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The synthesis of brominated PHA (polyhydroxy alkanoates) and its polymer-analogous conversion

Mona M. Chaudhary

THE SYNTHESIS OF BROMINATED PHA (POLYHYDROXY ALKANOATES) AND ITS POLYMER – ANALOGOUS CONVERSION

by

MONA M. CHAUDHARY

A THESIS

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ABSTRACT
The School of Graduate Studies
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Name of Candidate Mona M. Chaudhary

Title: The synthesis of Brominated PHA (Poly Hydroxy Alkanoates) and its Polymer – Analogous Conversion

Poly β-hydroxyalkanoates (PHAs) are a diverse class of natural polyesters produced by bacteria as a form of energy storage material. One of the most versatile PHA-producing organisms is *Pseudomonas oleovorans*, which is used in this study to produce poly β-hydroxy-octanoate-co-β-hydroxy-11-bromo-undecanoate (PHOBr). PHOBr was produced when grown in batch cultures on equimolar mixtures of high growth substrates (sodium octanoate) and substrates that yield functional side chains (11-bromoundecanoic acid).

The click chemistry reaction between PHOBr and sodium azide and propargyl benzoate was simulated using small molecule models. The viability of the small molecule model reactions revealed the possibility of click chemistry reaction. The halide (bromine) terminated side chains of PHOBr were first converted into azides followed by formation of triazole ring with propargyl benzoate.

This study has made important contributions to the field: It is the first demonstration of PHA with azide-terminated side chains, and thereby forming triazole click compound with propargyl benzoate.

Abstract Approval: Committee Chair C. Ded 10/17/12

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF TERMS

ATCC	American Type Culture Collection
11-BrUA	11-bromoundecanoic acid
FTIR	Fourier transform infrared spectroscopy
OA	octanoic acid
NMR	nuclear magnetic resonance
PHA	poly(β -hydroxyalkanoate)
lclPHA	long chain length poly(β -hydroxyalkanoate)
mclPHA	medium chain length poly(β -hydroxyalkanoate)
sclPHA	short chain length poly(β -hydroxyalkanoate)
PHB	poly-(β -hydroxybutyrate)
PHOBr	poly(β -hydroxyoctanoate)-co-(β -hydroxy-11-bromoundecanoate)
SO	sodium octanoate
TMS	tetramethylsilane

CHAPTER I

Introduction

1.1. Bacterial Polyesters Bibliographic Study

The study of bacterial growth and biopolyester production with a functionalized bromo terminal group which could be analogously converted to an azide and there upon perform click chemistry with an alkyne was the aim of this research project. Azide-alkyne click chemistry is a very useful tool in the context of drug delivery, pharmaceuticals, and material science.

First, the specific natural biopolymer used at the core of the work must be introduced. The native sources and basic characteristics of the microorganism, along with historical uses and applications will be addressed. And secondly, the recent attempts in the field of click chemistry of azide and alkyne cyclo addition will be addressed.

So far no systematic studies has been performed to synthesize 1,2,3 triazole click compound starting with brominated PHA. This study focused on the formation of brominated PHA from octanoic acid (OA) and 11-Bromo undecanoic acid (11-BrUA). Once the polymer was produced, the terminal bromine groups were converted to an azide and underwent a click reaction with propargyl benzoate.

1.2. Bacterial polyester

1.2.1 Definition, discovery and natural occurrence of PHA

Poly(β-hydroxyalkanoates) (PHA) are a large class of biopolyesters produced by microorganisms. PHAs were first discovered in 1926 by the French microbiologist Lemoigne, who reported that the bacterium *Bacillus megaterium* accumulated intracellular inclusion bodies, which were later identified to be a polyester: poly(β-hydroxyalkanoate) (Lemoigne 1926). This new material was found to be a homopolymer of 3-hydroxybutyric acid linked through ester bonds between the 3-hydroxyl group and the carbonyl group of the next monomer, yielding poly(β-hydroxybutyrate) (PHB) (Figure 1.1).

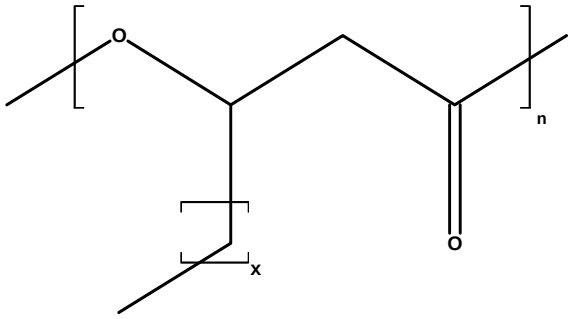


Figure 1.1. General structure of PHA. If x = 0, then the structure is that of PHB.

The chiral center gives an optically active structure. All known bacterially synthesized PHAs exhibit *R*-configuration, and are therefore 100% isotactic. All forms of PHA have the general structure shown in Figure 1.1. Depending on the carbon supply in the local environment of the microorganism, the side chain can consist of a variety of chemical structures, differing in the side chain lengths and chemical compositions. This

variation in side chain length has become a convenient way to classify PHAs which are now separated into three classes:

1) short chain length PHA (scl-PHA carbon numbers of monomers ranging from C_3 to C_5), 2) medium chain length PHA (mcl - PHA,C_6-C_{14}), and 3) long chain length PHA (lcl - PHA, >C_{14}) (Lee 1996). Further work has shown over 90 genera of archae and bacteria, in both aerobic and anaerobic environments, able to produce a form of PHA (Findlay 1983; Steinbuchel 1992). The largest classes of PHA-producers are Gram-negative bacteria which are able to generate varying forms of this polymer when fed on a wide range of carbon sources including sugars such as glucose and fructose, fatty acids, glycerol, and aliphatic hydrocarbons such as n-alkanes and alkanoic acids (Lenz 2005).

Natural habitats for PHA-producing microorganisms tend to be aqueous, often surrounded by oil-based contamination. PHAs of varying lengths have been isolated and purified from marine sediment, freshwater algal mats and activated sewage sludge (Findlay 1983; Capon 1983; Wallen 1974)

PHAs possess several interesting and potentially commercially useful properties, some of which stem from their biological origin. Due to their stereoregulartiy, all PHAs produced by microorganisms are inherently biodegradable. The PHA degradation products are β-hydroxy acids.

The simplest PHA, PHB, was the first PHA to be investigated in materials applications. It has a high melting temperature (T_m) of 175°C and high crystallinity (~80%), which limits its utility (Witholt 1999). PHB has similar physical properties to polypropylene (PP), but with the notable exception of extension to break and solvent

resistance (Table 1.1). As the field progressed, it was discovered that microorganisms were capable of producing copolymers consisting of PHB and five-carbon poly(β-hydroxyvalerate) (PHV), which were found to have superior properties including lower degree of crystallinity, which resulted in a soft, malleable material with a larger processing window. PHBV copolymers were found to have application as a biodegradable alternative to PP. However, despite initial promise, PHBV applications have been limited to specialized medical devices (Ueda 2003). The main reason for the lack of success of PHAs in the packaging market is their comparatively high price.

Table 1.1. Physical property comparison of PHB and PP (Howells 1982).

Property	PHB	PP
Tm (°C)	175	176
Crystallinity (%)	80	70
Mw (kDa)	50	20
Tg (°C)	4	-10
Density (gcm-3)	1.25	0.905
Tensile strength (Mpa)	40	38
Extension to break (%)	6	400
UV resistance	good	poor
Solvent resistance	poor	good

Pseudomonas strains were later discovered to produce mcl-PHA from a multitude of carbon sources, and it has been shown that chemical side-chain functionality can be maintained throughout the fermentation and polymerization processes (Lenz et al.). Various aspects of this process will be discussed hereafter, including culture design, metabolism, polymer processing, characterization, and modification.

1.2.2. Bacterial growth and polymer production patterns

mcl-PHAs are accumulated by a number of gram-negative bacteria, predominately fluorescent *Pseudomonads* (Huisman 1989; Timm 1990). The most versatile and well-studied strain of this class is *Pseudomonas oleovorans*. In general, PHA production in nature results when the organism senses environmental stress in the form of low trace elements, nitrogen, or oxygen supplies. Polyesters of this type are then generated as a reserve material, with some organisms able to accumulate as much as 70% of their dry weight as PHA (Doi 1990). Some engineered recombinant strains are able to accumulate up to 90% (Zinn 2001). To relieve the stress of low nutrient supply, the polyester is easily hydrolyzed within the organism, generating a stable supply of reducing equivalents required for its metabolism. This behavior of accumulating intracellular carbon and energy storage is best understood in the context of a normal, non-stressed growth pattern common to most bacteria, which consists of four distinct phases as described by Sparks (2008) (see Figure 1.2) (Doi; Zinn)

1) lag phase

In this phase, the cells are primarily adapting to the new environmental conditions. They begin to sense the general identity and relative concentrations of the nutrient supply in the medium and synthesize specific enzymes from their genome and accumulated cofactors that allow them to begin the growth process. This phase is characterized by very little cell division, which is reflected in the low and constant optical density of liquid cultures.

2) log phase

In this phase, the cells have synthesized the necessary enzymes and cofactors required for survival in the specific conditions, and have begun balanced and rapid cell division. The term "log" is used to describe this growth, because, depending on the bacterial strain and the composition of the medium, cell numbers can double in an increasingly short time, resulting in logarithmic growth. This phase is characterized by a steady increase in the optical density of liquid cultures.

3) stationary phase

In this phase, the cells have sensed a substantial decrease in the carbon-source concentration in the environment. As a result, cell division is halted and remaining nutrients are devoted to survival. This phase is characterized by a leveling in the optical density of liquid cultures.

4) death phase

In this phase, the cells have exhausted the nutrient supply in the medium. The high rate of cell growth and metabolism in the log and stationary phases has caused waste to accumulate to a toxic level. The number of live cells decreases exponentially, essentially the reverse of the log phase. Although cells are dying, the death phase cannot normally be observed using optical density measurements, because the amount of scattering contributed by the cell debris is equal to the contribution of the living cells. The result is a relatively constant series of optical density readings.

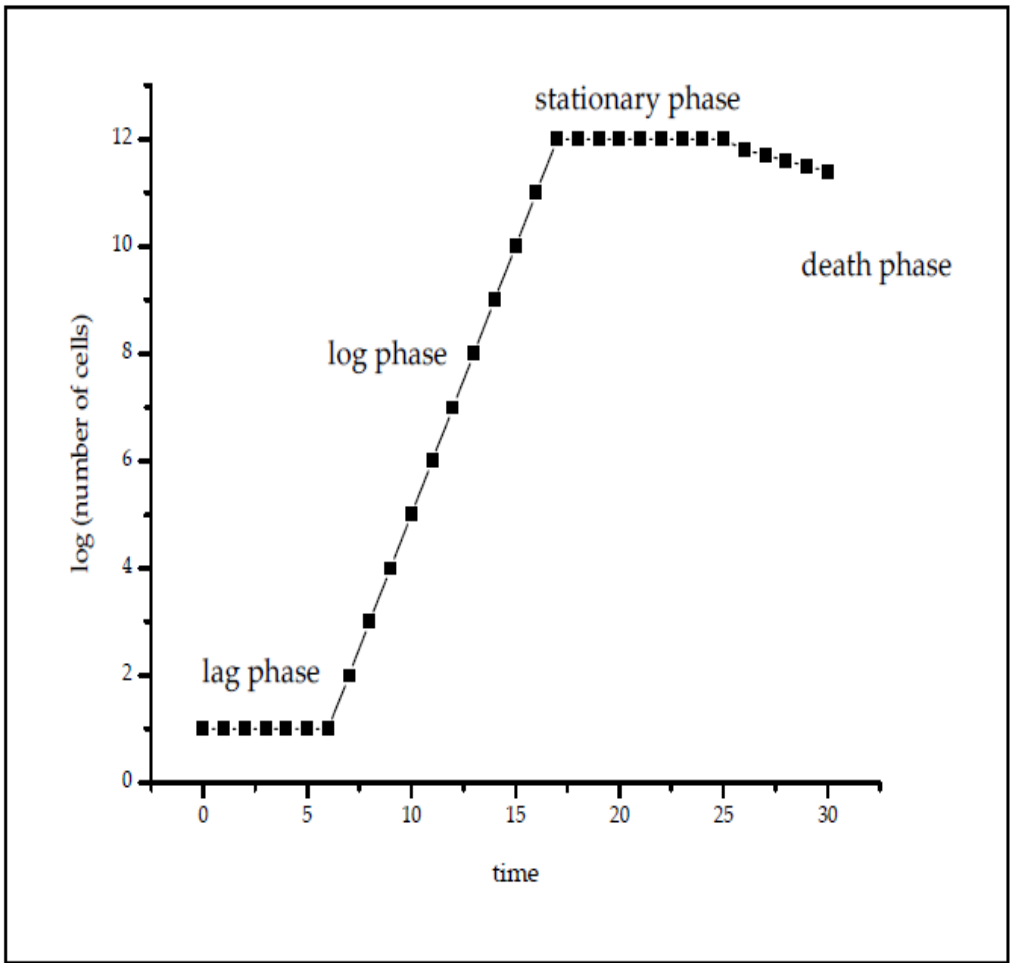


Figure 1.2. General bacterial growth curve (Doi; Zinn)

It is within this context that bacterial polymer production is best examined. Just as cell growth and increase in biomass are function of the available nutrient supply, polymer is produced in a likewise responsive manner. PHA is produced by microorganisms in response to a limited nutrient supply (environmental stress) in the presence of an excess supply of carbon. Although several nutrient concentrations are vital for cell survival such as carbon, nitrogen, and oxygen (in the case of aerobic bacteria), in laboratory or production facilities it is most often the nitrogen concentration that is limited to induce polymer production, as the amount of nitrogen can be readily

controlled by the addition of ammonium salts. In the environment the lack of any nutrient or trace metal can induce stress and

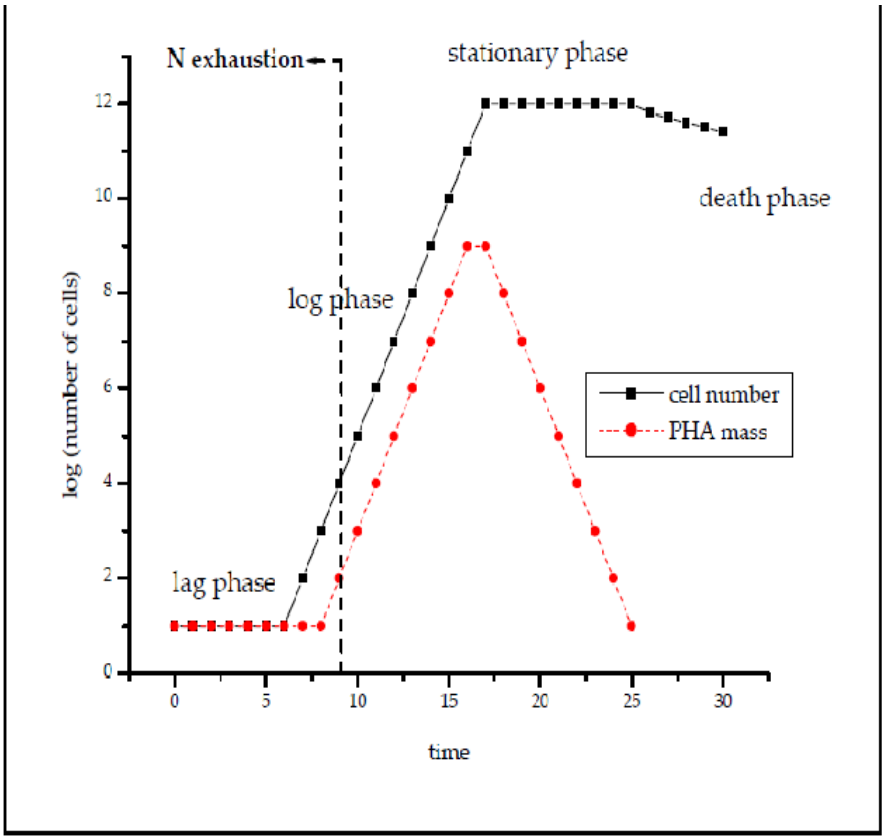


Figure 1.3. PHA-production pattern overlayed onto general bacterial growth curve. (Doi, Zinn)

therefore it induces polymer production. This limiting concentration of a single vital nutrient in the presence of excess carbon supply causes the cells to prepare for the exhaustion of this nutrient. They do so by efficiently utilizing the available carbon supply by producing PHA. In Figure 1.3, a graph of typical PHA production with time is overlaid onto the previous general bacterial growth curve.

Individual cells begin to produce PHA via a polymerase enzyme as they sense a drop in an essential nutrient, nitrogen, in the above example. In order to accumulate PHA in the laboratory or for commercial purposes, the point at which this occurs must be identified for each strain and carbon source(s). The polymer is stored inside large intracellular containers called inclusion bodies, which serve to protect the polymer from degradation by the cytoplasm. The polymerases are located on the membrane that coats the surface of these inclusion bodies. Thus, the growing hydrophobic polymer chains accumulate into the interior of the sequestered inclusion bodies. Polymer production continues until the carbon supply is completely exhausted. At this point, the organism begins to digest the polymer via a second enzymatic reaction, that is, hydrolysis, which guarantees the survival of the cell. This hydrolysis causes rapid polymer consumption by depolymerase enzymes, which are also located on the inclusion body membranes. After the polymer supply is exhausted, cells begin to die in larger numbers.

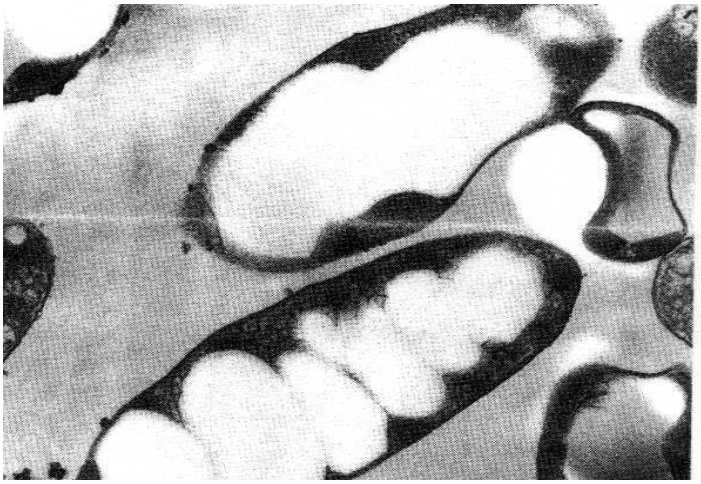


Figure 1.4 PHA accumulating microorganism *Alcaligenes eutrophus* now *Ralstonia eutropha* (MS, CH-540, UAH)

1.2.3 Intracellular seclusion

PHA is accumulated within the inclusion bodies inside the cell. Within the inclusion bodies, the polymer is not crystalline, but rather an amorphous elastomer (Bernard 1988; Bernard 1999). This gathering within inclusion bodies separates the polymer from the cell lumen, and as a result, the osmotic pressure of the cell is not changed significantly (Anderson 1990). The number of inclusion bodies per cell appears to be strain-specific. *P. oleovorans*, for example, has been found to have one or two large inclusion bodies (Klinke 1999), while *Ralstonia eutropha* produces between 8 and 12 inclusion bodies of variable sizes (Ballard 1987). Although the structures of the inclusion bodies vary with the organism, inclusion bodies containing mcl-PHA tend to be larger than those of PHB or scl-PHA, while the surface consists of a phospholipids layer separating two crystalline protein layers (Stuart 1995). Four types of proteins of varying functions are associated with the inclusion bodies: (i) PHA polymerases, (ii) PHA depolymerases, (iii) phasins (granule-stabilizing proteins), and (iv) proteins with unclear functions (M. Zinn 2001). The purpose of the surface locations of these proteins stems from the mechanism by which the organism synthesizes and isolates the polymer. In a model proposed by Ellar, *et al*., PHA synthesis occurs from the polymerases, and as the hydrophobic polymers are produced, they aggregate together with the polymerases to form an outer shell (Ellar 1968). As the polymer is synthesized, it is directed to the core of the newly formed inclusion body where it is protected from degradation and stored for later utilization by the organism. The inclusion body membrane serves a protective

function for the polymer, keeping the hydrophobic polymer sequestered from the aqueous cytoplasm (Merrick 1961; Merrick 1964; Stuart 1995).

1.2.4 Synthesis of mcl-PHA: The β-oxidation pathway

In *P. oleovorans*, PHA is synthesized from alkanoic acids intracellularly via the β-oxidation pathway (Figure 1.5) (Doi 1990). Alkanes are first transported into the cell, then converted to alkanoic acids in a series of steps involving alkane dehydrogenase, alkanol dehydroxylase, and alkanal dehydrogenase (Figure 1.5, steps I-III). The alkanoic acid is converted to the acetyl CoA derivative by acetyl CoA synthetase at the expense of one ATP (Figure 1.5, step IV). A double bond is introduced between the α and β carbons by acyl CoA dehydrogenase (Figure 1.5, step V). The β carbon is then hydroxylated by enoyl CoA hydratase (Figure 1.5, step VI). Polymerization proceeds, which is catalyzed by PHA polymerase. Polymerization continues until the removal of the CoA function by water results in chain termination (Figure 1.5, step VII). The introduction of monomer units with two fewer carbon atoms than that of its parent molecules occurs as well due to the β-oxidation and the thiolytic cleavage of the acid used as a growth substrate. In some cases, an additional two carbon atoms are introduced, possibly by the condensation of acetyl-CoA and acyl-CoA by acetyl-CoA synthetase (Doi 1990; Huisman 1989).

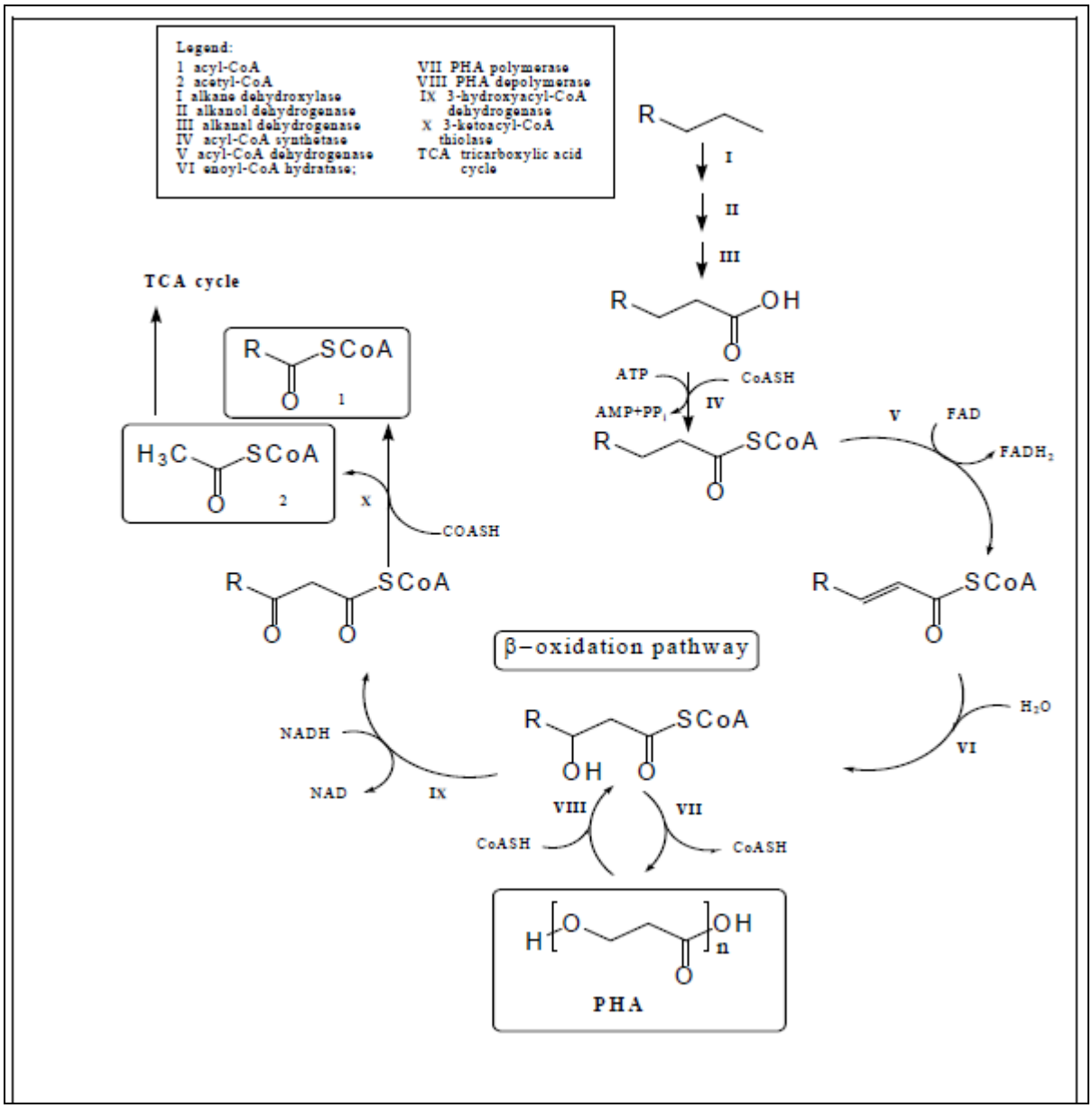


Figure 1.5. The β-oxidation pathway utilized by *P. oleovorans* to produce PHA (Doi 1990, Huisman 1989).

1.3. PHA copolymers

The most simple PHA, PHB homopolymer is the most abundant bacterial polyester. Despite the attractive biodegradable properties, its relatively poor mechanical properties make its applications limited. In the mid 1980s, Imperial Chemical Industries (ICI) discovered that they could produce the copolymer poly(β-hydroxybutyrate)-*co*-(β-hydroxyvalerate) (PHBV) by adding propionate to the fermentation broth, and that incorporating a small molar amount of β-hydroxyvalerate units into the polymer improved its flexibility and reduced brittleness (Poirier 1999). Copolymers with varying side chain lengths and functional group termination can be synthesized. This variability is a direct function of the carbon sources available to the bacterium in its local environment.

Over the past decade, PHB and other PHA copolymers have been found to possess properties advantageous for a wide variety of applications, particularly in the biomedical field. These include sutures, staples, bone plates, surgical mesh, orthopedic pins, bone marrow scaffolds, skin substitutes, wound dressings, and bulking and filling agents (Chen 2006). These specific applications often require materials with precise properties and materials that can be tuned as necessary are particularly interesting. For example, by incorporating just 25% β-hydroxyvalerate units into PHB homopolymer, the T_m can be lowered from 177° to 137°C, the T_g lowered from 4° to -6°C, and the tensile strength may be decreased from 43 to 20 Mpa (Doi 1995). By lowering the T_m and T_g, the processing window is increased, and processing can be performed without degradation.

In addition, as the molar concentration of hydroxyvalerate units is increased, Young's modulus is significantly decreased and impact strength is significantly increased due to the increase in flexibility imparted by the incorporation of random "disruptions" in crystallinity. The use of these materials in biological applications is also advantageous; as Sevastianov, *et al*. found, films of these PHBV copolymers in contact with blood, although causing coagulation and activation of the complement system, do not induce the homeostasis system by cellular response (Sevastianov 2003).

1.4. PHAs containing functionalized side chains

P. oleovorans microorganisms are able to produce PHA from a relatively large number of carbon sources including alkanes, alkenes, alkanoic acids, alkenoic acids, and many derivatives of these substrates. Although relatively large PHA yields may be obtained by polymer synthesis in batch or continuous cultures, using carbon sources such as *n*-alkanes and alkanoic acids, the resulting polymers have side chains terminated with unreactive methyl groups. In order to create more interesting molecules in terms of structure for further modification at the side chains, the PHA side chains must contain a certain amount of reactive functional groups. To obtain such derivatives, *n*-alkanes or alkanoic acids containing the desired terminal group must be utilized. Due to this versatility and their desirable biomedical properties, PHAs have been synthesized containing subunits with side chains terminated in phenyl, bromine, aryl, alkyl, ester, hydroxyl, branched methyl, and unsaturated groups (Fritzsche 1990; Kim 1992; Hazer 1996; Scholz 1994; Shah 2000; Hazer 1994; Fritzsche 1990; Fritzsche 1990). However, polymer yield is typically negligible when cultures are grown on such feed sources alone.

By co-feeding the desired derivatized acids with "good" growth substrates such as octanoic and nonanoic acids, copolymers containing monomer units with reactive side chains may be obtained in reasonable yields. The resulting polymers retain properties derived from the polyester backbone, including stereoregulartiy, hydrophobicity, biodegradability, and biocompatibility, but gain the ability to be modified to bring properties such as reactivity, the ability to cross-link, cellular targetting and labeling (Figure 1.6). Besides the pathway depicted in Figure 1.6, it is also possible for the β-oxidation to start at the R-end (functionalized end) of the carbon source as opposed to the acid end. This process ends in ultimate loss of the functional group, as it is cleaved in order to incorporate the carbonyl function. This depends on the chemical structure of the carbon source and the environmental conditions in which the β-oxidation pathway is prevalent (Scholz 1994). For example, methyl ester functions are lost much more frequently than phenyl functions, as ester hydrolysis is readily catalyzed. Large ring structures are sterically hindered from interacting with the active sites of the enzymes.

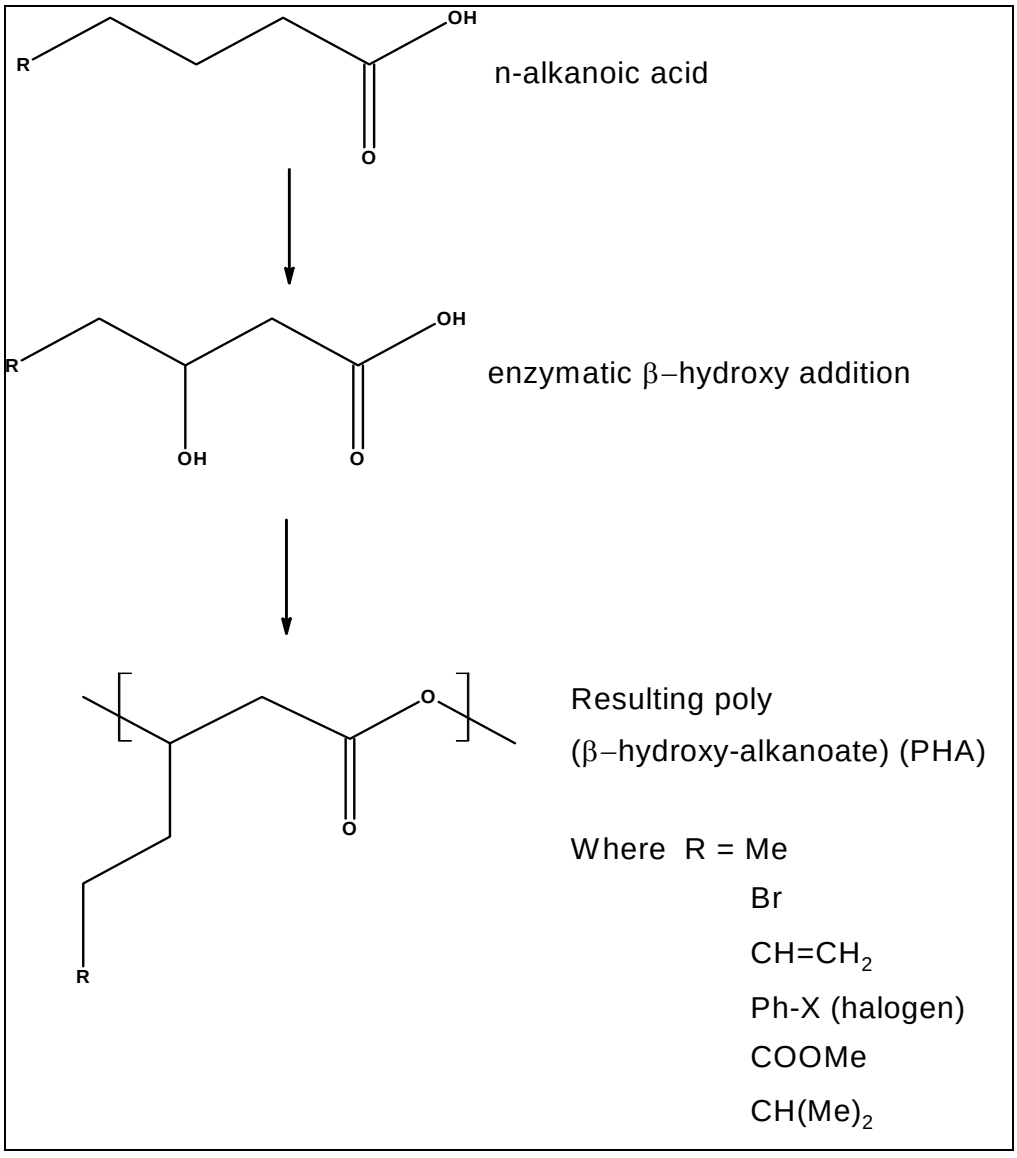


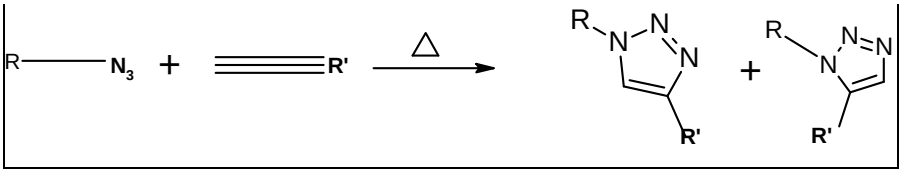
Figure 1.6. Pathway from n-alkanoic acid feed source to PHA. (Sparks 2008)

1.5 Click Chemistry

Click Chemistry is a term that was introduced by K. B. Sharpless in 2001 to describe reactions that are high yielding, wide in scope, create only byproducts that can be removed without chromatography, are stereospecific, simple to perform, and can be conducted in easily removable or benign solvents. This concept was developed in parallel with the interest within the pharmaceutical, materials, and other industries in capabilities for generating large libraries of compounds for screening in discovery research. Several types of reaction have been identified that fulfill these criteria, thermodynamically-favored reactions that lead specifically to one product, such as nucleophilic ring opening reactions of epoxides and aziridines, non-aldol type carbonyl reactions, such as formation of hydrazones and heterocycles, additions to carbon-carbon multiple bonds, such oxidative formation of epoxides and cycloaddition reactions.

For example, an examination of the azide-alkyne cycloaddition (a variation of the Huisgen 1,3-dipolar cycloaddition reaction between terminal alkyne and azides) shows that it fulfills many of the prerequisites. They combine high efficiency (usually above 95%) with a high tolerance of functional groups and solvents, as well as moderate reaction temperatures (25–70 ^{o}C) (Binder and Sachsenhofer, 2007).

Many of the starting monosubstituted alkynes and organic azides are available commercially, many others can easily be synthesized with a wide range of functional groups, and their cycloaddition reaction selectively gives 1,2,3-triazoles.



(K.C. Nicolaou, Tamsyn Montagnon, 2008)

Click chemistry does not replace existing methods for drug discovery, but rather, it complements and extends them. It works well in conjunction with structure-based design and combinatorial chemistry techniques, and, through the choice of appropriate building blocks, can provide derivatives or mimics of 'traditional' pharmacophores, drugs and natural products [Kolb, H.C. *et al.* 2001; Sneader, W. (1996), Bemis, G.W. and Murcko, M.A. (1996)]. Through the use of only the most facile and selective chemical transformations, click chemistry simplifies compound synthesis, providing the means for faster lead discovery and optimization. A click reaction must be of wide scope, giving consistently high yields with a variety of starting materials. It must be easy to perform, be insensitive to oxygen or water, and use only readily available reagents. Reaction work-up and product isolation must be simple, without requiring chromatographic purification.

Although vinyls have been known for decades, the synthesis and polymerization of vinyl-1,2,3-triazoles [Kevin, Anderson, Sizovs, Cortez, Waldron, Haddleton, and Reineke, (2012)] have recently gained attention driven by the striking development of the copper-catalyzed azide-alkyne cycloaddition, the most widely applied example of the click chemistry philosophy. Indeed, a broad library of vinyl-1,2,3-triazole-based

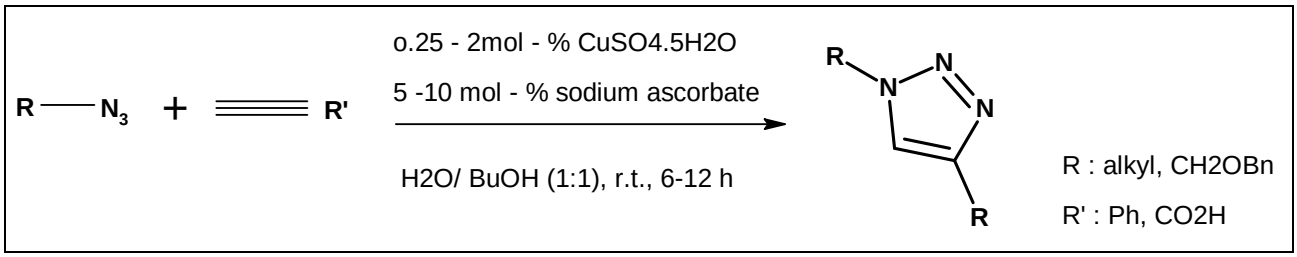
monomers and polymers carrying aliphatic, aromatic, heterocyclic or functional groups has been reported yet.

1,2,3-Triazoles have been extensively studied as compounds possessing important biological activities. An efficient approach for the synthesis of a new class of triazolophanes by 1,3-dipolar cycloaddition reaction of highly reactive organic azides with alkynes using Cu(I)-catalyzed azide–alkyne cycloaddition methodology ("click chemistry") has been developed. Huisgen's 1,3-dipolar cycloaddition of alkynes and azides yielding triazoles is, undoubtedly, the premier example of a "click reaction" [Huisgen, (1984)]. Azides and alkynes are easy to install, and, despite being among the most energetic species known, they are also among the least reactive functional groups in organic chemistry.

As per our knowledge, the terminal end group of polymer PHOBr (synthesized by *P. oleovorans)* has not been converted into an azide with subsequent click reaction with propargyl benzoate (alkyne). This conversion and click chemistry was the final goal of this project as this chemistry will be useful in the field of pharmaceutical, natural products, nano technology areas. All the compounds were characterized by spectral analyses.

1.6 Mechanism of the Copper-Catalyzed Azide-Alkyne Cycloaddition (CuAAC)

As one of the most efficient click reactions to date, the copper-catalyzed azide-alkyne cycloaddition features an enormous rate acceleration of 10^7 to 10^8 compared to the uncatalyzed 1,3-dipolar cycloaddition. In the absence of an appropriate catalyst, this reaction is usually quite slow as alkynes are poor 1,3-dipole acceptors. However, in the presence of copper(I), which can bind to terminal alkynes, cycloaddition reactions are dramatically accelerated, it succeeds over a broad temperature range, is insensitive to aqueous conditions and a pH range between 4 and 12, and tolerates a broad range of functional groups. Pure products can be isolated by simple filtration or extraction without the need for chromatography or recrystallization.

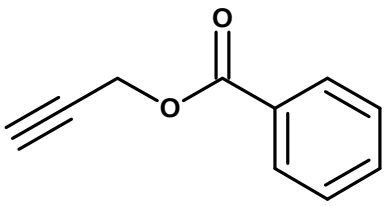


F. Himo, T. Lovell, R. Hilgraf, V. V. Rostovtsev, L. Noodleman, K. B. Sharpless, V. V. Fokin, J. 2005

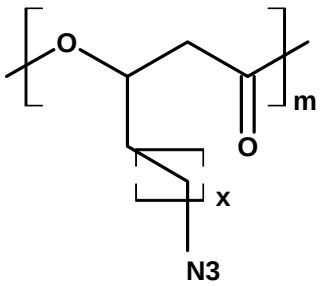
The active Cu(I) catalyst can be generated from Cu(I) salts or Cu(II) salts using sodium ascorbate as the reducing agent. Addition of a slight excess of sodium ascorbate prevents the formation of oxidative homocoupling products. Disproportionation of a Cu(II) salt in presence of a Cu wire can also be used to form active Cu(I) (K. C. Nicolaou, Tamsyn Montagnon, (2008).

The detailed mechanism for the reaction has been suggested by F Himo, T Lovell, R Hilgraf (Figure 1.7) as follows: Copper is a 1st row transition metal. It has the electronic configuration [Ar] $3d^{10}$ $4s^{1}$. The copper (I) species generated *in situ* forms a π-complex with the triple bond of a terminal alkyne. In the presence of a base, the terminal hydrogen, being the most acidic is deprotonated first to give a Cu acetylide intermediate. It has been suggested that the transition state involves two copper atoms. One copper atom is bonded to the acetylide while the other Cu atom serves to activate the azide. The metal center coordinates with the electrons on the nitrogen atom. The azide and the acetylide are not coordinated to the same Cu atom. The azide displaces one ligand (L) to generate a copper-azide-acetylide complex . At this point cyclization takes place. This is followed by protonation; the source of proton being the hydrogen which was pulled off from the terminal acetylene by the base. The product is formed by dissociation and the catalyst ligand complex is regenerated for further reaction cycles.

Propargyl benzoate



Azido PHA



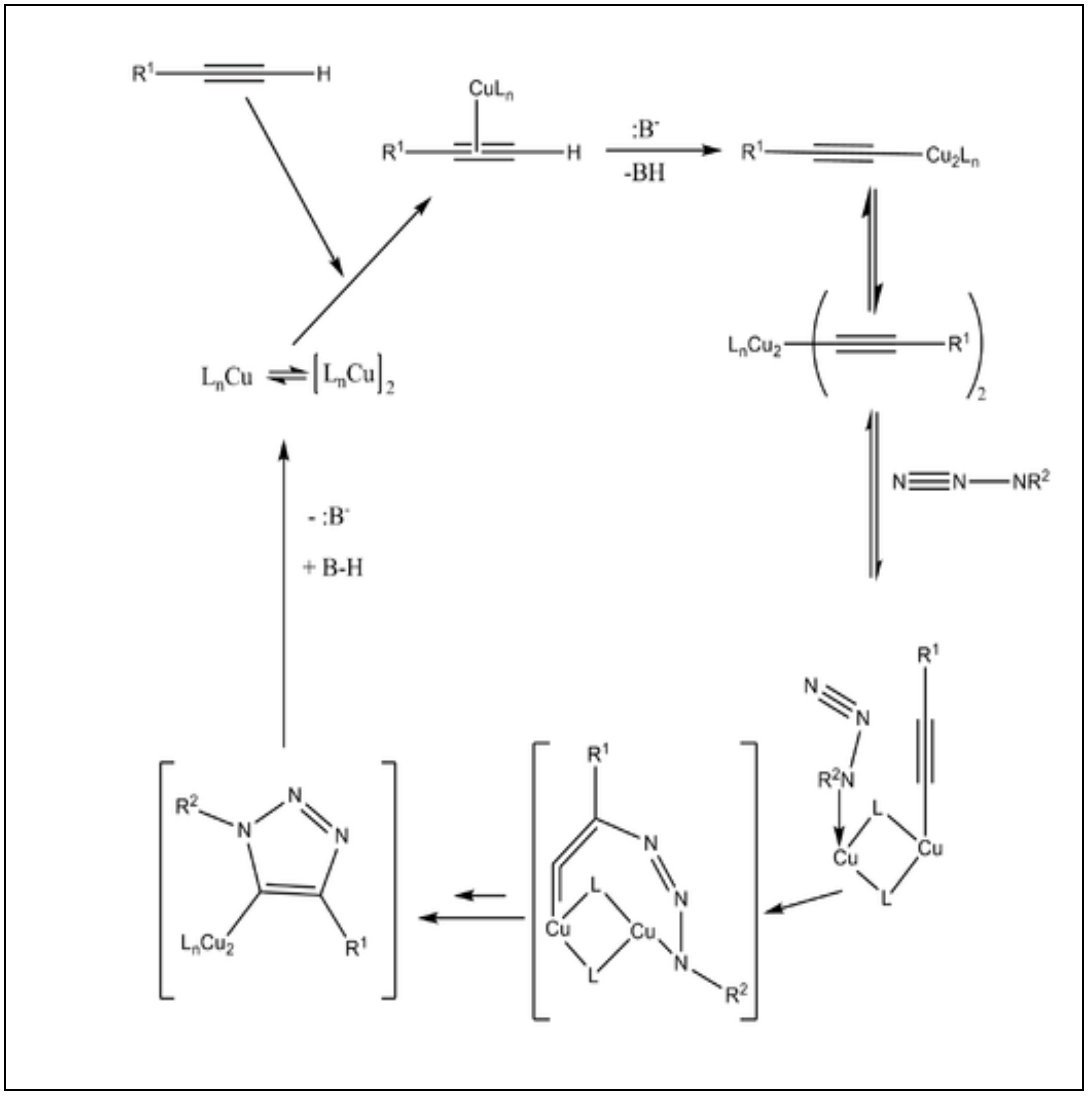


Figure 1.7 Copper catalyzed click reaction
F Himo, T Lovell, R Hilgraf (2005)

CHAPTER II

Experimental Techniques

2.1 Introduction

The main goal of this project was to synthesize a copolymer by feeding *P. oleovorans* an equimolar mixture of sodium octanoate and 11-Bromoundecanoic acid. Polymer yield is typically lower when cultures are grown on functionalized sources only. By co-feeding the derivatized acids with carbon sources that support high cell growth, copolymers are obtained that contain repeat units that carry reactive side chains. The initial carbon source ratio was not reflected in the degree of functional side groups present in the resulting copolymers. As per our hypothesis PHA with repeat unit of OA and 11-BRUA was synthesized. Although the bacteria were fed equal proportions of OA and 11-BRUA, the repeat units of PHO and PHOBr in the co-polymer were not produced in equal ratio.

Once the copolymer was synthesized with bromine as a terminal funcitional group the next goal was to aim for simple chemical modification of the side-chain of PHA. The first task was the analogous conversion of the functional group with an azide and then to perform click reaction with the alkyne group, such as propargyl benzoate or propargyl acetate.

2.2 Materials

P. oleovorans was obtained from the original cultures obtained from ATCC and the first culture was prepared according to guidelines. All salts and carbon sources were purchased from Sigma-Aldrich (St.Louis, MO) and used as received. All supplies for the fermentation including 250 mL centrifuge bottles, Eppendorf pipettes and tips, were purchased from Fisher Scientific Co. Non culture lab ware was sterilized before use. The incubator-Shaker (New Brunswick Scientific C24) was used for fermentation.

For subsequent studies *P. oleovorans* cultures were maintained in 2% agar culture plates at 4°C containing 15 mM octanoic acid (OA), 1.1 g of ammonium phosphate, 5.8 g of potassium phosphate (dibasic), 3.7 g of potassium phosphate (monobasic), 1 mL of microelement solution, 10 mL magnesium sulfate solution. Culture plates were maintained for 7-10 days and then replaced.

In order to determine the optimal batch culture volume, and growth time for PHA-producing *P. oleovorans* cultures, several growth experiments were performed as explained in section 2.3.2.

2.3 Methods: Bacterial growth

2.3.1 Preculture

100 mL preculture was prepared according to the chemicals listed in table 2.1. Preculture was autoclaved (Sterilmatic auto clave) at 121 ^{o}C for 15 min. 100mM, 12.037 g of $MgSO_4$ solution was autoclaved in a separate flask and added to the preculture after sterilization to prevent salt precipitation. Following sterilization, 15mM, 0.247g of sodium octanoate were added, along with 100mM, 1 mL $MgSO_4$. After the preculture was cooled down to room temperature or 30 ^{o}C, bacteria were inoculated by 3 loops full from the agar/Na octanoate plate by sterile procedure. The preculture was set in the New Brunswick Scientific C24 Incubator-Shaker at 30 ^{o}C and shaken at 225 rpm. After 12 to 15 hours the preculture was ready to use as inocula for the cultures. For growth curve determination, periodically 1 mL sample of the preculture was taken and diluted with 7 mL of deionized water and its optical density was measured using a Beckman Spec 20 Specrophotometer set at a wavelength of 640 nm referenced to distilled water. Once a strong optical density above 4.0 was reached the preculture was ready for inoculation.

Table 2.1 Composition of E* medium for growth and polymer production in *P. oleovorans* (Brandl 1988). Agar plates contained identical compositions with the addition of 2% (wt/wt) agar. Fermentations were typically performed in 100-mL, 200-mL, and 1-L cultures.

Preculture

100 mL DI water	
$(NH_4)_2HPO_4$ (g)	0.11
K_2HPO_4 (g)	0.58
KH_2PO_4 (g)	0.37
Sodium octanoate (g)	0.247
100 mM $MgSO_4$ (mL)	1
microelement soln (mL)*	0.1

microelement solution (per 1.0 L of 1.0 M HCl):

2.78 g $FeSO_4 \cdot 7H_2O$
1.98 g $MnCl_2 \cdot 4H_2O$
2.40 g $CoSO_4 \cdot 6H_2O$
1.67 g $CaCl_2 \cdot 2H_2O$
0.17 g $CuCl_2 \cdot 2H_2O$
0.29 g $ZnSO_4 \cdot 7H_2O$

Table 2.2 Composition of PHOBr Culture fermentation, 200 mL

(7.5 mM SO:7.5 mM 11-BrUA)

200 mL DI water	
$(NH_4)_2HPO_4$ (g)	0.22
K_2HPO_4 (g)	1.16
KH_2PO_4 (g)	0.74
Sodium octanoate	0.125
11-bromoundecanoic acid (g)	0.199
100 mM $MgSO_4$ (mL)	2
microelement soln (mL)*	0.2

Table 2.3 Composition of PHOBr Culture fermentation, 1L

(7.5 mM OA:7.5 mM 11-BrUA)

1 L DI water	
$(NH_4)_2HPO_4$ (g)	1.1
K_2HPO_4 (g)	5.8
KH_2PO_4 (g)	3.7
octanoate	1.25
11-bromoundecanoic acid (g)	1.99
100 mM $MgSO_4$ (mL)	10
microelement soln (mL)*	1

2.3.2. PHOBr production by *P. oleovorans*

One 100-mL flask of preculture containing E* medium and 15 mM concentration of sodium octanoate was prepared as per section 2.3.1. Three 200-mL cultures were prepared in 500 mL dented shake flasks containing 0.22 g ammonium phosphate, 1.16 g potassium phosphate (dibasic), 0.74 g potassium phosphate (monobasic), 0.2 mL microelement. It was autoclaved for 20 minutes at 121 ^{o}C. Once the culture cooled down to 30 ^{o}C, 15 mM concentration of carbon sources at a 50:50 (OA:11BrUA) (0.125g: 0.199g) and $MgSO_4$ was added to each flask. After 14 hrs of growth of the preculture as described in section 2.3.1, 20 mL (10%) of the preculture was used to innoculate the fresh 200-mL culture as a liquid inoculum. The 200-mL cultures were grown in parallel for polymer harvest. Cultures were monitored periodically for the growth curve by taking 1 mL sample of cultures and diluting it to 1:7 ratio with DI water from each flask, followed by measuring its OD using a spectrometer, set at 640 nm. At a series of time points 0 h, 2 h, 11 h, 13 h, 15 h OD was measured. The measurement was recorded and the data plotted as optical density as a function of time. This experiment was repeated several times to pin point the stationary and lag phase.

2.3.3. Determination of polymer content in PHOBr

Once the growth curve was established, several other batches of culture were harvested at different optical densities for example at 2.0, 2.2, 2.4, 2.5, 2.6, 2.8, 3.0, 3.2, 3.4 to determine the polymer content and composition by NMR. Cultures were centrifuged in 200-mL centrifuge bottles using JLA-16250 rotor at 3700 rpm for 25 min at 4 ^{o}C. The post spin supernatant was discarded and the cell pellet was collected from the each bottle. The collected biomass was frozen at -20 ^{o}C and subsequently lyophilized for 24 h. The dry biomass was placed in a 50-mL or 250-mL round bottom flask to which 30 mL or 150 mL dichloromethane was added. The polymer was extracted by refluxing for 48 hrs under stirring and resulting polymer solution was separated from the cell debris by filtering through Whatman #5 filter paper, inside funnel. The polymer solution was concentrated under reduced pressure in a Beckman rotary evaporator, and then precipitated by adding the concentrated viscous solution drop wise to cold (-15 ^{o}C) methanol under stirring. The resulting sticky polymer was dried under vacuum at room temperature for 24 h and stored under reduced pressure in a vacuum oven at room temperature.

2.3.4. *P. oleovorans* growth in larger culture volumes

The growth of *P. oleovorans* in larger culture volumes was investigated in comparison to previous cultures grown in smaller volumes. 1L and 100 mL containing E* medium and 7.5 mM of SO and 7.5 mM of 11 BrUA were prepared and inoculated from plates. Growth was monitored as described above by recording optical densities. By scaling up the culture volume the time for growth was almost doubled. Cultures were

centrifuged in 1-L centrifuge bottles using JLA-9.1 rotor at 3700 rpm for 40 min at 4 ^{o}C. Followed the same process as described in section 2.3.3 to harvest the polymer.

2.3.5 Synthesis of 11 Azido Undecanoic acid (Small molecule)

Due to the very low yield of the PHOBr, it was decided to perform click chemistry by a small molecule model. To a solution of 11-BrUndecanoic (1 g) in 100 mL of DMF, sodium azide (0.5g) was added. The mixture was allowed to stir at 60 ^{o}C for 48 h (Source Fernandez-Suarez, Baruah, Martizez-Hernandez, T.Xie, Baskin, Bertozzi and Ting).

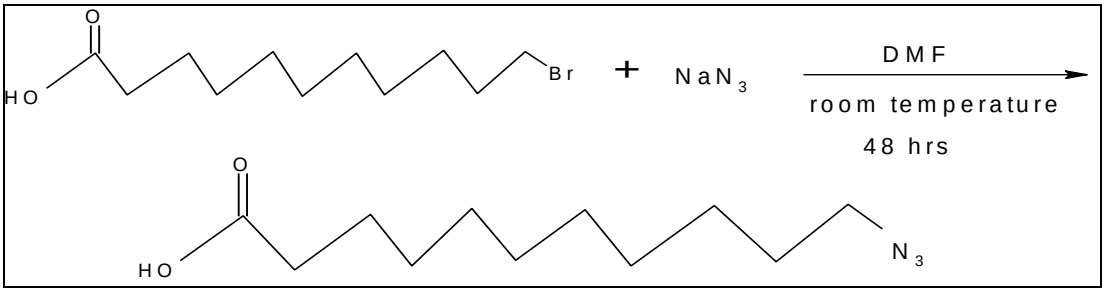


Figure 2.1 Reaction scheme of 11-Azidoundecanoic acid

The progress of the reaction was monitored by FT-IR. Prominent azide peak was observed at 2094 cm^{-1} and was visible in the FT-IR spectrum (figure is provided in the results and discussion chapter). DMF was removed under reduced pressure. The azido-PHA was pale yellow/brownish in color and sticky with 30% yield. The sample was left to dry under the hood overnight before carrying out click chemistry.

2.3.6 Synthesis of Azido Click Compound (Small molecule)

Once the functional bromine group was substituted by azide (S_N2 reaction), this azide underwent "click reaction" with an alkyne group. It was decided that propargyl benzoate and/or propargyl acetate would be used as as alkyne.

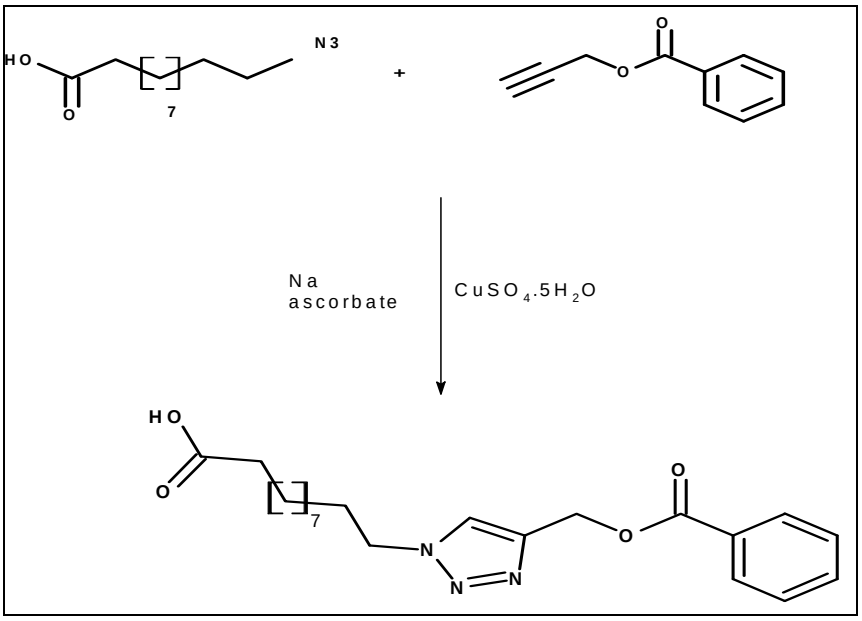


Figure 2.2 Reaction scheme of Azido click compound

In a round bottom flask 11-azidoundecanoic acid (0.091 g), and propargyl benzoate (0.512 g) was dissolved in THF (20 mL). Freshly prepared aqueous solution (2mL) of sodium ascorbate (0.068 g) was added followed by aqueous solution (2mL) of copper sulphate pentahydrate (0.017 g) was added. The ratio of azide and alkyne was 1:8. The mixture was stirred for 2 days at 80 ^{o}C temperature. The reaction mixture was cooled to room temperature. Functionalized small molecule was precipitated in hexane and allowed to dry in hood. 1,3-Triazole click compound (small molecule) was established by running NMR and correlating the data to available literatures.

2.3.7. Synthesis of 11-AzidoPHA

To a solution of 11-BrPHA (100 mg) in 20 mL of DMF, sodium azide (0.22 mg) was added. The mixture was allowed to stir at 60 ^{o}C for 144 hr (6 days). (Fernandez-Suarez, Baruah, Martizez-Hernandez, T.Xie, Baskin, Bertozzi and Ting, 2007).

The progress of the reaction was monitored by FT-IR. On the sixth day prominent azide peak at 2094 cm^{-1} was visible in the FT-IR spectrum. Spectra is provided in

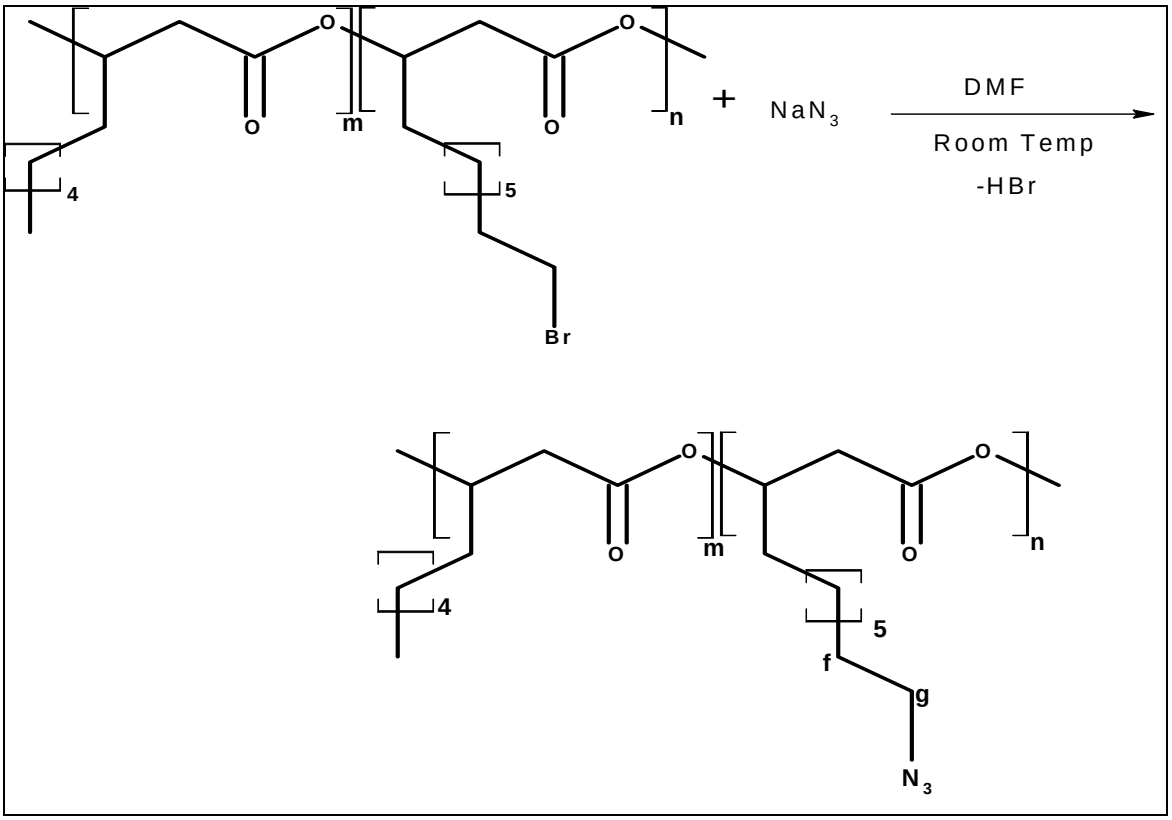


Figure 2.3 Reaction scheme of 11- Azido PHA

the results and discussion chapter (Chapter III). DMF was removed under reduced pressure. The azido-PHA was pale yellow/brownish in color and sticky. The sample was left to dry under the hood overnight before carrying out click chemistry.

2.3.8 Synthesis of PHA Click Compound

Once the functional bromine group was substituted by azide (S_N2 reaction), now this azide underwent "click reaction" with an alkyne group. It was decided that propargyl benzoate and/or propargyl acetate would be used as alkyne.

In a round bottom flask PHA-N_3 (0.10 g), and propargyl benzoate(0.512 g) was dissolved in THF (20 mL). Freshly prepared aqueous solution of sodium ascorbate (0.068 g in 2 mL) was added followed by aqueous solution of copper sulfate pentahydrate (0.017 g in 2mL). The ratio of azide and alkyne was 1:8. The mixture was stirred for 2 days at 80 ^{o}C.

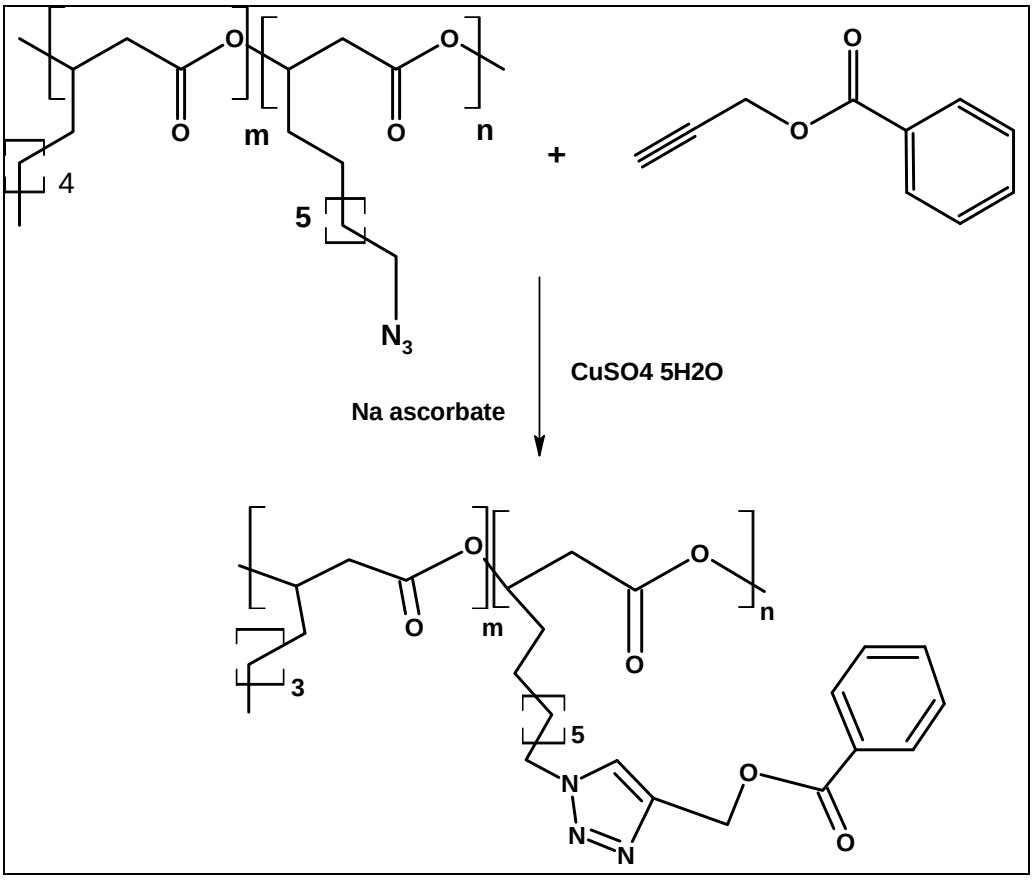


Figure 2.4 Reaction scheme of PHA click compound

The reaction mixture was cooled to room temperature. Functionalized polymer was first treated with minimal amount of water and then centrifuged. The precipitate was frozen overnight and then lyophilized for 24hrs. The dried sample was dissolved in dichloromethane and then centrifuged. The collected sample was dissolved in ether and filtered. 1,3 triazole click compound was established by running NMR and correlating the data to available literature (Anderson,Kevin ; Sixovs, Antons; Cortez Mallory; Waldron, Chris; Haddleton DM; Reineke, Theresa M. 2012).

2.4 Characterization of the polymer

2.4.1 FT-IR : Polymer was studied by the FT-IR and the peaks of ester bond, methyl, methylene groups were recorded. The PHA extracted from the organism was analyzed by FT-IR spectroscopy (JASCO FT/IR). The range of the FT-IR used in this study, for experimental data analysis was set at 4000-650 cm^{-1} to confirm the functional groups of the extracted polymer. About 5 mg of the sample was placed directly on the crystal to be analyzed by the attenuated total reflectance (ATR). ATR uses a property of total internal reflection sampling technique used in conjunction with infrared spectroscopy which enables samples to be examined directly in the solid or liquid state without further preparation. The results of FT-IR spectroscopy are shown in the results and discussion pages. The result obtained is similar to that of other researchers.

2.4.2 Nuclear magnetic resonance (NMR) characterization

Following parameters were used consistently while runing all the NMR samples during this project.

	Parameter	Value
1	Title	mc_Bromo_PHA_PROTON_01
2	Comment	mc_Bromo_PHA
3	Origin	Varian
4	Owner	
5	Site	
6	Spectrometer	inova
7	Author	
8	Solvent	cdcl3
9	Temperature	25.0
10	Pulse Sequence	s2pul
11	Experiment	1D
12	Number of Scans	16
13	Receiver Gain	30
14	Relaxation Delay	1.0000
15	Pulse Width	0.0000
16	Acquisition Time	2.7304
17	Acquisition Date	2012-04-10T16:01:45
18	Modification Date	2012-04-10T16:01:46
19	Spectrometer Frequency	499.92
20	Spectral Width	6000.6
21	Lowest Frequency	-505.1
22	Nucleus	1H
23	Acquired Size	16384
24	Spectral Size	32768

A purified polymer sample was dissolved in deuterated chloroform containing TMS as an internal reference. NMR experiments were performed using a Varian Unity Inova 500 MHz spectrometer equipped with indirect detection pulsed field gradient. All measurements were conducted at 25°C. Proton spectra were taken of the purified polymer, 11 bromoundecanoic acid, and 1,3-triazole click compound.

CHAPTER III

Results and Discussion

3.1. Bacterial growth

The main goal of the project was to produce PHAs from sodium octanoate and 11-bromoundecanoic acid to obtain a reactive side chains with bromine as the terminal functional group, to perform click reactions. Polyhydroxyalkanoates are synthesized by numerous bacteria as intracellular carbon and energy source. These are accumulated in the cytoplasm of cells as granules under conditions of nutrient imbalance. Accumulation usually occurs when carbon is in excess and if at least one nutrient that is essential for growth, is limiting. *Pseudomonas oleovorans* is one of the most versatile microorganisms in its ability to produce PHAs from a variety of carbon substrates, including many different *n*-alkanes, *n*-alkenes, alkanoic acids, and alkenoic acids. The literature suggests that functionalized bacterial PHAs are obtained in higher yields by using co-feeding techniques, that is, polymer is produced by cultures containing combinations of high-growth carbon sources (*e.g.,* OA or NA), and those carbon sources that provide desirable side chain functionality (*e.g.*, 11BrUA) (Fritzsche 1990; Kim 1992; Hazer 1994; Scholz 1994; Kim 1995; Bear 1997).

PHNBr has been previously synthesized (Kim 1992; Fritzsche 1990) from nonanoic acid and 11-bromoundecanoic acid. Synthesis of PHAs from octanoic acid and 11-bromo undecanoic acid was not successful (Kim 1992), as the culture did not grow even after 48hrs. During this current project it was decided to produce a copolymer using

octanoic acid and 11-bromo undecanoic acid as a trial and if it worked then proceed further and make ample amount of copolymer PHOBr. Although, PHOBrs production was successful, the yield was low.

In these prior reports, bacterial cultures were grown for polymer production using continuous culture chemostats. Such procedures routinely allow the production of ~10 g of polymer in similar time scales from 12 L fermentors. Since this capability was not available for this study, growth and polymer production curves were developed from small-scale shake flask experiments. Studies were performed using a range of volumes and temperature in order to find the optimal conditions for maximum polymer production using such a batch culture approach.

For this project, the polymer, producing substrate OA was chosen. Growth of polymer and its production curves were developed in the lab from small-scale shake flask experiments. Three 200-mL flasks of culture were grown in parallel to determine the growth curve of bacteria (Figure 3.1). The purpose of running three experiments at the same time was to ascertain the growth time of bacteria when it was fed only OA. By comparing the data from the three experiments it was determined that *P. oleovorans*, when fed only on OA, has log phase at around 5 hrs, stationary phase at 7 hrs and death phase at 10 hrs.

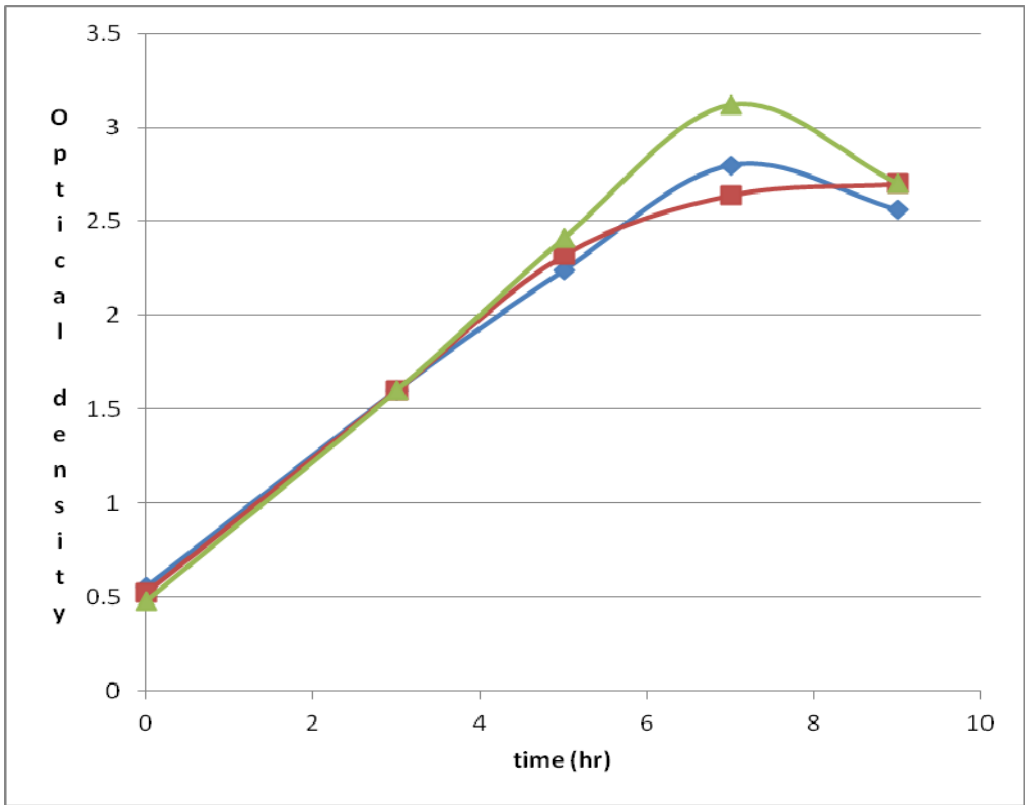


Figure 3.1. Growth curves for 200-mL cultures to produce PHO. The cultures were inoculated from a 14 hr pre-culture on 100% (15 mM) octanoic acid and were grown on a 100% octanoic acid. (three parallel cultures)

Once the growth curve of bacteria was established with octanoic acid, the same experiment was run with equimolar mixture of 11-bromoundecanoic acid (7.5 mM) and PHA producing substrate OA (7.5 mM) because the first goal of the project was to make a copolymer with a functional side chain.

The highest yield of polymer was observed in 15 mM equimolar mixture of OA and 11BrUA (50:50) between 2.5 and 2.7 optical density. Octanoic acid is the carbon source that supports strong cell growth and polymer production. Once the data were collected it was plotted and the similarity in the shapes of the growth curves seem to

suggest that the cultures grow first on OA and once this carbon source is used up by the bacteria, the growth slows down as shown by the dip in the chart (Figure 3.2) and then the bacteria starts to feed on 11-BrUA (functionalized carbon source) and continues growth till it reaches the stationary phase (around 17 h) and then starts to die.

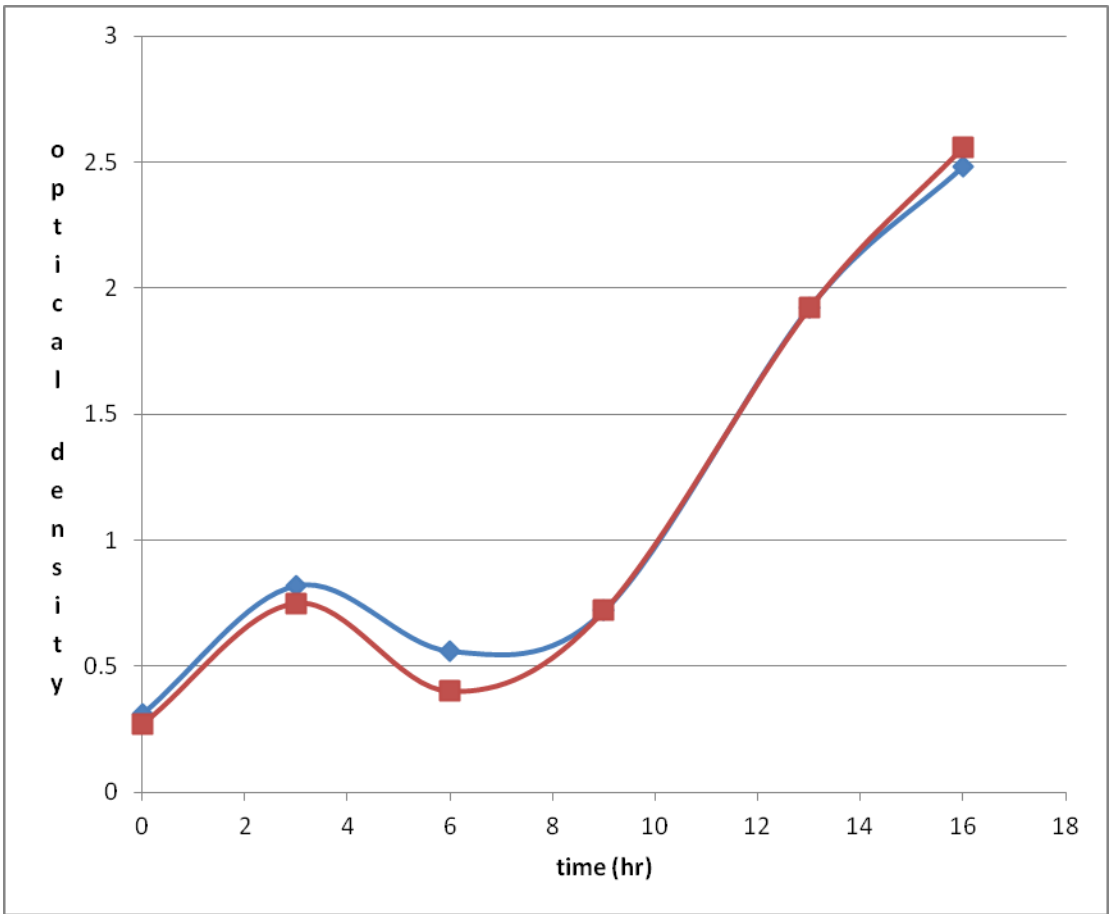


Figure 3.2 . Growth curve of two 200-mL cultures to produce PHOBr. The dip in the curve shows the exhaustion of OA source. Later on the growth is on 11-BrUA till it reaches the maximum growth.

Once the polymer production was scaled up to 1-L culture the same trend was observed as of 200-mL, except that the time to reach maximum optical density between 2.5 to 2.7 to harvest polymer scaled up to 24 h as shown in Figure 3.3.

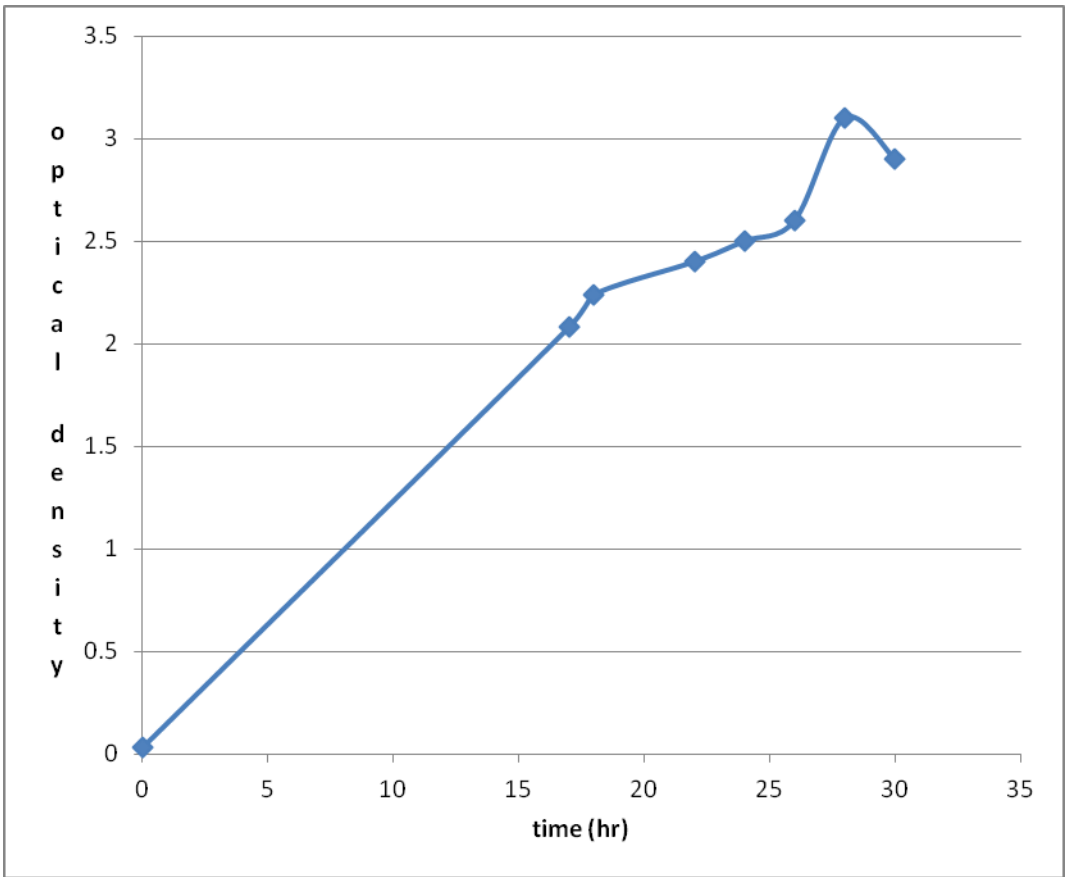


Figure 3.3 . Growth curve of 1-L cultures to produce PHOBr.
The curve shows the growth of the bacteria on OA and 11-BrUA. The dip in the curve is not witnessed in this figure as the first reading was taken at 15 hours.

PHOBr production was induced in *P. oleovorans* by supplying the culture with equimolar mixtures of growth and polymer-producing octanoic acid (OA) and

functionalized bromine-terminated 11-bromoundecanoic acid (11-BrUA). Batch cultures containing equimolar mixtures of OA and 11-BrUA (15 mM total) were inoculated with pre cultures grown on 100% OA. A typical growth curve behavior was observed with an 11h lag time, (Figure 3.2), followed by an exponential growth phase that reached a maximum optical density of 3.0 after a total growth time of 25 hr.

Effect of temperature on growth of PHOBr and the content of bromine in the synthesized copolymer PHOBr (determined by NMR ^{1}H data) respectively for 200 mL cultures and 1L culture is shown in Table 3.1, and Table 3.2. As the temperature was raised to 32 ^{o}C the time to reach maximum density was shorter but the bromine content in the polymer was less. By raising the temperature to 35 ^{o}C, the harvesting time was almost reduced by half but the amount of polymer harvested was very low. Harvesting of ample amount of polymer with good quantity of bromine content in the polymer depends not only on temperature but also on the time and optical density at which it is harvested.

Table: 3.1 Effect of temperature on growth of PHOBr and the content of bromine in the synthesized copolymer PHOBr in 200 mL Culture

Temp	Time (hrs)	Abs	Dilution(1:7)	OD	Bromine content (%)	PHOBr (mg/200mL)
30 ^{o}C	16	0.35	1:7	2.50	~30	10
32 ^{o}C	9 hrs	0.39	1:7	3.12	~12	6
30 ^{o}C	14	0.34	1:7	2.72	~20	10

Table:3.2 Effect of temperature on growth of PHOBr and the content of bromine in the synthesized copolymer PHOBr 1-L Culture

Temp	Time (hrs)	Abs	Dilution(1:7)	OD	Bromine content (%)	PHOBr (mg/L)
30 ^{o}C	23	0.39	1:7	3.12	~20%	12
30 ^{o}C	22	0.31	1:7	2.5	~30%	20
35 ^{o}C	17	0.37	1:7	3.0	~18%	10
30 ^{o}C	22	0.43	1:7	3.4	~12	8
32 ^{o}C	20	0.32	1:7	2.5	~30	23
35 ^{o}C	6	0.42	1:7	3.2	0	0

3.2. PHA Purification

PHA produced by the bacteria is segregated in inclusion bodies inside the cell. To obtain pure PHA, the biomass was collected by centrifugation. Harvesting bacterial cells enhances the stress on bacteria by removing their natural aqueous environment and can lead to accelerated degradation. Therefore, harvesting must not only be conducted at low temperature, but also as quickly as possible. In addition, the biomass must be frozen immediately after recovery from centrifugation. The frozen cell mass was lyophilized thus yielding the dry biomass. During the lyophilization step, it is not expected that the cells further degrade the polymer, as the cells go from the frozen to the dry state, neither of which support metabolism. The dry cells were lyophillized and PHA was extracted by refluxing with dichloromethane. Organic solvent extraction ruptures the cell membrane as well as the membrane surrounding the inclusion bodies. The resulting polymer solution was concentrated, and the PHA precipitated by adding the CH_2Cl_2 solution drop-wise into cold methanol. Sufficiently pure PHA was obtained, as evidenced in the ^1H NMR spectrum and the FT-IR spectroscopy. Typical yield for PHOBr was recorded to be approximately 20 mg/L.

In an attempt to increase the overall yield per set of batch cultures, the cell growth was measured on 1-L cultures compared to 200-mL cultures, containing 50% octanoic acid and 50% 11-Bromo undecanoic acid. It was observed in the experiment that the 1-L cultures took longer to reach the log phase. This effect is explained due to the relative difference in the size of the total culture volume. The growth in 1-L culture compared

favorably with the 200-mL cultures. The exponential growth phase was reached after 15 h for the 200-ml culture as compared to 22 hrs for the 1-L culture.

3.3. PHOBr Characterization

3.3.a ^{1}H NMR of PHOBr

The representative ^{1}H NMR spectra for PHOBr (Figure 3.4) and 11-bromoundecanoic acid (figure 3.5) are included below. The experiments were conducted in $CDCl_3$ and chemical shifts were assigned using TMS as in internal reference. Characteristic PHOBr peaks were observed at the appropriate chemical shifts, including main chain methylene protons (c, 2.5 ppm) and methine protons (bound to chiral carbon) (a, 5.1 ppm) and side chain protons (d, 1.2 ppm; 1.5 ppm). Side chain protons unique to PHOBr were observed at -C$\underline{H}_2$-CH_2-Br (f, 1.8 ppm), and –C$\underline{H}_2$-Br (e, 3.4 ppm). The percent of brominated side chains was determined based on the integration of the –CH_2-Br peaks at 3.4 ppm in relation to the chiral proton peaks at 5.1 ppm. The side chain terminal functional group contents of PHOBr was calculated to be between 15% and 30 % . The content of side chain terminal functional group in polymer formed from NA and 11 BrUDA is higher 37% to 45% (Kim et al 1992, Sparks 2007).

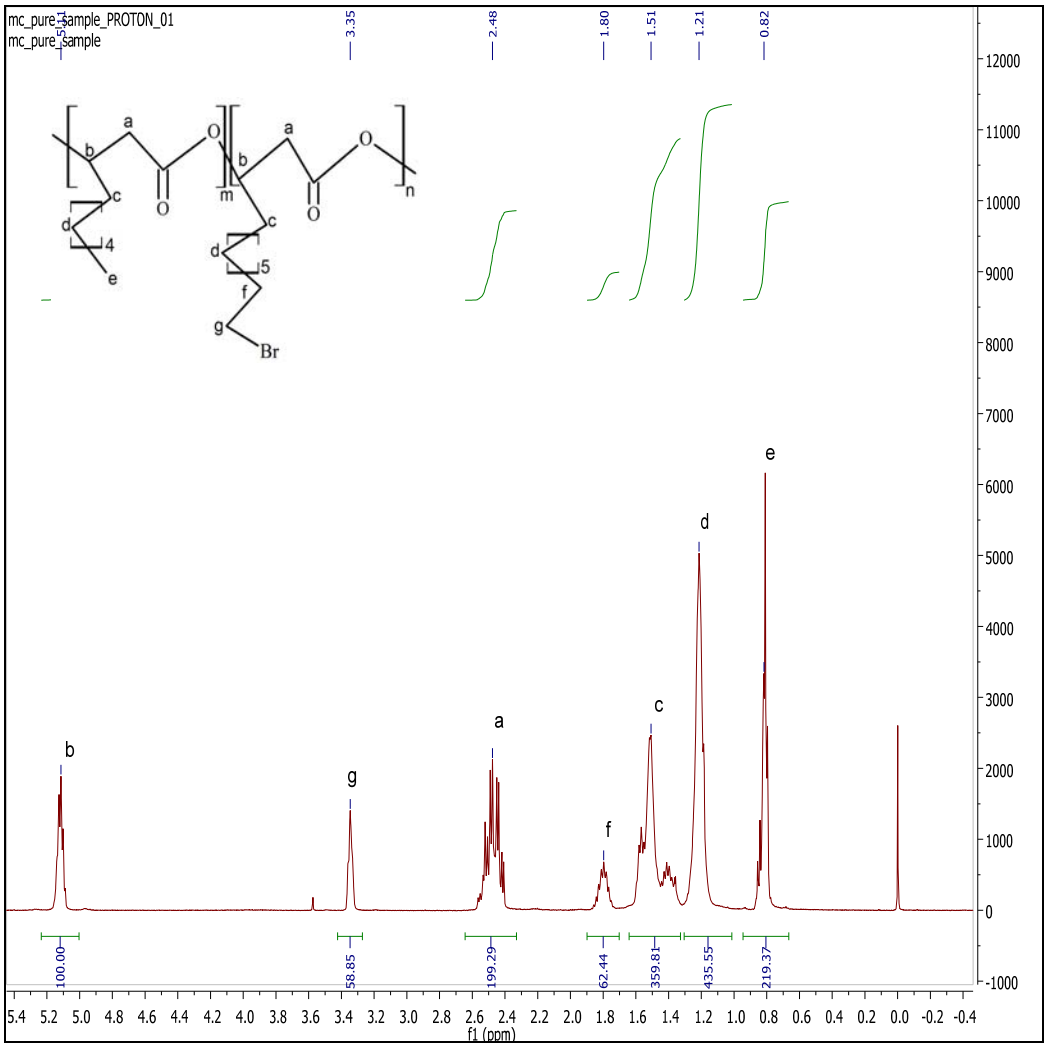


Figure 3.4 ^{1}H NMR spectrum of PHOBr.

The representative ^{1}H NMR spectrum for 11-bromoundecanoic acid (Figure 3.5) is included below. The experiments were conducted in $CDCl_3$. Characteristic protons were observed in the ^{1}H nmr spectrum. –C<u>H</u>$_2$-Br (a 3.4 ppm), -C<u>H</u>$_2$-CH$_2$-Br (f, 1.8 ppm), methylene protons (d, 2.5 ppm) next to the carbonyl group of acid, and rest of the protons (c, 1.6 ppm-1.3 ppm). Protons unique to 11-Bromoundecanoic were observed at the appropriate chemical shifts. Integration of all 21 protons can be observed.

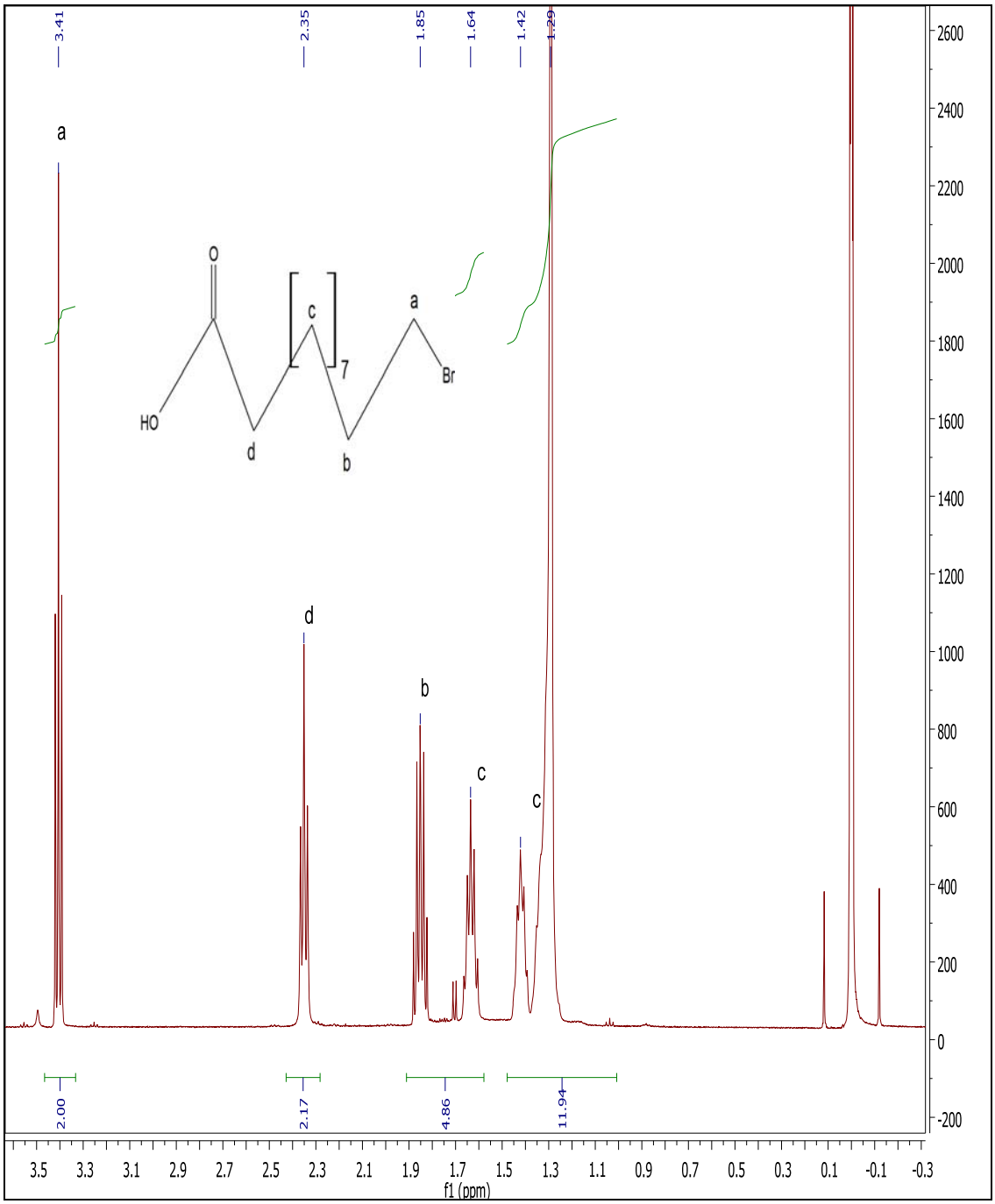


Figure 3.5. ^{1}H NMR spectrum of 11-Bromo Undecanoic Acid

Spectra of 11-bromonundecanoic acid (Figure 3.5) and PHOBr (figure 3.4) can be compared and it is observed that the –C<u>H</u>$_2$-Br peak is found at the same 3.4 ppm chemical shift, while the -C<u>H</u>$_2$-CH$_2$-Br at 1.8 ppm. The spectrum of 11-BrUDA was used to verify the peak assignments for the PHOBr polymer.

3.3.b. FT-IR Characterization of PHOBr

Polymer was studied by the FT-IR and the peaks of ester bond, methyl, methylene groups were noticed at 2900-2800 cm^{-1} The PHA extracted from the organism was analyzed by FT-IR Spectroscopy (JASCO FT/IR). It was used under the following conditions: spectral range, 4000-650 cm^{-1} to confirm the functional groups of the extracted polymer. The functional groups of the extracted PHOBr granules were identified by C=O group. The results of FT-IR spectroscopy are shown in Figure 3.6. The result obtained is similar to that of other researchers (De Smet et al., 1983; Castillo et al., 1986). Peak of CH_2-Br function is not visible in this spectrum as these functional peaks are reported to be visible around $400cm^{-1}$. The scale of the FT-IR instrument being used in this current study was from 4000 cm^{-1} to 650 cm^{-1}

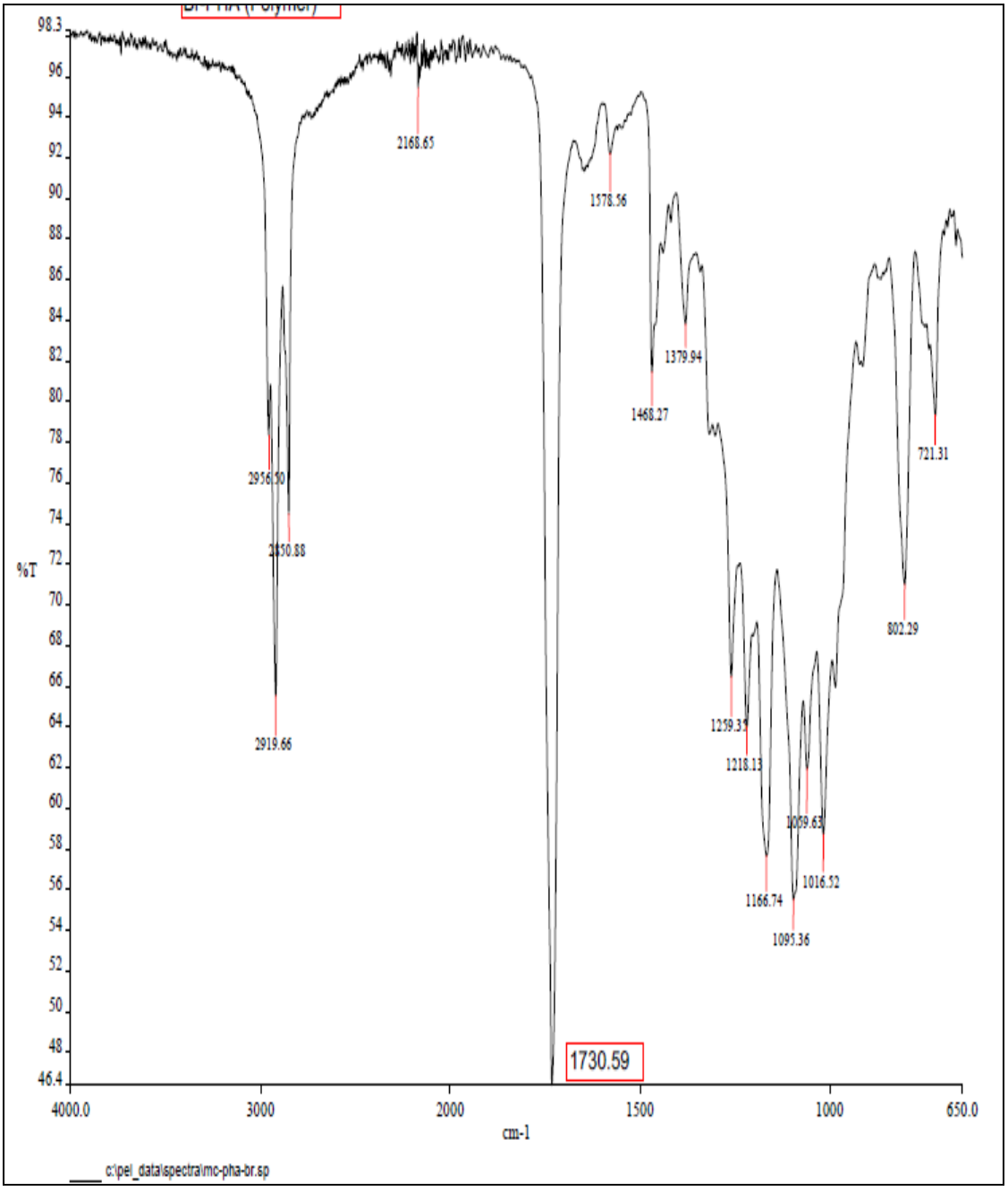
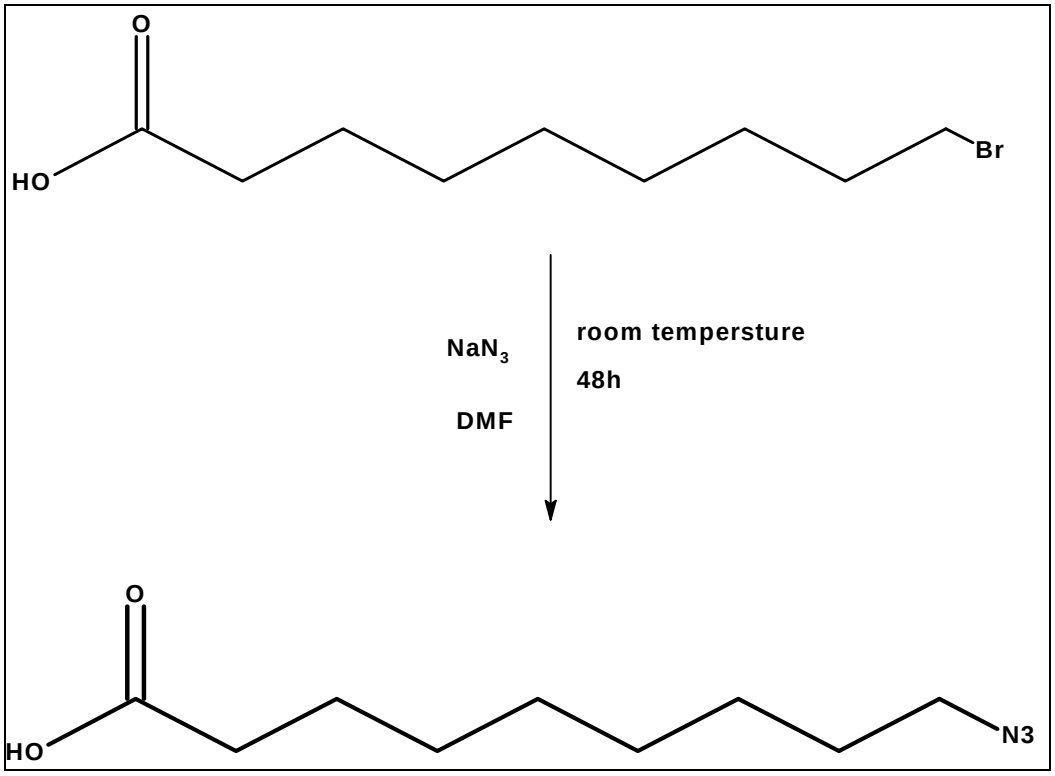


Figure 3.6 FT-IR of polymer PHOBr

3.4 Chemical conversion of brominated polymer

3.4.1 Model transformation of 11-Br-undecanoic acid into 11-azido undecanoic acid

In order to best utilize the synthesized polymer preliminary work was conducted on a small molecule (11-bromoundecanoic acid). Specifically the conversion of the bromo groups into an azide and with a subsequent "click chemistry" reaction was studied. It was hypothesized 11-bromoundecanoic acid, should undergo S_N2 reaction with NaN_3 (sodium azide) and form 11-azidoundecanoic acid. The azide then underwent 1, 3-dipolar cycloaddition click chemistry with propargyl benzoate or propargyl acetate to form the corresponding triazoles.



11-Azidoundecanoic acid

3.4.1.a ^{1}H NMR spectrum of 11-Azido Undecanoic Acid

11-Azido-undecanoic acid was analyzed by ^{1}H NMR (Figure 3.7). Chloroform-d ($CDCl_3$) was used as the solvent and tetramethylsilane (Me_4Si) as internal standard. The following peaks were observed 3.26ppm (a,2H $C\underline{H_2}N_3$), 2.33ppm (d,2H $C\underline{H_2}$-COOH), 1.61ppm(d,4H $C\underline{H_2}$-CH_2-N_3 and $C\underline{H_2}$-CH_2COOH), and 1.26(c,12H 6*$C\underline{H_2}$) ppm. Peaks at 2.10 and 2.04 could not be identified. The spectra was comparable to the literature published by Fernandez-Suarez group (2007).

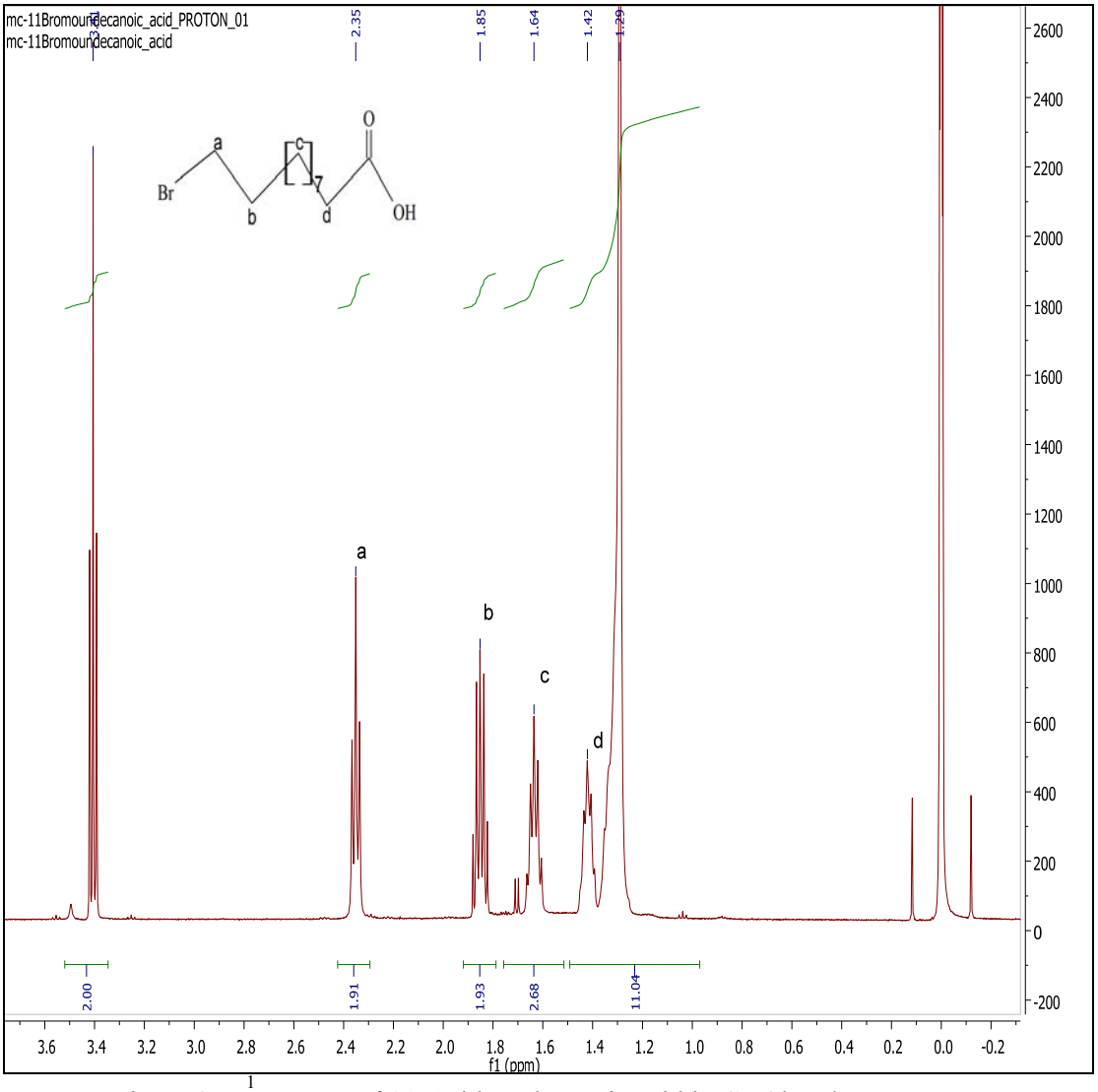


Figure 3.7 ^{1}H NMR of 11-Azidoundecanoic acid in $CDCl_3$ solvent.

3.4.1.b FT-IR Characterization of 11-Azidoundecanoic acid

In the FT-IR strong peak was observed at 2094 cm^{-1} which is the indicative of formation of azide (see Figure 3.8). It was further supported by observation of the azide stretch at 2094 cm^{-1} in the FTIR spectrum (Gacal, Koz, Yagci).

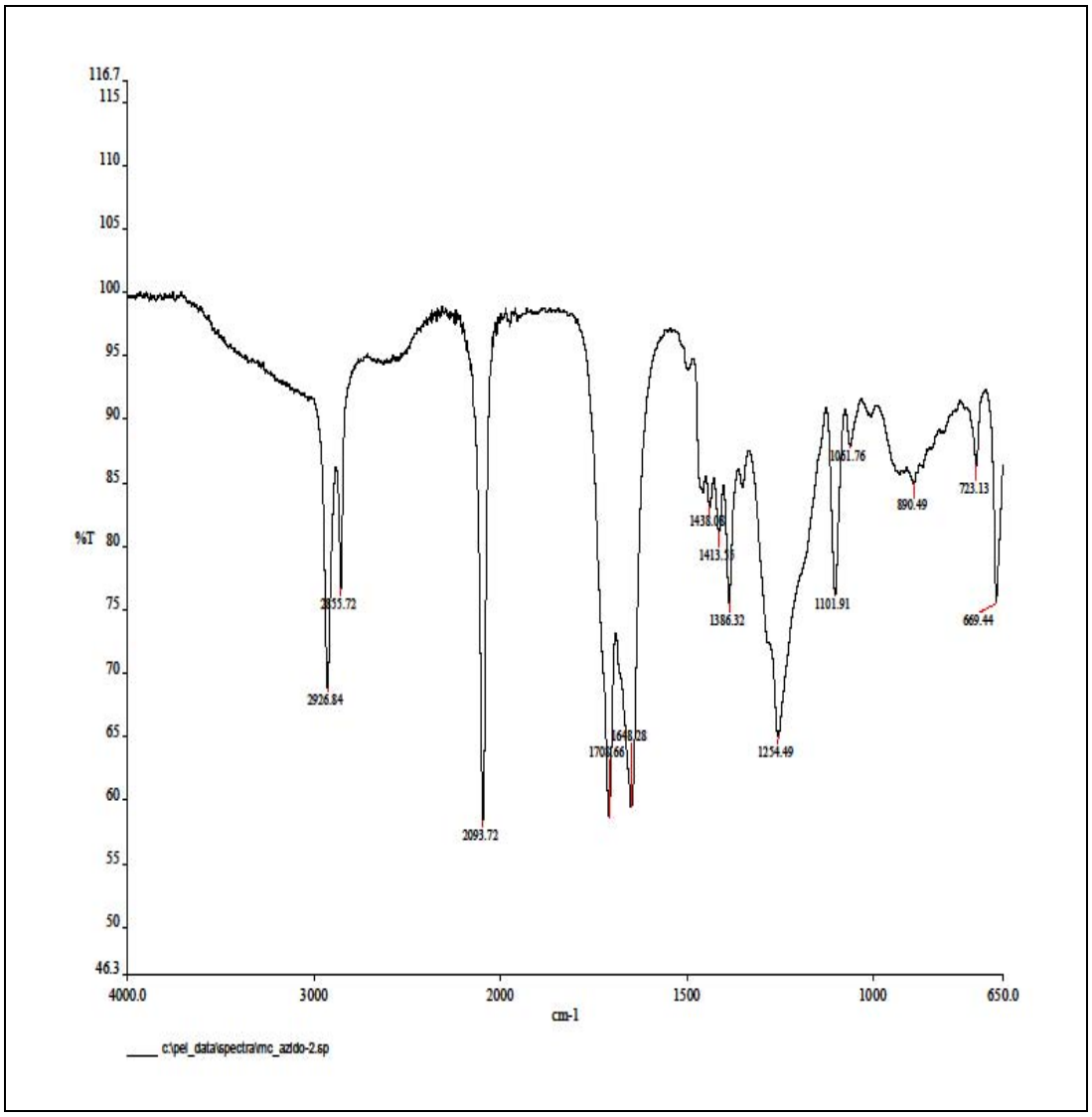


Figure 3.8 FT-IR of 11-Azido Undecanoic Acid

3.4.2 Conversion and characterization of 11-azido-undecanoic acid to 1,3 triazole click compound

After the successful synthesis of 11-azido undecanoic acid the next step was to perform click chemistry with an alkyne. Propargyl benzoate and Propargyl acetate were chosen as the alkyne substrates to click with the azide.

Equal molar quantities of 11-azidoundecanoic acid and propargyl benzoate were added in presence of $CuSO_4.5H_2O$ and Na ascorbate in THF as a solvent (Figure 3.9). $CuSO_4.5H_2O$ acts as a catalyst which binds with the ligands and catalyzes the formation of the triazole. Na ascorbate reduces Cu(II) to Cu(I). The mechanism has been explained in chapter 2. This experiment was run for five days and monitored by FT-IR for the disappearance of azide peak and appearance of carbonyl peak signifying the formation of triazole compound. The azide peak was still visible while the carbonyl peak of the propargyl benzoate was not. As equal molar ratio of 11-azido-undecanoic acid and propargyl benzoate did not work it was decided to run four parallel experiments. In the first experiment the ratio of 11-azido-undecanoic acid to propargyl benzoate was 1:2, in the second experiment the ratio was 1:4, and in the third experiment the ratio was 1:8. The fourth experiment was run with 11-azido-undecanoic acid and propargyl acetate in 1:8 ratio with DMSO as solvent.

All of the above mentioned four experiments were run at 60 ^{o}C for 60 hrs. The azide peak was monitored periodically by running FT-IR spectra of the samples. With the progress of reaction time, azide peak for all of the above mentioned experiments diminished over time. After about 60 h, the azide peak was not noticeable in the experiment number three where the ratio between the 11 azido-undecanoic acid and

propargyl benzoate was 1:8 (Figure 3.10). For experiment first, and second (figure 3.11 and 3.12) the azide peak was still observed. In experiment four (11-azido undecanoic acide : propargyl acetate) along with the azide peak, carbonyl peak of propargyl acetate was not observed (Figure 3.14). Further research needs to be done to find out as to why the ester bond breaks.

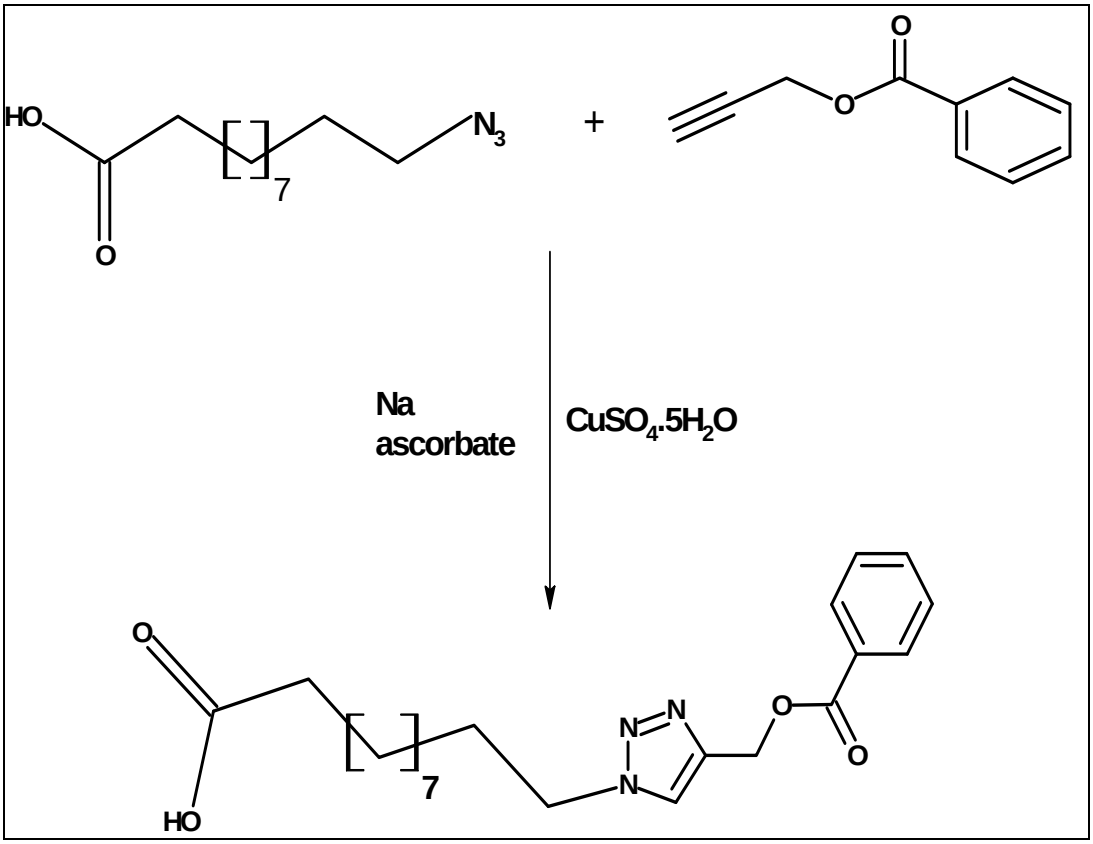


Figure 3.9 Chemical reactions between 11-azidoundecanoic acid and propargyl benzoate.

3.4.2.a FT-IR Characterization of Azido 1,3 triazole clickcompound (small molecule)

In the FT-IR spectrum, azide peak was not observed at 2093 $cm^{-1,}$ which was the characteristic of azides. The experiment (1:8) was run continuously for 60 hrs at ~60 ^{o}C.

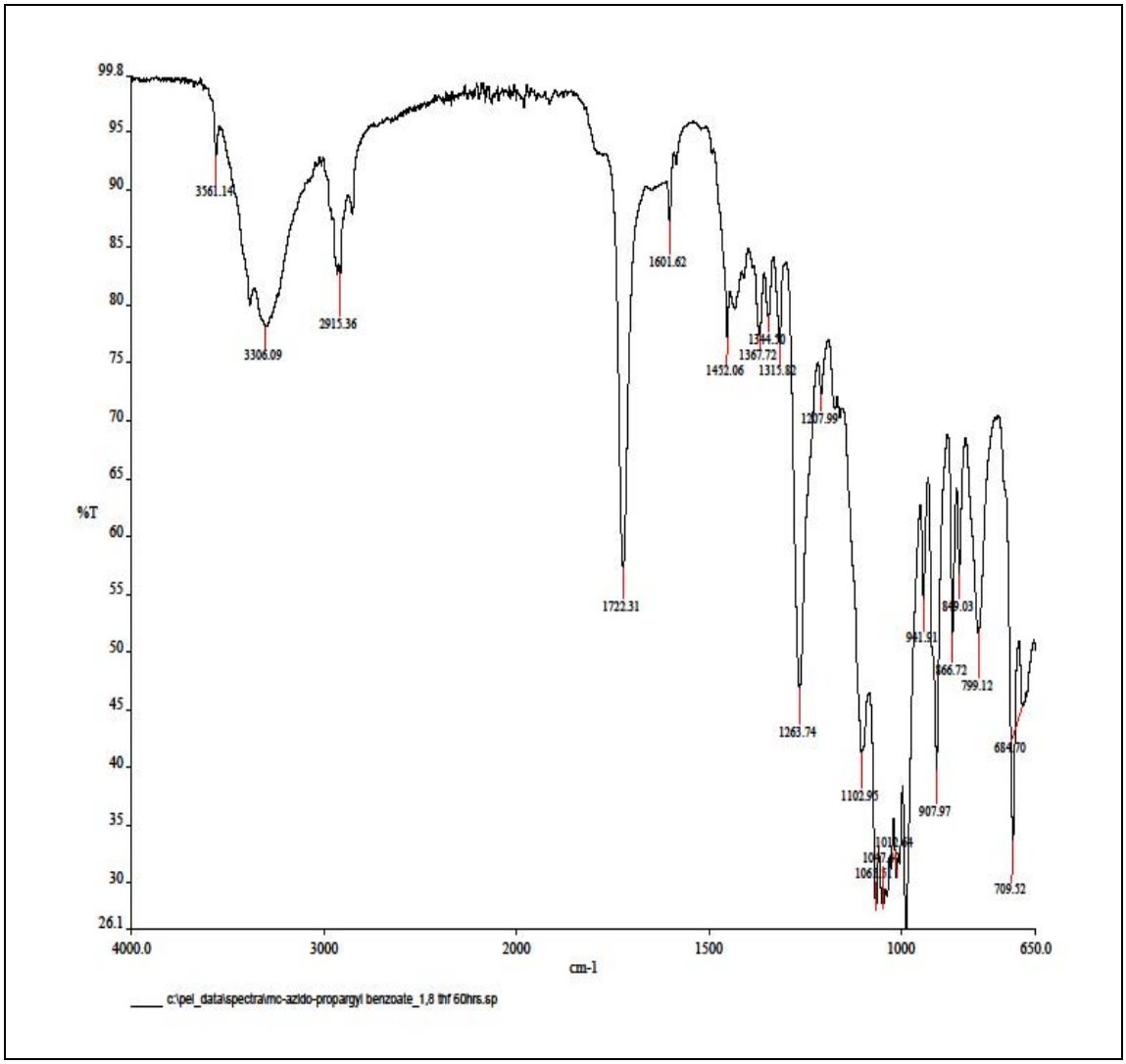


Figure 3.10 FT-IR spectrum of 1,3 triazole click compound 11-azido undecanoic acid and propargyl benzoate (1:8)

In the FT-IR spectrum,(Figure 3.11) azide peak was still observed at 2096 $cm^{-1,}$ which was the characteristic of azides. The experiment (1:2) was run continuously for 60 hrs at

~60 ^{o}C.

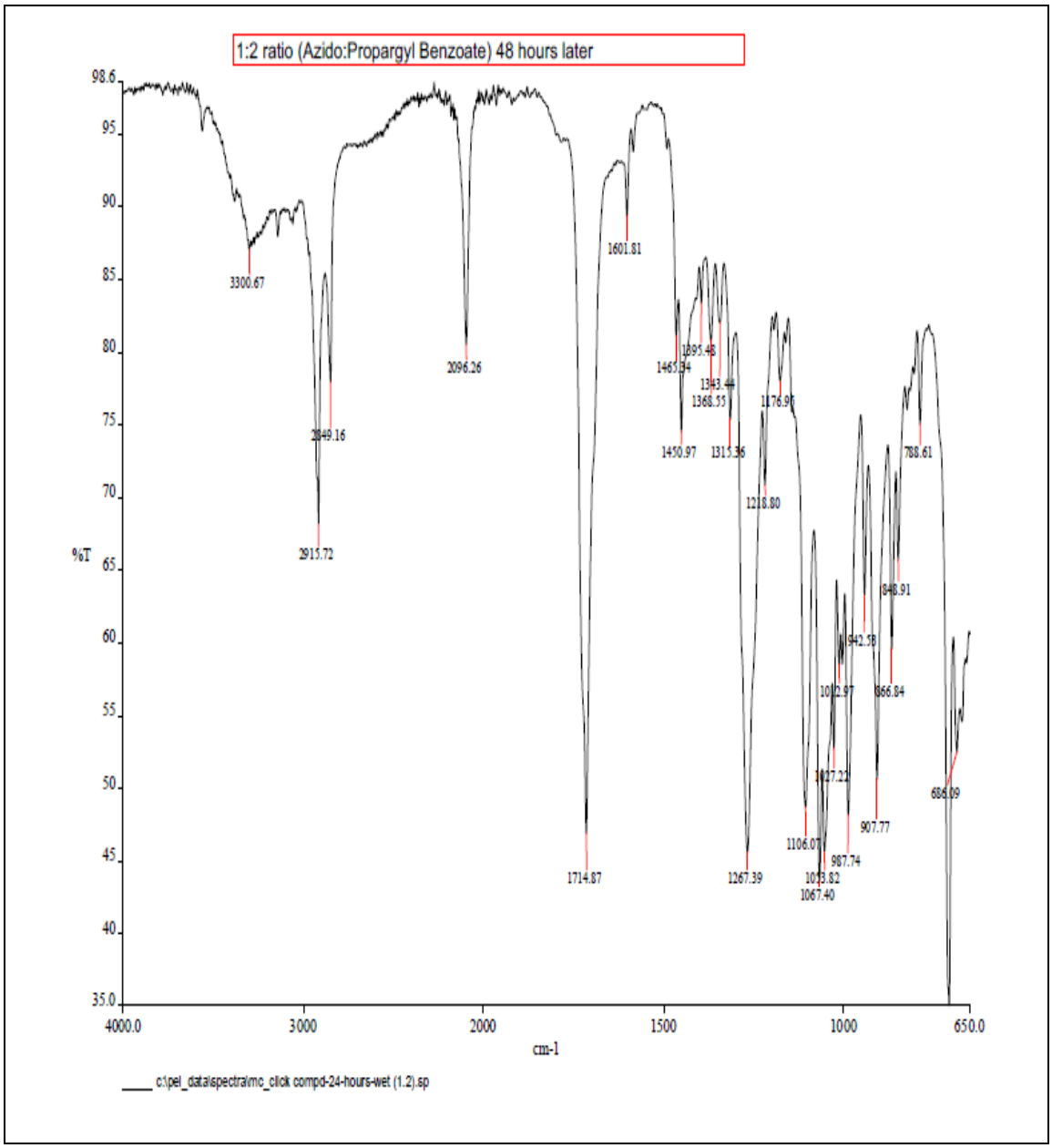


Figure 3.11 FT-IR spectrum of 11-azido undecanoic acid and propargyl benzoate (1:2)

In this FT-IR spectrum (Figure 3.12), azide peak was still observed at 2093 $cm^{-1,}$ which was the characteristic of azides. The experiment (1:4) was run continuously for 60 hrs at ~60 ^{o}C.

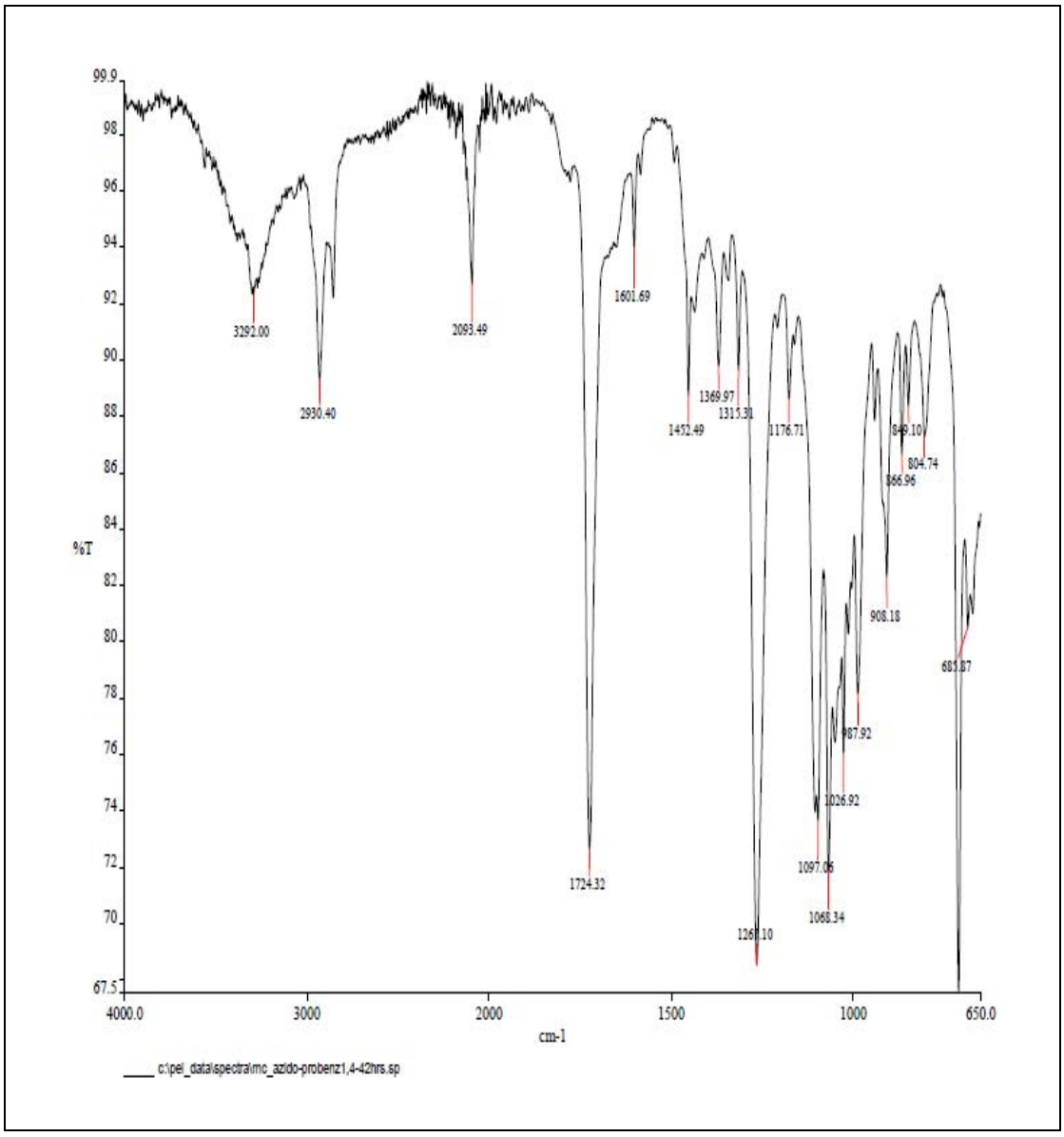


Figure 3.12 FT-IR spectrum of 11-azido undecanoic acid and propargyl benzoate (1:4)

In the following FT-IR spectrum (Figure 3.13), neither the azide peak nor the carbonyl peak of ester bond of propargyl acetate was observed. This experiment had 11-azido undecanoic acid and propargyl acetate in 1:8 ratio and the experiment was run for 60 hrs at ~60 ^{o}C.

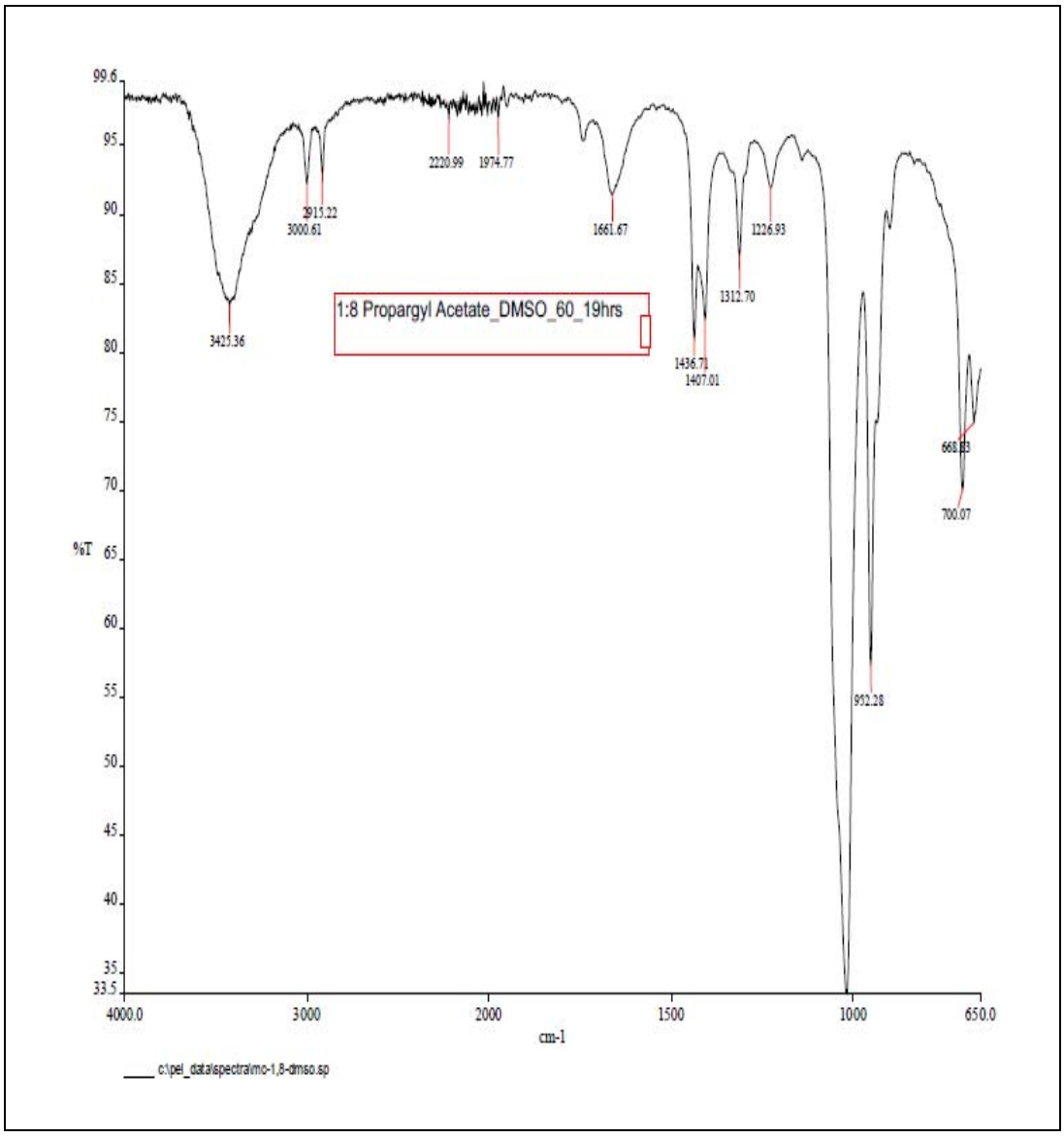


Figure 3.13 FT-IR spectrum of 11-azido undecanoic acid and propargyl acetate (1:8) in DMSO

3.4.2.b ^{1}H NMR spectrum characterization of Azido 1,3 triazole click compound

The ^{1}H NMR spectrum of 1,3-triazole click compound (Figure 3.14) is included below. The experiments were conducted in $CDCl_3$ and chemical shifts were assigned using TMS as internal reference. This spectrum is not integrated. Spectrum of 11-azidoundecanoic acid (Figure 3.7) and spectrum of 1,3 trizole click compound (Figure 3.14) are comparable. In both the spectra protons at 2.3 ppm (h,2H $C\underline{H_2}$-COOH), at 1.61(g 2H $C\underline{H_2}$-CH_2-N_3), at 1.91 ppm and $C\underline{H_2}$-CH_2COOH), and 1.26 (c,12H 6*$C\underline{H_2}$) ppm. The characteristic peaks of –$C\underline{H_2}$-N_3 at 3.26ppm disappears and new peaks of triazole was observed at (e,7.7ppm), with the phenyl –CH- peaks at ~ 8 ppm (a,c,b), -$C\underline{H_2}$-N_3 triazole ring (f, 4.34ppm), -CH_2 of the propargyl benzoate (d, -$C\underline{H_2}$-O-CO-, 5.34). At 1.26 ppm 12 protons of -11azido undecanoic acid were observed and in this triazole spectrum the same protons are present at 1.29 ppm.

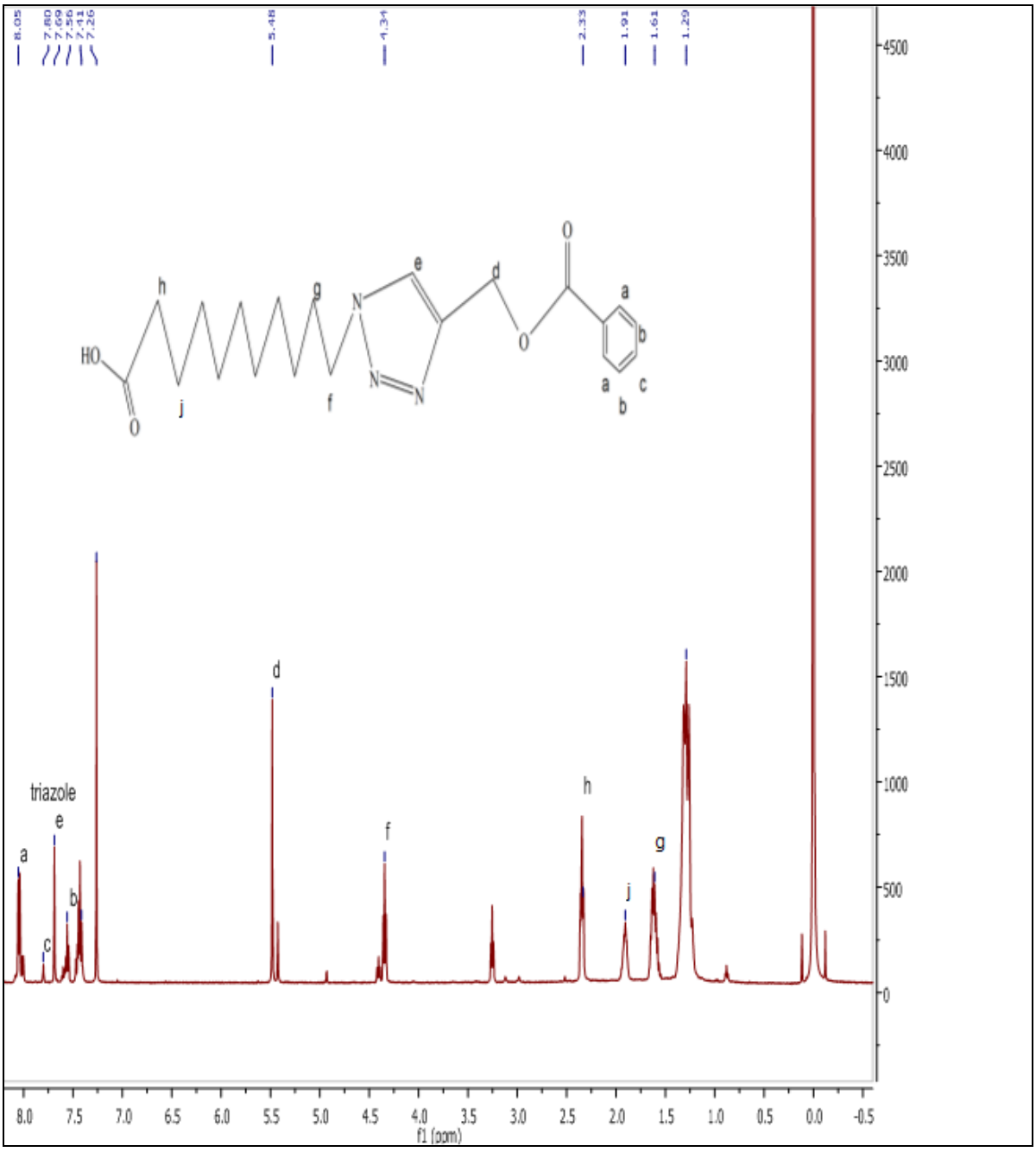


Figure 3.14 ^{1}H NMR of Azide 1,3 triazole click compound

3.5 Conversion of PHOBr into PHOAzide and Characterization

The Huisgen cycloaddition is the reaction of a dipolarophile with a 1,3-dipolar compound that leads to 5-membered heterocycles. Examples of dipolarophiles are alkenes and alkynes and molecules that possess related heteroatom functional groups (such as carbonyls and nitriles). 1,3-Dipolar compounds contain one or more heteroatoms and can be described as having at least one mesomeric structure that represents a charged dipole. (Lundberg, Hawker, Sharpless and coworkers).

After the successful synthesis of 1,3 trizole click compound from the model molecule, 11-bromoundecanoic acid, the same steps were followed for the polymer - analogous conversion of the terminal bromine group of the polymer PHOBr into an azide and then to click it with an alkyne. Figure 3.16 shows the conversion of PHOBr into an azide-polymer in presence of NaN_3.

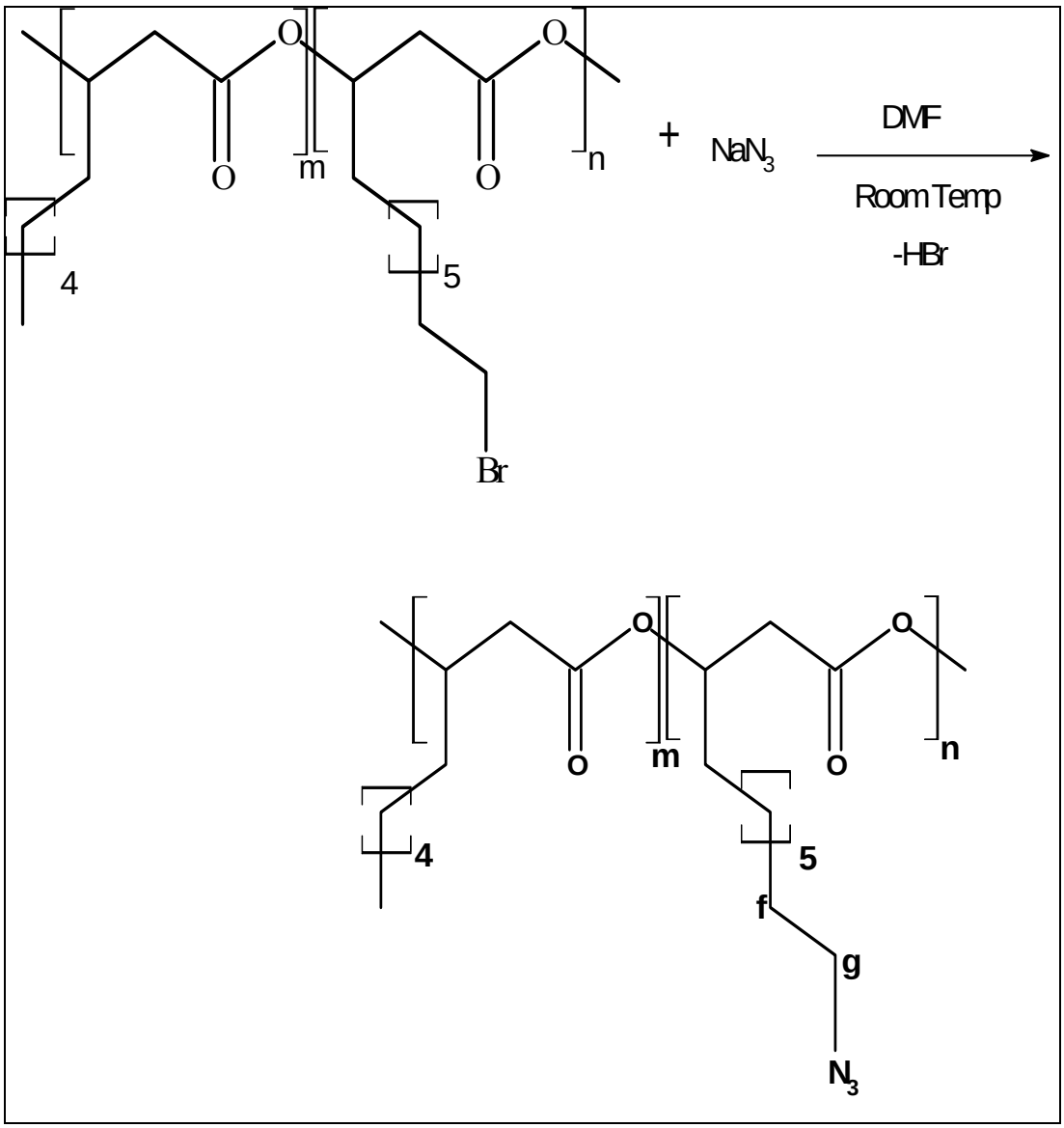


Figure 3.15 Chemical reaction between PHOBr and sodium azide and formation of azido-polymer

3.5.a FT-IR Characterization of PHO-Azide

In the following FT-IR spectrum of the azido-PHO, azide peak was observed at 2095 $cm^{-1,}$ which is the characteristic of azides. The ester bond of the polymer is also visible. ^{1}H NMR spectrum of this azido-polymer was not performed as the yield was very low ~10% and the sample were used for the click reaction.

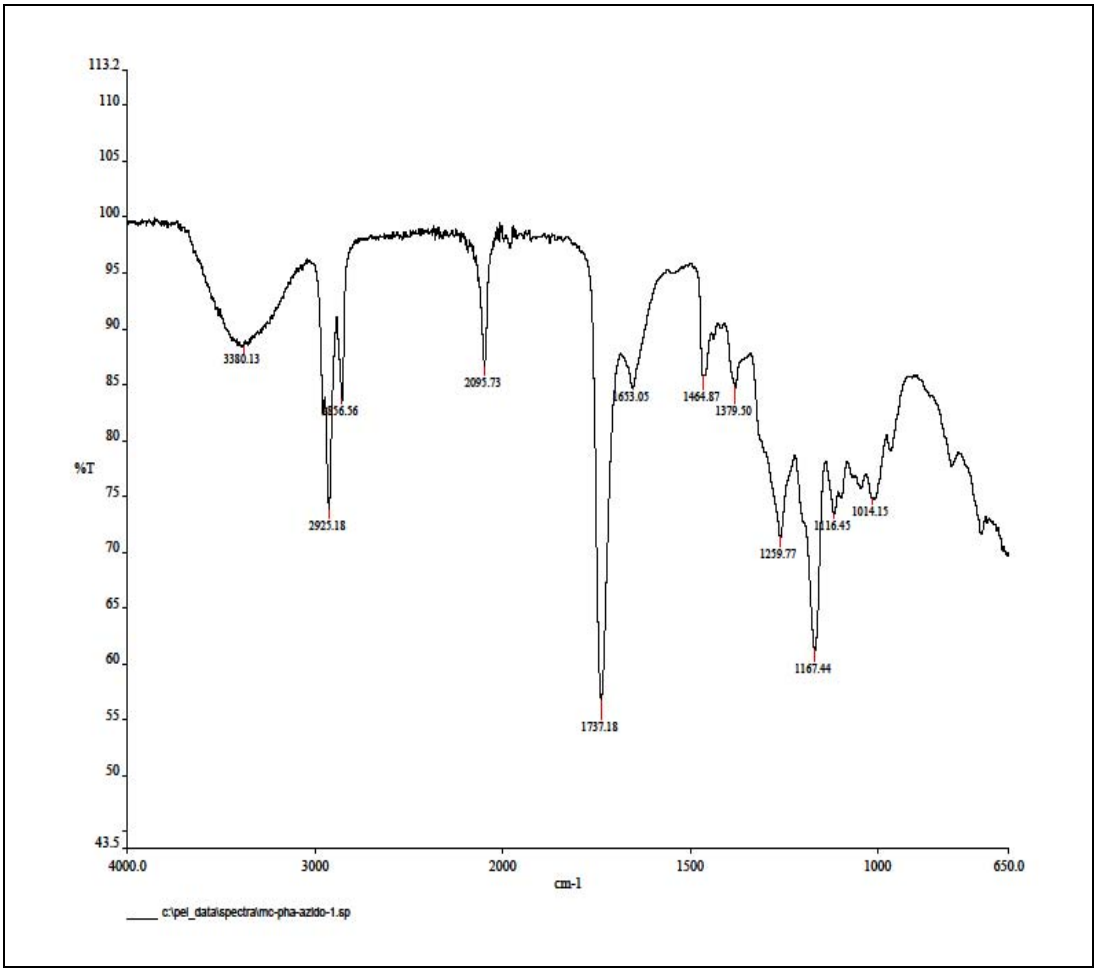


Figure 3.16 FT-IR spectrum of Azido-PHA

3.6 Conversion of Azido PHO into 1,3 triazole click PHA and Characterization

The amount of PHO-Azide recovered was of very low yield (15%). Approximately 10 mg of azido polymer was recovered, to this an 8-fold of propargyl benzoate was added in presence of $CuSO_4.5H_2O$ and Na ascorbate (Figure 3.17). Na ascorbate used was store bought. The experiment was run for 60 h and the disappearance of azide peak was monitored by running the FT-IR periodically. Crude sample (1,3 trizole PHA) was analyzed by ^1H NMR spectra to confirm the formation of the click compound.

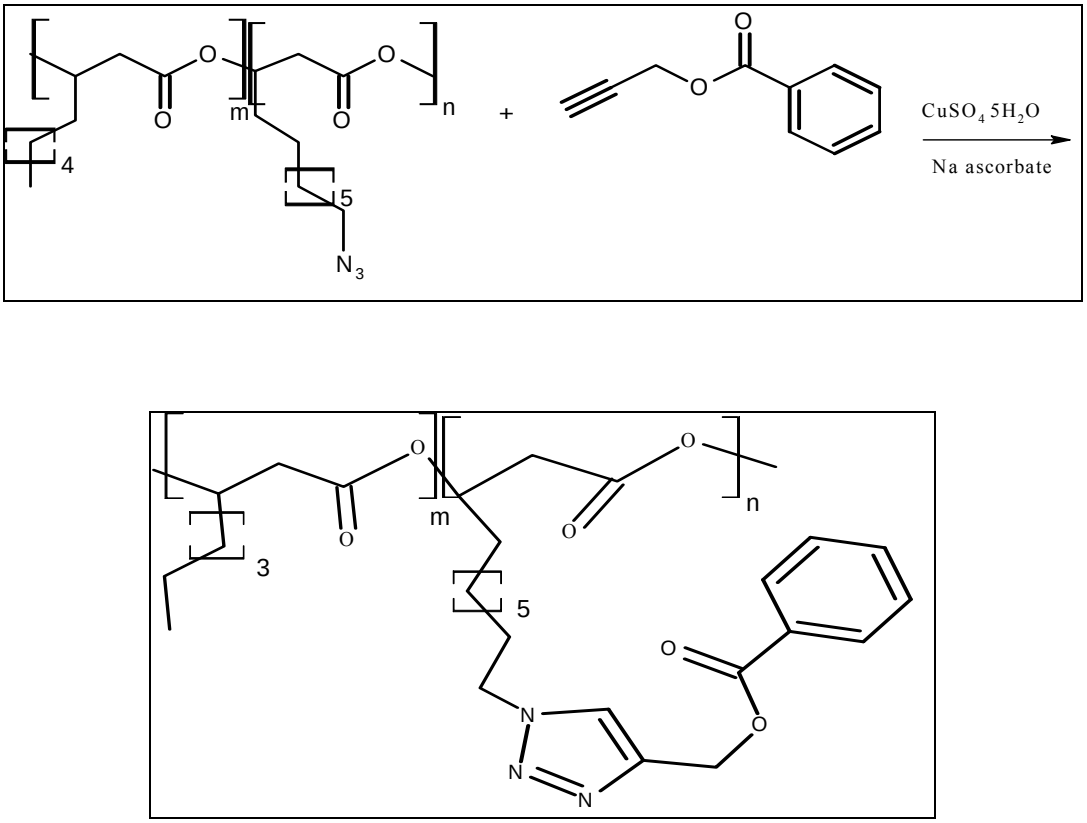


Figure 3.17 Chemical reaction between Azido-polymer and propargyl benzoate.

3.6.a FT-IR Characterization of PHO 1,3 triazole click compound

After running the experiment for two days, click reaction was successful as confirmed by the disappearance of the azide peak. The spectrum had the ester peak at 1723 cm^{-1} and CH_2, CH_3 peaks at 2915 cm^{-1} which are the characteristic peaks of the polymer. Triazole-benzoate peaks were located at ~1000 cm^{-1}.Triazole peaks as such are not specified in the FT-IR spectrum.

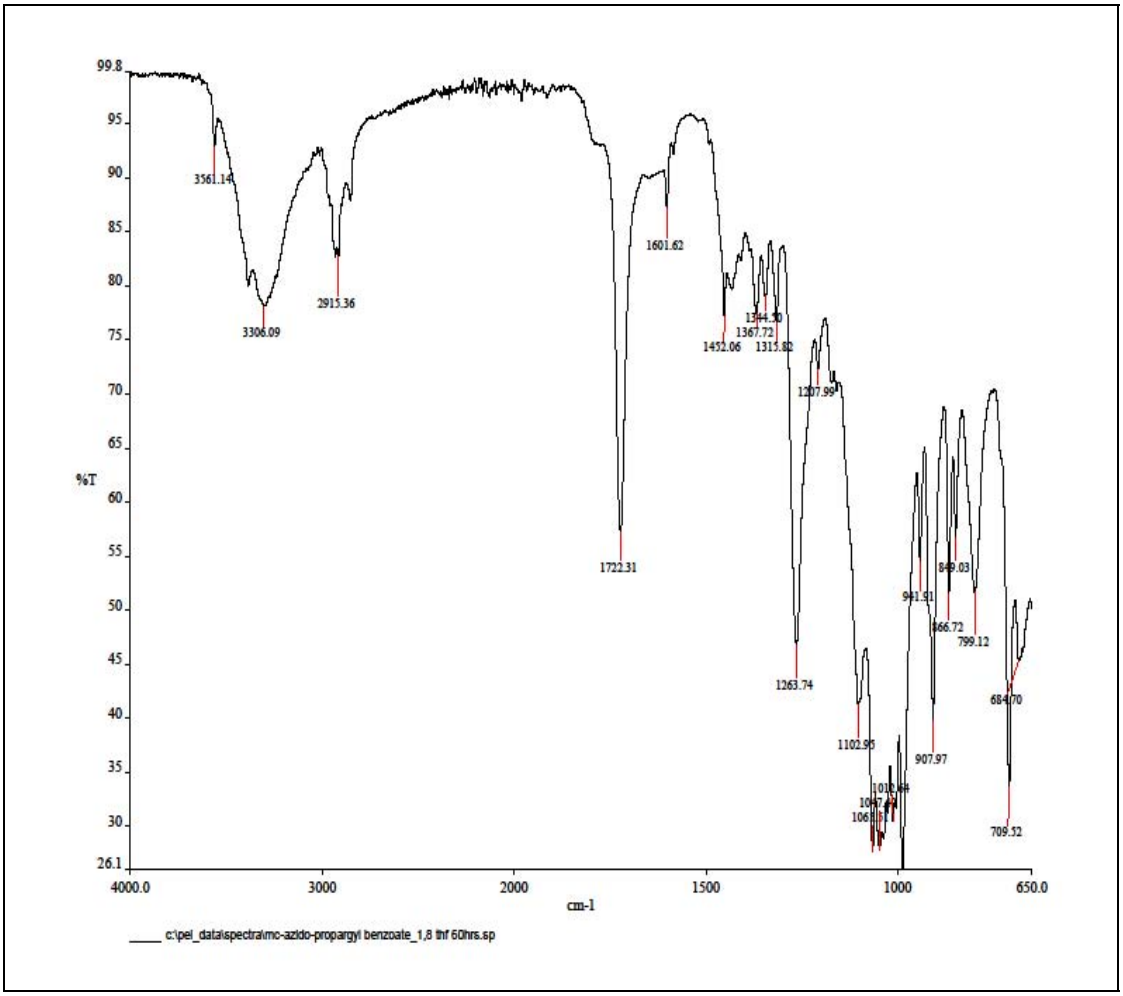
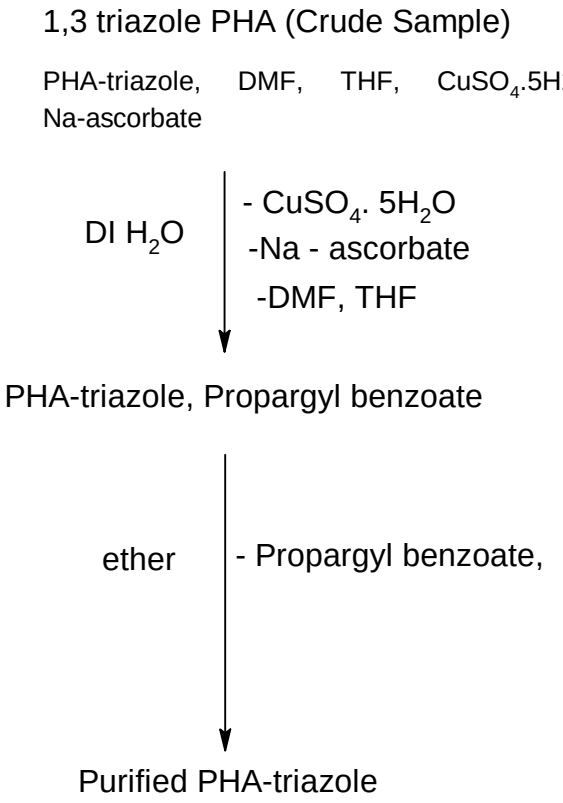


Figure 3.18 FT-IR spectrum of PHO - 1,3 triazole click compound

3.7 Purification of 1,3 triazole PHA click compound

Purification of the click compound was required as it had excess of Propargyl benzoate, solvents DMF and THF, and catalyst $CuSO_4.5H_2O$ and Na-ascorbate. The following purification scheme was followed



The crude sample was dissolved in DI water as $CuSO_4.5H_2O$ and Na - ascorbate were soluble in water. Na- ascorbate used in this experiment was store bought and did not completely dissolve in water. Polymer and propargyl benzoate are not water soluble. The sample was then centrifuged several times. Polymer, propargyl benzoate and part of Na-ascorbate and some left over solvents were in the solid phase which was collected

together. The liquid phase had $CuSO_4.5H_2O$ and Na - ascorbate . To this solid phase, ether was added as Propargyl benzoate, DMF and THF were soluble in ether, while the polymer was insoluble. The top layer which consisted of ether, propargyl benzoate, and the solvents were drawn out with the help of pipette and click compound was left in the hood to dry overnight. Yield ~10%. ^1H NMR spectra (Figure 3.19) was performed in deuterated chloroform to determine the formation of triazole compound.

3.7.a ^{1}H NMR spectrum characterization of 1,3 triazole PHA click compound

^{1}H NMR spectra was performed of the click compound to confirm the synthesis of the 1,3-triazole polymer. The sample (5mg) was dissolved in 1 mL of deuterated chloroform. Integration of this spectrum (Figure 3.19) was not done. The characteristic peaks of the triazole was observed at (h, –N–C<u>H</u>, 7.69 ppm), benzene ring (propargyl benzoate) peaks were found at approximately 8 ppm (a,c,b), –C<u>H$_2$</u>–COOH (a, 2.28 ppm), –CH$_2$-C<u>H$_2$</u>–N$_3$ of the trizole ring (g, 4.34 ppm), -C<u>H$_2$</u>-CH$_2$-N$_3$ (f, 1.68 ppm), –C<u>H$_2$</u>–CH$_2$–COOH (c, 1.96 ppm), –C<u>H$_2$</u>OCO of the propargyl benzoate (i, 5.40 ppm). Chiral proton 'b'of the polymer can also be seen at 5.38ppm, which is overlaid by C<u>H$_2$</u> proton 'i'. Consistency in the relevant peaks of the spectrum was observed for the small molecule spectrum (Figure 3.14), which was synthesized earlier as the model experiment and also correlated to the spectrum of 11-azidoundecanoic acid spectrum, Figure 3.7.

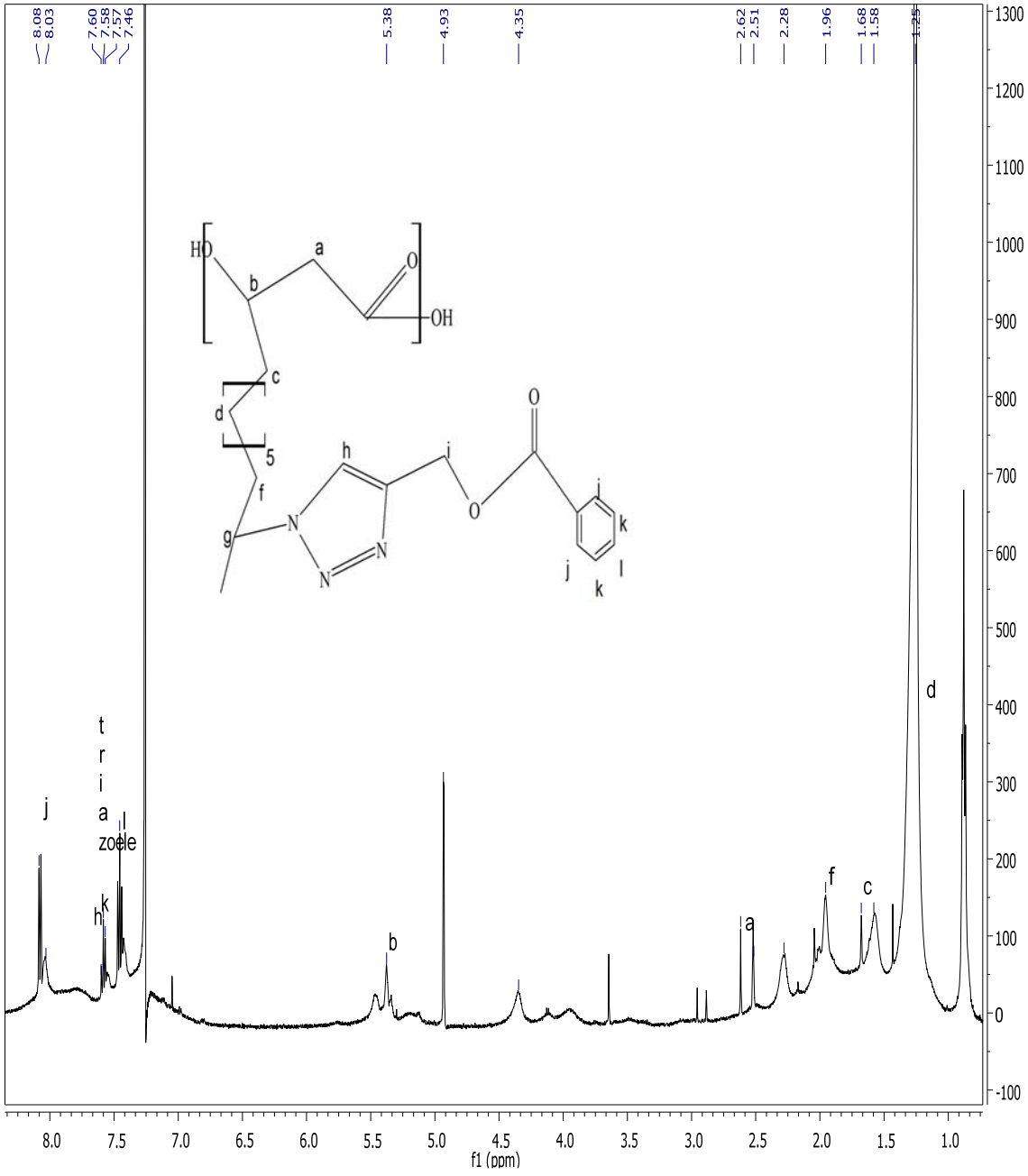


Figure 3.19 ^{1}H NMR spectra of 1,3 triazole PHA click compound

Literature on the type of PHA-triazole which was synthesized in this current project is not available. It is our understanding that this is the first time this PHA-triazole has been synthesized in any lab. Although in some references, triazoles and their ^{1}H NMR spectra are referenced (Chen-Chang Lee, GiovannaGrandinetti, Reineke, Lee, Yi-Teng Huang, Lee and Ke-PU Wu, 2012) which correlate with data that were collected by in this thesis. The synthetic work carried out in this thesis project and conversion of the PHOBr copolymer, into an triazole is the first of its kind.

CHAPTER IV

Future Work

The synthesized PHOBr was relatively a pure material, which was evident from the spectral data acquired from the NMR and FTIR; however the amount of polymer synthesized was very low. In future, the synthesis could be optimized and its application can be researched further. Due to the low yield (25%) of the PHOBr, the yield (10%) of 1,3 triazole click PHA synthesized with propargyl benzoate was of significantly low amount too! The purification of the triazole (crude sample) was performed and the outcome of the spectral data values was satisfactory and comparable to the spectra of published journals.

Due to the low yield of the synthesized click compound, its properties were not investigated. If PHOBr is synthesized in ample amount then the click compound can be synthesized in large amount, which will give opportunity for further investigation of its properties in the field of pharmaceutical , biomedical, biochemical applications.

As per our hypothesis propargyl acetate should have clicked to the azide just like propargyl benzoate, and should have retained its ester bonds. The reaction was performed using the small molecule model (11-azidoundecanoic acid). The reaction was not successful as the ester bond broke during the synthesis as the carbonyl peak was not visible in the spectral analysis (Figure 3.13).

With the tremendous growth and demand in the field of click chemistry this current research can be easily extended further by clicking several different alkynes to the

synthesized azidoPHA. A library of PHOBr-click compound can be created and new applications can be envisioned which can be very useful in the pharmaceutical, biomedical, biochemical and drug delivery industries. In short, as a result of this study, a new class of natural click compound polymer was created which has the potential to achieve a wide range of applications.

CHAPTER V

Conclusion

Biomaterials that are biocompatible and biodegradable are useful in a variety of applications. PHAs, which are produced as a natural response from bacteria is an example of biodegradable biomaterial. PHAs with chemically reactive functional groups located at the termini of its side chains have been synthesized before in this lab. As a logical extension of this idea, the present study sought to synthesize PHOBr with functional side chains of PHAs. In order to proceed with the chemical modification, the growth of *P. oleovorans* was examined on carbon source under a range of culture conditions.

Once the PHOBr was synthesized, its modification was evaluated using ^{1}H NMR spectra. The variance of PHBr content in the copolymer from one batch to another could not be concluded. The terminal alkyl group (Br) was efficiently substituted with an azide followed by Click reaction chemistry with an alkyne. This new molecule, 1,3-triazole PHA, represents the first example of azide-terminated PHA, as well as the first triazole PHA to be produced to our knowledge. Further study on the new product was not done because of the very low yield.

The current technology of bacterial polyesters extends only to those applications in which the biocompatible properties of the hydrophobic material may be utilized. With the successful synthesis of click compound with the PHOBr, it opens new horizon for the synthesis of different click PHA compounds.

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