Continuous Flow Chemistry – Applications in Chemistry Education and Bioconjugation

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Dissertation directed by

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Professor and Chair of Chemistry
DEDICATION

This work is dedicated in loving memory of my mother, Rose Sebeika, who taught me invaluable lessons about hard work, self-sacrifice, determination and inner strength. I love and miss you every day, and hope this achievement makes you proud.
ACKNOWLEDGMENTS

I would like to thank my family and friends, both at home and at Northeastern University, for all their love, support, and patience over these past five years. This work would not have been possible without having the best of the best on my side.

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ABSTRACT OF DISSERTATION

Applications of continuous flow chemistry have increased significantly over the past two decades, owing much success to adoption of the new methodology in both academic and industrial settings. The advantageous heating and mixing capabilities of flow systems make them attractive avenues for optimization of synthetic methods, manufacturing processes, and complex transformations. In recent years, flow devices have also been incorporated into the undergraduate teaching laboratory to introduce industrially relevant processes and stress the importance of green chemistry. Presented herein are both industrial and academic applications of flow chemistry toward expedited bioconjugation and discovery-based undergraduate laboratory experiments.

Antibody-drug conjugates (ADCs) are an emerging class of biopharmaceuticals that take advantage of the specificity of an antibody-antigen interaction to deliver a highly cytotoxic payload directly to cancer cells. The minimization of off-target binding should reduce debilitating side effects. Consistent methods of expedited chemical conjugation will be discussed, demonstrated using protein standards such as bovine serum albumin and the therapeutic antibody infliximab.

Additionally, an upper level undergraduate laboratory exercise has been developed to introduce students to microscale continuous flow reaction optimization and the green benefits associated with such methodology. The two-part guided inquiry activity allows students to probe their own knowledge of $S_N2$ reactions for the synthesis of benzyl azide and subsequent cyclization to produce 1,2,3-triazoles in a safe and convenient way.
TABLE OF CONTENTS

Dedication ii
Acknowledgements iii
Abstract of Dissertation v
Table of Contents vi
List of Figures xii
List of Schemes xv
List of Tables xviii
List of Abbreviations xxi
General Experimental Information xxv

Chapter 1: Technology-Assisted Methodology: Applications with Azides and Triazoles

1.1 Continuous Flow Chemistry 1
  1.1.1 Conventional vs. Flow Reactions 1
  1.1.2 Types of Flow Instrumentation 2
    1.1.2.1 Lab-on-a-Chip Microreactors 2
    1.1.2.2 Coil/Tube Reactors 6
    1.1.2.3 Catalyst Cartridge Reactors 7
  1.1.3 Advantages of Flow Chemistry 8
    1.1.3.1 Heating 9
    1.1.3.2 Mixing 9
    1.1.3.3 Green Chemistry 11
  1.1.4 Challenges with Flow Chemistry 13
  1.1.5 Applications of Flow Chemistry 14
1.1.5.1 Process Chemistry in the Pharmaceutical Industry 14
1.1.5.2 Methodology Development in Academia 16

1.2 Use of Azides and Triazoles in Heterocyclic Chemistry 18
  1.2.1 Azides and Triazoles as Pharmacophores 19
  1.2.2 Popular Applications of Organic Azides 20

1.3 Hazards of Working with Azides 20
  1.3.1 Azide Hazard Incident: Alcon Research 21
  1.3.2 Case Study: Industrial Work-Around to Use of Azides 21

1.4 Safer Alternatives to Working with Azides 22
  1.4.1 Continuous Flow Production of Azides 23
    1.4.1.1 Continuous Flow Application: 2H-azirines Using Photochemistry 24
    1.4.1.2 Continuous Flow Application: Click Chemistry in Flow (Tahir) 26
    1.4.1.3 Continuous Flow Application: Click Chemistry in Flow (Otvos) 28
    1.4.1.4 Continuous Flow Application: Tetrazole Formation (Palde) 31
    1.4.1.5 Continuous Flow Application: MCR Click Reaction (Bogdan) 33
  1.4.2 Safer Diazo Transfer Reagents 36
    1.4.2.1 Diazo Transfer Application: Polystyrene-Supported Sulfonyl Azide 37
    1.4.2.2 Diazo Transfer Application: Solution-based Salts 39
    1.4.2.3 Diazo Transfer Application: PEG-Supported Sulfonyl Azide 41
  1.4.3 Azides Generated In-Situ 43
    1.4.3.1 In-Situ Generated Azide Application: Click Chemistry (Quan) 43
    1.4.3.2 In-Situ Generated Azide Application: Click Chemistry (Kaboudin) 45
    1.4.3.3 In-Situ Generated Azide Application: Solid-Supported Click (Roy) 48
Chapter 3: Continuous Flow Functionalization of Proteins and Monoclonal Antibodies 132

3.1 Introduction 132

3.1.1 Antibody Drug Conjugates: The “Magic Bullet” of Chemotherapeutics? 132

3.1.2 Design Considerations for ADCs 134

3.1.2.1 Antibody/Target 134

3.1.2.2 Cytotoxin 135

3.1.2.3 Linker Chemistry 137

3.1.3 FDA Approved ADCs 139

3.1.3.1 Approval and Retraction of Mylotarg 139

3.1.3.2 Adcetris 141

3.1.3.3 Kadcyla 142

3.1.3.4 In the Pipeline 144

3.1.4 Methods of Analysis of ADCs 145

3.1.4.1 Drug to Antibody Ratio (DAR) via UV-Vis Spectroscopy 145

3.1.4.2 Verifying DAR and Conjugation Distribution via HIC LC-MS 146

3.1.4.3 Verifying DAR and Conjugation Distribution via SEC-MS 147

3.1.4.4 Verifying DAR and Conjugation Distribution via CE-MS 149

3.1.5. Site Specific Conjugation 151
3.1.5.1 THIOMAB: Engineered Reactive Cysteines 152
3.1.5.2 Non-natural Amino Acid Incorporation 153
3.1.5.3 N-terminal Serine Modification 154
3.1.5.4 Transglutaminases 154
3.1.5.5 Carbohydrates 156
3.1.6 Research Objectives 157
   3.1.6.1 Background on Infliximab (Remicade) 157
   3.1.6.2 Background on NIBRT mAb 158

3.2 Results and Discussion 158
   3.2.1 Proof of Principle – FSB 158
   3.2.2 BSA-FITC as a Model of Lysine Conjugation 160
   3.2.3 Infliximab as a Model of Lysine Conjugation to a mAb 163
   3.2.4 BSA-NPM as a Model of Cysteine Conjugation 165
   3.4.5 TCEP Reduction and Conjugation in One Step 166
   3.2.6 Synthesis of MC-DOX linker 168
   3.2.7 TCEP Reduction followed by Flow Conjugation 169

3.3 Conclusion and Future Directions 170
3.4 Experimental 170
3.5 References 182

CHAPTER 4: Applications in Chemistry Education and Bioconjugation – A Critical Perspective and Future Directions 192
4.1 Introduction 192
4.2 Practicality, Utility and Limitations of Continuous Flow Chemistry in Undergraduate Laboratories 192
4.2.1 Student Autonomy in the Laboratory
4.2.2 Cost of Equipment
4.2.3 Laboratory Logistics

4.3 Considerations for ADC Production Using Continuous Flow Chemistry
4.3.1 Protein and mAb Recovery from Microreactor
4.3.2 Assessment of Solvent Accessibility and Reproducibility
4.3.3 Feasibility of Scale Out Production

4.4 Future Directions

4.5 References

Appendix

Chapter 2 Spectra
Notes for Instructors
Chapter 3 Spectra
Permissions
# LIST OF FIGURES

**Chapter 1: Technology-Assisted Methodology: Applications with Azides and Triazoles**

| Figure 1.1 | A comparison of reaction set-ups for conventional and flow methodologies | 2 |
| Figure 1.2 | A generic lab-on-a-chip microreactor diagram | 3 |
| Figure 1.3 | The Chemtrix Labtrix Start (bottom) and S1 (top) systems used in this work | 4 |
| Figure 1.4 | A general schematic representation of a Chemtrix microreactor and an image of the model 3223 SOR glass microreactor | 6 |
| Figure 1.5 | A general coil reactor diagram | 6 |
| Figure 1.6 | A general catalyst cartridge diagram | 8 |
| Figure 1.7 | Laminar vs. turbulent flow in closed systems | 10 |
| Figure 1.8 | The staggered-oriented ridge (SOR) micromixers within the Labtrix microreactors | 10 |
| Figure 1.9 | A selection of natural products synthesized using continuous flow chemistry | 17 |
| Figure 1.10 | A selection of prescription drugs with azido and azide-derived pharmacophores | 19 |
| Figure 1.11 | The Conjure flow reactor developed for segmented flow synthesis of triazoles | 34 |

**Chapter 2: Implementing a Process Oriented Guided Inquiry Learning (POGIL) Undergraduate Laboratory Activity Utilizing Continuous Flow Chemistry**

| Figure 2.1 | Bloom’s Revised Taxonomy, as described in 2001 | 68 |
| Figure 2.2 | The different levels of inquiry, based on student involvement | 69 |
| Figure 2.3 | Typical flow of a POGIL class as described by Spencer et al | 73 |
| Figure 2.4 | Student interface of remote access to FutureChemistry | 79 |
| Figure 2.5 | The gas-phase apparatuses used in the transformation of propanol to propane | 80 |
| Figure 2.6 | Diagram of continuous flow reactor for triazole library synthesis a) two-step, one-pot reaction b) two-step sequential flow reaction | 84 |
| Figure 2.7 | Temperature optimization for the conversion of benzyl bromide to benzyl azide (Residence time = 750 seconds (12.5 min)) | 86 |
| Figure 2.8 | Temperature optimization for the conversion to benzyl triazole (Residence time = 500 sec, 8.33 min) | 88 |
| Figure 2.9 | Residence time optimization of conversion to benzyl triazole (Temp = 80 °C) | 88 |
| Figure 2.10 | Optimization for the one-pot, two-step triazole formation under flow conditions (Residence time = 45 sec (0.75 min)) | 91 |
| Figure 2.11 | Optimization for the one-pot, two-step triazole formation under flow conditions (Residence time = 225 sec (3.75 min)) | 91 |
| Figure 2.12 | Brillinta (ticagrelor) contains a triazolopyrimidine core that can be accessed readily by our methodology | 97 |

**Chapter 3: Continuous Flow Functionalization of Proteins and Monoclonal Antibodies**

| Figure 3.1 | The general structure of an antibody-drug conjugate | 134 |
| Figure 3.2 | The structure of an IgG antibody | 135 |
| Figure 3.3 | Four of the predominant toxins used in ADCs | 136 |
| Figure 3.4 | The structure of Mylotarg | 140 |
| Figure 3.5 | The structure of Adcetris | 142 |
| Figure 3.6 | The structure of Kadcyla | 143 |
| Figure 3.7 | Hydrophobic interaction chromatography theory | 146 |
| Figure 3.8 | Hydrophobic interaction chromatography (HIC) chromatogram, with an inset of UV-VIS spectra | 147 |
| Figure 3.9 | Size exclusion chromatography theory | 148 |
| Figure 3.10 | DAR distribution via ESI-MS, with an inset of the SEC chromatogram | 149 |
| Figure 3.11 | Capillary electrophoresis (CE) theory | 150 |
| Figure 3.12 | Comparison of RP-HPLC and CE-SDS chromatograms of an immunonoconjugate | 151 |
| Figure 3.13 | The THIOMAB conjugation model, utilized by Junutula et al | 153 |
| Figure 3.14 | Structure of the chimeric mAb infliximab (Remicade) | 157 |

**CHAPTER 4: Applications in Chemistry Education and Bioconjugation – A Critical Perspective and Future Directions**

| Figure 4.1 | Preparation of an in-house microfluidic device | 193 |
LIST OF SCHEMES

Chapter 1: Technology-Assisted Methodology: Applications with Azides and Triazoles

Scheme 1.1  Continuous flow synthesis of bis(indolyl)methane compounds under reduced catalyst loading 11
Scheme 1.2  High temperature, acid-free deprotection of Boc-intermediate using flow chemistry 12
Scheme 1.3  Kumada reaction scaled out using parallel reactors 13
Scheme 1.4  Convenient and efficient flow nitrations and fluorinations carried out at Pfizer 16
Scheme 1.5  Total synthesis of grossamide by Ley in 2005 18
Scheme 1.6  API synthesized on a large scale for commercial application 22
Scheme 1.7  Phenyl-substituted vinyl azides with electron deficient alkenes generated in flow 25
Scheme 1.8  2H-azirine formation optimized in flow using tandem photochemistry 25
Scheme 1.9  A β-CD functionalized microchip used for the first time in flow synthesis 26
Scheme 1.10  A homogeneous copper source and the use of a buffer allows for the high yielding synthesis of 1,4-substituted triazoles 29
Scheme 1.11  Flow process suppresses the formation of byproducts generated from nitrile hydration 31
Scheme 1.12  A library of 1,4-substituted triazoles generated in a copper flow reactor 34
Scheme 1.13  Polystyrene-bound benzene sulfonyl chloride used to conduct diazo transfer 37
Scheme 1.14  Route to crystalline diazo transfer agents 39
Scheme 1.15  Resin based route to azides 42
Scheme 1.16  Synthesis of resin-bound triazoles conducted at ambient temperature 43
Scheme 1.17  One pot triazole formation under mild conditions 44
Scheme 1.18  The arylboronic acid route to triazoles 46
Scheme 1.19  Immobilization of the copper source 49
Scheme 1.20  High yielding Cu click process 50
Scheme 1.21  The microwave-assisted, selective “click” synthesis of 1,2,3-triazoles in a multicomponent reaction system 52

Chapter 2: Implementing a Process Oriented Guided Inquiry Learning (POGIL) Undergraduate Laboratory Activity Utilizing Continuous Flow Chemistry

Scheme 2.1  Stilbene reactions to elucidate addition of bromine to an alkene 75
Scheme 2.2  Possible elimination products students must characterize to determine E2 mechanism 76
Scheme 2.3  The diastereoselective Grignard reaction adapted for a POGIL exercise 76
Scheme 2.4  Student-produced polystyrene flow device for the synthesis of azo dyes 78
Scheme 2.5  Synthetic route to methyl orange carried out using remote FutureChemistry instrument 79
Scheme 2.6  The two-step, gas-phase transformation of propanol to propane 79
Scheme 2.7  Continuous flow synthesis of HMF from fructose 81
Scheme 2.8  1,2,3-Triazoles and corresponding triazolopyrimidines via [3+2] cycloaddition chemistry 83
Scheme 2.9  Optimized route to 1-benzyl-1,2,3-triazole and its triazolopyrimidine derivative 89
Scheme 2.10  Synthetic route to access ticagrelor analogues 97
Scheme 2.11  Cyclopentyl analogue with a structure similar to ticagrelor 98
Chapter 3: Continuous Flow Functionalization of Proteins and Monoclonal Antibodies

Scheme 3.1  Traditional ADC linker strategies  138
Scheme 3.2  Lysine and cysteine conjugation methods for synthesizing ADCs  139
Scheme 3.3  The oxime ligation of auristatin derivatives using unnatural amino acids  154
Scheme 3.4  N-terminal serine modification for oxime ligation  154
Scheme 3.5  Transglutaminase-catalyzed functionalization of mAbs for ADC preparation  155
Scheme 3.6  The one pot chemoenzymatic modification of Herceptin glycoforms  156
Scheme 3.7  FSB conjugation to proof of principle protein standards  159
Scheme 3.8  Conjugation of FITC to bovine serum albumin (BSA) via lysine linkage  161
Scheme 3.9  Conjugation of FSB to infliximab under conventional and flow conditions  163
Scheme 3.10  TCEP reduction followed by NPM conjugation of BSA  165
Scheme 3.11  One-pot, two-step flow reduction/conjugation with FcM  167
Scheme 3.12  Synthetic route to MC-DOX linker-toxin  169
# LIST OF TABLES

## Chapter 1: Technology-Assisted Methodology: Applications with Azides and Triazoles

| Table 1.1 | Continuous flow applications in the pharmaceutical industry | 14 |
| Table 1.2 | Continuous flow optimization of 2H-azirine formation at room temperature | 25 |
| Table 1.3 | Scope of 1,2,3-triazoles synthesized via a β-CD functionalized glass microchip | 27 |
| Table 1.4 | Scope of β-aminocyclohexanecarboxylic acid derivatives produced via the copper column in flow | 30 |
| Table 1.5 | Tetrazoles formed using the modified Sharpless method in flow | 32 |
| Table 1.6 | Library of triazole products via the Conjure flow reactor | 35 |
| Table 1.7 | Comparison of diazo product yield and reaction time for PS and p-CBSA methods | 38 |
| Table 1.8 | Scope of azide synthesis from amines and imidazole-1-sulfonyl azide hydrochloride | 39 |
| Table 1.9 | Comparison of acids used to crystallize the diazo transfer salt | 41 |
| Table 1.10 | Triazoles formed from one-pot click reaction with a variety of aryl groups | 44 |
| Table 1.11 | Scope of triazoles synthesized from aryloboronic acids via azide intermediates | 46 |
| Table 1.12 | Scope of 1,4-substituted triazoles from aniline intermediates | 50 |
| Table 1.13 | Scope of 1,4-substituted triazoles from a regioselective MCR using microwave irradiation | 52 |

## Chapter 2: Implementing a Process Oriented Guided Inquiry Learning (POGIL) Undergraduate Laboratory Activity Utilizing Continuous Flow Chemistry

| Table 2.1 | Comparison of traditional general chemistry lecture to POGIL class | 74 |
| Table 2.2 | Series of continuous flow laboratory experiments developed by König | 81 |
Table 2.3  Temperature optimization of benzyl azide conversion (res time = 12.5 min)  85
Table 2.4  Residence time optimization of benzyl azide conversion (T=80 °C)  85
Table 2.5  Temperature optimization of benzyl triazole conversion (res time = 8.33 min)  86
Table 2.6  Residence time optimization of benzyl triazole conversion (T=80 °C)  87
Table 2.7  Temperature optimization of one pot, two step benzyl triazole conversion (res time 45 s (0.75 min))  90
Table 2.8  Temperature optimization of one pot, two step benzyl triazole conversion (res time 225 s (3.75 min))  90
Table 2.9  Investigation of the effect of starting material and base equivalents for the two-step, one-pot triazole synthesis  92
Table 2.10  Continuous flow synthesis of triazole analogs in (a) configuration using 1 eq of azide and 3 eq of cyanoacetamide and NaOH  93
Table 2.11  Continuous flow synthesis of triazole analogs in (b) configuration using 1 eq of azide and 2 or 3 eq of cyanoacetamide and NaOH, as shown in Column E and F, respectively.  95

Chapter 3: Continuous Flow Functionalization of Proteins and Monoclonal Antibodies

Table 3.1  Adapted from C&EN report, the top 20 selling drugs in 2014 are listed, with biologics in blue and small molecules in yellow  133
Table 3.2  Table of ADC clinical trials adapted from Advances in the Pharmaceutical Science Series report  144
Table 3.3  Conjugations of FSB to protein standards  159
Table 3.4  Optimization of continuous flow conjugations of BSA-FITC  162
Table 3.5  Conventional conjugation of FSB to infliximab  164
Table 3.6  Flow conjugation of FSB to infliximab  164
Table 3.7  Average NPM per BSA following TCEP reduction  166
<table>
<thead>
<tr>
<th>Table 3.8</th>
<th>Two-step, one pot flow reduction-conjugation with therapeutic mAbs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 3.9</td>
<td>Conventional and flow conjugation reactions of anti-IL8 with MC-DOX</td>
</tr>
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</table>
## LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>µL</td>
<td>microliter</td>
</tr>
<tr>
<td>µm</td>
<td>micrometer</td>
</tr>
<tr>
<td>$^{13}$C NMR</td>
<td>carbon-13 nuclear magnetic resonance</td>
</tr>
<tr>
<td>$^{1}$H NMR</td>
<td>proton nuclear magnetic resonance</td>
</tr>
<tr>
<td>4-MeOBP</td>
<td>4-methoxybiphenyl</td>
</tr>
<tr>
<td>ACN</td>
<td>acetonitrile</td>
</tr>
<tr>
<td>ADC</td>
<td>antibody-drug conjugate</td>
</tr>
<tr>
<td>AML</td>
<td>acute myeloid leukemia</td>
</tr>
<tr>
<td>AMPS</td>
<td>aminomethyl polystyrene</td>
</tr>
<tr>
<td>API</td>
<td>approved pharmaceutical ingredient</td>
</tr>
<tr>
<td>AZT</td>
<td>azidothymidine</td>
</tr>
<tr>
<td>BOC</td>
<td>tert-butoxy carbonyl</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CE</td>
<td>capillary electrophoresis</td>
</tr>
<tr>
<td>CTQ</td>
<td>critical thinking question</td>
</tr>
<tr>
<td>CuAAC</td>
<td>copper catalyzed azide-alkyne cycloaddition</td>
</tr>
<tr>
<td>CZE</td>
<td>capillary zone electrophoresis</td>
</tr>
<tr>
<td>DAR</td>
<td>drug-to-antibody ratio</td>
</tr>
<tr>
<td>DAST</td>
<td>diethylaminosulfur trifluoride</td>
</tr>
<tr>
<td>DBU</td>
<td>1,8-Diazabicyclo[5.4.0]undec-7-ene</td>
</tr>
<tr>
<td>DCM</td>
<td>dichloromethane</td>
</tr>
<tr>
<td>DHPMs</td>
<td>3,4-dihydropyrimidinones</td>
</tr>
</tbody>
</table>
DIPEA  diisopropylethyl amine
DMF  dimethylformamide
DMSO  dimethylsulfoxide
DNA  deoxyribonucleic acid
EEDQ  2-Ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline
EGFR  epidermal growth factor receptor
ESI  electrospray ionization
EtOAC  ethyl acetate
FA  formic acid
Fab  antigen binding region
Fc  constant region
FDA  Food and Drug Administration
FITC  fluorescein isothiocyanate
FSB  N-succinimidyl-4-fluorobenzoate
HC  heavy chain
HIC  hydrophobic interaction chromatography
HPLC  high performance liquid chromatography
HSA  human serum albumin
IgG  Immunoglobulin G
INS  insulin
INT  interferon
L  liter
LC  light chain
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>LC-MS</td>
<td>liquid chromatography-mass spectrometry</td>
</tr>
<tr>
<td>mAb</td>
<td>monoclonal antibody</td>
</tr>
<tr>
<td>MBHA</td>
<td>4-methylbenzhydrylamine</td>
</tr>
<tr>
<td>MC-DOX</td>
<td>maleimidocaproyl-doxorubicin</td>
</tr>
<tr>
<td>MCR</td>
<td>multicomponent reaction</td>
</tr>
<tr>
<td>MeOH</td>
<td>methanol</td>
</tr>
<tr>
<td>mL</td>
<td>milliliter</td>
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<tr>
<td>mm</td>
<td>millimeter</td>
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<tr>
<td>MMAE</td>
<td>monomethyl auristatin E</td>
</tr>
<tr>
<td>MMAF</td>
<td>monomethyl auristatin F</td>
</tr>
<tr>
<td>mmol</td>
<td>millimoles</td>
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<tr>
<td>MS</td>
<td>mass spectrometry</td>
</tr>
<tr>
<td>MsOH</td>
<td>methanesulfonic acid</td>
</tr>
<tr>
<td>MTBE</td>
<td>methyl tert-butyl ether</td>
</tr>
<tr>
<td>MTGase</td>
<td>microbial transglutaminase</td>
</tr>
<tr>
<td>MW</td>
<td>microwave</td>
</tr>
<tr>
<td>MWCO</td>
<td>molecular weight cut off</td>
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<tr>
<td>MYO</td>
<td>myoglobin</td>
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<tr>
<td>NBS</td>
<td>N-bromosuccinimide</td>
</tr>
<tr>
<td>NMP</td>
<td>N-methyl-2-pyrrolidone</td>
</tr>
<tr>
<td>NPM</td>
<td>N-pyrenyl maleimide</td>
</tr>
<tr>
<td>pAcPhe</td>
<td>p-acetylphenylalanine</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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</tr>
<tr>
<td>p-CBSA</td>
<td>(4-carboxybenzene-sulfonyl azide)</td>
</tr>
<tr>
<td>PEEK</td>
<td>polyether ether ketone</td>
</tr>
<tr>
<td>PEG</td>
<td>polyethylene glycol</td>
</tr>
<tr>
<td>PFA</td>
<td>perfluoroalkoxy alkane</td>
</tr>
<tr>
<td>PNGase F</td>
<td>peptide-N-Glycosidase F</td>
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<td>POGIL</td>
<td>Process-Oriented Guided Inquiry Learning</td>
</tr>
<tr>
<td>PTFE</td>
<td>polytetrafluoroethylene</td>
</tr>
<tr>
<td>RP-HPLC</td>
<td>reverse phase high performance liquid chromatography</td>
</tr>
<tr>
<td>SEC</td>
<td>size exclusion chromatography</td>
</tr>
<tr>
<td>SOR</td>
<td>static-oriented ridge</td>
</tr>
<tr>
<td>t-BuOH</td>
<td>tert-butanol</td>
</tr>
<tr>
<td>TCEP</td>
<td>tris(2-carboxyethyl)phosphine</td>
</tr>
<tr>
<td>TEA</td>
<td>triethylamine</td>
</tr>
<tr>
<td>TEAB</td>
<td>tetraethylammonium bromide</td>
</tr>
<tr>
<td>TG</td>
<td>transglutaminase</td>
</tr>
<tr>
<td>THF</td>
<td>tetrahydrofuran</td>
</tr>
<tr>
<td>THIOMAB</td>
<td>engineered antibody with reactive cysteine</td>
</tr>
<tr>
<td>TMS</td>
<td>trimethylsilyl</td>
</tr>
<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
</tr>
<tr>
<td>TsOH</td>
<td>p-toluenesulfonic acid</td>
</tr>
<tr>
<td>UV-Vis</td>
<td>ultraviolet-visible</td>
</tr>
<tr>
<td>Val-Cit</td>
<td>valine-citrulline</td>
</tr>
<tr>
<td>VOD</td>
<td>venoocclusive disease</td>
</tr>
</tbody>
</table>

xxiv
GENERAL EXPERIMENTAL INFORMATION

Chapter 2: All reagents and solvents were purchased from Fisher Scientific or Sigma Aldrich. THF was distilled from a sodium/benzophenone ketyl for the triazolopyrimidine synthesis. All continuous flow reactions were performed using the Chemtrix Labtrix S1 system (Chemtrix BV, NL) utilizing a T-mixer glass microreactors, which are commercially available through Chemtrix BV. All reagents were dissolved, loaded into 1 mL glass gas-tight syringes (SGE) and dispensed through the microreactor into the autosampler loaded with HPLC vials. The samples were analyzed by HPLC-UV (Waters Alliance; Agilent Eclipse Plus C18 Column; 254 nm), and yields for the continuous flow reactions were determined using a prepared calibration curve of each standard. \( ^1 \)H NMR spectra were recorded at 400 MHz, and \( ^{13} \)C NMR were recorded at 100 MHz on a Varian NMR instrument, and spectra was prepared using ACD/Labs (Toronto, Canada). High-resolution mass spectroscopy was obtained on a Waters 70-VSE (EI) or a Waters Q-ToF Ultima mass spectrometer (ESI) at the UIUC Mass Spectrometry Facility.

Chapter 3: All reagents and solvents were purchased from Fisher Scientific or Sigma Aldrich. All continuous flow reactions were performed using the Chemtrix Labtrix Start system (Chemtrix BV, NL) utilizing a T-mixer glass microreactors, which are commercially available through Chemtrix BV. All reagents were dissolved, loaded into 1 mL glass gas-tight syringes (SGE) and dispensed through the microreactor into the autosampler loaded with HPLC vials. \( ^1 \)H NMR spectra were recorded at 400 MHz, and \( ^{13} \)C NMR were recorded at 100 MHz on a Varian Mercury NMR instrument, and spectra was prepared using ACD/Labs (Toronto, Canada). High-resolution mass spectroscopy was obtained on a Waters 70-VSE (EI) or a Waters Q-ToF Ultima mass spectrometer (ESI) at the UIUC Mass Spectrometry Facility for analysis of synthesized
linkers. For ADC-linker analysis by LC-MS, samples were desalted using a Zeba (Thermo Scientific, Rockland, IL) spin desalting column (7 kDa MWCO). The LC-MS analysis was performed on a micro-LC 200 system (Eksigent, Framingham, MA) coupled with a 5600 ESI Triple ToF mass spectrometer (AB Sciex, Framingham, MA). Samples were injected on an Eksigent MicroLC ChromXP C4, 5μm, 300 Å, 50 × 0.5 mm column (Eksigent, Framingham, MA) set at 35 °C. Data analysis was performed with PeakView 1.0 and MagTran 1.03.

Myoglobin samples were desalted using a Zeba (Thermo Scientific, Rockland, IL) spin desalting column (7 kDa MWCO) prior to analysis. Antibody standards and ADCs were reduced by incubation with dithiothreitol (DTT). Briefly, 50 µL of a 1 mg/mL antibody or ADC solution in phosphate buffer saline (PBS) was mixed with equal amount of freshly prepared 40 mM DTT in water and then incubated at 37 °C for 1 hour with shaking. The reaction was quenched by a subsequent desalting step using a Zeba (Thermo Scientific, Rockland, IL) spin desalting column (7 kDa MWCO). Five microliters of sample was injected for LC-MS analysis.

The LC-MS analysis was performed on a micro-LC 200 system (Eksigent, Framingham, MA) coupled with a 5600 ESI Triple ToF mass spectrometer (AB Sciex, Framingham, MA). Samples were injected on an Eksigent MicroLC ChromXP C4, 5μm, 300 Å, 50 × 0.5 mm column (Eksigent, Framingham, MA) set at 35 °C. The mobile phase consisted of water with 0.1% FA (mobile phase A) and acetonitrile with 0.1% FA (mobile phase B). The following gradients were applied at a flow rate of 40 µL/min: precondition with 10% B for 5 min, linear gradient from 10% B to 50% B in 10 min, washing with 90% B for 2 min, and then re-equilibration with 10% B for a min before the next injection. The Triple ToF was operated in intact protein positive ion mode with an ionspray voltage of 5500V, a source temperature of 350 °C, Gas1/Gas2 set to 40 (arbitrary units) and a declustering potential set to 150 V.
Acquisitions were performed using TOF MS mode from m/z 500–4000, using an accumulation time of 0.1 s. Mass spectra data analysis was performed with PeakView ver. 1.0 and mass spectra deconvolution was performed with MagTran ver. 1.03.
CHAPTER 1: TECHNOLOGY-ASSISTED METHODOLOGY – APPLICATIONS WITH AZIDES AND TRIAZOLES

1.1. Continuous Flow Chemistry

Classical organic chemistry transformations have been carried out using batch (or conventional) conditions for decades. These closed system reaction set-ups include the use of round-bottom flasks, stir bars, and oil or sand baths for heating. Reactions conducted in this fashion suffer from poor mixing, inefficient heat transfer, and subsequently low yields. Undesired byproducts can form, and reaction conditions often do not afford complete conversion of starting material. As a result, the organic synthesis community, including process and medicinal chemists, have been searching for viable alternatives to traditional methodologies that improve in these areas. Continuous flow chemistry has been heavily investigated over the past two decades as a potential solution. This chapter will cover the components of a flow chemistry system, its advantages and disadvantages, and its applications as a safe and convenient method for the synthesis of azides and triazoles, which will be pertinent background for Chapter 2.

1.1.1. Conventional vs. Flow Reactions

Traditional batch reactions are designed using molar stoichiometric ratios of reagents. The reagents are combined in a reaction vessel, and subsequently stirred and monitored until completion at a given temperature. In contrast, as depicted in Figure 1.1, continuous flow processes are solution-based, and stoichiometry is dictated by both concentration and the rate at which the solutions flow relative to one another. The total time a molecule spends in the reactor is called the residence time, which can be varied based on flow rate of reagents and internal volume of the reactor.
1.1.2. Types of Flow Instrumentation

Based on the needs of the individual – both in terms of reaction scale and reaction conditions – there are numerous types of continuous flow reactors available. The main types of continuous flow reactors will be described below, along with a few examples of corresponding companies that manufacture these types of instruments. It is worth noting that although they will not be discussed in detail, flow devices are often fabricated in-house, as it is often necessary if the system needs to be highly specialized or reduced in cost.

1.1.2.1. Lab-on-a-Chip Microreactors

A common type of micro-scale reactor is a microchip-based system; a general scheme of a microreactor chip is shown in Figure 1.2. The microreactors are typically made of glass and have small channels through which solution-based reagents are pumped via a syringe pump.
The number of inlets and outlets is highly dependent on the reaction conditions, but are available in numerous configurations. Therefore, these systems are amenable to multi-step processes and in-line quenching. The reactions are run on a microliter per minute flow rate scale, and are capable of generating micrograms to milligrams of product within hours.

There are a few limitations of these systems in terms of reaction compatibility. The glass reactors would be etched by hydrofluoric acid, so it should therefore be avoided. The small channels of the microreactors are easily clogged if any reaction component precipitates out of solution, so care must be taken to ensure full solubility of reagents and products during the course of the reaction. Also, the tubing and fittings used in these systems are often the same as those used in high performance liquid chromatography (HPLC), so reagents need to be checked for compatibility, most commonly with polyether ether ketone (PEEK) and polytetrafluoroethylene (PTFE).

An example of a system relevant to this work is the Chemtrix line of products, as shown in Figure 1.3. Fabricated in the Netherlands, these systems are available in microscale (inner diameter 10-500 µm), meso scale (inner diameter 500 µm to several mm), and/or kilo scale (inner diameter > several mm). The microreactor systems, called the Labtrix Start and S1, consist of syringe pumps that are used to store solution-based reagents, a housing for the
microchips which can be heated or cooled to the desired temperature, and in the case of the S1, an auto-sampler that can be programmed via computer to control all reaction parameters with reaction recipes and collection instructions.

**Figure 1.3** The Chemtrix Labtrix Start (bottom) and S1 (top) systems used in this work (Reproduced with permission from C. Wiles of Chemtrix B.V.)

Based on the desired transformation, a variety of glass microreactors are available. The general setup of a static-oriented ridge (SOR) mixer chip is shown in **Figure 1.4.** The two
reactants are pre-heated before mixing together, which is an advantage over conventional reaction methods. The SOR mixer uses turbulent mixing to ensure a homogeneous reaction mixture before continuing on to the reaction channel. The time that the mixture spends in this region is called the residence time. Based on the flow rate employed, which is generally in the microliter per minute rage, the residence time can be easily varied. Reagents can also be controlled independently, so equivalence can be varied by changing the flow rate of one reagent relative to the other, or by preparing solutions of varied concentrations. If the reaction needs to be quenched in-line, the quenching reagent can be pre-heated and mixed with the reaction in another SOR mixer before exiting the chip for collection. A primary concern with these reactors is clogging, so the homogeneity of a given set of reagents, products and byproducts should be investigated before translating the system to flow.

Also depicted in Figure 1.4 is a sample of one design of the Chemtrix microreactors. Different microreactors can include diverse combinations of mixers, reagent and quench inlets, and reactor volumes. For reactions requiring solid-phase catalysis, chips are available with channels for embedded catalysts. The compatibility of most traditional organic reagents is very high. Also, when aiming to scale-up a reaction using their systems, the reaction conditions optimized at the micro scale can be used directly to kilo scale to produce larger quantities of the desired product, making this translatable technology highly desirable in chemical manufacturing and drug development.
1.1.2.2. **Coil/Tube Reactors**

Another micro to meso scale option is the use of a coil reactor; a general depiction of a coil reactor is shown in **Figure 1.5**. The coil/tube is typically made of material such as stainless steel or other inert metal alloys, or a polymer such as perfluoroalkoxy alkane (PFA). Reagent solutions are stored in reservoirs and using tubing and an HPLC or peristaltic pump, they flow through the heated or cooled reactor. Some of these materials such as PFA are acid-resistant, so they are amenable to some conditions that glass microreactors are not.\(^7\) \(^8\)

**Figure 1.4** A general schematic representation of a Chemtrix microreactor (left) and an image of the model 3223 SOR glass microreactor (right) (Reproduced with permission from C. Wiles of Chemtrix B.V.)

**Figure 1.5** A general coil reactor diagram
These systems are often used for meso scale processes, and the milliliter flow rates can generate milligrams to grams of product. Liquid-liquid reactors can be placed in-line for aqueous workups or for solvent switching. Polymer tubing allows for visualization of reagents, so clogs can be easily detected. The stainless-steel reactors can accommodate temperatures up to 250 °C. The coil reactor design can be heated or cooled to the desired temperature based on regulating the temperature of the air surrounding the coil. As a result, the uniform nature of the coil allows for superior temperature control over the full duration of the reaction.

1.1.2.3. Catalyst Cartridge Reactors

For transition metal catalysis, solubility can be a major concern. To circumvent this issue, catalyst cartridges are available. A general depiction of this reactor type is shown in Figure 1.6. A stainless steel cartridge is packed with the catalyst of interest, and the solution based reagents are pumped through. The cartridge is designed with a filter on each end so that there is no leakage of the catalyst into the reaction mixture. Pumps similar to and often compatible with the aforementioned coil reactor systems can be used for pumping these reagents. Care must be taken to ensure, as previously described, that no adducts or insoluble solids form within the tubing. The catalyst loading and activity should be monitored consistently; as catalyst turnover decreases, the effective quantity of catalyst may decrease, leading to reduced activity.
Figure 1.6 A general catalyst cartridge diagram

Hydrogenation reactors are an example of this type of process. Hydrogen gas for the reaction is generated \textit{in-situ} via water electrolysis, making the whole process easier and safer to handle. The substrate is combined with hydrogen and passed through a catalyst cartridge, and can be heated up to 100 ºC and pressurized to 100 bar. It is compact and can conveniently fit inside a fume hood.

The reactors are typically 30-100 mm in length. As the reaction mixture is collected, the catalyst remains bound to the cartridge, making the purification process much more streamlined. The particle size of the catalyst, as well as the length of the cartridge can be customized. Typical transition metals used for hydrogenation, like Pd/C and Raney Ni, are available, as well as more selective catalysts.

1.1.3. Advantages of Flow Chemistry

The following sections will detail some of the advantages of using continuous flow chemistry. Although not all reactions are amenable to continuous flow conditions, those that are improved by this methodology can attribute this success to one or more of the following principles.
1.1.3.1. Heating

One of the biggest advantages of continuous flow chemistry is the improved heating efficiency. In typical batch processes, a round bottom or other reaction vessel is equipped with a stir bar and submerged in an oil or sand bath. Due to the nature of heating one medium in order to subsequently heat another through a thick glass reaction vessel, temperature gradients are generated and therefore the heating is inconsistent. However, in a flow process on a chip, the high surface area to volume ratio allows for a precise and even heating or cooling throughout the entire residence time.\textsuperscript{21}

1.1.3.2. Mixing

The flow of a liquid within a defined space is related to its Reynolds number (Re). The Reynolds number is a ratio of inertial forces of the liquid to the viscosity of the liquid. As shown in Equation 1.1, where \( \rho \) is the density of the liquid, \( u \) is the velocity of the fluid in relation to the object in which it is flowing, \( L \) is the length of the flow, and \( \mu \) is the dynamic viscosity of the fluid.\textsuperscript{22}

\textbf{Equation 1.1} \[ Re = \frac{\rho u L}{\mu} \]

The superior mixing capabilities of continuous flow processes come from two different types of flow within the tubing/reaction channels: turbulent and laminar flow, as shown in Figure 1.7. Laminar flow occurs when the solution moves along its path in a consistent, even manner, and is characterized by a low Reynolds number, with a driving force on the velocity. The mixing observed when two solutions flow in this manner occurs via diffusion between the two layers.\textsuperscript{23} On a micro scale, laminar flow is the primary method observed,\textsuperscript{24} due to the low Reynolds number. Turbulent flow is characterized by higher velocities and therefore higher
Reynolds number, in which clusters of particles cause inconsistencies in mixing and swirling as a liquid passes through, which leads to lateral mixing between two solutions.\textsuperscript{23}

\textbf{Figure 1.7} Laminar vs. turbulent flow in closed systems

The design of the channels within a Chemtrix microreactor includes staggered-oriented ridge (SOR) micromixers to induce turbulent flow. As shown in \textbf{Figure 1.8}, these etched micromixers accelerate the mixing of the reagents within the chip. The design works well at both high and low flow rates, which is often difficult to achieve as laminar flow is favored at low flow rates.

\textbf{Figure 1.8} The staggered-oriented ridge (SOR) micromixers within the Labtrix microreactors (reproduced with permission from C. Wiles of Chemtrix)
1.1.3.3. **Green Chemistry**

From the standpoint of generating processes that reduce chemical waste, continuous flow chemistry presents a unique opportunity to implement these principles in terms of reaction conditions, optimization, and scaling. For example, flow reactions require less catalyst loading, as shown in **Scheme 1.1**. The increased surface area to volume ratio in flow allows the reactants to be exposed to more catalyst particles than they would normally, and thus the effective catalyst load is greater than the actual loading value. Traditional synthesis of bis(indolyl)methane compounds requires catalyst loading of 20 mol % and four hours reaction time,\textsuperscript{25} whereas the flow process developed by the Ley group reduces the loading to 5 mol % catalyst and one hour reaction time.\textsuperscript{26}

![Scheme 1.1 Continuous flow synthesis of bis(indolyl)methane compounds under reduced catalyst loading\textsuperscript{26}](image)

In addition, solvents and reaction mixtures can be heated beyond their boiling points due to the lack of empty head space created by the high surface area to volume ratio; commonly-used high boiling solvents like xylenes and diglyme can be replaced by greener solvents such as acetonitrile.\textsuperscript{27} As a result, previously un-reached temperature and pressure combinations can be
achieved. On a microscale, reaction components spend such a small amount of time in contact with the microreactor that highly exothermic reactions do not need to be carried out at low temperatures.\textsuperscript{28}

For example, Bogdan et al report efforts to access reaction temperatures beyond 250 °C, with the goal of reaching new, pharmaceutically-relevant reaction space.\textsuperscript{27} Scheme 1.2 shows their synthesis of complex carbamates via high-temperature, acid-free deprotection of a Boc-intermediate, which can be achieved at 300 °C in acetonitrile, despite the 80 °C boiling point. The entire process takes only ~8 minutes to achieve, with >99% conversion and a 91% isolated yield.

\textbf{Scheme 1.2} High temperature, acid-free deprotection of Boc-intermediate using flow chemistry\textsuperscript{27}

Scale up of batch processes require the use of large, multi-liter reactors, and therefore require re-optimization from the original conditions to account for the inefficient mixing and heat transfer. However, the solution-based nature of flow reactions allows a reaction to be scaled out, rather than scaled up. To scale out a reaction, a series of reactors are run in parallel at the
microscale conditions. Because many platforms have microscale, meso-scale, and/or kilo-scale instruments, a reaction can be optimized on the microscale, which generates less waste, and then use the optimized conditions to scale up to a higher-capacity instrument.

For example, the Kumada reaction for the synthesis of 4-methoxybiphenyl (4-MeOBP) was scaled out by Styring and Parracho. A batch synthesis of this material takes 24 hours at room temperature, and produces three byproducts. When optimized in a single 30 mm capillary reactor, similar conversion was attained in several minutes in comparison to the 24 hour batch reaction. To scale out, a parallel reactor with 120, 30 mm stainless steel capillaries was assembled and therefore the same conditions used for the single capillary were used. In the same 24 hour period, a total of 137 g of MeOBP was synthesized.

Scheme 1.3 Kumada reaction scaled out using parallel reactors

1.1.4 Challenges with Flow Chemistry

Despite the many advantages that flow chemistry affords, there are challenges associated with both the instruments and the adoption of the technology. Solubility, or lack thereof, tends to be one of the most prevalent concerns, especially on the micro scale, as cleaning out clogs in microreactors can be extremely elusive. Precipitation in a microreactor can happen very quickly, and if it is not observed by the user, could lead to damage of the microreactor. Chemists must also ensure compatibility of the desired reaction with the flow device, especially with corrosive or harsh reagents.
Willingness of chemists to embrace and adopt flow chemistry techniques is another less technical challenge, but remains prevalent in the field. Chemists who have decades of training in conventional chemistry do not have detailed training in flow systems, and can be hesitant to adopt new methods. This mentality plagued industrial settings at its onset, especially in regards to FDA regulations related to manufacturing. In the last 20 years, the advantages for each process are assessed and adopted if time, reagents, and money are saved.

1.1.5 Applications of Flow Chemistry

These advantages of continuous flow processing afford a variety of applications for these reactions. Two general fields of applications will be discussed briefly, followed by applications in a specific field in heterocyclic chemistry which will be pertinent to the subsequent material in these chapters.

1.1.5.1 Process Chemistry in the Pharmaceutical Industry

One of the most prevalent uses of flow chemistry is in the field of process chemistry, used by pharmaceutical, fine chemical and petrochemical companies. The industry benefits from flow processes because they are highly reproducible, easily scalable, and easily automated. Susanne et. al. from Pfizer published a Pharma Perspective on the utility of flow chemistry, which outlines the use of flow in process development at many different companies, as well as for challenging syntheses employing exothermic reactions or unstable reaction intermediates. Table 1.1 below details just a few of the many uses outlined in this work.

<table>
<thead>
<tr>
<th>Pharmaceutical Company</th>
<th>Reactions Investigated</th>
<th>Advantage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pfizer</td>
<td>Nitration of aromatic ring</td>
<td>Acid resistant</td>
</tr>
<tr>
<td>AstraZeneca</td>
<td>Nitration of pyrazole</td>
<td>Easy scale-up, reduced byproduct formation</td>
</tr>
<tr>
<td>Syngenta/Pfizer</td>
<td>Fluorination of aldehydes/alkyl groups</td>
<td>Selectflour/DAST to be heated</td>
</tr>
<tr>
<td>Company</td>
<td>Reaction</td>
<td>Benefits</td>
</tr>
<tr>
<td>------------------</td>
<td>------------------------------------</td>
<td>------------------------------------------------</td>
</tr>
<tr>
<td>Lonza</td>
<td>Organolithium opening of carbonyl</td>
<td>Facilitated workup/safer handling of reagents</td>
</tr>
<tr>
<td>Pfizer</td>
<td>Curtius rearrangement</td>
<td>Easy scale-up (optimization)</td>
</tr>
<tr>
<td>Johnson &amp; Johnson</td>
<td>Ring expansion</td>
<td>Easy scale-up (nitrogen gas released)</td>
</tr>
<tr>
<td>Organon</td>
<td>Moffatt-Swern oxidation</td>
<td>Temperature optimization (0º instead of -45 to -70 ºC)</td>
</tr>
<tr>
<td>AstraZeneca</td>
<td>NBS Bromination</td>
<td>Better solvent (DMF instead of CCl₄)</td>
</tr>
<tr>
<td>Pfizer</td>
<td>[3+2] dipolar cycloaddition</td>
<td>Faster, easy purification in-line</td>
</tr>
<tr>
<td>Merck</td>
<td>Biphasic phenol alkylation</td>
<td>Improved mixing</td>
</tr>
</tbody>
</table>

Two examples from Pfizer, a nitration and a fluorination, are shown below in Scheme 1.4. The nitration is carried out in a Vapourtec instrument that is acid sensitive, as the harsh conditions of this reaction were difficult to adapt to flow at the onset of flow development. The nitrated bromopyridine shown is routinely achieved at room temperature in 93% yield. The fluorination of the carbaldehyde using DAST as the fluorinating agent can be achieved in 73% yield at 80 ºC.³⁵
Scheme 1.4. Convenient and efficient flow nitrations and fluorinations carried out at Pfizer\textsuperscript{35}

1.1.5.2 Methodology Development in Academia

Another area of significant application of continuous flow chemistry is in reaction methodology development. Of note are efforts toward natural product synthesis, which are often plagued by challenging transformations, numerous steps, and low yields. In a review by flow pioneer Steven Ley and coworkers, uses of flow chemistry from single reaction to entire total synthesis are described.\textsuperscript{36} Figure 1.9 shows a selection of the natural products that have been synthesized using flow chemistry, either in part or in its entirety.
The work of Ley incorporates flow chemistry in unique and groundbreaking ways, one of which is the total synthesis of grossamide in 2005, as shown in Scheme 1.5. This strategy was developed before most of the commercial flow systems became available, and therefore was carried out on completely in-house developed equipment. This apparatus was engineered to be completely computer-driven, with switching between hand packed cartridges and reaction monitoring via UV and LC-MS. Another new component of this work is the use of silica-supported horseradish peroxidase (shown as Enzyme in Scheme 1.5) for oxidative dimerization. As a new standard of what could be accomplished using flow chemistry, a synthesis that typically took four days by conventional means was carried out in hours.

Figure 1.9 A selection of natural products synthesized using continuous flow chemistry
Scheme 1.5. Total synthesis of grossamide by Ley in 2005

1.2. Use of Azides and Triazoles in Heterocyclic Chemistry*

Organic azides are widely used as precursors to a large variety of functionalities and their derivatives, including triazoles, tetrazoles, and diazo compounds. These nitrogen-containing heterocycles are often the basis of biologically active pharmacophores with antibacterial, antifungal, antiviral, and anticancer properties. Efficient, simple, and safe synthetic routes to azides thus have importance and significance to medicinal and organic chemists alike. Azide derived heterocycles now have a storied history, improving the physicochemical and pharmacokinetic properties of lead compounds in medicinal chemistry.

* The subsequent sections are reproduced with permission from Sebeika and Jones.
1.2.1. Azides and Triazoles as Pharmacophores

Representative examples of drugs currently on the market with such core functionalities are shown in Figure 1.10. Desyrel (trazodone), first developed in the 1960s was approved in 1981 as an anti-depressant.\textsuperscript{67} Alfenta (alfentanil), an analog of fentanyl, is a potent analgesic first discovered in 1976, approved in 1986 and used as a muscle relaxant during surgery.\textsuperscript{68} An azide containing drug Retrovir (azidothymidine) commonly known as AZT, was the first FDA approved treatment for AIDS in 1987.\textsuperscript{69} Cozaar (losartan) was approved in 1995 as an anti-hypertensive.\textsuperscript{70} Fentrazamide, a rice herbicide, was developed in the early 2000s.\textsuperscript{71} Most recently, Brilinta (ticagrelor) was approved as a platelet aggregation inhibitor in 2011, emerging as a new competitor to Plavix.\textsuperscript{72}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{example.png}
\caption{A selection of prescription drugs with azido and azide-derived pharmacophores}
\end{figure}
1.2.2. Popular Applications of Organic Azides

Perhaps one of the most widely used applications of azides over the past decade is through “click chemistry,” a generic term for a high yielding cycloaddition reaction that takes place under mild conditions.\(^{73}\) In the case of the most popular variant (a copper-catalyzed Huisgen 1,3-dipolar cycloaddition) an azide reacts with an acetylene to yield 1,4-substituted triazole based heterocycles. Many of these products have been advanced as drug candidates and precursors. Click reactions can also be used for protein modification,\(^{74-77}\) in carbohydrate and glyco-chemistry,\(^{78-81}\) for bioconjugation,\(^{82-85}\) polymer synthesis\(^ {86-89}\) and DNA modification.\(^ {90-93}\)

The advent of click chemistry has generated a renewed interest in the use of organic azides.

1.3. Hazards of Working with Azides

Though they have a wide reaction scope and a storied history,\(^ {94}\) organic azides are often associated with a variety of hazards. They are typically unstable and in many cases, explosive. The explosive properties stem from thermal decomposition, releasing N\(_2\) gas and generating explosive force. If azides are exposed to strong acids, they can generate hydroazodic acid, HN\(_3\) which is harmful to the respiratory system.\(^ {95}\) In order to avoid the production of this acid, azide-containing molecules are typically not subjected to acidic work-ups. Azides are also metal sensitive.\(^ {96}\) Sodium azide (NaN\(_3\)) is a flammable solid, and can ignite on contact with a metal spatula. As a result, alternatives such as Teflon spatulas are commonly used.

Azides also have adverse interactions with chlorinated solvents\(^ {97}\) In the presence of dichloromethane, explosive byproducts such as diazidomethane have been observed. Azides with unsaturated carbon-carbon bonds, as well as structures containing more than one azido moiety are noted to pose a higher risk for explosion.\(^ {98}\) The explosive properties of azides do have some practicality, as they have also been developed for use in the explosives industry, as well as in
automobile airbags. However, these properties traditionally caused the pharmaceutical industry to circumvent the need to use azide chemistry at all, except in limited circumstances and on limited scale.

1.3.1. Azide Hazard Incident: Alcon Research

It is critical that all aspects of a chemical reaction be considered when scaling up a process involving azides. In a kilo-scale laboratory at Alcon Research (Fort Worth, TX), a 20 L rotary evaporator was at the center of an azide induced explosion. Residual dichloromethane from a previous process reacted with sodium azide to form diazidomethane, which unbeknownst to the chemists on hand, collected in the condenser overnight and caused an explosion when subsequently drained. Though no fatalities resulted, it underscored the incompatibility of azides and chlorinated solvents.

Some facilities will even go as far as to construct a dedicated “bunker” in which to carry out the azide step of a process. Starting materials and reagents are assembled in the bunker, and all intermediates completely isolated from any other reagents. The products are typically stored in a dedicated location after isolation then transferred to a separate plant where the remaining reaction steps are conducted.

1.3.2. Case Study: Industrial Work-Around to Use of Azides

Bristol-Meyers Squibb proposed some problem-solving strategies for the synthesis of a Boc-protected amide intermediate (4) that is generated via an azide transfer using trimethylsilyl azide (TMSN₃), as shown in Scheme 1.6. A concern with this reaction was that residual TMSN₃ would be carried over to the next step (a Staudinger reaction) where water would be added to the system. The water has the potential to react with TMSN₃ to produce toxic HN₃.
Possible remedies were to either run the reaction at high pH, thereby preventing the hydrolysis, or to precisely control the stoichiometry of reagents used. The first-generation route began with TMSN₃ as the limiting reagent. Though the reaction was completed in high yield, the more expensive starting material (1) was required in excess. The second-generation approach offered a better solution, where the stoichiometry of the TMSN₃ was not a factor. On completion of the first step, a solution of sodium hydroxide (NaOH) is added so that residual TMSN₃ reacts to produce NaN₃, trimethylsilanol (TMSOH) and eventually, hexamethyldisiloxane (TMS₂O). The NaN₃ is eliminated in the aqueous step of the work up, thereby eliminating the possibility of producing hazardous HN₃.

Scheme 1.6 API synthesized on a large scale for commercial application

1.4. Safer Alternatives to Working with Azides

In order to utilize azides to their full potential, much work has been performed to improve handling and processing of azides, in both microscale and macroscale laboratory settings. Substantial developments have been reported through technology-assisted synthesis, including use of continuous flow methods. Additionally, reaction methodology that results in the in situ
generation of azide intermediates has proven effective in eliminating the need for azide isolation, one of the most hazardous parts of any process. Improvements in the field of diazo-transfer reagents have also proved promising both in eliminating explosion risk, as well as improving the efficiency of the reactions themselves.

1.4.1. Continuous Flow Production of Azides

The field of continuous flow chemistry has grown and advanced considerably throughout the past decade.\textsuperscript{101-103} As described previously, flow chemistry takes advantage of a constant flow of reagent solutions, in contrast to the fixed amounts associated with a typical batch process. These processes can employ reactor chips, tubes, columns, or coils. Continuous flow processes provide consistent heating and mixing, unlike a batch process where conventional methods (viz. oil baths and stirring bars) do not always produce a reproducible and steady reaction environment. The surface area to volume ratios typical for flow reactions are very high (unlike in a batch reaction) which in the case of potentially explosive substances (e.g. azides) greatly reduces the prospect of bulk detonation.\textsuperscript{104}

Reactions can be optimized on a very small scale using a micro reactor such that limited amounts of reactants are used. This has the added benefit that the reagent costs and waste generation are minimal as the process is optimized. In some cases, a multi-step reaction that requires workup or purification can be carried out in a one-pot process, eliminating extraneous steps and saving both time and reagents. The consistency afforded through the precise control of flow process parameters allows for the accurate manipulation of reaction parameters. Accordingly, reactions can be more effectively optimized.\textsuperscript{105}

The optimized conditions identified from a microgram scale reaction can then be directly applied to the synthesis on a gram or kilogram scale. To produce a larger amount of product, a
flow reaction is merely “scaled out” rather than “scaled up.” Unlike under batch conditions where a reaction may work well on a small scale but falter once increased (a rule of thumb being 4X) a flow reaction produces a larger amount of product by “stacking” a series of reactors in parallel. This protocol provides a safer, (and greener) scaling process for reagents that could typically be hazardous on the larger scale (e.g. azides).106

1.4.1.1. Continuous Flow Application: 2H-azirines Using Photochemistry

In 2013, Cludius-Brandt et al. described a continuous flow synthesis of 2H-azirines from organic azides and alkenes employing photochemistry.107 Utilizing photochemistry in combination with flow provided a unique opportunity to take advantage of two easily controllable techniques in tandem. Under typical batch conditions, photochemistry requires the use of specialized glassware, and is often difficult to control precisely, especially on larger scales.108 Reactions that are typically disfavored under thermal conditions have the potential to be carried out successfully.109 Accordingly, if the reaction is to be performed on a large scale, a parallel set of reactors can be used in the scale up process, allowing for a uniform exposure to the light source while variables like the power of irradiation and flow rate can be manipulated with ease. The initial vinyl azides (5a-f) were obtained by first reacting with an alkene on a solid phase support using a bound iodine azide transfer reagent. The effluent was then subjected to a solid supported DBU to effect hydroiodic acid elimination to generate the vinyl azide.

The scope of the continuous flow process used to generate the 2H-azirines is shown in Scheme 1.7. The azide is combined with an electron-deficient alkene (6a-d) at room temperature using Teflon tubing to generate 2H-azirines (7). The concentration of reagents, ratio of reagents, solvent, and flow rate were varied.
Scheme 1.7. Phenyl-substituted vinyl azides with electron deficient alkenes generated in flow.  

Table 1.2 illustrates the optimization of one such azirine synthesis (10), where acrylonitrile (9) was reacted with a styryl azide bearing methyl ester functionality in the para position (8 Scheme 1.8). Optimal parameters produced a 96% yield under relatively benign conditions and minimal amounts of azide. Though the reactions performed under photochemical conditions required longer reaction times, the increase in yield and purity over thermal conditions was substantial.

<table>
<thead>
<tr>
<th>Entry</th>
<th>[9] (M)</th>
<th>Ratio (8:9)</th>
<th>Solvent</th>
<th>Flow (mL/min)</th>
<th>Isolated Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.025</td>
<td>1:10</td>
<td>toluene</td>
<td>0.05</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>0.025</td>
<td>1:10</td>
<td>benzene</td>
<td>0.05</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>0.025</td>
<td>1:10</td>
<td>CH₃CN</td>
<td>0.05</td>
<td>46</td>
</tr>
<tr>
<td>4</td>
<td>0.025</td>
<td>1:10</td>
<td>CH₃CN</td>
<td>0.1</td>
<td>53</td>
</tr>
<tr>
<td>5</td>
<td>0.012</td>
<td>1:10</td>
<td>CH₃CN</td>
<td>0.05</td>
<td>82</td>
</tr>
<tr>
<td>6</td>
<td>0.012</td>
<td>1:10</td>
<td>CH₃CN</td>
<td>0.1</td>
<td>74</td>
</tr>
<tr>
<td>7</td>
<td>0.012</td>
<td>1:10</td>
<td>CH₃CN</td>
<td>0.2</td>
<td>68</td>
</tr>
</tbody>
</table>
1.4.1.2. Continuous Flow Application: Click Chemistry in Flow (Tahir et al)

Click chemistry has gained widespread momentum since its advent in 2001. Tahir et al. reported the first click-flow process performed on a glass microchip, functionalized with β-cyclodextrin (β-CD). As indicated in Scheme 1.9, the copper catalyzed azide-alkyne cycloaddition (CuAAC) was carried out using mostly benzylic azides (11a-f) with both aromatic or aliphatic alkynes (12a-f). The functionalized glass microchip approach led to generation of a wide variety of 1,2,3-triazoles (13a-k) in very high yields, a sampling of which are indicated in Table 1.3. The scale up/out process has been effected using multiple microchips in parallel. The functionalized surface was found to be particularly stable, with no signs of degradation after 50 hours of use. The reaction can also be carried out at room temperature, which adds to the advantages of this process, reducing the risk of explosive events with the reactive azide species.

Scheme 1.9. A β-CD functionalized microchip used for the first time in flow synthesis.
Table 1.3. Scope of 1,2,3-triazoles synthesized via a β-CD functionalized glass microchip\textsuperscript{111}

<table>
<thead>
<tr>
<th>Entry</th>
<th>Azide</th>
<th>Alkyne</th>
<th>Product</th>
<th>Isolated Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><img src="image1" alt="Chemical Structure" /></td>
<td><img src="image2" alt="Chemical Structure" /></td>
<td><img src="image3" alt="Chemical Structure" /></td>
<td>99</td>
</tr>
<tr>
<td>2</td>
<td><img src="image4" alt="Chemical Structure" /></td>
<td><img src="image5" alt="Chemical Structure" /></td>
<td><img src="image6" alt="Chemical Structure" /></td>
<td>37</td>
</tr>
<tr>
<td>3</td>
<td><img src="image7" alt="Chemical Structure" /></td>
<td><img src="image8" alt="Chemical Structure" /></td>
<td><img src="image9" alt="Chemical Structure" /></td>
<td>99</td>
</tr>
<tr>
<td>4</td>
<td><img src="image10" alt="Chemical Structure" /></td>
<td><img src="image11" alt="Chemical Structure" /></td>
<td><img src="image12" alt="Chemical Structure" /></td>
<td>99</td>
</tr>
<tr>
<td>5</td>
<td><img src="image13" alt="Chemical Structure" /></td>
<td><img src="image14" alt="Chemical Structure" /></td>
<td><img src="image15" alt="Chemical Structure" /></td>
<td>98</td>
</tr>
<tr>
<td>6</td>
<td><img src="image16" alt="Chemical Structure" /></td>
<td><img src="image17" alt="Chemical Structure" /></td>
<td><img src="image18" alt="Chemical Structure" /></td>
<td>99</td>
</tr>
<tr>
<td>7</td>
<td><img src="image19" alt="Chemical Structure" /></td>
<td><img src="image20" alt="Chemical Structure" /></td>
<td><img src="image21" alt="Chemical Structure" /></td>
<td>99</td>
</tr>
</tbody>
</table>
1.4.1.3. Continuous Flow Application: Click Chemistry in Flow (Otvos et al)

Another application of click chemistry in flow was demonstrated by Otvos et al., through the use of a stainless steel block fitted with a copper-containing catalyst bed. The azide (14) and alkyne (15) reagents are pumped through the channel using a standard HPLC pump, as shown in Scheme 1.10. The block can be heated to the desired temperature, and the 1,2,3-triazole product (16a) is collected as effluent. The fact that this is a homogeneous copper source makes it particularly unique, in that most click reactions are carried out under heterogeneous conditions.
Scheme 1.10. A homogeneous copper source and the use of a buffer allows for the high yielding synthesis of 1,4-substituted triazoles.\textsuperscript{[112]}

The triazoles produced are β-aminocyclohexyl carboxylic acid derivatives (Table 1.4, 16a-f). This class of compounds has been shown to have antifungal properties, and are logical precursors for other synthetic endeavors. The reactions are typically run at relatively low azide concentration, so as to avoid the production of insoluble triazole byproducts. The reaction is also carried out in the presence of a buffer (N,N-diisopropylethylamine and acetic acid), which prevents the formation of byproducts and also increases the overall reactivity of the system. In terms of biological relevance, dicarboxylated triazole products have shown antitubercular activity,\textsuperscript{[113]} prompting the authors to generate a variety of molecules synthesized from internal alkynes. In unrelated applications, triazoles coupled to ferrocenes have been proposed as chemical probes,\textsuperscript{[114]} and have also attracted interest in polymer\textsuperscript{[115]} and peptide\textsuperscript{[116]} chemistries.

The process was amenable to scale up/out to yield gram quantities. In 100 min, the reaction of 1 equivalent of azide and 1.5 equivalents of the corresponding acetylene yielded 2.04
g of the triazole product, in overall 96% yield. The same copper catalyst was used throughout the entire run, suggesting that its versatility and long term stability would translate well to processes in an industrial setting.

**Table 1.4.** Scope of β-aminocyclohexanecarboxylic acid derivatives produced via the copper column in flow.¹¹²

<table>
<thead>
<tr>
<th>Entry</th>
<th>Azide (1 eq)</th>
<th>Acetylene (1.5 eq)</th>
<th>Triazole</th>
<th>Isolated Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>14a</td>
<td>15a</td>
<td>16a</td>
<td>96</td>
</tr>
<tr>
<td>2</td>
<td>14b</td>
<td>15a</td>
<td>16b</td>
<td>97</td>
</tr>
<tr>
<td>3</td>
<td>14a</td>
<td>15b</td>
<td>16c</td>
<td>97</td>
</tr>
<tr>
<td>4</td>
<td>14b</td>
<td>15b</td>
<td>16d</td>
<td>98</td>
</tr>
<tr>
<td>5</td>
<td>14a</td>
<td>15c</td>
<td>16e</td>
<td>93</td>
</tr>
<tr>
<td>6</td>
<td>14b</td>
<td>15c</td>
<td>16f</td>
<td>97</td>
</tr>
</tbody>
</table>
1.4.1.4. Continuous Flow Application: Tetrazole Formation (Palde et al)

In a similar vein, tetrazoles can also be synthesized using flow chemistry. The original reaction was pioneered by Sharpless, wherein sodium azide (NaN₃) reacted with nitriles in the presence of aqueous zinc bromide (ZnBr₂). However, this conventional process is not conducive to scaling up, as it utilizes large quantities of metal-containing compounds that are potentially toxic, and large excesses of azide in solution, which lends itself to the possibility of inadvertent hydroazodic acid (HN₃) generation.

As a result, a flow process was developed by Palde et al, with some distinct advantages (Scheme 1.11). Almost a 1:1 ratio of NaN₃ to nitrile (17) is utilized so as to reduce the possibility of explosive decomposition. The reaction is maintained under near-neutral pH to prevent generation of HN₃. An in-line quench feed of a NaNO₂ solution also helps eliminate any unreacted azide. Additionally, as a consequence of the flow synthesis not requiring the use of the ZnBr₂ promoter, the generation of the hydrated byproduct is reduced.

\[ \text{Scheme 1.11} \]

Flow process suppresses the formation of byproducts generated from nitrile hydration

Table 1.5 illustrates the scope of tetrazoles synthesized (18a-i) in high yield. The solvent ratio of 9:1 NMP/H₂O (NMP=N-methyl-2-pyrrolidone) proved optimal, and 1.05 equivalents of azide were sufficient to achieve high yield, but low enough to prevent excess azide exposure or
byproduct formation. The high pressure achieved by this flow system allows for the reaction to be run at high temperatures without increased risk of explosion.

The process was also able to be scaled up/out successfully, showing its applicability to small scale production. The reaction to produce the naphthyl-substituted tetrazole ran for 2.5 hours at 0.35 mL/min to produce 9.7 g of desired product in 96% yield. At such a rate, the system would be able to produce 116 g/day, which averages out to approx. 5 g/hr.

Table 1.5. Tetrazoles formed using the modified Sharpless method in flow

<table>
<thead>
<tr>
<th>Entry</th>
<th>Tetrazole</th>
<th>Isolated Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>18a</td>
<td>98</td>
</tr>
<tr>
<td>2</td>
<td>18b</td>
<td>95</td>
</tr>
<tr>
<td>3</td>
<td>18c</td>
<td>95</td>
</tr>
<tr>
<td>4</td>
<td>18d</td>
<td>85</td>
</tr>
<tr>
<td>5</td>
<td>18e</td>
<td>93</td>
</tr>
<tr>
<td>6</td>
<td>18f</td>
<td>96</td>
</tr>
<tr>
<td>7</td>
<td>18g</td>
<td>98</td>
</tr>
<tr>
<td>8</td>
<td>18h</td>
<td>77</td>
</tr>
<tr>
<td>Entry</td>
<td>Tetrazole</td>
<td>Isolated Yield (%)</td>
</tr>
<tr>
<td>-------</td>
<td>-----------</td>
<td>--------------------</td>
</tr>
<tr>
<td>9</td>
<td><img src="image" alt="18i" /></td>
<td>79</td>
</tr>
</tbody>
</table>

### 1.4.1.5. Continuous Flow Application: Multi-Component Click Reaction (Bogdan et al)

Bogdan et al have also applied flow chemistry to a multicomponent reaction (MCR) to produce a library of 1,2,3-triazoles. An alkyne is reacted with an alkyl halide in the presence of sodium azide, DMF, heat, and a copper catalyst. The azide is therefore generated in the process of the reaction and does not need to be isolated.

A copper flow reactor has been developed to meet this need. The Conjure flow reactor, as shown in **Figure 1.11**, takes advantage of a segmented flow method. In this method, perfluoromethyldecalin is used to separate each segment from one another. Thus, multiple reactions can be run sequentially through the reactor, which reduces reaction volumes and the quantity of waste. The reactor displays high efficiency, lending itself well to a library development scenario.

The reaction itself takes place on the reactor diskette. Here, a coil of copper or PTFE tubing runs between two copper plates, ranging from a volume of 2-10 mL. Each reaction segment was run on approximately a 400 µL volume, allowing for multiple segments to run in sequence. The copper reactor is sufficient for the reaction to proceed at high conversion rates such that no extra copper reagents are necessary. Also, the copper can be reused for hundreds of reactions, according to the authors, without loss of catalysis, adding to the green chemistry properties of this method.
Figure 1.11. The Conjure flow reactor developed for segmented flow synthesis of triazoles

The general reaction is shown in Scheme 1.12. A total of 6 different halides and 6 different acetylenes were reacted in combination with one another to afford a diverse triazole library. Aside from solution preparation, no extra chemical handling was necessary. The conditions were optimized for the reaction between 4-ethynyltoluene and 2-bromoethanol and then applied to the other systems.

\[
\begin{align*}
\text{R}_1 = & -\text{PhMe, -pyridine, -CH}_2\text{CH}_2\text{Ph, -CH}_2\text{OPhCOH, -4H-chromen-4-one, -isoquinolone} \\
\text{R}_2 = & \text{H, Me} \\
\text{R}_3 = & \text{Me, Et, -CH}_2\text{OH, oxetane, =CONHEt, -2-chloropyridine} \\
X = & \text{Br, Cl, I}
\end{align*}
\]

Scheme 1.12. A library of 1,4-substituted triazoles generated in a copper flow reactor

Table 1.6 shows a selection of these triazole products and their respective yields. Throughout all substrate combinations, the yields of the 30 library members ranged from 21-92% depending on the combination. Many reactions took as little as 5 minutes to achieve. The
process also was shown to have direct scale-up ability. In 12 minutes, 115 mg of compound 21c were isolated, which corresponds to 575 mg/hr, or 13.8 g/day.

Table 1.6. Library of triazole products via the Conjure flow reactor.\textsuperscript{118}

<table>
<thead>
<tr>
<th>Entry</th>
<th>Product</th>
<th>Isolated Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><img src="image" alt="21a" /></td>
<td>72</td>
</tr>
<tr>
<td>2</td>
<td><img src="image" alt="21b" /></td>
<td>72</td>
</tr>
<tr>
<td>3</td>
<td><img src="image" alt="21c" /></td>
<td>61</td>
</tr>
<tr>
<td>4</td>
<td><img src="image" alt="21d" /></td>
<td>76</td>
</tr>
<tr>
<td>5</td>
<td><img src="image" alt="21e" /></td>
<td>92</td>
</tr>
<tr>
<td>6</td>
<td><img src="image" alt="21f" /></td>
<td>63</td>
</tr>
<tr>
<td>7</td>
<td><img src="image" alt="21g" /></td>
<td>50</td>
</tr>
<tr>
<td>8</td>
<td><img src="image" alt="21h" /></td>
<td>32</td>
</tr>
</tbody>
</table>
1.4.2. Safer Diazo Transfer Reagents

Diazo transfer reagents are used to provide synthetic access to organic azides and diazo-containing compounds under safe(r) conditions. The products can be further utilized in olefination,\textsuperscript{119} insertion \textsuperscript{120} and cross coupling reactions,\textsuperscript{121} among many others. Diazo transfer reactions are particularly desirable because they often show a high degree of stereoselectivity with non-racemic substrates. A consequence of this is that in the case of reactions involving chiral amines the diazo group can act as an in situ protecting group, typically retaining the original configuration.\textsuperscript{122}

Sulfonyl azides have been conventionally used as diazo transfer agents, including tosyl azide (TsN\(_3\)) and triflyl azide (TfN\(_3\)).\textsuperscript{123} However, they have been reported to decompose under a variety of reaction conditions, generating a potential hazard and explosive concern. One potential byproducts is sulfuryl azide (N\(_3\)-SO\(_2\)-N\(_3\)), known as a particularly high risk for explosion,\textsuperscript{124} As a result, much effort has been placed into identifying sulfonyl azide derivatives that are more benign and can be used on a larger scale.

Efforts have been made in recent years to incorporate solid-phase synthesis into the process. Much research has been performed in developing polymer-supported diazo transfer
agents, as will be detailed in the following section. This popular alternative provides an active azide source while allowing for residual traces to be removed by filtration on reaction completion.

1.4.2.1. Diazo Transfer Application: Polystyrene-Supported Sulfonyl Azide (Green et al)

Green et al. utilized a polystyrene-supported benzene sulfonyl azide (23) to transfer the diazo group to an activated methylene (24), yielding diazo compound (25) (Scheme 1.13).\textsuperscript{125} The reaction proceeds at room temperature, of obvious safety advantage.

\[
\begin{align*}
\text{Polystyrene-bound benzene sulfonyl chloride used to conduct diazo transfer}^{125}
\end{align*}
\]

In order to determine the scope of the reaction, diazo transfer was carried out with both the polymer supported agent (23), as well as a common, solution based agent (4-carboxybenzene-sulfonyl azide, p-CBSA) to yield a variety of diazo compounds (25a-g). Such comparison highlighted both the utility and efficiency of the polymer-supported method (Table 1.7).
Table 1.7. Comparison of diazo product yield and reaction time for PS and p-CBSA methods

<table>
<thead>
<tr>
<th>Entry</th>
<th>Diazo Product</th>
<th>Isolated Yield from 20 (%)</th>
<th>Time (h)</th>
<th>Isolated yield from p-CBSA (%)</th>
<th>Time (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>25a</td>
<td>93</td>
<td>2.5</td>
<td>82</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>25b</td>
<td>65</td>
<td>2.5</td>
<td>67</td>
<td>12</td>
</tr>
<tr>
<td>3</td>
<td>25c</td>
<td>98</td>
<td>4</td>
<td>93</td>
<td>16</td>
</tr>
<tr>
<td>4</td>
<td>25d</td>
<td>75</td>
<td>16</td>
<td>80</td>
<td>16</td>
</tr>
<tr>
<td>5</td>
<td>25e</td>
<td>63</td>
<td>16</td>
<td>77</td>
<td>16</td>
</tr>
<tr>
<td>6</td>
<td>25f</td>
<td>NR</td>
<td>16</td>
<td>NR</td>
<td>16</td>
</tr>
<tr>
<td>7</td>
<td>25g</td>
<td>85</td>
<td>2.5</td>
<td>76</td>
<td>16</td>
</tr>
</tbody>
</table>

The polymer-supported reagent was generally successful, resulting in decreased reaction times or higher yields in most cases. Filtration of the remaining resin afforded product in good purity, lending itself to a safer handling of these often dangerous reagents.
1.4.2.2. **Diazo Transfer Application: Solution-Based Salts of Imidazole-Sulfonyl Azides (Goddard-Borger et al)**

In terms of *solution-based* diazo transfer, there have also been recent developments in alternatives to the standard TfN₃ reagents. Goddard-Borger *et al* reported a new agent, imidazole-1-sulfonyl azide (27), and its various salts (28a-f), as a safer alternative \(^{126}\) (**Scheme 1.14**). The intent was to identify a stable, crystalline solid that was less costly to prepare than existing alternatives (the hydrochloride).

![Scheme 1.14. Route to crystalline diazo transfer agents \(^{126}\)](image)

Some examples of the azides produced (30a-j) by reacting amines (29a-j) with imidazole-1-sulfonyl azide hydrochloride are shown in **Table 1.8**. Overall, the use of this salt lead to increased reaction yields and decreased reaction times. The reactions were conducted at room temperature, thus contributing to increased safety. It should be noted that the salt was also screened for the ability to add the diazo group to an activated methylene, but the products proved too volatile to give synthetically useful yields.

**Table 1.8. Scope of azide synthesis from amines and imidazole-1-sulfonyl azide hydrochloride**

<table>
<thead>
<tr>
<th>Entry</th>
<th>Amine</th>
<th>Product</th>
<th>Time (hr)</th>
<th>Isolated Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><img src="image" alt="29a" /></td>
<td><img src="image" alt="30a" /></td>
<td>12</td>
<td>84</td>
</tr>
<tr>
<td>2</td>
<td><img src="image" alt="29b" /></td>
<td><img src="image" alt="30b" /></td>
<td>12</td>
<td>85</td>
</tr>
</tbody>
</table>
Despite the improved properties of this reagent, some safety concerns arose regarding its storage. An explosive event was reported attributed to the concentrated alcohol that was used to crystallize the solid.\textsuperscript{127} Accordingly, the alcohol should be treated as if it were contained in solution of HN\textsubscript{3}.

The authors primary concern regarding the hazards associated with this diazo transfer agent stemmed from the use of HCl to form the salt. Accordingly, a variety of other acids were examined.\textsuperscript{128} As shown in Scheme 9, other acids scrutinized included sulfuric acid (H\textsubscript{2}SO\textsubscript{4}), methanesulfonic acid (MsOH), p-toluenesulfonic acid (TsOH), perchloric acid (HClO\textsubscript{4}), and

<table>
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<tr>
<th></th>
<th>[\text{\includegraphics[width=0.3\textwidth]{image1.png}}]</th>
<th>[\text{\includegraphics[width=0.3\textwidth]{image2.png}}]</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>(\text{\includegraphics[width=0.2\textwidth]{image1.png}}) \textsuperscript{29c} (\text{\includegraphics[width=0.2\textwidth]{image2.png}}) \textsuperscript{30c}</td>
<td>12</td>
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<tr>
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<td>12</td>
<td>66</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>(\text{\includegraphics[width=0.2\textwidth]{image1.png}}) \textsuperscript{29e} (\text{\includegraphics[width=0.2\textwidth]{image2.png}}) \textsuperscript{30e}</td>
<td>12</td>
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</tr>
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<td>(\text{\includegraphics[width=0.2\textwidth]{image1.png}}) \textsuperscript{29f} (\text{\includegraphics[width=0.2\textwidth]{image2.png}}) \textsuperscript{30f}</td>
<td>6</td>
<td>90</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>(\text{\includegraphics[width=0.2\textwidth]{image1.png}}) \textsuperscript{29g} (\text{\includegraphics[width=0.2\textwidth]{image2.png}}) \textsuperscript{30g}</td>
<td>6</td>
<td>83</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>(\text{\includegraphics[width=0.2\textwidth]{image1.png}}) \textsuperscript{29h} (\text{\includegraphics[width=0.2\textwidth]{image2.png}}) \textsuperscript{30h}</td>
<td>4</td>
<td>91</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>(\text{\includegraphics[width=0.2\textwidth]{image1.png}}) \textsuperscript{29i} (\text{\includegraphics[width=0.2\textwidth]{image2.png}}) \textsuperscript{30i}</td>
<td>4</td>
<td>78</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>(\text{\includegraphics[width=0.2\textwidth]{image1.png}}) \textsuperscript{29j} (\text{\includegraphics[width=0.2\textwidth]{image2.png}}) \textsuperscript{30j}</td>
<td>12</td>
<td>85</td>
<td></td>
</tr>
</tbody>
</table>
tetrafluoroboric acid (HBF$_4$). In order to compare the effectiveness of the various salts using the different acids, 4-aminobenzoic acid was converted to 4-azidobenzoic acid using each salt as shown in Table 1.9. The reaction yields relative to each salt show comparability among the series.

In order to assess stability of each salt, differential scanning calorimetry was used to determine the melting point and decomposition onset temperature for each member of the series. The lowest of all these temperatures was 88 °C, implying these reagents can all hypothetically be used up until that temperature (the authors advise not going beyond 60°C for drying or storing materials). The sensitivity of the materials to friction and impact was also determined. The UN Recommendations on the Transport of Dangerous Goods provides the standard for this attribute. The most sensitive compounds were the salts of HCl, HClO$_4$, and TsOH, with the others having either a very low sensitivity or total insensitivity.

With all properties and efficacy taken into account, it was determined that the HBF$_4$ and H$_2$SO$_4$ salts were the safest to handle. The HBF$_4$ salt had a longer shelf life, but the H$_2$SO$_4$ salt lent itself to an easier and more cost effective preparation.

Table 1.9. Comparison of acids used to crystallize the diazo transfer salt

<table>
<thead>
<tr>
<th>Acid</th>
<th>Isolated Yield of Salt (%)</th>
<th>Decomp. Temp. (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCl</td>
<td>63</td>
<td>102</td>
</tr>
<tr>
<td>H$_2$SO$_4$</td>
<td>63</td>
<td>131</td>
</tr>
<tr>
<td>MsOH</td>
<td>61</td>
<td>88</td>
</tr>
<tr>
<td>TsOH</td>
<td>70</td>
<td>114</td>
</tr>
<tr>
<td>HClO$_4$</td>
<td>57</td>
<td>140</td>
</tr>
<tr>
<td>HBF$_4$</td>
<td>64</td>
<td>146</td>
</tr>
</tbody>
</table>

1.4.2.3. Diazo Transfer Application: PEG-Supported Sulfonyl Azide (Castro et al)

More recently, Castro et al. have utilized imidazole-1-sulfonyl azide hydrochloride to prepare azido based solid supports (32a-d) from amine solid supports (31a-d). A variety of
resins were used, including polyethylene glycol (PEG), polystyrene (PS) and polyethylene glycol grafted onto polystyrene (PEG-PS), as shown in **Scheme 1.15**. These solid supports range from hydrophobic (PS) to hydrophilic (PEG).

Based on the solid support used, a variety of solvents and bases were examined in order to ensure optimal azide conversion. With a PS-based resin e.g. aminomethyl polystyrene (AMPS) or 4-methylbenzhydrylamine polystyrene (MBHA), the optimal solvent was DMSO, yielding almost quantitative yields without the presence of a copper catalyst. This served to confirm the utility and effectiveness of the diazo transfer agent (DIEA was used as a base with this support). With a more hydrophilic solid support, such as the PEG-PS Tentagel S NH$_2$ (TG S NH$_2$) or the PEG aminomethyl-ChemMatrix (AMCM), water was used as the solvent. K$_2$CO$_3$ was used as the base, yielding almost quantitative yields based on various equivalents of transfer agent.

![Scheme 1.15](image)

**Scheme 1.15.** Resin based route to azides \(^{130}\)

This azide was then used in a typical CuAAC reaction to yield solid bound 1,2,3-triazoles (34a-d), as shown in **Scheme 1.16**. This is a safer method because it allows for easy removal of the residual azide. The synthesis of this resin-bound triazole demonstrates its utility for subsequent synthesis beyond use of the pure azide itself.
Scheme 1.16. Synthesis of resin-bound triazoles conducted at ambient temperature \(^{130}\)

1.4.3. Azides Generated In-Situ

In order to avoid the hazardous workup and isolation of pure azides, new methodology has been developed to lend itself to a “one pot” synthesis. In this reaction environment, the intermediate (the azide) is generated in situ and carried on to a subsequent step, without the need for isolation or purification. This strategy alleviates the safety concerns associated with a particularly hazardous reagent, the operator avoiding having to directly handle or isolate it during the course of the reaction.

A number of reports underscore the practicality of using this approach for the situ generation of azides for “click” chemistry. Since the advent of the CuAAC reaction, which is far superior to the un-catalyzed variant,\(^{73}\) improvements have been sought to reduce the safety concerns associated with the azide components, as well as to utilize a heterogeneous copper source so as to avoid contamination and waste from a homogeneous copper salt.

1.4.3.1. In-Situ Generated Azide Application: Click Chemistry (Quan et al)

As an example Quan et al., used 3,4-dihydropyrimidinones (DHPMs) (35a-h) to generate azides \textit{in situ}, which proceed to react with alkynes in the presence of copper iodide to yield 1,2,3-triazoles (36a-h) via click chemistry (Scheme 1.17).\(^{131}\) To enhance yields further, copper acetate (Cu(OAc)\(_2\)•H\(_2\)O) and sodium ascorbate (NaAsc) were used in place of CuI.
The one pot conditions lent themselves to a range of aryl substrates, a representative portion of which are shown in Table 1.10. Both electron donating and electron withdrawing substituents were applicable using the Cu(OAc)$_2$•H$_2$O/NaAsc catalytic method. Though not illustrated, other studies involved thioketone analogs, and functionalization of the alkyne phenyl group. Such changes still resulted in reasonable reaction yields, underscoring the utility of the process.

**Table 1.10.** Triazoles formed from one-pot click reaction with a variety of aryl groups $^{131}$

<table>
<thead>
<tr>
<th>Entry</th>
<th>Aryl Group (Ar)</th>
<th>Product</th>
<th>Isolated Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><img src="image" alt="35a" /></td>
<td><img src="image" alt="36a" /></td>
<td>76</td>
</tr>
<tr>
<td>2</td>
<td><img src="image" alt="35b" /></td>
<td><img src="image" alt="36b" /></td>
<td>73</td>
</tr>
<tr>
<td>3</td>
<td><img src="image" alt="35c" /></td>
<td><img src="image" alt="36c" /></td>
<td>72</td>
</tr>
</tbody>
</table>
1.4.3.2. In-Situ Generated Azide Application: Click Chemistry (Kaboudin et al)

Kaboudin et al demonstrated a “one pot synthesis” of 1,2,3-triazoles via the in situ generation of azides from arylboronic acids. This method shows good functional group tolerance, low overall toxicity, and high product stability. It can be carried out in 4 h at room temperature, in the presence of air, and with water as a solvent.
The method relies on the generation of a copper(II)-β-cyclodextrin (Cu₂-β-CD) complex formed from copper(II) sulfate and β-cyclodextrin in a sodium hydroxide (NaOH) solution. The Cu₂-β-CD reacts with an arylboronic acid and sodium azide in water, followed by the subsequent aqueous click reaction with alkynes at room temperature, as shown in Scheme 1.18.

![Scheme 1.18](image)

Scheme 1.18. The arylboronic acid route to triazoles

A variety of arylboronic acids (37a-i) were explored, along with various alkyne click components (38a-i), as shown in Table 1.11. The longest reaction time was 6 h, and all were conducted at room temperature under ambient atmospheric pressure. The Cu₂-β-CD was required in only 5 mol%, adding to the ‘green’ advantages of the reaction. This reaction also catered for both aromatic and aliphatic alkynes, yielding a diverse spectrum of triazoles (39a-q).

Table 1.11. Scope of triazoles synthesized from arylboronic acids via azide intermediates

<table>
<thead>
<tr>
<th>Entry</th>
<th>Aryl Group (Ar)</th>
<th>Alkyne (R)</th>
<th>Triazole Product</th>
<th>Time (hr)</th>
<th>Isolated Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>37a</td>
<td>38a</td>
<td>39a</td>
<td>4</td>
<td>94</td>
</tr>
<tr>
<td>2</td>
<td>37b</td>
<td>38a</td>
<td>39b</td>
<td>2</td>
<td>94</td>
</tr>
<tr>
<td>3</td>
<td>37c</td>
<td>38a</td>
<td>39c</td>
<td>6</td>
<td>95</td>
</tr>
<tr>
<td></td>
<td>37e</td>
<td>38a</td>
<td>39d</td>
<td>2</td>
<td>96</td>
</tr>
<tr>
<td>---</td>
<td>-----</td>
<td>-----</td>
<td>-----</td>
<td>---</td>
<td>----</td>
</tr>
<tr>
<td>5</td>
<td>37e</td>
<td>38a</td>
<td>39e</td>
<td>4</td>
<td>94</td>
</tr>
<tr>
<td>6</td>
<td>37f</td>
<td>38a</td>
<td>39f</td>
<td>4</td>
<td>97</td>
</tr>
<tr>
<td>7</td>
<td>37g</td>
<td>38a</td>
<td>39g</td>
<td>4</td>
<td>96</td>
</tr>
<tr>
<td>8</td>
<td>37h</td>
<td>38a</td>
<td>39h</td>
<td>4</td>
<td>94</td>
</tr>
<tr>
<td>9</td>
<td>37i</td>
<td>38a</td>
<td>39i</td>
<td>4</td>
<td>94</td>
</tr>
<tr>
<td>10</td>
<td>37a</td>
<td>38b</td>
<td>39j</td>
<td>4</td>
<td>95</td>
</tr>
<tr>
<td>11</td>
<td>37a</td>
<td>38c</td>
<td>39k</td>
<td>4</td>
<td>95</td>
</tr>
<tr>
<td>12</td>
<td>37a</td>
<td>38d</td>
<td>39l</td>
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<td>13</td>
<td>37a</td>
<td>38e</td>
<td>39m</td>
<td>4</td>
<td>89</td>
</tr>
</tbody>
</table>
1.4.3.3. In-Situ Generated Azide Application: Solid-Supported Click Reaction (Roy et al)

An emerging trend has been to immobilize the copper catalyst on a solid support, thus making it easier to recover at the end of the reaction. Roy et al. report the use of a polymer support for the copper catalyst, which was constructed by binding copper (II) acetate to polystyrene-based imidazole ligand, denoted as Cu-PsIm (40). It is both inexpensive and simple to prepare, as shown in Scheme 1.19.
Scheme 1.19. Immobilization of the copper source

The goal for this catalyst was to establish a “one-pot” synthesis of 1,2,3-triazoles from an aryl starting material. The reaction was designed to be operative in water at room temperature with a minimal amount of azide and copper catalyst. In the most basic example, phenyl azide was formed in situ via the diazotization reaction of anilines, as shown in Scheme 1.20. The azide was then converted directly to the 1,4-substituted 1,2,3-triazole (42a-g) at room temperature via Cu-PsIm mediated click reaction with phenylacetylene. The scope of aryl amines (41a-g) is shown in Table 1.12. Substitutions at all positions on the aniline ring, with both electron donating and electron withdrawing substituents gave consistently good yield. Aside from the phenyl substitution, benzyl amine (Entry 7) was also studied, reacting in a similar manner to the phenyl substituted substrate. Though not shown, a variety of alkyne sources were also examined, all equally high yielding.
Scheme 1.20. High yielding Cu click process ⁱ³³

Table 1.12. Scope of 1,4-substituted triazoles from aniline intermediates ⁱ³³

<table>
<thead>
<tr>
<th>Entry</th>
<th>R</th>
<th>Product</th>
<th>Time (hr)</th>
<th>Isolated Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>H</td>
<td>42a</td>
<td>7</td>
<td>97</td>
</tr>
<tr>
<td>2</td>
<td>4-OMe</td>
<td>42b</td>
<td>7</td>
<td>97</td>
</tr>
<tr>
<td>3</td>
<td>2-NO₂</td>
<td>42c</td>
<td>8</td>
<td>92</td>
</tr>
<tr>
<td>4</td>
<td>3-OH</td>
<td>42d</td>
<td>8</td>
<td>94</td>
</tr>
<tr>
<td>5</td>
<td>2-I</td>
<td>42e</td>
<td>8</td>
<td>86</td>
</tr>
<tr>
<td>6</td>
<td>3-Cl</td>
<td>42f</td>
<td>7</td>
<td>90</td>
</tr>
</tbody>
</table>
This example utilizes benzylamine, as opposed to the previously listed anilines.

1.4.3.4 In-Situ Generated Azide Application: Microwave-Assisted Click (Appukkuttan et al)

Another approach to in situ generated azides is through the application of multi-component “click” reactions stemming from alkyl halide starting materials. Appukkuttan et al have described a microwave-assisted “click” process which allows for complete selectivity toward the 1,4-substituted triazole products, as shown in Scheme 1.21. The alkyl azide is generated in situ by a substitution reaction of the halide with sodium azide. The copper(I) species used for catalysis is also generated in situ by comproportionation of the Cu(0) and Cu(II) species in solution. The azide is subsequently reacted under microwave irradiation with acetylene to form exclusively the 1,4-substituted triazole. The microwave process not only resulted in high yields, but also in expedited reaction times.

A wide scope of alkyl halides (43a-h) were explored, as shown in Table 1.13. Though not pictured here, a variety of alkynes were also utilized. The products (44a-f) were easily isolated by crystallization and filtration. The yields are extremely high, above 84%, and were obtained in only 10-15 minutes. Direct comparison of the bromide and chloride leaving groups (Entry 1 and 2 of Table 1.13) generated the corresponding benzyl-substituted triazoles in almost identical yield at the same reaction time. Of other interesting note is a halide substrate not shown in the table – methyl iodide. The N-methyltriazole was formed in 89% yield after only 10 minutes. The corresponding methyl azide is particularly hazardous and volatile, so chemistry utilizing this compound is often very difficult to achieve. Adding a variety of substituents in all
locations relative to the halide showed no hindered effect of the reaction, resulting in a variety of functionalized rings that lend themselves to further elaboration.

\[
\begin{align*}
R_1 &= \text{Ph, -p-NO}_2\text{-Ph, -p-CN-Ph,} \\
o-\text{NO}_2\text{-Ph, -2,3,4-trimethoxy-Ph,} \\
m-\text{Cl-p-OMe-Ph, -CH}_2\text{Ph} \\
X &= \text{Br, Cl}
\end{align*}
\]

Scheme 1.21. The microwave-assisted, selective “click” synthesis of 1,2,3-triazoles in a multicomponent reaction system \(^{134}\)

Table 1.13. Scope of 1,4-substituted triazoles from a regioselective MCR using microwave irradiation \(^{134}\)

<table>
<thead>
<tr>
<th>Entry</th>
<th>Halide</th>
<th>Product</th>
<th>Time (min)</th>
<th>Isolated Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><img src="image1" alt="43a" /></td>
<td><img src="image2" alt="44a" /></td>
<td>10</td>
<td>93</td>
</tr>
<tr>
<td>2</td>
<td><img src="image3" alt="43b" /></td>
<td><img src="image2" alt="44a" /></td>
<td>10</td>
<td>91</td>
</tr>
<tr>
<td>3</td>
<td><img src="image4" alt="43c" /></td>
<td><img src="image5" alt="44b" /></td>
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<td>86</td>
</tr>
<tr>
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<td><img src="image7" alt="44c" /></td>
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<td>84</td>
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<td><img src="image8" alt="43e" /></td>
<td><img src="image9" alt="44d" /></td>
<td>10</td>
<td>87</td>
</tr>
</tbody>
</table>
1.5 Conclusion

Azide building blocks have enjoyed a rise in popularity in recent years, largely due to the advent of click chemistry. As a result, safer alternatives for handling pure azides have been a topic of considerable development. Primary concerns that have been addressed are their potentially explosive properties, specifically when a reaction generates hydroazodic acid or diazidomethane. Technology-assisted synthesis is an optimal platform to address this concern, particularly in the field of continuous flow chemistry. Use of flow conditions reduces the amount of reagent necessary for a reaction, as well as the reaction time, and allows for increased pressures, even mixing and heating, typically resulting in improved yields. Diazo transfer agents have also seen an increase in use, based on the emergence of safer protocols which use polymer-bound resins that can be easily removed from a reaction mixture. Lastly, reactions that generate azides in situ allow for the use of azide chemistry that circumvents potentially dangerous workup and isolation operations. In sum, use of modern technology in process design has opened the doors for the use of azides and diazo compounds in green syntheses where safety and efficacy
are greatly enhanced in comparison to traditional methods. The application of a method inspired by these principles will be described in Chapter 2.

1.6 References


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133. Roy, S.; Chatterjee, T.; Islam, S. M. Polymer anchored Cu(II) complex: an efficient and recyclable catalytic system for the one-pot synthesis of 1,4-disubstituted 1,2,3-triazoles starting from anilines in water. *Green Chem.* **2013**, *15* (9), 2532-2539.

134. Appukkuttan, P.; Dehaen, W.; Fokin, V. V.; Van der Eycken, E. A microwave-assisted click chemistry synthesis of 1,4-disubstituted 1,2,3-triazoles via a copper(I)-catalyzed three-component reaction. *Org. Lett.* **2004**, *6* (23), 4223-4225.
CHAPTER 2: Implementing a Process Oriented Guided Inquiry Learning (POGIL) Undergraduate Laboratory Activity Utilizing Continuous Flow Chemistry

2.1 Introduction

This chapter will detail efforts toward introducing students to green chemistry, a technique widely used by industrial chemists, and reaction optimization using continuous flow chemistry via a Process-Oriented Guided Inquiry Learning (POGIL) undergraduate laboratory experiment. First, a survey of inquiry-based learning will be presented, followed by a background into POGIL, as well as its previous applications in undergraduate laboratory courses. Then, the research project that developed the continuous flow reaction will be discussed, followed by the development a POGIL activity for upper-level undergraduates.

2.1.1 Types of Inquiry-Based Learning

The following sections will outline a background of four types of inquiry-based learning that are utilized in designing active learning activities. Active learning activities, as described by Bonwell, are “instructional activities involving students in doing things and thinking about what they are doing.” The author suggests that in order for students to take part in active learning, they “must engage in higher-order thinking tasks such as analysis, synthesis and evaluation.” These skills lie on the higher end of Bloom’s Taxonomy, which is depicted in Figure 2.1. The revised taxonomy was published in 2001, and is shown along with some of the actions students are carrying out at a given level of thought. As an educator, the taxonomy helps structure learning objectives of a course, an assignment, or an assessment from the side of what a student should be doing, rather than simply what chapters or what material should be covered. Traditional lectures can be modified to incorporate active learning into a given classroom via a variety of strategies, including the use of web-based response systems, clickers, peer instruction, and the subject of focus for this chapter: inquiry based learning.
Inquiry-based learning has four different levels, which will be discussed in the subsequent sections. The varying levels are dependent on how much of the material is provided to the student by the instructor and how much is generated by the student. Adapted from Tafoya, 

Figure 2.2 shows an overview of the levels of inquiry, with respect to participation of both student and instructor.\textsuperscript{15} As the level of inquiry increases, the extent of student involvement in the inquiry process increases. The four levels will be discussed in further detail in the next sections.
2.1.1.1 Confirmation Inquiry

Confirmation inquiry is the first level of inquiry, in which the instructor provides all materials relative to the activity, including the problem or question, the steps or procedure to solve the problem, with the outcome already known at the start. This type of inquiry is best utilized when the instructor’s aim is to reinforce a topic that has previously been covered, particularly by demonstrating the steps needed to take to solve a problem. An example of this type of inquiry is a laboratory experiment that emphasizes students collecting a set of data to confirm a known outcome. This method allows students to practice the act of collecting information and making the connection to the concept that was previously taught.

2.1.1.2 Structured Inquiry

The second level of inquiry is structured inquiry. In this level, the instructor provides a question to the students, while also providing a procedure or set of steps for the students to follow. Unlike confirmation inquiry, the students do not know the answer or solution ahead of time, and therefore are discovering it as they progress through the procedure.
many traditional laboratory experiments follow this type of protocol. The students are presented with a protocol, for example, to discover the correlation between two variables. Following the protocol given, the students carry out a set of experiments, varying each, and then answer a final question at the end of activity.

2.1.1.3 Guided Inquiry

Guided inquiry is the third level of inquiry, and is the most pertinent to the subsequent sections of this chapter. In this particular type of inquiry, the instructor presents students with a question or topic, and through a series of student produced or selected procedures/steps, students come to their own conclusions or generate an answer to the question that was proposed in the beginning of the exercise. This method of inquiry promotes student collaboration, as students typically work through these assignments in groups, where each student brings a unique perspective and his or her own prior knowledge to the group. Within this collaborative environment, students are “given opportunities to work with other students to formulate their ideas and also are encouraged to create a deeper understanding for themselves.” Guided inquiry will be discussed further in subsequent sections.

2.1.1.4 Open Inquiry

The fourth and perhaps most unstructured of all the levels of inquiry is open inquiry. In this model, students are given an open-ended opportunity to select a topic, choose how to explore it, and ultimately come up with an answer, conclusion or outcome. This type of inquiry ultimately plays into a topic in which a student has a particular interest or curiosity. For example, the topic given by the instructor may be as broad as “related to the topics discussed in class,” which requires students to utilize skills that are high on the Create category of Bloom’s Taxonomy, such as synthesizing, designing, planning, and producing. Open inquiry requires
that students have a broad prior knowledge, which often may not be applicable until a substantial amount of foundational material within a course has been covered.

2.1.2 Process Oriented Guided Inquiry Learning

Of relevance to this work is a specialized type of guided inquiry, called Process Oriented Guided Inquiry Learning (POGIL). POGIL was developed in the 1990s and primarily was implemented in chemistry classrooms. Three of the pioneers from Franklin and Marshall College in Lancaster, PA, John J. Farrell, Richard S. Moog, and James N. Spencer, founded the POGIL Project, a non-profit organization that promotes the use of POGIL, assists with grant writing, holds regional and national workshops, and provides support to instructors who are implementing the activities in the classroom.

A POGIL course does not consist of traditional style lectures. As outlined in one of their first reports on the subject,\textsuperscript{18} students work in small groups, ideally groups of four our five, to carry out a series of guided inquiry activities. At the end of the activity’s allotted time, the groups share their results with the class, which allows the full group to engage in discussion. The textbook is then read at home after working through the activities, and is essentially used to supplement and enforce the material, as opposed to its traditional use as a primary source of the material.

2.1.2.1 Roles in POGIL Classrooms

Each group member is assigned a role; some of the possibilities are the Manager, the Recorder, the Technician, the Reflector and the Presenter. Depending on the activity, some or all of these roles can be filled. The Manager is in charge of keeping track of time and ensuring that the group stays focused on the task at hand and accomplishes all required components of the activity. The Recorder maintains a record of the group members, roles, and the consensus
conclusions, points, and answers that the groups has finalized. The collective answers generated by the Recorder are submitted to the instructor at the end of class for feedback, clarification and any further explanation that may be needed. The Technician is responsible for carrying out any calculations that require the use of a calculator or manipulating any apparatus required by the activity. The Reflector observes the group, their interactions, and group dynamics, and jots down notes about these observations, often sharing them with the class at the end. Finally, the Presenter is spokesperson of the group, who will ultimately share out the group’s results with the class for a larger class discussion. These criteria can be slightly altered to fit an activity, and therefore should be considered guidelines rather than strict rules.

The instructor of the course will circle the room during the class period, listening to each group and making notes about his or her observations, points of confusion, and offering minimal, guiding questions if a group has encountered difficulty. It is to the discretion of the instructor whether to intervene when he or she notices a series of incorrect answers or a significant misunderstanding, and will typically be dependent on the group dynamic.

2.1.2.2 Structure of POGIL Activities

POGIL activities are often broken down into three main sections. First, the Model, Data and/or Information section gives the student some context for the topic that is going to be covered by the activity. This section may consist of a variety of media, such as text, figures, equations, tables, and/or data. Next, the Critical Thinking Questions (CTQs) guide students through interpreting data, developing connections and ultimately positing inferences or conclusions drawn from the aforementioned data. Finally, the Applications section contains exercises that require the use of knowledge, connections and inferences made in the first two sections, to apply that knowledge to a different set of circumstances. Depending on the length of
class and depth of discussion, these questions can also be carried out from home after the student has recorded the results of the CTQs. The class time can be structured as seen fit by the instructor. Figure 2.3 shows the proposed structure of a POGIL course in the model presented by Spencer, et al.

Figure 2.3 Typical flow of a POGIL class as described by Spencer et al

In terms of the efficacy of the POGIL course described by Spencer et al, a breakdown of a comparison of student grades in traditional general chemistry lecture versus POGIL general chemistry is shown in Table 2.1. In these two similarly sized cohorts, the percentage of students receiving an A or B in the course increased, while the students receiving a C stayed almost the same. More powerfully, the number of students who failed the course dropped significantly from 3.6% to 0.2%. In a combined number of students receiving a D, F, or withdrawing from the course went from 21.9% to 9.6%, a significant change. These courses were taught by the same
instructors with very similarly structured exams, which is crucial when making this type of comparison. Several other examples will be discussed in upcoming sections.

Table 2.1. Comparison of traditional general chemistry lecture to POGIL class, adapted from Spencer et al\textsuperscript{18}

<table>
<thead>
<tr>
<th>Period</th>
<th>n</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>W</th>
<th>F</th>
<th>D+W+F</th>
</tr>
</thead>
<tbody>
<tr>
<td>F90-S94 (Traditional)</td>
<td>420</td>
<td>19.3</td>
<td>33.1</td>
<td>25.7</td>
<td>9.0</td>
<td>9.3</td>
<td>3.6</td>
<td>21.9</td>
</tr>
<tr>
<td>F94-S97 (POGIL)</td>
<td>438</td>
<td>24.2</td>
<td>40.6</td>
<td>25.6</td>
<td>7.1</td>
<td>2.3</td>
<td>0.2</td>
<td>9.6</td>
</tr>
</tbody>
</table>

2.1.2.3 Design Considerations and Advantages of POGIL laboratory activities

An opportunity that is particularly relevant to this chapter is to employ POGIL in laboratory course. There are numerous reports of POGIL activities in literature, which will be discussed in the following section. Utilizing POGIL for a laboratory exercise affords students the chance to develop a hypothesis at the beginning of the experiment, collect data, and possibly change and adapt the hypothesis throughout the exercise. After supporting or refuting the developed hypothesis, students then apply the knowledge they have acquired, which conveniently for a lab exercise can come in the form of analyzing an unknown sample\textsuperscript{18}. Guiding questions can be used, similarly to the worksheets, to assist in the formulation of a hypothesis and design of experimental steps to test the hypothesis. In this way, students are not simply following recipe-like steps and being handed a conclusion, and are operating in a scenario that better mirrors the research process they may encounter in graduate school or industrial research.

2.1.2.4 POGIL Laboratory Examples in Literature

Examples of POGIL laboratory exercises have been documented from the late 1990’s. Exercises for laboratory courses such as general chemistry,\textsuperscript{19,25} inorganic chemistry,\textsuperscript{26} and
physical chemistry\textsuperscript{27, 28} have been reported, but of particular interest to this work are the applications in organic chemistry laboratory. 

One of the earliest applications of guided inquiry in an organic chemistry laboratory was pioneered as “discovery chemistry” by Jarret et. al. at College of the Holy Cross.\textsuperscript{24, 29} In these experiments, students form a hypothesis about a reaction using prior knowledge of, for example, reactions with similar substrates. In one example, the authors propose a reaction of an alkene with bromine, and ask students to predict the type of addition that will occur based on other addition reactions they’ve studied. Students then prove or disprove their hypothesis by reacting a series of stilbene molecules with bromine, as shown in Scheme 2.1. The students use melting point to determine which isomer has been formed, as the melting points are different by almost 100 °C. As a result, students will elucidate the mechanism of addition by determining the stereochemistry of the product.\textsuperscript{24}

\begin{center}
\begin{tikzpicture}

\node at (0,0) [draw] (cis-stilbene) {cis-stilbene};
\node at (2,0) [draw] (trans-stilbene) {trans-stilbene};
\node at (2,1) [draw] (br1) {Br$_2$};
\node at (2,2) (br2) {Br$_2$};

\draw [->] (cis-stilbene) -- (br1);
\draw [->] (trans-stilbene) -- (br2);

\node at (2,3) (c) {meso if syn addition (\pm) if anti addition};
\node at (2,4.5) (t) {c} {meso if syn addition (\pm) if anti addition};

\end{tikzpicture}
\end{center}

\textbf{Scheme 2.1} Stilbene reactions to elucidate addition of bromine to an alkene\textsuperscript{24}

In 2001, a group from Illinois Wesleyan University proposed a similar “discovery-oriented” approach to uncovering the stereochemistry of an E2 elimination reaction.\textsuperscript{30} Shown in \textbf{Scheme 2.2}, the reaction of trans-2-methylcyclohexyl tosylate with potassium tert-butoxide could possibly yield two possible isomers. Students make a prediction about the resulting
product, and often will incorrectly predict product A, as they are familiar with the Zaitsev rule and would postulate that the more substituted alkene would be favored. However, upon taking an NMR and GC of the product, students observe that exclusively product B is formed.

![Scheme 2.2](image)

**Scheme 2.2** Possible elimination products students must characterize to determine E2 mechanism

In a second week, students then perform the same reaction with the cis starting material, and after making similar predictions based on the previous week’s lesson, observe a distribution of products, favoring product A. This result allows students to consider factors beyond the bulkiness of the base and understand the anti-periplanar relationship between the proton being abstracted by the base and the leaving group in an E2 reaction.

Another example from Fordham University aims for students to probe the diastereoselectivity of Grignard addition to a ketone. As shown in **Scheme 2.3**, students react methyl magnesium iodide with an asymmetric ketone, (+)-benzoin, to form **Diastereomer A** and **Diastereomer B**. The 97:3 mixture (A to B) is purified by simple recrystallization and the product is characterized by melting point, as the two isomers have distinct melting points (104 °C and 94 °C respectively).

![Scheme 2.3](image)

**Scheme 2.3** The diastereoslective Grignard reaction adapted for a POGIL exercise
Thus, students discover the selectivity of the reaction in the process of isolating the product, as opposed to reading it out of a textbook. This observation leads to a further post-lab discussion of Cram’s rule, which provides rationale for the diastereoselectivity of addition to asymmetric ketones. In this example, students discuss the Cram Chelate Model, where the magnesium metal coordinates the oxygen atoms of both the carbonyl and the alcohol, yielding a rigid intermediate that necessitates the addition from the least sterically hindered face, thus resulting in a majority of Diastereomer A.

2.1.3 Examples of Continuous Flow Laboratory Experiments in Literature

Aside from the literature precedent for POGIL activities, there have been a number of continuous flow and microfluidic laboratory experiments reported. Some of these experiments have analytical, bioanalytical, and biochemical applications. In many undergraduate teaching laboratories, funds to purchase microfluidic devices from major manufacturers are not often available. In this case, there have been reports of small, student-fabricated microfluidic devices that can be used in the laboratory.

To highlight one of these examples, Feng et al report their student-fabricated devices for application in organic or inorganic chemistry. Students use Shrinky-Dinks, or polystyrene sheets, to print the channel of interest. A PDMS mixture is baked on top of the mold to generate the resulting microfluidic device. Once the custom device has been fabricated, it can be used for a variety of reactions, one of which is the synthesis of an azo dye, as shown in Scheme 2.4. A simple acidification of the resulting solution affords the solid product that students characterize by infrared spectroscopy and UV-Vis spectroscopy.
Scheme 2.4 Student-produced polystyrene flow device for the synthesis of azo dyes

Another unique experiment reported by van Rens et al utilizes a FutureChemistry® flow device called the FlowStart Remote, which allows students from VU University Amsterdam to control and monitor a flow device in real time remotely. The LabVIEW application on the computer is used as the interface, so there is no limitation on where in the world or what time of day this experiment can take place. A depiction of the student interface is shown in Figure 2.4. The students carried out the synthesis of methyl orange, as shown in Scheme 2.5. The temperature of the reaction was varied between 8-80 °C and the flow rate of each reagent adjusted as the students discovered the dependence of reaction yield on each of these variables. Students therefore had access to an instrument as well as an entire method of optimizing reactions that normally would be inaccessible, and were able to synthesize a compound with a potentially hazardous diazonium intermediate without having to isolate it.
Continuous flow reactions are typically thought of in the liquid form; however, examples using gas-phase reactions have been described. One example is the work of Mattson et al at Creighton University. The overall transformation is shown in Scheme 2.6, where propanol is converted to propane.
An acidified alumina-bead catalyst tube is used for the first reaction, the dehydration of propanol. Following the generation and purification of propene via a cold trap, a hydrogenation reaction is carried out in a catalyst tube charged with ceramic-supported palladium catalyst. In this tube, propane is formed and withdrawn via syringe. The three apparatuses used in this experiment are shown in Figure 2.5. Students then carry out ¹H NMR experiments to verify the production of the product.⁴³

![Image of apparatuses](image)

Figure 2.5 The gas-phase apparatuses used in the transformation of propanol to propane (Reproduced with permission)⁴³

Another example of a more traditional organic reaction is the flow dehydration of fructose to produce the biologically relevant intermediate 5-hydroxymethylfurfural (HMF), as
shown in Scheme 2.7. Using a glass-tube reactor fabricated in-house connected to compressed air, students react a mixture of fructose, tetraethylammonium bromide (TEAB) and sulfuric acid at 100°C to produce HMF. Purification and crystallization form the precipitated product at high conversion rates. Using HPLC and NMR, the product is characterized.\textsuperscript{44}

\[
\text{fructose} \xrightarrow{\text{Et}_4\text{NBr}} \xrightarrow{\text{H}_2\text{SO}_4} \text{HMF}
\]

**Scheme 2.7** Continuous flow synthesis of HMF from fructose\textsuperscript{44}

Finally, König et al, in a collaboration between Universität Regensburg and Cardiff University, developed a series of continuous flow experiments for implementation in the organic chemistry laboratory.\textsuperscript{45} Utilizing microreactors purchased from Little Things Factory, the authors propose six different reactions, details for which are described in Table 2.2. The emphasis on this series is industrially relevant reactions, as students are given exposure not only to flow chemistry methodology and instrumentation but also to a diverse set of reactions carried out regularly in industrial settings.

<table>
<thead>
<tr>
<th>Starting Material(s)</th>
<th>Product</th>
<th>Reaction Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-methoxyaniline, benzoyl chloride</td>
<td>N-(4-methoxyphenyl)benzamide</td>
<td>Amide bond formation</td>
</tr>
<tr>
<td>4-nitrophenyl acetate</td>
<td>4-nitrophenol</td>
<td>Hydrolysis</td>
</tr>
</tbody>
</table>
Condensation

benzil, 1,3-diphenylacetone
tetraphenylcyclopentadienone

Grignard

phenylmagnesium bromide, fluorenone
9-Phenyl-9H-fluoren-9-ol

Esterification

4-nitrophenol
4-nitrophenyl acetate

Bromination

anisole
4-bromoanisole

2.1.4 Work on 1,2,3-Triazole Formation Using Flow†

Triazoles and their fused heterocyclic derivatives have become important pharmacophores in drug discovery, with several FDA approved drugs currently on the market and many more in the pipeline.\textsuperscript{46-51} Brilinta (ticagrelor), developed by AstraZeneca and approved by the FDA in 2011 as a novel anti-platelet agent.\textsuperscript{52-54} Triazole chemistry has gained much popularity from the work of K. Barry Sharpless,\textsuperscript{55} with the copper-catalyzed Huisgen cyclization of azides with alkynes affording efficient access to substituted 1,2,3-triazoles.\textsuperscript{56} Coined, and popularized, as “click chemistry,” this reaction has many useful applications in synthetic,\textsuperscript{57-59} polymer\textsuperscript{60-62} and biological\textsuperscript{63-65} chemistries, among others. A similar core can be accessed via [3+2] cycloaddition

† The following sections have been reproduced with permission from Jones et al.\textsuperscript{1}
of functionalized azides with cyano-containing amides, such as that with cyanoacetamide, as shown in Scheme 2.8.

![Scheme 2.8.](image)

Scheme 2.8. 1,2,3-Triazoles and corresponding triazolopyrimidines via [3+2] cycloaddition chemistry

Given potential safety concerns regarding the use of organic azides in macroscale synthesis, a number of adaptations have been examined to mitigate risk, including the use of continuous flow methods. Continuous flow chemistry is an ever-growing and developing field with a variety of distinct advantages. The superior mixing capabilities are derived from a high surface-to-volume ratio, which allows for reactions to take place more efficiently, even employing temperatures above the boiling point of the solvent. Reactant heating is highly consistent, allowing for superb temperature control and reaction reproducibility. This methodology has the potential to allow the development of process conditions for the reduction of waste and a decrease in the direct handling of potentially hazardous materials. The nature of flow chemistry allows for reaction conditions optimized in a microscale environment to be directly scaled to the macroscale level, which is of particular importance when considering industrial processing.

Though continuous flow chemistry has been shown to be a highly effective method for azide synthesis, an obvious limitation still lies in the need to prepare and handle bulk quantities of the organic azide, which pose additional hazards in the case of low molecular weight variants. To address this issue, we elected to investigate the *in situ* production of organic azides
in a flow reactor, and then directly utilize this in line for the production of triazoles, as shown in 

Figure 2.6.

(a)

Figure 2.6 Diagram of continuous flow reactor for triazole library synthesis (a) two-step, one-pot reaction (b) two-step sequential flow reaction

2.1.4.1 Proof of Principle with Benzyl Bromide and Benzyl Azide

Using a Labtrix S1 flow system (Chemtrix BV, NL) equipped with a T-mixer microreactor, the conversion of benzyl bromide 1 to benzyl azide 2 was verified under flow conditions using HPLC-MS standards (for calibration curves, see Appendix). Table 2.3 and Table 2.4 show the results of the optimization of continuous flow conditions, including temperature and residence time. Under optimal conditions (Figure 2.7), essentially quantitative yields could be obtained within a few minutes at moderate reaction temperatures < 100 °C. Only product and recovered starting material was observed in the resulting chromatograms.
Table 2.3 Temperature optimization of benzyl azide conversion (res time = 12.5 min)

<table>
<thead>
<tr>
<th>Temp (°C)</th>
<th>Benzyl azide (% Conv)</th>
<th>Benzyl bromide (% Rem)</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td>49</td>
<td>51</td>
</tr>
<tr>
<td>30</td>
<td>84</td>
<td>16</td>
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<tr>
<td>35</td>
<td>86</td>
<td>14</td>
</tr>
<tr>
<td>40</td>
<td>88</td>
<td>12</td>
</tr>
<tr>
<td>45</td>
<td>88</td>
<td>12</td>
</tr>
<tr>
<td>50</td>
<td>88</td>
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<td>65</td>
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<td>4</td>
</tr>
<tr>
<td>70</td>
<td>97</td>
<td>3</td>
</tr>
<tr>
<td>75</td>
<td>quantitative</td>
<td>0</td>
</tr>
<tr>
<td>80</td>
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<td>0</td>
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<tr>
<td>85</td>
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<tr>
<td>90</td>
<td>quantitative</td>
<td>0</td>
</tr>
<tr>
<td>95</td>
<td>quantitative</td>
<td>0</td>
</tr>
<tr>
<td>100</td>
<td>quantitative</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 2.4 Residence time optimization of benzyl azide conversion (T=80 °C)

<table>
<thead>
<tr>
<th>Residence time (sec)</th>
<th>Benzyl azide (% Conv)</th>
<th>Benzyl bromide (% Rem)</th>
</tr>
</thead>
<tbody>
<tr>
<td>24</td>
<td>quantitative</td>
<td>0</td>
</tr>
<tr>
<td>30</td>
<td>quantitative</td>
<td>0</td>
</tr>
<tr>
<td>40</td>
<td>quantitative</td>
<td>0</td>
</tr>
<tr>
<td>60</td>
<td>quantitative</td>
<td>0</td>
</tr>
<tr>
<td>120</td>
<td>quantitative</td>
<td>0</td>
</tr>
<tr>
<td>600</td>
<td>quantitative</td>
<td>0</td>
</tr>
<tr>
<td>1200</td>
<td>quantitative</td>
<td>0</td>
</tr>
</tbody>
</table>
Figure 2.7  Temperature optimization for the conversion of benzyl bromide 1 to benzyl azide 2 (Residence time = 750 seconds (12.5 min))

With these results in hand, attention was then focused on the subsequent cycloaddition to form the 1,2,3-triazole 3 under flow conditions using benzyl azide 2 as a model substrate (Scheme 2.9). Optimization of temperature at constant flow rate (Table 2.5) and residence time at constant temperature (Table 2.6) again showed production of triazole, with only recovered starting material observed upon HPLC analysis. Quantitative conversion was found to be possible following optimization of the flow conditions (Figure 2.8 and Figure 2.9) evaluating the effect of reaction time (0.75 – 8.33 min) and temperature (25 – 100 °C) (n = 3).

Table 2.5 Temperature optimization of benzyl triazole conversion (res time = 8.33 min)

<table>
<thead>
<tr>
<th>Temp (°C)</th>
<th>Benzyl triazole (% Conv)</th>
<th>Benzyl azide (% Rem)</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td>61</td>
<td>39</td>
</tr>
<tr>
<td>30</td>
<td>69</td>
<td>31</td>
</tr>
<tr>
<td>35</td>
<td>72</td>
<td>28</td>
</tr>
</tbody>
</table>
### Table 2.6 Residence time optimization of benzyl triazole conversion (T=80 °C)

<table>
<thead>
<tr>
<th>Residence time (sec)</th>
<th>Benzyl azide (% Conv)</th>
<th>Benzyl bromide (% Rem)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>84</td>
<td>16</td>
</tr>
<tr>
<td>12.5</td>
<td>85</td>
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<td>16.67</td>
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<td>3</td>
</tr>
<tr>
<td>500</td>
<td>98</td>
<td>2</td>
</tr>
</tbody>
</table>
**Figure 2.8.** Temperature optimization for the conversion to benzyl triazole (Residence time = 500 sec, 8.33 min)

**Figure 2.9.** Residence time optimization of conversion to benzyl triazole (Temp = 80 °C)
Following the successful optimization of the separate reaction steps, a two-step continuous flow route, directly from the bromide 1 to the triazole 3, was investigated. Reaction screening of various temperatures at both 45 s (Table 2.7) and 225 s (Table 2.8) showed no byproduct formation, as only recovered starting material was observed. As temperature increased, greater conversion to triazole was observed, while increasing the residence time showed increased yield lower temperatures, as shown in Figures 2.10 and 2.11.

The number of equivalents of cyanoacetamide and sodium hydroxide were also probed with respect to the triazole transformation. Results are shown in Table 2.9. Notably, flow synthesis was able to convert in good yields the azide to triazole with only 1 eq. of cyanoacetamide and NaOH, while batch process required 2 eq. to afford similar yields. In order to confirm utility in the formation of pyrimidine derivatives of the product, subsequent conversion to the corresponding triazolopyrimidine 4 was investigated and conditions optimized, where T = 80 °C and flow rate = 20 μL/min (Scheme 2.9).
Table 2.7 Temperature optimization of one pot, two step benzyl triazole conversion (res time 45 sec (0.75 min))

<table>
<thead>
<tr>
<th>Temp (°C)</th>
<th>Benzyl triazole (% Conv)</th>
<th>Benzyl azide (% Rem)</th>
<th>Benzyl bromide (% Rem)</th>
</tr>
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<tr>
<td>25</td>
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<tr>
<td>95</td>
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<td>24</td>
<td>5</td>
</tr>
</tbody>
</table>

Table 2.8 Temperature optimization of one pot, two step benzyl triazole conversion (res time 225 sec (3.75 min))

<table>
<thead>
<tr>
<th>Temp (°C)</th>
<th>Benzyl triazole (% Conv)</th>
<th>Benzyl azide (% Rem)</th>
<th>Benzyl bromide (% Rem)</th>
</tr>
</thead>
<tbody>
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<td>2</td>
</tr>
<tr>
<td>60</td>
<td>67</td>
<td>29</td>
<td>4</td>
</tr>
<tr>
<td>65</td>
<td>75</td>
<td>25</td>
<td>0</td>
</tr>
<tr>
<td>70</td>
<td>78</td>
<td>22</td>
<td>0</td>
</tr>
<tr>
<td>75</td>
<td>80</td>
<td>20</td>
<td>0</td>
</tr>
<tr>
<td>80</td>
<td>79</td>
<td>21</td>
<td>0</td>
</tr>
<tr>
<td>85</td>
<td>84</td>
<td>16</td>
<td>0</td>
</tr>
<tr>
<td>90</td>
<td>85</td>
<td>15</td>
<td>0</td>
</tr>
<tr>
<td>95</td>
<td>85</td>
<td>15</td>
<td>0</td>
</tr>
</tbody>
</table>
Figure 2.10. Optimization for the one-pot, two-step triazole formation under flow conditions (Residence time = 45 sec (0.75 min))

Figure 2.11. Optimization for the one-pot, two-step triazole formation under flow conditions (Residence time = 225 sec (3.75 min))
Table 2.9. Investigation of the effect of starting material and base equivalents for the two-step, one-pot triazole synthesis

<table>
<thead>
<tr>
<th>Equivalents of cyanoacetamide/NaOH</th>
<th>Percent Conversion to Triazole</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>71</td>
</tr>
<tr>
<td>2</td>
<td>80</td>
</tr>
<tr>
<td>3</td>
<td>91</td>
</tr>
</tbody>
</table>

2.1.4.2 Synthesis of a Triazole Library Under Continuous Flow Conditions

With an effective route secured, the reaction scope was explored by forming a library of triazoles, thus confirming the versatility of the approach (Table 2.10). Entries 1, 2, 7, and 8 were successfully synthesized in a two-step, one-pot flow reaction in good to excellent yields. The azides were produced *in situ* with a residence time of 2.5 min, and the triazole subsequently prepared with a residence time of 1.9 min for a total reaction time of 4.4 min. The remaining entries were generated in a two-step sequential flow reaction, where the azide was first prepared in 6 min. Then a crude azide solution was re-injected into the chip, along with the cyanoacetamide and sodium hydroxide, to produce the triazole in 4.2 min, for a total reaction time of 10.2 min. Figure 2.6 details the flow reactor assembly for the production of the triazole library.

When the steps were carried out sequentially, as shown in Table 2.11, chlorinated substrates 7 and 18 produced insoluble salts during azide formation, likely due to the decrease in water present in that reaction system. Thus, when these substrates were reacted in a one-pot fashion, the amount of water in the system increased, and the reaction proceeded without solubility issues. Sulfur-containing substrates 9 and 11 suffered from low yields, which we attribute to the formation of an intermediate similar to 1-chloro-2-[(2-chloroethyl)sulfonyl]ethane (sulfur mustard). Extending the carbon chain to substrate 23 which contains a double bond may undergo its own 3+2 cycloaddition as a side reaction.
Table 2.10. Continuous flow synthesis of triazole analogs in (a) configuration using 1 eq of azide and 3 eq of cyanoacetamide and NaOH

<table>
<thead>
<tr>
<th>Entry</th>
<th>Substrate</th>
<th>Product</th>
<th>Percent Conversion to Azide (% Recovered SM)</th>
<th>Percent Conversion to Triazole</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><img src="5" alt="image" /></td>
<td><img src="6" alt="image" /></td>
<td>4</td>
<td>96</td>
</tr>
<tr>
<td>2</td>
<td><img src="7" alt="image" /></td>
<td><img src="8" alt="image" /></td>
<td>3 (2)</td>
<td>95</td>
</tr>
<tr>
<td>3</td>
<td><img src="9" alt="image" /></td>
<td><img src="10" alt="image" /></td>
<td>5 (95)</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td><img src="11" alt="image" /></td>
<td><img src="12" alt="image" /></td>
<td>12 (88)</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td><img src="13" alt="image" /></td>
<td><img src="14" alt="image" /></td>
<td>44 (33)</td>
<td>23</td>
</tr>
<tr>
<td>6</td>
<td><img src="15" alt="image" /></td>
<td><img src="14" alt="image" /></td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Chemical Structure</td>
<td>Chemical Structure</td>
<td></td>
<td></td>
</tr>
<tr>
<td>---</td>
<td>--------------------</td>
<td>--------------------</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>7</td>
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<td>8</td>
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<td><img src="image17" alt="Structure 17" /></td>
<td>19 (7)</td>
<td>74</td>
</tr>
<tr>
<td>9</td>
<td><img src="image19" alt="Structure 19" /></td>
<td><img src="image20" alt="Structure 20" /></td>
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<td>24</td>
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<tr>
<td>10</td>
<td><img src="image21" alt="Structure 21" /></td>
<td><img src="image22" alt="Structure 22" /></td>
<td>18</td>
<td>82</td>
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<td>11</td>
<td><img src="image23" alt="Structure 23" /></td>
<td><img src="image24" alt="Structure 24" /></td>
<td>77</td>
<td>23</td>
</tr>
</tbody>
</table>
Table 2.11. Continuous flow synthesis of triazole analogs in (b) configuration using 1 eq of azide and 2 or 3 eq of cyanoacetamide and NaOH.

<table>
<thead>
<tr>
<th>Entry</th>
<th>Substrate</th>
<th>Product</th>
<th>% Conversion to Azide</th>
<th>% Conversion to Triazole (2 eq)</th>
<th>% Conversion to Triazole (3 eq)</th>
</tr>
</thead>
<tbody>
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<td>99</td>
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<td>97</td>
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<td>67</td>
<td>76</td>
</tr>
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<td>75</td>
<td>73</td>
</tr>
<tr>
<td></td>
<td>Structure 1</td>
<td>Structure 2</td>
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<td>% 2</td>
<td>% 3</td>
</tr>
<tr>
<td>---</td>
<td>-------------</td>
<td>-------------</td>
<td>-----</td>
<td>-----</td>
<td>-----</td>
</tr>
<tr>
<td>6</td>
<td><img src="image1" alt="Structure 1" /></td>
<td><img src="image2" alt="Structure 2" /></td>
<td>100</td>
<td>75</td>
<td>73</td>
</tr>
<tr>
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<td><img src="image3" alt="Structure 1" /></td>
<td><img src="image4" alt="Structure 2" /></td>
<td>100</td>
<td>83</td>
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<tr>
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<td><img src="image12" alt="Structure 2" /></td>
<td>100</td>
<td>21</td>
<td>31</td>
</tr>
</tbody>
</table>

### 2.1.4.3 Synthesis of Ticagrelor Core Analogues

We expect this methodology to be of use in the design of triazole and triazolopyrimidine APIs and drug candidates. Aromatization and substitution on the 6-membered ring is a facile process, and can be used to gain access to advanced intermediates related to the cardiovascular
agent, Brilinta (ticagrelor), as shown in Figure 2.12. To synthesize these compounds, a three-step synthesis was completed to produce Brilinta analogues that can be further elaborated and tested to investigate anti-platelet properties. Scheme 2.10 displays the synthesis that was completed by the Jones Lab. Triazolopyrimidine 4 was chlorinated to give the dichlorinated species 25, then cyclopropylamine was selectively added to yield 26. Finally, the propyl sulfide group was added to afford the final analogue 27.

Figure 2.12. Brilinta (ticagrelor) contains a triazolopyrimidine core that can be accessed readily by our methodology

Scheme 2.10. Synthetic route to access ticagrelor analogues

In order to show the utility of this method for elaboration with an alkyl group similar to the ring of ticagrelor, (bromomethyl)cyclopentane (28) was used as a starting point, as shown in
Scheme 2.11. It was subjected to the same conditions as previously described to produce the 1,2,3-triazole (30), which was then carried on to the triazolopyrimidine (31). The price of the starting material (Matrix Scientific 1 g $83.20) makes it suitable for evaluation and optimization within continuous flow microreactors. Whilst detailed process optimization was not conducted, we see no reason why this method could not be further applied in an industrial setting.

Scheme 2.11. Cyclopentyl analogue with a structure similar to ticagrelor

In conclusion, a flow based method for the production of 1,2,3-triazoles and triazolopyrimidines has been developed. The process avoids the direct isolation of organic azides, adding to its enhanced safety features over the standard macroscale procedures. The flow process is highly efficient, and amenable to a wide range of substrates. This route can be applied to the synthesis of analogues of the core of ticagrelor, which can be further elaborated for investigation into anti-platelet properties.

2.1.5 Research Objectives

As previously described, use of continuous flow reactions have increased dramatically in the past two decades across many facets of chemistry. Therefore, the goal of this work is to expose students to this technology in the upper level organic chemistry laboratory with a POGIL
activity. This guided inquiry into the workings of flow will provide the fundamentals of the field, which students will discover as they work through the exercise. The exposure alone may give students a competitive advantage when applying for internships, co-ops, or jobs. We selected the Chemtrix equipment for teaching laboratory use, as it is user friendly and robust.

2.2 Results and Discussion

2.2.1 Student Materials

Why go with the flow?
A POGIL Activity for Advanced Organic Chemistry Laboratory
Developed by Meaghan M. (Sebeika) Fallano and Thomas R. Gilbert, Northeastern University, 360 Huntington Ave. Boston, MA 02115

INTRODUCTION

Typical organic reactions are carried out in round bottom flasks and are called conventional reactions. Because these reactions are typically heated in an oil bath and stirred with a magnetic stir bar, inconsistent mixing and temperature gradients are experienced. In contrast, continuous flow chemistry can be used, where reagents are prepared as solutions, loaded into syringes, and regulated syringe pumps are used to dispense reagents into the microreactor.
Continuous flow processes are solution-based, and stoichiometry is dictated by both concentration and the rate at which the solutions flow relative to one another. The total time a molecule spends in the reactor is called the residence time, which can be calculated using the volume of the microreactor and the flow rate at which the reagents are pumped into the system. Flow reactors have a high surface area to volume ratio, so the mixing and heating of these systems is extremely efficient.

Continuous flow chemistry is used in a variety of settings, from chemical and pharmaceutical manufacturing, to reaction development and optimization. For the next two weeks, we will be exploring the benefits of using solution-based continuous flow chemistry for the synthesis of 1,2,3-triazoles via a [3+2] cycloaddition of azides and alkynes. In the first week, we will synthesize benzyl azide using a nucleophilic substitution reaction, with sodium azide (NaN₃) acting as the nucleophile. In the second week, we will subsequently react the benzyl azide with cyanoacetimide to yield a 1,2,3-triazole.

**MODEL 1: Continuous flow reaction considerations**

Below is a diagram of a continuous flow microreactor, which is typically made from glass, with narrow reaction channels etched into it. (NOTE: This figure is not drawn to scale. The microreactors we will be using contain a maximum volume of 10-15 µL.)
Critical Thinking Questions

1. How does the diagram above differ from a typical reaction vessel, such as a round bottomed flask?

2. What considerations should a chemist consider regarding reagents and reaction preparation before deciding to undertake a flow reaction? What reagents or reactions might not be compatible with this system? Can you think of anything that would be incompatible with this system?

3. The reaction zone, shown as 5 in the diagram, is where the reaction takes place. The residence time is the time it takes for the reaction mixture to flow from the start to the end of the microreactor. What conditions would need to be known about the reaction to calculate the residence time? How would you set up the calculation?
MODEL 2: Synthesis of azide intermediates

Azides, denoted by the functional group N$_3$, are used as precursors to a variety of heterocyclic compounds used in many applications including pharmaceutical development and fine chemical manufacturing. The first compound we will synthesize is **benzyl azide**.

Critical Thinking Questions

1. Draw the most stable Lewis structure of benzyl azide (C$_7$H$_7$N$_3$). Add any non-zero formal charges.

2. Benzyl azide will be synthesized using sodium azide as a reagent. Based on the ionic nature of sodium azide and the structure of benzyl azide you drew, what type of reaction would produce benzyl azide as a product? **HINT**: Think back to the reactions you learned in Organic Chemistry I. Suggest a starting material you would need to carry out this transformation. Draw the mechanism of this reaction.

3. In what type of solvent (polar or nonpolar, aqueous or organic) are the reactants and products likely to be soluble?
4. An automobile airbag is inflated by the following decomposition reaction of sodium azide (NaN₃):

$$20 \text{NaN}_3(s) + 6 \text{SiO}_2(s) + 4 \text{KNO}_3(s) \rightarrow 32 \text{N}_2(g) + 5 \text{Na}_4\text{SiO}_4(s) + \text{K}_4\text{SiO}_4(s)$$

Why would azide intermediates be considered dangerous to handle and isolate, particularly due to decomposition?

5. Please ask the instructor to check the previous two questions. Upon completion, you will receive the protocol and results for the conventional (round bottom flask and traditional heating) reaction used to synthesize benzyl azide.

Based on the protocol described, propose a starting point for your flow reaction. Include your desired residence time, as determined by the flow rate, temperature, and equivalents of reagents. Calculate how long you will need to collect your reaction mixture to obtain 50 µL of reaction mixture. You will be using a microreactor with the following specifications:
EXPERIMENTAL PROTOCOL FOR AZIDE FLOW REACTION

1. Prepare solutions of the two reagents in the appropriate solvent(s). You will need a maximum of 1 mL of each solution.

2. Set the microreactor to the chosen temperature.

3. Once the desired temperature has been reached, fill the syringes with your solutions. Be sure to fill them both to the same volume level, as they will be attached to the same syringe pump.

4. Connect the syringes to the flow apparatus via the Leur Lock connectors. Place an empty HPLC vial on the end of the tubing coming out of the collection end of the apparatus.

5. Set the syringe pump to the desired flow rate. Ensure the handle is completely flush against the plunger of the syringes.

6. Start the syringe pump, and continue pumping reagents through until all the bubbles are out of the microreactor, and all solutions are flowing consistently.

7. After the flow has reached equilibrium, set a timer for your desired collection time. When ready, connect an HPLC vial containing 500 µL of water to the tubing coming out of the collection end of the apparatus and begin your collection.

8. When your time is up, stop the syringe pump and remove the HPLC vial from the tubing.

9. Return your solutions to their respective vials, and clean the syringes with water and acetone.

10. Fill one syringe with NMP and one with water. Set the flow rate to 20 µL/min and pump the solvents through the microreactor for 5 minutes to ensure it is clean for the next group.

11. Run your sample on the HPLC to obtain a chromatogram of your reaction mixture.
12. The chromatogram peak areas in this example are relative to the amount of each substance in the mixture. Integrate the peak areas, assuming the total area of all components is 1. Calculate your percent conversion.

13. You are going to choose a second set of conditions. Half the groups will vary temperature and half will vary residence time by selecting a new flow rate. Discuss your choice with the other groups varying the same parameter so that as many possible sets of conditions are covered.

14. Repeat the steps as above for your new set of conditions.

15. Once all groups are finished, report out all conditions with conversions.

**Critical Thinking Questions**

1. Based on the group results, plot two sets of data: residence time vs. percent conversion and temperature vs. percent conversion. What are the conditions that produced the best percent conversion? How do they compare to the conventional reaction?

2. Conventional reactions are based on mol quantities of reagents. Since the flow reactions are based on units of concentration (mol/L), what is the theoretical concentration of product in your vial?

3. Is this reaction more dependent on reaction time or temperature? How do you know?
4. Compare the flow reaction to the conventional reaction; what are the benefits you experience of using the flow process? Were there any drawbacks or challenges?

**MODEL 3: Synthesis of 1,2,3-triazoles**

Triazole moieties are used as precursors to a number of pharmaceutically relevant molecules. One of those drugs, ticagrelor (Brillinta) is shown below and is an inhibitor of platelet aggregation, and is used to prevent thrombic events, such as a heart attack or stroke. Today, we are going to synthesize a triazole core that can be cyclized and further modified to mirror the core of Brillinta.

![Triazole Core](image)

**Critical Thinking Questions**

1. The azide you made last week will be used to synthesize a benzyl-substituted triazole as shown below. You will receive a handout with the experimental protocol for this conventional process. Using that experimental protocol as a guide on how the product is isolated, how can we verify our product is being made in the flow system?
2. Consider the HPLC chromatogram from our reactions last week. Where will the product peak show up relative to the starting materials on the trace?

3. Based on the azide reactions and the conventional results for triazole synthesis, what conditions will you start with, in terms of temperature and flow rate to obtain the desired residence time?

4. In this reaction, we have three starting materials instead of two, however we will be using the same microreactor. Describe how you would set up this reaction using the available fittings.
EXPERIMENTAL PROTOCOL FOR TRIAOLE FLOW REACTION

1. Prepare solutions of the three reagents in the appropriate solvent(s). You will need a maximum of 1 mL of each solution.

2. Set the microreactor to the chosen temperature.

3. Once the desired temperature has been reached, fill the syringes with your solutions. Be sure to fill them all to the same volume level, as two of them will be attached to the same syringe pump.

4. Connect the syringes to the flow apparatus via the Leur Lock connectors. Place an empty HPLC vial on the end of the tubing coming out of the collection end of the apparatus.

5. Set the syringe pump to the desired flow rate. Ensure the handle is completely flush against the plunger of the syringes.

6. Start the syringe pump, and continue pumping reagents through until all the bubbles are out of the microreactor, and all solutions are flowing consistently.

7. After the flow has reached equilibrium, set a timer for your desired collection time. When ready, connect an HPLC vial containing 500 µL of water to the tubing coming out of the collection end of the apparatus and begin your collection.

8. When your time is up, stop the syringe pump, add 300 µL DMSO and ensure everything is dissolved. Take your HPLC vial for analysis and obtain the chromatogram.

9. Return your solutions to their respective vials, and clean the syringes with water and acetone.

10. Fill one syringe with NMP and one with water. Set the flow rate to 20 µL/min and pump the solvents through the microreactor for 5 minutes to ensure it is clean for the next group.
11. Obtain the percent conversion and percent remaining starting material from the HPLC chromatogram. Using these results, you are going to choose a second set of conditions. Half the groups will vary temperature and half will vary flow rate to change the residence time. Discuss your choice with the other groups varying the same parameter so that as many possible sets of conditions are covered.

12. Repeat the steps as above for your new set of conditions. Once all groups are finished, report out all conditions with conversions.

**Critical Thinking Questions**

1. Based on the group results, plot two sets of data: residence time vs. percent conversion and temperature vs. percent conversion. What are the conditions that yield the highest percent conversion for this reaction? How do they compare to the conventional reaction?

2. Copy those graphs and add a plot of the percent azide remaining vs. the same variables. What trend do you notice about this data?

3. Is this reaction more dependent on reaction time or temperature? How do you know?
4. Based on the conditions for both reactions, can you think of any ways to improve this process any further?

2.2.4 Materials for Instructors

Materials for instructors, including answers to questions, notes on consumables, protocols and chemicals, and representative data have been created (see Appendix).

2.3 Conclusions and Future Directions

In conclusion, a POGIL activity has been developed to help students learn the fundamentals of continuous flow chemistry. The next step in the process would be to pilot the experiment in the Northeastern University curriculum. An ideal audience for this experiment would be an upper division organic chemistry laboratory. The biggest limitation would be the fact that there is only one instrument and therefore group work will need to be carefully monitored. It would be advantageous for the instructor or any teaching assistants to practice using the apparatus before allowing students to proceed with the experiment. For smaller universities that cannot afford the $18,000-$35,000 for either a Start or S1 system, a long term goal would be to film students carrying out various sets of conditions along with yields, spectra and chromatograms, and creating an internet repository for students to access depending on the conditions they choose.
2.4 Experimental

2.4.1 General Methods

All reagents and solvents were purchased from Fisher Scientific or Sigma Aldrich. THF was distilled from a sodium/benzophenone ketyl for the triazolopyrimidine synthesis. All continuous flow reactions were performed using the Chemtrix Labtrix S1 system (Chemtrix BV, NL) utilizing a T-mixer glass microreactors, which are commercially available through Chemtrix BV. All reagents were dissolved, loaded into 1 mL glass gas-tight syringes (SGE) and dispensed through the microreactor into the autosampler loaded with HPLC vials. The samples were analyzed by HPLC-UV (Waters Alliance; Agilent Eclipse Plus C18 Column; 254 nm), and yields for the continuous flow reactions were determined using a prepared calibration curve of each standard. $^1$H NMR spectra were recorded at 400 MHz, and $^{13}$C NMR were recorded at 100 MHz on a Varian NMR instrument, and spectra was prepared using ACD/Labs (Toronto, Canada). High-resolution mass spectroscopy was obtained on a Waters 70-VSE (EI) or a Waters Q-ToF Ultima mass spectrometer (ESI) at the UIUC Mass Spectrometry Facility.

2.4.2. General procedure for batch synthesis of triazole library for spectroscopic analysis

![Chemical reaction diagram]

To a solution of benzyl bromide (1.00 g, 5.85 mmol) in N-methyl-2-pyrrolidinone (10 mL) was added sodium azide (0.76 g, 11.7 mmol). The reaction was stirred at 80 °C for 2 h. The reaction mixture was then cooled to 20 °C and quenched with MTBE (10 mL) and H$_2$O (15 mL). The organic layer was isolated and washed with 1M sodium bicarbonate (10 mL) and H$_2$O (10 mL), dried over MgSO$_4$, and concentrated in vacuo. To a solution of the crude azide in N-
methyl-2-pyrrolidinone (15 mL) was added cyanoacetamide (0.74 g, 8.77 mmol) and sodium hydroxide (0.35 g, 8.77 mmol). The reaction was stirred at 80 °C for 3 h. The reaction mixture was then cooled to RT, quenched with H2O (30 mL) and allowed to stir O/N at RT to allow for complete precipitation. The solid was then filtered and dried in vacuo to afford the product.

5-amino-1-benzyl-1H-1,2,3-triazole-4-carboxamide (3): light yellow powder (0.314 g, 40%). mp= 236-237 °C. (400 MHz, DMSO-d6): δ 7.45-7.17 (m, 5H), 7.09 (br s, 2H), 6.39 (br s, 2H), 5.41 (s, 2H). (100 MHz, DMSO-d6): δ 164.5, 145.0, 136.1, 128.9, 127.9, 127.6, 121.9, 48.5. HRMS (EI), m/z C10H11N5O (M)+ calcd. 217.0964, obsd. 217.0964.

![Reaction diagram]

To a solution of 2-bromobenzyl bromide (1.00 g, 4.00 mmol) in N-methyl-2-pyrrolidinone (10 mL) was added sodium azide (0.5021 g, 8.00 mmol). The reaction was stirred at 80 °C for 2 h. The reaction mixture was then cooled to 20°C and quenched with MTBE (10 mL) and H2O (15 mL). The organic layer was isolated and washed with 1M sodium bicarbonate (10 mL) and H2O (10 mL), dried over MgSO4, and concentrated in vacuo. To a solution of the crude azide in N-methyl-2-pyrrolidinone (15 mL) was added cyanoacetamide (0.3363 g, 4.00 mmol) and sodium hydroxide (0.1600 g, 4.00 mmol). The reaction was stirred at 80 °C for 3 h. The reaction mixture was then cooled to RT, quenched with H2O (30 mL) and allowed to stir O/N at RT to allow for complete precipitation. The solid was then filtered and dried in vacuo to afford the product.
5-amino-1-(2-bromobenzyl)-1H-1,2,3-triazole-4-carboxamide (6): white powder (0.58 g, 59%). mp= 215-216°C. (400 MHz, DMSO-d<sub>6</sub>): δ 7.69 (d, J=8 Hz, 1H), 7.53 (br s, 1H), 7.36 (t, J=7.6 Hz, 1H), 7.28 (t, J=7.2 Hz, 1H), 7.16 (br s, 1H), 6.63 (d, J=7.2 Hz, 1H), 6.45 (s, 2H), 5.44 (s, 2H). (100 MHz, DMSO-d<sub>6</sub>): δ 164.3, 145.4, 135.0, 132.8, 129.7, 128.1, 128.0, 121.9, 121.6, 48.9. HRMS (EI), m/z C<sub>10</sub>H<sub>10</sub>BrN<sub>5</sub>O (M)<sup>+</sup> calcd. 295.0069, obsd. 295.0067.

To a solution of 2-chlorobenzyl chloride (1.00 g, 6.21 mmol) in N-methyl-2-pyrrolidinone (10 mL) was added sodium azide (0.8073 g, 12.42 mmol). The reaction was stirred at 80 °C for 2 h. The reaction mixture was then cooled to 20 °C and quenched with MTBE (10 mL) and H<sub>2</sub>O (15 mL). The organic layer was isolated and washed with 1M sodium bicarbonate (10 mL) and H<sub>2</sub>O (10 mL), dried over MgSO<sub>4</sub>, and concentrated in vacuo. To a solution of the crude azide in N-methyl-2-pyrrolidinone (15 mL) was added cyanoacetamide (0.5221 g, 6.21 mmol) and sodium hydroxide (0.2484 g, 6.21 mmol). The reaction was stirred at 80 °C for 3 h. The reaction mixture was then cooled to RT, quenched with H<sub>2</sub>O (30 mL) and allowed to stir O/N at RT to allow for complete precipitation. The solid was then filtered and dried in vacuo to afford the product.

5-amino-1-(2-chlorobenzyl)-1H-1,2,3-triazole-4-carboxamide (8): light yellow powder (0.64 g, 49%). mp= 209-210°C. (400 MHz, DMSO-d<sub>6</sub>): δ 7.53 (m, 2H), 7.34 (m, 2H), 7.17 (br s, 1H), 6.73 (d, J=7.2 Hz, 1H), 6.50 (s, 2H), 5.49 (s, 2H). (100 MHz, DMSO-d<sub>6</sub>): δ 164.1, 145.2, 133.2, 131.6, 129.3, 129.2, 128.0, 127.3, 121.4, 48.3 (NMP impurity at 174.5, 49.2, 30.8, 29.7, 17.9).
HRMS (EI), m/z C₁₁H₁₃ClN₅O (M)+ calcd. 251.0574 obsd. 251.0579.

To a solution of 2-chloroethyl phenyl sulfide (1.00 g, 5.79 mmol) in N-methyl-2-pyrrolidinone (10 mL) was added sodium azide (0.7527 g, 11.58 mmol). The reaction was stirred at 80°C for 2 h. The reaction mixture was then cooled to 20 °C and quenched with MTBE (10 mL) and H₂O (15 mL). The organic layer was isolated and washed with 1M sodium bicarbonate (10 mL) and H₂O (10 mL), dried over MgSO₄, and concentrated in vacuo. To a solution of the crude azide in N-methyl-2-pyrrolidinone (15 mL) was added cyanoacetamide (0.4868 g, 5.79 mmol) and sodium hydroxide (0.2316 g, 5.79 mmol). The reaction was stirred at 80 °C for 3 h. The reaction mixture was then cooled to RT, quenched with H₂O (30 mL) and allowed to stir O/N at RT to allow for complete precipitation. The solid was then filtered and dried in vacuo to afford the product.

5-amino-1-(2-phenylthio)ethyl-1H-1,2,3-triazole-4-carboxamide (10): white powder (0.21 g, 20%). mp= 161-162 °C. (400 MHz, DMSO-d₆): δ 7.44 (br s, 1H), 7.36 (m, 4H), 7.23 (m, 1H), 7.09 (br s, 1H), 6.35 (br s, 2H), 4.35 (t, J=7.6 Hz, 2H), 3.37 (t, J=7.6 Hz, 2H). (100 MHz, DMSO-d₆): δ 164.5, 145.0, 135.1, 129.4, 128.9, 126.4, 122.0, 44.8, 31.3. HRMS (EI), m/z C₁₁H₁₃N₅OS (M)+ calcd. 263.0841, obsd. 263.0844.
To a solution of 3-chloropropyl phenyl sulfide (1.00 g, 5.36 mmol) in N-methyl-2-pyrrolidinone (10 mL) was added sodium azide (0.0.6963 g, 10.72 mmol). The reaction was stirred at 80 °C for 2 h. The reaction mixture was then cooled to 20 °C and quenched with MTBE (10 mL) and H₂O (15 mL). The organic layer was isolated and washed with 1M sodium bicarbonate (10 mL) and H₂O (10 mL), dried over MgSO₄, and concentrated in vacuo. To a solution of the crude azide in N-methyl-2-pyrrolidinone (15 mL) was added cyanoacetamide (0.4505 g, 5.36 mmol) and sodium hydroxide (0.2144 g, 5.36 mmol). The reaction was stirred at 80°C for 3 h. The reaction mixture was then cooled to RT, quenched with H₂O (30 mL) and allowed to stir O/N at RT to allow for complete precipitation. The solid was then filtered and dried in vacuo to afford the product.

5-amino-1-(3-(phenylthio)propyl)-1H-1,2,3-triazole-4-carboxamide (12): yellow oil (0.469 g, 47%). (400 MHz, DMSO-d₆): δ 7.47 (br s, 1H), 7.31 (m, 3H), 7.22 (m, 2H), 7.11 (br s, 1H), 6.34 (br s, 2H), 4.30 (t, J=6.8 Hz, 2H), 2.97 (t, J=7.2 Hz, 2H), 2.00 (t, J=7 Hz, 2H). (100 MHz, DMSO-d₆): δ 164.5, 145.0, 135.1, 129.4, 128.9, 126.4, 122.0, 44.8, 28.9, 28.1. HRMS (EI), m/z C₁₂H₁₅N₅O₅, (M)⁺ calcd. 277.0997, obsd. 277.0996
To a solution of β-Bromophenetole (1.00 g, 4.97 mmol) in N-methyl-2-pyrrolidinone (10 mL) was added sodium azide (0.6461 g, 9.94 mmol). The reaction was stirred at 80 °C for 2 h. The reaction mixture was then cooled to 20 °C and quenched with MTBE (10 mL) and H₂O (15 mL). The organic layer was isolated and washed with 1M sodium bicarbonate (10 mL) and H₂O (10 mL), dried over MgSO₄, and concentrated in vacuo. To a solution of the crude azide in N-methyl-2-pyrrolidinone (15 mL) was added cyanoacetamide (0.4179 g, 4.97 mmol) and sodium hydroxide (0.1915 g, 4.97 mmol). The reaction was stirred at 80 °C for 3 h. The reaction mixture was then cooled to RT, quenched with H₂O (30 mL) and allowed to stir O/N at RT to allow for complete precipitation. The solid was then filtered and dried in vacuo to afford the product.

**5-amino-1-(2-phenoxyethyl)-1H-1,2,3-triazole-4-carboxamide (14):** white powder (0.32 g g, 36%). mp= 188-189 °C. (400 MHz, DMSO-d₆): δ 7.45 (br s, 1H), 7.28 (m, 3H), 7.10 (br s, 1H), 6.93 (m, 2H), 6.35 (s, 2H), 4.55 (s, 2H), 4.32 (s, 2H). (100 MHz, DMSO-d₆): δ 164.6, 158.2, 145.4, 129.8, 121.9, 121.2, 114.7, 65.5, 45.2. (EI), m/z C₁₁H₁₃N₅O₂, (M)⁺ calcd. 247.1069, obsd. 247.1072.

![Chemical structure](image.png)

To a solution of α-bromo-para-xylene (1.00 g, 5.40 mmol) in N-methyl-2-pyrrolidinone (10 mL) was added sodium azide (0.7020 g, 10.80 mmol). The reaction was stirred at 80°C for 2 h. The reaction mixture was then cooled to 20 °C and quenched with MTBE (10 mL) and H₂O (15 mL). The organic layer was isolated and washed with 1M sodium bicarbonate (10 mL) and H₂O (10 mL), dried over MgSO₄, and concentrated in vacuo. To a solution of the crude azide in N-
methyl-2-pyrrolidinone (15 mL) was added cyanoacetamide (0.4540 g, 5.40 mmol) and sodium hydroxide (0.2160 g, 5.40 mmol). The reaction was stirred at 80 °C for 3 h. The reaction mixture was then cooled to RT, quenched with H₂O (30 mL) and allowed to stir O/N at RT to allow for complete precipitation. The solid was then filtered and dried in vacuo to afford the product.

5-amino-1-(4-methylbenzyl)-1H-1,2,3-triazole-4-carboxamide (17): white powder (0.59 g, 49%). mp= 222-223 °C. (400 MHz, DMSO-d₆): δ 7.49 (br s, 1H), 7.14 (m, 5H), 6.40 (s, 2H), 5.37 (s, 2H), 2.27 (s, 3H). (100 MHz, DMSO-d₆): δ 164.8, 145.2, 137.4, 133.4, 129.6, 127.9, 122.1, 48.5, 21.1. HRMS (EI), m/z C₁₁H₁₃N₅O (M)+ calcd. 231.1120, obsd. 231.1118.

To a solution of (2-Bromoethyl)benzene (1.00 g, 5.40 mmol) in N-methyl-2-pyrrolidinone (10 mL) was added sodium azide (0.7020 g, 10.80 mmol). The reaction was stirred at 80 °C for 2 h. The reaction mixture was then cooled to 20 °C and quenched with MTBE (10 mL) and H₂O (15 mL). The organic layer was isolated and washed with 1M sodium bicarbonate (10 mL) and H₂O (10 mL), dried over MgSO₄, and concentrated in vacuo. To a solution of the crude azide in N-methyl-2-pyrrolidinone (15 mL) was added cyanoacetamide (0.4540 g, 5.40 mmol) and sodium hydroxide (0.2160 g, 5.40 mmol). The reaction was stirred at 80°C for 3 h. The reaction mixture was then cooled to RT, quenched with H₂O (30 mL) and allowed to stir O/N at RT to allow for complete precipitation. The solid was then filtered and dried in vacuo to afford the product.
5-amino-1-phenethyl-1H-1,2,3-triazole-4-carboxamide (20): white powder (0.20 g, 26%).

mp = 201-202 °C. (400 MHz, DMSO-d₆): δ 7.42 (br s, 1H), 7.26 (m, 5H), 7.07 (br s, 1H), 6.33 (s, 2H), 4.35 (t, J=7.2 Hz, 2H), 3.04 (t, J=7.2 Hz, 2H), (100 MHz, DMSO-d₆): δ 164.6, 144.8, 138.0, 129.1, 128.6, 126.8, 121.9, 46.6, 34.5. HRMS (EI), m/z C₁₁H₁₃N₅O (M)⁺ calcd. 231.1120, obsd. 231.1118.

To a solution of 1-Bromo-3-phenylpropane (1.00 g, 5.02 mmol) in N-methyl-2-pyrrolidinone (10 mL) was added sodium azide (0.6526 g, 10.04 mmol). The reaction was stirred at 80 °C for 2 h. The reaction mixture was then cooled to 20 °C and quenched with MTBE (10 mL) and H₂O (15 mL). The organic layer was isolated and washed with 1M sodium bicarbonate (10 mL) and H₂O (10 mL), dried over MgSO₄, and concentrated in vacuo. To a solution of the crude azide in N-methyl-2-pyrrolidinone (15 mL) was added cyanoacetamide (0.4221 g, 5.02 mmol) and sodium hydroxide (0.2008 g, 5.02 mmol). The reaction was stirred at 80 °C for 3 h. The reaction mixture was then cooled to RT, quenched with H₂O (30 mL) and allowed to stir O/N at RT to allow for complete precipitation. The solid was then filtered and dried in vacuo to afford the product.

5-amino-1-(3-phenylpropyl)-1H-1,2,3-triazole-4-carboxamide (22): white powder (0.69g, 59%). mp = 196-197 °C. (400 MHz, DMSO-d₆): δ 7.44 (br s, 1H), 7.25 (m, 5H), 7.08 (br s, 1H), 6.31 (s, 2H), 4.14 (t, J=7.2 Hz, 2H), 2.58 (t, J=7.2 Hz, 2H), 2.00 (m, 2H). (100 MHz, DMSO-d₆):
δ 164.4, 144.6, 141.0, 128.4, 128.3, 126.0, 121.7, 44.9, 32.0, 30.1. HRMS (EI), m/z C_{12}H_{15}N_{5}O, (M)^{+} calcd. 245.1277, obsd. 245.1278.

To a solution of 3-Bromo-1-phenyl-1-propene (1.00 g, 5.07 mmol) in N-methyl-2-pyrrolidinone (10 mL) was added sodium azide (0.4056 g, 10.14 mmol). The reaction was stirred at 80 °C for 2 h. The reaction mixture was then cooled to 20 °C and quenched with MTBE (10 mL) and H_{2}O (15 mL). The organic layer was isolated and washed with 1M sodium bicarbonate (10 mL) and H_{2}O (10 mL), dried over MgSO_{4}, and concentrated in vacuo. To a solution of the crude azide in N-methyl-2-pyrrolidinone (15 mL) was added cyanoacetamide (0.4263 g, 5.07 mmol) and sodium hydroxide (0.2028 g, 5.07 mmol). The reaction was stirred at 80 °C for 3 h. The reaction mixture was then cooled to RT, quenched with H_{2}O (30 mL) and allowed to stir O/N at RT to allow for complete precipitation. The solid was then filtered and dried in vacuo to afford the product.

5-amino-1-cinnamyl-1H-1,2,3-triazole-4-carboxamide (24): tan solid (0.69 g, 59%). mp= 235-236 °C. (400 MHz, DMSO-d_{6}): δ 7.47 (br s, 1H), 7.24-7.45 (m, 5H), 7.10 (br s, 1H), 6.45 (m, 2H), 6.36 (s, 2H), 4.58 (d, J= 6 Hz, 2H). (100 MHz, DMSO-d_{6}): δ 164.3, 144.6, 135.9, 132.3, 128.7, 128.0, 126.5, 123.5, 121.7, 47.0 (NMP impurity at 174.5, 49.2, 30.8, 29.7, 17.9). HRMS (EI), m/z C_{12}H_{3}N_{5}O, (M)^{+} calcd. 243.1120, obsd. 243.1123.
2.4.3 General procedure for the continuous flow synthesis of triazole library compounds

**Benzyl azide (2):** To a Labtrix T-mixer chip (#3023), the following solutions were introduced: benzyl bromide in *N*-methyl-2-pyrrolidinone (0.5 M) and NaN₃ in aqueous *N*-methyl-2-pyrrolidinone (20% v/v, 0.5 M). The reaction was performed at temperatures ranging from 25-100°C and flow rates of 0.5 – 25 μL/min per syringe. Reaction mixtures were collected in 50 μL aliquots, repeated 3x, and diluted with H₂O (500 μL) prior to analysis by HPLC-UV.

5-amino-1-benzyl-1H-1,2,3-triazole-4-carboxamide (3): Using the setup in Figure 4a, to the Labtrix T-mixer chip (#3025), the following solutions were introduced: benzyl bromide in *N*-methyl-2-pyrrolidinone (0.5 M), NaN₃ in aqueous *N*-methyl-2-pyrrolidinone (20% v/v, 0.5 M), cyanoacetamide in aqueous *N*-methyl-2-pyrrolidinone (20% v/v, 0.75 M), and sodium hydroxide in H₂O (0.75 M). The reaction was performed at 80°C, and a flow rate of 1 μL/min. Reactions were collected in 50 μL aliquots, repeated 3x, and diluted with H₂O (450 μL) and DMSO (300 μL) prior to analysis by HPLC-UV.

5-amino-1-benzyl-1H-1,2,3-triazole-4-carboxamide (3): Using the setup in Figure 4b, to the Labtrix T-mixer chip (#3023), the following solutions were introduced: cyanoacetamide in
aqueous NMP (20% v/v, 0.75 M), sodium hydroxide in H₂O (0.75 M), and benzyl azide in aqueous NMP (20% v/v, 0.5 M). The reaction was performed at temperatures ranging from 25-100°C and flow rates of 0.5 – 25 μL/min per syringe. Reaction mixtures were collected in 50 μL aliquots in triplicate and diluted with H₂O (450 μL) and DMSO (300 μL) prior to analysis by HPLC-UV.

2.4.4 Synthesis of Ticagrelor analogues

3-benzyl-3,4-dihydro-5H-[1,2,3]triazolo[4,5-d]pyrimidine-5,7(6H)dione (4): To a solution of 5-amino-1-(phenylmethyl)-1H-1,2,3-triazole-4-carboxamide (3) (0.200 g, 0.92 mmol) and diethyl carbonate (0.326 g, 2.76 mmol) in dry THF (15 mL) was added potassium tert-butoxide (0.310 g, 2.76 mmol). The mixture was refluxed for 5 h. It was then cooled to room temperature, quenched with water (20 mL), and concentrated to ~ 20 mL in vacuo. The remaining aqueous solution was neutralized with 1N HCl to pH=6, and the precipitate was filtered, washed with water, and then air dried to afford the product as a beige solid (0.191 g, 85% yield). mp= 281-282 °C. (400 MHz, DMSO-d₆): δ 12.57 (br s, 1H), 11.30 (s, 1H), 7.41–7.28 (m, 5H), 5.63 (s, 2H). (100 MHz, DMSO-d₆): δ 156.9, 151.3, 142.5, 135.7, 129.5, 128.8, 128.3, 125.0, 50.6. HRMS (EI), m/z C₁₁H₉N₅O₂ (M)+ calcd. 245.0833, obsd. 245.0834.
**3-benzyl-5,7-dichloro-3H-[1,2,3]triazolo[4,5-d]pyrimidine (25):** To a cooled (-20 °C) solution of (4) (1.00 g, 4.11 mmol) and phosphoryl chloride (2.52 g, 16.4 mmol) in toluene (50 mL) was added dropwise 2,6-lutidine (0.441 g, 4.11 mmol). The reaction was refluxed for 5 h. After concentration in vacuo, the residue was dissolved in water (50 mL) and extracted with ethyl acetate (3 x 30 mL). The combined organic extracts were dried over MgSO₄ and concentrated in vacuo. The residue was purified by flash chromatography (SiO₂; 5% MeOH/CH₂Cl₂) to afford the desired compound as a yellow solid (0.069 g, 6.40%) mp = 83-84 °C. (400 MHz, DMSO-d₆): δ 7.41-7.28 (m, 5H), 5.83 (s, 2H). (100 MHz, DMSO-d₆): δ 157.8, 155.2, 151.2, 133.6, 133.5, 129.4, 129.3, 128.8, 51.7. HRMS (EI), m/z C₁₁H₇Cl₂N₅, (M)+ calcd. 279.0079, obsd. 279.0079.

**3-benzyl-5-chloro-N-cyclopropyl-3H-[1,2,3]triazolo[4,5-d]pyrimidin-7-amine (26):** To a stirring solution of (25) (0.185 g, 0.66 mmol) and cyclopropylamine (0.034 g, 0.60 mmol) in dry CH₂Cl (10 mL) was added N,N-diisopropylethylamine (0.077 g, 0.60 mmol). The reaction was stirred at room temperature for 5 hours, at which time an additional 0.9 eq of the cyclopropylamine and N,N-diisopropylethylamine was added. The reaction was stirred for another 16 hours and the additional 0.9 equivalent addition was repeated. The reaction mixture was stirred for an additional 2 hours (total reaction time = 24 h), and then the reaction mixture
was washed with water, dried with MgSO$_4$, then concentrated \textit{in vacuo}. The residue was purified by flash chromatography (SiO$_2$; 30% EtOAc:Hex) to afford the desired compound as a yellow oil (0.102 g, 52%). (400 MHz, CDCl$_3$): δ 7.45-7.27 (m, 5H), 5.71 (s, 2H), 2.32 (br s, 1H) 1.08 (d, $J = 6$ Hz, 1 H), 0.95 (m, 2H), 0.73 (br s, 2H). (100 MHz, CDCl$_3$): δ 159.5, 157.6, 156.1, 149.8, 134.6, 129.0, 128.7, 128.5, 128.4, 124.1, 50.5. HRMS (EI), m/z C$_{14}$H$_{13}$ClN$_6$, (M)$^+$ calcd. 300.0890, obsd. 300.0893.

3-benzyl-$N$-cyclopropyl-5-(propythio)-3\textit{H}-[1,2,3]triazolo[4,5-\textit{d}]pyrimidin-7-amine (27): To a cooled (0 °C) solution of sodium hydride (0.010 g, 0.40 mmol) in dry DMF (10 mL) was added propane thiol (0.030 g, 0.40 mmol) and allowed to stir until gas evolution ceased. The substrate (26) was then added in one portion, and the reaction mixture was allowed to warm to RT. After stirring overnight, the consumption of starting material was not complete, thus an additional 2 equivalents of propane thiol and sodium hydride was added. Upon an additional 3 hours of reaction time, the addition of 2 equivalents was repeated. The reaction was stirred for a total of 28 hours, at which time it was poured onto a saturated sodium carbonate solution (20 mL) and extracted with EtOAc (3 x 15 mL). The organic extracts were combined, washed with water (20 mL) and brine (20 mL), dried over MgSO$_4$, and concentrated \textit{in vacuo}. The residue was purified by flash chromatography (SiO$_2$; 5% EtOAc/CH$_2$Cl$_2$) to yield a yellow oil (0.091 g, 80%). (400 MHz, CDCl$_3$): δ 7.45-7.27 (m, 5H), 5.66 (s, 2H), 3.16 (br s, 2H), 1.79 (br s, 2H), 1.23 (s, 1H),
1.05 (m, 4H), 0.95 (m, 1H), 0.73 (br s, 2H). (100 MHz, CDCl₃): HRMS (EI), m/z C₁₇H₂₀N₇S, calcd. 340.1470, obsd. 340.1468.

5-amino-1-(cyclopentylmethyl)-1H-1,2,3-triazole-4-carboxamide (30): Following the same procedure for (3) produced the desired compound as slender yellow crystals (0.177 g, 52%).

mp= 226-227 °C. (400 MHz, DMSO-d₆): δ 7.41 (br s, 1H), 7.06 (br s, 1H) 6.28 (br s, 2H), 4.05 (d, J=8 Hz, 2H), 2.37 (m, 1H), 1.61 (m, 4H), 1.50 (m, 2H), 1.26 (m, 2H) (NMP impurity at 3.30, 2.70, 2.19, 1.90). (100 MHz, DMSO-d₆): δ 164.4, 144.6, 121.5, 49.5, 38.9, 29.5, 24.5. HRMS (ESI), m/z C₉H₁₅N₅O (M+H)⁺ calcd. 210.1277, obsd. 210.1355.

3-(cyclopentylmethyl)-3,4-dihydro-5H-[1,2,3]triazolo[4,5-d]pyrimidine-5,7(6H)-dione (31):

Following the same procedure for (4) produced the desired compound as a light yellow solid (0.035 g, 21% yield). mp= 294-295 °C. (400 MHz, DMSO-d₆): δ 12.4 (br s, 1H), 11.2 (s, 1H), 4.27 (d, J= 7.2Hz, 2H), 2.36 (m, 1H), 1.63 (m, 4H), 1.50 (m, 2H), 1.23 (m, 2H). (100 MHz, DMSO-d₆): δ 156.3, 150.7, 141.6, 124.1, 51.2, 39.0, 29.4, 24.5. HRMS (ESI⁺), m/z C₁₀H₁₃N₅O₂ (M+H)⁺ calcd. 236.1069, obsd. 236.1147.
2.5 References


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CHAPTER 3: Continuous Flow Functionalization of Proteins and Monoclonal Antibodies

3.1. Introduction to Chemotherapy

Despite the prevalence of a wide variety of chemotherapeutic agents for cancer, each has their own drawbacks. As their function dictates, the molecules are frequently extremely potent cytotoxins. Popular chemotherapeutic compounds include doxorubicin,\(^1\) methotrexate,\(^2\) and paclitaxel,\(^3\) amongst myriad others. These molecules target rapidly dividing cells like those in cancer, but can also attack other healthy cells that divide quickly, including blood, stomach and intestinal cells.\(^4\) The off-target cell killing results in many adverse effects to the patient, including nausea and vomiting,\(^5\) hair loss,\(^6\) and cognitive impairment or “chemo brain,”\(^7\) amongst many others. For decades, an alternative to this route of treatment has been sorely needed in the clinic.

3.1.1 Antibody Drug Conjugates: The “Magic Bullet” of Chemotherapeutics?

Originally proposed by German scientist Paul Ehrlich, the utility of a “magic bullet” would provide an alternative that would directly target cancerous cells, while bypassing healthy cells and leaving them intact.\(^8\) By acting as a “magic bullet”, antibody-drug conjugates (ADCs) provide a viable alternative to the issues related to traditional chemotherapy. As will be described in the subsequent sections, ADCs combine the specificity of a targeting antibody with the power of a cytotoxin that is not released until it reaches the cancer cell being targeted.\(^9\)\(^-\)\(^16\)

As the pharmaceutical industry has delved deeper into the world of biologic drugs, a targeted therapy has become more of a reality. Table 3.1 shows the top 20 selling drugs on the market for 2014, the most recent year for which complete, reliable data is available.\(^17\) Small molecules are highlighted in yellow and biologics in blue, which make up more than half of the chart.
Table 3.1 Adapted from C&EN report, the top 20 selling drugs in 2014 are listed, with biologics in blue and small molecules in yellow

<table>
<thead>
<tr>
<th>Rank</th>
<th>Name of Drug and Manufacturer</th>
<th>Sales (in $ billions)</th>
<th>Disease Treated</th>
<th>Type of Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Humira (adalimumab) AbbVie</td>
<td>11.00</td>
<td>Rheumatoid arthritis</td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>2</td>
<td>Enbrel (etanercept) Amgen</td>
<td>8.75</td>
<td>Rheumatoid arthritis</td>
<td>Fusion protein</td>
</tr>
<tr>
<td>3</td>
<td>Advair (fluticasone propionate and salmeterol) GlaxoSmithKline</td>
<td>8.30</td>
<td>Asthma, COPD</td>
<td>Small molecule</td>
</tr>
<tr>
<td>4</td>
<td>Remicade (infliximab) Johnson &amp; Johnson/Janssen</td>
<td>8.30</td>
<td>Rheumatoid arthritis</td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>5</td>
<td>Rituxan (rituximab) Roche/Genentech</td>
<td>8.00</td>
<td>Lymphoma, leukemia</td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>6</td>
<td>Lantus (insulin glargine) Sanofi</td>
<td>7.50</td>
<td>Diabetes</td>
<td>Protein (insulin analog)</td>
</tr>
<tr>
<td>7</td>
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<td>Cancer</td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>8</td>
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<td>Small molecule</td>
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<tr>
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<td>Diabetes</td>
<td>Small molecule</td>
</tr>
<tr>
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<td>PEG- GCSF (protein)</td>
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<td>Synthetic polypeptides</td>
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<td>17</td>
<td>Revlimid (lenalidomide) Celgene</td>
<td>4.25</td>
<td>Cancer</td>
<td>Small molecule</td>
</tr>
<tr>
<td>18</td>
<td>Lucentis (ranibizumab) Roche/Genentech</td>
<td>4.20</td>
<td>Macular degeneration</td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>19</td>
<td>Spiriva (tiotropium bromide) Boehringer Ingelheim</td>
<td>4.00</td>
<td>COPD</td>
<td>Small molecule</td>
</tr>
<tr>
<td>20</td>
<td>Nexium (esomeprazole) AstraZeneca</td>
<td>4.00</td>
<td>Gastroesophageal reflux disease (GERD)</td>
<td>Small molecule</td>
</tr>
</tbody>
</table>
3.1.2 Design Considerations for ADCs

As previously mentioned, ADCs are a targeted therapy made up of three parts, as shown in Figure 3.1. These three key components, the antibody, cytotoxin and linker, will be discussed in detail in the subsequent sections. The goal of the overall molecule is the site of interest in the body without being damaged or chemically altered, and release the cytotoxin only upon binding to the desired antigen. Therefore, the design considerations of each component are crucially important when proposing an ADC structure.

![Figure 3.1. The general structure of an antibody-drug conjugate](image)

3.1.2.1 Antibody/Target

Once the ADC enters the body, its goal is to take advantage of the specificity of an antibody-antigen binding interaction to deliver the cytotoxin exclusively to the tumor cells in question and not to other healthy cells. This off-target binding causes many of the negative side effects associated with traditional chemotherapy treatment. In many cancers, including breast cancer and lymphoma amongst many others, there are antigens that are overexpressed on the surface of cancer cells that serve as prime targets for ADC therapy.

The structure of a typical IgG antibody is shown in Figure 3.2. It consists of a light and heavy chain, joined by four inter-chain disulfide bonds. The Fab region contains the antigen binding site and therefore is variable depending on the variety of IgG. The Fc, or constant region, is structurally conserved within a species. Of relevance to this work are another variety of antibodies, called chimeric antibodies. These mAbs were originally developed from a non-human species, often a mouse. However, ingestion of mAbs produced from a non-human species
would generate a response from the human immune system and thus are engineered to contain human Fc regions to avoid this rejection. A specific chimeric antibody, infliximab, will be discussed in subsequent sections.

**Figure 3.2.** The structure of an IgG antibody

In breast cancer, HER2 is overexpressed in as many as a third of cases.HER2 is a member of the epidermal growth factor receptor (EGFR) family, and can promote tumorigenesis and ultimately lead to more fatal outcomes. In terms of lymphoma, CD-30, a tumor necrosis factor (TNF) receptor, is overexpressed on the surface of anaplastic large cell lymphoma (ALCL) cells and by Reed–Sternberg cells in Hodgkin lymphoma. As such, CD-30 is part of a signal transduction pathway that has been implicated in cell proliferation and survival.

### 3.1.2.2 Cytotoxin

One of the benefits of a directed therapy is the ability to use chemotherapeutic agents that are not traditionally safe for patients as is. A sampling of anticancer drugs that have been reported for use in ADCs is shown in **Figure 3.3**. For example, the enediyne calicheamicin, undergoes a Bergman cyclization upon binding DNA, thus cleaving the DNA double helix and
causing cell death.\textsuperscript{24} It is one of the most toxic cell-killing compounds to date, initiating apoptosis at concentrations less than 100 pM.\textsuperscript{25} Calicheamicin also kills cells indiscriminately, so when used on its own presents challenges in terms of toxicity. Maytansine derivatives like DM1 are tubulin binders that disrupt assembly of microtubules, subsequently causing cell death.\textsuperscript{26} DM1 is extremely potent, and this family of maytansins have been reported to be anywhere from 100-1000 times more potent than traditional standard of care.\textsuperscript{27} The auristatins, such as monomethyl auristatin E (MMAE), are derived from the original natural product dolastatin 10, and represent another potent class of tubulin inhibitors that have also shown to have antivasular properties.\textsuperscript{28} Doxorubicin, a topoisomerase II inhibitor, which interrupts DNA replication and causes cell death.\textsuperscript{29} It has been used to treat a number of different cancers, but suffers from grave side effects when used alone, such as congestive heart failure that results in a 50\% mortality rate if developed.\textsuperscript{30}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{toxins.png}
\caption{Four of the predominant toxins used in ADCs}
\end{figure}
3.1.2.3 Linker Chemistry

The final important component of designing an ADC is the linker that joins the drug to the antibody. Numerous strategies have been employed over the past two decades; some of the basic concepts will be discussed here, while site-specific methodologies will be covered in a subsequent section. The ideal linker must remain stable during purification, formulation, administration and circulation of the conjugate until the ADC reaches its target antigen. Then, and only then, can the linker be cleaved (cleavable linker) or the conjugate degraded by the cellular environment (non-cleavable linker) in such a fashion that the cytotoxin is released in its active or nearly active (prodrug) form and exclusively to the cancerous area. The integrity of the linker during circulation is important because premature cleavage would result in off-target effects, as well as insufficient dosage of cytotoxin to the target area.

A sampling of some of the more traditional linker types employed is shown in Scheme 3.1. Hydrazone linkers are stable at the pH of circulation in the blood (7.4-7.5), but will degrade upon an increase in pH when the conjugate enters an endosome following internalization (pH 5.5-6.2), and the lysosome where it is degraded and the drug is released (pH 4.0-5.0).\textsuperscript{31} Non-cleavable thioether linkages created by heterobifunctional crosslinkers undergo receptor mediated lysosomal degradation to release the drug.\textsuperscript{32} Dipeptide linkers, such as valine-citrulline, are designed to be cleaved by cathepsin B, a cysteine protease that is only found in meaningful concentrations in metastatic tumors.\textsuperscript{33}
Scheme 3.1 Traditional ADC linker strategies

As antibodies are such large biomolecules (average molecular weight around 150 kDa), they contain a number of amino acids that can be used as sites of attachment for chemotherapeutic payloads.\textsuperscript{34} The most common amino acid modifications are at lysine or cysteine residues. As shown in Scheme 3.2, the nucleophilic amine side chain of lysine can displace a leaving group on a linker, often a succinimide. The result is an amide bond linkage between the linker and the mAb. The light and heavy chains of a mAb are held together by four disulfide bonds between cysteine residues, so if one or more of these bonds are reduced, the resulting sulfur atom can undergo a Michael type addition to a maleimide to form the thioether linkage.
Although these methods of conjugation are successfully employed, there are limitations to these strategies. There are up to 100 lysine residues on average per mAb, and four disulfide bonds hold together the light and heavy chains. The result is a heterogeneous mixture in terms of number of sites bound, specificity and reproducibility of sites bound, and structural stability of the mAb. The implications of this heterogeneity will be discussed in upcoming sections, and serve as a basis for the research goals of this work.

3.1.3 FDA Approved ADCs

Since the concept of ADCs emerged, there have been three FDA approvals: Mylotarg (gemtuzumab ozogamicin) from Wyeth, Adcetris (brentuximab vedotin) from Seattle Genetics, and Kadcyla (trastuzumab emtansine) from Genentech. These three conjugates will be discussed, followed by a brief look into the pipeline for future ADCs.

3.1.3.1 Approval and Retraction of Mylotarg

Mylotarg (gemtuzumab ozogamicin) was the first ADC approved by the FDA. It was approved in 2000 for treatment of acute myeloid leukemia (AML) in patients who have relapsed from traditional chemotherapy and are over sixty years old. Mylotarg, depicted in Figure 3.4, consists of an anti-CD33 mAb, which binds CD33, which is expressed solely on the
surface of leukemia cells. An acid-labile hydrazone linker joins the anti-CD33 mAb to the toxin, calicheamicin.\textsuperscript{37} Initial comparisons of Mylotarg Phase II trials to traditional chemotherapy showed an interesting trend; the overall objective response rate (OR) was increased for Mylotarg if the patient had previous chemotherapy for a duration of 3-10.5 months. It showed a decreased OR if the patient had a previous chemotherapy duration of 19 months or longer. If the duration was between 10.5-19 months, the OR was equivalent.\textsuperscript{38} Therefore, the ideal usage for Mylotarg was in patients who received short term chemotherapy.

\begin{figure}
\centering
\includegraphics[width=0.5\textwidth]{mymolarg.png}
\caption{The structure of Mylotarg}
\end{figure}

After a year on the market, Mylotarg garnered a Black Box warning from the FDA, as 12\% of patients in one study who had not received stem cell replacement following treatment developed hepatic venoocclusive disease (VOD). VOD is a potentially fatal condition that causes liver enlargement, jaundice, and other forms of fluid retention.\textsuperscript{39} Based on results of a further
clinical trial which indicated a 5.5% mortality rate of patients who received Mylotarg vs. the 1.4% mortality rate in the control group.\textsuperscript{40} Pfizer (who had purchased Wyeth) elected to voluntary pull Mylotarg from the market at the urging of the FDA in 2010. In a very recent development in early 2017, Pfizer re-filed for approval in the US and EU, on the grounds that they had re-analyzed previous clinical trial data in conjunction with the results of a 2014 study that investigated the efficacy of Mylotarg in relation to the expression of 11 different genes and their mutations.\textsuperscript{41} The re-approval application, intended for AML patients 50-70 years of age, cites the need for a reduced dose of Mylotarg, so as to reduce side effects, while coupling it with low doses of traditional chemotherapeutics to maintain efficacy. A decision is not expected to be reached until early fall 2017.

\textbf{3.1.3.2 Adcetris}

Adcetris (brentuximab vedotin) was approved by the FDA in 2011 for the treatment of a relapsed form of Hodgkin’s lymphoma or relapsed refractory systemic anaplastic large cell lymphoma.\textsuperscript{42} As shown in Figure 3.5, Adcetris consists of brentuximab, an anti-CD30 antibody, and the toxin monomethyl auristatin E (MMAE). Binding of brentuximab to CD30 (a TNF receptor)\textsuperscript{22} degrades the TRAF2 protein, which is implicated in cell survival; a lack of TRAF2 leads to apoptosis.\textsuperscript{42} MMAE is bound to the mAb through a valine-citrulline dipeptide linker. Upon CD30 binding, the complex is internalized via endocytosis and subsequently enters the lysosome. Cathepsin B, a proteolytic enzyme, cleaves the valine-citrulline linkage and releases MMAE. While some of the free MMAE binds tubulin, as is its typical function, some diffuses out of the cell and can effect neighboring cells within the tumor.\textsuperscript{42} This phenomenon is called the bystander killing effect.\textsuperscript{43}
As the payload is linked through reduced interchain disulfide bonds, the drug-to-antibody ratio (DAR) is not consistent from molecule to molecule. The average DAR is 4 MMAE molecules per mAb, with a distribution from 0-8 in increments of two are observed. The DAR of this and other ADCs will be discussed in further detail in a subsequent section.

Figure 3.5 The structure of Adcetris

A Phase II clinical trial of patients with various Non-Hodgkin lymphoma patients showed a 40% OR, with 17 patients achieving partial or complete remission.\(^4^4\) Several side effects, including fatigue, immune system impairment, and peripheral sensory neuropathy, were observed.\(^4^5\) Adcetris also garnered a Black Box warning in 2012 for two noted cases of progressive multifocal leukoencephalopathy, a brain inflammation disease.\(^4^6\) A total course of Adcetris lasts 7-9 courses of treatment; each cycle is priced around $13,500 with a complete course ranging from $94,500-$121,500.\(^4^7\)

3.1.3.3 Kadcyla

Kadcyla (ado-trastuzumab emtansine) was approved in 2013 for the treatment of HER2 positive metastatic breast cancer. Approximately 15-25% of incidences of breast cancer are HER2 positive, meaning the HER2 receptor is overexpressed.\(^4^8\) Traditional treatment consists of a HER2 mAb (trastuzumab) in conjunction with a tyrosine kinase inhibitor (lapatinib), which has been shown to be more efficacious than simply trastuzumab alone, but also showed increased toxicity, by way of lower level side effects such as nausea and diarrhea, as well as higher grade
metabolic, gastrointestinal, and hepatotoxicity, with 8% of patients having to terminate their participation in the trial.\textsuperscript{49} As a result, alternative options that fuse the advantages of trastuzumab with traditional chemotherapy were needed.

Kadcyla, shown in Figure 3.6, contains the mAb trastuzumab (Herceptin) covalently bonded to a mertansine derivative, DM1. Some of the earlier attempts at developing these conjugates linked the drug and mAb via disulfide linkages. However, it was shown that the bond was being cleaved in the oxidative environment of endocytosis.\textsuperscript{50} So, a more stable linker (SMCC, shown in red) joins the mAb to DM1 via a thioether bond, which undergoes proteolytic degradation upon entering the lysosome. The conjugate is linked to the mAb via lysine residues that displace the succinimide moiety of SMCC.\textsuperscript{51} Therefore, a distribution of DAR is observed, with the average DAR of 3.5. As previously stated, the analytical implications of this distribution will be discussed in coming sections.

\textbf{Figure 3.6} The structure of Kadcyla

Promising results from an international Phase III clinical trial showed a substantially improved progression-free survival, as compared to standard lapatinib treatment, with a median survival time of 9.6 months as compared to 6.4 months.\textsuperscript{52} Also, the incidence of adverse events
reported were decreased with Kadcyla compared to lapatinib, with a reduction of almost 4% of patients. As recently as December 2016, a Phase I clinical trial studying clearance of Kadcyla in patients with HER2 positive breast cancer in conjunction with acute liver failure was reported. Results indicated that patients with mild to moderate liver failure did not clear DM1 any slower than those with normal liver function, as noted by no observed increase in DM1 in the blood following dosing. Therefore, Kadcyla presents an ever-growing advantage in the treatment of HER2 positive metastatic breast cancer, albeit with a price tag of approximately $94,000 for a 9-month course of treatment.

3.1.3.4 In the Pipeline

As of a recent 2015 report, there are over 40 ADCs in various stages of clinical trials. Treatment areas being targeted include a variety of cancers, such as breast, bladder, prostate, ovarian, lung, and renal cell cancers. Table 3.2 shows a sampling of the array of companies that are represented in the numerous stages of clinical trials. In the first half of 2014 alone, a total of 64 patent applications were granted to pharmaceutical companies for developing ADC technology. This extremely promising pipeline represents a unique opportunity for an emerging therapeutic area, albeit with an extremely competitive field.

Table 3.2 Table of ADC clinical trials adapted from Advances in the Pharmaceutical Science Series report

<table>
<thead>
<tr>
<th>Developing Organization(s)</th>
<th>Therapeutic Area(s)</th>
<th>Stage of Development</th>
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</thead>
<tbody>
<tr>
<td>Seattle Genetics</td>
<td>Further use of Adeceiris</td>
<td>Phases 2 + 3</td>
</tr>
<tr>
<td>Seattle Genetics</td>
<td>leukemia, breast cancer, bladder cancer, solid tumors</td>
<td>Phase 1</td>
</tr>
<tr>
<td>Genentech/Roche</td>
<td>Breast cancer</td>
<td>Phase 3</td>
</tr>
<tr>
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<td>Ovarian cancer, prostate cancer, non Hodgkin lymphoma</td>
<td>Phase 2</td>
</tr>
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<td>Further use of Kadcyla</td>
<td>Phase 2 + 3</td>
</tr>
<tr>
<td>Progenics</td>
<td>Prostate cancer</td>
<td>Phase 2</td>
</tr>
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### 3.1.4 Methods of Analysis of ADCs

Due to the heterogeneity of the species, varied charge distribution of the mAbs, and small mass of cytotoxin added relative to the large size of the mAbs, the analysis and characterization of these species remains a tremendous challenge in the field of ADCs. The subsequent sections will describe the traditional methods of analysis, along with their advantages and disadvantages.

#### 3.1.4.1 Drug-to-Antibody Ratio (DAR) via UV-Vis Spectroscopy

The drug-to-antibody ratio (DAR) is the average number of cytotoxin molecules conjugated per mAb. One of the simplest methods that is easily accessible to many researchers is UV-Vis spectroscopy. If the $A_{\text{max}}$ values of the drug and mAb are different, the Beer-Lambert Law can be used to determine the moles of both mAb and drug. For example, a mAb-doxorubicin conjugate was characterized using the $A_{\text{max}}$ of doxorubicin at 495 nm (where $\epsilon = 8030 \text{ cm}^{-1}\text{M}^{-1}$) to determine the mol of drug added.\(^{57}\) The number of mol of mAb were determined using the $A_{\text{max}}$ of the mAb at 280 nm, while correcting for the minor absorbance of the drug at the same wavelength, as shown in Equation 3.1.

#### Equation 3.1

$$[\text{mAb}] \left( \frac{\text{mg}}{\text{mL}} \right) = \frac{A_{280} - (0.724 \times A_{495})}{1.40}$$

The Val-Cit-MMAE linker-drug combination used in Adcetris has also been quantified using UV-Vis spectroscopy.\(^{58}\) The DAR (denoted here as MR, the molar ratio of drug to mAb) was calculated using Equation 3.2 with the corrected absorbance of both the mAb and the drug linker combination.
Equation 3.2

\[ MR = \frac{\varepsilon_{mAb}^{240} - R\varepsilon_{mAb}^{280}}{R\varepsilon_{drug}^{280} - \varepsilon_{drug}^{248}} \]

3.2.4.2 Verifying DAR and Conjugation Distribution via HIC LC-MS

Calculating DAR for conjugate mixtures yields the average drug loading, but for considerations of toxicity, efficacy and safety, it is important to understand the relative percentage of each conjugation state. One of the primary methods used to identify this distribution is hydrophobic interaction chromatography (HIC). HIC takes advantage of the aggregation of proteins and biomolecules when they are exposed to a high-salt, aqueous environment. As shown in Figure 3.7, the HIC column contains a matrix, on which a hydrophobic ligand is adsorbed. The solvent can be tuned via addition of salt or a small quantity of organic solvent, maintaining a weak interaction with the ligand until the sample is added. Upon addition of the sample, the hydrophobic regions of the protein will displace the solvent molecules and aggregate with the hydrophobic ligand.\(^{59}\)

**Figure 3.7** Hydrophobic interaction chromatography theory, adapted from McCue\(^ {59}\)

For analyzing biological samples, HIC differs from traditional reverse phase HPLC (RP-HPLC) in that the conditions used for separation (minimal organic solvent, relatively neutral pH) are not denaturing, so the protein structure remains intact throughout the process. This advantage is particularly useful for ADC characterization because despite reduction of disulfide bonds, the relative structure resulting from intra-molecular forces remains during analysis.\(^ {60}\)
A sample HIC chromatogram with corresponding UV-VIS spectra is shown in Figure 3.8. As the absorbance at 248 nm increases, the number of drugs conjugated per mAb increases. The DAR can be calculated using the UV-VIS method as previously described, but also can be determined via integration of the HIC chromatogram peaks, which show clean, baseline separation between conjugation states. This particular figure shows the conjugation of a mAb via disulfide reduction, so the DAR appears in increments of two, as two drug molecules are added per disulfide bond reduction. The non-denaturing conditions of HIC allow for identification of an 8-drug conjugate, despite the complete reduction of all four disulfide bridges.

![HIC chromatogram with UV-VIS spectra](image)

**Figure 3.8** Hydrophobic interaction chromatography (HIC) chromatogram, with an inset of UV-VIS spectra, reproduced with permission

### 3.1.4.3 Verifying DAR and Conjugation Distribution via Size Exclusion Chromatography-MS (SEC-MS)

Size exclusion chromatography (SEC), as the name would suggest, separates complex mixtures based on molecular size. As depicted in **Figure 3.9**, the column is packed with a porous material, typically a polymer. The sample is loaded onto the column, and as the solvent flows,
the smaller molecules (and often the solvent) interact with the porous materials to a higher degree than the larger molecules. As a result, the larger molecules elute first, gradually getting smaller until the solvent elutes last.

**Figure 3.9** Size exclusion chromatography theory, adapted from Striegel

Of advantage for ADC analysis, this technique is not denaturing. However, the difference between each conjugation states is such a small mass relative to the size of a large mAb that all the conjugation states elute in essentially one broad peak. So, the primary utility of this method is to separate the biomolecules from remaining small molecules, unconjugated drug, and solvent. Coupling SEC to a mass spectrometer allows for a deeper understanding of the conjugation distribution, as shown in **Figure 3.10**. This spectrum contains a lysine-conjugated ADC, so the conjugation states range from 0–7 drugs per mAb.
3.1.4.4 Verifying DAR and Conjugation Distribution via Capillary Electrophoresis (CE-MS)

Antibodies, which as previously discussed have an average molar mass of 150 kDa, have numerous variation in charge state, as they are comprised of many polarizable residues. In CE, a voltage is applied to a capillary, where mixtures separate based on their electrophoretic mobility. In the example shown in Figure 3.11, the flow of current runs from anode to cathode, and therefore, the electroosmotic flow inside the negatively charged capillary causes the positively charged ions to move more quickly and therefore elute first, followed by neutral molecules and finally negatively charged molecules. Capillary zone electrophoresis (CZE) incorporates not only the separation based on charge but also on size. The high surface-area-to-volume ratio makes this technique highly sensitive, and requires a small amount of sample to obtain clean results.
Figure 3.11 Capillary electrophoresis (CE) theory, adapted from Buszewski.⁶⁷

A comparison of a DTT-reduced conjugate by RP-HPLC (A-D) and a non-reducing sample of the same conjugate by CE (E-H) with denaturing sodium dodecyl sulfate (SDS) is shown in Figure 3.12. The heavy and light chain fragments are apparent in the RP-HPLC runs, and the ratios of each fragment can be used to calculate DAR. However, the non-reducing CE results are denatured by SDS, which provides information about which sites have been conjugated, as the denaturing takes place at sites that are not linked by covalent disulfide bonds.⁶⁸
As shown in the previous sections, the distribution of conjugation varies dramatically under conventional conditions. Most the aforementioned methods can separate on the basis of the number of drug molecules conjugated to a mAb, and some, including more complex mass spectrometric analyses, can begin to shed light onto the sites of conjugation. In general, these sites are not consistent, especially when there are numerous lysine residues or eight possible sulfide moieties after reduction. To combat this lack of specificity, which may have consequences in terms of mAb function and stability, much work has been done to investigate the possibility of site-specific conjugation, which is the ultimate goal of this work as well. This area of research is of particular importance when it comes to obtaining FDA approval for any of
these conjugates; being able to specify an exact DAR provides extremely consistent information for assessment of efficacy, toxicity, and manufacturing processes.

3.1.5.1 THIOMAB: Engineered Reactive Cysteines

One strategy for site-specific conjugation involves bioengineering methods of fine-tuning the amino acid sequence in a protein or antibody of interest. THIOMABs are one such class of antibodies. These mAbs are engineered to contain reactive cysteine residues in their constant Fab regions to be used as sites of conjugation, without disrupting the intrachain disulfide bridges. Early attempts at direct conjugation of THIOMABs proved unsuccessful, as they formed mixed disulfides with free cysteine or glutathione molecules.

To solve this problem, the following process, as shown in Figure 3.13, is used to generate the conjugate. The engineered mAb is partially reduced using TCEP, which cleaves the mixed disulfides as well as the interchain disulfide bonds, while maintaining the intrachain bonds that hold the light and heavy chains together. The interchain bonds are allowed to re-oxidize in air upon removal of the residual small molecules, or with the help of copper sulfate, affording a mAb with only the engineered sites free. Lastly, the drug linker portion is introduced for conjugation, yielding a conjugate with drug attached only at the desired sites.

The resulting conjugates have shown improved pharmacokinetic behavior, as the deconjugation rate from various sites can be studied with homogeneous species with fixed DAR. A THIOMAB conjugate with Val-Cit-MMAE has been reported for use against triple negative breast cancer. With a consistent DAR of 2, this conjugate showed suppressed tumor growth with single-dose administration, and presented no overt toxicity issues. As such, this ADC is viable and attractive candidate for further studies, as the patient is exposed to a fixed
quantity of cytotoxic drug with maintained antigen-binding, which also alleviates concerns regarding resistance and toxicity.

Figure 3.13 The THIOMAB conjugation model, utilized by Junutula et al.⁶⁹

3.1.5.2 Non-natural Amino Acid Incorporation

Another strategy for site-specific conjugation is the incorporation of non-natural amino acids into the mAb structure via genetic modification and subsequent production in yeast or mammalian cells. Herceptin (previously described for use in Kadcyla) has been modified to incorporate a fixed number of p-acetylphenylalanine (pAcPhe) residues at a chosen site on the mAb, and further reacted via oxime ligation to join a PEG-auristatin derivative,⁷² as shown in Scheme 3.3.
3.1.5.3 N-terminal Serine Modification

One additional strategy for a similar oxime ligation strategy is via engineering of an N-terminal serine residue on the VL domain of the light chain. Once expressed and purified, the serine residue can be oxidized under mild sodium periodate conditions to the corresponding aldehyde to participate in oxime ligation to generate the final ADC. The same PEG-auristatin analogue described previously can participate in oxime ligation, and has been shown to produce a consistent, site-specific ADC as shown in Scheme 3.4. The consistent DAR of 2 of this anti-EphA2 conjugate showed no inhibition of antigen binding and showed substantial tumor growth inhibition without affecting mouse body weight.

3.1.5.4 Transglutaminases

Another strategy for site-specific conjugation takes advantage of the biological function of bacterial transglutaminases. Transglutaminases (TGs) are acyl-transfer catalysts, that form linkages between glutamine and lysine residues, forming stable amide (isopeptide) bonds.
Though the lysine source is easily varied, even to the point that other amines are tolerated, the location of the glutamine residue is of importance; this enzyme is highly selective for accessibility on the surface of the protein in regions that have sufficient flexibility and a number of specific neighboring residues have been suggested.\textsuperscript{75} Although a mAb may contain numerous glutamine residues, it is important to note that they are often not in solvent-accessible regions; it may be necessary to engineer a glutamine residue into an advantageous location that may also facilitate deglycosylation of the mAb to increase mobility.\textsuperscript{74}

In terms of ADC applications, there have been many reported uses of TGs.\textsuperscript{76-79} One such example is described in Scheme 3.5. Deglycosylation of the mAb by peptide-N-Glycosidase F (PNGase F) affords a mAb with two solvent-accessible glutamine residues. Subsequent microbial transglutaminase-catalyzed formation of an isopeptide with an azide handle presents an opportunity for click chemistry to selectively add a toxin to those sites.\textsuperscript{80} This handle could also be used for radiochemistry labelling applications, making it an attractive option for a multifunctional linker.

**Scheme 3.5** Transglutaminase-catalyzed functionalization of mAbs for ADC preparation\textsuperscript{80}
3.1.5.5 Carbohydrates

A final strategy for site-specific conjugation involves the use of selective glycosylation reactions to modify mAbs at their glycosylated sites. As mAb engineering examples have shown, it is possible to tune the expression of glycosylation to a desired series of interest.\(^\text{81}\) The manipulation of glycosylation is achieved using knockouts for upregulation of production of desired glycans, or to use chemoenzymatic methods, that shorten the naturally occurring glycoforms, and subsequently add synthetic N-glycan oxazoline donors.\(^\text{82}\)

Upon engineering of the glycans of Herceptin, a one-pot endo-glycosidase-catalyzed chemoenzymatic synthesis was developed for the modification of N-glycan oxazolines, shown in Scheme 3.6 The product is an azide-handled Herceptin. With this mAb in hand, a site-specific click reaction as described in the previous section can be achieved to join a cytotoxic payload, such as MMAE.\(^\text{83}\)

Scheme 3.6 The one pot chemoenzymatic modification of Herceptin glycoforms, reproduced with permission\(^\text{83}\)
3.1.6 Research Objectives

Based on the ever-growing demand for new technology for the development of ADCs, the goal of this work is to show the potential applications of continuous flow chemistry in bioconjugation. The efficient mixing of flow chemistry can access traditionally difficult reaction space, so we hypothesize that it has the potential to improve the reaction times and yields. Proof of principle studies with protein standards and a therapeutic mAb (Remicade) will be described, followed by recent progress toward doxorubicin conjugates.

3.1.6.1 Background on Infliximab (Remicade)

The proof of principle work with a therapeutic mAb in this chapter was carried out using infliximab (trade name Remicade). Infliximab, shown in Figure 3.14, is a chimeric monoclonal antibody; this non-naturally occurring mAb is a hybrid of a human IgG Fc region and a mouse Fab region.84 Chimeric mAbs are advantageous because the human portion reduces the chance of any adverse immune responses, and the murine region carries specificity for the antigen.85

![Figure 3.14 Structure of the chimeric mAb infliximab (Remicade)](image)

Infliximab’s function is to bind transmembrane tumor necrosis factor-α (TNF-α). TNF-α is a cytokine, or chemical messenger, that is implicated in the spread of immune and inflammatory diseases such as rheumatoid arthritis,86 as well as the weight loss associated with
bacterial diseases and cancer.\textsuperscript{85} It was approved by the FDA in 1998 for the treatment of Crohn’s disease and has subsequently been used to treat colitis, psoriasis and rheumatoid arthritis.\textsuperscript{87} TNF-\(\alpha\) has been investigated as both a treatment and a target for cancer therapy,\textsuperscript{88} but the toxicity has been associated with its use as a treatment.\textsuperscript{89} Infliximab has also been shown to induce apoptosis in Jurkat T cells that express TNF-\(\alpha\), which serve as a model to study acute T-cell leukemia.\textsuperscript{90} As a result, it is in an attractive model system that is worth exploring.

3.1.6.2 Background on NIBRT mAb (anti-IL-8)

A second system that will be explored is an anti-interleukin-8 antibody provided by the National Institute for Bioprocessing Research and Training in Dublin, Ireland. Interleukin-8 is a chemokine that is also implicated in inflammation response, but has also been shown to increase proliferation, migration and survival of cancer cells.\textsuperscript{91} The function of IL-8 as an autocrine growth factor in the progression of colon cancer in particular has been explored.\textsuperscript{92} It is important to note the observed chemotherapeutic resistance developed as a result of IL-8 induced signaling within cancer cells.\textsuperscript{91} As a result, an anti-IL8 mAb that would interrupt this process would appear to be an intriguing option for development of a cancer treatment.

3.2 Results and Discussion\textsuperscript{†}

3.2.1 Proof of Principle – FSB

As a first proof-of-principle study shown in Scheme 3.7, N-succinimidyl-4-fluorobenzoate (FSB, 1) was conjugated to specimen protein standards using conventional and flow mediated conditions. Reagent 1 was selected as \(^{18}\text{F}\) labelled form has implications in positron emission tomography (PET) imaging\textsuperscript{94} allowing real-time biodistribution of conjugates to be monitored \textit{in vivo}. The standards utilized included serum albumins, insulin, interferon, and

\textsuperscript{†} Subsequent sections through Remicade work are reproduced with permission of Sebeika et al\textsuperscript{93}
myoglobin, and were carried out under various reaction conditions probing time, temperature, and the role of base triethylamine (TEA), as shown in Table 3.3.

![Scheme 3.7. FSB conjugation to proof of principle protein standards](image)

**Table 3.3. Conjugations of FSB to protein standards†**

<table>
<thead>
<tr>
<th>Entry</th>
<th>Protein</th>
<th>Temp (°C)</th>
<th>Time (min)</th>
<th>TEA</th>
<th># Tags</th>
<th>% Labelled</th>
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<td>0</td>
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<td>30</td>
<td>-</td>
<td>2</td>
<td>42</td>
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<tr>
<td>5</td>
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<td>50&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1</td>
<td>-</td>
<td>2</td>
<td>53</td>
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<tr>
<td>6</td>
<td>INS</td>
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<tr>
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<td>25</td>
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<td>15</td>
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<td>-</td>
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<td>62</td>
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<td>-</td>
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<td>62</td>
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<td>25&lt;sup&gt;b&lt;/sup&gt;</td>
<td>&lt;1</td>
<td>-</td>
<td>2</td>
<td>&gt;99</td>
</tr>
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<td>BSA</td>
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<td>&lt;1</td>
<td>-</td>
<td>4</td>
<td>&gt;99</td>
</tr>
</tbody>
</table>

† All reactions carried out with 3 eq FSB  
<sup>a</sup>Reactions were carried out under microwave irradiation.  
<sup>b</sup>Reaction conducted under flow conditions. HSA = human serum albumin, INS = insulin, INT = interferon, MYO = myoglobin, BSA = bovine serum albumin
After purification, the number of FSB tags per protein were determined by subsequent analysis using LC-MS with electrospray ionization. Due to the slightly basic nature of the buffer (pH = 8) the impact of base was negligible, and in some cases impeded conjugation. Temperature clearly plays a role, with a fine balance between chemical conversion and product degradation needing to be navigated. Based on prior experience working with sensitive substrates, the impact of microwave acceleration was probed and as expected had a beneficial impact (entries 5, 15). Spurred by this, an preliminary reaction was investigated using flow conditions and found to produce derivatized product within 1 min. at ambient temperature both in the case of myoglobin and the reagent protein BSA (entries 17-18). The findings are noteworthy and offer a potentially useful means for in vivo imaging of protein biodistribution via the $^{18}$F analog of 1 as the method (including workup and purification) is compatible with the $t_{1/2}$ of the isotope (~120 min).

**3.2.2 BSA-FITC as a Model of Lysine Conjugation**

With a flow method for protein derivatization revealed, we selected the reagent protein bovine serum albumin (BSA) as a standard from which to explore the conjugation parameters in greater depth. Fluorescein isothiocyanate (FITC), a commonly used fluorescent probe, was chosen for the conjugations, which were conducted under both conventional and flow conditions. FITC, which couples to BSA via lysine conjugation to the isothiocyanate was selected due to its high fluorescence intensity, allowing accurate reaction monitoring via spectrophotometric methods (Scheme 3.8).
Scheme 3.8. Conjugation of FITC to bovine serum albumin (BSA) via lysine linkage

Reaction time, temperature, and the number of equivalents of FITC were varied under both conventional and flow conditions. For the flow experiments, a Chemtrix Labtrix Start instrument was configured with the Chemtrix 3023 microreactor, with two inlets, one quench, and one outlet. Temperatures from 4-37 °C were studied in an effort to minimize denaturation (Table 3.4). After conjugation and purification, the average number of FITC tags was determined using UV-VIS spectrophotometry and application of the Beer-Lambert Law.
Table 3.4. Optimization of continuous flow conjugations of BSA-FITC

<table>
<thead>
<tr>
<th>Entry</th>
<th>Temp (°C)</th>
<th>Eq FITC</th>
<th>Conventional</th>
<th>Flow</th>
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</thead>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Time (min)</td>
<td>Avg. dye per protein (mol)</td>
</tr>
<tr>
<td>1</td>
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<td>3</td>
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<td>10</td>
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<td>5</td>
<td>720</td>
<td>3.03</td>
</tr>
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<td>27</td>
<td>37</td>
<td>10</td>
<td>720</td>
<td>2.32</td>
</tr>
</tbody>
</table>

Overall, use of flow conditions significantly accelerated protein tagging when comparing equivalent ratios of FITC to BSA under conventional conditions. For example, Entry 27 shows an increase in tagging from conventional to flow, while simultaneously decreasing the reaction time from 720 minutes to 300 seconds. Increasing temperature slightly increased the degree of tagging in general. Increasing the residence time for flow reactions did not uniformly result in an increase in tagging, while as expected increasing the equivalents of FITC had a pronounced impact. From this, one can conclude that degree of conjugation is less dependent on time, but
rather on the ratio of tag to protein in solution. In the case of reagent grade proteins and tags this is of little consequence, however in the case of antibody drug conjugations, where complex toxins are employed, this has obvious ramifications.

3.2.3 Infliximab as a Model of Lysine Conjugation to a mAb

With the utility of the flow methodology established, we wished to confirm its application for the derivatization of a therapeutic antibody. The chimeric antibody infliximab (Remicade), was chosen for this purpose and conjugation assessed under both conventional and flow reaction conditions, as detailed in Scheme 3.9.

Scheme 3.9. Conjugation of FSB to infliximab under conventional and flow conditions

Conjugation reactions under conventional conditions were accomplished by dissolving infliximab (1 mg/mL) in PBS buffer, followed by the addition of a solution of
FSB in DMSO. Upon completion of the reaction, the samples were purified and the number of FSB tags per protein were determined by reduction of the intact mAb to the heavy chain (HC) and light chain (LC), and subsequent analysis using LC-MS with electrospray ionization (Table 3.5). At ambient temperature, the increase in molar equivalents of FSB plays a larger role than increased reaction time (entries 1-4), with 10 eq extending tagging to the HC as well as the LC. Increasing the temperature to 37 °C (3 eq tag) results in tagging of the heavy chain as well (entry 5).

<table>
<thead>
<tr>
<th>Entry</th>
<th>Temp (°C)</th>
<th>Eq FSB</th>
<th>Time (hr)</th>
<th>FSB molecules added (LC)</th>
<th>FSB molecules added (HC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>25</td>
<td>3</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
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<td>3</td>
<td>1</td>
<td>1</td>
<td>2</td>
</tr>
</tbody>
</table>

Gratifyingly, repeating the reactions under optimized flow conditions (reactor setup as in Scheme 3.9) resulted in controlled derivatization within 1 min. (Table 3.6). Specifically, using 10 eq at ambient temperature or 5 eq at 37 °C resulted in tagging of two FSB groups within 60 seconds. Also noteworthy is the fact that additional residence time did not impact outcome, and no derivitization of the heavy chain of the mAb was detected under the reaction / workup conditions employed.

<table>
<thead>
<tr>
<th>Entry</th>
<th>Temp (°C)</th>
<th>Eq FSB</th>
<th>Residence time (s)</th>
<th>FSB molecules added (LC)</th>
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<tr>
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<td>60</td>
<td>0</td>
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<td>5</td>
<td>60</td>
<td>2</td>
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</table>
3.2.4 BSA-NPM as a Model of Cysteine Conjugation

After confirming BSA as a viable model for lysine conjugation both conventionally and with flow, it was also investigated as an option for cysteine conjugation, shown in Scheme 3.10, as it is readily available and reasonably priced. N-pyrenylmaleimide (NPM) was used as a fluorescent probe under conventional conditions following the TCEP reduction and the results were analysed using UV-Vis spectroscopy and the Beer-Lambert Law in accordance with literature precedent. The results of these reactions are shown in Table 3.7. Both DMSO and DMF were used as solvents for the NMP, but all reactions showed average values around 1 molecule per protein. As BSA has one free cysteine residue that is not participating in a disulfide bond, it was hypothesized that this site was undergoing conjugation and perhaps the other disulfide residues were not solvent accessible.

Scheme 3.10 TCEP reduction followed by NPM conjugation of BSA
Table 3.7 Average NPM per BSA following TCEP reduction

<table>
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<tr>
<th>Entry</th>
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<th>Solvent</th>
<th>Avg NPM per BSA</th>
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<td>0.977</td>
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3.2.5 TCEP Reduction and Conjugation in One Step

Given the utility of the two-step, one pot synthesis described in Chapter 2, the first attempts at cysteine conjugation in flow were undertaken using this same strategy. In collaboration with the National Institute for Bioprocess Research and Training (NIBRT) in Dublin, Ireland, a series of experiments (Table 3.8) were carried out using various therapeutic mAbs (bevacizumab, cetuximab, infliximab, rituximab, an trastuzumab) as model substrates with fluorescein-5-maleimide (FcM) (Scheme 3.11) as a probe following TCEP reduction. Much precipitation was observed in the microreactor, perhaps due to the ratio of aqueous/organic solvent required to carry out both transformations simultaneously, or the reduced residence time of a two-step, one-pot reactor in comparison to a single reaction carried out on its own. As shown in Table 3.8, the recovered concentration of mAb was relatively low, considering the
reactions were run at 2 mg/mL concentration. The extremely low recovery, combined with clogging issues, implied that a better starting point for cysteine conjugation would be with TCEP reduction carried out conventionally, followed by flow conjugation. TCEP reduction of disulfide bonds are typically achieved in about 30 min, so the additional time shouldn’t take away from the utility of the methodology.

Scheme 3.11 One-pot, two-step flow reduction/conjugation with Fluorescein-5-maleimide (FcM)

Table 3.8 Two-step, one pot flow reduction-conjugation with therapeutic mAbs

<table>
<thead>
<tr>
<th>mAb</th>
<th>Res Time (s)</th>
<th>Eq FcM</th>
<th>([\text{mAb}]_{\text{avg}}) produced (µg/mL)</th>
</tr>
</thead>
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<td>Bevacizumab</td>
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<td>234</td>
</tr>
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<td>13.3</td>
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### 3.2.6 Synthesis of MC-DOX linker

Although fluorescent probes allowed for validation of continuous flow as a method of bioconjugation at reasonable cost, it was ultimately the goal of this work to test this methodology on a therapeutically relevant ADC. As shown in **Scheme 3.12**, a non-degradable linker with
doxorubicin payload attached was synthesized (8, MC-DOX). After EEDQ mediated coupling of 6-maleimidohexanoic acid (3) and p-amino benzyl alcohol (4), a carbonate moiety with a good leaving group was added as a handle for attachment of doxorubicin (7).

![Scheme 3.12 Synthesis route to MC-DOX linker-toxin](image)

**3.2.7 TCEP Reduction followed by Flow Conjugation**

Using the NIBRT anti-IL8 mAb, a series of conventional vs. flow conjugation reactions with MC-DOX were carried out following a 30-minute reduction with TCEP. Although all reactions were repeated in triplicate, a representative sample of the conditions screened is shown in Table 3.9. Despite increased residence times, precipitation was still observed within the microreactors, despite the miscibility of DMSO and buffer. The samples were analyzed at NIBRT by SEC to determine purity (see Appendix for full spectra). In both conventional and flow reactions, minimal and unquantifiable conjugation was observed. Upon further research in literature, DMSO has been reported to expedite the oxidation of free sulfides to disulfide bonds. This result, in conjunction with the previous BSA results, may indicate that DMSO is not the ideal solvent for continuous flow conjugation, or may need to be prepared in a mixture with other miscible solvents or with buffer, without causing precipitation of the MC-DOX.
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<th>Time (min)</th>
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### 3.3 Conclusion and Future Directions

Based on these initial experiments, continuous flow microreactor technology is a viable option for exploring antibody-drug conjugations. The unparalleled efficiency of mixing and heating within these systems has demonstrated their ability to expedite reaction times and increase tagging with BSA and infliximab via lysine conjugation. Further solvent screens to reduce the amount of DMSO present in cysteine conjugation reactions will verify if the DMSO is reforming the disulfide bonds in the microreactor. Simultaneously, taking a step back to the smaller sulfide-reactive probes like FcM would be ideal for further studies, until the utility of flow for these reactions can be established. The next step for lysine conjugation in flow would be to design and/or synthesize a therapeutically relevant linker-drug conjugate to explore further.

### 3.4 Experimental

All reagents and solvents were purchased from Fisher Scientific or Sigma Aldrich. All continuous flow reactions were performed using the Chemtrix Labtrix Start system (Chemtrix BV, NL) utilizing a T-mixer glass microreactors, which are commercially available through
Chemtrix BV. All reagents were dissolved, loaded into 1 mL glass gas-tight syringes (SGE) and dispensed through the microreactor into the autosampler loaded with HPLC vials. $^1$H NMR spectra were recorded at 400 MHz, and $^{13}$C NMR were recorded at 100 MHz on a Varian NMR instrument, and spectra was prepared using ACD/Labs (Toronto, Canada). High-resolution mass spectroscopy was obtained on a Waters 70-VSE (EI) or a Waters Q-ToF Ultima mass spectrometer (ESI) at the UIUC Mass Spectrometry Facility for analysis of synthesized linkers.

For ADC-linker analysis by LC-MS, samples were desalted using a Zeba (Thermo Scientific, Rockland, IL) spin desalting column (7 kDa MWCO). The LC-MS analysis was performed on a micro-LC 200 system (Eksigent, Framingham, MA) coupled with a 5600 ESI Triple ToF mass spectrometer (AB Sciex, Framingham, MA). Samples were injected on an Eksigent MicroLC ChromXP C4, 5 µm, 300 Å, 50 × 0.5 mm column (Eksigent, Framingham, MA) set at 35 °C. Data analysis was performed with PeakView 1.0 and MagTran 1.03.

**Analytical methods for antibody-linker conjugate characterization**

*Sample reduction and desalting*

Myoglobin samples were desalted using a Zeba (Thermo Scientific, Rockland, IL) spin desalting column (7 kDa MWCO) prior to analysis. Antibody standards and ADCs were reduced by incubation with dithiothreitol (DTT). Briefly, 50 µL of a 1 mg/mL antibody or ADC solution in phosphate buffer saline (PBS) was mixed with equal amount of freshly prepared 40 mM DTT in water and then incubated at 37°C for 1 hour with shaking. The reaction was quenched by a following desalting step using a Zeba (Thermo Scientific, Rockland, IL) spin desalting column (7 kDa MWCO). Five microliters of sample was injected for LC-MS analysis.

*LC-MS sample analysis*

The LC-MS analysis was performed on a micro-LC 200 system (Eksigent, Framingham, MA) coupled with a 5600 ESI Triple ToF mass spectrometer (AB Sciex, Framingham, MA).
Samples were injected on an Eksigent MicroLC ChromXP C4, 5μm, 300Å, 50 × 0.5 mm column (Eksigent, Framingham, MA) set at 35 °C. The mobile phase consisted of water with 0.1% FA (mobile phase A) and acetonitrile with 0.1% FA (mobile phase B). The following gradients were applied at a flow rate of 40 µL/min: precondition with 10% B for 5 min, linear gradient from 10% B to 50% B in 10 min, washing with 90% B for 2 min, and then re-equilibration with 10% B for a min before the next injection. The Triple ToF was operated in intact protein positive ion mode with an ionspray voltage of 5500V, a source temperature of 350 °C, Gas1/Gas2 set to 40 (arbitrary units) and a declustering potential set to 150 V. Acquisitions were performed using TOF MS mode from m/z 500–4000, using an accumulation time of 0.1 s. Mass spectra data analysis was performed with PeakView ver. 1.0 and mass spectra deconvolution was performed with MagTran ver. 1.03.

**Experimental Procedures**

**Synthesis of N-succinimidyl-4-fluorobenzoate**

To a solution of 4-fluorobenzoic acid (2.0 g, 14.0 mmol) and triethylamine (2.89 g, 29.0 mmol) in acetonitrile (100 mL) was added disuccinimidyl carbonate (4.02 g, 16.0 mmol). The reaction was heated to 80 °C for 2 h, at which time the solvent was removed in vacuo. The resulting oil was diluted with a 1% sodium bicarbonate solution (100 mL) and extracted with chloroform (4 x 75 mL). The organic extracts were combined, dried over MgSO₄, and concentrated in vacuo. The reaction mixture was purified by silica gel chromatography (70:30 hexanes: EtOAc) to afford the product as a white solid (3.32 g, 60%). ¹H NMR (400 MHz, CDCl₃): 8.16 (m, 2H), 7.20 (m, 2H),
2.9 (br s, 4H); $^{13}$C NMR (100 MHz, CDCl$_3$): 169.33, 168.18, 165.61, 133.46, 121.44, 116.27, 25.72.

**Batch synthesis of FITC-BSA**

![Chemical structure](image)

To a solution of bovine serum albumin in phosphate buffer solution pH 8 (50 μL, 10 mg/mL) was added a 3, 5 or 10 eq solution of fluoroscein isothiocyanate in ACN (50 μL). The reaction was stirred at 4, 23, or 37 °C for 30 min, 4 h, or 12 h. The 27 experiments were immediately diluted with 400 μL of PBS pH 8, and a size-exclusion NAP5 column (Sephadex G-25 DNA grade) was used to remove any unreacted FITC. The sample was eluted with 1.0 mL of PBS to afford 100 μL of the reaction mixture. A spectrophotometer was used to analyze the samples at wavelengths of 288 and 493. The samples were diluted to a total volume of 1 mL for analysis. Calculations were completed based on the Beer-Lambert law.

**Flow synthesis of FITC-BSA**

To a Labtrix T-mixer chip (#3223), the following solution was introduced: bovine serum albumin in phosphate buffer solution pH 8 (10 mg/mL) and either a 3, 5 or 10 eq solution of fluoroscein isothiocyanate in ACN (50 μL). The reaction was performed at 4, 23, or 37 °C and 1-10 μL/min per syringe. Reaction mixtures were collected in 20 μL aliquots and diluted to 100 μL of PBS pH 8. A size-exclusion NAP5 column (Sephadex G-25 DNA grade) was used to remove any unreacted FITC. The sample was eluted with 1.0 mL of PBS to afford 100 μL of the
reaction mixture. A spectrophotometer was used to analyze the samples at wavelengths of 288 and 493. The samples were diluted to a total volume of 1 mL for analysis. Calculations were completed based on the Beer-Lambert law.

**Synthesis of FSB-myoglobin**

\[ \text{Myoglobin} + \text{FSB} \rightarrow \text{FSB-myoglobin} \]

\( P = \text{Protein} \)

To a solution of myoglobin (2 mg, 1 eq) in phosphate buffer solution (pH 8, 1 mL) was added \( N \)-succinimidyl-4-fluorobenzoate (FSB) (2 mg/mL in ACN, 3 eq). The reaction was stirred at room temperature for 5 min, 30 min, or 360 min, and immediately frozen or analyzed by ESI-MS.

**Analytical methods for Table 3.3**

After mass tagging, protein samples (200-500 pmols) were injected into an Alltech (Deerfield, IL) analytical in-line guard column, packed with POROS 20-R2 reversed-phase media (PerSeptive Biosystems). The column was connected to a Shimadzu SCL-10A VP HPLC flow water containing 0.05% formic acid, pH 2.6 at 50 μL/min coupled to a Waters LCT Premier mass spectrometer with a standard electrospray interface. Proteins were desalted (with water containing 0.5 % formic acid, pH 2.6) and then eluted directly into the mass spectrometer with a gradient of 15-98% acetonitrile (containing 0.05% formic acid, pH 2.6) in five minutes. Intact mass spectra were deconvoluted, and the ratio of modified to unmodified protein tested was determined using the software MagTran (Zhang, 1998). All intact MS experiments were conducted at least twice.
Microwave synthesis of FSB-myoglobin

To a solution of myoglobin (2 mg, 1 eq) in phosphate buffer solution (1 mL, pH 8) was added $N$-succinimidyl-4-fluorobenzoate FSB (2 mg/mL in ACN, 3 eq). The reaction was heated to 50 °C in a CEM Discover SP (Matthews, NC) for 1 and 5 minutes, and immediately frozen or analyzed by ESI-MS.

Synthesis of FSB-myoglobin

To a solution of myoglobin (2 mg, 1 eq) in phosphate buffer solution (pH 8, 1 mL) was added $N$-succinimidyl-4-fluorobenzoate (2 mg/mL in ACN, 3 eq). The reaction was stirred at 50 °C for 5 min, and immediately frozen or analyzed by ESI-MS.

Synthesis of FSB-myoglobin with base

To a solution of myoglobin (2 mg, 1 eq) in phosphate buffer solution (pH 8, 1 mL) was added $N$-succinimidyl-4-fluorobenzoate (2 mg/mL in ACN, 3 eq) and triethylamine (2 μL). The reaction was stirred at room temperature for 30 min or 360 min, and immediately frozen or analyzed by ESI-MS.
Synthesis of FSB-insulin

To a solution of insulin (2 mg, 1 eq) in phosphate buffer solution (pH 8, 1 mL) was added $N$-succinimidyl-4-fluorobenzoate (2 mg/mL in ACN, 3 eq). The reaction was stirred at room temperature for 30 min, and immediately frozen or analyzed by ESI-MS.

Microwave synthesis of FSB-insulin

To a solution of insulin (2 mg, 1 eq) in phosphate buffer solution (1 mL, pH 8) was added $N$-succinimidyl-4-fluorobenzoate (2 mg/mL in ACN, 3 eq). The reaction was heated to 50°C in a CEM Discover SP (Matthews, NC) for 1 and 5 minutes, and immediately frozen or analyzed by ESI-MS.

Synthesis of FSB-insulin with base

To a solution of insulin (2 mg, 1 eq) in phosphate buffer solution (pH 8, 1 mL) was added $N$-succinimidyl-4-fluorobenzoate (2 mg/mL in ACN, 3 eq) and triethylamine (2 µL). The reaction was stirred at room temperature for 30 min, and immediately frozen or analyzed by ESI-MS.

Synthesis of FSB-interferon-2α

To a solution of interferon-2α (2 mg, 1 eq) in phosphate buffer solution (pH 8, 1 mL) was added $N$-succinimidyl-4-fluorobenzoate (2 mg/mL in ACN, 3 eq). The reaction was stirred at room temperature for 30 min, and immediately frozen or analyzed by ESI-MS.
**Synthesis of FSB-interferon-2α with base**
To a solution of interferon-2α (2 mg, 1 eq) in phosphate buffer solution (pH 8, 1 mL) was added N-succinimidyld-4-fluorobenzoate (2 mg/mL in ACN, 3 eq) and triethylamine (2 μL). The reaction was stirred at room temperature for 30 min, and immediately frozen or analyzed by ESI-MS.

**Synthesis of FSB-human serum albumin**

![Diagram of FSB-human serum albumin](image)

To a solution of human serum albumin (2 mg, 1 eq) in phosphate buffer solution (pH 8, 1 mL) was added N-succinimidyld-4-fluorobenzoate (2 mg/mL in ACN, 3 eq). The reaction was stirred at room temperature for 30 min, and immediately frozen or analyzed by ESI-MS.

**Synthesis of FSB-human serum albumin with base**
To a solution of human serum albumin (2 mg, 1 eq) in phosphate buffer solution (pH 8, 1 mL) was added N-succinimidyld-4-fluorobenzoate (2 mg/mL in ACN, 3 eq) and triethylamine (2 μL). The reaction was stirred at room temperature for 30 min, and immediately frozen or analyzed by ESI-MS.

**Flow synthesis of FSB-myoglobin**

![Diagram of FSB-myoglobin](image)
To a Labtrix T-mixer chip (#3025), the following solution was introduced: myoglobin in phosphate buffer solution pH 8 (2 mg/mL) and either 3 eq solution of FSB in ACN. The reaction was performed at RT and 0.5 - 20 μL/min per syringe. Reaction mixtures were collected in 100 μL aliquots and frozen for analysis.

**Conventional synthesis of infliximab-fluorobenzoate**

To a solution of Infliximab (0.005 g, 1 eq) in phosphate buffer solution (pH 8) was added a 2 mg/mL solution of N-succinimidyl-4-fluorobenzoate in acetonitrile (12 μL). The reaction was stirred at room temperature for 1 h, and then immediately frozen.

**Flow synthesis of infliximab-fluorobenzoate**

To a Labtrix T-mixer chip (#3023), the following solution was introduced: infliximab in phosphate buffer solution pH 8 (2 mg/mL) and either 5 or 10 eq solution of FSB in DMSO. The reaction was performed at 23 or 37 °C and 0.5 - 5 μL/min per syringe. Reaction mixtures were collected in 50 μL aliquots and frozen for analysis.

**Conventional BSA-Pyrene Conjugation**
To a solution of bovine serum albumin (10 mg, 1 eq) in phosphate buffer solution (pH 8, 5 mL) was added TCEP (0.430 mg, 10 eq). The reaction mixture was stirred for 30 mins, at which time the reaction mixture was divided into five vials with 1 mL solution each. To each vial, N-pyrenylmaleimide as a soluiton DMSO or DMF (44.6 µL of a 2 mg/mL solution, 20 eq) was added. The reactions were stirred in the dark and 500 µL aliquots were taken at 30 minute intervals for purification by centrifugation in MWCO filter and the UV-Vis spectrum was obtained.

**Conventional conjugation of therapeutic mAbs with FcM**

To a solution of mAb (bevacizumab, cetuximab, infliximab, rituximab, and trastuzumab (2.5 mg, 1 eq) in phosphate buffer solution (pH 8, 1 mL) was added TCEP as a solution in HEPES buffer (10 eq). The reaction mixture was stirred for 30 mins, at which time the reaction mixture was divided in half. To each vial, fluorescein-5-maleimide was added (4 eq or 8 eq) as a solution in DMSO. The reactions were stirred in the dark for various time points and 83 µL aliquots were taken, quenched with 4 or 8 eq of benzyl mercaptan, and frozen for analysis.

**Flow conjugation of therapeutic mAbs with FcM**

To a Labtrix T-mixer chip (#3025), the following solution was introduced: therapeutic mAb ((bevacizumab, cetuximab, infliximab, rituximab, and trastuzumab) in PBS buffer (2.5 mg/mL, 1 eq), 10 eq TCEP in HEPES buffer, and either 4 or 8 eq FcM in DMSO. The reaction was
performed at RT and 0.5 - 20 μL/min per syringe. Reaction mixtures were collected in 50 μL aliquots, quenched with 2-mercaptopyrimidine and frozen for analysis.

**Synthesis of MC-DOX linker**

To a solution of 6-maleimidohexanoic acid 3 (0.200 g, 0.947 mmol) and 4-amino benzyl alcohol 4 (0.122 g, 0.994 mmol) in 3 mL DCM and 1.5 mL MeOH was added N-Ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ, 0.246 g, 0.994 mmol). The reaction mixture was stirred in the dark for six hours at room temperature to prevent decomposition, at which point the reaction mixture was concentrated in vacuo and purified by column chromatography (1:5 EtOAc/DCM) to afford the desired compound 5 as a yellow solid (0.2665 g, 89%) (400 MHz, DMSO-d$_6$): δ 9.80 (s, 1H), 7.53 (d, 2H), 7.22 (d, 2H), 7.03 (d, 2H), 5.09 (s, 1H), 4.43 (d, 2H), 3.40 (t, 2H), 2.27 (t, 2H), 1.61-1.48 (m, 4H), 1.24 (m, 2H). (100 MHz, DMSO-d$_6$): δ 170.98, 170.80, 137.82, 136.94, 134.34, 126.76, 118.68, 62.53, 36.88, 36.02, 27.71, 25.73, 24.52. MS (ESI+), m/z calcd. 316.14, obsd. [M+H] 317.04.

To a solution of 5 (0.1871 g, 0.592 mmol) and bis(4-nitrophenyl)carbonate 6 (0.5401 g, 1.78 mmol) in DMF (3 mL) was added diisopropylethylamine (DIPEA, 0.2062 mL, 1.19 mmol). The reaction mixture was stirred in the dark overnight at room temperature to prevent decomposition, at which point the reaction mixture was concentrated in vacuo, extracted with DCM to obtain an
oil, which was precipitated via azeotropic distillation with hexanes and diethyl ether to afford the desired compound 7 as a yellow solid (0.1083 g, 39%) (400 MHz, CDCl₃): δ 8.29 (d, 2H), 7.57 (d, 2H), 7.40 (d, 2H), 7.37 (d, 2H), 6.69 (s, 2H), 3.54 (t, 2H), 2.37 (t, 2H), 1.77 (m, 2H), 1.64 (m, 2H), 1.37 (m, 2H) with trace DCM (5.25) and DMF (8.02, 2.98, 290). (100 MHz, CDCl₃): δ 170.78, 170.55, 155.12, 152.07, 144.97, 138.31, 133.73, 129.47, 129.39, 124.97, 121.46, 119.45, 70.30, 37.13, 37.05, 27.80, 25.80, 24.41. MS (ESI+), m/z calcld. 481.15, obsd. [M+H] 482.01.

To a solution of 7 (0.0487 g, 0.1011 mmol) and doxorubicin-hydrochloride (0.0500 g, 0.09199 mmol) in DMF (2 mL) was added diisopropylethylamine (DIPEA, 0.032 mL, 0.1840 mmol). The reaction mixture was stirred in the dark for six hours at room temperature to prevent decomposition, at which point the reaction mixture was concentrated in vacuo and purified on silica gel (100% DCM-2.5% MeOH/DCM) to afford 8, MC-DOX as a bright red solid (0.0683 g, 83%) (400 MHz, CDCl₃): δ 8.05 (d, 2H), 7.80 (t, 2H), 7.46 (d, 2H), 7.20 (s, 1H), 6.67 (s, 2H), 4.98 (s, 2H), 4.76 (s, 2H), 4.55 (s, 1H), 4.13 (t, 2H), 4.09 (s, 3H), 4.01 (s, 1H), 3.86 (m, 2H), 3.74 (s, 1H), 3.66 (br s, 2H), 3.52 (m, 4H), 3.32 (d, 1H), 3.27 (d, 1H), 3.07 (d, 1H), 2.32 (m, 5H), 2.16 (m, 2H), 1.87 (m, 4H), 1.75 (m, 4H), 1.36 (t, 3H), 1.28 (m, 4H) with dichloromethane (5.30), grease (0.86, 1.26) and water (1.53) impurities. MS (ESI+), m/z calcld. 885.30, obsd [M+Na]. 908.20.

**Conventional Conjugation of MC-DOX to anti-IL8**

To a solution of anti-IL8 mAb (20 mg, 1 eq) in phosphate buffer solution (pH 8, 10 mL) was added TCEP as a solution in HEPES buffer (40.89 µL of a 2 mg/mL solution, 2.1 eq). The
reaction mixture was stirred for 30 mins, at which time the reaction mixture was divided into six different vials with 666 µL each. To each vial, MC-DOX (6 eq or 8 eq) as a solution in DMSO. The reactions were stirred in the dark for various time points and 580 µL aliquots were taken, quenched with 2-mercaptopurine, and frozen for analysis.

**Flow Conjugation of MC-DOX to anti-IL8**

To a Labtrix T-mixer chip (#3025), the following solution was introduced: Therapeutic anti-IL8 mAb in PBS buffer (2 mg/mL, 1 eq), 2.1 eq TCEP in HEPES buffer, and either 6 or 8 eq MC-DOX in DMSO. The reaction was performed at RT and 0.5 - 20 μL/min per syringe. Reaction mixtures were collected in 500 μL aliquots, quenched with 2-mercaptopurine and frozen for analysis.

**3.5 References**


CHAPTER 4: Applications in Chemistry Education and Bioconjugation – A Critical Perspective and Future Directions

4.1 Introduction

Continuous flow methodology has experienced both tremendous support and criticism from the chemistry community over the years. This chapter seeks to provide a critical perspective on this work, by identifying areas of concern in adopting flow chemistry within the fields of chemistry education and bioconjugation in conjunction with the benefits as previously described.

4.2 Practicality, Utility and Limitations of Continuous Flow Chemistry in Undergraduate Laboratories

Incorporating continuous flow methodology into the undergraduate chemistry laboratory curriculum provides students with the opportunity to gain exposure to technology that is widely used in many sectors of chemistry. While this benefit may lead to an increased awareness on the part of the student, there are several practical considerations that need to be discussed regarding student autonomy in the lab, cost of equipment and lab logistics.

4.2.1 Student Autonomy in the Laboratory

While POGIL activities are designed for students to learn concepts through their own decision making and analysis of results, transitioning this material to a laboratory setting requires more attention on the part of the instructor than through worksheet-based activities. The minimal guidance from the instructor in a paper-based POGIL activity may need to be increased in the laboratory, particularly in the case of this flow experiment. The instrument will need monitoring to ensure there are no clogs and that all parts are connected correctly. Students measuring out sodium azide will also require more close supervision. Though this breaks the mold of the POGIL model, the extra presence of the instructor is solely for logistics of set up and
preparation, and not regarding the actual chemical principles being discovered by the students. Therefore, a guided inquiry lab is a better balance for this dynamic than an entirely open inquiry experiment, where students lack the direction needed to use the equipment, coupled with the lack of knowledge of the question they have set out to answer.

4.2.2 Cost of Equipment

The Chemtrix instrument used in the development of this laboratory experiment can range in price from $18,000-$35,000, not including consumables. This cost may realistically be beyond the scope of what small, undergraduate-focused institutions would be able to afford for the teaching lab. The chemistry education literature details several examples of in-house fabricated flow devices, including microfluidic devices,\textsuperscript{1-4} gas-phase reactors\textsuperscript{5} and coil/tube based reactors.\textsuperscript{6} Figure 4.1 is an example of such a device, described by Teerasong et al.\textsuperscript{1} The microfluidic channel is created in a computer drawing program in the desired configuration. A photolithographic technique is used to create the design by polymerizing a monomer layer between two glass microscope slides using UV radiation. Their method takes approximately 30 minutes to complete, and takes advantage of commercially available materials. Therefore, students in an upper level setting could construct their own device using commercially available materials, and will need to think deeper into the function of each part of a flow apparatus, in order to construct it properly.

![Figure 4.1](image-url) Preparation of an in-house microfluidic device, reproduced with permission\textsuperscript{1}
4.2.3 Laboratory Logistics

Based on the previous discussion of cost, adoption of this laboratory exercise would accompany the purchase of a single Chemtrix instrument. Therefore, this experiment would be ideal for small, upper level courses for several reasons. Students are more likely to have been exposed to advanced organic chemistry reactions (preferably cycloadditions or heterocyclic chemistry) and some analytical chemistry (HPLC), which matches best with the various components of the exercise. Also, smaller class size will allow easier, faster turnover of the equipment, as its use will need to be staggered with multiple groups. For larger class sizes, in-house fabricated reactors may be a better alternative, as each group will be able to have their own device.

4.3 Considerations for ADC Production Using Continuous Flow Chemistry

Continuous flow conditions represent an expedited route to protein labelling using FSB and FITC as models of lysine conjugation. Although these initial results are promising, the first reactions using linker-toxin conjugates and therapeutic mAbs proved more challenging in terms of mAb recovery, precipitation and solvent accessibility, which all warrant further discussion, as well as the feasibility of scaling out this technology to produce ADCs on a larger scale.

4.3.1 Protein and mAb Recovery from Microreactor

The recovery of therapeutic mAbs described in Chapter 3 was less than 50%, and represents a significant challenge in moving forward with ADC conjugation in flow. In order to elucidate the cause of this low recovery, several methods can be utilized. Glass surfaces and biomolecules have well-documented adsorption interactions, namely through reactions between charged amines from the protein or mAb and silanol from the glass. A pepsin or SDS-mediated digest of any material remaining in the microreactor, followed by mass spec analysis, would
validate the presence of this interaction. Comparing the recovery of these reactions to those carried out on a microreactor made of an alternate surface would also shed some insight into this compatibility issue.

4.3.2 Assessment of Solvent Accessibility and Reproducibility

FSB conjugation to infliximab showed perceived light-chain selectivity using flow conditions. Further investigation to confirm this finding warranted, as the recovery of these reactions may have been limited due to the previously-described interaction with the glass surface. It would also be pertinent to understand the sites of conjugation of all the aforementioned reactions, not only within a single reaction but also with regards to reproducibility of a series of the same reactions. Understanding this relationship between reproducibility and solvent accessibility would allow for further investigation into the possible benefits of flow chemistry in this application.

4.3.3 Feasibility of Scale Out Production

In 2015, Kadcyla (ado-trastuzumab emtansine) generated $769 million in sales, and at a reported $94,000 per treatment, approximately 8200 courses of treatment were administered. Each course of treatment lasts 36 weeks, with treatments every three weeks, such that each course of treatment requires 12 doses. At the required dose of 3.6 mg/kg, with an average North American person weighing 80.7 kg, each treatment would require 3.5 g of ADC. In total, almost 30 kg of ADC is necessary for a total year of treatments. The microfluidic conditions alone are not sufficient to keep up with this demand, as a 10 µL/min flow rate generating a 1 mg/mL product would require 5460 yr to produce the desired quantity. Therefore, a kilo-scale reactor at a flow rate of 100 mL/min, or a series of meso scale reactors at 10 mL/min would be necessary to generate the amount of ADC necessary to meet the market demand.
4.4 Future Directions

Future work on these projects would require investigation into piloting the POGIL activity on a small scale with the Chemtrix instrument, as well as development of an in-house fabricated device that would allow the approach to reach a wider audience, from large research institutions to small undergraduate teaching colleges. A more in-depth mass spectrometric analysis of conjugation states needs to be carried out, along with analysis of recovered material from the microreactors, in order to understand any inhibitory interactions with the glass surface. From there, it may be pertinent to use other surface materials such as polymers or surfactants to carry out these transformations. On the whole, although flow chemistry presents a number of advantages, it is important to consider its limitations critically for each possible application.

4.5 References


Appendix Contents

Chapter 2

- Spectra
- Notes for Instructors

Chapter 3

- Spectra

Permissions
$y = 0.0092x + 0.0082$

$R^2 = 0.9992$

![Chemical Structure](image)

**Concentration (mg/mL)**

**Peak Area**
y = 0.0077x + 0.0188
R² = 1
y = 0.0171x + 0.0271
R² = 0.9991
$y = 0.0114x + 0.082$

$R^2 = 0.9983$
y = 0.0129x - 0.18
R² = 0.9954
Peak Area vs. Concentration (mg/mL) graph with the equation:

\[ y = 0.0117x + 0.0427 \]

and the coefficient of determination \( R^2 = 0.9997 \).
$y = 0.0093x + 0.0653$

$R^2 = 0.9957$
\[ y = 0.0134x + 0.2078 \]

\[ R^2 = 0.9994 \]
Why go with the flow? Notes for Instructors

A POGIL Activity for Advanced Organic Chemistry Laboratory

Developed by Meaghan M. (Sebeika) Fallano and Thomas R. Gilbert, Northeastern University,
360 Huntington Ave. Boston, MA 02115

Chemical and Equipment List

Chemicals:

- Benzyl bromide (CAS: 100-39-0) Acros Organics
- Sodium azide (CAS: 26628-22-8) Acros Organics
  - NOTE: Solid sodium azide can spark if agitated with metal spatulas. Please handle sodium azide with a PTFE/Teflon spatula. PTFE Coated Spatula FisherSci
- 2-Cyanoacetamide (CAS: 107-91-5) Acros Organics
- Sodium hydroxide (CAS: 1310-73-2) FisherSci
- 1-Methyl-2-pyrrolidinone (CAS: 872-50-4) FisherSci
- For water, deionized water can be used.
- Dimethyl sulfoxide (CAS: 67-68-5) FisherSci

Consumables

- HPLC Vials: HPLC Vials
- HPLC Vial Caps: Vial Caps
- Glass Pasteur pipettes
- Graduated cylinder
• Weigh paper

Equipment

• Chemtrix Labtrix Start system with reactor 3023, which includes fittings, tubing, reactor housing, syringe pumps, temperature controller
• SGE 1 mL gastight Leur lock syringes Syringes

INTRODUCTION

MODEL 1: Continuous flow reaction considerations

Continuous flow chemistry is used in a variety of settings, from chemical and pharmaceutical manufacturing, to reaction development and optimization. Today, we will be exploring the benefits of using solution-based continuous flow chemistry for the synthesis of 1,2,3-triazoles via a [3+2] cycloaddition of azides and alkynes.

Below is a diagram of a continuous flow microreactor, which is typically made from glass, with narrow reaction channels etched into it. (NOTE: This figure is not drawn to scale. The microreactors we will be using contain a maximum volume of 10-15 µL.) Reagents are loaded into syringes, and regulated syringe pumps are used to dispense reagents into the microreactor.
Critical Thinking Questions

1. How does the diagram above differ from a typical reaction vessel, such as a round bottomed flask?

   -A typical round bottomed flask is stirred with a stir bar and heated or cooled with a hot plate/oil bath or ice bath. Quantities are measured and added to the round bottom, and reacted for a set period of time. In this flow device, reagents are made into solutions and injected into the device using a syringe pump. The quantities are therefore related to each other based on concentration and not on mass.

2. What considerations should a chemist consider regarding reagents and reaction preparation before deciding to undertake a flow reaction? What reagents or reactions might not be compatible with this system? Can you think of anything that would be incompatible with this system?
Because this type of reaction is solution based, all reagents must be soluble in whatever solvent(s) are used, otherwise the channels will clog. Of particular note, incompatible reagents include insoluble solids, as well as hydrofluoric acid (HF) because fluoride ions will etch the glass and destroy the microreactor.

3. The reaction zone, shown as 5 in the diagram, is where the reaction takes place. The residence time is the time it takes for the reaction mixture to flow from the start to the end of the microreactor. What conditions would need to be known about the reaction to calculate the residence time? How would you set up the calculation?

In order to determine the residence time, the reactor volume (in µL) and the flow rate of all reagents (in µL/min) must be used. It should be noted that the flow rate used should be the combined flow rate of all reagents. For example, if the reactor volume was 10 µL, Reagent A was pumped at 10 µL/min and Reagent B was pumped at 10 µL/min, the total flow rate would be 20 µL/min.

The calculation would look like this:

\[10 \, \mu L \times \frac{\text{min}}{20 \, \mu L} = 0.50 \, \text{min or 30 sec}\]

**MODEL 2: Synthesis of azide intermediates**

Azides, denoted by the functional group N₃, are used as precursors to a variety of heterocyclic compounds used in many applications including pharmaceutical development and fine chemical manufacturing. The first compound we will synthesize is benzyl azide.

**Critical Thinking Questions**
1. Draw the most stable Lewis structure of benzyl azide (C₇H₇N₃). Add any non-zero formal charges.

![Lewis structure of benzyl azide](image)

2. Benzyl azide will be synthesized using sodium azide as a reagent. Based on the nature of sodium azide and the structure of benzyl azide you drew, what type of reaction would produce benzyl azide as a product? **HINT**: Think back to the reactions you learned in Organic Chemistry I. Suggest a starting material you would need to carry out this transformation? Draw the mechanism of this reaction.

*One could envision a SN2 reaction of a primary halide with an azide nucleophile to produce an alkyl azide. For example:*
3. In what type of solvent (polar or nonpolar, aqueous or organic) are the reactants and products likely to be soluble?

-Because we have polar organic molecules, as well as a salt as a byproduct, the best solvent system would include a polar solvent, as well as some percentage of water to ensure the sodium bromide does not clog the system.

4. An automobile airbag is inflated by the following decomposition reaction of sodium azide (NaN₃):

\[
20 \text{NaN}_3(s) + 6 \text{SiO}_2(s) + 4 \text{KNO}_3(s) \rightarrow 32 \text{N}_2(g) + 5 \text{Na}_4\text{SiO}_4(s) + \text{K}_4\text{SiO}_4(s)
\]

Why would azide intermediates be considered dangerous to handle and isolate, particularly due to decomposition?

_Azides, upon degradation, can release N₂ gas and thus, be explosive. Care should be used when handling azides._

5. Please ask the instructor to check the previous two questions. Upon completion, you will receive the protocol and results for the conventional (round bottom flask and traditional
heating) reaction used to synthesize benzyl azide.

Based on the protocol described, propose a starting point for your flow reaction. Include your desired residence time, as determined by the flow rate, temperature, and equivalents of reagents. Calculate how long you will need to collect your reaction mixture to obtain 50 µL of reaction mixture. You will be using a microreactor with the following specifications:

-Students will be given the information on the following page. Students may suggest the as similar conditions to conventional as possible (temperature 80ºC, relatively slow residence time, 1 eq bromide, 2 eq azide).

**EXPERIMENTAL PROTOCOL FOR AZIDE FLOW REACTION**

1. Prepare solutions of the two reagents in the appropriate solvent(s). You will need a maximum of 1 mL of each solution.

2. Set the microreactor to the chosen temperature.

3. Once the desired temperature has been reached, fill the syringes with your solutions. Be sure to fill them both to the same volume level, as they will be attached to the same syringe pump.
4. Connect the syringes to the flow apparatus via the Leur Lock connectors. Place an empty HPLC vial on the end of the tubing coming out of the collection end of the apparatus.

5. Set the syringe pump to the desired flow rate. Ensure the handle is completely flush against the plunger of the syringes.

6. Start the syringe pump, and continue pumping reagents through until all the bubbles are out of the microreactor, and all solutions are flowing consistently.

7. After the flow has reached equilibrium, set a timer for your desired collection time. When ready, connect an HPLC vial containing 500 µL of water to the tubing coming out of the collection end of the apparatus and begin your collection.

8. When your time is up, stop the syringe pump and remove the HPLC vial from the tubing.

9. Return your solutions to their respective vials, and clean the syringes with water and acetone.

10. Fill one syringe with NMP and one with water. Set the flow rate to 20 µL/min and pump the solvents through the microreactor for 5 minutes to ensure it is clean for the next group.

11. Run your sample on the HPLC to obtain a chromatogram of your reaction mixture.

12. The chromatogram peak areas in this example are relative to the amount of each substance in the mixture. Integrate the peak areas, assuming the total area of all components is 1. Calculate your percent conversion.

13. You are going to choose a second set of conditions. Half the groups will vary temperature and half will vary residence time by selecting a new flow rate. Discuss your choice with the other groups varying the same parameter so that as many possible sets of conditions are covered.
14. Repeat the steps as above for your new set of conditions.

15. Once all groups are finished, report out all conditions with conversions.

*Notes for Instructors: Ensure students discharge any air bubbles from syringes before connecting to the apparatus.*

**Critical Thinking Questions**

1. Based on the group results, plot two sets of data: residence time vs. percent conversion and temperature vs. percent conversion. What are the conditions that produced the best percent conversion? How do they compare to the conventional reaction?

*Below is a sample plot of percent conversion as a function of temperature.*
2. Conventional reactions are based on mol quantities of reagents. Since the flow reactions are based on units of concentration (mol/L), what is the theoretical concentration of product in your vial?

-Say you have 0.5 M BnBr and 0.5 M NaN₃. If you mix equal volumes of these two reagents together, as they combine in a 1:1 ratio, the product will be produced as a 0.25 M solution, because the volume of the mixture has been doubled.

3. Is this reaction more dependent on reaction time or temperature? How do you know?

-Will depend on the results from the conditions selected by students but hopefully they will point out that an increased residence time will increase the yield of the reaction, but that once you hit about 80 deg, the yield levels off, so there’s no reason to run the reaction at a higher temp than is necessary.

4. Compare the flow reaction to the conventional reaction; what are the benefits you experience of using the flow process? Were there any drawbacks or challenges?

-Advantages: quicker time, better conversion more quickly, easy set up

-Disadvantages: clogging, solution prep

**MODEL 3: Synthesis of 1,2,3-triazoles**

Triazole moieties are used as precursors to a number of pharmaceutically relevant molecules. One of those drugs, ticagrelor (Brillinta) is shown below and is an inhibitor of platelet aggregation, and is used to prevent thrombic events, such as a heart attack or stroke. Today, we
are going to synthesize a triazole core that can be cyclized and further modified to mirror the core of Brillinta.

Critical Thinking Questions

1. The azide you made last week will be used to synthesize a benzyl-substituted triazole as shown below. You will receive a handout with the experimental protocol for this conventional process. Using that experimental protocol as a guide on how the product is isolated, how can we verify our product is being made in the flow system?

\[
\text{PhN}_3 + \text{H}_2\text{NCON} \xrightarrow{\text{NaOH, NMP}} \text{PhN}_3\text{OCONH}_2
\]

-Since the product in the conventional reaction precipitates overnight with water, we should be able to see solid form once our product mixture hits the water in the HPLC
2. Consider the HPLC chromatogram from our reactions last week. Where will the product peak show up relative to the starting materials on the trace?

   - *Since a triazole is more polar than an azide, it will elute earlier on the HPLC chromatogram*

3. Based on the azide reactions and the conventional results for triazole synthesis, what conditions will you start with, in terms of temperature and flow rate to obtain the desired residence time?

   - *Most likely, students will start with their ideal conditions from the previous reaction. Usually, that’s approx. 80 deg and 1-5 µL/min.*

4. In this reaction, we have three starting materials instead of two, however we will be using the same microreactor. Describe how you would set up this reaction using the available fittings.

   - *There is a T connector that can be used to join the flow of both sodium hydroxide and cyanoacetimide*

**EXPERIMENTAL PROTOCOL FOR TRIAZOLE FLOW REACTION**
1. Prepare solutions of the three reagents in the appropriate solvent(s). You will need a maximum of 1 mL of each solution.

2. Set the microreactor to the chosen temperature.

3. Once the desired temperature has been reached, fill the syringes with your solutions. Be sure to fill them all to the same volume level, as two of them will be attached to the same syringe pump.

4. Connect the syringes to the flow apparatus via the Leur Lock connectors. Place an empty HPLC vial on the end of the tubing coming out of the collection end of the apparatus.

5. Set the syringe pump to the desired flow rate. Ensure the handle is completely flush against the plunger of the syringes.

6. Start the syringe pump, and continue pumping reagents through until all the bubbles are out of the microreactor, and all solutions are flowing consistently.

7. After the flow has reached equilibrium, set a timer for your desired collection time. When ready, connect an HPLC vial containing 500 μL of water to the tubing coming out of the collection end of the apparatus and begin your collection.

8. When your time is up, stop the syringe pump, add 300 μL DMSO and ensure everything is dissolved. Take your HPLC vial for analysis and obtain the chromatogram.

9. Return your solutions to their respective vials, and clean the syringes with water and acetone.

10. Fill one syringe with NMP and one with water. Set the flow rate to 20 μL/min and pump the solvents through the microreactor for 5 minutes to ensure it is clean for the next group.
11. Obtain the percent conversion and percent remaining starting material from the HPLC chromatogram. Using these results, you are going to choose a second set of conditions. Half the groups will vary temperature and half will vary flow rate to change the residence time. Discuss your choice with the other groups varying the same parameter so that as many possible sets of conditions are covered.

12. Repeat the steps as above for your new set of conditions. Once all groups are finished, report out all conditions with conversions.

**Critical Thinking Questions**

1. Based on the group results, plot two sets of data: residence time vs. percent conversion and temperature vs. percent conversion. What are the conditions that yield the highest percent conversion for this reaction? How do they compare to the conventional reaction?

*See below*

2. Copy those graphs and add a plot of the percent azide remaining vs. the same variables. What trend do you notice about this data?

![Graphs showing percent conversion and percent azide remaining vs. temperature](image)

*Here are some samples at different residence times where you can see the appearance of product and disappearance of starting material.*
3. Is this reaction more dependent on reaction time or temperature? How do you know?
   
   *This again will depend on the results chosen by the students, but you will likely see a similar topping off, such that the yield tops out around 80-90 deg.*

4. Based on the conditions for both reactions, can you think of any ways to improve this process any further?

   *-Since these reactions both operate at similar conditions, it is possible to do the entire process on one microreactor so as not to have to isolate the azide intermediate.*
**Figure S1** Representative deconvoluted mass spectrum (top panel) and full mass spectrum (bottom panel) for the flow synthesis of myoglobin with FSB
### Table S1  Summary results for the conventional synthesis of Infliximab with FSB

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Time (hr)</th>
<th>Equivalents of FSB</th>
<th>Reaction Temp (°C)</th>
<th>FSB molecules added (LC)</th>
<th>FSB molecules added (HC)</th>
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<tbody>
<tr>
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### Table S2  Summary of light chain masses detected in the conventional synthesis of Infliximab with FSB

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Unreacted light chain 23434 Da</th>
<th>Light chain with 1 FSB 23556 Da</th>
<th>Light chain with 2 FSBs 23678 Da</th>
<th>Light chain with 3 FSBs 23800 Da</th>
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<tr>
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Table S3  Summary results for heavy chain masses detected in the conventional synthesis of Infliximab with FSB

<table>
<thead>
<tr>
<th>Expt</th>
<th>Unreacted heavy chain peak 1</th>
<th>Heavy chain peak 1 w/ 1 FSB</th>
<th>Unreacted heavy chain peak 2</th>
<th>Heavy chain peak 2 w/ 1 FSB</th>
<th>Unreacted heavy chain peak 3</th>
<th>Heavy chain peak 3 w/ 1 FSB</th>
<th>Unreacted heavy chain peak 4</th>
<th>Heavy chain peak 4 w/ 1 FSB</th>
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Table S3 shows the summary results for heavy chain masses detected in the conventional synthesis of Infliximab with FSB. The table includes columns for Expt, Unreacted heavy chain peak 1, Heavy chain peak 1 w/ 1 FSB, Unreacted heavy chain peak 2, Heavy chain peak 2 w/ 1 FSB, Unreacted heavy chain peak 3, Heavy chain peak 3 w/ 1 FSB, Unreacted heavy chain peak 4, and Heavy chain peak 4 w/ 1 FSB. The values in the table represent the presence or absence of FSB at each peak, indicated by Y (yes) or N (no).
Figure S2  Representative deconvoluted mass spectrum (top panel) and full mass spectrum (bottom panel) of the light chain for the conventional synthesis of Infliximab with FSB
Figure S3  Representative deconvoluted mass spectrum (top panel) and full mass spectrum (bottom panel) of the heavy chains for the conventional synthesis of Infliximab with FSB (Table 4, Entry 3). Differences in deconvoluted masses versus tabulated mass (Table S2) are due to deconvolution of the low signal intensity full mass spectrum. *: One FSB molecule added. **: Two FSB molecules added.
**Table S4**  Summary of results for flow conjugation of infliximab-FSB

<table>
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<tr>
<th>Experiment</th>
<th>Residence time (s)</th>
<th>Equivalents of FSB</th>
<th>Reaction Temp (°C)</th>
<th>FSB molecules added (LC)</th>
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**Table S5**  Summary of light chain masses detected in flow conjugation of infliximab-FSB

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<th>Experiment</th>
<th>Unreacted light chain 23434 Da</th>
<th>Light chain with 1 FSB 23556 Da</th>
<th>Light chain with 2 FSBs 23678 Da</th>
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Figure S4 Representative deconvoluted mass spectrum (top panel) and full mass spectrum (bottom panel) of the light chain for the flow synthesis of Infliximab with FSB (Table 5, Entry 3)
BSA-NPM w/ DMSO

Absorbance vs. Wavelength (nm)

- BSA
- NPM w/ DMSO

Time Points:
- 30 min
- 1 hr
- 1 hr 30 min
- 2 hr
- 2 hr 30 min
- 3 hr
- 3 hr 30 min
- 4 hr
- 4 hr 30 min
- 5 hr
SEC for ADCs purification

MAbPac SEC-1 7.8 x 300 mm, 5 µm
(PN 088460, SN 001020
Lot# 300541, TFS 104 )
VanquishH LC4
- **Conventional reactions**
- **6 eq MC-DOX**

Cytotoxic drug

ADC
- Conventional reactions
- 8 eq MC-DOX
- Conventional reactions
- 8 eq MC-DOX

Cytotoxic drug

ADC

mAU

170216_003_ADC23-1_1min
170216_004_ADC23-4_15min
170216_005_ADC23-7_30min
170216_006_ADC23-10_1h
170216_007_ADC23-15_2h
170216_008_ADC23-16_5h
- Flow reactions
- 6 eq MC-DOX

Cytotoxic drug

ADC
- Flow reactions
- 8 eq MC-DOX

Cytotoxic drug

ADC
SEC for ADCs purification

MAbPac SEC-1 7.8 x 300 mm, 5 µm
(PN 088460, SN 001020
Lot# 300541, TFS 104)

Fraction Collector AFC 3000
mAb, 10 ug collected peak → Amicon® Ultra-0.5 10K MWCO

- Collect by time: 11.75-13.50min
- Delay time: 5 sec

AFC 3000

mAb, 10 ug

25µL inj volume

10ug/75µL

3.3ug
ADC 3 x 3µL injected (3 x 15 ug if solution contains 5µg/µL)

Collected and filter through Amicon 10k (0.5mL) for HIC analysis

Conventional Reaction ADC21-18_5hr

- Collect by time: 11.75-13.00min
- Delay time: 5 sec
Unconjugated DP12 mAb

mAb, 20 ug collected peaks → Amicon® Ultra-2.0 10K MWCO

For HIC analysis
60µg/ 90µL

AFC 3000
- Collect by time: 11.80-13.00min
- Delay time: 4.2 sec
Conventional Reaction ADC23-10_1hr
ADC collected peaks → Amicon® Ultra-2.0 10K MWCO

For HIC analysis
~100µg/ 80µL

AFC 3000
- Collect by time: 11.75-13.50min
- Delay time: 5 sec

Cytotoxic drug
Conventional Reaction ADC23-11_1hr

ADC collected peaks → Amicon® Ultra-2.0 10K MWCO

For HIC analysis ~100µg/ 90µL

AFC 3000
- Collect by time: 11.75-13.50min
- Delay time: 5 sec

Cytotoxic drug
HIC for ADCs DAR
MAbPac HIC-Butyl 4.6 x 100 mm, 5 µm
(PN 088558, SN 001154
Lot# 01425010, TFS 508)
VanquishH LC3
Unconjugated mAb 10 ug
duplicate injection
Unconjugated mAb 10 ug, duplicate injection

Unconjugated mAb 10 ug collected peak and filter through Amicon 10K-0.5
Unconjugated mAb 10 ug, collected peak and filter through Amicon 10K-0.5

ACD21-18_5h collected peak and filter through Amicon 10K-0.5
Unconjugated mAb 16 ug

Unconjugated mAb collected peaks and filter through Amicon 10K-2.0

ACD23-10_1h and ADC23-11_1h collected peaks and filter through Amicon 10K-2.0
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Peter Menzel, Bruce Mattson, Martin Pulce, et al

Journal of Chemical Education

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