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## Synthetic Studies of Potential New Ketogenic Molecules

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Synthetic Studies of Potential New Ketogenic Molecules

by

Mohammad Nazmus Sakib

A dissertation submitted in partial fulfillment  
of the requirements for the degree of  
Doctor of Philosophy  
Department of Chemistry  
College of Arts and Sciences  
University of South Florida

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O-acylated, hydrolysis

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## **Dedication**

My mother Rehana Khanam and brother Tanzilur Rahman Adnan.

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## List of Abbreviations

ADME	Absorption, distribution, metabolism and excretion
AOT	Sodium(bis-2-ethylhexyl) sulphosuccinate
ATP	Adenosine triphosphate
BHB	$\beta$ -Hydroxybutyrate
$\text{CDCl}_3$	Deuterated chloroform
$^{13}\text{C}$ NMR	Carbon-13 nuclear magnetic resonance
DABCO	1,4-Diazabicyclo[2.2.2]octane
DAG	Diacylglycerol
DCC	N,N'-Dicyclohexylcarbodiimide
DCM	Dichloromethane
DHA	Docosahexaenoic acid
DMA	N,N'-dimethylacetamide
DMAP	N,N'-Dimethylaminopyridine
DMF	N,N'-Dimethylformamide
DMSO	Dimethylsulfoxide
EPA	Ecosapentaenoic acid
equiv.	Equivalence(s)
ESI-TOF	Electrospray ionization-time of flight
EtOAc	Ethyl acetate
FAD	Flavin adenine dinucleotide
HIV	Human immunodeficiency virus
GC	Gas chromatography
$^1\text{H}$ NMR	Proton nuclear magnetic resonance
HPLC	High performance liquid chromatography
HRMS	High resolution mass spectrometry
$\text{K}_2\text{CO}_3$	Potassium carbonate
LCMS	Liquid chromatography mass spectrometry
LCT	Long chain triglyceride
MAG	Monoacylglycerol
MCT	Medium chain triglyceride
m-CPBA	m-chloroperoxybenzoic acid
MHz	Megahertz
mL	Milliliter
mmol	Millimole(s)
MR	1,2-bis[(2-mercaptopethyl)thio]-3-mercaptop-propane
MW	Microwave
NADH	Nicotinamide adenine dinucleotide hydride
NIH	National institutes of health
NMR	Nuclear magnetic resonance

PCl <sub>3</sub>	Phosphoroustrichloride
PKC	Protein kinase C
PLE	Porcine liver esterase
ppm	parts per million
PUFA	Polyunsaturated fatty acid
QTOF	Quadruple-time of flight
RT	Room temperature
SD	Standard deviation
TBAI	Tetrabutylammonium iodinde
TBHP	<i>tert</i> -butyl hydroperoxide
TG	Triglyceride
TDI	Tolylenediisocyanate
TEA	Triethylamine
TLC	Thin layer chromatography
UV	Ultraviolet
EXAFS	Extended X-ray absorption fine structure

## **Abstract**

The ketogenic diet has gained popularity in the past few decades among people and physicians due to its various health benefits. A significant amount of research has been done for the modification of the original ketogenic diet and investigation of its potential health benefits. However, synthesis of novel ketogenic compounds as a dietary supplement has not received the same level of attention. That is the primary objective of this research project.

The first chapter of this manuscript describes the synthesis of various short and medium chain esters of different polyols that have been done so far, and areas of their uses considering these molecules are the most significant candidates for identifying novel ketogenic compounds.

The second chapter discusses biochemistry of the ketogenic diet, synthesis and characterization of a series of molecules with an aim for their potential use as a ketogenic supplement.

The third chapter discusses the synthesized compounds' stability in different chemical environments, their enzymatic hydrolysis activity which mimics that likely to occur in the human body and finally testing of these compounds in animal models to examine their potential ketogenicity.

## **Chapter 1: A Literature Review of O-Acylated Polyol Derivatives Focusing on Short and Medium Chain Length and Their Uses**

### **1.1 Introduction**

Numerous families of O-acylated polyol derivatives have been reported in the scientific and patent literature and used for varying purposes. These compounds have been synthesized in the laboratory with different objectives including obtaining a novel compound for proprietary reasons, attaining functionality of different bio- and non-biological catalysts or as oxygen protecting groups for the innovation of a better synthetic scheme. Some of these compounds have been tested for possible biological properties and have shown promise in different areas such as monomers for polymerization, environmentally friendly pesticides or as biological prodrugs. A few of them provide neurological treatment benefits. However, none of these molecules have been tested for their functionality as a supplementary option for the ketogenic diet.

A ketogenic diet is a high fat, low carbohydrate diet where our body prioritizes metabolic breakdown of fats over carbohydrate, which is counter to our regular diet. Ketogenic diets have become very popular in the past few decades as a weight loss tool with other potential health benefits. In this dietary system, our body enzymatically hydrolyzes consumed fats, which are primarily triacylglycerides (triglycerides) of long-chain fatty acids (between 14 to 22 carbons).<sup>1</sup> However, medium-chain triglycerides (between 6 to 12 carbons) are considered more ‘ketogenic’ as they do not require active transport into cells and are absorbed by the liver directly. This

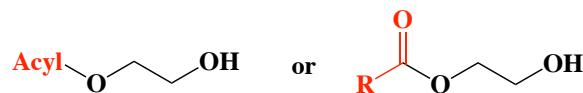
increases the rate of dietary triglyceride metabolism and induces greater ketogenic effect compared to long-chain triglycerides (LCT).<sup>2,3</sup>

This chapter reviews the chemistry literature on short and medium-chain length O-acylated polyol derivatives and their known biological and non-biological uses as well as gives an overview of areas of investigation for potential new ketogenic compounds. This might open up a new area of further scientific investigation of these molecules from the perspective of chemists and physiological scientists.

## 1.2 O-Acylated ethylene glycol derivatives and their uses

### 1.2.1 Monoesters

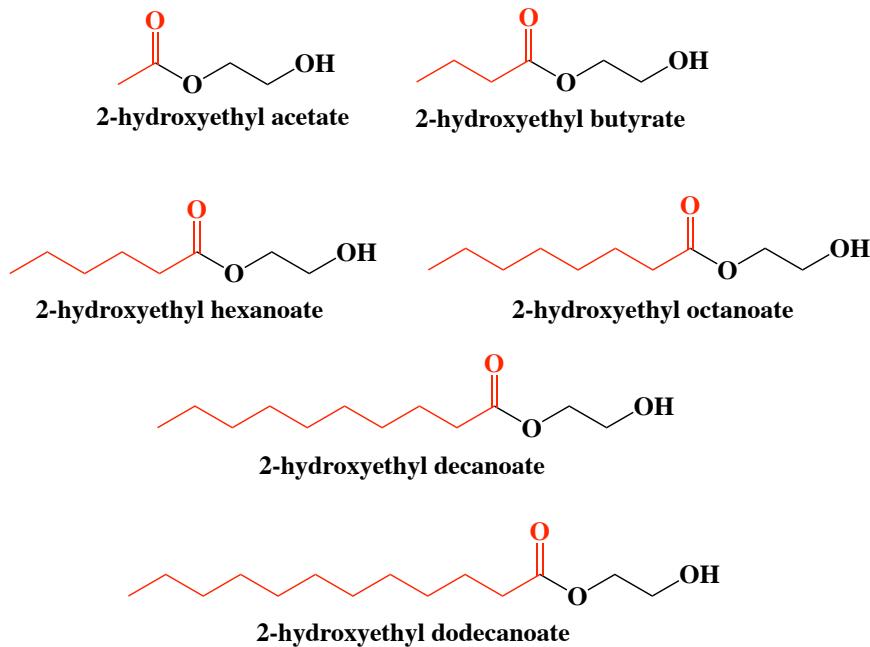
The simplest family of O-acylated polyol derivatives are monoacylated esters of ethylene glycol. These are reported schematically as shown in **Figure 1.1**, with a single acyl residue on one of the hydroxyl groups.



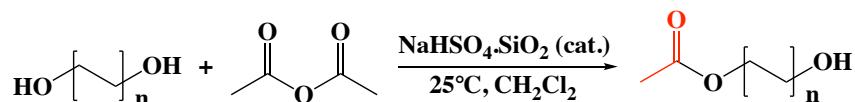
**Figure 1.1:** Generic structure of O-acylated ethylene glycol monoesters

Several O-acylated ethylene glycol monoesters have been reported in the scientific and patent literature, each varying in alkyl chain length (**Figure 1.2**).

2-Hydroxyethyl acetate was synthesized by Das *et al.* to design a synthetic route for highly selective and efficient monoacetylation of the symmetrical diols using NaHSO<sub>4</sub>.SiO<sub>2</sub> as a heterogenous catalyst. The reaction scheme (**Figure 1.3**) highlights monoacetate from a short reaction time (15-20 minutes) and excellent reaction yields.<sup>4</sup> The catalyst was prepared from NaHSO<sub>4</sub> and silica gel (finer than 200 mesh).

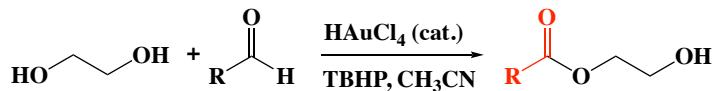


**Figure 1.2:** O-Acylated ethylene glycol monoesters in scientific literature



**Figure 1.3:** Scheme for mono acetylation of alcohol

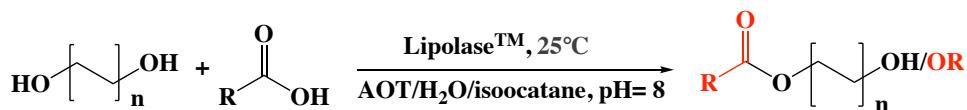
2-Hydroxyethyl butyrate was synthesized to establish a novel esterification method from aliphatic aldehydes and an alcohol using gold complexes as a homogenous catalyst. Hashmi *et al.* reported they were not convinced with the established homogenous gold catalyst oxidation reactions they examined, which showed precipitation of elemental gold. They were motivated to invent a “truly” homogeneous gold catalyst that can be used for these oxidation reactions. They also reported their reaction scheme was highly selective for forming monoesters from diols.<sup>5</sup> They used *tert*-butyl hydroperoxide (TBHP) as a test system of different Au<sup>III</sup> salts (**Figure 1.4**). An extended X-ray absorption fine structure (EXAFS) study was used to confirm their claim of a homogenous catalyst.



**Figure 1.4:** Scheme for 2-hydroxyethyl esters from aliphatic aldehydes

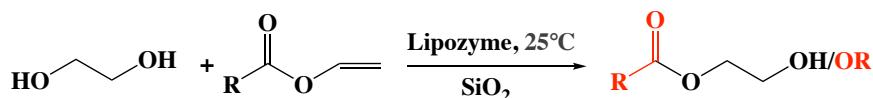
2-Hydroxyethyl octanoate and 2-hydroxyethyl decanoate are patented compounds and no description for their synthesis was provided.

2-Hydroxyethyl dodecanoate was synthesized by Stalmatis *et al.* to establish an esterification method for symmetrical diols in the presence of a lipase catalyst from *Humicola lanuginose*, in a water-in-oil microemulsion system stabilized with sodium(bis-2-ethylhexyl) sulphosuccinate (AOT) (**Figure 1.5**). Authors reported 2-hydroxyethyl dodecanoate has a nonionic surfactant property which might have potential use in the pharmaceutical, cosmetic and/or food industry.<sup>6</sup>



**Figure 1.5:** Scheme for esterification of diols catalyzed by lipase in microemulsions

In another study by Berger *et al.* this same compound was synthesized to establish an esterification method for symmetrical diols in presence of vinyl laurate and a lipase catalyst from *Mucor meihei* on a solid adsorbent silica gel support (**Figure 1.6**).<sup>7</sup>



**Figure 1.6:** Scheme for esterification from diol and vinyl laurate

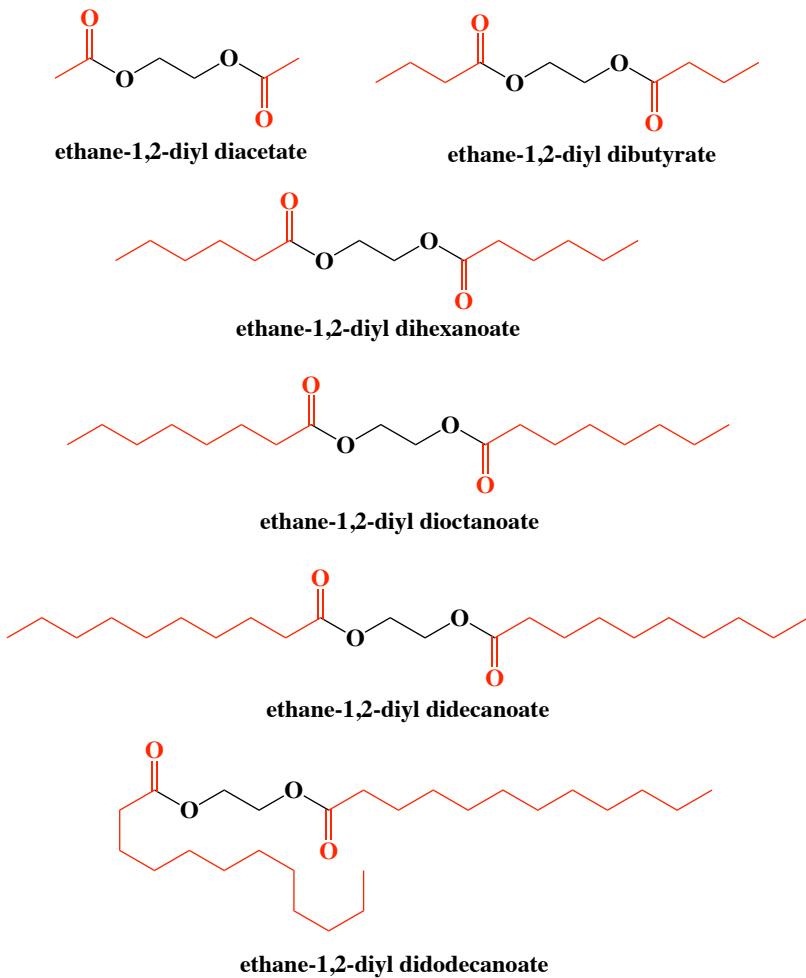
### 1.2.2 Diesters

The next class of O-acylated polyols ethylene glycol derivatives to be discussed are diesters, where two acyl groups are attached as shown in **Figure 1.7**. In all cases, acyl moieties are identical to each other.



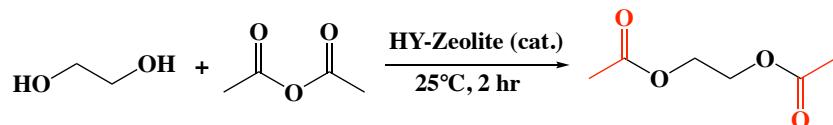
**Figure 1.7:** Generic structure of O-acylated ethylene glycol diesters

Six diesters of O-acylated ethylene glycol straight chain alkyl have been reported in the scientific literature.



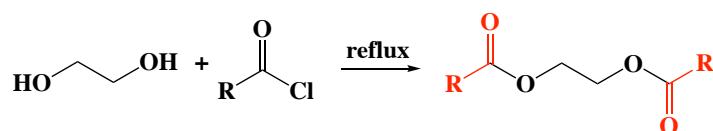
**Figure 1.8:** O-Acylated ethylene glycol diesters in scientific literature

Ethane-1,2-diyl dihexanoate was synthesized by Heravi *et al.* using a solventless reaction scheme for acylation of ethylene glycol with acetic anhydride in the presence of an HY zeolite ( $H_{50.895}Na_{1.005}Si_{140.1}Al_{51.9}O_{384}$ ) catalyst.<sup>8</sup> The authors reported HY zeolite is an extremely active and reusable catalyst for acylation of the alcohols (**Figure 1.9**).



**Figure 1.9:** Scheme for acylation of alcohols using HY-Zeolite catalyst

Kaul *et al.* synthesized a series of biorational glycol diesters including ethane-1,2-diyl dibutyrate, ethane-1,2-diyl dihexanoate, ethane-1,2-diyl dioctanoate and ethane-1,2-diyl didecanoate and tested their efficiency as an insecticide that inhibit the larval growth of second instars of *Spodoptera litura* and *Helicoverpa armigera*. According to their report these compounds are ecofriendly compounds that induce physiological toxicity of lepidopteran larvae via oral administration, at the same time showing low toxicity to beneficial insects.<sup>9</sup>

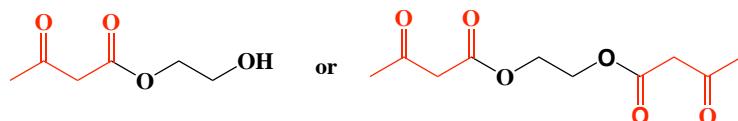


**Figure 1.10:** Scheme for acylation of alcohols using acid chloride

Ethane-1,2-diyl didodecanoate was synthesized by Stalmatis *et al.* and Berger *et al.* likewise 2-hydroxyethyl as previously mentioned in section 1.2.1. (page 4).<sup>6,7</sup>

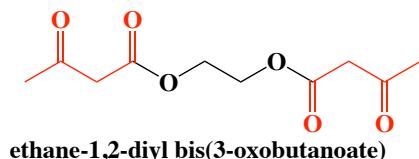
### 1.2.3 Acetoacetate esters

Acetoacetate esters of ethylene glycol have one or two a  $\beta$ -keto esters attached to the hydroxyl groups of ethylene (Figure 1.11):



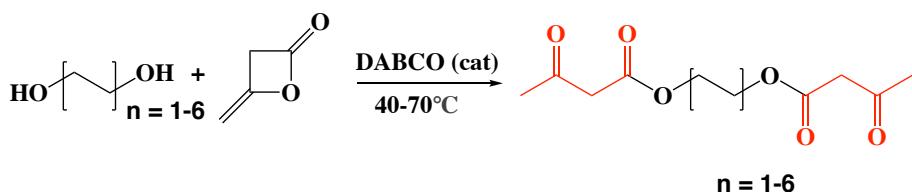
**Figure 1.11:** Generic structure of acetoacetate ester of ethylene glycol

Interestingly, only the diester was found in the patent literature.<sup>10</sup>



**Figure 1.12:** O-Acylated ethylene glycol acetoacetate ester in scientific literature

This compound was synthesized by acylating the two alcohol with diketene as the acylating agent using a solventless method with high product yield. This reaction condition does not require heat, rather requires use of a DABCO (1,4-Diazabicyclo[2.2.2]octane) as a catalyst. (Figure 1.13).<sup>10</sup>



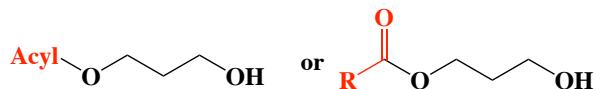
**Figure 1.13:** Scheme for preparation of acetoacetylated polyols

This method has been applied to a library of diester derivatives where n = 1-6.

## 1.3 O-Acylated 1,3-propanediol derivatives and their uses

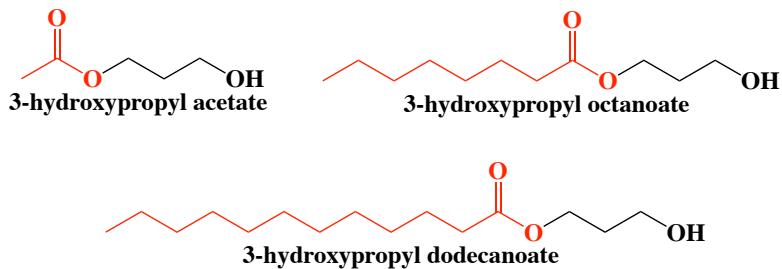
### 1.3.1 Monoesters

The next family of O-acylated diols of the 1,3-propanediol monoesters of which an ester group is attached to one of the hydroxyl groups (**Figure 1.14**).



**Figure 1.14:** Generic structure of O-acylated 1,3-propanediol monoesters

Three O-acylated 1,3-propanediol monoesters have been synthesized and reported in the scientific literature (**Figure 1.15**).



**Figure 1.15:** O-Acylated 1,3-propanediol monoesters in scientific literature

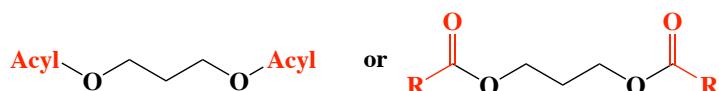
3-Hydroxypropyl acetate was reported in a patent publication and no detail description of the experimental method or intended use of the compound was provided.

3-Hydroxypropyl octanoate was synthesized by esterification of 1,3-propanediol via fermentation and reaction with an organic acid. Authors reported a series of compounds comprised of 1,3-propanediol, that were for potential use as synthesized following this method can be used as emulsifiers, surfactants, conditioners, thickeners, temperature stabilizers, chemical stabilizers, opacifiers, solvents, gelling agents, wetting agents, corrosion inhibitors, lubricants, antimicrobials or defoamers.<sup>11</sup>

3-Hydroxypropyl dodecanoate was synthesized by Stalmatis *et al.* using the method as for the synthesis of 2-hydroxyethyl, as provided in section 1.2.1. (page 4).<sup>6</sup>

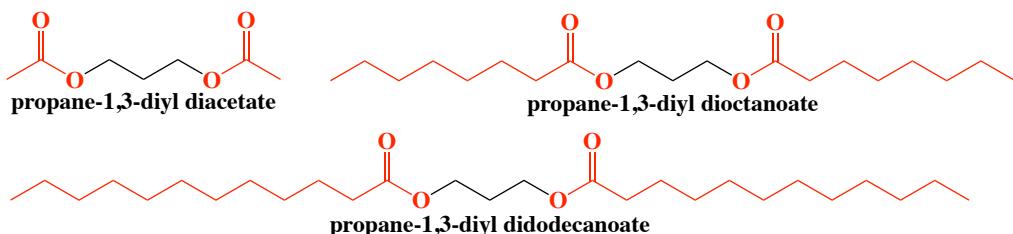
### 1.3.2 Diesters

Diesters of 1,3-propanediol have both of the hydroxyl groups are acylated (**Figure 1.16**).



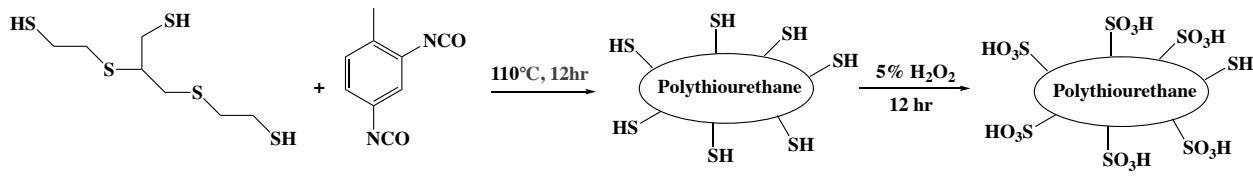
**Figure 1.16:** Generic structure of O-acylated 1,3-propanediol diesters

Three diesters of 1,3-propanediol have been reported in the literature (**Figure 1.17**).



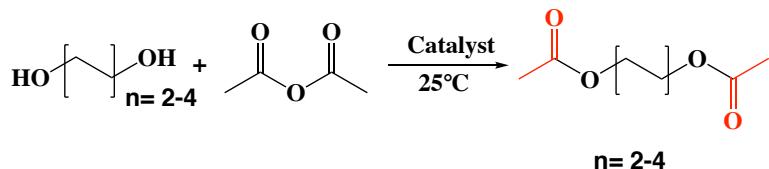
**Figure 1.17:**O-Acylated 1,3-propanediol diesters in scientific literature

Du *et al.* synthesized a novel polythiourethane-based acid and tested its catalytic activity for acylating 1,3-propanediol. The catalyst was synthesized by copolymerization of tolylenediisocyanate (TDI) and 1,2-bis[(2-mercaptopropyl)thio]-3-mercaptopropane (MR) using the synthetic scheme shown in **Figure 1.18**:



**Figure 1.18:** Synthetic route of the novel polyurethane-based catalyst

They reported this acid catalyst has high activity on acetylation of alcohols and offers the advantages of a short reaction time, solvent-free, high product yields and is considered a green acetylation method. As a part of their method the authors synthesized propane-1,3-diyli diacetate via acetylation, with other molecules in the compound library n = 2-4 (**Figure 1.19**).<sup>12</sup>



**Figure 1.19:** Synthetic scheme for preparation of diesters

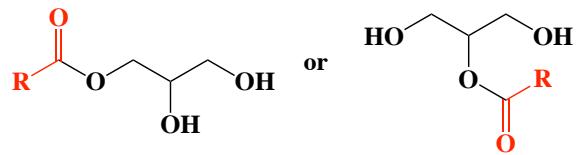
Propane-1,3-diyli dioctanoate was synthesized the same way as 3-hydroxypropyl octanoate as described in section 1.3.1 (page 8).<sup>11</sup>

Similar to the monoester, propane-1,3-diyli didodecanoate was synthesized by Stalmatis *et al.* as described in section 1.2.1. (page 4).<sup>6</sup>

## 1.4 O-Acylated glycerol derivatives and their uses

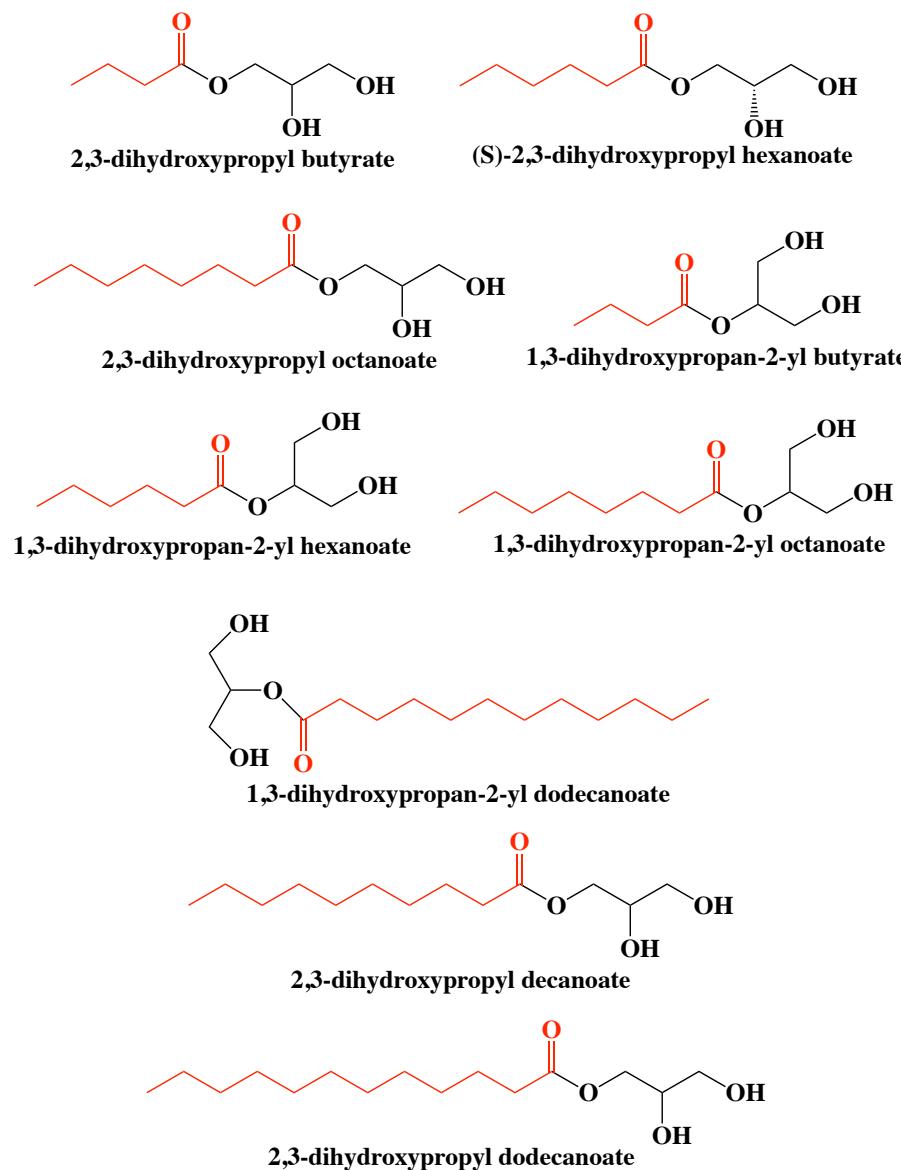
### 1.4.1 Monoesters

Monoesters of glycerol are where one of the hydroxyl group is acylated and other two remain free (**Figure 1.20**).



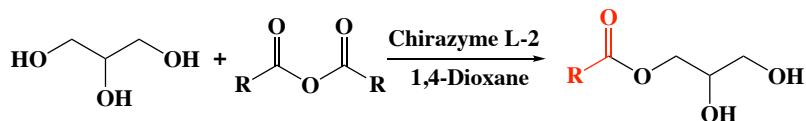
**Figure 1.20:** Generic structure of O-acylated glycerol monoesters

Nine monoesters of glycerol have been reported in literature, which are listed in **Figure 1.21.**



**Figure 1.21:** O-Acylated glycerol monoesters in scientific literature

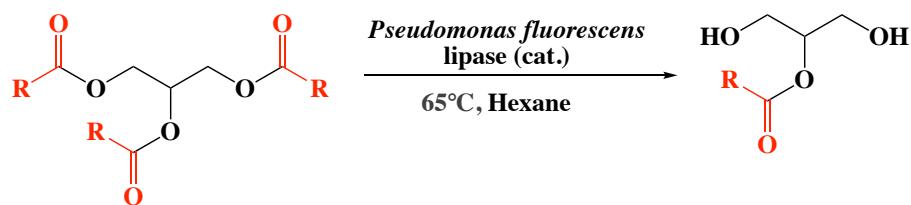
2,3-Dihydroxypropyl butyrate, (S)-2,3-dihydroxypropyl hexanoate and 2,3-dihydroxypropyl octanoate were synthesized by Batovska *et al.* using Chirazyme L-2 (*Candida antarctica*) catalyzed esterification of glycerol and aliphatic anhydrides (**Figure 1.22**).



**Figure 1.22:** Lipase-catalyzed esterification of glycerol with aliphatic acid anhydride

Six carbon aliphatic acid anhydride reactions are enantioselective for the S-enantiomer. Monoacylglycerols (MAGs) has been reported as emulsifiers with antimicrobial properties that can be used in the food, pharmaceutical and cosmetic industries. Enantiomerically pure compounds can be used as synthetic intermediates and building blocks in synthetic chemistry.<sup>13</sup>

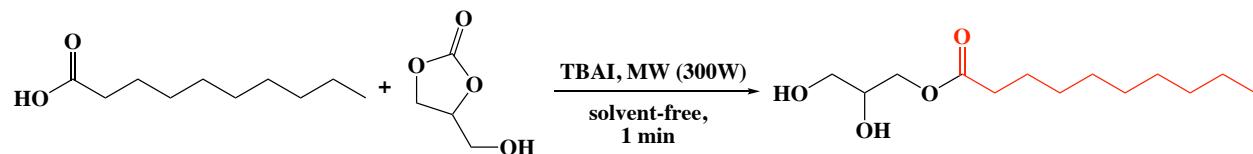
1,3-Dihydroxypropan-2-yl butyrate, 1,3-dihydroxypropan-2-yl hexanoate, 1,3-dihydroxypropan-2-yl octanoate and 1,3-dihydroxypropan-2-yl dodecanoate were synthesized by Lee *et al.* via celite-immobilized *Pseudomonas fluorescens* lipase-catalyzed regioselective alcoholysis of triglycerides (TG)<sup>14</sup> (**Figure 1.23**).



**Figure 1.23:** Lipase-catalyzed alcoholysis of glycerides

2,3-Dihydroxypropyl decanoate commonly known as 1-monocaprin, is used as a food additive for its antimicrobial and anti-viral activities against *Campylobacter jejuni*, *Escherichia coli* and human immunodeficiency virus (HIV). Park *et al.* synthesized this compound by

porcine liver carboxylesterases-catalyzed esterification in a reversed micellar system established with AOT (surfactant) and isoctane (reaction medium).<sup>15,16</sup> Mhanna *et al.* also synthesized this compound by microwave-assisted esterification in presence of tetrabutylammonium iodide (TBAI) as a catalyst in solvent-free conditions. This method required 1-5 minutes exposure in a microwave oven (at 300W) and offers high product yield (**Figure 1.24**).<sup>17</sup>

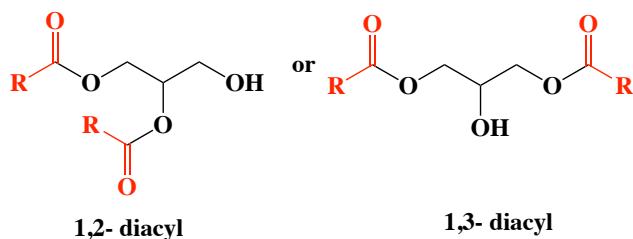


**Figure 1.24:** Scheme for the preparation of 2,3-dihydroxypropyl decanoate

2,3-Dihydroxypropyl dodecanoate was synthesized to study the kinetics of acyl migration in monoglycerides from the  $\beta$  to  $\alpha$  position for various acyl chain lengths. A 1:6.5 mixture of  $\alpha$ /  $\beta$ -monoacylglycerides rearranged to a 1:1 mixture after 24 hours of reaction.<sup>18</sup>

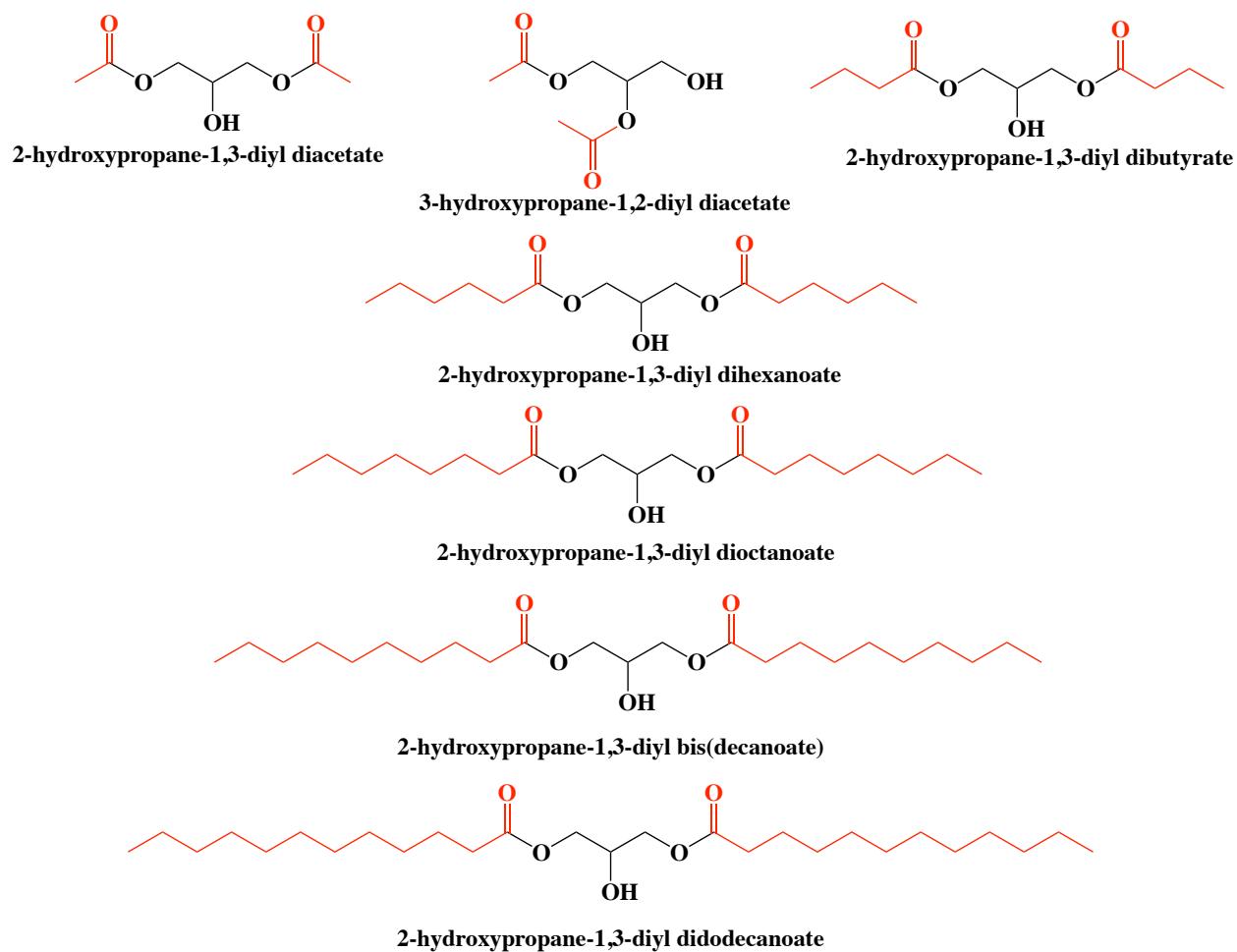
#### 1.4.2 Diesters

Glycerol diesters have two of the hydroxyl groups acylated and can be either 1,2- and 1,3- derivatives (**Figure 1.25**).



**Figure 1.25:** Generic structure of O-acylated glycerol diesters

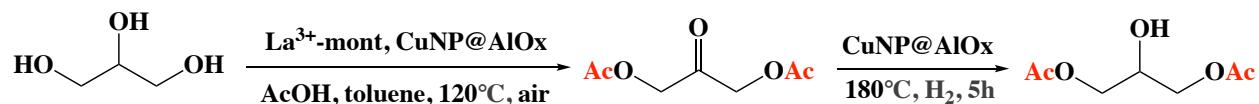
Seven glycerol diesters have been reported in the scientific literature (**Figure 1.26**).



**Figure 1.26:** O-Acylated glycerol diesters in scientific literature

Mizugaki *et al.* research showed that lanthanum cation-exchanged montmorillonite gave high selectivity towards formation of 1,2- and 1,3-diacylglycerols (DAG) from esterification of glycerol. Lanthanum montmorillonite is a layered clay mineral consisting of silica-alumina sheets with exchangeable La ions located in between layers. They also reported a metal nanoparticle catalyst made up of base Cu metal embedded in an AlO<sub>x</sub> matrix (CuNP@AlO<sub>x</sub>), selectively converts DAGs to 1,3-diacetylglycerols under successive oxidation-hydrogenation

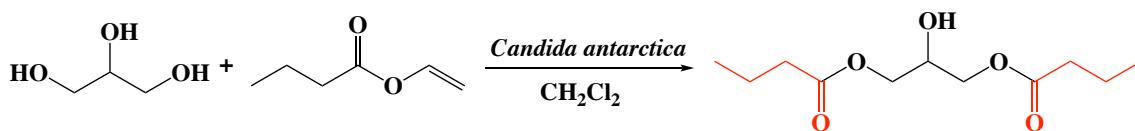
reactions with excellent yields. They synthesized 2-hydroxypropane-1,3-diyl diacetate upon combination of these two reusable heterogeneous catalysts (**Figure 1.27**).



**Figure 1.27:** Scheme for the preparation of 1,3-diacetyl glycerol

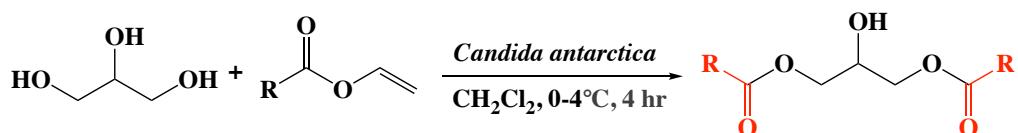
Authors have reported the application of diacylglycerides in pharmaceuticals, polymer, cosmetic and food industry.<sup>19</sup> In another research study, Zhou *et al.* synthesized these compounds for the reaction parameter optimization of the esterification reaction using three different solid acid catalysts namely, Amberlyst-15 (ion exchange resin), HZSM-5 and HUSY (acid zeolites). Their research showed that the optimum reaction condition for the production of a mixture of 2-hydroxypropane-1,3-diyl diacetate and 2-(hydroxymethyl)-3-oxobutyl acetate is to use glycerol and acetic acid in a 1:9 molar ratio, 110°C temperature in presence of Amberlyst-15 catalyst.<sup>20</sup>

2-Hydroxypropane-1,3-diyl dibutyrate was synthesized as a synthetic intermediate for the formation of monoacylglycerol. Whitten *et al.* reported a chemoenzymatic pathway for the selective synthesis of 2-monoacylglycerol with the reaction of glycerol and vinyl butyrate as the first step of the synthesis where *Candida antarctica* is as a catalyst (**Figure 1.28**).<sup>21</sup> As silica gel column purification is an inevitable condition of acyl group migration from carbon 2 to carbon 1 or 3 for monoacylglycerides,<sup>22</sup> the authors suggested a boric acid impregnated TLC plate and silica gel as a fruitful alternative.<sup>23</sup>



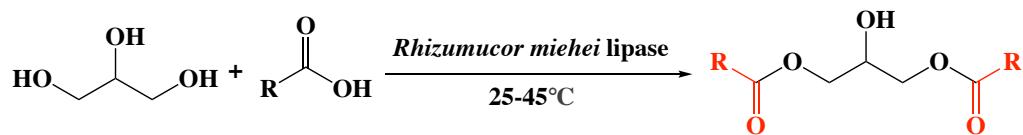
**Figure 1.28:** Scheme for the preparation of the 2-hydroxypropane-1,3-diyl dibutyrate

Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are marine fats known for treatment of cardiovascular and heart diseases<sup>24-26</sup> as well as inflammatory disorders,<sup>27-28</sup> mental disorders such as schizophrenia,<sup>29-30</sup> Alzheimer's dementia<sup>31</sup> and depression.<sup>32,33</sup> As dietary and health supplements, triacylglycerol with biologically active polyunsaturated fatty acid (PUFA) in the 2-position and medium chain length fatty acids in the terminal positions gained attention.<sup>34,35</sup> Magnusson *et al.* synthesized different compounds containing short chain acyls (C<sub>2</sub>, C<sub>4</sub>, C<sub>6</sub>) on the terminal hydroxyls and EPA or DHA on the middle of the secondary hydroxyls. As a synthetic intermediate they synthesized 2-hydroxypropane-1,3-diyl dihexanoate using *Candida antarctica* lipase enzyme as a catalyst (**Figure 1.29**).<sup>36</sup>



**Figure 1.29:** Scheme for the preparation of 1,3-diacylated glycerol

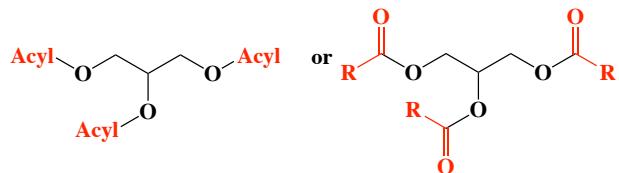
2-Hydroxypropane-1,3-diyl dioctanoate, 2-hydroxypropane-1,3-diyl bis(decanoate) and 2-hydroxypropane-1,3-diyl didodecanoate were also synthesized by the Rosu research group in Japan to prepare therapeutically and nutritionally valuable triacylglycerol. They also showed *Rhizumucor miehei* lipase enzyme can be used as a catalyst in a solvent-free reaction medium (**Figure 1.30**).<sup>37</sup>



**Figure 1.30:** Scheme for the preparation of 1,3-diacylated glycerol using fatty acid

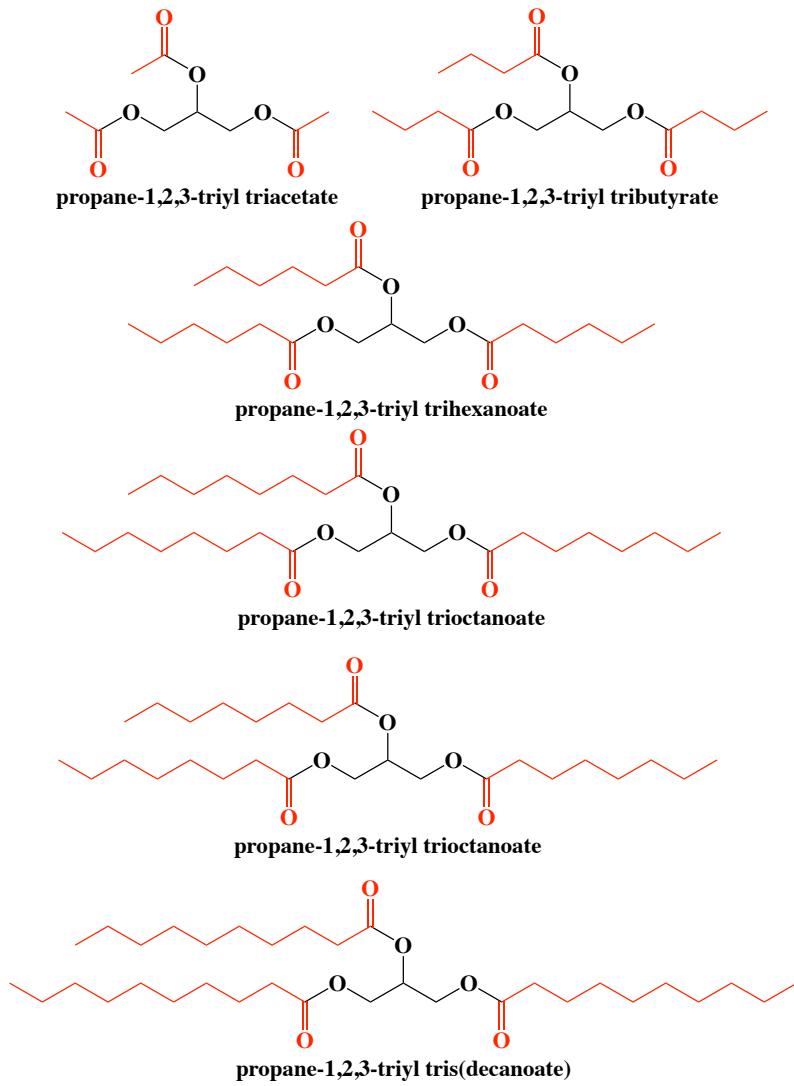
### 1.4.3 Triesters

O-Acylated triesters of glycerol having all three hydroxyl groups are acylated represent by far the largest diversity of acylated polyols and depicted in **Figure 1.31**.



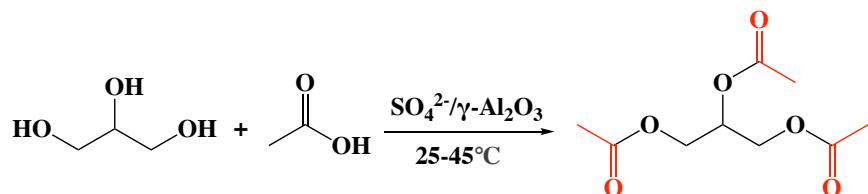
**Figure 1.31:** Generic structure of O-acylated triesters of glycerol

Six O-acylated triesters of glycerol have been reported (**Figure 1.32**).



**Figure 1.32:** O-Acylated glycerol triesters in scientific literature

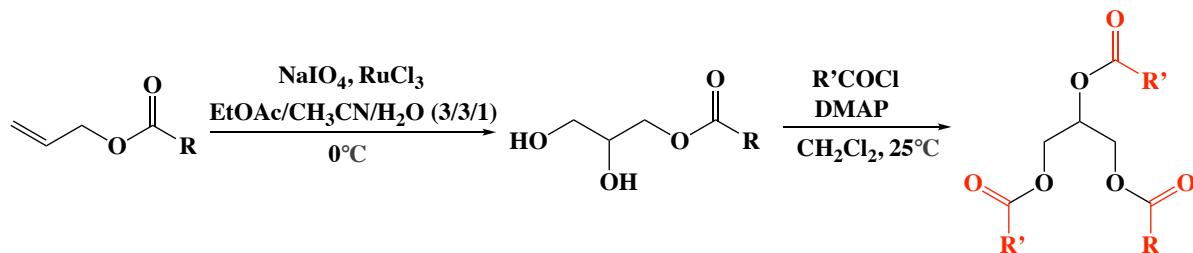
Propane-1,2,3-triyl triacetate is known as triacetin is mostly used as a humectant and an automotive fuel additive.<sup>38</sup> Ferreira *et al.* optimized the preparation of triacetin from glycerol and acetic acid in presence of sulfated-alumina catalyst.<sup>39</sup> (**Figure 1.33**)



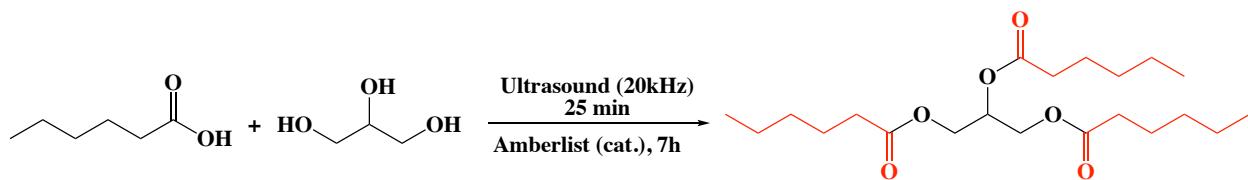
**Figure 1.33:** Scheme for the preparation of triacetin

Propane-1,2,3-triyl tributyrate is a patented oral prodrug that has antitumor activity<sup>40</sup> against colon cancer<sup>41,42</sup> and prostate cancer preventive agent.<sup>43</sup> This compound also showed reduction of antibiotic-induced intestinal injury<sup>44</sup> and lipopolysaccharide-induced liver injury in rats<sup>45</sup> as well as improvement of the growth and intestinal digestive and barrier functions in intrauterine growth-restricted piglets.<sup>46</sup>

Propane-1,2,3-triyl trihexanoate was synthesized by Nandini *et al.* as a member of their library of compounds for medium chain triacylglycerols, via RuO<sub>4</sub> catalyzed dihydroxylation of the allyl esterification method (**Figure 1.34**).<sup>47</sup> A sonication pre-treatment method with amberlyst-15 catalyst was also reported by Jadhav *et al.* for the synthesis of this molecule (**Figure 1.35**).<sup>48</sup> These compounds can be used in food, drugs and cosmetics.<sup>47</sup>

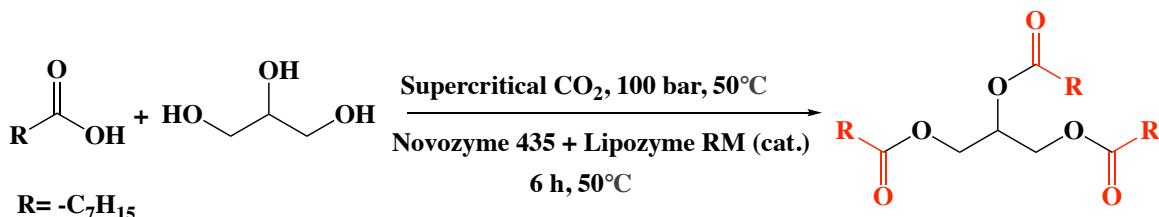


**Figure 1.34:** Scheme for the synthesis of propane-1,2,3-triyl trihexanoate



**Figure 1.35:** Scheme for the preparation of tricaproin

Propane-1,2,3-triyl trioctanoate (called tricaprylin) is another triglyceride synthesized by More *et al.* using Novozyme 435 lipase catalyst in supercritical carbon dioxide media (**Figure 1.36**). This compound has potential use in the pharmaceutical and cosmetic industry.<sup>49</sup>

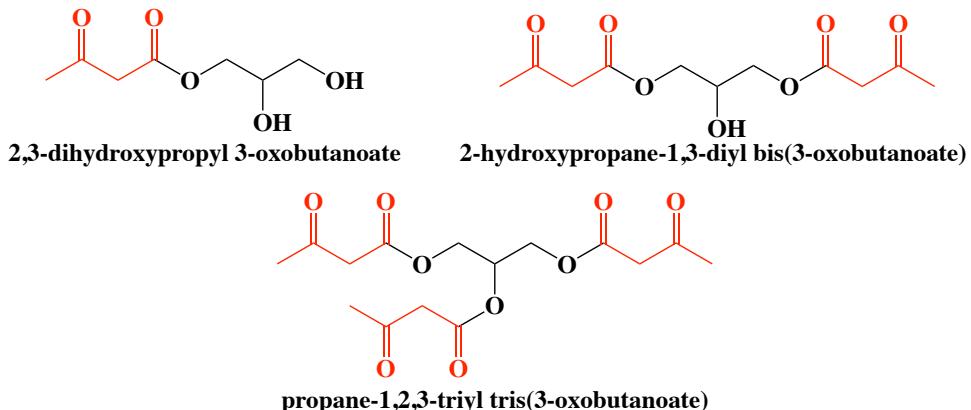


**Figure 1.36:** Scheme for the preparation of tricaprylin

Propane-1,2,3-triyl tris(decanoate) is commonly known as Tricaprin was synthesized by a traditional esterification method using acyl chloride and alcohol or DCC-DMAP catalysed coupling of carboxylic acid and alcohol. This triester may have application for the treatment of neurodegenerative diseases with a therapeutically effective dose.<sup>50</sup>

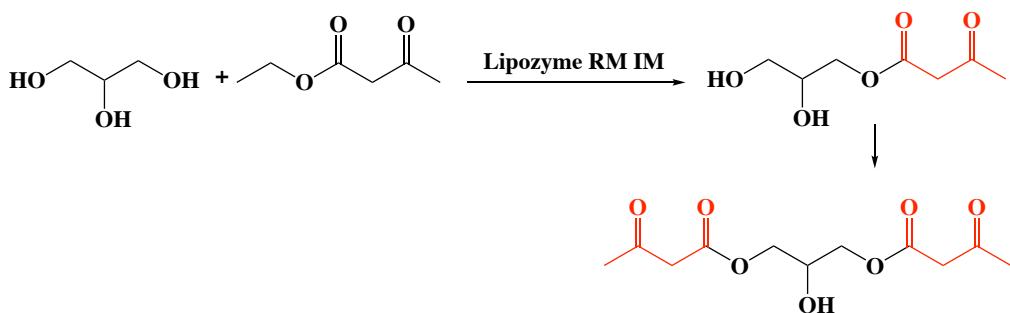
#### 1.4.4 Acetoacetate esters

Acetoacetate esters of glycerol are the group of molecules having a  $\beta$ -carbonyl ester attached to the hydroxyl groups of glycerol. All of the three O-acylated glycerol acetoacetates have been reported in the scientific literature are listed in **Figure 1.37**.



**Figure 1.37:** Generic structure of glycerol acetoacetate esters

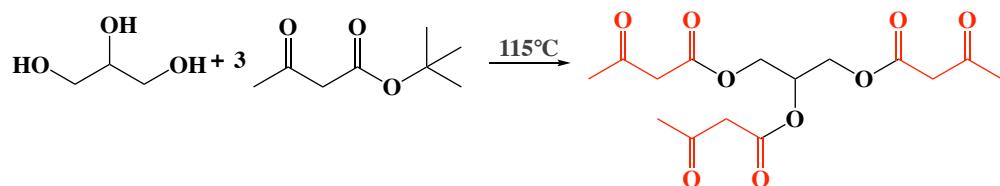
Mono and di-acetoacetyl glycerides have potential application as a prochiral building block in chemical synthesis as well as for cross-linked biopolymers. Traditional reaction conditions for acetoacetate esters of glycerol require high temperature and/or expensive catalysts. Haas *et al.* invented a milder reaction condition for the synthesis of glycerol mono and di-acetoacetates using immobilized *Rhizomucor meihei* lipase (Lipozyme RMIM) catalyst (**Figure 1.38**).<sup>51</sup>



**Figure 1.38:** Scheme for the preparation of glycerol mono- and di-acetoacetate esters

Nakajima *et al.* synthesized the glycerol triacetoacetate ester using diketene in presence of a base in a solventless reaction media was described in section 1.2.3 and **Figure 1.13**.<sup>10</sup> (page 7)

Sonnenschein *et al.* synthesized this compound with the reaction of glycerol and *tert*-butyl acetoacetate along (**Figure 1.39**), for use as a building for block producing foams and elastomers.<sup>52</sup>

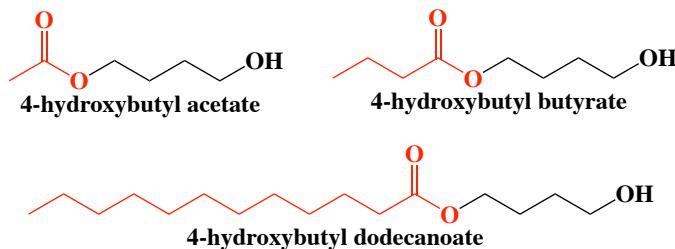


**Figure 1.39:** Scheme for the synthesis of glycerol tri-acetoacetate ester

## 1.5 O-Acylated 1,4-butanediol derivatives and their uses

### 1.5.1 Monoesters

Monoesters of 1,4-butanediol have been reported as well (**Figure 1.40**).



**Figure 1.40:** O-Acylated 1,4-butanediol monoesters in scientific literature

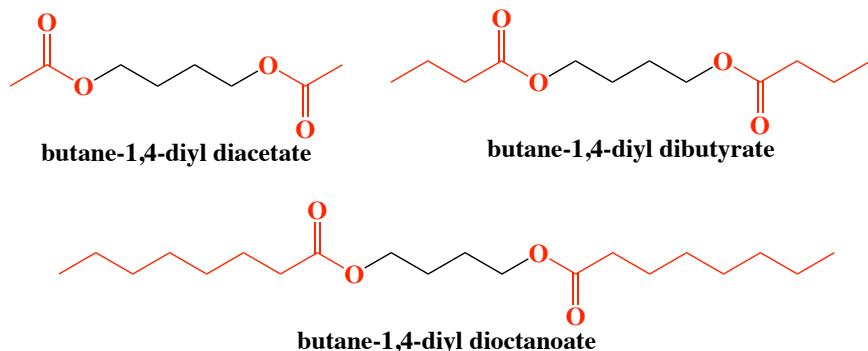
4-Hydroxybutyl acetate was synthesized by Das *et al.* was discussed in section 1.2.1. (page 2).<sup>4</sup>

4-Hydroxybutyl butyrate is a patented compound and no further detail is provided.

4-Hydroxybutyl dodecanoate was synthesized by Stalmatis *et al.* as described in section 1.2.1 (page 4).<sup>6</sup>

### 1.5.2 Diesters

Three of the O-acylated 1,4-butanediol diesters have been reported (**Figure 1.41**).

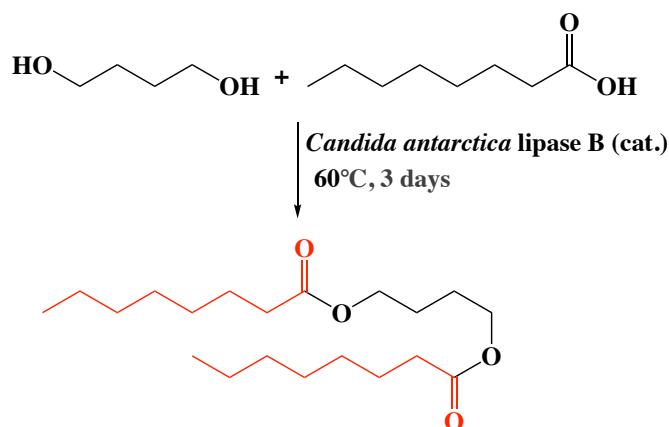


**Figure 1.41:** O-Acylated 1,4-butanediol diesters in scientific literature

Butane-1,4-diyl diacetate was synthesized along with propane-1,3-diyl diacetate by Du *et al.* for the invention of a novel polyurethane based solid catalyst as detailed previously in section **1.3.2** and **Figure 1.18.**<sup>12</sup> (page 10)

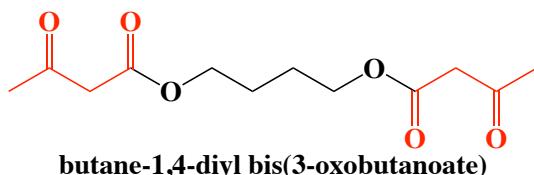
Butane-1,4-diyl dibutyrate is a patented compound and no further details are provided about its synthesis or its applications.

Butane-1,4-diyl dioctanoate was synthesized by Gallos *et al.* as part of their project to study elasticity of biobased materials. They synthesized this molecule by reaction of 1,4-butanediol and octanoic acid in the presence of *Candida antarctica* lipase B catalyst (**Figure 1.42**).<sup>53</sup>



**Figure 1.42:** Scheme for the preparation of butane-1,4-diyl dioctanoate

### 1.5.3 Acetoacetate esters



**Figure 1.43:** Generic structure of 1,4-butane diol acetoacetate esters

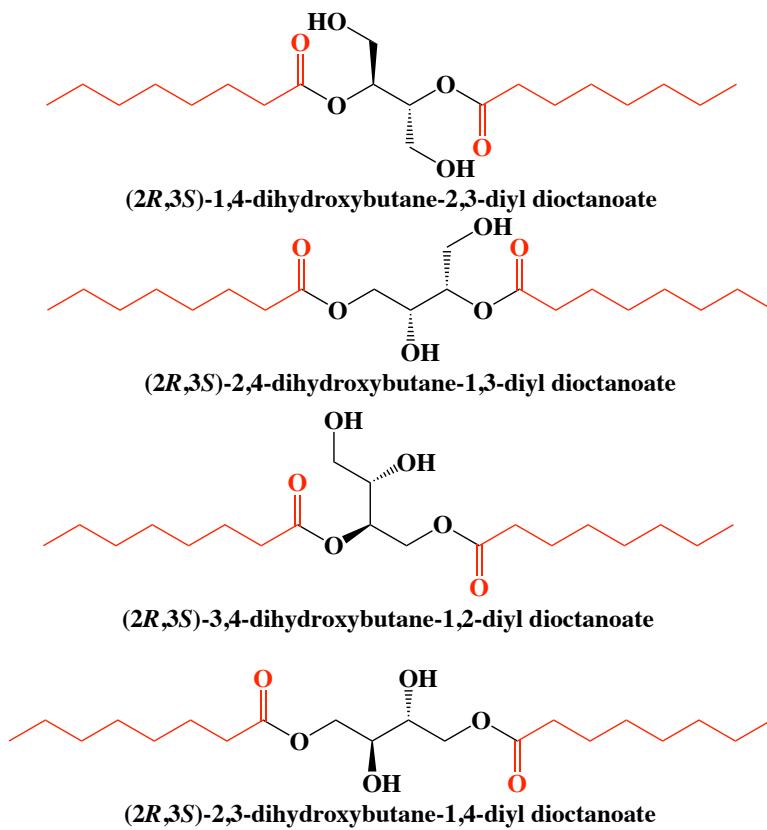
One O-acylated acetoacetate ester of 1,4-butane diol was reported in scientific literature (**Figure 1.43**). Butane-1,4-diyl bis(3-oxobutanoate) was synthesized by Nakajima *et al.* using a solventless preparation method with high product yield. This was described in section 1.2.3 (page 7).<sup>10</sup>

## 1.6 O-Acylated *meso*-erythritol derivatives and their uses

### 1.6.1 Diesters

Four diesters of *meso*-erythritol have been reported (**Figure 1.44**).<sup>54</sup>

Mamidi *et al.* synthesized a series of compounds including (2S,3S)-1,4-dihydroxybutane-2,3-diyl dioctanoate, (2S,3S)-2,4-dihydroxybutane-1,3-diyl dioctanoate, (2S,3S)-3,4-dihydroxybutane-1,2-diyl dioctanoate and (2S,3S)-2,3-dihydroxybutane-1,4-diyl dioctanoate and reported these compounds can act a ligand for C1 domain of PKC isoforms as a result for their molecular docking. Protein kinase C (PKC) of the serine or threonine family is a potential drug target for cancer treatment and other diseases. The two free hydroxyl groups reportedly increase



**Figure 1.44:** O-Acylated *meso*-erythritol diesters in scientific literature

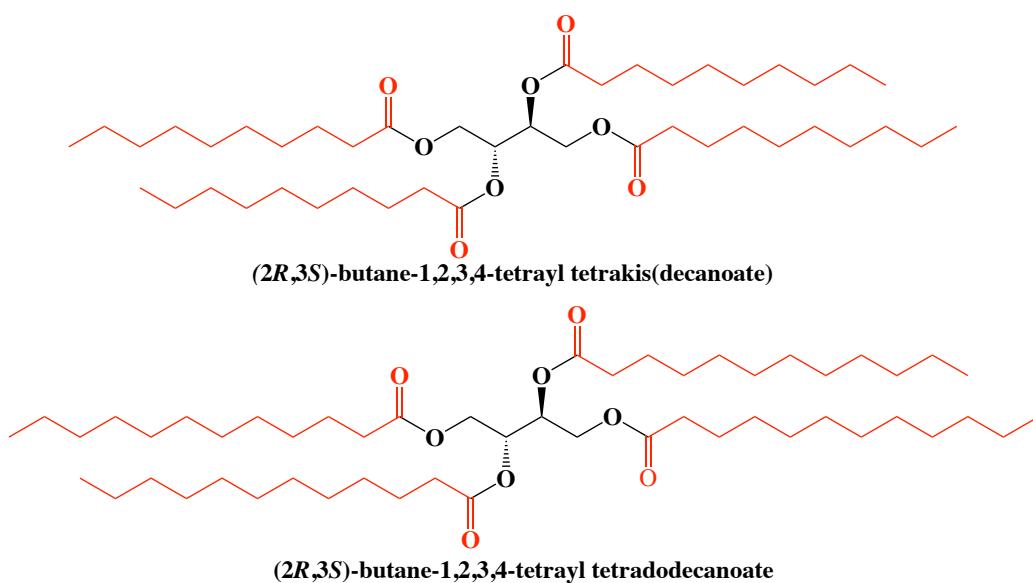
the binding affinity with PKC because of hydrogen bonding. (+)-Diethyl-L-tartrate was used as the starting material using benzyl protecting groups and Pd-C hydrogenation to later deprotect. For secondary hydroxyl group protection, acetal formation reaction and addition of acid to deprotect. Lithium aluminum hydride reduction was used to reduce to starting material ester

group to hydroxyl groups along with *N,N*'-dicyclohexylcarbodiimide (DCC) and *N,N*'-dimethyl aminopyridine (DMAP) mediated esterification reaction used for the esterification.<sup>54</sup>

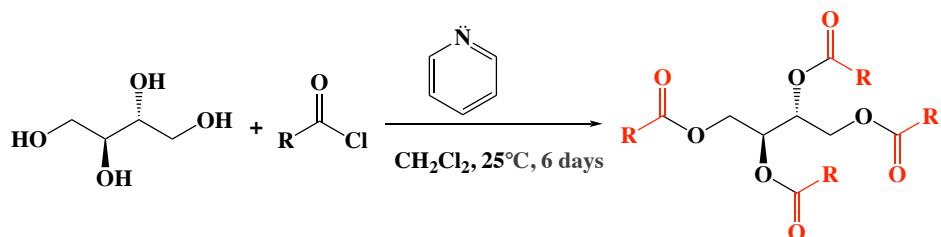
### 1.6.2 Tetraesters

Two examples of a butanetetraol tetraester have been reported, in the case of meso-erythritol (**Figure 1.45**).

Tetraesters of *meso*-erythritol might have similar potential application analogues to those of triglycerides. Ioannou *et al.* reported a route for complete esterification of erythritol (**Figure 1.46**).<sup>55</sup>



**Figure 1.45:** O-Acylated *meso*-erythritol tetraesters in scientific literature

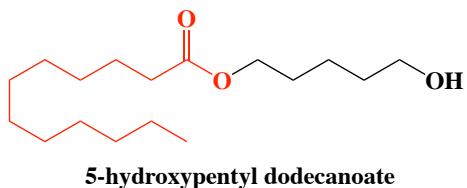


**Figure 1.46:** Scheme for the preparation of fully esterified erythritol

## 1.7 O-Acylated 1,5-pentanediol derivatives and their uses

### 1.7.1 Monoesters

One monoester of the O-acylated 1,5-pentane diol was reported in the scientific literature (**Figure 1.47**).<sup>6</sup>

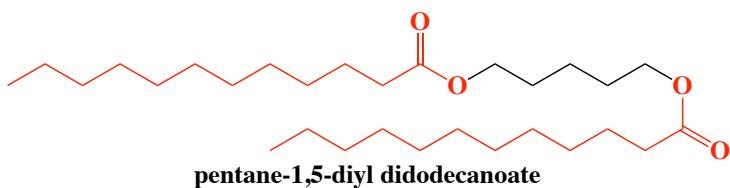


**Figure 1.47:** O-Acylated 1,5-pentanediol monoesters in scientific literature

5-Hydroxypentyl dodecanoate was synthesized by Stalmatis *et al.* by esterification of 1,5-pentanediol in presence of lipase from *Humicola lanuginose* (**Figure 1.5**).<sup>6</sup>

### 1.7.2 Diesters

One diester of 1,5-pentanediol has been reported (**Figure 1.48**).

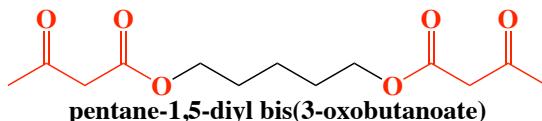


**Figure 1.48:** O-Acylated 1,5-pentanediol diester in scientific literature

Pentane-1,5-diyl didodecanoate was synthesized with 2-hydroxyethyl dodecanoate by Stalmatis *et al.* as a product of esterification in presence of catalyst from *Humicola lanuginose* (**Figure 1.5**).<sup>6</sup>

### 1.7.3 Acetoacetate esters

One acetoacetate ester of 1,5-pentanediol has been reported in a patent publication (Figure 1.49).<sup>10</sup>



**Figure 1.49:** O-Acylated 1,5-pentanediol acetoacetate esters in publication

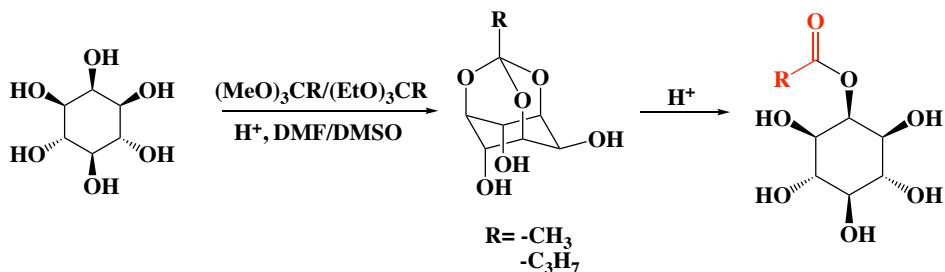
Pentane-1,5-diyl bis(3-oxobutanoate) was synthesized by Nakajima *et al.*, along with ethane-1,2-diyl bis(3-oxobutanoate) as described in section 1.2.3 (pages 7).<sup>10</sup>

## 1.8 O-Acylated *myo*-inositol derivatives and their uses

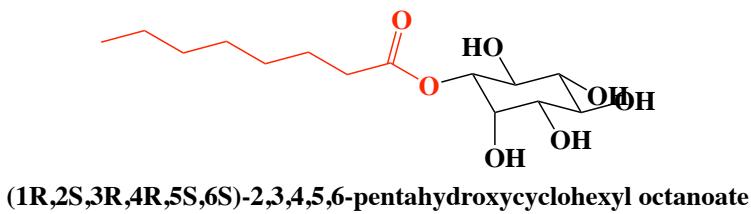
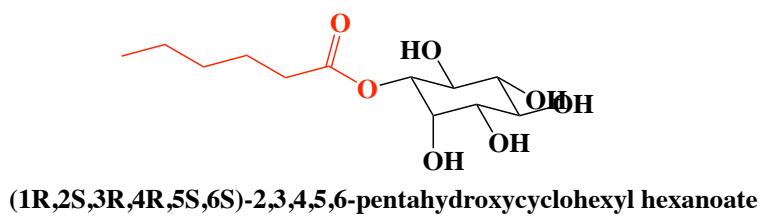
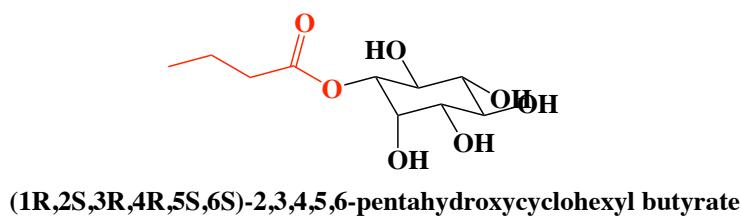
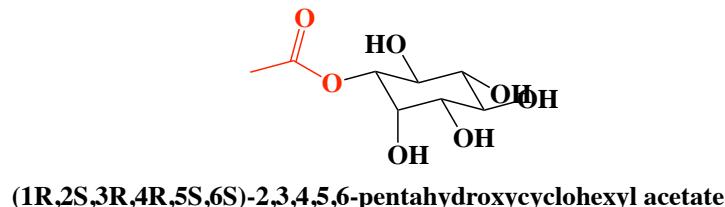
### 1.8.1 Monoesters

Four monoesters of *myo*-inositol have been reported as listed in Figure 1.51 (page 28).<sup>56</sup>

Godage *et al.* synthesized (1*R*,2*S*,3*R*,4*R*,5*S*,6*S*)-2,3,4,5,6-pentahydroxycyclohexyl acetate and (1*R*,2*S*,3*R*,4*R*,5*S*,6*S*)-2,3,4,5,6-pentahydroxycyclohexyl butyrate, as a synthetic precursor for the synthesis of 2-O-acyl inositol 1,3,4,5,6-pentakisphosphate (Figure 1.50):



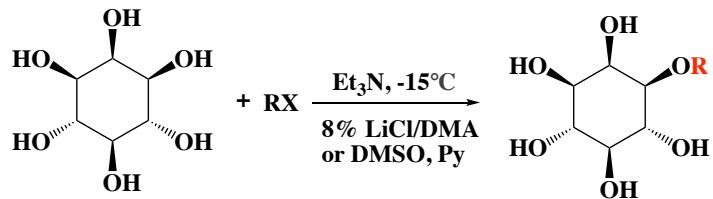
**Figure 1.50:** Scheme for the preparation of 2- and 4- carbon chain length monoesters of O-acylated *myo*-inositol



**Figure 1.51:** O-Acylated *myo*-inositol monoesters in scientific literature

The authors reported that this is a rapid and highly efficient method and the monoesterified product might have interesting biologically and anti-cancer properties.<sup>56</sup>

(1*R*,2*S*,3*R*,4*R*,5*S*,6*S*)-2,3,4,5,6-pentahydroxycyclohexyl hexanoate and (1*R*,2*S*,3*R*,4*R*,5*S*,6*S*)-2,3,4,5,6-pentahydroxycyclohexyl octanoate were synthesized by Watanabe *et al.* from *myo*-inositol by reaction with acid anhydride in a solution of *N,N*-dimethylacetamide (DMA) containing LiCl and dimethylsulfoxide (DMSO) (**Figure 1.52**).<sup>57</sup>

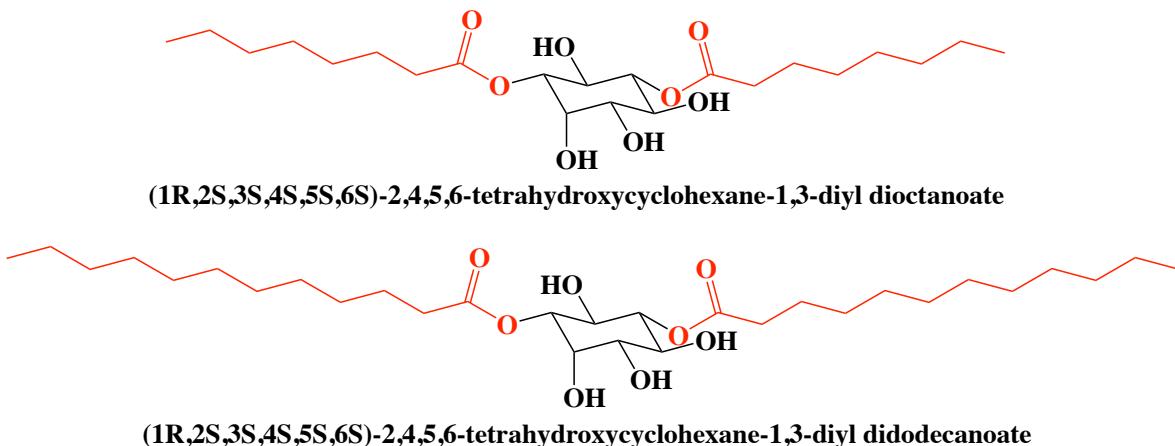


**Figure 1.52:** Scheme for the preparation of 6- and 8- carbon chain length monoesters of O-acylated *myo*-inositol

The authors reported that these compounds might be useful as surfactants and monomers for polymer synthesis.<sup>57</sup>

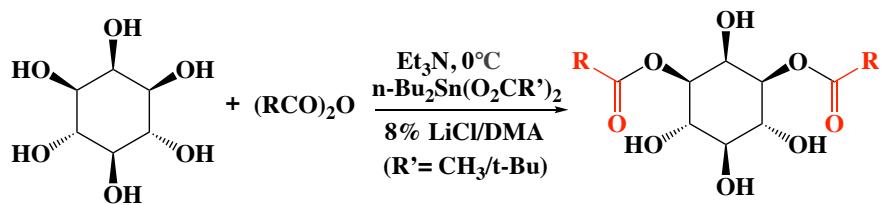
### 1.8.2 Diesters

Two of the diesters of *myo*-inositol have been reported as well (**Figure 1.53**).



**Figure 1.53:** O-Acylated *myo*-inositol diesters in scientific literature

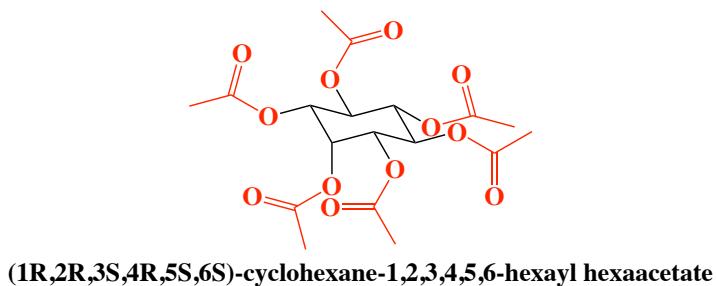
(1R,2S,3S,4S,5S,6S)-2,4,5,6-tetrahydroxycyclohexane-1,3-diyl dioctanoate and (1R,2S,3S,4S,5S,6S)-2,4,5,6-tetrahydroxycyclohexane-1,3-diyl didodecanoate were synthesized by Watanabe *et al.* and their synthetic scheme is shown in **Figure 1.54**.<sup>57</sup>



**Figure 1.54:** Scheme for the preparation of 1,3-diesters of *myo*-inositol

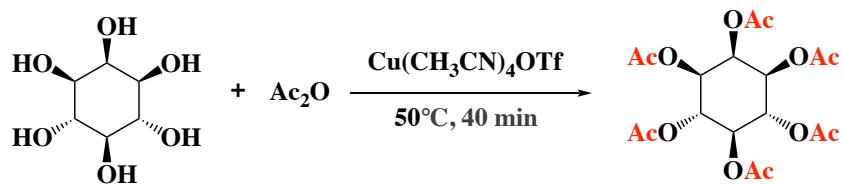
### 1.8.3 Hexaesters

One hexaester of *myo*-inositol has been reported (**Figure 1.55**).



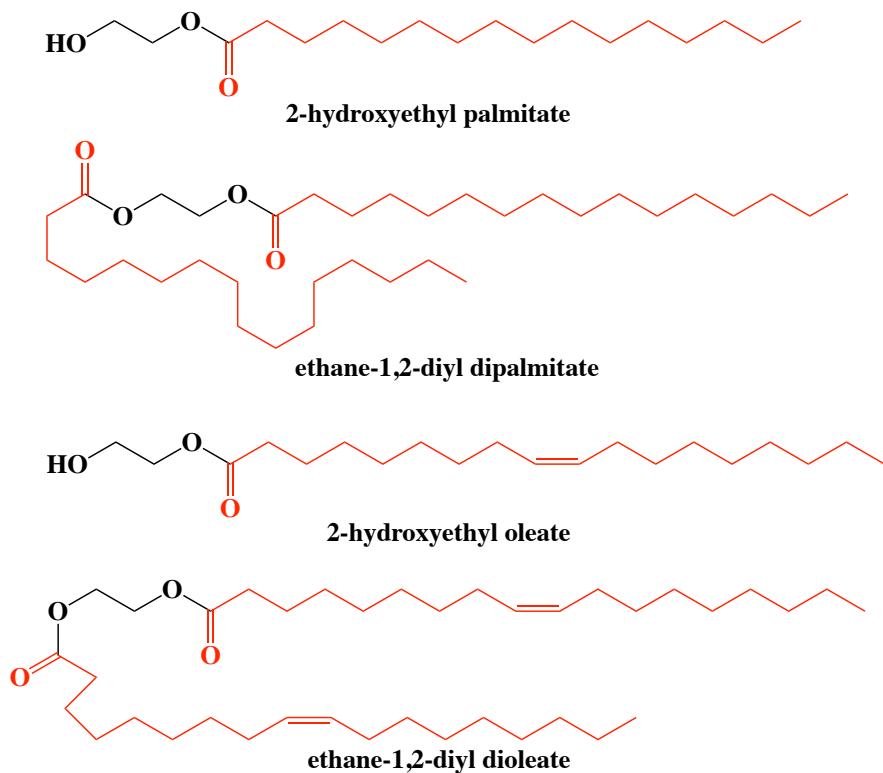
**Figure 1.55:** O-Acylated *myo*-inositol hexaester in scientific literature

(1R,2R,3S,4R,5S,6S)-cyclohexane-1,2,3,4,5,6-hexyl hexaacetate was synthesized by Mensah *et al.* by a simple and highly efficient synthetic method mediated by tetrakis(acetonitrile)copper(I) triflate ( $\text{Cu}(\text{CH}_3\text{CN})_4\text{OTf}$ ) catalyst (**Figure 1.56**).<sup>58</sup>



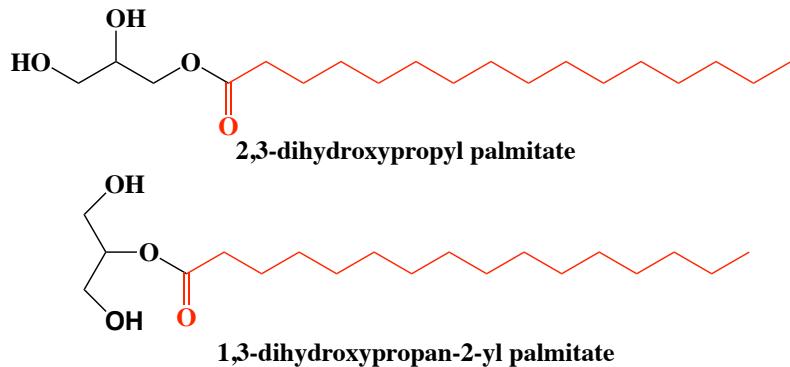
**Figure 1.56:** Scheme for the preparation of hexaacetate of *myo*-inositol

## 1.9 Miscellaneous long chain O-acylated polyol derivatives and their uses



**Figure 1.57:** Long chain ethylene glycol mono and diesters in scientific literature

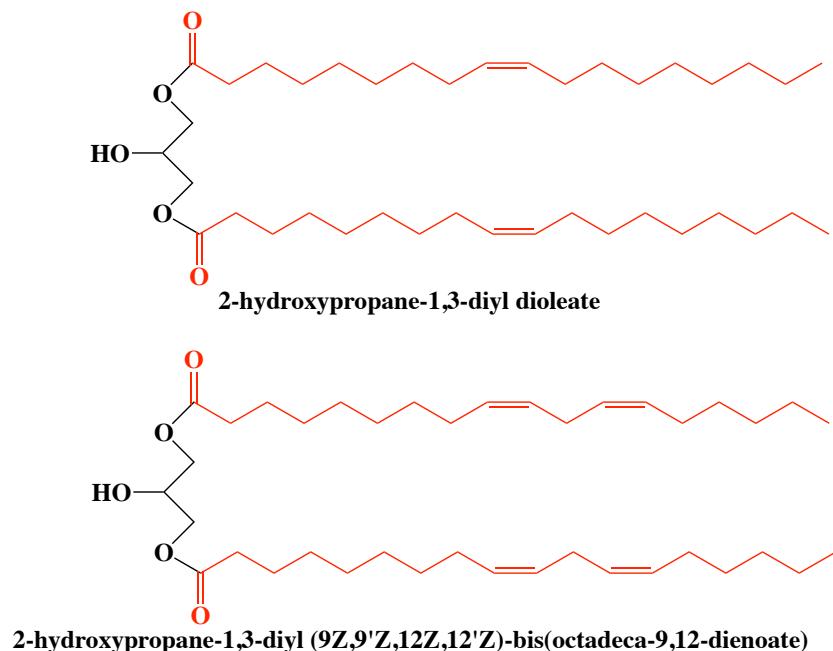
2-Hydroxyethyl palmitate, ethane-1,2-diyl dipalmitate, 2-hydroxyethyl oleate and ethane-1,2-diyl dioleate were synthesized by Stalmatis *et al.* research project as described in section 1.2.1 (page 4).<sup>6</sup> (Figure 1.57)



**Figure 1.58:** Long chain glycerol monoesters in scientific literature

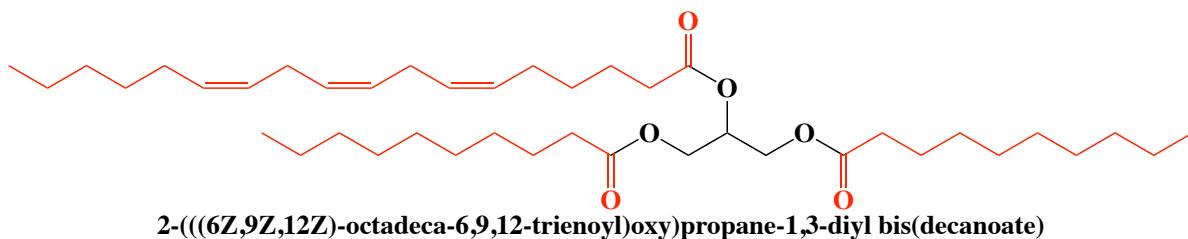
2,3-Dihydroxypropyl palmitate was synthesized by Boswinkel *et al.* as mentioned in section 1.4.1. (page 13).<sup>18</sup> (listed in **Figure 1.58**)

1,3-Dihydroxypropan-2-yl palmitate was synthesized by Lee *et al.* as previously mentioned in section 1.4.1. (page 12).<sup>14</sup> (listed in **Figure 1.58**)



**Figure 1.59:** Long chain glycerol diesters in scientific literature

2-Hydroxypropane-1,3-diyl dioleate and 2-hydroxypropane-1,3-diyl (9Z,9'Z,12Z,12'Z)-bis(octadeca-9,12-dienoate) were synthesized by Rosu *et al.* with other compounds as previously mentioned in section 1.4.2 last paragraph (page 16).<sup>37</sup> (listed in **Figure 1.59**)



**Figure 1.60:** Long chain glycerol triesters in scientific literature

2-(((6Z,9Z,12Z)-octadeca-6,9,12-trienoyl)oxy)propane-1,3-diyl bis(decanoate) was synthesized by Harbize *et al.* with the aim of therapeutic use for the treatment of neurodegenerative disease (**Figure 1.60**).<sup>50</sup>

### 1.10 Commercial availability of O-acylated derivatives

While not all of the molecules discussed in previous sections are commercially available, majority of them are. Here is the list of commercial vendors where compounds are available with price.

- BLD USA
- A2B Chem Product list
- Ambeed Inc. Product List
- Aurora building Blocks 3
- Aurora Building Blocks 4
- Aurora Building Blocks 6
- Aurora Building Blocks 8
- Aurora Building Blocks 11
- LabNetwork Compounds
- Atomax Chemicals Product List
- Enamine Stocks Building Blocks
- Alfa Chemistry Product List
- Hong Kong Chemhere Product list

### 1.11 Opportunities of O-acylated polyol derivatives as a keto diet supplement

When the human body consumes a high fat low carbohydrate diet, which is generally known as a keto diet, it shunts to an alternative metabolic pathway from glycogenesis to lipolysis for energy production.<sup>60</sup> In this process, long chain fatty acid glycerides hydrolyze enzymatically to release glycerol and free fatty acids. Glycerol goes through a metabolic pathway called gluconeogenesis, to produce glucose which is not sufficiently abundant to satisfy the bodily needs while on the ‘keto diet’. Free fatty acids go through a process called  $\beta$ -oxidation where

fatty acids enzymatically produce S-acetyl CoA by oxidizing fatty acid chain to a carbonyl group in the  $\beta$  position, then cleaving it off from the original structure. This S-acetyl CoA goes through Krebs cycle to produce energy in our body, which becomes primary energy supply while we are on the ‘Keto diet’.

### 1.12 Conclusion

There are numerous numbers of O-acylated polyol derivatives have been reported in the scientific and patent literature. A number of molecules have been synthesized in the laboratory set up for the invention of a new and efficient route for the synthesis of the molecules. A number of them were synthesized to evaluate functionality of new biological and non-biological catalysts, even only to measure the kinetic rate of the reaction. These molecules also contain a broad range of industrial and physiological applications that incorporates their use as emulsifiers, surfactants, conditioners, thickeners, temperature stabilizers, chemical stabilizers, opacifiers, solvents, gelling agents, wetting agents, corrosion inhibitors, lubricants, antimicrobials or defoamers, prodrug for cancer treatment and other neurological disorders. Unfortunately, none of these compounds have been tested for their use of as a ‘ketogenic molecule’.

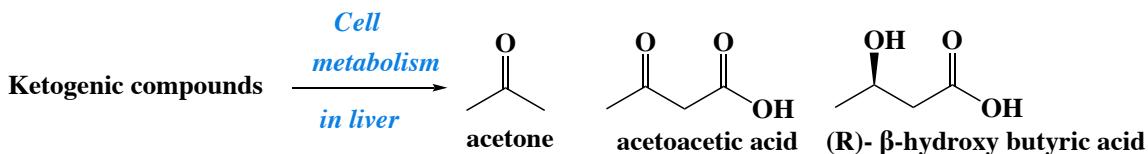
As mentioned earlier, medium chain triglycerides are considered to sustain more ketogenic properties, which is not as abundant as long chain glycerides we get from our dietary foods. O-Acylated medium chain polyol derivatives might be a great class of molecules to consider their potential use as a “keto diet” supplement. This research project is an effort to innovate synthetic route for the potential ketogenic molecules and test their ketogenic activity in laboratory experiments and biological trials. If this research project becomes successful, it might open up a new domain of thinking for the chemists and physiologists to continue further

investigation about these molecules of their other potential uses and addition of the new molecules in these class to enrich the library of ketogenic compounds.

## Chapter 2: Synthesis and Characterization of Potential Ketogenic Compounds

### 2.1 Introduction

Dietary fasting has been practiced in various religions for over thousands of years. For the past hundreds of years, dietary fasting has become popular among physicians for its practical use for the treatment of epileptic seizures.<sup>60-61</sup> At the same period of time, research has been conducted to manage diabetes and a high-fat, low-carbohydrate diet has been proposed<sup>62-64</sup> because of a diabetic patient's inability to catabolize glucose at a rate fast enough to satisfy calorific needs in the body.<sup>65</sup> Research has indicated that diabetes patients on a low-fat, high-carbohydrate diet get an increase of (R)- $\beta$ -hydroxybutyric acid and acetone in their blood (ketonemia) and urine (ketourea).<sup>66</sup> Another research study showed that modification of a diabetic patients' diet could be accomplished based on a relationship between acetoacetic acid and glucose in the blood.<sup>67</sup> Three molecules, namely acetone, (R)- $\beta$ -hydroxybutyric acid and acetoacetic acid are collectively known as "Ketone Bodies". Ketogenic compounds are molecules that can be catabolized in the body to release ketone bodies (**Figure 2.1**).



**Figure 2.1:** Ketogenic compounds

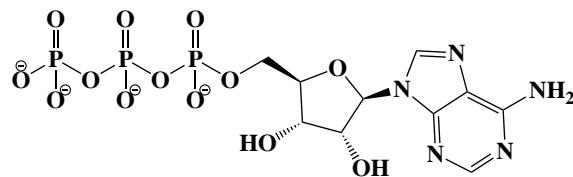
Physiologic ketosis is a normal response to low level glucose supply from a low carbohydrate diet or prolonged fasting, where ketone bodies serve as an energy source for the

brain. Normal diet provides a ketone body concentration of less than 0.3 mM in blood and urine, while this concentration can elevate up to 0.5 to 3 mM in dietary ketosis without any adverse effect in the body.<sup>68</sup>

As previously mentioned in **Chapter 1**, dietary ketosis produces ketone bodies upon enzymatic breakdown of long chain triglycerides (LCT), whereas medium chain triglycerides (MCT) are considered to contain more ketogenic capabilities as they do not require an active transport into cells and can be absorbed into the liver directly. These triglycerides are made up of a glycerol moiety with fatty acid groups attached to the hydroxyl groups. Fatty acid groups are the metabolic precursor of the ketone bodies, while glycerol acts as a carrier without directly participating in ketone body production. With that in mind, synthetic ketogenic compounds may be an effective dietary supplement. In this chapter, previously synthesized ketogenic compounds and new synthesis for novel potential ketogenic compounds are investigated.

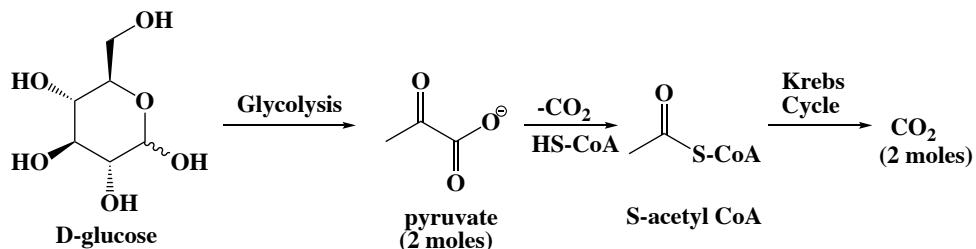
## 2.2 Biochemistry of the ketogenic diet

In the normal diet when carbohydrates are abundant, glucose is the main energy source of the body. Glucose present in the blood goes through a metabolic pathway called glycolysis, which produces pyruvate and adenosine triphosphate (ATP), the energy source of cells. (**Figure 2.2**).<sup>69</sup>



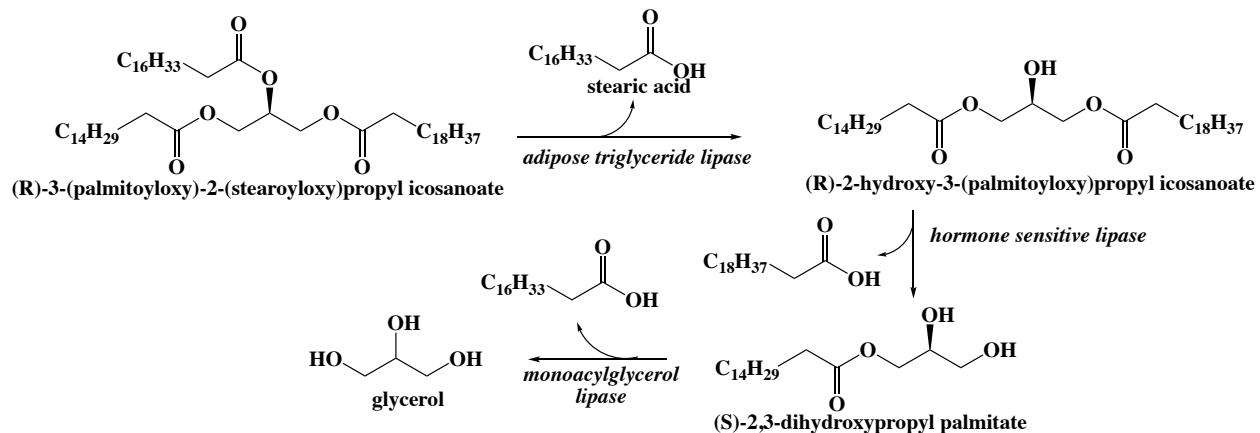
**Figure 2.2:** Adenosine triphosphate (ATP)

This pyruvate converts into S-acetyl Coenzyme A which goes into another metabolic pathway called the Krebs cycle which is the primary energy production route for most of the cells in our body (**Figure 2.3**).



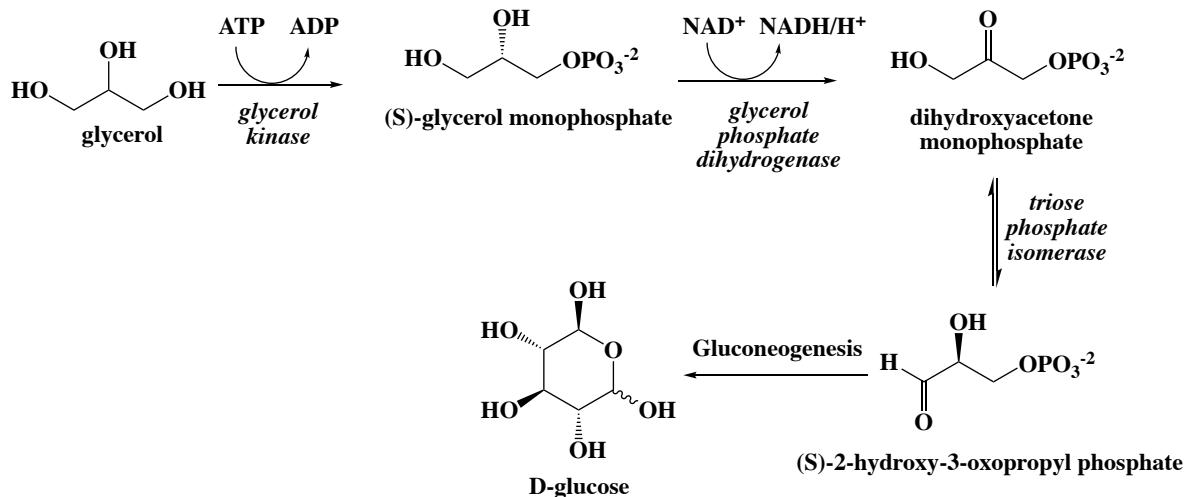
**Figure 2.3:** Metabolism of Glucose

When D-glucose is not available, triacylglycerides in the adipose cells and in the diet break down through lipolysis.<sup>70</sup> A schematic presentation of that process is depicted in **Figure 2.4**.



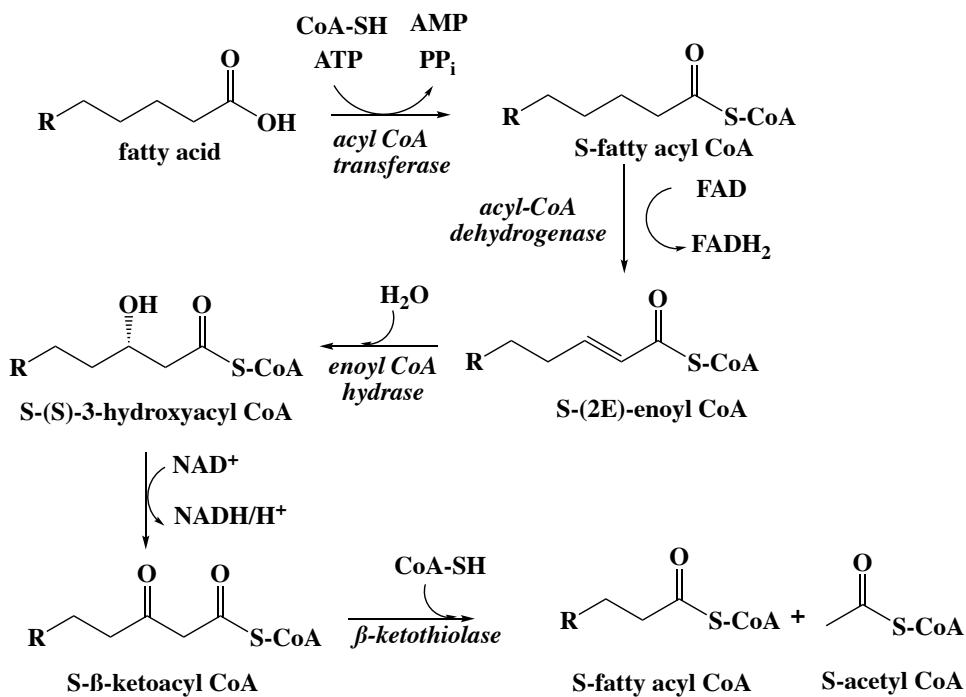
**Figure 2.4:** Lipolysis of triacylglycerides

Glycerol generated from this process goes through another enzymatic metabolic pathway, called gluconeogenesis, to produce glucose (**Figure 2.5**). However, this ‘new’ glucose is not sufficient to produce energy necessary for the body.



**Figure 2.5:** Process of glycerol conversion to glucose

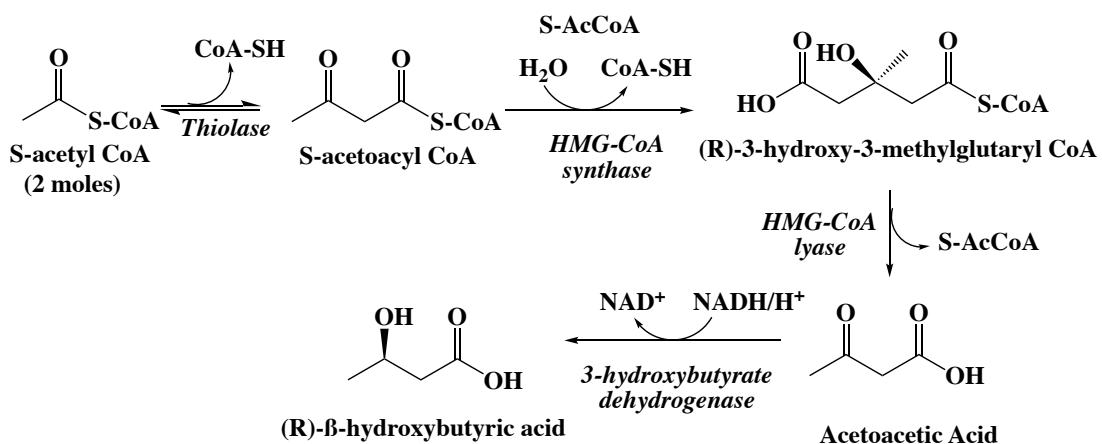
In this case, body shunts into an alternative pathway by breaking down fatty acid released from the lipolysis to produce S-acetyl CoA. A schematic presentation of  $\beta$ -oxidation of fatty acids is depicted in Figure 2.6.



**Figure 2.6:**  $\beta$ -Oxidation of fatty acids

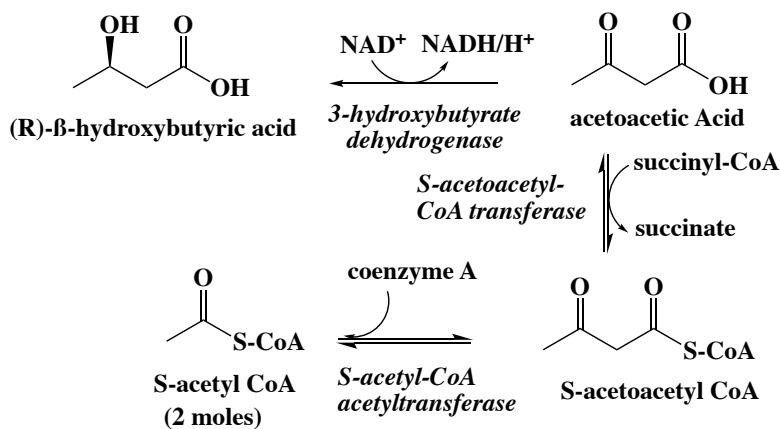
In this process, fatty acids are oxidized by flavin adenine dinucleotide (FAD) and nicotinamide adenine dinucleotide ( $\text{NAD}^+$ ) in the  $\beta$ -position in the S-acyl CoA chain, that is why this process is called  $\beta$ -oxidation. S-Acetyl CoA is released upon addition of CoA-SH and the acyl chain length becomes two carbons shorter. This S-Acetyl CoA goes in the Krebs cycle for energy production.

Fatty acids cannot cross the blood-brain barrier and because of that the brain cannot directly use fatty acids. This restriction is overcome by converting S-acetyl CoA to water soluble ketone bodies, which can be transported by blood, cross the blood-brain barrier and be used by neural cells as a source of energy. The process for the production of ketone bodies is called ketogenesis, which is depicted in **Figure 2.7**.



**Figure 2.7:** Ketogenesis for the production of ketone bodies

These ketone bodies can be transported by the blood and absorbed in different cells of the body where they can be converted back to S-acetyl CoA through a process called ketolysis (Figure 2.8). This S-acetyl CoA can enter into the Krebs cycle to produce energy in the form of ATP.



**Figure 2.8:** Ketolysis of the ketone bodies

### 2.3 Advantages of Ketogenic diet

High-fat diets have been implemented since the 1920's for a variety of human ailments. The modern ketogenic diet is considered to consist of a 4:1 ratio of fats to carbohydrates along with proteins, which may contain a maximum of 50 grams of carbohydrates per day.<sup>69</sup> Ketogenic diets have a long history of treatment for epileptic seizures.<sup>71</sup> Research have shown that ketogenic diets significantly reduce the number of occurrences of epileptic seizures, rate of occurrence and even make the patient completely seizure-free in some cases.<sup>72-79</sup> Continuation of research has shown that ketogenic diets have benefits more than reduction of epileptic seizures such as a tool for weight loss.<sup>80</sup> Ketogenic diet can improve insulin resistance in Type 2 diabetes, even reverse insulin resistance in cases where people are at risk of becoming diabetic.<sup>81-82</sup> Insulin serves as an inhibitor for lipolysis, β-oxidation of fatty acids and overall production of ketone bodies to an unsafe level which termed as ‘diabetic ketoacidosis’ where ketone bodies concentration rises above 10 mM.<sup>83</sup> When insulin resistance of the body increases, glucose conversion to triglycerides also increases, which may cause cardiovascular diseases.<sup>84-85</sup> Ketogenic diets my also lower the concentration of cholesterol and triglycerides in the blood of

people suffering from obesity, to reduce the risk of cardiovascular diseases.<sup>86-87</sup> Other physiological effects of a ketogenic diet are on neurological disorders.<sup>88-90</sup> Four weeks of strict ketogenic diet improves the score on the Unified Parkinson Disease Scale of people suffering from Parkinson disease,<sup>91</sup> and reduces the concentration of  $\beta$ -amyloid in the brain in mice models, which is considered as a risk factor for Alzheimer disease.<sup>92</sup> Further research has shown most that cancer cells consume glucose at a higher rate than normal cells<sup>94-95</sup> and increases the rate of glycolysis to enhance proliferation of cancer cells.<sup>96</sup> Ketogenic diets have shown inhibitory effects on the growth of tumor cells and have enhanced the effectiveness of radiation treatment in mice cancer models.<sup>97-99</sup> No clinical trial on humans, however, have been carried out.<sup>100-101</sup>

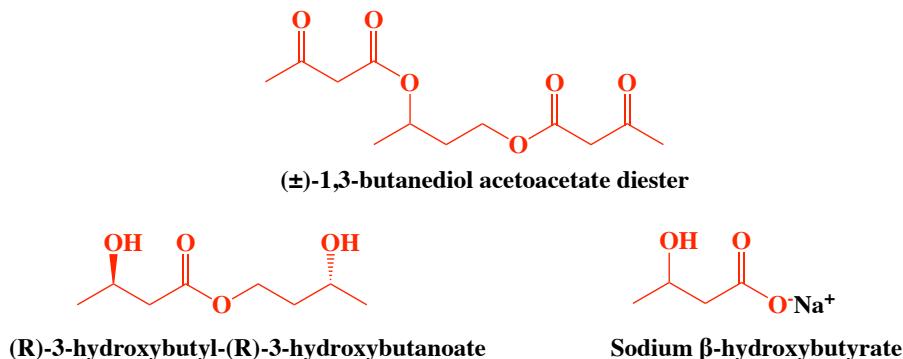
## **2.4 Advantages of synthetic ketogenic molecules**

There are numbers of potential account for developing synthetic ketogenic molecules as a dietary supplement rather than completely to a ketogenic diet. First, the ketogenic compounds can be rigorously purified and measured out for closer clinical analysis. This ensures a better regulation of intake and physiological observation of possible ketogenic effects. Ketogenic supplements may also avoid the use of other dietary components that may be deleterious, particularly carbohydrates. Traditionally keto diets include proteins, for instance, which are glucogenic and may alter the blood concentrations of glucose. Finally, ketogenic supplements may increase the diversity of available ketogenic substances for healthy foods and supplements.

## 2.5 Previous research on synthetic ketogenic compounds

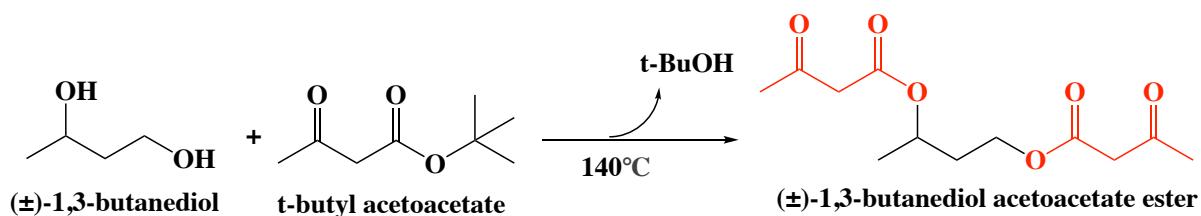
Three compounds have been investigated so far and shown to increase the concentration of ketone bodies in the blood to the level of dietary ketosis without switching into a ketogenic diet.

These compounds are listed below in **Figure 2.9**:



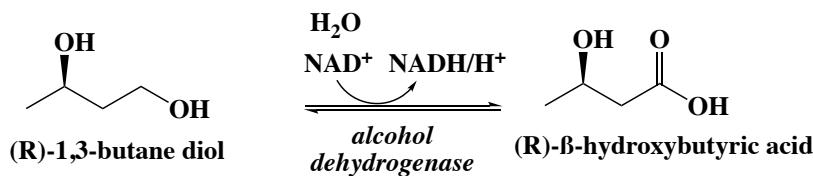
**Figure 2.9:** Synthetic ketogenic compounds

In 1995,  $(\pm)$ -1,3-butanediol acetoacetate diester was synthesized with the reaction of  $(\pm)$ -1,3-butanediol and t-butyl acetoacetate ester. The scheme for the reaction is depicted in **Figure 2.10**.<sup>102</sup>



**Figure 2.10:** Scheme for the preparation of  $(\pm)$ -1,3-butanediol acetoacetate diester

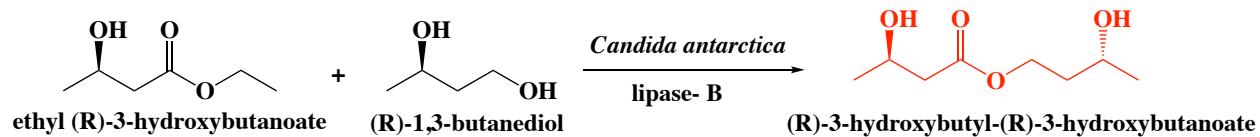
$(\pm)$ -1,3-Butanediol acetoacetate diester can be metabolically hydrolyzed to produce acetoacetic acid and  $(\pm)$ -1,3-butanediol. The R-enantiomer of 1,3-butanediol was shown to be oxidized to (R)- $\beta$ -hydroxybutyric acid in pigs and rats.<sup>103-104</sup> The metabolic pathway of oxidation of (R)-1,3-butanediol is shown in **Figure 2.11**.



**Figure 2.11:** Oxidation of (R)-1,3-butane diol in the liver

However, in perfused rat liver, S-enantiomer has been shown to increase ketone body concentrations, but its metabolic pathway remains undetermined.<sup>105</sup>

(R)-3-Hydroxybutyl-(R)-3-hydroxybutanoate was synthesized in 2010 as a drug and food additive via a *Candida antarctica* lipase-mediated transesterification reaction.<sup>106</sup> Ethyl-(R)-3-hydroxybutanoate and (R)-1,3-butanediol were used as starting materials to produce the product as depicted in **Figure 2.12**.



**Figure 2.12:** Scheme for the preparation of (R)-3-hydroxybutyl-(R)-3-hydroxybutanoate

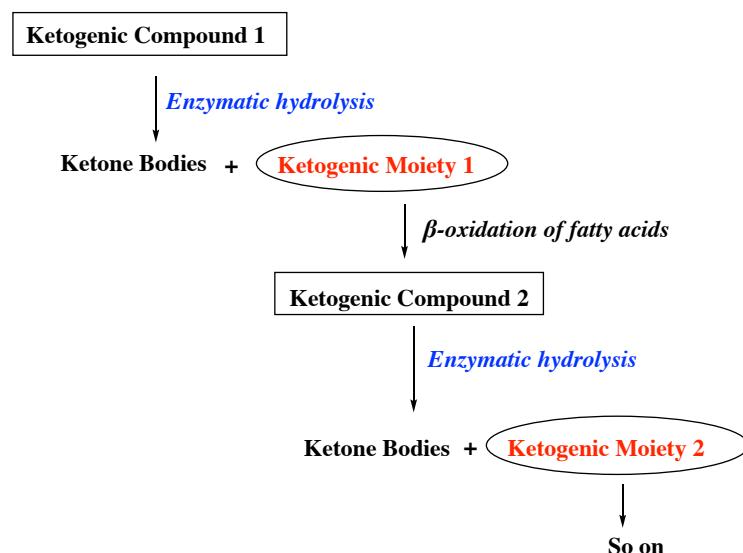
Research conducted in rat liver showed that (R)-3-hydroxybutyl-(R)-3-hydroxybutanoate metabolically hydrolyzes to (R)- $\beta$ -hydroxybutyric acid and (R)-1,3-butanediol. This (R)-1,3-butanediol can be oxidized to produce another equivalent of (R)- $\beta$ -hydroxybutyric acid.<sup>107</sup> Further research showed that (R)-3-hydroxybutyl-(R)-3-hydroxybutanoate induces ketosis in adult human upon daily administration for 28 days.<sup>108-109</sup>

The set of compounds tested for ketogenicity so far are the mineral salts of  $\beta$ -hydroxybutyrate, most commonly sodium and calcium. However, along with ketosis, adverse gastrointestinal effects have been noted due to the high concentration sodium cation.<sup>110</sup>

## 2.6 Synthesis and characterization of novel ketogenic compounds

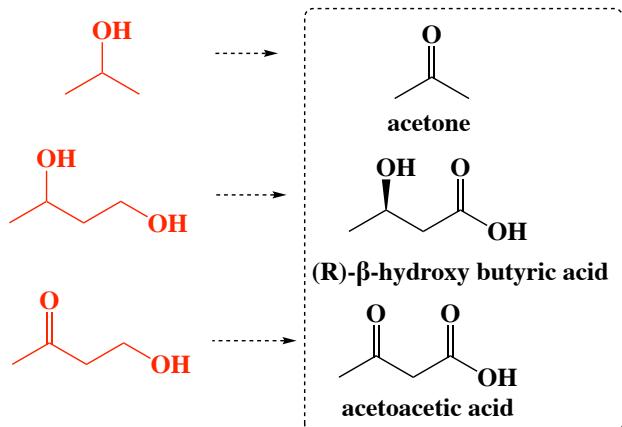
### 2.6.1 Ketogenic molecule design

While considering an over-arching design for new synthetic ketogenic compounds, a general thought process was followed which is applied to design for most of the prodrugs.<sup>111</sup> Most common prodrugs are synthesized having that in mind the parent molecule should have functional groups that are amenable to chemical prodrug derivatization. Moieties attached to the parent molecule should be safe and rapidly excreted from the body. The absorption, distribution, metabolism and excretion (ADME) should be well understood. Most common functional groups that are chosen to design bioactive molecule are carboxylic, hydroxyl, phosphate, amine and carbonyl groups due to their susceptibility to enzyme-mediated hydrolysis. Similarly, ketogenic compounds have to be in a structural format that can be enzymatically hydrolyzed to release ketone bodies or a suitable precursor to ketone bodies, that can further release ketone bodies via enzymatic transformation. Common functional groups such as esters work as a good linkage for the bioactive component and have been widely used for the design of bioactive compounds. A typical roadmap used to design new ketogenic molecules is outlined in **Figure 2.13.**



**Figure 2.13:** General design for new ketogenic molecules

In our cases, 2-propanol, 1,3-butanediol or 4-hydroxy-2-butanone were considered initial ketogenic building blocks (**Figure 2.14**).



**Figure 2.14:** Simplified hypothesis for new synthetic ketogenic compounds

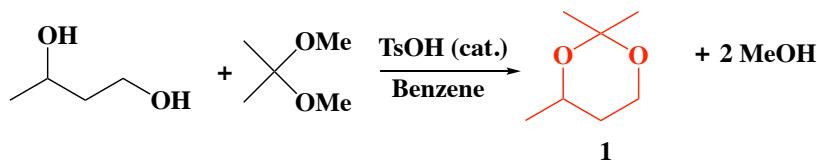
### 2.6.2 Materials for ketogenic compounds

All reagents were purchased from commercial vendors, either Fisher Scientific, Sigma-Aldrich, Oakwood Chemical or TCI Chemical Company. All solvents were purchased from Fisher Scientific Company. Thin layer chromatography (TLC) was carried out using SiO<sub>2</sub>-60, F-254 Merck plates. Flash column chromatography were performed using Dynamic Adsorbents Inc. flash chromatography silica gel (32-63μm). NMR spectroscopy was performed using a Varian Inova 400 MHz spectrometer, Bruker Neo 400 MHz spectrometer or Bruker Neo 600 MHz spectrometer. <sup>1</sup>H NMR (400 MHz, 600 MHz) and <sup>13</sup>C NMR (101 MHz, 151 MHz) spectra of samples in Chloroform-*d* (CDCl<sub>3</sub>). Chemical shifts were reported relative to CDCl<sub>3</sub> at 7.26 ppm for <sup>1</sup>H NMR and 77.00 ppm for <sup>13</sup>C NMR. Mass spectrometry was performed in Agilent LC-MS QTOF 6540 HRMS (ESI-TOF).

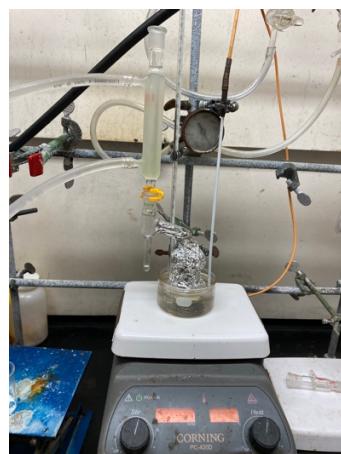
## 2.6.3 Experimental procedures for synthetic ketogenic compounds

### 2.6.3.1 Synthesis of cyclic acetal derivatives

#### 2.6.3.1.1 Synthesis of 2,2,4-trimethyl-1,3-dioxane (1)



Following the method<sup>112</sup> developed by Smith *et al.*, ( $\pm$ )-1,3-butanediol (8.9 mL, 0.1 mol) and 2,2-dimethoxypropane (12.249 mL, 0.1 mol) were dissolved in 20 mL of benzene. p-Toluene sulfonic acid, monohydrate (0.277g, 0.0014 mol) was added to the mixture. A Dean-Stark distillation condenser was connected to the flask and the mixture was heated to 70°C. Approximately 3 mL of methanol was collected in 30 minutes. The experimental set up was changed to a regular distillation and the reaction temperature was elevated to 130°C. 6.24 ml of 1 was collected as a yellow oil in 3 hours at a distillation head temperature of 117°C with a yield of 48%.



**Figure 2.15:** Experimental set up for the synthesis of 1

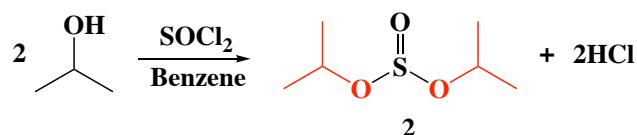
<sup>1</sup>H NMR (400 MHz, Chloroform-*d*) δ 3.90 (m, 2H), 3.75 (m, 1H) 1.50 (ddd, *J*= 5.9, 12.4, 12.4 Hz, 2H), 1.39 (s, 3H), 1.32 (s, 3H), 1.10 (d, *J*= 6.4 Hz, 3H)

<sup>13</sup>C NMR (151 MHz, Chloroform-*d*) δ 98.1, 65.0, 59.9, 32.9, 30.0, 22.3, 19.2

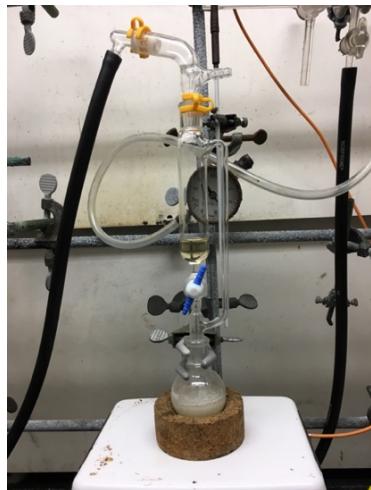
### **2.6.3.2 Synthesis of sulfite derivatives**

Sulfite derivatives of ketogenic compounds were prepared from the reaction of thionyl chloride and either 2-propanol, ( $\pm$ )-1,3-butanediol or 4-hydroxy-2-butanone in different equivalences and reaction conditions.

#### **2.6.3.2.1 Synthesis of diisopropyl sulfite (2)**



Following a method<sup>113</sup> reported by King *et al.*, 2-Propanol (17 mL, 0.22 mol) was added into a 100 mL round bottom flask and 10 mL of benzene was added. Thionyl chloride (7.3 mL, 0.10 mol) was added dropwise from a 25 mL dropping funnel under inert conditions over 25 minutes. The reaction was allowed to stir at room temperature for 5.5 hours. The solvent was evaporated by rotary evaporator. The resulting oil was dissolved in 15 mL of dichloromethane (DCM) and washed two times with water (5 mL) followed by once with 5% sodium bicarbonate solution (5 mL). The organic solvent was dried over anhydrous magnesium sulfate and then removed by rotary evaporation. To remove unknown black impurities, a distillation at ~10 mm of Hg pressure and 78°C was performed to give 9.13g of **2** as a clear oil in 55% yield.

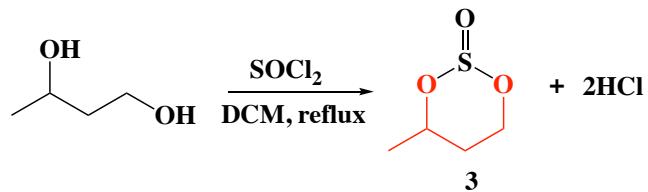


**Figure 2.16:** Experimental set up for the synthesis of **2**

<sup>1</sup>H NMR (400 MHz, Chloroform-*d*) δ 4.75 (septet, *J*= 6.4 Hz, 2H), 1.31 (d, *J*= 6.2 Hz, 6H), 1.28 (d, *J*= 6.4 Hz, 6H)

<sup>13</sup>C NMR (151 MHz, Chloroform-*d*) δ 68.0, 23.5

#### 2.6.3.2.2 Synthesis of 4-methyl-1,3,2-dioxathiane-2-oxide (3)



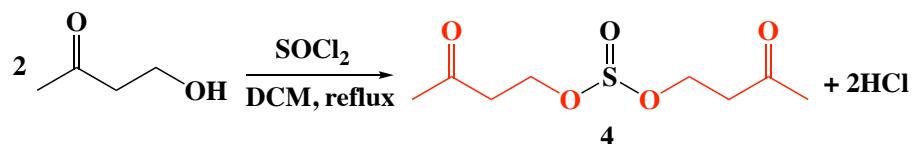
(±)-1,3-Butanediol (9 mL, 0.1 mol) was added into a 500 mL round bottom flask and dissolved in 70 mL DCM. The mixture was cooled to 0°C in an ice-bath while stirring. Thionyl chloride (7.3 mL, 0.1 mol) was added dropwise from a 25 mL dropping funnel under inert conditions. The reaction was allowed to stir at 40°C under reflux for 6 hours. The solution was washed two times with water (20 mL) and one time with 5% sodium bicarbonate solution (5 mL). The organic solvent was dried over anhydrous magnesium sulfate, filtered and removed using rotary

evaporator. To remove unknown black impurities the product was distilled at 60 mm Hg pressure and 98°C. 4.35g of **3** was collected as a distillate as a clear colorless oil in 32% yield.

<sup>1</sup>H NMR (600 MHz, Chloroform-*d*) δ 5.02 (tq, *J*= 2.4, 6.0 Hz, 1H), 4.89 (dt, *J*= 2.4, 12.6 Hz, 1H), 3.81 (ddd, *J*= 2.4, 3.6, 8.4 Hz, 1H), 2.11 (ddd, *J*= 4.8, 12.6, 13.2 Hz, 1H), 1.6 (ddd, *J*= 2.4, 3.0, 14.1 Hz, 1H), 1.20 (d, *J*= 6.6 Hz, 3H)

<sup>13</sup>C NMR (151 MHz, Chloroform-*d*) δ 64.2, 57.5, 33.2, 21.1

#### **2.6.3.2.3 Synthesis of bis-(3-oxobutyl) sulfite (4)**

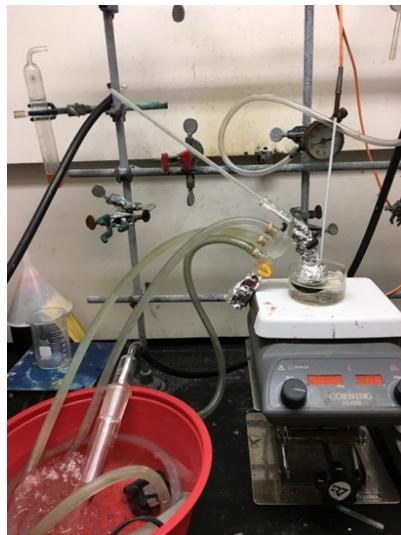


4-Hydroxy-2-butanone (1.7 mL, 0.02 mol) was added into a 25 mL round bottom flask and 5 mL of DCM was added. The solution was cooled to 0°C in an ice-bath while stirring. Thionyl chloride (2 mL, 0.01 mol) was added dropwise using a 2 mL syringe. The reaction was allowed to stir at 40°C under reflux condition for 6 hours. The reaction was rinsed with two partitions of 4 mL of water and three partitions of 2 mL of 5% sodium bicarbonate solution.

The solvent was dried over anhydrous magnesium sulfate, filtered and removed using rotary evaporation. Remaining crude product was purified by distillation at 30 mm Hg pressure and 55°C, which gave 0.49 g of **4** in a 22% yield.

<sup>1</sup>H NMR (400 MHz, Chloroform-*d*) δ 3.69 (t, *J*= 6.6 Hz, 4H), 2.89 (t, *J*= 6.4 Hz, 4H), 2.17 (s, 6H)

<sup>13</sup>C NMR (151 MHz, Chloroform-*d*) δ 205.2, 45.2, 38.2, 30.2



**Figure 2.17:** Experimental set up for the purification of 4

Limitation of 4: Compound turned black within 2 months.

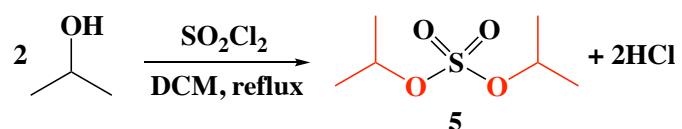


**Figure 2.18:** Appearance of 4 within 2 months

### **2.6.3.3 Synthesis of sulfate derivatives**

Sulfate derivatives of potential ketogenic compounds were prepared from the reaction of sulfonyl chloride and either 2-propanol or ( $\pm$ )-1,3-butanediol in different equivalence and reaction conditions.

#### **2.6.3.3.1 Synthesis of diisopropyl sulfate (5)**



2-Propanol (8.5 mL, 0.11 mol) was added into a 50 mL round bottom flask and 15 mL of DCM was added. the solution was cooled to 0°C in an ice-bath while stirring. Sulfuryl chloride (4.05 mL, 0.05 mol) was added dropwise using a 6 mL syringe. The reaction was stirred for 3 hours at 40°C under reflux condition. The solution was washed with two partition of 10 mL of water and one partition of 5% aqueous sodium bicarbonate solution. The solvent was filtered and removed by rotary evaporation, leaving 2.49 g of **5** in 27% yield.

<sup>1</sup>H NMR (600 MHz, Chloroform-*d*) δ 4.81 (septet, *J*= 6.0 Hz, 2H), 1.35 (d, *J*= 6.3 Hz, 12H)

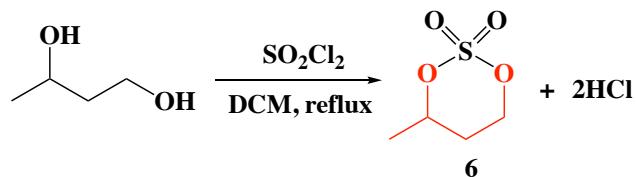
<sup>13</sup>C NMR (151 MHz, Chloroform-*d*) δ 79.8, 22.5

Limitation of **5**: Compound turned black within 2 months.



**Figure 2.19:** Appearance of **5** within 2 months

#### 2.6.3.3.2 Synthesis of 4-methyl-1,3,2-dioxathiane-2,2-dioxide (6)



(±)-1,3-Butanediol (0.89 mL, 0.01 mol) was added into a 25 mL round bottom flask and 6.5 mL of DCM was taken. The solution was cooled to 0°C in an ice-bath while stirring. Sulfuryl chloride (0.80 mL, 0.01 mol) was added dropwise using a 1 mL syringe. The reaction was allowed to stir at 40°C under reflux condition for 12 hours. The solution was washed with two

partitions of 4 mL of water and one partition of 5% aqueous sodium bicarbonate solution. The solvent was dried over anhydrous magnesium chloride, filtered and evaporated using rotary evaporator. The product was purified by flash column chromatography on silica gel using 1:9 ethyl acetate: hexane to give 0.68g of **6** in as clear liquid in 44% yield.

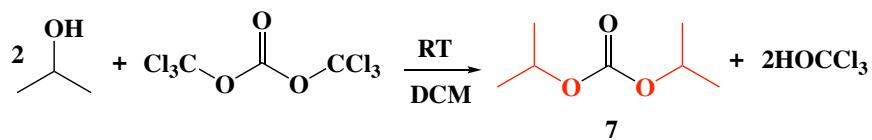
<sup>1</sup>H NMR (600 MHz, Chloroform-*d*) δ 4.68 (m, 1H), 4.62 (m, 1H), 4.11 (tq, *J*= 3.6, 3.6 Hz, 1H), 2.26 (m, 1H), 2.02 (m, 1H), 1.54 (d, *J*= 6.6 Hz, 3H)

<sup>13</sup>C NMR (151 MHz, Chloroform-*d*) δ 73.6, 53.2, 38.4, 25.3

#### **2.6.3.4 Synthesis of carbonate derivatives**

Carbonate derivatives can be hydrolyzed in the body by different esterase and a good candidate to potential ketogenic compounds. Carbonate derivatives were prepared from the reaction of triphosgene and either 2-propanol or ( $\pm$ )-1,3-butanediol in different equivalences and reaction conditions.

##### **2.6.3.4.1 Synthesis of diisopropyl carbonate (7)**

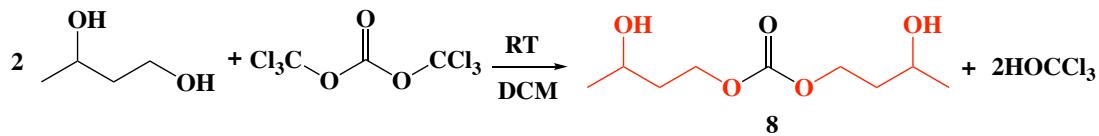


Following a method described by Liu *et al.*<sup>114</sup>, 2-propanol (2 mL, 0.02 mol) was added into a 25 mL round bottom flask and 5 mL of DCM was added. The solution was cooled to 0°C in an ice-bath while stirring. Triphosgene (3g, 0.015 mol) was slowly added while stirring in the ice-bath. After complete addition of triphosgene ice-bath was removed and reaction was continued for 6 hours at room temperature in inert condition. The solvent was rinsed with two partitions of 4 mL of water and one partition of 2 mL of 5% aqueous sodium bicarbonate solution, filtered and removed by rotary evaporator to give 1.1836 g of **7** in 81% yield.

<sup>1</sup>H NMR (600 MHz, Chloroform-*d*) δ 4.95 (septet, *J*= 6.1 Hz, 2H), 1.29 (d, *J*= 6.0 Hz, 12H)

<sup>13</sup>C NMR (151 MHz, Chloroform-*d*) δ 147.2, 75.3, 21.5

#### **2.6.3.4.2 Synthesis of bis-(3-hydroxy butyl) carbonate (8)**



(±)-1,3-Butanediol (2.09 mL, 0.02mol) was added into a 25 mL round bottom flask and 5 mL of DCM was added. The mixture was cooled to 0°C in an ice-bath while stirring. Triphosgene (2.96g, 0.01 mol) was slowly added while stirring in the ice-bath. After complete addition of triphosgene ice-bath was removed and reaction was continued for 24 hours at room temperature in inert condition. The solvent was washed with two partitions of 4 mL of water and one partition of 2 mL of 5% aqueous sodium bicarbonate solution, filtered and removed using rotary evaporation to give 0.93g of **8** in 45% yield.

<sup>1</sup>H NMR (600 MHz, Chloroform-*d*) δ 5.03 (m, 2H), 4.82 (m, 4H), 2.04 (m, 4H), 1.38 (d, *J*= 6.3 Hz, 6H)

<sup>13</sup>C NMR (151 MHz, Chloroform-*d*) δ 147.7, 74.8, 65.7, 34.1, 19.7

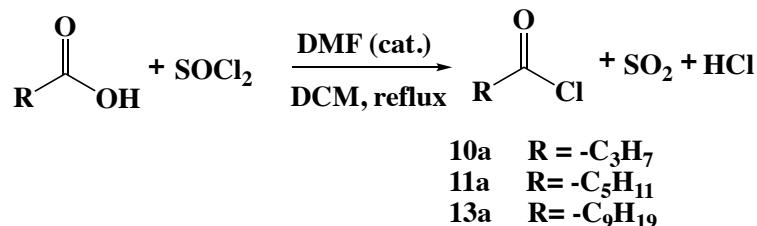
#### **2.6.3.5 Synthesis of ester derivatives**

Esters are the most common functional group used for prodrug design and approximately 49% of all marketed prodrugs are activated by enzymatic hydrolysis.<sup>115</sup> Esters mostly used to enhance passive membrane permeability with its lipophilicity, of water-soluble compounds<sup>116</sup>.

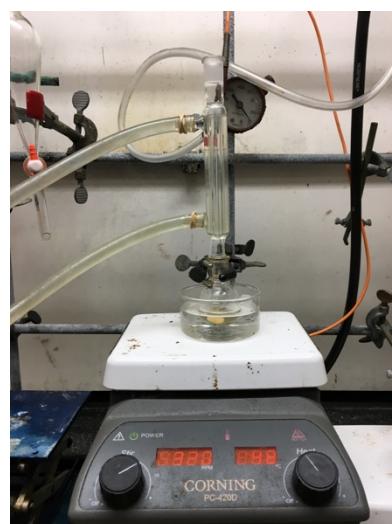
### **2.6.3.5.1 Synthesis of 3-oxo-butyl ester derivatives (9-13)**

An esterification method<sup>117</sup> used by Lin *et al.* is followed here with little modification by using thionyl chloride as an alternative to oxalyl chloride for the synthesis of potential ketogenic molecules.

**Scheme 2.1: Synthesis of acyl chloride**

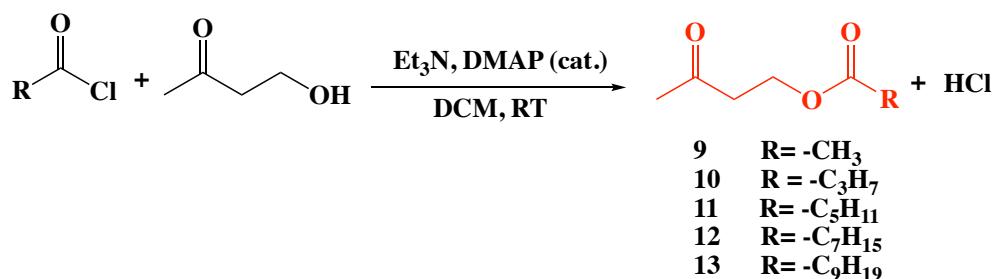


Carboxylic acids (1 equiv.) were dissolved individually in DCM in a 50 mL round bottom flask. A catalytic amount (0.03 equiv.) of *N,N'*-dimethylformamide (DMF) was added. Thionyl chloride (1 equiv.) was added dropwise from a 25 mL dropping funnel under nitrogen. The reactions were stirred at stirred at 40°C under reflux condition for 12 hours. The solvent was evaporated by rotary evaporation to give acyl chloride products with a yield of approximately above 90%.

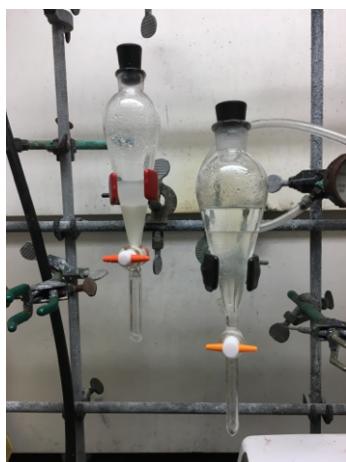


**Figure 2.20:** Experimental set up for the synthesis of **10a**

**Scheme 2.2: Synthesis of ester from acyl chloride**



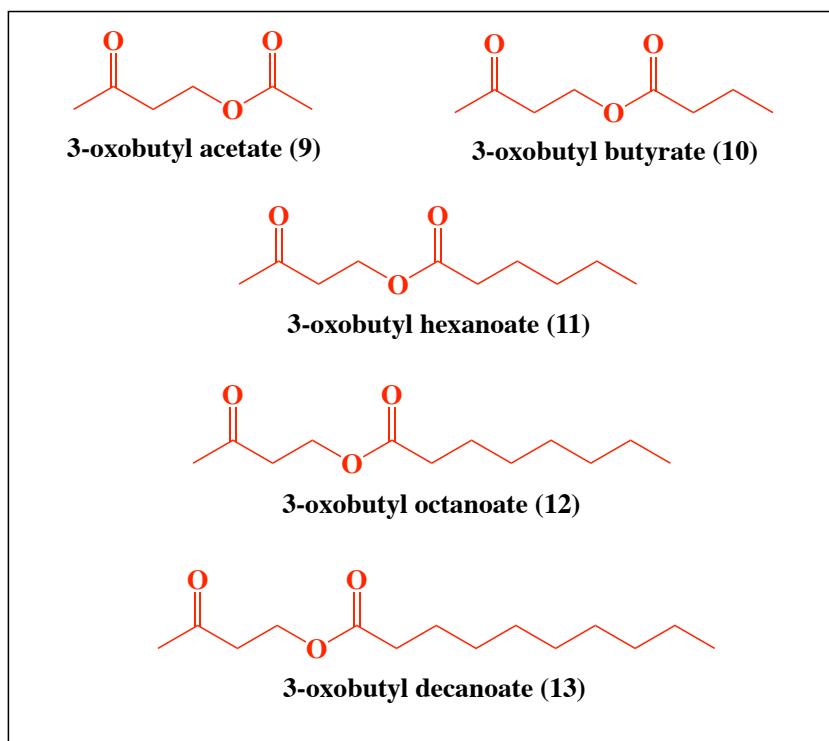
Acyl chlorides (1.2 equiv., 0.03 mol) were individually dissolved in 10 mL of DCM in a 50 mL round bottom flask. 4-Hydroxy-2-butanone (1 equiv., 0.025 mol) and a catalytic amount of *N,N'*-dimethylaminopyridine (DMAP, 0.3 mmol) was added and mixture was stirred for 10 minutes. Then solution was cooled to 0°C in an ice-bath while stirring. Triethylamine (TEA, 1.6 equiv., 0.04 mol) was dissolved in 5 mL of DCM and added dropwise to the solution from a 25 mL dropping funnel under inert condition while stirring the solution in ice-bath. The reaction was allowed to stir at room temperature for 24 hours. The solution was rinsed with two partitions of 6 mL of saturated ammonium chloride solution and three partitions of 9 mL of 5% aqueous ammonium bicarbonate solution. The solution was dried over anhydrous magnesium sulfate and filtered, then evaporated using rotary evaporation to give yellow oil in an approximate yield of 14-50%.



**Figure 2.21:** Experimental set up for the partitioning of **9** and **10**

**Table 2.1** Fatty acids used for 3-oxo-butyl ester syntheses

Fatty acid formula	IUPAC name	Product name	Product yield (Approximate)
CH <sub>3</sub> COOH	Acetic Acid	3-oxo-butyl acetate ( <b>9</b> )	33%
CH <sub>3</sub> (CH <sub>2</sub> ) <sub>2</sub> COOH	Butyric Acid	3-oxo-butyl butyrate ( <b>10</b> )	14%
CH <sub>3</sub> (CH <sub>2</sub> ) <sub>4</sub> COOH	Hexanoic Acid	3-oxo-butyl hexanoate ( <b>11</b> )	15%
CH <sub>3</sub> (CH <sub>2</sub> ) <sub>6</sub> COOH	Octanoic Acid	3-oxo-butyl octanoate ( <b>12</b> )	18%
CH <sub>3</sub> (CH <sub>2</sub> ) <sub>9</sub> COOH	Decanoic Acid	3-oxo-butyl decanoate ( <b>13</b> )	50%



**Figure 2.22:** Name and structure of the products synthesized from fatty acids

**3-oxo-butyl acetate (9):**

<sup>1</sup>H NMR (400 MHz, Chloroform-d) δ 4.30 (t, *J*= 6.2 Hz, 2H), 2.74 (t, *J*= 6.2 Hz, 2H), 2.16 (s, 3H), 2.01 (s, 3H)

<sup>13</sup>C NMR (151 MHz, Chloroform-d) δ 205.7, 170.8, 59.1, 42.0, 30.1, 20.8

**3-oxo-butyl butyrate (10):**

<sup>1</sup>H NMR (400 MHz, Chloroform-d) δ 4.27 (t, *J*= 6.4 Hz, 2H), 2.72 (t, *J*= 6.0 Hz, 2H), 2.22 (t, *J*= 7.8 Hz, 2H), 2.15 (s, 3H), 1.60 (m, 2H), 0.89 (t, *J*= 7.4 Hz, 3H)

<sup>13</sup>C NMR (151 MHz, Chloroform-d) δ 205.8, 173.5, 169.4, 59.0, 42.2, 37.0, 35.9, 30.2, 18.3, 17.7, 13.6

**3-oxo-butyl hexanoate (11):**

<sup>1</sup>H NMR (400 MHz, Chloroform-d) δ 4.25 (t, *J*= 6.4 Hz, 2H), 2.69 (t, *J*= 6.4 Hz, 2H), 2.37 (t, *J*= 7.6 Hz, 2H), 2.20 (t, *J*= 7.6 Hz, 2H), 2.12 (s, 3H), 1.57 (t, *J*= 6.8 Hz, 2H), 1.53 (t, *J*= 7.2 Hz, 2H), 1.26 (m, 8H), 0.83 (m, 6H)

<sup>13</sup>C NMR (151 MHz, Chloroform-d) δ 205.6, 178.6, 169.6, 59.0, 42.2, 35.2, 34.1, 31.2, 30.9, 30.2, 24.5, 23.8, 22.2, 13.8

**3-oxo-butyl octanoate (12):**

<sup>1</sup>H NMR (600 MHz, Chloroform-d) δ 4.26 (t, *J*= 6.6 Hz, 2H), 2.69 (t, *J*= 6.6 Hz, 2H), 2.37 (t, *J*= 7.7 Hz, 1H), 2.26 (t, *J*= 7.7 Hz, 1H), 2.20 (t, *J*= 7.8 Hz, 2H), 2.12 (s, 3H), 1.52 (m, 4H), 1.21 (m, 12H), 0.80 (m, 5H)

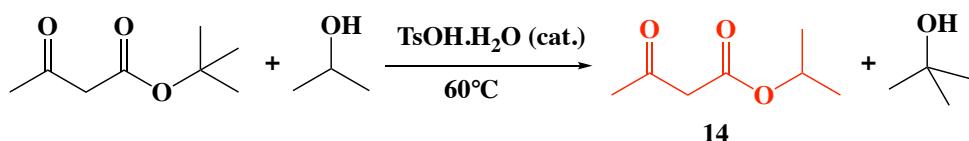
<sup>13</sup>C NMR (151 MHz, Chloroform-d) δ 205.8, 173.7, 59.0, 42.2, 34.1, 31.1, 30.2, 29.0, 28.8, 24.8, 22.5, 14.0

**3-oxo-butyl decanoate (13):**

<sup>1</sup>H NMR (600 MHz, Chloroform-d) δ 4.25 (t, *J*= 6.0 Hz, 2H), 2.70 (t, *J*= 6.0 Hz, 2H), 2.26 (t, *J*= 7.2 Hz, 2H), 2.20 (t, *J*= 7.8 Hz, 2H), 2.12 (s, 3H), 1.63 (m, 4H), 1.19 (m, 24H), 0.79 (t, *J*= 6.6 Hz, 6H)

<sup>13</sup>C NMR (151 MHz, Chloroform-d) δ 205.8, 179.5, 173.7, 59.0, 42.2, 34.1, 31.8, 29.3, 29.0, 24.8, 24.6, 22.6, 14.0

**2.6.3.5.2 Synthesis of isopropyl acetoacetate (14)**



t-Butyl acetoacetate (6.6 mL, 0.04 mol) was added into a 25 mL round bottom flask and 2-propanol (3.2 mL, 0.04 mol) was added. p-Toluene sulfonic acid.monohydrate (0.07 g, 0.4 mmol) was added. The reaction was allowed to stir for 8.5 hours at 60°C. The by product was collected in a Dean-Stark trap. 13 mL of DCM was added to the mixture and organic solvent was washed with 5% sodium bicarbonate solution and water. The solvent was removed by rotary evaporation to give 0.42 g of **14** as a clear liquid in a yield of 7.23%.

<sup>1</sup>H NMR (600 MHz, Chloroform-d) δ 4.99 (septet, *J*= 6.2 Hz, 1H), 3.35 (s, 2H), 2.20 (s, 3H), 1.18 (d, *J*= 6.5 Hz, 6H)

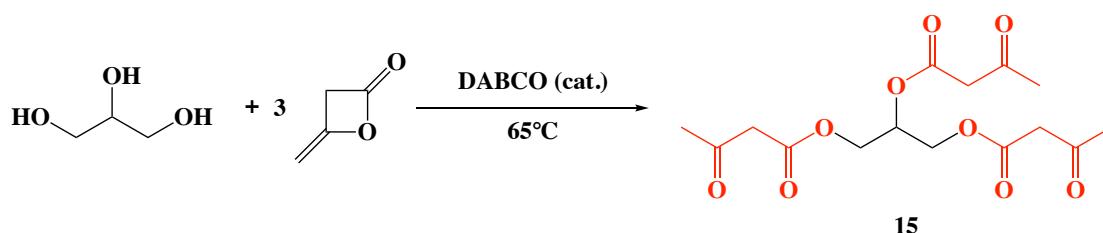
<sup>13</sup>C NMR (151 MHz, Chloroform-d) δ 200.8, 166.6, 68.9, 50.4, 30.0, 21.6

**2.6.3.6 Synthesis of acetoacetate esters derivatives of polyols**

As previously mentioned in section 2.5, ( $\pm$ )-1,3-butanediol acetoacetate diester was synthesized before and showed to increase ketone body concentration in the blood.<sup>118</sup> ( $\pm$ )-1,3-butanediol acetoacetate diester can release two equivalence of ketone bodies and ( $\pm$ )-1,3-butanediol can

further be oxidized to  $\beta$ -hydroxybutyric acid in liver to add another equivalence.<sup>103-105</sup> Polyols contain high tolerance in human body and can be derivatized to ester groups more easily because of the free hydroxyl groups. Additionally, glycerol can enter into gluconeogenesis to produce caloric value in the body. Monoesters of glycerol was synthesized before as a food supplement that can produce caloric value in the form of both carbohydrate and ketone bodies.<sup>119</sup> However, larger polyols and higher acetoacetate esters were never synthesized with an aim to test for ketogenic properties.

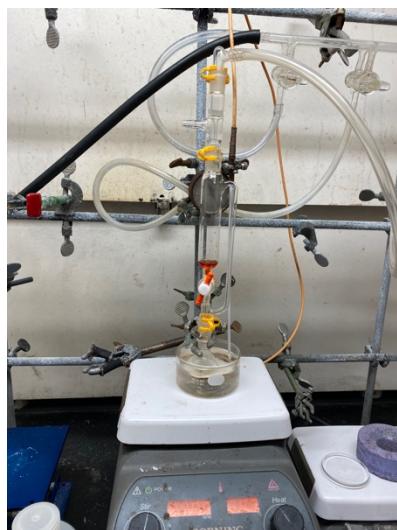
#### **2.6.3.6.1 Synthesis of propane-1,2,3-triyl tris(3-oxobutanoate) (15)**



Following the method from Nakajima *et al.*,<sup>10</sup> glycerol (0.7 mL, 10 mmol) was added into a 10 mL round bottom flask and 1,4-diazabicyclo[2.2.2]octane (DABCO, 0.0022g, 0.02 mmol) was added. The solution was heated to 65°C while stirring until DABCO completely dissolved. Diketene (2.69 mL, 35 mmol) was added from a 25 mL dropping funnel in 30 minutes under inert conditions (**Figure 2.23**). The reaction was allowed to stir for 2 hours at 65°C to give 3.1g of **15** in a yield of 67%.

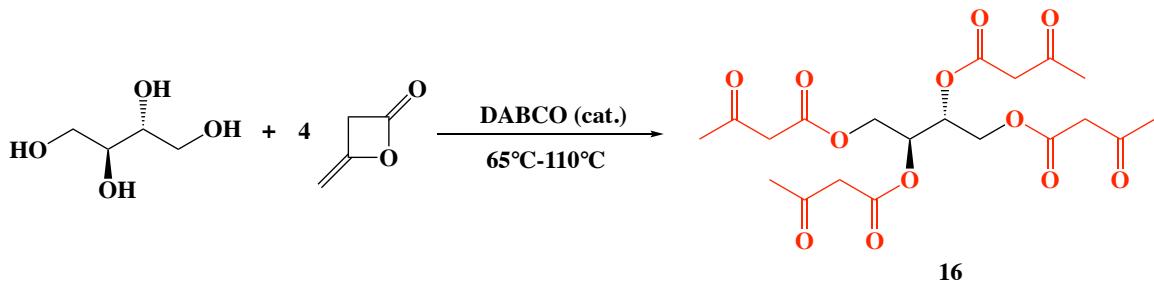
<sup>1</sup>H NMR (600 MHz, Chloroform-*d*)  $\delta$  5.31 (m, 1H), 4.32 (dd, *J*= 4.5, 11.4 Hz, 2H), 4.25 (dd, *J*= 6.0, 12.0 Hz, 2H), 3.48 (s, 6H), 2.22 (s, 9H)

<sup>13</sup>C NMR (151 MHz, Chloroform-*d*)  $\delta$  200.2, 166.6, 60.5, 62.4, 49.5, 30.3



**Figure 2.23:** Experimental set up for the synthesis of **15**

#### 2.6.3.6.2 Synthesis of (2*R*,3*S*)-butane-1,2,3,4-tetrayl tetrakis(3-oxobutanoate) (16)



Following the method from Nakajima *et al.*,<sup>10</sup> *meso*-erythritol (1.22g, 10 mmol) was added into a 25 mL round bottom flask and heated to 123°C until completely melted. DABCO (0.0011g, 0.01 mmol) was added and stirred until completely dissolved. Diketene (3.8 mL, 50 mmol) was added from a 25 mL dropping funnel under inert condition and slowly the temperature was reduced to 65°C over 1.5 hours. The reaction was continued to another 30 minutes to allow 4.45g of **16** in a yield of 97%.

<sup>1</sup>H NMR (600 MHz, Chloroform-*d*) δ 5.34 (m, 12H), 4.46 (m, 2H), 4.29 (m, 2H), 3.54 (s, 4H), 3.52 (s, 4H), 2.28 (s, 12H)

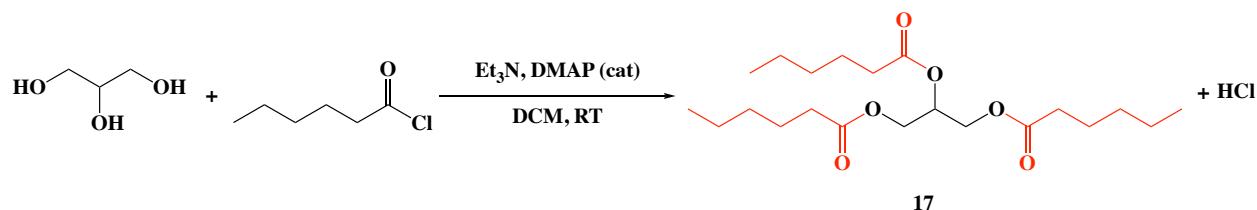
$^{13}\text{C}$  NMR (151 MHz, Chloroform-*d*)  $\delta$  199.9, 166.6, 165.9, 69.3, 62.1, 49.5, 30.2

HRMS (ESI) Calculated for  $\text{C}_{20}\text{H}_{26}\text{O}_{12}^+$  481.1322 [M+Na $^+$ ], found 481.1306

### **2.6.3.7. Synthesis of medium chain glycerides**

As previously mentioned in **Chapter 1**, even though dietary fats are primarily triacylglycerides (triglycerides) of long-chain fatty acids, medium chain triglycerides (MCT) are considered more ‘ketogenic’ as they do not require active transport into cells and are absorbed by the liver directly.<sup>1-3</sup> With that in mind, some medium chain glycerides two have been synthesized with an aim to test for ketogenicity.

#### **2.6.3.7.1 Synthesis of propane-1,2,3-triyl trihexanoate (17)**



Glycerol (0.73 mL, 0.01 mol) was added into a 50 mL round bottom flask and 8 mL of DCM was added. Hexanoyl chloride (2.79 mL, 0.02 mol) and DMAP (0.03g, 0.03 mmol) were added. The solution was cooled to 0°C in an ice-bath while stirring. TEA (4.2 mL, 0.03 mol) was added to 5 mL of DCM and the added to the solution from a 25 mL dropping funnel under inert condition. The reaction was allowed to stir for 24 hours. The solution was washed with two partitions of 6 mL of 5% aqueous ammonium chloride solution and 3 mL of 5% aqueous sodium bicarbonate solution. The solution was dried over magnesium sulfate, filtered and evaporated using rotary evaporator. To purify the product, a flash column chromatography (CC) was performed eluting with 9:1 hexane: ethyl acetate to give 0.50 g of **17** in a yield of 14%.

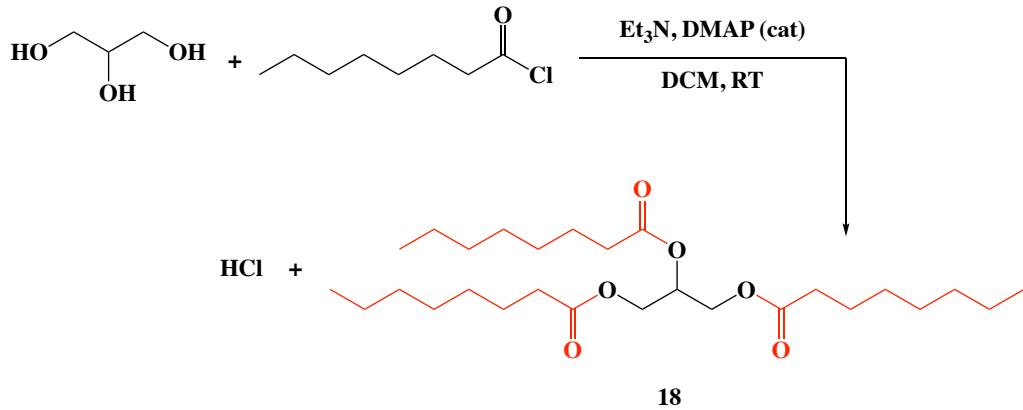


**Figure 2.24:** Column purification for the synthesis of **17**

<sup>1</sup>H NMR (600 MHz, Chloroform-*d*) δ 5.21 (m, 1H), 4.24 (dd, *J*= 4.2, 12.0 Hz, 2H), 4.07 (dd, *J*= 5.4, 12.0 Hz, 2H), 2.23 (m, 6H), 1.55 (m, 6H), 1.24 (m, 12H), 0.81 (t, *J*= 7.2 Hz, 9H)

<sup>13</sup>C NMR (151 MHz, Chloroform-*d*) δ 173.3, 68.8, 62.1, 34.0, 31.2, 24.5, 22.2, 13.9

#### 2.6.3.7.2 Synthesis of propane-1,2,3-triyl trioctanoate (18)



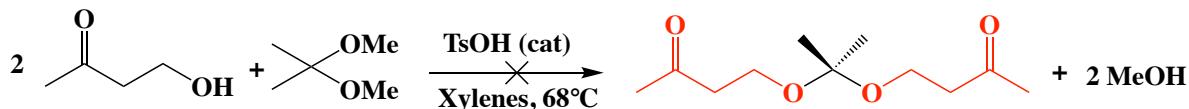
Glycerol (1 mL, 14 mmol) was added into a 100 mL round bottom flask and 10 mL of DCM was added. Octanoyl chloride (7.5 mL, 45 mmol) and DMAP (0.0033g, 0.03 mmol) were added to the solution. The solution was cooled to 0°C in an ice-bath while stirring. TEA (6.2mL, 45 mmol) and 10 mL of were mixed together and added to the solution from a 25 mL dropping

funnel under inert condition. The reaction was allowed to stir at room temperature for 48 hours. The solution was washed with two partitions of 10 mL of 5% aqueous ammonium chloride solution and three partitions of 15 mL of 5% aqueous sodium bicarbonate solution. The solution was dried over anhydrous magnesium sulfate and filtered. The organic layer was evaporated using rotary evaporation to give 4.19g of **18** in a yield of 64%.

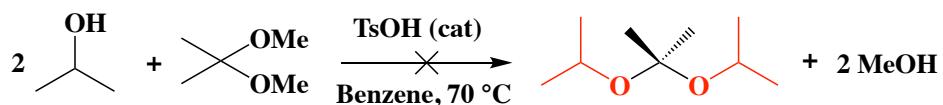
<sup>1</sup>H NMR (600 MHz, Chloroform-*d*) δ 5.20 (tt, *J*= 3.6, 5.1 Hz, 1H), 4.22 (dd, *J*= 4.2, 12.0 Hz, 2H), 4.07 (dd, *J*= 5.4, 12.6 Hz, 2H), 2.25 (m, 6H), 1.54 (m, 6H), 1.22 (m, 24H), 0.81 (t, *J*= 7.2 Hz, 9H)

<sup>13</sup>C NMR (101 MHz, Chloroform-*d*) δ 173.2, 68.6, 62.0, 34.0, 31.6, 29.0, 28.8, 24.8, 22.5, 13.9

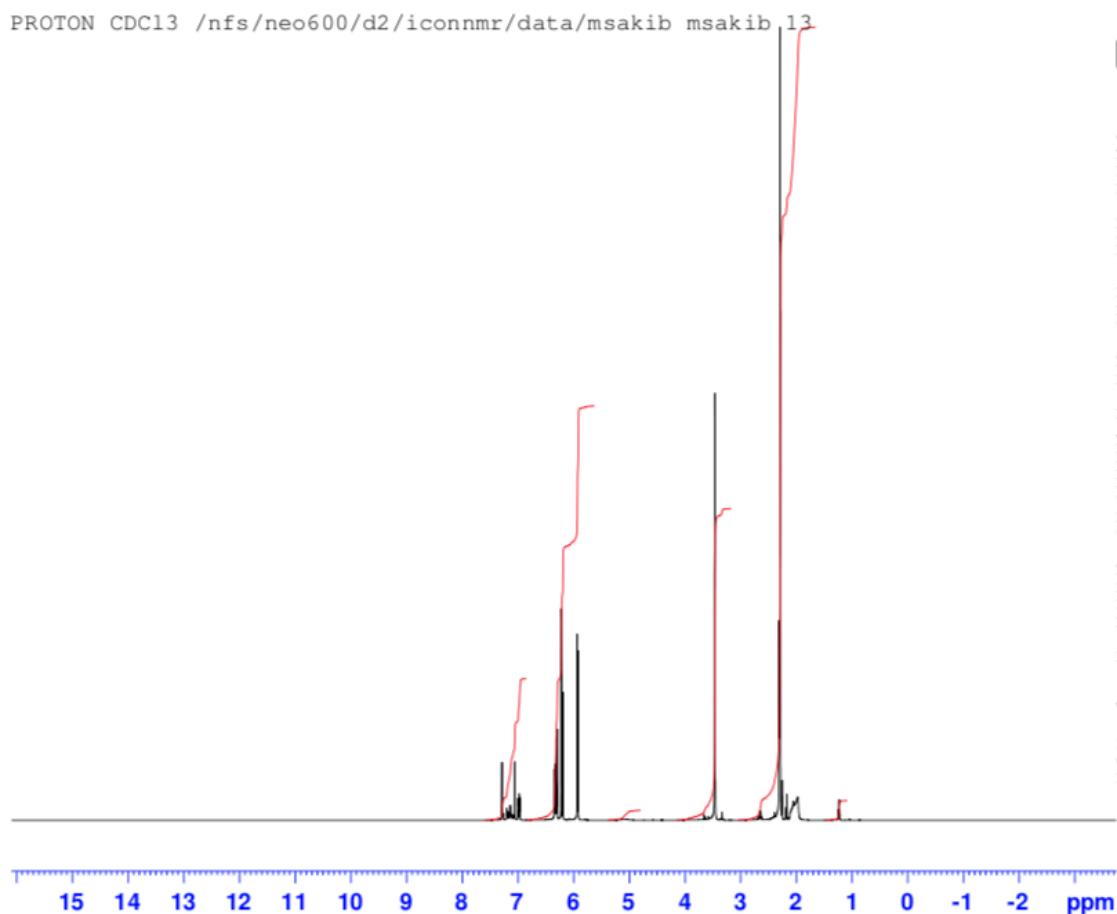
#### **2.6.3.8 Attempted synthesis of potential ketogenic compounds**



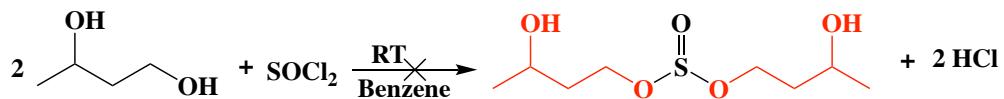
**4,4'-(propane-2,2-diylbis(oxy))bis(butan-2-one)** was attempted to be synthesized by reaction of 4-hydroxy-2-butanone (2 equiv.) and 2,2-dimethoxypropane (1 equiv.) in xylenes in the presence of p-toluenesulfonic acid catalyst at 68°C temperature under distillation set up. The reaction was allowed to go for 7.5 hours but no methanol by product came out as a distillate. The temperature was elevated to 120°C and distillate was collected at 100-110°C temperature in the distillation head, but <sup>1</sup>H NMR spectrum analysis did not show a match with the desired product (**Figure 2.25**).



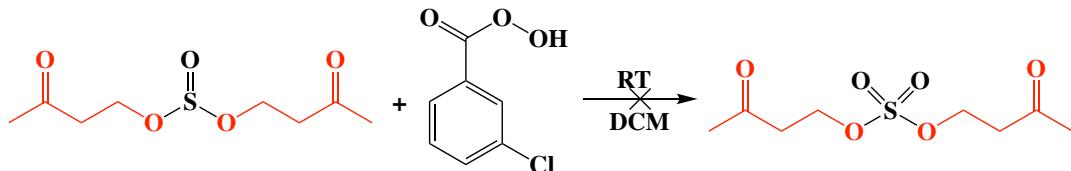
**2,2-diisopropoxypropane** was attempted to be synthesized similarly with the reaction of 2-propanol (2 equiv.) and 2,2-dimethoxy propane (1 equiv.) in benzene in presence of p-toluene sulfonic acid catalyst at 68°C temperature under distillation set up. The reaction was allowed to stir for 4 hours but no methanol by product came out as a distillate and the reaction color turned into brown.



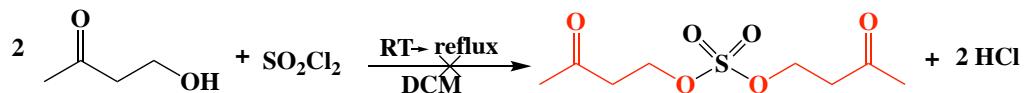
**Figure 2.25:**  $^1\text{H}$  NMR spectrum of the product obtained from the attempted synthesis of 4,4'-(propane-2,2-diylbis(oxy))bis(butan-2-one)



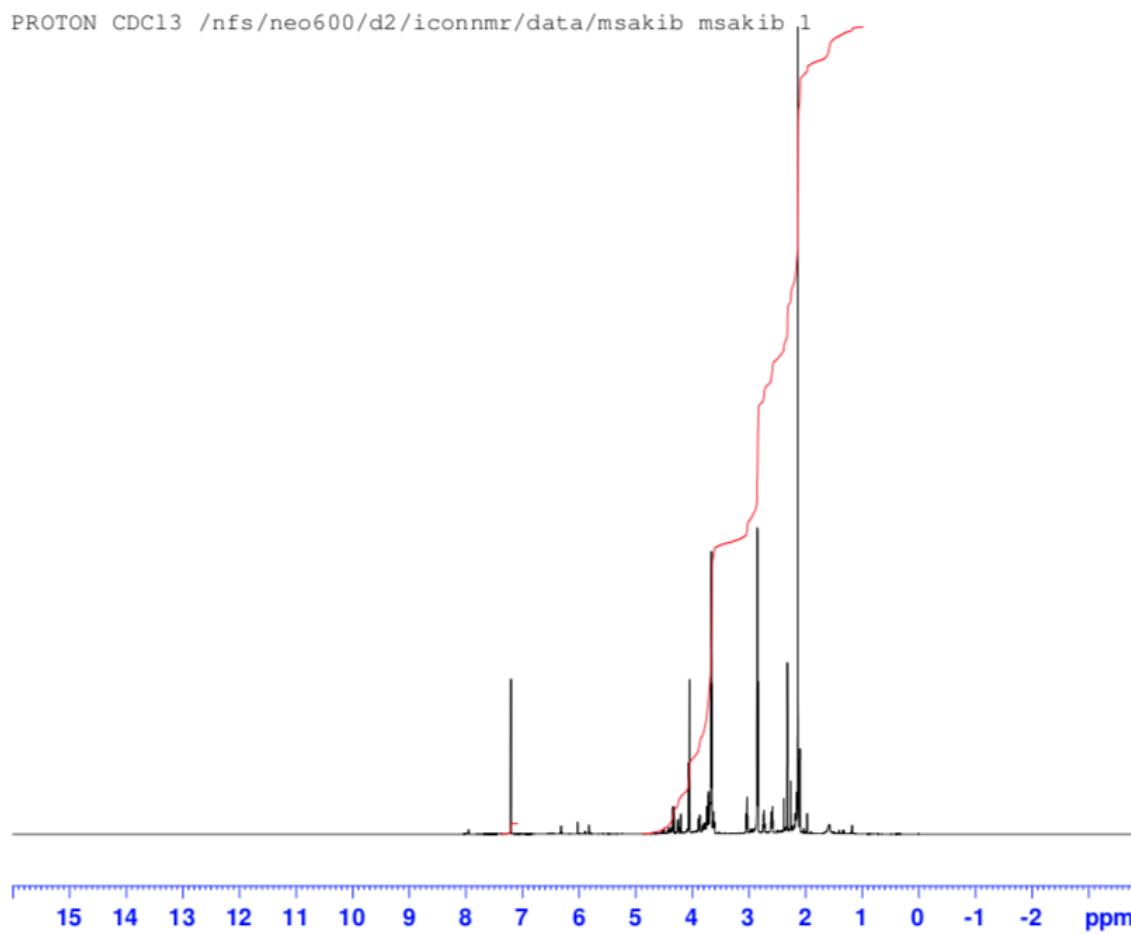
**Bis(3-hydroxybutyl) sulfite** was attempted to be synthesized with the reaction of ( $\pm$ )-1,3-butanediol (2 equiv.) and thionyl chloride (1 equiv.) in benzene. The reaction was allowed to stir at room temperature and  $^1\text{H}$  NMR analysis showed that the reaction produces cyclic sulfite only.



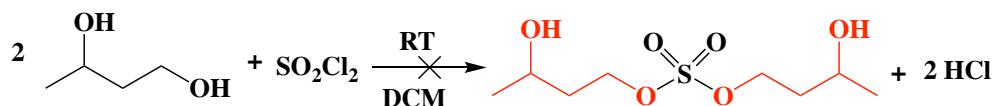
**Bis(3-oxobutyl) sulfate** was attempted to be synthesized with reaction of **4** (1 equiv.) and m-chloroperoxybenzoic acid (m-CPBA, 1 equiv.) in DCM at room temperature, but the reaction did not occur according to  $^1\text{H}$  NMR spectrum analysis.



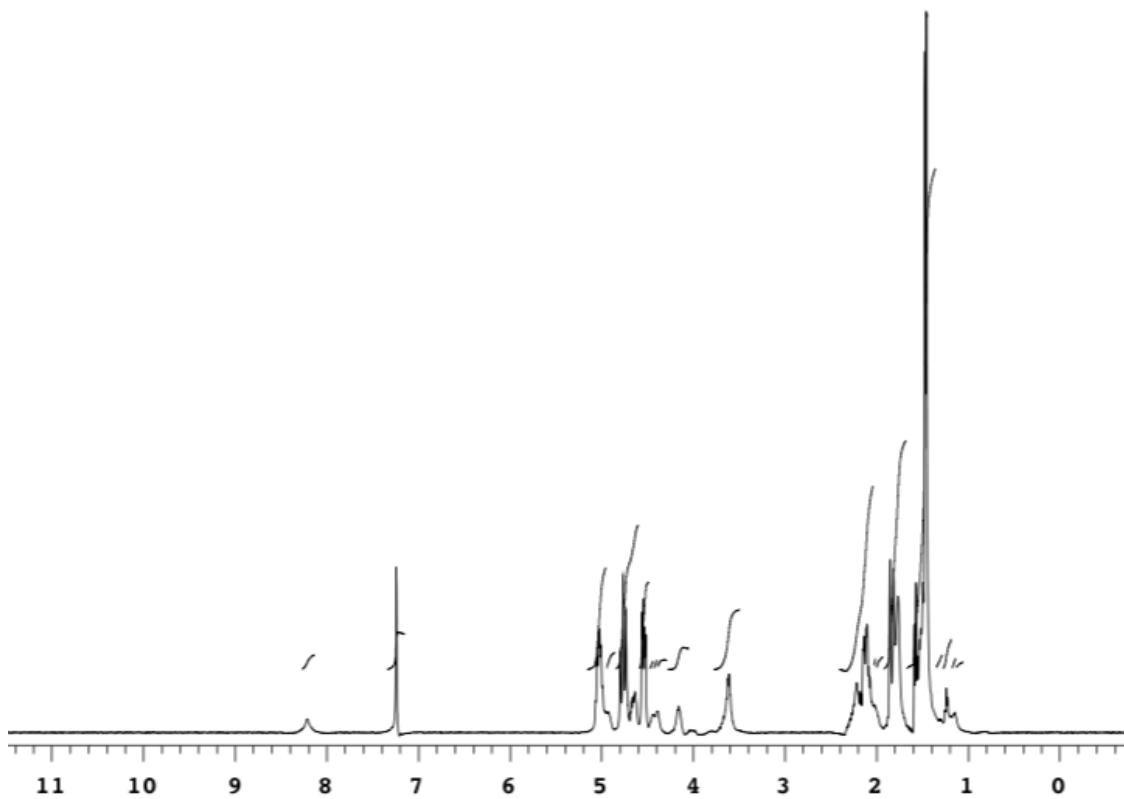
Again, this compound was attempted to synthesize with the reaction of 4-hydroxy-2-butanone (2 equiv.) and sulfuryl chloride (1 equiv.) in DCM starting room temperature to 40°C reflux condition for 48 hours. But  $^1\text{H}$  NMR spectrum analysis showed remaining of the majority of the starting material (**Figure 2.26**).



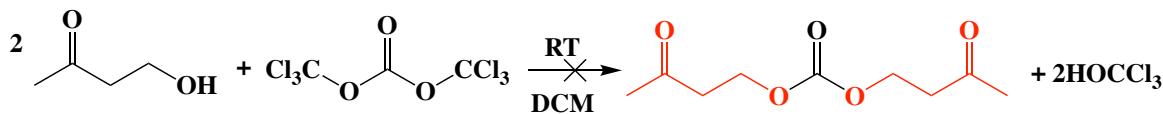
**Figure 2.26:**  $^1\text{H}$  NMR spectrum of the product obtained from the attempted synthesis of bis(3-oxobutyl) sulfate



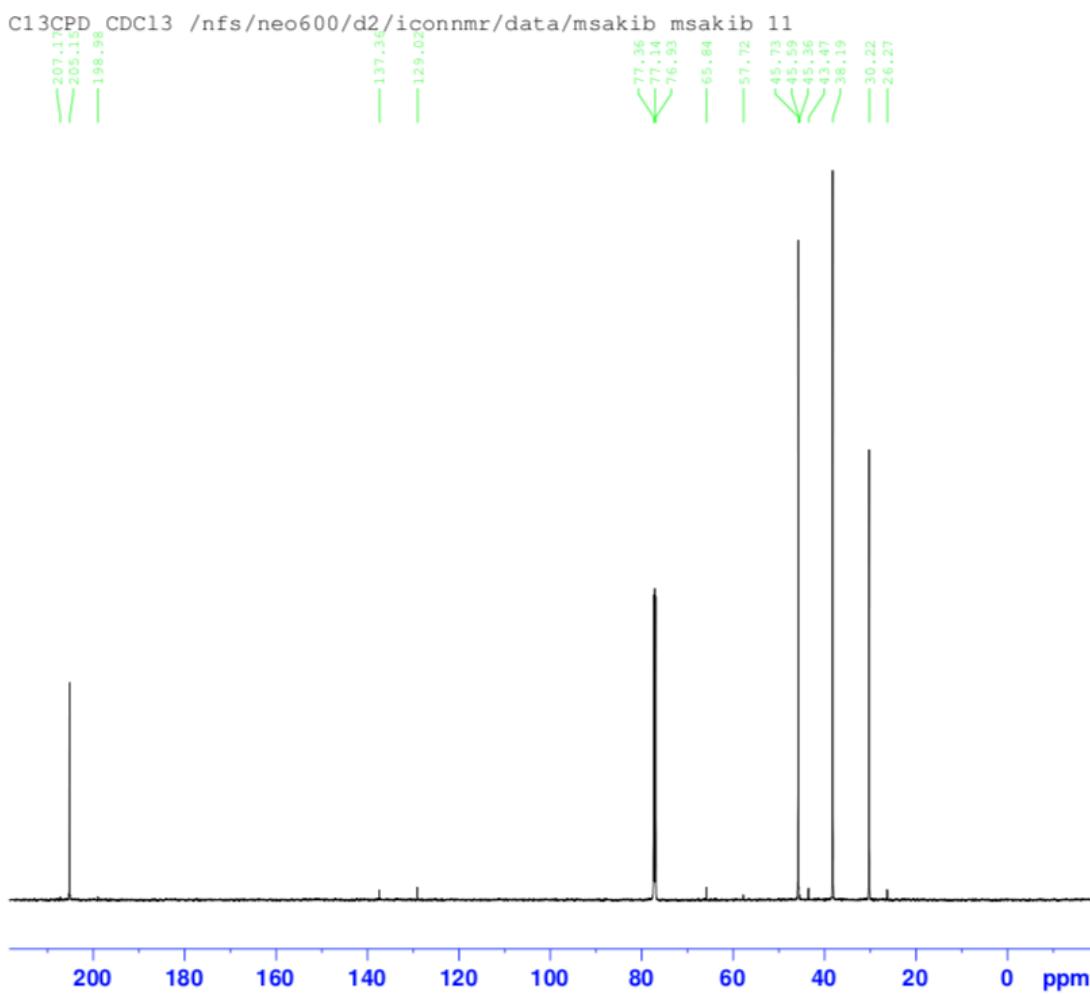
**Bis-(3-hydroxybutyl) sulfate** was attempted to be synthesized from the reaction of ( $\pm$ )-1,3-butanediol (2 equiv.) and sulfonyl chloride (1 equiv.) in DCM. After 24 hours of reaction,  $^1\text{H}$  NMR spectrum analysis showed the presence of cyclic sulfate byproduct and starting material in the mixture (Figure 2.27), but of the desired diol.



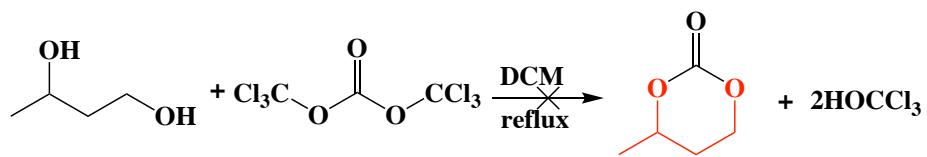
**Figure 2.27:**  $^1\text{H}$  NMR spectrum of the product obtained from the attempted synthesis of bis(3-hydroxybutyl) sulfate



**Bis-(3-oxo butyl) carbonate** was attempted to be synthesized with the reaction of 4-hydroxy-2-butanone (2 equiv.) and triphosgene (1 equiv.) in DCM. The reaction was allowed to stir for 6 hours at room temperature.  $^{13}\text{C}$  NMR spectrum analysis showed the presence of only the starting material (**Figure 2.28**).

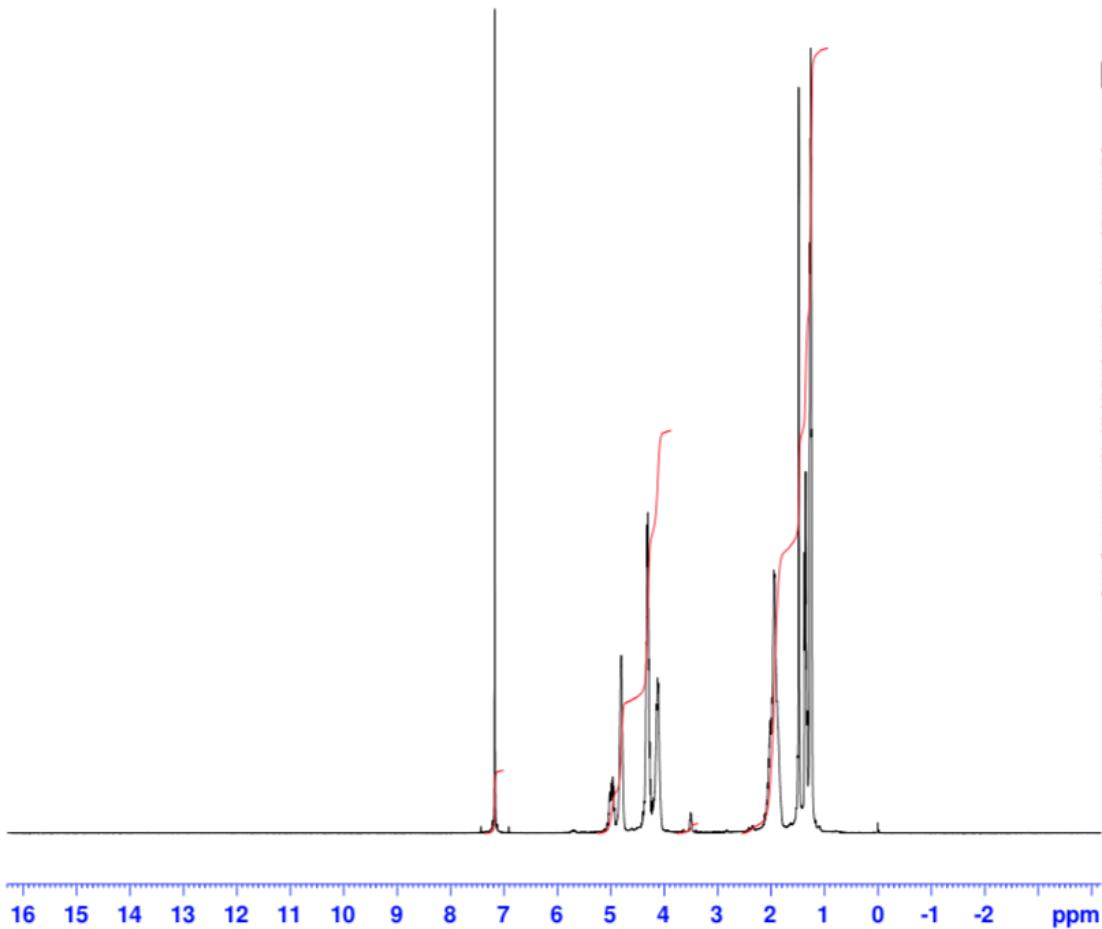


**Figure 2.28:**  $^{13}\text{C}$  NMR spectrum of the product obtained from the attempted synthesis of bis-3-oxo-butyl carbonate

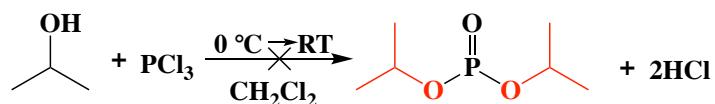


**4-Methyl-1,3-dioxan-2-one** was attempted to synthesize by the reaction of ( $\pm$ )-1,3-butanediol (1 equiv.) and triphosgene (1 equiv.) in DCM. The reaction was allowed at room temperature for 6 hours and at  $40^\circ\text{C}$  reflux condition for 12 hours.  $^1\text{H}$  NMR spectrum analysis showed the

product contains both the desired product and what seems likely is the bis-3-hydroxy butyl carbonate by product (**Figure 2.29**).

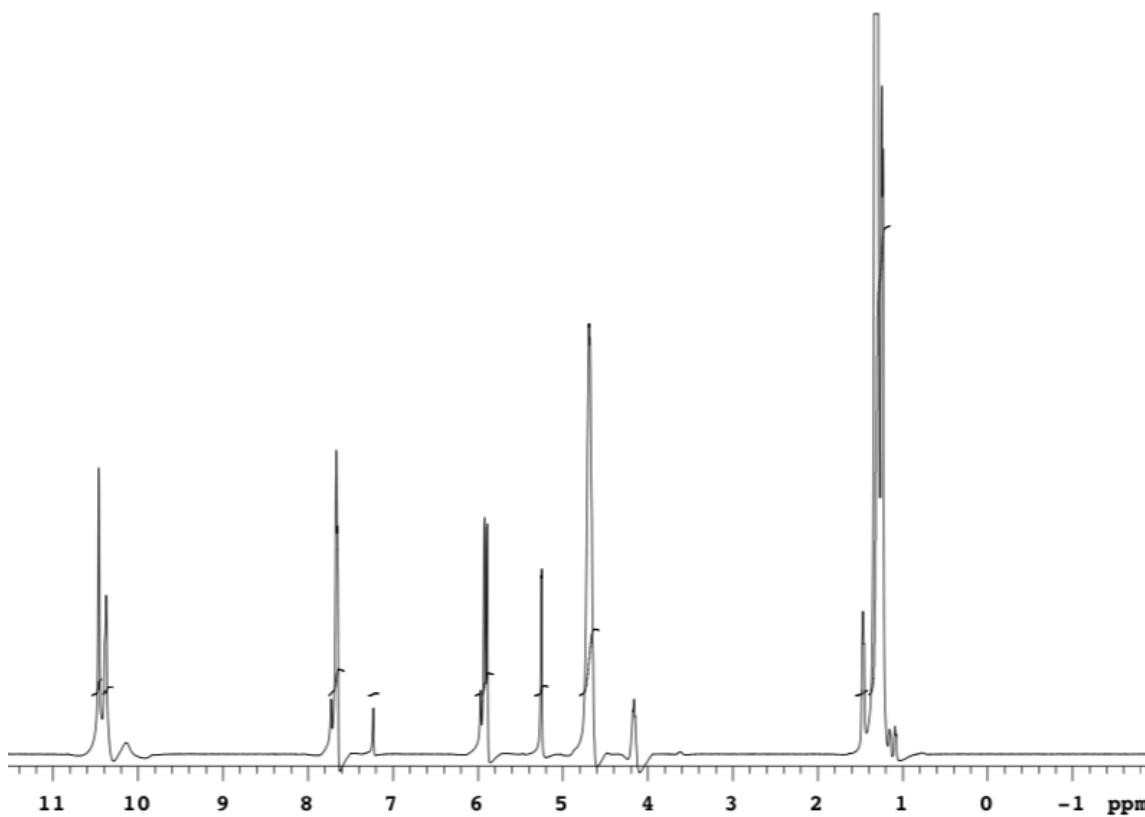


**Figure 2.29:**  $^1\text{H}$  NMR spectrum of the product obtained from the attempted synthesis of 4-methyl-1,3-dioxan-2-one

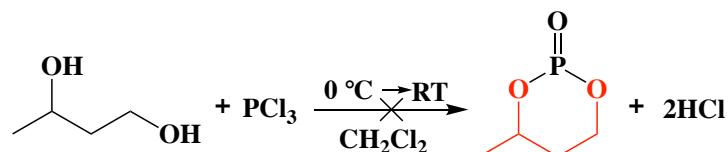


As phosphate group increase the water solubility of the molecule,<sup>120</sup> **diisopropyl phosphate** was attempted to be synthesized following a method developed by Fakhraian *et al.*<sup>121</sup> 2-Propanol (3 equiv.) and trichlorophosphine (1 equiv.) was allowed to stir starting from 0°C to room

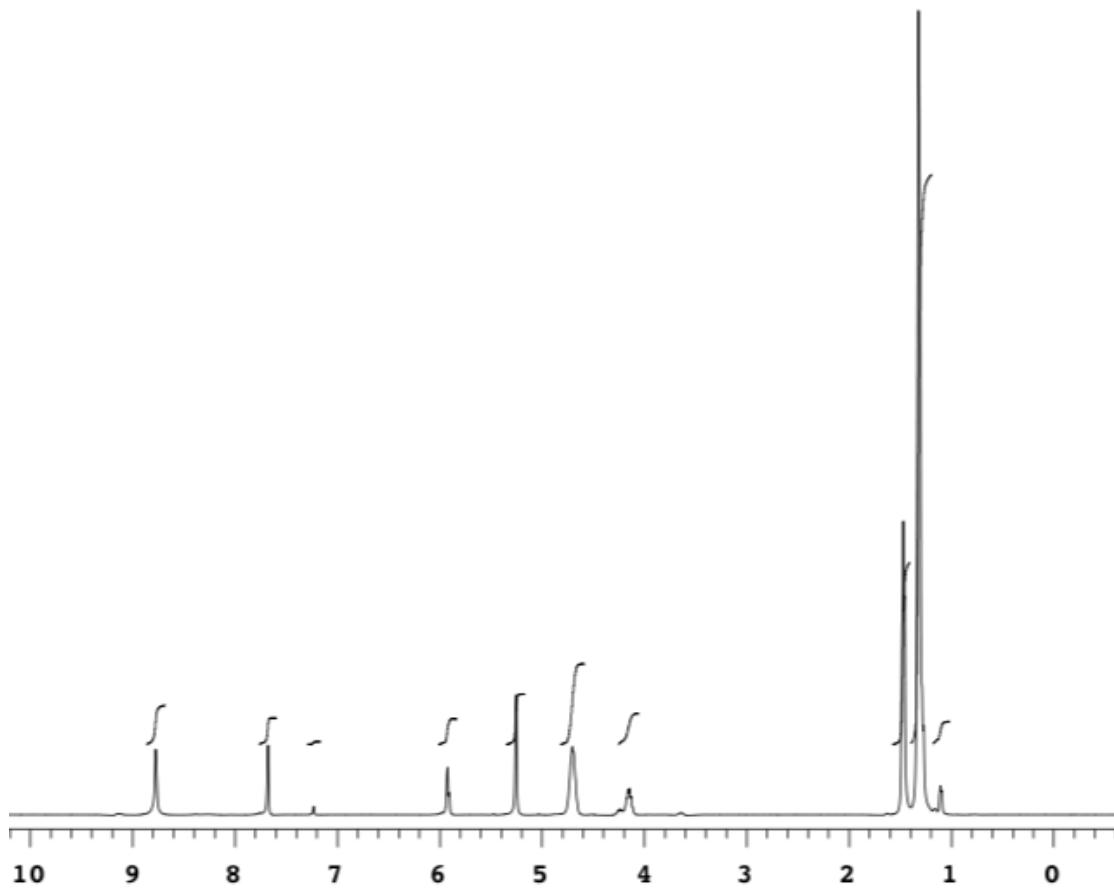
temperature in DCM, but the  $^1\text{H}$  NMR spectrum analysis of the product showed unrecognized signals for majority of the product (**Figure 2.30**).



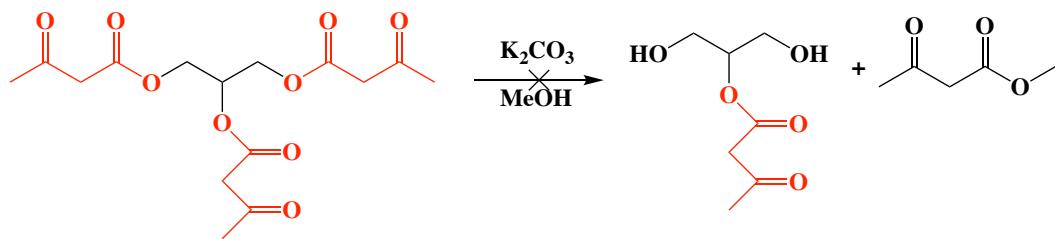
**Figure 2.30:**  $^1\text{H}$  NMR spectrum of the product obtained from the attempted synthesis of diisopropyl phosphate



**4-Methyl-1,3,2-dioxaphosphinane 2-oxide** was also attempted to be synthesized with the reaction of ( $\pm$ )-1,3-butanediol (2 equiv.) and triphosgene (1 equiv.) in DCM starting from  $0^\circ\text{C}$  to room temperature. Again, the  $^1\text{H}$  NMR spectrum analysis of the crude product mixture showed unrecognized signals along with what appears to be the desired product (**Figure 2.31**).



**Figure 2.31:**  $^1\text{H}$  NMR spectrum of the product obtained from the attempted synthesis of 4-methyl-1,3,2-dioxaphosphinane 2-oxide

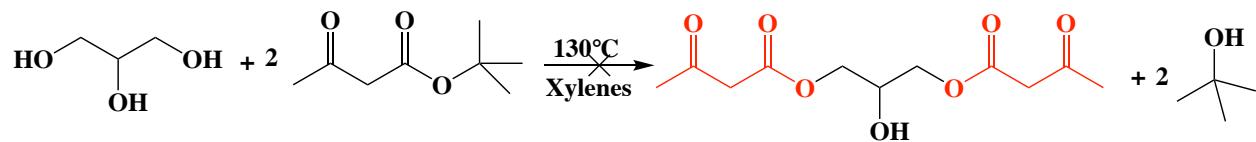


**1,3-Dihydroxypropan-2-yl 3-oxobutanoate** was attempted to be synthesized with the reaction of propane-1,2,3-triyl tris(3-oxobutanoate) (1 equiv.) and potassium carbonate (2 equiv.) in methanol. However, starting material was polymerized under this basic conditions (**Figure 2.32**).

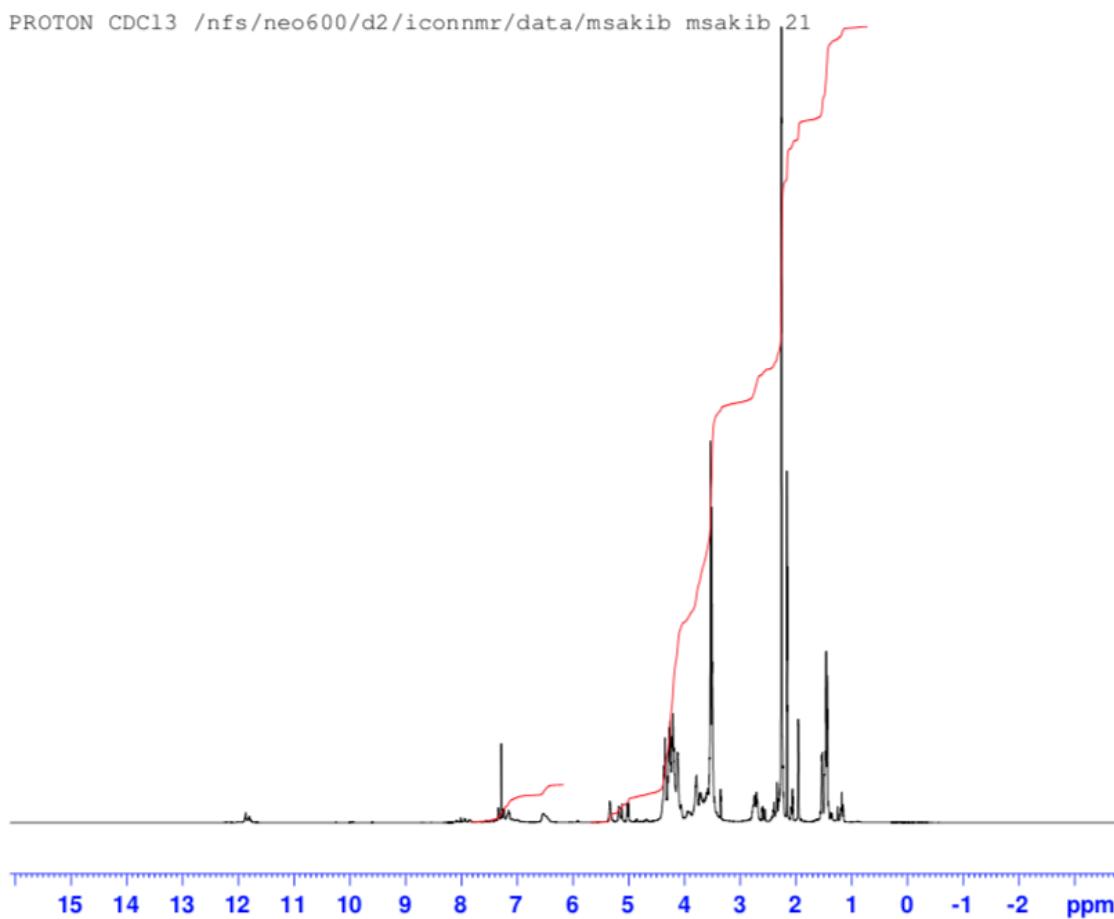


**Figure 2.32:** Pot material after reaction

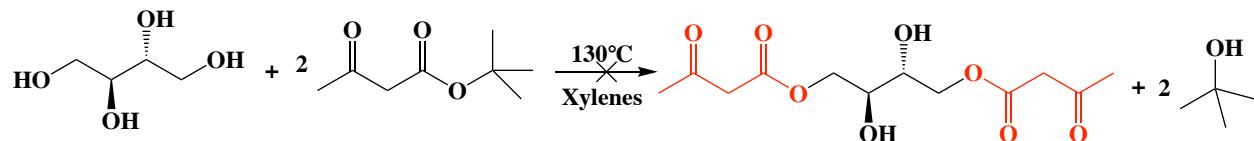
To increase water solubility, several diesters of the polyols were attempted to be synthesized using a method reported by Sonnenschein *et al.*<sup>52</sup>



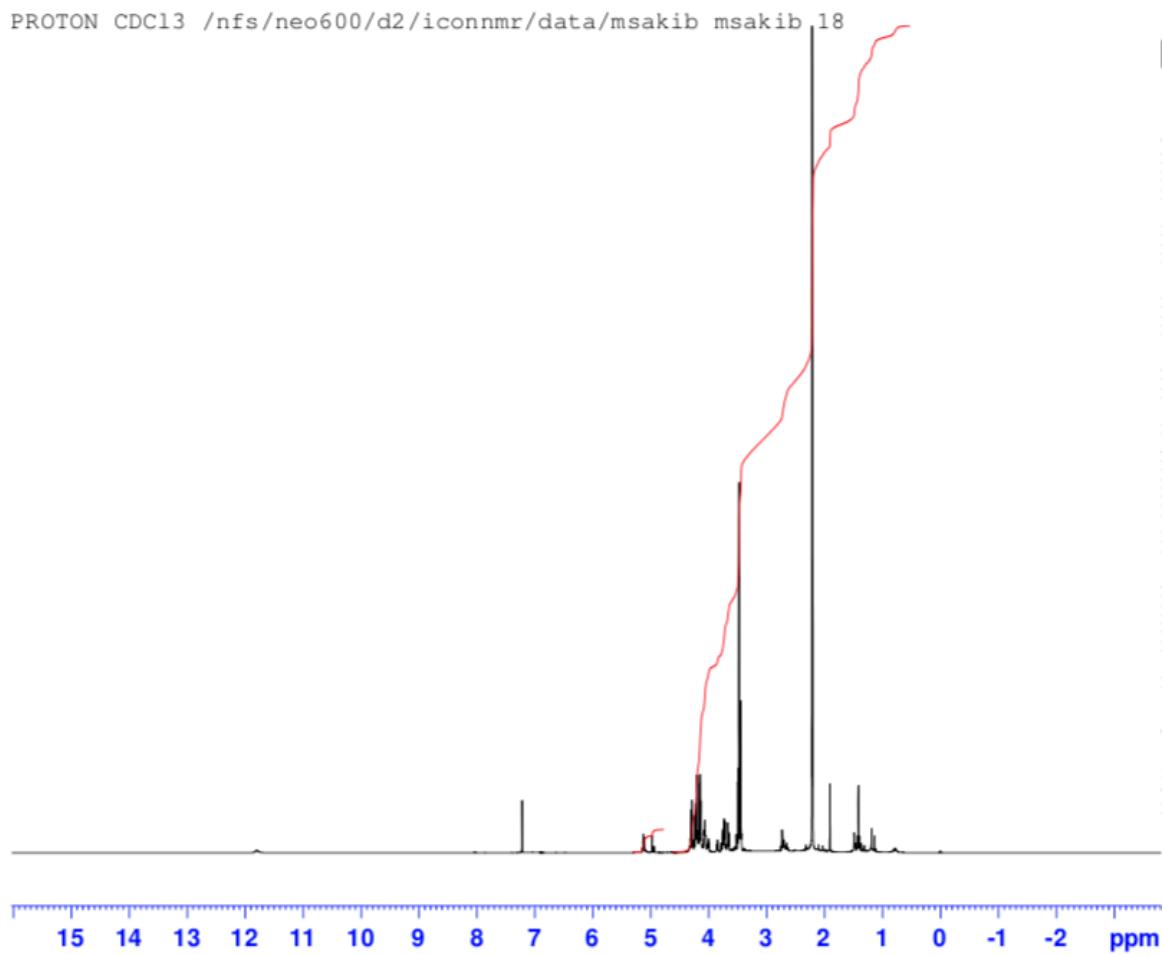
**2-Hydroxypropane-1,3-diyl bis(3-oxobutanoate)** was attempted to be synthesized with the reaction of glycerol (1 equiv.) and t-butyl acetoacetate ester (2 equiv.) in xylenes at 130°C for 5 hours. <sup>1</sup>H NMR spectrum analysis of the product showed a mixture of mono-, di- and tri acetoacetate esters (**Figure 2.33**).



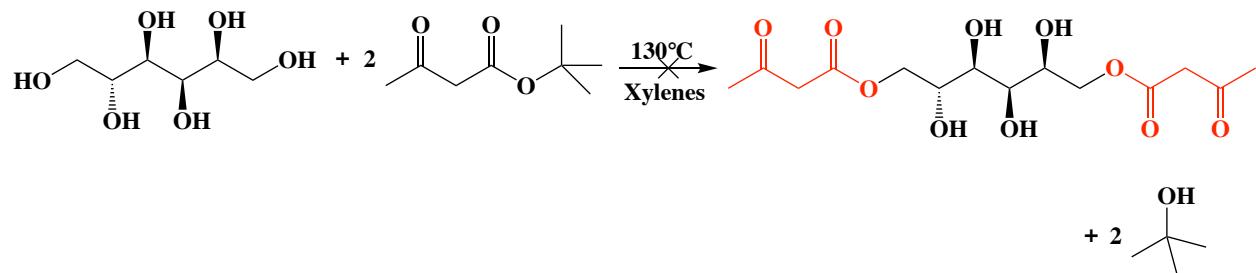
**Figure 2.33:**  $^1\text{H}$  NMR spectrum of the product obtained from the attempted synthesis of 2-hydroxypropane-1,3-diyl bis(3-oxobutanoate)



(**2R,3S**)-2,3-Dihydroxybutane-1,4-diyl bis(3-oxobutanoate) was attempted to be synthesized with the reaction of *meso*-erythritol (1 equiv.) and t-butyl acetoacetate ester (2 equiv.) in xylenes at 130°C for 5 hours.  $^1\text{H}$  NMR spectrum analysis of the product showed a mixture of mono-, di-, tri and tetraacetoacetate ester with unreacted -butyl acetoacetate ester (**Figure 2.34**).

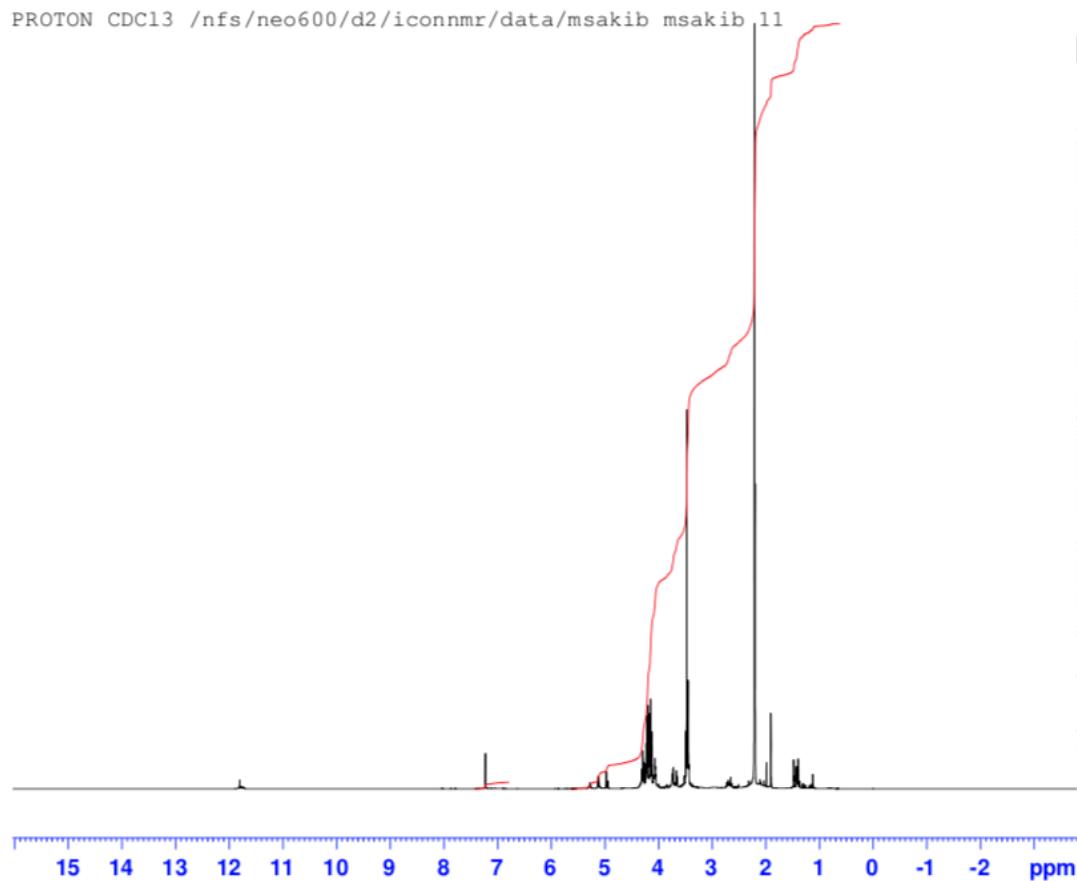


**Figure 2.34:**  $^1\text{H}$  NMR spectrum of the product obtained from the attempted synthesis of (2*R*,3*S*)-2,3-dihydroxybutane-1,4-diyl bis(3-oxobutanoate)

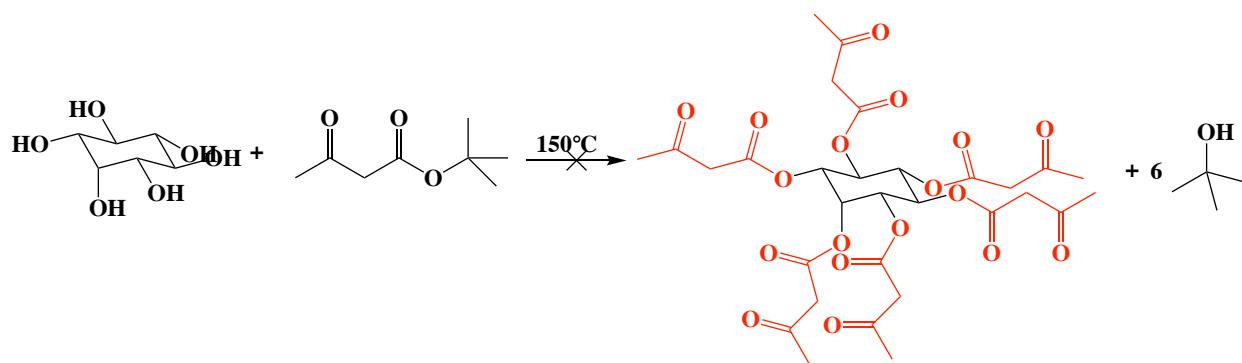


(2*R*,3*R*,4*R*,5*S*)-2,3,4,5-Tetrahydroxyhexane-1,6-diyl bis(3-oxobutanoate) was also attempted to be synthesize with the reaction of D-sorbitol (1 equiv.) and t-butyl acetoacetate (2 equiv.)

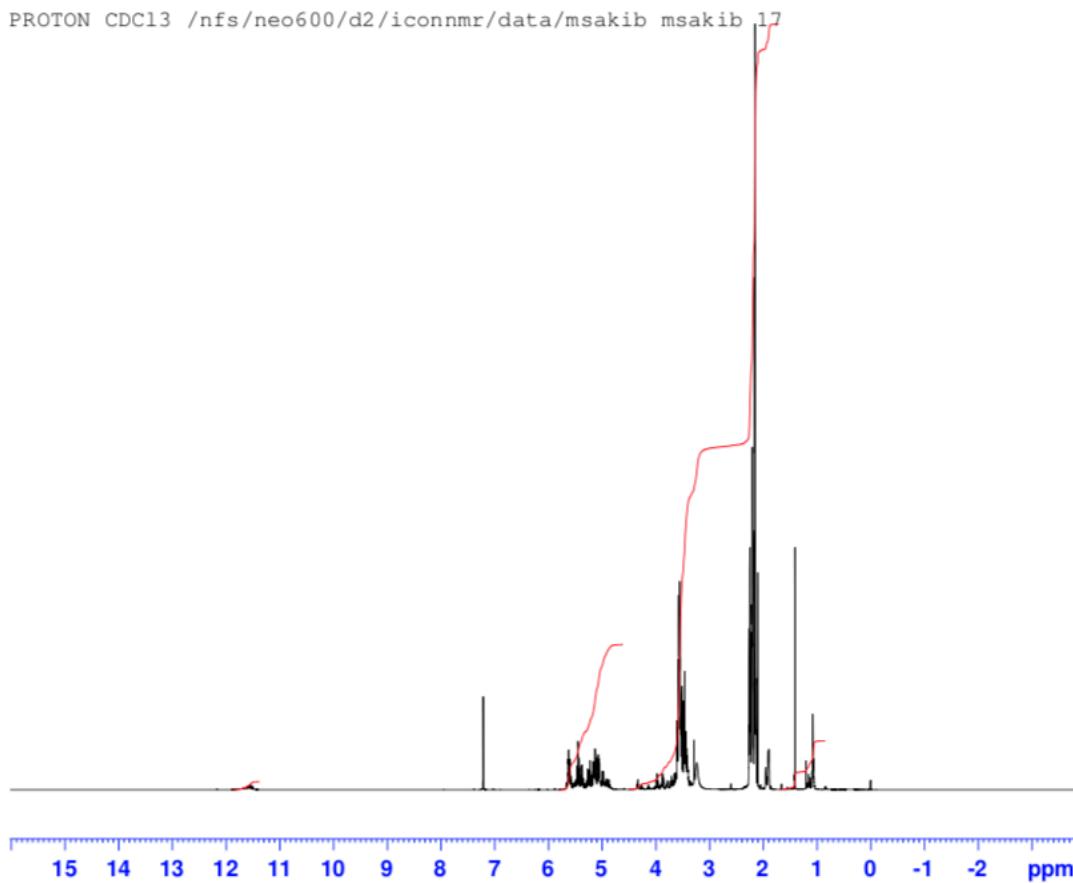
in xylenes at 130°C for 5 hours.  $^1\text{H}$  NMR spectrum analysis of the product showed a mixture of acetoacetate esters with unreacted -butyl acetoacetate ester (**Figure 2.35**).



**Figure 2.35:**  $^1\text{H}$  NMR spectrum of the product obtained from the attempted synthesis of  
 $((2R,3R,4R,5S)\text{-}2,3,4,5\text{-tetrahydroxyhexane}\text{-}1,6\text{-diyl bis}(3\text{-oxobutanoate})$



**(1R,2R,3S,4R,5S,6S)-Cyclohexane-1,2,3,4,5,6-hexayl hexakis(3-oxobutanoate)** was also attempted to be synthesized by reaction of *myo*-inositol (1 equiv.) and t-butyl acetoacetate ester (6 equiv.) at 150°C. After 5 hours of reaction, no t-butanol by product came over as distillate and <sup>1</sup>H NMR spectrum analysis of the reaction showed the reaction did not go full completion with unreacted -butyl acetoacetate ester (**Figure 2.36**).



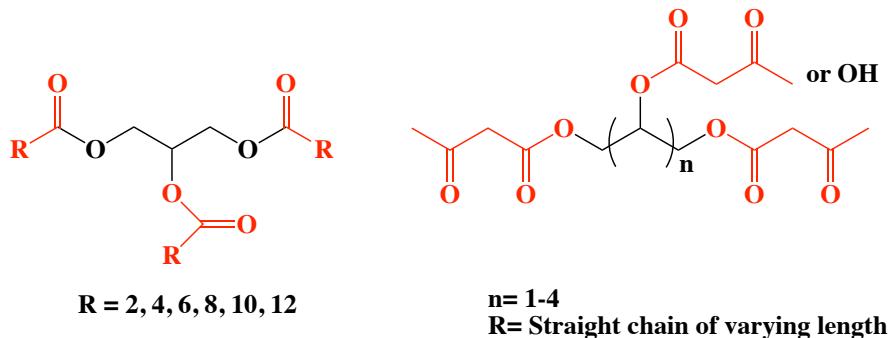
**Figure 2.36:** <sup>1</sup>H NMR spectrum of the product obtained from the attempted synthesis of **(1R,2R,3S,4R,5S,6S)-cyclohexane-1,2,3,4,5,6-hexayl hexakis(3-oxobutanoate)**

## 2.7 Conclusion

Common functional groups such as esters, carbonates and amides serve as an excellent linker for bioactive molecules. However, to derivatize ketogenic building blocks with common

functional groups is a challenge because of the need for high stability of the compounds during storage. Majority of the compounds decomposed over time during storage. Compounds from 4-hydroxy-2-butanone are very unstable at regular conditions and turned black during storage. Compounds from 1,3-butanediol also decomposed over time. The esters from 4-hydroxy-2-butanone decomposed over time as well and their stability is related to their chain length. The larger the chain length, the more stable the compound is.  $\beta$ -Ketoesters of polyols are difficult to synthesize without over esterification under thermal conditions and products decompose over time.

Based on the experimental data and observations, most potential structures to consider for ketogenicity are short to medium chain length glycerides and  $\beta$ -Ketoesters of various chain length polyols (**Figure 2.37**).



**Figure 2.37:** Most potential structures for ketogenicity

Although to ensure these compounds can be used as ketogenic molecules to increase ketone body concentration in the blood, further investigation is needed; these structures can serve as a milestone to explore more of the similar analogues to enrich the library of ketogenic molecules.

## **Chapter 3: Stability Analysis, Enzymatic Hydrolysis Activity and *in vivo* Testing of Potential Ketogenic Compounds**

### **3.1 Introduction**

Analysis of any bioactive compound requires development of a simple and cost-effective method which is reliable. While in the earliest history of the high-fat, low-carbohydrate diet, most of the testing were done in the clinical settings on patients of different diseases as mentioned in **Chapter 2** (section 2.3), but scientific methodology was not yet developed.

Monoacetoacetate esters were synthesized as a food supplement and tested for their efficacy to increase both glucose and ketone bodies.<sup>119</sup> Monoacetoacetate esters were administered to male Holtzman rats with 7 days of low-calory diet and blood samples were collected to measure the concentration of glucose, pyruvate, acetoacetate and  $\beta$ -hydroxy butyrate in the blood. Later, ( $\pm$ )-1,3-butanediol acetoacetate diesters were synthesized and tested in pigs and rats to test for ketogenicity.<sup>103-104</sup> Animals were enforced to fast and ketogenic compounds were administered intravenously in every 3 hours. Blood samples were collected every 5 hours to measure the serum concentrations of glucose, pyruvate, acetoacetate, both R- and S- $\beta$ -hydroxy butyrate and lactate. While importance was given mostly on the tolerance and effect of the compounds in the body, incremental changes of ketone bodies concentrations were observed for the experiments.

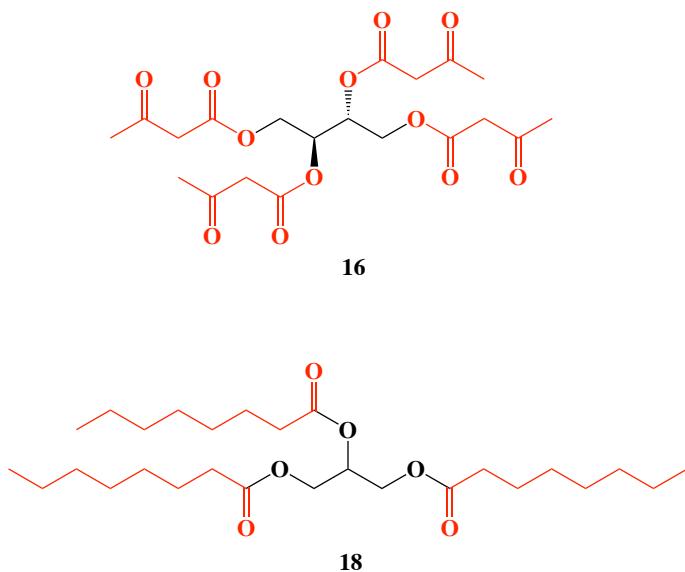
The first synthetic ketogenic compound tested for ketogenicity was (R)-3-hydroxybutyl-(R)-3-hydroxybutanoate, where test subjects were kept on a regular diet instead of fasting.<sup>106</sup>

This compound was tested on humans as a meal replacement beverage where 25% of calorific content was from ketogenic supplements. Different dosage ranging from 140-714 mg ketogenic compound / kg body weight were supplemented with commercial meal replacement drink and elevation of ketone body concentration in the blood was shown in a dose-dependent manner.<sup>108</sup>

In 2016, the D'Agostino research group focused on examining the effect of several ketonic supplements to induce ketosis by measuring ketone body concentration in the blood, while the subject was kept on the regular diet.<sup>118</sup> Medium chain triglyceride (MCT), a mixture of MCT and racemic mixture of sodium and potassium  $\beta$ -hydroxy butyrate salts, only racemic mixture of sodium and potassium  $\beta$ -hydroxy butyrate salts, ( $\pm$ )-1,3-butanediol and ( $\pm$ )-1,3-butanediol acetoacetate diester were all tested in Sprague-Dawley rats. Ketogenic substances were administered via intragastric gavage for 28 days and blood samples were collected in every 7 days intervals for testing. According to their report, all of the ketogenic supplements showed an increase of the ketone body concentration in the blood compared to the controlled group where no ketogenic substances were administered. At the same time, all the substances showed a reduction of blood glucose concentration compared to the control group rats, except for ( $\pm$ )-1,3-butanediol.

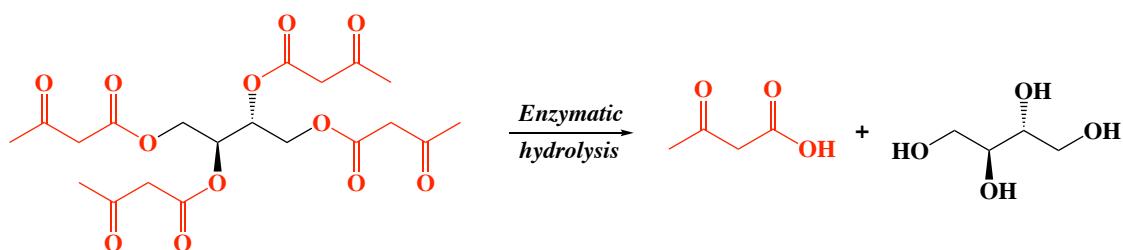
Testing a synthetic compound in an animal model requires facilities and approved experimental protocol. At the same time, samples required for testing in actual biological models need to be available on a larger scale compared to in vitro analysis. Additionally, biochemistry of the metabolism of the compound needs to be well understood to hypothesize to proceed to animal testing. Upon considering all these factors, two of the compounds were hypothesized to be a ketogenic supplement and have to be further tested for stability, enzymatic hydrolysis and *in vivo* testing in mice models. The selected compounds are (2R,3S)-butane-1,2,3,4-tetrayl

tetrakis(3-oxobutanoate) (**16**) and propane-1,2,3-triyl trioctanoate (**18**), which are showed in **Figure 3.1.**



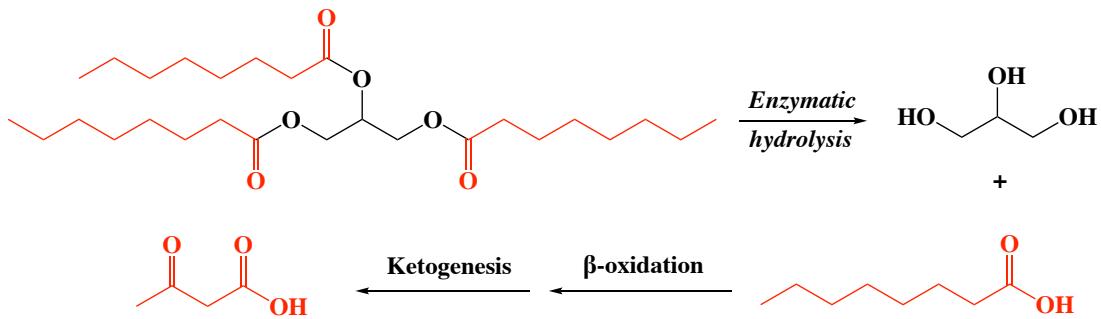
**Figure 3.1:** Selected compounds for stability analysis, enzymatic hydrolysis activity and *in vivo* testing as potential ketogenic compounds

For simplicity, **16** is referred to as erythritol tetra acetoacetate ester and **18** is referred to as glycerol tri octanoate in this Chapter. Upon digestion, **16** would be expected to hydrolyze enzymatically to acetoacetic acid and erythritol, as depicted in **Figure 3.2.**



**Figure 3.2:** Metabolism of erythritol tetra acetoacetate ester

Compound **18** would also hydrolyze enzymatically but would go through  $\beta$ -oxidation of fatty acids and ketogenesis to produce ketone bodies, as depicted in **Figure 3.3**.



**Figure 3.3:** Metabolism of glycerol trioctanoate

### 3.2 Studies of pH stability of ketogenic compounds

Ketogenic compounds can hydrolyze enzymatically, but at the same time might also undergo hydrolysis at various pH values. Stability analysis was done for **16** and **18** over a broad range of pH levels (2-10) to determine if they hydrolyze without enzymatic hydrolysis.

#### 3.2.1 Protocol for preparation of pH buffers solutions

A Fisherbrand<sup>TM</sup> AE150 pH meter was used to measure pH levels. Buffer solutions at pH= 2, 6, 8 and 10 were prepared. A 0.1M solution of potassium dihydrogen phosphate was prepared by dissolving 6.8g of potassium dihydrogen phosphate in 500 mL of DI water. A 0.05 mL sodium bicarbonate solution was prepared by dissolving 2.1g of sodium bicarbonate solution in 500 mL of DI water. The pH levels of buffer solutions were adjusted to the appropriate values by using either 0.2M hydrochloric acid (HCl) or 0.1M of sodium hydroxide (NaOH) solution before dilution.

A pH= 2 buffer solution was prepared by dissolving 0.034g of potassium dihydrogen phosphate in 200 mL of DI water and pH value was adjusted by 0.2M HCl solution.

A pH= 6 buffer solution was prepared by adding 11.2 mL of 0.1M NaOH solution to 100 mL of 0.1M potassium dihydrogen phosphate solution and diluting with 200 mL of DI water.

A pH= 8 buffer solution was prepared by adding 93.4 mL of 0.1M NaOH solution in 100 mL of 0.1M potassium dihydrogen phosphate solution and diluting with 200 mL of DI water.

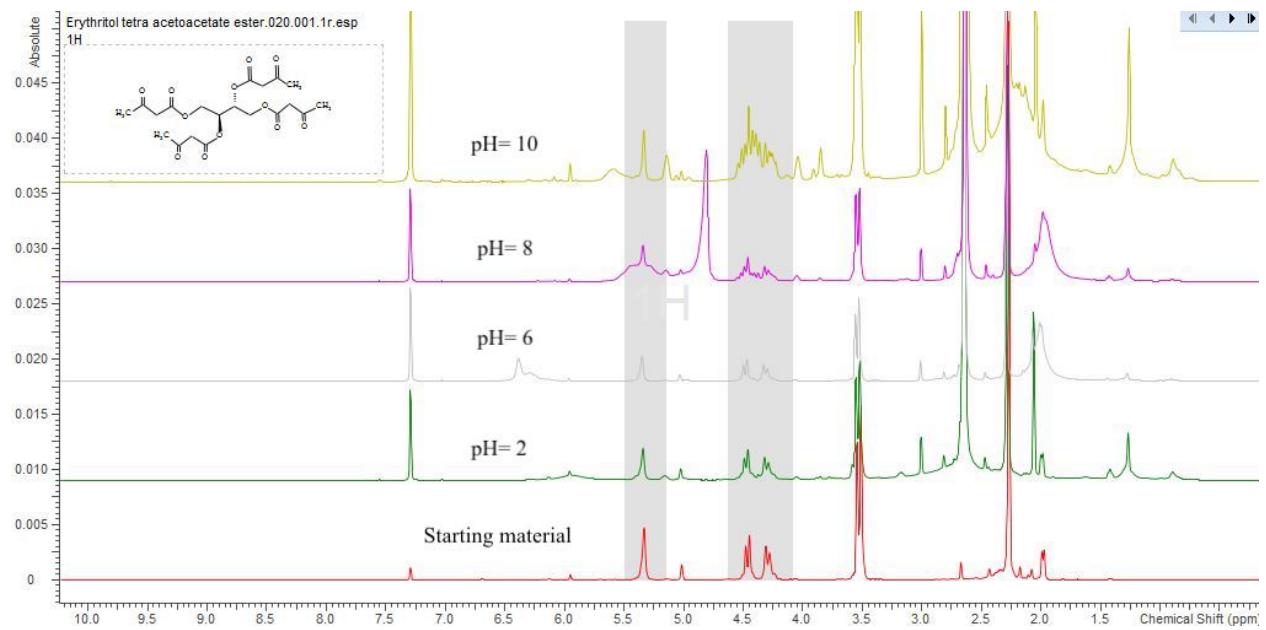
A pH= 10 buffer solution was prepared by adding 21.4 mL of 0.1M NaOH solution to 100 mL of 0.05 M sodium bicarbonate solution and diluting with 200 mL of DI water.

### ***3.2.2 Protocol for pH stability measurement for ketogenic compounds***

Four different samples of **16** (19.7 mg, 0.043 mmol) and **18** (20.2 mg, 0.043 mmol) were each dissolved in 0.4 mL of dimethylsulfoxide (DMSO) at room temperature. Buffer solutions at pH= 2, 6, 8 and 10 were added dropwise (0.3 mL). Sample vials were covered and wrapped with parafilm, then solutions of compound **16** were allowed to stir for 96 hours and solutions of compound **18** were allowed to stir for 72 hours. An aliquot (1 mL) from each sample were collected, rinsed three times with ethyl acetate (2 mL) and the organic layers were combined. The solvent was evaporated by rotary evaporation. Compounds were dissolved in CDCl<sub>3</sub> and analyzed on the 400 MHz NMR spectrometer.

### ***3.2.3 Effect of different pH levels on compound 16***

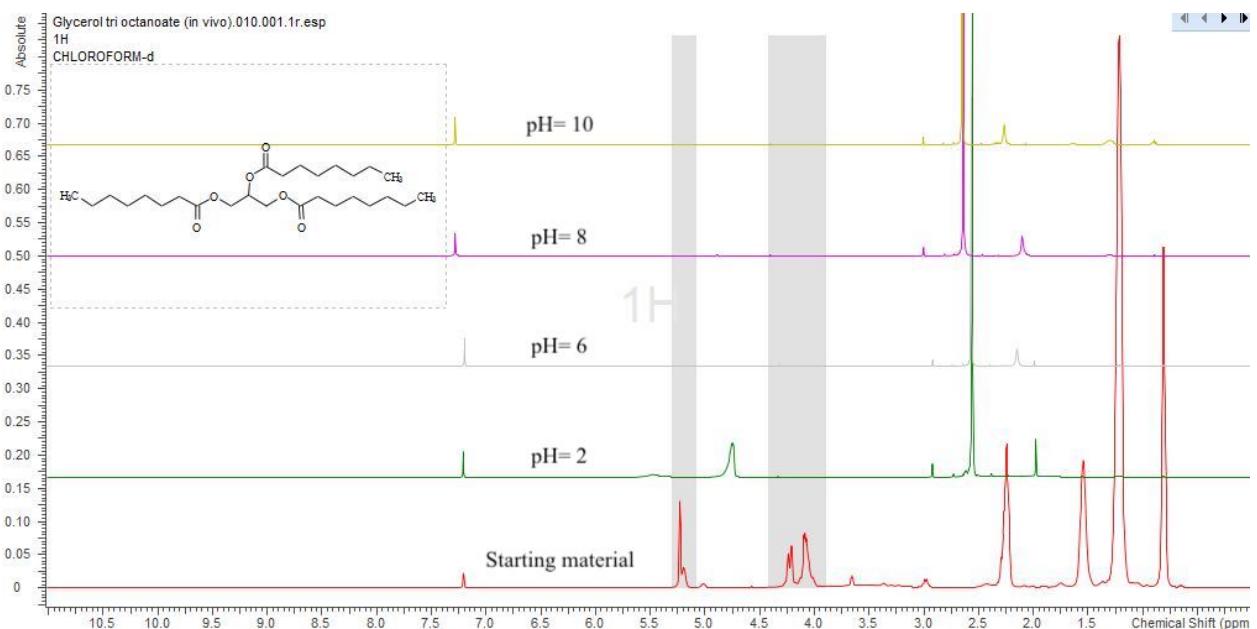
**Figure 3.4** illustrates the <sup>1</sup>H NMR spectrum of the samples after 96 hours exposure in different pH buffers. According to <sup>1</sup>H NMR spectral analysis, erythritol tetra acetoacetate ester (**16**) does not undergo hydrolysis under acidic media. No hydrolysis occurred at pH= 2 and pH= 6. However, partial hydrolysis occurred under basic condition at pH= 8 and pH= 10. Also, from the <sup>1</sup>H NMR signal integration it was observed that more hydrolysis occurred at pH= 10 than at pH=8. So, it can be concluded that the more basic the aqueous environment the more rapid ester hydrolysis occurs.



**Figure 3.4:**  $^1\text{H}$  NMR spectral analysis of erythritol tetra acetoacetate ester at different pH values

### 3.2.4 Effect of different pH levels on compound 18

Figure 3.5 illustrates the  $^1\text{H}$  NMR spectrum of the samples after 72 hours exposure to different pH levels.



**Figure 3.5:**  $^1\text{H}$  NMR spectral analysis of glycerol tri octanoate at different pH levels

According to  $^1\text{H}$  NMR spectral analysis, glycerol trioctanoate (**18**) is susceptible to hydrolysis in both acidic and basic media. **18** was completely hydrolyzed to release water soluble components, which washed away during aqueous-organic layer partitioning. None of the organic soluble starting material or hydrolysis products were observed.

### 3.3 Studies of enzymatic hydrolysis activity of ketogenic compounds

Enzymes are the most important factor to metabolize foods during digestion in the body. Therefore, enzymatic analysis is significant for bioactive compounds. However, various analytical techniques have been widely used such as UV spectroscopy, gas chromatography (GC), high performance liquid chromatography (HPLC), liquid chromatography-mass spectrometry (LC-MS) to do enzymatic assays. Protocols were developed and implemented for the testing of enzymatic hydrolysis activity and discussed here in **Chapter 3**.

Carboxylesterases are one of the key proteins to hydrolyze esters, amides and thioesters in the body.<sup>122-123</sup> In 1961, Alder and Kistiakowsky first isolated carboxylesterases from porcine liver (PLE).<sup>124</sup> To catalyze hydrolysis of meso diesters, use of PLE is very popular today.<sup>125-127</sup> Apart from these, for its excellent hydrolysis activity among wide range of substances, PLE is being very commonly used to determine enzymatic hydrolysis of different esters and amides.<sup>128</sup>

Lipases are another kind of enzymes widely used for the analysis of enzymatic hydrolysis of bioactive compounds. In 1991, *Burkholderia cepacian* lipase was cloned from *Burkholderia cepacian* bacteria and hereafter commercially used under the name of Amano PS lipase.<sup>129</sup> As lipase enzymes play an important role to hydrolyze dietary fats and can hydrolyze a wide range of substances, it has been considered to determine enzymatic hydrolysis of triacyl glycerides and novel synthetic ketogenic compounds.<sup>130,131</sup>

Therefore, protocols were developed to measure enzymatic hydrolysis activity of **16** and **18** using porcine liver esterase (PLE) and Amano PS lipase, and  $^1\text{H}$  NMR spectroscopy was used as an analytical method to track hydrolysis activity in different time intervals.

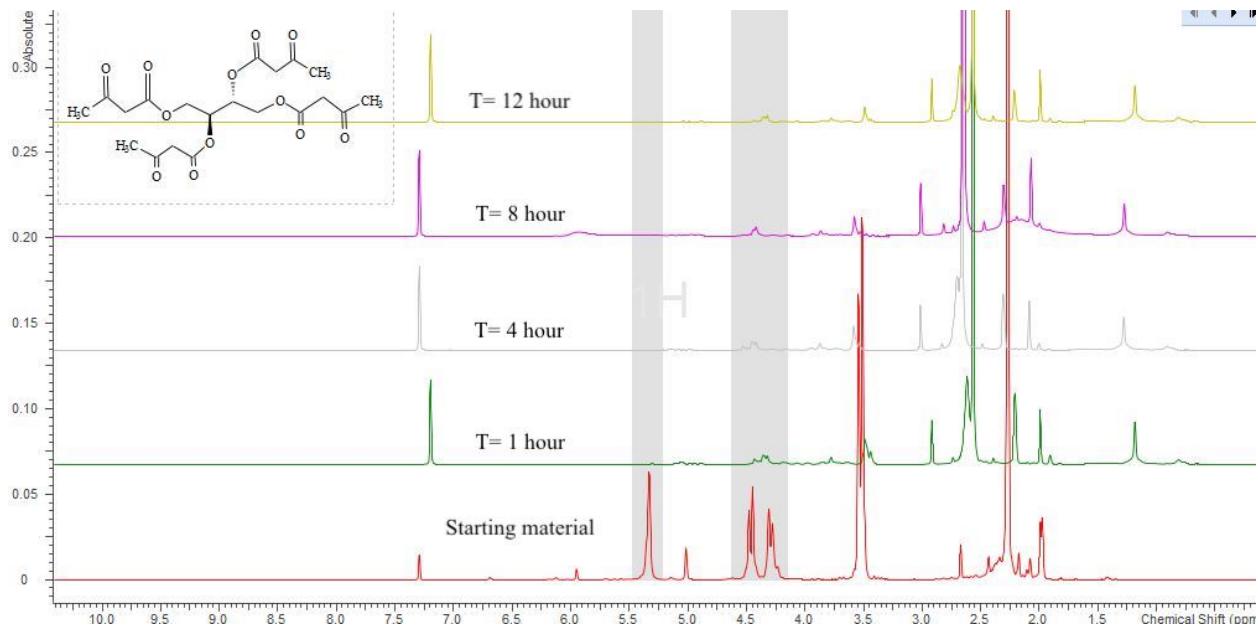
### ***3.3.1 Protocol for porcine liver esterase hydrolysis assay of ketogenic compounds***

9 mg of crude porcine liver esterase was dissolved in a 50 mM phosphate buffer solution (3 mL) at pH= 7 and allowed to stir for 30 minutes at 37°C to activate the enzyme. Compound **16** (19.69 mg, 0.043 mmol) and **18** (20.21 mg, 0.043 mmol) were each dissolved in 0.2 mL of DMSO and a 2 mL of 50 mM phosphate buffer solution at pH= 7 was added dropwise while stirring at 360 Hz. The spin was reduced to 120 Hz and solution was heated to 37°C. Porcine liver esterase solution (1 mL) added dropwise. Aliquots (1 mL) were collected at T= 1, 4, 8, 12 hours and rinsed three times with ethyl acetate (2 mL). The organic layers were combined and evaporated by rotary evaporator. The residue was dissolved in  $\text{CDCl}_3$  and analyzed using a 400 MHz NMR spectrometer.

### ***3.3.2 Porcine liver esterase hydrolysis activity on compound 16***

**Figure 3.6** illustrates the  $^1\text{H}$  NMR spectrum of the samples collected in different time intervals to evaluate porcine liver esterase hydrolysis activity on compound **16**.

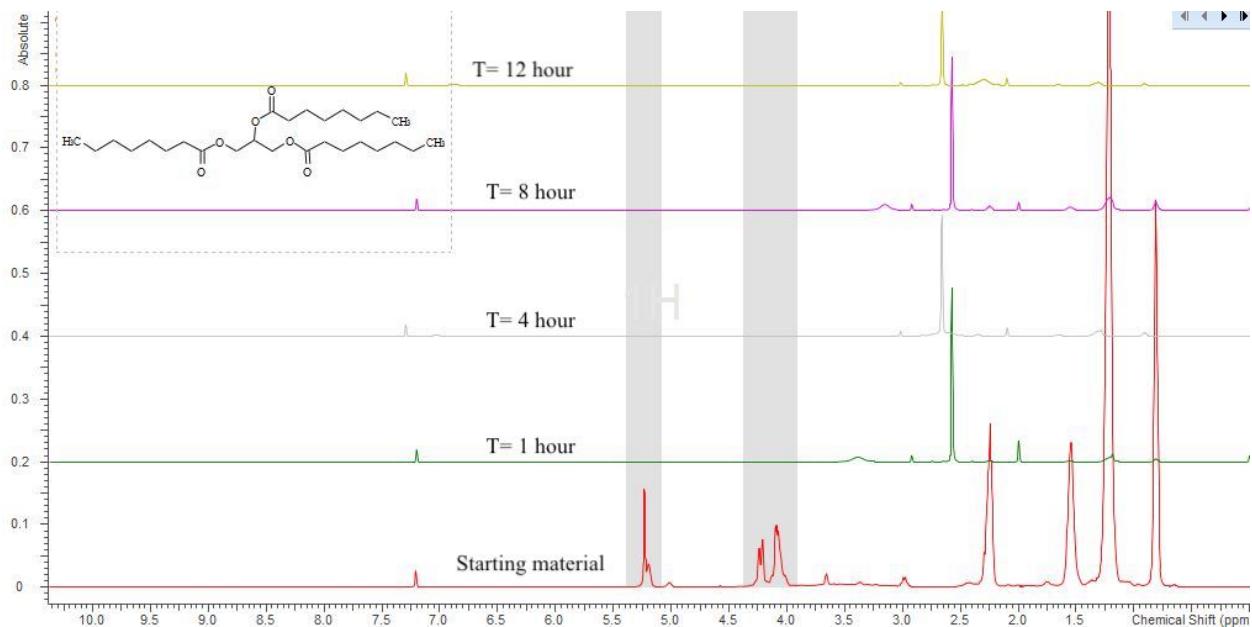
According to the  $^1\text{H}$  NMR spectral analysis, the secondary ester groups of **16** were largely cleaved within an hour and almost totally removed in 4 hours. Based on the signal integral values, 20% of the primary ester groups attached to **16** were cleaved within an hour and this number was increase to 60% after 12 hours.



**Figure 3.6:**  $^1\text{H}$  NMR spectral analysis of **16** in porcine liver esterase assay

### 3.3.3 Porcine liver esterase hydrolysis activity on compound 18

**Figure 3.7** illustrates the  $^1\text{H}$  NMR spectrum of the samples collected in different time intervals to evaluate porcine liver esterase hydrolysis activity on compound **18**.



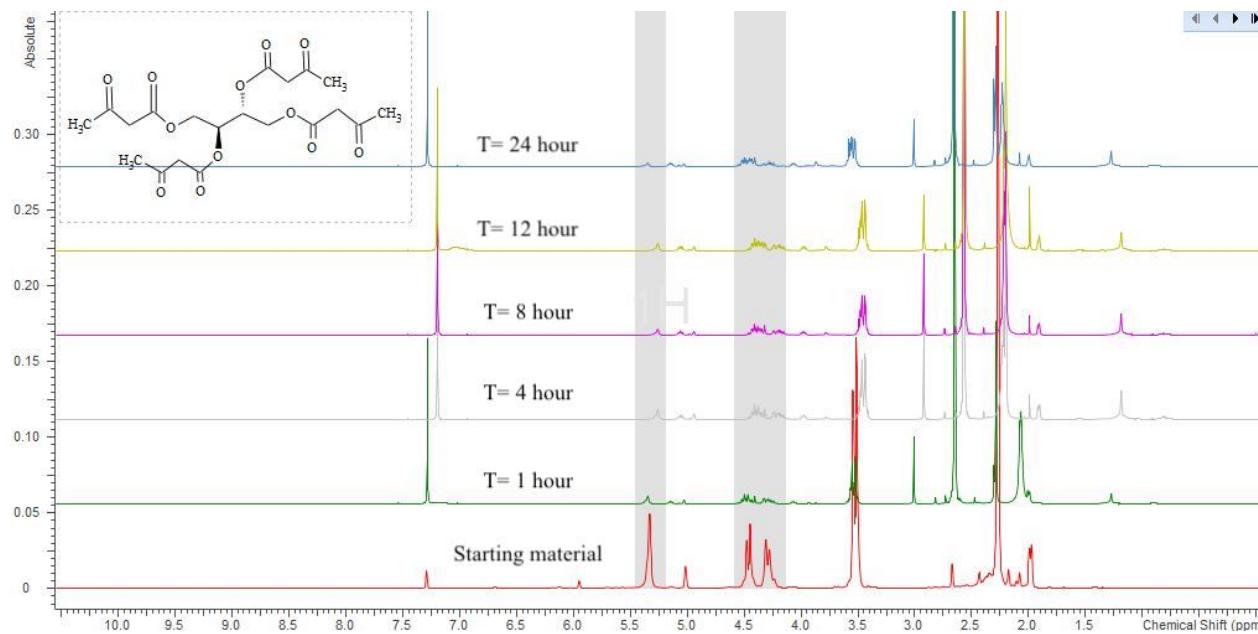
**Figure 3.7:**  $^1\text{H}$  NMR spectral analysis of **18** in porcine liver esterase assay

According to the  $^1\text{H}$  NMR spectral analysis, compound **18** was completely hydrolysed within 1 hour and no glycerol trioctanoate left in the solution.

### **3.3.4 Protocol for Amano PS lipase hydrolysis assay of ketogenic compounds**

6 mg of Amano PS lipase was dissolved in a 4 mL of 50 mM phosphate buffer solution at pH= 7 and allowed to stir for 30 minutes at 50°C to activate the enzyme. Compound **16** (19.69 mg, 0.043 mmol) and **18** (20.21 mg, 0.043 mmol) were each dissolved in 0.2 mL of DMSO and a 2 mL of 50 mM phosphate buffer solution at pH= 7 was added dropwise while stirring at 360 Hz. The spin was reduced to 120 Hz and solution was heated to 50°C. 2 mL of Amano PS lipase solution added dropwise. Different aliquots (0.5 mL) were collected at T= 1, 4, 8, 12, 24 hours and rinsed three times with ethyl acetate (2 mL). The organic layers were combined and evaporated by rotary evaporation. The residue was dissolved in  $\text{CDCl}_3$  and analyzed using a 400 MHz NMR spectrometer.

### **3.3.5 Amano PS lipase hydrolysis activity on compound **16****



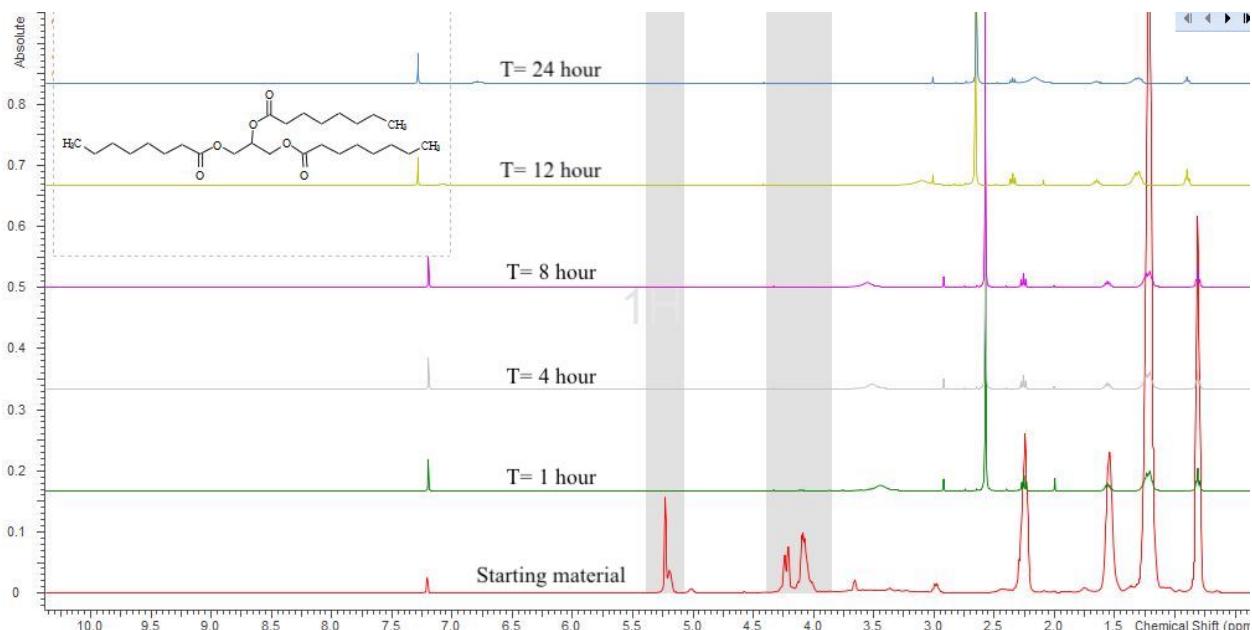
**Figure 3.8:**  $^1\text{H}$  NMR spectral analysis of **16** in Amano PS lipase assay

**Figure 3.8** illustrates the  $^1\text{H}$  NMR spectrum of the samples collected in different time intervals to evaluate Amano PS lipase hydrolysis activity on compound **16**.

Based on the  $^1\text{H}$  NMR spectral analysis, erythritol tetra acetoacetate ester started to be hydrolysed within 1 hour. According to spectral integration value, 50 % of the secondary ester groups of **16** were cleaved in 12 hours of the reaction. 25% of the primary ester groups also cleaved of the **16** in 12 hours reaction time.

### 3.3.6 Amano PS lipase hydrolysis activity on compound **18**

**Figure 3.9** illustrates the  $^1\text{H}$  NMR spectrum of the samples collected in different time intervals to evaluate Amano PS lipase hydrolysis activity on compound **18**.



**Figure 3.9:**  $^1\text{H}$  NMR spectral analysis of **18** in Amano PS lipase assay

According to the  $^1\text{H}$  NMR spectral analysis, all of the secondary ester groups of **18** were cleaved within an hour and glycerol trioctanoate was completely hydrolyzed in 4 hours. Due to high solubility of glycerol in water, signals did not appear on  $^1\text{H}$  NMR spectrum.

### **3.4 In vivo testing of potential ketogenic compounds in mice models**

Mice models have been used to determine efficacy of synthetic ketogenic compounds to induce ketosis. (*2R,3S*)-butane-1,2,3,4-tetrayl tetrakis(3-oxobutanoate) (**16**) and propane-1,2,3-triyl trioctanoate (**18**) are used for this *in vivo* analysis. (*2R,3S*)-butane-1,2,3,4-tetrayl tetrakis(3-oxobutanoate) (**16**) is referred as erythritol tetra acetoacetate ester and propane-1,2,3-triyl trioctanoate (**18**) is referred to as glycerol trioctanoate in all the data-table and graphs in this section.

#### ***3.4.1 Materials and methods for in vivo testing of synthetic ketogenic compounds***

##### **Ethics Declaration:**

All animal procedures were performed in compliance with the NIH Guide for the Care and Use of Laboratory Animals. Animals were used with permission of, and under the guidance of, the USF Institutional Animal Care and Use Committee (IACUC # IS00010024).

##### **Animals:**

Three pairs of C57/BL6 NJ mice were originally purchased from Jackson Laboratory (Bar Harbor, ME, USA) and allowed one-week acclimation period before establishing and propagating a breeding colony according to standard husbandry protocol. All mice were housed in a facility maintained at  $21 \pm 1$  °C with a relative humidity of  $55 \pm 10\%$  and with a 12 h light–dark cycle. 18 mice (9 male and 9 female) ages three to six months from this breeding colony were allowed standard rodent chow and water *ad libitum* preceding and during experimental procedures while remaining housed with original littermates.

##### **Experimental Design:**

Three male and three female C57/BL6 mice were randomly assigned to receive 4g/kg of one of three treatments administered by gavage technique: H<sub>2</sub>O control, (*2R,3S*)-butane-1,2,3,4-

tetrayl tetrakis(3-oxobutanoate) (**16**) and propane-1,2,3-triyl trioctanoate (**18**). Compounds were given for testing to Dr. Dominic D'Agostino's laboratory in the Department of Molecular Pharmacology and Physiology of University of South Florida.

On the day of testing, mice acclimated to the procedural room for 30 minutes prior to testing. During this time, the weights of the mice were taken to compute the proper volume of treatment to administer via gavage. The mice were provided standard rodent chow and water *ad libitum* to ensure their ketones were not >0.7mmol BHB at the baseline measurements so as to not confound the ketogenic effects of the treatments. After the acclimation period, baseline measurements of blood glucose and blood ketone levels were taken via small quantities of blood from the tail using approved methods (removal of scan of tail by small tail injury). Glucose and  $\beta$ -hydroxybutyrate (BHB) were measured using the Precision Xtra<sup>TM</sup> Blood Glucose & Ketone Monitoring System (Abbott Laboratories, Abbott Park, IL).

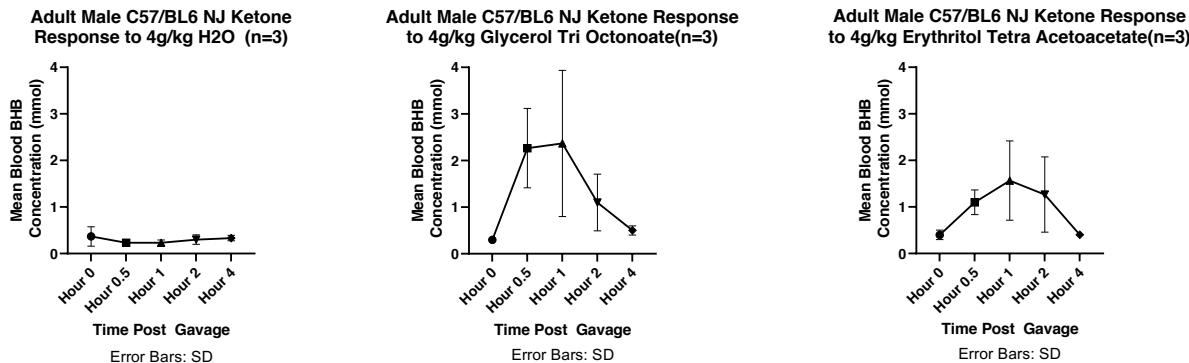
Immediately following baseline readings, each mouse was gavaged with their respective treatment via a 20-gauge gavage needle designated for mice. Following a 4g/kg dose, 4ml/kg of water, 3.76ml/kg of (2R,3S)-butane-1,2,3,4-tetrayl tetrakis(3-oxobutanoate) (**16**) and 4.36 ml/kg of propane-1,2,3-triyl trioctanoate (**18**) were given. Mice were stirred in the cage to ensure non-fasted state. Blood glucose and ketone measurements were taken at 0.5,1,2 and 4 hours post-gavage. The mice were monitored for any physical reaction to the compounds.

Statistics:

Graphpad Prism 9 was used to run multiple one-way ANOVAs with Tukey post-hoc test to address significant differences in blood glucose and blood ketone levels across the three groups, across time, and between genders. Alpha level was set to p<0.05 to address significance.

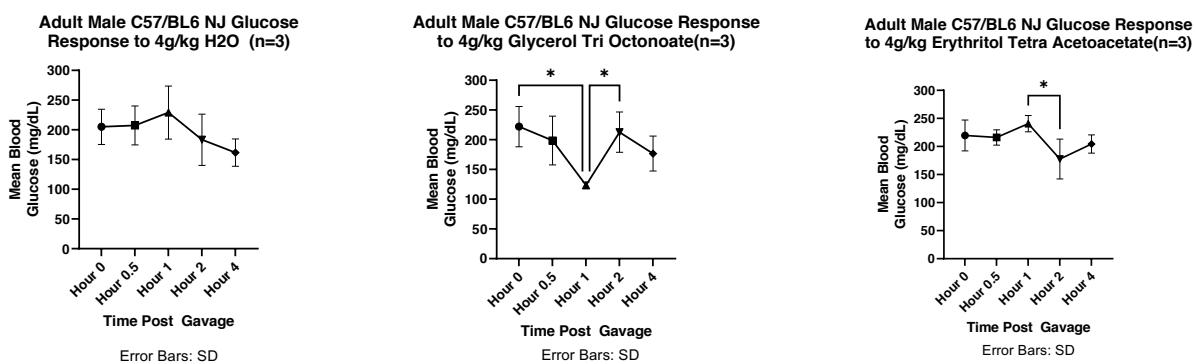
### 3.4.2 Effect of synthetic ketogenic compounds on mice models

All the experimental analysis was done on gender basis. Both ketone body concentrations and glucose concentrations were measured in different time intervals.



**Figure 3.10:** Concentrations of BHB in blood over time in male mice

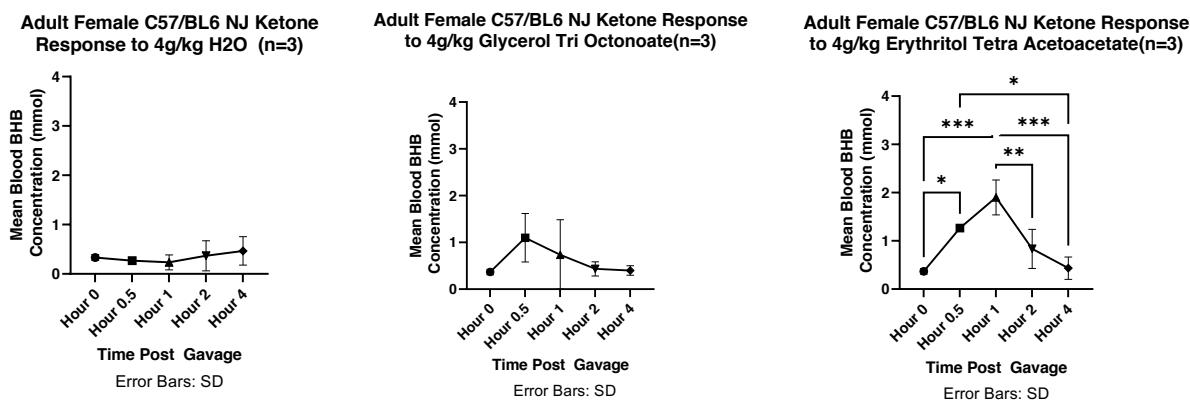
**Figure 3.10** illustrates effect of test compound **16** and **18** on ketone body concentration in the blood of male mice over time. Research data showed that after administration of test ketogenic compounds, all male mice induced ketosis even though concentrations of ketone bodies did not increase to a significant level.



**Figure 3.11:** Concentrations of glucose in blood over time in male mice

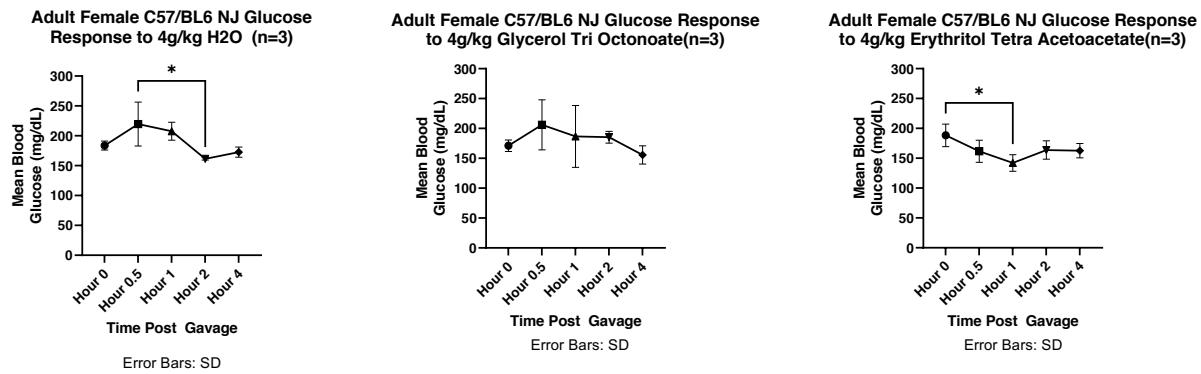
**Figure 3.11** illustrates effect of **16** and **18** on blood glucose level in male mice over time.

There is a significant glucose concentration lowering effect observed for both compound **16** and **18**. Blood glucose concentration dropped from 240 mg/dL to 175 mg/dL between T=1 hour and T=2 hour, after administration of test compound **16**. Similar effect was observed for test compound **18**. After administration glucose concentration in the blood reduced from 225 mg/dL to 110 mg/dL within an hour ( $p < 0.05$ ).



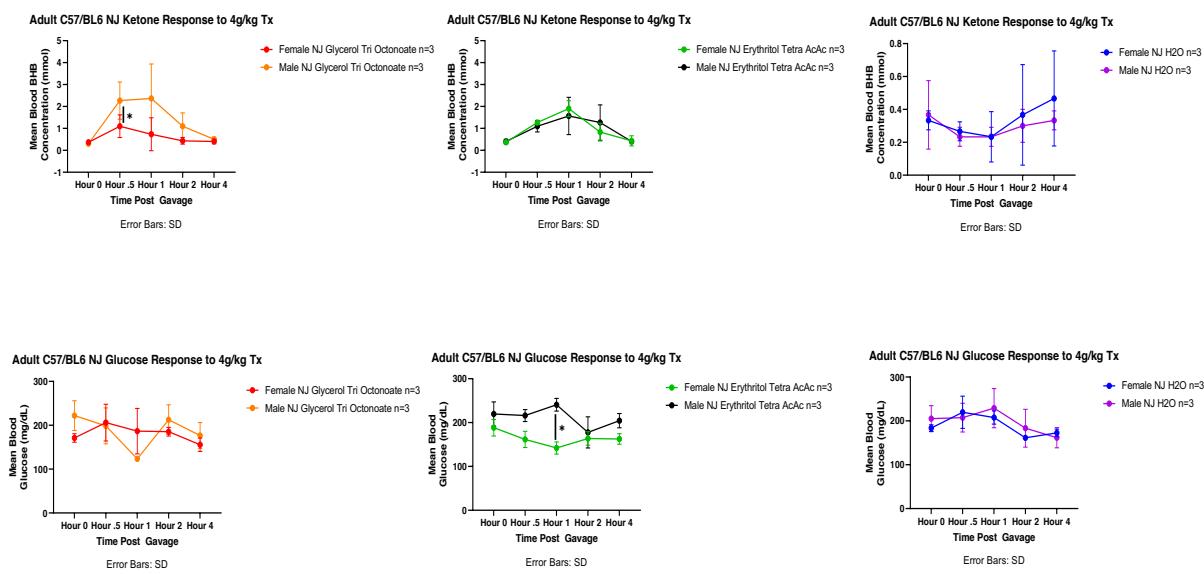
**Figure 3.12:** Concentrations of BHB in blood over time in female mice

**Figure 3.12** illustrates effect of **16** and **18** on ketone body concentrations in the blood of male mice over time. According to data, test compound **16** has significant effect on blood ketone body concentrations in female mice over time. Blood BHB concentration in mice was elevated up to 1.9 mmol from the control (0.3 mmol) within 1 hour ( $p < 0.001$ ). However, there was no significant effect on female mice after administration of test compound **18** over time.



**Figure 3.13:** Concentrations of glucose in blood over time in female mice

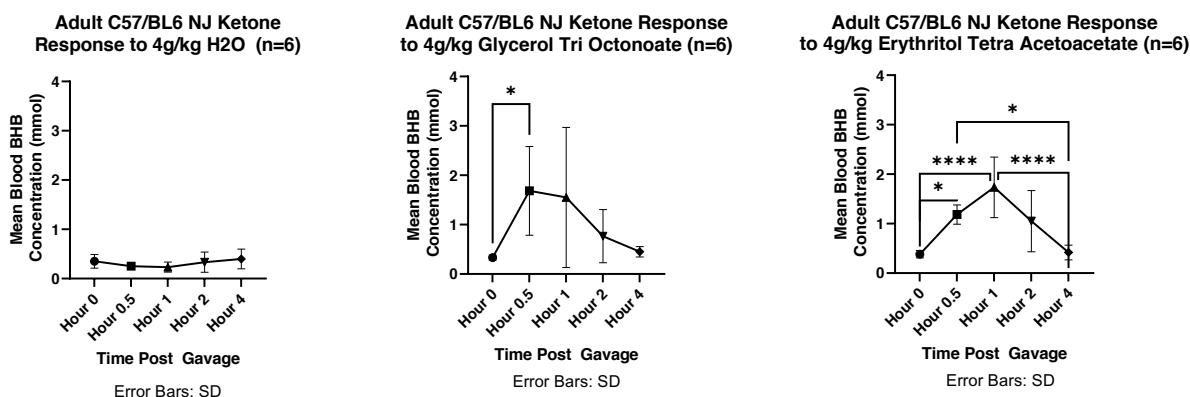
**Figure 3.13** illustrates effect of **16** and **18** on blood glucose level in female mice over time. According to the collected data, effect of test compound **16** and **18** is correlated to ketone body response data described in **Figure 3.11**, which clear indication of ketosis. Upon administration of test compound **16**, blood glucose concentration reduced from 190 mg/dL to



**Figure 3.14:** Blood BHB and glucose concentrations over time in male and female mice

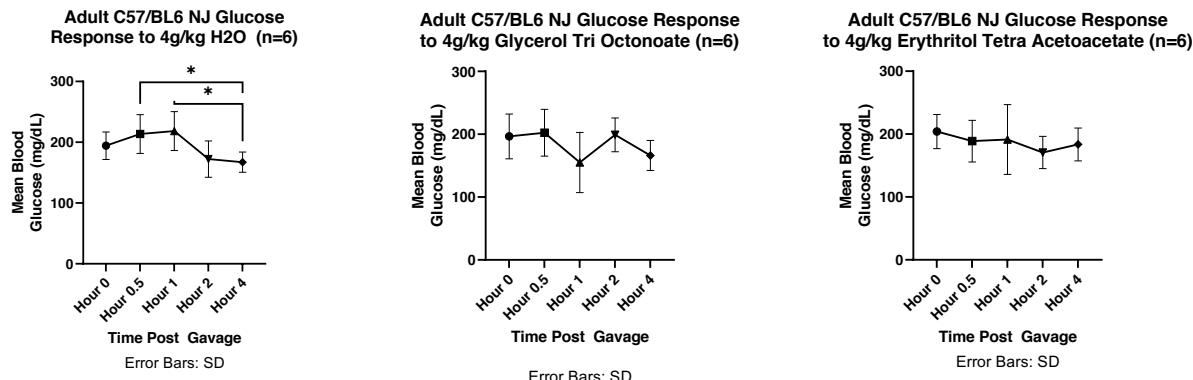
140 mg/dL within one hour while there was no significant reduction was observed upon administration of test compound **18**.

**Figure 3.14** depicts comparison of the effect of test compound erythritol tetra acetoacetate ester and glycerol trioctanoate on blood BHB and glucose concentrations in male and female mice. Interestingly, erythritol tetra acetoacetate ester increased ketone body concentrations with a correlation to the reduction of glucose concentrations in the blood more effectively in the female mice over male, whereas glycerol trioctanoate increased ketone body concentration with correlation to reduction of glucose concentration in the blood more effectively in the male mice over female.



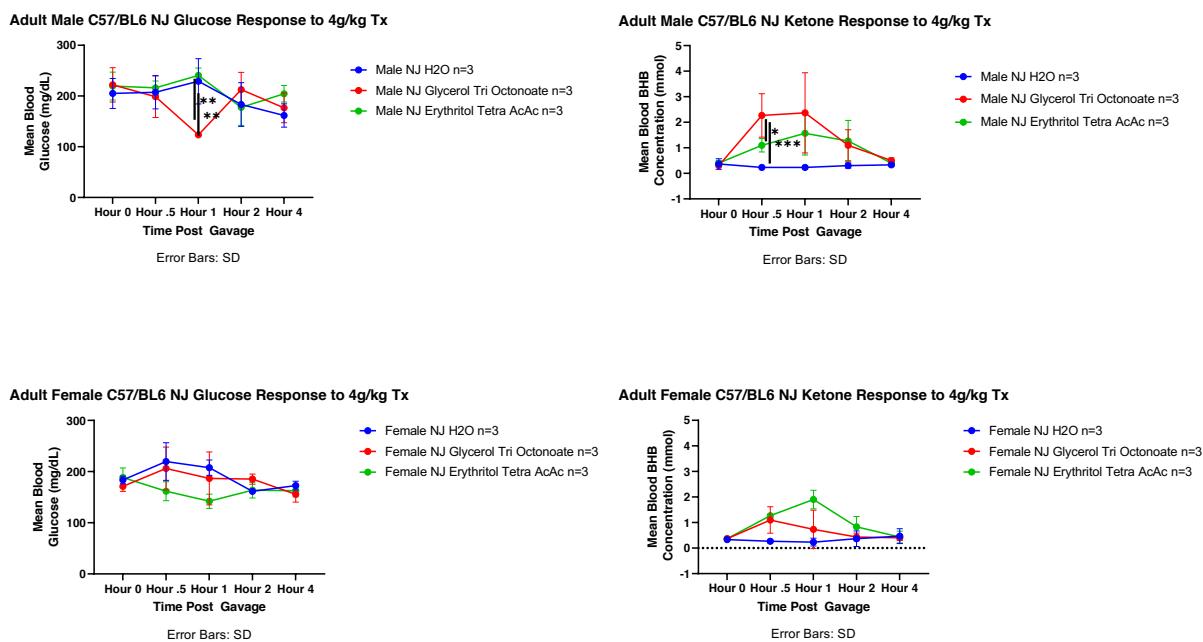
**Figure 3.15:** Comparison of the effect of synthetic ketogenic compounds on blood BHB concentrations over time in mice

**Figure 3.15** illustrates the comparison of the effect erythritol tetra acetoacetate ester and glycerol trioctanoate on blood BHB concentrations over time in mice. It was observed that while inducing ketosis glycerol trioctanoate elevated blood BHB concentration from 0.2 mmol to 0.75 mmol within half an hour, whereas erythritol tetra acetoacetate ester needed one hour for the same effect.



**Figure 3.16:** Comparison of the effect of synthetic ketogenic compounds on blood glucose concentrations over time in mice

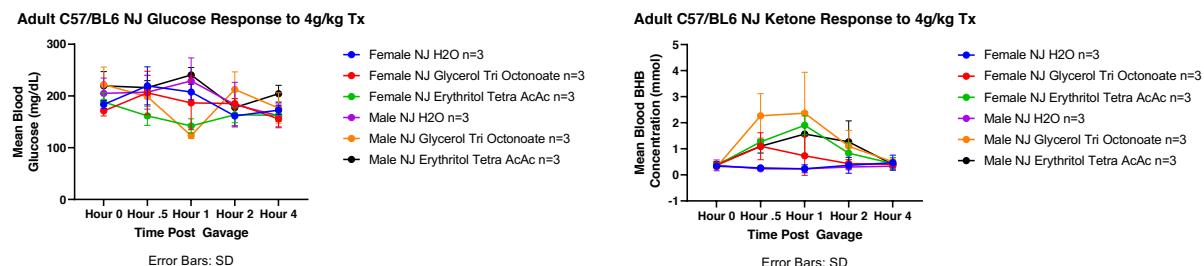
**Figure 3.16** illustrates the comparison of the effect of erythritol tetra acetoacetate ester and glycerol trioctanoate on the blood glucose concentrations over time in mice. It was found



**Figure 3.17:** Effect of synthetic ketogenic compounds on the blood BHB and glucose concentration over time in male and female mice

that there is a significant glucose concentration drop in one hour after the administration of glycerol trioctanoate.

**Figure 3.17** illustrates glycerol trioctanoate provides more ketogenic effect over time on male mice compared to the females, whereas erythritol tatraacetoacetate ester provides a more ketogenic effect over time on female mice compared to male.



**Figure 3.18:** Overall effect of synthetic ketogenic compounds on blood BHB and glucose concentration over time in mice

Upon considering the overall effect of these two test compounds, it can be concluded that erythritol tetra acetoacetate ester has a faster effect in terms of blood BHB concentration elevation over time, while glycerol trioctanoate induces ketosis for a longer period of time compared to erythritol tetra acetoacetate ester providing nearly same ketogenic effect (**Figure 3.18**).

### 3.5 Conclusion

According to this research hypotheses, ketogenic molecules can be synthesized in the laboratory and provide similar advantages as a food supplement without changing the regular diet. A number of molecules were synthesized in this project and two of those were selected and tested for ketogenicity. In compliance with the hypotheses, both of the compounds showed their

potential as a ketogenic supplement *in vitro* and *in vivo* testing. Compounds are susceptible to hydrolyze mostly under basic conditions as well as common carboxylesterase and lipase enzymes. Both of the test compounds induce ketosis on various group of mice models based on sex difference which justifies this research project hypotheses. This primary level of study and research outcome deserves further exploration of other potential ketogenic compounds from the perspective of synthetic organic chemists and consumers of ketogenic diet. According to this research outcome, various other fatty acid esters and acetoacetate esters of polyols are the most promising candidate for further investigation and exploration to enrich the library of novel synthetic ketogenic molecules.

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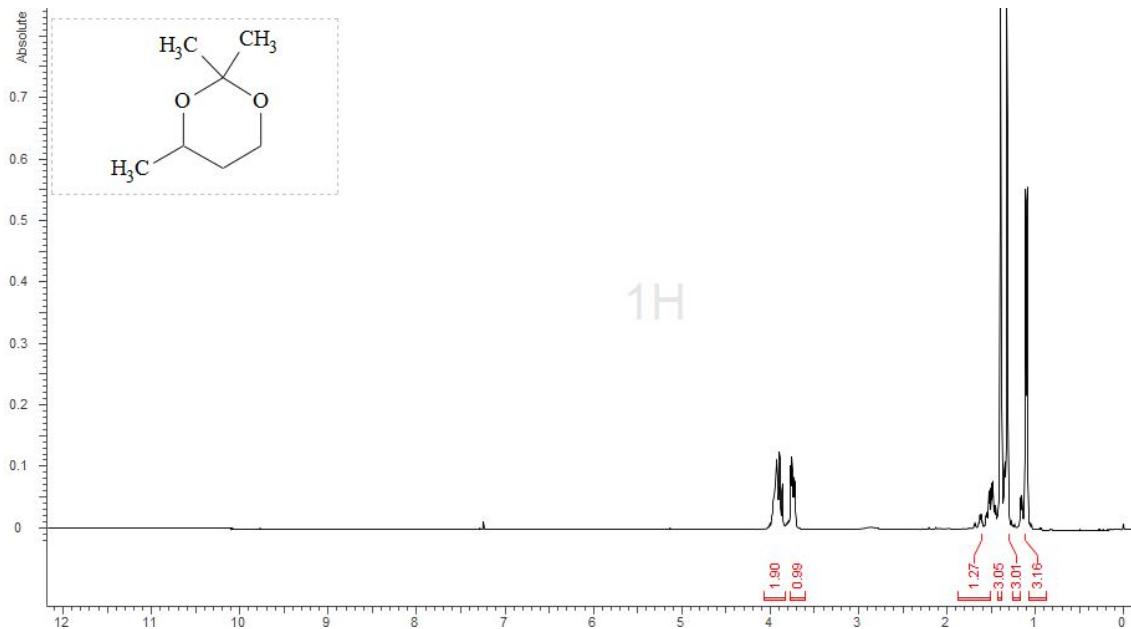
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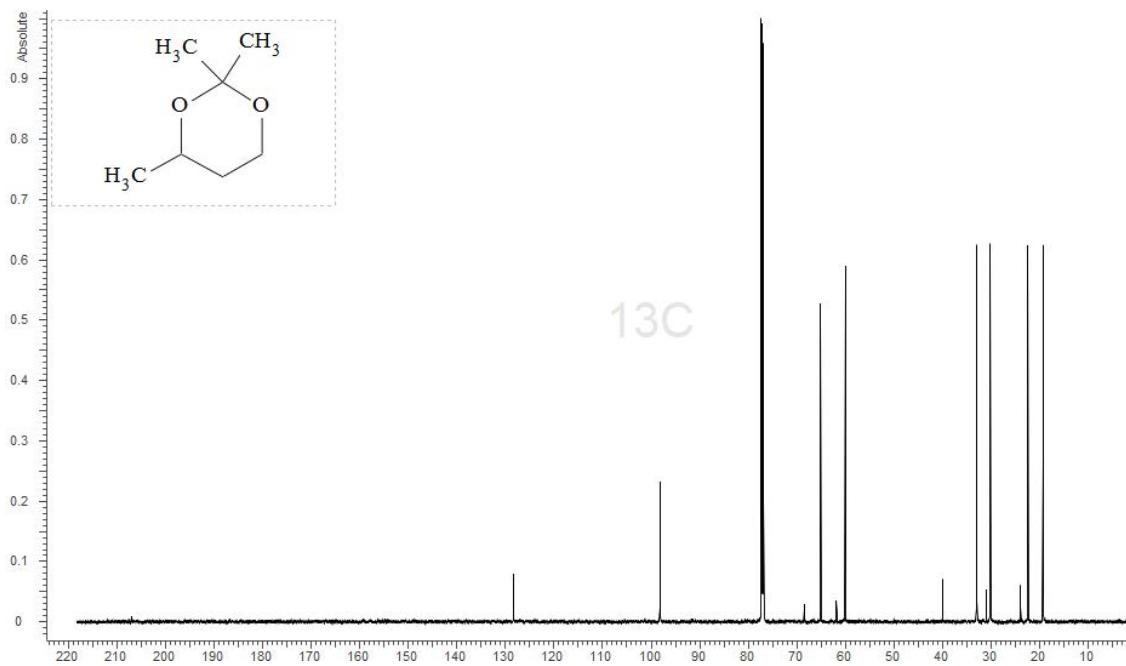
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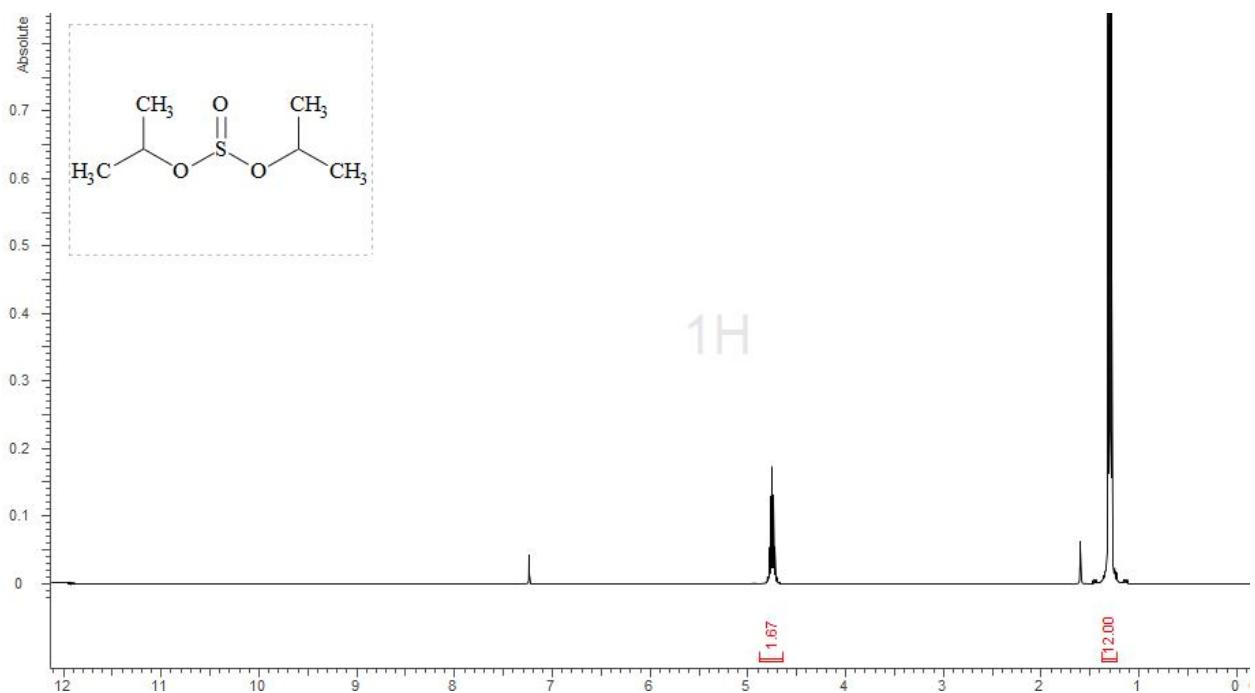
## Appendix A: Chapter 2 $^1\text{H}$ and $^{13}\text{C}$ NMR Spectra



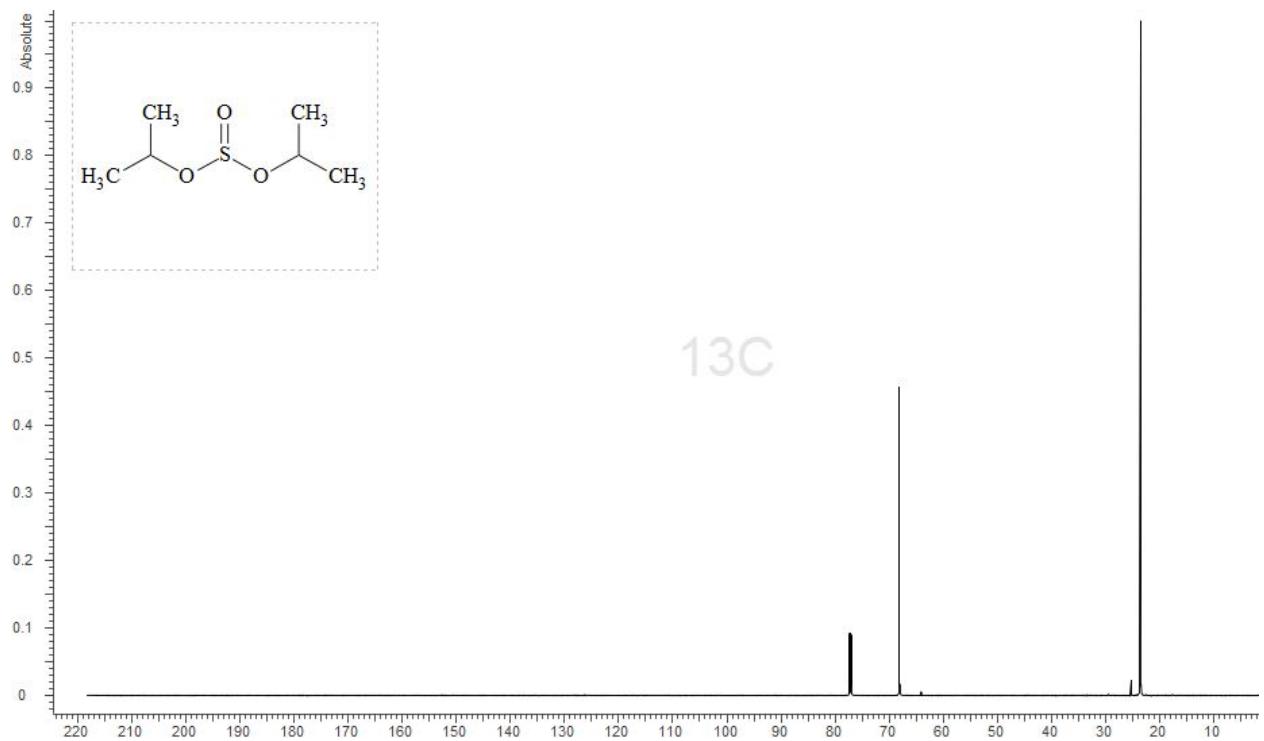
**Figure 4.1:**  $^1\text{H}$  NMR (400 MHz, Chloroform-*d*) spectrum of **1**



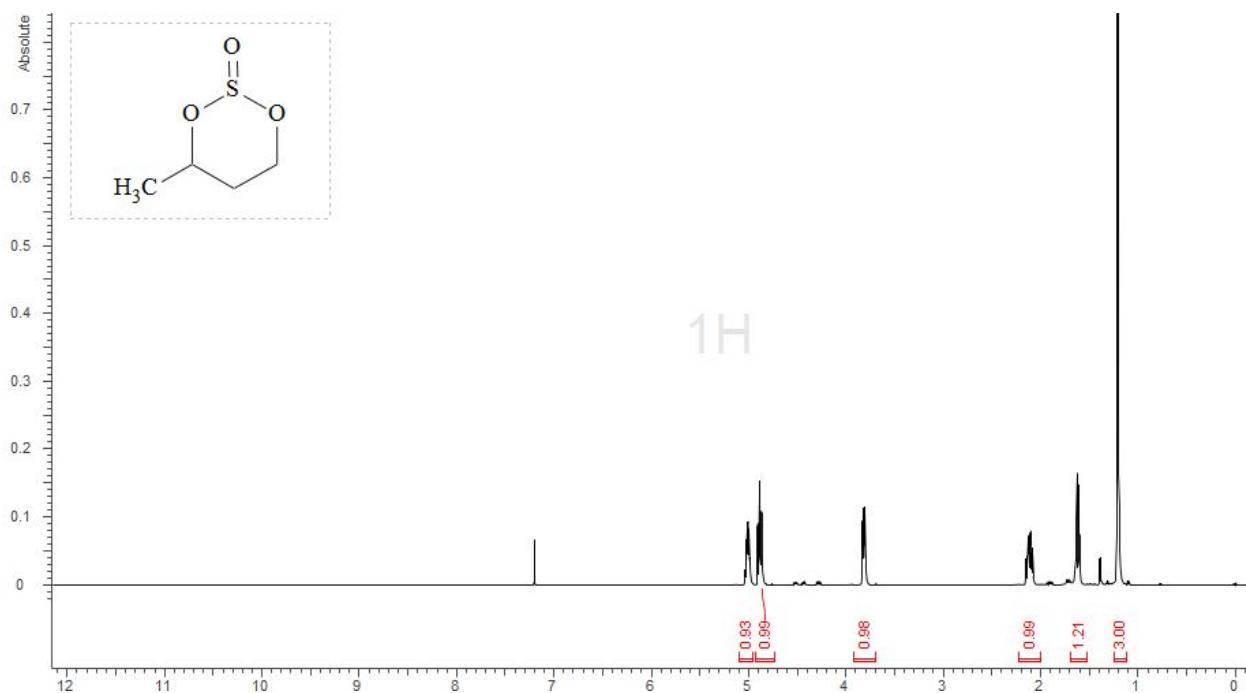
**Figure 4.2:**  $^{13}\text{C}$  NMR (151 MHz, Chloroform-*d*) spectrum of **1**



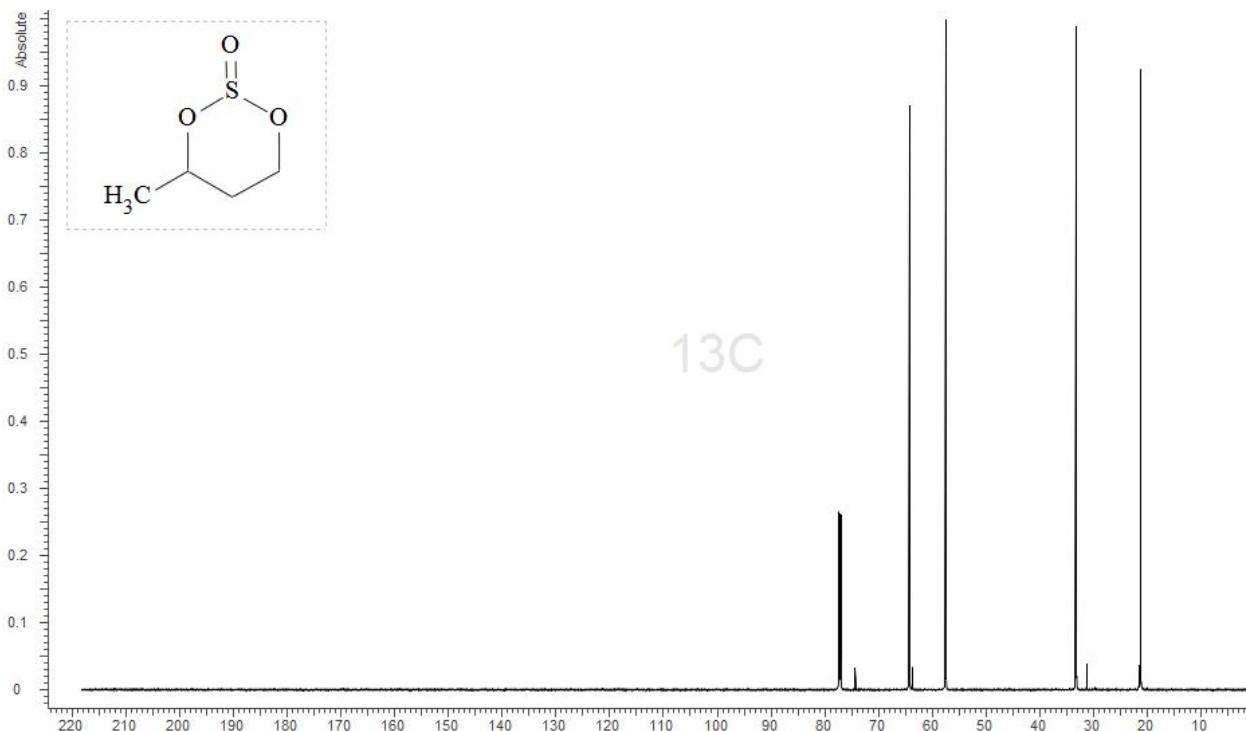
**Figure 4.3:**  $^1\text{H}$  NMR (400 MHz, Chloroform-*d*) spectrum of **2**



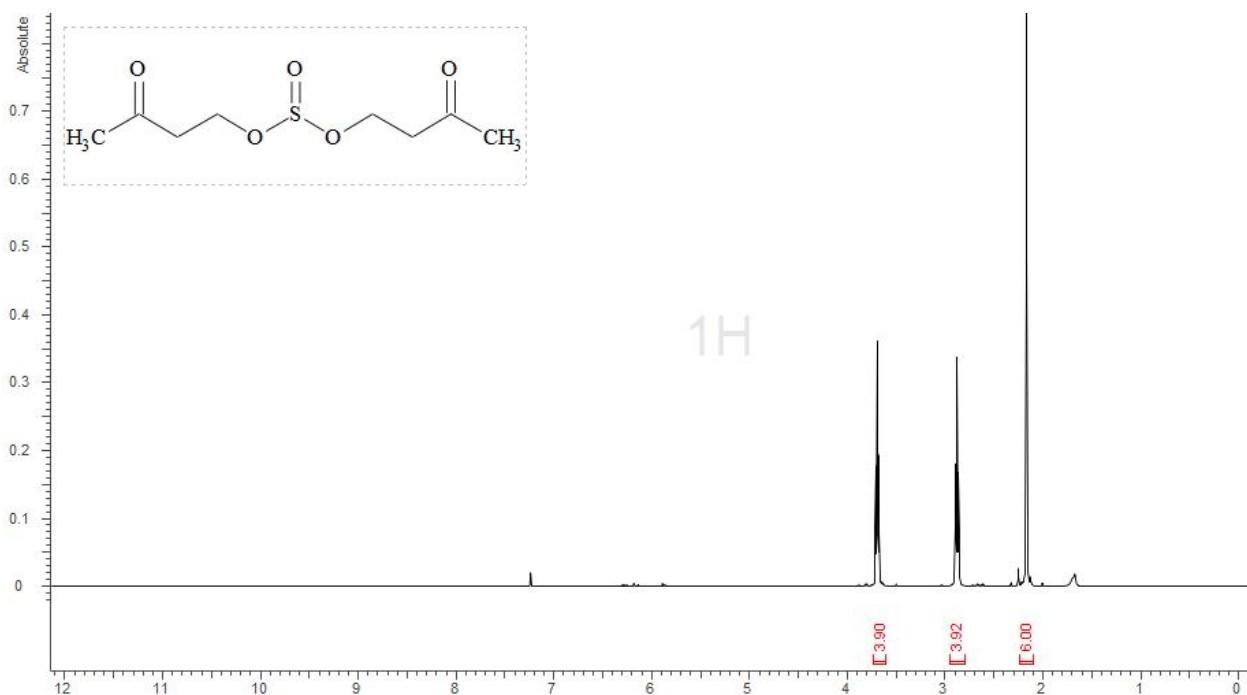
**Figure 4.4:**  $^{13}\text{C}$  NMR (151 MHz, Chloroform-*d*) spectrum of **2**



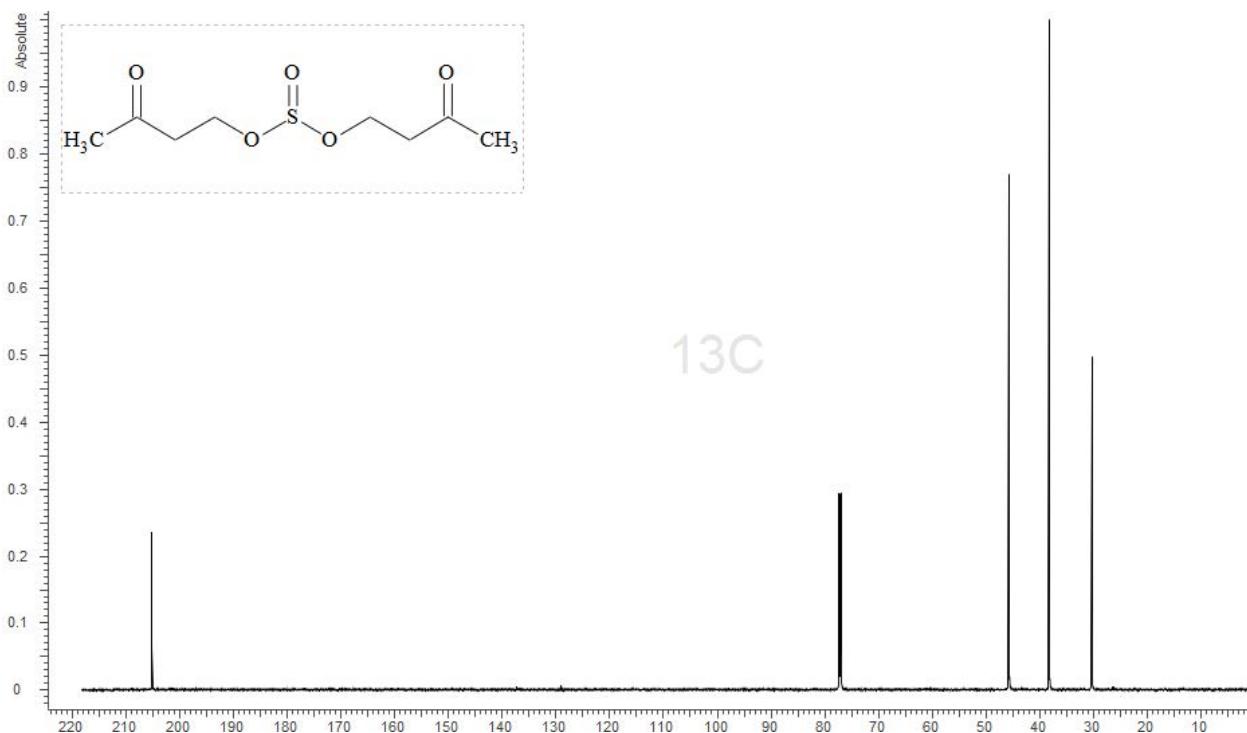
**Figure 4.5:**  $^1\text{H}$  NMR (600 MHz, Chloroform-*d*) spectrum of **3**



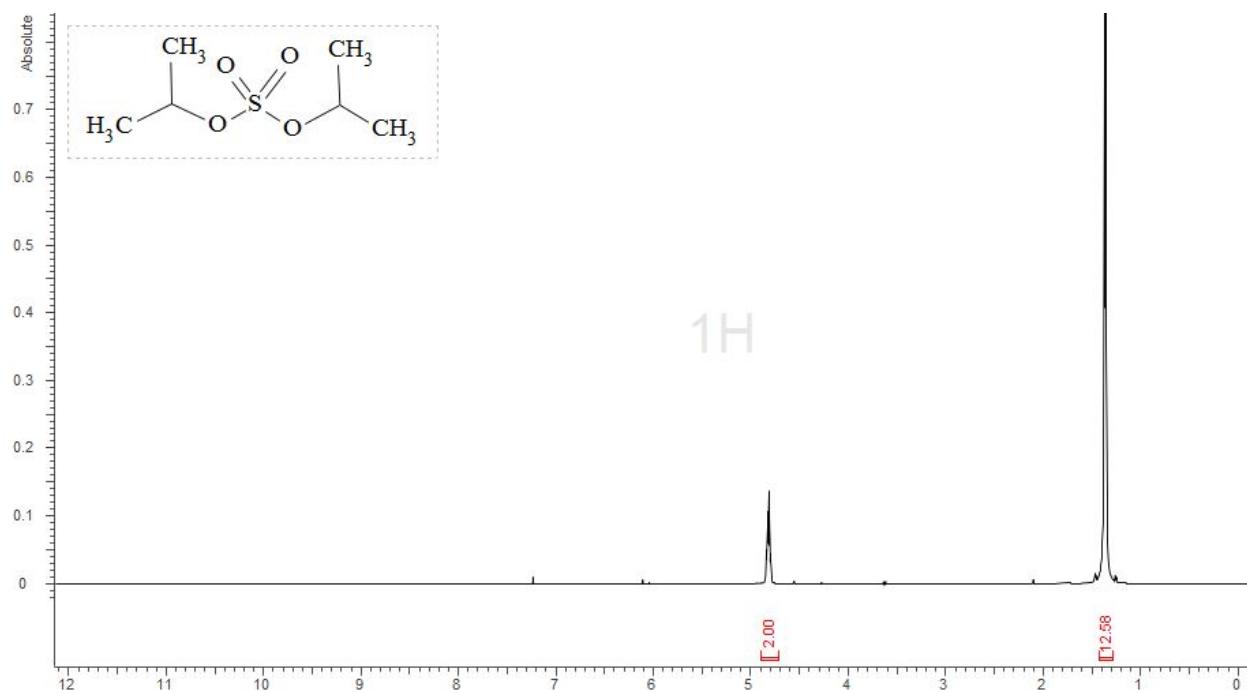
**Figure 4.6:**  $^{13}\text{C}$  NMR (151 MHz, Chloroform-*d*) spectrum of **3**



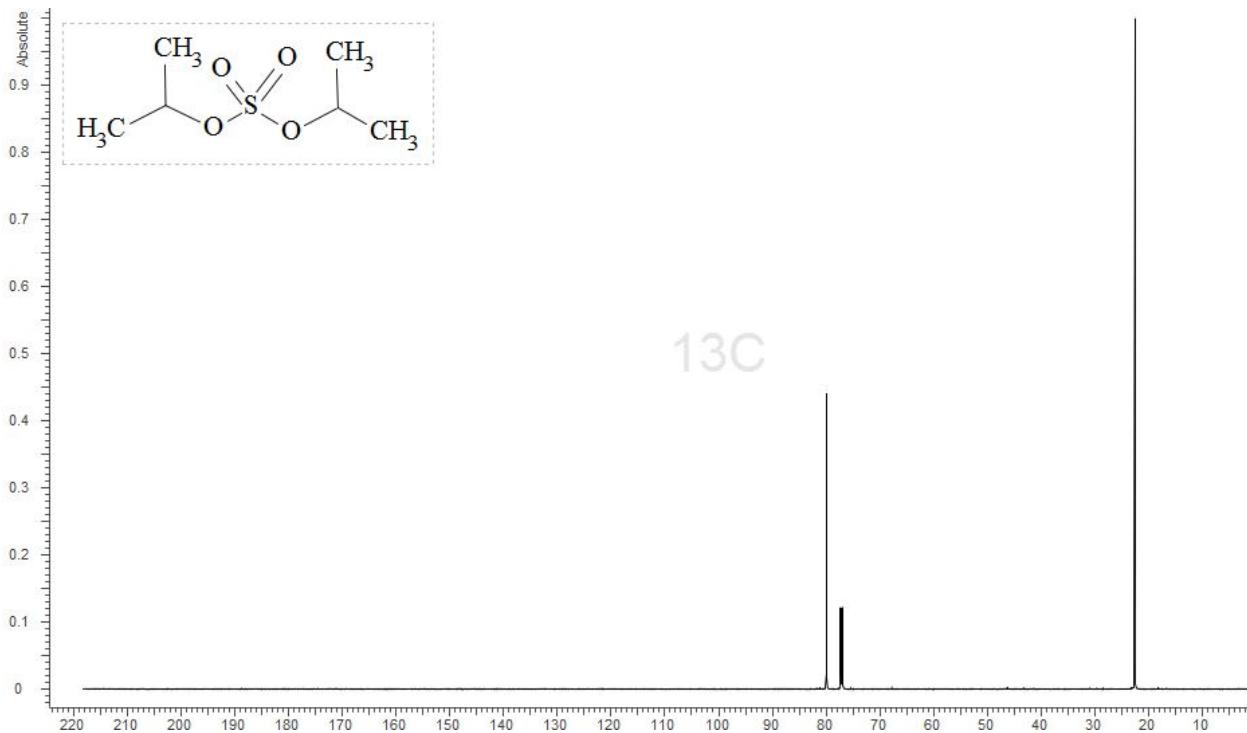
**Figure 4.7:**  $^1\text{H}$  NMR (400 MHz, Chloroform-*d*) spectrum of 4



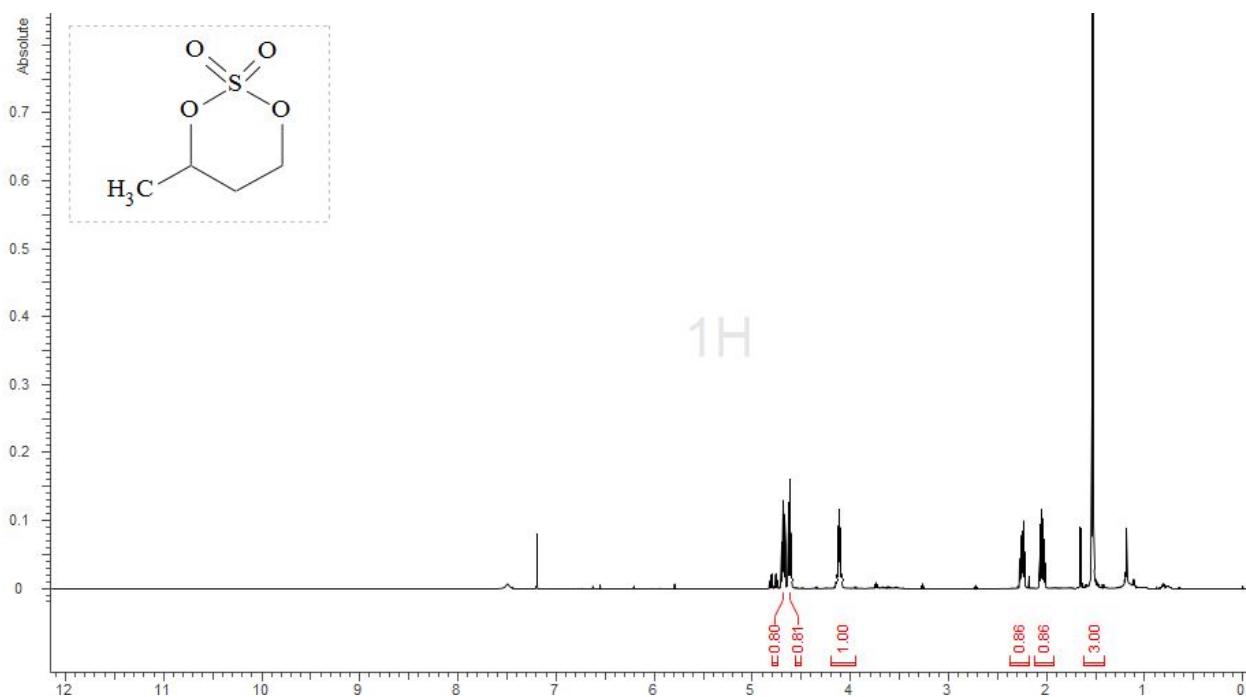
**Figure 4.8:**  $^{13}\text{C}$  NMR (151 MHz, Chloroform-*d*) spectrum of 4



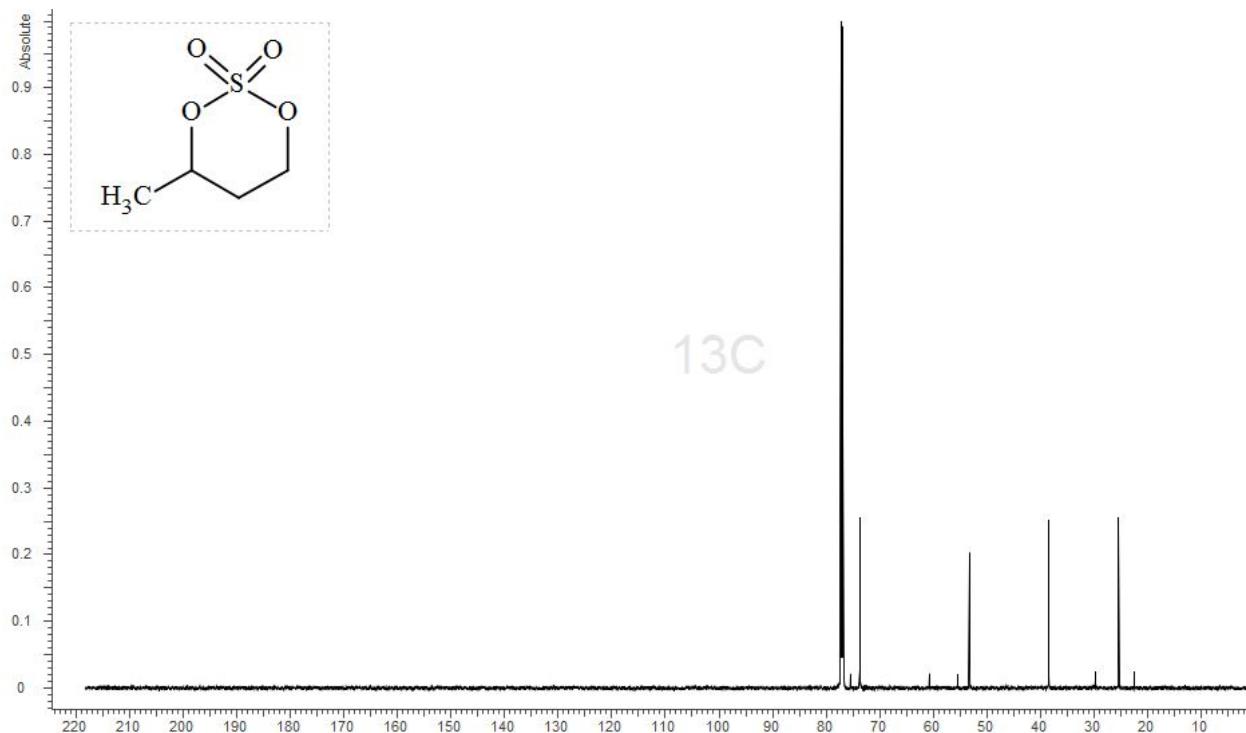
**Figure 4.9:**  $^1\text{H}$  NMR (400 MHz, Chloroform-*d*) spectrum of **5**



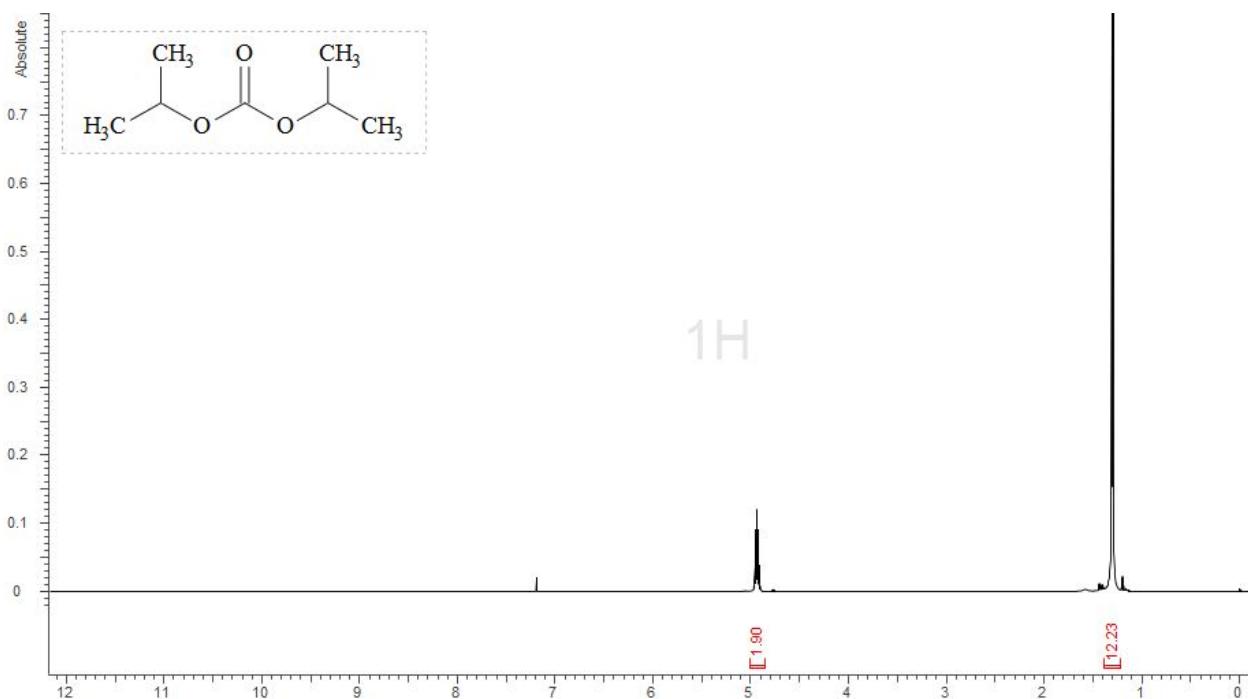
**Figure 4.10:**  $^{13}\text{C}$  NMR (151 MHz, Chloroform-*d*) spectrum of **5**



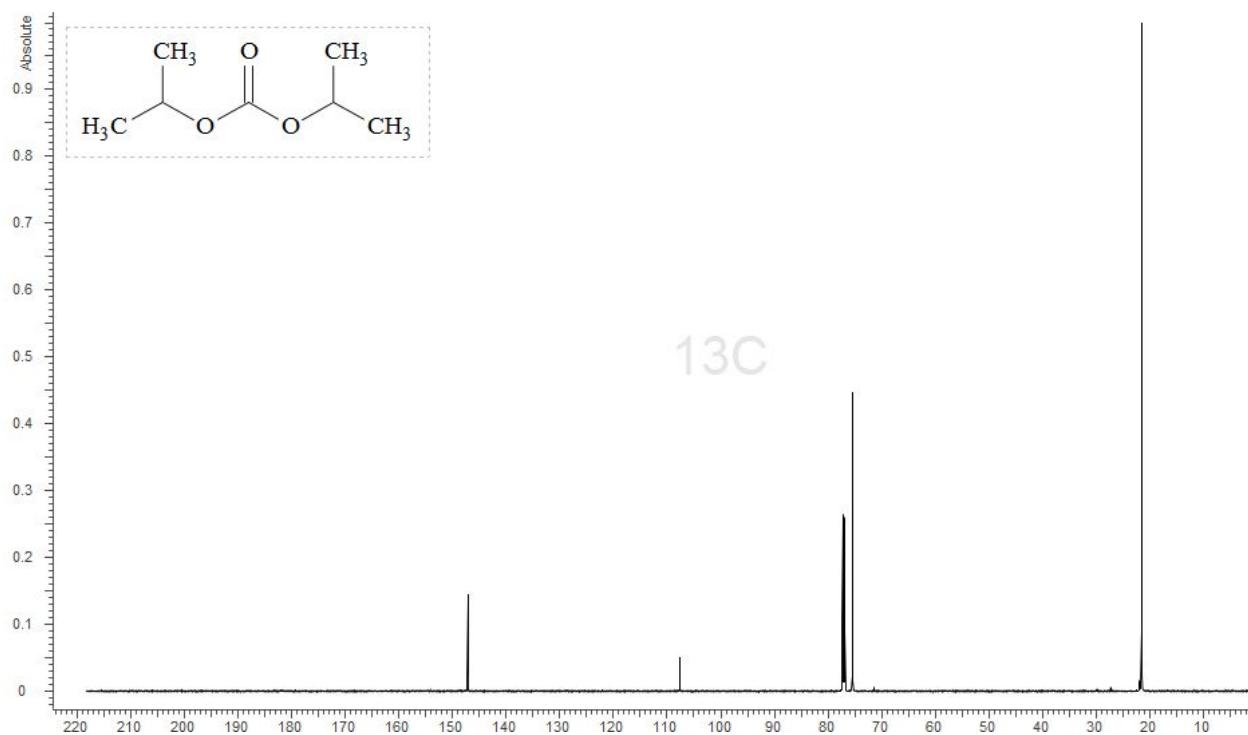
**Figure 4.11:**  $^1\text{H}$  NMR (400 MHz, Chloroform- $d$ ) spectrum of 6



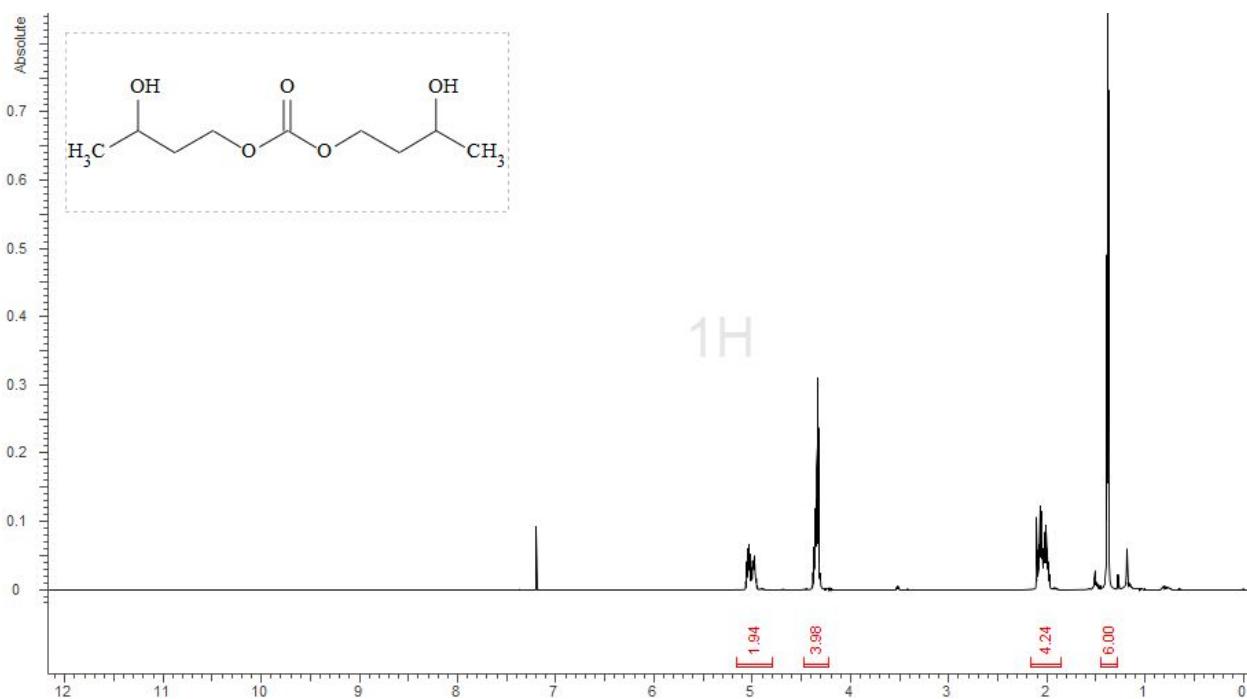
**Figure 4.12:**  $^{13}\text{C}$  NMR (151 MHz, Chloroform- $d$ ) spectrum of 6



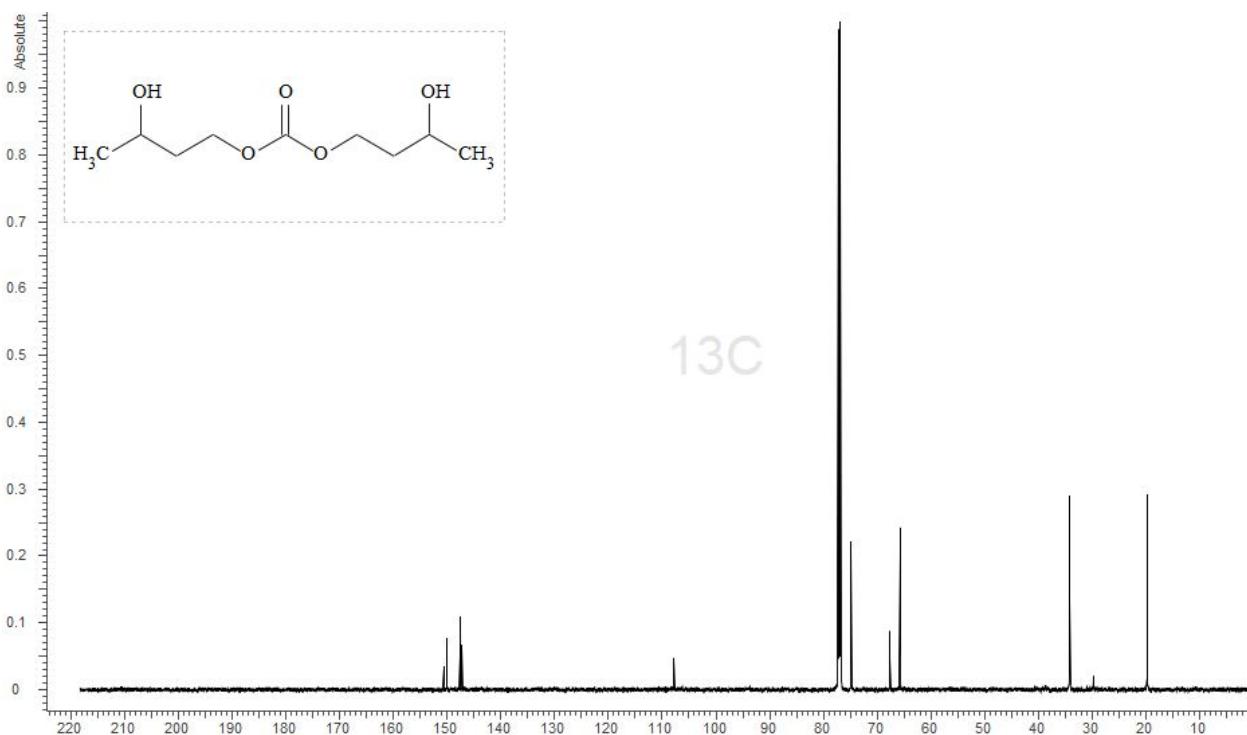
**Figure 4.13:**  $^1\text{H}$  NMR (600 MHz, Chloroform-*d*) spectrum of 7



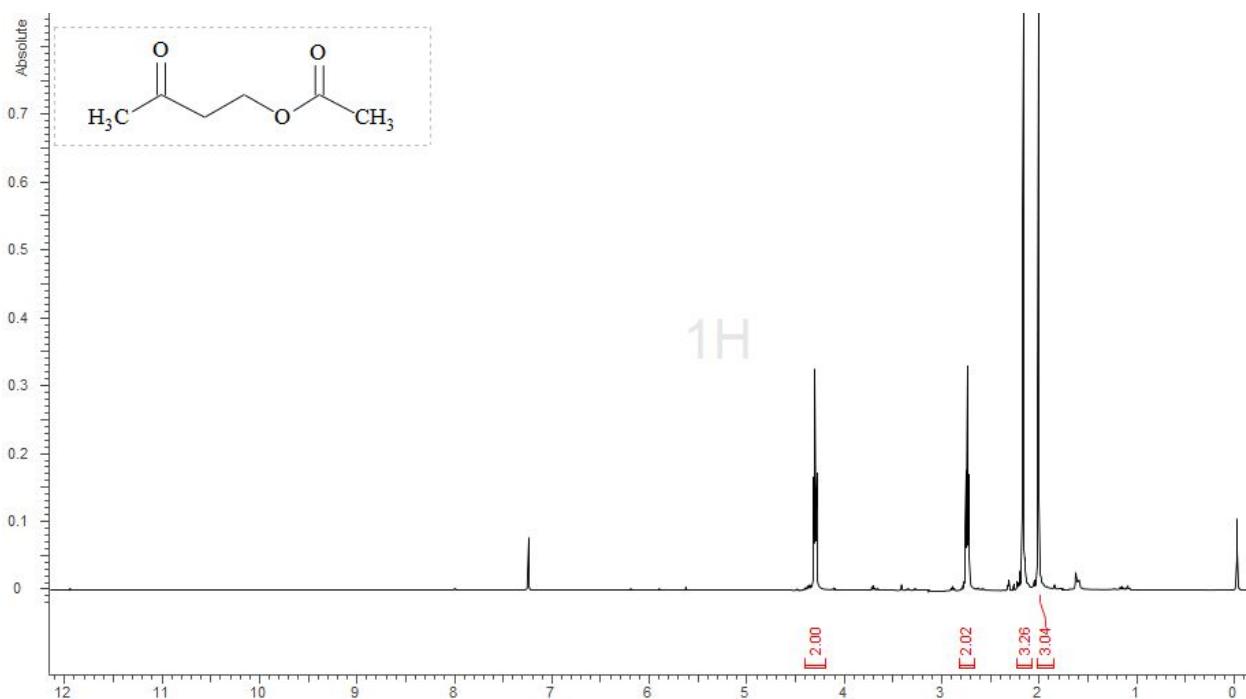
**Figure 4.14:**  $^{13}\text{C}$  NMR (151 MHz, Chloroform-*d*) spectrum of 7



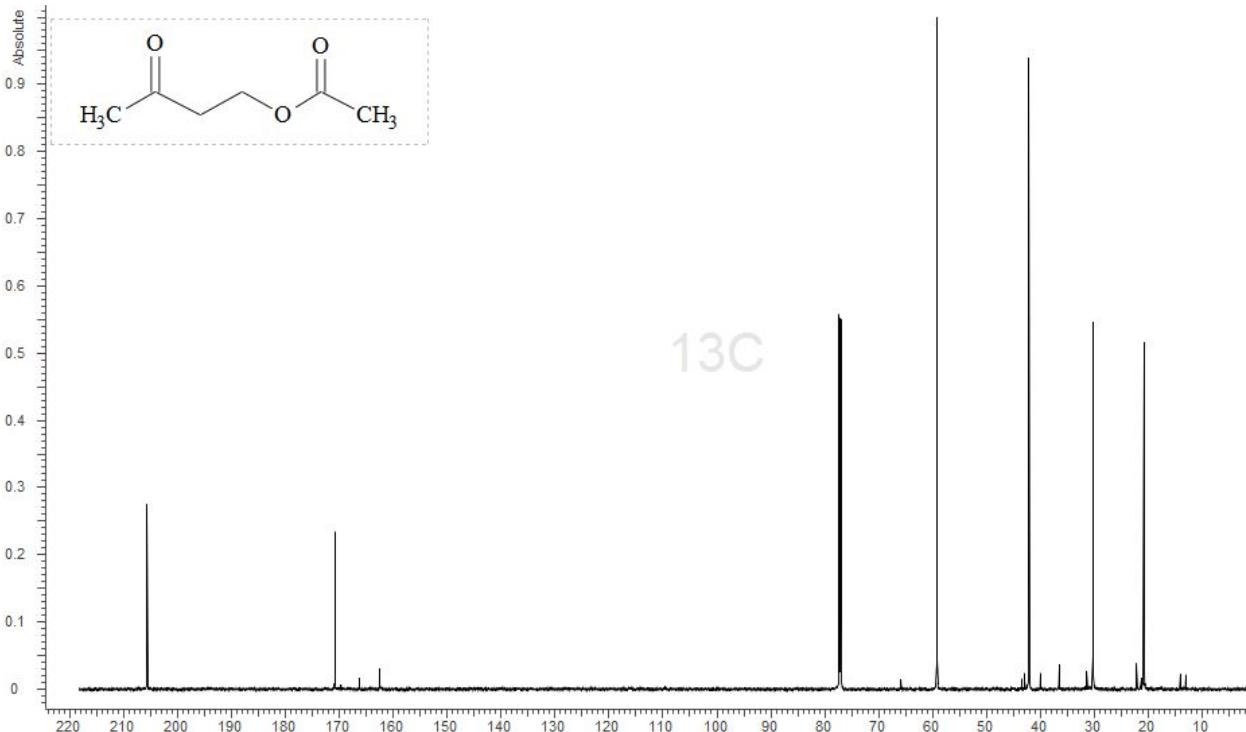
**Figure 4.15:**  $^1\text{H}$  NMR (600 MHz, Chloroform-*d*) spectrum of **8**



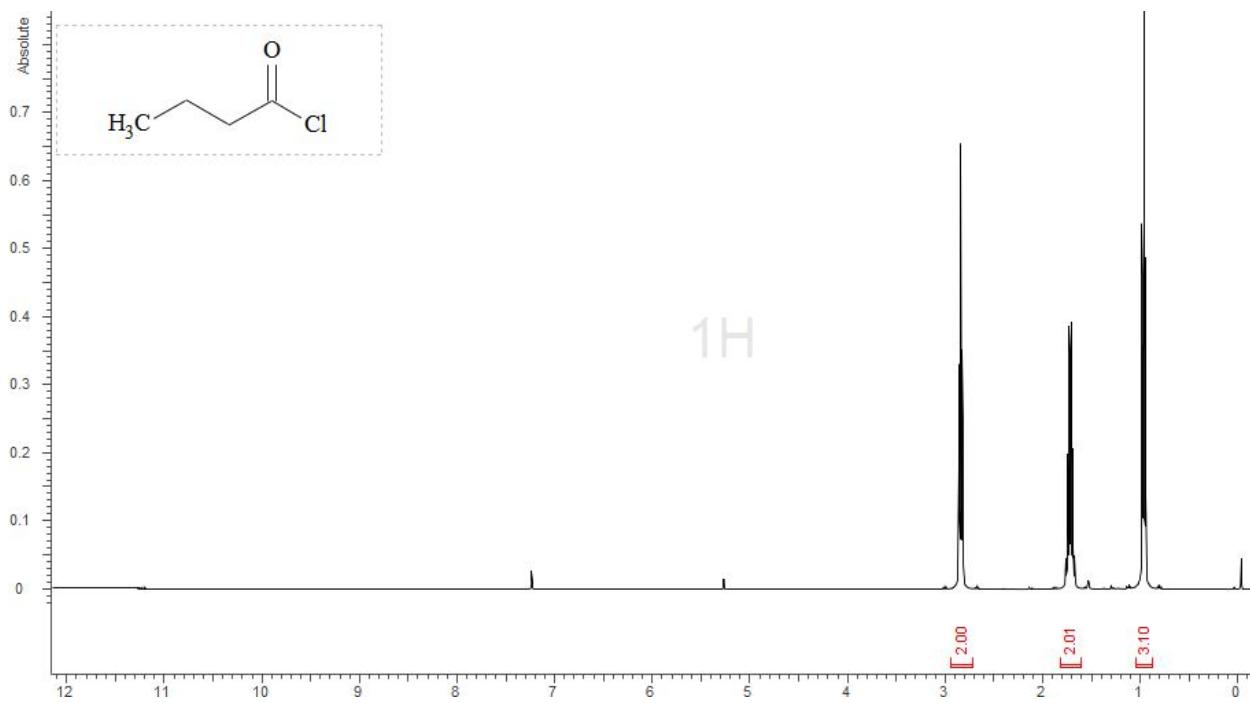
**Figure 4.16:**  $^{13}\text{C}$  NMR (151 MHz, Chloroform-*d*) spectrum of **8**



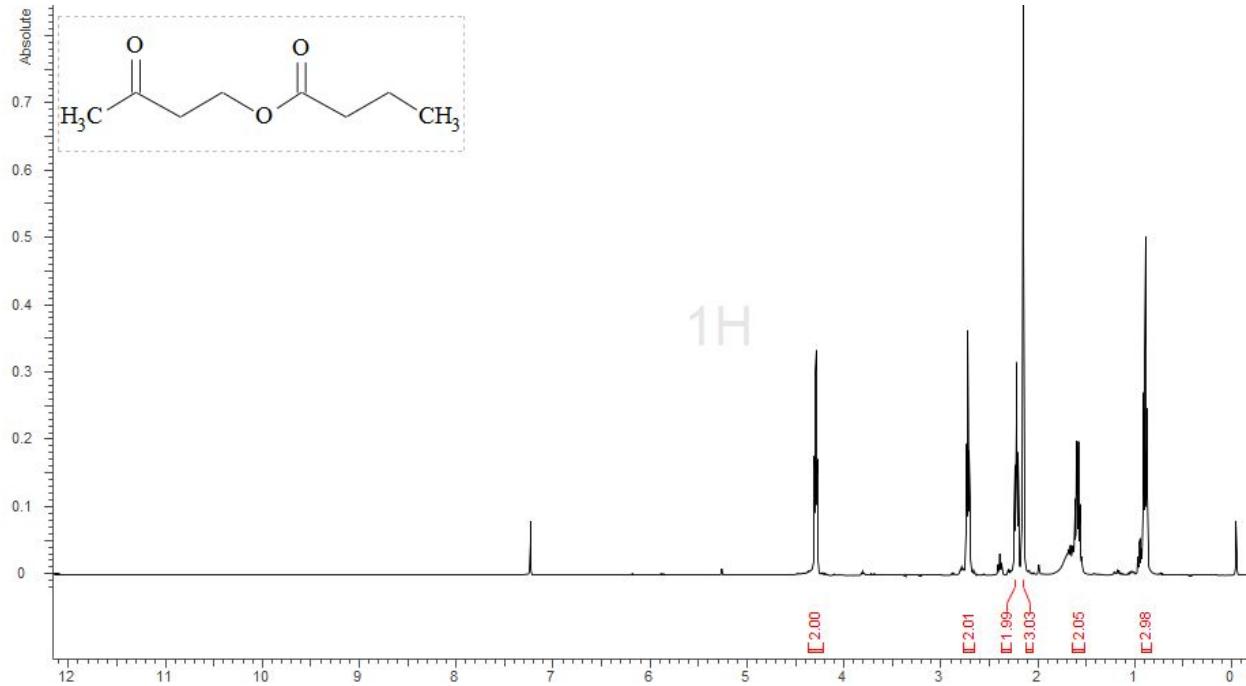
**Figure 4.17:**  $^1\text{H}$  NMR (400 MHz, Chloroform-*d*) spectrum of **9**



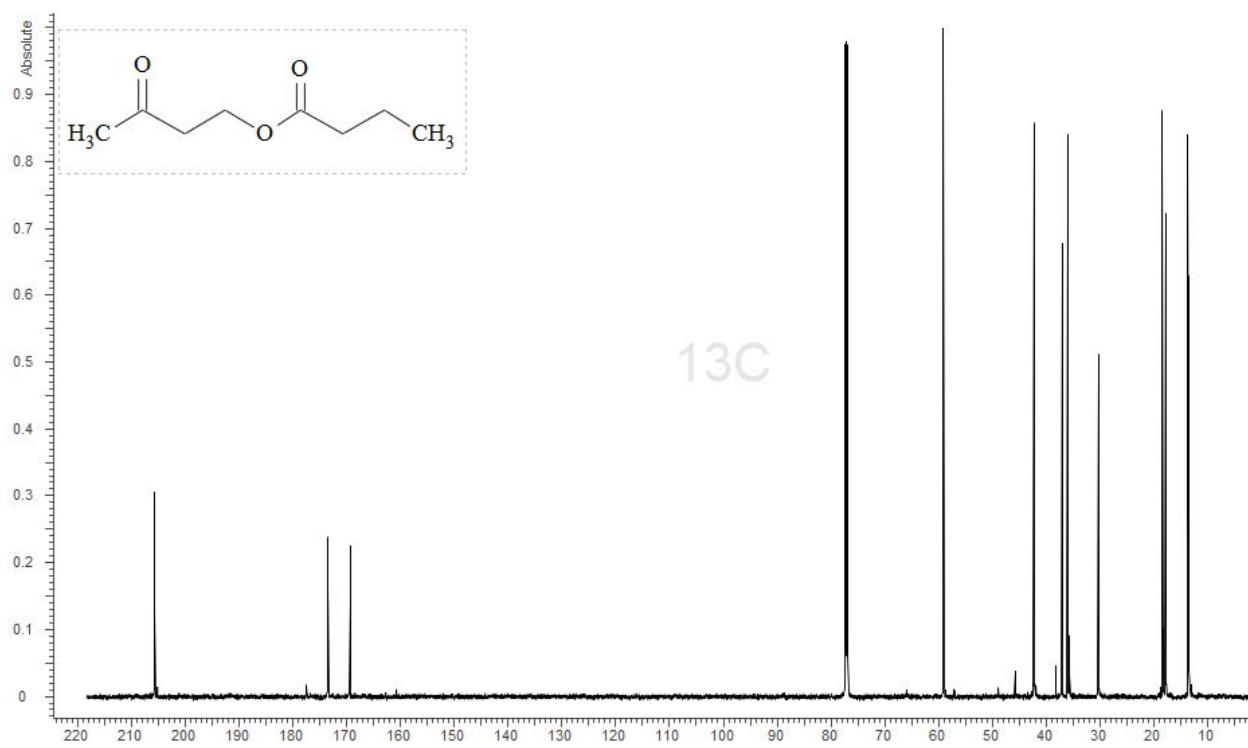
**Figure 4.18:**  $^{13}\text{C}$  NMR (151 MHz, Chloroform-*d*) spectrum of **9**



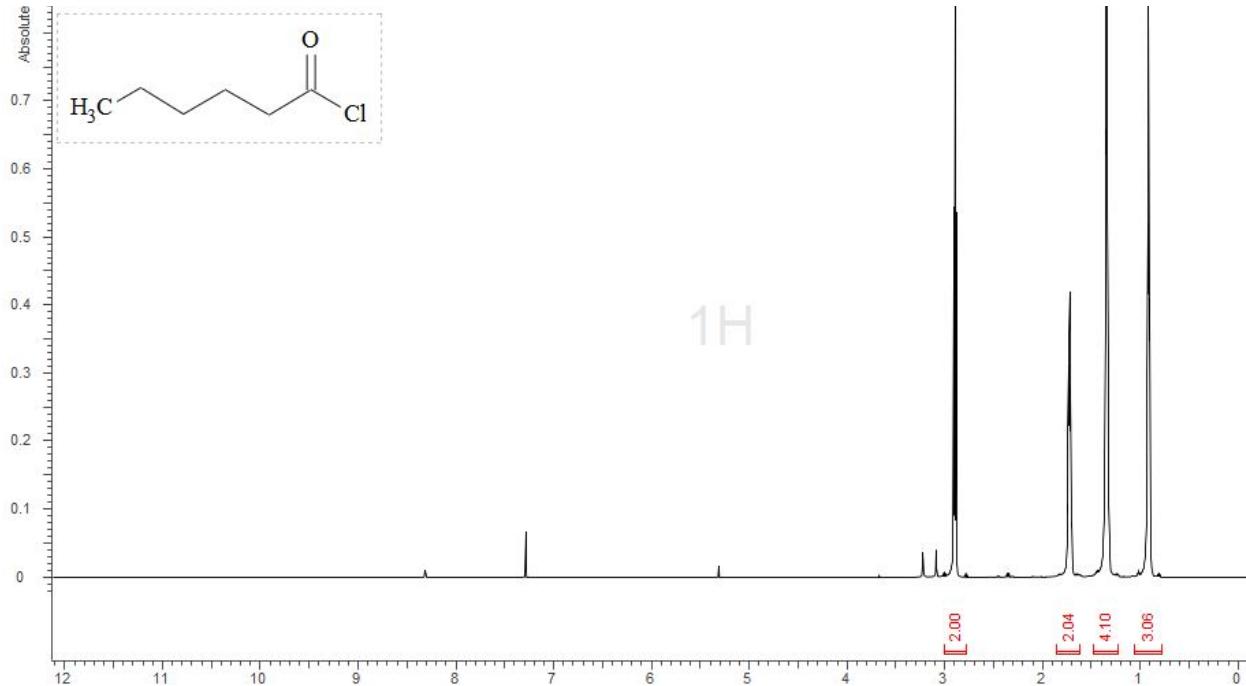
**Figure 4.19:**  $^1\text{H}$  NMR (400 MHz, Chloroform-*d*) spectrum of **10a**



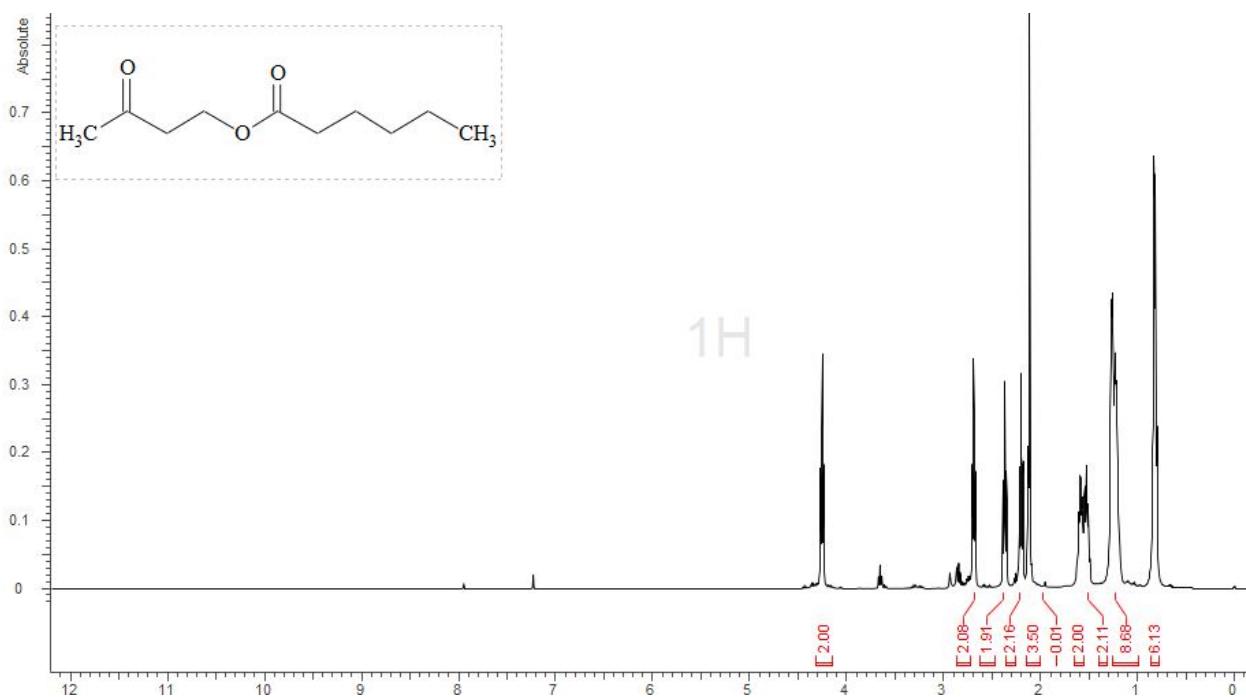
**Figure 4.20:**  $^1\text{H}$  NMR (400 MHz, Chloroform-*d*) spectrum of **10**



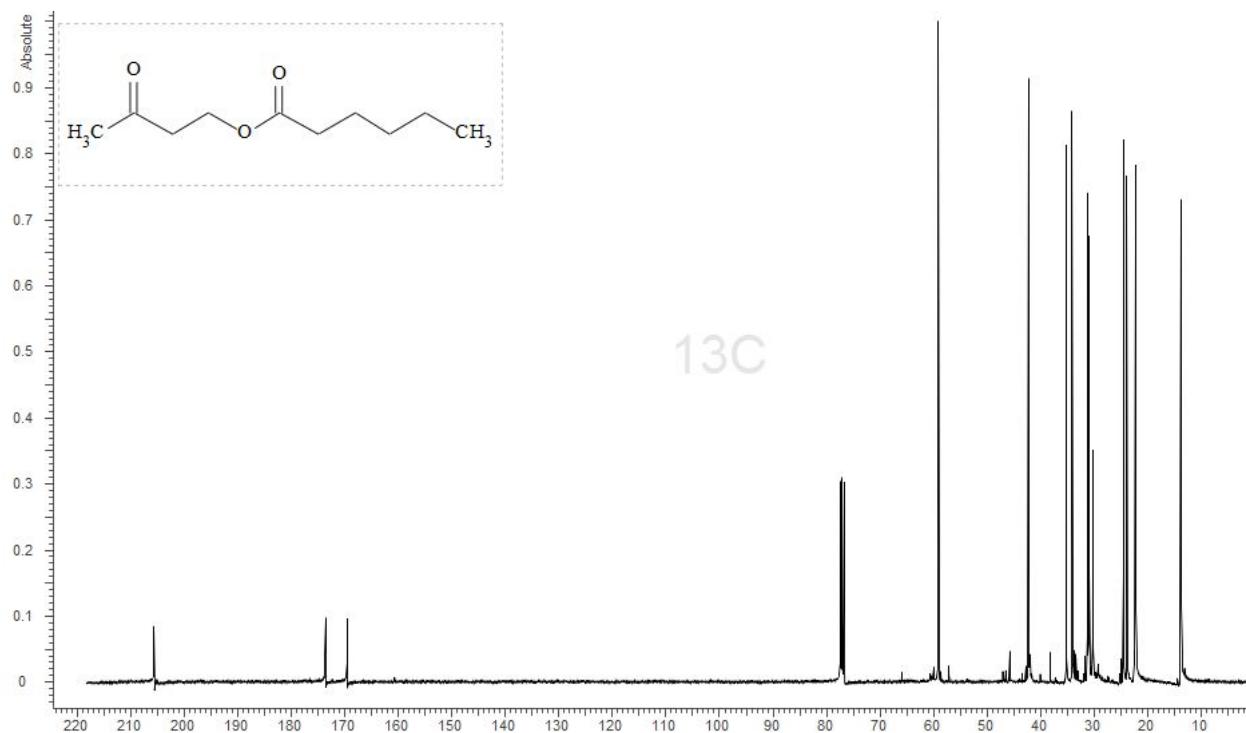
**Figure 4.21:**  $^{13}\text{C}$  NMR (151 MHz, Chloroform-*d*) spectrum of **10**



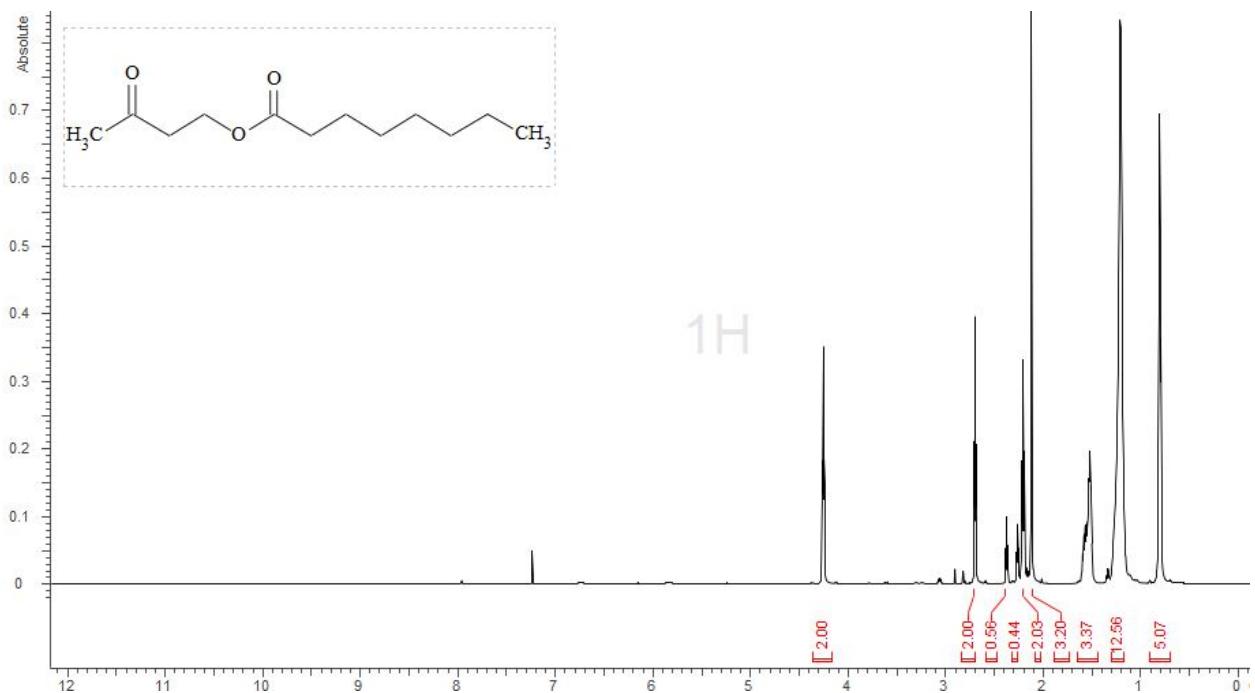
**Figure 4.22:**  $^1\text{H}$  NMR (600 MHz, Chloroform-*d*) spectrum of **11a**



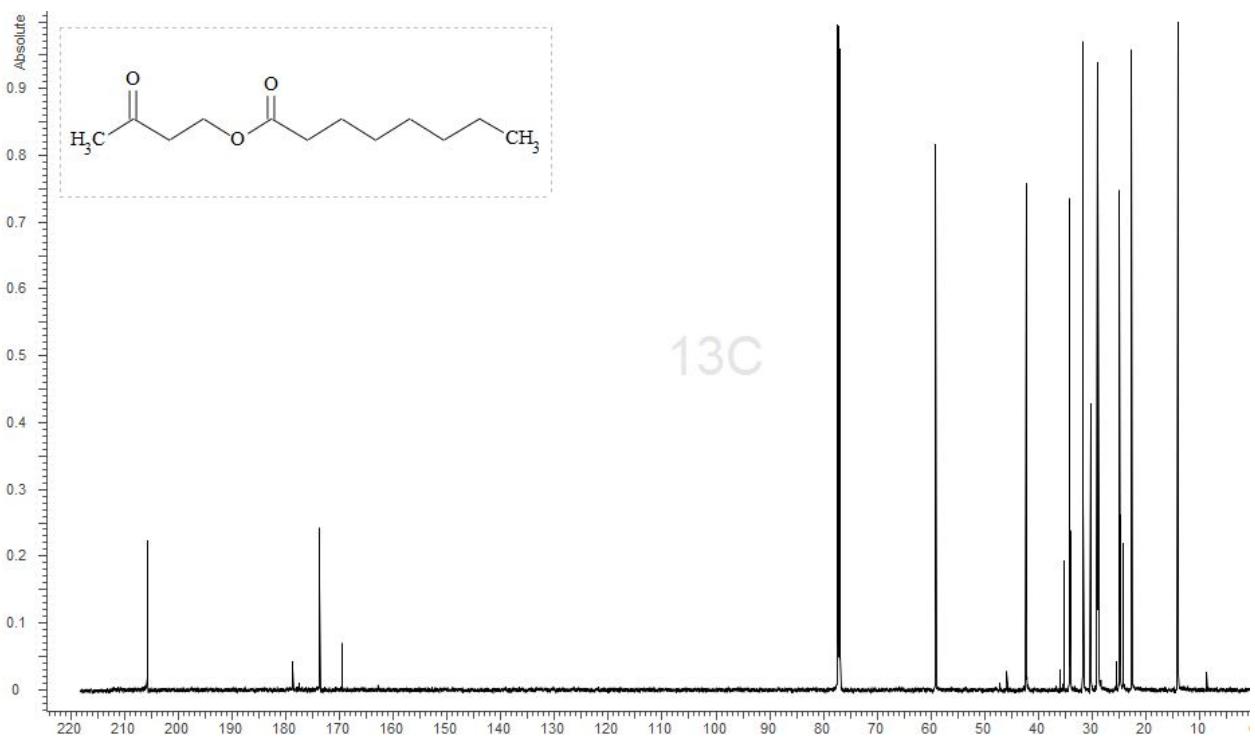
**Figure 4.23:**  $^1\text{H}$  NMR (400 MHz, Chloroform-*d*) spectrum of **11**



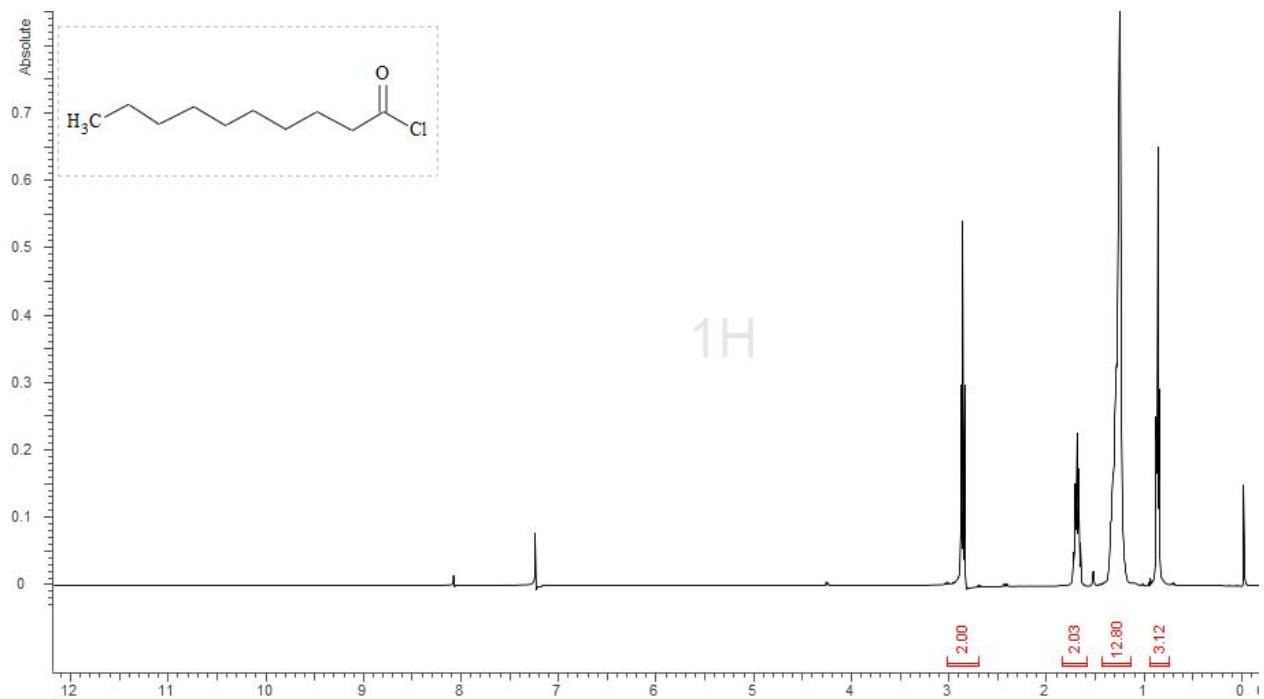
**Figure 4.24:**  $^{13}\text{C}$  NMR (151 MHz, Chloroform-*d*) spectrum of **11**



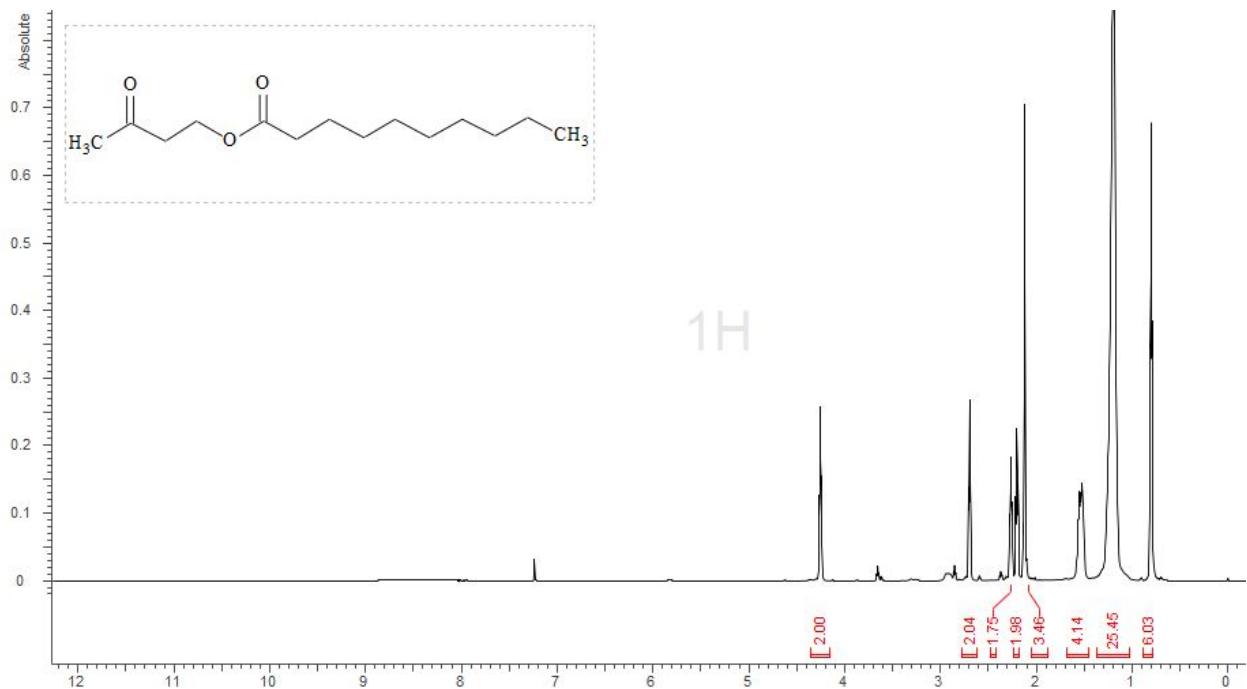
**Figure 4.25:**  $^1\text{H}$  NMR (600 MHz, Chloroform-*d*) spectrum of **12**



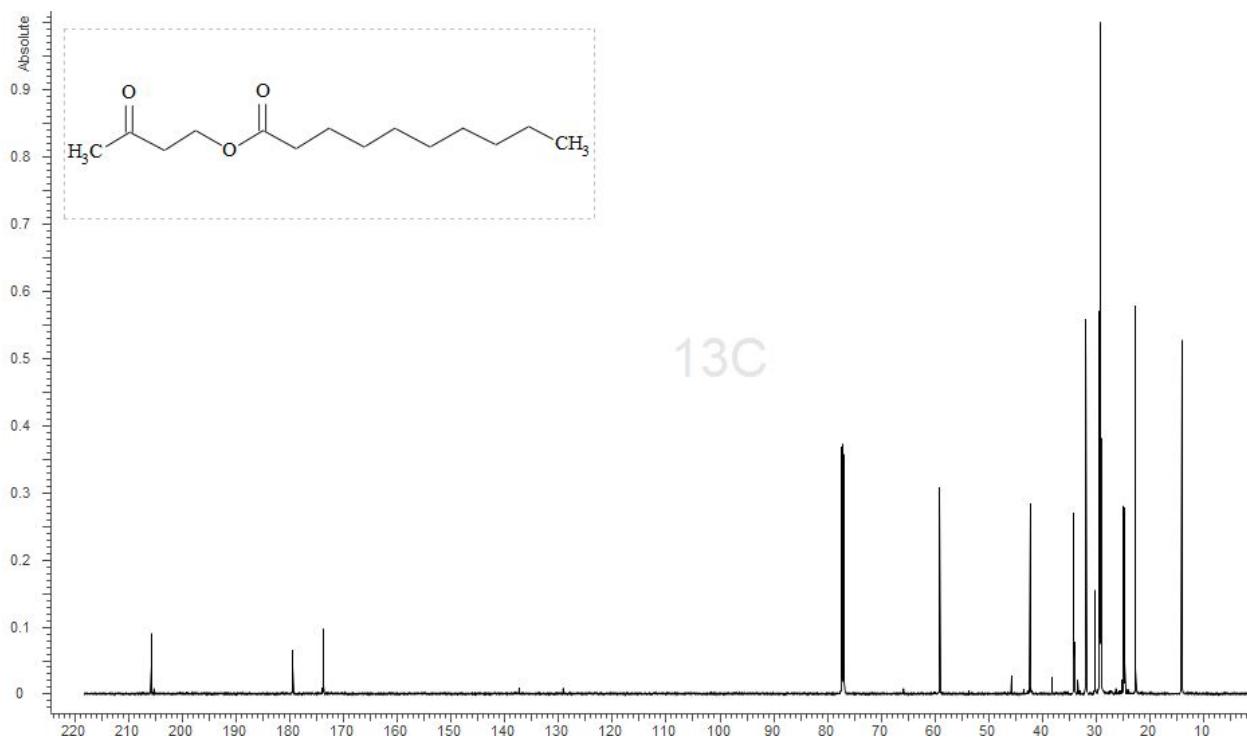
**Figure 4.26:**  $^{13}\text{C}$  NMR (151 MHz, Chloroform-*d*) spectrum of **12**



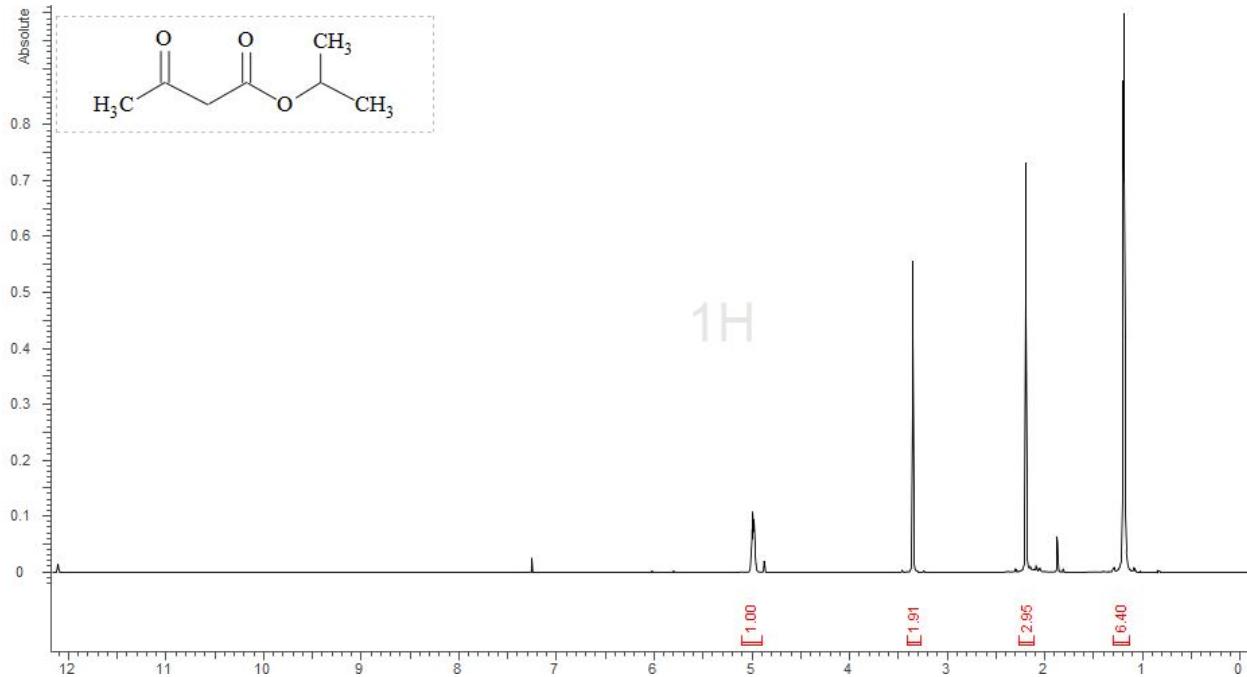
**Figure 4.27:**  $^1\text{H}$  NMR (400 MHz, Chloroform-*d*) spectrum of **13a**



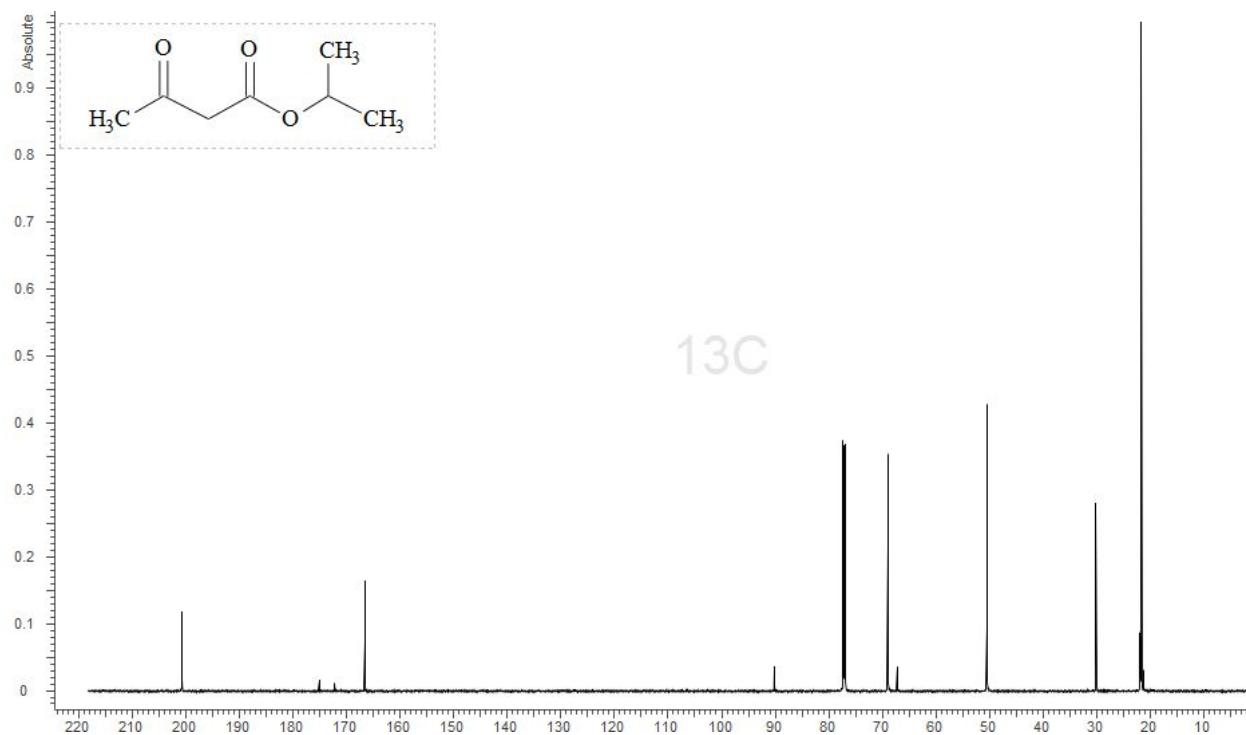
**Figure 4.28:**  $^1\text{H}$  NMR (600 MHz, Chloroform-*d*) spectrum of **13**



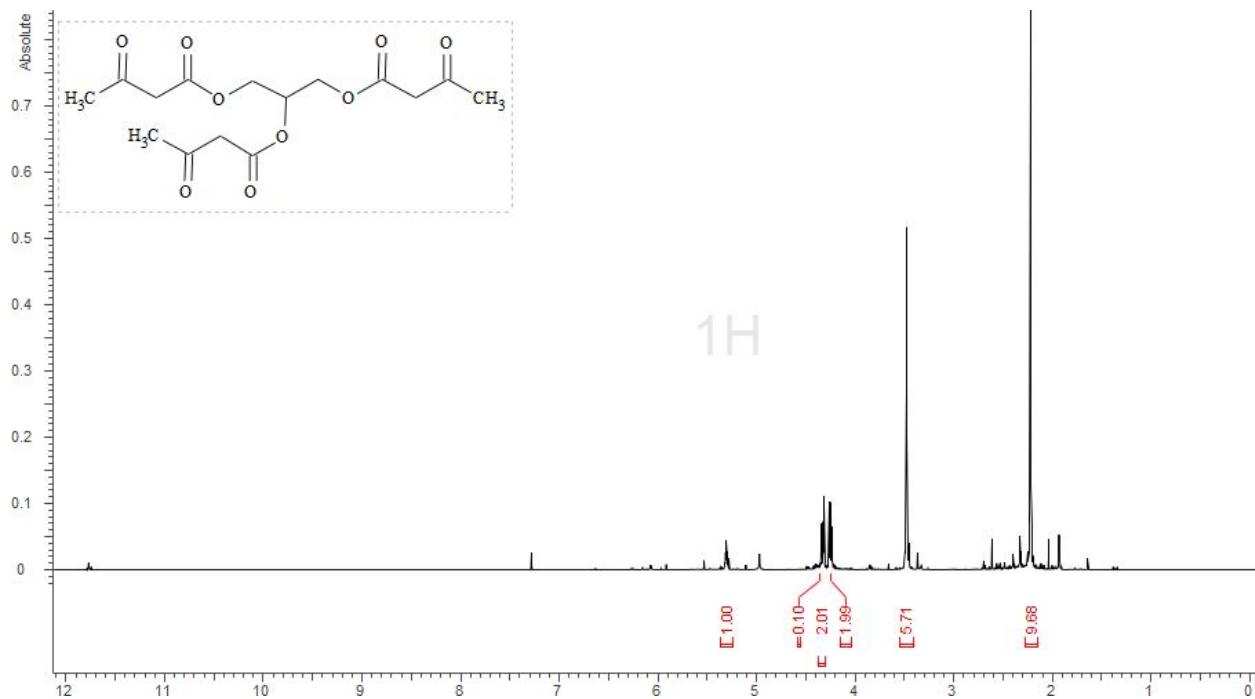
**Figure 4.29:**  $^{13}\text{C}$  NMR (151 MHz, Chloroform-*d*) spectrum of **13**



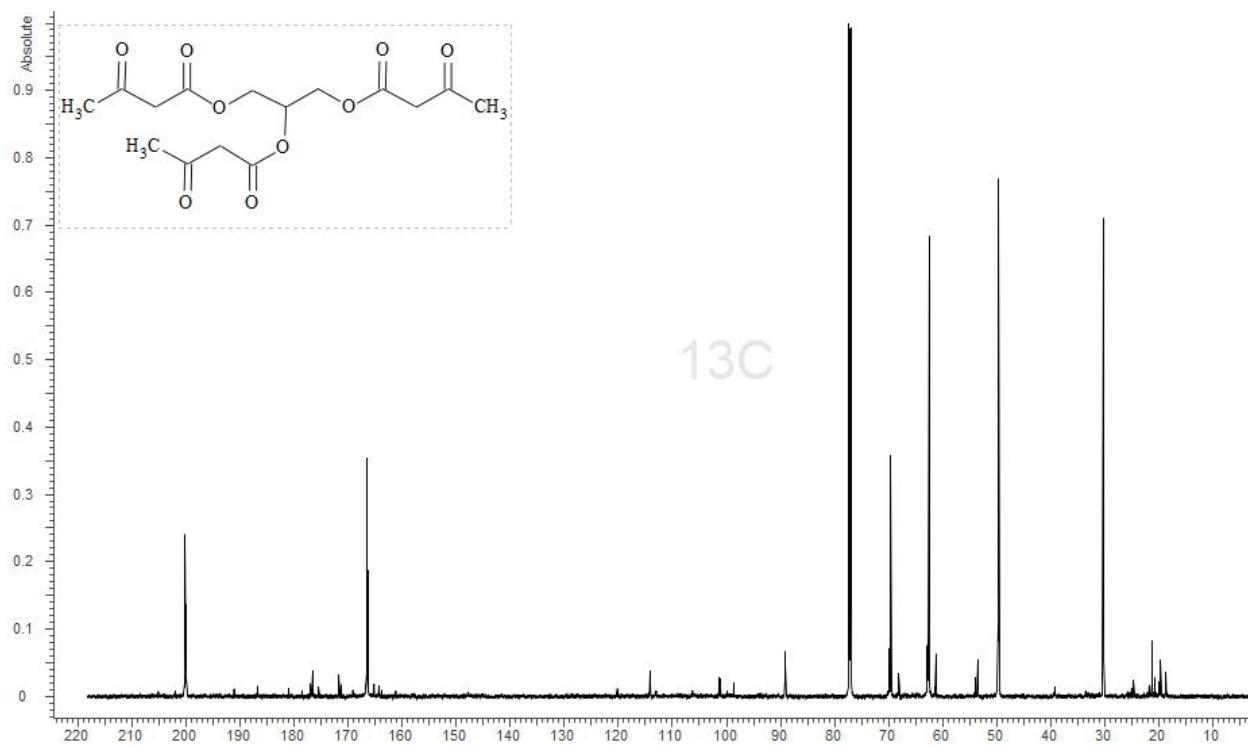
**Figure 4.30:**  $^1\text{H}$  NMR (600 MHz, Chloroform-*d*) spectrum of **14**



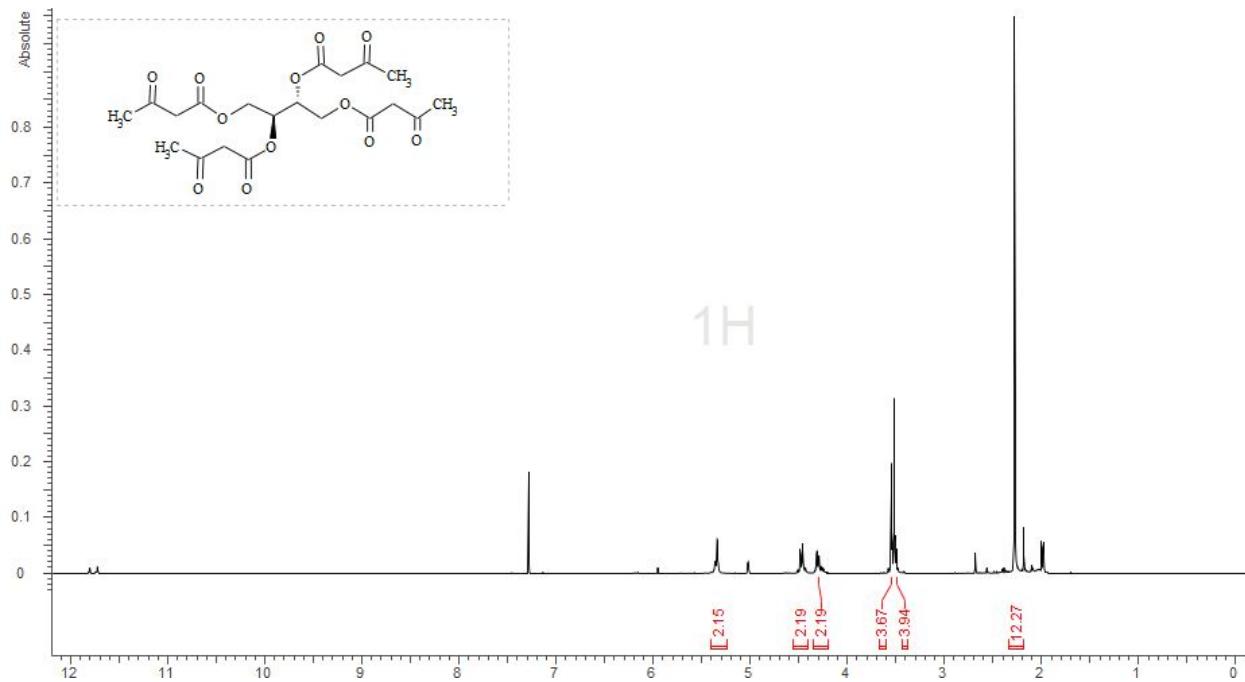
**Figure 4.31:**  $^{13}\text{C}$  NMR (150 MHz, Chloroform-*d*) spectrum of **14**



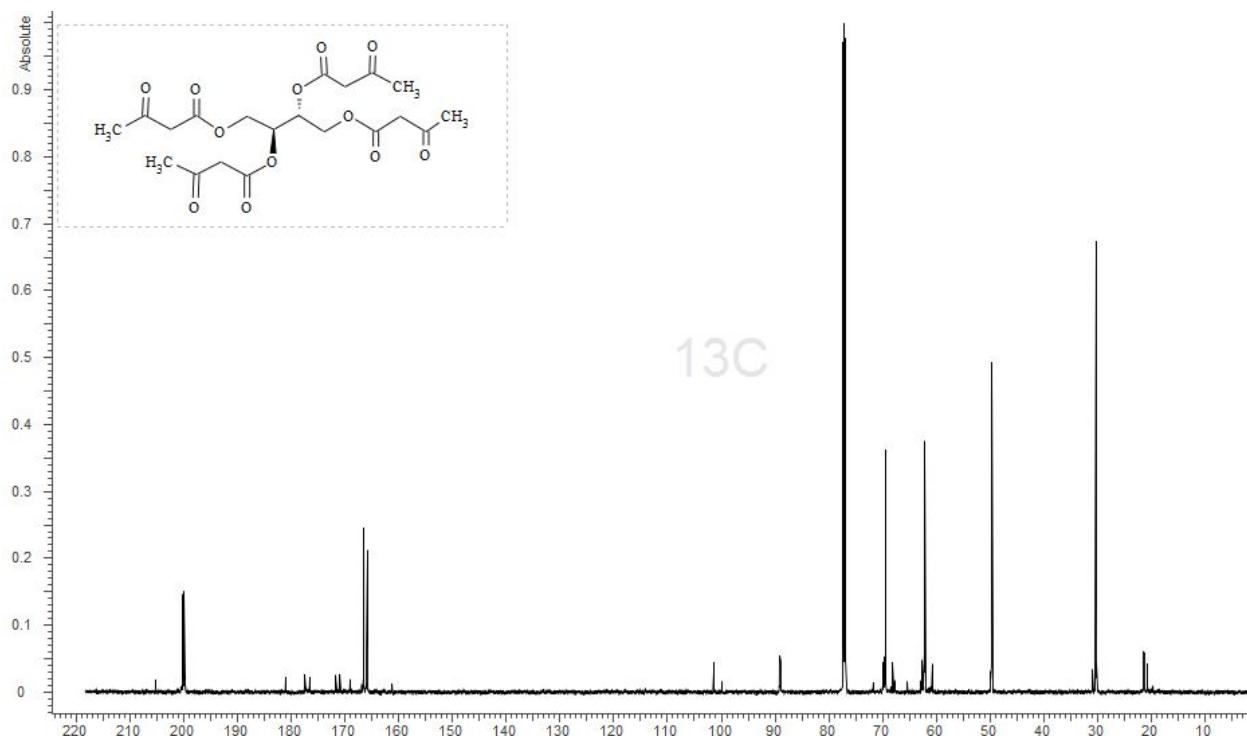
**Figure 4.32:**  $^1\text{H}$  NMR (600 MHz, Chloroform-*d*) spectrum of **15**



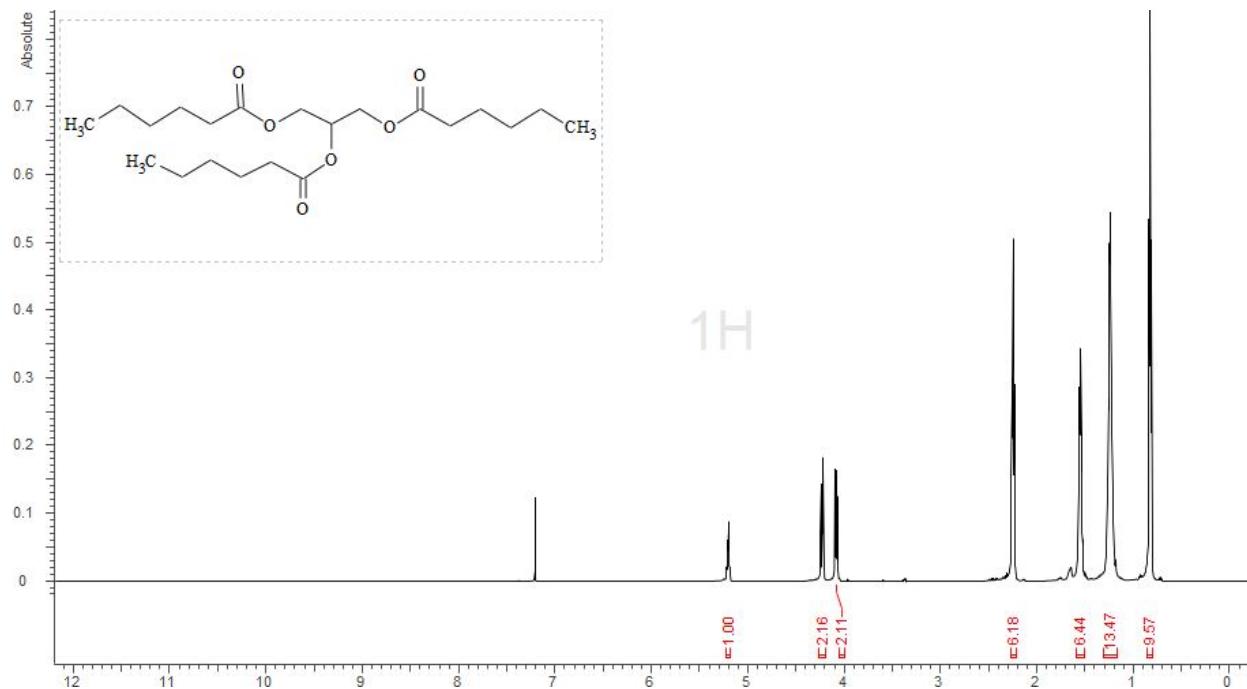
**Figure 4.33:**  $^{13}\text{C}$  NMR (150 MHz, Chloroform-*d*) spectrum of **15**



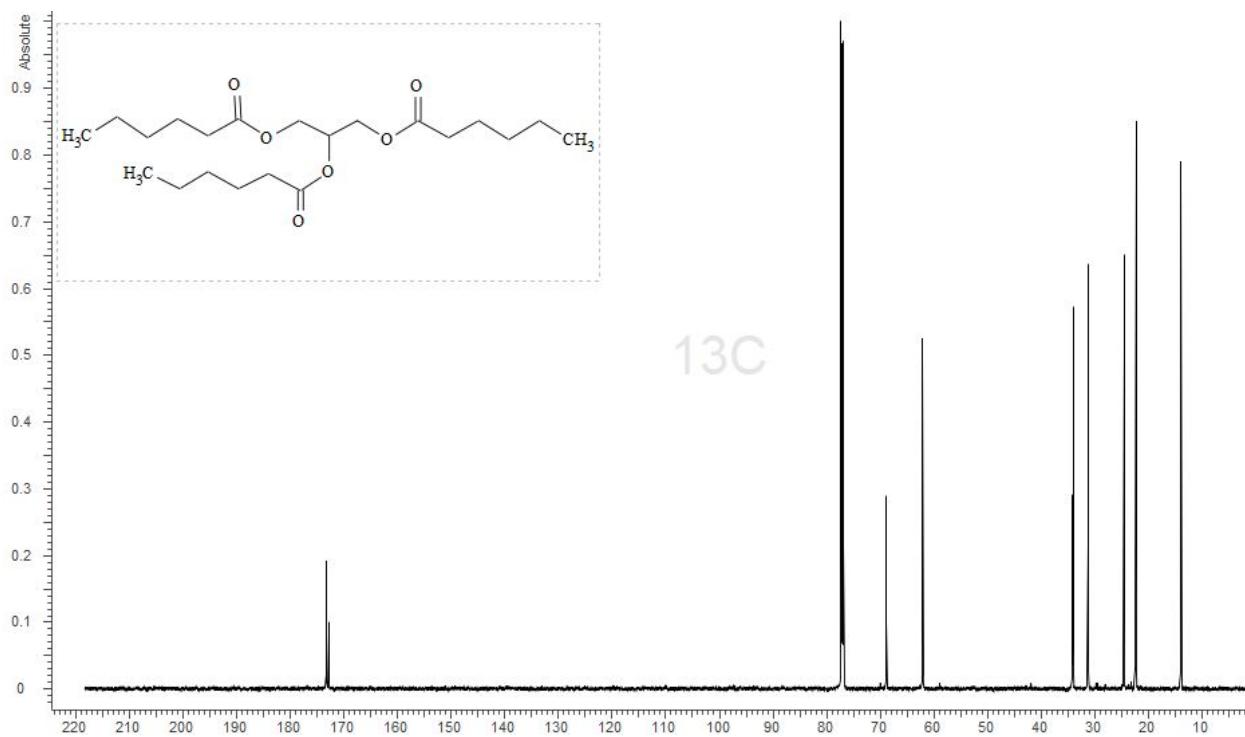
**Figure 4.34:**  $^1\text{H}$  NMR (600 MHz, Chloroform-*d*) spectrum of **16**



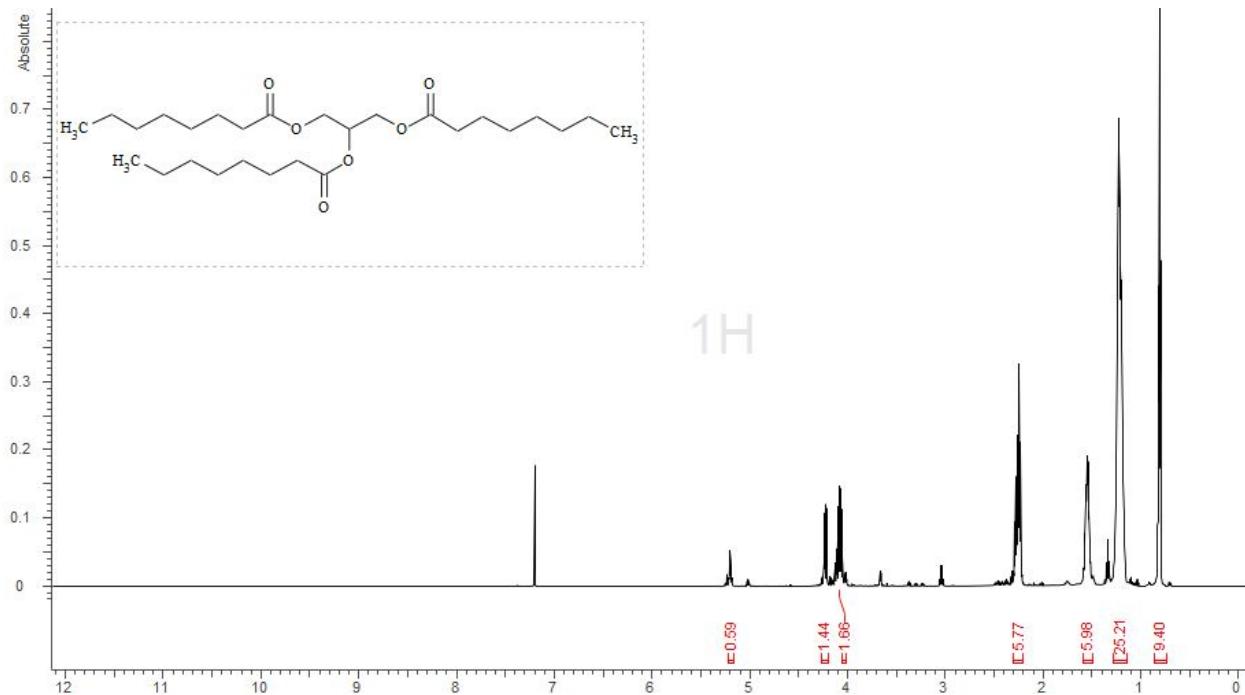
**Figure 4.35:**  $^{13}\text{C}$  NMR (150 MHz, Chloroform-*d*) spectrum of **16**



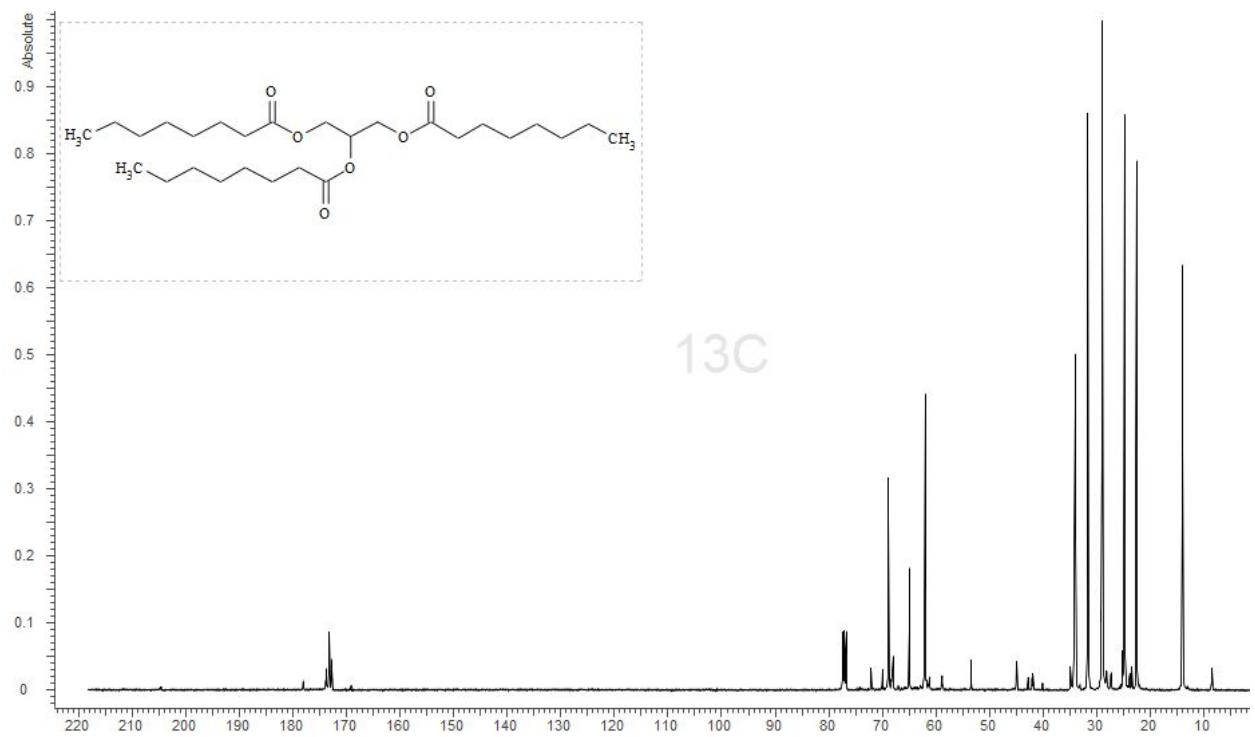
**Figure 4.36:**  $^1\text{H}$  NMR (600 MHz, Chloroform-*d*) spectrum of **17**



**Figure 4.37:**  $^{13}\text{C}$  NMR (150 MHz, Chloroform-*d*) spectrum of **17**



**Figure 4.38:**  $^1\text{H}$  NMR (400 MHz, Chloroform-*d*) spectrum of **18**



**Figure 4.39:**  $^{13}\text{C}$  NMR (101 MHz, Chloroform-*d*) spectrum of **18**