INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps. Each original is also photographed in one exposure and is included in reduced form at the back of the book.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.
NOTE TO USERS

The original manuscript received by UMI contains pages with slanted print. Pages were microfilmed as received.

This reproduction is the best copy available

UMI
DESIGN AND SYNTHESIS OF HEMITHIOINDIGO LIPIDS FOR PHOTO-CONTROLLED MEMBRANE FUSION

by

Pedro Jose Montoya Pelaez
B.Sc., Trent University, Peterborough, Ontario, 1992

A dissertation Submitted in Partial Fulfillment of the Requirements for the Degree of

DOCTOR OF PHILOSOPHY

In the Department of Chemistry

We accept this thesis as conforming to the required standard

Dr. T.M. Fyles, Supervisor (Department of Chemistry)

Dr. R. H. Mitchell, Department Member, (Department of Chemistry)

Dr. P.C. Wan, Department Member, (Department of Chemistry)

Dr. D. Paul, Outside Member (Department of Biology)

Dr. P. R. Cullis, External Examiner (University of British Columbia)

© PEDRO JOSE MONTOYA PELAEZ, 1999

University of Victoria

All rights reserved. Dissertation may not be reproduced in whole or in part, by photocopying or other means, without the permission of the author.
Abstract

The goal of this thesis was to design, synthesize and test a chemical switch for control of membrane fusion. Control of the shape of the molecules that comprise a membrane should induce a phase change in the membrane. According to current views of membrane fusion, the phase change should also facilitate formation of fusion intermediates hence should provoke membrane fusion. The design thus focused on synthetic lipid targets that have controllable shape changes. Specifically the incorporation of the hemithioindigo (HT) photochemical switch into the fatty acid chains of phospholipids was deemed a solution to the design problem.

The synthesis of four phosphatidylcholine (PC) analogues bearing two hemithioindigo moieties was accomplished. The successful synthesis starts from bromophenols. The bromide is extended to a nitrile via the Heck reaction with acrylonitrile. The thiophenol is converted to a thioindoxyl which is coupled with an aromatic aldehyde to produce the HT core. “Solventless” hydrolysis of the nitrile produces a carboxylic acid that can be coupled to a phosphoglycerol to give the target lipids. The synthetic process is both efficient and modular. All new compounds were characterized by NMR, MS and elemental analysis.

The photochemistry of various HT derivatives was studied to confirm the expected photoisomerization in both homogenous solutions and vesicle bilayers. Although the UV-Vis spectra become rather insensitive to the presence of different isomers, there is evidence to confirm the Z-E switching in a range of organic solvents and
in vesicles. Apparent bleaching of the HT-lipid may indicate a photochemical
dimerization reaction although isomerization would also be consistent with the data.

Fusion was explored by manufacturing PS vesicles with varying concentrations
and isomers of HT-lipid, and was monitored with the Terbium/Dipicolinic acid aqueous
contents mixing assay (Tb/DPA assay). The sensitivity of this assay was lower than
originally expected due to inner filter effects resulting in self-quenching the complex
luminescence. The available data suggest that the synthetic HT-lipids disturb the
membrane structure. Spontaneous fusion, apposition without metal cations, and contents
leakage are some of the observations of the complexity of this system. HT-lipids in one
population of vesicles are able to interact with a second population of vesicles,
presumably via membrane mixing. These results confirm that shape is a key factor in
the integrity of membranes, and that second generation HT-lipids have the potential to
control membrane fusion.

Examiners:

Dr. T. M. Fyles, Supervisor (Department of Chemistry)

Dr. R. H. Mitchell, Department Member, (Department of Chemistry)

Dr. P. C. Wan, Department Member, (Department of Chemistry)

Dr. P. R. Cullis, External Examiner (University of British Columbia)
TABLE OF CONTENTS

TITLE PAGE i
ABSTRACT ii
TABLE OF CONTENTS iv
LIST OF TABLES viii
LIST OF FIGURES ix
LIST OF SCHEMES xiii
LIST OF ABBREVIATIONS xv
ACKNOWLEDGEMENTS xix

CHAPTER 1. NATURAL MEMBRANES AND MEMBRANE FUSION 1
1.1 Introduction 1
1.2 Natural Membranes 2
1.2.1 Structures of Lipids 2
1.3 Model Membrane 6
1.3.1 Physical Properties: Polymorphism and Aggregated Morphology 6
1.4 Membrane Fusion 16
1.4.1 Steps to Fusion 16
1.4.2 Membrane Apposition 17
1.4.3 Models of Fusion 19
1.4.4 Biological Membrane Fusion 24

CHAPTERS 2. DESIGN OF CANDIDATE FUSION SWITCH 28
2.1 Synthetic Membranes: Examples and Limitations 28
2.2 Drug Delivery and Gene Transfection
2.3 Synthetic Amphiphile Fusion
2.4 Design of Fusion Switch

CHAPTER 3. SYNTHESIS OF THE HEMITHIOINDIGO LIPID ANALOGUES

3.1 Retrosynthetic Pathways
3.2 Thioether Synthesis
3.3 Williamson Ether Synthesis
3.4 The Heck Reaction
  3.4.1 Mechanism
3.5 Hemithioindigo Synthesis
  3.5.1 Mechanism
3.6 Phthalimidomethyl Protecting Group
3.7 The Nitrile Synthon
3.8 Hydrogenation of the Olefin
  3.8.1 Mechanism
3.9 Nitrile Hemithioindigo
3.10 Nitrile Hydrolysis of Hemithioindigo
3.11 Hemithioindigo Lipid Synthesis

CHAPTER 4. SYNTHESIS EFFICIENCY

4.1 Introduction
4.2 The Nature of the Synthetic Sequence
  4.2.1 Materials
5.4.4 Spontaneous Fusion 195

5.4.5 Fusion by Isomerization 199

5.5 Conclusion 201

CHAPTER 6. EXPERIMENTAL 203

6.1 General 203

6.2 General Procedures 204

6.2.1 General Procedures for Thioether Synthesis 204

6.2.2 General Williamson Ether Synthesis Procedure 204

6.2.3 General Procedure for Heck Reactions 204

6.2.4 General Hydrogenation Procedure 205

6.2.6 General Procedure for Hemithioindigo Synthesis 205

6.2.7 General Procedure for Nitrile Hydrolysis 206

6.2.8 General Procedure for Lipid Synthesis 206

6.3 Synthesized Compounds 207

APPENDIX 250

REFERENCES 273
LIST OF TABLES

Table 3-1: Heck Product Data of Aryl Bromide and Alkene, compiled from Cabri et al. 74
Table 3-2: Proton NMR of Hemithioindigo Derivatives (Core)/ppm 130
Table 3-3: Carbon NMR of Hemithioindigo Derivatives (Core)/ppm 131
Table 4-1: Real Yields of Path I 153
Table 4-2: Weight Summaries of Path I 153
Table 4-3: Comparison of Synthetic Pathways 160
Table 4-4: Real Weight Comparison between Path I and Path I-P 161
Table 5-1: Absorption Spectra of Hemithioindigo Compounds 168
LIST OF FIGURES

Figure 1-1: Membrane Fluid Mosaic Model of a Cell Membrane 3
Figure 1-2: Structures of Common Biological Lipids 4
Figure 1-3: The DDPC Gel-Liquid-Crystalline Phase Transition 8
Figure 1-4: Liquid Crystalline Phases of Lipids 9
Figure 1-5: Phosphatidylcholine Depicting Packing Parameters 12
Figure 1-6: Critical Packing Parameters (S) 13
Figure 1-7: Intrinsic Membrane Curvature 14
Figure 1-8: Intermembrane Forces (DLVO Theory) 17
Figure 1-9: Models for Membrane Fusion 20
Figure 2-1: Membrane Forming Module Concept 29
Figure 2-2: Aggregation Morphologies of Amphiphile 32
Figure 2-3: Amphiphile which form Bilayers in Organic Solvents 36
Figure 2-4: Relevant Molecules and their Photochromic Reactions 46
Figure 2-5: Minimized Molecular Models of Dihedral Angle Conformations 50
Figure 2-6: Modular Design of Targets 52
Figure 2-7: Molecular Model of Fusogen Isomerization 53
Figure 2-8: Molecular Models of Synthesized PC Analogues 28
Figure 3-1: Assigned $^1$H NMR Spectra of (a) 3.15, (b) 3.16, and (c) 3.17 63
Figure 3-2: Assigned $^{13}$C NMR Spectra of (a) 3.15, (b) 3.16, and (c) 3.17 64
Figure 3-3: Assigned NMR Spectra of 3.20, (a) $^1$H and (b) $^{13}$C 66
Figure 3-4: Assigned $^1$H NMR Spectra of (a) 3.24 and (b) 3.23 68
Figure 3-5: Assigned $^{13}$C NMR Spectra of (a) 3.24 and (b) 3.23 69
Figure 3-6: Assigned NMR Spectra of 3.29, (a) $^1$H and (b) $^{13}$C
Figure 3-7: Assigned NMR Spectra of 3.47, (a) $^1$H and (b) $^{13}$C
Figure 3-8: Assigned NMR Spectra of 3.49, (a) $^1$H and (b) $^{13}$C
Figure 3-9: Assigned NMR Spectra of 3.53, (a) $^1$H and (b) $^{13}$C
Figure 3-10: Assigned $^1$H NMR Spectra of (a) 3.59 and (b) 3.60
Figure 3-11: Assigned $^{13}$C NMR Spectra of (a) 3.59 and (b) 3.60
Figure 3-12: Assigned $^1$H NMR Spectra of (a) E-3.63 and (b) Z-3.63
Figure 3-13: Assigned $^{13}$C NMR Spectra of (a) E-3.63 and (b) Z-3.63
Figure 3-14: Assigned $^1$H NMR Spectra of (a) E-3.65 and (b) Z-3.65
Figure 3-15: Assigned $^{13}$C NMR Spectra of E/Z-3.65, (a) Full Spectrum and (b) Expanded Aromatic Region
Figure 3-16: Assigned NMR Spectra of 3.69, (a) $^1$H and (b) $^{13}$C
Figure 3-17: Assigned NMR Spectra of 3.71, (a) $^1$H and (b) $^{13}$C
Figure 3-18: Fine Coupling of Nitrile 3.71
Figure 3-19: Assigned NMR Spectra of 3.74, (a) $^1$H and (b) $^{13}$C
Figure 3-20: Assigned $^1$H NMR Spectra of (a) 3.75 and (b) Z-3.76
Figure 3-21: Assigned $^{13}$C NMR Spectra of (a) 3.75 and (b) Z-3.76
Figure 3-22: NMR Spectra of 3.80, (a) $^1$H and (b) $^{13}$C
Figure 3-23: Assigned NMR Spectra of Z-3.82, (a) $^1$H and (b) $^{13}$C
Figure 3-24: Assigned NMR Spectra of E-3.82, (a) $^1$H and (b) $^{13}$C
Figure 3-25: Aromatic Region Expanded $^1$H NMR Spectra of (a) Z-3.82 and (b) E-3.82
Figure 3-26: Aromatic Region Expanded $^{13}$C NMR Spectra of (a) Z-3.82 and (b) E-3.82
Figure 3-27: Assigned $^1$H NMR Spectra of (a) 3.88 and (b)Z-3.89 119

Figure 3-28: Assigned $^{13}$C NMR Spectra of (a) 3.88 and (b)Z-3.89 120

Figure 3-29: Unknown Product of 3.81 after Four Equivalents of NaOH in Ethylene Glycol (a) $^1$H and (b) $^{13}$C 125

Figure 3-30: Assigned NMR Spectra of 3.90, (a) $^1$H and (b) $^{13}$C 128

Figure 3-31: Assigned $^1$H NMR Spectra of PC-HT-6 3.106 136

Figure 3-32: Assigned $^{13}$C NMR Spectra of PC-HT-6 3.106 137

Figure 3-33: Assigned $^1$H NMR Spectra of PC-HT-m-6 3.107 138

Figure 3-34: Assigned $^{13}$C NMR Spectra of PC-HT-m-6 3.107 139

Figure 3-35: Assigned $^1$H NMR Spectra of PC-m-HT-6 3.108 140

Figure 3-36: Assigned $^{13}$C NMR Spectra of PC-m-HT-6 3.108 141

Figure 3-37: Assigned $^1$H NMR Spectra of PC-m'-HT-6 3.109 142

Figure 3-38: Assigned $^{13}$C NMR Spectra of PC-m'-HT-6 3.109 143

Figure 4-1: Example of a Plan Graph 146

Figure 4-2: Plan Graph of Path I with Real Yields 153

Figure 4-3: Plan Graph of Path III (Linear) 155

Figure 4-4: Plan Graph of Path I-P 157

Figure 4-5: Plan Graph of Path II 159

Figure 5-1: Absorption Spectra of Br-HT-6, 3.51, 5x10^{-5}M, in (a) Hexanes and (b) Dichloromethane 167

Figure 5-2: Absorption Spectra of (a) 4.8x10^{-5}M, NC-m'-HT-6, 3.89, in Hexanes and (b)3.3x10^{-3}M, NC-HT-6, 3.82, in CHCl3 169

Figure 5-3: Absorption Spectra of 1.7x10^{-5}M. NC-HT-m-6, 3.84, in Hexanes 170
Figure 5-4: Absorption Spectra of HT-Lipids in CHCl₃:
(a) 4.3 x 10⁻⁶ M PC-HT-6, 3.106
(b) 6.7 x 10⁻⁵ M PC-HT-m-6, 3.107
(c) 2.0 x 10⁻⁵ M PC- m-HT-m-6, 3.108

Figure 5-5: Absorption Spectra of PC-m'-HT-6 in CHCl₃:
(a) 1.65 x 10⁻⁵ M, Photoisomerization and
(b) 5.8 x 10⁻⁶ M, Thermal Reversion

Figure 5-6: Absorption Spectra of 4.3 x 10⁻⁵ MPC-HT-6, 5.15, in CF₃CH₂OH

Figure 5-7: Thermal Reversion of PC-HT-6 Bleaching in CF₃CH₂OH

Figure 5-8: NMR Spectra of Bleached PC-HT-6, 3.106: (a) ¹H and (b) ¹³C.

Figure 5-9: Photoisomerization of HT-Lipids in Vesicles

Figure 5-10: Fluorescence of Hemithioindigo Lipids

Figure 5-11: Figure 5-11. The Tb/DPA Fusion Assay. (a) Fluorescence Spectra of Tb(DPA)₃³⁺ and (b) Schematic of Vesicle Fusion.

Figure 5-12: The Effect of Concentration on the Tb⁺³/DPA Assay

Figure 5-13: Control for Fusion Assay (Ca²⁺ induced)

Figure 5-14: Ca²⁺ Induced Fusion with HT-Lipids

Figure 5-15: Spontaneous Fusion between PS:PC-m'-HT-6 and PS:PC Vesicles

Figure 5-16: Vesicle Leakage due to Calcium

Figure 5-17: Effect of Mg²⁺ on CF Vesicles

Figure 5-18. Attempted Fusion by Isomerization of PS:PC-HT-6 (8:2) Vesicles
LIST OF SCHEMES

Scheme 2-1: Property Directed Synthesis 45
Scheme 3-1: Retrosynthesis of PC-2-HT-6 59
Scheme 3-2: The Four Retrosynthetic Pathways Available to Form the Hemithioindigo Acid 60
Scheme 3-3: The Cannizzaro Reaction 67
Scheme 3-4: Mechanism of the Heck Reaction 72
Scheme 3-5: Coordination and Insertion Pathways 73
Scheme 3-6: Hemithioindigo Synthetic Pathways 77
Scheme 3-7: Tautomers of Indoxyl 3.35 78
Scheme 3-8: Possible Friedel-Crafts Products of Diacids 79
Scheme 3-9: Attempted HT Synthesis using Methyl Ester Protected Acid 81
Scheme 3-10: Synthesis of Indoxyl 3.47 83
Scheme 3-11: Synthetic Sequence for Path II (R=C_6H_{13}) 88
Scheme 3-12: Synthesis of Pthalimidomethyl Protected Acid 89
Scheme 3-13: Attempted Methods for Selective Deprotection of Ethyl Ester 93
Scheme 3-14: Mechanisms of Formation of 3.69 104
Scheme 3-15: Synthesis of Nitrile-HT Family 111
Scheme 3-16: Synthesis of 3.88 and 3.89 121
Scheme 3-17: Mechanism of Nitrile Hydrolysis 122
Scheme 3-18: Failed Attempts at Nitrile Hydrolysis 123
Scheme 3-19: Action of Hydroxide Anion on NC-HT-4.3.81 124
Scheme 3-20: Mechanism of Nitrile Hydrolysis by Phthalic Acid 126
Scheme 3-21: Nitrile Hydrolysis of HT Series 129
Scheme 3-22: Synthetic Pathways for Formation of the Phosphatidylcholine 132
Scheme 3-23: Phosphatidylcholine Homologue Synthesis from Natural Sources 133
Scheme 3-24: Synthesis of HT Lipid Membrane Analogues 134
Scheme 4-1: Path I of PC-HT-6, 3.106 150
Scheme 4-2: Path III (Linear) 155
Scheme 4-3: Synthesis of Path I-P 156
Scheme 4-4: Path II 158
Scheme 5-1: Mechanism of Photoisomerization and thermal Reversion of PC-HT-Lipids 171
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\gamma$</td>
<td>interfacial tension</td>
</tr>
<tr>
<td>$\nu$</td>
<td>hydrocarbon volume</td>
</tr>
<tr>
<td>$a_0$</td>
<td>optimal head group area</td>
</tr>
<tr>
<td>Ac</td>
<td>acetate</td>
</tr>
<tr>
<td>ANTS</td>
<td>aminonaphthalene trisulphonic acid</td>
</tr>
<tr>
<td>$^{13}$C NMR</td>
<td>carbon nuclear magnetic resonance</td>
</tr>
<tr>
<td>CF</td>
<td>carboxyfluorescein</td>
</tr>
<tr>
<td>CL</td>
<td>cardiolipin</td>
</tr>
<tr>
<td>COSY</td>
<td>correlation spectroscopy</td>
</tr>
<tr>
<td>DBU</td>
<td>1,8-diazabicyclo[5.4.0]undec-7-ene</td>
</tr>
<tr>
<td>DDAB</td>
<td>di-n-dodecyl dimethyl ammonium bromide</td>
</tr>
<tr>
<td>DDP</td>
<td>di(n-dodecyl) phosphate</td>
</tr>
<tr>
<td>DEPT</td>
<td>distortionless enhancement by polarization transfer</td>
</tr>
<tr>
<td>DMAP</td>
<td>N,N-dimethyl-4-aminopyridine</td>
</tr>
<tr>
<td>DMF</td>
<td>dimethylformamide</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethylsulphoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DODAC</td>
<td>dioctadecyl ammonium choride</td>
</tr>
<tr>
<td>DOPC</td>
<td>dioleoyl phosphatidylcholine</td>
</tr>
<tr>
<td>DOPE</td>
<td>dioleoyl phosphatidylethanolamide</td>
</tr>
<tr>
<td>DOPE-Me</td>
<td>N-methyl dioleoyl phosphatidyl choline</td>
</tr>
<tr>
<td>DOTMA</td>
<td>N-[2,3-(dioleoyloxy)propyl]-N,N,N-trimethylammonium</td>
</tr>
</tbody>
</table>
DPA dipicolinic acid
DPPC dipalmitoyl phosphatidylcholine
DPX p-xylylene bis(pyridinium bromide)
DSPC distearoyl phosphatidylcholine
DTP di(n-tetradecyl) phosphate
EDTA ethylenedinitrilotetraacetic acid
Et ethyl
FAB MS fast atom bombardment mass spectroscopy
Fe chain lateral pressure
Fh head lateral pressure
GPC glycerophosphocholine
GUV giant unilamellar vesicle
HA fusion protein hemagglutinin
HA1 binding site subunit of HA
HA2 fusion site subunit of HA
HC Hydrocarbon chain
Hf hexagonal phase
HII inverted hexagonal phase
HIV-1 human immunodeficiency virus-1
HRMS high resolution mass spectroscopy
HT hemithioindigo
ILA interlamellar attachment
IMI inverted micelle intermediate
L ligand
l rank
Lₜₜ lamellar phase
lₜ critical hydrocarbon chain length
LUV large unilamellar vesicle
Me methyl
MLV multilamellar vesicle
MS mass spectroscopy
n number of skeletal heavy atoms
NDB-PE N-(nitrobenzoxidazol)-PE
NMR nuclear magnetic resonance
NSF N-ethylmaleimide sensitive fusion protein
³¹P NMR phosphorus nuclear magnetic resonance
PA phosphatidyl acid
PC phosphatidylcholine
PCC pyridinium chlorochromate
PE phosphatidyl ethanolamine
PEG polyoxyethylene glycol
PG phosphatidyl glycerol
Ph phenyl
PI phosphatidyl inositol
PL phospholipid
PPh₃ triphenylphosphine
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>RET</td>
<td>resonance energy transfer</td>
</tr>
<tr>
<td>Rh-PE</td>
<td>N-lissamine rhodamine B sulfonyl-PE</td>
</tr>
<tr>
<td>S</td>
<td>critical packing parameter</td>
</tr>
<tr>
<td>S$_i$</td>
<td>sum of inverse yield</td>
</tr>
<tr>
<td>SNAP</td>
<td>synaptosome associated protein</td>
</tr>
<tr>
<td>SNARE</td>
<td>soluble N-ethylmaleimide-sensitive factor attachment protein receptor</td>
</tr>
<tr>
<td>SUV</td>
<td>small unilamellar vesicle</td>
</tr>
<tr>
<td>TES</td>
<td>2-[tris[(hydroxymethyl)methyl]-amino] 1-ethane sulfonic acid</td>
</tr>
<tr>
<td>$T_H$</td>
<td>transition temperature to the $H_{II}$ phase</td>
</tr>
<tr>
<td>TLC</td>
<td>thin layer chromatography</td>
</tr>
<tr>
<td>$T_m$</td>
<td>major phase transition</td>
</tr>
<tr>
<td>TMC</td>
<td>transmembrane contact</td>
</tr>
<tr>
<td>TMS</td>
<td>tetramethyl silane</td>
</tr>
<tr>
<td>TW</td>
<td>total weight manipulated</td>
</tr>
<tr>
<td>U</td>
<td>Van der Waals attraction</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>$V^E$</td>
<td>sum of electrostatic repulsion</td>
</tr>
<tr>
<td>W</td>
<td>total potential energy</td>
</tr>
<tr>
<td>$W_0$</td>
<td>total weight of starting material</td>
</tr>
<tr>
<td>x</td>
<td>inverse yield</td>
</tr>
<tr>
<td>y</td>
<td>yield of step</td>
</tr>
<tr>
<td>Z$_a$</td>
<td>lipid head group area in $L_a$</td>
</tr>
<tr>
<td>Z$<em>{H</em>{II}}$</td>
<td>lipid head group area in $H_{II}$</td>
</tr>
</tbody>
</table>
ACKNOWLEDGEMENTS

I would like to express my thanks to Dr. Tom Fyles for his support and guidance throughout this project. I would like to acknowledge the assistance of the technical staff of the chemistry department, in particular Mrs. Christine Greenwood and Dr. D. McGillivray.

I am grateful to all my co-workers, past and present, who have added color to this thesis. I am especially grateful to Todd, Dave R., Lynne, Xin, Daniela and Blair. I thank my fellow graduate students especially the S.P.s.

Finally, I have to thank Alexandra for all her help and for putting up with a morso.

Financial assistance from the University of Victoria was much appreciated.
1.1 Introduction

Living organisms can be considered to be thermodynamically open systems that exhibit positive entropy. Key to life is the control of material flowing to and from the organism. This flux control is what allows the highly ordered internal components of a cell to exist. Cells achieve this by possessing membranes that form the semi permeable barriers between the outside and the inside. The membranes define the cell, having as primary function the compartmentalization of biological space, that in turn allow life functions to be performed. The membranes control the flux of material through proteinous channels (ion channels), molecular recognition (antigens), chemical signaling, and endocytosis and exocytosis (fusion).

This chapter will focus on a chemical view of membranes, specifically summarizing the theories of how the supermolecular structure of the membrane is achieved and how the make-up of the molecular components dictates the former. The different structures of the molecules present in natural membranes will be documented, followed by a discussion on their common physical properties, concentrating their polymorphic behavior. Finally, membrane fusion will be defined, pertinent models will be presented, and fusion as it occurs in natural systems will be discussed. The overall direction of this thesis is to understand how fusion occurs as a result of the underlining structure of the membrane components. This knowledge can then be tested by the
development of a molecular system that can regulate the fusion process, based on a
chemical switch.

1.2 Natural Membranes

1.2.1 Structures of Lipids

The fluid mosaic model (Figure 1-1), developed by Singer et al, is the best
overall illustration of the various components that make up a membrane. The membrane
is composed of a bilayer of lipids, with associated proteins. The proteins are either
integral (spanning the whole membrane) or peripheral (embedded on the membrane
matrix). They can form supermolecular structures in order to fulfill their function. The
bilayer has a thickness that varies between 4-5nm. The major lipid components are the
phospholipids and cholesterol, the former defining the primary supermolecular assembly
of the membranes.

The molecular structures of the most common lipids are shown in Figure 1-2. These molecules are all amphiphiles, meaning that one end of the molecule is
hydrophobic, while the other is hydrophilic. The glycerophospholipids are the major
components of natural membranes. They are composed of a phosphate containing
hydrophilic headgroup, linked to a glycerol backbone that has attached to it, through ester
bonds, two hydrophobic fatty acids chains. Phospholipids are inherently chiral: only the
S isomer of the sn-2-carbon in the glycerol backbone exists in nature. The polar
headgroup has an overall neutral (PC and PE) or anionic charge (all other PLs). The fatty
acids vary from 14 to 24 carbons in length. On average the chain in position 1 of the
glycerol backbone is (more) saturated, while the chain residing in the 2 position has at
least one double bond and is two carbons longer. The unsaturations vary from 0-6 per
Figure 1-1 Membrane Fluid Mosaic Model of a Cell Membrane
Phospholipid backbone

a) Glycerophospholipids

\[
\begin{align*}
\text{Phosphatidic acid (PA):} & \quad -\text{OH} \\
\text{Phosphatidyl ethanolamine (PE):} & \quad -\text{O}^+\text{H}_3^+ \\
\text{Phosphatidyl choline (PC):} & \quad -\text{O}^+\text{H}_2\text{N}^+ \\
\text{Phosphatidyl glycerol (PG):} & \quad -\text{O}^+\text{H}_2\text{O} \\
\end{align*}
\]

b) Sphingolipids

\[
\begin{align*}
\text{Cardiolipin (CL):} & \quad -\text{OH} \\
\text{Phosphatidyl serine (PS):} & \quad -\text{O}^+\text{H}_2\text{N}^+ \\
\text{Phosphatidyl inositol (PI):} & \quad -\text{O}^+\text{H}_2\text{O} \\
\end{align*}
\]

Y = choline: Sphingomyelin

R: (un)saturated 11-23 chain hydrocarbons

c) Cholesterol

Figure 1.2 Structures of Common Biological Lipids.
chain and tend to be in the cis configuration. PC is the most common PL. In sphingomyelin, another common PL, the glycerol backbone is replaced by the sphingosine moiety. A single fatty acid is linked through an amide bond to the nitrogen of the sphingosine. When linked to the choline headgroup, this phospholipid is known as sphingomyelin. Without the phosphate group this lipid is known as ceramide. Ceramide forms membrane amphiphiles with polysaccharide headgroups, which are classed as glycolipids.

Natural membranes are not passive structures whose sole function is to be semi-permeable. There are huge differences in the actual PLs present in a membrane depending on the origin of the cell, as well as what type of cell (e.g. heart muscle mitochondria membranes have high proportion of CL compared to other tissue). There is substantial evidence that bacteria alter their PL composition depending on external stress, and that cells maintain an asymmetry in the lipid distribution between the two monolayers.

Cholesterol is the most important sterol found in natural membranes, and can represent up to 20% of the total lipid in some cells. The role of cholesterol in the biomembrane has been intensely studied. The cholesterol molecule positions itself with its polar hydroxy moiety at the hydrocarbon/water contact, where hydrogen bonding can take place. It is not as long as the average PL and thus increases the rigidity of the first nine fatty acid carbons, while leaving the remaining unaffected, or enhancing their fluidity.

The complexities of natural membranes make them very hard to study. It is common to look at simpler model systems, namely vesicles.
1.3 Model Membranes

Vesicles, or liposomes, are entities made solely from the lipid components of biomembranes. Three key elements define them: (i) they are a closed system, (ii) they enclose an internal aqueous space, and (iii) this internal compartment is separated from the external medium by a bilayer made up of discrete lipid molecules. Liposomes can be multilamellar or unilamellar. It is the latter that are of interest as models for the cell membrane. Unilamellar vesicles can be formed in three sizes: (i) small unilamellar vesicles (SUVs, 30-50 nm in diameter), (ii) large unilamellar vesicles (LUVs, 100-500 nm) and (iii) giant unilamellar vesicles (GUVs, 0.5-20x10^4 nm). SUVs and LUVs have been used the most in vesicle research. SUVs have different membrane behavior compared to LUVs because they are under curvature stress. They are in the minimum size range possible based on the maximum crowding the headgroups of the inner monolayer will tolerate. In egg PC SUVs, the area per headgroup in the inner monolayer is 18% less than in the outer, while the ratio of number of PLs of inner to outer is 1:2. GUVs have come of age with the aid of Menger and co-workers, allowing for their visualization under the light microscope. Menger’s study of “cytomimetic” chemistry has detailed transformations like aggregation, budding, fusion, and fission, as well as some understanding of vesicle morphology and how it relates to molecular events.

1.3.1 Physical Properties: Polymorphism and Aggregate Morphology

Membrane self assembly is a consequence of conflicting drives in lipid amphiphiles to hydrate and dissolve the headgroups, while isolating the hydrocarbon chains from the water. This conflict aligns the lipids into planar bilayer sheets, with the
polar groups facing the bulk aqueous phase, and the hydrophobic chains isolated in the middle. This is one example of the hydrophobic effect\textsuperscript{12}, in which energy is released from the bound water around each chain as it aggregates. The physical origins of hydrophobic interactions (attractions) and its converse, hydrophilic repulsion, are based on the net hydrogen binding interactions (or more general, Lewis acid base) energy of cohesion between surrounding water molecules\textsuperscript{13}.

Phospholipid thermodynamic properties have been reviewed on numerous occasions\textsuperscript{3,14-16}. The major phase transition, $T_m$, of a liposome, occurs when the gel state, having an all \textit{trans} configuration of the alkyl chains, changes to the liquid crystalline state that has a degree of \textit{trans-gauche} isomerization. Figure 1-3 shows a schematic of this transition for DPPC bilayers. In the gel phase the lipids are tilted with respect to the membrane plane, as the headgroup area occupied by choline ($48\text{Å}^2$) is greater than that of the of the two fatty acids ($39\text{Å}^2$), for DPPC. This way the chains are able to occupy more area, than if they were normal to the bilayer. When the chains melt to the $L_\alpha$ phase, the chains develop kinks due to \textit{gauche} configurations. Thus the area they occupy increases and their overall chain length decreases. Each PL has its unique $T_m$ which is dependent on the nature of the chains and the headgroup. Introduction of a \textit{cis} double bond forms a kink in the regular all \textit{trans} packing, lowering the $T_m$ markedly ($\approx 80 ^\circ\text{C}$ going from DSPC, $58.8 ^\circ\text{C}$ to DOPC, $-22 ^\circ\text{C}$)\textsuperscript{17}. The effect varies depending on the position of the double bond along the chain, the greatest lowering of $T_m$ taking place when the double bond is situated in the middle of the chain\textsuperscript{18}. Natural membranes possessing varied PLs have a very broad $T_m$ and low melting points insuring that the membrane is in a fluid state at physiological temperatures.
Figure 1.3. The DPPC Gel-Liquid-Crystalline Phase Transition

The fluid mosaic model (Figure 1-1) gives a very static image of a bilayer. One envisions a calm "sea of phospholipids" broken up by islands of protein. A better analogy would be the "sea" in the middle of a storm, of which the model is but an idealized average of this truly dynamic system. The gauche-trans rotations take place in the $10^{-12}$ s timeframe. As each leaflet is a liquid crystal, the individual movement of molecules in two dimensions is very rapid. The average diffusion coefficient of a lipid is $10^{-5}$ cm$^2$s$^{-1}$, which means that a lipid can travel 2 μm in 1 second. Thus, the lipid molecule could travel from one side of an average bacterium to the other in a second. Out of plane thermal excursions are also common, both having a local component (i.e. individual molecules) and long range undulations. On the other hand, transfer of lipid molecules between monolayers is slow as the flip-flop of a lipid molecule from one leaflet to the other occurs about once every 2-3 hours.

Nor does the sea of phospholipids form a random mixture. There is evidence of lateral heterogeneity in biomembranes, leading to the formation of different domains in the bilayer. A mixture of saturated and unsaturated PLs gives patches of saturated lipid alternating with unsaturated patches. Thus a hydrophobic peptide would preferentially...
Figure 1-4. Liquid Crystalline Phases of Lipids
insert into the saturated patches, suggesting the possibility of functional importance to these domains\textsuperscript{13}. Minor mismatches in the chain length of PLs, their chirality\textsuperscript{22}, as well as ether vs. ester linkages have been found to contribute to lateral heterogeneity \textsuperscript{23-25}.

The non-lamellar phases that phospholipids can form also seem to play a part in membrane properties. It is important to note that lipids form binary systems, i.e. their phase changes depend not only on the temperature, but also the water content. In this discussion, the lipid phases will be considered to always be in a fully hydrated state. X-ray diffraction was first used to identify the possible liquid crystalline phases of lipids/water: (i) the hexagonal phase $H_1$, (ii) the lamellar phase $L_{\alpha}$, (iii) the inverted hexagonal phase $H_{II}$, and (iv) a cubic phase\textsuperscript{26}. Figure 1-4 shows these four possible structures. The hexagonal phase, $H_1$ is characterized by the formation of lipidic tubes surrounded by water. In water rich mixtures this phase are related to micelles. The lamellar phase has been discussed in detail, as from it stems all membranes. The inverted hexagonal phase, $H_{II}$ is the formation of water tubes surrounded by lipid. The cubic phases consist of lipids packed in a highly symmetrical cubic lattice. The lipids are capable of converting from one phase to another, depending on their structure and the physical environment they are experiencing. For example, $L_{\alpha}/H_{II}$ transitions are influenced by the following factors: increasing headgroup size, ionization, or water content favors the lamellar phase, while increasing temperature and/or chain unsaturation favor the inverse hexagonal. This phase change is accompanied by decreases in the apparent molecular lengths\textsuperscript{27}. How this occurs is not clear. The hydration of the headgroups also changes. The $L_{\alpha}/H_{II}$ transition leads to approximately fewer bound water molecules/ lipid molecules in egg PE ($L_{\alpha}$, 28 °C, 6.9 H$_2$O/lipid molecule; $H_{II}$, 40
Structures identified as cubic phases have been found in living cells, making them of possible functional importance.

Israelachvili et al. developed a dimensionless parameter capable of predicting the morphology of the supermolecular structure adopted by amphiphiles based on their molecular structure. Figure 1-5 depicts the terms used to determine this Critical Packing Parameter (S), which is defined as:

\[ S = \frac{v}{a_0 l_c} \]

where \( v \) = hydrocarbon volume (calculated assuming hydrocarbon chain to be fluid, incompressible for maximum chain length).

\( a_0 \) = optimal headgroup area.

\( l_c \) = critical hydrocarbon chain length

This model relates the overall packing constraints (measured by S) of amphiphiles to predict the type of supermolecular assembly they will form (see Figure 1-6). If the overall amphiphile shape generated is conical (\( S < \frac{1}{2} \): large headgroup, small chain volume), then the \( H_1 \) phase is preferred, and micelles are predicted. A slightly wedge or cylindrical shape (\( \frac{1}{2} \leq S \leq 1 \)) leads to the \( L_\alpha \) phase: bilayers and vesicles; while an inverted cone (\( S < 1 \)) leads to the \( H_{II} \) phase. This simple model proves to have remarkable predictive power under thermodynamic equilibrium. The deficiencies of this model are that it does not take into account any attractive interaction energies between molecules and hence does not predict the formation of the cubic phase. Apparent exceptions to the rules have been reported. A notable one is the \( H_{II}-L_\alpha-H_{II} \) sequence of DOPE as water is removed. An alternative model based on bending, hydration, and interstitial energies accounts for this case. This model focuses on the interfacial tension acting on lipids.
Critical Chain Length. The Maximum Length Chain can Assume

\( l_c \): Molecular Area at Interface
\( V \): Volume of Hydrocarbon Chains

Figure 1-5. Phosphatidylcholine Depicting Packing Parameters
Figure 1-6. Critical Packing Parameter, S
Increased Hydration Increased Temperature Positive Mean Curvature Negative Mean Curvature

Figure 1.7. Intrinsic Membrane Curvature

There are three principal forces acting on a membrane layer (Figure 1-7a): The interfacial tension, $\gamma$, due to hydrocarbon/water contact, the lateral pressure ($F_h$) in the headgroup region, due to steric, hydrational, and electrostatic effects, which tend to be repulsive, except for H-bonding, and the repulsive lateral pressure due to gauche-trans rotations of the chains ($F_c$). Each monolayer has an intrinsic curvature, which has been related to the interfacial tension. When the temperature of the system increases, $F_c$ increases due to more disorder in the chains. This increase forces the chains to deviate from their preferred conformational state, leading to a preference for each monolayer to curve away from the alkyl chain region (Figure 1-7b), giving a negative mean curvature. Any deformation of the monolayer from this value exacts a cost on the free energy of the system. The extent of headgroup hydration has the same effect on $F_h$, resulting this time in positive curvature. When the hydration is increased, $F_h$ increases leading to a tendency for a curvature towards the chain region (Figure 1-7c), giving a positive mean
curvature. Neither of these curvatures can be jointly accommodated into the bilayer, as they would form voids. The equilibrium interfacial area per molecule thus ends up being a compromise that satisfies neither the preference of the chains nor of the headgroups. Each monolayer leaflet in a biomembrane thus finds itself in what is termed a state of frustration. Lipids in mixed systems tend to segregate, adjusting their composition to simultaneously minimize bending and hydrocarbon packing\textsuperscript{31}, but not eliminating the frustration.

The frustration energy can be relieved by a phase change. The transition from \( L_\alpha/H_{\Pi} \) allows the lipid monolayers to curl into an inverse cylinder. Even so the \( H_{\Pi} \) state still remains frustrated, as there is void space at the center of the aliphatic lattice which requires that the chains be of a certain length to occupy it completely. Chains can stretch beyond their optimal conformational state in an attempt to fill the required volume. The larger the radius of the water column the longer the chains have to be. Addition of alkanes to the bilayer was found to lower the \( L_\alpha/H_{\Pi} \) transitions as the free energy cost to the chains is now reduced\textsuperscript{31,32} The cubic phase allows for the development of curvature in the monolayers while not creating internal voids in the bilayer. These cubic phases have been observed experimentally to occur between neighboring lamellar and inverted hexagonal phases.

All lipids produce an intrinsic mean curvature in their aggregates. Molecules, like PE, which have a small headgroup area relative the chain area possess positive intrinsic curvature, and in a pure state will actually have a phase preference for the \( H_{\Pi} \) state. Other PLs that form \( H_{\Pi} \) phases under appropriate conditions are PS (pH < 3), PA (pH < 3;
Ca\textsuperscript{2+}) and CL (Ca\textsuperscript{2+}). These inverted phase-forming lipids are common in biological membranes, representing on average 30mol % of the total lipid.

The presence of lipids that can be seen to have a packing parameter of more than one, or a negative intrinsic curvature, hence favoring the $H_\text{II}$ phase in natural membranes, begs the question: "What are they doing there?". There is no apparent advantage to have them for the purpose of membrane stability since they actually destabilize the $L_\alpha$ phase. It is obvious that there are other functions that the lipids perform in the membrane. One candidate function is membrane fusion.

1.4 Membrane Fusion

Membrane fusion is a fundamental biological process that allows for the bulk transport of material in and out of the cells of a living organism. It is linked to many cellular processes like endocytosis, exocytosis, cell division, neuron signal transmission between cells, fertilization, and infection of cells by viruses. These processes are coupled to both integral and peripheral proteins that bring the two fusing membranes together. The actual fusion mechanisms are poorly understood. How the proteins destabilize the membranes and whether the lipids take an active part in the process has yet to be answered. To get a clearer picture of the process, one can look at the 'bare bones' steps needed to obtain membrane fusion in a protein free model system.

1.4.1 Steps to fusion

Membrane fusion can be divided into the following steps:

1) Trigger. The trigger is the initiating event that occurs either before or after the membranes are apposed. The actual sequence depends on the fusion system. Generally,
in model systems the trigger initiates apposition, while in natural systems, apposition is the more common first step.

2) Close approach of membranes: membrane apposition.

3) Lateral reorganization of membrane components and any initial interaction (e.g. recognition) between membrane components included.

4) Destabilization of membranes by formation of intermembrane intermediates.

5) Communication of the internal aqueous compartments and complete mixing of membrane components. Leakage may occur\textsuperscript{33}.

1.4.2 Membrane Apposition

\begin{figure}
\centering
\includegraphics[width=0.8\textwidth]{dlvo_intermembrane_forces}
\caption{Intermembrane Forces (DLVO Theory)}
\end{figure}

The interaction energy between two membranes can be described by the DLVO (Derjaguin, Landau, Verwey, and Overbeek) electrostatic double layer (Figure 1-8). It can be broken down to the following energy terms:
\[ W = V^E + U + V^H \]

where \( V^E \) is the electrostatic repulsion; \( U \) is the van der Waals attraction; and \( V^H \) is the hydration repulsion. An in depth analysis of these interactions can be acquired in several reviews\textsuperscript{33,34} on the whole topic of membrane fusion. Menger\textsuperscript{9} also suggests that a stiffness factor relating to the composition and the structure of the membrane molecules must be included.

For membrane fusion to occur, membranes must come in contact with each other. The interactions that come into play are the opposed attractive van der Waals forces and the repulsive electric forces, \( V^E \) and \( V^H \) (See Figure 1-8). The total energy generally goes through two minima, a shallow long distance secondary minimum, and a primary one of greater depth. For membrane fusion to occur, the primary minimum must be reached. The depth of this minimum is determined by the stability of the aggregate. Rand et al. have found that the hydration repulsion, \( V^H \), between the membranes is much greater than electrostatic repulsion, \( V^E \), making it the major barrier to apposition. The magnitude of the hydration force is dependent on the dielectric polarization of water. The headgroup regions of bilayers made of PC are relatively bulky compared to PE, hence they pack poorly and must be surrounded by more polarized water than PE. PE has zwitterionic compact surfaces and is able to form hydrogen bonds between its ammonium groups that allow it tighter packing and hence lower degree of hydration\textsuperscript{35}. This shows up in the interaction energies, e.g. the equilibrium distance for bilayer apposition of egg PE (19Å) is about 6Å closer than egg PC (25Å), and the energy minimum is 10 times deeper. Removal of this polarized water must be achieved to bring on fusion, making dehydration a key precursor to the process\textsuperscript{36,37}. 


Charge neutralization is another requirement for fusion to occur. When the two vesicles have an overall charge of the same sign, the electrostatic repulsion is higher and results in the energy minima being considerably decreased and, thus, the equilibrium distance increased. This charge can be screened by cations, of which the most effective is Ca\(^{2+}\). Charge neutralization and membrane dehydration are not the only conditions that are needed to induce fusion, the crucial final step being membrane destabilization.

1.4.3 Models of Fusion

Based on the premise that the lipids are key to membrane fusion in biological systems, models investigating the fusion of bilayers in pure phospholipid systems have been researched. Once the two membranes are apposed to each other the destabilization step must proceed in a fashion as to not compromise the structure of the liposome. Also, this process must not involve a rupture followed by a resealing of the membranes. This would result in a leaky process and lead to loss of the liposomes' content. Three different models of nonleaky intermembrane intermediates have been proposed (see Figure 1-9).

A) There are no defined fusion intermediates. The two apposed membranes undergo a local disordering of the PL which then leads to fusion. This is suggested as the possible pathway of fusion for anionic phospholipids, pure or in mixtures with zwitterionic or neutral lipids. Examples of this would be fusion of PA and PS induced by Ca\(^{2+}\).

The combination of Ca\(^{2+}\) with PS is particularly potent, totally dehydrating the bilayer and freezing the hydrocarbon chains of the molecules. This fusion is a result of bilayer rupture due to adhesion stress. Calcium ions reduce the proton acceptor capacity of the phospholipid membrane, in effect increasing the hydrophobicity of the surface.\(^{34}\)
Figure 1-9 Models for Membrane Fusion
This increased hydrophobicity seems to be the main driving force for the membrane fusion\textsuperscript{38} In a PC/PG system, addition of Ca\textsuperscript{2+} causes local phase separations by preferential binding of Ca\textsuperscript{2+} to the anionic PG lipids, creating a PG rich "contracted" domain, and a PC rich "expanded" domain. The latter experiences an increased hydrophobic effect, which results in a long range hydrophobic attraction between such regions. Upon contact these regions fuse\textsuperscript{39}. This suggests that fusion does not necessarily occur in the region of calcium binding.

This model is thought to be the least likely to have physiological importance, as the energy required to achieve a local defect is large and likely prohibitive. There is little potential for control, hence leakage is prevalent in these systems.

B) The Inverted Micelle Intermediate (IMO\textsuperscript{40-42}). The cubic phase is only accessible to the subset of lipids that are able to form inverted hexagonal (H\textsubscript{II}) phase. The initial defect between the two apposed membranes is thus postulated to be an inverted micelle intermediate (IMI), which forms at temperatures below the temperature of the transition to the H\textsubscript{II} phase (T\textsubscript{H}). At and above T\textsubscript{H} several IMIs aggregate in the plane of the apposed membranes, rapidly evolving to the H\textsubscript{II} phase. Liposome aggregation above the T\textsubscript{H} temperature thus leads directly to lysis. Another possible pathway open to the IMI, when in isolation, is to form another type of intermediate: the interlamellar attachment site (ILA), which in turn results in the formation of the cubic phase. The ILA can form into a variety of structures, all which assemble into distinct cubic forms.

Experiments with DOPE-Me (N-monomethyl-dioleoyl-phosphadityl-ethanolamine) liposomes fusion gave support for this mechanism. Isotropic \textsuperscript{31}P NMR
resonances were correlated to the beginning of a transition from the lamellar $L_a$ phase to an inverted cubic phase$^{43}$. This mechanism is also applicable to PE/PC membranes.

The rate and outcome of the $L_a/H_{II}$ transition depends on which of the following two processes is faster$^{41,42}$:

(i) aggregation of pairs of IMIs into $H_{II}$ precursors or

(ii) ILA formation from individual IMIs.

The kinetic model used assumes that in the vicinity of $L_a/H_{II}$ phase transition, the number of IMIs/cm$^2$ of apposed bilayers reaches a steady-state, $n_1^0$. Process (i) has thus a rate given by

$$r_{HII} = k_1 (n_1^0)^2$$

where $k_1$ is a 2$^{nd}$ order rate constant (of aggregation)

Process (ii) rate is

$$r_{ILA} = k_{ILA} n_1^0$$

where $k_{ILA}$ is a first order rate constant

If $k_{ILA} < k_1 n_1^0$ then the $H_{II}$ phase predominates

If $k_{ILA} \geq k_1 n_1^0$ then the ILA phase predominates

$k_{ILA}$ is sensitive to an equilibrium structure property of the lipid, $Z$, which determines which process predominates.

$$Z = \frac{\text{Lipid headgroup area in } L_a}{\text{Lipid headgroup area in } H_{II}}$$

For values of $Z \leq 1.2$ the ILA phase predominates. It is obvious that $k_{ILA}$ takes into account the curvature of the monolayers due to the headgroup lateral stress. The IMI is
the precursor to either H_{ll} or cubic phases. In effect, the Z parameter is a kinetic equivalent of the packing parameter (S).

The presence of "lipidic particles" in freeze-fracture micrographs in mixtures of L_{α} and H_{ll} preferring lipids was thought to be evidence for the IMI^{44}. There is now some doubt as to this assignment. Modern fast-freeze freeze fracture studies have revealed that the particles are not present during the initial rounds of fusion, suggesting that they are in fact the ILAs. These structures thus result from the fusion rather than being the causative agent.

C) The Stalk Model. Proposed by Chernomordik et al.{45}, the stalk model has the first intermediate being the formation of a semitoroidal connection between the two outer monolayers. The energy required for this connection is paid for by the out of plane thermal undulations that membranes possess. They are powerful enough to overcome the local hydration forces and bring the membranes together. At this close contact the hydration energies become prohibitive. The system adjusts by replacing these energies with the hydrophobic energy gained when the two contacting leaflets rupture^{46}. The rupture then expands radially to form a hemifusion intermediate, also known as the trans monolayer contact intermediate, TMC. The TMC is short lived and ruptures to form an ILA or pore in the bilayer^{47}.

Siegel developed his first fusion model to include the IMI as the first fusion intermediate. Further analysis, based on theoretical modelling, made him modify the model to favour the stalk intermediate, instead of the IMI^{47,48}. By defining the geometries of the possible intermediates, a comparison of their free energies was made. The stalk model was found to have lower energies than the IMI. The same kinetic model
as used for the IMI applies for the stalk model, except that the TMCs replace the IMIs. Thus an accumulation of TMCs still leads to the $H_{11}$ phase and leakage. The stalk model is presently considered to be the most likely mechanism of membrane fusion. It is clear that lipids having either negative curvature or an $S$ greater than 1 would stabilise the stalk intermediate, as the stalk intermediate has a net negative curvature.

As can be seen from the intermediates postulated in these models there is a requirement for negative curvature in all of them. It is thus fair to state that the presence of lipids that have negative intrinsic curvature in natural membranes could have as one of their functions the minimizing of the energy needed for these intermediates and the facilitation of fusion.

1.4.4 Biological Membrane Fusion

Even though membrane fusion is ubiquitous in cells, the systems that have been studied in detail are few. The main problems are the intricate nature of the systems, that have several proteins, co-factors, and prosthetic groups involved. Since some of the proteins are associated with the membrane, isolation and reconstitution in an artificial membrane has been problematic. Below are two examples of the best characterized natural systems.

The transport of cellular cargo from one organelle to another within membrane bound vesicles is a process that is governed by proteins that reside both on the vesicle and the target membrane. The involved proteins are known as NSF (N-ethylmaleimide sensitive fusion protein), SNAPs (synaptosome-associated protein), and SNAREs (soluble N-ethylmaleimide-sensitive factor attachment protein receptors). Recently all their structures have been determined (Sutton, 1998), as well as the pathway of the
protein assembly. The roles each protein plays in fusion mechanism, as well as that of Ca\(^{2+}\), if any, is far from elucidated, due to the complexity of the pathway.

The best characterized natural fusion processes have been related to viral infections of cells. In this process the viral envelope merges with the target cell membrane and delivers, after fusion, the viral nucleocapsid into the cytoplasm. By far the most studied is the influenza virus\(^43,50-52\).

The influenza virus fusion protein hemagglutinin (HA) extends about 135 Å above the viral membrane surface and consists of two subunits, HA\(_1\) and HA\(_2\), which contain the binding site and the fusion site, respectively. The initial step involves the attachment of an HA trimer to the cell membrane via the sialic acid binding sites on HA\(_1\). At low pH HA undergoes a conformational change in which the HA\(_2\) subunit exposes its amphipathic fusion peptide. This peptide is α-helical in areas adjacent to a lipid lined pore and random coil in the aqueous space\(^43\). The protein structure and the distance the HA\(_2\) peptide has to reach make it highly improbable that the fusion site actually interacts with the cell membrane. There is growing evidence that the HA structure undergoes drastic rearrangements which results in the formation of a long helix with the fusion peptide at the tip. The HA trimer is also thought to assume a tilted position that minimizes the distance the helix must cover\(^51\). The formation of an intermembrane intermediate of various structures has been proposed. Bentz\(^43\) has proposed that an aggregation of HA trimers, with their fusion peptides situated laterally, dehydrate the intermembrane space, which in turn promotes an IMI that either reverts to apposed membranes or progresses to an ILA. Siegel favours the stalk mechanism\(^50\). Recent work by Chernomordik alludes to the formation of a dimple in the membrane that, due to
binding stresses on the lipidic top of the dimple, facilitates fusion\textsuperscript{52}. The pore diameter size has been estimated to be around 4 nm. After the pore formation, the HAs diffuse apart, thereby widening the pore and leading to the final step of fusion, communication of the internal aqueous compartments of the cell to the inside of the viral capsid.

Whatever the mechanism HA uses for fusion, there is evidence that it is shared by other fusion proteins. Ebola and HIV-1 viruses have fusion proteins that share common structural features with influenza suggesting a common fusion mechanism\textsuperscript{53}. The sperm protein fertilin, thought to be responsible for membrane fusion during fertilization also shares common characteristics with viral fusion proteins\textsuperscript{51}. One must be careful in making such generalisations, as the NSF/SNAPs/SNAREs fusion mechanism does not have the common characteristics of those mentioned above.

There is also increasing evidence that the lipids are active participants in the fusion process, rather than in a secondary passive nature. It has been shown that membrane fusion events can be influenced by the membrane lipids\textsuperscript{46,54}. Introduction of lyso-lipids to a natural fusion system always leads to inhibition, while introduction of cis-unsaturated acids (arachidonic or oleic) leads to promotion\textsuperscript{46}. Both of these cases can be explained by looking at either the shape of the added amphiphiles or their intrinsic curvature. N-acyl derivatives of lipids have been found to accumulate under conditions of degenerative change of membranes, and are thought to imbue protection against such damage\textsuperscript{54}. A model study using N-stearoyl-PS liposomes found them to be more resistant to fusion by metal cations (Ca\textsuperscript{2+} or Mg\textsuperscript{2+}) than PS liposomes\textsuperscript{54}. Work by Epand et al. has shown that the viral fusion peptides can influence the morphology of the membranes: the influenza HA\textsubscript{2} protein, at pH 5, promotes the formation of cubic phases.
thus changing the kinetics of the $L_a/H_{II}$ phase change\textsuperscript{55}. In a further study with the feline leukemia virus fusion peptide, it was suggested that the peptide increases the negative curvature of the lipid system and decreases $T_{II}$\textsuperscript{56}.

In conclusion, one can appreciate the complexity not only of the natural membranes, but also of how they attain their functions. Irrespective of what protein system the cells use to cause fusion, they all must destabilize the apposed membranes, since simple apposition is not enough. The natural systems are further complicated by the need of the cell to regulate the fusion, as well as have positional control of where it occurs. This is not so critical in a viral system, hence the apparent simplicity. Nature would not reinvent the wheel each time it needed a fusion system (or membrane system as a case of point) and so one can assume that the fusion mechanisms available are not infinite. I believe that the lipids play a huge role in these possible mechanisms. The proteins that control membrane fusion do so by achieving a state of apposed membranes\textsuperscript{34} and by changing the properties of the lipids (namely their curvature) to induce membrane defects and hence destabilization. The goal of this thesis is to devise a strategy that would allow for the examination of the effects of modular changes to synthetic amphiphiles vis-à-vis membrane fusion. The strategies involved to date and the plan to achieve this goal are discussed in Chapter 2.
Chapter 2

Design of the Candidate Fusion Switch

2.1 Synthetic Membranes: Examples and Limitations

The phospholipids do not solely possess the ability to form bilayers, and their general structure is not the only way nature forms biomembranes. Archaebacteria have unique molecules that are best classified as bolaamphiphiles, containing two polar headgroups, linked together with two C₄₀ polyisoprenoid chains, which form the membrane as a monolayer⁵⁷, of which compound 2.1 is a representative example (isolated from Sulfolobus solfataricus)⁵⁸.

Kunitake et al. was able to form the first totally synthetic bilayer by sonicating di-\textit{n}-dodecyltrimethylammonium bromide (DDAB), 2.2, and since then there has been no looking back. The number of vesicle forming synthetic amphiphiles is vast and has been reviewed⁵⁹,⁶⁰. The main advantage that synthetic amphiphiles have over their natural counterparts is that they can be readily modified, and hence molecular design from structure to function is possible.
Figure 2-1. Membrane Forming Module Concept

Type A
Tail Connector Spacer Head

Type B
Rigid Segment Tail Connector Head

Type C
a) Modules of bilayer forming amphiphiles

Type D

Type E

Type F

Type G

Type H

b) Modules of bilayer forming bolaamphiphiles
The design principle of component amphiphiles and the molecular organization can be looked at in a module concept, as shown in Figure 2-1. The different modules can be classified into five categories: (i) tail (hydrophobic saturated or partially unsaturated hydrocarbon), (ii) headgroup (hydrophilic moiety), (iii) connector and (iv) spacer (linker between modules), and (v) rigid segment. One can then identify two families of molecules, amphiphiles (Figure 2-la) and bolaamphiphiles (Figure 2-1b), that can form a membrane assembly. These families can be divided into separate classes.

![Chemical structures](image)

Type A amphiphiles are made up of at least one headgroup, and two tails, the connector and linker not being essential. This covers the majority of natural phospholipids as well as synthetic bilayer forming amphiphiles. The simplest synthetic examples are the cationic dialkylammonium salts (like 2.2), which produce bilayers and vesicles, requiring tails to have at least 10 carbons. The headgroup can also be anionic (sulfonate, phosphate), neutral (oxyethylene oligamer), or zwitterionic. The size of the headgroup is important. Charged headgroup (positive, 2.3 or negative, 2.4) amphiphiles give bilayers, while the neutral analogue, 2.5 gives the H\textsubscript{II} phase. The deprotonated form of 2.5 transformed into vesicles. The connector module has been varied vastly and its
major function is to help promote the alignment of the alkyl chains. The spacer unit is important in determining the molecular orientation in the bilayer. The synthetic bilayers with identical chains display tilting normal to the bilayer, giving unsymmetrical chain packing. The gel to liquid crystal phase transitions can be affected by the conditions under which the membrane is produced. Amphiphiles of the homology of \( 2.2 \) (1-2wt% in \( \text{H}_2\text{O} \)), when sonicated give broad \( T_m \) peaks, but not when frozen (-50 °C); rather the \( T_m \) is sharp and occurs at higher temperatures. This suggests that the molecular organization of the sonicated samples is not uniform. Uniform bilayer organization can be maintained when the possibility of hydrogen bonding and/or dipolar interactions between component molecules exists.

Monoalkylated amphiphiles do not normally form membranes as the difference between the area occupied by the headgroup and chain is too great. The introduction of rigid segments (Type B) into monoalkyl surfactants, increases their effective area, restricts their conformations, and enhances intermolecular interactions, making the molecules better oriented, thus producing stable bilayer assemblies. Once again, a certain tail length is needed for amphiphiles to form membranes, and the variation of the tail to spacer ratio can affect the molecular orientation within the bilayer. This can be illustrated by examining the azobenzene-containing amphiphile homology \( 2.6 \). As illustrated in Figure 2-2, for short tailed \( 2.6 \) (\( n=8, m=10 \)) the morphology is interdigitated, and stable monolayers cannot be formed, while longer tailed \( 2.6 \) (\( n=12, m=10 \)) results in the H-aggregate. If the spacer is short
compared to the tail. 2.6 (n= 12, m= 5) then the J-aggregate results. Benzylideneaniline and salicylideneaniline were used to replace the azobenzene rigid segment of 2.6, and were found to have similar morphological aggregations. As long as the rigid segments are physically similar and the appropriate alkyl chain lengths are chosen, the aggregate morphology can be predicted. If the rigid segment is altered then other morphologies are possible. There is a correlation between the rigid segment's molecular structure and assembly patterns. Bent rigid segments give rise to curvatures that result in a radial molecular arrangement (rodlike or tubular). Double meta substitution at the rigid segment produces less organized aggregates. A disk morphology can be produced by an appropriate mix of amphiphiles (straight and bend rigid). Experiments using type A (2.2) amphiphiles mixed with 2.6 show that phase separation occurs as the temperature is lowered.

Type C amphiphiles having triple chains must allow the headgroup to protrude, while allowing compact chain packing to form membranes, and thus require connectors.
as a basic module. Trialkylmethylammonium amphiphiles thus do not give bilayers as they do not fulfill the above conditions. An ester connector is enough to relax these packing constraints.

Type D amphiphiles containing four alkyl chains form bilayers that are similar to the Type As. There is a strong dependence of chain alignment on the connector, which regulates the stability of the aggregate. Cardiolipin is a natural PL of this class, but CL does not form bilayers, as its headgroup is too small and the $H_{II}$ phase is favored.

Type C and D amphiphiles are less common, as they are synthetically more complicated to make and offer no advantages or special properties over Type A or B amphiphiles.

The bolaamphiphile family (Figure 2-1b) emulates the archaeabacteria lipids, which by their very nature form monolayer membranes, not bilayers. They can exist in different classes: Type E Head(H)-Spacer(S)-Head(H), where the spacer is a macrocycle; Type F, Head(H)-Spacer(S)-Head(H), where each head also has an alkyl tail attached; Type G, H-S-rigid segment(R)-S-H; and Type H, H-S-R-S-R-S-H. Type E closely resembles the natural bolaamphiphiles, but due to the macrocycle spacer are difficult to synthesize. The Type F are the synthetically simpler equivalents of the Type E. Type G uses the rigid segment to stabilize the aggregates as discussed for the Type B amphiphiles. All the three types (E, F, G) discussed aggregate into monolayer lamella while Type H forms rodlike structures. This is due to the middle linker in Type H molecules allowing the flexibility needed for bent molecular packing.
bolaamphiphiles that are symmetrical, like 2.7, have no inherent curvature and so do not form vesicles on their own. If cholesterol is added to 2.7 in a 1:3 ratio and the mixture is sonicated, then vesicles are formed. The cholesterol preferentially resides in outer leaflet and gives the curvature needed\textsuperscript{60}. Making the headgroups asymmetric, i.e. one head bigger than the other, as occurs with natural bolaamphiphiles, is another way to induce curvature.

Properties can also be modulated by stereochemistry. Membranes made from chiral components are arranged asymmetrically in fixed spatial dispositions and result in formation of higher order structures\textsuperscript{60}. S-2.8 forms bilayers which with time convert into a right handed helical morphology made up of twisted tapes. When heated they melt back into vesicles. The stereoisomer, R-2.8 does the same, with an opposite handed twist.

Racemic amphiphile 2.8 gives initially vesicles which evolve into rodlike structures. The spacer length is critical to helix formation. The amphiphile related to 2.8, having one carbon instead of ten in the spacer, does not form the helices. This property has been used to as a source for advanced materials, for example, in the development of lipid tubules. An in-depth study on tubule formation by 1,2-bis (tricosa-10, 12-diynoyl)-sn-glycero-3-phosphocholine 2.9, revealed that the structure requirements can be modulated by altering different components of the lipid\textsuperscript{63}:
headgroup (size and charge); chirality at the glycol linkage, linkage (ester, ether, none). and arrangement of the linkage (1,2 or 1,3); position of the diacetylene group, length of chain, and chain terminus (vinyl, nitrile, none). The ability to vary specific sections of the amphiphile has allowed control over the structure formation. It is important to state that the choice of solvent, temperature and counterions were also crucial to the formation of the tubules.

Amphiphiles containing fluorocarbon instead of hydrocarbon tails are another source of membranes. The same families and classes mentioned in Figure 2-1 are possible, with some or all of the hydrogens in the hydrocarbon tails replaced by fluorines. The aggregation morphologies are similar to their hydrocarbon (HC) counterparts, but some of their physical properties are different. The enthalpy change associated with the gel to liquid crystal phase transition, T_c, is smaller and membrane fluidity decreases with increasing number of fluorocarbons. The fluorocarbon bilayer is much less permeable than the HC layer to ions and small molecules. The fluorocarbon portions are not miscible with hydrocarbons or water. This cohesive force makes mixed amphiphiles, with both hydrocarbon and fluorocarbon, develop into better single walled vesicles. The vesicles have separate domains, or internal structure, that strengthens the vesicles.\textsuperscript{60}

The immicibility of fluorocarbons with hydrocarbons as been used by Kunitake et al.\textsuperscript{64} to create bilayer assemblies in aprotic organic solvents. The hydrophilic headgroup is replaced by a long tailed alkyl chain as shown in Figure 2-3.
Figure 2-3. Amphiphiles which Form Bilayers in Organic Solvents

Compounds 2.10-2.12 gave stable bilayer assemblies in benzene and chlorocyclohexane, that had diverse morphologies: tubes, tapes and rods\(^6\). The assemblies underwent bilayer to monomer phase transitions. These aggregates are not as stable as their aqueous counterparts, but more akin to aqueous micelles. The solvophobic effect responsible for aggregation in these systems has the common characteristic that the cohesive force of the medium molecules is stronger than that of the solute molecules. It is thus driven by an enthalpic force. The magnitude of the solvophobicity is related to the interfacial tension of a given liquid combination. The interfacial tension of water/fluorocarbon is 50-60 mN/m, of water/hydrocarbon is ~45 mN/m, and for hydrocarbon/fluorocarbon is 10-15mN/m. The stabilities of organic amphiphiles can be increased by components that are capable of hydrogen bonding or increased van der Waals interaction (as in 2.12, which is more stable that either 2.10 or 2.11).
The above summary of synthetic amphiphiles shows that not only can they display the same functions and morphologies as lipids in natural membranes, but novel ones as well. Other applications for these amphiphiles have been found in i) use as substitutes of natural lipids for protein and enzyme models and reconstruction, (ii) control of reactions by domain formation, and (iii) designed alignment of photonic, electronic and magnetic functional units. Applications involving membrane fusion will be discussed in the following sections.

2.2 Drug Delivery and Gene Transfection

Synthetic membranes have allowed the study of membrane fusion to encompass systems that are not found in the natural systems, specifically cationic amphiphiles. Synthetic vesicles have developed into fields of their own, where the most rapid growth has been in drug delivery and gene transfection research.

Amphiphilic systems in all their phases have been used as drug delivery vectors, but by far the most research has been in the use of vesicles. Vesicles made up of natural components were disappointing as delivery agents because they were cleared rapidly from the body and showed inability to deliver the drug to the cytoplasm. Most vesicles are rapidly removed from the systemic circulation by the macrophages of the liver, spleen, and bone marrow. The normal pathway of liposome cell uptake involves the endocytosis to the cell’s endosomes, followed by delivery to the lysosomes that can neutralize the drug. There has been some success using vesicles that are pH sensitive allowing them to degrade in the cell’s endosome, and hence, release their cargo before reaching the lysosomes. The next generation vesicles were coated with a hydrophilic polymer, notably polyoxyethylene glycol (PEG), which greatly increased their half-life in
the body (from minutes to days). These so-called "stealth liposomes" are thought to act by reducing the absorption and adhesion of immunoglobulins and antibodies that mark foreign objects in an organism defense mechanism. Amphiphiles have also been synthesized to take advantage of the enzymes present in the body. A normal substrate for a specific enzyme is incorporated as a mask of the function of one of the vesicle components. The enzyme can thus be used as a fusion trigger. In one example, N-acetyl-ala-ala-ala-DOPE is used to mask PE, allowing for stable vesicles, which are then rendered fusogenic when the peptide is cleaved by leukocyte elastase or proteinase K.

Specificity of drug delivery to cancer cells can be realized by targeting the enzymes that these cells have a propensity to produce. Neuroblastomas are known to exude large quantities of acetylcholinesterase. Compound 2.13 is able to form vesicles, and is also a substrate for acetylcholinesterase. The enzyme removes the acetate moiety unmasking a hydroxy group that then attacks one of the esters to form a six-membered lactone and eliminates an alkyl chain. With only one chain the amphiphile is not able to maintain the bilayer structure, and the vesicle eventually ruptures, releasing the drug.

Gene therapy involves the delivery of DNA to the nucleus of a cell as a means of correcting defective genes. DNA can thus be considered a drug that can treat or cure any hereditary disease. It is a field that is still at its infancy, which poses many technical problems. The major one is that the effectiveness of transfection must be vastly improved to be a viable therapeutic method. To this end, research is focused on understanding the success of the increased transfection rates of DNA in cationic
vesicles\textsuperscript{77-80}. This process is poorly understood, for all the steps have not been elucidated, and the fusion mechanism is unknown. DOTMA(2.14)/DOPE vesicles with DNA were the first successful complex and hence the most studied. When DOPE is replaced by DOPC  

\begin{center}
\includegraphics[width=0.5\textwidth]{structure.png}
\end{center}

transfection was inhibited. The nature of the linkages (ether/ester) does not seem to play a part in transfection. The initial lipid:DNA complex interactions are driven by electrostatics, where maximal uptake occurs when the charge ratio is \( \geq 1.5:1 \).\textsuperscript{81} The structure of the complex is debated. The complexes are very heterogeneous and dynamic, varying in size and shape depending on the ratio of cationic lipid to DNA\textsuperscript{77}. X-ray diffraction data of DOTAP(2.15)/PE complexed with DNA revealed that the DNA is sandwiched in highly ordered multilamellar structures made up of cationic bilayers. This lamellar structure could be converted into an \( H_{II} \) phase if the spontaneous curvature of the system were made more negative\textsuperscript{82}. The \( L_\alpha \) structures were found to bind stably with anionic vesicles, while the \( H_{II} \) structures, being more unstable, fused rapidly and released the DNA. The molecular shape factor for the transfection amphiphiles displays a tendency towards nonbilayer structures. These are thought to be needed for both the fusion and translocation processes in transfection, since in both, membrane destabilization has to occur. Curvature stress has been investigated as a factor in cationic fusion of DOTAP/PE. Cationic vesicles having differently unsaturated PEs were used to show that the stress
curvature was correlated to fusion. LUVs made from DOTMA/DOPE and DOTMA/DOPC can be made to fuse, using pCMV5 plasmid DNA. Addition of the plasmid to the DOTMA/DOPE LUVs causes an isotropic $^{31}$P NMR signal that is consistent with the presence of non-lamellar phases. The target cell's lipid composition effects on transfection (for DODAC/DOPE cationic vesicles) were also studied. Fusion was increased by increased negative surface charge and, for lipids in the $L_{\alpha}$ phase having increased chain unsaturation. The fusion between the lipids of the complexes and the cellular membranes does not determine the efficiency of transfection, suggesting that is determined by another process, such as transfer of the DNA to and into the nuclear membrane. Projects to design better transfection amphiphiles by synthesizing a homologous series have produced contrasting results. The best candidates from in vitro experiments did poorly in vivo, and vice versa. The model used did not predict the best transfection liposome, suggesting that designs should incorporate structures that are outside the model. PEG linked with ceramide has also been used to generate the “stealth liposomes” equivalent for transfection, which increases the circulation time of DOTMA/DOPE vesicles. Another avenue of gene therapy is to use viral fusogenic proteins to create virosomes. The success of the avenue is based on the virosomes being able to mediate fusion between liposomes and the target cell.
2.3 Synthetic Amphiphile Fusion

Phospholipid vesicles fuse on a very fast time scale. Some synthetic amphiphiles that undergo the vesicle fusion process have a propensity to be more controllable, and hence aggregation structures and intermediates are easier to study and define. DDAB vesicles were made to fuse in the presence of DPA. Only large vesicles fused, being susceptible to fusion when the bilayers were in the $L_\alpha$ state. A point defect mechanism is suggested. The DPA dehydrates the vesicle and forms trans complexes (similar to the $Ca^{2+}/PS$ system), which produce the defects. When synthetic vesicles fuse with liposomes two types of fusion are possible: (i) fusion with themselves (symmetric) or (ii) fusion with the lipid vesicles (asymmetric). Di(n-dodecyl)phosphate (DDP) and di(n-tetradecyl)phosphate (DTP) were able to perform asymmetric fusion with PS and PC as long as they were in the gel state, while the target vesicles were fluid. Both cases showed increase fusion rate by the addition of $Ca^{2+}$. Without the target vesicles the cationic vesicles transform into the $H_{II}$ phase, whereas with them larger vesicles are the predominant structure. Fusion was also possible with DOTMA/PE and PS/PE or PS/PC vesicles in which the electrostatic interaction between DOTMA and PS headgroups is enough to dehydrate the membranes and cause the fusion. Complimentary hydrogen binding headgroups have also been used successfully to induce aggregation and fusion. Vesicles independently containing 10% triaminopyrimidine (2.16) or barbituric acid (2.17), prepared in 1:9 of PC, mixed in a 1:1 ratio ultimately
gave larger vesicles\textsuperscript{91}. These headgroups form self-assembled, extended ribbons due to their double-faced nature. This likely follows the Ca\textsuperscript{2+}/PS fusion mechanism.

The chirality of the vesicle components can dictate the fusion behavior. The diastereomers, 2.18-2.20, of two phosphate groups differ in their aggregation behavior due to their spatial orientation\textsuperscript{92}. SUVs composed of either R,R (2.18) and S,S (2.19) isomers undergo fusion when Ca\textsuperscript{2+} is added, while the R,S (2.20) isomer undergoes fission. The vesicles formed by all isomers seem to have interdigitated alkyl chains before the addition of Ca\textsuperscript{2+}. The S,S and R,R aggregates form intermolecular complexes with the cation, which gives a stretched conformation inducing fusion. The R,S isomer forms a more compact intramolecular complex with the cation, which reorganizes the membrane into a highly curved bilayer that undergoes fission before finally transforming into tubular structures.

A novel method was employed by Smith et al. to mimic membrane fusion\textsuperscript{93}. They synthesized a lipid derivative that contained boronic acid, 2.21. Boronic acids
spontaneous form complexes with the vicinal diol groups of saccharides, like D-glucarate 2.22, which in turn are able to complex with Ca\(^{2+}\) ions. Vesicles made up of 1:1 2.21: PC after addition of 2.22 fused when Ca\(^{2+}\) was added. Without 2.22 no fusion was observed. The model proposed once again suggests the formation of calcium bridges between the vesicle membranes, much like the Ca\(^{2+}\)/PS system.

Morgan et al.\textsuperscript{94-96} are credited to have observed the first photo-induced fusion. They synthesized a group of PC liposomes that contained either one azo group or two incorporated into the fatty acid chains. When incorporated into DPPC (2.23) or DSPC analogues, the single azo-chain-PCs caused no loss of bilayer integrity, while enhancing water and proton permeability. Only the PC lipid with two azo groups (2.24) was able to cause fusion and leakage. Recently a study of this group of synthetic PC’s was undertaken where the position of the azo group was varied\textsuperscript{97}. A notable observation was that when predominantly azo-PC monomers and dimers were present in vesicles, very little CF leakage was observed when they were photoisomerized. When the azo-PC was present as an aggregate (domain) the photoisomerization destroys the vesicle and releases the dye.
2.4 Design of Fusion Switch

It should now be evident that the properties of amphiphile assemblies can be modulated by the molecular make-up of the components. This premise can be used to create a fusion switch, or fusogen. By altering the physical shape of the amphiphiles one can induce a change in the phase preference of the membrane that in turn favours fusion. In terms of the packing parameter model, the proposed switch involves a transformation of a molecule that prefers a lamellar aggregation (S ≤ 1) to one that prefers the H_{II} phase (S ≥ 1.2). The same idea implicitly applies for the intrinsic curvature model, in which the molecule is induced to change from a neutral curvature to a negative one, creating enough frustration to switch on fusion. Recall that S = \nu / a_{\theta} l_c. If one assumes that S=1 (for a membrane forming amphiphile, or one of neutral curvature), and that \nu will be constant, then it is obvious that to increase S either \(a_\theta\) or \(l_c\) must decrease. Most of the triggers to date have used a component that reduces \(a_\theta\) of the system to induce fusion: metal ions (Ca^{2+}), pH, temperature, and water content. Systems that affect \(l_c\) are limited to the azobenzene derivatives of Kunitake (2.6) and Morgan (2.23-2.24). The advantage of using a photochromic switch, as in the azobenzene cases, lies in the orthogonality of the process. Light is not one of the natural methods used to induce fusion. This added factor allows one to use it independently as a probe of the shape parameter.

There is a certain undefined quantity of disorder that a membrane can tolerate, as it is a homeostatic system, requiring the integrity of the system. Neither Kunitake’s azobenzenes or Morgan’s mono-substituted azo-PC’s totally disrupt the membrane. There is another factor that must be recognized. The presence of a photoswitch in the chains would more likely interact with “like molecules” in the membrane (short-range order).
which would lead to heterogeneity in the system (domain formation). In such a system the effect of a disruption will be magnified, as the change in the physical structure will have a synergistic effect on the similar molecules adjacent to the chromophore.

This thesis follows the structure of a property directed synthesis. In a property directed synthesis the desired function dictates the target. This is in contrast to conventional synthesis where a fixed structure defines the goal (product synthesis).

Scheme 2-1 describes a typical sequence of events for a property directed synthesis.

```
  Design Target
   ↓
Evaluate Devise Synthetic
     Functionality Plan Synthesis
       ↓
Synthesis
```

Scheme 2-1 Property Directed Synthesis

The sequence begins with the design of the target, incorporating the functional requirements. In our case a choice of the switch, the length of the molecule, and the regiochemistry are required to define a set of targets. A synthetic plan is then devised to achieve the targets, which leads directly to the actual synthesis. Since the actual structures of the targets are not crucial, one is able to evaluate the actual synthesis if any problems are encountered. This can result in a change of the structure of the targets or a new synthetic plan. Once one or more targets have been synthesized, they are tested to determine if the proposed function is accomplished. Knowledge acquired by testing the
a) Cis-trans Isomerization

![Chemical structures and reactions](image)

b) Heterocyclic Cleavage

![Chemical structures and reactions](image)

Figure 2-4. Relevant Molecules and their Photochromic Reactions
targets can thus lead to better targets and the loop is continued. The combination of a property directed synthesis with a modular design in effect produces a synthetic plan that incorporates multiple targets. The advantage gained is that the evaluation of the function can be processed faster, with respect to structure-activity studies. The disadvantages are the requirement of simple reactions that allow access to many targets.

There have been various reviews on photochemical switches and their possible applications\(^{98,99}\). Photochromic molecules can be classed by their photochemical reactions. The types include \textit{cis-trans} isomerization, electrocyclic reactions, tautomerization, homolytic and heterocyclic cleavages\(^{100}\). The required property is a change in the extended length, \(l_e\), of the switch, making the two most relevant photochemical reactions \textit{cis-trans} isomerization and heterocyclic cleavages. Examples of photochromic molecules relevant to this thesis are displayed in Figure 2-4. The left-handed isomer in the figure is typically the more stable. Azobenzenes have been extensively studied, as have been spiropyrans. Thioindigos are less common, but are presently seeing more research. The photochemical potential of hemithioindigos has just been scratched on the surface.

Being the most studied and best understood, azobenzene and spiropyrans have been the most widely used photoswitches\(^{101}\). They have been used to modulate metal binding, ion transport, and membrane potential to name a few examples. In both cases there are significant structural changes that take place when the chromophore isomerizes. Trans-azobenzene is planar, with no dipole, and has a 4 to 4' carbon distance of 9.0Å. The cis isomer has one ring occupying a plane tilted at 56° to the other, its dipole is 3.0 D, and its 4 to 4' length has decreased to 5.5Å\(^{100}\). Spiropyran is a neutral molecule, with
two rings linked by a tetrahedral sp³ carbon, making them orthogonal to each other. The merocyanine isomer exists as a planar zwitterion. This zwitterion is actually an equilibrium mixture of eight possible cisoid and transoid forms. The above properties of spiropyran make it a bad choice as a shape switch, since the predicted shape cannot be determined, and the electronic changes are probably too great. The formation of the zwitterion in the middle of the bilayer would cause catastrophic changes, in essence more than is required. Spiropyrans are also the most complex of the candidate molecules, and hence any modular synthesis would be complicated.

Azobenzene was the primary candidate for my work, especially since it had been shown to isomerize in membranes. But we were reluctance to use it. The fact that so few switches are well studied made us decide to give other switches a hard look. Other factors that contributed to the rejection of azobenzene as a switch were (i) the requirement of ultraviolet light for isomerization, which due to its higher energy, increases the chance of side reactions. (ii) The rate of the thermal backreaction can also be significant enough to make the detection of some cis isomers difficult. Isolation of certain isomers has been achieved, but special conditions tend to be employed. (iii) The solubility of azobenzene derivatives in PC/PA/cholesterol membranes was found to be very poor, in related work by C. Shan in this research group.¹⁰²

Thioindigo has recently gained popularity as an alternative photoswitch. It has been used to modulate metal binding, as well as an agent for doping liquid crystals, and selective membrane disruption. The trans form fluoresces, while the cis does not. There is a significant synthetic problem in using thioindigo as a switch. Thioindigo is an oxidised indoxyl dimer, making the differentiation of each indoxyl monomer a
requirement for the synthesis of the isomer. Furthermore, indoxyls are unstable compounds and tend to be used in situ. Recently this differentiation was performed by Lemieux et al.\textsuperscript{106}.

Hemithioindigos (HT) can be viewed as half thioindigo, half stilbene. They were first synthesized in 1909 by Friedlaender\textsuperscript{107}, their spectral properties were first studied by Mostoslarkii and Izmail’skii\textsuperscript{108-111} and by Reamonn and O’Sullivan in 1977\textsuperscript{112}, who further characterized the two HT isomers. The photochromism of HT is reversible. Both isomers have been isolated and the thermal stability of the less stable E-isomer is higher than the less stable Z-azobenzene counterpart. Preliminary studies in photofatigue\textsuperscript{113} show low degradation during the first 5000-20000 repetitions depending on substitution. This can be increased to 15000-50000 repetitions in the presence of 1,4-

diazabicyclo[2.2.2] octane, an \textsuperscript{1}O\textsubscript{2} scavenger.

Yamaguchi et al.\textsuperscript{113,114} were the first to study HT in molecular assemblies.

They synthesized a library of HTs of which 2.25 and 2.26 were tested in aqueous environments. Both dissolved in water, but 2.25 into monomers, while 2.26 formed aggregates. Compound 2.25 showed typical \textit{trans-cis} photochromism found in organic solvents, while 2.26 gave irreversible bleaching. The bleaching is believed to be due to [2+2] photodimerization of the C=C double bond at the center of the HT moiety. When they were incorporated into dialkylammonium vesicles (C\textsubscript{18} chains) in a 0.01 ratio, both gave Z to E isomerization without bleaching.
Figure 2-5. Minimized Molecular Models of the Dihedral Angle Conformations around the Double Bond
The above literature encompasses all that has been published of HT. The physical properties of HT are amenable to being a photoswitch. The use of hemithioindigos as a switch is also attractive due to its relative obscurity, allowing us to contribute not only by presenting a design for a modular fusogen, but also by adding another photochromic molecule to the list of potential switches.

To get a better idea of the stabilities of the HT isomers, we modeled the moiety by molecular mechanics. The dihedral angle around the double bond was varied at 45° intervals from the Z-isomer. These conformations were then minimized, their optimal energies calculated, and the plane tilt angle between the indoxyl and the benzylidene determined, as shown in Figure 2-5.

In all cases the dihedral angle reverts to a planar conformation, as would be expected. As can be seen by the models, there is a steric interaction between the ortho hydrogens of the benzylidene and the sulfur or oxygen of the indoxyl portion, greater the latter case. The planar E-isomer (2.30) lies 3 kcalmol\(^{-1}\) higher energy than the planar Z-isomer (2.27). They are both local minima. The global minima (4.3 kcalmol\(^{-1}\)) are the conformations 2.29 or 2.31 that have a plane tilt of 58°. Conformations 2.28 and 2.32 are intermediate local minima (6.3 kcalmol\(^{-1}\), 49° tilt angle). The benzene ring is free to rotate if there are no aromatic \(\Pi\)-bond interactions between it and the indoxyl. This is not the case in HT, but it is opposed by steric congestion. The benzene ring thus rotates, but not freely, going through at least the 3 defined minima presented here. These results would suggest that the HT cannot be viewed as a planar molecule.
Figure 2-6. Modular Design of Targets

Figure 2-7 illustrates the modular design of the candidate HT for a fusogen. The molecules are intended to replace the fatty acid component of phospholipids. The primary hemithioindigo target was chosen to be an analogue of Morgan's bis azo PC (2.24). This would allow the eventual testing of the replacement of the azobenzenes by the HTs. The HT is asymmetrical and thus the choice was made to investigate the linkage possibilities with the indoxyl side giving access to the linker, while the tail is accessed from the benzylidene end. This is the opposite symmetry of the amphiphiles synthesized by Yamaguchi et al. The length requirement can be adjusted by varying the length of the linker and the tail.

We developed a short-handed nomenclature to assist in identifying each structure.

The hemithioindigo is given the label HT, the length of the linker is represented by the number of carbons, while its stereochemical attachment is defined by its position relative to the sulfur atom. The tail is also labelled by a number that represents the length of the methylene carbons. Thus intermediate 2.33 would be abbreviated as HOOC-2p-HT-O-4.
Figure 2-7. Molecular Model of Fusogen Isomerization
This can be further condensed, as the most common linkages do not need to be explicitly stated, reducing the label to HOOC-HT-4. Figure 2-6 thus incorporates all the possible attachment sites on both the indoxyl and the stilbene portions of the HT. The stilbene portion will have no tail attachments in the ortho position as this has been previously found to hamper the isomerization reaction.  

The primary candidate fusogen, 2.33 (PC-HT-6), is shown in Figure 2-7. The depiction of the conversion of Z, Z-PC-HT-6 to E, E-PC-HT-6 seemed exaggerated and was thus modeled to get a better idea of the extent of the physical transformation. The modeled structures show a static minimum view of the process (which is in reality very dynamic), but one can still appreciate the vast difference in the two structures. The distance from carbonyl to terminal carbon for Z, Z-2.33 is 20.6 Å, while the E, E-2.33 isomer is only 17.4 Å. It is of interest to note that the HT benzene rings face each other, possibly due to Π-stacking interactions, in each isomer.

Figure 2-8 shows the molecular models of the 4 structural isomers of PC-HT-6 that were eventual synthesized (Chapter 3), in the Z, Z conformation. Not wanting to belabor the point, it is obvious, even if these static minima do not represent the "real" state of the molecules, that there are significant differences in their packing. PC-HT-\textit{m}-6, 2-35, shows an L-shaped conformation produced by the alignment of the alkyl tails. PC-\textit{m}-HT-6, 2-36, is too congested at the glycol linkage, resulting in splayed out tails, where no Π-stacking is apparent. PC-\textit{m}′-HT-6, 2-37, shows disorder in the indoxyl section of the HT, but the stilbene portion is able to stack up.

The critical packing parameter was hard to estimate for the above molecules and \textit{ab initio} estimations of the packing parameters are not trivial. The packing parameter S
Figure 2-8. Molecular Models of Synthesized PC Analogues
does not take into account interdigitation or tilting. Nevertheless, it has been used with phosphorylcholine amphiphiles made with one symmetrically branched alkyl chain to predict the formation of vesicles\textsuperscript{115}. The average PC $\alpha_o$ was used (60-70Å) to give predicted values for S of 0.6-0.7). The critical length can be estimated by looking at a fully extended molecular model of the tail. The volume for simple alkyl chains can be calculated by Tanford’s equations\textsuperscript{115}.

In our case we know that tilting will be an issue, and we have no way of calculating the volume occupied by the hemithioindigo moiety. Some very crude attempts at volume estimation were performed by making a CPK model of the PC-HT-6 wrapping it in plastic, and then measuring the amount of water displaced by the model. Natural PC was used as a point of reference. The raw results were normalized to the calculated S of PC, and gave predicted S of 0.9±0.2, and 1.2±0.2 for Z,Z-PC-HT-6 and E,E-PC-HT-6, respectively.

Another way to use the packing parameter is to look at the change in the length of the chains of each isomer, which is what is being varied. If one assumes an S = 1 for Z,Z-PC-HT-6 then, if the volume change is minimal, the S of the E,E isomer is simply the ratio of the two critical lengths, giving an S of 1.2. It thus seems that the E,E isomers of HT have on average a $\Delta S$ of 0.2 compared to the Z,Z. This should be enough to act as a fusion switch.

Now that the group of targets has been selected, the chemistry to synthesize this class of compounds must be evaluated. Chapter 3 describes this synthesis, followed by an assessment its synthetic efficiency in Chapter 4. The physical properties of the targets and the fusion assay results are discussed in Chapter 5.
Chapter 3

Synthesis of the Hemithioindigo Lipid Analogues

This chapter describes the synthesis of potential hemithioindigo fusogens. Incorporated into this section are the descriptions of the key chemical reactions, focusing on their attributes and limitations. Where not otherwise discussed, all the NMR assignments of the chemical species were deduced using DEPT, COSY, HETCOR and calculated $^{13}$C NMR chemical shifts$^{116,117}$. 
3.1 Retrosynthetic Pathways

The original goal of the project was to develop a synthetic pathway that was simple. The onus was on the synthesis of candidate fusogens spending as little time as possible developing new chemistry. The decision was taken to borrow as much synthetic know-how from previous papers on the subject.

We can devise a retrosynthetic analysis of the target, Scheme 3-1. The synthetic phosphotidylcholine PC-2-HT-6, 3.1, is logically made via the ester linkages of the glycerol backbone, from two hemithioindigo fatty acid analogues, 3.2 and glycerolphosphatidylcholine, 3.3. The HT acid can be synthesized by condensation of benzaldehyde bearing an alkyl tail, 3.4 and the indoxyl bearing a suitably protected acid, 3.5. The indoxyl formation from Friedel-Crafts acylation has to incorporate differentiation between the two acid moieties, hence there is a need to protect one. The indoxyl moiety is known to be unstable, which makes the attachment of the linker, after its formation, an unfavorable pathway. The Williamson ether synthesis affords the appropriate alkyl tail, while the Heck reaction yields the linker. Iodine is the preferred halide for Heck reactions, due to its higher reactivity, but bromine suffices for most reactions. Bromothiophenol, 3.11, is commercially available in all its isomeric forms, making it the source of different attachments for the linker. The bromophenylthioacetic acid needed for indoxyl formation can be accessed by thioether synthesis from bromothiophenol and chloroacetic acid.

This retrosynthesis illustrates all the important reactions, but it is only one of the possible combinations available. Scheme 3-2 shows, in pictorial form, the other possibilities. The hemothioindigo acid is formed by three key reactions, labeled a (Heck).
Scheme 3-1. Retrosynthesis of PC-2-HT-6
Scheme 3.2 The Four Retrosynthetic Pathways Available to Form the Hemithioindigo Acid
b (Friedel-Crafts followed by condensation) and c (Williamson ether synthesis). The indoxyl synthon is introduced via the thioether synthesis of starting material, d. There are four possible sequences that these reactions can be done. The pathway described in Scheme 3-1 is shown as path A. This pathway is the most convergent of the four. Path D is the most linear, forming the HT moiety first, followed by the addition of the alkyl tail and then the linker, or vice versa. Paths B and C bypass the need to differentiate between carboxylic acids since the HT moiety is already formed. Now that all the pathways are visualized, their inherent compatibility and hence efficiency can be investigated.

3.2 Thioether Synthesis

Thioethers are generally prepared by reaction of alkali salts of thiols with alkyl halides in polar organic solvents\(^1\text{19}\). When 4-bromothiophenol was reacted with K\(_2\)CO\(_3\), and chloroacetic acid in DMF, the yield was low and there were two side products. A much cleaner method\(^1\text{19}\) was used, in which 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) is employed as the base, in a non-polar solvent, benzene. This method simplifies the work-up, as the ammonium salt is filtered and removal of the solvent gives the product. High purity is achieved by distillation (Kugelrohr conditions) resulting in yields of 77\%-100\%.

\[
\begin{align*}
\text{Br} & \quad \text{SH} & + & \quad \text{Br} & \quad \text{O} & \quad \text{O} & \quad \text{CH}_3 \\
\text{3.11. 4-Br} & \quad \text{3.14} & \quad \text{3.15. 4-Br} & \quad \text{3.16. 3-Br} & \quad \text{3.17. 2-Br}
\end{align*}
\]

The assigned NMR spectra of 3.15, 3.16, and 3.17 are shown in Figure 3-1 (\(^1\text{H}\)) and Figure 3-2 (\(^{13}\text{C}\)). All three compounds share the same ethyl acetate fragment, as can
be seen by \( H_3-H_c, C_a-C_e \) and \( C_{b(1)} \) having the same peaks. The spectrum of 3.15 shows the typical \textit{para} substitution AB spin system: a doublet of doublets. The spectrum of 3.16 should have one singlet, two doublets and a triplet in the aromatic region, but \( H_d \) and \( H_f \) have very similar chemical shifts, and also have a small coupling constant with \( H_g \). The spectrum of 3.17 offers no surprises, two doublets (\( H_d, H_g \)) and two triplets (\( H_e, H_f \)). The \(^{13}\text{C} \) NMR spectra of all three isomers vary only in the aromatic carbons. The \(^{13}\text{C} \) assignments were made from a combination of HERCOR and calculated \(^{13}\text{C} \) chemical shifts.
Figure 3-1. Assigned $^1$H NMR Spectra of (a) 3.15, (b) 3.16 and (c) 3.17
Figure 3-2  Assigned $^1$C NMR Spectra of (a) 3.15, (b) 3.16 and (c) 3.17
The Williamson ether synthesis is the method of choice to synthesize unsymmetrical ethers. The reaction is simply an $S_N2$ attack of the metal alkoxide or phenoxide on a primary halide or tosylate. The common bases used are NaH and Ag$_2$O, while newer methods have employed KOH/DMSO$^{120,121}$, HgO/F$_4$BH$^{122}$ and quaternary ammonium salts$^{123}$.

We are interested in O-alkylation of phenols, which is notably harder than that of alcohols since the phenolate anions are less nucleophilic and this is exacerbated by use of less reactive electrophiles (e.g. long chain halides). Phenolate anions can also give C-alkylations. We choose the potassium hydroxide/dimethyl sulfoxide system$^{120,121}$ as the process is very simple, is inexpensive, does not give C-alkylation, and reportedly has moderate to high yields.

\[
\text{KOH/DMSO} + \text{RX} \rightarrow \text{OR}
\]

3.18

4-hydroxybenzaldehyde 3.18 was added to a slurry of DMSO and KOH powder, followed by the alkyl halide. This mixture was stirred at room temperature, or higher for the more sluggish reactions, for 30 min. Water was added to quench the reaction, upon which the crude product was extracted into CH$_2$Cl$_2$. To remove any DMSO in the organic layer, it was washed with water and then dried, and concentrated. The crude product afforded was purified by Kugelrohr distillation. The yields obtained were mediocre, but were tolerated since making a large amount of the ether was simple. It was
Figure 3.3 Assigned NMR Spectra of 3.20, (a) $^1$H and (b) $^{13}$C.
later discovered that 3.21 had been previously synthesized using KOH in DMF with a 76% yield, as well as a phase transfer catalyst in 97% yield. Improvements in this synthetic step are thus possible.

The assigned NMR spectra of 3.20 are shown in Figure 3-3 (1H/13C). The aldehyde proton, H₆, has a chemical shift of 9.82 ppm, while the symmetry of the para substituted benzene gives the typical A₂B₂ doublet of doublets for H₈ and H₉. The presence of the downfield resonance of H₆, in conjunction with its spin multiplicity (triplet), proves the addition of the aliphatic tail.

\[ \text{Scheme 3-3 The Cannizzaro Reaction} \]

When 3-hydroxybenzaldehyde 3.22 was used instead of 3.18 the product obtained was not the expected alkylated aldehyde, 3.23, but the alcohol 3.24 (Scheme 3-3). The assigned NMR spectra of 3.23 and 3.24 are shown in Figure 3-4 (1H) and Figure 3-5 (13C).
Figure 3-4 Assigned $^1$H NMR Spectra of (a) 3.24 and (b) 3.23
Figure 3-5 Assigned $^{13}$C NMR Spectra of (a) 3.24 and (b) 3.23
In the proton spectrum of 3.24 one can see that the aliphatic tail added as expected, but the lack of an aldehyde proton, in conjunction with appearance of two unexpected peaks at 4.62 ppm (H₁) and 2.00 ppm (H₂) imply the reduction of the aldehyde to an alcohol. The $^{13}$C NMR spectrum of 3.24 reiterates the lack of a carbonyl functional group, while suggesting an alcohol (C₈). This is confirmed by the MS, which gives a parent ion of m/z 206.

It seems that a Cannizzaro reaction took place in conjunction with the alkylation. This disproportionation reaction yields one equivalent of alcohol and one of carboxylic acid. The acid 3.25 remained in the water during organic extraction. This unique reaction occurs by nucleophilic addition of hydroxide ion to the aldehyde, giving a tetrahedral intermediate, which expels a hydride ion, which is in turn accepted by a second equivalent of aldehyde. This reaction normally only takes place with benzaldehyde. The results illustrate how subtle changes in the geometry can produce totally different chemical results, due to electronic effects.

The alcohol 3.24 was oxidized to the aldehyde 3.23 using pyridium chlorochromate (PCC), following a published general procedure. When comparing the NMR spectra of 3.23 and 3.24, one can see that the aldehyde proton is evident in 3.23 (H₁) while lacking all features associated with the alcohol.
3.4 The Heck Reaction

The Heck reaction, or the palladium-catalyzed coupling of haloarenes and haloalkenes with alkenes, was discovered by Richard F. Heck more than two decades ago. Since then the Heck reaction has matured into a powerful synthetic tool. Much work has been done in elucidating the mechanism, and controlling the substrate selectivity, as well as regio- and stereoselectivity.

\[
R^1-X + R^2 \xrightarrow{\text{Pd(0)}} R^1R^2 + \text{ products}
\]

3.4.1 Mechanism

Scheme 3-4 displays the catalytic cycle, adapted from Meijere et al. and Cabri et al. The catalytically active species is the coordinately unsaturated 14-electron complex, \( \text{L}_2\text{Pd}(0) \). The ligands are usually monodentate or bidentate phosphines. This complex is generated \emph{in situ}, either by loss of two ligands from tetrakis(triphenylphosphine) palladium (0) complex or by reduction of palladium (II) salts by the reaction medium. The most commonly used catalyst is palladium acetate with added \( \text{PPh}_3 \).

The first step of the catalytic cycle, A, involves the oxidative addition of \( RX \) to the palladium (0) complex. If \( X=I \), the presence of ligands is not required.

The second step of the mechanism, B, is the coordination and insertion of the alkene species. Two possible reaction pathways exist (Scheme 3-5). In path I the olefin coordinates via dissociation of a neutral phosphine ligand, while in path II coordination...
Scheme 3.4. Mechanism of the Heck Reaction
occurs via dissociation of the anionic ligand. In both cases the insertion process is stereoselective as it occurs in a syn manner requiring the metal, ethylene and R₂ group to be coplanar. Path I, where the coordination complex (3.26) is neutral, possessing one neutral ligand and one anionic ligand is found in systems where X is a halide, as the Pd-I(Br) bond is strong compared to that of the phosphine ligand. Path II is followed when X is a triflate, and bidentate phosphine ligands are used. The Pd-OTf is highly liable, and hence the cationic complex (3.27) has two neutral phosphine ligands associated with it.

![Diagram](image_url)

**Scheme 3-5. Coordination and Insertion Pathways**

In complex 3.26, steric factors always favor the migration of the R₂ group to the less substituted carbon with formation of linear products. However when the reaction proceeds via path II (anion dissociation) electronic factors predominate. The R₂ group in this case selectively migrates onto the carbon with lower charge density. This allows for the preferential formation of branched products (Table 3-1). Complex 3.27 can give asymmetric induction when the diphosphine is chiral while complex 3.26 cannot.
After insertion the generated alkyl palladium species undergoes an internal rotation (Step C), allowing for the essential syn orientation of a $\beta$ hydrogen needed for elimination (Step D).

The catalytic circle is closed by regeneration of the active species by reductive elimination of HX (Step E) in the presence of a base (typically Et$_3$N, 'Pr$_2$NEt, etc).

<table>
<thead>
<tr>
<th>Alkene</th>
<th>Path I</th>
<th>Path II</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>a</td>
<td>b</td>
</tr>
<tr>
<td>$\text{R} = \text{COOR, CONH}_2, \text{CN}$</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>$\text{Ph}$</td>
<td>100</td>
<td>60</td>
</tr>
<tr>
<td>$\text{OH}$</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>$\text{OH}$</td>
<td>90</td>
<td>5</td>
</tr>
<tr>
<td>$\text{OH}$</td>
<td>75-85</td>
<td>10</td>
</tr>
<tr>
<td>$\text{OEt}$</td>
<td>Mixture of isomers</td>
<td>100</td>
</tr>
<tr>
<td>$\text{OAc}$</td>
<td>Mixture of products</td>
<td>5</td>
</tr>
<tr>
<td>$\text{OnBu}$</td>
<td>Mixture of isomers</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>80</td>
<td>20</td>
</tr>
</tbody>
</table>

Table 3-1. Heck Product Data of Aryl Bromide and Alkene, compiled from Cabri et al$^{127}$

The synthetic plan (Schemes 3-1 and 3-2) uses the Heck reaction as the key linking C-C process between the spacer and the HT. The possibility of variation in the
spacer, both in length and functionality was part of the design and the Heck reaction seemed to offer these possibilities.

![Chemical structure of 3.15, 3.28, and 3.29](image)

All the preliminary work involved in elucidating the synthetic pathway used 3.15 as the aryl halide, as it is the cheapest reagent and has the simplest NMR spectrum compared to its isomers. The procedure followed was Heck's standard recipe\textsuperscript{126}; using Pd(OAc)\textsubscript{2}, PPh\textsubscript{3}, and NEt\textsubscript{3}. This method afforded mediocre yields of only the trans isomer 3.29 (33%). The assigned NMR spectra of 3.29 are shown in Figure 3-6 (\textsuperscript{1}H/\textsuperscript{13}C). The chemical shifts of H\textsubscript{g} and H\textsubscript{h}, and the coupling between them (J = 15.9 Hz) confirm the presence of the trans isomer. In the Heck reaction bulky alkenes tend to give trans isomers due to steric reasons. I do not consider methyl acrylate to be bulky which suggests that the insertion step is congested, or/and very sensitive to alkene size.
Figure 3-0  Assigned NMR Spectra of 3.29. (a) $^1$H and (b) $^{13}$C.
3.5 Hemithioindigo Synthesis

The synthetic pathway to HT has historically followed the sequence of Scheme 3-6:

![Scheme 3-6 Hemithioindigo Synthetic Pathways]

The (phenyl)thio acetic acids, 3.30, are converted to acid chlorides, and a Friedel-Crafts cyclization affords the indoxyl, 3.31. Knoevengel condensation of aromatic aldehyde 3.32 with the indoxyl, either acid\(^{107,108,111}\), or base\(^{113}\) catalyzed yields the thermodynamically stable Z-isomer HT 3.33, and some thioindigo as a minor product 3.34.
3.5.1 Mechanism

Three distinct reactions occur: (i) formation of the acyl chloride, (ii) Friedel Crafts acylation and (iii) Knoevenagel condensation.

(i) The acid displaces, in $S_{N}2$ fashion, a chloride ion on the thionyl chloride. This ion attacks the carbonyl, forming a tetrahedral intermediate, that breaks down to release $SO_{2}$ and $HCl$ gases, leaving behind the acid chloride.

(ii) Two mechanisms are likely for Friedel-Crafts acylation depending on the conditions. The active species is most often the acyl cation, catalyzed by a Lewis acid ($AlCl_{3}$), either as an ion pair or free ion species. The acylium ion is attacked by the arene, forming an arenium ion intermediate, which expells a proton to regain aromaticity, thus yielding the indoxyl.

(iii) The indoxyl is easily deprotonated by a mild base to give a carbanion, which then adds to the carbonyl. The oxygen acquires a proton and then dehydration results in HT formation.

![Diagram of tautomers of indoxyl]

3.35a 3.35b

Scheme 3-7. Tautomers of Indoxyl 3.35

The indoxyl can exist in two tautomeric forms: the ketone and the enol, (Scheme 3-7). A former graduate student, Binqi Zeng, synthesized 3.36, and obtained a mixture of ketone and enol. He was able to shift the indoxyl to the enol form by recrystallizing the indoxyl from boiling water. Indoxyl 3.36 is an exception to the general stability.
is considered to having an indefinite shelf life\textsuperscript{103}. All other indoxyls are hard to work with. They are unstable to O\textsubscript{2}, and even under inert conditions (Ar, vacuum, low temperatures) some decomposition occurs.

The indoxyl group has been protected as 3.37, and has a longer benchlife, but subsequent condensation gives poorer yields of HT\textsuperscript{111}.

\begin{equation}
\text{HO} \quad \text{O}
\end{equation}

\textbf{3.36}

\begin{equation}
\text{\textbf{3.37}}
\end{equation}

\begin{equation}
\text{HO} \quad \text{O}
\end{equation}

The diester 3.29 was tested as a possible candidate for indoxyl formation, and hence HT production, as shown in Scheme 3-8. Compound 3-29 was saponified to the dicarboxylic acid, 3.38, then activated for Friedel-Crafts acylation by converting it to the

\begin{equation}
\text{\textbf{3.40}}
\end{equation}

(a) KOH, MeOH, reflux, 3 h/H\textsuperscript{+}. (b) SOCl\textsubscript{2}, reflux, 1 h. (c) AlCl\textsubscript{3}, r.t., 40 min./H\textsubscript{2}O

\textbf{Scheme 3-8. Possible Friedel-Crafts Products of Diacid. 3.29}
diacid chloride, 3.39. The acylation with two possible reactive sites begs the question of side reactions. It is unlikely that the same molecule will cyclize twice as the addition of the first acyl group deactivates the benzene ring, while the double bond in the trans stereochemistry poses severe steric hindrance, eliminating the possibility of this acyl cyclizing first, 3.41. This does not prevent intermolecular acylation, as both substituents on 3.39 activate the ring. If the rate of intermolecular acylation is much faster than the intermolecular rate then this may not be a significant factor. One of the possible products of this intermolecular acylation, 3.42, is shown in Scheme 3-8.

When diacid chloride 3.39 was reacted with AlCl₃ the reaction produced an insoluble brown solid, which MS confirmed to contain the indoxyl 3.40, yet no purification was possible. When the crude product was condensed with benzaldehyde no HT was produced. This method is too heavy handed. More control is required, in the form of protecting groups.

The simplest possible protecting group is an ester. Esters are not immune to attack by the Lewis acid, AlCl₃, but under mild conditions the rate of this attack compared to that of the acid chloride was thought to possibly minimize any side reaction. I thus synthesized 3.44. Compound 3.15 was hydrolyzed to acid 3.43, which underwent palladium coupling with methyl acrylate to give acid-ester 3.44. The yield was once again low (~30%) and some attempt was made to improve it. A report by Heck¹²⁸ documents the poor performance of aryl bromides with strongly electron donating substituents, when undergoing the Heck reaction, under standard conditions. This was attributed to the competing formation of tetraarylphosphonium salts of the aryl bromide, ArPh₃⁺Br⁻. Two solutions are offered, substitution of PPh₃ by tri-o-tolyphospine, or use
of the corresponding aryl iodide thus eliminating the need for the phosphine ligands. I replaced triphenylphosphine by tri-o-tolylphosphine but only succeeded in lowering the reaction yield. Synthesis of the aryl iodide is explored later in this report.

\[
\begin{align*}
\text{Br} & \quad \begin{array}{c}
\text{S} \quad \text{O} \\
\text{R}
\end{array} \\
& \quad \begin{array}{c}
\text{O} \\
\text{H}
\end{array} \\
& \quad \begin{array}{c}
\text{O} \\
\text{S}
\end{array}
\end{align*}
\]

\[3.44\]

\(3.15\). \(R = \text{Et}\)

\(3.43\). \(R = \text{H}\)

(a) \(\text{KOH, MeOH, reflux, } 3 \text{ h} / \text{H}^+\). (b) \(\text{Methyl Acrylate, Pd (Ac)}_2, \text{PPh}_3, \text{NEt}_3\)

(c) \(1. \text{SOCl}_2, \text{reflux, } 1 \text{ h} \quad 2. \text{AlCl}_3, \text{r.t., } 40 \text{ min} / \text{H}_2\text{O}\)

Scheme 3-9. Attempted HT Synthesis using a Methyl Ester Protected Acid

Friedel-Crafts acylation of the acid chloride of 3.44 produced mainly starting material, while condensation yielded no HT. More elegant methods are thus needed for acid differentiation.

I turned my focus onto paths II and III in Scheme 3-2 that synthesize the HT moiety first, avoiding the presence of two acids, followed by the Heck reaction to add the linker. Thus as seen in Scheme 3-10 (p-bromophenyl)thio acetic acid, 3.43, was converted to the acid chloride by refluxing with \(\text{SOCl}_2\), and then cyclized, with \(\text{AlCl}_3\), using several solvents (1,2-dichlorobenzene, \(\text{CS}_2\), and 1,2-dichloroethane). Dichloroethane was used in subsequent reactions as the solvent of choice, since
Figure 3-7 Assigned NMR Spectra of 3.47: (a) $^1\text{H}$ and (b) $^{13}\text{C}$.
dichlorobenzene was hard to remove, and in CS₂ the reagents were sparingly soluble. In most cases the indoxyl, 3.47, was contaminated with an intense purple compound, the thioindigo, 3.48, which was invisible in the NMR spectra. Purification attempts (recrystallization and chromatography) resulted in decomposition. Storing 3.47 under Ar, in the freezer, did not stop the decomposition.

Scheme 3-10. Synthesis of Indoxyl 3.47

The reaction can be followed by NMR. The methylene protons shift downfield from 3.63 ppm in 3.43 to 4.01 ppm in 3.46. Likewise the methylene carbon shifts from 36.5 (3.43) to 48.4 ppm (3.46). The carbonyl carbon shifts from 175.0 ppm to 169.6 ppm. The NMR spectrum of the indoxyl 3.47 is shown in Figure 3-7(¹H/¹³C). Only the keto tautomer is present. The coupling constants allow definitive assignments to be made: H₆ (J = 8.5 Hz) couples to H₇, which also exhibits a small coupling constant (J = 1.8 Hz) to H₆. The methylene protons, H₅, have now been shifted upfield to 3.81 ppm. The carbon spectrum is more complicated, as the molecule is less symmetrical than 3.43.
Figure 3-8. Assigned NMR Spectra of 3.49, (a) $^1$H and (b) $^{13}$C.
with 8 resonances, having one secondary carbon, C\text{a}, three tertiary carbons, C\text{b}, C\text{c}, and 
C\text{d}, and four quaternary carbons, C\text{c}-C\text{h} (DEPT). C\text{a} has shifted upfield to 39.8 ppm.

while the carbonyl, C\text{h}, is now downfield at 198.5 ppm.

\begin{equation}
\begin{array}{c}
\text{Br} \\
\text{S} \\
\text{O} \\
\text{Br} \\
\text{3.47}
\end{array}
+ 
\begin{array}{c}
\text{O} \\
\text{OR} \\
\text{3.47}
\end{array}
\begin{align}
3.18 \ R & = H \\
3.19 \ R & = C_2H_5 \\
3.20 \ R & = C_6H_{13}
\end{align}

3.49 \ R & = H \\
3.50 \ R & = C_2H_5 \\
3.51 \ R & = C_6H_{13}

The Knoevengal condensation, when performed on “aged” (4 days) indoxyl and 
benzaldehyde, gave poor HT yields (0-17%). Reacting \textbf{3.47 in situ} increases this yield 
(66-74%). The NMR of the HT, \textbf{3.49}, is shown in Figure 3-8 (\text{^1H/^{13}C}).

Literature \text{^1H NMR spectra on HTs have assigned}^{112,113} \text{the } \beta\text{-proton to } \delta \ 7.81-7.92 \text{ ppm. Full assignment of all proton peaks is shown in Figure 3-8a. The } \beta\text{-proton can}
either be at 7.90 ppm or 7.86 ppm, but these can be distinguished. The peak at 7.90 ppm 
is coupled, which would not be expected for H\text{c}, while the spectrum of the indoxyl moiety 
(Figure 3-7a) has already shown that some meta coupling exists between H\text{f} and H\text{c}. The 
\beta\text{-proton can thus be assigned to 7.86 ppm. There are no literature precedents for the }^{13}C 
NMR data. Assignment of the carbon peaks is shown in Figure 3.9b. The only tentative
assignments are of the quaternary carbons $C_g$, $C_h$, $C_i$, and $C_j$, determined by comparing the calculated $^{13}C$ peaks, and observing the effect of substitution on the HT core (see Table 3-3 at the end of this Chapter).

One can synthetically add the HT tail after the formation of the hemithioindigo moiety (Path III, Scheme 3-3), but two disadvantages exist, the primary one being that this is a linear strategy, and hence compromises the efficiency of the synthesis. The second one is that the yield associated with the step is generally poor (37-56% for various analogues$^{113}$). Nevertheless this method was attempted and in my hands the yield of Br-HT-4, 3.22, was even more pitiful (16% yield).

3.49

The synthetic strategy employed for the rest of the HT compounds thus has the appropriate tail already added to the hydroxybenzaldehyde (Path I and II, Scheme 3.2), as discussed in the Williamson Ether Synthesis section.

Br-HT-OH 3.49 did not undergo palladium coupling with methyl acrylate. To see whether the phenol group was interfering with the reaction, I tried the Heck reaction with Br-HT-6, 3.51. Acetonitrile was added to the standard preparation of the Heck reaction to dissolve 3.51. The product 3.53 was successfully synthesized in low yields (22%). The NMR of the HT 3.53 is shown in Figure 3-9 ($^1H$/$^{13}C$).

This strategy (Path II) now runs into fundamental difficulties. It is not possible to selectively hydrogenate the double bond in the linker without touching the one in the HT moiety. To solve this setback, the HT double bond must be protected. Such a pathway
Figure 3-9 Assigned NMR Spectra of 3.53, (a) $^1$H and (b) $^{13}$C.
inherently adds more steps to the overall synthesis, introduces untested chemistry, and thus was not pursued further.

Path I is now the sole contender to achieve the target. Two strategies were explored in parallel (i) use of appropriate protecting groups to differentiate the carboxylic acids and (ii) use of a synthon that can be functionally manipulated to yield a carboxylic acid, in essence a masked acid. These will be addressed in the following sections.

3.6 Phthalimidomethyl Protecting Group

The choice of the protecting group is critical as it must fulfill the following criteria. It must be unreactive to the Heck reaction, to hydrogenation, to SOCl₂, to Friedel-Crafts acylation, and the deprotection method should leave the HT-moiety
untouched. The *N*-phthalimidomethyl protecting group was chosen after careful consultation of the reactivity charts of commonly used protecting groups\(^\text{129}\). It fulfills the above criteria and has the mildest deprotection possibilities (as compared to some candidate amide forming protecting groups).

Scheme 3-12 shows the synthetic route taken to produce the differentially protected carboxylic acids. *N*-hydroxymethylphthalimide 3.55 is converted to *N*-chloromethylphthalimide 3.56 by refluxing in SOCl\(_2\). The acrylic acid is then protected by reacting 3.56, in the presence of dicyclohexylamine (other bases like triethylamine and pyridine were not effective), in DMF, yielding ester 3.58. This ester was coupled with 3.15 under standard Heck conditions giving the *trans* product 3.59, which was then hydrogenated affording 3.60. The assigned NMR spectra of 3.59 and 3.60 are shown in
Figure 3-10 Assigned $^1$H NMR Spectra of (a) 3.59 and (b) 3.60
Figure 3-11 Assigned $^{13}$C NMR Spectra of (a) 3.59 and (b) 3.60
Figure 3-10 (¹H) and Figure 3-11 (¹³C). The *trans* stereochemistry of 3.59 can be clearly seen by the coupling between H₉ and H₈ (J=16.2 Hz). The phthalimido protons Hᵢ and Hⱼ have two coupling constants (³J= 5.9 Hz and ⁴J= 2.9 Hz). In 3.60 the double bond has clearly been hydrogenated, as seen by the appearance of two triplets H₉ and H₈. The ¹³C NMR spectra of these two compounds tells the same story: the vinyl carbons C₉ and C₈ of 3.59 are no longer present in the spectrum of 3.60, concurrent with the presence of two new alkyl carbons, C₄ and C₅.

The selective deprotection of the ethyl ester while leaving the phthalimidomethyl ester untouched was not considered to be problematic. Under literature conditions for the removal of ethyl esters (LiBr, pyridine¹²⁹) the phthalimidomethyl ester remains untouched¹³⁰. The attempted deprotection under the described conditions yielded starting materials (Scheme 3-13). Harsher conditions of LiF in DMF resulted in the very slow deprotection of the phthalimidomethyl ester¹³¹. Different deprotection conditions were attempted: (i) TMSCl, NaI, CH₃CN¹³¹ gave selective phthalimidomethyl deprotection and (ii) K-Ot-But/H₂O¹³² deprotected both the groups. It thus became obvious that for this system to work, the ethyl ester must be replaced by a more labile protecting group, such as a tert-butyl ester, which could be deprotected by p-toluenesulphonic acid¹³⁰, without touching the phthalimidomethyl ester. This method could still succeed, but the amount of manipulation needed, plus the ultimate success of the masked acid strategy (below), brought this investigation to a conclusion.
Scheme 3-13. Attempted Methods for Selective Deprotection of Ethyl Ester

3.7 The Nitrile Synthon

The idea of using the nitrile group as a masked carboxylic acid came directly from investigating substrates with functionality commonly used in the Heck reaction (see Table1-1). Acrylonitrile has historically given fair to good yields\(^{126}\), and the transformation of the nitrile group to a carboxylic acid is well known.

Acrylonitrile was thus reacted with 3.15 under standard Heck conditions to yield mixed isomers of 3.63. The two isomers can be separated by tedious chromatography, as silica does not discriminate well between the two. For this particular project there is no...
Figure 3-12. Assigned $^1$H NMR Spectra of (a) E-3.63 and (b) Z-3.63
Figure 3-13 Assigned $^{13}$C NMR Spectra of (a) E-3.63 and (b) Z-3.63
need to separate the isomers, as in the next step the double bond is reduced.

Nevertheless the separation was performed to get a well characterized material. Three different solvent systems were tested: hexanes/ether, hexanes/ethyl acetate, and toluene/ethyl acetate. None gave total separation of the isomers. The hexanes/ether system, in conjunction with extensive centrifugal chromatography, showed a minor preference for the trans isomer over the cis isomer, and 1:9 ratio of trans:cis isomer was obtained. The assigned NMR of trans-3.63 and cis-3.63 are shown in Figure 3-12 (1H) and Figure 3-13 (13C). The coupling between trans protons H_r and H_s is 16.7 Hz, while in the cis it is 12.1 Hz. The aryl protons in trans-3.63, H_d and H_c, form an unresolved 2nd order AB singlet, while the cis-3.63 a typical doublet of doublet is formed (J= 8.1 Hz).

The 13C NMR spectra overall show minor differences, most notable by C_r-C_h, associated with the vinyl linker which is found shifted upfield by 2 ppm.

\[
\begin{align*}
\text{Br} & \quad \text{S} & \quad \text{O} & \quad \text{O} & \quad \text{Cul/C} & \quad \text{3.15} \\
& \quad \text{S} & \quad \text{O} & \quad \text{O} & \quad \text{3.64}
\end{align*}
\]

The initial yields of this reaction were 40-50%. Improvements were attempted by exchanging the bromine in 3.15 by iodine. Iodine is a more reactive haloarene substrate in the Heck reaction, but it cannot be obtained by direct halogenation. This halogen exchange has been performed using Ni and KI\textsuperscript{133} and Cul supported by alumina or charcoal\textsuperscript{134}. Nickel cannot be used with 3.15 as the metal will be poisoned by the sulphur, so this method was not attempted. Copper(I) iodide supported on charcoal gave very lackluster results. The conversion was never above 20%, and the recovery of total
Figure 3-14. Assigned $^1$H NMR Spectra of (a) E-3.65 and (b) Z-3.65.
Figure 3.15 Assigned $^{13}$C NMR Spectra of E/Z-3.65, (a) Full Spectrum and (b) Expanded Aromatic Region
substrate was about 60%. In retrospect, even if the conversion had been more favourable, it is vastly inefficient, requiring large excesses of reagents. Based on a report\textsuperscript{135} stating an increase in the life time of the palladium catalyst by increasing the pressure ($\approx 120$ psi), I investigated the reaction yield at 60 psi, but obtained no notable improvement. An effective improvement was accomplished by increasing the amount of catalyst present from a 1\% mol equivalents to a 5 \% mol equivalents. This increased the yield to 78 \%.

\[
\begin{align*}
&\text{Br} & \text{S} & \text{O} & \text{CO} & \text{O} & \text{CH}_3 \\
&\text{C}_6 & \text{H}_4 & & & & \\
\end{align*}
\]

\begin{center}
3.16
\end{center}

3.16

\begin{align*}
\text{Br} & \quad \text{S} & \quad \text{O} & \quad \text{CO} & \quad \text{O} & \quad \text{CH}_3 \\
\text{C}_6 & \text{H}_4 & & & & \\
\end{align*}

\begin{center}
3.65
\end{center}

The other two isomers of 3.15 were also coupled to acrylonitrile. 3.16 gave 3.65 in a 60 \% yield. Total separation of the isomers was not performed, but analysis of samples that were partially enriched in one of the isomers allowed assignment of the NMR spectra of both, as seen in Figure 3-14 ($^1$H) and 3-15 ($^{13}$C). Several partially resolved NMR spectra of different molar fractions were used to determine which peaks were associated with which isomer. It can be seen that the most affected protons are $H_{d-g}$.

From \textit{trans} ($J = 16.9$ Hz) to \textit{cis} ($J = 12.1$ Hz): $H_d$ and $H_e$ are shifted upfield by 0.23 ppm and 0.40 ppm, respectively; $H_f$ and $H_g$ are both deshielded by the proximity of the nitrile group in the cis isomer, leading to a downfield shift of 0.25 ppm. The $^{13}$C NMR spectrum shows that the carbon atoms associated with the linker, $C_{d-e}$, $C_j$, are all shifted slightly upfield for the E-isomer. The carbons on the aryl moiety show subtler effects: $C_{e-g}$ which feel the proximity of the nitrile group, are found downfield; $C_h$ and $C_i$ are slightly upfield, while $C_i$ and $C_k$ are virtually unaffected.
Figure 3-16  Assigned NMR Spectra of 3.69, (a) $^1$H and (b) $^{13}$C.
The main product of the Heck reaction of substrate 3.17 with acrylonitrile was not the simple coupling expected. The product had no NMR peaks related to a vinyl group, but showed a new methyl peak, and gave the same parent ion as 3.65 and 3.66 in the MS. It was hypothesized that these products would have to be one of the following structures, above, 3.67-3.69. The NMR of this product is shown in Figure 3-16 ($^1H/^{13}C$). It is not possible to distinguish between the three structures, 3.67, 3.68 and 3.69. In compound 3.68 $H_d$ would split into a triplet, but in reality it is a doublet, so this can be discounted as a possible structure. The 1st order spin system for hydrogens $H_c-H_e$ of 3.67 and 3.69 is $A_2MX$ for both, giving similar coupling: $H_c$ couples with $H_e$ and splits into a doublet; $H_c$ also couples with $H_d$ and thus it splits is a doublet of triplets: $H_d$ is a doublet. $H_e$ in the spectrum is a triplet with satellite doublets, signifying that this system is actually 2nd order, while it is not possible to determine the coupling of other mentioned protons since...
Figure 3-17. Assigned NMR Spectra of 3.71, (a) $^1$H and (b) $^{13}$C.
Hb, also resonates 4.2 ppm and masks the multiplicity. In the 13C NMR the predicted NMR chemical shifts of 3.67 and 3.69 (Scheme 3-14) compared to the experimental results are also ambiguous.

The ester (3.67 or 3.69) was hydrolyzed to give the acid (3.70 or 3.71), whose NMR spectrum can be found in Figure 3-17 (1H/13C). It is now possible to see the rest of the coupling: Ha (2.72 ppm, 2nd order coupling); Hb (4.09 ppm, dt, J=6.6 Hz, J=3.3 Hz); and Hc (4.15 ppm, d, J=3.3 Hz). The two Ha protons are not magnetically equivalent and thus the spin system is in reality ABMX. HaA and HaB have similar chemical shifts and hence their spectrum resembles a coalescing AB system, which has been split by another proton, HbM. The identity of the product still remains to be solved though.

Figure 3-18. Fine Coupling NMR Undecoupled 13C of Nitrile 3.71

This was accomplished by running an undecoupled 13C spectrum and looking at the fine coupling of the nitrile Cb (see Figure 3-18). This signal has the form of a triplet of doublets having the respective coupling constants J=9.3 Hz and J=4.7 Hz. These couplings are between the Ha and Hb hydrogens and Cb. If the product were 3.70 then since Hb is 2 bonds away compared to Ha which is 3 bonds away, it would have the larger coupling constant. This is not the case, indicating that the HaS are closer to the nitrile than the Hb and thus that the product is indeed compound 3.71.
The possible mechanism that explains compound 3.69 is shown in Scheme 3-16.

The two α protons adjacent to the sulphur are acidic enough to be deprotonated by the base Et3N. The intermediate anion is stabilized by both the sulphur and the oxygen. A Micheal-type attack on the alkene results in ring closure. The product is formed when by picking up a proton from the solution.

Investigating the possibilities of increasing the chain length of the linker, I coupled allyl cyanide with 3.15, and got the unexpected mixed isomers of branched 3.74 as the main product. There was a very minor amount of (<1%) linear product, 3.72, and none of the expected branched product 3.73 (see Table 3-1). Compound 3.73 is presumed to be formed as the major product, but then it isomerizes to compound 3.74. The β-elimination (Step D, Scheme 3-7) is a reversible process, and if the coordinated olefin is slow in dissociating then the thermodynamic product is formed. It thus seems that using nitriles may be limited to allowing only two carbon spacers. Using 4-pentenenitrile
Figure 3-19. Assigned NMR Spectra of 3.74. (a) $^1$H and (b) $^{13}$C
as a five carbon spacer was not attempted, and since the nitrile is further away, maybe in this case the major product would be linear (as expected, see Table 3-1).

\[
\begin{align*}
\text{Compound 3.74 can be partially separated by exhaustive chromatography. The NMR of E-3.74 is shown in Figure 3-19 (}^1\text{H}/^{13}\text{C}). The methyl peak H}_d \text{ is at 2.41 ppm and 2.23 ppm, while H}_c \text{ is found at 5.58 (5.60 predicted) ppm and 5.35 (5.23 predicted) ppm, for Z and E-3.74 respectively.}
\end{align*}
\]

### 3.8 Hydrogenation of the Olefin

The hydrogenation of the \(\alpha, \beta\)-unsaturated esters and nitriles was performed using Wilkinson’s catalyst, tris (triphenylphosphine)chlororhodium (I)\(^{136}\). This method has the advantage of being able to reduce the carbon double bond without affecting the ester and nitrile functional groups.

#### 3.8.1 Mechanism\(^{137,138}\)

The exact details of the mechanism, i.e. the actual identity of the species involved, depends on the alkenes and reaction conditions. RhCl(PPh\(_3\))\(_3\) can lose a PPh\(_3\) ligand,
forming a solvated species, RhCl(PPh$_3$)$_2$(solvent) or dimerize via halide bridges, but the catalytic cycle can be still be viewed as following the sequence described below, irrespective of the actual species.

The first step involves the oxidative addition of H$_2$ to generate the metal dihydride. The dihydride must dissociate a ligand to have an open site available to coordinate the alkene. Once the alkene has coordinated, the hydrogen inserts in a syn manner, to yield an intermediate alkyl, which undergoes a syn reductive elimination, giving the alkane and regenerating the catalyst.

The rate of hydrogenation depends on the steric bulk around the alkene, generally following the order: monosubstituted $>$ disubstituted $>$ trisubstituted $>$ tetrasubstituted $\approx$ 0.

The hydrogenation step proved to be extremely sluggish. Initial attempts at hydrogenating **3.63** involved 3.5 mol% catalyst, 1:1 benzene:ethanol solvent, at 65 psi for 2 days. The maximum conversion was 63%. Heating the sample to 80°C did not affect the result, and increasing the reaction time to 5 days seemed to have a negligible effect. By increasing the length of the reaction to 10 days I was finally able to achieve full hydrogenation. Other hydrogenation methods were investigated as possible pathways for this design.
Figure 3-20 Assigned $^1$H NMR Spectra of (a) 3.75 and (b) Z-3.76
Figure 3-21. Assigned $^{13}$C NMR Spectra of (a) 3.75 and (b) Z-3.76
Heterogeneous catalysis, for example using Raney nickel, was not feasible due to the sulfur in the substrate poisoning the catalyst. Two methods attempted were using Bis-(dimethylglyoximato)cobalt(II)\(^{139}\) and sodium hydrogen telluride\(^{140,141}\). Neither proved to be satisfactory in replacing the standard method. The former failed completely while the latter gave poor conversion, and high loss of starting material. Although Wilkinson’s catalyst produces a slow reaction, the transformation is clean and hence all other hydrogenations were performed using this methodology.

The assigned NMR spectra of 3.75 and 3.76 are shown in Figure 3-20 (\(^{1}\)H) and Figure 3-21 (\(^{13}\)C). The conversion can be clearly seen. In the proton NMR the doublet olefinic proton peaks have vanished and have been replaced by two triplets at 2.86-2.90 ppm and 2.54-2.58 ppm (H\(_d\) and H\(_e\) respectively), while in the carbon NMR these saturated carbons resonate at 31.2-30.8 ppm and 19.0-18.9 ppm (C\(_d\) and C\(_e\) respectively).
3.9 Nitrile Hemithioindigos

Ester hydrolysis of 3.75 to yield the acid 3.77 went as expected. When using this acid, 3.77, as the starting material for HT synthesis, isolation of the indoxyl was not possible. The acid chloride was formed without any incident (methylene protons shifted from 3.64 ppm to 4.02 ppm; methylene carbon shifted from 36.5 ppm to 48.5 ppm; carbonyl carbon shifted from 175.2 ppm to 169.8 ppm). NMR spectra of the Friedel-Crafts' crude product did not suggest the presence of the indoxyl, rather of unreacted acid; yet if this crude product was condensed with benzaldehyde, HTs, 3.80-3.84 were
Figure 3-22: NMR Spectra of 3.80, (a) $^1$H and (b) $^{13}$C
Figure 3-23. Assigned NMR Spectra of Z-3.82, (a) $^1$H and (b) $^{13}$C.
produced. Under normal cyclization conditions (23°C for 40 min.) this sequence would sometimes fail, and hence the reaction was heated at 60°C for an hour instead to give more reproducible results. The yield of this sequence of steps varied from mediocre to very poor.

The assigned NMR spectrum of the NC-HT-OH 3.80, and NC-HT-6 3.82 are shown in Figure 3-22 (1H/13C), and Figure 3-23 (1H/13C), respectively. Replacing the bromine in HT-3.49 for an alkyl in 3.80 changes the position of peaks associated with Hf, Hg, and Hh. Hh is less deshielded and thus is found upfield from where it was in 3.49 (7.90 ppm to 7.76 ppm). The two protons Hf and Hg in 3.80 form an ABX spin system, where X is Hh (J_{ab} = 8.1 Hz, J_{ax} = 0, and J_{bx} = 1.1 Hz). In 3.82 there is no meta coupling, and so the similar protons, Hk and Hl form an AB spin system, and display second order coupling: a singlet with two small satellite peaks. These can be deconvoluted to give chemical shifts of 7.45 ppm and 7.48 ppm for Hk and Hl, respectively.

To obtain the E- HT isomer a sample of 3.82 was dissolved in chloroform and irradiated in a Rayonette reactor, under Ar bubbling, at 350 nm for 30 min. The solution turn from pumpkin orange to a dark red. Under a dim red light, the solution was concentrated, and the isomers were separated using centrifugal chromatography (1:3 hexanes/chloroform). The assigned NMR of the E-3.82 is shown in Figure 3-24(1H/13C). There are minor differences in the aliphatic region of the isomers. It is better to compare the expanded spectra associated with the HT moiety, of both isomers, as shown in Figure 3-25(1H) and Figure 3-26(13C).
Figure 3-24. Assigned NMR Spectra of E-3.82. (a) $^1$H and (b) $^{13}$C.
Figure 3-25. Aromatic Region Expanded $^1$H NMR Spectra of (a) Z-3.82 and (b) E-3.82.
Figure 3-26. Aromatic Region Expanded $^{13}$C NMR Spectra of (a) Z-3.82 and (b) E-3.82.
As expected the β-proton $H_j$ is affected the most, moving upfield by 0.78 ppm from 7.93 ppm to 7.15 ppm, while $H_i$ is shifted downfield by 0.43 ppm from 7.64 ppm to 8.19 ppm, due to the lack of influence of the oxygen in the former and its influence in the latter.

When the isomers of acid 3-76, 3.85, and 4-hexyloxybenzaldehyde 3.20, were used as starting material for synthesis of the HT moiety, the two possible indoxyl structural isomers 3.86 and 3.87 were formed. They could not be isolated and were used in situ to give HT products 3.87 and 3.89. They had distinct $R_f$'s (TLC, 1:1 chloroform/hexanes: 3.88 $R_f = 0.28$, 3.89 $R_f = 0.15$) and were separated, with relative ease, using centrifugal chromatography (1:3 chloroform/hexanes). The assigned NMR spectra of 3.88 and 3.89 are shown in Figure 3-27($^1$H) and Figure 3-28($^{13}$C). There are
Figure 3.27 Assigned $^1$H NMR Spectra of (a) 3.88 and (b) Z-3.89
Figure 3-28  Assigned $^{13}$C NMR Spectra of (a) 3.88 and (b) Z-3.89
two simple ways to tell the isomers apart. The signal for Hᵣ is found to resonate more
downfield in HT 3.88 compared to 3.89. The proximity of the oxygen is responsible for
this effect. The splitting of the protons Hₖ-Hₘ is also characteristic: a doublet, triplet,
doublet, respectively for 3.88; a singlet, doublet, doublet, respectively for 3.89.

Scheme 3-16. Synthesis of 3.88 and 3.89

(a) 1. SOCl₂, reflux, 1 h 2. AlCl₃, 60°C, 40 min / H₂O (b) 3-20. piperidine
3.10 Nitrile Hydrolysis of Hemithioindigos

The hydrolysis of a nitrile moiety to a carboxylic acid is a well-known transformation that is generally taught in second year organic chemistry. It is one of the best methods of producing carboxylic acids, as nearly all nitriles undergo the reaction. It is commonly used to elongate a chain by one carbon and to synthesize amino acids, starting from an aldehyde, sodium cyanide and ammonium chloride. One can erroneously conclude that such a reaction would not pose any major obstacle.

Scheme 3-17. Mechanism of Nitrile Hydrolysis

Nitriles can be hydrolyzed under either basic or acidic conditions. The mechanism for alkaline hydrolysis is shown in Scheme 3-17. The hydroxide nucleophile attacks the polar C=N bond, forming a hydroxy imine, which rapidly tautomerizes to the amide. This amide is further hydrolyzed by another hydroxide ion, following the $B_{AC^2}$ mechanism, through a tetrahedral intermediate, to produce the acid salt and ammonia.
Scheme 3-18. Failed Attempts at Nitrile Hydrolysis

This step proved to be the most difficult to surmount in the synthesis. The initial attempts to transform the nitrile 3.82 into a carboxylic acid were not successful, as shown in Scheme 3-18. Conditions attempted range from the most common to the very obscure. The basic hydrolysis was attempted under different conditions: (i) NaOH, H₂O, MeOH¹⁴⁴,¹⁴⁵, (ii) NaOH or KOH, H₂O, ethylene glycol¹⁴⁶,¹⁴⁷. Using an excess of the base seems to break the molecule apart. NMR spectra (¹H, ¹³C), as well as MS, seems to indicate the presence of the aldehyde, suggesting that the double bond in HT is susceptible to nucleophilic attack by the hydroxide ion. When NC-HT-4 was refluxed at 120°C for 6 h, with 4 equivalents of base (NaOH or KOH) in ethylene glycol, upon acidification a fine red precipitate resulted. The NMR of this product is shown in Figure 3-29 (¹H/¹³C). The changes in the proton spectrum are most pronounced in the aromatic
region. The β-proton is no longer present; H1 has moved downfield from 7.75 to 8.39 ppm, while Hj and Hk are now split (from 7.45 ppm to 7.67 ppm and 7.52 ppm). There is a small (J = 1.4 Hz) meta coupling between Hk and Hr which helps to assign these peaks. The 4 aryl protons, Hg and Hr, have also been shifted downfield slightly from 6.97 ppm and 7.63 ppm to 7.00 ppm and 7.67 ppm, respectively. The MS of this product gives a molecular ion at m/z 379 that is the starting nitrile with an additional 16 mass units, namely oxygen. The 13C spectrum has a low signal to noise ratio, as with time the compound would form aggregates in the NMR tube. Adding some DMSO helped to delay this. The carbonyl Cγ is has been shifted upfield to 174.2 ppm, the nitrile is untouched, and there are an appropriate number of carbon signals (13) for the HT skeleton to be intact. All these data when analyzed seem to suggest that a hydroxide has replaced the β-proton, by an unknown oxidation step (Scheme 3-19).

Scheme 3-19. Action of Hydroxide Anion on NC-HT-4. 3.81
Figure 1: NMR Spectra of Molecules X, Y, and Z.

Chart 1: ppm Values for Molecule X.
Attempts at acid hydrolysis (i) HCl/H$_2$O, (ii) H$_2$SO$_4$/H$_2$O, and (iii) HBr/CH$_3$COOH$^{148}$ proved to be unsuccessful. The nitrile was insoluble and would sublime onto the walls of the reaction flask. When either DMF or DMSO was added to conc. HCl the nitrile did dissolve, yet after 12 hours at 80°C it still remained unchanged. Hydrolysis using 100% H$_3$PO$_4$ was also attempted, but under these conditions ether cleavage also occurs, as shown by evidence in the MS. Heading towards the more obscure, a paper by Chemat et al.$^{149}$ describes a method employing CuCl$_2$·2H$_2$O to transform nitriles to carboxylic acids under solventless conditions (240°C, 4 bar, 1 h). This did not work either, and according to the proposed mechanism$^{149}$(a succession of oxido-reduction reactions) this was not surprising as the hydroxide ion is formed and this would attack the HT.

![Scheme 3-20. Mechanism of Nitrile Hydrolysis by Phthalic Acid](image-url)
Another paper by Chemat el al\textsuperscript{150} described another method for “dry” hydrolysis of nitriles, using dicarboxylic acids, namely phthalic acid. The conditions are similar to that of the CuCl\textsubscript{2}·2H\textsubscript{2}O method. Rounds and co-workers\textsuperscript{151} described a similar method in which either tetrafluorophthalic or tetrachlorophthalic acid is used in the solventless hydrolysis at lower temperatures (170\textdegree C), in sealed pressure tubes, for longer reaction times (5 days). The reaction mechanism is shown above in Scheme 3-20.

The initial attempt used phthalic acid and gave mixed results. At this point the scale of synthesis was becoming a problem. Working typically in the 20-50mg scale available high pressure bombs or pressure tubes were too big, heating mantles produced heterogeneous heat, and hence temperature control of the sample was poor. The reaction produced pockets of sublimed nitrile and charred material. Manufacturing spherical thick-walled Carius tubes 3 cm in diameter, sealing the reagents under vacuum, and then heating the tube in a steel shield that was in a metal bath solved these problems. The reaction was left at 260\textdegree C for one hour, and gave upon work-up a red solid. +FAB-MS of this compound gave the right parent ion (m/z 382), but the $^1$H spectrum showed signs of another side product.

The reaction was repeated with tetrafluorophthalic acid, at lower temperature (160\textdegree C) for 4 days. The resulting solid, on cooling, was dissolved chloroform, leaving behind most of the tetrafluorophthalimide as an insoluble white solid. Final purification by centrifugal chromatography on silica, using methanol, was unsuccessful as the product stuck and could not be regenerated. Gel permeation chromatography (LH-20, 4:3 CHCl\textsubscript{3}: MeOH) was used instead to afford the pure compound as a bright yellow compound. The NMR spectrum of the acid 3.90 is shown in Figure 3-30 ($^1$H/$^{13}$C). The proton spectrum
Figure 3-30  Assigned NMR Spectra of 3.90, (a) $^1$H and (b) $^{13}$C
does not show any significant change from the starting nitrile. Figure 3-30b clearly shows the absence of the nitrile carbon in conjunction with the presence of the acid (176.9 ppm). The presence of the product was confirmed by mass spectra: +LSIMS (411.1, [M+1]+) and -LSIMS (409.1, [M-1]).

![Chemical reaction diagram]

Yield (\%)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Yield</th>
<th>Reaction Condition</th>
</tr>
</thead>
<tbody>
<tr>
<td>HOOC-2-HT-6</td>
<td>3.90</td>
<td>90</td>
</tr>
<tr>
<td>HOOC-2-HT-8</td>
<td>3.92</td>
<td>71</td>
</tr>
<tr>
<td>HOOC-2-HT-{-m}-6</td>
<td>3.93</td>
<td>88</td>
</tr>
<tr>
<td>HOOC-2-m-HT-6</td>
<td>3.94</td>
<td>86</td>
</tr>
<tr>
<td>HOOC-2-m'-HT-6</td>
<td>3.95</td>
<td>83</td>
</tr>
</tbody>
</table>

Scheme 3-21. Nitrile Hydrolysis of HT Series

Using the same procedure, I was able to hydrolyze the series of nitrile HTs to yield the respective acids, as shown in Scheme 3-21.

All the NMR spectra of the hemithioindigos synthesized can now be compared. The NMR assignments of the core hemithioindigo moiety of all the HTs synthesized are displayed in Table 3-2 ('H) and Table 3-3 ('C). It can be seen quite clearly in these tables that there are certain atoms that remain fairly insensitive to the substitution pattern of the HT, as well to variation in solvent. The most important characterizing signal is that of C-7', which varies between in the 'H: 7.81-7.95 (\(\Delta = 0.14\)) ppm, and in the 'C: 133.6-134.9 (\(\Delta = 1.3\)) ppm. C-2, C-3, and C-1' are also insensitive, and thus can be used as identifiers for the presence of HT in a sample.
<table>
<thead>
<tr>
<th>Compound</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>2'</th>
<th>3'</th>
<th>4'</th>
<th>6'</th>
<th>7'</th>
<th>Solvent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Br-HT-OH, 3.49</td>
<td>7.90</td>
<td>7.61</td>
<td>7.29</td>
<td>7.64</td>
<td>6.94</td>
<td>10.5</td>
<td></td>
<td></td>
<td>7.86</td>
<td>DMSO</td>
</tr>
<tr>
<td>Br-HT-4 and 6, 3.50-51</td>
<td>8.02</td>
<td>7.63</td>
<td>7.36</td>
<td>7.62</td>
<td>6.96</td>
<td>7.91</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NC-HT-OH, 3.80</td>
<td>7.77</td>
<td>7.60</td>
<td>7.69</td>
<td>7.61</td>
<td>6.90</td>
<td>10.4</td>
<td></td>
<td></td>
<td>7.82</td>
<td>CD,OD</td>
</tr>
<tr>
<td>NC-HT-4, 6 and 8, 3.81-3.83</td>
<td>7.76</td>
<td>7.51</td>
<td>7.42</td>
<td>7.64</td>
<td>6.97</td>
<td></td>
<td></td>
<td></td>
<td>7.93</td>
<td>CDCl_3</td>
</tr>
<tr>
<td>HOOC-HT-6 and 8, 3.90, 3.92</td>
<td>7.77</td>
<td>7.41</td>
<td>7.63</td>
<td>6.96</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>7.91</td>
<td>CDCl_3</td>
</tr>
<tr>
<td>NC-HT-m-6, 3.84</td>
<td>7.77</td>
<td>7.47</td>
<td>7.26</td>
<td>7.36</td>
<td>6.95</td>
<td>7.20</td>
<td>7.91</td>
<td></td>
<td></td>
<td>CDCl_3</td>
</tr>
<tr>
<td>HOOC-HT-m-6, 3.93</td>
<td>7.77</td>
<td>7.47</td>
<td>7.38</td>
<td>7.26</td>
<td>7.33</td>
<td>6.95</td>
<td>7.20</td>
<td>7.90</td>
<td></td>
<td>CDCl_3</td>
</tr>
<tr>
<td>NC-m-HT-6, 3.88</td>
<td>7.82</td>
<td>7.08</td>
<td>7.32</td>
<td>7.59</td>
<td>6.94</td>
<td></td>
<td></td>
<td></td>
<td>7.86</td>
<td>CDCl_3</td>
</tr>
<tr>
<td>HOOC-m-HT-6, 3.94</td>
<td>7.84</td>
<td>7.12</td>
<td>7.33</td>
<td>7.63</td>
<td>6.97</td>
<td></td>
<td></td>
<td></td>
<td>7.90</td>
<td>CDCl_3</td>
</tr>
<tr>
<td>NC-m'-HT-6, 3.89</td>
<td>7.09</td>
<td>7.42</td>
<td>7.60</td>
<td>6.95</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>7.81</td>
<td>CDCl_3</td>
</tr>
<tr>
<td>HOOC-m'-HT-6, 3.95</td>
<td>7.09</td>
<td>7.41</td>
<td>7.63</td>
<td>6.97</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>7.87</td>
<td>CDCl_3</td>
</tr>
<tr>
<td>Me-OOC-m'-HT-6, 3.53</td>
<td>8.05</td>
<td>7.68</td>
<td>7.51</td>
<td>7.64</td>
<td>6.98</td>
<td></td>
<td></td>
<td></td>
<td>7.95</td>
<td>CDCl_3</td>
</tr>
</tbody>
</table>

Table 3-3. Proton NMR of Hemithioindigo Derivatives (Core)/ppm
<table>
<thead>
<tr>
<th>Compound</th>
<th>2</th>
<th>3</th>
<th>3a</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>7a</th>
<th>1'</th>
<th>2'</th>
<th>3'</th>
<th>4'</th>
<th>5'</th>
<th>6'</th>
<th>7'</th>
<th>Solvent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Br-HT-OH, 3.49</td>
<td>144</td>
<td>186.0</td>
<td>132.0</td>
<td>128.5</td>
<td>118.9</td>
<td>137.7</td>
<td>126.4</td>
<td>125.7</td>
<td>124.5</td>
<td>133.5</td>
<td>116.4</td>
<td>160.5</td>
<td>134.8</td>
<td>DMSO</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Br-HT-I and 6, 3.50-51</td>
<td>144.6</td>
<td>187.2</td>
<td>132.5</td>
<td>129.6</td>
<td>119.3</td>
<td>137.5</td>
<td>125.2</td>
<td>127.2</td>
<td>126.4</td>
<td>133.2</td>
<td>115.2</td>
<td>161.2</td>
<td>134.9</td>
<td>CDCl₃</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NC-HT-OH, 3.80</td>
<td>143.4</td>
<td>187.2</td>
<td>130.3</td>
<td>126.2</td>
<td>137.1</td>
<td>136.1</td>
<td>124.4</td>
<td>Hidden</td>
<td>124.7</td>
<td>133.4</td>
<td>116.4</td>
<td>160.2</td>
<td>134.3</td>
<td>CD₂OD</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NC-HT-I, 6 and 8, 3.81-83</td>
<td>145</td>
<td>188.2</td>
<td>131.4</td>
<td>126.3</td>
<td>135.5</td>
<td>135.2</td>
<td>124.3</td>
<td>127.7</td>
<td>126.6</td>
<td>133.1</td>
<td>115.1</td>
<td>161.1</td>
<td>134.3</td>
<td>CDCl₃</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HOOC-HT-I, 6 and 8, 3.90, 3.92</td>
<td>144.1</td>
<td>188.5</td>
<td>131.1</td>
<td>126.3</td>
<td>137.8</td>
<td>135.5</td>
<td>123.9</td>
<td>127.9</td>
<td>126.7</td>
<td>133.1</td>
<td>115.1</td>
<td>161.0</td>
<td>134.0</td>
<td>CDCl₃</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NC-HT-m-6, 3.84</td>
<td>145.2</td>
<td>188.3</td>
<td>131.0</td>
<td>126.4</td>
<td>135.7</td>
<td>135.6</td>
<td>124.3</td>
<td>130.5</td>
<td>135.3</td>
<td>123.6</td>
<td>130.0</td>
<td>118.6</td>
<td>159.5</td>
<td>116.1</td>
<td>134.1</td>
<td>CDCl₃</td>
</tr>
<tr>
<td>HOOC-HT-m-6, 3.93</td>
<td>144.2</td>
<td>188.6</td>
<td>130.7</td>
<td>126.5</td>
<td>138.0</td>
<td>135.9</td>
<td>123.9</td>
<td>Hidden</td>
<td>135.4</td>
<td>123.5</td>
<td>129.9</td>
<td>117.0</td>
<td>159.5</td>
<td>116.1</td>
<td>133.9</td>
<td>CDCl₃</td>
</tr>
<tr>
<td>NC-m-HT-6, 3.88</td>
<td>145.5</td>
<td>187.7</td>
<td>129.9</td>
<td>127.1</td>
<td>125.7</td>
<td>146.7</td>
<td>123.4</td>
<td>127.4</td>
<td>126.4</td>
<td>132.9</td>
<td>115.0</td>
<td>160.9</td>
<td>133.9</td>
<td>CDCl₃</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HOOC-m-HT-6, 3.94</td>
<td>146.6</td>
<td>188.2</td>
<td>129.4</td>
<td>127.1</td>
<td>126.0</td>
<td>148.3</td>
<td>123.5</td>
<td>127.7</td>
<td>126.7</td>
<td>133.0</td>
<td>115.1</td>
<td>160.9</td>
<td>133.9</td>
<td>CDCl₃</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NC'-m-HT-6, 3.89</td>
<td>147.6</td>
<td>189.0</td>
<td>127.2</td>
<td>141.0</td>
<td>127.7</td>
<td>134.4</td>
<td>123.3</td>
<td>127.4</td>
<td>126.5</td>
<td>132.9</td>
<td>115.0</td>
<td>160.9</td>
<td>133.9</td>
<td>CDCl₃</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HOOC'-m-HT-6, 3.95</td>
<td>147.4</td>
<td>189.2</td>
<td>127.5</td>
<td>143.9</td>
<td>127.4</td>
<td>134.3</td>
<td>122.5</td>
<td>127.6</td>
<td>126.8</td>
<td>133.0</td>
<td>115.1</td>
<td>160.9</td>
<td>133.6</td>
<td>CDCl₃</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Me-OOC'-m-HT-6, 3.53</td>
<td>147.8</td>
<td>187.9</td>
<td>131.4</td>
<td>126.0</td>
<td>132.1</td>
<td>134.8</td>
<td>124.3</td>
<td>127.4</td>
<td>126.5</td>
<td>133.2</td>
<td>115.2</td>
<td>161.2</td>
<td>134.0</td>
<td>CDCl₃</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 3-4. Carbon NMR of Hemithioindigo Derivatives (Core)/ppm
3.11 Hemithioindigo Lipid Synthesis

Scheme 3-22. Synthetic Pathway for Formation of the Phosphatidylcholine Headgroup.

The four literature routes to synthesis the phospholipid headgroup are shown in Scheme 3-22. The glycerol backbone with the acyl groups attached, ROH, is treated with phosphoryl chloride 3.96 to give alkyl phosphorodichloridates 3.97 (A). To get from 3.97 to the target one has a choice. 3.97 can be (i) condensed with bromoethanol, followed by amination\textsuperscript{152-154}, (ii) condensed with choline tosylate\textsuperscript{155,156}, or (iii) cyclized with ethylene glycol, and subsequently opened by nucleophilic attack\textsuperscript{157-160}. The fourth route (B) treats 3.96 with N-methylethanolamine, affording 2-chloro-3-methyl-1,3,2-\lambda\textsubscript{3}-oxazaphospholane 3.100, to which ROH is then coupled, yielding 3.101, which is oxidized and then cleaved with dimethylsulfate to yield the target, 3.102\textsuperscript{161,162}. 
Synthetic details on each route will not be discussed, as they can be found in the literature.

On a purely synthetic efficiency basis, route B is preferable, as the heavy ROH is added later along the synthetic sequence. There is no real advantage in the de novo synthesis of headgroups for natural lipids, as they are more accessible from natural sources. The routes are summarized here as they allow the synthesis of unnatural headgroups, and hence future optimal target manipulation.

Glycerophosphocholine (GPC) 3.103 is commercially available as the CdCl₂ complex and synthetic pathways exist to acylate it with fatty acids (and synthetic homologues)\textsuperscript{163,164}. The most common reagents are acid chlorides, anhydrides and mixed anhydrides (Scheme 3-23). The acid chloride route is not advisable as the reagent has to be used in large excess, forms side products and hence has low yields. Gupta et al.\textsuperscript{164}, using anhydrides in only a 1.2 mol equivalent per OH group, with $N,N$-dimethyl-4-aminopyridine (DMAP) as a catalyst, were able to synthesize phospholipids, at room
temperature, from GPC, lysolecithins, and N-protected lysophosphatidylethanolamines. in high yields (75-90%). The use of mixed anhydrides (prepared from pivaloyl chloride and acid) further economizes the use of the reagent, leading to nearly stoichiometric amounts of fatty acid needed.

Scheme 3-24. Synthesis of HT Lipid Membrane Analogue

The strategy employed in the synthesis of the photofusogens follows that of Whitten et al. HOOC-2-HT-6, 3.90 was reacted with pivaloyl chloride 3.104 and NEt₃, in dry CH₂Cl₂, at room temperature for 35 hours. The resultant crude mixed anhydride was dried under vacuum to remove excess pivaloyl chloride and amine. The GPC-CdCl₂ complex was rendered anhydrous by repeated evaporation of added dry benzene, and then had DMAP and the mixed anhydride added to it, in dry CH₂Cl₂. The mixture was allowed to stir for 23 h. Monitoring the reaction with TLC, one could easily tell from the intense colour, when all starting material had been converted to the product. The crude
product was concentrated and eluted through a Rexyn-I-300 (cationic and anionic) column to remove the cadmium and the DMAP. It was then purified by size exclusion chromatography, using Sepahdex LH-20, yielding a yellow solid upon concentration.

The NMR spectrum of PC-HT-6 3.106 is shown in Figure 3-31 ($^1$H) and Figure 3-32 ($^{13}$C). The $^1$H NMR shows the presence of the two HT esters, as all the peaks are doubled (except for the aliphatic tails). The peaks associated with the headgroup are characteristic of PC$^{97,165}$: H$_j$ (5.19 ppm), H$_l$ (4.11 ppm), H$_m$ (3.86 ppm) and H$_a$ (3.38 ppm). H$_i$ and H$_l$ show up as an unresolved broad peak at 4.36 ppm (lit. H$_i$, H$_l$ = 4.41, 4.33 ppm), while H$_k$ is partially hidden by the H$_f$ protons at 3.96 ppm (lit. H$_k$ = 3.94-4.02 ppm). The $^{13}$C NMR does not show the doubling up for all the HT carbons, instead this is limited to the ester linker (C$_{bb}$, C$_b$, and C$_h$: 172.2, 171.9; 29.8, 29.1; 34.9, 35.1 ppm respectively). The PC headgroup carbons can be assigned from literature precedent$^{153}$: C$_a$ (54.3 ppm), C$_b$ (66.2 ppm, $^{3}J_{CP}$ = 4.8 Hz), C$_c$ (59.5 ppm), C$_d$ (63.5 ppm), C$_e$ (70.7 ppm, $^{3}J_{CP}$ = 6.1 Hz), and C$_f$ (63.1 ppm).

The same procedure was employed in the synthesis of another three lipid isomers, by employing the different HT carboxylic acids, to yield PC-HT-m-6 3.107 (91 %), PC-m-HT-6 3.108 (87 %), and PC-m'-HT-6 3.109 (92 %). Their NMR spectra are shown in Figure 3-33 ($^1$H) 3.107, Figure 3-34 ($^{13}$C) 3.107, Figure 3-35 ($^1$H) 3.108, Figure 3-36 ($^{13}$C)3.108, Figure 3-37 ($^1$H) 3.109, and Figure 3-38 ($^{13}$C) 3.109. In all cases the NMR spectra are a combination of the HT acids (as summarized in Tables 3-2 & 3-3) and the PC headgroup. All four isomers had identical parent ion m/z 1042, [M+1]$^+$, by +FAB MS. In concentrated samples the lipid also shows the dimerized parent m/z 2083 (i.e. [M$_2$ + 1]$^+$).
Figure 3-31 $^1$H NMR Spectra of PC-HT-6 3.106.
Figure 3-32. $^{13}$C NMR Spectra of PC-HT-6 3.106.
Figure 3-33. $^1$H NMR Spectra of PC-HT-m-6 3.107.
Figure 3-34. $^{13}$C NMR Spectra of PC-HT-m-6 3.107.
Figure 3-35. $^1$H NMR Spectra of PC-$m$-HT-6 3.108.
Figure 3-36. $^{13}$C NMR Spectra of PC-\textit{m}-HT-6 3.108.
Figure 3-37. $^1$H NMR Spectra of PC-\textsuperscript{m'-}HT-6 3.109.
Figure 3-38. $^{13}$C NMR Spectra of PC-m-HT-6 3.109.
The synthesis described in this Chapter was directed to the goal of "rapid" production of HT candidate fusogens. In reality the synthesis took much longer than expected. The end result is a synthetic pathway that although not rapid, allows for the production of HT-lipid derivatives in about 3 weeks.

The discussion above focused on individual products and their characterization by NMR. The synthetic products were also examined by UV-Vis and elemental analysis. The data are given in Chapter 6. In addition a number of related compounds were prepared. Their NMR spectra are given in the Appendix.
Chapter 4

Synthetic Efficiency

4.1 Introduction

The quality of a synthesis (degree of excellence) has been hard to judge, as there are no set criteria for an objective analysis. The quality of a synthesis is linked to the following factors: (i) synthetic efficiency, (ii) environmental impact, and (iii) molecular complexity. This chapter will focus on the first factor. The environmental impact of the synthesis can be considered a crucial component of the synthetic efficiency, so it will be discussed briefly, if just to elucidate the areas where research is needed. The molecular complexity of a synthesis has been previously investigated\textsuperscript{166} and will not be discussed further.

The method normally used to compare different syntheses of the same compound has been simplistic at best. It has been couched in one term: overall yield. Traditional organic synthesis judges a synthetic pathway by the overall yield of the longest straight sequence of steps. The synthesis with the highest yield is thus judged the most efficient. The greatest problem with this methodology is that it only takes into account the yield starting from one of the reagents, and disregards any yields from shorter pathways. This is particularly misleading with highly convergent sequences. An alternative method was published by Hendrickson\textsuperscript{167}, in which, with the aid of graph theory, he examined efficiency taking into account the nature of the synthetic sequence, the amounts of starting materials and reagents, and the time needed to carry out the synthetic operations.
4.2 The Nature of the Synthetic Sequence

The synthesis plan devised by Hendrickson divides the nature of the synthetic sequence into the order in which the reactions are done and the type of connections made. There are two types: the construction reactions, which build the target skeleton; and the refunctionalization reactions, which alter the existent functional groups without changing the skeleton. The construction reactions can be further sub-divided into affixation reactions, which unite separate synthons, and cyclizations, which create skeletal rings. A graph plan can be used to examine the synthetic sequence. Every point the graph represents an isolable compound (starting material, intermediate, and target) and the connecting lines the type of transformation.

Figure 4-1 shows an example of the plan graph. There are \( k \) starting materials or synthons, and on the graph they are numbered \((i = 1 \rightarrow k)\). They are assigned a rank, \( l \), which corresponds to the number of steps they are away from the target, and hence the
target has a rank \( l = 0 \). The longest linear sequence of steps is called the main line (which would typically have been used to calculate the overall yield). Horizontal lines between points represent refuctionalizations and cyclizations, while diagonal lines are affixation reactions. A particular type of refuctionalization, that of the protection and deprotection is also identified. Figure 4-1 is a hypothetical synthesis involving 3 synthons that is completed in 7 steps. These steps can be divided into two refuctionalizations \((i = 1, l = 5 \text{ to } 4; i = 2, l = 3 \text{ to } 2)\), two affixations \((i = 1,2, l = 3 \text{ to } 2; i = 1, 3, l = 2 \text{ to } 1)\), a protection \((i = 2, l = 4 \text{ to } 3)\), a deprotection \((i = 1, l = 1 \text{ to } 0)\), and a cyclization \((i = 1, l = 4 \text{ to } 3)\).

Central to this analysis is the calculation of the total amount of starting material, \( W \), used in the synthesis. The overall yield from one starting material is the inverse of the amount of that material required to yield one mole of the target. A route that has an overall yield of 5%, for example, would need 20 mol \((1/0.05)\) of starting material to produce a mole of target. All amounts required from all \( k \) starting materials are taken into account in this analysis.

When the real yields of each step, \( y \), in a synthesis are known, calculation of its performance is possible by obtaining the inverse yields, \( x \), and the sum of the inverse yields, \( S_i \). If the yields are not know then one assumes an average yield of 80% per step\(^{167}\). This assumption is based on the idea that most trial synthetic reactions generally allow for the development of procedures that provide at least an 80% yield on average over several steps.
4.2.1 Materials

When comparing two synthetic paths the number of skeletal heavy atoms \((n_i)\) of a synthon \(i\) is used as a measure of the size of the synthon. According to Hendrickson\(^{167}\), its actual molecular weight is \(M_i = F n_i\), where \(F\) is a constant estimated to between 14 and 40. The weight of starting material thus needed to carry a synthon \(i\) through a synthetic path of length \(l_{ij}\) to one mole of target is \(W_{ij} = M_{ix}^{\ell_{ij}} = F n_{ix}^{\ell_{ij}}\). The total weight of all starting materials needed is given by the equation \(W_{a} = F \Sigma n_{ix}^{\ell_{ij}}\). A handier version of this equation is \(W = \Sigma n_{ix}^{\ell_{ij}}\), where \(W\) is a relative weight \((W = W_a/F)\), in which the average value of \(F\) is assumed to cancel out when two plans are compared.

4.2.2 Reagents

The reagent criterion is left in molar amounts rather than weight terms, as the variation of the molecular weights of reagents is very high. If one assumes a 1:1 stoichiometry of starting materials to reagents the estimate can be calculated as follows. The main line \((i = 1)\) incorporates all the intermediates of its line to the target. To include all the intermediates not in the main line, e.g. in convergent plans, where the synthons initiate other sublines, a new term \((\ell_{ij})\) is introduced. Each subline thus begins at rank \(l_i\) and ends at rank \(l_{ij}\), the rank of the last independent intermediate before juncture to a prior line. For a linear sequence the sum of molar amounts used is \(S_l = \Sigma x\). To count the reagents only once the sum of the difference of molar amounts has to be calculated between rank \(l_i\) and \(l_{ij}\): \(R = \Delta S = \Sigma (S_{li} - S_{li'})\) where \(l_{ij} = 0\) and \(S_0 = 0\).
4.2.3 Time

The total time required to execute a synthesis is dependent on the number of steps, \( s \), how long each step takes, and the amount of material manipulated. Although it is clear that some reactions take longer than others, an average effective time, \( T_0 \), will be assumed for each step. The total time required to reach the target becomes \( T = sT_0 \mu \), where \( \mu \) is an upscaling factor that takes into account the weight being manipulated. Powers\(^{168} \) found that \( T \approx W^z \) for nucleotide and nucleic acid synthesis, where \( z = 0.3 \). This is equivalent to saying that it takes twice as long to manipulate a tenfold increase of weight. In order to compute the upscaling factor the weight of each synthon each time it is part of an intermediate reaction is needed. This requires the total weight manipulated, \( TW \), which is \( TW = F\sum n_iS_i \), but \( F \) is a constant and thus is cancelled giving relative weights: \( TW = \sum n_iS_i \). The total weight divided by the number of steps gives an average weight per step, \( TW/s \). Total time now becomes \( T = s(TW/s)^{0.3} \). This allows for relative time required to be measured and is useful when comparing syntheses.

4.3 Synthetic Efficiency of Lipid Synthesis: PC-HT-6 Pathways

4.3.1 Path I

Judging the synthetic efficiencies of the lipid synthesis allows one to compare the different pathways employed (see Scheme 3-2), as well as the ability to plan future work in expanding the lipid homologous series. The PC-HT-6 synthesis was used as the standard. Path I, the sequence that was ultimately used, is shown in the Scheme 4-1. Each starting material/synthon is assigned its number, \( i \), and rank, \( l \). Ranks of the intermediates are also shown. The corresponding Hendrickson synthetic efficiency graph
Scheme 4-la: Path I of PC-HT-6, 3.106
Scheme 4-1b: Path I of PC-HT-6, 3.106
was constructed as shown in Figure 4-2. The yields of each individual step are indicated above each intermediate. Only the yield of the HT from the acid is known \( l = 6 \) to 3), so this 67 % yield is deconvoluted into 100 %, 41 % and 80 % for the acid chloride, indoxyl and condensation reactions, respectively. The acid chloride was formed with great ease, and minimum manipulation was required indicating a near quantitative process. The indoxyl formation is the low yielding reaction in this sequence, and thus by assigning the Hendrikson average 80 % yield value to the condensation step we get 41 % for the former. There are 11 steps in the synthesis: 5 affixations, 1 cyclization, 4 refunctionalizations, and 1 deprotection, as shown in the plan graph.

Table 5-1 gives a summary of the yields, \( y \), the inverse yields, \( x \), and the sum of inverse yields, \( S_x \), for each step. The ‘overall yield’ for the synthesis from bromothiophenol is \( y_{10} \), i.e. 10.4 %. These data were used to calculate the weight and the reagent amounts in Table 5-2. This table summarizes for each starting material/synthon, \( i \), the number of skeletal heavy atoms, \( n \), the rank, \( l \), its relative weight, \( n_r \), the relative total weight manipulated, \( n_S_i \), and the relative amount of reagent required \( S_i \). The sum of the relative weights of starting materials yields the total weight of starting material used, \( W \), while the total of weight manipulated, \( TW \), is the sum of \( n_S_i \). Assuming a 1:1 ratio of reagent:starting material, the amount of reagent required is the sum of \( S_i \).

With these data at hand, we are now in the position to evaluate different synthetic pathways to the same lipid. Three other pathways, outlined in Scheme 4-2, Path III (Linear Synthesis), Path I (with protecting groups), and Path II were analyzed using the Hendrickson approach. Their plan graphs are described and then they are compared. All the yield and weight data are tabulated in the appendix.
Figure 4-2 Plan Graph of Path I with Real Yields.

Table 4-1: Real Yields of Path I

<table>
<thead>
<tr>
<th></th>
<th>x</th>
<th>y</th>
<th>S_i</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.18</td>
<td>0.85</td>
<td>1.18</td>
</tr>
<tr>
<td>2</td>
<td>2.35</td>
<td>0.85</td>
<td>1.18</td>
</tr>
<tr>
<td>3</td>
<td>3.66</td>
<td>0.765</td>
<td>2.35</td>
</tr>
<tr>
<td>4</td>
<td>5.29</td>
<td>0.612</td>
<td>5.29</td>
</tr>
<tr>
<td>5</td>
<td>9.26</td>
<td>0.252</td>
<td>9.26</td>
</tr>
<tr>
<td>6</td>
<td>7.85</td>
<td>0.392</td>
<td>7.85</td>
</tr>
<tr>
<td>7</td>
<td>13.23</td>
<td>0.252</td>
<td>13.23</td>
</tr>
<tr>
<td>8</td>
<td>17.64</td>
<td>0.227</td>
<td>17.64</td>
</tr>
<tr>
<td>9</td>
<td>24.39</td>
<td>0.148</td>
<td>24.39</td>
</tr>
<tr>
<td>10</td>
<td>33.09</td>
<td>0.115</td>
<td>33.09</td>
</tr>
</tbody>
</table>

Table 4-2: Weight Summaries of Path I

<table>
<thead>
<tr>
<th></th>
<th>x</th>
<th>nx</th>
<th>S_i</th>
<th>nS_i</th>
<th>S-S_i</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>42.70</td>
<td>67.31</td>
<td>298.93</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>42.70</td>
<td>28.85</td>
<td>128.11</td>
<td>10</td>
<td>42.70</td>
</tr>
<tr>
<td>3</td>
<td>42.70</td>
<td>67.31</td>
<td>298.93</td>
<td>1</td>
<td>1.18</td>
</tr>
<tr>
<td>4</td>
<td>42.70</td>
<td>28.85</td>
<td>128.11</td>
<td>10</td>
<td>42.70</td>
</tr>
<tr>
<td>5</td>
<td>42.70</td>
<td>34.78</td>
<td>132.35</td>
<td>9</td>
<td>33.09</td>
</tr>
<tr>
<td>6</td>
<td>42.70</td>
<td>33.09</td>
<td>132.35</td>
<td>9</td>
<td>33.09</td>
</tr>
<tr>
<td>7</td>
<td>42.70</td>
<td>7.85</td>
<td>62.76</td>
<td>4</td>
<td>5.29</td>
</tr>
<tr>
<td>8</td>
<td>42.70</td>
<td>7.85</td>
<td>47.07</td>
<td>4</td>
<td>5.29</td>
</tr>
<tr>
<td>9</td>
<td>42.70</td>
<td>15.31</td>
<td>62.76</td>
<td>4</td>
<td>5.29</td>
</tr>
<tr>
<td>10</td>
<td>42.70</td>
<td>15.31</td>
<td>47.07</td>
<td>4</td>
<td>5.29</td>
</tr>
<tr>
<td>11</td>
<td>42.70</td>
<td>18.82</td>
<td>18.88</td>
<td>1</td>
<td>1.18</td>
</tr>
</tbody>
</table>

n_o = 72
W = 352
TW = 1357
R = 94.4
4.3.2 Path III (Linear Synthesis)

Knowledge of the advantages of a convergent synthesis over a linear one can be viewed as basic synthetic planning. An analysis of a linear synthetic plan of PC-HT-6 (Path III) is thus a good way to evaluate the data yielded by the Hendrickson method. Scheme 4-2 shows the altered synthetic pathway.

Only one reaction in the sequence has been changed: instead of performing the Williamson ether synthesis on the hydroxybenzaldehyde starting material, it is performed on the HT. The plan graph is shown in Figure 4-3. Since there are no convergent steps the highest rank $I = 11$, is equal to the number of synthetic steps. All the yields are the same as in Figure 4-2, although the reality of performing the ether synthesis on the HT gave much lower yields (18%). The importance in analyzing this sequence is to determine the effect of the nature of the pathway on the synthetic efficiency. Refer to Table A-1 and A-2 for yield data and weight data, respectively.
Scheme 4-2. Path III (Linear)

Figure 4-3. Plan Graph for Path III (Linear)
4.3.3 Path I-P (Protecting Groups)

The use of protecting groups in organic synthesis is very common. The effect of the use of protecting groups on synthetic efficiency can be looked at with the following synthetic plan for PC-HT-6, Scheme 4-3. A successful synthesis needs to incorporate two orthogonal protecting groups to enable the differentiation of carboxylic acids 1,3 and 2,4, as seen in Figure 4-4. The yields of the protecting steps are taken to be 80%, while the deprotections are taken to be 90% (striving to their equivalent steps in Figure 4-2). Protecting groups naturally add steps to the synthesis, normally two per group. In this synthesis only two are added overall, $s = 13$, since incorporated in Figure 4-2, is one
synthon already protected (bromo ethyl acetate, \( i = 2,4 \)) and the other (nitrile, \( i = 5, 6 \)) that has to be refunctionalized to yield the acid.

Refer to Table A-3 and A-4 for yield data and weight data, respectively.

4.3.4 Path II

The last synthetic pathway investigated involves the change of sequence of steps. The HT is formed first, and then the linker is added afterwards, Scheme 5-4. A protecting group is needed to preserve the HT double bond from dehydrogenation later down the synthetic sequence. This entails two additional steps, yet synthon 9 10 does not need to be protected, so the total number of steps is 12. Looking at the graph plan (Figure 4-5) an advantage becomes obvious. The formation of the basic HT moiety is of much higher yield than its equivalent in Figure 4-2. Here the yield for the 3 steps from acid to HT is 74 %, which was deconvoluted into 100 %, 92 %, and 80 % for acid
Scheme 4-4. Path II
chloride, indoxyl and condensation reactions. The protection and deprotection yields are assumed to be 80\%.

Figure 4-5. Plan Graph of Path II

Refer to Table A-5 and A-6 for yield data and weight data, respectively.
4.4 Comparison of all Synthetic Plans

Collating all the relevant data from the weight summaries of different plans allows one to compare their synthetic efficiencies. Table 5-3 displays the number of steps of each plan, s, the ‘overall yield’, OY, the total weight of starting materials, w, the total weight manipulated, TW, reagents amounts (1:1 ratio), R, and the relative time, T.

For clear comparisons, the data is normalized Path I.

Table 4-3. Comparison of Synthetic Pathways

<table>
<thead>
<tr>
<th>Plan</th>
<th>Synthetic Efficiency Data</th>
<th>Normalized Synthetic Efficiency Data</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>s</td>
<td>OY (%)</td>
</tr>
<tr>
<td>Path I</td>
<td>11</td>
<td>10.4</td>
</tr>
<tr>
<td>Path III</td>
<td>11</td>
<td>6.12</td>
</tr>
<tr>
<td>Path I-P</td>
<td>13</td>
<td>8.32</td>
</tr>
<tr>
<td>Path II</td>
<td>12</td>
<td>16.7</td>
</tr>
</tbody>
</table>

* $T = s(TW/s)^{0.3}$

The limitations of OY are now easily recognized. Path II (OY) compared to PC-HT-6 (OY) makes one believe that Path II is a superior synthesis. Hendrickson's data shows otherwise. Basically the same weight is needed, but the nature of the sequence dictates that it has to be manipulated more (TW is 14% higher), and hence more reagents are needed (11%), resulting in a 11% longer time. This was not predicted by OY, in fact quite the opposite.

The effect of a linear synthesis (Path III) as compared to the more convergent one results in a roughly 50% increase in $W$, $TW$, and $R$, leading to a 15% increase in $T$. $OY$ exaggerates the effect, indicating an increase of 70%.
The effect of protecting groups (Path I-P) is subtle. The weight increases slightly (6 %), due to the extra steps, which in turn augment the total weight 11 % and vastly affect the reagents needed (50 % increase). Stating that the weight stays nearly the same is ignoring the fact that the nature of protecting groups is to increase the $F$ of synthons, and hence an adjustment must be made for this. To get a better comparison the real molecular weights for Path I and the Path I-P plan were used to calculate $F$'s. This allows the real $W$ and $TW$ to be calculated. Refer to Table A-7 and A-8 for real weight data.

To calculate the Path I-P plan with real molecular weights we assume that the protecting groups are going to have a mass equivalent to the N-methylphthalimide protecting group. The protecting groups' mass is part of the total manipulated until the deprotection step, and hence an adjustment must be made in $TW$. As with calculating $R$, a subline, $l'$, is introduced that begins after the deprotection step, as well as $F'$, where $F' = F_{Path I-P} - F_{Path I}$, which allows the excess $TW'$ to be calculated. We are now in the position to compare the two synthetic plans, as shown in Table 5-4.

Table 4-4: Real Weight Comparison between Path I and Path I-P

<table>
<thead>
<tr>
<th>Plan</th>
<th>s</th>
<th>W</th>
<th>TW</th>
<th>T</th>
<th>Synthetic Efficiency Data</th>
<th>Standardized Data</th>
</tr>
</thead>
<tbody>
<tr>
<td>Path I</td>
<td>11</td>
<td>9534</td>
<td>38719</td>
<td>127</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Path I-P</td>
<td>13</td>
<td>17007</td>
<td>56218</td>
<td>160</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>s</th>
<th>W</th>
<th>TW</th>
<th>T</th>
<th>W</th>
<th>TW</th>
<th>T</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>1.78</td>
<td>1.45</td>
<td>1.26</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The addition of protecting groups increases the mean $F$ by 60 %. This has a profound impact on $W$ and $TW$. The weight of starting material required increases the same amount as the weight factor $F$. The total manipulated weight is less affected, as the protecting groups do not have to be carried right until the end of the synthesis. Needless
to say, total weight manipulated does increase ultimately resulting in a much higher relative time, 26%, the highest of all plans.

4.5 Conclusion on Synthetic Efficiency

Hendrickson's concept of synthetic efficiency is a very effective tool. It allows similar synthetic plans to be critically analyzed and brings to the surface several points that should be considered when devising a plan.

1) Linear plans will always be inferior to convergent ones. They always signify more W, WT and R. The change of two convergent steps to linear ones as in Path I to Path III increases, by 50%, all the three indicators, which in turn extends the time (by 15%) needed to perform the synthesis. The more convergent the synthesis, the more efficient.

2) An increase in total steps required to complete the synthesis has a profound influence. The main difference between Path I and Path II is that the latter has an extra step. It is important to note that using standard measures of synthetic efficiency, i.e. OY, Path II comes out on top, while analysis of the plan shows it substandard to Path I. Thus the general conclusion is to have the fewest steps possible, and that even a plan that has superior yields than one with fewer steps must be carefully scrutinized.

3) Protecting groups are a hidden bane. They increase the number of steps and at the same time increase F, resulting in higher W and WT. If protecting groups are used they must be added on at the last possible step and removed as soon as possible.

4) In the same vein as protecting groups, one must try to minimize F. Part of the convergence principle is the dissection of target in equal parts and the use of small
molecular weight starting materials. Protection of such synthons increases F dramatically, as these groups tend to be of moderate mass. In Path I-P, synthons, \( i = 2, 4 \), \( F \) increases by 1.8 (99.3/55.7), while for \( i = 5, 6 \), it increases by 4.3 (57.8/13.3) compared to Path I. The smaller the starting material the greater the effect. One must conclude that protecting lower molecular weight synthons (when \( n \) is small) is more detrimental than protecting heavier ones. Another way of stating this is that protection should be avoided at the beginning of a synthesis and should be restricted to its intermediates.

5) Any heavy synthon (high \( n \)) should be placed at the lowest rank possible. This minimizes the \( W \) and WT required. The opposite is true for lower yielding steps. If they are unavoidable, they should be placed at the highest rank possible.

6) The plan graphs give a useful pictorial view of synthetic plans. They easily show where the bottlenecks in a plan are and might consider sequences not previously thought of. One can quickly tell that the Achilles' heel of Path I is the indoxyl step. Path II improves this step, giving a much higher yield, but the nature of the sequence is of poorer efficiency, as can be deduced by the calculations. This is even before any analysis resulting from the protection/deprotection step that Path II requires and its weight consequences.

7) One last point needs to be made. The overall yields of synthetic plans are poor criteria to judge them. It is a one-dimensional factor that relies only on the yields of the mainline. As has been shown here, not only can OY exaggerate the effect of a synthetic sequence (Convergence vs. Linear), but also misinform (as in Path II), as well as give no information at all (as with protecting groups).
4.5.1 Other considerations

The previous discussion has focused on the analysis of synthetic efficiency of several pathways reaching the same target. Targets of property directed syntheses will vary in structure, and hence to use this system in such cases requires further development of the technique. Lynn Cameron in her dissertation compared the synthetic efficiency of the ion channels she synthesized compared to the previous generation of targets\textsuperscript{169}. She reasoned that since the function is the common factor (i.e. to transport ions), normalization by the number of skeletal heavy atoms of the smaller target yields comparable parameters.

The assumption that the time per step, i.e. reaction, work-up methodology, and purification of compounds even out over a multistep synthesis seems to have limitations. This is of more importance when comparing functionally related compounds that are chemically dissimilar. One could hypothetically have one synthetic pathway that involves only recrystallizations, while the other uses column chromatography. By the sheer nature of the method used, the former will be performed faster than the latter.

Advances in synthetic methods must also be considered. Purification has blossomed in available techniques\textsuperscript{170}. For example: (i) solid phase synthesis can improve the yields of specific reactions, making them quantitative, and at the same time reducing the amount of time required on each step, as purification is trivial. On the other hand, all the reagents used tend to be used in high excess (at least ten-fold). (ii) Fluorous phases, which are insoluble in many organic phases, have been used to separate either products or reagents by placing a fluorous handle on the appropriate molecule. Again, the purification is
trivial, but one can treat the fluorous handle as a protecting group, bring along its associated problems with extra steps and extra weight.

A final detail that needs to be addressed is the environmental impact of the synthetic pathway. If a pathway is going to be deemed efficient it must address the production of waste and its disposal. How to elucidate the impact of a sequence, and then compare it to another, is no easy matter. The details of purification become crucial. Recrystallization produces significantly less solvent waste than column chromatography, while solid phase synthesis can speed up the overall time taken to perform a synthesis, yet is wasteful in terms of reagents and solvents. The nature of the solvents used is important, the best being water, as it needs no specialized disposal, compared to halogenated solvents, like chloroform, which do. But how to gauge the impact of using one or the other, even if all the details were known? I offer no solutions. There are no easy answers. Herein lies the challenge.
Chapter 5

Photochemical Properties and Membrane Fusion Assay

5.1 Absorption Spectra and Photoisomerization

5.1.1 Hemithioindigo Spectra

The accumulated literature on the absorption spectra of hemithioindigos have identified certain trends. In hexanes the Z-isomer normally has two maxima varying from 410 nm to 490 nm, depending on the substitution of the HT moiety. The E-isomer absorbs at wavelengths that are typically 20-30 nm longer than the Z-isomer. Other organic solvents give a more poorly resolved spectrum in which the original peak decreases in intensity and gains a shoulder. Figure 5-1 shows the typical absorption spectra of Br-HT-6, 3.51, in hexane and CH₂Cl₂. The solutions were prepared in daylight, and as can be seen by spectrum a in 5-1.1, the presence of both isomers can be detected. The thermodynamically stable Z-3.51 has three absorption maxima, one in the UV region, at 351 nm, and two in the visible region, at 422 and 446nm. The E-isomer has two distinct peaks, one in the UV at 362nm and one in the visible at 473nm. Irradiation in hexane, at 406 nm for a total of 20 min, produces a photostationary state enriched in the E-isomer. This can be converted totally to the Z-isomer by shining 480 nm light for 15 minutes. The spectrum of 3.51 in dichloromethane, as expected, is not as resolved. The absorption spectrum is now a broad peak with a shoulder at 425 nm and a λmax at 450 nm. When converted to the E-isomer with 406 nm light for 5 min (+15 min same result), the peak loses its shoulder at 425 nm, broadens from ~500 nm to 520 nm, and the λmax shifting slightly (455 nm).

Table 5-1 summarizes the absorption spectra of the family of hemithioindigo compounds synthesized. There are certain trends of interest. The para-HT-alkyl series
Figure 5.1 Absorption Spectra of Br-HT-6, 3.51, $5 \times 10^{-4}$ M, in (a) Hexanes and (b) Dichloromethane
<table>
<thead>
<tr>
<th>Compound</th>
<th>$\lambda_{\text{max}}$ / nm</th>
<th>$\varepsilon_0 \times 10^3$ / M$^{-1}$cm$^{-1}$</th>
<th>Solvent</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Z-isomer</td>
<td>E-isomer</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>E-isomer$^a$</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>E-isomer$^a$</td>
<td></td>
</tr>
<tr>
<td>Br-HT-OH 3.49</td>
<td>423, 446</td>
<td>b</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>451</td>
<td>b</td>
<td>23</td>
</tr>
<tr>
<td>Br-HT-4 3.50</td>
<td>422, 446</td>
<td>362, 473</td>
<td>22</td>
</tr>
<tr>
<td>Br-HT-6 3.51</td>
<td>455</td>
<td>461</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>12</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Br-HT-6 3.50</td>
<td>451</td>
<td>b</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Br-HT-6 3.51</td>
<td>455</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NC-HT-4 3.81</td>
<td>418, 441</td>
<td>357, 467</td>
<td>26</td>
</tr>
<tr>
<td>NC-HT-6 3.82</td>
<td>452</td>
<td>459</td>
<td>28</td>
</tr>
<tr>
<td>NC-HT-8 3.83</td>
<td></td>
<td></td>
<td>19</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HOOC-HT-6 3.90</td>
<td>441</td>
<td>357, 467</td>
<td>17</td>
</tr>
<tr>
<td>HOOC-HT-8 3.92</td>
<td>452</td>
<td>c</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>9.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NC-HT-m-6 3.84</td>
<td>437</td>
<td>455</td>
<td>8.8</td>
</tr>
<tr>
<td></td>
<td>437</td>
<td>455</td>
<td>8.5</td>
</tr>
<tr>
<td>HOOC-HT-m-6 3.93</td>
<td>446</td>
<td>c</td>
<td>7.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NC-m-HT-6 3.88</td>
<td>447</td>
<td>358, 471</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>444</td>
<td>467</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>453</td>
<td>c</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>10</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NC-m'-HT-6 3.89</td>
<td>422, 447</td>
<td>358, 471</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>454</td>
<td>c</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>421, 444</td>
<td>354, 468</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>10</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PC-HT-6 3.106</td>
<td>356, 453</td>
<td>d</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>361, 460</td>
<td>462</td>
<td>28</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PC-HT-m-6 3.107</td>
<td>310, 444</td>
<td>d</td>
<td>9.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PC-m-HT-6 3.108</td>
<td>352, 445</td>
<td>d</td>
<td>26</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PC-m'-HT-6 3.109</td>
<td>351, 451</td>
<td>454</td>
<td>19</td>
</tr>
</tbody>
</table>

Table 5-1. Absorption Spectra of Hemithioindigo Compounds. a) calculated assuming 100 % conversion. b) insensitive to photoisomerization. c) shoulder peak. d) no apparent change.
Figure 5-2. Absorption Spectra of (a) $4.8 \times 10^{-3}$ M NC-$m'$-HT-o, 3.89, in Hexane and (b) $3.3 \times 10^{-3}$ M NC-HT-o, 3.82, in CHCl. 
(3.81-3.83, 3.90, 3.92) have very similar UV-Vis spectra. Varying the length of alkyl tail has no effect on the absorption, as would be expected. Varying the functional group that is attached to the 5 position of the HT has no effect on the position of the absorption maxima, but does influence the extinction coefficient. The sequence followed in decreasing intensity is cyanoethyl > Br > carboxyethyl. Br-HT-OH, 3.49, the hemithioindigo without an alkyl chain, also has the same UV-Vis spectrum as its alkyl counterparts, but shows no effective change when irradiated with light. The meta-HT-alkyl series (3.88, 3.89, 3.94, and 3.95) have very similar UV-Vis spectra compared to the para series. There are minor shifts in the absorption maxima, while the extinction coefficients follow the same trend of the para series. The absorption spectra of NC-\textit{m}\textsuperscript{-}HT-6, 3.89, in hexanes, and that of NC-HT-6, 3.82, in CHCl\textsubscript{3} are shown in Figure 5-2.

![Absorption Spectrum](image)

**Figure 5-3. Absorption Spectra of 1.7 \times 10^{-5} M NC-HT-\textit{m}-6, 3.84, in Hexanes**

The \textit{HT-\textit{m}-6} series does not follow this trend. The UV-Vis spectrum of NC-HT-\textit{m}-6, 3.84 in hexanes is shown in Figure 5-3. The compound exists in its two isomeric forms but
the peaks are poorly defined. The Z-isomer now has two blue-shifted absorption maxima, a broad UV peak at 305 nm and a visible one at 437 nm, while retaining a shoulder at 420 nm. The E-isomer shows no change in the UV peak, while giving a broad peak at 455 nm. The peaks found in the previous examples between 325 nm and 375 nm are no longer present. The nitrile 3.84 did not isomerize to the more enriched Z-isomer solution when the sample was irradiated at 480 nm. The sample in chloroform gives the same results. The results are similar for the acid, 3.93. These are the first HTs to have a substituent in the meta position of the benzyliidene moiety. It is clear that the effect on the electronics of HT is quite profound. These compounds also have lower extinction coefficients than their para isomers.

5.1.2 Hemithioindigo Lipid Spectra

The effect of having the two HT chromophores next to each other in the PC homologues was investigated. There are four possible cis-trans isomers of the PC-HT lipids as shown in Scheme 5.1. One can assume that the Z, Z isomer predominates in a dark solution, and is in equilibrium with the other three isomers of which the E, E-HT is a very minor component.

```
Dominant Z, Z-HT
Minor Z, E-HT
Minor E, Z-HT
Very Minor E, E-HT

Depleted Z, Z-HT
406 nm
Enriched Z, E-HT
480 nm
Enriched E, Z-HT
Major E, E-HT

\( k_2 \)
\( k_1 \)
```

Scheme 5-1. Mechanism of Photoisomerization and Thermal Reversion of PC-HT Lipids.

When the sample is irradiated with 406 nm light, the dominant Z, Z-HT should transform into enriched E, E and the mixed Z, E isomers. Once the irradiation has taken
place, one can safely assume that the E, E isomer is now the major component. To revert to the Z, Z isomer the E, E isomer must pass either through the Z, E-HT, or the E, Z-HT, which should form at similar rates ($k_1$). This would be followed by the isomerization of the second hemithioindigo moiety, which might have a different rate constant $k_2$.

Dilute solutions of PC-$m'$-HT-6, 3.109, and PC-HT-$m$-6, 3.107, in hexanes gave very broad peaks. It was not possible to study the isomerization in hexanes with these compounds, as they precipitated when exposed to the light. Thus the four PC-HT lipids were dissolved in chloroform and their isomerization was investigated. The spectra have broader shapes compared to their monomer counterparts. Lipids 3.106, 3.107 and 3.109 have slightly shifted absorption maxima, while 3.108 shows a blue-shift of 8 nm. Since each sample is a mixture of isomers the calculated extinction coefficients are only apparent values, but still provide some information on whether the two HT moieties in the molecules are communicating with each other. From the tabulated data in Table 5-1, these apparent extinction coefficients are all higher than the acid monomers, but the increase varies. In both 3.106 and 3.108 the increase is approximately threefold, while in PC-$m'$-HT-6, 3.109, case it is twofold. PC-HT-$m$-6, 3.107, shows a moderate increase of a factor of 1.4 times.

In Figure 5-4a the isomerization results of PC-HT-6, 3.106, are shown. Once the initial spectrum was recorded, the sample was left in the dark for 30 minutes to see if any growth occurred. This results in a slight increase of the peak at 460 nm. The sample showed apparent bleaching when irradiated at 406 nm. Irradiating at 480 nm had no effect on the spectrum but after 2 hours in the dark the sample did show an increase. Lipid 3.107 also shows bleaching when irradiated with 406 nm light, and was insensitive to 480 nm light, as seen in Figure 5-4b. The lipid PC-$m'$-HT-6, 3.108, when irradiated at 406 nm showed a
Figure 5-4. Absorption Spectra of HT-lipids in CHCl₃: (a) $4.3 \times 10^{-6}$ M PC-HT-6 3.106, (b) $6.7 \times 10^{-5}$ M PC-HT-m-6 3.107, and (c) $2.0 \times 10^{-5}$ M PC-m-HT-6 3.108
minor increase in both absorption peaks, concurrent with the development of a side-band at 480 nm. It was also insensitive to 480 nm light (Figure 5.4c).

The only PC-HT that showed clear evidence of isomerization in CHCl₃ was PC-\textit{m'-}HT-6. Figure 5.5a shows that the lipid undergoes both Z, Z → E, E and E, E → Z, Z photoisomerizations. After irradiation at 406 nm the spectrum has its UV absorption maximum red shifted slightly by 4 nm (351 → 355 nm), concurrent with an increased intensity, while the visible peak has decreased in intensity, grown a broad shoulder, and red shifted slightly (451 → 454 nm). This behavior is similar to that obtained with the HT monomers in CHCl₃. The photoconversion from the E, E-isomer back to the Z, Z-isomer does not regenerate the original absorption. This state is still more enriched in the Z isomers, as can be seen from the lower intensity and slight shoulder. The thermal conversion of E, E to Z, Z was followed after 15 minutes at 406 nm, as shown in Figure 5-5b. After one and a half hours most of the sample has reverted to the Z, Z form.

The apparent bleaching that we are observed with the other 3 PC-HT samples can be explained in two different ways. The transformation from a Z enriched state to an E enriched state would decrease the intensity of the primary absorption peak. If there is no difference in the absorption maxima of each state, then what would be observed is apparent bleaching. The insensitivity to 480 nm light is due to similar absorption of the individual stereoisomers. The return of intensity through a thermal pathway (as in PC-HT-6) is indicative of such a system. The other possibility is that the aforementioned photochemical [2+2] cycloaddition is taking place\textsuperscript{113,171}. In this scenario the double bond at the center of the HT moiety photodimerizes to form a cyclobutane. In general [2+2] cycloadditions are
Figure 5-5. Absorption Spectra of PC-\textit{m}'-HT-6 in CHCl$_3$:
(a) 1.65 x 10$^{-5}$ M, Photoisomerization, and
(b) 5.8 x 10$^{-6}$ M, Thermal Reversion
not reversible, and thus this pathway would result in "real" bleaching of the compounds. No intensity recovery would be expected.

The isomerization of PC-HT-6 was studied in another solvent system, CF$_3$CH$_2$OH, in order to study whether the bleaching was solvent dependent. Figure 5-6 shows that isomerization does indeed take place, but the change in the spectra are minute. Irradiation with 406 nm light decreases the primary peak and elongates the shoulder. Irradiation with 480 nm light seems to have no effect. This suggests that isomerization is also taking place in CHCl$_3$.

![Absorption Spectra of 4.3 x 10^-5 PC-HT-6, S.15, in CF$_3$CH$_2$OH.](image)

The dimerization process has still not been excluded, and could actually be working in conjunction with the isomerizations. To get a clearer picture on which process was taking place the thermal reversion of the PC-HT-6 bleaching was investigated. Figure 5-7 shows the results for a 4.3 x 10^-3 M solution of PC-HT-6 in CF$_3$CH$_2$OH. After the initial absorption
1.5 - 1.0 - 0.5 - 0.0

- Initial Absorption
- After 30 min. in 350 nm light
- 45 min. later
- 95 min. later
- 155 min later
- 245 min. later
- 365 min. later
- 545 min later
- 1555 min later

Linear Fit for Bleaching Recovery

\[
g(t) = 0.00114 t^{-0.480} + 0.00114 \pm 4\%
\]

- Figure 5-7. Thermal Reversion of PC-HT-6 Bleaching in CF₃CH₂OH
spectrum was recorded, the solution was placed in a Rayonette reactor with eight 350 nm lamps for 30 minutes. There is far more energy output in this setup compared to the usual irradiation at 406 nm. This was expressly done to force the production of the dimer.

If one assumes that the absorption at 461 nm is due entirely to the PC-HT-6, then the absorbance at 461 nm can be directly related to the PC-HT-6 concentration. The difference in the absorption after the bleaching can also be related to a concentration. Now if dimerization is taking place then the following equation describes the process:

\[
B_2 \xrightarrow{\Delta} 2B
\]

where B is PC-HT-6 and B_2 signifies the dimer. When bleaching occurs the concentration of the dimer is half that of PC-HT-6 associated with the drop in absorption. A first order rate constant is expected, so by using the integral method, if \([B_2]^0\) is the concentration at \(t = 0\) and \([B_2]^t\) is the concentration at time \(t\), then a plot of \(\ln([B_2]^0/[B_2]^t)\) against time should give a linear plot. The return of the peak at 461 nm was thus monitored and the linear fit for the intermediate values is shown in the inset of Figure 5-7. The rate constant is \(1.14 \times 10^{-3}\) min\(^{-1}\) which yields a half-life of 480 min. (\(t_{1/2} = \ln(2)/\text{rate constant}\)). I did not include the initial or the last value, 1555 minutes later, in the fit. The sample was left overnight and measured the next morning, and then 4 hours later, which showed no change. This value remains the same as long as the sample is left in the dark. Only after exposure to light for another two days did the original absorption finally return. Including these points it is clear that the plot is not linear. This suggests that the kinetics may have a simple dimerization component, but that there is at least one other slower pathway that is involved. This effect could be due to the
Figure 5.8  NMR Spectra of Bleached PC-HT-6, 3.106, (a) $^1$H and (b) $^{13}$C.
orientation of the pendant groups in the dimerization, that on thermal reversion have different rates of reaction.

Recalling Scheme 5-1, if this apparent bleaching is actually due to differences in absorbance of the different isomers in solution, then the two-component thermal recovery can be explained by different rate constants associated with $k_1$ and $k_2$. The faster rate would be $k_1$, associated to the first HT moiety isomerizing from E to Z, while the slower one is related to the second HT moiety flipping back to Z.

In search of more data on which process is taking place, I bleached (same set-up: Rayonet, 8x350 nm lamps, 30 min.) a more concentrated solution of PC-HT-6, in CDCl$_3$ and recorded its NMR spectra, as shown in Figure 5-8. The $^1$H spectrum (5-8a) has transformed into broad unresolved peaks. These are not due to a highly concentrated sample, as is evident by the signal to noise ratio in the baseline. The $^{13}$C spectrum shows the opposite: very little difference between the pure sample (see Figures 3-31 and 3-32) and the irradiated one. The most significant changes occur between $\delta = 60-70$ ppm, but all these peaks are associated with the PC headgroup, suggesting that the chemical environment that the glycerol linkage is experiencing is different. The dimerization [2+2] photobleaching process would affect the two peaks associated with the double bond the most ($\delta = 143.6$ ppm ($C_\alpha$) and 133.5 ppm ($C_\beta$)), but these peaks are still clearly present. Their intensities remain in the same ratio when compared to other peaks. These data suggest that isomerization is the predominant process and if dimerization is occurring it is a minor pathway. The broadening in the NMR must be related to some as yet unexplored aggregation process.

The photoisomerization of the HT-lipids was also attempted in PS vesicles. Figure 5-9 shows the results. The PC-HT-6 (5-9a) showed minor shoulder increase at 480 nm.
Figure 5.9 Photoisomerization of HT-lipids in Vesicles: (a) PS:PC-HT-6 (9:1), (b) PS:PC-HT-8:2 (8:2) and (c) PS:PC-8:2-HT-6 (9:1).
coupled with a decrease in the peak maximum when irradiated with 406 nm light. This pattern is consistent with the data obtained in the single solvent systems. Irradiating with 480 light seems to bring back the parent peak (or could be due to thermal reversion). PC-HT-m-6 shows minor “bleaching” (5-9b). PC-m'-HT-still retains its isomerization capabilities in vesicles as can be seen in Figure 5-9c.

The conclusion is that the HT moiety is capable of isomerizing in a membrane. This can be most clearly seen in the case of PC-m'-HT. There is a problem in monitoring the process though, as the UV-Vis. changes from one isomer to another are minor. The evidence accumulated so far suggests that if photochemical dimerization is occurring, it is minor. Still, more kinetic studies are needed to understand the thermal processes taking place.

5.2 Fluorescence

![Fluorescence of Hemithioindigo Lipids](image)

Figure 5-10. Fluorescence of Hemithioindigo Lipids
Hemithioindigos do not show intense fluorescence at room temperature. The fluorescence of the HT-lipids was recorded to determine whether it would affect the fusion assay results. Figure 5-10 shows the spectra of two of the lipids. In hexane there is a weak broad peak that has a maximum at 530 nm, irrespective of the actual isomer. When the solvent is changed to CHCl₃ the peak becomes blue-shifted to 480 nm and a small satellite peak becomes apparent at 580 nm. It is apparent that this fluorescence is low intensity and should not contribute to the fusion assay.

5.3 Fusion Assay

5.3.1 Selection of Assay

The most widely used fusion assays use fluorescence as the monitoring technique. The fluorescent probes can be placed in the lipid membrane, or in the aqueous contents of the vesicle.

The most common lipid mixing assay employs the fluorescence resonance energy transfer (RET) between $N$-(nitrobenzoxadiazol)-PE (NDB-PE) and $N$-(lissamine rhodamine B sulfonyl)-PE (Rh-PE). The both probes can be incorporated into one vesicle population, which results in low fluorescence as the Rh-PE quenches the NDB-PE. As fusion occurs with unlabelled vesicles there is a decrease in the efficiency of the resonance energy transfer, due to the dilution of the probes, and the fluorescence increases (probe-dilution method). The probe can also be incorporated into separate vesicle populations (probe-mixing), resulting in fusion being monitored by a decrease in the fluorescence signal due to RET. This method has been shown to give false-positive results in the absence of fusion, for example due to vesicle aggregation and outer monolayer mixing (hemi-fusion).
Aqueous contents mixing assays are not sensitive to aggregation or hemi-fusion, since a signal occurs only when the two internal aqueous spaces intermix. The most common methods used are the aminonaphthalene trisulfonic acid/p-xyylene bis(pyridinium) bromide (ANTS/DPX)\(^{172,175}\) and the terbium/dipicolinic acid (Tb/DPA)\(^{172,176}\) assays. The ANTS/DPX assay relies on the collisional quenching of ANTS fluorescence by the DPX, and thus encapsulating the probes in different vesicle populations produces a decrease of the ANTS signal upon fusion. The Tb/DPA assay is based on an increase of fluorescence intensity of Tb of four orders of magnitude\(^{177}\) when the metal complexes with DPA. The probes are thus encapsulated in separate vesicle populations that, upon fusion, show an increase of fluorescence.

The Tb/DPA assay was chosen to study the fusion properties of the HT lipids because it shows a pronounced fluorescence increase when fusion occurs, compared to the ANTS/DPX assay which involves a decrease. This makes the assay inherently more sensitive and the signal easier to detect. The following internal solutions are the standard literature preparations for LUVs\(^{172}\):

(i) Tb vesicles: 2.5 mM TbCl\(_3\), 50 mM Na citrate, 2mM L-Histidine and TES buffer, adjusted to pH 7.4.

(ii) DPA vesicles: 50 mM Na dipicolinate, 20 mM NaCl, 2mM L-Histidine and TES buffer, adjusted to pH 7.4.

The Tb is chelated with citrate so that it does not interact with the phospholipid bilayer, while the DPA is in 20 fold excess to ensure that all the Tb is chelated during the fusion reaction. The Tb(DPA)\(_3\)\(^{3-}\) complex occurs between a pHs 5 (pK\(_a\) of DPA) and 10, making the buffer system at pH 7.4, appropriately mid-range\(^{177}\). Figure 5-11 shows a schematic of the fusion
Figure 5-11. The Tb/DPA Fusion Assay. (a) Fluorescence Spectra of Tb(DPA)$_3^{3+}$ and (b) Schematic of Vesicle Fusion.

(a) 25 μM Tb$^{3+}$, 500 μM DPA.

(b)
process, as well as the excitation and emission spectra of the Tb(DPA)$_3$\(^{3+}\). The complex shows two absorption peaks at 271 nm and 276 nm. There are three fluorescence peaks at 491 nm, 545 nm and 582 nm. The assay is normally done with excitation at 276 nm and monitored at 545 nm. The external buffer is composed of 0.1 mM EDTA, 100 mM NaCl, 2mM L-Histidine and TES buffer, adjusted to pH 7.4. The EDTA strongly interferes with the Tb/DPA complexation, thus quenching any fluorescence due to vesicle leakage.

5.3.2 Vesicle Preparation

Xin Zhou, a post-doc in the Fyles' group, originally developed the Tb/DPA assay protocol. He used the tested method, particular to the group, of manufacturing vesicles. A 9:1 PS:PC chloroform solution containing 10 mg of lipid was evaporated to dryness. The dry lipid was dissolved in anhydrous diethyl ether and the internal stock solution was added to form a two-phase mixture. This mixture was sonicated to give a cloudy, homogenous solution. The ether was then slowly removed by evaporation at a controlled pressure. Once the ether has been removed the resultant solution was injected into a sizer that passes first through a 1.0 \(\mu\)m Nucleopore filter and then a 0.1 \(\mu\)m filter, under nitrogen pressure. Once the vesicles had been sized they were purified by gel filtration on a Sephadex G-25 column yielding LUVs. This method would typically have 80 % of the entrapped volume associated with 300 nm diameter vesicles and the remaining 20 % with 100 nm vesicles.

The system was very time consuming, and the fusion assay demands at least the production of two different vesicle populations, so a faster and even more homogenous method was employed. In this revised procedure, multilamellar vesicles were manufactured using the freeze-thaw method. The lipid, in chloroform, was dried and internal stock solution was added, as well as glass beads to enhance the removal of the lipid stuck to the glass walls.
The flasks were hand-rotated until all lipid had been removed from the walls, at 30 °C, until a homogeneous milky white suspension was present, and then left to stand for another 30 minutes. The vesicles were then subjected to ten freeze-thaw cycles using a CO₂/ethanol bath, to ensure solute equilibration between trapped and bulk solutions. The MLVs were then extruded at room temperature 19 times through a 19 mm polycarbonate filter (Avestin) with 0.1 μm pore size, using a Basic Liposofast device (Avestin), to produce homogeneous LUVs. This extrusion device is a machined housing that secures the filter between two 0.5 mL syringes. The samples were forced back and forth from one syringe to another. The odd number of passages avoids contamination of LUVs from any material that might not have been sized. Untrapped internal solution was removed using Sephadex G-25 columns. Light scattering results consistently gave a Gaussian distribution of LUVs of 130 nm ± 20 nm.

5.4 Results

5.4.1 Original proposal

The original goal of the fusion assay was to use it as a tool to not only monitor the fusion occurring, but also its kinetics. Once the knowledge was well established, then the effect of the HT lipids on the assay could be investigated. The proposed sequence was to create a population of vesicles that were primed for fusion, test the fusion dynamics using normal triggers (i.e. Ca²⁺), and then compare these with fusion caused by isomerization of the HT lipid. The vesicles would need to be apposed by Mg²⁺ before-hand. The amount of HT-lipid, as well as the isomer, present in the vesicles can be varied, thus allowing for some conclusions on the effect of shape and the sensitivity of the membrane to this. A major difference between this method compared to other previous assays is that there is
differentiation of the trigger population. Only half of the vesicle population has the HT lipid, and thus, the effect of fusion must be transmitted from this "active" population to the "passive" one. This is potentially a better model for a drug delivery system, as its fusogenisity depends on internal factors and not on the target cell.

This lofty goal was tempered to one of observations of the system. It soon became obvious that not enough was known about what processes were taking place. In conjunction with the normal variables of liposome assays which are hard to quantify, the variety of effects and the number of variables made it difficult to systematically explore. In addition, reproducibility was a serious problem as the knowledge of which factors were critical was lacking. These variables include (i) the source of natural lipid, (ii) the initial distribution of vesicle populations, (iii) the effect of age on vesicles, (iv) the exact morphology of vesicles with HT-lipid incorporated, and (v) the effect of ambient light on the HT-vesicles. That being said, I present what I consider to be the relevant observations in hope that they shed some light on the system.

5.4.2 Re-examination of the Assay

A great amount of time and effort was spent trying to get the TB/DPA assay to function according to the specifications of previous reports. The signal that was produced when PS:PC (9:1) vesicles were triggered to fuse with Ca$^{2+}$ was at best a two fold increase of the baseline fluorescence, substantially less than the factor of $10^4$ predicted from the fluorescence change of the unchelated Tb$^{3+}$. We investigate each component of the assay separately to nail down the problem. All the assay constituents were tested for impurities, while the vesicle preparation was reworked to give consistent LUV's of 130 nm diameter.
Figure 5-12. The Effect of Concentration on the \( \text{Tb}^{3+} / \text{DPA} \) Assay
None of these changes made a difference. We then focused on the actual chelation reaction taking place between Tb$^{3+}$ and DPA.

The standard Tb/DPA fusion has a metal to ligand ratio of 1:20, that is, with an excess of ligand. This assures that when fusion occurs, the Tb$^{3+}$ experiences an excess of ligand to force complete complexation in its 1:3 ratio, thus yielding data that is only dependent on the concentration of the metal. This is only valid as long as the complex fluorescence does not vary with the Tb$^{3+}$ and DPA concentrations. The problems we were having with the assay led us to question this assumption. Figure 5-12 displays the results. The fluorescence was monitored at the 545 nm peak, the Tb$^{3+}$ and ligand ratios corrected for the addition volume changes. The fluorescence, at higher Tb$^{3+}$ concentrations (≥0.5 mM) depends drastically upon the concentration ratio of metal to ligand. In both the buffered and the unbuffered case the fluorescence decreases before reaching a 1:3 ratio and by the 1:6 ratio it is nearly totally quenched. When the initial Tb$^{3+}$ concentration is 0.04 mM the profile is quite different. The maximum is reached at a 1:8 ratio, and the sample is more resistant to increases in the DPA concentration. Back titration of Tb increased the fluorescence with the same slope profile, while in the unbuffered case at higher Tb concentration (0.5 mM) no effect was observed (not shown). At even lower initial Tb concentrations (0.004 mM) the curve now becomes insensitive to the DPA concentration after reaching the saturation point of 3 DPA per Tb$^{3+}$. The quenching of Tb/DPA at millimolar concentrations has been attributed to inner filter effects.$^{172,176}$ The bulk phase concentration of Tb$^{3+}$ and DPA in a vesicle experiment is in the micromolar scale, but inside the vesicles the concentrations are effectively 2.5 mM for Tb Tb$^{3+}$ and 50 mM for DPA. Thus some self-quenching is inevitable. It seems from literature precedent that fluorescence is still detected, but the raw
data of the actual magnitude the fluorescence increases is rarely reported. Most data comes as a percentage of possible fusion, that is a ratio of observed fluorescence changes.

From the above data, I decided to decrease the concentration of DPA in the vesicles to 10 mM. The assay now gave a maximum fluorescence that was ten fold greater for Ca$^{2+}$ induced PS/PC vesicle fusion. While this signal was far superior to those previously obtained, it is still far from the four orders of magnitude mentioned in the literature.$^{177}$
5.4.3 Preliminary Results

Figure 5-13 shows the typical control experiment employed to check the Tb/DPA assay. When 20 µL of 0.1 M Ca\(^{2+}\) (4 mM final concentration) was added to PS:PC vesicles there was a sudden increase in the fluorescence, which once it reaches a maximum, starts to fall as the vesicles become leaky. The Tb\(^{3+}\)/DPA complex leaks into the external solution, where EDTA binds the Tb\(^{3+}\) and the signal falls. This fusion threshold concentration for Ca\(^{2+}\) is in agreement with the literature values (PS LUVs 2.4 mM Ca\(^{2+}\))\(^{34}\)

![Graph showing fluorescence over time](image)

**Figure 5-13. Control for Fusion Assay (Ca\(^{2+}\) induced)**

The effect of HT-lipid in the system was investigated as shown in Figure 5-14. The first major difference is that the initial fluorescence is much lower than in Figure 5-12 (~20% of control). In all cases in Figure 5-14 the samples undergo an initial jump in fluorescence when the missing vesicle population component is added. The profile is affected by the presence of different HT-lipids. In Figure 5-13a, PC-HT-6 (in a 1:9 ratio with PS) shows a
Figure 5.14  
Ca^{2+} Induced Fusion with HT-lipids
slight jump in fluorescence at a concentration of 2 mM of Ca\(^{2+}\) (10 µL of 0.1 M Ca\(^{2+}\) is added), but then remains flat. Addition of a second aliquot of Ca\(^{2+}\) (4 mM total concentration) causes the onset of fusion, which is followed by some leakage that levels off. The spike at the end of the fusion fluorescence rise is an unexplained event. The same experiment done with a lower concentration of PC-HT-6 (1:19 with respect to PS) is shown in Figure 5-14b. This experiment was slightly different in that the population of vesicles with HT-lipid had DPA encapsulated in them, while the “passive” population (PS:PC, 9:1) had Tb. The addition of Ca\(^{2+}\) causes an increase in fluorescence as before (Figure 5-14a), as does the second. The third injection of Ca\(^{2+}\) causes the fluorescence to jump, and the vesicles to precipitate as a fine suspension. Changing the HT-lipid to PC-\(m^\prime\)-HT-6 (1:9 with respect to PS), gives a decrease in fluorescence component upon the first addition of calcium (Figure 5-14c). This then gives the expected rise in fluorescence indicating fusion which then tapers off without apparent leakage.

These three figures illustrate “typical” behavior that is generally consistent with expectation. Addition of Ca\(^{2+}\) eventually induces fusion although the actual amount required varies with the differences in the lipid and composition. The drop in fluorescence in 5-14c is consistent with some vesicle damage occurring on Ca\(^{2+}\) addition.
5.4.4 Spontaneous Fusion ?!

When PS:PC vesicles (9:1, with DPA inside) were added to PS:PC-\(m'\)-HT-6 vesicles (Tb inside), there was a spontaneous increase in the fluorescence (Figure 5-15), which gave what seemed to be non-leaky fusion. I used detergent to destroy the vesicles to insure that there was enough EDTA in the external buffer, and that I was not just experiencing leakage. Unfortunately this result could not be reproduced with any other vesicle batch, and remains an observation.

Since no Ca\(^{2+}\) had been added to the above system, what was the trigger? Terbium is known to act as a fusion trigger for PS SUVs at concentrations as low as 8-10 \(\mu M\)\(^{34,38}\). The citrate present in the assay inhibits any association of the Tb\(^{3+}\) with the membranes\(^{176}\), and 1.0 mM EDTA is present in the elution buffer during gel filtration to prevent binding of Tb\(^{3+}\) to the outside of the vesicles. The stock Tb\(^{3+}\) vesicles, even if they
do leak, should not interact with the membranes as the EDTA affinity for the metal would chelate any Tb gone astray. In a typical experiment using 2 x 100 µL of the respective vesicle populations (at 5 mg lipid/mL), and 0.3 mL of buffer, for a total volume of 0.5 mL, the bulk concentration of the Tb^{3+} can be calculated to be 15 µM (at typical encapsulation value of 11.1 nmol Tb^{3+}/µmol of lipid^{176}). There is thus a possibility that if Tb^{3+} were to leak, even though it would be complexed by EDTA, apposition would be induced by the complex.

A more obvious leakage problem was the possibility of photoisomerization taking place during the manufacturing of the vesicles. This is only a problem during the final step, where the vesicles have already been sized and are passed through the Sephadex column to remove any excess of internal components. From the observations gathered so far, it seems that the HT-lipid vesicle populations are significantly more leaky than normal vesicles. This could be due to the isomerization or poor packing in the membrane. The HT lipids do not form vesicles on their own. An attempt to make 100% PC-HT-6 liposomes resulted in cloudy solutions (after sonication) that would clog up the sizing filters. Attempts to make vesicles using a 1:1 ratio of PS:PC-\( m’\)-HT-6 was also not successful. I was able to make CF-containing vesicles with PS:PC-HT-6 (3:2) and PS:PC-\( m’\)-6 (8:2) but they did not retain the dye after a period of 4 hours.

These observations beg another question. If these vesicles are so leaky, then why would there be any fluorescence signal, as EDTA can just as easily reach the internal compartment of the vesicles?

A concurrent experiment using 5(6)-carboxyfluorescein (CF)^{179} was employed to determine whether the addition of HT-lipid had any effect on the leakage. The CF vesicles were prepared in the same manner as those of the TB/DPA assay. The sample was excited at
430 nm and monitored at 518 nm. At high concentrations, the fluorescence of CF is negligible due to self-quenching. Only when CF escapes the confines of the vesicle's interior into the bulk solution, does its effective concentration decrease and is thus able to fluoresce.

Figure 5-16. Vesicle Leakage Due to Calcium

Figure 5-16 shows the effect of vesicle leakage with Ca\(^{2+}\), as it is modulated with HT-lipid. Addition of Ca\(^{2+}\) (final concentration 2 mM) to PS:PC, 9:1 (CF) vesicles shows a slight jump in fluorescence, followed by a gradual increase in the baseline. Another injection of the cation (4 mM [Ca\(^{2+}\)]) has a moderate effect on that slope. The result corroborates that fusion is taking place in the Tb/DPA assay (Figure 5-13) with very little leakage. When a population of CF vesicles has PS:PC-HT-6 added, followed by Ca\(^{2+}\) (total concentration 2 mM) there is catastrophic leakage. The vesicles lose all their integrity. It is apparent that the HT-lipid containing vesicles are able to communicate with the CF population (i.e. appose and mix) without the aid of ordinary factors.
Figure 5-17 The Effect of Mg\(^{2+}\) on CF vesicles
The results shown in Figure 5-17 document the effect of adding Mg$^{2+}$ to the CF vesicles. Figure 5-17a shows the control experiment in which Mg$^{2+}$ is added until significant leakage was observed. The vesicles become leaky at 18 mM Mg$^{2+}$, and precipitate as a fine suspension. Triton was added to confirm that the integrity of the vesicles was intact in the suspension. In 5-17b PS:PC-HT-6, 8:2 (DPA) vesicles are added to the CF vesicles, followed by irradiation at 406 nm for 10 min, which results in no apparent leakage. Mg$^{2+}$ was then added to appose the vesicles. The solution remained clear. After 15 minutes the sample was submitted to another 10 minutes of 406 nm light. The sample now had a globular appearance, but did not exhibit any leakage.

The Ca$^{2+}$ and the Mg$^{2+}$ results in the presence of HT-lipid seem to be at odds with each other. In the presence of Ca$^{2+}$ the integrity of the CF vesicles is somehow compromised, while with the Mg$^{2+}$ experiments there is a preservation effect, making the vesicle membranes harder to disrupt. The difference is that the Mg$^{2+}$ experiments were irradiated while the Ca$^{2+}$ was not. During the irradiation there seems to be a change in the composition of the vesicles, and in some of their properties.

5.4.5 Fusion by Isomerization

Figure 5-18 shows the results of attempted fusion by isomerization. Initially the cuvette had 0.1 ml of DPA vesicles (PS/PC-HT-6, 8:2) and 0.3 ml of the buffer. When 0.1 ml of Tb vesicles were added the fluorescence increased nearly two-fold, reached a maximum, then decreased to a stable value. At this point I was not sure if this effect was documenting "real" instantaneous fusion, reaching a maximum, followed by slow leaking. There was no decrease in the intensity as would be expect with leakage, but a slight increase.
Irradiating the vesicles at 406 nm seemed to have only transient effects. The fluorescence went down slightly, but then climbed back up to the stable value. This effect could be explained by partial quenching of some of the Tb$^{3+}$/DPA complex by EDTA as the membrane is disrupted. Once the isomerization is over the membrane recovers its integrity while vesicles are fusing, hence the increase in the fluorescence. Calcium ions were then added to determine whether the maximum fusion signal had been achieved. In both additions of Ca$^{2+}$ the fluorescence decreased and did not regain its previous maximum. The cation seemed be rupturing a fragile sub-population of vesicles. Since the vesicles are now definitely apposed, they were irradiated one more time. The solution at the end of the irradiation now had a globular appearance, similar to that documented in the Mg$^{2+}$/HT-lipid CF experiments. There is an increase of fluorescence, but in a ragged fashion. Adding more calcium had no clear effect. The vesicles were then lYZed with Triton-X, but the amount of
detergent needed was greater than what was normally used. The globules did not disintegrate as is usually expected, being abnormally resistant to the detergent. The fluorescence is totally quenched once the vesicles had been completely lysed.

5.5 Conclusion

There are no smoking guns that prove that the hemithioindigo lipids can serve as fusion triggers by modulating their critical packing parameter or curvature. Nevertheless there are enough observations here to warrant continuing investigation of these novel lipids:

(i) Figure 5-14 is as expected for Ca^{2+} induced fusion.
(ii) unexplained spontaneous fusion
(iii) apposition without cations
(iv) formation of globular aggregates

In hindsight, the Tb/DPA assay was the wrong choice to monitor the fusion. A better choice would have been the NBD-PE and Rh-PE lipid mixing assay. This assay has the potential of giving unambiguous results on the interactions taking place between the “passive” and “active” vesicle populations. More research needs to be performed on elucidating the exact morphology of the vesicles with HT-lipid incorporated in them. \(^{31}\text{P NMR}\) has been previously used to identify lamellar, inverted hexagonal, and cubic phases. Preliminary attempts at obtaining \(^{31}\text{P NMR}\) to determine the phase(s) present were not successful, as there was not enough HT-lipid available to get well-resolved spectra. The lipid should also be modified so that they spontaneously form vesicles while making them more susceptible to a phase change. This will involve lengthening of the tails and conversion of the headgroup
from PC to PE. This change should enhance the intrinsic curvature of the novel lipid, which in turn may reveal some answers to the questions that this project has posed.
Chapter 6
Experimental

6.1 General

Proton NMRs were recorded with a Bruker AC300 (300 MHz), or a Bruker AMX360 (360 MHz) spectrometer in CDCl₃, CD₃OD or DMSO. All spectra were referenced to the central solvent line (δ 7.24 ppm, δ 3.31 ppm, and δ 2.50 ppm for CDCl₃, CD₃OD and DMSO respectively, relative to TMS). Carbon NMR spectra were recorded with Bruker AC300 (75.47 MHz), or Bruker AMX360 (90.57 MHz) in CDCl₃, CD₃OD or DMSO referenced to the central solvent line (δ 77.0 ppm, δ 49.0 ppm, δ 39.5 ppm, for CDCl₃, CD₃OD and DMSO respectively, relative to TMS). Phosphorous NMR spectra were recorded with Bruker AMX360 (145.63 MHz) in CDCl₃ reference to 85% H₃PO₄.

The spin multiplicity splittings are reported as: s, singlet, d, doublet, t, triplet, q, quartet, qn, quintet, sx, sextet, m, multiplet and br, broad. CI mass spectra were recorded with a Finnegan 3300 GC-MS instrument. LSIMS (m-NBA as matrix) and HRMS spectra were recorded with a Kratos Concept-H instrument. Elemental analyses were performed by Canadian Microanalytical Services, New Westminster, B.C. Melting points were taken on a Reichert hotstage microscope (uncorrected). TLC was carried out using Eastman Kodak silica gel on polyester sheets with fluorescent indicator. Centrifugal chromatography was carried out using a Harrison Research Chromatotron, model 7924T. The plates were prepared with silica gel (Merck, TLC grade 7749) purchased from Aldrich containing gypsum binder and fluorescent indicator. DMF and DMSO were dried by refluxing, with CaH₂ and then distilled under vacuum. Et₃N, CH₃CN and CH₂Cl₂ were dried by refluxing with CaH₂ and then distilled. (CH₂Cl)₂ was dried by
refluxing with P₂O₅ and then distilled. Solutions in organic solvents were dried with MgSO₄, and then concentrated using a rotary evaporator.

All the spectra associated with the compounds are in Chapter 3 or in the Appendix, as noted. The following are known compounds: 3.15¹⁸⁰, 3.19-3.21¹⁸¹,¹⁸², 3.43 and 3.58¹⁸³. Their full data is provided here as the literature data was usually incomplete.

6.2 General Procedures

6.2.1 General Procedure for Thioether Synthesis

A solution of bromothiophenol (230 mmol) and ethyl bromoacetate (240 mmol, 1.05 eq) in 60 mL of benzene was refluxed, under N₂. DBU (240 mmol, 1.05 eq), in 10 mL of benzene, was added over 10 minutes and the solution was refluxed for 2 hours.

The DBU salt precipitate was filtered and the filtrate was concentrated to yield a light yellow liquid. This crude product was distilled, (Kugelrohr, 5 x 10⁻² atm), yielding a clear liquid (b.p. 120°C).

6.2.2 General Williamson Ether Synthesis Procedure

A suspension of KOH (130.8 mmol, 4 equi.) in 50 mL of DMSO had hydroxybenzaldehyde (32.7 mmol), and alkylhalide (49.0 mmol, 1.5 eq.) added to it. This suspension was stirred vigorously at room temperature for 30 minutes, and then quenched with water (150 mL). The product was extracted with CH₂Cl₂ (2x75 mL), and then washed with water (3x75 mL), dried, and concentrated to a yellow liquid. The crude was purified by distillation (Kugelrohr, 5 x 10⁻² atm), affording a clear liquid.
6.2.3 General Procedure for Heck Reactions

A mixture was prepared containing aryl bromide (1eq), freshly distilled alkene (1.5eq), NEt₃ (1.5eq), Pd(OAc)₂ (0.05 eq.) and P(Ph)₃ (0.10 eq.). The flask was purged with argon, sealed with a septum, sonicated to a fine suspension, and then heated at 100°C for 24 hours. The solid was triturated twice with ether. Filtration with fine ashless paper and concentration of filtrate resulted in a golden solution. The purification procedure for this crude varied with compound and is described individually.

6.2.4 General Hydrogenation Procedure

A solution of alkene (30.1 mmol) and Wilkinson's catalyst, Rh(PPh₃)₃Cl (1.05 mmol, 0.035 eq.), in 150 mL of degassed 1:1 benzene/ethanol was hydrogenated at rt (23°C), at 50 PSI, for 9 days. The solution was concentrated and the residue was triturated with ether, filtered and concentrated again. The purification procedure varied with compound and is described individually.

6.2.5 General Ester Hydrolysis Procedure

NaOH (38.0 mmol, 1.2 eq), in 25 mL of MeOH, was added to a solution of ester (31.7 mmol) in 75 mL of MeOH and refluxed for 3 h. The acid salt was concentrated and dried under vacuum. The resultant solid was dissolved in H₂O and then acidified to pH < 1. The white free acid precipitate was filtered and dried.

6.2.6 General Procedure for Hemithioindigo Synthesis

The phenyl thioacetic acid (7.81 mmol) in an excess of SOCl₂ (5 mL) was refluxed, under N₂, for 1 h. The excess thionyl chloride was removed under vacuum to
afford the acid chloride. The acid chloride, in 15 mL of (ClCH₂)₂, was cooled in a salt ice
bath to below 0°C. AlCl₃ (9.4 mmol, 1.2 eq.) was added to the cooled solution, under
rapid stirring, over a 2 min. period. The solution was left in ice bath for another 10 min.
removed and left stirring at rt (23 °C), for a further 40 minutes. The reaction was
quenched with ice/water, and product was extracted with CH₂Cl₂, dried, concentrated, and
put under vacuum for 15 minutes. This yellow solid thioindoxyl slowly decomposed and
so was used as soon as possible.

A solution of the indoxyl, benzaldehyde (8.2 mmol, 1.05 eq.) and 2 drops of
piperidine in 50 mL of benzene was refluxed for 2 h. The purification procedure varied
with compound and is described individually.

6.2.7 General Procedure for Nitrile Hydrolysis

The nitrile (0.323 mmol) and tetrafluorophthalic acid (0.323 mmol, 1 eq.) were
added to a Carius tube and sealed under vacuum. The vessel was heated at 170°C for 5
days. The solid crude product was dissolved in CHCl₃/MeOH and was passed through a
LH-20 column with CHCl₃/MeOH, 4:3, as eluent, to give a yellow solid.

6.2.8 General Procedure for Lipid Synthesis

A solution of acid (0.290 mmol), (CH₃)₃CCOCl (3.0 mmol, 10 eq.), and NEt₃ (1.4
mmol, 5 eq.) in CH₂Cl₂ was stirred, under N₂, for 35 h. The reaction was monitored by
TLC (CHCl₃/MeOH/H₂O, 65:25:4). The crude mixed anhydride was concentrated and
dried under vacuum, for 8 hours, to remove unreacted (CH₃)₃CCOCl and NEt₃.
In the meantime, the GPC-CdCl₂ complex (0.115 mmol, 0.4 eq.) was rendered anhydrous by repeated (x2) evaporation of added dry benzene. The mixed anhydride was dissolved in 5 mL of CH₂Cl₂ and added to a suspension of GPC-CdCl₂ and DMAP (0.230 mmol, 0.8 eq) in CH₂Cl₂ (10 mL) and stirred for 23 h. The solution was concentrated and eluted through an ion exchange column of Rexyn-I-300 to remove the CdCl₂ and DMAP (CHCl₃/MeOH/H₂O, 65:25:4). The crude product was concentrated and then re-dissolved in CHCl₃/MeOH, 4:3, and passed through a Sephadex LH-20 column to yield the pure product as a yellow solid.

6.3 Synthesized Compounds

3.15 [(4-Bromopheny)thio]acetic acid, ethyl ester. CAS 65251-10-7

The general procedure for thioether synthesis was followed. The amounts of reagents used: 4-bromothiophenol (4.34g, 230mmol) and ethyl bromoacetate (4.01g, 240mmol, 1.05 eq) in 60mL of benzene, DBU (3.65g, 240mmol, 1.05 eq), in 10mL of benzene added over 10 minutes. Yield:5.75g, 209mmol, 91%.

¹H NMR 300 MHz (CDCl₃): δ = 7.34 (d, J=8.4 Hz, 2H), 7.21 (d, J=8.4 Hz, 2H), 4.11 (q, J=7.2 Hz, 2H), 3.55 (s, 2H), 1.16 (t, J=7.3 Hz, 3H). Refer to Figure 3-1.

¹³C NMR 75.47 MHz (CDCl₃): δ = 169.1, 134.3, 132.0, 131.4, 120.8, 61.5, 36.3, 14.1. Refer to Figure 3-2.
Analysis: calculated for C_{19}H_{11}O_{2}SBr (%): C 43.65; H 4.03; O 11.63; S 11.65; Br 29.04. Found C 43.54; H 4.03; O 11.25; S 11.97; Br 29.21.

3.16 [(3-Bromophenyl)thio] acetic acid, ethyl ester

\[ \text{Br} \quad \text{S} \quad \text{O} \quad \text{O} \quad \text{C} \quad \text{O} \quad \text{O} \]

The general procedure for thioether synthesis was followed. The amounts of reagents used: 3-bromothiophenol (4.92g, 262mmol), ethyl bromoacetate (4.34g, 260mmol), 1 eq.) in 100mL of benzene, DBU (3.95g, 260mmol, 1 eq.) in 50 mL of benzene added over 20 minutes. Yield: 5.54g, 201mmol, 77%.

\(^1H\) NMR 300 MHz (CDCl\textsubscript{3}): \( \delta = 7.49 \) (s, 1H), 7.27 (m, 2H), 7.09 (t, J=8.1Hz, 1H), 4.12 (q, J=7.4Hz, 2H), 3.59 (s, 2H), 1.18 (t, J=7.4Hz, 3H). Refer to Figure 3-1.

\(^13C\) NMR 75.47 MHz (CDCl\textsubscript{3}): \( \delta = 169.2, 137.5, 131.9, 130.3, 129.8, 128.0, 122.8, 61.7, 36.2, 14.1 \). Refer to Figure 3-2.

Analysis: calculated for C\textsubscript{19}H\textsubscript{11}O\textsubscript{2}SBr (%): C 43.65; H 4.03; O 11.63; S 11.65; Br 29.04. Found C 44.06; H 4.07; O 11.14; S 11.96; Br 28.77.

3.17 [(2-Bromophenyl)thio] acetic acid, ethyl ester

\[ \text{Br} \quad \text{S} \quad \text{O} \quad \text{O} \quad \text{C} \quad \text{O} \quad \text{O} \]

The general procedure for thioether synthesis was followed. The amounts of reagents used: 2-bromothiophenol (10.33g, 547mmol) and ethyl bromoacetate (9.12g, 260mmol), 1 eq.) in 100mL of benzene, DBU (3.95g, 260mmol, 1 eq.) in 50 mL of benzene added over 20 minutes. Yield: 11.24g, 201mmol, 77%.

\(^1H\) NMR 300 MHz (CDCl\textsubscript{3}): \( \delta = 7.52 \) (s, 1H), 7.27 (m, 2H), 7.09 (t, J=8.1Hz, 1H), 4.12 (q, J=7.4Hz, 2H), 3.59 (s, 2H), 1.18 (t, J=7.4Hz, 3H). Refer to Figure 3-1.

\(^13C\) NMR 75.47 MHz (CDCl\textsubscript{3}): \( \delta = 169.2, 137.5, 131.9, 130.3, 129.8, 128.0, 122.8, 61.7, 36.2, 14.1 \). Refer to Figure 3-2.

Analysis: calculated for C\textsubscript{19}H\textsubscript{11}O\textsubscript{2}SBr (%): C 43.65; H 4.03; O 11.63; S 11.65; Br 29.04. Found C 44.06; H 4.07; O 11.14; S 11.96; Br 28.77.
547mmol, 1 eq.) in 250mL of benzene, DBU (8.33g, 547mmol, 1 eq.) in 75mL of benzene added over 30 minutes. Yield: 15.04g, 547mmol, 100%.

$^1$H NMR 300 MHz (CDCl$_3$): $\delta = 7.52$ (d, J= 8.1Hz, 1H), 7.34 (d, J=8.1Hz, 1H), 7.23 (t, J=7.4Hz, 1H), 7.03 (t, J=8.1Hz, 1H), 4.14 (q, J=6.6Hz, 2H), 3.64 (s, 2H), 1.20 (t, J=6.6Hz, 3H). Refer to Figure 3-1.

$^{13}$C NMR 75.47 MHz (CDCl$_3$): $\delta = 168.9, 136.2, 129.3, 127.8, 127.5, 123.9, 61.6, 35.4, 13.9$. Refer to Figure 3-2.

Analysis: calculated for C$_{10}$H$_{11}$O$_2$SBr (%): C 43.65; H 4.03; O 11.63; S 11.65; Br 29.04. Found C 43.87; H 4.10; O 11.07; S 12.09; Br 28.87.

3.19 4-Butoxybenzaldehyde CAS 5736-88-9

![4-Butoxybenzaldehyde](image)

The general procedure for Williamson ether synthesis was followed. The amounts of reagents used: 4-hydroxybenzaldehyde (4.00g, 32.7mmol), bromobutane (6.72g, 49.0mmol, 1.5 eq), and KOH (7.35g, 131.2mmol, 4 equi.) in 50mL DMSO, stirred at rt, for 30 min. Distillation at 80°C afforded the product (2.78g, 15.6mmol, 48%).

$^1$H NMR 300 MHz (CDCl$_3$): $\delta = 9.85$ (s, 1H), 7.80 (d, J=8.1Hz, 2H), 6.96 (d, J=8.1Hz, 2H), 4.01 (t, J=6.6, 2H), 1.77 (qn, J=8.1Hz, 2H), 1.48 (sx, J=7.4, 2H), 0.96 (t, J=7.4, 3H). Refer to Figure A-1.

$^{13}$C NMR 75.47 MHz (CDCl$_3$): $\delta = 190.6, 164.1, 131.8, 129.6, 114.6, 67.9, 30.9, 19.0, 13.7$. Refer to Figure A-2.
Cl MS $m/z$ (relative intensity): 219 ([M+C$_2$H$_5$]$^+$, 6), 207 ([M+C$_2$H$_5$]$^+$, 22), 179 ([M+H]$^+$, 100).

3.20 4-Hexyloxybenzaldehyde

![Chemical structure of 4-Hexyloxybenzaldehyde]

The general procedure for Williamson ether synthesis was followed. The amounts of reagents used: 4-hydroxybenzaldehyde (4.00g, 32.7mmol), bromohexane (5.41 g, 32.7mmol, 1 equi.), and KOH (7.35g, 131.2mmol, 4 equi.) in 50mL DMSO, stirred at 40°C, for 40 min. Distillation at 120°C afforded the product (4.28g, 20.8mmol, 64%).

$^1$H NMR 300 MHz (CDCl$_3$): $\delta = 9.82$ (s, 1H), 7.77 (d, J=8.8Hz, 2H), 6.94 (d, J=8.8Hz, 2H), 3.98 (t, J=6.6, 2H), 1.76 (qn, J=6.6Hz, 2H), 1.43 (qn, J=6.6Hz, 2H), 1.31 (m, 4H), 0.87 (t, J=7.4, 3H). Refer to Figure 3-3.

$^{13}$C NMR 75.47 MHz (CDCl$_3$): $\delta = 190.7, 164.2, 131.9, 129.6, 114.6, 68.3, 31.4, 28.9, 25.5, 22.5, 13.9$. Refer to Figure 3-3.

Cl MS $m/z$ (relative intensity): 247 ([M+C$_2$H$_5$]$^+$, 4), 235 ([M+C$_2$H$_5$]$^+$, 16), 207 ([M+H]$^+$, 100).

3.21 4-Octyloxybenzaldehyde CAS 24083-13-4

![Chemical structure of 4-Octyloxybenzaldehyde]
The general procedure for Williamson ether synthesis was followed. The amounts of reagents used: 4-hydroxybenzaldehyde (1.00g, 8.20mmol), iodoctane (1.97g, 8.20mmol, 1 equi.), and KOH (1.84g, 32.9mmol, 4 equi.) in 25mL DMSO, stirred at rt. for 30 min. The distillation (120°C) gave an impure product, and thus chromatography (silica, 50g, 1:3 ethyl acetate:hexanes) was employed to yield a thick clear liquid (330mg, 1.41mmol, 17%) 

\(^1\text{H NMR } 300 \text{ MHz (CDCl}_3)\): \(\delta = 9.86 \text{ (s, } 1H, -\text{CHO}), 7.81 \text{ (d, } J=8.8\text{ Hz, } 2H), 6.97 \text{ (d, } J=8.8\text{ Hz, } 2H), 4.02 \text{ (t, } J=6.6\text{ Hz, } 2H), 1.79 \text{ (qn, } J=6.6\text{ Hz, } 2H), 1.45 \text{ (m, } 2H), 1.30 \text{ (m, } 8H), 0.87 \text{ (t, } J=7.4\text{ Hz, } 3H). \text{ Refer to Figure A-1.} 

\(^13\text{C NMR } 75.47 \text{ MHz (CDCl}_3)\): \(\delta = 190.8, 164.2, 132.0, 129.7, 114.7, 68.4, 31.8, 29.3, 29.2, 29.0, 25.9, 22.6, 14.1 \text{ Refer to Figure A-2.} 

\text{Cl MS } m/z \text{ (relative intensity): 263 ([M+CsH}_3]^+, 20), 235 ([M+H]^+, 100)} \n
3.24 3-Hydroxymethyl-1-hexyloxybenzene 

The general procedure for Williamson ether synthesis was followed. The amounts of reagents used: 3-hydroxybenzaldehyde (4.00g, 32.7mmol), bromohexane (5.41g, 32.7mmol, 1 equi.), and KOH (7.35g, 131.2mmol, 4 equi.) in 50mL DMSO, stirred at 40°C, for 40 min. Kugelrohr distillation at 120°C afforded the product (2.74g, 13.2mmol, 40%).
$^1$H NMR 300 MHz (CDCl$_3$): $\delta = 7.23$ (t, $J=8.1$, 1H), 6.90 (s, 1H), 6.89 (d, $J=8.1$ Hz, 1H), 6.81 (d, $J=8.8$ Hz, 2H), 4.62 (d, $J=4.4$ Hz, 2H), 3.94 (t, $J=6.6$, 2H), 2.00 (s, 1H), 1.76 (qn, $J=6.6$ Hz, 2H), 1.45 (m, 2H), 1.34 (m, 8H), 0.90 (t, $J=7.4$, 3H). Refer to Figure 3-4.

$^{13}$C NMR 75.47 MHz (CDCl$_3$): $\delta = 159.3$, 142.43, 129.5, 118.8, 113.7, 112.8, 67.9, 65.2, 31.5, 29.2, 25.7, 22.6, 14.0. Refer to Figure 3-5.

Cl MS $m/z$ (relative intensity): 209 ([M+H]$^+$, 19), 207 ([M-H$_2$O]$^+$, 22), 191 ([M-H$_2$O]$^+$, 100).

3.23 3-Hexyloxybenzaldehyde

To a solution of PCC (2.25g, 10.4mmol, 1.5 equi), in 20mL of CH$_2$Cl$_2$, a solution of 3.24 (1.45g, 6.97mmol) in 10mL of CH$_2$Cl$_2$ was added, and stirred, under N$_2$, for 90 min. Ether was added (50mL), the solution decanted, filtered through a Florosil pad, and concentrated to afford the product (1.19g, 5.78mmol, 83%) as a clear oil.

$^1$H NMR 300 MHz (CDCl$_3$): $\delta = 9.94$ (s, 1H), 7.42 (s, 1H), 7.40, 7.36, 7.15 (m, 1H), 3.98 (t, $J=6.6$, 2H), 1.77 (qn, $J=6.6$ Hz, 2H), 1.44 (m, 2H), 1.31 (m, 8H), 0.88 (t, $J=7.4$ Hz, 3H). Refer to Figure 3-4.

$^{13}$C NMR 75.47 MHz (CDCl$_3$): $\delta = 192.2$, 159.7, 137.7, 129.9, 123.2, 121.9, 112.7, 68.3, 31.5, 29.0, 25.6, 22.5, 14.0. Refer to Figure 3-5.
3.29 (2E)-3-[4-(1-Thia-5-oxa-3-oxohexyl)phenyl]prop-2-enoic acid

The general procedure for Heck reactions was followed. The amounts of reagents used: 3.15 (8.35, 30.4mmol), freshly distilled methly acrylate (3.14g, 36.5mmol, 1.2eq.) and NEt₃ (3.69g, 36.5mmol, 1.5eq.), Pd(OAc)₂ (0.067g, 0.3mmol, 1% eq.) and P(Ph)₃ (0.16g, 0.6mmol, 2% eq.). The mixture was heated at 100°C for 72 h.

The crude product (TLC, 1:1 ether:hexanes, Rₜ = 0.53) was purified by chromatography (flash silica, 200g, 60:40 ether:hexanes) and gave a white solid (3.16g, 11.2mmol, 37%), m.p. = 47-48 °C.

¹H NMR 360 MHz (CDCl₃): δ = 7.62 (d, J=16.0Hz, 1H), 7.43 (d, J=8.3Hz, 2H), 7.34 (d, J=8.3Hz, 2H), 6.38 (d, J=16.0Hz, 1H), 4.16 (q, J=7.1Hz, 2H), 3.78 (s, 3H), 3.66 (s, 3H), 1.22 (t, J=7.1Hz, 3H). Refer to Figure 3-6.

¹³C NMR 90.57 MHz (CDCl₃): δ = 169.3, 167.4, 143.9, 138.3, 132.5, 128.7, 128.5, 117.6, 61.8, 51.7, 35.7, 14.1. Refer to Figure 3-6.

+LSIMS (mNBA as matrix) m/z (relative intensity): 281 ([M+1]⁺, 60), 280 (M⁺, 100), 249 ([M-CH₃OH]⁺, 43), 207 (M-CO₂C₂H₅, 35), 175 (30). Analysis: calculated for C₁₉H₁₆O₄S (%): C, 59.98; H, 5.75; O, 22.83; S, 11.44. Found C, 59.80; H, 5.77; O, 23.00; S, 11.43.
The general procedure for ester hydrolysis was followed. The amounts of reagents used: NaOH (0.54 g, 13.6, 2.5 eq), in 25 mL of MeOH, was added to a solution of 3.29 (1.53 g, 5.44 mmol) in 50 mL of MeOH and refluxed for 2h. The acid salt was concentrated and dried under vacuum. The resultant solid was dissolved in H₂O and then acidified to pH < 1. The white free acid precipitate was filtered and dried (1.10 g, 4.62 mmol, 85%), m.p. = 219-220 °C.

¹H NMR 300 MHz (DMSO):  δ = 12.63 (br s), 7.62 (d, J=8.8 Hz, 2H), 7.55 (d, J=15.4 Hz, 1H), 7.32 (d, J=8.8 Hz, 2H), 6.50 (d, J=15.4 Hz, 1H), 3.88 (s, 2H). Refer to Figure A-3.

¹³C NMR 75.47 MHz (DMSO):  δ = 170.5, 167.7, 143.3, 138.9, 131.5, 128.8, 127.0, 118.6, 34.2. Refer to Figure A-3.

3.43 [(4-Bromophenyl)thio]acetic acid CAS 3406-76-6
The general procedure for ester hydrolysis was followed. The amounts of reagents used: NaOH (1.52 g, 38.0, 1.2 eq), in 25 mL of MeOH, was added to a solution of 3.15 (8.72 g, 31.7 mmol) in 75 mL of MeOH and refluxed for 3 h. The acid salt was concentrated and dried under vacuum. The resultant solid was dissolved in H₂O and then acidified to pH < 1. The white free acid precipitate was filtered and dried (3.65 g, 14.8 mmol, 39%), m.p. = 117-119 °C.

^1H NMR 300 MHz (CDCl₃): δ = 7.41 (d, J=8.8 Hz, 2H), 7.27 (d, J=8.8 Hz, 2H), 3.63 (s, 2H). Refer to Figure A-4.

^13C NMR 75.47 MHz (CDCl₃): δ = 175.0, 133.6, 132.3, 131.7, 121.5, 36.5. Refer to Figure A-4.


**3.44 (2E)-3-[4-(Carboxymethylthio)phenyl]prop-2-enoic acid, methyl ester**

\[ \text{O} \quad \text{S} \quad \text{OH} \]

The general procedure for Heck reactions was followed. The amounts of reagents used: 3.43 (1.94g, 7.85mmol), freshly distilled methly acrylate (0.81g, 9.43mmol, 1.2eq.) and NEt₃ (0.93g, 11.8mmol, 1.5eq.), Pd(OAc)₂ (0.046g, 0.2mmol, 2.5 % eq.) and P(Ph)₃.
(0.092 g, 0.4 mmol, 5 % eq.). The mixture was heated at 100°C for 24 h. The crude product was afforded by ether extraction (2 x 25 mL) of the acidic aqueous reaction medium, dried, and concentrated. Recrystallization from 95 % ethanol gave a white solid (200 mg, 0.8 mmol, 10 %).

\[ \text{H NMR 360 MHz (DMSO): } \delta = 12.9 \text{ (br s, 1H), } 7.63 \text{ (d, } J=8.3 \text{ Hz, 2H), } 7.60 \text{ (d, } J=16.1 \text{ Hz, 1H), } 7.32 \text{ (d, } J=8.3 \text{ Hz, 2H), } 6.58 \text{ (d, } J=16.1 \text{ Hz, 1H), } 3.88 \text{ (s, 2H), } 3.70 \text{ (s, 3H).} \]

Refer to Figure A-5.

\[ \text{\carbon{1} NMR 90.57 MHz (DMSO): } \delta = 170.4, 166.7, 143.8, 139.3, 131.3, 128.8, 126.9, 117.1, 56.1, 34.1. \]

Refer to Figure A-5.

**3.47 5-Bromobenzo[b]thiophen-3(2H)-one**

![Chemical structure](image)

The general procedure for hemithioindigo synthesis was followed up to the indoxyl production. The amounts of reagents used: acid **3.43** (1.93 g, 7.81 mmol), excess SOCl\(_2\), followed by AlCl\(_3\) (1.12 g, 9.4 mmol, 1.2 eq.).

Acid Chloride:

\[ \text{H NMR 300 MHz (CDCl\(_3\)): } \delta = 7.45 \text{ (d, } J=8.1 \text{ Hz, 2H), } 7.30 \text{ (d, } J=8.1 \text{ Hz, 2H), } 4.01 \text{ (s, 2H).} \]

Refer to Figure A-6.

\[ \text{\carbon{12} NMR 75.47 MHz (CDCl\(_3\)): } \delta = 169.6, 133.2, 132.5, 131.9, 122.7, 48.4. \]

Refer to Figure A-6.
Thioindoxyl:

$^1$H NMR 300 MHz (CDCl$_3$): $\delta = 7.86$ (d, $J=1.8$ Hz, 1H), 7.61 (dd, $J=8.5$, 1.8 Hz, 1H), 7.29 (d, $J=8.5$ Hz, 1H), 3.81 (s, 2H). Refer to Figure 3-7.

$^{13}$C NMR 75.47 MHz (CDCl$_3$): $\delta = 198.5$, 153.0, 138.3, 132.6, 129.3, 125.9, 39.8. Refer to Figure 3-7

CI MS $m/z$ (relative intensity): 271, 269 ([M+C$_3$H$_7$]$^+$, 5), 259, 257 ([M+C$_2$H$_3$]$^+$, 11), 231, 229 ([M+H]$^+$, 100).

3.49 2-(4-Hydroxyphenylmethylene)-5-bromobenzo[b]thiophen-3(2H)-one

![Chemical structure of 3.49](image)

The general procedure for hemithioindigo synthesis was followed. The amounts of reagents used: The amounts of reagents used: acid 3.49 (1.93 g, 7.81 mmol), excess SOCl$_2$, followed by AlCl$_3$ (1.12 g, 9.4 mmol, 1.2 eq.), then 4-hydroxybenzaldehyde (1.0 g, 8.2 mmol, 1.05 eq.). Once the solution was cool a bright orange solid precipitated. The suspension was washed with Na$_2$S$_2$O$_3$ (5%, 25 mL x 2). filtered and dried under vacuum (1.72g, 5.16 mmol, 66%). The product was re-crystallized from 95 % EtOH, m.p. = 282 °C.

$^1$H NMR 300 MHz (DMSO): $\delta = 10.46$ (s, 1H), 7.90 (d, $J=2.2$ Hz, 1H), 7.86 (s, 1H), 7.84 (dd, $J=8.8$, 2.2 Hz, 1H), 7.74 (d, $J=8.1$ Hz, 1H), 7.64 (d, $J=8.8$ Hz, 2H), 6.94 (d, $J=8.8$ Hz, 2H). Refer to Figure 3-8.
$^{13}$C NMR 75.47 MHz (DMSO): $\delta = 186.0, 160.5, 144.0, 137.7, 134.8, 133.5, 132.0, 128.5, 126.4, 125.7, 124.5, 118.9, 116.4$. Refer to Figure 3-8.


3.50 2-(4-Butyloxyphenylmethylene)-5-bromobenz[b]thiophen-3(2H)-one

To a solution of 3.49 (0.52 g, 1.56 mmol), and bromobutane (0.213 g, 1.56 mmol, 1 eq.) in 15 mL of DMF, Cs$_2$CO$_3$ (0.61 g, 1.9 mmol, 1.2 eq.) was added. The suspension was refluxed at 80°C for 3 h. The solution was concentrated and then water and chloroform were added. The organic layer was dried and concentrated to give a red crude product. The product was recrystallized from chloroform/hexanes to yield yellow crystals (0.10 g, 0.25 mmol, 16%), m.p. = 134-135 °C.

$^1$H NMR 300 MHz (CDCl$_3$): $\delta = 8.02$ (d, $J=1.9$Hz, 1H), 7.91 (s, 1H), 7.61 (m, 3H), 7.35 (d, $J=8.3$Hz, 1H), 6.96 (d, $J=8.8$Hz, 2H), 4.01 (t, $J=6.5$Hz, 2H), 1.78 (qn. $J=6.5$Hz, 2H), 1.49 (sx, $J=7.4$Hz, 2H), 0.97(t, $J=7.4$Hz, 3H). Refer to Figure A-7.

$^{13}$C NMR 75.47 MHz (CDCl$_3$): $\delta = 187.2, 161.3, 144.6, 137.5, 134.9, 133.2, 132.5, 129.6, 127.2, 126.4, 125.2, 119.3, 115.2, 67.9, 31.1, 19.2, 13.8$. Refer to Figure A-7.

3.51 2-(4-Hexyloxyphenylmethylene)-5-bromobenzo[b]thiophen-3(2H)-one

The general procedure for hemithioindigo synthesis was followed. The amounts of reagents used: acid 3.43 (0.98 g, 4.3 mmol), excess SOCl₂, followed by AlCl₃ (0.61 g, 5.2 mmol, 1.2 eq.), then 3.20 (0.89, 4.3 mmol, 1 eq.). The solution was concentrated and the residue was recrystallized from CH₂Cl₂/hexanes to yield a yellow solid (1.34 g, 3.2 mmol, 74%), m.p. = 129-131 ℃.

¹H NMR 300 MHz (CDCl₃): δ = 8.02 (d, J=2.2 Hz, 1H), 7.91 (s, 1H), 7.63 (d, J=8.1 Hz, 1H), 7.62 (d, J=8.8 Hz, 2H), 7.36 (d, J=8.1 Hz, 1H), 6.96 (d, J=8.8 Hz, 2H), 4.00 (t, J=6.6 Hz, 2H), 1.79 (qn, J=7.4 Hz, 2H), 1.45 (qn, J=7.4 Hz, 2H), 1.34 (m, 4H), 0.89 (t, J=6.6 Hz, 3H). Refer to Figure A-8.

¹³C NMR 75.47 MHz (CDCl₃): δ = 187.2, 161.2, 144.6, 137.5, 134.9, 133.2, 132.5, 129.6, 127.2, 126.4, 125.2, 119.3, 115.2, 68.3, 31.5, 29.0, 25.6, 22.6, 14.0. Refer to Figure A-8.

+LSIMS (mNBA as matrix) m/z (relative intensity): 419, 417 (M+H⁺, 100). Analysis: calculated for C_{31}H_{31}O_{2}BrS: (%) C, 60.43; H, 5.07. Found C, 60.97; H, 5.51.
3.53 2-(4-Hexyloxyphenylmethylene)-5-(methoxycarbonylethenyl)benzo[b]thiophen-3(2H)-one

The general procedure for Heck reactions was followed. The amounts of reagents used: 5.51 (0.393 g, 0.94 mmol), methyl acrylate (0.4 mL, 1.0 mmol, 1.1 eq.), NEt$_3$ (0.11 g, 1.0 mmol, 1.1 eq.), Pd(OAc)$_2$ (0.018 g, 0.08 mmol, 8.5% eq.) and P(Ph)$_3$ (0.045 g, 0.17 mmol, 17% eq.) in 15 mL of CH$_3$CN were placed in a flask, heated at 110°C for 4 h. The crude was purified by centrifugal chromatography (CHCl$_3$), eluting unreacted 3.51, followed by 3.53 (0.090 g, 0.21 mmol, 22%), m.p. = 179-181 °C.

$^1$H NMR 300 MHz (CDCl$_3$): δ = 8.05 (d, J=1.5 Hz, 1H), 7.95 (s, 1H), 7.69 (d, J=15.4 Hz, 1H), 7.68 (d, J=8.1 Hz, 1H), 7.64 (d, J=8.8 Hz, 2H), 7.51 (d, J=8.1 Hz, 1H), 6.98 (d, J=8.8 Hz, 2H), 6.49 (d, J=15.4 Hz, 2H), 4.01 (t, J=6.6 Hz, 2H), 3.80 (s, 3H), 1.79 (qn, J=7.4 Hz, 2H), 1.46 (qn, J=6.6 Hz, 2H), 1.33 (m, 4H), 0.90 (t, J=6.6 Hz, 2H). Refer to Figure 3-9.

$^{13}$C NMR 75.47 MHz (CDCl$_3$): δ = 187.9, 167.1, 161.2, 147.8, 143.2, 134.8, 134.0, 133.2, 132.1, 131.4, 127.4, 126.5, 126.0, 124.3, 118.5, 115.2, 68.3, 51.8, 31.5, 29.1, 25.6, 22.6, 14.0. Refer to Figure 3-9.
+LSIMS (mNBA as matrix) m/z (relative intensity): 423 ([M+1]^+, 60), 289 (100).

Analysis: calculated for C_{33}H_{26}O_{4}S: (%) C, 71.06; H, 6.20; O, 15.15; S, 7.57. Found C, 71.37; H, 6.16; O, 14.98.

3.58 (N-Phthalimido)methyl acrylate CAS 40459-70-9

![Molecule](image)

A solution of acrylic acid (3.47, 48.1 mmol) and dicyclohexylamine, (8.71 g, 4.81 mmol, 1 eq), in 150 mL of DMF was heated to 65°C, in an inert atmosphere. N-chloromethylphthalimide (9.41 g, 48.1 mmol) in 100 mL of DMF was added over 10 minutes and the solution was stirred for a further 30 minutes. The ammonium salt precipitate was filtered and the supernatant concentrated. The crude product was recrystallized from toluene yielding a white solid (5.97 g, 25.8 mmol, 54%), m.p. = 142-143 °C.

^1H NMR 300 MHz (CDCl₃): δ = 7.91 (AB, J=5.2, 3.0 Hz, 2H), 7.76 (AB, J=5.2, 3.0 Hz, 2H), 7.42 (ABX, J=16.9, 1.5 Hz, 1H), 6.07 (ABX, J=16.9, 10.3 Hz, 1H), 5.85 (ABX, J=10.3, 1.5 Hz, 1H), 5.78 (s, 2H). Refer to Figure A-9.

^13C NMR 75.47 MHz (CDCl₃): δ = 166.7, 164.8, 134.6, 132.2, 131.7, 127.4, 124.0, 60.8. Refer to Figure A-9.

3.59 (N-Phthalimido)methyl 4-(1-thia-4-oxa-3-oxohexyl) cinnamate

The general procedure for Heck reaction was followed. The amounts of reagents used: 3.15 (2.38 g, 8.66 mmol), 3.58 (2.00 g, 8.66 mmol, 1 eq.), NEt$_3$ (0.87 g, 8.66 mmol, 1 eq.), Pd(OAc)$_2$ (0.10 g, 0.48 mmol, 5.5% eq.) and P(Ph)$_3$ (0.35 g, 1.3 mmol, 15% eq.) in 20 mL of CH$_3$CN were placed in a flask, heated at 105°C for 15 h.

The solid was dissolved in CH$_2$Cl$_2$ (100 mL) and the solution was washed with H$_2$O (50 mL x 2), and the concentrated. The crude product was recrystallized from ethyl acetate/hexanes and purified further using centrifugal chromatography (CHCl$_3$) to give a white solid (1.66 g, 3.90 mmol, 45%), m.p. = 130 °C.

$^1$H NMR 300 MHz (CDCl$_3$): $\delta$ = 7.91 (AB, J=5.9, 2.9 Hz, 2H), 7.76 (AB, J=5.9, 2.9 Hz, 2H), 7.62 (d, J=16.2 Hz, 1H), 7.42 (AB, J=8.1 Hz, 2H), 7.27 (AB, J=8.1 Hz, 2H), 6.32 (d, J=16.2 Hz, 1H), 5.82 (s, 2H), 4.14 (q, J=6.6 Hz, 2H), 3.65 (s, 2H), 1.19 (t, J=6.6 Hz, 3H). Refer to Figure 3-10.

$^{13}$C NMR 75.47 MHz (CDCl$_3$): $\delta$ = 169.2, 166.7, 165.5, 145.2, 138.8, 134.6, 132.1, 131.7, 128.6, 128.5, 123.9, 116.5, 61.7, 60.8, 35.5, 14.0. Refer to Figure 3-11.
CI MS \( m/z \) (relative intensity): 259 ([M+\( \text{C}_2\text{H}_5 \])^+, 14), 226 ([M+1]^+, 52), 408 ([M-\( \text{H}_2\text{O} \])^+, 100), 160 ([methylphthalimide]^+, 88). Analysis: calculated for \( \text{C}_{22}\text{H}_{19}\text{O}_6\text{NS} \) (%):

- C, 62.11; H, 4.50; N, 3.29. Found C, 61.67; H, 4.50; N, 3.31.

3.60 (N-Phthalimido)methyl 4-(1-thia-4-oxa-3-oxohexyl) dihydrocinnamate

The general procedure for hydrogenation was followed. The amounts of reagents used: 3.58 (2.00 g, 4.70 mmol) and Rh(PPh\(_3\))\(_2\)Cl (0.124 g, 0.134 mmol, 0.03 eq) in 150 mL of degassed 1:1 benzene/ethanol was hydrogenated at rt, at 60 PSI, for 10 days. The crude product was purified on a silica column (1:1, ethyl acetate/hexanes) to give a clear liquid (1.90 g, 4.45 mmol, 95%).

\(^1\)H NMR 300 MHz (CDCl\(_3\)): \( \delta = 7.89 \) (AB, \( J=5.5 \), 2.9 Hz, 2H), 7.77 (AB, \( J=5.5 \), 2.9 Hz, 2H), 7.26 (d, \( J=8.1 \) Hz, 2H), 7.08 (d, \( J=8.1 \) Hz, 2H), 5.68 (s, 2H), 4.11 (q, \( J=6.6 \) Hz, 2H), 3.62 (s, 2H), 2.88 (t, \( J=7.4 \) Hz, 2H), 2.59 (t, \( J=7.4 \) Hz, 2H), 1.18 (t, \( J=6.6 \) Hz, 3H). Refer to Figure 3-10.

\(^{13}\)C NMR 75.47 MHz (CDCl\(_3\)): \( \delta = 171.3, 169.5, 166.5, 139.0, 134.5, 132.4, 131.5, 130.4, 128.8, 123.8, 61.3, 60.6, 36.8, 35.1, 30.0, 13.9 \). Refer to Figure 3-11.

+LSIMS (mNBA as matrix) \( m/z \) (relative intensity): 427 (M\(^+\), 28), 354 ([M-\( \text{CO}_2\text{C}_2\text{H}_4 \])^+, 10), 280 ([M-phthalimide]^+, 20), 267 ([M-methylphthalimide]^+, 30), 209 (24).
3.63 \[\text{(4-(2-Cyanoethenyl)phenyl)thioj acetatic acid, ethyl ester}\]

The general procedure for Heck reactions was followed. The amounts of reagents used: 3.15 (6.37g, 23.2 mmol), freshly distilled acrylonitrile (1.84g, 34.8mmol, 1.5eq.) and NEt\textsubscript{3} (3.51g, 34.5mmol, 1.5eq.), Pd(OAc)\textsubscript{2} (0.27g, 1.2mmol, 5% eq.) and P(Ph)\textsubscript{3} (0.63g, 2.4mmol, 10% eq.).

The crude product (TLC, 1:1 ether:hexanes, R\textsubscript{f} = 0.33) was purified by chromatography (silica, 100g, 55:45 ether:hexanes) to give a yellow liquid that solidified on standing. Kugelrohr distillation (5 x 10\textsuperscript{-3} atm), gave a white solid between 140-165\textdegree C (4.46g, 18.0mmol, 78%). The isomers could be separated by repeated centrifugal chromatography (1:3 ether:hexanes), \textit{trans}-.m.p. = 44-45 \textdegree C.

\textsuperscript{1}H NMR 300 MHz (CDCl\textsubscript{3}): \textit{Trans}. \(\delta = 7.30 \text{(m,3H)}, 5.78 \text{(d, J=16.7Hz, 1H)}, 4.12 \text{(q, J=7.1, 2H)}, 3.64 \text{(s, 2H)}, 1.17 \text{(t, J=7.1Hz, 3H)}.~\textit{Cis}. \(\delta = 7.67 \text{(d, J=8.4Hz, 2H)}, 7.30 \text{(m,3H)}, 6.99 \text{(d, J=12.1Hz, 1H)}, 5.35 \text{(d, J=12.0Hz, 1H)}, 4.12 \text{(q, J=7.1, 2H)}, 3.65 \text{(s, 2H)}, 1.17 \text{(t, J=7.1Hz, 3H)}.~\) Refer to Figure 3-12.
$^1$C NMR 75.47 MHz (CDCl$_3$): Trans. $\delta = 168.8, 149.2, 139.5, 131.0, 127.9, 127.5, 117.9, 95.6, 61.5, 34.9, 13.8$. Cis. $\delta = 168.8, 147.4, 139.3, 131.0, 129.2, 127.6, 117.1, 94.3, 61.5, 34.9, 13.8$. Refer to Figure 3-13.

CI MS $m/z$ (relative intensity): 288 ([M+C$_2$H$_5]$), 276 ([M+C$_2$H$_5$]$, 17$), 248 ([M+1]'$, 100$), 174 (M-CO$_2$C$_2$H$_5$, 16$). Analysis: calculated for C$_{13}$H$_{13}$O$_2$NS (%): C 63.14; H 5.30; N 5.67. Found C, 63.77; H, 5.27; N, 6.05.

3.65 [(3-(2-Cyanoethenyl)phenyl)thio] acetic acid, ethyl ester

![Chemical structure](image)

The general procedure for Heck reactions was followed. The amounts of reagents used: 3.16 (3.16, 11.5mmol), acrylonitrile (0.61g, 11.5mmol, 1eq.), NEt$_3$ (1.16g, 11.5mmol, 1eq.), Pd(OAc)$_2$ (0.026g, 0.12mmol, 1% eq.) and P(Ph)$_3$ (0.060g, 0.24mmol, 2% eq.) were placed in a flask.

The crude product was fractionated on a column (silica, 60g, 2:1 ether:hexanes), followed by centrifugal chromatography (1:3 ether:hexanes), yielding 1.71g of product 3.65 (6.92mmol, 60%). Separation of isomers was achieved by tedious centrifugal chromatography.

$^1$H NMR 300 MHz (CDCl$_3$): Trans. $\delta = 7.46$ (s, 1H), 7.42 (d, J=7.35Hz, 1H), 7.29 (m, 2H), 5.86 (d, 16.9Hz), 4.14 (q, J=6.7Hz, 2H), 3.62 (s, 2H), 1.19 (t, J=6.6Hz, 3H). Cis. $\delta = 7.71$ (s, 1H), 7.66 (d, J=8.1Hz, 1H), 7.42 (d, 1H), 7.29 (m, 1H), 7.06 (d.
J = 11.8 (1H), 5.46 (d, J = 12.5 Hz, 1H), 4.14 (q, J = 6.7 Hz, 2H), 3.65 (s, 2H), 1.19 (t, J = 6.6 Hz, 3H). Refer to Figure 3-14.

$\text{^13}C$ NMR 75.47 MHz (CDCl$_3$): Trans. $\delta = 169.2, 149.5, 136.6, 134.22, 131.7, 129.5, 127.8, 125.6, 117.7, 97.2, 61.6, 36.0, 13.9$. Cis. $\delta = 169.2, 147.8, 136.3, 134.18, 131.5, 129.6, 129.4, 126.8, 116.9, 96.0, 61.5, 36.1, 13.9$. Refer to Figure 3-15.


3.69 2-(Carboxyethyl)-3-(cyanoethyl)-2H, 3H-benzo[b]thiophene

The general procedure for Heck reactions was followed. The amounts of reagents used: 3.17 (9.56 g, 34.8 mmol), acrylonitrile (2.76 g, 52.2 mmol, 1.5 eq.), NEt$_3$ (5.26 g, 52.6 mmol, 1.5 eq.), Pd(OAc)$_2$ (0.41 g, 1.8 mmol, 5% eq.) and P(Ph)$_3$ (0.95 g, 3.6 mmol, 10% eq.) were placed in a flask.

The crude was fractionated by chromatography (silica, 100 g, 2:1 ether:hexanes), followed by centrifugal chromatography (1:3 ether:hexanes), yielding 3.27 g of product (13.2 mmol, 38%).

$^1$H NMR 300 MHz (CDCl$_3$): $\delta = 7.18$ (m, 4H). 4.19 (m, 4H), 7.76 (t, J = 6.6 Hz, 2H), 1.27 (d, J = 8.8 Hz, 3H). Refer to Figure 3-16.
\[ ^{13} \text{C NMR} \ 75.47 \text{ MHz (CDCl}_3\): } \delta = 170.4, 138.28, 138.24, 129.2, 125.5, 124.6, 122.3, 117.4, 62.2, 53.1, 46.2, 21.7, 14.0. \text{ Refer to Figure 3-16.} \]

\[ \text{Cl MS } m/z \ (\text{relative intensity}): 288 ([M+C,H]^{+}, 9), 276 ([M+C,H]^{+}, 23), 248 ([M+1]^{+}, 100). \text{ Analysis: calculated for C}_{11}H_{11}O_{2}NS (\%): C, 63.14; H, 5.30; N, 5.67. Found C, 62.85; H, 5.21; N, 5.81. ]

3.71 2-Carboxy-3-cyanethyl-2H, 3H-benzo[b]thiophene

\[ \begin{array}{c}
\text{O} \\
\text{S} \\
\text{OH} \\
\text{N}
\end{array} \]

The general procedure for ester hydrolysis was followed. The amounts of reagents used: 8 (0.120 g, 0.486 mmol) and NaOH (0.1 g, xs.) in 15 mL of MeOH, was refluxed for 3 h. After acidifying the aqueous salt solution, the product was extracted with CH\(_2\)Cl\(_2\) (25 mL x 2), dried and concentrated to give a white solid (0.080 g, 0.365 mmol, 75%), m.p. = 142-144 °C.

\[ ^{1} \text{H NMR} 300 \text{ MHz (CDCl}_3\): } \delta = 9.14 (\text{br s, 1H}), 7.20 (\text{m, 4H}), 4.15 (\text{d, } J=2.9 \text{ Hz, 1H}), 4.09 (\text{dt, } J=6.6, 3.7 \text{ Hz, 1H}), 2.72 (\text{t, } J=6.6 \text{ Hz, 2H}). \text{ Refer to Figure 3-17.} \]

\[ ^{13} \text{C NMR} 75.47 \text{ MHz (CDCl}_3\): } \delta = 175.9, 138.1, 137.7, 129.4, 125.7, 124.7, 122.4, 117.2, 52.8, 46.1, 21.8. \text{ Refer to Figure 3-17.} \]

\[ +\text{LSIMS (mNBA as matrix) } m/z \ (\text{relative intensity}): 220 ([M+H]^{+}, 36), 219 (M^{+}, 97), 192 ([M-CN]^{+}, 174 ([M-CO]^{+}, 147 ([M-CO-CN]^{+}, 100). \text{ Analysis: calculated for C}_{5}H_{11}O_{2}NS \text{ (\%): C, 59.71; H, 5.02; N, 6.33. Found C 59.92; H 5.00; N 6.48.} \]
3.74 [(4-(1-Methyl-2-cyanoethenyl)phenyl)thio] acetic acid, ethyl ester

The general procedure for Heck reactions was followed. The amounts of reagents used: 3.15 (5.00 g, 18.2 mmol), allyl cyanide (1.22 g, 18.2 mmol, 1 eq.), NEt\textsubscript{3} (1.84 g, 1.82 mmol, 1 eq.), Pd(OAc)\textsubscript{2} (0.21 g, 0.92 mmol, 5% eq.) and P(Ph)\textsubscript{2} (0.49 g, 1.8 mmol, 10% eq.) were placed in a flask.

The crude was fractionated by chromatography (silica, 200 g, 1:3, ethyl acetate:hexanes), followed by centrifugal chromatography (1:3 Et\textsubscript{2}O:Hexanes), yielding 1.85 g of product (7.11 mmol, 39%).

\textsuperscript{1}H NMR 300 MHz (CDCl\textsubscript{3}): \textit{Trans}. \(\delta = 7.36\) (br s, 4H), 5.58 (s, 1H), 4.16 (q, \(J=7.4\) Hz, 2H), 3.66 (s, 2H), 2.41 (s, 3H), 1.21 (t, \(J=7.4\) Hz, 3H). \textit{Cis}. \(\delta = 7.48\) (d, \(J=8.8\) Hz, 2H), 7.39 (d, \(J=8.8\) Hz, 2H), 5.35 (d, \(J=1.8\) Hz, 1H), 4.16 (q, \(J=7.4\) Hz, 2H), 3.66 (s, 2H), 2.23 (d, \(J=1.8\) Hz, 3H), 1.22 (t, \(J=7.4\) Hz, 3H). Refer to Figure 3-19.

\textsuperscript{13}C NMR 75.47 MHz (CDCl\textsubscript{3}): \textit{Trans}. \(\delta = 169.1, 158.5, 138.4, 135.9, 128.4, 126.3, 117.5, 95.1, 61.7, 35.4, 19.9, 14.0\). \textit{Cis}. \(\delta = 169.3, 159.7, 137.9, 135.7, 128.5, 127.6, 117.4, 95.3, 61.7, 35.7, 24.4, 14.0\). Refer to Figure 3-19.

Cl MS \textit{m/z} (relative intensity): 290 [(M+\textit{C\textsubscript{6}H\textsubscript{15}})\textsuperscript{-}], 19) 261 [(M+1)\textsuperscript{-}], 100). Analysis: calculated for C\textsubscript{14}H\textsubscript{15}O\textsubscript{2}NS (%): C, 64.34; H, 5.79; N, 5.36. Found C, 65.00; H, 5.75; N, 5.25.
3.75 \([4-(2\text{-Cyanoethyl})\text{phenyl}]\text{thio} \text{acetic acid, ethyl ester}\)

The general procedure for hydrogenation was followed. The amounts of reagents used: 3.63 (7.43 g, 30.1 mmol) and Wilkinson’s catalyst, \(\text{Rh}(\text{PPh}_3)_3\text{Cl}\) (0.97 g, 0.105 mmol, 0.035 eq.), in 150 mL of degassed 1:1 benzene/ethanol. The crude product was distilled (Kugelrohr, \(5 \times 10^3\) atm) at 160-180°C, to yield product as a yellowish liquid. For higher purity 3.75 was passed through a silica column (1:9, ethyl acetate/ toluene) giving a clear liquid (4.85 g, 19.5 mmol, 65%).

\(^1\text{H} \text{NMR} 300 \text{MHz (CDCl}_3\text{):} \delta = 7.32 (d, J=8.3\text{Hz}, 2\text{H}), 7.12 (d, J=8.3\text{Hz}, 2\text{H}) 4.11 (\text{qua.}, J=7.0\text{Hz}, 2\text{H}), 3.57 (s, 2\text{H}), 2.86 (t, J=7.3\text{Hz}, 2\text{H}), 2.54 (t, J=7.3\text{Hz}, 2\text{H}), 1.17 (t, J=7.0\text{Hz}, 3\text{H})\). Refer to Figure 3-20.

\(^{13}\text{C} \text{NMR} 75.47 \text{MHz (CDCl}_3\text{):} \delta = 169.4, 136.7, 133.7, 130.2, 128.8, 118.8, 61.3, 36.4, 30.8, 18.9, 13.8\). Refer to Figure 3-21.

\(\text{CI MS } m/z \text{ (relative intensity):} 290 ([M+C\text{H}_3\text{J}^+], 5), 278 ([M+C\text{H}_3\text{J}^+], 19), 250 ([M+H]^+, 100), 176 ([M-CO}_2\text{C}_2\text{H}_4\text{J}^+], 25). \text{HRMS for } M^+, C_{13}\text{H}_{15}O_2\text{NS, } m/e: 249.0824, \text{ found 249.0823}.\)
3.76 [(3-(2-Cyanoethyl)phenyl)thio] acetic acid, ethyl ester

\[
\text{N} \text{=\text{\textbullet}} \text{S} \text{\textlongrightarrow} \text{O} \text{\textlongrightarrow} \text{S} \text{\textlongrightarrow} \text{O} \text{\textlongrightarrow} \text{S} \text{\textlongrightarrow} \text{O}
\]

The general procedure for hydrogenation was followed. The amounts of reagents used: 7 (1.71 g, 6.93 mmol) and Rh(PPh₃)₃Cl (0.22 g, 0.24 mmol, 0.035 eq) in 120 mL of degassed 1:1 benzene/ethanol was hydrogenated at rt, at 60 PSI, for 10 days. The crude product was purified on a silica column (1:1, ether:hexanes) to give a clear liquid (1.10 g, 4.42 mmol, 64%).

\(^1\text{H} \text{NMR 300 MHz (CDCl}_3\text{):} \delta = 7.25 \text{ (m, 3H), 7.06 \text{ (d, J=7.4 Hz, 1H), 4.14 \text{ (q, J=6.6 Hz, 2H), 3.62 \text{ (s, 2H), 2.90 \text{ (t, J=7.4 Hz, 2H), 2.58 \text{ (t, J=7.4 Hz, 2H), 1.20 \text{ (t, J=6.6 Hz, 3H). Refer to Figure 3-20.}}}}

\(^13\text{C} \text{NMR 75.47 MHz (CDCl}_3\text{):} \delta = 169.5, 138.9, 135.8, 129.4, 129.3, 128.2, 126.7, 118.8, 61.6, 36.3, 31.2, 19.0, 14.0. \text{Refer to Figure 3-21.}}

\text{CI MS m/z (relative intensity): 278 ([M+C}_2\text{H}_4\text{]}^+, 28), 250 ([M+H]^+, 85), 204 ([M-OC}_2\text{H}_5\text{]}^+, 37), 176 ([M-CO}_2\text{C}_2\text{H}_3\text{]}^+, 100). Analysis: calculated for C}_13\text{H}_{15}\text{O}_2\text{NS (\%): C, 62.60; H, 6.10; N, 5.60. Found C, 62.58; H, 6.05; N, 5.55.}}

3.77 [(4-(2-Cyanoethyl)phenyl)thio] acetic acid

\[
\text{N} \text{=\textbullet} \text{S} \text{\textlongrightarrow} \text{O} \text{\textlongrightarrow} \text{S} \text{\textlongrightarrow} \text{O} \text{\textlongrightarrow} \text{S} \text{\textlongrightarrow} \text{O} \text{\textlongrightarrow} \text{S} \text{\textlongrightarrow} \text{O}
\]
The general procedure for ester hydrolysis was followed. The amounts of reagents used: 3.75 (1.50 g, 6.01 mmol) and NaOH (0.29 g, 7.22 mmol, 1.2 eq.) in 50 mL of MeOH, was refluxed for 3 h. The product was a white solid (1.20 g, 5.43 mmol, 90%), m.p. = 106-107 °C.

\[ ^1H \text{NMR 300 MHz (CDCl}_3\text{): } \delta = 7.38 \text{ (d, } J=8.3\text{Hz, 2H), 7.17 (d, } J=8.3\text{Hz, 2H), 3.64 (s, 2H), 2.92 (t, } J=7.3\text{Hz, 2H), 2.59 (t, } J=7.3\text{Hz, 2H). Refer to Figure A-10.} \]

\[ ^{13}C \text{NMR 75.47 MHz (CDCl}_3\text{): } \delta = 175.2, 137.2, 133.4, 130.6, 129.2, 118.9, 36.5, 31.1, 19.2. \text{ Refer to Figure A-10.} \]

\[ \text{Cl MS } m/z \text{ (relative intensity): 262 ([M+}C_3H_7\text{]^+}, 10), 250 ([M+}C_2H_5\text{]^+}, 26.), 222 ([M+H]^+}, 17), 204 ([M-H}_2\text{O}]^+}, 78), 176 ([M-CH}_2\text{CO}_2\text{]^+}). \text{ Analysis: calculated for C}_9\text{H}_11\text{O}_2\text{NS: } \text{(%): C } 59.71; \text{ H 5.02; N 6.33. Found C 59.92; H 5.00; N 6.48.} \]

3.80 2-(4-Hydroxyphenylmethylene)-5-(2-cyanoethyl)benzo[b]thiophen-3(2H)-one

The general procedure for hemithioindigo synthesis was followed. The amounts of reagents used: acid 3.77 (0.23 g, 1.04 mmol), excess SOCl\(_2\), followed by AlCl\(_3\) (0.61 g, 5.2 mmol, 1.2 eq.), then 4-hydroxybenzaldehyde (0.20, 1.6 mmol, 1.5 eq.). The AlCl\(_3\) (0.210 g, 1.58 mmol, 1.2 eq.) in 10 mL of ClCH\(_2\)_2, was cooled in a salt ice bath to below 0°C. The acid chloride in 10 mL of ClCH\(_2\)_2, was added to the cooled solution, under rapid stirring, over a 2 min. period. The solution was left in ice bath for another 10 min. removed and heated to 60°C for a further 1 h. The indoxyl intermediate was unstable and
was used in situ. Once the solution was cool a brown solid precipitated out. The 
suspension was washed with Na₂S₂O₃ (5%, 25 mL x 2), filtered, and recrystallized from 
MeOH (0.20 g, 0.65 mmol, 62%), m.p. = 287 °C.

Acid Chloride:

\[ ^1H \text{ NMR } 300 \text{ MHz (CDCl}_3\text{)}: \delta = 7.40 (d, J=8.5 \text{ Hz}, 2\text{H}), 7.19 (d, J=8.5 \text{ Hz}, 2\text{H}), 4.02 (s, 2\text{H}), 2.92 (t, J=7.4 \text{ Hz}, 2\text{H}), 2.59 (t, J=7.4 \text{ Hz}, 2\text{H}). \]

Refer to Figure A-11.

\[ ^13C \text{ NMR } 75.47 \text{ MHz (CDCl}_3\text{)}: \delta = 169.8, 138.3, 132.1, 129.4, 118.8, 48.5, 31.1, 19.1. \]

Refer to Figure A-11.

Hemithioindigo:

\[ ^1H \text{ NMR } 300 \text{ MHz (CD}_3\text{OD)}: \delta =10.4 (\text{br s}, 1\text{H}), 7.82 (s, 1\text{H}), 7.77 (d, J=1.1 \text{ Hz}, 1\text{H}), 7.69 (d, J=8.1 \text{ Hz}, 1\text{H}), 7.61 (d, J=8.6 \text{ Hz}, 2\text{H}), 7.60 (dd, J=8.1 \text{ Hz}, 1.1 \text{ Hz}), 6.90 (d, J=8.6 \text{ Hz}, 2\text{H}), 2.93 (t, J=6.6 \text{ Hz}, 2\text{H}), 2.83 (t, 6.6 \text{ Hz}, 2\text{H}). \]

Refer to Figure 3-22.

\[ ^13C \text{ NMR } 75.47 \text{ MHz (CD}_3\text{OD)}: \delta =187.2, 160.2, 143.4, 137.1, 136.1, 134.3, 133.9, 133.4, 130.3, 126.2, 124.8, 124.4, 120.1, 116.4, 29.8, 18.0. \]

Refer to Figure 3-22.

Cl MS m/z (relative intensity): 308 ([M+H]⁺, 100). Analysis: calculated for 
C_{18}H_{13}O_{3}NS: (%): C, 70.34; H, 4.26; N, 4.56. Found C, 69.62; H, 4.24; N, 4.50.

3.81 2-(4-Butlyoxyphenylmethylene)-5-(2-cyanoethyl)benzo[b]thiophen-3(2H)-one

![Chemical Structure](image-url)
The general procedure for hemithioindigo synthesis was followed. The amounts of reagents used: acid 3.75 (0.293 g, 1.32 mmol), excess SOCl₂, followed by AlCl₃ (0.210 g, 1.58 mmol, 1.2 eq.), then 3.19 (0.235 g, 1.32 mmol, 1 eq.). The AlCl₃, in 10 mL of (ClCH₂)₂, was cooled in a salt ice bath to below 0°C. The acid chloride in 10 mL of ClICH₂Cl was added to the cooled solution, under rapid stirring, over a 2 min. period. The solution was left in an ice bath for another 10 min, removed and heated to 60°C for a further 1 h. The indoxyl intermediate was unstable and was used in situ. Once the solution was cool a yellow solid precipitated out, which was filtered and dried under vacuum (0.216 g, 0.595 mmol, 45%), m.p. = 158°C. If no precipitation occurred then the solution was concentrated and chromatographed (silica, 3:1, CHCl₃/hexanes) to afford the product.

\[ \text{\textsuperscript{1}H NMR 300 MHz (CDCl₃): } \delta = 7.91 (s, 1H), 7.75 (s, 1H), 7.63 (d, J=8.8 Hz, 2H), 7.45 (m, 2H), 6.97 (d, J=8.8 Hz, 2H), 4.01 (t, J=6.3 Hz, 2H), 2.99 (t, J=7.4 Hz, 2H), 2.65 (t, J=7.4 Hz, 2H), 1.78 (qn J=7.4 Hz, 2H), 1.48 (sx, J=7.4 Hz, 2H), 0.97 (t, J=7.4 Hz, 3H). \]

Refer to Figure A-12.

\[ \text{\textsuperscript{13}C NMR 75.47 MHz (CDCl₃): } \delta = 188.2, 161.0, 145.0, 135.5, 135.2, 134.3, 133.1, 131.3, 127.6, 126.6, 126.2, 124.2, 118.6, 115.1, 67.9, 31.1, 30.9, 19.2, 19.1, 13.8. \]

Refer to Figure A-12.

+LSIMS (mNBA as matrix) \( m/z \) (relative intensity): 364.1 (M+H⁺, 100).

Analysis: calculated for C₂₃H₁₉O₂NS: (%) C, 72.70; H, 5.83; N, 3.86. Found C, 71.92; H, 5.80; N, 3.99.
3.82 \(2-(4-\text{Hexyloxyphenylmethylene})-5-(2-\text{cyanoethyl})\text{benzo[b]thiophen-3(2H)}\)-one

\[
\begin{array}{c}
\text{N} \\
\text{O}
\end{array}
\]

\text{Z-3.82}

The preparation procedure is similar to that of 3.81. The amounts of reagents used: 3.75 (0.600 g, 2.71 mmol), excess SOCl\(_2\); AlCl\(_3\) (0.45, 3.4 mmol, 1.2 eq.), 75 mL (ClCH\(_2\)Cl); 3.20 (0.57 g, 2.8 mmol, 1 eq.), 60 mL of benzene, 4 drops of piperidine.

Product is a yellow solid (0.360g, 0.92 mmol, 34%), m.p. = 129-131 °C.

\text{Z-Isomer:

\(^1\text{H NMR 300 MHz (CDCl}_3\): \(\delta = 7.93\) (s, 1H), 7.76 (s, 1H), 7.64 (d, \(J=8.8\) Hz, 2H), 7.48, 7.45 (AB, \(J=8.5\) Hz, 2H), 6.97 (d, \(J=8.8\) Hz, 2H), 4.00 (t, \(J=6.6\) Hz, 2H), 3.00 (t, \(J=7.4\) Hz, 2H), 2.65 (t, \(J=7.4\) Hz, 2H), 1.79 (qn, \(J=6.6\) Hz, 2H), 1.44 (qn, \(J=6.6\) Hz, 2H), 1.34 (m, 4H), 0.90 (t, \(J=6.6\) Hz, 2H). Refer to Figures 3-23 and 3-25.

\(^{13}\text{C NMR 75.47 MHz (CDCl}_3\): \(\delta = 188.2, 161.1, 145.0, 135.2, 134.3, 133.1, 131.4, 127.7, 126.6, 126.3, 124.3, 118.8, 115.1, 68.3, 31.5, 31.0, 29.0, 25.6, 22.6, 19.2, 14.0. Refer to Figures 3-23 and 3-26.

+LSIMS (mNBA as matrix) \(m/z\) (relative intensity): 392.0 ([M+1]⁺, 100). HRMS (+LSIMS) C\(_{24}\)H\(_{35}\)O\(_2\)NS: calcd. 392.1684, found 392.1670. Analysis: calculated for C\(_{24}\)H\(_{35}\)O\(_2\)NS: (%): C, 73.63; H, 6.44; N, 3.58. Found C, 73.20; H, 6.45; N, 3.61.

The E-isomer was synthesized by irradiating a chloroform solution of Z-31, in a Rayonet reactor, at 350nm for 30 min. The solution was concentrated and the isomers were separated by centrifugal chromatography (1:3 hexanes:chloroform), under red light.
$^1$H NMR 300 MHz (CDCl$_3$): $\delta = 8.19$ (d, $J=8.8$ Hz, 2H), 7.68 (d, $J=1.5$ Hz, 1H), 7.44, 7.34 (AB, $J=8.1$ Hz, 2H), 7.15 (s, 1H), 6.91 (d, $J=8.8$ Hz, 2H), 4.00 (t, $J=6.6$ Hz, 2H), 2.98 (t, $J=6.6$ Hz, 2H), 2.62 (t, $J=6.6$ Hz, 2H), 1.78 (qn, $J=6.6$ Hz, 2H), 1.45 (qn, $J=6.6$ Hz, 2H), 1.34 (m, 4H), 0.90 (t, $J=6.6$ Hz, 2H). Refer to Figures 3-24 and 3.25.

$^{13}$C NMR 75.47 MHz (CDCl$_3$): $\delta =$ 185.4, 161.5, 144.4, 139.4, 134.8, 134.7, 133.9, 133.1, 130.2, 127.0, 126.2, 123.8, 118.7, 114.1, 68.1, 31.5, 31.0, 29.0, 25.6, 22.6, 19.2, 14.0. Refer to Figures 3-24 and 3-26.

3.83 2-(4-Octyloxyphenylmethylene)-5-(2-cyanoethyl)benzo[b]thiophen-3(2H)-one

The preparation procedure is similar to that of 3.81. The amounts of reagents used: 3.75 (0.29 g, 1.30 mmol), excess SOCl$_2$; AlCl$_3$ (0.210, 1.6 mmol, 1.2 eq.), 40 mL (ClCH$_2$)$_2$; 3.21 (0.280 g, 1.20 mmol, 0.92 eq.), 40 mL of benzene, 4 drops of piperidine. Product is a yellow solid (0.212g, 0.506 mmol, 42%), complex melting.

$^1$H NMR 300 MHz (CDCl$_3$): $\delta =$ 7.92 (s, 1H), 7.76 (s, 1H), 7.64 (d, $J=8.8$ Hz, 2H), 7.51, 7.42 (AB, $J=8.5$ Hz, 2H), 6.97 (d, $J=8.8$ Hz, 2H), 4.00 (t, $J=6.6$ Hz, 2H), 3.00 (t,
J=7.4 Hz, 2H), 2.65 (t, J=7.4 Hz, 2H), 1.79 (qn, J=6.6 Hz, 2H), 1.45 (m, 2H), 1.28 (m, 8H), 0.87 (t, J=6.6 Hz, 2H). Refer to Figure A-13.

$^{13}$C NMR 75.47 MHz (CDCl$_3$): δ = 188.2, 161.1, 145.0, 135.6, 135.2, 134.3, 133.1, 131.4, 127.7, 126.6, 126.3, 118.7, 115.2, 68.3, 31.8, 31.0, 29.3, 29.2, 29.1, 26.0, 22.6, 19.3, 14.1. Refer to Figure A-13.

$^+LSIMS$ (mNBA as matrix) m/z (relative intensity): 420 ([M+1]$^+$, 100). Analysis: calculated for $C_{28}H_{29}O_{2}NS$: (%): C, 74.43; H, 6.97; N, 3.34. Found C, 73.88; H, 7.11; N, 2.96.

3.84 2-(3-Hexyloxyphenylmethylene)-5-(2-cyanoethyl)benzo[b]thiophen-3(2H)-one

The preparation procedure is similar to that of 3.81. The amounts of reagents used: 3.75 (0.450 g, 2.04 mmol), excess SOCl$_2$; AlCl$_3$ (0.43, 3.2 mmol, 1.5 eq.), 50 mL (CICH$_3$)$_2$; 3.23 (0.42 g, 2.04 mmol, 1 eq.), 50 mL of benzene, 4 drops of piperidine. The product is a yellow solid (0.150 g, 0.384 mmol, 19%), m.p. = 158 °C.

$^1$H NMR 300 MHz (CDCl$_3$): δ = 7.91 (s, 1H), 7.77 (s, 1H), 7.47(AB,s, 2H), 7.36 (t, J=8.1 Hz, 1H), 7.26 (d, J=9.6 Hz, 1H), 7.20 (t, J=2.2 Hz, 1H), 6.95 (dd, J=8.1, 1.5 Hz, 1H), 4.00 (t, J=6.6 Hz, 2H), 3.00 (t, J=7.4 Hz, 2H), 2.65 (t, J=7.4 Hz, 2H), 1.81 (qn, J=6.6 Hz, 2H), 1.48 (qn, J=6.6 Hz, 2H), 1.34 (m, 4H), 0.90 (t, J=6.6 Hz, 3H). Refer to Figure A-14.
$^{13}$C NMR 75.47 MHz (CDCl$_3$): $\delta = 188.3, 159.5, 145.2, 135.7, 135.6, 135.3, 134.1, 131.0, 130.5, 130.0, 126.4, 124.3, 123.6, 118.6, 117.1, 116.1, 68.2, 31.6, 30.9, 29.1, 25.7, 22.6, 19.2, 14.0. Refer to Figure A-14.

Analysis: calculated for C$_{24}$H$_{23}$O$_2$NS: (%): C, 73.63; H, 6.44; N, 3.56. Found C, 73.45; H, 6.50; N, 3.60.

3.85 [(4-(2-Cyanoethyl)phenyl)thio] acetic acid

The general procedure for ester hydrolysis was followed. The amounts of reagents used: 3.76 (1.10 g, 4.39 mmol) and NaOH (0.21 g, 5.38 mmol, 1.2 eq.) in 50 mL of MeOH, was refluxed for 3 h. After acidifying the aqueous salt solution, the product was extracted with CH$_2$Cl$_2$ (25 mL x 2), dried and concentrated to give a white solid (0.820 g, 3.71 mmol, 84%), m.p. = 77-78 °C.

$^1$H NMR 300 MHz (CDCl$_3$): $\delta = 10.5$ (br s, 1H), 7.27 (m, 3H), 7.09 (d, J=6.6 Hz, 1H), 3.66 (s, 2H), 2.90 (t, J=7.4 Hz, 2H), 2.59 (t, J=7.4 Hz, 2H). Refer to Figure A-14.

$^{13}$C NMR 75.47 MHz (CDCl$_3$): $\delta = 175.3, 139.0, 135.2, 129.6, 129.5, 128.5, 127.1, 118.8, 36.2, 31.2, 19.1$. Refer to Figure A-14.

Cl MS m/z (relative intensity): Analysis: calculated for C$_{9}$H$_{11}$O$_2$NS: (%): C, 59.71; H, 5.02; N, 6.33. Found C, 59.72; H, 5.05; N, 6.07.
3.88 2-(4-Hexyloxyphenylmethylen)-6-(2-cyanoethyl)benzo[b]thiophen-3(2H)-one

The preparation procedure is similar to that of 3.81. The amounts of reagents used: 3.85 (0.820 g, 3.71 mmol), excess SOCl₂; AlCl₃ (0.62 g, 4.7 mmol, 1.3 eq.), 60 mL (CH₂Cl₂); 3.20 (0.280 g, 1.20 mmol, 0.92 eq.), 50 mL of benzene, 4 drops of piperidine. 3.88 elutes first on silica column (3:1, CHCl₃/hexanes), followed closely by 3.89. Both are yellow solids. 3.88 (0.240 g, 0.614 mmol), m.p. = 98-99 °C, and 3.89 (0.252 g, 0.644 mmol). Overall yield: 34%.

¹H NMR 300 MHz (CDCl₃): δ = 7.86 (s, 1H), 7.82 (d, J=8.1 Hz, 1H), 7.59 (d, J=8.8 Hz, 2H), 7.32 (s, 1H), 7.08 (d, J=8.1 Hz, 1H), 6.94 (d, J=8.8 Hz, 2H), 3.96 (t, J=6.6 Hz, 2H), 2.97 (t, J=7.4 Hz, 2H), 2.65 (t, J=6.6 Hz, 2H), 1.76 (qn, J=6.6 Hz, 2H), 1.43 (qn, J=6.6 Hz, 2H), 1.32 (m, 4H), 0.88 (t, J=6.6 Hz, 3H). Refer to Figure 3-27.

¹³C NMR 75.47 MHz (CDCl₃): δ = 187.7, 160.9, 146.7, 145.5, 133.9, 132.9, 129.9, 127.4, 127.1, 126.4, 125.7, 123.4, 118.5, 115.0, 68.1, 31.6, 31.4, 29.0, 25.6, 22.5, 18.7, 13.9. Refer to Figure 3-28.

+LSIMS (mNBA as matrix) m/z (relative intensity): 392 ([M+H⁺]. 100). Analysis: calculated for C₂₂H₂₂O₂NS (%): C, 73.63; H, 6.44; N, 3.58. Found C, 73.44; H, 6.56; N, 3.61.
3.89 2-(4-Hexyloxyphenylmethylene)-4-(2-cyanoethyl)benzo[b]thiophen-3(2H)-one

See 3.88 for procedure and yield.

$^1$H NMR 300 MHz (CDCl$_3$): $\delta = 7.81$ (s, 1H), 7.60 (d, $J=8.8$ Hz, 2H), 7.42 (m, 2H), 7.09 (d, $J=7.4$ Hz, 1H), 6.95 (d, $J=8.8$ Hz, 2H), 3.98 (t, $J=6.6$ Hz, 2H), 3.40 (t, $J=7.4$ Hz, 2H), 2.75 (t, $J=6.6$ Hz, 2H), 1.78 (qn, $J=6.6$ Hz, 2H), 1.45 (qn, $J=6.6$ Hz, 2H), 1.33 (m, 4H), 0.90 (t, $J=6.6$ Hz, 3H). Refer to Figure 3-27.

$^{13}$C NMR 75.47 MHz (CDCl$_3$): $\delta = 189.0, 160.9, 147.6, 141.0, 134.4, 133.9, 132.9, 127.7, 127.4, 127.2, 126.5, 123.3, 119.2, 115.0, 68.2, 31.5, 29.0, 28.3, 25.6, 22.5, 18.0, 14.0$. Refer to Figure 3-28.

+LSIMS (mNBA as matrix) $m/z$ (relative intensity): 392 ([M+1]$^+$, 100). Analysis: calculated for C$_{24}$H$_{20}$O$_2$NS: (%) C, 73.63; H, 6.44; N, 3.58. Found C, 73.29; H, 6.48; N, 3.54.

3.90 2-(4-Hexyloxyphenylmethylene)-5-(2-carboxyethyl)benzo[b]thiophen-3(2H)-one

![Chemical structure image]
The general procedure for nitrile hydrolysis was followed. The amounts of reagents used: 3.82 (129 mg, 0.323 mmol) and tetrafluorophthalic acid (81 mg, 0.323 mmol, 1 eq.). The product was a yellow solid (119 mg, 0.290 mmol, 90%), m.p. = 109 °C.

$^1$H NMR 300 MHz (CDCl$_3$): $\delta$ = 7.91 (s, 1H), 7.77 (s, 1H), 7.63 (d, $J$=8.8 Hz, 2H), 7.41 (AB, s, 2H), 6.96 (d, $J$=8.8 Hz, 2H), 4.00 (t, $J$=6.6 Hz, 2H), 2.99 (t, $J$=7.4 Hz, 2H), 2.70 (t, $J$=7.4 Hz, 2H), 1.79 (qdn, $J$=6.6 Hz, 2H), 1.45 (m, 2H). 1.33 (m, 4H), 0.89 (t, $J$=6.6 Hz, 3H). Refer to Figure 3-30.

$^{13}$C NMR 75.47 MHz (CDCl$_3$): $\delta$ = 188.5, 176.9, 161.0, 144.1, 137.8, 135.5, 134.0, 133.1, 131.1, 127.9, 126.7, 126.3, 123.9, 115.1, 68.3, 35.0, 31.5, 29.9, 29.1, 25.6, 22.6, 14.0. Refer to Figure 3-30.


3.92. 2-(4-OctyloxyphenylmethyIene)-5-(2-carboxyethyl)benzo[b]thiophen-3(2H)-one

The general procedure for nitrile hydrolysis was followed. The amounts of reagents used: 3.83 (55 mg, 0.13 mmol) and tetrafluorophthalic acid (32 mg, 0.13 mmol, 1 eq.).
eq.) heated at 180°C for 5 days. Product was a yellow solid (40 mg, 9.3 x 10^{-2} mmol, 71%), complex melting.

\(^1\)H NMR 300 MHz (CDCl\(_3\)): \(\delta = 7.91 (s, 1H), 7.76 (s, 1H), 7.62 (d, J=8.8 \text{ Hz}, 2H), 7.40 (AB, s, 2H), 6.95 (d, J=8.8 \text{ Hz}, 2H), 3.99 (t, J=6.6 \text{ Hz}, 2H), 2.97 (t, J=7.4 \text{ Hz}, 2H), 2.70 (t, J=7.4 \text{ Hz}, 2H), 1.78 (qn, J=6.6 \text{ Hz}, 2H), 1.44 (m, 2H), 1.28 (m, 8H), 0.87 (t, J=6.6 \text{ Hz}, 2H). Refer to Figure A-15.

\(^{13}\)C NMR 75.47 MHz (CDCl\(_3\)): \(\delta = 188.5, 177.9, 161.0, 144.1, 137.9, 135.5, 134.0, 133.1, 127.9, 126.7, 126.3, 123.9, 115.1, 68.3, 35.2, 31.8, 29.9, 29.3, 29.2, 29.1, 26.0, 22.6, 14.1. Refer to Figure A-15.

+LSIMS (mNBA as matrix) m/z (relative intensity): 439 ([M+1]^+, 100). Analysis: calculated for C\(_{26}\)H\(_{30}\)O\(_4\)S: (%) C, 71.20; H, 6.89; O, 14.59; S, 7.31. Found C, 70.97; H, 7.09; O, 12.73; S, 8.08.

3.93 2-(3-HexyloxyphenylmethyIene)-5-(2-carboxyethyl)benzo[b]thiophen-3(2H)-one

The general procedure for nitrile hydrolysis was followed. The amounts of reagents used: 3.84 (105 mg, 0.263 mmol) and tetrafluorophthalic acid (66 mg, 0.263 mmol, 1 eq.) heated at 160°C for 3 days. Product was a yellow solid (95 mg, 0.232 mmol, 88%), m.p. = 116-117°C.
$^1$H NMR 300 MHz (CDCl$_3$): $\delta$ = 7.90 (s, 1H), 7.77 (s, 1H), 7.47, 7.38 (AB, $J$=8.5 Hz, 2H), 7.33 (t, $J$=8.1 Hz, 1H), 7.26 (d, $J$=8.1 Hz, 1H), 7.20 (s, 1H), 6.95 (d, $J$=8.1 Hz, 2H), 4.00 (t, $J$=6.6 Hz, 2H), 3.00 (t, $J$=7.4 Hz, 2H), 2.71 (t, $J$=7.4 Hz, 2H), 1.80 (qn, $J$=6.6 Hz, 2H), 1.47 (m, 2H), 1.35 (m, 4H). 0.91 (t, $J$=6.6 Hz, 3H). Refer to Figure A-16.

$^{13}$C NMR 75.47 MHz (CDCl$_3$): $\delta$ = 188.6, 178.0, 159.5, 144.2, 138.0, 135.9, 135.4, 133.9, 130.7, 129.9, 126.5, 123.9, 123.5, 117.0, 116.1, 68.2, 35.1, 31.6, 29.8, 29.1, 25.7, 22.6, 14.0. Refer to Figure A-16.

+LSIMS (mNBA as matrix) m/z (relative intensity): 411 ([M+1]$^+$, 100). Analysis: calculated for $C_{29}H_{28}O_4S$: (%) C, 70.22; H, 6.38. Found C, 70.10; H, 6.46.

$^{3.94}$ 2-(4-Hexyloxyphenylmethylene)-6-(2-carboxyethyl)benzo[b]thiophen-3(2H)-one

![Chemical Structure](image)

The general procedure for nitrile hydrolysis was followed. The amounts of reagents used: 3.88 (184 mg, 0.469 mmol) and tetrafluoro-phthalic acid (112 mg, 0.469 mmol, 1 eq.) heated at 140°C for 4 days. Product was a yellow solid (166 mg, 0.405 mmol, 86%), m.p. = 134-135 °C.

$^1$H NMR 300 MHz (CDCl$_3$): $\delta$ = 7.90 (s, 1H), 7.84 (d, $J$=8.1 Hz, 1H), 7.63 (d, $J$=8.8 Hz, 2H), 7.33 (s, 1H), 7.12 (d, $J$=8.1 Hz, 1H), 6.97 (d, $J$=8.8 Hz, 2H), 4.00 (t, $J$=6.6 Hz, 2H).
Hz, 2H), 3.03 (t, J=7.4 Hz, 2H), 2.73 (t, J=7.4 Hz, 2H), 1.79 (qn, J=6.6 Hz, 2H), 1.45 (qn, J=6.6 Hz, 2H), 1.33 (m, 4H), 0.89 (t, J=6.6 Hz, 3H). Refer to Figure A-17.

$^{13}$C NMR 75.47 MHz (CDCl$_3$): $\delta$ = 188.2, 177.4, 160.9, 148.3, 146.6, 133.9, 133.0, 129.4, 127.7, 127.1, 126.7, 126.0, 123.5, 115.1, 68.2, 34.7, 31.5, 30.8, 29.1, 25.6, 22.6, 14.0. Refer to Figure A-17.

Analysis: calculated for C$_{34}$H$_{36}$O$_4$S: (%) C, 70.22; H, 6.38. Found C, 69.76; H, 6.66.

3.95 2-(4-Hexyloxyphenylmethylene)-4-(2-carboxyethyl)benzo[b]thiophen-3(2H)-one

The general procedure for nitrile hydrolysis was followed. The amounts of reagents used: 3.89 (193 mg, 0.492 mmol) and tetrafluorophthalic acid (118 mg, 0.492 mmol, 1 eq.) heated at 168°C for 4 days. Product was a yellow solid (168 mg, 0.410 mmol, 83%), m.p. = 142-144 °C.

$^1$H NMR 300 MHz (CDCl$_3$): $\delta$ = 7.87 (s, 1H), 7.63 (d, J=8.8 Hz, 2H), 7.41 (m, 2H), 7.09 (d, J=7.4 Hz, 1H), 6.97 (d, J=8.8 Hz, 2H), 4.01 (t, J=6.6 Hz, 2H), 3.44 (t, J=7.4 Hz, 2H), 2.73 (t, J=7.4 Hz, 2H), 1.77 (qn, J=6.6 Hz, 2H), 1.64 (qn, J=6.6 Hz), 1.34 (m, 4H), 0.90 (t, J=6.6 Hz, 3H). Refer to Figure A-18.
$^{13}$C NMR 75.47 MHz (CDCl$_3$): $\delta$ = 189.2, 178.1, 160.9, 147.4, 143.9, 134.3, 133.6, 133.0, 127.6, 127.5, 127.4, 126.8, 122.5, 115.1, 68.3, 34.5, 31.5, 29.1, 27.6, 25.7, 22.6, 14.0.

+LSIMS (mNBA as matrix) $m/z$ (relative intensity): 411 ([M+H]$^-$, 100), 393 ([M-H$_2$O]$^-$, 69). Refer to Figure A-18.

3.106 1,2 – Bis[2-(4-hexyloxyphenylmethylene)benzo[b]thiophene-3-(2H)-one-5-ylethanoyl] phosphatidyl choline

![Chemical Structure]

The general procedure for lipid synthesis was followed. The amounts of reagents used: 3.90 (119 mg, 0.290 mmol), (CH$_3$)$_3$CCOCll (0.30 mL, 3.0 mmol, 10 eq.), and NEt$_3$ (0.10 mL, 1.4 mmol, 5 eq.) in CH$_2$Cl$_2$ was stirred, under N$_2$, for 35 h. GPC-CdCl$_2$ (51.0 mg, 0.115 mmol, 0.4 eq and DMAP (29 mg, 0.230 mmol, 0.8 eq) in CH$_2$Cl$_2$ (10 mL) and stirred with mixed anhydride for 23 h. Product was a yellow solid (61 mg, 0.059 mmol, 85%), complex melting.

$^1$H NMR 360 MHz (CDCl$_3$): $\delta$ = 7.77 (s, 1H), 7.76 (s, 1H), 7.64 (s, 1H), 7.63 (s, 1H), 7.56 (d, $J$=3.6 Hz, 2H), 7.54 (d, $J$=2.8 Hz, 2H), 7.33 (m, AB, 2H), 7.31 (br s, AB, 2H), 6.91 (d, $J$=3.6 Hz, 2H), 6.89 (d, $J$=2.8 Hz, 2H), 5.19 (m, 1H), 4.36 (m, 3H), 4.11
(dd. J=12.1, 7.0 Hz, 1H), 3.96 (m, 6H), 3.86 (m, 2H), 3.38 (s, 9H), 2.86 (m, 4H), 2.61 (t, J=7.8 Hz, 2H), 2.54 (t, J=7.4 Hz, 2H), 1.75 (m, 4H), 1.41 (m, 4H), 1.32 (m, 8H), 0.89 (m, 6H). Refer to Figure 3-31.

$^{13}$C NMR 90.57 MHz (CDCl$_3$): $\delta$ =188.2, 172.2, 171.9, 160.8, 143.7, 138.01, 137.97, 135.5, 135.4, 133.55, 133.51, 132.9, 130.9, 130.7, 127.8, 127.7, 126.6, 126.5, 126.0, 123.0, 114.9, 70.7 (d, J=6.1 Hz), 68.1, 66.2 (d, J=4.8 Hz), 63.5, 63.1, 59.5, 54.3, 35.1, 34.5, 31.5, 29.8, 29.1, 25.6, 22.6, 14.0. Refer to Figure 3-32.

$^{31}$P NMR 145.63 MHz (CDCl$_3$): $\delta$ = -0.8.

+LSIMS (mNBA as matrix) $m/z$ (relative intensity): 1042.4 ([M+1]$^+$, 100). LSIMS: 1041.3 ([M-1]$^-$, 100). HRMS (+LSIMS) $C_{56}H_{89}NO_{12}S_2P$: calcld. 1042.3999, found 1042.3993.

$[\alpha]_D^0 = +16.6^\circ $cm$^2$g$^{-1}$ (c. 0.046, CHCl$_3$)

3.107 1,2 – Bis[2-(3-hexyloxyphenyl)methylene]benzo[b]thiophene-3-(2H)-one-5-ylethanoyl] phosphatidyl choline
The general procedure for lipid synthesis was followed. The amounts of reagents used: 3.93 (70 mg, 0.171 mmol), (CH$_3$)$_3$CCOCl (0.20 mL, 2.0 mmol, 10 eq.) and NEt$_3$ (0.06 mL, 0.8 mmol, 5 eq.) in 10 mL of CH$_2$Cl$_2$, was stirred for 38 h. GPC-CdCl$_2$ (30 mg, 0.068 mmol, 0.4 eq.) and DMAP (17 mg, 0.14 mmol, 0.8 eq) in 10 mL of CH$_2$Cl$_2$, was stirred with mixed anhydride (5 mL CH$_2$Cl$_2$) for 23 h. Product was a yellow solid (41 mg, 0.039 mmol, 91%), complex melting.

$^1$H NMR 360 MHz (CDCl$_3$): $\delta$ = 7.76 (s, 1H), 7.75 (s, 1H), 7.65 (s, 1H), 7.63 (s, 1H), 7.31 (m, 6H), 7.18 (m, 2H), 7.11 (s, 2H), 6.89 (m, 2H), 5.19 (m, 1H), 4.38 (d, $J$=12.0 Hz, 1H). 4.31 (m, 2H), 4.11 (dd, $J$=12.0, 7.1 Hz, 1H), 3.95 (m, 6H), 3.81 (m, 2H), 2.91 (s, 9H), 2.87 (m, 4H), 2.61 (t, $J$=7.3 Hz, 2H), 2.54 (t, $J$=7.6 Hz, 2H), 1.76 (m, 4H), 1.44 (m, 4H), 1.33 (m, 8H), 0.87 (m, 6H). Refer to Figure 3-33.

$^{13}$C NMR 90.57 MHz (CDCl$_3$): $\delta$ = 188.0, 171.8, 171.5, 159.1, 143.6, 137.89, 137.86, 135.6, 135.5, 135.00, 134.96, 133.24, 133.21, 130.29, 130.25, 130.18, 129.5, 125.9, 123.5, 123.1, 116.6, 115.7, 70.6 (d, $J$=6.1 Hz), 67.7, 66.1 (d, $J$=6.1 Hz), 62.9, 59.0, 54.1, 34.8, 34.6, 31.2, 29.5, 28.8, 25.4, 25.3, 22.2, 13.7. Refer to Figure 3-34.

$^{31}$P NMR 145.63 MHz (CDCl$_3$): $\delta$ = 1.3.

+LSIMS (mNBA as matrix) $m/z$ (relative intensity): 2084.6 ([dimer+1]$, 5)$, 1042.3 ([M+1]$^+$, 100). HRMS (+LSIMS) C$_{56}$H$_{60}$NO$_{12}$S$_2$P: calcd. 1042.3999, found 1042.3993.

$[\alpha]_D$ = +7.0°cm$^2$g$^{-1}$ (c. 0.028, CHCl$_3$)
3.108 1,2 – Bis[2-(4-hexyloxyphenylmethylene)benzo[b]thiophene-3-(2H)-one-6-ylethano|y|] phosphatidyl choline

The general procedure for lipid synthesis was followed. The amounts of reagents used: 3.94 (114 mg, 0.278 mmol), (CH$_3$)$_3$CCOCl (0.30 mL, 3.0 mmol, 10 eq.) and NEt$_3$ (0.10 mL, 1.4 mmol, 5 eq.) in 12 mL of CH$_2$Cl$_2$, was stirred for 42 h. GPC-CdCl$_2$ (50.8 mg, 0.114 mmol, 0.41 eq.) and DMAP (28.8 mg, 0.236 mmol, 0.85 eq) in 10 mL of CH$_2$Cl$_2$, stirred with mixed anhydride (5 mL CH$_2$Cl$_2$) for 26 h. Product was a yellow solid (64 mg, 0.061 mmol, 87%), complex melting.

$^1$H NMR 360 MHz (CDCl$_3$): $\delta =$ 7.722 (s, 1H), 7.715 (s, 1H), 7.69 (m, 2H), 7.48 (m, 4H), 7.21 (s, 1H), 7.17 (s, 1H), 7.02 (d, J=8.1 Hz, 1H), 6.96 (d, J=8.1 Hz, 1H), 6.85 (m, 4H), 5.16 (m, 1H), 4.36 (d, J=9.8 Hz, 2H), 4.24 (m, 2H), 4.08 (m, 3H), 3.88 (q, J=6.6 Hz, 4H), 3.75 (m, 2H), 3.31 (s, 9H), 2.85 (m, 4H), 2.60 (m, 4H), 2.53 (t, J=7.6 Hz, 4H), 1.71 (m, 4H), 1.38 (m, 4H), 1.29 (m, 8H), 0.86 (t, J=6.6 Hz, 3H). Refer to Figure 3-35.

$^{13}$C NMR 90.57 MHz (CDCl$_3$): $\delta =$ 187.71, 187.61, 172.0, 171.7, 160.8, 148.4, 148.3, 146.3, 146.2, 133.55, 133.47, 132.8, 129.0, 127.5, 127.4, 126.7, 126.46, 126.40, 125.8, 125.7, 123.36, 123.26, 114.9, 71.9 (d, J=7.4 Hz), 68.1, 66.1 (d, J=6.1 Hz), 63.2,
59.2 (d, J=4.9 Hz), 54.2, 34.7, 34.5, 31.5, 30.82, 30.76, 29.0, 25.5, 22.5, 13.9. Refer to Figure 3-36.

$^{31}$P NMR 145.63 MHz (CDCl$_3$): δ = 1.2.

+LSIMS (mNBA as matrix) m/z (relative intensity): 2084.6 ([dimer+1]$^+$, 10), 1042.3 ([M+1]$^+$, 100). HRMS (+LSIMS) C$_{56}$H$_{69}$NO$_{12}$S$_2$P: calcd. 1042.3999, found 1042.4005.

$[\alpha]_D^1 = +7.3^\circ$ cm$^{-1}$ g$^{-1}$ (c. 0.023, CHCl$_3$)

3.109 1,2 - Bis[2-(4-hexyloxyphenylmethylene)benzo[b]thiophene-3-(2H)-one-4-ylethanoyl] phosphatidyl choline

The general procedure for lipid synthesis was followed. The amounts of reagents used: 3.95 (124 mg, 0.302 mmol), (CH$_3$)$_3$CCOCl (0.31 mL, 3.1 mmol, 10 eq.) and NEt$_3$ (0.10 mL, 1.4 mmol, 5 eq.) in 12 mL of CH$_2$Cl$_2$, was stirred for 42 h. GPC-CdCl$_3$ (55.7 mg, 0.125 mmol, 0.41 eq.) and DMAP (33.9 mg, 0.278 mmol, 0.92 eq) in 10 mL of CH$_2$Cl$_2$, stirred with mixed anhydride (5 mL CH$_2$Cl$_2$) for 26 h. Product was a yellow solid (73 mg, 0.070 mmol, 92%), complex melting.

$^1$H NMR 360 MHz (CDCl$_3$): δ = 7.65 (s, 1H), 7.61 (s, 1H), 7.48 (m, 4H), 7.24 (m, 4H), 6.97 (m, 2H), 6.85 (m, 4H), 5.22 (m, 1H), 4.33 (m, 3H), 4.12 (dd, J=12.0, 7.1 Hz.
1H), 3.91 (m, 6H), 3.83 (m, 2H), 3.37 (s, 9H), 3.30 (m, 4H), 2.61 (m, 4H), 1.73 (m, 4H), 1.41 (m, 4H), 1.31 (m, 8H), 0.87 (m, 6H). Refer to Figure 3-37.

$^1$C NMR 90.57 MHz (CDCl$_3$): $\delta =$ 188.7, 172.6, 172.3, 160.6, 147.0, 143.9, 143.8, 134.2, 133.0, 132.8, 127.4, 127.32, 127.28, 127.22, 127.17, 126.73, 126.67, 122.2, 114.9, 70.8 (d, J=7.3 Hz), 68.1, 66.3 (d, J=6.1 Hz), 63.3, 63.0, 59.3, 54.4, 34.4, 34.3, 31.5, 29.1, 27.4, 25.6, 22.6, 14.0. Refer to Figure 3-38.

$^{31}$P NMR 145.63 MHz (CDCl$_3$): $\delta =$ -0.8.

+ LSIMS (mNBA as matrix) $m/z$ (relative intensity): 2084.6 ([dimer+1]$^+$, 15), 1042.3 ([M+1]$^+$, 100). HRMS (+LSIMS) C$_{56}$H$_{60}$NO$_{12}$S$_2$P: calcd. 1042.3999, found 1042.3999.

$[\alpha]_D = +25.8^\circ$cm$^2$g$^{-1}$ (c. 0.0433, CHCl$_3$)
Appendix

Figure 4-3: Plan Graph of Path III

Table A-1: Yields of Path III

<table>
<thead>
<tr>
<th>i</th>
<th>y</th>
<th>x</th>
<th>S_i</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.85</td>
<td>1.176</td>
<td>1.18</td>
</tr>
<tr>
<td>2</td>
<td>0.85</td>
<td>1.176</td>
<td>2.35</td>
</tr>
<tr>
<td>3</td>
<td>0.765</td>
<td>1.307</td>
<td>3.66</td>
</tr>
<tr>
<td>4</td>
<td>0.45</td>
<td>2.222</td>
<td>5.88</td>
</tr>
<tr>
<td>5</td>
<td>0.36</td>
<td>2.778</td>
<td>8.66</td>
</tr>
<tr>
<td>6</td>
<td>0.148</td>
<td>6.757</td>
<td>15.42</td>
</tr>
<tr>
<td>7</td>
<td>0.148</td>
<td>6.757</td>
<td>22.17</td>
</tr>
<tr>
<td>8</td>
<td>0.133</td>
<td>7.519</td>
<td>29.69</td>
</tr>
<tr>
<td>9</td>
<td>0.0863</td>
<td>11.58</td>
<td>41.28</td>
</tr>
<tr>
<td>10</td>
<td>0.0673</td>
<td>14.86</td>
<td>56.14</td>
</tr>
<tr>
<td>11</td>
<td>0.0612</td>
<td>16.34</td>
<td>72.48</td>
</tr>
</tbody>
</table>

Table A-2: Weight Summaries of Path III

<table>
<thead>
<tr>
<th>i</th>
<th>n</th>
<th>l</th>
<th>x</th>
<th>nx</th>
<th>S_i</th>
<th>nS_i</th>
<th>l'</th>
<th>S_T</th>
<th>S-S_T</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>7</td>
<td>11</td>
<td>16.34</td>
<td>114.4</td>
<td>72.48</td>
<td>507.4</td>
<td>0</td>
<td>0.00</td>
<td>72.48</td>
</tr>
<tr>
<td>2</td>
<td>3</td>
<td>11</td>
<td>16.34</td>
<td>49.0</td>
<td>72.48</td>
<td>217.4</td>
<td>11</td>
<td>72.48</td>
<td>0.00</td>
</tr>
<tr>
<td>3</td>
<td>7</td>
<td>11</td>
<td>16.34</td>
<td>114.4</td>
<td>72.48</td>
<td>507.4</td>
<td>1</td>
<td>1.18</td>
<td>71.30</td>
</tr>
<tr>
<td>4</td>
<td>3</td>
<td>11</td>
<td>16.34</td>
<td>49.0</td>
<td>72.48</td>
<td>217.4</td>
<td>11</td>
<td>72.48</td>
<td>0.00</td>
</tr>
<tr>
<td>5</td>
<td>4</td>
<td>10</td>
<td>14.86</td>
<td>59.4</td>
<td>56.14</td>
<td>224.6</td>
<td>10</td>
<td>56.14</td>
<td>0.00</td>
</tr>
<tr>
<td>6</td>
<td>4</td>
<td>10</td>
<td>14.86</td>
<td>59.4</td>
<td>56.14</td>
<td>224.6</td>
<td>10</td>
<td>56.14</td>
<td>0.00</td>
</tr>
<tr>
<td>7</td>
<td>8</td>
<td>5</td>
<td>2.78</td>
<td>22.2</td>
<td>8.66</td>
<td>69.3</td>
<td>5</td>
<td>8.66</td>
<td>0.00</td>
</tr>
<tr>
<td>8</td>
<td>6</td>
<td>4</td>
<td>2.22</td>
<td>13.3</td>
<td>5.88</td>
<td>35.3</td>
<td>4</td>
<td>5.88</td>
<td>0.00</td>
</tr>
<tr>
<td>9</td>
<td>8</td>
<td>5</td>
<td>2.78</td>
<td>22.2</td>
<td>8.66</td>
<td>69.3</td>
<td>5</td>
<td>8.66</td>
<td>0.00</td>
</tr>
<tr>
<td>10</td>
<td>6</td>
<td>4</td>
<td>2.22</td>
<td>13.3</td>
<td>5.88</td>
<td>35.3</td>
<td>4</td>
<td>5.88</td>
<td>0.00</td>
</tr>
<tr>
<td>11</td>
<td>16</td>
<td>1</td>
<td>1.18</td>
<td>18.8</td>
<td>1.18</td>
<td>18.9</td>
<td>1</td>
<td>1.18</td>
<td>0.00</td>
</tr>
</tbody>
</table>

n_o = 72
W = 536
TW = 2127
R = 143.8
Figure 4-4: Plan Graph of Path I-P

Table A-3: Yields of Path I-P

<table>
<thead>
<tr>
<th>l</th>
<th>y</th>
<th>x</th>
<th>S1</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.85</td>
<td>1.176</td>
<td>1.18</td>
</tr>
<tr>
<td>2</td>
<td>0.85</td>
<td>1.176</td>
<td>2.35</td>
</tr>
<tr>
<td>3</td>
<td>0.765</td>
<td>1.307</td>
<td>3.66</td>
</tr>
<tr>
<td>4</td>
<td>0.612</td>
<td>1.634</td>
<td>5.29</td>
</tr>
<tr>
<td>5</td>
<td>0.252</td>
<td>3.968</td>
<td>9.26</td>
</tr>
<tr>
<td>5'</td>
<td>0.392</td>
<td>2.551</td>
<td>7.85</td>
</tr>
<tr>
<td>6</td>
<td>0.252</td>
<td>3.968</td>
<td>13.23</td>
</tr>
<tr>
<td>7</td>
<td>0.227</td>
<td>4.405</td>
<td>17.64</td>
</tr>
<tr>
<td>8</td>
<td>0.148</td>
<td>6.757</td>
<td>24.39</td>
</tr>
<tr>
<td>9</td>
<td>0.115</td>
<td>8.696</td>
<td>33.09</td>
</tr>
<tr>
<td>10</td>
<td>0.104</td>
<td>9.615</td>
<td>42.70</td>
</tr>
<tr>
<td>10'</td>
<td>0.0920</td>
<td>10.87</td>
<td>43.96</td>
</tr>
<tr>
<td>11</td>
<td>0.0832</td>
<td>12.02</td>
<td>54.72</td>
</tr>
</tbody>
</table>

Table A-4: Weight Summaries of Path I-P

<table>
<thead>
<tr>
<th>i</th>
<th>n</th>
<th>l</th>
<th>x</th>
<th>nx</th>
<th>S1</th>
<th>nS1</th>
<th>l'</th>
<th>ST</th>
<th>S-S1</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3</td>
<td>11</td>
<td>12.02</td>
<td>36.06</td>
<td>54.72</td>
<td>164.2</td>
<td>0.00</td>
<td>54.72</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>7</td>
<td>10</td>
<td>9.615</td>
<td>67.31</td>
<td>42.70</td>
<td>298.9</td>
<td>10.00</td>
<td>42.70</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>11</td>
<td>12.02</td>
<td>36.06</td>
<td>54.72</td>
<td>164.2</td>
<td>1.18</td>
<td>53.54</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>7</td>
<td>10</td>
<td>9.615</td>
<td>67.31</td>
<td>42.70</td>
<td>298.9</td>
<td>10.00</td>
<td>42.70</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>4</td>
<td>10</td>
<td>9.615</td>
<td>38.46</td>
<td>43.96</td>
<td>175.8</td>
<td>9.00</td>
<td>33.09</td>
<td>10.87</td>
</tr>
<tr>
<td>6</td>
<td>4</td>
<td>10</td>
<td>9.615</td>
<td>38.46</td>
<td>43.96</td>
<td>175.8</td>
<td>9.00</td>
<td>33.09</td>
<td>10.87</td>
</tr>
<tr>
<td>7</td>
<td>8</td>
<td>5</td>
<td>2.551</td>
<td>20.41</td>
<td>7.85</td>
<td>62.08</td>
<td>4.00</td>
<td>5.29</td>
<td>2.55</td>
</tr>
<tr>
<td>8</td>
<td>6</td>
<td>5</td>
<td>2.551</td>
<td>15.31</td>
<td>7.85</td>
<td>47.14</td>
<td>4.00</td>
<td>5.29</td>
<td>2.55</td>
</tr>
<tr>
<td>9</td>
<td>8</td>
<td>5</td>
<td>2.551</td>
<td>20.41</td>
<td>7.85</td>
<td>62.08</td>
<td>4.00</td>
<td>5.29</td>
<td>2.55</td>
</tr>
<tr>
<td>10</td>
<td>6</td>
<td>5</td>
<td>2.551</td>
<td>15.31</td>
<td>7.85</td>
<td>47.14</td>
<td>4.00</td>
<td>5.29</td>
<td>2.55</td>
</tr>
<tr>
<td>11</td>
<td>16</td>
<td>1</td>
<td>1.176</td>
<td>18.82</td>
<td>1.18</td>
<td>18.90</td>
<td>1.18</td>
<td>0.00</td>
<td></td>
</tr>
</tbody>
</table>

n = W 7W 1516 140.2
Figure 4-5: Plan Graph of Path II

Table A-5: Yields of Path II

<table>
<thead>
<tr>
<th>i</th>
<th>y</th>
<th>x</th>
<th>S_i</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.85</td>
<td>1.176</td>
<td>1.18</td>
</tr>
<tr>
<td>2</td>
<td>0.85</td>
<td>1.176</td>
<td>2.35</td>
</tr>
<tr>
<td>3</td>
<td>0.68</td>
<td>1.471</td>
<td>3.66</td>
</tr>
<tr>
<td>4</td>
<td>0.442</td>
<td>2.262</td>
<td>6.27</td>
</tr>
<tr>
<td>5</td>
<td>0.345</td>
<td>2.899</td>
<td>9.17</td>
</tr>
<tr>
<td>6</td>
<td>0.276</td>
<td>3.623</td>
<td>12.79</td>
</tr>
<tr>
<td>7</td>
<td>0.221</td>
<td>4.525</td>
<td>17.32</td>
</tr>
<tr>
<td>8</td>
<td>0.203</td>
<td>4.926</td>
<td>22.24</td>
</tr>
<tr>
<td>9</td>
<td>0.141</td>
<td>7.092</td>
<td>24.41</td>
</tr>
<tr>
<td>10</td>
<td>0.203</td>
<td>4.926</td>
<td>27.17</td>
</tr>
<tr>
<td>11</td>
<td>0.183</td>
<td>5.464</td>
<td>32.63</td>
</tr>
<tr>
<td>12</td>
<td>0.167</td>
<td>5.988</td>
<td>38.62</td>
</tr>
</tbody>
</table>

Table A-6: Weight Summaries of Path II

<table>
<thead>
<tr>
<th>i</th>
<th>n</th>
<th>l</th>
<th>x</th>
<th>nx</th>
<th>S_i</th>
<th>nS_i</th>
<th>l'</th>
<th>S_i</th>
<th>S-S_i</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>7</td>
<td>11</td>
<td>5.988</td>
<td>41.92</td>
<td>38.62</td>
<td>270.4</td>
<td>0</td>
<td>0</td>
<td>38.62</td>
</tr>
<tr>
<td>2</td>
<td>3</td>
<td>11</td>
<td>5.988</td>
<td>17.96</td>
<td>38.62</td>
<td>115.9</td>
<td>11</td>
<td>11</td>
<td>0.00</td>
</tr>
<tr>
<td>3</td>
<td>7</td>
<td>11</td>
<td>5.988</td>
<td>41.92</td>
<td>38.62</td>
<td>270.4</td>
<td>1</td>
<td>1.18</td>
<td>37.44</td>
</tr>
<tr>
<td>4</td>
<td>3</td>
<td>11</td>
<td>5.988</td>
<td>17.96</td>
<td>38.62</td>
<td>115.9</td>
<td>11</td>
<td>11</td>
<td>0.00</td>
</tr>
<tr>
<td>5</td>
<td>8</td>
<td>8</td>
<td>7.092</td>
<td>56.74</td>
<td>24.41</td>
<td>195.3</td>
<td>7</td>
<td>17.32</td>
<td>7.09</td>
</tr>
<tr>
<td>6</td>
<td>6</td>
<td>8</td>
<td>7.092</td>
<td>42.55</td>
<td>24.41</td>
<td>146.5</td>
<td>7</td>
<td>17.32</td>
<td>7.09</td>
</tr>
<tr>
<td>7</td>
<td>8</td>
<td>8</td>
<td>7.092</td>
<td>56.74</td>
<td>24.41</td>
<td>195.3</td>
<td>7</td>
<td>17.32</td>
<td>7.09</td>
</tr>
<tr>
<td>8</td>
<td>6</td>
<td>8</td>
<td>7.092</td>
<td>42.55</td>
<td>24.41</td>
<td>146.5</td>
<td>7</td>
<td>17.32</td>
<td>7.09</td>
</tr>
<tr>
<td>9</td>
<td>4</td>
<td>5</td>
<td>2.899</td>
<td>11.59</td>
<td>9.17</td>
<td>36.7</td>
<td>5</td>
<td>9.17</td>
<td>0.00</td>
</tr>
<tr>
<td>10</td>
<td>4</td>
<td>5</td>
<td>2.899</td>
<td>11.59</td>
<td>9.17</td>
<td>36.7</td>
<td>5</td>
<td>9.17</td>
<td>0.00</td>
</tr>
<tr>
<td>11</td>
<td>16</td>
<td>1</td>
<td>1.176</td>
<td>18.82</td>
<td>1.18</td>
<td>18.9</td>
<td>1</td>
<td>1.18</td>
<td>0.00</td>
</tr>
</tbody>
</table>

n_p \ W | TW | K
72  | 360 | 1548 | 104.4
### Table A-7: Weight Summaries of Path I with Real Molecular Masses

<table>
<thead>
<tr>
<th>i</th>
<th>n</th>
<th>Mw</th>
<th>x</th>
<th>Fnx</th>
<th>S₁</th>
<th>FnS₁</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>7</td>
<td>189</td>
<td>27.0</td>
<td>9.615</td>
<td>1817</td>
<td>42.70</td>
</tr>
<tr>
<td>2</td>
<td>3</td>
<td>167</td>
<td>55.7</td>
<td>9.615</td>
<td>1606</td>
<td>42.70</td>
</tr>
<tr>
<td>3</td>
<td>7</td>
<td>189</td>
<td>27.0</td>
<td>9.615</td>
<td>1817</td>
<td>42.70</td>
</tr>
<tr>
<td>4</td>
<td>3</td>
<td>167</td>
<td>55.7</td>
<td>9.615</td>
<td>1606</td>
<td>42.70</td>
</tr>
<tr>
<td>5</td>
<td>4</td>
<td>53</td>
<td>13.3</td>
<td>8.696</td>
<td>461</td>
<td>33.09</td>
</tr>
<tr>
<td>6</td>
<td>4</td>
<td>53</td>
<td>13.3</td>
<td>8.696</td>
<td>461</td>
<td>33.09</td>
</tr>
<tr>
<td>7</td>
<td>8</td>
<td>122</td>
<td>15.3</td>
<td>2.551</td>
<td>311</td>
<td>7.85</td>
</tr>
<tr>
<td>8</td>
<td>6</td>
<td>165</td>
<td>27.5</td>
<td>2.551</td>
<td>421</td>
<td>7.85</td>
</tr>
<tr>
<td>9</td>
<td>8</td>
<td>122</td>
<td>15.3</td>
<td>2.551</td>
<td>311</td>
<td>7.85</td>
</tr>
<tr>
<td>10</td>
<td>6</td>
<td>165</td>
<td>27.5</td>
<td>2.551</td>
<td>421</td>
<td>7.85</td>
</tr>
<tr>
<td>11</td>
<td>16</td>
<td>257</td>
<td>16.1</td>
<td>1.176</td>
<td>302</td>
<td>1.18</td>
</tr>
</tbody>
</table>

\[(\Sigma F)/11 = W = TW = 26.7 = 9534 = 38719\]

### Table A-8: Weight Summaries of Path I-P with Real Molecular Masses

<table>
<thead>
<tr>
<th>i</th>
<th>n</th>
<th>Mw</th>
<th>x</th>
<th>Fnx</th>
<th>S₁</th>
<th>FnS₁</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>7</td>
<td>11</td>
<td>189</td>
<td>27.0</td>
<td>9.615</td>
<td>1817</td>
</tr>
<tr>
<td>2</td>
<td>3</td>
<td>10</td>
<td>298</td>
<td>99.3</td>
<td>12.020</td>
<td>3582</td>
</tr>
<tr>
<td>3</td>
<td>7</td>
<td>11</td>
<td>189</td>
<td>27.0</td>
<td>9.615</td>
<td>1817</td>
</tr>
<tr>
<td>4</td>
<td>3</td>
<td>10</td>
<td>298</td>
<td>99.3</td>
<td>12.020</td>
<td>3582</td>
</tr>
<tr>
<td>5</td>
<td>4</td>
<td>10</td>
<td>231</td>
<td>57.8</td>
<td>9.615</td>
<td>2221</td>
</tr>
<tr>
<td>6</td>
<td>4</td>
<td>10</td>
<td>231</td>
<td>57.8</td>
<td>9.615</td>
<td>2221</td>
</tr>
<tr>
<td>7</td>
<td>8</td>
<td>5</td>
<td>122</td>
<td>15.3</td>
<td>2.551</td>
<td>311</td>
</tr>
<tr>
<td>8</td>
<td>6</td>
<td>5</td>
<td>165</td>
<td>27.5</td>
<td>2.551</td>
<td>421</td>
</tr>
<tr>
<td>9</td>
<td>8</td>
<td>5</td>
<td>122</td>
<td>15.3</td>
<td>2.551</td>
<td>311</td>
</tr>
<tr>
<td>10</td>
<td>6</td>
<td>5</td>
<td>165</td>
<td>27.5</td>
<td>2.551</td>
<td>421</td>
</tr>
<tr>
<td>11</td>
<td>16</td>
<td>1</td>
<td>257</td>
<td>16.1</td>
<td>1.176</td>
<td>302</td>
</tr>
</tbody>
</table>

\[(\Sigma F)/11 = W = TW = \Delta TW = 42.7 = 17007 = 61687 = 56218\]
Figure A-1  Assigned $^1$H NMR Spectra of (a) 3.20 and (b) 3.21
Figure A-2  Assigned $^{13}$C NMR Spectra of (a) 3.20 and (b) 3.21
Figure A.3  Assigned NMR Spectra of 3.38, (a) $^1$H and (b) $^{13}$C.
Figure A-4  Assigned NMR Spectra of 3.43, (a) $^1H$ and (b) $^{13}C$. 
Figure A-6  Assigned NMR Spectra of Acid Chloride of 3,43, (a) $^1$H and (b) $^{13}$C
Figure A-7. NMR Spectra of 3.5. (a) $^1$H and (b) $^{13}$C.
Figure A-8: Assigned NMR Spectra of 3.5I. (a) $^1$H and (b) $^{13}$C.
Figure A.1. Assigned NMR Spectra of 3.58, (a) $^1$H and (b) $^{13}$C.
Figure A-10 Assigned NMR Spectra of 3.77. (a) $^1$H and (b) $^{13}$C.
Figure A-11  Assigned NMR Spectra of 3.78, (a) $^1H$ and (b) $^{13}C$. 
Figure A-12. NMR Spectra of 3.81, (a) $^1$H and (b) $^{13}$C
Figure A-13  Assigned NMR Spectra of 3.SJ, (a) $^1$H and (b) $^{13}$C
Figure A-14 Assigned NMR Spectra of 3,84. (a) $^1$H and (b) $^{13}$C
Figure A-15  Assigned NMR Spectra of 3,85, (a) $^1$H and (b) $^{13}$C.
Figure A-10  Assigned NMR Spectra of 3.92. (a) $^1$H and (b) $^{13}$C.
Figure A-17  Assigned NMR Spectra of 3.93. (a) $^1$H and (b) $^{13}$C.
Figure A-18  Assigned NMR Spectra of 3.94. (a) $^1$H and (b) $^{13}$C.
Figure A-19 Assigned NMR Spectra of 3.95. (a) $^1$H and (b) $^{13}$C
References


32) Tate, M. W.; Gruner, S. M. *Biochemistry* 1987, 26, 231-236.


169) Cameron, L. The Design and Synthesis of Macrocycles for the use as Components of Ion Transporters; University of Victoria: Victoria, 1997.


