.3). Upon completion, the solution was acidified using 1 M HCl. After an ethyl acetate extraction, the organic layers were dried with MgSO$_4$, filtered and
concentrated. The crude product was purified by column chromatography (silica gel, 1:9 methanol/DCM). A 48% yield was obtained. $^1$H NMR (500 MHz, chloroform-$d$) δ ppm 0.98 (t, $J=7.32$ Hz, 3 H) 1.66 - 1.86 (m, 2 H) 4.31 - 4.47 (m, 1H) 5.13 (s, 2 H) 5.26 (d, $J=7.81$ Hz, 1 H) 7.29 - 7.41 (m, 5 H).

(2R,3R)-2-(((benzyloxy)carbonyl)amino)-3-methylpentanoic acid

59: One equivalent of 1 M NaOH (2 mL) was added dropwise to a room temperature solution of D-isoleucine (0.20 g, 1.52 mmol) in dioxane (2 mL). An additional equivalent of NaOH (2 mL) was added alternately with dibenzyl dicarbonate (0.43 g, 1.52 mmol) over thirty minutes. The reaction was stirred for 2 hours and monitored by TLC (1:9 methanol/DCM: product $R_f$ 0.3). Upon completion, the solution was acidified using 1 M HCl. After an ethyl acetate extraction, the organic layers were dried with MgSO$_4$, filtered and concentrated. The crude product was purified by column chromatography (silica gel, 1:9 methanol/DCM). A 52% yield was obtained. $^1$H NMR (500 MHz, chloroform-$d$) δ ppm 0.79 - 1.08 (m, 6 H) 1.19 (dd, $J=13.92$, 7.08 Hz, 2 H) 1.46 (ddd, $J=12.94$, 7.57, 4.88 Hz, 1 H) 4.38 (dd, $J=9.03$, 4.64 Hz, 1 H) 5.10 (d, $J=2.44$ Hz, 2 H) 5.46 (d, $J=9.28$ Hz, 1 H) 7.25 - 7.42 (m, 5 H) 9.27 - 9.98 (m, 1 H).

(2R,3S)-2-(((benzyloxy)carbonyl)amino)-3-methylpentanoic acid

60: One equivalent of 1 M NaOH (1 mL) was added dropwise to a room temperature solution of D-allo-isoleucine (84.0 mg, 0.64
mmol) in dioxane (2 mL). An additional equivalent of NaOH (1 mL) was added alternately with dibenzyl dicarbonate (0.183 mg, 0.64 mmol) over thirty minutes. The reaction was stirred for 2 hours and monitored by TLC (2:98 methanol/DCM: product R$_f$ 0.5). Upon completion, the solution was acidified using 1 M HCl. After an ethyl acetate extraction, the organic layers were dried with MgSO$_4$, filtered and concentrated. The crude product was purified by column chromatography (silica gel, 2:98 methanol/DCM) to provide a white solid (20%). $^1$H NMR (500 MHz, chloroform-d) $\delta$ ppm 0.88 (d, $J$=6.84 Hz, 3 H) 0.96 (t, $J$=7.32 Hz, 3 H) 1.25 (dt, $J$=14.04, 7.39 Hz, 2 H) 1.47 (dt, $J$=13.79, 7.02 Hz, 1 H) 4.50 (dd, $J$=9.03, 3.66 Hz, 1 H) 5.05 - 5.18 (m, 2 H) 5.23 (d, $J$=9.28 Hz, 1 H) 7.27 - 7.46 (m, 5 H).

(S)-2-(((benzyloxy)carbonyl)amino)-2-cyclopropylacetic acid

61: One equivalent of 1 M NaOH (2 mL) was added dropwise to a room temperature solution of L-α-cyclopropylglycine (0.20 g, 1.73 mmol) in dioxane (2 mL). An additional equivalent of NaOH (2 mL) was added alternately with dibenzyl dicarbonate (0.497 g, 1.73 mmol) over thirty minutes. The reaction was stirred for 2 hours and monitored by TLC (1:9 methanol/DCM: product R$_f$ 0.3). Upon completion, the solution was acidified using 1 M HCl. After an ethyl acetate extraction, the organic layers were dried with MgSO$_4$, filtered and concentrated. The crude product was purified by column chromatography (silica gel, 1:9 methanol/DCM) to provide a white solid in 68% yield. $^1$H NMR (500 MHz, chloroform-d) $\delta$ ppm 0.37 - 0.48 (m, 1 H) 0.48 - 0.68 (m, 2 H) 0.88 (t, $J$=6.84
(S)-2-(((benzyloxy)carbonyl)amino)-2-cyclopentylacetic acid

62: One equivalent of 1 M NaOH was added dropwise to a room temperature solution of L-α-cyclopentylglycine (0.20 g, 1.39 mmol) in dioxane (2 mL). An additional equivalent of NaOH was added alternately with dibenzyl dicarbonate (0.399 g, 0.1.39 mmol) over thirty minutes. The reaction was stirred for 2 hours and monitored by TLC (1:9 methanol/DCM: product Rf 0.3). Upon completion, the solution was acidified using 1 M HCl. After an ethyl acetate extraction, the organic layers were dried with MgSO₄, filtered and concentrated. The crude product was purified by column chromatography (silica gel, 1:9 methanol/DCM). A 49% yield was obtained. ¹H NMR (500 MHz, chloroform-d) δ ppm 1.26 (t, J=7.32 Hz, 1 H) 1.28 - 1.38 (m, 1 H) 1.38 - 1.49 (m, 1 H) 1.49 - 1.59 (m, 1 H) 1.59 - 1.68 (m, 1 H) 1.68 - 1.75 (m, 1 H) 1.77 (dd, J=12.45, 7.57 Hz, 1 H) 2.21 - 2.36 (m, 1 H) 4.15 - 4.27 (m, 1 H) 4.35 (t, J=7.81 Hz, 1 H) 5.12 (s, 2 H) 5.22 (d, J=7.81 Hz, 1 H) 7.28 - 7.43 (m, 5 H).

(R)-2-(((benzyloxy)carbonyl)amino)-2-(thiophen-2-yl)acetic acid

63: One equivalent of 1 M NaOH was added dropwise to a room temperature solution of S-2-thienylglycine (0.20 g, 1.62 mmol)
in dioxane (2 mL). An additional equivalent of NaOH was added alternately with dibenzyl dicarbonate (0.464 g, 0.162 mmol) over thirty minutes. The reaction was stirred for 2 hours and monitored by TLC (1:9 methanol/DCM: product R_f 0.3). Upon completion, the solution was acidified using 1 M HCl. After an ethyl acetate extraction, the organic layers were dried with MgSO_4, filtered and concentrated. The crude product was purified by column chromatography (silica gel, 1:9 methanol/DCM) to provide a white solid (76%). ^1H NMR (500 MHz, chloroform-d) δ ppm 5.13 (s, 2 H) 5.49 - 5.61 (m, 1 H) 5.64 - 5.81 (m, 2 H) 6.99 (br. s, 1 H) 7.12 (br. s, 2 H) 7.29 (d, J=4.39 Hz, 1 H) 7.36 (br. s, 5 H).

(S)-2-(((benzyloxy)carbonyl)amino)-2-(thiophen-2-yl)acetic acid

64: One equivalent of 1 M NaOH was added dropwise to a room temperature solution of R-2-thienylglycine (0.20 g, 1.62 mmol) in dioxane (2 mL). An additional equivalent of NaOH was added alternately with dibenzyl dicarbonate (0.464 g, 0.162 mmol) over thirty minutes. The reaction was stirred for 2 hours and monitored by TLC (1:9 methanol/DCM: product R_f 0.3). Upon completion, the solution was acidified using 1 M HCl. After an ethyl acetate extraction, the organic layers were dried with MgSO_4, filtered and concentrated. The crude product was purified by column chromatography (silica gel, 1:9 methanol/DCM) to yield a white solid (70%). ^1H NMR (500 MHz, chloroform-d) δ ppm 5.13 (s, 2 H) 5.49 - 5.61 (m, 1 H) 5.64 - 5.81 (m, 2 H) 6.99 (br. s, 1 H) 7.12 (br. s, 2 H) 7.29 (d, J=4.39 Hz, 1 H) 7.36 (br. s, 5 H).
(2S,3S)-(S)-1-((2S,3S)-3-hexyl-4-oxooxetan-2-yl)tridecan-2-yl-2-(((benzyloxy)carbonyl)amino)-3-methylpentanoate

65: Alcohol 56 (367.4 mg, 1.04 mmol) was added to a stirred solution of carboxybenzyl protected amino acid (0.55 mg, 2.07 mmol) in anhydrous DCM. The solution was cooled to 0 °C and EDCI (496.5 mg, 2.59 mmol) and DMAP (316.4 mg, 2.59 mmol) were added. Completion of the reaction was determined by TLC (2:8 ethyl acetate/hexane: product Rf 0.65, starting material Rf 0.55). After aqueous workup, the organic layers were dried with MgSO₄, concentrated and purified by column chromatography (silica gel, 2:8 ethyl acetate/hexane) to provide a white solid (70%). ¹H NMR (500 MHz, chloroform-d) δ ppm 0.83 - 1.04 (m, 12 H) 1.09 - 1.59 (m, 30 H) 1.69 - 1.85 (m, 2 H) 1.85 - 1.95 (m, 1 H) 1.98 (dt, J=14.65, 4.64 Hz, 1 H) 2.18 (dt, J=14.65, 7.32 Hz, 1 H) 3.11 - 3.30 (m, 1 H) 4.24 - 4.37 (m, 2 H) 4.94 - 5.05 (m, 1 H) 5.12 (s, 2 H) 5.24 (d, J=8.79 Hz, 1 H) 7.28 - 7.46 (m, 5 H).

(2S,3R)-(S)-1-((2S,3S)-3-hexyl-4-oxooxetan-2-yl)tridecan-2-yl-2-(((benzyloxy)carbonyl)amino)-3-methylpentanoate

66: Hydroxy lactone 56 (58.4 mg, 0.16 mmol) was added to a stirred solution of carboxybenzyl protected amino acid 57 (0.58 mg, 0.22 mmol) in anhydrous DCM. The solution was cooled to 0 °C and EDCI (62.8 mg, 0.33
mmol) and DMAP (40.3 mg, 0.33 mmol) were added. Completion of the reaction was determined by TLC (1:9 ethyl acetate/hexane: product R\textsubscript{f} 0.45, starting material R\textsubscript{f} 0.30). After aqueous workup, the organic layers were dried with MgSO\textsubscript{4}, concentrated and purified by column chromatography (silica gel, 1:9 ethyl acetate/hexane) to provide a white solid (45%). \(^1\)H NMR (500 MHz, chloroform-\textit{d}) \(\delta\) ppm 0.81 - 0.93 (m, 9 H) 0.97 (t, \(J=7.32\) Hz, 3 H) 1.09 - 1.40 (m, 18 H) 1.40 - 1.53 (m, 4 H) 1.53 - 1.87 (m, 8 H) 1.95 (m, \(J=4.39\) Hz, 3 H) 1.98 - 2.05 (m, 1 H) 2.18 (dt, \(J=15.02, 7.39\) Hz, 1 H) 3.17 - 3.26 (m, 1 H) 4.23 - 4.33 (m, 1 H) 4.43 (dd, \(J=8.79, 3.42\) Hz, 1 H) 4.95 - 5.05 (m, 1 H) 5.12 (s, 2 H) 5.19 (d, \(J=8.79\) Hz, 1 H) 7.29 - 7.50 (m, 5 H).

(S)-(S)-1-((2S,3S)-3-hexyl-4-oxooxetan-2-yl)tridecan-2-yl-2-((benzyloxy)carbonyl)amino)butanoate

67: Hydroxy lactone 56 (46.3 mg, 0.13 mmol) was added to a stirred solution of carboxybenzyl protected amino acid 58 (0.42 mg, 0.18 mmol) in anhydrous DCM. The solution was cooled to 0 °C and EDCI (50 mg, 0.26 mmol) and DMAP (32.0 mg, 0.26 mmol) were added. Completion of the reaction was determined by TLC (1:9 ethyl acetate/hexane: product R\textsubscript{f} 0.45, starting material R\textsubscript{f} 0.40). After aqueous workup, the organic layers were dried with MgSO\textsubscript{4}, concentrated and purified by column chromatography (silica gel, 1:9 ethyl acetate/hexane) to yield a white solid (40%). \(^1\)H NMR (500 MHz, chloroform-\textit{d}) \(\delta\) ppm 0.84 - 0.92 (m, 6 H) 0.96 (t, \(J=7.57\) Hz, 3 H) 1.20 - 1.34 (m, 22 H) 1.35 - 1.85 (m, 8 H) 1.93 (m, \(J=4.88\) Hz, 2 H) 2.02 (m,
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J=6.84 \text{ Hz}, 1 \text{ H}) 2.17 (dt, J=14.89, 7.69 \text{ Hz}, 1 \text{ H}) 3.11 - 3.27 (m, 1 \text{ H}) 4.24 - 4.39 (m, 1 \text{ H}) 4.41 - 4.55 (m, 1 \text{ H}) 4.96 - 5.08 (m, 1 \text{ H}) 5.12 (s, 2 \text{ H}) 5.25 (d, J=7.81 \text{ Hz}, 1 \text{ H}) 7.29 - 7.44 (m, 5 \text{ H}).
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\((2R,3R)-(S)-1-((2S,3S)-3-hexyl-4-oxooxetan-2-yl)tridecan-2-yl-2-(((benzyloxy)carbonyl)amino)-3-methylpentanoate\)

\(68:\) Hydroxy lactone 56 (51.8 mg, 0.146 mmol) was added to a stirred solution of carboxybenzyl protected amino acid 59 (0.60 mg, 0.23 mmol) in anhydrous DCM. The solution was cooled to 0 °C and EDCI (56 mg, 0.292 mmol) and DMAP (35.8 mg, 0.292 mmol) were added. Completion of the reaction was determined by TLC (2:8 ethyl acetate/hexane: product \(R_f\) 0.55, starting material \(R_f\) 0.40). After aqueous workup, the organic layers were dried with MgSO\(_4\), concentrated and purified by column chromatography (silica gel, 2:8 ethyl acetate/hexane) to provide a white solid (22%). \(^1\)H NMR (500 MHz, chloroform-\(d\)) \(\delta \) ppm 0.79 - 0.93 (m, 9 H) 0.97 (t, \(J=7.32 \text{ Hz}, 3 \text{ H}) 1.18 - 1.40 (m, 26 H) 1.41 - 1.53 (m, 2 H) 1.52 - 1.59 (m, 1 H) 1.62 - 1.70 (m, 1 H) 1.70 - 1.86 (m, 2 H) 1.85 - 1.97 (m, 1 H) 2.01 (m, \(J=4.88, 4.88 \text{ Hz}, 1 \text{ H}) 2.20 (dt, \(J=14.53, 7.14 \text{ Hz}, 1 \text{ H}) 3.17 - 3.28 (m, 1 H) 4.30 - 4.37 (m, 1 H) 4.40 (dd, \(J=9.03, 3.66 \text{ Hz}, 1 \text{ H}) 4.97 - 5.07 (m, 1 H) 5.11 (d, \(J=3.91 \text{ Hz}, 2 \text{ H}) 5.18 (d, \(J=8.79 \text{ Hz}, 1 \text{ H}) 7.28 - 7.52 (m, 5 H).\)
(2R,3S)-(S)-1-((2S,3S)-3-hexyl-4-oxooxetan-2-yl)tridecan-2-yl-2-
(((benzyloxy)carbonyl)amino)-3-methylpentanoate

69: Hydroxy lactone 56 (16.5 mg, 0.0465 mmol) was added to a stirred solution of carboxybenzyl protected amino acid 60 (13.58 mg, 0.051 mmol) in anhydrous DCM. The solution was cooled to 0 °C and EDCI (18.0 mg, 0.093 mmol) and DMAP (11.4 mg, 0.093 mmol) were added. Completion of the reaction was determined by TLC (2:8 ethyl acetate/hexane: product R<sub>f</sub> 0.55, starting material R<sub>f</sub> 0.40). After aqueous workup, the organic layers were dried with MgSO<sub>4</sub>, concentrated and purified by column chromatography (silica gel, 2:8 ethyl acetate/hexane) to provide a white solid (39%). <sup>1</sup>H NMR (500 MHz, chloroform-d) δ ppm 0.79 - 0.93 (m, 9 H) 0.97 (t, J=7.32 Hz, 3 H) 1.18 - 1.40 (m, 26 H) 1.41 - 1.53 (m, 2 H) 1.52 - 1.59 (m, 1 H) 1.62 - 1.70 (m, 1 H) 1.70 - 1.86 (m, 2 H) 1.85 - 1.97 (m, 1 H) 2.01 (m, J=4.88, 4.88 Hz, 1 H) 2.20 (dt, J=14.53, 7.14 Hz, 1 H) 3.17 - 3.28 (m, 1 H) 4.30 - 4.37 (m, 1 H) 4.40 (dd, J=9.03, 3.66 Hz, 1 H) 4.97 - 5.07 (m, 1 H) 5.11 (d, J=3.91 Hz, 2 H) 5.18 (d, J=8.79 Hz, 1 H) 7.28 - 7.52 (m, 5 H).

(S)-1-((2S,3S)-3-hexyl-4-oxooxetan-2-yl)tridecan-2-yl-1-
(((benzyloxy)carbonyl)amino)cyclobutanecarboxylate

70: Hydroxy lactone 56 (15.0 mg, 0.042 mmol) was added to a stirred solution of carboxybenzyl protected amino acid (11.6 mg, 0.046 mmol) in anhydrous DCM. The solution
was cooled to 0 °C and EDCI (24.3 mg, 0.13 mmol) and a catalytic amount of DMAP were added. Completion of the reaction was determined by TLC (2:8 ethyl acetate/hexane: product R_f 0.5, starting material R_f 0.40). After aqueous workup, the organic layers were dried with MgSO_4, concentrated and purified by column chromatography (silica gel, 2:8 ethyl acetate/hexane) to yield a white solid (61%). ^1^H NMR (500 MHz, chloroform-d) δ ppm 0.53 - 1.11 (m, 6 H) 1.14 - 1.39 (m, 21 H) 1.39 - 1.50 (m, 2 H) 1.56 (s, 2 H) 1.60 - 1.69 (m, 1 H) 1.69 - 1.87 (m, 2 H) 1.91 - 2.09 (m, 4 H) 2.10 - 2.21 (m, 1 H) 2.22 - 2.48 (m, 3 H) 2.61 (br. s, 2 H) 3.13 - 3.27 (m, 1 H) 4.27 - 4.40 (m, 1 H) 4.96 - 5.06 (m, 1 H) 5.09 (s, 2 H) 5.39 (d, J=15.14 Hz, 1 H) 7.35 (br. s, 5 H).

(S)-1-((2S,3S)-3-hexyl-4-oxooxetan-2-yl)tridecan-2-yl-1-(((benzyloxy)carbonyl)amino)cyclohexanecarboxylate

71: Hydroxy lactone 56 (35.0 mg, 0.098 mmol) was added to a stirred solution of carboxybenzyl protected amino acid (31.0 mg, 0.12 mmol) in anhydrous DCM. The solution was cooled to 0 °C and EDCI (37.8 mg, 0.197 mmol) and a catalytic amount of DMAP were added. Completion of the reaction was determined by TLC (2:8 ethyl acetate/hexane: product R_f 0.60), starting material R_f 0.40). After aqueous workup, the organic layers were dried with MgSO_4, concentrated and purified by column chromatography (silica gel, 2:8 ethyl acetate/hexane) to yield a white solid (62%). ^1^H NMR (500 MHz, chloroform-d) δ ppm 0.80 - 0.95 (m, 6 H) 1.12 - 1.40 (m, 22 H) 1.40 - 1.57 (m, 7 H) 1.57 - 1.68 (m, 6 H) 1.70 - 1.78 (m, 2 H) 1.79 - 1.88 (m, 2
(S)-(S)-1-((2S,3S)-3-hexyl-4-oxooxetan-2-yl)tridecan-2-yl-2-(((benzyloxy)carbonyl)amino)-2-cyclopropylacetate

72: Hydroxy lactone 56 (133 mg, 0.375 mmol) was added to a stirred solution of carboxybenzyl protected amino acid 61 (112 mg, 0.45 mmol) in anhydrous DCM. The solution was cooled to 0 °C and EDCI (210 mg, 1.13 mmol) and a catalytic amount of DMAP were added. Completion of the reaction was determined by TLC (1:9 ethyl acetate/hexane: product \( R_f \) 0.30), starting material \( R_f \) 0.20). After aqueous workup, the organic layers were dried with MgSO\(_4\), concentrated and purified by column chromatography (silica gel, 1:9 ethyl acetate/hexane) to yield a white solid 52%). \(^1\)H NMR (500 MHz, chloroform-\( d \)) \( \delta \) ppm 0.37 - 0.48 (m, 1 H) 0.48 - 0.68 (m, 2 H) 0.88 (t, \( J=6.84 \) Hz, 6 H) 0.98 - 1.12 (m, 1 H) 1.14 - 1.50 (m, 28 H) 1.57 - 1.86 (m, 4 H) 1.91 - 2.03 (m, 1 H) 2.12 - 2.25 (m, 1 H) 3.14 - 3.26 (m, 1 H) 3.71 - 3.82 (m, 1 H) 4.24 - 4.37 (m, 1 H) 4.97 - 5.06 (m, 1 H) 5.10 (d, 2 H) 5.29 (d, 1 H) 7.29 - 7.44 (m, 5 H).
(S)-(S)-1-((2S,3S)-3-hexyl-4-oxooxetan-2-yl)tridecan-2-yl-2-(((benzyloxy)carbonyl)amino)-2-cyclopentylacetate

73: Hydroxy lactone 56 (218 mg, 0.616 mmol) was added to a stirred solution of carboxybenzyl protected amino acid 62 (188 mg, 0.677 mmol) in anhydrous DCM. The solution was cooled to 0 °C and EDCI (354 mg, 1.84 mmol) and a catalytic amount of DMAP were added. Completion of the reaction was determined by TLC (1:9 ethyl acetate/hexane: product Rf 0.40), starting material Rf 0.30). After aqueous workup, the organic layers were dried with MgSO₄, concentrated and purified by column chromatography (silica gel, 1:9 ethyl acetate/hexane) to yield a white solid (50%). ¹H NMR (500 MHz, chloroform-d) δ ppm 0.88 (t, J=6.84 Hz, 6 H) 1.14 - 1.49 (m, 28 H) 1.50 - 1.60 (m, 6 H) 1.64 (d, J=3.91 Hz, 4 H) 1.70 - 1.86 (m, 1 H) 1.87 - 2.03 (m, 1 H) 2.09 - 2.21 (m, 1 H) 2.21 - 2.33 (m, 1 H) 3.11 - 3.27 (m, 1 H) 4.28 (br. s, 2 H) 4.94 - 5.04 (m, 1 H) 5.11 (s, 1 H) 5.19 (d, 1 H) 7.29 - 7.43 (m, 5 H).

(S)-(S)-1-((2S,3S)-3-hexyl-4-oxooxetan-2-yl)tridecan-2-yl-2-(((benzyloxy)carbonyl)amino)-2-cyclohexylacetate

74: Hydroxy lactone 56 (200 mg, 0.561 mmol) was added to a stirred solution of Z-L-threonine (180 mg, 0.617 mmol) in anhydrous DCM under nitrogen. The solution was cooled to 0 °C and EDCI (322 mg, 1.68 mmol) and a catalytic amount of DMAP were added. Completion of the reaction was determined by
TLC (15:85 ethyl acetate/hexane: product R$_f$ 0.50), starting material R$_f$ 0.40). After aqueous workup, the organic layers were dried with MgSO$_4$, concentrated and purified by column chromatography (silica gel, 1:9 ethyl acetate/hexane) to provide a white solid (10%). $^1$H NMR (500 MHz, chloroform-$d$) $\delta$ ppm 0.90 (t, $J$=6.10 Hz, 6 H) 0.98 - 1.20 (m, 2 H) 1.20 - 1.52 (m, 28 H) 1.52 - 1.92 (m, 10 H) 1.94 - 2.05 (m, 1 H) 2.12 - 2.28 (m, 1 H) 2.30 - 2.41 (m, 1 H) 3.12 - 3.30 (m, 1 H) 4.20 - 4.37 (m, 2 H) 4.96 - 5.07 (m, 1 H) 5.13 (s, 2 H) 5.25 (d, 1 H) 7.31 - 7.44 (m, 5 H).

(R)-(S)-1-((2S,3S)-3-hexyl-4-oxooxetan-2-yl)tridecan-2-yl-2-(((benzyloxy)carbonyl)amino)-2-(thiophen-2-yl)acetate

$^{75}$: Hydroxy lactone 56 (100 mg, 0.282 mmol) was added to a stirred solution of 63 (98.0 mg, 0.338 mmol) in anhydrous DCM. The solution was cooled to 0 °C and EDCI (162 mg, 0.846 mmol) and a catalytic amount of DMAP were added. Completion of the reaction was determined by TLC (15:85 ethyl acetate/hexane: product R$_f$ 0.50), starting material R$_f$ 0.40). After aqueous workup, the organic layers were dried with MgSO$_4$, concentrated and purified by column chromatography (silica gel, 1:9 ethyl acetate/hexane) to yield a white solid (31%). Starting material was recovered. $^1$H NMR (500 MHz, chloroform-$d$) $\delta$ ppm 0.91 (t, $J$=6.84 Hz, 6 H) 0.99 - 1.11 (m, 2 H) 1.11 - 1.21 (m, 6 H) 1.21 - 1.42 (m, 18 H) 1.57 (s, 2 H) 1.66 - 1.88 (m, 2 H) 1.94 - 2.07 (m, 1 H) 2.16 - 2.33 (m, 1 H) 3.18 - 3.30 (m, 1 H) 4.27 - 4.47 (m, 1 H) 4.94 - 5.09 (m, 1 H) 5.14 (s, 2 H) 5.60 (d, $J$=7.32 Hz, 1 H)
5.75 (d, J=6.84 Hz, 1 H) 7.00 (m, J=4.39 Hz, 1 H) 7.08 - 7.16 (m, 1 H) 7.38 (br. s, 6 H).

(S)-(S)-1-((2S,3S)-3-hexyl-4-oxooxetan-2-yl)tridecan-2-yl-2-(((benzyloxy)carbonyl)amino)-2-(thiophen-2-yl)acetate

76: Hydroxy lactone 56 (108 mg, 0.304 mmol) was added to a stirred solution of 64 (97.0 mg, 0.33 mmol) in anhydrous DCM under nitrogen. The solution was cooled to 0 °C and EDCI (175 mg, .912 mmol) and a catalytic amount of DMAP were added. Completion of the reaction was determined by TLC (15:85 ethyl acetate/hexane: product R_f 0.50), starting material R_f 0.40). After aqueous workup, the organic layers were dried with MgSO_4, concentrated and purified by column chromatography (silica gel, 1:9 ethyl acetate/hexane) to yield a white solid (40%). Starting material was recovered. 

^1H NMR (500 MHz, chloroform-d) δ ppm 0.91 (t, J=6.84 Hz, 6 H) 0.99 - 1.11 (m, 2 H) 1.11 - 1.21 (m, 6 H) 1.21 - 1.42 (m, 18 H) 1.57 (s, 2 H) 1.66 - 1.88 (m, 2 H) 1.94 - 2.07 (m, 1 H) 2.16 - 2.33 (m, 1 H) 3.00 - 3.12 (m, 1 H) 3.93 - 4.05 (m, 1 H) 4.94 - 5.03 (m, 1 H) 5.14 (br. s, 2 H) 5.60 (d, J=6.84 Hz, 1 H) 5.65 (d, J=6.84 Hz, 1 H) 6.96 - 7.03 (m, 1 H) 7.09 - 7.15 (m, 1 H) 7.38 (br. s, 6 H).
(2S,3S)-(S)-1-((2S,3S)-3-hexyl-4-oxooxetan-2-yl)tridecan-2-yl-2-amino-3-methylpentanoate

**77:** A stirred solution of 65 (46.3 mg, 0.077 mmol) in THF (2.0 mL) was hydrogenated in the presence of 10% Pd/C (15 mg, 14.2 mmol) at room temperature and atmospheric pressure for 2.5 hours. Completion of the reaction was determined by TLC (3:7 ethyl acetate/hexane: product R_t 0.3, starting material R_t 0.8). The resulting mixture was filtered through a short pad of celite. The filtrate was concentrated to provide a white solid and used directly in the next step.

(2S,3R)-(S)-1-((2S,3S)-3-hexyl-4-oxooxetan-2-yl)tridecan-2-yl-2-amino-3-methylpentanoate

**78:** A stirred solution of 66 (59.8 mg, 0.099 mmol) in THF (2.75 mL, 17.8 mmol) was hydrogenated in the presence of 10% Pd/C (18.9 mg) at room temperature and atmospheric pressure for 2.5 hours. Completion of the reaction was determined by TLC (3:7 ethyl acetate/hexane: product R_t 0.3, starting material R_t 0.8). The resulting mixture was filtered through a short pad of celite. The filtrate was concentrated to provide a white solid and used directly in the next step.
(S)-(S)-1-((2S,3S)-3-hexyl-4-oxooxetan-2-yl)tridecan-2-yl 2-aminobutanoate

79: A stirred solution of 67 (21.4 mg, 0.037 mmol) in THF (1.0 mL) was hydrogenated in the presence of 10% Pd/C (6.76 mg, 6.4 mmol) at room temperature and atmospheric pressure for 2.5 hours. Completion of the reaction was determined by TLC (3:7 ethyl acetate/hexane: product R_f 0.3, starting material R_f 0.8). The resulting mixture was filtered through a short pad of celite. The filtrate was concentrated to provide a white solid and used directly in the next step.

(2R,3R)-(S)-1-((2S,3S)-3-hexyl-4-oxooxetan-2-yl)tridecan-2-yl-2-amino-3-methylpentanoate

80: A stirred solution of 68 (19.1 mg, 0.031 mmol) in THF (<1.0 mL) was hydrogenated in the presence of 10% Pd/C (6.0 mg, 5.7 mmol) at room temperature and atmospheric pressure for 2.5 hours. Completion of the reaction was determined by TLC (3:7 ethyl acetate/hexane: product R_f 0.3, starting material R_f 0.8). The resulting mixture was filtered through a short pad of celite. The filtrate was concentrated to provide a white solid and used directly in the next step.
(2R,3S)-(S)-1-((2S,3S)-3-hexyl-4-oxooxetan-2-yl)tridecan-2-yl-2-amino-3-methylpentanoate

81: A stirred solution of 69 (16.86 mg, 0.028 mmol) in THF (<1.0 mL) was hydrogenated in the presence of 10% Pd/C (5.0 mg, 4.7 mmol) at room temperature and atmospheric pressure for 2.5 hours. Completion of the reaction was determined by TLC (3:7 ethyl acetate/hexane: product Rf 0.3, starting material Rf 0.8). The resulting mixture was filtered through a short pad of celite. The filtrate was concentrated to provide a white solid and used directly in the next step.

(S)-1-((2S,3S)-3-hexyl-4-oxooxetan-2-yl)tridecan-2-yl-1-aminocyclobutanecarboxylate

82: A stirred solution of 70 (15.0 mg, 0.026 mmol) in THF (<1.0 mL) was hydrogenated in the presence of 10% Pd/C (4.5 mg, 4.25 mmol) at room temperature and atmospheric pressure for 2.5 hours. Completion of the reaction was determined by TLC (3:7 ethyl acetate/hexane: product Rf 0.3, starting material Rf 0.8). The resulting mixture was filtered through a short pad of celite. The filtrate was concentrated to provide a white solid and used directly in the next step.
(S)-1-((2S,3S)-3-hexyl-4-oxooxetan-2-yl)tridecan-2-yl-1-aminocyclohexanecarboxylate

83: A stirred solution of 71 (37.7 mg, 0.06 mmol) in THF (<1.0 mL) was hydrogenated in the presence of 10% Pd/C (14 mg, 13.3 mmol) at room temperature and atmospheric pressure for 2.5 hours. Completion of the reaction was determined by TLC (3:7 ethyl acetate/hexane: product R_f 0.3, starting material R_f 0.8). The resulting mixture was filtered through a short pad of celite. The filtrate was concentrated to provide a white solid and used directly in the next step.

(S)-(S)-1-((2S,3S)-3-hexyl-4-oxooxetan-2-yl)tridecan-2-yl-2-amino-2-cyclopropylacetate

84: A stirred solution of 72 (115.0 mg, 0.196 mmol) in THF was hydrogenated in the presence of 10% Pd/C (37 mg, 34.9 mmol) at room temperature and atmospheric pressure for 2 hours. Completion of the reaction was determined by TLC (3:7 ethyl acetate/hexane: product R_f 0.3, starting material R_f 0.8). The resulting mixture was filtered through a short pad of celite. The filtrate was concentrated to provide a white solid and used directly in the next step. A quantitative yield was assumed.
(S)-(S)-1-((2S,3S)-3-hexyl-4-oxooxetan-2-yl)tridecan-2-yl-2-amino-2-cyclopentylacetate

85: A stirred solution of 73 (65.0 mg, 0.097 mmol) in THF was hydrogenated in the presence of 10% Pd/C (20 mg, 18.86 mmol) at room temperature and atmospheric pressure for 2 hours. Completion of the reaction was determined by TLC (3:7 ethyl acetate/hexane: product R_f 0.3, starting material R_f 0.8). The resulting mixture was filtered through a short pad of celite. The filtrate was concentrated to provide a white solid and used directly in the next step. A quantitative yield was assumed.

(S)-(S)-1-((2S,3S)-3-hexyl-4-oxooxetan-2-yl)tridecan-2-yl-2-amino-2-cyclohexylacetate

86: A stirred solution of Cbz-protected 74 (23.2 mg, 0.037 mmol) in THF was hydrogenated in the presence of 10% Pd/C (10 mg, 9.43 mmol) at room temperature and atmospheric pressure for 2 hours. Completion of the reaction was determined by TLC (3:7 ethyl acetate/hexane: product R_f 0.3, starting material R_f 0.8). The resulting mixture was filtered through a short pad of celite. The filtrate was concentrated to provide a white solid and used directly in the next step. A quantitative yield was assumed.
(R)-(S)-1-((2S,3S)-3-hexyl-4-oxooxetan-2-yl)tridecan-2-yl-2-amino-2-(thiophen-2-yl)acetate

87: Dimethyl sulfide (0.2 mL, 2.26 mmol) and boron trifluoride diethyl etherate (0.122 mL, 0.98 mmol) were added to a stirred solution of Cbz-protected molecule 75 (65 mg, 0.103 mmol) in anhydrous DCM. The reaction was allowed to stir at room temperature for 1.5 hours before a second equivalent of dimethyl sulfide (0.166 mL, 2.26 mmol) was added. After an additional hour, the reaction was deemed complete by TLC (3:7 ethyl acetate: hexane). It was subsequently quenched with water and 10% ammonium hydroxide. After three DCM extractions, the combined organic layers were washed with water and brine, dried with MgSO₄ and concentrated. The white solid and was used directly in the next step.

(S)-(S)-1-((2S,3S)-3-hexyl-4-oxooxetan-2-yl)tridecan-2-yl-2-amino-2-(thiophen-2-yl)acetate

88: Dimethyl sulfide (0.126 mL, 1.72 mmol) and boron trifluoride diethyl etherate (0.075 mL, 0.6 mmol) were added to a stirred solution of Cbz-protected molecule 76 (40 mg, 0.063 mmol) in anhydrous DCM. The reaction was allowed to stir at room temperature for 1.5 hours before a second equivalent of dimethyl sulfide (0.103 mL, 1.40 mmol) was added. After an additional hour, the reaction was deemed complete by TLC (3:7 ethyl acetate/hexane). It was subsequently
quenched with water and 10% ammonium hydroxide. After three DCM extractions, the combined organic layers were washed with water and brine, dried with MgSO₄ and concentrated. The white solid was used directly in the formylation step.

5.4.4 Other Endocannabinoid Protein Assays.

**Cannabinoid Receptor Binding:** Assays were performed by the previously reported methods.²⁹,³⁸ Palmitylsulfonyl fluoride (ₙ-C₁₆H₃₃SO₂F) had an apparent IC₅₀ of 440 nM, which correlates well with the literature report²² for rCB1 (520 nM). All other standards (including RHC80267 and SD41) did not bind to the cannabinoid receptors, analogous to the previous reports for THL 3,³⁹,⁴⁰ JZL184,²¹ URB597,³⁴,⁴¹ and PMSF.²²

**Inhibition of rat FAAH:** Assays of the inhibition of fatty acid amide hydrolase (rFAAH) used the reported coumarin amide reporter compound⁴², were validated with standard compounds including the selective FAAH inhibitor URB597.⁴³ The N-terminal his-tagged rFAAH deletion sequence used was expressed in an E. coli cell line provided by the Cravatt group.²³ The rFAAH coumarin ester substrate fluorescence assay demonstrated URB597 to have an apparent IC₅₀ of 4.9 nM. This is comparable to inhibition of rat membrane preparations used for the hydrolysis of tritiated anandamide.³⁴,³⁷,⁴⁴ Palmitylsulfonyl fluoride (ₙ-C₁₆H₃₃SO₂F) had an apparent IC₅₀ of 6.3 nM in the fluorescent rFAAH assay (approximately 2 µM in the hMGL assay detailed below) which correlates well with the IC₅₀ of 7 nM using the
radiolabeled \( N \)-arachidonoylethanolamine (anandamide) substrate.\(^{22}\) All other standards THL \(^3\),\(^{39,40,45}\) JZL184,\(^{21}\) PMSF,\(^{22,46}\) and RHC80267\(^{45}\) have been reported to be poor inhibitors of FAAH activity.

**Inhibition of hMGL:** Assays of the inhibition of monoacylglycerol lipase (hMGL) used the 7-hydroxy-6-methoxy analog\(^{47}\) of the reported coumarin ester.\(^{48}\) The assays were validated with standard compounds including the MGL inhibitor JZL184.\(^{49}\) The \( N \)-terminal His-tagged full length human monoacylglycerol lipase used was expressed in *E. coli*.\(^{50}\) The coumarin substrate fluorescence assay demonstrated JZL184 to have an apparent IC\(_{50}\) of 57 nM that is comparable to human recombinant MGL expressed in COS7 cells where the IC\(_{50}\) of JZL184 was reported to be 2 to 6 nM with the endogenous substrate 2-AG.\(^{21,51}\) All other standards, THL \(^3\),\(^{39,45,52}\) JZL184,\(^{21}\) URB597,\(^{41,53}\) palmitylsulfonyl fluoride (\( n \)-C\(_{16}\)H\(_{33}\)SO\(_2\)F),\(^{53}\) PMSF,\(^{53}\) and RHC80267,\(^{45}\) were reported to be poor inhibitors of MGL activity.

### 5.5 Conclusions:

Diacylglycerol lipase has been an underexplored facet of the endocannabinoid system that requires additional research. Natural substrate, simple \( \beta \)-lactone and THL-based analogs were synthesized in an effort to further explore this biosynthetic enzyme. The natural substrate \( 7-20 \) and simple \( \beta \)-lactone-based compounds \( 33-35 \) were not shown to inhibit DGL at 10 \( \mu \)M. However, the THL analogs \( 39-50 \) were qualitatively characterized by potency and selectively comparable with literature reports. This series of compounds confirmed the conclusions established by Di Marzo
et al in 2008; requiring incorporation of a small, S-configured, N-formylated amino acid moiety for marked potency.
5.6 References:


(38) Bisogno, T. *Biochimica et Biophysica Acta* 2006, 1761, 205.


Figure 5.5

(5) N-formyl-L-leucyl ester (THL)

Figure 5.6

(6) N-formyl-L-isoleucyl analog
Figure 5.7

(40) *N*-formyl-L-α/β-isoleucyl analog

Figure 5.8

(39) *N*-formyl-α-aminobutyryl analog
(41) N-formyl-D-allo-isoleucyl analog

(43) N-formyl-D-isoleucyl analog
Figure 5.11

(44) N-formyl cyclopropyl analog

Figure 5.12

(45) N-formyl cyclopentyl analog
Figure 5.13

(49) N-formyl thienyl analog

Figure 5.14

(46) N-formyl cyclohexyl analog
Figure 5.15

(35) trans β-lactone

Figure 5.16

(34) cis β-lactone
Figure 5.17

(33) β-lactone
CHAPTER 6

FUTURE DIRECTIONS
Conclusions/Future Directions:

The endocannabinoid system is a multifaceted entity with enormous therapeutic potential. The work outlined in this thesis was diverse and contributed to the advancement of the field. The common theme among the chapters was the biosynthesis and degradation of lipid messenger 2-AG by DGL and MGL respectively.

Biochemical studies of monoacylglycerol lipase provided significant quantities of active enzyme necessary for elucidation of Ser$^{122}$'s role in catalysis. Over-expressed MGL was also utilized in additional MS and NMR studies. Synthesis of radiolabeled 2-AG utilizing lipases was important for assay of DGL inhibitors and for assessing the activity of DGL membrane preparations.

Investigation of DGL inhibitors created a starting point for further research. Natural substrate-based inhibitors and simple $\beta$-lactone compounds were investigated. THL-based inhibitors were found to inhibit DGL$\alpha$ at 10 $\mu$M. However, additional data needs to be collected in order to determine IC$_{50}$ values. Once an SAR analysis can be performed, a new generation of molecules can be designed and synthesized.

Efforts to implement a fluorescent assay were positive but require further work to improve its photochemical properties. A carbamate scaffold was designed and tested in various assays and proved to be selective against FAAH and MGL. Additional fluorophore moieties will be investigated in order to increase activity of the substrate for DGL$\alpha$. The benefit of implementing a MS-based assay for assessment of DGL inhibitors is also being investigated. The research presented in this thesis is considered a great starting off point for future research and consideration.