UNIVERSITY OF CALIFORNIA, SAN DIEGO

Development of a Polylactic Acid (PLA) Polymer with an Acid-Sensitive N-ethoxybenzylimidazole (NEBI) Crosslinker as a Drug Delivery System

A thesis submitted in partial satisfaction of the requirements for the degree of

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in

Chemistry

by

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Professor Emmanuel Theodorakis
Professor James Whitesell

2012

This thesis of Leibniz Fangtinq Hang is approved, and is acceptable in quality and form for publication on microfilm and electronically:

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Chair

University of California, San Diego

2012

DEDICATION

This work and thesis is dedicated to my late grandparents: Truong Quang Hang and Phuong Hang. They placed great emphasis and value on hard work and education. They always encouraged me to study hard and to reach for my goals. Together, they also taught me the true meaning of perseverance with their prolonged and valiant fight against heart and pulmonary diseases, respectively. It was a privileged to be raised by such great people.

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LIST OF ABBREVIATIONS

2M6NBA	2-methyl-6-nitrobenzoic anhydride
ACN	acetonitrile
cat.	catalytic
CuAAC	copper-catalyzed azide–alkyne cycloaddition
DDS	drug delivery system
DCM	dichloromethane
DMAP	4-dimethylaminopyridine
DMF	*N,N*-dimethylformamide
DMSO	dimethyl sulfoxide
Et_2O	diethyl ether
Et_3N	triethylamine
EtOAc	ethyl acetate
HCl	hydrochloric acid
Hrs	hours
HEPES	2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid
HPLC	high performance liquid chromatography
IC_{50}	half-maximal inhibitory concentration
kDa	kilodaltons
MeOH	methanol
MES	2-(*N*-morpholino)ethanesulfonic acid
MS	mass spectrometry
NBS	*N*-bromosuccinimide

NEBI	*N*-ethyoxybenzylimidazole
NMR	nuclear magnetic resonance
PDI	polydispersity index
PLA	poly-lactic acid
PTSA	*p*-toluenesulfonic acid
SRB	sulforhodamine B
ROP	ring opening polymerization
RT	room temperature
THF	tetrahydrofuran
TsOH	tosylic acid

ACKNOWLEDGMENTS

I would like to acknowledge Professor Jerry Yang for all of his support and patience during my graduate studies. Despite my lack of experience, he offered me the opportunity to join his laboratory and introduced me to the world of organic chemistry. While navigating through these new frontiers, he steered me along my path in science and research.

I would also like to thank my family: Dad, Mom, Newton and Erin and my girlfriend, Tracy, for their continuous mental [and financial] support throughout my academic career. Over the last few years, they encouraged me to pursue higher levels of education and to strive for excellence. They also showed great patience and encouragement whenever I faced the struggles of graduate school. I owe much of success to them, and I am forever thankful.

Furthermore, many of the more experienced members helped me through my growing pains when I first joined the Yang Lab. Their leadership and guidance has not been forgotten. I would like to thank Dr. Mark Rubinshtein, Dr. Alice Luong, Dr. Christina Capules, Dr. Lila Habib, Dr. Xiaobei Zhao and Yuchen Cao for their insight and feedback. They helped ease the transition into graduate research by being great examples of researchers, colleagues and friends. I would like to especially thank Alice for her demonstrative encouragement and research advice and to Mark for his early mentorship in chemistry.

Among the later members of the Yang Lab, Kevin Cao and Tim Chung always were available for tennis to alleviate some stress. These tennis outings would become a staple in our group, and they were some of my favorite memories of the Yang Lab. Kevin Sibucao always had interesting perspectives and was a great source for conversation. Kate's passion for chemistry was always exhibited and on display in her stories about norbornene derivatives (both exo and endo). Dan and Jessica always provided amusement and fun. Young Hun's cheerful personality and friendliness were great and welcomed additions to our group.

Dr. Mark Rubinshtein and Dr. Alice Luong provided the preliminary work in chapters 2 and 3 in the development of functionalized PLA and NEBI linker, respectively. Using their work as a starting point, I was able to combine and merge their projects to produce a new drug delivery system. Lastly, I would like to thank Kevin Cao for teaching me how to use the HPLC for my analytical studies. Their contributions to my research were greatly appreciated and will be reflected in any subsequent publications for my drug delivery system.

ABSTRACT OF THE THESIS

Development of a Polylactic Acid (PLA) Polymer with an Acid-Sensitive N-ethoxybenzylimidazole (NEBI) Crosslinker as a Drug Delivery System

by

Leibniz Fangtinq Hang

Master of Science in Chemistry

University of California, San Diego, 2012

Professor Jerry Yang, Chair

This thesis describes the development of an alkyne-functionalized poly-lactic acid (PLA) polymer with an acid-sensitive *N*-ethoxybenzylimidazole (NEBI) linker conjugated to doxorubicin as a drug delivery system (DDS). It also entails the specific qualities of the drug delivery system as a chemotherapeutic regiment.

Chapter 1 introduces why drug delivery systems are needed and their desirable properties. In particularly, chapter 1 discusses the roles of [polymeric] carriers and linkers in drug delivery. It also covers some of the drug-delivery strategies specific to chemotherapy. Chapter 2 presents the synthetic route and methodology to produce functionalized PLA, specifically with terminal alkyne moieties. It also covers different strategies to synthesize lactide derivatives with

different functional groups. Chapter 3 introduces the synthetic scheme to producing bi-functional *N*-ethoxybenzylimidazole (NEBI) derivatives. These novel linkers previously displayed accelerated rates of hydrolysis at lower pH. NEBI derivatives also serve as acid-sensitive linkers in the synthesis of my drug delivery system. Chapter 4 discusses the characteristics of my drug delivery system including incorporation percentages, payload, hydrolysis rate and cytotoxicity. The future directions of this project are also elaborated on in that chapter.

Chapter 1

Introduction to Common Desirable Characteristics of Drug Delivery Systems

Section 1.1 The Need For Drug Delivery Systems

In recent years, extensive research has gone towards the enhancement of conventional or "free" drugs by employing various types of drug delivery systems (DDS).[1-4] The basis for the use of drug-delivery systems is the possibility for improved pharmacokinetics. Pharmacokinetics describes metabolite kinetics within the body and often are analyzed in the following aspects: liberation, absorption, distribution, metabolism, excretion and toxicity.[5] Plasma levels of drugs are typically characterized by initial rising concentrations of the active drug, followed by decreasing concentrations by various mechanisms including metabolism, degradation and transport.[6]

Significant problems with drug regiments [along with wasted materials] typically arise outside the ranges of the minimally effective and the maximally safe regions.[6] Poor pharmacokinetics and other factors limit the efficacy or safety of "free" drugs such as solubility, tissue damage on extravasation, rapid degradation, and lack of selectivity.[1] For example, paclitaxel exhibits poor water solubility and requires the solubilizing agent and possible allergen, Cremophore EL, to be co-administered, intravenously.[7] Accidental extravasation and tissue necrosis may also occur from prolonged infusions of cytotoxic, topoisomerase II inhibitors such as "free" doxorubicin.[8] Furthermore, poor selectivity of drugs such as doxorubicin leads to a myriad of problems including dose-limiting, side effects and suboptimal, therapeutic effects.[1] The employment of drug delivery systems aims to address many of these complications.

Section 1.2 The Role of Carriers in Drug Delivery

One of the principal ways of incorporating drugs into drug delivery systems is through the use of a carrier. Carrier-based drug delivery offers protective encapsulations of drugs, improved bioavailability, and possibly tailored release.[9] Two of the predominant types of small-particle carriers include lipid-based and polymer-based complexes for drug delivery.[1] Among the particulate-based carriers, drug-delivery systems composed of lipids and polymers often display desirable pharmacokinetics.[1,9]

Microparticles and nanoparticles exhibit a variety of desirable characteristics for a carrier. They are capable of encapsulating thousands of drug molecules for increased payload.[1,10] In addition to the increasing delivery concentrations, small particles display significantly more robust activity against plasma clearance.[10] Some mechanisms proposed for the improved resiliency involve the protection offered by the carrier against acidity, heat, enzymatic degradation, and immune response.[9,11] Additional, different carriers used in drug delivery may also significantly augment the solubility of lipophilic drugs by improving the partition coefficient by several folds.[12,13] The difference in partitioning and solubility may translate to the delivery of higher concentrations of drugs to their target. In combination, these attractive properties significantly increase the bioavailability of the drug.

The application of small particles and conjugated carriers to encapsulate "free" drugs can also increase their biocompatibility. For example, sequestering

"free" doxorubicin inside suitable lipid micelles and liposomes helps resolve some of solubility concerns and increases retention.[14] Furthermore, in polymer-drug conjugates and drug-entrapped liposomal systems, the drugs are often rendered inactive and unavailable in their pro-drug form.[15] In the case of doxorubicin, encapsulation minimizes many of its adverse side-effects including inadvertent extravasation.[1]

It is important to consider that failure to release the drug in a timely fashion may reduce efficacy, while overly rapid release of the drug simply results in therapeutic activity similar to the "free drug".[15,16] However, properly adjusted rates of release of encapsulated and conjugated drugs can result in improved therapeutic characteristics over the "free" drug.[17] In one instance, toxicity of native amphotericin B prohibits the application of elevated levels of drug concentration. However, once sequestered within a liposome, the delivery system suppresses the overactive cytotoxic activity, and the controlled, sustained release results in an increase of the maximum daily dose and cumulative dose by over 5 folds.[17]

With respect to chemotherapeutics, drug delivery systems and particle-based carriers offer an additional advantage over "free" drugs—the EPR effect.[18] The enhanced permeability and retention effect (EPR effect) describes the increased vascular permeability factors exhibited by tumor cells.[19] These permeability factors include higher levels bradykinin, nitric oxide, peroxynitrite, prostaglandins, vascular endothelial growth factor (VEGF), proteases (kallikrein) and other cytokines like tumor necrosis factor.[19] These factors contribute to the observed phenotypes that

macromolecules remain at high levels in circulating blood near solid tumors.[19] Increased accumulation and poor clearance of proteins, macromolecules, and lipid particles in the interstitial space of tissue of solid tumors are hallmarks of the EPR effect.[20,21]

One of the proposed mechanisms for the enhanced retention involves a disparity in circulation and diffusion out of the interstitial space between solid tumors and normal cells.[21] Poor, "leaky" vasculatures around tumor cells were observed and provide a physiological explanation for the EPR effect.[3] Inherent genetic alterations and epitopic diversification of cancer cells may also influence the observed phenotypes.[18,22] Many particle-based drug delivery systems aim to employ the EPR effect to increase chemotherapeutic efficacy and specificity.[4,19] Due to increased accumulation of the drug vehicle in the interstitial space, the EPR effects can provide a "passive" targeting that discriminates between solid tumors and normal cells.[1,4,19,23]

Section 1.3 Biocompatible, Polymeric Types of Biomaterial for Drug Delivery

Currently, an assorted variety of drug-encapsulated polymers is commercially available.[6,24] Several types of encapsulated drugs include birth control, narcotic antagonist, diabetes regiments, various steroids, and chemotherapeutics.[6,24] Polymeric carriers offer many advantages general to many types of carriers including continuous maintenance of plasma drug levels, reduction of aforementioned harmful

side effects, protection of degradation and clearance, sustained administration of smaller concentrations, and the possibility of greater overall concentration in payload.[6] Perhaps most importantly, many polymeric carriers are approved by the American Food and Drug Administration for medical use.[6,25]

Polymeric drug delivery systems or "polymer therapeutics" can offer different advantages in comparison to other types of drug delivery systems.[18] Polymer-based micelles/carriers and polymer-drug conjugates can exhibit lesser sensitivity to solubility, electrostatic interactions, and pH gradients when compared with many liposomal counterparts.[26] Furthermore, *in vivo*-stable, amphipathic, liposomal, drug delivery systems typically have difficulty rapidly releasing their encapsulated drug.[9]

Sustained release of drugs can also be achieved by the assistances of polymeric carriers.[6] In these instances, the carriers serve as "drug reservoirs" or "drug matrices," and the drugs are released in a controlled fashion typically by diffusion. Their release rates and partition coefficients can be accurately described mathematically.[6] The extensive research in polymeric carriers resulted in highly controlled and tuned systems capable of variable release. Grafting different moieties to produce different copolymers can also modify and adjust release rates to match the specific drugs.[27,28]

The advent of amphipathic copolymers also allows carriers to display tunable physical properties, water solubility and various critical micelle concentration

(CMC) values.[9,29-31] Common copolymer substituents typically incorporated to improve solubility include PEG (polyethylene glycol) and PEO (polyethylene oxide) groups.[9,27,28,32] The accessible enhancement of polymer-based carriers by modification with the water-solubilizing units also offers an additional advantage—protection.[4] Water-solubility groups can stifle clearance by mononuclear phagocytic systems (MPS) by hindering the contact of macromolecules and nanoparticles with phagocytic cells.[4] These grafted polymeric carriers resolve many of the issues that previously restricted "free" drug efficacy.

In regard to anti-cancer drug regiments, polymer therapeutics often effectively utilized the pathophysiological uniqueness of the EPR effect.[18] The molecular weight and size of nanoparticles can contribute to greater "passive" accumulation in areas of increased vasculatures.[4,32] In conjunction with the resiliency that polymeric carriers display against plasma clearance, long-circulating macromolecules like polymeric nanoparticles effectively accumulate in tumors.[32] Aside from increased "passive" targeting, the discrimination of the EPR effect between tumor cells and normal cells may also minimize severe systemic side effects of the anti-cancer agent.[18] Polymeric carriers' exhibition of biocompatibility and synergy with the EPR effect suggest that they are excellent candidates to deliver chemotherapeutic drugs.[4,18,24,32]

Section 1.4 Logic of Acid-Sensitive Linkers for Triggered Chemotherapeutic Drug Release

Aside from diffusion-based release of drugs by carriers, research has also gone towards activated release by various mechanisms.[33] For controlled, triggered releases, various degradable linkers have been proposed to work in tandem with applicable carriers to form an applicable, triggered drug delivery system.[24] Chemotherapeutics agents [and other drugs] may be covalently attached to different carriers by cleavable linkers such as redox-sensitive linkers, disulfide-linkers, enzymatic-sensitive linkers, ultra-sound linkers, photodynamic linkers, and acid-sensitive linkers.[33-35] The various choices for cleavable linkers offer more tailored, triggered releases for specific targets.

In conjunction with the EPR effect, an acid-sensitive linker acts as a logical choice for the delivery of chemotherapeutic agents because of the pH differences between compartments in endocytosis. Endosomes serves as routes for drug delivery due to their decreased pH after endocytosis.[36] Macromolecules such as ligands, toxins, and pathogens undergo the same generic entry into the cell: concentration at cell surface, exposure to acidic, endosomal environment, and then delivery to lysosomes or escape to the cytoplasm. The differences between physiological pH and endosomal pH (7.4 and 5.0-6.0, respectively) can trigger the cleavage of drug-polymer conjugates with acid-sensitive linkers.[36,37]

Furthermore, at the decreased pH, the content within the endosomes may change conformation to destabilize the endosomal membranes.[36,37] These changes lead to increased permeability of the membrane and escape of the endosomal content.[36,37] The added effect of the membrane destabilization results in the possibility of increased delivery of the contents of the cleaved drug delivery system to the cytoplasm of the cell. A triggered release of drugs [and the activation of the pro-drug] at endosomal-suitable pH would take advantage of the increased permeability of endosomes.

Section 1.5 General Advantages of PLA and *N*-ethoxybenzylimidazoles (NEBI) as Carriers and Linkers, Respectively

This thesis will focus on the development of a chemotherapeutic, drug delivery system that utilizes poly-lactic acid (PLA) as a carrier with an acid-sensitive, *N*-ethoxybenzylimidazole linker. While other polymeric [and liposomal] carriers have also proven useful in drug delivery systems, poly-lactic acid polymers and copolymers exhibit a wide range of desirable properties as a drug delivery carrier.[1,3,4,14,18,25] Poly-lactic acid polymers were one of the originally proposed carriers at the starting point of the development of the polymeric drug delivery systems.[24] Poly-lactic, poly-glycolic acid copolymers (PLGA) are among the most popular biodegradable, polymeric carrier because of their extensive history of clinical experience, favorable degradation characteristics and the possibility of

sustained drug delivery.[25] Furthermore, by controlling different parameters such as molecular weight, ratio of lactic acid and copolymer, PDI value, hydrophobicity, and drug concentration, the drug delivery system and its physical properties can possibly be tuned to specific conditions.[2,38,39]

One of the major advantages of biodegradable polymers is the biocompatibility of the individually degraded monomers.[2,40] Poly-lactic acid polymers degradation, both *in vitro* and *in vivo*, by the normal enzymatic, metabolic pathway of lactic acid, has been well characterized over the past several decades.[41-45] After hydrolysis, the monomeric subunits of lactic acid enter the tricarboxylic acid cycle, where it is metabolized to carbon dioxide and water.[46] Aside from its biocompatibility, poly-lactic acid (PLA) can be efficiently produced from the fermentation of renewable resources such as corn-starch, making it a reasonably cost-efficient choice as a polymeric carrier.[41,42,44] For these reasons, poly-lactic acid (PLA) and its copolymer with glycolic acid (PLGA) received American Food and Drug Administration (FDA) approval to be used as a carrier for drug delivery.[25]

To enhance the effects of the biodegradable PLA carrier, different acid-sensitive linkers were considered for use. An ideal acid-sensitive linker for a chemotherapeutic drug delivery system would demonstrate an increased rate of hydrolysis and release at endosomal pH (5.5), but slower and more moderate hydrolysis at physiological pH (7.4). Considering that both an overly slow and sluggish release typically results in decreased efficacy and that molecules localize in endosomes and lysosomes for only a few hours, an ideal linker should have a tunable

rate of hydrolysis with a half-life of a few hours at endosomal pH.[14,15,47] Although several acid-sensitive linkers have been synthesized, the tunable aspect of the *N*-ethoxybenzylimidazole (NEBI) linker synthesized by Kong *et al* and Luong *et al* were particularly appealing.[48,49]

Briefly, Kong *et al* produced a tunable, acid-sensitive linker that displayed a wide range of half-lives of hydrolysis, ranging from 1 hour to several months at pH 5.5.[48] Just as important, they also showed that linker exhibits sluggish hydrolysis at pH 7.4 with approximately a 10-fold longer half-life on many of the variations of the linker. Moreover, Luong *et al* also demonstrated the possibility of utilizing the linker in conjunction with a targeting carrier to create a complete, chemotherapeutic, drug delivery system.[49] The DDS showed a significant increase in cellular uptake of the chemotherapeutic agent, presumably from a non-receptor mediated pinocytotic mechanism.[49] By also employing the EPR effect, the cellular uptake of the doxorubicin conjugates could possibly be further improved. Although the NEBI linker has yet to be optimized in cellular toxicity studies, the possibilities for adjusted rates of release makes the NEBI linker an excellent candidate for additional investigation with a PLA backbone carrier.

Chapter 2

Poly-lactic Acid (PLA) and Functionalized Derivatives of PLA

Section 2.1 Brief Introduction

Poly-lactic acid (PLA) polymers and copolymers potentially display a wide range of attractive characteristics useful for a drug delivery carrier.[1,3,4,14,18,25,50] Poly-lactic acid polymers were one of the originally proposed carriers at the starting point of the development of the polymeric drug delivery systems.[24] Poly-lactic copolymers (PLGA) are among the most popular biodegradable polymers as a carrier because of their extensive history of clinical experience, favorable degradation characteristics and the possibility of sustained drug delivery.[25] Furthermore, by tuning different variables such as molecular weight, ratio of lactic acid and copolymer, PDI value, hydrophobicity, and drug concentration, the physical properties, drug release, and biodistribution can possibly be adjusted to specific conditions.[2,38,39] Due to its biodegradable and biocompatibility advantages, poly-lactic acid (PLA) and its copolymer with glycolic acid (PLGA) received FDA approval to be used as a carrier for drug delivery.[25]

Section 2.2 Various Previous Syntheses of Functionalized Poly-Lactic Acid

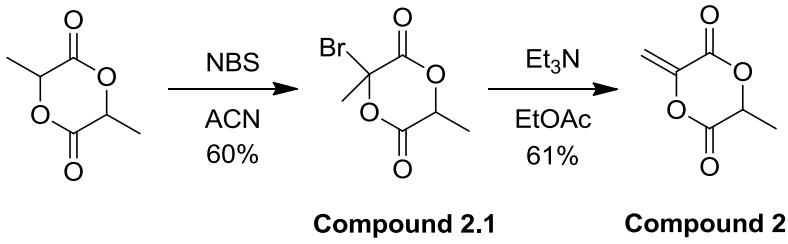
Considering the benefits of using PLA polymers in drug delivery systems [and biodegradable materials], extensive research in creating functionalized lactic acid, lactide (cyclic dimers of lactic acid), and PLA has taken place over the past few decades.[29-31,51-57] Functionalized PLA with molecular handles such as propargyl groups, dienophile acceptors, azido-groups, carboxylic acids, and protected alcohols,

allow for the possibility of drug conjugation. Among the various PLA derivatives with molecular handles, those with propargylic, azido, and dienophile acceptors groups appeared to be particularly attractive candidates for further modification because of the orthogonal nature of their chemistry. Although the functionalized PLA with protected alcohols and carboxylic acids showed large promise and the ability to be further modified, the choice was made to explore the advantages of the chemically orthogonal handles in favor over these polymers.[54-56]

Hillmyer and coworkers synthesized a poly-lactic acid, poly-norbornene copolymer with the intention of providing additional toughening of poly-lactic acid (PLA) by increasing its glass temperature (T_g).[31,58] The norbornene handle proved to be capable of further modification by ring-opening metathesis polymerization (ROMP) and cycloaddition with azide-containing molecules.[29,31] To the best of my knowledge, there has not yet been an example of a drug conjugated to the functionalized polymers proposed by poly-lactic acid, poly-norbornene copolymers proposed by Hillmyer and coworkers. To the best of my knowledge, a drug-delivery system has also not yet been proposed from the norbornene derivative of poly-lactic acid (PLA).

An intermediate of interest in the synthesis of norbornene derivative of lactide is the methylene-lactide molecule (compound 2.2). This methylene-lactide intermediate exhibits the ability to undergo Diels-Alder addition with various dienes.[31,58] Initially, the methylene-lactide derivative appeared to be a promising candidate for Michael-Addition or another type of nucleophilic addition. My efforts

to synthesized methylene-lactide from a modify protocol proposed by Hillmyer and coworkers were successful. With an adjusted protocol, I was able to reproduce the results [and intermediates] reported by Hillmyer and coworkers in comparable yields. The synthesis involves a radical bromination of commercially-available lactide by *N*-bromosuccinimide (NBS) to produce a brominated lactide (compound 2.1), followed by elimination of the halogen by triethylamine to produce the methylene lactide (compound 2.2).



Synthetic Scheme 2.1: *The synthetic schematic for compound 2.1 and compound 2.2 (methylene lactide)*.

Unfortunately, investigation by our research group into its reactivity with various nucleophiles resulted in undesirable ring-opening degradation of the molecule. In the investigation, methylene lactide was treated with alkyl and aryl nucleophiles (thiols, alcohols and amines). It showed sensitivity to nucleophilic attack to carbonyls, but no physical evidence of Michael-Addition or a useful form of nucleophilic addition. Various conditions were tried including different solvents [and the lack of solvent] and at different temperatures, but all attempts concluded with the ring-opening of the lactide. The results of the ring-opening addition by

nucleophiles were dimers of pyruvate and thioesters, esters and amide derivatives of lactate, respectively. For those reasons, further exploration in the nucleophilic addition to methylene lactide was abandoned in favor of the chemically orthogonal nature of other molecular handles.

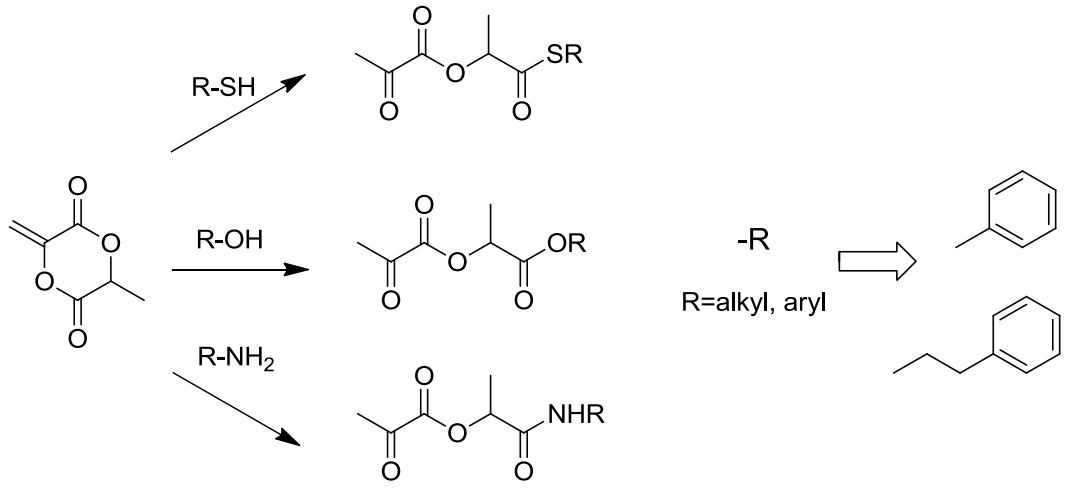
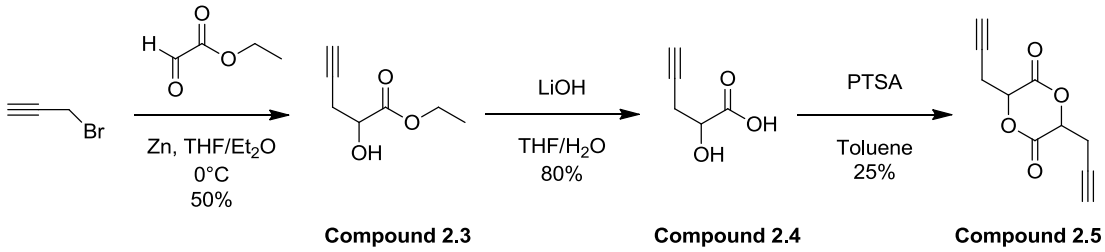


Figure 2.1: *The ring-opening degradation of methylene lactide (compound 2.2).*

Baker and coworkers also synthesized a "clickable" poly-glycolide homopolymer and a block copolymer with poly-lactic acid from synthesized lactic acid with a propargylic moiety.[30] In their efforts to create a thermo-responsive polymer, they illustrated that their functionalized polymers exhibited the ability to undergo copper-catalyzed azide-alkyne cycloaddition (CuAAC).[30] Furthermore, the acetylene-functionalized poly-lactide block copolymer also illustrated the ability to conjugate and graft azido-paclitaxel derivatives via CuAAC.[59] To the best of my knowledge, the acetylene-functionalized poly-lactic acid polymers conjugated to cytotoxic drugs have not yet been characterized by any biological assays.

My efforts to reproduce the synthesis of functionalized propargylic lactic acid [and lactide] were successful. Using a modified protocol of the synthesis proposed by Baker and coworkers, I was able to synthesize the desired monomers and dimers in comparable yields. Propargyl bromide was reacted with ethyl glyoxylate in a Reformatsky-like reaction to create ethyl 2-hydroxypent-4-ynoate (compound 2.3). Hydrolysis of the ethyl ester of compound 2.3 resulted in the α-hydroxy, carboxylic acid of compound 2.4. Dimerization of compound 2.4 was achieved by azeotropic removal of water under the presence of acid, which resulted in the synthesis of the propargylic homodimer (compound 2.5).



Synthetic Scheme 2.2 *The synthetic schematic for compound 2.3, compound 2.4 (propargylic-functionalized lactic acid derivative) and compound 2.5 (propargylic-functionalized homolactide).*

Section 2.3 Passerini-type Condensation for Generating Functionalized Sidechains of Lactic Acid and Glycolic Acid (α-Hydroxy Acid)

Yang and coworkers also reported synthesizing "clickable" copolymers with propargylic-functionalized poly-lactic acid and poly-lactic acid.[52] Unlike the two previous syntheses where the goals were to develop specific polymers [with unique physical properties] with the capability of being further functionalized, the intent of the reported synthesis by Yang and coworkers was to develop a facile method and procedure to develop generically functionalized poly-lactic acid and poly-glycolic acid from α-hydroxy, acyl-indole intermediates.[52] These α-hydroxy, acyl-indole intermediates were synthesized by reacting 1-(2,2-dimethoxyethyl)-2-isocyanobenzene (compound 2.6) with different aldehydes in an Passerini-like condensation.[60]

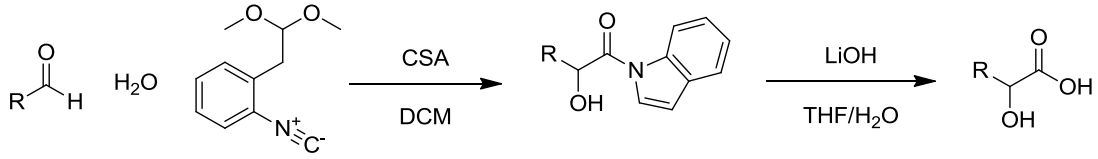
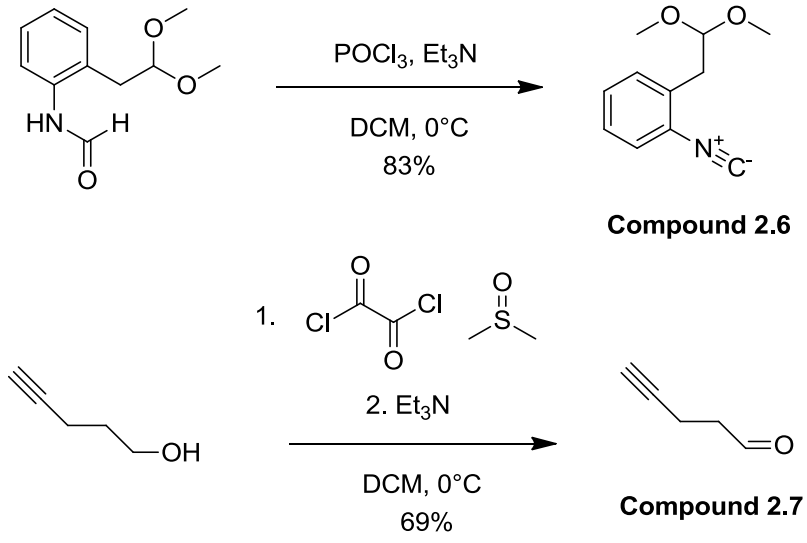


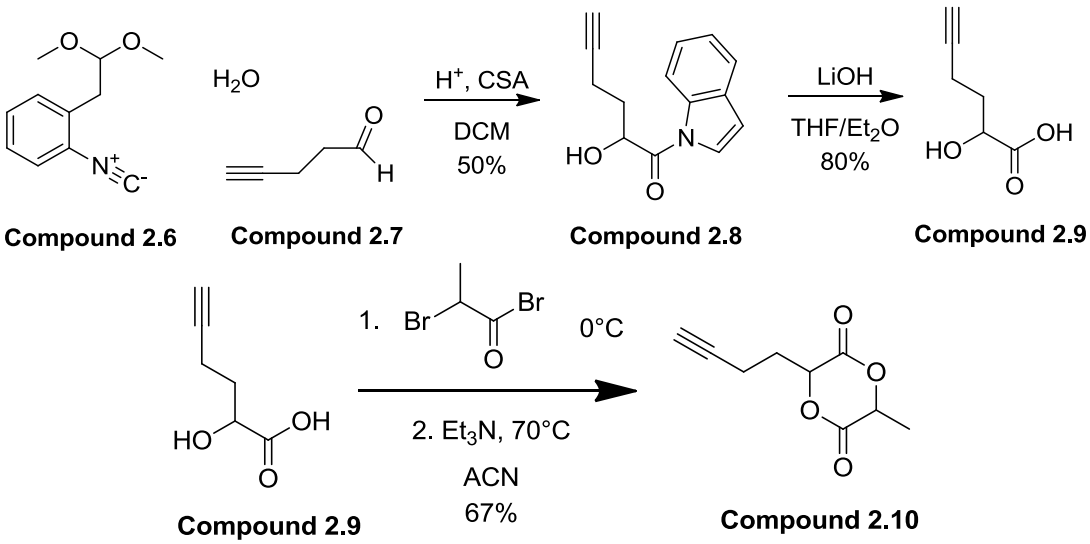
Figure 2.2: *General scheme to produce functionalized lactic acid derivatives from α-hydroxy, acyl-indole derivatives, which were made using a convertible isocyanide.*

My intent to reproduce the results reported by Yang and coworkers and to synthesize the proposed propargylic-functionalized lactic acid from its precursor proved to be successful. 1-(2,2-dimethoxyethyl)-2-isocyanobenzene, the convertible

isocyanide (compound 2.6), was synthesized from *N*-(2-(2,2-dimethoxyethyl)phenyl)formamide via a dehydration reaction, according to the synthesis reported by Kobayashi and coworkers.[60] Dr. Mark Rubinshtein, a previous Ph.D. student in the Yang group, synthesized the aforementioned formamide for the purposes of these experiments.[60] 4-pentyn-1-al, the propargylic aldehyde precursor (compound 2.7), was produced from commercially-available pent-4-yn-1-ol via the Swern oxidation.[52,61,62]



Synthetic Scheme 2.3: *The synthetic schematic for compound 2.6 (convertible isoyanide) and compound 2.7 (4-pentyn-1-al).*

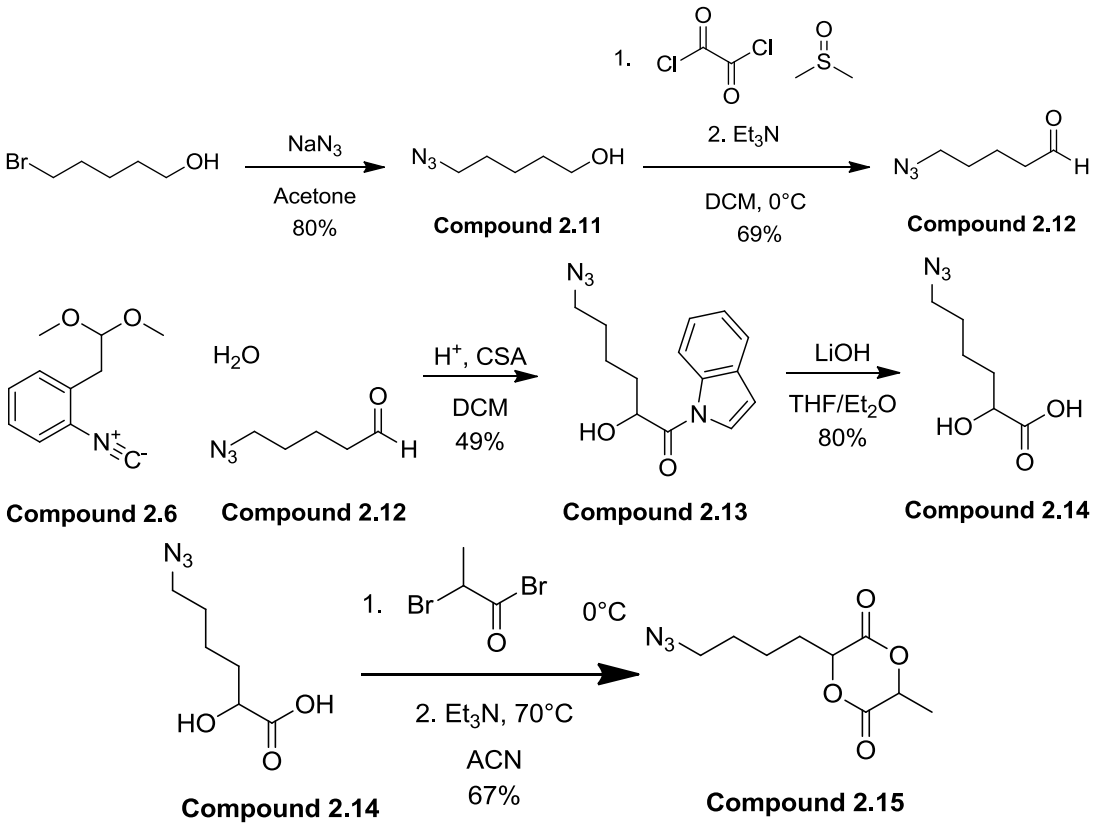


Synthetic Scheme 2.4: *The synthetic schematic for compound 2.10 (propargylic-functionalized hemi-lactide) from compound 2.6 (convertible isocyanide) and compound 2.7 (4-pentyn-1-al).*

Reacting the propargylic aldehyde (compound 2.7) with the convertible isocyanide (compound 2.6) and H_2O under the presence of acid, produced the propargylic, α-hydroxy, acyl-indole. The acyl-indole moiety easily hydrolyzes under the presence of base to produce the resulting propargylic-functionalized lactic acid (compound 2.8) in good yield. Treating the propargylic functionalized lactic acid with commercially-available 2-bromopropanoyl bromide resulted in a propargylic hemi-lactide (compound 2.9).

While all three different synthetic routes produced lactide-derivatives with molecular handles capable of further modification, I found that the methods and syntheses developed by Yang and coworkers was the most appealing because they

offer the chemical flexibility to add other desirable functional groups.[29,30,52,58,59] Yang and coworkers also reported the synthesis of functionalized azido-hemi-lactide capable of azide-alkyne addition as well. My attempts to reproduce their results to gain access to different polymers also proved to be successful.



Synthetic Scheme 2.5: *The synthetic schematic for compound 2.15 (azido-functionalized hemi-lactide) from compound 2.6 (convertible isocyanide) and compound 2.12 (5-azidopentan-1-al).*

5-azidopentan-1-ol (compound 2.11) was synthesized by the nucleophilic substitution of commercially-available 5-bromopentan-1-ol with sodium azide. The

bi-functionalized azido-alcohol was oxidized to the corresponding aldehyde (compound 2.12) using a Swern oxidation.[52,61,62] Reacting the azido-functionalized aldehyde with the convertible isocyanide (compound 2.6) resulted in the desired α-hydroxy, acyl-indole with the azide moiety (compound 2.13). The acyl-indole moiety easily hydrolyzes under the presence of base to produce the resulting azido-functionalized lactic acid (compound 2.14) in good yield. Treatment of the azido-derivative of lactic acid with commercially-available 2-bromopropanoyl bromide resulted in the functionalized hemi-lactide with an azido molecular handle (compound 2.15).

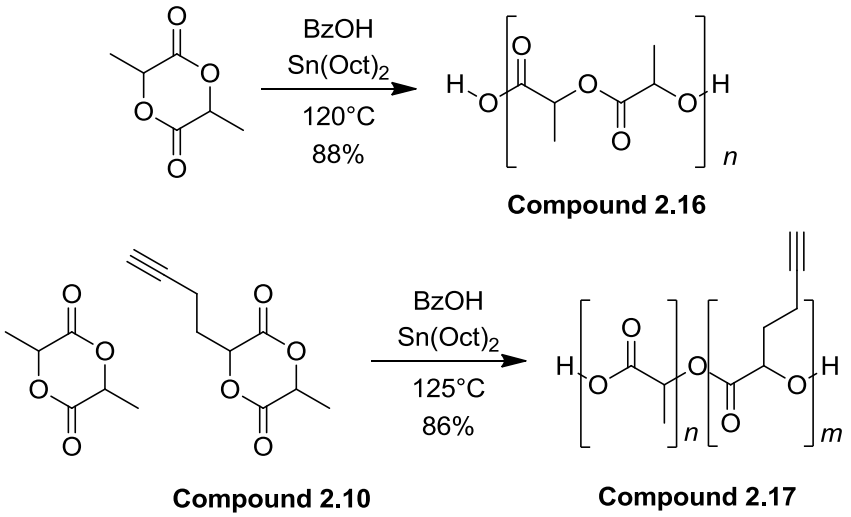
Considering the versatility of the facile synthetic procedure developed by Yang and coworkers, I chose to proceed further with the polymerization of the lactic acid, glycolic acid and their derivatives using modified procedures of their methods. Even though I decided to utilize a poly-lactic acid (PLA) polymer capable of undergoing cycloaddition with azido-functionalized molecules as my carrier for drug delivery, I still wanted the flexibility to easily synthesize different lactic acid derivatives for the future.

Section 2.4 Ring Opening Polymerization (ROP) of Lactide and Its Derivatives

Poly-lactic acid polymers can be synthesized in a ring-opening polymerization (ROP) from lactide (cyclic dimers of lactic acid) and its derivatives using both metal and metal-free catalysts. [13,29-31,45,52,56,58,59,63] Although there can be arguments made for the advantages of the different types of catalysts, stannous octoate (tin(II) 2-ethylhexanoate) has become a popular choice because it received approval from the American Food and Drug Administration (FDA) as a suitable food additive.[63,64] A proposed mechanism for its catalysis is that the stannous octoate facilitates a coordination-insertion with the carbonyl portion of lactide and the hydroxyl group of an available alcohol.[63,64] The ring-opening of lactide by an alcohol results in the availability of another alcohol for further polymerization.

My attempts to polymerize native lactide using a modified version of the procedure detailed by Yang and coworkers and Baker and coworkers yielded comparable results.[30,52] The ring-opening polymerization of native lactide produced unfunctionalized poly-lactic acid (compound 2.16) in good yield. Using a similar procedure, I was able to also copolymerize native lactide with propargylic-functionalized hemi-lactide (compound 2.10) to synthesize a PLA-based copolymer with propargylic functionalities (compound 2.17) in similar yields. The propargylic moiety in the copolymer (compound 2.17) offers a molecular handle for further modification. In the next section of this thesis, I will detail the synthesis of a

bifunctional linker to graft a cytotoxic drug to the derivatized poly-lactic acid (PLA) copolymer carrier. The overall conjugated, modified PLA copolymer will serve as the complete drug delivery system.



Synthetic Scheme 2.6: *The synthetic schematic for compound 2.16 (poly-lactic acid) and compound 2.17 (modified poly-lactic acid with alkyne side chains) from the ring-opening polymerization of lactide and functionalized lactide derivatives.*

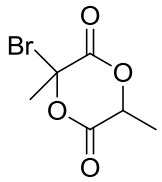
Section 2.5 Experimentals

All reagents were purchased from Acros Organic, Spectrum Chemicals, Sigma-Aldrich, TCI America, or Alfa Aesar and used without further additional purification unless specified. All solvents used for reactions were dried on alumina columns and distilled prior to use. Solvents used for chromatography were ACS technical grade and used without further purification. Distilled water (18.2 μΩ/cm) was filtered through a NANOPure DiamondTM (Barnstead) water purification system before use in synthesis or purification. NMR spectra were recorded on a Varian 400 (FT, 400 MHz, ^{1}H; 100 MHz, ^{13}C) spectrometer and a JEOL ECA-500 (FT, 500 MHZ ^{1}H) spectrometer. HR-MS (high-resolution mass spectra) were obtained in the Department of Chemistry & Biochemistry, University of California, San Diego (UCSD) using a ThermoFinnigan MAT900XL-MS. ESI-MS (electrospray ionization mass spectra) were obtained using ThemoFinnigan LCQDECA-MS.

Molecular weight (MW) and polydispersity index (PDI) of polymers were calculated using size-exclusion chromatography (Phenomenex Phenogel 5u 10, 1K-75K, 300 x 7.80 mm in series with a Phenomex Phenogel 5u 10, 10K-1000K, 300 x 7.80 mm (0.05 M LiBr in DMF, 0.75 mL/min at 60°C)) using a Hitachi-Elite LaChrom L-2130 pump equipped with a UV detector (Hitachi- Elite LaChrom L-2420), a multi-angle light scattering detector (DAWN-HELIOS: Wyatt Technology) and a refractive index detector (Hitachi L-2490). Data analysis was performed using the ASTRA software package. The characterizations and measurements of the

polymers were performed under the same conditions and in the same fashion as those indicated by Yang and coworkers.[52]

Synthesis of 3-bromo-3,6-dimethyl-1,4-dioxane-2,5-dione (compound 2.1)[31]

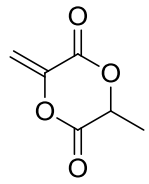


Compound 2.1

3-bromo-3,6-dimethyl-1,4-dioxane-2,5-dione (compound 2.1) was synthesized from the radical bromination of 3,6-dimethyl-1,4-dioxane-2,5-dione (lactide). Prior to use, commercially-available *N*-bromosuccinimide (yellow powder) was recrystallized in distilled H_2O. The resulting solid was isolated as white, flakey crystals. D,L lactide (892. mg, 6.19 mmol) and recrystallized *N*-bromosuccinimide (1.21 g, 6.80 mmol) were dissolved in acetonitrile (40 mL) and heated to reflux under N_2. A solution of benzoyl peroxide (45 mg, 190. mmol) dissolved in acetonitrile (3 mL) was added to the reaction dropwise by a syringe. The reaction was allowed to stir for more than 2 hours under reflux. Afterwards, the red reaction solution was allowed to cool to room temperature and then solids were filtered off. The solvent in the filtrate was evaporated under reduced pressure and a red-yellow solid was formed. The resulting solid was dissolved in ethyl acetate (80 mL) and the solution was washed and decolorized in saturated sodium bisulfite solution (4x 20 mL) and saturated NaCl (1x 20 mL). The washed organic layer was dried over

Na_2SO_4 and then the solvent was evaporated under reduced pressure. The resulting solid were recrystallized in toluene to produce white crystals in 60% yield. Characterization of 3-bromo-3,6-dimethyl-1,4-dioxane-2,5-dione (compound 2.1): ^{1}H-NMR ($CDCl_3$, 400 MHz) δ 5.5 (q, 1H), 2.35 (s, 3H), 1.74 (d, 3H)

Synthesis of 3-methyl-6-methylene-1,4-dioxane-2,5-dione (methylene lactide, compound 2.2)[31]

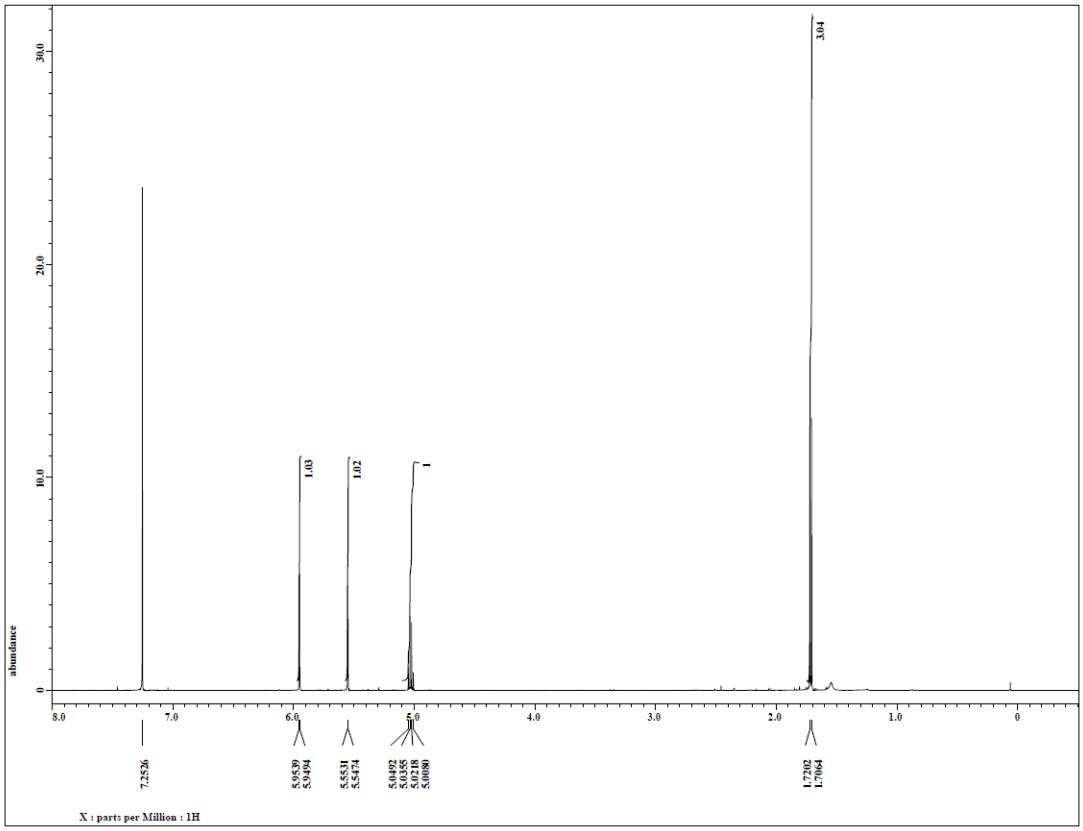


Compound 2.2

3-methyl-6-methylene-1,4-dioxane-2,5-dione (methylene lactide, compound 2.2) was synthesized from the elimination of hydrogen bromide from 3-bromo-3,6-dimethyl-1,4-dioxane-2,5-dione (compound 2.1) using triethylamine (Et_3N). Prior to use, commercially-available, reagent grade triethylamine was distilled over CaH_2. 3-bromo-3,6-dimethyl-1,4-dioxane-2,5-dione (6.05 g, 27.1 mmol) was dissolved in ethyl acetate (40 mL). The reaction flask was flushed with N_2 and cooled to 0°C using an ice bath. Triethylamine (4.2 mL, 30. mmol) was added dropwise by a syringe. The reaction solution was allowed to stir at 0°C for 1 hour.

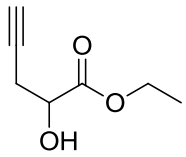
Afterward, the reaction solution was allowed to stir for another hour at room temperature. Upon completion of the reaction, an additional 60 mL of ethyl acetate was added. The final solution was washed with 0.1 M HCl (3x, 20 mL) and saturated

NaCl (1x 20 mL). The washed organic layer was dried over Na_2SO_4 and then the solvent was evaporated under reduced pressure. The resulting solid were recrystallized in toluene to produce a white powder in 61% yield. Characterization of 3-methyl-6-methylene-1,4-dioxane-2,5-dione (compound 2.2): ^{1}H-NMR ($CDCl_3$, 500 MHz) δ 5.952 (d, 1H), 5.550 (d, 1H), 5.029 (q, 1H), 2.35 (s, 3H), 1.713 (d, 3H)



Spectrum 2.2: *^{1}H-NMR spectrum of compound 2.2*

Synthesis of ethyl 2-hydroxypent-4-ynoate (compound 2.3)[30]



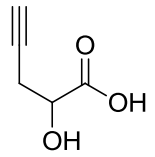
Compound 2.3

Ethyl 2-hydroxypent-4-ynoate was synthesized by a Reformatsky-like reaction of propargyl bromide with ethyl glyoxylate. A slurry of zinc (13.8 g, 211 mmol) and anhydrous THF (21 mL) were placed in a round-bottom flask. Propargyl bromide (80% by weight in toluene, 450 μL, 4.04 mmol) was added to the reaction flask to activate the zinc. The reaction flask was flushed with N_2 and cooled to 0°C using an ice bath. A solution of THF (30 mL), diethyl ether (42 mL), ethyl glyoxylate (17.6 mL of ~50% by weight in toluene, ~9 g, ~90 mmol), and propargyl bromide (15.8 mL, 142 mmol) was added dropwise to the stirred slurry with an addition funnel. The color reaction mixture eventually grew darker and turned black. The resulting slurry was allowed to stir overnight under N_2. The reaction flask gradually warmed from 0°C to room temperature.

Afterwards, the slurry was filtered over Celite and the solvent was evaporated under reduced pressure to produce a green gel. The gel was the dissolved in ethyl acetate (100 mL) and washed with 2 M HCl (3x 30 mL). The aqueous layer was pooled and back extracted with ethyl acetate (1x 20 mL). The organic layers were combined, dried over Na_2SO_4 and then the solvent was evaporated under reduced

pressure. The resulting yellow oil was further purified by column chromatography in the following solvent system: 4 EtOAc: 1 Hexanes. The final oil was yellow and viscous and isolated in 50% yield. Characterization of ethyl 2-hydroxypent-4-ynoate (compound 2.3): ^{1}H-NMR ($CDCl_3$, 500 MHz) δ 4.285 (m, 3H), 3.093 (d, 1H), 2.686 (m, 2H) 2.045 (t, 1H), 1.307 (t, 3H)

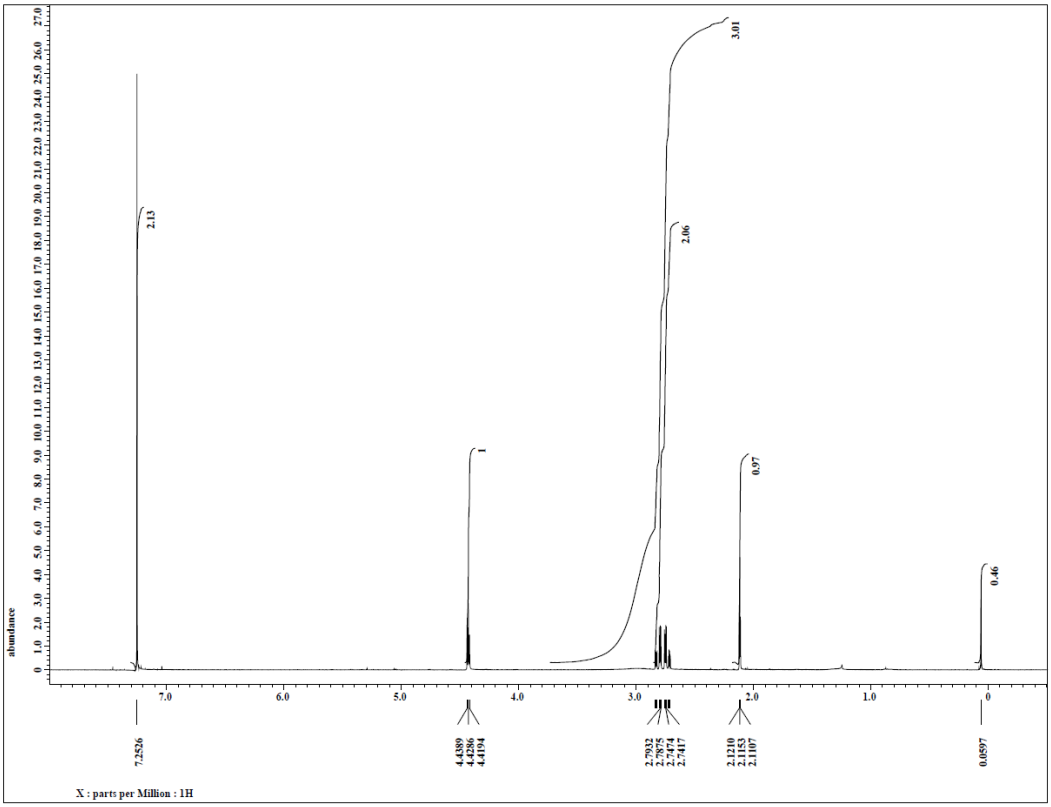
Synthesis of 2-hydroxypent-4-ynoic acid (compound 2.4)[52]



Compound 2.4

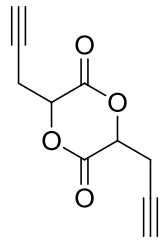
2-hydroxypent-4-ynoic acid (compound 2.4) was synthesized by the hydrolysis of ethyl 2-hydroxypent-4-ynoate (compound 2.3) by lithium hydroxide monohydrate. A reaction solvent system of THF (75 mL) and H_2O (50 mL) was prepared for use in the reaction. 2-hydroxypent-4-ynoate (7.0 g, 49 mmol) was dissolved in 25 mL of the reaction solvent system in a round-bottom flask, cooled to 0°C using an ice bath, and stirred vigorously. A mixture of lithium hydroxide monohydrate (4.13 g, 98.5 mmol) and reaction solvent (100 mL) was prepared for use in the reaction. The mixture of lithium hydroxide was added to the reaction mixture in four installments of 25 mL. More H_2O (25 mL) was then added to dissolve the remaining lithium hydroxide monohydrate. The reaction flask was allowed to stir overnight and gradually warmed from 0°C to room temperature.

Afterwards, the solvent was evaporated under reduced pressure. The resulting slurry was acidified with 6 M HCl (50 mL) and stirred for 1 hour. The aqueous solution was extracted with ethyl acetate (6x 30 mL) and diethyl ether (12x 30 mL) and the organic layers were pooled. The combined organic layer was dried over Na_2SO_4 and then the solvent was evaporated under reduced pressure to produce a brown, viscous oil. The oil was further purified by a silica plug using ethyl acetate as the eluent. After the silica plug purification, the subsequent oil was recrystallized twice in dichloromethane at 0°C. After the purification, the white crystals were collected, and the resulting yield was 80%. Characterization of 2-hydroxypent-4-ynoic acid (compound 2.4): ^{1}H-NMR (CDCl3, 500 MHz) δ 4.429 (t, 1H), 2.767 (m, 2H), 2.115 (t, 1H)



Spectrum 2.4: *^{1}H-NMR spectrum of compound 2.4*

Synthesis of 3,6-di(prop-2-yn-1-yl)-1,4-dioxane-2,5-dione (compound 2.5)[30]

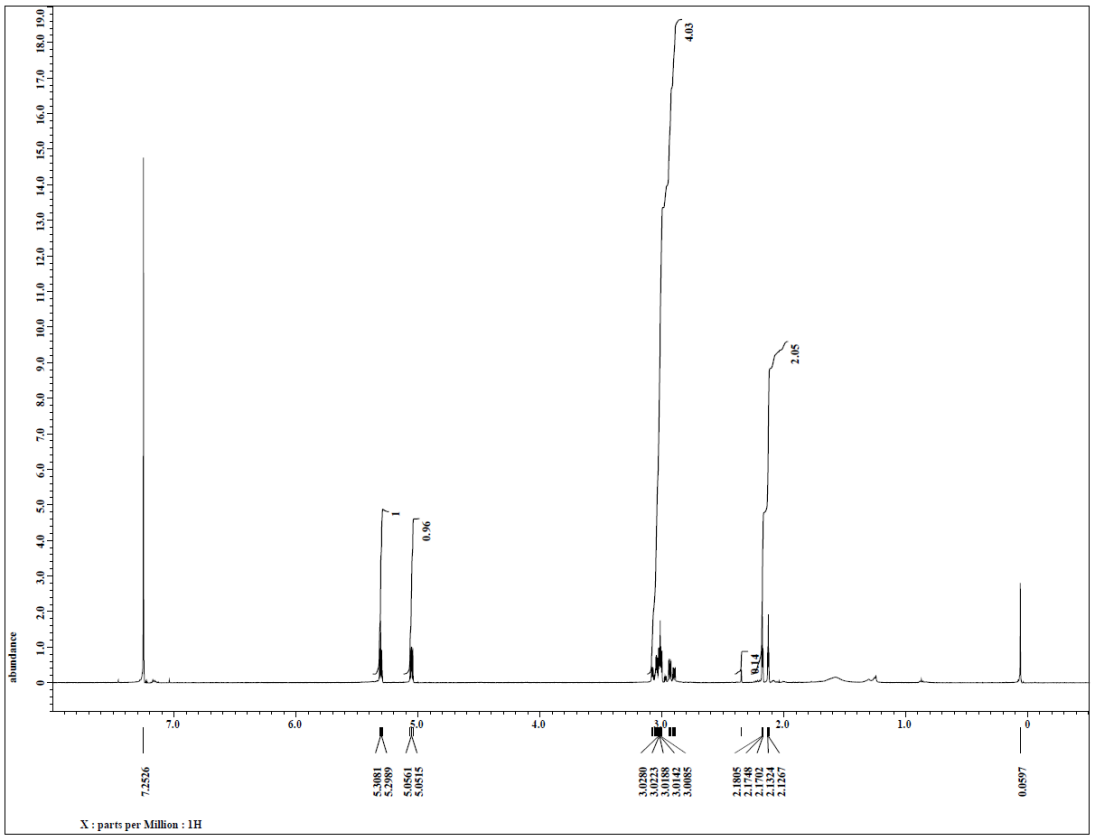


Compound 2.5

3,6-di(prop-2-yn-1-yl)-1,4-dioxane-2,5-dione (compound 2.5) was synthesized from the dehydration of 2-hydroxypent-4-ynoic acid (compound 2.4) by the azeotropic removal of H_2O. 2-hydroxypent-4-ynoic acid (2.8 g, 25 mmol) and *p*-

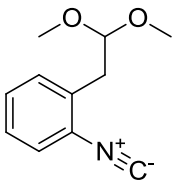
toluenesulfonic acid monohydrate (233 mg, 1.22 mmol) and toluene (280 mL) were added to a round-bottom flask. The reaction mixture was heated to reflux and H_2O was removed azeotropically using a Dean-Stark Trap. The flask was allowed to stir under reflux and N_2 for 3 days.

Afterward the reaction flask was allowed to cool to room temperature, the solids in the reaction solution were filtered and the solvent in the filtrate was removed under reduced pressure. The resulting slurry was dissolved in ethyl acetate (150 mL) and washed with saturated $NaHCO_3$ (4x 30mL) and saturated NaCl (1x 30mL) and dried over Na_2SO_4. The subsequent oil was recrystallized in toluene to produce white crystals as a *meso/rac* isomer mixture in 25% yield. Characterization of 3,6-di(prop-2-yn-1-yl)-1,4-dioxane-2,5-dione (compound 2.5): ^{1}H-NMR (CDCl3, 500 MHz) δ 5.303 (t, 1H), 5.054 (dd, 1H), 3.016 (m, 4H), 2.172 (d, 1H), 2.129 (d, 1H)). ESI-MS (m/z, M+MeOH+Na^+): 247.1



Spectrum 2.5: *[1]H-NMR spectrum of compound 2.5*

Synthesis of 1-(2,2-dimethoxyethyl)-2-isocyanobenzene (convertible isocyanide, compound 2.6)[52,60]

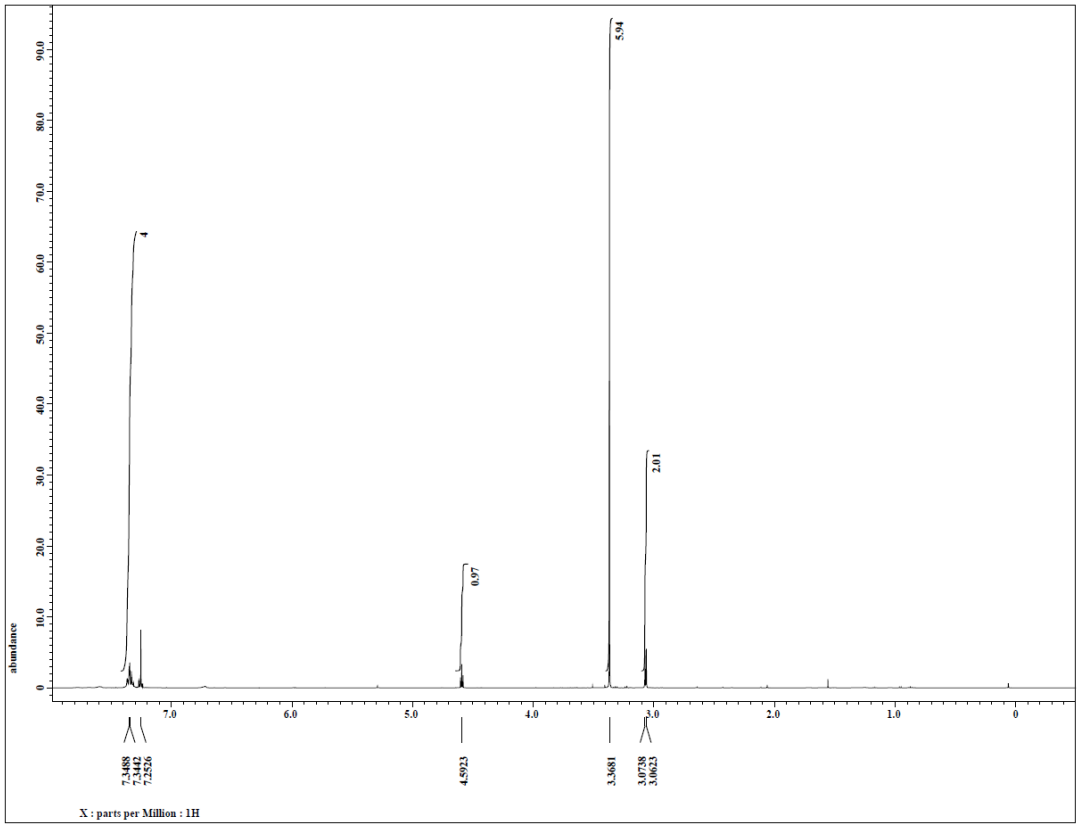


Compound 2.6

1-(2,2-dimethoxyethyl)-2-isocyanobenzene (convertible isocyanide, compound 2.6) was synthesized from the dehydration of *N*-(2-(2,2-

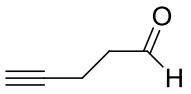
dimethoxyethyl)phenyl)formamide by phosphorus oxychloride, $POCl_3$. *N*-(2-(2,2-dimethoxyethyl)phenyl)formamide was originally synthesized for use by Dr. Mark Rubinsthein according to the procedure by Kobayashi and coworkers.[60] *N*-(2-(2,2-dimethoxyethyl)phenyl)formamide (20.6 g, 98.5 mmol) and triethylamine (69.9 mL, 502 mmol) were dissolved in dichloromethane (250 mL). The reaction flask was flushed with N_2 and cooled to 0°C using an ice bath. Phosphorus oxychloride (13.8 mL, 148 mmol) was added dropwise to the reaction mixture by an addition funnel. The reaction flask was allowed to stir overnight and gradually warmed from 0°C to room temperature.

Afterwards, the reaction mixture was filtered through Celite to resolve emulsions and colloids. The filtrate was poured into saturated $NaHCO_3$ (750 mL) and extracted with dicholormethane (16x 50ml). The organic layers were pooled, dried over Na_2SO_4, and then the solvent was evaporated under reduced pressure. The resulting, dark-red oil was further purified by column chromatography in the following solvent system: 49 EtOAc: 49 Hexanes: 2 Et_3N. The final, dark-red oil was isolated in 83% yield. Characterization of 1-(2,2-dimethoxyethyl)-2-isocyanobenzene (convertible isocyanide, compound 2.6): ^{1}H-NMR (CDCl3, 500 MHz) δ 7.337 (m, 4H), 4.5923 (t, 1H), 3.3681 (s, 6H), 3.065 (d, 2H)



Spectrum 2.6: *^{1}H-NMR spectrum of compound 2.6*

Synthesis of 4-pentyn-1-al (compound 2.7)[52,62]



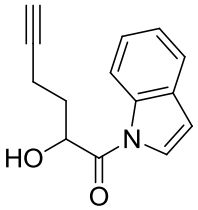
Compound 2.7

4-pentyn-1-al (compound 2.7) was synthesized via a Swern oxidation of commercially-available 4-pentyn-1-ol. Oxalyl chloride (3.4 g, 27 mmol, 2.3 mL) dissolved in dichloromethane (100 mL) in a round-bottom flask. The reaction flask was flushed with N_2 and cooled to -78°C using an acetone/dry ice bath. Dimethyl

sulfoxide (4.4 g, 56.3 mmol, 4.0 mL) was dissolve in dichloromethane (10 mL) and added dropwise to the reaction solution. The mixture was stirred at-78°C and N_2 for 30 minutes to activate the DMSO. Afterwards, a solution of 4-pentyn-1-ol (2.0 g, 24 mmol, 2.2 mL) dissolved in dichlormethane (20 mL) was added dropwise to the reaction vessel. After the addition, the reaction mixture was allowed to stir at -78°C and under N_2 for 1 hour. Triethylamine (14 g, 19 mL, 140 mmol) was then added dropwise, and the reaction mixture was allowed to stir at -78°C and under N_2 for 2 hour, followed by 1 hour at room temperature.

Upon completion, the reaction mixture was diluted with dichlormethane (100 mL), washed with 1M HCl (5 x 30 mL) and saturated NaCl (1 x 30) and over dried over Na_2SO_4. The solvent was then evaporated under reduced pressure. The residue was purified by Kugelrhor distillation and the resulting colorless oil was isolated in 69% yield. Characterization of 4-pentyn-1-al (compound 2.7): ^{1}H-NMR (CDCl3, 400 MHz) δ 9.79 (t, 1H), 2.70 (tt, 2H), 2.50 (m, 2H), 1.99 (t, 1H), 1.64 (s, 5H).

Synthesis of 2-hydroxy-1-(1H-indol-1-yl)hex-5-yn-1-one (compound 2.8)[52]

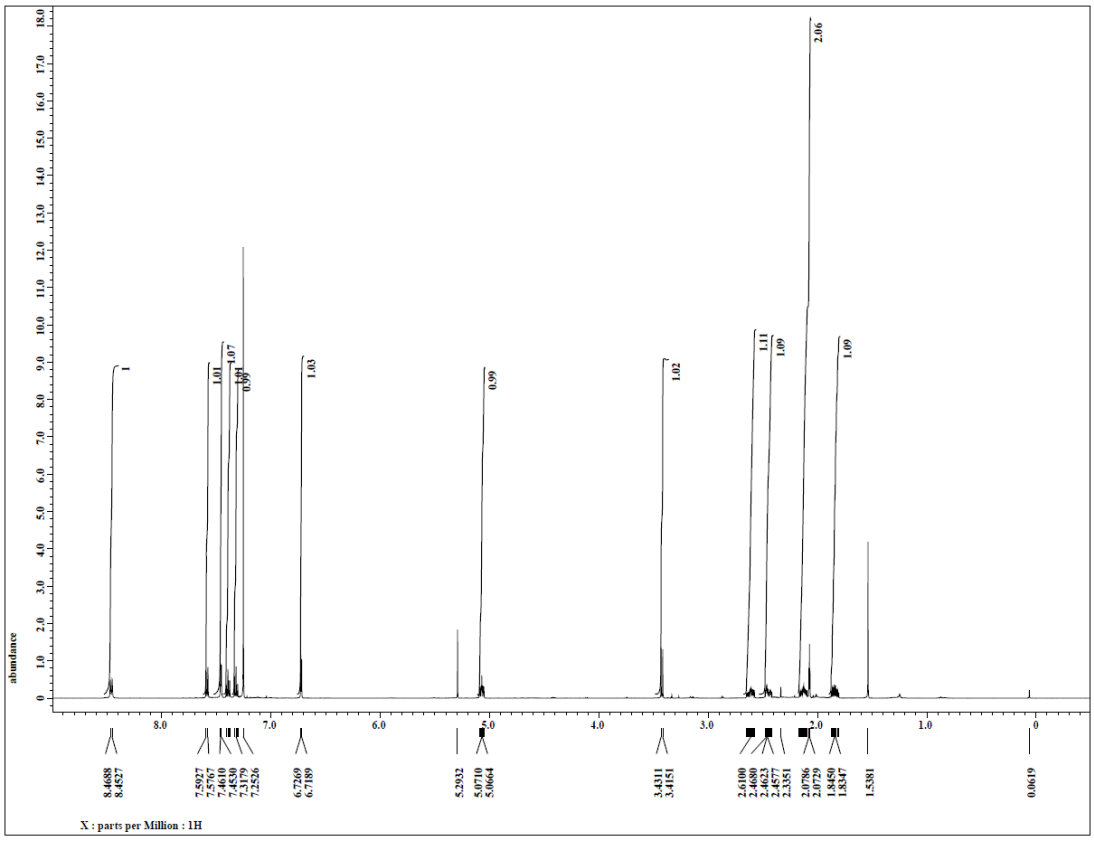


Compound 2.8

2-hydroxy-1-(1H-indol-1-yl)hex-5-yn-1-one (compound 2.8) was synthesized from a Passerini-like reaction with 1-(2,2-dimethoxyethyl)-2-isocyanobenzene

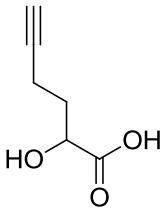
(convertible isocyanide, compound 2.6) and 4-pentyn-1-al (compound 2.7). 1-(2,2-dimethoxyethyl)-2-isocyanobenzene (390 mg, 2.0 mmol), 4-pentyn-1-al (0.20 g, 2.4 mmol) and distilled H_2O (0.070 ml, 0.070 g, 3.9 mmol) were dissolved in dry dichloromethane (10 mL) and allowed to stir at room temperature under N_2. D,L-camphorsulfonic acid (90 mg, 0.4 mmol) was then added to the reaction flask. The reaction was allowed to stir overnight at room temperature under N_2.

Afterwards, the solvent was evaporated under reduced pressure, and the resulting oil was purified by column chromatography in the following solvent system: 85 Hexanes: 15 EtOAc. The resulting, colorless oil was isolated in 50% yield. Characterization of 2-hydroxy-1-(1H-indol-1-yl)hex-5-yn-1-one (compound 2.8): ^{1}H-NMR (CDCl3, 500 MHz) δ 8.460 (d, 1H), 7.584 (d, 1H), 7.457 (m, 1H), 7.38 (m, 1H), 7.285 (m, 1H), 6.722 (d, 1H), 5.069 (m, 1H), 3.432 (d, 1H), 2.610 (m, 1H), 2.462 (m, 1H), 2.12 (m, 2H), 1.840 (m, 1H).



Spectrum 2.8: *[1]H-NMR spectrum of compound 2.8*

Synthesis of 2-hydroxyhex-5-ynoic acid (compound 2.9)[52]

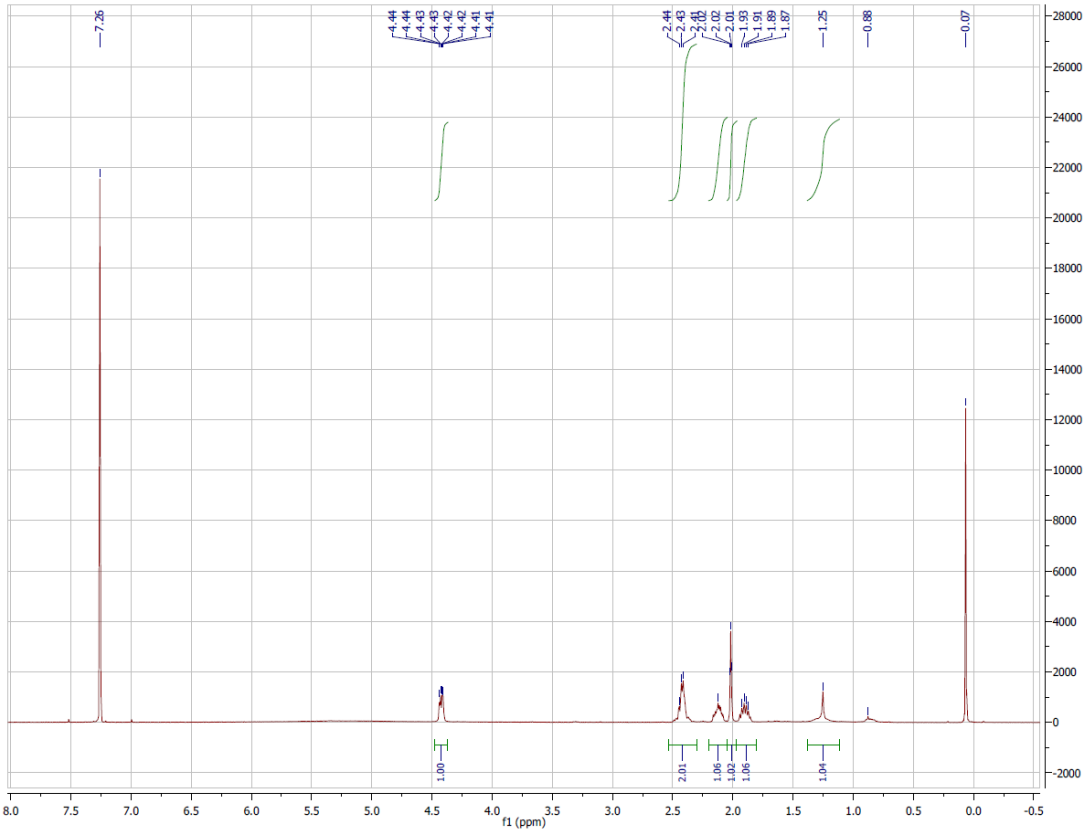


Compound 2.9

2-hydroxyhex-5-ynoic acid (compound 2.9) was synthesized from hydrolysis of 2-hydroxy-1-(1H-indol-1-yl)hex-5-yn-1-one (compound 2.8) by LiOH. 2-hydroxy-1-(1H-indol-1-yl)hex-5-yn-1-one (2.0 g, 8.8 mmol) was dissolved in THF

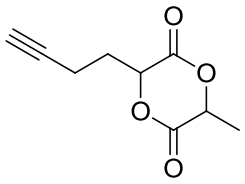
(60 mL), stirred, and cooled to 0°C using an ice bath. 1.0 M LiOH (20. mL, 20. mmol) was added to the reaction flask and the solution was allowed to warm gradually to room temperature. The reaction flask was stirred for 2 hours and then 1.0 M NaOH (10. mL, 10. mmol) was added to reaction solution to ensure a basic pH. The aqueous phase was extracted with ethyl acetate (3x 20 mL) to remove indole byproducts, and the organic layer was pooled.

The combined organic layers were back-extracted by 1.0 M NaOH (3x 10 mL) to recover purified functionalized lactate. Extracted aqueous layer was acidified by 6 M HCl to pH 2 to ensure protonation of the lactate salt. The acidified aqueous layer was extracted with ethyl acetate (6x 50 mL). The organic layer with the purified and recovered product was dried over Na_2SO_4, and then the solvent was evaporated under reduced pressure. The recovered yellow oil was used without further purification in 80% yield. Characterization of 2-hydroxyhex-5-ynoic acid (compound 2.9): ^{1}H-NMR (CDCl3, 500 MHz) δ 4.42 (m, 1H), 2.41-2.44 (m, 2H), 2.01 (m, 1H) 1.93-1.87 (m, 1H), 1.25 (m, 1H).



Spectrum 2.9: *^{1}H-NMR spectrum of compound 2.9*

Synthesis of 3-(but-3-yn-1-yl)-6-methyl-1,4-dioxane-2,5-dione (compound 2.10)[52]

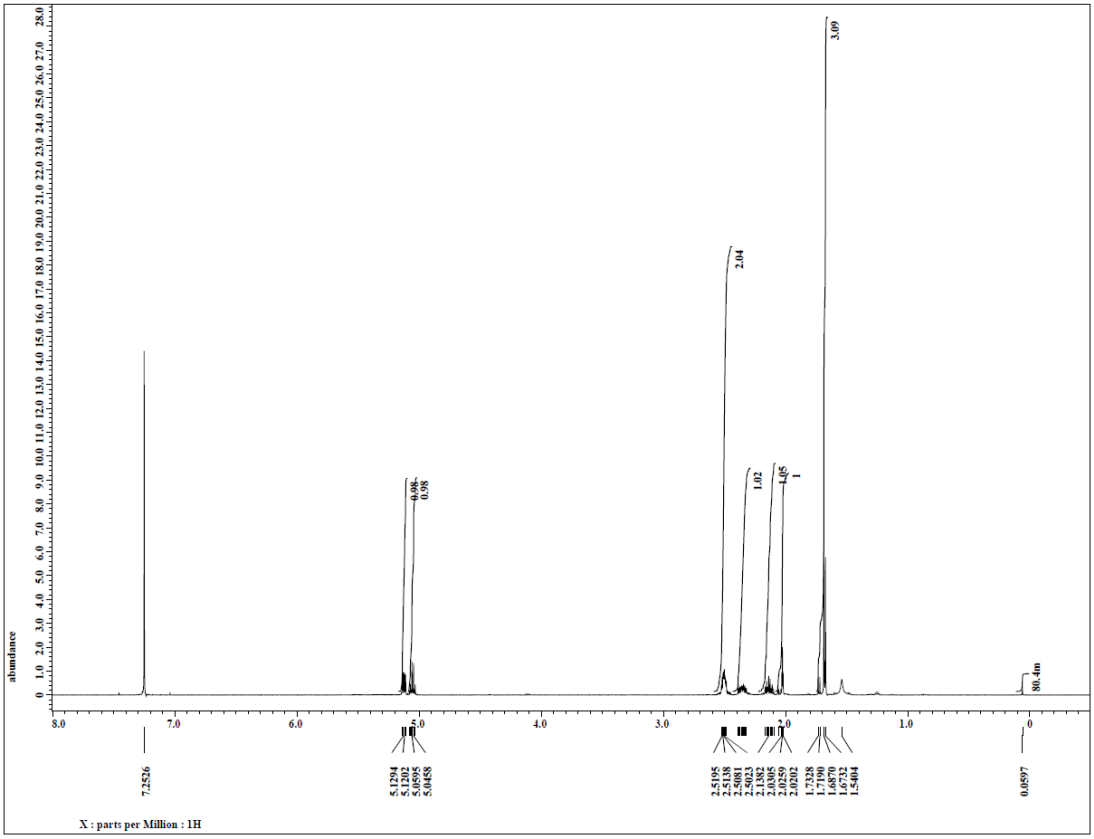


Compound 2.10

3-(but-3-yn-1-yl)-6-methyl-1,4-dioxane-2,5-dione (compound 2.10) was synthesized from the cyclization of 2-hydroxyhex-5-ynoic acid (compound 2.9) with commercially-available 2-bromopropanoyl bromide using a modified procedure

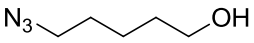
described by Yang and coworkers.[52] 2-hydroxyhex-5-ynoic acid (1.0 g, 7.8 mmol) and triethylamine (870 mg, 1.2 mL, 8.6 mmol) were dissolved in HLPC grade acetonitrile (25 ml), and the reaction flask was flushed with N_2 and cooled to 0°C using an ice bath. 2-bromopropanoyl bromide (0.90 mL, 8.6 mmol) was then added to the cooled reaction mixture and the solution was stirred at 0°C under N_2 for 1 hour. More triethylamine (870 mg, 1.2 mL, 8.6 mmol) was added and the reaction flask was allowed to stir at 70°C under N_2 for 3 hours.

Afterwards, the reaction flask was allowed to cool to room temperature and the solvent was evaporated under reduced pressure. The slurry was dissolved in ethyl acetate (50 mL) and then washed with 1 M HCl (3x 10 mL), H_2O (1x 10 mL), and saturated NaCl (1x 10 ml). The washed organic layer was dried over Na_2SO_4, and then the solvent was evaporated under reduced pressure. The recovered brown residue was additionally purified by column chromatography in the following solvent system: 80 Hexanes: 20 EtOAc to give a yellow oil, which solidified upon storage at -80°C. The purified solid was isolated at 67% yield. Characterization of 3-(but-3-yn-1-yl)-6-methyl-1,4-dioxane-2,5-dione (compound 2.10): ^{1}H-NMR (CDCl3, 500 MHz) δ 5.125 (dd, 1H), 5.052 (q, 1H), 2.520-2.502 (m, 2H), 2.38-2.32 (m, 1H), 2.18-2.10 (m, 2H), [(1.725, m), (1.680, m), 3H]



Spectrum 2.10: *^{1}H-NMR spectrum of compound 2.10*

Synthesis of 5-azidopentan-1-ol (compound 2.11)[52]



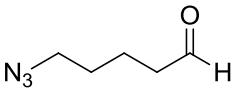
Compound 2.11

5-azidopentan-1-ol (compound 2.11) was synthesized from the nucleophilic substitution of commercially-available 5-bromopentan-1-ol with sodium azide. 5-bromopentan-1-ol (7.0 g, 42 mmol) and sodium azide (8.5 g, 130 mmol) was dissolved in distilled acetone (100 mL). The reaction flask was flushed with N_2, heated to reflux, and allowed to stir overnight. The reaction was followed by TLC

analysis. After completion of the reaction, the reaction flask was allowed to cool to room temperature, and the solvent was evaporated under reduced pressure.

The yellow residue that remained was dissolved in ethyl acetate (300 mL) and washed with deionized H_2O (4x 50 mL) and saturated NaCl (1x 50 mL). The washed organic layer was dried over Na_2SO_4, and then the solvent was evaporated under reduced pressure. The resulting yellow oil was isolated in 80% yield, and then used immediately without further purification.

Synthesis of 5-azidopentanal (compound 2.12)[52,62]



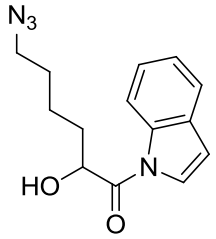
Compound 2.12

5-azidopentanal (compound 2.12) was synthesized from the Swern oxidation of freshly prepared 5-azidopentan-1-ol (compound 2.11). Oxalyl chloride (4.8 g, 38 mmol, 3.3 mL) dissolved in dichloromethane (150 mL) in a round-bottom flask. The reaction flask was flushed with N_2 and cooled to -78°C using an acetone/dry ice bath. Dimethyl sulfoxide (6.6 g, 84 mmol, 6.0 mL) was dissolve in dichloromethane (14 mL) and added dropwise to the reaction solution. The mixture was stirred at-78°C and N_2 for 30 minutes to activate the DMSO. Afterwards, a solution of 5-azidopentan-1-ol (4.4 g, 34 mmol) dissolved in dichlormethane (30 mL) was added dropwise to the reaction vessel. After the addition, the reaction mixture was allowed to stir at -78°C and under N_2 for 1 hour. Triethylamine (19 g, 26 mL, 190 mmol) was

then added dropwise, and the reaction mixture was allowed to stir at -78°C and under N_2 for 2 hour, followed by 1 hour at room temperature.

Upon completion, the reaction mixture was diluted with dichlormethane (100 mL), washed with 1M HCl (5 x 50 mL) and saturated NaCl (1 x 50) and over dried over Na_2SO_4. The solvent was then evaporated under reduced pressure. The residue was purified by column chromatography, and the resulting yellow oil was isolated at 69% yield. Characterization of 5-azidopentanal (compound 2.12): ^{1}H-NMR (CDCl3, 400 MHz) δ 9.78 (t, 1H), 3.31 (t, 2H), 2.50 (m, 2H), 1.76-1.59 (m, 4H).

Synthesis of 6-azido-2-hydroxy-1-(1H-indol-1-yl)hexan-1-one (compound 2.13)[52]



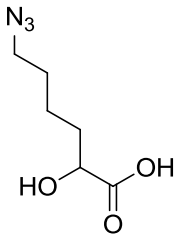
Compound 2.13

6-azido-2-hydroxy-1-(1H-indol-1-yl)hexan-1-one (compound 2.13) was synthesized from a Passerini-like reaction with 1-(2,2-dimethoxyethyl)-2-isocyanobenzene (convertible isocyanide, compound 2.6) and 5-azidopentanal (compound 2.12). 1-(2,2-dimethoxyethyl)-2-isocyanobenzene (3.4 g, 18 mmol), 5-azidopentanal (2.7 g, 21 mmol) and distilled H_2O (0.65 ml, 36 mmol) were dissolved in dry dichloromethane (100 ml) and allowed to stir at room temperature under N_2.

D,L-camphorsulfonic acid (0.90 g, 4.0 mmol) was then added to the reaction flask. The reaction was allowed to stir overnight at room temperature under N_2.

Afterwards, the solvent was evaporated under reduced pressure, and the resulting oil was purified by column chromatography in the following solvent system: 80 Hexanes: 20 EtOAc. The resulting, colorless oil was isolated in 49% yield. Characterization of 6-azido-2-hydroxy-1-(1H-indol-1-yl)hexan-1-one (compound 2.13): ^{1}H-NMR (CDCl3, 400 MHz) δ 8.46 (d, 1H), 7.58 (d, 1H), 7.47-7.30 (m, 3H) 6.72 (d, 1H), 4.85 (m, 1H), 3.49 (d, 1H), 3.28 (t, 2H), 1.96-1.93 (m, 1H), 1.79-1.58 (m, 5H).

Synthesis of 6-azido-2-hydroxyhexanoic acid (compound 2.14)[52]



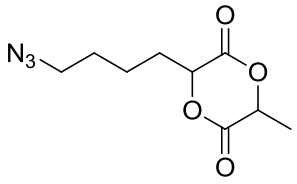
Compound 2.14

6-azido-2-hydroxyhexanoic acid (compound 2.14) was synthesized from hydrolysis of 6-azido-2-hydroxy-1-(1H-indol-1-yl)hexan-1-one (compound 2.13) by LiOH. 6-azido-2-hydroxy-1-(1H-indol-1-yl)hexan-1-one (2.4 g, 8.8 mmol) was dissolved in THF (60 mL), stirred, and cooled to 0°C using an ice bath. 1.0 M LiOH (20. mL, 20. mmol) was added to the reaction flask and the solution was allowed to warm gradually to room temperature. The reaction flask was stirred for 2 hours and

then 1.0 M NaOH (10. mL, 10. mmol) was added to reaction solution to ensure a basic pH. The aqueous phase was extracted with ethyl acetate (3x 20 mL) to remove indole byproducts, and the organic layer was pooled.

The combined organic layers were back-extracted by 1.0 M NaOH (3x 10 mL) to recover purified functionalized lactate. Extracted aqueous layer was acidified by 6 M HCl to pH 2 to ensure protonation of the lactate salt. The acidified aqueous layer was extracted with ethyl acetate (6x 50 mL). The organic layer with the purified and recovered product was dried over Na_2SO_4, and then the solvent was evaporated under reduced pressure. The recovered yellow oil was used without further purification in 80% yield. Characterization of 6-azido-2-hydroxyhexanoic acid (compound 2.14): ^{1}H-NMR (CDCl3, 400 MHz): δ 4.29 (dd, 1H), 3.31 (t, 2H), 1.93-1.86 (m, 1H), 1.79-1.48 (m, 5H).

Synthesis of 3-(4-azidobutyl)-6-methyl-1,4-dioxane-2,5-dione (compound 2.15)[52]



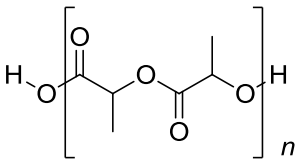
Compound 2.15

3-(4-azidobutyl)-6-methyl-1,4-dioxane-2,5-dione (compound 2.15) was synthesized from the cyclization of 6-azido-2-hydroxyhexanoic acid (compound 2.14) with commercially-available 2-bromopropanoyl bromide using a modified procedure described by Yang and coworkers.[52] 6-azido-2-hydroxyhexanoic acid (1.4

g, 8.1 mmol) and triethylamine (940 mg, 1.3 mL, 9.3 mmol) was dissolved in HLPC grade acetonitrile (25 mL), and the reaction flask was flushed with N_2 and cooled to 0°C using an ice bath. 2-bromopropanoyl bromide (0.90 mL, 8.6 mmol) was then added to the cooled reaction mixture, and the solution was stirred at 0°C under N_2 for 1 hour. More triethylamine (940 mg, 1.3 mL, 9.3 mmol) was added and the reaction flask was allowed to stir at 70°C under N_2 for 3 hours.

Afterwards, the reaction flask was allowed to cool to room temperature and the solvent was evaporated under reduced pressure. The slurry was dissolved in ethyl acetate (50 mL) and then washed with 1 M HCl (3x 10 mL), H_2O (1x 10 mL), and saturated NaCl (1x 10 mL). The washed organic layer was dried over Na_2SO_4, and then the solvent was evaporated under reduced pressure. The yellow residue was additionally purified by column chromatography in the following solvent system: 85 Hexanes: 15 EtOAc to give a yellow, viscous oil. The purified oil was isolated at 67% yield. Characterization of 3-(4-azidobutyl)-6-methyl-1,4-dioxane-2,5-dione (compound 2.15): ^{1}H-NMR (CDCl3, 400 MHz) δ 5.09-4.99 (m, 1H), 4.95-4.87 (m, 1H), 3.32 (t, 2H), 2.19-1.96 (m, 2H), 1.80-1.72 (m, 7H).

Polymerization of lactide to form unfunctionalized poly-lactic acid (PLA, compound 2.16)[30,52]

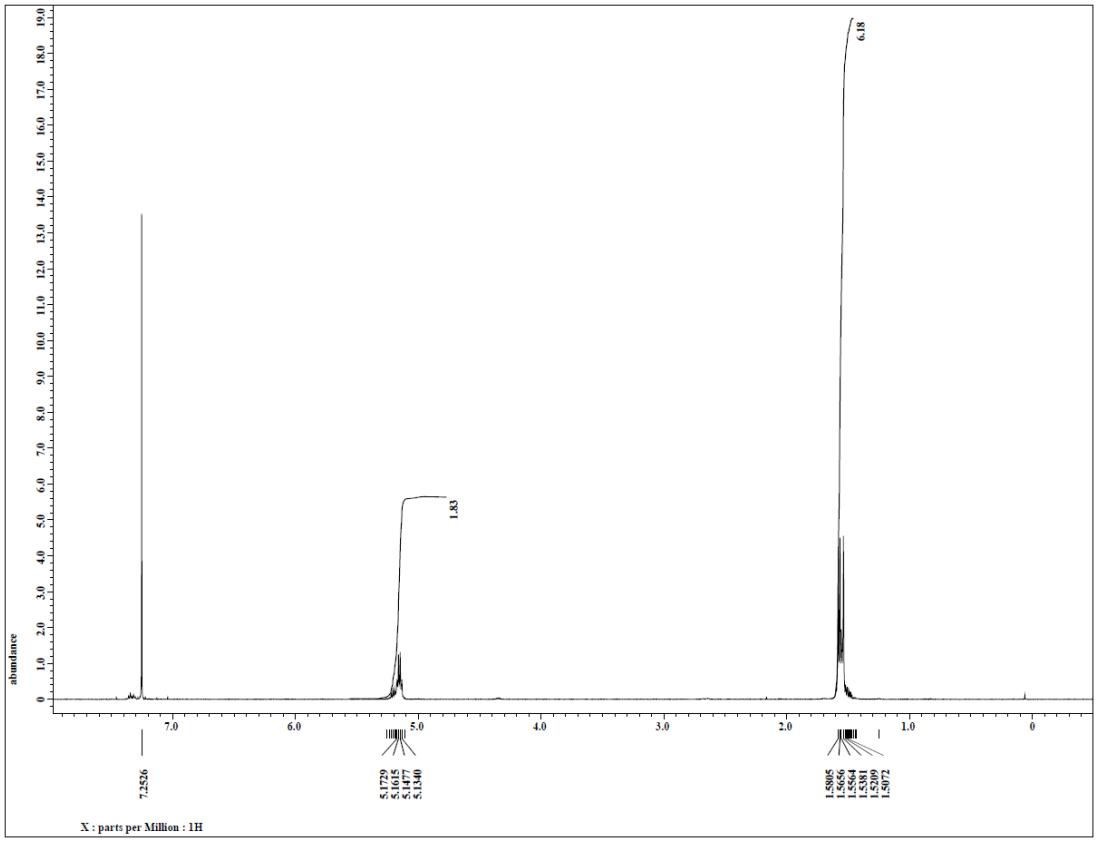


Compound 2.16

Unfunctionalized poly-lactic acid (PLA, compound 2.16) was synthesized by the ring-opening polymerization of native lactide in a modified version of the protocol reported by Yang and coworkers.[52] Fresh solutions of 0.20 M $Sn(Oct)_2$ and benzyl alcohol in anhydrous toluene were prepared prior to use in the polymerization. $Sn(Oct)_2$ (1.00 mL of 0.20 M solution) and benzyl alcohol (1.00 mL of 0.20 M solution) was added to a scintillation vial and the solvent was removed *in vacuo*. D,L lactide (1.44 g, 10.0 mmol) was added to the reaction vial. Afterwards, the vial was tightly sealed, flushed with N_2, heated to 125°C in a silicone oil bath, and then stirred. After one hour, the melted lactide became noticeably more cloudy and viscous, and the stir bar gradually began to struggle to stir. After 4.5 hours, the reaction vial was allowed to cool to room temperature. A portion of the crude was isolated for analysis by ^{1}H-NMR to measure percent conversion.

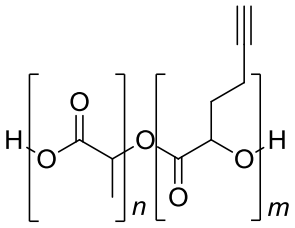
The remainder of the crude polymer was dissolved in chloroform and precipitated out in ice-cold methanol. The precipitated solid or gel was collected and immediately dried under reduced pressure. This purification process was repeated three times, and the purified, white polymer was isolated in resulted in 86% yield.

Afterwards, an aliquot of the purified polymer was dissolved in DMF, filtered through a Whatman Anontop 10 filter (0.2 μm) and analyzed by gel permeation chromatography (GPC) and static light scattering (SLS). Characterization of Poly-lactic acid (PLA, compound 2.15): ^{1}H-NMR (CDCl3, 500 MHz) δ 5.27-5.13 (m, 2H), 1.58-1.51 (m, 6H), Percent Conversion: >99%



Spectrum 2.16: *^{1}H-NMR spectrum of compound 2.16*

Copolymerization of lactide and propargylic-functionalized lactide to form poly-lactic acid, poly-acetylene-functionalized lactic acid copolymer (compound 2.17)[52,62]

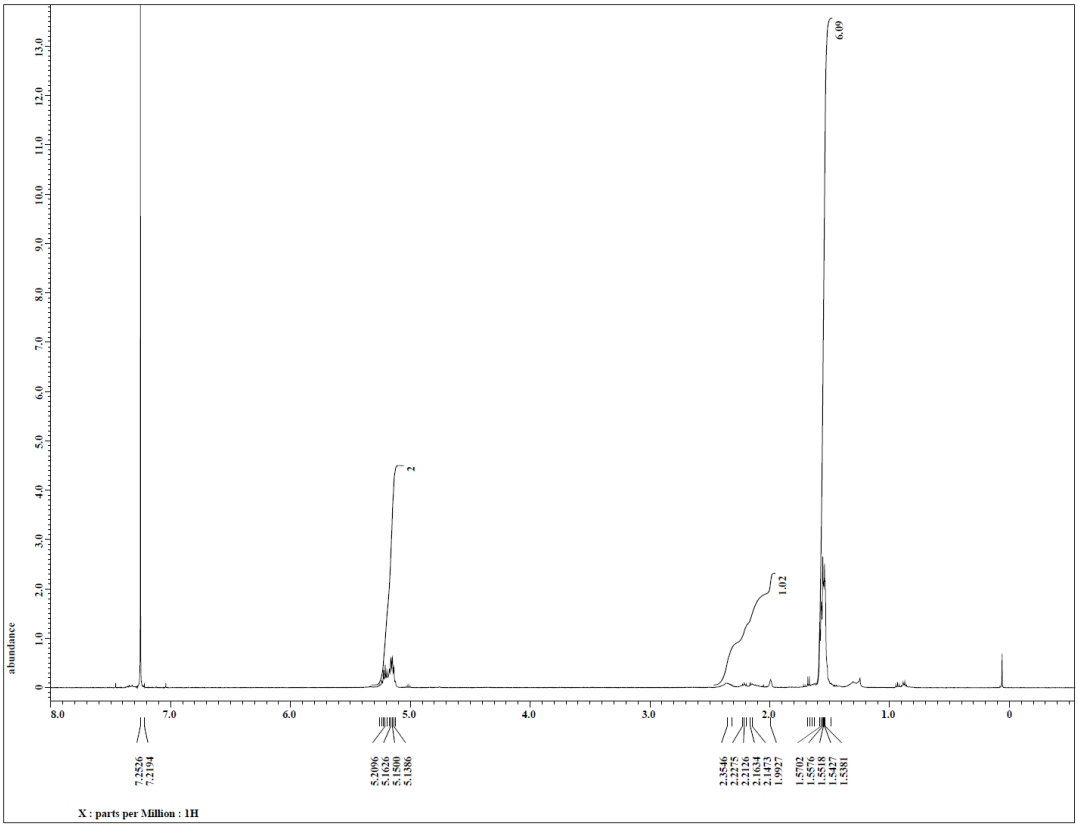


Compound 2.17

Poly-lactic acid, poly-acetylene-functionalized lactic acid copolymer (compound 2.17) was synthesized by the ring-opening polymerization of native lactide and 3-(but-3-yn-1-yl)-6-methyl-1,4-dioxane-2,5-dione (compound 2.10) in a modified version of the protocol reported by Yang and coworkers.[52] Fresh solutions of 0.20 M $Sn(Oct)_2$ and benzyl alcohol in anhydrous toluene were prepared prior to use in the polymerization. $Sn(Oct)_2$ (1.00 mL of 0.20 M solution) and benzyl alcohol (1.00 mL of 0.20 M solution) was added to a scintillation vial and the solvent was removed *in vacuo*. D,L lactide (1.15 g, 8.00 mmol) and 3-(but-3-yn-1-yl)-6-methyl-1,4-dioxane-2,5-dione (360 mg, 2.0 mmol) were added to the reaction vial. Afterwards, the vial was tightly sealed, flushed with N_2, heated to 125°C in a silicone oil bath, and then stirred. After one hour, the melted lactide and hemi-lactide became noticeably more cloudy and viscous and the stir bar gradually began to struggle to stir. After 4.5 hours, the reaction vial was allowed to cool to room temperature. A

portion of the crude was isolated for analysis by ^{1}H-NMR to measure percent conversion.

The remainder of the crude polymer was dissolved in chloroform and precipitated out in ice-cold methanol. The precipitated solid or gel was collected and immediately dried under reduced pressure. This purification process was repeated three times, and the purified, white polymer was isolated in resulted in 88% yield. Afterwards, an aliquot of the purified polymer was dissolved in DMF, filtered through a Whatman Anontop 10 filter (0.2 μm) and analyzed by gel permeation chromatography (GPC) and static light scattering (SLS). Characterization of Poly-lactic acid, poly-acetylene-functionalized lactic acid copolymer (compound 2.17): ^{1}H-NMR (CDCl3, 500 MHz) δ 5.27-5.13 (m, 2H), 1.58-1.51 (m, 6H), [(2.35, 2.21, 2.51, 1.99), 0.5H] Percent Conversion: >99%, MW: 57.6 kDa, PDI: 1.017, Percent Incorporation: 1 Propargylic Groups: 10 Methyl groups. Average number of functionalized side chains per polymeric strand: 69.



Spectrum 2.17: *^{1}H-NMR spectrum of compound 2.17*

Chapter 3

N –ethoxybenzylimidazole as an Acid-Sensitive Linker for Drug Delivery

Section 3.1 Desirable Characteristics and Previous Work in Other Acid-Sensitive Linkers

Although the advent of particulate carriers possibly resolves many of the issues that limit the efficacy of free drugs [including low solubility, poor partitioning, phagocytic plasma clearance, rapid degradation, small payload, extravasation and tissue necrosis], the implementation of carriers also introduces other variables and concerns.[1,4,9-14] In polymer-drug conjugates and drug-encapsulated liposomal systems, the drugs are [intentionally] rendered inactive and unavailable in their pro-drug form.[15] An undesirable consequence of the tempered activity of the pro-drug or sequestered drug is that the drug delivery system exhibits reduced efficacy if the active form of the drug is not released in a timely fashion.[15,16] However, when properly adjusting the rate of drug-release and activation, the therapeutic characteristics can be vastly improved over the "free" drug.[1,17]

Due to the significant role that drug activation and release plays in the efficacy of drug delivery systems, various cleavable linkers have been proposed for triggered drug release from carriers.[24,33] Some of these cleavable linkers include redox-sensitive linkers, disulfide-linkers, enzymatic-sensitive linkers, ultra-sound linkers, photodynamic linkers, and acid-sensitive linkers.[33-35] With respect to chemotherapeutic agents, acid-sensitive linkers particularly emerged as a favorable and popular choice due to the significant, compartmental changes in pH during endocytosis.[36] One of the main advantages of acid-sensitive linkers is the targeted

release or activate of the drug [or pro-drug] within the endosomes (pH 5-6), but not outside the cell (pH 7.4).[36]

In conjunction with the EPR (enhanced permeation and retention) effect where higher concentrations of macromolecules accumulate in the interstitial space of solid tumors, increased cellular uptake by various mechanisms including endocytosis have been observed.[20,21,65]. To improve the efficacy of the chemotherapeutic drug delivery system, some acid-sensitive, degradable linkers include *cis*-aconityl, hydrazone, acetal, ketal, and oxime linkers conjugated to anti-cancer agents have been developed to promote and improve drug release and escape from the acidic, endosomal compartments.[34,65-71]

Section 3.2 Previous Work on the *N*-ethoxybenzylimidazole (NEBI) Linker

Although different acid-sensitive linkers have been developed and implemented, I found the versatile *N*-ethoxybenzylimidazole (NEBI) linker, developed by Yang and coworkers, to be the most attractive because its derivatives display a wide range of half-lives.[48] Even though the *N*-ethoxybenzylimidazole (NEBI) linker demonstrates accelerated rates of degradation at lower pH [like other acid-sensitive linkers], there still exists great variability in these hydrolysis rates and half-lives between the NEBI derivatives.[48] Depending on the different substituents [and their placement] on the benzyl ring of the NEBI compound, the half-life of

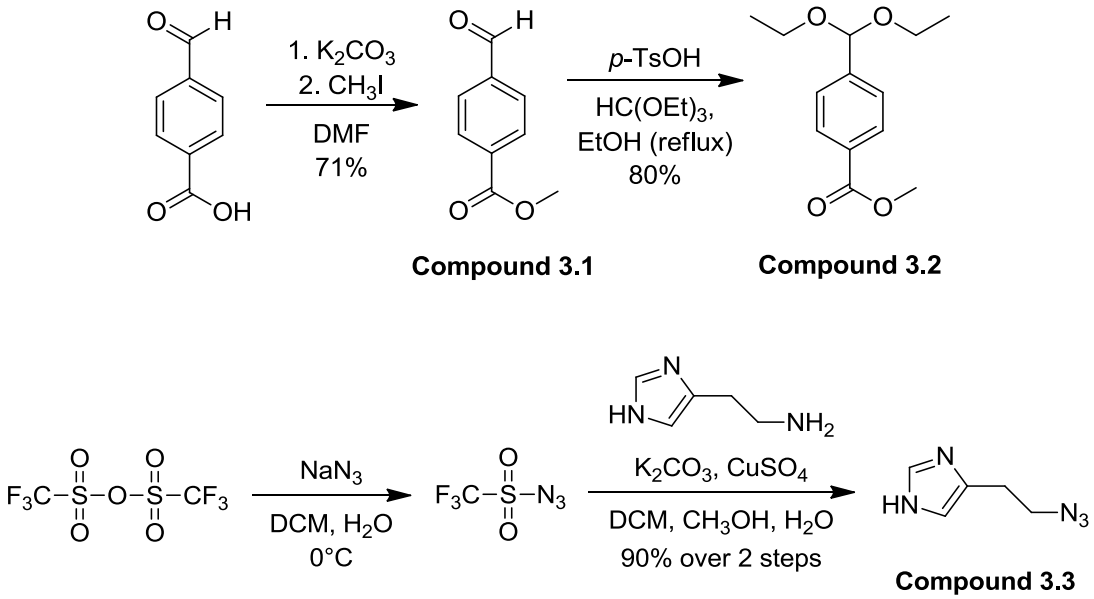
hydrolysis of the NEBI compound can vary considerably—ranging from less than an hour to thousands of hours.[48]

Despite displaying attractive variability in degradation rate between NEBI derivatives, Yang and coworkers still show that the hydrolysis rate of the basic NEBI compound [with only hydrogens on the benzyl ring] is robust to buffer concentration, multiple Lewis acids and fetal bovine serum.[48,49] After covalently attaching doxorubicin to the NEBI linker to create the pro-drug conjugate, the NEBI conjugate retained its acid-promoted hydrolytic properties.[49] Furthermore, the doxorubicin analog that is released from the NEBI linker upon hydrolysis still demonstrate cytotoxic properties.[49]

Luong *et al* reported the development of a drug delivery system of the NEBI-doxorubicin, pro-drug conjugate covalently linked to a human serum albumin (HSA) carrier.[49] They found that the complete drug delivery system with the carrier exhibited increased cytotoxicity relative to the released form of the drug, the doxorubicin analog.[49] The increased cytotoxicity could be related to the observed, increased cellular uptake (~5.5 folds) of the complete drug delivery system relative to the doxorubicin analog.[49] As a whole, Yang and coworkers illustrated that the *N*-ethoxybenzylimidazole (NEBI) linker can serve as a viable linker in the delivery of chemotherapeutic agents with perhaps the possibility of further optimization and specificity in the future.

Section 3.3 Synthesis of the Basic N-ethoxybenzylimidazole (NEBI) Linker

In the development of a model drug delivery system (DDS) of doxorubicin with a cleavable, NEBI linker, I chose to synthesize a bi-functional, NEBI linker with both an azide and carboxylic acid moiety (compound 3.5) from a modify procedure of those reported by Yang and coworkers.[48,49] The azide moiety allows for the covalent attachment to the carrier via copper-catalyzed azide-alkyne cycloaddition (CuAAC) reaction, while the carboxylic acid functionality allows for the attachment of the drug via an amide bond with doxorubicin. One of the advantages of selecting these functional groups involves the orthogonal nature of their chemistry relative to each other.

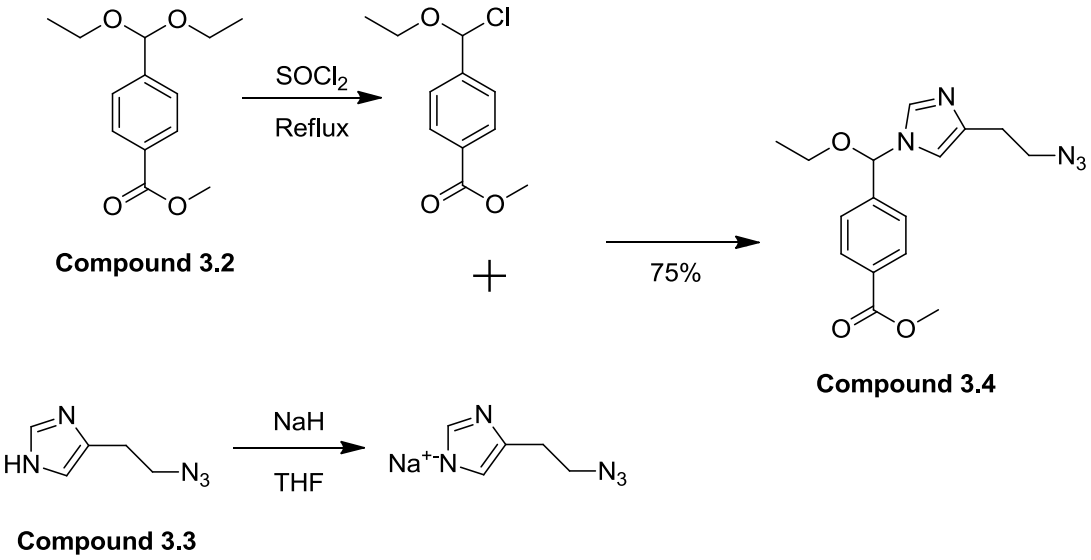


Synthetic Scheme 3.1: *The synthetic schematic for the synthetic precursors of the NEBI linker, compound 3.2 and compound 3.3 from commercially-available starting material.*

In the synthesis of the NEBI linker, two important precursors need to first be synthesized—the benzyl acetal (compound 3.2) and the azido-functionalized imidazole (histazide, compound 3.3). Commercially-available 4-formylbenzoic acid was reacted with iodomethane under basic conditions to esterify and protect the carboxylic acid group—producing methyl 4-formylbenzoate (compound 3.1). Refluxing methyl 4-formylbenzoate (compound 3.1) with anhydrous ethanol and triethyl orthoformate under acidic conditions produced the desired ethyl acetal (compound 3.3).

The azido-functionalized imidazole precursor, 4-(2-azidoethyl)-1H-imidazole (histazide, compound 3.3) was synthesized by a diazotransfer onto commercially-

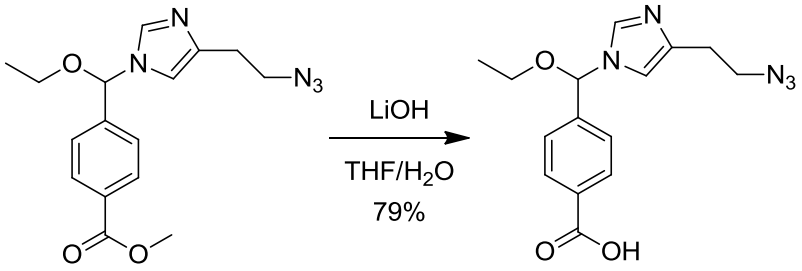
available histamine.[72] The diazotransfer agent, trifluoromethanesulfonyl azide or triflyl azide, was created *in situ* by reacting trifluoromethanesulfonic anhydride or triflyl anhydride with sodium azide. It was used immediately to convert the amine on histamine to an azide in good yield.



Synthetic Scheme 3.2: *The synthetic schematic for the ester-protected NEBI linker from the coupling of the synthetic precursors, compound 3.2 and compound 3.3.*

Once the benzyl acetal precursor (compound 3.2) and the azido-functionalized imidazole precursor (compound 3.3) are synthesized, a methyl ester derivative of the NEBI linker (compound 3.4) can be created from a nucleophilic substitution reaction between the two precursors. Both precursors need to first be activated prior to the nucleophilic substitution. The benzyl acetal (compound 3.2) is activated by thionyl chloride to produce methyl 4-(chloro(ethoxy)methyl)benzoate, making it more susceptible to nucleophiles, while the azido-functionalized imidazole

(compound 3.3) is deprotonated by sodium hydride to make it a better nucleophile. The activated forms of compound 3.2 and compound 3.3 are immediately reacted with each other to produce methyl 4-((4-(2-azidoethyl)-1H-imidazol-1-yl)(ethoxy)methyl)benzoate (compound 3.4) in good yield.



Compound 3.4 **Compound 3.5**

Synthetic Scheme 3.3: *The synthetic schematic for the bi-functional NEBI linker (compound 3.5) from the deprotection of the ester moiety of compound 3.4.*

The complete bi-functional (azide and carboxylic acid) NEBI linker (compound 3.5) is synthesized from the saponification of the methyl ester precursor (compound 3.4) with lithium hydroxide. Afterwards, both functional groups on the NEBI linker are now available for further modification. The goal is to link doxorubicin (the drug) and a modified poly-lactic acid polymer with alkyne side-chains (the carrier) with this cleavable linker.

An interesting observation made when attempting to characterize both the protected and deprotected NEBI linkers (compound 3.4 and 3.5) provided further insight into the mechanism of hydrolysis for the NEBI compounds. Previously, Yang and coworkers proposed that the rate determining step of the hydrolysis is the

heterolytic cleavage of the carbon-nitrogen bond at the benzyl position of the NEBI linker.[48] Kinetic studies of different NEBI linkers suggest that the degradation is first-order with respect to the concentration of the linker and zero-order with respect to the concentration of the buffer agent, supporting the proposed mechanism.[48] To the best of my knowledge, there previously has not been spectrometric evidence presented for the proposed intermediate. However, when characterizing compound 3.4 and compound 3.5 under electrospray ionization (ESI)-positive mode for mass spectrometry, I found spectrometric evidence (Figure 3.1) for the presence of the proposed, charged, resonantly stabilized intermediate. It provides further credence to the mechanism proposed by Yang and coworkers.

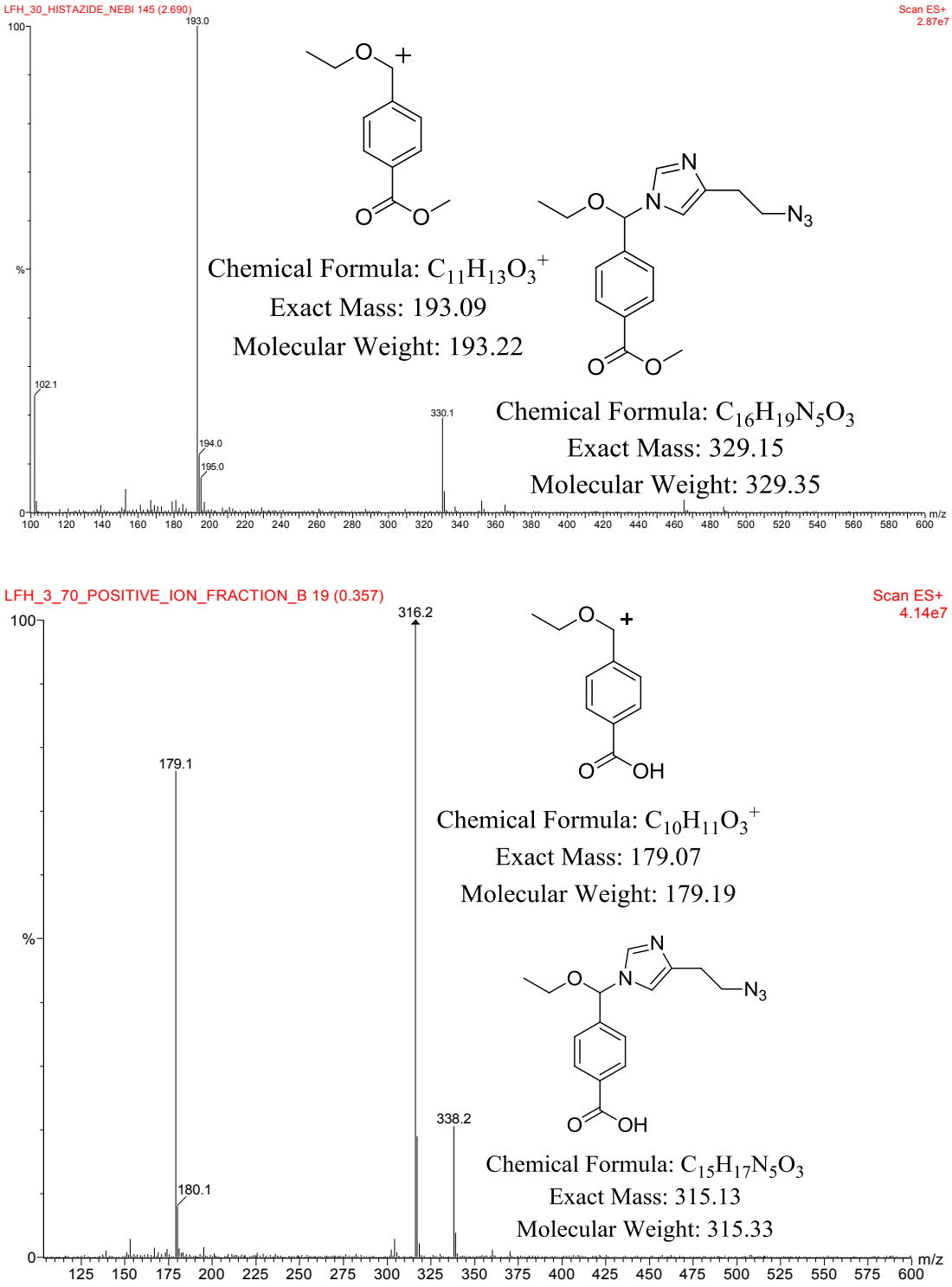
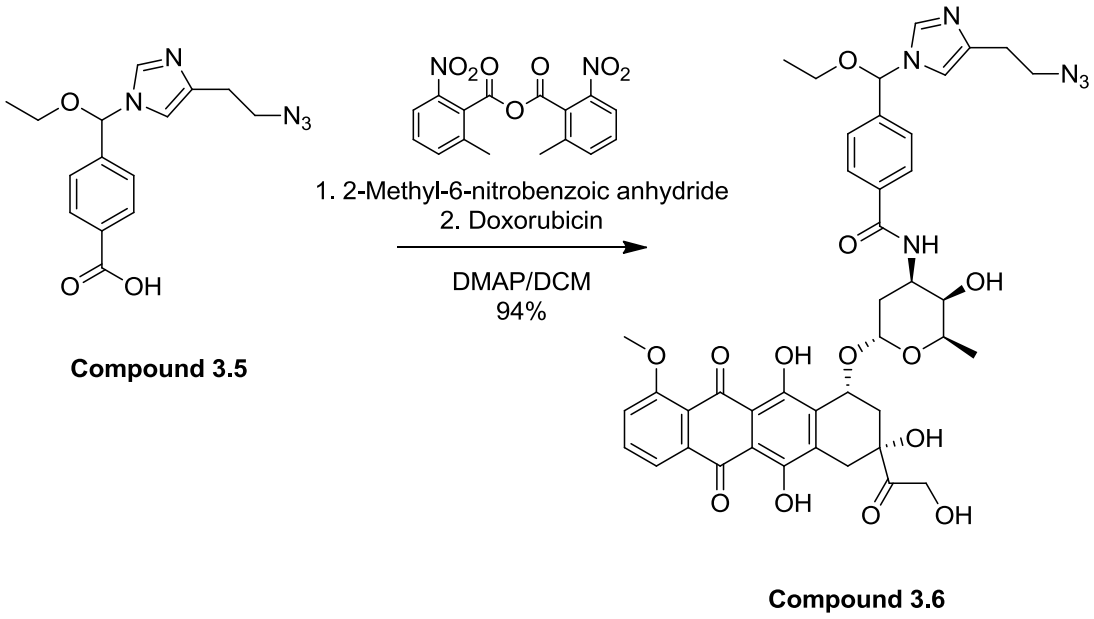


Figure 3.1: *Spectrometric evidence of the heterolytic cleavage of two NEBI molecules (compound 3.4 and compound 3.5).*

Section 3.4 Conjugation of Doxorubicin to a *N*-ethoxybenzylimidazole (NEBI) Bi-functional Linker to Synthesis a Pro-Drug of Doxorubicin with a Functional Molecular Handle

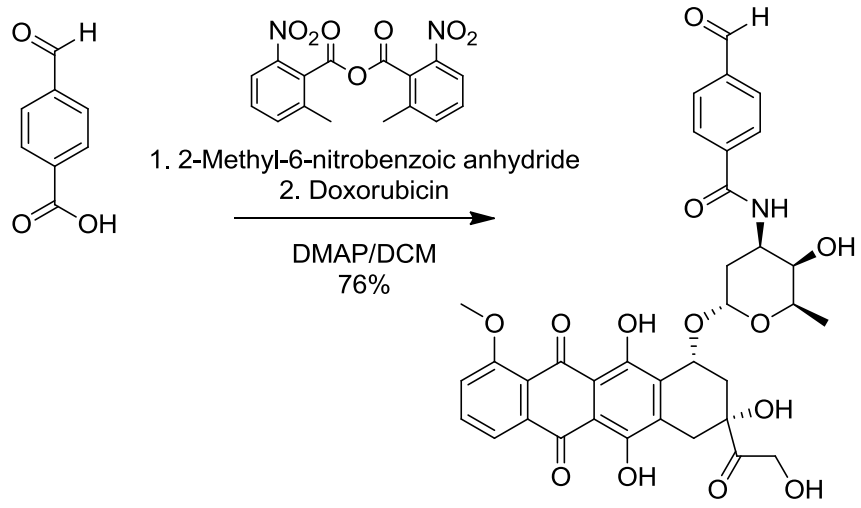
After synthesizing the active bi-functional NEBI linker (compound 3.5), a drug can then be conjugated to the NEBI linker to produce the corresponding pro-drug. Doxorubicin can be covalently attached to the linker via an amide bond. The carboxylic acid of the NEBI linker (compound 3.5) was activated with 2-methyl-6-nitrobenzoic anhydride (MNBA) and subsequently treated with doxorubicin to produce the functionalized NEBI-doxorubicin pro-drug in good yield (compound 3.6). Additionally, doxorubicin was conjugated to the carbonyl of the NEBI linker (compound 3.5) instead of the azido-moiety because of the relative ease of the chemistry and reaction.



Compound 3.5

Compound 3.6

Synthetic Scheme 3.4: *The synthetic schematic for the pro-drug, compound 3.6 (NEBI-doxorubicin conjugation) with a functionalized azido-molecular handle.*



Compound 3.7

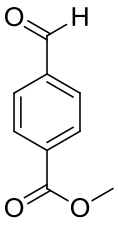
Synthetic Scheme 3.5: *The synthetic schematic for the active cytotoxic agent of the drug delivery system, compound 3.7 (doxorubicin-benzaldehyde analog).*

As control molecule, the active form of the drug (compound 3.7) after hydrolysis of the NEBI linker was also synthesized in a similar fashion. The carboxylic acid group of 4-formylbenzoic acid was activated for amide bond formation by 2-methyl-6-nitrobenzoic anhydride (MNBA) and subsequently treated with doxorubicin to produce the active doxorubicin analog in good yield. Furthermore, Yang and coworkers previously demonstrated that the active form of the drug (compound 3.7) retains cytotoxicity against cancer cells, making it a logical choice as a control molecule for this work.[48,49]

Section 3.5 Experimentals

All reagents were purchased from Acros Organic, Spectrum Chemicals, Sigma-Aldrich, TCI America, or Alfa Aesar and used without further additional purification unless specified. All solvents used for reactions were dried on alumina columns and distilled prior to use. Solvents used for chromatography were ACS technical grade and used without further purification. Distilled water (18.2 μΩ/cm) was filtered through a NANOPure Diamond^{TM} (Barnstead) water purification system before use in synthesis or purification. NMR spectra were recorded on a Varian 400 (FT, 400 MHz, ^{1}H; 100 MHz, ^{13}C) spectrometer and a JEOL ECA-500 (FT, 500 MHZ ^{1}H) spectrometer. HR-MS (high-resolution mass spectra) were obtained in the Department of Chemistry & Biochemistry, University of California, San Diego (UCSD) using a ThermoFinnigan MAT900XL-MS. ESI-MS (electrospray ionization mass spectra) were obtained using ThemoFinnigan LCQDECA-MS.

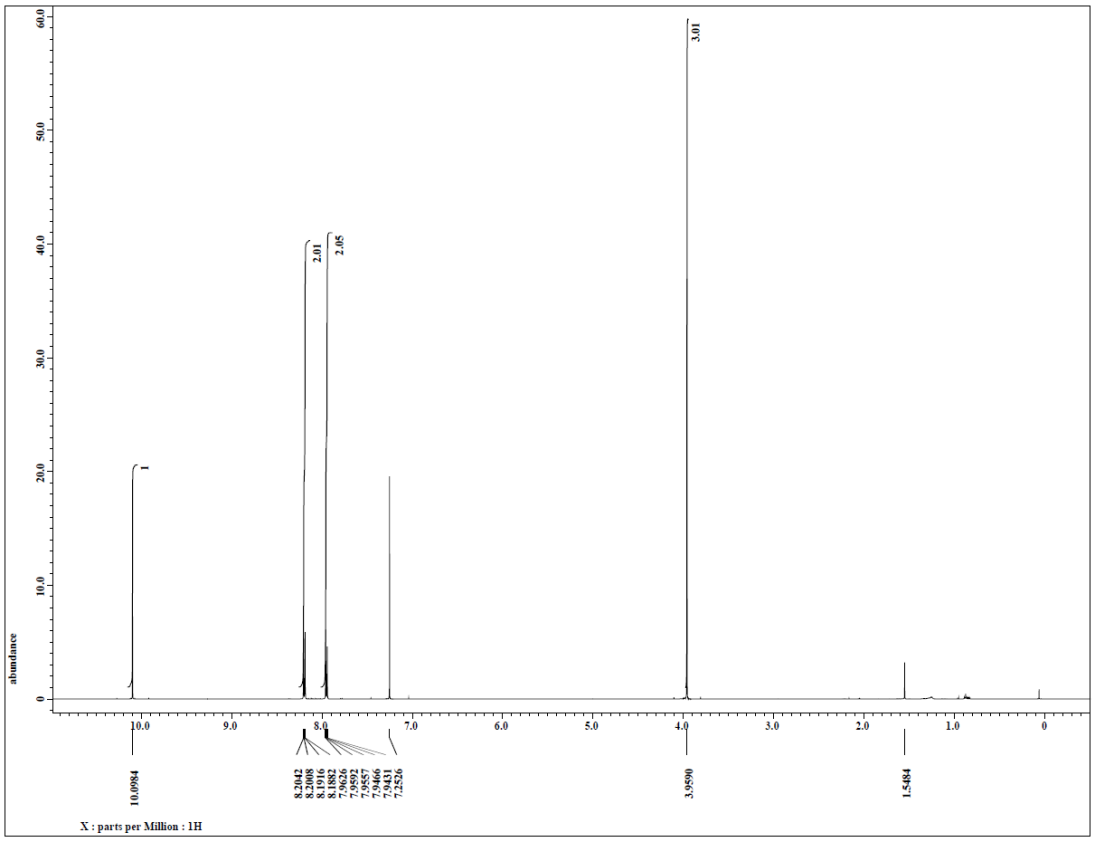
Synthesis of methyl 4-formylbenzoate (compound 3.1)



Compound 3.1

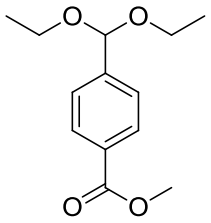
4-formylbenzoic acid (12.0 g, 79.9 mmol) and methyl iodide (21.29g, 9.34 mL, 150. mmol), and potassium carbonate (6.22 g, 45.0 mmol) were dissolved in

DMF (30. mL) and stirred overnight. The solvent was then removed under reduced pressure. The oil was then dissolved in distilled H_2O (300. mL). The aqueous solution was extracted with DCM (4 x 75 ml). The organic layers were pooled and washed with saturated sodium chloride (75 ml). The combined organic layers were then dried over sodium sulfate, and the solvent was removed under reduced pressure. The white solids were recrystallized in Et_2O to form white, needle crystals in 71% yield. Characterization of methyl 4-formylbenzoate (compound 3.1): ^{1}H-NMR ($CDCl_3$, 500 MHz) δ 3.9590 (s, 3H), 7.9557 (d, 2H), 8.1962 (d, 2H) 10.0984 (s, 1H)



Spectrum 3.1: *^{1}H-NMR spectrum of compound 3.1*

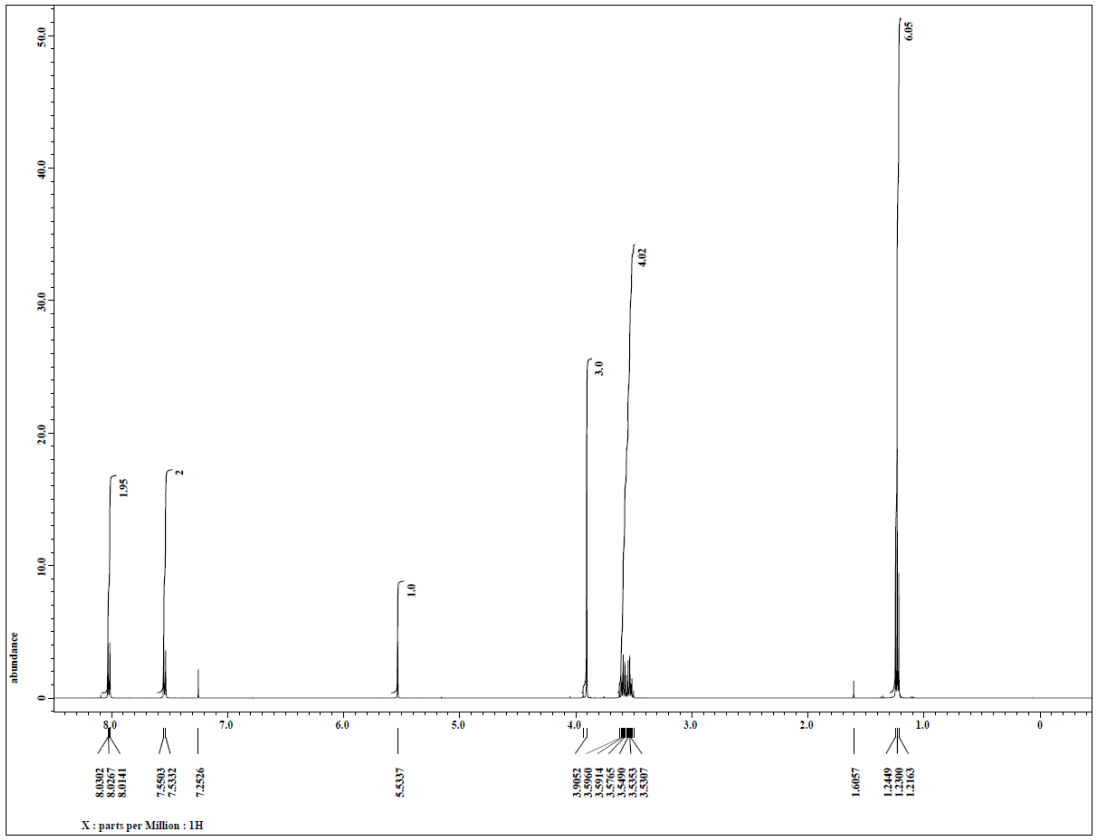
Synthesis of methyl 4-(diethoxymethyl)benzoate (compound 3.2)[49]



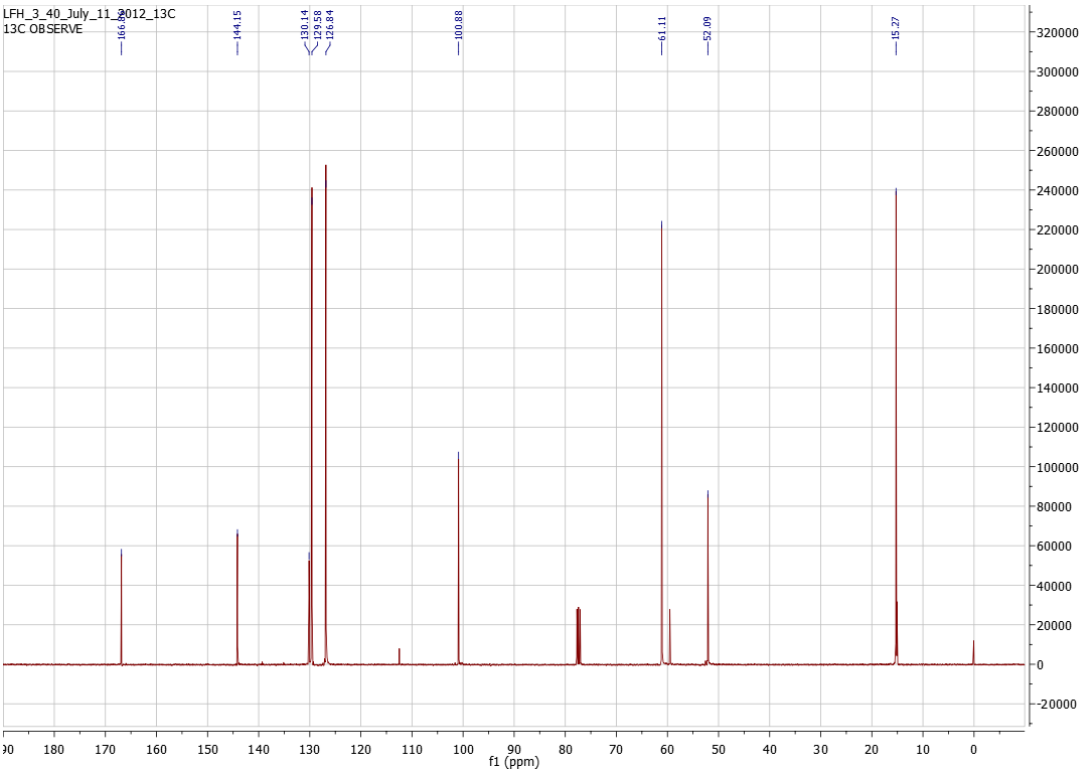
Compound 3.2

Methyl 4-(diethoxymethyl)benzoate (compound 3.2) was prepared by the addition of ethanol of methyl 4-formylbenzoate in the presence of triethyl orthoformate and catalytic amounts of *p*-toluenesulfonic acid (*p*-TsOH). Methyl 4-formylbenzoate (1.2 g, 7.3 mmol), triethyl orthoformate (3.25 g, 3.65 mL, 21.9 mmol), and *p*-toluenesulfonic acid (1.4 mg, 7.3 μmol) were dissolved in distilled ethanol (7.0 mL, 190 mmol). The solution was heated to reflux under N_2 for 24 hours. After removal of ethanol under reduced pressure, the oil was dissolved in DCM (50 mL). The organic layer was washed with saturated sodium bicarbonate solution (3x 10 mL) and saturated NaCl (1x 10 mL). The organic layer was dried over anhydrous sodium sulfate. TLC analysis shows the disappearance of the starting material and the presence of a new spot in the following solvent system: 4 EtOAc: 1 Hexanes. The oil was further purified by column chromatography in the same solvent system and then isolated in 80% yield. Characterization of methyl 4-(diethoxymethyl)benzoate (compound 3.2): ^{1}H-NMR ($CDCl_3$, 500 MHz) δ 1.23000 (t, 6H), 5.3765 (m, 4H), 5.5337 (s, 1H) 7.5418 (d, 2H), 8.0267 (d, 2H) ^{13}C-NMR

($CDCl_3$, 500 MHz) δ 166.86, 144.15, 130.14, 129.58, 126.84, 100.88, 61.11, 52.09, 15.27.

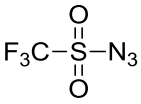


Spectrum 3.2.1: *^{1}H-NMR spectrum of compound 3.2*



Spectrum 3.2.2: *^{13}C-NMR spectrum of compound 3.2*

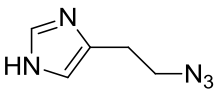
Synthesis trifluoromethanesulfonyl azide (trifyl azide)



Trifluoromethanesulfonyl azide (triflyl azide) was prepared *in situ* following a modified version of the procedure reported by Pelletier and coworkers.[72] Considering the possible explosiveness and toxicity of a large scale reaction with sodium azide, both a large blast shield and a respirator mask were employed in this synthesis as precaution. A solution of sodium azide (57.0 g, 880 mmol) was dissolved in distilled H_2O (144. mL) and dichloromethane (240. mL) and then cooled

to 0°C using an ice bath. Trifluoromethanesulfonic anhydride (29.6 ml, 164.8 mmol) was added dropwise over 10 minutes with vigorous stirring. After the addition of trifluoromethanesulfonic anhydride, the mixture received an additional two hours of stirring to ensure the completion of the reaction. The mixture was placed in a separatory funnel and the organic phase was removed. The aqueous layer was extracted with DCM (3 x 50 mL). The organic fractions containing trifluoromethanesulfonyl azide were pooled and washed once with saturated sodium carbonate (100 mL). The washed organic layer containing trifluoromethanesulfonyl azide was used without further purification.

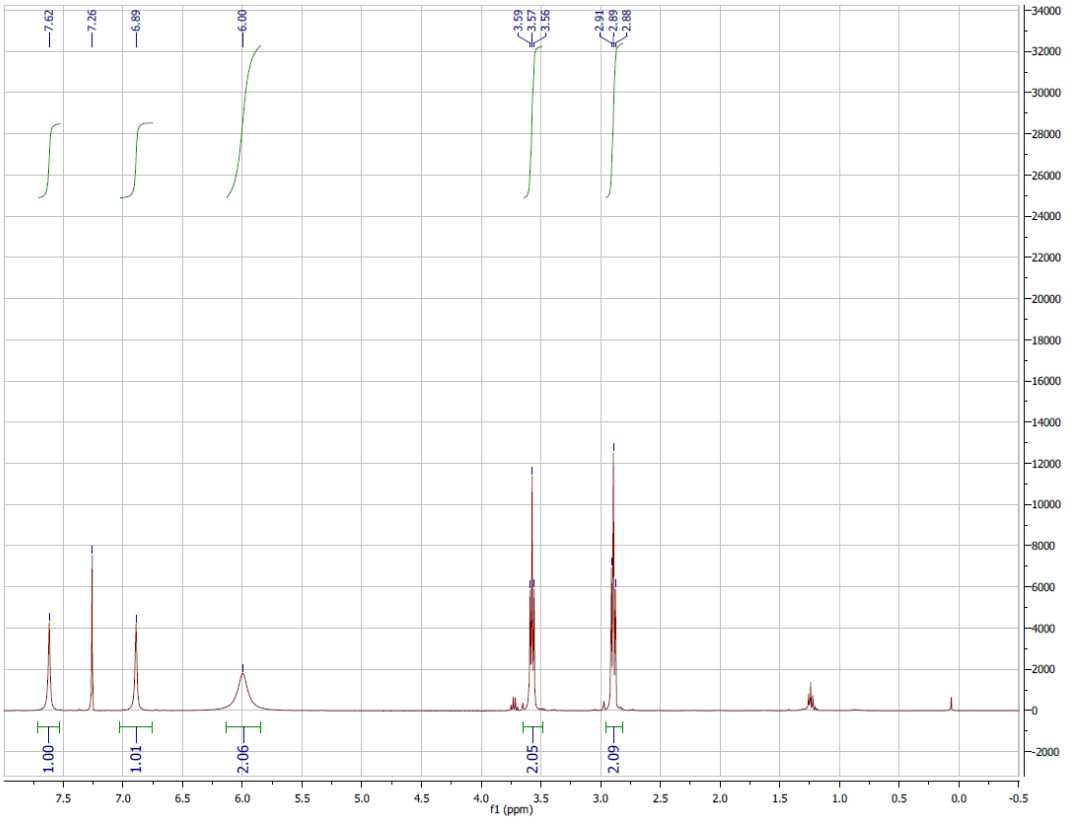
Synthesis of 4-(2-azidoethyl)-1H-imidazole (histazide, compound 3.3)[49]



Compound 3.3

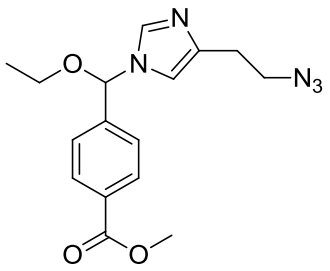
4-(2-azidoethyl)-1H-imidazole (histazide, compound 3.3) was prepared from commercially-available histamine•2HCl and triflyl azide prepared *in situ*. Histamine•2HCl (16.0 g, 89.6 mmol), potassium carbonate (18.5 g, 133.6 mmol), and $CuSO_4$•2 H_2O (230. mg, 921 μmol) were dissolved in a solution of distilled H_2O (60. mL) and (240. mL) of methanol. The pH of the solution was adjusted to 8.6 to ensure basicity. Prepared trifluoromethanesulfonyl azide in DCM (~350. mL) was added and the mixture was stirred in an opened container and at room temperature overnight. The aqueous layer was extracted with EtOAc (3 x 300. mL). The organic layers were pooled, filtered over Celite, and dried over sodium sulfate. The solvent

was then removed under reduced pressure. Histazide (compound 3.3) was isolated by column chromatography using a 96: 4: 2 eluent mixture of DCM: MeOH: Et_3N. The corresponding band was yellow and UV-active. The purified oil of histazide was isolated [along with residual triethylamine] in 80% yield. Characterization of 4-(2-azidoethyl)-1H-imidazole (histazide, compound 3.3): ^{1}H-NMR (CDCl3, 400 MHz) δ 2.89 (t, 2H), 3.57 (t, 2H), 6.00 (s, 2H), 6.89 (s, 1H), 7.62 (s, 1H). ESI-MS (*m/z*, $M+H^+$): 137.99



Spectrum 3.3: *^{1}H-NMR spectrum of compound 3.3*

Synthesis of methyl 4-((4-(2-azidoethyl)-1H-imidazol-1-yl)(ethoxy)methyl)benzoate (compound 3.4)[49]



Compound 3.4

Methyl 4-((4-(2-azidoethyl)-1H-imidazol-1-yl)(ethoxy)methyl)benzoate (compound 3.4) was synthesized from reacting methyl 4-(diethoxymethyl)benzoate (compound 3.2) with 4-(2-azidoethyl)-1H-imidazole (histazide, compound 3.3) in a nucleophilic substitution reaction. Methyl 4-(diethoxymethyl)benzoate with 4-(2-azidoethyl)-1H-imidazole needed to first be activated to become methyl 4-(chloro(ethoxy)methyl)benzoate for the reaction to occur. 4-(2-azidoethyl)-1H-imidazole also needed to first be deprotonated into its sodium salt by sodium hydride for the reaction to proceed.

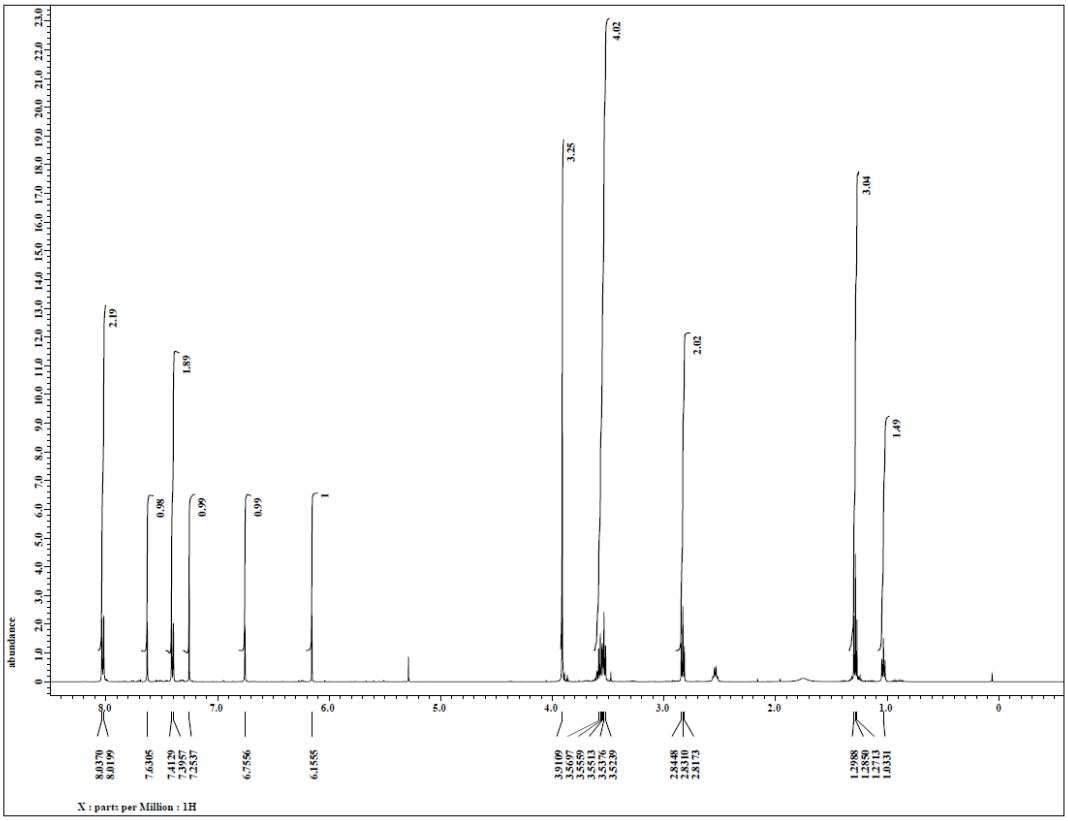
Methyl 4-(diethoxymethyl)benzoate was first converted to methyl 4-(chloro(ethoxy)methyl)benzoate using thionyl chloride. Methyl 4-(diethoxymethyl)benzoate (4.06 g, 17.1 mmol) and thionyl chloride (1.627 ml, 22.4 mmol) were added neatly to a dry reaction flask. The solution was heated to reflux under N_2. The solution gradually turned more yellow in color. After 1 hour, excess thionyl chloride was removed under reduced pressure and the resulting yellow oil

methyl 4-(chloro(ethoxy)methyl)benzoate intermediate was used without further purification.

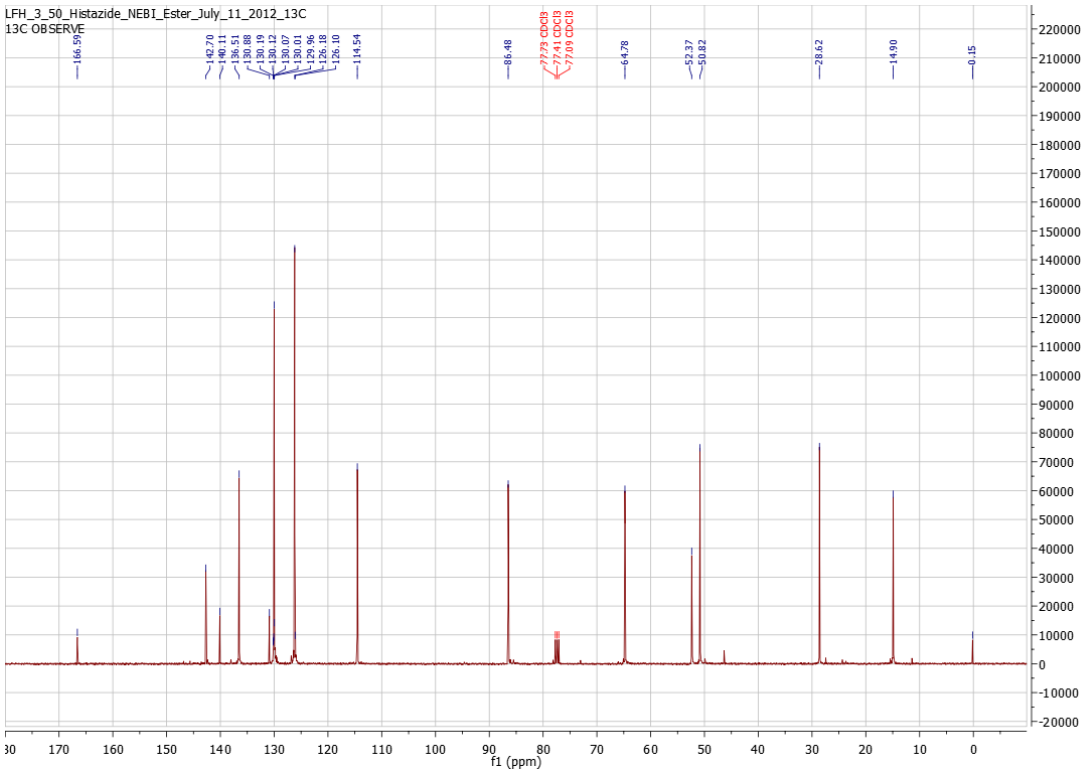
In a separate reaction flask, 4-(2-azidoethyl)-1H-imidazole (1.73 g, 12.6 mmol) was dissolved in dry, distilled THF (100. mL). Sodium hydride (605 mg, 25.2 mmol) was added to the THF solution containing histazide. The evolution of gas (presumably H_2) was immediately observed. The resulting mixture was stirred at room temperature for 1 hour.

The methyl 4-(chloro(ethoxy)methyl)benzoate intermediate was then dissolved in dry, distilled THF (100. mL) and then added dropwise (100 mL in ~10 minutes) to the reaction flask with the deprotonated 4-(2-azidoethyl)-1H-imidazole solution. The overall reaction flask was flushed with N_2 and the content was allowed to stir overnight. Afterwards, the solvent was removed under reduced pressure, and the resulting oil was dissolved in diethyl ether (100 mL). The crude product dissolved in diethyl ether was washed with saturated sodium carbonate (4x 50 mL) and saturated NaCl (1x 50 mL). The aqueous layers were pooled and back-extracted with diethyl ether (1x 50 mL). The combined organic layer was dried over Na_2SO_4, and the solvent was removed under reduced pressure. The resulting oil was purified by column chromatography using the following solvent system: 95 DCM: 3 MeOH: 2 Et_3N (R_f = 0.29). The produce was isolated in 75% yield. Characterization of methyl 4-((4-(2-azidoethyl)-1H-imidazol-1-yl)(ethoxy)methyl)benzoate (compound 3.4): ^{1}H-NMR (CDCl3, 500 MHz) δ 8.0285 (d, 2H), 7.6305 (s, 1H), 7.4043 (d, 2H), 6.7556 (s, 1H), 6.1555 (s, 1H), 3.9109 (s, 3H), 3.5697-3.5239 (m, 4H) 2.8310 (t,

2H), 1.2850 (t, 3H). ^{13}C-NMR (CDCl3, 500 MHz) δ 166.59, 142.70, 140.11, 136.51, 130.88, 130.12, 126.18, 114.54, 86.48, 64.78, 52.37, 50.82, 28.62, 14.90. ESI-MS (*m/z*, $M+H^+$): 330.1

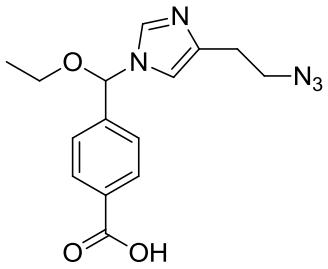


Spectrum 3.4.1: *^{1}H-NMR spectrum of compound 3.4*



Spectrum 3.4.2: *^{13}C-NMR spectrum of compound 3.4*

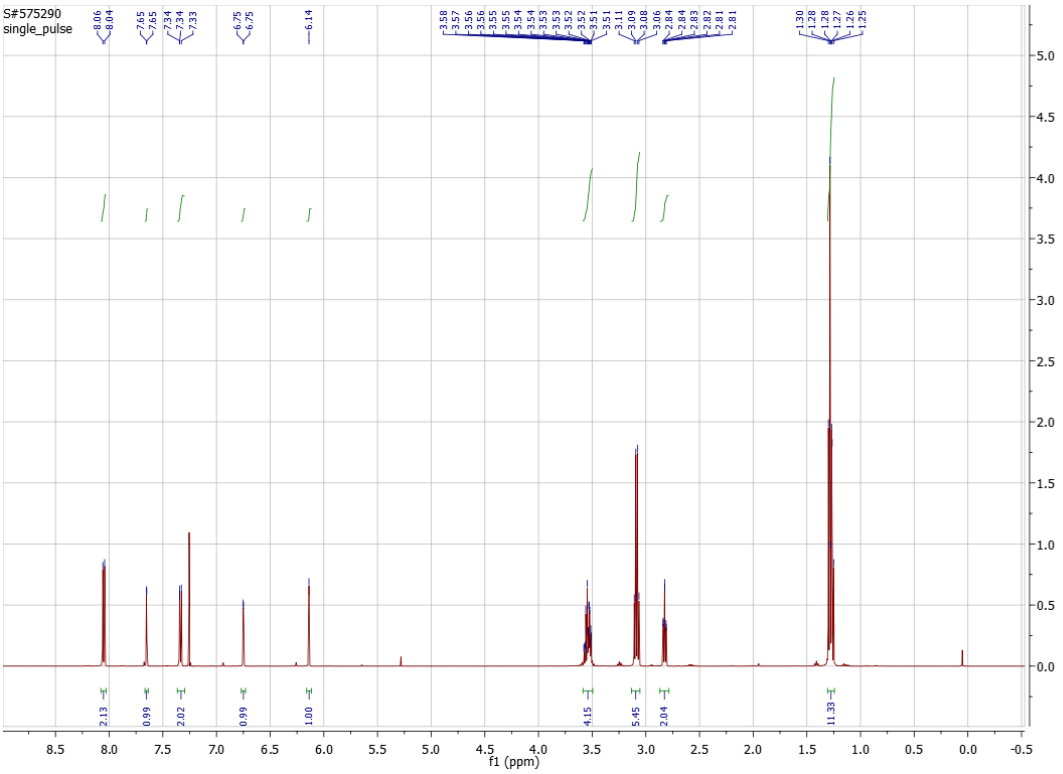
Synthesis of 4-((4-(2-azidoethyl)-1H-imidazol-1-yl)(ethoxy)methyl)benzoic acid (compound 3.5)



Compound 3.5

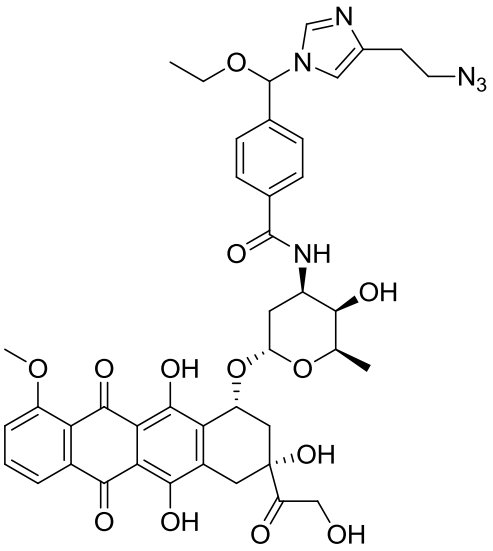
4-((4-(2-azidoethyl)-1H-imidazol-1-yl)(ethoxy)methyl)benzoic acid (compound 3.5) was synthesized from the saponification of methyl 4-((4-(2-

azidoethyl)-1H-imidazol-1-yl)(ethoxy)methyl)benzoate (compound 3.4) using lithium hydroxide. Methyl 4-((4-(2-azidoethyl)-1H-imidazol-1-yl)(ethoxy)methyl)benzoate (0.200 g, 0.608 mmol) was dissolved in dry THF (20 mL). Afterwards, an aliquot of 2.0 M LiOH (1.3 mL, 2.6 mmol) was added to the reaction solution. Distilled H_2O is added dropwise to the reaction mixture until all LiOH is dispersed in a colloid. The reaction solution was stirred overnight, and the reaction was followed by TLC analysis in the following solvent system: 95 DCM: 3 MeOH: 2 Et_3N. Afterwards, the solvent was removed under reduced pressure and the residue was purified by a silica plug under the following gradient solvent system: 95 DCM: 3 ACN: 2 Et_3N to 80 DCM: 18 ACN: 2 Et_3N. The product was isolated [along with triethylamine] in 79% yield. Characterization of 4-((4-(2-azidoethyl)-1H-imidazol-1-yl)(ethoxy)methyl)benzoic acid (compound 3.5): ^{1}H-NMR (CDCl3, 500 MHz) δ 8.05 (d, 2H), 7.65 (s, 1H), 7.34 (d, 2H), 6.75 (s, 1H), 6.14 (s, 1H), 3.58-3.51 (m, 4H) 2.83 (t, 2H), 1.28 (t, 3H). ESI-MS (*m/z*, $M+H^+$): 316.2, (*m/z*, $M+Na^+$): 338.2, (*m/z*, M-H): 314.1.



Spectrum 3.5: *^{1}H-NMR spectrum of compound 3.5*

Synthesis of 4-((4-(2-azidoethyl)-1H-imidazol-1-yl)(ethoxy)methyl)-N-((2R,3R,4R,6S)-3-hydroxy-2-methyl-6-(((1R,3R)-3,5,12-trihydroxy-3-(2-hydroxyacetyl)-10-methoxy-6,11-dioxo-1,2,3,4,6,11-hexahydrotetracen-1-yl)oxy)tetrahydro-2H-pyran-4-yl)benzamide (NEBI-doxorubicin conjugate, compound 3.6)

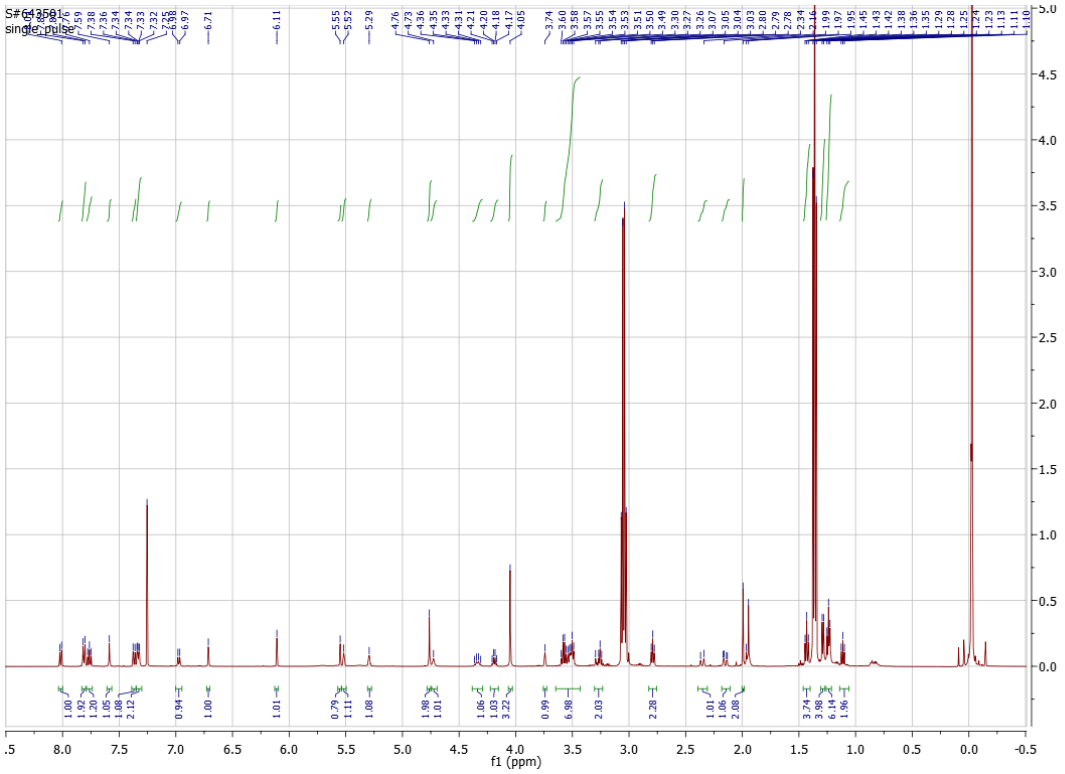


Compound 3.6

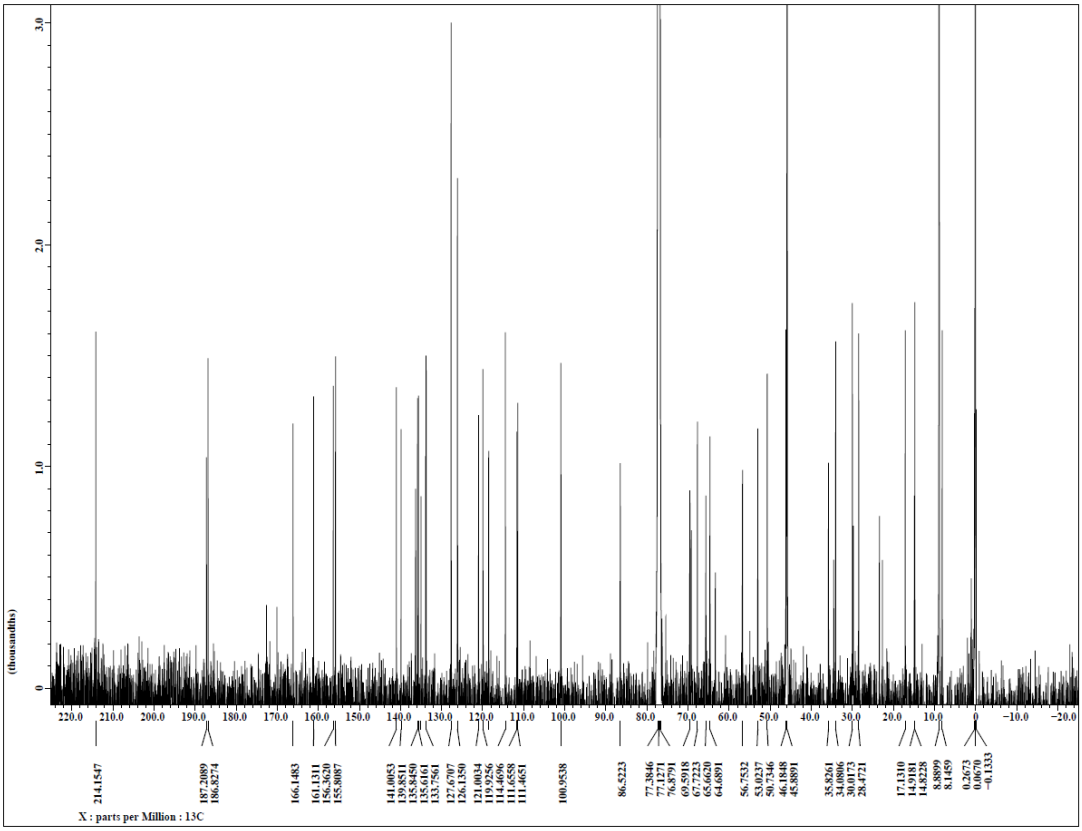
The NEBI-doxorubicin conjugate (compound 3.6) was synthesized by the covalent attachment of doxorubicin to the bi-functional NEBI linker (compound 3.5) via an amide bond [after activation]. 4-((4-(2-azidoethyl)-1H-imidazol-1-yl)(ethoxy)methyl)benzoic acid (16.2 mg, 38.9 μmol) was dissolved in distilled DMF (8.5 mL). 2-methyl-6-nitrobenzoic anhydride (13.37 mg, 38.9 μmol) and 4-dimethylaminopyridine (0.40 mg, 3.2 μmol) was then added to the reaction vial. Triethylamine (20. μL, 140 μmol) was then added to the reaction mixture, followed

by the addition of doxorubicin•HCl (19 mg, 33 μmol). Afterwards, the reaction vial was flushed with N_2 and the content was allowed to stir overnight.

Afterwards, the solvent was evaporated under reduced pressure. The residue was then purified by column chromatography in the following solvent system: 96 DCM: 2 MeOH: 2 Et_3N. The product was isolated [along with triethylamine] in 94% yield. Characterization of NEBI-doxorubicin conjugate (compound 3.6): ^{1}H-NMR: δ 8.02 (d, 1H), 7.81 (d, 1 H), 7.76 (t, 1H), 7.59 (s, 1H), 7.37 (d, 1H), 7.33 (dd, 2H), 6.98 (d, 1H), 6.71 (s, 1H), 6.11 (s, 1H), 5.55 (s, 1H), 5.52 (s, 1H), 5.29 (s, 1H), 4.76 (1s, 2H), 4.73 (s, 1H), 4.34 (q, 1H), 4.19 (q, 1H), 4.05 (s, 3H), 3.74 (s, 1H), 3.60-3.49 (m, 7H), 2.79 (t, 2H), 2.34 (d, 1H), 2.15 (dd, 1H), 1.99 (s, 2H), 1.43 (t, 4) 1.29 (d, 4) 1.24 (m, 6H) 1.11 (t, 2H). ^{13}C-NMR: δ 214.1547, 187.2089, 186.8274, 166.1483, 161.1311, 156.3620, 155.8087, 141.0053, 139.8511, 135.8450, 135.6161, 135.0057, 133.8801, 133.7561, 127.6707, 126.1350, 121.0034, 119.9256, 118.5234, 114.4696, 111.6558, 111.4651, 100.9538, 86.5223, 69.5918, 67.7223, 65.6620, 64.6891, 56.7532, 53.0237, 50.7346, 46.1848 , 35.8261, 34.0806, 30.0173, 28.4721, 17.1310, 14.9181, 14.8228, 8.1459. ESI-MS (*m/z*, $M+H^+$): 841.3

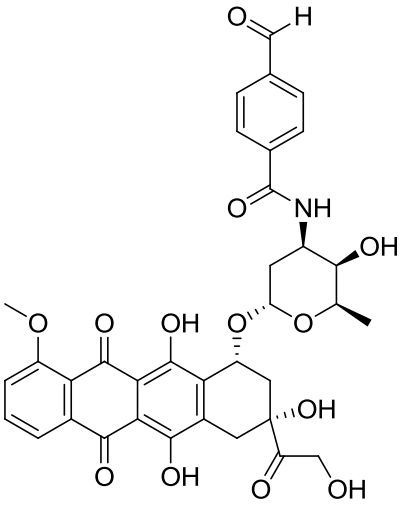


Spectrum 3.6.1: *^{1}H-NMR spectrum of compound 3.6*



Spectrum 3.6.2: *^{13}C-NMR spectrum of compound 3.6*

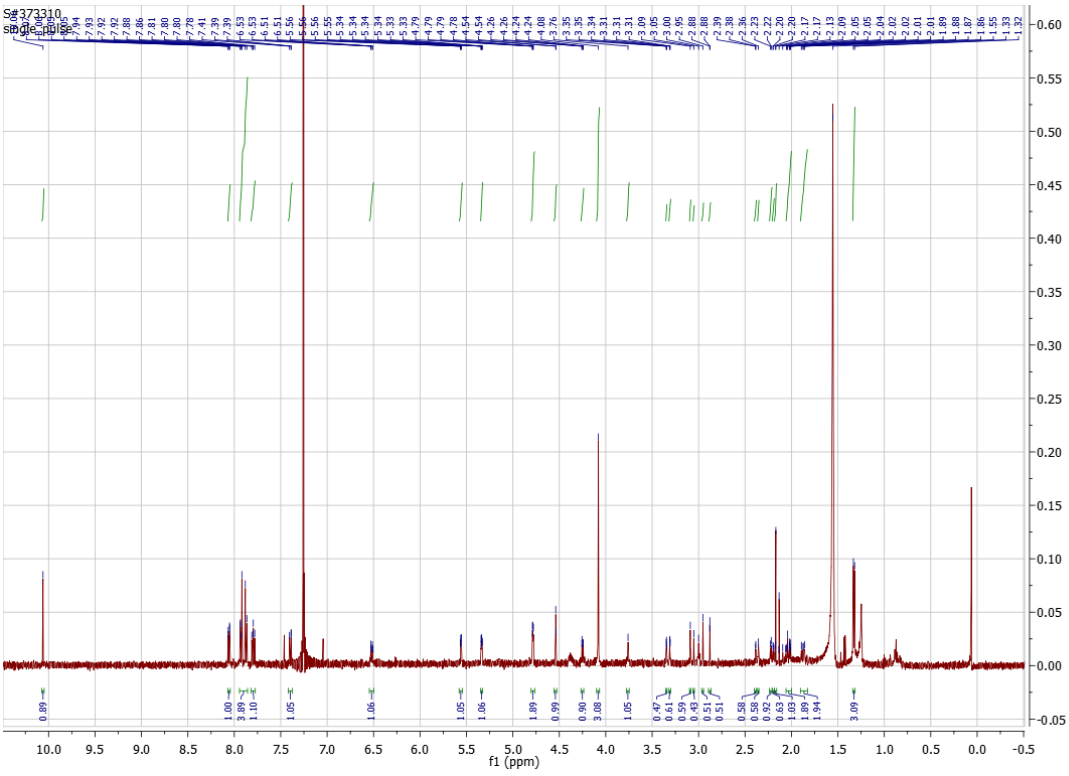
Synthesis of 4-formyl-N-((2R,3R,4R,6S)-3-hydroxy-2-methyl-6-(((1R,3R)-3,5,12-trihydroxy-3-(2-hydroxyacetyl)-10-methoxy-6,11-dioxo-1,2,3,4,6,11-hexahydrotetracen-1-yl)oxy)tetrahydro-2H-pyran-4-yl)benzamide (compound 3.7)



Compound 3.7

The *p*-formylbenzoyl-doxorubicin analog (compound 3.7) was synthesized by the covalent attachment of doxorubicin to 4-formylbenzoic acid via an amide bond [after activation]. 4-formylbenzoic acid (6.16 mg, 41.4 µmol) was dissolved in distilled DMF (8.5 mL). 2-methyl-6-nitrobenzoic anhydride (14.3 mg, 41.4 µmol) and 4-dimethylaminopyridine (0.42 mg, 3.4 µmol) was then added to the reaction vial. Triethylamine (21. µL, 150 µmol) was then added to the reaction mixture, followed by the addition of doxorubicin•HCl (20. mg, 34 µmol). Afterwards, the reaction vial was flushed with N_2 and the content was allowed to stir overnight.

Afterwards, the solvent was evaporated under reduced pressure. The residue was then purified by column chromatography in the following solvent system: 97 EtOAc: 3MeOH. The product was isolated [along with triethylamine] in 76% yield. Characterization of *p*-formylbenzoyl-doxorubicin analog (compound 3.7): ^{1}H-NMR: δ 10.06 (s, 1H), 8.06 (d, 1H), [7.92 (d), 7.87 (d), 4H], 7.80 (t, 1H), 7.40 (d, 2H), 6.52 (d, 1H), 5.56 (d, 1H), 5.34 (d, 1H), 4.79 (d, 1H), 4.54 (s, 1H), 4.25 (m, 1H), 4.08 (s, 3 H), 3.76 (s, 1H), 3.3 (d, 1H), 3.07 (d, 1H), 2.91 (d, 1H), 2.38 (d, 1H), 2.22 (d, 1H), 2.17 (s, 1H), 2.06-2.01 (m, 2H), 1.88 (dd, 2H), 1.33 (d, 3H). ESI-MS (*m/z*, M+Na^{+}): 698.2, (*m/z*, M+MeOH+Na^{+}): 729.9.



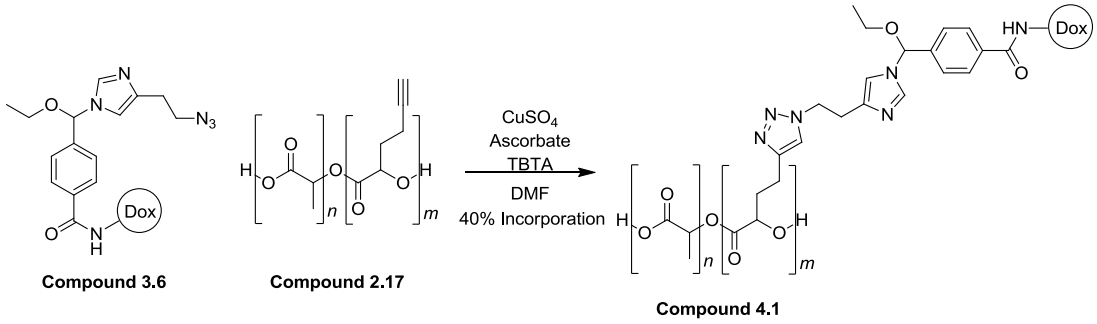
Spectrum 3.7: *^{1}H-NMR spectrum of compound 3.7*

Chapter 4

Doxorubicin Conjugated to Poly-lactic Acid (Carrier) via a *N*-ethoxybenzylimidazole (NEBI) Linker as a Drug Delivery System

Section 4.1 Synthesis of the Drug Delivery System

Previously in chapters 2 and 3, the synthesis of a modified poly-lactic acid (PLA) carrier (compound 2.17) with alkyne side-chains and a functionalized *N*-ethoxybenzylimidazole (NEBI) linker covalently attached to doxorubicin was described in detail (compound 3.6). The NEBI-doxorubicin conjugate (compound 3.6) serves as a pro-drug with an azido-molecular handle to link to the propargylic-functionalized carrier (compound 2.17). The modified PLA carrier (compound 2.17) and the NEBI-doxorubicin conjugate (compound 3.6 were covalently linked via a copper-catalyzed azide-alkyne cycloaddition (CuAAC) reaction as shown in synthetic scheme 4.1.



Synthetic Scheme 4.1: *The synthetic schematic for the active drug-delivery system (compound 4.1) from the coupling of the pro-drug (compound 3.6) with the poly-lactic acid carrier (compound 2.17)*

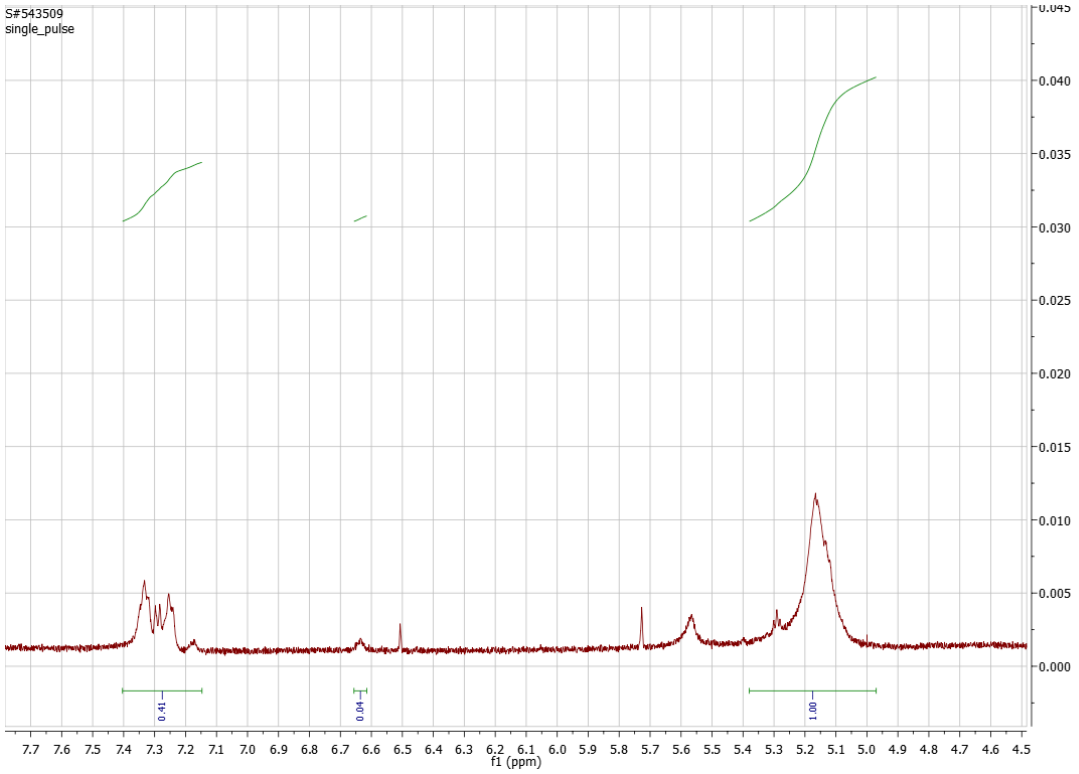


Figure 4.1: *^{1}H-NMR analysis of compound 4.1 (the active drug-delivery system) to quantify incorporation of doxorubicin.*

Based on ^{1}H-NMR analysis of the aromatic region (~7.15-7.40 ppm) and the benzylic position (~6.60-6.65) of the product, the integration indicates that the ratio of hydrogens between the two regions are approximately ten to one, consistent with the actual ratio of aromatic and benzylic hydrogens. Previously in chapter 2, ^{1}H-NMR analysis showed that there was approximately one functionalized alkyne side-chain for every ten lactic acid monomers. If the maximum number of NEBI-doxorubicin conjugates are covalently attached to the polymers, then the ratio of benzylic hydrogens to the alpha hydrogens of the poly-lactic acid (which corresponds to one lactic acid monomer) backbone should be 1:10. Since there are

ten aromatic hydrogens for each benzylic hydrogen, the maximum ratio of aromatic hydrogens to alpha hydrogens of lactic acid should be one to one. When comparing the actual integration of the aromatic region and the benzylic region with those of the alpha hydrogens on the poly-lactic acid backbone, the analysis indicates that the NEBI-doxorubicin conjugate was incorporated in ~40% of the maximum value (10%). The overall incorporation was therefore ~4%, meaning that there was 1 pro-drug doxorubicin for every 25 lactic acid monomer.

Furthermore, previously in chapter 2, gel permeation chromatography and static light scattering analysis indicated that the molecular weight of the modified PLA polymer was approximately, 57.6 kDa. Using the fact that there is approximately one alkyne-functionalized side-chain for every ten lactic acid monomers and the average molecular weight of the entire polymer, the average number of propargylic functional groups on each polymeric strand can be determined. By dividing the average molecular weight of the polymer by the weighted values of the lactic acid monomers and the alkyne-functionalized lactic acid monomers, it was determined that there are approximately 69 propargylic-molecular handles on average for each polymeric strand. In conjunction with the fact that there is approximately 40% incorporation of doxorubicin of all alkyne-side chains, it was also determined that there are approximately 28 doxorubicin pro-drugs incorporated into each strand of modified poly-lactic acid (PLA).

Section 4.2 Hydrolysis Studies of the Drug Delivery System.

Yang and coworkers previously showed a NEBI (*N*-ethoxybenzylimidazole) linker conjugated to doxorubicin exhibited acid-promoted hydrolytic properties.[48,49] They calculated the half-life of the pro-drug conjugate to be ~55 hours at pH 5.5 and at 37ºC.[48,49] To the best of my knowledge, the hydrolysis rate of a complete drug delivery system with a NEBI-doxorubicin conjugate covalently linked to a carrier has not yet been studied. To investigate the hydrolysis rate of the complete drug delivery system (compound 4.1) consisting of the modified poly-lactic acid polymer covalently linked to the NEBI-doxorubicin conjugate, a hydrolysis study with a modified protocol of the procedure used by Yang and coworkers was conducted.[48,49]

The drug delivery system (compound 4.1) was dissolved in DMSO (20% by volume) and buffer, incubated at 37ºC and room temperature and monitored at pH 5.5 (18.6 mM MES Buffer) and 7.4 (18.6 mM HEPES buffer) via analytical high-performance liquid chromatography (HPLC). Compound 4.1 was found to hydrolyze over time to give *N*-(formylbenzoyl)doxorubicin (compound 3.7). Compounds 4.1 and compound 3.7 were monitored by the absorption of the doxorubicin moiety at 470 nm using an Agilent 1100 series HPLC machine.

By plotting the natural logarithm of the final concentration divided by the initial concentration of compound 4.1, the slope of the regression line can be used to determine the half-life of the compound under the specific conditions (Figure 4.2) The half-lives of hydrolysis of compound 4.1 at pH 5.5 were calculated to be ~200

hours at 37ºC and ~650 hours at room temperature, respectively. The half-life of hydrolysis of compound 4.1 at 37ºC and pH 7.4 was calculated to be ~1230 hours. Due to the poor R^2 value (0.72) of the regression line of the hydrolysis at room temperature and pH 7.4, the precise half-life under those conditions cannot be determined accurately. However from the scatter plot, the half-life at pH 7.4 and at room temperature is determined to be significantly slower than the other conditions.

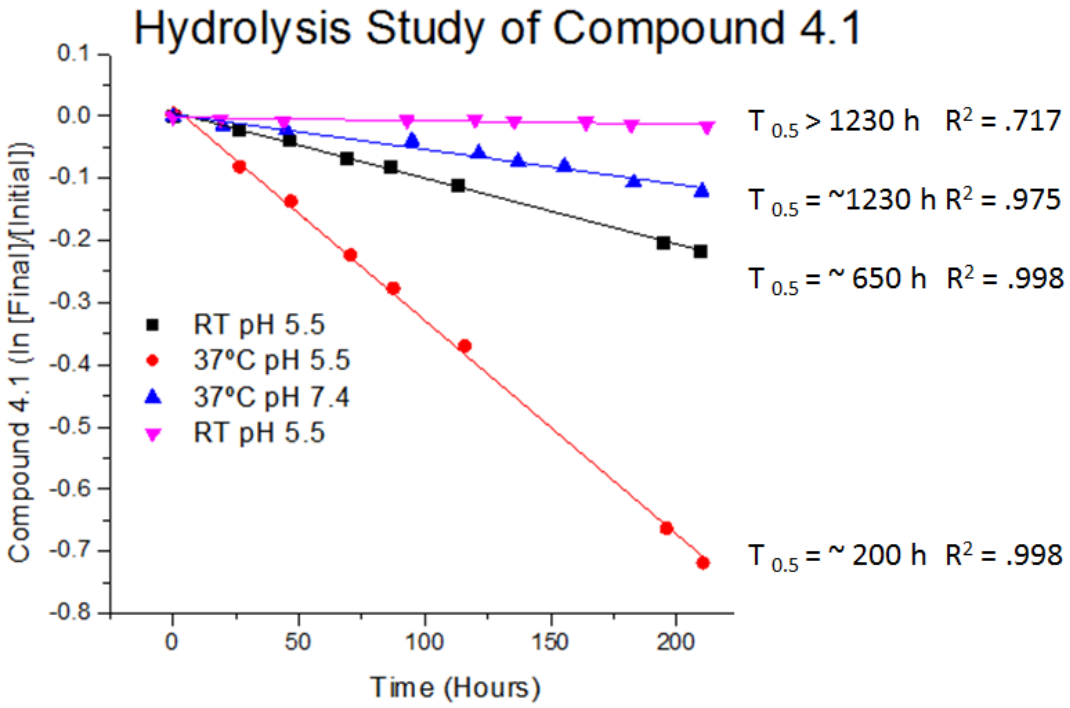


Figure 4.2: *Hydrolysis studies of the drug delivery system at pH 5.5 and pH 7.4*

Section 4.3 Cytotoxicity of Poly-Lactic Acid and Its Propargylic Derivative

To the best of my knowledge, the cytotoxicity of the modified poly-lactic acid polymer with alkyne side-chains (compound 2.17) has not yet been characterized. To accurately gauge the potency of the drug delivery system, the inherent cytotoxicity of the carrier without the drug must first be accounted for and analyzed. Although different types of cell viability assay may be applicable to characterize the cytotoxicity of the drug delivery system, the SRB (sulforhodamine B) assay was chosen for consistency and comparative analysis with the results reported by Yang and coworkers.[49]

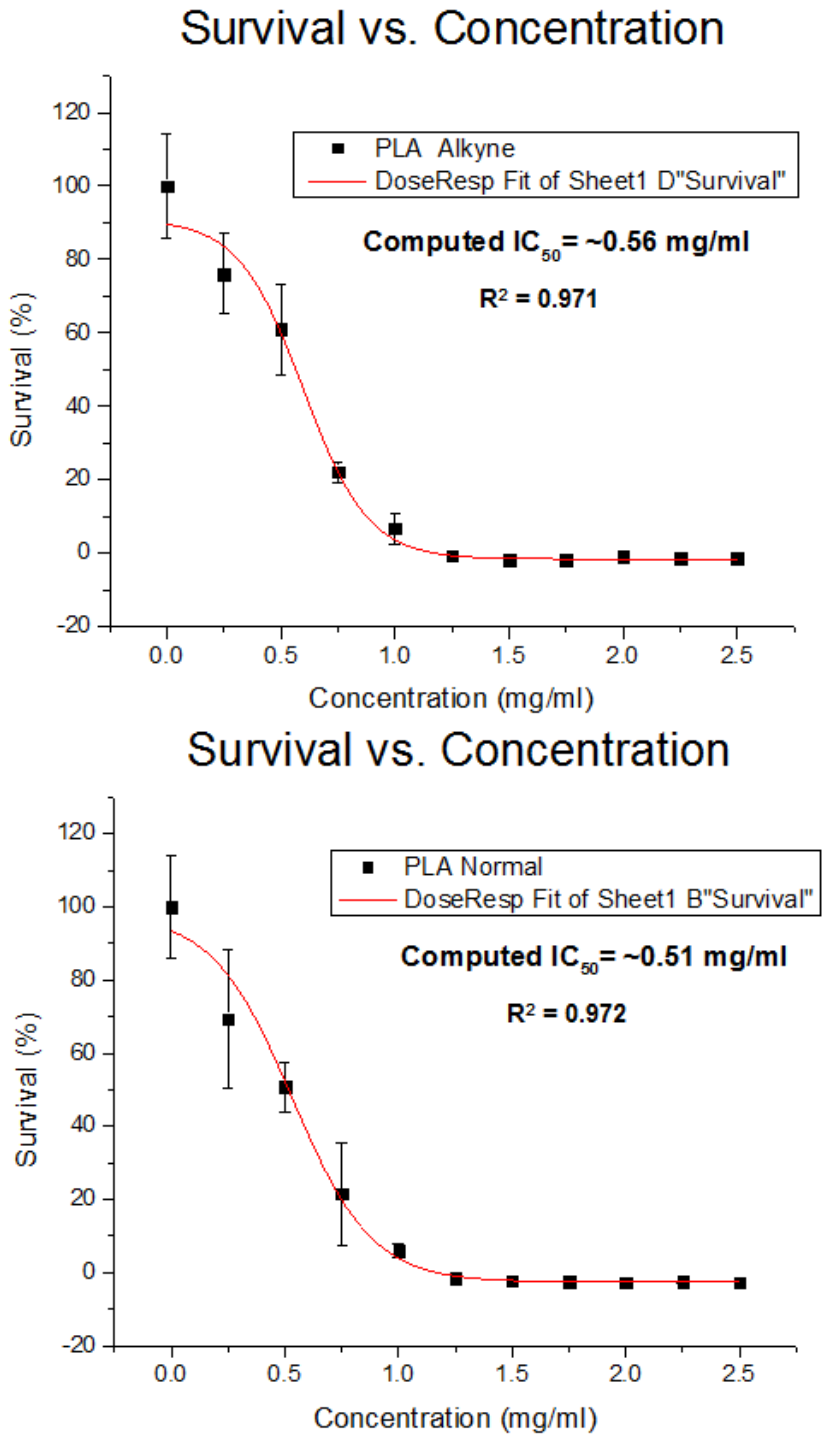


Figure 4.3: *SRB assay of the functionalized and unfunctionalized poly-lactic acid polymers to quantify the inherent cytotoxicity of the carrier.*

When using an SRB cell viability assay to measure the cytotoxicity of both the unmodified PLA polymer (compound 2.16) and the modified PLA polymer (compound 2.17) with alkyne side-chains, the approximate IC_{50} values (half-maximal inhibitory concentration) can be computed (Figure 4.3). Human ovarian carcinoma 2008 cells were exposed to increasing concentrations of compound 2.16 and compound 2.17 in 96 well plates. After 72 hours, the remaining cells were washed with 1x phosphate buffer saline, fixed with trichloroacetic acid and, then treated with sulforhodamine B. The absorption at 515 nm of the plates were measured and used to calculate the survival percentage and its error.

An appropriate dose-responsive (sigmoidal) fit was then applied to the scatter plot and the IC_{50} values of both compounds were calculated from the fit. The IC_{50} values were computed to be ~0.56 mg/ml for the modified PLA polymer with alkyne side-chains (compound 2.16) and ~0.51 mg/ml for the unmodified PLA polymer, respectively. The R^2 values calculated for both compounds were above 0.97, indicating a good and appropriate fit. The cytotoxicity studies show that compound 2.16 and 2.17 have comparable toxicity and have relatively high IC_{50} values. It is also worth noting that both polymers exhibited relatively poor solubility in the aqueous media during the performance of these studies.

Section 4.3 Cytotoxicity of the Complete Drug Delivery System

After quantifying the cytotoxicity of the functionalized PLA-based carrier, the cytotoxicity of the complete drug delivery system (compound 4.1) can now be evaluated against the control molecules, the active form of the drug (compound 3.7) and the unmodified version of the drug (doxorubicin•HCl). Although the carrier already shows mild toxicity at 250 μg/ml, the highest concentration of alkyne-functionalized poly-lactic acid used in the drug delivery system will only be at 1.0 μg/ml. For the purposes of the SRB assay, the cytotoxicity of the carrier was assumed to be negligible at such low concentrations.

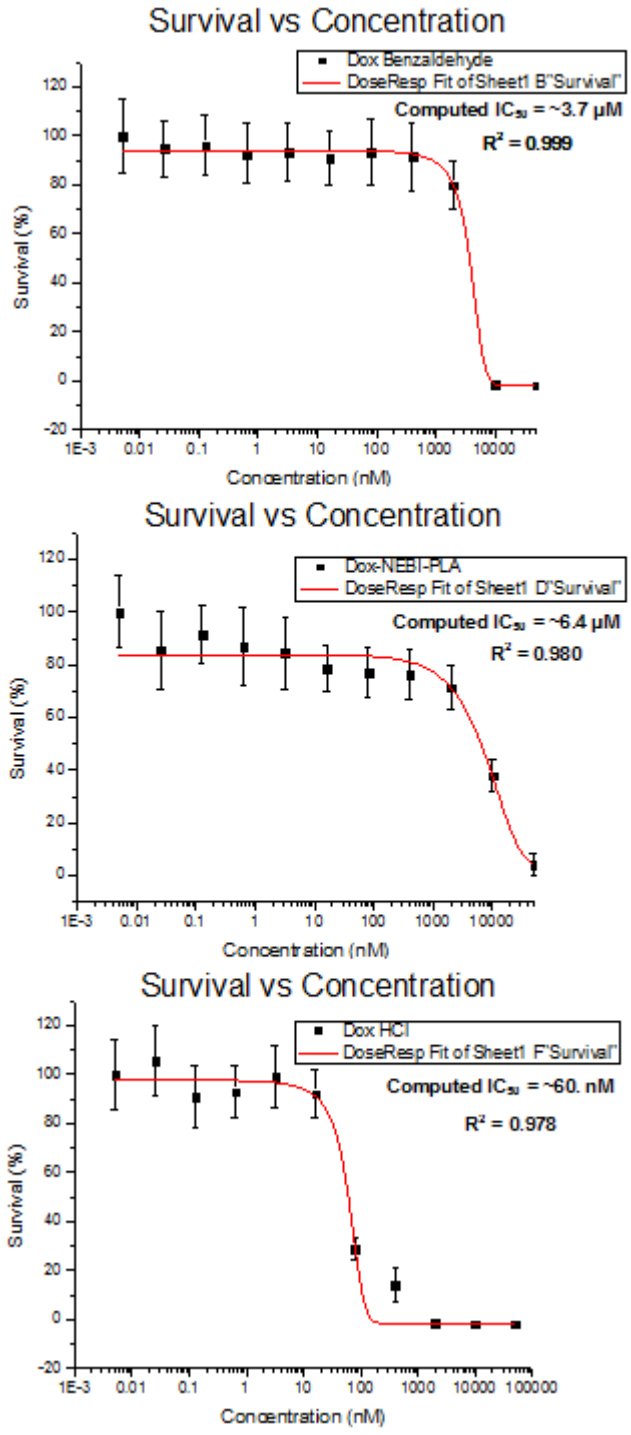


Figure 4.4: *SRB assay of the drug delivery system (DDS), doxorubicin analog and commercially-available doxorubicin to quantify the cytotoxicity of the DDS.*

When using an SRB cell viability assay to measure the cytotoxicity of the complete drug delivery system (compound 4.1), the active form of the drug ("Dox Benzaldehdye," compound 3.7) and the commercially-available doxorubicin•HCl, the approximate IC_{50} values (half-maximal inhibitory concentration) can be computed from the fit of the sigmoidal curve (Figure 4.4). Human ovarian carcinoma 2008 cells were exposed to increasing concentrations of the complete drug delivery system (compound 4.1), the active form of the drug ("Dox Benzaldehdye," compound 3.7) and the commercially-available doxorubicin•HCl in 96 well plates. After 72 hours, the remaining cells were washed with 1x phosphate buffer saline, fixed with trichloroacetic acid and, then treated with sulforhodamine B. The absorption at 515 nm of the plates were measured and used to calculate the survival percentage and its error.

An appropriate dose-responsive (sigmoidal) fit was then applied to the scatter plot and the IC_{50} values of both compounds were calculated from the fit. The IC_{50} values were computed to be ~3.7 μM for the active form of the drug ("Dox Benzaldehdye," compound 3.7), ~6.4 μM for complete drug delivery system (compound 4.1) and ~60 nM for the commercially-available doxorubicin•HCl. The R^2 values calculated for all compounds were above 0.97, indicating a good and appropriate fit. The cytotoxicity studies show that compound 3.7 and 4.1 had somewhat comparable toxicity being in the same order of magnitude while the commercially-available doxorubicin•HCl shows significantly more potency.

Yang and coworkers previously determined that the IC_{50} value of compound 3.7 to be 5.8 μM using the same cell line and also using an SRB assay.[49] In comparison with the results recently obtained, both studies produced IC_{50} values within the same order of magnitude. However, Yang and coworkers reported that their drug delivery system (doxorubicin covalently linked to human serum albumin via a NEBI linker) exhibited far more cytotoxicity than compound 4.1, perhaps suggesting that the carrier type may influence the efficacy of NEBI-linker based drug delivery systems. It is also worth noting that the drug delivery system (compound 4.1) only exhibited moderate solubility relative to the control molecules.

4.5 Conclusion

Based on ^{1}H-NMR analysis, the objective to create a model drug delivery system using the modified poly-lactic acid polymer (compound 2.17) with alkyne side chains as a carrier, the *N*-ethoxybenzylimidazole (NEBI) and doxorubicin conjugate (compound 3.6) was successfully met. Furthermore, ^{1}H-NMR integrations also indicated that there was 40% incorporation of doxorubicin to the possible alkyne side-chains of the polymer and a total of 4% incorporation of doxorubicin to each polymeric strand on average. Using the percent incorporation of doxorubicin in conjunction the molecular weight of polymer (determined from GPC and SLS analysis) and the known molecular weight of the lactic acid monomers, it was determined that each polymeric strand contained approximately 28 molecules of

doxorubicin on average. By using the propargylic-functionalized poly-lactic acid as a carrier, the payload of doxorubicin that can be possibly delivered was increased.

Hydrolysis studies of the drug delivery system (compound 4.1) showed that cleavage of the NEBI linker and the subsequent release of the active doxorubicin analog (compound 3.7) is facilitated and stimulated at lower pH [and higher temperatures]. Despite having the pro-drug conjugated to the propargylic-functionalized PLA carrier (compound 2.17), the release of the drug delivery system can still be modeled by an exponential decay function. The half-life of the drug delivery system was calculated to be approximately 200 hours at endosomal pH (5.5) and physiological temperature (37ºC) The calculated half-life is notably less than reported half-life of a NEBI-doxorubicin conjugate (~55 hours) under the same conditions.[48,49]

An SRB cell viability assay was employed to quantify the toxicity of poly-lactic acid (compound 2.16) and modified poly-lactic acid with alkyne side-chains (compound 2.17). Their respective IC_{50} values were determined to be 0.51 mg/ml (compound 2.17) and 0.56 (compound 2.16), indicating that the two polymers have comparable toxicity. It was also determined that the concentration of the carrier in the drug delivery system at its highest concentration (~1.0 μg/ml) will garner an insignificant amount of toxicity relative to the released drug.

Using the same SRB assay, the respective IC_{50} values of the drug delivery system (compound 4.1), the active form the drug (doxorubicin analog, compound

3.7) and commercially-available doxorubicin•HCl were calculated to be ~6.4 μM, ~3.7 μM and ~60. nM, respectively. The calculated IC_{50} value of compound 3.7 was comparable to the results by Yang and coworkers.[49] Although the cytotoxicity of the drug delivery system (compound 4.1) was comparable to the active form of the drug, it was expected to demonstrate even higher potency.

4.6 Future Directions

After successfully synthesizing a model of a drug delivery system that contains a modified poly-lactic acid polymer (compound 2.17) with alkyne side chains as a carrier and doxorubicin conjugated to the drug delivery system via an acid-sensitive NEBI linker, the focus of the research can now shift towards optimizing the drug delivery system. Considering that the drug delivery system exhibited many desirable properties including increased payload, acid-promoted release and comparable cytotoxicity, research now needs to be conducted on the factors that may further increase the potency of the drug delivery system. Specifically, solubility, cellular uptake and hydrolysis are parameters worth investigating and improving because they may affect its efficacy.

To determine which variables limit the potency of the drug delivery system, a cellular uptake study needs to be performed. If the drug delivery system shows significantly less uptake, then the solubility of the carrier may need to be enhanced by grafting polyethylene glycol or poly-ethylene oxide groups to the polymer. If the drug delivery system shows comparable uptake or even greater uptake, then the

hydrolysis rate of the compound at pH 5.5 and 37ºC needs to be accelerated. Having an electron-withdrawing carbonyl at the para position relative to the benzyl group may diminish the hydrolysis rate and suppress the release and it may need to be addressed. Other possible modifications may include using different derivatives with different substituents on the NEBI linker for future models of drug delivery systems. Fortunately, Yang and coworkers compared the relative hydrolysis rates of different NEBI linker derivatives which should aid the selection of the appropriate derivative.

Considering the observed solubility issues of the carrier and the tempered hydrolysis rate of the drug delivery system, there are different variables that can be addressed to improve efficacy. However, by choosing both a versatile carrier and linker, different variation of the drug delivery system may be more easily synthesized. Having synthesized the prototype of the drug delivery system which demonstrated many desirable characteristics, the synthesized model drug delivery system can now be used to help identify different problematic issues and help direct research towards the appropriate future modifications.

4.7 Experimentals

All reagents were purchased from Acros Organic, Spectrum Chemicals, Sigma-Aldrich, TCI America, or Alfa Aesar and used without further additional purification unless specified. All solvents used for reactions were dried on alumina columns and distilled prior to use. Solvents used for chromatography were ACS technical grade and used without further purification. Distilled water (18.2 μΩ/cm) was filtered through a NANOPure Diamond^{TM} (Barnstead) water purification system before use in synthesis or purification. NMR spectra were recorded on a Varian 400 (FT, 400 MHz, ^{1}H; 100 MHz, ^{13}C) spectrometer and a JEOL ECA-500 (FT, 500 MHZ ^{1}H) spectrometer. HR-MS (high-resolution mass spectra) were obtained in the Department of Chemistry & Biochemistry, University of California, San Diego (UCSD) using a ThermoFinnigan MAT900XL-MS. ESI-MS (electrospray ionization mass spectra) were obtained using ThemoFinnigan LCQDECA-MS. Kinetic analysis by reverse-phase high performance liquid chromatography (RP-HPLC) was performed with an Agilent 1100 Series HPLC using an analytical reverse-phase column (SPHERI-5 Phenyl 5 micron, 250x4.6mm). Purification of PLA conjugates were performed with Spectra/Por Dialysis Membrane Tubing (MWCO: 6-8 kDa).

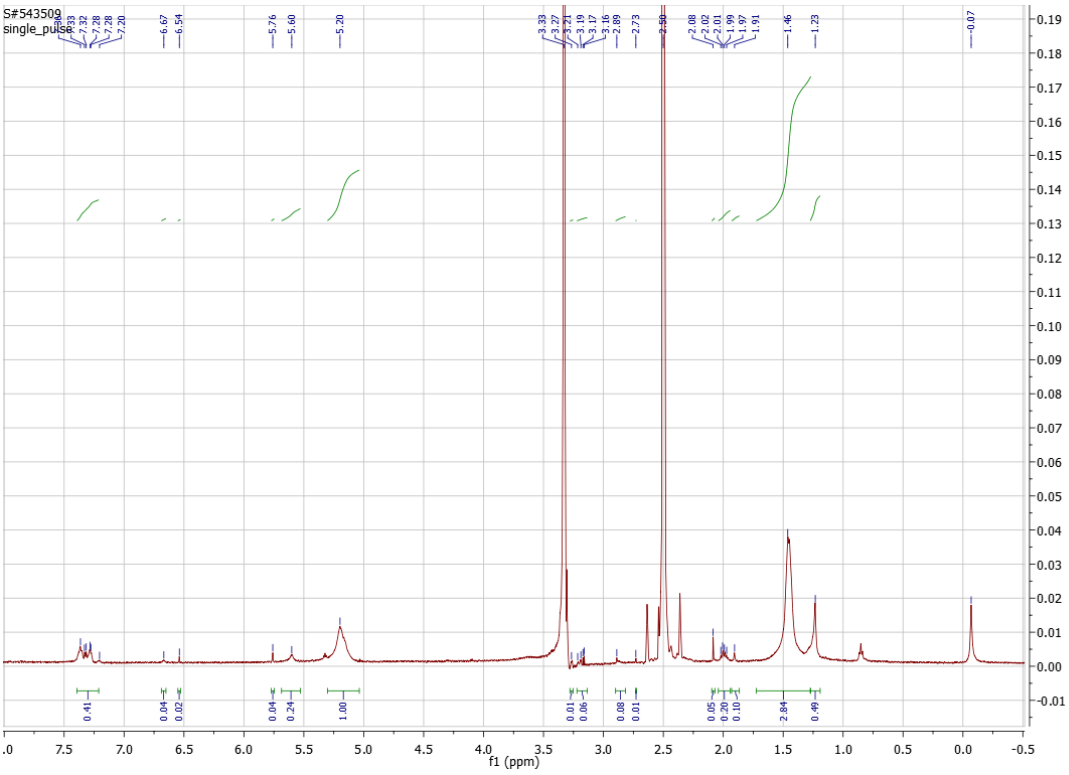
Synthesis of Doxorubicin-NEBI-PLA Conjugate (Complete Drug Delivery System, compound 4.1)

The complete drug delivery system was synthesized by the conjugation of propargylic-functionalized poly-lactic acid copolymer (compound 2.17) with the

NEBI-doxorubicin conjugate with the azido molecular handle (compound 3.6) via a copper catalyzed azide-alkyne cycloaddition (CuAAC). The alkyne-functionalized poly-lactic acid copolymer (9.5 mg, 11 μmol of alkyne) and the NEBI-doxorubicin conjugate (11.5 mg, 13.7 μmol) were dissolved in distilled DMF (6 mL). A slurry of sodium ascorbate (0.8 mg, 4 μmol), copper (I) iodide (0.8 mg, 4 μmol), Tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl]amine (2.4 mg, 4.5 μmol) and triethylamine (1 μL) were stirred in distilled DMF (2 mL) and then added to the reaction vial. The reaction mixture was allowed to stir overnight.

The content of the reaction was then transferred to a dialysis bag (Spectra/Por Dialysis Membrane Tubing (MWCO: 6-8 kDa)). The dialysis bag is then stirred in a dialysis chamber with dichloromethane as the solvent. The solvent in the dialysis chamber is changed at the 4 hour mark, the 10 hour mark and then allowed to stir overnight. Afterwards, the solution remaining in the dialysis bag is transferred to a scintillated vial, and the solvent is removed under reduced pressure. The residue is then dissolved in chloroform and precipitated out in ice-cold methanol. The residual solvent is then immediately removed under reduced pressure to produce a purple solid. ^{1}H-NMR analysis indicates that the NEBI-conjugate is incorporated in ~40% of all alkyne-functionalized side chains. Characterization of Doxorubicin-NEBI-PLA Conjugate (Complete Drug Delivery System, compound 4.1): ^{1}H-NMR δ 7.4-7.2 (m, 0.41 H), 6.67 (broad peak, 0.04 H), 6.54 (s, 0.02 H), 5.76 (s, 0.04 H), 5.60 (broad peak, 0.24 H), 5.40-5.20 (broad peak, 1.00 H), 3.27 (broad peak, 0.01 H), [3.21,

3.19, 3.17, 3.16, 0.06 H], 2.9-2.8 (broad peak, 0.08 H), 2.73 (broad peak, 0.01 H), 2.08 (s, 0.05 H), 2.08-1.97 (m, 0.20 H), 1.91 (s, 0.10 H).



Spectrum 4.1: *^{1}H-NMR spectrum of compound 4.1*

General Procedures for Hydrolysis Studies[49]

The complete drug delivery system, compound 4.1 (0.3 µmol) was dissolved in 1.0 mL of 18.6 mM MES buff (pH=5.5) or 18.6 mM HEPES buffer (pH=7.4) containing 20% DMSO by volume and incubated at 37ºC. Another set of samples were prepared in the same fashion, but these samples were left out in room temperature. The hydrolysis of compound 4.1 at pH=5.5 and pH=7.4 was monitored by RP-HPLC by injections of small aliquots (20 µL) of the solution at multiple

intervals. The chromatograms are analyzed at λ=470 nm. The products were eluted with an isocratic solvent mixture of 70% methanol, 30% H_2O and 0.1% trifluroacetic acid with a flow rate of 1 mL/min. The retention time of 4.1 and 3.7 were approximately 15 mins and 8 mins, respectively. The rates of hydrolysis of the NEBI moiety to release compound 3.7 was determined by comparing the relative integrated HPLC peak areas of compound 4.1 and compound 3.7 at 470 nm at each time point.

General Procedure for SRB Cell Viability Assay[49]

Human ovarian carcinoma 2008 cells were plated into each well of a 96 well plate (3000 cells/well) using RPMI-1640 media with 10% fetal bovine serum. The cells were incubated at 24 hours at 37ºC with 5% CO_2. After a 24 hour incubation period, cells were dosed with various concentrations of a cytotoxic agent (compounds 2.16, compound 2.17, compound 3.7, compound 4.1 or commercially-available doxorubicin•HCl) on a 96 well plate. Repeat the dosing of cytotoxic agents at various concentrations for each other compound of interest on other 96 well plates. The cells on the 96 well plates were allowed to incubate with the molecules for 72 hours.

After the incubation period, the cells were washed with phosphate buffer saline (3x 200 μL) at pH 7.4 (Mediatech, 46-013CM). The washed cells were then fixed with a solution of PBS (200 μL) and 50% trichloroacetic acid (50 μL) and allowed to incubated at 4ºC for over 1 hour. After fixation, cells were washed with H_2O (5x 200 μL) and then allowed to dry.

A 0.4% sulforhodamine B (SRB, Sigma Aldrich, S1402) solution in 1% acetic acid was added to the cells and incubated for an hour at room temperature. The 96 well plates were covered with aluminum foil and precautions were taken to avoid the exposure of light to the SRB solutions. The SRB-treated cells were then washed with 1% acetic acid (4x 200 μL) and then allowed to dry. Tris base solution (100 mM, 200 μL) was then added to each well and gently stirred for 15 mins. Afterwards, a microplate reader is used to read the absorbance of each well at 515 nm. The absorbance values are then converted to survival percentages and a dose-responsive, sigmoidal curve is fitted onto the scatter plot of the survival percentages.[49] Using the generated dose-responsive sigmoidal curve equation, the corresponding IC_{50} values can be computed for each cytotoxic agent.

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