Synthesis of Phospholipids for the Study of Cell Surface Protein Binding and Activation

Senior Honors Project

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Michael Best, Advisor

This project is dedicated to Dr. Michael Best, who I suspect will cure cancer one day, and then show up early the next day to get started on another disease.

Abbreviation	Full Name
CH_2Cl_2	dichloromethane
DCC	N,N'-dicyclohexylcarbodiimide
DDQ	2,3-dichloro-5,6-dicyanobenzoquinone
DMAP	4-(dimethylamino) pyridine
DMF	N,N'-dimethylformamide
Et_3N	triethyl amine
HCl	hydrochloric acid
KCN	potassium cyanide
KI	potassium iodide
KOH	potassium hydroxide
$LiAlH_4$	lithium aluminum hydride
m-CPBA	meta-chloroperoxybenzoic acid
MeCN	acetonitrile
$MeSO_2Cl$	methanesulfonyl chloride
NaH	sodium hydride
NaN_3	sodium azide
NH_4Cl	ammonium chloride
PMBCl	para methoxy benzyl chloride
$POCl_3$	phosphoryl chloride
THF	tetrahydrofuran
TMSBr	trimethylsilyl bromide
TsCl	toluene-2-sulfonyl chloride

Part I

Introduction

The motivation for Part I of this project was a desire to study the interaction which occurs between proteins and the surface of a living cell during physiological events. Because phospholipids are the molecules which compose the majority of a cell's membrane[1], the approach taken in the study was to synthesize phospholipids which had specific useful properties that could be exploited in future studies. In particular, the goal was to produce phospholipids which were "tagged" with an azide group ($-N=N^-=N^+$) in a certain portion of the molecule. The attractiveness of the azide molecule stems from the fact that it is extremely versatile, and it can be selectively modified without causing significant disruption to the biological system surrounding it.[2-3]

The azide tag can be placed in several positions throughout the phospholipid molecule. The goal of this study was to attach the azide tag at the head group of the molecule in the interest of mapping binding sites on a protein which binds to the target. In the future, two methods will be employed to accomplish this. In the first method, the azide tag is modified to a photo-crosslinking group, which binds to the protein at the site of interest. In the second method, the protein is modified so that it contains an alkyne in close proximity to a binding site. The alkyne group creates a linkage to the phospholipid via a 1,3 dipolarcycloaddition, known commonly as "click" chemistry.[4] Following purification, the result would be a covalent lipid-protein adduct in which the phospholipid is linked to the protein via the alkyne adjacent to the binding site.

Background

Phospholipids represent a portion of a large class of biological molecules known as lipids, which are characterized by being either hydrophobic or amphipathic (having both hydrophobic and hydrophilic nature). A phospholipid is defined, simply, as a lipid with a phosphate group attached to it.[5] Due to their low solubility in water, lipids provide an effective barrier between polar and nonpolar substances. Consequently, lipids are an ideal constituent for a biological cell's membrane.[6] Some of the simplest lipids in nature are called fatty acids. Fatty acids consist of a hydrocarbon chain with a carboxyl group at one end. The carboxyl group is called the fatty acid's "head," while the hydrocarbon chain is called its "tail." Fatty acids can contain saturated hydrocarbon chains with no double bonds, or unsaturated chains, which contain at least one double bond.[7]

Phospholipids have been divided into two major classes, glycerophospholipids and sphingophospholipids. It is a glycerophospholipid which is the immediate target of this study. Glycerol is a relatively small molecule containing a three carbon chain, with each of the carbons having a hydroxyl group attached to it. Glycerol is pictured in **Figure 1**. When the glycerol is esterified to two fatty acids at the first and second carbon atoms, and a phosphate group is esterified at the third carbon, the molecule is known as a glycerophospholipid.[8]

The simplest glycerophospholipid is called phosphatidic acid. Phosphatidic acid is the parent compound of all glycerol based phospholipids.[9] It is pictured in **Figure 2**. Phosphatidic acid (PA) and diacylglycerol (DAG) have been shown to be an intermediate structure in the formation of many important phospholipids; and have also been shown to be involved in a diverse group of physiological and pathophysiological events, including

protein regulation, immune response, regulation of calcium, and cancer metastasis.[10-11] The goal of this study is to present a method in which phospholipids such as PA can be easily synthesized and examined in the process of binding with a protein. Such a method for developing synthetic compounds which mimic natural membrane lipids would have broad utility in studying the interactions that occur between cells and proteins. Synthetically produced phospholipids, when compared to those taken from living cells, provide the advantage of being highly specified, as well as easier to manipulate in studies such as microarray analysis.

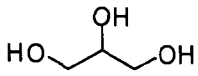


Figure 1

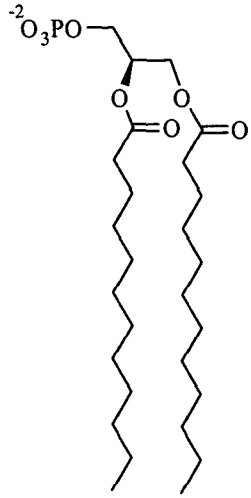
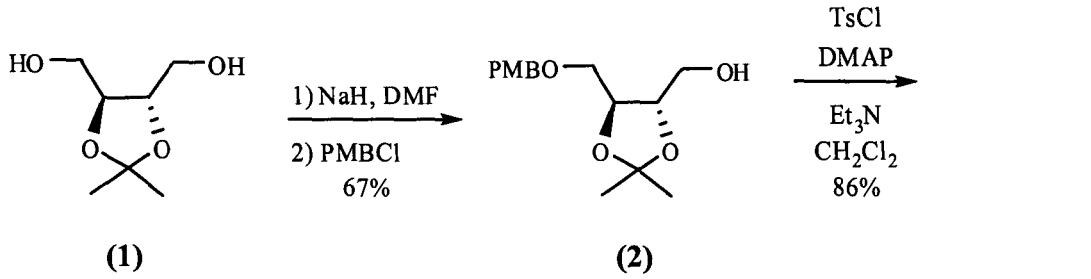


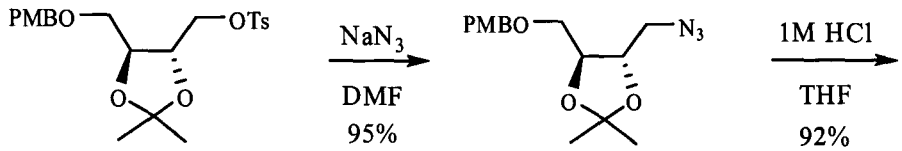
Figure 2

Experimental

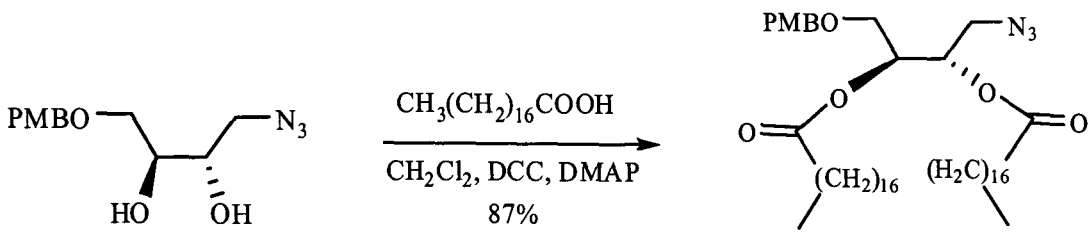
Scheme 1- Introduction of Head Group Tag



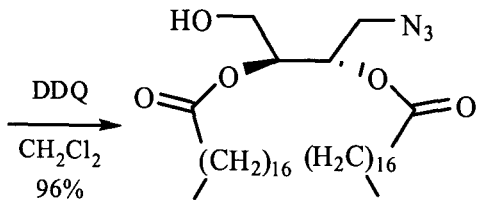
(1) (2)



(3) (4)

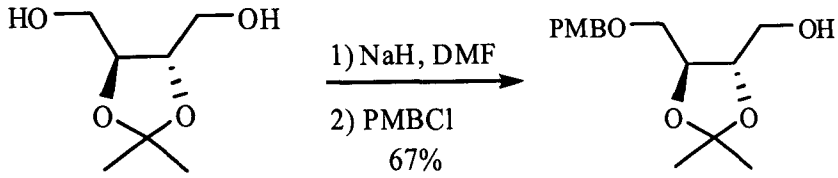


(5) (6)



(7)

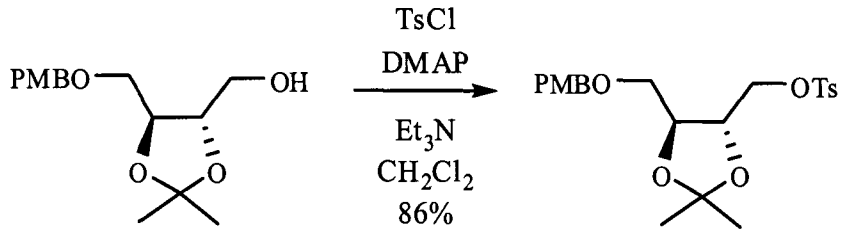
Reaction 1



(1) **(2)**

Sodium hydride (123 mg) was added to a 100 mL reaction flask, and the temperature was reduced to 0° Celsius. 20 mL of DMF was added to the flask and stirring was begun. **Compound (1)** (500 mg) was dissolved in 20 mL of DMF in a vial. The solution was transferred to an addition funnel, and added dropwise at 0° C. The reaction was warmed to 25° C for approximately 15 minutes, and then re-cooled to 0° C. PMBCl (460 mL) was added to the reaction, and it was allowed to stir at room temperature for approximately 72 hours. The reaction was quenched with H_2O and washed twice with CH_2Cl_2. The combined CH_2Cl_2 extracts dried over magnesium sulfate and filtered. The solvent was removed using a rotary evaporator. Yield: 579 mg (67%).

Reaction 2

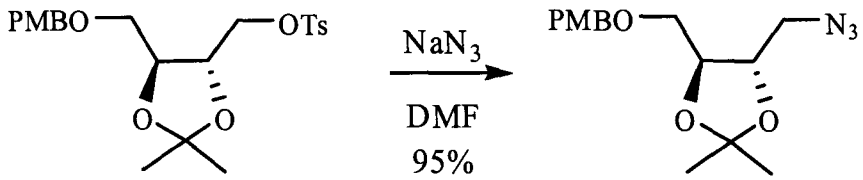


(2) **(3)**

Compound (2) (579 mg) was combined with DMAP (251 mg), TsCl (781 mg), Et_3N (572 μL), and CH_2Cl_2 (20 mL) in a reaction flask with stirring. The reagents were stirred at 25° C for two days. The product was extracted twice with CH_2Cl_2 and the

combined extracts were dried with magnesium sulfate, filtered, and the solvent was removed using a rotary evaporator. The product was purified using column chromatography with silica gel, 20% ethyl acetate/ hexanes (150 mL), 40% ethyl acetate/ hexanes (200 mL), and 75% ethyl acetate/ hexanes (100 mL). **Compound (3)** was present in fractions 11-14. Yield: 765 mg (86%).

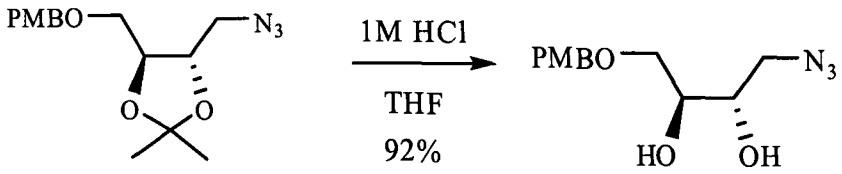
Reaction 3



(3) **(4)**

Compound (3) (765 mg) was added to NaN_3 (228 mg) and DMF (20 mL) in a flask. The reactants were stirred at 70° C for approximately 48 hours. The contents of the reaction flask were purified using column chromatography with silica gel, 10% ethyl acetate/ hexanes (200 mL), and 25% ethyl acetate/ hexanes (200 mL). **Compound (4)** was present in fractions 11-15. Yield: 507 mg (94%).

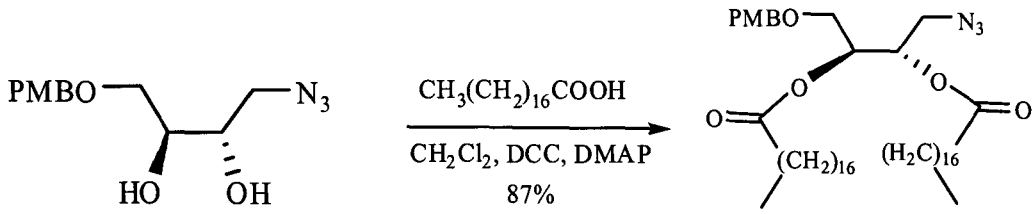
Reaction 4



(4) **(5)**

Compound (4) (507 mg) was combined with 1M HCl (10 mL) and THF (10 mL) in a flask with stirring. The reactants were stirred at 25° C for approximately 96 hours. The reaction mixture was quenched with $NaHCO_3$, and extracted once with CH_2Cl_2. The extract was dried over magnesium sulfate and filtered. The solvent was removed using a rotary evaporator. The product of the reaction was purified using column chromatography with silica gel, 50% ethyl acetate/ hexanes (200 mL), 75% ethyl acetate/ hexanes (100 mL), and ethyl acetate (100 mL). **Compound (5)** was present with slight overlap in fractions 6-9. Yield: 379 mg (86%).

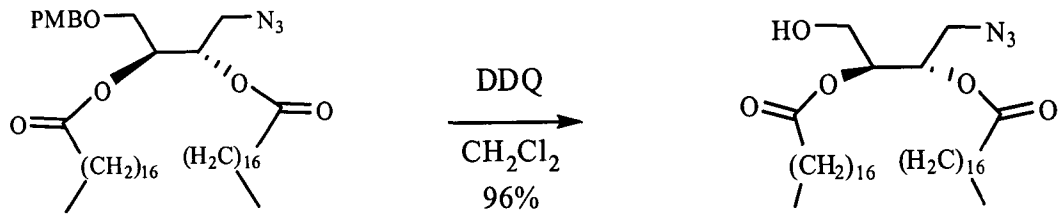
Reaction 5



(5) **(6)**

Compound (5) (87 mg) was combined with stearic acid ($CH_3(CH_2)_{16}COOH$) (278 mg), DCC (202 mg), DMAP (44 mg), CH_2Cl_2 (10 mL) in a flask with stirring. The reactants were stirred at 25° C for approximately 72 hours. The product of the reaction was purified using column chromatography with silica gel, hexanes (200 mL), 10% ethyl acetate/ hexanes (200 mL), and 30% ethyl acetate/ hexanes (100 mL). **Compound (6)** was present in fractions 17-19. Yield: 225 mg (87%).

Reaction 6



(6) **(7)**

Compound (6) (225 mg) was combined with DDQ (128 mg), CH_2Cl_2 (5 mL), and H_2O (500 µL) in a flask with stirring. The reactants were stirred at 25° C for approximately 24 hours. The reaction mixture was quenched with saturated $NaHCO_3$. The mixture was extracted twice with CH_2Cl_2. The combined extracts were dried over magnesium sulfate and filtered. The solvent was removed using a rotary evaporator. The product was purified using column chromatography with silica gel, 20% ethyl acetate/ hexanes (200 mL), and 30% ethyl acetate/ hexanes (200 mL). **Compound (7)** was present in fractions 13-16. Yield: 135 mg (71%).

Results and Discussion

Compound (7) was the initial target of this study. The utility of **Compound (7)** is vast, with several lipid probes available via one-step syntheses. In the continuation of this study, the compound has been tagged with several fluorophores, including multiple Forster Resonance Energy Transfer (FRET) pairs. With these pairs in place, it is possible to measure the distance between specific lipids during protein binding events. It should also be noted that the presence of the azide tag at the head group of the lipid is

advantageous due to its close proximity to proteins during binding events. This is especially useful when attempting photo-crosslinking studies.[12]

In initial investigations, lipid derivatives of **Compound (7)** imitate well the activities of natural diacylglycerols. They appear to follow the binding pattern of natural lipids when bound to protein kinase C, a known diacylglycerol binding receptor. Thus, it can be inferred that **Compound (7)** and its analogs will be powerful tools for the study of protein-membrane interactions in the future.

Part II

Introduction

The goal of Part II of this project was to produce a synthesis for a particularly interesting molecule, 1,12-dimethylbenzo[c]phenanthrene-5,8-dicarboxylic acid (DBP). This molecule is **Compound (20)** shown in Scheme 2. DBP is a di-substituted carboxylic acid analogue of [4] helicene, which is shown in **Figure 3**. Helicenes are a class of molecules which takes its name from the helical structures they form due to the steric interactions between their terminal rings[13]. These steric interactions cause helicenes to exhibit chiral behavior, despite the absence of asymmetric carbon atoms.[14]

In this project, the goal is to outline a method for producing a [4] helicene analogue. Following such a synthesis, the extended goal of the project is to study DBP and to determine certain of its thermodynamic properties.

Background

The name "helicene" was first introduced in 1955 by the great Melvin S. Newman to describe molecules with helical structure consisting of *o*-condensed aromatic rings.[15] Newman introduced the simple nomenclature which is still used to describe the helicenes which have only carbon atoms in their backbone, called "carbohelicenes." In Newman's system, [n] helicene is composed of n *ortho*-condensed aromatic rings.

Helicenes are considered to be special molecules in organic chemistry. Their uniqueness results from the fact that they are one of a relatively few known non-polymeric molecules that exhibit helical structure.[16] Molecules of this kind are relatively rare, since larger molecules such as deoxyribonucleic acid (DNA) are much more apt to display helical structure in their geometric arrangement.

The helical structure of a helicene is a result of the intramolecular steric interactions between its terminal rings and their substituents. Note **Figure 4**. In molecule **A**, due to their close proximity, rings 1 and 5 thermodynamically "prefer" to be non-coplanar. This exemplifies a steric hindrance, implying that **A** is at its lowest energy when rings 1 and 5 are angled away from one another. This angling produces the helical spiral exhibited by [5] helicene.

Smaller helical molecules such as helicene are of interest for several reasons. A major reason stems from the fact that helicenes possess a chiral nature; in other words, they exhibit right- or left-handedness.[17] This phenomenon is illustrated in **Figure 4**. In molecule **A**, benzene ring 1 is considered to be in the plane of the page, while rings 2 through 5 are not in the plane. The molecule can be imagined to be spiraling up and out towards the reader. Note that if ring 5 is considered closest to the reader, the rest of molecule **A** spins away in a counter-clockwise manner. Thus, by convention, **A** is the (−) or M form of [5] helicene. By contrast, molecule **B** spins down and away from the reader in a clockwise manner, making it the (+) or P form of [5] helicene.[18]

Molecules **A** and **B** are enantiomers, or non-super imposable mirror images of one another. This makes them distinguishable from one another despite the fact that their molecular formulas are identical. Experimentally, **A** will rotate a plane-polarized beam of light by a certain amount, and this is known as **A**'s specific rotation. Molecule **B** will rotate plane-polarized light by the same amount, but in the opposite direction.

[5] Helicene can racemize, or interconvert from **A** to **B**, and vice versa. The study of how helical molecules interconvert between enantiomers is a topic that has been

studied for years and continues to occupy chemists.[19-21] In this study, the future goal is to study the thermodynamics of the interconversion of (+) and (−) DBP.

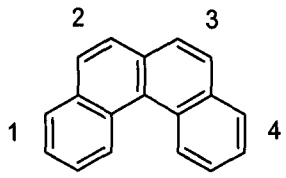


Figure 3

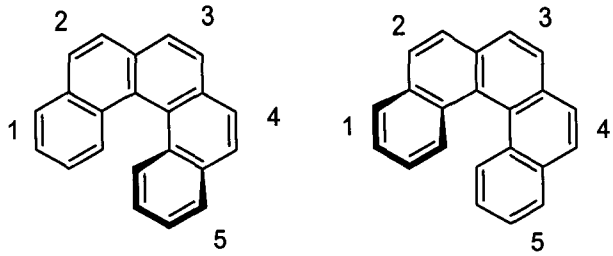
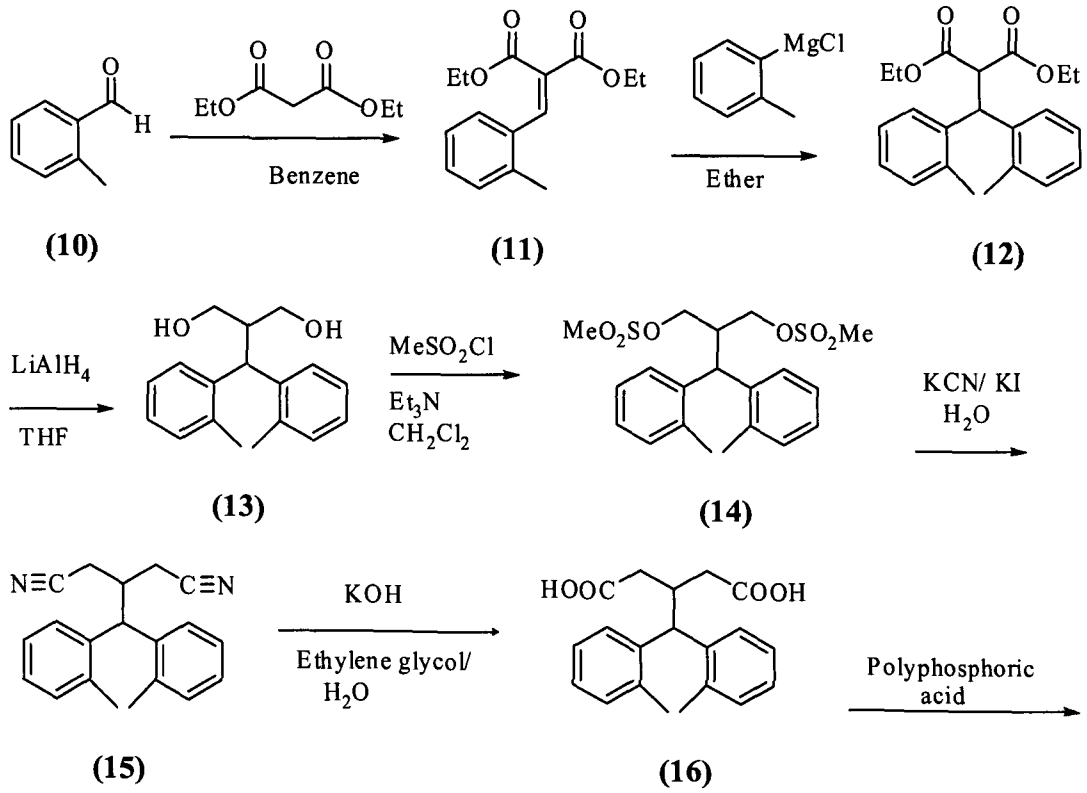
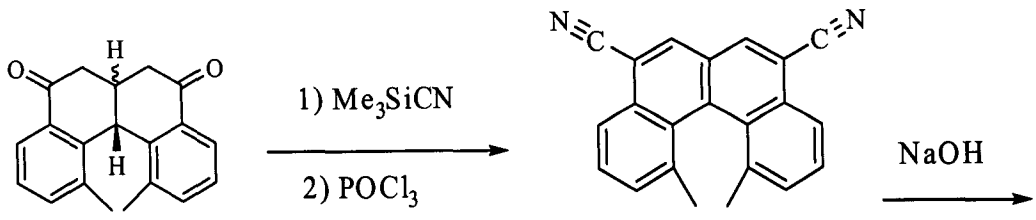


Figure 4 −,+ [5] Helicene

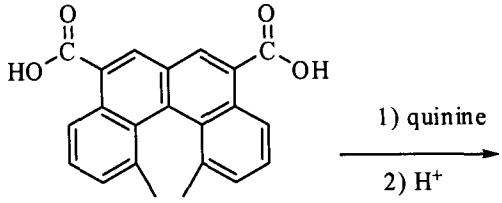
Experimental

Scheme 2- Proposed Synthesis of DBP

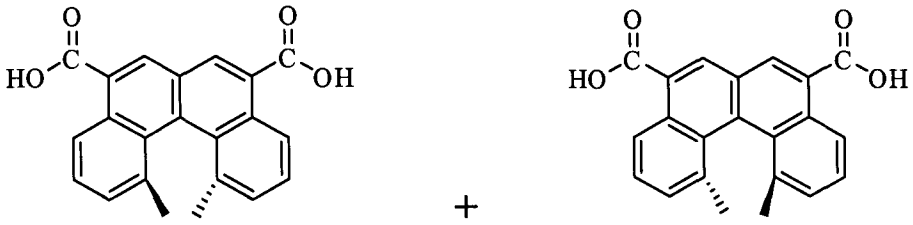




(17) **(18)**

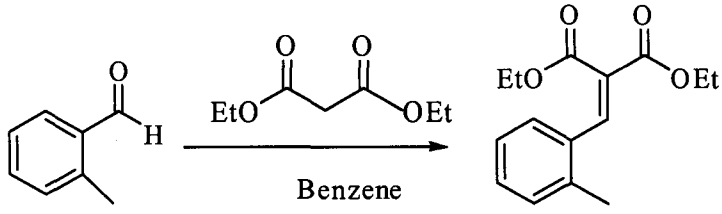


(19)



(+) (20) **(−) (20)**

Reaction 9

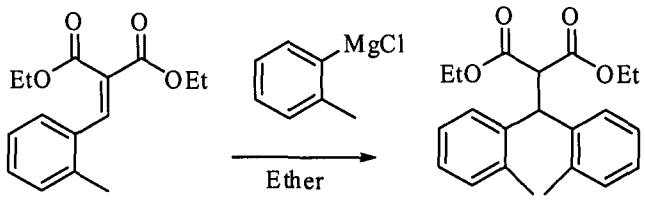


(10) **(11)**

Compound (10) (2-tolualdehyde) (5 g) was combined in a reaction flask with diethyl malonate (7.33 g), benzoic acid (.275 g), and piperidene (317 μg) and dissolved in benzene (8.31 mL). The reagents were stirred and heated to 85° C, and the flask was

fitted with a Dean Stark trap to monitor the amount of H_2O produced by the reaction. An additional 15 mL of benzene was added to the flask, and the reaction was allowed to run for approximately 24 hours. The solvent was removed via a rotary evaporator, and the product was purified using column chromatography with hexanes (150 mL), and 10% ethyl acetate/ hexanes (300 mL). **Compound (11)** was present in fractions 17-24. The product was distilled to further purify it. Yield: 3.618 g.

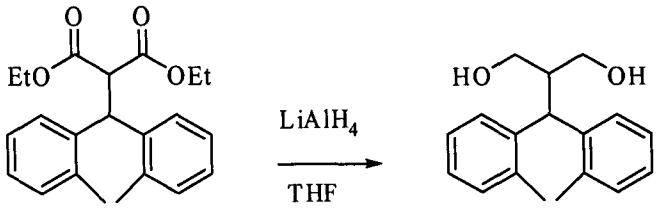
Reaction 10



(11) **(12)**

Compound (11) was dissolved in ether (75 mL). The flask was lowered to 0° C and a 1M solution of phenylmagnesium chloride (25 mL) was added to the flask. The reactants were stirred under nitrogen for approximately 48 hours. The reaction was quenched with a saturated solution of NH_4Cl (50 mL) added dropwise. H_2O (25 mL) was added to the flask, and the mixture was extracted with ether, dried over $MgSO_4$, filtered, and the solvent was removed via a rotary evaporator. The product was purified using column chromatography with silica gel and hexanes (600 mL). **Compound (12)** was present in fractions 9-23. Yield: 4.33 g.

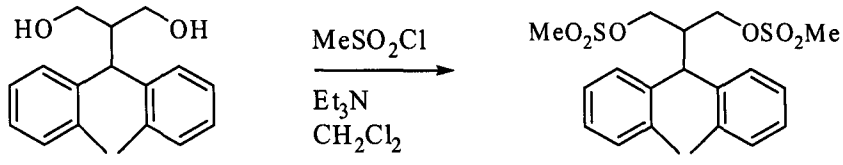
Reaction 11



(12) **(13)**

$LiAlH_4$ (107 mg) was suspended in THF (12 mL) in a reaction flask with stirring at 0° C. **Compound (12)** (500 mg) was dissolved in THF (3 mL) and added dropwise via an addition funnel over 30 minutes. The reaction was stirred at 0° C for 60 minutes, and then at 25° C for an additional 60 minutes. The flask was re-cooled to 0° C, and then quenched by adding H_2O (3 mL) dropwise, followed by 15% NaOH solution, and H_2O (9 mL). The mixture was stirred vigorously for 20 minutes. $MgSO_4$ was added to "dry" up the H_2O, and the mixture was stirred for an additional 60 minutes. The $MgSO_4$ was filtered from the product, and the solvent was removed via a rotary evaporator. The product was purified using column chromatography with silica gel, 70% ethyl acetate/ hexanes (300 mL), and ethyl acetate (200 mL). **Compound (13)** was present in fractions 13-18. Yield: 216 mg.

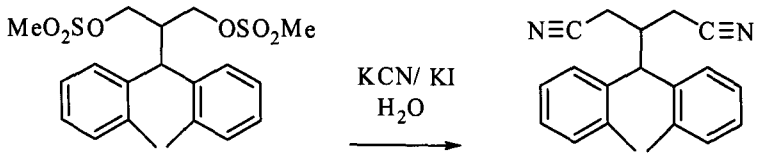
Reaction 12



(13) **(14)**

Et_3N (203 mg) and CH_2Cl_2 were combined with stirring in a flask at 0° C. Compound (13) (181 mg) was added, followed by $MeSO_2Cl$ (192 mg). Allowed the reaction to stir, and monitored the disappearance of starting material via thin-layer chromatography. The reaction was stopped after 2 hours. Poured contents of the flask into cold H_2O and extracted with ethyl acetate. Washed products with 3M HCl solution and saturated NaCl solution. Dried over $MgSO_4$, filtered, and removed solvent via a rotary evaporator. Yield: 269 mg.

Reaction 13



(14) **(15)**

Compound (14) (226 mg) was dissolved in a flask with DMF (7 mL). A solution of KCN (173 mg), KI (18 mg), and H_2O (3.5 mL) was added to the flask with stirring. The reaction was stirred at 90° C for 4.5 hours. The temperature was reduced to 60° C, and the contents of the flask were poured onto ice while being stirred vigorously. The product was extracted with ether and washed with successive portions of 3M HCl and saturated NaCl solution. The product was dried over $MgSO_4$ and filtered, and the solvent was removed via a rotary evaporator.

Results and Discussion

The synthesis outlined in Part II of this study has led as far as **Compound (15)**. Five steps remain in the synthesis, and the expected product is **Compound (20)** (DBP). Following the successful production of DBP, the focus of the project will shift to completing studies that will determine some of the unknown properties related to the compound's helical structure.

Several studies have been done to determine the kinetic properties of the inversion from the (+) to the (−) form of certain molecules[19-21], and one goal would be to perform a similar study on (+),(−) DBP. It has been theorized that the substitution of methyl groups at carbons 1 and 4 will increase the barrier to inversion of DBP relative to [4] helicene.[22] A possible goal in the future would be to test this assertion. A further goal would be to ascertain the effect of the substituted carboxylic acid groups at carbons 2 and 3 on the specific rotation of the molecule. In summation, the project has a bright future.

Acknowledgments

My sincere thanks go to all of the members of the Best Lab. Special acknowledgment goes to Matt Smith, whose work is a major portion of Part I of this project (I hope I failed to hinder his progress on most days); and Justin Reno, who is an inspiration to us all.

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